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Submerged Monoxenic Culture Medium Development for *Heterorhabditis bacteriophora* and its Symbiotic Bacterium *Photorhabdus luminescens*: Protein Sources

Cho, Chun-Hwi[1], Kyung Sook Whang[2], Randy Gaugler[3], and Sun Kyun Yoo[4]*

[1]*KAFCO Biochemistry Research Institute, Choogchung, and* [2]*Department of Microbial and Nano Materials, Mokwon Uniersity, Daejeon 302-729, Korea*
[3]*Department of Entomology, Rutgers University, New Brunswick, NJ 08901-8524, USA*
[4]*Department of Food Science and Biotechnology, Joongbu University, Chungnam 312-702, Korea*

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Most medium formulations for improving culture of entomopathogenic nematodes (EPN) based on protein sources have used enriched media like animal feed such as dried egg yolk, lactalbumin, and liver extract, among other ingredients. Most results, however, showed unstable yields and longer production time. Many of the results do not show the detailed parameters of fermentation. Soy flour, cotton seed flour, corn gluten meal, casein powder, soytone, peptone, casein hydrolysates, and lactalbumin hydrolysate as protein sources were tested to determine the source to support optimal symbiotic bacteria and nematode growth. The protein hydrolysates selected did not improve bacterial cell mass compared with the yeast extract control, but soy flour was the best, showing 75.1% recovery and producing more bacterial cell number (1.4×10^9/ml) than all other sources. The highest yield (1.85×10^5 IJs/ml), yield coefficient (1.67×10^6 IJs/g medium), and productivity (1.32×10^7 IJs/l/day) were also achieved at enriched medium with soybean protein.

Keywords: Soy flour, *Heterorhabditis bacteriophora*, *Photorhabdus luminescens,* protein, protein hydrolysate

Entomopathogenic nematodes (EPN) of the families Steinernematidae and Heterorhabditidae have emerged as effective biological control agents for soil-dwelling stages of many insect pests [14, 17]. They retain many positive aspects, such as their broad host range, safety to non-target organisms, and exemption from environmental registration in many countries [15]. The free-living, non-feeding, and development-arrested infective juveniles (IJs) of *Heterorhabditis bacteriophora* possess the symbiotic bacterium *Photorhabdus luminescens* in the lumen of their pharynx and intestine [20, 21]. They penetrate the insect host *via* natural body openings including the mouth, anus, and spiracles and release the associated bacteria into the host's body cavity [3, 22]. The bacteria multiply rapidly to cause a lethal septicemia within 24 to 48 h and create a suitable environment for reproduction and development of nematodes [3].

Nematodes can be mass-produced by two processes, *in vivo* and *in vitro* culture methods. The *in vivo* process is very simple and requires only minimal initial investment [13]. However, this process lacks any economy of scale; the labor, equipment, and material costs increase as a linear function of production capacity [24]. As present, although some companies are producing IJs by *in vivo* culture, the yield and productivity are too variable. Therefore, *in vitro* culture technology is the best option for mass production of EPN ever since as early as 1930 Rudolf Glaser recognized the value of developing the artificial culture method. As of this time, most research has focused on identifying essential nutrient sources for a liquid fermentation technique for large-scale production of nematodes [8–10, 18, 26, 27]. Most reports for medium formulation based on protein sources have used enriched media like animal feed such as dried egg yolk, lactalbumin, and liver extract, among other ingredients [7, 11, 12]. Most results, however, showed unstable yields and longer production time. Although these protein sources support the nematode production, many of the results do not show the detailed parameters of fermentation. Therefore, ongoing efforts to optimize protein sources in liquid culture media are critical to increasing nematode production yields. In this study, we assessed how protein sources including their hydrolysates influence the yield and productivity of *H. bacteriophora* and their symbiont *P. luminescens* in liquid culture.

**Corresponding author*
Phone: +82-41-750-6206, 82-10-7234-0112; Fax: +82-41-750-6422;
E-mail: skyoo@joongbu.ac.kr

H. bacteriophora was maintained in a last instar *Galleria mellonella* and its symbiotic bacteria were isolated by following the method of Yoo *et al*. [27]. Active nematodes of IJ stage were collected within 4 days of emergence and filtered to remove dead nematodes before use. Symbiotic bacteria were isolated by streaking infected *G. mellonella* hemolymph onto tryptic soy agar containing 25 ppm bromothymol blue [16]. Bacteria culture was established on lipid agar plates at 25°C for 48 h. These stock cultures were stored at 4°C and subcultured biweekly.

Monoxenic cultures were performed on lipid agar plates, before transfer to liquid culture. The medium composition of lipid agar was the following components: yeast extract 5 g, canola: olive oil [50:50 (w/v)] 25 g, agar 20 g, cholesterol 0.2 g, liver extract 0.1 g, NaCl 4.0 g, $MgSO_4$ 0.5 g, $CaCl_2$ 0.3 g, and KCl 0.3 g, per 1 l distilled water. The medium was homogenized, adjusted to pH 7.0, and sterilized for 25 min at 121°C. Symbiotic bacteria were first cultured on lipid agar plate at 25°C for 24 h. Infective juveniles were surface-sterilized using 0.1% (w/v) benzethonium chloride, washed three times with sterile distilled water, and added to fresh bacterial lawns grown on lipid agar plates. About 5 days after at 25°C, the plates were mostly packed with IJs.

Soybean flour, cotton seed flour, corn gluten meal, casein powder, soytone, peptone, casein hydrolysates, and lactalbumin hydrolysates as protein sources were tested to determine the source to support optimal symbiotic bacteria and nematode growth. For each protein source, liquid culture was conducted in three 250 ml culture flasks containing 40 ml of the minimal medium. Minimal media were used so that the quality of each protein source was tested against a control containing 0.5% yeast extract. All proteins were assessed at a concentration of 3.5% (w/v). The media were autoclaved at 121°C for 25 min. Each flask was seeded with 24-h-grown bacterial culture [2% (v/v)] and incubated at 25°C and 200 rpm for 24 h. After 24 h, each flask was inoculated with surface-sterilized IJs (4,000/ml) and incubated for 14 days at 25°C and 200 rpm.

For all experiments, nematode and bacterial yields were determined using the following methods. Nematode juvenile stages (J2, J3, J4, and infective juveniles), hermaphrodites, and gravid adults were counted every two days, using a stereomicroscope unless stated otherwise. The growth of *P. luminescens* was determined by measuring bacterial cell number using a hemocytometer. 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 stereomicroscope (Nikon, Japan). 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, USA), and the numbers of first generation adult hermaphrodites were counted. Percent recovery was calculated as the proportion of recovered to inoculated IJs/ml ×100.

The submerged fermentation for EPN production employs two-step processes; the symbiotic bacteria are initially cultured in complex media, and then nematodes are cultured. The liquid culture of *Heterorhabditis* as a cruiser type of nematode has been intimidating owing to its inconsistent yield and productivity and prolonged process time [10, 26]. The parameters and factors influencing yield and productivity cover culture pH, temperature, dissolved oxygen, medium composition, quality of symbiotic bacteria, and degree of recovery [6, 11, 16, 19, 26].

Many researchers have investigated the media components commonly used for EPN production. As protein sources, examples include organs of various domestic animals, peptone, beef extracts, egg yolk, milk, and yeast extracts and many of the results do not show the detailed parameters of fermentation [7, 11, 12]. In particular, the significance of symbiotic bacterial culture has been ignored. Protein sources, protein hydrolysates (peptonized casein and lactalbumin, peptone, and soytone) rich in free amino acids and proteins (casein, corn gluten meal, cottonseed flour, and soy flour), supported symbiotic bacterial growth with different aspects (Fig. 1). Changes in protein hydrolysates did not improve bacterial cell mass compared with the yeast extract control. Most protein hydrolysates showed similar bacterial cell numbers. Soy flour was the best, producing more bacterial cell number (1.4×10^9/ml) than all other sources. The significance of quality and mass of symbiotic bacteria has been greatly recognized in the EPN production industry because higher quality and mass of symbiotic bacteria improve the yield and productivity of nematodes [2, 17]. We found that soy flour as a protein source supported the best growth of symbiotic bacteria. This is not unusual because symbiotic bacteria produce a

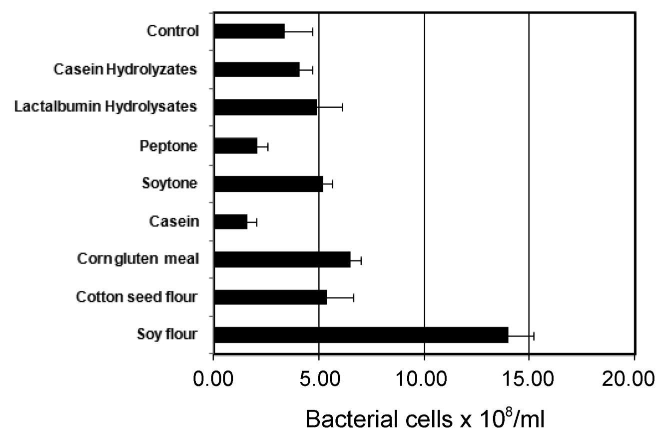

Fig. 1. The growth of *Photorhabdus luminescens.*
Bacteria were cultured for 24 h in shaker culture at 25°C, 200 rpm, and culture pH 7.0.

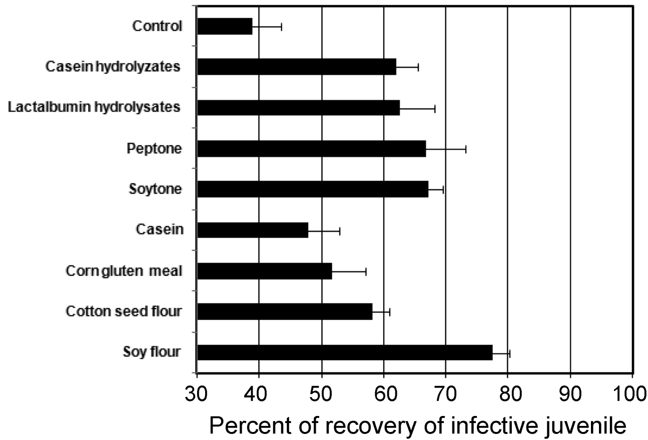

Fig. 2. Recovery of infective juveniles of *Heterorhabditis bacteriophora* after 6 days in liquid culture.
Infective juveniles were inoculated into 24-h-old bacterial cultures of *Photorhabdus luminescens* and cultured at 25°C, 200 rpm, and culture pH 7.0.

larger amount of various hydrolytic enzymes including protease [4, 5, 27].

We found that recovery of inoculum correlated with IJ yield (Fig. 2 and Table 1). Although changes with protein hydrolysates did not improve bacterial growth, they increased the percent of recovery and IJ yield (Table 1). High recovery is critical to improve nematode yield. Poor and unsynchronized recovery is the major reason for inconsistent yields [10]. Low recovery effectively decreases the initial inoculum, resulting in lower yield, and unsynchronized recovery produces inconsistent yield. The production of food signal to induce the recovery depends on the density of the bacterial cells [23]. However, the correlation recorded for the IJ recovery and bacterial number was low. This indicates that other factors possibly containing the protein hydrolysates also influence the IJ recovery. Soy flour was the best, same as the result of bacterial growth, recording 75.1%. This explains that hydrolysates of soy flour by hydrolytic enzymes of symbiotic bacteria might contain unknown material like a food signal [10].

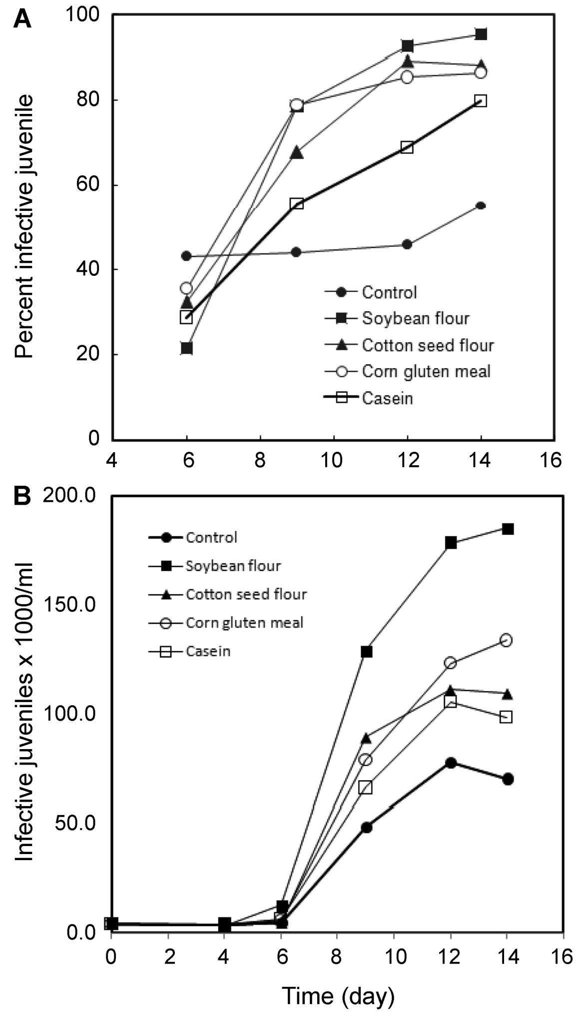

Fig. 3. Effects of protein sources (soybean flour, cotton seed flour, corn gluten meal, and casein) on the accumulation (**A**) and production (**B**) of *Heterorhabditis bacteriophora* infective juveniles in monoxenic culture.
Fermentation was conducted at 25°C, 200 rpm, and culture pH 7.0.

The media enriched with protein sources had a significant impact on IJs production (Fig. 3 and 4). Infective juveniles

Table 1. Fermentation parameters of *Heterorhabditis bacteriophora* with various protein sources.

Protein sources	Yield (IJs/ml×10^3)	Yield Coefficient (IJs/g medium×10^4)	Productivity (IJs/l /day×10^5)
Soybean flour	185.2±5.4	167.5±3.4	132.3±5.2
Cotton seed flour	111.2±4.3	100.5±2.9	79.4±2.1
Corn gluten meal	133.5±3.2	120.6±5.3	95.6±1.8
Casein	105.2±3.9	95.1±2.7	75.2±3.2
Soytone	165.6±4.5	149.7±4.8	118.3±4.1
Peptone	156.9±4.8	141.8±5.5	112.1±3.7
Casein hydrolysate	102.4±3.8	92.6±3.5	73.5±4.1
Lactalbumin hydrolysate	124.5±4.7	112.6 ±2.9	88.9±3.8

Bacteria and nematodes (monoxenic culture) were grown in a shaker at 25°C, 200 rpm, and culture pH 7.0. The yield, yield coefficient, and productivity were determined 12 days after nematode inoculation.

accumulation was sigmoidal and occurred within a single generation. The recovery was completed within 4 to 5 days post-inoculation at all protein sources. After recovery, hermaphrodites appeared at all protein sources. The lag phase lasted to 6 days and was followed by an exponential growth phase that lasted to 12 days. During the growth phase, the proportion of IJs increased rapidly at all protein sources (Fig. 3A and 4A). Most of the hermaphrodites had died by day 12 and the IJs accumulation had peaked (Fig. 3B and 4B). The complex protein hydrolysates such as soytone and peptone and soybean flour supported better production of nematodes than the other sources (Table 1). This indicates that there is a direct correlation between recovery and nematode production. The highest average yield (1.85×10^5 IJs/ml), yield coefficient (1.67×10^6 IJs/g medium) and productivity (1.32×10^7 IJs/l/day) were achieved at enriched medium with soybean protein. The parameters of these results were consistently obtained in one 12-day fermentation and exceed previously published results for this nematode species. Surrey and Davis [25] reported a maximum of 10^5 IJs/ml from batch fermentation in 15 to 20 days. Han [16] reported a maximum yield of 2×10^5 IJs / ml within 12 days. Furthermore, IJs proportion increased rapidly from 21.5% to 95.2%, indicating good developmental synchrony. Therefore, we can conclude that a protein source like soybean protein promotes nematode production because it improves the symbiotic bacterial growth and nematode recovery.

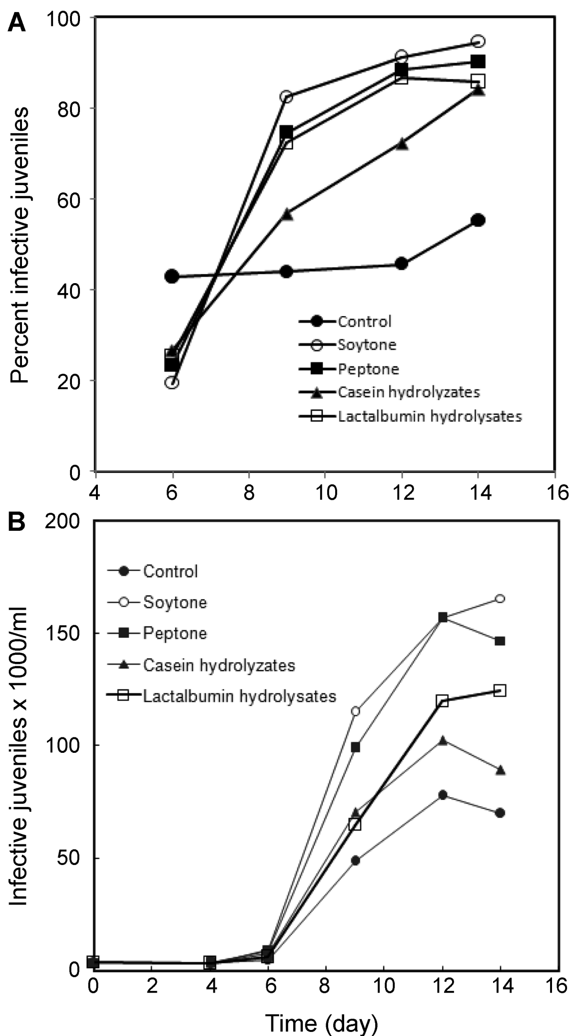

Fig. 4. Effects of protein sources (soytone, peptone, casein hydrolysate, and lactalbumin hydrolysate) on the accumulation (**A**) and production (**B**) of *Heterorhabditis bacteriophora* infective juveniles in monoxenic culture.

Fermentation was conducted at 25°C, 200 rpm, and culture pH 7.0.

Acknowledgments

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