

# Development of proper microbiological technique for experimentation with *Vibrio fischeri*

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## Abstract:

The methodology described in the following pages addresses difficulties encountered in recent months concerning reproducibility in experimentation performed with the photobacterium *V. fischeri*, strain ATCC 7744. This methodology encompasses conditioning growth media, making frozen glycerol stocks, preparing experimental cultures from a frozen stock, and using aseptic technique.

## Introduction

The focus of my research in Dr. Steve Hagen's lab has been to characterize the growth and light production, in liquid cultures, of the photobacterium *Vibrio fischeri*. *Vibrio fischeri* is a marine bacterium that, in the wild, lives as a symbiont in the light organ of certain fish and squid species, including the Hawaiian bobtail squid *Euprymna scolopes*. We use *V. fischeri* strain ATCC 7744 in the lab and are beginning work with its relative *V. Harveyi* strain 392 [MAV], which are both purchased from and preserved through freeze-drying by the American Type Culture Collection (ATCC).

*V. fischeri* is a bacterium that, in nature, produces light (bioluminesces) when concentrated in dense cultures in the light organ of a host creature, but not when in more dilute environments such as the ocean. *V. fischeri* produces light to help its host disguise its shadow from predators below, but when outside of the host, it is wasteful for the bacteria to continue expending energy on light production. *V. fischeri* therefore manages its light production using a form of cell-to-cell communication known as quorum sensing through which the bacteria can monitor population density.

FIGURE 1:

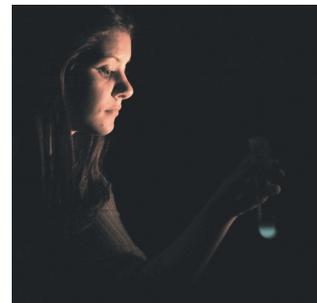


Figure 1: Summer 2006 REU student Rachel West holding a bioluminescent culture of *V. fischeri*

Quorum sensing, and therefore light production, is regulated by the LuxIR operon (group of several genes). The quorum-sensing mechanism of *Vibrio fischeri* as follows

FIGURE 2:

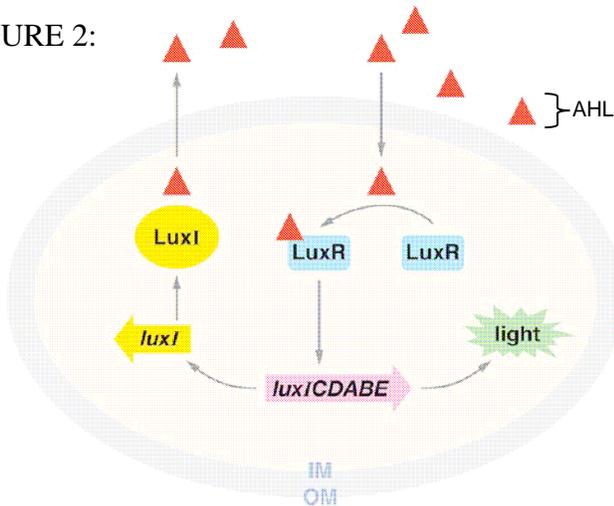


Figure 2: *V. fischeri*'s genetic quorum sensing mechanism as diagrammed by Waters and Bassler. [1]

(figure 2). A cell produces a small amount of a chemical, *N*-acyl homoserine lactone (AHL), known as the autoinducer. AHL is coded for in the LuxIR gene sequence and can diffuse freely through the cell membrane. If there are many other *V. fischeri* cells in the surrounding environment, the concentration of AHL in the culture becomes quite high. AHL then diffuses back into the cytoplasm where it triggers the LuxIR genes. The LuxIR genes code for two functions: the

production of more AHL, and the production of luciferase, an enzyme responsible for light production. As a result, even more AHL is concentrated in the culture, and the light production effects are amplified.

My overarching goal for the summer has been to try to characterize bioluminescence in a liquid culture of *V. fischeri* as a function of the AHL concentration in solution. What has occupied most of my time, however, is preparing a methodology for handling *V. fischeri* in a way that will yield reproducible and accurate results for my measurements. Having a reliable methodology is imperative to make progress, so my work this summer has been a starting point for both my specific experiment with AHL concentrations, and my fellow lab member's *V. fischeri* experiments that are already in progress.

The methodology I have been developing addresses specific procedures for conditioning liquid growth media (broth), preserving bacteria at liquid-nitrogen temperatures, and reproducibly inoculating experimental cultures. It is necessary to condition broth before use to remove any chemicals which might interfere with the natural behavior of the quorum-sensing mechanism. In addition, the bacteria are preserved in glycerol stocks, frozen dormant at liquid-nitrogen temperatures, so that each

experiment is started from the same strain in the same way. With this method, we do not need to worry about mutations that would occur if the bacteria were instead serially subcultured into fresh broth. The following sections justify and describe the new methodologies.

### Optical Density Measurements and Bacterial Culture Growth Phases

Bacteria, by definition of being alive, are ever changing, reproducing, and interacting with their environment. There are three phases in the collective growth of a

FIGURE 3:

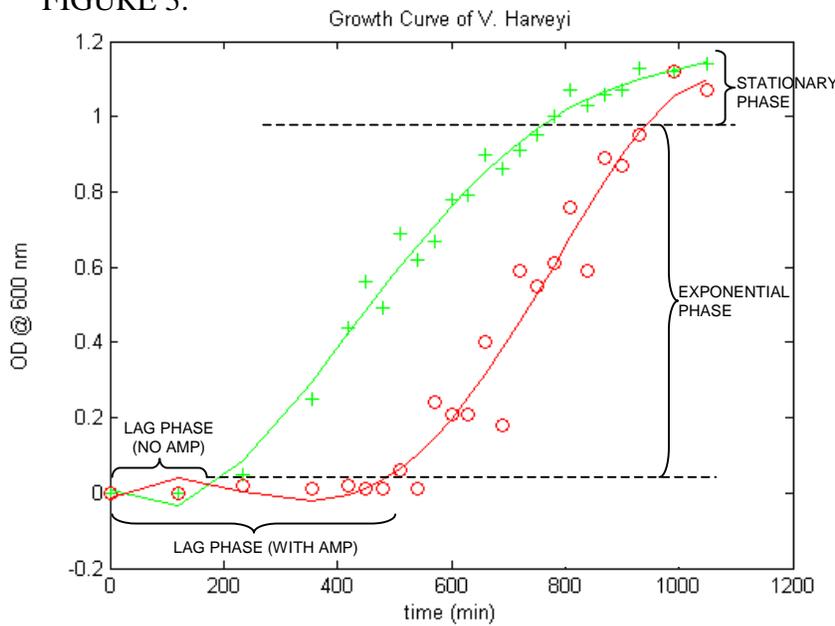


Figure 4: Two growth curves are diagramed, the green one represents a *V. harveyi* culture grown without ampicillin, and the red curve represents a *V. harveyi* culture inoculated at the same time as the green one, but with a concentration of 100ug ampicillin per mL of broth. Both use a quadratic fit. (note: OD path length = .4 cm

culture of cells: lag phase, exponential (or log) phase, and stationary phase. Each phase reflects the average state of health of the individual cells within the culture. Figure 3 diagrams the growth of a *V. fischeri* culture in liquid growth media in terms of its optical density (OD). OD has units of absorbance (A), and for the purposes of

the paper should be assumed to be measured at 600nm, with a 1-cm path length unless stated otherwise.

Optical density measurements are the primary method used in the lab for determining population densities in liquid cultures. OD is measured in a UV-1601 Shimadzu spectrophotometer, and is proportional to the length of the path that the light travels through the sample. OD also varies depending on the wavelength of light used to make the measurement. Specifically,  $OD = \log_{10}(I_0/I)$ , where  $I_0$  is the intensity of the light incident upon a sample, and  $I$  is the intensity of the transmitted light (that is, the light not

absorbed or scattered by particulates such as cells in the sample). See Figure 4. The effects of light scattered by salts and other particulates that are characteristic of the liquid growth medium (broth) used can be subtracted from data by assigning the baseline value of zero to the OD of sterile broth.

At values less than approximately 0.7A/cm, OD is a linear

function of population density in a culture. Above 0.7A/cm, however, OD is less correlated with population growth, because the culture has so many cells that the light gets scattered multiple times by the bacteria, and the OD no longer proportional to population density. During exponential phase growth, a *V. fischeri* population doubles approximately every hour (see figure 6). Exponential phase cells can be up to 4 times larger than lag or stationary phase cells, because they have replicated their chromosome, and are preparing to divide, undergoing binary fission. Larger cells scatter light differently, so this changing cell structure influences OD measurements. Exponential phase growth is defined as growth where the  $\log_{10}(\text{OD})$  is a linear function. It is ideal to work with exponential phase cells in experimentation.

The lag phase of bacterial growth is variable depending on a variety of factors. Figure 4 shows the effect an ampicillin concentration of 100ug/mL has on the length of the lag phase. Stationary phase will last until the culture consumes all of its resources, or pollutes its environment to levels of toxicity with waste excretions, at which point the culture will die, cells will lyse, and OD values will quickly decrease.

### Conditioning Photobacterium Broth

It is common in microbiology labs for researchers to prepare their own growth media. As a result, different *V. fischeri* labs have experimented with different recipes, and a wide variety of media are in use, although most have the aim of mimicking *V. fischeri*'s

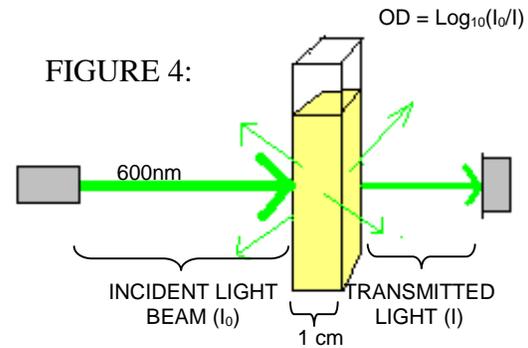


Figure 3: 600nm wavelength light passes through 1 cm of a sample. Some of the light is either absorbed or scattered. The light that is transmitted through the sample is detected and used to calculate optical density which can be correlated with population density.

natural environment, the ocean. Common ingredients in most media include Tryptone, yeast extract, glycerol, and roughly 3% NaCl by weight.

Although different researchers use different media recipes, many cite a common observation of inhibition of light production [2-4] in *V. fischeri* that can be attributed to the growth media itself. Light production is inhibited in *V. fischeri* for several hours, until the bacteria metabolize the chemical inhibitor present in the liquid media. Although no one has identified the specific inhibitor, it has been found that the effects can be eliminated through the conditioning of broth prior to use in experimentation. Researchers whose papers are particularly relevant to the work done in Dr. Hagen's lab condition their broth by growing a related Vibrio species, *V. harveyi* strain 392 [MAV], in the broth first [3,4]. *V. harveyi*, after being grown to a mid-exponential phase OD, consumes the majority of the inhibitor, and is then removed from the broth by filtration. If sub-micron pore size filter paper is used, this filtration also sterilizes the broth (bacterial cells are generally  $>1 \mu\text{m}$ ). I am currently in the process of trying to grow *V. harveyi* effectively so that I can begin conditioning broth prior to experimentation.

### **Preserving Bacteria at Liquid-Nitrogen Temperatures:**

One of my concrete tasks for the summer was to monitor the optical density of a sample through lag phase and exponential growth into stationary phase. All measurements were made in an automated system built by Jonathan Young, another undergraduate in the lab. This system requires growing the bacteria in a small-volume (~3mL) plastic disposable cuvette with a magnetic stirbar and stirplate, rather than the standard larger-volume glass culture tube positioned on an orbital shaker. With few exceptions, I was unable to get *V. fischeri* to grow at all in the automated system, despite using the same growth medium and the same inoculation procedures. Macroscopic properties such as container volume and material should, ideally, have no significant effect on culture growth. In trying to fix the growth problem, Dr. Hagen and I identified two errors in lab procedure that might have been causing finicky growth behavior in our bacteria. One error is in the way that we culture the bacteria directly from glycerol stocks. The other concerns the health of the glycerol stocks themselves.

When making a healthy frozen glycerol stock, three factors that hold significant weight are the phase of growth in which the bacteria are frozen, the percentage of glycerol concentrated in the frozen solution, and the rate at which the stocks are frozen. Ideally the bacteria should be in late exponential phase growth, and suspended in 30% glycerol, 70% photobacterium broth solution, at which point they should be dipped directly into liquid nitrogen (-196°C) so that they freeze solid in under 15 seconds. Including glycerol in the solution prevents the formation of ice crystals that could damage the bacterial cells during the freezing process [5-6].

The *V. fischeri* stocks that have been used for all experimentation in the lab since the project was started were frozen when far into the stationary phase. The percent concentration of glycerol in solution was probably around 15%, although this was not properly recorded at the time those stocks were made. Also, the bacteria were frozen by placement in a -80°C freezer, rather than by submersion in liquid nitrogen. Because the procedure used to make these stocks was not ideal, I made new frozen glycerol stocks this summer, from *V. fischeri* Dr. Hagen purchased from the ATCC.

Since working in the lab, I have made new stocks of both *V. fischeri* and *V. harveyi*. The *V. fischeri* stocks were frozen with 30% glycerol in solution, and frozen by direct submersion in liquid nitrogen somewhere in between the OD's of 0.74A/cm and 0.83A/cm. The uncertainty in OD at the freeze time comes from the growth that occurs during the 20 minutes or so that it takes to prepare the stocks and freeze them.

The *V. harveyi* stocks were frozen in 30% glycerol but were not frozen during exponential growth due to an error in accounting for the path length of the cuvette used to monitor the OD. In the near future the *V. harveyi* stocks will be remade correctly.

### **Preparation of Experimental Cultures**

The current procedure for growing experimental cultures is as follows. Twenty-four to thirty-six hours in advance of an experiment, a lab assistant trying to start a culture adds 20 µL of the antibiotic ampicillin to 10 mL of liquid growth medium (photobacterium broth purchased from Carolina Scientific) in a ~60 mL capacity large glass culture tube. He or she then scrapes bacteria from a frozen stock with a plastic pipette tip, and drops it into the broth/ampicillin solution. To assure that the cells get

enough oxygen to grow optimally, the culture is left, oriented at a tilt, on an orbital shaker.

Using this procedure, researchers in the lab have had difficulty getting reproducible growth and bioluminescence in liquid cultures. Often, two cultures started at the same time and in the same manner will not grow to similar optical densities after a given period of time and will not produce the same intensity of bioluminescence. This inconsistency can be traced to both our methodology and the state of health of the bacteria after they thaw.

When scraping a frozen glycerol stock for use in inoculations, the quantity of viable cells removed by the pipette tip can vary by orders of magnitude. Factors influencing this variability include the volume of ice shavings scraped, the amount of water vapor that condenses on the stock when it is removed from the liquid nitrogen freezer, and the distribution of cell density within the stock itself. It is important to note that preservation of bacteria at liquid nitrogen temperatures is a harsh process (see section on Preserving Bacteria at Liquid Nitrogen Temperatures), and that freshly thawed bacteria may not behave optimally as a result.

After consulting microbiologist Paul Gulig [6], who studies *Vibrio vulnificus*, a pathogenic relative of *Vibrio fischeri*, Dr. Hagen and I reevaluated and revised our culturing procedure as follows. After healthy glycerol stocks have been made, a lab assistant should scrape shavings from the frozen stock onto solid growth media, (Solid growth medium is often made by adding 15 g of agar, a gelatinous polysaccharide obtained from the cell walls of red algae or seaweed, to a liter of liquid growth media.)

FIGURE 5:

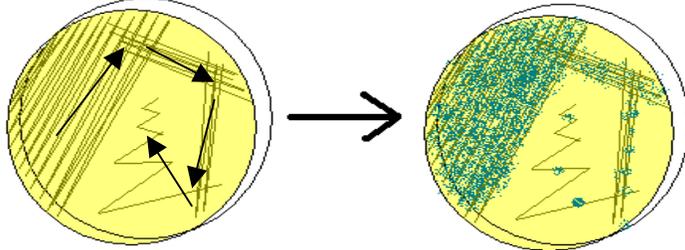


Figure 5: Streaking an agar plate. On the left the bacteria are streaked out on the agar, and on the right is a diagram of what the plate should look like after a day or two of growth. The different sections of streaks are intended to dilute the cells so that by the last streak, you grow only single colonies, as opposed to the lawn of bacteria that grows from the first streak.

Using a sterile wire inoculating loop, or a sterile wooden stick, he or she then streaks the melted shavings across the plate as diagrammed in figure 5. After a day or two, when the agar plate has grown a lawn of bacteria, he or she places it

in a refrigerator ( $\sim 5^{\circ}\text{C}$ ) to halt growth. This plate should last for weeks in good health, and will provide a consistent base from which to start all experiments during that time period. The evening before an experiment, the lab assistant starts a liquid culture by touching a sterile steel inoculating loop to the lawn of bacteria (not to an isolated colony, because these may represent genetic mutants) on the refrigerated agar plate, and then dips the loop into the sterile broth. The lab assistant then leaves this culture in a rack on the counter to grow overnight (referred to as a standing overnight), and in the morning it should have grown to a low OD of approximately 0.2 per centimeter. He or she then transfers a standard volume (approx. 10uL) of the standing overnight culture into clean broth. This new culture should pass into exponential phase growth within a few hours, at which point it will be ready for use in experimentation. This procedure ensures that each experimental culture is made in the same way, and starts with healthy, representative cells.

One significant difference between our current culturing procedure and this new one is the use of the antibiotic ampicillin. A concentration of  $100\mu\text{g/mL}$  ampicillin in the culture prevents the growth of contaminants. Although *V. fischeri*'s natural resistance allows it to grow in broth laced with ampicillin, the antibiotic still affects on the growth and behavior of the culture (see figure 4). For this reason, ampicillin was left out of the new procedure, and contamination will in the future be prevented through the use of aseptic (or sterile) technique as described in the following section.

The amount of time it takes for a final experimental culture described in the new procedure to pass into exponential phase growth is specific to the species and strain of bacteria. If the procedure is done correctly, each experimental culture for a specific species of bacteria will grow the same way, as described by a characteristic growth curve. The growth curves of *V. fischeri* and *V. harveyi*, collected from cultures that glycerol stocks were made from, are displayed in figure 6. These cultures were not inoculated using the described procedure, so the lag phase length is not representative. When the new procedure has been implemented, however, a more accurate curve can be measured. To be able to implement the procedure, it is necessary to be able to grow both *V. fischeri* and *V. harveyi* on agar plates. For reasons currently unknown, we have not yet been successful in this venture.

FIGURE 6:

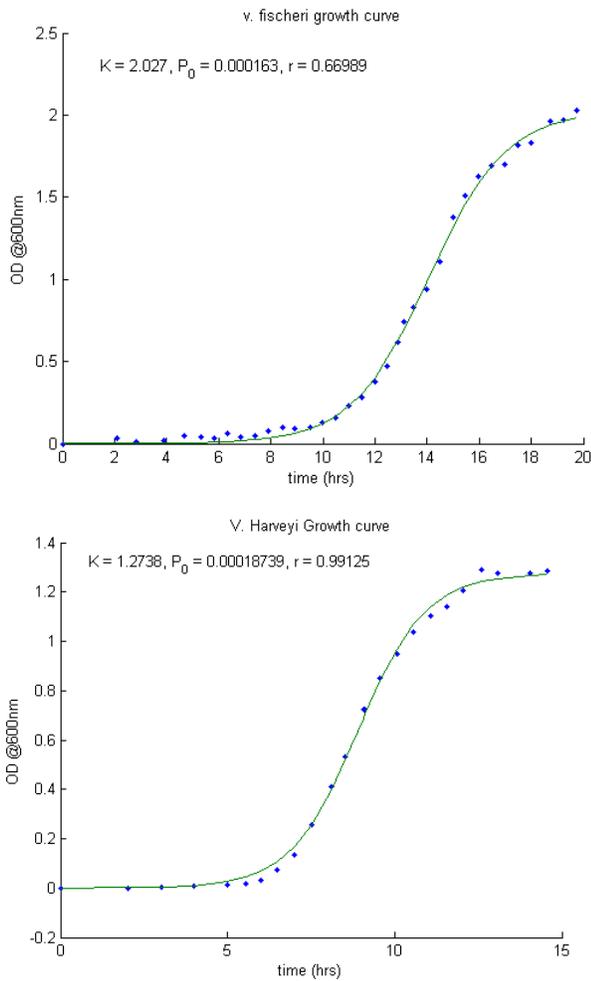


Figure 6: Growth curves for both *V. fischeri* and *V. harveyi* fit to a logistics curve:

$$P(t) = (K * P_0 * e^{rt}) / [K + P_0 * (e^{rt} - 1)]$$

where  $P(t)$  represents population or OD, and the other parameters are specified in graph.

NOTE: *V. harveyi* OD measured with a 0.4cm pathlength, making all values 2/5 of what they would have been for a 1 cm pathlength.

### Sterile Procedure – Aseptic technique

The focus of aseptic technique is on keeping the lab bench where work with cultures is done as clean as possible, and making sure that anything that comes in contact with a culture has been properly sterilized.

We have recently obtained a 10-quart pressure cooker, which has been used to sterilize pipette tips and wooden sticks, as well as plastic and glass centrifuge tubes. In general, it is good practice to sterilize anything that will be in contact with a culture so as not to risk contamination of an experiment. For our purposes, the pressure cooker serves the same function as the more formal sterilizing machine, the autoclave, which is basically an automated pressure cooker with a drying cycle. To sterilize either a liquid or a solid properly, the sample must remain at 121°C (250°F) and 15 psi for the time (minimum) indicated in Table 1. The

pressure cooker accomplishes this effectively.

TABLE 1:

	Liquids		Solids
Volume	<200 mL	>200 mL	any
minimum timer setting*:	15 min	20-30 min	>30 min

To improve aseptic technique in experimentation, I have set aside a separate work space under a Pure Aire flow hood that is strictly for use with bacterial cultures. Before any work is done, the lab hood is turned on, and the lab bench is wiped down with ethanol to sterilize it. Pipettors, which are used to accurately manipulate small ( $\mu\text{L}$ )

volumes of liquids, are never dipped into culture tubes past their disposable tips (which have been sterilized in the pressure cooker) because the shaft of a pipettor is not sterile. Steel wire inoculating loops and metal spatulas are dipped in ethanol and flamed to ensure sterility immediately before use with cultures.

The biggest threat for contamination is from the air and from the experimenters themselves. Experimenters therefore always wear gloves and try to hold the culture away from their body. When experimenters are not using a culture, the culture is always capped.

## **Conclusion**

This summer has been spent trying to educate myself and the other researchers in the lab on proper microbiological technique. Because bacteria are alive, they cannot be just thawed out and used as a chemical would. They must be coaxed into healthy behavior and treated in a reproducible manner so that they will behave in reproducible ways during experimentation. The methodology I have been developing works to achieve this end.

## **Acknowledgements**

I would like to thank the National Science Foundation for financially supporting me during my research and for running the national REU program. I would also like to thank Kevin Ingersent and Kristin Nichola for directing UF's REU program, and providing me with housing for the summer. Thanks also to Steve Hagen, the principal investigator, who gave me a position in his lab, and has been infinitely patient and helpful throughout my term of employment. Many thanks to OJ Ganesh, and Pablo Perez, for always being willing to talk me through ideas, and even spend long hours helping me with my work. Finally, thanks to Paul Gulig and his graduate students Crystal and Rosaline for all their guidance.

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