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Exploration of Fish Gut Associated Actinobacteria for its Anti-Microbial and Anti-Quorum Sensing Properties

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ABSTRACT

The study was undertaken to explore the gut associated actinobacteria from marine fish with specific to antimicrobial and anti-quorum sensing activities. A total of 25 actinobacterial strains were isolated from fish gut samples using starch casein agar and Kuster's agar medium. About 12 morphologically different strains recovered from *Rastrelliger kanagurta* (Indian mackerel), *Decapodiformes* (squid), *Lutjanus campechanus* (red snapper) and *Caranx melampyqus* (bluefin trevally) were screened for the antimicrobial activity against *Staphylococcus aureus MTCC96, Escherichia coli MTCC739, Candida albicans*, and quorum sensing inhibition (QSI) against *Chromobacterium violaceum*. The actinobacterial strain SQA4 from *Decapodiformes* (squid) showed both antimicrobial and QSI activity. Strain SQA4, which showed wide range of activity, were selected as the potential strain for further studies. Bioactive metabolites production, strain SQA4 produced bioactive metabolites compound earlier on ISP2 agar when compared to ISP2 broth. Crude antimicrobial from the strain SQA4 was produced by adopting agar surface fermentation and extracted using ethyl acetate. Based on the studied phenotypic characteristics, actinobacterial strain SQA4 was identified as *Streptomyces sp*. In optimization, it was found that various sugars and nitrogen compounds and minerals were found to influence the antiquorum sensing compound production. BLAST and phylogenetic tree analysis of SQA4 shows 99% similarity to sequence of *Streptomyces maritimus.* Further studies like purification and structure elucidation of the compound will be done in future. Thus, the findings suggested that the fish-associated actinobacteria is a promising source for potential bioactive compounds for developing novel therapeutic drugs.

Keywords: *Actinobacteria, fish gut, Anti-quorum sensing, Antimicrobial activity and phylogenetic analysis*

1. INTRODUCTION

Quorum sensing (QS), a communication system in bacteria which exists not only in the same species but also among different species, could regulate many microbial physiological activities, as well as the relationship among different bacteria. In response to the bacterial cell density, the production and sense of some small signal molecules, usually N-acylhomoserine lactones (AHL) in Gram-negative bacteria and oligopeptides in Gram-positive bacteria, the expression of many genes is regulated by bacterial quorum sensing system and in turn the bacterial behavior [1,2]. Since many bacterial physiological activities regulated by quorum sensing system are detrimental, such as virulence factor production and biofilm formation in pathogens, possible function of quorum sensing inhibitors (QSIs) started to be notified [3,4]. Microbial resources are reported as promising source for novel metabolites including Quorum sensing inhibitors. Actinobacteria are Gram positive bacteria that constitute one of the largest bacterial phyla and they are ubiquitously distributed in both aquatic and terrestrial ecosystems. Actinobacteria produce an different form of secondary metabolites with high pharmacological and industrially interest .With the identification of actinomycin, various anti-infection agents like antibiotics have been found from Actinobacteria, particularly from the genus *Streptomyces*

Actinobacteria are the group of Gram positive bacteria which contain high guanine plus cytosine (G+C) in their DNA. They play a vital role in biogeochemical cycling of organic and recalcitrant materials in the environment. Among the microbial resources, members of the phylum actinobacteria are well recognised as a source for novel secondary metabolites. About two third of antibiotics

commercially available in the market are produced by members of the phylum actinobacteria notably the genus Streptomyces. Actinobacterial members are widely distributed in terrestrial and aquatic environments including marine ecosystems [5]. Even the marine sediments are the richest source for bioactive actinobacteria, distribution of actinobacteria in marine organisms like fishes are also documented [6,7,8]. However, reports on anti-quorum sensing activity of fish gut associated actinobacteria are very few [7]. More over, it has been postulated that, emphasis on under-explored niches leads to the discovery of novel bioactive compounds. The present study is an attempt for isolation of fish gut associated actinobacteria from marine fishes to understand their antimicrobial and quorum sensing inhibition properties.

1.1. Related Work

In India, there are very few studies on fish associated actinobacteria and its bioactive activity testing. There are no reports on QSI from fish associated actinobacteria in our country.

Actinobacteria were isolated from different organs viz. skin, gills and gut contents of three species of fishes viz. *Mugil cephalus*, *Chanoschanos* and *Etroplus suratensis* from the Vellar estuary, situated along the southeast coast of India. Among the three fishes, *M. cephalus* harboured highest number of actinobacterial population in all the three body parts examined followed by *C. chanos* and *E. suratensis*. Out of the 40 strains isolated, only six strains (LA-2, LA-8, LA-15, LA-20, LA-29 and LA-35) showed significant L-asparagianse activity and the strains were tentatively identified as *Streptomyces aureofasciculus* (LA-2), *S. chattanoogenesis* (LA-8), *S. hawaiiensis* (LA-15), *S. orientalis* (LA-20), *S. canus* (LA-29) and *S. olivoviridis* (LA-35[6].

Nearly 87 isolates of Streptomyces were found to be associated with the gut of marine ornamental fishes namely *Chaetodon collare* (Red tail butterfly) and *Archamia fucata* (Orange-lined cardinal). Among them, only seven strains showed bioactivity against *Vibrio cholerae*. The seven strains were characterized by conventional methods and the studies strongly suggested the strains belong to the genus *Streptomyces sp*[8].

Later on gut microbiome has focused by many researchers and research is now moving quickly in this field. [9] in 2018 made a work related to the gut microbiota of marine fish. However, it is a field that has had historical interest and has grown significantly along with the expansion of the aquaculture industry and developments in microbiome research. Much recent focus has been on nutritional manipulation and modification of the gut microbiota to meet the needs of fish farming, while trying to maintain host health and welfare. However, the diversity amongst fish means that baseline data from wild fish and a clear understanding of the role that specific gut microbiota play is still lacking. In this review, the factors shaping marine fish gut microbiota and highlight gaps in the research.

In current years, approaches on microbial flora associated with fish gut had deepened our knowledge of the complex interactions occurring between microbes and host fish. [10] investigated on fish gut microbiome, stated that, gut microbiome not only reinforces the digestive and immune systems in fish but is itself shaped by several host-associated factors. Unfortunately, in the past, majority of studies have focused upon the structure of fish gut microbiome providing little knowledge of effects of these factors distinctively and the immense functional potential of the gut microbiome. Recently gained insights into the diversity and functions of the fish gut microbiome. They have also delved on the current approaches that are being employed to study the fish gut microbiome with an aim to collate all the knowledge gained and make accurate conclusions for their application based perspectives. The literature reviewed indicated that the future research should shift towards functional microbiomics to improve the maximum sustainable yield in aquaculture.

Later, the GI microbiota of fish has become a frontier research field. In 2019, [7] explored to the gut-associated actinobacteria from two marine fish with special reference to antimicrobial and anti-quorum sensing activity. A total of 40 actinobacterial strains were isolated from fish gut samples using starch casein agar and Kuster's agar medium. About 14 morphologically different strains recovered from *Rastrelliger kanagurta* (Indian mackerel) and *Panna microdon* (Panna croaker) were screened for the antimicrobial activity against *Staphylococcus aureus* MTCC96, *Escherichia coli* MTCC739, *Salmonella enterica*, *Candida albicans*, and quorum sensing inhibition (QSI) against *Chromobacterium violaceum* and *Serratia marcescens*. Strain IM20, which showed wide range of activity. Thus, he suggested that the fish-associated actinobacteria is a promising source for antimicrobial compounds for developing novel therapeutic drugs.

2. METHODOLOGY

2.1 isolation and identification of actinobacteria from marine fish gut

2.1.1 Sample collection and isolation of fish gut actinobacteria

Marine fish, namely, *Rastrelliger kanagurta* (Indian mackerel), *Lutjanus campechanus* (red snapper), *Caranx melampyqus* (Bluefin trevally), *decapodiformes* (squid) were collected from Kasimedu area, Chennai (Lat: 13.1251°N, Long: 80.2955°E) coastal area, Tamil Nadu. The samples were transported to the laboratory within the minimum possible time to avoid external microbial contamination. After transportation to the laboratory, the fish gut was removed. One gram of homogenates pooled intestinal segments was mixed with 100 ml of 0.85% saline in 250 ml conical flask and the flask was kept in shaker incubator at 55°C for 30 minutes, which

favours isolation of actinobacteria by reducing most unwanted Gram negative and other spore-forming bacteria [11]. All the media used in this study were properly sterilized by autoclaving at 121°C for 20 mins.

2.1.2 Isolation of actinobacteria

The pretreated samples were added into 90 ml of sterile distilled water and serially diluted upto 10^{-5} dilutions using sterile distilled water blank. Starch casein agar (SCA) and Kusters agar medium was prepared in 50% sea water and supplemented with cycloheximide (100 µg/ml) and nalidixic acid (20 µg/ml) to retard the growth of fungi and bacteria other than actinobacteria, respectively. About 0.1 ml of aliquot from 10^{-3}, 10^{-4} and 10^{-5} dilutions were transferred into SCA and Kusters agar plates and spreaded using sterile L rod. All the plates were incubated at 28±2°C and observed from 5^{th} day onwards for upto one month. Colonies with suspected actinobacterial morphology were enumerated, recovered and purified using yeast extract malt extract agar (ISP2 medium) (Shirling and Gottileb, 1966). Pure actinobacterial cultures were maintained as slant stock on ISP2 agar as well as in 30% glycerol broth at 4°C. All the media used in this study were prepared in 50% filtered sea water unless otherwise stated [12].

2.1.3 Characterization and dereplication of actinobacteria

Actinobacterial cultures were inoculated into ISP2 agar plates and incubated for 10 days at 28°C. Cultural characteristics recorded include growth, consistency, aerial mass colour, presence of reverse side pigment and soluble pigment production[13]. Micromorphological characteristics were studied by adopting slide culture method [5]. About 2 ml of ISP2 agar medium inoculated with actinobacterial spores were poured as a thin layer over the surface of sterile microscopic slides. The slides were kept in sterile petriplates and incubated at 28°C for 10 days. Then the slides were observed under bright field microscope at 40x magnification. The recorded microscopic characteristics include presence of aerial mycelium, substrate mycelium, mycelial fragmentation and spore chain morphology. Based on the results of growth pattern of actinobacteria on ISP2 agar medium and microscopic appearance, similar actinobacterial isolates were discarded and different isolates were selected for further investigations. The selected isolates were grouped into Streptomycetes and non-Streptomycetes / rare actinobacteria.

2.2 In-Vitro Screening Of Fish Gut Actinobacterial Cultures For Antimicrobial And Antiquorum Sensing Activity And Selection Of Potential Actinobacterial Strain

2.2.1 Test pathogens

Test pathogens used in this study are *Staphylococcus aureus* MTCC96, *Escherichia coli* MTCC739,*Candida albicans* MTCC227 and *Chromobacterium violaceum* MTCC 2656. These are maintained in nutrient agar plate for future screening.

2.2.2Testing of Antimicrobial activity

Antimicrobial activity of actinobacterial cultures were tested by adopting agar plug method [14].Fresh culture of actinomycetes strains were grown on ISP2 agar media a 37°C for 15 days. Test pathogens were swabbed onto the nutrient agar plates using a sterile cotton swab. Agar plug with 6mm diameter were cut with sterile cork borer and placed on surface of nutrient agar plates seeded with *Staphylococcus aureus* MTCC96, *Escherichia coli* MTCC739, and *Candida albicans* MTCC227. The prepared plates were incubated at 37°C for 24hrs. The inhibition zone of bacterial growth was observed and recorded in millimeter[15]. The actinobacterial strain showing the maximum zone of inhibition was selected as the potential strain for future studies.

2.2.3Testing of anti-quorum sensing activity

Actinobacterial strains were assessed for QS-inhibiting violacein production of the reporter strain *Chromobacterium violaceum* MTCC 2656 by above agar plug method. The appearance of turbid halo pigment less areas was assigned as QSI effect[7].

2.3 Production and Extraction Of Bioactive Compounds From Potential Actinobacterial Strain

2.3.1Production of bioactive compounds

Bioactive compound from actinobacterial cultures was produced by agar surface fermentation [14]. All the actinobacterial cultures were inoculated into ISP2 agar plates and incubated at 28^0C for 10 days for the production of secondary metabolites. During incubation, the extracellular metabolites are secreted into the agar medium.

2.3.2 Effect of fermentation method on bioactive compound production

Effect of solid-state and submerged fermentation on bioactive compound production by the potential strain SQA4 was investigated. Spores of the SQA4 actinobacterial strain was inoculated into five ISP2 agar plates and 100 ml of ISP2 broth. ISP2 agar plates were incubated at 28^0C for 12 days. ISP2 broth containing flasks were incubated in rotary shaker with 95 RPM for 12 days. For every 24 hours, agar plug from YEME agar plates were taken and tested against *C. Violaceum*[16].

2.3.3Production and extraction of bioactive compound by agar surface fermentation

Since the strain SQA4 showed activity only by agar plug method but not in well diffusion method, the actinobacterial strain produced the bioactive compounds only in solid culture but not in liquid culture till 12th day of fermentation. Hence, for further bulk production of bioactive compound from strain SQA4 agar surface fermentation method used. After incubation, mycelial growth was removed aseptically using sterile spatula. The agar medium was cut into pieces and extracted overnight at room temperature using different solvents (1:2 ratio) such as methanol, chloroform, ethyl acetate and n-hexane. The scraped mycelium was also suspended in methanol for extraction of intracellular bioactive compounds. The solvent extracts were concentrated using rotary evaporator and quantified[17].

2.3.4 Testing of active extract against C. violaceum

Antiquorum sensing activity of different solvent extracts was tested against *C.Violaceum*by disc diffusion method[18]. Filter paper disc with 5mm in diameter were impregnated with crude extract obtained from different solvents and dried. Bacterial inoculum was prepared using sterile nutrient broth and inoculated onto nutrient agar plates using sterile cotton swabs. The extract impregnated disc was placed over the nutrient agar plates. Zone of inhibition was measured after 24 hours of incubation at 37°C and expressed in millimetre in diameter.Solvent extract which showed maximum activity against the test pathogen was used to extract large quantity of bioactive compounds from 1000 ml of ISP2 agar medium.

2.4 Optimization of Culture Conditions for the Production Secondary Metabolites

In the classical medium optimization technique, one factor at a time (OFAT) experiments ,only one factor or variable is varied at a time while keeping other variables constant. This method is still in use even today, during the initial stages of medium formulation for the production of new metabolite .The effect ofvarious cultural and environmental conditions on productions of secondary metabolites by the strain SQA4 was studied . The influence of fermentation media on the production of secondary metabolites was studied by cultivating the strain SQA4 in different media concentrations .Basal medium of glucose 1%, yeast extract 1%, NaCl 0.1%, pH 7. In order to determine the effect of various carbon, nitrogen, mineral sources and pH on the secondary metabolite production ,basalmedium was supplemented with various types of carbon , nitrogen , mineral sources and pH[7].

2.5 Characterization and Taxonomy of Potential Actinobacterial Strain

2.5.1 Micromorphology

Micromorphology of potential actinobacterial strain was studied by adopting Transplanting embedding technique. Briefly a rectangular hole was dug out of ISP2 agar plate using sterile knife. Then the spore of the actinobacterial strain was inoculated on the edge of the hole under aseptic condition. A sterile coverslip was placed over the inoculated actinobacterial culture agar. The plate was incubated at 28ºC for 7-14 days. Then the cover slip was aseptically removed using sterile forceps and placed over a clean microscopic slide and the coverslip was fixed by using cellophane tape. Micromorphology pf potential strain was observed under bright field microscope under 10x and 40x magnifications(Balagurunathan et al., 2010).

2.5.2 Cultural characteristics

Cultural characteristics was studied by inoculating the growth of potential strain into different composition of ISP (International Streptomycetes Protocol) .This includes media such as ISP1 (Tryptone yeast extract), ISP3(Oatmeal agar), ISP4 (Inorganic salts_starchagar), ISP5 (Glycerol_asparagine agar), ISP6 (Yeast_iron agar) and ISP7(Tyrosine agar). All the media were prepared by following the guidelines described by[13]. All the plates were incubated at 35ºC for 10 days. Cultural characteristics records include nature of the growth, consistency, aerial mass colour, mycelium growth, presence of reverse side pigments and details of soluble pigment production if available.

2.5.3 Physiological characteristics

Basal agar medium recommended by [13] was used for carbon, nitrogen, mineral and different pH utilization studies. After sterilization of basal agar medium by autoclaving at 15 lb for 15 minutes, about 1% of various ether sterilized sugars were added and poured into petri plates. About one ml of well grown culture of actinobacterial strain SQA4 was inoculated on different sugar containing basal agar plates. Growth was recorded after 8 days of incubation at 28°C. Sugars used in this study include glucose, starch, lactose , mannitol, inositol, rhamnose and raffinose(Hi media). The same procedure was used to study the utilization of nitrogen sources by the potential actinobacteria strains. Effect of different nitrogen sources like yeast extract, malt extract, peptone, KNO3 and soybean meal was also studied using the above procedure.

Effect of pH on growth was determined by inoculating the actinobacterial strain SQA4 onto ISP2 agar medium adjusted to different pH values viz. 6,7,8 and 9. All the plates were observed for growth after 10 days of incubation at 28°C. All the plates were observed for growth after 10 days of incubation.

Effect of minerals on growth was determined by inoculating the actinobacterial strains onto ISP2 agar medium supplemented with different minerals are $MnCl_2$, $MgSo_4$,$CaCl_2$ and $FeSo_4$ at 0.1% concentration. All the plates were observed after 10 days of incubation at 28°C[19].

2.6 Phylogenetic analysis

2.6.1 Sequence Analysis

The genomic DNA of actinobacterial strain was isolated using the InstaGeneTM Matrix Genomic DNA isolation kit. Mycelial growth of actinobacterial strain was picked from ISP2 agar plates and suspended in 1ml of sterile water in a microfuge tube. The content was centrifuged for 1 minute at 10,000 rpm to remove the supernatant and the cells were pelleted. Then 200 µl of Insta Gene matrix was added to the pellet and incubate at 56 °C for 15 minutes and vortexed at high speed for 10s [20]. The mixture was heated in a boiling water bath for 10 minutes and then centrifuged at high speed to separate the matrix. Twenty microliters of the resulting DNA was used in a 50_µl PCR reaction to construct a 16S rRNA gene library. General primers for bacteria 27F (5'_ AGAGTTTGATCCTGGCTCAG_3') and 1492R (5'_ TACCTTGTTACGACTT_3') were used to amplify 16S rRNA genes from DNA 21 extracted from the actinobacterial strain. Final concentrations for 50-µl PCR reactions were as follows: 2 µl diluted DNA (10–100 ng) (or 20 µl for DNA extracted using InstaGeneTM Matrix), 0.2 µM of each primer, 0.2 mM dNTPs, 5 units of Taq polymerase and 1X Taq polymerase buffer. The reaction conditions were: 94 ºC for 3 min, 35 cycles at 94 ºC for 30 s, 55 ºC for 1.5 min, and 72 ºC for 2.5 min, and a final extension at 72º C for 5 min. By using Montage PCR clean up kit (Millipore), the unincorporated PCR primers and dNTPs have been removed. Single-pass sequencing was performed on each template using 518F/800R primers (518F 5'_CCAGCAGCCGCGGTAATACG-3' and 800R 5'_ TACCAGGGTATCTAATCC_3'). Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (Applied Biosystems).

2.6.2 16s rRNA Sequencing

The 16S rRNA sequence of actinobacterial strains were subjected to BLAST similarity search tool. By comparing the sequence similarity with non redundant database of nucleotide sequences deposited at NCBI (http://www.ncbi.nlm.nih.gov/) through BLAST program, closely related homologs were identified. The program MUSCLE 3.7 was used for to process the multiple alignments of sequences. The stability of relationship was admittance by performing bootstrap analysis for 1000 replicates. Divergence times for all branching points in the topology were calculated with the Real Time method using the branch lengths contained in the inferred tree. Bars around each node represent 95% confidence intervals were computed [21]. All positions containing gaps and missing data were eliminated. The evolutionary history was inferred by using the Neighbour Joining method in the MEGA 6 software. Identification of species through sequence similarity between query sequence and reference sequence based on criteria, for 99% or above similarity assigned to reference species, 99-95% similarity assigned to corresponding genus, less than 95%, assigned to corresponding family [22]. Pure genomic DNA was isolated from the cell and it was amplified with 16S rRNA gene. Sequencing analysis of sequence obtained by ABI 3130 Genetic Analyzer was subjected for BLAST search in GenBank. The partial 16S rRNA nucleotide sequence of the potential actinobacteria strain SQA4 was deposited to GenBank database.

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Actinobacteria From Marine Fish Gut

3.1.1 Sample collection from coastal area

Samples were collected from the kasimedu Beach, Chennai coastal area and gut was removed from the fishes and homogenized in a surface sterilized mortar and pestle (**Figure 1 and 1A**).

Indian mackerel (*Rastrelliger kanagurta*)

Red snapper(*Lutjanus campechanus*)

Bluefin trevally (*Caranx melampygus)*

sheer fish (S*comberomorus guttatus*)

Figure 1 : collection of different types of fish gut samples

Figure 1A: processing of gut from the fish samples

3.1.2 Isolation of actinobacteria from fish gut

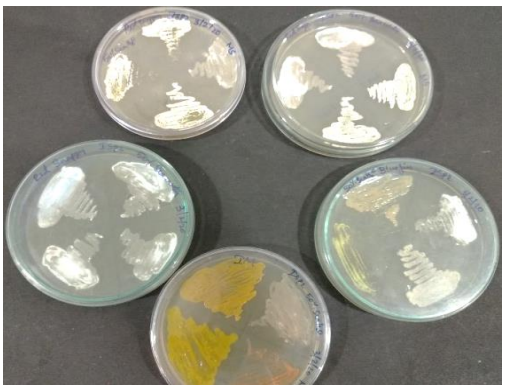


Figure 2: Isolation of actinobacterial strains

Totally 25 strain were isolated from fish gut. In that 12 morphologically different actinobacterial colonies were selected, sub cultured and maintained on ISP2 agar plates (Figure 2) (Table 1). Both aerial and substrate mycelium was produced by all the actinobacterial isolates. Based on their phenotypic characteristics all isolates were identified as Streptomycetes. Researchers reported that about 40 actinobacterial strains which belonged the Streptomyces species were isolated from the estuarine fish (*M. cephalus*, *C. chanos* and *E. suratensis*) gills and the skin [6]. [7] reported 40 actinobacterial strains were isolated from the gut of marine fishes *R. kanagurta*, and *P. microdon* and screened for antimicrobial and anti- quorum sensing activity. According to Squid and Red snapper studies on fish gut actinobacteria were less reported.

Table 1: No. of strains isolated from the fish samples

Fish samples	No. of strains isolated
Squid (*Decapodiformes)*	**4** [SQA1, SQA2, SQA3, SQA4]
Red snapper (*Lutjanus campechanus*)	**3** [RSA1, RSA2, RSA3]
Bluefin trevally (*Caranx melampygus*)	**2** [BFA1, BFA2]
Indian mackerel (*Rastrelliger kanagurta)*	**3** [IM1, IM2, IM3]
TOTAL	**12** Strains

3.1.3 Characterization of actinobacterial strains

During retrieval and preservation, All the actinobacterial strains has showed excellent growth on

ISP2 agar plates. 95% of the actinobacterial cultures showed the presence of substrate mycelium. About 97% of the cultures showed the presence of both aerial mycelium (AM) and substrate mycelium (SM) in which majority of them were Streptomyces species. All the plates were observed for growth, consistency, aerial mycelium (AM), substrate mycelium (SM), aerial mycelia colour (AMC), reverse side pigment (RSP) and soluble pigment (SP) production (**Figure 3**) (**Table 2**).

Figure 3. Morphology of different actinobacterial strains

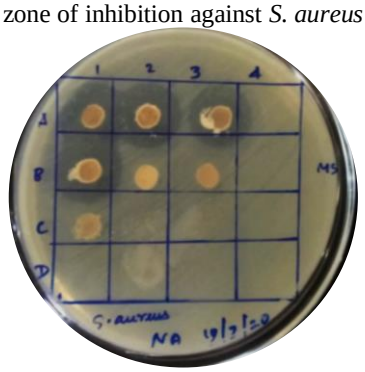
Table 2. Morphological characteristics of actinobacterial strains

S.No	Strain	Cultural characteristics						
1	SQA1	+++	Powdery	++	++	yellow	Nil	Whitish grey
2	SQA 2	+++	Powdery	++	++	yellow	Nil	Whitish grey
3	SQA 3	+++	Powdery	++	++	yellow	Nil	Whitish grey
4	SQA4	+++	Powdery	++	++	yellow	Nil	Whitish grey
5	IM1	++	Powdery	+	+	Nil	Nil	White
6	IM2	++	Powdery	+	+	Nil	Nil	White
7	IM3	++	Powdery	+	+	Nil	Nil	White
8	BFA1	+++	Smoothy	+	+	Nil	Nil	White
9	BFA2	++	Smoothy	+	+	Nil	Nil	White
10	RSA1	++	Powdery	++	+	Nil	Nil	Black
11	RSA2	+++	Powdery	++	+	Nil	Nil	Black
12	RSA3	++	Powdery	++	+	Nil	Nil	Black

3.2. In-Vitro Screening of Fish Gut Actinobacterial Cultures for Antimicrobial and Antiquorum Sensing Activity and Selection of Potential Actinobacterial Strain

3.2.1 Testing of antimicrobial activity

A total of 12 strains were preliminarily screened for their antimicrobial activity by agar plug method against clinical pathogens. Of the 12 strains tested, strain SQA4 from *decapodiformes* (squid) showed maximum zone of inhibition against *S. aureus* MTCC96 (18mm), *E. coli* MTCC739 (18 mm) and *C.albicans* (22 mm) all the strains (**Figure 4**) (**Table 3**). Above all the strains were screened with triplicate.A very few studies on the isolation and screening of antimicrobial activity of fish associated actinobacteria have been carried out[6,23,24,7]. In this study, SQA4 fish gut actinobacteria showed promising activity against clinical pathogens. Previously antimicrobial activity of fish(squid) gut associated actinobacteria was not reported.



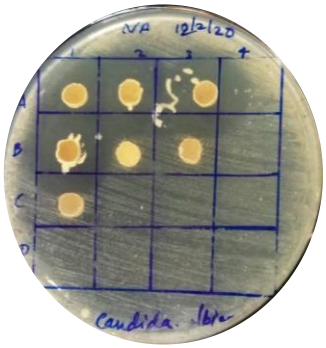


Figure 4: Antimicrobial activity of actinobacterial strains against clinical pathogens

Table 3. Antimicrobial activity of actinobacterial strains by agar plug method

S.no	Strains	Zone of inhibition in mm		
		S. aureus	***E.coli***	***C. albicans***
1	IM1	-	-	-
2	IM2	-	-	-
3	IM3	-	-	-
4	RSA1	-	-	-
5	RSA2	-	-	-
6	RSA3	-	-	-
7	SQA1	17mm	16mm	20mm
8	SQA2	16mm	17mm	19mm
9	SQA3	17mm	17mm	20mm
10	SQA4	18mm	18mm	22mm
11	BFA1	-	-	-
12	BFA2	-	-	-

3.2.2 Testing of anti-quorum sensing activity

In anti-quorum sensing screening activity, strains obtained from the squid fish has showed promising zone of inhibition against *C.violaceum* MTCC 2656 than the other strains. The strains SQA1, SQA2, SQA3 and SQA4 inhibited violet pigment formation of *C.violaceum* without affecting the bacterial growth (**Figure 5**) (**Table 4**).Other strains obtained from the Indian mackerel, bluefin trevally and Red snapper were showed poor activity against *C.violaceum.* From the screening, strain SQA4 has showed 22 mm of zone of inhibition, it has been considered as maximum zone of inhibition(mm). Above all the strains were screened with triplicate (**Figure 6**). A very few studies on the isolation and screening of anti-quorum sensing activity of fish associated actinobacteria have been carried out [7]. In this study, SQA4 fish gut actinobacteria showed promising anti-quorum activity. Study on anti-quorum sensing activity of fish (squid) gut associated actinobacteria was less. So,strain SQA4 selected as a potential strain and taken for further studies (**Figure 7**).

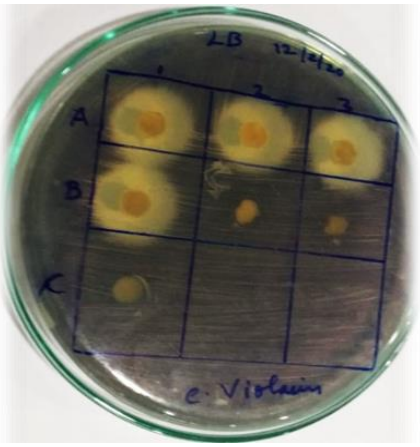


Figure 5. Anti-quorum sensing activity of actinobacteria strains

Table 4. Anti-quorum sensing activity of actinobacterial strains

S. no	Strain. No	(Zone of inhibition in mm) Anti-Quorum sensing activity tested on ***C. violaceum***
1	IM1	-
2	IM2	-
3	IM3	-
4	RSA1	-
5	RSA2	-
6	RSA3	-
7	SQA1	16mm
8	SQA2	17mm
9	SQA3	17mm
10	SQA4	22mm
11	BFA1	-
12	BFA2	-

Figure 6. Triplicate test of potential strain SQA4 against *C.violaceum*

Figure 7. potential strain SQA4

3.3 Production and Extraction of Bioactive Compounds from Potential Actinobacterial Strain

3.3.1 Effect of medium consistency and incubation period of bioactive compounds

The selected SQA4 actinobacterial strain was tested for bioactive metabolite production in submerged and solid medium. Bioactive compound production in solid and submerged medium was assessed from 1-10 days of incubation using *C.violaceum* as test organisms (**Figure 8**).Strain SQA4 produced bioactive metabolites on the 2nd day of incubation when it was grown on ISP2 agar medium, whereas the same strain has showed no activity till the 10th day of incubation when it was grown on ISP2 broth. When compared with liquid fermentation, solid-state fermentation has showed good growth and promising activity till the end of 10th day. In the present study, the bioactive metabolite from strain SQA4 was well produced by agar surface fermentation (**Figure 9**) (**Table 5**).

Figure 8. Solid and submerged state fermentation of SQA4 strain

Figure 9. Production and extraction of bioactive compounds from SQA

Table 5: Effect of medium consistency and incubation period of bioactive compounds

Days	**Strain**	**Solid state fermentation** Zone of inhibition in mm		**Submerged fermentation** Zone of inhibition in mm	
1st	SQA4	+	-	+	-
2nd	SQA4	++	17mm	+++	-
3rd	SQA4	+++	20mm	+++	-
4th	SQA4	+++	20mm	+++	-
5th	SQA4	+++	20mm	+++	-
6th	SQA4	+++	22mm	+++	-
7th	SQA4	+++	22mm	+++	-
8th	SQA4	+++	22mm	+++	-
9th	SQA4	+++	22mm	+++	-
10th	SQA4	+++	22mm	+++	-

Note : + - Growth, - No growth

The mass production of potential SQA4 strain is carried out in the solid state fermentation since the consistency and stability of the potential strain is shown in YEME agar fermentation with maximum zone of inhibition in 22 mm.

3.3.2 Extraction of bioactive compounds from potential actinobacterial strain

The SQA4 strain is streaked onto the ISP2 agar plates and incubated at 27ºC for 10 days until the bioactive secondary metabolite's are completely absorbed into the surface agar medium. Then the mycelium growth of the SQA4 strain is scraped out completely from the agar media. The agar media is cut into pieces and submerged into ethyl acetate solvent until the secondary metabolites are drained into the ethyl acetate solvent(Figure 10). In anti-quorum sensing activity testing the ethyl acetate extract of the strain showed promising inhibition against the *C.violaceum.*

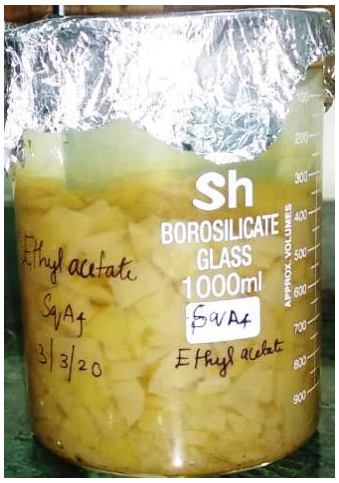


Figure 10: Extraction of bioactive compounds from SQA4

3.4 Characterization and Taxonomy of Potential Strain

3.4.1. Micromorphology

Under bright field microscopic observation, the strain SQA4 showed the presence of aerial and substrate mycelium with fragmentation (Figure11),

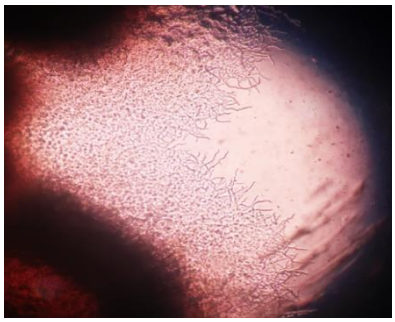


Figure 11. Micromorphology of strain SQA4

3.4.2 Cultural characteristics

Strain SQA4 showed good growth on ISP1, ISP3, ISP4, ISP5, ISP6 and ISP7 medium while moderate growth was observed on ISP1 medium (Table 6).

Table 6. Cultural characteristics of SQA4 strain on different ISP media composition

S.no	ISP media	Growth of actinobacterial strain SQA4
1	Tryptone yeast extract agar (ISP 1 medium)	Moderate
2	Oat Meal agar (ISP 3 medium)	Excellent
3	Inorganic salts - starch agar (ISP4 medium)	Good
4	Glycerol asparagine agar (ISP 5 medium)	Excellent
5	Peptone yeast extract iron agar (ISP6 medium)	Good
6	Tyrosine agar (ISP 7 medium)	Good

3.4.3 Physiological characteristics

The study of carbon utilization by the SQA4 strains was done using ISP2 media supplemented with different sugar sources like glucose, lactose, mannitol, inositol, rhamnose and raffinose. SQA4 was grown well in all the carbon sources.

Effects of nitrogen sources on growth were determined by inoculating the SQA4 strains into ISP2 media supplemented with peptone, yeast, malt, potassium nitrate, soybean extract. All the plates were seen well growth.

Effects of minerals on growth were determined by inoculating the SQA4 strains into ISP2 media supplemented with sodium chloride, calcium chloride, manganese chloride, ferrous sulphate and magnesium sulphate. All the media were showed good growth, In that only $FeSo_4$ has showed no growth.

Effects of pH on growth were determined by inoculating the SQA4 strains into ISP2 media adjusted to different pH values 6 to 9. All the plates were showed good activity. Strain SQA4 were able to grow well utilizing wide range of carbon sources, nitrogen sources, mineral sources and at different Ph. Based on the cultural characteristics, micromorphology and the utilization of strain in different composition media the strain SQA4 has been identified as Streptomyces species (Table 7).

Table 7. Growth of SQA4 strain in carbon, nitrogen, mineral sources and pH

Characteristics	Variables	Growth
Utilization of carbon sources	Starch	+++
	Mannitol	+++
	Inositol	+++
	Raffinose	+++
	Lactose	+
	Rhamnose	+++
Utilization of nitrogen sources	Soybean extract	+++
	KNO_3	++
	Peptone	++
Utilization of mineral sources	$CaCl_2$	+++
	$MgSo_4$	+++
	$FeSo_4$	-
	$MnCl_2$	+++
pH	pH 6	+++
	pH 7	+++
	pH 8	++
	pH 9	+++

Note: + - Growth

3.4.4 Optimization of culture conditions for the production of secondary metabolites

Among the various factors tested most of the carbon, nitrogen and minerals sources were found to influence the anti-quorum sensing activity of strain SQA4 (Table 8).

Table 8. Effect of culture conditions on anti-quorum sensing activity of potential strain SQA4

Variables	Media	Anti-QS activity of SQA4 against *C. violaceum* Zone of inhibition in mm
Utilization of ISP media	ISP1	-
	ISP3	-
	ISP4	-
	ISP5	-
	ISP6	-
	ISP7	-
Utilization of carbon sources	Lactose	17mm
	Starch	18mm
	Mannitol	9mm
	Inositol	17mm
	Rhamnose	17mm
	Raffinose	17mm
Utilization of nitrogen sources	Peptone	-
	KNO_3	-
	Soybean	-
Utilization of mineral Sources	Magnesium sulphate	10mm
	Calcium chloride	16mm
	Ferrous sulphate	-
	Manganese chloride	17mm
Different pH	pH 6	20mm
	pH 7	22mm
	pH 8	18mm
	pH 9	8mm

3.4.5 Phylogenetic Analysis

The strain SQA4 was subjected to 16sRNA analysis. NCBI BLAST analysis of SQA4 shows 99% similarity to sequence of *Streptomyces maritimus*, which further confirms distinct phylogenetic tree constructed with *Streptomyces*. sp SQA4 is chosen as *Streptomyces maritimus* (**Figure 12**).

Figure 12. Phylogenetic tree construction with Streptomyces sp. SQA4 designated as *Streptomyces maritimus*

4. CONCLUSION

The search for novel bioactive agents is an important element in battling threat posed by the multi-drug resistant bacteria. In this study, focus has been given to explore marine fish gut associated actinobacteria and their potential to yield QSIs of therapeutic value Gut microbiota of fish has promising source for novel anti-quorum sensing agents. In our study, Strain SQA4 has showed promising anti-microbial as well as good anti-quorum sensing activity.So that ,strain SQA4 has selected as a potential actinobacterial strain. Due to slow progress in identification and recording of novel class of antibiotics, substitute approaches such as blocking cell-cell communication (quorum sensing) is strain SQA4 as a novel anti-infective drug target in future. Further studies like purification and structure elucidation of the compound will be done in future.

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