J. Microbiol. Biotechnol. (2001), **11**(4), 644–648

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Medium Concentration Influencing Growth of the Entomopathogenic Nematode *Heterorhabditis bacteriophora* and its Symbiotic Bacterium *Photorhabdus luminescens*

YOO, SUN KYUN[1]*, IAN BROWN[2], NANCY COHEN[2], AND RANDY GAUGLER[2]

[1]*Engineering Research Institute, Chonnam National University, Gwang-Ju 500-757 Korea*
[2]*Department of Entomology, Rutgers University, New Brunswick, NJ 08901-8524 USA*

Received: February 24, 2001
Accepted: June 14, 2001

Abstract The biological control potential of entomopathogenic nematodes (EPN) can be enhanced by improved culture efficiency. Optimization of the media is a key factor for improving *in vitro* mass production of entomopathogenic nematodes. This study reports the effect of medium concentration. The medium is a combination of carbohydrates, lipids, proteins, salts, and growth factors, on the growth of *Heterorhabditis bacteriophora* and its symbiotic bacterium *Photorhabdus luminescens*. The overall optimal medium concentration for nematode recovery, hermaphrodite size, bacterial mass, infective juveniles (IJs) yield, and doubling time was 84 g/l. At this concentration rate, the doubling time of IJs production and the biomass of symbiotic bacteria was 1.6 days and 12.8 g/l, respectively. The maximum yield of 2.4×10^5 IJs/ml was attained within a one-generation cycle (eight days). The yield coefficient was 2.8×10^6 IJs/g medium, and the maximum productivity was 3.1×10^7 IJs per day. Medium concentration affected two independent factors, recovery and hermaphrodite size, which in turn influenced the final yield.

Key words: *Heterorhabditis bacteriophora*, *Photorhabdus luminescens*, fermentation, recovery, productivity, yield

Entomopathogenic nematodes (EPN) of the families Steinernematidae and Heterorhabditidae are recognized as effective biocontrol agents of insects [26]. They associate symbiotically with specific bacteria. IJs of *H. bacteriophora* carry the symbiotic bacterium *P. luminescens* in the lumen of their pharynx and intestine. They penetrate the insect host via natural body openings and release bacteria into the host's hemocoel [4, 25]. The bacteria multiply rapidly to cause a lethal septicemia, and the death of host occurs within 48 h. The nematodes ingest the bacteria and degraded host tissues, which provide essential nutrients needed for reproduction and development [4].

Much interest in EPN culture technology has increased with a strong demand for environmentally safe insecticides. Two processes, *in vivo* and *in vitro* cultures, have been developed commercially for mass production of EPN. *In vivo* culture has been used at the cottage industry level since minimal capital investment and expertise are required. In addition, a high yield coefficient further enhances the feasibility of *in vivo* culture for a small-scale production [2]. However, this process limits scale-up for mass production, that is sensitive to the physiological condition and pathogen load of the insect host [13]. For these various reasons, researchers have developed *in vitro* liquid culture processes amenable to the sterilization and scale-up process [7, 17, 24, 28]. As of this time, most research has focused on identifying essential nutrients for nematodes such as proteins [8], lipids [3], and additional growth factors [18, 19, 31]. As the significance of symbiotic bacteria for nematode growth has been revealed, monoxenic culture techniques have been developed [1, 4] and it is known that symbiotic bacterial quality [5], food signals [15], monoxenic inocula [22], and inoculum concentration of infective juveniles [16] significantly impact yield and productivity. In this study, we investigate how a complex medium concentration influences *H. bacteriophora* recovery, and the growth of adults (hermaphrodites). In addition, we investigated IJs of *H. bacteriophora* production kinetics, yield, and productivity in monoxenic culture fermentation.

Materials and Methods

Nematode and Bacterial Isolation

Wild-type *H. bacteriophora* was isolated from turfgrass plots in central New Jersey, U.S.A., using the method

*Corresponding author
Phone: 82-62-530-1992; Fax: 82-62-530-1973;
E-mail: skyoo@chonnam.chonnam.ac.kr

described by Bedding and Akhurst [5] and maintained in last instar *Galleria mellonella* by the method of Dutky *et al.* [11]. Active IJs were collected by modified White traps [21] which were used to initiate a monoxenic culture. The symbiotic bacteria were isolated by modifying the method of Akhurst [4]. Infected *G. mellonella* larvae were bled by cutting the legs, and hemolymph was dropped onto a sterile Petri dish. Hemolymph was streaked onto tryptic soy agar (Difco, Detroit, U.S.A.) containing 25 ppm bromothymol blue. The plates were incubated at 25°C for 48 h. A single phase 1 bacterial colony, chosen by the method of Hu and Webster [20], was transferred to the lipid agar [10] and incubated at 25°C for 48 h. These stock cultures were stored at 10°C and subcultured biweekly.

Media and Monoxenic Culture

The enriched liquid culture medium was prepared by using the following components per one liter of distilled water: 20 g soy flour, 5 g yeast extract, 5 g lactalbumin hydrolyzate, 20 g canola oil, 0.02 g cholesterol, 0.01 g liver extract, 5.0 g NaCl, 0.5 g $MgSO_4$, 0.3 g $CaCl_2$, and 0.3 g KCl. After adjusting the pH level to 7.0, media were homogenized, and then autoclaved for 25 min. Monoxenic culture of bacteria and nematodes was first established on lipid agar plates before being transferred to the liquid culture. IJs collected from White traps were surface-sterilized by using 0.1% (w/v) methyl benzethonium chloride and washed three times with sterilized distilled water, and then transferred to bacterial lawns on lipid agar plates. The plates were incubated at 25°C until covered with nematodes (about five days). Bacterial liquid culture was prepared by transferring a loop of colonies from stock culture into 40 ml of tryptic soy broth in each of the several 250-ml flasks. These culture flasks were incubated at 25°C and 200 rpm in a shaking incubator (NBS, New Brunswick, U.S.A.) until bacterial growth reached the early stationary phase. Next, nematodes grown on a lipid agar plate were aseptically transferred into the bacterial liquid culture flasks. The flasks were incubated under the established culture conditions for approximately ten days. These infective juveniles in monoxenic culture were used as a seed culture for all experiments.

Medium Concentration

We established several test concentrations, ranging from 0.5× to 4× the standard. An initial concentration of 224 g/l was dissolved in distilled water and homogenized. This mixture was serially diluted to 168, 84, 56, and 28 g/l. Aliquots of 40 ml were dispensed in triplicate into 250-ml culture flasks. These flasks were inoculated with 5% (v/v) bacterial culture and incubated at 25°C and 200 rpm in a shaking incubator for 24 h. Four thousand IJs/ml from seed liquid culture were inoculated into each bacterial culture flask, and these flasks were incubated under the previously established conditions.

Analytical Methods

To count nematodes during the fermentation process, samples of 100 µl from each treatment were diluted 100× in M9 buffer [6], and nematodes in 100 µl subsamples were counted under a stereo microscope (Nikon, Japan). Bacterial cell numbers were converted to the cell mass by using a standard curve, which was determined as follows. Several 40-ml samples of the bacterial culture were centrifuged at 4,000 rpm for 20 min (model CS-1R centrifuge with S4180 rotator, Beckman, CA, U.S.A.). The supernatant was discarded and cells were pooled. The cell pellets were washed twice with distilled water, and washed cells were resuspended to various densities. For each density, the bacterial number was determined by using a hemocytometer (0.02 mm depth and 1/400 mm^2). Counted aliquots were then transferred to aluminum weigh boats and oven-dried at 80°C to achieve a constant weight.

To determine nematode recovery, 100 µl samples were taken from culture flasks and diluted with distilled water. Diluted 100-µl subsamples were examined microscopically with the addition of Giemsa stain (EM Science, Gibbstown, U.S.A.), and the numbers of first generation adults (hermaphrodites) were counted. Percent recovery was calculated as the proportion of recovered to inoculated IJs/ml ×100.

To determine the size of first-generation adults, ten mature first-generation hermaphrodites were selected from each treatment. These were transferred into Petri dishes (60×15 mm) containing phosphate 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 lengths were determined with the aid of a video editor (Premiere 5.0, Adobe, U.S.A.).

To determine the number of IJs generated from hermaphrodites, twenty mature hermaphrodites of various sizes were chosen from the productive treatments. These were individually placed into wells of a 24-well plate filled with 500-µl aliquots of distilled water. After two days, infective juveniles generated from each hermaphrodite were carefully counted.

Statistical Analysis

Data were analyzed by ANOVA followed by a Student-Newman-Keul's multiple comparison test (α=0.05). Data are presented as mean±standard error of the mean.

RESULTS AND DISCUSSION

Industrial production of EPN generally employs a two-step fermentation process in *in vitro* liquid culture. The symbiotic bacteria are initially cultured in a complex media, then nematodes are aseptically inoculated. Liquid culture with *Heterorhabditis* has been limited due to its unstable yield and

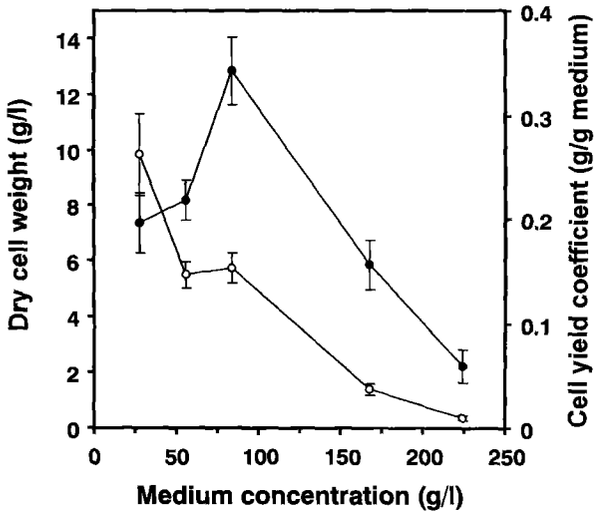

Fig. 1. Effect of medium concentration on growth of *Photorhabdus luminescens*.
Bacteria grown for 24 h in shaker culture at 25°C, 200 rpm, and culture pH 7.0. ●, Dry cell weight; ○, Yield coefficient (g dry cell weight/g dry media).

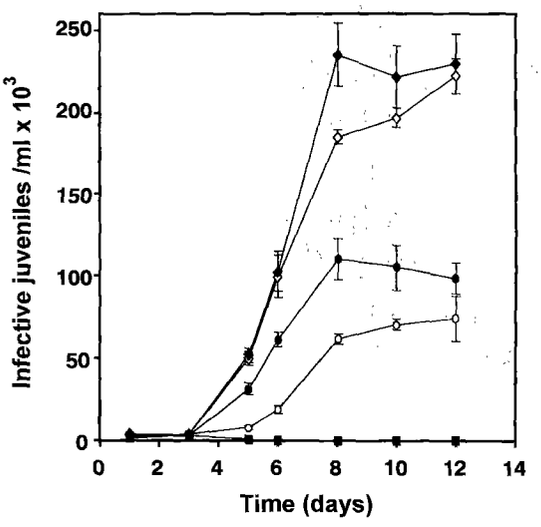

Fig. 2. Production time course of *Heterorhabditis bacteriophora* infective juveniles.
Two-step fermentation proceeded in shaker culture at 25°C and 200 rpm for 12 days. ○, 28 g; ◇, 56 g; ◆, 84 g; ●, 168 g; ■, 224 g.

prolonged process time [12, 30]. Critical factors influencing yield have included medium composition, oxygen supply, shear sensitivity, temperature, quality of symbiotic bacteria, recovery, and inoculum size [3, 8, 14, 16, 18, 19, 22, 23, 31, 32]. Our results indicate that medium concentrations significantly affect ($P<0.05$) the combined growth of *P. luminescens* and *H. bacteriophora*, which in turn influences yield and productivity (Figs. 1 and 2).

Biomass of symbiotic bacteria influences yields of IJs tremendously in both *in vivo* and *in vitro* cultures [20, 29]. Strauch and Ehlers [29] reported that bacterial mass influenced recovery of infective juveniles in a liquid culture. *H. bacteriophora* relate closely in their development and reproduction with the biomass of their symbiotic bacteria, *P. luminescens*. Therefore, optimization of *P. luminescens* growth is a prerequisite for improving *H. bacteriophora* yield. A generalized bacterial growth model exhibits saturation kinetics, followed by growth inhibition, as the nutrient concentration increases [9, 15, 32]. The growth of *P. luminescens* followed this model (Fig. 1). Bacterial mass increased with increasing medium concentration up to 84 g/l, beyond which growth was inhibited. Bacterial mass production did not correlate well with the bacterial yield coefficient, which peaked at the lowest concentration and declined thereafter.

Three to four days after the nematode inoculation, hermaphrodites were readily apparent at medium concentrations of 28 to 168 g/l, while only a few small adults were present at 224 g/l, resulting in a negligible infective juvenile production (Fig. 2). Infective juveniles were produced without any notable difference at medium concentrations of 56 and 84 g/l. In contrast, production was suppressed at the lowest and highest medium concentrations. The highest yield, 2.4×10^5 IJs/l, and productivity, infective juveniles 3.0×10^7/l/day, were achieved at 84 g/l in 8 days after the nematode inoculation (Table 1). The maximum yield, productivity, and production rates exceed previously published results for this nematode species. Surrey and Davies [30] reported

Table 1. Fermentation parameters of *Heterorhabditis bacteriophora* with various medium concentrations[1].

Concentration (g/l)	Doubling time[2] (days)	Yield (infective juveniles/ ml×10³)	Yield coefficient (infective juveniles/ g medium×10⁴)	Productivity (infective juveniles/ l per day×10⁵)
28	5.3	74.1	220.8	72.4
56	2.3	222.3	330.6	238.9
84	1.6	235.0	279.7	305.2
168	3.8	105.3	70.0	130.2
224	NC[3]	NC	NC	NC

[1]Bacteria and nematodes were grown in a shaker at 25°C, 200 rpm, and culture pH 7.0. Yield, yield coefficient, and productivity were determined eight days after nematode inoculation.
[2]Doubling time was obtained from the log phase of the infective juvenile production.
[3]NC: not counted.

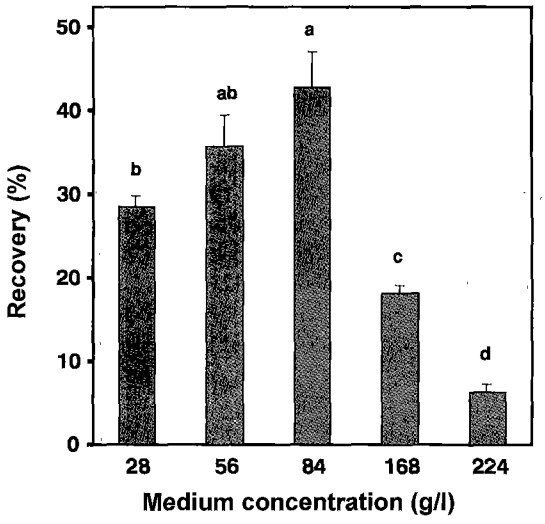

Fig. 3. Recovery of *Heterorhabditis bacteriophora* after 5 days in liquid culture.
Infective juveniles were inoculated into 24-h old bacterial cultures of *Photorhabdus luminescens*. Recovery values were significantly different among treatments ($P<0.05$). [a-d] Means with different superscripts differ (Student-Newman-Keul's multiple comparison test).

a maximum of 10^5 IJs of *H. bacteriophora* from batch fermentation in 15 to 20 days. Han [16] reported a maximum of 2×10^5 IJs/ml within 12 days in flask cultures inoculated with 5,600 IJs/ml.

We found that the recovery of inoculum and the average size of first-generation adults (hermaphrodites) correlate with infective juvenile yield. Strauch and Ehlers [29] reported that, in monoxenic liquid culture, unstable recovery causes inconsistent yield and productivity. Recovery responded positively with increasing medium concentration up to an optimal concentration, then decreased rapidly. In fact, the observed recovery trend corresponded to infective juvenile yield and productivity (Table 1).

An interesting result was that infective juvenile recovery at 28 g/l was greater than at 168 g/l (Fig. 3), but that the superior recovery did not translate to enhanced yield. Because hermaphrodites were of a similar size range at the lower and higher concentrations (Fig. 4B), the discrepancy in yield could not be attributed directly to nutritional deficiency of the lowest medium concentration in the first generation. However, the reproductive adult size and fecundity could have been reduced in subsequent generations, as it is in the case for *H. megidis* [12]. Hermaphrodite size varied less at the intermediate concentrations, and the size range obtained at these concentrations correlated with the greatest fecundity (Fig. 4A). The increase in fecundity with hermaphrodite length explains the observed differences in corresponding yields.

Maximal infective juvenile yield and productivity occurred at a medium concentration of 84 g/l. Fermentation parameters, as indicated by doubling time, suggest this concentration

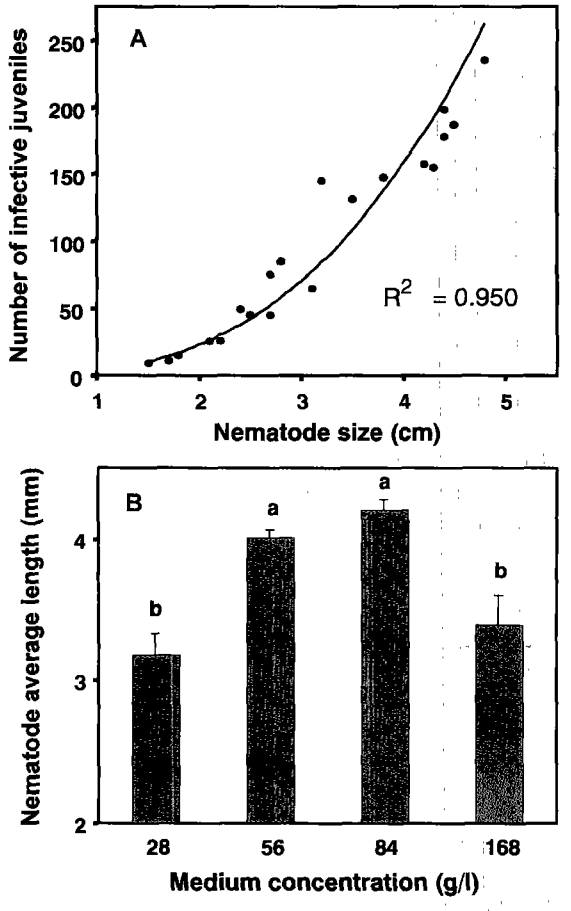

Fig. 4. Lengths of first-generation hermaphrodites of *Heterorhabditis bacteriophora* grown in liquid culture (A).
Lengths were significantly different among treatments ($P<0.05$). Number of infective juveniles produced from *Heterorhabditis bacteriophora* hermaphrodites of different lengths (B). Infective juveniles were inoculated into 24-h old bacterial cultures of *Photorhabdus luminescens* and were grown for one generation (about 8 days). [a-d]Means with different superscripts differ (Student-Newman-Keul's multiple comparison test).

as a key factor for optimizing the growth environment. Furthermore, nematode recovery, hermaphrodite size, and bacterial mass also peaked at this concentration, with only a modest reduction in the yield coefficient and with a concomitant decrease in time until it reached maximum production. Therefore, we can conclude that an increase in the medium concentration of 56 g/l to 84 g/l provides a simple and efficient step toward optimization of mass production of this entomopathogenic nematode species.

Acknowledgments

This research was funded in part by grants, which were received from the Tri-State Turfgrass Research Foundation, the Golf Course Superintendants Association of America, and the Rutgers Center for Turfgrass Science.

References

1. Abu Hatab, M. and R. Gaugler. 1997. Growth-mediated variations in fatty acids of *Xenorhabdus* sp. *J. Appl. Microbiol.* **82:** 351–358.
2. Abu Hatab, M., R. Gaugler, and R. U. Ehlers. 1998. Influence of culture method on *Steinernema glaseri* lipids. *J. Parasitol.* **84:** 215–221.
3. Abu Hatab, M. and R. Gaugler. 1999. Lipids of *in vivo* and *in vitro* cultured *Heterorhabditis bacteriophora*. *Biol. Contr.* **15:** 113–118.
4. Akhurst, R. J. 1980. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. *J. Gen. Microbiol.* **12:** 303–309.
5. Bedding, R. A. and R. J. Akhurst. 1975. A simple technique for the detection of insect parasitic rhabditid nematodes in soil. *Nematologica* **21:** 109–110.
6. Brenner, S. 1974. The genetics of *Caenorhabditis elegans*. *Genetics* **77:** 71–94.
7. Buecher, E. J. and E. L. Hansen. 1971. Mass culture of axenic nematodes using continuous aeration. *J. Nematol.* **3:** 199–120.
8. 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.
9. Doelle, H. W. and P. F. Greenfield. 1985. Fermentation pattern of *Zymononas mobilis* at high sucrose concentrations. *Appl. Microbiol. Biotechnol.* **22:** 411–415.
10. Dunphy, G. B. and J. M. Webster. 1989. The monoxenic culture of *Neoaplectana carpocapsae* DD-136 and *Heterorhabditis heliothidis*. *Revue de Nematologie* **12:** 12–123.
11. Dutky, S. R., J. V. Thompson, and G. E. Cantwell. 1964. A technique for the mass propagation of the DD-136 nematode. *J. Insect Pathology* **6:** 417–422.
12. 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*. *BioControl* **43:** 77–86.
13. Friedman, M. J. 1990. Commercial production and development. pp. 153–172, *In* Gaugler, R. and H. K. Kaya (eds.), *Entomopathogenic Nematodes in Biological Control*, CRC, Boca Raton, FL, U.S.A.
14. 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.
15. Giraud, E., B. Lelong, and M. Raimbault. 1991. Influence of pH and initial lactate concentration on the growth of *Lactobacillus plantarium*. *Appl. Microbiol. Biotechnol.* **36b:** 96–99.
16. Han, R. C. 1996. The effects of inoculum size on yield of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* in liquid culture. *Nematologica* **42:** 546–553.
17. Hansen, E. L. and W. S. Cryan. 1966. Continuous axenic culture of free-living nematodes. *Nematologica* **12:** 138.
18. Hieb, W. F. and M. Rothstein. 1968. Sterol requirement for reproduction of a free-living nematode. *Science* **160:** 778–779.
19. Hieb, W. F., E. L. R. Stokstad, and M. Rothstein. 1970. Heme requirement for reproduction of a free-living nematode. *Science* **168:** 143–144.
20. Hu, K. and J. M. Webster. 1998. *In vitro* and *in vivo* characterization of a small-colony variant of the primary form of *Photorhabdus luminescens* MD (Enterobacteriaceae). *Appl. Environ. Microbiol.* **64:** 3214–3219.
21. Kaya, H. and S. P. Stock. 1997. Techniques in insect nematology. *In* Lacey, L. A. (ed.). *Manual of Techniques in Insect Pathology*, pp. 281–324, Academic Press, San Diego, CA, U.S.A.
22. Lunau, S., S. Stoessel, A. J. Schmidt-Peisker, and R. U. Ehlers. 1993. Establishment of monoxenic inocula for scaling up *in vitro* cultures of the entomopathogenic nematodes *Steinernema* spp. and *Heterorhabditis* spp. *Nematologica* **39:** 385–399.
23. Pace, G. W., W. Grote, D. E. Pitt, and J. M. Pitt. 1986. Liquid culture of nematodes. Patent, International Publication Number WO 86/1074.
24. 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.
25. 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.
26. Poinar, G. O. Jr. 1990. Taxonomy and biology of Steinernematidae and Heterorhabditidae. pp. 23–61, *In* Gaugler, R. and H. R. Kaya (eds.), *Entomopathogenic Nematodes in Biological Control*, CRC Press, Boca Raton, FL, U.S.A.
27. Schmidt, T. M., K. Kopecky, and K. H. Nealson. 1988. Bioluminescence of the insect pathogen *Xenorhabdus luminescens*. *Appl. Environ. Microbiol.* **55:** 2607–2612.
28. Stoll, N. 1953. Axenic cultivation of the parasitic nematode, *Neoaplectana glaseri*, in a fluid medium containing raw liver extract. *J. Parasitol.* **39:** 422.
29. Strauch, O. and R. U. Ehlers. 1998. Food signal production of *Photorhabdus luminescens* inducing the recovery of entomopathogenic nematodes *Heterorhabditis* spp. in liquid culture. *Appl. Microbiol. Biotechnol.* **50:** 369–374.
30. Surrey, M. R. and R. J. Davies. 1996. Pilot-scale liquid culture and harvesting of an entomopathogenic nematode, *Heterorhabditis bacteriophora*. *J. Invert. Pathol.* **67:** 92–99.
31. Vanfleteren, J. R. 1974. Nematode growth factor. *Nature* **248:** 255–257.
32. Wang, D. L. C., C. L. Cooney, A. L. Demain, P. Dunnill, A. E. Humphrey, and M. D. Lilly. 1978. *Fermentation and Enzyme Technology*. John Wiley & Sons, New York, U.S.A.