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Lab-scale *in vitro* Mass Production of the Entomopathogenic Nematode *Heterorhabditis bacteriophora* Using Liquid Culture Fermentation Technology

Devang Upadhyay[1, *], Sivanadane Mandjiny[2], Rebecca Bullard-Dillard[3], Meredith Storms[4], Michael Menefee[5], Leonard D. Holmes[1]

[1]Sartorius Stedim Biotechnology Laboratory, Biotechnology Research and Training Center, the University of North Carolina at Pembroke, Pembroke, USA
[2]Department of Chemistry and Physics, the University of North Carolina at Pembroke, Pembroke, USA
[3]School of Graduate Studies and Research, the University of North Carolina at Pembroke, Pembroke, USA
[4]College of Arts & Sciences, the University of North Carolina at Pembroke, Pembroke, USA
[5]Thomas Family Center for Entrepreneurship, the University of North Carolina at Pembroke, Pembroke, USA

Email address:
danny.uncp@gmail.com (D. Upadhyay)

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Abstract: The objective of this research is to develop fermentation methodology for the production of the biocontrol agent *Heterorhabditis bacteriophora*. Deployment of this organism will reduce the use of chemical insecticides which threaten the environment. This study shows how to produce the entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora* and its bacterial symbiont *Photorhabdus luminescens* utilizing an *in vitro*, monoxenic liquid culture. EPNs were cultured in three different bioreactor working volumes of 1.5, 4 and 7 liters with initial nematode inoculation concentrations of approximately $2x10^3$/mL. Liquid nematode media was conditioned with the bacterial symbiont 24 hours prior to nematode inoculation. Within three days after inoculation, infective juveniles (IJs) developed into self-fertilizing hermaphrodites and eventually produced IJ offspring. Maximum nematode densities were obtained seven days post-nematode inoculation. All three working volumes (1.5, 4 and 7 liters) produced final yields of $4.6x10^4 \pm 2000$ IJs/mL, $4.2x10^4 \pm 2200$ IJs/mL and $3.9x10^4 \pm 2000$ IJs/mL, respectively. *In vitro* scale-up technology can be further optimized for production of this biocontrol agent by improving media formulation, process parameters, bioreactor design and inoculation times that will maximize nematode yield.

Keywords: *Heterorhabditis bacteriophora, Photorhabdus luminescens*, Entomopathogenic Nematode, Fermentation Technology, Biocontrol Agent

1. Introduction

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* have long been recognized as effective biocontrol agents [1, 2]. These microscopic round worms may be used to control a wide variety of economically important agricultural pests and act as potent bioinsecticides in an effectual, environmentally suitable approach. Their potency relies upon a symbiotic partnership between a fatal insect pathogen bacterium and the host-seeking nematode [3]. The bacterium *Photorhabdus luminescens* is symbiotically associated with *Heterorhabditis bacteriophora* [4]. Upon penetration of these EPN into a host insect body via the insect's mouth, anus, spiracles, or integument, they release their symbiont into insect hemolymph where the bacteria are proliferated [5, 6]. Developmentally arrested infective juveniles then recover to develop through their growth cycle. The bacteria produce toxins and antimicrobial compounds which kill the insect host within 48 h while creating an appropriate environment for nematode development and reproduction. The nematodes feed upon the proliferating bacteria and the decomposing

insect cadaver. There, these EPN develop, mate, and produce eggs. Subsequently a new generation of IJs is produced, which upon depletion of nutrients, exit the host to search for new insect targets [2, 7].

EPNs have been monoxenically cultured *in vitro* and consequently scaled-up to commercial production levels [8]. Because of non-competitive costs compared to chemical insecticides and production inconsistencis, EPN commercialization has not been fully achieved [7, 9]. Evolution of liquid culture technology may be crucial as a solution for the achievement of high yield, short fermentation cycle and high quality product [7].

Present production of entomopathogenic nematodes is sufficient for laboratory use and small-scale field application. But for large scale, it is necessary to develop technology for providing high quality and quantity product at reasonable costs [1, 10].

In vitro liquid culture technology of these nematodes has been studied by a number of research and commercial organizations [2, 11]. The establishment of artificial media for liquid culture technology is a foremost step toward commercial production of these EPNs [12].

EPN mass production with *in vitro* liquid culture technology has challenges. Importantly, adequate dissolved oxygen must be provided with minimum agitation to prevent shearing of nematodes. Shearing has been addressed in several ways: (1) using downward sparging and restricted agitation and (2) by airlift reactor or (3) using baffles positioned inside the bioreactor [13]. Special protocols on the bioreactor operation can enhance the possibility of sexual reproduction [14]. However, in the case of *Heterorhabditis bacteriophora,* sexual mating is no concern because the first generation is hermaphroditic. Subsequent generations have amphimictic forms, which cannot easily mate in liquid culture [15, 16]. As a result, mass production yield of *Heterorhabditis* spp. using liquid culture process rests on the probability of nematode life cycle recovery. The exit from the developmentally arrested third juvenile stage (IJ3) is called "recovery." 100% recovery of *Heterorhabditis* has been reported using *in vivo* methods, whereas up to 85% recovery in liquid culture process has been achieved [5, 17]. Nutritional factors such as lipid content and environmental factors (aeration and temperature) effect recovery efficiency in a bioreactor. Low and unsynchronized IJ recovery, media composition, inoculum concentration and species of nematodes are significant factors governing the final yield of nematode in liquid culture processes [5, 13, 18]. Consequently, process time may be as long as three weeks, although several species can reach maximum IJ production within two weeks or less [13].

In the present study authors present mass production technology to produce the EPN *Heterorhabditis bacteriophora* at lab-scale by *in vitro* liquid culture fermentation. Bioreactors of working volumes of 1.5, 4 and 7 liters were used. In this paper, a successful scale-up methodology has been reported for the mass production of *Heterorhabditis bacteriophora* within a short processing period of 7 days at all volumes.

2. Materials and Methods

2.1. Insects and Nematode

The last instars of the Lepidopteron insect *Galleria mellonella* obtained from Carolina Biological Supply Company (Burlington, NC USA) and the nematode *Heterorhabditis bacteriophora* obtained from Gardening Zone (Camearillo, CA USA) were used throughout this study.

2.2. Bacterial Isolation, Culture and Growth Condition

In vivo culture of symbiont *Photorhabdus luminescence* was developed by the method of Inman and Holmes (2012b) [19]. Sanitized *Heterorhabditis bacteriophora* IJs (400-500 IJs/ ml) were dropped with a sterile wiper on a petri dish containing 5 to 10 *G. mellonella* and incubated at 28°C for 48-72 hours in the dark. Then, infected dead *G. mellonella* were surface sanitized by submerging them in 70% ethanol for few seconds and the cadavers of dead larvae were aseptically dissected and a loop full of haemolymph was streaked on nutrient agar (NA) (g/L: 5 peptone, 3 beef extract or yeast extract, 5 NaCl and 15 agar) and diagnostic agar medium NBTA (g/L: 8 nutrient agar; 0.025 bromothymol blue; 0.040 2, 3, 5 triphenyl-tetrazoliumchloride) and incubated at 28°C [19, 20]. Phase I cells of *Photorhabdus luminescence* confirmed using NBTA media and bioluminescence were selected and sub-cultured onto nutrient agar until pure colonies of uniform size and morphology were obtained. Phase I absorbs bromothymol blue and produced dark blue colonies by reducing TTC (Triphenyl Tetrazolium chloride), whereas cells of phase II do not absorb bromothymol blue, but reduce TTC and produce red colonies [21, 22]. A single colony was transferred to 2x NB broth (in g/l: 5 peptone, 3 beef extract or yeast extract, 5 NaCl) and incubated at 28°C at 150 rpm on orbital shaker (Certomat IS, Sartorius, Germany) for 24 hours.

2.3. Preparation of Production Media "Chicken Media"

Table 1. *Composition of Production Media "Chicken Media".*

Chicken legs	4 legs/ 1000 mL
Trehalose	1% (10 gm/1000mL)
boiled Chicken Tissues	1% (10 gm/1000mL)
K_2HPO_4	25 mMol (4.35 gm/1000mL)
pH	7.5

For the culturing of the nematodes, production media "Chicken Media" was prepared according to Table. 1. Four chicken legs per 1000 mL water were boiled for two hours producing chicken broth which was subsequently separated from the boiled chicken legs using a metal kitchen strainer. Then, 1% of boiled chicken tissue was added to the chicken broth and homogenized using with a blender. Trehalose (1%) and 25mMol K_2HPO_4 were mixed to homogenized chicken broth and final volume was adjusted according to the respective bioreactor's working volume. Finally, the media

was adjusted to pH 7.5 (before autoclave) and autoclaved in the bioreactor at 121°C for 15 minutes.

2.4. Nematode Mass Production

For monoxenic liquid cultures of the bacto-helminthic complex, the production media (basic nutrients; chicken tissue; lipid; at physiological pH) was pre-inoculated with 5% culture of 24 hour *P. luminescens* grown at 28°C and 150 rpm into 2x NB in Sartorius Stedim Biostat bioreactors A + (2L & 5L volume capacity) and B + (10L volume capacity) with working volume 1.5L, 4L and 7L respectively. The growth parameters in the reactor were set as follows: temperature, 28°C; agitation, 200 rpm; pH, 7.30 and dissolved oxygen, 30%. Meanwhile, the nematodes were surface sanitized using 0.125% Hyamine and subsequently washed with sterile tap water to remove all Hyamine. When the bacterial growth reached early stationary phase (20-24hr), bioreactors were inoculated with above sanitized nematodes at densities approximately 2000 IJs/mL and cultivated at 28°C on 7.3 pH. The agitation was maintained constant at 200 rpm in each bioreactor during initial bacteria growth, reduced to 100 rpm, 150 rpm and 200 rpm in bioreactors A + and B + respectively. After nematode inoculation, dissolved oxygen was maintained at 30% during bacterial culture and increased to 40% for nematode mass production. The bioreactors operated in batch mode for 7-8 days.

2.5. Determination of Nematode Yield

Culture samples were collected from the bioreactors periodically to determine nematode development and density using samples diluted to 10 to 1000 times with sterile tap water. Samples of required dilution in triplicate were used for counting the total number of the nematodes in each bioreactor using a compound microscope at 4x or 10x magnification. The averages of counted numbers of nematodes were multiplied by respective dilution factors.

2.6. Harvesting of Nematodes

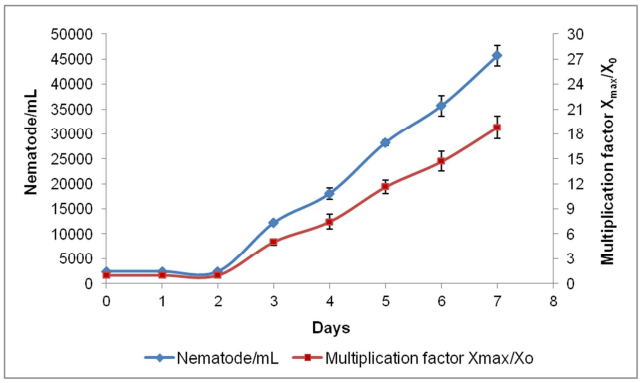
On day 7 or 8, high concentrations of IJs were observed in the production media. The media was collected and centrifuged at 2500 rpm for 3 minutes to isolate nematodes in pellet form. The supernatant was discarded and the pellet was washed with sterile water to remove bacteria from the nematode culture which was subsequently centrifuged at 500 rpm for 5 minutes to separate nematodes from bacteria. This washing step was repeated 3-4 times. The nematodes were collected as pellets and supernatant containing bacteria was discarded to obtain the final product IJs. The IJs were intended for packaging and storage or utilized for inoculation in a subsequent batch process.

2.7. Statistical Analysis

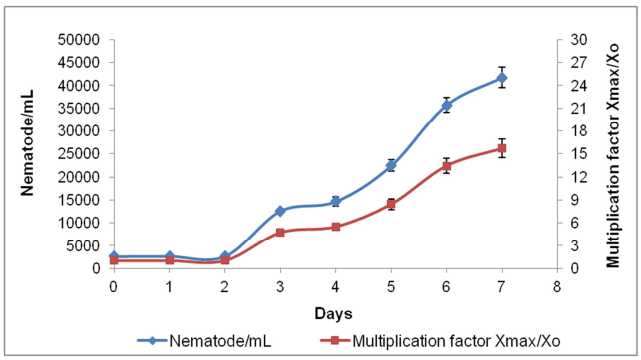
The nematode number from all the three reactors on the day of harvesting was compared on the basis of ANOVA test in excel program of Microsoft office.

3. Results and Discussion

Fig 1 A, B and C provide results of fermentation studies of *Heterorhabditis bacteriophora* mass production at three scales: A+ (2L & 5L total volume capacity) and B+ (10L total volume capacity).



(A)



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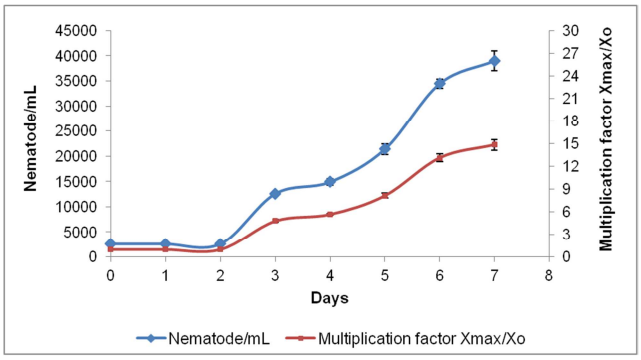

(C)

Figure 1. *Comparison of nematode mass produced in (A) Bioreactor A+ (2L Volume capacity) (B) Bioreactor A+ (5L Volume capacity) and (C) bioreactor B+.*

A lag phase of about 2 days was followed by nematode recovery and development. The culture condition of bioreactor A+ (1.5L working volume) is given in fig. 1A. The inoculum density was 2400±150 IJs/mL. Hermaphrodites were observed at the end of the day 2 which subsequently developed to adults during day 3. On day 4, the first juvenile stages of the F1 generation and eggs were observed. Adults which had not laid eggs into the production media

commensed intra-uterine birth causing maternal death (*endotokia matricida*), where all eggs developed into IJs within the maternal female adult body. Approximately $2.8x10^4$ ± 550 nematodes/mL of F1 generation and adults with *endotokia matricida* were counted on day 5. Upon depletion of nutrients within the female adult body, IJs were released from *endotokia matricida* into the production media on days 6 and day 7. Maximum density of $4.6x10^4$ ± 2000 IJs/mL was reached at day 7.

The other bioreactors' A+ (4L working volume) and B+ (7L working volume) culture conditions are presented by figures B and C respectively. Both A+ and B+ inoculum was approximately 2600 IJs/mL. The development and growth patterns of nematodes in both bioreactors was similar to the A+ (1.5 working volume) bioreactor. The nematodes recovered within 2 days. At day 3, recovered IJs rapidly developed into egg producing adults. On day 5, nematode densities were 22400±1200 nematodes/mL and 21400±1000 nematodes/mL in bioreactors A+ and B+ respectively including the female adults with *endotokia matricida*. The maximum nematode density was seen in both bioreactors after 7 days post-nematode inoculation, similar to A+ (1.5 working volume). The final nematode density in the 5A+ and B+ batches were $4.2x10^4$ ± 2200 IJs/mL and $3.9x10^4$ ± 2000 IJs/mL respectively. Ehlers et al. (1998) reported the mass production of *H. megidis* with their bacterial partner *P. luminescens* using a 10L internal loop bioreactor and produced maximum number of IJs ($4.1x10^4$/mL) on day 20 of processing [23]. Compared to the cited study a slightly higher yield was obtained in the A+ (1.5L working volume) and an almost similar yield in other two bioreactors on day 7 of incubation in the present study.

The mass production strategy would be more efficient for commercialization by shortening the recovery period of IJs [2]. Recovery is crucial to achieve an economically feasible nematode mass production process [5]. Surrry and Davies (1996) reported 7 and 14 days long lag phase for *H. bacteriophora* mass production at pilot scale using 20L and 500 L bubble column fermenters respectively [2]. In this study, IJs reached the adult reproductive stage by day 3. Nematodes were harvested after one generation to avoid unsynchronized growth.

Bioreactor A+ (1.5L working volume) produced a nematode yield with multiplication factor $X_{max}/X_0 \approx 19$ where X_{max} = 46000, X_0 = 2400 IJs/mL. A+ (4L working volume) and B+ produced a nematode yield with multiplication factors approximately 16 and 15 respectively. The number of nematodes obtained from the reactors with smaller capacity (A+ and 5A+) was found to be higher. Statistically significant (p=0.02) difference was only found in case of A+ and B+. Increasing agitation speed had a negative effect on yield. The nematode yield of each bioreactor is also compared on the basis of the agitation rate (Fig. 2). As the agitation speed increased (from 100 rpm, 150 rpm and 200 rpm in bioreactor A+, 5A+ and B+, respectively) the final yield were found to decrease. The agitation speed was varied between bioreactors with a purpose of gaining enough dissolved oxygen and to provide optimal conditions for nematode reproduction and suppressing of shear stress. Friedman et al (1992) discovered that adults were more sensitive to shear stress compared to young juveniles and thus nematode development and final yield would diminish at high agitation speed [24].

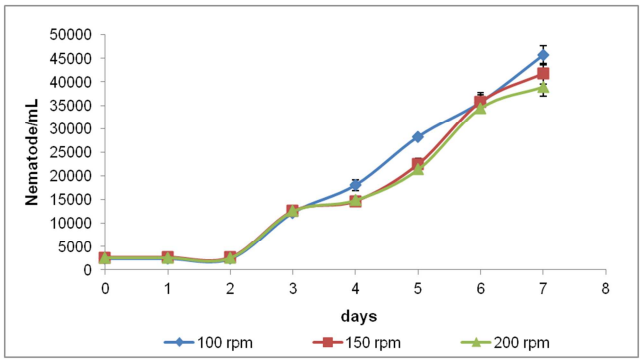

Figure 2. *Effect of agitation rate on mass production of nematodes.*

4. Conclusion

An efficient recovery phase of Infective Juveniles is important for mass production of beneficial nematodes such as *Heterorhabditis bacteriophora*. In the present study the liquid media was formulated and preconditioned to shorten the nematode recovery time. To the best of our knowledge harvesting of Infective Juveniles on 7th day is a first report in the entomopathogenic nematode mass production research in liquid suspension bioreactors. Additional research will achieve higher yield. Media optimization, nematode inoculum concentration and bioreactors parameters will increase efficiency.

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