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Combination of molecular and traditional approaches to identify ice-ice disease causing bacteria on the seaweed *Kappaphycus alvarezii* (Doty)

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ABSTRACT

The main cause of the disease is that bacteria thrive and attack seaweed by secreting carrageenan hydrolyzing enzyme, making it more vulnerable to other microbial pathogens. In this study, the bacterial composition of healthy and ice-ice samples of *Kappaphycus alvarezii* were analyzed by sequencing one hundred 16S rRNA gene of random clones from relevant gene libraries. In addition, the conventional method was used to isolate bacteria from the ice-ice disease seaweed and identify carrageenan hydrolyzing bacteria. Our results displayed a significant difference in the number of bacterial operational taxonomic units (OTUs) between the healthy (11 OTUs) and disease samples (31 OTUs). Bacterial composition in the healthy algal samples was dominated by two genera *Cobetia* and *Roseobacter, which* accouted for 39% and 21% of the total, respectively. In contrast, the ice-ice disease algal sample was dominated by the genera *Pseudoalteromonas* and *Phaeobacter,* which accouted for 14% and 12% respectively, closely followed by three genera *Alteromonas*, *Salinimonas* and *Psychrobacter*. After screening bacterial isolates from the ice-ice disease sample for carrageenan, two types of isolates were visually observed to produce this enzyme, indicated by a clear halo zone on 0.5% carrageenan agar plate. Two carrageenan-producing isolates were subsequently identified and named as *Alteromonas* sp. IKS 3 and *Tenacibaculum* sp. IKS4. In the pathogeny test, at 5×10^4 CFU/mL, the isolates IKS3 and IKS4 caused an ice-ice disease syndrome to 75.33% and 59.66% *K. alvarezii* seaweed respectively, higher than that of the control (22%, without any isolate infection), indicating that *Alteromonas* sp. IKS3 and *Tenacibaculum* sp. IKS4 was an ice-ice disease that caused bacteria on the *K. alvarezii* seaweed.

Keywords: *Alteromonas,* carrageenase*,* ice-ice disease, *Kappaphycus alvarezii*, *Tenacibaculum, Vibrio*.

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INTRODUCTION

Seaweed is currently used in many applications, including food for human consumption and animal feed, as source of hydrocolloid process, cosmetics, experimental medicine, fertilizer, biofuel and others [1]. Seaweed is harvested worldwide with an estimated 97% coming from aquaculture.

Carrageenan is a gelling agent extracted from red seaweeds and is used for multiple applications in food processing and other industrial purposes. The two genera *Kappaphycus* (colloquially known as 'cottonii') and *Euchema* ('spinosum') are primary red seaweed sources for carrageenan production, contributing over 90% of the global output [2]. The carrageenan seaweeds were also introduced and cultivated in more than 20 countries, including India, Madagascar, Tanzania, and East Timor but the production scale were remarkably smaller. *Kappa* carrageenan, a polysaccharide with alternating units of *D*-galactose and 3,6-anhydro-galactose, extracted from *K. alvarezii* is stronger than that extracted from *Euchema denticulatum*, *iota* carrageenan. In 1993, Vietnam started cultivation of the carrageenan-producing seaweed, *K. alvarezii* (Doty) Doty [3], and has rapidly expanded the cultivation in the middle and south regions of the country due to the ease of cultivation, the simple farming approach, the short cycle, the low initial capital investment, the processing techniques.

During farming seaweed, numerous unexpected critical problems such as tropical storms, disease outbreaks or predators would occur that cause a significant decrease in biomass production. One main problem in seaweed farming is the disease, e.g., ice-ice and epiphyte infestation [4]. Doty and Alvarez (1975) presented that ice-ice disease was characterized by bleaching and softening of the thallus, followed by the disintegration of the affected tissue [5], causing a decrease in the carrageenan yield and reducing the quality of carrageenan viscosity and gel strength [6]. There are two leading causes of ice-ice disease. The first is unfavorable environmental conditions, including high or low temperatures, high or low salinity, high or low light intensity, and insufficient nutrients, and so forth [4]. The second is the opportunistic microorganisms that thrive and attack when seaweed is stressed and become more vulnerable to microbial pathogens [4].

Although the ice-ice problem was first reported in 1974 during commercial farming in the Philippines, a few studies has been conducted on the microbial causative agent. Noticeably, Largo et al., (1995) [7] pointed out that *Vibrio* P21 and *Cytophage* P25 caused early ice-ice whitening of *K. alvarezii* when the seaweed was subjected to experimental environmental stress. Largo suggested that pathogenic bacteria was considered as the secondary causative agent. These bacteria attack and degrade seaweed tissue by hydrolytic carrageenase enzyme. After this published result, by conventional method, the scientists from Indonesia, the Phillipines, and India have tried to isolate causative bacteria from ice-ice infected thallus of *K.alvarezii* and investigate their pathogenicity. From their results, more ice-ice causative bacteria were indicated and showed to belong to the different species, including *Alteromonas macleodii*, *Pseudoalteromonas issachenkonii* and *Aurantimonas coralicida* [8], *P.carrgenovara* [9] and *Vibrio* sp. ABI-TU15 [10]. It was also known that microbial communities associated with seaweed were host-specific, but their pattern was temporally and spatially variable [11]. This would explain the incidence of various causative bacteria in ice-ice disease occurrence at different farming sites.

Recently, using the Illumina sequencing method, Kopprio (2021) analyzed the bacteria associated with carrageenan seaweeds farmed in Khanh Hoa province of Vietnam and showed a significant dissimilarity in bacterial compositions between healthy and ice-ice infected seaweed, with a dominance of genus *Vibrio* and *Alteromonas* observed in infected *K. alvarezii* [12]. This result provides a good insight into the potential role of diverse groups of bacteria in seaweed life, especially the ice-ice disease. However, conventional methods including bacteria isolation, carrageenan evaluation, and pathogenicity tests, are needed to ensure and specify the virulence of the bacterial isolates. Thus, in this study, we aim to investigate and specify causative bacterial isolates involved in ice-ice infected *K. alvarezii* farmed in Khanh Hoa, Vietnam, using conventional methods combined with sequencing analysis of partial 16S rRNA gene library constructed in plasmid. Specifically, we ask (i) which dominant bacterial group is present in healthy and diseased thallus and (ii) if bacterial isolates from diseased thallus possess carrageenan activity and induce ice-ice disease in the seaweed.

MATERIALS AND METHODS

Sample collection and preparation

The seaweed *K. alvarezii* used in this study was collected in the farming site at Van Phong Bay (N12°40′16′′, E109°16′′41′′, Khanh-Hoa province of Vietnam in May 2021. Seaweed thalli were thoroughly rinsed with autoclaved seawater to remove loosely attached epibionts or sand particles and transported to the laboratory in zip-log bags in cold condition for processing within 24 hours of collection.

Figure 1. Image of the seaweed collected in the farming site at Van Phong bay. The red arrow indicates the ice-ice disease point on the thallus

Two types of seaweed, *K. alvarezii,* were sampled, including (i) healthy and (ii) ice-ice disease thalli (Fig. 1). Five replicates of healthy and apparently ice-ice-infected thalli were collected. From individual thallus, healthy or infected parts (~100 mg each) were excised with razor blades and subsequently used to prepare healthy or disease samples, each containing five agal parts from the five corresponding thallus. The samples were then used bacterial community DNA extraction. Each excised part of the individual thallus was used to isolate associated bacteria within one day of collection.

DNA extraction, 16S rRNA gene cloning and sequencing

DNA extraction of associated bacteria: Bacterial community DNA was extracted following the protocol of Griffiths [13]: first with hexadecyltrimethylammonium bromide (CTAB, Carl Roth), and later with phenol-chloroform-isoamyl alcohol (25:24:1, Sigma Aldrich). After a centrifugation step at 16,000 g at 4°C for 10 min, the upper phase containing DNA was precipitated with 2 volumes of 30% polyethylene glycol 6,000 (Sigma-Aldrich) 1.6 M NaCl at 4°C for 2 hours. The solution was then centrifuged at 17,000 g at 4°C for 90 min and the pellet was washed with cold 70% ethanol, vacuum-dried and resuspended in Tris-EDTA buffer.

DNA extraction of bacterial isolates: genomic DNA was extracted following Moore's protocol [14]. In brief, the bacterial cell was suspended in 564 μL Tris-EDTA buffer containing 10 μL of lysozyme (100 mg/mL). The mixture was incubated for 30 min. at 37°C followed by adding 6 μL of protease K (10 mg/mL), and 30 μl of SDS (10%). Subsequently, the mixture was incubated at 37°C for 30 minutes, and the suspension becomes relatively clear and viscous. One hundred μL of NaCl (5M) was added and mixed thoroughly by gentle inversion followed by incubation at 65°C for 2 minutes. CTAB/NaCl solution (preheated at 65°C) was added and mixed thoroughly (do not vortex) and incubation was carried out at 65°C for 10 min. The DNA solution was extracted with 800 μL phenol/chloroform/isoamyl alcohol (25:24:1) solution, followed by centrifugation at 15,000 g for 5 min. DNA in the upper phase was precipitated after adding 0.7 volumes of ice-cold isopropanol. Then, the DNA pellet was obtained by centrifugation at 15,000 g for 10 min. After washing with 70% EtOH, the DNA pellet was resuspended in 50 μL TE buffer and stored at 4°C for immediate use and at -20°C for long-term storage.

PCR amplification of 16S rRNA genes was performed using the universal bacterial primer 27F (AGA GTT TGA TC(A/C) TGG CTC AG) and 1492R (TAC GGYTAC CTT GTT ACG ACT) with the following condition: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturing at 94°C for 30 seconds, annealing at 52°C for 1min and extension at 72°C for 90 seconds, and a final extension at 72°C for 10 min. After amplification, PCR products were examined in 0.8% agarose gel, and expected bands were recovered and purified using MEGAquick-spin™ Plus (iNtRON).

Two clone libraries for the 16S rRNA gene were constructed using purified 16S rRNA amplicons from healthy and disease agal samples cloned into the plasmid PLUG-Prime®TA cloning vector (iNtRON Biotechnology), following the manufacturer's instruction. Competent *E. coli* DH10B was transformed by Wu [15]. Clones were screened for α-complementation with X-Gal (5-bromo-4-chloro-3-indolyz-β-D-galactopyranoside) as a substrate on Luria Bertani (LB) agar plates containing ampicillin (100 μL/mL). Recombinant plasmids from white color clones were isolated and purified using a DNA-spin DNA purification kit (iNtRON Biotechnology) under manufacturer's instruction. The plasmids were digested with restriction endonuclease *Hind*III, resulting in two DNA bands of about 2.7 kb and 1.5 kb to confirm the insertion of the 16S rRNA gene. For each clone library, one hundred purified plasmids were sequenced in one direction using bacterial 27F primer by 1st BASE DNA Sequencing (Malaysia). These obtained sequences were aligned and clustered into operation taxonomic units (OTU) at ≥ 97% identity using the Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). The representative sequence of each OTU was used for BLAST search into GenBank to find the related sequences for taxonomic classification.

Sequence analysis and data processing

The sequence chromatograms were analyzed using Bioedit program to obtain good quality sequences. The 16S rRNA gene sequence was compared with sequences available in the GenBank database using the BLASTn program (http://www.ncbi.nlm.nih.gov/blast). Bacterial sequences with high similarity from the clear taxonomic isolates were recorded for further analysis. Phylogenetic tree based on 16S rRNA gene sequences was constructed using the MEGA7 software and the Neighbor-joining method [16].

Isolation of bacteria associated with seaweed

The alga parts were kept in ice-cold plastic bags to ensure the survival of associated bacteria. In the laboratory, each part was immersed in 250 μL sterile seawater and vigorously shaken 100 times, followed by mechanical grinding of sample using mortar and pestle to make a concentrated bacteria suspension. The suspension then was diluted up to 10^{-6} prior to plating using sterile seawater. A volume of 50 μL of samples of each diluent (10^0 to 10^6) was spread onto Marine Agar 2216 (Zobell marine agar, MA, HiMedia) plate and incubated at 25°C for five days with 24 h interval observation. Colonies were selected based on the distinct morphological trait of each observed colony in MA plates. Bacterial colony purification was done by re-streaking single isolated colonies in MA plate and stored at 4°C in MA slants, and monthly sub-culturing. For long term storage, bacteria were kept in marine broth 2216 medium containing 30% glycerin at -80°C. For determination of occurrence percentage of a bacterial group, for each agal part, one MA plate exhibiting adequate bacterial density of approximately 80–150 colonies was used for counting number of colonies with similar morphology. Abundance rate of each bacterial group was calculated using a following equation:

$$Abundance\,rate(\%)=\frac{\sum_{i=5}n}{\sum_{i=5}N}*100\%$$

where: n: number of bacterial colonies of similar morphology observed on one selected MA plate; *N*: number of total bacterial colonies observed on the selected MA plate; *i*: 5, five infected samples.

For taxa identification, one representative of the group were used for 16S rRNA gene sequencing in one direction using bacterial 27F primer. Only 16S rRNA gene of the potential ice-ice causative isolates were sequenced using two primers 27F and 1492R.

Detection of carrageenan producing bacteria

All the bacterial isolates obtained were evaluated for their ability to degrade carrageenan according to the protocol established by Ohta and Hatada [17]. Briefly, the bacteria were grown for 2–3 days on the MA plate at 25°C, and subsequently, the active bacteria were streaked on the MA plate containing 0.5% carrageenan (sigma Aldrich). The plates were then incubated for 2–3 days at 25°C to induce the enzyme production. After flooding the agar plates with a 10% cetylpyridinium chloride (CPC) solution, the bacteria-producing clear zones were identified as carrageenan-producing bacteria. As a polyanionic polysaccharide, carrageenan can be precipitated with CPC, a cationic detergent. All the experiments were performed in duplicate. In addition, to examine the extracellular carrageenan, bacteria were cultured in Zobell marine broth (MB) at 37°C and 200 rpm for 1 day. Bacterial cells were separated from the culture by centrifugation at 7,000 rpm for 10 min in a cooling centrifuge at 4°C. The clear supernatant was considered the crude carrageenan source and used for extracellular property test using the agar well diffusion method. In brief, a volume of 50 ul crude enzyme was added to a premade hole on the agar plate which contained 0.5% carrageenan in 50 mM potassium phosphate buffer pH 7.4 supplemented with 2% NaCl. The plates were incubated at 37°C overnight. Carrageenan activity was visualized as a halo of clearing against a milk-white background after flooding the plate with 10% CPC.

Pathogenity test using potential causative bacteria

In this experiment, the algae *K. alvarezii* was cultured in non-axenic conditions. Healthy and main branches of *K. alvarezii* thalli were washed with natural seawater, cut into approximately 12 cm long pieces, and maintained for 7 days in a 200 L-filled concrete tank in a flow-through water system for acclimatization. The maintaining factors included water temperature 25°C, irradiance 130 μmol photons $m^{-2}s^{-1}$, controlled lighting 12 h light/12 h dark cycle, salinity ~3.5%, and pH 8.0. After acclimation, seaweed fragments (150 explants) in good condition were selected for disease induction, with 50 pieces randomly distributed in each 40 L glass tank. The seedlings were tied to plastic mesh panels and placed at the bottom of the tank.

Two suspective pathogenic isolates were used for pathogenicity tests based on their presence on disease thalli and carrageenan degrading activities. Bacteria were cultured in MB medium at 37°C until cell density reached 10^8/mL (~ 24 h). The tank was inoculated with each bacterial isolate at approximately 5×10^4 CFU/mL density and labeled according to the bacterial treatment type. The control tank was not inoculated with any of the bacterial isolates. During culturing, thalli were then 3-days intervals observed for sign of ice-ice disease. The number of infected pieces was calculated and recorded towards the final day of the experiment. The experiments were performed in triplicate.

To compute for incidence, the number of infected pieces was divided by the total number of pieces in each tank.

$$\%\,ice-ice\,incidence=\frac{No.\,of\,infected\,piecies}{Total\,no.\,of\,\,piecies}\times 100\%$$

The statistical analysis

Statistical data analysis is performed using Microsoft Excel 10 Data were expressed as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Bacterial composition of clone libraries

One hundred cloned 16S rRNA genes from each library generated using healthy and ice-ice disease algal samples were sequenced in the forward direction resulting in approximately 750 bps length. Using the Clustal Omega tool, the sequences with ≥ 97% identities were grouped in one operation taxonomic unit (OTU). Subsequently, a representative sequence of each OTU was blasted in the GenBank database to examine the nucleotide similarity. There was a significant difference in the number of bacterial OTUs between the two clone libraries created from the healthy and disease thalli samples (Fig. 2). It was calculated that a number several 31OTUs were found from 100 sequences of the disease clone library, a higher number than a healthy one that contained 11 OTUs (Fig. 2). Unknown OTUs referred to sequences which did not show a clear identity with any bacteria at a certain taxa level. The bacterial composition in the disease sample was more complicated and diverse. Two genera, *Cobetia* and *Roseobacter,* were shown to dominate in the heathy sample, with estimation at 39% and 21%, respectively, but less abundant in the disease sample. Instead, *Pseudoalteromonas* and *Phaeobacter* were the two genera most found in the disease sample with estimation at 14% and 12%, respectively, closely followed by the three genera *Alteromonas*, *Salinimonas* and *Psychrobacter* observed at 7%, 8% and 6% in total count, respectively. Notably, several genera, including *Vibrio* [10], *Cytophaga* [7], and *Alteromonas* [8], reported as ice-ice causative agents found in disease sample were not observed in the healthy sample. Three bacterial phyla existed in the clone library from the disease algal sample, including *Proteobacteria*, *Bacteroidota* and *Spirochaetota*, at the proportions of 87%, 12%, and 1%, respectively (data not shown). However, only the phyla *Proteobacteria* was observed in the clone library from the healthy one.

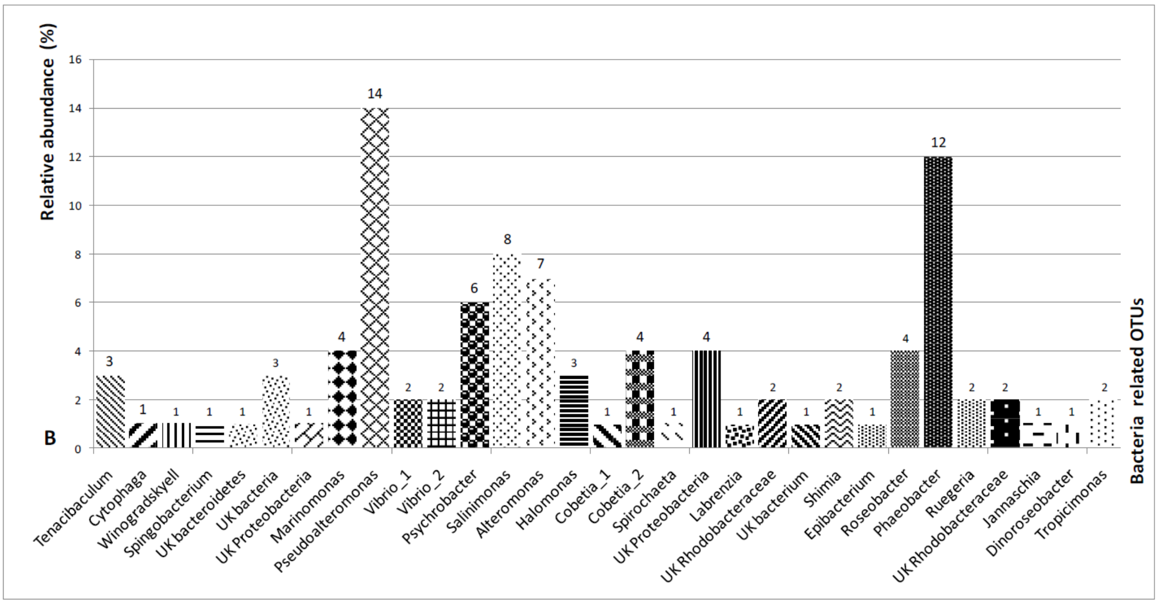

Figure 2: Relative abundance (%) of bacterial OTUs in healthy (A) and ice-ice disease (B) algal sample *K. Alvarezii*

Recently, Riyaz (2024) [9] studied a bacterial community associated with ice-ice disease *K. alvarezii* using the free method, the MiSeq-Illumina platform (Riyaz, 2024)[18]. These authors analyzed the bacterial composition of the distinct parts of the disease fragments and reported the remarkable difference in bacterial taxomomy and abundance. The infected thallus was dominated by *Cobetia*, *Halomonas,* and *Marinobacte*r, with relative abundance of 81%, 13%, and 6%, respectively. In the infected tip, the genus *Bacillus* and *Paenibacillus* dominated at 82% and 18%, respectively [9]. However, microbiome analysis of the infected seaweed demonstrated the abundance of the genera including *Psychrobacter*, *Marinobacter*, and *Cobetia* at occurrence percentages of 52%, 32%, and 15%, respectively. Our result on bacterial composition by analyzing partial 16S rRNA gene has showed that the common genera *Cobetia*, *Psychrobacter*, *Phaeobacter,* and *Roseobacter* were found remarkably dominant in both type of the samples (healthy and diseased) (Fig. 2). To a certain extent, our finding agreed well with that of Riyaz on the dominant bacterial genus in *K. alvarezii* [18]. In another study [12], using Illumina Miseq sequencing of the 16S rRNA gene, Kopprio (2021) studied bacterial community of three red seaweeds, including *K.avalrezii*, *K. striatus* and *Euchema denticulatum,* which were farmed in Vietnam. Genera *Vibrio*, *Alteromonas*, *Cobetia,* and unknown bacteria were most abundant in diseased *K.alvarezii* [12]. The healthy parts of this seaweed hosted mostly *Vibrio*, *Ruegeria*, *Porphyrobacter*, *Rhizobiaceae*, *Epibacterium,* and unknown genera.

In the present study, remarkable differences in microbial composition between the healthy and diseased samples of the seaweed samples revealed bacterial involvement in the pathogenesis of ice-ice disease. However, microbiological and biochemical methods must be applied to investigate the symptoms and the causative bacteria to understand major causative bacteria at the first onset of the ice-ice disease. These methods include bacterial isolation, detection of carrageenan hydrolyzing enzyme, and pathogenicity test.

Bacterial Isolates associated with ice-ice disease seaweed

Five replicates of ice-ice fragments from five individual thalli were used to isolate bacteria. For each ice-ice sample, one plate with a suitable colony density of about 50–100 colonies was selected for isolating and calculating the bacterial distribution. Based on the difference in morphological features, 14 types of bacteria were recorded. All of them were taxonomically identified based on partial 16S rRNA sequences, and their closest sequences and related taxa were showed in Table 1.

The bacterial groups present in all infected samples were dominated by *Vibrio*, *Alteromonas*, *Pseudoalteromonas*, *Cobetia, Tenacibacullum*, and *Ruegeria*, with varying relative abundance. The remaining groups were only detected in individual samples with a lower observed frequency.

Of 14 bacterial geneva found in the disease thallus, 12 geneva are known as actual marine bacteria except *Priestia* and *Paracoccus*, which inhabit a wide range of habitats, both terrestrial and marine ecosystems. The most observed isolates belonging to the genera *Vibrio*, *Cobetia*, *Alteromonas*, *Tenacibacullum*, *Pseudoalteromonas*, and *Ruegeria* were also recorded as major groups in 16S rRNA gene library of the diseased thallus. This result indicated that the traditional microbial method enabled the successful isolation of dominated bacterial groups identified using uncultured method. Several studies were conducted on isolating bacteria from ice-ice disease *K. alvarezii* [8, 18, 19]. In the present study, some fourteen genera found associated with diseased thallus demonstrated more diverse groups as compared to studies of Magun [19] (5 genera found), Azis [20] (4 genera found), Riyaz [21] (2 genera found), and Syafitri (7 genera found) [8]. Yusriyyah isolated fourteen bacteria belonging to 7 genera, including *Vibrio*, *Alteromonas*, *Phaeobacter*, *Pseudoalteromonas*, *Cobetia*, *Celeribacter*, and *Labrenzia* from K.*alvarezii* [22]. The fourteen representatives obtained in the present study are a good input source for determining ice-ice causative agent in subsequent work.

Table 1. Bacterial groups associated with ice-ice disease *K. alvarezii* and their molecular and morphology characteristics

No.	Colony morphology	Number of colonized sample; abundance rate (%)	Representative Isolate	Closest species	Similarity (%); E value	Accession number	Family/Genus
1	Circular, entire margin, wet, translucent, convex, light cream	5/5; (21.3)	IKS1	*Vibrio alginolyticus*	100; 0.0	CP054700.1	*Vibrionaceae/ Vibrio*
2	Circular, entire, shiny, convex, opaque	5/5 (24.5)	IKS2	*Cobetia marina*	100; 0.0	MH259982.1	*Halomonadaceae/Cobetia*
3	Circular, entire margin, umbonate, shiny, opaque/white	5/5 (15.6)	IKS3	*Alteromonas australica*	99.85; 00	MZ298714.1	*Alteromonadaceae/ Alteromonas*
4	Irregular, wet, shiny, flat, iridescent	5/5 (11.1)	IKS4	*Tenacibacullum mesophilum*	100; 0.0	MN481027.1	*Flavobacteriaceae/ Tenacibacullum*
5	Circular, entire, shiny, translucent, white	5/5 (9.5)	IKS5	*Pseudoalteromonas shioyasakiensis*	100;0.0	MK439592.1	*Pseudoalteromonadaceae/ Pseudoalteromonas*
6	Circular, entire, convex, smooth, translucent	5/5 (8.0)	IKS6	*Ruegeria mobilis*	100; 0.0	KP843693.1	*Rhodobacteraceae/Ruegeria*
7	Small, circular, convex, shiny, beige	3/5 (2.1)	IKS7	*Marinobacter hydrocarbonoclasticus*	100; 0.0	MT325887.1	*Alteromonadaceae/ Marinobacter*
8	Irregular, wrinkled, opaque, undulate	1/5 (0.42)	IKS8	*Priestia araabhattai*	100; 0.0	CP129011.1	*Bacilaceae/Priestia*
9	Small, circular, shiny, raised, orange	2/5 (1.26)	IKS9	*Paracoccus zeaxanthinifaciens*	100;0.0	NR_025218.1	*Rhodobacteraceae/ Paracoccus*
10	Small, circular, entire, convex, smooth, opaque	1/5 (1.26)	IKS10	*Psychrobacter adeliensis*	99.71; 0.0	MT110671.1	*Moraxellaceae/ Psychrobacter*
11	Small, circular, entire, translucent, beige	1/5 (0.61)	IKS11	*Shimia aestuarii*	99.16; 0.0	MW828576.1	*Rhodobacteraceae /Shimia*
12	Small, circular, entire, smooth, opaque	3/5 (3.1)	IKS12	*Epibacterium mobile*	100; 0.0	MH746055.1	*Rhodobacteraceae/ Epibacterium*
13	Small circular, convex, smooth, yellow	2/5 (0.63)	IKS13	*Mesonia sediminis*	99.54;0.0	MT585163.1	*Flavobacteriaceae/Mesonia*
14	Circular, entire, convex, smooth, light yellow	1/5 (0.41)	IKS14	*Thalassospira povalilytica*	99.37; 0.0	MH746072.1	*Thalassospiraceae/ Thalassospira*

Screening carrageenan activity

The actual marine bacterial genera *Vibrio*, *Alteromonas*, *Pseudoalteromonas*, *Cobetia*, *Psychrobacter*, *Marinobacter*, *Bacillus*, *Micrococcus*, etc., were frequently associated with *K. alvarezii*. Among them, *Alteromonas* and *Pseudoalteromonas* [8], *Bacillus* and *Vibrio* [9] and *Vibrio* [10] were reported to be the ice-ice causative bacteria on *K. alvarezii* based on the carrageenanse or pathogenity test. The ice-ice disease is known to be caused by a particular pathogen bacteria attack, especially when the seaweed is in a weak growth condition (under stress), such as water quality parameter changes, unfavor temperature or low (or high) salinaty level. These phenomena indicate that a bacterial attack is secondary factor after the seaweed confront a harsh environmental stressor. The carrageenan hydrolyzing bacteria are most considered as a major trigger of ice-ice symptom because they produce carrageenanse that break down the seaweed wall (mainly consist of carrageenan), enabling secondary invasion of opportunistic bacteria.

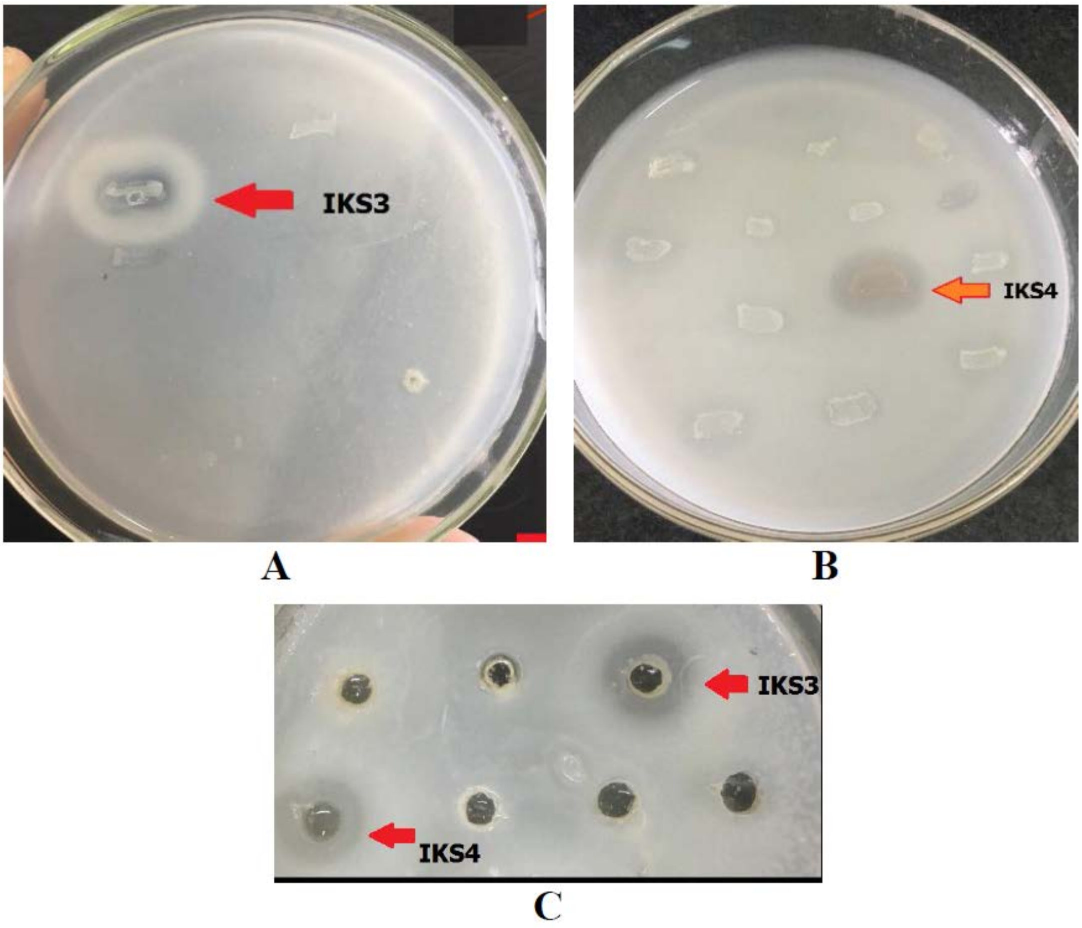

Figure 3: Carrageenan hydrolyzing activity of fourteen selected bacterial isolates (A, B) and extracellular carrageenanse property (C). Carrageenan hydrolysis is indicated by red arrows

The fourteen representative bacterial isolates were tested for their carrageenan hydrolyzing capacities. Only two isolates, ISK3 and ISK4, were detected to produce carrageenan and generate clear halo zones on the carrageenan agar plate (Fig. 3). In addition, the two positive strains showed extracellular carrageenan (Fig.3C), an important property of bacteria to metabolize carrageenan distributed outside cellular membrane. The other bacterial isolates showed no or unremarkable halo zone and thus were considered negative strains regarding carrageenan producers. Nearly the full length (approximately 1,500 bps) of the 16S rRNA gene of the two positive isolates were subsequently sequenced and used for molecular identification and phylogenetic tree construction. Regarding 16S rRNA sequence comparison, the strain ISK3 showed 98.98% identity with *Alteromonas* sp. MR31D (AN: HQ439508) and the strain ISK4 showed 100% identity with *T. mesophilum* F2 (AN: MN481027). The phylogenetic trees of the ISK3 and ISK4 were constructed based on the similarity of the 16S rRNA gene sequence (Fig. 4). Notably, representative members of the genera *Vibrio* [22, 23] and *Pseudoalteromonas* [22, 24] being reported to produce carrageenan did not show carrageenan activity in our test. The finding of carrageenan activity in the present work supports the notion that the capability to degrade red algae cell wall carrageenan is likely common in *Alteromonadaceae*. However, the *Tenacibacullum* bacterium, for the first time, is proven to produce extracellular carrageenan, confirming a novel property of this genus. Recently, Riyaz [21] studied the microbiome of ice-ice diseased *K. alvarezii* and obtained *Bacillus* and *Vibrio* strains that hydrolyzed carrageenan. However, the enzymatic action on carrageenan seemed unclear with lugol's iodine stain [9].

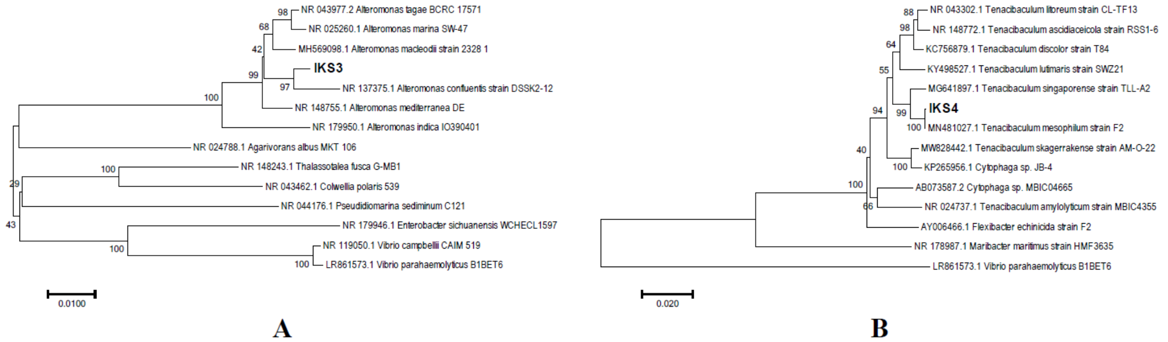

Figure 4. Neighbour-Joining phylogenetic tree based on 16S rRNA gene sequence shows the relationships between the isolate IKS3 (A), IKS4 (B) with other related genera. Numbers at nodes are levels of bootstrap support (%). Scale bar represents nucleotides substitution per 100 nucleotides

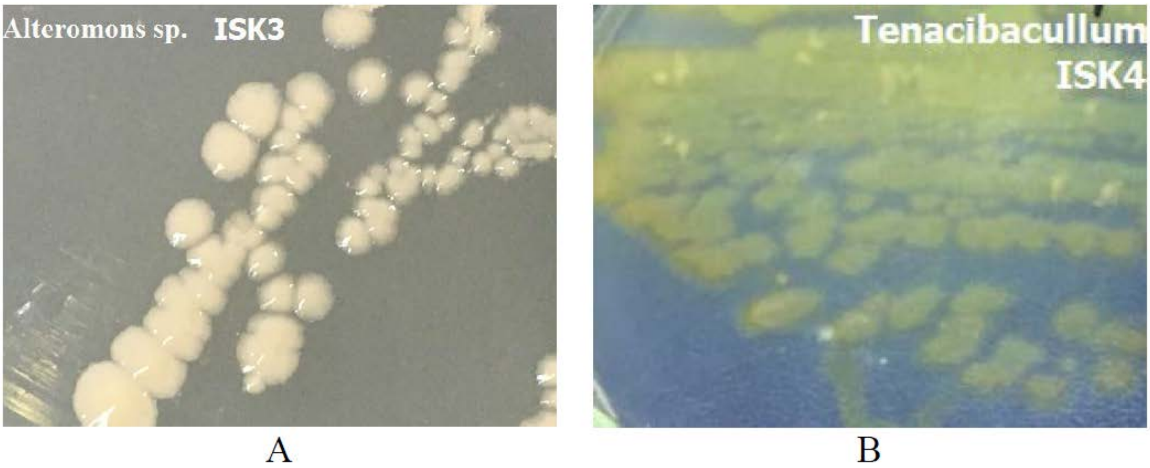

Figure 5. colony images of the carrageenase producing bacteria. *Alteromonas* sp. ISK3 (A) and *Tenacibacullum* sp. ISK4 (B)

Colony images of the two isolates, *Alteromonas* sp. ISK3 and *Tenacibacullum* sp. ISK4, was showed in Figure 5.

Pathogeny test

Alteromonas sp. IkS3 and *Tennacibacillum* sp. IKS4, the two bacterial strains colonizing all five ice-ice infected pieces and showing carrageenan degrading activity, were selected for pathogeny test. The two bacterial strains induced apparent ice-ice infection observed in three replicate tanks as early as day 3 of culture. In the control tank, only one piece in three replicate-tanks were observed ice-ice symptom after 6 days of culture and the ice-ice had an outbreak since day 12 of culture. The number of infected pieces treated with two isolates has increased remarkably as culture time progressed. At the end of culturing (day 21), the strain *Alteromonas* caused the ice-ice infection to 75.33 ± 4.16% of agal pieces, followed by the strain *Tenacibacullum*, which caused infection to 59.66 ± 2.51%. The lowest infection was observed in the control tank without bacterial treatment, with only 22 ± 4% algae pieces got infected until the end of culturing. Figure 6 shows the highest incidence of infection, at all interval times of culturing, in the tank treated with *Alteromonas* IKS3, followed by *Tenacibacullum* IKS4. The lowest value of incidence was observed for the control tank. Statistical analysis results (*t*-test, paired two samples for means) showed a significant difference in ice-ice infection percentage of the tank treated with *Alteromonas* sp. IKS3 (value $P(T \le t)$ two-tail = 0.00641) or *Tenacibaculum* sp. IKS4 (value $P(T \le t)$ two-tail = 0.00641) in comparison with control (no bacterial treatment). Besides, *Alteromonas* sp. IKS3 caused more severe damage to the seaweed than *Tenacibacullum* sp. IKS4 is expressed by higher ice-ice disease percentage (Fig. 6). Previously, a pathogeny test [24] of bacterial infection on *K. alvarezii* was conducted with 9 bacterial strains belonging to the genera *Alteromonas*, *Bacillus*, *Pseudoalteromonas*, *Aurantimonas*, *Rhodococcus*. The author(s) concluded that *A. macleodii* was the most severe causative agent among tested bacteria [24]. In another pathogenic study, Largo (1995) [7] reported that of 10 tested bacteria, *Vibrio* sp.11 and *Cytophaga* sp.25 severely caused ice-ice disease to *K.alvarezii* althouth their carrageenase detection had not been conducted. From *K. alvarezii*, 13 bacterial strains were isolated and evaluated for their agalytic activity using phytoplanton cells [17]. Most of the isolates belonged to *Pseualteromonas* and *Vibrio*; five showed algalytic activity [17].

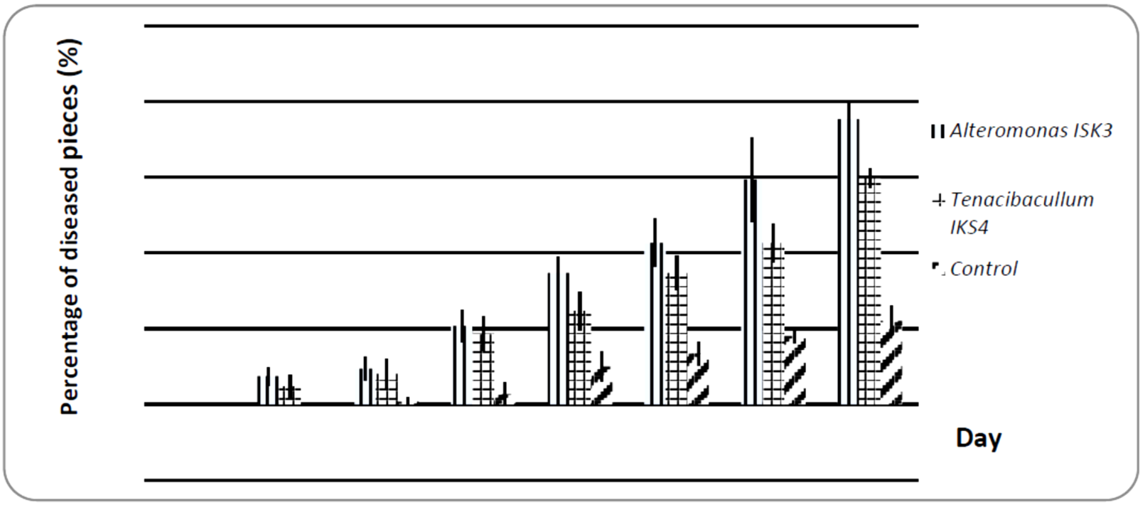

Figure 6. Percentage of diseased algal pieces infected with selected bacteria

CONCLUSION

The main idea was to use a non-culture-based approach combined with microbial and biochemical methods to understand the microbial communities of healthy and ice-ice diseased *K. alvarezii* and to identify disease-causative bacteria. The results reveal that bacterial groups in ice-ice diseased samples are more diverse than healthy samples. Some 14 bacterial groups were isolated, including two isolates named *Alteromonas* sp. ISK3 and *Tenacibacullum* sp. ISK4 showed carrageenan degrading activity. Results of the pathogenicity test of these two isolates on *K. alvarezii* showed that the isolate ISK3 and ISK4 caused the ice-ice disease to 75.33 ± 4.16% and 59.66 ± 2.51% algal samples, respectively.

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