World Journal of Fish and Marine Sciences 3 (2): 151-158, 2011
ISSN 2078-4589
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Occurrence of Sponges Associated *Streptomyces* and its Antimicrobial Activity

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Abstract: Sponges are host organisms for various symbiotic microorganisms such as Archea, Bacteria, Cyanobacteria and Microalgae. Sponges associated microorganisms are sources of wide variety of useful natural products like cytotoxins, antifouling agents, antibiotics, anti-inflammatory and antiviral compounds. Nearly 96 strains of *Streptomyces* were found to be associated with four species of marine sponges but only 28 stains were selected for antimicrobial activity. Among that four strains showed antagonistic property against pathogens like *Vibrio cholerae, E. coli* and *Psuedomonas* spp. by spot inoculation method. The inhibition zones of 10-15 mm were recorded. *In vitro* screening of the submerge culture extracts showed more than 10 to 30 mm of inhibition zone against *Vibrio cholerae, E. coli* and *Psuedomonas* spp. If these symbiotic *Streptomyces* strains from which some natural products are derived can be cultured, the *Streptomyces* strains could be used in a mass production of the bioactive compounds. The results indicated that *Streptomyces* strains isolated from of marine sponges produced antibacterial substances against harmful pathogens.

Key words: Marine sponges % Actinomycetes % *Streptomyces* % Antibacterial substances

INTRODUCTION

Marine sponges are rich source of structurally unique natural compounds, several of which have shown a wide variety of biological activities [1]. It is well known that even excellent drug candidates from sponges are often not developed because those sponges are rare and difficult to collect or both. Sponges harbour a rich diversity of marine organisms in their tissues [2]. Numerous products from marine invertebrates show striking structural similarities to metabolites of microbial origin, suggesting that micro organisms are the true source of these metabolites or are initially involved in their biosynthesis [3]. It has been suggested that the growth of these useful microorganisms may be under the control of sponge host [4]. This growth of beneficial microorganisms is termed gardening. Farming may occur frequently among sponges. Application of fluorescence *in-situ* hybradization with rRNA targeted oligo-nucleotide probes [5], on thin section of sponge tissues enables domain or even species-specific determination of symbiotic bacteria particularly in the sponges. The sponge will consume a substantial part of this primary production by direct ingestion of the symbionts or in the form of excreted metabolic products such as glycerol or glycogen [6, 7]. It has been estimated that 80% of the total energy requirements of the sponge *Phyllospongia lammellosa* was produced by its phototrophic symbionts [8]. It is generally assumed that the interior of the sponge body is continuously oxygenated, due to the efficient pumping of water through the aquiferous system [9, 10]. Hence sponges are not likely to harbour anaerobic micro organisms. However, the presence of facultative anaerobic bacteria in sponges has been demonstrated [11-13] and the recent discovery of sulfate-reducing bacteria [14, 15] and other symbiotic archea in sponges show that anaerobic microbial process may take place in sponge's tissues. The search for new bioactive substances has been remarkably successful and approximately two third of naturally occurring antibiotics including many medical importance have been isolated from actinomycetes [16] and majority from the genus *Streptomyces*. Compared to terrestrial forms, aquatic *Streptomyces* are important sources for the discovery of novel antibiotics. *Streptomyces* has wide application for antiviral, antibacterial, antitumour, auti-helminthic, insecticidal, immuno-modulator, Immuno-Suppressant etc. Occurrence of enzymes, L-asparaginase, which is employed in the treatment of tumours and acute lymphatic leukemia, has been reported in bacteria, fungi, streptomycetes [17-19].

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Thus an alternative strategy targeting the micro-organisms preferably *Streptomyces* associated with sponges for the screening of bioactive natural products and it may prove to be an effective approach to the requirement of antibiotics from Streptomycetes, since no literature is available pertaining to this aspect. In the present study, an attempt was made on isolation of *Streptomyces* strains associated with marine sponges and tested for their antagonistic potential against harmful pathogens.

MATERIALS AND METHODS

Collection of Sponges: Four varieties of sponges were collected from Kovalam Coast which is situated on the West coast of Kerala, India about 14 Km to the South of Thiruvananthapuram at 8° 23' N latitude and 76° 57' E longitude. Samples of sponges were collected in sterile polythene bags. Then the samples were transported to the laboratory with minimum possible time to avoid the external microbial contamination and excessive proliferation. After bringing them to the laboratory all the epiphytic faunas were removed. Then it was washed with sterile sea water to remove the bacteria originating from environmental sea water.

Fixation and Preservation of Sponges: Numerous standard histological fixatives works well for sponges, but the simplest and most reliable is 10% formalin in seawater. Buffer additions are generally not necessary. After fixation of few hours to a few days, the sample must be transferred to and stored in at least 70% alcohol [20].

Spicule Preparation: For spicule preparation, boil a fragment of sponge in a test-tube with about ten times the volume of fuming nitric acid (HNO_3) until the cellular material is dissolved and liquid is clear. Fill the sample tube with distilled water and let the spicule settle to the bottom at least 2 hours (process may be speeded up by gentle centrifugation). Remove the water by pipette and change three times. After the third water change, rinse with 95% alcohol in the same way again three times. After the last change suspend spicule in 1-2 ml alcohol and pour on to a slide. Let the alcohol to evaporate and then dry the slide at 60°C. (Burning of alcohol may cause unwanted clustering of spicule) [20].

Isolation of *Streptomyces* from Marine Sponges: The sponges were divided into small pieces with the help of sterile scissors. Then crushed separately in a sterile mortar and pestle and it was introduced into separate 99 ml blanks prepared with 100% sea water in a conical flask and serial dilation was done. Sterile Petri-dishes were taken and 1ml of aliquot which was serially diluted up to 10^2 was pipette into the Petri-dishes and we followed pour plate technique. The sterilized medium is poured in the Petri dish which contains the aliquot, at an ear bearing temperature. The Petri-dish was rotated in clock-wise as well as anticlockwise directions for even distribution and allowed to cool. After solidification of the medium the Petri-dishes were inverted and incubated for 3-7 days at room temperature (28±2°C). The selective medium used is Kuster's agar, the best medium allowing good development of actinomycetes micelles while suppressing bacterial growth which was containing starch or glucose as the carbon sources with casein and asparagines or nitrate as the nitrogen source, which is enriched with amino acids, vitamins, yeast and sediment extracts [21].

Enumeration and Maintenance of Cultures: In seven selective media number of colonies of *Streptomyces* spp was found were sub-cultured in Kuster's agar slants. Later they were kept in Refrigerator (4°C) till further analysis was to be carried out [22].

Characterization of Selected *Streptomyces* spp: The selected *Streptomyces spp* strains were characterized according to the method employed by collaborators in International *Streptomyces* Project [23].

Colour Determination: For colour determination the cultures were streaked on Kusters agar media and their combinations and then they were observed after 4-7 days. The aerial and substrate mycelial colour were recorded.

Pigmentation of Mycelia and Spore Morphology: The cultures were grown on a Petri dish containing casein-starch-peptone-yeast extract (CSPY) agar medium with a cover slip inserted at an angle of 45°. The cover slip was removed after 7 days of incubation, air dried and observed under scanning electron microscope [24].

Utilisation of Carbon Sources: The cultures were inoculated to test tubes containing 10 ml of basal mineral salt medium to which sterilised carbon sources (xylose, arabinose, rhamnose, fructose, galactose, raffinose, mannitol, inositol, sucrose, glucose) were added to a final concentration of 1 %. The tubes were incubated at 28 °C and after 7 days the growth of the cultures were observed. Glucose was used as positive control.

Sodium Chloride Tolerance: Sodium chloride at various concentrations (1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9 and 10 %) was added to 5 mL of the basal medium. The medium was inoculated with the cultures and incubated at 28 °C for 7 days. The biomass thus obtained was separated from the broth, dried and weighed. The dry weight of the biomass was expressed in grams.

Physiological and Biochemical Characteristics: were studied according to the procedures described previously [25, 26].

Anti Microbial Assay: The selected *Streptomyces* strains isolated were inoculated in Glycerol aspargine medium for seven days. After seven days, using 1 ml of chloroform for 40 minutes, arrest the growth of inoculated colonies, then it was over laid with 5ml sloppy agar containing seeded microbes like *E.coli, Psedomonous* spp, *Vibrio* spp and incubated for 24 to 48 hours at 37°C. Diameter of the incubation zone was recorded in millimetres [27].

***In vitro* Screening of Isolates for Antimicrobial Activity (Disc Method):** Isolates that showed activity against test microorganisms were inoculated as submerged culture in 500 ml Erlenmeyer flasks containing 100 ml of the liquid medium (0.8 g NaCl, 1 g NH_4Cl, 0.1 g KCl, 0.1 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.04 g $CaCl_2.2H_2O$, 2 g glucose, 3 g yeast extract in 1 litre of distilled water, pH 7.3). These cultures were grown in a rotary shaker at 200 rpm, 28°C for 120 h under the standard conditions of aeration and agitation. The resultant cultures were centrifuged for 15 min. The culture filtrates were solvent extracted with ethyl acetate (1:1) in the separating funnel and shaken vigorously for 20 min. The upper organic layers were collected and evaporated to dryness in a vacuum evaporator at 40°C. A crude gummy extract were obtained. The crude extracts were suspended in ethyl acetate at concentration of 1 mg/ml for antimicrobial studies. Sterile filter paper discs 6 mm in diameter (HiMedia, India) were impregnated with 50 ìl (50 ìg crude extract) suspensions, dried and placed onto the plates previously seeded with test microorganisms. Then the plates were kept at 4°C for at least 2 h to allow the diffusion of crude extracts. Then they were incubated for 24 h at 37°C for bacteria and 48 h at 28°C for fungi. The diameters of inhibition zones were then measured [28].

RESULTS AND DISCUSSION

In the present study, a total of four species of sponges were collected from Kovalam coast, Kerala, India. For identification of sponges, the sponge tissues were preserved in 10% formalin and photographed and then spicule were separated and fixed. These spicules are later drawn and photographed through Camera Lucida microscope. Each species has unique spicules and according to that species classification was done [29]. Four species sponges were identified as *Mycale mytilorum* (Annandale), *Tendania anhelans* (Lieberkuhn), *Callispongia diffusa* (Ridely) and *Dysidea fragilis* (Montagu) belonging to the class Demospongiae Sollas. They are classified according to specific spicule of each species and they have monoaxonic and tetraaxonic silicious spicules [30, 31]. They are structurally coloured. Earlier findings reported that these marine sponges have high concentration and storage of secondary metabolites [32].

The isolation of *Streptomyces* was carried out using the seven selective media of Kuster's agar. Nearly ninety six colonies of *Streptomyces* were isolated four species of marine sponges. Among that twenty eight strains were selected based o n their mycelial colouration (Table 1).

Table 1: Isolation of *Streptomyces* spp. associated with marine sponges using different media

	Total number of *Streptomyces* colonies (10^2/g)			
Microbial medium	*Callispongia diffusa*	*Mycale mytilorum*	*Tendania anhelans*	*Dysidea fragilis*
KA	2	10	5	3
KA+AA	2	5	8	6
KA+V	4	4	3	2
KA+AA+V	3	2	2	1
KA+Y	1	1	4	1
KA+Y+S	2	2	1	2
KA+S	4	4	8	4

KA-Kuster's agar, AA-amino acid, V-vitamin, Y-yeast extract, S-sediment extract

Table 2: Mycelial colour characteristics of *Streptomyces* spp. associated with marine sponges using different combination of Kusters media

Kuster's agar media and combination	Mycelial colouration of *Streptomyces* strains							
	Callispongia diffusa		*Mycale mytilorum*		*Tendania anhelans*		*Dysidea fragilis*	
	Aerial	Substrate	Aerial	Substrate	Aerial	Substrate	Aerial	Substrate
KA	Olive green	White	Olive green	White	Olive green	White	Greenish ash	Pale white
KA+AA	White	Light brown	Olive green	White	Olive green	White	Olive green	White
KA+Y	Olive green	Pale green	Olive green	White	Dark green	Yellow	Yellow	White
KA+AA+V	Creamy white	Pale yellow	Olive green	White	Olive green	White	Reddish brown	Pale brown
KA+V	White	Yellow	Pale yellow	Reddish brown	Yellow	White	Pale orange	White
KA+V+AA	Olive green	White	White	Pale yellow	Pale red	White	Olive green	yellow
KA+S	Brown	White	Olive green	White	Olive green	White	Olive green	White

KA-Kuster's agar, AA-amino acid, V-vitamin, Y-yeast extract, S-sediment extract

Table 3: Spore morphology and carbon utilization of *Streptomyces* strains (+ Positive results,-Negative results, ± Doubtful results, RF-Rectiflexibilis, S-Smooth)

Streptomyces strains	Spore surface	Spore chain	No carbon source (negative control)	D-xylose	L-arabinose	D-Rhamnose	D-fructose	D-galactose	Raffinose	mannitol	D-Inositol	Sucrose	D-glucose (positive control)
AQBCD10	RF	S	-	+	+	-	+	-	-	-	-	-	+
AQBMM34	RF	S	-	+	-	-	+	-	-	-	+	-	+
AQBTA79	RF	S	-	-	+	-	+	+	-	-	-	±	-
AQBDF94	RF	S	-	+	-	-	+	-	-	-	+	-	+

Table 4: Physiological and biochemical characteristics of *Streptomyces* strains (+ positive results,-negative results)

Parameters	AQBCD10	AQBMM34	AQBTA79	AQBDF94
Starch hydrolysis	-	-	+	+
Production of H_2S	+	+	-	-
Degradation of cellulose	-	-	+	+
Liquefaction of gelatin	+	+	+	+
Coagulation of milk	+	+	+	-
Peptonization of milk	+	+	+	-
Degradation of urea	-	-	+	+
Citrate utilization	+	-	+	+
Indole production	+	+	+	+
Catalase	+	-	-	-

Out of 28 strains, four strains exhibited positive antagonism against the tested pathogens. These strains were characterised by the methods recommended by International *Streptomyces* Project. In controversy, because of filter feeding mechanism of species which leads to passage way for microbes to enter the body, leads to deposition of microbes inside. That's what maximum colouration occurs since sponges cannot synthesize their own food. So they get their secondary metabolite deposition through various types of microbes associated which are used for defense mechanism. The usage of enrichment in selective medium for isolation of *Streptomyces* associated with marine sponges was first attempted in the present work. The enriched media were supplied with extra vitamin, amino acids, yeast extract and sediment extract along with selective media and the results indicated the microbe which expressed are much more coloured than the native one. This may be reason that these sources trigger or activate the particular genes for the expression of much more bioactivity than native one [33]. Aerial and substrate mycelia showing different colouration was resulted (Table 2).

The highlighted four strains (AQBCD10, AQBMM34, AQBTA79, AQBDF94) in Table 3 were selected for morphological, physiological and biochemical characterization. The four colonies were slow growing, chalky, folded and aerobic. The strains were acid-fast negative and found to be Gram-positive. Studies on spore morphology revealed that the four strains bore rectus flexibiles hyphae and smooth sporephores (Table 3).

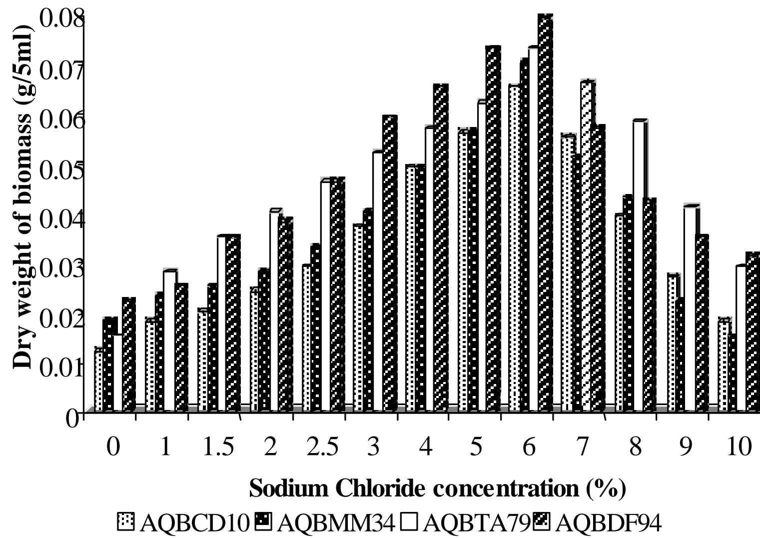

Fig. 1: Sodium chloride tolerance of the strains at varying concentrations

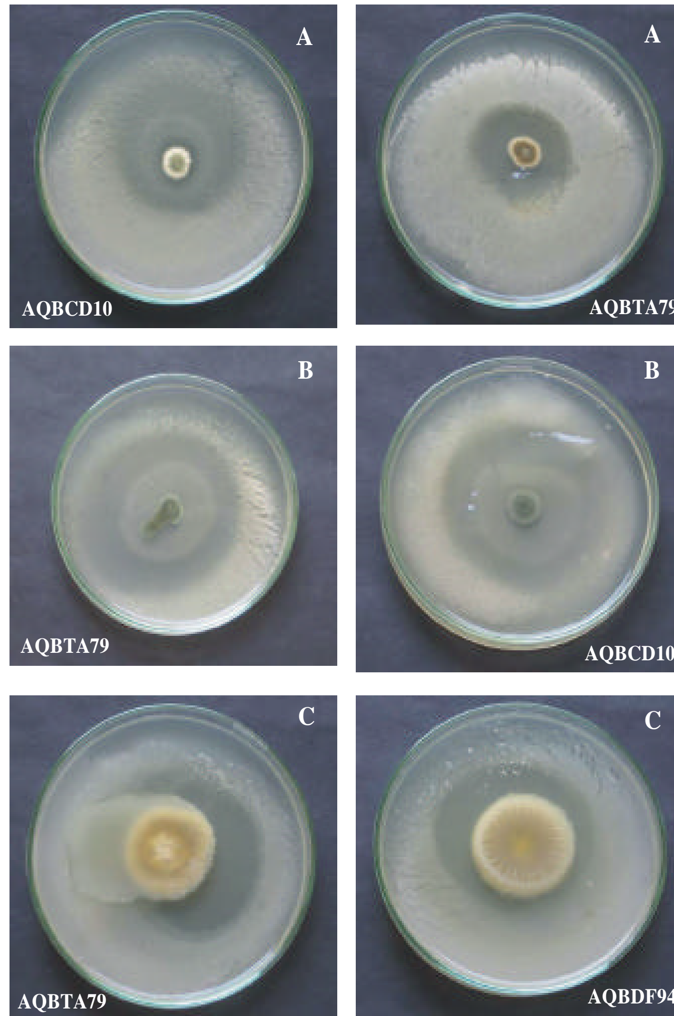

Fig. 2: Antibacterial activity of selected *Streptomyces* (AQBCD10, AQBMM34, AQBTA79, AQBDF94) against human pathogen *Vibrio cholerae* (A), *E.coli* (B), *Pseudomonous spp.* (C)

All the strains possess spore diameter of 2ìm. The strains showed typical morphology of *Streptomycetes* when analyzing the shape and spore chains under scanning electron microscope when compared with the earlier reports [24].

The nutritional characteristics of the strains were studied using criteria like carbon utilization and sodium chloride tolerance. The utilization of carbon sources is displayed in Table 3. The strains grew well in media containing fructose, glucose and xylose but the strains did not assimilate to Rhamnose, Raffinose and mannitol. At the sodium chloride concentration of 6 %, the strains showed profuse growth and exhibited maximal biomass (Fig. 1). The results of physiological and biochemical characteristics of the strains are displayed in Table 4. All the strains were able to grow in 22-45°C and pH 4-10. All the strains were able to liquefy gelatine and indole production. Solidification of milk and peptone cannot be done by the strain AQBDF94. Strains AQBTA79 and AQBDF94 were able to hydrolyze starch and cellulose but were unable to produce hydrogen sulphide. Degradation of urea was effectively done by AQBTA79 and AQBDF94. Positive utilization of citrate was confirmed in the strains AQBCD10, AQBTA79 and AQBDF94, except AQBMM34. Lastly, the catalyse activity was effectively seen only strain AQBCD10. The strains characterized by physiological and biochemical properties, nutritional uptake and all the four isolates fitted the *Streptomyces* genus as reported by several investigators [23]. The identification of the *Streptomyces* is a very complex process. The *Streptomyces* classification system was mainly dependent on characteristics like the form of spores, melanoma and use of carbon [25].

The antagonistic properties of *Streptomyces* spp against human pathogens like *Vibrio cholerae, E. coli* and *Psuedomonas* spp were investigated. The four strains showed maximal inhibition zonation of 10 to 15mm (Fig. 2). The maximal bio-activity of *Streptomyces* associated with sponges occurred. This can be compared with antibiotic production against bacteria *Bacillus subtilis*, *E. coli* and *Vibrio* spp [34]. Later, *In-vitro* screening of the culture extracts was carried out by disc method which resulted in the occurrences of more than 10 to 30 mm diameters of inhibition zones against *Vibrio cholerae, E. coli* and *Psuedomonas* spp. Recently, there are reports on the isolation of *Streptomyces* associated with marine sponges and its bioactive potential against bacterial and fungal pathogens [35]. The results almost correlated with the previous findings in which they investigated the antimicrobial activity of 74 *Streptomyces* isolates from soil [28]. Isolation and screening *Streptomyces* from the forest areas of Assam for antimicrobial metabolites were also earlier investigated [36].

It is concluded that marine sponge-associated *Streptomyces* strains represent a promising source of antibacterial agents against human pathogens. The result of the antibacterial studies and spectral and thin layer chromatographic analysis revealed that these strains were effective producers of bioactive metabolites against specific target pathogens. There are very limited reports on use of *Streptomyces* associated with marine sponges as antibacterial agents against pathogens. The discovery of new classes of antibiotics is highly necessary due to the increased incidence of resistant pathogens to drugs that are currently in use. Antibiotics developed from marine microbes are particularly important because they have high potency when compared with terrestrial counterparts.

ACKNOWLEDGEMENT

The authors wish to thank Department of Aquatic Biology and Fisheries, University of Kerala, for providing necessary facilities.

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