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ANTIMICROBIAL POTENTIAL OF MARINE ACTINOMYCETES ISOLATED FROM MANGROVE SWAMP AREAS

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ABSTRACT

The present study was undertaken to isolate, determine the inhibitory profile against shrimp test pathogens and identify the actinomycete isolates with prominent activity. A total of 47 Actinomycetes were isolated from 45 samples which included seawater, marine sediment and swab samples of submerged substrates from the Mangrove swamp area located along the coast of Thoothukkudi, Tamil Nadu, India. High number of actinomycetes were isolated from the mangrove sediment samples (26) followed by swabs (12) and seawater (9). 34 (72%) actinomycetes exhibited antagonism to the shrimp test pathogens, *Vibrio alginolytics*, *V. harveyi*, and *V. parahaemolyticus* to varying degrees. 12 isolates exhibited prominent inhibitory activity against the shrimp test pathogens. The isolate A_{10} displayed maximum inhibitory activity, with ≥ 20 mm, zone of growth inhibition against all the three shrimp test pathogens. In the color series, high number of antagonistic actinomycetes belonged to gray color series (21) followed by white color series (7) and violet color series (1). The antagonistic actinomycete isolate A_{10} exhibited prominent inhibitory activity against all the shrimp test pathogens and hence was subjected to standard chemotaxonomic and light microscopy investigations and was identified to be belonging to the genus *Streptomyces* spp. The results of the present study indicate that, Mangrove swamp areas are one of the good sources of inhibitory marine actinomycetes. Also, because these isolates displayed antagonism against the shrimp pathogens, they could be used as bio-control agents in shrimp aquaculture systems for preventing the outbreak of shrimp diseases caused by bacterial shrimp pathogens such as *Vibrio alginolyticus*, *V. harveyi* and *V. parahaemolyticus.* Also, novel, anti-microbial compounds can be extracted from these inhibitory marine actinomycetes for controlling shrimp diseases caused by the antibiotic resistant shrimp bacterial pathogens.

KEYWORDS: Mangrove Swamp, Antagonistic Actinomycetes, Cross-Steak Assay, Shrimp Pathogens, Streptomyces

INTRODUCTION

As the shrimp production from capture fisheries is dwindling, shrimp culture industry is under tremendous pressure to enhance the production and due to indiscriminate use of therapeutic drugs in the industry, shrimp bacterial pathogens are becoming more resistant to the conventional therapeutic drugs used for their control (Karunasagar *et al.*, 1994). Hence, the shrimp culture industry is suffering from heavy financial losses due to such uncontrollable disease outbreaks. Hence there is an urgent need for the search of novel antibacterial compounds with activity against drug resistant shrimp pathogens to alleviate the financial loss incurred by the shrimp farmers due to disease outbreaks. Since, the world oceans occupy more than 70% of the Earth's surface and since the diverse and unique ecosystems in the marine

environment are the richest sources of micro-organisms with unique physiological capabilities, marine actinomycetes antagonistic to shrimp pathogens are the ray of hope for the aquaculture industry. Unique and diverse classes of bioactive compounds have been isolated from marine microorganisms including actinomycetes when compared to their terrestrial counterparts (Bernan *et al.*, 1997). Also, Actinomycetales, a single taxonomic group representing actinomycetes, contributes to most of the commonly used antibiotics (Sanglier *et al*., 1996). Hence, in the present study, efforts were made to isolate marine actinomycetes from mangrove ecosystem along the Thoothukkudi coast, Tamil Nadu, determine their antagonistic profile against the shrimp bacterial pathogens, find out their color series and identify the actinomycete isolates with prominent inhibitory activity.

MATERIALS AND METHODS

Collection of the Samples

Water, sediment and swab samples were collected from the mangrove swamps of Thoothukkudi coast, Tamil Nadu. Water samples were collected aseptically in 50 ml sterile, glass, screw cap vials. Sediment samples were collected in sterile polypropylene bags. Swab samples were collected from marine submerged substrates and dropped into 10 ml sterile seawater in 50 ml glass, screw cap vials.

Isolation of Marine Actinomycetes

The sediment samples were air dried at room temperature for 24-48 hours and then used. Ten fold serial dilutions were carried out with aged sea water for all the samples and the dilutions were mixed in a vortex mixer and were spread plated on modified 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
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 actinomycete isolates with a typical chalky to leathery appearance (IMTECH, 1998) were selected. The isolates were also subjected to Gram staining, Acid fast staining and to light microscopy (NIKON, Japan) for ascertaining their filamentous nature, with width of hyphae being 0.5 – 2 µ, their nature of aerial and substrate mycelium (Cappucino and Sherman, 2004). Those isolates which were Gram-positive, non-acid fast with aseptate hyphae were picked up and purified onto Starch Casein Agar (SCA) plates. The purified isolates were then sub-cultured on SCA slants, incubated at room temperature for 6-7 days and stored at refrigeration temperature till further use.

Determination of the Antagonistic Profile of Marine Actinomycetes against Shrimp Bacterial Pathogens

The antagonistic profile of the actinomycete isolates was determined against selected shrimp test pathogens as per

the modified cross-streak assay of Lemos *et al*. (1985). Modified Antibiotic Assay Medium (AAM) medium (Table 2) was used and the actinomycete isolates were streaked across the diameter on AAM plates with a width of the streak being 8-10 mm.

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 an incubation period of 5-7 days at room temperature, young cultures of the shrimp test pathogens, *Vibrio alginolyticus, V. harveyi* and *V. parahaemolyticus* 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 clear zones near the central strip indicate the inhibitory activity of actinomycete isolates and the 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 mycelial color of the actinomycete isolates as well as the antagonistic actinomycete isolates was observed and recorded.

Identification of Antagonistic Marine Actinomycetes

The antagonistic actinomycete isolates with prominent activity were identified using the standard taxonomic schemes of IMTECH (1998) and Goodfellow (1989).

Light Microscopy

The actinomycete isolates with higher antagonistic activity were sub-cultured with SCA medium by employing the Cover slip culture technique. The aerial and substrate mycelial nature of these antagonistic actinomycete isolates was observed and recorded with a trinocular compound microscope (Nikon, Japan) by using a novel, indigenously designed cover slip holder for scanning the field (Cappucino and Sherman, 2004).

Chemotaxonomy

Xanthine, casein, 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 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. 0.2% (w/v) ninhydrin in acetone was used 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 standards and identified. The characteristic cell wall sugars of the antagonistic actinomycete isolates were also detected with the help of 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. Aniline phthalate reagent (prepared using aniline 2 ml, phthalic acid 3.3g and water saturated butanol 100 ml) was used for visualization. The plates were developed by heating the plates at 100^0C for 5 minutes. The Rf values of samples were calculated, compared with standards and the sugars in the samples were identified.

RESULTS AND DISCUSSIONS

Isolation of Actinomycetes

In the present study, a total of 47 actinomycetes were isolated from 45 samples which included water, sediment and swab samples from the mangrove swamp (Table 3).

Table 3: Number of Actinomycetes Isolated from Different Marine Samples

Samples/Actinomycetes		Numbers
Water	No. of Samples	15
	No. of Actinomycete Isolates	9
Sediment	No. of Samples	15
	No. of Actinomycete Isolates	26
Swabs	No. of Samples	15
	No. of Actinomycete Isolates	12
Total	**No. of Samples**	**45**
	No. of Actinomycete Isolates	**47**

Rosmine and Varghese (2016) reported the isolation of 50 actinomycete strains from estuarine and mangrove sediments. On the contrary, Karthik *et al*. (2010) reported a total of 100 actinomycetes from 20 marine sediment samples. High number of actinomycetes were isolated from the mangrove sediment samples (55%) in the present study (Table 3). Sahu *et al.*(2007) reported that the mean population density of actinomycetes was higher in sediment samples than in other samples.

Inhibitory Activity of the Actinomycete Isolates against various Fish Test Pathogens

Among 47 actinomycete isolates, 34 (72%) actinomycetes exhibited antagonism to the shrimp test pathogens to varying degrees. The results of the present study are in agreement to those of Vanajakumar *et al.* (1991) who reported that 75% of the marine actinomycete isolates were inhibitory to various test organisms. In contrast to the results of the present study, 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*. Out of 34 antagonistic actinomycete isolates, 12 isolates exhibited prominent inhibitory activity against the shrimp test pathogens, *Vibrio alginolytics*, *V. harveyi*, and *V. parahaemolyticus* (Table 4) in the present study.

Table 4: Inhibitory Profile of Antagonistic Actinomycetes from Different Marine Sampling Stations against Shrimp Test Pathogens

Antagonistic Actinomycete Isolates	Shrimp Test Pathogens (Zone of Inhibition in mm)		
	V.alginolyticus	*V. harveyi*	*V. parahaemolyticus*
A_1	14	18	19
A_2	10	13	6
A_3	18	12	11
A_4	R	18	15
A_6	16	19	15
A_8	18	15	R
A_9	15	12	14
A_{10}	22	25	24
A_{12}	13	16	12
A_{13}	R	8	12
A_{14}	5	7	14
A_{16}	13	10	7

However, Dharmaraj, S. (2011) reported that among 94 actinomycete isolates, only seven actinomycete isolates were antagonistic to the shellfish pathogens, *V. alginolyticus*, *V. harveyi*, and *V. parahaemolyticus*. In another study, Rosmine and Varghese (2016) reported that only two of the isolates out of 50 actinomycetes were antagonistic to the shrimp bacterial pathogens, *V. alginolyticus*, *V. harveyi*, and *V. parahaemolyticus*. Nine of the 12 antagonistic actinomycete isolates inhibited 100% the shrimp test pathogens to varying degrees, in the present study. In the present study, the isolate A_{10} exhibited prominent inhibitory activity, with a zone of growth inhibition of ≥ 20 mm against all the three shrimp test pathogens, *V. alginolyticus*, *V. harveyi*, and *V. parahaemolyticus* (Table 4) (Fig. 1).

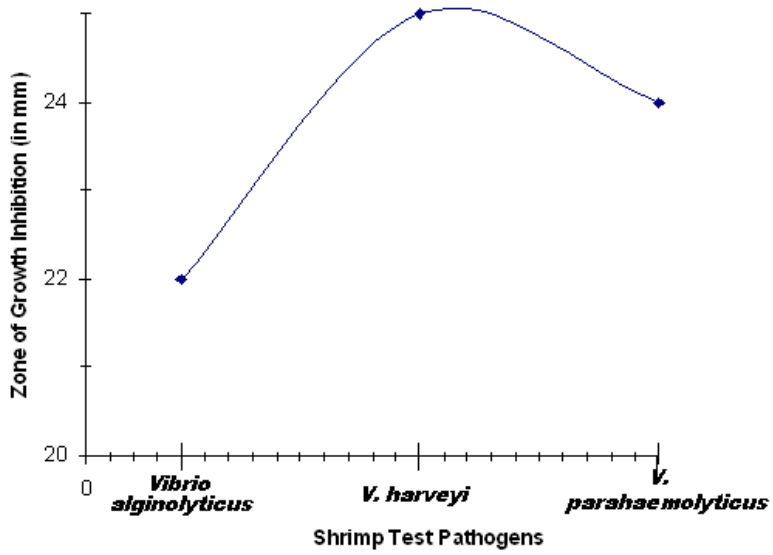

Figure 1: Inhibitory Activity of the Highly Antagonistic Actinomycete Isolate A_{10} in Terms of the Zone of Growth Inhibition (in Mm)

In a study, Sahu *et al.* (2007) reported that the marine actinomycete strain, MKS-24 was very active against all the three shrimp test pathogens *Vibrio alginolytics*, *V. harveyi*, and *V. parahaemolyticus*.

Color-Series of the Actinomycetes

In the present study, a high percentage (55%) of the marine actinomycetes belonged to white color series followed by gray (36%) and the lowest percentage (9%) was represented by violet color series (Fig 2-A). Vanajakumar *et al.* (1991) also reported 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. Among the antagonistic actinomycetes, a high percentage (73%) belonged to gray color series followed by white color series (24%) and Violet color series (3%) in the present study (Fig. 2-B). On the contrary, Dharmaraj (2011) reported that 57% of the inhibitory actinomycetes belonged to white color series followed by gray color series (29%) and yellow color series (14%). On the other hand, Rosmine and Varghese (2016) reported that white and gray color series were equally inhibitive and observed that 50% of the inhibitory actinomycetes belonged to white color series and another 50% had gray colored aerial mycelium.

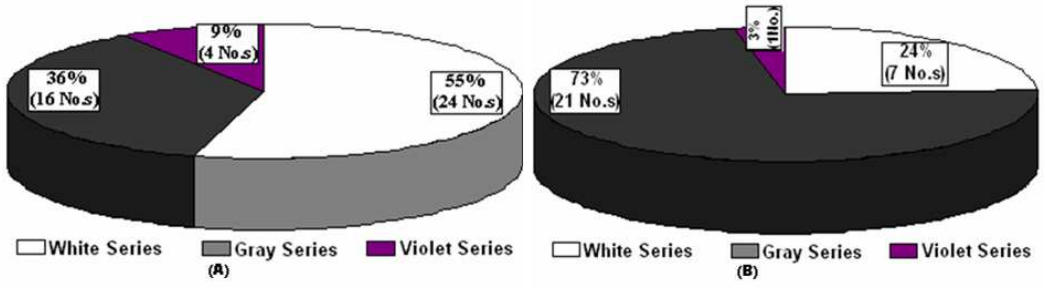

Figure 2: Color Series of (A) Actinomycetes (B) Antagonistic Actinomycetes

The results of the present study are in agreement to those of Sathiyaseelan and Stella (2011) who noted that majority (57%) of the inhibitory strains belonged to gray series followed by white (29%) color series and yellow (14%) color series.

Identification of Actinomycete Isolates with Antagonistic Activity

The isolate A_{10} from the Mangrove swamp area exhibited a high level of inhibitory activity against all the shrimp test pathogens, in the present study. Hence, the strain A_{10} was subjected to standard chemotaxonomic and light microscopy investigations and was identified to be belonging to the genus *Streptomyces* spp. (Table 5). Studies by many workers, revealed that 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. However, in a separate study, Dharmaraj, S. (2011) reported that all the seven antagonistic actinomycete strains belonged to the genus *Streptomyces* spp.

Table 5: Chemo-Taxonomic Tests Used for the Identification of Antagonistic Marine Actinomycete Isolate A_{10}

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"

CONCLUSIONS

It can be inferred from the results of the present study that Mangrove swamp areas are one of the good sources of inhibitory marine actinomycetes. Also, the inhibitory profile of the antagonistic marine actinomycetes against the shrimp test pathogens revealed that these antagonistic marine actinomycetes can be used as bio-control agents in shrimp aquaculture systems in preventing the outbreak of diseases caused by bacterial pathogens such as *Vibrio alginolyticus*, *V. harveyi* and *V. parahaemolyticus* in the shrimp culture systems. Also, the novel, anti-microbial compounds can be extracted from these antagonistic marine actinomycetes for controlling shrimp diseases caused by the antibiotic resistant shrimp bacterial pathogens.

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