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OCCURRENCE AND ANTIBACTERIAL ACTIVITY OF ACTINOMYCETES ISOLATED FROM MARINE SAMPLES FROM THOOTHUKKUDI, TAMIL NADU, ON THE EAST COAST OF INDIA, AGAINST FISH PATHOGENS

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ABSTRACT
The study was undertaken to isolate marine actinomycetes, detect their inhibitory activity, determine their anti-microbial profile, identify and determine the inhibitory spectrum of the crude extract. The actinomycete strains were isolated from seawater, marine sediment and swab samples of submerged substrates from three different sampling stations, Thermal Beach, Hare Island and Near Shore Area, located along the east coast of Thoothukkudi, Tamil Nadu, India. Significantly higher values of $1.50 \pm 0.16 \times 10^5$ and $1.56 \pm 0.25 \times 10^6$ CFU per ml/g of water and sediment samples respectively from the Thermal Beach were recorded for the bacterial population when compared to those of Near Shore Area and Hare Island ($p<0.05$). The actinomycete population was also observed to be significantly higher, $5.46 \pm 0.22 \times 10^4$ and $5.53 \pm 0.15 \times 10^5$ CFU per ml/g in the water and sediment samples respectively from the Thermal Beach when compared to those of Near Shore Area and Hare Island samples ($p<0.05$). Among 87 actinomycete strains isolated from 74 marine samples, a total of 64 antagonistic actinomycetes were detected by spot-inoculation assay. Samples from Thermal Beach yielded the highest number of antagonistic actinomycetes (60.94%) in comparison to the isolates of Hare Island (26.56%) and Near Shore Area (12.50%). Eight, highly antagonistic actinomycetes were tested for their inhibitory spectrum against the selected test fish pathogens, *Aeromonas hydrophila*, *A. sobria*, *Vibrio fischeri*, *V. vulnificus, Edwardsiella tarda* and *Pasteurella* spp. by cross-streak assay. The actinomycete isolate A_{55} from the Thermal Beach area exhibited prominent inhibitory activity with a zone of growth inhibition of 20 mm against 83.33% of the test fish pathogens. A high percentage (54%) of the marine actinomycetes belonged to white color series followed by gray (37%) and the lowest percentage (9%) was represented by violet color series. Among the antagonistic actinomycetes, a high percentage (53%) belonged to white color series followed by gray color series (44%) (Fig. 4). Violet color series represented only 3% among the antagonistic actinomycetes. The isolate A_{55} with a high level of antagonism against all the test fish pathogens was identified to be belonging to the genus *Streptomyces* spp. At 50 µg/ml concentration, the ethyl acetate crude extract of the spent medium of the actinomycete isolate A_{55}, recorded the highest zone of growth inhibition of 20 mm against three of the test fish pathogens and 11-19 mm zone of inhibition against three of the test fish pathogens. While, at 100 µg/ml concentration, the crude extract of A_{55} registered the highest zone of growth inhibition of 20 mm against four of the test fish pathogens and 11-19 mm zone of inhibition against two of the test fish pathogens. The results of the present study indicate that high number of antagonistic actinomycetes producing novel antimicrobial compounds can be isolated from the marine environments with high organic load and also that marine antagonistic actinomycetes can be used as bio-remediation agents for the suppression of proliferation of disease causing microbes thereby preventing the outbreak of diseases in aquaculture systems.

KEY WORDS: marine, antagonisitic, actinomycetes, fish pathogens, TLC, *Streptomyces*, crude extract, cross-streak assay.

INTRODUCTION
Fish production through capture fisheries has been dwindling steadily and this has led to exertion of the enormous pressure on the Indian aquaculture industry. This has resulted in continuing efforts for intensification of the aquaculture practices. Intensive aquaculture systems generate tremendous stress on culture species and make them more susceptible to diseases. The lack of scientific awareness coupled with the need to overcome problems somehow, has led to indiscriminate use of antibiotics in the aquaculture industry. This has consequently led to emergence of new diseases as well as drug-resistant strains of fish and shell fish microbial pathogens (Karunasagar *et.al.*, 1994). Hence, finding novel anti-microbial compounds with therapeutic potential is the need of the hour. World oceans occupy more than 70% of the Earth's surface and because of this enormous nature, the marine environment, support diverse and unique ecosystems. These unique ecosystems are the richest sources of micro-organisms with unique physiological capabilities. Marine microorganisms have been found to produce unique and diverse classes of bioactive compounds when compared to their terrestrial counterparts (Bernan *et al.,* 1997). Actinomycetales, a single taxonomic group, has been observed to contribute to most of the commonly used antibiotics (Sanglier *et al*., 1996). Diverse classes of antimicrobial compounds like, Aminoglycosides, Anthracyclines, Chloramphenicol, -lactams, Macrolides

and Tetracyclines have been isolated from Actinomycetes which are the gram-positive, filamentous bacteria (Okami and Hotta, 1988). This group of bacteria alone contributes to about 3,477 antibiotics (Labeda and Shearer, 1990). The origin of almost 80% of the world's antibiotics can be traced to actinomycetes (Pandey *et al*., 2004). Marine antagonistic actinomycetes represent a fairly untapped resource of novel antimicrobial compounds and these compounds can be can be used in aquaculture for controlling various microbial diseases of fish and shell fish (Patil *et al*., 2001 b). Marine antagonistic actinomycetes can also be used as biocontrol agents to control fish/ shell fish pathogens in an eco-friendly manner (Patil *et al*., 2001 a). Hence, the present study was carried out with the objectives of isolation of the actinomycetes from different marine samples, their detection for inhibitory activity, determination of their inhibitory profile against various selected fish & shell fish test pathogens, their identification using standard chemotaxonomic schemes and testing the inhibitory activity of the crude extract of the anti-bacterial compound against selected test fish pathogens.

MATERIALS & METHODS

Collection of samples

Marine water, sediment and swab samples were collected from the three sampling stations, Thermal Beach, Hare Island and Near Shore Area located along the east coast of Thoothukkudi, Tamil Nadu, India (Fig. 1).

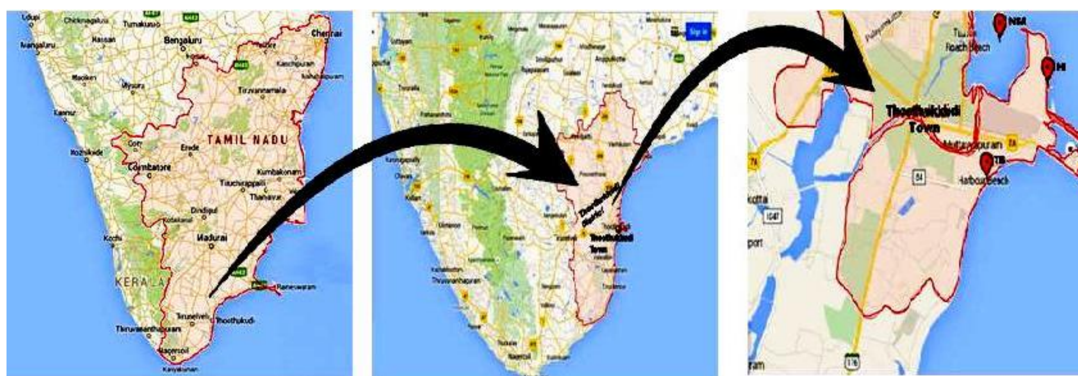

FIGURE 1: Sampling stations along the east coast of Thoothukkudi town, Thoothukkudi District, Tamil Nadu state, India (*Balloons indicate the exact locations of the sampling; TB- Thermal Beach, HI- Hare Island, NSA- Near Shore Area).

Water samples were collected aseptically in sterile, 50 ml, cylindrical, screw-capped glass bottles, from the beach area. Sterile polypropylene bags were used for the aseptic collection of sediment samples from the beach area. Petersen Grab sediment sampler was used for the collection of near shore sediment samples. The sediment samples from the Grab were transferred aseptically into sterile polypropylene bags. Sterile cotton swabs were used for the collection of swab samples from various submerged substrates such as corals, rocks and seaweeds and the sawabs were stored in sterile, 50 ml, cylindrical, screw-capped glass bottles with sterile aged seawater. All the collected samples were brought to the laboratory within an hour of collection and used immediately. Marine sediment samples were air dried for 4-5 days and then used. Water and swab samples were thoroughly mixed using a Cyclomixer to disperse the adhering bacterial cells and then used.

Isolation of marine actinomycetes

Aged seawater was used both as diluent and bacterial cell suspension medium. Ten fold serial dilutions were carried out and the dilutions were thoroughly mixed with the help of a vortex mixer for a minute. Inoculation was done using spread plating onto a selective medium, Starch-Casein Agar (SCA)(Hi-Media Pvt. Ltd., Mumbai) (Table 1) with antifungal agents (filter sterilized), Cycloheximide and Ketoconazole @ 50µg/ml (Hi-Media Pvt. Ltd. Mumbai) each.

TABLE 1: Composition of Starch Casein Agar (SCA) (g/l)

Soluble starch	10.0
Vitamin free casamino acids	0.3
Calcium Carbonate $CaCO_3$	0.02
Fe3SO4.7H2O	0.01
KNO3	2.0
MgSO4.7H2O	0.05
NaCl	5.0
Agar	18.0
*D/w	Make upto 1L
pH	7.1±0.1

*When distilled water was used instead of aged seawater, NaCl @ 0.5% was added.

The plates were then incubated at room temperature (30± 2°C) for 5 – 7 days. The selection of actinomycete isolates was done based on their colony morphology with a typical chalky to leathery appearance (IMTECH, 1998) followed by gram staining, acid fast staining. Light microscopy (NIKON, Japan) was carried out to check the isolates for filamentous nature, width of hyphae (0.5 – 2 μ), nature of aerial and substrate mycelium (Cappucino and Sherman, 2004). The Gram-positive, non-acid fast isolates with aseptate hyphae were picked up and purified onto Starch Casein Agar (SCA) plates. The purified isolates were sub-cultured on SCA slants, incubated at room temperature for 6-7 days and stored at refrigeration temperature till further use.

Detection of antagonistic marine actinomycetes

Primary screening of purified actinomycete isolates for their inhibitory activity, against a sensitive strain of *Escherichia coli* (MTCC 739, IMTECH, Chandigarh) was carried out by employing a modified spot inoculation method of James *et al*.(1996). Spot inoculation of actinomycete isolates was carried out at the center of the Antibiotic Assay Medium (AAM) (Hi-Media Pvt. Ltd. Mumbai) (Table 2) plates. After incubation for 6-7 days at room temperature, the plates were flooded with an overnight broth culture of the sensitive strain of *E. coli* and incubated at 37^{0}C for 24-48 hours. The antagonistic nature of actinomycete strains was detected by the presence of clear zones of growth inhibition of the sensitive *E. coli* strain, around their colony. Based on the extent of the zone of inhibition, the degree of antagonism of actinomycete isolates was evaluated on a 5-point scale. Antagonistic actinomycete isolates with higher antagonistic activity were used for the secondary screening against selected fish and shell fish test pathogens.

TABLE 2: Composition of Antibiotic Assay Medium (AAM) (g/l)

Peptic digest of Animal tissue	6.0
Yeast extract	3.0
Beef extract	1.5
NaCl	5.0
Agar	15.0
*D/w	Make up to 1L
pH	7.9±0.2

*When distilled water was used instead of aged seawater, NaCl @ 0.5% was added.

Determination of the inhibitory profile of marine actinomycetes against fish bacterial pathogens

Modified cross-streak assay of Lemos *et al*. (1985) was employed during the secondary screening of the detected, antagonistic actinomycete isolates for the determination of their inhibitory profile against selected test fish pathogens. Modified Streptomycete Antibiotic Activity agar (SAA) medium (Table 3) was used and the actinomycete isolates were streaked across the diameter on SAA plates with a width of the streak being 8-10 mm.

TABLE 3: Composition of Streptomycete Antibiotic Activity Agar (SAA) medium (g/l)

D-Glucose	15.0
Glycerol	2.5 ml
Soybean meal	15.0
Yeast extract	1.0
Calcium Carbonate	1.0
NaCl	5.0
Agar	15.0
*Aged Seawater	1000 ml
pH	6.7 ± 0.1

*When distilled water was used instead of aged seawater, NaCl @ 0.5% was added.

After an incubation period of 5-7 days at room temperature, young cultures of the fish test pathogens, *Aeromonas hydrophila, A. sobria, V. fischeri, Edwardsiella tarda, Pasteurella sp., and Vibrio vulnificus* were streaked perpendicular to the central strip of the actinomycete culture apart by 1-2 mm from the central strip. The plates were then incubated at room temperature for 24 h. The absence of growth near the central strip indicated the inhibitory activity of actinomycete isolates and clear zones of growth inhibition of various test pathogens was measured in millimeters (mm). The AAM agar plates with only the test pathogens served as control.

Color series of actinomycete isolates

The aerial mycelium of the actinomycete isolates as well as the antagonistic actinomycete isolates was observed for its color and was recorded.

Identification of antagonistic marine actinomycetes

Standard chemotaxonomic schemes of IMTECH (1998) and Goodfellow (1989) were followed for the identification of actinomycete isolates with greater inhibitory activity.

Light microscopy

Sub-culture of the actinomycete isolates with prominent antagonistic activity was done by employing the cover-slip culture technique using SCA medium. The nature of aerial and substrate mycelium of these antagonistic actinomycete isolates was observed and recorded with a trinocular compound microscope (Nikon, Japan) with camera attachment by using a novel, indigenously designed cover slip holder for scanning the field (Cappucino and Sherman, 2004).

Biochemical tests and determination of cell wall chemotypes

The biochemical tests, casein, xanthine, urea, xylose and lactose utilization tests were carried out (Schaal, 1985). Thin Layer Chromatographic (TLC) analysis of the extracted cell wall amino acids was carried out using cellulose coated TLC sheet, LL-Diamino Pimelic Acid (DPA), meso-DAP, DD-DAP isomer standards, Glycine standard (Qualigens, India) and methanol: water: 6 N HCl : Pyridine (80: 26: 4: 10 v/v) as mobile phase. The plates were sprayed with 0.2% (w/v) ninhydrin in acetone for visualization. The plates were heated at 105^0C for 5 minutes. The Rf values of amino acids in the samples were calculated, compared with the standards and identified. Characteristic sugars present in the cell wall of the antagonistic actinomycete isolates were also detected by TLC. Silica gel coated TLC sheets were used with Glucose, Mannose, Rhamnose, Galactose, Ribose, Arabinose, Xylose as sugar standards (Qualigens, India), and acetonitrile:water (92.5:7.5 v/v) as mobile phase. Visualization was done by spraying aniline phthalate reagent (prepared using aniline 2 ml, phthalic acid 3.3g and water saturated butanol 100 ml) and heating the plates at 100^0C for 5 minutes for visualisation. The Rf values of samples were calculated, compared with standards and the sugars in the samples were identified.

Extraction of antibacterial substances from the antagonistic marine actinomycetes

Modified Soybean Yeast extract Glucose (SYG) (Vanajakumar *et al.,*1991) broth medium (Table 4) was inoculated with the highly inhibitory actinomycete isolate, A_{15} in 2 litre Erlenmeyer flasks with 1 litre of the broth medium and incubated at room temperature in a rotary shaker at 200 rpm for a period of 7 days.

TABLE 4: Composition of Soyabean Yeast extract Glucose (SYG) medium (g/l)

Soya peptone	10.0
Yeast extract	4.0
Glucose	4.0
*Aged Seawater	1000 ml
pH	7.1 ± 0.1

*When distilled water was used instead of aged seawater, NaCl @ 0.5% was added.

The actinomycete culture was then killed by exposure to chloroform vapours for a period of 1 hour with agitation in a rotary shaker. The culture was then centrifuged for 15 min at 10,000 rpm. The extraction procedure described by Okamoto *et al*. (1986) was modified and adopted. The clear supernatant with the antibacterial substance was mixed with 1 litre of ethyl acetate solvent and the contents were subjected to vigorous shaking for 1 hour to achieve complete extraction. The contents were allowed to settle down for a minute and poured into a separating funnel for the separation of the two phases. The upper solvent phase containing the bioactive compound was collected in a separate flask. Then, 500 ml of ethyl acetate was again added to lower aqueous phase and kept for extraction. Again the upper solvent phase was collected. Like this the ethyl acetate extraction was repeated one more time with 500ml of ethyl acetate and all the ethyl acetate fractions were pooled together and concentrated to dry powder using a rotary evaporator under vacuum at 40°C. The dry powder of this crude extract with the bioactive compound was resuspended in ethyl acetate to the concentration of 1mg/ml.

Determination of inhibitory level of crude extracts of the antibacterial substances

Filter paper discs of 6 mm diameter were punched out of Whatman filter paper (HiMedia, India) and sterilized in an autoclave. The sterile filter paper discs were impregnated with 100 µL (100 µg of crude extract) of crude extract, dried and placed at the center of the modified SYG agar plates previously seeded with the fish test pathogens. One fish test pathogen per plate was used. The sterile filter paper discs impregnated with ethyl acetate alone and later dried, were used as control. *Aeromonas hydrophila*, *A. sobria*, *Vibrio fischeri*, *Edwardsiella tarda*, *Pasteurella sp.* and *Vibrio vulnificus* were used as the fish test pathogens. The plates were then incubated for 24 h at room temperature and the zone of growth inhibition indicated by the clear zone around the filter paper discs was measured in millimeters.

Statistical analysis

The calculated means were expressed as mean ± standard error. Normality of the data was tested by Box-Plot method. Wherever needed, arcsine and logarithmic (to the base 10) transformations were carried out. Analysis of Variance was performed to find out the significant difference between the mean values of different treatments ($P<0.05$).

RESULTS

Total bacterial population and the actinomycetes population

The population of bacteria was observed to be $1.96 \pm 0.16 \times 10^4$ and $6.42 \pm 0.18 \times 10^5$, $1.52 \pm 0.13 \times 10^4$ and $2.05 \pm 0.19 \times 10^5$ CFU per ml/g of water and sediment samples from Near Shore Area and Hare island respectively (Table 5). Significantly high values of $1.50 \pm 0.16 \times 10^5$ and $1.56 \pm 0.25 \times 10^6$ CFU per ml/g of water and sediment samples respectively from the Thermal Beach were recorded for the bacterial population when compared to those of Near Shore Area and Hare Island ($p<0.05$). The actinomycete population was observed to be significantly higher, $5.46 \pm 0.22 \times 10^4$ and $5.53 \pm 0.15 \times 10^5$ CFU per ml/g in the water and sediment samples respectively from the Thermal Beach when compares to the actinomycete population from other samples and other sampling stations ($p<0.05$)(Table 5).

TABLE 5. Comparison of total bacterial population with actinomycete population

Sampling Station	Nature of Sample	No. of Samples	Total bacterial population (CFU per ml/g)	Actinomycete population (CFU per ml/g)
Thermal Beach	Water	4	1.50 ± 0.16 x $10^{5\,k*}$	5.46 ± 0.22 x $10^{4\,d}$
	Sediment	18	1.56 ± 0.25 x $10^{6\,i}$	5.53 ± 0.15 x $10^{5\,c}$
Hare Island	Water	5	1.52 ± 0.13 x $10^{4\,j}$	1.17 ± 0.16 x $10^{3\,l}$
	Sediment	15	2.05 ± 0.19 x $10^{5\,g}$	5.16 ± 0.16 x $10^{3\,e}$
Near Shore Area	Water	4	1.96 ± 0.16 x $10^{4\,h}$	2.99 ± 0.14 x $10^{3\,f}$
	Sediment	10	6.42 ± 0.18 x $10^{5\,b}$	8.62 ± 0.15 x $10^{3\,a}$

*Values with the same superscripts are not significantly different ($p<0.05$)

Isolation of actinomycetes

A total of 15 actinomycetes from 13 water samples, 52 from 43 sediment samples and 20 from 18 swab samples were isolated. A total of 45, 27 and 15 actinomycetes were isolated from the Thermal Beach, Hare Island and Near Shore Area respectively. 87 actinomycetes were isolated in total from all the samples and all the stations together (Table 6).

TABLE 6. Number of Actinomycetes isolated from different marine samples from different sampling stations

Samples/Actinomycetes		Sampling Stations		
		Thermal Beach	Hare Island	Near Shore Area
Water	No. of Samples	4	5	4
	No. of Actinomycete Isolates	8	5	2
Sediment	No. of Samples	18	15	10
	No. of Actinomycete Isolates	27	14	11
Swabs	No. of Samples	2	12	4
	No. of Actinomycete Isolates	10	8	2

Detection of antagonistic marine actinomycetes

Only 64 isolates were found to be antagonistic to the sensitive *E. coli* strain (MTCC 739), out of 87 actinomycete isolates from the three sampling stations, constituting to 73.56% of antagonistic isolates during the primary screening (Table 7). Samples from Thermal Beach yielded the highest number of antagonistic actinomycetes (60.94%) in comparison to the isolates of Hare Island (26.56%) and Near Shore Area (12.50%) (Table 7).

TABLE 7: Number of antagonistic actinomycete isolates from different marine sampling stations as detected by spot inoculation method against sensitive *E. coli* (MTCC 739) strain and represented on a 5-point scale

Sampling Stations	Number of antagonistic actinomycetes (On 5-Point Scale)				
	1	2	3	4	5
Thermal Beach	10	7	7	7	8
Hare Island	6	4	3	2	2
Near Shore Area	3	1	2	1	1

*Zone of inhibition on a 5-Point Scale **:** I- 1 to 5mm; II- 6 to 10mm ; III- 11 to 15mm; IV- 16 to 19mm; V- 20 mm

Inhibitory activity of the actinomycete isolates against various fish test pathogens

A total of eight actinomycete isolates with prominent inhibitory activity out of 64 antagonistic actinomycetes, were subjected to secondary screening to determine their inhibitory profile against the selected fish test pathogens. The isolate A_{55} from the Thermal Beach area exhibited prominent inhibitory activity with a zone of growth inhibition of 20 mm against 83.33% of the fish test pathogens (Table 8) (Fig. 2). Growth inhibition of 20 mm was noticed from five of the eight antagonistic actinomycete isolates against the test fish pathogens. One of the inhibitory actinomycete isolates exhibited a zone of growth inhibition of 20 mm against three of the test fish pathogens and two isolates exhibited a zone of growth inhibition of 20 mm against two of the test fish pathogens. One of the inhibitory actinomycete isolates exhibited a zone of growth inhibition of 10-19 mm against 83.33% of the test fish pathogens while two of the inhibitory actinomycete isolates exhibited a zone of growth inhibition of 10-19 mm against 50% of the test fish pathogens (Table 8).

TABLE 8 : Inhibitory activity profile of antagonistic actinomycete isolates from different marine sampling stations against different test fish pathogens

Sampling Station	Antagonistic Actinomycete Isolates	Fish Test Pathogens* (Zone of inhibition in mm)					
		T1	T2	T3	T4	T5	T6
Thermal Beach	A_{55}	24	27	25	14	28	21
	A_{60}	6	8	10	4	6	12
	A_{80}	12	15	8	20	16	5
	A_{84}	12	15	11	14	10	8
	A_{85}	8	12	4	16	24	21
Hare Island	A_{103}	5	22	10	5	0	24
	A_{114}	0	4	0	5	0	0
Near Shore Area	A_{129}	11	22	24	28	12	12

*Test Fish Pathogens: T1- *Aeromonas hydrophila*; T2- *A. sobria*; T3- *Vibrio fischeri*; T4 - *Edwardsiella tarda*; T5- *Pasteurella sp.*; T6- *Vibrio vulnificus*

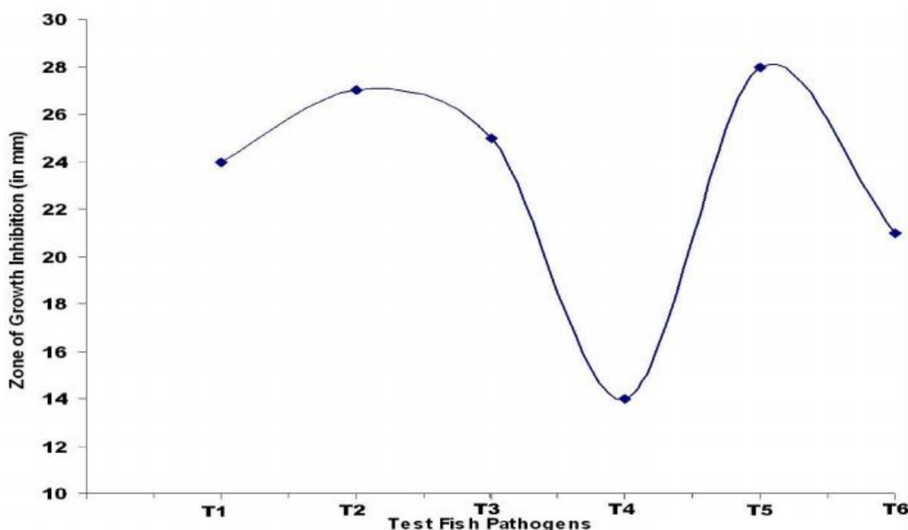

FIGURE 2: Inhibitory profile of antagonistic actinomycete strain A_{55} isolated from Thermal Beach

Color-series of the actinomycetes

47 actinomycete isolates displayed white aerial mycelium, while, 32 isolates exhibited gray and 8 isolates displayed violet aerial mycelium (Fig. 3A).Among the antagonistic actinomycetes, 34 isolates were found to be with white aerial mycelium, while, 28 isolates displayed gray and 2 isolates exhibited violet aerial mycelium (Fig. 3B).

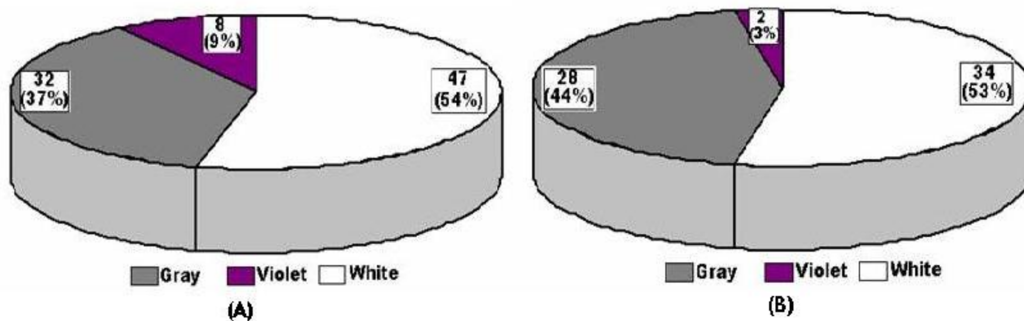

FIGURE 3. Color-series of (**A**) actinomycete isolates (**B**) antagonistic actinomycete isolates

Identification of actinomycete isolates with antagonistic activity

The actinomycete isolate A_{55} from the Thermal Beach area exhibited a high level of inhibitory activity among the antagonistic actinomycete isolates, against all the test fish pathogens and hence was subjected to standard chemo-taxonomical tests for identification.

TABLE 9. Chemo-taxonomic tests used for the identification of antagonistic marine actinomycete isolate A_{55}

Test/Analysis			Result
Light, Compound Microscopy		Gram's Reaction	Gram +ve
		Acid-Fast Staining	Non acid-fast
		Cellular Nature	Filamentous, Asepatate hyphae with hyphal width -0.5 - 2 µ Aerial hyphae- bearing spores in spirals
Biochemical Tests		Casein decomposition	+
		Xanthine decomposition	+
		Urea decomposition	+
		Acid from Xylose	+
		Acid from Lactose	+
TLC Analysis	Cell wall amino acid	LL-DAP and Glycine	Present - "Cell wall chemotype-I"
		Meso-DAP	Absent
		DD-DAP	Absent
	Whole cell sugar pattern		No diagnostic sugar present – "Sugar pattern – C"

Light microscopy

The light, compound microscopy studies of the highly inhibitory actinomycete strain A_{55} revealed, filamentous, asepatate hyphae which were Gram +ve and non-acid fast. The hyphal width was micro-metrically determined to be 0.5 - 2 µ and the aerial hyphae were bearing spores in spirals (Table 9).

Biochemical tests and determination of cell wall chemotypes

Cell wall hydrolysate of the strain A_{55} was subjected to TLC analysis and revealed the presence of LL-DAP & glycine and hence the isolate was classified under Cell Wall Chemotype-I. TLC analysis of the whole cell sugar of the isolate revealed the absence of characteristic sugars and hence was classified under the Sugar Pattern- C (Table 9). From the above results, the isolate A_{55} was identified to be belonging to the genus *Streptomyces* spp.

Determination of inhibitory level of crude extracts of the antibacterial substances

At 100 µg/ml concentration of the crude extract, the highest inhibition of 28 mm was recorded against the test fish pathogen, *A.sobria*, followed by 26 mm against *V. fischeri*. Also the lowest inhibition was observed to be 18 mm against *Edwardsiella tarda* at the same concentration (Table 10). Whereas, at 50 µg/ml concentration of the crude extract, the highest inhibition of 22 mm was recorded against the test fish pathogen, *A.sobria*, followed by 21 mm against *A. hydrophila* and *V. fischeri*. While, the lowest inhibition of 13 mm was observed against *Pasteurella* sp. at the same concentration of the crude extract (Table 10).

TABLE 10. Inhibitory activity of ethyl acetate crude extract of the mycelium and spent medium of *Streptonyces* spp. strain A_{55} against fish bacterial pathogens

Crude Extract Concentration (in µg)	Test Pathogens* (Zone of inhibition in mm)					
	T_1	T2	T3	T_4	T_5	T6
50	21	22	21	14	13	18
100	23	28	26	18	19	22

*Test Fish Pathogens: T1- *Aeromonas hydrophila*; T2- *A. sobria*; T3- *Vibrio fischeri*; T4 - *Edwardsiella tarda*; T5- *Pasteurella sp.*; T6- *Vibrio vulnificus*

DISCUSSION

Total bacterial population and the actinomycetes population

Significantly lower populations of the actinomycetes were observed in the present study, when compared to that of bacteria both sample-wise and sampling station-wise ($p<0.05$)(Table 5). Goodfellow and Williams (1983) also reported that, the actinomycetes represent only a small fraction of the bacterial population isolated from the marine environment. Significantly high number of bacteria as well as actinomycetes were observed in the sediment samples when compared to those of water samples in the present study ($p<0.05$)(Table 5). Actinomycetes were found in seawater to a much lesser extent when compared to the sediments samples in another study (Grein and Meyers,1958).

Isolation of marine actinomycetes

High number of actinomycetes were isolated from marine sediment samples (60%) when compared to swab samples (23%) and sea water samples in the present study (17%)(Table 6). It was reported that the mean population density of actinomycetes was higher in sediment samples than in the water samples (Sahu *et al.*, 2007). In the present study, in total, 46 actinomycetes were isolated from all the samples and all the stations (Table 6). Karthik *et al.* (2010) reported the isolation of a total of 100 actinomycete strains from 20 marine sediment samples from the Nicobar islands. In another study, 42 actinomycete strains were isolated from estuarine and mangrove sediments (Rosmine and Varghese, 2016).

Detection of antagonistic marine actinomycetes

During the primary screening of the present study, 73.56% of the total number of actinomycetes isolated, were

detected to be antagonistic against the sensitive *E. coli* strain (MTCC 739) (Table 7). Sahu *et al*. (2007) also reported that 61% of the actinomycete isolates showed antibacterial activity against various shrimp pathogens. On the contrary, only 4% of the actinomycete isolates were found to be inhibitory in a study by Rosmine and Varghese (2016). However, Remya and Vijayakumar (2008) also reported that only 33% of the actinomycete isolates had antimicrobial activity. This may be due to the differences in the concentration of organic matter present in the samples of different sampling stations. Of the three sampling stations, samples from the Thermal Beach, yielded the highest number of antagonistic actinomycetes (60.94%) in comparison to the isolates of Hare Island (26.56%) and Near Shore Area (12.50%)(Table 7). The reasons for high incidence of antagonistic actinomycetes in the samples of Thermal Beach when compared to those of the other two stations in the present study, may be due to the presence of high organic load in the samples of Thermal Beach due to various anthropogenic activities because of tourism. This might lead to high competition between actinomycetes and other bacterial species for nutrients & space. Only the actinomycete strains with inhibitory property thrive in high numbers in such micro-environments with high bacterial load, by secreting highly diverse classes of anti-microbial compounds (Walker and Colwell, 1975).

Determination of inhibitory profile of marine actinomycetes

During the secondary screening in the present study, eight selected antagonistic actinomycete isolates with prominent activity, inhibited the test fish pathogens to varying degrees (Table 8). 75% of the actinomycete isolates inhibited 100% of the fish test pathogens, 62.5% of the actinomycete isolates inhibited 83.33% of the fish test pathogens in the present study, while, 25% of the actinomycete isolates inhibited 33.33% of the fish test pathogens (Table 8). Okazaki and Okami (1972) reported that only 27% of marine actinomycete strains were antagonistic to various test pathogens. However, in another study, Vanajakumar *et al.*(1991) also reported that, 75% of the marine actinomycete isolates were inhibitory to various test organisms. On the contrary, Sahu *et al.* (2007) reported that only 23% of the marine actinomycetes exhibited varying degrees of antagonistic activity against shrimp pathogens with prominent activity against V. *harveyi*. In the present study, prominent inhibitory activity was exhibited by the actinomycete isolate A_{55} from the Thermal Beach area with a zone of growth inhibition of 20 mm against 83.33% of the test fish pathogens (Fig. 2). Sahu *et al.* (2007) observed that the marine actinomycete strain MKS-24 was very active against all the three shrimp test pathogens *Vibrio alginolytics*, *V. harveyi*, and *V. parahaemolyticus*. Five of the eight antagonistic actinomycete isolates tested, exhibited a zone of growth inhibition of 20 mm against at least one of the test fish pathogens, in the present study. In a study, it was reported that one actinomycete isolate AN1 displayed significant antibacterial activity against the test pathogens, *Staphylococcus aureus*, *Bacillus cereus* and *Salmonella paratyphi* A with the zones of inhibition, 19 mm, 17 mm and 16.5 mm respectively (Abirami *et al.*, 2013).

Color Series of Actinomycetes

A high percentage (54%) of the marine actinomycetes belonged to white color series followed by gray (37%) and the lowest percentage (9%) was represented by violet color series, in the present study (Fig 3). Vanajakumar *et al.* (1991) also noted that the white colour series of actinomycetes were dominant followed by the gray, yellow and red color series. Also, in another study, it was reported that very few *Streptomyces* were found to belong to violet colour series (Williams *et al*.,1989). On the contrary, Hatano (1997) observed that, a high percentage (77%) of the isolates of *Streptomyces* belonged to gray color series and the remaining to other color series. In the present study, among the antagonistic actinomycetes, a high percentage (53%) belonged to white color series followed by gray color series (44%) (Fig. 4). Violet color series represented only 3% among the antagonistic actinomycetes. It was reported by Dharmaraj (2011) that, 57% of the inhibitory actinomycetes belonged to white color series, only 29% belonged to gray color series and 14% of them had yellow colored aerial mycelium. On the other hand, Rosmine and Varghese(2016) reported only two color series among the inhibitory actinomycetes and that 50% of them belonged to white color series and another 50% had gray colored aerial mycelium.

Identification of antagonistic marine actinomycetes

The actinomycete isolate A_{55} from the Thermal Beach area exhibited a high level of inhibitory activity against all the fish test pathogens in the present study. Hence, the strain A_{55} was subjected to light microscopy investigations including standard chemo-taxonomical tests and was identified to be belonging to the genus *Streptomyces* spp. (Table 9). The actinomycete isolates with good inhibitory activity were identified to be belonging to the genus *Streptomyces* spp. in other studies by many workers (Sahu *et al*.,2007; Karthik *et al.,* 2010; Abirami *et al.*, 2013). However, in their study, Parthasarathi *et al*. (2012) identified 66% of the antagonistic actinomycetes to be belonging to the genus *Streptomyces* spp., 18% of the isolates to *Nocardiopsis* spp., 11% of the isolates to *Micromonospora* spp. and 5% of the isolates to the genus *Actinopolyspora* spp.

Inhibitory levels of crude extract of the antibacterial substance from *Streptomyces* against fish bacterial pathogens

At 50 µg/ml concentration of the crude extract of the actinomycete isolate A_{55}, in the present study, the highest zone of growth inhibition of 20 mm was recorded against three of the test fish pathogens and 11-19 mm zone of inhibition against three of the test fish pathogens (Table 10). Dharmaraj (2011) also reported that, at 50 µg/ml concentration of crude extract, 29% of the actinomycete isolates exhibited > 30 mm zone of growth inhibition against two pathogens, 14% of the isolates exhibited > 30 mm zone of growth inhibition against one pathogen, 43% of the isolates exhibited 21-30 mm zone of growth inhibition against two pathogens, while 43% of the isolates exhibited 21-30 mm zone of growth inhibition against one pathogen. However, at 100 µg/ml concentration of the crude extract of the actinomycete isolate A_{55} in the present

study, the highest zone of growth inhibition of 20 mm was recorded against four of the test fish pathogens and 11-19 mm zone of inhibition against two of the test fish pathogens (Table 10). In the present study, ethyl acetate extract of the spent medium exhibited the antimicrobial properties. Similar to the results of the present study, ethyl acetate crude extract of the actinomycete strain RM 17 showed maximum zone of inhibition of 20 mm against only one pathogen, 16-19 mm against three of the test pathogens and 11-15 mm against only one of the test pathogens. While, the ethyl acetate crude extract of the strain RM42 exhibited maximum zone of inhibition of 20 mm against two of the test pathogens, 16-19 mm against two test pathogens and 11-15 mm against one of the test pathogens (Remya and Vijayakumar, 2008). It can be interpreted from the results of the present study that, higher concentrations of the ethyl acetate crude extract of the *Streptomyces* spp. strain, inhibit the fish test pathogens to a greater degree than at lower concentrations. Also, the results of the present study indicate that high number of antagonistic actinomycetes producing novel antimicrobial compounds can be isolated from the marine environments with high organic load, since the high carbon to nitrogen (C:N) ratio supports the growth of antagonistic actinomycetes. This may be due to the fact that the presence of high organic load favours the growth of other bacteria too, creating competition for space and nutrients among actinomycetes and other bacteria. This may trigger the secondary metabolite pathways responsible for the production of anti microbial compounds by the antagonisitic actinomycetes for the suppression of the other bacteria and for their proliferation & survival. From the results of the present study, it can be recommended that marine antagonistic actinomycetes may be used as bio-remediation agents for the suppression of proliferation of disease causing microbes thereby preventing the outbreak of diseases in aquaculture systems.

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REFERENCES

Abirami, V. M., Khanna, G. and Kannabiran, K.(2013) Antibacterial activity of marine *streptomyces* sp. isolated from Andaman & Nicobar islands, India. Int. J. of Pharma and Bioscience. 4, 280 – 286.

Arasu, M. V., Asha, K. R. T., Duraipandiyan, V., Ignacimuthu, S., Agastian, P. (2012) Characterization and phylogenetic analysis of novel polyene type antimicrobial metabolite producing actinomycetes from marine sediments: Bay of Bengal India. Asian Pac J of Trop. Biomed. 2, 803-810.

Baarn, R.B., Gandhi, N. M. and Freitas, Y. M. (1966) Antibiotic activity of marine microorganisms: the antibacterial spectrum. Helgolan. Wiss. Meer. 13, 188-191.

Bernan, V. S., Greenstein, M. and Maiese, W. M. (1997) Marine microorganisms as a source of new natural products. Adv. Appl. Microbiol. 43, 57-90.

Davo, A. P., Fenical, W. and Jensen, P. R. (2008) Comparative actinomycete diversity in marine Sediments. Aquat. Microb. Ecol. 52, 1–11.

Dharmaraj, S. (2011) Antagonistic potential of marine actiono-bacteria against fish and shellfish pathogens. Turk. J. Biol. 35, 303-311.

Goodfellow, M. and Williams, S. T. (1983) Ecology of actinomycetes. Ann. Rev. Microbial. 37, 189 – 216.

Goodfellow, M. (1989) Suprageneric classification of Actinomycetes. In: Krieg, **N.** R., Ludwig**,** W., Whitman**,** W., Hedlund**,** B.P., Paster**,** B.J., Staley**,** J.T., Ward**,** N., Brown**,** D., Parte, A. (Eds.), Bergey's Manual of Systematic Bacteriology, Vol. 4, Williams and Wilkins Company, Baltimore, pp.2333-2339.

Grein, A. and Meyers, S.P. (1958) Growth characteristics and antibiotic production of actinomycetes isolated from littoral sediments and materials suspended in seawater. J. Bacteriol. 76, 454-463.

Hatano, K. (1997) Actinomycete population in mangrove rhizospheres. IFO Res. Commun. 18, 26-31.

Hotta, K., Yoshida, M., Hamada, M. and Okami, Y. (1980) Studies on new aminoglycoside antibiotics, istamycins, from an actinomycete isolated from a marine environment. J. Antibiot. 33, 1515-1520.

IMTECH (1998) Labortary manual on Actinomycetes: isolation, screening, identification and gene cloning *Streptomyces*. IMTECH, Chandigarh.

James, S.G., Holmstrom, C. and Kjelleberg, S. (1996) Purification and characterization of a novel antibacterial protein from the marine bacterium D2. Appl. Environ. Microbiol. 62, 2783-2788.

Jensen, P. R., Dwight, R. and Fenical, W. (1991) Distribution of actinomycetes in near-shore tropical marine sediments. Appl. Environ. Microbiol. 57, 1102-1108.

Jianyou, L., Jianrong, X. and Yongheng, C. (2011) Isolation and identification of two marine-derived *Streptomyces* from marine mud of coast and offshore Zhuhai, and bioactive potential for plant pathogenic Fungi. Afr. J. Biotechnol. 10, 11855-11860.

Karthik, L., Kumar, G. and Rao, K.V.B. (2010) Diversity of marine actinomycetes from nicobar marine sediments and its antifungal activity. Int. J. Pharm. Pharm. Sci. 2, 199-203.

Karunasagar, I., Pai, R., Malathi, G. R. and Karunasagar, I. (1994) Mass mortality of *Penaeus monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. Aquaculture, 128, 203-209.

Korn-Wendisch, F. and Kutzner, H.J. (1992) The family streptomycetaceae. In: Balows, A., Truper, H. G.., Dworkin, M., Harder, W. and Schleifer, K. H. (Eds.), The Prokaryotes. Vol. I, Springer - Verlag, New York, pp.921-995.

Labeda, D.P. and M.C. Shearer (1990) Isolation of actinomycetes for biotechnological applications. In: Labeda, D. P. (Ed.), Isolation of biotechnological organisms from nature. McGraw-Hill Publishers, New York, pp. 1-19.

Lemos, M.L., Toranzo, A.E. and Barja, J.L. (1985) Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. Microb. Ecol. 11, 149-163.

Okami, Y. and Hotta, K. (1988) Search and discovery of new antibiotics, In: Goodfellow M, Williams, S. T., Mordarski, M. (Eds.), Actinomycetes in Biotechnology. Academic Press Inc., San Diego, pp. 33-67.

Okami, Y., Okazaki, T., Kitahara, T. and Umezawa, H. (1976) Studies on marine microorganisms. V. A new antibiotic, aplasmomycin, produced by a streptomycete isolated from shallow sea mud. J. Antibiot. 29, 1019-1025.

Okamoto, M., Yoshida, K., Nishikawa, M., Ando, T., Iwami, M., Kohsaka, M. and Aoki, H. (1986) FR-900452, A specific antagonist of platelet activating factor (PAF) produced by *Streptomyces phaeofaciens*. J. Antibiot. 39, 198-204.

Okazaki, T. and Okami, Y. (1972) Studies on marine microorganisms. II. Actinomycetes in Sagami Bay and their antibiotic substances. J. Anibiot. 25, 461-466.

Pandey, A., Ali, I., Butola, K. S., Chatterji, T. and Singh, V. (2011) Isolation and characterization of actinomycetes from soil and evaluation of antibacterial activities of actinomycetes against pathogens. Int. J. Appl. Biol. Pharm. Technol. 2, 384-392.

Parthasarathi, S., Sathya, S., Bupesh, G., Samy, R. D., Mohan, M. R., Kumar, G. S., Manikandan, M., Kim, C. J. & Balakrishnan, K. (2012) Isolation and Characterization of Antimicrobial Compound from Marine *Streptomyces hygroscopicus,* BDUS 49. World J Fish Mar. Sci. 4, 268-277.

Patil, R., Jeyasekaran, G. and Shanmugam, S.A. (2001) Occurrence and activity of marine actinomycetes against shrimp bacterial pathogens. Appl. Fish. Aquaculture. 1, 79-81.

Patil, R. Jeyasekaran, G., Shanmugam, S. A. and Shakila, R. J. (2001) Control of bacterial pathogens associated with fish diseases by antagonistic marine actinomycetes isolated from marine sediments. Indian J. Mar. Sci. 30, 264-267.

Remya, M. and Vijayakumar, R. (2008) Isolation and characterization of marine antagonistic actinomycetes from west coast of India. Med. Biol. 15, 13-19.

Rosmine, E. and Varghese, S. A. (2016) Isolation of actinomycetes from mangrove and estuarine sediments of Cochin and screening for antimicrobial activity. J. Coastal Life Med. 4, 207-210.

Sahu, M. K., Murugan, M., Sivakumar, K., Thangaradjou, T. and Kannan, L. (2007) Occurrence and distribution of actinomycetes in marine environs and their antagonistic activity against bacteria that is pathogenic to Shrimps. Isr. J. Aquacult – Bamid. 59:155-161.

Sanglier, J. J., Haag, H., Huck, T. A. and Fehr, T. (1996) Review of actinomycetes compounds 1990 – 1995. Exp. Opin. Invest. Drugs. 5, 207-223.

Sathiyaseelan, K. and Stella, D. (2011) Isolation, identification and antimicrobial activity of marine actinomycetes isolated from Parangipettai. Recent Res. Sci. Technol. 3, 74-77.

Schaal, K.P. (1985) Identification of clinically significant actinomycetes and related bacteria using chemical techniques. In: *Chemical methods in bacterial systematics*. Goodfellow, M. and D.E. Minnikin (Eds.) Academic Press Inc., London, pp.359-378.

Vanajakumar, N. Selvakumar and R. Natarajan (1991) Antagonistic properties of actinomycetes isolated from molluscs of the Porto Novo region, South India. In: *Bioactive compounds from marine organisms*. Thompson, M.F., R. Sarojini and R. Nagabhushanam (Eds.). Oxford and IBH Publishing Co., New Delhi, pp.267-274.

Walker, J.D. and R.R. Colwell (1975) Factors affecting enumeration and isolation of actinomycetes from Chesapeake Bay and South eastern Atlantic Ocean sediments. *Mar. Biol.,* 30: 193-201.