Terrestrial actinomycetes from diverse locations of Uttarakhnad, India: Isolation and screening for their antibacterial activity

Vijay Kumar¹, Gajraj Singh Bisht¹*, Omprakash Gusain²

¹Department of Microbiology, Sardar Bhagwan Singh Post Graduate Institute of Biomedical Sciences and Research, Balawala, Dehradun, Uttarakhand, India, 248161. ²Department of Zoology and Biotechnology, H.N.B. Garhwal University, Srinagar, Uttarakhand, India.

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ABSTRACT

Background and Objective: Uttarakhand region is less explored, but possess a great biodiversity. This diversity can be explored for isolation and characterization of new actinomycetes strains for seeking antimicrobial molecules. It can therefore be predicted that novel bioactive metabolite producing actinomycetes can be discovered to combat multidrug resistant bacterial pathogens.

Materials and Methods: Variations in the viable count of actinomycetes were accessed in different altitudes. Actinomycetes were isolated, indentified and screened for their antibacterial activity.

Results: The highest viable counts of actinomycetes were recorded in valleys followed by mid hills and high hills. A total of 512 actinomycetes were isolated which were found to belong the 14 different genera of actinomycetes. Mainly the genus Streptomyces was dominant in all the soil samples. Out of 512 isolates recovered, 23.44% exhibited antibacterial activity against one or more tested bacterial pathogens. Of these 56.67% showed activity against Gram-positive bacteria, 26.67% against Gram-negative bacteria while 16.67% showed broad spectrum activity. Isolate DV1S and GR9a-5 showed highest antibacterial properties against several multi-drug resistant bacterial pathogens and were identified using polyphasic approach. DV1S and GR9a-5 were found to be most closely related with S. massasporeus NBRC 12796ᵀ and Nocardia nova JCM 6044ᵀ respectively.

Conclusion: The results of this study strongly support the idea that the viable count of actinomycetes varied greatly with altitude. The actinomycetes species isolated from valleys, mid hills and high hills possess significant capacity to produce compounds which are active against several drug resistant bacterial pathogens.

Keywords: Actinomycetes, Altitudinal variation, Antibacterial activity, Streptomyces

INTRODUCTION

The emergence of multidrug resistant among common bacterial pathogens is a serious problem. Therefore, there is a continuous need for new molecules to combat these pathogens. This need for new antibiotics for the past few decades has been met largely by semisynthetic tailoring of natural product scaffolds discovered in the middle of the 20th century (1). As the soil-derived microorganisms have been intensively screened as a source of therapeutically important molecules over a half century (2), the frequency of discovering structurally new compounds is decreasing these years. These findings seem to imply that the easily accessible microorganisms in soil had been exhausted and there is a need to seek unutilized microorganisms from unexplored sources. It is likely that the diversity of secondary metabolites relies more or

* Corresponding author: Gajraj Singh Bisht Ph.D
Address: Department of Microbiology, Sardar Bhagwan Singh Post Graduate Institute of Biomedical Sciences and Research, Balawala, Dehradun, Uttarakhand, India, 248161.
Tel: +91-971-9148874
E-mail: grsbisht@gmail.com

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less on the isolation source, namely, the habitat of the producers (3). On the basis of these facts, new actinomycetes strains that generate active compounds have been recently isolated from novel sources including saline, ocean, mangrove forests and niche habitats such as caves, beehives, solitary wasp mud nest, earthworm castings, pristine forests, lakes, rivers and other wetlands (4-9). New species of the microorganisms have the potential to produce new metabolites, which justifies the isolation of new species for drug discovery purposes (10). In addition, the isolation of diverse strains of actinomycetes provides information for exploitation and utilization of resources produced by this group of microorganisms (11).

To cope up with the demand for new pharmaceutical compounds and to combat the antibiotic resistant pathogens, researchers have been forced to look for novel microorganisms in unexplored environments. Therefore, the soil samples from different regions of the Himalayan state of Uttarakhand was collected for isolation and screening. Uttarakhand region though less explored, but possess a great biodiversity. This diversity can be explored for isolation and characterization of native actinomycetes for antibacterial molecules. Nevertheless, detailed studies on occurrence and distribution of actinobacteria in valleys, mid hills, and high hills of Uttarakhand are still lacking. As per our knowledge we are first time reporting the culturable actinobacterial diversity and their antibacterial activities from these sites.

MATERIALS AND METHODS

Collection of soil samples. Soil samples were randomly collected from different locations of Uttarakhand, India. The sampling sites are given in Table 1. Three samples were collected from each site and were carefully taken with spatula after removing 2-3 mm top soil and kept in sterile polypropylene bags. The collected samples were taken to the laboratory for isolation of actinomycetes. Totally, 60 soil samples (3 from each site) were collected from different areas / locations of Uttarakhand.

Isolation and screening of actinomycetes for their antibacterial activity. Isolation of actinomycetes was done according to the method described previously (9). The preliminary antibacterial activity was checked by agar disc method (12). The bacterial cultures used in the study Staphylococcus aureus MTCC96, Micrococcus luteus MTCC106, Bacillus subtilis MTCC441, Escherichia coli MTCC2939, Pseudomonas aeruginosa MTCC424, Acinetobacter baumannii MTCC1425 (resistant to cephotaxime and streptomycin) and Mycobacterium smegmatis MTCC6 were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India while clinical isolates of S. aureus (methicillin resistant S. aureus), E. coli (resistant to co-trimoxazole, bacitracin, erythromycin, cephalothin and penicillin-G) and Acinetobacter sp. (resistant to cephotaxime and nitrofuratoin) from Departmental culture collection, Department of Microbiology, Sardar Bhagwan Singh PG institute of Biomedical Sciences and Research, Balawala, Dehradun, India.

Extraction of metabolites from solid agar media. The isolates showing activities in plug were selected for extraction with n-hexane, ethyl acetate and methanol according to method described previously (8). The Petriplates left after cutting the agar plugs were flooded with n-hexane, ethyl acetate and methanol separately in each plate and left at room temperature for an hour, then crushed with glass rod and filtered with Whatman no.1. The solvents were evaporated under vacuum and dried.

Antibacterial activity of extracted product. The anti-bacterial disk diffusion assay was carried out on Mueller–Hinton agar (HiMedia) plates following the method described previously (13). The stock (25 mg/ml in DMSO) of actinomycete extract was prepared. The 10 µl of extract was impregnated on sterile discs (6 mm diameter, Whatman paper) and allowed to dry for 30 min. The discs were transferred to the surface of bacterial lawn. The disks containing solvent (DMSO) served as negative control. The disk containing antibiotic rifampicin (5 µg/disc, HiMedia) and vancomycin (30 µg/disc, HiMedia, India) were used as positive controls. The plates were then incubated for 24 h at 37ºC, and the zone of bacterial growth inhibition around disk was measured. The assay was repeated twice, and mean of the three experiments was recorded.

Identification of Actinomycetes. All strains were characterized morphologically and physiologically according to the methods described in the International Streptomyces project (14) and Bergey’s Manual of Systematic Bacteriology (15). The spore
chain morphology and spore surface ornamentation was examined by scanning electron microscopy according to the method described previously (16). The cell wall diamiopimelic acid isomers, whole cell sugars analysis and 16S rDNA sequence analysis was done as described in previous study (8).

Statistical Analysis. The one-way analysis of variance (ANOVA) was carried out on the viable count of actinomycetes (CFU g⁻¹) to see the significant difference among three altitudes (valleys, mid hills and high hills). Further Post Hoc analysis was done to see the significance difference between altitudes (valleys, mid hills and high hills). The software used for computation of statistics was SPSS version 16.

RESULTS

Uttarakhand hills (including foot hills) though less explored but possesses a great biodiversity had been selected for the present study. A total of 60 soil samples were randomly collected from 20 different locations of Uttarakhand, India. The pH of soil samples ranged between 6.5 and 8.6. When considering the pH of the soil samples relatively highest viable count of actinomycetes were recovered at alkaline (Above 7.5) while lowest from acidic soil. Highest viable count of actinomycetes were recorded in valleys (1.19 ×10⁵ ± 34.94) followed by mid hills (4.6 × 10⁴ ± 15.02) and low hills (3.6 × 10⁴ ± 11.36) (Table 2). The significant difference in the viable count of actinomycetes were recorded for three different altitude (F (2,177) = 231.76, p < 0.000). On the basis of different macromorphology, a total of 512 actinomycetes were isolated. Out of 512 isolate, 23.44% (n = 120) exhibited anti-
bacterial activity against one or more tested bacterial pathogens. Of these, 56.67% (n = 68) showed activity against Gram-positive bacteria (*S. aureus, B. subtilis, M. luteus*), 26.67% (n = 32) against Gram-negative bacteria (*E. coli, P. aeruginosa, A. junii*) while 16.67% (n = 20) showed broad spectrum (both Gram-positive and Gram-negative) activity. Some of the active isolates are depicted in Table 4. Isolates DV1S and GR9a-5 were found to be most prominent in the terms of desirable activities, hence taken for further studies. These isolates were found to have promising antibacterial properties against several drug resistant bacterial pathogens (Table 4) and were identified using polyphasic approach. The aerial mycelium of DV1S have open spiral with spiny spores (Fig. 1) while the isolate GR9a-5 lack aerial mycelium with spores (Fig. 2). The physiological characteristics of strain DV1S and GR9a-5 are given in Table 5. Chemotaxonomic tests showed that whole-cell hydrolysates of isolate DV1S were rich in LL-diaminopimelic acid (LL-DAP),

**Table 2. Variations in viable count of actinomycetes with altitudes.**

| Sites                  | Plate count (CFU g⁻¹) × 10⁷ | No. of isolates | Active isolates |
|------------------------|-------------------------------|-----------------|-----------------|
| Valleys (below 700 m)  | 119.3 ± 34.94*               | 203             | 29.55%          |
| Mid hills (800-2000 m) | 46.48 ± 15.02*               | 190             | 21.05%          |
| High Hills (above 2000)| 36.52 ± 11.36*               | 119             | 16.88%          |
| Total                  | 512                           |                 | 23.43%          |

Mean ± standard deviation; * viable counts of actinomycetes are significantly different in three different sites (one way ANOVA, Tukey HSD, P < 0.05).

**Table 3. Distribution of genera of actinomycetes from soil samples collected from different regions of Uttarakhand.**

| Sampling area | No. of isolates | Number of actinomycetes isolated |
|---------------|-----------------|----------------------------------|
|               | A    B    C    D    E    F    G    H    I    J    K    L    M    N |                  |
| Rishikesh     | 55    34    11    2    1    1    3    -    -    1    1    -    -    -    1 |                  |
| Dehradun      | 69    30    9    10    7    3    2    4    2    -    -    2    -    -    - |                  |
| Shrinagar     | 49    31    9    5    -    3    -    1    -    -    -    -    -    -    - |                  |
| Deoprayag     | 30    14    3    7    2    -    1    1    -    -    1    -    -    1    - |                  |
| Bageshwar     | 10    8    -    2    -    -    -    -    -    -    -    -    -    -    - |                  |
| Narendernagar | 20    18    -    -    1    -    -    -    -    -    -    -    -    -    - |                  |
| Almora        | 21    16    1    2    2    -    -    -    -    -    -    -    -    -    - |                  |
| Uttarkashi    | 18    12    -    2    2    -    -    -    1    1    -    -    -    -    - |                  |
| Chamba        | 27    25    -    1    1    -    -    -    -    -    -    -    -    -    - |                  |
| Pauri         | 39    20    9    5    2    3    -    -    -    -    -    -    -    -    - |                  |
| Tehri         | 35    21    2    5    1    1    1    -    2    1    1    -    -    -    - |                  |
| Ranikhet      | 20    15    2    -    1    -    -    -    -    -    -    -    -    -    - |                  |
| Munsyari      | 20    18    2    -    -    1    1    -    -    -    -    -    -    -    - |                  |
| Gagnani       | 9     5     1    1    -    1    -    -    -    -    1    -    -    -    - |                  |
| Jhala         | 6     4     -    -    1    -    -    -    -    -    -    1    -    -    - |                  |
| Dharali       | 23    15    1    -    -    -    -    -    -    2    2    1    1    1    - |                  |
| Lanka         | 10    9     -    -    -    -    1    -    -    -    -    -    -    -    - |                  |
| Gangotri      | 13    12    -    -    1    -    -    -    -    -    -    -    -    -    - |                  |
| Badrinath     | 21    17    1    2    1    -    -    -    -    -    -    -    -    -    - |                  |
| Mana          | 17    10    4    -    1    -    1    1    -    -    -    -    -    -    - |                  |
| Total         | 512   334   53    46    23   14   10   7    6    5    5    4    3    1    1 |                  |

- No isolates were recovered; A, *Streptomyces*; B, *Streptosporangium*; C, *Actinomadura*; D, *Nocardia*; E, *Nocardiodes*; F, *Saccharopolyspora*; G, *Thermoactinomycetes*; H, *Amycolatopsis*; I, *Micromonospora*; J, *Mycobacteria*; K, *Intrasporangium*; L, *Planobispora*; M, *Nocardiopsis*; N, *Geodermatophilus*. 
while no characteristic sugar indicated a chemotype I. The whole-cell hydrolysates of isolate GR9a-5 were rich in meso- diaminopimelic acid (meso-DAP), along with arabinose and galactose indicated a chemotype IV. On the basis of chemotaxonomic, morphological, and physiological properties of the isolate DV-1S and GR9a-5 are in line with its classification in the genus *Streptomyces* and *Nocardia* respectively. The 16S rDNA sequences (900 bp) of the strains DV1S and GR9a-5 were determined and submitted to GenBank under the accession number HM991289, HM991288. Isolate DV1S shares a sequence similarity of 99.6% with *S. massasporeus*. Its position among the type strains of *Streptomyces* is shown in Fig. 3. While the isolate GR9a-5 shared 16S rRNA gene sequence similarity of 99.33% with *Nocardia nova* JCM 6044T. Its position among the type strains of *Nocardia* is shown in Fig. 4.

**DISCUSSION**

Searching new antibiotics has increased worldwide because of the serious problem of antibiotic resistance among the microbes. The recent discovery of novel primary and secondary metabolites from taxonomically unique population of actinomycetes suggest that these organisms could add a new dimension to microbial natural product research. The history of new drug discovery processes shows that novel skeletons have, in the majority of cases, come from natural sources (17). On the basis of above facts new actinomycetes strains that generate active compounds have been recently isolated from novel sources including saline, ocean, mangrove forests and niche habitats such as caves, pristine forests, lakes, rivers, and other wetlands (18). Today, the emphasis is on the exploration of unusual and previously ignored ecosystems (19). Actinomycetes from several unexplored environments have been intensively studied in last few decades for novel and potent molecules (5). In this context, Uttarakhand hills (including foot hills) though less explored but possesses a great biodiversity had been selected for this study. This diversity can be explored for isolation and characterization of native actinomycetes for...
**Table 4.** Antibacterial activity of some promising isolates.

| Isolates    | S. aureus MTCC 96 | S. aureus (MRSA) MTCC441 | B. subtilis MTCC2939 | E. coli clinical MTCC 424 | E. coli MTCC 6 | P. aeruginosa clinical MTCC1425 | A. baumanii MTCC1425 | Acinetobacter sp. clinical MTCC 6 |
|-------------|------------------|--------------------------|----------------------|--------------------------|----------------|--------------------------------|---------------------|----------------------------------|
| RK-1*       | 16.33 ±1.24      | 18.33±0.47               | 14.66±1.24           | 11.66±1.24               | 11.33±0.47     | -                              | -                   | -                                |
| DV1D*       | 17.00±0.81       | 12.33±1.24               | -                    | 17.33±1.67               | 18.33±0.47     | -                              | -                   | -                                |
| DV1H*       | -                | 19.33±1.24               | 15.33±1.24           | 15.00±0.81               | -              | -                              | -                   | -                                |
| DV1S*       | 24.00±0.81       | 23.66±2.05               | 32.33±0.47           | 16.66±0.94               | 11.66±0.94     | -                              | 23.66±1.88          | 20.33±0.47                      | 21.66±0.47          |
| BR-3*       | 16.66±0.47       | 18.00±0.00               | 21.66±0.47           | 15.00±1.41               | -              | -                              | -                   | -                                |
| TRII (g)*   | 11.00±0.81       | 13.66±0.47               | 9.66±1.67            | 9.66±0.47                | -              | 12.66±0.94                     | -                   | -                                |
| JH 1*       | 30.33±0.94       | 25.00±0.81               | 31.00±0.81           | -                        | -              | -                              | -                   | -                                |
| JH 2*       | 19.00±1.41       | 16.66±0.47               | 29.66±0.94           | 11.33±0.47               | -              | -                              | -                   | -                                |
| GR9a-5**    | 16.00±1.41       | 13.33±0.94               | 20.00±1.24           | 16.00±0.47               | 15.00±1.81     | 14.00±0.94                     | 19.33±0.81          | 15.00±1.00                      | 16.33±0.94          |
| GR4-3*      | 16.33±1.24       | -                        | 29.33±0.47           | -                        | -              | -                              | -                   | -                                |
| Rifampicin 5 µg/disc | 22.33±0.47 | 31.66±1.24 | 17.67±0.47 | 28.33±0.47 | 21.00±0.00 | - | 19.33±0.94 | 17.66±0.47 | 23.66±0.94 |
| Vancomycin (30 µg/disc) | 24.00±0.81 | 18.66±0.94 | 21.33±0.94 | - | - | - | - | - | ND |

* Extracted with ethyl acetate; **extracted with methanol; Average of triplicate ± standard deviation; ND, not determined, the diameter of the filter paper disks (6 mm) is included; (–) not active; values are mean ± standard deviation of three experiments in replicate.

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**Fig. 3.** Neighbour-joining phylogenetic tree based on 16S rDNA gene sequences showing the relationships between strains DV-1S and the most closely related type strains of *Streptomyces*.
antimicrobial molecules. As per our knowledge this is the first exhaustive screening program done in the Uttarakhand region for isolation and screening of actinomycetes. The results of the present study revealed that the viable count of actinomycetes varied greatly with altitude. The significant differences in the viable count of actinomycetes were recorded for three different altitudes. This is in accordance with the previous reports from Sikkim, India (20). The actinomycetes isolated from these sites were belonged to the 14 different genera of actinomycetes. The total number of actinomycete genera recovered is much lower than that in similar studies performed in other geographic areas (21-23). Mainly the genus \textit{Streptomyces} was dominant in all the soil samples; however some rare genera were also recovered in the study, but in low frequency. When considering the pH of the soil samples highest percentage of actinomycetes were recovered at alkaline while lowest from acidic soil, supporting the earlier reports (24).

The results of antibacterial activity (23.44\%) in present study were different from those of other authors showing 53–61\% in Algerian soil (25). The antibacterial results were also comparable with that described by Barakate \textit{et al.} (26) studying the activity of Moroccan soil actinomycetes and those of other authors showing 16\% isolates showing antimicrobial activity in soil of Turkey (27). Highest percentages of actinomycetes were found to belong the genus \textit{Streptomyces} (67\%). This high frequency of antimicrobial activities among \textit{Streptomyces} species has been previously observed in other soil and aquatic isolates (28-29). The results of the present study were also comparable with a previous study of actinomycetes in terrestrial soil from Doon Valley (30).

Isolate DV1S and GR9a-5 showed highest antibacterial properties against several multi-drug resistant bacterial pathogens and were identified using polyphasic approach. DV1S and GR9a-5 were found to be most closely related with \textit{S. massasporeus} NBRC 12796\(^{T}\) and \textit{Nocardia nova} JCM 6044\(^{T}\) respectively. These isolates can be differentiated from type strains in a number of cultural and physiological tests. Isolate DV1S produced grey aerial and brown reverse mycelium with spiral spore chains with spiny spore surface. It utilizes only glucose and arabinose and gave positive result for \(\text{H}_2\text{S}\) production. It can tolerate a salt concentration of 7\% (w/v). In contrast \textit{S. massasporeus} produced grey to violet aerial and violet reverse mycelium with spiral spore chains with smooth spore surface and utilizes the glucose, arabinose, sucrose, inositol, manose, and fructose. It can tolerate a salt concentration of 5\% (w/v) (31). Similarly, \textit{S. indiaensis} (previously it was classified as \textit{Streptosporangium}) produced grey aerial and brown reverse mycelium with spiral spore chain with
Characteristics | DV-1S | GR9a-5
--- | --- | ---
Acid fast | - | +
Aerial mycelium | Greyish pink | Pink
Reverse | Brown | Colourless
Diffusible pigment | +, Brown | -
Melanin pigment | - | -
Sporulation | Good | -
Spore chain | Open spirals | Fragmentation
Starch hydrolysis | + | +++
Casein hydrolysis | - | -
Gelatin hydrolysis | - | -
Oxidase | - | +
Catalase | - | +
C- utilization
Dextrose | +++ | +++
Rhamnose | - | +++
D-Maltose | ++ | +++
L-Arabinose | - | -
L-Sucrose | - | +
L- Raffinose | - | -
Cellubiose | - | +
D-Mannose | - | +
N- Utilization
L-Arginine | - | -
L- Valine | ++ | +++
L- Serine | - | +
L- Phenylalanine | +++ | +
L- Threonine | +++ | +
L- Methionine | +++ | +++
Hydroxyproline | +++ | +++
L- Histidine | ++ | +
Pottasiun nitrate | +++ | +
Nitrate reduction | - | +++
Growth at temp.
4°C | - | -
15- 37°C | + | +
45°C | - | +
Growth at NaCl (w/v)
0-3 % | +++ | +++
4-6 % | ++ | -
7% | + | -
Growth at pH
4 | - | +
7-10 | + | +

smooth spore surface and utilized glucose, arabinose, sucrose, inositol, mannose, fructose, and rhamnose. It can tolerate a salt concentration of 5%, w/v (32). *S. hawaiensis* produced blue to grey aerial and reverse
yellow to brown mycelium with spiral spore chain with spiny spore surface. It utilized glucose, arabinose, inositol, mannose, fructose, rhamnose and raffinose (33). Hence it may be a new strain of *Streptomyces*.

Similarly, the strain GR9a-5 can be differentiated from type strains in various biochemical characteristics. *N. nova* utilized rhamnose, sucrose, inositol, L-proline, L-serine, and L-valine whereas D- mannose, L-phenyl alanine and L- leucine were not utilized (34). In contrast strain GR9a-5 utilized rhamnose, sucrose, D-mannose, L-valine, L-serine while L-arabinose, L-raffinose, fructose, inositol, xylose, salicin, trehalose and L-arginine were not utilized. *N. jiangxiensis* utilized D-arabinose, D-cellobiose, D-fructose, glucose, inositol, D-lactose, D-maltose, D-raffinose, D-ribose, D-sorbose, D-sucrose, D-trehalose and D-xylose are used as sole carbon source. It does not degrade starch and Tween 80 (35) while, they were degraded by the strain GR9a-5. Hence, it may represent a new strain of *Nocardia*.

The results of this study strongly support the idea that the viable count of actinomycetes varied greatly with altitude. Highest viable counts of actinomycetes were recorded in valleys followed by mid hills and High hills. The actinomycetes species isolated from valleys, mid hills and high hills possess a significant capacity to produce compounds having unique antibacterial activity. The isolates DV1S and GR9a-5 were found to be most prominent in the terms of desirable activities. These isolates were found to have excellent antimicrobial potential against several drug resistant bacterial pathogens. Results obtained from this work are promising and hence merit further studies concerning purification, characterization and identification of the active secondary metabolites.

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REFERENCES

1. Clardy J, Fischbach MA, Walsh CT. New antibiotics from bacterial natural products. *Nat Biotechnol* 2006; 24: 1541-1550.
2. Fenical W. Chemical studies of marine bacteria: developing a new resource. *Chemical Reviews* 1993; 93: 1673-1683.
3. Igarashi Y. Screening of novel bioactive compounds from plant-associated actinomycetes. *Actinomycetol* 2004; 18: 63-66.
4. Mukku VJ, M Speuling, H Laatsch and E Helmke. New butenolides from two marine streptomycetes. *J Nat Prod* 2000; 63: 1570-1572.
5. Mitra A, Santra SC, Mukherjee J. Distribution of actinomycetes, their antagonistic behaviour and the physio-chemical characteristics of the world’s largest tidal mangrove forest. *Appl Microbiol Biotechnol* 2008; 80: 685-695.
6. Promnua Y, Kudo T, Chantawannakul P. Actinomycetes isolated from beehives in Thailand. *World J Microbiol Biotechnol* 2009; 25: 1685-1689.
7. Radhakrishnan M, Suganya S, Balagurunathan R, Kumar V. Preliminary screening for antibacterial and antitubercular activity of actinomycetes from less explored ecosystems. *World J Microbiol Biotechnol* 2010; 26: 561-566.
8. Kumar K, Bharti A, Gupta VK, Gusain OP, Bisht GS. Actinomycetes from solitary wasp mud nest and swallow bird mud nest: isolation and screening for their antibacterial activity. *World J Microbiol Biotechnol* 2012a; 28: 871-880.
9. Kumar K, Bharti A, Negi YK, Gusain OP, Pandey P, Bisht GS. Screening of actinomycetes from earthworm castings for their antimicrobial activity and industrial enzymes. *Braz J Microbiol* 2012b; 43: 205-214.
10. Shomura T, Yoshida J, Amano S, Kojima M, Inouye S, Niida T. Studies on Actinomycetales producing antibiotics only on agar culture. I. Screening, taxonomy and morphology productivity relationship of *Streptomyces halstedii*, strain SF 1993. *J Antibiot* 1979; 32: 425-427.
11. Williams ST, Vickers JC (1988) Detection of actinomycetes in the natural environment problems and perspectives, In: *Biology of Actinomycetes* Ed Y Okami, T Beppu, H Ogawara. Japan Scientific Societies Press, Tokyo, pp. 265-270.
12. Xie Z, Xu Z, Shen W, Pei-Lin, Cen P. Biosynthetic pathways of milidomycin and a rapid, cost-effective agar plug method for screening high yielding mutants of milidomycin, *World J Microbiol Biotechnol* 2005; 21: 1433-1437.
13. Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic sensitivity testing by a standardised single disk method. *Am J Clin Pathol* 1966; 45: 493-496.
14. Shirling EB, Gottlieb D. Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* 1966; 16: 313-340.
15. Locci R (1989). *Streptomyces* and related genera. In: *Bergey’s manual of systematic bacteriology Vol. 4*. Ed, ST Williams, ME Sharp, JG Holt. Williams and Wilkins, Baltimore, pp. 2451-2506.
16. Kumar V, Bharti A, Gusain O, Bisht GS. Scanning
electron microscopy of *Streptomyces* without use of any chemical fixatives. *Scanning* 2011a; 33: 1-4.
17. Bevan P, Ryder H, Shaw I. Identifying small molecule lead compounds: the screening approach to drug discovery. *Trend Biotechnol* 1995; 13: 115-121.
18. Mitra A, Santra SC, Mukherjee J. Distribution of actinomycetes, their antagonistic behaviour and the physico chemical characteristics of the world’s largest tidal mangrove forest. *Appl Microbiol Biotechnol* 2008; 80: 685-695.
19. Cragg GM, Newman DJ, Snader KM. Natural products in drug discovery and development. *J Nat Prod* 1997; 60: 52-60.
20. Pandey A, Palni LMS. Microbes in Himalayan soils: Biodiversity and potential applications. *J Sci Ind Res* 1998; 57: 668-673.
21. Wang Y, Zhang ZS, Ruan JS, Wang YM, Ali SM. Investigation of actinomycete diversity in the tropical rainforests of Singapore. *J Ind Microbiol Biotechnol* 1999; 23: 178-187.
22. Hayakawa M, Yamamura H, Nagakawa Y, Kawa Y, Hayashi Y, Misonou T, Kaneko H, Kikushima N, Takahashi T, Yamasaki S et al. Taxonomic diversity of actinomycetes isolated from Swine Manure compost. *Actinomycetol* 2010; 24: 58-62.
23. Muramatsu H, Murakami R, Ibrahim H, Murakami K, Shahab N, Nagai K. Phylogentic diversity of acidophilic actinomycetes from Malaysia. *J Antibiot* 2011; 64: 621-624.
24. Taber WA. Evidence for the existence of acid-sensitive actinomycetes in soil. *Can J Microbiol* 1960; 6: 503-514.
25. Sabhaou N, Boudjella H, Bennadji A, Mostefaoui A, Zitouni A, Lamari L, Bennadji H, Lefebvre G, Germain P. Les sols des oasis du Sahara algerien, source d’actinomyces rares producteurs d’antibiotiques. *Se´cheresse* 1998; 9: 147-153.
26. Barakate M, Ouhdouch Y, Oufioud K, Beaulieu C. Characterization of rhizosporic soil streptomyces from Moroccan habitats and their antimicrobial activities. *World J Microbiol Biotechnol* 2002; 18: 49-54.
27. Oskay M, Tamer A, Azeri C (2004) Antimicrobial activity of some actinomycetes isolated from farming soils of Turkey. *Afr J Biotechnol* 2004; 3: 441-446.
28. Ouhdouch Y, Barakate M, Finance C. Actinomycetes of Moroccan habitats: isolation and screening for antifungal activities. *Eur J Soil Biol* 2001; 37: 69-74.
29. Abo-Shadi MAA-R, Sidkey NM, Al-Mutrafy M. Antimicrobial agent producing microbes from some soils in the world’s largest tidal mangrove forest. *Int J Syst Evol Microbiol* 2002; 52: 1225-1228.
30. Mathpal V, Bisht GS. Antimicrobial activity of actinomycetes isolated from Malaysian soils. *J American Sci* 2010; 6: 915-925.
31. Shinobu R, Kawato M. On *Streptomyces massasporeus* nov. sp. *Bot Mag (Tokyo)* 1959; 72: 283-288.
32. Gupta KC. A new species of the genus *Streptosporangium* isolated from Indian soil. *J Antibiott* 1965; 18: 125-127.
33. Cron MJ, Whitehead, DF, Hooper IR, Heinemann B, Lein J. Bryamycin, a new antibiotic. *Antibiot Chemother* 1956; 6: 63-67.
34. KimSB,Goodfellow M.Streptomyces thermospinisporus* sp. nov., a moderately thermophilic carboxydotrophic streptomyces isolated from soil. *Int J Syst Evol Microbiol* 2002; 52: 1225-1228.
35. Cui Q, Wang L, Huang Y, Liu Z, Goodfellow M. *Nocardia jiangxiensis* sp. nov. and *Nocardia mitunensis* sp., isolated from acidic soils. *Int J Syst Evol Microbiol* 2005; 55: 1921-1925.