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Microbiome Analysis and Characterization of Carrageenan in Infected Farmed *Kappaphycus sp.*

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Abstract: ***Marine living resources are considered as an asset for their valuable materials for the society and the derived by products like drug, food, materials, etc. The occurrence of ice-ice disease in farmed Kappaphycus alvarezii has tremendous implication on the carrageenan industry. Microbiome plays the major role in determining the health of seaweed. This study will attempt to know the variation in the microbiome population in both healthy and infected seaweed, to determine the role of any uncultured bacterial population in inducing the ice-ice disease in farmed Kappaphycus alvarezii. This disease may lead to poor quality yield of carrageenan and this polymer will further studied in detail. Thus, samples were collected from coastal region of Rameswaram. To determine the effect of these diseases on the quality of carrageenan, homogenized solution of thalli from healthy and disease K. alvarezii will be investigated using FTIR, GC-MS, and Plant sectioning method of analysis. Genomic DNA of bacterial population present in the diseased part of K. alvarezii was isolated followed by PCR amplification of 16S rRNA gene sequence in V3-V4 region. The obtained gene sequence is subjected to advanced BLAST analysis. After identification the phylogenetic comparison will be done by using NGS (Next Generation Sequencing) and the sequence will be submitted to NCBI at GenBank as a repository.***

Keywords: Carrageenan, Ice-Ice disease, NGS

1. Aim and Scope

Microbiome analysis on farmed seaweeds helps to know the microbial community present in and around the seaweed. Qualitative analysis of carrageenan extracted from seaweed helps to determine the effects of ice- ice.

a) To identify the bacteria, present on the seaweed *Kappaphycus alvarezii* infected by *ice-ice* diseases.
b) Molecular identification of bacteria by amplifying V3-V4 hyper variable region of 16 SrRNA gene.
c) To identify the uncultivable bacteria present in the micro biome of infected seaweed.
d) To find the microbial community of the infected farmed seaweed using Illumina platform.
e) To determine the effects of *ice-ice* diseases on seaweed especially on the quality of carrageenan product by the seaweed.

2. Methodology

a) Sample Collection (Seaweed)

Sample seaweeds both infected and healthy of *K. alvarezii* were collected from cultivation farms at Rameswaram (9° 17' 00" N, 79° 10' 55" E) after collection, the infected and healthy seaweed materials were collected and stored into sterile borosilicate bottles containing autoclaved seawater and brought in chilled condition, to the laboratory and stored in 4° C. Each sample was thoroughly cleaned and washed to remove the excess salt.

b) Drying of Seaweed

Both healthy and infected sample of *K. alvarezii* were cut into smaller pieces of about 1 cm and dried in hot oven for overnight at 60°C. These dried samples were further used for extraction of carrageenan.

c) Extraction and Purification of Carrageenan

Carrageenan from each sample were extracted by adding 10 g of dried seaweed in 700 mL of distilled water and kept it boiling for about 3 hours. The filtration was performed on the algal material using a steel mesh filter. The product was purified by Ethanol precipitation method. The product was then lyophilized using a Lark Penguin Classic Plus 4kg freeze drier.

d) 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, 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 0.1 aliquots from 10^{-1} to 10^{-7} dilution were spread onto Zobell 2216E marine agar medium. Then, they were incubated upside down at room temperature for 24 hours. Based on morphological features, colonies were randomly picked and purified by making streak plates.

e) Preparation of Inoculum

From the 24 hours incubated ZoBell marine agar slant of each test organism a loop full of the microorganism was inoculated in nutrient broth at pH 7.4 so as to activate the bacterial strains used as test organisms. The broths were kept for incubation at 37°C for 24 hrs to allow microorganism to grow till the log phase.

f) DNA Extraction and Purification

This DNA Extraction and Purification was conducted by using HIMEDIA DNA Extraction kit. Selected colonies were inoculated and after incubated to grow the bacteria culture. And then DNA Extraction method is performed as follow:

Experimental Protocol:

- Bacteria cell pellet were collected in a collection tube and allowed to thaw at room temperature.
- Resuspension of pellet in 180µl of lysis solution I.
- 20µl of proteinase K solution were added and mixed further, incubated for 30 min at 55ºC.
- 20µl of RNase A solution were added and vortex for 10-15 seconds, and incubated for 5 min at room temperature (15-25ºC).
- 200µl of lysis solution II were added and vortex for 15 seconds, and incubated for 10 min at 55ºC.

Binding

- 200µl of 100% ethanol were added to the lysate and mixed by vortexing.
- Lysate were transferred to Spin Column and Centrifuged at 10,000 rpm for 1 min. The supernatant was discarded and spin were placed into a new collection tube.

Prewash

- Prewashed by adding 500µl of prewash solution to the column.
- Centrifuged at 10,000 rpm for 1 min. The supernatant was discarded and placed the column was placed in new collection tube.

Wash

- 00µl of wash solution were added to the column and centrifuged at 13,000 rpm for 3 min.
- The supernatant was discarded and the spin were dried.

DNA Elution

- Transfer the spin to new collection tube and 200µl of elution buffer were added and incubated for 5 min at room temperature.
- Centrifuged at 10,000 rpm for 1 min to elute DNA. Isolated DNA were amplified using thermocycler.

Storage of the eluate with purified DNA

- The eluate contains pure genomic DNA. For short term storage (24-48 hours) of the DNA 2-8ºC is recommended. For long term storage, 20ºC or lower temperature (-80ºC) is recommended. Avoid repeated freezing and thawing of sample which may cause denaturing of DNA. The elution buffer will help to stabilize the DNA at these temperatures.

Quantification of DNA by Nanodrop

The eluted DNA was quantified in the Eppendorf Bio Spectrometer Kinetic at 260/280 nm. The purity and the concentration of the eluted DNA was determined based on the absorbance at 260/280 nm.

Specific Amplification of 16S rRNA Partial Sequence

A clean work surface was used for all PCR preparations. PCR mix was prepared in a laminar air flow chamber. The 16s rRNA amplification reaction mixture (10µl) consisted of 2X master mix (Amplicon, Denmark) with 10 ng of template DNA and 10 pmol of each primer. PCR amplification was performed in Eppendorf AG - 22331 nexus gradient PCR machine.

Initial Denaturation	25 Cycles			Final Extension
95°C 5 Minutes	Denaturation 94°C 1 Minute	Annealing 55°C 1 Minute	Extension 72°C 1 Minute	72°C 5 Minutes

The reaction mixture was incubated in thermocycler. Initial denaturation of DNA at 95°C for 5 minutes followed by 25 cycles of denaturation at 95°C for 1 minutes, annealing of DNA at 55°C for 1 minutes, 72°C for 1 minute's extension of DNA (dependent upon the length of fragment to be amplified; typically, 1 minutes per 1000 nucleotides to be amplified), followed by a final extension at 72°C for 5 minutes and finally set to hold at 4°C until the samples were removed.

Composition of 2X Taq Master Mix RED

- Tris-HCl pH 8.5
- $(NH4)_2SO4$ and 3 mM $MgCl_2$,
- 0.2% Tween 20
- 0.4 mM of each dNTP
- 0.2 units/µl Amplicon Taq DNA polymerase
- Inert red dye and stabilizer

The polymerase chain reaction was carried out with a reaction mixture of 10µL containing as follow:

2X Taq Master Mix RED	Template DNA	Forward Primer (27F)	Reverse Primer (1472R)	Sterile Water (DEPC)
5µL	1µL	1µL (1:10)	1µL (1:10)	2µL

The amplified PCR products were electrophoresed along with 1 kb Ready to Use DNA marker on 1.2% gel at 100 volts for 45 min at room temperature using 1X TAE buffer (Tris– acetate 40 mM pH 8.0, EDTA 1mM, pH 8.0). The PCR products were stained with ethidium bromide and visualized and photographed using Gelstan gel documentation system

Agarose Gel Electrophoresis of Amplified 16SrRNA partial sequence

Agarose gel of 1.6% prepared by dissolving 0.8g of agarose in 50 mL of 1X TAE buffer. After melting it in microwave it was cooled to add Ethidium Bromide and allow to polymerize for 30 minutes in a tray about 3 - 5 mm thick. The amplicons were loaded in the wells and electrophoresis run in 1X TAE buffer at 50V until the inert red dye reaches $3/4^{th}$ of the gel. After electrophoresis, the gel was visualized in UV transilluminator.

Preparation Of Solutions Electrophoresis Buffer

50X stock TAE buffer

Tris base : 25 g
Glacial acetic acid : 50 ml
0.5 M EDTA : 4 g
The solution was made up with distilled water to 1000 ml and diluted to 1X for use.

TAE working buffer (1X concentration)

40m M Tris acetate pH 8.0 1mM EDTA pH 8.0
The amplified PCR products were

Sample buffer (50 ml)
0.1 M EDTApH 8.0 : 2.0 ml of 0.5 M stock
0.001 Tris HCL pH 8.0 : 0.1 ml of 1 M stock
0.25% Bromophenol blue : 25 mg
50% Glycerol : 45 ml
To this 2.9 ml of distilled water was added.

Ethidium Bromide Solution
10 mg of it was dissolved in 1 ml of 1X TAE buffer.

1% Agarose gel
1g of it was dissolved in 100 ml 1X TAE buffer.

16S rRNA SEQUENCE ANALYSIS
The amplified DNA product of 500bp were given for sequencing for Sanger dideoxy sequencing. The sequence that showed >91% sequence identify were retrieved and saved in FASTA format. The FASTA file was opened and multiple sequence alignment was done using the ClustalW tool (Larkin *et al.,* 2007) using Bio Edit software. The multiple sequence alignment was saved in *.meg format and loaded in MEGA X for the construction of phylogenetic tree. The phylogenetic tree was constructed with default parameters using maximum parsimony (character based method), minimum evolution and neighbor joining (distance based method) algorithm. A bootstrap value of 1000 replicates was used to construct a consensus dendrogram. The strain was identified and classified based on the percentage of identity (%), i.e. the sequence showing >95% sequence identity.

Web based servers
BLAST: BLAST (Basic local alignment search tool) is a statistically driven method that finds the regions of similarity between the query sequence and the repository sequence. These are called segment pairs and consist of gapless alignments of any part of two sequences.

CLUSTAL W: This is basic multiple alignment algorithm consisting of three main stages:

- All pairs of sequences are aligned separately in order to calculate a distance matrix giving the divergence of each pair of sequence
- A guide tree is calculated from the distance matrix;
- The sequence is progressively aligned according to the branching order in the guide tree.

Phylogenetic Analysis
Multiple sequence alignments were produced using CLUSTAL W algorithm available in MEGA X (Tamura *et al.,* 2013). Phylogenetic relationships were inferred using the neighbour joining method (saitou *et al.,* 1987) with bootstrapping (Felsenstien, 1985). Evolutionary distances were computed using the P-distance method (Nei et al., 2000) using MEGA X Bootstrap values (1000 iterations) were calculated based on >70% majority rule and confidence limits were placed at the major nodes of the tree.

FT-IR SPECTROSCOPIC ANALYSIS
The lyophilized sample of carrageenan from both healthy and infected seaweed and commercial carrageenan (SIGMA-ALDRICH) were analysed in fourier transform infrared spectroscopy to identify organic and polymeric materials present in the sample. Two milligrams of the sample were mixed with 200mg KBr and pressed into a homogenous pellet and then the pellet was immediately put into the sample holder of Bruker Alpha FT-IR spectrometer in which the spectra bands were recorded in the range of 4000 cm^{-1} to 400 cm^{-1}

GC-MS Analysis
The lyophilized carrageenan samples from both healthy and infected samples were dissolved in methanol and kept in water bath at 60°C for 15 minutes. Then placed in a ultrasonicator for 5 minutes and filtered using 0.45 microns filters. The Clarus 680 GC was used in the analysis employed a fused silica column, packed with Elite-5MS (5% bi-phenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250µm df) and the components were separated using Helium as carrier gas at a constant flow of 1 mL/min. The injector temperature was set at 260°C during the chromatographic run. The 1µL of extract sample injected into the instrument the oven temperature was as follows: 60 °C (2 min); followed by 300 °C at the rate of 10 °C min^{-1}; and 300 °C, where it was held for 6 min. The mass detector conditions were: transfer line temperature 240 °C; ion source temperature 240 °C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The fragments from 40 to 600 Da. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST library.

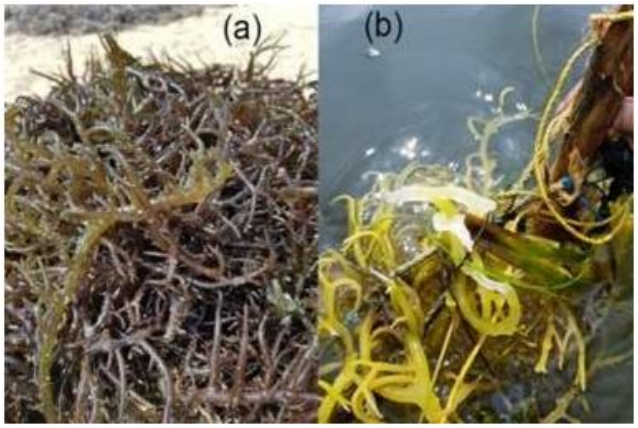
Microbiome Analysis
The thallus of infected seaweed was placed in polypropylene vials and kept in a cooler box along with dry ice were submitted to RTL Genomics, Texas, USA. Triplicate infected samples were collected from the same field in which the microbiome analysis was performed. The microbiome of the infected seaweed was analysed in Illumina MiSeq platform. The universal primer 337F and 805R for bacteria were used for the amplification targeting 16S region. The samples were processed to extract all the genomic DNA and sequenced in Illumina MiSeq sequencer and MiSeq Reagent Kit v3 were used. The raw sequencing data generated on Illumina has been pre-processing MOTHUR which is an open source bioinformatics pipeline for performing microbiome analysis from raw DNA sequencing data. The data analysis pipeline consisting of denoising, chimera checking, FASTQ file generation, quality checking, sequence clustering, taxonomic identification and data analysis.

3. Result & Discussion

Sample Collection (Seaweed)
Sample seaweeds both infected and healthy of *K. alvarezii* were collected from cultivation farms at Andaman & Niccobar. The sample was collected at 30°C in the cultivation farm and the temperature were determined by thermometer. The salinity of the water is 38% and pH of the sea water is 8 which were identified using refractometer and digital pH metre respectively. The humidity is 65% at sample collection site and it was identified by Digital hygrometer. The seaweed materials were collected and stored into sterile borosilicate bottles containing autoclaved seawater and brought in chilled condition, to the laboratory and stored in

4° C. Each sample was thoroughly cleaned and washed to remove the excess salt

Extraction of Carrageenan



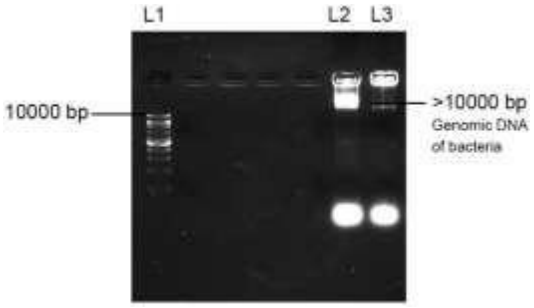
The collected seaweed sample were dried in hot air oven at 60°C for overnight. The Carrageenan were extracted by modified form of Wilson G. Mendoza *et al.,* 2002. The extracted carrageenan was purified andlyophilized. The product appears to be white in colour and in powder in form.

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 and 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. Aliquots of 0.1 ml from 10^{-1} to 10^{-6} dilution were spread on ZoBell 2216E marine agar medium. Then, they were incubated upside down at room temperature for overnight. Number of colonies were counted after incubating at room temperature (25 to 28°C) for up to 2 days (ZMA). Two bacterial strains were isolated from infected seaweed sample in which is observed in 10^{-3} dilution of ZMA plates. Colonies were picked and purified by making streak plates. The isolated pure colonies were maintained using ZoBell marine broth.

DNA Extraction and Purification

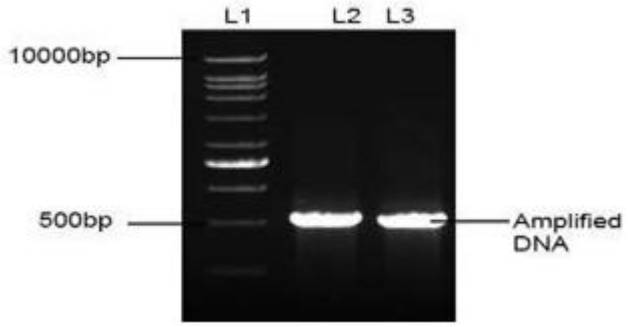
The bacterial genomic DNA were isolated using HiPurA bacterial genomic DNA purification kit from the two bacterial strains. The DNA were eluted and the concentration of DNA were quantified using Nanodrop. After the baseline was corrected, the absorbance was measured at 260 nm. The DNA concentration (µg/µL) of the DNA samples are 4.92 and 6.96. From the concentration of the DNA the absorbance was measured in A260/A280 ratio for the DNA samples are 1.78 and 1.89 with dilution factor 50(µg/mL). The result obtained for the two DNA samples are permissible to carry out further analysis like PCR.



Fluorogram of extracted Genomic DNA from the isolated bacteria, Agarose Gel (0.8%) L1- 1kb DNA ladder, L2- Sample 1, L3- Sample 2

Specific Amplification of 16SrRNA Partial Sequence

The isolated DNA samples were amplified specifically on V3 - V4 region using 337F and 805R universal primer for bacteria with an amplicon size of 500bp. The amplified DNA were further confirmed by agarose gel electrophoresis.



Fluorogram of amplified DNA on V3-V4 region, Agarose Gel (1.2%) L1- 1kb DNA Ladder, L2- Sample 1, L3- Sample 2

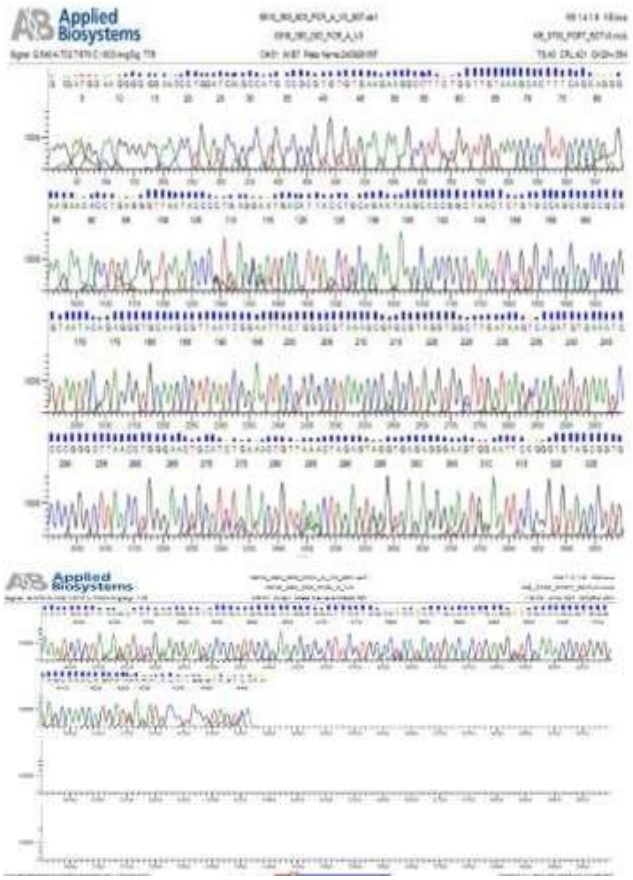
Sequence Analysis

The amplified product of Bacterial strains was sequenced through Sanger dideoxy sequencing method (Eurofins Genomics Pvt. Ltd.). The sequence was obtained in FASTA format were submitted in BLASTn to find out the homologous species with >95% sequence identity from non-redundant database.

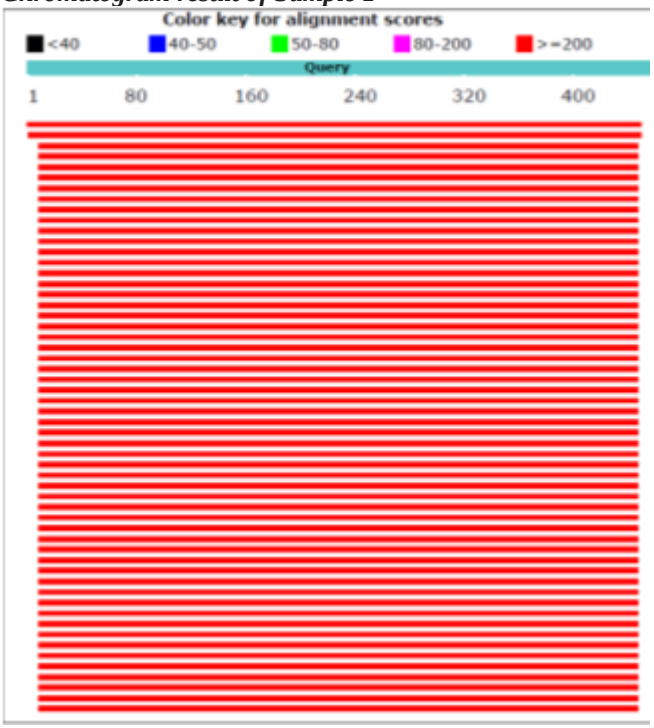
Sequencing Sample 1

>Sample 1
GGGATGGAAGGGGGGAACCCTGGATCCAGCC
ATGCCGCGTGTGTGAAGAAGGCCTTTCTGGTT
GTAAAGCACTTTCAGCAGGGAAGAACACCTGA
GGGTTAATACCCCTGAGGAATGACATTACCTG
CAGAATAAGCACCGGCTAACTCTGTGCCAGCA
GCCGCGGTAATACAGAGGGTGCAAGCGTTAA
TCGGAATTACTGGGCGTAAAGCGAGCGTAGG

TGGCTTGATAAGTCAGATGTGAAATCCCCGGG
CTTAACCTGGGAACTGCATCTGAAACTGTTAA
ACTAGAGTAGGTGAGAGGGAAGTGGAATTCC
GGGTGTAGCGGTGAAATGCGTAAAGATCTGAA
GGAATACCGATGGCGAAGGCGGCTTCCTGGC
CTCATACTGACACTGAGGTTCGAAAGCGTGGG
TAGCAAACAGGAATAAAGAATACCTTGGGTAG
TCAAA



Chromatogram result of Sample 1



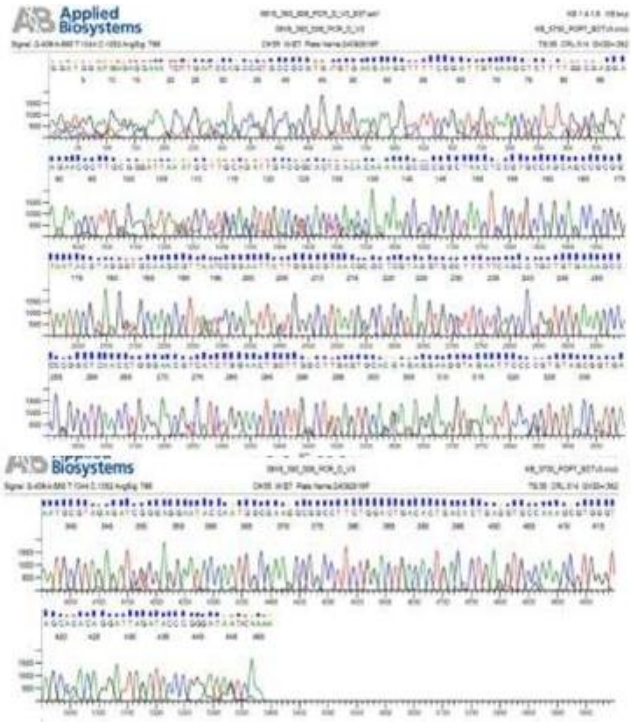
Distribution of top 100 BLAST Hits on 100 subject sequence for Sample 1

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Psychrobacter celer strain E2 16S ribosomal RNA gene, partial sequence	858	858	97%	0.0	94.04%	KM007057.1
Psychrobacter sp. ZJHO2-24 16S ribosomal RNA gene, partial sequence	858	858	97%	0.0	94.04%	KF569828.1
Uncultured bacterium clone SL-129 16S ribosomal RNA gene, partial sequence	858	858	97%	0.0	94.04%	KF571758.1
Psychrobacter sp. D7080 16S ribosomal RNA gene, partial sequence	858	858	97%	0.0	94.04%	FJ161381.1
Psychrobacter celer strain 3-24 16S ribosomal RNA gene, partial sequence	851	851	97%	0.0	93.81%	MK206166.1
Psychrobacter celer strain SCAU-250 16S ribosomal RNA gene, partial sequence	851	851	97%	0.0	93.81%	MH864387.1
Psychrobacter celer strain CHZ-32-Nd 16S ribosomal RNA gene, partial sequence	851	851	97%	0.0	93.81%	MH804584.1
Psychrobacter celer strain M73 16S ribosomal RNA gene, partial sequence	851	851	97%	0.0	93.81%	MH213234.1
Uncultured bacterium clone Shelves_A_132 16S ribosomal RNA gene, partial sequence	851	851	97%	0.0	93.81%	MF992427.1
Uncultured bacterium clone Filter_A_15 16S ribosomal RNA gene, partial sequence	851	851	97%	0.0	93.81%	MF991715.1

Homology search for 16S rRNA gene of Sample 1 against nr/nt database

Phylogenetic tree based on neighbour joining method with the Psychrobacter celer of 16s rRNA of sample 1

>Sample 2
GGATGGAATGGAGAGGAAATCCTTGAATCCAGCCA
CTGCCGCGTGATGTGAAGAAGGTTTTCGGATTGTA
AAGCTCTTTTGGCGAGGAAGAACGCTTGCGGGATT
AAATGCTTGCAGATTGACGGCACTCACACAAAAAG
CCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT
ACGTAGGGTGCAAGCGTTAATCCGGAATTATTGGG
CGTAAAGCGCTCGTAGGTGGCTTCTTCAGCCTGAT
GTGAAAGCCCCCGGCTCAACCTGGGAACGTCATCT
GGAACTGCTTGGCTTGAGTGCACGAGAGGAAGGTA
GAATTCCCCGTGTAGCGGTGAAATGCGTAGAGATC
GGGAGGAATACCAATGGCGAAGGCGGCCTTCTGG
ACTGACACTGACACTGAGGTGCCAAAGCGTGGGTA
GCACACAGGATTAGATACCCGGGATAATACAAAA

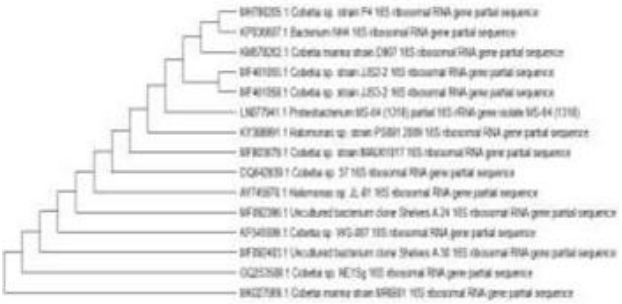


Chromatogram result of Sample 2.

Distribution of top 100 BLAST Hits on 100 subject sequence for Sample 2

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Cobetia sp. NE15g 16S ribosomal RNA gene, partial sequence	543	543	82%	4e-151	90.45%	GQ253889.1
Cobetia sp. WG-007 16S ribosomal RNA gene, partial sequence	544	544	92%	1e-150	90.12%	KF346886.1
Cobetia sp. strain IMAUE1017 16S ribosomal RNA gene, partial sequence	540	540	94%	2e-149	89.74%	MF800879.1
Cobetia sp. strain JJS2-2 16S ribosomal RNA gene, partial sequence	540	540	93%	2e-149	89.88%	MF461088.1
Cobetia sp. strain KMM 6096 16S ribosomal RNA gene, partial sequence	538	538	93%	7e-149	89.88%	MK067632.1
Cobetia sp. strain DZ-03-12-car 16S ribosomal RNA gene, partial sequence	538	538	93%	7e-149	89.88%	MK077260.1
Cobetia sp. strain SA031 16S ribosomal RNA gene, partial sequence	538	538	93%	7e-149	89.88%	MH067429.1
Cobetia marina strain CKC16023 16S ribosomal RNA gene, partial sequence	538	538	93%	7e-149	89.88%	MK063862.1
Cobetia marina strain CKC15023 16S ribosomal RNA gene, partial sequence	538	538	93%	7e-149	89.88%	MK063861.1
Cobetia marina strain CKC14023 16S ribosomal RNA gene, partial sequence	538	538	93%	7e-149	89.88%	MK063859.1

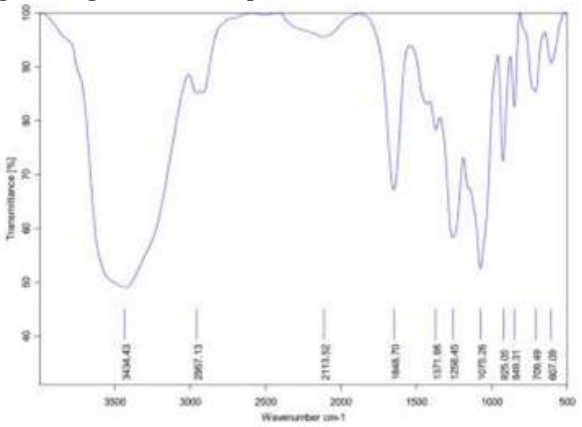
Homology search for 16S rRNA gene of Sample 2 against nr/nt database



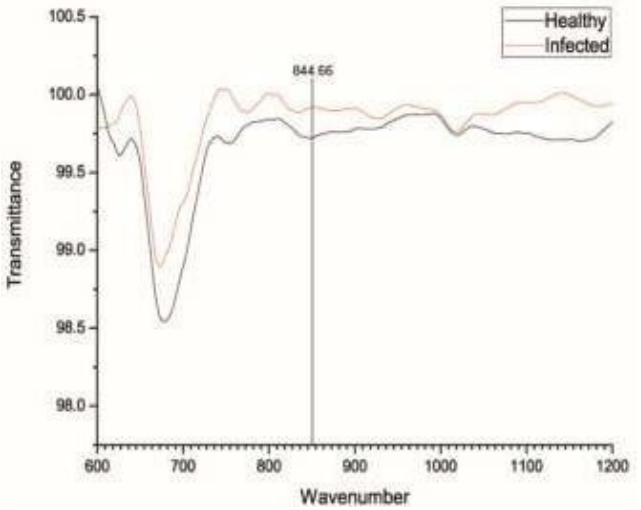
Phylogenetic tree based on neighbour joining method with the Cobetia marina of 16s rRNA of sample 1

FT-IR SPECTROMETER ANALYSIS

FT-IR spectrometer results were analysed and compared with commercial carrageenan and the extracted carrageenan from both healthy and infected sample. The absorption peaks observed in healthy sample at 844.6 cm^{-1} which is found to be D-galactose-4-sulphate but no absorption peaks were found in infected sample this confirms the absence of D- galactose-4-sulphate in sample of carrageenan extracted from infected seaweed. The former peak, which was observed at 840 cm–1 to 850 cm^{-1} to be D-galactose-4-sulphate (Dewi *et al.*, 2015; Webber *et al.*, 2012). In commercial carrageenan the peak for D-galactose-4-sulphate were found at 849.31 cm^{-1} which confirmed the range of D-galactose-4-sulphate to be 840 cm^{-1} to 850cm^{-1}.



FT-IR spectra of commercial carrageenan which shows a peak at 849.31 cm^{-1} to be D-galactose-4- sulphate.



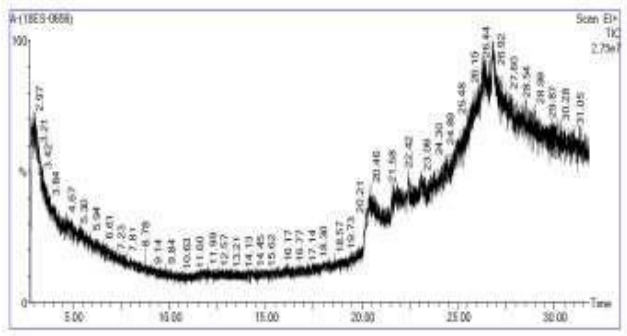
FTIR spectra of Healthy and Infected carrageenan sample.

The absorption peak of D-galactose-4-sulphate at 844.6 cm^{-1} is present in carrageenan extracted from healthy sample and no absorption peak found in the range of 840 cm^{-1} to 850 cm^{-1} in carrageenan extracted from infected sample.

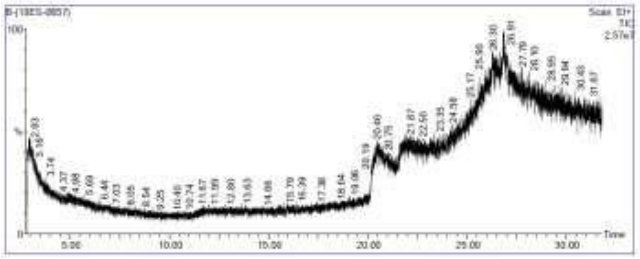
GC-MS ANALYSIS

The carrageenan is composed of major components namely, galactose and 3, 6 anhydro-D-galactose as well as the sulphur content. The chemical profile of the carrageenan was compared with NIST library and the metabolites present in the samples were identified by GC-MS. Based on the mass spectral values molecular weight and the nature of the metabolite was identified.

The GC-MS spectra show 13 peaks in both healthy and infected extracted carrageenan sample. At the retention time of 27.464 min the spectrum of the component comparing with NIST library which matches to 3,4-anhydro-D-galactosan in the healthy sample and at 27.739 min a peak detected in infected sample in which it found to be 2,3-anhydro-D-galactosan by comparing the spectrum with NIST library. Carrageenan is a disaccharide which composed of two anhydrosugars linked with glycosidic linkage.



GC-MS analysis of carrageenan extracted from healthy sample



GC-MS analysis of carrageenan extracted from infected sample

Sample	Retention Time	Compound	Peak area %
Healthy	27.464	3,4-anhydro-D- galactosan	3.585 %
Infected	27.739	2,3-anhydro-D- galactosan	3.421 %

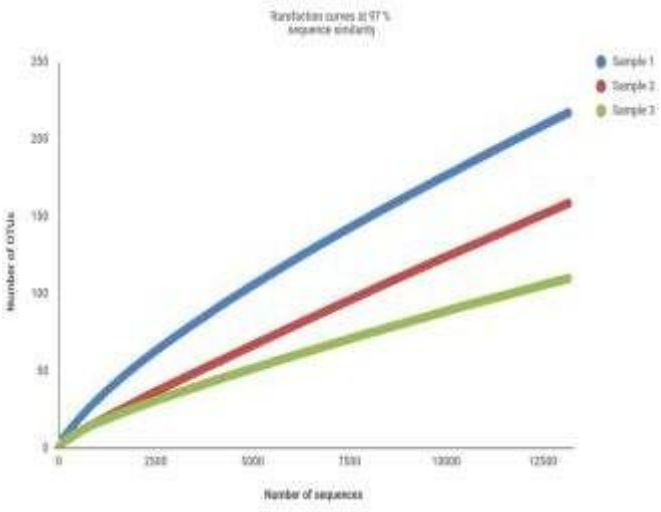
Comparison of retention time and compounds obtained with NIST library in GC-MS analysis

Microbiome Analysis

The cooler box and dry ice maintained -70°C until the sample received by the RTL Genomics, USA. The sample is processed to isolate all genomic DNA of bacteria present in the infected seaweed thallus. Targeted 16S region microbiome analysis conducted on Illumina MiSeq platform in which amplification of V3 - V4 hypervariable region has been amplified using 337F and 805R universal primer. The amplicons were sequenced in Illumina MiSeq sequencer using MiSeq Reagent Kit v3. A raw sequencing data were obtained from the sequencer and data analysis has been successfully completed in MOTHUR a bioinformatics pipeline for microbiome analysis from a raw DNA sequencing data. The data has been denoised and checked for chimeric 16S rRNA sequence. This data has been checked for quality based on:

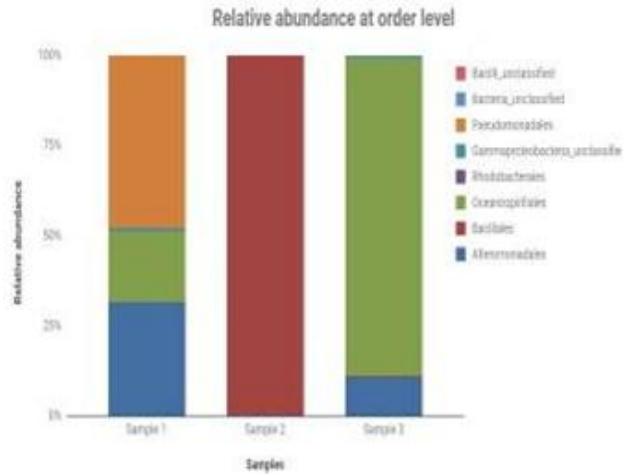
- Sequences must be at least half the expected length given the primer sets used.
- Sequences must contain a valid error free barcode.

The passed sequence is condensed into single FASTA formatted sequence. OTU selection program has been done in order to select OTUs based on certain methodologies.

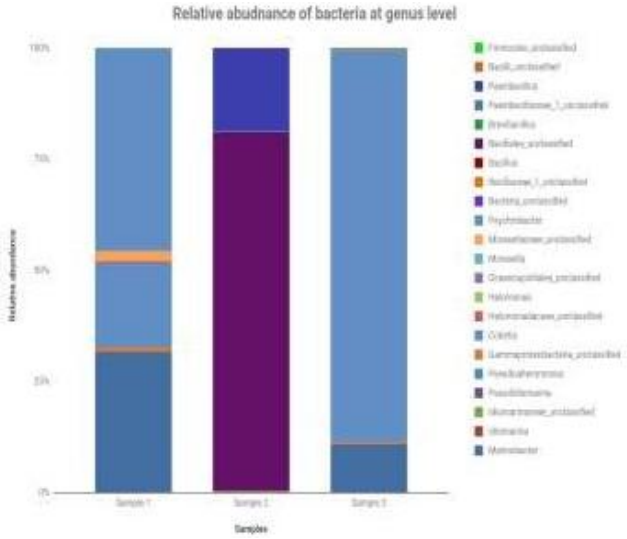


Sequence similarity curves obtained as an outcome of successful DNA sequencing. Upscaled OTUs denoted the cluster of similar sequence variants

Taxonomic identification has been done using USEARCH global alignment program in which the data is identified using a database of high- quality sequences derived from NCBI. The processed data subjected to diversity analysis program in which the microbial diversity among infected seaweed has been successfully obtained.

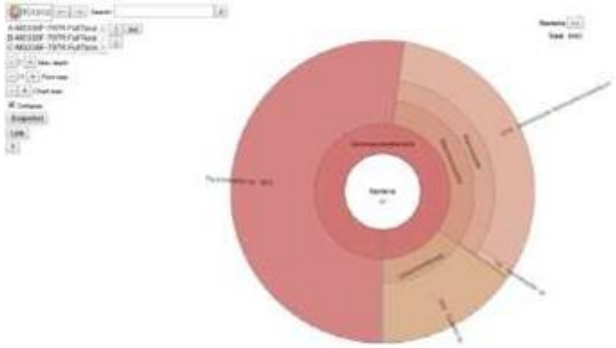


Order level relative abundance.
Order: Pseudomonadales rich in sample 1; Order: Bacillales rich in sample 2;
Order: Oceanospirillales rich in sample 3

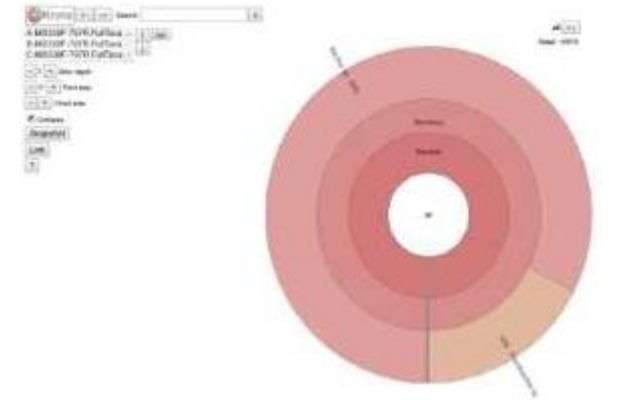


Genus level relative abundance.
Genus: Psychrobacter rich in sample 1; Genus: Bacillus rich in sample 2;
Genus: Cobetia rich in sample 3

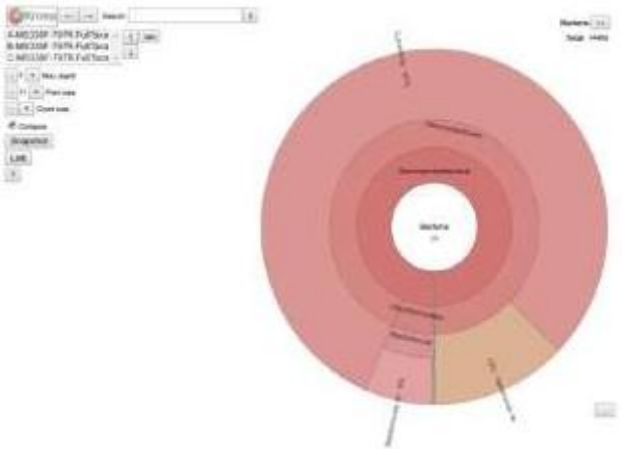
Krona visualization has been created which represent the whole taxonomic identification data up to genus level.



Krona full taxonomical visualization for sample 1 indicates 52% of Psychrobacter sp. in sample 1



Krona full taxonomical visualization for sample 2 indicates 82% of Bacillus sp. in sample 2



Krona full taxonomical visualization for sample 3 indicates 81% of Cobetia sp. in sample 3

4. Summary and Conclusions

The work is based on the analysis of microbiome present in the infected seaweed of *Kappaphycus alvarezii.* These seaweeds are farmed in the purpose of yielding carrageenan. *Ice-ice* diseases reduce the yield of carrageenan ranging from 25% to 40% (Trono 1993). Few previous works on *ice-ice* diseases reported that the diseases is caused by several microorganisms. In which the attempt to identify the microbiome present in the infected seaweed, targeting on bacteria. Bacteria from the infected seaweed were isolated from the infected thallus of the infected farmed seaweed. Infected region of thallus appears to be white and hardened, bacteria were isolated by homogenize the infected thallus and diluted in distilled water up to 10^{-4} concentration. The diluted sample is cultured by ZoBell marine agar medium by spread plate method 100 µL of diluted sample were spread on the marine agar medium using glass rod. This procedure was taken place in a laminar air flow chamber in order to achieve the expected result of isolating various marine microbes. The plates were incubated at 37℃ for 24 hours. Few colonies were isolated and purified by streak plating method and the purified colonies were picked randomly and cultured in a ZoBell marine broth to identify the species.

This research work was also targeted on the quality of carrageenan produced by the infected seaweed. A comparative analysis of carrageenan between healthy and infected seaweed was carried out. Carrageenan from both

healthy and infected samples were extracted and analysed in FT-IR and GC-MS. Carrageenan is composed of two saccharides linked by glyosidic bond. The extracted sample were subjected for IR spectra analysis in which the functional group present in the sample were identified. D-galactose-4-sulphate is present in the carrageenan whereas healthy sample show a peak for the D-galactose-4-sulphate at 844.6 cm^{-1} and infected sample has no absorbance in that range. This shows that the functional groups of carrageenan have some change in infectedsample

This work analysed the micro biome present in the infected farmed *Kappaphycus alvarezii* and analysed the quality of carrageenan present in the infected seaweed. This study will help in better understanding of micro biome which takes place in the occurrence of *ice-ice* diseases and quality of carrageenan on the infected seaweed.

References

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