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DIFFERENT CORAL PATHOGENS CAUSING DISEASE THROUGH BIOFILM FORMATION AND WAYS TO STUDY AND DISRUPT IT

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by

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ABSTRACT

DIFFERENT CORAL PATHOGENS CAUSING DISEASE
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Coral pathogens have been causing irreversible damage to the coral communities all around the world. Specific genera of bacteria like *Vibrio* have been studied throughout the years, however, researchers continue to find new species. Some pathogens' biofilm formation ability negatively impacts the corals. We attempted to optimize the biofilm assay in a specific species *Vibrio coralliilyticus* in order to learn more about the conditions and variables that affect biofilm formation and to develop a set of conditions that the pathogen deems as ideal. The immediate goal of this research is to be able to quantify biofilm and find factors that affect its formation.

Once we have this information, we want to find out if it is possible to disrupt these harmful biofilms using coral compounds. This is necessary for understanding host defense

mechanisms against biofilm, which will also help in the advancement of this research and its future application to coral diseases.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

CHAPTER 1

INTRODUCTION

1.1 Coral Pathogens

Various pathogens have been causing the demise of coral communities. It is predicted that irreversible damage will occur to the coral reefs within this century due to climate change, disease and other factors that continue to affect our planet daily. One factor that has been studied is disease caused by pathogens targeting coral communities. However, it is difficult to track and document the pathology and specific causes for many different coral diseases (1). Some of the most prominent pathogens that serve as the causative agents for coral diseases are members of the bacterial genera *Vibrio* and *Nautella*.

1.1.1 Vibrio coralliilyticus

Researchers have found evidence of disease in corals in the form of bleaching. This bleaching was found to occur depending on the temperature. In addition to bleaching, signs of necrosis were found in coral reefs. Add a temperature of 24°C to 25°C, evidence of bleaching was found in the coral after two to three weeks of exposure to these temperatures. However, when the temperature was increased 27°C to 29°C, the corals showed more severe damage and rapid necrosis. Both of these conditions combined are referred to as white syndrome. Alongside *Vibrio coralliilyticus*, two other species of *Vibrio* also can also cause this syndrome to manifest in different hosts like *Pocillopora damicomis* and *Pachyseris speciosa* (1)

1.1.2 Vibrio harveyi

Several coral diseases are caused by pathogens like *V. harveyi* and other pathogens present in coral reefs that essentially makeup the coral-pathogen pathosystem. The ability of these pathogens to form biofilms can indicate irreversible damage in the already suffering coral reef communities. The whole process of how infection occurs is not completely understood and is the subject of research experiments done in labs here at UTA. To understand how infection occurs, we should be able to mimic the conditions where the infection is taking place. Various factors need to be taken into consideration when attempting these experiments: incubation temperature, bacterial concentration, the type of media, amount of salt, type of salt, etc., and finding the optimal conditions for each is one of the subjects of our research.

1.1.3 Nautella italica

Nautella italica, was isolated from a marine electroactive biofilm and was extensively studied by researchers from three different European countries. However, not only did they isolate one strain of that species, they were able to isolate five different strains. These scientists were able to analyze the phenotypic and genotypic differences between these strains and concluded that they belong to a new genus and species that has not been discovered before (2). Evidently, studying biofilms proves extremely important seeing as novel species of bacteria could be found living there.

1.2 Biofilm Formation

Certain bacterial species can exist in aggregated communities on various surfaces forming biofilms. Several methods have been outlined for the study and observation of early biofilm formation which includes the adherence of the bacteria to the surface and

their aggregation into microcolonies. However, the outlined techniques are specific for biofilms that are inert, making it easier to visualize under a microscope and even with the naked eye using stains like crystal violet to stain the bacteria. Small changes in the parameters used for the screens can result in differences that are observed and noted (3).

1.2.1 Significance of the biofilms in healthcare

The study of biofilms also proves necessary in healthcare, specifically with the use of medical devices that are placed inside the body cavity. Biofilms pose a serious problem for these patients since these devices can serve as a surface for their formation. Biofilm-forming bacteria show resistance to any antibiotics or drugs given to treat them, which increases the chances of the patient developing a serious infection that cannot be treated with antibiotics. Therefore, most of the time the device that has the biofilm growth will be removed completely from the patient to treat the infection (4).

CHAPTER 2

LITERATURE REVIEW

2.1 Role of *Vibrio coralliilyticus* in Coral Diseases

Strains of *Vibrio* were isolated from an organism called *Pocillopora damicornis* in the Red Sea, specifically from the Eliat reef (1). These isolated strains revealed not only bleaching, but necrosis as well. This new isolated strain was then referred to as *V. coralliilyticus* and was proven to be closely related to other species in the *Vibrio* genus such as *V. shilonii*, which is a causative agent for white syndrome. After various experimental infections at different temperature ranges, it was revealed that this new strain was temperature-dependent and caused bleaching and necrosis at different temperatures (5). At a temperature of 27°C, an enzyme called metalloprotease was isolated from the *P. damicornis* indicating rapid tissue damage. Following these experiments, *V. coralliilyticus* was found in many different parts of the world and was listed, in addition to other pathogens, as a causative agent for the white syndrome (Fig. 2.1) in regions all throughout the Indo-Pacific (1).

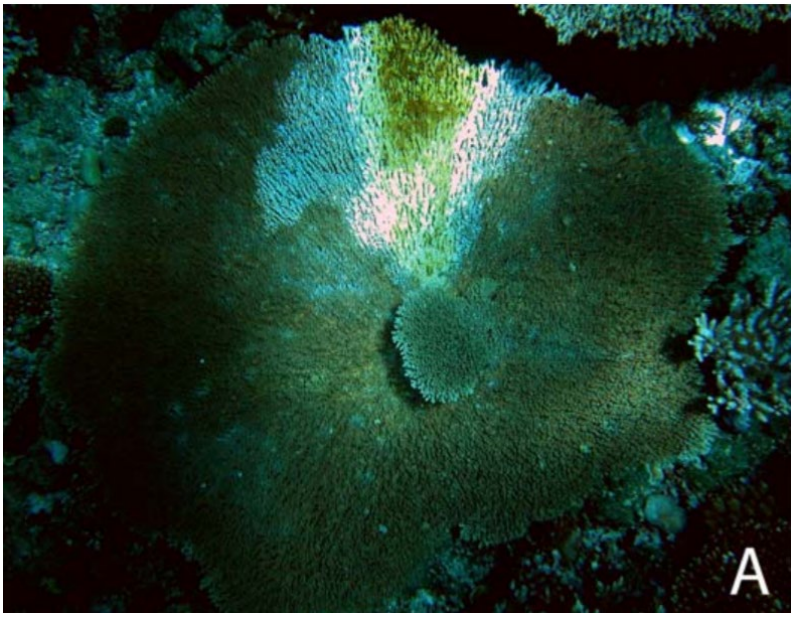

Figure 2.1: White syndrome in *Acropora* sp.
(image courtesy of Meir Sussman) (1)

This strain has also been isolated from different organisms in the Caribbean, Mediterranean, England, and Brazil. When comparing the 13 different strains of *V. coralliilyticus* that have been isolated, phenotypic and phylogenetic differences indicate that there is a high level of diversity among genomes. What the researchers were searching for is a link between the strains or the possibility of a clonal population structure that may indicate there is an epizootic strain circulating. An epizootic strain would explain the presence of this species in regions all over the world (1).

2.2 Role of *Vibrio harveyi* in Corals

Aside from white syndrome, there are different diseases caused by coral pathogens like the ones previously discussed. Another disease that has been commonly studied is seen in the genus *Montastrea* in the Caribbean reef-building coral: yellow band/blotch disease (YBD). This disease manifests as yellow blotches, as the name describes, that are pale and spread in a band fashion (Fig. 2.2) the more the disease progresses (1). Different

experiments and studies revealed that species of *Vibrio*, including *V. harveyi*, attack the coral tissue of the *Montastrea* and other corals that are present within the same region. These attacks cause the blotching and banding due to the disruption of the algal cells. Researchers analyzed genetic material and clone libraries to compare normal, non-diseased corals and corals with YBD. Interestingly, they found the healthy corals actually had strains of *V. harveyi* but the number of strains found did not correlate with disease. Although the presence of *V. harveyi* was not indicative of disease, it could be an indication of another factor impacting the health of the coral. Their presence could be related to environmental stressors or even bleaching. Furthermore, later studies revealed that in general there is an increase in *Vibrio* species presence in the *Montastrea* species during bleaching, but once the coral recovers, the levels of *Vibrio* species return to normal (1).

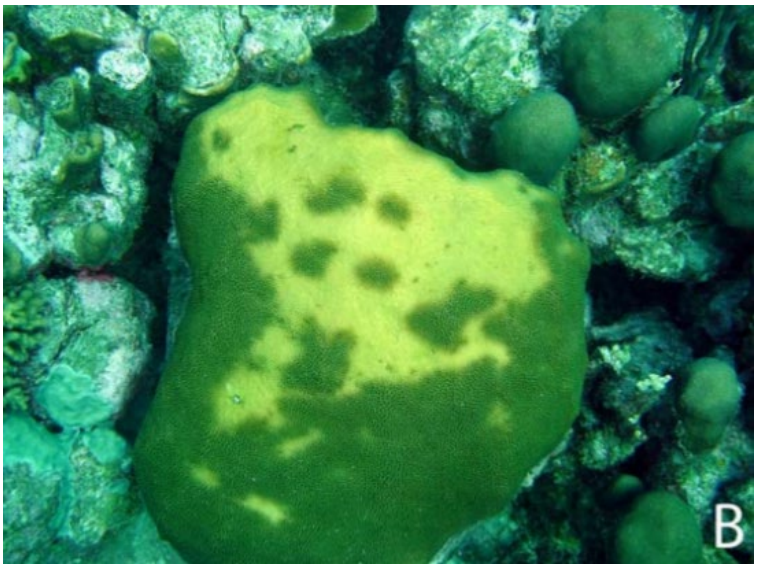

Figure 2.2: Yellow blotch/band diseases on Montastrea sp. (image courtesy of James Cervino) (1)

2.3 Anti-Biofilm Compounds Using *Staphylococcus aureus*

Two different experiments were carried out using *Staphylococcus aureus* due to its various implications on health. The first experiment uses a fairly similar concept to our intended research. The biofilm assay was used to study how the bacteria formed the biofilm and what was able to disrupt in order to synthesize anti-biofilm compounds to use in drugs and medication to treat infections in hospitals. This experiment uses coral-associated actinomycete (CAA) as the anti-biofilm compound tested for its ability to disrupt biofilms (6). As for the other experiment, it uses a similar anti-biofilm compound from corals, coral-associated bacterial extracts (CAB), but took it a step further with trying to inhibit virulence factors other than the biofilm that were being produced by *S. aureus* (7).

CHAPTER 3

METHODOLOGY

As the coral pathogen, we will use the bacteria *Vibrio coralliilyticus* to find the optimal conditions for biofilm formation, develop a working biofilm assay, and experiment with the conditions and length of incubation to optimize the assay. This experiment was completed in phases and in order to move on to the next phase, the preceding task must be successfully fulfilled.

3.1 Optimizing Bacterial Growth Media

The goal is to mimic the conditions where the corals reside; therefore, marine agar and broth would be suitable for growth. Multiple batches of marine broth and agar were required to achieve the ideal concentration of media in salt water. To make marine broth, suspend 37 g of the marine broth powder into 1 L of salt water. Bring to a boil on a hot plate, while stirring aggressively to dissolve all the powder. Autoclave the flask at 121°C for 20 minutes. The broth should be sterilized and ready for use, however, as a safety precaution to ensure no contamination, the flask can be placed in an incubator at 40°C overnight. To make the marine agar, the broth powder was used, however, 17 g of solidifying agent were also added to the 1 L of salt water. Bring to a boil while stirring aggressively, and autoclave under the same conditions as the broth. Once the flask has been autoclaved, the media is sterilized and ready to be poured into petri dishes aseptically. Once the media has solidified in the plates, they should be moved to the incubator for a day and then moved to a fridge for storage.

3.2 Creating Bacterial Stocks and Fresh Cultures

The sample of *V. coralliilyticus* was received in a small glass ampoule that require careful opening. The tip of the vial was heated with a Bunsen burner. A few drops of water were added to the tip to crack the glass tip. The remainder of the tip was carefully removed. With sterile forceps, the insulating material and cotton were carefully removed to obtain the bacterial culture. Four stock cultures were prepared and incubated for 24 hours at 40°C on TSA (Fig. 3.1) and two tubes of TSB. To ensure that the sample is not contaminated, one of the stock cultures was used for a Gram staining. To maintain bacterial cultures for *V. coralliilyticus* for an extended period of time, cryovials were prepared using 50% glycerol and sterile TSB (Fig. 3.2) and stored at -80°C.

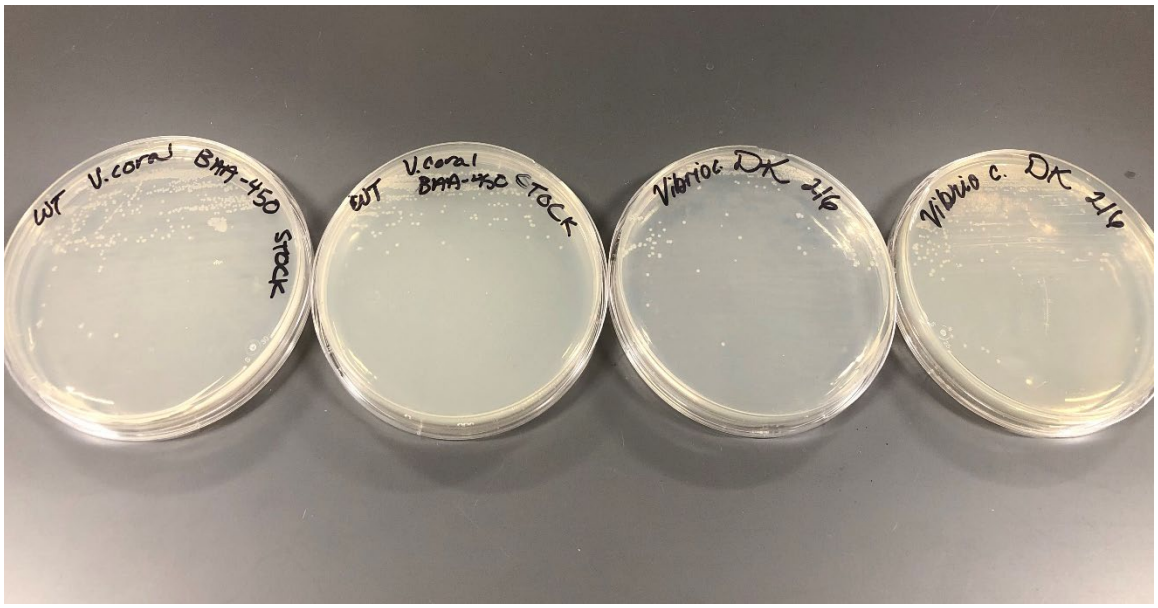

Figure 3.1: Stock cultures of *V. coralliilyticus* on TSA

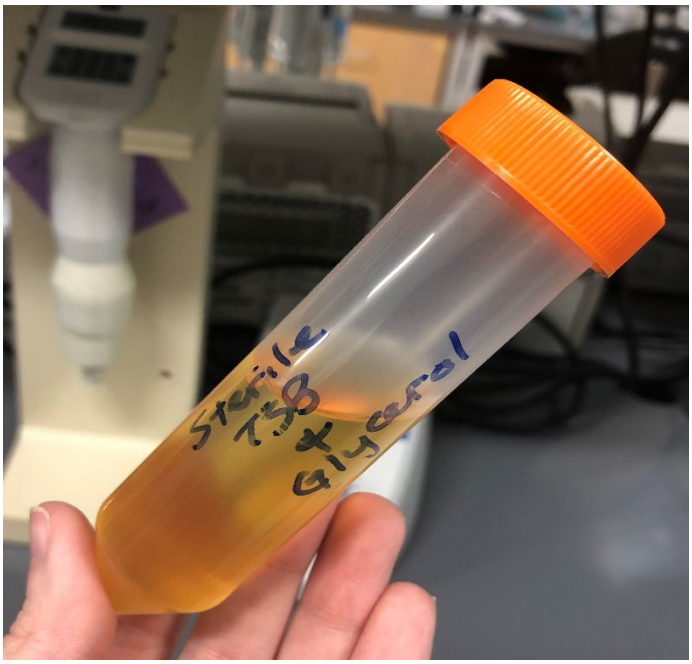

Figure 3.2: Prepared glycerol and TSB solution for cryovials

3.3 Dilution Growth Curves

Two colonies of *V. coralliilyticus* were inoculated into 10 mL of marine broth and the tube was labeled "control". Eleven tubes were obtained and filled with 5 mL's of sterile marine broth. The tubes were labeled 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, and blank. The control tube was vortexed, and 5 mL's were transferred to the 1:2 tube aseptically. The process was repeated for the remainder of the tubes except for the blank. Using a multichannel pipettor, the media was transferred to a 96-well plate with one dilution per column. The first column began with the control, then 1:2 and so on with the last column being the blank. The pipette was changed between every transfer and the tubes were vortexed. The Gen5 program was used to obtain a growth curve for the bacteria with the different dilutions and the plate was incubated for 12 hours at 30°C and the optical density was measured every 10 minutes.

3.4 Running and Optimizing the Biofilm Assay

To run the biofilm assay and work on optimizing it, the bacteria *Pseudomonas aeruginosa* was used. Two colonies were grown to stationary phase for 6 hours in 10 mL of TSB. 5 mL's of is transferred into a tube labeled "control". Eleven more tubes were obtained, filled with 5 mL's of TSB, and labeled 1:2 to $1:10^3$ and one blank. The remaining 5 mL's of the inoculated bacteria was transferred into the 1:2 tube, vortexed and the process was repeated with the remainder of the tubes. The blank only has sterile media. Using a multichannel pipette, 100 uL of the control was transferred into each well in the first column of a 96-well round bottom plate. The same was repeated for the remainder of the tubes until the entire plate was full. The plate was incubated at 30°C for 6 hours. After the incubation, the plate was shaken over a hazardous waste bin to remove any liquid media or bacteria. Three trays, half filled with water, were set up on a sterile bench. The plate was submerged in the first tray and the water was shaken out over the waste bin. 125 uL of 0.1% crystal violet was transferred into each well and stained for 10 minutes. Then plate was shaken over the waste bin again, submerged in the second tray, shaken over the waste bin, submerged in the third tray, and then shaken over the waste bin another time. The plate was then inverted and left to air dry. 200 uL's of 30% acetic acid were added to each well and left there for 15 minutes with the plate lid on. Using the pipettor, the contents of each well were mixed while transferring the contents to another 96-well flat bottom plate (3). The optical density of the plate was measured using the Gen5 program at 640 nm, and the data was analyzed in Excel with averages of each column and a bar graph.

Optimizing the assay required multiple trials with varying incubation lengths and temperatures. The biofilm protocol had to be adjusted countless times to reach the optimal

conditions that *P. aeruginosa* grows in. After developing a working biofilm protocol, the biofilm assay was run using the *V. coralliilyticus* in an attempt to find the optimal conditions under which this pathogen will grow and form biofilm. The same steps were carried out with varying incubation times and temperatures.

CHAPTER 4

RESULTS

4.1 Optimizing Growth Media

Making sure that media being used is free of contamination and will consistently work at that specific concentration is crucial in running a scientific experiment. Since corals live in seawater, it would be preferable to run the experiment in conditions that simulate those of the host, therefore, marine broth would be the closest to that. Finding the right concentration of powder to use while maintaining the volume is challenging. There is fine line between the broth being too dilute and having enough nutrients but without the turbidity affecting the optical density. The growth curve in Figure 4.1 was reached using the successful broth with minimal turbidity. Finding that balance was crucial in order for us to move forward with the experiment using the *V. coralliilyticus*.

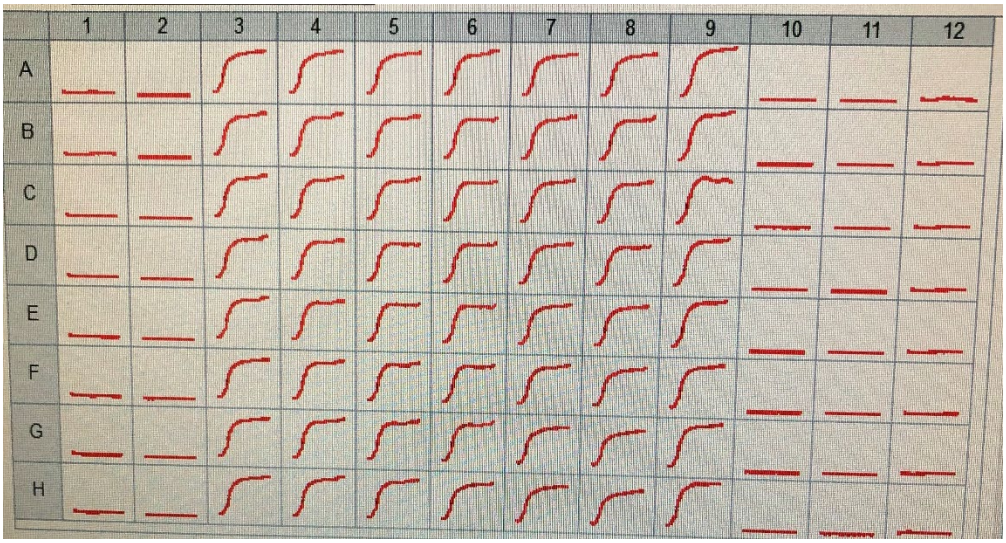

Figure 4.1: Growth curve for *V. coralliilyticus*

4.2 Creating Cryovial Stocks

After creating the cryovials, it is essential to ensure that the stock was correctly stored, and this is done by inoculating a TSA or marine agar plate with the contents from the cryovial. If there was growth following the incubation, and the growth matched that of the known stocks, then the cryovial stocks were successful and should remain intact for future use in the -80°C freezer, which was the case for our *V. coralliilyticus* cryovials.

4.3 Dilution Growth Curves

In order to carry out further experimentation with the *V. coralliilyticus*, finding the lowest concentration of marine broth in which the pathogen was able to grow is essential. By doing the serial dilutions growth curve, we were able to see the concentration at which growth was present, however, subsequent dilutions would have minimum to no growth due to the lack of nutrients.

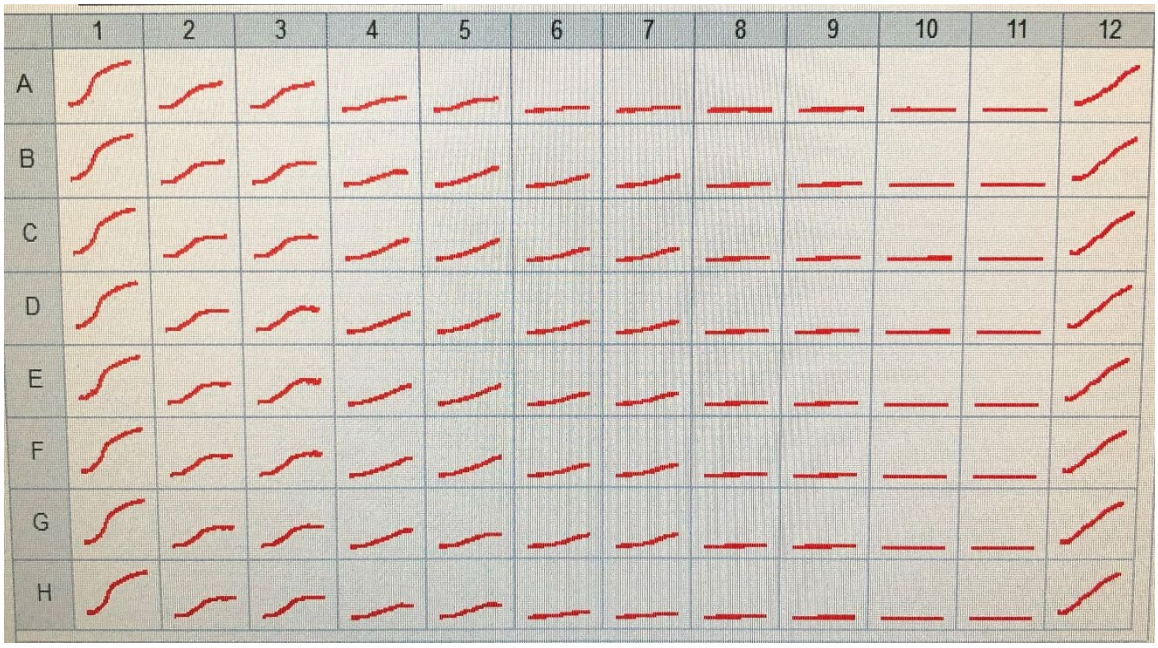

Figure 4.2: Serial dilution growth curves of *V. coralliilyticus*

From the growth curve (Fig. 4.2) we concluded that a 1:8 dilution, columns 6 and 7, would be the dilution of choice since there was still evident growth even if it were not in significant numbers.

4.4 Running and Optimizing the Biofilm Assay

Optimizing the biofilm assay for *P. aeruginosa* was complete and the protocol by which it was made possible was also completed (Fig 4.3). The R^2 value is approaching 1, indicating a good confidence level. However, optimizing the biofilm assay for *V. coralliilyticus* proved to be more challenging. Countless trials have revealed the different incubation times and temperatures at which biofilm formation is not at its optimum. When running a biofilm assay, the optical density should be highest for the control column and lowest in the blank, therefore creating an inversely proportional relationship between optical density and dilution factor.

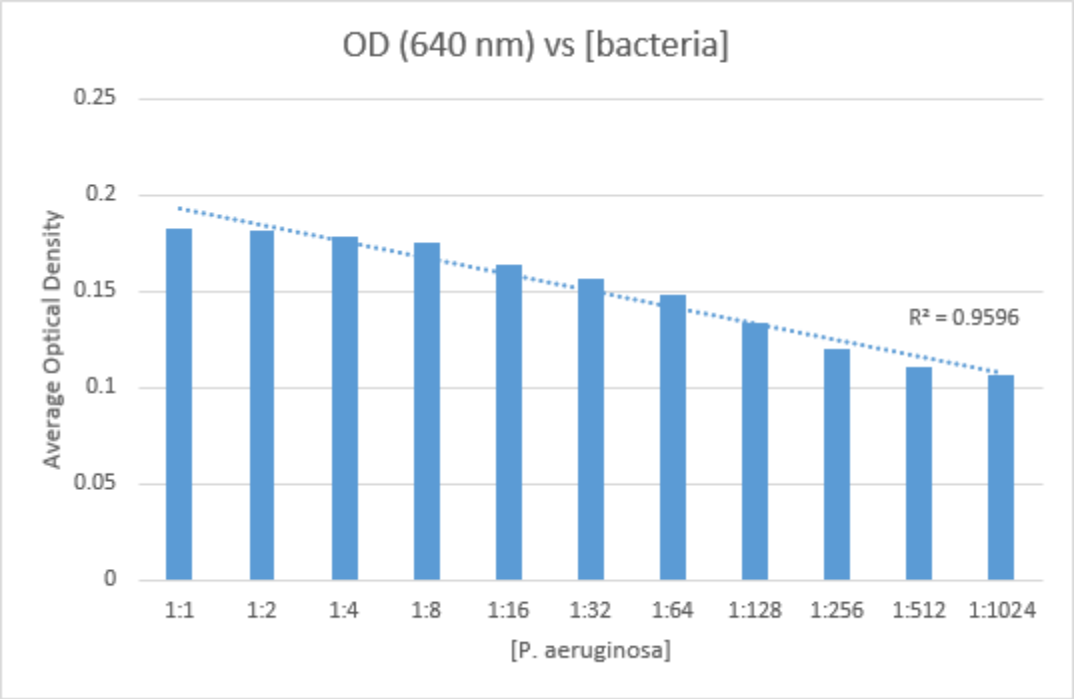

Figure 4.3: Optical density vs dilution graph for *P. aeruginosa*

CHAPTER 5

CONCLUSION

Coral communities are facing various different stressors that are endangering their existence and causing irreversible damage. Bacterial genera like *Vibrio* and *Nautella* are known pathogens that cause coral diseases such as white syndrome and YBD. *V. coralliilyticus* is a species of *Vibrio* that uses biofilm formation as a defense mechanism against environmental stressors, leaving behind diseased corals. Although the exact conditions for optimal biofilm formation for this species remains unknown, it continues to be the subject of research and further studies. Although the *V. coralliilyticus* biofilm assays were not optimized, we are moving towards optimizing the assay through trial and error and eliminating steps and variables that were revealing themselves to be unnecessary with every passing run. Understandably, it would be more difficult to find the ultimate conditions under which a coral pathogen would optimally function, especially since their environment is not easy to replicate or mimic. Other aspects of the research like perfecting the marine broth and eliminating its turbidity, running successful growth curves, performing serial dilutions, and creating a protocol for a biofilm assay are all things that are just as important as the research itself.

The information yielded from the biofilm assays can also be helpful in setting up an infection model for the specific species of coral that is being studied. If we have optimized the conditions for a coral pathogen to form biofilms, we can induce infection in the corals under those optimized conditions, with the lowest concentration of media, and

be able to study that infection model in depth with more known variables that can decrease the possibility of error. The reason it is difficult to create, and therefore study, these models, is due to the fact that their original conditions are extremely challenging to simulate 100%, especially for the pathogen. This is what makes this experiment essential for moving forward with larger experiments that study the actual diseases and infection process of coral reefs.

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BIOGRAPHICAL INFORMATION

Dena Kamel is earning an Honors Bachelor of Science in Microbiology, with minors in Business Administration and Biochemistry. She is a 3-year graduate and is planning to apply to medical school this summer. She has always dreamed of pursuing a career in medicine and is extremely excited to take this new step in her academic and professional career. Dena is involved with various organizations including the Student National Medical Association and the Honors College Council. She hopes to one day have the means to join an organization like Doctors Without Borders and serve the underserved and disadvantaged communities.