Antimicrobial Activity of Callyspongia sp From Culasi, Antique Against Ice-Ice Promoting Bacteria, Bacillus cereus, Brevundimonas diminuta, and Vibrio alginolyticus

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Abstract – Seaweeds are one of the Philippines' major exports. However, seaweed farms are often damaged by ice-ice, a disease condition that turns the branches of the seaweeds into white, fragile branches that easily come off. Previous studies have shown that *Bacillus cereus*, *Brevundimonas diminuta*, and *Vibrio alginolyticus* are known to be associated with and causes ice-ice disease. Methods known to use other organisms to control or inhibit the bacteria that cause the disease are polyculture cultivation and bioprotection. *Callyspongia sp.* was concluded to have potential as bioprotector in seaweed cultivation; however, other studies suggest that sponges at different locations may result to different antimicrobial activity due to the different symbionts present in different environments, which are responsible to the production of many chemical compounds. This study tested the antibacterial activity of *Callyspongia sp.* from Mararison Island, Culasi, Antique against *Bacillus cereus*, *Brevundimonas diminuta*, and *Vibrio alginolyticus*. *Callyspongia sp.* crude extract was extracted using 200 mL of methanol for every 25 g of *Callyspongia sp.* Broth dilution test was used and serial dilution was performed to produce an eight-fold concentration. Each bacterium was inoculated into the treated test tubes containing the crude extract and three replicates were used. Data was collected using a spectrophotometer and was analyzed using ANOVA. Results showed that the crude extract had significant effects in the growth of *Bacillus cereus* and *Vibrio alginolyticus* especially in 4.8x10-3 g/mL and 4.8x10-4 g/mL concentrations and for *Brevundimonas diminuta*, extract concentration 4.8x10-3 g/mL significantly inhibited its growth. Results show that *Callyspongia sp.* has the ability to inhibit the growth of bacteria associated with and causing ice-ice disease and has the potential to be used as a bioprotector in seaweed cultivation.

Introduction. – Seaweeds are one of the Philippines major exported products, which constitutes 60 percent of the Philippines aquaculture produce. In fact, the Philippines was once considered as the worlds leading supplier of seaweeds, exporting up to 400,000 metric tons of fresh seaweeds which comprises about 80 percent of the total world supply [1].

Seaweed farms are often damaged by ice-ice, a disease condition that turns the branches of the seaweeds into white, fragile branches that easily come off with little disturbance [2]. It has caused a negative growth of 42.8 percent in the aquaculture production of Zamboanga and a drop of 22 percent in the aquaculture industry of the Philippines in 2003 [3]. It has been reported by Largo (2002) that seaweed farms in Bohol, Batangas, and Iloilo have ceased to exist due to ice-ice disease. According to statistics, the Philippines had a total seaweed production of 84,500 tons and semi-refined carrageenan was only 2,592 metric tons in 2010 [4].

Ice-ice outbreaks are often associated with environmental stress such as change in salinity, temperature, and amount of light received by the organisms. When the seaweeds are stressed, they release organic substances that attract bacteria belonging to the *Vibrio- Aeromonas* and *Cytophaga-Flavobacterium* complexes, a group of bacteria that has the ability to break down cells and cause diseases. The most common bacteria discovered to promote ice-ice disease are *Vibrio sp.* and *Cytophaga sp.* [5]. Results from the study of Aris (2011) showed that bacterial species isolated from *Kappaphycus alvarezii* suffering from

ice-ice disease were *Vibrio alginolyticus*, *Brevundimonas cepacia*,*Flavobacterium meningosepticum*, *Brevundimonas diminuta* and *Plesiomonas shigelloides*, while in the study by Tokan et al. (2014), they were able to identify the specific species of bacteria that causes ice-ice disease namely, *Brevundimonas nigricaciens*, *Brevundimonas fluorescens*, *Vibrio granii*, *Bacillus cereus* and *Vibrio agar liquefaciens*.

One of the methods done in order to control ice-ice is planting other species of seaweeds such as *Achantophora spicifera*, *Caulerpa racemosa*, etc, in the same field to control the bacteria promoting ice-ice disease [6]. This technique is called polyculture cultivation, a process where different species of plants are cultivated in the same field resulting in the reduction of pests [7]. However, species such as Achantophora spicifera and Caulerpa racemosa are considered pests or invasive plants due to their fast growth. Although they may protect the seaweed farm from ice-ice disease, their invasive growth could potentially hinder the seaweed farms growth instead [8] [9].

Similar to polyculture cultivation, another method is to use live organisms in order to suppress a pest or pathogen to an acceptable level. These organisms serve as bioprotectors, biological control, or biosecurity. The goal of using biological control is to suppress pests or pathogens and to restore the native plant community [10]

In order to determine the potential of an organism as a bioprotector against ice-ice disease promoting bacteria, it must be a natural source of antibacterial compounds, chemical compounds that can kill bacteria, which it naturally releases into its environment [6].

Tokan and Lodo (2008) showed that three species of sponges, *Callyspongia biru*, *Callyspongia subarmigera* and *Callyspongia sp.* have antimicrobial effect against *Escherichia coli* and *Staphylococcus aureus*. Consequently, another study was conducted in order to determine whether *Callyspongia* species have antibacterial effects or potency against ice-ice causing bacteria and can be a potential bioprotector against ice-ice disease on seaweeds. It was observed that the three types of *Callyspongi*a showed different effects on the inhibition of bacteria. *Callyspongia biru* showed the most potent antibacterial activity especially having the most vulnerability against *Acinetobacter*. Based on the observations, *Callyspongia* has the potential to be used as a bioprotector to biologically control ice-ice disease [6]. Currently, there are no other known biological controls other than *Callyspongia* that are used against ice-ice disease on seaweeds.

A study of Qian et al. (2006) demonstrated that two *Callyspongia* species from the same genus that were collected from two bio-geographically different zones accommodated distinct bacterial communities but produced relatively similar secondary metabolites. Based on this, it can be inferred that at different locations, sponges may have a different composition of microbial symbionts, which are known to be responsible to the production of many chemical compounds extracted from sponges [11] [12]. There is also the possibility that different environments may cause the differences between metabolic activities of sponges [13]

This study aims to determine the antimicrobial activity of Callyspongia sp. found in Mararison Island, Culasi, Antique against ice-ice disease promoting bacteria and its potential as a bioprotector in cultivation of seaweeds.

Methods. – *Acquisition of bacteria.* Cultures of *Bacillus cereus*, *Brevundimonas diminuta*, and *Vibrio alginolyticus* were purchased from the National Institute of Molecular Biology and Biotechnology (BIOTECH) in Los Baos, Laguna, and were stored in the refrigerator at 4C. Under the laminar flow hood, *Brevundimonas diminuta* was directly inoculated into a test tube containing Tryptic Soy Broth while *Bacillus cereus* and *Vibrio alginolyticus* were directly inoculated into test tubes containing 6 percent NaCl Nutrient Broth in order to obtain subcultures. The test tubes were covered with Parafilm and were incubated at an optimal temperature of 30C for two days.

Media Preparation. In a 500 mL media bottle, two batches of broth media were prepared to obtain a total of 620 mL of 6 percent NaCl Nutrient broth and one batch was prepared to obtain 330 mL of Tryptic Soy Broth. The prepared broth was transferred into their respective test tubes, 33 for TSA and 62 for 6 percent NaCl NB. Each test tube was capped using cotton balls, covered with aluminum foil and gathered for sterilization. The prepared media was autoclaved at optimal temperature of 121C and pressure of 15 psi for 15 mins, cooled and stored in the refrigerator prior to usage.

Collection of samples. One kg of Callyspongia sp. was collected eight to ten meters underwater in the seas of Mararison Island, Culasi, Antique by local divers. They were then cleaned and transported to Philippine Science High School - Western Visayas Campus.

Drying. The collected samples were sundried for 6 hours under sunlight and air dried in the laboratory at night for two days until no more moisture was present in the sponge. They were then minced into the finest possible particle that can be obtained.

Extraction. The crude extract was extracted by using 200 mL of methanol for every 25 g of dried Callyspongia sp. The mixture was brought to the Department of Science and Technology (DOST) to collect the extract by evaporating the methanol using a rotary evaporator. The remaining evaporation was done in Philippine Science High School Western Visayas Campus using a water bath at 40C. The final amount collected was 0.53 grams and diluted to obtain an initial concentration of 4.8×10^{-4} g/mL.

Antibacterial Screening. Serial dilution was performed in order to dilute the extract in the prepared broths in 8-fold concentrations. The bacteria was then inoculated into the treated test tubes. The test tubes were then incubated for 18-24 hours under optimal temperature of 30C. The turbidity of each test tube was measured using UV-2100 Spectrophotometer with a wavelength of 600 m.

Statistical Analysis. One-way ANOVA was used to find the significant difference between the treated and untreated groups for every concentration. Using the LSD Post-hoc Test, each concentration were compared with each other to determine whether they are significantly different from each other.

Results and Discussion. – The mean absorbances of *Bacillus cereus* treated with the different concentrations of crude extract are shown to be lower than that of the untreated group. The first two concentrations exhibited the lowest absorbance while concentrations $4.8x10^{-6}$ g/mL and $4.8x10^{-8}$ g/mL exhibited the highest as shown in Figure 1. After statistical analysis using One-Way ANOVA, the extract showed an effect in the growth of Bacillus cereus. All of the concentrations showed a significant difference when compared to that of the untreated group. The LSD post-hoc test showed that absorbance reading for concentrations $4.8x10^{-5}$ g/mL to $4.8x10^{-10}$ g/mL are not significantly different from each other and among all the concentrations, absorbance is significantly lower when Bacillus cereus is exposed to extract concentrations of $4.8x10^{-3}$ g/mL and $4.8x10^{-4}$ g/mL.

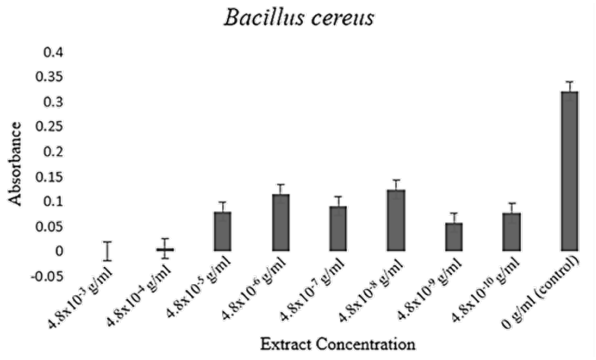

Fig. 1: Absorbance level means of both treated and untreated groups in different concentrations against *Bacillus cereus*.

The results of the treatment against *Vibrio alginolyticus* show that the means were relatively lower than that of the untreated group. The one with the highest concentration ($4.8x10^{-3}$ g/mL) showed the lowest absorbance while the lowest concentration ($4.8x10^{-8}$ g/mL) exhibited the highest as shown in Figure 2. Statistical analysis using One-Way ANOVA showed that all of the concentrations had significant effect on the growth of *Vibrio alginolyticus*. Similar to the results of *Bacillus cereus*, the LSD post-hoc test showed that absorbance reading for concentrations $4.8x10^{-5}$ g/mL to $4.8x10^{-10}$ g/mL are not significantly different from each other and among all the concentrations, absorbance is significantly lower when *Vibrio alginolyticus* is exposed to extract concentrations of $4.8x10^{-3}$ g/mL and $4.8x10^{-4}$ g/mL.

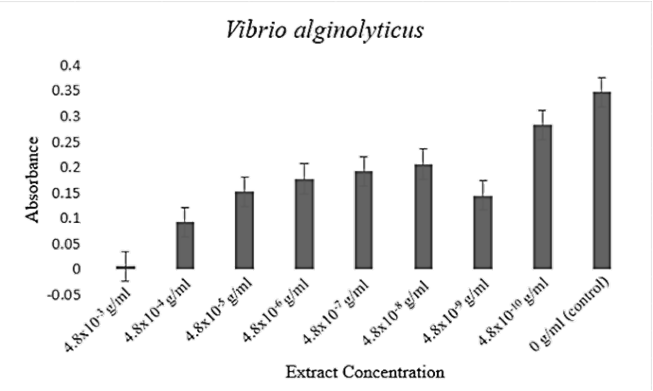

Fig. 2: Absorbance level means of both treated and untreated groups in different concentrations against *Vibrio alginolyticus*.

The means of the treated group for *Brevundimonas diminuta* were all lower than the untreated group. The lowest absorbance values were from concentrations one ($4.8x10^{-3}$ g/mL), five ($4.8x10^{-7}$ g/mL), and six ($4.8x10^{-8}$ g/mL) while the highest ones were from concentrations two ($4.8x10^{-4}$ g/mL) and three ($4.8x10^{-5}$ g/mL) as shown in Figure 3. One-way ANOVA showed that the mean absorbance reading of *Brevundimonas diminuta* treated with different extract concentrations are all significantly lower than the untreated group. The LSD post-hoc test showed that absorbance reading for concentrations $4.8x10^{-4}$ g/mL to $4.8x10^{-6}$ g/mL and $4.8x10^{-9}$ g/mL to $4.8x10^{-10}$ g/mL are not significantly different from each other and among all the concentrations, absorbance is significantly lower when the bacteria is exposed to extract concentrations of $4.8x10^{-4}$ g/mL, $4.8x10^{-7}$ g/mL, and $4.8x10^{-8}$ g/mL.

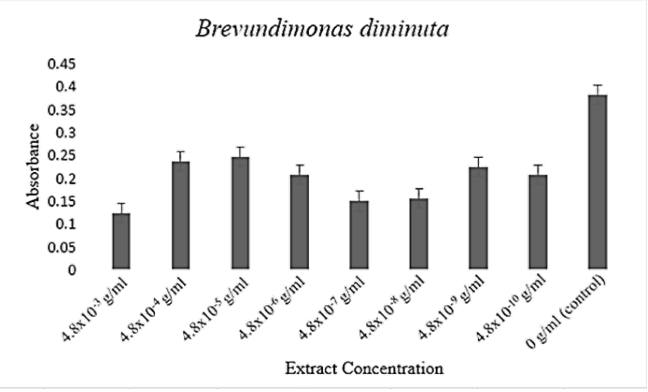

Fig. 3: Absorbance level means of both treated and untreated groups in different concentrations against *Brevundimonas diminuta*.

Previous studies have shown that Callyspongia sp. has

anti-larval, antifungal and antimicrobial activity. Different species of Callyspongia sp has inhibited the growth of microorganisms such as tubeworms, fungus and bacteria [14] [15] [16] [17]

Diverse and unique bioactive metabolites have been isolated from sponges. There are various antibiotics that are active against human pathogens and other bacteria foreign to sponges. Examples of these are plakortin from *Plakortis halichondroides*, manoalide from *Luffariella variabilis*, furospongin-1 from *Spongia officinalis*, and aerothionin from *Aplysina gerardogreeni* [18] The group of secondary metabolites, 3-alkylpiperidine alkaloids, serve as a chemical marker for the order where *Callyspongia sp.* belongs. It has been recorded that these compounds exhibit antifungal, antimicrobial, and anticancer activity [19] However, sponges from different locations have varying metabolic activities [13]. This is due to the different bacterial communities found in varying environments [20] and this may affect the composition of the microbial symbionts, which are known to be responsible to the production of many chemical compounds extracted from the sponges [11] [12]. This poses a possibility that different sponges from different places will not have the same effect on the same bacteria. The results have shown that the sponge collected from Mararison Island, Culasi, Antique have antimicrobial activity against certain ice-ice disease-causing bacteria.

The sponge extract notably had the highest bacterial inhibition against *Bacillus cereus* and the lowest against *Vibrio alginolyticus*. The resistance of *Vibrio alginolyticus* against the sponge extract may be due to it being a gram-negative bacteria. Gram-negative bacteria are known to have more layers of cell membranes which make it harder for antibiotics to penetrate the bacteria and inhibit the bacterial colonies growth. Meanwhile, gram-positive bacteria are more susceptible to antibiotics due to having only a single layer of cell membrane - even with its thick peptidoglycan layer [21] [22]. In a study by Mustapha et al (2013), it has been shown that *Vibrio alginolyticus* is generally resistant to antibiotics like penicillin and vancomycin. Its resistance is also more prominent when compared to other strains from the same family like *Vibrio cholerae*. *Bacillus cereus* is a gram-positive bacteria making it less resistant to the extract as compared to the other two bacteria which are gram-negative. This has also been shown in the study of McCaffrey and Endean (1985), where the gram-positive bacteria were generally more susceptible to the sponge extracts than the gram-negative bacteria as exhibited by the greater inhibition of *Thorecia vasiforis* against *Staphylococcus aureus*, a gram-positive bacteria, than *Pseudomonas aeruginosa*, a gram-negative bacteria . Contrastingly, the studies of Bergquist and Bedford (1978) and Amade et al. (1982) have exhibited that the gram-negative strains were more sensitive to the antimicrobial activity of sponges than the gram-positive bacteria. This has led them to propose that sponges contain active constituents that make them improve the capturing and digestion of particulate organic matter by prompting the slight clustering of vulnerable bacteria, therefore enlarging the size of particles that are captured at the choanocyte chambers. This theory of Bergquist and Bedford, however, seems unlikely due to the contrasting results from the study of McCaffrey and Endean (1985) as well as in an earlier study conducted by Burkholder and Ruetzler (1969). Out of the three bacteria that were tested, only *Vibrio alginolyticus* exhibited a trend in relation with the concentration of the extract. Meanwhile, the other two - *Brevundimonas diminuta* and *Bacillus cereus* - had fluctuating sets of data despite having all of them having significant differences when compared to the untreated counterparts. With the help of One-Way ANOVA, it has been shown that there are sets of data with greater standard deviations compared to others. This may be due to the presence of erroneous data. According to Dr. Ananya Mandal [10], biosecurity or bioprotection refers to measures that are taken to stop the spread or introduction of harmful organisms to human, animal and plant life. The measures taken are a combination of processes and systems that have been put in place by bioscience laboratories, customs agents and agricultural managers to prevent the use of dangerous pathogens and toxins. Its goal is to protect human health and to increase and protect agricultural produce through the prevention, control and management of biological risk factors. In order to determine the potential of an organism as a bioprotector against ice-ice disease promoting bacteria, it must be a natural source of antibacterial, a chemical compound that can kill bacteria, which it naturally releases into its environment [6]. Studies have shown that sponges naturally have antimicrobial compounds which they release in their surrounding environment. It has been observed that the sponge surfaces, including surrounding waters and nearby substrates where these sponges are located, rarely get affected by fouling organisms. However, some of these antimicrobial substances are shown to be selective and specific to certain strains of bacteria and other microorganisms [18]. With the results of the study, it can be said that Callyspongia sp. from Mararison Island, Culasi, Antique has the potential to be a bioprotector in the cultivation of seaweeds as it exhibited potent antibacterial activity against the strains of bacteria that cause ice-ice disease. However, it should be considered that the antibacterial compounds from the sponge were extracted from it and a high concentration is needed in order to effectively inhibit the growth of the pathogens. Although at lower concentrations, the extract can still significantly reduce the pathogens, the antimicrobial activity is still lower when compared to high concentrations. There may be a difference as to how the sponge will act when it is put in the natural environment. The antimicrobial compounds it will release in its surroundings may not be the same concentration as the extract needed to inhibit the growth of the bacteria that causes ice-ice disease.

Conclusion. – Callyspongia sp. from Mararison Island, Culasi, Antique has the potential to be a bioprotector against ice-ice disease causing bacteria. It has shown potent antimicrobial activity against the bacterial strains, *Bacillus cereus*, *Vibrio alginolyticus*, and *Brevundimonas diminuta*. The most effective extract concentrations against *Bacillus cereus* are $4.8x10^{-3}$ g/mL and $4.8x10^{-4}$ g/mL; for *Vibrio alginolyticus* extract concentrations $4.8x10^{-3}$ g/mL and $4.8x10^{-4}$ g/mL significantly inhibited its growth; and for *Brevundimonas diminuta*, the most effective concentration is $4.8x10^{-3}$ g/mL.

Recommendations. – In order to further isolate the specific compounds that causes the antimicrobial activity, it is recommended to perform thin-layer chromatography on the sponge extract.

Additionally, a more accurate method for determining the antimicrobial activity of the sponge extract is to use the disk diffusion assay since it measures the zone of inhibition which effectively quantifies the bacterial growth.

Furthermore, the spicules of the collected sponge should be investigated by an expert to validly confirm its taxonomic identity. Moreover, field experiments can be done in order to test whether this antimicrobial activity is also exhibited in the natural environment. Instead of extracting the sponges, the secreted enzymes may be tested to determine its antimicrobial activity in a natural setting.

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