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Antagonistic activity of marine actinomycetes from Arabian Sea coast

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ABSTRACT

The sponge-microbial association is a potential chemical, ecological phenomenon, which provides sustainable source of supply for developing novel pharmaceutical leads. The present study was carried out to investigate the in vitro antimicrobial activity of sponge associated actinomycetes and actinomycetes isolated from marine soil. Sixty three marine actinomycetes strains were isolated from the sponge and soil samples collected from two different stations from Arabian sea, south west coast of India. The counts of actinomycetes were found maximum in sponges during south west monsoon season. The antimicrobial screening showed that, five Streptomyces sp. exhibited antimicrobial activity against eye pathogens, antibiotic sensitive and resistant bacterial pathogens.

Keywords: Actinomycetes, Antimicrobial activity, Bacterial pathogens, Marine sponges, Marine soil.

INTRODUCTION

Ocean has been considered as a rich source of compounds possessing novel structures and biological activities. Biologically active molecules isolated from marine flora and fauna have applications in pharmaceuticals, nutritional supplements, cosmetics, agrochemicals, molecular probes, enzymes and fine chemicals [1]. Actinobacteria are a group of morphologically and physiologically diverse, Gram-positive bacteria having DNA with a high GC (>55 mol%) content [2]. The actinomycetes are a successful group of bacteria that occur in a multiplicity of natural and man-made environment. Actinomycetes are easily isolated from the marine environment and actinomycetes are important microorganisms since more than 90% of practical antibiotics originate from them and two thirds of 10,000 biologically active substances of microbial origin are produced by them. Important secondary metabolites, including antibiotics, herbicides and growth-promoting substances are produced by several members of the actinomycetes [3].

Since marine organisms live in a significantly different environment from those of the terrestrial organisms, it is reasonable to expect that their secondary metabolite will differ considerably. The presence of large amounts of microorganisms within the mesophyl of many demosponges has been well documented [4, 5]. The present investigation was mooted out to explore the antibacterial potential of sponge associated actinomycetes from Arabian sea.

MATERIALS AND METHODS

Sample collection

The sponge and soil samples were collected for a period of one year (February 2009 – January 2010) from the Chinnamuttam coast (Lat 08° 06' N and Long 77° 34' E, Station - I) and Pallam coast (Lat 08° 06' N and Long 78° 26' E, Station - II) in the Arabian sea. To avoid cross contamination, only unbroken samples were used for microbiological analysis. The sponge samples were placed in sterile sip-lap bags containing seawater and immediately transported to laboratory. To remove loosely associated microorganisms from inner and outer sponge surfaces, the sponge samples were kept for 3 × 30 min in one liter each of filter sterilized (0.20µm pore size, Sartorius Stedim Biotech GmbH, Germany) seawater in separate containers.

Isolation of actinomycetes

Isolation and enumeration of actinomycetes were performed on starch casein agar medium within 6 hours after sampling. One gram of samples was cut into small pieces using sterilized scissor and put into 99ml of presterilized 50 percent seawater blank. This setup was kept over the rotary shaker at 100rpm for one hour for the proper detachment of adhering propagules from the sponge tissues. Simultaneously gram of soil was serially diluted and plated in triplicate on starch casein agar medium (Soluble starch: 10g; Casein: 1g; Agar: 18g; Aged seawater: 500ml; Distilled water: 500ml; pH 7.2±0.2; autoclaved at 15lbs for 15 min; Nalidixic acid: 20µg/ml; Nystatin: 25µg/ml; Cycloheximide: 100µg/ml) [6] using spread plate method. The plates were incubated in an inverted position for 7-10 days at 28±2°C [7]. Based on the colour and morphological differences, powdery colonies were counted and restreaked thrice in a yeast extract malt agar (ISP2) (Glucose: 4g; Yeast extract: 4g; Malt extract: 10g; Agar: 18; Aged seawater: 500ml; Distilled water: 500ml; pH 7.2±0.2; autoclaved at 15lbs for 15 min) medium to get an axenic culture. The spore stocks were prepared from the culture grown on ISP2 medium and stored in refrigerator for further identification and antagonistic studies [8].

Test organisms

Ten antibiotic sensitive pathogens *Staphylococcus aureus, Vibrio cholerae, Proteus vulgaris, Salmonella enteritidis, Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis, Bacillus megaterium, Klebsiella pneumoniae, Candida albicans* and five antibiotic resistant human pathogens *Streptococcus sp., Klebsiella sp., Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae* and ten eye pathogens *Escherichia coli, Proteus sp., Pseudomonas sp., Klebsiella sp., Staphylococcus aureus, Acinitobacter sp., Streptococcus pneumoniae, Streptococcus viridians, Streptococcus pyogens, Staphylococcus epidermidis* identified from different sources in Government hospitals maintained in our laboratory were chosen for the present study.

Primary screening

The antagonistic activity was tested by following Cross streak assay method [9] [10]. Single streak (4–6 mm diameter) of the actinomycetes strains were streaked on the surface of the modified nutrient agar plates (Glucose: 5g; Peptone: 5g; Beef extract: 3g; Sodium chloride: 5g; Agar: 15g; Distilled water: 1000ml; pH 7.0 ± 0.2; autoclaved at 15lbs for 15 min) and incubated

at room temperature (28 ± 2°C) for 5-7 days [11]. After obtaining a ribbon-like growth, the over night culture of chosen bacterial pathogens were streaked at perpendicular to the original streak of actinomycetes and incubated at 28 ± 2°C. Control plate was also maintained without inoculating actinomycetes to assess the normal growth of bacteria and fungi. Actinomycetes isolates which showed prominent and broad spectrum activity were used for secondary screening.

Secondary screening

Promising antagonistic strains were subjected for the mass cultivation for the extraction of required quantity of antimicrobial metabolites. A loopful inoculum of chosen actinomycetes strains were inoculated into 500 ml Erlenmeyer flask containing 100 ml of yeast extract – malt extract broth (ISP2) and kept at 28°C for 72 h with continuous shaking. Twenty milliliter of the broth culture was then transformed to 1000 ml of Glycerol Asparagine broth (ISP5) [Glycerol: 10g; Asparagine: 1g; Dipotassium hydrogen phosphate: 1g; Sodium chloride: 5; Trace solution: 1ml (Trace solution - $FeSO_4$: 0.1g; $MnCl_2$: 0.1g; $ZnSO_4$: 0.1g; distilled water: 100ml); Distilled water: 1000ml; pH 7.2 ± 0.2; autoclaved at 15lbs for 15 min) and incubated for 7 days under continuous shaking in a rotatory shaker. Filtered cell free broth (pH 7.2) was adjusted to pH 5.0 using 1N hydrochloric acid mixed with equal volume of ethyl acetate in a separating funnel to extract the bioactive compound. The upper organic phase was concentrated in a vacuum evaporator at 40°C for 24 h and a reddish brown coloured crude extract was obtained. This process was repeated three times to obtain complete extraction of crude extract. 50µg concentration of soluble crude extract was impregnated on to Whatman No.1 filter paper disc (6 mm diameter) and placed on Muller Hinton Agar (HiMedia Laboratory Private Limited, Mumbai, India) which was previously inoculated with test organisms. The sterile disc impregnated with ethyl acetate was used as a control and all the plates were incubated at 37°C for 24 h. After incubation, the zone of inhibition appearing around the discs were measured and recorded as zone of inhibition in millimeter diameter.

Identification of potential actinomycetes

The generic level identification of potential actinomycetes was carried out [11]. The morphological, cultural, physiological, biochemical, colour and carbon utilization were carried out using standard procedure and were identified based on the keys of Bergey's manual of determinative bacteriology [12].

RESULTS AND DISCUSSION

Selection of potential antagonistic actinomycetes depends upon the huge diversity of strains and sample size. To explore the diversity of promising strains, the present investigation has carried out for the isolation of actinomycetes in soil and sponge samples from Arabian sea which was not explored so far. The culturable actinomycetes associated with sponge samples from Chinnamuttam (54×10^2 CFU/g) and Pallam (189×10^2 CFU/g) were found maximum during the month of August. Similarly the actinomycetes counts in soil were found maximum during the month of August in Station I (5×10^2 CFU/g) and in Station II (11×10^2 CFU/g). The seasonal distribution of sponge associated actinomycetes counts were found maximum in both sponge and soil samples during South West monsoon in Station I&II (Table 1). 63 strains were (MSUKR-1 to MSUKR-63) selected based upon the morphological characteristics (Data not shown) irrespective of the collection site and seasons.

Table 1: Counts of actinomycetes in sponge and soil samples

Month of Collection	Season	No. of sponge samples	No. of actinomycetes (CFU × 10^2 /g)			
			Station I		Station II	
			Sponge	Soil	Sponge	Soil
Feb-2009	Non-monsoon	5	21±0.72	2±0.27	67±1.24	0
Mar-2009		5	36±0.93	2±0.31	179±2.49	10±0.54
Apr-2009		5	7±0.12	0	0	0
May-2009		5	32±	1±0.21	23±0.58	0
Jun-2009	South-West monsoon	5	50±1.06	0	22±0.79	1±0.15
July-2009		5	40±0.94	0	32±0.59	1±0.34
Aug-2009		5	54±1.71	5±0.35	189±2.68	11±0.57
Sep-2009		5	0	0	21±0.48	0
Oct-2009	North-East monsoon	5	24±0.54	2±0.16	0	0
Nov-2009		5	8±0.16	0	0	0
Dec-2009		5	9±0.27	0	42±0.85	0
Jan-2010		5	12±0.38	0	13±0.48	0

Values are the average of three replicates

Table 2. Species level identification of potential strains of biologically active sponge associated actinomycetes

Characteristics	Name of the actinomycetes species				
	MSUKR29	MSUKR39	MSUKR44	MSUKR46	MSUKR57
True mycelium	Present	Present	Present	Present	Present
Facultative anaerobe	+	-	+	+	+
Acid fastness	-	-	-	-	-
Spores in aerial mycelium	+	+	+	+	+
Spores in substrate mycelium	-	-	-	-	-
Sporangium on aerial mycelium	-	-	-	-	-
No. of spores on aerial mycelium	21 – 50	13 – 20	13 – 20	21 – 50	15 – 17
Shape of spores	Globose	Globose	Globose	Globose	Globose
Spore motility	+	-	-	-	+
Cell wall type	1	1	1	1	1
DAP isomer	LL-DAP	LL-DAP	LL-DAP	LL-DAP	LL-DAP
Spore chain morphology	Recti-flexibles	Recti-flexibles	Recti-flexibles	Recti-flexibles	Recti-flexibles
Spore surface	Smooth	Smooth	Smooth	Smooth	Smooth
Aerial mycelium colour	White	White	White	Grey	Grey
Reverse side colour	-	-	-	Brown	-
Melanin pigment	-	-	-	-	-
Growth at 45°C	+	-	+	+	+
Halophilic	-	+	+	+	+
Alkalophilic	+	+	-	-	-
Utilization of carbon sources					
Glucose	+	+	+	+	+
Arabinose	+	+	+	+	+
Sucrose	+	-	+	-	+
Xylose	-	+	-	-	-

Inositol	+	-	-	+	-
Mannitol	+	+	+	+	+
Fructose	+	+	+	+	+
Rhamnose	+	+	+	-	+
Raffinose	+	-	+	+	+
Cellulose	-	-	-	-	-

DAP – Diaminopimelic acid ; + : Positive ; - : Negative; MSUKR29 : Streptomyces albogroseolus (MSUKR29) ; MSUKR39 : Streptomyces aureocirculatus (MSUKR39); MSUKR44 : Streptomyces raceochromogenes (MSUKR44) ; MSUKR46 : Streptomyces achromogenes (MSUKR46); MSUKR57 : Streptomyces furlongus (MSUKR 57)

All the actinomycetes were tested against ten antibiotic sensitive human pathogens, ten human eye pathogens and five antibiotic resistant pathogens to choose the wide spectrum activity of antagonistic actinomycetes by cross streak assay. It revealed that, 33 strains showed antagonistic activity against at least one antibiotic sensitive human pathogen, 31 strains showed antagonistic activity against at least one eye pathogens and 43 strains showed antagonistic activity against at least one human antibiotic resistant pathogens (Data not shown). Five sponge associated marine actinomycetes which showed potential antimicrobial activity were further subjected for the secondary screening by antimicrobial disc diffusion assay. The chosen actinomycetes strains were identified as *Streptomyces albogroseolus* (MSUKR-29), *Streptomyces aureocirculatus* (MSUKR-39), *Streptomyces raceochromogenes* (MSUKR-44), *Streptomyces achromogenes* (MSUKR-46) and *Streptomyces furlongus* (MSUKR-57) (Table 2).

Table 3. Antimicrobial susceptibility of antagonistic actinomycetes metabolites against human pathogens

Test pathogens	**Zone of inhibition in mm diameter**				
	***Streptomyces albogroseolus* (MSUKR 29)**	***Streptomyces aureocirculatus* (MSUKR 39)**	***Streptomyces raceochromogenes* (MSUKR 44)**	***Streptomyces achromogenes* (MSUKR 46)**	***Streptomyces furlongus* (MSUKR 57)**
Antibiotic sensitive pathogens					
Staphylococcus aureus	8±0.28	7±0.25	-	8±0.75	-
Vibrio cholerae	8±0.35	10±0.62	7±0.49	-	8±0.39
Proteus vulgaris	8±0.75	8±0.58	7±0.85	7±0.48	-
Salmonella enteritidis	8±0.64	-	8±0.53	7±0.41	8±0.68
Pseudomonas aeruginosa	8±0.75	-	10±0.82	7±0.53	8±0.39
Escherichia coli	10±0.51	8±0.65	10±0.72	8±0.57	-
Bacillus subtilis	7±0.42	8±0.55	10±0.67	7±0.29	7±0.34
Bacillus megaterium	8±0.38	7±0.25	10±0.32	7±0.41	9±0.52
Klebsiella pneumoniae	8±0.29	-	7±0.51	-	7±0.37
Candida albicans	8±0.24	-	8±0.45	-	8±0.72
Eye pathogens					
Escherichia coli	10±0.76	8±0.45	8±0.41	7±0.34	8±0.50
Proteus sp.	10±0.61	8±0.56	8±0.40	8±0.65	-
Pseudomonas sp.	-	-	-	-	8±0.69
Klebsiella sp.	7±0.28	-	-	-	8±0.47
Staphylococcus aureus	10±0.64	8±0.26	10±0.47	10±0.71	8±0.54
Acinitobacter sp.	8±0.76	-	10±0.39	-	-

Streptococcus pneumoniae	12±0.92	10±0.84	12±0.95	12±0.68	10±0.67
Streptococcus viridians	8±0.84	-	-	-	10±0.74
Streptococcus pyogens	8±0.57	7±0.23	10±0.57	8±0.51	9±0.61
Staphylococcus epidermidis	9±0.70	8±0.45	8±0.42	8±0.36	-
Antibiotic resistant pathogens					
Streptococcus sp.	8±0.58	-	-	-	7±0.35
Klebsiella sp.	12±0.90	10±0.73	8±0.52	7±0.38	7±0.62
Pseudomonas aeruginosa	7±0.26	8±0.39	7±0.68	-	7±0.41
Staphylococcus aureus	7±0.51	-	8±0.37	-	-
Streptococcus pneumoniae	7±0.43	-	7±0.58	-	-

– : No activity; Values are the average of three replicates

The maximum zone of inhibition was noticed against human antibiotic sensitive pathogens by the crude extract obtained from *Streptomyces albogroseolus* (MSUKR29) (10mm dia) against *Escherichia coli*, *Streptomyces aureocirculatus* (MSUKR 39) against *Vibrio cholerae* and *Streptomyces raceochromogenes* (MSUKR44) against *Escherichia coli, Pseudomonas aeruginosa*, *Bacillus subtilis* and *Bacillus megaterium*. Likewise, the maximum zone of inhibition was noticed by the extract from *Streptomyces albogroseolus* (MSUKR29), *S. raceochromogenes* (MSUKR44) and *S. achromogenes* (MSUKR46) against *Streptococcus pneumoniae* (12mm dia), followed by *Staphylococcus aureus* (10mm dia) eye pathogens. But, *Streptomyces albogroseolus* (MSUKR29) alone showed maximum zone of inhibition (12mm dia) against antibiotic resistant *Klebsiella sp*. (Table 3). Generally Streptomyces *albogroseolus* showed wide spectrum of antimicrobial sensitivity against all the chosen bacterial pathogens.

Marine organisms produce many of the pharmaceutically active natural compounds. Identification of potential drugs from living organisms particularly from marine organisms could solve several hurdles in the management of bacterial and fungal diseases. Among the marine organisms, marine animals have been identified as a good source for drug development. The Arabian coast of India is a hot spot of diverse marine floral and faunal assemblages particularly sponges, sea cucumber, sea anemones, sea urchin, soft corals and diverse number of seaweeds. Most of these immaculate resources have not been explored for bioprospecting and microbial ecological studies. The marine sponges have been proven to be rich source of biologically active and pharmacologically valuable natural products, with a high potential to become effective drugs for therapeutic use [13].

Marine invertebrates have developed highly specific relationship with numerous associated microorganisms and these associations are of recognized ecological and biological importance [14, 15]. It has been reported that, the ratio of microorganisms with antimicrobial activity from marine sponges were higher than other sources [16-21], which suggest that sponge associated microorganisms might play a chemical defence role for their host. Actinobacteria produce over half of the bioactive compounds in the Antibiotic Literature Database [22].

A total of 106 strains of actinomycetes were isolated from sponge samples collected from the Yellow sea, China and in that, 78 actinomycetes strains were belonged to *Streptomyces sp* [23]. But in the present study, 63 strains were isolated from 120 sponge samples were investigated and it reveals that, five species of *Streptomyces sp*. showed promising antimicrobial sensitivity against all the tested human pathogens. Many researchers found that, *Streptomyces sp.* isolated from sponges have been shown to have antimicrobial activity [17, 19, 24]. Streptomycetes have

received important in recent years because of their bioactive compounds for industrial applications [25]. The actinomycetes counts were found maximum during the southwest monsoon and non-monsoon periods compared to northeast monsoon in both the collection sites. This could have been the result of higher load and reproductive spores in the study area brought by runoff water from surroundings.

It is also interesting to note that, 31 strains out of 63 isolates showed sensitivity against *Candida albicans*. However, actinomycetes strains isolated from the sediment of Parangipettai coast did not showed sensitivity against *Candida albicans* [26] and hence it is confirmed that sponge associated actinomycetes have unique novel antimicrobial compounds than the sediment microflora. It is also observed by the present study that both the Gram positive and Gram negative strains were equally inhibited by the tested strains of actinomycetes. The Gram positive pathogens were highly inhibited than the Gram negative pathogens [19]. It is also inferred by the present study that eye pathogens were inhibited maximum by the sponge associated actinomycetes followed by antibiotic sensitive and resistant pathogens. The antimicrobial nature of extracts from the sponge associated actinomycetes due to the presence of biochemical constituents in the extracts and they live in a biologically competitive environment with unique conditions of pH, temperature, pressure, oxygen, light, nutrients and salinity, which is especially rich in chlorine and bromine elements.

CONCLUSION

It is concluded from the present study that, more actinomycetes were found in marine sponges and the ethyl acetate extract of sponge associated actinomycetes possess significant antimicrobial activity against human eye pathogens, antibiotic sensitive and resistant pathogens. Among the isolated sponge associated actinomycetes, *Streptomyces sp.* appears to be the dominant potential strains. It is confirmed from the present study, sponge associated actinomycetes are good sources of unique natural bioactive metabolites.

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