# STUDENT STUDY PROJECT

# ON

# Isolation of Antagonistic *Actinomycetes* species from Rhizosphere


**Submitted By**

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## KAKATIYA GOVERNMENT COLLEGE

**[Re- Accredited with NAAC 'A' Grade]**

## Hanamkonda, Warangal (U)District- Telangana State

## CERTIFICATE

This is to certify that students of the B. Sc –MICROBIOLOGY – Second year has been successfully completed the project for the Jignasa programme entitled "**Isolation of Antagonistic Actinomycetes species from Rhizosphere** from the department of Microbiology ,Kakatiya government college,hanamkonda.

**Head of the Department** **Principal**

## ACKNOLEDEMENTS

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We are great full to commissioner, CCE, Hyderabad for conducting programmes like Jignasa and inculcating scientific approach in our studies.

**INDEX**

**TITLE: ISOLATION OF ANTAGONISTIC ACTINOMYCETES SPECIES FROM RHIZOSPHERE**

# 1. Hypothesis

Soil is the critical component of the earth system functioning for the production of food, fodder, fiber and also maintains environment quality. The plant microbe interaction in the rhizosphere is one of the major factors regulating the health and growth of plants. Actinomycetes are common filamentous soil microorganisms important in maintaining a satisfactory biological balance in the soil, largely because of the ability to produce antibiotics.

Actinomycetes secrete low levels of antibiotic compounds as their secondary metabolites. Many of them are effective against bacteria, fungi and actinomycetes which maintain natural soil health. This is a continuous process which can inhibit or kill some of the plant pathogens in that vicinity. These are also known to produce several antifungal and antibacterial compounds that are being exploited commercially for the control of several microbial plant and animal diseases.

# 2. Aims and Objectives

## 2.1. AIM

**ISOLATION OF ANTAGONISTIC ACTINOMYCETES SPECIES FROM RHIZOSPHERE.**

## 2.2. Objectives

- To dectect antagonistic actinimycete from rhizospheric soil.
- To screen for the property
- To isolate in pure culture.
- To maintain for further study

# 3. Review of Literature

The Rhizosphere contains a large and majority of the soil biota (more than 10 folds of bulk soil). The plant microbe interaction in the rhizosphere is one of the major factors regulating the health and growth of plants. It is also widely acknowledged that root exudates govern which organisms reside in the rhizosphere; therefore any change in the quality of rhizosphere exudates will modify the soil biodiversity and cause changes in populations of both deleterious and beneficial microorganisms. Soil bacteria living in the rhizosphere can enhance plant growth by several mechanisms like antagonism against plant pathogens, solubilization of phosphates [1], production of phytohormones [2,3], siderophores production as in Kloepper *et al.*, 1980b; Raaska *et al.*, 1993 [4], antibiotic production (Schneider *et al.*, 1996 [5], inhibition of plantethylene synthesis (Glick *et al.*, 1998 [6] and induction of plant systemic resistance to pathogens (Kloepper *et al.*,1999 [7]. Most of the plant growth promoting rhizobacteria (PGPR) inhibit the deleterious plant pathogens by involving synthesis of proteins, peptides etc. The study of rhizosphere is important as far as control of soil pathogens which pass through the rhizosphere and infect root system.

Biological control is a common phenomenon in a soil ecosystem. It is a site for complex diverse microbe mediated processes. Several microorganisms like Actinomycetes secrete low levels of antibiotic compounds as their secondary metabolites. Many of them are effective against bacteria, fungi and actinomycetes which maintain natural soil health. This is a continuous process which can inhibit or kill some of the plant pathogens in that vicinity. Actinomycetes are common filamentous soil microorganisms important in maintaining a satisfactory biological balance in the soil, largely because of the ability to produce antibiotics. They are also known to be actively involved in degradation of complex organic materials in soils and contribute to the biogeochemical transformations. Most of the actinomycetes are capable of producing wide variety of cell wall degrading enzymes like chitinases, glucanases, cellulases, hemicellulases, amylases etc. These are also known to produce several antifungal compounds that are being exploited commercially for the control of several microbial plant diseases.

# 4. Research Methodology

## 4.1. Soil sampling: Soil sampling

The study area covers gardens of Kakatiya Government college, hanamkonda, Warangal district, Telangana State, India. The rhizospheric soil samples were collected by shaking the roots vigorously to separate the loosely bound bulk soil. The soil samples are collected from 0-15 cm depth using a 5 cm diameter soil corer *Amith Kishore Singh et al.,* 2013[8]. After removal of plant debris, the soil samples were sieved using 2mm mesh size sieve and air dried. Then they were labeled and transported to the laboratory in polyethylene bags and stored at $4^0$C, and were further used for the isolation of antagonistic Actinomycetes.

### 4.1.1.Isolation of actinomycetes by Crowed plate method

The rhizospheric soil (1gm) was suspended in 10 ml of sterile 0.85% NaCl solution, serially diluted ($10^{-1}$ to $10^{-6}$), centrifuged at 500 rpm for 20 minute to disperse the spore chains. The suspension was allowed to settle for 1hr and plated on to Starch Casein Agar (SCA) [21]. The plates were incubated at $28\pm2^0$C for 84 hrs. The plates were observed intermittently during incubation for whitish pin point colonies with a zone of inhibition around them. The pin point colonies with inhibitory zone were selected and purified by multiple streaking methods. The isolated eight types of actinomycetes colonies from soil sample were maintained on SCA slants at 4 $^0$C [20].

## 4.2. Primary screening by Giant colony technique

### 4.2.1.Selection of phytopathogenic fungi and pathogenic bacteria:

Four Fungal cultures of agriculture importance (*Alternaria alternata, Fusarium moniliformae, Macrophomena phaseolina,* and *Aspergillus niger*) were used to determine the antifungal activity of the isolated actinomycetes strain. The phytopathogenic fungi were obtained from Department of pytopathology Agricultural University, Rajendranagar, Hyderabad. Four pathogenic bacterial cultures (*Escherichia coli, Staphylococcus aureus, Proteus vulgaris, Pseudomonas pyogenes*) were collected from Kakatiya Medical College Warangal, Telangana State and used to determine the antibacterial activity of the isolated actinomycetes strains.

### 4.2.2.Giant colony technique

Single streak of each Actinomycete was made on Modified nutrient agar (glucose 5gm, peptone 5gm, beef extract 3gm, NaCl 5gm, agar 15 gm at pH 7) and incubated at 28±2$^{o}$C for 4 days to test antibacterial activity. After observing a ribbon like growth of the Actinomycetes, the pathogenic bacterial cultures (*Escherichia coli, Staphylococcus aureus, Proteus vulgaris, Pseudomonas pyogenes*) were streaked at right angles to the original streak of each actinomycete and incubated at 37$^{o}$C. The inhibition zone was measured after 24 h [19]. Four Fungal cultures of agriculture importance (*Alternaria alternata, Fusarium moniliformae, Macrophomena phaseolina,* and *Aspergillus niger*) were used to determine the antifungal activity of the isolated actinomycetes strain. To test the antifungal activity single streak of actinomycetes were made on Kuster's agar [21] and the test fungal pathogens were streaked at right angles to the original streak of each Actinomycete and incubated at 28±2$^{o}$C. The inhibition zone was measured after 7days of incubation [12].

### 4.3. Secondary screening of selected strains by Well Diffusion method

Five isolates which shown most effect on phytopathogenic fungi, were selected for secondary screening. It was carried out by Well Diffusion method.

#### 4.3.1.Preparation of fermentation broth

The strains were cultured on Starch Casein Agar slants at 28±2$^{0}$C for 2 weeks for sporulation. The mature spores were inoculated in Starch Casein Broth. The fermentation set up was incubated on rotary shaker at 200 rpm for 10 days at 28±2$^{0}$C. The fermented broth was centrifuged at 10,000 rpm at 4$^{0}$C for 20 min. The supernatant was filtered using 0.2 µm filters and the filtrate was collected as antibiotic sample [11].

#### 4.3.2 Testing of antibiotic sample from antagonistic Actinomycetes

To determine the antagonistic activity the phytopathogenic fungi were cultured in Asthana Hakuer's broth [12] at 28$^{0}$C for 5 days. The cultures were swapped on Potato Dextrose Agar (PDA). Four wells (6 mm) were prepared in each seeded agar plate and each well was filled with 100 µl of the fermentation broth of the selected strains. These PDA plates were incubated at 28±2$^{0}$C for 5 days. After the incubation the diameter of the inhibition zone was measured. Depending on the zone of inhibition, one strain was selected .

### 4.4. Detection of morphology

**Determination of Colonial characteristics of AS-II**

**Aerial mass color**

The color of the mature aerial mycelium of AS was recorded as per directions given by ISP 2 (Shiriling and Gottlieb, 1996). The color of the mycelia may be recorded as white, yellow, gray, red, blue, and violet. When the aerial mass color falls between two color series, both the colors were recorded.

**Reverse side pigments**

The test strains were divided in to two groups, according to their ability to produce characteristic pigments on the reverse side of the colony, namely distinct when produce (+) red, orange, green, blue or violet and not distinctive or none (-) when they produce yellow, brown, olive or yellowish brown pigments. The reverse side of the colonies were observed for presence or absence of pigments. The observations were recorded.

**Diffusible pigments**

The test strains were divided into two groups by their ability to produce soluble pigments other than melanin. When colored pigments like red, orange, green, yellow, blue, and violet are produced, it was recorded as positive (+) and there is no distinct color negative (-). $P^H$ sensitivity of the diffusible pigments was assessed by noting any color change induced in the medium by the addition of alkali (Shriling and Gottlieb, 1996). The space around the colonies were observed for diffusible pigments and observations were noted.

**Melanin pigment production**

Based on the production of melanoid pigments i.e., Greenish brown, Brownish black, or distinct brown or pigment modified by other colors in the medium, the strains were grouped as melanoid pigment producers (+) and non-producers (-). This was determined after four days of incubation on Peptone Yeast Extract Iron Agar and Tyrosine agar .

**Gram stain**

A loop full culture of AS-II grown on SCA was smeared and heat fixed. The smear was covered with crystal violet for 30 seconds then washed with distilled water for a few seconds and flooded with Gram's iodine solution. After 30 seconds, the slide was washed and decolorized with 95% ethyl alcohol. The slide was washed with distilled water and counter

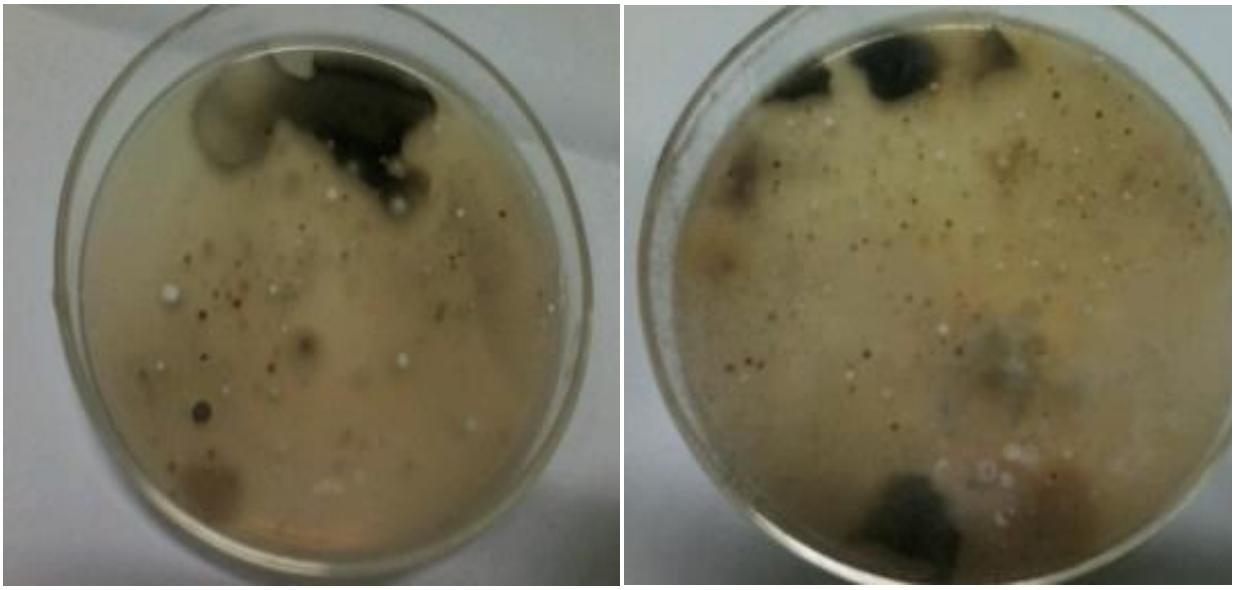
stained with safranine solution for 30 seconds and observed for their Gram color and mycelia morphology and results were recorded.

## 5. Results and Analysis

### 5.1: Isolation of Actinomycetes by Crowed plate method

The antagonistic actinomycetes were isolated by crowded plate method by maintaining triplicates of SCA (Starch Casein Agar) plates for soil samples. Whitish pin point colonies with the zone of inhibition were observed in a good number on SCA plate with $10^{-5}$ dilution.(Photo-1). Eight colonies were selected. All the selected cultures were sub cultured to get pure cultures. All the colonies from rhizophere soil sample were named as ASI, ASII, ASIII, ASIV, ASV, ASVII and AS VIII. Further, all these colonies isolated were screened for their antagonistic activity against test phytopathogenic fungi and pathogenic bacteria.

**Crowded plate method (Photo-1)**



### 5.2. Primary screening by Giant colony technique

The eight isolates from rhizosphere were tested for antagonistic activity against phytopathogenic fungi, and pathogenic bacteria by giant colony technique ( Photo-2)and zone of inhibition was compared among the isolates. The order of the isolates for their antagonistic

activity was ASII, ASI, ASIII, ASVII, ASIV, ASV, ASVI, and ASVIII (Table 1). The three isolates (ASII, ASIII, AS I) with better activity were selected for further screening to select one strain.

**Giant colony method (Photo-2)**



**Table 1: Zone of inhibition against Fungi and Bacteria on Giant colony Technique by isolates**

| **Test fungi** | **Zone of inhibition (mm)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **AS I** | **AS II** | **AS III** | **ASIV** | **ASV** | **AS VI** | **AS VII** | **AS VIII** |
| *A. alternata* | 3 | 3 | 5 | 2 | 3 | 2 | 3 | 2 |
| *F. moniliformae* | 4 | 4 | 2 | 3 | 2 | 1 | 2 | 1 |
| *M. phaseolina* | 4 | 6 | 3 | 2 | 1 | 1 | 2 | 2 |
| *A. niger* | 3 | 6 | 2 | 1 | 3 | 2 | 1 | 2 |
| **Test Bacteria** | | | | | | | | |
| *E .coli* | 4 | 6 | 3 | 3 | 4 | 3 | 3 | 3 |
| *S. aureus* | 3 | 7 | 2 | 3 | 2 | 2 | 3 | 3 |
| *P. pyogens* | 6 | 9 | 4 | 3 | 3 | 3 | 3 | 4 |
| *p. valgaris* | 4 | 6 | 2 | 2 | 3 | 3 | 2 | 3 |

### 5.4. Secondary screening of selected strains by Well Diffusion method

**Secondary screening of selected strains by Well Diffusion method**

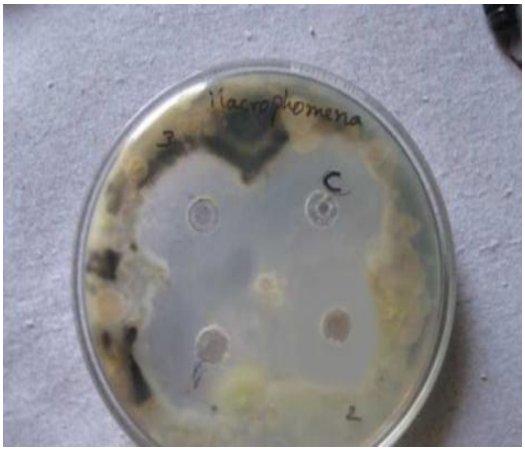
Three selected isolates from rhizospheric soils were further screened for their antagonistic activity against test fungi by Well Diffusion method. The zone of inhibition for each of the isolates was measured and compared to get one best isolate from rhizospheric environment (Table 2)

**Table 2: Antifungal activity of the isolate by Well Diffusion method**

| Test fungi | Zone of inhibition (mm) | | |
|---|---|---|---|
| | AS 1 | AS II | AS III |
| *A. alternata* | + | ++ | ++ |
| *F. moniliformae* | ++ | ++ | + |
| *M. phaseolina* | + | +++ | + |
| *A. niger*, | ++ | + | ++ |

Weak inhibition 5-9mm(+), moderate inhibition10-19mm(++),strong inhibition>20mm(+++).

**Photo 3 and 4: Zone of inhibition against *M. phaseolina* and *A. alternata*by ASII (1), AS I (2) ASIII (3) and control (C)**





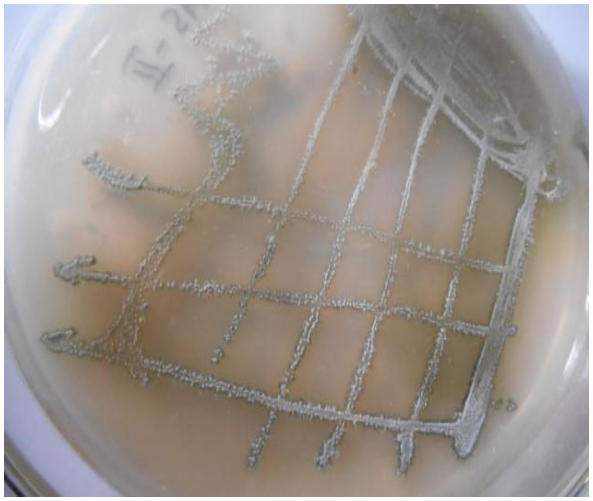
Depending on the results obtained from primary and secondary screening ASII from soil rhizospheric environment were found to have good antagonistic property.

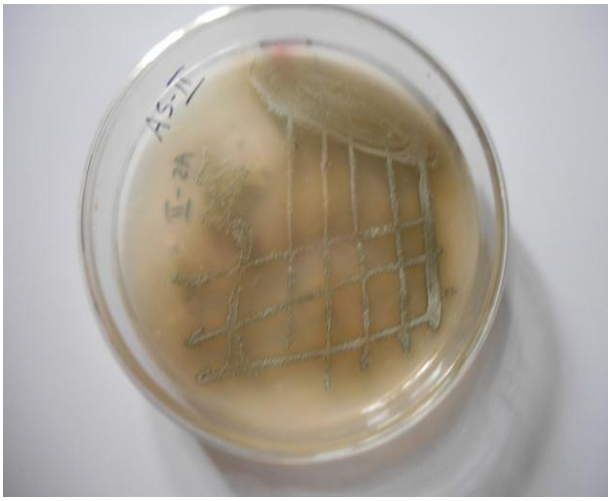
**Photo 5 : Zone of inhibition against *M. phaseolina* by *AS-II***


ASII was found to have strong inhibition against *M.Phaseolina* and moderate inhibition against *A. alternata*, *F. moniliformae*, *A. niger*, whereas ASI and ASIII have lesser activity comparatively. The most effective colony was streaked onto the fresh Starch casein Agar medium and incubated for 72 hrs at room temperature these culture plates are maintained as stock cultures by preserving at refrigeration temperature. (Photo 6 and 7).

**5.5 Morphology**

The spore mass of AS II was found to be Dark grey in color (Photo 6). There is dark brown to black coloration on the reverse side of the culture (Photo 7). Diffusible pigments were produced in different media with varied colors. The aerial and substrate mycelia were well developed branched and unfragmented. The substrate mycelia were firmly attached. At all stages of growth the cells were found as Gram positive. Fine structure of AS II was studied using compound microscope (100X).





## 6.Conclusion

In the present study antagonistic Actinomycetes was isolated from the rhizospheric soil. The isolated strain was studied for morphological characters and determined its antifungal activity by using basic techniques. This isolated strain *AS-II* has good antifungal activity against all the test fungi and has shown highest activity against *M. phaseolina* which was a common fungal plant pathogen in the rhizosphere and causative agent of several root rots. Determination of optimum conditions for the fermentation product, it's other applications, molecular characterization of antibiotic substance was the scope of this study.

## 7. suggestions

- Determination of optimum conditions for the fermentation product, it's other applications, molecular characterization of antibiotic substance was the scope of this study.
- Soil is the resource for several antagonistic microorganisms which can be exploited for the control of plant and human pathogens.
- The new antibiotic should be discovered as it is several years science the discovery of new antibiotic.
- There is an urgent necessity for the new antibiotic in the present scenario of drug resistance.

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**Students at work:**


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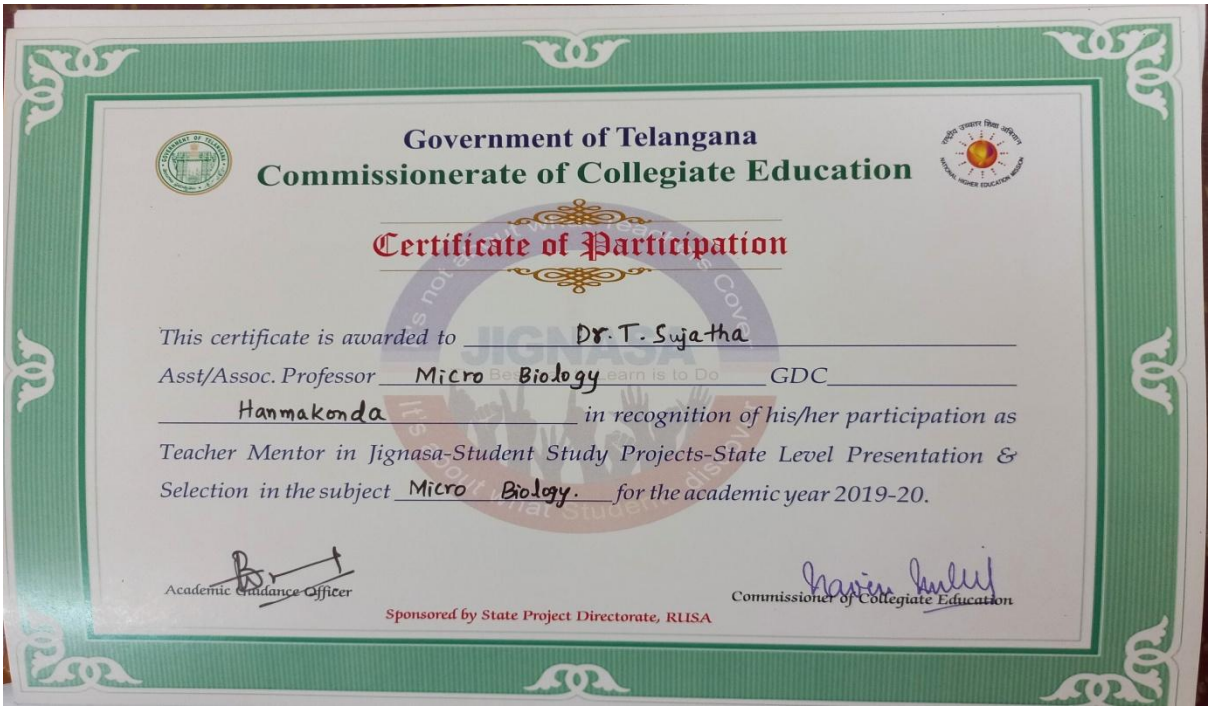

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