Mass Production of the Beneficial Nematode *Steinernema carpocapsae* Using Solid State Fermentation

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***Abstract*—*Steinernema carpocapsae* is a microscopic entomopathogenic nematode (EPN) that may be used as an alternative to chemical pesticide. This species creates a symbiotic relationship with the bacteria *Xenorhabdus nematophila*. This biological control agent has many advantages compared to chemical pesticides as it does not harm either the environment or humans. *Steinernema carpocapsae* is a vector for the bacteria to infect the targeted insect pest. The bacteria kills the host within 24-48 hours. This paper focuses on the mass production of beneficial nematodes using solid state fermentation. The purpose of the experiment was to find the optimum conditions to mass produce the nematode efficiently. Maximizing yield with the minimalized nutrients will increase the cost efficiency of production, making it a more affordable attractive alternative to harmful chemical pesticides.**

Index Terms*—beneficial nematode, solid state fermentation, *Steinernema carpocapsae*, *Xenorhabdus nematophila

I. Introduction

Steinernema carpocapsae are nematodes that evolved a symbiotic relationship with the bacteria, *Xenorhabdus nematophila*, which belongs to the family Enterobacteriaceae. The bacteria live inside the nematode, which serves as a vector for the bacteria into the insect host. In return, the bacteria kill the insect by secreting protein toxin(s) into the hemolymph and bioconverting the insect into nutritional components for both the nematode and the bacteria [1]. The secreted antimicrobials are speculated to be a defense mechanism used to ward off competing microbes [2]. *S. carpocapsae* has become a center of attention for bio-ag researchers because it is safe and can kill harmful insects within 24-48 hours [3]. *Steinernema carpocapsae* "is a natural and effective alternative to chemical pesticides, and have no detrimental effect on non-target species" [4]. *Steinernema carpocapsae* is known to attack its host by "ambushing" migrating insects [5], [6]. The diversity of insects affected by the biological control agent is narrow compared to the diversity of those insects controlled by chemical pesticides. The symbiotic pair have low heat tolerance and cannot survive the internal body heat of humans [7].

The bacteria life cycle has two phenotypes, Phase I and Phase II. Phase I bacteria are infective, whereas Phase II are non-infective cells [8]. Reports demonstrate that stressful conditions increase the production of stable Phase II cultures. Mechanisms which cause phase variation are yet to be identified [9]-[12]. Unlike culturing nematodes in suspension where pH control is possible, control of pH on solid state media after inoculation is not possible. Studies of pH effects on nematode culture will be important as this technology continues to advance.

The purpose of this research was to mass produce the nematode using a solid state fermentation process. Media composition was modified to determine optimal growth conditions. The media formulation contained nutrient broth, beef extract, yeast extract, peptone, agar, olive oil, and canola oil. Oil was necessary as demonstrated in earlier studies [13]. The concentration of the media was modified to 0.5x, 1x, 1.5x, and 2x. The nematode inoculum concentrations were also varied. Reducing the production cost of the beneficial nematode is as important as having a high nematode final yield. Cheaper mass production of the beneficial nematode will facilitate their use as a replacement of agricultural chemical pesticides.

II. Materials and Methods

A. Isolation of *Xenorhabdus nematophila*

Galleria mellonella insect larvae were used to isolate the nematode symbiont *X. nematophila* [14]. Infected larva were dead and turned dark brown color after 24 hours. The color change verified that the biocontrol agent killed the larvae. Bacterial isolation was performed by the method of Inman and Holmes [15]. Deceased insect larvae were sterilized by plunging them four times into isopropanol, followed by rinsing with sterilized water. Air-dried, sanitized larva were dissected to isolate *X. nematophila* from the hemolymph [13]. The hemolymph was transferred onto nutrient agar bromothymol blue tetrazolium chloride agar (NBTA) plates to differentiate Phase I from Phase II cells. Blue colonies indicate Phase I cells [15]. NBTA contained per liter: 8.0 g nutrient agar;

Manuscript received May 21, 2018; revised September 7, 2018.

doi: 10.18178/joaat.5.4.276-280

25 mg bromothymol blue; 40 mg 2,3,5-triphenyltetrazolium chloride (TTC). Blue colonies were sub-cultured and streaked onto a 2xNutrient Broth (Carolina Biological, USA) media agar plate and incubated at 28℃. The bacteria were periodically transferred onto fresh 2xNB plates throughout the study to preserve the culture.

B. Sanitization of Steinernema carpocapsae

Steinernema carpocapsae nematodes were obtained from Arbico Organics (Tucson, AZ. USA) and used throughout this study. The nematodes were sanitized as follows: Nematodes were gently shaken overnight in sterile tap water at 150 rpm and room temperature. To sanitize the nematodes, 0.125% Hyamine® (Sigma) was added and incubated for 20 minutes on shaker [16]. The sanitized nematodes were transferred into 50 mL sterile tubes and centrifuged for 5 minutes at 500 rpm and the pellet was collected. The process was repeated 10-12 times to remove dead bacteria from the supernatant. A sample was collected every 3 cycles and gram stained to check the presence of bacterial load.

C. Preparation of Solid Media

All glassware and equipment were washed thoroughly using de-ionized water and autoclaved to remove contamination. Fig. 1 shows the small, medium, and large glass plates used during this experiment. The surface areas for small, medium, and large glass plates were approximately 400 cm2, 500 cm2 and 700 cm2 respectively, and fermentation media volumes were 400 mL, 500 mL and 700 mL respectively. The depth of solid media bed was maintained at 1 cm in all glass plates. The fermentation media was composed of 1% beef extract, 1% yeast extract, 2% peptone, 1% olive oil, 1% canola oil, agar and pH was adjusted to 7.5. The media was blended for five minutes to ensure all the ingredients were mixed well and then poured into the plates. The loaded plates were covered with aluminum foil and autoclaved at 121 ℃ for 30 minutes.

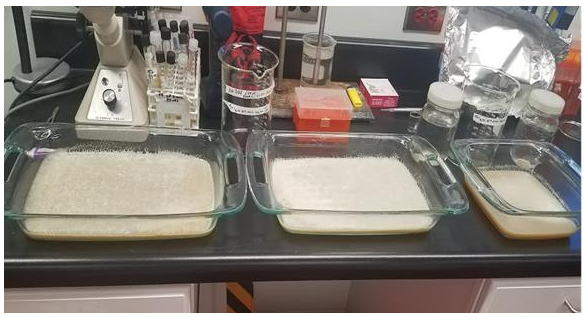

Figure 1. Plates used during the experiment (large, medium, and small)

D. Inoculation of Sanitized Steinernema carpocapsae

The presence of a whitish lawn coating the media surface indicated bacterial growth. Phase I cells of *X. nematophila* were confirmed by gram staining. Surface sanitized *S. carpocapsae* were then aseptically introduced to the bacteria. The nematode inoculum concentrations were adjusted according to the experiments. The incubation period necessary to obtain the first generation growth cycle of nematodes was observed on day 13 at room temperature.

E. Harvesting and Packing the Nematodes

First generations of *Steinernema carpocapsae* were harvested from the plates. Harvesting was accomplished by adding sterile tap water on top of the solid media and washing the nematodes by gentle shaking. The nematodes were counted and centrifuged at 2500 rpm. The pellets were packed in 5mm open cell sponge material and stored at 4 ℃.

F. Nematode Yield

Nematode yield was determined using a gridded sedwick rafter counting cell® (Wildco) by serial dilution [17]. The final fold of nematodes was calculated as the ratio of harvest concentration to inoculum concentration. For example: 1.8 $x10^7$ nematodes (yield)/ $5.0x10^5$ nematodes (inoculum) = 36 folds

III. Results and Discussion

A. Effect of Nematode Inoculum Concentration on Yield

Fig. 2 shows that nematode inoculum concentrations increase or decrease the final nematode yield. Increasing nematode density will increase metabolic waste and competition for food. To illustrate: inoculating 250 nematodes per cm^2, resulted in 110 folds. Microscopic examination showed the adults were larger, contained more eggs and yielded higher production of nematodes. When the nematode inoculum was doubled to 550 nematodes per cm^2, the final folds decreased by 50%. Johnson *et al.* (2016) reported 25 fold yield of the beneficial nematode *Heterorhabditis bacteriophora* by inoculating 900 nematodes per cm^2 of solid media surface which correlates closely with this report [17]. Upadhyay *et al.* (2015) reported 19 folds of the entomopathogenic nematode *Heterorhabditis bacteriophora* using liquid culture fermentation technology [18]. However, in terms of absolute production, solid state fermentation yielded 110 maximum fold, but relatively small final yield. Liquid medium technology has the advantage of nematodes using three dimensions, whereas solid state fermentation is limited to two dimensions (the upper surface of the media.)

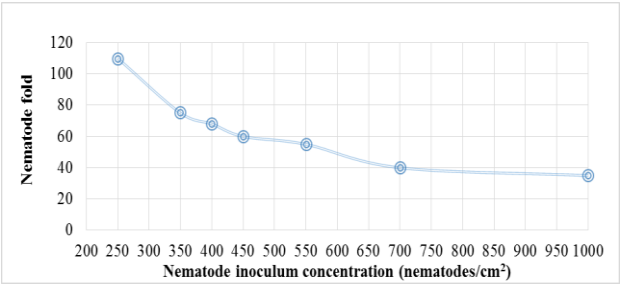

Figure 2. Nematode yield as a function of nematode density

Few nematodes were observed within the solid media matrix. Higher nematode density, less availability of nutrients and more waste metabolites may have repressed

the nematodes' ability to produce eggs. From a mass production perspective, fold is not always the preferred measurement of success. Another approach to commercialization might be to shorten the 'recovery period' of IJs [19], [20]. The exit from the developmentally arrested third juvenile stage (IJ3) is called "recovery." Time of recovery may be important to achieve an economically feasible production process. Nematode recovery is dependent on various factors including bacterial phase variant, media formulation and bacterial density [21], [22]. Finally, nematode and bacterial density are certainly factors affecting final nematode yield [21], [22].

B. *Effect of Fermentation Media Concentration on Nematode Yield*

In this experiment, approximately 1500 nematodes/cm^2 were inoculated onto 700 cm^2 plates containing different concentrations of fermentation media. Results are reported in Fig. 3. Final yield is related to nutrient concentration. Doubling media concentration resulted in decreased yield.

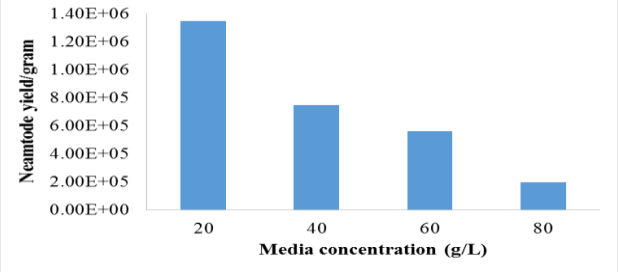

Figure 3. Nematode yield of *Steinernema carpocapsae* per gram of media ingredients/Liter

The 0.5x concentration (20 g/L) of original fermentation media concentration was observed to maximize final yield per gram amount of media used. Johnson *et al.* reported (2016) 5.5x10^5 *H. bacteriophora* IJs per gram of nutrients using same nutrient ingredients of this study [17]. Whereas, Somwong and Petcharat (2012) achieved 3.04x10^5, 2.45x10^5 and 2.98x10^5 IJs/gram *Steinernema carpocapsae* using different media ingredients including dog food, powdered fish and silkwarm pupa respectively in their study [23]. Fresh chicken was used as media by Tabassum and Shahina (2004) who reported 7.5x10^4 IJs/gram *H. indica spp* [14]. Both lipid quality and quantity affect the final nematode yield [24]. Hence, 1% olive oil and 1% canola oil were used in growth media throughout this study. Optimization of lipid concentration can be conducted to obtain maximum yield of nematodes in future studies. Nematode production can be commercialized by optimizing culture conditions, inoculum size, and incubation period as well as by verifying media elements and concentrations.

To many researchers, beneficial nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae in vitro* mass production on a large scale is challenging and cumbersome due to various obstacles [25]. The factors responsible for making nematode mass production difficult are: (a) phase shifting of the bacterial symbiont; (b) concentrations of inoculum (bacterial/nematode); (c) fermentation parameters pH, temperature, oxygen concentration, etc. (d) aseptic handling; (e) low percentages of nematode copulation. Contamination is also a challenge in mass production of nematodes. The advantage of growing *X. nematophila* prior to the nematode inoculation on solid media is the increase of secreted antimicrobial compounds, thereby preventing contamination [26]. Success in nematode mass production requires growing the bacterial symbiont within the media prior to nematode inoculation [27].

Nematode yield is dependent on the concentration and composition of media components [28]. Yoo *et al.* reported that media solution containing high sources of mono-unsaturated fatty acids and few saturated fatty acids favor optimal growth and development of nematodes. Yoo *et al.* developed a media with the mixture of olive and canola oil for the growth of *Heterorhabditis bacteriophora*. High lipid concentration promotes long term food supply. However the bacteria have limited ability to convert mono-unsaturated fatty acids into usable energy [24]. In the fermentation media, peptone was used as a principal source of organic nitrogen and yeast/beef extracts provided amino acids, peptides, vitamins and carbohydrates to support growth [28].

IV. Conclusion

This study provides valuable guidance on implementing a solid state fermentation technology for mass production of *S. carpocapsae*. The research demonstrates principles to achieve highest yield of nematodes. As nematode inoculum concentration increases, the nematode final fold decreases, though nematode final yield increases. Finally, nematodes as a biological control agent are important because insects build resistance toward chemical pesticides. Not only is the bio-control agent safe for the environment and humans, but it does not harm beneficial insects. It is concluded that increasing the nutrient concentration did not benefit nematode mass production. Lowering the nutrients may help to decrease production cost. Future solid media studies for making production cheaper may focus on the use of natural raw material.

Acknowledgment

The authors thank to Farm Bureau of Robeson County and UNCP Department of Chemistry and Physics for financial assistance.

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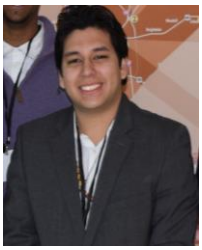

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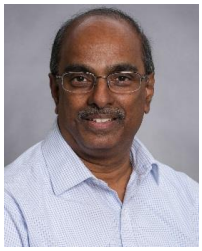

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