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Antibacterial Activity of *Streptomyces* Species (T7) Isolated from Kanjamalai Hills of South India

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ABSTRACT

The emergence of drug resistance to antimicrobial agents is the biggest threat to public health. Hence novel antibiotics from soil microorganisms are timely needed for the control of several pathogenic microorganisms. Actino bacteria from soil samples collected from Kanjamalai hills of South India were isolated using starch casein agar medium. Based on the cultural morphology, a total of 12 actinobacterial strains were selected for further investigations. In primary antimicrobial screening by cross streak method, actinobacterial strain T7 showed broad-spectrum antibacterial activity. Bioactive metabolites from actinobacterial strain T7 was produced by adopting submerged fermentation using soy bean meal medium. In secondary antimicrobial screening by agar-well diffusion method, culture filtrate from strain T7 showed promising antibacterial activity. Further crude extract from the culture filtrate was extracted using ethyl acetate and tested for antibacterial activity by agar- well diffusion method, which showed good activity against *Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Shigella dysenteriae and Serratia marcescens.* Based on the studied phenotypic characteristics actinobacterial strain T7 was identified as *Streptomyces* species. Findings of the present study concluded that Kanjamalai hills was the potential ecosystem for antagonistic actino bacteria which deserves for bioprospecting. The antagonistic actinobacterium T7 could be source of antimicrobial bioactive substance since it showed antimicrobial activity against all the tested bacterial pathogens.

Keywords: Actinobacteria, Kanjamalai hills, antimicrobial screening, bioactive compound.

INTRODUCTION

Novel broad-spectrum antimicrobial agents are immediately needed to combat frequently emerging multi-drug resistant microorganisms. Even though significant progress is being made in the field of chemical synthesis, nature still remains the richest and the most versatile source for new antibiotics [1]. Screening of microbial products continues to represent an important route to the discovery of novel chemicals for development of new broad-spectrum therapeutic agents [2]. Actinobacteria, isolated from unexplored regions of the world may be the ultimate solution to this problem [3].

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Actinobacteria are ubiquitous in the world that surrounds us, but they seem to prefer the solid constituent of earth. Actinobacteria have been recognized as the prime sources of antibiotics, siderophores, antitumor agents, immunosuppressive agents, enzymes and enzyme inhibitors[4]. Moret han 16,500 of the naturally occurring antibiotics discovered are synthesized by this group of microorganisms[5].Actinobacteria have provided many important bioactive substances of good commercial value and continue to be regularly screened for new bioactive compounds. A search for literature revealed that more than 4,607 patents have been issued on actinobacteria related products and processes [6].

Recently, the rate of discovering new antibiotics from terrestrial actinobacteria has decreased but the rate of re-isolation of known actinobacteria and antibiotics is on the increase. This has led the researchers to explore unique environments for potentially new biosynthetic diversity. India with its vast geographical diversity offers unique environmental dynamics that support prospering of the diverse group of microorganisms which also include different kinds of actinobacteria [3].With a view to screen a unique environment, the present study was undertaken for bioprospecting of actinobacteria from Kanjamalai hills of South India with special reference to antibacterial activity.

MATERIALS AND METHODS

Collection of Soil Samples

About 100 g of soil samples were collected at the depth of 20 cm from four different stations of Kanjamalai hills (Latitude:11°62' N; longitude:78°05' E), Tamil Nadu, India. The soil sample was transported in sterile polythene bags to the laboratory within 2 hours of collection. Each sample was crushed, thoroughly mixed and sieved through a 2 mm pore size mesh and used for isolation of actinobacteria.

Pretreatment and Isolation of Actinobacteria

One gram of soil sample was shade dried for 1 week and transferred to a flask containing 99 mL of sterile distilled water and mixed thoroughly in a shaker for 30 minutes at 150 rpm. The soil suspension was further diluted up to 10^{-6} levels. 1 mL of the diluted suspension was spread over the surface of starch casein agar medium supplemented with cycloheximide (100 µg/mL) and nalidixic acid (20 µg/mL). The Petri plates were then incubated at 28°C and the colonies were observed from third day onwards and up to one month. Strains of actinobacteria were picked out and purified by repeated streaking on yeast extract-malt extract agar (ISP-2).Purified actinobacterial isolates were then inoculated on ISP-2 agar slants, labelled, incubated and then stored at 4°C in the refrigerator for further analysis.

Primary Antimicrobial Screening

All the selected actinobacterial strains were screened for their antibacterial activity by cross streak method, using modified nutrient glucose agar (MNGA) medium [7] against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Shigella dysenteriae, Serratia marcescens and Bacillus cereus*. The actinobacterial strains were inoculated on MNGA medium as a straight line. After incubation at 28°C for 5 days, 18 hours old test bacterial pathogens were inoculated at right angle to the original streak of the actinobacterial strain and incubated at 37°C for 24 hours. Actinobacterial isolates which showed broad-spectrum antimicrobial activity were used for secondary screening.

Fermentation Process

The crude bioactive compounds were produced from the potential actinobacterial strain through submerged fermentation by adopting shake flask method. The actinobacterial strain T7 was grown in ISP-2 agar medium for six days at 28°C. Spores were then harvested and a homogenous spore suspension in 0.05% Tween 20 was prepared. Five millilitre of this suspension was transferred into each 100 mL of soybean meal medium [8] and incubated in a rotary shaker with 120rpm at 28°C for 7 days. After fermentation, broth was filtered using Whatman No.1 filter paper.

Secondary Antimicrobial Screening

Secondary antimicrobial screening of actinobacteria was detected by agar-well diffusion method on nutrient agar. The bacterial cultures were inoculated on nutrient agar plates using sterile cotton swabs. In each of these plates, 5 mm wells were cut out using well cutter and loaded with 100 µL of cell free culture supernatant and incubated at 37°C for 24 hours. After incubation, agar plates were observed for the presence of a zone of inhibition around the wells.

Extraction of Bioactive Compound

The culture filtrate of actinobacterial strain T7 was used for solvent extraction with ethyl acetate. The ratio of the filtrate and solvent (1:1 v/v) was taken in a separating funnel and shaken vigorously. Extraction was repeated three times with the same solvent. The solvent phase that contains the antibiotic was separated from the aqueous phase by separating funnel and evaporated to dryness in water bath at 80°C to 90°C. The crude extract thus obtained was stored at 4°C.

In-vitro Antibiosis

The crude extract (1mg) was dissolved in 1 mL of dimethyl sulfoxide : water (1:9) and was used for antimicrobial activity study using agar-well diffusion method [9].The18-hours old broth test bacterial cultures were inoculated on freshly prepared nutrient agar plates by using sterile cotton swab. Then 5 mm well was made on the nutrient agar plates using a well cutter and loaded with 100 µL of crude extract and incubated at 37°C for 24 hours during which antimicrobial activity was indicated by the presence of a zone of inhibition surrounding the well. Antibacterial activity was expressed as the mean of diameter of the inhibition zones (mm) produced by the secondary metabolites.

Phenotypic Characterization of Potential Actinobacterial Strain

Spore chain morphology

The sterilized cover slips were carefully inserted at an angle of 45 degrees in to the solidified ISP-2 medium in Petri dish, until about half of the cover slip was buried in the medium. The actinobacterial isolates were inoculated along the line where the medium meets the upper surface of the cover slip. After incubation for 7 to 10 days, the cover slip was carefully removed and placed downwards on the slide and directly examined under the high power of a microscope [10].

With regard to spore chains, the strains could be grouped into 'sections'. The species belonging to the genus *Streptomyces* were divided into three sections, namely rectiflexibiles (RF), retinaculiaperti (RA) and Spirales(S). When a strain forms two types of spore chains, both were noted [11].

Colour of aerial spore mass

The aerial mass colour of mature sporulating aerial mycelium was recorded after incubation for seven, 14 and 21 days at 25°C in oat meal agar (ISP-3) plates. The aerial mass colour was classified according to Nonomura [12] in the following colour series: white (W), gray (Gy), red (R), yellow (Y), green (Gn), blue (B) and violet (V). When the aerial mass color falls between two colours series, both the colours were recorded. If the aerial mass color of a strain to be studied showed intermediate tints, both the colour series were noted

Colour of substrate mycelium

The colour of the substrate mycelium was recorded after incubation for seven, 14 and 21 days at 25°C in yeast extract- malt extract agar (ISP-2) and oat meal agar (ISP-3) plates. The substrate mass colour was assigned according to Szabo and Marton [13] in the following colour groups: yellow-brown, yellow-brown + red (or orange), yellow brown + blue or violet and yellow-brown + green. Cultures forming characteristic pigments were recorded as distinctive (+). Absence of characteristic pigment was recorded as not distinctive or none (-). A colour with low chroma such as olive, pale yellow, or yellowish brown occurs, it was included in the latter group (-).

Soluble colours other than melanoid pigmentation

The test was carried out on yeast extract-malt extract agar (ISP-2), oat meal agar (ISP-3), inorganic salts-starch agar (ISP-4) and glycerol-asparagine agar (ISP-5). The inoculated plates were observed for soluble pigments other than brown or black. Cultures forming red, orange, green, yellow, blue and violet pigments were recorded as positive. Absence of soluble colours other than melanoid pigments were recorded as negative [12].

RESULTS AND DISCUSSION

Pretreatment and Isolation of Actinobacteria

A total of 12 different actinobacterial strains were recovered from soil sample, using starch casein agar medium supplemented with cycloheximide(100 µg/mL) and nalidixic acid (20 µg/mL). Colonies having characteristic features such as leathery, powdery appearance with convex, concave, or flat surface and color ranging from white, gray to pinkish and yellowish were selected. This observation indicated

that starch casein agar medium with antibacterial and antifungal antibiotics are effective for the isolation of actinobacteria. It is well-known that pre-treatment and selective media are needed to avoid isolates of interest being overrun by unwanted fast growing gram-negative bacteria and fungi [14].

Primary Antimicrobial Screening

The actinobacterial isolates were screened for antibacterial activity by cross streak method against clinical pathogens (Figure 1).Out of twelve actinobacterial strains, isolate T7 was able to produce inhibitory bioactive compounds against *Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Shigella dysenteriae* and *Serratia marcescens. Klebsiella pneumoniae* and *Bacillus cereus* were found to be resistant to the bioactive compound. Actinobacterial strain T7 was selected for its broad-spectrum of antimicrobial activity for further studies.

Screening of microbial strains for antagonistic activity is prerequisite for any natural product drug discovery programme. Crowded plate method, agar overlay method, cross streak method and agar plug method are some common methods which are in practice from ancient times for primary screening for the detection of antagonistic activity [15]. In the present study, cross streak method was used for primary antimicrobial screening which was simple, less laborious and user friendly.

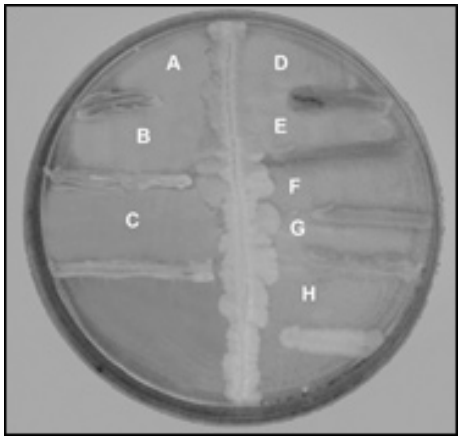

Figure 1: Primary antimicrobial screening of actinobacterial strain T7.

A: Escherichia coli, B: Staphylococcus aureus, C: Bacillus cereus, D: Proteus mirabilis, E: Klebsiella pneumoniae, F: Pseudomonas aeruginosa, G: Serratia marcescens, H: Shigella dysenteriae

Fermentation Process and Secondary Antimicrobial Screening

During submerged fermentation, actinobacterial strain T7 showed good growth in soy bean meal medium. The cell free culture supernatant of actinobacterial strain T7 showed antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Shigella dysenteriae* and *Serratia marcescens* (Figure 2).

In most of the studies, the antimicrobial activity of crude compound was tested only after extraction from culture broth using solvents like ethyl acetate [16]. In the present study, before extraction using solvents, the cell free supernatant was tested for antimicrobial activity by agar-well diffusion method which showed that the antibacterial activity of potential actinobacterial strain is due to the production of extracellular bioactive compounds.

Table 1: Antibacterial Activity of Ethyl Acetate Extract of Actinobacterial Strain T7

S.No	Test Microorganisms	Antibacterial activity (Diameter of zone of inhibition in mm)
1	*Staphylococcus aureus*	20
2	*Escherichia coli*	18
3	*Proteus mirabilis*	20
4	*Pseudomonas aeruginosa*	12
5	*Shigella dysenteriae*	21
6	*Serratia marcescens*	22

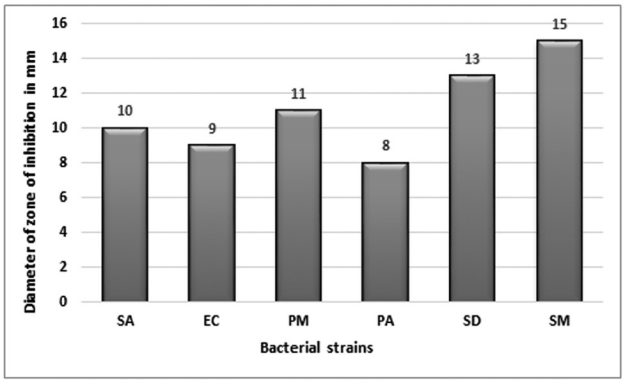

Figure 2: Secondary antimicrobial screening of actinobacterial strain T7.

SA: Staphylococcus aureus, EC: Escherichia coli, PM: Proteus mirabilis, PA: Pseudomonas aeruginosa, SD: Shigella dysenteriae, SM: Serratia marcescens

Extraction of Bioactive Compound and *In-vitro* Antibiosis

Ethyl acetate extract of actinobacterial strain T7 showed good antibacterial activity against *Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Shigella dysenteriae* and *Serratia marcescens* (Table 1, Figure 3). Mohanraj et al[15] have also used ethyl acetate in their studies for the extraction of extra cellular bioactive compounds from actinobacteria,

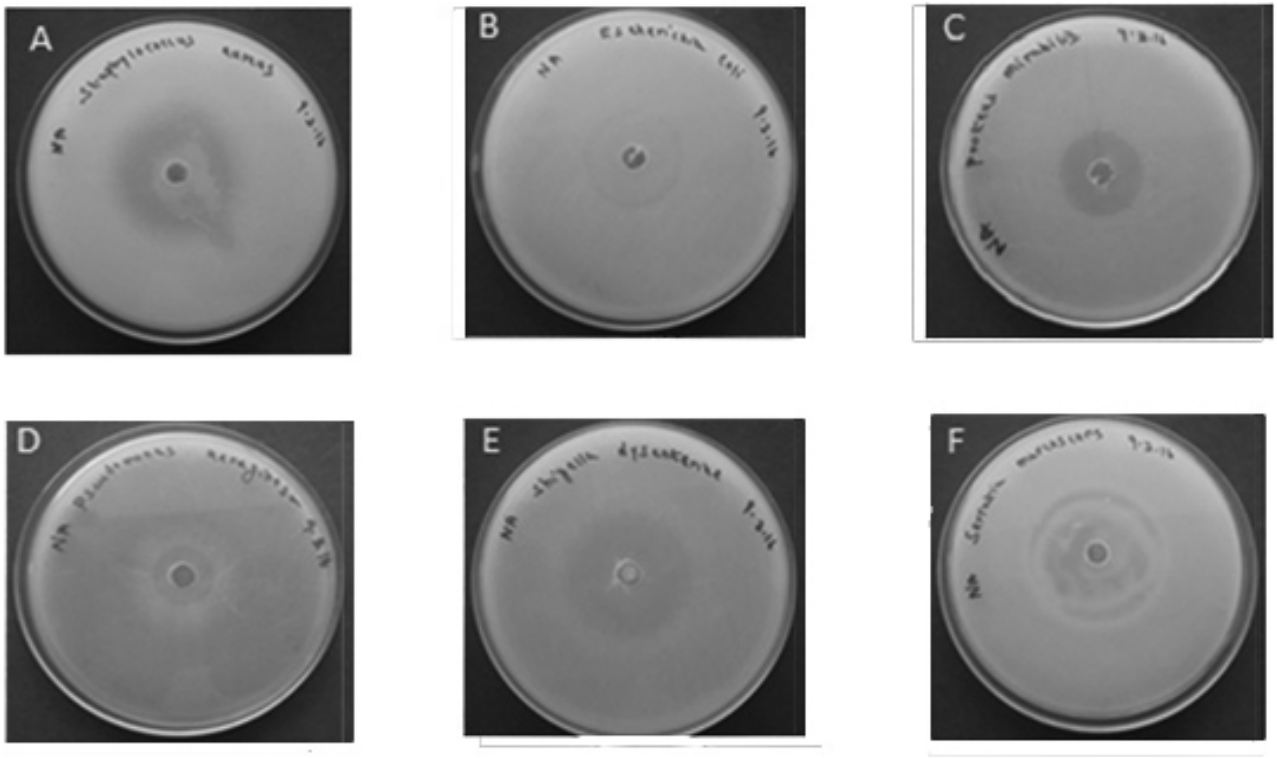

Figure 3: Antibacterial activity of actinobacterial strain T7 against bacterial pathogens.

A: Staphylococcus aureus, B: Escherichia coli, C: Proteus mirabilis, D: Pseudomonas aeruginosa, E:Shigella dysenteriae, F: Serratia marcescens

Phenotypic Characterization of Potential Actinobacterial Strain

The actinobacterial isolate T7 showed good growth on ISP-2, ISP-3, ISP-4 and ISP-5 media and moderate growth on ISP-1 medium. The morphological characteristics were studied by light microscopy on the 7th, 14th and 21st day culture in ISP-2 media. The actinobacterium produced straight to flexible hyphae and long chain rectiflexible (RF) spores (Figure 4). The organism produced yellow-white (YW) aerial mycelium, yellow reverse pigment and did not produce soluble pigment and melanoid pigment. Based on the phenotypic characteristics the strain T7 was identified as *Streptomyces* species (Table 2).

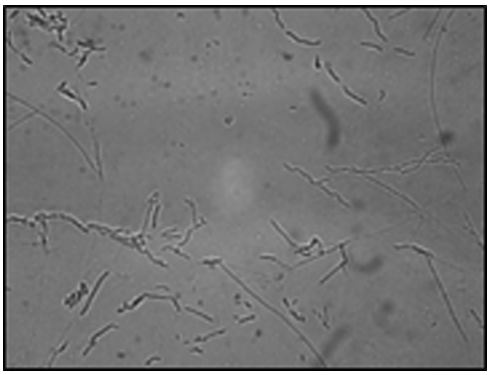

Figure 4: Light micrograph of actinobacterial strain T7 showing rectiflexibiles (RF) Spore Chain Morphology

Table 2: Phenotypic Characteristics of Actinobacterial strain T7

S.No	Characteristics	Responses of Actinobacterial strain T7
Morphological Characteristics		
1	Cell shape	Mycelial
2	Aerial mycelium	+
3	Substrate mycelium	+
4	Spore chain morphology	Rectiflexibiles (RF)
Cultural and Physiological Characteristics		
5	Colony consistency	Powdery
6	Aerial mass colour	Yellow-White
7	Reverse side pigment	Yellow
8	Soluble pigment	-
9	Melanoid pigment	-
10	Growth on ISP-1medium	Moderate
11	Growth on ISP-2 medium	Good
12	Growth on ISP-3 medium	Good
13	Growth on ISP-4 medium	Good
14	Growth on ISP-5 medium	Good

+ Positive; - Negative

CONCLUSION

The findings of the present study showed that Kanjamalai hills in South India was the potential ecosystem for antagonistic actinobacteria which deserves for bioprospecting. *Streptomyces* species (T7) have a great potential to produce bioactive compounds against bacterial pathogens enabling the discovery of new antibiotics and hence merit in future studies.

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