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Growth Optimization of *Photorhabdus luminescens* Isolated from Entomopathogenic Nematode *Heterorhabditis bacteriophora*

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The yield of infective juveniles of *Heterorhabditis bacteriophora* (Tf strain) *in vitro* monoxenic liquid culture was improved significantly as the amount of symbiont biomass, *Photorhabdus* sp. strain Tf, increased. To investigate the influence of abiotic factors on the growth and biomass production of *Photorhabdus* sp. strain Tf, triplicate flask cultures were performed. The optinal temperature and medium pH for the growth of *Photorhabdus* sp. strain Tf were 30°C and between pH 5.5-7.3, respectively. Aeration also improved greatly growth and yield of biomass of *Photorhabdus* sp. strain Tf. *Photorhabdus* sp. strain Tf in batch fermentation showed growth-associated pattern in terms of pigment production, and the pH of culture medium rose steadily until growth stopped during the fermentation. Both pigment production and culture pH rise would be useful parameters indicating a reliable growth of *Photorhabdus* sp. strain Tf.

Key words: *Heterorhabditis bacteriophora*, symbiont, *Photorhabdus* sp., *in vitro* culture, pigment

The Gram-negative rod *Photorhabdus luminescens* is the only known bioluminescent terrestrial bacterium and a symbiotic bacterium of entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora*[4]. The free-living, non-feeding, and developmentally arrested infective juveniles (IJs) of *H. bacteriophora* carry *P. luminescens* in the lumen of their pharynx and intestine[11]. After entering into hemocoel of insect host via natural body openings, IJs release the bacteria into the host's hemolymph and recover to get into the developmental step[7]. Once released, *P. luminescens* proliferates rapidly to cause host death within 24 to 48 h and creates suitable environment for reproduction and development of nematode. The nematode and bacterial symbiont continue to grow and multiply within the infected insect until the emergence of new generation of infective juveniles carrying the bacterial symbionts to initiate a new infection cycle in another insect.

As the demand for environmentally benign insecticides has increased, interest in EPN production has raised even thought their full potential as an insecticide has been hardly realized due to non-competitive cost compared with chemical insecticides. Currently, EPN can be mass-produced by *in vivo* or *vitro* methods. Because *in vivo* process lacks the economy of scale-up, *in vitro* process such as monoxenic liquid culture technique has been developed[6]. EPN is mainly produced using a two-stage fermentation: symbiotic bacterial culture is first established to produce biomass and metabolites such as food signals, and then IJs are aseptically inoculated[13,15]. The quality of metabolites and the quantity of biomass of symbiotic bacteria have been known to influence greatly the yield of infective juveniles of nematode *in vivo* and *vitro* culture [9,13]. Strauch and Ehlers [13] reported that bacterial mass influenced the degree of recovery of IJs. Especially, *H. bacteriophora* relate closely their development and reproduction with biomass of their symbiotic bacteria, *P. luminescens* [8]. Therefore, optimization of *P. luminescens* growth is a prerequisite for improving the yield of *H. bacteriophora*. The present study was designed to evaluate the significance of biomass of *Photorhabdus* sp in mass production of IJs of *H. bacteriophora* and growth optimization of *Photorhabdus* sp in liquid culture.

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Materials and Methods

Bacterial Isolation and Culture Conditions

To isolate a symbiotic bacteria *Photorhabdus* sp.strain Tf, we infected last-instar *Galleria mellonella* with IJs of

H. bacteriophora at 25°C[5]. After one day post-infection, the larvae infected were bled onto a sterilized Petri dish by cutting legs. The hemolymph was streaked onto NBTA (nitroblue tetrazolium chloride agar) plate[1]. The plate was incubated at 25°C for 48 h. Only primary form bacteria were selected as described by Boemare *et al*[4]. The selected bacteria were maintained on tryptic soy agar plate (Difco, MI, USA) at 10°C, and subcultured every two week. A working culture was prepared by suspending a loopful of colony in 50 ml of tryptic soy broth (Difco, MI, USA) containing 2% (w/v) sucrose in a 250 ml-culture flask, and then was incubated at 25°C and 200 rpm on orbital shaker (NBS, NJ, USA).

Monoxenic Culture

The mass of *Photorhabdus* sp. was produced in liquid culture medium containing the following components per one liter of distilled water: soy flour 20 g , yeast extract 5 g, lactalbumin hydrolyzate 5 g, canola oil 20 g, cholesterol 0.02 g, liver extract 0.01 g, NaCl 5.0 g, $MgSO_4$ 0.5 g, $CaCl_2$ 0.3 g, and KCl 0.3 g. The media components except the salts were supplied from Sigma Chemical Company, MO, USA. The liquid medium was homo-genized by an agitation using a magnetic stirrer overnight. The homogenized liquid medium pH was adjusted 7.0 prior to sterilization. Monoxenic culture was prepared using the method of Yoo et al.[15].

Optimization of Growth and Biomass Production of *P. luminescens*

Triplicate culture experiments were used to optimize various process parameters, such as initial culture temperature, initial medium pH, aeration intensity, and medium concentration. For optimal growth and biomass production optimization, unless otherwise specified, the following medium composition was used for all culture studies: 3% (w/v) tryptic soy broth containing 2% (w/v) sucrose. Inoculum, 2% (v/v) was provided from 24 h-old working culture. To determine of optimal temperature, triplicate culture flasks containing medium (pH 7.0) were incubated at temperature range of 15 to 35°C in temperature controlled incubators. The determination of pH optima was performed at the range of pH 4.5 to 8.3. To determine the aeration effect, bacterial culture was performed at 100, 200, and 300 rpm, and at each condition oxygen transfer rate (K_La) was determined using values of the actual amount of dissolved oxygen measured by a dissolved oxygen meter (YSI model 58 with YSI 5730 dissolved oxygen probe, Yellow Springs Instrument, Ohio, USA). Control culture was done without aeration (standing). Five different medium concentrations, ranging from 3% to 28% (w/v), were tested to determine the optimum substrate concentration for bacterial growth and biomass production. The medium was prepared by combining 3% (w/v) tryptic soy broth with different sucrose concentrations.

Analysis

To count nematode during the fermentation, samples of 100 μl from each treatment were diluted 100× in M9 buffer and then nematodes in 100 μl subsamples were counted under a stereo microscope (Nikon, Japan). The growth of *Photorhabdus* sp. was determined by measuring the biomass as described previously by Otts and Day[10]. Dry weight cells were determined by weighing drying washed cells in tared aluminum dishes at 105°C for 12 h before weighing them. Values of optical density measured at 660 nm were converted into the weight of dried biomass; an absorbance of 4.0 was equal to 5.92 mg/ml dry weight of bacteria. The red color intensity produced was spectrophotometrically measured at 490 nm. The data were analyzed using ANOVA, and Students-Newman-Keul's multiple comparison test (α=0.05) was used for mean comparisons. Data are presented as mean ± standard error of the mean.

Results and Discussion

The significance of quality of symbiotic bacteria has been greatly recognized in the EPN production industry because higher quality of bacterial culture improves the yield of IJs *in vitro* production[9]. The quality of symbiotic bacteria is specified into two groups, primary and secondary form, according to their morphology and biochemical characteristics. The primary form produces larger amount of various hydrolytic enzymes[3,14], antibiotics[2], pigments [12], and bioluminescent[11]. Many researchers reported that primary form bacteria significantly improved the EPN growth, eventually increased the yield of IJs. Recently, Han and Ehlers[8] suggested that the growth of symbiotic bacteria, *P. luminescens*, related closely to *H. bacteriophora* development and reproduction. In the present study, further work was achieved to determine the role of bacterial mass in nematode production. We performed two-step fermen-

tation, in which *Photorhabdus* sp. grew first at complex media and then IJs were inoculated until first generation [15]. As the data shows the yield of IJs *in vitro* culture is significantly dependent of the amount of symbiont (Fig. 1). The increased more biomass triggered to excrete more food signals inducing the recovery of IJs [13]. Therefore, this result strongly suggests that a process improvement for the higher bacterial biomass production should be considered in the EPN production.

The bacterial growth and biomass production by *Photorhabdus* sp. strain Tf varied significantly dependening on

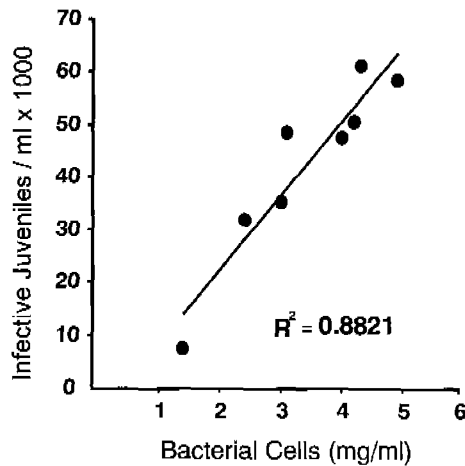

Fig. 1. Correlation between bacterial mass of *Phtorhabdus* sp. strain Tf and the yield of infective juveniles of *Heterorhabditis bacteriophora*.

the initial culture temperature (Fig. 2). The specific growth rate and biomass yield coefficient increased gradually with increasing culture temperature up to 30°C and both declined rapidly at 35°C. Values of the specific growth rate between 25 and 30°C was not significantly different, but biomass yield coefficient at 30°C was greater than that at 25°C. The optimum growth temperature of *Photorhabdus* sp. strain Tf was different from the previous report where constant specific growth rate was observed over a temperature range of 24 to 25°C in a complex medium[3,10,14]. The specific growth rate and biomass yield coefficient of *P. luminescens* sp. strain Tf were influenced by significantly by the initial pH of culture medium (Fig. 3). The specific growth rate increased rapidly when the medium pH was increased from 4.5 to 5.5, and rose slightly to 7.3, but declined over this pH. Changing the medium pH from 5.5 to 7.3 did not significantly alter the biomass yield coefficient. This result indicates the necessity of keeping the medium pH in the range of 5.5 to 7.3 to ensure high biomass yield coefficient in batch fermentation. The growth of *Photorhabdus* sp. strain Tf affected significantly by intensity of aeration represented by oxygen transfer rate and shaking speed (rpm) (Fig. 4). The average of specific growth rate and biomass yield coefficient increased continuously from to 200 rpm, but remained constant at higher agitation speed (300 rpm). Increasing agitation speed from 0 to 100 rpm enhanced 2 and 4 fold of the growth rate and biomass

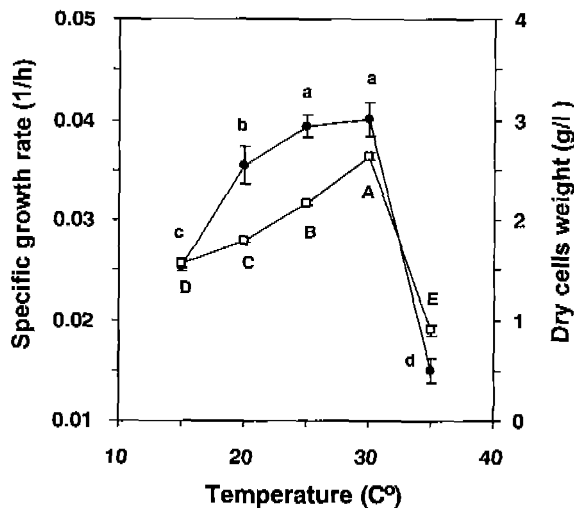

Fig. 2. Effect of culture temperature on the specific growth rate and bacterial mass of *Phtorhabdus* sp. strain Tf. Batch culture was conducted at pH 7.0 and 200 rpm on shaker for 24 h.
Symbols: specific growth rate (●) and biomass (□).

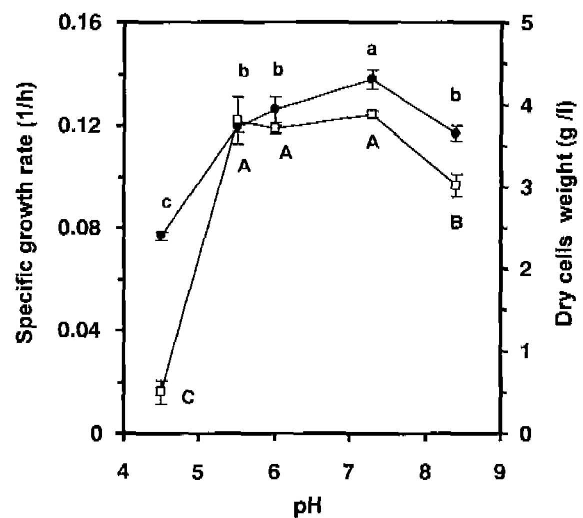

Fig. 3. Effect of culture pH on the specific growth rate and bacterial mass of *Phtorhabdus* sp. strain Tf. Batch culture was conducted at 25°C and 200 rpm on shaker for 24 h.
Symbols: specific growth rate (●) and biomass (□).

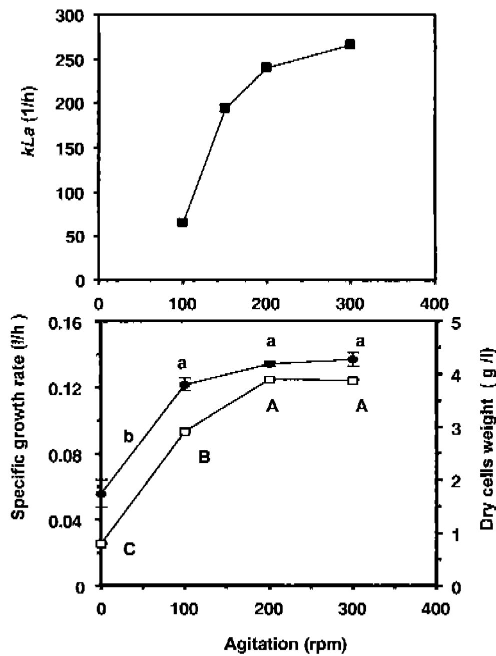

Fig. 4. Effect of aeration on the specific growth rate and bacterial mass of *Phtorhabdus* sp. strain Tf. Batch culture was conducted at 25°C, pH 7.0 and 200 rpm on shaker for 24 h.
Symbols: specific growth rate (●) and biomass (□).

production of *P. luminescens*, respectively. The specific growth rate did not changed significantly above 100 rpm, whereas cell yield coefficient at above 200 rpm was significantly higher at 100 rpm. Agitation with higher 200 rpm did not enhance the growth and cell yield coefficient. These dada indicate that aeration might be a critical factor to improve specific growth rate and biomass yield coefficient. In order to study growth repression by sucrose concentration, different surose amount ranging from 3 to 28% in a complex medium was used in a batch fermentation. The specific growth rate and biomass yield coefficient varied significantly, depending on different initial sucrose concentration (Fig. 5). The specific growth rate decreased gradually from 18 to 28%, but its average value was not significantly different in a medium containing 3 to 18% sucrose. Biomass yield coefficient increased up to 13% of sucrose concentration, but decreased at higher concentration of sucrose. This result indicates that about 13% sucrose concentration might ensure maximum biomass and specific growth rate in batch fermentation. Our result confirms that biomass yield coefficient exhibits saturation-type kinetics as the nutrient concentration is increased, but higher than 13% sucrose suppresses the growth *P. luminescens* sp.

Pigment presence and color produced by symbiotic

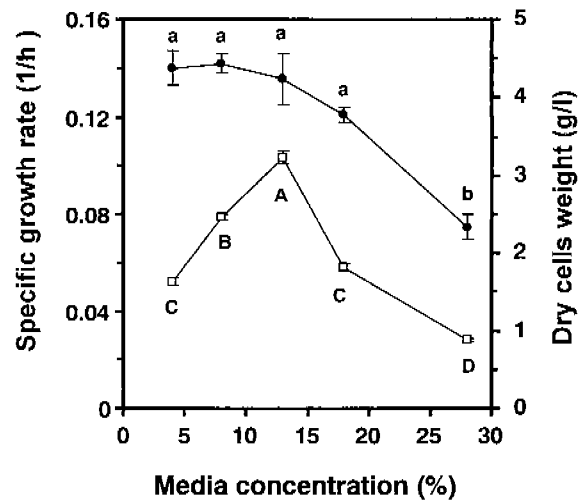

Fig. 5. Effect of media concentration on the specific growth and bacterial mass of *Phtorhabdus* sp. strain Tf. Batch culture was conducted at 25°C, pH 7.0 and 200 rpm on shaker for 24 h.
Symbols: specific growth rate (●) and biomass (□).

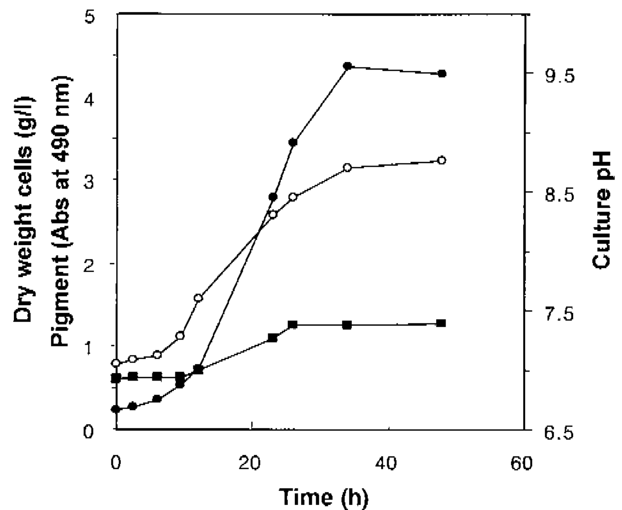

Fig. 6. Growth kinetics of *Phtorhabdus luminescens* on batch culture. Culture conditions were at 25°C, pH 7.0, and 200 rpm on shaker for 24 h.
Symbols: Pigment pH (■), Culture pH (○), and dry weight cells (●).

bacteria of nematodes can be used as a taxonomic study. Secondary form of *P. luminescens* lacks many of the primary form characteristics and cannot support nematode reproduction[9]. Of metabolites of primary form bacteria, pigment can be used as a growth indicator if the pattern of pigment production linked to the bacterial growth in the liquid culture. Very little is known about the kinetic relationship between bacterial growth and pigment pro-

duction except that maximum food signal inducing nematode recovery is produced at the end of log phase of bacterial growth[13]. We found that pigment was not detected at lag phase of 10 h, but its production was increased rapidly at log phase, and peaked at stationary phase of growth (Fig. 6). Culture pH was increased constantly during the period of fermentation and reached pH 8.7 at stationary phase. We suggest that culture pH and pigment color *in vitro* liquid culture could be reliable growth indi-cators for *P. luminescens,* and this parameter can be used in the two-step fermentation process for mass production of entomopathogenic nematodes.

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국문초록

병원성 선충 *Heterorhabditis bacteriophora*에서 분리된 공생 박테리아 *Photorhabdus luminescens*의 생장조건

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공생 박테리아*Photorhabdus* sp. strain TF 균체량이 증가함에 따라서 병원성 선충 *Heterorhabditis bacteriophora*의 Infective Juveniles의 생산이 증가 되었다. 이 공생 박테리아의 성장 최적 조건은 각각 배양 온도 30°C 그리고 초기 배지 pH 5.5 부터 7.3 사이에서 얻어졌다. 통기 상태에서 박테리아 균체의 생산과 성장이 촉진됨이 밝혀졌다. *Photorhabdus* sp. strain TF 박테리아의 성장 중 생산된 색소와 배양액의 pH의 변화는 박테리아의 성장 정도 나타내어 액체배양에서 병원성 선충 *Heterorhabditis bacteriophora*의 접종시기로 표시로서 이용할 수 있을 것이다.

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