

Characterization and identification of isolated bacteria from ice-ice disease infected seaweed *Kappaphycus alvarezii*

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Ice-ice disease occurs in cultivated algal seaweed *Kappaphycus alvarezii* due to pathogenic bacterial infections. This seaweed has rich source of carrageenan widely known as the kappa carrageenan. Generally, ice-ice disease leads to whitening of the branches initiated with colour changes of the thalli, which become transparent in the end. This study was aimed to isolate and identify the bacteria based on morphology and biochemical characterization on ice-ice diseased *K. alvarezii* from three different places, namely, Kottapatinam, Thondi and Rameswaram. The bacterium was isolated in Zobell Marine Agar (ZMA) and thiosulfate-citrate-bile saltsucrose (TCBS) agar. Morphological and biochemical characterizations revealed that the isolated bacteria causing ice-ice disease were closely related to the genera *Bacillus* in ZMA and *Vibrio* species in TCBS. Total viability count, physical and chemical properties of the bacteria by gram staining and morphological analysis were done for all species isolated from three places.

[**Keywords:** *Kappaphycus alvarezii*; *Vibrio* sp.; *Ice-ice* disease]

Introduction

Solieriaceae is a red algal family in Order Gigartinales of Class Rhodophyceae. In this family, there are many valuable species including *Betaphycus gelatinus*, *Kappaphycus alvarezii* and *Euclidean denticulatum* which generally have multi-axial thalli and often inhabit the warm seas. *K. alvarezii* is often cultivated in the upper part of the sub littoral zone, from just below the low tide line to rocky substrates where water flow is slow to moderate. *Kappaphycus* is an important red alga cultivated in south-east Asia and India as raw material both for the extraction of the phycocolloid, carrageenan, and as food¹.

There are two kinds of diseases in seaweed: Infectious and non-infectious type. The former involves a transmissible infectious agent (bacteria, fungi, virus, etc.), while the latter is induced by physiogenic factors such as extremes of temperature, salinity, light intensity or pollution. Microorganisms have been identified as the colonizers of seaweeds, and bacteria have been regarded as the primary colonizer². This bacteria-seaweed association often disturbed the production of *K. alvarezii*, which is often damaged by the occurrence of ice-ice disease. According to Mendoza³, ice-ice disease led to significant decrease in seaweed production and decrease in carrageenan yield

ranging from 25-40% compared to the healthy crop. Bacteria are known to be the main component of seaweed epibionts with higher diversity and had implications in the development of the disease⁴. It has been further found that non-pigmented bacterium and a yellow bacterium, *Cytophaga-Flavobacterium* complex and *Vibrio-Aeromonas* complex were causative microbes of the whitening of *K. alvarezii*. However, very little is known about seaweed disease and its status in India. The disease is spreading along with the extension of cultivation area and environmental degradation. Due to regular outbreaks and economic loss caused by bacterial community, several strategies have been attempted worldwide to stop spreading of seaweed diseases. However, those approaches were demonstrated ineffective and there were very little success in controlling the disease on large spatial scale. This study was conducted to determine the incidence and infection of ice-ice disease in *Kappaphycus alvarezii*, as well as the environmental factors that triggered the disease. To understand the causative agent of *K. alvarezii* ice-ice disease in Rameswaram (RH), Thondi (TH) and Kottaipatinam (KT), microbiological and biochemical techniques were applied to investigate the symptom and the causative bacteria.

Materials and Methods

Sampling site

Infected samples of *K. alvarezii* were collected from cultivation farms at Kottapatinam (N 9° 58' 51.6842", E 79° 12' 9.4613"), Rameswaram (9° 17' 16.8" N, 79° 18' 46.8" E) and Thondi (9.7438°N, 79.0185°E) in August 2017 (Fig 1). After collection, the infected seaweed materials were stored into sterile borosilicate bottles containing autoclaved seawater and brought in chilled condition to the laboratory and stored at 4 °C.

Isolation of bacteria from infected seaweed

One gram of infected seaweed sample was grinded using mortar and diluted in 10 mL of sterilized seawater. The sample was vortexed to homogenize the solution. Following this, 1 mL of the solution was diluted into 9 mL of sterilized seawater to make 10⁻¹ seaweed dilution. The processes were repeated until 10⁻⁶ seaweed dilution. The 100 µL aliquots from 10⁻¹ to 10⁻⁷ dilution were spread onto Zobell marine agar (ZMA) and incubated at room temperature for 24 h. Based on morphological features, colonies were randomly picked and purified by making streak plates.

Identification of Isolated Bacteria by Gram staining method

Thin smear of bacterial cultures was prepared on clear, dry glass slide and allowed to air dry and fixed by gentle heat. Gram staining was done for isolated bacterial cultures according to the protocol and procedures using Gram staining kit (Himedia K001). The slides were air-dried or blot-dried between sheets of clean bibulous paper and examined under oil immersion objective.

Identification of Isolated Bacteria by Biochemical test method

Bacteria isolated from *ice-ice* disease infected *K. alvarezii* were subjected to biochemical test using KB001 HiIMViC Biochemical Test Kit which is a combination of 12 tests for identification of Gram negative species. It contains sterile media for Indole,

Methyl red, Voges Proskauer's, Citrate utilization tests and eight carbohydrates, namely, glucose, adonitol, arabinose, lactose, sorbitol, mannitol, rhamnose and sucrose, Reagents supplied with kit included: Kovac's Reagent (R008) for Indole test Methyl Red Indicator (I007), Barritt Reagent A (R029) and Baritt Reagent B (R030) for VP test.

Results

Sampling site

Table 1 describes hydrological parameters namely, salinity, temperature, relative humidity and pH of three different sites. It shows that salinity, relative humidity and pH are higher in TH and KT sites than in RM site, whereas it's opposite with respect to temperature parameters, it's higher in RM site and lower in TH and KT sites.

Isolation of bacteria from infected seaweed

The *ice-ice* disease infected *K. alvarezii* is shown in Fig. 1. Growth of white milky bacterial colonies is observed in RMZMA01, THZMA01, KTZMA01-Zobell Marine Agar plates (Fig. 2a) and yellow colonies in TCBS plates of RMTCBS01 and THTCBS01 plates, but no colonies are observed in



Fig. 1 — Collection of *K. alvarezii* sample from sampling site (A); (B & C) *Ice-Ice* disease infected *K. alvarezii* (B & C); Healthy sample of *K. alvarezii* (D).

Table 1 — Physiological features and conditions of three sample sites

S.No.	Features	Rameshwaram(RM)	Thondi (TH)	Kottapattinam(KT)
1.	Salinity (ppt)	34	36	36
2.	Temperature	38.5° C	33.1° C	32.7° C
3.	Relative Humidity	60%	73%	69%
4.	pH	8.1	8.3	8.3

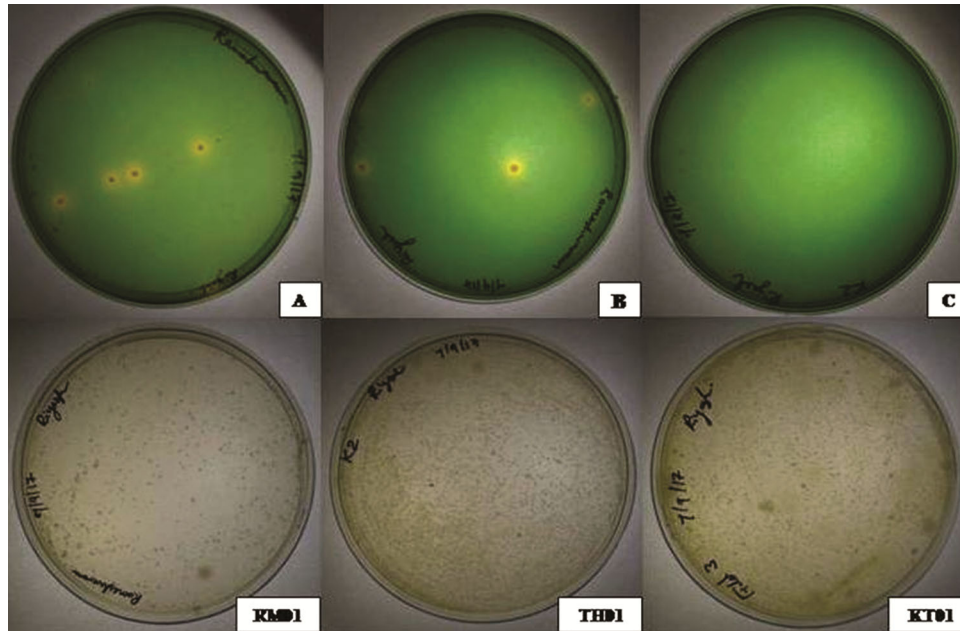


Fig. 2 — Isolation of bacteria in TCBS Agar from *ice-ice* disease infected *K. alvarezii* thalli (a); Isolation of bacteria in ZMA from *ice ice* disease infected *K. alvarezii* thalli (b).

KTTCBS01 plates. Further plates are stored in 4 °C (Fig. 2b).

Identification of Isolated Bacteria by Gram staining method

The slides observed in light microscope showed rod-shaped bacteria with purple colour from ZMA culture plates for KTZMA01, THZMA01 plates and RMZMA01 cultures which elucidates that these cultures are Gram positive. The THTCBS01 and RMTCBS01 bacterial colonies from TCBS plates showed pink colour, with rod and comma shape, respectively which elucidates that these bacteria are Gram negative (Fig. 3).

Identification of isolated bacteria by biochemical test method

The results of biochemical test are given in Table 2. The test is based on pH change and substrate utilization. On incubation, the bacteria underwent metabolic changes which are indicated as colour change in the media that can be either interpreted visually or after addition of the reagent.

Discussion

Diseases are generally caused by low salinity, high temperature and light intensity. When the plant is under stress whitening of the branches occurs, which results in crop loss. Using colony morphology on

selected agars and Gram-stain morphology, a number of 1-step biochemical or enzymatic tests can be identified by the skilled microbiologists. These results are often available more quickly and are as accurate as those derived from conventional methods. To support the integrated prevention of ice-ice disease, information about bacterial pathogens and the availability of fast and accurate detection are required. The development of ice-ice disease in *K. alvarezii* depends on several factors to which the seaweed was exposed. Largo et al., 1995 found that the combined effect of stress and abiotic agents, such as opportunistic pathogens are primary factors of the ice-ice disease⁵. The infection of the seaweed by these pathogenic bacteria may depend on their ability to successfully attach to the seaweed surface.

The degree of infection during August was high in this study in TH and KT. This increase could be related to the changes of environmental conditions in the coast, mainly, increasing amount of pollution due to increasing human activities near the farms and increasing temperatures. This result confirms the findings of Largo et al., 2006⁶, wherein high temperature during the summer months (sometimes reaching more than 30 °C in some places during low tides), coupled with high light intensity and low water movement are the key factors that trigger ice-ice development in the cultivation ground.

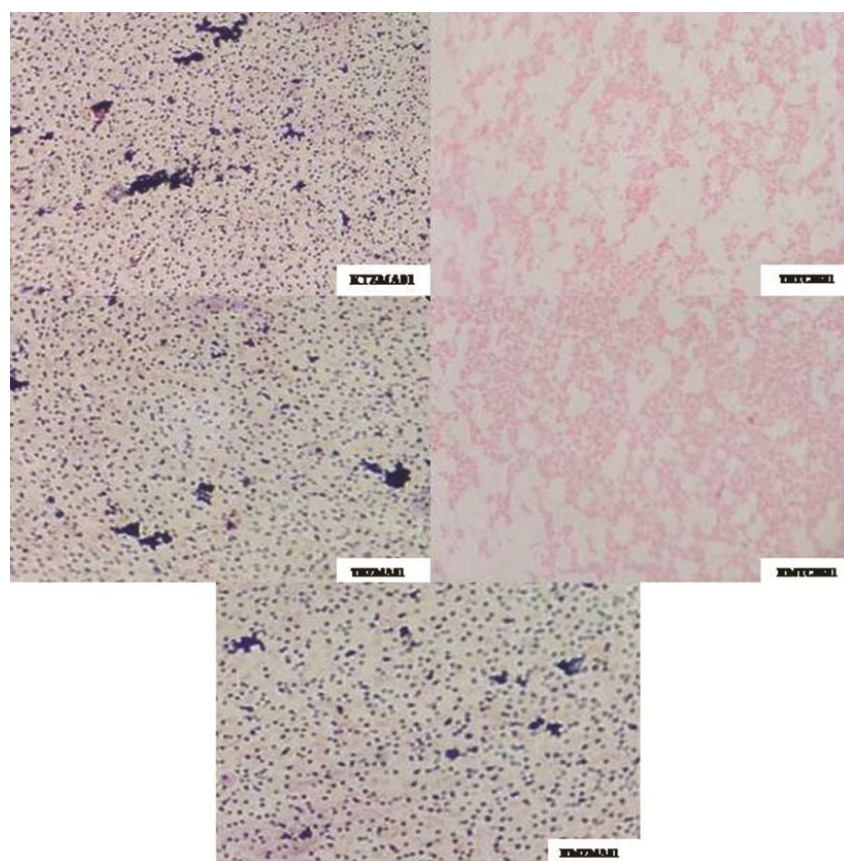


Fig. 3 — Identification of bacterial isolates from *ice-ice* diseased *K. alvarezii* by Gram staining method.

Table 2 — Identification of Isolated Bacteria by Biochemical test method

S.No.	Test	KTZMA01	THTCBS01	THZMA01	RMTCBS01	RMZMA01
1.	Indole	-	-	-	-	-
2.	Methyl Red	+	+	-	+	-
3.	Voges Proskauers	-	-	+	-	-
4.	Citrate Utilization	+	-	+	-	-
5.	Glucose	+	+	+	+	+
6.	Adonitol	-	-	-	-	-
7.	Arabinose	+	-	+	-	+
8.	Lactose	-	-	-	-	-
9.	Sorbitol	-	-	-	-	-
10.	Mannitol	+	+	-	+	-
11.	Rhamnose	-	-	-	-	-
12.	Sucrose	-	+	-	+	-

Conclusion

Morphological and biochemical characterizations of the isolated bacteria from ice-ice disease infected branches of the seaweed *Kappaphycus alvarezii* from KT, TH and RH revealed that ice-ice disease is caused by bacteria closely related to the genera *Bacillus* in ZMA and *Vibrio* in TCBS. It can be concluded that isolates obtained are Gram positive and Gram negative bacteria areas. This study

emphasises the need for further confirmation by molecular techniques with pathogenicity tests.

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