Mass Production of the Beneficial Nematode, *Heterorhabditis bacteriophora*, in Submerged Culture.

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Introduction

Nematodes have a common life cycle that is comprised of five developmental stages that are separated by a molting process in which a new cuticle is formed and the old one shed. When the stage five adults reproduce, the fertilized eggs hatch into stage 1 larvae (J1s) and under appropriate growth conditions they transition into stage 2 larvae (J2s). When nutrients are available, the J2 larvae normally transition to feeding stage 3 larvae (fJ3s) then onto stage 4 (J4s) and then complete the cycle to the adult stage. However, when conditions are not favorable for growth and development the J2s develop into infective stage 3 juveniles (IJs). During the transition to the infective stage the cuticle of the J2 remains and becomes the sheath. The sheath completely encloses the IJs to prevent desiccation. IJs are infective to insects and complete their life cycle to the adult stage at the expense of the insect host.

IJs of *Heterorhabditis bacteriophora* are very effective for insect control and are easily identifiable. These infective stage nematodes usually live on soil particles that are covered by surface water. This infective stage requires a great deal oxygen and water for survival. However, they do not feed, so they utilize their own stored food reserves for energy. Another aspect of *H. bacteriophora* is the significance of its symbiont, *Photorhabdus luminescens. P. luminescens* is primarily responsible for the nematodes' entomopathogenic properties and antimicrobial agents that fend off other microorganisms while the nematodes develop within the decaying insect carcass.

Abstract

The goal of this work is to mass produce, in liquid media, a submerged culture of the beneficial nematode, *Heterorhabditis bacteriophora*. Initial shake flask cultures were scaled up to 10 L in a Sartorius-stedim Biostat B® Plus fermentation system. The culture conditions that were controlled by the system include pH, pO_2, agitation, and temperature. Microscopic observations and nematode density counts were collected over a four week period to evaluate the growth and development of the nematodes in liquid culture. To evaluate nematode development, the nematode life cycles were used as markers. The system was inoculated with approximately 1,000 infective stage 3 juveniles (IJs) per mL of media and nematode development was observed within three days after inoculation.

Purpose

The main objective of this project is to mass produce *H. bacteriophora* in a liquid suspension that can be used as a biological pesticide to control insect crop damage.

Figure 1 – *Galleria mellonella* infected with *H. bacteriophora*

Methodology

1. The essential phase 1 variant of *P. luminescens* was cultured in 300 mL of nutrient broth and incubated for 2 days at 30°C on a shaker incubator until the relative luminescence units (RLUs) reached $1.3x10^4$.
2. Nematode growth media (NGM) was prepared with 2% egg yolk, 1% yeast extract, 3.5% corn oil and 3% soytone and was autoclaved.
3. The NGM was then added aseptically to the Sartorius-stedim Biostat B® Plus fermentation vessel and added to this the 300 mL culture of phase 1 *P. luminescens* (5% inoculum).
4. The parameters of the fermentation system are set for the culturing of the bacteria. Parameters were set pH=7, stir rate = 60 rpm, pO_2 = 60%, and air flow = 10 L/min and was allowed to run overnight.
5. Over the course of the night the pO_2 dropped, so to correct this problem the stir rate was increased to 400 rpm to maintain the pO_2 at 60%.
6. The bacteria were allowed to grow until the bacterial cell density reached approximately $1.0x10^7$cells per milliliter (RLUs = $2.7x10^4$).
7. *H. bacteriophora* nematodes were added to the vessel on the third day at a concentration of approximately 1,000 nematodes per milliliter.
8. Nematode density counts were performed daily for the first week then every two days thereafter.
9. Representative samples taken from the vessel were diluted 4 fold and 100 µL of the diluted sample was used to determine the nematode density count. To determine the number of nematodes per milliliter the following equation was used.

# of nematodes counted x dilution factor x 10 = # of nematodes/mL

Data Analysis/Results

Nematode growth and development was determined throughout the experiment using microscopic nematode counts that entailed the life stages of the nematodes as markers. Figure 1 depicts a Greater Wax Moth larva (*Galleria mellonella*) that was infested with *H. bacteriophora*. Figures 2 – 7 shows microphotographs taken during nematode density counts of the life stages of the nematodes using an inverted microscope. Chart 1 shows the approximation of the total population growing within the fermentation vessel based upon the life stages of the nematodes. At the beginning of the experiment roughly 800 nematodes per milliliter was used to inoculate the vessel and towards the end of the experiment the nematode density count was approximately 560 nematodes per milliliter.

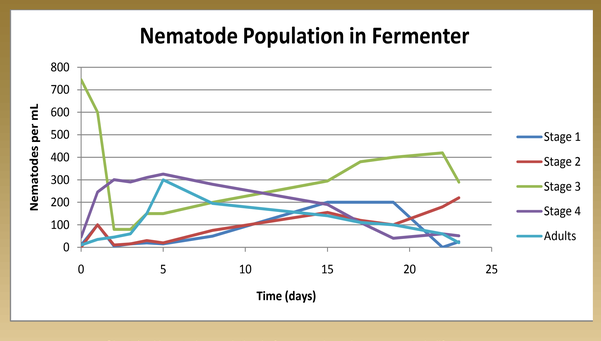

Chart 1 – Nematode population in fermenter based upon nematode life stages.

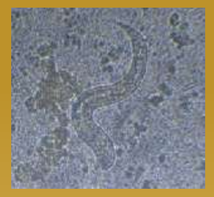

Figure 2 – *H. bacteriophora* Stage 1

Figure 3 – *H. bacteriophora* Stage 2

Figure 4 – *H. bacteriophora* Stage 3 (infective juvenile)

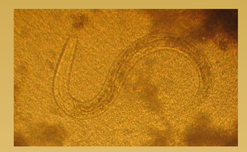

Figure 5 – *H. bacteriophora* Stage 4

Figure 6 – *H. bacteriophora Stage 5* (adult hermaphrodite)

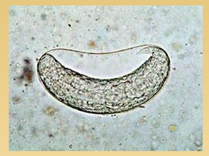

Figure 7 – *H. bacteriophora* egg

Figure 8 – Fermenter after four weeks of culturing

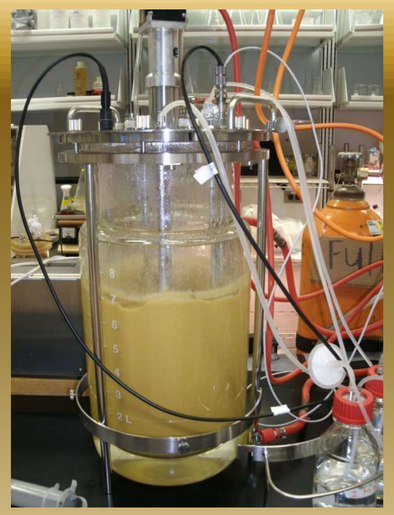

Figure 8 – Fermenter just after inoculation of the bacterial symbiont *P. luminescens*

Discussion/Conclusion

Culturing soil-living nematodes in liquid suspension is very difficult and the demands of growth is overwhelming. When culturing nematodes in a fermentation vessel it is ideal to set important parameters such a pH, pO_2, stir rate, and temperature. Delineating from those set parameters will result in a culture crash. The media components is also vital and of most importance is the use of the phase 1 variant of *P. luminescens*, the bacterial symbiont of *H. bacteriophora*. It has been determined that the media should contain 2% egg yolk, 1% yeast extract, 3.5% corn oil and 3% soytone.

According to the results in Chart 1, growth and development was occurring, but not at an exponential rate. The results suggests that more experimentation is needed to optimize the current media and procedural parameters for enhancing the growth and development of the nematodes.

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