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Identification Particles in Bacteria that Cause Disease in Seaweed *Kappaphycus Alvarezii*at Arakan Village, South Minahasa, North Sulawesi using Scanning Electron Microscopy and Energy DispersiveX-ray Spectroscopy

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Abstract. *Kappaphycus alvarezii* seaweed is widely cultivated in Indonesia and has important economic value.Nevertheless, this seaweed is easily attacked by diseases. This study was to determine the *ice-ice* triggers in seaweed farming locations. It was conducted at Arakan Village, South Minahasa, North Sulawesi. We performed the bacteria isolation on infected *thallus* of *Kappaphycus alvarezii and Staphylococcus arlettae* caused the infection. *Staphylococcus aureus infection is* used to be found on the skin and mucous membranes of a healthy human, while *Staphylococcus epidermis* inhabits only the skin of healthy humans. Approximately 30% of the normal healthy population was effected by *Staphylococcus aureus* as it asymptomatically colonizes human hosts. How this *Staphylococcus arlettae*(mainly found in pork farm) infected *Kappaphycus alvarezii* that is on high salinity environment requires in add depth study.We completed the experiment by usingScan Electron Microscopy–Energy Dispersive X-ray Spectroscopy (SEM-EDX), analysis on infected *thallusKappaphycus alvarezii* and on series of bacteria isolation to observe the morphology and the element contents *Staphylococcus arlettae* bacteria has successfully attached to the surface of *Kappaphycus alvarezii* and caused infection. This proves that there have been environmental changes that have stimulated pathogenic bacteria *Staphylococcus arlettae* in the area where *Kappaphycus alvarezii* are cultivated.The mechanism of biosorbtion by microbes that can live in environments that are contaminated with Pb metal is active uptake. This mechanism occurs simultaneously in line with the consumption of metal ions for the growth of microorganisms. *Staphylococcus arlettae* that are resistant to heavy metals due to the ability to detoxify the influence of heavy metals in the presence of protein or granular material. From the results of the study using SEM-EDX of algae and bacteria*Staphylococcus arlettae*, it can be concluded that content of the particle of metal in *thallus* triggers bacteria to live on the seaweed as energy source.

Keywords: Bacteria, disease, infection, *Kappaphycus alvarezii*, seaweed, *Staphylococcus arlettae*

INTRODUCTION

Seaweed, the main commodity of cultivation in Indonesia is *Eucheuma cottonii* which is now known as *Kappaphycus alvarezii.* The first step in the success of seaweed cultivation is the factor of land feasibility (Gerung *and* Ngangi, 2017). The potential of seaweed commodities as a whole is large, but the use of land in this cultivation is not optimal, especially for areas that geographically have the potential for seaweed cultivation (Noor,2015). *Kappaphycusalvarezii* has the prospect of seaweed cultivation which is very profitable if it is developed as a basic material for the production of various needs in the industrial world (Surni,2014). The success factor of a seaweed cultivation, in addition to being determined by the right cultivation method, is necessary to pay attention to a good environmental quality, in addition to other factors that influence it, such as the quality of seaweed that will be used in cultivation. The success rate of seaweed farming activities is greatly influenced by environmental factors, including salinity (Ariyanti, *et.al* 2016). However, the main obstacle in seaweed cultivation is the prevalence of*ice-ice* disease that causes the seaweed stems to break up and eventually die. The prevalence of *ice-ice* can be caused by water quality conditions in declining waters (Ngangi, 2012).

Seaweed is categorized into poikilothermic organisms where growth and metabolism are strongly influenced by environmental conditions.If conditions such as salinity, temperature, current and light intensitychanged then seaweed will be easily stressed (Hurtado,2013).When stressed, seaweed will release organic substances that cause *thallus* slimy. This condition stimulates the bacteria to grow around it and causes diseases that result in seaweed production decrease. (Mendoza, *et al*., 2002 and Lachnit *et al*., 2009).

The results of several studies show that the causes of diseases in the cultivation on seaweed are the bacteria. However, infectious information on seaweed *Kappaphycusalvarezii* which are the main commodities in seaweed cultivation, has not been widely known and researched .Therefore, this research can provide more data and information for further research, in relation to reducing the level of pathogenicity when cultivating *Kappaphycusalvarezii*.The purpose of this study was to analyze the content of particles contained in seaweed that is infected by disease and bacteria that cause disease in seaweed, in Arakan Village, South Minahasa, North Sulawesi (Fig. 1)

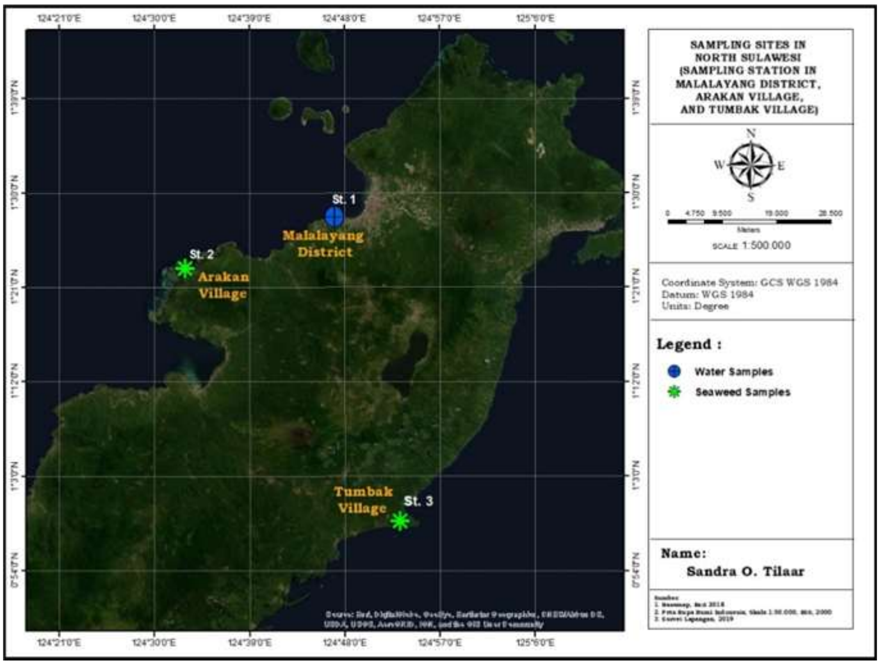

Figure 1. Sampling location Arakan Village, South Minahasa, North Sulawesi

Extract Preparation for Bacterial Isolation

First, *thallus* (0.5 mm) of *Kappaphycus alvarezii* was homogenized in 0.1 mL of sterile seawater and crushed with mortar to get the solution. The supernatant solution was placed in media Nutrient Algae(NA) plate. This sample was incubated on 37°C for 48 hours.Furthermore, the sample was diluted to 10^{-1} µL, 10^{-2} µL, 10^{-3} µL and 10^{-4} µL. The solution of concentration 10^{-3} µL and 10^{-4} µL was inoculated again into mediaNA plate and incubated at 37˚C for about 48 hours. Growing bacterial isolates were isolated based on their morphological characteristics following method of bacterial identification by Cappucino *and* Sherman (1998).

The bacterial isolates were regrown in Nutrient Broth media and incubated at 37° C for ± 24 hours. Bacteria that have grown are indicated by the presence of turbidity in the medium as much as 6 ml of centrifuge at 4° C for 5 minutes at a speed of 14,000 rpm (Sari et al., 2013). The supernatant resulting from the centrifuge is then discarded and the pellets that are left behind are bacterial isolates which will continue to the Deoxyribonucleic Acid (DNA) isolation stage.

Bacterial DNA was isolated using DNeasy Blood and Tissue Kit (QIAGEN). DNA isolation was started by adding 180 µl ATL Buffer into 1.5 ml ependorph which already contained DNA pellets. Next, add 20 µl of Proteinase K and vortex for 15 seconds then incubate at 55° C for 1 hour. In the incubation process, bacteria samples are vortexes every 15 minutes. After 1 hour, add 200µl Buffer AL and vortex for 15 seconds. Samples were incubated again at 70° C for 10 minutes. Next, add 200 µl of 100% ethanol and vortex for 15 seconds. The next step is to move the sample solution to the DNeasy Mini Spin Column which is above the 2 ml Collection Tube. The sample is put into centrifuge at a speed of 8000 rpm for 1 minute and drain the liquid that is at the bottom. Next, add 500 µl AW 1 and centrifuge for 1 minute at 8000 rpm and then drain the liquid at the bottom. Add 500 µl of AW2 and centrifuge for 4 minutes at 14,000 rpm and after that drain the liquid again at the bottom. After that, move the DNeasy mini spin column to the top of the Elution Tube and add 200µl Buffer AE to the center of the DNeasy mini spin column and then incubate at room temperature for 1 minute. The final step, centrifuge the sample at a speed of 8000 rpm for 1 minute. DNA has been in the elution tube and has been isolated.

Application of Scan Electron Microscopy – Energy Energy-dispersive X-ray Spectroscopy (SEM-EDX)

Analysis for Scan Electron Microscopy. We applied Scan Electron Microscopy (SEM) with SNE 4500M which is integrated with the Energy Dispersive X-ray Spectroscopy (EDX). The SEM-EDX was used to determine the morphology and the composition of particles found in seaweed and bacteria. For the morphological observation process and particle data, seaweed samples were cut into small pieces. In terms of bacterial samples, they were taken from agar plates and affixed to the specimen holder using carbon double types. Previously, the sample was vacuumed to remove water content, so that the filament became optimal for analysis.

RESULTS AND DISCUSSION

Results

Isolation and Identification of Bacteria

Seaweed of *Kappaphycus alvarezii* in Indonesia are famous and easy to cultivate with varieties of methods (Parenrengi *and* Sulaeman, 2007; Nursidi *et.al*, 2017; Kasim *and* Mustafa, 2017; Rama *et al*., 2018). In fact, the effort to cultivate *Kappaphycus alvarezii* organically has begun in Boalemo District, Gorontalo Province, Indonesia (Tuiyo, 2016). Although these algae are easy to cultivate and have high economic value, they are easily attacked by diseases. We can see at Figure 2A : fresh and healthy seaweed. Figure 2B is seaweed infected by bacteria. Based on the isolation results from seaweed infected by *ice ice* in Arakan village waters, 3 bacterial isolates were obtained. These bacterial isolates were obtained after three times of purification . The morphology of each bacterial isolate originating from seaweed can be seen in Figure 3.

A B

Figure 2.*Kappaphycus alvarezii*

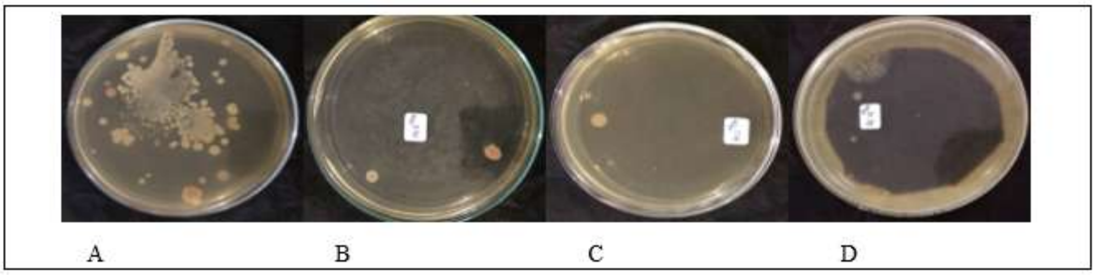

Figure 3. (A)Bacterialcolony (B); Bacterial isolation through different (C) Bacterial Isolation 2nd and (D) Bacterial Isolation 2nd

These bacteria produce hormones such as in dole acetic acid, solubilize phosphate, fix atmospheric nitrogen and synthesize side raphes and anti-microbial products. Concurrently, heavy metal reduction by bacteria may occur directly via enzymatic reduction or complex formation with metabolites (*Cervantes and Campos-Garcia, 2007;* Khan *etal*. 2009). For identification of this isolate, 16 S rDNA sequencing was carried out. A 1.5 kb 16S rDNA fragment was amplified using consensus primers and sequenced. The sequence showed 99% similarity to *Staphylococcus arlettae*. On the basis of the 16SrRNAgenesequenceanalysis, the isolate was identified as *Staphylococcus arlettae*strain LCR34 and submitted with NCBI Gen Bank with accession No.FJ976543.1.

Serial studies in Asian region had been conducted on *Kappaphycusalvarezii* regarding disease infected by several disease agents (Vairappan *et.al*. 2008; Solis *et al*., 2010; Pang *et al*., 2015; Syafitri *et.al*. 2017).Syafitri *et al*. (2017) revealed that on molecular characterization, the disease on this kind of algae mainly by bacteria which comes from the genera of *Alteromonas*, *Bacillus*, *Pseudomonas*, *Pseudoalteromonas*, *Glaciecola*, *Aurantimonas*, and *Rhodococcus*. *Bacillus* was found as agar-digester by forming a clear depression around their colony and/or liquefaction of agar on *Gracilariopsis heteroclite* (Martinez *and* Padilla, 2017). In the case of *Kappaphycus alvarezii*,bacterial pathogens belonging to the *Vibrio-Aeromonas* complex and the *Cytophaga-Flavobacterium* complex demonstrate the seaweed-bacteria interaction (Largo *et al*. 2012). We found the new strain of bacteria infection to *Kappaphycus alvarezii* called*Staphylococcus arlettae.*Studied in several strains on *Staphylococcus arlettae*bacteria showed that these have high remarkable survivability amidst the physicochemical pressures present, both intracellular and externally (Onyango *and* Alreshidi, 2018). *Staphylococcus aureus* normally is found on the skin and mucous membranes of a healthy human, while *Staphylococcus epidermis* inhabits only the skin of healthy humans.

Scan Electron Microscopy–Energy Dispersive X-ray spectroscopy(SEM-EDX) Analysis of*Kappaphycus alvarezii*

Structure morphology of *Kappaphycus alvarezii* infected by bacteria is shown in Figure 4.The particle content is shown in Figure 5.Tissues are showing signs of decomposition and bright color. The dominant particle were Si, Mg, Al and Ca. Mineral contents for healthy algae are K, Ca, P, S, Mn, Fe, Cu, Zn, Ni, Br, Na, Mg and Al, with the higher amount of Na, K, Ca and Mg for macro minerals (Ruperez, 2002; Tillaivasan, 2019).Thus, infected seaweed causes its mineral content to decrease.

Scan Electron Microscopy–Energy Dispersive X-ray spectroscopy(SEM-EDX) Analysis of Bacterial Isolates.

Results of analysis of SEM-EDX on bacteria isolation are shown on Figure 5-8. The research study, *Staphylococcus arlettae* bacteria has successfully attached to the surface of *Kappaphycus alvarezii* and caused infection. The mineral content of the bacteria increased by Figure 6-8. This result proves that there have been environmental changes that have stimulated pathogenic bacteria *Staphylococcus arlettae* in the area where *Kappaphycus alvarezii* wascultivated. This phenomenon is known as dysbiosis (Fernandes *et al*., 2012; Zozaya-Valdes *et al*., 2015; Egan and Gardiner, 2016). Currently, Syafitri *et.al*.(2017) conducted a study by *Kappaphycus alvarezii* in Karimunjawa Island, North Java Sea, Indonesia. They found that *Alteromonas macleodii*, *Pseudoalteromonas issachenkonii* and *Aurantimonas coralicida* caused pathogenity in *Kappaphycus alvarezii*.

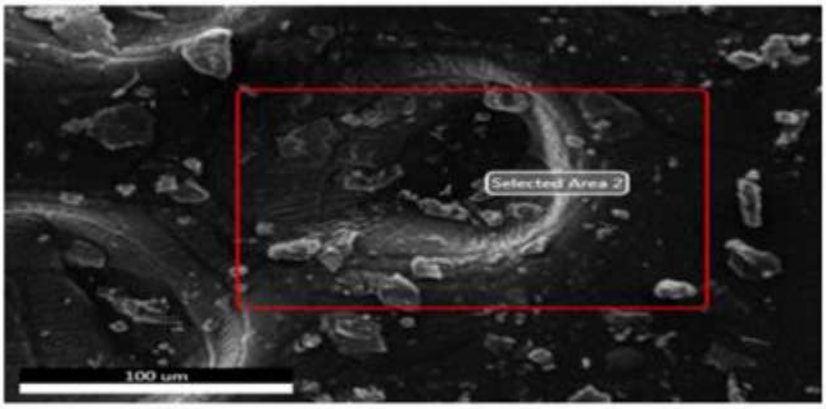

Figure 4. Morphological structure of *Kappaphycus alvarezii* was infected by Bacteria

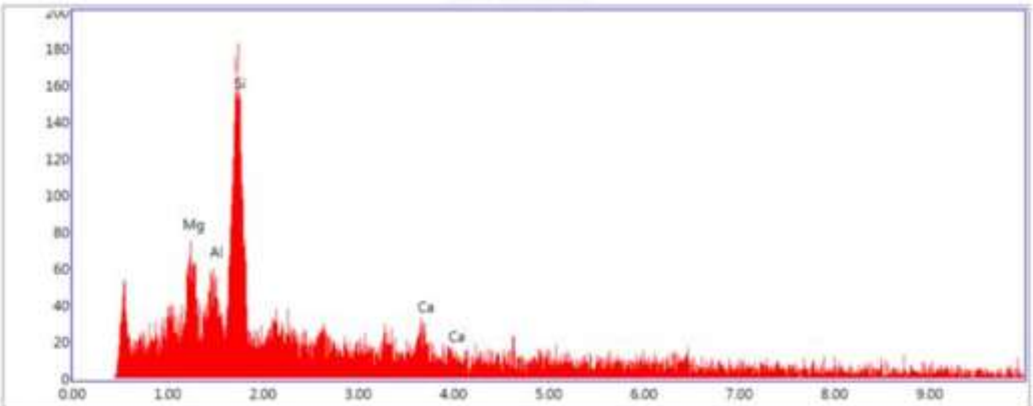

Figure 5. Particle composition in *Kappaphycus alvarezii* was infected by Bacteria

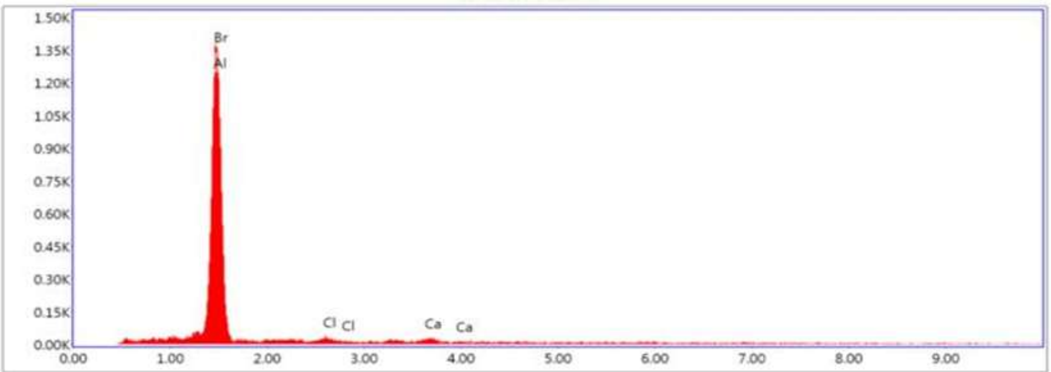

Figure 6. Element content on Bacteria Isolation 1, *Kappaphycus alvarezii*

Bacteria *Staphylococusarlettae* are able to grow in metal-contaminated media and have the ability to accumulate metals in their cells.Metal ions are positively charged, so they are electrostatically bound to the cell surface. The interaction between metal ions and bacterial cells wall shows the role of carboxyl groups in peptidoglycan and phosphoryl groups in the secondary polymers of the teikic and theicuronic acids (Solikha & Dwianita, 2013). The mechanism of biosorbtion by microbes that can live in environments that are contaminated with Pb metal and thisis active uptake. This mechanism occurs simultaneously in line with the consumption of metal ions for the growth of microorganisms (intracellular accumulation of metal ions) (Fakhrudin *et al*., 2008). Bacteria are resistant to heavy metals due to the ability to detoxify the influence of heavy metals in the presence of protein or granular material (Adi & Nana, 2010).

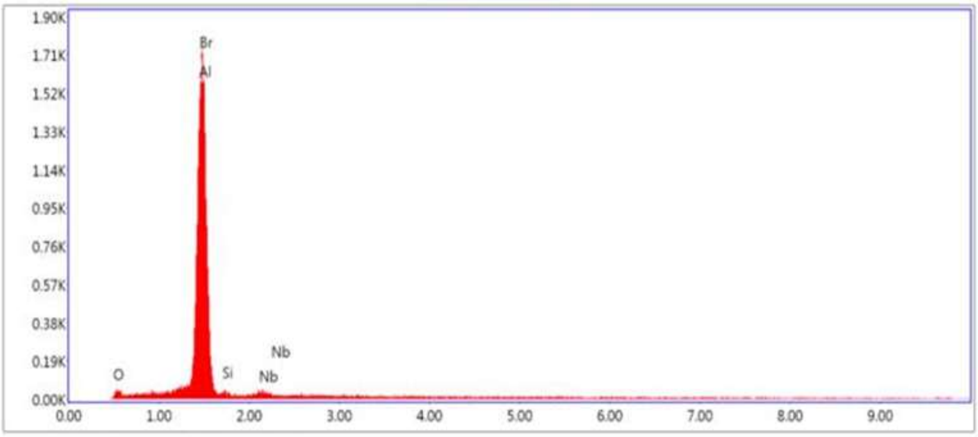

Figure 7. Element content on Bacteria Isolation 2 *Kappaphycus alvarezii*

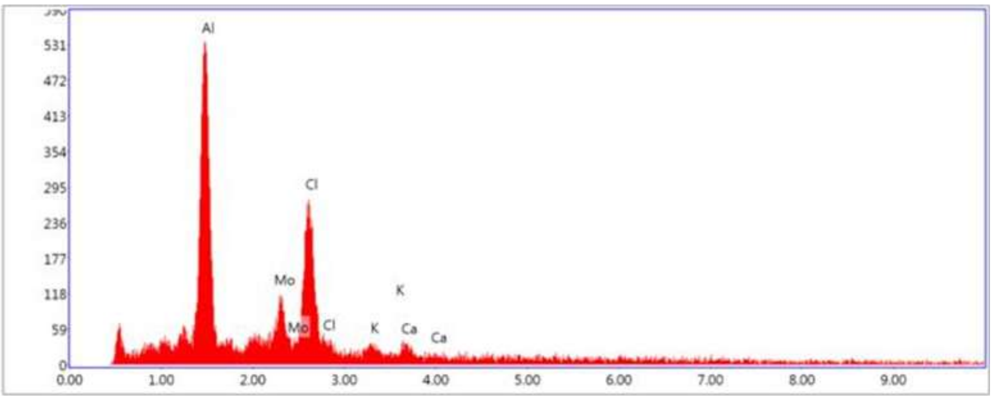

Figure 8. Element content on Bacteria Isolation 3 *Kappaphycus alvarezii*

Discussion

Metal enters to seaweed influenced by the compounds that exist on the seaweed, where the compound is able to absorb metal ions into the cells.The entry of metallic elements into plants resulting in compounding, between metals and proteins and polysaccharides that subsequently able to penetrate the cells wall and enter into the cytoplasm. (Sukma Qumain *et.al.*2016). *Ice-ice* is an effect of growing old seaweed and nutritional deficiencies, characterized by the occurrence of red spots/patches in some of the old *thallus* being pale yellow and eventually gradually become white and eventually become crushed or fall (Runtuboy, 2004). From the results of the study using SEM-EDX on algae and bacteria, it can be concluded the presence of metal content that causes damage to the *thallus* triggers bacteria to live on the seaweed *thallus*. The morphological change of *Kappaphycus alvarezii* can result from the inability of biosorption and seaweed growth ability (Bambang Yulianto *et al*. 2006). *Kappaphycus alvarezii* undergoes morphological changes due to bacterial infection. The symptoms of *ice-ice* disease are generally characterized by bleaching at the base of *thallus*, and young *thallus* end, which begins with a change of the color of *thallus* to clear white or transparent, as well as being pale and on some branches to rot (Largo *et.al*.1995). *Thallus* becomes fragile and easily . When seaweed is stressed it will facilitate pathogenic infections. On a stressful state, seaweed will liberate the organic substance that causes *thallus* to slid and stimulate bacteria to grow abundantly.

From the results of the study using SEM-EDX on algae and bacteria, it can be concluded that the presence of metal content causes damage to the *thallus* triggers bacteria to live in the seaweed *thallus*.*Staphylococcus arllettae* , a type of Gram-positive bacteria, from the genus *Staphylococcus* bacteria. These bacteria are commonly found from isolated textile plant waste, and can be degraded color (Francisco. E. *et.al*, 2009).Metal particles on seaweed are suspected to trigger the emergence of *Staphylococcus arllettae* bacteria. The damage of the seaweed *thallus* does not always result from changes in weather, temperature or salinity. Water quality could also result in damages of the *thallus* then stimulate the occurrence of bacteria. Since bacteria are good bioaccumulation for metals, *Staphylococcus arllettae*can absorb metal, such as Arsen. The trigger of *Kappaphycuss alvarezii* disease in Arakan Village is caused by the *Staphylococcus arlettae* bacteria. These bacteria use seaweed as a host and reduce weight on the seaweed, so that the seaweed metabolism is inhibited.

When the seaweed experiences stress it will facilitate pathogen infection. In a state of stress, the seaweed will release organic substances that cause *thallus* slimy and stimulate bacteria to grow. Bacteria found in the seaweed in Arakan waters are pathogenic bacteria that have the potential to cause disease, namely *Staphylococcus arlettae.*

Based on the results, it can be concluded that the bacteria found in *thallus* seaweed are*ice-ice* disease in the Arakan Village, South Minahasa, North Sulawesi is bacteria *Staphylococcus arlettae.* From the SEM-EDX analysis of results on seaweed taken in Arakan Village, South Minahasa,North Sulawesi, there are some metal content in *Kappaphycuss alvarezii*seaweed and *Staphylococcus arlettae*bacteria.

Ice-ice disease in seaweed cultivation is one of the biggest obstacles for cultivators. Regular monitoring of seaweed cultivation by fishermen or by the local government is one way to find out the conditions/problems faced by farmers. The problems faced can be considered in taking preventive actions.

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