Molecular identification of new bacterial causative agent of ice-ice disease on seaweed *Kappaphycus alvarezii*

Marlina Achmad, Alimuddin Alimuddin, Utut Widyastuti, Sukenda Sukenda, Emma Suryanti, Enang Harris

Background. Ice-ice disease is still a big challenge for seaweed farming that is characterized with "bleaching" symptom. Bacteria are suspected as cause of ice-ice disease on seaweed Kappaphycus alvarezii. The 16S rRNA gene sequencing is current technique used for bacterial phylogeny and taxonomy studies. This study was aimed to identify bacterial onset of ice-ice disease on K. alvarezii.

Methods. Eight sequenced isolates from Indonesia were identified and characterized by biochemical tests and sequenced by 16S rRNA gene as target. The isolates sequence compared to the strains of bacteria from GenBank. DNA sequences are analyzed with ClustalW program and phylogeny were performed using the result generated by Mega v.5. The micropropagules (2-4 cm) was soaked in seawater containing 106 cfu/ml of bacteria to determine the pathogenicity. Onset of ice-ice symptoms was visually observed every day. Histology are analyzed to show tissue of micropropagule post-infection by bacteria.

Results. Identification of bacteria employed biochemical tests and 16 SrRNA gene sequence analysis. The results reveal eight species of bacteria, namely: Shewanella haliotis strain DW01, 2 Vibrio alginolyticus strain ATCC 17749, Stenotrophomonas maltophilia strain IAM 12323, Arthrobacter nicotiannae strain DSM 20123, Pseudomonas aeruginosa strain SNP0614, Ochrobactrum anthropic strain ATCC 49188, Catenococcus thiocycli strain TG 5-3 and Bacillus subtilis subsp.spizizenii strain ATCC 6633. In term of groups, bacteria S. haliotis, V. alginolyticus, S. maltophilia, P. aeruginosa and C. thiocycli are the in Gammaproteobacteria group and O. anthropi is in the Alphaproteobacteria group, A. nicotianae and B. subtilis is in the of Proteobacteria group both of are Actinobacteria and Firmicutes group Low GC respectively. The results showed that the fastest onset of ice-ice symptoms was caused by S. maltophilia (five hours post-infection), while the slowest it was caused by V. alginolyticus (44 hours post-infection). Other bacteria give rise to ice-ice symptoms for 15-21 hours post-infection. Thus, S. maltophilia also showed number of bleaching spot of higher than others. However, V. alginolyticus showed increased width of bleaching 2.29 mm2 greater than S. maltophilia.

Discussion**.** Bacteria *S. haliotis* and *V. alginolyticus* were found in healthy thallus, while others were found in bleaching thallus. Indicator of bleaching this is the first study shows *S. maltophilia* association to ice-ice disease on *K. alvarezii*. Interaction of temperature with different disease-causing pathogens ice-ice on seaweed is unreported and it is thought-provoking to examine in further research. In addition, the bacteria isolated in this study is potentially used to hold the assembly seaweed ice-ice disease through the challenge test.

Molecular Identification of New Bacterial Causative Agent of Ice-Ice Disease on Seaweed *Kappaphycus alvarezii*

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ABSTRACT

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Keywords: bacteria, ice-ice, *Kappaphycus alvarezii*, micropropagule, pathogenicity

Introduction

Seaweed, especially, *Kappaphycus alvarezii* is an important commodity for aquaculture in Indonesia. The *K. alvarezii* is a source of kappa-carrageenan used for foods, cosmetics, pharmaceuticals and photographies (Yu et al. 2002).

Ice-ice disease is still a big challenge for seaweed farming, for certain season. This disease is characterized with white symptom “bleaching or whitening” on upper tissue of the seaweed and it can be caused from different opportunistic pathogenic bacteria (OPB) (Largo et al. 1995a;

Vairappan et al. 2001; Aris 2011). The difference of the source bacterial isolate can be cause variation species different founded it.

Pathogenicity test against disease-free seaweed has not been done. The OPB existed in *K. alvarezii* thallus are *Vibrio* sp. (P11) and *Cytophaga* sp. (P25) (Largo et al. 1999) and caused pathogenic in the aseptic *K. alvarezii*. *K. alvarezii* disease-free can be produced through tissue culture. The success of tissue culture has been reported by Sulistiani and Yani (2014). Maintaining callus for two months can generate mikropropagule (Reddy et al., 2008). The mikropropagule is potentially used to test candidate disease-causing bacterial pathogenicity ice-ice.

The development of bacterial species identification techniques is rapidly grew. The 16S rRNA gene sequencing is current technique used for bacterial phylogeny and taxonomy studies. There are three main reasons using this gene: a) exist in almost every bacterium, b) steady function over time has not changed, suggesting that random change of sequence is more accurate and c) the 16S rRNA gene (1,500 bp) is large enough for an information purpose (Patel 2001; Widow and Abott 2007). The aim of this research was to identify biochemical, pathogenic and molecular bacterial candidate cause ice-ice disease through 16S rRNA gene sequencing as the target.

Materials and Methods

Collection of seaweed sample

K. alvarezii was collected from seaweed farming at Bulukumba Beach, South Sulawesi, Indonesia (Figure 1) on January 2015, salinity and temperature ranges of location were 30-32 ppt and 28-29^{o}C, respectively. Samples were chilled in a cooler box and transported to the laboratory within twelve hours.

Isolation and identification of bacteria using biochemical test

Thallus of *K. alvarezii* with and without ice-ice disease were used as sample for isolation of bacteria. One gram of thallus crushed to produce liquid, then 0.1 ml of the liquid distributed into petridishes containing solid media of sea water complex (SWC) consists of 5 g bacto-peptone, 5 g yeast extract, 3 ml glycerol, 250 ml distilled water, 750 ml sterile seawater and 20 g bactoagar. The bacteria were cultured at 28°C for 24 hours. Isolates were scratched several times to obtain pure isolates. Then, colony types were biochemically evaluated and identified.

Extraction of DNA genome

Bacterial genomic DNA was extracted using Presto ™ mini kit gDNA bacteria (Geneaid, Taiwan). Gram-negative bacteria cell were lysed using proteinase K-contained GT buffer (20 mL) and incubated at 60°C for 10 minutes. Gram-positive bacteria cell were lysed using lysozyme-contained GT buffer (4 mg/ml) and incubated at 37°C for 30 minutes. Then, it was added proteinase K (20 mL) and incubated at 60°C for 10 minutes.

DNA was dissolved using 100 mL elution buffer. DNA concentration was measured and confirmed using Genquant (Teare et al. 1997) and separated using 1% agarose gel electrophoresis. Visualization of DNA was obtained using stained ultraviolet light and dye ethidium bromide stain. Finally, DNA solution were stored at -20°C until next processing.

PCR amplification and nucleotide analysis

Amplification of 16S rRNA gene was performed using a universal primer (Marchesi et al. 1998), which is 63F (5'-CAG GCC CAC TAA GTC ATG CAA-3 ') and 1387R (5'-GGG CGG GTA WGT CAA GGC-3'). The PCR program used pre-denaturation of 94°C for two minutes, 30 cycles of amplification at a denaturation of 92°C for 30 seconds, annealing of 55°C for 30 seconds, extension of 72°C for 1 minute and a final extension of 75°C for 20 minutes. PCR results were separated using 1% agarose gel electrophoresis to confirm the amplification

product. Then, PCR results were purified using a PCR clean-up and gel extraction (Geneaid), then products were sequenced using ABI3730XL machine.

Sequencing results were edited using Bioedit v 7.4 program (Tamura et al. 2011; Azanza et al. 2013). Later, sequences were analyzed using the basic local alignment search tool (Altschul et al. 1990; Azanza et al. 2013) for identification of bacteria isolates and related other organisms sequences. 16S rRNA sequences were associated to bacteria strain in the Gene Bank.

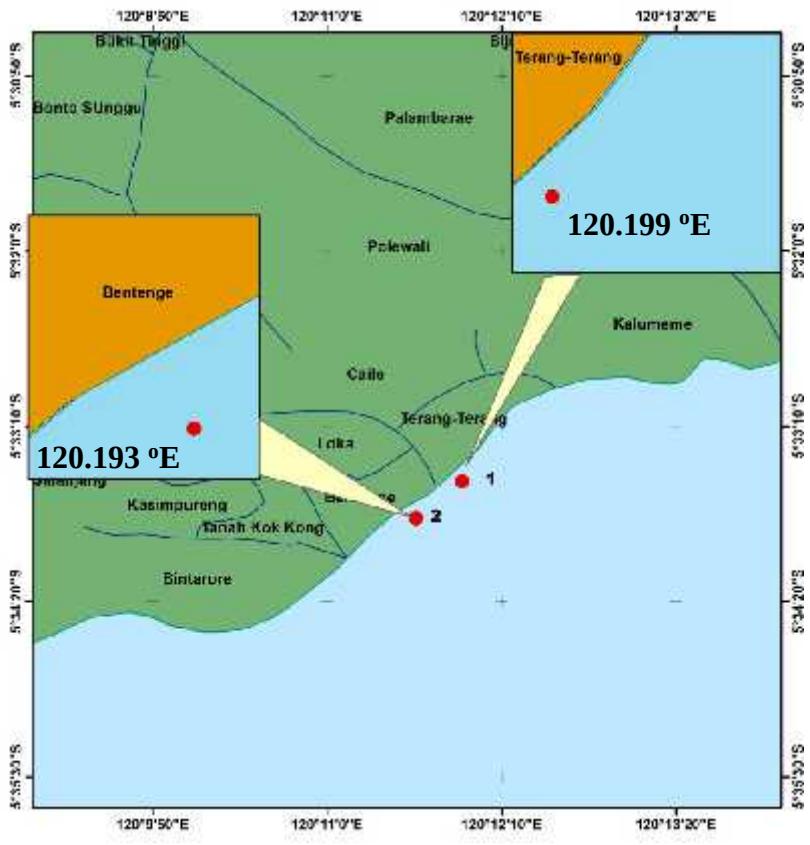

Figure 1. Sampling sites in South Sulawesi, Indonesia.

Phylogeny analysis

The results of DNA sequences, previously be aligned (alignment) with nucleotide sequences, were performed using ClustalW program. Phylogeny analysis was made using the MEGA v.5, 0 program (Azanza et al. 2013) with sample repetition bootstrap 1000 times.

Pathogenicity test

Pathogenicity test was conducted using micropropagule samples produced by SEAMEO Biotrop. The micropropagule regenerates embryogenic callus and it is produced from tissue culture technology (Sulistiani and Yani 2014). Before pathogenicity test, according to Vairappan et al. (2001), media and micropropagule are held for three days to ensure free contamination with microorganisms. The bacterial suspension (10^6 cfu/ml) of 0.3 ml was inserted to micropropagule media at a bottle chicken. Each bottle was consisted two parts of 2-4 cm MP length. Each type of bacteria is made for three bottles. Bacteria and MP inoculations were aseptically performed at laminar air flow (LAF). Then It was shaken using a shaker bottle, 100 rpm speed and incubated for 7 days at 20°C, 14:10 hours (light: dark) photoperiod. Daily MP conditions were observed to determine the initial appearance of ice-ice disease symptoms. Histological MP was made according to Aris (2011) with: twice fixation in neutral buffered formalin (BNF), then five times of dehydration in different alcohol (70, 90 and 100%.), then, 2-3 times of cleaning in toluene, impregnation liquid paraffin and planting in paraffin for final dyeing preparations. Preparates were stained using hematoxylin and eosin stains. Data were analyzed descriptively.

RESULT

Identification of bacteria isolates

Algal-surface bacteria were successfully removed and cultured in SWC media. Table 1. shows biochemical and physiological characteristics of bacteria isolates. There are six bacteria species were group of Gram-negative bacteria, such as *Shewanella* sp., *Vibrio* sp., *Stenotrophomonas* sp., *Pseudomonas* sp. and *Ochrobactrum* sp., while two isolates were group of Gram-positive bacteria, namely *Arthrobacter* sp. and *Bacillus* sp. These bacteria belong to characteristics of rod shape and motile.

Phylogenetic diversity of bacteria isolates

Nucleotide sequences of 16S rRNA gene analyses were correlated with biochemical characters. The bacteria were identified to species level among strains of *Shewanella haliotis* DW01, *Vibrio alginolyticus* strain ATCC 17749, *Stenotrophomonas maltophilia* strain IAM 12323, *Pseudomonas aeruginosa* strain SNP 0614, *Ochrobactrum anthropi* strain ATCC 49188, *Arthrobacter nicotianae* strain DSM 20123 and *Bacillus subtilis* subsp. spizizenii strain ATCC 6633. The similarity of 16S rRNA gene nucleotide sequence between bacterial isolates with Gene Bank database are more than 90% (see Table 2.).

Table 1. Biochemical and physiological characteristics of bacteria isolates in seaweed *K. alvarezii*

Characteristics	**Shw**	**Vib**	**Ste**	**Arb**	**Psd**	**Ocb**	**Cac**	**Bal**
Gram stain	-	-	-	+	-	-	-	+
Cell shape	r	r	r	c	r	r	c	r
Colony								
Form								
Circular	+	-	-	+	-	-	+	-
Irregular	-	+	+	-	+	+	-	+
Margin								
Entire	+	-	-	+	-	-	+	-
Undulate	-	-	+	-	-	+	-	-
Filamentous	-	-	-	-	-	-	-	+
Curled	-	+	-	-	+	-	-	-
SIM	+	+	+	+	+	+	-	+
Catalase	+	+	+	-	+	+	+	+
Oxidase	-	+	-	-	-	-	-	+
OF test	-	O	-	-	O	-	-	-

Notes: c: Coccus; r: Rod; O: Oxidation; F: Fermentation; +: Positive; −: Negative. Shw: *Shewanella* sp.; Vib: *Vibrio* sp., Ste: *Stenotrophomonas* sp., Arb: *Arthrobacter* sp., Psd: *Pseudomonas* sp., Ocb: *Ochrobactrum* sp., Ctc: *Catenococcus* sp., Bal: *Bacillus* sp.

Table 2. The 16S rRNA gene nucleotide sequences of bacterial isolates on ice-ice disease at seaweed *K. alvarezii* with similarity percentage to Gene Bank database.

Isolate	Bacteria	Identity %	Query/ Subject	Accession Number
NA1	*Shewanella haliotis* strain DW01	98%	1197/1227	NR_117770.1
NB2	*Vibrio alginolyticus* strain ATCC 17749	97%	1196/1229	NR_117895.1
TA8	*Stenotrophomonas maltophilia* strain IAM 12323	97%	1102/1136	NR_041577.1
TA9	*Arthrobacter nicotianae* strain DSM 20123	96%	1183/1231	NR_026190.1
TA2	*Pseudomonas aeruginosa* strain SNP0614	97%	1277/1316	NR_118644.1
TA4	*Ochrobactrum anthropi* strain ATCC 49188	95%	1075/1134	NR_074243.1
UB3	*Catenococcus thiocycli* strain TG 5-3	98%	1195/1220	NR_104870.1
UA5	*Bacillus subtilis subsp. spizizenii* strain ATCC 6633	95%	1234/1294	NR_118486.1

Notes: NA1 as bacteria were isolated from healthy thallus (N), site of sampling (A), number of sample(1); TA8 as bacteria were isolated from surface of the ice-ice thallus (T), site of sampling (A), number of sample (8); UB3 as bacteria were isolated from tip of the ice-ice thallus (U), site location (B), number of sample (3)

This study showed that molecular identification of bacteria isolates identified interesting species that have not been reported from previous study. The bacteria species showed a high similarity accordance to Gene Bank database and supported by bootstrap values for phylogeny tree (Figure 2). Majority of the bacteria isolates were group of *Proteobacteria* division, gamma subdivision, i.e *Shewanella haliotis* DW01, *Vibrio alginolyticus* strain ATCC 17749, *Stenotrophomonas maltophilia* strain IAM 12323, *Pseudomonas aeruginosa* strain SNP 0614 and *Catenococcus thiocycli* strain TG 5-3 whereas alpha subdivision is *O. anthropi* strain ATCC 49188. However, there are two species of bacteria outside the division *Proteobacteria* both of are *B. subtilis* subsp. spizizenii strain ATCC 6633 (*Firmicutes*) and *A. nicotiannae* strain DSM 20123 (*Actinobacteria*).

Identified putative species from this study include bacteria related to *Stenotrophomonas* sp., *Pseudomonas* sp., *Shewanella* sp., *Ochrobactrum* sp., *Bacillus* sp. *Athrobacter* sp., and 2 species allied to *Vibrio* sp.

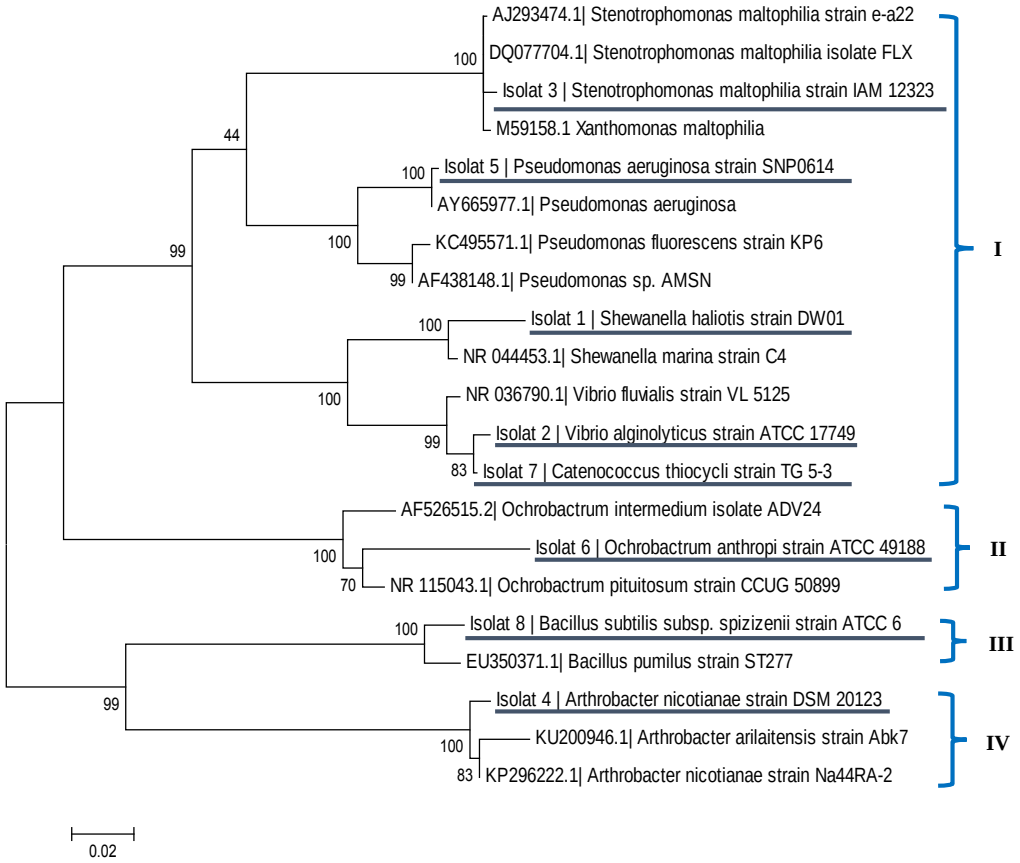

Figure 2. A maximum likelihood tree based on the 16S rRNA gene fragments of the bacterial isolates with bootstrap supports generated with 1000 times resampling. Accession numbers precede the identity of the reference sequences. Based on phylogeny tree, bacteria ice-ice disease on seaweed from Indonesia divided into four classification: I. Gammaproteobacteria; II. Alphaproteobacteria; III. Firmicutes; IV. Actinobacteria

Pathogenicity test

Pathogenicity test showed that when initial symptoms are different from bacterial bleaching, see Figure 3. The bacteria cause rapid bleaching is *S. maltophilia* (5 hourspostinfection, hpi), followed by *O. Anthropi* (15 hpi) and *P. aeruginosa* at 17 hpi. The bacteria *S. maltophilia* showed number of spots and wide of bleaching, which are 17 spots and 1.87 mm^2 respectively, higher compared to other isolates (see Figure 4).

On the other hand, *Stenotrophomonas maltophilia* isolate from disease thallus showed the fastest time of beginning symptom within five hours which total spot and wide bleaching significantly affected on *K. alvarezii*.

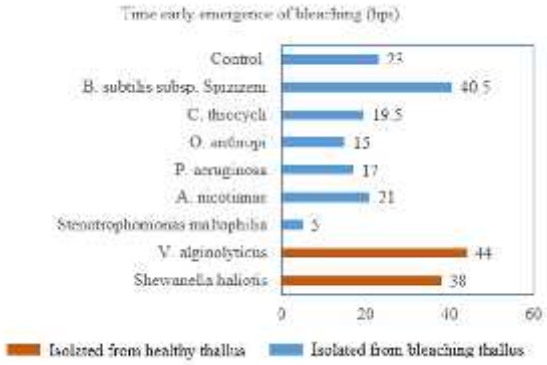

Figure 3. Time early emergence of bleaching on seaweed *K. alvarezii*. This graphic compared among bacterial of emergence the bleaching on seaweed.

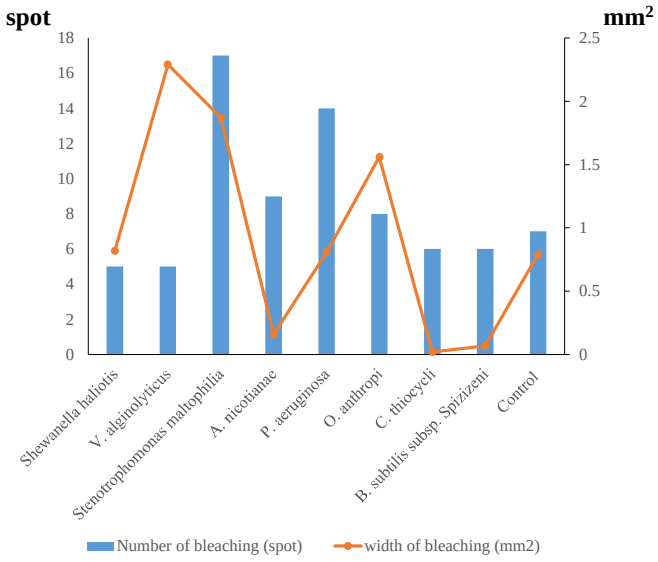

Figure 4. Indicators of ice-ice symptom on seaweed *K. alvarezii*. There are two axis that showed number of bleaching (spot) as primary axis and width of bleaching (mm^2) as secondary axis.

Histology

Morphology (Figure 5) and histology (Figure 6) showed different micropropagules that are challenged with the bacteria. Cell walls and protoplasm in healthy-looking tissue were intact

without apparent damage (Figs. 6A, 6B and 6C). The micropropagule showed less severe symptoms generally have a habit of attacks at one place, namely one (on the tip of micropropagule), and severe bleaching is indicated with symptoms at almost of surface the micropropagule. Histologically also showed difference micropropagules networks indicated light worse to heavy worse after treatment pathogenicity test (Figure 4D-4F).

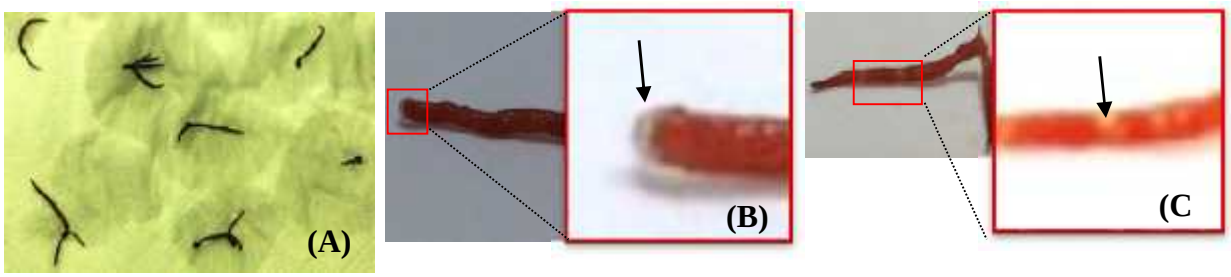

Figure 5 Morphology of micropropagule *K. alvarezii* pascainfection by bacteria. (A) Before experimented, and post-infection by bacterial with not severed areas (B), and severed areas (C). Black arrow showed the bleaching symptom.

Discussion

Bacterial identification results are generally in the group of *Gammaproteobacteria*, a group of bacteria associated with a cause of a disease. Bacteria *Gammaproteobacteria* group is bacteria essential for health study. A number of genome studies have been conducted to identify unique proteins in bacterial species *Gammaproteobacteria* associated with virulence (Van Sluys et al., 2002; Gao et al., 2009).

V. alginolyticus bacterium has a close relationship with the bacterium *S. haliotis* and this is in line with the Kita-Tsukamoto et al. (1993) and Thompson et al. (2004) who found that based on partial 16S rRNA sequences selected from 50 species, included in the family *Vibrionaceae*, are generally species of Vibrio and Aeromonas species, Deleya, Escherichia, Marinomonas, Pseudomonas and Shewanella. Isolates of *C. thiocycli* also is in one branch of V. *alginolyticus*. Based on the taxonomic level *C. thiocycli* goes into family of *Vibrionaceae* (GBIF 2016).

Thus, *S. maltophilia* is a primary bacterium candidate cause a disease of ice-ice on *K. alvarezii*. This type of bacteria is the first reported disease associated with ice-ice. Bacteria *Stenotrophomonas* sp are microorganisms that have widely spread (Brooke 2012) and been reported as opportunistic pathogens (Falagas et al. 2009). However, *S. maltophilia* isolated from the seaweed *Laminaria saccharina* is a source of active antibiotics (Wiese et al. 2009).

Other bacteria were isolated, in this study, and reported in several species of seaweed. Epibiotic bacterium *P. aeruginosa* as the brown alga *Padina tetrastromatica* (Ravisankar et al. 2013). Type *O. anthropi* discovered a bacterium degrade alginate in brown algae (Zhou et al., 2008), *C. thiocycli* and *B. subtilis* has been isolated from seaweed *Sargassum* spp. (Susilowati et al. 2015), *A. nicotiannae* reported has been isolated from biofilm on surface of marine organisms and algae (Lee et al. 2003). However, these bacteria have not been reported as a major trigger bacterial ice-ice disease in seaweed *K. alvarezii*.

Also, in this study, bacteria *O.anthropi* and *P.aeruginosa* are the second and third bacterium after *S. maltophilia* cause ice-ice. Yet, other bacteria that have been reported to be the cause of ice-ice disease, namely Cytophaga-Flavobacterium (Largo et al. 1995a), *Vibrio sp.* (Largo et al. 1995a; Aris 2011), *Pseudomonas* sp., *Plesiomonas* sp. and *Flavobacterium* sp. (Aris 2011). *Pseudomonas* sp. also has been isolated from the seaweed *K. alvarezii* and known cause disease ice-ice (Largo et al. 1995b; Aris 2011). However, *S. maltophilia* and *O. anthropi* have not been found in seaweed *K. alvarezii* troubled ice-ice.

The gentlest bacterium is *V. alginolyticus* with an average time of beginning to appear bleaching is 44 hours post-infection. This time is much longer than control showing early symptoms of bleaching, 23 hours post-infection. The emergence of symptoms of bleaching is on control allegedly due to stress during handling of treatment. Generally, the color changes to white seaweed originated from former talus end cutting (Sulistiani and Yani 2014).

Bacteria *S. haliotis* and *V. alginolyticus* were isolated from healthy thallus, so both of these bacteria were not suspected to be associated with ice-ice disease, they are good bacteria and this result similar with Zadeh et al. (2010) that *Shewanella* algae found in healthy digestive system of black tiger shrimp *Penaeus monodon*, it is a probiotics candidate. Adding probiotic *S. haliotis* on feed could improve growth and disease resistance on white spots over vaname shrimp *Litopenaeus vannamei* (Hao et al. 2014). Species of *V. alginolyticus* are also reported as effective bio control of diseases caused by pathogenic bacteria on tiger shrimp larvae (Austin et al. 1995; Widanarni et al. 2003) and shrimp vanname (Gomez-Gil et al. 2000). Unfortunately, this bacterium has a different story on seaweed. It is found as pathogens in *K. alvarezii*. It has been identified by Aris (2011) on seaweed thallus, length with 1 mm, bleaching phenomenon first seen in 12 hours post-infection with the bacterium *V. alginolyticus* PNGK 1. Yet, *V. alginolyticus* strain isolated from *K. alvarezii* was apparently different with *V. alginolyticus* strain reported by Aris (2011).

The protoplasm content is higher in a healthy micropropagule and did not seriously than severe micropropagule. Protoplasm indicates occurrence of severe chronic the micropropagule. This is in line with those reported by Quere et al. (2015) that algae disease characterized by reduced number of cell protoplasm.

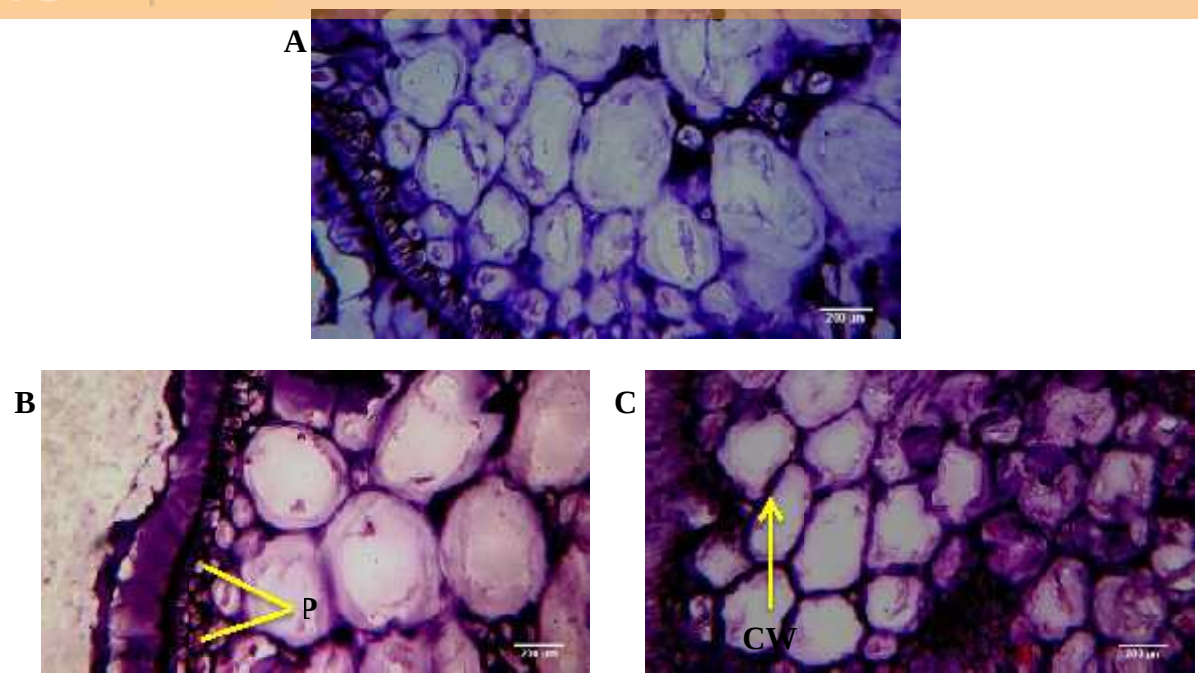

Figure 6 Longitudinal histological sections showing of micropropagule, *K. alvarezii*. (A) Display without infection (control), post-infection by bacteria, overview with not severed areas (B), and severed areas (C). P, content protoplasm; CW, cell wall

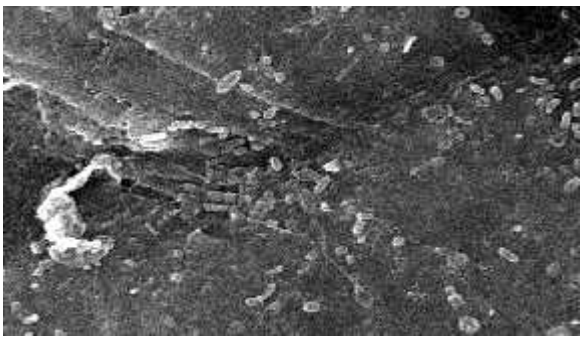

Figure 7 Scanning electron micrographs illustrating the presence of surface bacteria and the condition of the surface cellular layers of the control thallus of *K. alvarezii*. Magnify 5,000x

On ecological component, the occurrence of ice-ice on seaweed is predominantly influenced by unsteady temperature, salinity and light intensity (Largo et al. 1995b). In this study, micropropagule as product of tissue culture disease-free use and challenge test carried out under the same conditions at the indoor laboratory. The result is negative and control micropropagule also showed symptoms of bleaching at 23 hours post-infection. It came out that stress during the preparation of the micropropagule cause of bleaching. Interaction of pathogenic bacteria

with environmental stress-related illnesses ice-ice on *K. alvarezii* was conducted with 20 ppt salinity and it decreased concentration of bacteria 10^3 and 10^4 cfu/ml (Largo et al. 1995a). However, interaction of temperature with different disease-causing pathogens ice-ice on seaweed is unreported and it is thought-provoking to examine in further research. In addition, the bacteria isolated in this study is potentially used to hold the assembly seaweed ice-ice disease through the challenge test.

CONCLUSION

Among eight isolates of seaweed ice-ice disease tested on pathogenicity concluded that the leading candidate *Stenotrophomonas maltophilia* triggers ice-ice disease on *K. alvarezii*.

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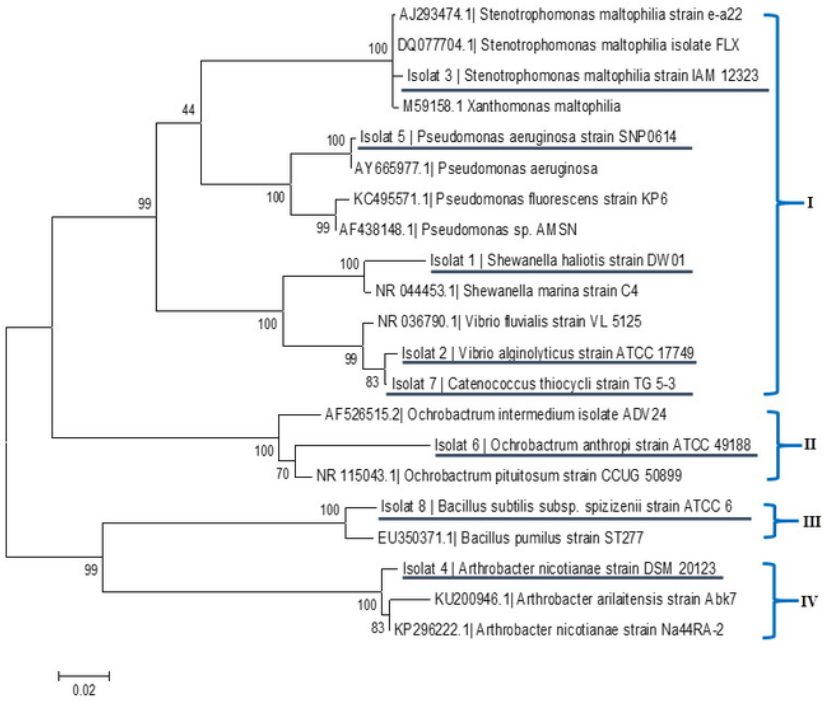
Table 2 - Figure of sequencing

Isolate	Bacteria	Identity %	Query/ Subject	Accession Number
NA1	*Shewanella haliotis* strain DW01	98%	1197/1227	NR_117770.1
NB2	*Vibrio alginolyticus* strain ATCC 17749	97%	1196/1229	NR_117895.1
AT8	*Stenotrophomonas maltophilia* strain IAM 12323	97%	1102/1136	NR_041577.1
AT9	*Arthrobacter nicotianae* strain DSM 20123	96%	1183/1231	NR_026190.1
AT2	*Pseudomonas aeruginosa* strain SNP0614	97%	1277/1316	NR_118644.1
AT4	*Ochrobactrum anthropi* strain ATCC 49188	95%	1075/1134	NR_074243.1
BU3	*Catenococcus thiocycli* strain TG 5-3	98%	1195/1220	NR_104870.1
AU5	*Bacillus subtilis subsp. spizizenii* strain ATCC 6633	95%	1234/1294	NR_118486.1

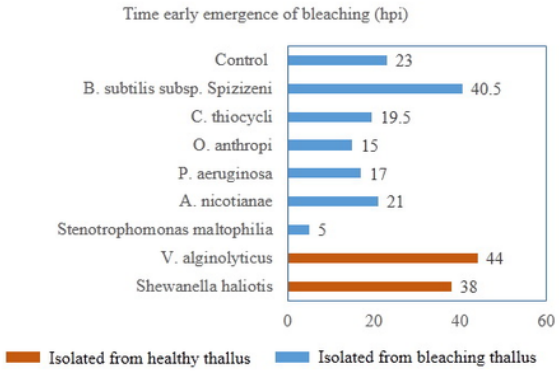
2

Figure 2 - figure of phylogeny tree



3

Figure 3 - Figure of time early emergence of bleaching



4

Figure 4 - figure of others indicator of bleaching