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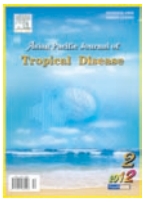

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Antagonistic activity of marine sponge associated *Streptomyces* sp. against isolated fish pathogens

G. Palani Selvan[1], S. Ravikumar[1*], A. Ramu[2], P. Neelakandan[1]

[1]*School of Marine sciences, Department of Oceanography and Coastal Area studies, Alagappa University, Thondi Campus, Thondi, Ramanathapuram District, Tamil Nadu, India*

[2]*School of Chemistry, Department of Inorganic Chemistry, Madurai Kamaraj University, Madurai District, Tamil Nadu, India*

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ABSTRACT

Objective: To investigate the antibacterial potential of the marine actinomycetes isolated from sponge samples. **Methods:** Thirty six marine sponge samples were collected from Palk Strait and further used for actinomycetes isolation by using serial dilution. The antibacterial activity was carried out by using cross streak assay method. Moreover, most potential strain also subjected to MIC and MBC techniques and the isolated potential strain was identified by molecular tools. **Results:** The maximum counts (26 x 10^2 CFU/g) were observed in the month of May and minimum counts (1 x 10^2 CFU/g) were noticed in April. A total of 21 actinomycetes were isolated and their antibacterial potential was assessed by using cross streak method. Among the 21 actinomycetes, the ACT-21 showed sensitivity against all the isolated fish pathogens. Further, the MIC and MBC results reveal that, the ACT-21 showed sensitivity at the concentration ranged between 500 μg/mL-1500 μg/mL. The phylogenetic analysis suggested that, the potential isolate ACT-21 (accession no: JF899543) showed maximum similarity index (>98%) with *Streptomyces* sp. **Conclusions:** It is concluded from present study that, the crude extracts of sponge associated actinomycetes could be used as an effective antibacterial agent for the management of disease free fish culture system.

1. Introduction

In recent years, fishery sector is growing worldwide rapidly. World fisheries and aquaculture production increased as 142 and 53 million in 2008 respectively and valued at around US$99 billion in 2008[1]. However, fish diseases mainly caused by bacteria, fungus, virus and protozoan *etc*. Particularly, bacterial diseases are responsible for heavy mortality in wild and cultured fish[2]. Several aquaculture industries used the chemotherapy for the treatment of various microbial diseases. However, resistance may enter at least one of the tested pathogens. Due to the increase in the outbreak of bacterial diseases in the aquaculture industry and the development of bacterial resistance, new antibacterial agents are required. The terrestrial plant *Cinnomum verum* showed antibacterial and antifungal activity against isolated ornamental fish pathogens[3,4]. Moreover, the marine plants *viz*., seagrasses, seaweeds and mangrove plants have the potency to cure various microbial diseases[5-13]. The routine plant treatments against the microbial diseases lead to loss of biodiversity. Marine sponges are one of the important component of benthic communities[14]. Marine invertebrates have developed highly specific relationships with numerous associated microorganisms and these associations are of recognized ecological and biological importance[12]. Especially, sponge associated actinomycetes having most potential biological activities *viz*., antibacterial[15,16], antiplasmodial[14], antifungal[17] and antifouling activities[18]. However, studies related with antibacterial property of sponge associated actiomycetes against bacterial fish pathogens are limited. In this connection, the present study has been made an attempt to find out the novel antibacterial agent from sponge associated actinomycetes.

*Corresponding author: Dr.S.Ravikumar, School of Marine sciences, Department of Oceanography and Coastal Area studies, Alagappa University, Thondi Campus, Thondi-623409, Ramanathapuram District, Tamil Nadu, India
Tel: 04561-243470
E-mail:ravibiotech201321@gmail.com

2. Materials and Methods

2.1. Sample collection

Thirty six sponge samples (MSP1-MSP36) were collected through by-catch from Thondi coast of Palk Strait (9 °44′ 12″ N and 79 °10′14″ E) during the month of August 2008-July 2009. The samples were sealed in a pre-sterile sip-lap plastic bag and stored in an ice-box and immediately transferred to the laboratory.

2.2. Isolation of actinomycetes

One gram of sample was mixed with 100 mL of presterilized 50 percent seawater blank with 100 r/min for 1 h. About 1 mL of the sample was plated on starch casein agar medium (Soluble starch: 10g; Casein: 1g; Agar: 18g; Aged seawater: 500ml; Distilled water: 500ml; pH 7.2±0.2; autoclaved at 15lbs for 15 min; Nalidixic acid: 20 μg/mL; Nystatin: 25 μg/mL; Cycloheximide: 100 μg/mL) by using spread plate method. Triplicates were maintained and the plates were incubated for 7-10 days at (28±2) ℃[19]. The colonies were selected based on the colour and morphological differences, powdery colonies were counted and restreaked thrice in a yeast extract malt agar (ISP2) (Glucose: 4g; Yeast extract: 4g; Malt extract: 10g; Agar: 18 g; Aged seawater: 500ml; Distilled water: 500 mL; pH 7.2± 0.2).

2.3. Fish pathogens

Five fish pathogens *viz.*, *Bacillus* sp. RPAUOCAS1, *Bacillus* sp. RPAUOCAS2, *Bacillus cereus* RPAUOCAS3, *Bacillus* sp. RPAUOCAS4 and *Bacillus* sp. RPAUOCAS5 were isolated from infected fish *Mugil cephalus* and identified by conventional method. The isolated strains were deposited in NCBI under accession number JF899538, JF899539, JF899540, JF899541 and JF899542 respectively (data not shown).

2.4. Primary screening

The antagonistic activity of actinomycetes was tested by cross streak assay method[20]. Single streak of the isolated actinomycetes strains were streaked on the surface of the Mueller Hinton agar (HIMEDIA, MUMBAI) plates and incubated at room temperature of (28±2) ℃ for 5-7 days. After obtaining a ribbon-like growth, the overnight culture of chosen bacterial fish pathogens were streaked perpendicular to the original streak of actinomycetes and incubated at (28 ±2) ℃ at 24 h. Control plate was also maintained without actinomycetes to assess the normal growth of bacteria. The potential actinomycetes strains were also subjected for secondary screening.

2.5. Secondary screening

A loopful inoculum of potential actinomycete strain (ACT-21) was inoculated into 500 mL Erlenmeyer flask containing 100 mL of yeast extract-malt extract broth (ISP2) and kept at 28℃ for 72 h with continuous shaking. Twenty milliliter of the broth culture was then transferred to 1000 mL of glycerol asparagine broth (ISP5) (Glycerol: 10g; Asparagine: 1g; Dipotassium hydrogen phosphate: 1g; Sodium chloride: 5; Trace solution: 1ml (Trace solution-$FeSO_4$: 0.1g; $MnCl_2$: 0.1g; $ZnSO_4$: 0.1g; distilled water: 100ml; pH 7.2±0.2) and incubated for 7 days under continuous shaking in a rotatory shaker. Filtered cell free broth (pH 7.2) was adjusted to pH 5.0 using 1N hydrochloric acid mixed with equal volume of ethyl acetate in a separating funnel and the upper organic phase was concentrated in a vacuum evaporator at 40℃ for 24 h. The biochemical constituents such as sugars, phenol, proteins, amino acids, quinines, tannins and alkaloids were analyzed[21].

2.6. Minimum Inhibitory Concentration (MIC)

Five hundred microlitre (500 μL) of varied concentration of extracts (125, 250, 500, 1000 and 1500 $\mu g.\ mL^{-1}$) were mixed with 450 μL nutrient broth and 50 μL of 24 h old bacterial inoculum. Whole setup in triplicate was also incubated at 37℃ for 24 h in thermostat shaker. After incubation, the tubes were then examined for microbial growth by turbidity observations.

2.7. Minimum Bactericidal Concentration (MBC)

A loopful inoculum of broth culture from each MIC tubes were streaked on the nutrient agar plates and then incubated at 37 ℃ for 24 h. MBC was recorded as the lowest concentration that prevents the growth of bacterial pathogens on this solid media.

2.8. 16S rRNA gene amplification and sequencing

Genomic DNA was isolated by using standard method[15] and amplified by PCR with universal primers F243 (5′-GGA TGA GCC CGC GGC CTA-3′) and R513GC (5′-CGG CCG CGG GCT GCT GGC ACG TA-3′). The reaction mixture contained 25 to 50 ng of DNA, Ex *Taq* PCR buffer, 1.5 mmol/L $MgCl_2$, 10 mmol/L deoxynucleoside triphosphate mixture, 50 pmol of each primer and 0.5 IU of Ex *Taq* polymerase. PCR conditions consisted of an initial denaturation at 94 ℃ for 5 min; 30 cycles at 94℃ for 1 min, annealing 58℃ for 1 min and 72℃ for 1 min and final 5 min extension at 72℃ . The amplification products were examined by agarose gel electrophoresis and purified by using a QIA quick PCR clean up kit with the protocol suggested by Qiagen Inc. The 16S rRNA partial gene was sequenced by using the PCR products directly as sequencing template with above mentioned primers. All sequencing reactions were carried out with an ABI 3730 automated DNA sequencer (Applied Biosystems, Monza, Italy).

2.9. Construction of phylogenetic tree

The retrieved gene sequences were compared with other bacterial sequences by using NCBI BLAST. Multiple sequences alignment and the phylogenetic tree were constructed with MEGA 4.0 software (http://www.megasoftware.net) by using

neighbour joining (NJ) method with 1000 replicates as bootstrap value and NJ belongs to the distance -matrix method[15].

3. Results

The result of the present study reveals that, 21 different sponge associated actinomycetes were isolated from sponge samples and identified with their morphological characteristics (Table 1). The counts of actinomycetes were found maximum (26 x 10^2 CFU/g) in the month of May and minimum (1 x 10^2 CFU/g) counts were noticed in April. The counts of actinomycetes were not reported in months of August, October, November and March (Fig. 1). However, the actinomycetes counts were found maximum (18 x 10^2 CFU/g) during the summer season (Fig.2). The antibacterial assay revealed that, 6 actinomycetes showed antibacterial activity and the results are depicted in table 2. Among them, the ACT-21 showed antibacterial sensitivity against all the tested pathogens. The MIC and MBC values of ACT-21 ranged between 500-1500 μg/mL (Table 3&4). Preliminary biochemical analysis of the cell free extracts (ACT-21) was showed the presence of alkaloids and quinines (Table 5). The BLAST analysis of the

Table 1.
Morphological characteristics of isolated actinomycetes (ACT)(Out of the 36 sponge samples during 12 months collection period)

S. No.	Strain No.	Colour of aerial mycelium	Reverse side colouration	Diffusible pigment	Size of the colony (mm)
1.	ACT-01	Ash with white	Yellow	-	4
2.	ACT-02	Orange	White	-	3
3.	ACT-03	Yellow white	Yellow	-	3
4.	ACT-04	White	Dull yellow	-	2
5.	ACT-05	Ash white	Dull white	-	3
6.	ACT-06	Greyish	Ash	-	5
7.	ACT-07	Dull ash	Dull yellow	-	6
8.	ACT-08	Ash with white	Dull yellowish white	-	4
9.	ACT-09	White	Dull yellow	-	3
10.	ACT-10	Dull greyish white	Butter white	-	1
11.	ACT-11	Gray	Light green	-	3
12.	ACT-12	Gray	Light gray	-	3
13.	ACT-13	Gray	Butter white	-	3
14.	ACT-14	Dull ash with white	Dull brown	Light pink	4
15.	ACT-15	Milky white	Butter white	-	2
16.	ACT-16	White	Butter white	-	3
17.	ACT-17	Dull white	Butter white	-	4
18.	ACT-18	Ash	Butter white	-	3
19.	ACT-19	Dark ash	Dull ash	-	5
20.	ACT-20	Ash	Brown	Pink	3
21.	ACT-21	Ash	Dull yellow	-	3

Table 2.
Antibacterial activity of sponge associated actinomycetes against fish pathogens

Sponge associated actinomycetes	Bacterial fish pathogens				
	Bacillus sp. RPAUOCAS1	*Bacillus* sp. RPAUOCAS2	*Bacillus cereus* RPAUOCAS3	*Bacillus* sp. RPAUOCAS4	*Bacillus* sp. RPAUOCAS5
ACT-5	+	++	++	+++	-
ACT-7	+	++	++	-	+
ACT-15	-	+	+	-	-
ACT-16	+++	+	+	+	-
ACT-18	++	++	+	+	-
ACT-21	+++	+++	+++	+++	++

"+++"- high, "++"-medium, "+"-low, "-"- absence of inhibition

Table 3.
Minimum inhibitory concentration of crude extract obtained from sponge associated actinomycete (ACT-21).

Fish pathogens	Concentration of the Extract (μg/mL)				
	125	250	500	1000	1500
FPAU01	+++	++	-	-	-
FPAU02	+++	++	-	-	-
FPAU03	+++	++	-	-	-
FPAU04	+++	++	-	-	-
FPAU05	+++	++	-	-	-

"+" indicates intensity of sensitivity "-" indicates no sensitivity

Table 4.
Minimum bactericidal concentration of crude extract obtained from sponge associated actinomycete (ACT-21)

Fish pathogens	Concentration of the Extract (μg/mL)				
	125	250	500	1000	1500
FPAU01	+++	++	-	-	-
FPAU02	+++	++	-	-	-
FPAU03	+++	++	-	-	-
FPAU04	+++	++	-	-	-
FPAU05	+++	++	-	-	-

"+" indicates intensity of sensitivity "-" indicates no sensitivity

ACT-21 showed maximum similarity (>98%) with *Streptomyces* sp. and the accession number is JF899543 (Fig.3).

Table 5.
Biochemical constituents of sponge associated actinomycete (ACT-21)

Biochemical constituents	Sponge associated actinomycete (ACT-21)
Sugars	-
Phenol	-
Proteins	-
Amino Acids	-
Alkaloids	+
Tannins	-
Quinines	+

"+" present, "+"- absent

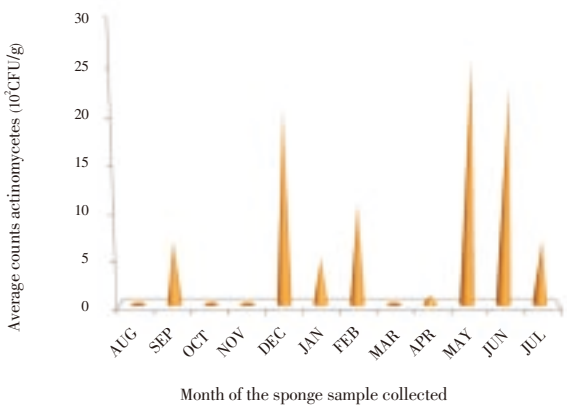

Figure 1. Monthly variation of associated actinomycetes counts in marine sponges

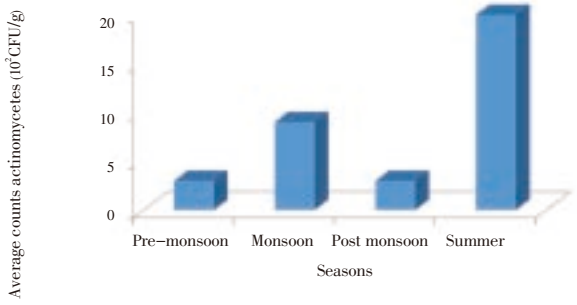

Figure 2. Seasonal variation of associated actinomycetes counts in marine sponges

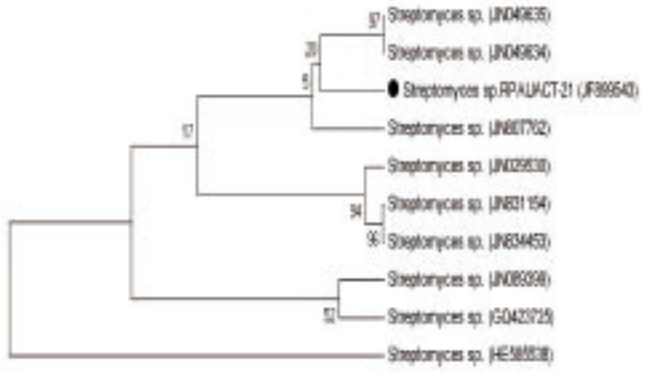

Figure 3. Neighbor Joining phylogenetic tree analysis (16s rRNA) of the isolated actinomycete (ACT-21).

4. Discussion

Natural products from microbes have been an important source for the novel drugs. Generally, bacteria have proven with the vast majority of novel compounds have been discovered till date[14]. Secondary metabolites of actinomycetes possess a wide range of biological activities[14,16]. The genus *Streptomyces* alone produces a large number of bioactive compounds[22]. In the present study the maximum of 21 actinomycetes strains were isolated from sponge samples during summer season (May-July). This might be due to the availability of huge amounts of particulate nutrients introduced into the sea during monsoon by land runoff could deposit the nutrients during summer[23]. Burja and Hill[24] reported that, 228 strains of bacteria, 25 fungal strains, 3 actinomycetes strains and 9 strains of cyanobacteria were isolated from 10 individuals of sponge samples from Australian Great Barrier Reef. The antibacterial potential of actinomycetes suggested that, the maximum antibacterial activity was identified with ACT-21 strain against all the tested pathogens. Joseph et al.[15] and Krishnakumar[25] reported that, the sponge associated actinomycetes showed sensitivity against clinical pathogens and antibiotic sensitive human pathogens. In addition that, the sponge associated *Streptomyces* sp. showed maximum inhibition of Parasitemia[14]. Marine sediment actinomycetes exhibited excellent antibacterial activity against antibiotic resistant bacteria[26]. The antibacterial activity of cell free extract from *Streptomyces* sp. (ACT-21) might be due to the presence of active secondary metabolites such as alkaloid and quinine. Jen Cheng et al.,[27] reported that, the alkaloid isolated from actinomycete *Acrocarpospora* showed good antibacterial and antifungal activity against *S. aureus*, *B. subtilis*, *P. aeruginosa*, *E.coli*, *A.niger*, *C.albicans* and *S.cereviacea*. In addition several authors reported that, the anticancer and antimicrobial activity of actinomycetes due to the presence of active secondary metabolites such alkaloids and quinines[21,23,28]. The MIC and MBC values of the most promising strains of actinomycetes (ACT-21) showed that, the ACT-21 showed sensitivity at the concentration ranged between 500-1500 μg/mL. Gandhimathi et al.[29] reported that, the minimum inhibitory concentration and minimum bactericidal concentration of Nocardiopsis dassonvillei showed sensitivity against tested pathogens at 300-600 μg/mL. Similarly, Ravikumar et al.[5] reported that, the ethanol extracts of *Syringodium isoetifolium* root extracts exhibited MIC and MBC value at 1000 μg. μL^{-1} against 3 bacterial fish pathogens *viz.*, *Aeromonas hydrophila*, *Bacillus subtilis* and *Serratia* sp. The construction of phylogenetic tree was used to find out the relationship between the organisms. It reveals that, the ACT-21 (*Streptomyces* sp. JF899543) was proved to have good antibacterial activity. Similarly, the other species (*Streptomyces* sp. JN049635 and *Streptomyces* sp. JN049634) from the same phylogenetic group was also proved antifungal and nematocidal activity (unpublished NCBI nucleotide data) (Fig 3). Similarly, Ravikumar et al. [28] reported that, the marine sediment *Streptomyces* sp. showed excellent anticancer activity against MCF-7 and MDA-MB-231 cell lines. Several studies proved that, natural products from marine sponges are produced by microorganisms, which are associated commensally or symbiotically with marine invertebrates[30]. To confirm this hypothesis, there has been a great deal of interest in isolating microorganisms with bioactivities from sponge and in recent years, a number of novel compounds with biological activity have been discovered through cultivation of sponge associated microorganisms[14,23,25]. It is concluded from the present findings that, the sponge associated actinomycete could be

used as a potential antibacterial agent for the management of fish diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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