A NEW MEDIUM FOR LIQUID FERMENTATION OF *STEINERNEMA FELTIAE*: SELECTION OF LIPID AND PROTEIN SOURCES

Luis G. Leite[1]*, David I. Shapiro-Ilan[2]*, Selcuk Hazir[3], and Mark A. Jackson[4]

[1]Instituto Biologico, APTA, CP 70, Campinas, SP 13001-970, Brazil. Tel: +55-19-32522942; Fax: +55-19-32522942; [2]USDA-ARS, SEFTNRL, Byron, GA 31008, USA; [3]Adnan Menderes University, Faculty of Arts and Sciences, Department of Biology, Aydin-Turkey; [4]USDA-ARS, NCAUR, Peoria, IL, 61604, USA. *Corresponding authors: lgleite@biologico.sp.gov.br and david.shapiro@ars.usda.gov

ABSTRACT

Leite, L. G., D. I. Shapiro-Ilan, S. Hazir, and M. Jackson. 2016. A new medium for liquid fermentation of *Steinernema feltiae*: Selection of lipid and protein sources. Nematropica 46:147-153.

Entomopathogenic nematode production in liquid fermentation still requires improvements to maximize efficiency, yield, and nematode quality. The objective of this study was to develop a more suitable liquid medium for mass production of *S. feltiae* by selecting lipid and nitrogen sources, and by assessing the impact of glucose. Seven sources of lipid (corn oil, palm oil, fish oil, peanut oil, sunflower oil, canola oil, and pork lard) and six of nitrogen (egg yolk, yeast extract, egg white, soy extract, beef extract, and fish collagen) were tested at a concentration of 4% (v/v) and 1.25% (w/v), respectively. Additionally, egg yolk and egg white selected in the previous experiment, were tested solely (both at 1.25% w/v) and combined (at 0.625% w/v each) in media with or without glucose (2.5% w/v), supplemented also with yeast extract (2.3% w/v), NaCl (0.5% w/v), peanut oil (4% v/v), and agar (0.2% w/v). For the lipid and nitrogen source experiments, pork lard and yeast extract provided the lowest yields. The combination of egg yolk + egg white increased nematode yield compared to these nitrogen sources tested individually. Also, the addition of 2.5% glucose increased nematode yields.

Key words: biocontrol, entomopathogenic nematode, *in vitro* production, liquid fermentation..

RESUMEN

Leite, L. G., D. I. Shapiro-Ilan, S. Hazir, y M. Jackson. 2016. Un nuevo medio para la fermentación líquida de *Steinernema feltiae*: Selección de las fuentes de lípidos y proteinas. Nematropica 46:147-153.

La producción de nematodos entomopatógenos en fermentación líquida requiere todavía de mejoras que maximicen su eficacia, productividad y calidad de los nematodos. El objetivo de este estudio fue desarrollar un mejor medio líquido para la producción en masa de *S. feltiae* mediante la selección de fuentes de lípidos y nitrógeno, así como evaluar el impacto de la glucosa. Siete fuentes de lípidos (aceite de maíz, aceite de palma, aceite de pescado, aceite de cacahuete, aceite solar, aceite de colza, y grasa de cerdo) y seis de nitrógeno (yema de huevo, extracto de levadura, clara de huevo, extracto de soja, extracto de carne de ternera, y colágeno de pescado) se ensayaron a concentraciones de 4% (v/v) y 1.25% (p/v), respectivamente. Adicionalmente, la yema de huevo y la clara de huevo seleccionadas en el experimento anterior, se ensayaron individualmente (ambas a 1.25% p/v) y combinadas (a 0.625% p/v cada una) en medio con o sin glucosa (2.5% p/v), suplementado con extracto de levadura (2.3% p/v), NaCl (0.5% p/v), aceite de cacahuete (4% v/v), y agar (0.2% p/v). En los experimentos para las fuentes de lípidos y nitrógeno, la grasa de cerdo y el extracto de levadura proporcionaron las menores productividades. La combinación de yema de huevo + clara de huevo incrementó la producción de nematodos en comparación con estas mismas fuentes de nitrógeno ensayadas individualmente. Además, la adición de glucosa al 2.5% incremento la producción de nematodos.

Palabras clave: biocontrol, nematodo entomatopatógeno, producción, *in vitro*, fermentación líquida.

Entomopathogenic nematodes (EPNs) from the genera *Steinernema* and *Heterorhabditis* have been identified as commercially viable biological control agents used for suppression of a vast array of economically important insect pests of a wide range of agricultural and horticultural crops (Ehlers, 1996; Hazir *et al.*, 2003). The free-living, non-feeding, and developmentally-arrested infective juveniles (IJs) possess symbiotic bacteria *Xenorhabdus* spp. (for steinernematids) and *Photorhabdus* spp. (for heterorhabditids) in the lumen of their pharynx and intestine (Poinar, 1975; Poinar, 1990). The nematodes penetrate the insect host via natural body openings (mouth, anus, and spiracles) or through the cuticle, and release the associated bacteria into the host's body cavity (Poinar and Thomas, 1966; Akhurst, 1980). The bacteria multiply rapidly to cause a lethal septicemia or toxemia within 24 to 48 hr and create a suitable environment for reproduction and development of nematodes (Lewis and Clarke, 2012).

The nematodes feed upon the proliferating bacteria and the decomposing insect cadaver (Strauch and Ehlers, 1998). The nematodes complete 1-3 generations within the insect host and then IJs exit to search for new insect targets (Stock, 2015). The IJ is adapted to be durable and survive in the soil environment until it can find a suitable insect host (Lewis and Clarke, 2012; Shapiro-Ilan *et al*., 2014). *Steinernema feltiae* Filipjev is one of the most studied nematodes among the EPNs, and it is used widely in biocontrol applications such as for suppression of fungus gnats, an insect pest of worldwide importance on ornamental, nursery plants, and mushrooms (Jess *et al*., 2005; Tomalak *et al*., 2005).

Entomopathogenic nematodes have been mass produced by *in vivo*, using living insects, and *in vitro*, using the bacteria culture as source of food, with the liquid fermentation process allowing the large-scale production. Despite great progress in the past couple of decades, EPNs full potential has hardly been realized due to non-competitive costs compared with chemical insecticides and inconsistent nematode quality. Both issues may be addressed by improving liquid culture technology. Improving media is a viable option. A standard nematode production medium should contain carbon sources such as sugars, sugar alcohols and lipids, and a nitrogen source (Pace *et al*., 1986; Friedman *et al.*, 1989; Han *et al*., 1993; Surrey and Davies, 1996; Ehlers *et al*., 1998). Lipids support for 60% of the total energy for the non-feeding IJs (Selvan *et al*., 1993) while several nitrogen sources including dried egg yolk, lactalbumin, and liver extract improved nematode production (Buecher *et al*., 1970; Friedman, 1989; Friedman, 1990). Therefore, efforts to optimize media composition, specifically by selecting key ingredients such as lipid and nitrogen sources, are critical to increasing IJ yields. The objective of this study was to develop a more suitable liquid media for mass production of *S. feltiae* by evaluating various sources and combinations of lipid and nitrogen sources, and by assessing the impact of glucose addition to the selected lipid/nitrogen combinations. Glucose has been demonstrated to increase yields in *Heterorhabditis bacteriophora* (Gil *et al*., 2002). Thus, we hypothesized the addition of glucose would also enhance *S. feltiae* yields. The experiments were conducted using shake flasks, but the results may also be applicable for bioreactor conditions.

Three experiments on *in vitro* liquid culture production of *S. feltiae* were conducted; the first one to assess sources of lipids, the second one to assess sources of nitrogen, and the third one to assess the combination of the best sources mixed with glucose.

Bacterial isolation and nematode axenization

Bacterial isolation and nematode axenization were done according to Leite *et al*. (2016).

Base medium

For the lipid and nitrogen experiments, the base medium contained yeast extract (2.3% w/v), egg yolk (1.25% w/v), NaCl (0.5% w/v), and corn oil (4% v/v). This medium was selected as a high-yielding composition for production of *S. feltiae* in a previous study conducted by Chavarría-Hernández and Torre (2001). This medium was tested with 0.2% agar, which improved the nematode yield in our previous research (Leite *et al.*, 2016).

Sources of lipid

In the first experiment, seven lipid sources (corn oil, palm oil, salmon oil, peanut oil, sunflower oil, canola oil, and pork lard) were tested at a concentration of 4% (v/v) in the base media described above. Three replications were established for each treatment, with each replication consisting of an Erlenmeyer flask (150 mL) containing 50 mL of liquid medium. The media were inoculated with the bacteria *Xenorhabdus bovienii* previously grown in tryptic soy broth (4% w/v) + yeast extract (0.5% w/v) medium (TSB + ye) by adding 1 mL (5 x 10^9 cells) of culture to each flask. The *X. bovienii* cultures were grown in an orbital shaker (G 25, New Brunswick Scientific) at 280 rpm and 25°C for 2 days. After this period, each flask was inoculated with nematodes by adding 10,000 2-day-old juveniles (suspended in 0.3 mL of TSB + ye medium) that had hatched from the surface-sterilized eggs. The initial concentration of the nematodes in the production media was 200

juveniles/mL.

The flasks were kept in the shaker, and evaluations were done 2 days after bacteria inoculation by counting bacteria cells and 28 days after nematode inoculation by counting nematode populations. For the bacterial counts, 25-µl (0.025 mL) samples were taken from each flask and diluted in 40 mL water in a 50-mL centrifuge tube. After the tubes were closed and vortexed, the bacterial suspension was counted in a Neubauer slide using a compound microscope (×400). For nematode counts, 1 mL samples were taken from each flask and diluted in 500 mL water in a 1,000-mL beaker. While the suspension was stirring, a 2-mL sample was taken for counting nematode in a Peter's slide using a stereomicroscope (×16). Two samples were measured for each replicate, and the entire experiment was repeated once in time.

Sources of nitrogen

In the second experiment, 12 media formulations, which consisted of 6 nitrogen sources (egg yolk, yeast extract, egg white, soy extract, beef extract, or fish collagen) at a concentration of 1.25% (w/v) added to the base medium with 2 different lipid sources were tested. One group of nitrogen sources was supplemented with corn oil and another with peanut oil (both at 4% v/v). The two lipid sources chosen for this experiment were selected based on the highest yield of nematodes in previous experiment (see Results section). The methodology used was the same as described for the previous experiment. The entire experiment was repeated once in time.

Nitrogen sources and sugar

For this experiment, six treatments were tested. Egg yolk and egg white (selected based on the highest yield of nematodes in previous experiment–see Results section) were tested solely (both at 1.25% w/v) and combined (at 0.625% w/v each) in media with or without glucose (2.5% w/v), supplemented also with yeast extract (2.3% w/v), NaCl (0.5% w/v), peanut oil (4% v/v), and agar (0.2% w/v). The methodology used was the same as described for the previous experiment. The entire experiment was repeated once in time.

Statistical analyses

Data from repeated trials were pooled and variation among trials was treated as a block effect. For all experiments, treatment effects were detected using ANOVA. If the F value was significant, then treatment differences were further elucidated with Tukey's test. Additionally, in the factorial experiment involving lipids and nitrogen sources and nitrogen combinations, with or without glucose, if the effects of the factorials were found to act independently (no interaction detected between them), then the analysis focused solely on these main effects, and simple effects were not elucidated further (Cochran and Cox, 1957). Alternatively, if significant interactions were detected among main effects, then the analysis focused on simple effects. Data were square root transformed prior to analysis (Southwood, 1978); non-transformed means are presented in the results section. All statistical comparisons were done using SAS version 9.0 software, with a P value ≤ 0.05 indicating significance.

For bacterial growth (Fig. 1), no differences among lipid sources were detected ($F = 1.82$; df = 6, 34; $P = 0.1155$). For nematode production (Fig. 1), pork lard supported the lowest nematode yield, differing significantly from the other treatments ($F = 8.15$; df = 6, 34; $P < 0.0001$). Pork lard, which contains abundant saturated fatty acids, also produced the lowest yields of *H. bacteriophora* according to Yoo *et al.* (2000). These authors observed that, at room temperature, pork lard is semi-solid during fermentation and may make these lipids unavailable during liquid culture growth.

Peanut oil is the cheapest oil among those tested and provided numerically the highest yield of nematodes (although not significantly different from other treatments, except from pork lard). Peanut oil is low in saturated fats, free from cholesterol, and contains the essential fatty acid

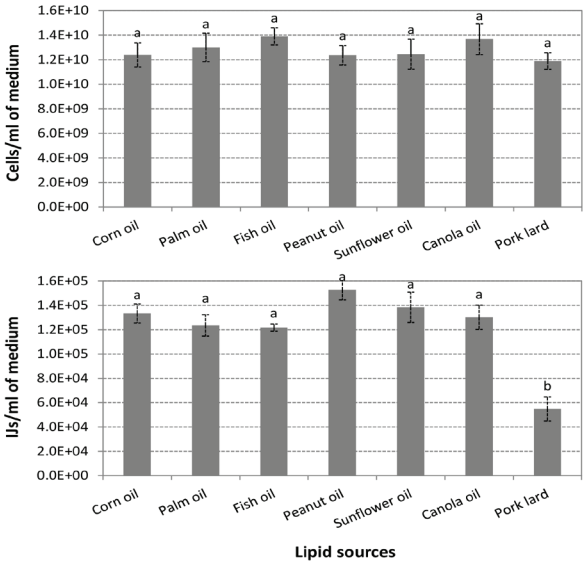

Fig. 1. Yield of *Xenorhabdus bovienii* 2 days post inoculation, and of *Steinernema feltiae* 28 days after nematode inoculation in liquid media consisting of egg yolk (1.25% w/v), yeast extract (2.3% w/v), NaCl (0.5% w/v), agar (0.2% w/v), and different sources of lipid (4% v/v). Different letters above bars indicate statistical differences (ANOVA & Tukey's test; $P < 0.05$).

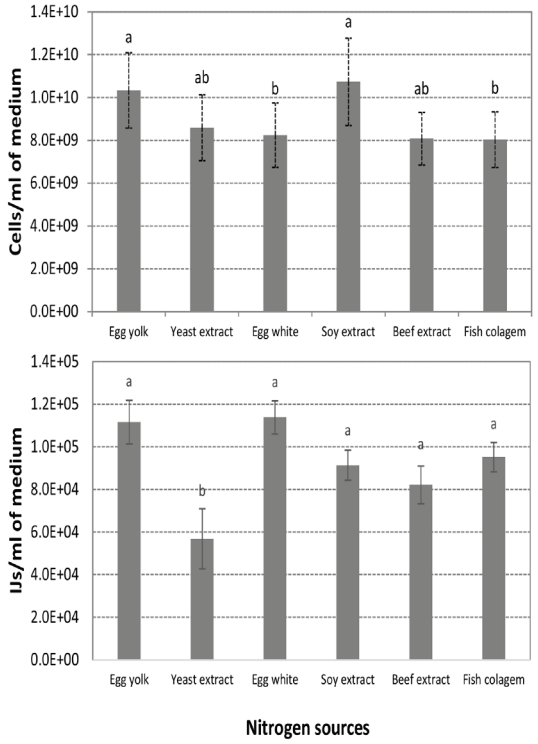

Fig. 2. Yields of *Xenorhabdus bovienii* 2 days post inoculation, and of *Steinernema feltiae* 28 days after nematode inoculation in liquid media consisting of yeast extract (2.3% w/v), NaCl (0.5% w/v), agar (0.2% w/v), and different sources of nitrogen (1.25% w/v). Yields were averaged across media with corn oil and peanut oil (both at 4%). Different letters above bars indicate statistical differences (ANOVA & Tukey's test; $P < 0.05$).

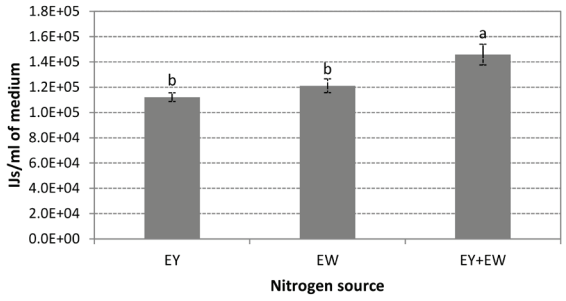

Fig. 3. Yields of *Steinernema feltiae* 28 days after nematode inoculation in liquid media consisting of yeast extract (2.3% w/v), NaCl (0.5% w/v), peanut oil (4% v/v), agar (0.2% w/v), and with egg yolk (EY) or egg white (EW), tested alone (both at 1.25% w/v) or combined (at 0.625% w/v for each one). Different letters above bars indicate statistical differences (ANOVA & Tukey's test; $P < 0.05$).

linoleic acid - omega-6, one of the most abundant fat content in insects (18.2%) (Finke, 2004). Canola oil, which was selected as the best oil to grow *H. bacteriophora* (Yoo *et al*., 2000), showed similar results as other lipid sources in our study, but is more expensive compared to peanut oil.

For bacteria and nematode counts, there was no interaction between the lipids (corn and peanut oil) and the nitrogen sources tested (bacteria $F = 80.81$; df = 5, 59; $P = 0.5092$; nematodes $F = 4.62$; df = 5, 59; $P = 0.3895$). Thus, the nitrogen sources were analyzed across lipid sources (Fig. 2), which did not differ significantly from each other for bacterial ($F = 1.06$; df = 1, 59; $P = 0.308$) and nematode counts ($F = 0.25$; df = 1, 59; $P = 0.62$). For bacterial counts, egg yolk and soybean powder provided the highest yields, differing significantly from egg white and fish collagen ($P = 0.0005$), but not from yeast extract and beef extract. For nematode counts, only yeast extract differed significantly ($P < 0.0001$) by supporting the lowest yield.

According to Cho *et al.* (2011), soybean flour supported the highest yield of *H. bacteriophora* by improving symbiotic bacterial growth and nematode recovery. In our study, soybean powder did not exhibit superior results except when compared with egg white and fish collagen for bacteria growth, and with yeast extract for nematode growth (Fig. 2). The medium containing yeast extract as the sole source of nitrogen at a high rate (3.55% w/v) supported lower yields compared to those containing lower concentrations of yeast extract (2.3% w/v) but supplemented with other sources of nitrogen (1.25% w/v). These results suggest that the combination of nitrogen sources may provide higher yields of nematodes compared to sole sources. To confirm this hypothesis, we assessed the combination of two nitrogen sources that provided the highest nematode yields, egg yolk and egg white, as a complement to yeast extract.

For the combination nitrogen sources and sugar experiment, the analyses of bacteria and nematode yields showed no interaction between the nitrogen sources and the glucose factor (For bacteria $F = 0.57$; df = 2, 29; $P = 0.8284$. For nematode $F = 6.37$; df = 2, 29; $P = 0.9767$). Thus, the analysis focused on the main effects.

For bacterial growth, there were no significant differences among nitrogen sources ($F = 0.57$; df = 2, 29; $P = 0.2486$) (ranging from 2.6×10^{10} to 3.1×10^{10} cells/ml), and no effect of glucose addition was detected ($F = 0.57$; df = 1, 29; $P = 0.755$) (2.9×10^{10} for medium with and without glucose).

For nematode yields, the combined nitrogen source EY+EW significantly increased the yield ($F = 6.37$; df = 2, 29; $P = 0.0003$) compared to EY and EW, which did not differ from each other (Fig. 3). Also, the addition of 2.5% glucose increased

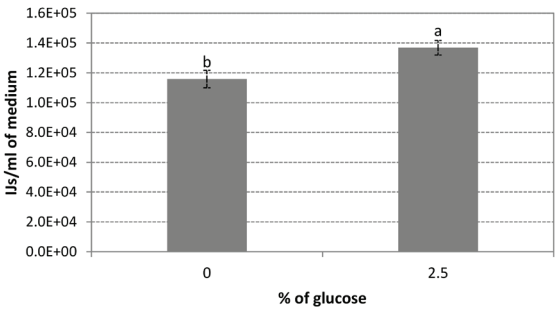

Fig. 4. Yields of *Steinernema feltiae* (IJs) 28 days after nematode inoculation in liquid media consisting of yeast extract (2.3% w/v), NaCl (0.5% w/v), peanut oil (4% v/v) and agar (0.2% w/v), with or without glucose (2.5% w/v). Yields were averaged across media with egg yolk (1.25% w/v), egg white (1.25% w/v), or a mixture of both sources (at 0.65% w/v for each one). Different letters above bars indicate statistical differences (ANOVA & Tukey's test; $P < 0.05$).

nematode yields ($F = 6.37$; df = 1, 29; $P = 0.0005$) (Fig. 4).

Several studies used egg yolk as a source of nitrogen for nematode production (Friedman *et al.*, 1989; Surrey and Davies, 1996; Chavarría-Hernández *et al*., 2003; Chavarría-Hernández, 2010; Chavarría-Hernández *et al*., 2011), but no studies have tested egg white. Egg yolk and egg white provided a significant increase in nematode yield when mixed together, suggesting they complement each other for nematode nutrition. The yolk makes up about 34% of the liquid weight of the egg. It contains all of the fat in the egg and a little less than half of the protein. With the exception of niacin and riboflavin, the yolk contains a higher proportion of the egg's vitamins. The yolk also contains more calcium, copper, iron, manganese, phosphorus, selenium, and zinc than the white. The white contains more than half the egg's total protein, a majority of the egg's niacin, riboflavin, magnesium, potassium and sodium, and none of the fat.

Chavarría-Hernández *et al*. (2001) obtained the highest yield of *S. feltiae* in a medium containing egg yolk (1,25%) and yeast extract (2.3%) when compared to a medium containing lower concentrations (1% egg yolk and 0.5% yeast extract) but supplemented with soybean flour (2%). Thus, it is clear that the components of combined ingredients and their concentrations are important in determining potential benefits.

The addition of glucose significantly increased the production of *S. feltiae*, especially when supplemented with egg yolk + egg white, increasing nematode production to 157,125 IJs/mL with more than 98% IJs 27 days post nematode inoculation. Studies suggest that a standard nematode production medium should contain a carbon source such as glucose or glycerol (Pace *et al*., 1986; Friedman *et al*., 1989; Han *et al*., 1993; Surrey and Davies, 1996; Ehlers *et al*., 1998). According to Gil *et al.* (2002), glucose is a superior carbon source for bacterial growth whereas canola oil is optimal for the nematode production. Higher yields of *H. bacteriophora* were obtained when glucose was initially added and oil was fed to the culture after the bacterial growth phase and concurrent with nematode inoculation. However, in our study, glucose did not significantly increase bacterial growth, but increased nematode yield, suggesting it is important for nematode production. How glucose is used by the nematode is not clear since the bacteria produce exoenzymes that may interact with media components before the nutrient is available to the nematode. Furthermore, the bacteria can synthesize an array of nutrients that the nematodes cannot, and therefore the nematodes rely on the bacteria to provide these nutrients (Ferreira and Malan, 2014).

The experiments were conducted using shake flasks, but the results may also be applicable for bioreactor conditions. We grew *S. feltiae* successfully in a 5-liter fermenter using this selected new medium, obtaining 150,000 IJ/ml (unpublished data). Additional research is needed to assess other media components and combinations as well as the use of the developed medium for production of other EPNs species.

ACKNOWLEDGMENTS

The authors would like to thank the São Paulo Research Foundation (FAPESP) (Grant 2014/00651-4) for supporting the first author. The research was partially supported by the USDA national program for Crop Production (Project Number: 6042-21220-012-00D). We also thank Stacy Byrd for her valuable technical assistance.

LITERATURE CITED

Akhurst, R. J. 1980. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes Neoaplectana and Heterorhabditis. Journal of General Microbiology 12:303-309.

Buecher, E. J., E. L. Hansen, and E. A. Yarwood. 1970. Growth of nematodes in defined medium containing hemin and supplemented with commercially available proteins. Nematologica 16:403-409.

Chavarría-Hernández, N. C., and M. Torre. 2001. Population growth kinetics of the nematode, *Steinernema feltiae*, in submerged monoxenic culture. Biotechnology Letters 23: 311–315.

Chavarría-Hernández, N., A. I. Rodríguez-Hernández, F. Pérez-Guevara, and M. Torre.

2003. Evolution of culture broth rheological properties during propagation of the entomopathogenic nematode, *Steinernema carpocapsae*, in submerged monoxenic culture. Biotechnology Progress 19:405-409.

Chavarría-Hernández, N., E. Ortega-Morales, A. Vargas-Torres, J. C. Chavarría-Hernández, and A. I. Rodríguez-Hernández. 2010. Submerged monoxenic culture of the entomopathogenic nematode, *Steinernema carpocapsae* CABA01, in a mechanically agitated bioreactor: Evolution of the hydrodynamic and mass transfer conditions. Biotechnology and Bioprocess Engineering 15:580-589.

Chavarría-Hernández, N., G. Maciel-Vergara, J. C. Chavarría-Hernándezb, J. Castro-Rosasc, B. R. Rodríguez-Pastrana, M. Torre-Martínezd, and M. I. Rodríguez-Hernández. 2011. Mass production of the entomopathogenic nematode, *Steinernema carpocapsae* CABA01, through the submerged monoxenic culture in two internal-loop airlift bioreactors with some geometric differences. Biochemical Engineering Journal 55:145–153.

Cho, C. H., K. S. Whang, R. Gaugler, and S. K. Yoo. 2011. Submerged monoxenic culture medium development for *Heterorhabditis bacteriophora* and its symbiotic bacterium *Photorhabdus luminescens*: Protein Sources. Journal of Microbiology and Biotechnology 21(8):869–873.

Cochran, W.G., and G. M. Cox. 1957. Experimental Designs, 2nd ed. New York: John Wiley & Sons.

Ehlers, R. U., S. Lunau, K. Krasomil-Osterfeld, and K. H. Osterfeld. 1998. Liquid culture of the entomopathogenic nematode bacterium complex *Heterorhabditis megidis/ Photorhabdus luminescens*. Biological Control 43:77-86.

Ehlers, R. U. 1996. Current and future use of nematodes in biocontrol: Practice and commercial aspects with regard to regulatory policy issues. Biocontrol Science and Technology 6:303–316.

Ferreira, T., and A.P. Malan. 2014. *Xenorhabdus* and *Photorhabdus*, bacterial symbionts of the entomopathogenic nematodes *Steinernema* and *Heterorhabditis* and their *in vitro* liquid mass culture: A review. African Entomology 22:1-14.

Finke, M. D. 2004. Nutrient Content of Insects. Pp. 1563-1575 *in* J. I. Capinera (ed.). Encyclopedia of Entomology. Dordrecht, Netherlands: Kluwer Academic Publishers.

Friedman, M. J. 1990. Commercial production and development. Pp. 153-172 *in* R. Gaugler and H. K. Kaya (eds.). Entomopathogenic Nematodes in Biological Control. Boca Raton: CRC.

Friedman, M. J., S. E. Langton, and S. Politt. 1989. Mass production in liquid culture of insect-killing nematodes. Patent, International Publication Number WO 89/04602.

Gil, G.H., H. Y. Choo, and R. Gaugler. 2002. Enhancement of entomopathogenic nematode production in *in-vitro* liquid culture of *Heterorhabditis bacteriophora* by fed-batch culture with glucose supplementation. Applied Microbiology and Biotechnology 58:751–755.

Han, R. C., L. Cao, and X. Liu. 1993. Effects of inoculum size, temperature and time on *in vitro* production of *Steinernema carpocapsae* Agriotos. Nematologica 39:366–375.

Hazir, S., H. K. Kaya, S. P. Stock, and N. Keskin. 2003. Entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) for biological control of soil pests. Turkish Journal of Biology 27:181–202.

Jess, S., H. Schweizzer, and M. Kilpatrick. 2005. Mushroom Applications. Pp. 191-213 *in* P. S. Grewal, R. U. Ehlers, and D. I. Shapiro-Ilan (eds.). Nematodes as Biocontrol Agents. Oxfordshire, UK: CABI Publishing.

Leite, L. G., D. I. Shapiro-Ilan, S. Hazir, and M. A. Jackson. 2016. The effects of nutrient concentration, addition of thickeners, and agitation speed on liquid fermentation of *Steinernema feltiae*. Journal of Nematology 48:126-133.

Lewis, E. E., and D. J. Clarke. 2012. Nematode parasites and entomopathogens. Pp. 395-424 *in* F.E. Vega, and H. K. Kaya, eds. Insect Pathology, second ed. Amsterdam, Netherlands: Elsevier.

Pace, G. W., W. Grote, D. E. Pitt, and J. M. Pitt. 1986. Liquid culture of nematodes. Patent, International Publication Number WO 86/1074.

Poinar, G. O. Jr. 1975. Description and biology of a new insect parasitic rhabditoid, *Heterorhabditis bacteriophora* n. gen., n. sp. (Rhabditida; Heterorhabditidae N. Fam.). Nematologica 24:463-470.

Poinar, G. O., Jr. 1990. Taxonomy and biology of Steinernematidae and Heterorhabditidae, Pp. 23-61 in R. Gaugler, and H. R. Kaya, eds. Entomopathogenic Nematodes in Biological Control. Boca Raton: CRC Press.

Poinar, G. O., Jr., and G. M. Thomas. 1966. Significance of *Achromobacter nematophilus*, Poinar and Thomas (Achromobacteraceae: Eubacteriales) in the development of the nematode, DD-136 (Neoaplectana sp. Steinernematidae). Parasitology 56:385-390.

Selvan, S., R. Gaugler, and E. E. Lewis. 1993. Biochemical energy reserves of entomopathogenic nematodes. Journal of Parasitology 79:167-172.

Shapiro-Ilan, D., R. Han, and X. Qiu. 2014. Production of entomopathogenic nematodes. Pp. 321-355 *in* J. A. Morales-Ramos, M. G.

Rojas, and D. I. Shapiro-Ilan (eds.) Mass Production of Beneficial Organisms. Oxford: Elsevier.

Southwood, T. R. E. 1978. Ecological Methods: With Particular Reference to the Study of Insect Populations. Amsterdam, Netherlands: Springer.

Stock, P. 2015. Diversity, biology and evolutionary relationships. Pp. 3-27 *in* R. Campos-Herrera (ed.). Nematode Pathogenesis of Insects and Other Pests. New York: Springer.

Strauch, O., and R. Ehlers. 1998. Food signal production of *Photorhabdus luminescens* inducing the recovery of entomopathogenic nematodes *Heterorhabditis* spp. in liquid culture. Applied Microbiology and Biotechnology 50:369-374.

Surrey, M. R., and R. J. Davies. 1996. Pilot-scale liquid culture and harvesting of an entomopathogenic nematode, *Heterorhabditis bacteriophora*. Journal of Invertebrate Pathology 67: 92-99.

Tomalak, M., S. Piggott, and G. B. Jagdale. 2005. Glasshouse Application. Pp. 147-166 *in* P. S. Grewal, R. U. Ehlers, and D. I. Shapiro-Ilan (eds.) Nematodes as Biocontrol Agents. Oxfordshire, U.K.: CABI Publishing.

Yoo, S., I. Brown, and R. Gaugler. 2000. Liquid media development for *Heterorhabditis bacteriophora*: lipid source and concentration. Applied Microbiology and Biotechnology 54:759-763.

Received: *Accepted for publication:*
3/III/16 *23/V/2016*
Recibido: *Aceptado para publicación:*