

Quorum Sensing in *Vibrio Fischeri*

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July 2006

Abstract

Vibrio fischeri is a bioluminescent bacterium that participates in a communication phenomenon known as quorum sensing. The variation in the light output of these organisms individually will give insight into biological gene noise. For a study of noise to succeed, *V. fischeri* must be cultured in conditions that induce maximal intensity and duration of light output. The purpose of this project was to identify all pertinent environmental factors and characterize their effects on the luminescence of *V. fischeri*.

Introduction

Quorum sensing is a recently identified means of communication amongst bacteria in communities. When a population reaches a critical concentration, gene expression is altered more or less simultaneously in all cells. Often this alteration results in a behavior that is beneficial to the species only when a large number of bacteria are present. Through quorum sensing, single-celled organisms are able to function much like multi-celled beings.

The subject of this study of quorum sensing is the marine bacterium *Vibrio fischeri* (Fig. 2), a Gram-negative species that becomes bioluminescent in concentrated communities. *V. fischeri* has a symbiotic relationship with several species of fish and squid; when dense populations of the bacteria inhabit a nutrient-rich area known as the

light organ of the host, they emit a bluish-green light, helping the host to hunt or protect itself. However, light production is a very energy-expensive activity; it does not benefit an individual three-micron-long cell to luminesce when floating alone in the ocean. Thus, the biological mechanism that monitors luminescence is dependent on cell density: it involves the concentration of a self-produced chemical, N-(β -ketocaproyl)-L-homoserine lactone (of chemical formula $C_{10}H_{15}NO_4$). This chemical, commonly called the *V. fischeri* autoinducer, diffuses freely through the cell membrane. The bacteria harbor two proteins for regulation of the autoinducer: LuxI is the autoinducer synthase, and LuxR is a transcription factor for the genes that control light production. LuxR becomes stable when it binds to the autoinducer; in the absence of autoinducer, it degrades quickly. This binding occurs when a sufficient amount of autoinducer accumulates in the environment, resulting in three simultaneous effects: activation of the light-production gene, production of the enzyme luciferase, and synthesis of LuxI. Induction of this quorum-sensing circuit, which occurs at autoinducer concentrations as low as 0.5 ng/ml, causes both autoinducer synthesis and light production to increase exponentially [1]. The chemical reaction whose energy release results in the emission of a photon is as follows:



This reaction between reduced luciferin ($FMNH_2$), the long-chain fatty aldehyde tetradecanal ($RCHO$), and molecular oxygen is catalyzed by luciferase and results in luciferin (FMN), the oxidized form of the aldehyde (the corresponding acid $RCOOH$), and water (H_2O) [2,3].

The examination of gene noise is the ultimate objective of the Hagen Lab's study of the quorum sensing mechanism in *V. fischeri*. Optical tools will be used to characterize

the variance in the light output of single cells. Logistically, examination with a sensitive photometer would be feasible only if a culture were emitting light in both a relatively intense and steady fashion. For example, in order to observe a single bioluminescent cell with a CCD camera, the rate of photon emission must be sufficiently high to create a visible image. It is also desirable that this rate of emission be relatively constant (or at least decaying in a predictable fashion) to allow for both sufficient observation time and ease in separating the effects of noise from extraneous system changes. Thus, the first step in this project was to identify all environmental factors that affect luminescence, and then to vary all such parameters to create maximal intensity and duration of light production.

Bacterial Strains and Culturing Conditions

Two different strains of *Vibrio fischeri* were initially used to study luminescence characteristics: the wild-type variety (Carolina Biological), and ATCC 7744 (American Type Culture Collection). The latter is ampicillin resistant [4]. The use of this antibiotic protected cultures against contamination by extraneous species. All experimental work was conducted with ATCC 7744 once it was obtained.

The medium selected for culture was a liquid photobacterium broth (Carolina Biological). The photobacterium broth differed from Luria Burtani (LB) broth (widely used to culture such common bacteria as *E. coli*) in two significant ways. First, to simulate seawater, the broth contained several salts, most notably NaCl, which was present at the significant concentration of 0.58 M. Additionally, glycerol replaced the quantity of glucose typically found in LB. Both glycerol and glucose act as bacterial

carbon sources, but glucose has been found to inhibit the production of light in *V. fischeri* [2,5].

The high salt concentration caused the broth to separate within an hour of mixing, with white sediment settling on the bottom of culture tubes. The mixed broth was thus quite opaque, making accurate measurements of optical density impossible. Since optical density is the best indicator of bacterial growth in a large culture, this left us without a way to correlate light output with population. Filtering with a 0.47 μm pore size filter yielded a clear broth comparable in appearance to LB. Though this clear broth was more conducive to monitoring growth than was the cloudy broth, the characteristics of *V. fischeri* in the two broths differed, as will be discussed below under “Maximizing Luminescence.”

The development of a viable stock was necessary to ensure a continued supply of available bacteria. Since ATCC 7744 was received in freeze-dried form, the strain was rehydrated and deposited in 10 mL of broth medium. The culture tube was shaken at 200 rpm on an orbital shaker (VWR LabShop) for a period of 24 hours, after which time bright luminescence was visible. 850 μL of this culture were transferred to a plastic stock tube, and to this 150 μL of glycerol were added, to prevent the formation of damaging ice crystals. The individual stock tubes were stored at -80 degrees Celsius.

All subsequent cultures were inoculated directly from this frozen stock to ensure genetic similarity. A clean pipette tip was used to lightly scrape the stock surface, and then the tip was dropped into a test tube containing a standard volume of 10 ml of liquid broth. Ampicillin (Fisher Scientific), which was stored in a frozen stock solution of concentration 50 mg/ml in distilled water, was added to cultures in a final concentration

of 100 µg/ml to suppress the growth of competing bacterial species. A minimum time period of 24 hours was then allowed for cultures to reach peak luminescence. Cultures were constantly shaken for aeration at 200 rpm and kept at room temperature (22-26 degrees Celsius). The pH remained in the range 6.8-7.0.

Maximizing Luminescence

V. fischeri strain ATCC 7744 emits a mint-green colored light when viewed in the dark. A spectrofluorometer was used to collect a wavelength spectrum of the emission, which is shown in Fig. 2. The peak emission wavelength was identified as 493 nm.

Five factors have significant impact on the light emission of ATCC 7744: oxygen supply, type of broth medium, age of the culture, ampicillin concentration, and autoinducer concentration. Assays attesting to the effects of the first four variables were performed with a timed measurement on the fluorimeter, using 1 cm cuvettes. Due to a tight time constraint on luminescence (imposed by oxygen deprivation), emission was recorded for a single wavelength (495 nm) in all timed assays, rather than for the entire range of *V. fischeri*'s emission spectrum.

This time constraint was a readily identified, and not easily overcome, difficulty in the reliable quantification of *V. fischeri*'s light emission. Most cultures, even those in the phase of maximum light production (about 24-56 hours after inoculation), are not visibly luminescent in the dark until they are agitated. After being shaken, light emission generally returns to almost zero within a relatively short period of time, ranging from several seconds to nearly one minute. The surface is the last area of the culture to darken, indicating that a lack of oxygen inhibits light production. Indeed, as noted previously, the

chemical reaction in *V. fischeri* that produces a photon requires molecular oxygen. As a culture ages, it becomes increasingly starved for oxygen, and the decay of light output occurs more and more rapidly. Cultures were grown on an orbital shaker for aeration, but the difficulty in providing oxygen to cultures while in the fluorimeter limited emission measurements significantly.

Fig. 3 shows the decay of light output for a culture at various ages throughout the period of maximum luminescence. It is evident from the initial intensities of the cultures (near time equal to zero) that light output increases with age until the culture is about 48 hours old. After this age, light emission decreases, until it stops entirely at about 64 hours (data not shown). At all ages, the light output can be separated into two stages of behavior: a plateau of relatively constant emission, followed by an exponential decay in the emitted intensity. The length of the plateau decreases as the culture ages. This indicates that the bacteria have exhausted the oxygen infused in the culture, and therefore metabolize the oxygen supplied by shaking more quickly. The stage of rapid light output decay can be modeled accurately by performing an exponential regression. In the equation that results, the decay constant provides a good basis for comparison among cultures. The absolute value of the decay constant grows as the culture ages, from about 0.26 absorbance units for the youngest culture shown to about 0.57 absorbance units for the oldest (see Fig. 3). Once again, this value may be connected with oxygen levels in the culture.

It should be noted that the time between the cessation of shaking and the start of emission measurement in Fig. 3 is 10 seconds. Such lag times are of importance: for cultures that have not reached (or have surpassed) their prime luminescence phase, a

majority of the light output is typically lost in the first few seconds after shaking stops. This quick decay of light emission renders quantification of maximum light output difficult. Due to this problem, increasing the constancy of light emission became a priority in addition to increasing the intensity.

Unexpectedly, we discovered that the type of broth used had just such an effect on the bacteria cultures. While using clear broth cultures to monitor optical density, we noted that although dimmer, luminescence in clear broth cultures did not decay nearly as quickly as in their cloudy counterparts. Figure 4 displays emission spectra from two 48-hour-old cultures. The clear culture is not quite half as bright, but its light output decays by only 9% in the first 30 seconds of measurement, whereas the cloudy culture does not retain even 1% of its original light over the same time period. Possible explanations for this difference are that the clear broth is not capable of supporting as many cells as is the cloudy broth, or that the growth rate in cloudy broth is retarded by the absence of certain nutrients. Optical density (OD) measurements, displayed in Fig. 5 for a wavelength of 600 nm, indicate that *V. fischeri* in clear culture does not grow exponentially. Although the turbidity of cloudy broth prevents similar OD measurements in cloudy broth, OD in similar photobacterium broths (i.e., containing similar nutrients) is recorded as growing exponentially during this time period [6,7]. Thus, it is probable that the decreased light emission and longer intensity plateau in clear broth cultures are the result of different growth patterns and bacterial populations than those that occur in cloudy broth. With fewer cells, oxygen and nutrient levels would not be exhausted as quickly in clear broth, allowing for a longer period of light production.

The last factor that has been tested in correlation with luminescence is concentration of ampicillin. The minimum concentration necessary to ward off growth of other bacterial species (about 5 $\mu\text{g/ml}$) and the maximum concentration that can be withstood by *V. fischeri* (about 200 $\mu\text{g/ml}$) place constraints on the use of ampicillin. However, between these limiting quantities, the antibiotic could be used as a tool to manipulate light output. Fig. 6 shows the effect of different concentrations of ampicillin on light output over time. Higher concentrations yielded decidedly greater light intensities, although the length of the constant intensity plateau was shortened. The increase in the rate of decay during the exponential decay phase of light output at greater concentrations is the same effect observed increasingly in cultures as they age. Therefore, we would speculate that cultures with maximum concentrations of ampicillin (100 $\mu\text{g/ml}$) grow at a greater rate than those with lower concentrations (and are thus comparable to older cultures). This could be attributable to the absence of contamination and therefore of competition with other bacterial species for nutrients. The greater cell density in these cultures causes the same oxygen-starvation effects as previously observed. For the purposes of this project, the creation of very bright cultures was preferred to very long-lasting ones, and so the highest concentration of ampicillin tested was chosen for standard use.

Discussion

The final parameter whose relation to light output must be examined is the concentration of autoinducer. The first assay that will be done to this effect is the addition of different quantities of synthetic autoinducer (purchased from Sigma Chemical Co.) to

cultures. Chiefly, the correlation between autoinducer concentration and light output will be characterized, and the amount of time needed for a culture to respond to a changing concentration must be measured. We will determine the concentration that results in optimal light intensity, which previous researchers have found to be about 200 nM [7,8], and test whether there is an upper limit on the amount of the chemical a culture can healthily sustain.

In addition to these autoinducer assays, I hope that the conclusion of this project will include the further development of a mathematical relationship between the two stages of light emission (i.e., the plateau and decay stages) and the amount of oxygen present in a culture.

With the exception of autoinducer concentration, recommendations can be made for the control of all major factors affecting light emission. Maximal light intensity and duration is induced in *V. fischeri* in an environment of constant oxygen concentration and high ampicillin concentration. Cultures should be examined within 24-48 hours after inoculation. Due to both the ease in calculating optical density and the much greater duration of bioluminescence, I would recommend the culturing of *V. fischeri* in clear broth over cloudy broth.

The results of this project provide information about limiting characteristics of *V. fischeri* cultures, which must be accounted for in the design of the optical techniques and device that will be used to examine single cells. For example, we now know that not only must an optically trapped cell remain in culture (to maintain autoinducer levels), but a device must be included to constantly supply oxygen, or luminescence will cease in a

matter of seconds. Such recognitions will hopefully expedite the overall experimental process, laying the foundations for the study of gene noise.

Acknowledgments

I would like to thank Dr. Steve Hagen for welcoming me into his lab and providing insight at every turn, and OJ Ganesh for his daily patience, support, and humor. I am grateful to my fellow REU student Lauren Riggs, who was extremely generous with her time and input. And, of course, many thanks are owed to Dr. Kevin Ingersent and the National Science Foundation for providing the research opportunity.

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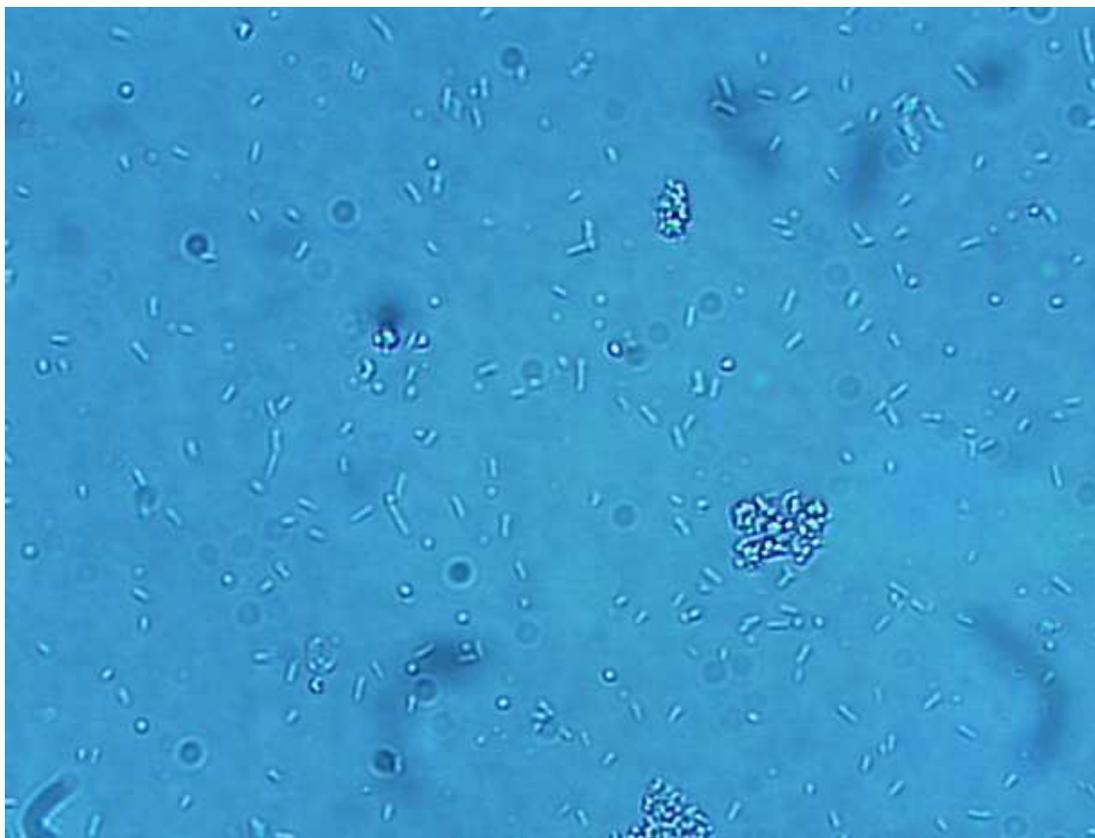


Fig. 1. Photomicrograph of individual *V. fischeri* cells.

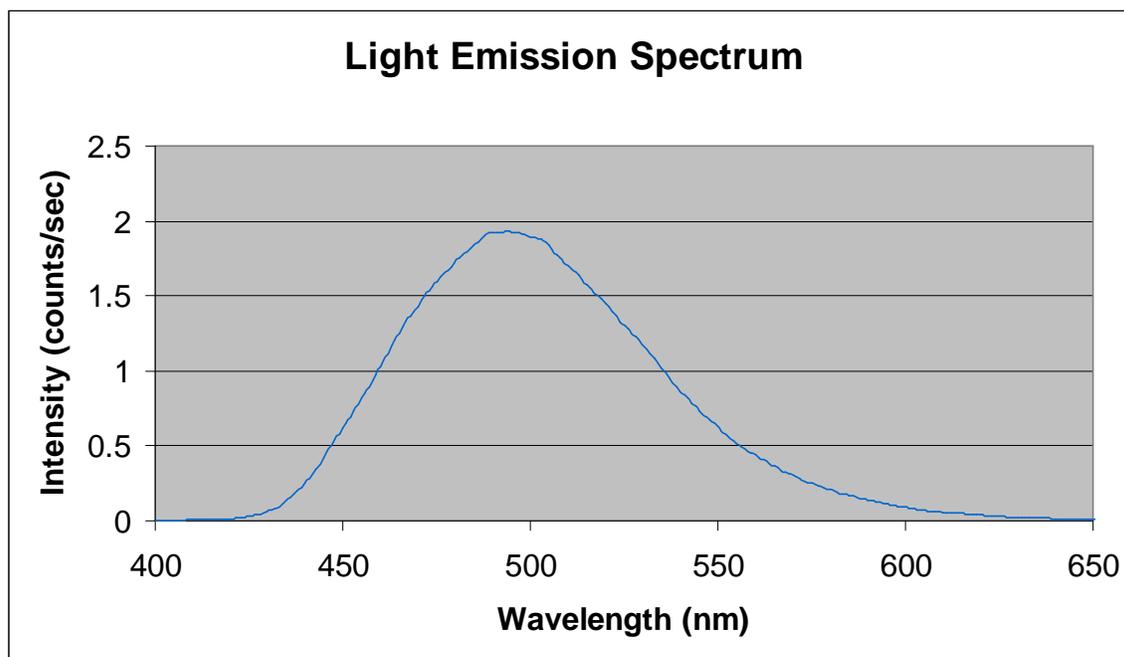


Fig. 2. Wavelength spectrum of *V. fischeri* light emission.

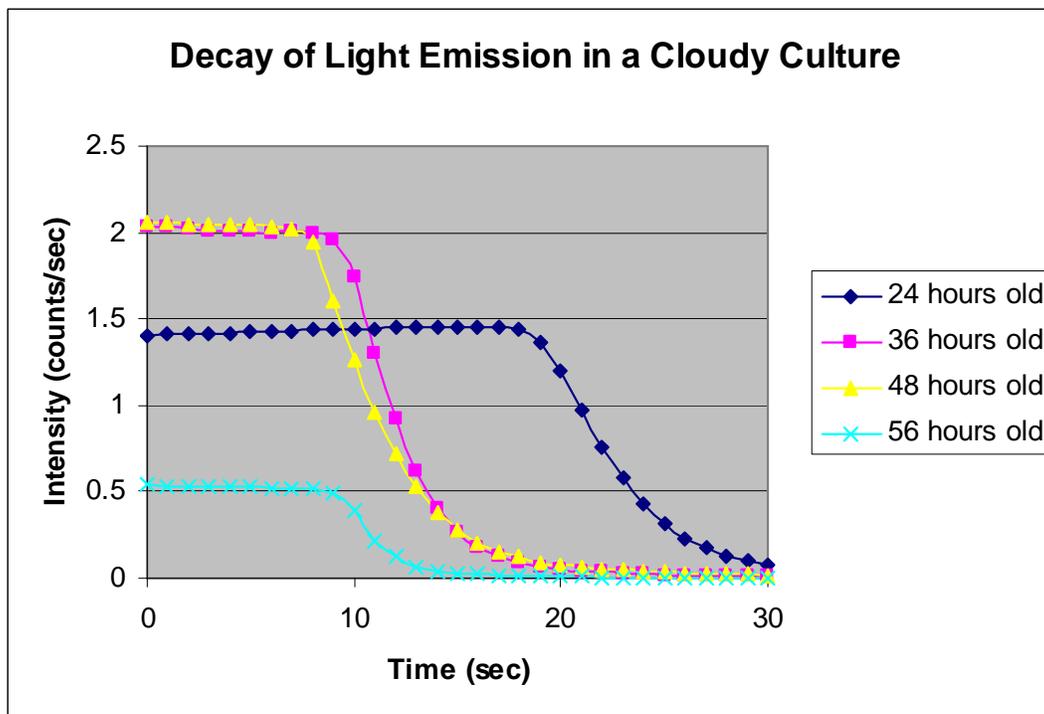


Fig. 3. Light emission of a cloudy culture at various ages, monitored over a period of 30 seconds. Lag time is 10 seconds.

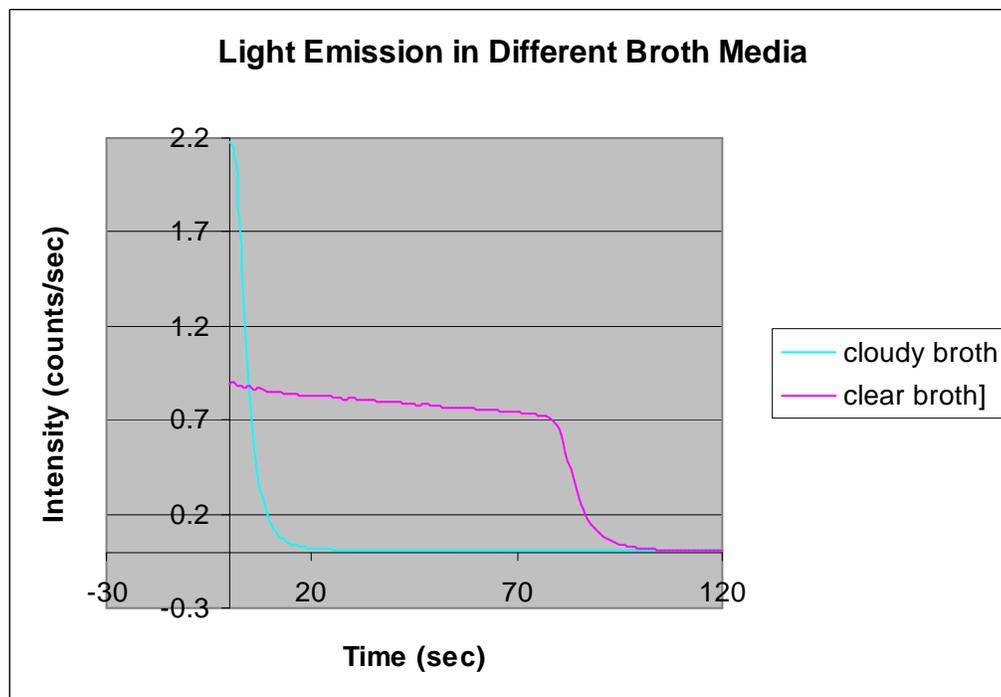


Fig. 4. Light emission of cloudy and clear cultures 48 hours after inoculation. Lag time is 15 seconds.

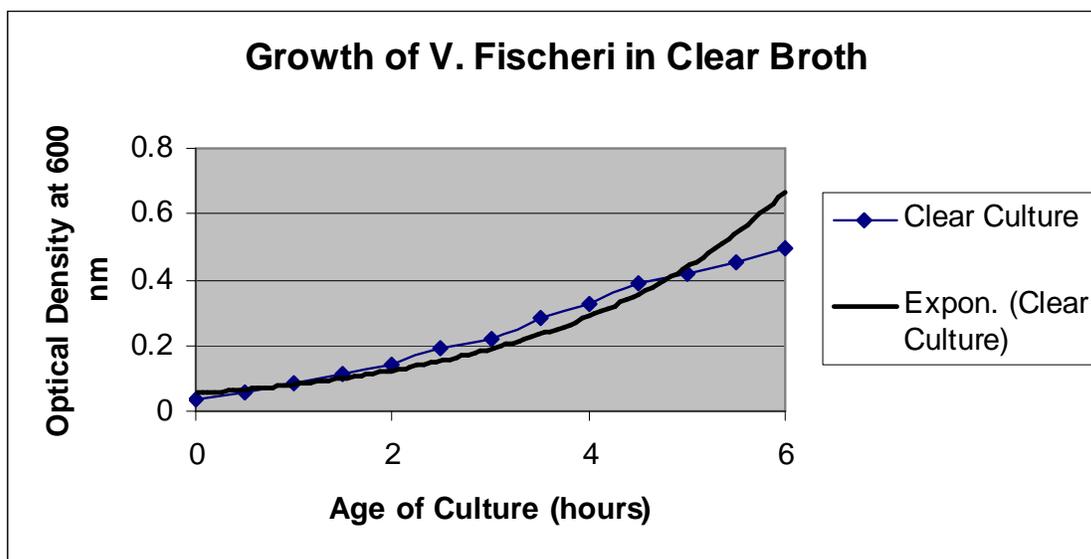


Fig. 5. Optical density of a clear culture for the first six hours after inoculation. The exponential curve, which would typically model bacterial growth trends well, does not approximate growth in this type of broth.

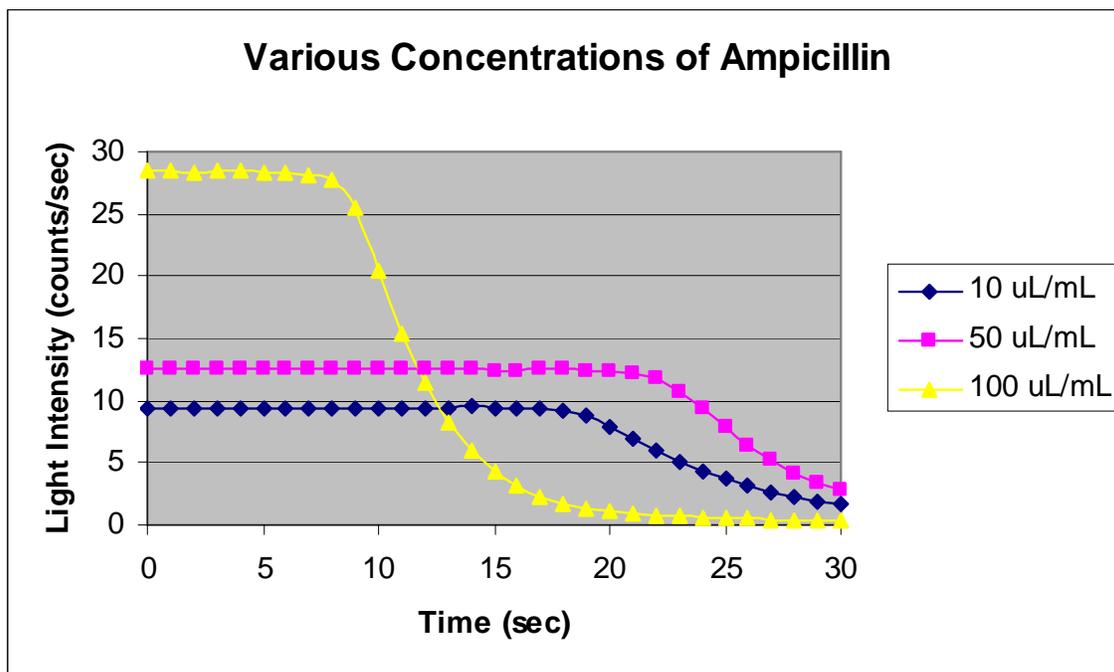


Fig. 6. Light emitted by cloudy broth cultures containing various concentrations of ampicillin. Lag time is 10 seconds.