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# A preliminary study on the antimicrobial activity of marine actinomycetes

## Authors Info

**P. Dhevagi[1]*, A. Brundha[2], K. Geetha[2] , R. Gobu[2] , K.A.A. Manju[2] and E. Poorani[3]**

[1]Department of Environmental Sciences, Tamil Nadu Agricultural University, Coimbatore -641 003, India

[2]Department of Environmental Sciences, Tamil Nadu Agricultural University, Coimbatore-641 003, India

[3]Department of Biotechnology, Government College of Technology, Coimbatore-641 013, India

*Corresponding Author Email : devagisivaraj@gmail.com

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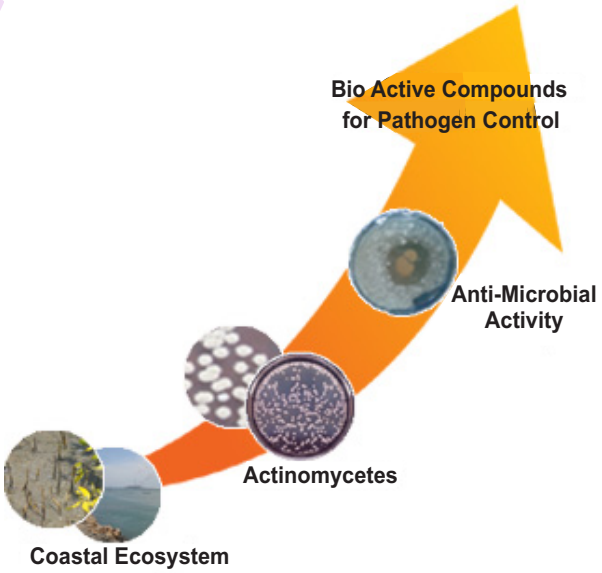
## Abstract

**Aim:** Marine actinomycetes are considered as an unexplored source of metabolites with novel properties. During the past few years, the studies on marine actinomycetes highlighted their potential as source of numerous antibiotics. An experimental study was planned to harness the potential of marine actinomycetes as a source of bioactive compounds to control plant diseases.

**Methodology:** Sediment samples from Thoothukudi coastal ecosystem were collected and analyzed for biological properties. The isolates with different morphological characteristics were selected and about 108 isolates were subjected to cross streak method of screening against fungal cultures like *Pyriculariae oryzae, Aspergillus niger*, *Fusarium solani, Trichoderma, and Macrophomina sp* and bacterial strains like *Bacillus cereus, Erwinia carotovora, Serratia sp., Flavobacterium sp* and *Pseudomonas fluorescens*. Solvent extract of isolates producing significant zone of inhibition were screened against plant pathogens.

**Results:** Many isolates produced an inhibition zone of 2 to > 10mm against the fungal and bacterial cultures. The isolate TS3 obtained from TS sediments showed more than 10 mm inhibition zone against *Erwinia caratovora*, *Pyriculariae oryzae, Aspergillus niger* and *Trichoderma.* The isolates which showed more than 10 mm antifungal and antibacterial activity were selected for further screening. Ehtylacetate extract of RPS5 showed an inhibition zone of 9 to 14 mm and 18 to 22 mm, respectively, against bacterial and fungal pathogens. Similarly, HCS 6 isolates showed an inhibition zone of 9 to 12 mm against bacterial pathogens and 16 to 22 mm inhibition zone, respectively, against fungal pathogens.

**Interpretation:** Considering the environmental impacts of chemical fungicides, control of pathogens through biological means helps to overcome the worsening problem caused by chemicals. The solvent extract of RPS 5 and HCS 6 isolates showed significant reduction in the growth of pathogenic organisms, which paves way for commercial exploitation of the isolates to control plant pathogens.



## Introduction

Marine environment is being increasingly studied in search for novel microbial products. The varied and extreme environmental conditions in the marine ecosystem could be the reason why almost every group of organisms isolated from marine environment has unique structures and properties. The focus and frequency of metabolites isolation from the terrestrial Actinomycetes is decreasing gradually, in contrast to the screening of the Actinomycetes from marine environment that have gained attention. Today researchers have isolated approximately 11,000 marine derived products (Sahu *et al.,* 2007a and b; Vimal *et al.,* 2009) with marine Actinomycetes being responsible for the production of about half of the discovered bioactive secondary metabolites. Sujatha Peela *et al.* (2005), Dhevagi and Poorani (2006) isolated Actinomycetes capable of producing bioactives from the sediments of South East Coastal regions of India.

Plant diseases contribute to severe agricultural losses (nearly 50%), especially in developing countries. Fungal and bacterial attacks in agricultural plants are the major contributors in the subtropical and tropical regions (Pohanka, 2006a). The mycotoxins produced from them are harmful to humans and livestock. The effectiveness of the current fungicides and bactericides has become less, besides becoming toxic to humans and other non-target organisms, and also serve as a base for pathogenic resistance development (Zaitlin *et al*., 2004; Dahiya *et al*., 2006; Gohel *et al*., 2006; Pohanka, 2006b). Considering these worsening problems, the micro-organisms or their secretions serve as an attractive alternative against these plant pathogens. Therefore, the biological control tactics to control diseases is a significant approach that can't be ignored and they facilitate sustainable agriculture. In this regard, Actinomycetes as known to produce novel antibiotics deserve to be studied.

The antagonistic activity of marine Actinomycetes has been reported by Krishnaraj and Mathivanan (2011). Many Actinomycetes have been tested, however very limited work has been done in the use of antibacterial and antifungal compounds against plant pathogens (Patil *et al*., 2001). Hence, an attempt was made to isolate actinomycetes with antibacterial and antifungal activities from the coastal sediments of Thoothukudi, Tamil Nadu.

## Materials and Methods

**Collection of marine sediments :** Marine sediment samples were collected from six different places viz., Threspuram (TS), Meenavar Kuppam (MK), Beach road near Roach Park (RP), Harbor camp (HC), Deep sea sediment (D1, D2) from Thoothukudi district of Tamil Nadu. To minimize variability, subsamples of minimum five was taken and mixed together as composite sample. Depending on the location, Grab sampler or Core sampler method was used for collecting samples. The samples were collected in alcohol rinsed Peterson Grab sampler (RISC, 1999), then transferred to sterile polythene bags and transported to laboratory for further analysis. Grab sampler was used for collecting samples from D1 and D2 and in case of TS, RP, HC and MK core sampling method was used. The depth at which sample was collected varied from 1 feet in case of rhizosphere samples to 20 feet in case of deep sea sediments. The TS sediment was clay with fine organic materials and MK, D1 and D2 sediments were found to be muddy sand types. Samples RP and HC were from rhizosphere zone of mangrove plants. The sediment samples were analyzed for physico-chemical properties as per the method suggested by APHA (2005).

**Isolation of marine actinomycetes :** Enrichment of sediment was done by transferring one gram of sediment sample to 100 ml of sterile seawater complex broth. It was then incubated at 37° C for 14 days in an incubator cum shaker (Ellaiah and Reddy, 1987). After incubation, the enriched sediment suspension was diluted. One ml of the diluted suspension was spread over the surface of the Sea Water Complex Agar medium, incubated and observed for number of colonies.

Seven media viz., nutrient agar and Kenknight agar (Cappucino and Sherman, 2002) , Sea water complex agar (Parsons *et al*.,1984), Actinomycetes isolation agar (Hi media laboratories), Modified $M_9$ medium (Dharmaraj, 2011), Modified Nutrient Agar (Rathna kala and Chandrika, 1993) and Starch casein agar (Shirling and Gottlieb, 1966) were selected and the efficacy of these, in isolation and growth of actinomycetes from the marine sediment samples was determined. The pH of the medium was adjusted to 7.4. Further more to prevent bacterial and fungal contaminants, Cyclohexamide (50 mg $l^{-1}$) and Nalidixic acid (20 mg $l^{-1}$) were added. The plates were incubated at 32$^0$C and colonies were observed. Individual colonies were purified by repeated streaking on SCA medium and maintained on slants at 4 ºC.

**Isolates used for the study :** Actinomycetes: The pure isolates obtained from the above procedure were preserved on agar slants until further use.

**Bacterial cultures :** Bacterial cultures *viz*., *Bacillus cereus, Erwinia carotovora, Serratia sp., Flavobacterium sp and Pseudomonas fluorescens* obtained from MTCC, Chandigarh were cultured and maintained at 4 ºC on nutrient agar slants.

**Fungal cultures :** Fungal isolates *Pyriculariae oryzae, Aspergillus niger, Fusarium solani, Trichoderhma sp* and *Macrophomina sp* obtained from the Department of Plant Pathology, TNAU were used for testing the antagonistic activity. The fungi were cultured on potato dextrose agar plates.

**Screening of actinomycete isolates for antimicrobial activity primary screening :** Primary screening for antibacterial activity

of isolates was determined by cross streak method (Arifuzzaman *et al.,* 2010). Actinomycete isolates were inoculated in straight line on Mueller Hinton agar plate and incubated at 32.0 ºC for six days. Then test organisms were inoculated perpendicular to the Actinomycetes and these plates were incubated at 37.0 ºC for 24 hrs. At the end of incubation, the antibacterial activity was observed by determining the distance of inhibition between target bacteria and Actinomycetes colony margins. Those isolates which showed maximum inhibition zone were selected for secondary screening

Antifungal activity was measured by conventional spot inoculation method with slight modification. Pure Actinomycete strains were spot inoculated and then inverted over 2 ml of chloroform for 45 min in a fume hood to kill the organisms. Killed colonies were overlaid with 10 ml of Potato Dextrose Agar, previously inoculated with one ml of test organisms ($1\times10^6$ spores $ml^{-1}$). The antifungal activity around the colonies was recorded after incubation. Control plates were also maintained without inoculating Actinomycetes to assess the normal growth of the pathogens. Both negative and positive control plates were maintained.

**Secondary screening :** Secondary antimicrobial screening of the promising isolates was done by disc diffusion assay (Bradshaw, 1979). The promising isolates were inoculated in SCA broth and kept on shaker. After 96 hrs of shaking, the culture broth was centrifuged at 10, 000 rpm for 10 min. The supernatant, thus obtained, was mixed with ethyl acetate, chloroform, butanol (1:1 v/v) and shaken vigorously for 30 min in a separating funnel. The contents were allowed to stabilize for 48 hrs. After 48 hrs, the solvent phase was separated from aqueous phase. The solvent extracts were concentrated and used for testing antimicrobial activity (Atta and Ahmad, 2009)

Wells (6.0mm diameter, 2.0cm apart) were formed using sterile cork borer in the test organism inoculated plates and 50 µl of solvent extract was loaded into each well for antagonistic activity against different pathogens. Uniform diffusion of extract was allowed by incubating the plates at 4 ºC for 2 hours (Gulve and Deshmukh, 2012), and the zone of inhibition excluding disc diameter indicated the antimicrobial activity.

**Characterization of promising isolates :** The promising isolates showing both antibacterial and antifungal activities were characterized by standard methods (Shirling and Gottlieb, 1966; Pridham and Gottlieb, 1948).

## Results and Discussion

Marine sediment samples were collected and characterized from six different coastal zones of Thoothukudi. A pH range of 8.4 to 9.0 with considerable organic carbon content was observed in all the six samples. The total number of organisms were also high which is in line with the findings of Sugumar *et al.* (2008) who showed the presence of potential risk due to pollution in the coastal waters along Thoothukudi. After enrichment and incubation, the sediment samples were plated on Sea Water Complex Agar from which individual colonies were purified and stored. Maximum numbers of isolates were obtained from RP (28) and HC (24) samples and least population were observed in TS (12), which might be attributed to the difference in the sampling locations. The occurrence of Actinomycetes in the marine sediments was reported by Kathiresan *et al.* (2005). Patil *et al.* (2001) isolated a total of 133 Actinomycetes isolates from Tuticorin coast of Southeast Coastal areas of India. Thus, mangrove sediments can be a potential source for marine Actinomycetes as stated by Krishnaraj and Mathivanan (2011) and Valli *et al.* (2012).

Dhevagi and Poorani (2006) used sea water complex broth for culturing the actinomycetes. Least population was observed in modified M9 medium and Kenknight agar, both of which are not effective for enumeration of Actinomycetes, in contrary, to Rathinakala and Chandrika (1993), who observed that in Kuster's agar, when acetic acid was used, the colonies were countless showing selective enrichment of Actinomycetes over control medium. Lateef and Oloke (2003) observed that Lactose minimal agar is good for isolation of Actinomycetes, as it inhibited the growth of bacteria and fungi. Patro *et al.* (2014) developed medium composition for Industrial production of L asparaginase. Baskaran *et al. (*2011) reported that the number of colony forming units were high in AIA.

Different morphology was observed in different media. In sea water complex agar medium (SWCA), large sized white cottony growth was observed. In AIA medium, small white colored colonies were observed. Large white colored colonies with fibrous growth surrounding the colonies were observed in SCA medium. Since the colony morphology and size was large in case of SCA, this medium can be a suitable medium for the isolation of Actinomycetes from the sediments.

All the samples had isolates with antibacterial activities and more than 80% of the isolates exhibited less inhibition zone (less than 2mm or none) (Table 1). Test fungi were suppressed with an inhibition zones ranging from 2 mm to 15 mm (Table 2). In all the samples, more than 50 % of the isolates showed an inhibition zone of > 2 mm and in many cases no inhibition zone at all. The isolates showing inhibition zone of >10 mm antifungal and antibacterial activity was termed as promising isolates. Of the isolates tested, promising isolates showing both bacterial and fungal antagonistic activity (TS3, MKS4, RPS5, HCS6, DS7, and DS8) were selected and used for secondary screening.

Microbe based bio-control methods are an alternative way to control diseases in place of agrochemicals (Bressan, 2003; Jayasinghe and Parkinson, 2008). An isolate EPD 27 with L

**Table 1 :** Antibacterial activity as measured by zone of inhibition (mm) of Actinomycetes isolates from Thoothukudi coastal ecosystem of Tamil Nadu

| Sampling location | Total no of isolates | Zone of Inhibition (mm) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Bacillus cereus* | | | *Erwinia caratovora* | | | *Serratia sp* | | | *Flavobacterium sp* | | | *Pseudomonas fluorecens* | | |
| | | < 2 | 5-10 | >10 | < 2 | 5-10 | >10 | < 2 | 5-10 | >10 | < 2 | 5-10 | >10 | < 2 | 5-10 | >10 |
| TS | 12 | 10 | 1 | 0 | 5 | 2 | 0 | 8 | 0 | 0 | 8 | 1 | 0 | 5 | 2 | 0 |
| MK | 13 | 5 | 3 | 1 | 7 | 1 | 0 | 4 | 2 | 0 | 8 | 0 | 0 | 6 | 1 | 0 |
| RP | 28 | 8 | 8 | 1 | 13 | 4 | 1 | 9 | 8 | 1 | 7 | 2 | 2 | 9 | 3 | 1 |
| HC | 24 | 14 | 1 | 0 | 3 | 0 | 1 | 8 | 4 | 2 | 12 | 5 | 1 | 8 | 1 | 1 |
| D1 | 6 | 4 | 2 | 0 | 5 | 3 | 0 | 4 | 1 | 0 | 2 | 2 | 1 | 4 | 2 | 0 |
| D2 | 15 | 5 | 2 | 0 | 6 | 2 | 1 | 6 | 0 | 0 | 7 | 0 | 0 | 4 | 1 | 1 |

**Table 2 :** Antifungal activity as measured by zone of inhibition (mm) of Actinomycetes isolates from Thoothukudi coastal ecosystem of Tamil Nadu

| Sampling location | Total no of isolates | Zone of Inhibition (mm) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Pyriculariae oryzae* | | | *A. Niger* | | | *Fusarium solani* | | | *Trichoderma sp* | | | *Macrophomina sp* | | |
| | | < 2 | 5-10 | >10 | < 2 | 5-10 | >10 | < 2 | 5-10 | >10 | < 2 | 5-10 | >10 | < 2 | 5-10 | >10 |
| TS | 12 | 3 | 2 | 1 | 4 | 2 | 1 | 10 | 0 | 0 | 7 | 3 | 1 | 2 | 5 | 0 |
| MK | 13 | 6 | 2 | 2 | 8 | 2 | 1 | 12 | 2 | 0 | 4 | 2 | 1 | 4 | 0 | 0 |
| RP | 28 | 12 | 6 | 3 | 11 | 4 | 3 | 12 | 4 | 2 | 11 | 7 | 1 | 8 | 6 | 3 |
| HC | 24 | 7 | 4 | 2 | 9 | 3 | 0 | 4 | 5 | 1 | 09 | 8 | 4 | 12 | 3 | 1 |
| D1 | 15 | 8 | 2 | 1 | 6 | 2 | 0 | 6 | 4 | 1 | 10 | 4 | 0 | 6 | 2 | 1 |
| D2 | 16 | 4 | 2 | 0 | 12 | 1 | 0 | 11 | 2 | 1 | 7 | 4 | 0 | 8 | 2 | 0 |

**Table 3:** Antibacterial activity as measured by zone of inhibition (mm) of promising Actinomycetes isolates from Thoothukudi coastal ecosystem of Tamil Nadu

| Promising Isolates | Zone of Inhibition (mm) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Bacillus cereus* | | *Erwinia caratovora* | | *Serratia sp* | | *Flavobacterium sp* | | *Pseudomonas fluorecens* | |
| | CE | EE | CE | EE | CE | EE | CE | EE | CE | EE |
| TS3 | 5 | 10 | 5 | 8 | 5 | 9 | 5 | 10 | 5 | 10 |
| MKS4 | 5 | 5 | 4 | 9 | 5 | 9 | 5 | 9 | 7 | 9 |
| RPS5 | 7 | 14 | 5 | 9 | 5 | 9 | 5 | 12 | 8 | 10 |
| HCS6 | 9 | 12 | 5 | 9 | 5 | 10 | 5 | 10 | 5 | 9 |
| DS7 | 9 | 10 | 4 | 9 | 4 | 9 | 4 | 9 | 4 | 10 |
| DS8 | 5 | 10 | 5 | 10 | 4 | 10 | 4 | 9 | 5 | 5 |

CE : Crude Extract; EE- EthylAcetate Extract; ChE: Chloroform Extract

asparaginase activity was isolated from marine sediments (Poorani *et al.,* 2009). *Streptomyces coeruleorubidus*, a marine sediment isolate shows good antogonistic effect on the newly emerging diseases (Raghava Rao *et al.,* 2012). Among 38 isolates tested, 17 isolates were found to be antibacterial compound producers (Sweetline *et al.,* 2012). Antibacterial activity of 107 marine actinomycetes isolated from near sea shore sediment and seawater from Konkan coast of Maharashtra was studied and found that they produced antagonistic effect against many pathogens (Gulve and Deshmukh, 2012). In contrary to the above report, the present study showed lowest activity against the bacterial pathogens.

Extract collected from culture filtrates collected by solvent extraction method was used for secondary screening with disc diffusion method. Many species of Actinomycetes, especially those belonging to the genus *Streptomyces* are well known biocontrol agents inhibiting plant pathogenic fungi (Og *et al.,* 2008; Silva Sousa *et al.,* 2008). In the present study also, marine Actinomycetes with antagonistic activity against the bacterial and fungal pathogens was observed (Table 3 and 4). The inhibition zone was more than 10 mm in all the promising isolates and Nurettin and Aysel, (2003) also reported *Streptomyces antibioticus* (MU106, MU107) and *S. rimosus* (MU114) with an inhibition zone >20 mm.

All the six isolates showed antagonistic activity at different level, but the isolate RPS 5, HCS 6 had good antagonistic activity. Although the exact mechanisms by which these actinomycetes isolates operate to reduce disease incidence is not elucidated, biocontrol agents inhibited hyphal growth of fungal pathogens, which might be due to chitinase production. Mangrove sediments harbouring marine actinomycetes were found effective against four phytopathogenic organisms was reported by earlier worker (Kathiresan *et al.,* 2005). Dhanasekaran *et al.* (2008) reported ethyl extract of Actinomycetes isolated from North Cyprus soil exhibiting antifungal activity. However, intense study is needed to assess the MIC of the different extracts.

The potential antagonistic activity of streptomyces found in this study against pathogenic bacteria and fungi shows their suitability as a biocontrol agent. Therefore, the preliminary screening may open an avenue for further exploration of antagonistic behaviour of marine actinomycetes followed by development and formulation of active principle as drug.

The isolated Actinomycete colonies showed fast growth without any pigmentation. The colonies were white, dull white and grey colored growth without any motility. All the isolates were Gram positive and acid fast negative. Holt *et al.* (1994) also stated gram positive nature of Actinomycetes and Vanajakumar *et al*. (1995) reported that white colour series of actinomycetes. Temperature for growth ranged from 37 to 40 ºC and grew at 5 % NaCl. All the isolate hydrolysed casein and starch but not utilized cellulose. All the isolates preferred glucose as carbon source but also grow in mannitol and fructose containing media. Differences were observed between the isolates in case of nitrogen utilization. L asparagine was utilized by all the isolates and L Phenylalanine was not utilized. TS 3, DS 7 and DS 8 strongly utilizes L asparagines. Vasavada *et al.* (2006) showed the use of optimum pH,salinity and carbon sources. Finally, after all these characterisation studies , results were compared and matched with the keys given for 458 species of actinomycetes included in ISP (International Streptomyces Project) and was tentatively identified as *Streptomyces* sp, but it needs molecular confirmation

The present study concludes that antimicrobial activity produced by the marine actinomycetes showed promising antibacterial and antifungal activity against many plant pathogens.

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