

## Evaluating the Antibacterial Potential of *Streptomyces* sp.

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### Abstract

**Background and Objectives:** Marine bacteria are recognized as an untapped resource of bioactive metabolites of diverse structure and functions. The present research is focused on the screening of the antagonistic potential of bacteria isolated from marine sponge against human and shrimp pathogens.

**Methods and Findings:** In this study, a total of 51 strains of bacteria isolated from five species of marine sponges sourced from the Vizhinjam littoral (Indian Ocean) were screened for possible antagonistic activity against human and shrimp pathogens. Of the 51 bacterial isolates, twelve strains isolated from marine sponges, *S. officinalis* and *D. nigra* showed different ranges of activity. The results of agar-well diffusion assay showed that, out of the twelve strains, MAPS 15 strain showed the highest activity which subdued the growth of all the tested 20 bacterial pathogens. The molecular characterization based on partial 16S rRNA sequence revealed that the active isolate MAPS 15 was *Streptomyces* sp.

**Conclusion:** This study serves as a basis for further research on the possibility of utilizing *Streptomyces* sp. for the development of human and veterinary grade antibiotics.

**Keywords:** Marine sponge isolates; *Streptomyces*; Secondary metabolites; Antagonistic activity

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### Introduction

The available statistics indicate marine invertebrates still to be virgin reservoir with rich novel biomedical leads [1]. Nevertheless, it's difficult to procure adequate and reliable supplies of these compounds from the nature. Hence, to resolve this tangle, new research focusing on marine microorganisms has been gaining considerable attention [2]. The majority of these organisms have been well known to produce bioactives which assist them in the colonization of surfaces including those of their host animals. During the last few decades probing for therapeutically active substances has riveted on marine organisms as it is relatively easy to isolate, purify and produce in bulk-scale [1]. In contrast with terrestrial organisms, marine organisms produce a cornucopia of bioactive secondary metabolites [3]. The secondary metabolites by marine bacteria isolated from different seas have been reported since long [3]. The majority of these bacterial strains were isolated from seawater and marine sediments. Bacteria associated with marine invertebrates have been found to display significant bioactivity including antifouling, antibacterial and cytotoxic activities. And there are innumerable studies on marine medical exploration and ecology investigation

about sponge associated bacteria and fungi [4-7]. Investigation of marine bacteria isolated from marine algae and sponge has revealed many of them having the ability to preclude the growth of human and shrimp pathogens [4,8]. In the light of this, the

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present research is focused on the screening of antagonistic bacteria isolated from marine sponge against human and shrimp pathogens.

## Methods

### Collection of sponges

The sponge specimens were collected off the Vizhinjam littoral (80°21'N, 77°00' E) by snorkeling at a depth of between 1 to 3 m. Upon collection, the sponges were soaked in aged natural sea water for 2 h to remove slackly associated microorganisms [4]. Afterwards, the drained samples were kept in a sterile incubator oven for 1 h at 40°C to dry the surface, and frozen at 20°C.

### Isolation of the marine sponge-associated microbes

A dollop of sponge tissue was excised using a sterile scalpel from the internal mesohyl region. The tissue samples were then homogenized with sterile sea water in a tissue homogenizer. Dilution series were prepared from the homogenates and each dilution (~500 µL) was inoculated by spread plate on to the different media such as Zobell marine agar (ZMA) or nutrient agar (1.5% NaCl). These media were supplemented with Nalidixic acid and cyclo hexamide to inhibit the other bacterial and fungal contamination, respectively. The inoculated Petri-plates were incubated for 14 days at 27°C after which colony-forming units (CFU) were counted. Counts between 30 and 200 CFUs were used for analysis. On the basis of morphological features, different colony types were randomly picked and purified by making streak plates on NA plates supplemented with 1.5% of NaCl. The diversity of colonies was examined based on morphological characteristics such as size, color, opacity, texture, form, elevation, margin and

surface [9]. Single colonies were transferred at least three times until considered pure. Strains were stored frozen at 4°C in Zobell Marine Broth.

### Antimicrobial screening of sponge-associates by cross streak method

The potential antagonistic bacteria were selected by screening the sponge isolates against a battery of human and shrimp pathogens (Table 1). The human and shrimp pathogens with MTCC number were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India. While the *Vibrio* isolates were culled from *Penaeus monodon* smote with Vibriosis [10]. All the test organisms were maintained on nutrient agar slants at 4°C before being used in the inhibitory assays. The antimicrobial activities of the sponge associated bacteria were performed by cross-streak method [11]. Mueller-Hinton agar plates were prepared and inoculated with sponge isolates by a single perpendicular streak of inocula in the centre of the Petri-dish and incubated at 27°C for 4 days. The plates were seeded with test organisms by a perpendicular streak at a 90° angle to the line of the sponge isolates. Antagonism was observed based on the inhibitory interaction between the sponge isolates and test organisms. The reference standard (Nalidixic acid) was used for the detection of strain sensitivity/resistance. Inhibition activities and colonization effect are noticed at 24, 48, 72 and 96 hours. All the tests were conducted in triplicates.

### Antimicrobial screening of sponge-associates by agar well diffusion method

The bacterial strains which displaying the antagonistic activity in cross-streak method was further confirmed with the agar well-diffusion method as described by Manilal et al. [4]. The sponge bacterial strains were grown in a nutrient broth (Merck, Germany), incubated at 25°C for 18h, and adjusted to an approximate concentration of 10<sup>8</sup> CFU ml<sup>-1</sup>. The Mueller Hinton agar plates were prepared and uniformly swabbed with respective pathogens. Thereafter, it was punched with a five millimeters diameter wells and filled with 120 µl of the respective microbial cell suspension (CS). The well with broth used for culture was considered as negative control. The Petri-dishes were incubated at 37°C for 24h. After incubation, plates were examined for inhibition zones. The assay was performed in triplicates of individual Petri-dishes. Clear zone of inhibition formed around wells were considered as indicative of antimicrobial activity. The inhibitory activity was measured by calculating the area of clear zone. The antibiogram was statistically analyzed for the determination of Skewness among the tested bacterial strains.

### Biochemical profiling of sponge isolates

Biochemical characterization of the potential sponge isolates were performed using the methods described by Lalucat et al. [12] and Lechevalier [13] respectively. Morphological characterization of the sponge isolates were determined by motility test, Gram's staining and Ziehl Nielsen staining. Physiological characterization was performed by checking the hydrolytic activity and carbohydrate fermentation of the potential antagonistic strains. The cultural characteristics of the sponge isolates were determined by naked eye examination of 14 days old cultures that were grown on different media such as

**Table 1** Panel of pathogens used for antimicrobial assay.

Group	Species
Human pathogens(MTCC)	<i>Staphylococcus aureus</i> (MTCC 96)
	<i>Bacillus amyloliquefaciens</i> (MTCC 10441)
	<i>Shigella flexneri</i> (MTCC 1457)
	<i>Micrococcus luteus</i> (MTCC 106)
	<i>Vibrio mimicus</i> (MTCC 4434)
Shrimp pathogens (MTCC)	<i>V. alginolyticus</i> (MTCC4439)
	<i>V. vulnificus</i> (MTCC 1145)
	<i>V. parahaemolyticus</i> (MTCC 451)
	<i>V. alcaligenes</i> (MTCC 4442)
	<i>V. fischeri</i> (MTCC 1738)
	<i>V. harveyi</i> (MTCC 3438)
	<i>V. anguillarum</i> (VB 10)
Shrimp vibrio isolates	<i>V. alginolyticus</i> (VB 11)
	<i>V. vulnificus</i> (VB 14)
	<i>V. parahaemolyticus</i> (VB 12)
	<i>V. harveyi</i> (VB 15)
	<i>V. fischeri</i> (VB 17)
	<i>V. splendidus</i> (VB 22)
	<i>V. campbellii</i> (VB 23)
<i>Ph. damselae</i> (VB 26)	

Actinomyces agar, Actinomycetes isolation agar (AI agar), Yeast Malt Extract agar (YME agar), Inorganic salt agar International *Streptomyces* Project Agar (ISP-4agar), Oat meal agar, Oat meal with glycerol medium and Chitin agar. The micro-morphology and sporulation were observed by light microscopy. Antibiotic sensitivity was also examined using selected antibiotics. Colours of aerial and substrate mycelium were determined with the ISCC-NBS centroid colour charts (US National Bureau of Standard, 1976). Hemolytic activity was examined using blood agar plates with 5% human blood. Plates were analyzed for hemolysis after incubation at 37°C for 24 h. The clear zone of clearance around the colony was considered as positive. All the culture media were prepared and treated as per the manufacturer guidelines.

### Molecular identification of the active sponge isolate (MAPS 15)

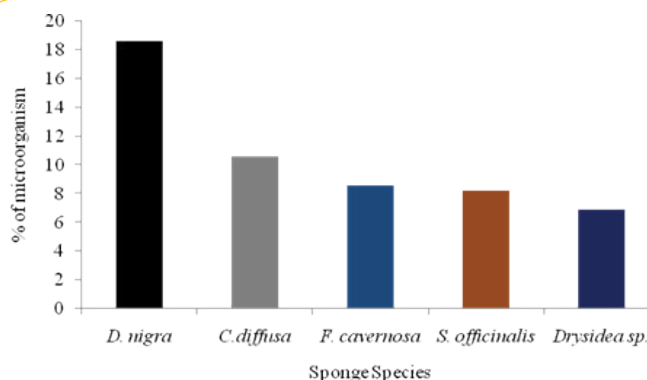
Bacteria with highest and broadest rank of antimicrobial activity and non-hemolytic property was chosen for partial sequencing and phylogenetic analysis. Molecular identification of the potential candidate sponge isolate was based on partial 16S ribosomal RNA gene sequencing. The MAPS 15 bacterial strain was cultured in marine broth at 25°C, and genomic DNA was extracted by the CTAB/NaCl method. The universal eubacterial 16S rRNA gene primer (5'-GAGTTTGATCCTGGCTCAG-3'; 5'-AGAAAGGAGGTGATCCAGCC-3') was used for the PCR amplification of bacterial 16S ribosomal RNA gene. The amplified 16S ribosomal RNA gene product was cloned by the TA cloning method using a TOPO TA cloning kit as per the manufactures instruction (Invitrogen) for sequencing. The obtained 16S ribosomal RNA gene sequence from the active isolate MAPS 15 was compared with sequences of other bacteria by using BLAST (Basic Local Alignment Search Tool) [14] to analyse pair wise homology. The sequences obtained were deposited in GenBank, EMBL in Europe and the DNA Data Bank of Japan with an accession number JQ723478.

### Statistical analysis

All the data were expressed as mean ± standard deviation (S.D.). Mean values were assessed using one way analysis of variance (ANOVA) using SPSS for Windows version 20.0 (Statistical Package for Social Services, Chicago, IL, USA).

## Results

Based on the colony morphology observed and stability in sub-culturing, a total of 51 strains of bacteria were segregated from



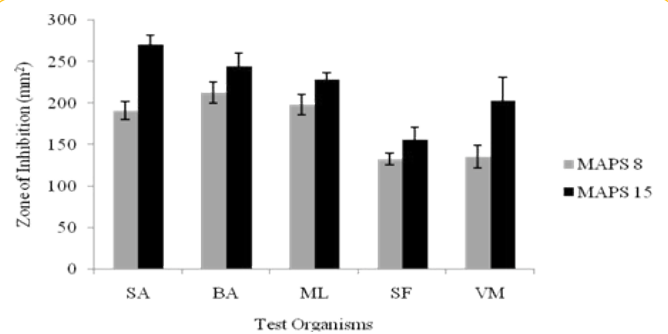
**Figure 1:** Percentage diversity of microbes isolated from different sponge species.

five different species of marine sponges and designated as MAPS (Marine Actinomycetes Probiotic Strain). The highest number of bacterial strains was isolated from *D. nigra* (18.6%), followed by *C. diffusa* (10.6%), *F. cavernosa*, (8.6%), *S. officinalis* (8.2%), and *Drysidea sp.* (6.9%) (Figure 1 and Table 2). Among the media used, ZoBell marine agar (ZMA) was found to be the most appropriate medium for the isolation of sponge-associated bacteria. Sixteen strains of bacteria were isolated on ZMA.

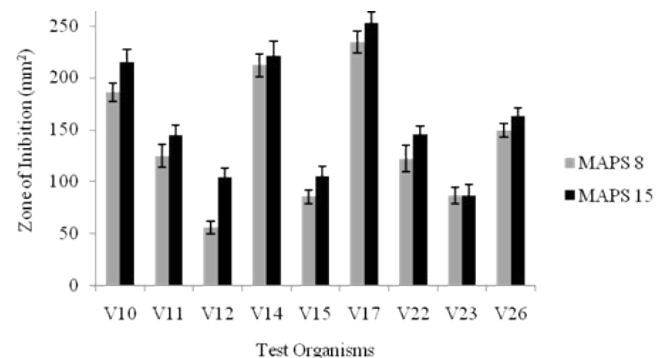
**Table 2** Bacterial strains isolated from marine sponges.

Marine sponges	Strains
<i>Dendrilla nigra</i>	9 (MAPS 1 to MAPS 9)
<i>Spongia officinalis</i>	14 (MAPS 10 to MAPS 23)
<i>Fasciospongia cavernosa</i>	10 (MAPS 24 to MAPS 33)
<i>Callyspongia diffusa</i>	11 (MAPS 34 to MAPS 44)
<i>Drysidea sp.</i>	7 (MAPS 45 to 51)

\*MAPS-Marine actinomycetes probiotic strain.



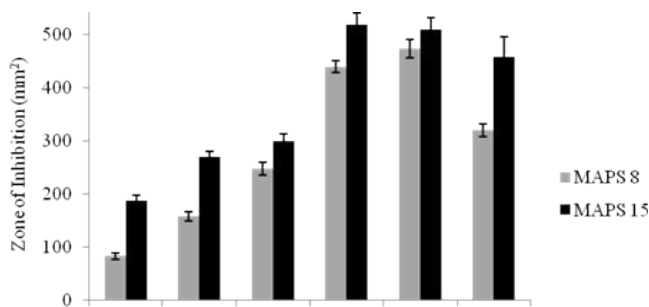
**Figure 2:** Antibacterial potential of sponge isolates MAPS 08 and MAPS 15 against human pathogens. The activity index was calculated as mm<sup>2</sup> area based on the diameter of halo displayed. [MTCC cultures- SA-*S. aureus*; BA-*B. amyloliquefaciens*; ML-*M. luteus*; SF-*S. flexneri*; VM-*V. mimicus*].



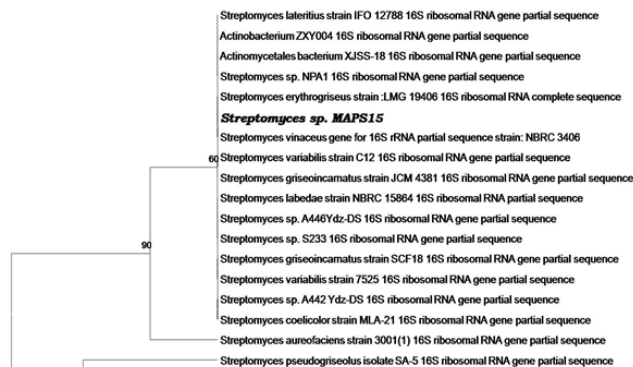
**Figure 3:** Antibacterial potential of sponge isolates MAPS 08 and MAPS 15 against shrimp *Vibrio* isolates. [VB10- *V. anguillarum*; VB11- *V. alginolyticus*; VB12- *V. parahaemolyticus*; VB14- *V. vulnificus*; VB15- *V. harveyi*; VB17-*V. fischeri*; VB22- *V. splendidus*; VB23- *V. campbellii*; VB-26 *Ph. damsela*].

### Antagonistic activity of the marine sponge isolates

In the preliminary antibacterial screening, it was observed that among the fifty one bacteria, only twelve strains of bacteria isolated from marine sponges, *S. officinalis* and *D. nigra* exhibited activity against at least one of the tested pathogens while other strains produced no results at all. A higher percentage of antagonistic strains were isolated from *S. officinalis*. The activity was meager for the rest of the strains isolated from *D. nigra*. On the basis of results from the cross streak method, only two isolates (MAPS 08 and MAPS 15) were further subjected to agar-well diffusion assay against a battery of human and animal pathogens. Both isolates surpassed the growth of 20 tested bacterial pathogens including type culture of human and shrimp pathogens and vibrio isolates. The anti biogram of MAPS 08 and MAPS 15 revealed that the cell suspension contained a broad-spectrum bactericidal agent. The antibacterial potentials of MAPS 08 and MAPS 15 are depicted in the **Figures 2-4**. Of the two isolates, MAPS 15 showed the highest activity. The CS of



**Figure 4:** Antibacterial potential of sponge isolates MAPS 08 and MAPS 15 against MTCC Vibrio cultures. [VH- *V. harveyi*; VP- *V. parahaemolyticus*; VV- *V. vulnificus*; VAC- *V. alginolyticus*; VA- *V. alcaligenes*; VF- *V. fischeri*].



**Figure 5:** UPGMA bootstrapping phylogenetic tree of the sponge isolate MAPS 15 and their closest NCBI (BLASTn) based on the 16S rRNA gene sequences.

**Table 3** Morphological characterization of sponge isolates (MAPS 08 and MAPS 15)

Strains	Motility	Grams staining	Ziehl Nielsen staining
MAPS 08	*NM	**G +ve rod	Aerial mycelium with two branches, conidia present
MAPS 15	NM	G +ve rod	Mycelium with branches, conidia present

\*NM-Non motile, \*\*G<sup>+</sup>- Gram Positive

**Table 4** Physiological characteristics (Hydrolytic activity) of antagonistic strains (MAPS 08 and MAPS 15).

Strain	Starch	Gelatin	Cellulose	Tributyryn	Chitin
MAPS 08	+	+	+	+	+
MAPS 15	+	+	+	+	-

**Table 5** Carbohydrate fermentation of antagonistic strains (MAPS 08 and MAPS 15).

Strains	Glucose	Mannitol	Sucrose	Gelatin	Glycerol
MAPS 08	+	-	-	-	+
MAPS 15	+	-	-	-	+

MAPS 15 displayed greater inhibitory effects against the growth of human and shrimp pathogens. The striking inhibition values were recorded against shrimp pathogens such as *V. alginolyticus* (519.98 ± 23.2 mm<sup>2</sup>) and *V. alcaligenes* (510.98 ± 22.2 mm<sup>2</sup>). The antibacterial potential of MAPS 15 against the human pathogens was in the range of 156.2 ± 14.4 to 270.6 ± 11.8 mm<sup>2</sup>. The highest inhibitory zone of 270.6 ± 11.8 mm<sup>2</sup> was manifested against *S. aureus* whereas the least inhibitory zone was produced against *S. flexneri* (156.2 ± 14.4 mm<sup>2</sup>). Similarly, the CS of MAPS 08 produced an activity ranged between 132.7 ± 7.3 and 212.9 ± 12.3 mm<sup>2</sup> against the human pathogens and 55.8 ± 5.9 to 234.08 ± 10.3 mm<sup>2</sup> of inhibitory zone against the shrimp Vibrio isolates and 83.3±5.8 to 473.86 ± 17.3 mm<sup>2</sup> for shrimp MTCC cultures. Based on the highest and broadest activity spectrum, MAPS-15 was chosen for further studies.

### Bio physiochemical profiling of the sponge-associated antagonistic bacteria

The morphological, physiological and biochemical profile of the sponge isolates (MAPS 08 and MAPS 15) are presented in **Tables 3-6**. The results of the morphological characteristics showed that both the strains were non motile gram positive rod shaped microorganisms. The elaborated cultural characteristics of the antagonistic strains were shown in **Table 7**. Several media such as Zobell Marine Agar, Actinomyces agar, Actinomycete isolation agar, Yeast malt extract agar, Inorganic salts starch medium, Oat meal agar, Oat meal with glycerol medium and chitin agar were used to determine the cultural characteristics of the isolates. The MAPS 08 strain showed maximal growth in all the media used whereas it showed moderate growth in yeast malt extract agar, inorganic salt starch media and chitin agar.

The strain MAPS 08 appeared as pale yellow colored complex aerial mycelia with white colored mass of spores. The strain showed positive results for starch, gelatin, cellulose, tributyrin and chitin hydrolysis. It was positive for glucose and glycerol



**Table 6** Biochemical characteristics of antagonistic strains (MAPS 08 and MAPS 15).

Strains	Citrate	TSI	MR	VP	Oxidase	Catalase	Indole	Hemolytic activity
MAPS 08	+	+	+	-	+(Weak)	-	-	+
MAPS 15	+	+	+	-	+	-	-	-

TSI– Triple Sugar Iron agar test; MR– Methyl Red; VP– Voges Proskauer, +-Positive, – -Negative.

**Table 7** Cultural characteristics of antagonistic strains (MAPS 08 and MAPS 15).

Strains	Actinomyces agar	Actinomycetes isolation agar	*YME agar	**ISP–agar	Oatmeal agar	Oat meal with glycerol medium	Chitin agar
MAPS 08	Aerial pale yellow, large mycelium surrounded, yellowish brown centered powdery colonies.	Aerial white and substrate yellow powdery colonies.	Aerial and substrate muddy white color pin headed colonies.	Pale yellow partially slimy and dry colonies.	Ash color colonies, less growth.	Yellow color mucoid flat colonies.	Ash with yellow slimy colonies.
MAPS 15	Aerial and substrate ash with slimy colonies.	Aerial white and substrate yellow powdery colonies.	Observed less growth pale white color powdery colonies.	Ash with white color minute pin headed dry colonies.	Ash with yellow color mucoid colonies, less growth.	Dark yellow Color mucoid, flat colonies	No growth.

\*YME-Yeast Malt Extract, \*\*ISP-4-Inorganic salt agar International *Streptomyces* Project Agar (ISP).

**Table 8** Antibiotic sensitivity of the antagonistic strains (MAPS 08 and MAPS 15).

Strain	Ampicillin	Tetracycline	Ciprofloxacin	Chloramphenicol
MAPS 08	*R	R	S	S
MAPS 15	R	**S	S	S

\*R– Resistant; \*\*S-Sensitive

fermentation and was negative for mannitol, sucrose and gelatin. The isolates are able to produce enzyme oxidase, but unable to produce positive result for catalase. The MAPS 08 strain was found to be positive for citrate, TSI and MR. It was negative for VP and indole. The antibiotic sensitivity pattern of the isolate is shown in **Table 8**. The strain was sensitive to ciprofloxacin and chloramphenicol. It showed prominent resistance against ampicillin and tetracycline. A distinct characteristic of MAPS 08 was its ability to produce haemolysin. It exhibited significant hemolytic activity. Therefore, this strain is excluded from further molecular identification. The strain MAPS 15 showed maximal growth in all the media used whereas it showed moderate growth in inorganic salt starch media, less growth in yeast malt extract agar and showed no growth in chitin agar. The strain MAPS 15 appeared as ash colored slimy colony with complex aerial mycelia. The strain showed positive results for starch, gelatin, cellulose, tributyrin but showed negative result for chitin hydrolysis. It was positive for glucose and glycerol fermentation and was negative for mannitol, sucrose and gelatin. The isolates are able to produce enzyme oxidase, but are unable to produce positive result for catalase. The MAPS 15 strain was found to be positive for citrate, TSI and MR. It was negative for VP and indole test. The antibiotic sensitivity pattern of MAPS 15 is shown in **Table 8**. The strain was sensitive to tetracycline, ciprofloxacin and chloramphenicol. It showed prominent resistance against ampicillin. In hemolysis assay, MAPS 15 showed a negative result.

### Molecular identifications of the sponge isolate MAPS 15

The genomic DNA isolated and subjected to 16S rRNA amplification for the identification of the potent antagonistic producer. A PCR product of length 1200 bp was purified using the GeneiPure™ quick PCR purification kit and sequenced by Sigma Aldrich. Taxonomic affiliation of the 16S rRNA sequences of the isolate MAPS 15 was retrieved from classifier program of Ribosomal Database Project II release 10.0 (<http://rdp.cme.msu.edu/>). The 16S rRNA sequence of the isolate was blasted using megablast tool of GenBank (<http://www.ncbi.nlm.nih.gov/>). This revealed that the isolate was a *Streptomyces* strain. Representative of maximum homologous (98-99%) sequences of each isolate were obtained from seqmatch programme of RDPII and were used for the construction of phylogenetic affiliation (**Figure 5**). The 16S rRNA sequence of the isolate MAPS 15 was further analyzed using NCBI BLASTn tool with a query to limit the search to the closest relatives such as *Streptomyces erythrogriseus* LMG 19406 SCF18 and *Streptomyces vinaceus* NBRC 3406. Representatives of maximum homologous sequences from the search were used for the construction of phylogenetic tree using UPGMA algorithm. The 16S rRNA gene partial sequence obtained in this study was submitted to the GenBank database with assigned accession numbers JQ723478.

## Discussion

It is a long-familiar concept that sponges harbour verdant types of microbial communities with versatile biopotency. Recent years have witnessed the origination and development of novel bio-engineering techniques on the production of bioactive metabolites from marine sponge associated microbes [3]. The studies on bioactivity screening of microorganisms such as this provide a base line data for the identification of possible antagonistic candidates. In the present study, antagonistic effects of sponge isolates on the growth of human and shrimp pathogens were determined. The highest proportion of antagonistic activity was found among the isolates from sponges such as, *S. officinalis* and *D. nigra*. Among the bacterial strains, MAPS 15 isolated from *S. officinalis* exhibited the highest and broadest activity spectrum. The results of agar well diffusion assay with MAPS 15 demonstrate that an extracellular product might be responsible for inhibitory action. The results ensured that sponge-associated microorganisms are highly potential resource of bioactive natural products owing to their competition for nutrition, space and light [15-17]. The results of the *invitro* assays agree with former studies showing antagonistic activity of bacteria isolated from

other sponge species [18]. Scores of studies affirmed that marine bacteria could reduce the growth of pathogenic human and animal bacteria. However, data pertinent to the repression of *Vibrios* by marine bacteria are limited [8]. Moreover, *Vibrios* has been associated with food poisoning in human who consumed tainted shell fishes particularly the species such as *V. parahaemolyticus* and *V. vulnificus*. The molecular characterization based on partial 16S rRNA sequence revealed that the active isolate MAPS 15 was *Streptomyces* sp. Species of the genus *Streptomyces* are well known producers of metabolites with antimicrobial activity [19]. In conclusion, the sponge isolate, *Streptomyces* sp. exerts significant antagonistic activity against all the tested human and shrimp pathogens. The antagonistic efficacy exhibited by the *Streptomyces* sp. against pathogens envisaged the future use of sponge-associated bacteria for developing human and veterinary grade antibiotics.

## Competing Interests

The authors have no conflicts of interest to disclose.

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