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Research Article

IN VITRO ANTIMICROBIAL ACTIVITY OF MARINE *ACTINOBACTERIA* ISOLATES FROM PULICAT LAKE, TAMIL NADU, INDIA

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ABSTRACT

Objectives: The present investigation is undertaken with an aim to check the antimicrobial potential of marine *Actinobacteria* isolated from marine sediment samples collected from Pulicat Lake, Tamil Nadu.

Methods: In this study, various physical and chemical pretreatment methods were used for selective isolation of *Actinobacteria* including air drying, incubation of soil samples with calcium carbonate, incorporation of antibacterial, antifungal antibiotics in the media and plating on *Actinobacteria*-specific media. A combination of such methods facilitated isolation of slow growing *Actinobacteria* with a simultaneous reduction of other free-living bacterial and fungal contaminants. The primary and secondary screening was done to ascertain the antimicrobial potential of these isolates.

Results: The results obtained from primary screening was about 110 isolates, were tested against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans,* and *Aspergillus niger*, out of these, 31 isolates showed remarkable antimicrobial activity. Based on the results of primary screening, 31 isolates were subjected to secondary screening from which two isolates with a strong and broad spectrum of antimicrobial activity were selected for further studies.

Conclusion: The present study is useful as the isolation of most of the antimicrobial compounds from different *Actinobacteria* species showed partial resemblance to earlier reported antibiotic chemical structures. Further, chemical characterization of the isolated compound from producer isolates may yield more effective analogs or hybrid bioactive molecules.

Keywords: *Actinobacteria*, Antimicrobial activity, Pulicat lake.

INTRODUCTION

Marine microorganisms are progressively fetching an important source in the search for industrially important molecules. Today, both academic and industrial interests in marine microorganisms are on the upsurge, because unique and biologically active metabolites have been reported from marine organisms [1-3]. *Actinobacteria* exist in innumerable ecological habitats such as soil, fresh water, backwater, lake, compost, sewage, and marine environment [4]. *Actinobacteria* have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds. Most of the antibiotics in use today are derivatives of natural products of *Actinobacteria* and fungi [5,6]. Hence, it is known that the *Actinobacteria* are perhaps the most important group of organisms studied extensively for the discovery of drugs and other bioactive metabolites program [7,8].

The *Actinobacteria* are known to produce diverse antibacterial and antifungal compounds, which are being exploited commercially for the control of several plant diseases [9]. Since the late 1980s, the number of novel compounds isolated from terrestrial microorganisms has gradually reduced. To cope up with the demand for new pharmaceutical compounds and to combat the antibiotic resistant pathogens, researchers have been forced to look for novel microorganisms in unwonted environment. Relatively, the Bay of Bengal, an arm of the Indian Ocean has scarcely been explored for microbial diversity and microbial metabolites. Hence, there is an immense possibility to discern new marine *Actinobacteria* in the Bay of Bengal to discover novel bioactive compounds [10]. Consequently, this study was targeted to investigate the diversity of industrially important marine *Actinobacteria* in Pulicat Lake, which connect the Bay of Bengal with the eventual objective of discovering novel bioactive compounds.

METHODS

Area of study

The study area encircled the Pulicat Lake in the north of Tamil Nadu. Pulicat Lake is the second largest brackish water lagoon in India, which runs parallel to the Bay of Bengal. It is located at 60 km Northeast of Chennai and is separated from the Bay of Bengal by Sriharikota Island in the state of Andhra Pradesh. The lake is about 360 km^2 in size, and its depth varies from 1 to 6 m (Fig. 1).

Collection of sample

A total of 20 sediments were collected from different locations in and around Pulicat Lake, Tamil Nadu, India. Collections of soil samples were made in 3-5 m depth from each site within the plot using a grab sampler and were pooled in sterile polypropylene bags. The collected soil samples were mixed thorough and stored in small sterilized airtight polyethylene bags and maintained at room temperature till they reached the laboratory. The collected samples were brought to the laboratory for isolation of marine *Actinobacteria* and the location, nature of sample, and pH were documented.

Determination of sediment pH

To determine the soil, pH was measured directly. 10 g of each sediment sample was suspended in 40 ml of double distilled water. It was kept in shaker for 1 hr. After being left to settle, the pH was measured in the supernatant of the soil suspension [11].

Pretreatments for *Actinobacteria* isolation [12]

All the marine sediment samples were subjected to preheat treatment before serial dilution. Pretreatment was performed by incubating the sediment samples with 0.1% $CaCO_3$ at 60°C for 1 hr in oven.

Fig. 1: Area of study and sample for isolation

Isolation of marine *Actinobacteria*

One gram of pretreated sediment samples was suspended in 100 ml of sterile saline and mixed well in a shaker. Five different medias starch casein agar (SCA) [13], yeast extract-malt extract agar (YMA, ISP2) [14], glycerol asparagine agar (GA agar, ISP5) [14], Bennet's agar (BA agar), potato dextrose agar (PDA) was used for the isolation of *Actinobacteria*. From each sample, 0.1 ml suspension was drawn and plated over the surface of the media namely SCA, PDA, BA, ISP2, and ISP5. All the plates were incubated at 28±2°C for 5 weeks. The spread plated petri plates were incubated at room temperature (28±2°C) and monitored periodically over 5 weeks for the development of *Actinobacteria* growth. The *Actinobacteria* colonies were recognized by powdery, thick, and leathery features; initially, white colonies that adhered strongly to the agar surface [14]. For every plate that formed *Actinobacteria* colonies, the total number of colonies observed was counted, and representatives of all morphotypes were obtained in pure culture by repeated transfer from a single colony. Each isolate was given a unique alphanumeric accession number. The pure cultures of marine *Actinobacteria* were sub-cultured in SCA slants and incubated at room temperature for 5-7 days to achieve good sporulation.

Growth characteristics of marine *Actinobacteria*

All the isolates of marine *Actinobacteria* were grown on SCA at room temperature and the growth rate was monitored every day up to 28 days. The isolates, which showed good growth in 7 days, were considered as fast growers and those that showed good growth between 7 and 10 days were classified as moderate growers and the slow growers took more than 10 days for their growth. In addition to growth, mycelial color, and pigment production were monitored in all the isolates of marine *Actinobacteria* and documented.

Fermentation of marine *Actinobacteria* in production media

Based on the literature reviews and our laboratory reports recommended to grow all the marine *Actinobacteria* in metabolites production medium proposed by Balagurunathan and Subramanian [15].

Culturing of the marine *Actinobacteria*

A total volume of 100 ml medium was prepared in a 250 ml Erlenmeyer (EM) flask separately for all the 110 isolates and sterilized at 121°C for 15 minutes using stainless steel autoclave (Goley, Mumbai, India). After sterilization media were inoculated with a loopful of marine *Actinobacteria* individually. The inoculated EM flasks were kept at orbital shaker for 7 days at room temperature (28±2°C). After 7 days of incubation, the bacterial biomass were filtered using four lines of muslin cloth and centrifuged at 5000 rpm at room temperature (28±2°C).

Extraction of secondary metabolites

The cell free supernatant was used for the secondary metabolite isolation. Based on the literature survey, the solvent ethyl acetate was most used solvent because of its high polar and non-miscible nature. The equal volume of ethyl acetate was used to separate the secondary metabolites which have been dissolved in culture filtrate and kept in shaking condition for 4 hrs. The separating funnel was used for the secondary metabolites separation. The upper organic phase was separated and used for the further evaporation. The evaporation of the solvent was done using the rotary evaporator (Buchi, India). After the evaporation of solvent, the crude extract was kept under refrigeration until further use.

Test-microorganisms

Test organisms were obtained from The Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh. The selected human pathogenic microorganisms used in the antimicrobial study were *Staphylococcus aureus* MTCC 96, *Pseudomonas aeruginosa* MTCC 1688, *Candida albicans* MTCC 227, and *Aspergillus niger* MTCC 282, stock cultures of bacteria and fungi were maintained at 4°C on nutrient agar and PDA slant. Active cultures for experiments were prepared by transferring a loop full of culture from the stock cultures into the test tubes containing nutrient broth/PD broth, which were incubated at 18-24 hrs at 37°C.

Screening of the marine actinobacterial crude extract for antimicrobial activity

All the 110 marine actinobacterial isolates were screened for antibacterial and antifungal activity by agar well diffusion method. About 25 ml of molten Mueller Hinton Agar was poured into a sterile petri plate (HiMedia, Mumbai, India). The plates were allowed to solidify, after which 18 hrs grew (optical density adjusted 0.6) 100 µl of above said pathogenic bacteria cultures were transferred onto the plate and made culture lawn using sterile L-rod spreader. After 5 minutes setting of the bacteria, the test samples were dissolved in ethyl acetate at various concentrations (i.e., 25, 50, 75, and 100 µg/well) poured into the respective wells. The solvent ethyl acetate loaded served as negative control and streptomycin served as positive control. The plates were incubated at 37°C in a 40 W fluorescent light source (~400 nm) for 24 hrs. The antibacterial activity was determined by measuring the diameter of the zone of inhibition around the well using antibiotic zone scale (HiMedia, Mumbai, India).

PDA was used for the determination of antifungal activity. A similar preparation was made of the above procedure except the culture lawn instead of 5 mm fungal disc was cut and placed on the center point of the agar plate. Clotrimazole amended well served as positive control.

RESULTS

Isolation of *Actinobacteria*

Different selective media (SCA, BA, YMA, GA and PDA) were used to grow the *Actinobacteria* and the appearance of actinobacterial colonies varied on these media. SCA, YMA, and GA agar were found to be the most effective for promoting selective isolation of *Actinobacteria*. From these sediment samples, 110 different isolates of marine *Actinobacteria* was isolated. Isolates were purified by restreaking on SCA medium at least 2-3 times. Purified colonies were stored in SCA slants prepared by 2% agar strength and stored at 4°C in a refrigerator for monthly preservation. Each isolate was given a unique alphanumeric accession number starting from RA01 to RA110 (Figs. 2 and 3).

Screening of *Actinobacteria* for inhibitory activity

A total of 110 isolates were subjected to the primary screening of antibacterial (*P. aeruginosa* and *S. aureus*), anti-candidal (*C. albicans*), and antifungal (*A. niger*) activity and the results are shown in Table 1.

Primary screening

All the isolates (110) were subjected to the antimicrobial activity against four sensitive strains *P. aeruginosa* MTCC 1688 (Gram-negative rod), *S. aureus* MTCC 96 (Gram-positive coccus), *C. albicans* MTCC 227 (yeast), and *A. niger* MTCC 282 (fungus). Among these (110 isolates), 31 isolates were found to show activities against sensitive strains. The percentage of isolates which had shown activities against *P. aeruginosa, S. aureus*, *C. albicans*, and *A. niger* were found to be 12%, 40%, 20%, 8%, respectively (Fig. 4).

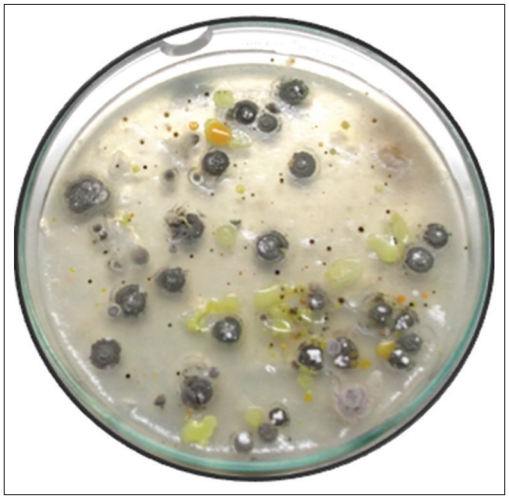

Fig. 2: Isolation plate of marine *Actinobacteria*

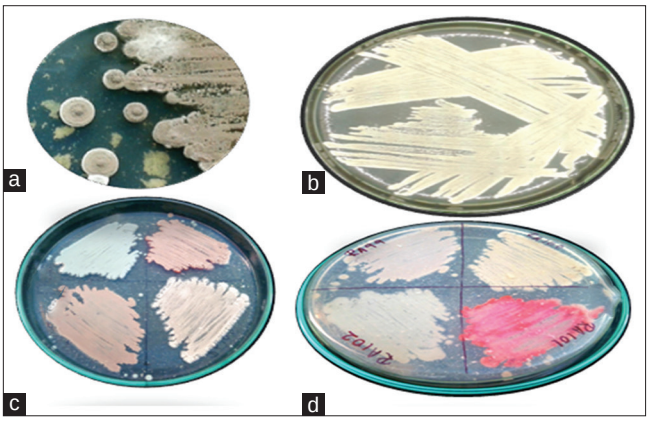

Fig. 3: Colony morphology and pigment production of selected marine *Actinobacteria* isolates; (a) Single colony of *Actinobacteria*, (b) purified culture of *Actinobacteria* (c and d) mycelial color and pigment formation in selected marine *Actinobacteria*

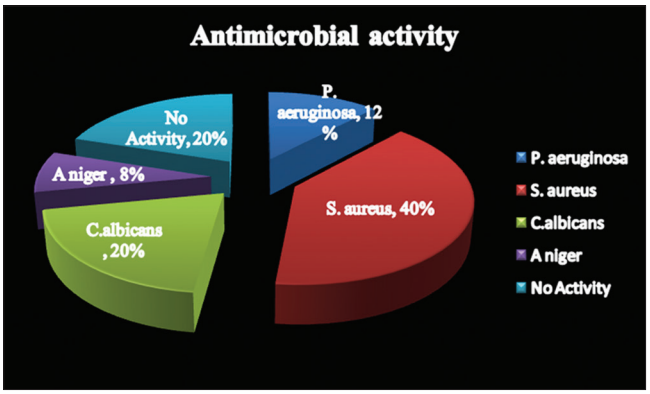

Fig. 4: Antimicrobial activity of marine *Actinobacteria*

Secondary screening

A total of 31 isolates of marine *Actinobacteria* were selected for secondary screening on the basis of their showing substantial inhibition of at least two or more sensitive organisms during primary screening. From the 31 potential isolates, two isolates (RA03 and RA86) with strong and broad spectrum activity were selected for further study (Table 2).

Table 1: Preliminary screening of marine *Actinobacteria* for antimicrobial activity by well diffusion method

Isolate code	Antimicrobial activity (zone of inhibition in mm*)			
	S. aureus MTCC 96	*P. aeruginosa* MTCC 1688	*C. albicans* MTCC 227	*A. niger* MTCC 282
RA01	0	0	9±1	0
RA02	0	0	0	0
RA03	0	0	13±1	2±3.47
RA04	0	0	12.66±4.93	0
RA05	4.66±4.04	0	15.66±3.78	0
RA06	0	0	10±1	0
RA07	0	0	12.66±3.05	0
RA08	4.66±4.16	0	3.33±5.77	0
RA09	3.33±5.77	0	0	0
RA10	2.66±4.61	0	19.66±2.51	0
RA11	3±2.64	0	0	0
RA12	2.33±4.04	0	15.66±5.68	0
RA13	4.66±4.16	3.33±5.77	18±4	2.33±2.51
RA14	4±3.60	0	14.33±1.52	0
RA15	0	0	14±2	0
RA16	3.66±3.21	0	13.33±1.52	0
RA17	0	8.66±1.52	15±2.64	0
RA18	6±2	0	15.66±2.51	0
RA19	1.66±2.88	0	2.66±4.61	0
RA20	6±1	8.66±2.08	12±1	1.33±2.30
RA21	0	0	11.33±2.51	2.33±2.08
RA22	0	0	0	0
RA23	4±3.60	0	12±2	0
RA24	2.66±4.61	0	14.66±8.50	0
RA25	0	0	0	0
RA26	0	0	10±4.35	0
RA27	0	0	15±7	0
RA28	1.66±2.88	0	0	3.66±3.21
RA29	1.33±2.30	0	11.66±1.15	0
RA30	2±3.46	0	14±5.29	0
RA31	4.66±4.50	0	12.66±3.05	0
RA32	4±4	0	10.66±0.57	0
RA33	1.56±2.88	3±5.19	10±3.60	0
RA34	3.66±3.21	2.66±4.61	0	0
RA35	1.66±2.88	0	8.66±4.16	0
RA36	4.66±4.16	3±5.19	16.33±5.50	0
RA37	7.33±1.52	0	11±2	0
RA38	5.33±4.72	8.33±1.52	17.33±5.50	2.33±4.04
RA39	2.33±4.04	3.33±5.77	11.33±1.15	0
RA40	4.66±4.16	0	17.66±6.80	0
RA41	4.33±3.78	0	21±1.73	0
RA42	6.33±1.52	0	15±4.35	0
RA43	0	2.66±4.61	18.66±5.68	2.66±4.61
RA44	4.66±4.16	0	0	0
RA45	0	0	6.66±6.11	0
RA46	0	0	9±1	0
RA47	5±4.35	0	18.66±8.32	1.66±2.88
RA48	1.33±2.30	3±5.19	19.66±6.11	2±3.46
RA49	2.33±4.04	0	14±1	0
RA50	6.66±1.52	0	8±7.21	0
RA51	6.66±1.52	0	11.33±1.52	2.33±2.08
RA52	0	0	14±2	0
RA53	6±1	0	16.33±2.08	0
RA54	2.33±4.04	0	11.33±1.52	0
RA55	2±3.46	2.66±4.61	13±3.60	0
RA56	1.66±2.88	0	17.33±4.50	0
RA57	5.33±5.03	0	13.66±1.52	2.66±4.61
RA58	10.33±1.52	0	10.33±2.51	2±2
RA59	2.33±4.04	0	15.66±3.78	0
RA60	7±1	0	3.33±5.77	0
RA61	4.66±4.16	0	11±2	0
RA62	2.33±4.04	3±5.19	15.66±6.42	0
RA63	2±3.46	0	12.66±1.52	0
RA64	9.33±1.52	0	16.33±3.05	2.33±4.04
RA65	3.66±3.21	0	13.33±1.52	0
RA66	7±2	8±1	11±1	0.66±1.15

(Contd....)

Table 1: *(Continued)*

Isolate code	Antimicrobial activity (zone of inhibition in mm*)			
	S. aureus MTCC 96	*P. aeruginosa* MTCC 1688	*C. albicans* MTCC 227	*A. niger* MTCC 282
RA67	4.66±4.16	0	3.66±6.35	0
RA68	9.66±1.52	0	0	2.33±4.04
RA69	2±3.46	0	8±7	0
RA70	8±2	0	15±5.29	0
RA71	6.66±1.52	0	16±3.60	0
RA72	3.33±1.52	0	7.33±6.65	0
RA73	2±3.46	3.66±6.35	0	0
RA74	4±3.60	0	3.33±5.77	0
RA75	5.66±2.08	0	11.33±9.86	2.33±2.51
RA76	2±3.46	0	15.33±2.08	0
RA77	4.33±3.78	0	7.33±6.42	0
RA78	2±3.46	4±6.92	0	2.66±4.61
RA79	4.33±1.52	0	11.33±1.52	0
RA80	6±1	0	14±2	0
RA81	1.66±2.88	3±5.19	14.33±3.51	0
RA82	2.33±4.04	6±5.29	18±5.29	0
RA83	3.66±3.21	0	8±7.21	0
RA84	5.33±0.57	8.66±1.52	15±2.64	0
RA85	2±3.46	0	15.66±3.78	0
RA86	4.33±3.78	2.66±4.61	17.33±3.05	3±5.19
RA87	5±4.35	8.66±1.15	8.66±8.08	2.66±4.61
RA88	8±1	0	0	0
RA89	2±3.46	8.33±1.52	7.66±6.80	1.66±2.88
RA90	8.33±1.52	3±5.19	21.66±6.02	1.66±1.52
RA91	3.66±3.21	3.33±5.77	9.66±1.52	0
RA92	7.66±1.52	5±4.35	11±2	2±3.46
RA93	2±3.46	2.66±4.61	5.66±5.13	1.66±2.88
RA94	4.33±4.04	0	9±1	0
RA95	2±3.46	0	2.66±4.61	1.33±2.30
RA96	8.33±1.52	0	18±5	1.33±1.52
RA97	9±1	9.66±2.88	12.66±1.52	2.66±4.61
RA98	8.33±1.52	±	13.66±1.52	2±3.46
RA99	7±2	7.66±1.15	7.66±6.80	1.66±2.88
RA100	0	2.66±4.61	12±1	0
RA101	7.33±1.52	2.66±4.61	3.33±5.77	2±3.46
RA102	1.66±2.88	8.33±1.52	6.33±5.68	2.33±4.04
RA103	2±3.46	0	7.66±6.80	0
RA104	1.66±2.88	0	3±5.19	0
RA105	2±3.46	0	13.33±1.52	0
RA106	2±3.46	0	12.66±1.52	0
RA107	1.33±2.30	0	11.66±0.57	0
RA108	0	0	12±1	0.66±1.15
RA109	2.66±4.61	0	13±6.24	0
RA110	1.66±2.88	2.66±4.61	0	0

*Values are mean of triplicates with standard deviation,
S. aureus: *Staphylococcus aureus, P. aeruginosa*: *Pseudomonas aeruginosa, C. albicans*: *Candida albicans, A. niger*: *Aspergillus niger,* MTCC: Microbial Type Culture Collection

Table 2: Secondary screening of marine *Actinobacteria* for antimicrobial activity by well diffusion method

Isolate code	Antimicrobial activity (zone of inhibition in mm*)			
	S. aureus MTCC 96	*P. aeruginosa* MTCC 1688	*C. albicans* MTCC 227	*A. niger* MTCC 282
RA03	37±18.5	24±12	33±16.5	17±8.5
RA13	18±9	23±11.5	28±14	6±3
RA20	15±7.5	0	30±15	9±4.5
RA21	13±6.5	16±8	18±9	0
RA28	0	15±7.5	27±13.5	12±6
RA36	18±9	0	30±15	9±4.5
RA38	16±8	20±10	28±14	6±3
RA43	17±8.5	18±9	33±16.5	12±6
RA47	18±9	0	32±16	11±5.5
RA48	18±9	0	32±16	0
RA51	14±7	15±7.5	31±15.5	0
RA57	13±6.5	14±7	25±12.5	0
RA58	15±7.5	18±9	27±13.5	9±4.5
RA64	22±11	18±9	29±14.5	7±3.5
RA66	15±7.5	13±6.5	0	6±3
RA68	14±7	15±7.5	26±13	10±5
RA75	18±9	14±7	26±13	0
RA78	15±7.5	20±10	29±14.5	0
RA86	24±12	21±7.5	33±16.5	17±8.5
RA87	15±7.5	14±7	26±13	9±4.5
RA89	14±7	20±10	28±14	12±6
RA90	13±6.5	19±9.5	30±15	12±6
RA92	15±7.5	20±10	24±12	7±3.5
RA93	18±9	18±9	26±13	6±3
RA95	14±7	15±7.5	24±12	6±3
RA96	0	0	27±13.5	0
RA97	14±7	0	26±13	0
RA98	15±7.5	0	30±15	8±4
RA99	0	13±6.5	32±16	9±4.5
RA101	13±6.5	18±9	21±10.5	6±3
RA102	15±7.5	15±7.5	32±16	8±4

*Values are mean of triplicates with standard deviation,
S. aureus: *Staphylococcus aureus, P. aeruginosa*: *Pseudomonas aeruginosa, C. albicans*: *Candida albicans, A. niger*: *Aspergillus niger*, MTCC: Microbial Type Culture Collection

DISCUSSION

Soil is the major repository of microorganisms that produce antibiotics. *Actinobacteria* are well known for the production of antimicrobial compounds. *Actinobacteria* were isolated from marine sediment samples. It has been reported in earlier studies that actinomycetes isolated from diverse ecological habitats are producers of the novel and diverse chemical entities [16-22]. Sediment samples were taken from a depth of 3 to 5 m of the top layer. It has been known that top sediments are rich in organic matter, and microbes were most of the biological activities occur. The population of actinomycetes decreased significantly with soil depth as there is decrease in organic substrate as well as aeration is poor in deeper layers of soils [23,24].

Sediment samples were subjected to different physical and chemical pretreatment methods for selective isolation of actinomycetes. These have been developed and used by researchers for selective isolation of actinomycetes, and some of these are genus specific [25,26]. Air drying of samples was done for a variable duration depending on the moisture content. Drying of the soil samples kills contaminant Gram-negative bacteria, whereas desiccation resistant actinomycetes survive [27-29]. Therefore, the number of actinomycete colonies on isolation plates increased. Incubation of soil samples with calcium carbonate also effectively decreased the number of contaminant microbes, but actinomycetes selected were morphologically alike as reported earlier [30-32].

A range of selective media was tested for facilitating isolation of *Actinobacteria*. SCA, YMA, and GA agar were found to be the most effective in the study. These media have also been used by other research groups in isolation of actinomycetes [33]. It was found that the addition of glycerol and asparagine in the isolation media favored the growth of actinomycetes [34]. Zhang and Zhang used glycerol asparagine as the medium for selective isolation of actinomycetes [35]. Previous authors reported that SCA be effective for the isolation of *Actinobacteria*, similar results were obtained in this study [28,36]. Antibiotics nalidixic acid and clotrimazole were added to the isolation media for decreasing occurrence of contaminant bacteria and fungi without affecting the growth of *Actinobacteria*.

Subramani and Narayanasamy isolated that 208 marine *Actinobacteria* using starch the casein agar medium in which 111 isolates exhibited antimicrobial activity against human pathogens whereas our study revealed 110 isolates against the antimicrobial activity. During the primary screening, 110 isolates were randomly chosen and subjected

to antimicrobial analysis. Among isolates, 12%, 40%, 20%, 8% isolates had shown activities against *P. aeruginosa, S. aureus*, *C. albicans*, and *A. niger*, respectively. The results of primary screening revealed that most of the isolates were active against Gram-positive bacteria (*S. aureus)* as compared to Gram-negative bacteria, yeast and fungus (*P. aeruginosa, C. albicans* and *A. niger*). The reason for differential activity against Gram-positive and -negative bacteria might be due to the morphological differences between them [37]. This kind of difference in the activities of *Actinobacteria* against pathogenic strains has been reported earlier [38-41]. Depending on the results of primary screening, 31 isolates were selected for secondary screening. From the 31 potential isolates, two isolates (RA03 and RA86) with a strong and broad spectrum of antimicrobial activity were selected for further study.

CONCLUSION

In this study, marine sediment samples were collected from 20 different points in Pulicat Lake North - East coast region of Chennai, Tamil Nadu. The *Actinobacteria* from these collected samples were isolated using a combination of physical and chemical pretreatment methods in five different selective isolation media. The isolates were screened for antimicrobial activity; isolates showed noticeable activity not only against Gram-positive bacteria but also against Gram-negative bacteria, yeast, and fungi. Primary and secondary antimicrobial analysis helped in the selection of potential strains for further studies. These strains can further be subjected to strain improvement and combinatorial biosynthesis for the production of more effective bioactive molecules.

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