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RESEARCH ARTICLE

Mass-culture technique of a South African *Heterorhabditis bacteriophora* isolate, using *in vitro* liquid culture

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Heterorhabditis bacteriophora is an entomopathogenic nematode (EPN), and together with its mutualistic bacteria, is a highly effective insect biocontrol agent. The preferred method for large-scale production involves *in vitro* liquid culture, whereby the nematode and bacteria are cultivated in an artificial medium that replicates the haemocoel conditions found within the insect host. Although the mass-culturing method for *H. bacteriophora* has proven successful in other countries, it has still to be implemented as a local commercial product in South Africa, despite its considerable potential for pest control. Several factors impact on the success of an *in vitro* liquid culture, including the bacterial inoculum density and the ingredients used in the culture media. Thus, this study aimed to develop an in vitro liquid culture protocol for a local isolate of *H. bacteriophora*. Switching from soy powder to egg yolk powder significantly increased the yield of infective juveniles (IJ) during culture, despite there being no differences in IJ recovery between days 2 to 4 after nematode inoculum. Furthermore, the bacterial inoculum density exerts a significant influence on recovery and yield, with the use of a 2% (v/v) inoculum concentration showing the most favourable results. Bacterial cell density is crucial for IJ recovery, as it provides the food signal that activates the IJ. The success obtained with this liquid culture technique for *H. bacteriophora* paves the way for the optimisation of various additional liquid culture parameters, including nutrients levels, oxygen concentrations and cost-effective ingredients.

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INTRODUCTION

Heterorhabditis bacteriophora Poinar is an entomopathogenic nematode (EPN) belonging to the family Heterorhabditidae (Poinar 1975; Thomas and Poinar 1979). EPNs, which kill insects within a period of 24-48 h after penetration, belong to the families Heterorhabditidae and Steinernematidae, with symbiotic relationships with the bacteria *Photorhabdus* spp. and *Xenorhabdus* spp., respectively (Ehlers 2001; Han and Ehlers 2001). The nematode, together with its symbiotic bacteria, is ideal to use as a biocontrol agent (Surrey and Davies 1996). The majority of the EPNs life cycle occurs in the infected host insect, with the only free-living stage being the infective juvenile (IJ) (Shapiro-Ilan and Gaugler 2002). The IJ is an arrested, non-feeding survival stage, which is an adapted third-stage juvenile (J3) that moves through the soil in search of a host insect. The IJs, which use chemosensory signals to seek out their target host insect (Lewis et al. 2006), enter the insect through natural openings and the soft parts of the insect's cuticle (Bedding and Molyneux 1982). Upon entering the insect host, the IJs release the symbiotic bacteria that is stored in their gut. The insect then dies from septicaemia, with a food signal being released resulting in the activation of the IJs concerned (Golden and Riddle, 1984; Strauch and Ehlers 1998). For *H. bacteriophora*, the IJs develop into fourth-stage juveniles, and then into hermaphroditic females, as the first generation in the insect. The hermaphroditic females then produce juveniles that develop into amphimictic adults, or IJs, depending on the food availability (Strauch et al. 1994). Abundant food resources lead to the production of multiple generations. However, when the food resources become depleted, the J3 stage ceases feeding, retaining the symbiotic bacteria in the gut, and developing into arrested IJs (Hirao and Ehlers 2010). The subsequent IJs then leave the host insect's cadaver, in search of a new host, where their life cycle continues.

Heterorhabditis bacteriophora is an ideal candidate for use as a biocontrol agent, due to them being widely distributed (Bhat et al. 2020), posing no threat to non-target organisms, and infecting a variety of important agricultural pest insects (Kaya and Gaugler 1993). Thus, implementing the use of *H. bacteriophora* in an integrated pest management (IPM) system is an environmentally friendly alternative to reducing the use of chemical pesticides. Due to its potential as a biopesticide, *H. bacteriophora* is currently being mass-cultured and used in field applications (Shapiro-Ilan et al. 2012; Han et al. 2024) To mass-culture EPNs on a commercial scale, *in vitro* liquid culture is the preferred method (Ehlers 2001; Islas-López et al. 2005; Chavarría-Hernández et al. 2006;). In an *in vitro* liquid culture system, the nematode and its associated bacteria are cultivated in a medium that is designed to replicate the environment found inside an insect. The subsequent IJs that are produced in the *in vitro* liquid culture process are harvested and formulated into a product that can be applied in the field. Noteworthily, monoxenic cultures should be established to eliminate the risk of contamination, and to ensure that only the nematode and its symbiotic bacteria are present in the culture media. Doing so can be achieved through the sterilising of the eggs, instead of the IJs (Lunau et al. 1993). The IJs possess an additional sheath layer and a digestive system that might retain bacterial contaminants, when surface sterilisation is the sole method employed, potentially resulting

in contamination. Using a monoxenic *in vitro* liquid culture can significantly increase the yields produced (Ogier et al. 2023).

A crucial factor that impacts on yield, and on the success of an *in vitro* liquid culture technique, is the liquid media employed (Hatab and Gaugler 1999). Since the *in vitro* liquid culture aims to replicate the insect's hemocoel environment, the culture medium should contain nutrients closely resembling those found within an insect. The source of protein, carbohydrates, nitrogen, and mineral salts are all important to both the nematode and the associated bacteria concerned (Selvan et al. 1993; Cho et al. 2011; Dunn et al. 2021a). The bacteria involved feed on the culture media, while the nematode feed off the bacterial cells. *Heterorhabditis bacteriophora* has a complex relationship with its *Photorhabdus*-associated bacteria, as the nematode cannot develop in the absence of the *Photorhabdus* bacteria involved. High cell densities result in increased IJ recovery, or 'activation' percentage, which, in turn, result in higher yields being achieved (Inman et al. 2012). Both field and laboratory efficacy studies have been conducted to assess whether the species is likely to prove effective in managing important agricultural pest insects in South Africa (Malan et al. 2011; Malan and Moore 2016; Steyn et al. 2021), with *H. bacteriophora* having shown potential to control such pest insects. Currently, this species is being imported as a biopesticide.

Odendaal et al. (2016) demonstrated that the imported *H. bacteriophora* species exhibited reduced effectiveness in comparison to the local isolates during field efficacy trials, which underscores the necessity of mass-culturing local EPNs, rather than importing exotic nematode/bacteria combinations as currently is the case in South Africa (Hatting et al., 2019). Local species are better adapted to the environment, tending to present a reduced risk to the indigenous biota, which holds significant importance within the South African context. South Africa, which is known for being an ecological hotspot, has strict environmental laws in place when it comes to the importation of exotic species.

EPNs, including *Steinernema yirgalemense* Nguyen, *Steinernema jeffreyense* Malan et al. and *Heterorhabditis zealandica* Poinar ('green' isolate MJ2C) (Booysen et al. 2022), have been successfully mass-cultured in South Africa (Dunn et al. 2021b; Dunn and Malan 2023a, b; Dunn and Malan 2024; Ferreira et al. 2014). *Steinernema yirgalemense* and *S. jeffreyense* have been successfully mass-cultured, using the *in vitro* liquid method, and have been upscaled to benchtop bioreactors (Dunn et al. 2021b; Dunn and Malan 2023b; Dunn and Malan 2024).

The objective of the current study was to establish a standardised *in vitro* liquid mass-culture method, capable of effectively producing a local isolate of *H. bacteriophora*. The research involved focused on examining the two key parameters of such production: the protein and the bacterial inoculum density, with their respective influence on the activation of the IJs and on the subsequent IJ yield, as obtained from the *in vitro* liquid culture process.

METHODS AND MATERIALS

Maintenance of nematode isolate

The *H. bacteriophora* isolate SGI_170, selected for the mortality bioassay and the *in vitro* liquid culture was obtained from the Agricultural Research Council (ARC), and it was originally isolated from Fouriesburg, Free State province, South Africa (Hatting et al. 2009) The IJs were maintained and recycled using *in vivo* methods, through *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae. The infected larvae were placed on a modified White trap until the emergence of the IJs (White 1927). The IJs, which were then collected into cell culture flasks, were stored at 14 °C. The flasks were shaken weekly to ensure oxygen circulation.

Establishing a monoxenic culture

Monoxenic cultures were established using a modified method by Lunau et al. (1993). Bacterial lawns were made on Wouts (1981) plates (lipid agar: 16 g/l nutrient broth [Bacto], 5 g/l canola oil, 12 g/l agar) using the monoxenic-verified bacteria of the isolate SGI_170. The Wouts' plates were left to incubate for 48 h at 28 °C. *In-vivo*-produced *H. bacteriophora* (non-sterile) IJs were placed in Eppendorf tubes and centrifuged at 10 000 rpm for 2 min. The excess fluid was then removed, with the remaining pellet containing the IJs being inoculated onto the bacterial lawns. The plates were then incubated at 25 °C for 3-4 days, or until hermaphroditic females were present on the plates, which were rinsed with Ringer's solution, washed several times and sieved through a 90-µm sieve to remove the other life-stages involved. The hermaphrodites were then harvested into a beaker, where they were allowed to settle. The excess water was then siphoned off from the beaker, with 1 ml of the nematode suspension being pipetted into Eppendorf tubes. The Eppendorf tubes were then centrifuged at 10 000 rpm for 2 min, with the supernatant then being removed, and the hermaphroditic females being crushed, using a macerating tool to release the eggs. The eggs were then surface-sterilised, using an annexing solution (1.25 ml NaOH (16% w/v solution), 2.25 ml bleach [household Jik], 6.5 ml distilled water) for 5-8 min consistently, turning the Eppendorf tubes slowly. After 5-8 min, the Eppendorf tubes were centrifuged at 10 000 rpm for 2 min, and the supernatant was removed. To rinse the sterilised eggs, 1000 µl sterile water was pipetted into the Eppendorf tubes and centrifuged at 10 000 rpm for 30 sec. The process was then repeated to remove the sterilising solution completely. The supernatant was removed to leave only the egg pellet behind in the Eppendorf tubes.

In a sterile 24-well plate, 1 ml of tryptic soy broth (TSB) (0.9 g TSB, 30 ml distilled water) was pipetted into every alternate well. The sterilised eggs of each Eppendorf tube were then added to the TSB, leaving four wells with TSB empty, as a control measure. The 24-well plates were then sealed and incubated at 25 °C for two days. Two days prior to the egg extraction and sterilisation process, 200 µl bacteria, proliferated in TSB, were added to flasks containing 30 ml complex media (CM) (20 g/l egg yolk powder, 15 g/l yeast extract, 4 g/l NaCl, 0.35 g/l KCl, 0.15 g/l $CaCl_2$, 0.1 g/l $MgSO_4$, 36 ml canola oil, 1 L distilled water). The culture media was then placed on an orbital shaker at 140 rpm for 48 h in a 28 °C incubating chamber. After 48 h, the two-day-old juveniles were added to the culture media and placed on a 140-rpm orbital shaker at 25 °C, until a new cohort of IJs was present (approximately 30 days). Once the IJs were present, the flasks could then be used as inoculum for further studies.

Recovery and yield

Recovery was observed up to five days post inoculation, when signs of *endotokia matricida* were observed. To establish recovery, 200 µl samples of the culture media were extracted from the flasks daily. Each sample was diluted to 20 ml, using distilled water. One ml of the diluted sample was pipetted into a glass counting chamber. The samples were observed under a stereomicroscope, to be able to establish whether the IJs recovered, and the life stages present were counted, using an analogue tally counter.

Recovery was determined using the following equation:

$$\text{Recovery \%} = \frac{\left[J4 + \text{Adults}\left(\text{Hermaphroditic adults} + \text{Females} + \text{Males}\right)\right]}{\text{Total population}\left(IJs + J4 + \text{Adults}\right)}$$

Yield was observed 14 days post inoculation. To establish the yield, a 200-µl sample was taken from each flask and diluted to 20 ml. Using a 20-µl pipette, three rows, each consisting of five 10 µl drops, were pipetted onto a Petri dish. The number of IJs

per drop, which was observed under a stereomicroscope, was counted using an analogue counter. The average was calculated (the total divided by the number of rows), multiplied by a factor of 20, and then by a factor of 100 to establish the number of IJs present per ml sample.

Protein source

Two powdered proteins sources were compared, namely soy powder and egg yolk. Each treatment had three flasks, plugged with non-absorbent cottonwool, and wrapped in aluminium foil to prevent contamination. The culture flasks were then autoclaved at 121 °C for 21 min. After being autoclaved and cooled under a laminar flow, 200 µl of bacteria was added to the CM (2% v/v), with the bacteria concerned being allowed to proliferate in 30 ml TSB at 28 °C on a 140-rpm mini orbital shaker, 48 h prior to IJ inoculation. The culture media containing the symbiotic bacteria were then placed in a 28 °C incubating chamber, on a mini orbital shaker, at 140 rpm for 48 h. After 48 h, IJs from the established inoculum flasks were inoculated into the soy and egg yolk powder culture media, with the initial inoculum used containing 5 000 IJs. Once the flasks were inoculated, they were placed on orbital shakers at 140 rpm at 28 °C. Recovery and yield were then observed, with the whole process being repeated on completion of the first trial.

Effect of bacterial inoculum density

Twelve culture flasks were inoculated with the associated bacterial species at four different concentrations (2%, 5%, 8%, 10% (v/v)) to establish the optimum bacterial concentration to increase the extent of both the recovery and the yield, with each treatment using three flasks. The symbiotic bacteria were obtained from the 1 ml stock cultures stored at -80 °C, and then inoculated into a 30-ml TSB flask. The flask was incubated at 28 °C, on a mini orbital shaker, at 140 rpm for 48 h. After 48 h the bacteria were inoculated at four different concentrations, being 2%, 5%, 8% and 10% (v/v), into flasks containing 30 ml of the culture media CM with 20 g of egg yolk/L. Thereafter, the flasks were incubated for 48 h at 28 °C, on mini orbital shakers at 140 rpm. After 48 h, 5000 IJs were inoculated into each of the 12 flasks and shaken at 140 rpm at room temperature, 25 °C. Recovery and yields were observed post inoculation. This trial was repeated two weeks later.

Statistical analysis

The data from the trials and their repeated trials were pooled together when no significant difference was found in the main effects. A normality test was done to establish whether the data was parametric. A repeated-measures analysis of variance (ANOVA) was done for the recovery data, comparing the egg and soy powder culture media, with a significant value of $\alpha = 0.05$. To determine significant differences in recovery between the egg and soy powder on the different days, a least significant difference (LSD) post-hoc test was done. To compare the IJ yields achieved for the egg and soy powder, a t-test was undertaken. For the recovery data of the bacterial concentrations concerned, a repeated-measures ANOVA was done, with the interactions between the different treatment groups being significant at $\alpha \leq 0.05$. To determine significant differences between the different treatment groups used, an LSD post-hoc test was done. A one-way ANOVA was done to compare the IJ yields of the four bacterial concentrations used, and an LSD post-hoc test was performed to establish the significant differences between the four treatment groups concerned. All statistical analyses were conducted in Statistica, ver. 14 (Tibco Software Inc., 2020).

RESULTS

Protein source

The analyses using the repeated-measures ANOVA showed no significant difference between the mean recovery percentage and the number of days post inoculation ($F_{2,20} = 0.947$; $p = 0.405$) (Figure 1). For the egg protein, the mean IJ recovery was 32.7% ± 12.63% on day 2, 45% ± 9.53% on day 3, and 57% ± 20.91% on day 4. Recovery between days 2, 3 and 4 showed a significant ($p < 0.05$) increase. The flasks containing soy powder had a lower mean IJ recovery than did the flasks containing the egg powder. The mean recovery for the soy flasks was 13.4% ± 5.62% on day 2, 25% ± 23.96% on day 3, and 28.4% ± 18.47% on day 4. Between the two protein powders, significant differences were present in recovery on day 4 ($p < 0.01$). After 14 days post inoculation, the average yield for the flasks containing culture media with egg protein was 134 800 IJs/ml, with it being 76 417 IJs/ml for the culture flasks containing soy powder ($t_{14} = 4.606$, $p < 0.01$) (Figure 2).

Effect of bacterial inoculum density

The repeated-measures ANOVA showed a significant difference in mean IJ recovery between the different days post inoculation ($F_{6,40} = 3.680$, $p < 0.005$) (Figure 3). However, there were no differences in mean recovery between the bacterial treatment concentrations, except on day 3, when the 5% (v/v) treatment had a significantly lower mean recovery. The mean recovery on day 2 for the treatment groups were, 28.36% ± 6.54% for the 2% (v/v) inoculation, 29.09% ± 4.82% for the 5% (v/v) inoculation, 28.47% ± 6.15% for the 8% (v/v) inoculation, and 24% ± 3.15%

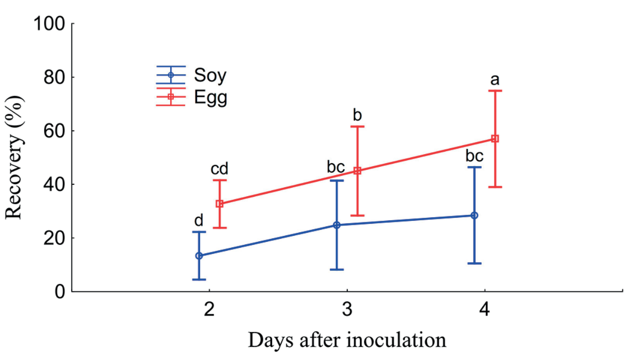

Figure 1: Mean IJ recovery rate (95% confidence interval) of the *Heterorhabditis bacteriophora* (SGI_170) *in vitro* liquid cultured in 30-ml culture media (repeated measures ANOVA: $F_{2,20} = 0.947$; $p = 0.405$). The same letters above the bars indicate no significant differences ($p > 0.05$) between the egg yolk and soy protein powder, and the IJ recovery rate.

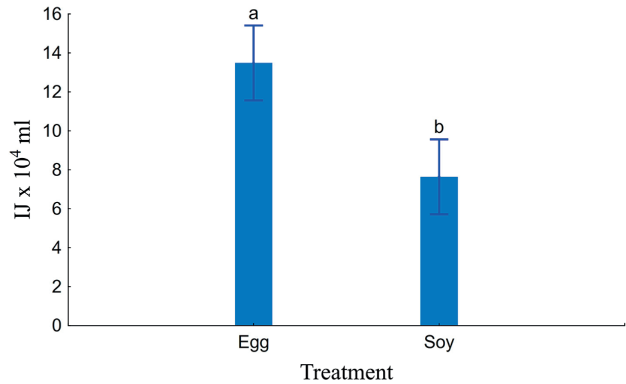

Figure 2: Mean infective juvenile (IJ) yield (95% confidence interval) 14 days post inoculation of the *Heterorhabditis bacteriophora* (SGI_170) *in vitro* liquid cultured in 30-ml culture media (*t*-test: $t_{14} = 4.606$, $p < 0.01$). Different letters above the bars indicate significant differences ($p < 0.05$) between the egg yolk and soy protein powder, and the IJ yield.

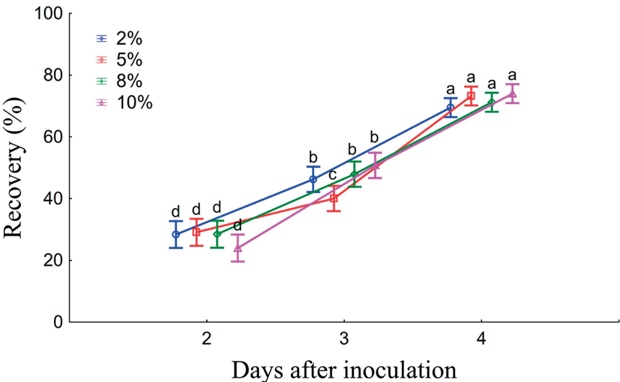

Figure 3: Mean infective juvenile (IJ) recovery rate (95% confidence interval) of the ***Heterorhabditis bacteriophora*** (SGI_170) ***in vitro*** liquid cultured in 30 ml culture media (repeated measures ANOVA: $F_{6,40} = 0.947$; $p = 0.405$). Different letters above the bars indicate significant differences ($p < 0.05$) between the bacterial initial inoculum and the IJ recovery rate.

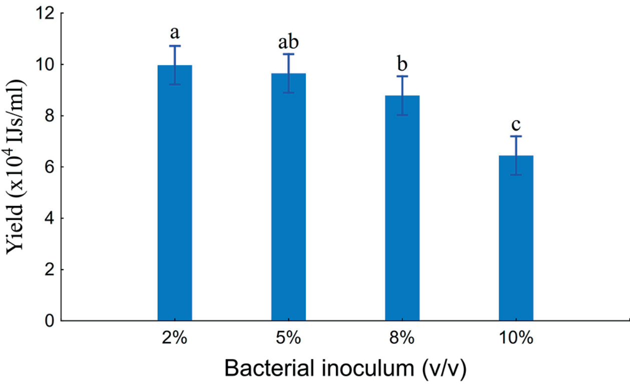

Figure 4: Mean infective juvenile (IJ) yield (95% confidence interval) 14 days post inoculation of the ***Heterorhabditis bacteriophora*** (SGI_170) ***in vitro*** liquid cultured in 30-ml culture media (one-way ANOVA: $F_{3,20} = 19.454$; $p < 0.01$). The same letters above the bars indicate no significant differences ($p > 0.05$) between the bacterial initial inoculum and the yield.

for the 10% (v/v) inoculation. On day 3, the mean recovery was 46.20% ± 4.36% for the 2% (v/v) inoculation, 40.03% ± 4.23% for the 5% (v/v) inoculation, 48% ± 6.49% for the 8% (v/v) inoculation, and 50% ± 4.14% for the 10% (v/v) inoculation. Lastly, on day 4 the mean recovery was 69.5% ± 2% for the 2% (v/v) inoculation, 73.23% ± 3.35% for the 5% (v/v) inoculation, 71.22% ± 4.57% for the 8% (v/v) inoculation, and 74% ± 2.20% for the 10% (v/v) inoculation. After 14 days, a yield of 99 667 IJs/ml was achieved for the 2% (v/v), 96 500 IJs/ml for the 5% (v/v), 87 777 IJs/ml for the 8% (v/v), and 64 445 IJs/ml for the 10% (v/v) bacterial inoculum. A one-way ANOVA showed significant differences between the mean yields of the different treatment concentrations ($F_{3,20} = 19.454$, $p < 0.01$) (Figure 4). There were no significant differences ($p = 0.542$) between the mean yield of the 2% and 5% (v/v) bacterial inoculum. The mean yield of the 2% (v/v) inoculum was higher than the 8% ($p = 0.030$) and 10% ($p < 0.001$) (v/v) inoculum. Between the 5% and 8% (v/v) inoculum, there were no significant ($p = 0.103$) differences, however the 5% inoculum was significantly higher ($p \leq 0.001$) in yield than the 10% inoculum. The 10% (v/v) bacterial inoculum had a significantly ($p \leq 0.001$) lower yield than the other three inoculum treatments.

DISCUSSION

Even though there were no initial differences in the IJ recovery for *H. bacteriophora* over days 2 to 3, a significant difference was indicated in day 4 between the soy and egg yolk powder, with the IJ yield increasing when the protein source was switched from soy powder to egg yolk powder. To achieve high yields of IJs, one can supplement the medium culture with a variety of proteins, or even with a combination of proteins. Soy flour powder is predominantly used in combination with casein and lactalbumin hydrolysate to achieve increased IJ yields (Yoo et al. 2000). Similarly, Cho et al. (2011) also investigated the use of different protein sources to increase the IJ yields in a monoxenic *in vitro* liquid culture. The liquid media containing soybean flour produced the highest yield, 185 200 IJs/ml, 12 days post inoculation. This protein also had the highest bacterial cell density, and the highest recovery compared to the other protein sources investigated (Cho et al. 2011). Dunn and Malan (2023a) similarly showed that *Heterorhabditis zealandica* Poinar preferred a soy-based protein over egg yolk. This preference resulted in increased recovery rates and production of hermaphrodites, with the use of soy. Such a finding underscores the necessity of establishing a dependable protein source, using the preculture flask method before the initiating of mass production.

Bacterial cell density is important for the recovery of the IJ, as the food signal induces the activation of the IJ to feed on the bacterial cells (Cho et al. 2011). However, due to the variability in growth of the nematodes, the presence of a high recovery rate usually indicates a high yield. Protein sources, like casein hydrolysate and lactalbumin hydrolysate, had a recovery of over 60%, but had a lower yield after 12 days, compared to the other protein sources tested, although the yields achieved were still above 100 000 IJs/ml (Cho et al. 2011). Dunn et al. (2021b) demonstrated that the IJ yield is crucial to reducing culture cost, since it leads to the more efficient utilisation of resources in the process than would otherwise be the case.

The bacterial inoculation concentrations showed similar recovery and yields 14 days post inoculation. The 10% concentration was the only treatment with a significantly lower IJ yield than was experienced with the other concentrations. Therefore, it seems that a threshold of optimal inoculation exists when it comes to the bacteria, suggesting that the bacteria might be competing with the nematodes when the bacterial inoculation is too high. In the study done by Cho et al. (2011), the protein that produced the highest cell density was found to improve the IJ yields 14 days post inoculation. Other studies also found a positive correlation between the bacterial cell density/growth and increased IJ yields after production (Jeffke et al., 2000; Han and Ehlers, 2001). Even though a high bacterial density could potentially lead to higher recovery, and increase the yields produced during the *in vitro* liquid culture, the culture medium needs to be optimal for both the nematode and the bacteria. Thus, finding the optimum bacterial inoculum is important. In the present study, inoculating 2% (v/v) of the bacteria into the culture media was found to be the more optimal inoculum. Even though, there was no significant differences between the 2% and 5% inoculum, the 2% inoculum would be the preferred inoculum density, as adding more volume to the flasks could potentially impact on the oxygen transfer occurring in the culture medium, which is already a constraint, due to the high viscosity of the culture media for *H. bacteriophora*. Once the optimum bacterial inoculum has been established for the EPN species, the bacterial cell density can be optimised to improve IJ yields. Such optimisation can be done through investigating the temperatures at which the bacteria can be grown to increase the bacterial cell density. Although the optimal growth temperature for *Photorhabdus* bacteria is 28 °C, some strains can grow at higher temperatures (Boemare 2002). Thus, to increase the cell density of the bacteria concerned, the optimal temperature range should be investigated. The exploration for such an investigation should occur during the growth phase of the bacteria, as this

phase of the bacteria is important for the initial recovery of the IJs (Gaugler and Han 2002; Dunn and Malan 2024). If competition exists for resources in the culture medium, one organism might be negatively affected, which would also be likely to influence the other organisms, as they are in a continuum and are mutually symbiotic. Reduced oxygen concentrations, which negatively impact on the growth of *H. bacteriophora*, are likely to result in reduced yields by the end of the production period.

A South African *H. bacteriophora* isolate has now been successfully mass-cultured, using *in vitro* liquid culture techniques. This is the first time that a local *H. bacteriophora* species associated with an unknown bacterial symbiont (Claasen et al. 2024) has been mass-cultured, using *in vitro* liquid culture techniques, in South Africa. Using egg yolk powder as the main protein source in the CM resulted in yields surpassing 120 000 IJs/ml. A yield of 100 000 IJs is required for mass-culture production to be considered successful. To increase the yields in the shake flasks for upscaling to a benchtop bioreactor, the *in vitro* liquid culture production requires optimisation. Optimisation can occur through changing or improving parameters such as the nutrients, the mechanical aspects, like the oxygen concentration, and the prevention of a second generation after 14 days. Production can also be optimised by means of reducing the costs involved, which can be achieved by means of using more affordable ingredients for the liquid media, without compromising the quality and quantity of the *in vitro* liquid culture-produced nematodes concerned.

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AUTHOR CONTRIBUTION

Nicholle Claasen: writing – original draft, writing – review and editing, formal analysis; Murray Dunn: conceptualisation, writing – review and editing; Antoinette Malan: conceptualisation, writing review and editing, supervision.

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