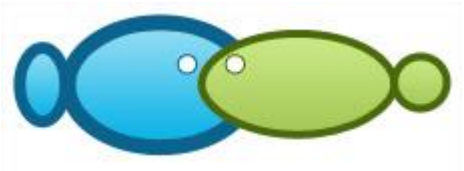

Isolation, culture characteristics and identification of bacteria from *Kappaphycus alvarezii* (Doty, 1987) attacked by ice-ice

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Abstract. The main problem in the efforts to increase the production of *Kappaphycus alvarezii* is the intensity of the ice-ice disease caused by pathogenic bacteria. This study aims to isolate and identify bacteria in the *Kappaphycus alvarezii* seaweed attacked by ice-ice and to determine the level of pathogenicity of the isolated bacteria. The isolated bacteria were grown on Sea Water Complete (SWC) and Thiosulphate Citrate Bile Salt Sucrose (TCBS) media. Identification was done by using API 20 E and API 20 NE analysis techniques. The results obtained showed that bacteria on *Kappaphycus alvarezii* attacked by ice-ice are *Vibrio alginolyticus, Pseudomonas cepacia, Flavobacterium meningosepticum, Pseudomonas diminuta* and *Plesiomonas shigelloides*, with *V. alginolyticus* being the most present.
Key Words: ice-ice disease, pathogenicity, seaweed, *Vibrio alginolyticus*.

Introduction. *Kappaphycus alvarezii* seaweed is one of the main commodities of aquaculture that is economically important and has been cultivated commercially (Bixler & Porse 2010; Bindu & Levine 2011; Valderrama et al 2015). *Kappaphycus alvarezii* cultivation has grown very rapidly with the demand increase for carrageenan in many countries (Barbosa de Barros-Barreto et al 2013; Hurtado et al 2015; Porse & Rudolph 2017; Adharini et al 2018; Hessami et al 2020). However, there are several problems encountered in the development of *Kappaphycus alvarezii* cultivation.

Disease attack in algae cultures is a major problem because it can harm aquaculture efforts resulting in decreased production due to seaweed death (Largo et al 1995). The main disease that attacks cultured seaweed is ice-ice (Mendoza et al 2002; Vairappan 2006; Solis et al 2010; Luhan et al 2014). Ice-ice causes a decrease in seaweed farming production ranging from 70 to 100% (Loureiro et al 2009). The disease was observed in seaweed producing countries such as the Philippines (Hurtado et al 2006), Vietnam (Hung et al 2008), Tanzania (Msuya & Porter 2014), Malaysia (Vairappan et al 2008), and Indonesia (Zainuddin et al 2019).

Bacteria play a role in the development of the ice-ice disease in seaweed cultivation (Largo et al 1995). Usually, the bacteria that cause ice-ice in *Kappaphycus alvarezii* are *Vibrio, Aeromonas, Cytophaga, Flavobacterium, Pseudomonas* and *Bacilllus* (Largo et al 1995; Yulianto 2002). This study aims to determine the bacteria that cause ice-ice in seaweed cultivated on Panggang Island, Indonesia. This study aims to determine the bacteria that cause ice-ice in seaweed cultivated on Panggang Island, Indonesia. This study also approached the level of pathogenicity of bacteria that were successfully isolated.

Material and Method

Sampling. Samples thallus of *K. alvarezii* attacked by ice-ice were collected from the waters of Panggang Island, DKI Jakarta Province, Indonesia (05°44'30.7''S;

106°36'04''E). Five thallus samples were collected randomly. The thallus surface was cleaned with 70% alcohol. Next, the thallus was placed into a sterile plastic bag. All samples were placed in a cool box and transported for further analysis in the laboratory. Bacterial analysis was carried out at the Fish Disease Laboratory, Bogor Sempur Freshwater Aquaculture Research Center.

Isolation and identification of bacteria. 1 g of thallus was crushed. From the scoured liquid, 0.1 mL was collected and spread on a petri dish containing Sea Water Complete (SWC) and TCBS (Thiosulphate Citrate Bile Salt Sucrose) agar. The results of bacterial isolation were then selected several times to obtain pure isolates. After pure bacterial isolates were obtained, the type of colony was evaluated. The identification of bacteria was based on physiological and biochemical characteristics, by the analysis of API 20 E and API NE 20 (Biomerieux 2009, 2010).

Bacterial dilution. The bacteria used for the pathogenicity test were obtained from the diseased seaweed. Each bacterial species was prepared to have 10^6 CFU mL^{-1} concentration. To obtain a bacterial concentration of 10^6 CFU mL^{-1}, bacteria was first cultured in SWC and TCBS media for 18-24 h with a shaker incubator at 29°C. 1 mL was taken from the bacterial culture and placed in an Ependorf tube to be centrifuged at 5000 rpm. Washing of bacteria was repeated three times with PBS (Phosphate Buffer Saline) solution. The yielded bacteria was diluted based on the volume of the infectious test container to reach 10^6 CFU mL^{-1}.

Pathogenicity test of bacterial isolates. *K. alvarezii* samples used were healthy seaweed with a wet weight of 35 g obtained from a *K. alvarezii* seaweed farm in Kupang, East Nusa Tenggara. Before the pathogenicity test, the seaweed was acclimatized for 3 days. This acclimatization was carried out to adapt seaweed to new environmental conditions. During acclimatization, 5 mL of liquid organic fertilizer (POC NASA) per the aquarium volume of 80 L was added. The test container was equipped with aeration. The bacteria used for the pathogenicity test were obtained from isolation from ice-ice infected seaweed. After obtaining bacterial concentrations of 10^6 CFU mL^{-1}, each type of bacteria was introduced in healthy seaweed containers by immersion. The parameters observed were changes in the morphology of the thallus, namely the color and weight of the thallus. Observations were made at 3-hour intervals for 3 days (72 hours).

Results and Discussion

Bacterial isolates from K. alvarezii. Bacterial colonies isolated from infected seaweed thallus can be seen in Figure 1.

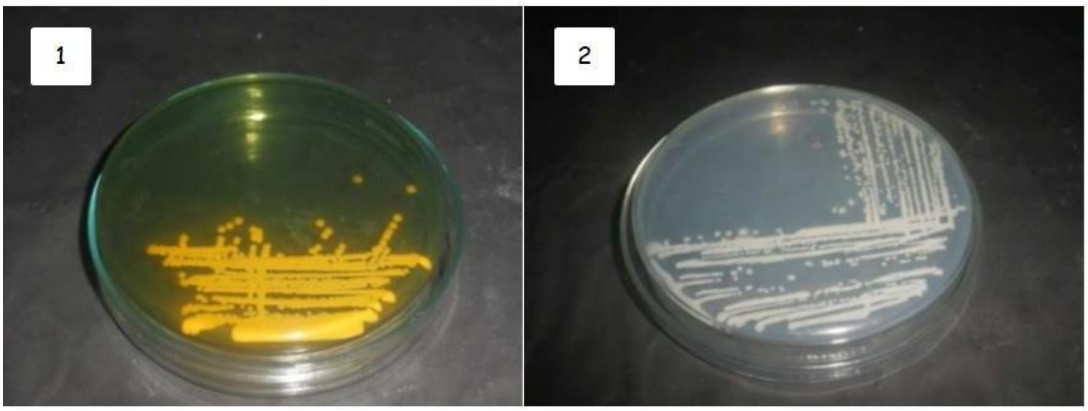

Figure 1. Colonies of bacteria successfully grown on TCBSA (1) and SWC (2).

Identification results obtained are *V. alginolyticus, P. cepacia, F. meningosepticum, P. diminuta, P. shigelloides*.

Pathogenicity of isolated bacteria. The pathogenicity test was conducted *in vitro*. The results of the pathogenicity test show that the transmission of disease agents in the healthy seaweed thallus caused changes in its morphology, especially color changes (Figure 2 and 3).

Figure 2. Thallus discoloration due to transmission of *F. meningosepticum, P. cepacia, P. diminuta and P. shigelloides* (d - day).

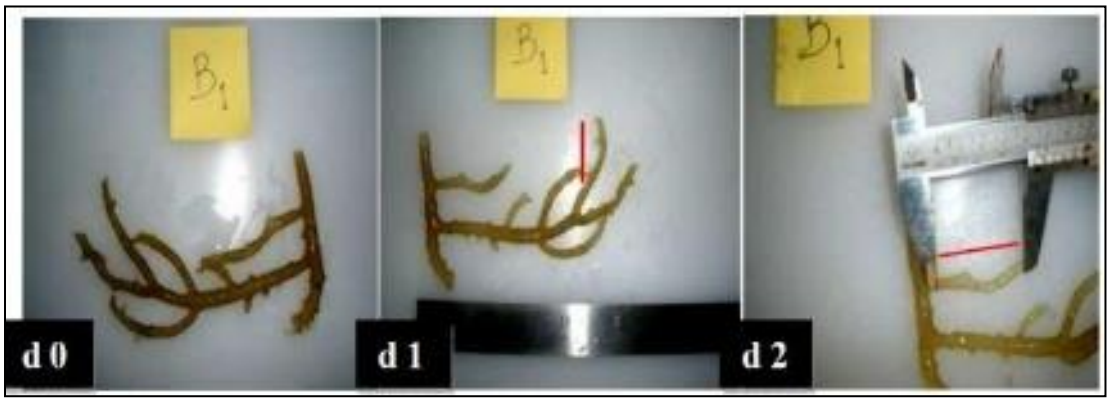

Figure 3. Thallus discoloration due to *V. alginolyticus*.

Changes in color and the appearance of discharge at the end of the thallus was followed by changes in wet weight (g). The observations on wet weight can be seen in Figure 4.

F. meningosepticum is rod-shaped and gram-positive bacteria. It is able to hydrolyze arginine, decarboxylase lysine, and cannot utilize citrate as a carbon source. It can deaminase tryptophan, have gelatin enzymes, oxidize glucose, mannitol, sorbitol. It does not produce enzymes in O/F test. It does not form nitrites, and does not acidify sucrose. Colonies of this bacteria are round, smooth, convex and yellowish in color. From the results of isolation and identification, these bacteria are present in the thallus and the cultivation media presented a yellow pigmented colony.

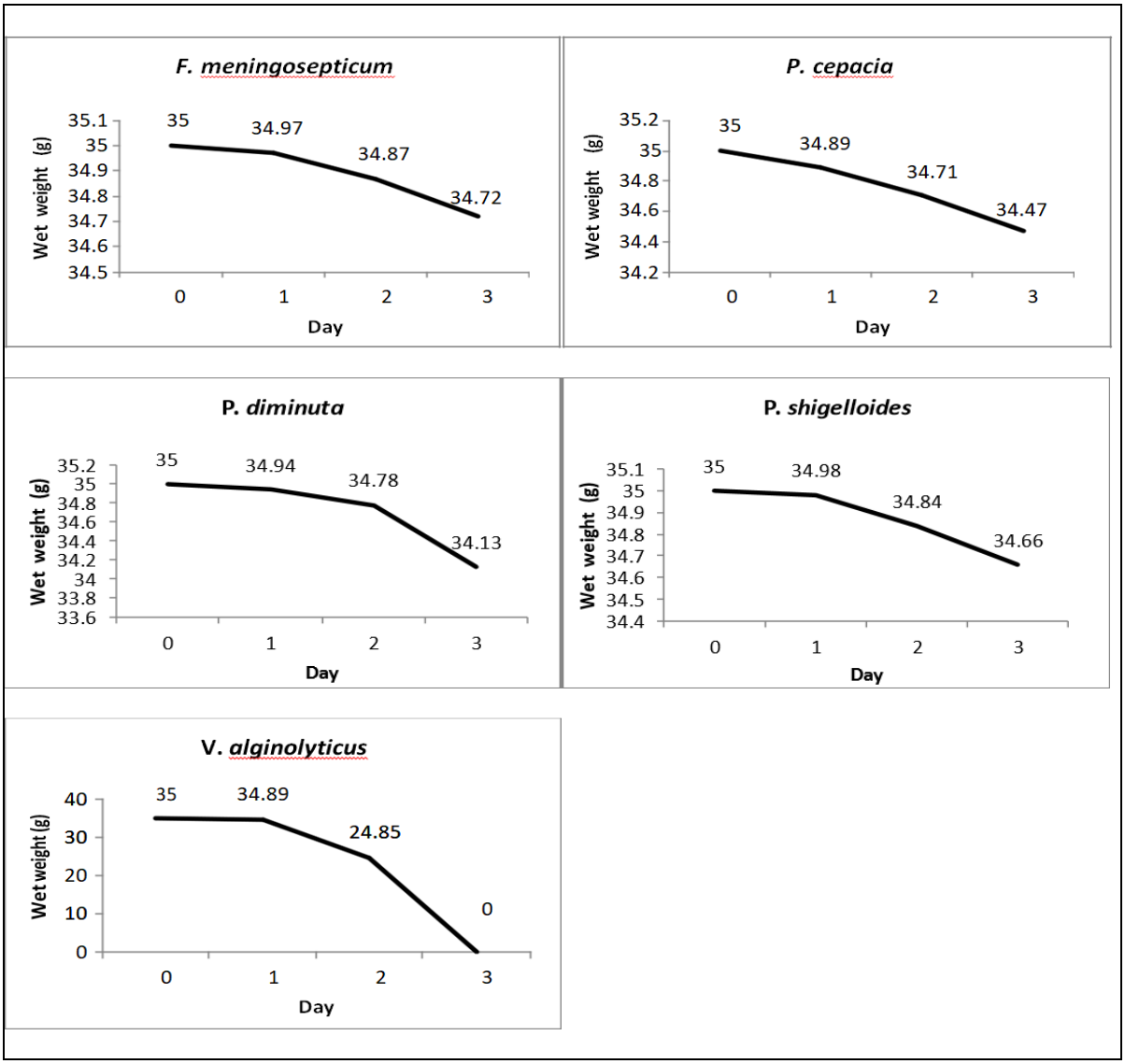

Figure 4. Wet weight of thallus of *Kappaphycus alvarezii* infected with bacteria causing ice-ice (72 hours).

F. meningosepticum is found in fresh water, seawater and soil (Ceyhan & Celik 2011). Bacterial species from the family Flavobacteriaceae are pathogenic bacteria that can enter aquaculture activities and cause a decrease in production (Loch & Faisal 2015). Flavobacterial disease was reported for the first time in 1922 (Davis 1922). Until now, it has attacked various fish such as *Ictiobus bubalus, Leopomis* spp., *Cyprinus carpio, Micropterus salmoides, M. dolomieu, Pomixis* spp., *Leopomis gulcosus, Perca flavescens, Morrinchrysopus, Salvelinus fontinalis, Pimephalus notatus, Ictalurus punctatus,* and *Ameiurus* spp. (Loch & Faisal 2015). Bacteria are also the agents causing ice-ice disease in *K. alvarezii* seaweed (Largo et al 1995) and were successfully isolated in *Gracilariopsis lemaneiformis* (Sun et al 2012) and *Gracilaria verrucosa* (Zainuddin et al 2019). Table 1 presents some characteristics of bacteria isolated from seaweed attacked by ice-ice.

Table 1

Characterization of bacteria isolated from seaweed thallus attacked by ice-ice

Results	*Identification*					
Morphology Colony shape	Round	Round	Round	Round		Round
Cell shape	Rod	Rod	Rod	Rod		Round
Media/Color	SWC/Yellow	SWC/White	SWC/White	SWC/White		TCBSA/Yellow
Gram	+	+	+	+		-
	Physiological and biochemical properties					
OF - O/F	+	+	+	+		-
Motility	-	+	+	+		+
	Other characteristics					
ONPG	-	-	+	-	NO3	+
ADH	+	+	+	+	TRP	+
LDC	+	+	+	+	GLU	+
ODC	+	+	-	-	ADH	-
CIT	+	-	-	-	URE	-
H2S	-	+	-	-	ESC	-
URE	-	+	-	-	GEL	+
TDA	+	+	+	+	PNPG	-
IND	+	+	+	+	GLU	+
VE	+	-	-	+	ARA	-
GEL	+	+	+	+	MNE	-
GLU	+	+	+	+	MAN	+
MAN	+	+	+	+	NAG	-
INO	-	-	-	-	MAL	-
SOR	-	-	+	-	GNT	-
RHA	-	-	-	-	CAP	-
SAC	+	+	+	+	ADI	-
MEL	-	-	+	-	MLT	+
MAY	-	-	+	-	CIT	-
ARA	-	-	+	-	PAC	-
OX	+	+	+	+	OX	+
MCC	+	+	+	+		
Bacterial similarity	*Flavobacterium meningosepticum*	*Pseudomonas cepacia*	*Pseudomonas diminuta*	*Plesiomonas shigelloides*	*Vibrio alginolyticus*	

Note: SWC - Sea Water Complete; TCBSA - Thiosulphate Citrate Bile Salt Sucrose Agar; OF - Glucose; O/F - fermentation-oxidation; ONPG - Ortho NitroPhenyl-ßDGalactopyranosidase; ADH - Arginine Dihydrolase; LDC - Lysine DeCarboxylase; ODC - Ornithine DeCaboxylase; CIT - Citrate Utilization; URE - Urease; TDA - Tryptophane DeAminase; IND - Indole production; VE - acetoin production; GEL - Gelatine; GLU - Glucose: fermentation/oxidation; MAN - Mannitol: fermentation/oxidation; INO - Inositol: fermentation/oxidation; SOR - Sorbitol: fermentation/oxidation; RHA - Rhamnose: fermentation/oxidation; SAC - Saccharose: fermentation/oxidation; MEL - Melibiose: fermentation/oxidation; MAY - Amygdalin: fermentation/oxidation; ARA - Arabinose : fermentation/oxidation; OX - Cytochrome-Oxidase; MCC - MacConkey medium growth; TRP - Tryptophan: Indole Reaction; ; ESC - Hydrolysis of esculine; MNE - assimilation of mannose; NAG - assimilation of N-acetyl glucosamine; MAL - maltose assimilation; GNT - assimilation of potassium-gluconate; CAP - capric acid assimilation; ADI - assimilation of adiptic acid; MLT -c malic acid assimilation; PAC - assimilation of phenylacetic acid; PNPG - Galactosidase.

The results of identification (Table 1) show that there are two species of the genus *Pseudomonas*, namely *P. cepacia*, and *P. diminuta*. *P. cepacia* is rod-shaped and gram-positive, able to hydrolyze arginine and lysine decarboxylase. It cannot decryboxylase ornithine, and is able to produce H_2S compounds, urea, tryptophane deaminase. It has gelatin enzymes, it oxidizes glucose, mannitol, sucrose, cytochrome. It is motile and can grow on MacConkey media. *P. cepacia* is a bacterial pathogen in plants (Jacobs et al 2008) and can be found in aquatic and soil environments (Zanetti et al 2000; Peeters et al 2016).

P. diminuta is rod-shaped and gram-positive, able to hydrolyze arginine. It can decarboxylase lysine and ornithine. It is unable to produce H_2S compounds, unable to produce urea or tryptophan deaminase. It is able to produce acetoin, possess gelatin

enzymes, oxidizes glucose, mannitol, sucrose compounds, cytochrome. It is motile. It can grow on MacConkey media. *P. diminuta* is a pathogenic bacterium found in aquatic and soil environments and attacks plants (Segers et al 1994; Lu et al 2013).

Pseudomonas genus is a disease-causing agent that attacks aquaculture activities both in freshwater and seawater (Blanco et al 2002; Toranzo et al 2005; Tripathy et al 2007; Li et al 2019). *Pseudomonas* bacteria have attacked various species of fish such as *Oreochromis niloticus* (Eissa et al 2010), *Epinephelus coioides* (Al-Marzouk 1999), *Oncorhynchus mykiss* (Seifzadeh & Rabbani-Khorasgani 2020)*, Salmo salar, S. trutta, Dicentrarchus labrax, Sparus aurata, Scophthalmus maximus, Anguilla anguilla, A. japonica,* and *Clupea harengus* (López-Romalde et al 2003). *Pseudomonas* bacteria have also been successfully isolated from seaweed (Rungprom et al 2008; Kumar et al 2013; Ravisankar et al 2013).

This research succeeded in isolating *P. shigelloides* from seaweed. *P. shigelloides* is rod-shaped and gram-positive. It has the ß-galactose enzyme, and is able to hydrolyze arginine, decarboxylase lysine, deaminase tryptophan, produce indole, have gelatin enzymes, oxidize glucose, mannitol, sorbitol, sucrose, melibiosa, ammonia, amigladladder, amigladine, arabinose. It is motile. It can grow on MacConkey media. *P. shigelloides* is a bacterium found in fresh, brackish, and marine waters (Janda et al 2016). *P. shigelloides* is found in various economically important aquaculture commodities such as oysters *Crassostrea gigas* and clam *Protothaca staminea* (Gu & Levin 2006), *O. mykiss* (Huber et al 2004), *Hippoglossus hippoglossus* (Herrera et al 2006), *Hypophthalmichthys molitrix* (Behera et al 2018), *O. niloticus* (Nadirah et al 2012), *Litopenaeus vannamei* (Govahi et al 2014), blue crab *Callinectes sapidus* (Marshall et al 1996).

V. alginolyticus is round and gram negative. *V. alginolyticus* is able to utilize nitrate, glucose, tryptophan, mannitol, have gelatin enzymes, oxidize cytochrome, and are motile. *V. alginolyticus* is one of the bacteria of the genus *Vibrio* in the marine, coastal and estuary environments (Narracci et al 2013). *V. alginolyticus* is a disease-causing agent in the cultivation of *E. coioides* (Cui et al 2010) and Asian seabass *Lates calcarifer* (Sharma et al 2013), as well as other important aquaculture commodities such as clam *Ruditapes decussatus* (Gómez-León et al 2005) and *Perna perna* (Bronzato et al 2018), *L. vannamei* (Cheng et al 2005; Dashtiannasab et al 2012), seahorse *Hippocampus kuda* (Adiputra et al 2005). *V. alginolyticus* was found to be associated with *K. alvarezii* (Azizi et al 2018) and *Eucheuma spinosum* (Saraswati & Darmasetiyawana 2016). The V*ibrio* genus is also found in seaweed *Sargassum myriocystum* (Chakraborty et al 2016) and *G. verrucosa* (Zainuddin et al 2019).

The observations on pathogenicity (Figure 4) show that *V. alginolyticus* had the highest level of pathogenicity to healthy seaweed thallus. Each bacterial soaking treatment showed a decrease in wet weight. *V. alginolyticus* infection produced the highest wet weight reduction. This infection resulted in the seaweed thallus dying on the second day.

Transmission of disease agents in healthy seaweed thallus causes changes in color. The discoloration in this study did not differ for each bacterial treatment (Figure 2), except for *V. alginolyticus*, which showed a change in the color of the thallus to white (chlorosis) (Figure 3).

Changes in seaweed thallus were seen on the first day after the bacterial infection with *V. alginolyticus,* characterized by increased mucus production covering the thallus, discoloration, wilting and the appearance of black spots around the thallus branches. Entering the second day, the black spots turned transparent because of the loss of pigment. The discoloration of the thallus represents the beginning of the bleaching phenomenon. This incident is in line with the observations of Largo et al (1995), Ask et al (2003) and (Sulu et al 2003). Discoloration (depigmentation) and reduction in wet weight occur due to bacterial infection of the pathogen that causes the ice-ice disease in seaweed thallus. These changes are increasing along with an increase in the time of bacterial activity in secreting virulence factors to the host. According to Hurd et al (1994) and Largo et al (1995), ice-ice attacks intensified with the time of pathogenic bacterial infection in seaweed thallus, resulting in failure of seaweed cultivation.

Conclusions. Bacteria species that were successfully isolated from the thallus of *K. alvarezii* attacked by ice-ice disease in the management of seaweed cultivation in the waters of Panggang Island were *V. alginolyticus, P. cepacia, F. meningosepticum, P. diminuta and P. shigelloides. V. alginolyticus* shows the highest level of pathogenicity when compared with other bacteria.

Conflict of Interest. The authors declare that there is no conflict of interest.

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Received: 10 July 2020. Accepted: 23 October 2020. Published online: 30 December 2021.
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How to cite this article:
Aris M., Tamrin, Labenua R., 2021 Isolation, culture characteristics and identification of bacteria from *Kappaphycus alvarezii* (Doty, 1987) attacked by ice-ice. AACL Bioflux 14(6):3806-3816.