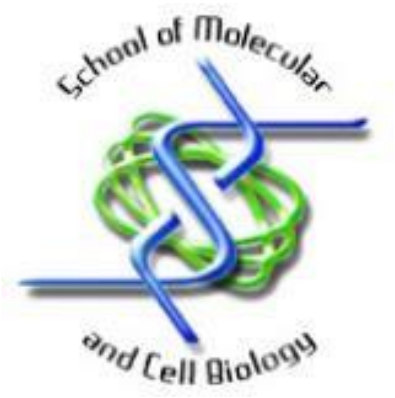

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Evaluating the productivity of selected *in vitro* culture techniques for the production of a locally isolated entomopathogenic nematode

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A dissertation submitted in fulfilment of the requirements for the degree of

Master of Science

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Declaration

I, Ofentse Matlhabe, am a student registered for the degree of Master of Science (MSc) at the University of the Witwatersrand in the academic year 2018.

I hereby declare the following:

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Signature of candidate.... O. Matlhabe..... Date: 22/05/2018

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Contents

Declaration ii

Acknowledgements iii

Abstract viii

List of figures x

List of tables xiii

List of abbreviations xiv

Chapter 1 1

Literature Review 1

1.1. Introduction 1

1.1.1. Chemical control 1

1.1.2. Integrated Pest Management strategies 1

1.1.3. Biological control 2

1.2. An overview of Entomopathogenic nematodes 3

1.2.1. Life cycle and mechanism of insect pathogenesis 4

1.2.2. Bacterial symbionts of entomopathogenic nematodes 5

1.3. Members of the *Oscheius* genus as EPNs 7

1.4. *Serratia* genus as an insecticidal partner of *Oscheius* EPNs 8

1.5. Production methods for EPNs 9

1.5.1. *In vivo* production 9

1.5.2. *In vitro* production (monoxenic and axenic culturing) 10

1.5.3. *In vitro* production (liquid and solid-state production) 10

1.6. Study Rationale 11

1.6.1. Effects of EPNs in biocontrol 11

1.6.2. Commercialisation of EPNs in South Africa 11

1.7. Aims and Objectives 12

1.7.1. Aim 1: To isolate and identify entomopathogenic nematodes found in soil samples 12

1.7.2. Aim 2: To isolate and identify bacterial symbionts of entomopathogenic nematodes 13

1.7.3. Aim 3: To evaluate the productivity of selected *in vitro* culture techniques for the production of entomopathogenic nematodes 13

References 14

Chapter 2 23

Isolation and Identification of a South African entomopathogenic nematode 23

2.1. Introduction 23

2.2. Methods and Materials 24
2.2.1. *In vivo* culturing of EPN on *Galleria mellonella* 24
2.2.2. Isolation of entomopathogenic nematodes (EPNs) 26
2.2.3. Nematode surface sterilisation 29
2.2.4. Molecular identification of isolated nematodes and phylogenetic analysis 30
2.3. Results and Discussions 31
2.3.1. Isolation of entomopathogenic nematodes 31
2.3.2. Molecular identification and phylogenetic analysis 33
2.4. Conclusions 36
Reference 37
Chapter 3 41
Isolation and Identification of the bacterial species associated with *Oscheius* L8 MCB 41
3.1. Introduction 41
3.2. Methods and Materials 43
3.2.1. The isolation, culturing and phenotypic identification of EPN bacterial symbionts 43
3.2.2. Molecular identification and phylogenetic analysis of the nematode bacterial symbiont 44
3.2.3. *In vitro* monoxenic cultures in lipid agar plates (for further confirmation of bacterial symbiont) 46
3.3. Results and Discussions 46
3.3.1. Isolation and phenotypic identification of the bacterial symbionts 46
3.3.2. Molecular identification of bacterial symbionts 47
3.3.3. Monoxenic cultures of EPN for confirmation of bacterial symbionts 48
3.4. Conclusions 50
References 51
Chapter 4 56
Evaluating the effects of *In vitro* axenic and monoxenic culturing techniques and growth media supplementation on the production of *Oscheius* L8 MCB 56
4.1. Introduction 56
4.2. Methods and Materials 58
4.2.1. Culturing bacterial symbiont in various media 58
4.2.2. *In vitro* production of *Oscheius* L8 MCB (monoxenic vs. axenic production) 59
4.2.3. Media supplementation studies 59
4.2.4. Data analysis 61
4.3. Results and Discussions 61
4.3.1. Culturing bacterial symbiont in various culture media 61

4.3.3. Media composition studies for the effects of glucose and canola oil 65
4.4. Conclusions 69
Reference 70
Chapter 5 74
Monitoring of life cycle stages of axenic cultures of *in vitro* produced *Oscheius* L8 MCB semi-solid state and liquid state and observing the effects of *in vitro* production on nematode efficacy against *Galleria mellonela* 74
5.1. Introduction 74
5.2. Methods and Materials 76
5.2.1. Monitoring of life cycle stages of axenic cultures of *Oscheius* L8 MCB during *in vitro* production in semi-solid state and liquid state. 76
5.2.2. Harvesting of nematodes from *In vitro* cultures 79
5.2.3. Comparative dose-response assays and Emergence studies for nematode infectivity and efficacy 80
5.2.4. Data analysis 82
5.3. Results and Discussions 83
5.3.1. Monitoring of life cycle stages of axenic cultures of *Oscheius* L8 MCB during *in vitro* production in semi-solid state and liquid state. 83
5.3.2. Comparative dose-response assays and Emergence studies for nematode infectivity and efficacy 92
5.4. Conclusions 98
Reference 100
Chapter 6 103
Conclusions and future works 103
Appendix I 106
***Galleria mellonella* rearing media** 106
Appendix II 107
Nematode Genomic DNA extraction (Protocol from Puregene® DNA Purification Kit, Genera systems 2003) 107
Preparation of 0.1% jik solution for infective juvenile sterilization 108
PCR condition for amplification of 18S rDNA region (35 cycles) 108
Appendix III 109
Media for isolating symbiotic bacteria associated with entomopathogenic nematodes 109
Gram stain procedure 110
Appendix IV 111
Isolation of genomic DNA from bacterial cells (Protocol from ZR Fungal/Bacterial DNA Kit, catalog # D6005) 111
PCR conditions for amplification of bacterial 16S rDNA region (35 cycles) 112

Appendix V 113
Recipes for *in vitro* culture media 113
Appendix VI 115
***Oscheius* L8 MCB consensus sequence** 115
Appendix VII 116
***Serratia* marcescens L8 MCB consensus sequence** 116
Appendix VIII 117
Complete 24 hour bacterial growth curve of *S. marcescens* L8 MCB 117
Appendix IX 118
Axenic cultures of *Oscheius L8 MCB* in Solid and liquid state shake flasks 118
An illustration of a male nematode of *Oscheius* L8 MCB. 118
Appendix X 119
Tables of statistics 119

Abstract

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* have gained interest as biocontrol agents of insect pests due to their ability to search and kill soil dwelling insects. Some members of the *Oscheius* genus have been shown to have insecticidal abilities as a result of their association with bacteria in the *Serratia* genus. This has led to the consideration of the *Oscheius* as an EPN genus. The biocontrol potential serves as an incentive for studying EPNs and various production methods for their commercial use as biopesticides. A putative EPN was isolated from soil samples collected at Brits in the North West Province of South Africa. 18S rDNA based identification indicated that it belonged to the *Heterorhabditis* genus however, phylogenetic analysis and symptoms on *Galleria mellonella* larvae suggested that the nematode may belong to the *Oscheius* genus. The bacterial symbiont associated with this nematode was found to be a strain of *Serratia marcescens,* through 16S rDNA based sequencing and phylogenetic analysis. Various *in vitro* production methods were evaluated for their effect in the production of *Oscheius* L8 MCB. These include monoxenic and axenic culturing, growth media supplementation, and production in solid and liquid state. Axenic cultures were found to produce high maximum yields of EPNs (78 600 EPNs/ml) compared to monoxenic cultures (53 833 EPNs/ml). It was concluded that axenic culturing was efficient for the production of this species. *Oscheius* L8 MCB was cultured in NB supplemented with varying concentrations of oil and glucose. Supplementation of NB with 2% canola produced a significant amount of EPNs in reduced culture times, but NB supplemented with 4% canola oil and 25mg/ml glucose increased nematode yield but prolonged the culture time. It is noted that media composition (with regards lipid and carbohydrate content) plays an important role in nematode yield and culture time and thus optimization of these components is critical for efficient nematode production. Solid state and liquid state production of *Oscheius* L8 MCB showed that solid state cultures allowed for early IJ production, whereas liquid state culturing produced the highest IJ yield (36 000 IJs/ml). The reduced culture period, makes solid state production more cost effective and preferable for mass production. However, liquid production can still be used as it offers the benefits of high nematode yeild and efficient recovery of nematodes from culture. The *in vitro* methods of EPN production have been reported to have an effect on the efficacy of the EPNs against insect hosts. Dose-response assays showed that *in vivo* produced EPNs resulted in high *G. mellonella* larvae mortality at lower concentrations compared to solid and liquid *in vitro* methods. Larvae infected with *in vivo* produced *Oscheius* L8 MCB produced a high

number of emerging IJs compared to larvae infected with EPNs produced using axenic *in vitro* culturing methods. The differences between mortality and IJ emergence in larvae infected with solid state and liquid state cultured EPNs were marginal. Therefore, it is concluded that axenic culturing methods may reduce the efficacy of *Oscheius* L8 MCB against insect hosts.

List of figures

Figure 1.1. The schematic diagram of the general life cycle of EPNs

Figure 1.2. Schematic flow diagram of the occurrence of primary and secondary forms of EPN bacterial symbionts.

Figure 2.1. Adult moth (A) and larvae (B) of *Galleria mellonella*

Figure 2.2. Culture bottle containing Adult moth and larvae of *Galleria mellonella*

Figure 2.3. Insect baiting method using *Galleria mellonella* as bait for the isolation of EPN from soil samples

Figure 2.4. Illustration of the White Trap method used to isolate EPN from insect hosts.

Figure 2.5. Illustration of a sand bioassay used to test for insect-pathogenicity and for the *in vivo* production of EPN.

Figure 2.6. Dead larvae infected by the putative isolate L8 MCB. Larvae showed no distinctive colour change, but putrefaction was absent.

Figure 2.7. Maximum-likelihood tree based on the analysis of 18S rDNA sequence data showing the Phylogenetic relationships of Unknown isolate L8 MCB to members of the Heterorhabditis genus with *Caenorhabditis elegans* as an outgroup.

Figure 2.8. Maximum-likelihood tree based on the analysis of 18S rDNA sequence data showing the Phylogenetic relationships of Unknown isolate L8 MCB to members of the *Oscheius* genus with *Caenorhabditis elegans* as an outgroup.

Figure 3.1. NBTA (A) and McConkey (A) plates of the bacterial symbiont L8B MCB alleged to be associated with *Oscheius* L8 MCB.

Figure 3.2. Gram stain of the supposed isolated bacterial symbiont of *Oscheius* L8 MCB. The isolated bacterium is a gram negative one.

Figure 3.3. Maximum-likelihood tree based on the analysis of 16S rDNA sequence data showing the Phylogenetic relationships of the unknown L8 MCB isolate to members of the *Serratia* genus with *E. coli* as an outgroup

Figure 3.4. Monoxenic cultures of *Oscheius L8 MCB* in lipid agar plates containing *S. marcescens* L8 MCB.

Figure 4.1. Bacterial growth curves of *S. marcescens* L8 MCB isolate in nutrient broth (NB), NB1 and NB2 over a 14-hour time.

Figure 4.2. Growth of *Oscheius* L8 MCB in axenic and monoxenic cultures over a 15 day time period.

Figure 4.3. Growth of *Oscheius* L8 MCB in NB1 variations.

Figure 4.4. Growth of *Oscheius* L8 MCB in NB2 variations.

Figure 4.5. Growth of *Oscheius* L8 MCB in different nutrient broth variations.

Figure 5.1.1 An illustration of a solid/ semi-solid culture flask used to produce *Oscheius* L8 MCB

Figure 5.1.2 Illustration of a liquid state culture flask to produce *Oscheius* L8 MCB

Figure 5.2. An illustration of 24 well plate used in the comparative dose-response assay

Figure 5.3. A-E show Light microsccope images of different life cycle stages of *Oscheius* L8 MCB as observed NB2 *in vitro* culture.

Figure 5.4. Growth of different stages of *Oscheius L8 MCB* in solid culture over a period of 15 days (A). B represents nematode composition at days that have the peak number of IJ in solid state culture.

Figure 5.5. Growth of different stages of *Oscheius L8 MCB* in liquid culture over a period of 15 days (A). B represents nematode composition at days that have the peak number of IJ in solid state culture.

Figure 5.6. The comparison between the production of infective juveniles of *Oscheius* L8 MCB in solid state and liquid state cultures.

Figure 5.7. Dose-response assay at different time points for comparing *Oscheius L8 MCB* cultured *in vivo* and *in vitro* (solid and liquid).

Figure 5.8.1 Probit mortality obtained after 48 hours at each log concentration tested for *Oscheius L8 MCB* produced using *in vivo* and *in vitro* (solid and liquid) culture techniques

Figure 5.8.2. Infectivity of *Oscheius* L8 MCB against *Galleria mellonella*, at a concentration of 100IJ/larvae over the cause of 96 hours.

Figure 5.9. Mean number of emerging IJ progeny from larvae that had been infected with IJ from different culture sources at different doses.

List of tables

Table 2.1. 18S rDNA primer sequences used for nematode identification

Table 2.2. Results for nematode isolation from soil samples

Table 3.1. 16S rDNA primer sequences used for the identification and phylogenetic analysis of the bacterial symbiont associated with the isolated nematode

Table 4.1. Different NB1 variations used for axenic *in vitro* production of the EPN isolate

Table 4.2. Different NB2 variations used for axenic *in vitro* production of the EPN isolate

Table 5.1. LD_{50} values of *Oscheius L8 MCB* cultured using *in vivo* and *in vitro* methods

Table 5.2. LT_{50} values of *Oscheius L8 MCB* cultured in different production methods

List of abbreviations

BLAST- Basic Local Alignment Search Tool

DNA- Deoxyribose Nucleic Acid

EM- *Endotokia Matricida*

EPN- Entomopathogenic nematode

EPNs- Entomopathogenic nematodes

GF- Gravid Female

HF- Hermaphroditic Female

IPM- Integrated Pest Management

ITS- Internal Transcribed Spacer

NB- Nutrient Broth Variation

NB1- Nutrient Broth Variation 1

NB2- Nutrient Broth Variation 2

NBTA- Nutrient bromothymol blue agar

PCR- Polymerase Chain Reaction

rDNA- Ribosomal Deoxyribose Nucleic Acid

Chapter 1

Literature Review

1.1. Introduction

1.1.1. Chemical control

Insect pests cause serious damage to agricultural crops, thus resulting in major annual yield losses which then further result in substantial financial losses on a global scale (Oerke, 2006). Not only do they pose a financial threat, they also pose a threat to global food security for an increasing human population (Parsa *et al*., 2014). Since the 1940s, synthetic chemical pesticides have been produced for the effective control of various insect pests of both agricultural and horticultural crops (Kogan, 1998). However, regardless of their low cost of production and high efficacy in controlling insect pests, chemical pesticides have serious negative effects such as environmental pollution, undesired effects on non-target organisms (including animals and humans), and the selection of resistant insects which cause secondary pest outbreaks, thus leading to more crop losses (Guedes *et al*., 2016; Bravo *et al*., 2011; Devine and Furlong, 2007).

The use of chemical pesticides is still relevant irrespective of the negative effects of their use because they remain the most effective tool for pest management (Aktar *et al*., 2009). However, the increasing number of resistant insect pests is outweighing the development and production of chemical pesticides because of increased costs of development and the banning of such pesticides due to strict regulations laid out by several governments in countries such as Australia, USA, South Africa and several European countries, for the development of chemical pesticides (Ehlers, 1996; Hazir *et al*., 2003; Klein *et al*., 2011; Sjoberg *et al*., 2015). Therefore, there is a need for the use of other tools in the integrated pest management programmes for control of insects.

1.1.2. Integrated Pest Management strategies

Integrated pest management (IPM), is defined as resource management based on reasonable ecological principles, aimed at the promotion of the judicious and integrated use of various strategies (chemical, mechanical, cultural and biological) for the management of pests and pest resistance (Young, 2017). The aforementioned definition of IPM is one of many,

however, the central theme these definitions carry is that IPM involves the coordinated integration of various complementary methods for the suppression of pests in a safe, cost-effective and environmentally friendly manner (Ehrler, 2006). IPM programmes have been used since the 1960s as a means of crop protection to reduce the negative effects associated with chemical control (Parsa *et al*., 2014).

Most IPM programmes have been aimed at controlling insect pests because of the significant losses brought about by insects, and the development of resistance to chemical pesticides by insect pests of various crops (Godfray *et al.,* 2010). The various strategies employed in IPM to control insects include behavioural, physical (use of physical means such as barriers), chemical control and biological control (Kogan, 1998; Guedes *et al*., 2016; Young, 2017). Among the stated means strategies for the control of insects, biological control is the most advantageous because it provides effective and sustainable control (Young, 2017).

1.1.3. Biological control

Biological control of insect pests has and is being used in Europe, North America, Australia, China and Japan as an alternative to minimize the negative effect brought about by synthetic chemical pesticides (Van Zyl and Malan, 2014). Biological control is defined as the action of natural enemies of a host or pest, which results in the maintenance of host population at levels that are lower than would normally occur in the absence of those enemies (Ehler, 1990). It is also defined as the control of a pest by the deliberate use of a living organism to maintain the pest population at a lower level than would occur in the absence of used biological agent (DeBach, 1964).

Biological control can either be natural or applied, whereby natural biological control involves the suppression of pest populations by co-evolved natural enemies without any human intervention, whereas applied biological control involves human intervention to enable or enhance natural enemy activity in the suppression of pest population (Hazir *et al*., 2003). Applied biological control can be further divided into classical and augmentative biological control. Classical biological control involves the introduction of exotic natural enemies against an exotic or native pest (Goldson *et al*., 2014), whereas augmentative biological control entails the enhancement of the effectiveness of natural enemies already present in a particular environment, either through the manipulation of the natural enemy itself or the surrounding environment (Naranjo *et al*., 2015; Hazir *et al*., 2003). Synthetic chemical control and Biological control must be used in together as part of an integrated pest

management program as the use of only one measure of pest control may not be fully effective in the management of insect pest population in agricultural crops (Bravo *et al*., 2011).

In the biological control of insect pests, arthropod predators and parasites have been used as natural enemies, however, microorganisms (Bacteria, fungi, viruses and protozoa) have gained more interest as biological insecticides and are being used for the augmentative biological control of insect pests (Lacey *et al*., 2015; Hazir *et al*., 2003). Of the different microbial biological pesticides, *Bacillus thuringiensis* has been the most successful microbial biopesticide used for the control of certain insect orders (Bravo *et al*., 2011). However, entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis*, along with their respective bacterial symbionts of the genera *Xenorhabdus* and *Photorhabdus* have gained interest as microbial pesticides due to their ability to infect and kill a wide range of insect species (Kaya and Gaugler, 1993; Ali *et al*., 2011).

1.2. An overview of Entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) are organisms that have the ability to infect and kill insect hosts (Hatting *et al*., 2009). EPNs of the genera *Steinernema* and *Heterorhabditis* belonging to the respective families, Steinernematidae and Heterorhabditidae of the order Rhabditida (Hazir *et al*., 2003; Tabassum and Shahina, 2002). These EPNs and their associated mutualistic bacteria, naturally occur in soil and can kill host insects within 48-72 hours upon infection (Nguyen *et al*., 2006). Bacteria of the genera *Xenorhabdus* and *Photorhabdus* are found to have formed symbiotic associations with *Steinernema* and *Heterorhabditis* respectively, in order to cause death in a wide variety of insects (Hazir *et al*., 2003; Lephoto *et al*., 2016).

Apart from their ability to rapidly kill insect hosts, the advantages of EPNs are that they have a broad spectrum host range (Gaugler, 1988), are safe towards vertebrates, non-target organisms, plants and the environment (Akhurst, 1990; Boemare *et al*., 1996), can be used along with a number of chemical pesticides (Ramakuwela, 2015), are easy to mass produce *in vivo* and *in vitro* (Leite *et al*., 2017), and since soil is one of the environments in which it is difficult to achieve biocontrol, EPNs are effectively used for biocontrol of insect pest because they are highly adapted to soil (Klein, 1990). The disadvantages associated with EPNs are their narrow tolerance to environmental conditions, and their broad specificity, which may

also affect beneficial insects (Kaya, 1993). However, narrow tolerance to environmental conditions can be alleviated by changing the formulation of EPNs, whereas infection of beneficial insect has not yet been observed (Kaya, 1993; Boemare *et al*., 1996). Therefore, EPNs can be used for the biocontrol of insect pests in agricultural and horticultural crops.

1.2.1. Life cycle and mechanism of insect pathogenesis

The mechanisms of insect pathogenesis in *Steinernema* and *Heterorhabditis* are similar, irrespective of the difference in the phylogeny of these two genera (Blaxter *et al*., 1998). EPNs are obligate parasites that have a free-living, non-feeding, third stage infective juvenile (IJ), which infects the larvae of insect host in soil environments (Poinar, 1979). This is the only stage that occurs outside of a host insect in the life cycle of EPNs, and it is covered by a second stage cuticle to adapt to the harsh conditions in the soil environment (Hazir *et al*., 2003). The infective juveniles move through the soil to seek and find host insect using chemo-receptors (Riga, 2004).

When the IJ encounters the insect, it enters the host insect via natural openings such as the mouth, spiracles and the anus, or through inter-segmental membranes of the host cuticle for Heterorhabditids, whereby penetration into the haemocoel of the insect host involves the use of a dorsal tooth (Kaya and Gaugler, 1993; Popiel and Hominick, 1992). The IJ then continues to release its associated mutualistic bacteria from its gut into the haemocoel of the host insect through the mouth or the anus for Heterorhabditids and Sterneinermatids respectively (Ciche and Ensign, 2003; Poinar, 1966). Upon exposure to the haemocoel, the bacteria reproduce and release antimicrobial substances that kill the host via septicaemia, and prevent the cadaver from undergoing putrefaction (Hazir *et al*., 2003). These substances include toxins, exo-enzymes and antimicrobial agents (Van Zyl and Malan, 2014). After the bacterial symbiont has colonised the cadaver, the IJ will then continue to feed on the mutualistic bacteria and the metabolised host tissue to develop and reproduce (Strauch and Ehlers, 1998).

During development and reproduction, the IJ develops into the 3rd stage feeding juvenile, and further into a fourth stage juvenile (J4) before developing into the first generation of adults (Ehlers, 2001). For *Steinernema*, the first generation of adults comprises amphimictic (cross-fertilising) males and females, followed by subsequent amphimictic generations (Gaugler and Georgis, 1991). Whereas for *Heterorhabditis*, the first-generation adults are automictic (self-fertilising) hermaphrodite females succeeded by amphimictic generations (Poinar, 1990;

Kaya and Gaugler, 1993). EPNs may have 1-3 generations in the host, depending on the host size (Hazir *et al*., 2003). But when nutrients are depleted, a new generation of free-living IJs is produced and these IJs leave the cadaver to search for new insect hosts to start the life cycle once again (Stock, 2015). Figure 1 depicts the typical life cycle of EPNs.

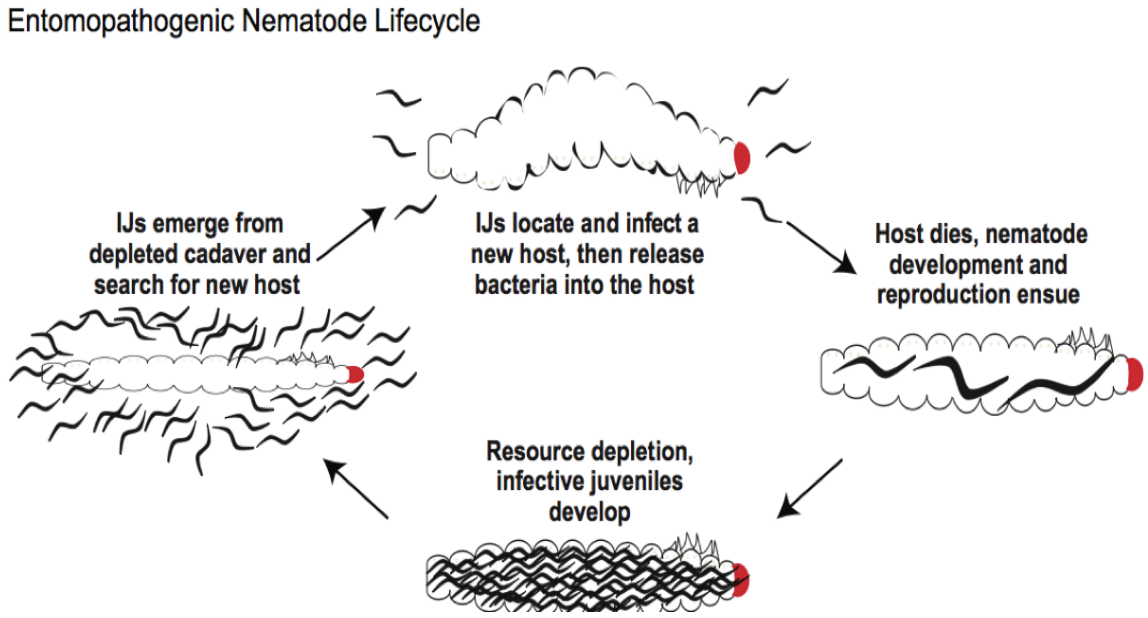

Figure 1.1. The schematic diagram of the general life cycle of EPNs. The cycle involves the predation and infection of an insect host by infective juveniles and the release of bacteria into host haemocoel, the death of the host and the development and reproduction of EPN, and the emergence of IJs from the cadaver to search for a new host, upon nutrient depletion. Courtesy of Dillan *et al*., (2012).

1.2.2. Bacterial symbionts of entomopathogenic nematodes

EPN-bacteria interactions are mutualistic interactions because the nematode partner protects the associated bacteria from the harsh conditions of the external environment; penetrates into the haemocoel of the host, and inhibits antibacterial proteins of the host (DeBach, 1964; Tanada and Kaya, 1993). Whereas, the bacterial partner provides a suitable environment for the nematode by killing the host and preventing the growth of other microorganisms; and it provides nutrition to the nematode partner by converting host tissue into a food source, and by being the food source itself (Ehler, 1990; Kaya and Gaugler, 1993). EPN-bacteria interactions are specific since a certain species of nematode can only be associated with a certain species of bacterial species (Hazir *et al*., 2003).

Bacteria of the genera *Xenorhabdus* and *Photorhabdus* are associated with EPNs of the genera *Steinernema* and *Heterorhabditis* respectively (Hazir *et al*., 2003; Lephoto *et al*., 2016). These bacteria all belong to the bacterial family Enterobacteriaceae, which consists of gram-negative, non-spore forming, and facultative anaerobes, capable of respiratory and fermentative metabolism (Brenner, 1999). *Xenorhabdus* and *Photorhabdus* can be distinguished by the fact that *Photorhabdus* is capable of bioluminescence and catalase activity, whereas *Xenorhabdus* is not (Adams *et al*., 2006).

Irrespective of their differences, entomopathogenic nematode associated bacteria occur in two phases, which are phenotypic variant cell forms called the primary form (phase I) and secondary form (phase II) (Forst and Clarke, 2002). The primary form is associated with EPNs, and it produces antibiotics, develops large intracellular inclusions composed of crystal proteins, it is found to be pathogenic and it favours EPN development and reproduction, whereas the secondary form occurs when the bacteria are in a non-growth stage, weakly produce antibiotics and inefficiently develops inclusions (Forst and Clarke, 2002). Figure 2 shows the biphasic nature of EPN bacterial symbionts.

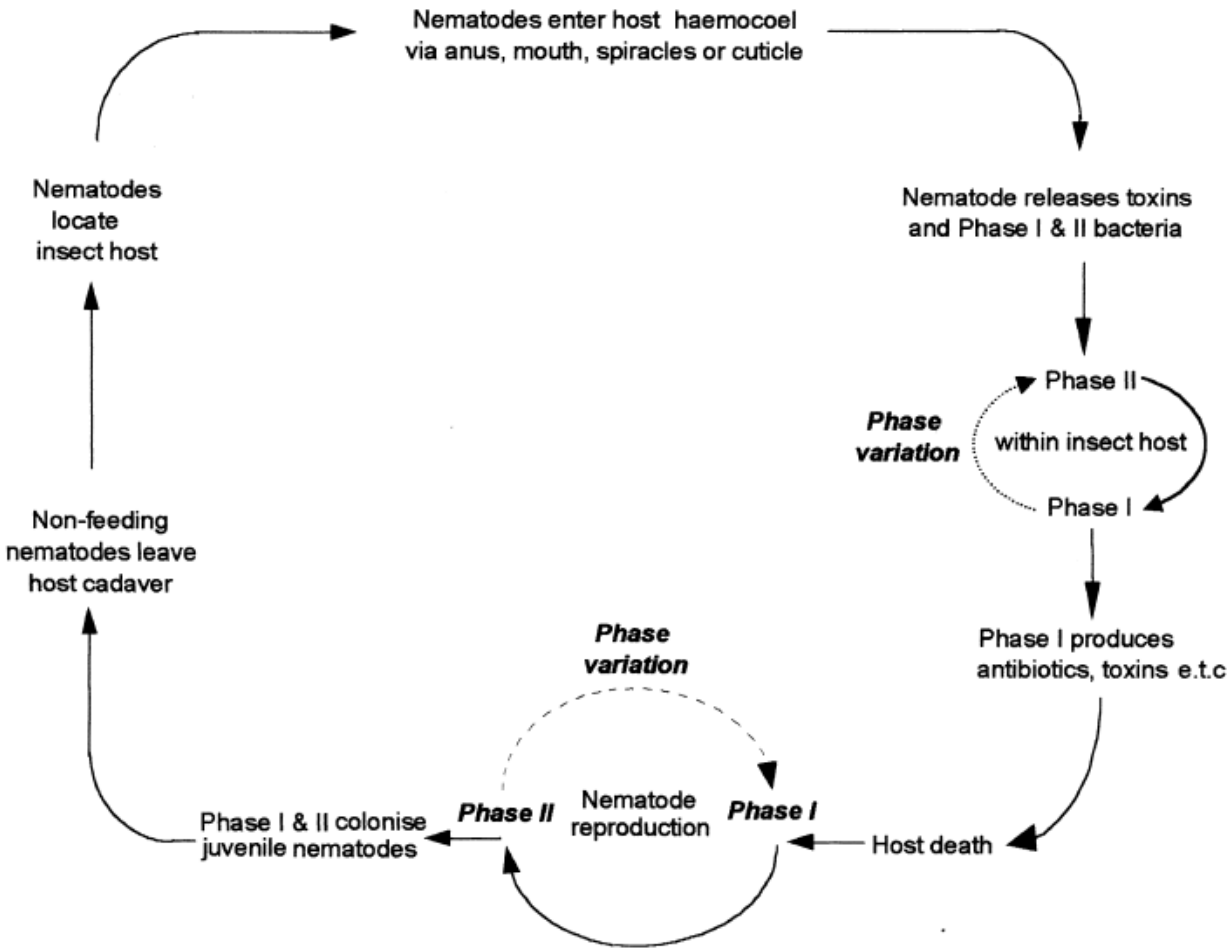

Figure 1.2. Schematic flow diagram of the occurrence of primary and secondary forms of EPN bacterial symbionts. Phase one promotes EPN development and reproduction. Courtesy of Ouwama, (2001).

1.3. Members of the *Oscheius* genus as EPNs

The nematode genus, *Oscheius*, is a genus consisting of soil-dwelling nematodes that belong to the Rhabditae family (Felix *et al*., 2001). This genus was first established by Andrassy (1976) as a sub-genus of *Rhabditis*. However, it was later recognised as an autonomous genus by Tabassum and Shahina, (2002). It is divided into two major sub-groups, *Dolichura* and *Insectivora*, which can be differentiated by the difference in size, whereby *Dolichura* species are smaller in size compared *Insectivora* species (Sudhaus, 1976; Sudhaus and Hooper, 1994). The reproductive modes of the *Oscheius* genus range between dioecious (reproduction between amphimictic adults at any given generation) and androdioecious (reproduction between automictic hermaphrodites and amphimictic males) reproduction (Denver *et al*., 2011).

Oscheius share a similar life cycle and mode of reproduction with *Caenorhabditis* species, *C. elegans* and *C. briggssae*, involving four juvenile stages (J1-J4) and a third stage juvenile that is like the dauer juveniles of *C. elegans* (Felix, 2006; Denver *et al.*, 2011). *Oscheius* nematodes are considered free-living nematodes, however, some members of the genus have insecticidal abilities (Campo-Herrera *et al*., 2015; Dillman *et al*., 2012). Campo-Herrera *et al*., (2015) argued that *Oscheius* species, act as scavengers of insects rather than entomopathogenic nematodes after observing the colonisation of freeze-killed *G. mellonella* cadaver by *Oscheius* species, *O. orinici* and *O. tipulae*. However, this does not hold true for *O. chongmingensis* (Liu *et al*., 2012), *O. carolinensis* (Torres-Barragan *et al*., 2011) which meet the conditions described by Dillman *et al*., (2012), for defining an entomopathogenic nematode. The conditions state that for a nematode to be considered as an EPN, it should have a mutualistic relationship with bacteria, whereby the relationship results in insect pathogenesis; and that insect death should occur in less than 5 days (Dillman *et al.,* 2012). These two *Oscheius* species form relationships with bacteria, particularly those of the *Serratia* genus to bring about insect death (Liu *et al*., 2012; Torres-Barragan *et al*., 2011). Other species of the *Oscheius* genus that have been classified as EPNs include *Oscheius safricana* (Serape, 2015) and *Oscheius* sp. TEL-2014 (Lephoto *et al*., 2016), which were also described as forming associations with bacteria in the *Serratia* genus.

1.4. *Serratia* genus as an insecticidal partner of *Oscheius* EPNs

The *Serratia* genus belongs to the Enterobacteriaceae family of the class γ-proteobacteria (Zhang *et al*., 2009). Bacteria in the *Serratia* genus are broadly distributed in the environment, with some being pathogens of invertebrates and vertebrates (Petersen and Tisa, 2013). Some species, like *Serratia entomophila* and *Serratia marcescens*, have been shown to have insecticidal activity (Bidari *et al*., 2018). *S. entomophila* has been developed into a commercial biopesticide product due to its insecticidal abilities (Jackson, Boucius and Thaler, 2001). Bidari *et al* (2018) screened *S. marcescens* against larvae of lepidopteran pests, *Plodia interpunctella* and *Ephestia kuehniella* and observed that this bacterial species possess insecticidal activity towards the larvae of lepidopteran insects. Torres-Barragan *et al*., (2011) found that *Oscheius carolinensis* was associated with four bacterial species, *S. marcescens*, *Enterococcus mundtii*, *Achromobacter xylosoxidans*, and *Providencia rettgeri*, however, they suggested that *S. marcescens* contributes to the entomopathogenicity of *O. carolinensis*

because they had observed that this bacterial species resulted in 100% mortality when screened against the larvae of *Helicoverpa zea*.

Indole derivatives of *Photorhabdus* and *Xenorhabdus* have been shown to suppress the growth of insect larvae and various microorganisms (Hu and Webster, 2000; Sunder and Chang, 1993; Paul *et al*., 1981). Serepa *et al*., (2015) showed that *Serratia marcescens* strain MCB, a bacterial symbiont of *Oscheius safricana*, produces indole derivative compounds, tryptophan and indole-3-acetic acid (IAA), which are similar to those produced by *Photorhabdus* spp. and *Xenorhabdus* spp. suggesting that *S. marcescens* strain MCB uses mechanisms that are similar to those used by bacterial species of the aforementioned genera for causing insect death. Studies such as these indicate that the *Oscheius-Serratia* insecticidal complex may share similar mechanisms with the *Heterorhabditis-Photorhabdus* and *Steinernema-Xenorhabdus* complexes with regards to insect pathogenicity.

1.5. Production methods for EPNs

The ability of EPNs to kill insects within 48-72 hours makes them attractive as an effective means of controlling insect pests in agricultural and horticultural crops (Van Zyl and Malan, 2014). The biopesticides potential of EPNs serves as an incentive for their mass production on a commercial scale for the biological control of insect pests (Salma and Shahina, 2012). EPNs have been cultured using either *in vivo* or *in vitro* technology (Shapiro-Ilan and Gaugler, 2002; Shapiro-Ilan *et al.*, 2014).

1.5.1. *In vivo* production

In vivo culturing methods make use of insects for the culturing of EPNs, whereby the host insect (usually *G. mellonella*) is reared on an artificial diet that allows the reproduction fattening of the insect (Kaya and Stock, 1997; Woodring and Kaya, 1988). This will, in turn, allow for the production of large numbers of high-quality EPNs (Shapiro-Ilan and Gaugler, 2002). *In vivo* culturing, methods often make use of the White trap for the collection of EPNs from insects (Dutkey *et al*., 1964; Kaya and Stock, 1997). Regardless of the high quality of EPNs produced by *in vivo* culturing, the requirement of intensive labour and insect hosts by these methods are not cost-effective, and therefore, confine *in vivo* culture methods to the small-scale production of EPNs (Shapiro-Ilan and Gaugler., 2002; Kaya and Stock, 1997). In contrast, *in vitro* production methods are not limited by the requirement of labour and insect hosts, meaning that *in vitro* production is the only economically reasonable means for large-

scale commercial production of EPNs (You *et al*., 2000; Ehlers and Shapiro-Ilan, 2005). The nutrient composition of the culture medium has to be taken into consideration prior *in vitro* production of EPNs because it could affect the final yield (Friedman, 1990). The nutrients that have been deemed as essential for the *in vitro* culturing of nematodes include proteins, lipids, and growth factors (Yoo *et al*., 2001; Abu Hatab and Gaugler, 1999; Vanfleteren, 1974).

1.5.2. *In vitro* production (monoxenic and axenic culturing)

In vitro production methods may be monoxenic (solid or liquid) or axenic (solid or liquid). Monoxenic cultures involve the production of nematodes in the presence of their bacterial symbiont in the growth medium (McMullen and Stock, 2014). The nematodes and the bacterial symbionts are the only organisms that are present in the culture, whereby EPNs to feed on both the growth media and bacterial symbionts thus favouring EPN reproduction (Strauch *et al*., 1994; Bedding, 1981). Axenic culture technique, on the other hand, involves the culturing of EPN in the absence of their bacterial symbiont in the growth medium, whereby the nematode may feed and reproduce on the artificial media (McMullen and Stock, 2014; Michnori *et al*., 2016). Upon realising the biopesticide potential of *Neoplactema glaseri*, Glaser, (1940) *in vitro* produced *Neoplactema glaseri* in axenic cultures, Hansen *et al*., (1968) *in vitro* produced *Neoplactema carpocapsae* in axenic cultures. However, it was later discovered later that bacterial symbionts play an important role in the reproduction of EPNs and monoxenic cultures have since been the used for *in vitro* production of EPNs (Poinar and Thomas, 1966; Hara *et al*., 1981; Bedding, 1981).

1.5.3. *In vitro* production (liquid and solid-state production)

In vitro production methods aimed at the large-scale commercial production of EPNs have been based on solid state and liquid state culturing (Shapiro-Ilan and Gaugler, 2002; Ehlers and Shapiro-Ilan, 2005). The most common method for solid-state culture is based on the method by Bedding, (1981), which involved the monoxenic culturing of *Steinernema* spp. on crumbled polyurethane sponges coated with poultry offal. High yields of up to 2 billion EPNs could be cultured per 3 kg bag of media using this method (Gaugler and Georgis, 1991; Bedding 1981; 1984). The disadvantages of *in vitro* solid-state culturing for mass commercial production include difficulty in online monitoring and the threat of contamination during upstream and downstream processing (Leite *et al*., 2017; Shapiro-Ilan and Gaugler., 2002; Shapiro-Ilan *et al*., 2014). Even though solid-state culturing offers improved scalability,

research based on liquid production has been conducted to further increase production scale with reasonable costs of production (Surrey and Davies, 1995; Ehlers and Shapiro-Ilan, 2005). Friedman *et al*., (1989) displayed the potential of liquid *in vitro* production for increased scalability when they produced *Steinernema carpocapsae* at maximum yields of 100 000 IJs/ml in 15 000 litre fermenters. The high yields of EPNs produced by liquid culturing makes it the most economically reasonable means for large-scale commercial production of EPNs (Yoo *et al*., 2000). However, EPNs have been commercially produced using both liquid and solid state *in vitro* methods in Australia, Europe, Asia, and North America (Leite *et al*., 2017; Shapiro-Ilan and Gaugler, 2002; Gaugler and Georgis, 1991).

1.6. Study Rationale

1.6.1. Effects of EPNs in biocontrol

Insect pests cause serious damage to agricultural and horticultural crops worldwide, causing major yield losses, resulting in substantial financial losses (Oerke, 2006). In response to crop damage from insect pests, chemical pesticides have been used for the control of insect pests. However, synthetic chemical pesticides contribute towards environmental pollution and affects non-target and beneficial organisms (Bravo *et al*., 2011). Biological pest control has been used to reduce the negative effects of chemical pesticides in the integrated pest management program, whereby biological organisms are used to control insect pest populations (Ehler, 1990). Entomopathogenic nematodes are one of the organisms that have been used in the biological control of pest insects, as they are able to infect and kill insect host within two to three days, and have a broad host range (Nguyen *et al*., 2006). It is for this reason that EPNs of the genera *Steinernema* and *Heterorhabditis*, along with their associated mutualistic bacteria have been commercially produced and used for pest control in Europe, North America, Australia, Japan and China (Leite *et al*., 2017; Ehlers & Jokinen 1996).

1.6.2. Commercialisation of EPNs in South Africa

In South Africa, the importation of EPNs from other countries is tightly regulated by Department of Agriculture, Forestry and Fisheries (DAFF), through the South African Agricultural Pests Act 36 of 1983, which prohibits the importation of foreign organisms unless a permit is provided, and a full-impact study is conducted to justify importation (Klein *et al*. 2011). This regulatory policy calls for the need to develop indigenous EPNs for the control of local pests. Cryptonem™ a product by River BioScience, is the only

entomopathogenic nematode product that has been commercially available (Steyn and Daneel, 2008). It is a powdery formulation of *Heterorhabdidtis bacteriophora* aimed at controlling the false codling moth, banded fruit weevil and other weevil species, codling moth, and sciarid flies (River BioScience website).

Different studies have been conducted in order to find and identify other local EPNs that are better adapted to local environmental conditions (Van Zyl and Malan, 2014). Apart from *Heterorhabditis bacteriophora*, nematode species that have been identified in SA thus far are *S. citrae, S. khoisanae, S. yirgalemense, H. zealandica, H. noenieputensis*, and *Oscheius* sp. TEL-2014 and *O. safricana* (Malan *et al*., 2011; 2014; Lephoto *et al*., 2016; Serepa, 2015). Even though these EPNs have been found to be effective against pest insects, their development as a commercial product is still a prospect as the focus is aimed at optimizing *in vitro* production (Ferreira, 2011; Van Zyl and Malan, 2014). Amongst these studies that are aimed at finding EPN in local soil is our current study, which aims to find and identify local a EPNs for potential biological control of local insect pests, in addition, this study aims to culture the isolated EPN using various *in vitro* production methods to monitor the productivity of these methods, and also the observe the effects of selected culturing techniques on nematode efficacy.

1.7. Aims and Objectives

1.7.1. Aim 1: To isolate and identify entomopathogenic nematodes found in soil samples

Objectives:

- Collect soil sample in Brits, North West Province, South Africa
- Insect baiting of collected soil samples with *Galleria mellonella* in order to isolate and recover EPNs
- The isolation of EPN infective juveniles (IJs) from larval cadavers.
- Perform sand bioassays for confirmation the insect pathogenicity of recovered IJs.
- Maintenance of EPNs by *in vivo* culture on *Galleria mellonella.*
- Molecular identification based on the sequence of the 18S rDNA PCR amplicon.
- Construction of 18S rDNA based phylogenetic trees

1.7.2. Aim 2: To isolate and identify bacterial symbionts of entomopathogenic nematodes

Objectives:

- Surface sterilisation of infective juveniles
- Isolation and culturing of bacterial symbionts
- Molecular identification based on the sequence of the 16S rDNA PCR amplicon.
- Construction of 16S rDNA based phylogenetic trees.

1.7.3. Aim 3: To evaluate the productivity of selected *in vitro* culture techniques for the production of entomopathogenic nematodes

Objectives:

- Culturing of the bacterial symbiont in different Nutrient Broth based media in Erlenmeyer shake flasks
- Culturing EPN in monoxenic and axenic cultures
- Culturing EPN in various nutrient broth based media compositions using the selected culture technique to observe the effects of media composition on EPN production
- Maintaining cultures of EPN in shake flasks using the selected culture technique.
- Evaluation of nematode development and IJ production in solid (bi-phasic) state and liquid state culture
- Comparative dose-response assay using *G. mellonella* larvae
- Emergence monitoring

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Chapter 2

Isolation and Identification of a South African entomopathogenic nematode

2.1. Introduction

Entomopathogenic nematodes (EPNs) are insect pathogens that have been extensively studied due to the important role that they play in the biological control of soil insect pests in agricultural ecosystems (Stock, 2015; Blanco-Perez *et al*., 2017). EPNs have various advantages that allow them to be used as effective biological control agents. These advantages include the ability to search and kill their insect host within 2-3 days of infection; the ability to infect a broad range of hosts; high adaptation to soil environments; and safety to the environment and non-target organisms (Jaffuel *et al*., 2017; Hazir *et al*., 2002; Klein, 1990; Grewal, 2002).

Due to their biocontrol abilities, the two EPN genera, *Heterorhabditis* and *Steinernema*, along with their respective bacterial symbionts of the genera *Photorhabdus* and *Xenorhabdus*, have been extensively studied and have been shown to share a similar life cycle and mechanism of pathogenesis (Lephoto *et al*., 2016; Blanco-Perez *et al*., 2017). The shared lifestyle and method of pathogenesis is a result of convergent evolution because these genera have distinct phylogenies (Poinar, 1993; Blaxter *et al*., 1998). Insect Pathogenesis involves a free-living parasitic stage (IJ) which finds and enters a suitable host and releases its bacterial symbiont into the haemocoel of the insect. The bacteria proceed to produce toxins which cause septicaemia, resulting in the death of the insect (Dillman *et al*., 2012). Thereafter, the EPN and bacteria reproduce for a few generations in the insect cadaver up until nutrients are depleted. In response to nutrient depletion, IJs are formed and they leave the cadaver in search of new hosts (Stock, 2015). Recently, some members of *Oscheius* genus, along with their bacterial symbionts in the *Serratia* genus, have been reported to cause insect death in a manner which is similar fashion, and thus are also considered EPNs (Lephoto *et al*., 2016).

The status of *Oscheius* as an entomopathogenic genus is controversial, as some researchers like Campos-Herrera *et al*., (2015), consider nematodes of the *Oscheius* genus as free-living nematodes that may act as scavengers that compete with EPNs for insect cadaver. However, some insecticidal nematodes of the *Oscheius* genus are considered EPNs based on the

conditions outlined by Dillman et al., (2012) for defining EPNs. The conditions state that for a nematode to be considered an entomopathogenic nematode, it should have an insecticidal relationship with bacteria, causing insect death in less than 5 days. Entomopathogenic *Oscheius* species that have been defined include *Oscheius chongmingensis*, *Oscheius carolinensis*, *Oscheius* sp. TEL-2014 and *Oscheius* sp. MCB (Liu *et al*., 2012; Ye *et al*., 2010; Lephoto *et al*., 2016; Serepa *et al*., 2015)

EPNs have been stated to be ubiquitous and widely distributed in the soil in all continents except Antarctica (Popiel and Hominick 1992; Hominick 2002). This ubiquitous occurrence of EPNs in soils and their biopesticide potential have served as a driving force behind several studies that are aimed at isolating and identifying EPNs. Bedding, (1990) outlined that the use of indigenous nematodes as effective agents for the control of indigenous pests because they are better adapted to the local environment. Therefore, the aim of this study is to isolate and identify a local entomopathogenic nematode for its biopesticide potential.

2.2. Methods and Materials

2.2.1. *In vivo* culturing of EPN on *Galleria mellonella*

The use of greater wax moth species, *Galleria mellonella* as a host species for the cultivation of entomopathogenic nematodes was developed by Duty (1959). This insect species belong to the Lepidoptera order and Pyralidae family. Greater wax moth species in their natural form are considered as a pest that causes serious damage to honey-bee colonies (Williams, 1978).

G. mellonella is a preferred host species for the cultivation of EPN due to its high susceptibility to the EPN, its commercial availability and ease of adjustment to being reared on artificial diets (Kaya and Stock, 1997; Van Zyl and Malan, 2014). In addition, larger sizes of the late-instar larval stages of *G. mellonella* allow for the production of high numbers of EPN (Flanders *et al.,* 1996), hence their use in this study.

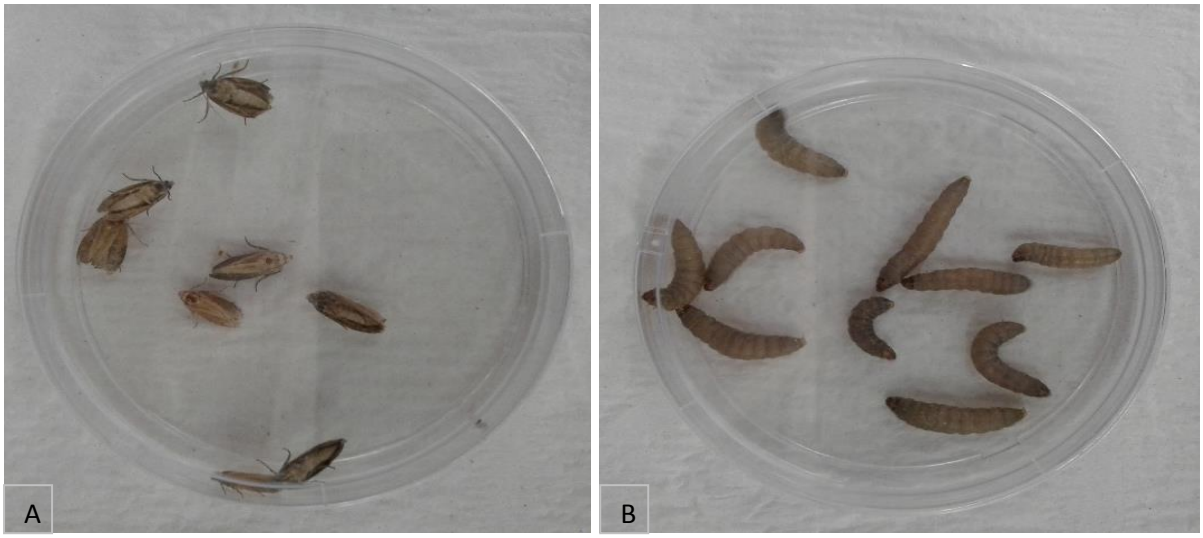

Figure 2.1. Adult moth (A) and larvae (B) of *Galleria mellonella*

G. mellonella were reared on an artificial diet based on the one outlined by Woodring and Kaya (1988). The diet consisted of a mixture of 500 g blended cereal (Bokomo Pronutro®), 200 ml honey, 200 ml glycerol, 5 teaspoons of yeast extract, 1 g sodium benzoate (preservative) and boiling water (See Appendix I for details). The mixture was made up to 3 times the amount of the given units, and then it was autoclaved at 121^{o}C and 15psi for 20 minutes. After cooling, wax paper (which serves as an egg-laying medium for the larvae) was used to wrap the mixture and was fed to *G. mellonella* adults and larvae in 3L Consol® glass bottles in a growth chamber at temperatures ranging from 25^{o}C- 28^{o}C (figure 2.2). Wax moth feeding was performed at 7-day intervals and the sub-culturing of the adult moth to new sterile bottles was performed at monthly intervals to prevent contamination. However, if bacterial/fungal contamination was observed, the contaminated cultures were discarded, and the glass bottles were cleaned with soap water containing bleach and thereafter sterilised by autoclaving at 121^{o}C and 15psi for 20 minutes.

Cultured *G. mellonella* larvae were used for insect-baiting, verification of entomopathogenicity, and maintaining *in vivo* cultures of EPN.

Figure 2.2. Culture bottle containing Adult moth and larvae of *Galleria mellonella*

2.2.2. Isolation of entomopathogenic nematodes (EPNs)

2.2.2.1. Soil sample collection

Soil samples were collected from eight locations around the Brits (25.6100^{o} S, 27.7960^{o} E) region in the North-West Province, South Africa. Hand spades that had been sterilised with 70% ethanol were used to collect soil samples from the depths of about 20cm underneath morula trees and around other shrubs in the region (spades were sterilised in-between sampling). Twenty soil samples were collected in 1 L ice cream tubs and were analysed and observed for the presence of entomopathogenic nematode species. In order to do this, the insect-bait technique was used as outlined by Bedding and Akhurst (1975).

2.2.2.2. Insect-bait technique

The insect-bait method makes use of last instar larvae of the wax moth, *Galleria mellonella* as bait in order to attract entomopathogenic nematodes (Bedding and Akhurst, 1975). Collected soil samples were slightly moisturised by adding sterile distilled water (dH_2O). This allowed for the movement and pathogenicity of nematodes in the soil since they need a

water film to carry out their motility (Georgis and Gaugler 1991). After soils were moisturised, 6th instar *G. mellonella* larvae were buried in the soil whilst some were placed over the soil samples in tubs (figure 2.3). The tubs were closed and were kept at 25°C ± 3°C for seven days. This was done with the expectation that the presence of *G. mellonella* in the soil would attract infective juveniles of EPN thus resulting in infection and eventual larval death (Bedding and Akhurst, 1975). After seven days at room temperature (25°C ± 3°C), insect cadavers that were suspected of being infected by EPNs were recovered and rinsed by spraying with 70% ethanol and transferred to White traps for the extraction of infective juveniles (Poinar, 1979).

Figure 2.3. Insect baiting method using *Galleria mellonella* as bait for the isolation of EPN from soil samples

2.2.2.3. Nematode extraction (White trap)

White traps were used for the collection and recovery of infective juveniles escaping from infected larvae cadavers (White, 1927). The method takes advantage of the natural process that occurs when IJs leave a nutrient depleted cadaver in search of new hosts (White, 1927). Modified White traps, as outlined by Kaya and Stock, (1997), were prepared by placing an overturned petri dish with a diameter of 5 cm, covered with a circular filter paper (Whatman No. 1) of the same size, inside a larger petri dish with a 9 cm diameter. Distilled water (dH_2O) was added to the larger petri dish, but not to the full amount but just enough to saturate the filter paper, then one or more *G. mellonella* larvae cadaver were placed in the middle of the filter paper (figure 2.4). White traps were left for 7-14 days at 25°C ± 3°C to

allow for emergence. When infective juveniles emerged from the cadaver and moved into the water, they were collected for use in tests to confirm insect pathogenicity of the putative entomopathogenic nematodes.

Figure 2.4. Illustration of the White Trap method used to isolate EPN from insect hosts.

2.2.2.4. Sand bioassays for confirmation of insect pathogenicity and *in vivo* culturing of G. mellonella

Sand bioassays were used in order to confirm pathogenicity, proving that the EPNs are responsible for the infection of *Galleria mellonella* larvae, the infective juveniles collected from the White traps were used to inoculate *Galleria mellonella* larvae (Steyn and Cloete, 1989). River sand was collected, sieved and then sterilised by autoclaving at 121^{o}C for 20 minutes. The infective juveniles trapped in the water film from the White traps were transferred to 9 cm diameter petri dishes containing sterile sand. The moisture content in the sand was adjusted to ~8% (w/w) and then one or more *Galleria mellonella* larvae are added to the plates (figure 2.5). The plates were inverted in order to maximise contact between the larvae and alleged EPN in the sand. After 3-7 days, plates were monitored for larval death. Cadavers were rinsed with dH_2O before they were transferred to White traps in order to recover IJs. The recovered IJs were transferred to moist sterile sand and last-instar larvae were added. Larval death at this point served as confirmation that the isolated EPNs were the causal agent of infection and death in larvae.

The above-mentioned procedure for proving pathogenicity was subsequently used for *in vivo* culturing of the EPN isolate. EPN produced from *in vivo* culturing were used for EPN DNA extraction, bacterial symbiont isolation, *in vitro* production experiments, and dose-response bioassays.

Figure 2.5. Illustration of a sand bioassay used to confirm insect-pathogenicity and for the *in vivo* production of EPN.

2.2.3. Nematode surface sterilisation

Surface sterilisation of nematodes was carried out on isolated IJs as outlined by Kaya and Stock (1997). It was performed in order to remove EPN microbial surfactants, which could be contaminants during EPN DNA extraction, EPN bacterial symbiont isolation and *in vitro* production.

IJs from White traps were transferred to 50 ml Falcon tubes and were left to sediment by gravity. Excess water was removed and IJs were immersed in 20 ml of 0.1% sodium hypochlorite solution (see Appendix II for preparation) for 1 hour. Excess sodium hypochlorite was removed and IJs were transferred to a different Falcon tube using a 3 ml disposable Pasteur pipette. Twenty millilitres (20 ml) of 0.1% sodium hypochlorite was added to the tube to immerse IJs for a further 3 hours. After the immersion period, IJs had formed sediment and the excess sodium hypochlorite was removed and IJs were rinsed three times using 4 ml of sterile distilled water per rinse. Surface sterilisation was performed under

the sterile conditions of a Laminar flow (Scientific). Sometimes, Ringer's solution was used instead of dH_2O because it provides isotonic conditions which allow IJs to live longer as compared to water.

2.2.4. Molecular identification of isolated nematodes and phylogenetic analysis

2.2.4.1. Genomic DNA extraction

Nematode Genomic DNA extraction was carried out by using the DNA extraction was carried out using the Puregene® DNA Purification Kit, Gentra Systems 2003 as outlined in Appendix II. The quantity and purity (260/280 nm absorbance ratio) of DNA was measured by using the Nanodrop® ND-1000 spectrophotometer. Isolated DNA was stored at 4^oC and later sent to Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa (Inqaba Biotec) for PCR and sequencing. The reason PCR was done at Inqaba was because outsoursing reduced the costs of performing this method.

2.2.4.2. Polymerase Chain Reaction (PCR)

PCR was used on the extracted DNA in order to amplify the internal transcribed spacer (ITS) region iin the 18S rRNA of the EPNs using the universal primers TW81 and AB28 (table 2.1) as suggested by (Joyce *et al*., 1994). PCR conditions are outlined in Appendix II. The ITS region was amplified because it is a short DNA sequence with a high copy number and also because there is high variation in this region allows for the distinction of closely related species (Hominick *et al*., 1997). PCR was not performed in the lab, but isolated nematode genomic DNA was sent to Inqaba Biotec for PCR and subsequent DNA sequencing. The reason PCR was done at Inqaba was because outsoursing reduced the costs of performing this method.

Table 2.1. ITS primer sequences used for nematode identification

Primer	Sequence 5'-3'
TW81 (forward)	GTTTCCGTAGGTGAACCTGC
AB28 (reverse)	ATATGCTTAAGTTCAGCGGGT

2.2.4.3. Sequencing and sequence identification

PCR products of the ITS region of the 18S rDNA using the TW81 and AB28 universal primers were used for sequencing. Sequencing was carried out on an ABI 3500XL Genetic Analyzer, POP7™ (ThermoScientific) machine, which allows for decreased turnaround time and improves the quality of the generated sequences (Inqaba Biotec website, 2018). FinchTV Version 1.4.0 was used to analyse and edit the generated sequence (Geospiza, Inc. 2004).

After sequence analysis, the unknown sequence was identified by making use of NCBI nucleotide BLAST (basic local alignment search tool). NCBI-BLAST for nucleotides compares the unknown DNA sequence with known DNA sequences from the genomic database, GenBank®.

2.2.4.4. Phylogenetic analysis

Phylogenetic studies were performed to determine the phylogeny of the isolated nematode species in relations to other EPN. MEGA version 7.0.26 (Kumar *et al*., 2016) was used to construct a phylogenetic tree of the EPN isolate using the DNA sequence that had been analysed and identified by FinchTV V 1.4.0 and NCBI-BLAST respectively. A phylogenetic tree was constructed by comparing the 18S rDNA sequences of the EPN isolate with those of known entomopathogenic nematode species. Sequences of other EPN species were collected from GenBank®.

MEGA7 was used to align sequences and construct maximum-likelihood phylogenetic trees using the bootstrap method with 1000 replications. The bootstrap is a statistical model that was used to place confidence intervals on constructed phylogenies (Felsenstein, 1985).

2.3. Results and Discussions

2.3.1. Isolation of entomopathogenic nematodes

Larvae death was only observed in 1 tub of 20 1L tub soil samples that had been collected from the 8 locations (5 % EPN recovery) in the Brits region when the insect baiting technique was used. After putative EPN were isolated from cadavers using White traps, sand bioassays showed that these isolated nematodes were pathogenic to *Galleria mellonella*. An interesting observation that was made was that the dead larvae infected by this putative EPN did not display any red-maroon or brown distinctive colour change associated with *Heterorhabditis* and *Steinernema* respectively, thus it was assumed that it is most likely an *Oscheius* species

(figure 2.6). Nematodes emerging from *G. mellonella* larvae were viewed under a dissection microscope (Olympus SZ40, Japan).

Table 2.2. Results for nematode isolation from soil samples

Number of locations + for putative EPN	Soil sample Location number	Soil type	Vegetation type	Assigned code for referral
1 out of 20	Location 8	Loamy Soil	Morula field	L8 MCB

Figure 2.6. Dead larvae infected by the putative isolate L8 MCB. Larvae showed no distinctive colour change, but putrefaction was absent.

The percentage recovery of putative EPNs (5%) in soil samples collected from the Brits region (1 of 20 sub-locations) is likely to have been affected by the sampling strategy used (Table 2.2). Sampling involved collecting moist soil close to different types of vegetation to increase the chances of extracting EPNs as they would most likely be near plants with the aim of finding their hosts (Van Tol *et al*., 2001). Myers *et al*., (2015) reported a 68% recovery of *Heterorhabditis* spp. from sandy soil within 100 metres of the Ocean in the Hawaiian Islands (187 sites of 275) using *G. mellonella* as bait. The reason for their high EPN recovery percentage may be that the soil type at their sampling sites comprised sandy soil, which has more spaces between sand particles, giving EPNs high motility between sand particles. Whereas the sampling sites for our current study consisted mainly of loamy type soil, which

has less inter-particle spaces compared to sand, thus restricting the movement of EPNs. Another reason could be agricultural practice, unlike the sampling sites were chosen by Myers *et al*., (2015), the sampling sites in this study were subject to agricultural practices (Marula farming), which could have negatively affected EPN populations. However, other factors that might have resulted in high EPN recovery include geographical location (tropical vs savannah biome), and soil conditions (pH and nutrient availability).

The use of *G. mellonella* as bait is effective because it is a susceptible host, however, it may not be a preferred host for some nematodes such as *Steinernema scapterisci*, which has been found to not reproduce in this insect as a host (Nguyen and Smart, 1992). Torres-Barragan *et al* (2011) showed that *Oscheius carolinensis* caused high mortality in *Pieris rapae* larvae (100%) compared to *G. mellonella* larvae (40%). Therefore, use of one type of insect species for baiting is limiting, and thus to improve the recovery rate from the soil it would be better to use a variety susceptible insect hosts that are not from the same order to improve the chances of isolating nematodes that are better adapted to infecting other insect orders. However, doing so is laborious and it would most likely be expensive to maintain cultures of multiple insect species that are potentially susceptible.

Therefore, in order to reduce costs associated with the use of multiple hosts, nematodes could be directly isolated from soil using methods such as sucrose centrifugation (Jenkins, 1964) and followed by the insect-baiting technique (in sterile soil) to select for EPNs as was done by Jaffuel *et al* (2016) when extracting EPN from 96 plots of different soil types (63% EPN recovery). The use of sucrose centrifugation does not take away the importance of the insect baiting technique for isolating EPN, but it does increase the chances of isolating various EPN that might not infect the selected susceptible host. However, regardless of the recovery rate, a putative entomopathogenic species was successfully isolated from soil samples using the insect baiting method, and sand bioassays proved that the unknown nematode isolate L8 MCB was responsible for the infection. Molecular techniques were used to identify the EPN isolate.

2.3.2. Molecular identification and phylogenetic analysis

In order to confirm the identity of the EPN isolate, genomic extraction was performed, and the DNA was sent to Inqaba Biotec for PCR and sequencing using the primers for the 18S rDNA region. After the sequence was analysed using FinchTV, NCBI BLAST was used to identify the sequence. The BLAST search showed that the unknown sequence was 99%

identical (Expect value = 0.0) to the sequence of *Heterorhabditis* sp. H1 (accession number KM387406.1), a species that has not yet been characterised. The unknown sequence was aligned with ITS rRNA sequences of member species of the genus *Heterorhabditis* using MEGA 7 (figure 2.7). These sequences were obtained from Genebank in order to analyse the phylogeny of unknown L8 MCB.

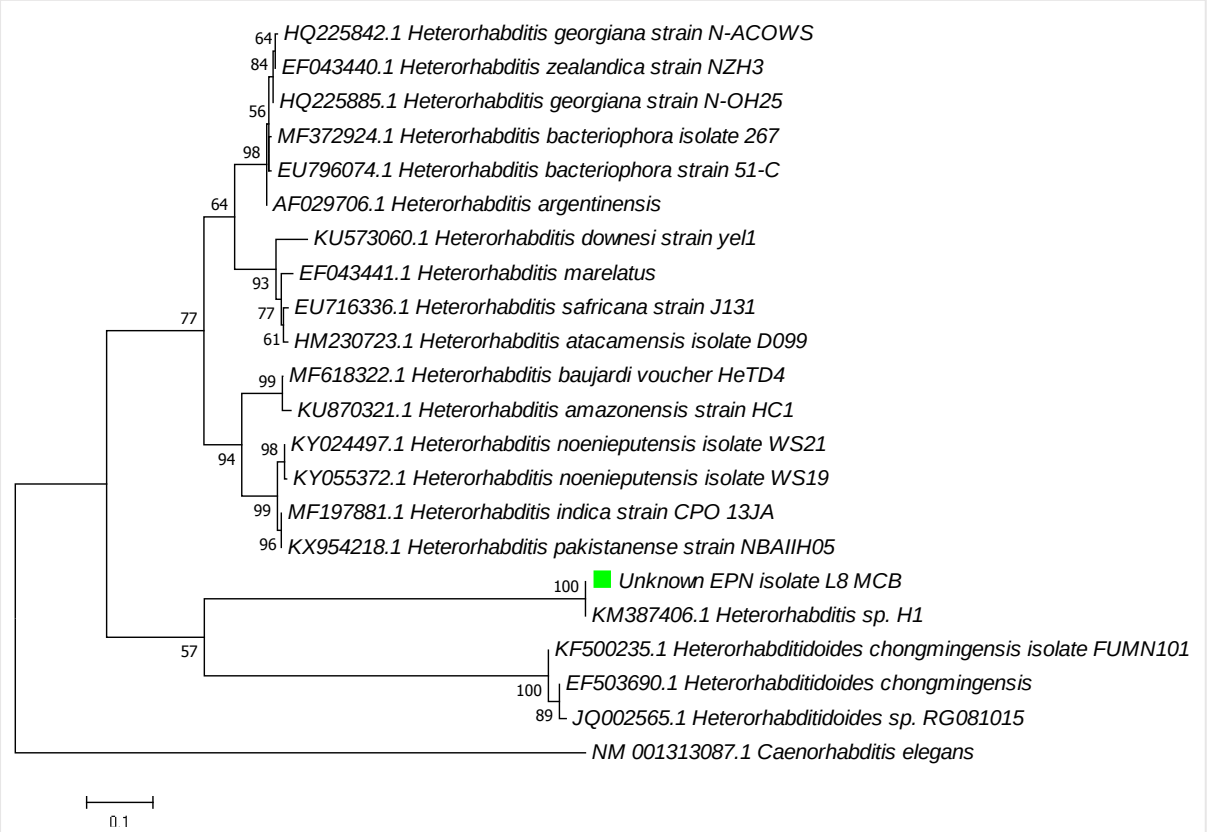

Figure 2.7. Maximum-likelihood tree based on the analysis of 18S rDNA sequence data showing the Phylogenetic relationships of Unknown isolate L8 MCB to members of the Heterorhabditis genus with *Caenorhabditis elegans* as an outgroup.

The phylogenetic analysis of EPN isolate L8 MCB supports the relation between isolate L8 MCB and the BLAST sequence *Heterorhabditis* sp. H1 (100% bootstrap percentage). However, an interesting observation is that isolate L8 MCB does not group with other typical *Heterorhabditis* spp. in the clade supported by 77% bootstraps, instead, it groups *Heterorhabditidoides chongmingensis* strains with a marginally significant bootstrap of 57%.

The ancestor species of Unknown L8 MCB and *Heterorhabditidoides chongmingensis* clade has had a few speciation events since it split from the ancestor that gave rise to other *Heterorhabditis* spp. and it is hypothesised that the splitting of these ancestral species came

about with difference in association with bacterial symbionts because members of the *Heterorhabditis* spp. clade are typically Heterorhabtids which associate with the *Photorhabdus* genus whereas *Heterorhabditidoides chongmingensis* species have been shown to be associated with *Serratia* species (Zhang *et al*., 2009). In addition, *Heterorhabditidoides chongmingensis* were recently characterised as being members of the *Insectivora* group of the *Oscheius* genus by Yen *et al*., (2010), therefore it is most likely that EPN isolate L8 MCB is also a member of the *Oscheius* genus. However, to see if the unknown nematode isolate is an *Oscheius*, an 18S rDNA based maximum likelihood tree comparing unknown L8 MCB with *Oscheius* species was constructed (figure 2.8). The *Oscheius* sequences were obtained by finding closely related members of *Heterorhabditidoides chongmingensis* on NCBI-BLAST and downloading the sequences from Genebank.

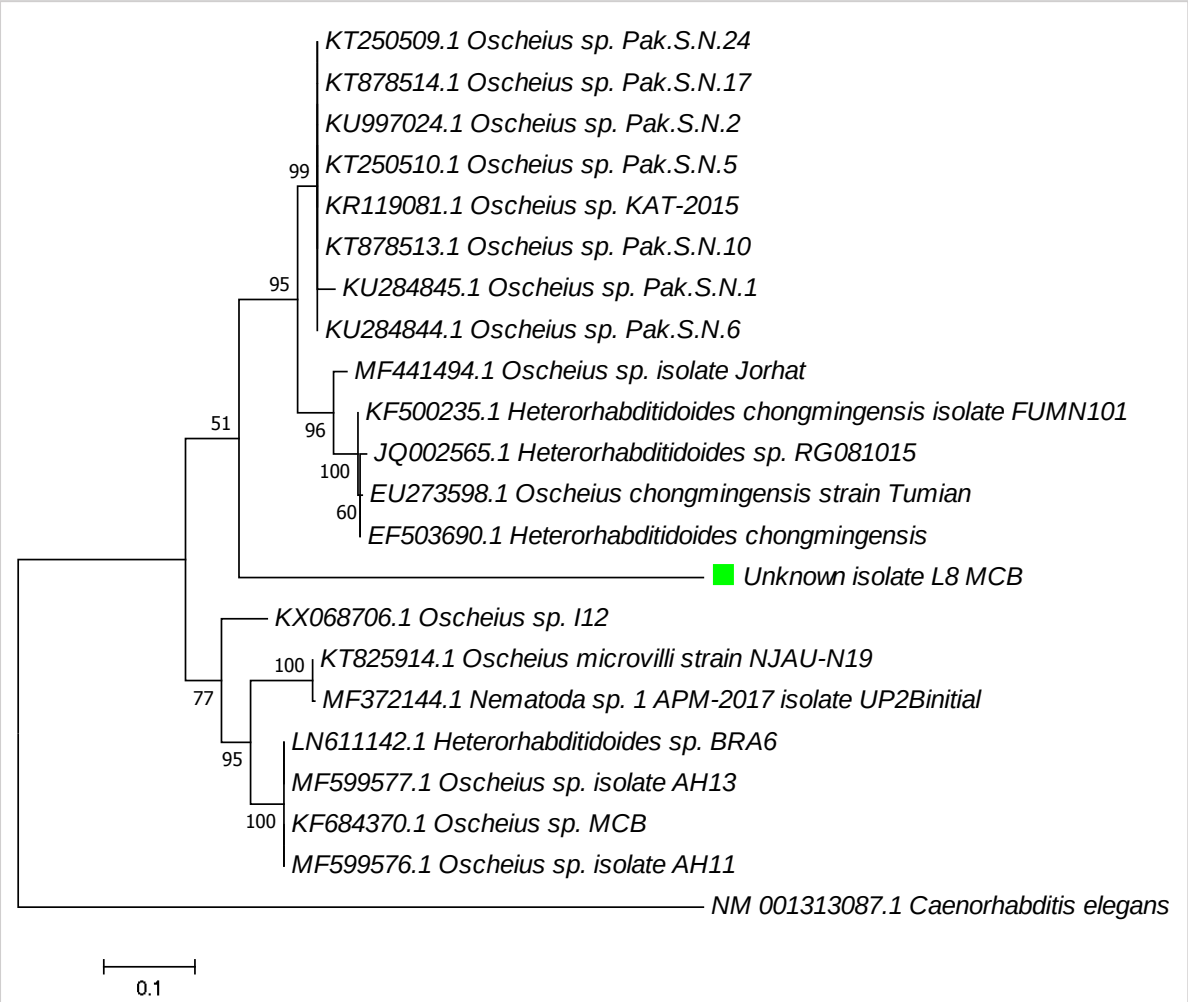

Figure 2.8. Maximum-likelihood tree based on the analysis of 18S rDNA sequence data showing the Phylogenetic relationships of Unknown isolate L8 MCB to members of the *Oscheius* genus with *C. elegans* as an outgroup.

When the phylogeny of the unknown EPN isolate L8 MCB was compared to members of the *Oscheius* genus, it was observed that this isolate does not group with any other in this genus, indicating that it might be a new species (Figure 2.8). Unknown L8 MCB forms a clade with *Oscheius* members in the group containing *Oscheius chongmingensis*, supported by a marginally significant bootstrap of 51%, indicating that the isolate may be an *Oscheius* species. Since the identification of this species is not fully convincing, the use of additional biomarkers (5.8S, 28S and 18S), whole genome sequencing, and morphological characterisation could be used the confirmation of the identification of this nematode species. However, for the sake of referral in this text, this nematode species will be called *Oscheius* L8 MCB.

2.4. Conclusions

The insect-bait technique was used to successfully isolate a putative entomopathogenic nematode species from soil samples collected at the Brits region, in the North West Province, South Africa and sand bioassays confirmed pathogenicity to *G. mellonella* thus confirming that the nematode species was entomopathogenic in nature. Infection symptoms in the larvae lacked the significant colours changes associated with *Heterorhabditis* and *Steinernema*, indicating that the nematode might be an *Oscheius* species. Molecular identification based on the 18S rDNA showed the nematode to be a *Heterorhabditis* species, however, this was contradicted by 18S rDNA based phylogenetic analysis, which grouped the unknown species with members of the *Oscheius* genus; this concurs with the phenotypic symptoms observed in infected larvae. Therefore, it is concluded that EPN isolate L8 MCB is most likely an *Oscheius* species. However, studies such as whole genome sequencing and morphological characterisation are needed for the confirmation of this species as an Oscheius species, but in this text, isolate L8 MCB will be referred to as *Oscheius* L8 MCB. It should be noted that this referral does not in any way infer any taxonomic value to this species, but only used for referring to the isolate.

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Chapter 3

Isolation and Identification of the bacterial species associated with *Oscheius* L8 MCB

3.1. Introduction

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* along with their respective mutualistic bacterial symbionts of the genera *Xenorhabdus* and *Photorhabdus* have gained interest as biopesticides for the control of insect pests due to their ability to search and kill soil-dwelling pests (Poinar, 1990; Boemare *et al*., 1993; Grewal *et al*., 2005; Yan *et al*., 2012). The ability to bring about insect death is a result of their symbiotic association with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, which belong to Enterobacteriaceae, a family of gram-negative bacteria, whereby the difference between these genera is that *Photorhabdus* bacteria display bioluminescence and are catalase positive, whereas *Xenorhabdus* bacteria lack bioluminescence and are catalase negative (Boemare, 2002; Adams *et al*., 2006).

The bacterial symbionts play an important role in pathogenesis and also in nematode reproduction within the host (Goodrich-Blair and Clarke, 2007). In Heterorhabtids, *Photorhabdus* spp. colonise the whole intestine, whereas for Steinernematids, *Xenorhabdus* spp. are kept in a specialised intestinal vesicle (Ciche and Ensign, 2003; Forst and Clarke, 2002). During the invasion of the insect haemocoel by the parasitic stage (third stage infective juvenile), the juveniles release bacteria through regurgitation for *Heterorhabditis* spp. or through defaecation for *Steinernema* spp. (Ciche and Ensign, 2003; Wouts, 1991).

Prior to the release of bacterial symbionts, *Steinernema* nematodes evade the host immune system by the release of enzymes that aid the release of bacteria, whereas *Heterorhabditis* nematodes produce antiphagocytic factors that prevent phagocytosis by host cells (Boemare and Akhurst, 1999; Silva *et al*., 2002). Upon release into the haemocoel, the bacteria produce substances that are involved in host immune suppression and septicaemia leading to insect death (Hazir, 2003). The substances include toxins, enzymes and antimicrobial agents (Van Zyl and Malan, 2014). *Photorhabdus luminescence* has been shown to produce Tc toxins which show insecticidal activity that is similar to that of δ-endotoxins of *Bacillus thuringiensis* which are involved septicaemia and host bioconversion (Bowen *et al*., 1998;

Forst and Nealson 1996; Waterfield *et al*., 2001). These bacterial secretions that result in insect death are part of the type III secretion system, a system encoded for by at least 20 virulence genes, which are involved in the movement of bacterial proteins to eukaryotic cells (Cornelis and Van Gijsegen, 2000; Brugirard-Ricaud *et al*., 2004).

After bacterial toxins result in insect death, the bacteria produce various compounds that prevent the growth of other organisms that might compete for the nutrient resources provided by the cadaver. These compounds include scavenger deterrent factors (SDF), which are unknown compounds but are believed to protect the cadaver from invading scavengers (Lewis *et al*., 2015; Campos-Herrera *et al*., 2015). Antimicrobial compounds such as antibiotics, bacteriocins and lumicins are also produced to prevent the cadaver from being colonised by other microorganisms such as bacteria and fungi (Boemare *et al*., 1997; Thaler *et al*., 1995; Sharma *et al*., 2002). Bacterial symbionts, along with their secretions, play an important role in the selection for its mutualistic nematode partner in the cadaver, as they eliminate competing organisms. This selection allows the growth and reproduction for several generations of the nematode partner, as a result of high nutrient availability in the form of bio-converted host tissue and cells of the bacterial symbiont (Dillman *et al*., 2012). The nematode feeds on the bacteria not only as a form of nutrition but also to allow the bacterial symbionts to colonise the intestinal tissues of the nematode.

When nutrients are depleted in the cadaver, nematodes develop to form the third stage infective juvenile (IJ), harbouring the bacterial symbiont cells in its intestine (Forst and Clarke, 2002). This stage will actively seek out new hosts. Therefore, the importance of the role played by the bacterial symbionts has called for the need to isolate and identify these bacterial symbionts.

EPNs of the *Oscheius* genus are believed to form associations with insecticidal members of the *Serratia* genus (Serepa *et al*., 2015). This genus includes insecticidal bacterial species such as *Serratia entomophila* and *Serratia marcescens* which are known to secrete virulence factors that lead to insect death (Bidari *et al*., 2018; Jackson *et al*., 2001; Ishii *et al*., 2012) The bacterial symbionts of *Oscheius* spp. have not been as extensively studied as those of *Heterorhabditis* and *Steinernema*, however it is believed that they follow a similar mechanism as those of the latter genera with regards to their pathogenicity towards insects, and reproduction (Lephoto *et al*., 2016).

Indole derivatives of *Photorhabdus* and *Xenorhabdus* have been shown to suppress the growth of insect larvae and various microorganisms (Hu and Webster, 2000; Sunder and Chang, 1993; Paul *et al*., 1981). Serepa *et al*., (2015) showed that *Serratia marcescens* strain MCB, a bacterial symbiont of *Oscheius safricana*, produces indole derivative compounds, tryptophan and indole-3-acetic acid (IAA), which are similar to those produced by *Photorhabdus* spp. and *Xenorhabdus* spp. suggesting that *S. marcescens* strain MCB has similar mechanisms to bacterial species of these genera. Studies such as these indicate that the *Oscheius-Serratia* insecticidal complex may share similar mechanisms with the *Heterorhabditis-Photorhabdus* and *Steinernema-Xenorhabdus* complexes with regards to pathogenicity and reproduction. A local nematode was isolated and identified as *Oscheius L8* MCB however, its bacterial symbiont is unknown. Therefore, the aim of this study is to isolate the bacterial symbiont of *Oscheius* L8 MCB.

3.2. Methods and Materials

3.2.1. The isolation, culturing and phenotypic identification of EPN bacterial symbionts

3.2.1.1. Isolation and storage of the nematode bacterial symbiont

Infective juveniles from White traps were surface sterilised to avoid contamination with bacteria that are not involved in the killing of the insects. Sterile IJs in Ringer's solution were transferred to sterile nutrient broth (see Appendix V for preparation) in an Eppendorf tube. The IJs were homogenised with the nutrient broth (NB) by crushing with a sterile plastic pestle to allow the release of bacteria into the NB. The homogenate was transferred to McCartney bottles containing nutrient broth and incubated in a shaker for 24-48 hours at 25^{o}C in order to allow for bacterial growth.

After incubation, the bacteria were streaked on Nutrient bromothymol blue agar (NBTA) and MacConkey agar (see Appendix III for preparations), to allow the selective growth of gram-negative bacteria, in the current case, the agars selected for either *Xenorhabdus* spp., *Photorhabdus* spp. or *Serratia* spp., which are bacteria that are symbiotically associated *Steinernema* spp., *Heterorhabditis* spp. and *Oscheius* spp. respectively. The bacteria were subsequently streaked on NBTA plates every 24 hours up until pure isolated bacterial colonies were observed. To further confirm that the isolated bacteria are gram-negative, a Gram stain was performed. For storage, single colonies of phase I bacteria from NBTA were

transferred to NB in three 250 ml Erlenmeyer flasks. The flasks were placed on a shaker at 180 rpm and incubated at 25^{o}C. From each flask, a loopful of bacterial culture was transferred to McCartney bottles containing 5 ml NB and 17% (v/v) glycerol. Bottles were stored at -20^{o}C. For use, cultures were thawed at room temperature (25^{o}C ± 3^{o}C). All bacterial work was performed under sterile conditions of a laminar flow (Scientific) and an open flame.

3.2.2. Molecular identification and phylogenetic analysis of the nematode bacterial symbiont

3.2.2.1. Bacterial genomic DNA extraction

Bacterial genomic DNA was extracted using the ZR Bacterial DNA Kit, # D6005 as outlined in appendix IV. The quantity and purity (260/280 nm absorbance ratio) of DNA was measured by using the Nanodrop® ND-1000 spectrophotometer and it was found to have a concentration of 375ng/µl. Isolated DNA was stored at 4^{o}C and later sent to Inqaba Biotec for PCR and sequencing. As an alternative to genomic extraction, isolated colonies in NBTA plates were sent to Inqaba Biotech for colony PCR and sequencing.

3.2.2.2. PCR of bacterial 16S rDNA gene

PCR was used to amplify the 16S rDNA region of the bacterial DNA (See Appendix IV for PCR conditions). This is because this gene sequence is highly conserved across all bacteria, the gene has a stable function, and even though this sequence is conserved, the conservative primary structure includes sequences that are variable enough to distinguish between bacterial species (Janda and Abbot, 2007). PCR was carried out using the universal primers, EUB968 and UNIV1382 (table 3.1) outlined by Kim *et al*., (2009). However, it was not performed in the lab, but isolated DNA was sent to Inqaba Biotech for PCR and subsequent DNA sequencing using the aforementioned universal primers. The reason PCR was done at Inqaba was because outsoursing reduced the costs of performing this method.

Table 3.1. 16S rDNA primer sequences used for the identification and phylogenetic analysis of the bacterial symbiont associated with the isolated nematode

Primer	Sequence (5'-3')
EUB968 (forward)	GAA GAG TTT GAT CAT GGC TC
UNIV1382 (reverse)	AAG GAG GTG ATC CAG CCG CA

3.2.2.3. Sequencing of bacterial 16S rDNA and identification of bacteria

PCR products of the 16S rDNA region using the EUB968 and UNIV1382 universal primers were used for sequencing. Sequencing was carried out on an ABI 3500XL Genetic Analyzer, POP7™ (ThermoScientific) machine, which allows for decreased turnaround time and improves the quality of the generated sequences (Inqaba Biotec website, 2018). FinchTV Version 1.4.0 was used to analyse and edit the generated sequence (Geospiza, Inc. 2004).

After sequence analysis, the unknown sequence was identified using NCBI nucleotide BLAST, which compares the unknown sequence to those in the GenBank® database.

3.2.2.4. Phylogenetic analysis and tree construction

Phylogenetic studies were performed to determine the phylogeny of the isolated nematode species in relations to other bacteria. MEGA version 7.0.26 (Kumar *et al*., 2016) was used to construct a phylogenetic tree of the EPN isolate using the DNA sequence that had been analysed and identified by FinchTV V 1.4.0 and NCBI-BLAST respectively. A phylogenetic tree was constructed by comparing the 16S rDNA sequences of the EPN isolate-bacterial symbiont with those of known EPN associated bacterial species. Sequences of other EPN species were collected from GenBank®.

MEGA7 was used to align sequences and construct maximum-likelihood phylogenetic trees using the bootstrap method with 1000 replications. The bootstrap is a statistical model that was used to place confidence intervals on constructed phylogenies (Felsenstein, 1985).

3.2.3. *In vitro* monoxenic cultures in lipid agar plates (for further confirmation of bacterial symbiont)

The method for monoxenic culturing of EPN on lipid agar plates (Wouts,1981) was developed by Kaya and Stock, (1997) for cross-breeding studies, however, in this study, it was used for further confirmation that the isolated bacteria was the bacterial symbiont of the EPN isolate. Lipid agar plates were prepared as outlined in appendix V. A single colony of Phase I bacteria from NBTA plates were transferred to nutrient broth (NB) in a 250 ml flasks. The flask was placed on a shaker at 180 rpm and incubated at 25^{o}C. After incubation, 100 µl aliquots of culture in the flask were transferred to 5 lipid agar plates. The aliquots were spread in each plate and the cultures were left to grow for 24 hours at 25^{o}C. Twenty (20) surface sterilised IJs of the EPN isolate were then added to the plates using a steel needle. Plates were sealed and left for 3-7 days at 25^{o}C. EPN growth and reproduction on plates confirmed that the isolated bacteria were symbiotically associated with the EPN isolate.

3.3. Results and Discussions

3.3.1. Isolation and phenotypic identification of the bacterial symbionts

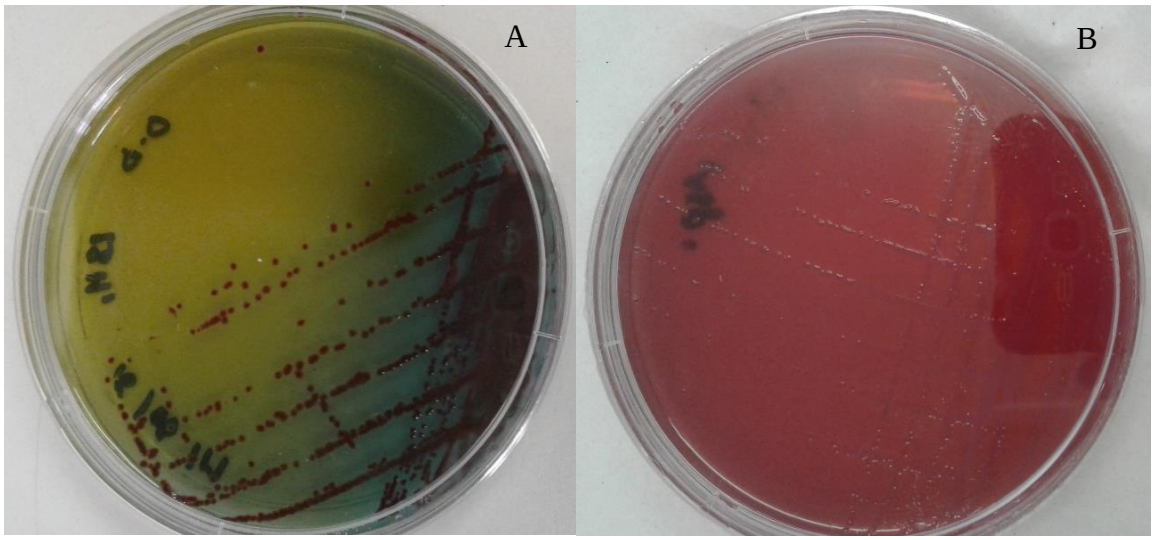

Figure 3.1. Nutrient bromothymol blue agar (A) and McConkey agar (A) plates of the bacterial symbiont L8B MCB alleged to be associated with *Oscheius* L8 MCB.

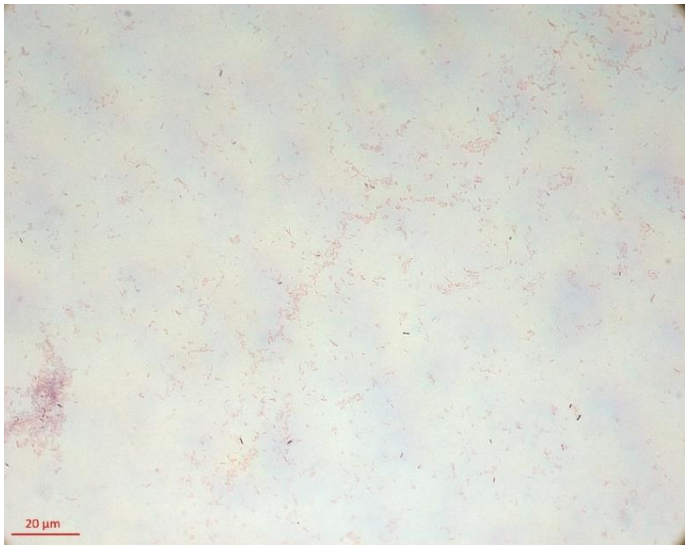

Figure 3.2. Gram stain of the supposed isolated bacterial symbiont of *Oscheius* L8 MCB (1000X magnification). The isolated bacterium is gram-negative.

In NBTA, the isolated bacterial colonies have red pigmentation with clear zones around them. Colonies also absorbed the dye as indicated by the change in colour of the plates from green to blue. In McConkey plates, bacterial colonies form pink colonies. The colony traits in figures 3.1 A and B are bacterial traits that are associated with gram-negative bacteria. These results were further confirmed by a Gram stain showing that the isolated bacterium is gram negative (figure 3.2).

3.3.2. Molecular identification of bacterial symbionts

After genomic isolation of bacterial DNA, 16S rDNA based PCR and sequencing were performed at Inqaba Biotec. The BLAST of the sequence edited by FinchTV showed that the sequence was 100% identical to that of *Serratia marcescens* (Expect value = 0.0)

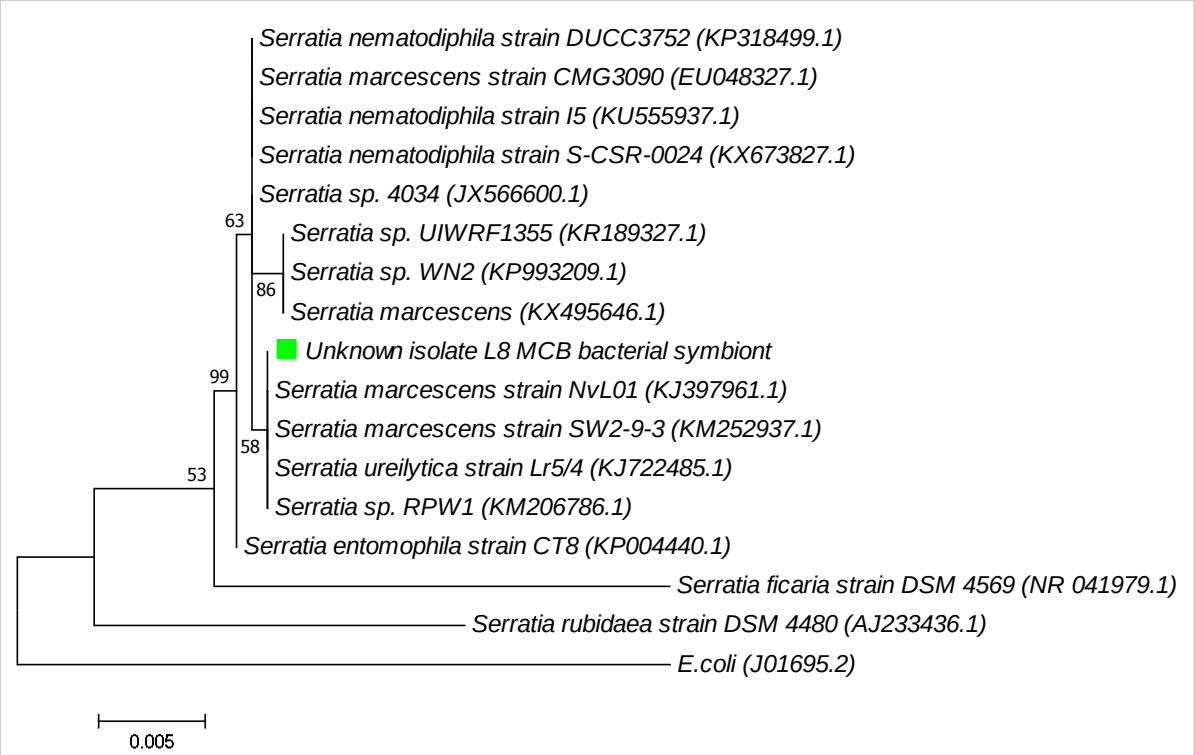

Figure 3.3. Maximum-likelihood tree based on the analysis of 16S rDNA sequence data showing the Phylogenetic relationships of the unknown L8 MCB isolate to members of the *Serratia* genus with *E. coli* as an outgroup

Phylogenetic analysis showed that the isolated bacteria was a member of the *Serratia* genus as shown by the 99% bootstraps that support this relationship. The unknown species forms a group with strains of *Serratia marcescens*, however, with a low bootstrap of 58%, indicating that it is a different strain of *Serratia marcescens*. Therefore, the unknown bacterium believed to be putatively associated with EPN isolate *Oscheius* L8 MCB is most likely a strain of *Serratia marcescens* and it will be referred to *S. marcescens* L8 MCB in this text. It should be noted that this referral does not infer any taxonomic value to the species.

3.3.3. Monoxenic cultures of EPN for confirmation of bacterial symbionts

Monoxenic cultures of *Oscheius L8 MCB* were attempted in the presence of *S. marcescens* L8 MCB to confirm if there is a symbiotic relationship between the two organisms.

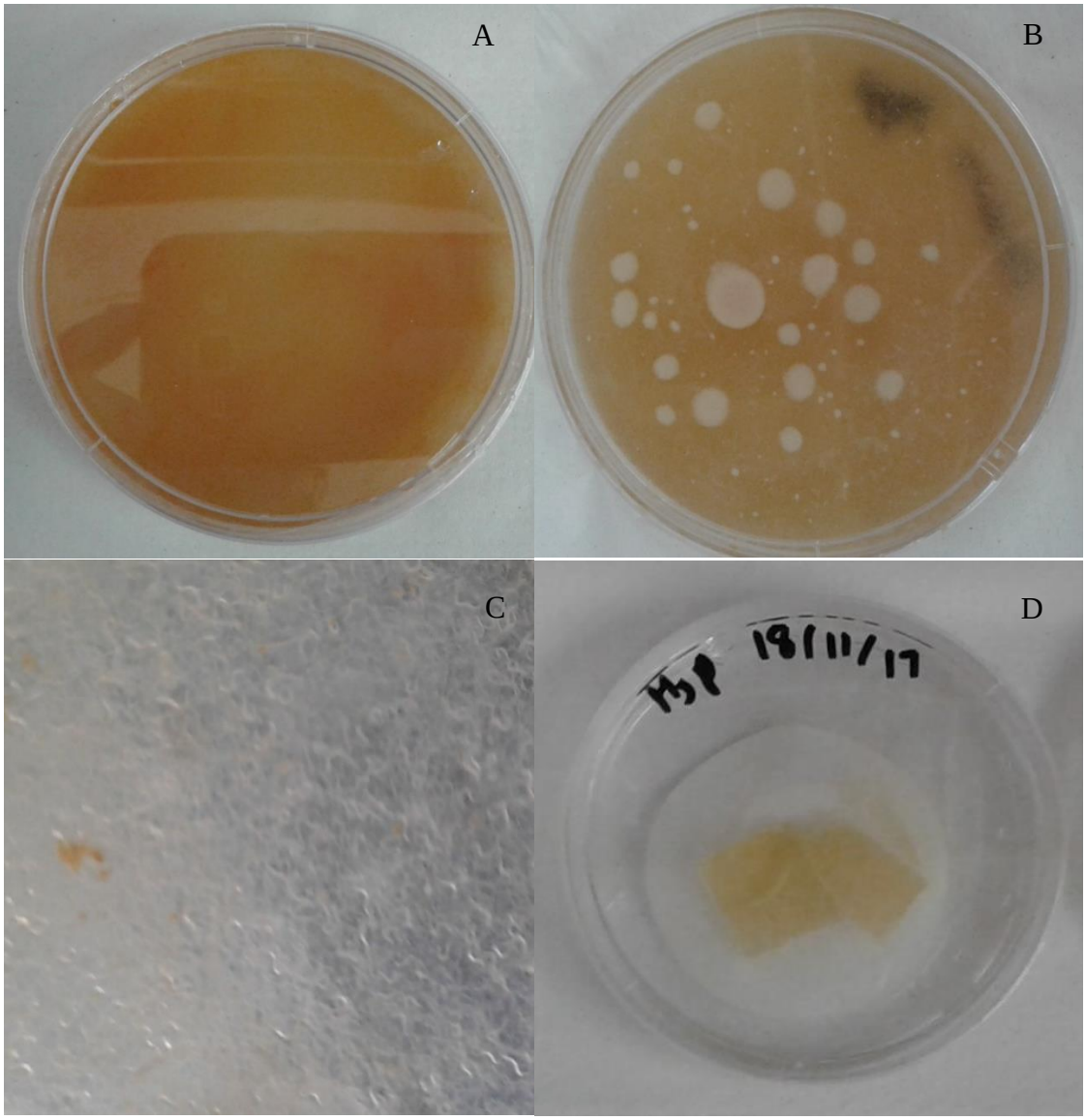

Figure 3.4. Monoxenic cultures of *Oscheius* L8 MCB in lipid agar plates containing *S. marcescens* L8 MCB. The figure shows a bacterial spread plate prior to the addition of the nematode isolate (A), whereas frame (B) and (C) indicated the growth of the EPN on monoxenic cultures in the presence of *S. marcescens* L8 MCB. D shows a White trap for the isolation of EPNs from lipid agar.

The absence of a bacterial lawn as shown in figure 3.4.C indicates that *Oscheius* L8 MCB has fed on the bacteria and was able to grow and reproduce. This growth confirms that *Serratia marcescens* L8 MCB is the bacterial symbiont that is associated with *Oscheius* L8 MCB. These findings concur with those of various studies that have shown that *Oscheius* species associate with *Serratia* species. The study by Serepa *et al.*, (2016) showed that *Serratia marcescens* strain *MCB* the symbiont of *Oscheius safricana*. MCB; Yen *et al*., (2010) showed

that *Serratia* spp. are symbionts of *Oscheius chomingensis*; and Torres-Baragan *et al.*, (2011) showed that *Oscheius carolinensis* is facultatively associated with *Serratia marcescens* spp. However, the relationship between *Oscheius* spp. and *Serratia* spp. is not as specific as those of *Heterorhabditis* spp. and *Steinernema* spp. along with their respective bacterial symbionts, *Photorhabdus* and *Xenorhabdus*.

Torres-Barragan *et al.*, (2011) also found *Oscheius carolinesis* to be associated with four types of bacteria which include *Serratia marcescens, Enterococcus mundtii, Acromobacter xylosoxidans,* and *Providencia retgerri*. However, they suggested that *S. marcescens* was the main contributor to the insecticidal abilities of *O.carolinensis*. Kumar *et al*., (2012) showed that *Oscheius* spp. formed an insecticidal complex with *Bacillus cereus,* which secretes antimicrobial compounds against bacteria and fungi. *Serratia* species such as *S. entomophila* and *S. marcescens* produce insecticidal compounds (Jackson *et al*., 2007; Bidari *et al*., 2018) and thus, it is likely that these gram-negative bacteria are the insecticidal members of the *Oscheius*-bacterium complex.

3.4. Conclusions

The bacterial species believed to be associated with the EPN species, *Oscheius* L8 MCB was isolated from surface sterilised nematodes and the species was identified to be a *Serratia* species that is most likely a strain of *Serratia marcescens* using the 16S rDNA gene. This species was referred to as *S. marcescens* L8 MCB, and it allows for the growth of *Oscheius* L8 MCB in monoxenic cultures. In addition, *Oscheius* L8 MCB cultured in the presence of *S. marcescens* L8 MCB was shown to be pathogenic to *G. mellonella*. Therefore, it is concluded that the isolated bacteria, *S. marcescens* L8 MCB, is indeed the bacterial symbiont of the nematode isolate *Oscheius* L8 MCB.

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Chapter 4

Evaluating the effects of *In vitro* axenic and monoxenic culturing techniques and growth media supplementation on the production of *Oscheius* L8 MCB

4.1. Introduction

Entomopathogenic nematodes (EPNs) are soil-dwelling insect pathogens that belong to the Steinernematidae and Heterorhabditidae families (Kaya and Stock, 1997; Lacey *et al*., 2015). These nematodes form mutualistic relationships with gram-negative γ-Proteobacteria belonging to the *Xenorhabdus* and *Photorhabdus* genera to bring about insect death (Dolinski *et al*., 2012; Dillman *et al*., 2012). Features that make EPNs to be attractive biological control agents include the ability to search and kill their insect host within 2-3 days of infection; the ability to infect a broad range of hosts; high adaptation to soil environments; and safety to the environment and non-target organisms (Jaffuel *et al*., 2017; Hazir *et al*., 2002; Klein, 1990; Grewal, 2002). The advantages possessed by EPN as biocontrol agents have shifted attention towards their commercial production. EPNs can be produced using *in vivo* and *in vitro* because they are amenable to both methods of production (Lacey *et al*., 2015; Shapiro-Ilan *et al*., 2014)

In vivo culturing methods involve the use of a susceptible insect host to culture EPNs of choice (Woodring and Kaya, 1988; Kaya and Stock, 1997). *In vivo* production is based on the White trap method, whereby insects are inoculated with nematodes, and after insect death, larvae are placed in White traps and insects are harvested from these White traps (Woodring and Kaya, 1988; Shapiro-Ilan *et al*., 2002). The advantages of *in vivo* production are that it is less prone to contamination, it needs less technical expertise and that it produces high-quality EPNs (Gaugler and Han, 2002; Gaugler and Georgis, 1991; Yang *et al*., 1997). The disadvantage of this method is that it is laborious and thus impractical for large-scale production.

In vitro methods, involve the culturing of nematodes in artificial media, in the absence of the insect host (Kaya and Stock, 1997; Shapiro-Ilan and Gaugler., 2002). *In vitro* culturing of EPNs in artificial media may affect EPN quality, however, it is able to overcome the disadvantages associated with *in vivo* culturing, and thus it is a preferred option for

commercial production (Yang *et al*., 1997; Shapiro-Ilan and Gaugler., 2002). Upon realising the biopesticide potential of *Neoplactema glaseri*, Glaser, (1940) *in vitro* produced the nematode in axenic cultures, however, chemical pesticides were of more interest at the time, and thus less attention was given to this work (Van Zyl and Malan, 2014). Later on, in the 1960s when interest in biopesticides was rising due to the introduction of integrated pest management (IPM), Hansen *et al*., (1968) *in vitro* produced *Neoplactema carpocapsae* in axenic cultures. However, it was later discovered later that bacterial symbionts play an important role in the reproduction of EPNs and monoxenic cultures have since been the used for *in vitro* production of EPNs (Poinar and Thomas, 1966; Hara *et al*., 1981; Bedding, 1981).

In vitro culturing of EPNs in monoxenic cultures involves the production of nematodes in the presence of their bacterial symbiont in the growth medium (McMullen and Stock, 2014); whereby the nematodes and the bacterial symbionts are the only organisms that are present in the culture. This allows the EPN to feed on both the growth media and bacterial symbionts thus favouring EPN reproduction (Strauch *et al*., 1994; Bedding, 1981). Axenic culture technique, on the other hand, involves the culturing of EPN in the absence of their bacterial symbiont in the growth medium, whereby the nematode is expected to feed and reproduce on the artificial media (McMullen and Stock, 2014; Michnori *et al*., 2016).

Pure axenic cultures of *Steinernema* spp. have been produced, however, artificial media that allow for the axenic production of *Heterorhabditis* have not been found (Boemare *et al*., 1997). Most EPNs in the *Heterorhabditis* and *Steinernema* genera are incapable of reproduction in the absence of their bacterial symbionts, indicating that bacteria provide nutritional factors that promote nematode reproduction, however, these nutritional factors are unknown (Lunau *et al*., 1993).

In addition to nutrients provided by the EPN bacterial partner, the nutrient composition of the medium used for *in vitro* production is also an essential factor in EPN *in vitro* production as it could determine the final yield (Friedman, 1990). The nutrients that have been deemed as essential for the culturing of nematodes include proteins, lipids, and growth factors (Yoo *et al*., 2001; Abu Hatab and Gaugler, 1999; Vanfleteren, 1974). However, among these nutrients, lipids are nutrients of interest because they contribute 60% of the total energy metabolised by the non-feeding IJ stage (Selvan *et al*., 1993; Abu Hatab and Gaugler, 1997). Therefore, since lipids play an essential part in IJs, the optimization of lipid composition in liquid culture is important for increasing IJ yields. Gil *et al*., (2002) showed that the

culturing of *Heterorhabditis bacteriophora* in growth culture medium containing canola oil supplemented with glucose increased nematode yield, showing the importance of glucose supplementation.

Insecticidal members of the *Oscheius* have recently been described as EPNs. The insecticidal species of this genus that have been defined include *Oscheius chongmingensis*, *Oscheius carolinensis*, *Oscheius* sp. TEL-2014 and *Oscheius* sp. MCB (Liu *et al*., 2012; Ye *et al*., 2010; Lephoto *et al*., 2016; Serepa *et al*., 2015). Insecticidal *Oscheius* spp. are believed to have formed mutualistic relationships with members of the *Serratia* genus to bring about insect death (Dillman *et al*., 2012; Serepa *et al*., 2015). Since the *Oscheius* genus has been found to have entomopathogenic species, no studies have been conducted on the *in vitro* production of these nematodes as potential biopesticides. *Oscheius* L8 MCB is an insecticidal nematode that was isolated from Brits in the North West Province of South Africa. This nematode was found to be associated with a *Serratia* bacterium referred to as *S. marcescens* L8 MCB. This study aims to report on the *in vitro* culturing of *Oscheius* L8 MCB in monoxenic and axenic cultures, to understand the effects of the bacterial symbiont, on nematode growth. In addition, the study aims to evaluate the effects of culture media supplementation (with canola oil and glucose) on the yields of Oscheius L8 MCB in axenic liquid cultures.

4.2. Methods and Materials

4.2.1. Culturing bacterial symbiont in various media

Prior to monoxenic and axenic culturing, bacterial growth in artificial media was compared in order to find the medium that allows for optimal bacterial growth. The artificial media compared were nutrient broth (NB) and its variations, NB1 (NB+ 4% canola oil) and NB2 (NB+ 4% canola oil (w/v) + 25mg/ml glucose). Consult Appendix V for the preparations of NB variations.

Fifty millilitres (50 ml) of NB were inoculated with a single colony of *S. marcescens* L8 MCB from an NBTA plate in a 250 ml Erlenmeyer flask. The flask was incubated on a shaker at 180 rpm for 24 hours at $25C^{o} \pm 2C^{o}$. Close to the end of the incubation period, 9 shake flasks were prepared for the addition of the different NB variation media. Of the 9 flasks, 3 contained NB, 3 contained NB1 and 3 contained NB2. After the 24h incubation, 100µl aliquots of the bacterial symbiont were transferred to each of the shake flasks and were

placed on a shaker at 120 rpm for 24 hours at 25C^{o} ± 3C^{o}. Bacterial growth was monitored by measuring the absorbance at 600nm at 1-hour intervals for 24 hours using a spectrophotometer. The media that was found to yield more bacterial growth was NB2 and it was further used for the comparison of nematode production in monoxenic and axenic cultures.

4.2.2. *In vitro* production of *Oscheius* L8 MCB (monoxenic vs. axenic production)

4.2.2.1. *In vitro* monoxenic cultures

A single colony of *S. marcescens* L8 MCB isolated from NBTA plate was used to inoculate 50 ml of NB in a 250 ml Erlenmeyer flask. The mixture was then incubated for 24 hours at 25C^{o} ± 2C^{o} on a shaker at 120 rpm. After the incubation period, 100µl aliquots of the bacterial symbiont in NB were transferred to each of the three 250 ml flasks containing 50ml NB2 (the flasks had been prepared a few hours earlier). The inoculated flasks were then incubated on a shaker at 120 rpm for 24 hours at 25C^{o} ± 3C^{o}.

At the end of the 24h incubation period, 100 surface sterilised IJs of the *Oscheius* L8 MCB isolate were added to each flask. The flasks were placed on a shaker at 120 rpm for 15 days at 25C^{o} ± 3C^{o}. EPNs were enumerated under a dissection microscope at 24h intervals for 15 days. Enumeration of EPNs in each flask was carried out by taking the average of nematodes counted in five 20 µl drops of culture, and if the number of EPNs was too high to count, dilution was incorporated to make counting easier. Enumeration only differentiated between live EPNs and dead EPNs, life cycle stages were not taken into consideration. This experiment was carried out in triplicates.

4.2.2.2. *In vitro* axenic cultures

Fifty millilitres (50 ml) of NB2 was prepared in three 250ml Erlenmeyer flasks and 100 surface sterilised IJs of the *Oscheius* L8 MCB were added to each flask. The flasks were placed on a shaker at 120 rpm and incubated for 15 days at 25C^{o} ± 3C^{o}. The enumeration of EPNs was carried out as mentioned in 4.2.2.1. The experiment was carried out in triplicates.

4.2.3. Media supplementation studies

Media supplementation studies were performed in order to observe the effects of canola and glucose supplementation on the production of EPN in axenic cultures as they were found to produce higher numbers of EPNs compared to monoxenic cultures.

4.2.3.1. Culture in canola oil (nutrient broth 1 variations)

Fifty millilitres (50 ml) of NB1 variations (table 4.1) were prepared in three 250ml Erlenmeyer flasks (consult appendix V for preparations) and axenic culturing of *Oscheius* L8 MCB was carried out as shown in 4.2.2.2. The enumeration of EPNs was carried out as mentioned in 4.2.2.1. The experiment was carried out in triplicates.

Table 4.1. Different NB1 variations used for axenic *in vitro* production of the EPN isolate

NB1 variation	**% canola oil**
NB1a	0.4
NB1b	1
NB1c	2
NB1d	4

4.2.3.2. Culture in canola oil + glucose (nutrient broth 2 variations)

Fifty millilitres (50 ml) of NB2 variations (as shown in table 4.2) were prepared in three 250ml (consult appendix V for preparations) and axenic culturing of *Oscheius* L8 MCB was carried out as shown in 4.2.2.2. The enumeration of EPNs was carried out as mentioned in 4.2.2.1. The experiment was carried out in triplicates.

Table 4.2. Different nutrient broth variations (NB2) used for axenic *in vitro* production of the EPN isolate

NB2 variation	**% canola oil**	**Glucose concentration (mg/ml)**
NB2a	0.4	2.5
NB2b	1	6.125
NB2c	2	12.5
NB2d	4	25

4.2.4. Data analysis

The data for comparing monoxenic and axenic culturing was analysed using Student's T-test in Microsoft Excel 2013. The differences with $P< 0.05$ ($\alpha=0.05$) were considered significant whereby the null hypothesis is that there is no difference in EPN production between monoxenic and axenic culturing.

4.3. Results and Discussions

4.3.1. Culturing bacterial symbiont in various culture media

Bacterial symbionts play an important role in the reproductive aspect of EPNs, with optimal bacterial growth favouring nematode reproduction. Therefore, the bacterial growth of *S. marcescens* L8 MCB was studied prior to nematode culturing in order to find the nutrient broth variation medium that would allow for optimal bacterial growth, which would subsequently promote EPN reproduction. *S. marcescens* L8 MCB was grown in NB, NB1 and NB2 and bacterial growth was monitored for 24 hours. However, growth for up to 14 hours is shown (figure 4.1) because the stationary phase had been observed by this time and all the recorded optical density after this time point represent background noise in a form of dead cell sediments and cellular secretions. The 24 h growth curve can be observed in appendix VIII.

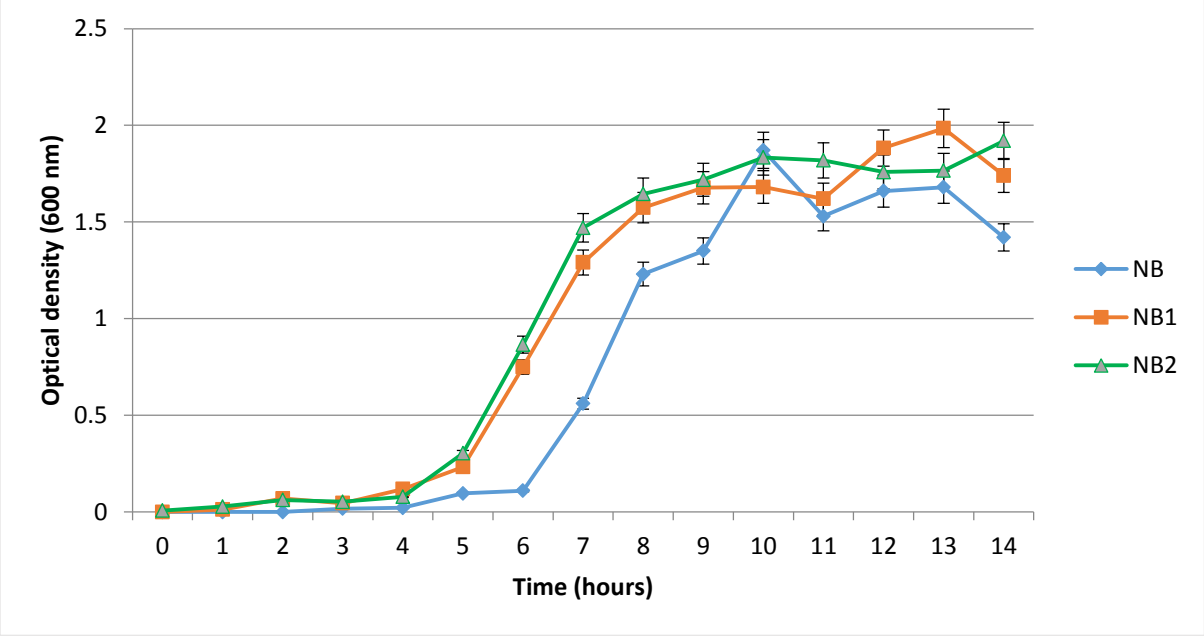

Figure 4.1. Bacterial growth curves of *S. marcescens* L8 MCB isolate in nutrient broth (NB), NB1 and NB2 over a 14-hour period. Error bars represent the standard error of the mean (SEM±) of triplicates.

In NB1 and NB2 (figure 4.1), the log phase occurs between 5 and 8 hours, compared to that of NB, occurring between 6 and 9 h at this point, there is rapid bacterial growth. The stationary phase begins at 9 h for NB1 and NB2, and 10 h for NB. At this point growth/reproduction slows down and later after this phase, the bacterial population enters the death phase, represented by declining absorbance on the graph. This phase is characterised by a decline in reproduction as a result of limited mineral resources.

The main observation in figure 4.1 is that bacteria growing in both NB1 and NB2 have higher absorbance than those cultured in NB, meaning that there are more bacterial cells in these two media than NB at any point on the growth curve except at 10 hours. This is due to the fact that the supplementation of NB with canola oil and glucose provided additional forms of nutrition to the bacteria that they promoted bacterial reproduction. Comparing NB1 and NB2, it was observed that except for the point at 5 hours, most points from the log phase to the stationary phase had higher absorbance for NB2, meaning that NB2 produced more bacterial cells. Therefore, NB2 was selected for producing *Oscheius* L8 MCB in axenic and monoxenic cultures.

4.3.2. *In vitro* production of *Oscheius* L8 MCB (monoxenic vs. axenic production)

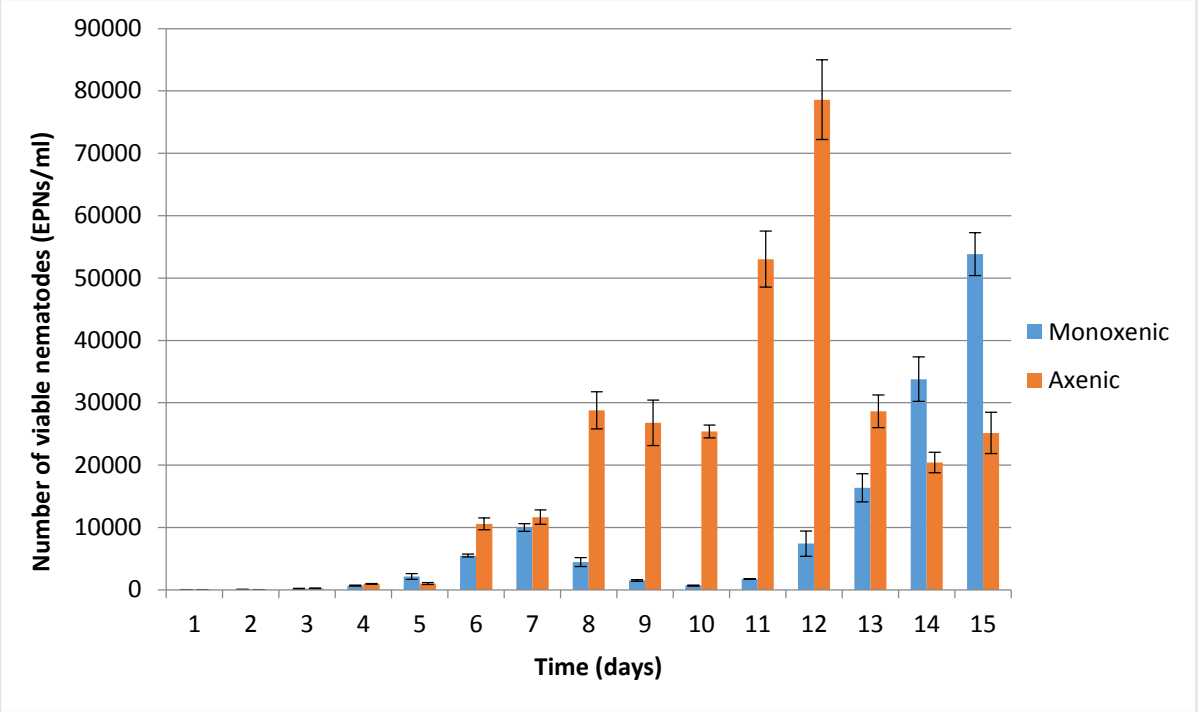

Figure 4.2. The growth of *Oscheius* L8 MCB in axenic and monoxenic cultures over a 15 day time period. Error bars represent the standard error of the mean (SEM±) of triplicates.

Oscheius L8 MCB was cultured using monoxenic and axenic methods in order to determine the productivity of these methods in culturing the nematode. Both culture methods used for production showed lag in production from day 1-5 (Figure 4.2). This lag phase could be associated with the acclimatisation of EPNs to the artificial medium.

The growth of *Oscheius* L8 MCB in monoxenic cultures seems to be stable given the smooth and bimodal nature of growth (figure 4.2). The initial peak at day 7 (10 017 EPNs/ml) may represent the first generation of EPNs offspring reproduced by the initial IJs used for inoculation. The decline in EPNs numbers after this point (day 8- day 10) could have been due to the inability of some of the offspring to adapt to the conditions of the artificial culture mediun (incompetent nematode offspring) or that the bacterial elements present at this point in culture were not competent enough to allow for EPNs reproduction, maybe due phase variation change (involving bacteria changing to the less favourable secondary phase), however, it is also noted that phase variation has not been studied in bacterial symbionts associated with the *Oscheius* genus. Other reasons for this decline in viable EPNs are

unknown. The second peak observed at the end of the culture period (Day 15: 53 833 EPNs/ml SEM ±3435 EPNs/ml) could have been due to the presence of competent bacteria, which might have produced secondary metabolites that favour EPN reproduction. Another reason is that this second generation of EPNs is better adapted to artificial media.

Nematode growth in axenic cultures also seemed to be also stable giving a single peak day 12, with a high yield of 78 600 EPNs/ml SEM ± 6379 EPNs/ml (figure 4.2). The peak was a result of offspring produced by a large number of adult members from one or two generations that succeeded the initial generation used for inoculation. The sharp decline observed might have been due to nutrient depletion or the decline in the quality of the medium at this point.

Figure 4.2., along with table in Appendix X show that axenic culturing significantly produced more viable EPNs than monoxenic culturing at reduced culture periods, whereas monoxenic culturing significantly produced more EPNs than axenic cultures later in the given culture period. In addition, axenic culturing not only produced more EPNs at reduced culture time, but it produced more EPNs in the overall culture period. These observations were found to be interesting because they contradict various literature that have shown that monoxenic culturing is a favourable method for nematode production because high numbers of EPNs are produced as a result of the presence of symbiotic bacteria, which plays an important role in nematode reproduction (Bedding, 1981; Surrey and Davies, 1995; Lunau *et al*., 1993) Aumann and Ehlers, 2001).

Lunau *et al* (1993), reported that liquid axenic cultures of *Steinernema carpocapsae* reached densities of 18 000 EPNs/ml in 1L shake flasks containing 200 ml basal medium, which is lower than the reported 100 000 IJs/ml reported by Friedman *et al*., (1989) for this species under liquid monoxenic cultures. They also reported lack of growth and poor growth of *Heterorhabditis* species (*H.bacteriophora* and *H. megidis*) under axenic conditions. Strauch and Ehlers, (1998) showed that the growth and development of *Heterorhabditis* spp was significantly increased by the presence of *Photorhabdus luminescens*, which provided a food signal that promoted nematode recovery from the developmentally arrested IJ stage.

Therefore, since current observations show that axenic culturing favours the growth of *Oscheius* L8 MCB compared to monoxenic culturing, it is hypothesised that this high productivity observed was a result of surface sterilised IJs in axenic cultures managing to release competent bacterial cells into the medium, whereby these bacterial cells reproduced and released secondary metabolites that favour EPN reproduction. In terms of monoxenic

cultures, it is believed that the poor growth might have been due to the presence of incompetent bacteria present initially in culture (bacteria used for inoculation might have reduced competency due to extended periods of artificial culturing), which negatively affected nematode growth.

On the case of surface sterilisation it should be noted that this method is not efficient for the production of bacteria-free nematodes as suggested by Lunau *et al*., (1993). In some instances in the current study, nematode cultures often failed (both monoxenic and axenic) due to the presence of contaminating bacteria in culture due to inefficient surface sterilisation, therefore methods such as alkaline lysis as suggested by Lunau *et al*., (1993) for the production of axenic cultures could be used. However, surface sterilised axenic nematodes still did manage to reproduce in NB2 liquid media. However, cultures may not have been purely bacteria free.

The EPN-bacterium relationship of the insect parasitic members of the *Oscheius-Serratia* complex has not been extensively studied. The role of the bacterial symbionts of Oscheius is still poorly understood and thus more studies need to be conducted for the better understanding of the role they play in nematode nutrition and reproduction.

4.3.3. Media composition studies for the effects of glucose and canola oil

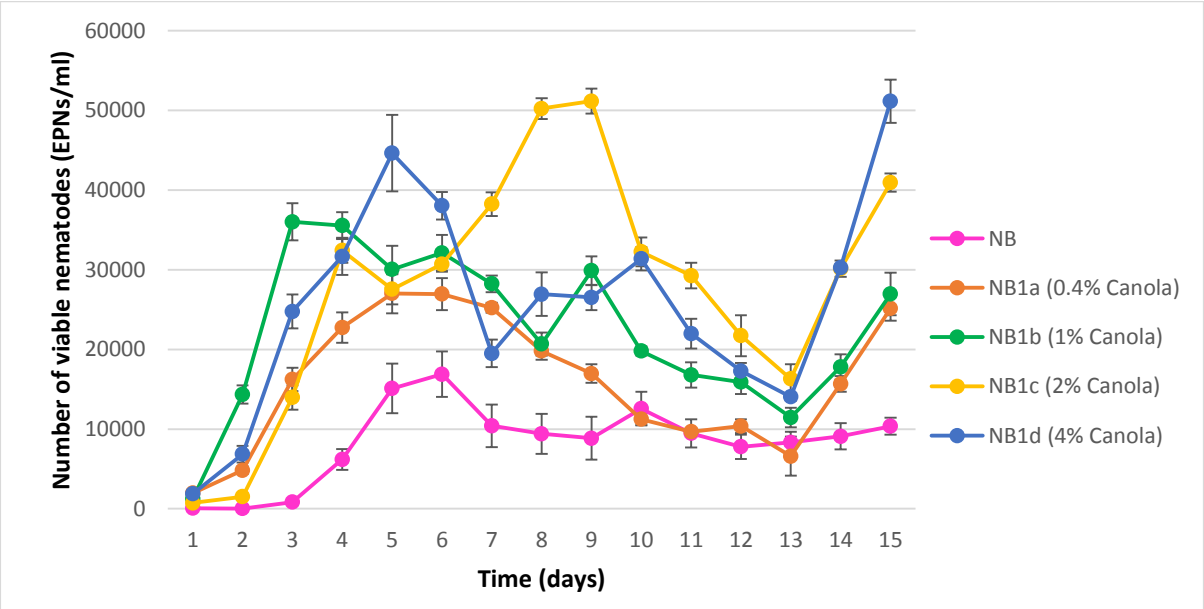

Figure 4.3. The growth of *Oscheius* L8 MCB in NB1 variations. The nutrient broth was supplemented with different canola oil concentrations. Error bars represent the standard error of the mean (SEM±) of triplicates.

In figure 4.3.., it is seen that all NB1 variations supplemented with canola oil significantly produced more EPNs compared to nutrient broth alone, except for NB1a between day 10 and day 13. The NB1 variations that produced high yields of EPNs (exceeding 40 000 EPN/ml) were NB1c (51 170 EPNs/ml SEM ± 1577 EPNs/ml at day 9) and NB1d (51 158 EPN/ml SEM ± 2717 EPNs/ml at day 15). This might suggest that increasing concentrations of canola will increase yields of EPNs. However, it should be noted that this is always not the case as the figure also shows that NB1c produced high nematode yields compared to NB1d at the respective time intervals of their initial peaks. This may mean that there is an optimal amount of canola that should be used to obtain high nematode yields within a range of given concentrations.

The culturing of *Oscheius* L8 MCB in NB1 variations showed that production follows a bimodal (NB1a and NB1c) and a trimodal (NB1b and NB1d) mode of growth as shown by two peaks and three peaks respectively, whereby time points at the end of the culture period are also considered to be peaks (figure 4.3.). This variation in mode of growth of *Oscheius* L8 MCB indicates that this species may not follow a stable in culture. Regardless of the mode of growth, it was observed that the lag phase in all the four NB1 variations is shorter than that of NB (control). This means that the presence of canola oil somehow managed to catalyse the transition of non-feeding juvenile stages to feeding stages (day 1-2), that that went to reproduce as seen by the exponential phase (day 2-5). However, the mechanism by which this catalysis takes place is not known, but it can only be surmised that the fatty acids contained in canola oil may be readily recognised as a food signal by the non-feeding juvenile stages. The signal then triggers the juveniles to release competent bacteria that also feed on the media and allow the culture conditions to be favourable for growth.

From the bimodal and trimodal nature of *Oscheius* L8 MCB cultures in NB1 variations, it is noticed that the initial peaks are observed at different time points. It is also noted that there is no trend with regards to delay in reaching peak EPN with increased canola concentration. This serves to further emphasise the instability of *Oscheius* L8 MCB in artificial culture.

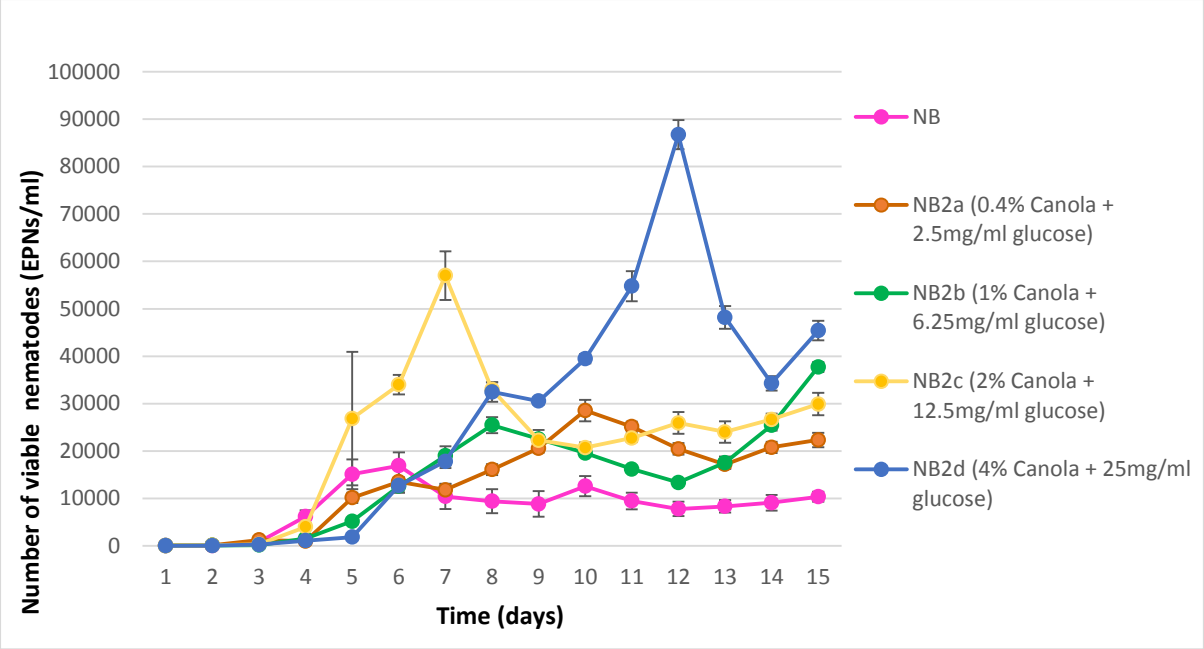

Figure 4.4. The growth of *Oscheius* L8 MCB in NB2 variations. The nutrient broth was supplemented with different canola oil and glucose concentrations. Error bars represent the standard error of the mean (SEM±) of triplicates.

Figure 4.4. shows that all NB2 variations supplemented with different concentrations of canola and glucose resulted in increased nematode numbers compared to NB alone. This is because canola oil and glucose provide more minerals in the form of lipids and carbohydrates. The NB2 variations that produced maximum yields of EPNs (exceeding 40 000 EPNs/ml) were NB2a (57 000 EPNs/ml SEM ± 1263 EPNs/ml at day 7) and NB2d (86 730 EPNs/ml SEM ± 2065 EPNs/ml at day 12) and reproduction in these variations only give a single peaks which represent a new generation of juveniles spawn as a result of the abundance in nutrients.

What has also been observed is that the lag phases for all NB2 variations are longer compared to the control. This might be because NB2 is complex as it has both canola and glucose, the combination of these two compounds may affect the manner in which non-feeding juvenile stages perceive as a food signal. For figure 4.3., it was hypothesised that the reason non-feeding juvenile stages progress to become the feeding and reproductive stages is that they may perceive fatty acids from canola oil as a food signal as it is a source of lipids, which are the primary nutrient derived from the host. However, with regards to glucose, it may not be readily seen as a food signal as it is not found readily in this form in the natural host, hence the prolonged lag phase (Figure 4.4).

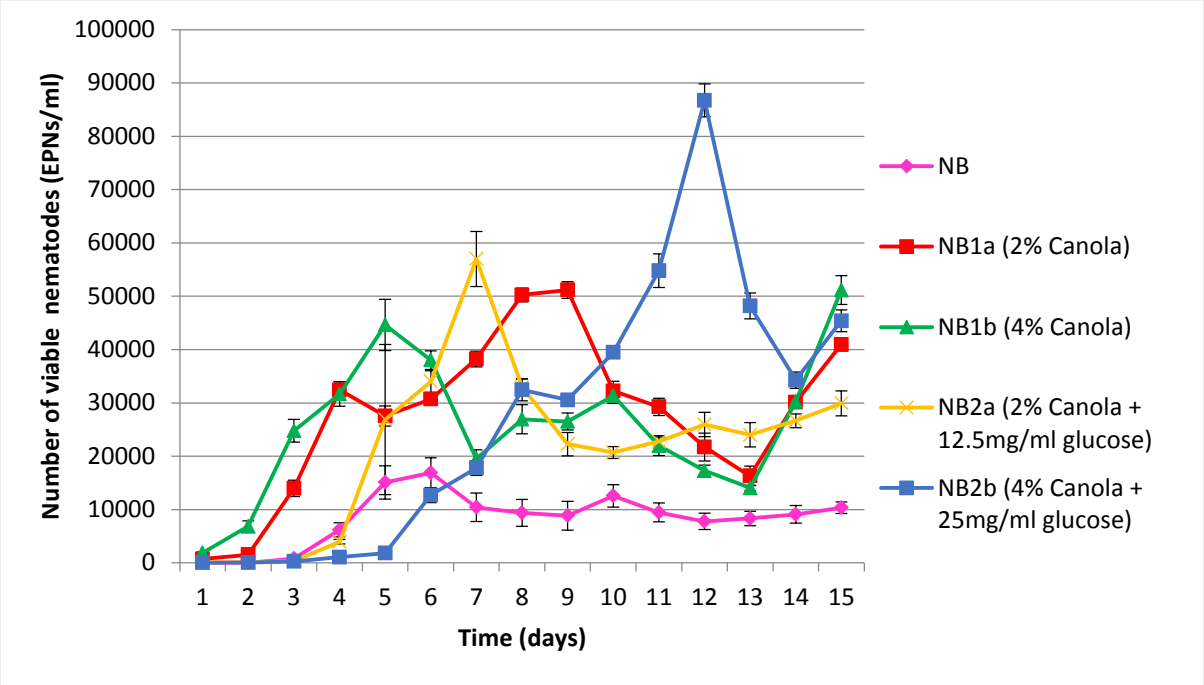

Figure 4.5. The growth of *Oscheius* L8 MCB in different nutrient broth variations. A comparison of nematode growth in NB1 and NB2 variations. Error bars represent the standard error of the mean (SEM±) of triplicates.

The two NB variations that produced a high number of EPN in figures 4.3 and 4.4 were combined in a single graph for comparison (figure 4.5). What can be gathered from this graph is that culturing EPNs in canola alone or in canola with a moderate amount of glucose can reduce culture times at the expense of high, whereas culturing EPNs at high concentrations of both canola and glucose will result in high yields of EPNs, however, this bears the cost of increased culture periods. The high yields observed concur with the observations by Gil *et al*., (2002), who reported that nematode yields increased by 43%, when cultured in media containing both canola oil and glucose, compared to media with canola oil alone (from 2.53×10^5 to 3.62×10^5 infective juveniles). On the commercial scale, reduced culture times and stable cultures are preferred as they reduce the cost of maintaining culture. Therefore, in the case of figure 4.5., the type of NB variation that would favour stable cultures and reduced culture times would be NB1a (NB+ 2% canola) because it produced EPNs in a more stable manner (no sudden sharp peaks) and it did so at reduced culture times. However, the appeal of high yields associated with NB2d cannot be ignored, even though they come at prolonged cultures times.

4.4. Conclusions

S. marcescens L8 MCB optimally grew in NB2 media, showing that this bacterium is able to efficiently metabolise both canola and glucose. The axenic culturing of *Oscheius* L8 MCB favoured the production of the nematode compared to the monoxenic. The reason for this high production is hypothesised involve the regurgitation of competent bacteria by nematodes in axenic cultures which favoured EPN growth, unlike the bacteria in monoxenic cultures, which might have lost fitness due to prolonged *in vitro* culturing. The role of bacterial symbionts of *Oscheius* EPNs play in nematode growth and reproduction is still poorly understood and thus more studies are needed for further understanding of this role. However, regardless of the role the bacterial symbionts play, *Oscheius* L8 MCB was grown efficiently in axenic cultures compared to monoxenic, therefore this method can be used for the production of these nematodes and most likely other *Oscheius* species.

The axenic culturing of *Oscheius* L8 MCB in nutrient broth supplemented with various concentrations of canola oil and canola oil with glucose showed that supplementation of the media with these compounds, irrespective of the concentration, increased nematode production. However, supplementation of NB with 2% canola produces a significant amount of EPNs in reduced culture times, but NB supplemented with 4% canola oil and 25mg/ml glucose increased nematode yield but prolonged the culture time. It is noted that media composition (with regards lipid and carbohydrate content) plays an important role in nematode yield and culture time and thus optimization of these components is critical for efficient nematode production.

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Chapter 5

Monitoring of life cycle stages of axenic cultures of *in vitro* produced *Oscheius* L8 MCB semi-solid state and liquid state and observing the effects of *in vitro* production on nematode efficacy against *Galleria mellonela*

5.1. Introduction

Entomopathogenic nematodes (EPN) of the genera *Heterorhabditis* and *Steinernema* along with their respective bacterial symbionts in the *Photorhabdus* and *Xenorhabdus* genera are insect pathogens that have been extensively studied due to the important role that they play in the biological control of soil insect pests in agricultural ecosystems (Stock, 2015; Blanco-Perez *et al*., 2017). Some members of the recently discovered *Oscheius* genus have been found to form insecticidal associations with members of the *Serratia* genus, thus igniting interest in this genus as an EPN genus (Tabassum, K.A., and Shahina, F. 2002; Serepa *et al*., 2015; Lephoto *et al*., 2016).

The biopesticide potential of EPNs calls for the shifting of focus towards the commercial production of these nematodes, as an alternative to chemical pesticides. Although high quality nematodes can be cultured in using *in vivo* methods, *in vitro* production methods, are the only economically reasonable means for large-scale commercial production of EPNs (Ehlers and Shapiro-Ilan, 2005).

I*n vitro* solid-state production of EPN was first performed in petri dishes (2 dimensional systems), containing various media such as those based on dog food, animal products and enriched media such as lipid agar (Hara *et al*., 1981; Wouts 1981). However, these two-dimensional systems were limited in terms of large scale production. The scalability of *In vitro* solid culture was improved when Bedding, (1981) developed a 3-dimensional culture system that involved the monoxenic culturing of *Steinernema* spp. on crumbled polyurethane sponges coated with poultry offal. Up to 2 billion EPNs could be cultured per 3 kg bag of media using this method (Bedding 1984; Gaugler and Georgis, 1991).with considerably with the invention of a three- dimensional rearing system involving nematode culture on crumbled polyether polyurethane foam (Bedding, 1981).

Even though the 3D culturing system developed by Bedding, (1981) offered improved scalability compared to 2D systems, research based on liquid production has since been conducted to further increase production scale with reasonable costs of production (Surrey and Davies, 1995; Ehlers and Shapiro-Ilan, 2005). Friedman *et al*., (1989) displayed the potential of *in vitro* liquid production for increased scalability when they produced *Steinernema carpocapsae* at maximum yields of 100 000 IJs/ml in 15 000 litre fermenters. However, EPNs have been commercially produced using both liquid and solid-state methods in Australia, Europe, Asia, and North America (Shapiro-Ilan and Gaugler, 2002; Gaugler and Georgis, 1991). In South Africa, *Heterorhabditis bacteriophora* has been the only one EPN product that has been commercially available under the name "Cryptonem", produced by River BioScience (Steyn and Daneel, 2008).

Certain studies have shown that the culturing of a nematode using *in vivo* or *in vitro* methods may affect the quality of EPNs in terms of efficacy in controlling insects and EPN shelf life. Bilgrami *et al*., (2006) has shown that strains of *Heterorhabditis bacteriophora* and *Steinernema carpocapsa*e that were *in vivo* produced over 20 serial passages in *Galleria mellonella* displayed reduced virulence. Gaugler and Georgis (1991) reported that *in vitro* liquid cultured H. bacteriophora displayed reduced efficacy against the Japanese dung beetle *Popillia japonica* as compared to *in vivo* produced *H. bacteriophora*. However, they also found that in there was no difference in efficacy between *in vivo* and *in vitro* produced *S. carpocasae*, indicating the efficacy is not only dependent on the culture method used but also dependent on the EPN species.

It is widely accepted the knowledge of the life cycle and reproduction mode of a nematode is required for effective *in vitro* culturing because it can be used for the effective *in vitro* culturing (Strauch *et al*., 1994; Ferreira and Malan, 2014). The life cycles and reproductive modes of EPNs in the *Heterorhabditis* and *Steinernema* genera have been extensively studied and observed using *in vivo* and *in vitro* culture methods. The natural life cycle of EPNs in these genera is similar but the difference is in the reproductive mode (Strauch *et al*., 1994).

Steinernema species have a dioecious mode of reproduction, whereby reproduction is only through amphimictic adults across generations (Poinar, 1990). The exception is *S. hermaphroditum*, whereby Griffin *et al*., (2001), showed that this species makes use of an androdioecious mode of reproduction, involving automictic hermaphroditic females that can be fertilised by males (Denver *et al*., 2011). However, regardless of the difference

reproductive mode, copulation for *Steinernema* species involves males coiling around their female partners. This helix-type copulation is the main factor behind the success of *in vitro* (solid and liquid) production *Steinernema* species (Ferreira and Malan, 2014).

Heterorhabditis species have a heterogonic mode of reproduction that involves the occurrence of various reproductive modes across generations (Poinar; Denver *et al*., 2011). Infective juveniles of *Heterorhabditis* spp. develop into first generation of adults characterised only by automictic female hermaphrodites, which is then succeeded by amphimictic generations of adults in the abundance nutrients. The hermaphroditic stages are only formed form infective juveniles (Ehlers *et al*., 1998). Copulation for members of this genus involves the gliding of males along their female partners, attaching their spicules to the vaginal area of the females (Stauch *et al*., 1994; Ferreira and Malan, 2014). This type of copulation was found to be limiting in terms of liquid *in vitro* production of the nematodes because males fail to attach to females in liquid media, meaning that reproduction is based on only the automictic hermaphrodite stage.

Members of the *Oscheius* genus are diverse in their modes of reproduction, however, dioecious and androdioecious reproduction are common in this genus (Denver *et al*., 2011). Since the i*n vitro* production of *Oscheius* EPNs has not been studied, the aim of this study is to culture *Oscheius* L8 MCB in axenic *in vitro* semi-solid and liquid state, to observe the effects of these culture methods on the production of different life cycle stages (with preference given to the 3rd stage infective juvenile due to its insect parasitic nature), and to observe the effects of *in vitro* production on the efficacy of the nematode against *G. mellonella*.

5.2. Methods and Materials

5.2.1. Monitoring of life cycle stages of axenic cultures of *Oscheius* L8 MCB during *in vitro* production in semi-solid state and liquid state.

Production of EPNs using the axenic culture technique was carried out in liquid and semi-solid/bi-phasic in order to observe the effects of both culture techniques on the quantity and quality of EPNs produced. During semi-solid and liquid state culture, different life cycle stages of the EPNs isolate were monitored.

5.2.1.1. Solid state cultures

Polyurethane sponge crumbs in three 250ml Erlenmeyer flasks were infused with 50 ml NB2 and autoclaved at 121C^{o} for 20 minutes. After cooling, 100 surface sterilised IJs of *Oscheius* L8 MCB were added to the flasks. The flasks were placed on a shaker at 120 rpm and incubated for 15 days at 25C^{o} ± 2C^{o}. The media infused sponges formed the solid phase whereas the excess NB2 collected at the bottom of the flask formed a liquid phase, allowing this model to be a bi-phasic (figure 5.1.1.). The excess NB2 at the bottom of the flasks allowed for the collection of EPNs from the sponges. It played an important role in nematode enumeration because EPNs used for counting were collected from this phase.

For enumeration, EPNs were collected by swirling flasks to allow EPNs to collect at the bottom of the flasks. Enumeration of EPNs in each flask was carried out by taking the average of nematodes counted in five 20 µl drops of culture, and if the number of EPNs was too high to count, dilution was incorporated to make counting easier. Counting was performed under a light microscope at 10X and 40X objectives (Nikon Alphaphot 75. Japan) instead of a dissection microscope in order to monitor the different life cycle stages of the nematode isolate. The experiment was carried out in triplicates. The words, solid, semi-solid, or bi-phasic are used inter-changeably to refer to this method of culture.

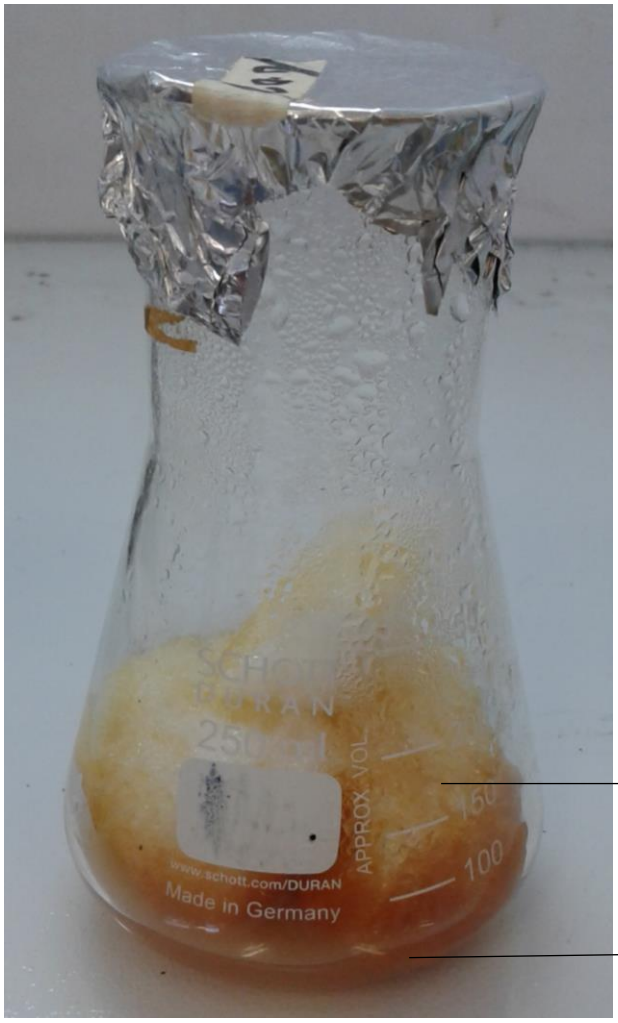


Figure 5.1.1. An illustration of a solid/ semi-solid culture flask used for the production of *Oscheius* L8 MCB.

5.2.1.2. Liquid state cultures

Three 250ml Erlenmeyer flasks with 50 ml NB2 were prepared and 100 surface sterilised IJs of the EPN isolate were added to the flasks (figure 5.1.2). The flasks were placed on a shaker at 120 rpm and incubated for 15 days at 25C^{o} ± 2C^{o}. Enumeration was carried out as mentioned for the solid state (5.2.1.1.). The experiment was carried out in triplicates.

Figure 5.1.2. Illustration of a liquid state culture flask for the production of *Oscheius* L8 MCB.

5.2.2. Harvesting of nematodes from *In vitro* cultures

Nematodes that were produced *in vitro* were harvested for future use in bioassays. The harvesting of EPN in liquid state was different from that of solid, thus the harvesting methods are outlined as follows:

5.2.2.1. Harvesting entomopathogenic nematodes produced in liquid culture flasks

Harvesting was carried out by transferring cultures to 50 ml Falcon tubes and centrifuging the cultures at 300 rpm for 3 minute in order to sediment nematodes. The supernatant (NB2 media) was removed and the nematodes were rinsed by adding 15 ml of distilled water (dH_2O), centrifuging at 300 rpm for 1 minute, and removing the water supernatant. Rinsing was repeated two more times before the EPN were suspended in Ringers solution.

5.2.2.2. Harvesting entomopathogenic nematodes produced in solid culture flasks

Nematode harvesting in each flask was carried out by transferring culture sponges and liquid media from the flask to 50 ml Falcon tubes. Water was added to each tube to a total volume of 35 ml. In the cases where the tubes contained sponges, the tubes were vortexed at low speeds, to promote movement of nematodes from the sponges into the water. After vortexing, dH_2O was added to make up a volume of 45 ml in all tubes. The tubes were centrifuged at 300 rpm for 3 minute in order to sediment nematodes. After sedimentation, supernatants were discarded and nematodes in tubes were pooled into a single falcon tube. Rinsing was carried out as outlined in the liquid state harvest. After rinsing, EPN were suspended in Ringers solution.

Harvested EPN were stored in Ringers solution at $25C^{o} \pm 2C^{o}$ for few days before use in bioassays. This was done in order to starve the nematodes.

5.2.3. Comparative dose-response assays and Emergence studies for nematode infectivity and efficacy

5.2.3.1. Comparative dose-respondent assays (bioassays)

Comparative dose-respondent assays as outlined by Glazer and Lewis, (2000) were conducted for *in vivo*, *in vitro* solid and *in vitro* liquid produced EPN to observe if the axenic *in vitro* production of EPN affects their ability to infect and cause mortality in *G.mellonella* larvae

Bioassays involved the exposure of G. mellonella larvae to different concentrations of nematode IJ ranging from 5 to 200 IJs/larvae. Insect mortality was recorded at 24h intervals over a period of 96h. The assays were carried out in 24-well plates with each well having an area of ca. $2cm^2$ (See figure 5.2) to limit the movement of larvae and keep it near the IJs (Kaya and Stock 1997). Ten (10) wells were assigned to a single EPN concentration on a single plate, meaning that a single 24-well plate was used for two concentrations. Each well that was assigned a concentration was filled with 0.5 g ± 1 g of sieved and sterilised river sand. Each well was then inoculated with 5, 10, 25, 50, 100 and 200 IJs per 100 µl of autoclaved dH_2O.

IJ concentrations in water were prepared by centrifuging the IJs in Ringers solution at 300 rpm for 1 minute to sediment the IJs. The supernatant was removed and IJs were suspended

in 5 ml dH_2O. IJs were evenly mixed by pipetting in and out using a micropipette. Five aliquots of 20 µl of IJ suspension were transferred to a 9 cm petri dish and IJs were counted under a dissection microscope. The concentration of nematode per millilitre was calculated by multiplying the average of the five 20 µl IJ counts by 50.

In order to adjust the concentrations of IJs to those used in this bioassay the following formula was used: $[(i/c)-1] \times V= Va$. Where i is the initial concentration= IJs/20 µl dH_2O, c is the final concentration= IJs/100 µl dH_2O, V is the volume of nematode suspension (ml) = 5 ml and Va is the amount of dH_2O to be added (if value is positive) or subtracted (if the value is negative). For concentrations that were lower than 50 IJs/100 µl (5, 10 and 25 IJs/ 100 µl), IJs were transferred to 100 µl dH_2O as is suggested by Glazer and Lewis (2000) in order to get accurate concentrations.

After sterile sand and IJs were added to the well plates, single last instar G.mellonella larvae were added to each well. G.mellonella larvae were also added to control wells, which contained only sterile sand and 100 µl EPN-free dH_2O. Wells were sealed with glass covers and Parafilm® in order in order to prevent the larvae from escaping. Wells were kept at $25C^o \pm 3C^o$ and larvae mortality was observed at 24, 48, 72 and 96 hours. Upon death, individual cadavers were transferred to White traps in order to perform emergence studies.

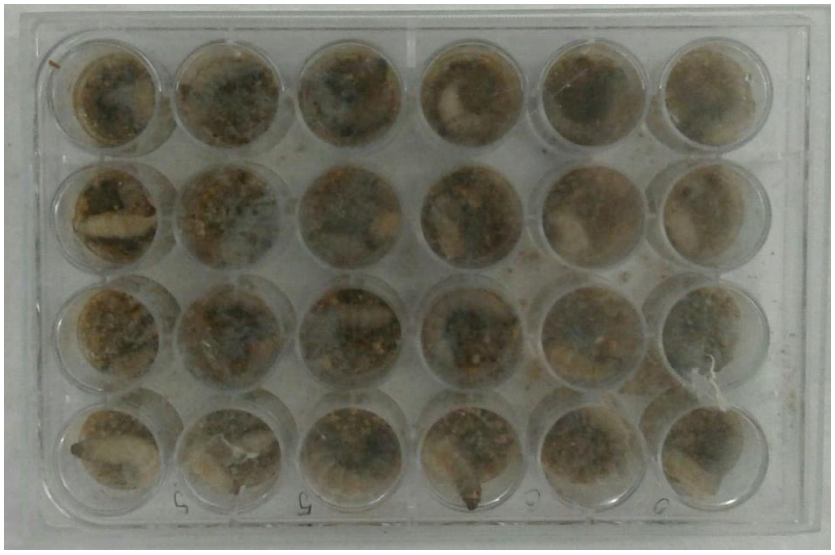


Figure 5.2. An illustration of 24 well plate used in the comparative dose-response assay

5.2.3.2. Emergence studies

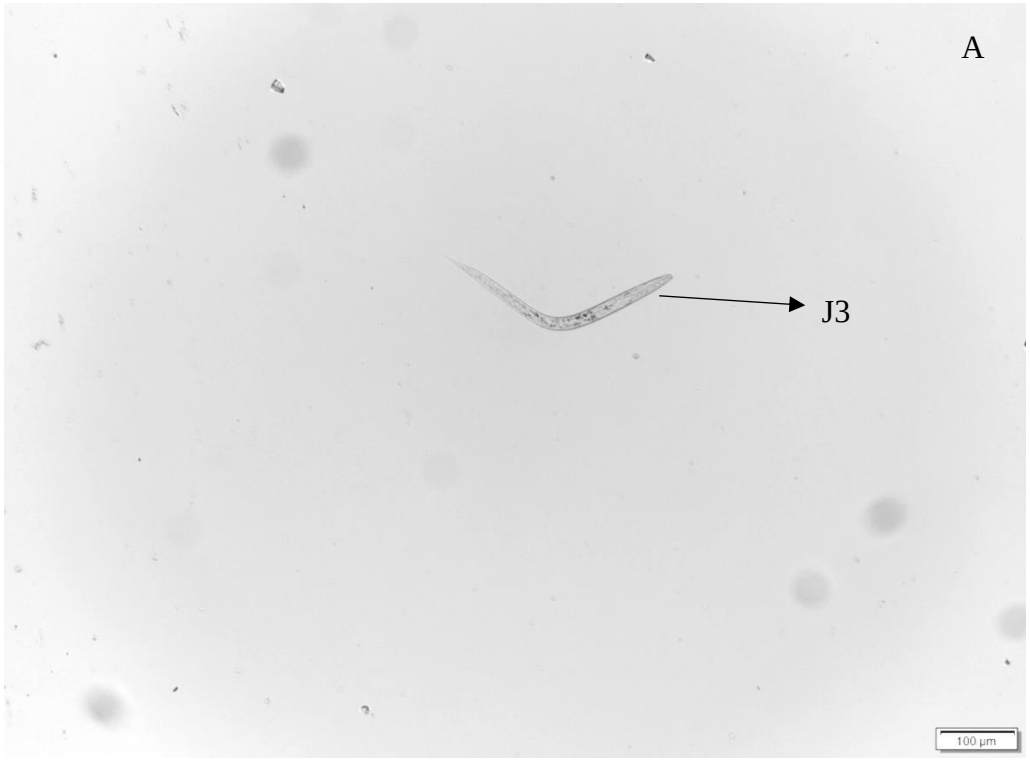
The number of IJs that emerge from an insect cadaver may determine the ability of an EPN to spread and persist in a field thus IJ emergence may affect field efficacy. Upon death of larvae comparative dose-response assays (section 5.2.3.1), individual cadavers were transferred to White Traps and left for 15 days at $25C^{o} \pm 3C^{o}$. In order to count IJs that emerged from each larvae, water (containing IJs) from White traps was transferred to 50 ml Falcon tubes. Tubes were left to stand for 1 hour to allow IJs to sediment. Excess water was removed and aliquots of 10 ml fresh dH_2O were added to each tube and IJs were enumerated.

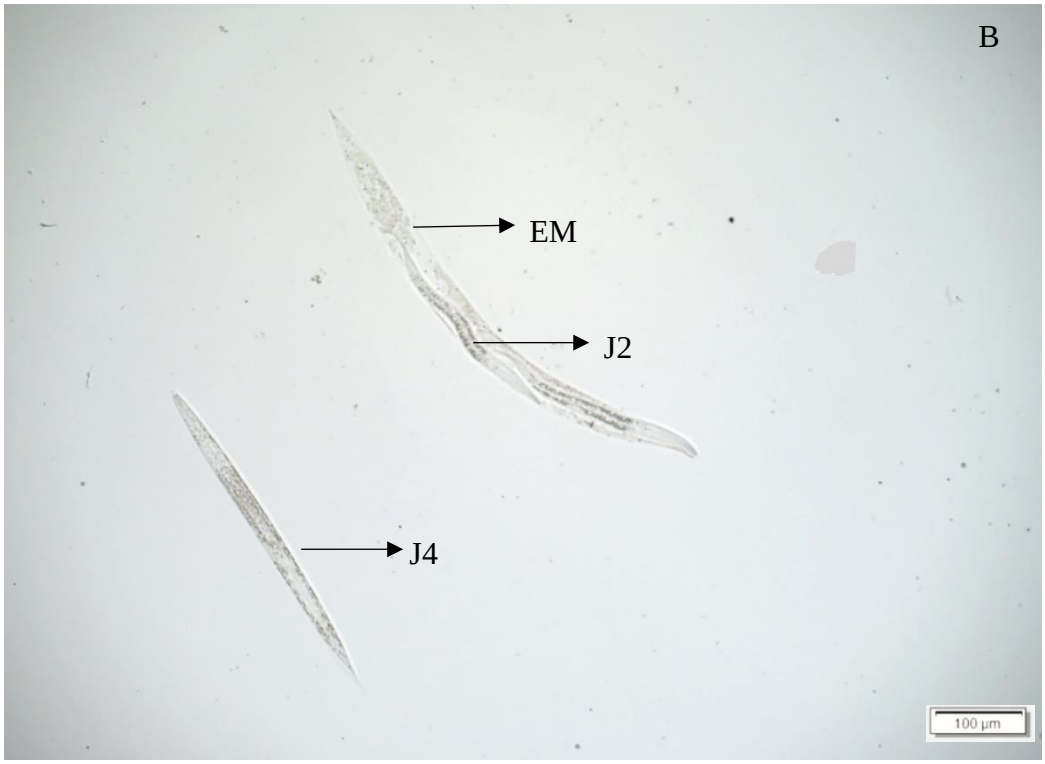
5.2.4. Data analysis

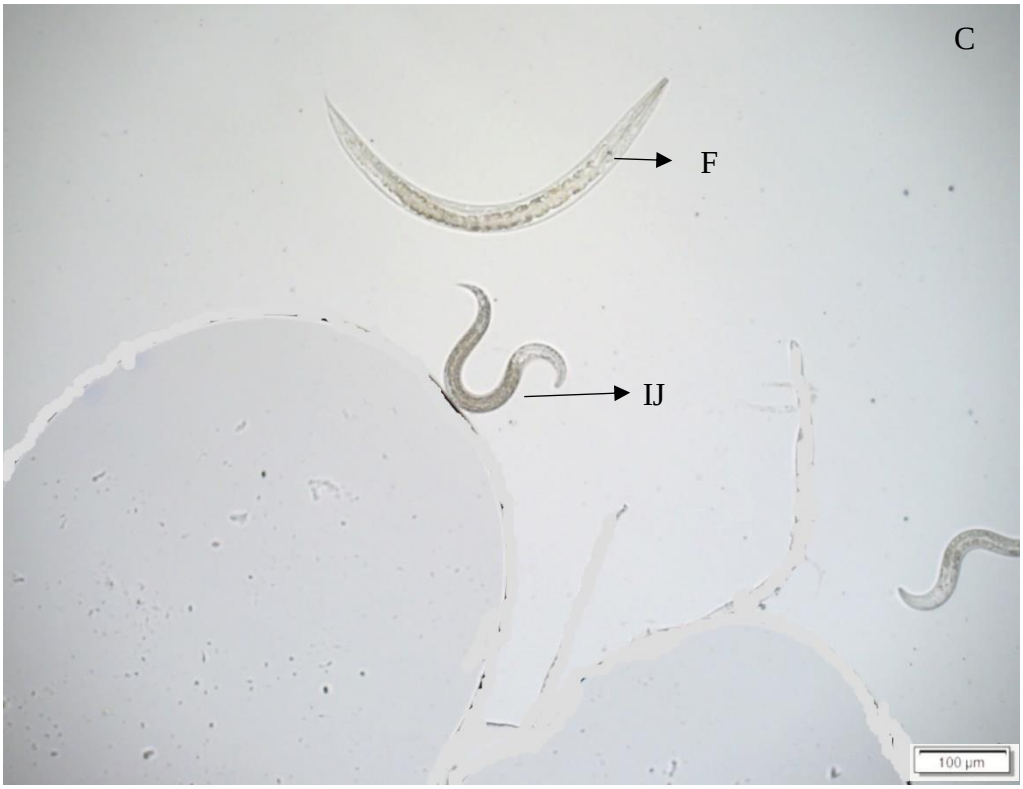
The data for comparing solid and liquid state IJ production was analysed using Student's T-test in Microsoft Excel 2013. The differences with $P< 0.05$ ($\alpha=0.05$) were considered significant whereby the null hypothesis is that there is no difference in IJ production between axenic solid and liquid culturing. The data for Percentage mortality data were analysed using two-way ANOVA (Excel, Microsoft Inc. 2013). The differences with $P< 0.05$ were considered significant. Before ANOVA, mortality data were Arcsine transformed. Data for the control bioassays were not used because insect mortality was not observed. One-way ANOVA was used for mean number of emerging IJs.

5.3. Results and Discussions

5.3.1. Monitoring of life cycle stages of axenic cultures of *Oscheius* L8 MCB during *in vitro* production in semi-solid state and liquid state.



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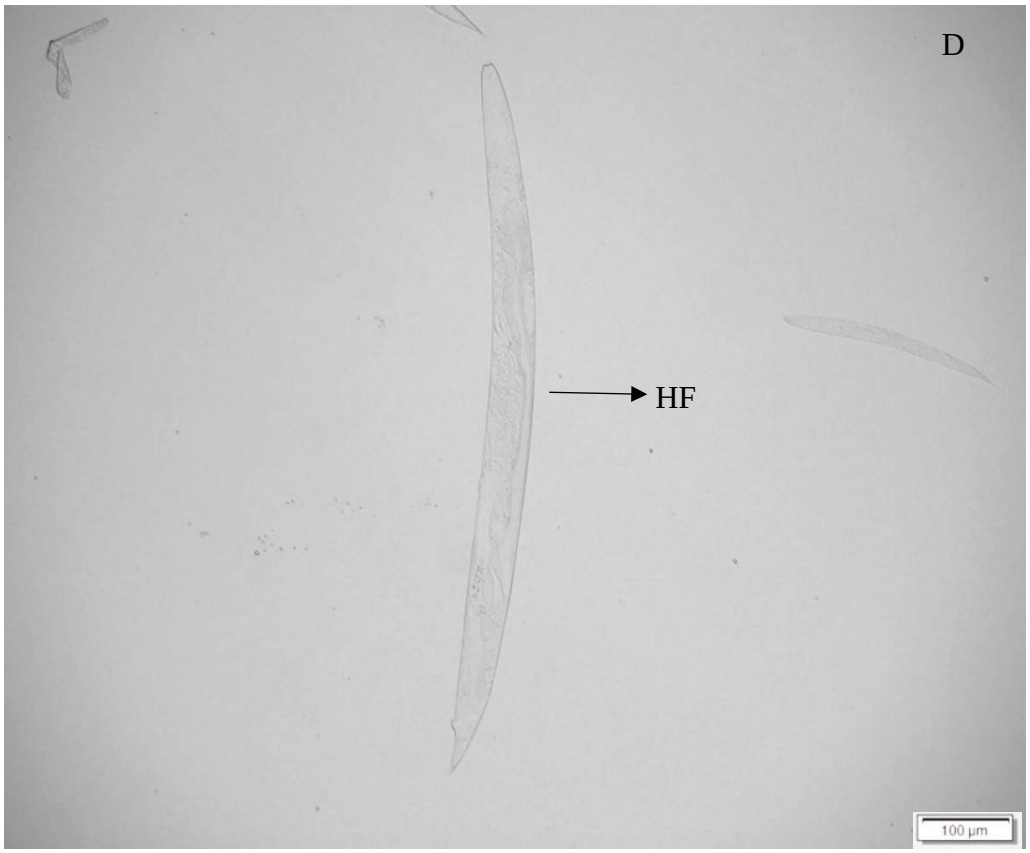
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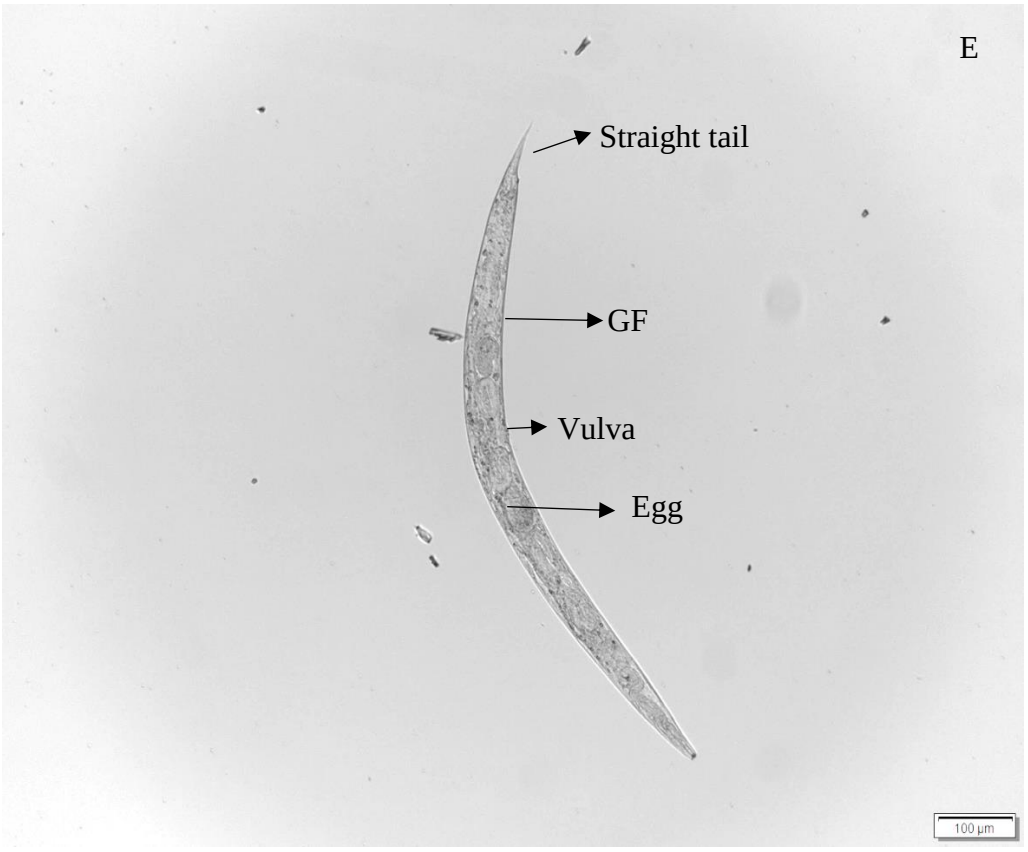


Figure 5.3. A-E show Light microsccope images of different life cycle stages of *Oscheius* L8 MCB as observed NB2 *in vitro* culture. Stages include J2, J3 and J4 (feeding juvenile stages), IJ (non-feeding infective juvenile), F (young female), HF (hermaphroditic female), GF (gravid female), and EM (*Endotokia matricida*). All images were taken using the 10X objective and the scale bar represents 100µm.

Figure 5.3., shows the different life stages associated with the isolated EPN *Oscheius* L8 MCB. The life cycle stages of *Oscheius* L8 MCB were deduced using the illustration provided by Ehlers, (2001) showing the different life cycle stages of *Heterorhabditis* spp., and the difference in size classes. J2 represents the 2nd juvenile (figure 5.3. B), a feeding stage that occurs prior development of the 3rd stage juvenile, it is characterised be its small size (~250µm in length) and reduced fat content and depending on the availabity of nutrients, it may develop into the IJ or the 3rd stage feeding juvenile. The J3 (3rd stage juvenile) developmental stage is characterised by either the non-feeding IJ or feeding J3. These stages were were approaximately similar in size (~350 µm in length), however the difference was that the IJ had a high fat content (figure 5.3. C) compared to J3(figure 5.3. A) which has developed feeding structures. J3 develops into feeding J4 (figure 5.3. B), a stage that occurs before the adult stages, it is characterised by under developed adult features and its smaller in

size compared to the adult stages (~410 µm in length). Adult stage consisted of male, female and hermaphroditic female stages. Female stages (figure 5.3. C and E) were observed by the presence of the vulva, straight tails and relatively larger sizes compared to the male stages (~550µm or more in length). Hermaphroditic stages were characterised by their large sizes (~830µm) compared other adult stages. *Endotokia matricida* is a stage that is associated with nutrient depletion, whereby depleted nutrients in the surrounding environment of the nematode cause eggs in the gravid female to feed of the nutrients in the gravid female thus resulting death of the female (figure 5.3. B)

The adult male and J1 stages of *Oscheius* L8 MCB could not obtained because the imaging light microscope had a malfuntional in the 40X objective and thus, repair and maintanance made it impossible to obtain these stages. However J1 stages were noted by their transparency, coiling and small sizes in relation to other stages. Male species were noted by their smaller sizes in relation to females, and the presence of spicule and gubernaculum around the tail area (See appendix IX for illustration of the male stage). *Oscheius* L8 MCB could not be morphorlogicaly characterised due to malfuntions in the light and the scanning electron microscopes. Regardless of the state of imaging microscopes, a conventional light microscopes were used to monitor life cycle stages in the solid and liquid stage culturing of *Oscheius* L8 MCB.

Oscheius L8 MCB was *in vitro* cultured axenically in solid state and liquid state and various life cycle stages were monitored in order to observe the effects of both culture methods on the production of EPNs, with focus special on IJ production because it is the insect parasitic stage. Therefore, stages were grouped together in relation to the role they play in reproduction and infecticity. J1 and J2 were grouped together because there is little variability in size between the stages and because they occur prior to the formation IJ or J3 stages.The IJ stage was not grouped with any other because this is the stage of interest as it is parasitic to insects. J3 was grouped together with J4 as they represent feeding juvenile stages and thus representative of recovery from the non-feeding stage. There was no distinction made between amphimictic and hermaphroditic females bacause they are stages responsible for egg production, thus the type of gravid females was not specified.

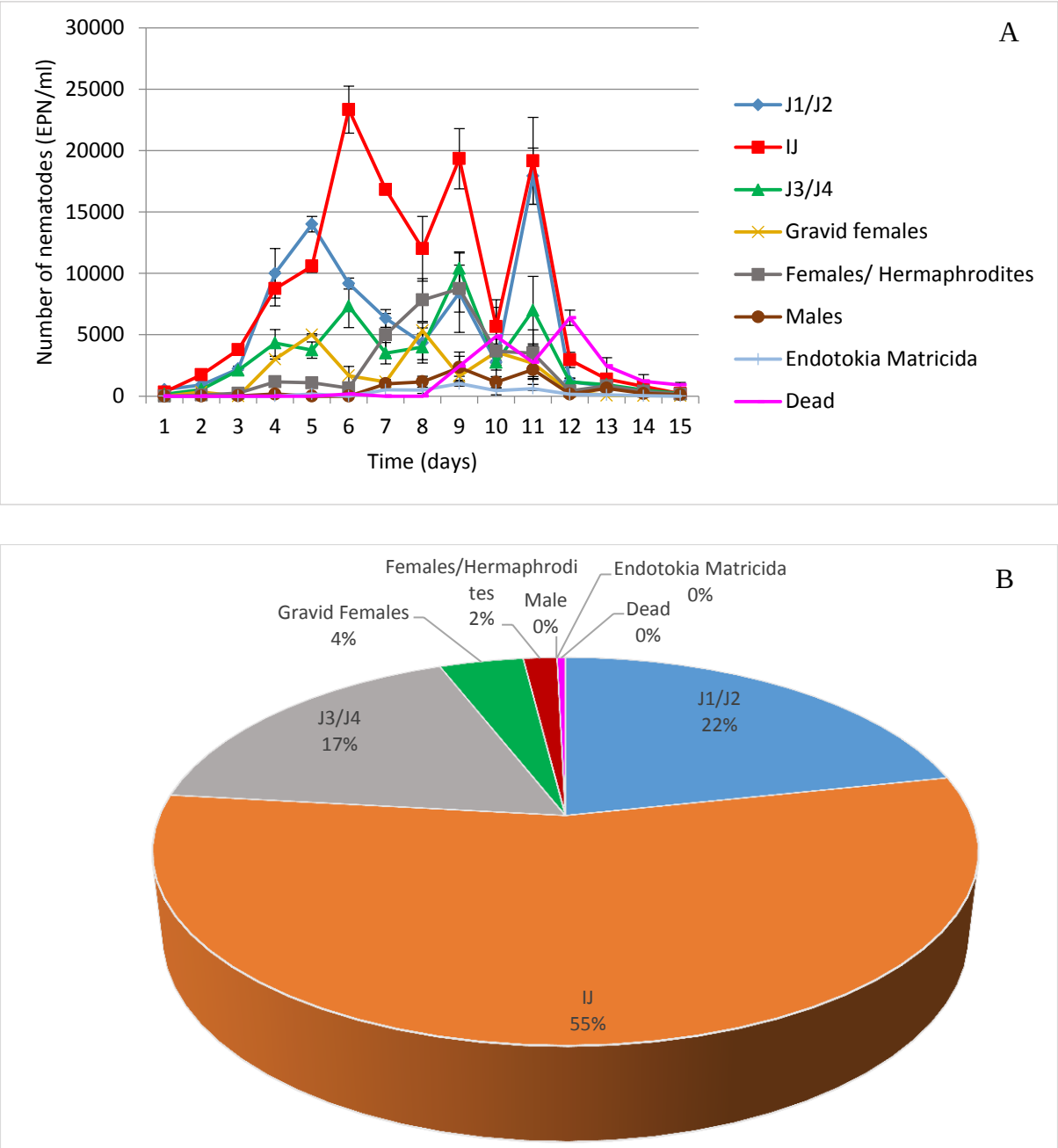


Figure 5.4. Growth of different stages of *Oscheius L8 MCB* in solid culture over a period of 15 days (A). B represents nematode composition at days that have the peak number of IJs in solid state culture. Error bars represent the standard error of the mean (SEM±) of triplicates.

In the solid state (figure 5.4 A) the recovery of inoculated IJs from the non-feeding state occurs during day 1 because by day 2 there J3/J4 (the hall mark stage of recovery). This early recovery may be attributed to improved aeration as a result of increased surface area to volume ratio provided by the polyurethane sponge. The growth of the juvenile stages and gravid female stages are trimodal in nature as these stages peak three times in the culture period, thus it is most likely that nutrient availability was the main driver of reproduction. It is possible that the trimodal nature may have come about as a result of selective nutrient use, whereby nematodes selectively metabolised a favoured compound before using other compounds as nutrients. It is hypothesised that EPNs selectively shifted their mode of nutrition from canola (lipid source), to glucose and then later made use of the remaining minerals in nutrient broth. This is because EPNs cannot make their own lipids and thus would normally extract lipids from their host as a primary source of nutrients for the formation of IJs formation and this case, lipids are taken from the artificial media.

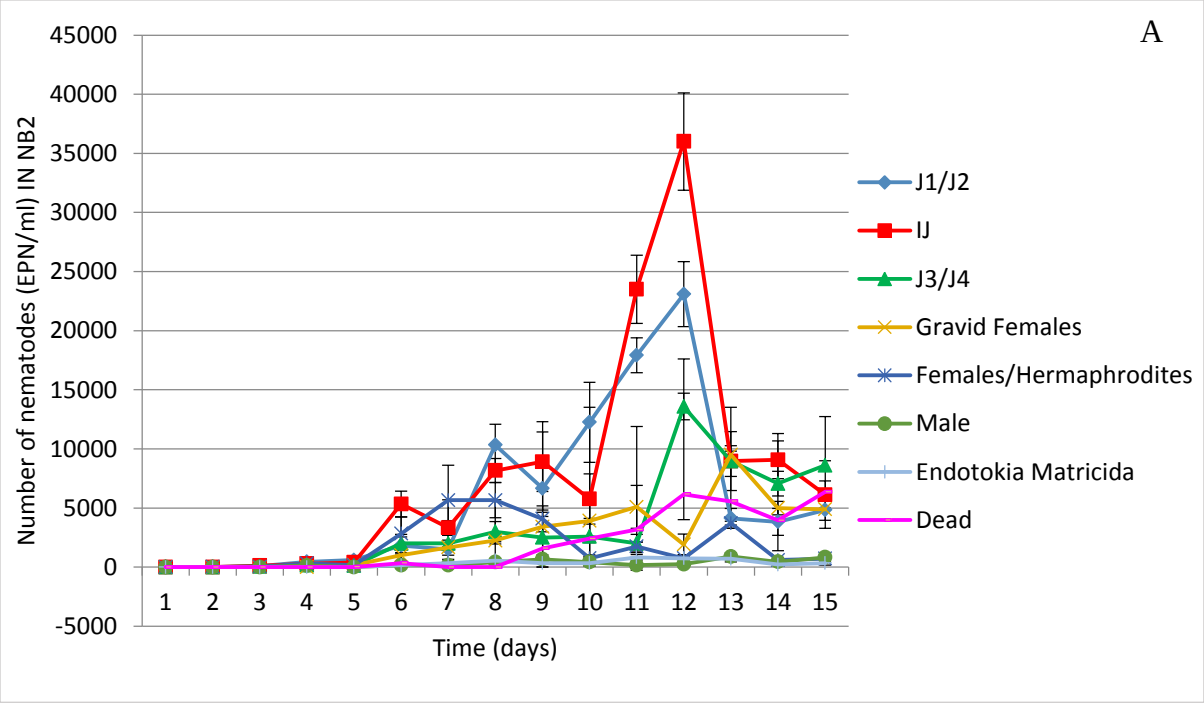
Except for day 5 gravid females peak a day prior the peaking juvenile stages, indicating that they produce eggs which develop to J1 within a day. The mode of reproduction prior to day 6 may have been automictic because adults were primarily hermaphrodites and also because male phenotypes were only observed later, from day 7. The presence of males initially resulted in an increase in gravid females a day later, indicating that reproduction at this point might have been amphimictic, however, copulation was not visually observed. Irrespective of amphimictic reproduction, self-fertilizing female hermaphrodites were still present in culture, therefore, reproduction at this point could have been both automictic and amphimictic. The existence of *endotokia matricida* from day 7 (although at low numbers) could have been a product of depleted nutrient content in the form of canola (lipids). This stage contributed to IJ formation from day 9 onwards.

Juvenile stages exist in high numbers throughout the culture period. These high numbers might be attributed to the type of method that was used for harvesting nematodes used for counting. In solid/bi-phasic cultures, EPNs used for counting were collected from the culture medium collected at the bottom of the shake flasks (liquid-phase). Therefore, it is likely that throughout the culture period, juvenile stages were able to easily move through the pores of the polyurethane sponges into the liquid media due to their smaller sizes in relation to adult EPNs. IJ were more capable of this movement due to their high motility and nutrient seeking abilities. Therefore, the nematode harvesting method is most likely to have resulted in the over-estimation of juvenile stages and the under-estimation of adult stages. However, it is

also possible that the high numbers of juvenile stages were a result of reproduction as a result of nematode response to nutrient availability and adequate aeration, and thus, IJ formation could have been the result of nutrient decline at a particular time point.

The sharp fluctuations in life cycle stages, the presence of juvenile stages (J1/J2) as early as day 2, and the coexistence of self-fertilising hermaphrodites with amphimictic adults indicate an instability in culture. This instability could have stemmed from the IJ inoculum, whereby it is possible that the inoculum might have consisted of small amounts of developed individuals (such as gravid females), which could have led to production of J1 stages early in culture thus causing the EPN stages to develop in an unsynchronized fashion. Another factor that might have led to instability is high sample variability and the low rate of culture success when using surface sterilised IJs.

The decline of all life cycle stages observed from day 12 was the result of fungal contamination that was usually observed in most samples at this point in the culturing period (see Appendix IX). The contaminated media (liquid phase) at this point was murky and thick and the polyurethane sponge had fungi that was assumed to be a *Fusarium* species due its green colour. The obscurity caused by the murkiness in the media resulted in increased dilutions to increase the visibility of nematodes. Higher dilutions might have resulted in the underestimation of life cycle stages.



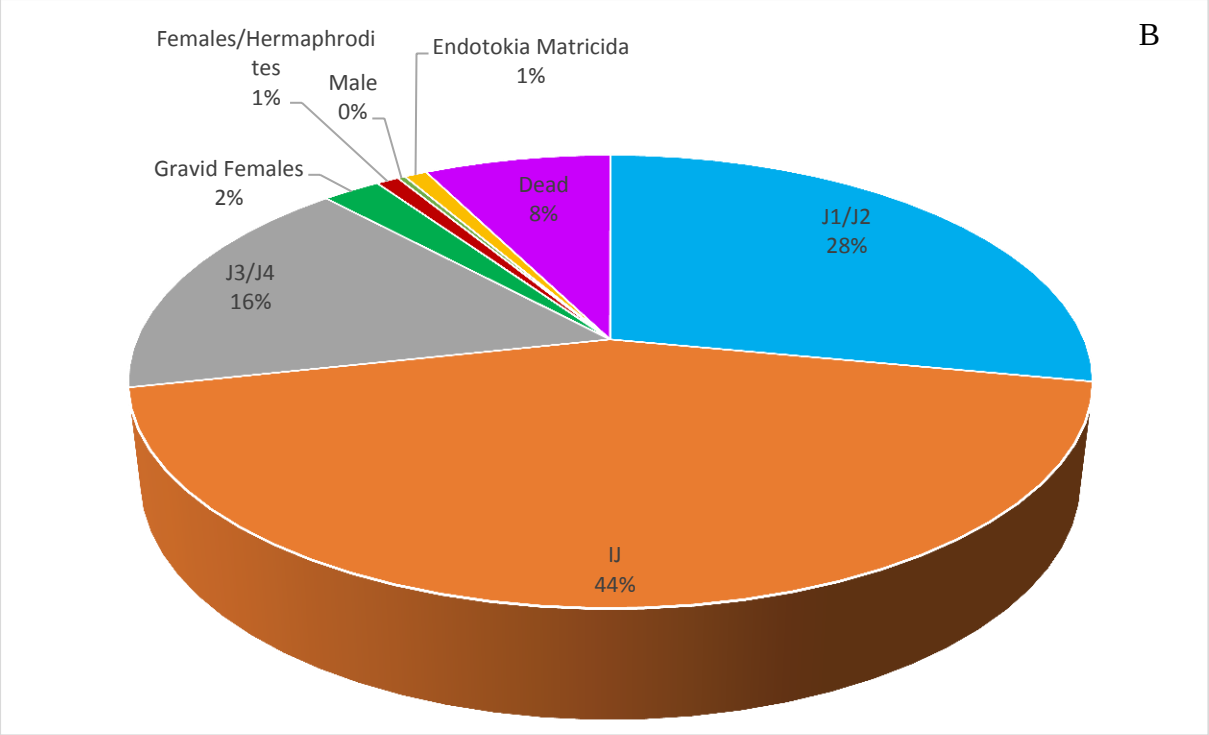


Figure 5.5. Growth of different stages of *Oscheius L8 MCB* in liquid culture over a period of 15 days (A). B represents nematode composition at days that have the peak number of IJ in solid state culture. Error bars represent the standard error of the mean (SEM±) of triplicates.

In liquid state, (figure 5.5. A), there is a prolonged lag phase for all life cycle stages (5 days), as a result of reduced aeration compared the solid/bi-phasic state and the adaptation of EPN to liquid media. Recovery occurs during day 5 as there are J3/J4 stages present by day 6 in liquid culture. Females produced between day 5 and 10 were believed to be mostly hermaphroditic because the male phenotypes were still absent at these points, and thus these females develop to be gravid females a day later (day 6-11) and reproduction at these time points was most likely automictic. Amphimictic males occur late in culture (day 9 onwards) and at low occurrence rates thus it is unlikely that reproduction was amphimictic. The sharp drop observed in all life cycle stages from day 12 could be attributed to decline in media quality at later time points which resulted in death of nematodes. However, in the graph it is seen that death has not significantly increased in relation to the decline in life cycle stages. This decline can be explained by the deterioration and rupture of dead nematodes observed in the culture media. This phenomenon was observed mostly occurring in adult stages. Another reason for the decline in all life cycle stages could have been due to presence of contaminants/ by-products that resulted in the murkiness of the medium (see Appendix IX).

These may have caused the underestimation of stages as a result of increased dilutions in order to increase nematode visibility.

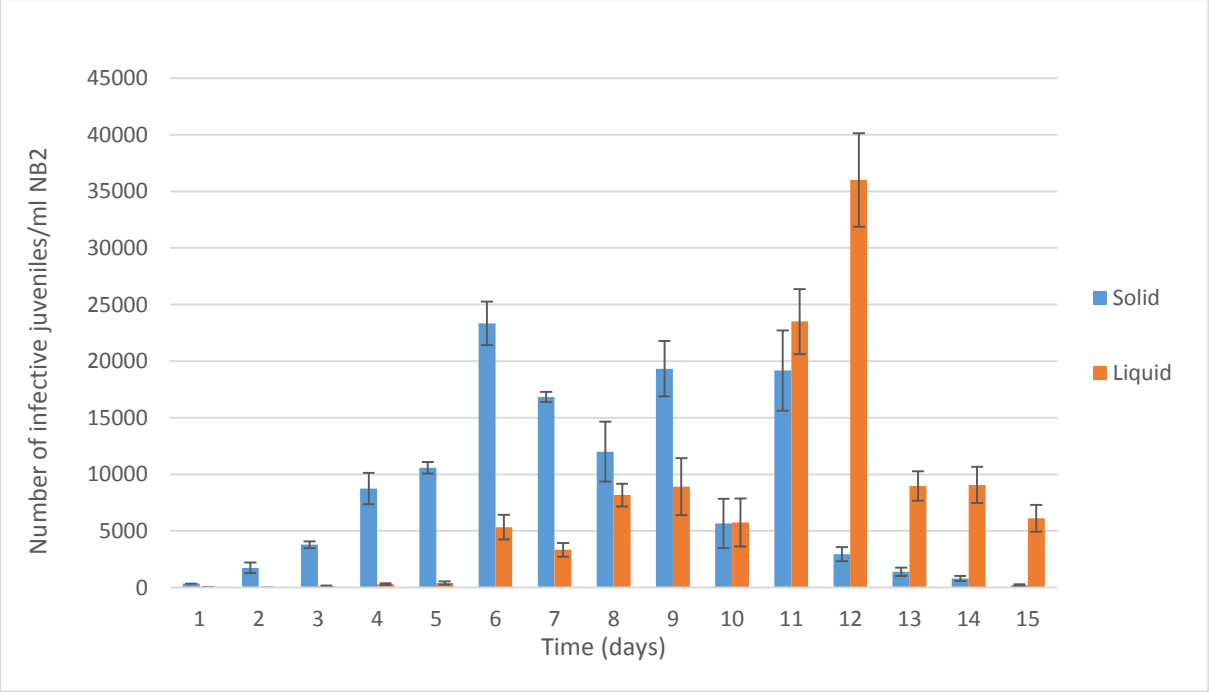


Figure 5.6. The comparison between the production of infective juveniles of *Oscheius* L8 MCB in solid state and liquid state cultures. Figure was derived from 5.4 (A) and 5.5 (A). Error bars represent the standard error of the mean (SEM±) of triplicates

It should be stated that the use of surface sterilised IJ for establishing axenic cultures in both solid and liquid states was challenging and not always a successful endeavour due to the presence of remnant bacterial contaminants most likely found under the nematode cuticle. Whenever possible, axenic cultures of EPN should be obtained through the alkaline lysis of gravid females as outlined by Kaya and Stock (1997). However, in the case of the current study, *Oscheius* L8 MCB production was carried through EPNs that were axenised using surface sterilisation. The concurrent occurrence of automictic hermaphrodites and amphimictic adults in both culture methods could have stemmed from presence of other EPN stages in the inoculum generation. This prevented efficient monitoring of life cycle stages and the determining of the mode of reproduction followed by *Oscheius* L8 MCB, however reproduction is surmised that the nematode follows either heterogonic or androdioecious mode of reproduction due to the existence of hermaphrodite stages throughout the culture period.

The production of *Oscheius* L8 MCB infective juveniles between solid state and liquid state was compared (Figure 5.6 and table 2 in Appendix X). It was observed that solid/bi-phasic cultures significantly produced more IJ earlier in culture (day 2-7 and day 9) compared to liquid state, which significantly produced more EPN later in the culture period (day 12-15). Early production of IJ in the solid state was most likely a result of improved aeration which resulted in high reproduction that led to nutrient depletion at faster rates thus triggering early IJ formation. However, it was observed that liquid cultures produced the highest infective juvenile yield at its peak (36 000 IJs/ml SEM ± 1178 IJs/ml) compared solid state (23 333 IJs/ml SEM ± 1922 IJs/ml) although later in culture times. Axenic solid-state production of *Oscheius L8 MCB* is believed to be a better method for *in vitro* of production for this species for early IJ production and in relatively adequate numbers. This reduction in culture time would be cost effective in commercialisation. However, potential threat to process maximisation are contamination and poor EPN extraction from the solid state. Axenic liquid production offers the benefits of high nematode yeild and efficient recovery of nematodes from culture at the expense of prolonged culture periods. These observations made with regards to EPN/IJ production using *in vitro* solid state and liquid state culture methods agree with those outlined by Shapiro-Ilan and Gaugler (2002) when they analysed the benefits and draw backs of nematode production technologies.

5.3.2. Comparative dose-response assays and Emergence studies for nematode infectivity and efficacy

Since nematode yield does not translate to increased infectivity, comparative dose-response bioassays were used to compare the infectivity of *in vivo* and *in vitro* axenic cultures of *Oscheius* L8 MCB that were produced in solid state and in liquid state against *G. mellonella*

5.3.2.1. Comparative dose-response assay

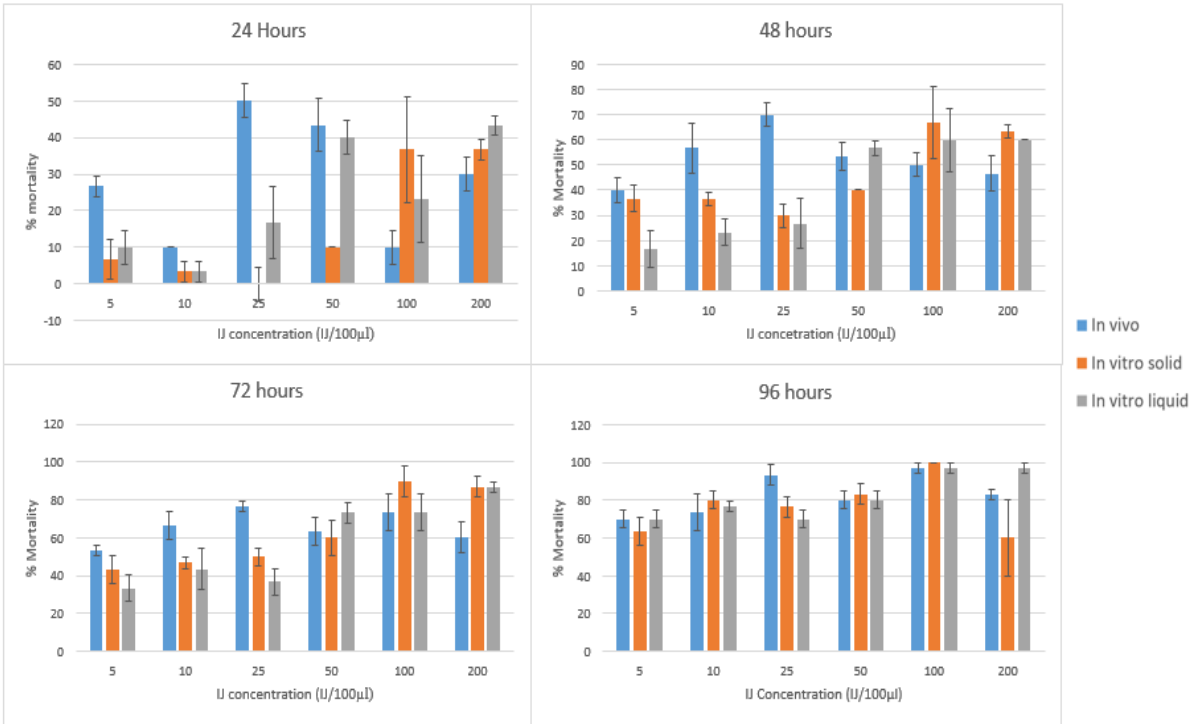


Figure 5.7. Dose-response assay at different time points for comparing *Oscheius L8 MCB* cultured *in vivo* and *in vitro* (solid and liquid).

In figure 5.7, at 24 hours, *in vivo* produced EPNs significantly resulted in more larvae mortality at lower EPN concentrations (5-25 IJs/ larvae) than EPNs cultured using both axenic *in vitro* methods. At this time point the only significant difference between mortality caused by the *in vitro* production methods was observed at 25 and 50 IJs/ larvae (see table 3 in Appendix X). *In vivo* cultured EPNs had already caused 50% mortality at a concentration of 25. At this time interval, there was no correlation between EPN concentration and mortality.

At 48 hours, there was no significant difference in larvae mortality between EPNs cultured *in vivo* and *in vitro* except for concentrations of 10 and 25 IJs/larvae. There were significant differences in mortality between *in vitro* solid and liquid cultured nematodes observed at concentrations of 5, 10 and 50IJs/larvae (see table 3 in Appendix X). At this point, correlation between IJ concentration and infectivity is observed for *in vitro* cultured EPNs, whereby increases in concentrations of IJs/larvae resulted in increased insect mortality. The highest insect mortality at this point (70%) was reached by *In vivo* produced EPN at a concentration

of 25 IJs/ml. Insect mortality over 50% had been reached EPN produced at all methods but at different concentrations.

At 72 hours, there was no significance in larvae mortality caused by EPNs from all methods at IJ concentrations of 5, 50 and 100 IJs/larvae (see table 3 in Appendix X). *In vivo* produced EPNs, significantly produced more insect mortality than *in vitro* cultured EPNs at low concentrations (10 and 25 IJs/ml) whereas *in vitro* produced EPN resulted in more insect mortality than *in vivo* produced EPN at a high concentration of 100 IJs/larvae, suggesting that *in vitro* culturing may reduce efficacy against insects. There was no significance in insect mortality between both *in vitro* produced EPN across all concentrations of IJs. Insect mortality exceeding 80% are reached by *in vitro* cultured EPN.

At 96 hours there was no significant difference between insect mortality in EPNs produced through the three different culture methods, at all concentrations except for at 25 IJs/larvae (*In vivo* cultured nematodes resulted in mortality that was significantly higher, reaching 100%) and at 200IJs/larvae (solid *in vitro* mortality is significantly higher, reaching 100%). Larvae mortality of ~100% is observed at for all culture conditions at a concentration of 100IJs/ larvae.

The efficacy of nematode cultures produced using *in vivo* and axenic *in vitro* methods, varies according to the concentration and infection period. *Oscheius L8 MCB* IJs reach 100% insect mortality after 96 hours irrespective of the EPN culture conditions, however, this mortality is concentration dependent. The observation that *in vivo* produced EPN reach 100% mortality at low concentrations compared to its *in vitro* counterparts suggests that the *in vitro* culturing may reduce the efficacy EPNs. There was not much variation observed between *in vitro* solid and *in vitro* liquid produced EPN in terms of infectivity. This could be due to the use a single type of medium (NB2d) used, which provided similar amounts of nutrients to EPN for IJ formation. The reduced infectivity associated with *in vitro* culturing methods could be attributed to the reduced amount of lipids provided by NB2 which may affect IJ fitness. Whereas, *in vivo* cultures have high amounts of lipids provided by a lipid rich host. The cause of 100% mortality of *Oscheius* L8 MCB at 96 hours meets another hallmark of an EPN, as prescribed by Dillman *et al*., (2012), that a nematode must kill its host within 5 days of infection.

In order to determine the lethal dose needed to achieve 50% insect mortality (LD_{50}) of *Oscheius* L8 MCB, probit analysis was performed for the dose response assay at 48 hours

because it is at this time point that atleast 50% mortality has been achieved and also because there is still variabiltity in concentrations tested. The last reason could be that EPN LD_{50} isussually determined at this time point by other studies (Malan *et al*., 2011; James *et al*., 2018).

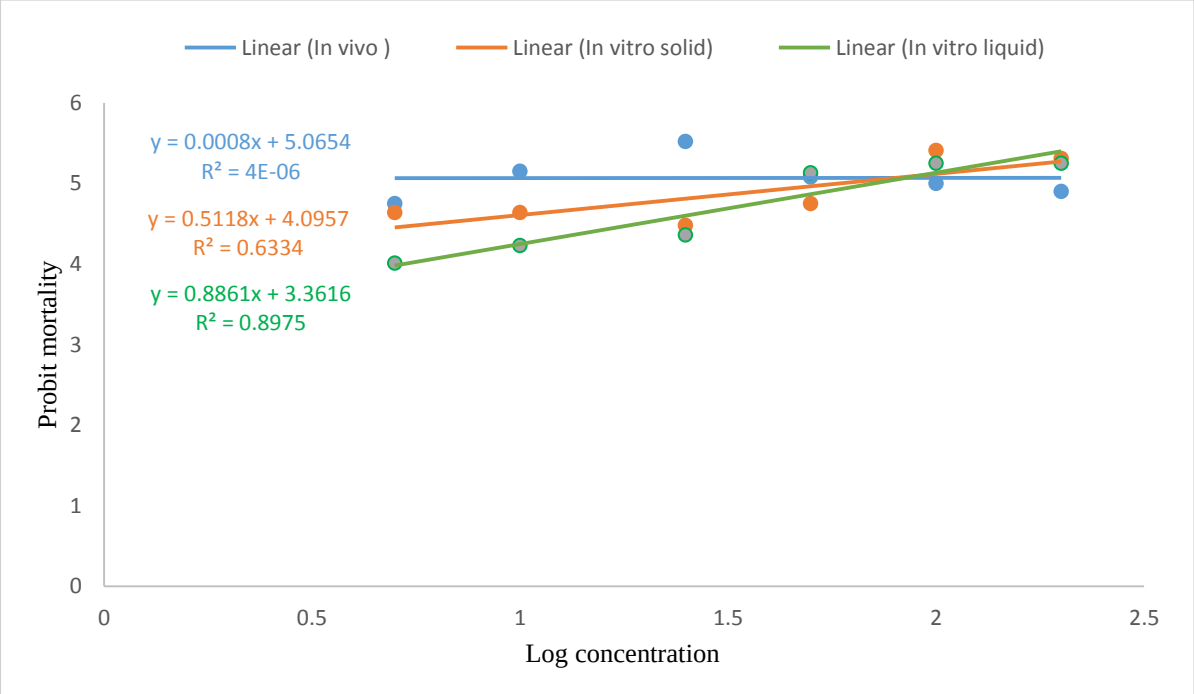


Figure 5.8.1. Probit mortality obtained after 48 hours at each log concentration tested for *Oscheius L8 MCB* produced using *in vivo* and *in vitro* (solid and liquid) culture techniques.

Table 5.1. LD_{50} values of *Oscheius L8 MCB* cultured using *in vivo* and *in vitro* methods

Culture method	LD_{50}
In vivo	Undetermined
In vitro solid	58.47~ 59 IJs
In vitro liquid	70.63~71 IJs

The LD_{50} of *Oscheius* L8 MCB was dependent on culture condition and it is reduced in soild cultures, indicating that solid *in vitro* EPNs could be more infective than liquid state (figure 5.8.1). LD_{50} could not be determined for *in vivo* produced EPNs because at least 50% mortality was reached at low concentrations and because there was no correlation between

EPN concentration and insect mortality. The virulence of an entomopathogenic nematode can also be expressed as the rate of mortality (Caroli *et al*., 1996), therefore, the infectivity of *Oscheius* was measured by the time it takes to kill *G. mellonella* larvae. The concentration of 100 IJs/ml was used because it is at this concentration *Oscheius* L8 MCB, cultured both *in vivo* and *in vitro*, reaches 100% insect mortality.

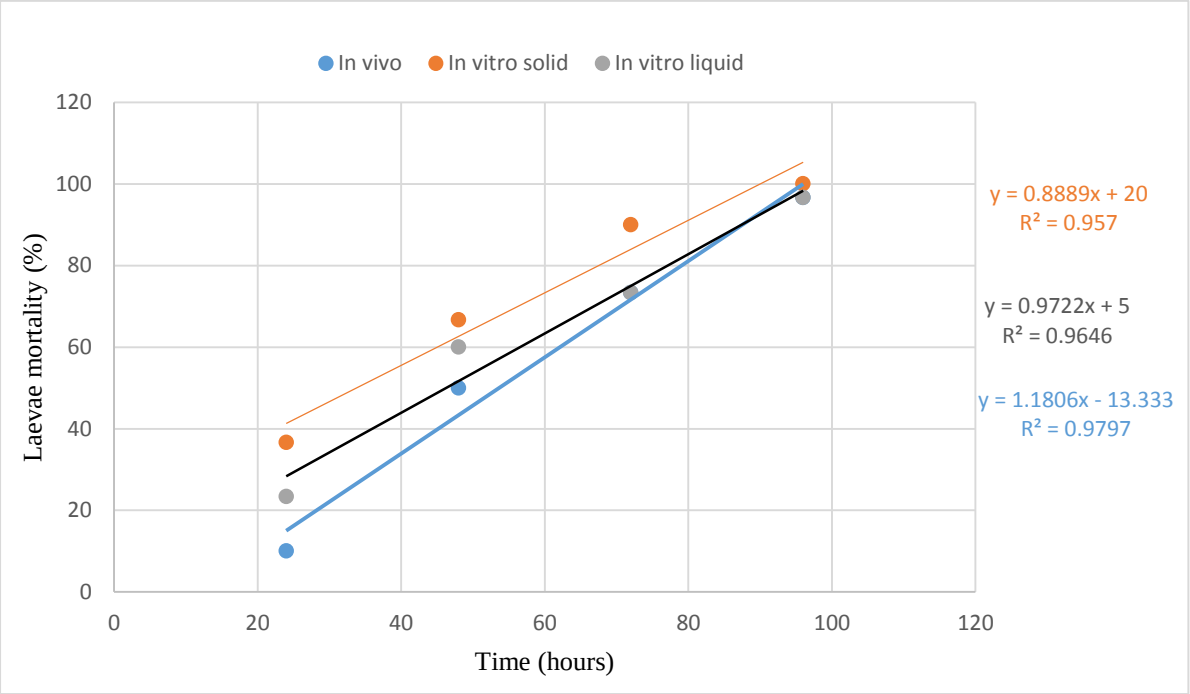


Figure 5.8.2. Infectivity of *Oscheius* L8 MCB against *G. mellonella*, at a concentration of 100IJs/larvae over the cause of 96 hours.

Table 5.2. LT_{50} values of *Oscheius L8 MCB* cultured in different production methods

Culture method	LT_{50} (hours)
In vivo	53
In vitro solid	33.75
In vitro liquid	46.29

Figure 5.8.2 and table 5.2 both show that in *Oscheius* L8 MCB IJs produced using *in vitro* methods results have a high infectivity rate than their *in vivo* produced counterparts when applied at 100IJs/larvae. This is because *in vivo* produced *Oscheius* L8 MCB, perform well at

lower concentrations, and thus high concentrations of *in vivo* IJs, it is likely that there is competition among IJs in larvae, resulting in reduced infectivity (Woodring and Kaya, 1988). However, regardless of the difference in infectivity rates *Oscheius* L8 MCB results in insect death in less than 5 days and thus qualifies as an EPN. However, infectivity needs to be tested against a less susceptible insect because *G. mellonella* is highly susceptible and thus does not give sufficient insight into the pathogenicity of EPNs (Georgis and Gaugler, 1991). Nevertheless, the emergence of infective juvenile in *G. mellonella* cadaver was monitored in order to observe the effects of *in vitro* culturing and the dose of infection on nematode reproduction in insect hosts. In addition, the number of emerging IJs may be associated with field efficacy and persistence, because increased number of progeny increases the chances of finding hosts. Thus, emergence studies can be used to give some insight into field efficacy and persistence of *Oscheius* L8 MCB.

5.3.2.2. Emergence studies

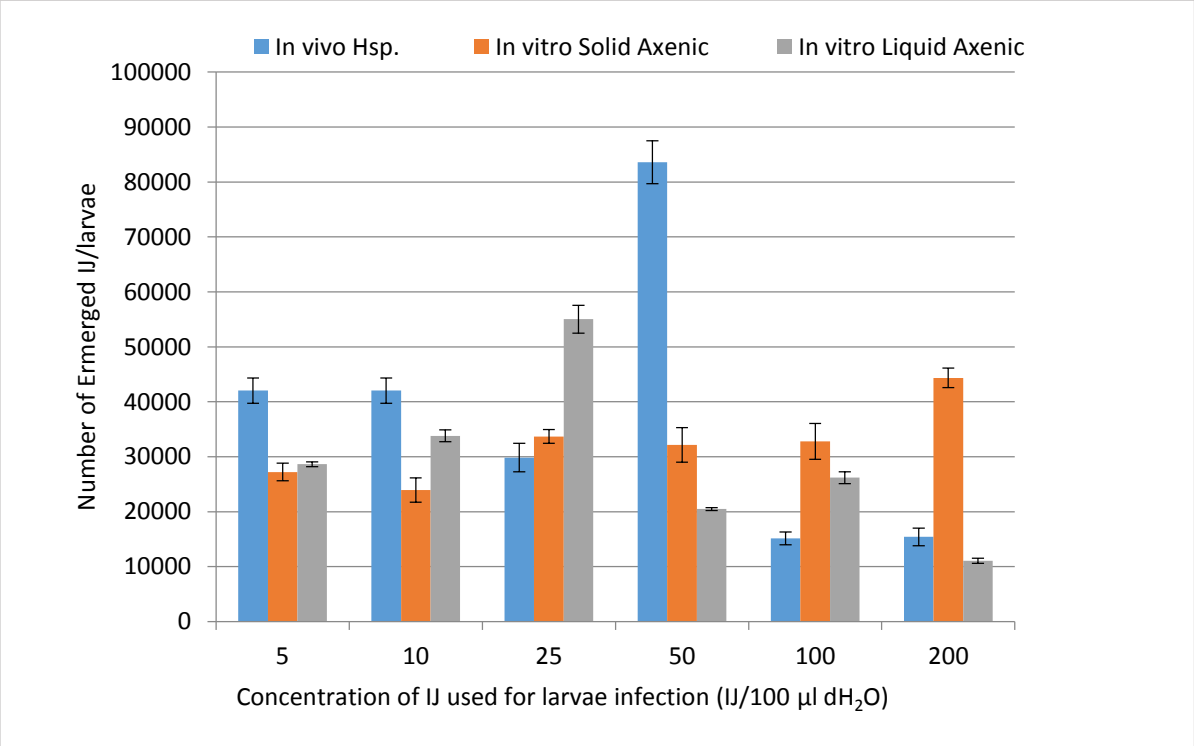


Figure 5.9. Mean number of emerging IJ progeny from larvae that had been infected with IJs from different culture sources at different doses. Error bars represent the standard error of the mean.

In figure 5.9, *G. mellonella* cadaver that had been infected with IJs from *in vivo* cultures significantly produced more IJ progeny at infection compared to *in vitro* IJs at concentrations

of 5, 10, and 50 IJs/ml, with the latter concentration giving the highest number of progeny (IJs/ml) compared to all observations (see table 4 in Appendix X). The high progeny production observed for in vivo, may be due to the fact that *in vivo* produced EPN are better adapted to the host environment compared to *in vitro* produced EPNs. The low EPN production observed for cadaver infected with *in vivo* produced IJs at high nematode concentrations (100 and 200 IJs/ml) may be due to intra-species competition within the larvae reducing IJ reproduction, as a result of reduced nutrient availability (Woodring and Kaya 1988). *In vitro* liquid cultured EPNs at 10 and 25 IJs/ml resulted in more progeny in G. mellonella cadaver that solid state produced, however, solid state cultured nematode resulted in a consistent production of IJ progeny across all concentration, making it more efficacious than the liquid *in vitro* produced EPNs. However, when comparing progeny from *in vivo* IJ cadaver to their *in vitro* counterparts, it is seen that *in vivo* produced IJs produce more progeny. Therefore, it is concluded that axenic *in vitro* production may reduce the efficacy of *Oscheius* L8 MCB and possibly other *Oscheius* EPNs.

5.4. Conclusions

Both the solid and liquid axenic culture methods used for the production of *Oscheius* L8 MCB involved the parallel occurrence of automictic hermaphrodites and amphimictic adults as a possible result of the presence of other nematode adult stages in the inoculum IJ population. This prevented efficient monitoring of life cycle stages and the determining of the mode of reproduction followed by *Oscheius* L8 MCB, however reproduction in this nematode is surmised to be either a heterogonic or androdioecious mode of reproduction due to the existence of hermaphrodite stages throughout the culture period.

The high aeration rate in solid state cultures allowed for early nematode and IJ development when compared to the liquid state. This reduces the culture time, therefore making this method more cost effective and preferable for mass production. However, amenability to contamination, and reduced extraction efficiency are limiting to this method. To avoid the risks associated with *in vitro* solid-state production methods, liquid production can be used as it offers the benefits of high nematode yeild and efficient recovery of nematodes from culture, however it does so at the expense of prolonged culture periods.

The comparative dose-response assay showed that *in vivo* produced EPNs resulted in high G. mellonella larvae mortality at lower concentrations compared to *in vitro* cultured EPNs, and

the reason for this observation was that *in vivo* produced larvae are already adapted to G. mellonella insect hosts. In addition, the difference in mortality between *in vitro* solid and *in vitro* liquid produced was insignificant. These observations suggested that axenic *in vitro* production may reduce the efficacy of *Oscheius* L8 MCB. Probit analysis was attempted to determine the LD_{50} of *Oscheius* L8 MCB, however it was found that there is no clear correlation between IJ concentration and larvae mortality. Thus, the infectivity rate (LT_{50}) was taken into consideration in order to determine the infectivity of *Oscheius* L8 MCB. With this it was found that regardless of the culture method used, *Oscheius* L8 MCB resulted in 100% larvae death in 96 hours, thus qualifying this nematode as an EPN based on the conditions by Dillman *et al*., (2012).

Emergence studies which showed that *in vivo* produced EPN reproduce more efficiently in G. mellonella than the *in vitro* culture methods (solid and liquid), indicating that axenic *in vitro* production reduced efficacy, supporting the observations from the dose-response assay. Therefore, it is concluded that axenic *in vitro* production may reduce the efficacy of *Oscheius* L8 MCB and possibly other *Oscheius* EPNs.

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Chapter 6

Conclusions and future works

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* along with their respective bacteria in the genera *Xenorhabdus* and *Photorhabdus*, have insecticidal abilities resulting in the death of an insect in 48-72 hours. This ability of EPNs to bring about insect death sparked interests aimed at their inundative application as biocontrol agents.

Some members of the recently discovered *Oscheius* genus have been found to form insecticidal associations with members of the *Serratia* genus, thus igniting interest in this genus as a potential EPN. Species of this genus considered as EPNs include *Oscheius chongmingensis* and *Oscheius carolinensis* due to their ability to kill insect hosts in less than 5 days.

The biopesticide potential of EPNs has proved to be an incentive for studies focused on their isolation and identification, and commercial production for biological control. The current study, was also aimed at identifying a local entomopathogenic nematode for the potential use as a biocontrol agent. A local nematode was successfully isolated from local soil in the Brits region of North West province. The nematode was identified to be a member of the *Oscheius* genus due to the lack of distinctive colour change in infected larvae and phylogenetic analysis supporting this discovery. It was concluded that whole genome sequencing and morphological were required for the full characterisation and classification of this organism. However, of this nematode was referred to as *Oscheius* L8 MCB based on the phylogenetic analysis. Phylogenetic analysis showed that *Oscheius* L8 MCB was associated with a strain of *Serratia marcescens*, referred to as *S. marcescens* L8 MCB. This growth of *Oscheius* L8 MCB in monoxenic cultures consisting of *S. marcescens* L8 MCB and the ability to infect insect hosts after culturing proved that this bacterial species is indeed the insecticidal symbiont of the *Oscheius* species.

The discovery of the role played by bacterial symbionts play an important role in the reproduction of *Steinernema* and *Heterorhabditis* EPNs has led to the extensive use of monoxenic for *in vitro* production of EPNs. However, the role of bacteria for the in vitro culturing of *Oscheius* EPNs has not been studied. Therefore studies were conducted to observe the role played by *S. marcescens* L8 MCB on the growth of *Oscheius* L8 MCB, by

culturing the nematode using monoxenic and axenic culturing methods. *S. marcescens* L8 MCB was shown to optimally grew in nutrient broth 2 (4% canola oil + 25 mg/ml glucose) media, showing that the bacteria is able to efficiently metabolise both canola and glucose. Therefore NB2 media was used for the *in vitro* production of *Oscheius* L8 MCB in monoxenic and axenic cultures. The axenic culturing of *Oscheius* L8 MCB found to favour the production of the nematode compared to the monoxenic. The reason for this high production was surmised to involve the regurgitation of competent bacteria by nematodes in axenic cultures which favoured EPN growth, unlike the bacteria in monoxenic cultures, which might have lost fitness due to prolonged *in vitro* culturing. The role of bacterial symbionts of *Oscheius* EPNs play in growth and reproduction is still poorly understood and thus more studies are needed for further understand of this role. However, regardless of the role the bacterial symbionts play, *Oscheius* L8 MCB was grown efficiently in axenic cultures compared to monoxenic, therefore this method can be used for the production of these nematodes and most likely other *Oscheius* species. It should be noted that axenic EPN cultures obtained from surface sterilisation may not be efficient because sometimes cultures failed to grow. However, regardless of the inefficiency in the EPN sterilisation technique, surface sterilised *Oscheius* L8 MCB still managed to grow *in vitro*.

Growth culture components such as lipids and carbohydrate content have been previously shown to affect nematode yield in monoxenic production of EPNs. However, these components have not been studied in the *in vitro* axenic culturing of an *Oscheius* species. Therefore, studies were conducted to observe the *in vitro* axenic culturing of *Oscheius* L8 MCB in nutrient broth supplemented with various concentrations of canola oil, and canola oil with glucose to observe the effect of these compounds on nematode yield. It was observed that supplementation of the media with these compounds, irrespective of compound concentration, increased nematode production. However, supplementation of NB with 2% canola produces a significant amount of EPNs in reduced culture times, but NB supplemented with 4% canola oil and 25mg/ml glucose increased nematode yield but prolonged the culture time. It is noted that media composition (with regards lipid and carbohydrate content) plays an important role in nematode yield and culture time and thus optimization of these components is critical for efficient nematode production.

In vitro production of *Steinernema* and *Heterorhabditis* EPNs has been based on monoxenic production is solid state and liquid state, for improved scalability. Since *Oscheius* L8 MCB was shown to grow better in axenic cultures, a study was conducted to observe the effects of

solid state and liquid state production on the yields of this nematode. The axenic *in vitro* cultures in solid and liquid used for the production of *Oscheius* L8 MCB showed the parallel occurrence of automictic hermaphrodites and amphimictic adults (most likely as a result of the presence of other nematode adult stages in the inoculum IJ population). This prevented efficient monitoring of life cycle stages and the determining of the mode of reproduction followed by *Oscheius* L8 MCB, however reproduction in this nematode is surmised to be either a heterogonic or androdioecious mode of reproduction due to the existence of hermaphrodite stages throughout the culture period.

The high aeration rate in solid state cultures allowed for early nematode and IJ development when compared to the liquid state. This reduces the culture time, therefore making this method more cost effective and preferable for mass production. However, amenability to contamination, and reduced extraction efficiency are limiting to this method. To avoid the risks associated with *in vitro* solid state production methods, liquid production can be used as it offers the benefits of high nematode yeild and efficient recovery of nematodes from culture, however it does so at the expense of prolonged culture periods

In vitro methods of EPN production may have an effect on the efficacy of the EPNs against insect hosts. The comparative dose-response assay and Emergence studies showed that *in vivo* produced EPNs caused high mortality in *G. mellonella* larvae at lower concentrations and also that these larvae resulted in a high number of emerging IJs compared to EPNs produced using axenic *in vitro* culturing methods. The differences between mortality and IJ emergence in larvae infected with solid state and liquid state cultured EPNs were marginal. Therefore it is concluded that axenic culturing methods may reduce the efficacy of *Oscheius* L8 MCB, and possibly other *Oscheius* species, against their respective insect hosts.

Future works

- Fully describing *Oscheius* L8 MCB with the aid of whole genome sequencing and morphological characterisation (using both light and scanning electron microscopy).
- Using alkaline lysis of gravid females for obtaining pure axenic eggs, in order to study the life cycle and mode of reproduction of *Oscheius* L8 MCB *in vitro*
- To test the efficacy of *in vivo* and *in vitro* reared *Oscheius* L8 MCB against a less susceptible host.

Appendix I

Galleria mellonella rearing media

Components:

500g Bokomo ProNutro (banana flavour)

200ml pure natural honey

200ml glycerol

5 teaspoon yeast extract

200ml boiled distilled water

1 teaspoon benzoate

1. Mix honey, glycerol and ProNutro together.
2. Add yeast extract, boiling water and benzoate to ProNutro mixture.
3. Mix contents thoroughly.
4. Place mixture in tin foil and seal adequately.
5. Autoclave at 121˚C and 15 psi for 25 minutes.

Appendix II

Nematode Genomic DNA extraction (Protocol from Puregene® DNA Purification Kit, Genera systems 2003)

1) Rinse surface sterilized infective juveniles three times using approximately 4ml distilled water per wash.

2) Pellet nematodes in a microfuge tube by spinning at 14000rpm for 10 minutes. Place on ice for 30 seconds. Remove excess water.

3) Re-suspend nematode pellet in 1 ml distilled water and transfer the nematode suspension to a 1.5 ml microfuge tube on ice.

4) Centrifuge at 13000-16000 rpm for 3 minutes than place the tube on ice for atleast 30 seconds and discard the supernatant.

5) Add 600µl Cell Lysis Solution (from kit) and invert several times.

6) Add 3µl Proteinase K solution (from kit) and invert 25 times. Incubate at 55˚C for 3 hours to overnight, until the tissue particulates have dissolved. Invert periodically.

7) Add 3µl RNase A Solution (from kit) to the cell lysate, invert 25 times and incubate at 37˚C for 15-30 minutes.

8) Cool the sample to room temperature.

9) Add 200µl Protein Precipitation Solution (from kit) to the RNase A treated cell lysate.

10) Vortex at high speed for 20 seconds.

11) Centrifuge at 13000-16000 rpm for 3 minutes. A tight protein pellet should form. If this pellet is not visible repeat step 10, followed by incubation on ice for 5 minutes, than repeat step 11.

12) Pour the supernatant containing the DNA into a 1.5ml centrifuge tube containing 600µl 100% Isopropanol.

13) Invert gently 50 times.

14) Centrifuge at 13000-16000 rpm for 1 minute, the DNA will be visible as a white pellet.

15) Pour off the supernatant and drain the tube on clean absorbent paper.

16) Add 600µl 70% Ethanol and invert the tube to wash the pellet.

17) Centrifuge at 13000-16000 rpm for 1 minute and carefully pour off the ethanol. Pour slowly as the pellet may be loose.

18) Invert and drain the tube on absorbent paper again and allow to air dry for 10-15 minutes.

19) Add 100µl DNA hydration Solution (from kit).

20) Rehydrate the DNA by incubating the sample 1 hour at 65˚C. Tap the tube to aid dispersing the DNA.

21) Store DNA at 4˚C.

Preparation of 0.1% jik solution for infective juvenile sterilization

971.43 ml distilled water

28.57 ml 3.5% jik

1. Autoclave distilled water.
2. Mix jik and distilled water in a 1 L Schott bottle.
3. Autoclave at 121˚C and 15 psi for 15 min.

PCR condition for amplification of 18S rDNA region (35 cycles)

Initial denaturation: 94^{o}C for 5 min

Denaturation: 95^{o}C for 60 sec

Annealing: 64^{o}C for 60 sec

Extension: 72^{o}C for 120 sec

Final extension: 72^{o}C 10 min

Appendix III

Media for isolating symbiotic bacteria associated with entomopathogenic nematodes

NBTA (adapted from Akhurst, 1980)

1 litre nutrient agar

0.04g triphenyltetrazolium chloride (TTC)

0.025g bromothymol blue (BTB)

1. Mix nutrient agar and BTB.
2. Autoclave at 121˚C and 15 psi for 15 min.
3. Add TTC, just before pouring into petri dishes, however ensure the autoclaved medium is less than 50˚C. TTC will break down if added when medium is too hot.
4. Swirl to mix.
5. Dispense into sterile petri dishes and leave to solidify.

McConkey agar (commercially available)

Composition (g/l)
20.0g Peptone

10.0g Lactose

1.5g Bile Salts

5.0g Sodium Chloride

0.03g Neutral Red

0.0001g Crystal Violet

13.5g Agar

1. Weigh out MacConkey agar powder and suspend in 1000ml distilled water.

2. Boil whilst stirring until completely dissolved.
3. Autoclave at 121ºC and 15 psi for 15 min.
4. Cool to 45 – 50ºC.
5. Mix well, dispense into sterile petri dishes and leave to solidify.

Gram stain procedure

1. Place slide with heat fixed smear on staining tray.
2. Gently flood smear with crystal violet and let stand for 1 minute.
3. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
4. Gently flood the smear with Gram's iodine and let stand for 1 minute.
5. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
6. Decolorize using 95% ethyl alcohol. Tilt the slide slightly and apply the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear. Be careful not to over-decolorize.
7. Immediately rinse with water.
8. Gently flood with safranin to counter-stain and let stand for 45 seconds.
9. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
10. Blot dry the slide with bibulous paper.
11. View the smear using a light-microscope under oil-immersion.

Appendix IV

Isolation of genomic DNA from bacterial cells (Protocol from ZR Fungal/Bacterial DNA Kit, catalog # D6005)

1) Pick an isolated bacterial colony from a previously streaked NBTA plate and suspend in a ZR BashingBead™ Lysis Tube.

2) Vortex at maximum speed for 5 minutes.

3) Centrifuge the ZR BashingBead™ Lysis Tube in a microcentrifuge at 10 000 x *g* (rpm) for 1 minute.

4) Transfer up to 400µl supernatant to a Zymo-Spin ™ IV Spin Filter in a Collection Tube and centrifuge at 7000 rpm for 1 minute.

5) Add 1200µl of Fungal/ Bacterial DNA binding buffer to the filtrate in the Collection Tube from Step 4.

6) Transfer 800µl of the mixture from Step 5 to a Zymo-Spin™ II Column in a Collection Tube and centrifuge at 10000rpm for 1 minute.

7) Discard the flow through from the Collection Tube and Repeat Step 6.

8) Add 200µl DNA Pre-Wash Buffer to the Zymo-Spin™ II Column in a new Collection Tube and centrifuge at 10000rpm for 1 minute.

9) Add 500µl Fungal/Bacterial DNA Wash Buffer to the Zymo-Spin™ II Column and centrifuge at 10000rpm for 1 minute.

10) Transfer the Zymo-Spin™ II Column to a clean 1.5 ml microcentrifuge tube and add 100µl DNA Elution Buffer directly to the column matrix. Centrifuge at 100000rpm for 30 seconds to elute the DNA.

PCR conditions for amplification of bacterial 16S rDNA region (35 cycles)

Initial denaturation: 95^{o}C for 3 min

Denaturation: 94^{o}C for 30 sec

Annealing: 60^{o}C for 45 sec

Extension: 72^{o}C for 90 sec

Final extension: 72^{o}C 7 min

Appendix V

Recipes for *in vitro* culture media

Lipid agar media (adapted from Woodring and Kaya, 1997)

10 g Honey

5 g Yeast extract

25 g nutrient agar

2.5 ml cod liver oil

2 g MgCl2.6H2O

1. Mix ingredients together in distilled water.
2. Autoclave at 121˚C and 15 psi for 20 min and asceptically pour into petri dishes.

Nutrient broth (commercially available)

Composition (g/l)

1g Meat extract

2g Yeast extract

5g Peptone

8g Sodium chloride

1. Weigh out nutrient broth powder and suspend in 1000ml distilled water.
2. Mix well and dispense adequate amounts into volumetric flasks.
3. Autoclave at 121˚C and 15 psi for 15 min.

Nutrient broth variation one

Nutrient Broth

0.4, 1, 2 or 4% (w/v) Canola oil

1. Weigh out nutrient broth powder and suspend in desired volume of distilled water.
2. Mix well and dispense adequate amounts into Erlenmeyer flasks.
3. Add desired percentage (w/v) of Canola oil to each Erlenmeyer flask containing nutrient broth.
4. Autoclave at 121˚C and 15 psi for 15 min.

Nutrient broth variation two

Nutrient Broth

0.4, 1, 2 or 4% (w/v) Canola oil

2.5, 6.125, 12.5 or 25mg/ml glucose

1. Weigh out nutrient broth powder and suspend in desired volume of distilled water.
2. Add desired amount glucose.
3. Mix well and dispense adequate amounts into volumetric flasks.
4. Add desired percentage (w/v) Canola oil to each volumetric flask containing nutrient broth.
5. Autoclave at 121˚C and 15 psi for 15 min.

Appendix VI

Oscheius L8 MCB consensus sequence

>*Oscheius* L8 MCB

TCATCGGATACAGAGATGGGTTGTAGATGCTAGCTGGTATGAATAGGGATTTGGTCCTTGTTCG
GCATCTATAGCCAGTGAAATCCCTGAGCAGTGTGCTCACTTGGTAACGTGCAGACTCTTTTCAGGG
AGGTCTGATAAGCGTTACCGATCTCTCTTTCGAGAGATTGAGCTTAATTCGAGCAGCTACGCTCAG
CACTGTAGTTTTATTGCTGGCTAACGCTTTGCCTGTGCATAGCAGCCTTAATGACCGTAGCTTTATT
GGCGGCAGTAAAACAAATCTTTTTGAGCAGATAATTTAGTATACTTTCACGTTAGTGAAATAACTT
ATCAGCTTATGTGTTGGATCGGTTGATTCGTAGATCGATGAAAAACGCAGCTAGCTGCGTTATATA
CCACGAATTGCTTAAAAATTGAGTGGTAAAATTTTGAACGCATAGCGCCACAGGGTTTACTCTGTG
GCACGCCTGGTTCAGGGTTGTATTATCTACTACCGAATGCCTGATTCGGTTAGTCGAGCGGCTATC
TATTCACGTAGATACCCGTTCTAGTTCAGAATTGATCGCAACGTTTCTATTTCTATAGATACGGAAG
GCGTGAGTGAACACGACCACTGCATTCGAAGCAGCTTGATTGAGCTAATGCTTTGAACGCACGCTA
GTAAAGTGGTGAGTTTGCTCTAATGCTTTGCATTTGCAACCTGAGCTCAGACGTGATTACCCGCTG
AT

Appendix VII

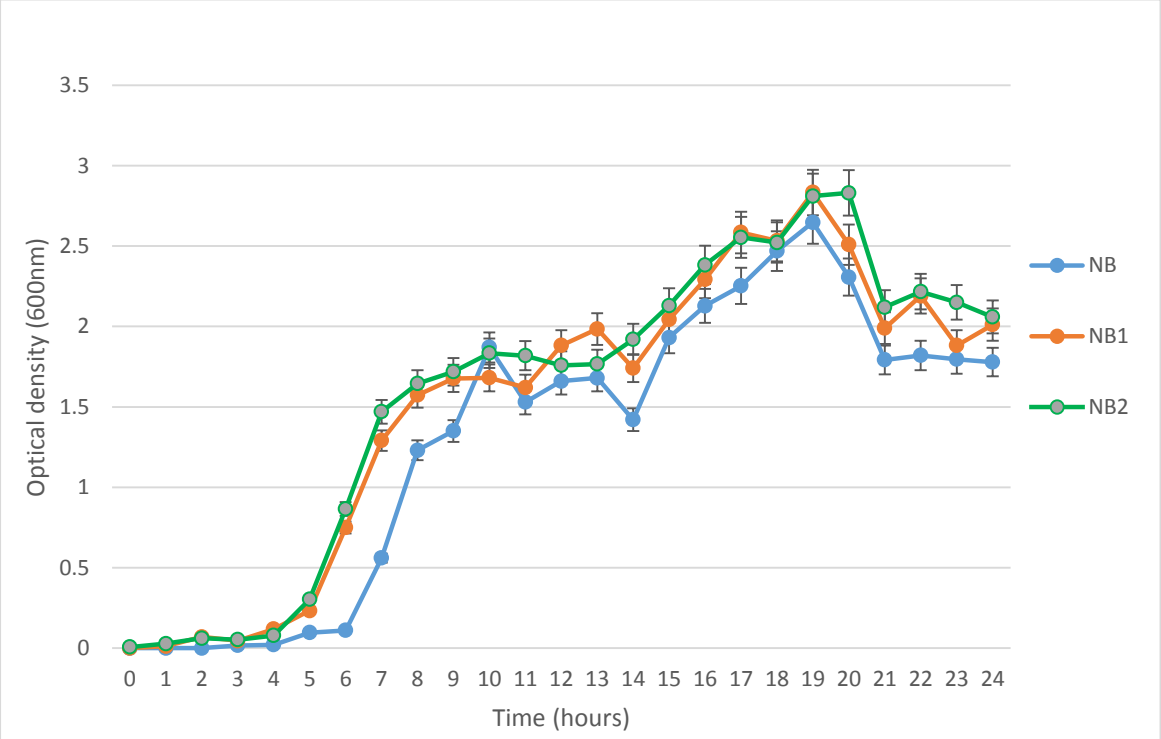
Serratia marcescens L8 MCB consensus sequence

>Serratia L8 MCB

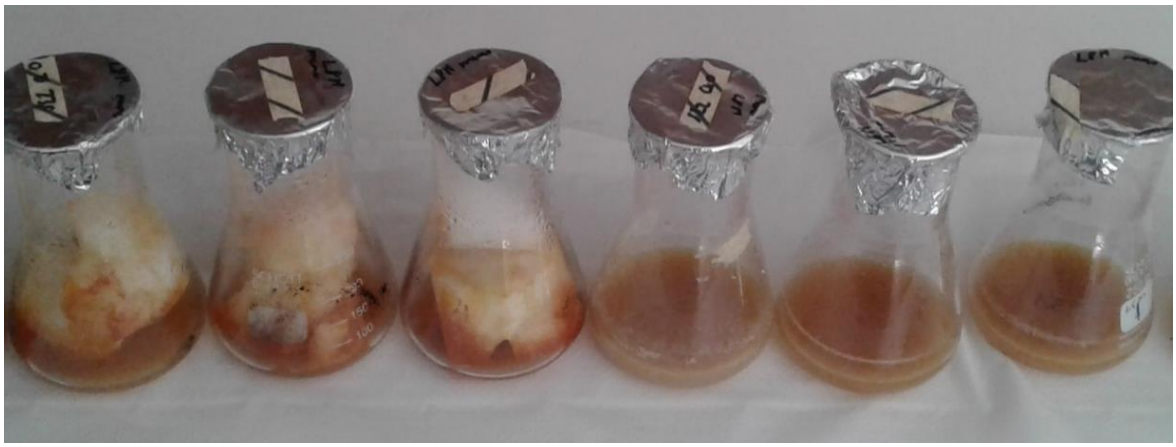
TTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAATCGACATCGTTTACAGC
GTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTCGTC
CAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAAT
TCTACCCCCCTCTACGAGACTCTAGCTTGCCAGTTTCAAATGCAGTTCCCAGGTTGAGCCCGGGGA
TTTCACATCTGACTTAACAAACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCA
CCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGAGTAACGTCAATT
GATGARCGTATTAAGCTCACCACCTTCCTCCTCGCTGAAAGTGCTTTACAACCCGAAGGCCTTCTTC
ACACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGT
AGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTC
GCCTAGGTGAGCCATTACCCCACCTACTAGCTAATCCCATCTGGGCACATCTGATGGCAAGAGGCC
CGAAGGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGTATTAGCTACCGTTTCCAGTAGTTATCCCC
CTCCATCAGGCAGTTTCCCAGACATTACTCACCCGTCCGCCGCTCGTCACCCGGGGAGCAAGCTCC
CCTGTGCTACCGCTCGACTTGCATGTGTTAAGCCTGCCGCCAGCGTTCAATCTGAGT

Appendix VIII

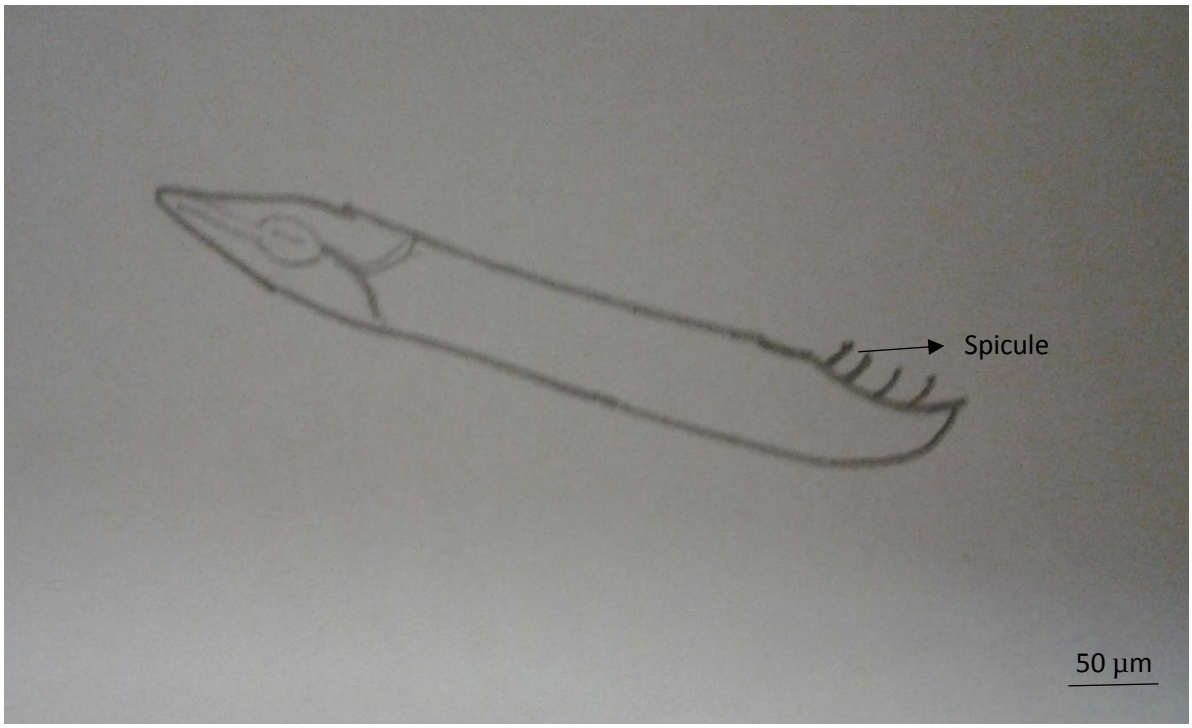
Complete 24 hour bacterial growth curve of *S. marcescens* L8 MCB



Appendix IX



Axenic cultures of *Oscheius L8 MCB* in Solid and liquid state shake flasks



An illustration of a male nematode of *Oscheius* L8 MCB.

Appendix X

Tables of statistics

Table 1. P-values of derived from T-tests comparing *Oscheius* L8 MCB production in axenic and monoxenic cultures. (P-values < 0.05 indicate that the result is significant).

Day	T	P-value
1	1.236051	0.284063
2	-1.02553	0.36308
3	1.006967	0.37092
4	2.544943	0.063646
5	-1.92756	0.126168
6	4.207797	0.01361
7	1.043436	0.355655
8	6.47547	0.002931
9	5.670705	0.00477
10	19.98616	3.7E-05
11	9.325088	0.000736
12	8.686355	0.000967
13	2.882324	0.044908
14	-2.77817	0.049912
15	-4.90935	0.00799

Table 2. P-values derived from T-tests comparing IJ production in solid and liquid state. (P-values < 0.05 indicate that the result is significant).

Day	T	P-value
1	34.45775	4.23E-06
2	3.742667	0.020074
3	12.41532	0.000242
4	6.068061	0.003725
5	19.40781	4.16E-05
6	8.140806	0.001239
7	18.11215	5.46E-05
8	1.352941	0.24749
9	2.959461	0.041577
10	-0.02733	0.979507
11	-0.9497	0.396041
12	-7.92351	0.001373
13	-5.60364	0.00498
14	-5.12157	0.006879
15	-5.00447	0.007467

Table 3. P-values and F-values obtained from two-way ANOVA for comparative dose response assay at different time points. (P-values < 0.05 indicate that the result is significant).

Time point post infection (hours)	Difference in cultures method		Difference in concentration of IJs		Interactions between culture method and concentration	
	P- value	F-value	P-value	F-value	P-value	F-value
24	0.00661	5.788	0.00025	6.375	0.0313	3.578
48	0.06595	2.935	0.00364	4.294	0.03125	2.325
72	0.30217	1.238	2.41E-05	8.405	0.02036	2.524
96	0.47060	0.770	4.38E-05	7.861	0.04519	2.153

Table 4. P-values and F-values for emergence obtained through one-way ANOVA. (P-values < 0.05 indicate that the result is significant).

IJ concentration at infection	P-value	F-value
5	0.001062	26.402
10	0.001981	20.888
25	0.000554	33.518
50	9.49E-06	138.70
100	0.001769	21.804
200	7.49E-06	150.36