ISOLATION AND EVALUATION OF SOIL ACTINOMYCETES FOR THEIR ANTIMICROBIAL ACTIVITY

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Abstract: Quick emergence of antibiotic resistance in pathogenic microorganisms, predominantly multi drug resistant (MDR) bacteria, underlines the need to hunt for new antimicrobial substances. Actinomycetes are one of the most striking sources of new bioactive metabolites. The main objective of the present study was isolation, purification, and characterization of actinomycetes from garden soil samples, having antimicrobial activity against selected pathogenic strains of bacteria. Soil samples were collected from different niche habitats of Maharashtra such as Pune, Karjat, Dadar, Shahad, and Ulhasnagar and different strains of soil actinomycetes were selectively isolated purified and preserved. Isolates were morphologically and biochemically characterised. Isolated actinomycetes were further evaluated for their antimicrobial activity. Totally 15 actinomycete isolates were tested for antagonistic activity against seven pathogenic microorganisms. Out of fifteen only five isolates showed antimicrobial activity against different pathogenic bacteria. All five isolates were most active against *Coreynebacterium diphtheria,* 3 isolates were active against *Bacillus.* Isolates SA2 was highly active, while SA1, SA3 and SA4 were less active and SA5 was least active against the pathogenic microorganisms. SA2 was found most promising and it inhibits six out of seven pathogenic bacteria. Isolates having antimicrobial activity could be used in the development of new antibiotics for pharmaceutical purposes.

Keywords: Soil, Actinomycetes, agar well diffusion method, antibiotics, drug resistance, antimicrobial activity, pathogenic bacteria.

Introduction: Soil microorganisms provide an excellent resource for the isolation and identification of therapeutically important products. Among them, actinomycetales are an important group. The actinomycetes are Gram positive bacteria having high G+C (>55%) content in their DNA [1]. They were originally considered to be an intermediate group between bacteria and fungi but now are recognized as prokaryotic organisms which are widely distributed in soil, water and found colonizing plants [2][3]. Actinomycetes have been well known for the production of secondary metabolite. Many of the presently used antibiotics such as streptomycin, gentamicin, rifamycin and erythromycin are the product of actinomycetes. Almost 80% of the world's antibiotics are known to come from actinomycetes [4]. The emergence of multidrug resistant among common bacterial pathogens is a serious problem. WHO recently identified antimicrobial resistance as one of the three greatest threats to human health. Resistance to first- or even second-line antibiotics forces the use of more expensive second or third line antibiotics. In the European Union, the annual cost associated with antibiotic resistant infections is estimated to be €1.5 billion (based on 2007 euros) [5], while in the United States the value is estimated to be greater than $16 billion

(based on 2000 dollars)[6]. Therefore, there is a continuous need for new molecules to combat these pathogens. On the basis of above facts new actinomycetes strains that generate active compounds have been recently isolated from novel sources including saline, ocean, mangrove forests and niche habitats such as caves, beehives, solitary wasp mud nest, earthworm castings, pristine forests, lakes, rivers and other wetlands. New species of the microorganisms have the potential to produce new metabolites, which justifies the isolation of new species for drug discovery purposes. So, we need to screen more and more actinomycetes from different habitats for antimicrobial activity in the hope of getting some new actinomycetes strains that produce antibiotics, which have not been discovered yet and are active against drug-resistant pathogens [6]. The aim of this study was to isolate some novel actinomycetes from different soils and evaluate their antibacterial activity.

Materials and methods:

Soil sampling and pre treatment:

Soil samples were collected from different niche habitats of Thane district, Maharashtra were collected from different locations of Pune, Karjat, Dadar, Shahad, Ambernath and Ulhasnagar such as field, dam, residential area and near well. All samples were collected between February and June 2013. Samples were collected by inserting a sterilized polyvinyl corer into the sediments. The corer was surface sterilized with 70% alcohol before sampling at each location. Each collection was made from 6-10 inches depth of the surface of ground. These samples were placed in sterile poly bags, sealed tightly, and transported immediately to the laboratory. These soil samples were air dried for 3-4 hours at 45°C, crushed, and sieved prior to use for isolation purpose. The samples were placed in polyethylene bags, closed tightly and stored in a refrigerator.

Isolation of pure culture of actinomycetes:

Actinomycete strains were isolated as pure culture by using standard microbiological method [7]. One gram of each soil samples collected from respective locations were inoculated into 100ml of sterile actinomycetes isolation broth and incubated at rotary shaker at room temperature for 3-4 days. After incubation, serial dilution of the inoculated soil was performed till 10^{-8} concentration. Starch casein agar medium was used for the isolation of Actinomycetes. Spread plate technique was used for the isolation of pure colonies of actinomycetes. Three starch casein agar media plates with the following dilutions $1:10^6$, $1:10^7$ and $1:10^8$ were used. From those dilutions 0.1ml of aliquot was spreaded thoroughly. After spreading, plates were incubated at room temperature for one week. After incubation, tough leathery, dry, rough and powdery colonies of actinomycetes were observed. These typical colonies were selected and purified. The purified actinomycetes isolates were preserved on starch casein agar slants at 4°C.

Characterization of isolates:

The potent actinomycetes isolates selected from primary screening were characterized by morphological, biochemical and physiological methods. The morphological method consists of macroscopic and microscopic characterization. Macroscopically the actinomycetes isolates were differentiated by their colony characters, e.g. size, shape, colour, consistency etc. For the microscopy, the isolates were grown by cover slip culture method [8]. They were then observed for their mycelial structure and conidiospore and arthrospore arrangements on the mycelia under microscope (1000X). The observed morphology of the isolates was compared with the Actinomycetes morphology provided in Bergey's manual of determinative

bacteriology, ninth edition [9] for the presumptive identification of the isolates. Various biochemical tests performed were catalase, oxidase, citrate utilization, nitrate reduction, starch hydrolysis, acid production from sugar, and the physiological test included motility.

Morphological characterization:

The morphology of Actinomycete isolates were characterised by Slide Culture Method [9]. This method is carried out aseptically containing sterile petriplate and sterile slide, on which 1ml actinomycetes isolation agar was poured at the centre of the slide and incubated for 2 days at room temperature. The growth was observed under 10X and 40X and organism's morphology was studied with respect to colour, hyphae, aerial and substrate mycelium, branching and the nature of colony [10].

Biochemical characterization:

fter preliminary studies, the actinomycetes which showed zones of inhibition against standard test pathogens were selected for biochemical studies [11]. Biochemical tests generally used are sugars fermentation test, IMViC test, gelatin hydrolysis test, starch hydrolysis test, catalase test, motility test, oxidase test and triple sugar iron test [12].

Test organisms:

Seven test organisms were used to test the antibiotic activity of the isolates. Three of them were Gram positive and five were Gram negative bacteria. Grampositive species were *Corynebacterium diphtheria*, *Bacillus spp* and *Staphylococcus aureus*. Gram-negative strains were *Escherichia coli, Serretia marcescen , Salmonella typhi* and *Salmonella paratyphi B*. They were maintained in nutrient agar slants at 4^{o}C.

Antibacterial activity of isolates:

The screening method of isolates consists of two steps: primary screening and secondary screening. In primary screening, the antibacterial activity of pure isolates was determined by Single line streak method on nutrient agar plate. Antibacterial activity of cell free extracts was determined by agar well diffusion method on Mueller Hinton Agar medium [13].

Determination of antibacterial activity by single line streak method (Primary screening):

Preliminary screening for antibiotic activity of the isolates was done by using streak-plating technique on nutrient agar medium. Each pure isolates were streaked individually on different agar plates in a single line. The plates were then incubated at room temperature for 5 days to allow the isolates to secrete antibiotics into the medium. After the incubation period, the properly diluted test organisms were cross streaked along the line of fully grown isolates. Each streaking was started near the edge of the plates and streaked toward the *Streptomyces* growth line. The plates were then incubated for 12 hrs at 37^{o}C, and the zone of inhibition was measured using a millimeter scale [14].

Determination of the antibacterial activity by agar well diffusion method (Secondary screening):

Preparation of cell free extract:

The actinomycetes were grown on actinomycetes broth at room temperature for 3-4 days. After incubation, 10ml of respective actinomycetes suspention were taken in to the centrifuge tube and centrifuged it at 10000 rpm for 20 min and supernatant was taken for determination of antimicrobial activity of actinomycetes.

Determination of antibacterial activity: Antibacterial activity of cell free extracts was determined by agar well diffusion method. [13]. Mueller Hinton Agar medium was bulk seeded

with gram positive and gram negative organism and their plates were prepared. The optical density of cell suspension of all pathogens was adjusted at 1.04 at 530nm. Wells on Mueller Hinton Agar plate were bored by sterilized 1000 µl micro tip. Then 100 µl of each cell free extract inoculated into the well respectively and incubated at 37°C for 24 hrs. Zone of inhibition were observed [15].

Results and discussion:

Actinomycetes have been intensively studied in several underexplored environments, niche, and extreme habitats in various parts of the world in the last few years. Yet, there is no report regarding isolation of actinomycetes from Thane region, (Maharashtra). Therefore, an attempt has been made to isolate the actinomycetes from this unexplored region in order to find novel species.

Totally 15 actinomycetes strains were isolated from soil samples of different areas based on the gram staining and colony morphology. Out of them 5 isolates were found active against bacterial pathogens in primary screening by single line streak method. All the selected isolates were found Gram positive and had different morphological structures. The biochemical properties of actinomycetes isolates were determined and recorded in Table 1and 2. The identification of strain was performed by physiological characterisation, biochemical tests, microscopic observation at 10X and 40X. Based on their mycelial and cellular morphology observed under microscope (1000X), the antibacterial actinomycetes isolates were identified as:

1. SA 1 was identified as *Rothia*.
2. SA 2 was identified as Jonesia.
3. SA 3 was identified as *Actinobispora*.
4. SA 4 was identified as Kineosporia.
5. SA 5 was identified as Nocardiods.

These actinomycetes were identified with the help of Bergy's manual of 9th edition.

Table-I: Sugar fermentation by different isolates of Actinomycetes.

Actino - mycetes	Glu	Lac	Rah	Mal	Ara	Suc	Xyl	Gal
SA 1	-	-	-	-	-	-	-	-
SA 2	-	-	-	-	-	-	-	-
SA 3	+	-	-	-	-	-	-	-
SA 4	-	-	-	-	-	-	-	-
SA 5	+	-	-	+	-	-	-	-

Abbreviations: - Glu: Glucose, Lac: Lactose, Rah: Rhamnose, Mal: Maltose, Ara: Arabinose, Suc: Sucrose, Xyl: Xylose, Gal: Galactose

Key: + indicates positive result and - indicates negative result

Table-II: Biochemical characterization of actinomycetes isolates												
Actinomycetes	**Sta**	**Gel**	**Mot**	**TSI**				**Ind**	**MR**	**VP**	**Cat**	**Oxi**	**SC**
				S	**B**	**G**	**H_2S**						
SA 1	+	-	-	-	-	-	+	-	-	-	+	-	+
SA 2	+	-	+	P	Y	-	-	-	-	+	+	+	-
SA 3	+	-	+	-	-	-	-	-	-	-	+	-	
SA 4	-	-	+	-	-	+	+	-	-	-	+	-	+ (FB)
SA 5	-	-	+	P	Y	+	-	-	+	-	+	+	+

Abbreviations :- Sta:Starch, Gel: Gelatinase, Mot:Motility, TSI:Triple Sugar Iron, S:Slant, B:Butt, G:Gas, Ind: Indole, MR: Methyl Red, VP: Voges –Proskauer, Cat: Catalase, Oxi: Oxidase, SC :Simmon Citrate Slant, FB: Faint Blue, P:Pink and Y: Yellow.

Key: + indicates positive result and - indicates negative result

Antimicrobial activity of isolates:

Five out of 15 actinomycetes isolates showed antimicrobial activity against one or more test bacteria in primary screening by single line streak method. These isolates of soil actinomycetes were named as SA1, SA 2, SA 3, SA 4 and SA 5 and selected for secondary screening by agar well diffusion method. The anti-bacterial activity of selected soil actinomycetes isolates were determined and recorded in Table 3 and Fig1.

In Secondary screening, all selected actinomycetes isolates SA 1, SA 2, SA 3, SA 4 and SA 5 were found active against Gram positive bacteria only while SA 2 was found active against both Gram positive and Gram negative bacteria.

1. Isolate SA 1 exhibited the activity against *Coreynebacterium diphtheria, Bacillus spp.* (zone of inhibition 16mm and 15mm respectively).
2. Isolate SA 2 exhibited the activity against *Serretia marcescens, Salmonella typhi, Salmonella paratyphi B, Coreynebacterium diphtheria, Ecsherichia coli and Bacillus spp (*Zone of inhibition 17, 15, 17, 45, 16, and18 mm respectively.
3. Isolate SA 3 exhibited the highest activity against *Coreynebacterium diphtheria* (zone of inhibition 33mm) and *Staphylococcus aureus* (zone of inhibition 20mm).
4. Isolate SA 4 exhibited activity against *Coreynebacterium diphtheria, Bacillus spp.* (zone of inhibition 38mm and 20mm respectively).
5. Isolate SA 5 exhibited the activity against *Coreynebacterium diphtheria* (zone of inhibition 27mm).

Table-III: Antimicrobial Activity (Agar Well Diffusion Method)		
Actinomycetes isolates	**Pathogenic bacteria**	**Zone of Inhibition (mm)**
SA 1	*Coreynebacterium diphtheria* *Bacillus spp.*	16mm 15mm
SA 2	*Serretia marcescens* *Salmonella typhi* *Salmonella paratyphi B* *Coreynebacterium diphtheria* *Ecsherichia coli* *Bacillus spp.*	17mm 15mm 17mm 45mm 16mm 18mm
SA 3	*Staphylococcus aureus* *Coreynebacterium diphtheria*	20mm 33mm
SA 4	*Coreynebacterium Diphtheria* *Bacillus spp.*	38mm 20mm
SA 5	*Coreynebacterium diphtheria*	27mm

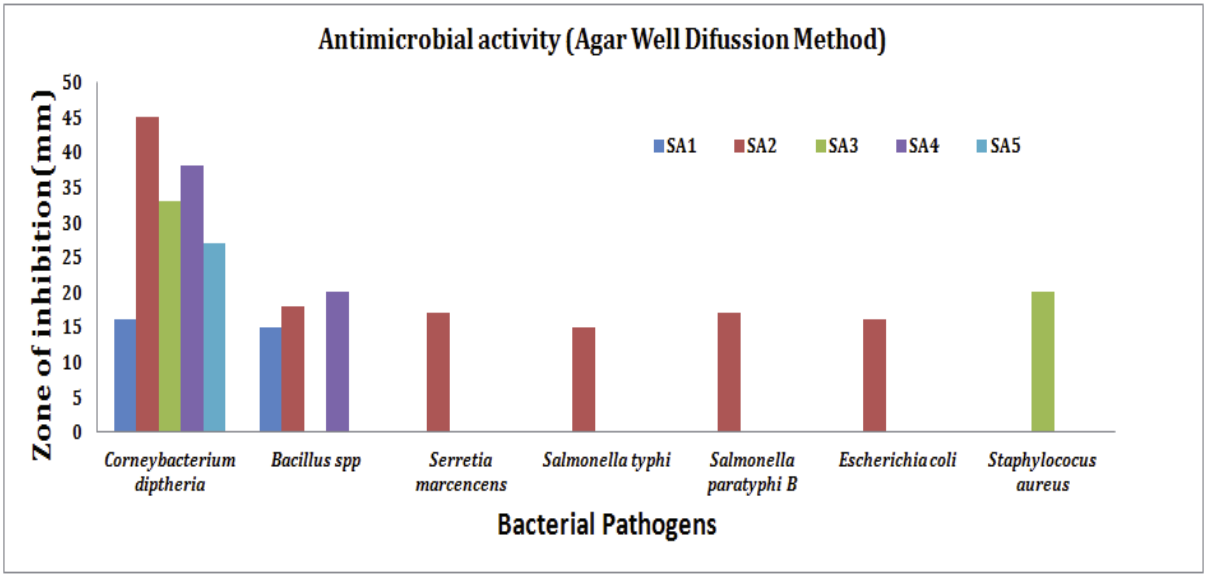

Fig.-1 Graphical representation of Agar Well Difussion Method **Pathogens Vs Zone of inhibition (mm)**

Fig-1 Results of antibacterial activity of actinomycetes isolates (SA1, SA 2, SA 3, SA 4 and SA 5) checked on Mueller Hinton agar media by agar well diffusion method. Zone of inhibition recorded in

millimetre.

Conclusion: 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 reisolation 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. Antibiotics are the most important bioactive compounds for the treatment of infectious diseases. But now, because of the emergencies of multi drug resistant pathogens, there are basic challenges for effective treatment of infectious diseases. Thus, due to the burden for high frequency of multidrug resistant pathogens in the world, there has been increasing interest for searching effective antibiotics from soil actinomycetes in diversified ecological niches. Actinomycetes are the most widely distributed groups of microorganisms in nature which primarily inhabit the soil. They are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about more than half of the discovered bioactive secondary metabolites notably antibiotics, antitumor and immunosuppressive agents, and enzymes.

Present study is undertaken to determine biodiversity of actinomycetes in soils of different regions and selectively isolate them to evaluate their antagonistic activity. The main goal is the selective isolation and screening of new strains of actinomycetes in search of novel antagonistic method. The results showed that, all the isolates exhibited the highest activity against *Corynebacterium diphtheria*. The identification of strain was performed by physiological characterisation, biochemical tests and microscopic observation. Most of the actinomycetes isolates showed positive results for catalase, oxidase, starch utilization, TSI, MR-VP, sugar fermentation (glucose and maltose) and motility test while the isolates showed negative results for indole and gelatin utilization tests. Present study will establish the rich actinomycetes diversity of the region. So, further intensive studies are required on the actinomycetes diversity of exclusive biotopes in Thane district, which could form an important input into pharmaceutical industries. Most promising actinomycetes isolates were tested for antagonistic activity against pathogenic Gram positive and Gram negative microorganisms. Isolates having antibacterial activity could be used in the development of new antibiotics for pharmaceutical or agricultural purposes. The results of the present study may be significant to initiate many researchers in this particular area to look for different sources of samples for searching new bioactive secondary metabolites like antibiotics. Results obtained from this work are promising and hence merit further studies concerning purification, characterization and identification of the active secondary metabolites.

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