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REVIEW ARTICLE

Mass Production of the Beneficial Nematode *Heterorhabditis bacteriophora* and Its Bacterial Symbiont *Photorhabdus luminescens*

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Abstract Entomoparasitic nematodes (EPNs) are being commercialized as a biocontrol measure for crop insect pests, as they provide advantages over common chemical insecticides. Mass production of these nematodes in liquid media has become a major challenge for commercialization. Producers are not willing to share the trade secrets of mass production and by doing so, have made culturing EPNs extremely difficult to advance existing technologies. Theoretically, mass production in liquid media is an ideal culturing method as it increases cost efficiency and nematode quantity. This paper will review current culturing methodologies and suggest basic culturing parameters for mass production. This review is focused on *Heterorhabditis bacteriophora*; however, this information can be useful for other nematode species.

Keywords Beneficial nematodes · *Photorhabdus luminescens* · *Heterorhabditis bacteriophora* · Mass production

Introduction

The use of beneficial or entomoparasitic nematodes (EPNs) as a biocontrol measure for insect pests is increasing because they provide several advantages: (i) more effective than chemical agents; (ii) persist within soil; (iii) sustainability; and (iv) safe for user and environment [1–5]. The problem of insect resistance to chemicals has created a genuine interest in EPNs and therefore their market viability is increasing. Currently, these "products" are produced by a few companies and the increasing use of integrated pest management is creating pressures for producers to keep up with customer demands. It is the aim of this review to highlight the methodologies utilized in the mass production of *Heterorhabditis bacteriophora*.

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Nematode Products as Biocontrol Agents

EPN producers/retailers offer multiple products depending on application whereas nematodes can be purchased in pack quantities ranging from 5 to 500 million. The producer/distributor ships nematodes cold-packed and recommends that they be stored at 4 °C if they are not used immediately. From our experience, *H. bacteriophora* can remain viable for one month at this temperature (unpublished data). Producers may package EPNs on a variety of solid substrates (clay, vermiculite, alginate gels, diatomaceous earth) [5].

Nematodes are roundworms (Fig. 1) that exhibit different lifestyles that include free-living, predaceous or parasitic. Biodiversity of these animals is extensive; however, EPNs strictly belong to the families Steinernematidae and Heterorhabditidae. Many species of nematodes have been identified from these families; however, only few of them are commercially available [6]. Steinernematidae and Heterorhabditidae nematodes are of interest as they are the only nematodes that can parasitize a broad range of insects; however, they will only persist as insect hosts are available. EPNs have a high degree of safety for plants, livestock and

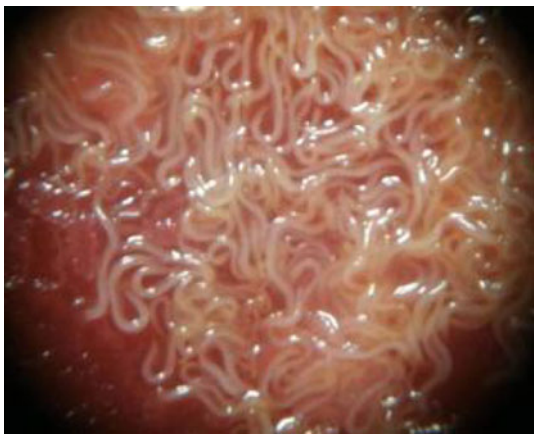

Fig. 1 Nematodes of *H. bacteriophora* growing on a lawn of *Photorhabdus luminescens*

humans making them an attractive environmental choice [7, 8]. The likelihood of the nematode becoming a human pathogen is extremely remote because sudden genetic mutations of either the nematode or bacterium would disrupt symbiosis and neither organism would flourish. Furthermore, no life-threatening risks have been associated with the use of beneficial nematodes.

Research has investigated the utilization of liquid cultures of the bacterial symbionts in order to circumvent the problems with nematode rearing; however, this approach may present safety and/or environmental problems when applied directly to crops [9]. In another approach, the use of cloned toxin genes from bacterial symbionts as an alternative to the *Bt* toxin was discussed. Other approaches included toxin preparations as potential oral insecticides; however, there is concern for non-target organisms [10, 11].

One of the main issues of mass producing EPNs is yield. Batch requirements are problematic for Heterorhabditidae nematodes; however, they can be reared on solid media, but large-scale production is not cost efficient [12–14]. A more efficient approach is to utilize bioreactors containing liquid media [15, 16]. Bioreactors are expensive; however, scale-up is much easier and economical (Fig. 2).

The Symbiotic Relationship

Heterorhabditis bacteriophora nematodes only exist symbiotically with its bacterial symbiont *Photorhabdus luminescens*. *P. luminescens* benefits from this relationship by utilizing *H. bacteriophora* as a vector and ultimately bioconverts the insect into nutrients that can be utilized by both partners. *P. luminescens* also creates an environment for nematode reproduction within the insect [17–19]. Nematodes can enter the insect larvae through many of the

Fig. 2 Culturing *H. bacteriophora* in liquid media: (*Left*) Fermentor prior to *Photorhabdus* inoculation. (*Right*) After 21 days of rearing nematodes

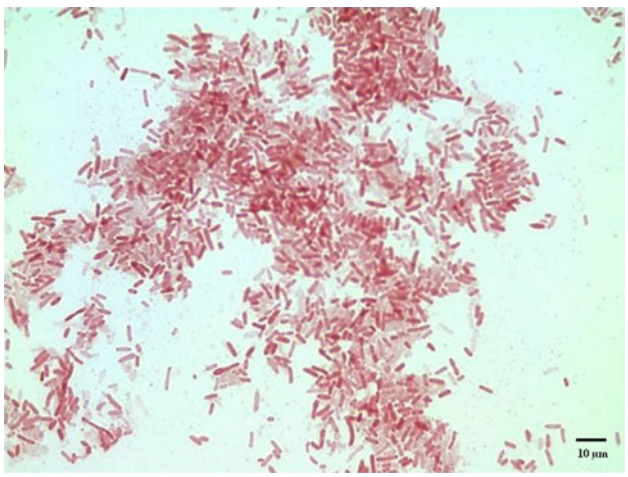

Fig. 3 Gram stain of *P. luminescens* (1,000×)

insect's orifices including cuticle penetration. Upon entry, the bacteria are regurgitated into the insect hemocoel where they proliferate causing insect mortality within 2 days [20, 21].

Photorhabdus Luminescens

Photorhabdus luminescens is a Gram-negative, bioluminescent, pigment producing, phase varying entomopathogen (Fig. 3). This bacterium is not free-living and can only be isolated from its nematode symbiont or infected insects. When viewed under electron microscopy, *P. luminescens* is 5 microns in length and 1 micron in width (Fig. 4) suggesting that *H. bacteriophora* can feed upon them (Fig. 5).

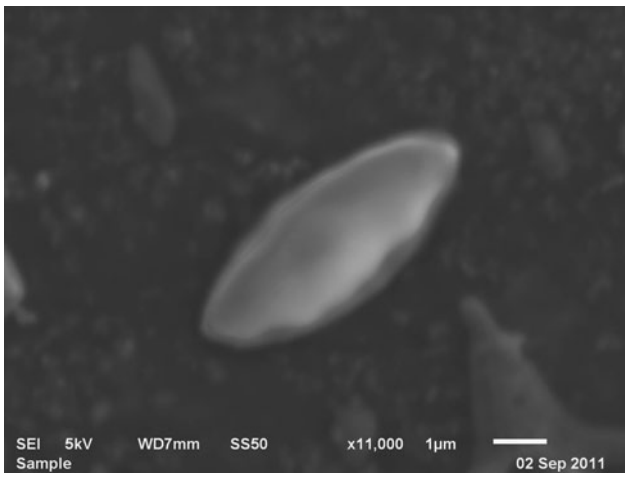

Fig. 4 Scanning electron micrograph of *P. luminescens*

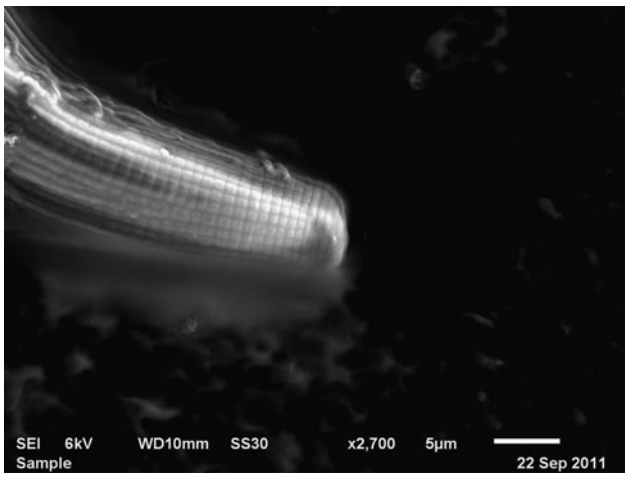

Fig. 5 Scanning electron micrograph of nematode buccal region

P. luminescens is considered to be a model to study symbiosis, host-pathogen interactions and phase variation. Research has suggested that a single infective juvenile nematode may carry ten cells of *P. luminescens*, but only three cells are necessary to cause mortality [22]. Other research shows that *P. luminescens* produces several biologics that are responsible for bioconversion, protection, and nematode transmission [23–27]. Recent studies indicate that insect colonization is due to protective mechanisms against insect macrophages [28]. The *makes caterpillars' floppy* toxin that is produced is essential for insect mortality as it causes the insect to lose body turgor [29]. *P. luminescens* contains a *lux* operon that is similar to that of the genus *Vibrio*; however, the *lux* operon is not associated with quorum sensing [30]. *Photorhabdus* is genetically related to *Xenorhabdus* where they form a unique group of entomopathogenic bacteria; however, luminosity and pigmentation is used to differentiate between the genera [31].

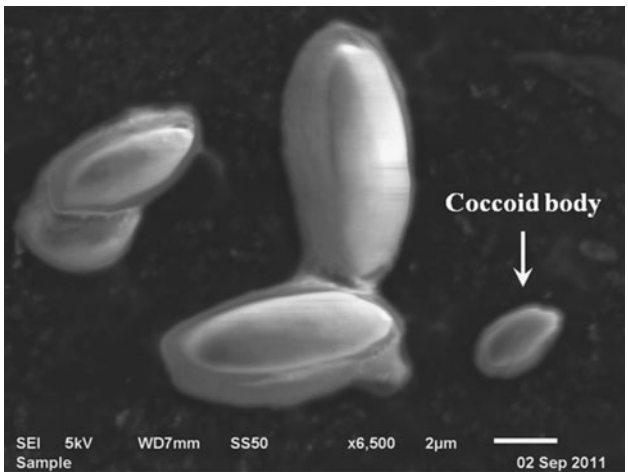

Fig. 6 SEM micrograph depicting phase I cells and the associated coccoid bodies (*arrow*)

Two related species of *P. luminescens* have been identified: *P. temperata and P. asymbiotica*. *P. luminescens* and *P. temperata* are known to be associated with *H. bacteriophora* and other species; however, *P. asymbiotica* is associated with a new species *H. gerrardi* [32, 33]. Research has shown that the ability of *P. luminescens* to support a culture of *Heterorhabditis* spp. is strain and species specific; however, nematode reproduction is influenced by the phase variant [34–36]. Strauch and Ehlers [37] suggested that the bacteria provide a "food signal" that modulates nematode development; however, this signal is yet to be identified [38].

Photorhabdus luminescens undergoes phase variation, meaning that it can shift to a more stable state. This process produces primary and secondary forms (phase I and phase II) which differ morphologically and physiologically [39–41] such as the association of coccoid bodies with phase I cells (Fig. 6). *P. luminescens* adapts by shifting between these two distinct phenotypes [42]. Each phenotype is continuously generated, but the phenotype with the best fitness increases [43–45].

Optimum temperature and pH for culturing *Photorhabdus* is 28 ±2 °C and 7.30 ± 0.01, respectively [46]. There are different media formulations for culturing *Photorhabdus*. Nutrient broth (5 g digested gelatin, 3 g beef extract) supplemented with 2.5 % trehalose (NBT) adjusted to pH 7.3 works well for routine culturing. Trehalose, a multifunctional sugar, is utilized because of its abundance within the insect and has been suggested to stabilize characteristics such as luminosity [47, 48]. Upon sequencing, Duchaud et al. [26] reported that the genome of *P. luminescens* does encode a trehalose operon consisting of a repressor protein (treR), a trehalose permease (treB) and a trehalose 6-phosphate hydrolase (treC). Presently, the metabolism of trehalose and trehalose

6-phosphate in both phase variants has yet to be described. Inman and Holmes [47] demonstrated that *P. luminescens* cultured in 1.5 % trehalose did not form acidic end products when compared to 1.5 % glucose cultures. This suggests that trehalose, at best, is slowly metabolized. Biological phase characteristics (Table 1) are used to differentiate phase I and II cells. Identifying and culturing phase I cells is critical as phase II-shifted bacteria can impact nematode reproduction and yield; therefore phase II cells should be avoided [49].

Table 1 Phase characteristics of *Photorhabdus luminescens* [56]

Characteristic	Phase I	Phase II
Relative luminosity	+++	+
Red pigmentation	Pos	Neg
Yellow pigmentation	Pos	Pos
Protease activity	Pos	Neg
Lipase activity		
Tween 80	Pos	Neg
Tween 40	Pos	Pos
Antimicrobial production	Pos	Neg
Inclusion bodies	Pos	Neg
Colony description on:		
NBTA	Maroon-elevated colonies with feathered edges. Media assumes a blue tint	Transparent-elevated colonies that assumes color of media
Nutrient agar	Red-elevated colonies. Red pigment diffuses	Yellow-elevated colonies. Yellow pigment does not diffuse

+++ Highly luminescent; + minimal luminosity, *Pos* positive; *Neg* negative

Heterorhabditis bacteriophora

In 1975, *H. bacteriophora* was described as a new species, genus, and family in the order Rhabditida and was found that infective juveniles were similar to the dauer stage of *Caenorhabditis elegans*. Also, this "new" species (Fig. 7) is able to transmit a bacterium into the insect [50]. Since then, eleven species of *Heterorhabditis* have been isolated, described, and studied [51, 52]. The life cycle of *H.*

Fig. 7 Scanning electron micrograph of *H. bacteriophora*

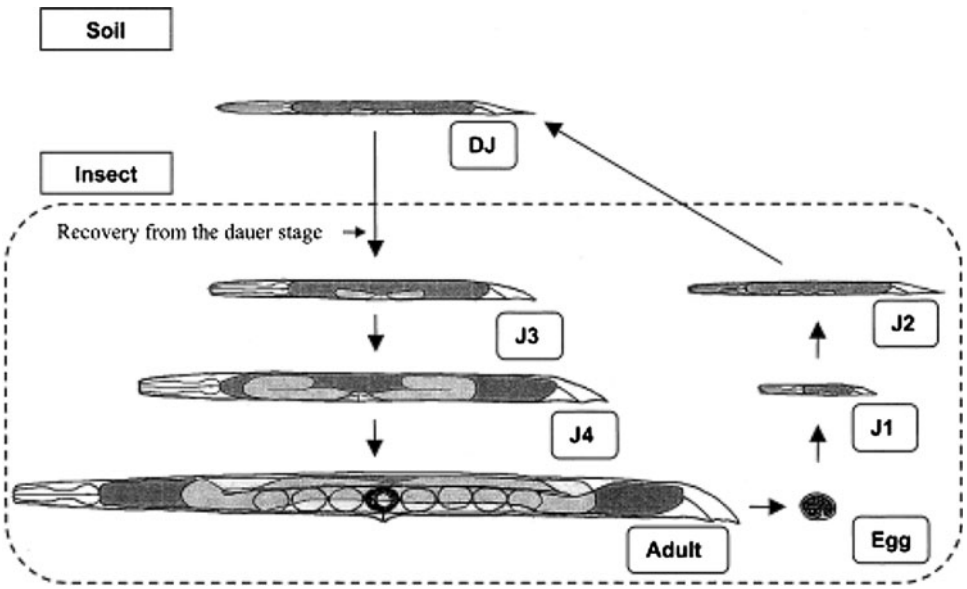

Fig. 8 Life cycle of *H. bacteriophora*. Used with permission [49]

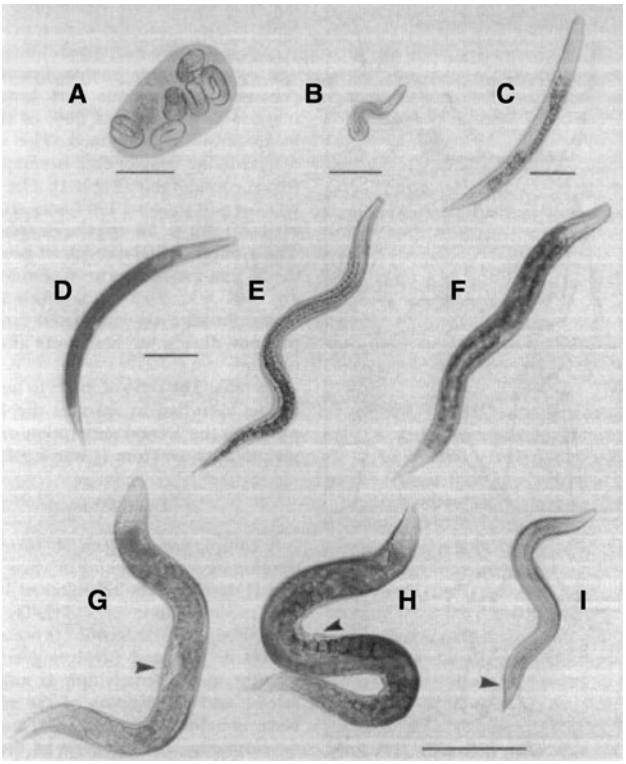

Fig. 9 Developmental stages of *H. bacteriophora*. *A* Eggs; *B* Stage 1; *C* Stage 2; *D* Feeding stage 3; *E* Infective stage 3; *F* Stage 4; *G* Hermaphrodite (*arrow* undeveloped vulva); *H* Mature female (*arrow* protruding vulva); *I* Adult male nematode (*arrow* specula). Scales: *A* = 12 μm; *B* = 25 μm; *C* = 10 μm; *D–F* = 50 μm; and *G–I* = 100 μm. Figure used with permission [53]

bacteriophora is an intriguing process (Fig. 8) that consists of five growth stages (Fig. 9). The infective stage is the only stage that is free-living. Han and Ehlers [49] reported that *H. bacteriophora* develops slowly when phase II bacteria are used because they are not retained by IJs and ultimately do not support growth and reproduction [53].

Mass Production in Liquid Media

There are several ways that cultures of *H. bacteriophora* can be grown: shake flasks, stirred bioreactors [54], airlift bioreactors [55] and in vivo production. Nematode inoculum densities can vary between 300–4,000 nematodes per milliliter. This section focuses on the use of bench-scale stirred reactors and the parameters used: (i) bacterial culture; (ii) media composition; (iii) oxygen availability/agitation; and (iv) temperature/pH.

Bacterial Culture

Photorhabdus luminescens is isolated from the nematode to ensure that the bacterial strain is specific. The simplest way

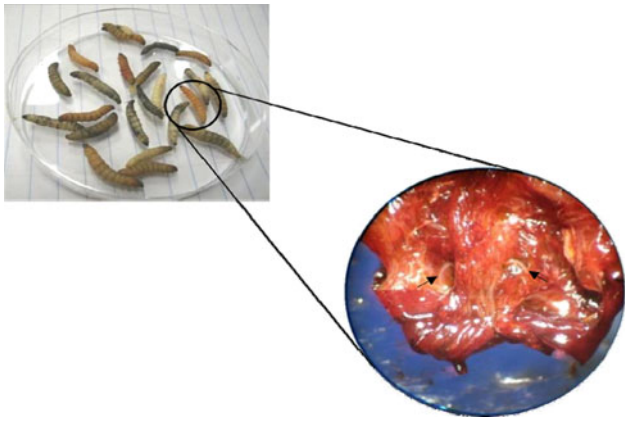

Fig. 10 Dissection of a *G. mellonella red* coloration is due to pigment production. Adult nematodes were seen (*arrows*)

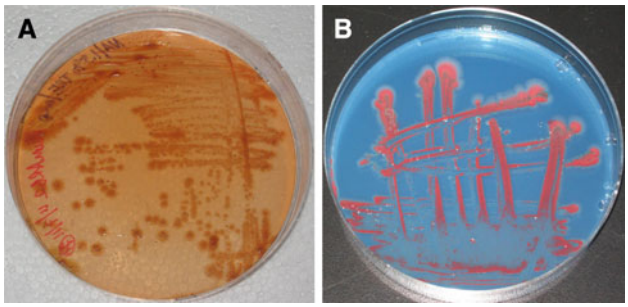

Fig. 11 *P. luminescens* on **a** NAT and **b** NBTA plates

to isolate the bacteria is to inoculate an insect with IJs. After 2 days, red pigmented larvae are placed into a clear microcentrifuge tube and luminosity measured [56]. These cadavers are aseptically dissected (Fig. 10) and the infected hemolymph is streaked onto nutrient agar containing trehalose (NAT).

After three days, presumptive phase I colonies on NAT media are cultured onto NBTA plates for phase confirmation (Fig. 11). Luminosity is measured by suspending a portion of a colony into a milliliter of water in a clear micro-tube. Colonies exhibiting high luminosity and red pigmentation are predominately phase I cells and can be utilized to prepare NBT seed cultures. Seed cultures are then scaled-up for reactor inoculation. Since the nematode culture will be growing for a few weeks in the bioreactor, sterility is critical. Time and effort spent maintaining purity will prevent many processing problems. Since *P. luminescens* is naturally resistant to ampicillin, the addition of ampicillin reduces potential contamination [57]. Performing Gram stains, measuring luminosity and monitoring pH are convenient ways to monitor culture health.

Trehalose (Fig. 12), a multifunctional sugar, is abundant in many insects and fungi. Trehalose is also found in *C. elegans*; however, the role of trehalose is not yet understood [58]. Trehalose serves many biological

purposes as it is known to: (i) stabilize cellular constituents during environmental stresses such as high osmolarity and (ii) play a role in bacterial carbohydrate metabolism [59–63].

Culture Media

There has been a wide-range of media employed for co-culturing *P. luminescens* and *H. bacteriophora* (Table 2), however, NBT is a good formulation for the bacteria. Cultures of *P. luminescens* grown in NBT will produce red pigmentation and strong bioluminescence. We also found that pigmentation varies with media composition (Figs. 13, 14) and pH [47]. The phase I inoculation concentration should be ~2.5 % of the growth media volume and tested for luminosity and contamination prior to fermentor

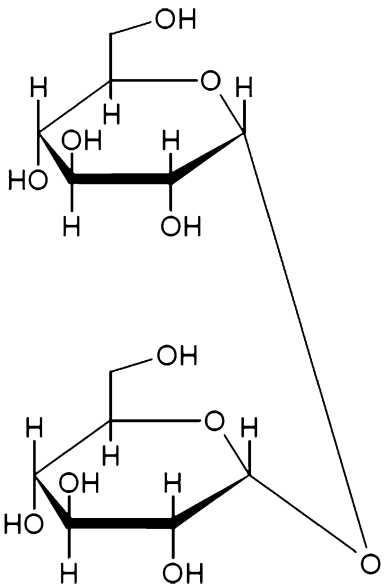

Fig. 12 Structure of trehalose

Fig. 13 Media pigmentation *left to right*: Fluka NB *gray*; Carolina NB *orange*; Carolina NB + glucose *yellow*; Carolina NB + lipid *brick red*. (Color figure online)

Fig. 14 An upscaled culture of *P. luminescens*

Table 2 Liquid media formulations for mass production

Medium	Scale	Features	Reference
Complex liquid media	Bench to pilot	Chicken egg	[69]
Swine kidney/bovine fat	0.5 L	Polyether/urethane sponges	[35]
Lipid culture media	50 mL	Increasing lipids	[66]
Chicken offal	10 L	Paddle-stirred	[54]
Nutrient roux	0.25 L	Polyurethane foam; soy flour	[12]
Modified wouts' 1	20 L	Air lift	[69]
Modified wouts' 2	20 and 500 L	Milk powder	[69]
Liquid culture medium	5 L	Increased aeration rates	[37]
Liquid nematode media	10 L	Variable agitation rates; egg yolks	[16]
Nematode growth media	0.5–2 L	Cholesterol, liver extract,	[12]
Liquid chicken extract	2 and 10 L, predicted 30 L	Culture stabilizer; biological buffer	(unpublished data)

upscale. After inoculation, *P. luminescens* is grown for 24 h at 28 °C, with an air flow rate of 1 vvm and agitation of 100 rpm [64].

The medium for the co-culture is much more complex than bacterial media alone [65]. Published formulations contain varying concentrations of the following: (i) peptone; (ii) yeast extract; (iii) beef extract; (iv) lipid; (v) carbohydrate; (vi) cholesterol; and (vii) salts. These media are rich in proteins and lipids because *P. luminescens* secretes lipases and proteases to degrade these molecules into nutrition for both the nematode and itself. The proteins and lipids found within the media are required due to the longevity of the production process [66]. It is important that growth conditions must satisfy both *H. bacteriophora* and *P. luminescens* because as IJs develop, they will require *P. luminescens* for nutrition. *P. luminescens* is the diet for all nematode stages except the IJs, which do not feed, but require *P. luminescens* for the food/development signal [20]. If the bacteria are maintained in the phase I state, the nematodes will develop and produce progeny resulting in maximum yields.

Oxygen Availability and Agitation

Oxygen is poorly soluble in water; therefore, oxygen transfer to the media is critical. Gas to liquid transfer is influenced by the viscosity and volume of the medium, stirrer design/speed and flow rates [67]. The fragile nature of adult nematodes requires low impeller velocities to prevent disruption; therefore, a compromise between agitation and oxygen flow must be reached. Oxygenation is crucial because bacterial demands will compete with those of the nematodes; therefore, an oxygen flow of 1 volume of gas to 1 volume of media per min (vvm) is recommended to prevent the build-up of CO_2 [67]. When culturing EPNs, the dissolved oxygen should be high, approximately 40 %, for juveniles and approaching 60 % for adults and the agitation should not be greater than 60 rpm [37].

Temperature and pH

Nematodes and bacteria grow best at 28 °C ± 2 °C [46]. Other temperatures may not produce satisfactory results due to the phase sensitivity of the bacteria (unpublished data). We recommend that the media pH be buffered around 7.30 because it has been observed that the pH increases to 8.0 after 48 h when a buffer is not used [47].

Discussion

Nematode reproduction in vivo is stimulated by the environment inside the insect. Culturing in vitro requires media conditioning with the bacterial symbiont. Others have shown that best production occurs when nematodes are inoculated during late logarithmic growth of phase I cells [46]. After 7 days in culture, juvenile populations should begin to recover (unpublished data). This first generation feeds upon *P. luminescens* and develop into hermaphrodites. The self-fertilized eggs of the hermaphroditic nematode will hatch internally to give rise to offspring that will feed upon and develop within the maternal nematode (termed *endotokia matricida*). It is through this process that 80 % of the hermaphroditic nematodes will bear live young after 12 days post-inoculation [68]. These nematodes will emerge from the hermaphroditic nematode in the J3 stage and the life cycle continues. It is clear that the hermaphrodite transmits the symbiotic bacteria required by the infective juveniles. Furthermore, eggs that are produced by sexual reproduction are laid outside of the adults and will hatch within the media; however, in liquid culture adults may or may not be able to reproduce sexually based upon culturing parameters mainly agitation [38]. Development of infective juveniles (IJs) is induced by nutrient depletion of the maternal nematode, insect or media.

Mass producing beneficial nematodes in liquid culture is a challenging task; however, understanding both the nematode and its bacterial symbiont in liquid culture increases the chance of success. For starters, understanding the symbiotic relationship provides information about how they will respond when cultured together. Another daunting aspect of mass production technologies is the role of phase variation in the bacterial symbiont. The phase I variant is crucial to the life cycle of the nematode because the bacteria have the ability to (1) produce toxins and enzymes that kill and bioconvert the insect into nutritional components for both symbionts, (2) produce and excrete antimicrobials to ward off contaminating microbes, (3) produce bioluminescence; however, its function is yet to be determined, (4) secrete "food signals" for nematode recovery, and (5) serve as the main food source of the nematodes. All of the above mentioned processes must occur to create the optimal environment for nematode reproduction.

To mass produce beneficial nematodes in liquid culture, a liquid media formulation must be created that can be utilized by the variant bacteria to produce the necessary reproductive environment for the nematodes. To date, most liquid media consists of high concentrations of proteins and lipids. The addition of a biological buffer and a culture stabilizer may benefit the culturing process. The pH of the media being utilized should be tolerable by both symbionts (pH 7.0–7.3). Another process parameter, dissolved oxygen (DO) is also important. The nematode oxygen demand is dependent on the current nematode growth stage. It is recommended that the DO should be 40 % when the

prevalence of juveniles are seen and up to 60 % when adults are in abundance. Agitation, another important parameter in fermentation, should be avoided due to its negative impact on gravid adult nematodes; however, if agitation is desired the speed should not exceed 60 rpm. Published yields of IJs vary considerably with average counts of around 20,000 to 40,000 per mL. Based upon our current media formulation and process parameters we can achieve IJ counts of 50,000 per mL (unpublished data) which exceeds the data range obtained by other researchers. Therefore, consistency in nematode yields continues to be a present-day challenge.

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