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Characterization and Identification of Bacteria Isolated from Seaweed *Gracilaria verrucosa* (Linn., 1758) Infected by Ice-ice

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Abstract Ice-ice disease in cultivated algae occurs due to pathogenic bacterial infections. Generally, ice-ice disease is characterized by whitening of the branches and initiated with the color changes of the thalli becomes transparent. This study was aimed to isolate and identify bacteria on seaweed *Gracilaria verrucosa* infected by ice-ice. Isolated bacteria was inoculated in Tryptic Soy Agar (TSA) and Thiosulphate Citrate Bile Sucrose (TCBS). Morphological and biochemical characterization of the bacterial isolates revealed eight species of bacteria were found in infected thalli including *Acinetobacter* sp., *Pseudomonas* sp., *Bacillus* sp., *Micrococcus* sp., *Corinoverm* sp., *Cytophaga* sp., *Vibrio mimicus* and *V. Campbelii*, but not all identified bacteria are pathogens on *Gracilaria verrucosa*. The pathogenic bacteria were *Acinetobacter* sp, *Pseudomonas* sp, *Bacillus* sp, *Cytophaga* sp. and *Vibrio* sp.
Keywords *Gracillaria verrucosa*; *Pathogenic bacteria; Ice-ice disease; Indonesia*

Introduction

Seaweed is an aquaculture commodity that has a high economic value. Production of seaweed *Gracilaria* was estimated to increase concurrent with the increasing demand of seaweed for food industry, pharmacy and cosmetics. *Gracilaria* has been cultivated worldwide. *Gracilaria verrucosa* and *G. gigas* are commonly cultured in brackish water ponds in Indonesia such as in South Sulawesi (Jeneponto, Takalar, Sinjai, Bulukumba, Wajo, Palopo, Bone and Maros Regencies), North Coast of Java (Serang, Tangerang, Bekasi, Karawang, Brebes, Pemalang, Tuban and Lamongan Regencies) and West Lombok (DKP, 2005).

The development of *Gracilaria* cultivation in ponds, in addition to increase the productivity and income of breeding farms, it is also expected to improve the quality of pond environment.. By cultivating the *Gracilaria* sp in brackish-water ponds the production of oxygen is increasing due to the increasing of the photosynthesis and the decreasing of the nitrite due to the nitrification process. In the other hand the production of the brackish-water ponds (milkfish and shrimp) can be increased. Seaweed is one of alternatives that can be used to improve the pond water environment, because it has ability to absorb nutrients, so that can be used as a biofilter, bioakumulator as well as biomonitoring pollution that occurs in aquaculture pond waters (Komarawidjaja, 2005). Thus, the causative agent of ice-ice disease can affect the sea weed as a host of bacteria.

One of the constraints experienced in the process of cultivation of seaweed is disease. The attack of ice-ice disease increased in line with pathogenic bacterial infection of the seaweed thalli. The condition is caused by the increasing of pathogenic bacteria activity in secrete virulence factors. The development of the pathogenic bacteria activity on seaweed thalli result in white patches on the thallus and gradually become brittle and eventually thallus fracture, resulting in decreased production of seaweed range of 70-100% (Vairappan *et al*. 2008).

The results of the identification on the seaweed *Kappaphycus alvarezii* infected by ice-ice disease is caused by a pathogenic bacteria namely; *Vibrio alginolyticus, Pseudomonas cepacia, Flavobacterium meningosepticum, Pseudomonas diminuta and*

Plesiomonas shigelloides (Aris, 2011). Types of bacteria that cause ice-ice disease are *Pseudomonas nigricaciens, Pseudomonas fluorescens, Vibrio granii, Bacilllus cercus and Vibrio agarliquefaciens* Yulianto (2002).

The mmanagement of good seaweed cultivation and free of ice-ice disease are the means in improving production technology of *G.verrucosa*. The disease needs to be studied through isolation, identification and control of pathogenic bacteria in *G. Verrucosa*. .

1 Materials and Methods

1.1 Place of Experimental Seaweed

Seaweed *Gracilaria verrucosa* was taken from brackishwater pond at Takalar Regency, South Sulawesi Province, Indonesia. Ice-ice infected thalli of *G. verrucosa* were washed with sterile water and placed into sterile plastic bags. Samples were then placed in the cool box and transported to laboratory. Isolation and biochemical characterization of bacteria were carried out in Marine Microbiology Laboratory, Faculty of Marine Sciences and Fisheries, Microbiology and Immunology Laboratory, Faculty of Medicine, Hasanuddin University, and Institute of Brackishwater Aquaculture Development, Maros.

1.2 Bacterial Isolation and Identification

Isolation and identification of bacteria was conducted from ice-ice infected branches of seaweed *Gracilaria verrucosa*.

One gram of ice-ice infected branches of *Gracilaria verrucosa* was homogenized in 9 ml sterile NaCl 0.9% and dilution series up to 10^{-6} were made up. One milliliter of aliquot from each dilution series was spread-plated onto tryptic soy agar (TSA) and thiosulphatecitrate bile sucrose (TCBS) agar plates and then incubated at 30℃ for 24 h. For purification and identification, differentially isolated colonies were randomly picked and streaked onto the TSA medium. Identification of bacteria was conducted based on the morphology of colonies and biochemical characterizations.

1.3 Biochemical Test

Identification of bacteria was done through the biochemical test to find out the behavior or characteristic of the bacteria according to Capuccino and Sherman, (1987), that included the Gram staining, OF, oxidase test, catalase test, SIM test (indole, motile, gas, H_2S), TSIA test (butt, slant, H_2S, gas), MR, VP, King A and King B. The nature or characteristic of bacteria by using medium TCBS (*Thoisulphate Citrate Bile Salt Sucrose Agar).*

Characterization is one of the activities carried out to observe the results of bacterial isolation (isolates). Characterization activities can be carried out based on the nature of cytology (cell form, movement or motility, Gram nature), morphology, and physiological nature. Test morphological natures include the natures of colonies, such as size, shape, color and edges, while the physiological nature such testing starch hydrolysis test, hydrolysis of fat, protein hydrolysis and catalase test. Test of Gram staining was conducted to group bacteria into 2 big groups according to its cell wall structure namely Gram-positive and Gram-negative bacteria. The staining was important stage in the bacteria characterization and identification (Lay, 1994). The catalase test was conducted to find out whether a bacteria has catalase enzyme in which the enzyme oxidized H_2O_2 (Cappuccino and Sherman, 1987).

2 Results

2.1 Bacterial Isolation and Identification

The results of the observation on the thalli showed that the gross signs of the thalli infected with ice-ice disease were initiated with translucent appearance of

The top of the thalli

The bottom of the thalli

The center of the thalli

All parts of the thalli

Figure 1. Gross signs of the thalli infected with ice-ice disease

the proximal, median or distal end of the thalli leading to whitening of the whole thalli (Figure 1). Santoso (2008) reported that ice ice infection in seaweed attacked the base of thallus, stem and the tip of thallus that caused the tissue become white and soft.

There were eight bacterial isolates with different colonial morphologies found in the infected thalli of the seaweed (Table 1).

Bacterial isolate was successfully isolated from the seaweed *G. verrucosa* infected by ice-ice. Morphology of bacteria colony was almost entirely circular, except at Gv.1 isolates. Almost all isolates were *entire* colony margins, except Gv.2, Gv.3 and Gv.4 which were *serrate*, elevation is generally *convex*, except Gv.4 and Gv.5 are *flat*. The color of the colony on Gv.1 and Gv.5 isolates where white, the isolates Gv.2 was yellow, Gv.4, Gv.7 and Gv.8, were cream, and orange on Gv.3 and Gv.6 isolates. These data were appropriate with funding of Cappucino and Sherman (1987), that bacterial colonies shape in general *circular, irreguler, filamentous, rhizoid*, the elevation was *raised, convex, flat, umbonate, crateriform.* Margin shapes *entire, undulute, filiform, curled and lobate.*

2.2 Characteristic of Bacteria

The result of gram staining of ice-ice bacteria isolates in *G. verrucosa* was obtained that bacteria isolates Gv.1, Gv.2, Gv.6, Gv.7, and Gv.8 belonged to gram-negative. While isolates Gv.3, Gv.4 and Gv.5 belonged to Gram-positive. The result of catalase test showed that isolates Gv.1, Gv.2, Gv.3 and Gv.4 showed positive catalase reaction, while isolates Gv.5 and Gv.6 showed negative reaction (Table 2).

The SIM test (gas, H_2S) and TSIA (gas, H_2S) showed that all isolates were negative. It was caused by the formation of H_2S marked by the black sign in the medium and the formation of gas marked by the rupture of medium at the lower end of the glass tube.

Biochemical characterization of the isolates showed the presence of *Acinetobacter* sp. at Gv.1, *Pseudomonas* sp. at Gv.2, *Bacillus* sp. at Gv.3, *Micrococcus* sp. at Gv.4, *Corinoverm* sp. at Gv.5, *Cytophaga* sp. at Gv.6 (Table 2), *Vibrio mimicus* at Gv.7 and *Vibrio Campbelii* at Gv.8 (Table 3) in the ice-ice disease infected thalli.

3 Discussion

3.1 *Acinetobacter* sp.

The results of staining obtained show that *Acinetobacter sp*. included in the gram-negative marked in red on the bacterial cell. The results of characteristic (Table 2) showed that surface of the colony is shiny, not motile, oxidase negative, does not produce pigment, catalase positive, and white.

Table 1. Colonial morphology of bacteria isolated from *Gracilaria verucosa* infected with ice-ice diseases.

Isolates	Media	Colonial morphology			
		Shape	Margin	Elevation	Colour
Gracilaria verrucosa 1 (Gv.1)	TSA	Irregular	Entire	Convex	White
Gracilaria verrucosa 2 (Gv.2)	TSA	Circular	Serrate	Convex	Yellow
Gracilaria verrucosa 3 (Gv.3)	TSA	Circular	Serrate	Convex	Cream
Gracilaria verrucosa 4 (Gv.4)	TSA	Circular	Serrate	Flat	Yellow
Gracilaria verrucosa 5 (Gv.5)	TSA	Circular	Entire	Flat	White
Gracilaria verrucosa 6 (Gv.6)	TSA	Circular	Entire	Convex	Orange
Gracilaria verrucosa 7 (Gv.7)	TCBS	Circular	Entire	Convex	Yellow
Gracilaria verrucosa 8 (Gv.8)	TCBS	Circular	Entire	Convex	Yellow

Table 2 Biochemical characterization of bacteria isolated from ice-ice infected seaweed *Gracilaria verrucosa* and inoculated in *Tryptic Soy Agar* (TSA).

Characteristic		***GV.1***	***Gv.2***	***Gv.3***	***Gv.4***	***Gv.5***	***Gv.6***
Gram'stain		-	-	+	+	+	-
OF		-	-	-	-	-	-
Oxidation		-	-	+	-	+	+
Catalase		+	+	+	+	-	-
SIM	Indol	-	+	-	-	-	-
	Motile	-	+	+	+	-	-
	Gas	-	-	-	-	-	-
	H_2S	-	-	-	-	-	-
TSIA	Butt	Red	Red	Red	Red	Red	Red
	Slant	Red	Red	Red	Red	Red	Red
	H_2S	-	-	-	-	-	-
	Gas	-	-	-	-	-	-
MR		-	+	-	-	-	-
VP		-	-	-	-	-	-
King A		-	+	+	+	-	+
King B		-	+	+	+	-	+
Genus		*Acinotebacter sp.*	*Pseudomonas sp*	*Bacillus sp*	*Micrococus sp*	*Corinoverm sp*	*Cythopaga sp*

Generally, *Acinetobacter* can be found in freshwater (Allen *et al*., 1983 *in* Austin and Austin, 1993) and marine environment (Austin, 1982 *in* Austin and Austin, 1993). These bacteria may have ability to cause diseases in aquatic organisms (Roald and Hastein, 1980 *in* Austin and Austin, 1993). `

3.2 *Pseudomonas* sp.

Pseudomonas sp. is a rod Gram negative bacteria, motile (some have polar flagellum) and catalase positive. Generally, colonial bacteria are yellow, facultative anaerobic or aerobic, can grow at 4–43 ℃. Most species have an optimum temperature around 30 ℃ (Buchanan and Gibbons, 1974).

Pseudomonas generally can be found in the soil, freshwater and seawater (Buchanan and Gibbons, 1974). These bacteria frequently isolated from plant and animal surfaces. Some members of these genera considered as a true pathogen in plants (Todar, 2013). *Pseudomonas* grows in injured tissues and produces toxins leading to the death of surrounding cells (Moorman, 2013). Some species such as *Pseudomonas. aeruginosa*, are opportunistic pathogen releasing extracellular protease that can infect host tissues (Buell *et al.* 2003).

Amiluddin (2007) identified five bacteria causing ice-ice disease on *K. alvarezii* including *Bacillus cereus,*

Table 3. Biochemical characterization of bacteria isolated from ice-ice infected seaweed *G.verrucosa* and inoculated in *Thiosulphate Citrate Bile Sucrose Agar* (TCBS).

Characteristic	Gv.7	Gv.8
Swarming	-	-
Luminescence	-	-
VP Test	-	-
Arginin Dihydrolase	-	-
Gas From Glucose	-	-
Growth at 400C		-
Lysine Decarboxylase	+	+
Pigmentation	-	-
Amylase	+	+
Sucrose	-	-
Indole	-	-
Ornithin Decarboxylase	+	+
Putrescine	-	-
Ethanol	-	-
Serine	-	-
Heptaniate	-	-
Xantine	-	-
Aminobutirate	-	-
Arabinose	-	-
Cellubiose	-	-
Glucuronate	-	-
Ketoglutarate	-	-
L-Alanine	-	+
Leucine	-	-
Propionate	-	-
Genus	Vibrio sp.	Vibrio sp.
Species	Vibrio mimicus	Vibrio campbelli

Vibrio granii, V. liquefaciens, P. nigricaciens, and P. fluorescens. Darmayanti *et al.* (2003) also isolated *Pseudomonas* sp., *Vibrio* sp. and *Aeromonas* sp. in healthy and infected thalli of cultured *K. alvarezii* in Pari Island. In addition, *Pseudomonas* sp. was found on cultivated seaweed in coastal water in Takalar Regency, South Sulawesi, Indonesia

3.3 *Bacillus* sp

Bacillus is a Gram negative bacteria, rod, motile (some non-motile), catalase positive and oxidase positive. *Bacillus* may form endospore, aerobic and facultative anaerobic (Feliatra, 2004). *Bacillus* can be isolated from soil and water including seawater. These bacteria can be found as a pathogen, opportunistic saprophytes. Some species produced extracellular enzymes that have ability to hydrolyze proteins and polysaccharides (Pelczar *et al.* 1975).

Bacillus sp. produced spores that are heat resistant and have ability to degrade xylan and carbohydrates (Cowan and Steel, 1973). *Bacillus* sp. has ability to grow at temperature more than 50 ℃ and less than 5 ℃, at high concentration of salt (> 10%), resistant to pasteurization, produce spores and has higher proteolytic activities than other microbes. *Bacillus* sp. resistant to environmental conditions like resistance of heat, acid and extreme salt content. Kinds of *Bacillus* produce extracellular enzymes which can hydrolyze protein and polysaccharide complex.

3.4 *Micrococcus* sp

Micrococcus is a Gram positive bacteria, sphere (cocci) with a size of 0.5–3.0 μm in diameter. It is aerobic and anaerobic and has an optimum temperature of 25–37℃ to grow. These bacteria can be found as a pathogen, opportunistic saprophytes and commensal organism. The role of this bacteria in the ground is as decomposers to decompose organic material into its composing constituent that can be used directly by plants.

3.5 *Cytophaga* sp

Cytophaga is a rod Gram negative bacteria. Some member within this group can cause diseases in fish. These bacteria can utilize cellulose and agar/chitin. This microbe can produce cell membranes containing endogluconases and periplasmic exogluconases that have activity in degrading cellulose. These bacteria can attack cellulose, but they cannot produce cellulose. This enzyme can only bind to the cell envelope , mix with mucus and is released during movement. This bacterium inhibits soil and water. The colony of these bacteria is yellow or orange and can be grown in the medium containing cellulose. Largo *et al.* (1995), who isolated bacteria in *Kappaphycus* obtained 10 bacteria strains *Cytophaga* sp. (*Cytophaga-Flavobactroium).*

3.6 *Vibrio* sp

Vibrio have a characteristic of curved rod Gram negative bacteria with a size of 1 – 3 × 0.4 – 0.6 μm,

non-forming spore and capsule, and motile with a polar flagella. This saprophytic microbe becomes pathogenic when the environment quality depleted. According to Kabata (1988), *Vibrio* is a pathogenic bacteria that commonly cause problems to the aquatic organisms. Largo et al. (1995) isolate *Vibrio* sp from the infected *K. alvarezii*. Recently, a study on ice-ice infected *K.alvarezii* revealed the presence of *V. alginolyticus*, *P. cepacia*, *P. diminuta*, *Plesiomonas shigelloides* and *Flavobacterium meningosepticum* in the infected thalli (Aris, 2011). Furthermore, Bockemihi *et al.* (1986), Chowdury *et al*. (1989) *in* Eaves (1994) reported that *Vibrio mimicus* was found in brackish water and fresh water.

Largo *et al*. (1999) explained the mechanism of the *Vibrio* infection in seaweed thallus was when the seaweed under stress. The *Vibrio* would breed in the cell wall by using polysaccharide as the media or carbon source. Furthermore, Lin *in* Yulianto (2009) explained that after 2-3 days, *Vibrio* entered the tissue until the medullary seam by pumping the caraginase enzyme then caused the thallus became pale/white and its tissue was soft and easily broken.

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