Isolation, antifungal activity and characterization of soil actinomycetes

Akansha Saxena*, Ramraj Upadhyay, Dhaneshwar Kumar and Naveen Kango
Department of Applied Microbiology, Dr. Harisingh Gour University, Sagar, Madhya Pradesh, India

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Actinomycetes were isolated from eight samples of two different habitats, i.e., garden soil and cultivated field soil. Isolation of actinomycetes was carried out on soil extract agar medium using serial dilution method and forty seven isolates were obtained. All these actinomycetes were then assessed for their antifungal activity against two fungal pathogens i.e., Candida albicans and Aspergillus niger by cross streak method. Eleven actinomycetes were found to inhibit A. niger. Out of 11 actinomycetes, only 6 showed antifungal activity against both test organisms. On the basis of screening, one potential actinomycete GS 22 was selected for further studies. The antibiotic was extracted with methanol from cellular extract of actinomycete GS 22. The extract of GS 22 culture filtrate produced a clear zone of 27 mm against C. albicans by agar well diffusion method. Actinomycete strain GS 22 was further characterized on the basis of morphological, biochemical and physiological characteristics and identified using probabilistic identification of bacteria (PIBWin) software as Streptomyces phaeochromogenes.

Keywords: Actinomycetes, antifungal activity, characterization, Streptomyces

Introduction

Actinomycetes are ubiquitous, slow-growing, filamentous bacteria having high G+C content in their DNA. They are commercially important group of microorganisms producing wide range of bioactive molecules such as antibiotics, immunosuppressive agents, cosmetics, vitamins, nutritional materials, herbicides, pesticides and enzymes. A wide range of enzymes such as protease, lipase, chitinase, inulinase and asparaginase are well reported from actinomycetes. Actinomycetes are the major producers of antibiotics. Antibiotics produced from actinomycetes are known to possess antibacterial, antifungal, neuritogenic, anticancer, antialgal, antimalarial and anti-inflammatory activities. Majority of these secondary metabolites are obtained from a single genus Streptomyces.

Actinomycetes are a potential source of most clinically relevant antibiotics available today. Antifungal antibiotics play a significant role in the control of mycotic diseases. The development of newer antifungal antibiotics with greater potency has been a major challenge to the pharmaceutical industry today due to rapidly increasing fungal infections in human society, particularly in immune-compromised individuals and development of drug resistance to the currently used antifungal agents available in the market. The need of novel metabolites necessitates the screening of a large number of new actinomycetes strains. The objectives of the present study were to isolate actinomycetes from soil habitats, their screening for antifungal antibiotic production and extraction of antifungal metabolite from potential actinomycete strain. Further the potential actinomycete was characterized and identified on the basis of cultural, morphological, physiological and biochemical features.

Material and Methods

Sampling Area

Soil samples were collected from two different habitats i.e., garden soil (GS) and cultivated field soil (CFS) from different localities of Sagar, Madhya Pradesh (23º 50'N latitude and 78º 40'E longitude). The samples were collected in sterilized polythene bags, from a depth not exceeding 6 inches. All the samples were dried at room temperature and were given pretreatment at 45°C for 24 h before isolation.

Isolation of actinomycetes

Isolation of actinomycetes was performed by serial dilution method on soil extract agar medium. The composition of soil extract agar medium was as follows: glucose 1.0 gm, KH₂PO₄ 0.5 gm, soil extract 100 ml, agar-agar 20 gm. Tap water was added to make up volume upto 1000 ml. The medium was sterilized by
autoclaving at 15 lbs pressure for 20 min at 121°C. One gram of dried soil sample was serially diluted in sterile distilled water up to $10^{-7}$ dilutions and vortexed for few minutes. Then 0.1 ml of each dilution was plated over the surface of the plates containing soil extract agar medium using a sterile spreader. The plates were then incubated at 28°C for 7 to 10 days and observed at regular intervals for appearance of colonies. The colonies showing characteristics of actinomycetes (rough, chalky, powdery appearance, radiating growth and leathery texture etc.) were purified onto the soil extract agar medium plates. Pure isolated actinomycetes were sub-cultured on yeast extract malt extract agar slants \(12\) (g/L: glucose 4, yeast extract 4, malt extract 10, agar-agar 20) and allowed to grow for 7-14 days at 28°C. After incubation, slants were stored at 4°C for further use.

Test organisms
All the actinomycetes were tested for their antifungal activities against *Aspergillus niger* (GNCC 2006) and *Candida albicans* (GNCC 1008) obtained from the Gour Nodal Culture Collection (GNCC) of Department of Applied Microbiology, Dr. Harisingh Gour University, Sagar, Madhya Pradesh.

Screening of actinomycetes for antifungal activity
The antifungal activity of isolated actinomycetes was performed by a *Cross streak method*\(^{13}\) against aforesaid test fungi. Sterile Petri plates of yeast extract malt extract agar medium (ISP-2) were prepared. The suspension of test actinomycetes was prepared in 5 ml of 0.01% sterile tween-80 solution by transferring a loopful culture from 14 days colonies of actinomycetes, grown on ISP-2 medium slants. Plates were streaked with loopful suspension of the actinomycetes culture towards periphery of Petri plates and incubated at 28°C for 7 days. After 7 days Petri plates were re-inoculated with the freshly prepared suspension of test organisms at right angle to the growing actinomycetes colony. The Petri plates were again incubated at 28°C for 48 h to allow the growth of test organisms. Antagonistic activity of all tested actinomycetes against *A. niger* and *C. albicans* were recorded as total inhibition of growth (TIG), growth inhibition and retardation (GIR), growth retardation (GR) and no inhibition (NI).

Production of antifungal antibiotic
Potential actinomycetes selected on the basis of cross streak method were further inoculated on beef extract broth\(^{14}\) for antibiotic production. For this 25 ml of above medium was taken in 150 ml Erlenmeyer flask and sterilized by autoclaving at 15 lbs pressure for 25 min at 121°C. Spore suspension was prepared. The production medium was inoculated with spore suspension of test actinomycete and incubated at 28°C on a rotary shaker (120 rpm) for 108 h.

Extraction of antifungal metabolite
After incubation, the contents of the flasks were centrifuged at 7,000 rpm for 15 min and the supernatant was stored in screw cap tubes at 4°C for further studies. Cells obtained as pellet were lysed open using a homogenizer. The homogenate obtained was treated with 50% (v/v) methanol by shaking at 120 rpm for 1 h at 28°C for extraction of the antibiotic. The contents of the flask were again centrifuged at 7000 rpm for 15 min. The supernatant thus obtained was stored in screw cap tubes covered with black paper sheet at 4°C for further studies.

Localization of antifungal antibiotic
Localization of antifungal metabolite both in the supernatant and cellular extract was done using agar well diffusion method\(^{15}\). Yeast extract malt extract agar medium plates were prepared and a well was bored in the centre of each plate with the help of sterilized cork borer. Spore suspension of *C. albicans* was spread with a sterilized spreader. Supernatant and cellular extract (200 µl each) of potent test actinomycetes were poured in the wells separately and incubated at 28°C for 48 h. After incubation, the plates were observed for zone of inhibition around each well.

Characterization of potent actinomycete
The potential actinomycete strain was further characterized on the basis of cultural, morphological, biochemical and physiological features according to standard protocols of International *Streptomyces* Project\(^{12}\) and its identity was established using PIBWin software. Cultural characteristics such as colour of substrate and aerial mycelia, growth pattern and production of diffusible pigment were studied by culturing potent strain on ISP-2 medium\(^{16}\). Morphological studies were carried out using coverslip culture technique\(^{17}\) in which cover slips were inserted at 45° in inoculated medium for 14 days. Biochemical tests such as Indole, methyl red, Voges-proskauer (VP) citrate utilization, H₂S production, nitrate reduction, starch hydrolysis, gelatin hydrolysis, pectin hydrolysis, lipolysis, lecithinase activity, elastin degradation, xanthine degradation and arbutin degradation were also performed\(^{12}\).
The ability to utilize different carbon sources (i.e., sucrose, meso-inositol, mannitol, raffinose, D-melezitose, adonitol, D-melobiase, dextran, xylitol, glucose and fructose) was determined on basal salt agar medium to which sterilized carbon source was added to obtain a final concentration of 1% (w/v). The inoculated plates were incubated at 28°C for 21 days. Plates without carbon source were treated as control. Utilization of nitrogen sources was also studied using the method as given in Bergey’s Manual of Systematic Bacteriology, Volume IV. Different nitrogen sources (i.e. tyrosine, histidine, phenyl-alanine, valine, cysteine and hydroxyl-proline) were supplemented in basal medium to obtain a final concentration of 0.1% (w/v). Nitrogen free basal medium was used as a control. The inoculated plates were incubated at 28°C for 7-15 days. Physiological characteristics such as growth at different temperatures (4-42°C), effect of NaCl concentration (2-12%), effect of pH (4-11), effect of inhibitors and antibiotic sensitivity against ten different antibiotics (Hi-Media, Mumbai, India), Neomycin (N 50), Rifampicin (R 50), Oleandomycin (O 100), Penicillin G (10 IU), Cephaloridine (C 30), Kanamycin (K 30), Lincomycin (L 2), Norfloxacin (N 10) and Tobramycin (T 10) were also tested.

Identification of actinomycete

Identification of potential actinomycetes strain up to species level was done using PIBW (Probabilistic identification of bacteria for windows) software based on various morphological, physiological and biochemical features. This programme contains matrices of known strains and provides probable identification of unknown bacterial strains attaining minimum ID score.

Results and Discussion

Isolation of actinomycetes

Actinomycetes are the most economically and biotechnologically valuable prokaryotes responsible for the production of bioactive secondary metabolites. It is well known that actinomycetes are one of the major components of the microbial population present in soil. As soil is the major repository of microorganisms, therefore soil samples were collected from different locations of Sagar (M.P.) and processed for isolation. In the present study, eight samples were collected from two different habitats for isolation of actinomycetes. Samples used for isolation included four samples of garden soil and four samples of cultivated field soil.

A total of 47 actinomycetes were isolated which included 31 isolates from garden soil and 16 isolates from cultivated field soil. All the samples of garden soil and cultivated field soil were found positive for the presence of actinomycetes. The occurrence of actinomycetes in soil samples from different sites is presented in Table 1. Maximum number of actinomycetes was isolated from samples of garden soil. This may be due to high availability of organic matter in these samples.

Screening of actinomycetes for antifungal activity

All the isolated actinomycetes were studied for antifungal activity by cross streak method as shown in Figure 1. Results indicated that out of 47 test actinomycetes, only 11 isolates showed antifungal activity
against *A. niger* (Table 2). Out of these only 6 isolates (i.e. GS 1, GS 7, GS 15, GS 18, GS 22 and CFS 22) exhibited antifungal activity against both *A. niger* and *C. albicans*. Maximum antifungal antagonists were recorded from garden soil. These results were similar with the observations of earlier workers\(^{22, 23}\) that antagonist actinomycetes are abundant in garden soil enriched with compost. About 36 test actinomycetes showed no inhibition against both pathogenic fungi. Only two actinomycetes, GS 18 and GS 22 were found to be strong antagonists producing a zone of inhibition of more than 10 mm against both fungi. Test actinomycete GS 22 showed growth inhibition of 14 mm and was further examined for production and localization of antifungal antibiotic.

**Localization of antifungal antibiotic**

Agar well diffusion assay of culture filtrate and intracellular extract of test actinomycete GS 22 demonstrated that antibiotic produced is intracellular. Intracellular extract of GS 22 produced a zone of 27 mm against *C. albicans* while there was no such inhibition with extracellular cell-free culture filtrate. The result of localization of antifungal antibiotic by actinomycete strain GS 22 is shown in Figure 2.

**Morphological, biochemical and physiological features of actinomycete strain GS 22**

Test actinomycete GS 22 exhibited good growth on yeast extract malt extract agar medium. Strain GS 22 developed creamish substrate mycelia and white aerial mycelia. The details of morphological and biochemical features of actinomycete GS 22 are given in Table 3. Morphological characteristics are considered important in identification of various genera of actinomycetes. Spore chain morphology was studied using coverslip culture technique. Test actinomycete GS 22 exhibited spiral spore chain morphology. Thus, initial morphological details showed that potential isolate belongs to the genus *Streptomyces*.

Actinomycete GS 22 was negative for citrate utilization, indole production and VP test. Test actinomycete GS 22 failed to reduce nitrate to nitrite and showed a weak positive result for methyl red test, starch hydrolysis and lecithinase activity. Production of \(\text{H}_2\text{S}\) was studied using Triple sugar iron (TSI) agar

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Test Actinomycetes</th>
<th><em>A. niger</em></th>
<th><em>C. albicans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>GS 1</td>
<td>GIR (3)</td>
<td>GI (2)</td>
</tr>
<tr>
<td>2.</td>
<td>GS 7</td>
<td>GR</td>
<td>GI (10)</td>
</tr>
<tr>
<td>3.</td>
<td>GS 9</td>
<td>GI (2)</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>GS 12</td>
<td>GR</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>GS 14</td>
<td>GR</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td>GS 15</td>
<td>GIR (1)</td>
<td>GI (5)</td>
</tr>
<tr>
<td>7.</td>
<td>GS 17</td>
<td>GR</td>
<td>-</td>
</tr>
<tr>
<td>8.</td>
<td>GS 18</td>
<td>GR</td>
<td>GIR (12)</td>
</tr>
<tr>
<td>9.</td>
<td>GS 19</td>
<td>GR</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>GS 22</td>
<td>GIR (1)</td>
<td>GI (14)</td>
</tr>
<tr>
<td>11</td>
<td>CFS 2</td>
<td>GR</td>
<td>GI (10)</td>
</tr>
</tbody>
</table>

*TIG - Total inhibition of growth; GIR - Growth inhibition and retardation; GR - Growth retardation; - No inhibition. Data given in parentheses indicate ‘zone of inhibition’ in mm.
medium. GS 22 showed H2S production on this medium. The isolate was found positive for elastin, xanthine and arbutin degradation.

Carbon and nitrogen source play important role in metabolism24. A wide range of nitrogen sources supplemented in basal medium were utilized by selected isolate GS 22. It showed excellent growth with L-histidine as nitrogen source. Similar findings regarding L-histidine as suitable nitrogen source for thermophilic *Streptomyces* have been reported earlier25. Isolate GS 22 showed weak growth on L-cysteine, L-valine, L-phenylalanine and L-hydroxyproline. Among carbon sources, test actinomycete GS 22 exhibited excellent growth on fructose. Glucose was found to be the next best carbon source for growth of this actinomycete. It showed poor growth on sucrose, meso-inositol, mannitol, L-rhamnose, raffinose, D-melezitose, adonitol, D-meliobiose, dextran, xylitol, and sorbitol. It is well documented that different cultural conditions influence the growth of actinomycetes 26.

Antibiotic susceptibility profile showed that test actinomycete GS 22 was found resistant to Oleandomycin (100µg/ml), Penicillin G (10 units), Cephaloridine (30 µg/ml) and Norfloxacin (10 µg/ml). It was found sensitive against Neomycin (50 µg/ml), Rifampicin (50 µg/ml), Kanamycin (30), Lincomycin (2) and Tobramycin (10) antibiotics. The actinomycete strain GS 22 was found to be most sensitive against Rifampicin and Tobramycin which produced 36 mm and 29 mm zones of inhibition, respectively (Table 4).

The assessment of physiological characteristics revealed that isolate GS 22 grew well at 25°C and 37°C while the test isolates grew poorly at 42°C. The ability of this isolate to grow at various salt concentrations was also tested. It exhibited optimal growth at 2% NaCl while poor growth was observed at 5% -7% NaCl concentrations. It failed to grow at higher (9-12%) NaCl concentrations. Actinomycete GS 22 showed luxuriant growth in the pH range of 9.0-11.0 indicating its alkalophilic nature while poor growth was observed in the pH range of 4.0-7.0.

Isolate GS 22 showed good growth in presence of 0.001% (w/v) potassium tellurite and was found to be resistant against this inhibitor. It also exhibited weak growth on phenol (0.1%). The isolate was found to be sensitive to sodium azide (0.01%) and thallous acetate (0.001%) and showed no growth.

### Table 3

| Morphological and biochemical characteristics of *S. phaeochromogenes* GS 22 |
|-------------------------------------------------|-------------|
| **Morphological Characteristics**               |             |
| Spore chain                                     | Spiral      |
| Color of spore mass                             | White       |
| Substrate mycelium                              | Creamish    |
| Mycelial pigment                                | Negative    |
| Diffusible pigment produced                     | Positive    |
| Melanin on peptone iron agar                    | Negative    |
| Melanin on tyrosine agar                        | Negative    |
| **Biochemical Characteristics**                 |             |
| Test                                            | Result      |
| Indole production, Voges-proskauer, Citrate     | Negative    |
| utilization, Pectin hydrolysis, Nitrate          |             |
| reduction, Starch hydrolysis                    |             |
| Methyl red, Lecithinase activity, Gelatin        | Weak positive|
| hydrolysis, Lipolysis, Hydrogen sulphide production, Elastin degradation, Xanthine degradation, Arbutin degradation |

### Table 4

| Physiological characteristics of actinomycetes strain GS 22 |
|-------------------------------------------------|-------------|
| Antibiotic Sensitivity (Zone of inhibition in mm)  |
| 1. Neomycin (50) 12                              |
| 2. Rifampicin (50) 36                            |
| 3. Oleandomycin (100) R                          |
| 4. Penicillin G (10 I.U.) R                       |
| 5. Cephaloridine (30) R                          |
| 6. Kanamycin (30) 21                             |
| 7. Lincomycin (2) 10                             |
| 8. Norfloxacin (10) R                            |
| 9. Tobramycin (10) 29                            |
Identification using PIBWin Software

Primary identification of test actinomycete was done on the basis of spore chain morphology. On the basis of morphological characteristics it was identified to belong to genus *Streptomyces*. Several workers\(^{27,28}\) reported dominance and frequency of *Streptomyces* in various soil types. Further this isolate was identified using PIBWin programme based on morphological characters, pigment production, biochemical and physiological features, growth in presence of inhibitors and sensitivity to different antibiotics etc. Actinomyxete strain GS 22 was identified as *Streptomyces phaeochromogenes* with I.D. score 0.999 using PIBWin software.

Conclusions

Preponderance of actinomycetes was noticed in garden and cultivated soil samples. Out of 47 isolates, only 11 inhibited *A. niger*. Out of these, 6 isolates exhibited antifungal activity against both *A. niger* and *C. albicans*. Intracellular extract of the isolate GS 22 showed strong inhibition of *C. albicans* indicating intracellular presence of the antibiotic. Based on morphological and biochemical characteristics isolate GS 22 was identified as *Streptomyces phaeochromogenes*. Further characterization of the antifungal active principle is underway.

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References


