

## SELECTIVE ISOLATION OF RARE ACTINOMYCETES PRODUCING NOVEL ANTIMICROBIAL COMPOUNDS

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### ABSTRACT

Microbial pathogens are developing resistance against existing antibiotics, stressing the urgency for discovery of new therapeutic compounds. Actinomycetes, alone produce 70-80% of the available antibiotics. The chances of isolating undiscovered strains from the terrestrial habitats have diminished so that the search for novel products has switched to rare genera of actinomycetes from normal habitats or to discovery of strains/species found in unusual habitats. Rare genera of actinomycetes can be selectively isolated using various physical and chemical pre-treatment methods. Although preliminary identification of strains can be made on the basis of morphology, yet reliable classification of actinomycetes may not be possible using traditional approaches based on morphological and physiological features alone. Therefore rapid molecular methods including Restriction fragment length polymorphism (RFLP), Pulse field gel electrophoresis (PFGE), Amplified ribosomal DNA restriction analysis (ARDRA), Random amplified polymorphic DNA (RAPD) and genus specific primers would be extremely useful to discern between the rare genera of actinomycetes from the common ones. Primary and secondary screening of isolates is done to determine their antimicrobial potential both qualitatively and quantitatively. Chemical structures of active metabolites are ascertained using chromatographic and spectrophotometric techniques. Thin layer chromatography (TLC) helps in determining the  $R_f$  values of the compound fractions while Bioautography helps in pinpointing the exact bioactive fraction(s). Nowadays, TLC and spraying reagent techniques have been replaced by more efficient techniques for frequent detection of antibiotics. Databases are available for retention times and UV-visible absorbance spectra of standards. Therefore, culture filtrates and/or extracts from broth cultures of novel actinomycetes can be compared with those of databases to determine the novelty of compounds and to avoid rediscovery of already known compounds.

**Keywords:** Rare actinomycetes, Antimicrobial compounds, Traditional and Molecular approaches, Screening methods, Chromatographic analyses, Spectrophotometric analyses

### I. Introduction

A large number of actinomycetes have been isolated and screened from soil in the past several decades, accounting for 70-80% of relevant secondary metabolites available commercially [1]. Consequently the possibility of isolating novel actinomycete strains from the terrestrial habitats have diminished so that the search for novel products has switched in emphasis to rarer genera of actinomycetes or to well characterized ones that are found in unusual environments. The logic behind these approaches is that such strains may be producers of novel bioactive compounds. Microbial screening programs have started

taking into account the ecological significance of antibiotic producing microorganisms [2-4].

It is difficult to isolate rare actinomycete genera by using conventional isolation methods. Novel genera can be isolated taking into account several factors during the isolation procedure, such as the selection of ecological habitats for sample collection, chemical and physical pre-treatment of the samples, use of specific selective media, fine-tuning of culture conditions and genus-specific methodologies for screening of isolates [5,3].

After isolating an actinomycete, it is initially identified on the basis of morphological characters so as to have a preliminary

determination of the genus. Actinomycetes can be observed under the light microscope using coverslip culture [6-7] and slide culture techniques [8]. Strains are observed for several characters such as presence or absence of aerial mycelium, fragmentation or non fragmentation of substrate and aerial mycelium, presence of sclerotia, spore chain morphology and color of spore mass [8].

It is important to avoid strain duplication by an accurate identification of isolates. However taxonomic characterization based only on morphological and biochemical characteristics, is tedious and not always reliable [9]. There is therefore a need to develop molecular methods that used in conjunction with the earlier techniques would help in differentiating between the rare and common genera of actinomycetes [10].

A number of nucleic acid fingerprinting molecular methods have been developed to differentiate between related species and strains of actinomycetes. Restriction fragment length polymorphism (RFLP) of genomic DNA followed by pulse field gel electrophoresis (PFGE) is useful for identifying genetic variation among microorganisms [11]. Amplified ribosomal DNA restriction analysis (ARDRA) is the derivative of RFLP and employs digestion of amplified ribosomal DNA with different restriction enzymes. This method is an accurate and rapid strain identification tool for establishing relationships between closely related actinomycete strains [12]. The random amplified polymorphic DNA (RAPD) is a PCR based technique which can detect DNA polymorphism using a single primer of arbitrary nucleotide sequence in the absence of specific nucleotide sequence information. This method is a powerful tool for eliminating duplicate strains in microbial screenings [13]. Rare actinomycetes are isolated infrequently, due to their low occurrence in the natural ecosystems. Availability of genus specific oligonucleotides have been extremely important for the rapid detection of several rare genera including *Actinoallomurus*, *Actinopolyspora*, *Amycolatopsis*,

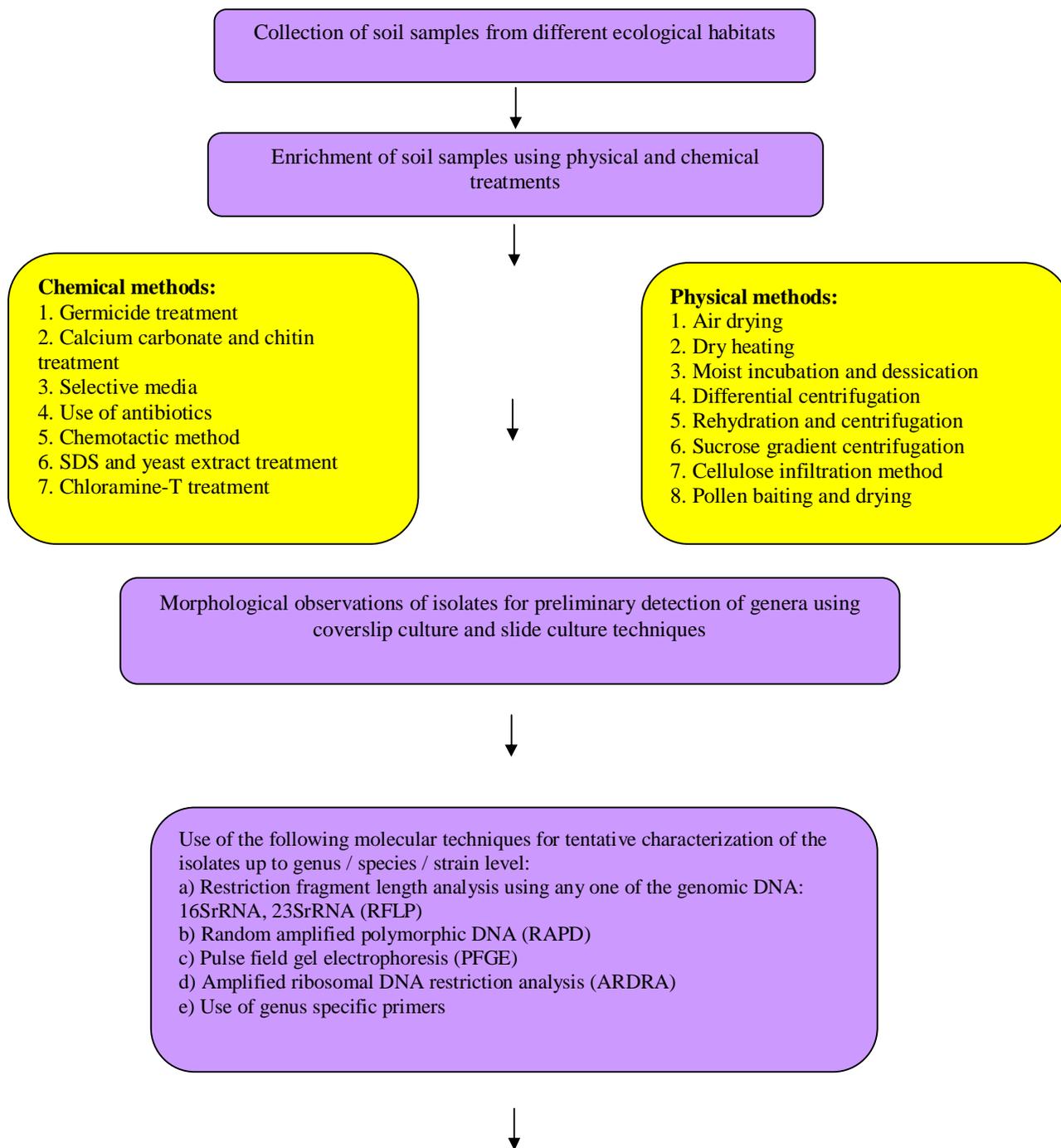
*Dactylosporangium*, *Gordonia*,  
*Micromonospora*, *Nocardiopsis*,  
*Saccharomonospora*, *Saccharopolyspora*,  
*Saccharothrix*, *Streptomonospora*,  
*Streptomyces* [14-24]

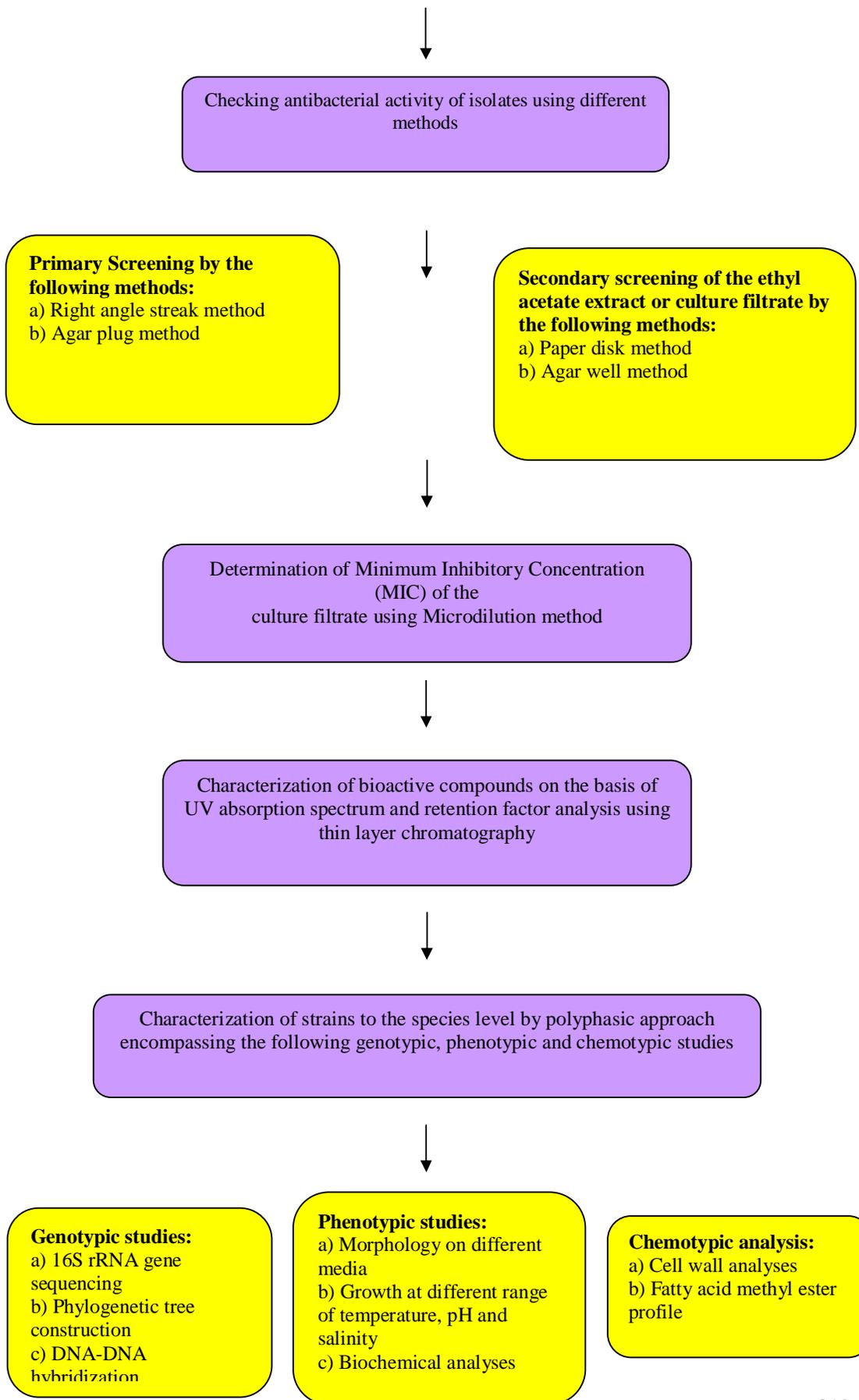
Actinomycetes are among the largest producers of commercially important metabolites. The antimicrobial potential of isolates is tested by primary screening techniques including cross streak method, right angle streak method, and agar plug method [25-27]. Selected strains are subjected to secondary screening methodologies to quantify activities of the antimicrobial metabolites. Paper disk method [28] and agar well method [29-30, 26] are used for secondary screening using either the active culture filtrate or the organic solvent extracts of the culture broths. The screening method may also be extended to determine the minimum inhibitory concentrations (MICs) of the active fractions against a range of indicator microbes [31-32]. Thin layer chromatography (TLC) helps in determining the  $R_f$  values of the compound fractions while Bioautography helps in pinpointing the exact bioactive fraction(s) [33-35]. Verification of retention factor values and analyses of absorbance maxima of the active metabolites can be done using the techniques of high performance liquid chromatography (HPLC) and Spectrophotometry [36-37].

The commercially relevant strains are finally characterized by molecular and traditional techniques encompassing genotypic, phenotypic and biochemical studies. These include 16S rRNA sequencing, phylogenetic tree construction, DNA-DNA hybridization, morphological studies on different media, growth at different ranges of temperature, pH and salinity, biochemical analyses, cell wall analyses and fatty acid methyl ester analyses [38-39]

The following flow chart highlights the sequence of events followed for the selective isolation of rare genera of actinomycetes with potential commercial importance

Figure 1: Steps followed for the selective isolation of rare actinomycetes with potential commercial importance.





Soil actinomycetes are prolific producers of antibiotics. However, this group of microbes has been tapped to such an extent that the chances of isolating a novel strain with a unique metabolite producing capability are slim [40]. A useful approach to address this problem is to explore unusual ecosystems such as sediments from deep sea water, hyper saline areas, hot sulfur springs, glacier regions to name a few, as a source of novel actinomycetes. Microorganisms from such unusual environments are expected to be potential producers of novel and useful secondary metabolites [41]. The rationale behind such strategies is to increase the probability of discovering novel chemical moieties by screening rare actinomycetes which are under-represented in the previous natural product screening programs [42]. A compilation of actinomycetes isolated from diverse ecological habitats has been presented in Table 1.

## **II. Actinomycetes isolated from unusual environments**

Marine environment represents one such largely untapped ecosystem from which rare actinomycete genera having a potential for producing novel metabolites have been discovered [4, 43-44]. Actinomycetes can be isolated from alkaline and acidic environments and have the potential of producing novel enzymes, enzyme inhibitors and antibiotics [45-46]. Rare actinomycete genera can also be isolated from activated sludge scum. Sewage and fecal samples from various sources are an important repository of actinomycetes [47-48]. Thermophilic bacteria can be collected from many environments like hot sulphur springs, solar-heated soils, sites of hot industrial discharges, desert soils, coal mining or compost pits [49-52]. In contrast are the psychrophilic microbes isolated from glacial samples that are known to be producers of novel bioactive compounds [53]. Radiation resistant actinomycetes occur in various irradiation habitats [54]. Novel actinomycetes have been isolated from the surfaces of acidic and heavy metal galleries of mining areas [55-57].

Diversity of microbes in the vicinity of cultivated plants is a direct result of their interaction with and adaptation to the toxins produced by the plants. Result is that these microbes have specific secondary metabolite producing profiles. Rare actinomycetes have been reported from rhizosphere of plants, agricultural soils, preserved areas, forest soils and uncultivated arid soils [58-59, 4]. Pathogenic actinomycete strains have also been isolated from animals and humans [60-61].

The use of MDR pathogens against known antibiotics makes it imperative to isolate rare genera and under exploited species of common actinomycete genera since they have the potential to produce novel metabolites. But it is difficult to isolate rare actinomycetes by conventional dilution plate methods. As a result isolation methodologies now employ a combination of physical and chemical pretreatment techniques and use of genus specific selective media. As a consequence of these treatments, there is elimination of non filamentous bacteria from samples, suppression in growth of the rapidly proliferating common *Streptomyces* species that in turn promotes growth of slow growing rare actinomycetes [62-67]

## **III. Physical and chemical pretreatment methods for selective isolation of actinomycetes**

Following is a brief summary of the various physical and chemical pretreatment methods applied on soil samples to facilitate isolation of rare actinomycetes as well as new species of *Streptomyces*:

Spores of some rare actinomycete genera including *Streptosporangium* and *Microbispora* can withstand treatment with sporicidal chemicals like phenol, benzethonium chloride and chlorhexidine gluconate. On the other hand, spores of the common actinomycetes succumb to the same treatment. Consequently the above mentioned chemicals can be easily used for the selective isolation of rare actinomycetes [68-69]. Addition of calcium carbonate and chitin to the growth medium has also been known to enhance actinomycete growth. These inorganic/organic substances can

be utilized by actinomycetes as sources of carbon and nitrogen [6, 70-71]. Appropriate selective media containing macromolecules like casein, chitin, humic acid are widely used for promoting growth of rare actinomycetes present in the soil samples and simultaneously suppressing/hindering the contaminant bacterial/fungal colonies [68, 50, 69, 17, 72]. Addition of antibacterial and antifungal antibiotics such as anisomycin, cycloheximide, gentamicin, kanamycin, nalidixic acid, novobiocin, nystatin, penicillin, primaricin, polymyxin, rifampicin, streptomycin, tunicamycin and vancomycin to the isolation media enhances the selection of members of the family actinomycetales [69,17,72]. Selective isolation of sporulating actinomycetes known to produce motile spores is done by the use of xylose, chloride,  $\gamma$ -collidine, bromide and vanillin. These substances act as chemoattractants for accumulating spores of *Actinoplanes*, *Dactylosporangium* and *Catenuloplanes* [5]. Treatment of soil suspension with sodium dodecyl sulphate (SDS) and yeast extract increases the count of actinomycete colony forming unit (cfu's) and decreases the count of the contaminating microbes. The latter can be further decreased by adding any antifungal antibiotic for instance nalidixic acid into the isolation medium. A combination of yeast extract and heat treatment enhances germination of spores of rare actinomycetes. Simultaneously, SDS also acts as a germicide for the non-actinomycete cells while nalidixic acid suppresses growth of fungal spores without affecting the actinomycetes [69,3]. Selective isolation of genera *Herbidospira*, *Microbispora*, *Microtetrastpora* and *Streptosporangium* can be done by chloramine treatment of soil suspensions. Chlorination is known to suppress growth of contaminant bacteria and promotes the growth of these rare microbes when plated on humic acid-vitamin enriched media [69]. When soil is air-dried, other bacterial colonies are suppressed while the growth of the slow-growing actinomycete colonies is enhanced [5]. The aerial spores of most actinomycete genera are found to resist desiccation and show a

slightly higher resistance to dry heat compared to the resistance shown by Gram negative bacteria. Pre-treatment of soil by drying and heating stimulates the isolation of rare actinomycetes by eliminating most unwanted gram negative bacteria [69,70,73]. Actinomycetes producing motile zoospores can be separated from non motile genera including *Streptomyces* with the help of differential centrifugation [3,74]. Soil samples are treated with phosphate buffer to liberate zoospores. Streptomycetes and other non-motile actinomycetes are eliminated by centrifugation that facilitates selective isolation of motile actinomycetes, which are thereby retained in the supernatant and can be spread on appropriate medium such as HV agar containing nalidixic acid and trimethoprim [5]. Sucrose gradient centrifugation is a new method that can be used for selective isolation of certain actinomycete genera. Yamamura et al. (2005) [48] have used the sucrose gradient centrifugation for selective isolation of *Nocardia* spp. In conjunction with the use of HV agar containing cycloheximide, chlorotetracycline and nalidixic acid. Selective isolation of actinomycetes can also be achieved by placing a suitable barrier between the growing bacterial colonies and the agar surface which would allow only the filamentous actinomycetes to penetrate to the underlying agar while retaining other bacteria on the filter surface [5]. Pollen grains of pinus act as useful baits for isolating certain actinomycete genera including *Actinoplanes*. These bacteria colonize the pollens floating on the surface of soil suspension. Contaminants are eliminated by desiccation of sporangia and the spores are liberated upon immersion in water. Appropriate volume of the enriched zoospore sample is plated on HV agar containing nalidixic acid [68,5]. On the other hand glass rods coated with paraffin wax have been used as bait for selective isolation of *Nocardia* [75]. Details of the various physical and chemical pretreatment methods have been presented in Table 2.

#### **IV. Morphological identification of isolates**

Microbes are largely characterized on the basis of their morphological characters. The macroscopic and microscopic studies of an actinomycete growing on agar can provide useful and rapid clues for identification of their respective genus. Macroscopic characters include colony characteristics such as size, shape, color, consistency on different media, the absence or presence of aerial mycelium and extent of spore formation. Cultures are observed for microscopic features including fragmentation or non fragmentation of substrate and aerial mycelium, presence of sclerotia, spore chain morphology and spore surface ornamentation. On the basis of spore chains, the strains can be placed into groups. For example, the species belonging to the genus *Streptomyces* are divided into three groups broadly i.e. rectiflexibiles, retinaculiperti and spirales. Characteristics of the spore bearing hyphae and spore chains can be determined by light microscopy using coverslip culture [6,7] and slide culture techniques [8]. Actinomycetes are also observed by the phase-contrast microscopy for study of spore surface ornamentation [7]. Genera of purified isolates can be identified based on morphological comparisons to the existing description of known genera as given in Bergey's Manual of Determinative Bacteriology [76].

#### **V. Molecular techniques for selective isolation of actinomycetes**

##### **A. Limitations of strain identification based on morphology and chemical tests**

In microbial screening programs, a large number of isolates are screened to increase the probability of finding novel antimicrobial compounds, since it is generally known that morphologically similar appearing strains have more chances of producing the same secondary metabolites compared to morphologically distinct strains [77]. Therefore strain discrimination is important to save efforts, time and resources. However, there are limitations in the use of traditional morphological methods

alone for identifying strains. Firstly, some genera of actinomycetes do not produce distinct aerial mycelium or show specific coloration, therefore morphological analysis of such strains would not help in their distinction as separate genera. Secondly, many actinomycete genera show morphological variation at different stages of growth when cultured on various media causing confusion during their taxonomic identification [78]. As a result, reliable identification of actinomycete genera may not be possible. Thirdly, different strains of actinomycetes belonging to the same genus appear morphologically alike on the isolation plates and would be eliminated during screening programs decreasing the chances of recovering potentially useful isolates [77].

Furthermore, the traditional biochemical methods and chemotypic studies including cell wall analyses, polar lipids analyses and fatty acid methyl ester analyses which are used for the identification of the aerobic filamentous actinomycetes require extensive labor and time. In many cases such methods cannot identify an isolate to the level of a single genus [9].

Another strategy that should be adopted during screening programs is to avoid rediscovery of known compounds derived from the commonly occurring soil *Streptomyces* species [1]. *Streptomyces* spp. have been extensively screened and exploited for secondary metabolite production [1,80] Therefore, the probability of isolating novel antimicrobial compounds from species of *Streptomyces* is very low. The chances of isolating new bioactive compounds from rarer, non-streptomycete actinomycetes appear more promising [80,69,16]. Therefore methods which can distinguish *Streptomyces* from morphologically similar actinomycetes as well as identify rare actinomycetes up to the genera level would be extremely useful [81,10].

##### **B. Typing studies based on RFLP-PFGE approach**

A number of nucleic acid fingerprinting molecular studies have been developed to differentiate between closely related species and strains belonging to the same genus.

Restriction Fragment Length Polymorphism (RFLP) is a typing method for creating a restriction profile of the DNA based on random distribution of restriction sites in the genome. Pulsed Field Gel Electrophoresis (PFGE) then size fractionates the generated genomic fragments. DNA fingerprint of every strain/species is distinct depending on the specificity of the restriction enzyme used and bacterial genome sequence [82,78,81]. Chromosomal aberrations such as DNA deletions, insertions, or rearrangements within a particular strain accumulated over time can be detected by DNA fingerprinting studies [82]. Relationships among strains of different *Streptomyces* species have been determined using RFLP methods. One limitation of this method however is, that often complex fingerprints are generated due to presence of a large number of low molecular weight fragments hampering elucidation of taxonomic relationships. Low Frequency Restriction Fragment Analysis (LFRFA-PFGE) is a technique that can be used for analysing large DNA fragments useful in identification of microbial genetic variation [11].

### C. ARDRA as a molecular typing tool

An important derivative of RFLP is Amplified Ribosomal DNA Restriction Analysis (ARDRA) that employs digestion of amplified rDNA with different restriction enzymes for typing of microbes [38]. ARDRA is useful in taxonomic identification at strain/species level [97,113]. Medically important species of aerobic actinomycetes belonging to the genera *Actinomadura*, *Actinoplanes*, *Gordonia*, *Nocardia*, *Rhodococcus*, *Saccharomonospora*, *Saccharopolyspora*, *Streptomyces* and *Tsukamurella* have been identified using ARDRA [58]. A method has been used by Muharram et al., 2010 [81] by means of which environmental *Streptomyces* isolates can be rapidly identified by restriction digestion of amplified 16SrDNA using selected restriction endonucleases. Non-streptomycete species can be identified rapidly to a specific genus or a small subgroup of genera. Strains can also be

distinguished by analysis of conserved regions in the 16S and 23SrRNA genes flanking the 16S-23SrDNA spacer region [83].

### D. Typing studies based on RAPD approach

A method is required for elimination of duplicate strains when screening a large number of isolates for search of new metabolites. The random amplified polymorphic DNA (RAPD) method is a PCR based DNA polymorphism detection system in which a single primer of arbitrary nucleotide sequence is used for random amplification of DNA segments. Sequence similarity at the 3' end of the primers allows their binding at the complementary sequence sites in the target DNA producing a species specific pattern of amplification bands. DNA polymorphisms can be detected using these primers even in the absence of specific nucleotide sequence information. RAPD is a simple, rapid and inexpensive method for comparative genomic fingerprinting of a large number of isolates and for identification of conserved regions of genomes [11,84,78]

### E. Use of Genus-specific primers in typing studies

Members of actinomycete genera *Saccharomonospora*, *Pseudonocardia*, *Saccharopolyspora* and *Streptomonospora* occur infrequently in the environment. Their isolation and characterization on the basis of morphological, biochemical and chemotaxonomic techniques has limitations. As a result, genus-specific oligonucleotides have been designed for the rapid identification of these genera and several other genera including *Actinoallomurus*, *Actinopolyspora*, *Amycolatopsis*, *Dactylosporangium*, *Gordonia*, *Micromonospora*, *Nocardiopsis*, *Pseudonocardia*, *Saccharomonospora*, *Saccharopolyspora*, *Saccharothrix*, *Streptomonospora*, *Streptomyces* [49,15-24] (Table 3). Nucleotide sequence, especially of the 16SrRNA genes are analyzed for a wide range of collection and wild type strains

belonging to a particular genus and the conserved regions are used for the construction of genus specific primers. Such primers are subsequently used for direct identification of members of rare genera and in assessing their occurrence in various habitats.

#### **V. Antimicrobial analysis of isolated actinomycetes**

Microbial metabolites account for a majority of drugs available in the market [80]. Among bacteria, Actinomycetes are known for the production of different classes of antibiotics including aminoglycosides, anthracyclins, glycopeptides,  $\beta$ -lactams, macrolides, nucleosides, peptides, polyenes, polyethers, terpenes and tetracyclines [85] which possess wide range of biological activities [80,43]. The genus *Streptomyces* particularly is a prolific producer of secondary metabolites with several of its species having been isolated and screened from soil extensively [86-88]. Consequently, it is difficult to isolate novel *Streptomyces* from the soil. Emergence of multidrug resistant pathogens has triggered the need for discovery of new antibiotics with unique modes of action as exemplified by the following case studies [89]. Emergence of methicillin-resistant *Staphylococcus aureus* causes serious infections. Vancomycin was earlier used to circumvent the problem of methicillin resistant *Staphylococcus aureus*, but unfortunately vancomycin resistant *S. aureus* have been reported in hospitals [63]. Penicillin, ampicillin, streptomycin or gentamicin are used in various combinations for the treatment of infections caused by enterococci. Some strains have developed resistance to aminoglycosides,  $\beta$  lactam antibiotics and it leads to the failure of combination therapy [64-66]. Occurrence of Penicillin resistant *Streptococcus pneumoniae* (PRSP) is on rise worldwide [67]. MDR-tuberculosis strains exist owing to development of resistance by the causative agent towards important anti-TB drugs, including isoniazid and rifampicin [62]. Effective treatment of the infections caused by these organisms is yet to

be established. Thus, the need for the discovery and development of new and effective antimicrobial agents is a priority.

Actinomycetes from terrestrial habitats have been screened and characterized extensively. Therefore, rarer genera of actinomycetes or those from unusual environments need to be isolated stressing the consideration of microbial ecology and their role in natural ecosystems during formulation of strategies for novel antibiotic discovery [2-4]. The rationale for these approaches is that these isolates may produce novel metabolites. Actinomycete colonies isolated from varied ecological habitats are analyzed for biosynthesis of new antibiotics by the following screening methodologies.

#### **Primary screening**

Primary screening of isolates is done to ascertain the potential of strains with respect to production of antimicrobial secondary metabolites. During primary screening process a large number of isolates are screened against a range of sensitive strains. On the basis of primary screening results, isolates showing substantial antimicrobial activities are selected for subsequent secondary screening programs. The isolates are subjected to the bioassay techniques of Cross streak and Agar plug tests. Modifications of these procedures are being routinely used in different labs:

**1) Cross streak method-** Actinomycete isolate is inoculated on the center of the appropriate medium plate and incubated for approximately a week at 28-30°C. Later the plates are seeded with sensitive organisms in a radial arrangement around the actinomycete strains streaked like spokes of a wheel. The microbial interactions can be analyzed by the size determination of the inhibition zones. Bioassay of isolates for inhibitory action can also be carried out by using a modified cross streak method. The sensitive organisms are streaked at right angles to the line of growth of the producer isolate inoculated towards one side of the plate and grown for a week at 28-30°C. After appropriate incubation, the extent of

inhibition of the various sensitive organisms can be measured [26,27].

**2) Agar plug method-** The isolate is inoculated on an agar plate. Culture growth, restricted towards one end of the plate is allowed to occur for a week. Radial agar plugs are cut out from the culture plate and placed atop a second agar plate already over-layered with a known concentration of sensitive strain. Inhibition zones of the pathogenic strains around the plugs can be measured within 24-48 hours for bacteria and yeasts and on the 7<sup>th</sup> day for phytopathogenic fungi [25,30,90]. In order to perform a double check, the actinomycete culture plate (from which the plug was removed) can be subjected to the cross streak test. Known volume of the sensitive culture can be streaked along the row of wells and the extent of inhibition of the culture tract due to the presence of antibacterial metabolite can be noted.

Isolates are selected for secondary screening on the basis of their inhibition of at least two or more of the sensitive organisms during primary screening.

### Secondary screening

Active bioactive compounds are recovered from the culture broth by fermentation. Production of metabolite can be increased by optimizing the conditions during fermentation. After 7-10 days of incubation, the fermented broth is filtered through a membrane filter (0.20-0.45 $\mu$ m pore size) or Whatman No.1 filter to separate the cellular components from the culture filtrate and it can be either directly used for the determination of antimicrobial activity against the sensitive organisms or subjected to the following treatment.

Bioactive compounds can be recovered from filtrate by organic solvent extraction method. Culture supernatants are extracted with an equal volume of appropriate organic solvent like ethyl acetate, methanol or n-butanol for 1-2 hours [91-96]. The organic fraction obtained is allowed to evaporate under vacuum to dryness using a rotary evaporator at 70°C-90°C to

remove all traces of the organic solvent. The residue obtained is weighed; a stock solution of known concentration is prepared and used for antimicrobial analyses, minimum inhibitory concentration and to perform bioautography.

Secondary screening is performed by the disk activity assay and by the agar well diffusion method against standard pathogenic organisms:

**1) Disk activity method-** This method can be used for comparative antimicrobial analyses of various bacterial fermented broths [28]. Sterile paper disks are loaded with known concentrations of bioactive compounds or defined volume of culture filtrate and placed on a lawn of sensitive strain seeded plate. Relative activities of different bioactive compounds can be determined from diameter of the zones of inhibition.

**2) Agar well diffusion method-** In this method, wells are aseptically bored in sensitive strain seeded plate. The wells are filled with defined volumes of culture filtrates or with the organic solvent extracted broths. The relative activities of metabolites are determined based on the diameter of zones of inhibition formed in the adjacent solidified medium [29,30,26].

Secondary screening of potent actinomycetes is done to confirm the results of primary screening. It is possible that isolates show non-corresponding activities in primary and secondary screening programs. Antimicrobial activities also vary on solid medium and in culture broth. Isolates which show activities on solid medium may not show activities in liquid medium or vice versa. Therefore, potential of isolates for antimicrobial production should be tested on both solid and liquid medium [97-98]].

The screening method may be extended to determine the minimum inhibitory concentrations (MIC) of the active fractions against a range of indicator microorganisms.

### 3) Minimum inhibitory concentration (MIC) test

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an

antimicrobial compound that inhibits the visible growth of a sensitive strain after the appropriate incubation period. Minimum inhibitory concentrations (MICs) of antimicrobial agents are determined, *in vitro*, by means of agar and broth dilution methods [31,32]. In this test, pathogenic strains are tested for their ability to show visible growth on a series of agar plates (agar dilution) or in microplate wells of broth (broth microdilution) containing suitable dilutions of the antimicrobial agent [31]. The MIC endpoint is recorded after an overnight incubation as the lowest concentration of residue at which there is no visible growth of the pathogenic strain.

### Characterization of bioactive compounds

Bioactive compounds are characterized by using the techniques of chromatography and spectrophotometry. Paper or thin layer chromatography (TLC) is used to separate the metabolites produced by the strain and then visualized under UV light (254 and 365 nm) or by staining with reagents such as Anisaldehyde/H<sub>2</sub>SO<sub>4</sub>, Hydroxyl ammonium-Fe(III) chloride, Iodine, Orcin-Fe(III) chloride, Vanillin/H<sub>2</sub>SO<sub>4</sub> to name a few that helps in visualizing the complete secondary metabolite pattern of an actinomycete strain [27,35]

Chromatographic techniques followed by bioautography are very useful in the identification of bioactive fractions from the metabolite mixture [94,34]. Novelty of bioactive molecules can be determined by comparing their R<sub>f</sub> values with those of known antibiotics from databases to eliminate the well characterized compounds during the screening programs. In this way, already characterized compounds can be eliminated from the further studies. R<sub>f</sub> values of some antibiotics determined by means of thin layer chromatography are depicted in Table 4. Nowadays, TLC and spray reagent techniques have been replaced by a more efficient reversed-phase HPLC technique coupled with computerized diode-array detection [99,36]. Culture filtrates and raw extracts of actinomycete strains are analyzed under

standardized HPLC conditions, running commercially available antibiotics alongside as reference compounds. HPLC-UV-Vis Databases are created and maintained in which retention times and UV-visible absorbance spectra of standards (Table 5) are stored and compared to test samples to determine the novelty of compounds [99,100].

### Conclusions:

Infectious diseases are the leading health problems in developing countries. The advent of MDR resistant pathogens is a major obstacle in the treatment regime of these diseases. Multidrug resistance is currently an urgent focus of research and new bioactive compounds are necessary to combat the MDR microbes. Attempts therefore are being made continuously to develop novel drugs against infectious diseases. Actinomycetes are widely reported for production of several antibiotics which are used therapeutically. Soil actinomycetes have been screened extensively for discovery of new antibiotics, increasing the chances of re-isolation of known compounds. It is therefore important that a new group of rare and uncommon actinomycetes from unusual ecosystems should be explored as a potential source of new therapeutic compounds. Isolation of rare actinomycetes is difficult by the application of conventional isolation procedures. Various types of physical and chemical pretreatment methodologies have been devised for isolating desirable rare actinomycete genera. The use of these genus oriented methods in industrial screening programmes has provided a significant impetus towards the discovery of new bioactive compounds. A large number of antimicrobial compounds have been isolated from *Streptomyces* spp. while rare and novel species of this genus are expected to contain as yet undiscovered bioactive metabolites. Different molecular approaches can also be used to rule out the commonly occurring *Streptomyces* species. These techniques include ARDRA, RFLP-PFGE, RAPD and genus specific primers. R<sub>f</sub> values and absorption spectrum of culture filtrates and/or extracts from broth

cultures of novel actinomycetes are compared with those of already discovered antimicrobial compounds. This is done to circumvent the problem of recharacterization of known bioactive molecules and to help in screening novel compounds. Use of the above mentioned strategies should make feasible the discovery of novel antimicrobial compounds.

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S.No.	Actinomycete	Habitat
1.	<i>Actinomadura</i> , <i>Actinosynnema</i> , <i>Amycolatopsis</i> , <i>Arthrobacter</i> , <i>Frankia</i> , <i>Geodermatophilus</i> , <i>Gordonia</i> , <i>Kitasatospora</i> , <i>Micromonospora</i> , <i>Mycobacterium</i> , <i>Nocardioides</i> , <i>Nocardiopsis</i> , <i>Nonomurea</i> , <i>Psuedonocardia</i> , <i>Rhodococcus</i> , <i>Saccharopolyspora</i> , <i>Salinispora</i> , <i>Streptomyces</i> , <i>Streptosporangium</i> , <i>Tsukamurella</i> , <i>Verrucosipora</i> , <i>Williamsia</i>	Marine
2.	<i>Actinomadura</i> , <i>Amycolatopsis</i> , <i>Bogoriella</i> , <i>Kribbella</i> , <i>Microbispora</i> , <i>Micromonospora</i> , <i>Nocardia</i> , <i>Nonomurea</i> , <i>Nocardiopsis</i>	Acidic and alkaline soils
3.	<i>Bifidobacterium</i> , <i>Rhodococcus</i> , <i>Nocardia</i> , <i>Gordonia</i>	Sewage and fecal samples
4.	<i>Actinomadura</i> , <i>Micromonospora</i> , <i>Microtetraspora</i> , <i>Saccharomonospora</i> , <i>Streptomyces</i> , <i>Thermoactinomyces</i>	Hot springs
5.	<i>Actinomadura</i> , <i>Amycolatopsis</i> , <i>Microbispora</i> , <i>Microtetraspora</i> , <i>Nocardia</i> , <i>Nocardiopsis</i> , <i>Saccharothrix</i>	Desert
6.	<i>Amycolatopsis</i> , <i>Laceyella</i> , <i>Planifilum</i> , <i>Saccharomonospora</i> , <i>Streptomyces</i> , <i>Thermoactinomyces</i> , <i>Thermobifida</i> , <i>Thermoactinomyces</i> , <i>Thermomonospora</i> , <i>Thermus</i>	Compost piles
7.	<i>Arthrobacter</i> , <i>Friedmanniella</i> , <i>Modestobacterium</i> , <i>Nocardia</i> , <i>Nocardiopsis</i> , <i>Pseudonocardia</i> , <i>Streptomyces</i>	Glacial samples
8.	<i>Streptomyces</i>	Radiation treated
9.	<i>Amycolatopsis</i> , <i>Arsenicicoccus</i> , <i>Catellatospora</i> , <i>Fodinicola</i>	Mining fields
10.	<i>Actinoplanes</i> , <i>Couchiplanes</i> , <i>Catenuloplanes</i> , <i>Microbispora</i> , <i>Saccharopolyspora</i>	Agricultural fields
11.	<i>Amycolatopsis</i> , <i>Gordonia</i> , <i>Nocardia</i> , <i>Rhodococcus</i>	Clinical samples of humans and animals

**Table 1** Actinomycetes isolated from diverse ecological habitats

## SELECTIVE ISOLATION OF RARE ACTINOMYCETES PRODUCING NOVEL ANTIMICROBIAL COMPOUNDS

S.No.	Treatment	Genera selected
Chemical treatment		
1.	Phenol, benzethonium chloride and chlorhexidine gluconate	<i>Microbispora</i> , <i>Streptomyces</i> , <i>Streptosporangium</i>
2.	Quaternary ammonium compounds	<i>Mycobacterium</i> , <i>Rhodococcus</i>
3.	Calcium carbonate, chitin	<i>Streptomyces</i>
Isolation Medium		
4.	LSV-SE agar	<i>Microtetraspora</i>
5.	Humic acid vitamin agar (HV agar) with Chloramine T treatment	<i>Herbidospora</i> , <i>Microbispora</i> , <i>Microtetraspora</i> , <i>Streptosporangium</i>
6.	Hair hydrolysate vitamin agar	<i>Actinoplanes</i> , <i>Microbispora</i> , <i>Micromonospora</i> , <i>Streptosporangium</i>
7.	Sodium propionate, Phenol treatment, Chitin medium	<i>Streptomyces</i>
8.	Charcoal-yeast extract medium with polymyxin, anisomycin, and vancomycin	<i>Nocardia</i>
9.	HV agar containing nalidixic acid with SDS and yeast extract treatment	<i>Actinomadura</i> , <i>Microbispora</i> , <i>Micromonospora</i> , <i>Microtetraspora</i> , <i>Streptomyces</i> , <i>Streptosporangium</i> , <i>Nocardia</i>
Antibiotics		
10.	Nalidixic acid, Penicillin G	<i>Rhodococcus</i>
11.	Kanamycin, nalidixic acid, trimethoprim	<i>Actinokineospora</i>
12.	Gentamicin	<i>Streptosporangium</i> , <i>Actinomadura</i> , <i>Micromonospora</i>
13.	Novobiocin	<i>Actinoplanes</i> , <i>Thermoactinomyces</i>
14.	Penicillin, Nalidixic acid	<i>Saccharothrix</i>
15.	Rifampicin, Streptomycin, Kanamycin	<i>Actinomadura</i>
16.	Tunicamycin	<i>Micromonospora</i>
Chemotaxis		
17.	Xylose, chloride, $\gamma$ -collidine, bromide and vanillin, HV agar with nalidixic acid	<i>Actinoplanes</i> , <i>Catenuloplanes</i> , <i>Dactylosporangium</i>
Dry heat treatment		
18.	Dry heat treatment, HV agar with nalidixic acid and kanamycin	<i>Microbispora</i> , <i>Actinomadura</i>
19.	Dry heat, LSV-SE agar with kanamycin, nalidixic acid and norfloxacin	<i>Microtetraspora</i>
20.	Dry heat treatment, HV agar with nalidixic acid	<i>Spirilliplanes</i>
21.	Heat treatment, Gauze 1 medium with nystatin, nalidixic acid and rubomycin	<i>Actinomadura</i>
Centrifugation		
22.	Centrifugation, HV agar with nalidixic acid and trimethoprim	<i>Actinoplanes</i> , <i>Actinokineospora</i> , <i>Actinosynnema</i> , <i>Catenuloplanes</i> , <i>Cryptosporangium</i> , <i>Dactylosporangium</i> , <i>Geodermatophilus</i> , <i>Kineospora</i> , <i>Sporichthya</i>
23.	Sucrose gradient centrifugation, HV agar with nalidixic acid	<i>Nocardia</i>
Cellulose infiltration method		
24.	Cellulose infiltration method, Modified Bennett medium containing cycloheximide and canadacidin	<i>Streptomyces</i> , <i>Micromonospora</i> , <i>Nocardia</i> , <i>Actinoplanes</i>
Baiting		
25.	Pollen grains of Pinus	<i>Actinoplanes</i>
26.	Paraffin	<i>Nocardia</i>

**Table 2** Physical and chemical methods developed for the selective isolation of rare actinomycetes from soil

## SELECTIVE ISOLATION OF RARE ACTINOMYCETES PRODUCING NOVEL ANTIMICROBIAL COMPOUNDS

S. N.	Genus	Sequence of primers	
		Forward Primer	Reverse Primer
1.	<i>Amycolatopsis</i>	5'GTATCGCAGCCCTCTGTACCAGC 3'	5' GGTGTGGGCGACATCCACGTTGT 3'
2.	<i>Gordonia</i>	5' CGACCTGAGAGGGTGATCG 3'	5' ATAACCCGCTGGCAATACAG 3'
3.	<i>Nocardopsis</i>	5' TCTCTTGGGGTTGACGGTAG 3'	5' TAAATGACCTCACATCTCT 3'
4.	<i>Pseudonocardia</i>	5' GTGGAAAGTTTTTCGGCTGGGG 3'	5' GCGGCACAGAGACCGTGGAAAT 3'
		5' GTGGAAAGTTTTTCGGTGGGG 3'	5' GCGGCACAGAAAACCGTGGAAAT 3'
5.	<i>Saccharomonospora</i>	5' CGTCTGCCGTGAAAACCTGCGGC 3'	5' CGGCACGGGACACGTGMACAGC 3'
6.	<i>Saccharopolyspora</i>	5' GGTGACGGTAGGTGTAGAAG 3'	5' GTGGAACCCATCCCCACACC 3'
			5' GTGGAAMCAGTCCCCACACC 3'
7.	<i>Saccharothrix</i>	5' AAGGCCCTTCGGGGTACACGAG 3'	5' TCGACCGCAGGCTCCACG 3'
8.	<i>Streptomonospora</i>	5' TCTGTGCGGGTTGACGGTAG 3'	5' CGAAGGCACCCCTCCGTCTC 3'
9.	<i>Streptomyces</i>	5' ACAAGCCCTGGAAACGGGGT 3'	5' CACCAGGAATCCGATCT 3'
		5' ACAAGCCCTGGAAACGGGGT 3'	5' ACGTGTGCAGCCCAAGACA 3'

**Table 3** Genus specific primers for isolation of actinomycete genera

S.No	Antibiotic	Producing organism	Solvent system	R <sub>f</sub> value	Detection system	Biological activity
1.	Alborexlin (Poyether) [103-104]	<i>Streptomyces albus</i>	Ethy acetate:cyclohexane (40:60)	0.40	Sulphuric acid and heated to 110° C	Antibacterial
2.	Amphotericin B (Polyene) [105,103]	<i>Streptomyces nodosus</i>	Butanol:ethanol:acetone:32 % ammonium hydroxide (2:5:1:3)	0.41	UV	Antifungal
3.	Bluensomycin (Aminocyclitol) [103,106]	<i>Streptomyces hygrosopicus</i>	Chloroform:methanol:17% aq. ammonia (2:1:1)	0.01	Bioautography	Antibacterial
4.	Chalcomycin B (Macrolide) [107,103]	<i>Streptomyces bikiniensis</i>	Ethyl acetate (100)	0.33	Sulphuric acid and heated to 130° C	Antibacterial Antifungal
5.	Cycloheximide (Piperidine) [103,108]	<i>Streptomyces griseus</i>	Isopropanol:ethyl acetate (2:98)	0.7	Molybdic acid-ceric sulphate-sulphuric acid water (2.5:1.0:11.8:88 w/w/v/v)	Antifungal
6.	Glivocarcin V (Glycoside) [109]	<i>Streptomyces gilvotanareus</i>	Chloroform:methanol (9:1)	0.38	UV	Antitumor
7.	Kanamycin (Aminoglycoside) [103,110]	<i>Streptomyces kanamyceticus</i>	Chloroform:methanol:17% aq. ammonia (2:1:1)	0.75	Ninhydrin	Antibacterial
8.	Novobiocin (Aminocoumarin) [103,108]	<i>Streptomyces niveus</i>	Methyl ketone:ethyl acetate:water (9.3:2.6:1)	0.91	UV	Antibacterial
9.	Thienamycin (β-lactam) [111]	<i>Streptomyces cattleya</i>	Ethanol:water (70:30)	0.49	a) Bioautography b) Ninhydrin	Antibacterial
10.	Tylosin (Macrolide) [103,112]	<i>Streptomyces fradiae</i>	Ethyl acetate: methanol (85:15)	0.60	a) UV b) Saturated solution of silver sulphate in 10% sulphuric acid.	Antibacterial

**Table 4** R<sub>f</sub> values of different antibiotics in various solvent systems using thin layer chromatography.

## SELECTIVE ISOLATION OF RARE ACTINOMYCETES PRODUCING NOVEL ANTIMICROBIAL COMPOUNDS

S.No.	Antibiotic	Solvent	Absorption maxima (nm)
Amino acid Derivatives, oligo and polypeptide antibiotics			
Amino acid derivatives			
1.	Azaserine	-	250
2.	Duazomycin (Diazomycin A)	Buffer pH 7.0	245 and 275
3.	DON (6-diazo-5,oxo-L-norleucin)	Buffer pH 7.0	224 and 274
$\beta$ -lactam antibiotics			
4.	A(16886-11WS-3443-B)	Water	260
5.	Cefamycin A	HCl	287
6.	Cefamycin A	Water	220
7.	Cefamycin A	Water	285
8.	Cefamycin A	NaOH	280
9.	Cefamycin B	HCl	305
10.	Cefamycin B	Water	226
11.	Cefamycin B	Water	305
12.	Cefamycin B	NaOH	328
13.	Cefamycin C	Water	242
14.	Cefamycin C	Water	264
15.	Cephalosporin C	Water	250
16.	Desacetoxycephalosporin C	Water	260
17.	Deacetylcephalosporin C	Water	260
18.	7-Methoxycephalosporin C	Water	242
19.	7-Methoxycephalosporin C	Water	265
20.	7-Methoxydesacetylcephalosporin C	Water	241
21.	7-Methoxydesacetylcephalosporin C	Water	264
22.	SF-1623	Water	271
23.	WS-3442-D	Buffer pH 6.5	263
Polypeptide and cyclic polypeptide antibiotics			
24.	Capreomycin	Water	268
25.	Capreomycin	0.1 M NaOH	289
26.	Capreomycin IA	0.1 M NaOH	268
27.	Questiomycin A	0.1 M NaOH	237, 422 and 434
28.		Methanol	328, 428 and 589
29.	Quinomycins A-E	-	240-245 B/w 315 and 325
30.	Quinomycin D	Water	243 and 320
31.	Triostin A and B	-	240-245 B/w 315 and 325
Depsiptide antibiotics			
32.	Staphylomycin C	Ethanol	305
33.	Valinomycin	Hexane	281
Purine and Adenine nucleosides			
34.	Angustmycin A (Decoyinine)	Acid	260
35.	Angustmycin C (psicofuranine)	Acid	259
36.	Angustmycin C (psicofuranine)	Basic	261
37.	Aristeromycin	Neutral	212, 258
38.	Cordycepin	Neutral	258
39.	Cordycepin	Acid	259
40.	Cordycepin	Basic	260
41.	Puromycin	Neutral	266
42.	Puromycin	Acid	267

## SELECTIVE ISOLATION OF RARE ACTINOMYCETES PRODUCING NOVEL ANTIMICROBIAL COMPOUNDS

43.	Puromycin	Basic	275
44.	Spongoin	Neutral	267
45.	Spongoin	Acid	249, 274
46.	Spongoin	Basic	268
Pyrrolopyrimidine Nucleosides			
47.	Sangivamycin	Neutral	229
48.	Sangivamycin	Acid	228
49.	Sangivamycin	Basic	227
50.	Toyocamycin	Neutral	231
51.	Toyocamycin	Acid	232
52.	Toyocamycin	Basic	227
53.	Tubercidin	Acid	272
54.	Tubercidin	Basic	270
Pyrimidine Nucleosides			
55.	Amicetin	Neutral	305
56.	Amicetin	Acidic	316
57.	Amicetin	Basic	322
58.	Blasticidin S	Neutral	232
59.	Blasticidin S	Acidic	274
60.	Blasticidin S	Basic	266
61.	Polyoxins	Neutral	270-290
62.	Polyoxins	Acidic	275-290
63.	Polyoxins	Basic	262-271
Antibiotics with a naphthacene skeleton (Tetracyclines)			
64.	Chlorotetracycline	Water	230, 275, 367.5
65.	Chlorotetracycline	0.1 N HCl	230, 262.5, 267.5
66.	Chlorotetracycline	0.1 N NaOH	255, 285, 345
67.	Chlorotetracycline	0.1 N H <sub>2</sub> SO <sub>4</sub>	228, 265, 368
68.	Oxytetracycline	Methanol	270, 370
69.	Oxytetracycline	Methanol 0.01 N HCl	270, 359
70.	Oxytetracycline	Methanol 0.01 N NaOH	245, 264, 375
71.	Tetracycline	Methanol	269, 365
72.	Tetracycline	0.1 N HCl	220, 268, 355
73.	Tetracycline	0.1 N NaOH	225, 255, 285, 345
74.	Tetracycline	0.1 N H <sub>2</sub> SO <sub>4</sub>	218, 268, 355
Polyene and Polyene Antibiotics			
75.	Agrocybin	Methanol	216, 224, 269, 286, 304 and 325
76.	Isomycomycin	Ether	246, 257.5, 267, 287.5, 305.5, 324 and 347
77.	Mycomycin	-	256, 267 and 281
78.	Nemotin	Hexane	208, 208.5, 236.5, 249, 262.5 and 278
Macrolide antibiotics (Large Lactones)			
Twelve membered lactones			
79.	Methymycin	-	223-225 and 332
Fourteen membered macrolides			
80.	Antimycin A	Ethanol	225 and 321
81.	Elaiohylin	Methanol	252
82.	Erythromycin	Ethanol	289
83.	Picromycin (Albomycetin)	Ethanol	225 and 293
Macrocyclic Di and Tetralactone			
16 Membered macrolides			
84.	Carbomycin A	Ethanol	238 and 327
85.	Deltamycin A	Ethanol	240
86.	Leucomycin	Ethanol	230-232 and 285
87.	Spiramycin	Ethanol	232
Polyene macrolides			
Tetraene			

## SELECTIVE ISOLATION OF RARE ACTINOMYCETES PRODUCING NOVEL ANTIMICROBIAL COMPOUNDS

88.	Amphotericin A	-	291, 304, 318.5
89.	Natamycin (Pimaricin)	-	289.5, 302.5, 317.5
90.	Nystatin	-	291, 304, 318.5
Pentaenes			
91.	Flavomycoin	-	320, 337 and 355
92.	Surgumycin	-	317, 333 and 351
Heptaene antifungal antibiotics			
93.	Amphotericin B	-	363, 382, 406
94.	Aureofungin	-	359, 379, 402
95.	Candicidin	-	360, 379, 402
96.	Candidin	-	364, 383.5, 407.5
97.	Fungimycin (Perimycin)	-	361, 383, 406
98.	Hamycin	-	363, 383, 406
99.	Heptafungin	-	360, 380, 402
100.	Levorin A	-	356, 379, 398
101.	Levorin B	-	362,381,404
102.	Mycosheptin	-	362, 382, 406
103.	Trichomycin	-	361.5, 382, 404
104.	Trichomycin A	-	363, 384.5, 407
Ansa Macrolides			
105.	Gledanamycin	Methanol	225, 304 and 400
106.	Rifamycin B	Buffer pH 7.3	223,304 and 425
107.	Rifamycin SV	Buffer pH 7.3	223, 314 and 445
108.	Rifamycin W	-	540
109.	Streptovaricins	-	245, 260, 320 and 420
Oxygen containing Heterocyclic Compounds			
110.	Griseofulvin	Methanol	232, 292 and 324

**Table 5:** Absorption spectra of reference antibiotics [101-102]