Biodiversity of Symbiotic Microorganisms of *Caulerpa racemosa* from Lemukutan Island, Indonesia and Its Antibacterial Activity

Warsidah\(^1\,*\), Rizky\(^1\), Mega Sari Juane Sofiana\(^1\), Ikha Safitri\(^1\), Sukal Minsas\(^1\), Melia Trianasta\(^1\), Susi Sumanti\(^1\)

\(^1\)Department of Marine Science Universitas Tanjungpura, Pontianak, Indonesia

*Corresponding author. Email: warsidah@fmipa.untan.ac.id*

**ABSTRACT**

Symbiotic microorganisms usually have the same secondary metabolite activity as their host. The aims of this study were determine the biodiversity of symbiotic microorganisms from *Caulerpa racemosa* grown in Lemukutan Island, Indonesia and evaluate their antibacterial activity. Thirty eight bacterial and eighteen fungal were isolated. All isolates showed antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. All fungal isolates were identified from spore morphology using a microscope and, the best bacterial isolates with highest antibacterial activity (IB21 and IB47) were identified using biochemical tests, such as gram staining, citrate test, MR-VP test, carbohydrate fermentation test, fermentative oxidation test, oxidation test, catalase test, indole test, motility test, urease test and hydrogen production test (H2S). The genera of isolated fungi were identified as genus *Trichocladium*, *Aspergillus*, *Chaetomium*, *Coprinus*, *Cladorrhinum*, *Hymeochaete*, *Rhizopus*, *Tremella*, *Zygorhynchus*, *Mucor*, and *Bjerkandera*. Meanwhile the best bacteria IB21 and IB47 were putatively identified as member of genus *Corynebacterium* and *Neissiria*, respectively.

**Keywords:** Antibacterial Activity, Biodiversity, Caulerpa racemosa, Microorganism.

**1. INTRODUCTION**

Indonesian marine waters have a high biodiversity of marine organisms, such as macroalgae. It was found 555 species out of 8,000 species of total world macroalgae biodiversity [1]. The diversity and community of macroalgae influenced by oceanographic, topographic, and biological factors [2]. Macroalgae are classified into three groups, namely brown (Phaeophyta), red (Rhodophyta) and green macroalgae (Chlorophyta). Macroalgae have a crucial role in primary productivity, absorbing pollutants, producing organic matter and oxygen for another aquatic biota. In addition, the ecological role of macroalgae is habitat for feeding, spawning, and nursery grounds [3] for other organisms.

Macroalgae is one of the marine resources in bioactive compounds. Macroalgae from the waters of West Kalimantan, namely *Eucheuma spinosum* from Lemukutan Island and *Padina pavonica* Hauck from Kabung Island have antioxidant activity [4],[5]. Antibacterial and antioxidant properties of *Caulerpa racemosa* and *C. lentillifera* have also been reported [6]. These bioactive compounds are secondary metabolites produced by macroalgae as a form of self-defense from unfavorable environmental conditions and pathogenic microbes. Bioactive compounds can also obtained from symbiotic microorganisms. Proksch [7] have reported that the bioactive compounds of symbiotic microorganisms are identical to those of the host. Therefore, symbiotic microorganisms have the potential as a source of bioactive compounds. Symbiont bacteria are more effective to use than crude extract of macroalgae because they are easy to culture in the laboratory, thus they can avoid excessive use of natural materials [8].

The bioactivity of bacterial and fungal symbionts has been widely reported. Antibacterial of CR2 bacterial isolates associated with *C. racemosa* was active against *Pseudomonas aeruginosa* and HM isolates associated with *Halimeda macroloba* were active against *Escherichia coli* and *P. aeruginosa* [9]. Bacteria associated with green macroalgae from Singkawang waters obtained 3 isolates of bacteria associated with *C. racemosa* and 4 isolates of bacteria from *Caulerpa taxifolia* had antibacterial activity [10]. Fungi symbionts...
(FSUr-1, FSUr-2, FSUr-3) from Ulva reticulata from Takalar, South Sulawesi also showed antibacterial and antifungal activity [11] Aspergillus nomius associated with Bornetella sp. showed antibacterial against E. coli and Staphylococcus aureus [12]. The bioactivity of this symbiotic microorganisms indicates that the microorganism has great potential as an antibacterial. Therefore, the aimed of this research are determine the biodiversity of the symbiotic microorganisms of C. racemosa and its antibacterial activity.

2. MATERIALS

Materials used in this research were NB (Nutrient Broth), NA (nutrient agar), seawater, PDA (Potato Dextrose Agar), and Zobell 2216E, C. racemosa, bacteria test (S. aureus and E. coli), alcohol, aquades, set of biochemical test materials and phytochemical reagent kits, chloramphenicol (50 mg/L), and 1% sodium hypochlorite.

3. METHODS

3.1. Sampling

Samples of macroalgae C. racemosa was collected from Lemukutan Island, Indonesia, in 6 and 7 December 2020. The sample was taken at a depth of 0.9 - 1.3 meters with coordinate N 00° 46’48.46’’ E 108° 42’23.91’’ (Figure 1). The sample was put into a sterile plastic bag containing seawater and then stored in a cool box containing ice cubes for further analysis in the laboratory.

3.2. Isolation of Microorganisms

Isolation of fungi and bacteria was conducted using two methods, namely serial dilution, and direct method. The sample washed with flowing water and then the surface was sterilized by immersing the sample in 1% sodium hypochlorite solution for 5 minutes, 70% ethanol for 1 minute and in the end washed with sterile distilled water [20]. Sample crushed using a mortar and put into an erlenmeyer and added 100 mL sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution). The sample suspension from the stock solution was taken 1 mL and then put into 9 mL of sterile seawater (stock solution).

3.3. Screening Antibacterial Activity Test

Antibacterial activity test of fungal isolates was carried out using the agar diffusion methods. Colonies of endophytic fungal isolates of green macroalgal C. racemosa were grown for 7 days then cut (6 mm in diameter) and placed on NA which had been spreading with bacterial test and incubated at 37°C for 2 days. Antibacterial activity showed by formation of inhibition zone [19].

Antibacterial activity test of bacterial isolates was carried out by cross streak method. Colonies of bacteria were grown perpendicular to bacteria test and incubated at 37°C for 1x24 hours. Antibacterial activity indicated by the formation of a clear zone [25].

3.4. Characterization of the Isolate Microorganism

Identification of endophytic fungal isolates were carried out based on identification book Pictorial Atlas of Soil and Seed Fungi Morphologies of Cultured Fungi and Key to Species, [22] and referring to the journal [21], [23], and [24]. Thus, macroscopic (colony color, and colony shape) and microscopic observations were conducted under a light microscope with a magnification of 100x [20].

Identification of bacterial isolates were using biochemical tests, namely gram staining, citrate test, MR-VP test, carbohydrate fermentation test (D(+)-glucose, sucrone, lactose, maltose, and D(+)-mannitol), fermentative oxidation test, oxidase test, catalase test, indole test, motility test, urease test and hydrogen sulphide test (H2S).
3.5. Fermentation of Bacterial Suspension

Fermentation test used isolates using two bacteria with the highest antibacterial activity such as IB21 and IB47. The suspension of bacteria was grown in NB media, then agitated at 170 rpm for 24 hours. The suspension was centrifuged at 3,000 rpm for 30 minutes to separate the supernatant. The supernatant was used for phytochemical tests [35].

3.6. Phytochemical Activity Test of Bacterial Suspension

Phytochemical tests were conducted following to method by Masrani [37] to identify flavonoids, alkaloids, steroid/sterpenoids, saponins, and phenolics compounds.

4. RESULT AND DISCUSSION

4.1. Sampling and Isolation of Microorganisms

Samples of C. racemosa were taken under temperature of 29.53°C, salinity of 32.37 ppt, pH of 8.046 and DO of 4.63 mg/L. A total of 38 of bacteria and 18 of fungi were isolated (Figure 2). Furthermore, the isolates were tested for antibacterial activity.

4.2. Screening of Antibacterial Activity

Screening of antibacterial activity of symbiotic microorganisms were conducted (Table 1). Two bacterial isolates (IB21 and IB47) showed highest antibacterial activity based on the formation of highest clear zone diameter. This is accordance with the research of Yap et al [6] C. racemosa had antibacterial activity against pathogenic bacteria (E.coli and S.aureus). Similar research carried out by Rahaweman et al. [14] 13 fungal isolates from macroalgae Caulerpa spp., Halimeda spp., and Sargassum spp. Kepulauan Seribu, Indonesia has antibacterial activity against pathogenic bacteria S. aureus and E. coli. Similar research was also conducted too by Ismail et al. [13]. A total of 26 bacterial isolates from the macroalga Padina pavonica had antibacterial activity against 12 pathogenic bacteria such as S. aureus, E. coli, A. salmonicida, A. hydrophila, E. xiangfangensis, E. faecium, Micrococcus sp., S. typhimurium, Streptococcus sp., V. alginolicus, V. proteolyticus, and V. vulnificus. Another 19 isolates bacterial and fungi can inhibit only one of gram positive or negative pathogenic bacterial (Table 1). This could be due to differences in composition and structure of peptidoglycan in pathogen bacterial cell wall (E. coli and S. aureus) which can affect antibacterial activity [15].

4.3. Characterization of Microorganism

The biochemical test of IB21 (Table 2) showed positive result in metabolite products, namely: methyl red, O/F and simon citrate. IB21 was motile and gram-positive. Positive result in enzyme characteristic test, namely: oxidase and catalase, and negative of lactose test in carbohydrate fermentation. IB21 was bacilli. The results of the identification of bacteria based on Bergey’s Manual of Determinative Bacteriology (1994) isolates IB21 suspected of the genera Corynebacterium. The biochemical test of IB47 (Table 2) show positive result in metabolite products, namely: indole, methyl red and O/F. IB47 had positive result in enzyme characteristic test, namely: oxidase and catalase, and positive result on sucrose and D(+)‐glucose. IB47 was coccus, motile and gram-negative. The results of the identification, IB47 suspected of the genera Neissiria. Corynebacterium and Neissiria were obtained from marine environments such as algae [33], [38].

Endophytic fungal of C. racemosa from Lemukutan Island waters were identified macroscopically and microscopically. Macroscopic identification was carried out by looking at the color differences of fungi isolated colonies and light microscope with magnification of 100X (Table 3). The hyphae characteristics obtained from microscopic observations were matched with standard micrographs from identification books to determine the genus [22]. Based on the identification results, the genera of fungal isolates are Trichocladium, Aspergillus, Chaetomium, Coprinus, Cladorrhinum, Hymoochaeta, Rhizopus, Tremella, Zygorhynchus, Mucor, and Bjerkandera. The most dominant genus of endophytic fungal isolates obtained was the genera Aspergillus. (Table 3). Aspergillus commonly found in marine, such as macroalgae from different aquatic origins [26], [27], [28]. It was strengthened by the research of [29], [30], [31], that endophytic fungal of C. racemosa and sponges from India identified as the genera Aspergillus, Penicillium, Cladosporium, Monascus, and Schizophyllum. Other endophytic fungal genera and species were also identified by Ahamed and Murugan [28]; Handayani et al [32] from macroalgae namely Chaetomium and Trichoderma harzianum.
Table 1. Screening of Antibacterial Activity

| No. | Isolates | Medium | E. coli | S. aureus |
|-----|----------|--------|---------|-----------|
| 1   | IB02     | NA     | +       | ±         |
| 2   | IB04     | NA     | +       | ±         |
| 3   | IB06     | NA     | +       | -         |
| 4   | IB08     | NA     | ±       | -         |
| 5   | IB09     | NA     | +       | -         |
| 6   | IB10     | NA     | ±       | ±         |
| 7   | IB13     | NA     | +       | -         |
| 8   | IB17     | NA     | -       | ±         |
| 9   | IB18     | NA     | -       | -         |
| 10  | IB21     | NA     | +       | +         |
| 11  | IB24     | NA     | ±       | ±         |
| 12  | IB25     | NA     | +       | -         |
| 13  | IB29     | NA     | ±       | +         |
| 14  | IB32     | NA     | ±       | ±         |
| 15  | IB38     | Zobell 2216E | +       | -         |
| 16  | IB39     | Zobell 2216E | -       | +         |
| 17  | IB45     | Zobell 2216E | ±       | -         |
| 18  | IB46     | Zobell 2216E | -       | -         |
| 19  | IB47     | Zobell 2216E | +       | +         |
| 20  | IB48     | Zobell 2216E | +       | -         |
| 21  | IF01     | NA     | ±       | ±         |
| 22  | IF02     | NA     | -       | ±         |
| 23  | IF03     | NA     | +       | ±         |
| 24  | IF04     | NA     | -       | ±         |
| 25  | IF05     | NA     | -       | ±         |
| 26  | IF06     | NA     | -       | ±         |
| 27  | IF07     | NA     | ±       | ±         |
| 28  | IF08     | NA     | -       | ±         |
| 29  | IF09     | NA     | -       | ±         |
| 30  | IF10     | NA     | +       | +         |
| 31  | IF11     | NA     | ±       | ±         |
| 32  | IF12     | NA     | ±       | ±         |
| 33  | IF13     | NA     | +       | +         |
| 34  | IF14     | NA     | +       | +         |
| 35  | IF15     | NA     | +       | ±         |
| 36  | IF16     | NA     | ±       | ±         |
| 37  | IF17     | NA     | ±       | ±         |
| 38  | IF18     | NA     | ±       | ±         |

Note: IB: Bacterial, IF: Isolate Fungi, +: positive antibacterial activity, -: negative antibacterial activity, ±: has mist zoon.

Figure 3. Antibacterial activity test of (1) bacterial isolates and (2) fungal isolates; (A) bacterial isolates, (B) pathogenic bacteria, (C) fungal isolates.

Table 2. Characterization Result of Bacterial Isolates

| Test                          | Result  | IB21 | IB47 |
|-------------------------------|---------|------|------|
| Morphology                    |         |      |      |
| Gram staining                 | Positive|      | Negative|
| Motility                      | +       |      | -    |
| Shape of Bacterial            | Bacilli |      | Coccus|
| Carbohydrate                  |         |      |      |
| Fermentation                  |         |      |      |
| D(+)-Glucose                  | +       |      | +    |
| Sucrose                       | +       |      | +    |
| D(+)-Mannitol                 | +       |      | -    |
| Lactose                       | -       |      | -    |
| Maltose                       | +       |      | -    |
| Enzyme                        |         |      |      |
| Urease                        | -       |      |      |
| Catalase                      | +       |      | +    |
| Oxidase                       | +       |      | +    |
| Metabolite Products           |         |      |      |
| H₂S Production                | -       |      | -    |
| Indole                        | -       |      | +    |
| Methyl Red                    | +       |      | +    |
| Voges Proskauer               | -       |      | -    |
| Simmons citrate               | +       |      | -    |
| O/F                           | +       |      | +    |

Genus | Corynebacterium Neisseria

Note: +: positive test/growth; -: negative test/growth

Table 3. Characterization Result of Fungi

| Isolates | Colony Colour | Genera |
|----------|---------------|--------|
| IF01     | Green         | Trichocladium sp. |
| IF02     | Brown         | Aspergillus sp. |
| IF03     | Light Brown   | Chaetomium sp. |
| IF04     | White         | Coprinus sp. |
| IF05     | Dark Brown    | Cladorrhinum sp. |
| IF06     | White Silver  | Aspergillus sp. |
| IF07     | Yellowish-brown| Hymeochaete sp. |
| IF08     | Silver        | Rhizopus sp. |
| IF09     | Yellowish-brown| Hymeochaete sp. |
| IF10     | White         | Tremella sp. |
| IF11     | White         | Zygorhynchus sp. |
| IF12     | White         | Aspergillus sp. |
| IF13     | Yellowish White| Mucor sp. |
| IF14     | Purple        | Bjerkandera sp. |
| IF15     | Dark yellow   | Chaetomium sp. |
| IF16     | Silver        | Aspergillus sp. |
| IF17     | Gray          | Chaetomium sp. |
| IF18     | Dark green    | Aspergillus sp. |

4.4. Phytochemical Activity Test

IB21 isolate has alkaloids and saponin compound (Table 4). The IB47 isolate has alkaloids, saponins and phenolics. Secondary metabolites in bacteria are formed during the stationary phase and along with the change in energy sources from macromolecules to bioactivity [36][37].
Table 4. Phytochemical Activity Test Result

| Phytochemical Test | Isolat    |
|--------------------|-----------|
|                    | IB21 | IB47 |
| Flavonoid          | -    | -    |
| Alkaloid           | -    | -    |
| Meyer              | -    | +    |
| Dragendorf         | +    | +    |
| Terpenoid          | -    | -    |
| Steroid            | -    | -    |
| Saponin            | +    | +    |
| Phenolic           | -    | +    |

Note: +: positive, -: negative

5. CONCLUSIONS

The conclusions of this research were:
- The isolates of 38 bacterial isolates and 18 fungal isolates were isolated from *C. racemosa*. Bacterial and fungal isolates had antibacterial activity against *S. aureus* and *E. coli*. Only two bacterial isolates namely IB21 and IB47 showed highest antibacterial activity. These two selected bacteria were characterized using biochemical test and they were identified as member of genus *Corynebacterium* and *Neisseria*, respectively. IB21 has positive in dragendorff (alkaloids) and saponins, IB47 has alkaloids, saponins and phenolics. Fungal isolates *Trichocladium*, *Aspergillus*, *Chaetomium*, *Coprinus*, *Cladorrhinum*, *Hymeochaete*, *Rhizopus*, *Tremella*, *Zygorychus*, *Mucor*, and *Bjerkandera*.

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