*International Journal Of*

**Recent Scientific Research**

**ISSN: 0976-3031**

**Volume: 6 (12) December -2015**

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THE OFFICIAL PUBLICATION OF
INTERNATIONAL JOURNAL OF RECENT SCIENTIFIC RESEARCH (IJRSR)
http://www.recentscientific.com/ recentscientific@gmail.com

ISSN: 0976-3031

Available Online at http://www.recentscientific.com

*International Journal of Recent Scientific Research*
*Vol. 6, Issue, 12, pp. 7684-7688, December, 2015*

*International Journal of Recent Scientific Research*

**RESEARCH ARTICLE**

# EFFECT OF ENVIRONMENTAL FACTORS ON GROWTH KINETICS OF *Photorhabdus luminescens* PHASE-I CELLS USING A 2L A+ SARTORIUS STEDIM BIOSTAT ® FERMENTATION SYSTEM

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**ARTICLE INFO**

***Article History:***

Received 05th September, 2015
Received in revised form 08th October, 2015
Accepted 10th November, 2015
Published online 28st December. 2015

***Key words:***

Heterorhabditis bacteriophora, *Photorhabdus luminescens,* specific growth rates, doubling times, growth kinetics

**ABSTRACT**

*Photorhabdus luminescens*, a pigment-producing enteric bacterium and symbiont of *Heterorhabditis bacteriophora*, was studied in batch cultures to determine the specific growth rates and doubling times. The purpose of this experiment is to investigate the growth kinetics of *Photorhabdus luminescens* Phase-I in a bioreactor containing complex growth media at different environmental conditions. Fermentation parameters were controlled utilizing a Sartorius Stedim Biostat® A+bioreactor systemover the course of the bacterial growth. The reactor parameters included pH, temperature, agitation and aeration. The specific growth rates and doubling times of *P. luminescens* were determined. The study showed that the parameters of 1.0 vvm, pH 7.8, 28°C and 300 RPM had the highest specific growth rate 0.59 $h^{-1}$ and the lowest doubling time 1.1 hr. The bacterium, *P. luminescens* growth was best under those conditions.



## INTRODUCTION

*Photorhabdus luminescens* is a Gram-negative, bioluminescent, pigment-producing enteric bacterium, which is pathogenic to insects and has the capability to undergo Phase variation (Figure 1) [Inman and Holmes, 2012; Bowen *et al*., 2012]. This bacterium is able to shift between two metabolically different states; Phase-I and Phase-II. The Phase-I variant of *P. luminescens* exists as a symbiont where it plays a critical role in the life cycle of the nematode, *Heterorhabditis bacteriophora* [Gerdes *et al*., 2015; Han and Ehlers, 2001]. Phase-I variants occur in the infective-stage nematodes and produces an extracellular protease, an extracellular lipase, antibiotic substances, and intracellular protein crystals. The Phase- Ivariant also has the trait of bioluminescence and red pigmentation (Figure 2) [Williamson and Kaya, 2003].

Bacterial growth can be followed after bacteria are inoculated into a liquid medium and the bacterial density is plotted over time [Madigon *et al*., 2011]. There are four distinct Phases of bacterial growth: (1) lag phase; (2) log phase; (3) stationary

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phase and (4) death or decline phase. The lag phase is the period when bacteria are adjusting to the conditions in the liquid medium and there is little or no cell growth. This is followed by the log phase when bacterial cells begin to divide and cell growth is exponential. The number of cells produced is higher than the number of cells dying. During stationary phase bacterial growth begins to stabilize (number of cells produced equals the number of cells dying) and bacterial cell division begins to decrease. In the final phase, death (decline) phase, bacteria cell population decreases (number of cells dying is higher than the number of cells produced) and cells lose their ability to divide [Madigon *et al*., 2011]. Microbial growth kinetics is the relationship between the specific growth rate (μ) of a microbial population and the substrate concentration (s) or the environmental parameters [Kova and Egli, 1998].

The symbiotic relationship between *H. Bacteriophora* and *P. luminescens* is complex [Patterson *et al*., 2015]. Infective adolescent nematodes provide their bacterial symbionts with: (1) protection from environmental conditions; (2) a route to gain access to "food" (e.g. insect hemolymph); and (3) a method of transportation (a vector) from host to host. *P. luminescens* gives numerous advantages to its nematode accomplice: (1) protection from insect immunity, (2) growth factors from bioconversion of the insect, (3) production of antimicrobials that protect the insect cadaver from other invading organisms, (4) secretion of "food signals" that cause nematodes to develop and (5) being the fundamental nourishment source [Bowen *et al.,* 2012].

Scientists have been searching for alternative biocontrol insecticides because of the fear that chemical pesticides can cause harm to humans and the environment. Recent experiments have introduced *P. luminescens* and its symbiotic nematode, *H. bacteriophora* as a potential alternative to chemical poisons [Peel *et al*., 1999; Morgan *et al*., 1997]. The symbiotic pair does not stimulate insect resistance nor does it show toxicity to humans, wild life or plants [Rodou *et al*., 2010; Waterfield *et al*., 2004, Ffrench-Constant *et al*., 2007]. It also displays a wide range of insect hosts [Akhurst and Smith, 2002; Georgisand Gaugler, 1991; Georgis *et al*., 1991].

In order to mass-produce *Heterorhabditis bacteriophora*, its bacterial symbiont *Photorhabdus luminescens*, must be mass-produced as well [Inman *et al*., 2012]. To grow P. luminescens, you must first grow it in nutrient medium up to the stationary phase. After approximately 24 hours, infective juvenile nematodes, which are suspended in enriched nutrient broth (eNB), are inoculated into the bacterial culture. To check the entomopathogenicity, *Galleria mellonellais* infected with the nematodes along with its symbiont, and if *G. mellonella* death occurs within 48 hours, the insect pathogenicity of the H. Bacteriophora-*P. luminescens* combination is confirmed [Singh *et al*., 2012; Kooliyottil *et al*., 2013].

Based upon the preceding information, the present study describes the growth kinetics of *P. luminescensin* a bioreactor under different environmental conditions: pH, temperature, agitation and aeration to determine the optimum condition for achieving aximum bacterial growth rate. This study is crucial to develop protocols for mass production of the nematode *Heterorhabditis bacteriophora*. It is to the authors' knowledge that there are no reports regarding the effect of various environmental conditions to study growth kinetics of *P. luminescens* Phase-I cells in a bioreactor.

## MATERIALS AND METHODS

### *Bacterial Isolation and Scale up*

*P. Luminescens* was isolated from nematode (*Heterorhabditis bacteriophora*) infected larvae of Wax Moths (*Galleria mellonella*) by the method of Inman and Holmes [Inman and Holmes, 2012]. Five to ten larvae of *G. mellonella* were surface cleaned with 10% isopropanol solution and set onto sterile filter paper containing *Heterorhabditis* nematodes. These infected *G. mellonella* were placed at room temperature for 48 to 72 hours in the dark. Then, infected dead *G. mellonella* were surface sanitized by submerging them in 70 % ethanol for 3-5 seconds and the dead larvae were aseptically dissected. A loop of infected hemolymph was streaked onto nutrient agar (NA) plates for the isolation of Phase-I cells. Plates were incubated at 28°C for two days until red-pigmented bacterial colonies appeared confirming the presence of Phase-I cells (Figure 3).

According to Boemare and Akhurst, the adsorption of bromothymol blue in the presence of triphenyltetrazolium chloride the NBT Amedia and the blue colonies that form on the plates suggest that there is the existence of Phase-I cells [Boemare and Akhurst, 1988]. Red colonies on the NBTA media suggest that there is Phase-II cells present. The red from the NA plates, with the production of luminescence suggests the existence of Phase-I cells [Boemare and Akhurst, 1988]. NBTA contained per liter: 8.0 g nutrient agar; 25 mg bromothymol blue; 40 mg 2,3,5-triphenyltetrazolium chloride (TTC); Nutrient agar contained per liter: 5 g peptone; 3 g beef extract; 15 g agar [Upadhyay *et al*., 2013]. A single colony was transferred to 5 mL 2x NB broth and incubated at 28°C at 150 rpm on an orbital shaker. After the 24 hours, the 2x NB culture was pigmented red. To upscale the culture, 1 mL of the Phase-I culture was added to a flask containing 50 mL of 2x NB [Boemare and Akhurst, 1988].

### *Culture Media &Fermenter Preparation and Experimental Designs*

Liquid media are sometimes used when a large number of bacteria are required. The media used for these experiments in the fermenter was 2xNutrient Broth containing per liter: 3 g beef extract and 5 g enzymatic digest of gelatin [Singh *et al*., 2012]. The working volume of NB in the bioreactor was a total of 1.5 liters. The pH was adjusted before auto claving. The vessel and all reactor parts were sterilized to remove all contaminants. After cool down, the bacterial inoculums was antiseptically added to the reactor (Figure 4). Experimental designs were set according to Table 1 to check the effect of environmental parameters on growth kinetics of *Photorhabdus luminescens* Phase-I cells.

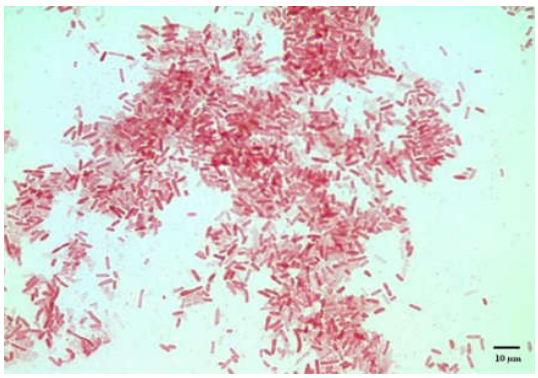

**Figure 1** Gram-stain of *Photorhabdus luminescens (*1,000 x). [Inman and Holmes, 2012]

**Figure 2** 1.5 L bioreactor containing phase- I *Photorhabdus luminescens*

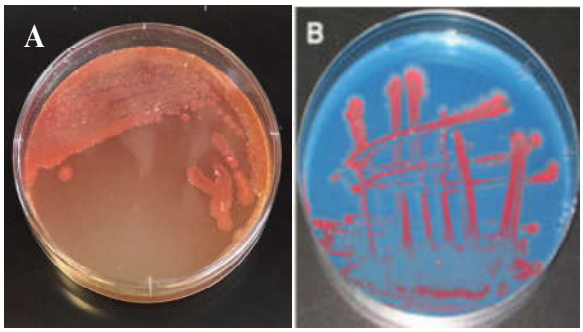


**Figure 3** Phase-I *Photorhabdus Luminescens* on A) Nutrient Agar and Phase-II *Photorhabdus Luminescens B*) NBTA plates. [Inman *et al.,* 2012]

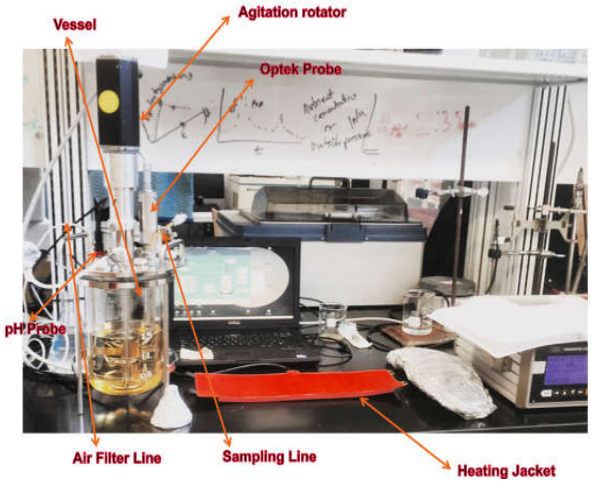


**Figure 4** Photo of assembled bioreactor. The bioreactor is ready for *P. luminescens* bacteria inoculation.

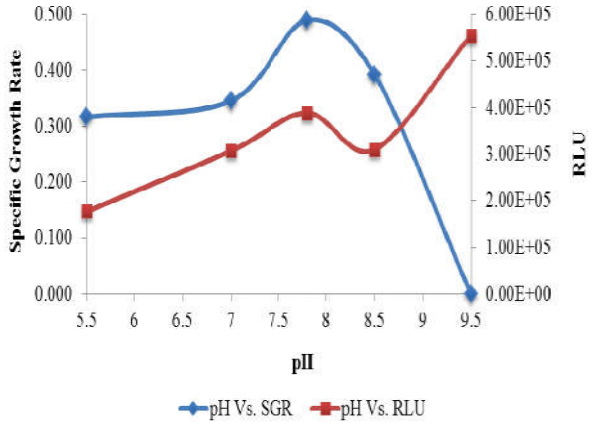


**Figure 5** Effect of pH on bacterial growth and bioluminescence

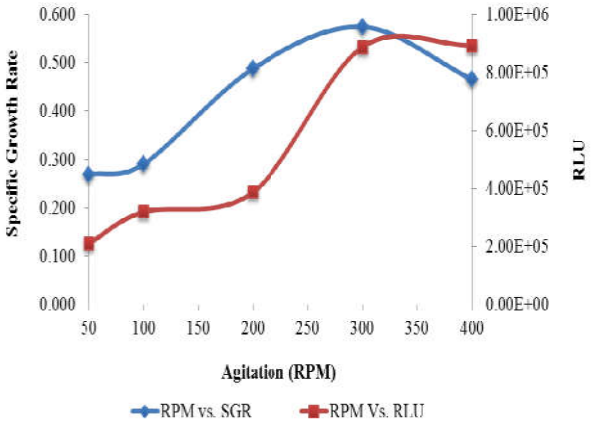


**Figure 6** Effect of agitation on bacterial growth and bioluminescence

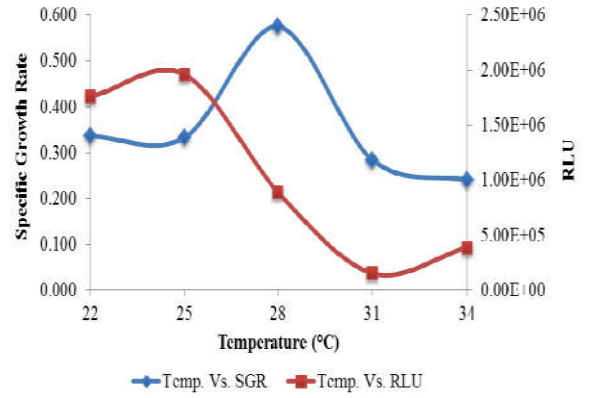


**Figure7** Effect of temperature on bacterial growth and bioluminescence

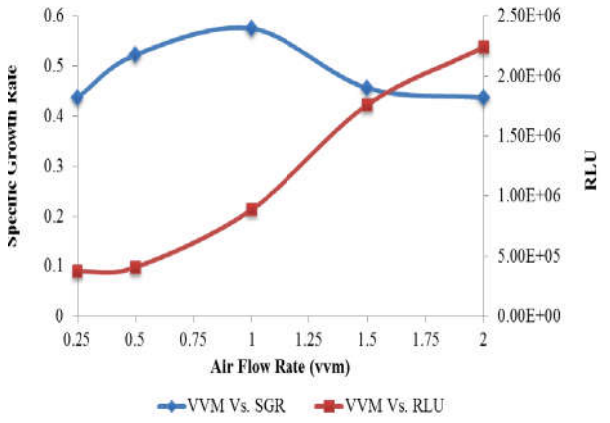


**Figure 8** Effect of air flow rate on bacterial growth and bioluminescence

**Table 1** Experimental Designs

| Parameters | Design 1 | Design 2 | Design 3 | Design 4 |
|---|---|---|---|---|
| PH | 5.5, 7.0, 7.8, 8.5, 9.0, 9.5 | 7.8 (Best Condition) | 7.8 | 7.8 |
| Temp (°C) | 28 | 28 | 22, 25, 28, 31, 34 | 28 (Best Condition) |
| RPM | 200 | 50, 100, 200, 300, 400 | 300 (Best condition) | 300 |
| Air Flow Rate (vvm) | 1 | 1 | 1 | 0.25, 0.5, 1 (Best Condition), 1.5, 2.0 |

***Determination of Specific Growth Rates (SGR) and Doubling Times (DT)***

Specific growth rate (SGR) (μ) is defined as the increase in bacterial cell mass per unit time during the exponential phase [Singh *et al*., 2012]. The most common units are in reciprocal hours ($h^{-1}$); however, it can also be expressed in reciprocal seconds ($s^{-1}$) or minutes ($min^{-1}$). Doubling time (DT) is the time it takes for the growth of the bacteria in a culture to double. The specific growth rate for each batch was determined by plotting natural log of concentration units as a function of time [Bowen *et al*., 2012]. The density data was collected by the Optek®cell density recorder. Specific growth rates and doubling times were calculated by using Microsoft ®Excel software. All data were entered into Excel and a scatter plot graph was made to determine SGR and DT. The SGR was determined by using the data points on the graph that had linear growth. The doubling time was determined by using the equation:

$$\mu = \frac{\ln(2)}{td}$$

Where, μ is SGR and $t_d$ is doubling time [Madigon *et al*., 2011].

***Determination of RLU***

Since *P. luminescens* is a bioluminescent bacterium, the luminescence was measured to determine Phase variation based on pigmentation. Relative luminosity units (RLUs) were measured utilizing a Turner Biosystems Modulus™ single-tube luminometer using 1 mL samples of each culture [Inman and Holmes, 2012].

## RESULTS AND DISCUSSION

Design1. Fermentation conditions were: 28°C; 200 rpm; 1vvm. The pH ranged from 5.5 to 9.5. Maximum specific growth rate (0.49 $hr^{-1}$) was observed at pH 7.8. The slowest growth rate (0.32 $h^{-1}$) was observed at pH 5.5 and increased with pH up to pH 7.8 (Figure 5). No growth was observed at pH 9.5. Inman *et al.* (2012) reported optimum growth at pH 7.3. With respect to bacterial doubling times (DT), the shortest doubling time (1.4 h) was observed at pH 7.8; and as expected the longest doubling time (2.2 h) was measured at pH 5.5. At pH 9.5 doubling time was not calculated. Using nutrient broth media, Singh *et al*. (2012) reported a specific growth rate of 0.36 $h^{-1}$ and a doubling time of 2.1 h at pH 7.2. All bioluminescence measurements (RLU) were taken at approximately 20 hours after inoculation during stationary Phase. As with the growth pattern above, RLU increased from pH 5.5 ($1.7 \times 10^5$) to pH 7.8 ($3.9 \times 10^5$). All cells showing bioluminescence were Phase-I with rod morphology. The culture at pH 9.5 showed no bioluminescence due to lack of growth.

Design 2. Agitation was varied from 50 rpm to 400 rpm at optimal pH and temperature conditions determined above (pH 7.8 and 28°C). Aeration was maintained at 1 vvm. The maximum specific growth rate was measured at 0.59 $hr^{-1}$ at 300 rpm. At 50 rpm the specific growth rate was 0.27 $hr^{-1}$ (Figure 6). The doubling time at 300 rpm was 1.1 hr. Figure 6 shows that bioluminescence (RLU) increased with agitation speed, maximum 300 rpm ($8.9 \times 10^5$). In another report by Jallouli *et al.* (2012) investigating agitation effects on *Photorhabdus temperata*, optimal growth was observed at 500 rpm using a 3-Liter bioreactor whereas in this study a 2-Liter vessel was used.

Design 3. Fermentation conditions were 7.8 pH, the optimum pH condition for maximum bacterial growth as described above. The other parameters were 200 rpm and 1 vvm air. The temperature was varied from 22°C to 34°C. Previous research [Inman *et al*., 2012] reported the optimum temperature for *Photorhabdus luminescens* growth to be 28°C. The highest specific growth rate (0.58 $hr^{-1}$) was observed at 28°C in this research study (Figure 7). The minimum SGR (0.24 $hr^{-1}$) was observed at 34°C. The shortest doubling time (1.2 hr) was measured at 28°C. Bioluminosity ranged from $1.5 \times 10^5$ to $8.9 \times 10^5$ with the maximum at 28°C and lowest at 31°C. Inman *et al* concluded that bioluminescence variation may also be a result of Phase shifting at temperatures above and below the optimum growth temperature of 28°C.

Design 4. Under the optimized conditions above: (a) pH 7.8 (b) temperature 28°C and (c) Agitation 300 rpm. The air flow rate was varied between 0.25 vvm to 2.0 vvm and optimal growth was observed at 1 vvm. Bacterial growth was inhibited at flow rates greater than 1 vvm. However, bioluminescence continued to increase beyond 1 vvm to a maximum $2.3 \times 10^6$ (Figure 8). This was the highest RLU over entire study design. Previous research in our laboratory with *Photorhabdus luminescens* has consistently demonstrated higher bioluminescence as air flow increased [Wilson and Hastings, 2013; Belur *et al*., 2013].

## CONCLUSION

In conclusion, this study was completed to understand *P. luminescens* and its microbial growth kinetics. This study investigated how *P. luminescens* responds to different environmental conditions in a bioreactor. In the mass production of the nematode *Heterorhabditis bacteriophora*, the symbiont *P. luminescens* must have good cell growth [Gerdes *et al*., 2015]. Rapid cell growth is necessary for faster recovery and better yield of the nematodes during mass production in bioreactors. In conclusion, *P. luminescens* with the parameters of 1.0 vvm, pH 7.8, 28°C, and 300 RPM had the highest specific growth rate 0.59 $h^{-1}$and the lowest doubling time1.1hr. This study could help to further progress using *H. bacteriophora* as an alternative for using chemical pesticides.

***Acknowledgments***

Financial support was provided, in part, by the: Farm Bureau of Robeson County, North Carolina; University of North Carolina at Pembroke (UNCP) Office of the Provost and Academic Affairs; UNCP Department of Chemistry and Physics; North Carolina Biotechnology Center (NCBC) and UNCP Thomas Family Center.

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ISSN 0976-3031