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Endosymbiotic Perspectives, Bioactive Compound Identification and its Protein Profile from Chosen Marine Sponges

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Abstract: Today, one of the emerging source of small molecule drug lead in the world's ocean. Based on the present findings, the sponge extract from *Zygomycale parishi* have potential anti-microbial activity against pathogenic bacteria and fungi. Endosymbiotic bacteria and actinomycetes were isolated from the sponge. Marine actinomycetes are recognized as the rich source of secondary. In this study, it is shown that extracts from the sponge derived actinomycetes also provide good source for the discovery of anti-microbial compounds. Endosymbiotic actinomycetes shows excellent activity against Pseudomonas species and its activity against *Bacillus subtilis* are feeble. Protein profiling reveals the band which indicates that the fraction obtained might have responsible potential anti-microbial activity. In SDS-PAGE protein profile study shows on crude toxins yielded nine bands in *Halichondria panicea* aqueous extract ranging from 14.3-116 kDa with three well-defined bands at 19.5, 39.0 and 66.2 kDa. The compound responsible for the anti-microbial activity in both sponge and its endosymbiotic actinomycetes has to be isolated and can be used for the drug development against diseases.

Key words: Endosymbiotic actinomycetes, marine sponge, *Zygomycale parishi*, *Denrilla membranosa*, drug, India

INTRODUCTION

The marine environment, particularly with sponges is a rich source of new bioactive metabolites 287 novel metabolites was isolated from marine sponges in 2008 (Blunt *et al*., 2010). The availability of biomass is a limiting factor for isolating marine natural products. The widespread isolation of typical microbial metabolites from sponges leads to the hypothesis that these metabolites are in fact the products of microbial metabolism (Zhang *et al*., 2006). Sponges are grouped in to 4 classes, the Hexactinellida (glass sponges), the Calcarea (calcarious sponges), Sclerospongiae (coralline or tropical reef sponges) and the Demospongiae. The widespread isolation of typical microbial metabolites from sponges leads to the hypothesis that these metabolites are in fact the products of microbial metabolism described by Fortman and Sherman (2005), Palpandi *et al*. (2007) and Nagasathya and Thajuddin (2008). The isolation of secondary metabolite-producing bacteria from sponges and of microbial secondary metabolism gene clusters from the metagenome of sponges has led to the general understanding that these metabolites are in many cases, the products of microbial symbionts and are not derived from the microbial diet of sponges (Kennedy *et al*., 2009).

A number of reports have been published on the isolation of actinobacteria from marine organisms (Li, 2009; Izumikawa *et al*., 2010). Very recently, several researchers were reported and investigated the screening bioactive substances from these marine-derived actinobacteria has yielded several new bioactive metabolites (Lin *et al*., 2010; Li *et al*., 2011; Sujatha and Joseph, 2011). The occurrence of large scale of bioactive compounds is not common to all living organisms but restricted to certain taxonomic groups.

Among marine animals, reef's invertebrates are the most prolific producers of secondary metabolites and have become sources of great interest to natural product chemistry, since they provide a large proportion of bioactive compounds with different biological activities described by Radjasa *et al*. (2007).

Sponges have been recognized as rich sources of novel compounds that are potential interest to mankind. Another fact that has been recognized is that virtually all sponges contain endosymbiotic microorganisms and these symbionts often contribute considerably to the total sponges biomass (Bharath *et al*., 2008). Sponges have been shown to harbor a large number of bacteria in some cases >60% of the tissue volume. Microorganisms detected so far in sponges include archaea, heterotrophic

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bacteria, cyanobacteria, red and green algae, dinoflagellates and diatoms (Hentschel *et al*., 2002, 2003; Hill, 2004; Javanshir *et al*., 2008). Marine sponges were provided with the largest number of marine derived secondary metabolites including some of the most interesting pharmacologically important drug candidates (Lam, 2006). A series of kalihinols, deterpenes isolated from the Philippine marine sponge *Acanthella cavernosa*, as potential bacterial foliate biosynthesis inhibitors. Very few researchers have been studied this kind of sponge species and its bioactivities. There is very ample of researches been developed. Since, the present study makes an attempt to find out the bioactive potential of sponge extract and associated actinomycetes.

MATERIALS AND METHODS

Preparation of crude extracts: The sponges collected in methanol containers were squeezed in a tissue homogenizer depending on the nature of the sponge species which was used for extraction. In the case of Mycale and Dendrilla species, they were cut into small pieces and squeezed to prepare the crude extract using a mortar and pestle using methanol as solvent. They were extracted thrice and the combined extract was concentrated in a rotary vacuum evaporator at room temperature. Thus, concentrated crude extract was collected in air tight plastic containers and kept in refrigerator. The aqueous extract of sponge was prepared by squeezing the sand-free specimens in triple distilled water. The resultant solution was filtered and dialyzed using dialysis membrane against D-glucose to remove the excess water. The supernatant obtained was lyophilized and stored at 4°C in a refrigerator for further use as aqueous crude extract for SDS-PAGE.

Anti-bacterial activity of sponge extract against pathogenic bacteria

Preparation of sponge extract: From an initial 10 g sample of dried sponge tissue, methanolic and chloroform-methanol (2:1) extraction, yielded, respectively 1.970 and 0.520 g of crude toxin while aqueous extraction yielded 3 g.

Estimation of protein: The crude protein contents were found to be 0.096 and 0.192 mg mL^{-1}, respectively in methanolic and chloroform-methanol extracts and 0.124 mg mL^{-1} in aqueous extract. It ranged from below detectable levels to 0.014 mg mL^{-1} in methanolic extract fractions and from below detectable levels to 0.002 mg mL^{-1} in aqueous extract.

Microorganism material: The sponge-associated actinobacterium *Streptomyces microflavus* was isolated from the inner tissue of the marine sponge Hymeniacidon perlevis collected from the inter-tidal beach of the Yellow Sea at Dalian, China (38°52'N, 121°41'E) in March, 2003. Sponge specimens were placed in plastic bags containing seawater and immediately transported to the laboratory. Actinobacteria identification was carried out by the method reported by Zhang *et al*. (2006).

Filter Paper Disc Method: The anti-microbial susceptibility testing was done by Kirby-baeur Disc Diffusion Method. This method allows the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that result from diffusion of the agent into the medium surrounding the disc. In this procedure, filter paper discs of uniform size (6 mm) are impregnated with specified concentrations of two methanolic sponge extract and then placed on the surface of an agar plate that has been seeded with the organisms to be tested. The medium of choice is Mueller-Hinton agar with pH of 7.2-7.4 which is poured into plates to uniform depth of 5 mm and refrigerated on solidification. Label the covers of each plate with the name of test organisms to be inoculated. Bacterial colonies were allowed to grow overnight at 37°C then the inhibition zone around the disc was measured.

Anti-fungal activity of sponge extract against fungi: Petri dishes with Rose Bengal agar medium were inoculated with four isolated fungi. Round paper discs of 6 mm were dipped in to 0.001 mL of each methanolic sponge extract and placed in the centre of the inoculated petri dish. Fungal colonies were allowed to grow overnight at 20°C and then the inhibition zone around the disc was measured.

Sponge was collected by scuba divers from South West coast of India. Voucher specimens were either preserved in 70% ethanol immediately upon collection or freeze-dried for chemical characterization. Freeze-dried specimens were prepared for histology by dehydration in a very weak solution of detergent for 24 h followed by preservation in 70% ethanol. Histological sections and spicule preparations were made as in the research of Kelly-Borges and Vacelet. Specimens are stored for the long term in 70% ethanol. Voucher specimens representing material that was chemically characterized were registered with the Natural History Museum and specimens are deposited in their sponge collections.

The sponges collected in methanol containers were squeezed in a tissue homogenizer depending on the nature of the sponge species which was used for extraction. In the case of Mycale species, they were cut into small pieces and squeezed to prepare the crude extract using a mortar and pestle using methanol as solvent. They were extracted thrice and the combined

extract was concentrated in a rotary vacuum evaporator (Buchi, Flawil and Switzerland) at room temperature. Thus, concentrated crude extract was collected in air tight plastic containers and kept in refrigerator.

SDS-PAGE analysis of protein profile: One dimension Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel Electrophoresis (PAGE) was carried out. The protein content of methanolic crude extract and aqueous crude extract were dissolved in 300 μL of sample buffer and the samples were loaded into several lanes of 30% gradient gel along with the molecular weight standards. The process adapted a power supply of 80 volts for 3 h. After electrophoresis, the gel was stained with silver staining.

Procedure for silver staining: After running one dimensional gel incubate the gel in Fixer (40% ethanol, 10% acetic acid, 50% water) for 1 h. Wash the gel in H_2O for atleast 30 min. Sensitized the gel in 0.02% sodium thiosulphate for only 1 min, wash gel in H_2O for 3×20 sec. Place the gel in a new staining tray wash gel in H_2O for 1 min. Develop the gel in 30% sodium carbonate, 0.05% formaldehyde. Change developer solution immediately after when it turns yellow. Terminate when the staining is sufficient. Wash the gel in H_2O for 20 sec. Terminate staining in 5% acetic acid for 5 min. Leave the gel at 4°C in 1% acetic acid for storage.

The aqueous extract of sponge was prepared by squeezing the sand-free specimens in triple distilled water. The resultant solution was filtered and dialyzed using dialysis membrane against D-glucose to remove the excess water. The supernatant obtained was lyophilized and stored at 4°C in a refrigerator for further use as aqueous crude extract for SDS-PAGE.

The crude extracts were purified by ammonium sulphate precipitation. During ammonium sulphate precipitation, the salt has to be preventing increase of high local concentration. Ammonium sulphate was used for precipitation of total proteins at -90% saturation or for precipitation of proteins using different saturations of salt 40% saturation was done to precipitate the proteins from the culture. The solution was equilibrated for approximately one day in cold condition to ensure complete precipitation and then the precipitate was collected by centrifugation and it was further purified by dialysis method using dialysis membrane. Thus prepared were used for 1 dimension Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel Electrophoresis (PAGE) was carried out. The protein content of methanolic crude extract and aqueous crude extract were dissolved in 300 μL of sample buffer and the samples were loaded into several lanes of 30% gradient gel along with the molecular weight standards. The process adapted a power supply of 80 volts for 3 h. After electrophoresis, the gel was stained with silver staining.

Table 1: Quantification of endosymbiotic actinomycetes DNA from *Zygomycale parishii* and *Dendrilla membranosa*

Endosymbiotic actinomycetes	OD of endosymbiotic bacteria	
	Zygomycale parishii (nm)	*Dendrillamembranosa*(nm)
	1.790	1.742
ES2	1.723	1.776
ES3	1.754	1.845
ES4	1.664	1.734
ES5	1.690	1.800

Quantification of DNA from the sponge: DNA extraction of *Zygomycale parishi* was carried by Lysis Buffer with Cetyl Trimethyl Ammonium Bromide (CTAB). Homogenization of sponge fragments in 1:3 (weight:volume) solution of 2% CTAB in 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl plus 1 μL β-mercaptoetanol and 10 μg μL^{-1} proteinase K. The suspension was incubated at 50°C for at least 1 h and centrifuged at 3000 g/10 min. The supernatant was extracted twice with one volume of chloroform:isoamyl alcohol (24:1). Precipitate DNA from the homogenate by the addition of 0.8 volumes of isopropanol plus 1/10 volume of 3M sodium acetate pH 5.2, followed by centrifugation at 10000 g/10 min at 4°C. The pellet was washed in 70% ethanol, air dried, dissolved in sterile water plus 20 μg mL^{-1} RNAse A and incubated for 1 h at 37°C. The concentration and purity of bacterial and sponge DNA were determined spectrophotomectrically for this purpose, DNA absorbance was measured at 260 nm (DNA μg g^{-1} sample; 1 A 260 = 50 μg mL^{-1} DNA) and protein impurities were checked at 280 nm (Table 1).

RESULTS AND DISCUSSION

Anti-bacterial activity of *Zygomycale parishii* and *Dendrilla membranosa* against pathogenic bacteria: Present study revealed that the tested in marine sponge *Zygomycale parishii* possessed potential anti-bacterial activity against *Azetobacter* sp., *B. cereus*, *B. subtilis*, *Glucanobactor oxydens*, *Pseudomonas aeruginosa* (Table 2, Fig. 1). While tested by the disc diffusion method, methanolic sponge extract of *Zygomycale parishii* showed significant activity against *B. subtilis* and *Glucanobactor oxydans* produced minimum inhibitory activity 18.7 mm. Among the five species of bacteria sponge methanolic extract showing highly significant anti-bacterial activity against *P. aeruginosa*. Another sponge of *Dendrilla membranosa* extracts showed maximum anti-bacterial activity of 18 mm, *Azetobacter* sp., followed by *Glucanobactor oxydans* 16 mm. Though, optimum as well as least activity

observed 14 mm in case of *B. subtitlis* and 12 mm in case of *B. cereus*, respectively. Of these two marine sponges, *Zygomycale parishii* extract consist of similar zone of inhibition observed 18 mm in *B. subtitlis* and *B. cereus.* Furthermore, biochemical characterization was observed results were shown on Table 3.

Anti-fungal activity of *Zygomycale parishii* and *Dendrilla membranosa* against fungi: Here, it was found that the extracts successfully prevent the growth of fungi. The inhibitory zone produced by extract against *Aspergillus aculeatus* 12 and 18 mm, *Aspergillus niger* was 12.5 and 15 mm, *Candida tropicalis* is 9 and 16.5 mm and *R. micchii* for 14 and 16 mm, respectively. Among this, *Zygomycale parishii* showed more activity against *R. micchii* (14 mm) followed by *Aspergillus niger* (12.5 mm) *and Candida tropicalis* (9 mm). The extract shows feeble activity against *Aspergillus aculeatus* (12 mm). In case of *Dendrilla membranosa*, it had higher activity against all the four fungi.

Anti-bacterial activity of endosymbiotic actinomycetes from *Z. parishii* and *Denrilla membranosa*: The anti-bacterial activity screening was performed by agar well method against the standard test organisms such as *B. subtilis*, *P. aeruginosa*, *P. fluorescens.* Actinomycetes have high activity against *Pseudomonas aeruginosa* and *pseudomonas fluorescens* while its activity was feeble against *Bacillus subtilis* (Fig. 2). By

Table 2: Bioactivity of endosymbiotic actinomycetes from two marine sponges, *Z. parishii* and *D. membranosa*

Bacteria	ESAcA1	ESAcA2	ESAcB1	ESAcB2
Bacillus subtilis	8	9.2	7.5	10.2
Pseudomonas aeroginosa	14	12.5	10.7	18.0
Pseudomonas fluorescens	12	18.5	20.2	13.8

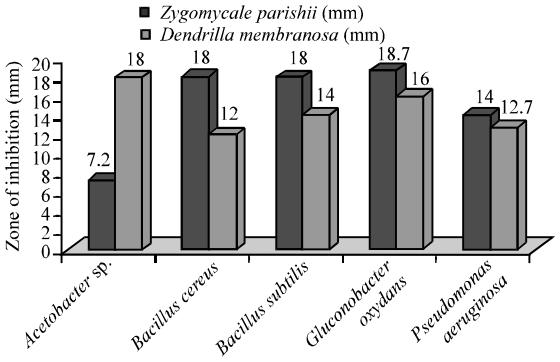

Fig. 1: Anti-bacterial activity of sponges (*Z. parishii* and *D. membranosa*) against bacteria

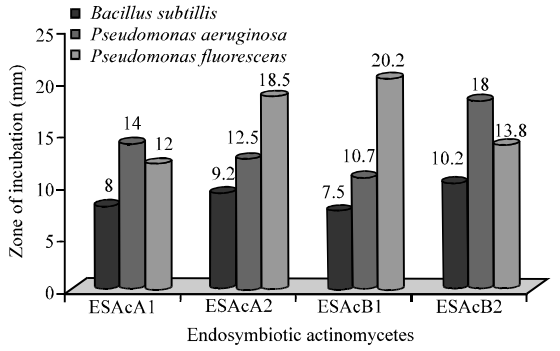

Fig. 2: Anti-bacterial activity of endosymbiotic actinomycetes from *Z. parishii* and *Denrilla membranosa*

Table 3: Biochemical characterization of endosymbionts from *Z. parishii*

Bacterial isolates	Casein	Catalase	Citrate	Indole	H_2S	MR	Oxidase	Urease	VP
ESA1 Endosymbionts from *Zygomycale parishii*	+	-	+	+	-	+	-	+	+
ESA2	+	-	-	-	-	-	-	+	-
ESA3	+	-	+	+	-	++	-	+	+
ESA4	+	-	-	+	-	++	+	+	+
ESA5	-	-	-	+	-	++	-	+	+
ESA6	-	-	+	+	-	++	+	+	-
ESA7	+	-	-	+	-	-	+	+	-
ESA8	+	+	-	+	-	+	-	+	-
ESA9	+	+	+	+	-	+	-	+	+
ESA10	+	-	-	-	-	+	-	+	+
ESA11	+	-	+	-	-	+	-	+	-
ESA12	+	+	+	+	-	-	+	+	+
ESA13	+	+	+	+	+	++	-	+	+
ESA14	+	+	+	+	-	+	-	+	+
ESA15	+	-	-	+	-	+	-	+	+
ESAcA1	+	+	+	-	-	+	-	+	+
ESAcA2	+	-	+	+	+	+	-	-	+
ESAcA3	+	-	+	+	-	+	-	-	-
ESAcA4	+	+	+	+	+	++	+	+	-
ESAcA5	+	-	+	-	+	++	-	+	+

(+): Positive; (-): No response; (++): Maximum

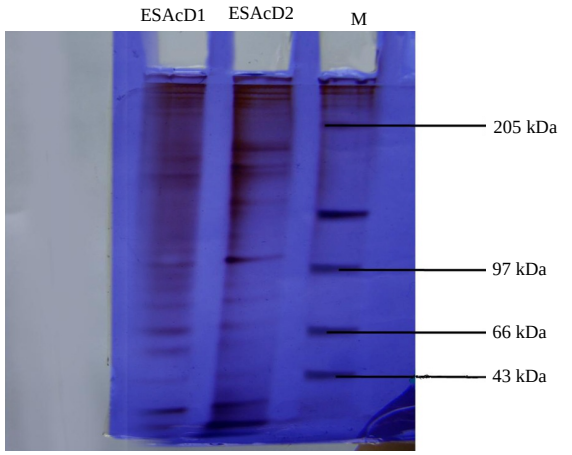

Fig. 3: SDS-PAGE analysis of endosymbionts from two different experimental sponges

SDS-PAGE on 12% gel, crude toxins yielded nine bands in *Halichondria panicea* aqueous extract, ranging from 14.3-116 kDa with three well-defined bands at 19.5, 39.0 and 66.2 kDa (Fig. 3). Moreover, the appeared banding patterns (higher molecular weight) were randomly present on the ESAcD2 compared with ESAcD1. In case of lower molecular weighed bands range between 43-97 were visibly appeared in ESAcD1 but in ESAcD2 having lower banding patterns were looking faded in nature. Though, 97 kDa band alone clearly visible in ESAcD2 than remaining bands.

In the present study, sponges *Zygomycale parishii* and *Dendrilla membranosa* collected from Vizhinjam coastal area, produced potent anti-microbial extracellular products. This property indicates that the species might have defense mechanisms in the host sponge. These sponges has extreme potent of anti-fungal and anti-bacterial activity (Chelossi *et al*., 2006). Previously, most of the researchers have been studied. Despite the crude of aqueous and chloroform extracts at different concentration of 5, 10 and 15 mg mL^{-1} were tested against 6 species of bacteria such as *Pseudomonas* sp., *Streptococcus aureus*, *Vibrio parahaemolyticus*, *V. cholerae*, *E. coli* and *V. parahaemolyticus* and 3 species of fungus namely; *A. flavus*, *A. niger* and *Candida albicans*. From this results agreed with the researchers viewed by Ankudey *et al*. (2008) and that the crude aqueous extract inhibit the growth of *V. cholerae* where as in the chloroform extract a clear inhibition zone were observed only against *Pseudomonas* sp. The present study indicated the closely related bacterial species present with in these sponges reported the findings about the endosymbiotic relationship with the sponges (Montalvo *et al*., 2005). Among the ten isolates, four strains of white series were selected and characterized by conventional methods and assessed for their antagonistic activity against fish pathogens like *Aeromonas hydrophila* and *Vibrio* sp. All the strains showed inhibitory activity against these fish pathogens (Safaein *et al*., 2009).

CONCLUSION

In this study depicted screening for pharmacologically active agents from marine actinomycetes, the researchers found that petroleum ether and ethyl acetate culture broth of *Nocardiopsis* sp. VITSVK5 (FJ973467) was found to be active against selected microbial pathogens (Boobathy *et al*., 2009). Furthermore, the present study also been agreed by Radjasa *et al*. (2007) with them opinion that marine bacteria associated with sponge *Haliclona* sp. showed strong growth inhibition against indicator microorganisms. Moreover, marine bacteria are emerging as an exciting species for the discovery of new classes of therapeutics and it could provide the drugs needed to sustain us for the next 100 years in our battle against drug resistant infectious diseases (Molinski, 2004; Williams, 2009) and which exhibited weak anti-fungal activity against *Alternaria alternate* (Usama *et al*., 2010). Indian marine sediment samples few potential bioactive Nocardiapsis have been reported. A protease-producing, crude oil degrading marine *Nocardiopsis* sp., NCIM 5124 have been reported (Dixit and Pant, 2000).

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