Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 22, issue 3, 2012, pp. 451-455


# SPATIOTEMPORAL VARIATION AND ANTIBACTERIAL ACTIVITY OF ACTINOMYCETES ISOLATED FROM HIGH ALTITUDE GRASSLAND SOILS OF TROPICAL MONTANE FOREST – KERALA, INDIA

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**ABSTRACT.** TThe invention of novel antibiotics and other bioactive microbial metabolites continues to be an important aim in new drug discovery programmes. Actinomycetes have the potential to synthesize lots of diverse biologically vigorous secondary metabolites and in the last decades actinomycetes became the most productive source for antibiotics. Therefore in the present study we analyze the antibacterial activity of the actinomycetes isolated from grassland soil samples of Tropical Montane forest. A total of 33 actinomycete strains isolated were characterized and screened for antibacterial activities using well diffusion method against six specific pathogenic organisms. Identification of the isolates revealed that the majority of them were belonging to *Streptomycetes* followed by *Nocardia*, *Micromonospora*, *Pseudonocardia*, *Streptosporangium*, *Nocardiopsis* and *Saccharomonospora*. Among the 33 isolates, Gr1 strain showed antagonistic activity against all checked pathogens. Nine strains showed antibacaterial activity against *Listeria, Vibrio cholera, Bacillus cereus, Staphylococcus aureus* and *Salmonella typhi* and only 2 strains (Gr1and Gr25) showed antagonism to *E. coli*. The overall percentage of activity of actinomycetes isolates against each pathogenic bacterium was also calculated. While 63.63% of the actinomycetes were antagoinistic against *Listeria, Vibrio cholerae*, and *Bacillus cereus*, 60.6% of them were antagonistic to *Staphylococcus aureus*. Very few isolates (6.06%) showed antibacterial activity against *E. coli.* In general most of the actinomycetes isolates were antagonistic to gram-positive bacteria such as *Listeria, Bacillus* and *Staphylococcus* than Gram-negative bacteria *Vibrio cholerae, E. coli and Salmonella*.

**Keywords:** Tropical montane forest, Soil, Actinomycetes, Antibacterial activity, Antibiotics

## INTRODUCTION

Actinomycetes, the gram-positive filamentous bacteria, are well known as a good source of microbial secondary metabolites-producer in drug discovery programs. They are widely distributed in soil, water and other natural environments and the population and types of actinomycetes in an ecosystem are determined by numerous physical, chemical and biological factors. They produce branching mycelium which may be of two kinds viz. substrate mycelium and aerial mycelium. Among actinomycetes, the streptomycetes are the dominant and the non streptomycetes are called rare actinomycetes, comprising approximately 100 genera.

After penicillin was discovered, the search for additional antibiotics focused on many fungi and bacteria. One particular group of microbe grabbed the attention of scientists, the actinomycetes. At present, 4000 antibiotic substance obtained from bacteria and fungi have been applied in medicine, out of which about 75% are produced form gram-positive actinomycetes (Miyadoh, 1993). A few decades after the introduction of antibiotics into clinical practice, resistance by pathogenic bacteria has become a major health concern. Indeed, while in the mid 1970s infectious diseases were considered virtually conquered (Breithaupt, 1999); many Gram positive bacteria and Gram negative opportunistic pathogens were becoming resistant to virtually every clinically available drug (Greenberg, 2003). The use of antimicrobial drugs for prophylactic or therapeutic purposes in human and veterinary or for agricultural purposes, have provided the selective pressure favouring the survival and spread of resistant organisms. In third world countries like India, irrational use of antibiotics is a major cause of resistance (Al-bari et al., 2007), so it is no doubt important to discover, safer and more effective antibiotics.

New microbial metabolites are permanently needed due not only to the increase in resistant pathogens, but also to the evolution of novel diseases and toxicity of currently used compounds (Demain, 1999). Therefore, several strategies have to be employed to find new bioactive drugs including the exploration for new compounds from well known and talented microorganisms, such as Actinomycetes, due to the fact that only a small range of their biosynthetic capacity is currently exploited (Zahner et al., 1995). Therefore, investigation of new ecosystems for isolation of actinomycetes is crucial for the discovery

of novel actinomycetes and subsequently newantibiotics. Recently, several studies reported the investigation of different habitats for isolation of novel actinomycetes as rich sources of bioactive compounds (Hozzein et al., 2008). For the present study we selected grassland soils of Eravikulam National park near to Anamudy, the top most peak in South India. These areas are yet poorly studied and represent diverse and largely unscreened ecosystem and the least investigated area for the isolation of potent antibiotic producing actinomycetes.

## MATERIALS AND METHODS

### *Study area*

The study area is located at the top areas of Eravikulam National park lies between 10º05'N - 10º20'N latitude and 77º0'E - 77º10'E longitude in Idukki district, Kerala, India (Anamudy Region) at an altitude of 1900 m to 2400 m above MSL. Most of the land in this area is covered by grass lands and shola. For the present study we selected six sites in grassland at different altitudes for sample collection.

### *Collection of sample*

The soil samples were collected from six sites (Gr1 – Gr6) of grassland at different altitude or different microclimate. Samples were collected at a depth of 15 to 20 cm from the surface after removing the top layer. For each of the sampling sites, sub-samples of soil were collected from different locations, pooled together and homogenized so as to obtain representative sample. Sampling was carried out throughout the year during post-monsoon, pre-monsoon and monsoon seasons. Samples were collected by a spade that is thoroughly cleaned and disinfected between sampling so as to prevent cross-contamination.

### *Isolation, Enumeration and maintenance of Actinomycetes*

Isolation and enumeration of Actinomycetes were carried by standard serial dilution plate technique. 10 g of soil was transferred to in 90 ml sterile distilled water and agitated vigorously. Different aqueous dilutions, $10^{-1}$ to $10^{-4}$ of the suspensions were prepared and spread plated on Kusters Agar. Nystatin (50 µg/ml) or Amphotericin (75µg/ml) and Streptomycin (25µg/ml) were added to the isolation media in order to prevent fungal and bacterial contamination respectively. The plates were incubated at room temperature for 2 to 3 weeks. After incubation Actinomycetes colonies were count and separate colonies were streaked on to Kusters Agar plates and incubated at room temperature for 4-6 days to obtain pure cultures of Actinomycetes. For determining the load of actinomycetes in the sample, colony forming units were determined.

### *Morphological and Biochemical characterization of isolates*

Actinomycete strains which are maintained as pure culture on Kusters Agar were characterized by morphological tests as per Bergeys Manual of Determinative Bacteriology (2000) and physiological tests (Gordon, 1967). The morphology of Actinomycetes strains was examined using slide culture technique (Bergeys Manual of Determinate Bacteriology, 2000). Sterile cover slips were placed at an angle in the Actinomycetes growth medium (Kusters Agar) and the mycelia adhering to cover slips were transferred to a slide and examined at 40x and 100x magnification using a light microscope (Olympus CH 20i).

### *Evaluation of antibacterial activity of the isolates using well diffusion method*

The young culture of the selected pathogens (Listeria, Vibrio cholerae, Bacillus cereus Staphylococcus, Salmonella and E. coli) were prepared in Nutrient Broth and lawn culture of different pathogens were prepared by swabbing young culture (16-18 h) in Glycerol Yeast Agar and waited for 15 minutes to absorb the culture to the medium. Agar wells (3 mm diameter) were punched in the plates using a sterile gel puncture.

Thirty µl of a four day old culture of all the isolated Actinomycetes strains in appropriate broth was pipetted in to the wells and plates were incubated for 24 h at room temperature. Zone of inhibition around the wells were recorded in mm.

## RESULTS AND DISCUSSION

### *Spatiotemporal variation of Actinomycetes load*

There was a temporal and seasonal variation of Actinomycetes load in the grassland soils of tropical montane forest. Actinomycetes load was higher during pre monsoon season followed by post monsoon and monsoon season. Among the six sites selected Gr 4 and Gr5 showed comparatively high load of actinomycetes during all the season and site Gr 3 and Gr 4 exhibited less count. Actinomycetes load was higher during pre monsoon, this indicate that decomposers are most active during the pre monsoon season because of better soil temperature which might favor the microbial activity. Load was lower in monsoon season perhaps may due to competition for nutrients by plants and also protozoal predation of microorganisms decreases the number (Bhatt and Pandya, 2006).

Spatial variation of the microbial load attribute to variation of the soil characteristics and vegetation structure due to the difference in terrine of the study area. A number of studies have shown that even small scale topographical landforms can alter environmental conditions, which in turn retard or accelerate the activity of organisms (Scowcroft et al., 2000). The effects of topographical landforms on species composition, productivity, environmental conditions, and soil characteristics have been well investigated (Barnes et al., 1998).

Seasonal variation in microbial population is due to change in climatic factors. Seasonally-variable environmental factors, like air temperature and soil moisture, may influence the soil microbial community and activity and control the organic matter decomposition process (Maire et al., 1999).



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***Diversity of Actinomycetes***

Identification of the isolates collected [33] revealed that most of them were belonging to Streptomycetes [15] followed by Nocardia [11], Micromonospora [2], Pseudonocardia [2], Streptosporangium [1], Nocardiopsis [1] and Saccharomonospora [1]. Many reports of actinomycetes diversity of tropical soils supports the present work. Studies of Wang et al. (1999) in the soils of rainforests of Singapore reports different actinomycete genera, among which Streptomyces, Micromonospora, Actinoplanes, Actinomadura, Nonomuria, Nocardia and Streptosporangium were the most abundant. Balagurunathan et al. (1996) reported most of the genus identified from this study from south Indian soil.

***Antibacterial potential of actinomycetes***

Among the actinomycete strains isolated, Gr1 (Streptomycetes) showed antibacterial activity against all the tested pathogenic organisms. Isolate Gr3, Gr4, Gr5 Gr6, Gr7, Gr9, Gr10, Gr11, and Gr16 showed antibacterial activity against five of the test organism. However, Gr28, Gr29, Gr32 and Gr33 strain did not show antibacterial activity against the pathogenic organisms tested. Gr1and Gr25 showed activity against E. coli, however, other strains were not active against E. coli. The result of antibacterial activity of actinomycetes revels that most of the isolates were antagonistic to gram-positive bacteria like Listeria, Bacillus and Staphylococcus than Gram-negative bacteria Vibrio cholerae, E. coli and Salmonella. The reason for different sensitivity between gram-positive and gram-negative bacteria could be ascribed to the morphological differences between these microorganisms, Gram-negative bacteria having an outer polysaccharide component makes the cell wall lipopolysaccharide components. This made the cell wall impermeable to lipophilic solutes; the Gram-positive should more susceptible having only an outer peptidoglycan layer that is not an effective permeability barrier (Scherrer and Gerhardt, 1971). This because the Gram-positive bacteria more sensitive to antibiotics produced by actinomycetes (Robbers et al., 1996). Pandey et al. (2007) and Thakur et al. (2007) studies also revealed that high percentage of inhibition was recorded against Gram positive bacteria while Gram negative bacteria were less inhibited. Wijittra et al. (2006) and Sahin (2002) studies revealed that the activity against E .coli was less.

## CONCLUSIONS

There was a temporal and spatial variation of actinomycetes in the grassland soils of tropical montane forest. Identification of the isolates revealed that most of them were belonging to Streptomycetes followed by Nocardia, Micromonospora, Pseudonocardia, Streptosporangium, Nocardiopsis and Saccharomonospora. Actinomycetes of the selected study area could be an interesting source of antibacterial substance. Further investigations are needed in order to determine which the metabolites responsible for the antibacterial activities are. As research on new antibiotics is a thrust area, the extraction of antimicrobial compounds from these isolates and detailed investigation assumes significance.

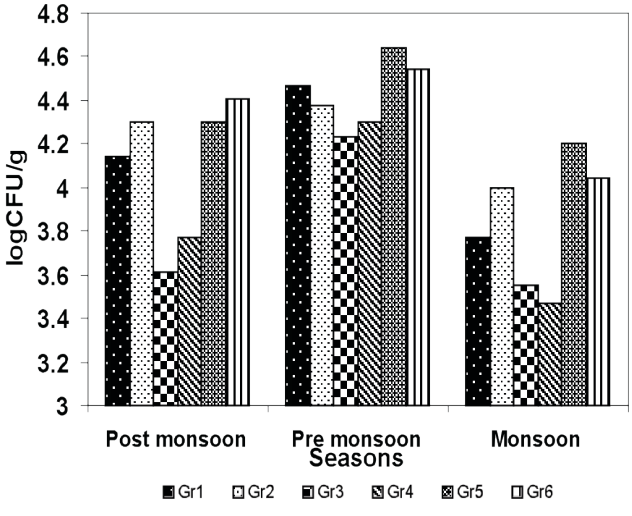


**Fig 1** Spatiotemporal variation of Actinomycetes load in Grassland soils of tropical montane forest

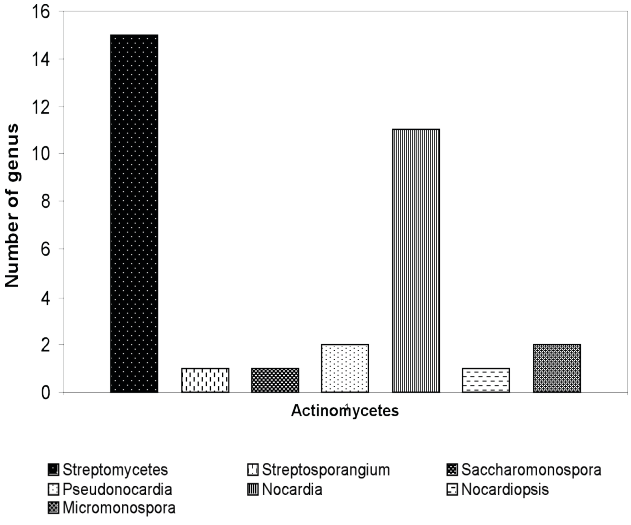


**Fig 2** Generic diversity of Actinomycetes in Grassland soils of tropical montane forest

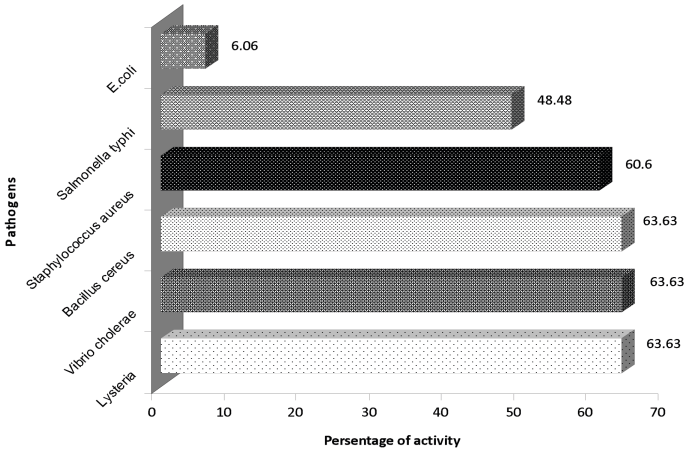


**Fig. 3** Percentages of antibacterial activity exhibited by actinomycete isolates from Grass land soils against specific pathogens

*Table 1*

**Antibacterial activity exhibited by actinomycete isolates**

| Isolates Name: | Diameter of inhibition zone (mm) against test microorganisms | | | | | |
|---|---|---|---|---|---|---|
| | *Listeria* | *Vibrio cholera* | *Bacillus cereus* | *Staphylococcus aureus* | *Salmonella typhi* | *E. coli* |
| Gr1 | 17 | 20 | 16 | 20 | 20 | 14 |
| Gr2 | 20 | 20 | 15 | 17 | 0 | 0 |
| Gr3 | 19 | 18 | 13 | 17 | 11 | 0 |
| Gr4 | 11 | 0 | 16 | 11 | 12 | 0 |
| Gr5 | 12 | 25 | 12 | 20 | 0 | 0 |
| Gr6 | 20 | 20 | 15 | 25 | 16 | 0 |
| Gr7 | 24 | 25 | 22 | 20 | 20 | 0 |
| Gr8 | 0 | 0 | 0 | 0 | 14 | 0 |
| Gr9 | 17 | 30 | 15 | 18 | 20 | 0 |
| Gr10 | 14 | 20 | 18 | 23 | 11 | 0 |
| Gr11 | 23 | 22 | 21 | 22 | 17 | 0 |
| Gr12 | 23 | 18 | 13 | 15 | 0 | 0 |
| Gr13 | 15 | 21 | 15 | 0 | 0 | 0 |
| Gr14 | 0 | 15 | 0 | 15 | 14 | 0 |
| Gr15 | 25 | 23 | 22 | 23 | 0 | 0 |
| Gr16 | 19 | 24 | 17 | 15 | 15 | 0 |
| Gr17 | 0 | 15 | 18 | 17 | 0 | 0 |
| Gr18 | 25 | 23 | 27 | 29 | 0 | 0 |
| Gr19 | 0 | 33 | 16 | 19 | 16 | 0 |
| Gr20 | 30 | 25 | 20 | 20 | 0 | 0 |
| Gr21 | 17 | 0 | 17 | 16 | 0 | 0 |
| Gr22 | 16 | 30 | 16 | 13 | 0 | 0 |
| Gr23 | 22 | 20 | 0 | 0 | 0 | 0 |
| Gr24 | 0 | 0 | 0 | 0 | 15 | 0 |
| Gr25 | 15 | 0 | 0 | 0 | 15 | 20 |
| Gr26 | 0 | 0 | 0 | 0 | 15 | 0 |
| Gr27 | 19 | 20 | 0 | 0 | 0 | 0 |
| Gr28 | 0 | 0 | 0 | 0 | 0 | 0 |
| Gr29 | 0 | 0 | 0 | 0 | 0 | 0 |
| Gr30 | 0 | 0 | 17 | 0 | 0 | 0 |
| Gr31 | 0 | 0 | 0 | 0 | 25 | 0 |
| Gr32 | 0 | 0 | 0 | 0 | 0 | 0 |
| Gr33 | 0 | 0 | 0 | 0 | 0 | 0 |



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*Vol. 22, issue 3, 2012, pp. 451-455*

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