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MASS PRODUCTION OF ENTOMOPATHOGENIC NEMATODE *HETERORHABDITIS BACTERIPHORA IN VIVO* AND *VITRO* CULTURE

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Abstract
The strategies of commercial development have been focused on the economy of scale for a process. The design of media has been recognized as a key in assuring mass production of entomopathogenic nematodes. Media optimization was conducted with insect host, proteins, lipids, and symbiotic bacteria mass. *G. mellonella* (insect host) produced about 290,000 infective juveniles per one. Complex media produced about 250,000 infective juveniles / ml in liquid culture within 8 days (one generation).

Introduction

Entomopathogenic nematodes Rhabdita: *Steinernematidae* and *Heterorhabdatidae* have been intensively studied due to their potential to control a wide range of insect pests (Kaya and Gaugler 1993). *Heterorhabditis bacteriophora* is among the most versatile nematodes of the more than 30 described entomopathogenic nematode species. Infective juveniles of *H. bacteriophora* carry the symbiotic bacteria, *Photorhabdus luminescens,* in their gut. Upon penetration of an insect, the developmentally arrested infective juveniles resume development (a process referred to as recovery); and bacteria are released into the host hemocoel (Poiner 1975; Akhurst 1980). The bacteria multiply rapidly, killing the host within 24 to 48 hr, and produce a suitable environment for nematode reproduction and development. The nematodes ingest the bacteria and degrade host tissues, which provide essential nutrients (Akhurst 1983).

Demand for environmentally benign insecticides has stimulated an interest in entompathogenic nematode mass culture. Production on a cottage industry level continues to be based on *in vivo* culture methods. Bedding's (1981, 1984) pioneering efforts in devising solid *in vitro* culture used media-impregnated plastic foam to provide a substrate for a large culture surface area. However, harvesting nematodes from foam is laborious and inefficient. The next advance in production came with the advent of Biosys and liquid culture production (Friedman 1990). This approach remains the preferred method for large-scale commercial production because it offers economies of scale not realizable with other production methods. Entomopathogenic nematodes have been commercial products for twenty years, yet in most markets they remain more a curiosity than legitimate pest management tool (Gaugler 1997). Two key reasons are lack of cost competitiveness with chemical insecticides and concerns of inconsistent nematode quality. Both issues may be addressed by improving liquid culture technology. One approach is adjusting media composition until it is optimal (i.e., high yield, short fermentation, good quality) for nematode development and reproduction (Buecher et al. 1970, Buecher and Hansen 1971; Vanfleteren 1974). We predict that nematode yield will be determined primarily by the constitutive quality of their primary food source: the bacteria. In this study, we assessed

how media sources influenced the yield and productivity of *H. bacteriophora* infective juveniles and their symbionts in liquid culture.

Materials and methods

Nematode and bacterial cultures

H. bacteriophora (Tf strain) was isolated from turfgrass in central New Jersey (USA). The nematodes were reared in last instar *Galleria mellonella* using the method of Dutky *et al.* (1967). Symbiotic bacteria were isolated using a modified method of Akhurst (1980). Primary phase bacteria were determined as described by Hu and Webster (1998).

Monoxenic culture preparation

Bacterial lawns were prepared by streaking over lipid agar plates. Infective juveniles were surface-sterilized using 0.1 % (w/v) hyamin and washed three times with sterile distilled water. Sterilized infective juveniles were inoculated over the bacterial lawn plates. The plates were incubated at 25°C for 8 days.

Monoxenic liquid culture.

Liquid culture medium was prepared using the following components per one liter of distilled water: soy flour powder 25 g, yeast extract 5 g, lactalbumin hydrolyzate 10 g, canola oil 25g, NaCl 4.0 g, $MgSO_4$ 0.5 g, $CaCl_2$ 0.3g, and KCl 0.3g. The medium was homogenized, adjusted to pH 7.0 prior to sterilization. Bacterial liquid culture was prepared by transferring stock culture into 40 ml of liquid media in 250 ml culture flasks. The flasks were incubated at 25°C and 200 rpm in a shaking incubator (NBS, New Brunswick, NJ, USA) until bacterial growth reached the early stationery phase. Nematodes were transferred into the bacterial liquid culture flask. The flasks were incubated until infective juveniles appeared (first generation). Infective juveniles produced were used with seed culture in liquid cultures for optimization of media.

Recovery

For determination of nematode recovery, 100 µl monoxenic culture was taken daily and diluted with distilled water (1:5 or 1: 10). With 100 µl samples of diluted culture, the recovered nematodes were observed using Giemsa solution (EM Science, NJ), which stains only infective juveniles. Recovery was calculated as the proportion of recovered to inoculated infective juveniles X100.

Size measurement of hermaphrodites

Ten mature hermaphrodites were randomly selected from each treatment, for a total of 40 hermaphrodites. These were transferred into Petri dishes (60 x 15 mm) containing buffer (Fisher Gram-Pac, pH 7.43). They were placed with a calibration scale under a stereo microscope onto which was mounted a video camera connected to a video cassette recorder (EVD, Sony, Japan). Hermaphrodite length was determined by use of a movie editor (Premiere 5.0, Adobe, USA).

Number of infective juveniles in hermaphrodite

Twenty mature hermaphrodites of various sizes were chosen haphazardly from all four treatments. These were placed individually into wells of 24-well plates that were filled

with 500 μl aliquots of distilled water. After two days, infective juveniles generated from each hermaphrodite were counted.

Analytical methods and data analysis

During fermentation, nematode juvenile stages (J2, 3, 4, and infective juvenile), hermaphrodites, and gravid adults, were counted using a stereomicroscope. The bacterial concentration was determined by cell counts using a haemocytometer (0.02 mm depth and 1/400 mm^2) and was converted to bacterial dry mass. The data were analyzed using ANOVA and Tukey's multiple range test (α =0.05) was used for mean comparisons. Data in the text are presented as mean ± standard error of the mean.

Results & Discussion

In vivo production

We isolated a prolific strain *Heterorhabditis bacteriophora*. The production of infective juveniles *in vivo* culture depended on the volume of host (Fig. 1). The average of infective juveniles produced was 937, 092 / g host. This strain produced an average of nearly 290,000 infective juveniles per *G. mellonella* (insect host).

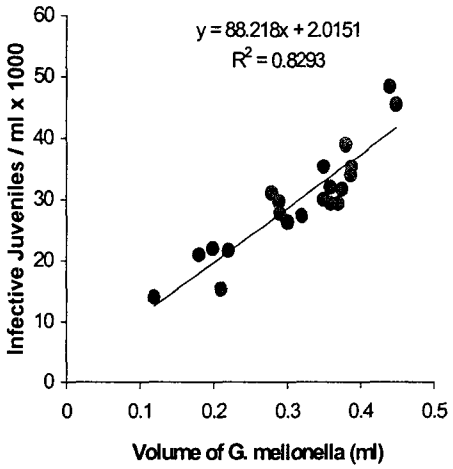

Fig. 1. The number of infective juveniles produced from *G. mellonella*

Media developments

Proteins, lipids, salts, and media concentrations were evaluated for nematodes and bacteria growth. Culture media for nematodes and their symbiotic bacteria require a complex media as nutrients. Growth kinetics of bacteria was previously reported; the bacteria demonstrated osmotolerant (growth at 20% sucrose or more). The culture conditions to build up the highest cell mass were obtained at 25°C, pH 6.5 to 7.0, and 10% (w/v) sucrose concentration. Casein, peptonized milk, peptonized lactalbumin, corn seed flour, soytone, peptone, corn gluten mea tested, soybean flour supported the best bacterial and nematode

growth (data not shown). Lipid have received a great attention as an essential nutrient. Lipid content and quality directly affect the quality of entomopathogenic nematodes and their symbiotic bacteria. Of canola, safflower, olive, lard, sesame, coconut, and oil mixture, canola, safflower, olive oil, and mixture of them that are abundant in monounsaturated fatty acids supported best growth of bacteria and nematodes. As lipid concentration increase 2.5 to 8.0% (w/v), recovery, yield, and productivity of infective juveniles improved. The greatest nematode yield concentration, 2.75 x 10^5 infective juveniles / ml, was achieved using media containing an 8% (w/v) lipid mixture within 8 days post-nematode inoculation (Fig. 2).

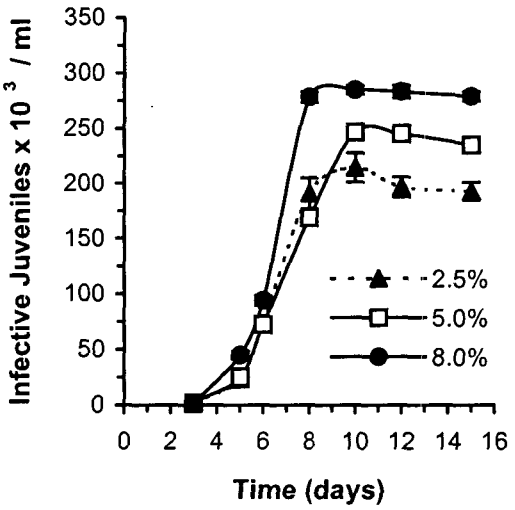

Fig. 2. The progress curve of infective juveniles of *Heterorhabditis bacteriophora* Tg in the monoxenic culture. All nematodes were counted until first generation ended. Fermentation was undertaken at 25°C, 250 rpm, and initial pH 7.0 on a shaking incubator. Each value is the mean ± standard error of three different replicate experiments.

Because procaryotic bacteria are deficient in sterols, synthetic media of nematodes could be enriched by adding extra sterols, specially cholesterol. Myoglobin, hemoglobin, cytochrom, and heme are also known as growth factors for reproduction of nematodes and can substitute effectively for the liver fraction. We prepared media fortified by cholesterol and liver extract. Cholesterol was dissolved into 95% alcohol and liver powder was dissolved in the distilled water. They are filter-sterilized prior to storage. The growth of nematodes improved two or three times by adding cholesterol and liver extract (data not shown).

We compared salts composition of present media with salts composition of media designed for in sect cell cultures. Growth inhibition was found at 0.8% (w/l) or more salt concentration. Present salt composition and concentration supported greater growth than any other chosen salt composition. Based on results from protein, lipid, growth factor, and salts sources study, media composition was modified from previous one (Table 1).

Table 1. Media composition of bacteria and nematode medium

Nutrients	Conc. (g/l)	Sterilization
Soybean protein	25.0	Autoclave
Protein hydrolyzates	10.0	Autoclave
Yeast Extract	5.0	Autoclave
Canola oil	20.0	Autoclave
Olive oil	20.0	Autoclave
Cholesterol	0.2	Filter
Liver extract	0.1	Filter
NaCl	4.0	Autoclave
$CaCl_2$	0.4	Autoclave
KCl	0.4	Autoclave

Media concentration

The effect of culture media concentration on the growth of nematodes were studied. Media were prepared with 28, 56, 84, 168, and 224 g/l concentration. Best growth of bacteria and nematodes were found at 84 g/l media concentration (Fig 3). Nematode and bacteria growth inhibition occurred at higher than 84 g/l media concentration. No infective juveniles grew at 224 g/l media concentration. Recovery varied at media concentration. Recovery was best at 84 g/l and then decreased as media increased (Fig 4.) The size of hermaphrodites in the first generation varied at different media concentration. The number of infective juveniles generated from hermaphrodite was dependent of their size (volume) (Fig.5). Comparing the size of hermaphrodites in the first generation, size was limited at low (28 g/l) and high (158 g/l) media concentration. The size was best and not significantly different at 56 and 84 g/l. The average size at these concentration was 4.2 cm.

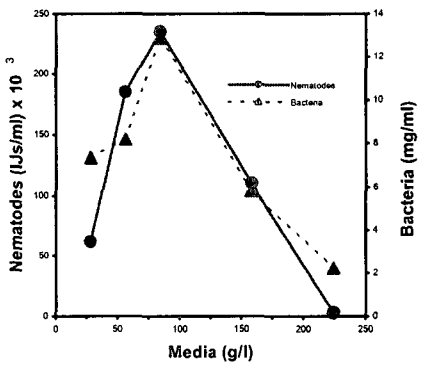

Fig. 3. Infective juveniles and bacterial mass at different media concentration.

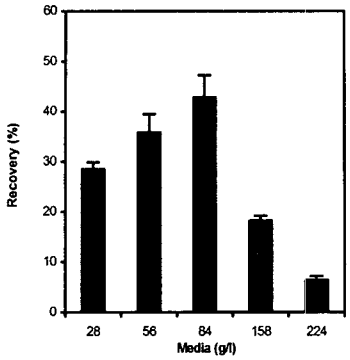

Fig. 4. Recovery of infective juveniles at different media concentration.

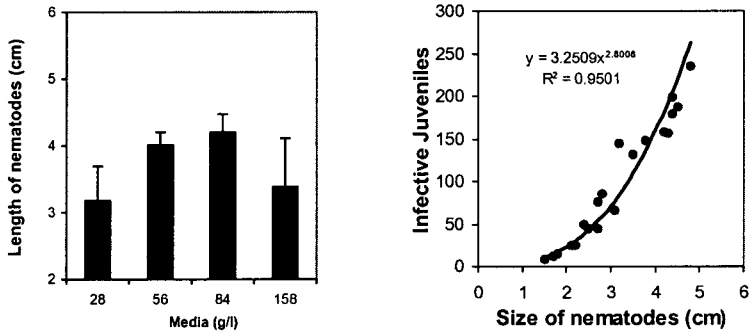

Fig. 5. The number of infective juveniles generated from different size of hermaphrodites (above). The length of hermaphrodites grown from different concentration of media. All hermaphrodites are from first generation stage (below).

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