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Bioactive potential and its innovative perspectives of Various Marine soil Actinomycetes

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ABSTRACT

A total of 25 different types of actinomycetes were isolated from the soils of marine sediment soil. All the isolated actinomycetes were characterized and identified based on the morphological, biochemical, cultural characteristics. Both primary and secondary screening methods were used to screen actinomycetes for antibacterial activity. The result of the screening revealed that all the isolates were against bacterial culture. But the best strain was found to be *Streptomyces sp* as they showed broad spectrum activity with big zone of inhibition, even though the strain *Staphylococcus* and *Streptomyces sp* showed augmented antibacterial activity against all the tested human bacterial pathogens. Comparatively, when they were treated with pathogenic microorganisms all the isolates produced to maximum and minimum zone of inhibition with its responsible broad spectrum of bioactivity. Moreover, LAM-4 and LAM-11 strains were clearly showed significant activity against both *Staphylococcus aureus* and *S. agaricus*. From the HPLC peaks which had antimicrobial activity of *Streptomyces sp* was identified to be at 3.296. This is similar to oxohexaene antibiotic whereas, the antimicrobial compound of *Streptomyces sp3* showed a retention time of 3.233 on HPLC, this peak was similar to Cephalexin. From the results highlight the most of the isolates inhibited growth of the Gram negative bacteria tested. All the antibiotic producing actinomycetes were isolated at different temperatures from marine soil. These microorganisms might have potential to produce a number of the most important medicines constantly developed.

Key words- Actinomycetes, pathogenic organisms, HPLC

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INTRODUCTION

Actinomycetes are gram positive bacteria frequently filamentous and sporulating with DNA rich in G+C from 55-75%[1].The name actinomycetes derived from Greek aktis (a ray) and mykes (fungus) was given to these organisms from initial observation of their morphology. They are responsible for the production of about half of the discovered bioactive secondary metabolites[2], antibiotics[3], Streptomyces is the dominant among actinomycetes. They are responsible for the production of about half of the discovered bioactive secondary metabolites[4]. Actinomycetes are the most widely distributed group of microorganisms in nature which primarily inhabit the soil[5]. They have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds[6]. The present study is aimed to isolate , identify and to optimize the nutritional and cultural characterization of actinomycetes obtained from soil sample and then to compare the effect of antimicrobial activity of actinomycetes with pathogenic bacterial orgaganisms. Finally to purify antibacterial metabolites from efficient strain and to detect antimicrobial compounds by advanced technique of HPLC. The crumple of the usual universal sources particularly marine soil sources and the rise of resistant pathogens utter the search for novel actinomycetes and new antibiotics. In this context, niche habitats such as caves, pristine forests, lakes, rivers, and other wetlands, high salt environments, marine ecosystems and endophytic niches are promising targets for survey of bioactive actinomycetes. Hence, the present work is Bioactive potential and its innovative perspectives of Various Marine soil Actinomycetes designed.

MATERIALS AND METHODS

The marine soil samples were collected from10 different localities of sea shore area. 25 different soil samples were collected from the surface to about 10 cm deep as a mixture for the isolation of actinomycetes.

Isolation of Actinomycetes from soil samples

Actinomycetes were isolated by spread plate technique following the serial dilution of soil samples on starch casein agar plates containing cycloheximide and nystatin (each at concentration of 50 µg/ml of medium) and incubated at 28°C for 7 days. The following media were also used for the isolation of actinomycetes: Starch caesin agar, Starch caesin nitrate agar, Actinomycetes Isolation agar, Oat meal agar etc. Isolation plates were incubated at 28-30˚C for 7-15 days for fast growing actinomycetes or up to 35 days for slow growing ones. Identification of Actinomycetes to genus level was conducted by first using morphological and Purification of

secondary metabolites from soil actinomycetes chemical criteria according to Bergey's Manual of Determinative Bacteriology [7].

Screening of Actinomycetes for antibacterial and activity

Primary screening

A modified cross- streak method[8] was used for antimicrobial activity .Single streak of actinomycetes was made on surface of the modified nutrient agar and incubated at 28°C. After observing a good stretch like growth of the actinomycetes on the plates, the overnight pathogenic bacterial strains, such as *Serratia spp*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella paratyphi*, *Klepsiella pneumionea*, *Streptococcus agalactiae*, *Pseudomonas fluorescens*, *Enterococcus faecalis*which (isolated from the admitted patients from Karakkonam Hospital) were streaked at right angles to the original streak of actinomycetes and incubated at 28°C and the incubation distance was measured after 24-48 hrs. A control plate was also maintained without inoculating the actinomycetes, to assess the normal growth of bacteria.

Secondary Screening

Determination of Antimicrobial activity

The antimicrobial activity was determined by agar well method purified extract obtained by the evaporation of the ethyl acetate extract. Then 25, 50, 75 and 100µl of it were loaded into well bored and test organism (0.5 McFarland turbidity standards) were swabbed on Muller Hinton agar plates. The plates were incubated at 37°C for 18-24 hours and examined. The diameter of the zones of complete inhibition was measured to the nearest whole millimeter. The formation of inhibition zone around the pathogenic strains is due to the production of secondary metabolites by actinomycetes isolates.

HPLC

The culture filtrate was extracted three times by the same volume of ethyl acetate. After the removal of cell mass, supernatant was analysed by Spectrophotometer at 220nm. This was carried out to for HPLC analysis. Culture filtrate extracted by ethyl acetate was analyzed by HPLC. 10 µl of sample was injected to C18 column (250mm X 4.6mm X 5mm). The flow rate was 0.50 ml/min. Sample was analysed at 220 nm wavelength. In order to purify the active fraction (methanol: chloroform 20: 80 extract); chloroform was added gradually (dropwise) until formation n of the first precipitate. The precipitate was separated by centrifugation. Each fraction was tested in inhibition test. Ammonium sulphate was also used for the extraction. Ammonium sulfate was grind in a glass mortar to be a fine powder that easily to dissolve. This powder was added gradually to the antagonistic solution. The solution was shacked using a vortex. The

formed precipitate was separated by centrifugation. The amount of ammonium sulfate was noted for each precipitate and calculated as saturation percent. Each precipitate was dissolved in water and re-precipitated again by ammonium sulfate as a purification step. Each precipitate was dissolved in methanol and centrifuged to remove any residue of ammonium sulfate. This step was repeated several times until the removing all amounts of ammonium sulfate. Each Precipitate was used for the inhibition test and analysed by HPLC.

Statistical Analysis

Oneway ANOVA was used to determine the significance of antibacterial broad spectrum activity of 12 actinomycetes with nine bacterial pathogens. Data's were analysed with a one way of ANOVA Test using pp Version-4 Window. Result with $P<0.05$ were considered as statistically significant

RESULT AND DISCUSSION

From the table-1 and 2 expressed the primary screening secondary metabolic activity of Actinomycetes showed highest antibacterial activity against *Staphylococcus aureus* (35) and *K. pneumonia* (39±0.72) (given in Figure.1). From the initial screening 60 cultures of various strains were obtained. While the 9 culture exhibited antagonistic activity against the few of the bacterial strains such as gram positive bacteria as well as negative organisms. Though, least percentage exhibited broad spectrum activity noted against both of the organisms.

Table: 1. Primary screening of antibacterial activity of Marine Actinomycetes against various Bacterial Pathogens.

Actinomycetes	Responsive tested organisms of Bacterial pathogens								
Isolates	S.e	S	S.a	E.c	S.pa	K. pneu	St.ag	P.f	E.f
LAM-1	+	-	++	+-	-	+	+	+	+
LAM-2	++	+	++	-	+	-	++	++	++
LAM-3	++	-	+	+	-	-	+	++	-
LAM-4	++	+++	+++	+++	++	-	+	+++	+++
LAM-5	-	-	+	+	-	-	+	+	-
LAM-6	++	-	++	-	-	+	+	-	-
LAM-7	-	+++	+++	++	+++	-	+++	+++	-
LAM-8	-	-	++	-	-	++	+	++	++
LAM-9	+	-	+	-	-	-	++	-	-
LAM-10	-	-	+	-	++	+	+	+++	-
LAM-11	++	+	++	++	-	-	++	-	++
LAM-12	-	++	++	-	++	-	++	-	-

S. e:Staphylococcus *epidermidis*, S:*Serratia spp.*, S.a: *Staphylococcus aureus*, E.c*: Escherichia coli*, S.pa *: Salmonella paratyphi A*, S.pb*: Salmonella paratyphi B*, St.ag *:Streptococcus agalactiae*, P.f *:Pseudomonas fluorescens*, E.f *:Enterococcus faecalis*

'+' Positive (noticed growth inhibition); '++' average inhibition; '+++' effective growth inhibition;'-' Negative (no growth inhibition);

Table: 2. Measurement of Zone of Inhibition (Antibacterial activity) from the marine soil actinomycetes against pathogenic bacteria

Micro org.	Name of the tested Organisms (Inhibition Zone Diameter in mm)								
	S. e	***S***	***S. a***	***E. c***	***S. pa***	***K. pneu***	***St. ag***	***P. f***	***E. f***
LAM-1	13±1.21	-	14±0.64*	13±3.54	-	11±0.50	13±2.36*	17±3.21	12±2.30
LAM-2	13±0.65	18±2.58	8±1.57	-	24±2.87	-	12±1.64**	14±1.98	10±4.11
LAM-3	12±108	-	35±0.02**	13±2.10	-	37±1.91	9±1.43	39±0.72	-
LAM-4	16±0.36	15±1.64	14±0.66	9±1.05	22±2.20	-	15±0.34**	10±1.56	11±3.60
LAM-5	-	-	11±1.49	9±1.08	--	27±2.32	12±1.41	6±2.03	-
LAM-6	11±0.58	-	13±2.33	-	-	15±3.21	13±0.95	-	-
LAM-7	-	30±0.36	31±0.23*	32±0.09	20±0.57	-	14±2.01*	11±0.31	-
LAM-8	-	-	24±1.77*	-	-	21±3.64	10±1.06*	3±0.65	12±0.53
LAM-9	4±2.23[Is]	-	20±1.32	-	-	-	9±0.68*	-	-
LAM-10	-	-	15±0.11*	-	16±2.36	13±1.49	7±0.31	16±2.30	-
LAM-11	12±2.10	14±0.98	15±**	11±1.54	-	-	22±1.54**	-	18±0.67
LAM-12	-	7±1.32	25±3.01**	-	15±1.27	-	10±5.20*	17±5.10	-

P < 0.05- Significant ,*** *P* < 0.001- Highly significant

[4]RIZD =Percentage of relative inhibition zone diameter at 25 µg/ml (compared to the respective antibiotic standard). Values are means of three replications.

NA = not applicable. (-) = no inhibition of growth at the concentrations tested.

Values are Means ± SEM of triplicates, Asterisks indicate values which are significantly different.

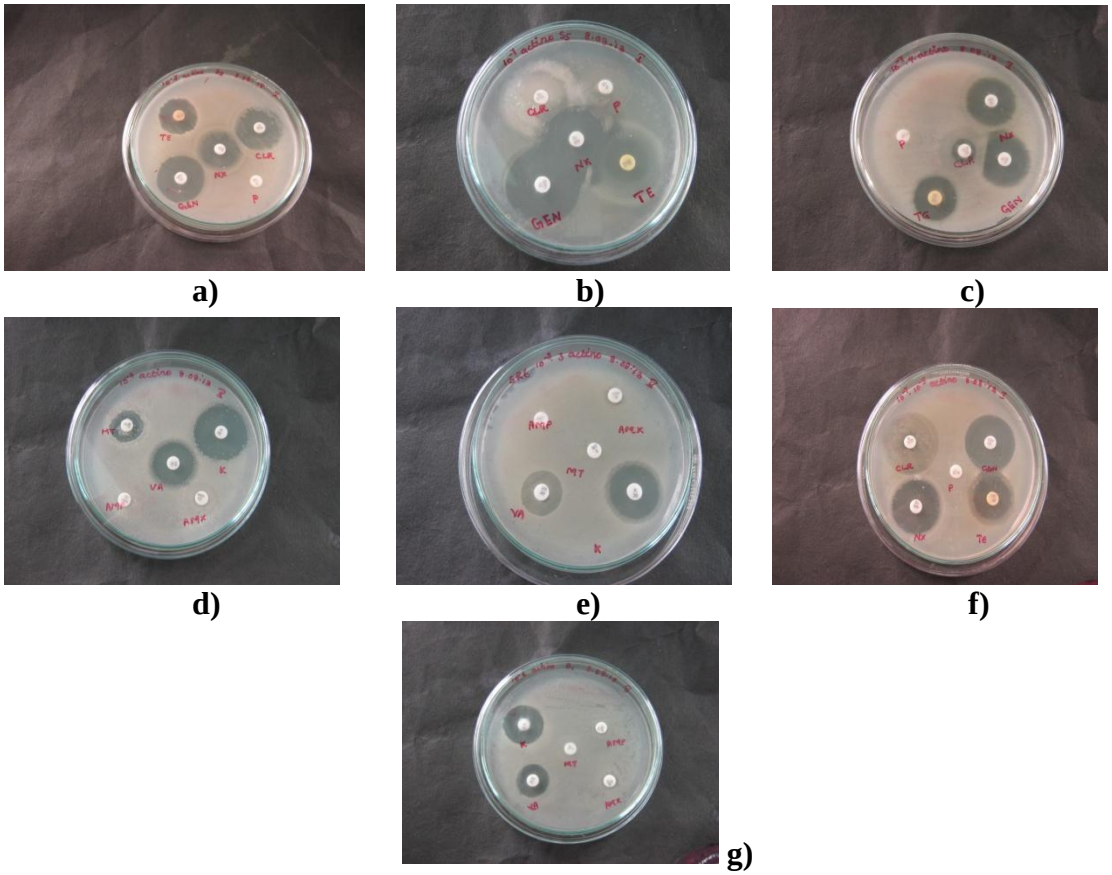
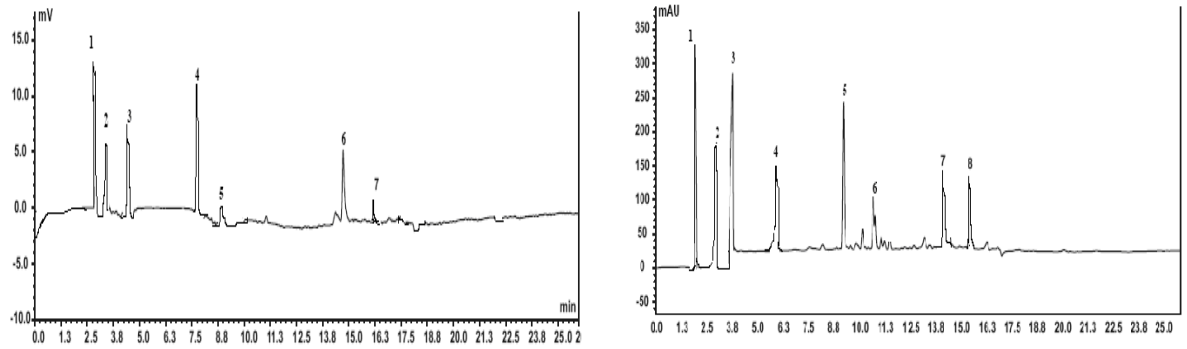


Figure-1: Antimicrobial activity of actinomycetes against various pathogenic bacterial organisms

Furthermore, the strain LAM-3 showed more activity against the tested bacteria zone diameter such as 35, 37 and 39 for Staphylococcus *aureus, K. pneumonea* and *Pseudomonas fluorescens* respectively. Whereas the specific strain of LAM-4 for capable to produce minimum to moderate inhibitory activity (in the form of zone) noticed against all the tested bacteria except *K. pneumonea.* In addition strain LAM-7 showed linearly increased inhibitory zone such as *Serratia* spp (30±0.36), *S. aureus* (31±0.23*) and *E. coli* (32±0.09). Interestingly, all the actinomycete strains had a remarkable activity against *Staphylococcus aureus* and *S. agalactiae*. Despite, Comparatively, when they were treated with pathogenic microorganisms all the isolates produced to maximum and minimum zone of inhibition with its responsible broad spectrum of bioactivity. Moreover, LAM-4 and LAM-11 strains were clearly showed significant activity against both *Staphylococcus aureus* and *S. agaricus.*

HPLC ANALYSIS

HPLC is being routinely used for the analytical estimation of various antibiotics. In the present investigation, HPLC profile of the antimicrobial compounds of *staphylococcus aureus* and *Streptomyces agalactiae* were performed by Rheodysne Column up to 15 min at 350 nm. The antimicrobial compounds of *S. aureus* showed absorption peaks at retention time (min) 1.235, 4.332, 2.381, 3.233, 5.214, 4.110 and 3.754. Likewise, the antimicrobial compounds of *Streptomyces agaricus* showed absorption peaks at retention time (min) 1.225, 2.393, 3.312, 6.383, 7.390, 5.874 and 8.369 .(figure 2,)



(A) **(B)**

Figure 2 . HPLC Chromatogram of antimicrobial compound of A) *Staphylococcus aureus* B) *Streptococcus agalactiae*

(Table 3 and 4). The peaks which had antimicrobial activity of *S. aureus* were identified to be at 3.223. This is almost similar to oxohexaene antibiotic. While, the antimicrobial compound of *Streptomyces agaricus* showed a retention time of 3.312 on HPLC, this peak was more or less similar to cephalaxine. Despite in S. aureus species consist of seven highlight peak among the

seven two unknown compound has been identified also that higher abundance responsible compound is 990 Mycophenolic compound. Similarly in *S. agaricus* totally eight compounds were identified among the eight two are unknown. Whereas maximum abundance denoted compound is macrolide.

Table: 3 *Chromatogram values* for *Staphylococcus aureus*

Num of peak	Name of the analyte	Retention time (min)	Abundance (%)
1	Hyal-2'-uronidase	1.235	400
2	unknown	4.332	458
3	unknown	2.381	800
4	Triglycine sortase	3.233	627
5	Mycophenolic compound	5.214	990
6	benzoquinones	4.110	624
7	Viridicatin	3.754	471

Table: 4. *Chromatogram values* for *S. agalactiae* analytes tracked

Number of peak	Name of the analyte	Retention time (min)	Abundance (%)
1	unknown	1.225	364
2	Peptide 401	2.393	547
3	Histidine-34	4.312	758
4	Roqufortine	6.383	798
5	macrolide	7.390	874
6	unknown	5.874	961
7	polyenes	8.369	821
8	Trp-containing peptide	9.324	654

Isolation of actinomycetes has always been faced with difficulties in comparison to their competitors like other bacteria and Purification of secondary metabolites from soil actinomycetes fungi[7]. Both primary and secondary screening methods were used to screen actinomycetes for antibacterial activity (denoted Table 1,2). The first screening was used to select the antibacterial isolates and determine the range of microorganism that was sensitive to the antibiotic. The secondary screening method was crucial to select the isolates for further studies. The result of the screening revealed that all the isolates were against bacterial culture. But the best strain was found to be *Streptomyces sp* as they showed broad spectrum activity with big zone of inhibition. Therefore the isolates were chosen for fermentation. Actinomycetes, particularly *Streptomyces* species are among the richest sources of antibiotics. Roughly 60% of biologically active compounds that have been developed for agricultural use originated from *Streptomyces*. Various groups of bioactive compounds such as macrolide, benzoquinones, aminoglycosides, polyenes, and nucleoside antibiotics are examples of agriculturally useful metabolites produced from *Streptomyces*. Previously this results also agreed by results proposed[10].

Further characterize that *Streptomyces sp 2*comes under aminoglycoside group, very much related to *Staphylococus* and the *Streptomyces sp* comes under the antibiotic cephalexin[11]. HPLC is being routinely used for the analytical estimation of various antibiotics. In the present investigation, HPLC profile of the antimicrobial compounds of *Streptomyces sp* were performed by Rheodysne Column (C-18) up to 10 min at 220 nm. The antimicrobial compounds of *Staphylococcus aureus* showed absorption peaks at retention time (min) by its respective abundance also with each peak having peculiar compounds. Similarly, the antimicrobial compounds of *Streptococcus agalactiae* showed absorption peaks at retention time (min). The peaks which had antimicrobial activity of *Streptococcus agalactiae* was identified with similar to oxohexaene antibiotic this kind of comparable research already been done by Harindran[12]. Later[13] also been reported another kind of soil actinomycetes that was a total of 110 actinomycetes isolates were isolated from the soil samples collected from the protected forest soil from two states in Northeast India[14, 15]. These were then characterized by conventional methods and assessed for their antagonistic activity preliminary against test microorganisms. Results of the present study also indicate that the higher number of actinomycetes was isolated from wasteland alkaline soil active against bacteria, where the human activity is very less for agriculture or other purpose and these actinomycetes can be useful for many applications such as control of infectious diseases and drug discovery.

CONCLUSION

Actinomycetes are unparalleled sources of bio-active metabolites including antibiotics, plant growth factors, and other substances. *Streptomyces* and other actinomycetes are major contributors to biological buffering of soils and have roles in organic matter decomposition conductive to crop production. Actinomycetes are known to produce bioactive substances, especially antibiotics that are effective against phytophathogenic fungi. The present study was an attempt to identify and pick out versatile actinomycetes strains that display antimicrobial activity against a variety of microbial pathogens intrinsically. These microorganisms produce some of the most important medicines forever developed. Hence the marine soil is an innovative goldmine of biodiversity has been amply justified by the richness of microbial diversity.

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