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Effects of Quorum Sensing and Media on the Bioluminescent Bacteria *Vibrio fischeri*

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Summary

Artificial light is a valuable and important resource. It allows people to work, move freely, and do chores even at night. Most people take it for granted in the developed world. However, thousands of villages in third-world countries do not have access to light, as they do not have dependable electricity, and batteries are expensive. Villagers that do have access to these often burn trash, including batteries, compact fluorescent lamp (CFL) bulbs, and plastic as a way to dispose of waste, thereby releasing toxic gas and chemicals, making these light sources potentially environmentally dangerous. With almost 750 million people still living in villages in India (1), the effects of this on the environment and people are catastrophic (2). In order to provide environmentally friendly light sources, bioluminescent organisms such as the species of bacteria called *Vibrio fischeri*, could be utilized; however, the natural light from bioluminescent organisms is not bright enough to be effective. In order to increase the light emitted from the bacteria V. fischeri, I altered two variables in separate experiments. In the first experiment, four different chemicals were added to the medium in which the bacteria grew: chitosan, fatty aldehyde, $FMNH_2$, and L-cysteine. Bacteria grown in chitosan media had the most luminescence overall, followed by the control, and the other three chemicals had around the same luminescence. In the second experiment, I changed the bacterial densities. Using McFarland standard as a measure of density, I tested densities of 0.5 McFarland (McF), 1 McF, 2 McF, 3 McF, and 4 McF. The most luminescent bacteria were those grown in 0.5 McF or 1 McF, with 4 McF being the least luminescent. The results also showed that the bacteria grown in chitosan medium were luminescent enough to illuminate three-fourths of an 8.5 inch by 11 inch paper when held next to it, but the density of bacteria had no significant effect on luminescence.

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Introduction

Bioluminescence is an organism's ability to produce light, and can be used as a light source in third world countries without access to electricity. Many countries, such as India, have villagers living without light available during the nighttime. When they do have access to electricity or bulbs, they often burn the used resources with the rest of their trash, and the smoke from burning bulbs, batteries, and plastic is incredibly toxic to the environment. The current study investigates the use of bioluminescence to create an eco-friendly and efficient solution.

Before starting the experiment, I explored three possible organisms: bioluminescent algae, bacteria genetically modified to produce a fluorescent protein, and the bioluminescent bacteria *V. fischeri*. Upon observation, *V. fischeri* proved to produce a bright and continuous grow, thus it was selected as the choice material to evaluate in this study.

Similar to fireflies and other bioluminescent organisms, *V. fischeri* produces an enzyme called luciferase. As shown in **Figure 1**, Luciferase oxidizes the catalyst $FMNH_2$, or riboflavin 5' monophosphate sodium salt hydrate, and the lipid fatty aldehyde, to produce FMN, or flavin mononucleotide, H_2O, and fatty acid (3). The reaction is exothermic and gives off photons of light (4). *V. fischeri* is a type of marine bacteria found in shallow coastal waters inside nocturnal bobtail squids, most commonly *Euprymna scolopes*, the Hawaiian bobtail squid (5). At night, the squid uses the bacteria's luminescence as camouflage (6). The light is strong enough to hide the shadow of the two-inch squid by mimicking moonlight in water with a blue-green glow (7).

V. fischeri bioluminescence depends on quorum sensing, a system where a gene is not expressed unless a minimum density of bacteria is achieved. The bacteria constantly produce chemicals called autoinducers, in this

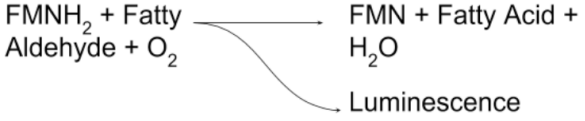

Figure 1: The chemical reaction of bioluminescence in *Vibrio fischeri*, taking place on the enzyme luciferase

	24 Hours				48 Hours				72 Hours			
	1	2	3	Avg	1	2	3	Avg	1	2	3	Avg
Control	46.0	58.0	66.0	56.7	57.0	51.0	57.0	55.0	41.0	46.0	54.0	47.0
Chitosan	47.0	54.0	64.0	55.0	59.0	72.0	78.0	69.7	43.0	43.0	50.0	45.3
Fatty Aldehyde	46.0	52.0	37.0	45.0	44.0	46.0	47.0	45.7	36.0	44.0	52.0	44.0
$FMNH_2$	44.0	53.0	49.0	48.7	51.0	57.0	58.0	55.5	43.0	46.0	36.0	41.7
L-Cysteine	49.0	53.0	58.0	53.3	44.0	47.0	64.0	51.7	40.0	50.0	51.0	47.0

Table 1: All the values of luminescence obtained for each media over 72 hours.

case homoserine lactone (or HSL), and the bacteria can also sense the levels of HSL in the surrounding water (8). If the density of bacteria is high enough, the HSL binds to the transcription factor TraR, which activates certain genes in the bacteria (9), causing it to produce luciferase (10). Because glowing in the daytime when the squid sleeps in the sand does not confer any benefit, the squid expels a majority of the bacteria every morning into the water. Thus, in the daytime, the bacteria in the squid and the surrounding water are not dense enough to glow, but the *V. fischeri* in the squid will continue to reproduce. By nightfall, once the squid is awake, the bacteria's density is high enough for it to produce luminescence (11).

In the current study, I explored strategies to increase the luminescence of the *V. fischeri*, as it does not produce enough luminescence on its own to be an efficient light source. This experiment was conducted in two parts: first, I tested how adding additional chemicals to the media affected the bacteria's luminescence. I added four different chemicals to the media. I hypothesized that $FMNH_2$ and fatty aldehyde would increase luminescence, as the *V. fischeri* would have more resources to carry out the chemical reaction.

In the second part of the experiment, I tested the effect of the initial bacterial density on the bioluminescence.

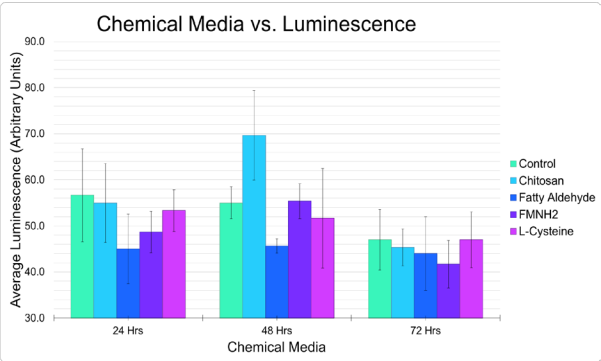

Figure 2: Chemical Media vs Luminescence. On the first day, the bacteria in the control medium produced the most luminescence, with chitosan in second, followed by L-cysteine, $FMNH_2$, and fatty aldehyde. On the second day, chitosan produced a significantly increased amount of luminescence. The last day yielded the same order as the first, with less luminescence. Error bars show one standard deviation.

Since quorum sensing plays a key role in *V. fischeri* bioluminescence, I hypothesized that luminescence would increase with density.

The first experiment found that chitosan had the most pronounced effect on luminescence. In the second experiment, luminescence was unaffected by the density of the *V. fischeri*.

Results

This study explored using bioluminescent bacteria as an artificial light source. The research investigated ways to increase the luminescence produced by the bacteria through adding chemicals to the media and changing the density in which the bacteria were grown. I grew the bacteria in five separate media with added chemicals (one was the control group, with just normal photobacterium media), as well as five separate densities.

I used photobacterium medium for the control group in the chemical experiment. For the density experiment, I also used photobacterium medium for all the groups. I performed both experiments in triplicate, and I photographed and measured the luminescence three times: at 24 hours, 48 hours, and 72 hours after subculturing, or after transferring the bacterial cells to fresh media from a previous culture.

I analyzed each image of the chemical experiment through an image processor, and obtained the values shown in **Table 1**.

Overall, the highest amount of average luminescence

24 Hours:	Control	Chitosan	Fatty Aldehyde	$FMNH_2$	L-Cysteine
σ	10.1	8.54	7.55	4.51	4.51

Total σ: 7.66

Source	SS	df	MS
Between Media	277	4.00	69.2
Within Media	544	10.0	54.4

The f-ratio value is 1.27. The p-value is 0.343.

48 Hours:	Control	Chitosan	Fatty Aldehyde	$FMNH_2$	L-Cysteine
σ	3.46	9.71	1.53	3.79	10.8

Total σ: 10.1

Source	SS	df	MS
Between Media	937	4.00	234
Within Media	479	10.0	47.9

The f-ratio value is 4.89. The p-value is 0.0190.

72 Hours:	Control	Chitosan	Fatty Aldehyde	$FMNH_2$	L-Cysteine
σ	6.56	4.04	8.00	5.13	6.08

Total σ: 5.57

Source	SS	df	MS
Between Media	60.7	4.00	15.2
Within Media	373	10.0	37.3

The f-ratio value is 0.406. The p-value is .800.

Table 2: The results of an ANOVA test on every value of luminescence in the media experiment.

	24 Hours				48 Hours				72 Hours			
	1	2	3	Avg	1	2	3	Avg	1	2	3	Avg
0.5 McF	55.0	56.0	62.0	57.7	49.0	50.0	54.0	51.0	45.0	36.0	56.0	45.7
1 McF	50.0	59.0	61.0	56.7	56.0	47.0	53.0	52.0	47.0	51.0	43.0	47.0
2 McF	51.0	60.0	68.0	59.7	47.0	50.0	51.0	49.3	46.0	41.0	42.0	43.0
3 McF	56.0	61.0	48.0	55.0	48.0	49.0	50.0	49.0	43.0	44.0	43.0	43.3
4 McF	52.0	53.0	51.0	52.0	45.0	49.0	50.0	48.0	40.0	47.0	41.0	42.7

Table 3: All the values of luminescence obtained for each density over 72 hours

produced was by bacteria in media with chitosan, at 48 hours. At 24 hours, the control group produced the most luminescence. At 48 hours, bacteria grown in chitosan medium produced significantly more luminescence than the other bacteria, and more than the control bacteria. At 72 hours, the luminescence was more or less the same across all conditions. Overall, in the media experiment, the bacteria displayed the most luminescence at either 24 or 48 hours, depending on the media. L-cysteine and $FMNH_2$ had little to no effect compared to the control, and fatty aldehyde appeared to decrease luminescence. The maximum luminescence overall occurred at 48 hours, with chitosan (**Figure 2**).

An analysis of variance (ANOVA) test on the data from the different media conditions returned the values shown in **Table 2**.

I repeated this for the separate densities. I used 5 densities: 0.5 McF as the control, as well as 1 McF, 2 McF, 3 McF, and 4 McF. Though these were the densities of the bacteria initially, they grew without limitations or restraint after subculturing. Density seemed to have no significant effect on luminescence, and luminescence declined each day.

When analyzed with an image processor, each bottle returned the values shown in **Table 3**.

Every density had the highest luminescence at 24 hours, less at 48 hours, and the least luminescence at 72

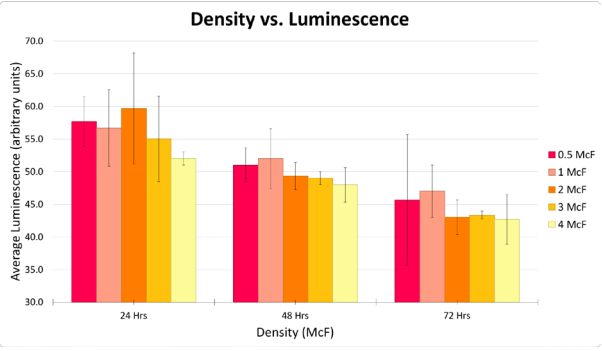

Figure 3: Density vs Luminescence. Throughout the experiment, the varying densities' results did not differ much, and luminescence decreased with time. Density also seems to cause a slight decline in luminescence, spiking at 2 McF on the first day, and 1 McF on the second and third day. Error bars show one standard deviation.

hours. However, there was not significant variation in the luminescence produced between the different density groups. At 24 hours, 2 McF had the most luminescence, followed by 0.5 McF, then 1 McF, 3 McF, and 4 McF with the least luminescence. Forty-eight hours after subculturing, the most luminescence came from the 1 McF density, second was 0.5 McF, then 2 McF, 3 McF, and 4 McF. On the last day, the densities ranged from most luminescent to least luminescent as follows: 1 McF, 0.5 McF, 3 McF, 2 McF, and lastly, 4 McF (**Figure 3**).

An ANOVA test on the different densities returned the values shown in **Table 4**.

On the second day, the bacteria grown in chitosan were bright enough to light up around three quarters of a paper (8.5 inch by 11 inch) held next to it in total darkness. Writing on it was also legible and easy to read. Under total darkness, the same effect on a sheet of paper could be achieved with a phone screen at 30 percent brightness. The control bacteria could only illuminate roughly a fifth to a quarter of the page, and text was difficult to read. The bacteria grown in different densities overall could light up between a fifth and a quarter of the paper, similar to the control bacteria.

Discussion

In the media experiment, the bacteria in the chitosan medium produced the most luminescence, possibly because chitosan, a starch, acted as a nutrient to the bacteria, giving them more energy. At 48 hours, the

24 Hours:	0.5 McF	1 McF	2 McF	3 McF	4 McF
σ	3.79	5.86	8.50	6.55	1.00

Total σ: 5.54

Source	SS	df	MS
Between Media	100.	4.00	25.1
Within Media	330.	10.0	33.0

The f-ratio value is 0.761. The p-value is 0.574.

48 Hours:	0.5 McF	1 McF	2 McF	3 McF	4 McF
σ	2.65	4.58	2.08	1.00	2.65

Total σ: 2.83

Source	SS	df	MS
Between Media	31.1	4.00	7.77
Within Media	80.7	10.0	8.07

The f-ratio value is 0.963. The p-value is 0.469.

72 Hours:	0.5 McF	1 McF	2 McF	3 McF	4 McF
σ	10.0	4.00	2.65	0.577	3.79

Total σ: 4.78

Source	SS	df	MS
Between Media	43.3	4.00	10.8
Within Media	276	10.0	27.6

The f-ratio value is 0.393. The p-value is 0.810.

Table 4: The results of an ANOVA test on every value of luminescence in the density experiment

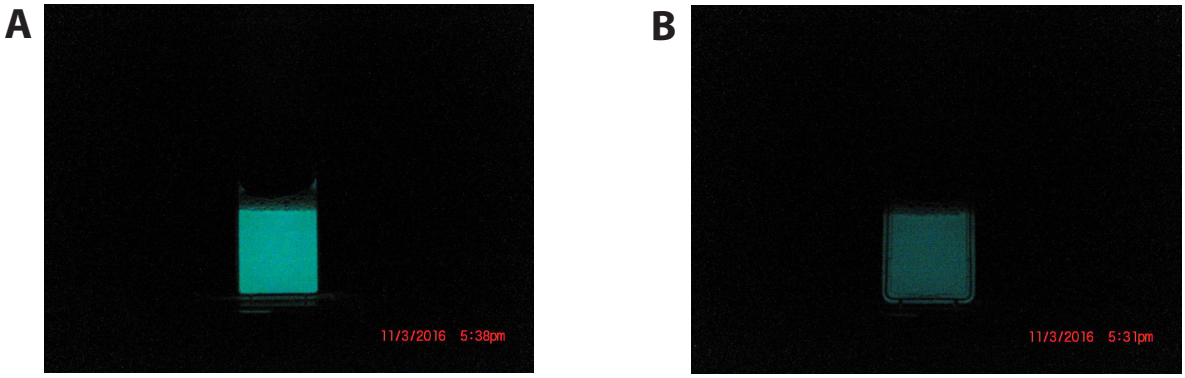

Figure 4: *V. fischeri* 48 hours after subculturing. (A) *V. fischeri* in the medium containing chitosan. (B) *V. fischeri* in the control Photobacterium medium. Photographed under total darkness.

bacteria grown with chitosan (**Figure 4a**) was significantly more luminescent than the control bacteria (**Figure 4b**). Future work could include measuring how much chitosan the bacteria consumed and attempt to correlate it to the bacterial luminescence. Since bacteria in the medium with chitosan did not display the most luminescence until 48 hours, I postulate that the bacteria need time to consume the chitosan, though this could be investigated in later studies.

L-cysteine might not have affected the luminescence because as an amino acid, it might have not been taken in or broken down as quickly as chitosan. The fatty aldehyde and $FMNH_2$, the two reactants of the chemical reaction for bioluminescence, might not have had any effect because in either group, only one reactant was increased in supply, but not the other. This likely meant that the bacteria were unable to increase the reaction in either case with the resources they had. In future experiments, the effects of adding both fatty aldehyde and $FMNH_2$ to the solution could be studied.

In the density experiment, contrary to my hypothesis, the luminescence decreased as density rose, although this difference was not statistically significant. For this result, I inferred that even though quorum sensing was at work, there were not enough resources for the high amounts of bacteria. A possibility is that the large density of bacteria used up the resources too quickly, and in the following days, the bacteria died. Further research investigating why increased densities had a negative effect on the bacterial luminescence could be done. Further research could test ways in which additional nutrients could affect the bacteria in different densities as well as test for growth, perhaps using plated bacteria on agar rather than broth. Another possible experiment could study the effects of adding homoserine lactose, the autoinducer in quorum sensing for *V. fischeri*, to the medium, to test if that could activate the bacteria's bioluminescence without increasing the densities.

Future works could also include researching ways to increase the longevity of the bacteria, rather than the brightness, and growing *V. fischeri* at the most successful densities in the most successful media, which would be the media containing chitosan. Ways to efficiently implement this bacteria prototype in the real world need to be further explored. In a real-world situation, the bacteria could be used in a hand-held container, in media. Villagers would still need to be able to create subcultures of the *V. fischeri* when needed and would need access to the media and containers. This could be an idea to explore in later study. In further research, a spectrometer could be used to measure luminescence.

This research does not include the field study. The experiment was conducted in a lab with the goal and purpose of determining which chemical media and density yielded more luminescence. It did not address how bioluminescence would practically be implemented in the real world.

My research contributes to creating environmentally friendly light sources for third world countries. This study proves that *V. fischeri* is a viable option to provide light, both as an alternative to ecologically harmful sources, as well as in cases where no light sources are accessible at night.

Methods

Before starting the experiments, I needed to test the bacteria to ensure that they were healthy and would glow in the control media. To make the control media, I used a recipe from Flinn Scientific for Photobacterium Media (12). The recipe called for chemicals in powder form, measured in grams. However, 1 molar solutions of each chemical needed were available, thus I converted grams to milliliters (mL). I divided the molar mass of the chemical plus the mass of the water in the solution by 1000 mL to find the density of the molar solution. I then

divided the number of grams called for in the recipe by this density to find the amount of mL that needed to be added to the media.

I used the above formula to get 10.2 mL of calcium chloride, 27.1 mL of magnesium chloride, 9.4 mL of potassium chloride, 96.5 mL of sodium chloride, and 28 mL of magnesium sulfate. I also added 5g of tryptone, 3g of yeast extract, and 700 mL of distilled water, as per the recipe. The molarity of the calcium chloride was 0.01020325 M $CaCl_2$, magnesium chloride was 0.0270528 M $MgCl_2$, potassium chloride was 0.00938967 M KCl, sodium chloride was 0.482546 M NaCl, and magnesium sulfate was 0.0279928 M $MgSO_4$.

I mixed these into water using a magnetic stirrer with a hot plate. After I autoclaved it, I poured the medium into 3 containers, with 30 mL in each. I put a 10 µL loop of *V. fischeri* into each container and I incubated them at room temperature on a rotating platform, as the bacteria had to be agitated in order to glow, for 24 hours. The next day, the bacteria were glowing and healthy. To take pictures of the bacteria, I built a cardboard box. I painted the insides black and cut a hole to insert the lens of the camera. On the top, I cut a slit for inserting the container of bacteria. While taking the pictures, I covered the slit with a black blanket so that pictures of the bacteria could be taken under total darkness, capturing only the luminescence of the bacteria.

For the media experiment, the independent variable was the media. I prepared five different photobacterium media with supplemental chemicals. The media mimicked the bacteria's natural environment inside the squid, and I used a container of regular photobacterium medium as a control. The first chemical was chitosan; which, in addition to being found in the bacteria's natural habitat, is a starch, or a carbohydrate, which would serve as a nutrient. L-cysteine, the second chemical I tested, is also found in their natural environment (13). The final two chemicals, $FMNH_2$ and fatty aldehyde, are both reactants in the chemical equation that causes the bioluminescence. I mixed each chemical into separate containers of photobacterium media.

The first batch of media was a control with no added chemicals. I included 500 µL chitosan per mL of media in the second batch of media, 167 µL of $FMNH_2$ in the third, 3.1 µL of fatty aldehyde in the fourth, and 300 µL of L-cysteine per mL of media in the fifth. I then autoclaved the media. I poured 30 mL of the media into the 50 mL containers, 3 containers for each type of media. I grew each group of bacteria in three bottles to increase statistical accuracy and eliminate outliers or mistakes. There were 15 containers total. I put a 10 µL loop of bacteria into each container. I then incubated the bacteria at room temperature after taping them to a rotating platform. Twenty-four hours after subculturing, I observed each container and took pictures of them in the cardboard box. I took pictures again 48 hours and 72 hours after subculturing.

In the second experiment, the initial density of the *V. fischeri* was the independent variable. I used the McFarland (McF) Standard, a measure of cell concentration, to measure the initial densities. The densities I used were 0.5 McF, the control, 1 McF, 2 McF, 3 McF, and 4 McF. 0.5 McF is roughly 1.5×10^8 cells per mL, 1 McF is 3×10^8 cells per mL, 2 McF is 6×10^8 cells per mL, 3 McF is 9×10^8 cells per mL, and 4 McF is 12×10^8 cells per mL. I poured 30 mL of autoclaved medium into 15 containers, with 30 mL of medium in each. I put a loop of bacteria into three of the containers and compared them to the 0.5 McF tube. I viewed different prints and colors through the tube to determine how similar the opacity and clarity of the tubes were. I added bacteria to the container until it matched the 0.5 McF tube. I repeated these steps for 1 McF, 2 McF, 3 McF, and 4 McF. Once again, I took pictures 24 hours, 48 hours, and 72 hours after subculturing.

I transferred all the digital pictures of the bacteria to a computer. To measure the light produced by the bacteria, I utilized an image processing software called ImageJ. Using ImageJ, I converted each picture taken of the bacteria into a black and white image. After selecting the area around the container of bacteria, the number of white or grey pixels in the image and the intensity of the whiteness in each pixel using RGBW values were measured, therefore effectively obtaining a numerical value of light produced. I then compared the light produced by the bacteria in the different media to each other. I repeated this process for the density section of the experiment.

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