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BIO-ACTIVITY OF MARINE ACTINOMYCETES AGAINST FOOD-BORNE HUMAN PATHOGENS

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ABSTRACT
The present study was undertaken to isolate and assess the anti-microbial profile of antagonistic actinomycetes against the seafood-borne human pathogens. A total of 133 actinomycetes were isolated from 129 samples. The samples which included seawater, marine sediment and swab samples were obtained from five different sampling stations, Mangrove swamp, Roche Park, Thermal Beach, Hare Island and Near Shore Area, located along the coast of Thoothukkudi, Tamil Nadu, India. Preliminary confirmation of the actinomycetes was done by gram's reaction, acid fast staining and cellular nature of the isolates through light, compound microscopy. Highest number of actinomycetes (45) was isolated from the Thermal Beach samples. The actinomycete isolates were assessed for their antagonistic activity against the seafood-borne human pathogens, *Salmonella typhi*, *S. typhimurium* and *Escherichia coli* by cross-streak assay. A total of 104 actinomycete isolates exhibited activity against the three pathogens to varying degrees. High percentage of antagonistic actinomycetes (86.67%) was isolated from the Thermal Beach samples. The results of the present study, indicate that novel antimicrobial compounds with activity against food-borne human pathogens can be extracted from the antagonistic actinomycetes from various marine environments.

KEY WORDS: marine, antagonisitic actinomycetes, food-borne human pathogens, color series, cross-streak assay.

INTRODUCTION

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, β-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*., 2011). Marine antagonistic actinomycetes represent a fairly untapped resource of novel antimicrobial compounds and these compounds can be can be used in controlling the diseases caused by various pathogens (Patil *et al.*, 2001). Hence, the present study was carried out with the objectives of isolation of the actinomycetes from different marine samples and screening them for activity against food-borne human pathogens.

MATERIALS & METHODS

Collection of samples

Marine water, sediment and swab samples were collected from the five sampling stations, Mangrove swamp, Roche Park, Thermal Beach, Hare Island and Near Shore Area located along the east coast of Thoothukkudi, Tamil Nadu, India. Water samples were collected aseptically in sterile, 50 ml, cylindrical, screw-capped glass bottles. 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, and 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.

Isolation of marine actinomycetes

Aged seawater was used as a diluent and tenfold 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
$Fe_3SO_4.7H_2O$	0.01
KNO_3	2.0
$MgSO_4.7\ H_2O$	0.05
NaCl	5.0
Agar	18.0
Aged Sea water	Make upto 1L
pH	7.1±0.1

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).

Ascertaining the isolates as Actinomycetes

Gram staining, acid fast staining and 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 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. 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 compound microscope (Nikon, Japan) by using a novel, indigenously designed cover slip holder for scanning the field (Cappucino and Sherman, 2004).

Assessement of antagonism of marine actinomycetes

Modified cross-streak assay of Lemos *et al*. (1985) was employed for the determination of the antagonistic profile of the actinomycete isolates against selected food-borne human pathogens. Modified Antibiotic Assay Medium (AAM) (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. After an incubation period of 5-7 days at room temperature, young cultures of the food-borne human pathogens, *Salmonella typhi*, *S. typhimurium* and *Escherichia coli* 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.

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
Aged Sea water	Make up to 1L
pH	7.9±0.2

Color series of actinomycete isolates

The color characteristics of the aerial mycelium of the actinomycete isolates play an important role in the identification of the isolates. The color of the aerial mycelium of the actinomycete isolates was observed and noted down.

RESULTS & DISCUSSION

Isolation of actinomycetes

A total of 133 marine actinomycetes from the five sampling stations, Mangrove swamp, Roche Park, Thermal Beach, Hare Island and Near Shore Area were isolated (Table 5) from a total of 129 marine samples which included seawater, marine sediment and swab samples.

TABLE 3. Number of Actinomycetes isolated from different marine samples from different sampling stations

Samples/Actinomycetes		Sampling Stations					Total
		Mangrove Swamp	Roche Park	Thermal Beach	Hare Island	Nearshore Area	
Water	No. of Samples	5	6	4	5	4	24
	No. of Actinomycete Isolates	3	6	8	5	2	24
Sediment	No. of Samples	15	19	18	15	10	77
	No. of Actinomycete Isolates	9	14	27	14	11	75
Swabs	No. of Samples	6	4	2	12	4	28
	No. of Actinomycete Isolates	5	9	10	8	2	34

High number of actinomycetes were isolated from marine sediment samples (75) when compared to swab samples (34) and sea water samples in the present study (24)(Table 5). In the present study, in total, 133 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). The picked up colonies were subjected to gram staining, acid-fast staining and light, compound microscopy studies to confirm the isolates as actinomycetes (Table 4).

TABLE 4. Tests used for the confirmation of actinomycetes

Test/Analysis	Result
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

Assessment of the antagonistic profile of marine actinomycetes

In the present study, of the 133 actinomycete isolates, a total of 104 actinomycetes exhibited antagonism to the three food-borne human pathogens to various degrees. About 8.65% of the actinomycete isolates exhibited >20 mm zone of growth inhibition against *Salmonella typhi*, 10.58% of the actinomycete isolates displayed >20 mm zone of growth inhibition against *S. typhimurium* and 16.35% of the actinomycete isolates revealed >20 mm zone of growth inhibition against *Escherichia coli* (Table 5) in the present study. The results of the present study are similar to the results of Sharma *et al.*, (2011) who observed that 7.84% of the actinomycete isolates displayed 15-20 mm inhibitory zone against *E. coli* and 2% of the actinomycete isolates exhibited >20 mm zone of inhibition against *E. coli*, 4% of the isolates displayed 15-20 mm zone of inhibition against *Salmonella typhi* and 2% of the isolates exhibited > 20 mm zone of inhibition against *Salmonella typhi.* In the present study, 34.61% of the total actinomycetes isolated, exhibited >10 mm zone of inhibition against *Salmonella typhi*, 25.97% exhibited >10 mm zone of inhibition against *S. typhimurium* and 34.62 % exhibited >10 mm zone of inhibition against *Escherichia coli.* Sharma *et al.,* (2011) also observed that 38% of the actinomycete isolates showed activity against one or more test pathogens. A total of 67 (57.68%) actinomycetes were observed to be active against *S. typhi*, 59 (56.74%) actinomycetes active against *S. typhimurium* and 63(60.58%) actinonomycetes exhibited their activity against *Escherichia coli* (Table 5) in the present study. The results of the present study are in agreement with those of Kumar *et al.* (2014), who observed that 64.86% of the actinomycete isolates, showed activity against *Salmonella typhi*-B, *S. typhi* and *S. typhimurium* to varying degrees. On the contrary, only nine out of 54 actinomycetes inhibited the human pathogens of *E. coli*, *S. typhi* and *S. typhimurium* in a study by Sengupta *et al.* (2015).

Table 5. Zone of growth inhibition displayed by marine actinomycetes against food-borne human pathogens

Test pathogens	Number of actinomycete isolates (zone of inhibition in mm)					
	< 1	1 – 5	5 – 10	10 – 15	15 – 20	> 20
Salmonella typhi	44 (42.31)	21 (20.19)	3 (2.88)	13 (12.50)	14 (13.46)	9 (8.65)
S. typhimurium	45 (43.27)	20 (19.23)	12 (11.54)	12 (11.54)	4 (3.85)	11 (10.58)
Escherichia coli	41 (19.42)	13 (12.50)	14 (13.46)	17 (16.35)	2 (1.92)	17 (16.35)

* Percentage values are given in paranthesis

In the present study, of the five sampling stations, samples from the Roche Park and Thermal Beach yielded the highest number of antagonistic actinomycetes. The reasons for high incidence of antagonistic actinomycetes in the samples of Roche Park and Thermal Beach when compared to those from other sampling stations may be the presence of high organic load in the samples of Roche Park and Thermal Beach due to various anthropogenic activities due to 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 by secreting highly diverse classes of anti-microbial compounds (Walker and Colwell, 1975).

Color-series of the actinomycetes

In the present study, 71(53.38%) actinomycete isolates displayed white aerial mycelium, while, 49 (36.84%) isolates exhibited gray aerial mycelium and 13 (9.77%) isolates displayed violet aerial mycelium (Table 6).

In a study, Vanajakumar *et al.* (1991) also reported that the white colour series of actinomycetes were dominant followed by the gray, yellow and red color series. Among the antagonistic actinomycetes, 52 (50%)isolates were found to be with white aerial mycelium, while, 46 (44.23%) isolates displayed gray and 6 (5.77%) isolates exhibited violet aerial mycelium (Table 7) in the present study.

TABLE 6. Color series of the actinomycetes from different sampling stations

Actinomycetes	Mangrove Swamp	Roche Park	Thermal Beach	Hare Island	Inshore Area	Total
White	9 (52.94)	15 (51.72)	23 (51.11)	16 (59.26)	8 (53.33)	71 (53.38)
Gray	6 (35.29)	11 (37.93)	16 (35.56)	10 (37.04)	6 (40)	49 (36.84)
Violet	2 (11.76)	3 (10.34)	6 (13.33)	1 (3.70)	1 (6.67)	13 (9.77)

*Percentage values are given in paranthesis

TABLE 7. Color series of the antagonistic actinomycetes from different sampling stations

Antagonistic Actinomycetes	Mangrove Swamp	Roche Park	Thermal Beach	Hare Island	Inshore Area	New Total
White	7 (53.85)	12 (48.00)	18 (48.65)	10 (52.63)	5 (50.00)	52 (50.00)
Gray	5 (38.46)	11 (44.00)	16 (43.24)	9 (47.37)	5 (50.00)	46 (44.23)
Violet	1 (7.69)	2 (8.00)	3 (8.11)	0 (0)	0 (0)	6 (5.77)

* Percentage values are given in paranthesis

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. In another study, Rosmine and Varghese(2016) reported that 50% of the inhibitory actinomycetes belonged to white color series and another 50% had gray colored aerial mycelium.

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