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

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## ABSTRACT

The present study was undertaken to isolate, detect inhibitory activity, determine the anti-microbial profile, identify and determine the inhibitory profile of the crude extract of the marine actinomycetes. Actinomycetes were isolated from seawater, marine sediment and swab samples of submerged substrates from two different sampling stations, Mangrove swamp and Roche Park located along the coast of Thoothukkudi, Tamil Nadu, India. Significantly higher values for the bacterial population of $3.99 \pm 0.16 \times 10^5$ and $9.84 \pm 0.14 \times 10^6$ CFU per ml/g were observed for water and sediment samples respectively, while the actinomycete population recorded significantly lower values of $1.77 \pm 0.21 \times 10^4$ and $7.84 \pm 0.13 \times 10^5$ CFU per ml/g of water and sediment samples respectively from Roche Park ($p<0.05$). A total of 36 antagonistic actinomycetes were detected among 46 actinomycete strains isolated from 55 marine samples by spot-inoculation assay. Nine, highly antagonistic actinomycetes were tested for their inhibitory profile against the selected test fish pathogens, *Aeromonas hydrophila*, *A. sobria*, *Vibrio fischeri*, *Vibrio vulnificus, Edwardsiella tarda* and *Pasteurella* spp. by cross-streak assay. 78% of actinomycetes among the nine antagonistic isolates inhibited 100% of the test fish pathogens. The isolate $A_{15}$ exhibited prominent inhibitory activity with a zone of growth inhibition of 20 mm against all the test fish pathogens. 53% of the marine actinomycetes belonged to white followed by 37% to gray and 10% to violet color series. The isolate $A_{15}$ with a high level of antagonism against all the test fish pathogens was identified to be belonging to the genus *Streptomyces* spp. The ethyl acetate crude extract of the actinomycete isolate $A_{15}$ at the concentration of 50 µg/ml, recorded the highest zone of growth inhibition of 20 mm against two of the test fish pathogens, whereas, at 100 µg/ml concentration, highest zone of growth inhibition of 20 mm was recorded against five 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.

## INTRODUCTION

The declining production from the capture fisheries has mounted enormous pressure on Indian aquaculture industry and this has led to the ever-intensification of the aquaculture practices. 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, the need for novel anti-microbial compounds with therapeutic potential is ever present. More than 70% of the Earth's surface is covered by the world oceans. Because of this enormous nature of the marine environment, it supports diverse and unique ecosystems, which are the richest sources of micro-organisms with unique metabolic and bioactive capabilities. These marine microorganisms are found to produce unique and diverse classes of bioactive compounds when compared to their terrestrial counterparts (Bernan *et al.,* 1997). It is observed that bacteria from a single taxonomic group, the Actinomycetales contribute most of the commonly used antibiotics (Sanglier *et al*., 1996). Actinomycetes are the gram-positive, filamentous bacteria and diverse classes of antimicrobial compounds like, Aminoglycosides, Anthracyclines, Chloramphenicol, -lactams, Macrolides and Tetracyclines have been isolated from this single group of 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. Novel antimicrobial compounds from the marine actinomycetes can be can be used in aquaculture for controlling various microbial diseases of fish and shell fish (Patil *et al*., 2001 b). These marine antagonistic actinomycetes can also be used as biocontrol agents to control fish/ shell fish pathogens in an eco-friendly manner (Patil *et al*., 2001a). Hence, the present study was carried out with the objectives of isolation of actinomycetes from different marine samples, detection of the antagonistic strains, determination of their inhibitory profile against various selected fish & shell fish test pathogens, their

identification using standard chemotaxonomic schemes, preparation of the crude extract of the anti-bacterial compound and testing its activity against the selected fish & shell fish test pathogens.

## MATERIALS & METHODS

### Collection of samples

Marine water, sediment and swab samples were collected from two sampling stations, Mangrove Swamp and Roche Park located along the coast of Tuticorin, Tamil Nadu, India (Fig. 1).

**FIGURE 1 :** Two sampling stations along the coast of Thoothukkudi town, Thoothukkudi District, Tamil Nadu state, India (*Balloons indicate the exact location of the sampling; RP- Roche Park, MS- Mangrove Swamp)

Water samples from the beach area were collected aseptically in sterile, 50 ml, cylindrical, screw-capped glass bottles. Beach sediment samples were collected in an aseptic manner in sterile polypropylene bags. Inshore sediment samples were collected using the *Petersen Grab* sediment sampler and brought aseptically in sterile polypropylene bags. Swab samples were collected from various submerged substrates such as corals, rocks and seaweeds using sterile cotton swabs and 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 actinomycete isolates were selected based on their colony morphology with a typical chalky to leathery appearance (IMTECH, 1998) followed by gram staining, acid fast staining and subjected to light microscopy (NIKON, Japan) 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

A modified spot inoculation method of James *et al*.(1996), was employed for the primary screening of purified actinomycete isolates for their inhibitory activity, against a sensitive strain of *Escherichia coli* (MTCC 739) (IMTECH, Chandigarh). Spot inoculation of actinomycete isolates was done at the center of the Antibiotic Assay Medium (AAM) (Hi-Media Pvt. Ltd. Mumbai) (Table 2).

**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.

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.

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

The antagonistic actinomycete isolates were subjected to secondary screening for the determination of their antagonistic profile against selected fish test pathogens as per the modified cross-streak assay of Lemos *et al*. (1985). 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 24h. 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 color of the aerial mycelium of the actinomycete isolates as well as the antagonistic actinomycete isolates was observed and recorded.

**Identification of antagonistic marine actinomycetes**

The identification of actinomycete isolates with greater inhibitory activity was carried out using the standard chemotaxonomic schemes of IMTECH (1998) and Goodfellow (1989).

**Light microscopy**

Cover slip culture technique was used for sub-culturing the actinomycete isolates with prominent antagonistic activity 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**

Casein, xanthine, urea, xylose and lactose utilization tests were carried out (Schaal, 1985). The extracted cell wall amino acids were subjected to Thin Layer Chromatographic (TLC) analysis using cellulose coated thin layer chromatography sheet, LL-Diamino Pimelic Acid (DPA), meso-DAP, DD-DAP isomer standards, Glycine (Qualigens, India) and methanol: water: 6 N HCl : Pyridine (80: 26: 4:10 v/v) as mobile phase. The plates were prayed with 0.2% (w/v) ninhydrin in acetone for visualization. The plates were heated at 105$^0$C for 5 minutes. The Rf values of amino acids in the samples were calculated, compared with 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$^0$C 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 bacterial population of $1.58 \pm 0.22 \times 10^4$and $2.60 \pm 0.17 \times 10^6$ CFU per ml/g of water and sediment samples respectively from the Mangrove swamp area were observed (Table 3). Whereas, significantly lower values of $3.70 \pm 0.70 \times 10^3$ and $6.20 \pm 0.84 \times 10^4$ CFU per ml/g of water and sediment samples respectively from the Mangrove swamp area were recorded for the actinomycete population. With respect to Roche Park sampling station, bacterial population of $3.99 \pm 0.16 \times 10^5$ and $9.84 \pm 0.14 \times 10^6$ CFU per ml/g were observed for water and sediment samples respectively while the actinomycete population recorded a significantly lower values of $1.77 \pm 0.21 \times 10^4$ and $7.84 \pm 0.13 \times 10^5$ CFU per ml/g of water and sediment samples respectively from Roche Park ($p<0.05$)(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) |
|---|---|---|---|---|
| Mangrove Swamp | Water | 5 | $1.58 \pm 0.22 \times 10^{4\ h*}$ | $3.70 \pm 0.70 \times 10^{3\ e}$ |
| | Sediment | 15 | $2.60 \pm 0.17 \times 10^{6\ f}$ | $6.20 \pm 0.84 \times 10^{4\ c}$ |
| Roche Park | Water | 6 | $3.99 \pm 0.16 \times 10^{5\ d}$ | $1.77 \pm 0.21 \times 10^{4\ g}$ |
| | Sediment | 19 | $9.84 \pm 0.14 \times 10^{6\ a}$ | $7.84 \pm 0.13 \times 10^{5\ b}$ |

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

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

| Samples/Actinomycetes | | Sampling Stations | |
|---|---|---|---|
| | | Mangrove Swamp | Roche Park |
| Water | No. of Samples | 5 | 6 |
| | No. of Actinomycete Isolates | 3 | 6 |
| Sediment | No. of Samples | 15 | 19 |
| | No. of Actinomycete Isolates | 9 | 14 |
| Swabs | No. of Samples | 6 | 4 |
| | No. of Actinomycete Isolates | 5 | 9 |

**Isolation of actinomycetes**

A total of nine actinomycetes were isolated from 11 water samples, 23 from 34 sediment samples and 14 from 10 swab samples. A total of 17 and 29 actinomycetes were isolated from the Mangrove swamp area and Roche park area respectively. A total of 46 actinomycetes were isolated from all the samples and all the stations (Table 6).

**Detection of antagonistic marine actinomycetes**

Out of 46 actinomycete isolates from the three sampling stations, only 36 isolates were found to be antagonistic to the sensitive *E. coli* strain (MTCC 739), constituting to 78.26% of antagonistic isolates during the primary screening (Table 7). Of the two sampling stations, samples from the Roche Park yielded the highest number of antagonistic actinomycetes (86.21%) in comparison to the isolates of Mangrove swamp area (64.71%) (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* ) | | | | | |
|---|---|---|---|---|---|---|
| | I | II | III | IV | V | Total |
| Mangrove Swamp | 4 | 2 | 1 | 1 | 3 | 11 |
| Roche Park | 6 | 5 | 4 | 5 | 5 | 25 |
| Total | 10 | 7 | 5 | 6 | 8 | 36 |

*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**

Among the 36 antagonistic actinomycetes, a total of nine isolates with prominent inhibitory activity were subjected to secondary screening to determine their inhibitory profile against the selected fish test pathogens. The isolate $A_{15}$ from the Mangrove swamp area exhibited prominent inhibitory activity with a zone of growth inhibition of 20 mm against all the fish test pathogens (Table 8) (Fig. 2). Three of the nine antagonistic actinomycete isolates tested, exhibited a zone of growth inhibition of 20 mm against 50% the test fish pathogens. Two of the inhibitory actinomycete isolates exhibited a zone of growth inhibition of 20 mm against any three of the test fish pathogens and two isolates exhibited a zone of growth inhibition of 20 mm against any three 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 three of the inhibitory actinomycete isolates exhibited a zone of growth inhibition of 10-19 mm against 66.67% 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 | Test Fish Pathogens* (Zone of inhibition in mm) | | | | | |
|---|---|---|---|---|---|---|---|
| | | T1 | T2 | T3 | T4 | T5 | T6 |
| Mangrove Swamp | $A_5$ | 14 | 13 | 21 | 22 | 0 | 21 |
| | $A_7$ | 6 | 7 | 22 | 23 | 11 | 20 |
| | $A_{11}$ | 5 | 11 | 4 | 8 | 6 | 10 |
| | $A_{15}$ | 28 | 21 | 23 | 29 | 26 | 24 |
| Roche Park | $A_{25}$ | 8 | 17 | 15 | 24 | 14 | 12 |
| | $A_{32}$ | 12 | 11 | 16 | 22 | 0 | 18 |
| | $A_{36}$ | 12 | 13 | 17 | 14 | 12 | 21 |
| | $A_{43}$ | 16 | 12 | 15 | 6 | 8 | 13 |
| | $A_{47}$ | 21 | 11 | 8 | 12 | 20 | 12 |

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

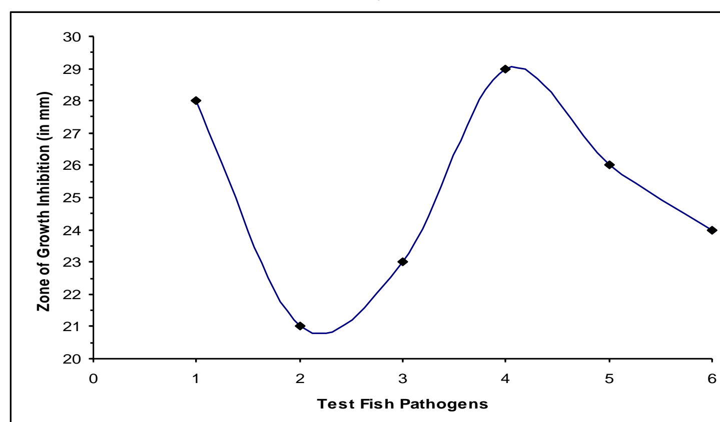


**FIGURE 2:** Inhibitory profile of antagonistic actinomycete strain A15 isolated from Mangrove Swamp

**Color-series of the actinomycetes**

Among the actinomycetes isolated, 71 isolates displayed white aerial mycelium, 49 isolates exhibited gray aerial mycelium and 13 isolates were observed to be with violet aerial mycelium (Fig. 3).Whereas, among the antagonistic actinomycetes, 52 isolates were found to be with white aerial mycelium, 44 isolates displayed gray aerial mycelium and 4 isolates exhibited violet aerial mycelium (Fig. 4).

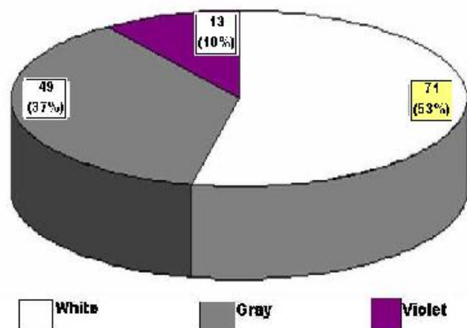


**FIGURE 3.** Color-series of the actinomycete isolates

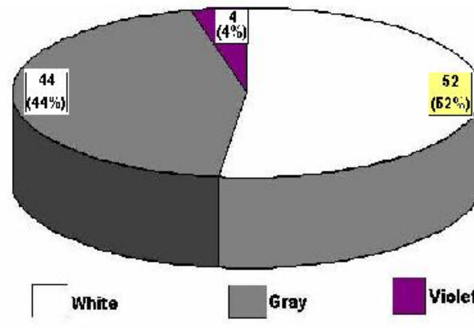


**FIGURE 4.** Color-series of the antagonistic actinomycete isolates

**Identification of actinomycete isolates with antagonistic activity**

Among the antagonistic actinomycete isolates, the isolate $A_{15}$ from the Mangrove swamp area exhibited a high level of inhibitory activity against all the fish test pathogens and hence was subjected to standard chemo-taxonomical tests.

**Light microscopy**

The light, compound microscopy studies of the highly inhibitory actinomycete strain $A_{15}$ 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**

TLC of the cell wall hydrolysate of the strain $A_{15}$ 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 $A_{15}$ was identified to be belonging to the genus *Streptomyces* spp.

**TABLE 9.** Chemo-taxonomic tests used for the identification of antagonistic marine actinomycete isolate $A_{15}$

| 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<br>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" |

**Determination of inhibitory level of crude extracts of the antibacterial substances**

At 100 µg/ml concentration of the crude extract, the highest inhibition of 26 mm was recorded against the test fish pathogen, *Vibrio vulnificus* followed by 25 mm against *V. fischeri*. Also the lowest inhibition was observed to be 15 mm against *Pasteurella* sp. at the same concentration (Table 10). Whereas, at 50 µg/ml concentration of the crude extract, the highest inhibition of 21 mm was recorded against the test fish pathogen, *V. vulnificus* followed by 20 mm against *A. sobria*. While, the lowest inhibition of 12mm 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 $A_{15}$ 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 | 19 | 20 | 19 | 17 | 12 | 21 |
| 100 | 24 | 23 | 25 | 21 | 15 | 26 |

*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

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

### Isolation of marine actinomycetes

In the present study, a high number of actinomycetes were isolated from marine sediment samples (50%) when compared to swab samples (30%) and sea water samples (20%)(Table 6). In a study, Sahu *et al.*(2007) also reported that the mean population density of actinomycetes was higher in sediment samples than in water samples. 46 actinomycetes were isolated from all the samples and all the stations in total in the present study (Table 6). A total of 100 actinomycete strains were isolated from 20 marine sediment samples from the Nicobar islands in a study by Karthik *et al*. (2010). In another study, 42 actinomycete strains were isolated from estuarine and mangrove sediments (Rosmine and Varghese, 2016).

### Detection of antagonistic marine actinomycetes

78.26% of the total number of actinomycetes isolated was detected to be antagonistic against the sensitive *E. coli* strain (MTCC 739) during the primary screening in the present study (Table 7). The results of the present study are in agreement to those of Sahu et al. (2007) who 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 two sampling stations, samples from the Roche Park yielded the highest number of antagonistic actinomycetes (86.21%) in comparison to the isolates of Mangrove swamp area (64.71%) (Table 7). The reasons for high incidence of antagonistic actinomycetes in the samples of Roche Park when compared to that of mangrove swamp, in the present study, may be due to the presence of high organic load in the samples of Roche Park 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

In the present study, during the secondary screening, nine selected antagonistic actinomycete isolates inhibited the selected fish test pathogens to varying degrees (Table 8). 78% of the actinomycete isolates inhibited 100% of the fish test pathogens while 22% of the actinomycete isolates inhibited 83% of the fish test pathogens in the present study (Table 8). 75% of the marine actinomycete isolates were inhibitory to various test organisms in another study (Vanajakumar *et al.*, 1991). On the contrary, Okazaki and Okami (1972) reported that only 27% of marine actinomycete strains were antagonistic to various test pathogens. However, 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*. The isolate $A_{15}$ from the Mangrove swamp area exhibited prominent inhibitory activity with a zone of growth inhibition of 20 mm against all the fish test pathogens (Fig. 2) in the present study. 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*. In the present study, three of the nine antagonistic actinomycete isolates tested, exhibited a zone of growth inhibition of 20 mm against 50% the test fish pathogens. In a study, Abirami *et al.* (2013) 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.

### Color Series of Actinomycetes

In the present study, a high percentage (53%) of the marine actinomycetes belonged to white color series followed by gray (37%) and the lowest percentage (10%) was represented by violet color series (Fig 3). The results of the present study are in agreement with those of Vanajakumar *et al.* (1991) who noted that the white colour series of actinomycetes were dominant followed by the gray, yellow and red color series. Also, in another study, Williams *et al*. (1989) reported that very few *Streptomyces* were found to belong to violet colour series. On the contrary, it was observed that a high percentage (77%) of the isolates of Streptomyces belonged to gray

color series and the remaining to other color series (Hatano, 1997). In the present study, among the antagonistic actinomycetes, a high percentage (52%) belonged to white color series followed by gray color series (44%) (Fig. 4). Violet color series represented only 4% among the antagonistic actinomycetes. Dharmaraj (2011) reported 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**

In the present study, the isolate $A_{15}$ from the Mangrove swamp area exhibited a high level of inhibitory activity against all the fish test pathogens. Hence the strain $A_{15}$ was subjected to light microscopy investigations including standard chemo-taxonomical tests and was identified to be belonging to the genus *Streptomyces* spp. (Table 9). In other studies by many workers, the actinomycete isolates with good inhibitory activity were identified to be belonging to the genus *Streptomyces* spp. (Sahu *et al*., 2007; Karthik *et al.,* 2010; Abirami *et al.*, 2013). However, Parthasarathi *et al*. (2012), in their study, 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**

In the present study, at 50 µg/ml concentration of the crude extract of the actinomycete isolate $A_{15}$, the highest zone of growth inhibition of 20 mm was recorded against two of the test fish pathogens and 16-19 mm zone of inhibition against three of the test fish pathogens and 11-15 mm against only one of the test fish pathogens (Table 10). Similar results were reported by Dharmaraj, 2011, who observed that the at the concentration of 50 µg/ml of crude extract from 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. In the present study, at 100 µg/ml concentration of the crude extract of the actinomycete isolate $A_{15}$, the highest zone of growth inhibition of 20 mm was recorded against five of the test fish pathogens and 11-15 mm zone of inhibition against one of the test fish pathogens (Table 8). 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). From the results of the present study, it can be inferred 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. 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. The results of the present study also indicate that marine antagonistic actinomycetes can also 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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