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Isolation and Screening of Actinomycetes producing Antibacterial compounds from different river sediments

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ABSTRACT

The history of antibacterial begins with the observations of Pasteur and Joubert, who discovered that one type of bacteria could prevent the growth of another. They did not know at that time that the reason one bacterium failed to grow was that the other bacterium was producing an antibiotic. Of course, in today's common usage, the term antibiotic is used to refer to almost any drug that attempts to rid your body of a bacterial infection. The past researches indicated that huge number of antibiotics was produced by Gram +ve ike bacteria known as Actinomycetes. So we can say that among all microbes more than 50% of the known antimicrobial compounds were produced by Actinomycetes only. In our study isolation and screening of Actinomycetes was performed by using different river sediments. Soil samples was collected from river Godaveri and Krishna and stored in the U.V. and alcohol sterilized Poly bags. Soil samples was serially diluted up to 10^{-6} and 1 ml from each dilution was plated on different isolation media like starch Casein agar, Albumin media and YMA media, consisting of antifungal agent Nystatin 50 µg/ ml, by pour plate technique. The plates were incubated at different temperature ranges 18 ^{0}C to 28 ^{0}C upto 7-14 days. Certain biochemical tests were performed for identification of different strains producing antibacterial compound. Some bacteria, including Gram positive and Gram negative were used to determine the antimicrobial activity against the isolated actinomycetes. The potent actinomycetes were characterized by morphological and biochemical methods. These were than Inoculated into different media like L.B. broth and Starch Casein Broth and Albumin Broth etc and were kept for incubation at different Temperatures ranging from 28^{0}C- 40^{0}C for the production of antibacterial compound upto18 days. Determination of antibacterial activities of pure actinomycetes cultures of S1, S2, S3 and S4 were performed by using streak -plate method. Mueller hinton agar plates will prepared and inoculated with actinomycetes cultures by a single streak of inoculums in the center of the Petri dish and will incubated at 27^{0}C for 4 days. Later, the plates will seeded with test organisms by a single streak at a 90^0 angle to actinomycetes strains. Antagonism was measured by the determination of the size of the inhibition zone. The antibacterial activity of compound was tested against different gram +ve and Gram –ve by the standard disc diffusion method and cup plate method. Standard streptomycin was used for comparison of the antibacterial activity. Nutrient agar was used as a bacteriological media. The minimum inhibitory concentration (MIC) was calculated. Purification of the compound was performed using TLC and Column chromatography using different solvent composition.

Keywords: Actinomycetes, river sediments, Nutrient Media, Identification.

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I. INTRODUCTION

Scope of Herbal Drugs:

India can play a major role in the coming years in the global market for herbal products based medicines, since there is a growing demand for plant based medicines and cosmetics, since pharmaceutical industry is plagued with increased cost of new drug development coupled with low serum rate. Scientific validation quality, quantity, consistency and good marketing network are quite essential for the growth of herbal plant industry in India. The absence of these in the country has affected growth of medicinal plant industry in the country. India has a big potential for the cultivation of herbal plants.

Both China and India share 38% in marketing of medicinal plants worldwide. While China's turnover in medicinal plants has been Rs.22,000crore, India's business is hardly about Rs.450 crore. In fact the country has a rich collection of medicinal plant in Uttaranchal, Himalayas, Kerala and North Eastern States, but hardly some medicinal plants have been marketed here.

- As against a demand of 35,000 tonnes of medicinal plants, the supply is around 5000 tonnes. While 98% of herbal plants depending upon forest production.
- In some Asian and African countries, 80% of the population depends on traditional medicine for primary health care.
- Herbal medicines are the most lucrative form of traditional medicine, generating billions of dollars in revenue. Traditional medicine can treat various infectious and chronic conditions: new antimalarial drugs were developed from the discovery and isolation of artemisinin from *Artemisia annua* L., a plant used in China for almost 2000 years.
- Counterfeit, poor quality or adulterated herbal products in international markets are serious patient safety threats.
- More than 100 countries have regulations for herbal medicines.

Who uses traditional medicine?

In some Asian and African countries, 80% of the population depends on traditional medicine for primary health care. In many developed countries, 70% to 80% of the population has used some form of alternative or complementary medicine (e.g. acupuncture). Herbal treatments are the most popular form of traditional medicine, and are highly lucrative in the international marketplace. Annual revenues in Western Europe reached US$ 5 billion in 2003-2004. In China sales of products totaled US$ 14 billion in 2005. Herbal medicine revenue in Brazil was US$ 160 million in 2007.

Actinomycetes [1] are a widely distributed and successful group of bacteria which have a number of properties which favor them in competition with other saprophytic microorganisms and ensure their survival under unfavorable environmental conditions. Actinomycetes form an integral part of any balanced microbial community in soil, the majority of isolates being Streptomycetes which manly exist in the form of dormant spores. These spores germinate in presence of suitable plant and animal remains to form a limited branching mycelium bearing short chain of spores. The spores are continuously washed into aquatic habitats where they accumulate in sediments.

The past researches indicated that huge number of antibiotics was produced by Gram +ve ike bacteria known as Actinomycetes. So we can say that among all microbes more than 50% of the known antimicrobial compounds were produced by Actinomycetes only. These are a specific type of class of prokaryotes forming thread like structure at some stage of their growth, so refereed as filamentous prokaryotes. This class or group is an actively produce of different types of enzymes, enzyme inhibitors, growth promoter and antibiotics etc.

Needs for New Medicines [2]

Nowadays human is facing great harming due to different diseases because a number of microbes got resistance against the available drugs.

These products have been exploited for human use for thousands of years, and plants have been the chief source of compounds used for medicine. Even today the largest users of traditional medicines are the Chinese, with more than 5,000 plants and plant products in their pharmacopoeia In fact, the world's best known and most universally used medicine is aspirin (salicylic acid), which has its natural origins from the glycoside salicin which is found in many species of the plant genera *Salix* and *Populus*. Examples abound of natural-product use, especially in small native populations in a myriad of remote locations on Earth. For instance, certain tribal groups in the Amazon basin, the highland peoples of Papua New Guinea, and the Aborigines of Australia each has identified More recently, the Benedictine monks (800 AD) began to apply *Papaver somniferum* as an anesthetic and pain reliever as the Greeks had done for years before Many people, in past times, realized that leaf, root, and stem concoctions had the potential to help them. These plant products, in general, enhanced the quality of life, reduced pain and suffering, and provided relief, even though an understanding of the chemical nature of bioactive compounds in these complex mixtures and how they functioned remained a mystery.

Number of antibiotics produced by major group of microorganisms [2]

Taxonomic groups	Number of antibiotics
Bacteria other than *actinomycetes*	950
Actinomycetes	4600
Fungi	1600

Above table showed that most of the drug mainly produced by Actinomycetes.

Important Microbes Producing Antibiotics [3]

S. N.	Name of microorganism	Name of antibiotics
1	*P. notatum*	Penicillin
2	*P. griseofulvum*	Griseofulvin
3	*P. chrysogenum*	Penicillin
4	*S. griseus*	Streptomycin
5	*S. venezuelae*	Chloramphenicol
6	*S. aureofaciens*	Chlortetracycline
7	*S. virdofaciens*	Aureomycin
8	*S. rimosus*	Oxytetracycline
9	*S. texas*	Tetracycline
10	*S. aureofaciens*	Dimethyl-chlortetracycline
11	*S. erythricas*	Erythromycin
12	*S. halstedii*	Carbamycin
13	*S. ambofaciens*	Ravomycin
14	*S. noursei*	Nystatin
15	*S. griseus*	Cycloheximide

II. MATERIALS AND METHODS

1. Collection of sample: The different samples of soil sediments were collected from river Krishna and Godaveri after making 2 cm depth and stored in sterile polybags.

2. Sterilization of polybags: Airtight polybags were purchased from market and these were sterilized after application of ethyl alcohol and keeping into U.V. light for 5 minutes.

3. Storing of sediments: Collected soil sediments first about 20 gm were kept in polybags and stored in refrigerator.

4. Preparation of samples for isolation of actinomycetes: The different soil samples were taken and these were serially diluted upto 10^{-6}. Each of sample were prepared using different test tubes.

5. Isolation of actinomycetes by using different nutrient media

The ingredients of media were accurately weighed for the each 500 ml of the three type's media i.e.

- Starch Casein Agar Media
- YMA Media
- Albumin Media

Weighed ingredient were dissolved in required quantity of distilled water and sterilized at 121 °C (15 lbs) for 15 min by using autoclave. After sterilization the antifungal Nystatin was added (50 µg/ml) then media were poured into Petri dishes under sterile condition (laminar air flow) and allow cooling for sufficient time for the solidification of media.

The surface sterilized plant parts were taken and crushed using sterile pestle and mortar and spread on the three of the media and kept at 28 °C for 2-3 weeks, growth of microbes were observed each day and produced actinomycetes colony were purified on the Petri dishes using streak methods on the same media.

6. Identification of different actinomycetes

Growth on different ISP media

Media composition were weighed and dissolved in water and sterilized at 121°C (15 lbs) for 15 min by using autoclave. After sterilization the media were poured into Petri-dishes under sterile condition (laminar air flow) and allow cooling for sufficient time for the solidification of media and after solidification isolated microbes were streaked on solidified media in zigzag fashion and kept for incubation in incubator at 37°C for about 24 hrs.

Gram's staining

1. The microbes' smears were taken on glass slide.
2. The smears were air dried.
3. Smears were covered with crystal violet for 30 seconds.
4. Covered each smear with Gram's Iodine solution for 60 seconds.
5. Washed off Iodine solution with 95% ethyl alcohol, ethyl alcohol was added drop by drop until no more colour flows from the smear.
6. The slides were washed with distilled water and drain.
7. Safranin was applied to smears for 30 seconds (counter staining).
8. The slides were washed with distilled water and blot dried with absorbent paper.
9. Let the stained slides air dry.
10. The slides were examined under microscope.

Isolation of actinomycetes producing antibacterial compound: [15]

Determination of antibacterial activities of pure actinomycetes cultures were perfomered by using streak - plate method. Mueller hinton agar plates will prepared and inoculated with actinomycetes cultures by a single streak of inoculums in the center of the Petri dish and will incubated at 27^0C for 4 days. Later, the plates will seeded with test organisms by a single streak at a 90^0 angle to actinomycetes strains. Antagonism will measured by the determination of the size of the inhibition zone.

Test microorganisms: [1]

Some bacteria, including Gram positive and Gram negative were used to determine the antimicrobial activity against the isolated actinomycetes.

Characterization of isolated actinomycetes from soil samples:[8]

The potent actinomycetes were characterized by morphological and biochemical methods. These were than Inoculated into different media like L.B. broth and Starch Casein Broth and Albumin Broth etc and were kept for incubation at different Temperatures ranging from 28^0C- 40^0C for the production of antibacterial compound upto18 days.

Purification of antibacterial compound: [17]

Purification of the compound was performed using TLC and Column chromatography using different solvent composition.

Isolation of actinomycetes producing antibacterial compound: [8]

Determination of antibacterial activities of pure actinomycetes cultures of S1, S2, S3 and S4 were performed by using streak -plate method. Mueller hinton agar plates will prepared and inoculated with actinomycetes cultures by a single streak of inoculums in the center of the Petri dish and will incubated at 27^0C for 4 days. Later, the plates will seeded with test organisms by a single streak at a 90^0 angle to actinomycetes strains. Antagonism was measured by the determination of the size of the inhibition zone.

Production of antimicrobial compound using different media like starch casein broth and L.B. broth: [7]

Only two actinomycetes S1 and S3 showing zone of inhibition showing were then Inoculated into different media like L.B. broth and Starch Casein Broth and Albumin Broth etc and will be kept for incubation at different Temperatures ranging from 28^0C- 40^0C for the production of antibacterial compound upto18 days.

Extraction of antimicrobial compound using different solvents:[9,]

All of above Broth of actinomycetes S1 and S3 were taken at the end of 7^{th} day and centrifuged at 10,000 rpm for 15 min to separate the mycelial biomass; the supernatant will obtained separated by filtration using Whatman filter paper. Certain solvents used for extraction of antibacterial compound like butanol, n-hexane, ethyl acetate, petroleum ether, chloroform, ethanol (1:1) ratio. Supernatant mixture was agitated for 50 min. with homogenizer and the solvent were separated from broth by separating funnel, Solvent present in the broth were separated by centrifugation at 5000 rpm for 15 min to remove traces of fermentive broth. All extracts obtained through this method were assayed for antibacterial study against different microbes using respective solvents as control by agar well diffusion method.

Antibacterial Screening:[9]

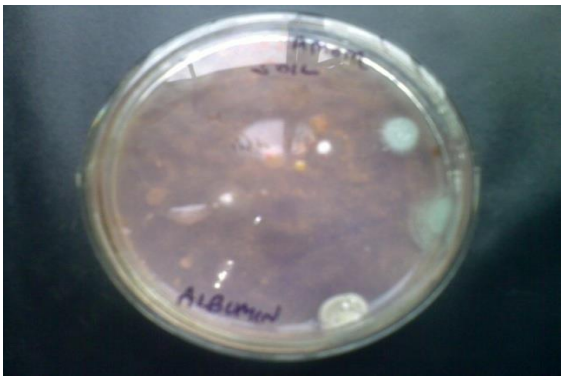
The antibacterial activities of compound were tested against different gram +ve and Gram –ve by the standard disc diffusion method and cup plate method. Standard streptomycin was used for comparison of the antibacterial activity. Nutrient agar was used as a bacteriological media. The minimum inhibitory concentrations (MIC) were calculated.

Purification of antibacterial compound: [10]

Purification of the compound was performed using TLC and Column chromatography using different solvent composition.

III. RESULTS AND DISCUSSION

Out of 12 sediments sample total 19 Actinomycetes were isolated. Out of 19 sediments sample only 4 showed antibacterial activities. These 4 Actinomycetes further allow to grow on ISP media.Out of 4 Actinomycetes 2 were isolated on Starch Casein Media from river sediments and named as S1 and S2, one was isolated from Godavari river sediment on Starch Casein Media named as S3 and 4th Actinomyctes was isolated from Yamuna river sediment on Starch Casein Media. The Actinomycetes isolated from sediments were shown in photograph.



Photograph 1: Showed actinomycetes growth on Albumin media

Morphological study:

The actinomycetes strains which showing anti-bacterial activity isolated from different river sediments samples (S1, S2, S3, and S4) were allowed to grow on the different types of ISP (International *Streptomycin* Project) media as follows.

a. ISP-2
b. ISP-4
c. ISP-5
d. ISP-6
e. ISP-7

- **Culture Characteristics of actinomycetes strain on ISP-2 and ISP-4 Media**

  The colour of mycelium above the Petri dishes and colour of media were shown in Table No.1.

- **Culture Characteristics of actinomycetes strain on ISP-5 and ISP-6 media**

  The colour of mycelium above the petri dishes and colour of media were shown in Table No.2.

- **Culture Characteristics of actinomycetes strain on ISP-7 media**

  The colour of mycelium above the petri dishes and colour of media were shown in given Table No.3.

❖ **Gram's Staining**

All the eight actinomycetes were found Gram's positive and showed the violet colour on the slide, isolated from different river sediments samples.i.e. S1, S2, S3 and S4.

Biochemical Test

There are certain biochemical test were performed for the identification of 4 strains of actinomycetes producing anti-bacterial compound given as-

- **Melanoid Formation**

The actinomycetes producing anti-microbial compound isolated from different soil samples showed different results, given in Table 4

- **Test for Nitrate Reduction**

The actinomycetes producing anti-microbial compound isolated from different soil samples showed different results, given in Table No.5

- **Test for acid production**

The actinomycetes producing anti-microbial compound isolated from different soil samples showed different results, given in Table No.6.

Table 1: Shows the Colour of mycelium and culture media on ISP-2 and ISP-4 media.

Actinomycets strain	ISP-2		ISP-4	
	Colour of mycelium	Reverse Side Colour	Colour of mycelium	Reverse Side Colour
S1	-	-	-	-
S2	-	-	-	-
S3	-	-	Yellow	Dark Brown
S4	-	-	-	-

The –ve sign indicate that actinomycetes are unable to grow on the ISP media because of their culture characteristics of growth on different media

Table 2: Shows the colour of mycelium and culture media on ISP-5 and ISP-6 media.

Actinomycetes strain	ISP-5		ISP-6	
	Colour of mycelium	Reverse Side Colour	Colour of mycelium	Reverse Side Colour
S1	-	-	-	-
S2	White	Brown	Orange white	Orange
S3	White	Dark brown	-	-
S4	-	-	-	-

The –ve sign indicate that actinomycetes are unable to grow on the ISP media because of their culture characteristics of growth on different media

Table 3: Shows the colour of mycelium and culture of media on ISP-7

Actinomycetes strain	ISP-7	
	Colour of Mycelium	Reverse Side Colour
S1	White	Brown
S2	-	-
S3	-	-
S4	Orange	Brown

The –ve sign indicate that actinomycetes are unable to grow on the ISP media because of their culture characteristics of growth on different media

Table 4: Shows the Melanoid formation test

Actinomycetes strain	Reaction
S1	+ve
S2	+ve
S3	+ve
S4	-ve

Table 5: Shows Nitrate reduction test

Actinomycetes strain	Reaction
S1	-ve
S2	-ve
S3	+ ve
S4	-ve

Table 6: Shows Acid production test

Actinomycetes strain	Reaction
S1	-ve
S2	-ve
S3	-ve
S4	-ve

Table 7: Shows Hydrogen sulphide test

Actinomycetes strain	Reaction
S1	-
S2	+
S3	+
S4	+

DISCUSSION

There were only two actinomycetes S1 and S3 showed strong antibacterial activity. S1 was isolated from Godavari river sediment on Starch Casein Media named as S3 Actinomyctes was isolated from Yamuna river sediment on Starch Casein Media.

There was one compound was extracted in Starch Casein Broth and named as A1

Calculation of λ max of the antibacterial compound: The λ max of two compounds A1, was calculated using Methanol and Ethanol respectively. λ max of A was 306

Analysis of the antibacterial compound: The physical analysis of the compound was performed as follows

- **Physical Analysis**

Colour of the compound: The colour of the A1 compound was found white.

State: Solid in nature (powder). The compound A1 was given for elucidation of structure.

Antimicrobial Assay:

S.N.	Name of microorganisms	Zone of inhibition (mm) C1
1.	*E. coli* ATCC 8739	3.9 mm
2.	*S. typhi* ATCC 23564	0 mm
3.	*S. aureus* ATCC 29736	4.0 mm
4	*M. luteus ATCC11880*	3.9mm
5.	*K. pneumoniae ATCC 10031*	0 mm
6.	*S. fecalis ATCC 8043*	3.5 mm
7.	*B. subtilis ATCC 6633*	6.3mm
8.	*S. boydi ATCC 9207*	3.9 mm
9.	*P. mirabilis ATCC 2124*	2.2 mm

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