

Microbial Kinetics of *Photorhabdus luminescens* in Glucose Batch Cultures

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ABSTRACT

Photorhabdus luminescens, an entomopathogenic bacterial symbiont of *Heterorhabditis bacteriophora*, was studied in batch cultures to determine the specific growth rates of the bacterium in various glucose concentrations. *P. luminescens* was cultured in a defined liquid medium containing various concentrations of glucose. Culture parameters were monitored and controlled utilizing a Sartorius stedim Biostat® A plus fermentation system. Agitation and air flow remained constant; however, the pH of the media was chemically buffered and monitored over the course of bacterial growth. Measurements of culture turbidity were obtained utilizing an optical cell density probe. Specific growth rates of *P. luminescens* were determined graphically and mathematically. The substrate saturation constant of glucose for *P. luminescens* was also determined along with the bacterium's maximum specific growth rate.

1. INTRODUCTION

Photorhabdus luminescens is a Gram-negative, bioluminescent, entomopathogenic bacterium that is found to be a bacterial symbiont of the nematode *Heterorhabditis bacteriophora* (Inman, Singh and Holmes, 2012). These symbiotic partners serve as a bacto-helminthic complex that is considered to be a safe alternative to chemical insecticides. Through symbiosis and entomopathogenicity, these organisms are capable of infecting a broad range of insect hosts belonging to the orders of Coleoptera, Diptera, and Lepidoptera (Ehlers and Hokkanen, 1996). The infective juveniles of *H. bacteriophora* carry many cells of the bacterial symbiont in their upper digestive tract and upon entrance into the insect hemocoel; the juveniles regurgitate the cells into the insect hemolymph.

As the cells proliferate, they produce biomolecules (toxins and digestive enzymes)

that kill and bioconvert the insect host into nutritional components for both organisms (Boemare, Laumond, and Mauleon, 1996). Furthermore, *P. luminescens* secretes pigments and antimicrobials to ward off other contaminating microbes and as a result, ideal conditions are created for nematode growth and development (Waterfield, Ciche and Clark, 2009).

The symbiotic relationship between *H. bacteriophora* and *P. luminescens* is very intimate and complex as both partners are needed to support each other in many aspects. In this relationship, infective juvenile 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 mode of transportation from host to host. On the other hand, *P. luminescens* provides many benefits to its nematode partner such as: 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 induce nematodes to develop; and 5) being the main food source. It is of worth to mention that this relationship may not sound like a true mutualistic association; however, the parasitism and pathogenicity refer to the effects of these two organisms (nematode and bacteria, respectively) on their primary host, the young insect larvae.

The mass production of nematodes for biocontrol purposes must incorporate the use of mid to large-scale fermentation systems along with optimized media that will support survival of the nematodes along with their bacterial symbionts for extended periods of time. Because *P. luminescens* is the main food source for the nematodes, it is crucial that the mass production medium be pre-inoculated with *P. luminescens* prior to nematode inoculation (Inman, Singh and Holmes, 2012). Furthermore, researchers also suggest that *P. luminescens* secretes a “food signal” that triggers the infective juveniles to continue their development (Strauch and Ehlers, 1998). Therefore, bacterial cultures at high cell densities are required to obtain maximum recovery of infective juveniles (Ehlers, Lunau, Krasomil-Osterfeld and Osterfeld, 1998).

In their paper, Jeffke et al. (2000) studied the growth of *P. luminescens* in glucose batch and fed-batch cultures. However, the authors did not report the necessary information needed to obtain a basic understanding of glucose utilization for this bacterium. The information needed to determine kinetics of glucose utilization includes specific growth rates of the bacterium at different glucose concentrations and the substrate saturation coefficient (K_s) for glucose. Furthermore, the researchers utilized an undefined medium for their research. Without the employment of a defined medium, determination of glucose

utilization is extremely difficult as the undefined medium contains other sources of utilizable carbon. Therefore, it is the main objective of this study to determine the microbial kinetics of *Photorhabdus luminescens* in batch cultures containing synthetic media. To obtain the necessary information for glucose utilization, batches of defined media were supplemented with different initial glucose concentrations. This investigation complements previously performed research (Jeffke et al., 2000) and also provides more information about glucose utilization in *P. luminescens*.

2. MATERIALS AND METHODS

2.1 Organisms and Media

Infective juvenile nematodes of *Heterorhabditis bacteriophora* were obtained from Arbico Organics, Tuscan, AZ and used to infect larvae of *Galleria mellonella* according to Inman and Holmes (2012). Isolation of the symbiotic bacterium, *Photorhabdus luminescens*, from the hemolymph of nematode-infected larvae of *G. mellonella* was performed as described by Ehlers, Stoessel and Wyss (1990). The nutrient agar used in this study contained (g/L): beef extract, 5; digested gelatin, 3; trehalose dihydrate, 15; and agar, 15. Isolated, red pigmented, bioluminescent colonies were upscaled to 3 mL broth tubes and finally to 100 mL shake flask containing nutrient broth. The composition of the nutrient broth was the same as nutrient agar with the exception of agar.

2.2 Equipment and Software

A two liter Sartorius stedim Biostat® A plus fermentation system and its associated software was used to control and monitor process parameters. The Sartorius stedim BioPAT® MFCS/DA 3.0 software was used to record and export the process

data. A Mettler Toledo 405-DPAS-SC-K8S/200 gel filled combination electrode was utilized to monitor pH. An optek-Danulat, Inc. ASD19-N optical density probe and fermentor control module was used to measure and record culture turbidity in the near infrared range of 840-910 nm. Microsoft® Excel (2007) was used to graphically approximate the specific growth rates of each glucose batch culture based upon culture turbidity.

2.3 Fermentation Media

Liquid media for both overnight and experimental cultures were based on the defined media formulation of Bleakley and Neelson (1988). The composition of the liquid media contained (mM): NaCl, 50; MgSO₄, 5; urea, 30; L-proline, 10; Na₂MoO₄•2H₂O, 0.025; MnCl₂•4H₂O, 0.025; FeCl₃•6H₂O, 0.025; HEPES, 50; KH₂PO₄, 100; and various glucose concentrations. Media components were prepared in deionized water and autoclaved within the appropriate vessels with the exception of glucose and potassium phosphate stock solutions, which were sterilized separately. During preparation of the medium components, the volumes of glucose and phosphate stocks needed to achieve the final media concentration of each were taken into account when preparing the other components. Prior to inoculation, the required volumes of each stock solution were aseptically added to the reactor. All solutions were adjusted to a final pH of 7.50 with 5 M KOH. The concentrations of glucose utilized in this study ranged from 1.8 mg/L to 198 mg/L. Proline is utilized in the kinetics medium as it: 1) acts as an osmoprotectant within insect hemolymph (Neelson, Schmidt, and Bleakley, 1990); 2) is believed to be a signal for regulation of phase I characteristics (Seydel, Lieb, Ehlers and Röck., 2000; Kontnik, Crawford and Clardy, 2010); and

3) is a requirement for *P. luminescens*. The bacterial strain used is a proline auxotroph as determined by initial shake flask cultures.

2.4 Process Setup and Parameters

Agitation of each batch culture was achieved with Rushton turbine stirrers set as a fixed rate of 100 rpm. Each batch was sparged with air at a constant flow rate of 1 vvm; while the process temperature was maintained at 28°C. Upon process equilibrium, the reactor was inoculated from a fresh 24 hour overnight of *P. luminescens* at a concentration of 1.67% of the media volume. Each overnight culture was cultivated in the defined medium containing minimal glucose concentrations to minimize excess glucose in the experimental media.

2.5 Analysis of microbial growth

2.5.1 Graphical approximations of specific growth rates

Turbidity data (time and concentration units) were downloaded from the data logger of the fermentor control module and imported into Microsoft® Excel as follows: Column A – time (h) and Column B – concentration units (cu). In Column C, the natural logs of the respective concentration units (ln cu) were calculated and entered utilizing Excel's natural log function. Data from Columns A and C from each batch were graphed into a single scatter plot.

Upon visual observation of the natural log curve, the exponential phase is seen as the sharp increase in the natural log data. Data points on the natural log curve that begin and end this phase are used as reference points as they are used as the limits for creating the linearized exponential phase, which is added to the same plot. A linear regression is performed on the natural log data between and including the limit

points. The line of best fit is determined for this linearization and the slope of this line reflects the specific growth rate for each batch under their respective conditions.

2.5.2 Calculations of specific growth rates

According to Stanbury, Whitaker and Hall (2003), the simplified equation for describing the exponential phase is shown in Equation (1), where x is the concentration of cells; t is time, in hours; and μ is the specific growth rate, reported as hour⁻¹.

$$(1) \quad \frac{dX}{dt} = \mu X$$

Because this equation is fitted to utilize cellular concentrations (biomass, cell numbers, etc.) it must be slightly modified to fit culture turbidity (Widdel, 2010). In theory, culture turbidity or optical density (OD) is directly proportional to cellular density (Equation (2)); where N is cell number and V is unit volume. However, a proportionality factor (α) is needed to accurately convert the cellular density into OD (Equation (3)). Therefore, the proportionality factor and the OD can be substituted for X in Equation (1) to become Equation (4)

$$(2) \quad \frac{N}{V} \sim OD$$

$$(3) \quad \frac{N}{V} = \alpha \cdot OD$$

$$(4) \quad \frac{d(\alpha \cdot OD)}{dt} = \mu (\alpha \cdot OD)$$

Upon integration, Equation (4) becomes Equation (5), where OD_0 is the initial optical density; OD_t is the optical density at some time; t is some time, in hours; e , the base of the natural logarithm; and μ is the specific growth rate.

$$(5) \quad (\alpha \cdot OD_t) = (\alpha \cdot OD_0) e^{\mu t}$$

Upon solving for μ , the proportionality factor is reduced to 1 and Equation (5) becomes Equation (6), where OD_{t_2} is the optical density at the time of recording, in cu; OD_{t_1} is the initial optical density, in cu; t_2 is the time of recording, in hours; and t_1 is the initial time, in hours.

$$(6) \quad \mu = \frac{\ln(\alpha \cdot OD_{t_2} / \alpha \cdot OD_{t_1})}{(t_2 - t_1)}$$

2.5.3 Calculating generation/ doubling times

The generation time takes into account the doubling of microbial growth, meaning that if the cellular concentration at time 2 is divided by the cellular concentration at time 1 and the result is equal to 2, then the doubling time is equal to the difference in time ($t_2 - t_1$). Furthermore, this concept can be included into Equation (6) to determine the specific growth rate based upon the doubling time (t_d). Upon concept substitution, Equation (6) yields Equation (7). Therefore, once the specific growth rate has been approximated, Equation (7) can easily be used to determine the doubling time.

$$(7) \quad \mu = \frac{\ln(2)}{t_d}$$

2.5.4 Calculating the glucose Ks value

Monod (1949) developed a hyperbolic model (Equation 8) that is used to describe the relationship between the specific growth rates (μ) of an organism to various concentrations of a limiting substrate (s). This model is based upon two main microbial parameters: the maximum specific growth rate (μ_{max}) and the constant of substrate saturation (K_s). The maximum specific growth rate can be biologically defined as the highest rate of growth that an organism can obtain for a particular substrate; whereas the substrate saturation constant represents the organism's affinity to the limiting substrate. The K_s value is defined as the value of the limiting substrate concentration that corresponds to the specific growth rate that is equal to one-half the maximum specific growth rate of the investigated organism.

$$(8) \quad \mu = \mu_{max} \frac{[s]}{K_s + [s]}$$

Since the Monod equation is extremely similar to the Michaelis-Menten equation for enzyme kinetics; equations that linearized the Michaelis-Menten model can also be applied to the Monod equation. These equations include the Lineweaver-Burk, Eadie-Hofstee and Hanes-Woolf. In this research, the Eadie-Hofstee equation is utilized because the resultant linearized equation specifically identifies the K_s value and the maximum specific growth rate.

To obtain the Eadie-Hofstee linearization of the Monod equation, the Monod equation (8) is inverted and multiplied by μ_{max} (9) and afterwards rearranged for μ_{max} (10). Finally, Equation 10 is then used to isolate μ (11). To create the Eadie-Hofstee plot, specific growth rates (μ) are graphed as a function of the

respective growth rate divided by the associated substrate concentration ($\mu/[s]$). As defined by Equation 11, the slope of the plot corresponds to the negative value of the saturation constant ($-K_s$) and the y intercept corresponds to the maximum specific growth rate (μ_{max}).

$$(9) \quad \frac{\mu_{max}}{\mu} = \frac{K_s + [s]}{[s]}$$

$$(10) \quad \mu_{max} = \frac{\mu K_s}{[s]} + \mu$$

$$(11) \quad \mu = -K_s \frac{\mu}{[s]} + \mu_{max}$$

3. RESULTS

3.1 Specific Growth Rates and Generation Times

Growth data of *P. luminescens* from each glucose batch were used to determine growth rates and generation times (Table 1). To calculate the growth rates utilizing Equation (6), the two limit points (lower and upper) from the graphical method (Figure 1) were used and substituted into the equation. Generation times were also determined based upon the approximated growth rates from each method utilizing Equation (7).

3.2 Glucose Ks Determination of *P. luminescens*

Specific growth rates were plotted as a function of the respective glucose concentration to create a Monod curve (Figure 2). Upon examination of this Monod curve, the maximum specific growth rate appears to approach a value of 1.0

h-1. To determine the bacterium's affinity for glucose (K_s) and its maximum specific growth rate when cultured in glucose, the Eadie-Hofstee equation (Equation 11) is employed. Plotting specific growth rates as a function of the growth rate divided by its respective glucose concentration results in a line of best fit. The equation of the best fit line approximates the K_s value along with μ_{max} . For *P. luminescens*, the K_s value for glucose is determined to be 1.95 mg/L with a maximum specific growth rate of 0.96 h⁻¹.

4. DISCUSSION

Understanding the rates and patterns of microbial growth is essential to successfully culture cells to high densities. In theory, cultures that progress at their maximum specific growth rate under set conditions will reach higher cell densities in less time. Monod (1949) describes that specific growth rates will increase with increasing substrate concentrations until the maximum specific growth rate for the organism is reached for the particular substrate.

In reference to the data obtained from this study, the experimental glucose concentrations (1.8, 13.1, 18, 198 mg/L) produced variable specific growth rates. By means of the Monod's description of the maximum specific growth rate, we conclude that glucose concentrations exceeding 198 mg/L (1.1 mM) allows for *P. luminescens* to grow at its maximum growth rate (μ_{max}). This result for the maximum specific growth rate does seem logical because the glucose concentration in the hemolymph of the insect host *Galleria mellonella* is ~1.1 mM (Wyatt and Kalf, 1957), which is represented by the 198 mg/L glucose batch. Therefore, pathogenicity and/or virulence may be related to the maximum rate of bacterial proliferation. Furthermore, Monod mentions the microbe's affinity to the substrate; otherwise known as the K_s

or substrate saturation coefficient. Monod describes this coefficient as the substrate concentration at one-half the maximum specific growth rate. Based upon this study, we determined that the K_s value of *P. luminescens* for glucose is 1.95 mg/L. This value is within range with other glucose K_s values for enteric bacteria such as *Escherichia coli* (4 mg/L) and *Klebsiella aerogenes* (9 mg/L) (Nielsen, 2006).

Jeffke et al. (2000) performed a glucose study with *P. luminescens* to maximize bacterial density. This study included both batch and fed-batch cultures where they utilized an initial glucose concentration of 0.22% (2,200 mg/L) for each batch mode. From the results of our study, *P. luminescens* cultured in 2,200 mg/L glucose should be growing at its maximum specific growth rate. However, Jeffke does not report the growth rate for the batch culture. On the other hand, Jeffke reports that under fed-batch conditions they were able to maintain the growth rate of 0.05 h⁻¹ by injecting glucose (0.2 – 0.4% concentrations) over a 50 hour period. This growth rate is extremely low when compared to the results of this study. Furthermore, the researchers utilized an enriched medium and to it the glucose was added. Through the utilization of this highly enriched medium, it is possible that the bacterial cells were stressed due to the high solute concentration and ultimately, negatively impacting the specific growth rate.

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Table 1 Comparison of graphical and calculated values of specific growth rates and doubling times

| Glucose (mg/L) | Graphical | | Calculated | |
|----------------|--------------------------------|--------------------|--------------------------------|--------------------|
| | Growth rate (h ⁻¹) | t _d (h) | Growth rate (h ⁻¹) | t _d (h) |
| 0 | 0 | 0 | 0 | 0 |
| 1.8 | 0.46 | 1.52 | 0.46 | 1.52 |
| 13.1 | 0.78 | 0.89 | 0.78 | 0.89 |
| 18 | 0.91 | 0.76 | 0.92 | 0.75 |
| 198 | 0.95 | 0.73 | 0.95 | 0.73 |

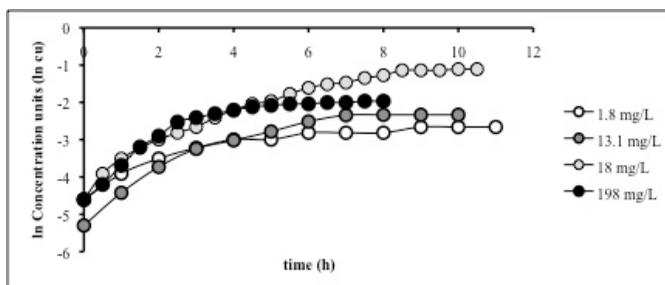


Figure 1: Natural log curves of *Photorhabdus luminescens* growth in batch cultures containing various concentrations of glucose. Specific growth rates (μ) for each batch are: 0.46 h⁻¹ (1.8 mg/L); 0.78 h⁻¹ (13.1 mg/L); 0.91 h⁻¹ (18 mg/L); and 0.95 h⁻¹ (198 mg/L).

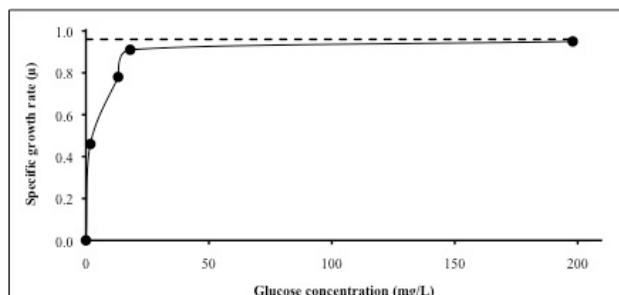


Figure 2: Monod plot depicting the hyperbolic relationship between glucose concentration and the specific growth rate of *Photorhabdus luminescens*. According to Monod, the maximum specific growth rate (dashed line) is the maximum rate that an organism can grow regardless of increasing the substrate concentration.

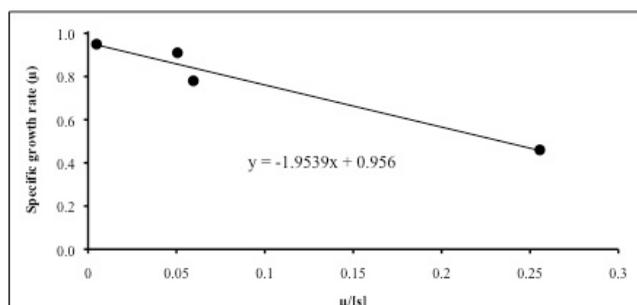


Figure 3: Eadie-Hofstee diagram used to determine the values of K_s and μ_{max} in regards to glucose utilization of *Photorhabdus luminescens*. Utilization of the Eadie-Hofstee equation determined that the K_s of *P. luminescens* for glucose is 1.95 mg/L with a maximum specific growth rate (μ_{max}) of 0.96 h⁻¹.

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