PROCESS OPTIMIZATION AND TECHNO-ECONOMICS ASSESSMENT OF NEMATODE BIOPESTICIDE PRODUCTION

By

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Declaration

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Summary

Entomopathogenic nematodes (EPNs) of the genera *Steinernema yirgalemense* are used as an agent in insect biocontrol programs, as they have proven to be safer and more effective than chemical pesticides. Successful commercialization of the nematode-bacteria symbiont as a biocontrol product depends on the ability to produce sufficient and viable quantities of these products at competitive prices for a full pest control program.

The general objective of the study was to conduct a comparative analysis of the techno-economics of two technological routes of nematode biopesticides, namely; *in vivo* and *in vitro*. The entomopathogenic nematode-bacteria complex of the strains was cultured through *in vivo* and *in vitro* methods, using the LOTEK system and a bioreactor, respectively. The pros and cons associated with the two processes in terms of production yields, cost of production, capital outlay, and technical expertise required, economies of scale and product quality were assessed. This was achieved through the design and construction of the LOTEK system, followed by basic testing through a series of cultivations with both the LOTEK and in a bioreactor. *In vitro* fermentations in a two-stage process was also conducted to compare batch fermentation systems with fed-batch systems. Using glucose and/or bacteria as feed pointers, a high proportion of infective juveniles (IJs) were recorded in fed-batch systems in comparison to traditional batch systems.

Based on the experimental and technical data obtained, an initial cost analysis through process modelling was performed to ascertain which of the process technologies offer lower cost of production at industrial scale. Techno-economic studies were conducted using the data obtained at the laboratory level, considering a required annual production rate to provide nematodes sufficient for treatment of 5680 hectares per annum. Techno-economics analysis indicated that the *in vitro* technology (bioreactor) was highly preferred for commercialization, based on the low cost of production in comparison to the *in vivo*. This was confirmed by economic indicators, with the calculated IRR, NPV and payback time of the project providing values of 24%, R 173 MRand and 7 years respectively, indicating the profitability of the project. A sensitivity analysis indicated that the selling price and the CAPEX had the greatest impact on the IRR%, compared to the contributing factors of production cost (such as media and utilities). Furthermore, a comparative cost analysis between the cost of production of nematode-based products estimated in this present investigations (R 8800 per hectare) and most conventional chemical pesticides on the market (R 410 per hectare) revealed no competitive price advantage of the former. Therefore, reducing the intensive use of conventional chemicals on the market by substituting with nematode products will require further process development to reduce the cost of the latter.

However, to realize the potential of EPNs as biological-control products in agriculture, also offering significant health benefits to consumers, requires cost-competitive production and application methods, in comparison to conventional pesticides in order to overcome the inherent high costs associated with the production technology

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Nomenclature

IJ	Infective Juvenile
TCI	Total Capital Investment
FCI	Fixed Capital Investment
EPN	Entomopathogenic Nematodes
CAPEX	Capital Expenditure
OPEX	Operating expenditure
IRR	Internal Rate of Return
PB	Pay-back time
PC	Production Cost

Glossary

Anhydrobiosis – condition during which some nematode species are able to undergo a loss of water and assume a coiled, shrunken posture, associated with survival of long periods of drought

Biological control – reduction of nematode populations by the activity of another organism, typically a fungus or bacterium, which parasitizes or is antagonistic to the nematode

Cuticle – the exoskeleton of nematodes

Host – any plant species on which the nematode can complete its life cycle; good hosts support high levels of reproduction whereas poor hosts support relatively low levels of reproduction

Infective Juvenile – an alternative stage of development for some nematode genera, specifically adapted for dispersal and survival of adverse conditions.

Infected – the establishment of an intimate relationship between the parasite and its hosts, characterized by an exchange of metabolites

Juvenile – immature stages (typically four in number, J1, J2, J3, J4) of nematodes, the preferred term to larva

Larva – immature stage of insects (the preferred term for nematodes is juvenile)

Management – strategies used to suppress nematode population densities and maintain them at densities at which there is little or no crop loss

Pathogen – an agent that causes disease

Pathogenesis – the act of causing disease

Patent infection - the invasion of a pathogen into a host, resulting in signs and symptoms distinct for that particular disease

Sedentary – nematodes that lose locomotion abilities as they mature, typically endoparasites or semi-endoparasites with greatly swollen bodies at maturity

Tropical climate – climates similar to that existing in the tropics, that region between the Tropic of Cancer and Tropic of Capricorn; characterized by warm temperatures year round, often humid with excessive rainfall

Vector - an organism that transmits or disseminates pathogens and parasites from one host to another

Virulence – referring to the ability of nematode pathotypes or races to parastize host genotypes that are resistant to other populations of that nematode specie

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Chapter 1

Introduction

1.1 Background

Agriculture remains one of the key driving forces behind the economic development of South Africa. It provides food resources and raw materials to feed both the growing population and non-agricultural sectors of the economy (Diao, Hazell and Thurlow, 2010). According to 2009 exportation reports released by South African Department of Agriculture (DAFF), the agro-industrial sector accounts for 12% of the nation's Gross Domestic Product (GDP), and the exportation of agricultural produce contributes 8% of the total export revenues in this country (Quinn *et al.*, 2011). In 2009/2010, the largest agricultural export products from South Africa were produce such as wine, maize, grapes, apples and citrus fruits, which generated a total GDP generation of $ 3.41 billion, and was consequently listed 31st on the global Gross Domestic Product (GDP) (Quinn *et al.*, 2011). In order to ensure the continual flow of revenue from the export of agriculture, practical strategies are necessary to deploy agricultural technologies that guarantee the incessant flow of food produce; as well as, legislating policies that safeguard productivity and food security.

One of the foreseeable global challenges against agriculture produce has been the activities of pests (Finizio and Villa, 2002). They have profound impacts on crops, and their control constitutes a significant portion of production costs. To minimize their destructive effects on crops, farmers resort to the intensive application of chemical pesticides which have lasting effects on the postharvest processing and consumption of agricultural produce (Pimentel and Huang, 1997). Moreover, their indiscriminate use poses serious health implications on consumers as well as besetting the integrity of our ecosystems (Eddleston *et al.*, 2008).

According to World Health Organization (W.H.O) report of 1999: "No consumer is entirely safe from the possible risks and health effects of pesticides'' (W.H.O, 1999). On the average, one million people in the world suffer various acute poisonings, hormonal disruption, diminished intelligence, reproductive abnormalities, immune suppression, and cancer annually due to pesticide poisoning (Gupta, 2004). Specifically, developing countries account for 99% of deaths arising from acute pesticides poisoning in the world (Village, 2000).

The term 'pesticides' embodies insecticides, fungicides, rodenticides, herbicides and heavy compounds (naphthalene, organophosphate, and captans) (Jeyaratnam, 1990). These hydrocarbon compounds when released into the environment, set up chains of petrochemical reactions that leak dangerous ions and metals into water bodies, aquatic organisms, phytoplankton, and soils, leading to an aftermath of pollution (Relyea, 2009; Roig *et al.*, 2011). All these negative aspects of chemical pesticides have steered a major concern from both the government and most research institutions to find alternative pathways that displace the intensive application of pesticides and embrace a more natural and benign approach to pests control. Biological controls through biological agents offer an attractive alternative to chemical pesticides, as a more natural yet potent approach to combat pests. Moreover, some insects have gained some degree of resistance to the pesticides presently utilized and therefore render their application ineffective (Chavarría-Hernández *et al*. 2011).

Integrated Pests Management (IPM) considers various biological control mechanisms along with complementary management approaches for the controlling of a vast spectrum of pests (Smart, 1995). It assesses different pest management and control strategies by evaluating the pros and cons associated with a particular control option with regards to the environment (Hoddle, 2000). The IPM systems, therefore, resort to the use of living organisms that prey on pests to reduce their population density on crop fields (C. Van Zyl and Malan, 2014). There is a growing body of literature that recognizes the role various groups of microbial control agents such as viruses, fungi, bacteria, and nematodes play in the control of pest arthropods, as they have proven to be non-toxic, sustainable and safe for use in the environment (Tofangsazie, Arthurs and Giblin-davis, 2012).

The use of entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* as one of the control strategies to pests on the field has been given much attention over the years as they have proven to be safer and more effective than chemical pesticides (Kaya and Gaugler, 1993). They are host specific and exhibit a high level of insecticidal potential against more than 200 commercial pests under laboratory conditions (Fischer-Le Saux *et al.*, 1999). Nematodes have no negative impact on other useful vertebrates, crops and mammals on the field, and thus allowing an exemption from Federal pesticide registration in the USA (Shapiro & Gaugler 2002). Notwithstanding the trade liberalization of using nematode products in the USA, almost all new strains of nematodes from other countries into South Africa are subject to the scrutiny and regulations by the DAFF, as they have to undergo a thorough study (DAFF, 2012). The introduction of foreign strains into any country can have adverse environmental effects as it can result in pollution if not properly controlled and managed (Chen and Dickson, 2004). Additionally, most exotic organisms fail to adapt climatically to their new ecological conditions, and thus

affecting their general performance (C. Van Zyl and Malan, 2014). All these have led to the surge of the development of an endemic strain that has a high insecticidal potential to combat pests in South Africa.

Studies into a model endemic EPN specie, *Steinernema yirgalemense*, have been intensified over the years and has been adopted into biocontrol programs due to its pathogenicity attributes and positive approach to mass-culture (Ferreira, 2013). However, their successful commercialization depends on the ability to produce sufficient and viable quantities at competitive prices for a full pest control program. Nematode production integrates two main production streams, i.e. the *in vivo* and the *in vitro* technologies. The *in vivo* method of production aims to simulate the natural environment of EPNs by their cultivation in the body of fictitious insect host, while the *in vitro* technology employs production of nematodes in liquid cultures utilizing bioreactors.

1.2 Motivation and Significance of Study

EPNs as a substitute to chemical insecticides is an attractive alternative for use in agriculture, although the high costs of production still limit commercial viability in comparison to low-cost chemical insecticides (Gaugler, 2002). The cultivation and production processes for nematodes at industrial scale remain under-explored, with insufficient technical data available at commercial scale, especially for the *in vivo* method. *In vivo* production entails a simple and traditional method based on the White's trap method where the nematodes are cultivated on petri dishes and shelves on fictitious hosts (Gaugler and Lewis, 2004). Noted challenges of this technology include the high cost of production, low yields, and high input of manual labour (Gaugler *et al.*, 2002a). Thus, the process is adopted by nematode producers with low capital investment and research institutions for academic purposes (Sharma, Sharma and Hussaini, 2011). This is as a result of limited information about nematode cultivation, and more importantly, enough data are not available on a commercial scale basis. Contrarily, *in vitro* production has been touted by many researchers and nematode producers as the appropriate pathway to mass-production due to the high yield of EPNs achieved using a simple complex media (Shapiro-Ilan, Han and Dolinksi, 2012). Two production media can be adopted, i.e. the solid and liquid media (Alexander, 2011). In both, the nematodes are produced in the presence of their symbiotic bacteria in bioreactors or mechanically agitated shake flasks (Gil, 2002). However, due to the strict process conditions associated with the cultivation route, it requires a high level of technical expertise along with huge capital investment to initiate production (Gaugler *et al.*, 2002b).

In order to realize the potential of EPNs as biological-control products in agriculture, also offering significant health benefits to consumers, requires cost-competitive production and application methods,

in comparison to chemical pesticides. Both the assessment of proposed production technologies, regarding yields of EPNs obtained, as well as the economics of alternative production processes (techno-economics) was addressed in this present study.

The study therefore sought to compare the techno-economics of two production processes for EPNs, i.e. the *in vivo* and *in vitro* methods. Technical data and process conditions on EPN yields were collated by undertaking series of cultivations of the nematode-bacteria complexes using a tray feeder system (*in vivo*), formerly proposed by (Gaugler *et al.*, 2002a) as the 'LOTEK' system, and submerged liquid culture in a bioreactor (*in vitro*). The advantages and disadvantages of the two processes in terms of yields of EPNs, the cost of industrial operational requirements, technical expertise and manual labour requirement, production cost, capital outlay, ease of achieving economies of scale and quality of final products from both methods were assessed.

This was achieved through the design and construction of the Tray Feeder System (TFS) followed by basic set of testing with the *in vivo* method, to demonstrate the functionality of the TFS. Similarly, EPNs production in a two-stage process (batch versus fed-batch) according to the *in vitro* method was performed in bioreactors, to access process performance and product yield. The technical data from the optimized laboratory for each of the methods was used for the core design of industrial facilities, allowing development of economic models to predict the comparative cost of production for the two processes. Both the technical and economic considerations ultimately informed on which of the two methods offers a viable route for technical implementation and scale-up

A successful completion of this study would provide enough hands-on information for potential investors with limited resources to venture into nematode production via any of the preferred route whiles individuals in low-value crops plantation would have enough information at their disposal to implement an on-site nematode production through the feasibility assessment of both technologies

1.3 Summary of Research Aims and Objectives

A detailed techno-economics study for nematode technology has never been done, especially for the comparison of *in vivo* and *in vitro* in South Africa. To the best of author's knowledge, this case has not been given considerable attention by other researchers and thus motivated the need for this present study. It represents one of the first investigations to develop technical and economic models based on results from optimized laboratory trials in the cultivation of *Steinernema yirgalemense* as a model organism. The specific aim of the study was to evaluate the techno-economics of nematode biopesticide production for the implementation in South Africa.

Based on the aforementioned aim, the following set objectives were identified:

1. To investigate the functionality of the LOTEK (TFS) system through a series of cultivations of nematodes in order to ascertain its viability of the *in vivo* method for commercial implementation. The outcome of this objective is intended to investigate the practicality of scaling-up production by modelling a room in a similar fashion as the LOTEK system.
2. To assess of the impact of different fermentation mode (batch versus fed-batch) on the growth of the nematode/bacteria complexes in fermenters to obtain higher EPN biomass from the *in vitro* method. The factors affecting reproducibility, and the impact of adding fresh bacteria and glucose supplement to existing batch cultures would be investigated.
3. To perform full economic assessments of the two production routes based on the modelled room technology (*in vivo*) and the bioreactor system (*in vitro*). This will enable comparative study between the two production technologies based on the cost of production in order to inform on which of the above technologies is viable for commercial implementation.

1.4 Outline of Thesis

The overall structure of the study takes the form of six chapters as summarized in figure

- Chapter 1 gives a general background and lays out the specific aims and objective of this study.
- Chapter 2 begins by laying down the theoretical dimensions of the research and looks at a summary of the fundamental biology and physiology of nematodes that influence successful production. The various production methodologies are also discussed.
- Chapter 3 summarizes the principal findings of the *in vivo* technology experiments through the LOTEK system
- Chapter 4 discusses the methodology and results of the *in vitro* experiments in bioreactors (batch cultures versus fed-batch cultures)
- The techno-economics of the *in vivo* and the *in vitro* cultures are addressed in Chapter 5 to review the anticipated production cost of replicating both the *in vivo* and the *in vitro* technologies on a large scale.
- The final Chapter 6 includes a discussion and implications of the findings in chapters 3, 4 and 5 on future research works in EPNs production via the known technologies. (Conclusions and recommendations based on the experimental results are spelled out and also propose steps to improve upon the production techniques in future).

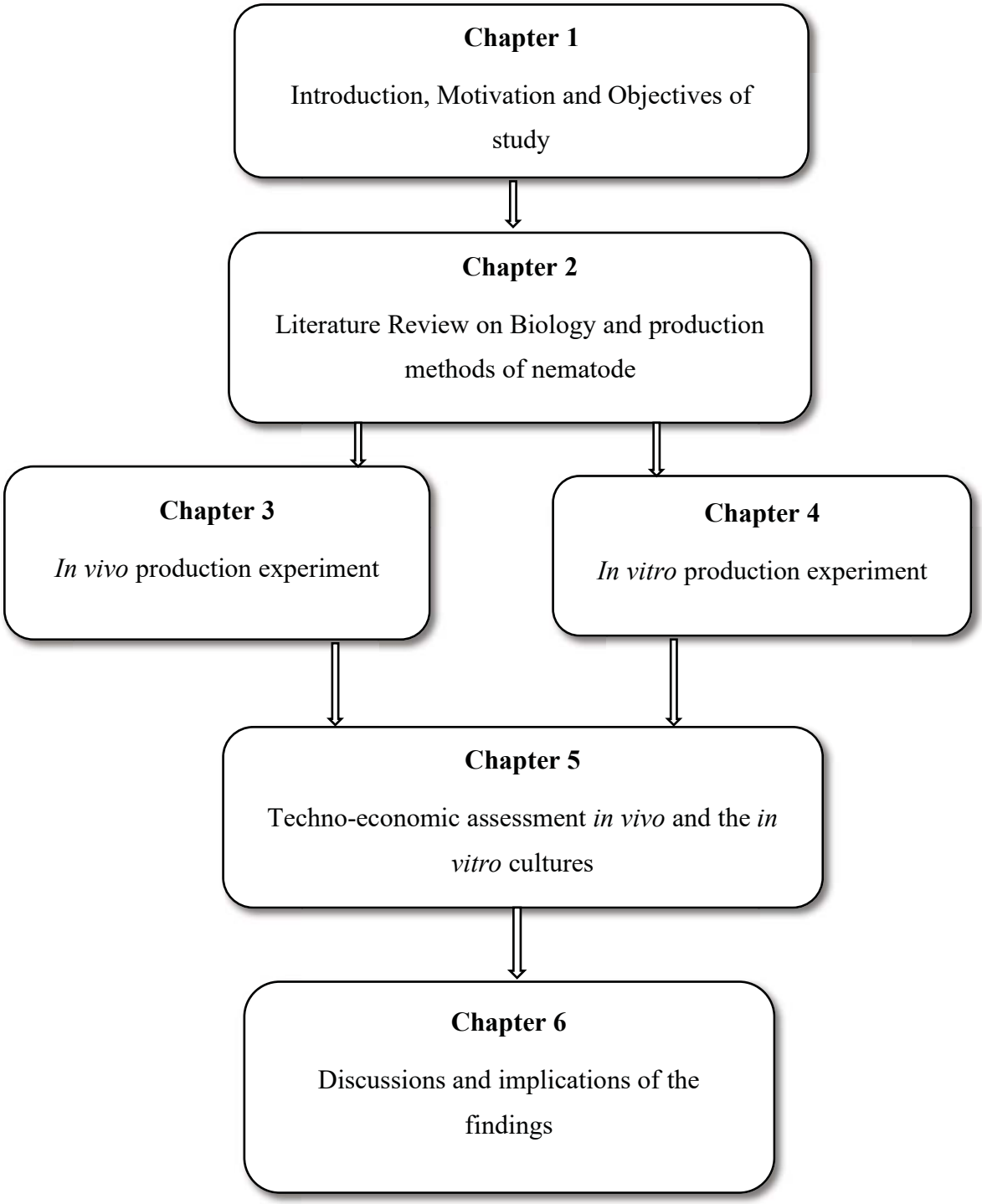

Figure 1-1: Flow diagram of thesis layout

Chapter 2

Literature Review

2.1. Introduction

This review was primarily focused on the biology of the EPNs in the families *Steinernema yirgalemense* and *Heterorhabditis,* since they are the two indigenous strains found in South Africa and possess high pathogenicity attributes (Ferreira, 2013; C. Van Zyl and Malan, 2014). The aim of this chapter was to understand the biological and functional behaviour of the nematode-bacteria complexes (*Steinernema yirgalemense*), in order to optimize for a higher yield and process efficiency, as the prerequisites for a successful mass production. The production methodologies of EPNs were therefore reviewed and the different process parameters that affect their successful commercialization were accordingly addressed.

2.2. Biology and Ecology of Entomopathogenic Nematode

Entomopathogenic nematodes (EPNs) are a group of microscopic, multicellular organisms located in the soil with no appendages, with an average length and width of 536.4µm and 25.3µm respectively (Poinar, 1990). The families of *Heterorhabditidae* and *Steinernematidae* are known to be pathogenic to pests, due to their obligate mutualistic relationship with the bacteria of genus *Photorhabdus* and *Xenorhabdus* respectively (Shapiro-Ilan, Han and Dolinksi, 2012).

EPNs are sampled and isolated from the soil using a baiting method described by Akhurst (1993) where an insect host is used as bait (preferably, *Galleria mellonella*) because of its high susceptibility to EPNs (Jawish, Al-assas and Basheer, 2015). Nematodes attack diverse invertebrate organisms, and their distribution around the globe is influenced by factors such as environmental conditions as well as the availability of a suitable host (Stock, Pryor and Kaya, 1999). Consequently, EPNs are mostly located in most tropical and temperate regions where the soil conditions are favourable for their development and growth (Grewal, Ehlers and Shapiro-Ilan, 2005). Table 2-1 shows the morphological features and effective temperature range for most nematodes widely cultivated in the USA.

Nematodes are known to be efficient in the control of the population density of some destructive pests (such as caterpillars, cutworms, crown borers, grubs, corn rootworm, crane fly, and beetles) in the soil (Klein, 1988). However, the vast majority of studies on nematodes hitherto have been quantitatively limited to only 11 species due to their virulence attributes and positive approach to mass production (Table 2-2) (Miles *et al*., 2014).

Table 2-1: Morphology and effective temperature range for widely cultivated nematodes in USA

Nematode species	Length(µm)	Width(µm)	Activity (°C)*
S. carpocapsae	558	25	8-32
S. feltiae	849	26	8-30
S. glaseri	1130	43	10-37
S. scapterisci	572	24	12-35
S. riobrave	622	27	10-39
H. bacteriophora	588	23	12-32
H. megidis	768	29	10-35

Source: (Chen *et al.*, 2003)

Table 2-2 Nematode species and the types of insects they suppress

Crop(s) Targeted	Pest Common Name	Efficacious Nematodes
Artichokes	Artichoke plume moth	Sc
Vegetables	Armyworm	Sc, Sf, Sr
Ornamentals	Banana moth	Hb, Sc
Bananas	Banana root borer	Sc, Sf, Sg
Turf	Billbug	Hb, Sc
Turf, vegetables	Black cutworm	Hb, Hd, Hm, Hmeg, Sc,
Berries, ornamentals	Black vine weevil	Hb, Sc, Sf
Fruit trees, ornamentals	Borer	Sc
Home yard, turf	Cat flea	Sr, Hb
Citrus, Ornamentals	Citrus root weevil	Sc, Sf, Sr
Pome fruit	Codling moth	Hb, Sc
Vegetables	Corn earworm	Hb

Source: (Gaugler and Bilgrami, 2004)

The performance of nematodes can be adversely affected under certain soil conditions. Various authors have discussed the effects of factors such as desiccation, high-temperature regimes and UV rays on the performance on EPNs in the soil (Shapiro-Ilan *et al*. 2012; (Jagdale & Grewal 2007); Sharma *et al*. 2011). Overall, these studies acknowledge the relevance of choosing an appropriate EPN candidate towards a target pest, considering the extremity of most soil conditions. Jagdale *et al*. (2007) also reported that, although most EPN strains show a high efficacy against target pests at controlled laboratory conditions, they fail to adapt to certain variable conditions in

the soil. Ultimately, there is a need of cultivating EPN strains whose efficacy and stability in the field is not aggregately affected by the environmental conditions (Jagdale and Grewal, 2007). Dillon *et al*., 2007 compiled a list of currently known nematode species and the types of specific insect pests which they have proven to suppress on commercial crop fields, as shown in Table 2-2 (Dillon *et al*., 2007).

2.3. Biology of the Symbiotic Bacteria

Steinermatids and *Heterorhabditis* share a common attribute in their morphology and behavioural characteristics as a result of convergent evolution (Grewal, Ehlers and Shapiro-Ilan, 2005). Both species retain a symbiotic bacteria in the intestines or gut during the third stage of their life cycle (Lewis *et al.*, 2006). The bacteria of the genus *Photorhabdus* and *Xenorhabdus* are Gram-negative enterobacteria strains that are symbiotically associated with the entomopathogenic nematode of the family *Steinernematids* and *Heterorhabditids* respectively (Medina-torres *et al.*, 2007). The nematodes at this stage are referred to as infective juveniles (IJs), and they have the ability to live independently outside the body of a host (Chavarría-Hernández *et al.*, 2001). The bacteria are responsible for the pathogenicity and virulence attributes of the nematodes (Valle *et al.*, 2011). However, they are unable to penetrate into the body cavity of a host dependently; hence, the nematodes act as vectors to aid the transportation of pathogenic bacteria into the body of a target host (Medina-torres *et al.*, 2007). Some bacterial strains in a specific class can be associated with two or more families of nematodes as indicated in Table 2-3.

Table 2-3: Various nematodes and their symbiotic bacteria.

Bacterial species	Nematode
X.beddingii	*Steinernema* spp.
X. bovienii	*S.feltaie*
	S. intermedia
	S. kraussei
	S. affins
X. nematophilus	*S. carpocapsae*
X. poinarii	*S. glaseri*
Xenorhabdus species	*S. longicardum*
	S. kushidai
	S. neocurtillis
	S.rara
Source: Bedding *et al.*.1993	

2.3.1. Phenotypic forms of Bacteria

According to Shapiro *et al.,* 2002, the nematode symbiont can exist on two phenotypic forms, i.e. Phase I and the Phase II. The phase I is a desirable phase during culturing, and it is associated with two major characteristics: the production of antibiotic substances and the absorption of dyes (Ehlers, 2009). Nematodes are highly efficient in suppressing hosts and reproduce significantly in liquid cultures when their bacterial partner is stabilized in the primary variant (Wang *et al.*, 2007). Nonetheless, they lose some important traits which are required for successful pathogenicity when they shift phases from phase I to phase II (Gerritsen, De Raay and Smits, 1992). Additionally, in the phase II variant, because there is a deterioration of essential traits the bacteria cannot produce antibodies in their primary cells to enhance their virulence and development mostly during *in vitro* liquid cultures.

The primary factors that induce phase shift processes are still not known. In an attempt to investigate the causes of phase shifting, Krasomil-Osterfeld (1995) was able to induce the secondary variant stage of the bacteria by cultivating them in media under stress conditions such as low osmotic strength, high temperatures, and low dissolved oxygen. Wang *et al*., (2007) also ascribed secondary factors such as contamination and subculturing practices as important causes for phase shifting especially in *in vitro* culture cycles. Subculturing practices affect important traits such as parasitizing abilities, fecundity, nictation and survival qualities of the nematodes (Han and

Ehlers, 2001). It is, therefore, important to prevent a phase shift (alteration in cell variants) in liquid cultures by selecting primary variants of strains to initiate culturing. Ehlers *et al*., (2001) noted how the adverse effects of phase variation are more pronounced in solid and liquid cultures that often results in low production figures.

Nematode producers verify phase change by the method described by Akhurst (1980). A streak of bacteria in phase I produces dark blue colonies by the absorption of bromothymol blue, while the phase II bacteria reduce triphenyl tetrazolium chloride (TTC) to produce colonies of red (Medina-torres *et al.*, 2007)

2.3.2. Isolation of Bacteria

The bacteria *Photorhabdus* and *Xenorhabdus* species can be isolated from the haemocoel of an insect or extracted from the infective juveniles (IJs) using a hand-held grinder . They can be cultured separately in an artificial growth media ranging from Luria –Bertani (LB)[1], NBTA agar, nutrient broth, yeast extract or in a natural medium (such as the haemolymph of an insect) (Han and Ehlers, 2000).

In formulating the growth media, the *Photorhabdus* strains do not require any vitamin or growth factor supplements, as they can grow in a standard medium with NH_4+ as a nitrogen source, and glucose, mannose or N-acetylglucosamine as a carbon source (Gaugler, 2002; Stock and Goodrich-Blair, 2012). Contrarily, *Xenorhabdus* species require supplementation of a cofactor such as nicotinic acid for their growth in a medium, as they lack nicotinamide adenine dinucleotide (NAD) for the synthesis of this cofactor (Stock and Goodrich-Blair, 2012). Additionally, the bacterial strains are sensitive to oxidants that build up in media exposed to light and are therefore mostly cultured or stored in a media that have been kept in the dark or to which pyruvate has been added to the final concentration (Gaugler, 2002). This step prevents the growth of contaminants such as *Escherichia coli* in the media (Stock and Goodrich-Blair, 2012). The optimal temperature for culturing is usually between 28-30^{0}C, as they do not thrive in higher temperatures (e.g., 38^{0}C) and cold storage (e.g., 4^{0}C) (Stock and Goodrich-Blair, 2012).

[1] See Appendix A

2.4. The Life Cycle of Nematode

Control of the EPNs life cycle within any production process is essential to ensure efficacy and pathogenicity of the final product during agricultural application. The reproductive life cycle of EPNs consists of five main stages excluding the egg stage with four infective juvenile stages in-between the egg stage and adult stage (Campbell and Gaugler, 1991). Throughout the life cycle of the nematode, each stage exhibits characteristics that are closely related to one other particularly in the juvenile stages (J1, J2, J3, and J4) (Gaugler and Bilgrami, 2004). Moulting processes characterize the transition from one stage to another where there is an exsheathment of the nematode cuticles (Grewal, Ehlers and Shapiro-Ilan, 2005). The free-living and non-parasitic stage of the nematode is the J3 stage, and it is a specially adapted stage of the nematode that is induced as a result of unfavourable conditions (such as lack of food) in the soil (Inman, Sunita and Holmes, 2012). Two unique features of the J3 stage in the life cycles of *Steinernema* and *Heterorhabditis* are that they carry the bacteria required for pathogenicity in the anterior part of their intestines and are capable of independent living (Hirao and Ehlers, 2010).

The life cycle of *Steinernema* is similar to *Heterorhabditis* except that the first generation and second generation offspring are developed as a result of copulation activities between adult male and female *Steinernema* nematodes (Kooliyottil *et al.*, 2013). Contrarily, the first generation of *Heterorhabditis* is predominantly IJs, which are developed as a result of eggs laid in the uterus of the parental hermaphroditic adult nematodes; a process referred to as *endotokia matricida* (Ehlers *et al.*, 1998). These fertilized eggs in the uterus are produced as a result of self-fertilization within the hermaphrodite nematodes. However, the third generation nematodes of *Steinernema* are solely as a consequence of *endotokia matricida* (Jeffke *et al.*, 2000).

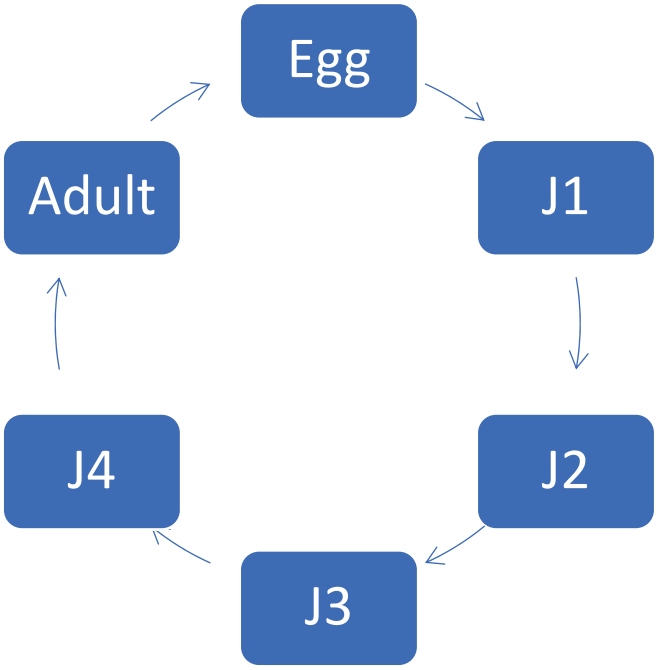

Figure 2-1: Life cycle of nematode

2.5. Pathogenesis of Nematodes

The J3 stage of IJs defines the required "product quality" of EPNs produced from selected production processes. The infective juveniles (IJs) in the J3 stage move through the soil seeking out insect hosts to invade their system via the mouth, anus or any natural openings (Glazer and Salame, 2000). Upon invading the host's body, the nematode provides an initial protection to the symbiotic bacteria by shielding it from the immune defence mechanisms of the insect (Gaugler and Bilgrami, 2004). The shielding process is made possible as a result of the nematodes releasing toxins that inhibit the inducible immune system of the host, thereby allowing the bacteria to proliferate inside the haemocoel of the insect. The haemocoel is a highly nutrient-rich part of the insect's system that provides nutrients and nourishment for bacteria proliferation (Grewal, Ehlers and Shapiro-Ilan, 2005). As the bacteria grow and reach the stationary phase, they induce conditions that are favourable for the nematode reproduction by secreting exoenzymatic complexes (such as lipases, phospholipases, and proteases) that suppress the defence mechanisms of the host (Lacey *et al.*, 2015). The bacteria also produce antimicrobial agents such as bacteriocins, xenocoumacin, xenorhabdins, and trans-stilbene that prevent the growth of other bacteria, fungi and yeasts that may compete with the nematode for nourishments (Wang *et al.*,

2011). Since the association between the nematode and its bacteria is mutualistic, the infective juveniles (IJs) in turn feed on the viable bacteria and the bio-converted host tissues, and exit from the developmentally arrested stage (J3), a process known as 'recovery,' to a fully functional organism (Kooliyottil *et al.*, 2013). Recovery is one of the most important steps in the life cycle of the nematodes (Chavarría-Hernández *et al.*, 2006). During recovery, the infective juveniles (J3) feed on the growing bacteria population and develop into juvenile stage four (J4) then to the adult stage (Hirao and Ehlers, 2010). At the adult stage, copulation occurs, leading to the reproduction of the first filial generation of nematodes (Ehlers, 2001).

Depending on the amount of nutrients available, one to three generations can be completed in an insect cadaver (C. Van Zyl and Malan, 2014). However, a full cycle takes about 7- 10 days to complete. (Smart and Sciences, 1995). Nematodes begin to move out in search of new hosts when conditions in the deteriorating cadaver become unfavourable as the offspring develop into IJs before exiting cadaver to initiate a new cycle of pathogenesis (Smart and Sciences, 1995).

A summary of the life cycle events in the bacto-helminthic nenmatode/bacteria association is given in Table 2-4.

Table 2-4: Life cycle events in bacteria -nematode symbiosis

Stage	Nematode Life cycle	Bacteria life cycle
I	Infective juvenile in the soil Search for insect host Infective juvenile enters insect haemocoel	Bacteria retained in nematode gut
II (Early)	Recovery in the haemocoel	Bacteria released into haemolymph, production of virulence factors, death of insect
II (Late)	Nematode reproduction	Bacteria in stationary phase Production of antibiotics, exoenzymes, crystal protein
III	Development of new infective juveniles	Colonization of the intestine of infective juveniles

Source: Gaugler (2002)

2.6. Behavioural attributes of Entomopathogenic Nematode

In this section, investigations are made to understand the basic ecological and behavioural aspect of nematodes which are relevant for successful biological control of soil pests.

2.6.1 Foraging strategy – Host Interaction

The foraging strategies used by nematodes in finding hosts account for their suitability in controlling most soil pests (Grewal *et al.*, 1994). The J3 stage within the life cycle of nematodes is the only free-living stage capable of independent living, and can persist in the ground for several weeks after application before finding a suitable host (Hirao and Ehlers, 2010). EPNs find their host with the aid of their well-developed chemoreceptors and mechanoreceptors, and thus, exhibit two main predatory lifestyles: Cruising and ambushing (Grewal, Gaugler and Selvan, 1993; C. Van Zyl and Malan, 2014). *H. bacteriophora* and *H. megidis* strains use cruising behaviours in search of sedentary hosts in the soil as they move from one point to another (Kooliyottil *et al.*, 2013). Furthermore, with the help of their well-developed chemoreceptors, cruisers can detect chemical signals such as CO_2 concentrations from nearby hosts and use their mechanoreceptors to attach to their hosts (Laznik and Trdan, 2013). Some of the external stimuli from the hosts that the nematode may be exposed to may include faeces or by-products from the metabolism of the insect. (Wilson, 2009)

On the other hand, *S. carpocapsae* and *S. scapterisci* strain wait patiently for their prey to cross their area of strike before attacking (Cheng, 2007). They are therefore referred to as 'ambushers.' The ambushing strategy is as a result of the nematodes attacking preys which are mostly mobile and larger than themselves (Williams *et al.*, 2013). However, some nematode strains such as *S. feltiae* and *S. riobrave* exhibit intermediate search behaviour depending on their proximity to the preys (Laznik & Trdan 2007; Brown *et al* . 2002). The penetration sites of nematodes on the body of the hosts can vary from one species to the other and may also depend on the nature of the host (Inman, Sunita and Holmes, 2012). Common penetration sites may include the cuticle, the mouth or the anus of the insect (Lewis *et al.*, 2006). Most nematodes avoid penetration through the mouth as they can be crushed by the huge mandibles of the insect (Lewis *et al.*, 2006). Table 2-5 below summarizes four common EPNs species based on their foraging strategy and the type of insect known to control

Table 2-5 Summary of foraging and behavioural strategies exhibited by insects

***Steinernema* spp.**	**Foraging strategy**	**Nictation**	**Jumping**	**Ranging to localized search by host contact**	**Attraction increased by host contact**
S. carpocapsae	Ambusher	Yes	Yes	No	Yes
S.feltiae	Intermediate	No	No	No	No
S. riobrave	Intermediate	No	Yes	No	No
S.glaseri	Cruiser	No	No	Yes	No

Source: (Grewal, Ehlers and Shapiro-Ilan,

2.6.2 Tolerance Level of EPNs

Desiccation and stress levels of EPNs differ from one species to another (Somvanshi, Koltai and Glazer, 2008). Some nematode species can persist under soil conditions for longer periods than others as a result of the differences in morphology (such as host's density), the type of nematode strain and more essentially the environmental conditions (Ferris, 1980). Extreme temperatures (less than 1^0C and greater than 35^0C) can affect the performance of nematode species in the soil (Shapiro-ilan *et al* . 2014). A study was done by Zyl *et al*., 2014 on the effects of temperature on the virulence level of *Heterorhabditis* spp. using *T. molitor* (wax moth) as a fictitious host. The results showed that exposure to high temperatures could have detrimental effects on the growth and virulence of nematodes. In a follow-up study, Brown *et al.* 2006 also investigated the factors that could affect the efficacy of nematodes by focusing on the optimal temperatures for maximum efficacy. It resulted that maximum effectiveness of nematodes in the soil is largely dependent on the type of nematode strain, as some species are known to be more heat-tolerant than others (Brown *et al* . 2006). For example, Shapiro-Ilan *et al*. 2014 reported that *H. indica* and *S. riobrave* (hot-tolerant) species can survive in high temperate regions than *S. feltiae* which are cold-tolerant.

In an attempt to reduce the effects of desiccation and preserve virulence, nematodes often undergo a reversible, physiologically arrested state of dormancy known as partial anhydrobiosis where they lose 95-98% of their body water and lower their body metabolism below detectable limits (Glazer and Salame, 2000). The nematodes are therefore protected from environmental extremes such as hypoxia and metabolic poisons that can reduce their survival rate in the soil (Jagdale & Ã 2007). Furthermore, the nematodes withstand high osmotic stress levels or low oxygen content in the soil by entering into a state of asmobiosis or anoxybiosis respectively (Glazer and Salame, 2000)

2.7. Production Technologies of EPN

EPNs as biopesticide can be produced via two main technological routes, namely *in vivo* and *in vitro* (submerged liquid culture or solid substrate) (McMullen II and Stock, 2014). The *in vivo* technology is adopted for laboratory and small scale cottage industries where the cost of labour is relatively cheaper, where capital and infrastructure is limited, and a low level of technical expertise is available (Shapiro-Ilan, Han and Dolinksi, 2012). However, for commercialization purposes and broad field-testing application, the *in vitro* submerged culture has been touted by many researchers (Friedman, Gaugler and Kaya, 1990; Shapiro and Gaugler, 2002) as the economically feasible route for EPN production, consequently generating a global interest specifically in ways to improve the production technology.

The success or failure of any production method depends on IJ yield that can be achieved as well as maintaining the required infectiveness of the nematode-bacterial symbionts produced (Shapiro and Gaugler, 2002). It is, therefore, important to ensure that cultures reach a high proportion of IJs as it is the only stage within the life cycle of the nematode capable of independent living and also required for pest control (Ehlers, 2001). After the production processes, a high proportion of non-IJs stages and adults in the broth require necessary additional downstream processing (DSP) of the culture broth. Inefficient DSP leads to contamination in the final products (Wilson, Pearce and Shamlou, 2001) and reduces the shelf life of the nematode product which is a challenge most nematode producers encounter after harvesting (Didik Sulistyanto, 2014)

2.7.1. *In vivo* Production

The *in vivo* technology involves the culturing of a particular strain in the body of a fictitious host utilizing shelves and trays lined with absorbent paper (Gaugler *et al.*, 2002a). Many authors (Dutky *et al.,* 1964; Kaya & Stock, 1997; and Gaugler *et al,* 2002) have investigated this production technique using different surrogate hosts (Table 2-6), albeit with some slight modification to white's (1927) design. Nonetheless, the underlying principles such as inoculation, harvesting and concentration processes remain homogenous (Shapiro-Ilan *et al.*, 2002).

Most *Steinernema* and *Heterorhabditis* are produced at the laboratory using the *G. mollonella* larvae as hosts, and it involves the adaptation of the White trap devised by White (1927). The White trap system involves an inverted watch glass placed over another petri dish lined with absorbent paper (e.g. filter paper or plaster of Paris) and sits in a large bowl filled with water to

create a humidified environment (Perez, Lewis and Shapiro-Ilan, 2003) Infected larvae are placed on the filter paper after 2-5 days of nematode infection and the progeny of infective juveniles (IJs) move into the water during emergence after 16- 21 days of incubation (Gaugler and Lewis, 2004). Incubation is done at 23-25^{o}C depending on the strain (Shapiro-Ilan, Han and Dolinksi, 2012). Central to White's design is the ability of the infective juveniles to migrate naturally from the infected cadaver into the surrounding water, where they are trapped and subsequently harvested (Gaugler *et al.*, 2002a).

Figure 2-2: White trap system devised by White (1927)

2.7.1.1. The In vivo Cultivation Process

The first practical step to *in vivo* production is the selection of suitable hosts (Lewis *et al.*, 2006). Hosts should show a high level of susceptibility towards the nematode species to be cultured (Parsa *et al*. 2006). The most common host used by small scale cottage industries is Wax Moth Larvae (WML), *Galleria mollonella*, which are highly susceptible to most EPNs species (Poinar and Grewal, 2012). They are widely cultivated in the USA, readily available, and comparatively cheaper as compared to other hosts such as yellow mealworm (*Tenebrio molitor*) (Shapiro-Ilan *et al.*, 2002). Hosts can be reared on artificial diets within a relatively short time (Laznik & Trdan

2007). However, diets for the rearing of larvae can vary according to the type of host species and thus account for the variations in costs analysis for different species (Gaugler and Lewis, 2004). More importantly, overall production cost estimates should be based on the yield per cost of hosts, and this could inform on the choice of hosts for cultivation in cottage industries (Shapiro and Gaugler, 2002).

Earlier researchers of the *in vivo* technology such as Shapiro-Ilan *et al*. 2002 studied the influence of host species and size on the final yield of EPNs. They investigated how the size of the IJs inoculated into the host can determine the final yield achieved. The size of IJs is inversely proportional to the final yield of nematodes achieved (Figure 2-3). The type of host also determines their susceptibility to infection levels (C. Van Zyl and Malan, 2014). However, the most important factors to consider when selecting hosts for cultivation are their amenability to culturing and consistencies to higher infection levels (Shapiro-Ilan *et al.*, 2002). Some host species are more susceptible to some specific EPN strains than others (Lacey *et al.*, 2005). For example, the larvae of the *Galleria mellonella* strain are predominantly used for cultivation, because they show a high-level susceptibility to most nematode strains widely cultivated Nonetheless, much emphasis must be placed on the yield and quality of nematodes produced from the host species.(Shapiro-Ilan, Han and Dolinksi, 2012). Hosts are often reared on artificial media mostly containing cereals and other ingredients, and they contribute substantially to the overall cost of production (van Niekerk and Malan, 2012). Blinova and Ivanova (1987) emphasized the importance of finding a trade-off between the type of host for the cultivation of EPNs and the cost of rearing them. The long-horned beetle (*Cerambycidae*) was found to be proficient to *Galleria* mellonella regarding the average yield of nematode obtained; however, low infection levels and the high cost of production rule out their use as ideal hosts (Gaugler and Lewis, 2004). In another study, Shapiro-Ilan *et al*., 2002 observed a relatively economical production of *H. bacteriophora* nematodes in *Tenebrio molitor* to their production in *G. mellonella* hosts, although their production efficiencies appeared to be the same for both hosts. Additionally, *Tenebrio molitor* was found to be less expensive to rear on a larger scale than *G. mellonella*. Lastly, the quality of IJs produced from the *in vivo* method is dependent on the choice of host. (Peters, 1996) Compatible hosts are likely to produce quality IJs, which will have high efficacy during field application and vice versa. Common hosts that are often adopted by nematode producers as fictitious hosts are shown in Table 2-6.

Table 2-6: Types of fictitious host used for *in vivo* production of nematodes

Common name	Botanical name
navel orange worm	*Amyelois transitella*
tobacco budworm	*Heliothis virescens*
pink bollworm	*Pectinophora gossypiella*
cabbage looper	*Trichoplusia ni*
beet armyworm	*Spodoptera exigua*
corn earworm	*Helicoverpa zea*
gypsy moth	*Lymantria dispar*
house cricket	*Acheta domesticus*

Source: (Gaugler and Lewis, 2004)

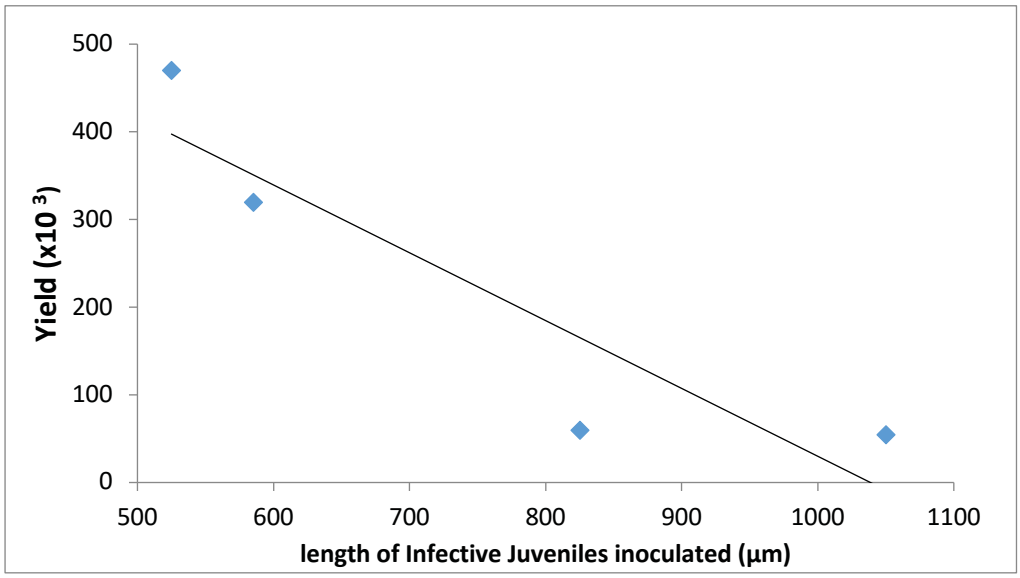

Figure 2-3: Relationship between infective juvenile size and production yield

[Redrawn from (Gaugler, 2002)

Following the selection of suitable hosts, they are inoculated and transferred to the White trap or incubator after 2-5 days of infection for incubation (Han and Ehlers, 2000). During this period, housekeeping practices (such as removal of non- infected or deteriorating larvae from the group) is done every three days to prevent possible contamination of the whole culture (Shapiro-Ilan, Han

and Dolinksi, 2012). Secondly, it is necessary to maintain a high level of humidity in the White traps especially on the absorbent paper to avoid desiccation of the emerging IJs (Grewal, Ehlers and Shapiro-Ilan, 2005). Nonetheless, the high humidity levels should be monitored in the course of production as too much water on the absorbent paper may trigger a premature emergence of nematodes (Gaugler *et al.*, 2002a). As a consequence of the natural migration of the IJs from the host cadavers into the White traps, harvesting of the IJs from the nematode-water suspension could be through simple filtration steps. An overview of *in vivo* production process is illustrated in Figure 2-4.

During production in shelves or metallic perforated trays, the selection of an absorbent paper is critical towards the success of the production. The absorbent paper serves as the first point of contact for the emerging nematodes and needs to be kept moist throughout the cultivation processes. Notwithstanding, the nature of the material should have enough interstitial openings to allow the passage of the nematodes. Common absorbent papers usually used include plaster of Paris, medical gauze and paper towel (Brown and Gaugler, 2004)

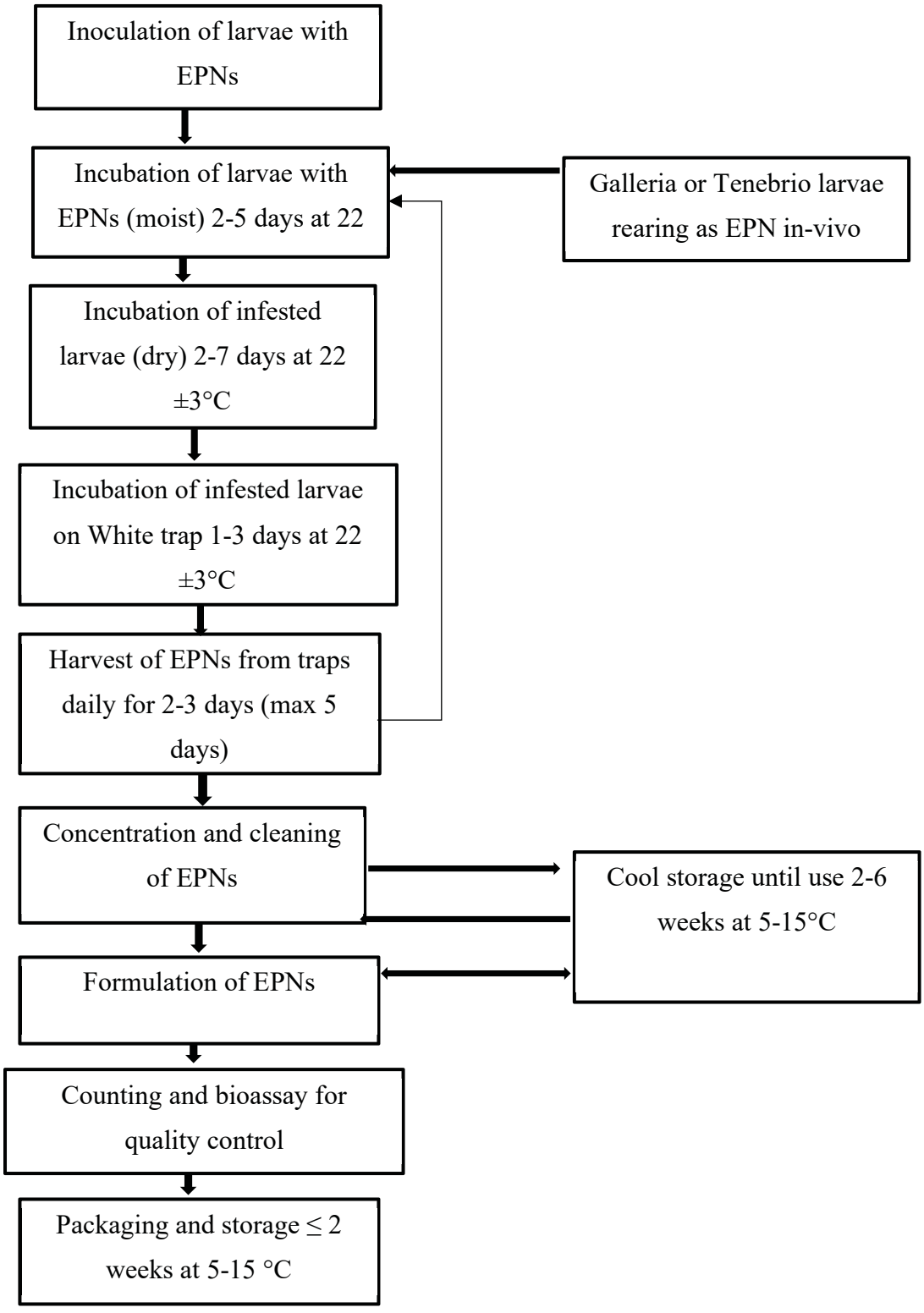

Figure 2-4: Schematic presentation of *In vivo* Cultivation Process

[redrawn from (Holmes *et al.*, 2015)**]**

2.7.1.1. Factors Affecting Yield and Survival of Nematode

Nematode yield and field efficacy can be well-affected by several biotic and abiotic factors (Lacey *et al.*, 2015). Abiotic factors such as the soil temperature and moisture content can affect the development and growth of nematodes as other parasitic fungi and predatory mites in the ground can also feed on the nematodes (Grewal, Ehlers and Shapiro-Ilan, 2005). Some of the factors that can affect nematode yield are discussed below:

2.7.1.2. Inoculation Techniques

The success of the *in vivo* cultures depends on the infectivity levels that could be achieved during the inoculation of the hosts with nematode suspensions (Brown *et al.*, 2002). Different inoculation methods are often adopted to various hosts as they respond differently to a specific inoculation technique. Ultimately, the goal of an inoculation technique is to break down the host defence mechanisms so as to achieve an acceptable level of infections that leads to patent infection. Lacey and Brooks (1997) defined patent infection as: 'the invasion of a pathogen into a host, resulting in signs and symptoms distinct for that particular disease''(Lacey, 2012). Poor infection levels waste insect host and demand a huge labour to remove them. Thus, parameters to optimize inoculation techniques can be implemented in the production process to ensure high patent infections (Shapiro-Ilan *et al.*, 2002).

Data from several studies also suggest that the *H. bacteriophora* nematodes achieve poor infection levels with *T. molitor* as a host. The poor infection is a result of the secretion of microbial protein, *Tenecin* 1, from the body of the host (Grewal, Ehlers and Shapiro-Ilan, 2005) The *Tenecin* 1 inhibits the growth of *H. bacteriophora* nematodes resulting in inconsistent levels of infection with *T. molitor* (Kaya and Hara, 1981). In an attempt to maximise infection levels, Brown *et al*. (2006) deployed physical and chemical stressors to compromise the penetration sites of the hosts. The physical stressors such as dry heat and water treatments were used to enhance the susceptibility of *H. bacteriophora* nematodes to *T. molitor*. Additionally, chemical stressors such as manganese and magnesium ions also guaranteed high successful infection during the inoculation process (Brown, Shapiro-ilan and Gaugler, 2006). However, the downside of these manipulation processes was the high mortality rate that occurred among insects host (Zyl & Malan 2014a; Brown *et al*. 2006)).

Additional parameters that can often lead to unsuccessful inoculation among insect hosts include inoculum dosage and host density (Shapiro and Gaugler, 2002). Low inoculum concentrations result in low mortality rate among hosts while in excess often lead to failed infections due to increased competition from microbes hidden under the cuticles of the IJs (Shapiro-Ilan *et al.*, 2002). Gaugler *et. al.* 2002 , therefore, recommended that intermediate levels of inoculum concentrations should be used to maximize yield per host and reduce competition (Wang *et al.*, 2007). Selvan *et al*., (1993) also reported that host density determines the degree of patent infections of the host, and the ability to produce patent infections decreases as host density increases in all nematode-host combinations and *vice versa*. Low patent infections occur because as the density of insect hosts increases, there is competition for oxygen and the lack of it leads to the evolution of ammonia from hosts bodies (A. C. Van Zyl and Malan, 2014). Ammonia has been shown to hinder nematodes growth and development of nematodes thereby affecting final yields (Gaugler *et al.*, 2002a).

Researchers have employed various inoculation techniques over the years in the quest to achieve a high patent infection among hosts. These methods include pipetting, immersion, shaking, and spraying (Shapiro-Ilan *et al.*, 2002). Nonetheless, the efficiencies of these techniques inevitably depend on the type of host species under cultivation. Gaugler *et al*. 2000 suggested that pipetting method is inefficient when cultivating *S. carpocapsae* in *G. mellonella* due to the low infection levels. However, it produces satisfactory results with *Tenebrio* and *H. bacteriophora*, and it is impractical for large-scale inoculation of insect host as it is time-consuming and inefficient (Kaya, 1978; Shapiro-Ilan *et al.*, 2002). Non-infected hosts need to be manually removed from the bulk to avoid future contamination of the whole culture.

Inoculation by the immersion method is ideally employed during mass inoculation of hosts (Gaugler *et al.*, 2002a). The hosts are carried on a perforated tray and dipped in a suspension of nematode solution for approximately 5-10 seconds. This method is considered to be time-efficient in comparison to other conventional methods such as pipetting (Yang *et al.*, 1997). Gaugler *et al*. 2002 proposed that a single worker can employ this technique to inoculate (>2 x 10^5) insects in an hour. However, the downside of this approach is the total number of nematodes required to prepare the suspensions (unpublished data). Additionally, the immersion method is seemingly not suitable for some host-nematode combination such as *H. bacteriophora* and *T. molitor*, especially when a higher level of infection is needed (Shapiro-Ilan *et al.*, 2002).

The shaking method is similar to the immersion method with a container being used in place of a perforated tray. The insect hosts are collected into the container having the nematode suspension which is then agitated for 1 min to ensure an even coverage of the nematode suspension on the host's body. Although efficient, the agitation should be gently done to reduce the impact forces on the host body as it can result in the rupturing of the host (Shapiro-Ilan, Han and Dolinksi, 2012).

In inoculation by spraying, the nematode inoculum is delivered unto the host's body through spray bottles. However, *in vivo* mass-producers can employ this technique in large scale production where the inoculum can be pumped and distributed through a network of pipes above the hosts (Grewal, Ehlers and Shapiro-Ilan, 2005).

2.7.1.3. Temperature and Soil Conditions

Nematode yield and field efficacy can be well-affected by environmental conditions such as desiccation and extreme temperatures (Jagdale and Grewal, 2007). At extreme soil conditions, the rate of energy utilization and basal metabolism of the nematodes increase, and this affect their performance as biocontrol agents (Hussaini, 1994). Temperature can significantly reduce the mobility of the nematodes and often reduce their pathogenicity attributes towards preys (Brown *et al.*, 2002). However, IJs mitigate these effects by using the second-stage cuticles which are often retained by the IJs during moulting processes as a sheath (Ferreira, 2013).

In a comprehensive study of the effects of soil temperatures on the performance of nematodes, Georgis (1990) noted the optimal surviving temperatures for most species in an open field is between 5 ^{0}C and 15 ^{0}C, whiles their production temperatures at the laboratory is typically between 18 ^{0}C and 28 ^{0}C. It is, therefore, important to choose EPN candidates that have a close temperature correlation with their geographic origin and the field of application for maximum efficacy (Gaugler and Georgis, 1991).

In another study conducted by Radová (2010), it was shown that moisture levels of the soil could have adverse effects on the virulence and reproduction rate of nematodes. In one-on-one bioassays sampled in soil, the virulence of the *S. feltiae* tested against *T. molitor* host increased significantly with increasing humidity (Kaya and Gaugler, 1993). This is because the *S. feltiae* are cold tolerant species, able to show a high efficiency towards target pest mostly in cold temperate regions. In another study conducted by Kaya (2000, cited in Radová, 2010), it was shown that the effect of moisture level content of the soil and desiccation is more pronounced in *S. carpocapsae* strains

than *S. feltiae* strains due to their foraging behaviours. Most *Steinernema* species exhibit nictation by standing on their tails and this result in exposing about 90 % of their bodies to the atmosphere. At higher temperatures, nictation abilities of the nematodes are significantly reduced and therefore affects their field efficacy (Grewal *et al.*, 1994).

Other factors that could hinder the achievement of a higher yield during the culturing of nematodes in the laboratory and small scale cottage industries is the relative humidity in the humidity-controlled chamber. A high humidified environment should be kept throughout the production cycle as it directly affects the growth kinetics of the nematodes. In the LOTEK system of rearing tool, automatic misters are used to ensuring a relatively high humidity. However, uncontrolled misting cycle causes cadavers to be water-saturated thereby affecting adequate oxygen circulation. This situation is apparent when dealing with host species such as *Tenebrio*.

2.7.1.4. Mass Production status of In vivo

The status of mass production through *in vivo* over the years has been the adoption of large Petri dishes for cultivation. However, this system has been inefficient as it reduces the efficiency associated with the harvesting of the nematodes during emergence. Innovative ways have been proposed by Gaugler (2002) with the implementation of the LOTEK system. This system enhances the production route by maximising harvesting efficiencies whiles reducing the amount of labour work. One of the hallmarks of *in vivo* is the cumulative cost of production with increasing production capacity (Friedman, Gaugler and Kaya, 1990). More often, *in vivo* technology is constrained by space within the humidity-controlled unit (e.g. incubator). Conditioning of infected worms, which is one of the prerequisite steps for a successful cultivation is a major challenge for most *in vivo* producers. It has the advantage of reducing the culturing time as well as improving the productivity of the nematodes. The implementation of a technology that can mitigate the constraint of space is likely to contribute significantly to the advances towards mass-production through the *in vivo* culture. However, the practicality of this approached is yet to be investigated.

2.7.2. In vitro Production

The Glaser group reported the first record of mass production via the submerged liquid culture when a medium containing unsterilized raw kidney extract was used to culture *Steinernema glaseri*

in an axenic medium on a shaker (Smart and Sciences, 1995). Their approach proved successful, but the final yield was too low for industrial commercialization. The axenic medium lacked the symbiotic bacteria needed for growth and development by the nematodes (Sharma, Sharma and Hussaini, 2011). Moreover, the cost associated with producing the medium was too expensive for efficient replication on a large scale (Grewal, Ehlers and Shapiro-Ilan, 2005). Nonetheless, this approach paved the way for mass production of nematodes. Bedding (1981) also reported significant progress in mass culture when *Steinernema* spp. was cultivated on a porous foam substrate using a polyether-polyurethane sponge on homogenate kidney extract (ESolr *et al*. 2003; Ehlers 2001). Bedding (1981) approach opened up solid-state production of EPNs (Ehlers 2001).

Recent advances in *in vitro* technology have facilitated investigations into parameters such as medium compositions, bacterial and nematode inocula, process conditions (temperature, availability of oxygen and pH) that could impact on the successful production (Inman, Sunita and Holmes, 2012). On medium formulations, many authors have reported on different ingredients, but on the whole, an ideal medium contains protein, nitrogen, carbohydrate, and fat source (Abu Hatab and Gaugler, 2001; Chavarría-Hernández *et al.*, 2006). Abu Hatab *et al.* 1997 worked on the effects of different diet composition on *in-vitro* produced nematodes, *Heterorhabditis bacteriophora*. Friedman *et al*. 1997 also emphasized the importance of some important nutritional composition in a medium. In general, the nutritional composition of a medium affects the dynamics of the bacteria and the nematode partner, by dictating their growth kinetics and development in the medium (Chavarría-Hernández *et al.*, 2006). Additional investigations carried out by Bailey (1986) revealed that the absence of a particular component of the medium could have detrimental growth effect on the nematodes rather than the presence of other nutrients. Alexander (2011) affirmed the work of Bailey (1986) by studying the effects of various compositional nutrients (such as nitrogen and carbohydrate) play in the development of nematodes in liquid cultures. Hernandez *et al*. 2005 also produced *Steinernema carpocapsae* in four different monoxenic media containing different concentrations of nitrogen, carbohydrates, and fats using agitated orbital bottles. It resulted that carbohydrates sources contributed to a greater percentage of the total IJs recorded in the study (Chavarría-Hernández *et al.*, 2001). Their works proved vital as it opened up further investigations into liquid medium optimization.

Two production routes can be adopted for *in vitro* technology; the *in vitro* solid culture and *in vitro* liquid culture (Abu Hatab, Gaugler and Ehlers, 1998). The initial steps towards *in vitro* production (using solid or liquid) involve the establishment of a monoxenic culture, which includes the

isolation of the symbiotic bacteria through surface sterilization of the IJs; and the establishment of bacteria-free nematodes (Shapiro-ilan, Han and Qiu, 2014). Details on the procedures involved in the surface sterilization of IJs are provided by Akhurst (1983). However, improper sterilization procedures introduce contaminants into the culture as some bacteria were able to hide under the cuticles of nematodes during the sterilization process (San-Blas *et al.*, 2014). Monoxenic cultures are now preferably established by the axenisation of nematode eggs from adult females, which can be stored on shakers at 200 rpm and 4 ^{o}C until used, and strain collections can be kept in liquid nitrogen to prevent deterioration of beneficial qualities (Han & Ehlers 2001). Moreover, storing the bacteria cultures in glycerol or deep frozen at -80 ^{o}C maintain their purity and genetics (Bai *et al.*, 2004). During culture, the bacteria are thawed and propagated in TSB medium for 24 hrs before the addition of the nematodes (Ferreira, 2013).

2.7.2.1. In vitro: Solid Culture

Bedding (1981) developed a three-dimensional monoxenic system that utilized a sponge matrix foam (polyether polyurethane) coated with homogenate of animal offal for the culturing of *Neoaplectana* species in flasks, since the existing two-dimensional culture system (e.g. petri dishes) proved uneconomical for a large-scale venture (Bedding, 1981). The sponge matrix provided a large surface area for efficient air flow and ease of migration of the nematodes during the emergence of infective juveniles (Somwong and Petcharat, 2012). Autoclaving the polyurethane foam along with the medium before the inoculation of the bacteria and the nematode is critical to maintaining sterility of the whole culture (Friedman, Gaugler and Kaya, 1990). The IJs were harvested after 3-5 weeks of incubation at 23-25^{0}C by submerging the foam in a bowl of water (Ehlers, 2009). The emerging nematodes are trapped in the surrounding water where they are subsequently harvested.

Bedding's (1981) accomplishment in mass production provided a breakthrough for scale-up possibilities and the development of liquid cultures for commercial purposes. Higher yield was recorded in comparison to previous attempts (Bedding, 1981). Notwithstanding, further advancement in production regarding scale-up possibilities required the use of large autoclavable bags to replace the Erlenmeyer flasks initially utilised (Alexander, 2011). Compressed air was delivered into the bags through air hoses to provide sufficient air for the living nematodes over the incubation period (Gaugler, 2002). One major problem encountered with Bedding's (1981) mass-cultivation system was the cost of customizing specialized microbial filters for the fitting of each

autoclavable bag for both the inlet and outlet passive aeration (Gaugler, 2002). Additionally, the high cost of solid material (dog-food agar, liver, kidney, and lipid agar) and animal products required for formulation of the media made the solid culture expensive for replication on a large scale (Shapiro and Gaugler, 2002). Moreover, standardizing the media to maintain consistent results also proved challenging, as the yield varied from one batch to another. In an attempt to reduce media formulation cost, Wouts (1981) developed an improved culture medium that was less expensive and contained some building blocks such as yeast extract, nutrients broth, vegetable oil, and soy flour (Shapiro-ilan *et al*. 2014). Wouts (1981) formulation method attracted attention among early researchers as it was perceived as a trade-off between media cost and final yields of the nematode. This study, therefore, adopts the medium for the propagation of nematodes in liquid fermenters.

A key advantage that most solid cultures have over the submerged liquid substrate technology is the quality of nematodes produced (Yang *et al.*, 1997). Nematodes from solid cultures contain a higher level of lipids that ensures an efficient pathogenicity and longevity (Abu Hatab and Gaugler, 2001). The lipids serve as a stored food for the nematodes during adverse soil conditions like drought (Gaugler and Georgis, 1991). Gaugler *et al*., 1991 carried out investigations on the efficacy of *Steinernema carpocapsae* and *H. bacteriophora* nematodes produced via *in vivo*, *in vitro* on solid culture and *in vitro* in the liquid substrate for the field control of the Japanese beetle and black vine weevil. The nematodes from the *in vivo* and *in vitro* solid cultures achieved better results than *H. bacteriophora* produced from submerged liquid culture when a one-on-one bioassay was conducted with *G. mellonella* larvae. The poor performance of *Bacteriophora* towards the target pest was as a result of the low lipid content of the medium in which they were propagated (Abu Hatab and Gaugler, 2001). In another study on the effects of lipids on field performance of nematodes, Shapiro-Ilan (2001) suggested that the quality of the nematodes produced in liquid cultures is dependent on media composition, as the nematodes are more sensitive to lipid contents of the medium.

Bedding's (1981) three-dimensional monoxenic culture is still in use for the mass-production of nematodes in countries such as China by a company called Century Horse Development Limited, due to the low cost of labour and some advanced improvement in the production route (Shapiro-Ilan, Han and Dolinksi, 2012). Solid cultures offer the higher prospect of achieving economies of scale when compared to *in vivo* technology at the long run (Grewal, Ehlers and Shapiro-Ilan, 2005). However, they are more prone to contamination during the transfer of media into bags. This is

probably one of the reasons most producers prefer the *in vitro* liquid cultures to solid cultures due to low risk of contamination associated with the production route (McMullen II and Stock, 2014).

2.7.2.2. *In vitro*: *Submerged Liquid Culture*

For large scale production of EPNs, *in vitro* culturing using submerged liquid substrate is an appropriate technology (Friedman, Gaugler and Kaya, 1990). The nematodes are propagated in the presence of their symbiotic bacteria in a nutritive medium for 3-5 weeks depending on the type of nematode species, utilizing either shake flasks or large fermenters (Alexander, 2011). The bacteria are first isolated from the IJs through surface sterilization technique and cultured separately on nutrient agar plates for 3-4 days (Ferreira, 2013). They are subsequently pre-incubated into nutritive media (liquid culture medium) which that has been previously autoclaved previously at 121 ^{0}C (15 mins) in order to produce food signals for the recovery of IJs (Ehlers *et al.*, 1998). Once the bacteria have passed the exponential growth phase (mostly 30-36 hours after inoculation), the nematodes are inoculated and propagated for 11-17 days before harvesting (Kooliyottil *et al.*, 2013).

The recovery process is the capability of the infective juveniles to resume growth and development after a period of diapause, as a result of favourable conditions (San-Blas *et al.*, 2014). Ehlers (2002) stresses the role bacteria play in the recovery processes of the IJs due to the lack of precursors in artificial media to stimulate any significant recovery. As a consequence, conditioning by pre-incubation of the bacteria before the inoculation of the nematodes is pivotal for the success of liquid cultures (Inman, Sunita and Holmes, 2012). The bacteria can degrade the complex media into a minute and easily available components for efficient assimilation by nematode/bacteria complex (Poinar and Grewal, 2012). Furthermore, conditioning heightens the amount of food signals secreted by the bacteria in the medium for a high recovery of the infective juveniles (Kooliyottil *et al.*, 2013). Unlike *in vivo* cultures, where there is a complete recovery of infective juveniles, the recovery rates in liquid cultures are mostly variable, typically between 18-90% (Ehlers, 2009). Recovery process impacts on the success of culture, and factors that trigger the full recovery in *in vitro* cultures remained under-explored (Johnigk, 2004).

After the cultivation process is over, the nematodes are cleaned and either stored or formulated using appropriate ingredients. Storage could be done in flat rectangular flask containing distilled water and can last up to 2 weeks at 14^{0}C (Kagimu, 2015) (unpublished). Oxygen is provided to

the nematodes by a means of sparger connected to a compressor. A summary of the production route is illustrated in Figure 2-5 below.

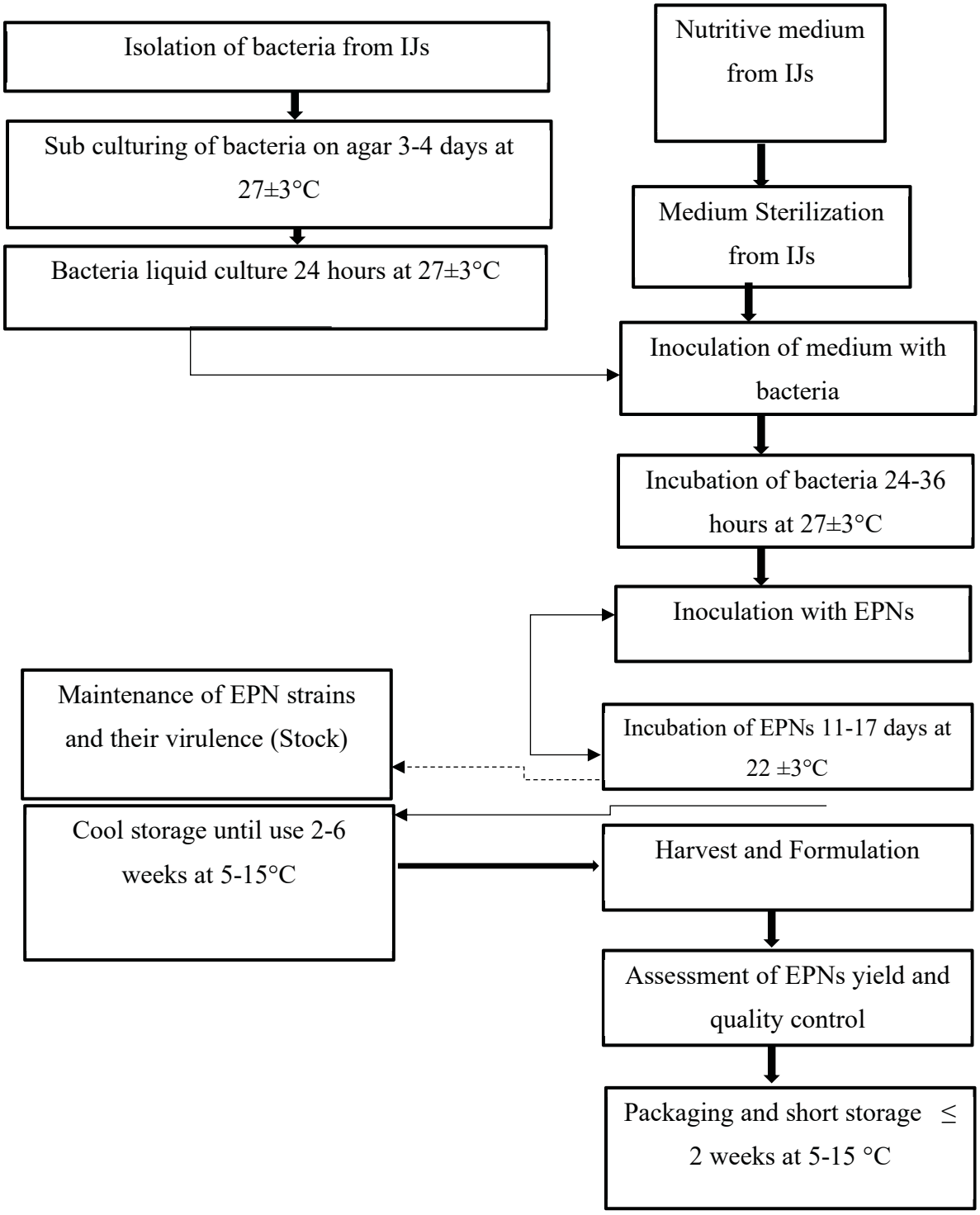

Figure 2-5: Schematic presentation of *in vitro* culture

[Redrawn from (Holmes *et al.*, 2015)**]**

2.7.2.3. Factors Affecting Commercial Production of in vitro

Factors influencing the successful commercialization of *in vitro* technology have been explored in various studies in areas such as recovery, process type, medium composition, reactor type, process variables, and inoculum cell concentration. These are necessary factors to consider for reliable and consistent nematode production.

2.7.2.4. Recovery of IJs

The success of liquid cultures is dependent on the rate of recovery of the inoculated IJs (Ehlers, 2009). In *Steinernematids,* the percentage of recovery determines the number of first females and males available for copulation activities and subsequent reproduction of filial generations (Grewal, Ehlers and Shapiro-Ilan, 2005). Nevertheless, variable recovery rates prolong culturing time and eventually affect process stability (e.g. low yields) (Johnigk, 2004). Ehlers (1998) suggested that the IJs react differently to food signals produced by the bacteria and that results in variable recovery among IJs. The bacteria only activate this signal upon entering the late logarithm growth phase during culturing (Inman, Sunita and Holmes, 2012). In an attempt to investigate factors that induce recovery of infective juveniles of entomopathogenic nematodes, Jessen *et al*. (2000) studied the effects of co-factors such as carbon dioxide on recovery rates during fermentations. They noted that various thresholds of carbon dioxide concentrations could trigger the recovery of IJs due to its synergistic effects. Eventually, they hypothesized that carbon dioxide enhances the quality and quantity of food signals produce by the bacteria in the media to help trigger a high recovery of *Heterorhabditis* nematodes in liquid cultures. Nonetheless, at higher concentrations of carbon dioxide, a significant decline in the response variable is frequently recorded. Secondly, Strauch and Elhers (1998) proposed that phase variation of the bacteria from the desired phase I to the undesirable phase II during cultures frequently lead to low recovery rates among IJs and impacts on final yields.

2.7.2.5. Fermentation Mode (Process Type)

Many authors (Jeffke *et al.*, 2000; Gil, 2002; Lim and Shin, 2013) have proposed numerous process modes for the production of EPNs, i.e. batch, fed-Batch and continuous. In batch fermentation processes, also referred to as the traditional/conventional cultures, the bacteria and nematode cell substrates are fed into the flasks or reactor vessels without the addition of further substrates to the fermenter (Alexander, 2011). The fermentation is then allowed to prolong until the development

of nematodes is over. Alexander (2011) noted that EPNs are known to grow faster under favourable conditions, especially in fermenters, and this results in a quick depletion of the symbiotic bacteria. As a consequence, batch cultures were subsequently moved to fed-batch cultures by the addition of new bacteria feed to the medium every 4 to 7 days during the 25 day fermentation period to make enough food available for the nematodes to sustain their growth. For continuous fermentation systems, samples of the medium are aseptically withdrawn while continuously adding a fresh feed to the existing fermentation broth (Lim and Shin, 2013). Alexander (2011) identified fed-batch systems to be effective than batch cultures due to high biomass achieved at the end of cultivation period.

2.7.2.6. Media Formulation

The nutritional demands of nematodes can be manipulated to optimize for higher yields (Abu Hatab, Gaugler and Ehlers, 1998). Many researchers (Abu Hatab, Gaugler and Ehlers, 1998; Chavarría-Hernández *et al.*, 2006) have directed efforts towards maximizing the production rates of nematodes by studying their nutritional requirements in liquid cultures. Moreover, different research groups have extensively studied the impact of some key nutrients such as carbon compounds, nitrogen, phosphorus, fat and oil on the growth and metabolism of the nematode/bacteria complexes (Islas-López *et al.*, 2005). Depending on the medium composition, some media can maintain the growth of the bacteria and the nematodes for longer periods than others (Chavarría-Hernández *et al.*, 2006). Alexander (2011) studied the abilities of different media to sustain the rapid development of bacteria by propagating *Photorhabdus* strain in liquid culture medium (LCM), egg yolk, lipid broth, and nutrients broth. There were remarkable differences in the cell densities of the bacteria and the nematodes in each of the medium tested. Abu Hatab and Gaugler (2001) also cultured *Heterorhabditis bacteriophora* in artificial liquid media containing different lipid sources (insect, beef or lard). They studied the effects the quality and quantity of lipid content in a medium can have on the production time, the yield and the final lipid contents of the nematode produced. The results showed that the medium supplemented with insect lipids recorded the highest population of nematodes compared to the medium supplemented with beef or lard. Additionally, the developmental rate of nematodes in the insect lipid medium was 1.7 times faster than the medium supplemented with beef or lard. All these studies emphasize the importance of finding the appropriate media for the propagation of the nematodes/bacteria complexes in order to obtain a higher yield. In formulating the nutritional compositions of liquid

cultures, the rationale is to mimic the natural conditions in the natural host so as to induce a higher recovery of the IJs (Hatab and Gaugler, 1997). In another study, Jeffke *et al*. 2000 investigated fed-batch cultures with glucose as a supplementary substrate to increase the bacteria cell density. The bacteria are prone to shift phase under poor cultivation conditions, such as low quality of the media in which they are propagated. However, the addition of the glucose at periodic intervals to the existing culture increased the cell biomass of the EPNs as there was abundant food available for the progeny to feed on (Jeffke *et al.*, 2000).

2.7.2.7. Reactor Types and Configuration

De La Torre (2003) reported that nematode propagation in pneumatic or mechanically agitated reactors increases the hydrodynamic forces in reactors. Higher inertial forces often lead to high turbulence as a result of the increased in Reynolds number (Re); consequently impairing the insemination cycles between adult male and female nematodes (Chavarría-Hernández *et al.*, 2011). This situation is mostly predominant in *Steinernema* male and female nematodes that often coil around each other during mating processes (Gaugler and Bilgrami, 2004). Contrarily, Ehlers *et al*., 2005 suggested that agitation and aeration scarcely affect the copulation activities of *Heterorhabditids* species in pneumatic or mechanically agitated reactors as the species are automictic (self-fertilizing). However, Pace (1986, cited in Johnigk *et al*. 2004) reported that shear stress from the tips of impellers (such as flat blades) cause the disruption of adult females in fermenters. They recommended that flat blade impellers should be replaced with Rushton impellers or the velocity of impellers should be reduced below 0.3 m/s to mitigate the effects of shear forces on nematodes, especially, the female nematodes that are more susceptible to shearing effects (De La Torre and Torre, 2003)

Additionally, past literature works in nematology have rarely discussed the hydrodynamic conditions that exist in nematode broths in fermenters. In an attempt to bridge this gap in knowledge, Nerves *et al*. 2011 studied the hydrodynamic forces that frequently occur in reactors during nematodes propagation. Their investigations revealed the extent at which higher inertial forces exerted by aeration and agitation strongly determine the distribution pattern of adult male and female nematodes in the reactor. At certain air flow rates, they observed a biased distribution of the latter in different zones of the external-loop reactor used. Alexander (2011) also made a similar observation when he compared an external recycle reactor with a standard fermenter during the cultivation of *Steinernema* species. The recycle loop section of the external-loop reactor, which

characterized a low liquid velocity zone, promoted sexual contact between the males and female nematodes, consequently recording a higher number of nematodes, particularly in that zone. It is, therefore, important to design reactors that offer a non-homogenous distribution pattern of nematodes and also reduce the hydrodynamic stress conditions that occur within them, as geometric differences between reactors affect yield (Chavarría-Hernández *et al.*, 2011).

2.7.2.8. Process variables

Temperature, pH, and dissolved oxygen are critical process parameters that may disturb the final EPN yields of a culture if monitoring and controlling tasks are neglected throughout the cultivation period (Chavarría-Hernández *et al.*, 2011). The optimal temperature is defined from the onset of production since a slight deviation from the optimal can affect production figures (Grewal, Ehlers and Shapiro-Ilan, 2005). The temperature for the incubation of the symbiotic bacteria is often different from that of the nematodes (generally between the temperatures of 28 ºC - 30 ºC depending on the strain.) However, the temperature is adjusted to 23 ºC-25ºC before the inoculation of the nematodes into the fermenter (Richou Han, 1993).

Like any bioprocess operation in fermenters, the dissolved oxygen content is dependent on the rate of agitation, airflow rate and the apparent viscosity of the culture broth (Garcia-Ochoa *et al.*, 2010). Moreover, the rheological behaviour of most fermentation broths change from dilatant to pseudoplastic with time, and this affects the oxygen transfer efficiency in the culture broth (Medina-torres *et al.*, 2007). Before the onset of culturing, the understanding of the specific oxygen demand of nematodes at various stages of their life cycle is essential for successful cultivation and future scale-up processes (Junker, 2004). Chavaria-Harnandez *et al*. (2014) studied the oxygen consumption of *Steinernema* nematodes with its corresponding symbiotic bacteria in a submerged liquid cultures. The study showed the mean oxygen consumption of the bacteria and the nematode is highest during the logarithm growth phase where the population of the infective stage 2 (J2) and infective stage 3 (J3) have reached a maximum in the broth. Conversely, oxygen consumption is minimal when the nematodes enter the stationary and death phases since their metabolism and growth rates are greatly reduced (Ferreira, 2013).

The pH of a medium progressively increases in the time-course of culturing due to the active metabolisms of the nematodes and their bacterial partner (Inman, Sunita and Holmes, 2012). Various researchers over the years have expressed diverse opinions on impacts of pH on the growth and final yields of the nematodes. Stoll (1952) noted that nematodes are most productive between

a pH of 6-6.5. Essen *et al*. 2000 also suggested that the effects of pH of culture broth on nematodes during cultivation can be neglected since the bacteria can metabolize the various components of the medium to provide a conducive growth environment for their survival and reproduction. Notwithstanding the diverse opinions of pH effects, both authors acknowledge that pH can be used as an online monitor to ascertain the health status of the culture since it affects the phase stability of the bacteria culture (Jessen *et al.*, 2000). The higher the pH of the medium, the higher the rate of mortality among the nematodes and bacteria Nonetheless, it is highly recommended that a buffer medium (pH 6-7.3) be ensured before the inoculation of the bacteria so as to prevent any phase changes (Richou Han, 1993).

2.7.2.9. Inoculum cell concentration

Studies into the optimal nematode concentrations have been investigated to establish the optimal nematodes and bacteria densities needed to ensure a significant and higher yields of EPNs. The bacteria serve as food for the nematode, and their concentration significantly reduces after the first two days of nematode inoculation due to recovery processes of the IJs (Shapiro and Gaugler, 2002). However, the cell density of the bacteria before inoculation can influence the growth dynamics of the nematodes as well as the success of the culture (Ehlers, 2009). Ehlers *et al.* 2009 studied the effects of different cell density of bacterial cells on IJs recovery in liquid cultures. Cell concentrations of $1x10^{10}$ ml^{-1}, $1x10^{9}$ $ml^{-1,}$ and $1x10^{8}$ ml^{-1} were investigated and their influence on recovery was examined. The results showed that the medium containing a higher bacterial cell density (10^{10} ml^{-1}) positively affected the time scale of the IJs recovery as well as their development to reproductive adults and *vice versa*. Moreover, Hirao *et al.* 2009 also suggested that initiating cultures by inoculating higher density of bacterial cells into the medium eventually lead to higher yields. However, this should be compensated for by inoculating a higher number of nematodes. This resulted in another study by Hirao *et al.* 2009 on the influence of initial nematode concentrations on the population dynamics of nematodes in liquid cultures. In theory, an initial nematode inoculum size should determine the number of parental females available in liquid cultures after recovery, as the lack can affect the final yields of the culture (Richou Han, 1993). Cultures containing high-density female nematodes have an increased level of fecundity with the likelihood that most of the IJs would occur within the first generation of filial nematodes (Hirao and Ehlers, 2010). One life cycle culture of the nematode is often desirable in cultures as compared to those that undergo two or more generational cycles before completion (Gaugler and Bilgrami,

2004). This shortens a process time of the culture, which is often advantageous in terms of reducing the probabilities of eventual contamination and high operating costs (Gil, 2002).

In order to understand the effects of initial nematode and bacterial densities on the final yields of culture, Han *et al*. (1993) observed an increase in final nematode biomass at higher densities of both nematode and bacteria inocula. However, they cautioned that the effects of a higher initial concentration of the former may not always lead to a high nematode population at the end for some nematode strains (such as S. *feltiae* and *S. carpocapsae*). The final yields of these species showed a negative response to increasing inocula size due to initial overcrowding of nematode population in the fermenter, and small inocula sizes tend to favour their maximum growth. Nonetheless, a population density of 3-6 x 10^3 nematodes per ml with a higher percentage of parental females (> 10^3) was recommended for inoculation to achieve positive results (Richou Han, 1993).

In general, on the subject of volumetric quantifications, the bacteria and nematode inocula constitute 0.5-1% and 10% of the total volume of the reactor respectively for all nematode species (Shapiro and Gaugler, 2002).

2.7.2.10. Contamination

In nematode liquid cultures, contamination occurs when bacteria hide under the cuticles of the IJs and surface sterilization processes are ineffectively done (Bedding, 1981). The foreign microbes compete with the nematode and the bacteria for the limited supply of oxygen in the flask or bioreactor (Grewal, Ehlers and Shapiro-Ilan, 2005)**.** This competition impacts on culture and leads to poor yields and downtime. However, recent works suggest that the washing the IJs with a solution of sodium hypochlorite during sterilization processes can reduce the growth of microbes and prolong the stability of the culture (Han & Ehlers 2001). Contamination can as well occur in nematode formulated products when they contain non-IJ stages, dead nematodes and debris, due to inefficient separation processes (ESolr, Gómez and Sánchez, 2003). Nonetheless, the addition of an antimicrobial agent (such as ampicillin) to the formulation ingredients mitigates the growth

of other microbes (Connick *et al.*, 1994). More importantly, housekeeping practises should be ensured around the surroundings to reduce potential contamination (sterile environment).

2.8. Comparison of Production Methods

Table 2-7 below summarizes the advantages and disadvantages associated with each production technology during the production of nematodes. *In vivo* offers several advantages regarding capital outlay, quality of epns and low-level expertise requirement. The *in vitro* solid is considered as an intermediate between the *in vivo* and *in vitro*- liquid

Table 2-7: Comparison of Production Methods

	Production Approach		
Issue	***In vivo***	***In vitro*-solid**	***In vitro*-liquid**
Capital outlay	Low	Intermediate	High
Required expertise	Nominal	Intermediate	Extensive
Ease of achieving quality	Easy	Difficult	Difficult
Labour required	High	Intermediate	Low
Economy of scale	Low	Intermediate	High
Ease of adaptation to new species	Easy	Difficult	Difficult

Source: Shapiro-ilan *et al*. 2014

2.9. Downstream Processing of Culture Broth

2.9.1. Separation Techniques/ Harvesting

Unlike *in vivo* production where the separation of IJs from harvested nematode-water suspensions is relatively easy, separation processes for *in vitro* cultures remains a bottleneck for most nematode producers due to the nature of broths after fermentation. The characteristics of the complex fermentation broth which usually contains different components including non-IJs stages, bacteria, cuticles and dead nematodes make it difficult to use a simple filtration technique to separate the viable IJs from the rest (Young, Dunnill and Pearce, 1998). The complex media contain oils, insoluble solids and waste materials that rule out conventional separation techniques including dead-end filtration. Preferably, a centrifuge could provide the best separation technique, although, the cost does not warrant the investment for niche or small-scale nematode producers. For *small-scale nematode* production, Pearce *et al.* 2002 proposed some conventional separation techniques

such as dead-end filtration, flotation, decantation, sedimentation and gravity settling for the recovery of the IJs from other life stages of nematodes in the fermentation broth. Occasionally, an appropriate separation method may depend on the physical characteristics of the particulate solids in the broth such as their density and settling rate. Although most niche producers employ filtration technique as the most viable option for the harvesting of nematodes, Pearce (2002) observed a constant blocking of the filters as a result of fine particles arising from the compositions of the medium, thereby making filtration inefficient when adopted for large scale harvesting of nematodes. For a large scale harvesting of nematodes, Surrey and Davies (1996) achieved a successful separation of IJs from culture broth during the production of *H. bacteriophora* nematodes using scroll and basket centrifuges. However, the viability of the nematodes was affected due to shear forces exerted by the centrifugal forces on the equipment. Notwithstanding, large producers of nematodes such as Biosys, use horizontal scroll decanters for the harvesting of several species of nematodes by manipulating the stress regimes to reduce the impact of shear forces during operations.

Wilson *et al*. 2003 reported a Vibrating Membrane Filter (VMF) apparatus for the recovery and concentration of viable IJs from spent media. This equipment works on the principles of oscillatory motion provided by a stack of circular disk filters. One problem reported so far concerning its operation is the frequent fouling of the vibrating membrane caused by fine particles of which needs to be replaced

Notwithstanding the method used for the production of the nematodes, separation steps are more often difficult when there are non-synchronous developmental stages within the nematode population (Ehlers, 2009). As such, producers determine the success of a culture by the proportion of infective juveniles per volume of media in comparison to other non-IJ stages.

Figure 2-6 below illustrates the general protocol for the harvesting of nematodes in commercial scale production using a centrifuge

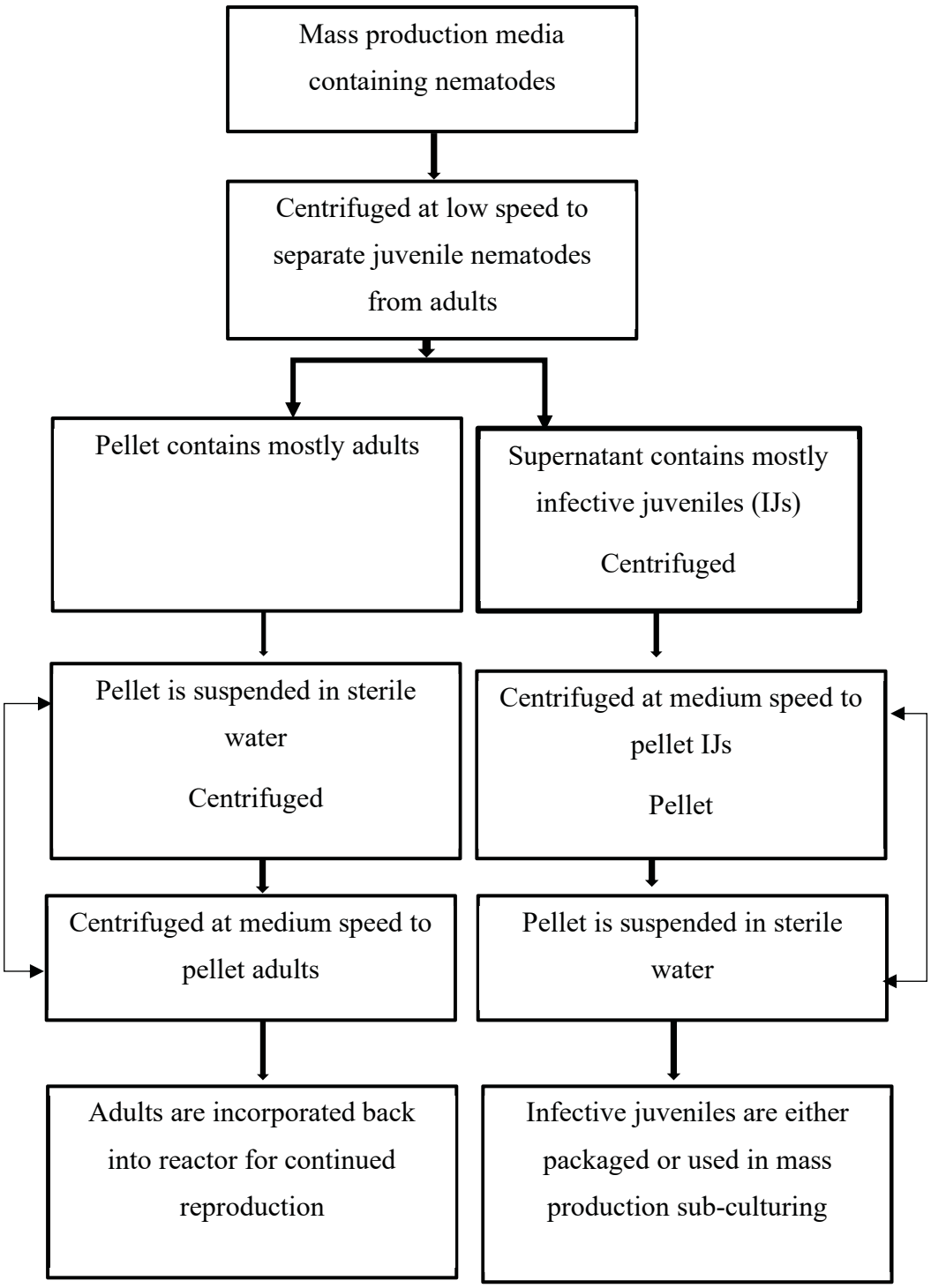

Figure 2-6: Schematic presentation of separation techniques of nematodes

[Redrawn from (Upadhyay *et al.*, 2013b)**]**

2.9.2. Storage of nematode

The nematode and the bacteria strains can be stored for future sub-culturing practices through cryopreservation technique (Bai *et al.*, 2004). The harvested nematodes may be retained in distilled water in flat bottles with adequate aeration at 5-14 ^{0}C for at most 2-3 weeks (Holmes *et al.*, 2015). Kagimu (2015) (unpublished) designed a storage system that allowed the storage of nematodes up to 2-3 weeks. Storing nematodes in a less oxygenated environment results in a high mortality rate due to the deprivation of oxygen. Oxygen is delivered to the storage tank by means of a sparger connected to a compressor. The bacteria stock is often stored at -80 ^{0}C to preserve desirable characteristics such as virulence and pathogenicity. However, to maintain the genetic stability and prevent strain deterioration of the nematode stocks, they are preserved in liquid nitrogen at -196 ^{0}C (Chen and Dickson, 2004; Gaugler and Lewis, 2004)

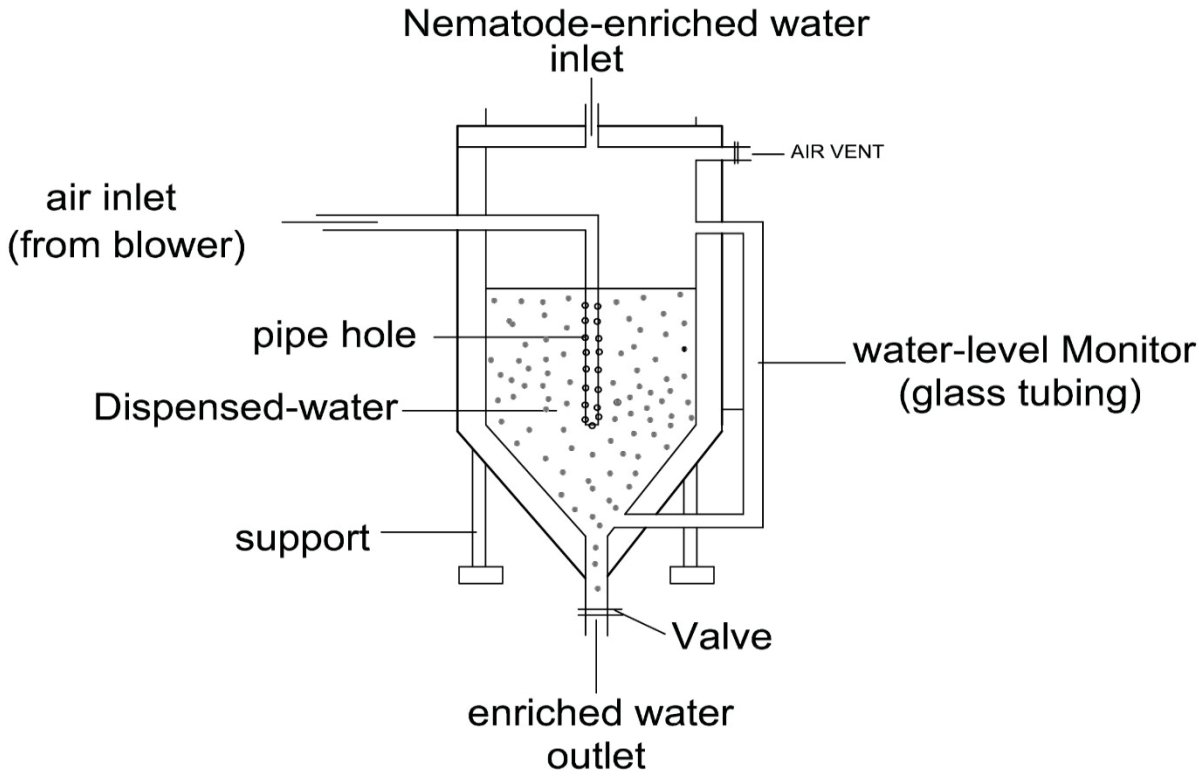

Figure 2-7 Aerated tank for nematode storage

[Redrawn from (Kagimu, 2015) **unpublished]**

2.10. Costing Models

The most relevant costing parameters of interest to potential investors are the capital and operating expenditures during the lifespan of a project. These costing models give investors an idea of the monetary investment needed to fund the project, and the rate at which they would have their investment yield margins at the end of the project life. (Towler and Sinnott, 2008)

For most generic solid-liquid processing plants, the cost of major equipment is estimated from past literature data and projected quotes from vendors. However, the costs of specific equipment are often scaled using an appropriate scale factor obtained from the Chemical Engineering Plant Cost Index to reflect their current price listing. Installation factors are obtained from (Towler and Sinnott, 2008) to evaluate the installed costs of equipment and capital cost expenditure separately. Working capital is often assumed as 15% of fixed capital expenditure for most projects (Towler and Sinnott, 2008).

In *in vitro* nematode production, the major cost includes the cost of establishing the nematode and bacteria inocula, labour, formulation ingredients, packaging, utilities, and other manufacturing costs.(Gaugler, 2002). However, the cost of insect and labour constitute ~90% of the total cost of production for *in vivo* technology. (Shapiro and Gaugler, 2002) The estimation of major equipment for a typical processing plant is summarized in Table 2-8 below

2.9.1. Economic Evaluations of a Project

Before making any investment into a project, a potential investor evaluates the attractiveness of the project to other alternatives such as the interest rate from financial institutions. Engineers perform a series cash flow analysis over the lifespan of the project to establish the soundness or viability of the investment (Towler and Sinnott, 2008). Before the generation of any profit margin for any project, money flows out of the company to settle debts accrued over the Fixed Capital Investments (FCI) such as building, land and equipment erection. Figure 2-8 below illustrates a typical cash flow diagram. The different regions highlight the trends of cash flow excluding taxes and depreciation during the lifespan of the project. Cash flow analysis gives the potential investors an idea of the time value of their investment [(often referred as Discounted Cash Flow Rate of Return (DCFROR)]. DCFROR is an evaluation method used to estimate the attractiveness of a project. The analysis uses free cash flow projections and discounts them at a specific interest rate to arrive at its enterprise value, which is then used to evaluate the potential for investment

Other economic indicators that influence the decision making of a project was briefly discussed by Lauer (2008) which include: Net Present Value (NPV) and Internal Rate of Return (IRR). The NPV value indicates the return on the investment over the lifetime of the project at a specified discount rate (Leibbrandt, 2010).The Total net flow cash flows for each year is discounted to present value which are then summed together and subtracted from the total Capital Investment (TCI). A positive NPV implies the project is worth investing whereas a negative value considers it not feasible at that discounted rate. The DCFROR value represents the maximum interest rate investors could pay and still break even at the end of the project, and it is dependent on the type of project under consideration (Towler and Sinnott, 2008).

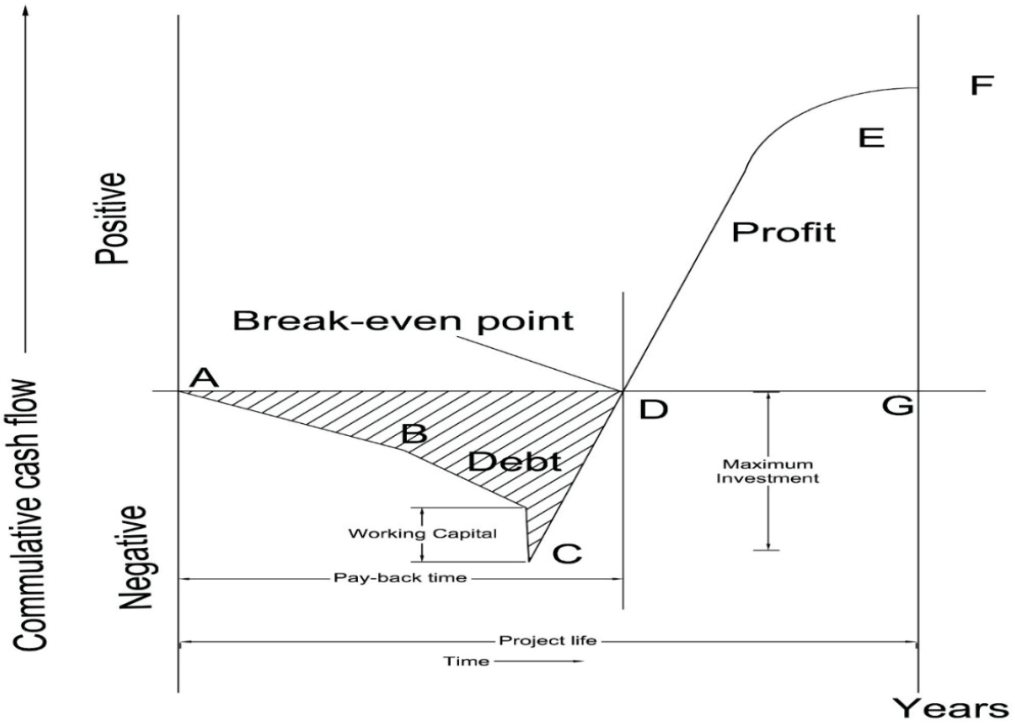

Figure 2-8: Project Cash flow diagram

[Redrawn from (Towler & Sinnot 2008)]

Table 2-8: Estimate of major equipment cost for a typical processing plant

	Cost Factor
FIXED CAPITAL COST C_{FC}	
Major equipment, total purchase cost	C_e
Equipment erection	0.5
Piping	0.6
Instrumentation and control	0.3
Electrical	0.2
Civil	0.3
Structures and buildings	0.2
Lagging and paint	0.1
Design and Engineering (D&E)	0.25
Contingency (X)	0.1
Total fixed capital cost $C_{FC} = C\ (1 + OS)(1 + DE + X)$	
ANNUAL OPERATING COST	
C_{FC} –Dependent Items	
Depreciation	
Maintenance Material	
Insurance	(0.01 * C_{FC})
Local Taxes	(0.02 * C_{FC})
Factory Expense	(0.05 * C_{FC})
Total	
Labour-Dependent Items	
a. Operating Labour	(Working hours * rate *
b. Maintenance Labour	(summed over all units)
c. Supervision	0.20 * (a + b)
d. Operating Supplies	(0.10 * a)
e. Laboratory	(0.15 * a)
Total	
Administration And Overhead Expense	0.6 * (a + b + c)
Raw Materials	
Other Consumables	
Utilities	
Waste	
Total Annual Operating Cost	
Source: (Towler and Sinnott, 2008)	

Table 2-9: Summary of the different phases of a project

Region	Characteristics of regions
A-B	Investment needed for the designing of the plant
B-C	Capital inputs for building of plant and maintaining operations (working capital)
C-D	Flow of revenue from sales
	Net cash flow remains positive
	Accumulation cash flow remains negative until the initial investment is paid off
D	Break-even point: the total sales at this stage are equal to the total expenditure. The time to reach this phase is referred to as the pay-back time.
D-E	Accumulative cash flow becomes positive as the investment is paid off. The plant is generating profits at this stage.
E-F	The cost of operations is not matching up with the total revenues being generated due to the low production volume of the plant and high cost of operations. This phase indicates the end life of the project.
F	The cumulative net cash flow at the end of the business life

Source : Towler & Sinnott 2008

2.11. Summary of Literature Findings

2.10.1. *In vivo* Technology

The prevalent lack of interest in *in vivo* technology by producers, investors and more importantly universities has resulted in a lack of innovation to possibly arrest the bottleneck in areas such as commercialization, harvesting and concentration procedures. Earlier preference by researchers towards the *in vitro* methodology limited any remarkable studies into *in vivo*, as the *in vitro* technology was conceived as the future of nematology. Notwithstanding, the *in vivo* technology has shown an immense growth rate over the past two decades, and it does not seem to be disappearing anytime soon. More *in vivo* companies are now being established along with innovative ways, in an attempt to increase capacity and shrink the high cost associated with the production methodology.

Noted challenges of the *in vivo* technology include the high cost of production, low yields, and high input of manual labour. Thus, the process is often limited to nematode producers with low capital investment and research institution for academic purposes. However, mechanization interventions have been suggested as an alternative approach to improve the efficiency by reducing the amount of manual labour involved in the production route. Gaugler (2002) proposed a rearing tool, LOTEK, that automates most of the manual operations, predominantly, the harvesting of the nematodes. Gravity flow of water harvests the emerging IJs from the perforated trays that carry the cadavers into a central storage tank for further downstream processing. Cultivation processes such as inoculation and incubation follow the same procedures as the White traps albeit with slight modifications. Notwithstanding, the Gaugler (2000) design is often constrained by space needed for the conditioning (such as incubator) of the infected worms, making it difficult to accommodate a large number of trays. Therefore based on the need for larger space for cultivation, part of this study proposes the adoption of the LOTEK system for replication in a room based on the recommendation of Holmes (2015). This proposal would be referred to as the 'room technology' in subsequent chapters. Although extensive studies were conducted by Gaugler (2002) to establish the potential of the LOTEK system to scale-up operations, limited information is however available to ascertain the cost implications of implementing such design in South Africa with regards to scale-up operations. In order to assess the feasibility of the LOTEK system before replicating on a larger scale demands series of cultivations of nematodes on the LOTEK system. The LOTEK system ensures a higher harvesting efficiency as compared to the traditional system White trap system as well as reducing the amount of labour involved in the production route (Brown and Gaugler, 2004)

Moreover, Holmes *et al*. 2015, recommended that *in vivo* producers should consider a room technology for commercialization. In this study, a full economic model based on room technology is developed to ascertain the feasibility of Holmes (2015) recommendation.

2.10.2. *In vitro* Technology

With i*n vitro* cultures, due to the variability and inconsistencies of nematode yields, the development of a stable cultivation process is warranted for a successful commercial venture. Culturing practices that can lead to the high biomass of IJs at the least cost of media should be investigated and implemented. Since the model organism (*S. yirgalemense*) is newly identified in South Africa, it is important to investigate factors that can hinder the development of a stable

bioprocess and leads to the achievement of quality and higher yields. Over the years, many researchers have investigated a range of fed-batch processes on *Heterorhabditis* strains but invariably little work on *Steinernema*tids strains. Using glucose and bacteria as feed pointers, they recorded an increased in the final concentration of the *Heterorhabditis* nematode. The experiment described in this thesis (Chapter 4) entails the possibility of cultivating *Steinernema yirgalemense* via two different fermentation systems, the batch and fed-batch, and their effects on the growth of the newly bacto-helminthic nematode complexes. Identified in South Africa

2.10.3. Economics Status of Nematode Technology

Notwithstanding the dearth of information on nematology, its production efficiency is however limited to studies such as media optimization, bioreactors design, optimization of operating parameters and elucidation of nematode/bacteria symbiosis. Technical information about cost evaluations on EPN production such as capital and operational expenditures remain under-explored, with insufficient data currently available for potential investors. Most researchers often make the assertion that, '*in vivo* or *in vitro* technology lacks or achieves some economies of scale'' without the backing of actual experimental data and figures to substantiate these claims. Even with the limited data available, they are inherently proprietary and not opened- source due to trade secrets and special competitive advantage each company wants to have over the other.

To help in this regard, the challenge is to develop economic models currently unavailable for full-scale implementation of nematode biopesticides. These models are developed to analyse the cash flows in nematode technologies using the yields achieved in this current study and benchmarked the results with past researchers. The best-case scenario would be used as a preferred route to develop the economic models. This study would provide hands-on information for potential investors willing to enter into nematode production.

Chapter 3

Experimental Production of EPNs with the In vivo Method

The objective of this Chapter was to adapt and construct the LOTEK system proposed by Gaugler *et al*. 2002, to allow a basic series of EPN production tests with the *in vivo* cultivation method. It was therefore aimed at demonstrating the functionality of the LOTEK system by moving production from petri dishes to an industrial level, and measuring the production yields that could be achieved with this set-up. The technical data from the laboratory trials will be used for the basic design of industrial facilities, allowing development of economic models to predict the comparative cost of production for the two production processes (*in vivo* and *in vitro*)

3.1 Introduction

In vivo pathway of producing nematodes involves the culturing of a specific strain of entomopathogenic nematode in the body of an insect host (Peters, 1996). In the laboratory and cottage industries, nematode producers rely on a two-dimensional system, the Whites trap system, for the production of the nematodes in petri dishes (90mm), shelves, and trays (White, 1927).

EPNs show positive directional responses to water, as such, nematode infected hosts are placed in the petri dishes, lined with filter paper and then placed on top of a water reservoir (Gaugler *et al.*, 2002a). The emerging infective juveniles migrate from the host cadaver into the water reservoir surrounding the dish in response to unfavorable conditions such as depletion of nutrients or overcrowding within the host body (Selvan, Campbell and Gaugler, 1993). The harvested nematodes are then collected from the water by filtration using 45 µm sieve, and total nematode yield is assessed (Gaugler *et al.*, 2002b). The White trap system is, therefore, is a low-scale approach of producing nematodes.

An attempt to scale-up production to industrial levels may involve the provision of large petri dishes; however, the diameter of the dishes cannot be increased beyond 150 mm as a result of the lateral migration of the emerging nematodes needed (Gaugler *et al.*, 2002b). Carne and Reed

(1964) also described an alternative apparatus where host cadavers were placed on perforated disks resting in the mouth of a large funnel for the emerging nematodes to migrate to the bottom. Nematodes are then collected by the opening a stopcock (Gaugler *et al.*, 2002b). Although this approach was an improvement upon the White trap system, it was considered as a crude approach of harvesting the nematode, as they were often deprived of oxygen for a long time before the cork is opened. This crude aspect of their apparatus contributed to the design not being adopted for nematode production on a commercial scale. Conversely, in an attempt to scale up *in vivo* production, Gaugler *et al*. (2002) described an automated harvesting equipment (LOTEK) where emerging nematodes are washed with misting water into a central bulk storage tank. This system increases the efficiency of harvesting by mitigating the need for lateral migration of the nematodes unlike the design of Carne *et al* (1964). The LOTEK system was therefore adopted for the series of *in vivo* cultivations in the present study, using *G mellonella* as fictitious hosts, to ascertain the functionality of the system to mass-production (Figures 3-2 and 3-3).

3.2 Materials and Methods

3.2.1. Mechanical Design of LOTEK

The design of the LOTEK was based on the four main steps involved in *in vivo* production route:

1. Inoculation of host organisms with infective juvenile nematodes
2. Incubation of the infected hosts
3. Harvesting of nematodes from depleted cadavers.
4. Collecting and concentrating of harvested nematodes

Incubation after inoculation is done at room temperature for 2-5 days before infected hosts are transferred into a humidity controlled incubator. Non-toxic materials such as aluminium, stainless steel, plastics and non-metallic objects are mostly the preferred materials for the construction of this unit as they pose no potential contamination to the nematodes. A brief description of the embodiment of the system as described by Gaugler (2002) is given below (Figure 3-2):

The unit consists of a perforated tray system, an inner collection tray, support base, and piping system. All these components are sized to fit into the open area within the humidity controlled incubator.

a) **The Perforated Tray system**

The perforated tray system carries the insect hosts during inoculation, incubation, and washing. It is a rectangular flat bottom piece with perforations that hold the insect host cadaver while allowing the passage of nematodes during washing. Perforation sizes of 1.6 mm are often considered in this design, as it permits the passage of the nematodes during emergence. The trays are constructed to be reusable and to offer easy cleaning options with materials such as aluminium used. The dimensions of the trays in this study (30 x 26 x 4 cm) allowed much better space of accommodating about 200 g (approximately 800 of insect hosts) per tray. In total, four trays could fit into the incubator with inner dimensions of the incubator (60x50x50 cm; volume of 0.15m^3) in this study. The trays are seated on metal rods projecting from the general support base of the unit. The sides of the perforated trays are made a little bit longer (4 cm) to limit the mobility of inoculated hosts.

b) The Piping system

The piping system is integrated into two parts: a) misting system; and b) washing system. The misting system is made up of PVC pipes with dispensing nozzles that are angularly positioned on either side of the perforated trays to supply misted water onto the trays (Figure 3-1). This ensures a high level of humidity in the unit to prevent desiccation of insect hosts. The nozzles are controlled by a timer that is configured to respond automatically to the humidity conditions of the incubator. The timer dispenses water from the nozzles at a flow rate of 60 ml/min for 3 minutes within every 6-hr intervals per day, to maintain at least 95% humidity in the incubator. The number of nozzles often used in an incubator can vary based on the flow rate of the water and the humidity conditions preferred, as humidity can be dependent of the host species under cultivation (Grewal, Gaugler and Selvan, 1993). However, the misting cycles can be adjusted during the cultivation to prevent the premature emergence of the nematodes.

For the harvesting of the nematodes from cadavers, the washing system is activated. The system constitutes a series of PVC pipes merged to create a network of pipes in a parallel configuration (Figure 3-1). This configuration offers a uniform flow distribution of water with reduced pressure drops in the lines. The water flows in one direction and subsequently splits into the multiple pipes as shown in Figure 3-1 below. Water is distributed evenly onto the trays as the configuration was merged above the tray system. End caps are placed at the distal ends of the pipes to cease water from gushing out at the ends, and force it to flow through the holes created in the sub-conduits. In this study, the washing of the nematodes was manually done by the opening of a valve to allow

the flow of water onto the trays for 3 minutes at 5-hr intervals for a total duration of 1-5-days during harvesting based on the harvesting procedure devised by (Shapiro *et al*. 2002).

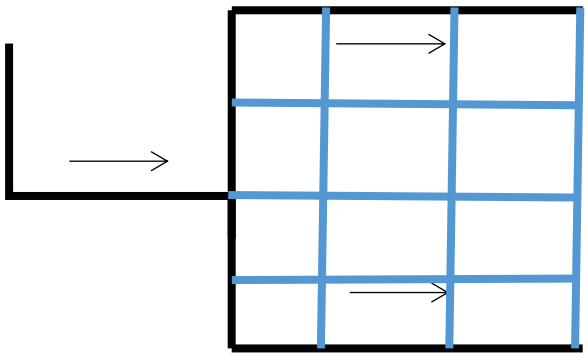

Figure 3-1: Parallel configuration of the piping system

c) The Collection Tray

The collection tray is positioned directly underneath the perforated tray systems, and it is anchored by a support piece that protracts horizontally from the general support base. Unlike the perforated trays, it has no perforations with its dimensions (35 x 30 x 7 cm) extending beyond the tray system. It has longer side walls in comparison to the perforated trays to help retain the nematode-enriched water during washing cycles. It is oriented in an angular position to ease the flow and hold up of dispensed water from the washing trays towards an outer drain tube connected to the storage vessel placed underneath the incubator. Outside the drain tube is a filter that removes residual debris such as dead hosts and worn out cuticles from the dispensed water.

d) The Support System

The support frame anchors the tray system, the piping system, and the collection tray. It has both vertical and horizontal support base which gives rigidity to the whole structure. Protruding from the vertical perpendicular section are horizontal rods which serve as a seat for the perforated trays.

The vertical rear of the support base provides apertures for the attachment of the piping system. The support system is mostly built from stainless steel to offer strength and has corrosion resistant properties. Additionally, it offers a lighter weight for easy portability in and out from the incubator unit. Removable fasteners made of plastics are used to tighten the connections between the pipes and the trays. This prevents the trays from toppling over during washings as it provided firmness.

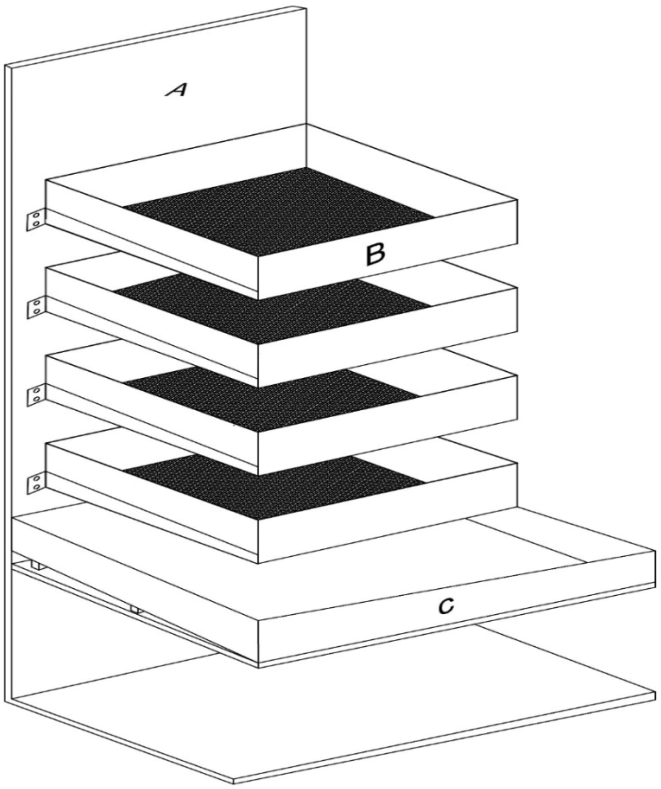

Figure 3-2: A pictorial drawing of the LOTEK unit

[redrawn from (Brown and Gaugler, 2004)]

(A- Support System, B- Perforated Tray, C- Collection Tray)

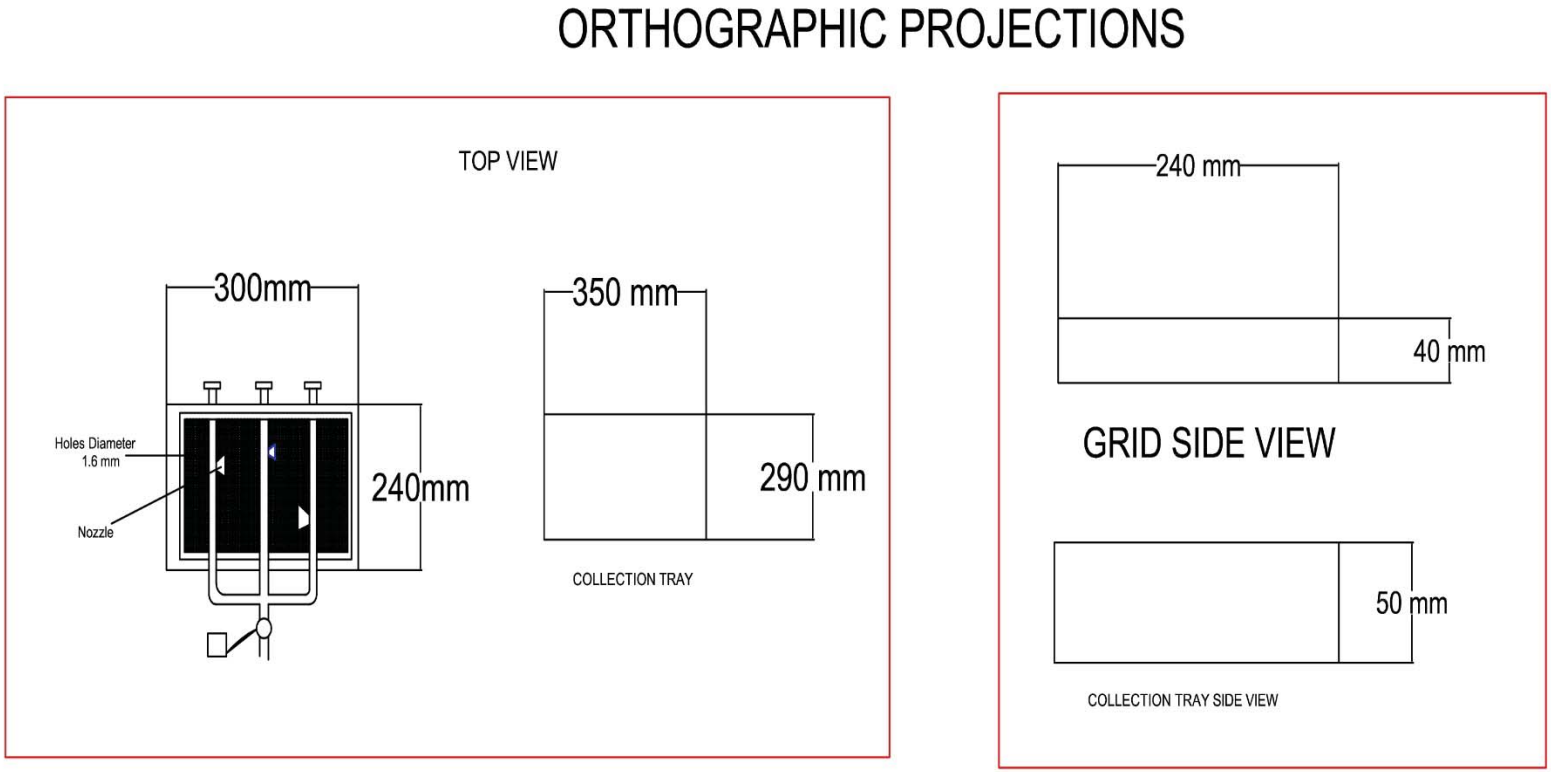

Figure 3-3 Orthographic projections of the LOTEK design

3.2.2. Source of Insect host and Nematodes

The insect hosts, *Tenebrio* (mealworm), were obtained from the Department of Conservation Ecology and Entomology, Faculty of AgriSciences, Stellenbosch University, which had been reared in the laboratory on a diet containing the following compositions: five parts brown bread flour; five parts Cerelac Nestle™ regular baby cereal; two parts wheat germ; two parts yeast; two parts glycerine; and one part honey. The starter culture of the MWL was purchased from a pet shop. All the ingredients were mixed with a beeswax comb (Bronskill, 1961; Woodring & Kaya, 1988). This allowed the accommodation of approximately 500 hosts per tray.

The nematodes, *S. yirgalemense*, were obtained from the Department of Conservation Ecology and Entomology at Stellenbosch University nematodes collection (Malan *et al*., 2006). Infective juveniles were produced through in vivo in mealworm larvae utilising petri dishes based on the White trap method (Shapiro-Ilan *et al.*, 2002). Emerging IJs were harvested and stored in distilled water placed in culture flasks at 14^{o}C. The IJs were used for inoculation processes within 30 days after harvesting.

Shapiro-Ilan *et al*. 2002 suggested that many factors including host density, nematode concentration, and method of inoculation could preclude the attainment of a high nematode yield during in vivo cultures. For the host density, Flanders et al (1990) showed that it does not affect the final yield of IJs significantly. However, hosts of similar sizes [(0.3 ± 0.005 g (±sd)] were used as recommended by (Shapiro-Ilan *et al.*, 2002) to mitigate any possible effect this parameter can have on the development of the IJs. Additionally, an inoculum concentration of 200 IJs per host has been suggested to cause the degree of patent infections of the nematodes (Shapiro-Ilan *et al.*, 2002; A. C. Van Zyl and Malan, 2014). This study, therefore, sought to investigate the effect of inoculation techniques on the final yield of IJs before mass production of the nematodes was conducted.on the LOTEK.

3.2.3. Comparison of inoculation methods

Two techniques were adapted for investigations, i.e., inoculation by immersion, and inoculation by spraying. The efficiency of both methods was established based on the degree of patent infections. In immersion technique, the 30 g of hosts were put on a tray and dipped in a suspension of nematodes with concentrations (4000 IJs/ml) for 5 seconds. Host inoculated by spraying received nematode suspension with the same concentration delivered through spray bottles

Experiment	Inoculation Technique
A	Immersion
B	Spraying

3.2.4. Assessment of yield

10 hosts were randomly selected from each of the experiment and transferred to the White trap system for harvesting. The data was analysed by using a one-way ANOVA with *post hoc* comparison of the means using Bonferroni's method in Statistica (Statsoft Inc. 2014).

3.2.5. Mass production on LOTEK

Based on the results from the previous experiments, production of IJs using the TFS was conducted. After successful inoculation, the infected hosts were transferred to the trays in a humidity controlled chamber, incubator, for conditioning. The system was equipped with 4 trays each carry at least 500 insect host. Humidity levels were maintained in the incubator by adjusting the timer that automatically regulates the misters delivering water at a flow rate of 50 ml/min. The trays were lined with absorbent paper (such as medical gauze) to provide a conducive and humid platform for the emerging nematodes. Efficient air flow through the incubator was ensured so as to avoid the accumulation of ammonia in the unit. A high concentration of ammonia can have a detrimental effect on the growth and development of the nematodes (San-Blas *et al.*, 2014). The experiment was repeated on different test dates to assess the maximum efficiency of the LOTEK system. The efficiency of the LOTEK system was assessed by setting up White traps to compare the average yield and the percentage of IJs per host that could be achieved from both systems

3.2.6. Harvesting

Harvesting of the nematodes was initiated after 12-17 days of incubation by activating the washing system. The water-induced the emergence of the nematodes, which are then washed into a central storage vessel placed beneath the unit. Washing was done continuously for 3-5 days after which the operation was discontinued due to the trivial numbers of nematodes being recovered. In total,

20 L of water was collected in each production run. This was then concentrated and the IJ host per insect is calculated by dividing the total IJ by the total host used per tray.

3.3 Results and Discussion

3.3.1. Comparison of inoculation methods

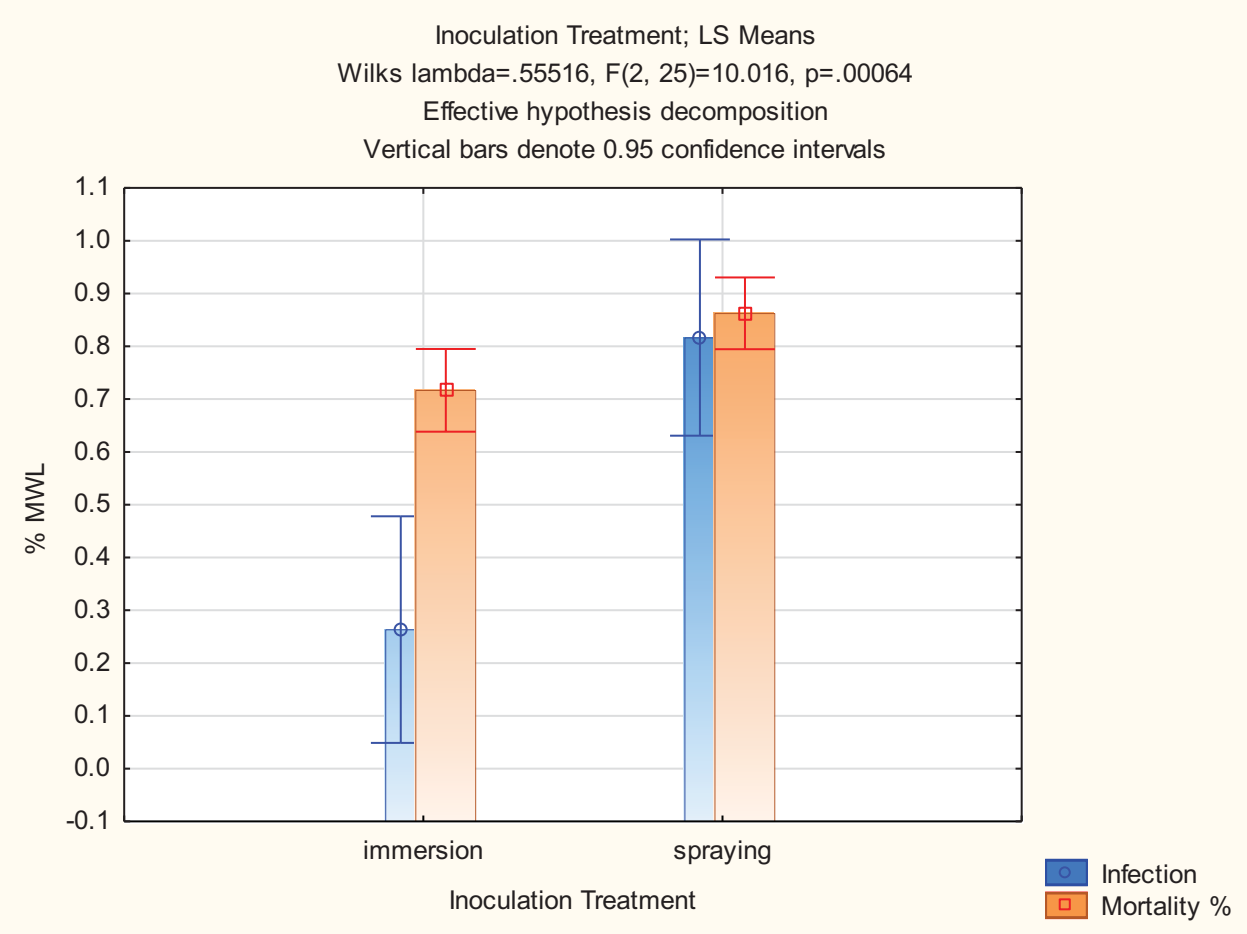

Figure 3-4: 4.Means and 95%confidence intervals for the means of mealworm larvae mortality and infection for each of the two inoculating treatments, using *S. yirgalemense*

From fig, there were significant differences between each of the inoculation treatments investigated (*P < 0.0005*). The spraying technique *(F = 0.2633; df = 26; P = 0.00462)*.caused more infections and mortality to the nematode-host combination tested than the immersion treatment *(F = 0.71667; df =262; P = 0.0076)*.Even though the infection rate of the immersion method was low (27% ± 3.85%), it achieved a comparatively a high mortality (70% ± 2.65%) as the spraying method (83 % ± 6.7%). However, mortality results cannot be used a selective factor on possible inoculation treatment as abiotic factors can also play a role in the death of hosts. Also, it is the degree of patent infections that determines the mortality and the average yield per host

Malan *et al* 2014 conducted similar investigations of inoculation treatment of MWL using H. bacteriophora nematode. They reported a mortality rate of 96.92% ± 1.75% with pipetting (similar to spraying) method whiles immersion levels were low at 56 ± 3.62%. Similarly, low infection levels were obtained for the immersion technique as even though a mortality rate was recorded in each nematode- host combination tested. This follows that the MWL is more susceptible to the nematodes when inocula are delivered onto their bodies by either the spraying or pipetting method. The spraying method was therefore chosen as a preferred treatment for the inoculation of the MWL on the LOTEK system.

3.3.2. Mass Production on LOTEK

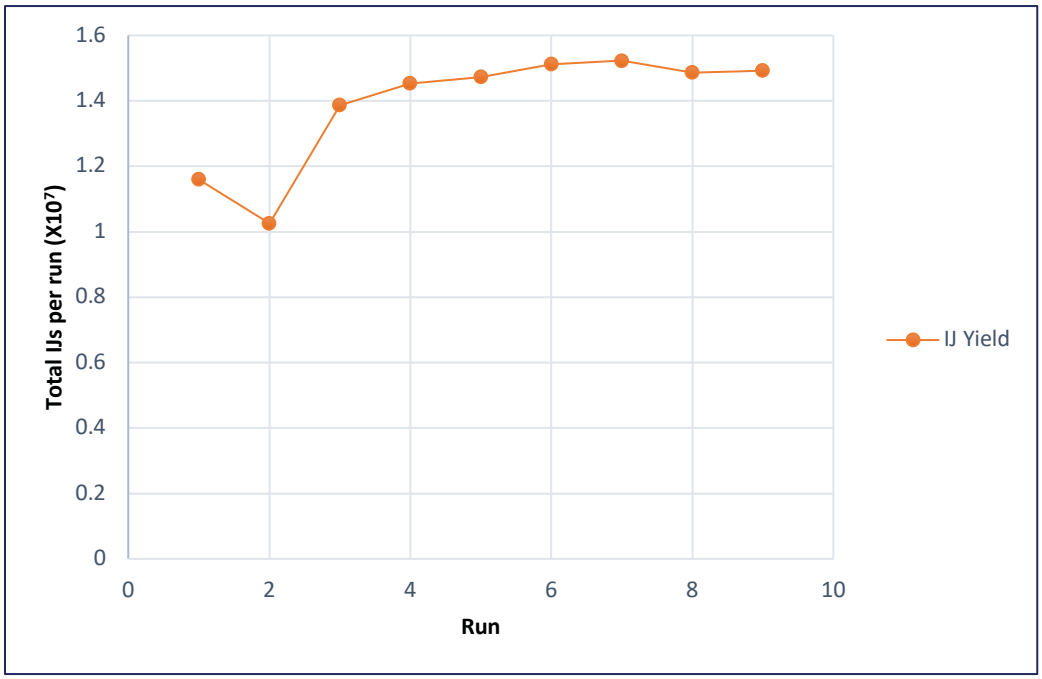

Figure 3-5: Total IJ yield obtained from nine runs conducted on the LOTEK

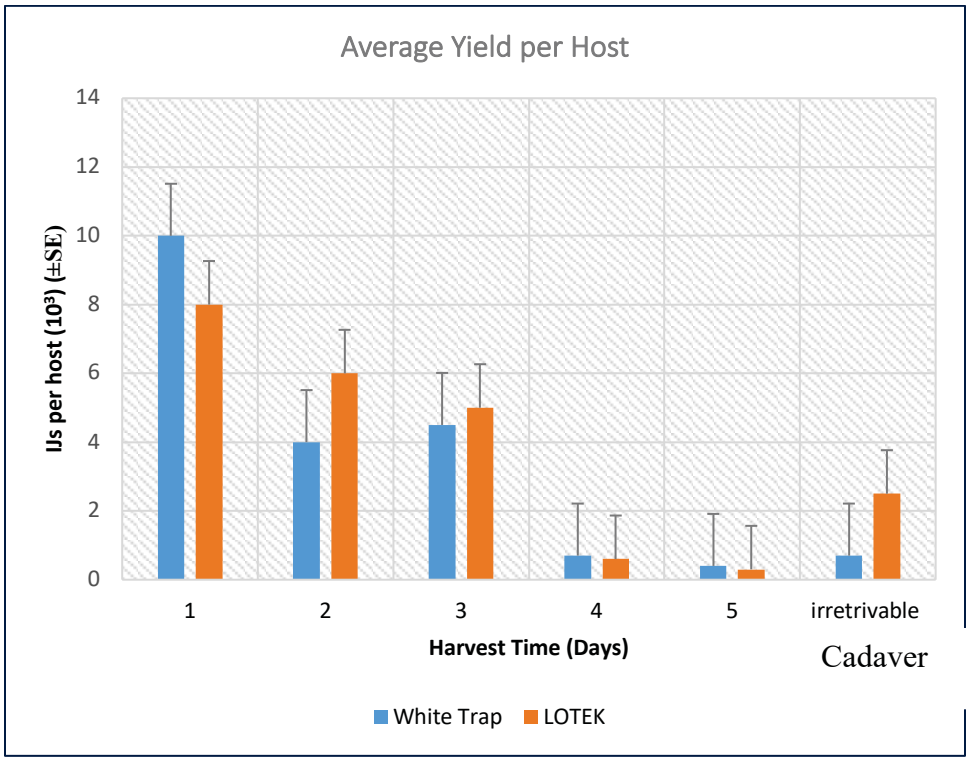

Figure 3-6: Yield of *Steinernema* infective juveniles from LOTEK and White trap systems recorded daily

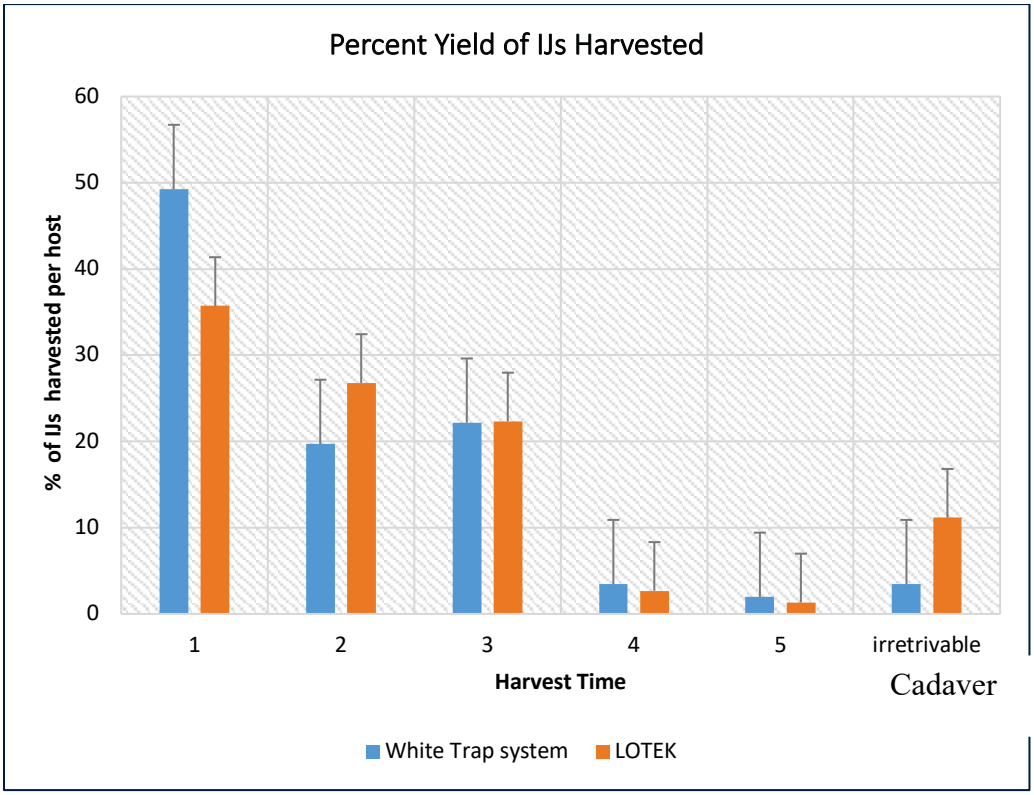

Figure 3-7: Percentage of yield of *Steinernema* infective juveniles harvested in *Tenebrio* from LOTEK

The total yield of IJs obtained in each run of the *in vivo* experiment with inoculum concentration of 200 IJs per host at 25^0C in a relative humidity-controlled unit is shown in Figure 3-6. Each data point on Figure 3-7 represents a complete set of *in vivo* run using the TFS. In all, a total of nine runs was investigated to assess the functionality of the LOTEK system with the White trap system setup as control experiments. With the exception of run 2 where the average number of the host on each tray was doubled (> 1000 insect host), the average number of host per tray was kept constant (<600 hosts per tray). The doubling of the host per tray was in an attempt to maximize the host density per area based on the yield results from run 1 ($1.2x10^7$ IJs). However, this action resulted in the lowest yield being recorded among all the experimental runs ($1.02x10^6$) (Figure 3-5). The increase might have led to overcrowding of the host on the trays resulting in the production of ammonia that affected the normal development of the nematodes (San-Blas *et al.*, 2014). In terms of IJs per host, the average yield of IJs obtained per host from each run in the LOTEK system is approximately 4.85-5.03 x 10^3 IJs per host (Figure 3-7). Comparatively, there were no appreciable differences between the average yields per host recorded for the White trap system (4.6-4.8 x 10^3 IJs per host) and the LOTEK system as shown in Figure 3-7.

Process performance of the LOTEK and White trap regarding harvest efficiency is shown in Figure 3-7. During the harvesting, about 52 % of the emergent nematodes were harvested from both systems on the first day, 91% on the second day and a trivial number of nematodes obtained after the third day (<2%) (Figure 3-7). However, for the LOTEK system, a lot of the nematodes upon dissection of cadavers remained irretrievable and did not emergence (< 9% emergence for White trap system and < 12% for LOTEK) (Figure 3-6). Additionally, a close examination of the LOTEK unit after a series of washings revealed less than 3% of the nematodes remained irretrievably trapped between the wet absorbent papers and the washing trays (Figure 3-2) after emergence (results not shown). The white recorded a lower loss of nematodes that were trapped between the filter paper and the petri dish (< 1%). Also, about 3% of the IJs were lost due to the splashing of water onto the sides of the trays that could not be recovered (results not shown). This loss in the TFS system is comparable to the loss results reported by Gaugler (2002) where approximately 2% of the IJs remained trapped within the unit.

Benchmarking the yield results in this study to those reported by Gaugler (2002) on the LOTEK system showed some remarkable differences regarding the IJs per host obtained in both studies. Gaugler (2002) reported excellent results by recording an average yield of 11-12 x 10^4 IJs per host during the cultivation of *H. bacteriophora* on *G. mellonella* cadavers in LOTEK, and 8-9 x 10^4 IJs

per host in the White trap system in comparison to $4x10^4$ IJs and $2\text{-}3.5x10^4$ IJs per host achieved for LOTEK and White trap system respectively. The difference in IJs per host in both studies could be ascribed to the dissimilar nematode and host strains cultivated in each study. However, in terms of the overall performance in yield, there is no appreciable difference between the LOTEK and the White. The low yield could also be as a result of the nematode-host combination. *Tenebrio* has been reported to perform poorly with certain nematode strain (Gaugler *et al.*, 2002a). Although *G. mellonella* often provides excellent results in vivo cultures at the laboratory, it was not adopted for the mass cultivation in this study. Jones (1968) pointed out that *Galleria* spins themselves into a cocoon as soon as they are inoculated with entomopathogenic nematodes especially when mass inoculation of the host is the performed. To prevent the cocoons from forming, the wax moth larvae are subjected to temperature treatment (Lacey *et al.*, 2005). This immobilizes the wax moth larvae preventing the spinning into cocoons. This preventive technique is cumbersome and time-consuming. Weighing up the above and taking cost of production into account, the choice of host for this study was mealworms. Furthermore, when producing mealworms on a large scale on wheat germ bran, other insects such as midges and vinegar flies are also attracted. The bran is frozen prior to production to eliminate mites, but the flying midges are impossible to keep out. Midges flying around the production facility lay their eggs in the entomopathogenic nematode culture resulting in contamination. The rate at which the midges contaminate the culture increases with the temperature of the mealworm production units. During summer, for example, there were more midgets in the environment contaminating the cultures as the TFS unit was standing in an open area with other equipment every time the unit (incubator) was opened, midges could potentially gain access. This could be the reason for the low EPN numbers as compared to Gaugler (2002).

Another plausible reason for the low yields recorded in this study could be ascribed to possible contamination (an anecdotal observation made in the lab). Other infections not necessarily from the nematodes often infect the worms during incubation. Contamination resulting from dead mealworms remaining on the tray is often inevitable. Removing these dead mealworms is painstaking and time–consuming. Notwithstanding the efficient housekeeping practises, contamination may have inevitably resulted in one way or the other including microbial activity on non – infective stages thereby reducing yields.

Additionally, the conditioning and misting regimes in the incubator could account also for the low yields. For the LOTEK system, although practical measures were put in place to mitigate uncontrollable misting regimes, this situation was still apparent in some cases. This resulted in

triggering a premature emergence, of the nematodes which could not be counted among the final yield of the LOTEK system. Additionally, the cadavers were saturated with the misting water on some occasions which might have affected the efficiency of oxygen transfer within the unit (Gaugler *et al.*, 2002a).

Since the nematode is a newly found biocontrol agent in south Africa and the lack of literature studies on this strain, it still remains unknown whether the low yield could partly due to the biological characteristic of the nematode with *Tenebrio* as a low yielding species or not. This demands further investigations with a variety of hosts to find the perfect host-nematode combination for a higher yield.

On the whole, the performance of the LOTEK system is comparable to the White trap with equal harvesting efficiency. The LOTEK system promises to increase the cost efficiency relative to scale-up and mass production operations. However, mechanization interventions that reduce the amount of labour and house-cleaning operations should be implemented to enhance the overall efficiency.

Chapter 4

Experimental Production of EPNs with the In vitro Method

4.1 Introduction

The families of *Steinernematids* and *Heterorhabditis* are used as biological control agents due to their mutualistic association with the gamma negative bacteria *Xenorhabdus* and *Photorhabdus* of the subclass Proteobacteria (McMullen II and Stock, 2014). They are known to be pathogenic to most commercial pest and safe for use in the environment as compared to chemical pesticides and thus generating a global interest in their cultivation and application (Gaugler *et al.*, 2000). However, for successful commercialization of these biopesticides in large quantities, there is a need for stable process parameters and optimization to ensure a higher but reproducible nematode yield. Most recent studies on nematode cultivation are focused on finding practical ways to improve upon the technical and process aspects of the *in vitro* liquid technology as it provides a promising route to commercialization regarding costs and ease of achieving economies of scale (Grewal, Ehlers and Shapiro-Ilan, 2005). Moreover, most researchers have described the *in vitro* technology as the most economical route for mass-production (Upadhyay *et al.*, 2013a).

Notwithstanding, challenges such as variability and irreproducibility of yield are often encountered during fermentations (Gerritsen, De Raay and Smits, 1992). These undesirable occurrences are attributed to various biological and operating conditions often encountered, and the principal among them is the rate of recovery of IJs after inoculation (Jessen *et al.*, 2000). The problems associated with recovery in *in vitro* cultures have received critical attention over the years as it hinders the achievement of a higher nematode biomass. Results from earlier studies by Ehlers et al. (2009) demonstrated a robust and consistent link between the quality and quantity of the bacterial cell density inoculated and the percentage of IJs recovery per run (Ehlers, 2009). The bacteria play a key role in the recovery of the nematode by secreting 'food signals' needed for their development. Elhers (2009) proposed that a higher bacterial cell density makes available enough food for the nematode during the long period of fermentation run.

Other factors known to be influencing variable recovery rate of IJs is phase changes in the bacterial symbiont after inoculation (Johnigk, 2004). Factors that trigger phase change is still unknown but some authors attribute it to subculturing practices, medium composition, and cultivation conditions (osmotic strength of the medium, oxygen availability, and temperature) (Glazer and Salame, 2000). The bacteria can change from phase I (desirable trait) to phase II (undesirable trait), and this can affect the final yields of EPNs (Han and Ehlers, 2001). In an attempt to find out how the medium composition can sustain the rapid growth of bacteria and also maintain the phase I, (Jeffke *et al.*, 2000) investigated the role glucose could play in stabilizing the phase variant of *Photorhabdus* bacteria. The addition of glucose at periodic intervals prevented the bacteria from shifting phases with a higher bacterial cell density being recorded. Additionally, they reported an improved 'food signals' secretion by the bacteria in the medium which directly affected the recovery of the DJs and the final nematode yield (Jeffke *et al.*, 2000).

This study is a carry-on investigation initiated by Ferreira et al. (2013) whom characterized the symbiote of *Steinernema yirgalemense*, and investigated the propagation of the symbiote in liquid batch cultures in a bioreactor (Ferreira, 2013). Most of the research works with this model organism were done under batch fermentation system, where the bacteria and the nematodes were inoculated into the fermenter without the addition of further substrates. However, there are still gaps needed to be filled to wholly understand the best growth conditions for the nematode and symbiotic bacteria for successful commercialization in South Africa. For example, Pace et al, (1986) and (Chavarría-Hernández *et al.*, 2011) studied how the effects of bioreactor design, shear stress and agitation can impact the growth of nematodes in stirred tank and internal loop bioreactors. Nerves et al. (2001) also investigated the effects of air flow rate on the yields of nematodes in external loop bioreactors. Alexander (2011) also studied how the yields of *H. bacteriophora* can differ considerably depending on the choice of the culturing method (batch, fed-batch and continuous) used. He studied the impact of adding fresh bacteria feed to an existing fermenter during the fermentation of *H. bacteriophora* nematode production. He hypothesized that the bacteria get depleted in the course of fermentation due to the rapid growth of the nematodes, and replenishing the media with fresh feed would boost the growth of the nematodes (Alexander, 2011).

This study is one of the first investigations into conversion of the *S. yirgalemense*-bacteria symbiote production system from batch to fed-batch cultures, by the further addition of one of two possible substrates: bacteria or glucose. Using glucose or bacteria as feeds during the course of the

fermentation, the growth of the bacteria and the nematodes was monitored, and their impact on nematode yield evaluated. The aim of the study was to assess the best production method that increases final IJ yield and also reduces the production time spent by nematodes in the fermenters. All experiments were conducted on two different test dates in the following manner.

1. Assessment of the growth of the bacteria partner of *S. yirgalemense*, *Xenorhabdus*, in batch and glucose fed-batch culture
2. Investigations into traditional batch fermentation of *S. yirgalemense*
3. The impact of adding fresh bacteria to existing batch cultures with bacteria as substrate

A comparative analysis of these experiments (batch, bacteria fed-batch, and glucose fed-batch) into areas such as final concentrations, quality of IJs, and the total process time, were discussed in the follow-up sections.

4.2 Materials and Methods

4.2.1. The fermenter Set-up

The Biostat® B plus – 5 L CC Twin fermenter (Sartorius BBI Systems GmbH, Switzerland) was a bench-top, double-walled glass bioreactor with a stainless-steel lid with a working volume of 3-L (Figure 4-1). Equipped in the fermenter are electrodes/sensors (Pt 100 temperature sensor, pH-electrode (Mettler-Toledo), and pO2-electrode), which were connected to a central process control unit and signal amplifier. Other accessories in the reactor included a gasing tube with micro sparger that distributed air from the top, a baffle that promoted efficient mixing within the reactor, a stirrer shaft fitted at the end of Ruston impeller for mixing of the culture medium, and a manual sampler for medium samples for analysis. The air was delivered into the fermenter via flexi-tube connected to an air pump through to the sparger. Sterilization of the air was done using a Cole-Palmer syringe filter, sterile G.D PTFE membrane, and PP housing connected to the fermenter via flexing tubing. The filters act as a sterile pressure release vent for CO_2 through the top of the condenser. The air pressure was kept at 10 kPa to prevent foaming and subsequent spillage of reactor content. However, foaming problems were mitigated by the addition of antifoam into the medium before autoclaving. The temperature in the reactor was kept constant with circulating water supplied via flexible tubing connected to a Julabo water bath (Labotec, South Africa).

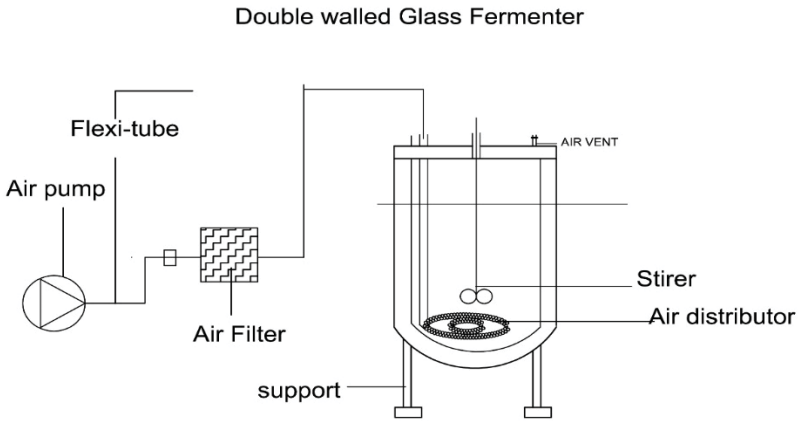

Figure 4-1 Schematic drawing of the fermenter Set-up

4.2.2. Media preparation

The experiment was carried out with *S. yirgalemense* strain and its symbiotic bacteria *Xenorhabdus,* which were obtained from the Entomology and Conservation Ecology Department, Stellenbosch University. Two culture media were prepared: Liquid Culture Medium (LCM) and trypticase soy broth (TSB) for nematodes propagation and bacterial propagation respectively. The LCM consisted of the following compositions (per L): 5.0 g yeast extract (Merck), 20.0 g soy powder (Health-Connections, River Park, South Africa), 4 g NaCl, 0.35 g KCl, 0.15 g $CaCl_2$, 0.1 g $MgSO_4$ (Merck), 46 ml vegetable oil per L (Ehlers *et al.*, 1998). The TSB (bioMerieux) medium for the bacterial propagation for 48 h at 30 ^{0}C composed of (per L): 17g tryptone, 3.0g soytone, 2.5g glucose, 5.0g NaCl (Wang *et al.* 2011).

4.2.3. Isolation of symbiotic bacteria

To obtain the symbiotic bacteria, they are isolated from the infected *Galleria* larvae by the use of a sterilized streaking rod. The rod is heated with a naked fire before it is inserted into the body of the cadaver. The extracted substance is then streaked onto the agar plates for incubation at 25-28 ^{o}C for 2 days. The plates are normally turned upside down to promote a successful growth of the bacteria. The plates are propagated in a nutritive medium (TSB) for 48 hours at 30 ^{o}C before they are used. Unused stock strains of the bacteria can be stored in 15% glycerol at -80^{o}C. When needed, they are thawed at room temperature before they are transferred into a TSB for propagation.

4.2.4. Axenisation of nematodes

To establish a bacteria free-IJs to use in monoxenic cultures, the infected *Galleria* showing signs of patent infections are dissected after four days of incubation by washing in 0.1% hyamine solution (Sigma) containing sterile blades. This last step isolates the eggs of the gravid females which are then transferred onto lipid agar plates. The plates are incubated for 4 days at 25°C while monitoring the growth of the cultures.

4.2.5. Establishment of Monoxenic cultures

To establish a monoxenic culture, the bacteria on the agar plate (cell density of 10^7 cfu ml^{-1}) are scooped from the lipid agar plate into a sterile TSB into an Erlenmeyer flask and pre-incubated at 30 °C on an orbital shaker at for 36 hours. The bacteria free IJs are inoculated after the period incubation at 25°C. Samples of the growing culture are aseptically withdrawn from the growing culture to assess the growth of IJs. Final harvest of the IJs can be done after 11 days of post nematode inoculation.

4.2.6. Culture conditions

Production of nematodes was carried out in the 5-L fermenter containing 3-L of the above LCM, which was prepared and autoclaved for 15 mins at 121 ^{0}C. The temperature was kept constant at 30 ^{0}C. The pH of the medium was kept at 7 by adjusting the profile with a solution of NaOH/KOH. The airflow was maintained at the maximum (1 l/min) throughout the fermentation cycle as agitation rate was maintained between 200 and 600 rpm to keep the dissolved oxygen content in the fermenter above 30%. The DO was monitored online by an oxygen probe (Mettler-Toledo) initially calibrated with nitrogen and compressed air. Foaming was controlled in the fermenter by the addition of anti-foam (0.03 v/v %) (Sigma, South Africa).

4.2.7. Growth of *Xenorhabdus* bacteria in batch and glucose fed-batch culture

Investigations were made to study the growth kinetics of the symbiotic bacteria under both batch and glucose fed-batch modes. The objective of this experiment was to obtain a high bacteria cell

density in a bioreactor by feeding glucose at periodic intervals. The bacteria serve as the primary source of food for the nematodes, and processes that can lead to an increase in the average cell density per ml need investigations. Two bioreactors were set up for investigations. An initial glucose concentration of (5 w/w %) was added to the LCM in both bioreactors. However, one of the reactors was moved from batch to fed-batch by the feeding of a stock solution of (10 g/l) at periodic intervals between 25 h to 56 h after the concentration of the glucose had been depleted (Table). The rationale was to maintain the concentration of the glucose between 0.2% and 0.4% over the period of 60 hours of the bacterial growth. This experiment followed the experimental principles Jeffke *et al.*2000 where the growth of *Photorhabdus luminescens* in batch and glucose fed-batch cultures was analysed. No IJs were inoculated into any of the bioreactors during the 60 h of the bacteria growth.

Table 4-1: Time-interval for the addition of glucose stock solution (10 g/L) to the fermenter

Culture Time (h)	23	26	29	32	35	38	41
Glucose solution (ml)	25	25	27	30	25	20	35
Culture Time (h)	44	47	50	53	56	59	62
Glucose solution (ml)	70	25	25	15	20	25	30

4.2.8. Growth of S. *yirgalemense* in batch and glucose fed-batch culture

The experiment was repeated on different test date by following the same protocol described above, but with the inoculation of IJs at this time. Two bioreactors were set up with the addition of glucose to the basal LCM in each. However, one of the bioreactors was moved to fed-batch with the continuous supplementation of stock of glucose solution until 2000 IJs/ml was inoculated into each bioreactor (batch and fed-batch). The inoculation was done after 36 hours of bacteria incubation when the cell concentrations of the batch and fed-batch systems have reached (45×10^7 cells ml^{-1}) and (18×10^7 ml^{-1}) respectively [point A in Figure 4-3]. Further stock of glucose solution was added to the fed-batch every 2 days after the inoculation of IJs. This decision was to assess the impact the glucose can have on of the nematodes (IJs/ml) in areas such as percent recovery, growth, and reproduction. The residual glucose concentrations during the period of fermentation are shown in figure (). Samples of the cultures (1000 µl) were aseptically withdrawn from each

culture every day to assess the parameters mentioned above. Samples collected were diluted in Ringer's solution (see Appendix), and 100 µl was fetched and analysed under an inverted microscope (Nikon TMS, Japan). The mean recovery rate of the IJs was calculated using equation 4-1(Johnigk, 2004) after 48 h to 75 h post-IJ inoculation or by dividing the total number of recovered IJs in the culture by the total IJs inoculated into the bioreactor (Gil, 2002). The mean population of IJs of *S. yirgalemense* in glucose fed batch monoxenic liquid cultures at 25°C, were counted over a period of 11 days and compared with IJs of the batch cultures fig (04).

$$\boldsymbol{Recovey\ rate}(\%) = \sum \frac{\boldsymbol{J4\ x\ 100}}{\boldsymbol{J1,J3,J4,}} \qquad \textbf{\{Equation 4-1\}}$$

Where J1, J3 and J4 are the infective juveniles at stage 1, 3 and 4 respectively.

4.2.9. Batch and fed-batch fermentations of nematodes with bacteria

Two 5L bioreactors were setup for this investigation. A 250 ml, 24 h cultured *Xenorhabdus* with an initial density of $10x10^7$ cfu ml^{-1} was pre-incubated in the LCM at 30°C before the IJs (2000 ml^{-1}) was inoculated into each of the bioreactors. The growth profile of the nematode with its symbionte was investigated under the batch system (no addition of substrate) and fed-batch (addition of bacteria). The cell density of *Xenorhabdus* was assessed on each day during the 12 days of fermentation at 25°C (fig). A fresh bacterial inoculum (50 ml) was prepared based on the procedure described in section (4.2.3) and aseptically pumped into the fed-batch system on day 4 and 8 (graph) due to the low cell counts on the said days. All culture conditions and parameters were kept constant as described in section (4.2.6) Assessment of IJ count and bacterial cell density were determined as described in the section (4.2.11). The culture was allowed to run to completion for 11-16 days before it was terminated.

4.2.10. Virulence of Nematode

The virulence of the nematodes produced from each experiment was assessed using a one-one-bioassay with *Galleria* as a host. Ten live hosts' larvae were placed in 12-well plates, and inoculum concentration of 200 IJs/host was prepared and delivered in each petri dish (50 µl) each. 20 replicates of this setup were prepared. Virulence and pathogenicity of the nematodes were evaluated based on the mortality rate of the host and penetration ability of the nematode (Somwong and Petcharat, 2012). .For the control experiment, water was inoculated onto the body of the host.

The plates were incubated at 25°C and the percent mortality was recorded after 24 h. The mortality data was pooled and analysed in Statistica software using one-way ANOVA after 24 h post exposure of the host to nematodes. Arcsine transformations were done to express the mortality data into percentages before statistical analysis was performed

$$\textbf{Percentage penetration} = \frac{\textbf{Number of nematodes penetrated into host}}{\textbf{total number of added neamtodes}} \textbf{ x 100} \qquad \textbf{\{Equation 4-2\}}$$

4.2.11. Assessment of yield

For experiment (A), samples of the growing culture were taken every 3-h intervals till 60 hrs of process time to assess the bacterial cell density [counting the bacterial colony forming units (cfu)], and also the residual concentrations of glucose in the culture. To assess the cell density of the bacteria during each cultivation, 10 µl of the samples was injected on LB agar (see Appendix) plates and the colony forming units (cfu) were counted. Microplate assay procedure based on glucose oxidase assay (Sigma-Aldrich, South Africa) was used to determine the residual concentrations of the glucose in the bioreactor in the course of the experiment (see Appendix for the procedure). Contamination of cultures was checked by streaking 10 µl of broth on NBTA according to Boemare and Akhurst (1998). All process conditions such as temperature, pH, agitation and air flow rate were maintained constant in both bioreactors as described in section (4.2.6)

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4.3 Results and Discussion

4.3.1 Growth of *Xenorhabdus* bacteria in batch and glucose fed-batch culture

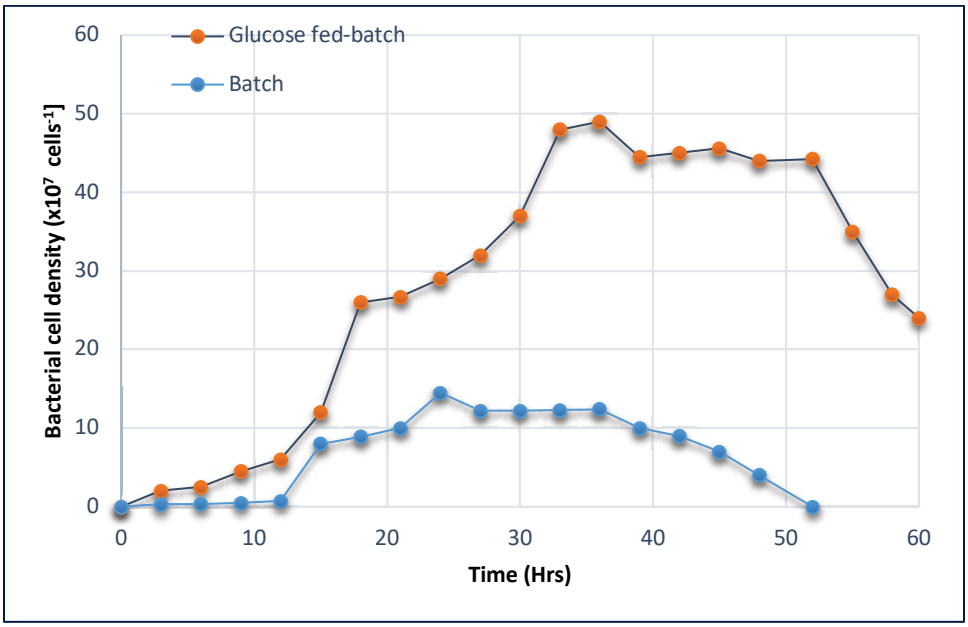

Figure 4-2: The cell growth, measured as bacterial density·(cfu ml^{-1}) of *Xenorhabdus* spp. in liquid medium, in three hour periods over a period of 60 h in a 5-L fermenter at 30˚C

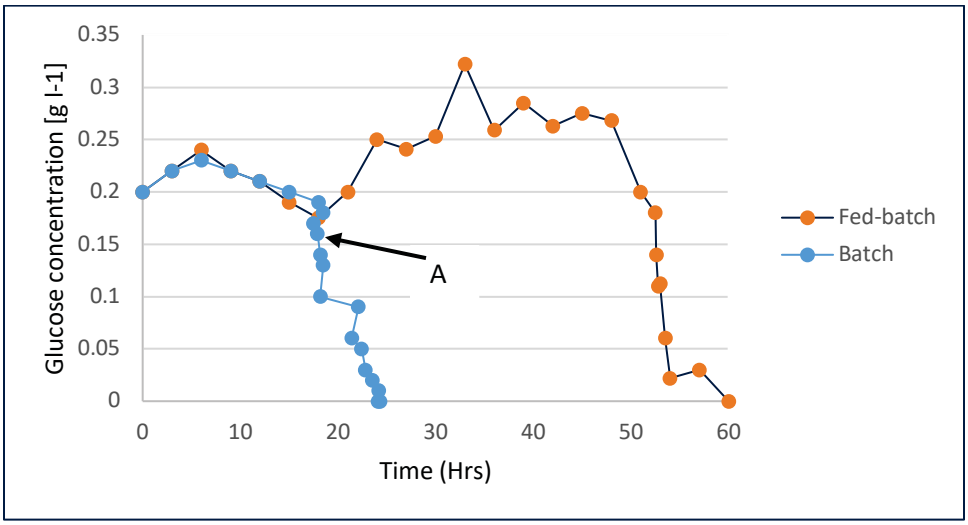

Figure 4-3: Time-course concentration of glucose in batch and fed-batch systems during the propagation of *Xenorhabdus* bacteria in LCM at 30˚C

A-Time of feeding the fed-batch culture with stock of glucose (10 g l^{-1})

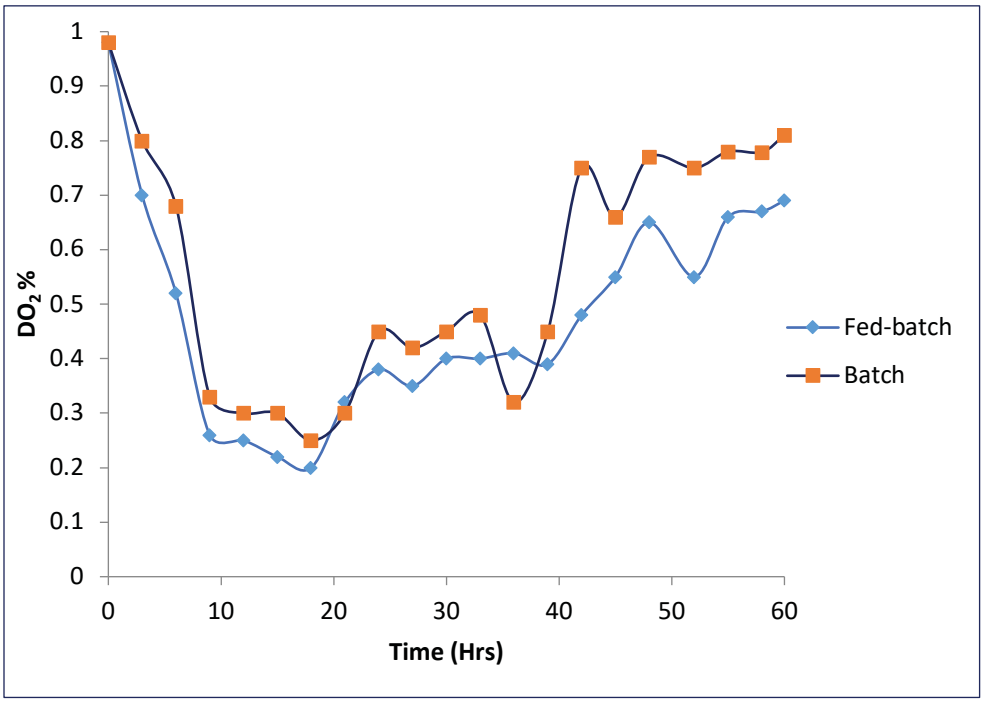

Figure 4-4: The dissolved oxygen (DO_2%) readings taken every 3 h, over a period of 60 h, in a 5-L fermenter, in which *Xenorhabdus* was cultured in the liquid medium at 30˚C under fed-batch and-batch systems]

Bacteria of cell density of 1 x10^7 cfu ml^{-1} was prepared through serial dilution process and inoculated into the bioreactors at 30^0C. The population density profile of the bacteria under batch and fed-batch systems, as well as the timing of glucose feeding for the glucose fed-batch, are represented in Figure 4-2 and Figure 4-3 respectively. In analysing the results of both cultures, it is important to identify the factors that promoted or hindered the achievement of a higher cell biomass. Johnigk *et al.* 2014 proposed that the dissolved oxygen (DO_2%) content in a fermenter can be used as an online monitoring tool to observe the growth rate of the bacteria, as it is indicative of their metabolism and growth rate. The DO_2 data was taken every 3 hours during the propagation of the bacteria in both systems (Figure 4-4). This was used to assess the growth performance of the bacteria at various stages of their growth profile. Also, the cell density of the bacteria was determined every 3- hours by taking samples from the bioreactor and assessing the cfu as described previously. The growth curve of the bacterial cells in both fermentation systems (Figure 4-2) were very similar to the each other (Figure 4-2), except for the stationary phase where the batch culture was shortened after 34 h of their growth. The lag phase of the bacteria in both cultures was surpassed after 10-12 hours before the onset of the exponential growth phase. This stage was characterized by the progressive decrease of the DO_2 content initially at 98% to 70% (Figure

4-4).The exponential growth phase for both cultures was short as it occurred between 12 hours to 23 hours, with a corresponding drastic decrease in the percent DO_2 in both cultures below the set point (30 %). However, this decline was compensated for by an increased in the stirring rate of the impeller to make enough oxygen available for the bacteria (200-300 rpm). The bacteria had started to grow at this stage. Data from cell counts also revealed a similar growth trend in population density of the bacteria in both cultures (12-15 x 10^7 cells ml^{-1}). Analysis of the glucose concentration profiles (Figure 4-3) also showed a continuous consumption of the glucose in the fermenter from 0 hr to 25 hrs after bacteria inoculation for both systems (from 0.25 g l^{-1} to 0.03 g l^{-1}). At this stage, the DO_2 content in the reactors had become constant (30%), and this could characterize the stationary phase of the bacterial growth. The introduction of further stock glucose solution (10 g l^{-1}) into only one of the bioreactors (fed-batch) was done between 24 to 56 hours after the glucose concentration in the batch cultures had been depleted (point A on Figure 4-3). This action did not result in any observable changes in DO_2 content as the bacteria had reached the stationary growth phase. This stage was characterized by a reduced need for oxygen by the bacteria and a gradual rise in DO_2 level in both systems. However, the stationary phase of the bacteria in the fed-batch system (Figure 4-2) was prolonged for longer hours before the onset of the death phase in comparison to the batch cultures. It occurred after 42 h, 8 h later after the occurrence of the stationary phase in the cultures that had no further addition of glucose substrate (batch culture) (Figure 4-2). The prolonged stationary phase of the fed-batch systems could be attributed to the quality of the glucose able to sustain the rapid growth of bacteria for an extended period before the onset of the death phase. Cell counts during the stationary growth phase showed an increase in the average cell density of the glucose fed-batch (48 x 10^7 cells ml^{-1}) in comparison to (15 x 10^7 ml^{-1}) achieved for the batch system, an overall percentage increase of 68% for the fed-batch culture. The increase in cell counts of the fed-batch cultures than the batch might be as a result of the extended growth of the bacteria owing to the extra supplementation of glucose (Gil, 2002). Thus, it served as a source of extra nutrients for the bacteria when some building blocks of the basal medium were limiting during the stationary phase of their growth (Gil, 2002).

Jeffke *et al.* 2000 made similar observations during the parallel investigations of batch and fed-batch cultures. The exponential growth phase of the bacteria for both systems ended early, between 16 and 18 hrs after the bacteria inoculation in comparison to the 23 hrs reported in this study. This deficit in time may be a consequence of the differences in the concentrations of the stock glucose solution added to the basal medium in both cultures. They added a stock glucose solution of (10%

w/w), while the basal medium in this study had (5% w/w).glucose. Gil *et al.* 2002 suggested that the bacteria can metabolize the glucose into essential components for easy assimilation into their bodies. The higher concentration of glucose used by Jeffke (2000) meant that the bacteria receive enough glucose throughout their development. Jonigk *et al.* 2014 also reported that at certain concentrations of glucose, the growth of the bacteria could be inhibited. It is still uncertain whether there were instances of substrate inhibition due to the 5% w/w glucose concentration adopted for this study. This highlights the need for further optimisation with regards to optimal glucose concentrations required for bacteria growth. The results achieved were encouraging as it increased the total density of the bacterial cells by 68% in comparison to the batch cultures.

4.3.2 Growth of *S. yirgalemense* in batch and glucose fed-batch culture

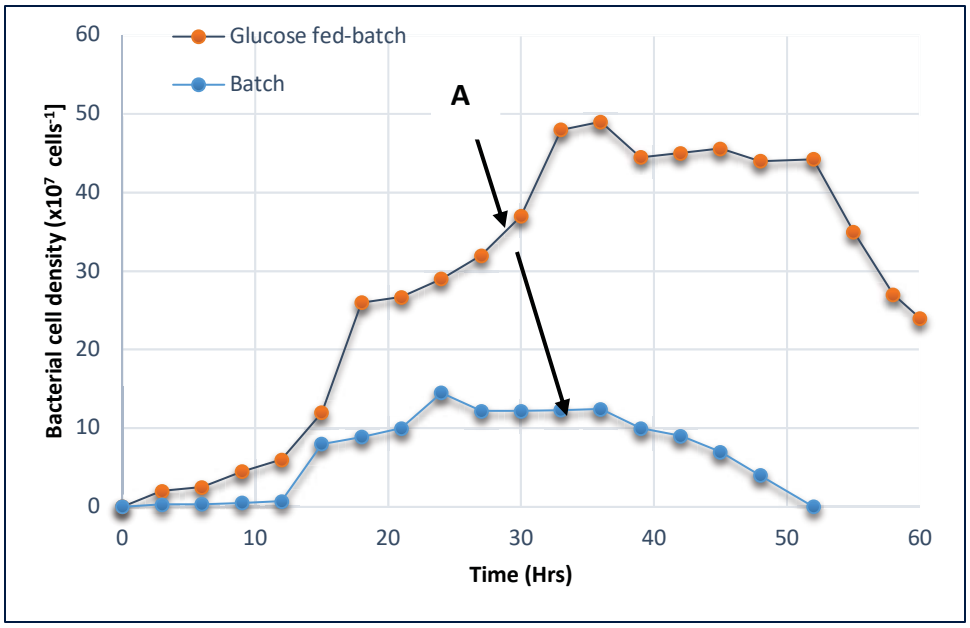

Figure 4-5: The cell growth, measured as bacterial density·ml-1 of *Xenorhabdus* spp. in liquid medium, in three hour periods over a period of 60 h in a 5-L fermenter at 30˚C

A-Time of inoculation of IJs into IJs into glucose fed-batch and batch systems [inoculum concentration of 2000 IJs per ml]

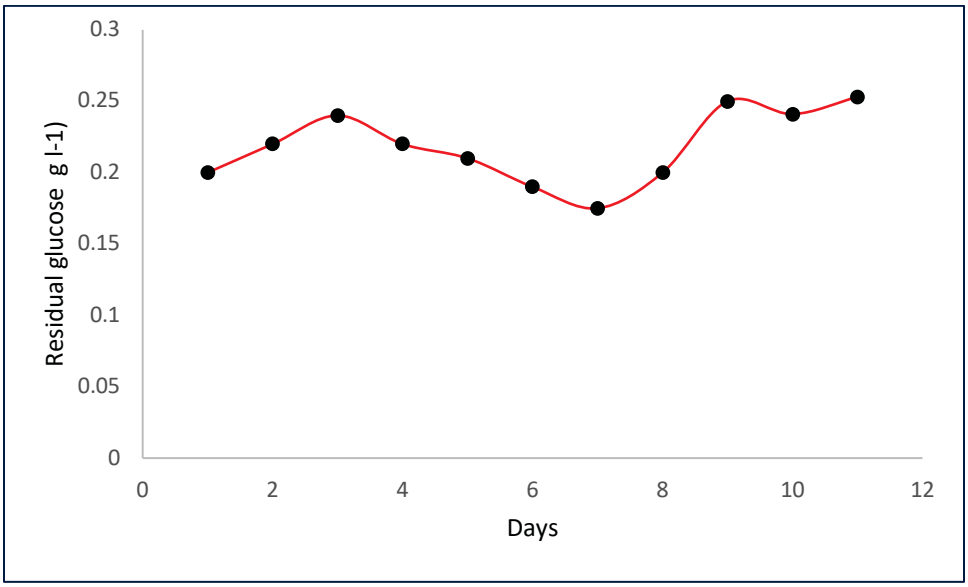

Figure 4-6: Time course residual concentrations of glucose in fed-batch cultures at a concentration of 10 g l^{-1} cultivated at 25°C

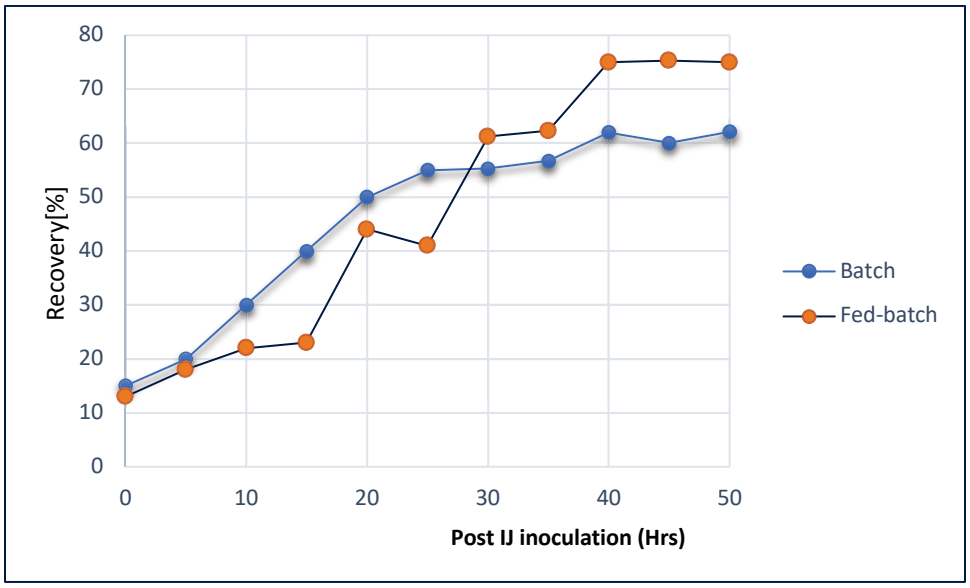

Figure 4-7: Mean IJ recovery (%) of S. *yirgalemense* in glucose fed-batch and batch monoxenic liquid cultures at 25°C, over a period of 48 hrs after IJ inoculation.

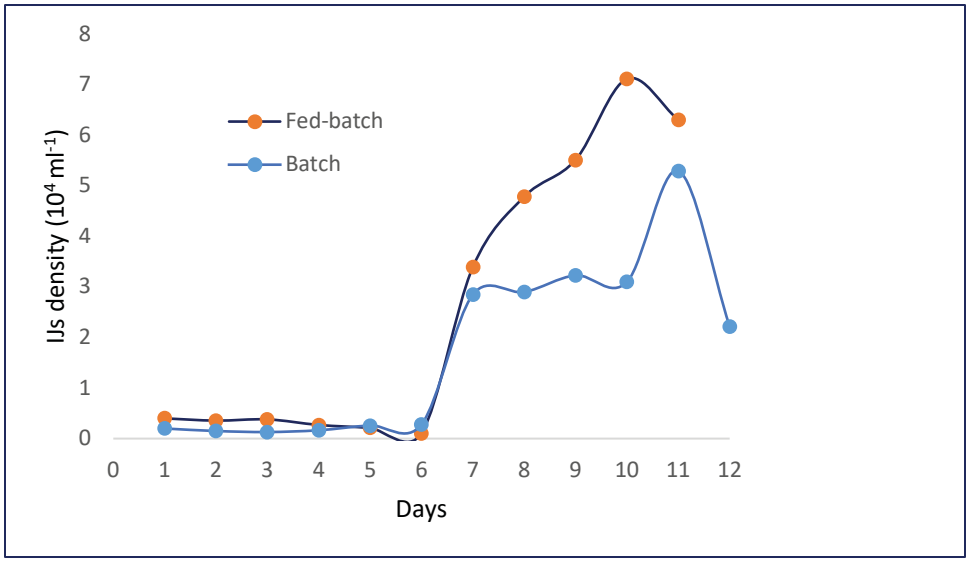

Figure 4-8: Mean population density of IJs of *Steinernema yirgalemense* in glucose fed-batch and batch monoxenic liquid cultures at 25°C, over a period of 11 days

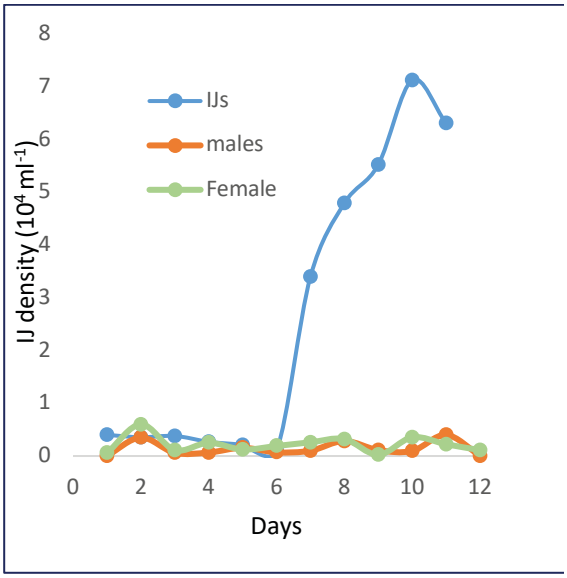

Figure 4-9 Mean population density of males, females, and IJs of *Steinernema yirgalemense* in glucose fed-batch monoxenic liquid culture at 25°C, over a period of 11 days.

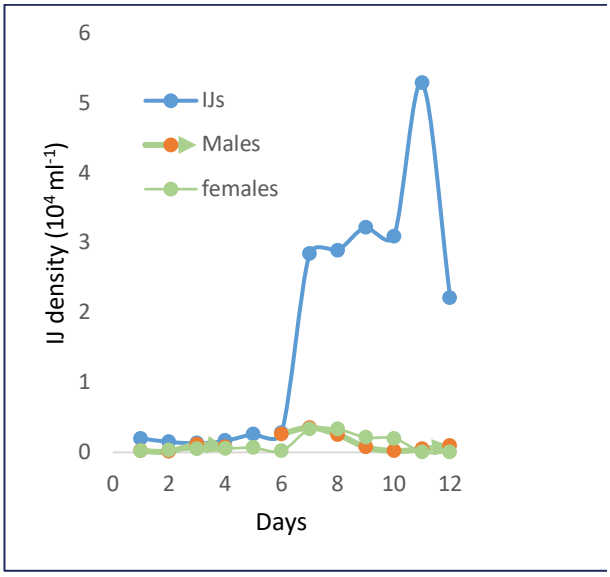

Figure 4-10: Mean population density of males, females and IJs of *Steinernema yirgalemense* in the batch monoxenic liquid culture at 25°C, over a period of 11 days.

The aim of this experiment was to investigate the impact of glucose on the growth and development of the nematodes, and more importantly, the final yield of the infective juveniles. The set-up of and the experimental procedure are previously described in section (4.2.8). The growth development of the IJs was assessed both in the batch and fed-batch fermenters simultaneously. The mean recovery rates of the IJs in the glucose fed-batch and batch cultures were observed at 68% and 75% respectively (Figure 4-7). At these recovery rates, the maximum population density of the IJs at the end of the cultivation for batch cultures reached an average of 53,000 IJs per ml while the fed-batch cultures recorded 71,000 IJs per ml (Figure 4-8), a percentage increase of 25%. The higher IJ yields obtained for the fed-batch could be attributed to the quality of 'food signal' excreted by the bacteria into the media as a result of the increased bacterial density. The concentration of the glucose was maintained between 0.2-0.3 g l^{-1} throughout the culture (Figure 4-6). This translated to the first males and females occurring on day 2 after IJ inoculation in the fed-batch cultures (710 males per ml and 790 females per ml) (Figure 4-8). The number of IJs and reproductive stages (adult males and females) of the nematodes in the fed-batch cultures also increased steadily from day 6, 9, 10 and 11 (4000 ml^{-1}) , with the maximum density of 71,000 IJs per ml occurring on day 10 (Figure 4-8). On the other hand, as a result of the low recovery rate recorded for the batch cultures (68%), the first females and males were observed on day 4 - 697 ml^{-1} and 281 ml^{-1} respectively (Figure 4-9). The second and third adult males and females were observed and also increased progressively from day 7 to 11 (2600 ml^{-1}), with the maximum density of 53,000 IJs per ml being observed on day 11 (Figure 4-8). For both cultures, there were two different days at which the maximum IJs occurred (day 10 for the fed-batch and 11 for batch cultures) (Figure 4-9 and Figure 4-10 respectively). The extended culture time of the batch cultures, compared to the fed-batches, may be attributed to the late times for the first male and female adults to occur. This may have delayed the mating cycles of the nematodes in the batch cultures. The early times of the occurrence of the first male and female nematodes in the fed-batch cultures could be attributed to the quality of the media to induce high recovery.

The results of the study were benchmarked with the experimental results reported by Gil *et al.* 2002 on glucose fed-batch cultures on *Heterorhabditis* nematodes. They reported a maximum yield of 3.62 $x10^5$ IJs per ml as against $7.1x10^4$ IJs per ml^{-1} obtained in this study. The difference in yield could be ascribed to so many factors including the different cultivation conditions, the size of IJ inoculum, the unit operation used (flask versus bioreactor) and the concentrations of glucose fed to the culture. They utilized Erlenmeyer flask for the propagation of the nematodes which in

reality provides suitable growth conditions for nematodes than in bioreactors (Ferreira, 2013). In bioreactors, the growth of the nematodes is subjected to the influence of agitation and higher inertial forces that consequently impair the mating cycles of the male and female nematodes (Chavarría-Hernández *et al.*, 2011). This negatively affects the final yields of a culture. Secondly, the initial concentration of IJs inoculated in the experimental work of Gil *et al.* 2002 was 5630 infective juveniles ml^{-1} in comparison to 2000 IJs per ml^{-1}, ~ 65% lower in average nematode density in this present investigation. Hirao *et al.* 2009 suggested that initiating cultures by inoculating higher inoculum size of nematode eventually lead to higher yields. Han *et al.* (1993) also observed an increase in final nematode biomass at higher densities of IJs inoculated. The lower density of IJs inoculated compared to that of Gil *et al.* 2002, therefore, hindered the achievement of a higher IJ yields in this study. Moreover, two different concentrations of glucose were fed to the bacteria during both studies. Gil *et al.* 2002 used a concentration of 25 g l^{-1}, while 10 g l^{-1} was added to the basal medium with intermittent feeding every 2 days. It is also possible that in the course of the glucose fed-batch fermentations in this investigation, there were instances of catabolite repression and substrate inhibition effects on the bacterial and nematode metabolism that contributed to the low yields in comparison to Gil *et al.* 2002. Thus, further optimization on the optimal glucose concentrations, time of feeding, as well as the feeding rate is warranted to achieve a higher yield, as it is one of the decisive factors for commercialization of nematode (Gil, 2002). Although the durations of both systems appear slender at the end, the production times can further be reduced to achieve efficient fermentations, minimize the overall cost of production and lessen the risk of eventual contamination associated with long production hours.

4.3.3 Batch and fed-batch fermentations of nematodes with bacteria

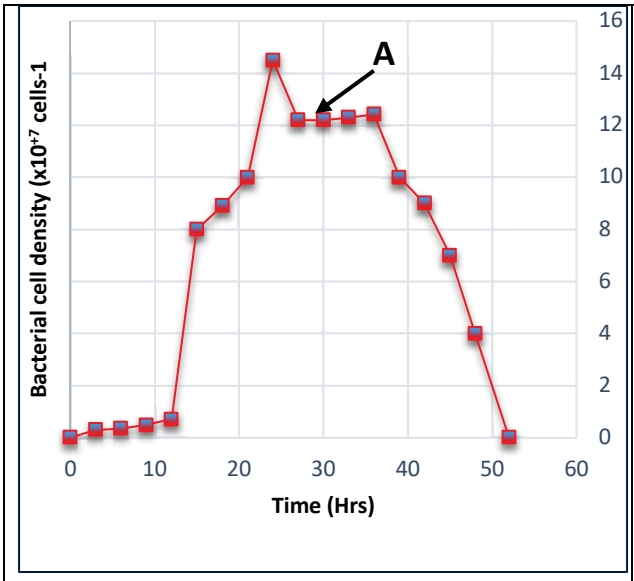

Figure 4-11:The formation of colony-forming units ml-1l of *Xenorhabdus* sp. in liquid culture before the inoculation of IJs

A-Point of inoculation of IJs

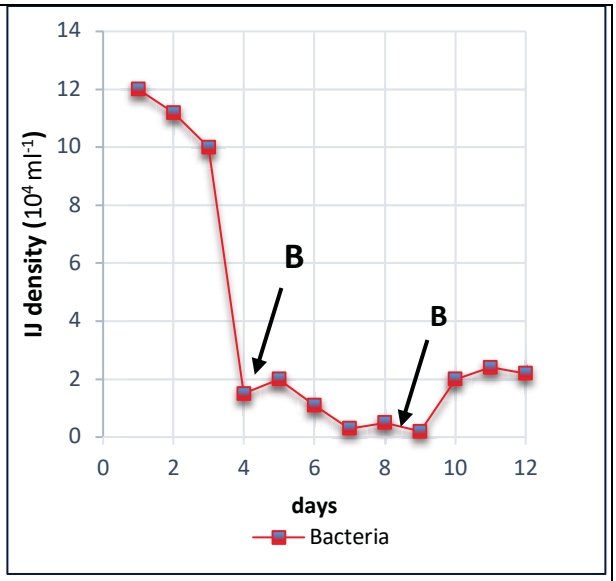

Figure 4-12: The depletion of colony forming units ml-1l of *Xenorhabdus* sp. in liquid culture measured over a period of 11 days after IJ inoculation

B-Point of low bacterial cell counts

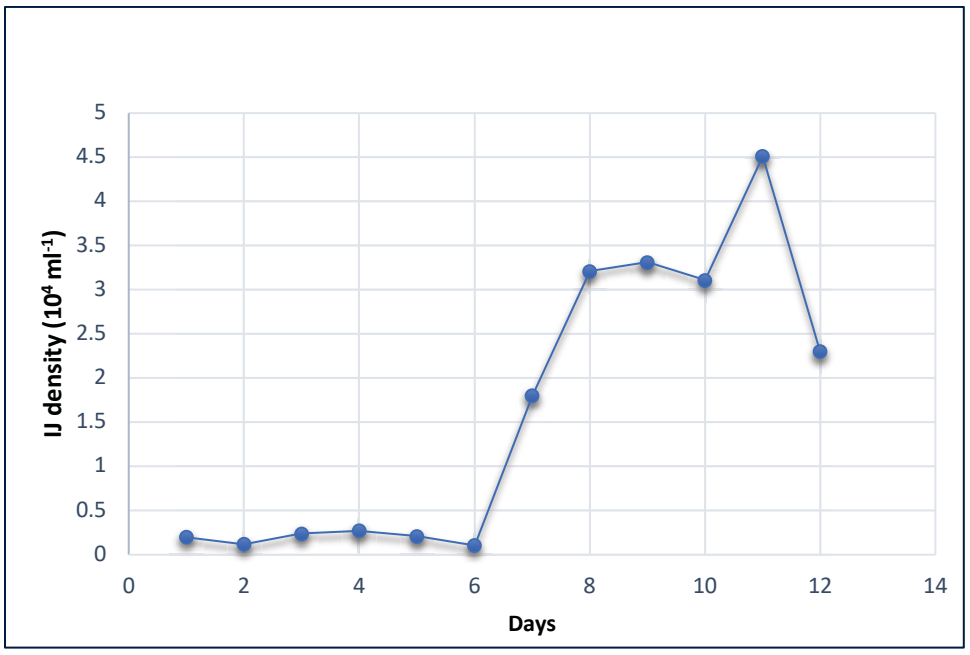

Figure 4-13: Mean population density of IJs of *Steinernema yirgalemense* in batch monoxenic liquid cultures at 25°C, over a period of 11 days

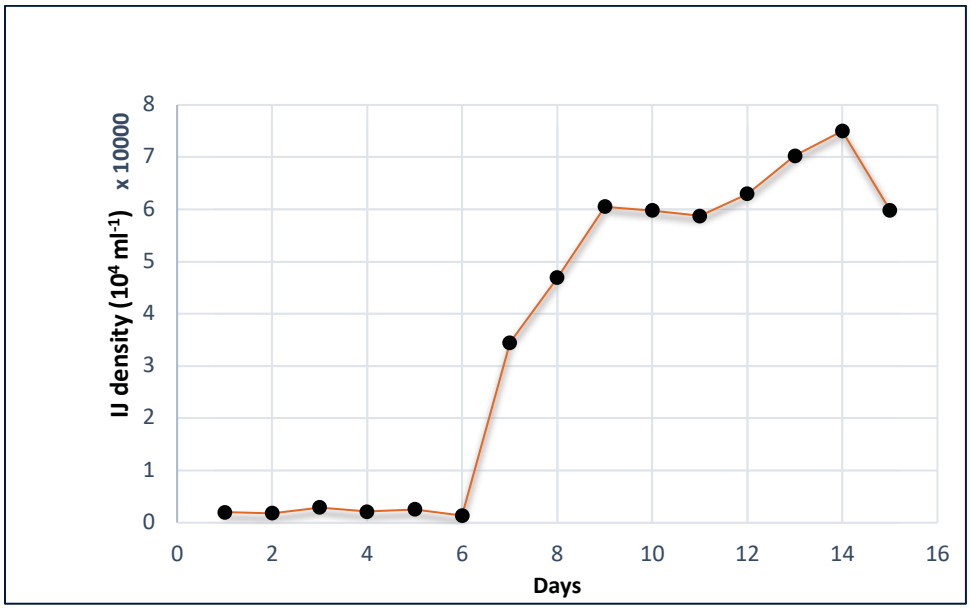

Figure 4-14 Mean population density of IJs of *Steinernema yirgalemense* in bacteria fed-batch monoxenic liquid cultures at 25°C, over a period of 11 days

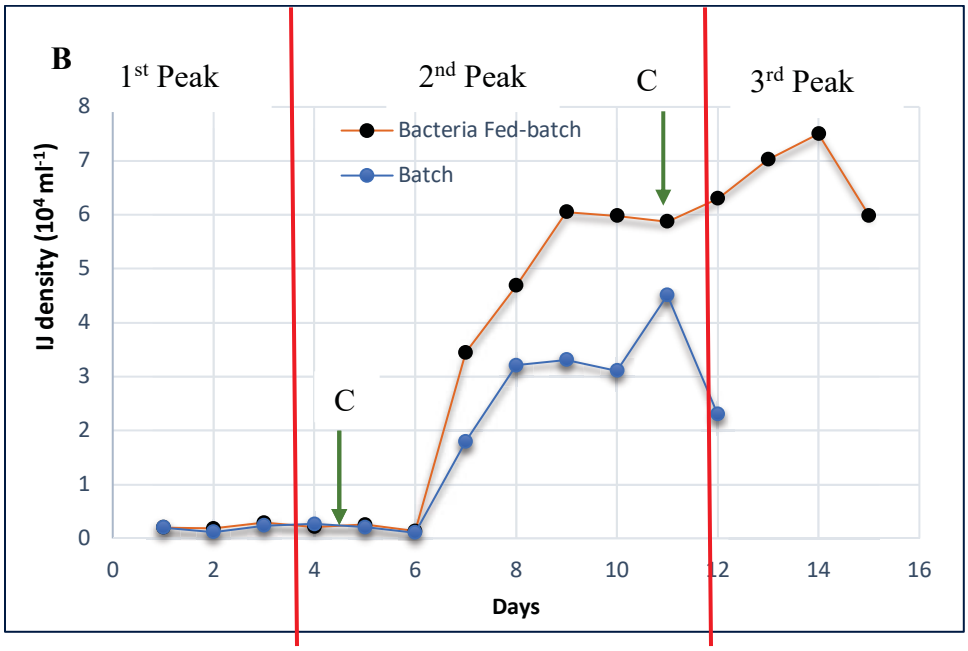

Figure 4-15: Comparison of growth level of infective juveniles counted over batch and fed-batch system

Maximum IJ count in batch culture at day 8 is 32100 IJs/ml

Maximum IJ count in fed-batch at day 8 after first bacteria inoculation 46900 IJs/ml

Maximum IJ count in fed-batch at day 16 after 2nd bacteria inoculation 75000 IJs/ml

C- Point at which a fresh feed of bacteria was added to the culture following low bacterial cell counts

The focus of this section was to examine the implications the addition of a fresh bacteria feed to existing cultures could have on the population dynamics of IJs in liquid cultures. Alexander (2011) suggested that due to the fast growth of the EPNs in an aerated environment, there is a quick depletion of the bacteria which serve as a food substrate for the nematodes. As a consequence, prior to this investigation, it was important to ascertain which days within the fermentation period has a low bacterial cell count in order to inform on when to inoculate a fresh feed of bacteria. Batch fermentations were therefore conducted to first address this issue before they were subsequently moved to fed-batch systems. The setup of the bioreactors and cultivation conditions follow the same experimental plan as discussed in the previous sections.

4.3.3.1. The Batch cultures

The measurement of the bacterial cell densities and the concentration of the IJs during the 11-days period of fermentation at 25°C is shown the Figure 4-12 and Figure 4-13 respectively. The bacteria was inoculated at an initial cell density of $2x10^7$ cfu per ml^{-1}, with the concentration reaching $1.2x10^8$ cfu ml^{-1} at the time of IJ inoculation (36h) (point A on Figure 4-11). After the inoculation of the nematodes (2000 IJs per ml), the average bacterial cell count measured over the 11-day period continued to decline linearly on each day (Figure 4-12). The quick depletion of the bacteria could be attributed to the feeding activity of the inoculated IJs. The bacteria produce food signals into the medium that cause the IJs to desist from the developmentally non-feeding state to a to a fully functional organism (Kooliyottil *et al.*, 2013). Following recovery, the infective juveniles (J3) feed on the growing bacteria population and develop into juvenile stage four (J4) then to the adult stage (Hirao and Ehlers, 2010). At the adult stage, copulation occurs, leading to the reproduction of the first filial generation (J1 and J2) of nematodes (Ehlers, 2001). The recovery rate was recorded at 60% after 4 days post-IJ inoculation. The measurement of the bacteria densities showed that the lowest cells count ($<1.5x10^7$ cfu per ml^{-1}) were on day 4, and 9 post IJ inoculation. The day 4 and 9 also coincided with the days the first and maximum females and males of the F1 generation occurred respectively (data not shown). The concentration of IJs recorded on day 8 was 32100 IJs/ml with a low bacterial cell density of $1.1x10^7$ cfu per ml^{-1}. The culture was allowed to run until day 11 where a maximum IJ concentration of ~45000 IJs/ml was recorded.

4.3.3.2. The influence of the bacteria on IJs: Fed-batch

Following the results of the batch cultures, further investigations were conducted to evaluate the impact of adding fresh bacteria inoculum to an existing culture. The bioreactor was set up following the same experimental procedures and growth conditions as the batch cultures, with the same densities of nematode (2000 IJs per ml) and bacterial cells ($2x10^7$ cfu per ml^{-1}) inoculated. A fresh feed of bacterial inoculum (50 ml) was prepared and added to the culture on day 4 and 11 post-IJ inoculation (point C in Figure 4-15. Samples of the culture were withdrawn on each day and the average IJ count recorded (Figure 4-14).

The results showed a remarkable increase in the IJ concentration in comparison to the numbers obtained for the batch cultures after day 8 of post-IJ inoculation. For the fed-batch cultures, the IJ concentration had reached ~ 47000 per ml after day 8 of post-IJ inoculation, an increase of ~32% in the concentrations recorded for batch cultures on the same day. This increase in IJ count provides enough evidence that the bacteria are not only significant in recovery processes but could be used to increase the average density of IJs. The maximum IJ count occurred on day 9 of the fermentation period ~61000 epns/ml, 2 days earlier than the batch cultures. Following the suggestion made by Ehlers *et al.* 2009 that the development of IJs in liquid cultures is as a result of unfavorable conditions such as over-population of nematodes in the medium. Overpopulation leads to the production of metabolic and nitrogenous wastes, such as urea, as a result of the active reproduction rates of the parental adults and the subsequent generation of nematodes (San-Blas *et al.*, 2014). This induces starvation conditions in the medium forcing the J3 stage to develop into infective juveniles. This hypothesis was tested by adding a fresh bacterium inoculum into the existing culture after day 11. The culture was allowed to run until day 16 where the concentrations of the IJs started to decline. The assessment of the culture showed a majority of J1 and J2 and a few J3 that were developed from the F1 parental adults. The second feeding of bacteria to the culture made enough food available to the nematodes as the bacterial cell density peaked thrice during the 16-days of the fed-batch fermentation cycle. The first peak occurred during the first 36h before the IJ inoculation, with the second and third peaks occurring after day 4 and 13 respectively (Figure 4-15). As a consequence, it resulted in two generational cycles with a maximum count of 75000 IJs/ml on day 16.

Benchmarking the results of this present investigation with those reported by Alexander (2011) during a fed-batch fermentation of Heterorhabditis nematodes shows a similar trend in terms of

the percentage of increase of the IJs from the day 11up to day 18. He reported an increase of 33% as compared to the 29% increase achieved in this study. It is plausible that the average number of IJs could increase further if the culture was allowed to prolong for more than 16 days. However, the percentage increase should be weighed against the cost incurred for running the bioreactors for the few extra days. Moreover, for mass production of nematodes, one generational cycle is often desirable in cultures as compared to two or more cycles (Gaugler and Bilgrami, 2004). Two or more generational cycles after production processes, often lead to a high proportion of non-IJs stages and adults that require necessary additional downstream processing (DSP) of the culture broth before formulation can be done. Inefficient DSP leads to contamination in the final products (Wilson, Pearce and Shamlou, 2001) and reduces the shelf life of the nematode product which is a challenge most nematode producers encounter after harvesting (Didik Sulistyanto, 2014).

Although the bacteria fed-batch cultures provided the highest yield in comparison to the previous investigations, the process time needed to reach the maximum IJ count (75000 IJ/ml) was longer (16 days) than any of the cultures investigated. Longer cultivation periods are often detrimental in terms of the probabilities of eventual contamination and high operating costs (Gil, 2002). The fed-batch liquid culture production could be further improved through optimisation procedures to increase the number of IJs per ml and decrease the population of non-infective juveniles.

4.3.4 Virulence Studies

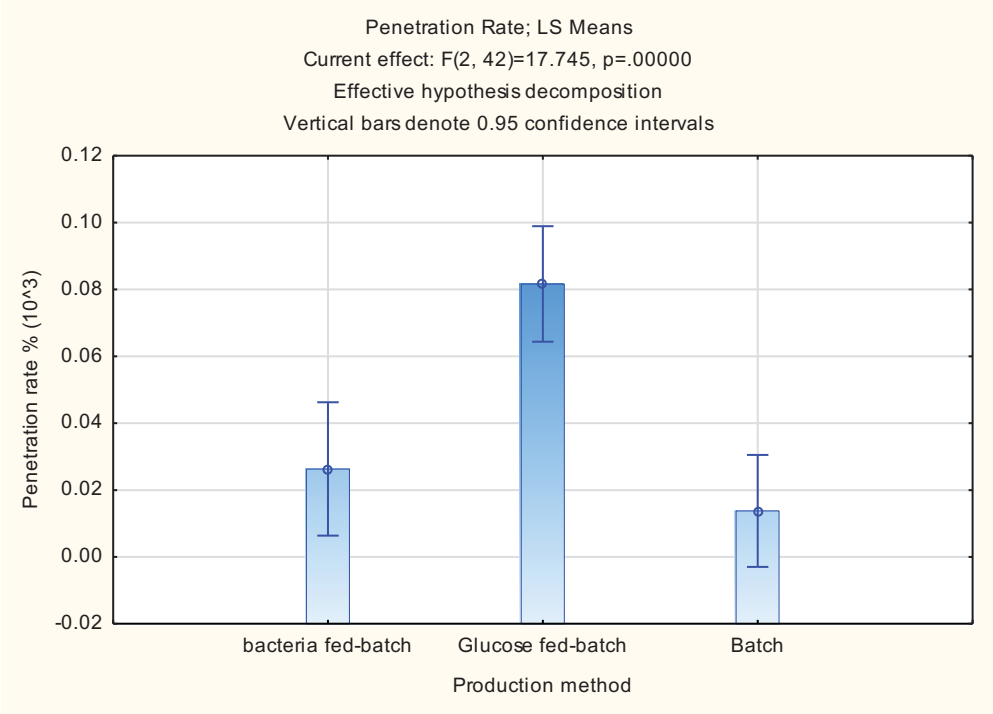

Figure 4-16: Comparison of the percent penetration rate of S. *yirgalemense* to *Galleria* at 250C for 24 hours

(One-way ANOVA; F (2, 13) = 1.888, P < 0.000025). Significant differences are indicated by different letters above vertical bars

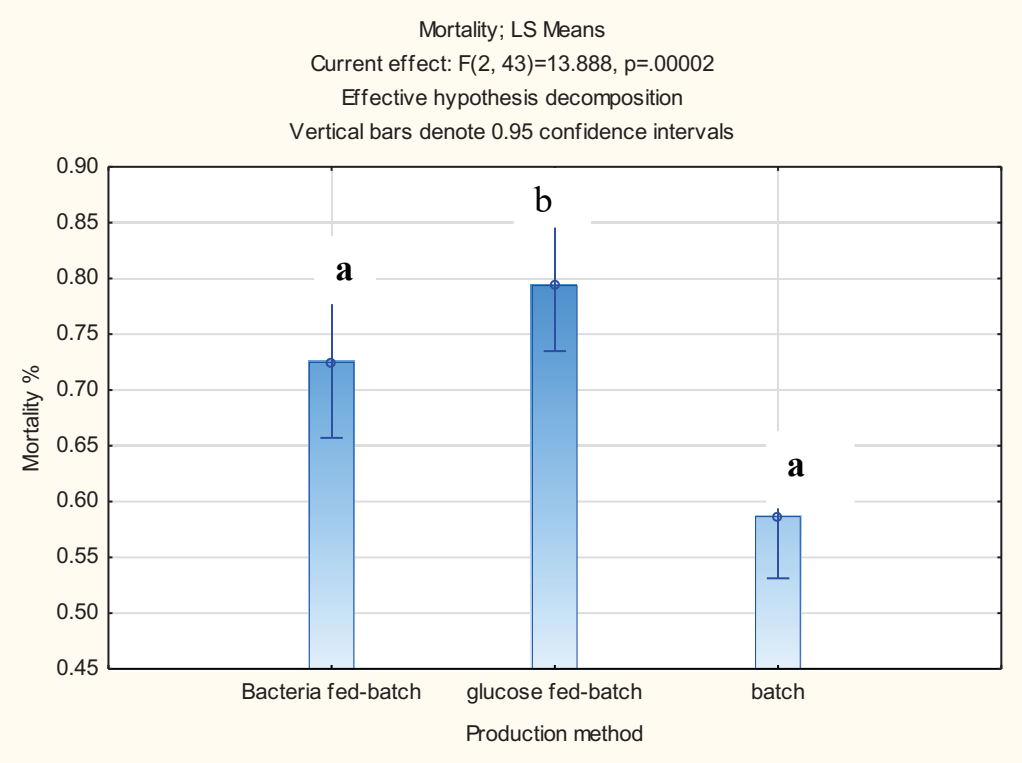

Figure 4-17: Comparison of the percent mortality of *Galleria* larvae exposed to S. *yirgalemense* at 25°C for 24 hours

(One-way ANOVA; F (2, 43) = 13.888, P < 0.00002). Significant differences are indicated by different letters above vertical bars

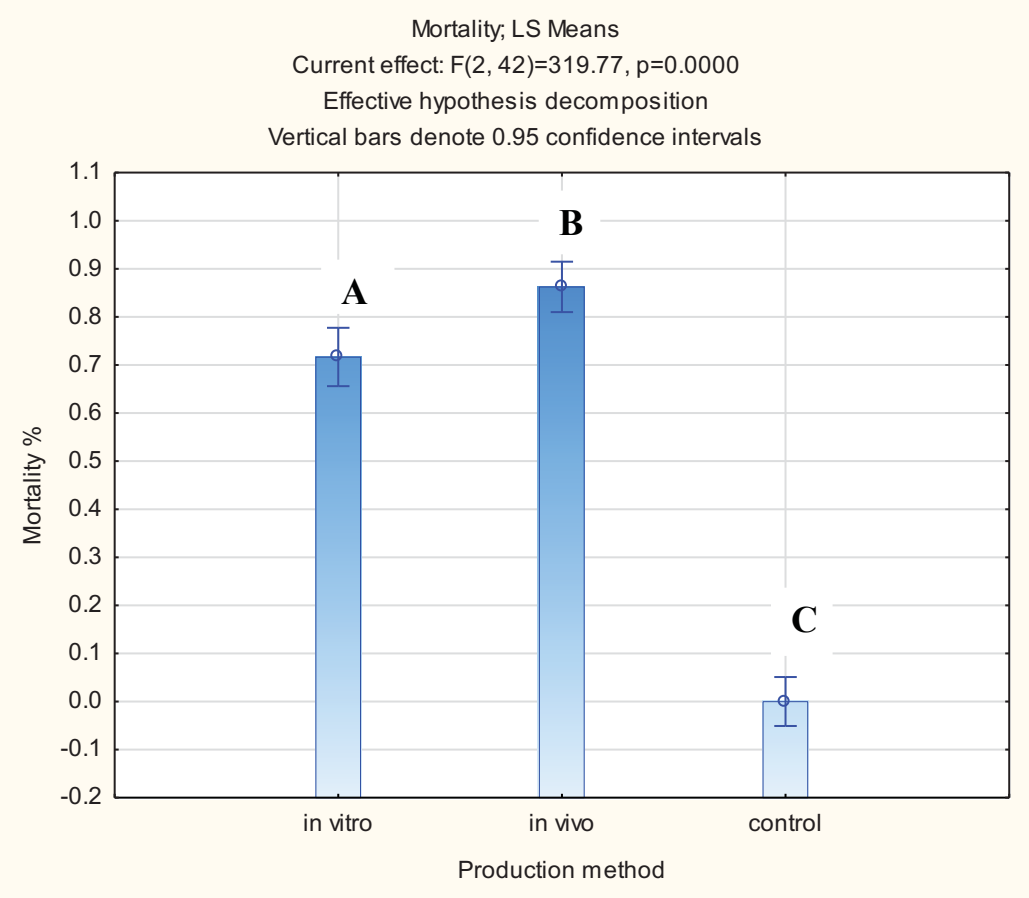

Figure 4-18: Comparison of the percent mortality of *Galleria* larvae exposed to S. *yirgalemense* at 25oC for 24 hours

(One-way ANOVA; F (2, 42) = 319.77, P < 0.00002). Significant differences are indicated by different letters above vertical bars

The virulence of the nematodes produced from each production methods was compared through a one-on-one bioassay with *Galleria* as described in section (4.2.10). Caroli *et al*. (1996) suggested that differences in pathogenicity of nematodes could be explained by their ability to penetrate hosts. The results from the one-way ANOVA showed significant differences between each treatment (fig), with the nematodes produced from glucose fed-batch *(F = 0.02633; df = 42; P = 0.000001)* achieving the highest penetration rate (Figure 4-16). There was no significant difference between bacteria fed-batch cultures and batch cultures in their abilities to penetrate host *(F = 0.8166; df = 42; P = 0.336)*.

The percent mortality of the hosts was assessed after nematodes from the three fermentation modes were exposed to *Galleria* at 25ºC. The results showed significant differences between the

nematodes cultivated from each of the systems (Figure 4-17). The mortality rate after 24 hours for glucose fed-batch, bacteria fed-batch, and batch cultures were 80, 73, and 60% respectively. The nematodes from the bacteria fed-cultures *(F = 0.02633; df = 42; P = 0.000124)* achieved a higher mortality rate than the batch treatment *(F = 0.01376; df = 42; P = 0.336)* probably due to the higher bacteria content in their bodies. Somwong (2012) suggested that a high number of bacteria within the body of nematodes quickens the proliferation rate of the bacteria in the body of the host, and caused more infections. Although the mortality rate recorded between the nematodes from the glucose fed batch *(F = 0.08166; df = 42; P = 0.00124)* and bacteria fed-batch cultures *(F = 0.02633; df = 42; P = 0.000124)* appear slender, the glucose fed-batch cultures caused more infections than the former. This highest performance of the nematodes produced from the glucose fed-batch could be attributed to the quality of the media in which they were propagated. According to Vanninen (1990), the virulence of nematodes is related to the total lipids content in their bodies. The feeding of glucose throughout the fermentation period might have increased the quality of the media, and thus amount of lipids in the bodies of the IJs in the media. This is the same reason why *in vivo* produced nematodes achieved a higher mortality (81%) in *G. mellonella* than the liquid cultures (72%). A comparison between the two in terms of their mortality rate showed significant differences *(F = 319.8; df = 27; P = 0.005)*.

The results emphasize the importance of finding the appropriate media for the propagation of the nematodes/bacteria complexes in order to obtain a high-quality nematode. In liquid cultures, the bacteria are prone to phase changes that make them lose some virulence attributes needed for pathogenicity and high field performance (Jeffke *et al.*, 2000). Abu Hatab and Gaugler (2001) cultured *Heterorhabditis bacteriophora* in artificial liquid media containing different lipid sources (insect, beef or lard). They studied the effects the quality and quantity of lipid content in a medium can have on the production time, quality of the IJs, and the yield nematode produced. The results showed that the medium supplemented with insect lipids recorded the highest population of nematodes compared to the medium supplemented with beef or lard. Additionally, the quality and developmental rate of nematodes in the insect lipid medium was 1.7 times faster than the medium supplemented with beef or lard. Moreover, the total lipid contents of a nematode define the shelf life, as it serves as a source of energy during the physiologically inactive state (Abu Hatab and Gaugler, 2001). San-Blas (2013) suggested that extreme temperature conditions increase the rate of metabolisms of the IJs in an open field, leading to the high rate of lipid consumption. Infective

juveniles produced fro *in vivo* often contain high lipids in their bodies and thus perform better than nematodes from liquid cultures.

4.3.5 Conclusion

The successful culturing of *S. yirgalemense in vitro* using fed-batch culture techniques resulted in gaining valuable information during this study. The average IJs per ml obtained in each investigation was affected by the type of fermentation mode adapted. On the whole, the fed-batch system with the glucose performed comparatively better in terms of total IJS/ ml, production time and mortality rate than the two other methods investigated (Table 4-2). Since the model organism (*S. yirgalemense*) is newly identified in South Africa, it is important to investigate factors that can promote the development of a stable bioprocess (liquid cultures) and also lead to the achievement of quality and higher IJ yields.

Table 4-2: Summary of experimental results for the three fermentation modes investigated

Experiment	Average yield (10^3 ml^{-1})	Recovery %	Days to Maximum IJ count	Mortality %
Glucose				
Batch	53	68	11	60
Fed-batch	71	75	10	80
Bacteria				
Batch	45	60		60
Fed-batch	61	-	9 (on 1st addition of bacteria inoculum day 4)	73
Fed-batch	75	-	16 (on 2nd addition of bacteria inoculumday 11)	73

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Chapter 5

Techno-Economics Assessments of Nematode Production

In this chapter, the two production methods are compared in terms of production cost, for a facility with a production capacity that is linked to the market opportunity. Technical process descriptions and performances based on the experimental work with the *in vivo* and *in vitro* production methods were used to determine the size of a production facility, required to service a production target of $4.26x10^{12}$ EPNs per year. The resulting basic engineering designs of facilities were used to estimate capital and operating costs as well as revenues, allowing the assessment of total production costs and investment viability for the two production routes, assuming that the process performances observed at lab scale will be reproduced at industrial scale.

5.1. Factory Design Criteria (*in vivo* and *in vitro*)

5.1.1. Site selection

The establishment of a nematode facility should be sited in locations that have proximity to agricultural farms, and have continuous access to water supply and electricity. Nematodes are sensitive to temperature changes during and after cultivation. Frequent power surges during culturing often lead to low yields and inferior nematode products. For the *in vitro* method, a minimum of 3 to 5 h of electricity is required at critical production stages each day while *in vivo* method need 1 to 3 hours (Holmes *et al.*, 2015). Electricity is needed for incubation, maintaining sterility in laminar flow cabinet during inoculation processes, and more importantly, maintaining strains and virulence stock. Additionally, production in both methods requires a high volumetric supply of water for the establishment of liquid culture media (*in vitro*), harvesting and cleaning (*in vivo* and *in vitro*). Holmes et al. 2015 pointed out that, irrespective of the production method, a nematode factory should be 120 to 180 m^2. This eases the flow of work, and it is also independent of the production output rate. The layout of a typical *in vivo* and *in vitro* mass production factory of EPNs according to Holmes et al. 2015 is discussed in section (5.3)

The amount of labour needed in the factory is dependent on the production method. *In vitro* requires personnel with a high technical expertise due to the strict process conditions associated with the production route. Contrarily, *in vivo* require semi-skilled labour due to the low level of operations with the production stream. Notwithstanding, both methods require at least a full-time entomologist who would assist in the cultivation processes.

5.2. Design Approach

The techno-economic evaluation is therefore performed in the following stepwise manner:

- The development of a process model
- Mass balances on the nematode
- Sizing and costing of major equipment (*in vivo* and *in vitro*)
- Profitability and sensitivity analysis

5.2.1. Process Description

In order to achieve a successful nematode production, detailed information on the entire cultivation process is warranted. This includes information on the production target, unit operations, equipment sizing as well as operating conditions that impact final yields of the nematode products.

5.2.2. Process Overview

The process of nematode production via the *in vitro* (liquid culture) and *in vivo* includes all the operations from the establishment of seed cultures, i.e. nematode and/or bacteria inocula to the final stage of formulation and packaging. A detailed description of each production step at the laboratory based on the experimental procedures described by Surrey et al. 1995 is given in materials and method section(4.2) of the in vitro experiments. Production processes such as formulation, assessment of yield, packaging, and storage are generic and do not depend on the production method used to obtain the nematodes.

5.2.3. Mass Production: *in vitro*

5.2.3.1. Process Overview

Based on the assumed production target, 10000L fermenter is required to produce sufficient nematode to achieve the production target. The seed cultures are carried through a series of scale-up in shake flasks and seed fermenters until enough IJs are obtained to establish seed inoculum for the 1000-L bioreactor. For the production of nematodes in a 10000 -L fermenter, a series of the complete production cycle (11-13 days) in seed reactors (10-L, 150-L and1000-L) are done in a cascade order before the final fermentation broth from the 1000-L fermenter is aseptically pumped into the 10000-L fermenter. The rule of thumb is to transfer the seed cultures once enough IJs have been produced to serve as inoculum for the preceding fermenter. This action greatly reduces the ideal production cycle of 57 days. Notwithstanding, prior to each transfer, the symbiotic bacteria ($\geq 10^7$ cfu ml^{-1}) must be pre-cultured in the subsequent reactor for 36 hours to produce the important 'food signals' needed for the recovery of the IJs in the LCM. The established bacterial culture ($\geq 10^7$ cfu ml^{-1}) must be checked for purity and phase stability before they are inoculated into the fermenter for pre-incubation at 30°C. The air flow rate is adjusted at this stage to ensure that enough oxygen is available for the bacteria. After 36 hours of incubation, the temperature of the culture is adjusted to 25°C before the growing culture is aseptically pumped into the next bigger fermenter. The culture is allowed to prolong for 11-13 days before the next scale-up operation. Ideally, a total production cycle through scale-up operations takes a maximum of 57 days to complete. Process parameters such DO content should be maintained at least 30% saturation, pH between 5.5 and 7 and temperature must be kept at 25 °C throughout the production cycle. Agitation is dependent on the production fermenter and should be maintained at a reasonable rate (100-1000 rpm) to reduce the effects of shearing on the nematodes. Additionally, sterility around culture should be maintained at all times to avoid contamination. After cultivation, the harvested IJs are cleaned and stored to initiate a new fermentation run. The flow chart of the scale-up procedures as well as the schedule of the fermenter to produce nematodes is illustrated in the fig below. With a complete cycle of 11-13 days (2 days for clean-up operations) in each fermenter and 330 working days per year, a total of 6 runs is estimated to be completed in per annum.

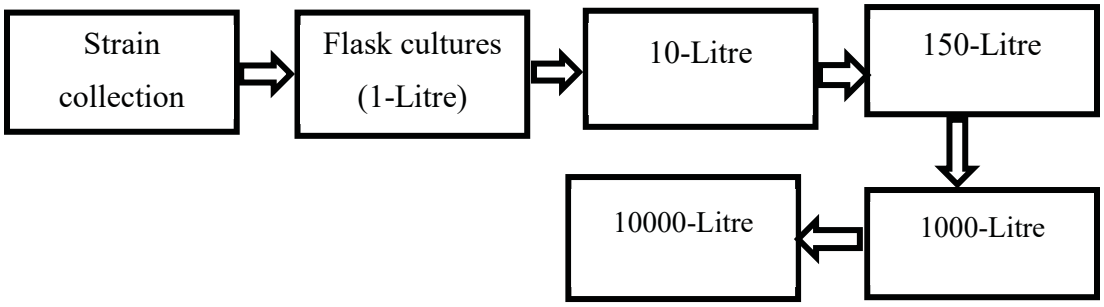

Figure 5-1: A flow chart of nematode production process

The duration per cycle for nematode production in the 10,000-L fermenter is greatly reduced after sufficient seed cultures (IJs) have been established for subsequent runs, as sub-culturing practices can be done at this stage. For example, fermentations in the 10-L seed fermenter can be stopped/removed from the production chain by inoculating directly into the 150-L fermenter. However, the entire production process is repeated when stock cultures become contaminated or lose some important attributes such as pathogenicity and virulence. The nematode and the bacteria strains can be stored for future sub-culturing practices through cryopreservation technique (Bai et al. 2004)

Downstream operations such as separation, formulation, and storage after production are critical for a successful nematode commercialization. The characteristics of the complex fermentation broth, which usually contains different components including non-IJs stages, bacteria, cuticles and dead nematodes need to be removed before formulation to avoid contamination. Nematodes are formulated to preserve their viability during transportation and also ensure a long shelf-life during storage. Storing nematodes in a less oxygenated environment results in a high mortality rate due to the deprivation of oxygen. The harvested nematodes may be retained in distilled water in flat bottles with adequate aeration at 5-14 ^{0}C for at most 2-3 weeks.

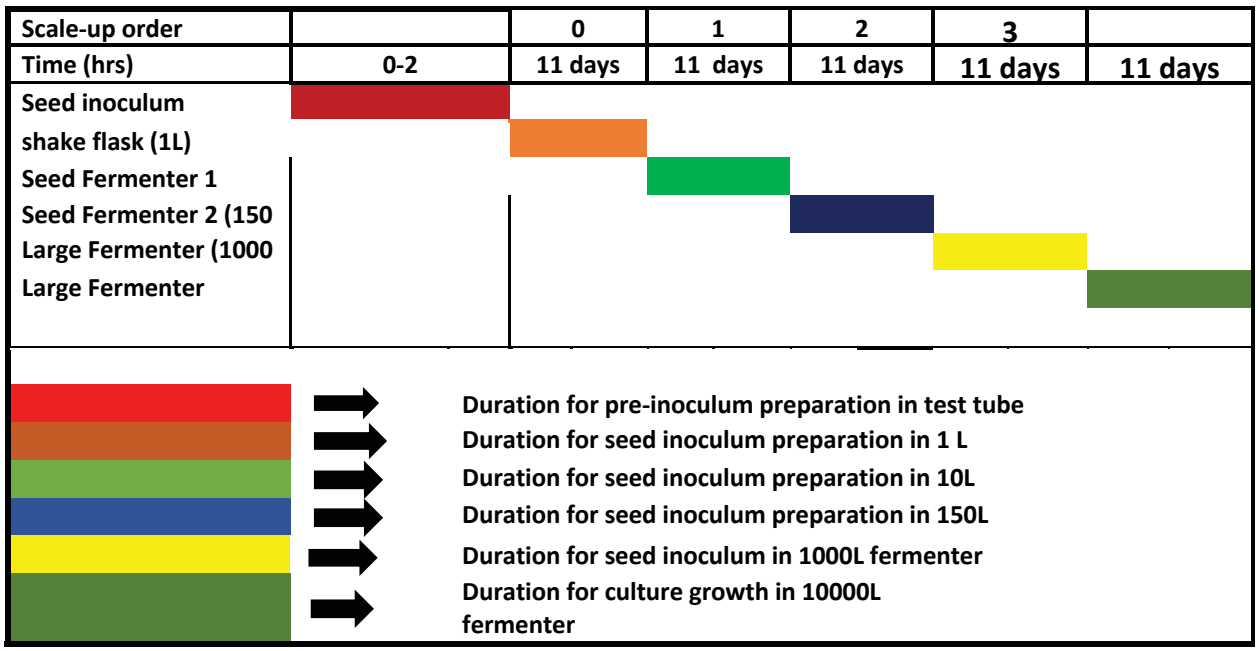

Figure 5-2: Production schedule for nematode production facility for a total production cycle of 57 day

5.2.3.2. Formulation of pre-inoculum and LCM

The nutritional requirements of nematodes include basic building blocks such as nitrogen, carbohydrate, protein, and fat and oil sources. The mass compositions of the various ingredients required for industrial formulation of the LCM in each production step for the propagation of the nematode are shown in Table. For production of nematode in a 1L Erlenmeyer flask at the laboratory, the various volumetric quantities of the pre-inoculum are used (Table 5-1)

Table 5-1: Pre-inoculum for establishing 1-L of monoxenic nematode culture

	Volume (ml)	Quantity (ml)	Total
Starter culture	50	2	100
Seed culture	100	1	100
Production culture	800	1	800
Total			**1000**

Table 5-2: Industrial Mass compositions of medium components for the formulation of production culture (LCM) at the various production stages during scale-up for the propagation of nematodes

Medium components	Conc (g/L)	Mass (g) per 10 L	Mass (g) per 150 L	Mass ('000g) per 1000 L	Mass ('000g) per 10000 L
Yeast extract	15	150	2250	15	150
Peptone	5	50	750	5	50
Soy powder	20	200	3000	20	200
NaCl	4	40	600	4	40
KCl	0.35	3.5	52.5	0.35	3.5
CaCl2	0.15	1.5	22.5	0.15	1.5
MgSO4	0.1	1	15	0.1	1
Veg oil	46	460	6900	46	460

Source: (Ehlers *et al.*, 1998).

5.2.4. Mass Production: *in vivo*

5.2.5.1. Process Overview

The mass production through the *in vivo* require a lot of hosts usually, the larvae of *Tenebrio* (mealworm) or *Galleria* that can be mass reared in-house on artificial diet (wheat bran). The starter culture of the host can be obtained at most pet shops. During rearing, foodstuffs such as apple, carrots and potato slices are placed on top to maintain moisture content within the rearing container (Nyamboli, 2008). *Tenebrio* Adult mealworms are transferred into a separate bowl to lay eggs. The eggs are collected and incubated at 25 ^{o}C to repeat the production cycle. This method is an adaptation from Woodring and Kaya (1998). The hosts are then inoculated with the infective juvenile stage of the nematode, followed by a period of incubation (2-4 days) before they are transferred into an incubator for 12-14 days conditioning process (Gaugler *et al.*, 2002b). During emergence period, the IJs are washed with gravity flow of water as described in the LOTEK system in chapter 3. The harvested IJs can either be formulated or stored in an aerated tank at the cooler room within the production facility. A block flow diagram of the *in vivo* production process, as well as process schedule, is shown in (figures) below. Based and the productivity results from the *in vivo* experimental trials conducted in the lab, the proposed facility can cater for 16 runs per annum to meet the production target of $4.26x10^{12}$ EPNs. The production process and the schedule for each run are shown in Figure 5-3 and Figure 5-4 respectively.

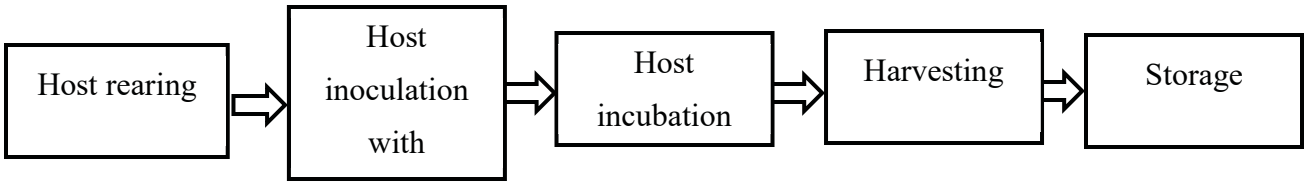

Figure 5-3: *In vivo* nematode production process

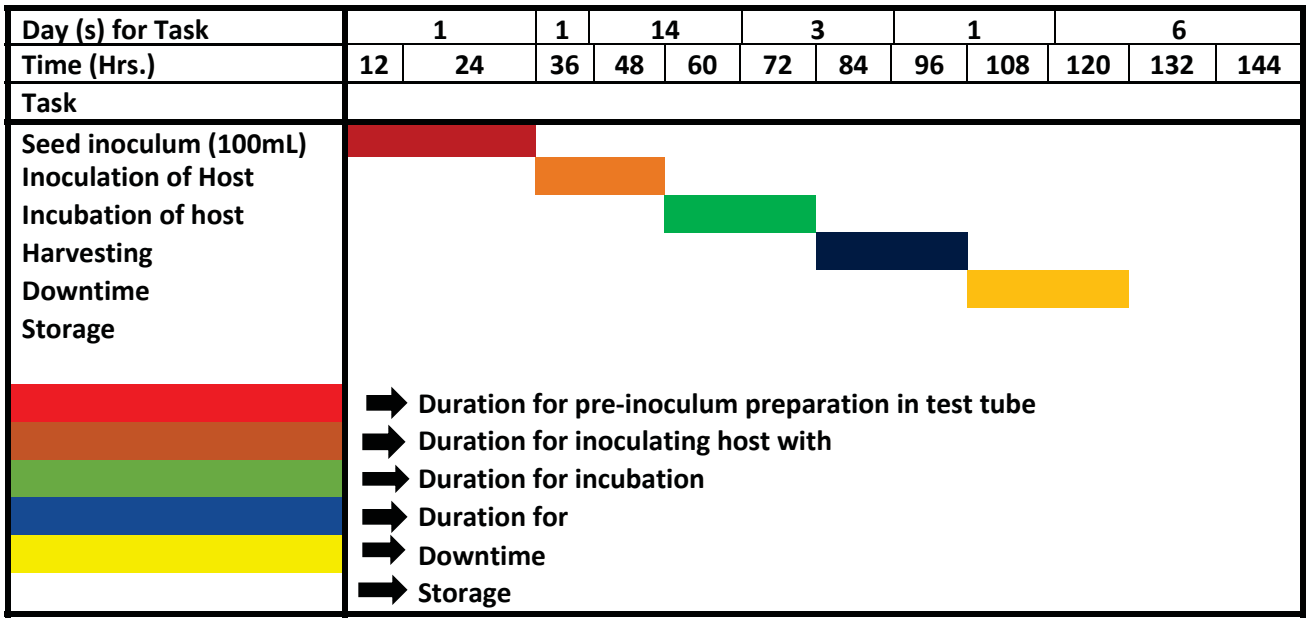

Figure 5-4: production schedule per run for nematode *in vivo* production facility.

The length of bars is not a reflection of the duration of each task

5.3. Criteria for External and Internal Factory Design for nematode production

5.3.1. Description of the Layout of In *In vivo* Factory

Office	Storage room	Host insect (adult) rearing room	Host insect (larva) rearing room	Autoclaving room	Media preparation room	Nematode culture room (on larvae)	Cool storage room	Harvesting Room	Formulation, packaging and quality control

Figure 5-5: Figure: Layout out option for establishing an *in vivo*- based production factory according to work flow for the rearing of *Tenebrio* on industrial scale

Thick wall indicates insulation from one room to another

The proposed facility for mass production of EPNs on *Tenebrio* is illustrated in Figure 5-5 based on the description given by Holmes *et al.* 2015. The host larvae rearing-room, as well as the nematode culture room (on larvae), must be designed as a temperature and humidity-controlled rooms. This saves on costs of purchasing many incubators for conditioning of the larvae. It should be positioned away from the direct effect of the sun within the facility as extreme temperature conditions during the summer can affect the normal performance of the air-conditioner as well as increasing the rate of energy utilisation. This reduces the overall cost of energy utilization per cycle of production. The nematode culture room (on larvae) and the cool storage room within the facility should be insulated from the other rooms to reduce the level of cross-contamination during production and storage. However, the insulation is not needed if the insect host is regularly sourced from the external producer. The cultivation room must be well-ventilated to mitigate the possibility of ammonia accumulation in the room due to the active metabolism of the nematodes and the host. Notwithstanding, the windows should be well-sealed to keep away flying insect such as midges and vinegar fly (anecdotal observation from the laboratory). Their activities on the host can impact negatively on the final production yield of a batch culture. Flat opened-trays designed in a similar fashion as the LOTEK system is used as a rearing tray for the host. These trays should be sized larger enough to accommodate the required number of host to achieve the production target. The media preparation room should be kept as far from the host insect rearing room and should be

insulated as contaminated inoculum reduces the level of patent infections and waste insects. The cool storage room should be kept at 8-14^{0}C at all times during storage by installing cooling systems. It must be well-insulated to reduce the influence of external environmental conditions on the humidity conditions in the room.

5.3.2. Description of the Layout of In *in vitro* Factory

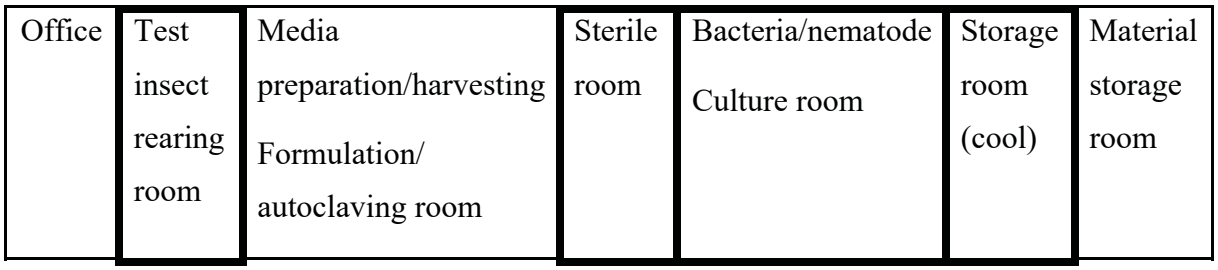

Figure 5-6: Figure: Layout options for establishing a liquid *in vitro* –based mass production factory for entomopathogenic nematodes according to workflow

**Thick wall indicates insulation*

Maintaining sterility in *in vitro* cultures is more critical than *in vivo* cultures. The production facility includes a sterile room, bacteria and nematode culture room, insect rearing room and media preparation room (Figure 5-6). The sterile room functions as the main production unit where *in vitro* mass culture of the nematode is done. It houses the production facility such as a bioreactor, laminar flow cabinet, media flask, and bottles. This room must always be connected to an incessant supply of water and electricity during cultivation periods to ensure successful runs. Ventilation in and out must be limited as all windows are often shut and well-sealed. Preferably, it must be isolated from other rooms in the facility especially the insect rearing room to reduce the level of cross-contamination. The bacteria and nematode culture room is where the seed cultures for initiating production are prepared. The room should be well-ventilated since it is often installed with orbital shakers for incubation of the seed cultures. Active cultures generate a lot of heat due to cell metabolism (Holmes *et al.*, 2015), and accumulation of heat in the room can affect the seed cultures. Incubation is done at 25-30^{o}C based on the species and often kept dark for the optimal growth of the bacteria cultures. Other room requirements common to both production methods

include the formulation and quality control room. The quality control room is often equipped with stereomicroscopes to assess the quality of the bacteria, as well as performing one-to-one bioassays to ascertain the virulence of the produced nematodes and the stock.

A summary of the various dimensions and room design for a typical industrial factory is given in Table 5-3 below.

Table 5-3: Room requirement for the *in vitro* and/or *in vivo* production process of EPN

Factory rooms	Temperature °C			water	Light	Sanity	Air/ventilation	land size
	Min	Opt	Max					
Media preparation room	n/i	n/i	n/i	Yes	Yes	n/i	some	20-
Host insect rearing room: Larvae (*Tenebrio*)	20	28	32	No	Little	Clean	A lot	>40
Host insect rearing room: Adult (*Tenebrio*)	20	28	32	No	Little	Clean	Some	10-
Autoclaving room	n/i	n/i	n/i	Little	Little	Clean	A bit	5-10
Clean (sterile) working room	10	n/i	30	n/i	Yes	Sterile	No	8-20
Bacteria culture room	15*	25*	28*	n/i	No	Very	Yes	15-
Nematode culture room	15*	25*	28*	n/i	No	Very	Yes	15-
Cool storage room (nematode storage)	4(0***)	8-15*	18	n/i	No	Very	No	5-20
Cool storage room or fridges (bacteria short storage	4	4*	10	n/i	No	Very	No	1-2
Deep freezer (bacteria long term storage 1-3 years)	n/a	-80	n/a	n/i	n/i	Very	n/i	n/a
Freeze dried storage (bacteria long term storage 1-3	n/i	≤ 5	20	No	No	N/A	No	n/a
Harvesting room/formulation and packaging room	4	22	25	Yes	Yes	N/I	Some	10-
Material/media storage room	4	22	28	No	Yes	Clean	No	8-16
Quality control room	4	22	28	No	Yes	Clean	No	8-16

n/i = not important, n/i = not applicable; * depends on species/strain; ** Not possible in areas with power cuts; *** Ice water fluid storage possible with some nematode species . All rooms need electricity supply. Rooms for bacteria work only needed for in-vitro method. Multiple insect rearing rooms only needed for in-vivo method, except of one small insect rearing room for quality control

Source: Holmes et al. 2015

5.4. Equipment Selection and sizing

5.4.1. *In vitro* Technology

The sizing of the production facility for *in vitro* is based on an annual production target of $4.26x10^{12}$ EPNs per year. A full production cycle of EPNs takes a maximum of 11-13 days to complete, with two days for establishing bacteria and nematode inocula. Based on the productivity from *in vitro* experimental work (71,000 EPNs/ml), six production cycles per annum in the 10000L fermenter is required to produce sufficient nematode to achieve the production target. This requires a total culture production volume of 60,000-L.

5.4.2. *In vivo* Technology

For mass production of EPNs for commercialization through the *in vivo*, a temperature and atmosphere controlled room are adopted to avoid the capital expenditure of many incubators. In order to meet the annual production target of $4.26x10^{12}$ EPNs using the yield 27000 IJs per ml results (27000 IJs per ml) from the LOTEK tray system, various assumptions were made to achieve the production target.

1. A room dimensions of 730 cm x 400 cm x 250 cm (29 m^2).
2. Each run produces 2.7x1011 EPNs based on an effective cultivation area (stacked-trays) of 150 m^2
3. An estimated total number of 3.7×10^7 insect host per annum is required to achieve the set production target.
4. Each production cycle takes a maximum of 21 days including downtime (Figure 5-4)
5. A standard application rate of EPNs of $5\text{-}7.0 \times 10^8$ IJs/hectare (e-nema, n.d.)

5.4.2.1. Tray System

Based on the production target, trays of dimensions (500 cm x 100 x 20 cm) are stacked together to form a trolley system to aid mobility from one point to another. Each trolley system contains ten stacked trays with a total surface area of 500,000 cm^2. On top of each trolley system is a network of pipes connected to a water supply that provides water for the washing of the emerging nematodes. Dispensed water is collected in a large tray, which is angularly placed underneath the perforated trays to ease the flow of water through a large diameter pipe connected to its side. Also, equipped on either side of the trays are nozzles that provide misted water onto the nematode-infested cadavers to keep them from desiccation. Additional units installed in the room include a temperature-controlled system (air-conditioner) and a relative humidity control system. A summary of the final design parameters and graphical presentation of the trays configuration are shown in Table 5-4 and Figure 5-7: Orthographic projections of the Room Technology.

Table 5-4: Summary of the final design parameters

Conditions in the Room	85% humidity 25°C
Room Dimensions	730 cm x 400 cm x 250 cm
Volume of harvested nematode water required	10 m^3
Estimated yield based on lab trials with LOTEK	27000 EPNs/ml
Number of EPNs required per run	2.7 x 10^{11} EPNs
Dimensions of Trays required	500 x 100 x 20 cm
Surface area of trays required for cultivation	1,500,000 cm^2
Estimated number of *Tenebrio* larvae required per tray	76000
Estimated number of insect hosts required on total trays per run	2,300,000
Estimated number of insect hosts required on total trays per annum	3.7 x 10^7

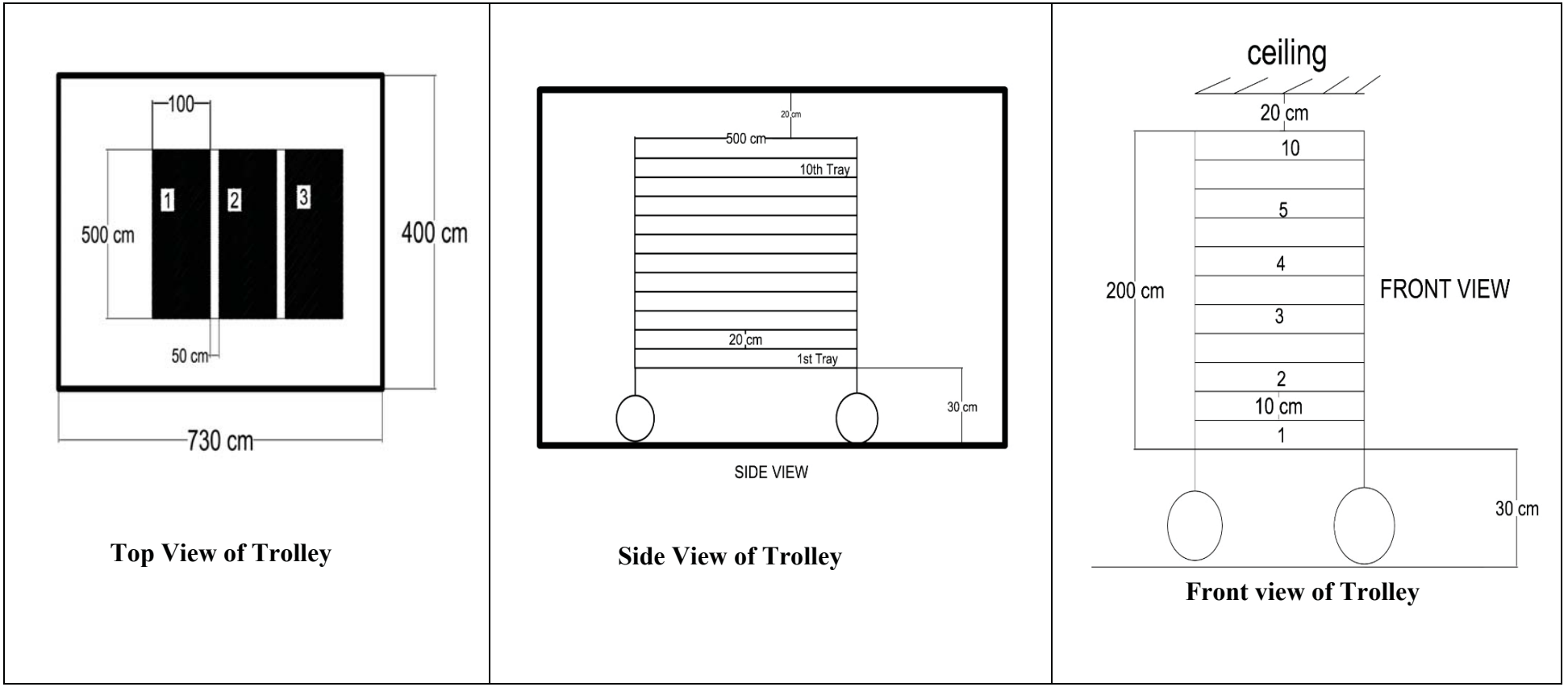

Figure 5-7: Orthographic projections of the Room Technology

5.5. Cost Evaluation of Processes

In this section, an investment case analysis is made by comparing the capital and operating costs of an *in vivo* and *in vitro* facility to potential revenues, and also to inform on the economic performance of each of the processes. The cost evaluation in this study was based on the following assumptions.

1. The plant life was estimated as 20 years
2. Total operational days per year of the plant is 330
3. Operational Hours per day is 24
4. Revenue flow is, based on the cumulative selling price per hectare (no escalation)
5. Tax requirement in South Africa is 28%
6. Working capital is estimated as 5% of Fixed Capital Investment (FCI) (Peters and Timmerhaus, 1991).
7. Average annual inflation rate in South Africa is 5.70% (July 2016)
8. The plant is 100% equity financed
9. Discount rate of 10%
10. Exchange rates $1 = 14.51 (Nov,2016)
11. Salvage value is zero at the end of the project life
12. The market selling price of EPNs per billion was set at R 1500 [Clement Owusu Prempeh, E-mail to Keith Danckwerts [Online], 18 Nov. Available E-mail: 19579500@sun.ac.za]

5.5.1. Costing of equipment (*in vivo* and *in vitro*)

Based on the production target and various unit operations requirement, the total capital expenditures (CAPEX) for *in vivo* and *in vitro* technologies was estimated at R 2.34 MRand (Table 5-5) and R 53.5 MRand (Table 5-6) respectively. The major contributing costs for the *in vivo* included the cost of building, land, temperature control system, trays, piping, and storage vessels. On the hand, the cost of fermenter, seed reactors, chiller unit constituted the bulk of the CAPEX of *in vitro*. Production units such as compressors, filtration unit, and formulating apparatus are common to each production technology, therefore contributed equally to the capital costs.

The cost factor for the estimation of major equipment was obtained from (Towler and Sinnott, 2008). Otherwise, quotations from trusted online traders such as Alibaba.com® and Gumtree.co.za were used.

The cost of some equipment which quotations could not be attained were estimated by updating the prices and capacities of similar equipment reported in the literature based on {Equation 5-1}and {Equation 5-2}

$$\text{New cost} = (\text{Base cost})\frac{(New\ year\ cost\ index)}{(Base\ year\ cost\ index)} \quad \textbf{\{Equation 5-1\}}$$

$$New\ cost = (Base\ cost)(\frac{New\ size}{Base\ size})^{n} \quad \textbf{\{Equation 5-2\}}$$

Where n is a scaling factor usually within the range of 0.6 to 0.7. A factor of 0.6 was used in most cases

Table 5-5: Capital cost estimation for the room technology (*in vivo*) producing 4.26x1012 EPNs per annum

Description		**Cost ('000**
Relative Humidity Control System		45
Air Conditioner		52
Building		132,
Concrete Slabs with Electrical Works		105
Total Plant Direct Cost (TPDC)		**334**
Total(Purchased equipment)		1,220
Cost Estimate	cost factor	cost
Design and Engineering	0.05	61
Utilities/offsite	0.1	122
Contractors fee	0.05	61
Contingency	0.05	61
Tray construction	0.2	244
Piping	0.1	122
Total Plant indirect cost (TPIC)		**671**
Purchased Equipment	nr	
Compressor	1	50
Storage vessels	2	50
Filtration unit	2	100
Chiller unit	1	800
Autoclave	1	70
Formulating unit	1	50
Total Purchased Equipment (TPE)		**1,220**
Fixed Capital investment (FCI)		**2,225**
Working capital		**111**
CAPEX		**2,336**

Table 5-6: Capital cost estimation for in-vitro 10000-L facility producing 4.26x1012 EPNs per annum

Cost Component	Cost factor	Cost (MRand)	Reference
Purchased equipment delivered	1	12.6	Sinnott (2008)
Installation/Equipment erection	0.3	3.7	Sinnott (2008)
Instrumentation and Control	0.4	5.1	Sinnott (2008)
Piping	0.7	8.8	Sinnott (2008)
Electrical	0.2	2,5	Sinnott (2008)
Civils	0.3	3.8	Sinnott (2008)
Lagging and Painting	0.1	1.3	Sinnott (2008)
Total Plant Direct Cost (TPDC)		**R 37.80**	
Design and Engineering	0.3	3.8	Sinnott (2008)
Utilities/overheads/offsite	0.5	6.3	Sinnott (2008)
Contractors fee	0.05	0.628	Sinnott (2008)
Contingency	0.1	1,3	Sinnott (2008)
Critical/Commissioning Spares	0.1	1.3	Sinnott (2008)
Total Plant indirect cost (TPIC)		**R 13.2**	
Purchased Equipment	**nr**	**Cost Estimate**	
Bioreactor	1	7.5	Alibaba.com®
Chiller unit	1	0.80	Alibaba.com®
Compressor	1	0.40	Alibaba.com®
Boiler	1	1	Alibaba.com®
Seed reactor (10, 150 L and 1000L) seed	3	1.5	Alibaba.com®
Storage vessels	2	0.3	Alibaba.com®
Filtration unit	2	1	Alibaba.com®
Autoclave	1	0.07	Alibaba.com®
Total Purchased Equipment (TPE)		**12.6**	
Fixed Capital investment (FCI)		**51.10**	
Working capital		**2.6**	
CAPEX		**R 53.7**	

Of the two production technologies undergoing the same production target per annum, the total purchase equipment (TPE) for the *in vivo* was substantially lower than the *in vitro* (35%). The big difference in equipment cost was also reflected in the fixed capital investment (FCI) since the equipment cost forms the foundation for the capital investment estimation. The *in vivo* only required simple trays and an incubation room. This offers a major advantage to *in vivo* systems relative to *in vitro* regarding the start-up capital, which is inherently low for the *in vivo* production method. It requires a low level of infrastructure for production as compared to *in vitro* (Shapiro-Ilan, Han and Dolinksi, 2012). For *in vivo*, the total plant direct cost (TPDC) contributed the least to the total fixed capital investment (FCI) (Figure 5-8). Conversely, TDPC was the major contributing factor for the high capital investment needed for *in vitro* technology. It contributed to about 59% of the total fixed capital investment (Figure 5-9). This was

due to the high cost of production equipment such as fermenters and seed reactors. A comparison of the capex of the two technologies is shown in Figure 5-10.

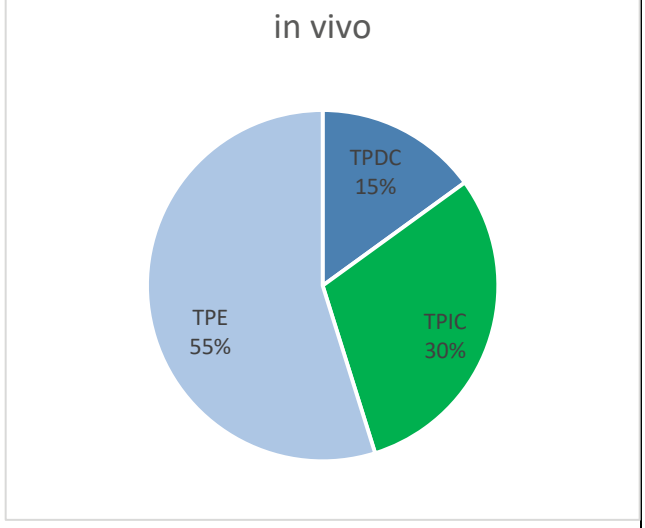

Figure 5-8: Cost breakdown of fixed capital investment (FCI) for annual production rate of $4.26x10^{12}$ EPNs via *in vivo* technology

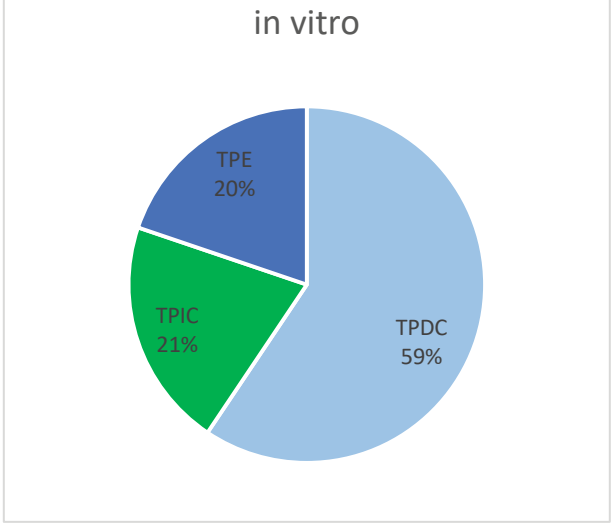

Figure 5-9: Cost breakdown of fixed capital investment (FCI) for annual production rate of $4.26x10^{12}$ EPNs via *in vitro* technology

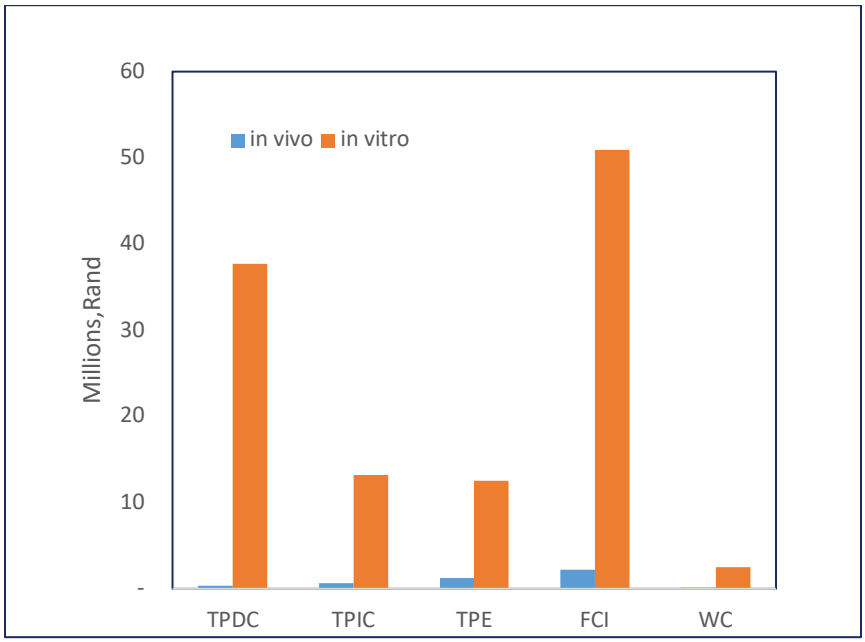

Figure 5-10: Comparison of Total Capital expenditure (CAPEX) for *in vitro* and *in vivo* production methods

5.5.2. Estimation of Operating Expenditure

Operational costs include the day to day costs of production which comprises the fixed and variable costs. For nematode production, the fixed cost includes the cost of land, principal and interest on loans, depreciations, insurance premiums, and salaries of workers. Variable cost changes in the course of production as it is dependent on production output. This includes the cost of feed, utilities, formulating ingredients and cultures (nematode and bacteria).

Variable operating cost such as utilities (electricity and water) was estimated based on the energy demand and utilisation of each processing unit. Cost rates for industrial water (18.24 Rand/kL) and electricity (87.34 c/kWh) were obtained from Department of water and sanitation, South Africa and Electricity companies respectively. Other operating costs such as taxes, insurance, were estimated as a percentage of ISBL and FCI (Table 5-7)

Table 5-7:Estimation of total annual operating cost for the EPNs production via *in vivo* and *in vitro* at $4.26x10^{12}$ EPNs per annum servicing 5680 ha/annum

			In vivo	***In vitro***
Item			**Cost (000 Rand/yr)**	
Production space			60	36
Utilities			260	252
Raw materials			210	516
Consumables			45	45
Formulation			200	200
Packaging and shipping			100	100
Waste treatment/disposal			7.32	1.2
Total variable operating cost			**980**	**1,130**
Labour and supervision				
Position	Salary	#	Total Salary	Total salary
Supervisor	900	1	900	900
Production labour	240	30	3,600	-
Production Labour	480	2	-	680
Maintenance Technician	200	1	204	200
Clerks & Secretaries	240	1	240	240
Total salaries			**4,900**	**2,000**
Maintenance	3.00%	of ISBL	37	225
Property Insurance & Tax	0.70%	of FCI	156	356
Total fixed operating cost			**5,137**	**2,600**
Total Operating Cost			**6,120**	**3,755**

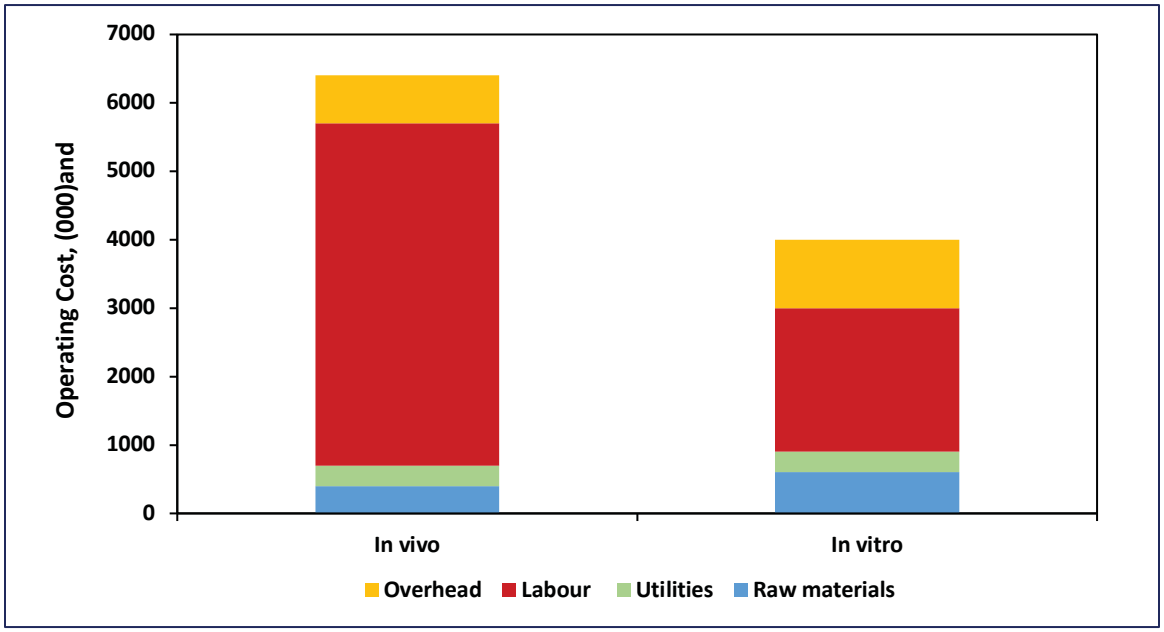

Figure 5-11: Breakdown of annual operational cost for the two production methods

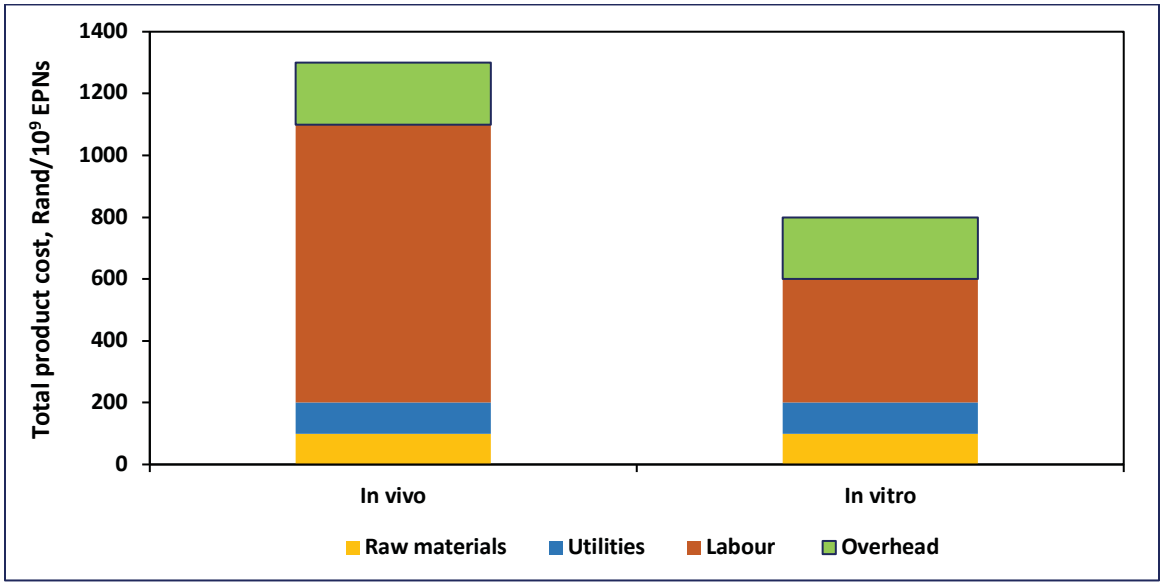

Figure 5-12: Total and Specific Operating cost for the production of billion EPNs

The annual operational cost of *in vivo* and *in vitro* at the same production rate per annum was estimated at 6.1 MRand and 3.7 MRand (Figure 5-11). Despite the high capital investment of in vitro technology (Table 5-6), the production cost per billion was 39% lower than the *in vivo* technology (Figure 5-12). The highest contributing factor to the high cost of production for the *in vivo* technology was the amount of labour involved in the production route (Figure 5-11). Based on the tray specifications to achieve the

annual production target ($4.2x10^{12}$ EPNs), a total of 30 workers (including a supervisor) was assumed to be ideal, with the supposition that at least one worker can manage a stack of trays with regards to house-cleaning operations. Operations such as removal of non-infected and contaminated hosts need to be performed manually during each production run so as to avoid a possible contamination of the whole culture. Labour, therefore, contributed to about 80% to the total cost of production for the *in vitro*. Secondly, the cost of rearing host also accounted for the high cost of production for *in vivo*. The cost of insect host was estimated based on the diet requirement for the cultivation of a lot of hosts. One kilogram of mealworms diet (wheat bran) for an in-house rearing of the host (4400 insect host) in the laboratory was estimated at a cost of R16 (Zyl and Malan, 2015). Consequently, the total cost of diet for rearing the required number of mealworms ($3.7x10^7$ larvae) to meet the annual production target was estimated at R210,000 (Table 5-7). This contributed to ~4% of the total production cost. Other minute factors included the cost of production space and utilities.

Factory space for both systems was estimated based on the recommendations of Holmes et al. 2015 on factory sizes for commercial production of EPNs (Table 5-3). A minimum of 200 m^2 of production space was estimated for *in vivo* technology due to the large need for space to accommodate the tray systems serving as the main production unit for *in vivo*. Conversely, *in vitro* required less production space. A maximum space of 120 m^2 as suggested by Holmes et al. 2015 for *in vitro* cultures was used as an estimate for calculations, and it contributed a little to the overall production cost.

For the *in vitro*, the major contributors to the total operating cost were the cost of maintenance, raw materials, and utilities. Media cost was estimated based on the cost of formulating ingredients per liter per run (Appendix). Also, the annual salaries of 3 skilled workers (including a supervisor) constituted the bulk of the total operating cost due to the little manual operations associated with the production route. This was reflected in the sensitivity analysis performed on the IRR with respect to labour Figure 5-14.

5.5.3. Discounted Cash Flow Analysis

In order to determine the viability of the two production methods, DCFROR assessment based on the earlier costing assumptions (section 5.5) was performed. This was done to calculate the selling price at which the NPV is zero at a minimum IRR of 10% through series of iterations. Investors are often interested in the value of the NPV to assess whether a particular project is worth investing or not. The results of the DDCFOR analysis for the two production methods are summarized in Table 5-8

Table 5-8: IRR and payback periods estimated for the two methods at an annual production target of 4.2x1012 EPNs (values obtained at NPV=0, base Nematode selling price of R 1500/billion EPNs)

	In vivo	*In vitro*
IRR, %	n/c*	24
Payback period, years	-	7
NPV, Rand	-12,876,953	193,244,209
Minimum selling price, Rand/hectare	1,662	1,064

*n/c-not calculated, cost of production > adopted maximum market selling price

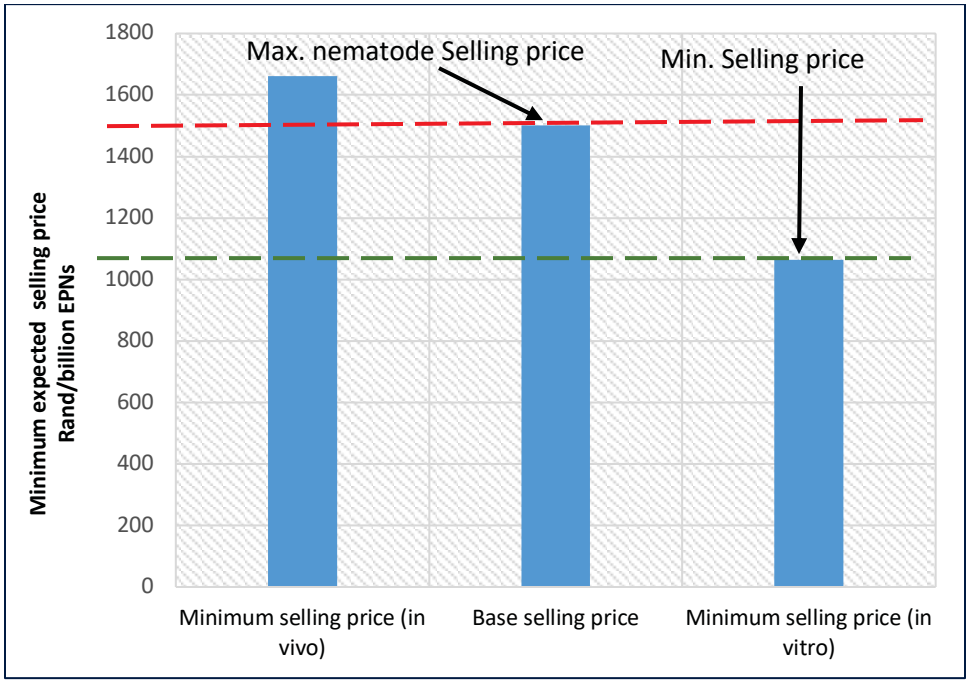

Figure 5-13: Minimum expected nematode price (NPV calculated at acceptable minimum IRR of 10%, IRR = discount rate at which NPV=0)

Table 5-8 summarizes the results from the profitability assessment of the two nematode technologies undergoing an annual target production of $4.26x10^{12}$ EPNs. From the results of the economic assessment, it was noted that under the *in vivo* the minimum selling price at NPV = 0 was higher than the current selling price of nematode products. This suggests *in vivo* technology is not viable in this case scenario. In contrast, at the field selling price of R1500/billion EPNs, the estimated minimum selling price of the *in vitro* (R1, 064) was 13% less than the prevailing market price. Thus, suggesting the economic viability of *in vitro* technology. It can also be deduced from the minimum selling price that a manufacturer

adapting the *in vitro* technology can afford to sell at a price lower than that of a manufacturer using the *in vivo* in the quest to penetrate the market.

Although *in vivo* had the least capital cost in comparison to *in vitro*, the total operating cost was higher than the latter. The costs of the principal component of production such as an insect, production space and labour increase as a linear function of the production capacity. Thus, making it impossible to achieve some economies of scale even at a higher production rate. *In vitro*, therefore, presents a viable approach to commercialisation even though the initial start-up capital is often high. The high NPV value obtained with a minimum 7 years at the set production target makes an investment into the project a worthwhile venture

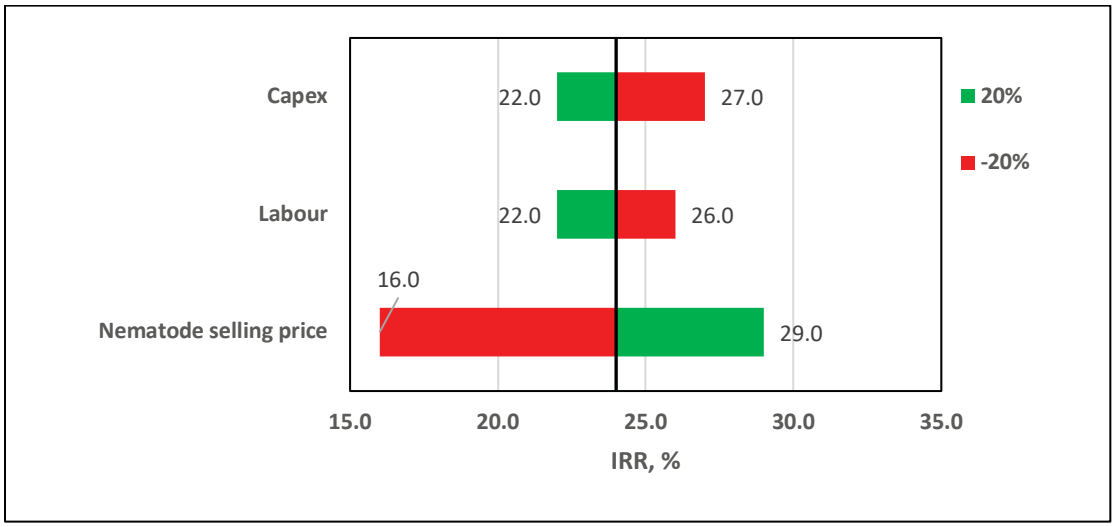

Figure 5-14: Economic sensitivity analysis of *in vitro* technology

The effect of capex, labour and nematode selling price on IRR was investigated in the sensitivity analysis for the *in vitro* culture because of its viability to commercialization (Figure 5-14). From the results, the nematode selling price was observed to have the greatest effect on IRR followed by Capex and labour in that order. A 20% increase in nematode selling price caused a corresponding 6% increase in IRR, whereas a 20% decrease caused an 8% change in IRR. Conversely, a 20% increase in capex caused a 2% decrease in IRR, and a corresponding decrease in Capex resulted in 3% increase in IRR. Gaugler suggested that capital investment of in vitro technology is the most influencing production parameter and should be targeted to reduce the overall production cost. At this estimated selling price of nematode products, the focus of replacing chemical pesticides with nematode biopesticide may not seem feasible due to the high cost of production. The most contributing factor to the production cost is the total capital investment

needed for in vitro cultures. The production cost per billion EPNs for the in vitro (R 880) is 52% higher than the cost of production (R419) for a low impact chemical insecticide (Gaugler, 2002). Even with *in vivo,* along with the low initial capital requirement, the cost of production was still high (R1460). Alternative approach to cut down the cost of operation should be explored. Suggestions such as delivering the nematode-infected insect cadavers directly into the soil removes the high cost associated with labour as little/no housekeeping operations are needed (Shapiro-Ilan *et al.*, 2001). Additionally, increase in the average nematode yield per square area of the trays (nematode/m^2) can further reduce the cost of production. This calls for optimization studies into parameters such as the concentration of nematode inoculum, host size, and density as more efficient methods likely to reduce the overall cost of production. Additionally, integrated approach should be exploited. Possibilities into toll productions should be explored to alleviate the high cost of capital investment associated with nematode production. Research Development and Expertise (RDE) into optimisation processes should also be put in place to improve the average yield per gram of medium and shorten the process time per batch of culture.

Chapter 6

Summary of Conclusions and Recommendations

Results from *in vitro* studies provided enough evidence that the growth of the nematode with its symbionte can be well-affected by the fermentation modes. The comparison of the different processes resulted in different maximum IJ densities being recorded after 11-days except for the fed-batch with bacteria that the culture was allowed to prolong for extra 5 days in order to achieve the focus of the study. The experiment with glucose showed that the cell density of the bacteria can be maximized through the feeding of glucose at periodic intervals during cultivations. One of the challenges encountered was the determination of the various growth phases of the bacteria in the time course of their development in the complex liquid culture media. Although this challenge was mitigated by collecting data on bacteria cell density and the rate of dissolved oxygen in the bioreactor, these parameters might often not be a true representation of the growth kinetics of the bacteria, especially, at the lag, exponential and stationary growth phases of the bacteria in the reactor. We recommend that a more reliable approach such as gas analysis would be used in future studies. Gas analysis provides information which can be taken online to compute metabolic parameters such as oxygen uptake rate (OUR) and CO_2 production that are true reflections of the metabolism of the bacteria.

Secondly, the glucose oxidase assay procedure adopted for the measuring of the residual concentration of the glucose in the bioreactor could preferably be conducted by analysing data on a parameter such as respiration quotient (RQ). Johnigk (2003) suggested that the utilisation of glucose by the bacteria during cultures leads to an RQ of 1 and drops < 1when depleted. This could provide reliable information on when to start feeding glucose into the system. Also, the difficulty encountered with the glucose assay was the preparation of standards to match the residual glucose in the complex media. The culture broth had to be serially diluted on several occasions before the spectrometer could read the correct absorbance to match the standards. The impact of the glucose was well-pronounced on the growth of the nematodes as well due the final IJ density recorded at the end of the run. However, it is still unclear the concentration regimes that can positively affect the nematodes. Response surface methodology (RSM) should be employed to investigate the optimal concentrations before the glucose is fed to the nematodes.

Investigations into fed-batch with bacteria also confirmed the hypothesis that the bacteria serve as the main food source for the nematodes. During culturing, observation was made on the gradual depletion of the bacteria during the time-course of fermentation especially after recovery on day 4 post-IJ inoculation. This informed on the addition of a fresh bacteria feed on the said date, which led to an increase of IJ count by ~40% in comparison to batch cultures at the end of the 11-day fermentation run. However, a further addition of bacteria after the 11 day fermentation in the quest to increase the IJ concentration contributed a little to the total IJs recorded at the end of the 16-day cycle. It resulted in a two generation cycle, with an increased densities of J1, J2, parental adults, and few IJs. As the main objective of liquid cultures is to reduce the production time while maximizing the final IJ count, a two generation cycle would be uneconomical for mass production if the final IJ yield does not offset the operational cost incurred for the few extra days. We recommend that, although bacteria can be used as a feed substrate to increase the average IJ count, the ideal timing for inoculation should be investigated further to prevent the bacteria from undergoing another undesirable generational cycle.

Recovery is still one of the bottlenecks associated with liquid cultures. A low recovery affects the final IJ yield, as asynchronous IJ development precludes optimal population management of the nematodes as well as shortening the process time of the culture. This situation was apparent to the batch cultures where a low recovery (<60%) was mostly recorded. The synergistic effects of CO_2 have been studied to induce a high recovery rate among IJs. Future studies should investigate this hypothesis further and probably adopt for such purpose. Additionally, a parameter such as pH could be employed to measure the amount of food signal produced by the bacteria in the LCM. Johnigk (2003) noted that at a high recovery of the IJs, the pH of the medium increases above the minimum set point, an indicative of the high food signal in the medium.

Data on the virulence of the nematodes expressed as percent mortality to *Galleria* host showed significant differences among the nematodes produced from the two main methods (*in vivo* and the *in vitro*). The nematodes from then *in vivo* recorded a higher mortality as compared to those obtained from *in vitro*. The reason being the high lipid content mostly found in the bodies of nematodes cultivated in the natural host in comparison to those propagated in artificial complex media. The supplementation of the glucose had a significant effect on the virulence of the nematodes, as it might have increased the total lipid content of the nematodes. This assumption was confirmed by the higher mortality rate achieved by the glucose fed-batch cultures than the nematodes from the batch cultures. Thus, we can confirm that lipid content of the nematode is related to their virulence and pathogenicity attributes. However, further research should be done to estimate the total lipid content of the nematodes through chromatography as it would

provide a concrete information on the virulence of nematodes in relation to the media in which they are propagated.

Finally, the techno-economic studies on both production methods investigated revealed that the *in vivo* is uneconomical when replicated on a commercial scale. The cost of central components such as production space, labour and insect increase linearly with production capacity, making it difficult to achieve any economies of scale in the long run. Evidently, at the assumed production target, the minimum selling price (R1460) was higher than the estimated minimum market selling price per billion of EPN produced (R2852), along with the negative NPV value obtained in the DCFROR analysis. However, with further innovations and research works, cooperative growers and small-scale field-testing researchers could still adopt this system as an ideal pathway for production of enough EPNs. This eliminates the need for significant investments needed for liquid cultures to achieve those targets. Also, avenues such as mechanisation interventions should be explored to streamline the cultivation process by reducing the amount of manual labour involved in the production route.

Contrarily, under the same assumptions, *in vitro* provided the best option towards commercialisation, although the initial capital cost of investment estimated was high. The cost of production was less than the *in vivo* (R880), with a minimum selling price of R1,062 at NPV =0. DCFROR analysis presented an IRR of 24% with a positive NPV (193,244,209). The high NPV value along with a payback time of 7 years positioned the *in vitro* technology as economical for commercialization in comparison to the *in vivo*. Moreover, achieving economies of scale is possible since the cost of a principal component of production (such as labour) is not proportional to production.

Future Works

To realize the potential of EPNs as biological-control products in agriculture, also offering significant health benefits to consumers, requires cost-competitive production and application methods, in comparison to conventional pesticides in order to overcome their inherent production costs associated with nematode production. Investigations into the engineering process parameters for successful cultivation is necessary for a future commercialization purpose. Specifically, research works into the following areas are warranted

1. Investigations into the impact of different fermentation mode on the growth of nematodes and the bacteria complexes have shown to adversely affect the final yields of the nematodes, however

without any optimisation procedures. Optimising a fed-batch culture by studying the effects of glucose along with other nutrients is a vital step towards nematode production. The optimal concentration that influences or hinders the achievement of a higher nematode yields needs to be investigated. The effects of such step on the quality (pathogenicity) and the shelf-life on nematode would need to be studied. Attainment of long shelf-life is one of the many challenges most nematode producers face with after formulation. This has given most conventional chemical insecticides an extra advantage over nematode products. Current nematode products already in the market have a shelf-life of~6 months and studies into factors that can influence a long product stability need to be addressed.

2. Series of *in vitro* experiments needs to be conducted in bioreactors to ascertain the optimal process parameters for liquid cultures. The cultivation of organisms in bioreactors is subject to a high number of uncontrolled and uncontrollable variables such as impacts of stirring rate, air flow rates, pH, the number of impellers and more importantly the gas flow rate. These parameters influence the outcome of the cultivation. Nematodes are known to be sensitive to the effects of shear stress and high inertial forces, and therefore, finding the appropriate regimes for the aeration rate for various volumes of bioreactors would be needed for future scale-down purposes to enhance the growth of the bacteria and the nematodes. In any aerobic bioprocesses, the growth of the organism is limited by the amount of oxygen that is made available during fermentation. Therefore, investigating the oxygen requirement by studying the volumetric mass transfer coefficient, K_La, is a crucial step in the design and developmental operations of bioreactors, for the successful cultivation of nematodes.

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Appendix

Standard Procedures

A. **Isolation of bacteria from infective juvenile nematodes using a hand-held grinder**

1. Transfer 1 ml of IJ suspension to a sterile 1.5-ml microfuge tube (a minimum of 300 IJs).
2. Pellet IJs by spinning for 3 min at maximum speed (at least 13,000 rpm.) in a microfuge.
3. Remove water and add 1 ml 1.0% bleach.
4. Incubate for 1 min at room temperature.
5. Pellet nematodes as in step 2.
6. Remove bleach and rinse with 1 ml sterile water.
7. Repeat wash steps 5 and 6 for a total of two washes.
8. Remove water from last wash and re-suspend the nematode pellet in 0.5e1.0 ml sterile distilled water.
9. Quantify IJ concentration by counting at least three 2- to 5-ml drops and averaging.
10. Dilute IJs to a 4 IJs/ml.
11. Move 50 ml of the IJ suspension into a sterile microcentrifuge tube containing 200 ml LB-broth.
12. Using a hand-held motor driven grinder and poly- propylene pestle (Kontes), grind nematodes for 2 min.
13. Plate immediately onto two LB-pyruvate plates using a flame sterilized spreader. a. Plate 50 ml of the homogenate onto the first plate. b. Dilute homogenate 1:3 in LB-broth and plate 50 ml on to a second plate.
14. Incubate plates overnight at 30^{o}C (or optimal temperature for symbiont).
15. Inspect plates for colonies. a. One of the two plates should contain countable colonies

b. Plates may need to be incubated for an additional day to produce colonies large enough for counting

B. **Luria-Bertani Agar**

5 g yeast extract, 10 g tryptone, 5 g NaCl, 20 g agar, 1 L H_2O

Procedure

1. Place in a 2-l flask with stir bar.
2. Add 1 L H2O.
3. Autoclave.

4. Stir while cooling.

Note: When cool enough to touch with hand add then any desired supplements (e.g. antibiotics) and pour into petri dishes.

C. **NBTA agar (for 1 L)**

8 g nutrient broth, 15 g agar, 0.25 g bromothymol blue, 1 L H_2O

Procedure

1. Add water into a 2-l Erlenmeyer flask.
2. Pour all solid ingredients into the water.
3. Autoclave.
4. Stir while cooling.
5. Add 0.04 g triphenyltetrazolium chloride (TTC).

Glucose Oxidase Assay Procedure

MANUAL ASSAY PROCEDURE:

Wavelength: 510 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: 25 ± 0.1°C
Final volume: 3.0 mL
Sample solution: glucose oxidase diluted to 0.01-0.08 U/mL
Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
solution 2 (POD mixture) solution 3 (D-glucose)	2.00 mL 0.50 mL	2.00 mL 0.50 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 5 min and start the reactions by addition of:		
sample solution distilled water	- 0.50 mL	0.50 mL -
Mix*, read the absorbances of the solutions (A_2) after exactly 20 min.		

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

CALCULATION:

Determine the absorbance difference (A_2-A_1) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{510\ nm}$/20 min for the sample being analysed. While the time course increase in absorbance on incubation of glucose oxidase with the assay reagents is linear (Figure 1), the standard curve relating glucose oxidase activity (mU/assay i.e./0.5 mL) to absorbance increase in 20 min at 510 nm is not perfectly linear (Figure 2). Consequently, activity values (mU/0.5 mL) are obtained by reference to the standard curve shown in Figure 2, and calculated as follows:

$$\textit{U/L of sample solution} = \text{mU/0.5 mL} \times 2000 \times \frac{1}{1000} \times D$$

1/1000 = conversion from mU to U

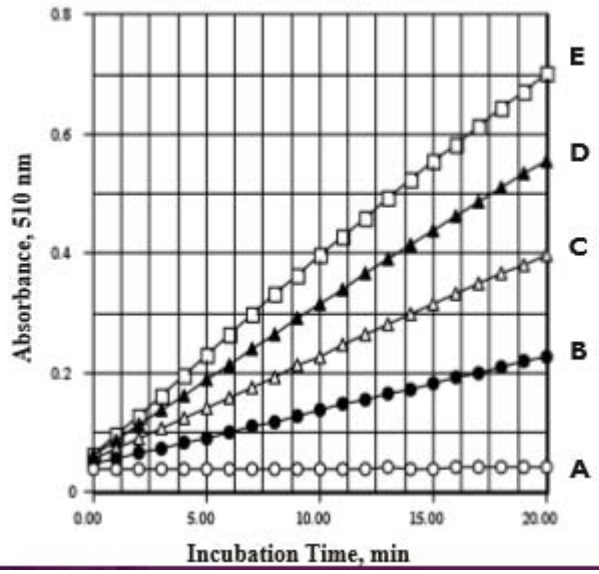
D = dilution factor (i.e. if sample is diluted 10-fold, D=10)

When analysing solid and semi-solid samples which are weighed out for sample preparation, the activity (U/g) is calculated from the amount weighed as follows:

Glucose oxidase activity (U/g of preparation)

$$= \frac{\text{GOX activity [U/L sample solution]}}{\text{weight}_{\text{sample}}\ [\text{g/L sample solution}]} \quad [\text{U/g}]$$

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears in the Megazyme web site (www.megazyme.com).



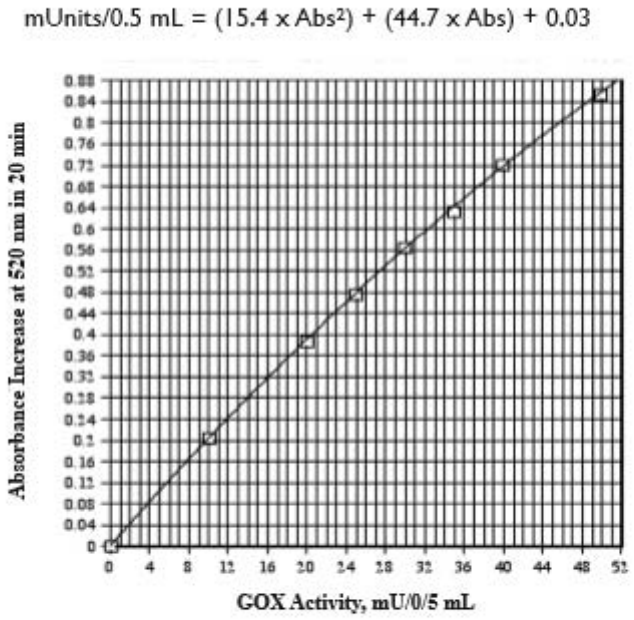


Figure 2. Standard curve relating glucose oxidase activity (mU/assay i.e./0.5 mL) to absorbance at 510 nm.

Residual concentrations of Glucose during Glucose fed-bactch

gram per litre	Time
0.2	0
0.22	3
0.24	6
0.22	9
0.21	12
0.19	15
0.175	18
0.2	21
0.25	24
0.241	27
0.253	30
0.322	33
0.259	36
0.285	39
0.263	42
0.275	45
0.268	48
0.2	51

0.18	52.5
0.14	52.6
0.11	52.8
0.112	53
0.06	53.5
0.022	54
0.03	57
0	60

Xenorhabdus Experiment in 5LBioreactor

Glucose Exp.			Batch Exp		Bacteria Fed-batch	
Time	Hrs	CFU	Hrs	CFU	Hrs	CFU
6am	0	1	0	0	0	0
7am	3	30000000	3	75000000	3	8E+07
8am	6	60000000	6	1.5E+08	6	2E+08
6am	9	90000000	9	2.25E+08	9	2E+08
7am	12	1.2E+08	12	3E+08	12	3E+08
8am	15	1.5E+08	15	3.75E+08	15	4E+08
6am	18	1.8E+08	18	4.5E+08	18	5E+08
7am	21	2.1E+08	21	5.25E+08	21	5E+08
8am	24	2.4E+08	24	6E+08	24	6E+08
6am	27	2.7E+08	27	6.75E+08	27	7E+08
7am	30	3E+08	30	7.5E+08	30	8E+08
8am	33	3.3E+08	33	8.25E+08	33	8E+08
6am	36	3.6E+08	36	9E+08	36	9E+08
7am	39	3.9E+08	39	9.75E+08	39	1E+09
8am	42	4.2E+08	42	1.05E+09	42	1E+09
6am	45	4.5E+08	45	1.13E+09	45	1E+09
7am	48	4.8E+08	48	1.2E+09	48	1E+09
8am	51	5.1E+08	51	1.28E+09	51	1E+09
6am	54	5.4E+08	54	1.35E+09	54	1E+09
7am	57	2600000	57	1.3E+08	57	1E+08
8am	60	120000	60	6000000	60	60000

Mean Count IJ density

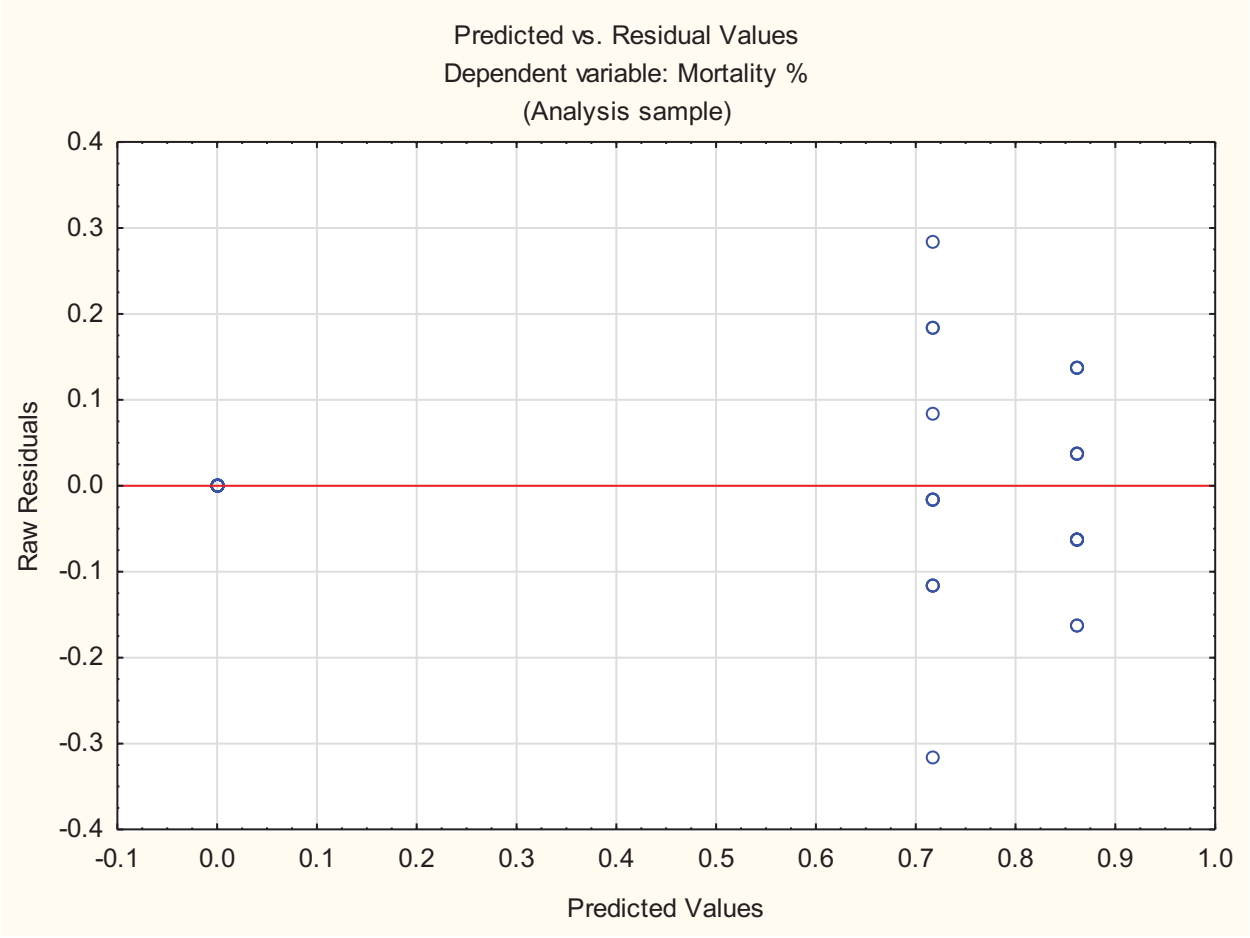
	Ijs in batch culture	Ijs in fed-batch cultues
Day 1	2000	2000
Day 2	8000	8000
Day 3	8500	8500
Day 4	15000	9200
Day 5	10000	40000
Day 6	20000	45000
Day 7	17000	46000
Day 8	19500	40500
Day 9	28000	48900
Day 10	35000	51000
Day 11	35000	51000
Day 12	32000	48500

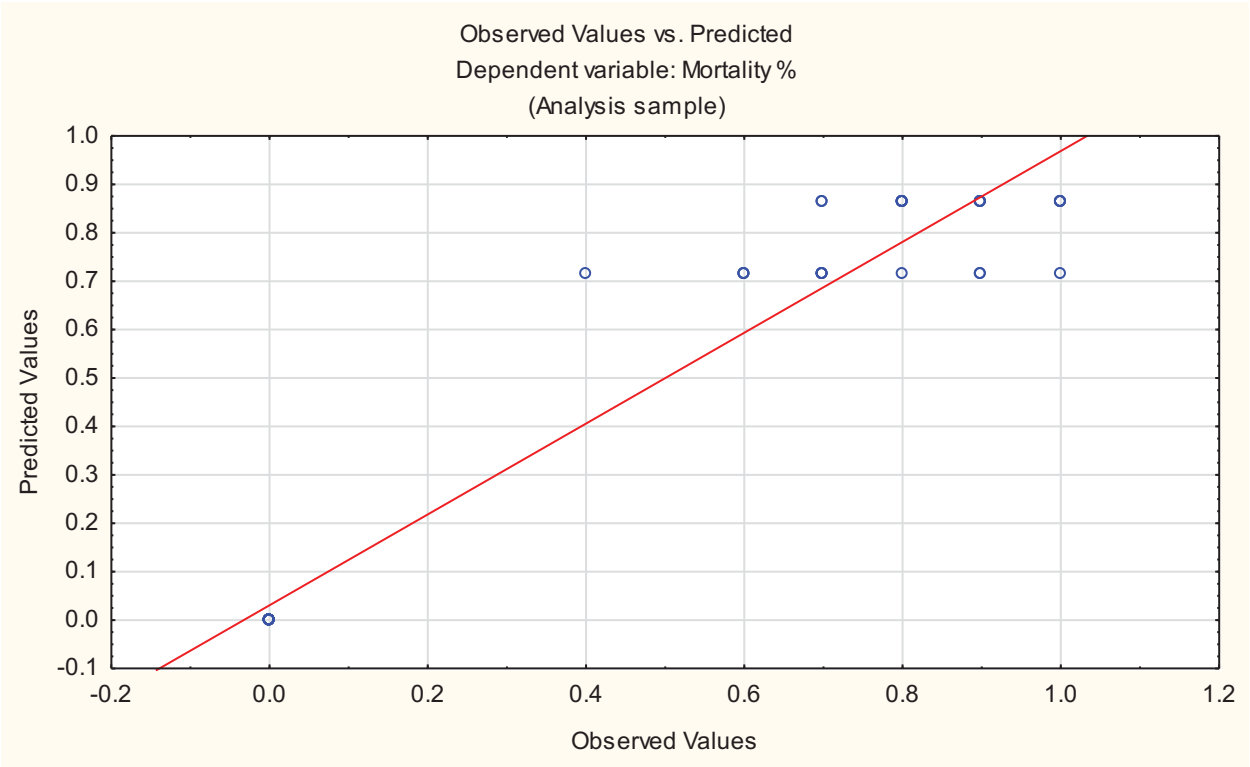
Virulence Study

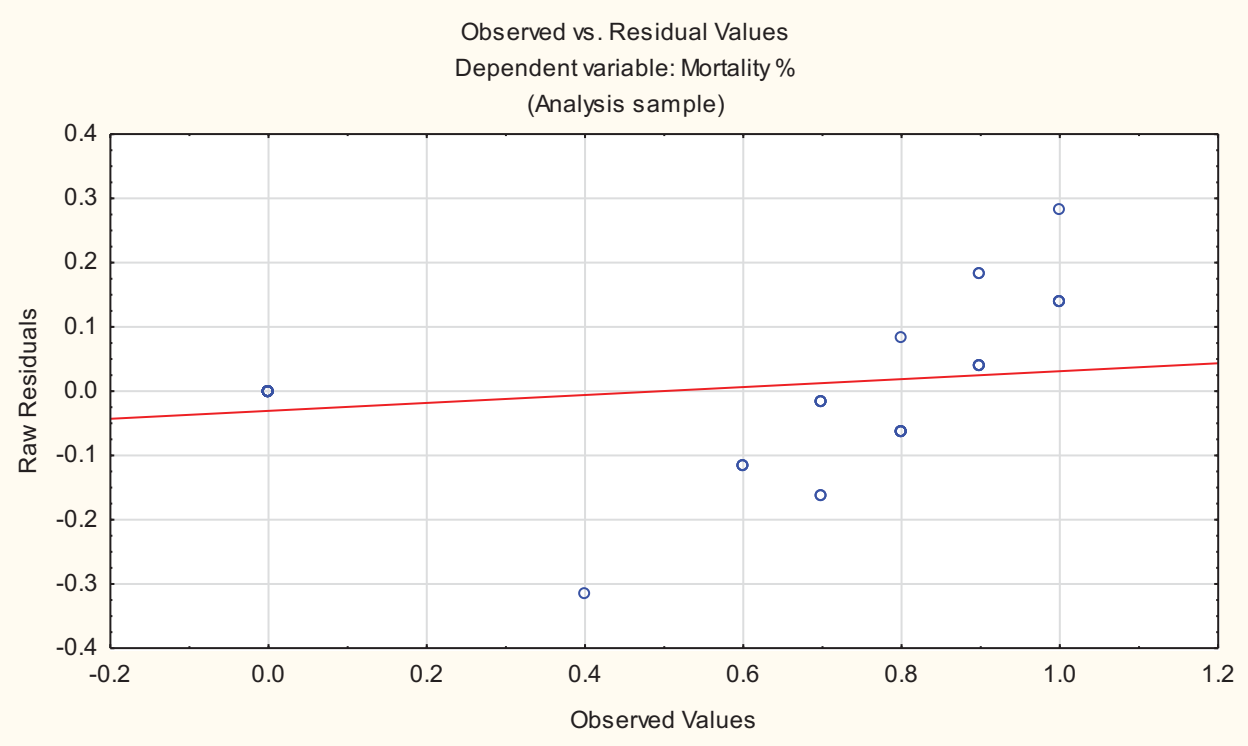
DISH	Treatment	Replica	Concentra	Temp	Dead	Mortality %	Dead Control	Proportio	Total larvae	#Infections	Total Nemat	Proportion Infection
1	*in vitro*	1	100	25	7	0.7	1	0.1	10	15	1000	0.015
2	*in vitro*	1	100	25	9	0.9	1	0.1	10	56	1000	0.056
3	*in vitro*	1	100	25	7	0.7	2	0.2	10	71	1000	0.071
4	*in vitro*	1	100	25	9	0.8	2	0.2	10	52	1000	0.052
5	*in vitro*	1	100	25	9	0.9	2	0.2	10	25	1000	0.025
6	*in vitro*	2	100	25	6	0.6	1	0.1	10	17	1000	0.017
7	*in vitro*	2	100	25	6	0.6	0	0	10	2	1000	0.002
8	*in vitro*	2	100	25	4	0.4	2	0.2	10	21	1000	0.021
9	*in vitro*	2	100	25	6	0.6	0	0	10	18	1000	0.018
10	*in vitro*	2	100	25	7	0.7	1	0.1	10	19	1000	0.019
11	*in vitro*	3	100	25	7	0.7	0	0	10	8	1000	0.008
12	*in vitro*	3	100	25	10	1	3	0.3	10	12	1000	0.012
47	*in vivo*	1	100	25	7	0.7	0	0	10	62	1000	0.061938062
48	*in vivo*	1	100	25	8	0.8	0	0	10	115	1000	0.114770459
49	*in vivo*	1	100	25	9	0.9	2	0.2	10	162	1000	0.161515454
50	*in vivo*	1	100	25	7	0.7	0	0	10	33	1000	0.032868526
51	*in vivo*	2	100	25	5	1	0	0	10	39	1000	0.038767396
52	*in vivo*	2	100	25	8	0.8	0	0	10	103	1000	0.102284012
53	*in vivo*	2	100	25	8	0.8	1	0.1	10	19	1000	0.018849206

Effect	Univariate Tests of Significance, Effect Sizes, and Powers for Per Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	0.072434	1	0.072434	61.74230	0.000000
Treatment	0.041635	2	0.020818	17.74485	0.000003

Cell No.	LSD test; variable Penetration Rate % (Msc.) Probabilities for Post Hoc Tests Error: Between MS = .00117, df = 42.000 Treatment	{1} .02633	{2} .08166	{3} .01376
1	bacteria fed-batch		0.000124	0.336002
2	Glucose fed-batch	0.000124		0.000001
3	Batch	0.336002	0.000001	







Mortality and Penetration test of *S. yirgalemense* in Tenebrio

Appendix B

PRODUCTIVITY AND DEMAND		
Yield of EPNs	72000	EPNs/ml
Number of EPNs required per ha	2500000000	EPNs
Vol of broth required per ha	34.7222222	L
In the first year		
Total land area covered	50	ha/month
Vol of broth required	1736.11111	L/month
Number of runs required	1.73611111	per month
After 5 years		
Total land area covered	1000	ha/month
Vol of broth required	34722.2222	L/month
Number of runs required	34.7222222	

OPEX

Medium Cost- Direct cost per run

Consumables per run	Per unit	Qty	Price
Tips yellow (bag 1000)	R 510.00	0.5	R 255.00
Tips blue (bag 1000)	R 510.00	1	R 510.00
Tips white (10 ml, bag 100)	R 1,098.00	0.5	R 549.00
Eppendorf tubes (1.5 ml, bag 500)	R 139.00	1	R 139.00
Conical tubes (50 ml, box 500)	R 1,356.00	0.5	R 678.00
Totals			R 2,131.00
VAT			R 298.34
Source : Quotation from Sigma-Aldrich®,			

Quotation for Liquid Culture Medium

Component	g/L	g	ZAR/g	Cost per run
Yeast extract	15	15000	0.39	5850
Soy powder	20	20000	0.016	320
NaCl	4	4000	0.135	540
KCl	0.35	350	0.241	84.35
$CaCl_2$	0.15	150	1.642	246.3
$MgSO_4$	0.1	100	0.486	48.6
Vegetable oil (ml/L)	46	46000	0.016	736
Total usable fermenter volume		1000	L	
			Total	R 7,825.25
	Medium cost		7.82525	ZAR/L

Source : Quotation from Sigma-Aldrich®,

Bacteria Growth Medium

Component	g/L	g	ZAR/g	Cost per run
Yeast extract	15	15000	0.39	5850
Soy powder	20	20000	0.016	320
NaCl	4	4000	0.135	540
KCl	0.35	350	0.241	84.35
$CaCl_2$	0.15	150	1.642	246.3
$MgSO_4$	0.1	100	0.486	48.6
Vegetable oil (ml/L)	46	46000	0.016	736
Total usable fermenter volume		1000	L	
			Total	R 7,825.25

Quotation					Quote:	1018
Attention: Emmanuel Anane / Eugene van Rensburg					Date:	26/08/2015
Company: Stellenbosch University					Fax:	021-808-2059
From: Christo	Vat Nr: 4650183835				Tel:	021-808-9485
Email: anane@sun.ac.za					Cell:	078-269-2964
DESCRIPTION		**Product Code**	**QTY**	**UNIT PRICE**		**PRICE**
Relative Humidity Control System		RHCS	1	R 45000.00	R	45000.00
Air Conditioner		AIRCON	1	R 52000.00	R	52000.00
Building		BUILD	1	R 132000.00	R	132000.00
Concrete Slabs with Electrical work		CONELEC	1	R 105000.00	R	105000.00
				Sub-total:		**334 000.00**
				VAT:		**46 760.00**
				Total:		**380 760.00**

Terms & conditions:

Acceptance of quotation: upon receipt of signed copy of quotation, this is deemed to be a confirmed order. Kindly fax to 086 559 2247

Payment terms: 50% deposit upon acceptance of quotation. Balance due immediately upon completion of installation

Signature of acceptance of payment terms: _____ **Date:** _____

All equipment installed by Misting SA, remains the sole property until paid for in full. Misting SA reserves the right to remove all installed equipment should payment not be made upon completion of installation.

All components carry a six month warranty on factory defects. Warranty excludes negligent use. Pumps carry a separate warranty. Damage caused by poor water quality cannot be claimed from MSA. **Not covered by guarantee/ warranty: Misting SA is not responsible for blocked nozzles. It is the clients responsibility to ensure that nozzles, and filters are cleaned as and when needed. A filter is added to capture fine particles, however the chemical consistency of the water eg. calcium build up, is out of our control**

No claims resulting from negligent or misuse of equipment can be claimed from Misting SA

Lead time is 10 working days from the date of receipt of payment

Please note: Due to the fluctuating Rand, quotations are only valid for 7 (seven) days from date of issue

BANKING DETAILS: *Simco Pty Ltd*
FNB, Branch: 200912
Acc: 6207 511 8242

***** Please note that a designated power supply & water point is required to complete installation*****

Signature of acceptance _____ **Date:** _____

Figure 0-1: Quotation for the cost of the 'room technology'