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Antibacterial activity of the symbiotic bacteria of green algae *Caulerpa racemosa* from Pulau Lima Indonesia

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Abstract. The use of symbionts is the most efficient way to obtain antibacterial bioactive compounds without the extraction of the host plant. *Caulerpa racemosa* or sea grapes are known to have symbiotic bacteria. This study was aimed to examine the antibacterial activity of symbiotic bacteria of *C. racemosa*. The research obtained two isolates from the inside of algae that formed an obstructive zone and of antagonist activity against *Staphylococcus aureus* and *Salmonella typhi* bacteria. Further analysis of the isolate coded CaD5₁ by paper disc diffusion showed that the bacteria produced an obstructive zone by 5.08 mm against *S. aureus* and had 50% *Minimum Inhibitory Concentration* (MIC) on both pathogenic bacteria. The bacteria reached their optimal growth rate at the 16th to 20th hour. The CaD5₁ were identified as gram negative bacteria, coccus, acid intolerant, not producing spores, not motile, and anaerobic facultative. Based on these key identification properties, the isolate CaD5₁ belongs to the genus *Neisseria*.

Keywords: antibacterial, *Caulerpa racemosa*, ITS2 markers, *Staphylococcus aureus*, wild seaweed

1. Introduction

Caulerpa racemosa is an algae type belonging to green algae which is still not fully utilized, although its existence is abundant in Indonesia [1]. *Caulerpa racemosa* can be consumed by the coastal communities as a vegetable or a food supplement. The utilization of symbiotic microorganisms is interesting as its ability to produce a compound is the reason they were active, a compound viable to be used to defend against the outbreak of severe pest attacks and other issues, according to of [2] an antibacterial compound produced by the bacterium that generally come from a secondary metabolite that is not used in the process of growth but for self-defense and the face of competition from microbes belong to another in getting nutrition, habitats, oxygen, light and others. The purpose of this research was to identify the disappearance of seaweed field samples, get isolates bacteria symbiont, and examine the potential of the compound antibacterial from bacteria.

2. Materials and Methods

The research consisted of two phases namely the research introduction and the advanced research. The preliminary study consisting from the sample, isolation symbiont bacteria, selection isolates producer antibacterial compound, advanced research was undertaken by identifying isolates bacteria symbiont elected. The scheme of the research methodology can be seen in figure 1.



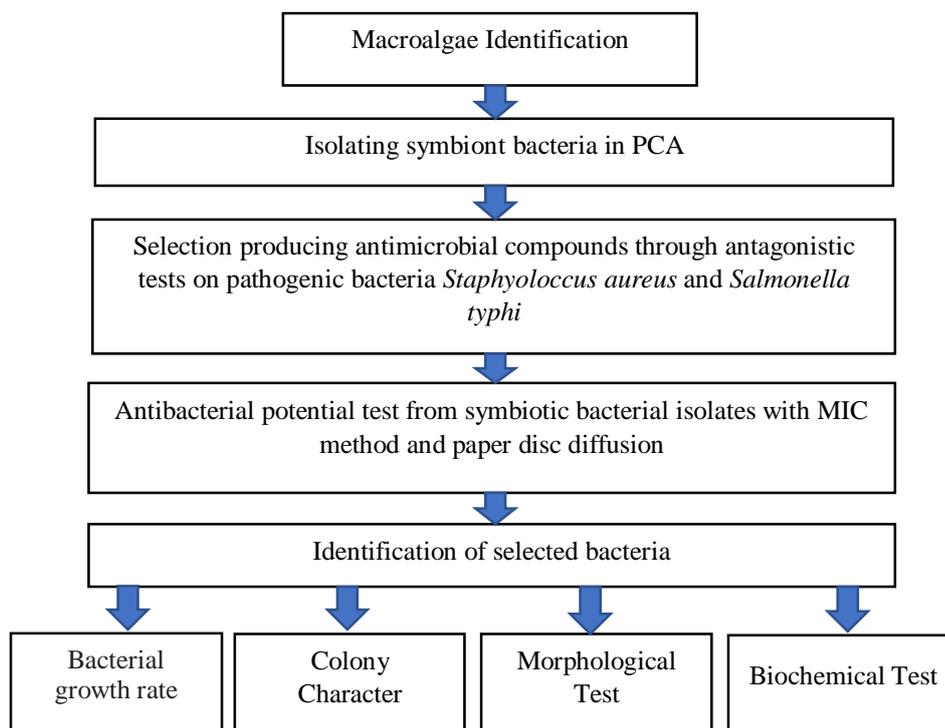


Figure 1. Scheme of Symbiont *C. racemosa* Bacterial Testing Method (modification [3]).

2.1 Macroalgae identification

The macroalgae sample identification was carried out by observing macroalgae morphology directly and matching it with the key to determination and literature.

2.2 Insulation of symbiotic bacteria

The isolation of symbiotic bacteria was carried out by refreshing. Refreshing samples were carried out on the surface of the algae and inside of the algae [3]. Refreshing part of the algae surface aimed to isolate the symbiotic bacteria that were on the surface while the refresher on the inside aimed to isolate symbiotic bacteria that were on the inside of the algae. Symbiotic bacterial growth is characterized by the appearance of clear zones after incubation. Colonies that produce clear zones will be isolated as symbiotic bacteria.

2.3 Selection of symbiotic bacteria producing antibacterial compounds

To determine the ability of bacterial isolates in inhibiting the growth of pathogenic bacteria carried out qualitatively the direct challenge test by spraying the isolate on the surface of the MHA (Mueller Hinton Agar) media which has been spread with the test bacteria (*Staphylococcus aureus* and *Salmonella typhi*). Then this was incubated for 48 hours at a temperature of $35 \pm 2^\circ \text{C}$. Isolates that form clear zones are best then inoculated to get some pure culture for further testing.

2.4 Antibacterial activity test against pathogenic bacteria

Symbiotic bacteria that have been identified before were tested for their antibacterial potential against pathogenic batteries of *Staphylococcus aureus* and *Salmonella typhi* using the MIC (Minimum Inhibitory Concentration) method and the diffusion method using paper discs. Symbiotic bacterial culture in the NB medium was incubated at $35 \pm 2^\circ \text{C}$ for 96 hours. A separation of biomass cells with the NB medium containing secondary metabolites of symbionous bacteria (supernatant) was carried

out by centrifugation at a speed of 3000 rpm at 25 °C for 1 hour, the resulting supernatant was used for screening symbiotic bacteria producing antibacterial compounds.

1) MIC method (Minimum Inhibitory Concentration)

This MIC method refers to [4] with modifications. The supernatant was diluted in an Eppendorf tube, supplemented with a Broth Nutrient media at concentrations of 100%, 75%, 50%, and 25%. After that, 4 petri dishes were prepared for the test of bacteria *S. aureus* and *S. typhi*. The surface of the cup was then divided into two equal fields, and labeled with the name of the bacteria that would be used in each area. Each dilution that had been made was inserted into petri dishes as much as 1 mL, and then added to it 9 mL of NA / Nutrient Agar. The petri dish was then shaken slowly so that the mixture was evenly mixed. Finally, it was frozen. The positive control consisted of 10 mL of NA and one dose of bacteria. The negative control only contained 10 mL NA. All test tubes were incubated at 35±20°C for 18-24 hours. The results of turbidity that occurred was observed and then compared with the positive and negative controls.

2) Screening symbiotic bacteria that have antibacterial potential

The supernatant was pipetted as much as 40 µl 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 *S. typhi* test which had been prepared on an NB medium was planted in a pour plating manner which was 1 mL pipetted into a petri dish and added with 10 mL liquid MHA with a temperature of 40 °C. The medium and suspension were homogenized by shaking the petri slowly to form number 8 and left for 15 minutes in a sterile laminary flow chamber to solidify. After having solidified, 1 piece of paper disc was inoculated with supernatant, 1 paper disc containing broth nutrient as a negative control, and 1 chloramphenicol as a positive control. The inhibitory potential was measured based on the clear zones seen around the disc paper after incubation at 35±2°C for 48 hours using a calliper.

2.5 Identification of symbiont bacteria

Selected symbiont bacteria were then identified by observing the growth rate by spectrophotometry, and phenotypic identification of bacteria by observing cell morphology, biochemical testing and then matching the key to bacterial determination.

3. Results and Discussion

The results of the sample refresher showed two isolates producing clear zones. The two isolates were derived from the algae in the petri dish diluting parts in algae 10⁻⁴ and 10⁻⁵ with the colony code CaD4₂ CaD5₁. The number of *Caulerpa racemosa* symbiotic bacterial isolates obtained in this study is not much different compared to that found in previous studies, namely [5] obtained 3 bacterial isolates that were symbiotic with *C. racemosa* from Bali waters. The results of the sample refresh can be seen in figure 2.

The inhibitory zone of CaD4₂ isolates was challenged by *S. aureus* bacteria formed at 24 hours of observation but at 48 hours of observation the inhibition zone was lost. In contrast to CaD5₁ isolates that were challenged with the same pathogenic bacteria produced a wider inhibitory zone and lasted up to 48 hours of observation. None of the symbiotic bacterial isolates tested for challenge with *S. typhi* showed no growth in inhibitory zones. In general, isolates with the CaD5₁ code were isolates that showed the best antagonistic activity against the test bacteria when compared with the CaD4₂ code. Thus it can be ascertained that CaD5₁ isolates have antagonistic properties with pathogenic bacteria.

The results of testing of the antimicrobial activity in two methods, namely paper disc and MIC (Minimum Inhibitory Concentration) diffusion, can be seen in figure 4.

The antagonistic test results of symbiotic bacterial isolates with pathogenic bacteria can be seen in figure 3.

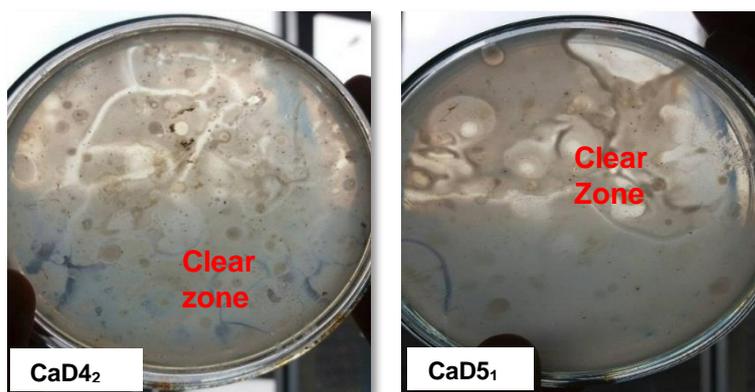


Figure 2. The isolation of *C. racemosa* symbiont bacteria on 10^{-4} and 10^{-5} dilution plates from the inside of the algae.

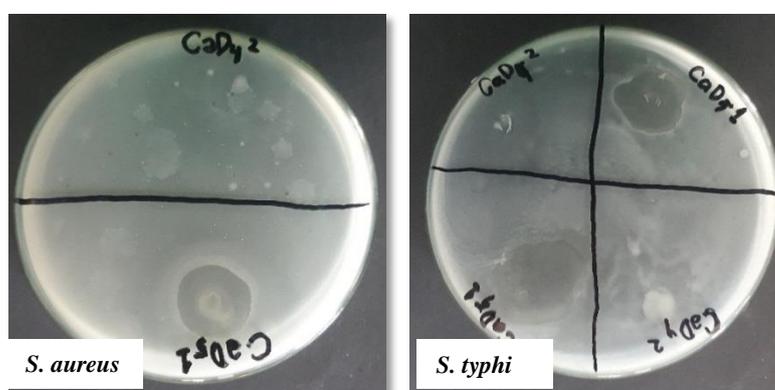
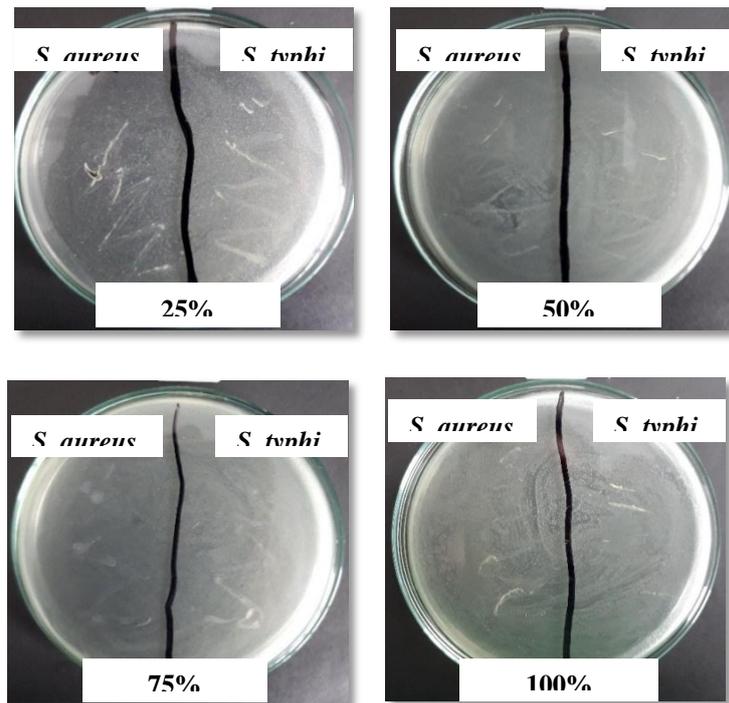


Figure 3. Antagonistic test results of symbiont bacterial isolates with *Staphylococcus aureus* and *Salmonella typhi* on PCA medium.

The identification of symbiont bacteria *C. racemosa* in this study was done by observing bacterial growth curves and phenotypic identification. Curve observation aims to determine the growth rate of symbiotic bacteria. Phenotypic identification is based on morphological observations such as cell shape and gram staining, physiological, metabolic (biochemical) or chemotaxonomic tests.

The measurement results of the bacterial growth rate of the turbidimetric method are directly proportional to the count of the cups in log cfu / mL units. The phase determination of the growth curve is based on the absorbance value curve. In the 8th hour on the growth curve shows the phase of adaptation (lag phase) that is the time needed by bacteria to adapt to their new environment. The adaptation phase lasts until 12 hours and then continues to the exponential phase (log phase) which at this time the cell will divide until the maximum number of cells is reached (a period of very rapid growth) [6]. In this observation, the exponential phase continues until the 16th hour. Then, the optimal phase of this observation occurs from the 16th to 20th hours. The next phase is the stationary phase that occurs in the 20th to 24th hours in which the living cell of bacteria or the result of division is the same as the number of dead cells so that the number of cells lives constantly, as if there is no growth. The symbiotic bacterial death phase begins at 24 hours and so on. In certain bacteria the death phase can be seen visually by observing color changes in the media, smell, and color of mucus [1]. *CaD5₁* isolate growth curve can be seen in figure 5.



Information :
 Left image: scratches from *S.aureus* bacteria
 Right image: scratches from *S.typhi* bacteria

Figure 4. Results of antimicrobial tests with MIC determination.

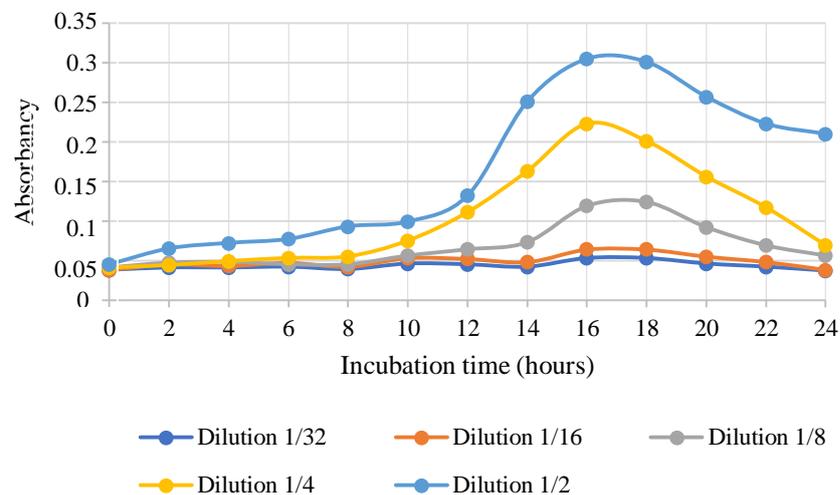


Figure 5. Growth curve of *C. racemosa* symbiotic bacteria by spectrophotometric method.

From a series of phenotypic tests on CaD5₁ isolates, a typical character from CaD5₁ isolates was obtained. Characters from CaD5₁ isolates can be seen in the following table 1. These characters can be used as a reference to identify further symbiont bacteria. Further identification can be done using the Identification Key Table from [7]. Based on the identification key from [7], it refers to the number 1 where the characterization indicates that the type of bacteria suspected to have similar characters is Neisseria. Specific reactions given by the bacteria tested.

Table 1. Characteristics of CaD5₁ isolates.

Characterization	CaD5 ₁
Strain	Gram Negative
Shapes	Coccus
Acid fast	Not acid resistant
Spores	Not Spore
Motility	Negative (-)
Catalase	Negative (-)
Glucose	Positive (+)
Mannitol	Positive (-)
Gelatine	Negative (-)
Urease	Negative (-)
Citrate	Positive (+)

4. Conclusion

The isolation results obtained two symbiotic bacterial isolates from the inside of the algae prove that potential isolates that can inhibit the *Staphylococcus aureus* test bacteria, CaD5₁ isolates and are declared bacteriocidal, based on the antibacterial activity test of selected CaD5₁ isolates by paper disc diffusion showed an inhibition zone of 5.08 mm while in testing the antibacterial activity with the MIC (Minimum Inhibitory Concentration) method, a minimum dose of supernatant against pathogenic bacteria was obtained as much as 50%. In observing the antibacterial growth rate it was found that bacterial growth reaches the optimal phase at 16 to 20 hours. Phenotypic identification results of CaD5₁ isolates included in the gram negative group, the form of coccus cells, not resistant to acid, not spore forming, not motile, and facultative anaerobes. Based on the identification key refers to the genus *Neisseria*.

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