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# Isolation and Screening of Streptomyces Species Producing Anti-Microbial Compounds Active against Multi-Drug Resistant Pathogens causing Respiratory Tract Infections

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## Abstract

**Background**: The members of the family Actinomycetaceae, are the largest known group of microbes that act as reservoirs of biologically active compounds i.e. antibiotics. **Aim**: The current study was carried out with an aim to isolate Actinomycetes from soil samples and screen for antibiotic producers effective against Multi-Drug Resistant (MDR) pathogens causing Respiratory Tract Infections (RTIs). **Methods**: Twenty-one isolates were obtained from various soil samples in our study, and were subjected to primary screening for antibiotic production by agar strip method, Wilkins overlay method, agar diffusion and disc diffusion assays. Identification of Actinomycetes was carried out on the basis of morphological, cultural, biochemical tests and cell wall characteristics. **Results**: Among the Actinomycetes isolated in our study, 12 isolates showed considerable antibacterial activity against test pathogens. Four isolates showed inhibition of pathogenic *S. aureus* but were ineffective against gram-negative and fungal pathogens. Three isolates inhibited gram-positive and fungal pathogens but were ineffective against gram-negative test pathogens. However, 5 isolates showed promising anti-microbial activity against gram positive and gram negative bacteria as well as fungal pathogens. Four of these 5 isolates were identified as *Streptomyces noboritoensis, Streptomyces nigrifaciens, Streptomyces griseorubiginosus* and *Streptomyces rubiginosohelvolus*. The remaining one isolate showed characteristics of a fungus. **Conclusion**: The antibiotic-producing strains isolated in our study can be a good candidate for broad-spectrum antibiotic production which is not only effective against MDR bacteria but also fungal pathogens causing RTIs.

**Keywords**
Actinomycetes, Agar strip method, Drug-resistant, Screening, Streptomyces.

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## INTRODUCTION

The Respiratory Tract Infections (RTIs) are the most common infections affecting the human population with an estimated 17.2 billion recorded cases to have occurred globally in 2015 alone [1]. Although it mostly occurs as un-complicated conditions affecting upper respiratory tract as common cold, laryngitis, pharyngitis/tonsillitis, rhinitis, sinusitis, and otitis media caused due to inflammation or viral infection, it can also present itself with complications of bacterial or fungal infections occurring naturally or as a secondary infection predisposed to primary viral infections [2, 3]. Conversely, the lower RTIs may be caused by bacterial, viral or fungal pathogens [4, 5].

Given the fact that majority of clinical occurrences of upper RTIs are of viral etiology, it can be argued that prescription of antibacterial agents in the treatment of the same has strongly and rapidly supported the evolution of antibiotic resistance in bacterial and fungal pathogens which were once believed to be comparatively harmless [6, 7]. Over the years, factors like inappropriate antibiotic therapy, availability of over the counter drugs and self-medication have influenced the development of antibiotic resistance among pathogens causing RTIs in general [8]. Although these reasons verily justify the clinical malpractices; on certain occasions of diagnostic uncertainty, patient expectations, as well as socio-cultural and economic pressures, it plays an important role in forced antibiotic prescriptions [9].

Considering either scenario; the fact remains that we face a genuine problem of fast spreading and evolving antibiotic resistance among pathogens today. To make matters worse, there are increasing numbers of reports on RTIs caused by Multi-Drug Resistant (MDR) pathogens including Methicillin-resistant *Staphylococcus aureus* (MRSA) and Extended spectrum β-lactamase (ESBL) producing Enterobacteriaceae [10, 11, 12]. As the name suggests MRSA strains show resistance to one of the most potent antibiotics against pathogenic *S. aureus* strains. The ESBLs are plasmid-mediated beta-lactamases capable of efficiently hydrolyzing penicillin's, β-lactam antibiotics, narrow spectrum cephalosporin's, many 3$^{rd}$ generation cephalosporin's (i.e., cefotaxime, ceftazidime), and monobactams (aztreonam). They often show resistance to β-lactamase inhibitors too [13, 14].

In order to combat these infections, alternative strategies are proposed like the use of phytochemicals as herbal remedies, phage therapy, vaccines, prebiotics, probiotics etc. However, the advantages offered by antibiotics over these strategies are far greater. Unfortunately, a decline in the rate of new antibiotic production is observed in the last few decades. This is due to the fear of development of resistance against the newly discovered antibiotic, as has occurred for most antibiotics today [15].

Actinomycetes, being an enormous reservoir of antimicrobial compounds, may be exploited for the discovery of a single novel compound with broad-spectrum activity against bacterial, fungal as well as viral pathogens which may or may not be resistant to current antibiotics. This strategy seems more promising than alternative treatment methods. Currently, almost two-thirds of the natural antimicrobial drug compounds are obtained from different genera of this family viz., Streptomyces, Nocardia and Micromonospora etc. More than 500 species and subspecies of Streptomyces have been described alone [16]. Common examples of antibiotics produced by Streptomyces include streptothricin (*Streptomyces lave*ndulae), neomycin and fradicin (*Streptomyces fradiae)*, aureomycin and terramycin (*Streptomyces flavus)*, streptomycin (*Streptomyces griseus*) [17].

Since its first discovery n 1950s, the number of antimicrobial compounds reported from Streptomyces sp. increased almost exponentially for the next two decades reaching a peak in 1970s [18]. This further supports the prospect of discovery of new and undiscovered potent compounds, which may aid in dealing with the current problem of antibiotic resistance among pathogens.

Keeping the current scenario of fast spreading antibiotic resistance in mind, the current study was carried out with an aim to isolate and identify antibiotic-producing Actinomycetes from soil samples effective against bacterial as well as fungal pathogens causing RTIs.

## MATERIALS AND METHODS

### Chemicals and media

All the chemicals and media used in our study were of analytical grade and purchased from SRL and Hi-media Laboratories, Mumbai, India.

### Pathogens used in our study as test isolates for determining the antibacterial activity of Actinomycetes

The nine test pathogens used in our study were obtained from Hinduja hospital, Mumbai. These strains consisted of a Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from sputum sample, MDR *Pseudomonas aeruginosa* and *Acinetobacter baumanii* isolated from tracheal secretion, Extended-spectrum β-lactamase producing *Escherichia coli, Klebsiella pneumoniae*

and *Klebsiella oxytoca* isolated from tracheal secretions, and MDR *Enterobacter aerogenes, Candida albicans* and *Candida glabrata* isolated from sputum samples.

**Enrichment and isolation of Actinomycetes from soil samples**

In our study, various soil samples were collected from different spots in the same location i.e. Maharashtra Nature Park, Dharavi. These samples included soil from compost bins, garbage dumps of iron sediments, rhizosphere region of plants, and lakes. For enrichment of actinomycetes, the soil samples were initially pre-treated by air drying and heating it to 110°C for 10min in order to suppress the growth of bacteria and fungi. The samples were then mixed with 0.05g Calcium carbonate ($CaCO_3$) and incubated overnight at Room Temperature (RT) i.e., around 30°C. The pre-treatment of rhizosphere soil was done by mixing the skin of tubers with 1:140 diluted solution of phenol. Further enrichment of soil samples was carried out by incubating it with substrates like powdered chitin and pollen membranes for 7 days at 25°C. One gram of the pre-treated and enriched soil samples was then serially diluted 100-folds up to $10^{-5}$ dilution using sterile saline. A 0.1mL aliquot of these dilutions was plated on to Casein Starch Agar (CSA) medium supplemented with 50µg nystatin, fluconazole, rifamycin and kanamycin antibiotics, and the plates were incubated at 30°C for 14days. After incubation, the colonies showing morphological similarity to Streptomyces sp. were purified by re-isolating it on Oatmeal Agar (OA) medium (ISP-3). The pure cultures thus obtained were maintained on Yeast Extract Malt Extract (YEME) agar (ISP-2) slants and stored at refrigerated conditions before further use.

**Screening of antibiotic activity by isolated Actinomycetes against test isolates**

Four different methods were used to check the antibiotic production by the isolated actinomycetes sp. against test organisms. These methods are described below.

1. Agar strip method
   This method was performed with slight modification to that described by Augustine et. al. (2005) [19]. A rectangular ISP-2 agar strip with matt growth of actinomycetes culture, obtained after incubation at 30°C for 4-5 days, was cut and placed on Sabouraud agar (SAB) and Nutrient agar (NA) plates streaked horizontally with fungal and bacterial cultures respectively. The plates were incubated for 24-48h and observed for inhibition of the test organisms surrounding the agar strip.
2. Wilkins overlay method
   Wilkins agar serves a dual purpose as an overlay medium. It eliminates the inhibition due to acid production which is evident due to the presence of bromothymol blue, and also defines the zones of inhibition produced. To carry out this procedure, a saline suspension of the test isolates (0.1O. $D_{540nm}$) showing antibacterial activity was prepared and spot inoculated on Casein Yeast Beef Peptone Agar (GCYBP) plates since it supported good growth and sporulation. The plates were incubated at 30°C for 4-5 days, after which it was overlayed with 10mL of molten Wilkins agar seeded with 1mL of 18h old test pathogens adjusted to 0.3 O.D. The overlayed medium was further incubated at 37°C for 24h to observe for visible zones of inhibition.
3. Agar well diffusion method
   The individual Actinomycetes test cultures were inoculated in 150mL of ISP-2 broth and incubated at 30°C for 4 weeks. A 5mL aliquot of above growth was removed and assessed for antibacterial and anti-fungal activity at intervals of 2days. The aliquots were centrifuged at 3000rpm/10min to remove cell mass and this supernatant was used as an antibiotic sample. In order to assess the antibacterial and antifungal activities, molten NA and SAB medium were seeded with test bacteria and fungi respectively and poured into sterile petri-plates. Following solidification of the medium, wells were punched into the agar using a sterile cork-borer (6mm diameter) and 0.1mL of the cell-free supernatant was added to these wells. Plates were refrigerated for 20min to allow pre-diffusion of the broth from the well into the agar medium and then incubated at 37°C for 24h [15].
4. Disc diffusion method
   This method was carried out similar to the previous method, except that filter paper discs dipped in cell-free supernatant of the fermentative broth were placed equidistantly on NA and SAB plates swabbed with test pathogens. The plates were incubated at 37°C for 24h and observed for zones of inhibition around the filter paper discs.

**Preliminary identification of selected Actinomycetes**

Identification of the most promising isolates was done on the basis of their morphological, cultural and biochemical characteristics described in the Bergey's manual [20]. The classification of species

was also carried out on the basis of cell wall amino acid and sugar profiles [21].

The cultural characteristics like size, shape, color, opacity, margin, elevation, surface consistency and pigmentation were examined on CSA medium. The gram nature and the microscopic morphological structure of the isolates were also noted. In addition, the pigments produced on different ISP medium viz., YEME (ISP-2) agar, OA (ISP-3), Inorganic salt starch (ISP-4) agar and Glycerol asparagine (ISP-5) agar were also studied.

The aerial and substrate mycelia were observed using the slide culture technique under 10X and 40X magnification. The biochemical characteristics were studied by checking for fermentation of various sugars viz., Dextrin, Fructose, Galactose, Glycerol, Glycogen, Inositol, Inulin, Lactose, Mannitol, Rhamnose, Soluble Starch, Sucrose, Trehalose and Xylose in base agar medium containing bromo-cresol purple as an indicator. The degradation of substrates like casein, tyrosine, urea and esculin were also studied. In addition, a nitrate reduction test was also carried out.

**Chromatographic analysis of whole cell hydrolysate**

Whole cell hydrolysates were prepared as described by Boone and Pine, 1968 and analyzed using paper and Thin Layer Chromatography (TLC) techniques [22]. A previously grown cell mass was harvested by centrifugation at 3000rpm and the hydrolysate was prepared by boiling it with 0.5mL of 0.1N NaOH in a tightly sealed screw-capped tube placed in a water bath for 1h. The cell deposits were washed four times with distilled water and recovered with centrifugation at 3000rpm. Finally, the extracted cell hydrolysate was suspended in minimal amounts of distilled water. Two-thirds of this solution was used for the detection of sugars and the remaining was used for the detection of amino acids. The samples were hydrolyzed for 2h with 6N HCl in a tightly sealed screw-capped tube, dried and concentrated. The residual HCl was removed by adding water and re-drying the sample three times. Samples were dissolved in 0.3mL distilled water for chromatography. The TLC was performed using Silica Gel H coated plates. The solvent system used for detection of sugar and amino acid profiles were water: pyridine: toluene (6:6:1) and n-propanol: ammonia: water (6:2:1) respectively. The hydrolysates of test isolates were spotted on TLC plates and Whatman filter paper No. 1, along with the reference standards for sugars (1% arabinose, galactose and rhamnose) and amino acids (glycine, aspartic acid and alanine). The sugar and amino acid spots were developed using 1% aniline hydrogen phthalate and 0.2% ninhydrin respectively [23, 24, 25].

## RESULTS AND DISCUSSION

**Enrichment and isolation of Actinomycetes from soil samples**

Actinomycetes have been isolated from various sources like composts, decaying organic matter, mud etc. However, the most common source for isolation of actinomycetes remains soil [26]. A total of 21 isolates were obtained after enrichment of actinomycetes from samples collected in our study. The pre-treatment of the soil samples ensured the absence of bacteria and fungi since normal soil flora can rarely tolerate exposure to high temperatures (i.e. 110°C for 10mins). Actinomycetes, on the other hand, show characteristic tolerance to adverse environmental conditions including high temperatures. The diverse physiology and metabolic flexibility observed among members of this family enable them to survive under hostile and unfavorable conditions [27]. Also, calcium carbonate and chitin are not readily utilized by other groups of micro-organisms except for actinomycetes. Hence incubation of test samples with these compounds helped in the enrichment of the same. The ability of actinomycetes to utilize chitin as a sole source of carbon and nitrogen has also been used for its isolation from the soil as well as aquatic habitats previously [28, 29]. Moreover, the actinomycetes can easily assimilate casein and starch in the CSA medium and grow in higher concentrations; thus reducing the growth of other groups of organisms that may be present in the samples. Furthermore, the presence of antibiotics i.e., nystatin, fluconazole, rifampicin and kanamycin in CSA plates prepared in our study, further ensured the absence of bacterial and fungal contaminants in the growth medium. The combination of antibacterial or antifungal agents prevents the growth of resistant bacteria from test samples. A study reported complete inhibition of 84% test fungi and partial inhibition of the remaining 16% on using more than one antibiotic in conjunction [30].

The current isolation protocol designed by means of nutritional selection, selective inhibition and pre-treatment of sample prevents the growth of competing microbes without adversely affecting the propagation of actinomycetes [29]. Considering these precautionary measures, the 21 colonies of Actinomycetes were selected on the basis of colony characteristics and preliminary examinations i.e., tenacious growth, pigmentation and chalky

appearance on the medium, and gram-positive nature.

**Screening of antibiotic activity by isolated Actinomycetes against test isolates**

The agar strip method was used as a primary screening method to assess the antibacterial activity of the test isolates. Table 1 represents the observed zones of inhibition by the test isolate against RTI causing pathogens used in our study. Nine out of twenty-one isolates failed to show any antibacterial activity against the test pathogens. Four isolates (isolate no. 2, 8, 10 and 15) showed inhibition of *S. aureus* but were ineffective against gram-negative bacterial and fungal pathogens. Three isolates (isolate no. 6, 17 and 18) inhibited gram-positive bacterial and fungal pathogens but were ineffective against gram-negative test pathogens. However, 5 isolates (isolate no. 3, 5, 7, 9 and 11) showed promising anti-microbial activity against gram positive and gram negative bacteria as well as fungal pathogens causing RTIs used in the current study. Among these, isolates 3 and 11 were isolated from compost garbage dump of iron sediments; isolates 5 and 9 from the soil of daswanti and tulsi plants, and isolate 7 from lake sediments. These isolates were further tested for antibiotic production by Wilkins's overlay technique, disc diffusion and agar diffusion assays. Table 2 represents the zone of inhibition observed by test isolates against RTI causing pathogens by these techniques.

The Wilkins's overlay technique showed the absence of yellow color near the zones of inhibition, hence confirming that the inhibition of pathogens by test isolates was due to the production of antibiotics and not due to the production of acids as a metabolic end product. Also, in certain cases, low concentration of antibiotics incorporated in disc diffusion method leads to false negative results. Hence in our study, disc diffusion, as well as agar diffusion assay was carried out to confirm the anti-microbial action of test isolates.

Among the 5 most promising isolates, few cultures (isolate 5 and 9) showed no antibacterial activity for up to 4 weeks of incubation in the growth medium. However, considerable antimicrobial activity was observed later. This may be due to the gradual increase in antibacterial activity on the utilization of carbon and nitrogen sources. Also, they showed negligible antimicrobial activity by agar diffusion and disc diffusion assays. However, considerable activity was observed by the agar strip method. This may be due to the slow growth rate of these isolates which resulted in lower biomass production and hence the low concentration of antibiotic to show potential by above techniques.

An active microbial strain of *Streptomyces levis* isolated from the agricultural soil of Narnaul, Haryana, India was found to produce extracellular active compound showing strong antimicrobial activity against *K. pneumoniae* MTCC 109, *P. aeruginosa* MTCC 741 and *S. aureus* MTCC 96 [31]. Also, Streptomyces sp. VITBRK2, capable of producing indolo antibacterial compounds actively inhibited MRSA and vancomycin-resistant Enterococci [32]. In another study, 36 novel compounds have been identified from 11 actinomycete strains isolated from plant roots, as well as from sediments of mangrove forests and oceans. Few examples include iminimycin- an iminium ion containing compound discovered from the culture broth of *Streptomyces griseus* OS-3601; mangromicins isolated from the culture broth of *Lechevalieria aerocolonigenes*; K10-0216 KA and KB steroids and pyrizomicin A and B isolated from unidentified actinomycetes [33]. Another interesting study reported the identification of new antibacterial compounds such as alchivemycin A and B, arcyriaflavin E or ciromicins obtained from fermentation broth containing co-cultures of different Rhodoccoci with the species of Streptomyces, Tsukamurella and Nocardiopsis [34, 35]. Another cryptic natural product keyicin was discovered when Micromonospora strain and Rhodococcus sp., were co-cultured [36].

**Preliminary identification of selected Actinomycetes**

Tables 3 and 4 represents the colony and biochemical characteristics of the 5 most promising actinomycetes isolated in our study. In addition to these characteristics, the pigments produced on different ISP medium were also noted since they can be observed more clearly as compared to other medium [20, 37]. The pigments produced on ISP media also help in species identification especially the melanoid pigments produced on ISP-5 [38].

All 5 potential isolates, obtained in our study, were non-motile gram-positive filaments. Isolate 3 showed melanoid pigments with grey spore mass on ISP agar medium whereas isolates 5, 7 and 11 showed colored spore mass without melanoid pigmentation. Isolate 9 showed red colored spore mass with yellowish brown pigmentation.

The slide culture technique (Figure 1) showed extensively branched aerial and substrate hyphae for isolate 3. The aerial spore chains appeared to be of the rectus flexibilis (straight to wavy) type and no spores were produced on the substrate hyphae. The

isolate 5 appeared as long branched aerial mycelia with spore chains like rectus flexibilis. The spores observed in the substrate mycelia for isolate 7 and 9 were also of rectus flexibilus type and aerial mycelia were found to be absent. In contrast to these 4 isolates, the isolate 11 showed conidia formation with sporangium which is a characteristic of fungus e.g., Penicillium. Also, it did not show glucose assimilation, and the observed colony characteristic was not powdery. On the contrary, it showed spore aerial mass with extensive mycelial fragmentation on the agar surface. Hence it can be concluded that isolate 11 does not belong to the actinomycetes group.

Hence, the preliminary morphological and biochemical examination of the cultures indicated that 4 out of 5 potential isolates, obtained in our study have characteristics of the genera Streptomyces. Further identification of species was carried out with the help of whole cell hydrolysate analysis.

In another study, 13 out of 31 Streptomyces sp. isolated from water and sediments of lake Tana, Ethiopia, showed antibacterial activity on primary screening. The aerial mycelium, substrate mycelium growth and pigmentation also showed distinct variations on different growth media used. The actinomycetes appeared as leathery, white powdery, creamy, pinpoint and powder colonies on CSA medium and hydrolyzed starch and urea [39]. Similarly, the formation of the substrate and aerial mycelia for Streptomyces sp., along with soluble pigment production in various ISP medium is reported in other studies [40, 41, 42].

**Chromatographic analysis of whole cell hydrolysate**

The Sugar pattern of the isolates could not be determined using TLC, hence paper chromatography was carried out using Whatman filter paper No. 1. The standard $R_f$ values for sugars and amino acids and that obtained for whole cell hydrolysates are represented in tables 5 and 6 respectively. The cell walls of the isolates 3, 5, 7 and 9 can be classified as Type I (Table 7). They also showed the absence of a clear sugar. The collective observation of cell wall composition and sugar utilization patterns led to the tentative identification of test isolates as *Streptomyces noboritoensis* (isolate 3), *Streptomyces nigrifaciens* (isolate 5), *Streptomyces griseorubiginosus* (isolate 7) and *Streptomyces rubiginosohelvolus* (isolate 9).

**Table 1: Zone of inhibition observed by test isolates against RTI causing pathogens by the agar strip method**

| Test isolates | Zones of inhibition observed against RTI causing pathogens "in mm" | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *S. aureus* | *P. aeruginosa* | *A. baumanii* | *E. coli* | *K. pneumoniae* | *K. oxytoca* | *E. aerogenes* | *C. albicans* | *C. glabrata* |
| 1 | No inhibition observed | | | | | | | | |
| 2 | 17 | No inhibition observed | | | | | | | |
| 3 | 18 | 12 | 14 | 30 | 20 | 25 | 13 | 25 | 12 |
| 4 | No inhibition observed | | | | | | | | |
| 5 | 15 | 12 | - | 10 | 0 | 11 | - | 12 | - |
| 6 | 15 | No inhibition observed | | | | | | 11 | - |
| 7 | 17 | 15 | 15 | 29 | 10 | 13 | 10 | 15 | 10 |
| 8 | 25 | No inhibition observed | | | | | | | |
| 9 | 17 | 12 | 12 | 12 | - | 10 | - | 10 | 0 |
| 10 | 15 | No inhibition observed | | | | | | | |
| 11 | 7 | 12 | - | 25 | - | 12 | - | 18 | 0 |
| 12 | No inhibition observed | | | | | | | | |
| 13 | No inhibition observed | | | | | | | | |
| 14 | No inhibition observed | | | | | | | | |
| 15 | 12 | No inhibition observed | | | | | | | |
| 16 | No inhibition observed | | | | | | | | |
| 17 | 25 | No inhibition observed | | | | | | 22 | 10 |
| 18 | 17 | No inhibition observed | | | | | | 10 | 12 |
| 19 | No inhibition observed | | | | | | | | |
| 20 | No inhibition observed | | | | | | | | |
| 21 | No inhibition observed | | | | | | | | |

**Table 2: Zone of inhibition observed by test isolates against RTI causing pathogens by Wilkins's overlay technique, disc diffusion and agar diffusion assays**

| RTI causing pathogens | Zone of inhibition observed by test isolates "in mm" | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3 | | | 5 | | | 7 | | | 9 | | | 11 | | |
| | *A | *B | *C | *A | *B | *C | *A | *B | *C | *A | *B | *C | *A | *B | *C |
| *S. aureus* | 18 | 19 | 19 | 15 | - | 10 | 17 | 23 | 23 | 17 | 10 | - | 7 | 20 | 20 |
| *P. aeruginosa* | 12 | 12 | 12 | 12 | - | 5 | 15 | 10 | 10 | 12 | 12 | - | 12 | 10 | 10 |
| *A. baumanii* | 14 | 20 | 20 | 0 | - | - | 15 | 20 | 20 | 12 | - | - | 0 | - | - |
| *E. coli* | 30 | 13 | 13 | 10 | - | - | 29 | 13 | 13 | 12 | - | - | 25 | - | - |
| *K. pneumoniae* | 20 | - | - | 0 | - | - | 10 | 12 | 12 | 0 | - | - | 0 | - | - |
| *K. oxytoca* | 25 | 30 | - | 11 | - | - | 13 | 10 | 25 | 10 | - | - | 12 | - | 20 |
| *E. aerogenes* | 13 | 14 | - | 0 | - | - | 10 | 14 | 14 | 0 | - | - | 0 | - | - |
| *C. albicans* | 25 | 25 | 25 | 12 | 10 | 10 | 15 | 29 | 29 | 10 | - | - | 18 | 22 | 22 |
| *C. glabrata* | 12 | 20 | - | 0 | - | - | 10 | - | - | 0 | - | - | 0 | 20 | 20 |

***A: Wilkins overlay technique; *B: Disc diffusion assay; *C: Agar diffusion assay**

**Table 3: Colony characteristics of the selected Actinomycetes isolates**

| Characteristic | Isolate 3 | Isolate 5 | Isolate 7 | Isolate 9 | Isolate 11 |
|---|---|---|---|---|---|
| Size | Discrete | 1mm | Pinpoint | 1mm | pinpoint |
| Shape | Irregular | Circular | Circular | discrete | circular |
| Colour | Green | Black | Cream | Green | White |
| Pigment | Yellow | - | - | - | Lime green |
| Margin | Irregular | Entire | Irregular | Irregular | Entire |
| Opacity | Opaque | Opaque | Opaque | Opaque | Opaque |
| Elevation | High Convex | Convex | Raised | Flat | Flat |
| Surface | Leathery | Smooth | Smooth | Leathery | Leathery |
| Consistency | Tough | Tough | Tough | Hard | Tough |
| Gram Nature | Gram +ve | Gram +ve | Gram +ve | Gram +ve | Gram +ve |
| Morphology | Filaments | Filaments | Filaments | Filaments | Filaments |
| Motility | Non-motile | Non-motile | Non-motile | Non-motile | Non-motile |
| **The pigment produced in different ISP medium** | | | | | |
| ISP-2 | Ochre yellow | Grey | Grey yellow | Grey white | Brown |
| ISP-3 | Grey | Grey | White | Grey | Red |
| ISP-4 | White | Grey | Yellow | Grey | Grey |
| ISP-5 | Red | Grey | Yellow-brown | Grey | Grey |

**Table 4: Biochemical characteristics of the selected Actinomycetes isolates**

| Tests performed | Isolate 3 | Isolate 5 | Isolate 7 | Isolate 9 | Isolate 11 |
|---|---|---|---|---|---|
| **Fermentation of sugars** | | | | | |
| Dextrin | + | - | + | - | - |
| Fructose | + | + | + | + | - |
| Galactose | + | + | + | - | + |
| Glucose | + | + | + | + | - |
| Glycerol | + | + | + | + | + |
| Glycogen | - | - | - | - | - |
| Inositol | + | + | + | - | + |
| Inulin | - | - | - | - | - |
| Lactose | + | + | + | + | - |
| Mannitol | + | + | + | + | - |
| Rhamnose | - | + | + | + | + |
| Soluble starch | - | - | - | - | - |
| Trehalose | + | + | + | + | + |

| Tests performed | Isolate 3 | Isolate 5 | Isolate 7 | Isolate 9 | Isolate 11 |
|---|---|---|---|---|---|
| Xylose | + | + | + | + | + |
| **Degradation of substrates** | | | | | |
| Tyrosine | - | - | - | - | - |
| Casein | + | + | + | + | + |
| Esculin | + | + | + | + | + |
| Urea | + | + | + | + | + |
| Gelatin | - | + | - | - | + |

**Table 5: Standard $R_f$ values for sugars and amino acids.**

| Sugars and amino acids | $R_f$ value |
|---|---|
| Arabinose | 0.40 |
| Galactose | 0.38 |
| Rhamnose | 0.63 |
| Aspartic acid | 0.25 |
| Glycine | 0.30 |
| Alanine | 0.35 |

**Table 6: $R_f$ values observed for cell-wall hydrolysates for sugar and amino acids**

| Sample | $R_f$ value for sugars | $R_f$ value for amino acids |
|---|---|---|
| **Isolate no 3** | 0.34 | 0.33 (glycine) |
| **Isolate no 5** | 0.33 | 0.32 (glycine) |
| **Isolate no 7** | 0.63 (Rhamnose) | 0.30 (glycine) |
| **Isolate no 9** | 0.60 (Rhamnose) | 0.31 (glycine) |
| **Isolate no 11** | 0.61 (Rhamnose) | 0.36 (alanine) |

**Table 7: Classification of Actinomycetes based on major constituents of the cell wall**

| Cell wall type | *DAP meso | *DAP LL | Glycine | Arabinose | Galactose |
|---|---|---|---|---|---|
| I | - | + | + | - | - |
| II | + | + | + | - | - |
| III | + | - | - | - | - |
| IV | + | - | - | + | + |

***Standards of meso DAP; LL-DAP were not available. The whole cell hydrolysates of the isolates if any could not be confirmed**

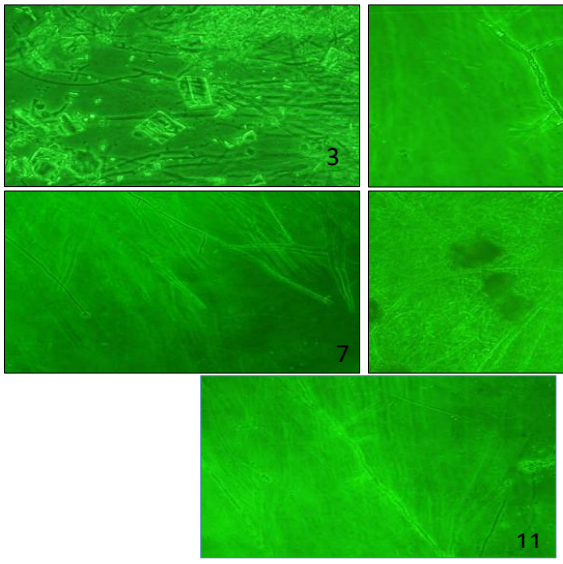


**Figure 1: Morphological characteristics of test isolates observed under 40X objective of phase contrast microscope by slide culture technique**

## CONCLUSION

The current study demonstrated the potential of 4 different Streptomyces species and a fungal culture, isolated from different soil samples, to be a potent antibiotic producer. Interestingly, these isolates can be targeted towards bacterial as well fungal MDR pathogens causing RTIs. Hence the antibiotics produced by the isolated Streptomyces species may be extremely helpful in treatment of complex infections and in emergency cases where the etiological agents are not known.

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