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Antimicrobial activities of secondary metabolites and phylogenetic study of sponge endosymbiotic bacteria, *Bacillus* sp. at Agatti Island, Lakshadweep Archipelago

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ABSTRACT

Twenty-one species of sponges were recorded under the class of Demospongiae and Calcareous sponges of which 19 species were new to Agatti reef. A total of 113 Sponge endosymbiotic bacterial strains were isolated from twenty-one species of sponges and screened for antimicrobial activity. Five bacterial strains of sponge endosymbiotic bacteria (SEB) namely SEB32, SEB33, SEB36, SEB43 and SEB51 showed antimicrobial activity against virulent marine fish pathogens such as *Vibrio alginolyticus*,*Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Aeromonas salmonicida*, *Flavobacterium* sp., *Edwardsiella* sp., *Proteus mirabilis* and *Citrobacter brackii*. The secondary metabolites produced by SEB32 from sponge *Dysidea fragilis* (Montagu, 1818) [48] was selected with broad range of antibacterial activity and subjected for production, characterization by series of chromatography techniques and spectroscopic methods. Based on the results of FT-IR and mass spectrometry, the active molecule was tentatively predicted as "Pyrrol" and the structure is Pyrrolo[1,2-*a*]pyrazine-1,4-dione, hexahydro- with molecular formula of C7H10N2O2. The LC_{50} of active molecule was 31 μg/ml and molecular weight of the metabolites was 154. The potential strain SEB32 was identified by gene sequence (GenBank Accession number JX985748) and identified as *Bacillus* sp. from GenBank database.

1. Introduction

Marine ornamental fish culture is a profitable industry in several countries of Asia, and its developing sector along the coastal states of Indian subcontinent. However, diseases are recognized as a major constraint as well as limiting factor for sustainable production of fishes in captive condition. It is well known that aquatic organisms come in direct contact with the ambient microbes continuously, which may act as opportunistic pathogens [38]. Therefore, it is very difficult to prevent diseases caused by opportunistic pathogens during the entire culture period. Medication of aquatic organisms cannot be restricted to the diseased individuals and as a result, resistant microbial strains may develop, which change the normal microbial composition leading to massive outbreaks of the disease. Initial management of such outbreaks by reducing the stocking density or carrying capacities is considered as uneconomical, due to increased production costs. The total sterility of rearing water in the entire culture period by pretreatment is practically impossible [3]. Although post-infection therapy using medicated feeds incorporated with antibiotics is possible to a certain extent [35]. Hence, cost-effective treatment technologies need to be developed for controlling/preventing such outbreaks especially due to opportunistic and secondary pathogenic invaders. Therefore, in the present study, due emphasis was given for the eco-friendly utilization of sponge endosymbiotic bacteria as source material for the development of novel metabolites to prevent the fish diseases.

The phylum Porifera has 7000 described and 15,000 estimated species worldwide, divided in three taxonomic classes: Calcarea, Hexactinellida and Demospongiae [23]. Sponges were invertebrate filter feeders, which live in areas with strong currents or wave

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action and important benthic fauna to builds coral skeleton in their reef surroundings. Most of the carnivorous animals avoid sponges because of the splinter-like spicules and toxic chemicals produced/sequestered by the sponges [39]. Symbiotic microbes in marine invertebrates can dramatically impact the ecosystem functioning by changing the phenotype of their hosts [17]. Sponges can host heterotrophic bacteria, archaeabacteria, cyanobacteria and unicellular algae [21]. Mainly bacteria and algae play key role in nutrition absorption, metabolic transport in sponges [46].

Bioactive metabolites are often present in minute concentrations and it may be produced by sponge or sponge endosymbionts. So far, increasing evidence has demonstrated that most of the sponge-derived natural products come from sponge-associated microorganisms [26,7]. Accordingly, the isolation and scale-up cultivation of sponge-associated microbes, which produce the bioactive natural products, may solve the current bottleneck problem of supply limitation [10]. Hence, the present study was carried out to find out the potential lead of endosymbiotic bacteria protection towards the inhibition of multi-drug resistant and emerging fish pathogens in aquatic systems.

2. Material and methods

2.1. Collection and identification of sponge samples

By skin dive and SCUBA diving methods at sub tidal habitats of 3 m to 40 m (deep photic zone) depth twenty-one species of sponges were collected at different locations of Agatti island (Lat. 10° 51′ N, Long. 72° 11′ E), Lakshadweep archipelago. An aliquot of 5 gms of each sponge samples were collected and kept into sterile polythene bags contains Phosphate Buffer Saline (5 mM, pH-8). Further, they were transported to the laboratory for the isolation of sponge endosymbiotic bacteria (SEB). For identification of sponge's underwater photographs and adequate documentation like locality, habitat, depth, surface features and colour etc were noted. Sponges were immersed for a while in 5% buffered formaldehyde. Then, the specimens were transferred and preserved immediately in 70% methanol or formalin for further identification. Calcareous sponges are not fixed or preserved in formalin. The sponges were identified using acid digestion method recommended by Hooper [23]. The confirmation of the sponge species was carried out by Dr. P. A. Thomas, Scientist (Retd) CMFRI (ICAR).

2.2. Isolation of sponge endosymbiotic bacteria and screening for antimicrobial activity

All the Twenty-one sponge samples were subjected to isolation of endosymbiotic bacteria followed by Gopi et al. [18]. Using colony morphology different types of bacterial colonies were isolated and were repeatedly streaked to obtain pure cultures and stored in Zobell agar slants at 4 °C for further studies. Bacterial strains named as SEB 1 to SEB 113 and antibacterial activity was determined by using culture filtrate of sponge isolates against fish pathogens followed [18] such as *Vibrio alginolyticus* (FP1), *Vibrio vulnificus* (FP2), *Vibrio parahaemolyticus* (FP3), *Aeromonas salmonicida* (FP4), *Flavobacterium* sp. (FP5), *Edwardsiella* sp. (FP6), *Proteus mirabilis* (FP7) and *Citrobacter brackii* (FP8) were obtained from the marine ornamental fish culture facility, Annamalai University, India.

2.3. Test against standard MTCC bacteria

Further, the potential sponge endosymbiotic bacterium SEB32 was selected and subjected to confirm the potential activity against standard MTCC (Microbial Type Culture Collection, IMTECH, Chandigarh) bacteria by well diffusion method. The standard MTCC cultures *via*, *Vibrio alginolyticus* (strain no. 4182), *V. vulnificus* (1145), *Aeromonas salmonicida* sub sp. *salmonicida* (1522), *Flavobacterium* sp. (4664) and *Edwardsiella* sp. (2400) were procured from IMTECH, Microbial type Culture Collection Gene bank, Chandigarh were used as positive control.

2.4. Extracellular production and optimization of solvent system for extraction of secondary metabolites

A seed culture of potential bacterial strain SEB32 was prepared in about 50 ml using Zobell marine broth [18] with pH-7.5, salinity-35 ppm (parts per million) in a shaker (30 °C/150 rpm) for 18 h. Inoculum was transferred to 500 ml Zobell marine broth containing 1 l Erlenmeyer flask and cultured with shaking (250 rpm) for 5–9 days at 28 °C. Cells were separated by centrifugation (4 °C/8671*g*). The active crude metabolites were extracted with various solvent systems like *n*-hexane, di-chloromethane, chloroform, ethyl acetate, methanol and DMSO and the extracted secondary metabolites are studied for its bioactivity by disc (25 μg/disc) diffusion method in addition with standard antibiotic discs (30 μg) of Ceftazidime (CAZ) and Cefotoxime (CTX) against fish pathogens. The solvent has been chosen for extraction of crude metabolites by using separating funnel.

2.5. Partial purification of secondary metabolites by thin layer chromatography

The secondary metabolites extracted using ethyl acetate showed maximum activity and selected for further purification. The crude metabolites were further obtained in large scale from the strain SEB32 by shake flask method. An aliquot of concentrated fractions were loaded on the activated silica gel TLC sheet 20 cm × 20 cm (Merck, Germany). The plates were developed using hexane: chloroform (80:20), chloroform: ethyl acetate (90:10), methanol: ethyl acetate (30:70) and ethyl acetate: methanol (90:10) solvents and were tested to determine appropriate solvent system. The methanol: ethyl acetate (30:70) was selected as suitable solvent system based on the fractionation of spots. The visible fractions were obtained and spots were located by exposing the plate to iodine fumes. Fractions having same number of spots with similar *Rf* values on TLC plate were pooled.

2.6. Bioassay-Agar overlay method

The developed chromatogram were placed in a square plate with cover and an inoculum of fish pathogens containing 10^6 CFU/ml in molten Mueller-Hinton agar was distributed over the plate. After solidification of the medium, the TLC plates were incubated overnight at 28 °C. Subsequently the bioautogram was sprayed with an aqueous solution of 2,3,5-triphenyltetrazolium chloride (TTC) and incubated at 37 °C for 4 h. Inhibition zones indicated the presence of active metabolites [32,24]. The corresponding active fraction was concentrated by using rotary evaporator subjected for further study.

2.7. Purification of crude metabolites by column chromatography

An aliquot of three grams of concentrated extract of SEB32 was further separated by column chromatography. Activated silica gel (230–400 mesh, MERCK) was packed on to a glass column (450 mm × 40 mm) with the maximum height of 30 cm using methanol:ethyl acetate (30: 70) solvent and 3 g of extract was loaded on the top of silica gel. Fractions eluted successively with 50 ml of methanol and ethyl acetate (30: 70). Purity of the active metabolites was confirmed by analytical TLC as well as by gas

chromatography analysis using the method described by Wilkins [45].

2.8. FT-IR spectral analysis

The molar concentration of chemical used in Perkin-Elmer, Lamda 2000 to detect the concentration of test sample, varied from 5×10^{-5} to 5×10^{-3} M (in saturated solutions) and of models from 5×10^{-4} to 5×10^{-1} M. Depending on concentration of metabolites and the IR region studied different cells were applied: quartz cells from 1 to 50 mm and KBr cells from 0.066 to 2.66 mm.

2.9. Gas chromatography and mass spectroscopy (GC–MS) characterization of crude extract

The methanol extract of purified metabolites was undergone for the detection of active molecules by GC–MS. It was analyzed using Perkin Elmer Clarus 500 gas chromatography equipped with an Elite-5 capillary column (5% Phenyl 95% dimethylpolysiloxane) (30 m × 250 μm) and mass detector turbomass gold of the company which was operated in EI mode. Helium was the carrier gas at a flow rate of 1 ml/min. The injector was operated at 290 °C and the oven temperature was programmed as follows; 70 °C for 6 min, then gradually increased to 280 °C at 10 min. The identification of components was based on comparison of their mass spectra with NIST 2005 Library.

2.10. Structure elucidation of purified molecule

Based on the above spectral characterization the active metabolites was structurally elucidated

2.11. Determine minimum inhibitory concentration of purified molecule

Minimum inhibitory concentration (MIC) of purified metabolites for each test microorganism was determined with liquid growth antimicrobial assay. The overnight culture of fish pathogens was adjusted with 0.5 Mcfarland tubes. The 0.5 mg of purified metabolites of SEB32 was dissolved in 2 ml solution was sterilized by filtration (Millipore 0.22 filter unit) and diluted into 500 μg, 250 μg, 125 μg, 62.5 μg, 31.25 μg, 31.25 μg/ml in sterile PBS (10 mM, pH 7.4). An aliquot of 100 μl of each diluted solution was incubated in sterilized 96-well plates with 100 μl of fresh media and 100 μl of test microorganisms disposed in each wells. An aliquot of 100 μl media and 100 μl of test organisms used as a negative control. 100 μl media and 100 μl of test organisms diluted standard antibiotic amoxicillin used as a positive control. MIC was considered the lowest antibiotic concentration that showed no increase in the optical density (OD_{600}) read at the microplate reader (Cyberlab, China) after every 3 h of stationary incubation. The experiments were carried out in triplicate.

2.12. Physiological and biochemical characteristics of potential bacteria

The potential bacteria SEB32 from sponges were identified based on the morphological and physiological properties listed in Bergey's manual of systematic bacteriology. The bacterial colonies were identified based on size, colour, form, margin, elevation and opacity. The Gram staining and motility test were performed to identify Gram +ve or −ve, shape and motility of the isolated bacteria. Indole, methyl red, vogues proskeur, citrate utilization, nitrate reduction, production of hydrogen sulphide, urease, catalase, oxidase and fermentation of glucose, galactose, fructose, lactose, glycerol, maltose, mannitol, raffinose, sucrose, xylose and glycerol were performed and identified the bacterial isolates [15].

2.13. ***16S*** *rDNA sequence analysis and identification*

2.13.1. Isolation of genomic DNA

The exponential phase culture of the potential bacteria SEB32 was centrifuged at 14,334*g* for 5 min. Supernatant was discarded and the pellet was dissolved in 567 μl TE buffer, 30 μl of 10% sodium dodecyl sulfate, and 3 μl proteinase K (60 μg). After 1 h incubation at 37 °C, 100 μl of 5 M NaCl was added and mixed thoroughly. The DNA was then purified as described by Altinok et al. [5].

2.13.2. Amplification of 16 rDNA

The 16S rDNA sequences were amplified by Polymerase Chain Reaction (PCR) using universal primers of 8f (3′-AGAGTTT-GATCCTGTGCTCAG-5′) and 1490r (5′-GACTTACCAGGGTATC-TAATCC-3′). The reaction mixture was prepared which consist of 5 μl of 10× buffer (Mg^{2+} free), 5 μl of 2.5 μM $MgCl_2$, 0.5 μl of dNTP mix (2.5 μM each), 1 μl of each primer 2 μl of template DNA and 0.5 μl of Taq DNA polymerase (5U/μl) (Genei, Bangalore), in a final volume of 50 μl. The reaction was performed in a thermal cycler with an initial de-naturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min annealing at 50 °C for 1 min, 72 °C for 90 s and final extension at 72 °C for 7 min. PCR products were electrophoresed on 1% agarose gel and documented under ultraviolet *trans*-illuminator. The PCR product was purified by using PCR purification kit (Genei, Bangalore).

2.13.3. Sequencing and phylogenetic analysis of 16 rDNA gene

The nucleotide sequences of the PCR products were determined by using the automated DNA sequencer with forward and reverse primers (Bioserve Pvt. Bio Technologies, Hyderabad). The sequences was compared with top 10 hits of gene sequence data stored NCBI by nucleotide BLAST. The partially sequenced 16S rDNA of about 1441 (SEB32) bp in length of sponge associated bacteria have been submitted in the GenBank public database and published (NCBI) with accession number. The matching sequence in the GenBank was determined and to infer possible phylogenetic affiliations by using Clustal X 1.8 software and phylogenetic tree was generated by "Neighbor joining" method with phylogenetic division or subdivision or classified category.

3. Result

3.1. Sponges of Agatti Island

Twenty-one species of sponges were collected, identified, classified under the Demospongiae and Calcareous sponges (dominant in the coral reef area). The most abundant sponges in Agatti reef comes under the order of Dictyoceratida which accounts for 7 species in 3 families of Dysideidae, Spongiidae and Thorectidae. Next in the abundance comes under the order of Poecilosclerida and Haplosclerida consists 3 species from 2 families of Microcionidae, Desmacellidae and 3 species from 2 families of Chalinidae, Callyspongiidae respectively. The order Astrophorida consist 2 species from 2 families of Ancorinidae, Geodiidae and order, Calcinea consist 2 species from Clathrinida. Along with low abundance of sponges comes under the orders of Calcaronea, Verongida, Hadromerida and Halichondrida consists each one species from Leucosolenidae, Pseudoceratinidae, Clionaidae and Halichondriidae respectively. The present study twenty-one species of sponges recorded, and 2 species namely *Spongia officinalis* and *Cliona celata* were recorded by Thomas [42], and the remaining 19 species are new records in Agatti Island.

3.2. Screening potential sponge endosymbiotic bacteria

About 113 culturable bacterial strains were isolated in Twenty-one species of sponges. On average, 7 different types of colonies were isolated from each sponge species, pure colonies were maintained using Zobell marine agar plates and total count of bacterial colonies was showed in Table 1. The bacterial isolates nearly 26 sponge associated bacteria alone showed inhibition against marine ornamental fish pathogens (Table 2). The active secondary metabolites secreted by SEB32 isolated from host sponge *Dysidea fragilis* [28] have selected with broad range of antibacterial activity against test fish pathogens and showed maximum zone of inhibition comparatively with other isolates.

3.3. Secondary metabolites from potential bacteria

Potential bacterial strain SEB32 (2000 ml) was produced by shake flask method using Zobell marine broth (pH-7.5, salinity-35, 28 °C/250 rpm) for 5–9 days. Culture was analyzed to determine good yield at alternative days (5th, 7th and 9th day). Based upon the quantity of yield at 9th day culture broth contain better yield of secondary metabolites. The ethyl acetate extract contains active secondary metabolites which showed significant antimicrobial activity against fish pathogens determined by paper disc method.

3.4. Thin layer chromatography

The solvent system of methanol: ethyl acetate (30: 70) was selected to separate crude metabolites using analytical TLC the sample spotted in line and showed clear spots on TLC plate were pooled and numbered (Fr.1–Fr.4). This crude metabolite was subjected to purify by column chromatography.

3.5. Purification of crude metabolites by column chromatography

The active metabolites dissolved in 50 ml of methanol loaded and eluted successfully and 9 fractions measuring 5 ml each were collected and concentrated. Fr.1–Fr.9 was tested for bioactivity by well diffusion method and concentrated active fraction (Fr.2) which showed inhibition against fish pathogens. The active fraction was further analyzed to obtain single molecule by using final step of TLC (Fig. 1) and confirmed the same.

3.6. FT-IR spectral analysis of purified metabolites

Total 9 functional groups were detected at the wave range of 3616 & 3510, 3402, 3288, 3178, 3047, 2927, 2829, 2436, 2353 & 2328 and 1832 (cm^{-1}). Based on the previous literature and standard protocol, the functional classes were characterized as alcohols, phenols, alkynes, carboxylic acid, alkenes, aldehydes, amine hydrohalides and phosphorus functions (Fig. 2).

3.7. GC–MS characterization of purified extract

The purified extract was analyzed using GC–MS in order to obtain chemical profiles, and were compared with NIST (2005) Library, single metabolites were identified through mass spectrum (Fig. 3). The molecular weight and nature of the substance was identified based on the spectral values of mass spectrometry.

3.8. Structure elucidation

Based on the results of UV, FT-IR, NMR (^{13}C and ^{1}H) and mass spectrometry the novel molecule was tentatively predicted as pyrrol and the structure is Pyrrolo[1,2-*a*]pyrazine-1,4-dione, hexahydro- metabolites and molecular formula of (C7H10N2O2). The molecular weight of the metabolites was predicted as 154. The structure was shown in Fig. 4.

3.9. MIC of purified molecule

The minimum inhibition concentration (MIC) of purified molecule was determined by broth dilution assay method. Different concentrations (500 μg, 250 μg, 125 μg, 62.5 μg, 31.25 μg and 31.25 μg/ml) of the lyophilized molecule were tested against the bacterial fish pathogens. The results observed MIC at all concentrations of 500 μg, 250 μg, 125 μg, 62.5 μg, 31.25 μg and 31.25 μg/ml showed positive results against tested pathogens. So, it can be concluded that the purified molecule was able to inhibit the growth of tested bacteria in the *in-vitro* studies and determined the minimum inhibitory concentration LC_{50} was 31.25 μg/ml.

Table 1
Total count of bacteria from 21 sponge species collected at Lakshadweep archipelago. Also the above table gives information depth and association of sponges in Lakshadweep archipelago.

S. No	Name	Depth (meters)	Habitat/ association	Total count cfu/gm
1	*Amorphinopsis excavans*	7	Coral	3×10^2
2	*Callyspongia tubulosa*	3	Coral rocks	2×10^3
3	*Clathira procera*	3	Coral	4×10^3
4	*Clathira* spp.	11	Coral rocks	2×10^4
5	*Clathrina* sp.	16	Coral rocks	3×10^2
6	*Cliona celata*	2	Coral rubbles	2×10^2
7	*Dysidea fragilis*	4	Sand	2×10^3
8	*Dysidea granulosa*	3	Sand	3×10^5
9	*Ecionemia acervus*	2	Live rock	3×10^4
10	*Haliclona nematifera*	5	Live rock	2×10^2
11	*Heteronema erecta*	3	Sand	5×10^2
12	*Hyattella cribriformis*	7	Sand	3×10^4
13	*Lamellodysidea herbacea*	2	Live rock	3×10^2
14	*Lanthella flabelliformis*	7	Live rock	6×10^2
15	*Leucetta chagosensis*	18	Coral rock	3×10^2
16	*Pericharax heterorhaphis*	5	Coral rubbles	4×10^2
17	*Psammaplysilla purpurea*	2	Live rock	3×10^3
18	*Sigmadosia fibulata*	2	Sand	5×10^2
19	*Sigmaxinella cf flabellate*	22	Rock	3×10^2
20	*Spongia officinalis*	4	Coral rock	4×10^2
21	*Stelletta* sp.	5	Coral rock	2×10^2

Table 2
This table proving 26 sponge associated bacteria (SEB) from 21 species of sponges found antimicrobial activity against fish pathogens. Based on wide range of bioactivity and high inhibition during *In-vitro* analysis the bacterium SEB32 was selected for the further production of bioactive compound and characterization.

SL. No	Sponge associated bacteria/Strain No.	Inhibition zone (in mm diameter)							
		Fish pathogens							
		FP1	FP2	FP3	FP4	FP5	FP6	FP7	FP8
1.	SEB11	13	10	10	11	10	9	–	11
2.	SEB14	–	11	9	11	12	–	9	–
3.	SEB16	–	–	–	–	–	7	–	–
4.	SEB17	12	14	12	13	12	8	–	–
5.	SEB21	14	11	12	13	10	–	6	11
6.	SEB22	12	–	–	–	–	–	–	–
7.	SEB24	–	–	–	09	11	–	9	6
8.	SEB31	10	–	–	–	–	–	–	–
9.	SEB32	**18**	**16**	**23**	**21**	**19**	**19**	**18**	**16**
10.	SEB33	14	13	15	14	15	15	17	15
11.	SEB36	16	13	15	17	15	17	18	15
12.	SEB41	–	–	14	11	9	–	–	–
13.	SEB42	11	–	14	–	8	–	11	–
14.	SEB43	17	15	19	17	14	17	15	15
15.	SEB51	15	17	16	19	18	15	14	11
16.	SEB52	–	11	14	11	9	15	–	–
17.	SEB53	12	–	14	16	11	–	8	–
18.	SEB54	13	11	–	–	–	–	–	–
19.	SEB81	6	–	8	–	–	–	–	–
20.	SEB83		9	11	13	–	–	–	–
21.	SEB92	12	15	11	–	8	–	12	–
22.	SEB93	9	11	14	–	9	–	11	–
23.	SEB122	–	15	16	12	14	13	–	–
24.	SEB133	9	13	11	7	9	11	12	8
25.	SEB171	13	11	8	8	12	15	11	9
26.	SEB172	–	9	9	13	11	10	10	8

Vibrio alginolyticus (FP1), *Vibrio vulnificus* (FP2), *Vibrio parahaemolyticus* (FP3), *Aeromonas salmonicida* (FP4), *Flavobacterium* sp. (FP5), *Edwardsiella* sp. (FP6), *Proteus mirabilis* (FP7) and *Citrobacter brackii* (FP8).

Fig. 1. TLC silica gel 60 (5 × 7.5 cm Merck, Germany), Aluminum backed showed single band from partially purified active molecule found at top of the gel. The rings found in middle of the gel show the running downstream to upstream.

3.10. Biochemical characters of potential bacteria

Biochemical characteristics of the potential bacteria SEB32 were gram +ve rods, Spores were found, catalase, MR-positive and citrate utilization was also positive, H_2S production, indole, VP were negative and sugars like sucrose, maltose, fructose and dextrose was utilized and sorbitol, glucose, mannitol, lactose, arabinose were not utilized. Amylase, lipase, gelatinase was positive and urease, phosphatase, litmus milk and lecithinase was not produced.

3.11. Isolation of genomic DNA and agarose gel electrophoresis

In the present investigation, the strain SEB32 was identified by using the PCR amplification of gene followed by the sequencing. The genomic DNA of the bacterial strains SEB32 was isolated and confirmed by agarose gel electrophoresis. The gene was amplified from the genomic DNA of the strain SEB32 under the optimal conditions. The amplified product was analyzed by resolving it in the 1% agarose gel. The gel shows a clear band of 1.5KB corresponding to the marker similar to that the DNA was amplified. 16S rDNA gene of SEB32 was sequenced and analyzed with NCBI database.

3.12. rDNA sequence of SEB32

Molecular identification of the gene shows 99% identity at 100% coverage with *Bacillus* sp. (SEB32). The sequenced gene of strain was identified as is 1441 bp in length, and exhibited 99% similarity with the gene of *Bacillus* sp. from GenBank database. According to the gene, a phylogenetic tree was generated (Fig. 5). The sequenced 16 S rDNA from sponge associated bacteria *Bacillus* sp. SEB32 from *Dysidea fragilis* have been assigned as *Bacillus* sp. (CASMBLAKS32)

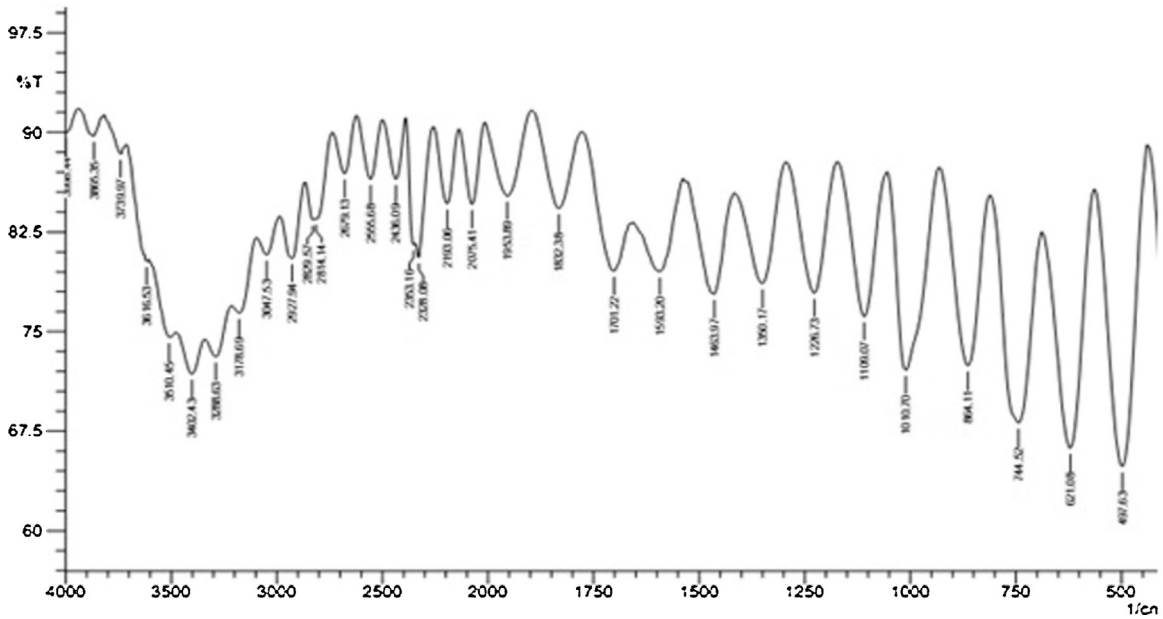

Fig. 2. Active Molecule contains functional groups were detected by FTIR at the wave range of 3616 & 3510, 3402, 3288, 3178, 3047, 2927, 2829, 2436, 2353 & 2328 and 1832 (cm^{-1}). Based on the previous literature and standard protocol, the functional classes were characterized as alcohols, phenols, alkynes, carboxylic acid, alkenes, aldehydes, amine hydrohalides and phosphorus functions.

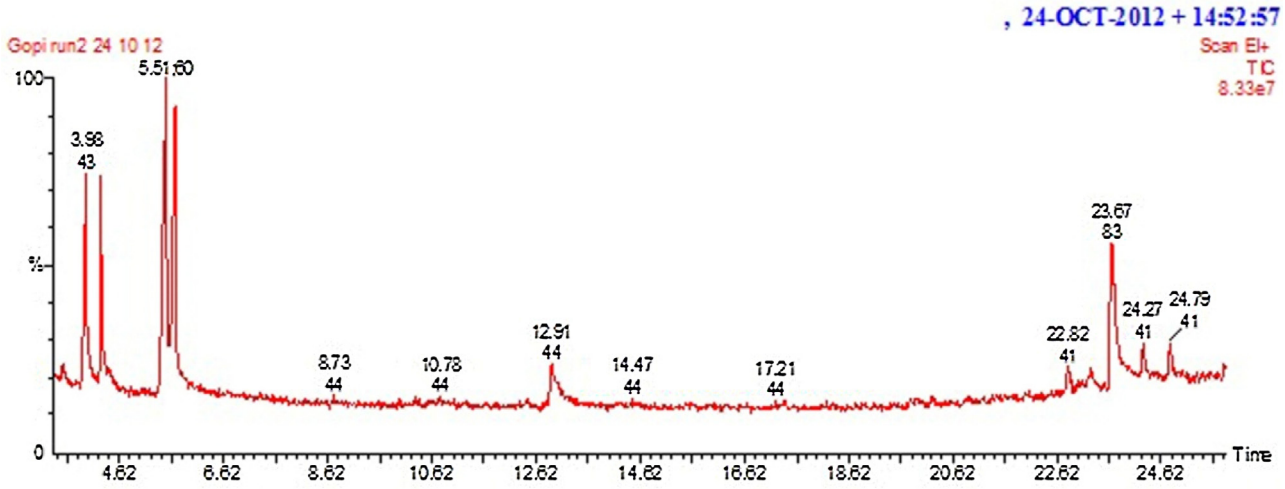

Fig. 3. Partially purified molecule analyzed by Gas Chromatogram-Mass Spectrum analysis of SEB32. Chromatogram (x-axis = Retention time; y-axis = % intensity/% abundance). The potential molecule of *Bacillus* sp. (CASMBLAK32) was found at the Retention time: 23.67, Peak area: 4442024, %peak area: 21.1465, the Molecule name is Pyrrolo[1,2-*a*]pyrazine-1,4-dione, hexahydro- identified using NIST library 2005.

and deposited in the GenBank public database with accession number JX985748.

4. Discussion

Multicellular organisms have developed defence systems to recognize invading microorganisms and eliminate them [6]. Fishes affected disease through intrinsic and extrinsic environmental factors and transportation of fishes for commercialization. Thus, the aquaculture industry has been overwhelmed with its share of diseases and problems caused by viruses, bacteria, fungi, parasites and other undiagnosed and emerging pathogens. So that there is a distinct need for the discovery of new safer and more effective antibacterial agents to control fish diseases.

Numerous natural products from marine invertebrates showed striking structural similarities to metabolites of microbial origin especially sponges, suggesting that microorganisms are the true source of these metabolites or are intricately involved in their biosynthesis [37]. Lakshadweep sea sponges harbor diverse bacteria with metabolic potential from biodiversity to biotechnological prospecting is unrevealed except antimicrobial potential of few sponges reported by Gopi et al. [19]. So, investigations related to sponge associated microorganisms have expanded. In this study, culturable bacteria associated with twenty-one species of sponges at Agatti reef alone are studied and they are Demospongiae and calcareous sponges were recorded. Thomas [42] who studied the sponge fauna of Lakshadweep recorded 91 species in 10 islands and only 6 species were recorded at Agatti. The present collection of twenty-one sponge species from Agatti Island of which Thomas [42] was recorded 2 species namely *Spongia officinalis* and *Cliona celata*, and the remaining 19 species are new records in Agatti Island. This is the third report on sponges in Lakshadweep waters,

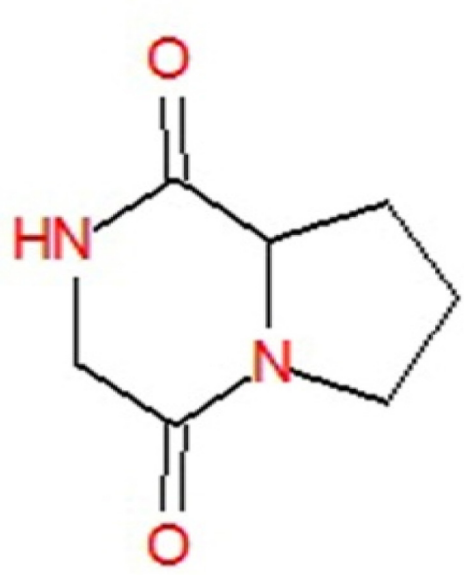

Fig. 4. The structure of active molecule Pyrrolo[1,2-*a*]pyrazine-1,4-dione, hexahydro-an extracellular molecule secreted by sponge bacteria *Bacillus* sp. (CASMBLAK32). Formula: C7H10N2O2 and molecular weight of the compound is 154.

the first being that of Thomas [42]. The present collection of total 113 culturable bacterial strains were isolated, screened against marine ornamental fish pathogens and selected potential bacterial endosymbiont of sponge. Sponge associated bacteria required unique nutrients to multiply inside the host sponge.

Fish diseases and various symptoms such as, septicemia, hemorrhaging at fins and body surface, tail rot, fin rot, accumulation of ascetic fluid in the abdominal cavity and exophthalmia caused by various pathogens [13,12]. Choudhury et al. [9] reported the antibacterial properties of organic extracts of 15 species of sponges collected from coastal waters of Gopalpur (Bay of Bengal) showed activity against six virulent fish pathogens. The present study describes, of the 113 sponge bacteria, 35% of bacteria only were showing antibacterial activity some of species specific against test pathogens, rest of the bacteria not showing any kind of inhibition against tested pathogens. Few of sponge bacteria were showed broad range of inhibition and releases potential secondary metabolites against tested pathogens. In this study, *Bacillus* sp. (SEB32) host sponge *Dysidea fragilis* [28] was selected with broad range of antibacterial activity and inhibited the growth of all tested fish pathogens and showed maximum zone of inhibition comparatively to others.

Marine sponges are known to be important sources of novel marine natural products. Sponge-associated microorganisms represent a treasure house of biodiversity for the discovery of marine natural products with biotechnological potential. 18 species of *Dysidea* analyzed to date, more than 400 originate from about

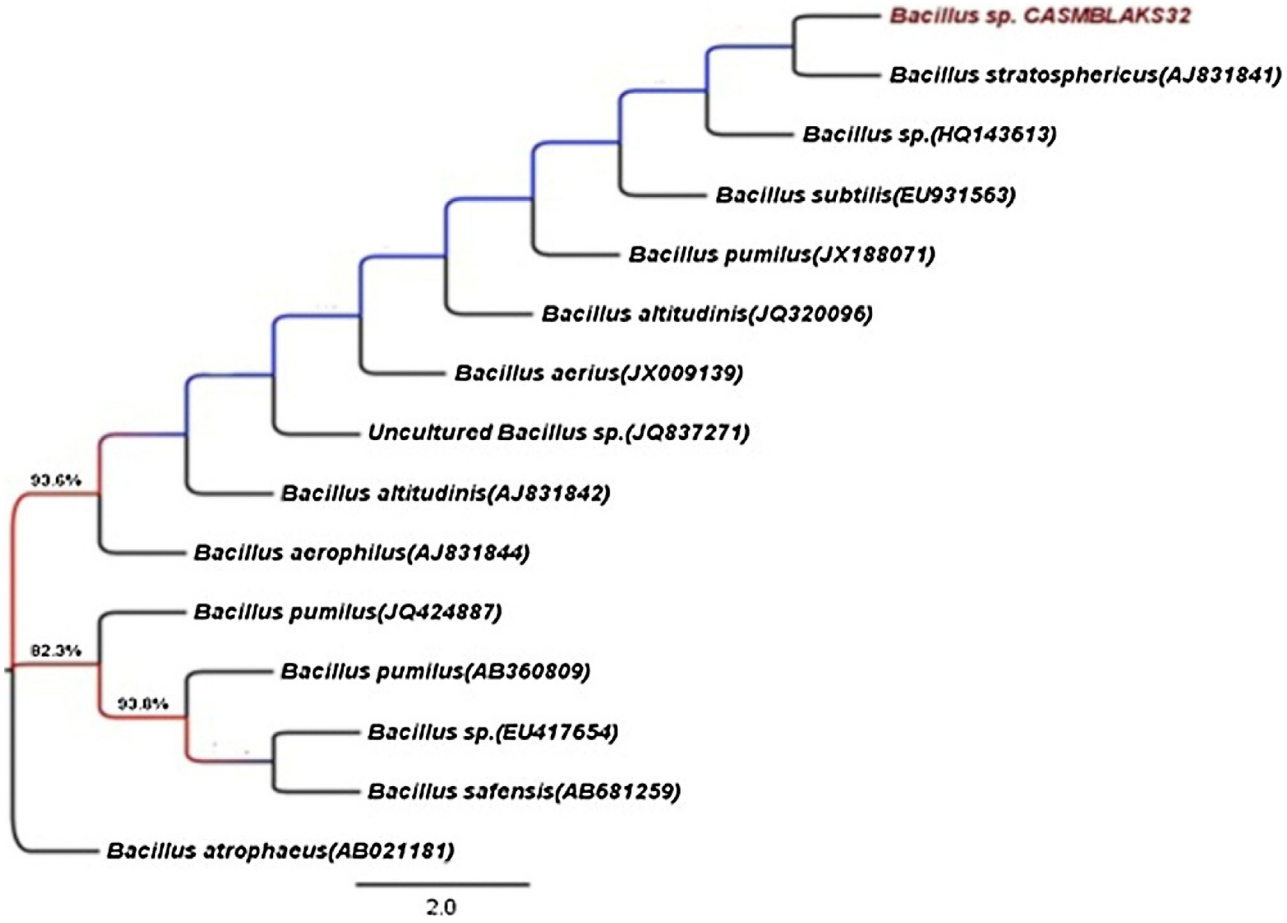

Fig 5. The phylogenetic tree was constructed using the neighbor joining method with 100 re-samplings in bootstrap analysis of sponge endosymbiotic bacteria (SEB 32) *Bacillus* sp. (CASMBLAK32). Bar scale 2.0. Pseudomonas.
KUMS3 was used as an out group. Queried sequence: *Acinetobacter* sp. TTAG (HM004071).

thirty as yet un-described *Dysidea* species [8]. Increasing importance on sponge species diversity is reflected in finding the diversity of secondary metabolites by sponges in recent times. Van Soest et al. [43] reviewed in aspect *Dysidea* is a particularly species rich taxon with more than 50 described species and many more undescribed species. *Dysidea* sponge-derived biologically active marine natural products include polybrominated phenyl ethers, chlorinated amino acid derivatives, furano-sesquiterpenes and polyhydroxy steroids [44,47,27].

As earlier reported by Anand et al. [4], 77 marine bacterial strains associated with four species of sponges (*Echinodictyum sp.*, *Spongia sp.*, *Sigmadocia fibulatua* and *Mycale mannarensis*), agar-overlay method was used to screen for antibiotic production. In this study, used the culture filtrates of isolated bacteria were screened for antimicrobial activity against fish pathogens by well diffusion method. Antimicrobials are thought to confer a selective advantage, when in competition with other bacteria populating for their survival in the same ecological niche. The functions of metabolites include the prevention of phagocytosis by predators and the establishment of symbiotic interactions with invertebrate hosts and their role as virulence factors (phytotoxins) in microbe plant interactions [40]. Many bacteria, fungi, actinomycetes and cyanobacteria associated with sponges were found to be the sources of antibiotics and other bioactive metabolites in marine environment. As reported earlier by Dhayanithi et al. [13], some of the bacterial pathogens from ornamental fishes identified as *A. hydrophila, Enterobacter sp.*, *E. coli*, *P. aeruginosa*, *Proteus sp.*, *Streptococcus sp.*, *V. cholerae*, *V. alginolyticus*, *V. parahaemolyticus* and *Yersinia enterocolitica* were controlled by neem extracts. Hentschel et al. [21] reported the antimicrobial activities of marine sponges *Aplysina aerophoba* and *A. cavernicola* with phylogenetic analysis.

Various studies have confirmed the predominance of gram-negative bacteria found in the marine environment particularly marine *Bacillus* species are often isolated from sediments and invertebrates [34]. In the marine environment, members of the genus *Bacillus*, known for the production of metabolites with antimicrobial, antifungal or generally cytotoxic properties were regularly isolated from invertebrates and thus display a high potential in the search for new antimicrobial substances [30]. The present study has been clearly shown that *Bacillus* sp. bacteria associated with sponge reflects the chemical defends in the host and useful in nutrition cycling both in host and surrounding ecosystem.

Sponge-associated microbial fermentation will provide a strategy to overcome the bottleneck problem of supply limitation in the research and development of sponge-derived marine natural products. This study was the first to optimize the cultivation of sponge-associated microbes for the production of marine natural products with pH-7.5, salinity-35 ppt at 28 °C/250 rpm for 9 days. The maximum bacteriocin like substance produced by *Bacillus* sp. strain P34 was obtained in a medium supplemented with soybean protein, but no antimicrobial activity was obtained by supplemented with fish meal, feather meal, whey, and grape waste [29]. Zobell marine broth was optimized for the production of secondary metabolites of *Bacillus* sp. and medium supplemented by peptic digest animal tissue, yeast extract for basic carbon and nitrogen source and other trace elements like sodium, ammonium, magnesium, ferric and strontium was provided for the microbial production as like in natural sea water. Symbiotic microbial functions that have been attributed to microbial flora include nutrient acquisition, stabilization of sponge skeleton, processing of metabolic waste and secondary metabolite production [22].

This study examined ethyl acetate extract of potential bacteria for their antibacterial activity against 8 pathogenic bacteria. Some of these test bacteria isolated from marine ornamental fish [13,12] have been described as potential aquatic pathogens or are involved in water contamination or finfish and shellfish diseases, it is of great economic importance, therefore, to find organisms possessing antibacterial activity against fish pathogens. The single molecule was subjected to antibacterial activity by disc diffusion method and LC_{50} value of purified fraction 31.25 μg/ml and the same was analyzed at 310 nm by UV–vis spectroscopy for purity confirmation. In the past two decades the scientific community has under laid objectives in search of secondary metabolites produced by many classes associated with diverse groups of sponges in various study area and they have reported prospective drugs showing antibacterial, antifungal, antiviral, antifouling and many other bioactivity for clinical and aquatic pathogens.

The purified metabolite was analyzed using GC–MS in order to obtain a basic idea of the metabolites present and to compare the chemical profiles of each peak. Several quorum-sensing signals were found in the purified fraction. Surprisingly, the metabolites of *Bacillus* sp. had some major peaks in common. The present study assumes that major peaks are relevant to the potent bioactivity shown by the extracts according to study carried out by [41], RIC (Reconstructed ion chromatogram) data [20] and the GC peaks were present at 16.302 corresponding to Pyrrolo (1, 2-*a*) pyrazine 1, 4-dione, hexahydro 3 (2-methyl propyl) and at 17.181, but the corresponding mass spectra could not be identified in the database.

Two new bromopyrrole alkaloids, damipipecolin (1) and damituricin (2), have been isolated from the Mediterranean sponge *Axinella damicornis*. The Mediterranean sponge *Axinella verrucosa* has been investigated for its alkaloid composition and has been found to produce a complex mixture of bromopyrrole alkaloids [1,2]. The species *Mycale cecilia* produces an array of metabolites characterized by possessing a pyrrole-2-carbaldehyde nucleus. The pyrrole containing metabolites of *M. cecilia* have shown activity as growth inhibitors of various tumor cell lines, in particular against the human prostate cancer cell line LNcaP [33]. In the present study peak area was also detected at 0.513 ppm, and this may be expected as active metabolites. Finally the pyrrol was predicted and the structure obtained. Pyrrolo[1,2-*a*]pyrazine-1,4-dione, hexahydro- formula (C7H10N2O2) with the molecular weight of MW:154.

SEB32 strain phylogenetically analyzed in this study and manual comparison with the compounds produced by certain bacteria reported in diverse species of sponges at various study area reported by De Rosa et al. [11], Anand et al. [4], Swagatika et al. [41], Jin et al. [25], Phelan et al. [36], Liu et al. [16] The genomic DNA of the bacterial strain SEB32 was isolated and confirmed by agarose gel electrophoresis. Molecular identification of the gene shows 99% identity at 100% coverage with *Bacillus* sp. The sequenced gene of strain (1441 bp in length) is the gene of *Bacillus* sp. from GenBank database.

In the previous studies, *Bacillus* sp. was found to produce a highly active metabolites reported by Anand et al. [4]. Many antibiotics including cyclic peptides, cyclic lipopeptides and novel thiopeptides have been reported from marine *Bacillus* sp. [31]. Until 2002, 12 bioactive metabolites were reported from marine *Bacillus* sp. [14], Bacillamide C production by the optimized cultivation of the *Bacillus atrophaeus* strain C89 associated with the South China Sea sponge *Dysidea avara* [25]. In the past two decades, the scientific community has under laid objectives in search of secondary metabolites produced by many classes associated with diverse groups of sponges in various study area and many scientists isolated *Bacillus* sp. from diverse group of sponges and obtained therapeutic enzymes, novel metabolites and bactericidal agent for aquatic pathogens [11,22,4,41,25,36,16]. In the present study, concluded potential bacteria SEB32 was confirmed as *Bacillus* sp. by sequence analysis and these bacteria

is potential producer of antimicrobial agents and can be used in pharmaceutical and aquaculture culture sectors.

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