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Isolation and identification of bacteria with antimicrobial activities from green algae (*Halimeda discoidea*) from Pulau Lima, Banten Bay, Indonesia

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Abstract. Bacteria associated with seaweed were involved in the production of metabolites associated with their host, so they tend to have nearly the same metabolites to defend themselves or as antibiotics. The aim of this study was to isolate and to identify bacteria with antimicrobial activities from the marine green algae (*Halimeda discoidea*). Laboratory analysis was for the isolation of bacteria, selection of bacteria with antimicrobial activities, antibacterial activity tests and Minimum Inhibitory Concentration (MIC). Results showed that six bacterial isolates were isolated from outside and inside of the algae, which have antimicrobial activities. One was selected for further work which has more antimicrobial activities. The bacteria showed antimicrobial activity against Gram-positive bacteria (*Staphylococcus aureus*) but not against Gram-negative bacteria (*Escherichia coli*). The bacterial growth curve was at an optimum phase at the 10th to 16th hour. Microscopic analysis and biochemical tests showed that isolated bacteria were a rod-shaped Gram-negative, acid-free, non-endospore, reacted positively to gelatin, citrate and carbohydrate, reacting negatively on motile, and urease. We conclude that the bacteria isolated from green algae which had more antimicrobial properties belongs to the genus *Pseudomonas guzei*.

Keywords: *Halimeda discoidea*, *Pseudomonas guzei*, symbiont bacteria

1. Introduction

Seaweed is one of the largest producers of biomass in the marine environment. They produce various active chemical metabolites in their environment, potentially as a tool to protect themselves against other sedentary organisms. Biogenic molecules infuse unique chemical diversity in seaweed compared to other plants [1]. In addition to its primary economic value, the secondary metabolite content of seaweed has the potential to produce diverse bioactive metabolites as antibacterial, antiviral, antifungal and cytostatic [2].

Antibiotics are drugs that are common in medicine. The inappropriate selection of antibiotics can have a negative impact, like the emergence of bacterial resistance and low antibiotic effectiveness against certain bacteria. Further research is needed to find new antibacterial metabolites.



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More than 300 secondary metabolites had been identified from green algae including the *Bryopsidales* [3]. Green algae *Halimeda* contains epigallocatechin (a type of antioxidant) in very high amounts (28µg/g dry weight). Furthermore, *Halimeda* is also rich in minerals such as Fe, Mn, Zn and Cu. Minerals are an important component of antioxidant enzymes. Zn, Cu and Mn minerals are essential minerals for superoxide dismutase activity [4].

10

2. Materials and Methods

2.1. Materials

The following material were used in the analysis include Green algae (*Halimeda discoidea*), pure culture of *S. aureus*, pure culture of *E. coli*, aquades, Agar, Nutrient Broth (Oxoid), Plate Count Agar (Oxoid), Mueller Hinton Agar (Oxoid), sterile sea water, 70% alcohol, 95% alcohol, crystal violet, iodine, safranin, immersion oil, carbolfuchsin dyestuff, alcoholic acid, Methylene Blue, Malachit Green solution, filter paper, tissue / cloth, cotton and paper disc.

2.2. Sampling

Samples of Green algae (*Halimeda discoidea*) were obtained from two stations (S 6°0'5.6356 and "E 106°9'15.3684) at Pulau Lima, Banten Bay. The distance from station 1 to station 2 was about 100 meters (figure 1). Taking samples at two stations, taking supporting data such as temperature of sea water, pH, salinity and current strength.

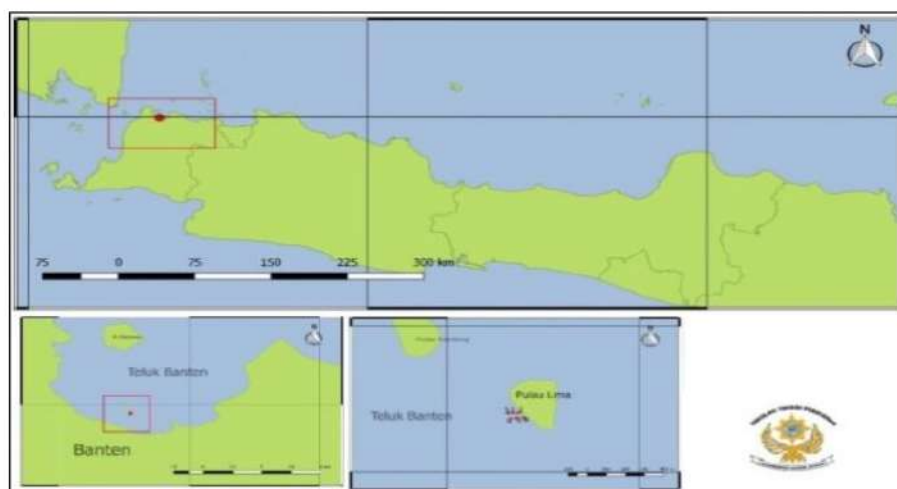


Figure 1. Sampling location, ● = station, ■ = island, ■ = sea.

2.3. Isolation of symbiotic bacteria that produce antibacterial compounds

Isolation bacteria from the surface of algae : 15 grams of algae is rinsed with 30 ml of sterile sea water. Then the rinse water was added to 30 ml of NB medium and then shaken using a shaker at room temperature for 24 hours. Isolation bacteria from the surface of algae : 15 ml of sterile sea water was added to 15 grams of rinsed algae and then crushed. The suspension was put into 30 ml of NB media and shaken using a shaker at room temperature for 24 hours. Samples that have been refreshed on NB media were diluted into 1:10 to 1:100.000 dilution, and each dilution was grown on PCA media, following 48 hours of incubation at 35° C for 48 hours. Bacteria that have a stable clear zone were isolated on strength seawater [5].

2.4. Qualitative challenge test

To identify the pathogenic antimicrobial activity of isolate bacteria, qualitative challenge tests were done [6]. One ml of test bacteria (*Staphylococcus aureus* and *Escherichia coli*) was mixed into 10 ml sterile liquid Plate Count Agar (PCA) and was let to stand for about 15 minutes to become solid. Bacteria was isolated from *Halimeda discoidea*. Sprayed on the surface of the media that had been

spread with the test bacteria (*Staphylococcus aureus* and *Escherichia coli*). Following 48 hours of incubation at 35°C.

2.5. Antibacterial test

2.5.1. Paper disc method. The supernatant (40 µl) was placed on a sterile paper disc containing a sterile watch glass and left for 1 hour in a sterile laminary flow chamber so that the supernatant was absorbed perfectly into the paper disc. The microbial suspension of *S. aureus* and *E. coli* test which have been prepared on NB medium was grown in pour plating. The medium and suspension were homogenized by shaking the petri dish slowly, and left for 15 minutes in the sterile laminary flow chamber to become solid. One piece of paper disc was inoculated with supernatant, 1 paper disc containing Nutrient Broth as a negative control, and 1 piece for chloramphenicol as a positive control. The inhibitory potential was measured based on the clear zones seen around the paper disc after incubation at 35°C for 24, 48 and 72 hours [7].

2.5.2. Minimum Inhibitory Concentration (MIC). The supernatant is diluted with 100%, 75%, 50%, and 25% of Nutrient Broth. Petri dishes were prepared for *S. aureus* and *E. coli* test bacteria (4 plates each). Each dilution (1 ml) is inserted into petri dishes, and mix with 9 ml of Nutrient Agar in the petri dish. Positive control consists of 10 ml of NA and one ose of bacteria. Negative control contains 10 ml of NA. All test tubes were incubated at 35°C for 24 and 48 hours [8].

2.6. Identification of selected symbiont phenotypes and bacterial genotypes

2.6.1. Spectrophotometric growth curve. Stratified dilution of bacterial symbionts was into 1/2, 1/4, 1/8, 1/16, 1/32. The wavelength is set at 686 nm. The cuvette was inserted into a *Spectrophotometer*. The calculation formula with MC Farland equation is as follows.

$$Y = 2.62 \times 10^9 \mu - 6.39 \times 10^7$$

$$y = \text{density (CFU / ml) and } \mu = \text{adsorbs (\AA)}$$

2.6.2. TPC growth curve. One ounce of bacterial culture that had been refreshed on the PCA medium was inoculated into 13 tubes containing 9 ml of sterile Nutrient Broth and incubated. Observation of the TPC value was carried out every 2 hours during a 24 hour period. Bacteria was planted into the Nutrient media and was then incubated at 35°C for 24 hours. Observation of the colony character was carried out with Gram staining, spore staining, Z-Neelsen staining, biochemical tests including motility, gelatin, citrate, urease, and carbohydrate. Estimation of bacterial types is based on the identification key [9] and DNA molecular test.

Samples of bacteria grown on PCA medium were incubated at 35°C for 3 days and then checked for purity and then used for the molecular identification process. Identification was performed using molecular analysis based on 16S rDNA fragments in bacteria. Isolation of bacterial genomic DNA was performed using PCR colony method [10]. The supernatant was taken and used as a DNA template on PCR amplification. Symbiont bacteria species was determined by molecular testing. The DNA of the symbiont bacteria isolates was amplified using primers 9F and 1541R. The DNA bands used were relevant to the resulting PCR product of about 1400 base pairs. Molecular identification was done through the partial genetic analysis of 16S rDNA. DNA extraction was performed using the modified GES method [11]. The analysis of nitrogen base sequence readings was done using an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). The next sequenced raw data was trimmed and assembled using the BioEdit program. Sequencing data that has been assembled in BLAST with genome data that has been registered in DNA Data Bank of Japan [12].

3. Results and Discussion

3.1. Isolation of bacteria

Of the 90 petri dishes incubated, 6 colonies shown inhibitory activity characterized by a clear zone around the colony and 3 of which originate from the outside the green algae, while the other 3 colonies are from the inside of the algae (figure 2). Bacteria isolates from the inside of brown algae have better antibacterial activity than bacterial isolates from its surface [5]. Symbiosis that produces secondary metabolites can be triggered because of biotic environmental barriers [13].

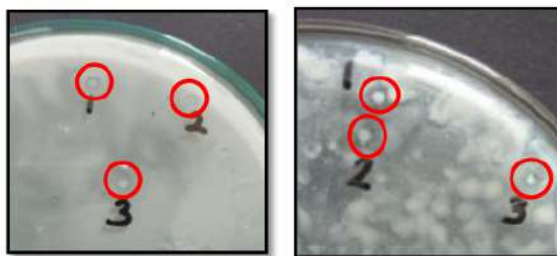


Figure 2. Colonies that shown inhibitory activity.

3.2. Qualitative challenge test

Clear zones appeared at 24 hours of incubation and was stable until 48 hours at a temperature of 37°C. The results of the direct challenge test were known that only 1 isolate of the 6 isolates tested bacteria showed consistency of inhibitory activity against test bacteria (*S. aureus* and *E. coli*) (figure 3). This isolate showed the best antagonistic activity against test bacteria when compared to other isolates and was chosen for further analysis.

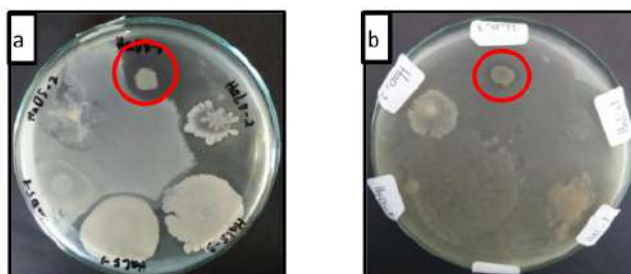


Figure 3. Antagonistic activity against *S. aureus*(a), and *E. coli* (b).

3.3. Antibacterial test

3.3.1. Paper disc method. Positive control had a wider clear zone compared to the clear zone by the supernatant in both test bacteria. Supernatant showed a 6 mm in diameter clear zone and chloramphenicol as a positive control showed a 17.68 mm in diameter clear zone in *Staphylococcus aureus* test bacteria. While in *Escherichia coli*, the supernatant did not shown clear zones and chloramphenicol showed a 13.84 mm in diameter clear zone (figure 4). So that the supernatant was bacteriostatic to Gram positive bacteria. The negative control did not show clear zones for the two test bacteria. Chloramphenicol at a dose of 0.03 mg will effectively produce a zone of inhibition of up to 18 mm so that bacteria can be said to be sensitive to antibiotics, 13-17 mm including intermediate, whereas if <12 mm is said to resistant to antibiotics [14].

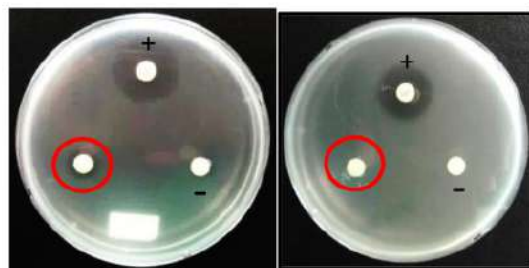


Figure 4. Antibacterial activity by supernatant (o), chloramphenicol (+), and negative control (-) against *S. aureus* (a) and against *E. coli* (b).

3.3.2. Minimum Inhibitory Concentration (MIC) test. The application of antibacterial compounds produced by selected seaweed associated bacteria showed different antibacterial activity on both test bacteria (*S. aureus* and *E. coli*). The *S. aureus* bacteria began to be inhibited by 25% of supernatant, while *E. coli* appeared to be stunted by 75% supernatant (figure 5). Gram negative has a thinner cell wall consisting of 10% peptidoglycan and high lipid content (11-12%). While Gram-positive bacteria have thick cell walls consisting of 60% -100% peptidoglycan and low lipid content (1-4%) [15].

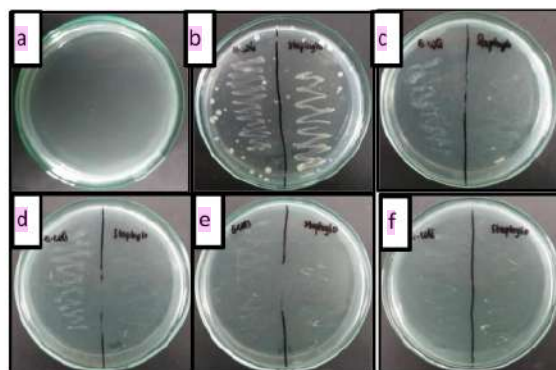


Figure 5. (MIC test results (Minimum Inhibitor Concentration), negative control (a), positive control (b), supernatant 25% (c), 50% (d), 75% (e), and 100% (f)).

The antibiotic MIC test against microbes was used to determine the sensitivity of microbes to antibiotics. MIC values are contrary to the sensitivity of the microbes tested. The lower the MIC value of an antibiotic, the greater the sensitivity of bacteria [16].

3.4. Identification of selected symbiont phenotypes and bacterial genotypes

3.4.1. Growth of bacteria. The lag phase or slow phase is experienced by bacterial symbionts at 0 to 8 hours of growth in both methods. This phase was time consuming because the symbiont bacteria needed time for adaptation to the environment or new medium. The adaptation phase was affected by several factors, including the medium and the growth environment and the number of inoculums.

The log phase (exponential phase) of symbiont bacteria was shown at 10th hours of incubation for all growth curves. In this phase, bacterial cell numbers double during each unit time period. This is because the bacteria have been able to adapt to the environment or medium. Generation time of most bacteria is between 20 minutes to 20 hours, but the rate of exponential growth varies between bacteria genera and was also influenced by culture conditions [17]. In the 16th to 22nd hours of incubation there was the stationary phase. This phase is the phase where the number of populations that grow is the same as the number of dead cells. During stationary phase, some of the cell die and lyse. These lytic products of cells can provide nutrients for other cells, and these divided and replace the dead ones.

The individual in this phase differ in certain biochemical components from cells in the exponential phase [17].

The number of cells at 24 hours of incubations shows a decrease (decline or death phase). Decline or death phase is the phase when the death rate exceeds the rate of reproduction, the cell population is in the death or decline phase [17]. In the death phase, the population of microorganisms has begun to experience death because nutrient in the medium was almost exhausted and the reserve energy in the cell was exhausted (figure 6).

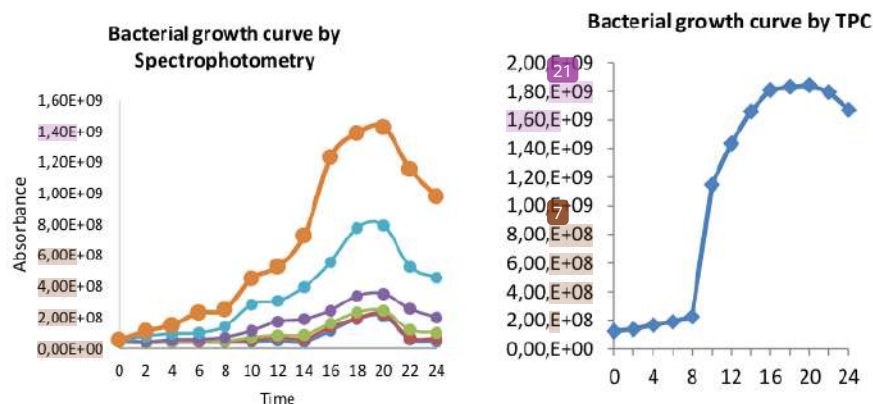


Figure 6. Growth curve of bacteria during incubation. Left: Bacterial growth curve by Spectrophotometry, $1/32$ dilution (—●—); $1/16$ dilution (—●—); $1/8$ dilution (—●—); $1/4$ dilution (—●—); $1/2$ dilution (—●—); without dilution (—●—). Right: Bacterial growth curve by TPC (CFU/mL).

3.4.2. Morphology and Biochemistry Test. Selected symbiont bacteria at 24 hours of incubation, inoculated into the Nutrient Broth medium and also at agar slant. In liquid (NB) media the nature of bacterial growth is on the surface, below the surface and bottom of the tube which can be seen clearly. The appearance of the medium with bacterial inoculation appears cloudy and forms a pellicle (thick membrane) on the surface of the medium. In various layers of surface there can be seen various kinds of growth, in some microbes visible formation of thick pellicles on the surface layer [14].

The growth of symbiotic bacterial isolates was observed in order to tilt looks fertile, looks like a tree and follows the direction of the scratch. Other characteristics that can be observed were having a marginal edge, when viewed from the side it looks convex, its consistency is not like mucus but quite thick and slightly greenish in color. Microbes that thrive on the surface of a medium will look more opaque compared to infertile growth [14].

Gram staining shows red cells or Gram negative and rod shaped. Gram negative bacteria have thinner cell walls (lipid content greater than peptidoglycan) than cell walls of Gram positive bacteria (figure 7a). Spore staining shows that symbiotic bacteria do not produce spores. Evidently with whole red vegetative cells without green cores (figure 7b). As for bacteria with the ability to produce spores, vegetative cells will be red and green in the middle. Bacterial spores can be stained by heating. Heating causes the outer layer of the spore to expand, so that the dye malachite green can enter [14]. The Z-Neelsen staining showed that symbiotic bacteria belonged to a group of bacteria that were not acid resistant shown with blue bacterial cells, while the acid resistant bacteria will be red (figure 7c).

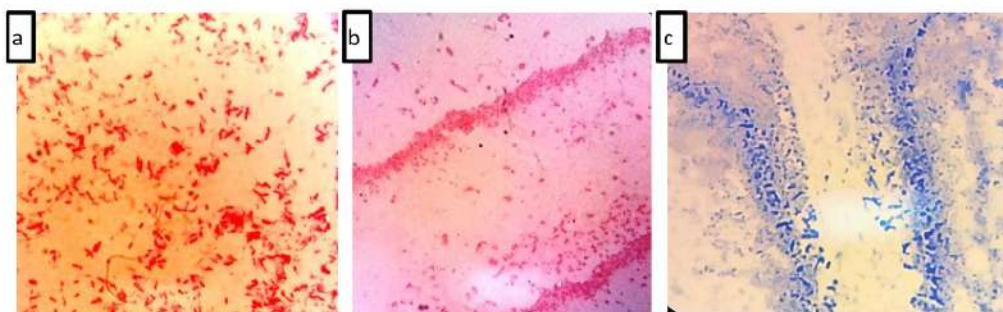


Figure 7. (a) Gram staining, (b) spore staining and (c) Z-Neelsen staining.

The characteristics of known symbiont bacteria from microscopic identification include the form of rods / bacilli, acid-resistant, non-spore-forming. Biochemical tests include symbiont bacteria is non-motile, aerobically grown, and positive carbohydrate tests. Based on the identification key from Cowan and Steel (1993) referring to a group of bacteria that are thought to have similar characteristics, to *Pseudomonas*.

3.4.3. Sixteen rDNA Molecular Examination. 16S rDNA molecular examination was carried out to determine species of symbiotic bacteria. Molecular identification is carried out through partial genetic analysis of 16S rDNA. PCR amplification results from 16S region of bacterial ribosome DNA. The sample PCR product was visualized using a gel documentation system measuring 1500 bp and the negative control did not contain the following DNA bands. The DNA tape used was relevant to PCR products produced around 1500 base pairs. Nitrogen base sequences sorted from symbiont bacterial isolates can be seen in figure 8. Based on secondary data obtained from IPBCC, sequence analysis of 16S rDNA isolates from European Union-A was identified as *Pseudomonas guezenei* which had similarities in 99%, at a maximum score of 2117 (total score of 2117), demand for 100% coverage, value of E 0.0 against bacterial taxa.

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TTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTTCGAAA
GGAACGCTAATACCGCATACGCTCTACGGGAGAAAAGTGGGGGATCTTCGGACCTCACGCTATCAG
ATGAGCCTAGGTCCGATTAGCTAGTTGGTGGGGTAATGGCCACCAAGGCGACGATCCGTAATCG
GTCTGAGAGGATGATCAGTCACACTGGAAGTGAACACGGTCCAGACTCCTACGGGAGGCAGCAG
TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTC
GGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTAC
CAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGCGGTAGGTGGTTCAGCAAGTTGGATGTGAAAAGCCCCGGGC
TCAACCTGGGAATTGCATCCAAAACACTGAGCTAGAGTACGGTAGAGGGTGGTGGAAATTCCTGT
GTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAAGTGGCGAAGGCGACCACTGGACTGAT
ACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGTACCCTGGTAGTCCACGCCGT
AAACGATGTCGACTAGCCGTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACCGGATAAGTCGA
CCGCTGGGGAGTACGGCCGCAAGGTTAAACTCAAATGAATTGACGGGGGCCGACAAAGCGGT
GGAGCATGTGGTTTAATTGGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCAGAGAATTT
CCAGAGATGGATTGGTGCCTTCGGGAACCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCTTGTCCTTAGTTACCAGACCTCGGGT
GGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATG
CCCCTACGGCCAGGGCTACACACGTGCTACAATGGTGGTACAAAGGGTTGCCAAGCCGCGAGG
TGGAGCTAATCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCG
GAATCGTAGTAATCGGA
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Figure 8. The sequence of nitrogen bases sequenced from symbiont bacteria, A = adenine, T = thiamine, G = guanine, C = cytosine.

4. Conclusion

These results demonstrated that 6 colonies showed inhibitory activity, 3 of which were originated from outside the green algae, while the other 3 colonies were from the inside of the algae. The results of the antibacterial test showed that 1 of the 6 isolates tested bacteria showed consistency of inhibitory activity against pathogenic bacteria *S.aureus* (Gram positive bacteria), meaning that it was bacteriostatic to Gram-positive bacteria. Selected isolates bacteria from *Halimeda discoidea* was Gram-negative bacteria, rod-shaped, non-spore-forming, not acid resistant and 16S rDNA examination results indicate that symbiont bacteria is a member of genus *Pseudomonas* and species *Pseudomonas guezenei*.

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