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STUDIES ON ANTIBIOTIC PRODUCING POTENTIAL OF ACTINOMYCETES ISOLATED FROM DUMP YARD SOIL AND SEDIMENTS FROM FRESH WATER RESERVOIR.

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ABSTRACT

Objective: To isolate and screen potential antibiotic producing Actinomycetes from hospital dump yard soil and sediments of Bhadrakali reservoir, Warangal. **Methods:** Soil dilution – plating method was used to isolate Actinomycetes. Cross-streak method for primary screening, well-diffusion method for secondary screening of crude extracts and TLC to detect secondary metabolites was performed. **Results:** Of the twelve isolates of soil and freshwater sediments, eight were potential producers of antimicrobial metabolites. One soil isolate and three aquatic Actinomycetes were effective inhibitors of pathogenic bacteria employed. **Conclusions:** The present study is aimed to screen Actinomycetes from dump yards and to screen sediments as novel sources of antibiotics. The isolation of potential secondary metabolites producing Actinomycetes may help to meet the need of search of new antimicrobials. Most of the isolates from water sediments exhibited good antimicrobial activity, which can be further used in new drug discovery.

KEYWORDS: Actinomycetes, cross streak method, secondary metabolites, antimicrobials.

INTRODUCTION

Antibiotics are antimicrobial compounds, which play a vital role primarily in treatment of bacterial infections, in Biochemistry, Molecular biology, Microbiology, Pharmacology, Organic chemistry and even in tissue culture techniques and screening methods. Due to their wide applications there is continuous search for novel antibiotics.[1] Actinomycetes comprise a diverse group of Gram-positive, aerobic, filamentous bacteria. They are known to produce extensive variety of secondary metabolites including most of the antibiotics. About 80% of world's antibiotics come from Actinomycetes, especially from *Streptomyces* and *Micromonospora*, which can be isolated from sediments. Only species of *Streptomyces* produces 70% of total antibiotics. Though Actinomycetes are found in various habitats like dump yards, soils, aquatic habitats such as streams, lakes, rivers, beach sands, marine and fresh water sediments, they are rarely isolated from untamed habitats like water sediments.[2,3]

Actinomycetes are bacteria of the family Actinomycetacea and are most economically and biotechnologically valuable prokaryotes known to produce 70-80% of relevant secondary metabolites. They are producers of several important bioactive compounds, especially antibiotics, anti-tumor agents, immunosuppression agents, enzymes etc. Though there is continuous production of synthetic chemicals as antimicrobials, Actinomycetes remain as relevant source of novel, natural antibiotics, which help combat problems associated with rapid spread of antibiotic-resistant pathogens causing life threatening infections.[4,5]

Though traditionally, terrestrial Actinomycetes were source of bioactive compounds for last 4 decades, recently these have been isolated from aquatic sediments. Literature reveals that about 7600 components have been produced from only *Streptomyces* species. But aquatic, especially marine Actinomycetes explored, like *Micromonospora* which produces Rifamycin, which have high commercial value in treatment.[6] Antibiotics truly known as wonder drugs and attack every type of microbial activity by interfering with DNA, RNA proteins and membrane function, electron transport, germination, sporulation etc. But due to emergence of drug resistance among pathogenic bacteria, there is a dire need of efficient, novel antimicrobial product with new mechanism of action.[7]

As Actinomycetes exist in various habits in nature, which also influences compounds produced by them, screening them from diverse habitats may help in

extracting some novel bioactive compounds. In present investigation Actinomycetes were isolated from various habitats like hospital dump yards, aquatic sediments and screened for their production of antimicrobial compounds which may lead researchers to valuable conclusions towards novel drug discovery.

2. MATERIALS AND METHODS

2.1. Collection of soil sample and sediments

Soil samples from hospital dump yards were collected in sterile plastic pouches, sealed and brought to the laboratory. Similarly sediments from Bhadrakali fresh water reservoir, were collected in sterile glass tubes and brought to the laboratory, 1g of each sample were added to 100ml of sterile water and serially diluted up to 6 fold under aseptic conditions.[8]

2.2. Isolation and culture conditions

Mc Beth scale starch mineral agar, starch casein agar, glycerol asparagine agar and nutrient agar medium were used for the preliminary isolation of antibiotic producing Actinomycetes. 1ml of each of the diluted sample(10^{-4}dilution) was added on to the solidified medium in Petri plates (spread plate method) and incubated at 37^0C for 1-2weeks. They were observed for colonies of Actinomycetes. After satisfactory growth they were isolated as pure cultures in Mc Beth scales slants and preserved at 4^0C for further use.[9]

2.3. Gram staining to study morphology

Each culture was studied by smear preparation, Gram staining and microscopic observation(100x). The structure, color, shape and texture of the colonies were studied by comparing with Bergey's manual of determinative bacteriology 9th edition, macroscopic observation of mycelium, color of conidiospores was done to identify the cultures.[10]

2.4. Screening for antibiotics

2.4.1. Primary screening by cross streak method

Antibacterial spectrum of isolated Actinomycetes from various samples were tested by modified cross-streak method, employing nutrient agar plates.[11,12] Test organisms used were Gram positive bacteria like, *Staphylococcus aureus*, *Bacillus subtilis* and Gram negative bacteria *Escherichia coli*, *Proteus vulgaris*. They are obtained as clinical samples from KMC, Warangal. In single line streak method, each pure culture of isolated Actinomycete was inoculated as a single streak down the middle of the plate and incubated for 4-7 days at 37^0C. After this period fresh subcultured test organisms were streaked perpendicular to the Actinomycete streak. The plates were incubated at 37^0C for 24 hrs.

2.4.2. Fermentation and extraction of crude extracts for secondary screening.

Most promising antibiotic producing Actinomycetes were selected based on inhibition in primary screening.[13,14] Eight different cultures were maintained as pure cultures and inoculated into Mc Beth scales broth, Casein broth, Asparagine broth and Nutrient broth. Conical flasks with 25ml different broth media were inoculated with pure cultures and shaken in shaker and incubated for 2-3 weeks at 37^0C. Then cultures were filtered, centrifuged. 0.1 ml of each was used for well-diffusion method to detect antimicrobial activity.

2.4.3. Well-diffusion method.

Each test organism was inoculated on solidified nutrient agar medium with 0.1 ml of bacterial cell suspension(10^4-10^5cells/ml). The inoculum was spread with the help of spreader. Plates were refrigerated for 2 hrs. After this wells were cut in the gel of agar. 0.1 ml of each of centrifuged Actinomycetes culture was added and plates were incubated for 24 hrs at 37^0c. Observed for the presence of inhibition zones.

2.5. Thin Layer Chromatography:

After growth in broth medium culture filtrate of each potent Actinomycete is concentrated and used for thin layer chromatography.[15,16] 2µl of each of the antimicrobial metabolite and standard antibiotic (Penicillin and Streptomycin) was applied separately on TLC sheet of required size. Chromatography was developed using Butanol: Acetic acid: Water as solvent system. The spots were observed under UV chamber and developed using iodine vapour.

2.5. Data analysis

All the experiments were performed in triplicate. The different inhibition zone measurements in triplicate were compared and noted as mean ± SD using MS-excel.

3. RESULTS

3.1. Sampling and isolation of Actinomycetes

Of total 12 samples six were from hospital dump yards, six were from fresh water sediments. They have been isolated as pure cultures in different types of media and their cultural characters are observed. That is shown in table 1 and figure 1.

In MC Beth scales medium there was good growth of all isolated Actinomycetes. Initial isolation was done in MC Beth agar as growth on nutrient agar was very slow. They exhibited colony colours ranging from white, grey, dark grey, orange, to dark brown.

3.2. Gram staining

Actinomycetes observed exhibited Gram positive, filamentous nature with spores of different morphologies.

3.3. Primary screening

Of 12 Actinomycetes isolated and subjected to primary screening through cross streak method, only 8 isolates were active against Gram positive and Gram negative bacterial pathogens. Four of them from fresh water sediments and four from hospital dump yards. Out of 8

isolates Wa1, H1 exhibited highest inhibition of pathogenic bacteria(table 2 and figure 2)

3.4. Secondary screening

After primary screening, 8 isolates with antimicrobial activity were subjected to secondary screening. MC Beth broth was used for submerged fermentation of Actinomycetes. Crude extract after fermentation and centrifugation were used against pathogenic bacteria by well-diffusion method. Wa1, Wa2, Bk3 were potent isolates which exhibited good inhibition of pathogenic bacteria employed. Crude extracts of Actinomycetes after 3 weeks incubation showed antibacterial activity. Zone of inhibition were observed and tabulated.(table 3 and figure 3). Standard antibiotics used are shown in fig 4.

3.5. TLC

Secondary metabolites produced after submerged fermentation were separated and detected using TLC. Results shown in table 4 and figure 5. When compared with standard antibiotics Penicillin and Streptomycin there were few spots with same Rf values. Some UV-detected components were seen with few extracts.

Table 1: Colony characteristics of Actinobacteria isolated from different sources. (Wa1, Wa2, Wa3 are from Waddepally water reservoir, BK1-BK3 are from Bhadrakali water reservoir, H1-H6 are from hospital dumpyards).

Actinomycete isolates	Culture medium	Growth	Color of aerial mycelium	Color of substrate mycelium
Wa1	MC Beth scales agar medium	Excellent	Whitish grey	White
Wa2	‘’	Excellent	Orange	White
Wa3	‘’	Good	Brown	White
BK1	‘’	Excellent	Brown	White
BK2	‘’	Good	White	White
BK3	‘’	Excellent	Brown	White
H1	‘’	Good	White	White
H2	‘’	Excellent	Dark grey	White
H3	‘’	Excellent	White	White
H4	‘’	Good	White	White
H5	‘’	Excellent	Grey	White
H6	‘’	Excellent	Brown	White

Table 2: Primary screening by Cross streak method (inhibition zone in mm). ND =Not detected.

Actinomecete isolates	*E.coli*	*Pseudomonas*	*Staphylococcus*	*Bacillus*
Wa1	30±0.2	30±0.2	45±0.4	40±0.5
Wa2	30±0.2	29±0.6	3±80.5	39±0.3
Wa3	ND	ND	ND	ND
BK1	28±0.8	ND	38±0.3	32±0.6
BK2	ND	ND	ND	ND
BK3	25±0.4	28±0.6	39±0.3	41±0.2
H1	30±0.2	30±0.8	40±0.5	41±0.4
H2	30±0.8	29±0.4	38±0.6	39±0.5
H3	20±0.4	38±0.5	37±0.3	31±0.4
H4	ND	ND	ND	ND
H5	28±0.4	32±0.8	35±0.6	38±0.5
H6	ND	ND	ND	ND

Table 3: Inhibition of pathogenic bacteria during secondary screening(inhibition zone in mms)

Actinomecete isolates	*E.coli*	*Pseudomonas*	*Staphylococcus*	*Bacillus*
Wa1	18±0.8	5±0.5	25±0.5	28±0.6
Wa2	10±0.2	8±0.2	15±0.9	13±0.8
BK3	6±0.8	8±0.2	12±0.8	11±0.9

Table 4: TLC for potential Actinomycetes broth culture samples and standard antibiotics.

Actinomycetes cultures	Rf values
Wa1	0.5
Wa2	0.8, 0.4(UV)
BK1	0.5
BK3	0.7, 0.45(UV)
H1	0.5
H2	0.5 0.4(UV)
H3	0.6 0.3(UV)
H5	0.4
Penicillin	0.8
Streptomycin	0.7

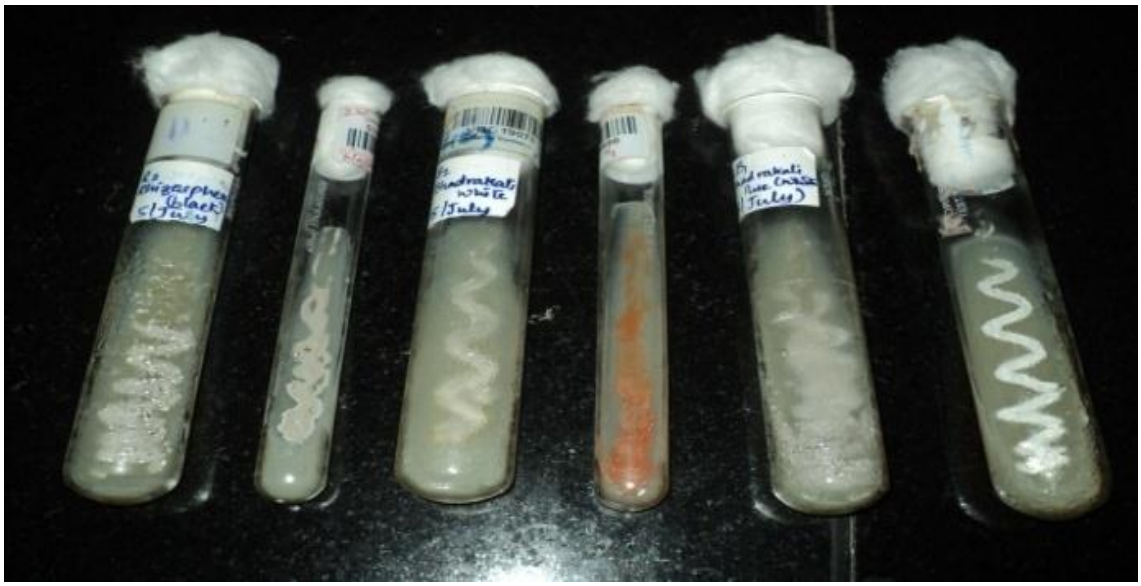


Figure 1: Colony characters of isolates.

Wa1

Wa2

Wa3

BK1

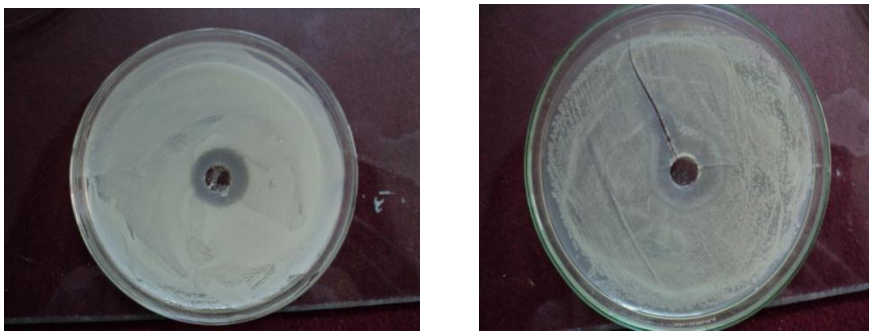
BK2

BK3

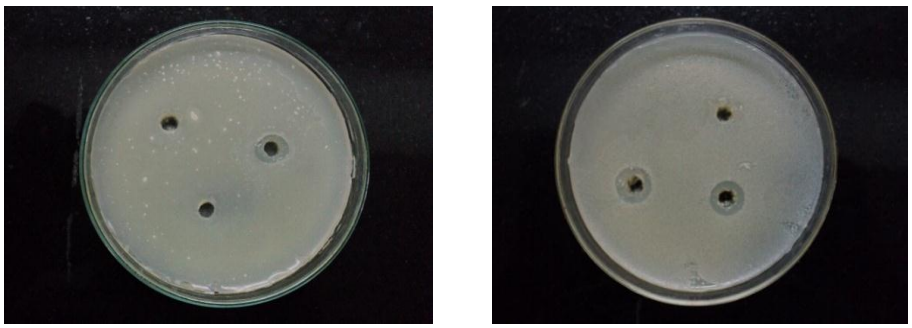
H1 H2 H3

H4 H5 H6

Figure 2: Cross streaking for primary screening of isolates.

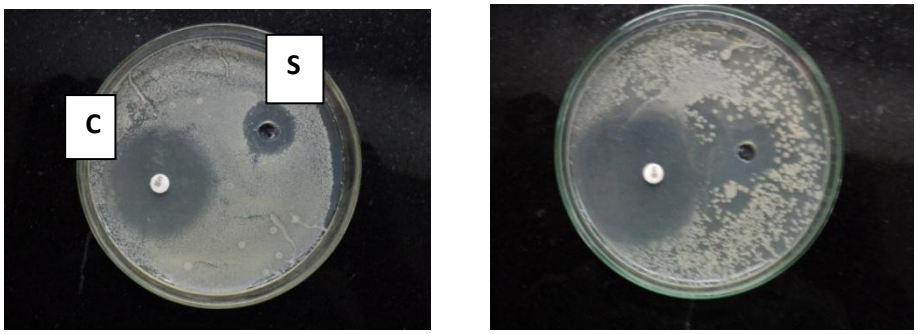


E.coli **inhibited by Wa1** ***Bacillus*** **inhibited by Wa1**



Staphylococcus **inhibited by Wa2** ***Bacillus*** **inhibited by BK3 and Wa1**

Figure 3: Inhibition of bacteria by Actinomycetes isolates during secondary screening.



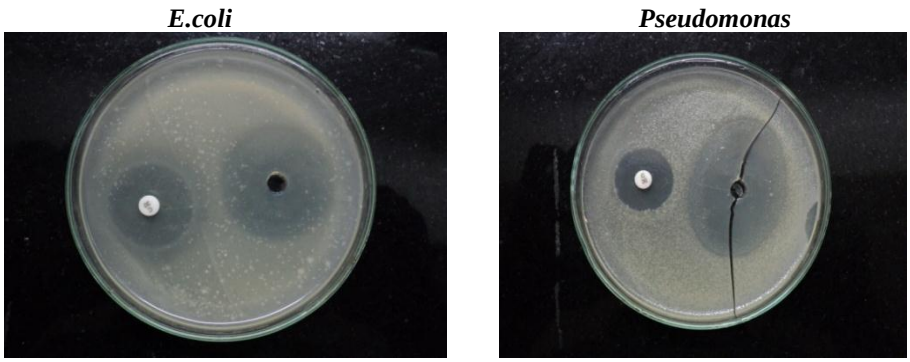


Figure 4: Inhibition zones of Standard antibiotics Chloramphenicol(C) and Streptomycin(S).

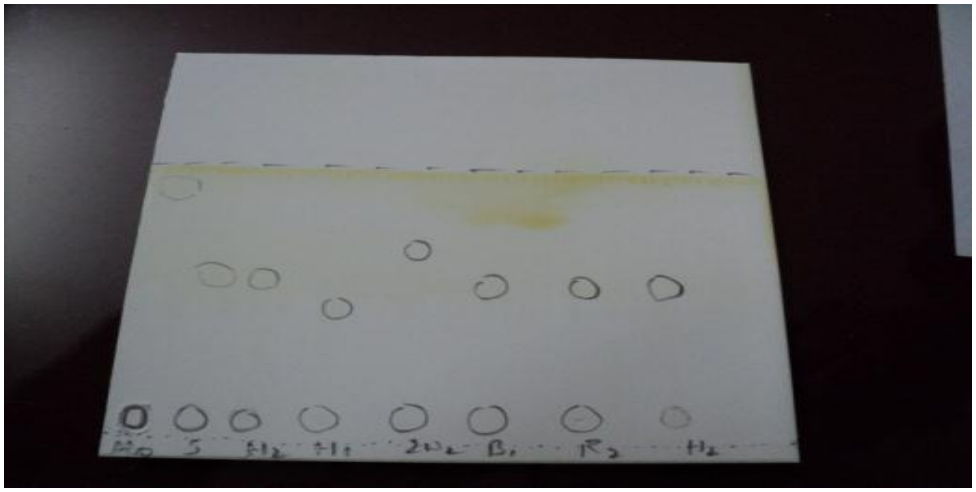


Figure 5: TLC for isolated Actinomycete culture extracts.

4. DISCUSSIONS

Though we have powerful experimental tools such as genomics, combinatorial chemistry and high-through put *in vitro* screening available for antibiotic discovery, the traditional method by screening extracts from Actinomycetes is still used as most antibiotics are direct natural products produced from Actinomycetes. Many genera especially *Streptomyces* has the capacity to produce secondary metabolites including antibiotics.[17] There are reports on new strain of *Streptomyces* species US7 MDCC 8723 which has good antibacterial and antifungal activity.[18] Actinomycetes isolates from soil(S114) and marine(MD2) exhibited potent antibacterial activity.[19]

Different antibiotics have different action spectra, the nature of antibiotics produced by Actinomycetes depends on the species isolated and its habitat. Even their growth in different media may influence the production of secondary metabolism.[20]

Of the Actinomycetes isolated, three from water sediments namely Wa1, Wa2, Bk3 were potential anti microbial producers and were effective during secondary screening. Of the different broth media used MC Beth and starch casein broth where more effective in cultivation of Actinomycetes. Some of the hospital soil dump yard isolates like H1,H2,H3 and H5 also exhibited inhibition of pathogenic bacteria during primary screening but not as potent producers like those of water sediments. While detection of secondary metabolites after submerged fermentation using thin layer chromatography, Wa2 and H3 showed the presence of components which matched with that of standard antibiotics. Their RF values were similar to that of Penicillin and streptomycin. Probably they are species of *Strepomycis.*

Though generally Actinomycetes are of great socio economic importance due to the production of bio active compounds, those from aquatic environments are gaining importance this is mainly because of diluting effect of water due to which aquatic Actinomycetes have to produce more of bio active compounds to be effective.[21]

All isolates of present study inhibited Gram positive bacteria more effectively than Gram negative bacteria. Though earlier lot of soil isolates exhibited potent activity as reported by Waksman[22], recently Actinomycetes from water sediments have become more popular as potential producers of antibiotics. Our study supports the need for new anti microbial compounds against drug resistant pathogens, which can replace old and invalidated antibiotics.

CONCLUSION

Results obtained emphasize the need for screening natural resources for the potential antimicrobial producers which aids in novel drug discovery for the future.

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