Identification of bacteria in seaweed (*Kapapphycus alvarezii)* affected by the ice-ice disease in Amal and Tanjung Tarakan waters, Indonesia

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Abstract. The ice-ice disease is caused by abiotic and biotic factors that appear macroscopically in the presence of white and soft parts of the infected seaweed. This disease is a major obstacle in the cultivation of seaweed (*Kappaphycus alvarezii*). The transmission is very fast and can cause the death of seaweed, resulting in considerable losses in cultivation. Ice-ice disease infection is seasonal, contagious and generally caused by a secondary bacterial infection. The purpose of this study was to identify bacteria that causes the ice-ice disease in *K. alvarezii*, during the growing season from August to October 2021. A total of five sampling points were used, including four stations in Amal waters and one in Tanjung Pasir Tarakan, Indonesia. Based on the results of the study, five genera of bacteria were identified in the infected samples collected from the five observation stations, namely *Vibrio* sp., *Pasteurella* sp., *Edwardsiella* sp., *Plesiomonas* sp., and *Chromobacterium* sp.
Key Words: bacteria, infection, water quality parameters.

Introduction. Seaweed, also known as algae, is one of the important fishery commodities in Indonesia because they have large export potential for processed products (Bixler & Porse 2011; Bindu & Levine 2011; Valderrama et al 2015). *Kappaphycus* spp. is a type of these aquatic plants widely cultivated in the aforementioned country as a source of carrageenan needed by food, cosmetic and pharmaceutical industries (Necas & Bartosikova 2013; Loureiro et al 2017). North Kalimantan Province (Kaltara) produced 489,699 tons of seaweed including *Kappaphycus alvarezii* in 2019. Nunukan produces the largest seaweed quantity, with an average annual rate of 337,122 tons, while the quantity in Tarakanand Bulungan is around 152,577 (BPS Kaltara 2020). The increase in seaweed cultivation has a positive correlation with the increasing income and number of farmers, which is supported by fairly good prices and markets.

Easy cultivation techniques and high market demand jointly encourage the government to promote seaweed as a strategic commodity in the fisheries revitalization program launched by the Ministry of Maritime Affairs and Fisheries. *K. alvarezii* is a seaweed type that is widely cultivated in the waters of Tarakan, Kaltara due to its relatively simple cultivation techniques. One of the problems affecting this plant is an ice-ice disease which has caused considerable losses to the producing community in Tarakan over the last 2 years. The spread of disease in the marine environment is expected to increase due to anthropogenic stress and has already affected marine biota, such as corals and seaweed (Jiasui et al 2021). Ice-ice disease is affected by abiotic and biotic factors characterized by the presence of white and soft parts of the talus infected seaweed. The occurrence of this disease is influenced by species, place, nutrients and seasons (Tahiluddin & Terzi 2021).

K. alvarezii is mainly affected by environmental changes, such as weak currents, very high temperatures, nutrient deficiencies and water clarity, leading to a decreased production (Webber et al 2012). Furthermore, it is often attacked by an ice-ice disease caused by certain pathogenic bacteria (Solis et al 2010; Ward et al 2019). This reduces the level of seaweed aquaculture ranging from 70 to 100% (Loureiro et al 2009). Cases of the ice-ice disease have been reported in several countries, namely the Philippines, Vietnam, Tanzania, Malaysia, and Indonesia (Hung et al 2009; Vairappan et al 2008; Msuya & Porter 2014).

Initial information about the ice-ice pathogens in seaweed is important. Studies have identified different bacteria genera and species as the initiators, but it is very rare to find a single pathogen of this disease. Hence, this study aimed to determine the bacteria types that attack *K. alvarezii* as the cause of ice-ice disease in Tarakan waters.

Material and Method

Sampling. The disease-attacked part of *K. alvarezii* (±50 g each) was collected from four stations in Amal waters and one in Tanjung Pasir Tarakan (Figures 1, 2). The samples were stored in clear glass bottles and placed in a cool box filled with ice, then transported to the Tarakan Fish Quarantine Center Laboratory.

Figure 1. Location of *Kappaphycus alvarezii* cultivated in the Amal and Tanjung Tarakan Waters, Indonesia (A); Samples of ice-ice disease attacked part of *Kappaphycus alvarezii* that was collected from 4 stations (B); In vitro isolation of bacteria from typical signs of ice-ice disease grown on TSA plates (C).

Figure 2. Thallus of *Kappaphycus alvarezii* cultivated in Amal and Tanjung Tarakan Waters, Indonesia showing typical signs of ice-ice disease.

Bacteria purification. Purification was conducted on the incubated samples where bacterial colonies were transferred using a loop needle based on the TSA media color difference and then incubated again for 24 hours in an incubator at 35ºC. Afterward, the samples were identified through biochemical tests which included the main tests.

Identification of bacteria. The pure cultures were grown on 3% TSA media in 6 Petri dishes for each isolate and incubated at 37°C for 24 hours. This was followed by gram staining and the main test, namely 3% KOH, 3% H_2O_2, and oxidase, the O/F Glucose and the Mio test were further performed (Dwyana & Gobel 2011). The results were matched with the literature (Barrow & Feltham 1993).

Results. The results of bacterial isolation in seaweed using 3% TSA media showed that the colonies had creamy and yellow color characteristics. The colony morphology included an irregular shape, entire edge, and convex elevation above the agar surface (Table 1). Colonies growing on 3% TSA media were observed visually after the incubation process for 24 hours and purification was continued based on the bacteria color, then they were incubated for 24 hours at a room temperature of 37°C. According to Mailoa & Setha (2011), their yellow color fermented sucrose and lowered the pH on TSA media.

Table 1

Bacterial isolation results on seaweed affected by ice-ice disease

No	*Isolate code*	*Colony forms*	*Elevation*	*Edge of colony*	*Color*
1.	RLA1	Irregular	Law convex	Entire	Cream
2.	RLA2	Irregular	Law convex	Entire	Yellow
3.	RLA3	Irregular	Law convex	Entire	Cream
4.	RLA4	Irregular	Law convex	Entire	Yellow
5.	RLTP	Irregular	Law convex	Entire	Yellow

The results showed several gram-negative bacteria that have a cellular morphology in form of rods/bacilli and cocci/round. The bacterial shape, called spherical ultra microcells in affected seaweed, changes from rods to cocci or spherical once their age increases and nutrient requirements for the culturing media reduce (Luhan et al 2015). By comparing the gram staining results to Achmad's et al (2016) study, the bacteria commonly found in seaweed samples are gram-negative with 2 cell forms, namely cocci and bacilli.

Four bacterial isolates obtained from the results were characterized in depth after colonies purification and the identity was determined with main and follow-up tests. Presumptive tests were carried out on each sample to facilitate the identification stage by knowing the cultured bacteria genus. These included the gram test which was used to determine whether the bacteria were gram-positive or negative, as well as catalase and oxidase tests as presented in Table 2.

Table 2

Gram positive, gram negative, catalase and oxidase tests and bacterial biochemical test for each sample

No	*Biochemical test*	*RL 1*	*RL 2*	*RL 3*	*RL 4*	*RLTP 1*
Presumptive test				Reaction		
1.	Gram test	-	-	-	-	+
2.	Catalase test	+	+	+	+	+
3.	Oxidase Test	+	+	+	+	+
Characteristic		RLA 1	RLA 2	RLA 3	RLA 4	RLTP 1
1.	Glucose medium	+	+	+	+	-
2.	Fermentative oxidation with paraffin	F	F	F	F	F
3.	Fermentative oxidation without paraffin	F	F	F	F	F
4.	Slanted TSIA	+	+	+	+	-
5.	Upright TSIA	-	-	-	-	-

No	Biochemical test	RL 1	RL 2	RL 3	RL 4	RLTP 1
6.	Gas TSIA	-	-	-	-	-
7.	H2S	-	-	-	-	-
8.	Motility	+	-	-	+	-
9.	Indole	-	-	-	-	-
10.	Ornithin	-	-	+	-	-
11.	Lysine	+	+	+	+	-
12.	Urea	-	-	-	-	-
13.	Citrate/Simon	-	-	-	-	-
14.	Arginine					
15.	MRVP MR Test	+	-	-	+	+
16.	MRVP VP Test	+	+	+ +	+	+
17.	Lactose	+	+	+	+	-
18.	Maltose	+	+	+	+	+
19.	Sorbitol	+	+	+	+	+
20.	Mannitol	+	+	+	+	-
21.	Sucrose	+	+	+	+	-
22.	Dulcitol	+	+	+	+	+
Organims identities		*Vibrio* sp.[1]	*Edwardsiella* sp.[3]	*Plesiomonas* sp.[3]	*Vibrio* sp.[2]	*Chromobacterium* sp.[3]

+ positive; - negative; F fermentative; [1]Barrow & Feltham (1993); [2]West et al (1986); [3]Holt et al (2000).

Water sampling was carried out at the same station during the collection of seaweed attacked by ice-ice disease. Moreover, water quality was observed as supporting data for the feasibility of seaweed cultivation, and to obtain an overview of ice-ice disease causes. This was used to determine the significant influence of water quality as a trigger for pathogen attacks on seaweed. According to Arisandi & Farid (2014), fluctuations in environmental parameters such as currents, temperature, and brightness at the cultivation site trigger stress and ice-ice occurrence in the aquatic plant for a long time. The results of water quality measurements can be seen in Table 3.

Table 3

The results of water quality measurements at each observation station

Parameters	*Unit*	*Station*				*Standard*
		1	*2*	*3*	*4*	
Total NH_3	mg L^{-1}	0.012	0.025	0.023	0.029	0.3
Phosphate	mg L^{-1}	0.007	0.001	0.014	0.017	0.015
Nitrate	mg L^{-1}	0.225	0.291	0.06	0.194	0.008

Discussion. Only observation results of the genus level were obtained based on all characteristics shown from biochemical tests, because to reach the bacterial species level, PCR and sequencing need to be performed. The seaweed samples identified consisted of 5 genera, namely *Vibrio* sp., *Pasteurella* sp., *Edwardsiella* sp., *Plesiomonas* sp., and *Chromobacterium* sp. The results also imply that the group of bacteria discovered in Tarakan waters was gram-negative. In several studies, gram-negative bacteria are known to be commonly found in seaweed and they cause ice-ice disease (Achmad et al 2016; Azizi et al 2018).

After the death of host cells, disease symptoms can be seen macroscopically. In general, bacteria from the genera *Vibrio*, *Pasteurella*, *Edwardsiella*, *Plesiomonas,* and *Chromobacterium*, produce cavities and wounds in seaweed thallus, which becomes brittle, leading to "rot disease". Bacterial attack of the *Vibrio* species on seaweed causes wounds and thallus bleaching (Vairappan et al 2010; Tahiluddin & Tarzi 2021). A disease with bleaching symptoms,also known as ice-ice, attacks the seaweed thallus tissue. The disease is initiated through the wounding process perpetrated by several types of fish and microbenthic. The wound generated becomes an area that is susceptible to bacterial colonies, hence opening up opportunities for pathogenic bacteria to enter and multiply. This condition leads to tissue damage and disease, then over time, it causes the thallus to become brittle and die (Ward et al 2021). Besides the macroscopic appearance, histology of the damaged thallus shows an association with necrosis or cell death (Riyaz et al 2021). Also, the seaweed cortex has an irregular shape, different from a healthy tissue cortex which is either round or oval (Maulani et al 2017).

Stress effect is a possible cause of the close relationship between seaweed and pathogens. The bacteria attack mechanism used is a form of algal-pathogenic interaction to cause new diseases in algae around the thallus area. The study of algal pathology is generally conducted from the association of one host-pathogen to another, which is triggered by environmental stress. The ice-ice disease is often described to be generated from complex interactions of the environment and pathogens (Ward et al 2021). The relationship between ice-ice disease-carrying agents, namely bacteria, and environmental stressors, such as low salinity and temperature elevation, has been studied. It was discovered that ice-ice symptoms are accelerated and exacerbated by the interaction of unstable cultivation environmental conditions (Azizi et al 2018). Diseases in seaweed are also caused by the interaction of several organisms such as bacteria, fungi, algal endophytes, and nematodes and worsened by increasing water temperature or extreme changes in salinity that cause stresses.

Pathogens cause disease by (i) invasion, (ii) multiplication within tissues, (iii) defense system resistance or attack, and (iv) infection of their hosts. Concerning these, pathogenicity and virulence are the two main things that are focused on and both are assumed to be intrinsic properties of microorganisms although pathogenicity is also recognized as absolute or not changing.

Nitrate measurement results are from 0.06 to 0.23 mg L^{-1} with an average value of 0.19 mg L^{-1}. The results of a research conducted by Nursidi et al (2017) showed that in the rainy season nitrate concentrations of 1.12 to 2.17 gave the best growth in *K. alvarezii*. This element is unstable in natural waters, in oxygen presence, and it is an intermediate form between ammonia and nitrite (nitrification) as well as between nitrate and nitrogen gas during denitrification (Effendi 2003). Almost all nitrate in marine waters comes from river flows produced by agricultural, aquacultural, industrial, and household or residential wastes, which indicates the level of fertility of the waters (Hamuna et al 2018). Naturally, the concentration of nitrogen nitrate in seawater is only a few mg L-1 and is one of the compounds that function in stimulating the growth of marine biota, so that it directly has an important role in primary production which has a close relationship with water fertility (Susana 2004). Wedemeyer (1996) recommends that nitrate levels in waters are less than 1.0 mg L^{-1}. Meanwhile, according to the Sea Water Quality Standard for Marine Biota, as stated in the Minister of Environment Decree No. 51 of 2004, the value is <0.008 mg L^{-1} (KLH 2004).

The laboratory analysis results for total ammonia parameters (NH^3-N) at the experimental site showed a concentration value between 0.012-0.029 mg L^{-1}. Ammonia (NH_3) and its salts are soluble in water. Besides, the element is generated in waters through the breakdown of organic nitrogen such as protein and urea. Other sources include inorganic nitrogen found in soil and water from the decomposition of organic matter such as dead aquatic plants & biota by microbes and fungi (Effendi 2003; Susana 2004). Sublethal effects of ammonia on fish greatly affect the diffusion process, causing a constriction of the gill surface. This decreases gas exchange speed, blood cells, levels of oxygen carried, physical resistance, and the immune system response against disease, leading to structural damage in various organs. Ammonia nitrogen levels in waters ought not to exceed 0.5 mg L^{-1} (Hamuna et al 2018). The Seawater Quality Standard for Marine Biota stipulates <0.3 mg L^{-1} for total ammonia in waters (KLH 2004). The concentration of ammonia at the observation stations is categorized as suitable for seaweed cultivation.

Phosphate concentration measurement during the study showed an average value of 0.013 mg L^{-1}, with a range of 0.011–0.024 mg L^{-1}, and was included in the very appropriate category (Hakanson & Bryhn 2008). Furthermore, it is a form of phosphorus that is used by plants, being a limiting factor for aquatic plants and algae which greatly affects water productivity levels. The presence of excessive phosphorus accompanied by nitrogen triggers explosive growth of algae in the waters, known as algae bloom (Susana 2004). Abundant algae form a layer on the water surface to inhibit oxygen and sunlight penetration. Phosphate levels stipulated in the Minister of Environment Decree No. 51 of 2004 concerning Seawater Quality Standards for Marine Biota are <0.015 mg L^{-1} (KLH 2004).

Conclusions. Based on the identification results for seaweed samples attacked the by ice-ice disease, five bacteria genera were found in Amal and Tanjung Tarakan waters, namely *Vibrio* sp., *Pasteurella* sp., *Edwardsiella* sp., *Plesiomonas* sp., and *Chromobacterium* sp. The results of the measurement of water quality parameters at the study site are still in the feasible category for seaweed cultivation.

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Conflict of interest. The authors declare no conflict of interest.

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