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# ANTIMICROBIAL ACTIVITIES OF ACTINOBACTERIA ISOLATED FROM MANGO ORCHARDS

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## ABSTRACT

*Attempts were made to isolate and enumerate actinobacteria from Mango orchards of Vissannapet, Krishna district of Andhra Pradesh. Soil samples were initially analysed for moisture content (11%), pH (7.2), organic carbon (1.10%) and total nitrogen (0.79%). Among the three culture media employed for isolation, yeast extract malt extract dextrose agar supported high colony counts as compared to Inorganic salts starch agar (ISP-4). Out of 30 strains isolated 13 strains exhibited antimicrobial activity. Antimicrobial activity of the secondary metabolites produced by two predominant actinobacterial strains (VL-RK_05 and VL-RK_09) among the 13 strains was high against Bacillus Megaterium and Xanthomonas campestris. The study clearly evidenced that the actinobacteria from mango orchards could exhibit strong antimicrobial potential. It is expected that the current attempt for the isolation of actinobacteria from Mango orchards will be useful for the identification of novel antibiotics effective against various pathogens.*

***KEY WORDS***

*Actinobacteria, Mango orchards, Antimicrobial activity, Secondary metabolites.*

## INTRODUCTION

Natural products and their derivatives are important sources of new medicines [1]. Natural product research is proven to be effective in yielding unique chemical structures with direct application in the treatment of diseases and serve as chemical scaffolds from which molecules with enhanced efficacy are derived. Natural products with industrial applications can be produced from primary or secondary metabolism of living organisms (plants, animals or microorganisms). Natural products obtained from microorganisms are one of the most important sources in the discovery of novel antibiotics successfully introduced in the market and still used today in clinical practice [2]. The total number of marketed drugs used in human therapy is approximately 3500 compounds of which 400-500 are of microbial origin [3].

Actinomycetes (Greek 'atkis'-a ray, 'mykes'-fungus) are Gram positive bacteria having high G+C (>55%) content in their DNA [4]. They are a diverse group of organisms characterized by a complex life cycle, belonging to the phylum Actinobateria that represents one of the largest taxonomic units among the 18 major lineages recognized within the domain Bacteria[5, 6]. Actinomycetes are morphologically diverse ranging from rod to coccoid, fragmenting hyphal forms to those with a highly differentiated branched mycelium[7]. Many of these bacteria also produce external spores. Majority of actinomycetes are free living, saprophytic bacteria, widely distributed in soil, water and

colonizing plants and have been identified as one of the major groups of soil population which may vary with the soil type [8, 9].

Despite the past success of antibiotic drug discovery, at least in the industrially developed world, infectious diseases remain the second-leading cause of death worldwide. Bacterial infections cause 17 million deaths globally, particularly in children and elders. Due to careless and promiscuous use of existing mainline antibiotics, there has been an increased resistance to antibiotics in organisms that are typical human pathogens. Half the percentages of Emerging Infectious Diseases are caused by bacteria that are multi drug resistant and are highly infectious with high mortality rate which are reffered as "superbugs". The therapeutic options for these microbes are reduced, and periods of hospital care are extended and more costly [10]. The emergence of drug resistant pathogens as well as the rise in diseases affecting the immune system greatly intensified the need to investigate new bioactive metabolites of natural origin for potential pharmaceutical and industrial applications. Therefore in the present study, actinobacteria which have been proven as an immeasurable source for chemical and biological diversity of natural products were isolated from unexplored soils of Mango orchards of Vissannapet, Krishna district and screened for the production of secondary metabolites.

## MATERIALS AND METHODS

### Soil characters

Soil samples were randomly collected from Mango orchards of Vissannapet, Andhra Pradesh, for the isolation of actinobacterial strains. The soil samples transported to the laboratory in sterile polythene bags were air dried at room temperature and initially analyzed for physico-chemical properties such as moisture content (%), pH, organic carbon (%) and total nitrogen content (%).

### Moisture content

To determine the moisture content, 10 g of soil sample was dried in a hot air oven at 105°C until a constant weight is obtained. The difference between the weights of pre-drying and post-drying was taken as the moisture content of the initial soil sample.

### Soil pH

The pH of the soil sample was determined with the help of digital pH meter of model Di-707 (Jackson, 1973) [11]. 20 g of soil sample was taken in a 100 ml beaker and 40 ml of distilled water was added. The suspension was mixed thoroughly and allowed to settle the suspended clay particles from the suspension for about 1h before recording the pH.

### Organic carbon

Organic carbon content of the soil sample was estimated by following Walkey and Black method (1934) [12]. 10 g of soil sample was taken into 500 ml Erlenmeyer flask. To this 10 ml of 1N $K_2Cr_2O_7$ was added and shaken gently to disperse the soil into the solution. 20 ml of conc. $H_2SO_4$ was added followed by vigorous agitation for 1min. 200 ml of distilled water was added and filtered. Few drops of O- phenanthroline indicator was added to the filtered solution and titrated against 0.5N $FeSO_4$. $7H_2O$. Sample without soil served as blank. The amount of organic carbon in the soil sample was calculated by using the following formula.

$$\text{Organic carbon (\%)} = \frac{(\text{milliequivalents of } K_2Cr_2O_7 - \text{milliequivalents of } FeSO_4.) \times 0.003 \times 100}{\text{Soil weight (g)}} \times f$$

$f$ = correlation factor = 1.33

**Total nitrogen**

Total nitrogen in the soil sample was determined by Micro-Kjeldahl method [13]. Finely sieved soil sample (100 g) was taken into a digestion flask and 2 g of $K_2SO_4$, 90 mg of mercuric oxide and 2 ml of conc. $H_2SO_4$ were added, mixed thoroughly and kept for digestion on heater. Before carrying out distillation process, limited addition of distilled water (5ml) to the flask was followed by the addition of sodium hydroxide-sodium thiosulphate solution. Ammonia was collected in boric acid. The distillate (20ml) collected was titrated against 0.02N HCl and appearance of violet color is the end point. Blank was maintained using equal volume of distilled water instead of distillate. Total nitrogen present in the soil sample was calculated by using the formula.

$$\text{Total nitrogen} = \frac{\text{HCl (ml) in sample} - \text{HCl (ml) in blank} \times \text{normality of acid} \times 14.01 \times 100}{\text{Soil weight (mg)}}$$

**Isolation of actinobacterial strains**

Soil samples collected from Mango orchards, at a depth of 6-10 cm were pretreated with calcium carbonate (1:1 w/w) and dried at 45°C for 1 h in order to reduce the incidence of bacteria and molds. Yeast extract malt extract dextrose agar (YMD), ISP-4 (Inorganic salts starch agar) and asparagine-glucose agar media were prepared, sterilized at 15 lbs pressure (121°C) for 15 min and poured into Petri plates under aseptic conditions. Antibiotics such as rifamycin and fluconozole (50 µg/ml) were added to the media just before pouring into Petri plates. Soil dilution plate technique was employed for the isolation of actinobacterial strains. The pretreated soil sample (1g) was suspended in 100 ml of sterile distilled water. Serial dilutions were prepared and 0.1ml of $10^{-4}$ and $10^{-5}$ dilutions were plated on International Streptomyces Project (ISP) media including YMD agar (ISP-2) and inorganic salts starch agar (ISP-4). The inoculated plates were incubated at 28 ± 2°C for 10 days. After incubation, actinobacterial strains distinguished from other microbial colonies by characteristics such as tough and leathery colonies partially submerged into the agar were isolated. The colonies were picked and the specks of the colonies were streaked over the YMD agar medium followed by incubation at 30°C for 7 days. Further, pure cultures were maintained on YMD agar slants and stored at 4°C for further study.

**Screening of actinobacterial strains for bioactive metabolites**

A total of 30 actinobacterial strains were isolated and screened for antimicrobial activity and found 13 strains were found predominant on ISP-2, ISP-4, and asparagine glucose agar with antimicrobial activity. The secondary metabolites produced by actinobacterial strains were extracted by the method of Elliah *et al.* (2005) [14]. The pure culture of the strains was transferred aseptically and individually into the seed medium (ISP-2 broth). After 48 h of incubation, the seed culture at a rate of 10% was inoculated into the production media of the same composition. The fermentation was carried out at 35°C for 5 days under agitation at 250 rpm. After 120h of incubation, the culture filtrates harvested from the flasks were extracted twice with ethyl acetate and the pooled solvent extracts were evaporated to dryness under vacuum to yield a crude residue. For the extraction of secondary metabolites, similar protocol was followed for all the strains. The residues were then dissolved in 0.2 ml of dimethyl sulphoxide (DMSO) and diluted with 0.8 ml of distilled water. Extracts thus collected were used for antibacterial assay.

Two predominant strains with strong antimicrobial activity designated as VL-RK_05 and VL-RK_09 were selected for further studies. Antimicrobial profile of both the strains was evaluated at every 24 h interval for 7 days by inoculating in YMD broth at 35°C. The cultures of ***Staphylococcus aureus*** **(MTCC 3160),** ***Bacillus megaterium*** **(NCIM 2187),** ***Shigella flexneri*** **(MTCC 1457),** ***Xanthomonas campestris*** **(MTCC 2286),** ***Proteus vulgaris*** **(ATCC 6380),** ***Pseudomonas aeruginosa*** **(ATCC 9027)** and ***Escherichia coli*** **(ATCC 9027)** grown overnight at 37°C were employed for antibacterial assay. ***Candida albicans*** **(MTCC 183)** was used as test fungus.

**Results and Discussion**

Characteristics of soil samples collected from Mango orchards, Vissannapet, Krishna district were analysed and the recorded parameters are presented in **Table 1**. The moisture content of the soil sample is 11%, pH 7.2, organic carbon- 1.1% and the total nitrogen is 0.79%. Soils with slightly alkaline or neutral pH together with 10-15% moisture content and high organic content were reported to support high incidence of actinobacteria [15, 16].

**Table 1: Characteristics of soil samples collected from Mango orchards, Vissannapet, Krishna district.**

| Soil Characters | Result |
|---|---|
| **Moisture content** | 11% |
| **Soil pH** | 7.2 |
| **Organic carbon** | 1.10% |
| **Total Nitrogen** | 0.79 |

**Table 2: Enumeration of actinobacterial colonies on different media**

| Media | Actinobacterial population/g of dry wt. of soil |
|---|---|
| **ISP-2** | $3.2\times10^4$ |
| **ISP-4** | $1.5\times10^4$ |
| **Asparagine glucose agar** | $1.8\times10^4$ |

**Isolation & screening of actinobacterial strains**

The air dried soil samples pretreated with calcium carbonate as suggested by El-Nakeeb and Lechevalier (1963) were employed for the isolation and enumeration of actinobacterial strains [17]. Out of several pretreatment procedures, pretreatment of soil samples with calcium carbonate was reported to be the most efficient technique for the preferential isolation of actinobacteria [18-20]. Serially diluted soil sample (0.1 ml) was plated on ISP-2, ISP-4 and asparagine-glucose agar media supplemented with rifampicin (50µg/ml) and fluconozole (50 µg/ml) and incubated at 30°C. After 10 days of incubation the actinobacterial population was enumerated. High counts of actinobacteria were recorded on ISP-2 followed by Asparagine glucose agar (**Table 2**).

A total of 30 actinobacterial strains designated as VL-RK_01 to VL-RK_30 were isolated and initially subjected to screening for their antimicrobial potential. Among the tested ones, 13 strains (Plate-1) exhibited antimicrobial activity. The antimicrobial spectrum of the 13 strains against test organisms is presented in **Table 3**. The strains designated as VL-RK_05 (Plate-2) and VL-RK_09 (Plate-3) exhibited high antimicrobial potential against the test microorganisms, hence selected for further study.

Antimicrobial profiles of the secondary metabolites produced by predominant actinobacteria strains viz., VL-RK_05 and VL-RK_09

in YMD broth were evaluated by extracting the culture filtrates at regular intervals of 24 h up to 168 h.

Data on the antimicrobial activity of the ethyl acetate extracts of the strain VL-RK_05 are recorded in **Table 4**. The metabolites collected from 120 h old culture of the strain VL-RK_05 exhibited high antibacterial activity against *Bacillus megaterium* and *Xanthomonas campestris* among the bacteria tested and *Candida albicans* (yeast). A gradual rise in the antibacterial spectrum was observed with increasing age of the culture up to 5 days. Thereafter, a subsequent decline in its antibacterial activity was noticed. Of all the test organisms, *B. megaterium* was highly sensitive to the metabolites of the strain.

The antimicrobial activity of the secondary metabolites produced by the strain VL-RK_09 is depicted in **Table 5**. The crude extract obtained from 120 h old culture of the strain exhibited activity on all the bacteria tested. Of all the test organisms, *B. megaterium* was highly sensitive to the metabolites produced by the strain. The metabolites produced by the strain could also inhibit *Candida albicans*.

**Table 3: Antimicrobial activity exhibited by different actinobacterial strains isolated from the Mango orchards**

| S.No | Code of Isolate | Antimicrobial activity recorded in terms of zone of inhibition in diameter (mm) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | ***Sa*** | ***Bm*** | ***Xc*** | ***Pv*** | ***Pa*** | ***Ec*** | ***Can*** |
| 1 | VL-RK_01 | 13 | 14 | 12 | 8 | 15 | 6 | 12 |
| 2 | VL-RK_02 | 5 | 9 | 7 | - | 5 | - | 9 |
| 3 | VL-RK_03 | 13 | 13 | 9 | 10 | 14 | 7 | 13 |
| 4 | VL-RK_04 | 9 | 10 | 8 | 5 | 7 | 5 | 8 |
| **5** | **VL-RK_05** | **15** | **16** | **16** | **12** | **18** | **9** | **16** |
| 6 | VL-RK_06 | 5 | 9 | 12 | 3 | 4 | 4 | 7 |
| 7 | VL-RK_07 | 6 | 7 | 10 | 2 | 5 | 3 | 5 |
| 8 | VL-RK_08 | 5 | 4 | 7 | - | 6 | - | 6 |
| **9** | **VL-RK_09** | **16** | **19** | **17** | **14** | **18** | **13** | **17** |
| 10 | VL-RK_10 | 12 | 15 | 15 | 9 | 15 | 8 | 13 |
| 11 | VL-RK_11 | 6 | 8 | 7 | 5 | 4 | 6 | 6 |
| 12 | VL-RK_12 | 14 | 15 | 13 | 10 | 14 | 7 | 12 |
| 13 | VL-RK_13 | 9 | 10 | 8 | 6 | 9 | 5 | 8 |

***Sa-Staphylococcus aureus; Bm-Bacillus megaterium; Xc-Xanthomonas campestris; Pv;Proteus vulgaris; Ec-Escherichia coli; Can-Candida albicans***

**Table 4: Antimicrobial activity of ethyl acetate extracts of the actinobacterial strain VL-RK_05 grown in ISP-2 broth.**

| S.No | Time of Incubation in Hrs. | Wt. of biomass mg/100ml | Antimicrobial activity of the strain VL-RK_05 in terms of Zone of inhibition(mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Sa* | *Bm* | *Sf* | *Xc* | *Pv* | *Pa* | *Ec* | *Can* |
| 1 | 24 | 48 | 0 | 3 | 2 | 4 | 3 | 4 | - | 4 |
| 2 | 48 | 76 | 6 | 8 | 7 | 8 | 6 | 8 | 2 | 6 |
| 3 | 72 | 123 | 0 | 10 | 8 | 10 | 7 | 11 | 6 | 8 |
| 4 | 96 | 203 | 12 | 14 | 13 | 14 | 9 | 13 | 8 | 9 |
| **5** | **120** | **223** | **16** | **21** | **16** | **20** | **15** | **16** | **13** | **17** |
| 6 | 144 | 223 | 13 | 16 | 13 | 15 | 10 | 13 | 7 | 13 |
| 7 | 168 | 163 | 11 | 12 | 9 | 11 | 7 | 10 | 5 | 9 |
| 8 | 192 | 143 | 10 | 8 | 7 | 9 | 5 | 8 | 4 | 4 |

*Sa-Staphylococcus aureus; Bm-Bacillus megaterium; Xc-Xanthomonas campestris; Pv-Proteus vulgaris; Ec-Escherichia coli; Can-Candida albicans*

**Table 5: Antimicrobial activity of ethyl acetate extracts of the actinobacterial strain VL-RK_09 grown in ISP-2 broth.**

| S.No | Time of Incubation in Hrs. | Wt. of biomass mg/100ml | Antimicrobial activity of the strain VL-RK_09 in terms of Zone of inhibition(mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Sa* | *Bm* | *Sf* | *Xc* | *Pv* | *Pa* | *Ec* | *Can* |
| 1 | 24 | 70 | 6 | 6 | 5 | 5 | 4 | 5 | 2 | 4 |
| 2 | 48 | 80 | 9 | 8 | 7 | 8 | 6 | 8 | 4 | 6 |
| 3 | 72 | 130 | 11 | 10 | 8 | 10 | 7 | 11 | 6 | 8 |
| 4 | 96 | 160 | 14 | 14 | 12 | 13 | 9 | 13 | 8 | 9 |
| **5** | **120** | **160** | **18** | **22** | **18** | **21** | **16** | **18** | **15** | **19** |
| 6 | 144 | 80 | 13 | 16 | 13 | 15 | 10 | 13 | 7 | 13 |
| 7 | 168 | 60 | 11 | 12 | 10 | 11 | 7 | 10 | 5 | 9 |
| 8 | 192 | 50 | 10 | 8 | 7 | 9 | 5 | 8 | 4 | 4 |

*Sa-Staphylococcus aureus; Bm-Bacillus megaterium; Xc-Xanthomonas campestris; Pv-Proteus vulgaris; Ec-Escherichia coli; Can-Candida albicans*

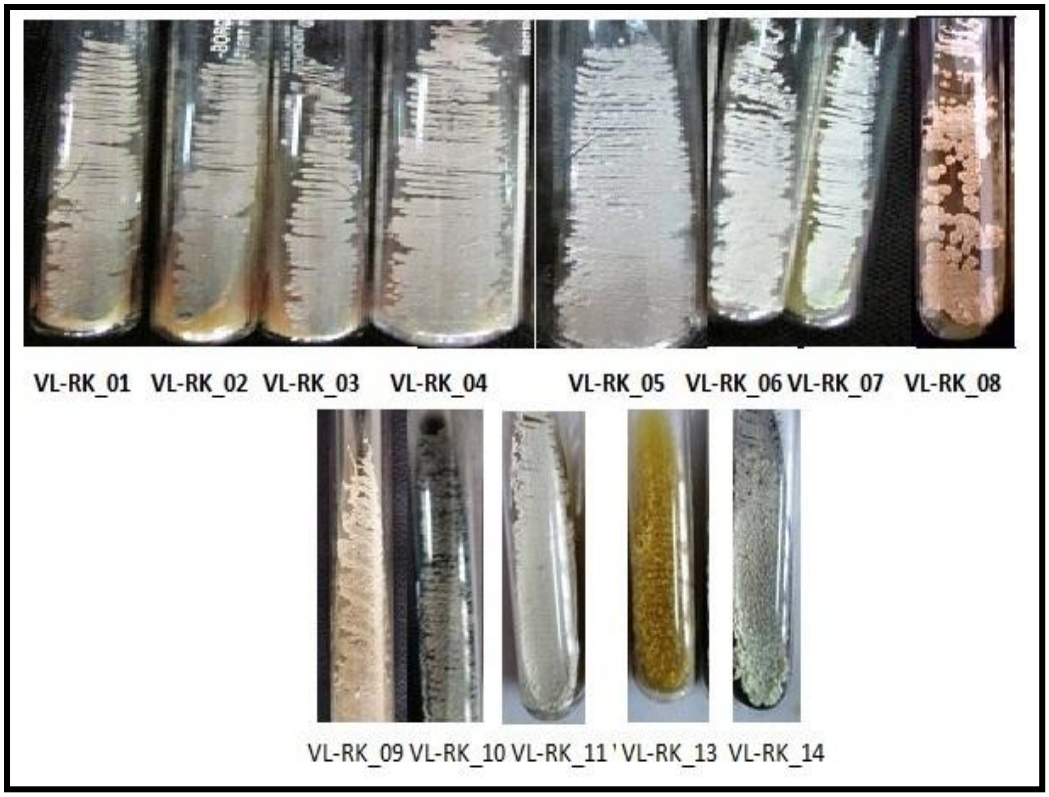


**Plate 1: Actinobacterial strains exhibiting antimicrobial activity (VL-RK_01 to VL-RK_13) isolated from Mango orchards.**

**Plate 2: Actinobacterial strain VL-RK_05 grown on ISP-2 medium**

**Plate 3: Actinobacterial strain VL-RK_09 grown on ISP-2 medium**

## DISCUSSION

Chemical defenses employed by microbes have proven to be the available sources of novel molecules providing lead compounds for drug discovery. Therefore, the search for potent metabolites of actinobacteria for continuous struggle against the multi-drug resistant pathogens as well as the emergence of new pathogens is going on progressively. In this regard, the actinobacterial strains opted in the present work may provide novel secondary metabolites which will act as the logical starting point for discovering new drugs to fight against the existing and emerging infectious diseases.

Actinobacterial strains VL-RK_05 and VL-RK_09 selected for the present study from soils of Mango orchards were screened for antibacterial metabolites. The secondary metabolites of five-day old cultures of both the strains were highly inhibitory to the test bacteria. Crude extracts of five day old cultures of *Rhodococcus erythropolis* VLK-12 [21] and *Streptomyces purpeofuscus* and *S. albidoflavus* were active against Gram positive as well as Gram negative bacteria and fungi [22]. Secondary metabolites extracted from five day old cultures of *Streptomyces* sp. CDRIL-312 [23] and *Streptomyces* spp. [24] exhibited good antifungal activity. Five day old culture of *Streptomyces clavuligerus* was reported to produce good yield of Clavulanic acid [25]. Hence, screening of actinobacteria still continues as a fruitful source for the extraction of diverse array of secondary metabolites and the strains VL-RK_05 and VL-RK_09 explored in the present study may elaborate potent metabolites which might form the basis for the synthesis of novel therapeutic drugs to avoid the ongoing crisis of multiple drug resistant pathogens.

## CONCLUSION

The present study is mainly involved in the isolation and enumeration of actinobacteria with antimicrobial potential from Mango orchards, Vissannapet, Andhra Pradesh. The study clearly evidenced that the actinobacteria from mango orchards could exhibit strong antimicrobial potential. It is expected that the current attempt for the isolation of actinobacteria from Mango orchards will be useful for the identification of novel antibiotics effective against various pathogens.

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