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DIVERSITY OF MARINE ACTINOMYCETES FROM NICOBAR MARINE SEDIMENTS AND ITS ANTIFUNGAL ACTIVITY

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ABSTRACT

Actinomycetes were cultivated using a variety of media and selective isolation techniques from 20 marine samples collected from the island of Nicobar. In total, 800 actinomycetes colonies were observed and 100 (12.5%) of these, representing the range of morphological diversity observed from each sample, were obtained in pure culture. The majority of the strains isolated (90%) required sea water for growth it indicating high degree of marine adaptation. The dominant actinomycetes recovered belonged to the genus of *Streptomyces*. These results support the existence of taxonomically diverse populations of actinomycetes in the Nicobar marine environment.

Keywords: Actinobacteria, Nicobar, Opportunistic fungi

INTRODUCTION

It is interesting that the world's oceans, which cover 70% of the earth's and include some of the most biodiversity ecosystems on the planet, have not been widely recognized as an important resource for novel actinobacteria. In fact, the distributions of actinobacteria in the sea remain largely undescribed, and even today, conclusive evidence that these bacteria play an important ecological role in the marine environment have remained elusive. An intriguing picture of the diversity of marine actinobacteria is beginning to emerge. Once largely considered to originate from dormant spores that washed in from land[1], it is now clear that specific populations of marine adapted actinobacteria not only exist but add significant new diversity within a broad range of actinobacterial taxa[2].

The first report on the marine actinobacteria was made by Nadson, when he observed and documented those in the salt mud. In 1969, Weyland carried out an extensive survey on the distribution on marine actinobacteria in the sediments of North Sea and Atlantic Ocean and suggested that the marine actinobacteria are the best sources for isolation of unique bioactive compounds compared to terrestrial ones[3]. After this, a number of researchers around the world have concentrated to isolate and identify the actinobacteria from the different marine habitats. So far 83 species of actinobacteria belonging to 28 genera were recorded from the marine environment. Among them, *Streptomyces sp.* is dominant. Most of the recorded genera are new to science. If in-depth studies will carried out, the marine actinobacterial diversity could be increased.

The marine environment is characterized by the hostile parameters such as high pressure, salinity, low temperature, absence of light etc. and the marine actinobacteria have adapted themselves to survive in this environment. They require Na^+ for growth because it is essential to maintain the osmotic environment for protection of cellular integrity. Oligotrophy is also one more adaptation because of the smaller amount of available nutrients. However, actinobacterial action promotes organic degradation, decomposition and mineralization processes in the sediments and in the overlying water column and releases of the dissolved organic and inorganic substances. The mineralization of organic matter, which

is derived from the primary producers, results in its being recycled, so that these substances are again available for the primary producers. Distribution of actinobacteria depends on changes in water temperature, salinity and other physicochemical parameters. Actinobacteria also serve as important source of food for a variety of marine organisms. Thus, actinobacteria not only maintain the pristine nature of the environment, but also serve as biological mediators through their involvement in biogeochemical processes.

MATERIALS AND METHODS

Sample collection and processing

Twenty marine samples were collected from the island of Nicobar. The sample consisted of 20 sediments nature from fine mud's to small rocks. The sediments were collected through scuba diving to the depth of 1-100 m. The samples were collected in plastic bags and immediately transported to the laboratory. The collected samples were processed using different method.

Method 1: Sediment was dried overnight in a laminar flow hood and when clumping occurred, grinds with mortar and pestle. An autoclaved foam plug (2 cm in diameter) was pressed onto the sediment and then press onto the surface of an agar plate in a serial dilution effect.

Method 2: Dried sediment was diluted with sterile sea water. The diluted sample was vortex mixed and this solution inoculated onto the surface of an agar plate and spread with a glass rod.

Method 3: Dried sediment was mixed with 3 ml of sterile sea water and heated to 55^0C for 6 mins and 100 µl of the suspension inoculated onto an agar plate.

Method 4: Wet sediment was frozen at -20^0C for 24 hrs and diluted in sterile sea water (1:3, 1:120 depending on particle size) and 100 µl of the suspension inoculated onto an agar plate.

Media and Isolation of actinobacteria

To increase the isolation of actinobacteria, 6 different types of media were selected from the literature (Table 1). All media supplemented with a final concentration of 50 µg ml^{-1} potassium dichromate 15 µg ml^{-1} nalidixic acid to facilitate the isolation of slow growing actinobacteria. Potassium dichromate inhibits fungal growth[4], while nalidixic acid inhibits many gram negative bacteria[5]. A 10 fold serial dilution series was made and plated in triplicate on agar plates. The plates were incubated at room temperature (28^0C) and monitored periodically over 3 months for actinomycetes growth.

Table 1: Composition of the 6 different media for isolation of actinobacteria

Medium	Composition	References
M1	10g of soluble starch, 4g of yeast extract 2g of peptone, 18g of agar.	Mincer et al
M2	6ml of glycerol, 1g arginine,1g K_2HPO_4,0.5g $MgSO_4$,18g agar	Mincer et al
M3	4g yeast extract,10g malt extract,4g glucose,18g agar	Webster et al
M4	4g beef extract, 4g peptone, 1g yeast extract,10g glucose,20g nacl,18g agar	Webster et al
M5	20g soluble starch,1g KNO_3,0.5g K_2HPO_4,0.5g $MgSO_4$, 0.01g $FeSO_4$, 20g NaCl	Labeda and Shearer
M6	10g glycerol,0.3g casein, 2g Kno_3,2g Nacl, 2g K_2HPO_4, 0.05g $Mgso_4$,0.02 g $CaCo_3$,0.01g $Feso_4$,15g agar	Kuster

Fermentation and extraction of secondary metabolites

One loop full of cultures were inoculated in to 50 ml SS medium in 250 ml Erlenmeyer flasks containing the sea water 50%, distilled water 50%, pH 7.5 and incubated for 2 days in rotary shaker incubator (200 rpm) at 28˚C. The seed inoculums (10%) were transferred into 200 ml production medium in 1 L Erlenmeyer flasks. The inoculated cultures in the production medium were incubated for 72 h on a rotary shaker (2000 rpm) at 28ºC. After fermentation the broth was centrifuged at 4000 rpm for 10 min and the filtrate was separated.

In Vitro Antifungal Assay

Antifungal activity of the crude extract was determined by using the standard method. The fungal cultures were maintained in 0.2% dextrose medium and the optical density 0.10 at 530 nm was adjusted using spectrophotometer. Each fungal inoculum were applied on plate and evenly spread on Sabouraud's Dextrose agar using a sterile swab. Agar diffusion assay was followed to evaluate the antimicrobial activity along with amphotericin B. The Petri plates were incubated at 30°C for 2 days. At the end of the 48 hrs, inhibition zones formed in the medium were measured in millimeters (mm). All experiments were done in three replicates.

Taxonomic Investigation

The genus of strains with good antagonistic activity against the pathogens was identified using cell wall composition analysis, micromorphological studies[6] and Bergey's manual of determinative bacteriology[7].

RESULTS AND DISCUSSION

A total of 20 samples were processed for actinomycetes isolation of which 100 (12.5%) yielded actinomycetes growth. The total number of actinomycetes colonies observed on all primary isolation plates was 800. Among 6 different actinobacteria isolation media tested in those 5 media only recovered actinobacteria. Significant differences in the total number of isolates recovered were observed among the 5 different media (Table 2). M2 produced the highest recovery with 40 isolates, followed by M1 and M6 (20 isolates), M3 (12 isolates) and M4 (8 isolates). These 6 media have been widely used to isolate actinobacteria from different terrestrial environments. Out of 100 strains 4 strains show potential antifungal activity.

From the present study, the sediment sample harbored highest number of actinomycetes population. Hence the sediment sample, good source for enumerating the actinomycetes population. On average, we observed 3.03 actinomycetes colonies per plate with that number increasing to 16.1 per plate when only considering those plates that yielded actinomycetes. Considering the total number of colonies in all the plates, the maximum number of colonies was enumerated in M2 medium followed by M3 medium and M6 medium. In 2001, Sivakumar reported higher number of actinomycetes in M6 medium[8] but in this study we revealed M2 medium enumerating more actinomycetes population so we recommended M2 medium is the most suitable one for isolating actinomycetes from marine sediments.

Table 2: Actinomycetes recovery using various isolation media

Medium	No. of plates inoculated	No. of plates with actinomycetes (%)	Total no. of actinomycetes observed	Mean no. of actinomycetes per plate	No. of actinomycetes isolated
M1	60	30	110	3.6	20
M2	60	20	322	16.1	40
M3	60	35	143	4.08	12
M4	60	33	100	3.03	8
M5	60	32	0	0	0
M6	60	36	125	3.47	20

Recent research has revealed that deep ocean sediments are a valuable source of new actinobacteria that are unique to the marine environment. As Dr. Rita Colwell, Director of the U.S. National Science Foundation, commenting on the importance of exploration and conservation of microbial diversity, has stated: “Hiding within the as-yet undiscovered microorganisms are cures for diseases, means to clean polluted environments, new food sources, and better ways to manufacture products used daily in modern society”[9].

Tuberculosis (TB), is a highly contagious disease of the lungs that was thought to have been virtually eliminated by the 1960s, but is now resurgent and kills nearly two million people worldwide every year. New fungal infections are occurring at a rate of one per second. Every year, millions of people die all over the world due to tuberculosis. Many countries are still under the clutches of tuberculosis resulting in morbidity and mortality.

The prevalence of secondary infection in the pathogenesis and symptomatology of pulmonary tuberculosis is evidenced by the extensive literature which has accumulated since Koch’s discovery of the primary and essential cause of tuberculosis infection. Subsequent invasion of the secondary fungi such as *Candida albicans, Aspergillus niger* and *Aspergillus flavus* resulting in the secondary infection which causes tissue destruction and ulceration which had already occurred as a result of primary infection[10].

In pulmonary tuberculosis it is this latter form of secondary infection which plays a vital role in blocking the direct access of air in to the alveoli and produce tissue symbiosis producing a passive mixed infection leading to chronic obstructive lung disease resulting in a fatal death. The mortality rate is 50-60 per cent in pulmonary tuberculosis is due to the invaders of secondary bacteria and fungi. Hence more attention is to be focused for the simultaneous treatment for secondary infections along with the tuberculosis.

In the present study all the 100 isolates obtained were screened for antifungal activity by agar well diffusion method. The colonies were designated as LK1-LK-100 based on their colony morphology and among them only 4 isolates (LK1, LK4, LK5 and LK6) exhibited the ability to produce antifungal secondary metabolite (Table 3). In this LK5 were taken for further study. The isolate LK5 showed activity against *Aspergillus niger, Aspergillus fumigatus* and *Candida albicans.* The genus of strains with good

Table 3: Secondary metabolites against opportunistic fungi

STRAINS	LK-1 (mm)	LK-4 (mm)	LK-5 (mm)	LK-6 (mm)
Pencillium sps	20	0	0	0
Aspergillus niger	10	0	46	46
Aspergillus fumigatus	0	14	38	44
Candida albicans	20	0	20	0

Zone of inhibition-diameter in mm

antagonistic activity against the pathogens was identified as *Saccharomonospora sps* using cell wall composition analysis, micro morphological studies and Bergey's manual of determinative bacteriology.

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