Antibacterial activity of Caulerpa racemosa *against pathogenic bacteria promoting "ice-ice" disease in the red alga* Gracilaria verrucosa

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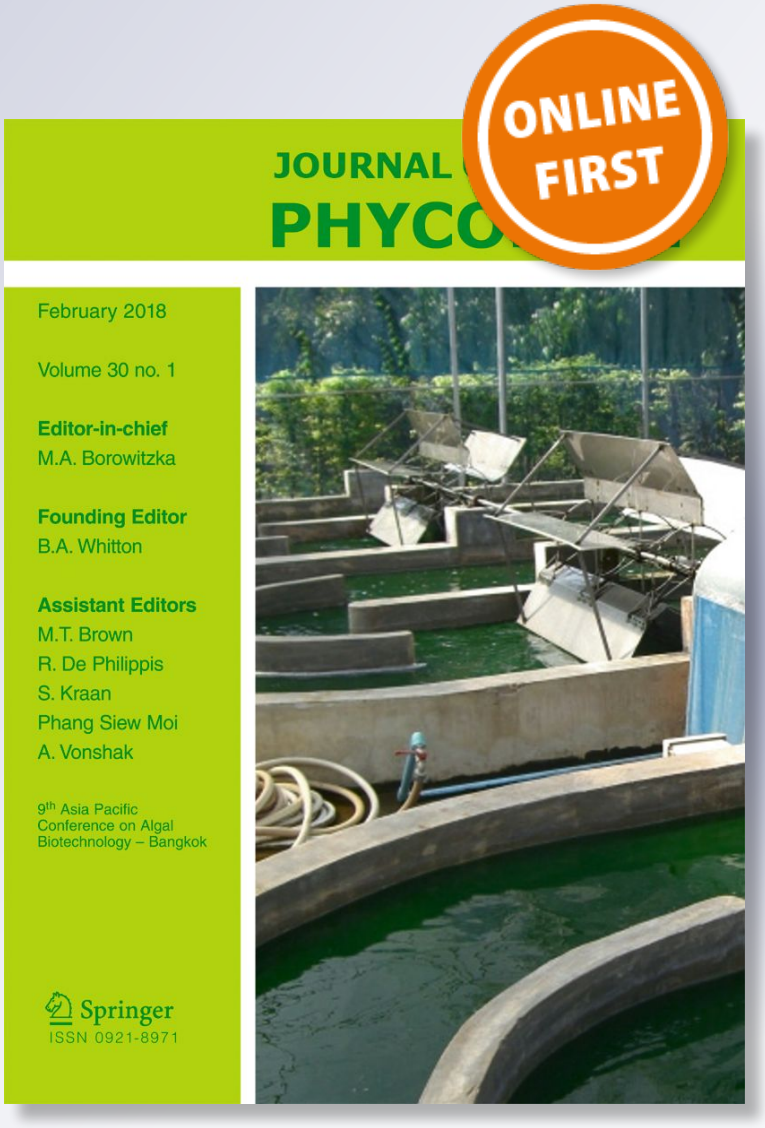

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Antibacterial activity of *Caulerpa racemosa* against pathogenic bacteria promoting "ice-ice" disease in the red alga *Gracilaria verrucosa*

Elmi Nurhaidah Zainuddin[1] · Hilal Anshary[1] · Huyyirnah Huyyirnah[1] · Ridha Hiola[1] · Dolores V. Baxa[2]

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Abstract

Increasing ocean temperatures associated with climate change have triggered the occurrence of diseases in marine resources such as macroalgae or seaweed. "Ice-ice" is one of the most devastating diseases affecting economically important seaweeds such as *Gracilaria* and *Eucheuma*. In this study, we investigate the bacterial composition of diseased and healthy *Gracilaria verrucosa*, a red seaweed cultured in brackish water ponds in Takalar, Indonesia. Morphologic and phenotypic characteristics showed that the isolates from diseased *Gracilaria* belong to various genera: *Vibrio*, *Chromobacterium*, *Flavobacterium*, *Pseudomonas*, and *Achromobacter*. Several bacteria were also isolated from healthy *Gracilaria* including *Corynebacterium*, *Serratia*, *Shigella*, *Micrococcus*, *Proteus*, and *Flavobacterium*. Using Koch's postulates, bacterial pathogenicity was established by bath exposure of naïve *G. verrucosa* to each of the bacteria isolated from diseased *Gracilaria* resulting in symptom characteristic of "ice-ice" disease. The antibacterial property of the green seaweed *Caulerpa racemosa* against the pathogenic bacteria was assessed using extracts that were prepared with solvents of various polarities such as hexane, chloroform, ethyl acetate, methanol, and methanol-water. The highest antibacterial activity was observed in methanol extracts *Caulerpa* while extracts using the other solvents showed moderate to low activities. These findings demonstrate the potential of *Caulerpa* to inactivate bacterial pathogens associated with "ice-ice" disease.

Keywords *Gracilaria verrucosa* · "Ice-ice" bacteria · Antibacterial activity · *Caulerpa racemosa*

Introduction

Indonesia is the second largest marine fishery producer in the world after China (FAO 2016) with a long coastline of 99,093 km and 13,466 islands (BIG 2013). Seaweed is one of Indonesia's prime commodities from among its diverse marine resources. Indonesia has the potential to develop seaweed cultivation since it is a tropical region penetrated by sun throughout the year. Seaweed production in Indonesia is at an average of 3 million tonnes per year that increased to 11 million tonnes in 2016 with projected trajectories of up to 13.4 million tons in 2017 (KKP 2016).

Although marine seaweeds are highly diverse in the Coral Triangle with an estimated 555 species identified in Indonesian waters, only five species are economically important including *Eucheuma*, *Gracilaria*, *Gelidium*, *Sargassum*, and *Caulerpa* (Indriani and Sumiarsih 1992; Kasim 2016). Nutrient-rich waters and bay regions are protected from large waves placing Indonesia at the forefront of *Kappaphycus alvarezii* and *Eucheuma* spp. production worldwide (FAO 2015). *Gracilaria* is among the red algal species that belong to the agar-producing group (Agarophyta) vitally utilized globally for food, medicine, and cosmetic industries (Kim and Chojnacka 2015). Agar is a phycocolloid widely used in microbiology for cell culture and as a medium for microbial growth (Jamilah 2013). Due to increasing world demand, *Gracilaria* production has increased to 3–5% per year (Bixler and Porse 2011).

Indonesia is the second biggest supplier of *Gracilaria* seaweed after China with a total production of 975,000 t (KKP 2013). Due to the increasing global demand and limited

✉ Elmi Nurhaidah Zainuddin
elmi18id@gmail.com; elmi18id@fisheries.unhas.ac.id

[1] Faculty of Marine Science and Fisheries, Hasanuddin University, Jl. Perintis Kemerdekaan, Km 10, Tamalanrea, Makassar 90245, Indonesia

[2] Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California, Davis, CA 95616, USA

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availability of stocks in nature, *Gracilaria* has been cultured in brackish water ponds in South Sulawesi, a province in Indonesia with a large pond area that is well suited for cultivation of *Gracilaria*, particularly *G. verrucosa*, which can tolerate a wide range of salinity between 15 to 30 ppt (Anggadiredja et al. 2008). Production volumes of about 780,820 t (78.02%) of the total national production of *Gracilaria* species makes South Sulawesi the largest supplier of *Gracilaria* in Indonesia (KKP 2013).

As primary producers, seaweeds play essential roles in marine ecosystems as food sources of herbivorous organisms, habitats for various microorganisms, and sanctuaries for early life stages of invertebrates (Bulleri et al. 2002; Fraschetti et al. 2006). Climate change has been associated with the spread of diseases in marine ecosystems including adverse effects to seaweed aquaculture (Harvell et al. 2009; Gachon et al. 2010). Herbivores and pathogens can have substantial effects on algal fitness, regulate population dynamics, and cause considerable damage in marine ecosystems. Such diseases or predation may also be highly destructive in managed ecosystems of *Saccharina* (*Laminaria*) *japonica* (Ishikawa and Saga 1989), *Pyropia yezoensis* (Fujita et al. 1972), and cultured *Eucheuma* and *Kappaphycus* (Ask and Azanza 2002; Hurtado et al. 2006).

Bacterial species previously associated with "ice-ice" disease in several seaweed species include *Vibrio*, *Aeromonas*, *Cytophaga*, and *Flavobacterium* (Loureiro et al. 2009). Also reported in diseased marine seaweeds are bacterial species such as *Chromobacterium*, *Acinetobacter*, *Flavobacterium-Cytophaga* and *Vibrio* (Nurjanna 2008). The agar-degrading bacterium, *Pseudoalteromonas gracilis* can cause damage in the seaweed *Gracilaria gracilis* by producing agarase enzymes (Schroeder et al. 2003). Diseases observed in *Gracilaria lemaneiformis* have also been associated with *Corynebacterium*, *Arthrobacter*, *Flavobacterium*, and *Cytophaga* (Sun et al. 2012). Seaweed disease is generally characterized by white discoloration, fractured, or hardened thallus commonly attributed to bacterial infections (Largo 2002). Physical and biological factors such as high-water temperatures, density, and low aeration are suspected to cause damage in *Gracilaria conferta* (Egan et al. 2014). To date, investigations on "ice-ice" disease in cultivated seaweeds are limited to the identification of pathogens causing the disease (Largo 2002; Yulianto and Mira 2009; Aris 2011). Studies that focus on the control of seaweed diseases, through prevention or treatment, have not been widely reported.

In addition to its commercially valuable phycocolloid content (agar, carrageenan, and alginate), seaweed has highly diverse bioactive compounds (Chanda et al. 2010). Seaweed produces various metabolites such as caulerpin, caulersin, and caulerpenyne, generating research interest due to their unusual structural features and remarkable pharmacological properties (Yang et al. 2014). Despite the intensive research about the antibacterial activity of seaweed in the fight against pathogenic bacterial infections in humans (Zainuddin 2010b), aquaculture organisms (Bansemir et al. 2006), and plants (Arunkumar et al. 2010), very little research has been conducted on antibacterial activity against pathogenic bacteria in seaweed.

This study will therefore aim to determine the pathogens causing "ice-ice" disease in cultivated *Gracilaria* and to assess the potential antibacterial activity of *Caulerpa racemosa* against the pathogens isolated from "ice-ice"-infected *Gracilaria*.

Materials and methods

Collection of seaweed samples

Samples of *Gracilaria verrucosa* (± 1 kg wet weight) were collected from a brackish water pond in Takalar, Indonesia (5° 24′ 58.4958″ S 119° 29′ 15.2406″ E). Diseased thalli were stored in a sterile air-tight plastic bag and transported to the laboratory. Healthy samples of *Gracilaria* (± 1 kg wet weight) were also collected and separately stored in air-tight plastic bags as above. Infected thalli of *G. verrucosa* showed white patches on the surface of the thallus, which is a typical symptom of "ice-ice" disease.

Caulerpa racemosa for extraction of antibacterial compounds against "ice-ice' bacteria were collected from Takalar waters (5° 35′ 8.862″ S 119° 29′ 5.337″ E). Seaweed samples were cleaned with seawater in situ, placed in plastic bags, and then transported in a cool box to the laboratory to preserve the active compounds during transportation.

Bacterial isolation from infected *Gracilaria verrucosa*

Diseased *Gracilaria* thalli were first washed with sterile distilled water and cut into small pieces. About 1 g of each sample was placed into a test tube containing 9 mL of 0.9% NaCl solution, homogenized with vortex for 5 min, and used for the stock solution. The stock solution was serially diluted (1:10) up to 10^{-6} in sterile 0.9% NaCl solution, 1 mL from each serial dilution was pipetted onto a TSA (Tryptic soy agar) plate and spread with a sterile glass spreader. Healthy *Gracilaria* thalli were processed in the same manner. Homogenates from healthy and infected *G. verrucosa* were inoculated onto plates and incubated at 30 °C for 24 h. For assessment of biochemical properties, a single well-isolated colony was selected, re-streaked onto TSA, and incubated at 30 °C for 24 h, as described above. Colonies representing the most abundant bacteria at first isolation from field samples were used for the biochemical characterization and pathogenicity tests.

Pathogenicity test of bacterial isolates by Koch's postulates

Healthy *Gracilaria* thalli that were used for the pathogenicity tests were decontaminated with 70% ethanol solution for 10 s, rinsed with sterile distilled water, and then placed in a sterile jar containing sterile seawater. Bacterial isolates (G1-G7) were cultured in TSB (Tryptic soy broth) in 30 °C incubator shaker for 72 h. The bacterial suspension (2 mL/jar) contained a dose of 1×10^6 cfu mL^{-1} and used for continuous bath exposure of healthy *G. verrucosa* (2–3 thalli/jar) for 14 days with aeration and fluorescent light (24-W bulb, 24:0 = L:D photoperiod) at room temperature. *Gracilaria* exposed to the bacterial isolates were observed daily for color change, morphology, and texture of the thallus. *Gracilaria* exposed to the same conditions as above but with sterile seawater without bacteria served as controls. The concept of Koch's postulates was used to assess whether the isolated bacteria from diseased thalli of *Gracilaria* caused "ice-ice" symptoms in healthy thalli. After 14 days of exposure of healthy thalli to each suspect bacterium, *Gracilaria* thalli exposed to each of the isolated bacterium were sampled and inoculated onto TSA to determine whether the re-isolated bacteria were identical to the original specific causative agent(s) isolated from field samples of diseased *Gracilaria*.

Identification of pathogenic bacteria in "ice-ice" infected *Gracilaria*

Growth at different salinity and water temperatures

The bacterial isolates that caused infections (white, brittle, or black thallus) following bath exposure of *Gracilaria* were assessed for differences in growth as influenced by salinity and temperature. Pathogenic bacteria were grown in TSB medium at salinity ranges of 15, 25, 30, and 35 ppt and incubated at 35 °C for 24 h. Different salinity ranges were prepared using NaCl dissolved in sterile distilled water. Bacterial growth was also assessed after 24 h of incubation at 5 °C (refrigerator), 25 °C (room temperature), and at 35 °C and 40 °C (incubator). Bacterial growth was measured for optical density (absorbance) at 600 nm after incubation for 24 h at the different salinities and temperatures.

Biochemical test

The bacterial isolates were established for pathogenicity to *Gracilaria* using Koch's postulates and were examined for biochemical characteristics as described in Cappucino and Sherman (1986) that includes Gram staining, catalase, motility, indole, TSIA (Triple sugar iron), gas and H_2S, urease, and growth on TCBS (thiosulfate-citrate-bile salts-sucrose) agar. These biochemical tests are described in detail in Cappucino and Sherman (1986), and the results were used to determine bacterial types according to Cowan and Steels Manual for the Identification of Medical Bacteria (Barrow and Feltham 2003).

Isolation of *Vibrio* spp. using thiosulfate-citrate-bile salts-sucrose (TCBS) agar

Bacterial isolates were inoculated onto TCBS, a highly selective medium for the isolation of *Vibrio* spp. and incubated for 24 h. Yellow or bluish green colonies that grew on TCBS were tentatively identified as *Vibrio* sp.

Preparation of *Caulerpa racemosa*

In the laboratory, *C. racemosa* was first rinsed with sterile seawater to prevent cell osmosis during the cleaning process. The seaweed sample was then washed with fresh or tap water to remove salts and finally rinsed with distilled water. The *Caulerpa* sample was drained overnight and assessed for wet weight followed by sun drying to obtain dry weight. Dried *Caulerpa* was homogenized into a fine powder using a blender, placed in air-tight plastic bag, and stored at room temperature until used.

Extraction of *Caulerpa racemosa*

Bioactive compounds were extracted from *C. racemosa* using kinetic maceration (Zainuddin 2010a, 2010b). Five solvents with increasing polarities (*n*-hexane, chloroform, ethyl acetate, methanol, and methanol-water) were used for the extraction. The extraction procedure was carried out by weighing 100 g of *C. racemosa* seaweed powder in an Erlenmeyer flask and added 200 mL of *n*-hexane and stirred for 24 h at room temperature. The solution was passed through Whatman No. 1 filter paper, and the supernatant was collected in a 1000-mL blue-cap bottle (Schott, Duran). The residue was extracted two more times with the same volume of solvent as described above. The supernatant from the three macerations was combined and evaporated on a rotary evaporator to obtain a 5–10 mL concentrated extract. The extract was kept on the small vial, dried at room temperature to yield a thick and oily crude extract, and stored air-tight at − 20 °C for further analysis. The residue from *n*-hexane extraction was dried in a fume hood for 24 h until the solvent was evaporated and successively re-extracted as described above with the next solvent in the order of polarity (chloroform, ethyl acetate, methanol, and methanol-water). Methanol-water extract was lyophilized or freeze-dried. All extracts were stored at ≤ 4 °C until used.

Antibacterial activity assay

The agar diffusion method was used for assessment of antibacterial activity as previously described (Zainuddin 2006, 2010b). Before use for the antibacterial assay, bacteria were rejuvenated by inoculating onto TSA plate and incubated at 37 °C for 24 h. After incubation, a loopful of the isolate was suspended in 2 mL 0.9% NaCl solution. The bacterial suspension (200 μL) was pipetted into a beaker containing 20 mL of warm (43 °C) TSA medium and gently mixed in a circular motion. The warm agar solution containing the bacterial inoculum was then poured into a sterile Petri dish and allowed to solidify. Before the antibacterial test, the crude *Caulerpa* extract was re-dissolved in each solvent and added a 50 μL at 2 mg concentration onto a sterile paper disc (6 mm diameter). The paper disc with *Caulerpa* extract-solvent was evaporated aseptically by laminar flow at room temperature until dry; the disc is placed on the agar surface inoculated with bacteria and incubated at 37 °C for 24 h. For negative control, discs contained only solvents for extraction while positive control discs were impregnated with a similar volume of tetracycline (30 μg). The antibacterial activities of *Caulerpa* extracts against each bacterial species were assessed by measuring the diameter of clear zones around the paper discs. All experiments were repeated two times and every time with three replicates.

Data analysis

Physical and biochemical properties of the bacteria were characterized, while antibacterial activity was analyzed by one-way ANOVA using statistical package SPSS 16 for Windows to determine the antibacterial effect of *Caulerpa* extract on bacteria that cause "ice-ice" disease in *G. verrucosa*. Differences in antibacterial activities among the *Caulerpa* extracts were assessed using Tukey's Honest Significant Difference test (HSD) multiple comparison procedure with adjusted p value at 0.05.

Results

Bacterial isolates from *Gracilaria verrucosa*

"Ice-ice"-diseased red seaweed *G. verrucosa* showed the presence of seven bacterial isolates (G1–G7), and their characteristics are summarized in Table 1. All isolates were Gram-negative and colonies were raised (G1, G2, G4, and G6), flat (G3, G5, and G7), smooth (G1, G3, G4, G5, and G7), and with rough edges (G2 and G6). Colony colors were white (G3, G5, and G6), cream (G4 and G6), and yellow (G1) to yellowish (G2).

In addition to the isolates obtained from diseased *G. verrucosa*, 12 bacteria were obtained from healthy *G. verrucosa*, and their characteristics are summarized in Table 2. Nine of the 12 isolates were Gram-negative, and only three were Gram-positive. Most of the isolates were rod-shaped, and one was coccus (GH-35). Colonies were either yellow (GH-11, GH-12, GH-35, GH-36, and GH-37), orange (GH-13, GH-32, and GH-33), or cream colored (GH-14, GH-15, GH-31, and GH-34).

Pathogenicity of isolated bacteria "ice-ice"

Koch's postulates were conducted to determine if the bacteria isolated from "ice-ice" infected *G. verrucosa* caused disease in healthy *Gracilaria*. All seven isolates (G1–G7) from diseased *Gracilaria* caused typical symptoms of "ice-ice" in healthy *G. verrucosa* following bath exposures for 14 days. Symptoms were similar to those observed in natural infections where the thallus were white, brittle, and broken, and some showed the presence of mucus. Symptoms of "ice-ice" as observed within 14 days of exposure occurred at different exposure times and types of bacteria. Onset of "ice-ice" clinical signs was immediately observed (3–4 days post exposure) in *Gracilaria* exposed to *Pseudomonas* (G5), and 5–6 days to *Vibrio* (G1 and G4), *Flavobacterium* (G2), *Chromobacterium* (G6), and 7–8 days to *Chromobacterium* (G3) and *Achromobacter* (G7). After 2 weeks of the experimental duration, the thallus of *Gracilaria* without "ice-ice" infection (control) remained intact, the color did not change, and mucus was not produced compared to *Gracilaria* groups exposed to each of the pathogen suspensions.

The growth of isolates from "ice-ice"-infected *Gracilaria* at different salinities and water temperatures

Growth at various salinity levels was assessed as characterized by optical density measurements at 600 nm. At 15, 25, and 30 ppt, five isolates (G1, G2, G3, G5, and G7) showed relatively increased growth (0.87–1.12 OD) whereas two isolates (G4 and G6) showed generally low growth (0.51–0.63 OD). At 35 ppt, growth was relatively high (0.87–1.02 OD) among four isolates (G1, G3, G5, and G6), while three isolates (G2, G4, and G7) generally had low growth (0.57–0.67 OD (Table 3).

Growth at various water temperatures was also assessed as characterized by optical density measurements at 600 nm. At 25 °C, most of the bacteria (G2, G3, G5, G6, and G7) grew optimally (0.89–1.10 OD) while two isolates (G1 and G4) showed poor growth (0.49–0.57 OD). Growth was slow or not observed at 5 °C in all seven isolates (G1–G7) (0 to 0.07 OD). At 35 °C, three isolates (G2, G3, and G7) showed optimal growth (0.87–0.97 OD), while four isolates (G1, G4, G5,

Table 1 Biochemical properties of bacteria isolated from "ice-ice"-diseased *Gracilaria verrucosa*

Biochemical test	*Vibrio* (G1)	*Flavobacterium* (G2)	*Chromobacterium* (G3)	*Vibrio* (G4)	*Pseudomonas* (G5)	*Chromobacterium* (G6)	*Achromobacter* (G7)
Gram stain	−	−	−	−	−	−	−
Indole	−	−	−	−	−	−	−
Motility	+	−	+	+	+	+	+
TSIA	A/K	K/K	K/A	K/A	K/A	K/A	K/A
Catalase	+	+	+	+	+	+	+
Urease	−	+	+	+	+	+	+
Gas	−	−	−	−	−	−	−
H_2S	−	−	−	−	−	−	−
TCBS	Grow	−	−	Grow	−	−	−

TSIA triple sugar iron agar (test on fermentation of glucose, lactose, and sucrose), *TCBS* thiosulfate-citrate-bile-salts-sucrose agar (highly selective medium for the isolation of *Vibrio* species), *A* acid, *K* alkaline

and G6) generally showed poor growth (0.51–0.64 OD). At the highest temperature tested (40 °C), only two isolates (G4 and G7) showed high growth (0.87–1.02 OD), while five isolates (G1, G2, G3, G5, and G6) showed minimal growth (0.57–0.65 OD) (Table 4).

Biochemical properties and identification of bacterial isolates

All of the seven isolates (G1–G7) from "ice-ice"-infected *G. verrucosa* were Gram-negative and produced catalase enzymes, and all were motile except one isolate (G2); all were indole negative as they were incapable of converting tryptophan into indole as indicated by the absence of a red layer on the top of the TSIA medium (Table 1).

Based on the TSIA test, five isolates (G3, G4, G5, G6, and G7) fermented glucose while two isolates (G1 and G2) did not utilize glucose. All seven isolates did not produce H_2S and gas. The gas formation was characterized by the breaking of the medium at the bottom of the test tube, while the H_2S was characterized by the presence of black color on the medium. Six bacterial isolates (G2, G3, G4, G5, G6, and G7) showed the presence of urease, while one isolate (G1) was negative for urease. The urease test indicates the ability of bacteria to hydrolyze urea to ammonia using urease enzymes, which raises the pH of the medium and changes the color of the culture from yellow (negative) to red (positive). Presumptive identification of *Vibrio* was found in two isolates (G1 and G4) based on growth on TCBS (thiosulfate citrate bile salt) agar.

In healthy *G. verrucosa*, nine isolates were Gram-negative (GH-13, GH-14, GH-15, GH-31, GH-32, GH-33, GH-34, GH-36, and GH-37) while three isolates were Gram-positive (GH-11, GH-12, and GH-35). In contrast to the isolates from diseased *Gracilaria*, all isolates from healthy *Gracilaria* were catalase positive. Most isolates were non-motile (GH-11, GH-12, GH-13, GH-15, GH-32, GH-33, GH-34, GH-35, GH-36, and GH-37) and only two were motile (GH-14 and GH-31). All isolates from healthy *Gracilaria* were indole negative, oxidase negative, lactose negative, H_2S negative, and ornithine positive. Most isolates did not produce urease and citrate, and only one produced urease (GH-34) and citrate (GH-14). Ten isolates were MR negative, and only two were MR positive (GH-31 and GH-32). The VP test showed that 5 of 12 isolates were VP positive (GH-14, GH-15, GH-31, GH-32, GH-33) and the rest of the isolates were VP negative (Table 2).

Extracts of *Caulerpa racemosa*

For the antibacterial assessments, the entire thallus of *C. racemosa* was extracted using kinetic maceration and stirring to facilitate the destruction of cell wall using solvents without heating (Zainuddin 2006). The maceration method is a simple soaking extraction using solvents and compounds that attach to the cell by the polarity system where the non-polar compounds are dissolved in a non-polar solvent while the polar compounds would dissolve in a polar solvent. The extraction process was carried out successively starting from the non-polar solvent (*n*-hexane) followed by the semi-polar solvents (chloroform and ethyl acetate), and ending with the polar solvents (methanol and methanol-water) to facilitate the separation or fractionation of compounds based on their polarity (Zainuddin 2006).

Based on the polarity of the solvents used for extraction, *C. racemosa* extracts showed variable yields: 146.5 mg of *n*-hexane extract at 0.03%, 360.8 mg of chloroform extract at 0.09%, 101.6 mg of ethyl acetate extract at 0.02%, and 372.3 mg of methanol extract at 0.09%. The methanol-water extract showed the highest yield of 475.8 mg at 0.11%.

Table 2 Biochemical properties of bacteria isolated from healthy *Gracilaria verrucosa*

Biochemical test	*Corynebacterium* (GH11, GH12)	*Flavobacterium* (GH13, GH32, GH33)	*Serratia* (GH14, GH31)	*Shigella* (GH15, GH36, GH37)	*Proteus* (GH34)	*Micrococcus* (GH35)
Gram stain	+	−	−	−	−	+
Indole	−	−	−	−	−	−
Motility	−	−	+ (GH14) − GH31)	−	−	−
TSIA	A/− (GH11) A/A (GH12)	A/A (GH13, GH32) KA (GH33)	K/A (GH14) A/A (GH31)	A/A	A/A	A/A
Catalase	+	+	+	+	+	+
Urease	−	− (GH13, GH33) + (GH32	− (GH14) + (GH31)	−	+	−
Gas	−	−	−	−	−	−
H_2S	−	−	−	−	−	−
TCBS	−	−	−	−	−	−
Oxidase	−	−	−	−	−	−
Lactose	−	−	−	−	−	−
Citrate	−	−	+ (GH14) − (GH31)	−	−	−
MR	−	− (GH13, GH33) + (GH32)	− (GH14) + (GH31)	−	−	−
VP	−	− (GH13) + (GH32, GH33)	+	+ (GH15) − (GH36, GH37)	−	−
Ornithin	+	+	+	+	+	+

TSIA triple sugar iron agar (test on fermentation of glucose, lactose, and sucrose), *TCBS* thiosulfate-citrate-bile-salts-sucrose agar (highly selective medium for the isolation of *Vibrio* species), *MR* methyl red, *VP* Voges-Proskauer (tests to aid in the identification of enteric Gram-negative bacilli), *A* acid, *K* alkaline

Antibacterial activity of *Caulerpa racemosa* extracts against the bacterial isolates from diseased *Gracilaria*

One-way ANOVA analysis showed that the five extracts of *C. racemosa* (*n*-hexane, chloroform, ethyl acetate, methanol, and methanol-water) had a significant effect ($p < 0.05$) on all isolate of "ice-ice" bacteria (G1, G2, G3, G4, G5, G6, and G7). Differences in antibacterial activity based on the Tukey test were observed among the five *C. racemosa* extracts against the seven isolates of "ice-ice" bacteria (Table 5).

The antibacterial activity of five extracts of *C. racemosa* against *Vibrio* (G1) showed that only three extracts (non-polar to the semi-polar group) showed activities from moderate to low, while the two polar extracts had no activity. The highest antibacterial activity was shown in *n*-hexane extract (10.92 mm) followed by chloroform extract (9.38 mm) and ethyl acetate extract (9.33 mm). Similar inhibitory properties were also detected on the five extracts of *C racemosa* against the isolate G2 (*Flavobacterium*) in which the highest antibacterial activity was demonstrated by the chloroform extract with a diameter inhibition zone of 9.28 mm. There was no difference in activity between the *n*-hexane (8.32 mm) and the ethyl acetate (8.28 mm) extracts.

The Tukey test showed variable antibacterial activity: *Caulerpa* extracts against *Chromobacterium* (G3), and other species (e.g., *Vibrio* and *Flavobacterium*) were high using polar extract but only low and no activity in semi-polar and non-polar extracts. In *Chromobacterium*, the highest activity was shown using methanol extract with an inhibitory zone diameter of 21.45 mm. No significant differences were observed between methanol-water (8.53 mm), and ethyl acetate (7.63 mm) extracts in inhibiting the growth of isolate G3. The five *C. racemosa* extracts against *Vibrio* (G4) also showed high to moderate activity in the polar extract (methanol and methanol-water), while low to no activities were detected on non-polar and semi-polar extracts. The highest antibacterial activity was demonstrated in methanol extract with an inhibition zone diameter of 23.25 mm which differed in the activities of the methanol-water (11.57 mm) and ethyl acetate (7.67 mm) extracts. The antibacterial activity of *C. racemosa* extract against isolate G5 was highest in methanol extract (20.32 mm), followed by moderate activity in the methanol-water extract (13.92 mm) and low or no activity in semi- and non-polar extracts. The diameter of inhibition zones of ethyl acetate and *n*-hexane extracts were 7.73 mm and 7.65 mm, respectively (Table 5).

Based on the Tukey test, the effect of *C. racemosa* extract on *Chromobacterium* (G6) showed that only polar extracts had high and moderate antibacterial activities, while the semi- and non-polar extracts had no activity. The methanol extract exhibited a high level of activity (19.82 mm) followed by a moderate level of activity in the methanol-water extract

Table 3 The growth of bacterial isolates from diseased *Gracilaria verrucosa* at different water salinities. Optical density (OD) of bacterial culture was measured at 600 nm

Salinity (ppt)	Bacterial isolates						
	Vibrio (G1)	*Flavobacterium* (G2)	*Chromobacterium* (G3)	*Vibrio* (G4)	*Pseudomonas* (G5)	*Chromobacterium* (G6)	*Achromobacter* (G7)
15	0.97	0.87	0.92	0.51	0.88	0.63	1.12
25	0.92	0.89	1.00	0.60	0.92	0.60	0.98
30	0.98	0.87	0.91	0.62	0.97	0.62	0.97
35	1.02	0.67	0.95	0.57	0.87	0.87	0.65

(12.10 mm). The antibacterial activity against *Achromobacter* (G7) showed that only methanol extract from *C. racemosa* had very high level of activity (inhibition zone diameter of 22.20 mm), while the other four extracts showed no activity (Table 5).

Discussion

Gracilaria verrucosa ranks second in seaweed production after *Kappaphycus alvarezii* in South Sulawesi province. In addition to being cultured along coastal areas, *Gracilaria* has been widely cultured in brackish water ponds. That makes South Sulawesi the largest producer of *Gracilaria* in Indonesia. Increased cultures of *Gracilaria* in ponds may also increase the occurrence of "ice-ice" diseases. "Ice-ice" disease was initially thought to be mainly due to stress from environmental factors. However, various studies showed that environmental stress might increase seaweed susceptibility to pathogens (Largo et al. 1995a). Bacteria that inhabit the water column or commensal with the seaweed may potentially transform into opportunistic pathogens in stressful environments (Largo 2002).

In our study, seven bacterial species were isolated from diseased *Gracilaria* grown in culture ponds at Takalar, South Sulawesi. Based on the results of morphological identification and biochemical tests, the characteristics of the seven isolates matched the description of the genus *Vibrio* (isolates G1 and G4), *Chromobacterium* (isolates G3 and G6), *Flavobacterium* (isolates G2), *Pseudomonas* (isolate G5), and *Achromobacter* (isolate G7).

Various studies have shown the diversity of bacteria isolated from "ice-ice" diseased seaweeds (Nasution 2005; Nurjanna 2008; Aris 2011; Achmad et al. 2016). All the isolated "ice-ice" bacteria obtained from *Gracilaria* were Gram-negative. These results are similar to the results from Maheswaran et al. (2013) where most pathogenic bacteria that commonly cause disease in aquaculture are Gram-negative bacteria, such as *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Flavobacterium columnare*, *Vibrio* spp., and *Pseudomonas* sp. Three of those bacteria are the same genera with our finding, such as *Vibrio*, *Flavobacterium*, and *Pseudomonas*. In another study, *Vibrio* sp. was found to be opportunistic as this bacterium was present in both healthy and diseased *Gracilaria changii* (Musa and Wei 2008). Our study showed that *Vibrio* was found only in diseased *Gracilaria* while *Flavobacterium* was isolated from both infected and healthy *Gracilaria*. Our results concur with previous studies demonstrating that *Vibrio* sp. was observed only in "ice-ice" infected seaweed (Largo 2002; Wang et al. 2008). Other studies isolated *Vibrio alginolyticus* and *Pseudomonas aeruginosa* from *Eucheuma spinosum* afflicted with "ice-ice" disease in the Kutuh Coastal Waters (Saraswati and Darmasetiyawana 2016).

Table 4 The growth of bacterial isolates from diseased *Gracilaria verrucosa* at different water temperatures. Optical density (OD) of bacterial culture was measured at 600 nm

Temperature (°C)	Bacterial isolates						
	Vibrio (G1)	*Flavobacterium* (G2)	*Chromobacterium* (G3)	*Vibrio* (G4)	*Pseudomonas* (G5)	*Chromobacterium* (G6)	*Achromobacter* (G7)
5	0.07	0.09	0.10	0.12	0.13	ng	ng
25	0.49	1.10	0.92	0.57	0.91	1.01	0.89
35	0.51	0.97	0.87	0.62	0.62	0.64	0.97
40	0.61	0.64	0.65	0.87	0.57	0.62	1.02

ng no growth

Table 5 Antibacterial activity of *Caulerpa racemosa* extracts against "ice-ice" bacteria from diseased *Gracilaria verrucosa*

Solvents for *Caulerpa* extraction	Inhibition zones (mean ± sd) (mm)*						
	Vibrio (G1)	*Flavobacterium* (G2)	*Chromobacterium* (G3)	*Vibrio* (G4)	*Pseudomonas* (G5)	*Chromobacterium* (G6)	*Achromobacter* (G7)
n-Hexane	$10.92^{b**} \pm 1.20$	$8.32^{b} \pm 1.06$	$6.00^{a} \pm 0.00$	$6.00^{a} \pm 0.00$	$7.65^{a} \pm 0.53$	$6.00^{a} \pm 0.00$	$6.00^{a} \pm 0.00$
Chloroform	$9.33^{b} \pm 0.12$	$9.28^{b} \pm 0.10$	$6.00^{a} \pm 0.00$	$6.00^{a} \pm 0.00$	$6.00^{a} \pm 0.00$	$6.00^{a} \pm 0.00$	$6.00^{a} \pm 0.00$
Ethyl acetate	$9.38^{b} \pm 0.99$	$8.28^{b} \pm 0.98$	$7.63^{ab} \pm 0.64$	$7.30^{a} \pm 0.70$	$7.73^{a} \pm 0.63$	$6.00^{a} \pm 0.00$	$6.00^{a} \pm 0.00$
MeOH	$6.00^{a} \pm 0.00$	$6.00^{a} \pm 0.00$	$21.45^{c} \pm 1.51$	$23.25^{c} \pm 1.06$	$20.32^{c} \pm 0.12$	$19.81^{c} \pm 1.50$	$22.20^{c} \pm 1.95$
MeOH:H_2O (1:1)	$6.00^{a} \pm 0.00$	$6.00^{a} \pm 0.00$	$8.53^{b} \pm 0.75$	$11.57^{b} \pm 1.64$	$13.92^{b} \pm 1.69$	$12.10^{b} \pm 2.05$	$6.00^{a} \pm 0.00$
Tetracycline 30 μg $disc^{-1}$	16.95 ± 0.74	9.27 ± 1.05	8.53 ± 0.54	13.12 ± 1.76	13.55 ± 0.53	8.15 ± 0.00	7.80 ± 0.61

*Inhibition zones include the disc diameter of 6 mm; the activity is categorized according to the diameter of the inhibition zone around the disc, ≥ 20 mm = highest, < 20–≥ 15 mm = high, < 15–≥ 10 mm = moderate, < 10 mm–> 6 mm = low, 6 mm = no activity

**Values (mean ± SD) within a column sharing a common superscript are not significantly different (one-way ANOVA, $p > 0.05$, $n = 3$)

Based on the criteria of Koch's postulates, bath exposures of healthy, uninfected *Gracilaria* to bacteria isolated from diseased *Gracilaria* showed symptoms of "ice-ice" disease at different days of incubation. The color and general appearance of *Gracilaria* thallus infected with the "ice-ice" bacteria were altered overtime. The onset of infection can occur when pathogenic bacteria attach to stressed thallus and develop on the cell wall by utilizing polysaccharide as a medium or carbon source (Largo et al. 1999). In our experiments, symptoms associated with "ice-ice" developed at different days of post exposure to the bacteria that ranged from 3 to 8 days of exposure. The red alga *K. alvarezii* inoculated with *P. issachenkonii* and *A. coralicida* showed symptoms after 3 days post infection (Syafitri et al. 2017) while other bacterial species can promote disease development in *K. alvarezii* at 5–15 h post infection (Achmad et al. 2016).

Although many epiphytes exist on the macroalgal surface as non-pathogenic symbionts (Correa and Craigie 1991), some secrete enzymes such as agarases and cellulases which are potentially harmful to *Gracilaria* (Polne-Fuller and Gibor 1987). As most marine bacteria exhibit agarolytic activity, it is hypothesized that they may be responsible for causing significant collapses in natural populations of *G. gracilis* with characteristic symptoms such as thallus-bleaching and subsequent fragmentation of macroalgal thalli (Jaffray 1998). Agar-lysing (agarolytic) bacteria include *Cytophaga*, *Flavobacterium*, *Alteromonas*, *Pseudomonas*, and *Vibrio* that were identified as potential infectious agents in *Gracilaria* (Agbo and Moss 1979; Jaffray 1998). Most bacterial isolates (30–39%) from *G. gracilis* are highly agarolytic suggesting that this characteristic may be responsible for causing macroalgal disease that is potentially associated with the proteolytic activity (Jaffray 1998). In our study, *Vibrio* and *Pseudomonas* that were isolated from diseased *Gracilaria* are agarolytic bacteria including *Flavobacterium* that was also found in both healthy and diseased *Gracilaria*. In the pathogenicity experiment, the thallus of *Gracilaria* infected with agar-liquefying bacteria, such as *Vibrio*, showed mucus production and frail or eroded. Unfortunately, the potential relationship between agarolytic capacity and bacterial pathogenicity in our study was not assessed.

Our study showed that "ice-ice" developed in *Gracilaria* in wide ranges of salinity and water temperature regimes. The results suggest that differences in bacterial composition associated with "ice-ice" disease are primarily dependent on water quality and other environmental conditions that may potentially affect the seaweed stress response to pathogens and disease severity (Largo et al. 1995a). Environmental stress due to water temperature, irradiance, and salinity can induce disease in cultivated seaweeds (Largo et al. 1995b). These environmental factors may have contributed to infections associated with "ice-ice" as observed in our study. Although *Vibrio* (G1) and *Chromobacterium* (G3) adapted to salinity based on optimal growth observed across various salinity gradients, growth was generally observed at 25 °C across all isolates, while another *Vibrio* (G4) species showed optimal growth at 40 °C. Higher temperatures can potentially increase the growth of agarolytic bacteria (Jaffray 1998). As the bacteria that we isolated from diseased *Gracilaria* are agarolytic, the higher temperature that we used may, in part, explain the optimum growth and pathogenicity ("ice-ice" symptoms) of bacteria at potentially due to agarose production and higher agarose enzyme activity.

Most of the bacteria that we observed in healthy *Gracilaria* belong to various genera including *Flavobacterium*, *Shigella*, *Corynebacterium*, *Serratia*, *Proteus*, and *Micrococcus*. However, we also found *Flavobacterium* sp. in "ice-ice"-infected *Gracilaria*. Previous study showed the isolation of *Flavobacterium* sp. and *Vibrio* sp. in healthy and diseased *K. alvarezii* (Aris 2011) suggesting that *Flavobacterium* in this and our study can be opportunistic in the presence of stressful environmental factors.

Seaweeds with secondary metabolites could be used as a natural antibacterial to prevent "ice-ice" diseases. Secondary metabolite compounds from seaweed have been shown to inhibit or kill pathogens that affect humans, cultured organisms, and land plants (Bansemir et al. 2006; Arunkumar et al. 2010; Zainuddin 2010b). In our study, we hypothesize that potential metabolite compounds could be associated with the antibacterial capacity of *Caulerpa* extracts based on growth inhibition of bacteria in "ice-ice" infected *Gracilaria*.

Although our study demonstrates that the five extracts of *C. racemosa* showed variable antibacterial activity against the pathogens associated with "ice-ice" disease in *Gracilaria*, the most inhibitory effect was observed in methanol extract suggesting the potential presence of bioactive compounds in the polar solvents in comparison to diverse compounds produced from *C. racemosa* in other geographic regions. Antibacterial compounds found in *C. racemosa* collected from the Red Sea (Hurghada, Egypt) include caulerpin, flexin, trifarin, or caulerpanyene (Salem et al. 2011). The crude extract of *C. racemosa* collected from the Makassar Strait contained phytochemical compounds, such as flavonoids, terpenoids, alkaloids, and phenols (Rusli et al. 2016). Chloroform extract of *C. racemosa* obtained from Moluccan waters contained steroids and terpenoids compounds (Sampulawa et al. 2017). The biologically active compounds of *C. racemosa* collected from Jepara Waters (Central Java) contained an alkaloid, tannin, phenol hydroquinone, flavonoid, steroid, and terpenoid (Utami 2014).

Since all isolated bacteria from *G. verrucosa* were Gram-negative bacteria, this finding indicated that methanolic extract of *C. racemosa* was effective in inhibiting the growth of Gram-negative bacteria. Previous studies showed that methanolic extract of *C. racemosa* obtained from Southeast Coast of India exhibited a broad spectrum of antibacterial activity against both Gram-negative and Gram-positive bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* (Kandhasamy and Arunachalam 2008).

The screening of antibacterial activities of methanolic extracts of *C. racemosa* and *C. prolifera* from Northern Cyprus showed that *C. racemosa* extract inhibited *S. aureus* and *E. coli* with inhibition zones of 15 and 12 mm diameter, respectively. While the methanolic extract of *C. prolifera* was only active against *E. coli* with 9.0 mm diameter of inhibition zone (Taskin et al. 2012). Selim et al. (2015) found that methanolic extract of *Caulerpa prolifera* from Suez Canal region, Egypt, was more effective against Gram-positive bacteria (*B. subtilis* and *S. aureus*) than Gram-negative bacteria, with a diameter of inhibition zones of ≥ 15 mm.

Bioactive compounds obtained from methanolic extracts could be alternatively used for bactericidal activity to help overcome the harmful impact of synthetic antibiotics on the environment. Polar solvents such as methanol can effectively extract organic compounds, some fats, and tannin where inhibitory activities against various bacterial species have been reported such as *S. aureus*, *P. aeruginosa*, and *E. coli* (Karthikeyan et al. 2015). A significant body of work demonstrates the antibacterial activity of methanol extract of *C. racemosa* against various bacterial species such as *P. aeruginosa* and other pathogenic bacteria (Nagaraj and Osborne 2014) and *Enterobacter aerogenes* (Etcherla and Narasimha Rao 2014). Previous studies indeed demonstrate the benefits of methanol extracted seaweed. For example, *Sargassum wightii*, *Padina tetrastomatica*, *Caulerpa racemosa*, *Agardhiella subulata*, and *Stoechospermum marginatum* that were tested against four pathogens (*E. coli*, *Klebsiella pneumonia*, *Salmonella typhi*, and *Vibrio cholera*) showed that the methanol extract of *C. racemosa* had the maximum antibacterial activity against *Vibrio cholerae* (Radhika et al. 2012).

In our experiment, methanolic extract of *C. racemosa* showed the highest activity against *Vibrio* (G4), *Achromobacter* (G7), *Chromobacterium* (G3), and *Pseudomonas* (G5) with diameter of inhibition zones of 23.25, 22.20, 21.45, and 20.32 mm, respectively. Through an antagonistic test, methanolic extracts of *C. racemosa* and *C. sertularioides* showed the highest activities against fish pathogen *Vibrio parahaemolyticus* with inhibition zones of 24.0 and 20.0 mm, respectively (Maheswaran et al. 2013). The methanol extract of *Caulerpa sertularioides* also demonstrated generally high antibacterial activity (Ernawati 2007) although variable activities were observed against the agents of shrimp vibriosis, *V. parahaemolyticus*, and *V. alginolyticus* (Esquer-Miranda et al. 2016). The lipidic extract (chloroform-methanol) of *Caulerpa cylindrica* showed the highest activity against *Vibrio* spp. (*Vibrio fischeri*, *V. inusitatus*, and *V. litoralis*) with the most upper inhibition zone of 0.9 cm (Stabili et al. 2016).

In addition to the methanolic extract of *C. racemosa*, the methanol-water extract in our study showed moderate activity against *Vibrio*, *Pseudomonas*, and *Chromobacterium* and no effect of non-polar and semi-polar extracts against all bacteria isolated from "ice-ice"-infected thalli. In contrast to our results, Raj et al. (2017) found that semi-polar extract (ethyl acetate) had a higher activity against all bacteria that they tested (*B. subtilis*, *Streptococcus pyogenes*, *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Vibrio cholerae*, *Shigella flexneri*, *Proteus mirabilis*, and *Proteus vulgaris*). The lipidic extract (chloroform-methanol) of *C. racemosa* collected from the coast of Tacloban City, Philippines, showed the highest antibacterial effectivity against *P. aeruginosa* (Seno et al. 2004). Chloroform extract of *C. racemosa* showed minimal inhibitory concentrations against *Streptococcus mitis*, *Bacillus anthracis*, and *B. cereus* at a level of 0.5% and 80% (Sampulawa et al. 2017). Ethanolic extract of *C. racemosa*

inhibited the growth of S. *typhimurium*, *S. enteritidis*, and *S. typhi* with the diameter of the inhibition zone of 1.5–15.5 mm (Utami 2014).

As shown in these studies together with our current results, methanol extracts of *C. racemosa* have great potential to inactivate various microorganisms including pathogens causing "ice-ice" diseases in seaweeds. We selected *Caulerpa* as an antibacterial agent is based on its ability to grow and to co-exist with *Gracilaria* and potentially with other seaweed species in brackish water environments in South Sulawesi. The potential release of secondary metabolites from *Caulerpa* into surrounding waters may protect *Gracilaria* from exposure to "ice-ice" pathogenic bacteria. Soaking *Gracilaria* seeds in *Caulerpa* extracts prior to pond culture is one practical application to diminish vulnerability to pathogens that cause "ice-ice" disease. Another method is via polyculture of *Caulerpa* with *Gracilaria verrucosa* in ponds.

Conclusion

Global climate change has been associated with increasing disease occurrence and spread across organisms living in marine waters, including seaweed (Harvell et al. 2009). Due to exposures to environmental stress, seaweed endurance is weakened increasing their vulnerability to opportunistic pathogens. Extreme environmental conditions have been suspected as a significant driver of "ice-ice" disease (Largo et al. 1995b). Opportunistic pathogens that co-exist as commensal to the seaweed may subsequently attack and cause disease in weakened host seaweeds due to exposure to extreme environmental stress (Largo 2002). Our study showed diverse bacteria from diseased *Gracilaria*, including one pathogen common to both healthy and infected seaweed, which caused disease in experimentally pathogen-exposed *G. verrucosa*. These results suggest that bacteria may initially maintain a commensal-symbiotic relationship with the seaweed host (Singh and Reddy 2014); however, environmental conditions can predispose the vulnerability of seaweeds to disease and potentially alter host-pathogen interaction from opportunistic to pathogenic.

South Sulawesi is the largest *Gracilaria* seaweed-producing region in Indonesia, and production is centered at Takalar Regency. Aquaculture of *Gracilaria* has a great potential to support the framework of an Indonesian seaweed industry. Increasing the demand for production of *Gracilaria* species for export and domestic agar industry in Indonesia requires finding a solution to improve the quantity and quality of *Gracilaria* species impacted mainly by "ice-ice" diseases. *Caulerpa racemosa* is another important marine resource in Indonesia that can be used as a natural antibiotic against "ice-ice" disease bacteria. The benefits of co-culturing *G. verrucosa* and *C. racemosa* including immersion of seaweed seed stocks in *Caulerpa* extracts before pond cultures are potential measures that warrant further investigation to help reduce the damage of "ice-ice" diseases in seaweed aquaculture. Seaweed disease research has mainly focused on discovering and identifying the agents associated with infections. This study will advance current knowledge on mitigating "ice-ice" diseases that impact seaweed species with great economic potential.

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