JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

PRIMARY CHARACTERIZATION OF SPONGE ASSOCIATED BACTERIA OF MARINE SPONGES- *HALICHONDRIA GLABRATA, CLIONA LOBATA, SPIRASTRELLA PACHYSPIRA* AND THEIR ANTIMICROBIAL PROPERTIES

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doi: 10.15414/jmbfs.201x.volume.issue.pages

ARTICLE INFO

Received 15. 11. 2013
Revised 20. 8. 2014
Accepted 20. 8. 2014
Published 1. 10. 2014

Regular article

OPEN ACCESS

ABSTRACT

Marine sponge associated bacterias have been recognized as an important and untapped resource for novel bioactive compounds. In the present study four strains of microorganisms were isolated from three different varieties of marine sponge viz. *Halichondria glabrata*, *Cliona lobata* and *Spirastrella pachyspir*a. They showed broad spectrum antimicrobial activity against both Gram positive and Gram negative indicator organisms. From the biochemical tests and cetrimide agar test, it was concluded that the Strain B isolated from *Cliona lobata* is a *Pseudomonas* species. Strain A (gram negative) culture product isolated from *Halichondria glabrata* showed the antibiotic activity against Gram positive (*B. subtillis*) and Gram negative (*S. typhi, P. vulgaris, E.coli*) organisms. The minimum inhibitory concentration for showing antibacterial activity on all the standard strain was found to be 40 µL of culture broth supernatant. This strain was further identified by ABIS software based on biochemical tests and confirmation of the strain was done after 16S r RNA gene sequencing. The strain showed close similarity with *E. coli* and *Enterobacteria* strains and most of the uncultured bacterium from different hosts, which confirmed its nature of being it a symbiont from sponge *Halichondria glabrata* with antimicrobial activity.

Keywords: 16S rRNA sequencing, Marine sponge, *Halichondria glabrata, Cliona lobata, Spirastrella pachyspira*

INTRODUCTION

Sponges are multicellular invertebrate and sessile filter feeders. They are abundant in the oceans as well as in freshwater habitats. Association of sponges with a wide variety of microorganisms has lead to great interest in them. These microorganisms are found to be a rich source of secondary metabolites, which exhibit a broad range of bioactivities such as antiviral, antimicrobial, anti-inflammatory, antitumor, cytotoxic, inhibition of enzyme activities, cell division and cardiovascular properties. Numerous studies concerning specific aspects of sponge bacterium associations were accomplished using distinct methods for the evaluation of the microbial diversity or the bioactivities (culture-dependent methods) or biosynthetic aspects of secondary metabolites of the associated bacteria (**Schneemann *et al.*, 2010**). The interactions between sponges and bacteria in the marine environment are poorly understood. It is generally believed that symbiotic interactions exist between sponges and microorganisms. Symbiotic functions that have been attributed to microbial flora include nutrient acquisition, stabilization of sponge skeleton, processing of metabolic waste and secondary metabolite production (**Hentschel *et al.*, 2002**). It has also been suggested that some of these bacteria chemically defend the host against microbial infection. The concentrations of many highly bioactive compounds in marine invertebrates are often very less, accounting for less than a millionth of the wet weight (**Proksch *et al.*, 2002**). Marine sponges are a rich source of structurally unique natural compounds, several of which have shown a wide variety of biological activities (**De Rosa *et al.*, 2003**). It is well known that even excellent drug candidates from sponges are often not developed because those sponges are rare, difficult to collect or both. Numerous natural products from marine invertebrates show striking structural similarities to metabolites of microbial origin, suggesting that microorganisms are the true source of these metabolites or are intricately involved in their biosynthesis (**Proksch *et al.*, 2002**). Production of antimicrobial compounds seems to be a general phenomenon for most bacteria. An admirable array of microbial defence systems are produced, including broad-spectrum classical antibiotics, metabolic by-products such as organic acids, and lytic agents such as lysozyme. In addition, several types of protein exotoxins, and bacteriocins, which are biologically active peptide moieties with bactericidal mode of action, were described. This biological arsenal is remarkable in its diversity and natural abundance, since some substances are restricted to some bacterial groups while other is widespread produced. The search for new antimicrobial agents is a field of utmost importance. The prevalence of antimicrobial resistance among key microbial pathogens is increasing at an alarming rate worldwide. Current solutions involve development of a more rational approach to antibiotic use and discover of new antimicrobials, but the problem of antibiotic resistance is increasing globally and may render the current antimicrobial agents insufficient to control at least some bacterial infections (**Motta *et al.*, 2004**). The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes. The rRNA gene is the most conserved (least variable) DNA in all cells. Portions of the rDNA sequence from distantly related organisms are remarkably similar. This means that sequences from distantly related organisms can be precisely aligned, making the true differences easy to measure. For this reason, genes that encode the rRNA (rDNA) have been used extensively to determine taxonomy, phylogeny (evolutionary relationships), and to estimate rates of species divergence among bacteria (**Janda *et al.*, 2007**). It is widely accepted that culture- based technique for the studying of bacterial community diversity because most of the bacteria are very difficult to culture by current and traditional techniques. The recent surge of research in molecular microbial ecology provides compelling descriptions on complete microbial community composition and can indicate possible nutritional requirements and physiological niches of many microorganisms according to information already available for known phylogenetic relatives. 16S rRNA gene based approaches have enormously enhanced our knowledge about the diversity of environmental microbial communities, such as marine sponge associated microbial diversity (**Webster *et al.*, 2001; Hentschel *et al.*, 2002**). This may also be helpful for the experimental manipulation of culture conditions to provide the correct growth conditions for targeted bacteria. Since past few years marine products have demonstrated potential antibiotic activity. Marine sponges and several micro organisms have symbiotic relationship. The main objective of the project was to

isolate and identify such micro organisms and to check its anti microbial activity using standard indicator strains.

MATERIAL AND METHODS

Collection of sponge samples

Marine sponges were collected by hand picking from inter tidal regions during low tide in the month of February, 2013 from western coast of Mumbai (19°4'20"N 72°49'57"E).Three sponge samples were collected from Khardanda and were sent for identification at Marine Biology Regional Centre, Zoological Survey of India, Chennai, and are registered there. Sample collection was done for a period of three months from the Mumbai coastal area. All the samples were sealed in a presterile plastic bag individually and stored in a -80° C for further use.

Isolation of microorganisms

Fresh samples of marine sponges were taken for the extraction of microorganism. 1g of sample was crushed with 15 mL of sterile artificial sea water in mortar and pestle. The sample was decanted into sterile test tube to give a dilution of the order of 1. From this, solution series of dilutions 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were prepared. These were then streaked on agar plates and incubated at 37° C for 24 hours.

Physiological and biochemical tests.

Isolated bacteria were tested for Gram reaction, motility, indole production, catalase production, triple sugar ion, gelatin hydrolysis, urease production, starch hydrolysis, cetrimide agar, 7% NaCl growth, for growth at 45° and 60°C, spore formation and cell morphology according to the methods described by **Kebede *et al*., (2007)**.

Antimicrobial Sensitivity Assay

Disc diffusion method was used to carry out the antimicrobial sensitivity assay. The isolated bacterias were allowed to grow for 48hrs, at 37°C in LB broth and were centrifuged to collect their supernatant after 24hrs and 48 hrs. The sterile filter paper discs were dipped and placed on the Mueller Hinton agar which was previously spreaded by standard pathogen bacterias viz., *Proteus vulgaris, Salmonella typhi, Bacillus subtilis, E. coli* and *Staphylococcus aureus.* All the plates were incubated overnight at 37°C under static conditions. The zone of inhibition around the discs was recorded in cm in diameter. Triplicate samples were maintained and readings were taken.

Genomic DNA isolation from bacteria

A single colony from each isolate was inoculated into 10 ml of the nutrient medium broth (kept in a 15 ml Falcon tubes) and incubated overnight at 37°C. The cultivated culture was harvested by centrifugation at 5,000 rpm for 5 min and the genomic DNA was isolated by a modified genomic DNA isolation protocol (**Sambrook and Russell, 2001**).

16S rRNA and sequence determination and phylogenetic analysis.

Sequencing of the 16S RNA were performed in collaboration. Sequences were submitted to the National Center for Biotechnology Information (Bethesda, MD) and clustal software for similarity searches through BLAST (http://www.ncbi.nlm.nih.gov/blast) and were compared with type strains by multiple alignments.

RESULTS AND DISCUSSION

The collected three sponge samples were identified as *Halichondria glabrata, Cliona lobata* and *Spirastrella pachyspira* at Zoological Survey of India, Chennai. Gram staining of all the strains isolated was done and the results obtained are tabulated in Table 1 and Fig 1 (Fig 1.1a, 1.1 b, 1.1 c and 1.1 d). Strain A isolated from *Halichondria glabrata* showed positive response to antibiotic studies. Various biochemical tests were performed and based on the results obtained the possible microorganisms were determined shown in Table 2. The cetrimide agar test was performed for the Strain B which later confirmed to be a *Pseudomonas* species and gave green color colony after incubation. Later all the biochemical tests were used to get the probable microorganism names by running Advanced Bacterial Identification Software (ABIS). It gave the list of microorganism with their possibilities and accuracy percentage listed in Table 3.

Antimicrobial studies

Among all the four strains studied for antibacterial properties, Strain A showed the positive result against all the tested pathogens except *Staphylococcus aureus.* Cup agar method was later taken up for this study; 80µL of supernatant was loaded and gave good antibacterial activity. Further, minimum inhibitory volume was determined. Standard 10µg Ampicillin disc was used as standard. Results are compiled in Table 4 and Table 5.

Genome Sequencing

The 16S rRNA sequence was obtained from Gene Ombio Technologies, Pune. The sequence of 772 basepairs was obtained. PCR amplification of partial 16S rRNA gene of sponge associated bacteria, purification of PCR products and sequencing analysis were performed according to the method of **Radjasa *et al*., (2007)**. The determined DNA sequences of strains were then compared for homology to the BLAST database at NCBI. The sequence is avaialable with Genbank accession no KF488586.1. Molecular identification based on 16S rDNA approach showed that the active isolates are the member of genera *Escherichia* and *Enterobacteria* (Fig 2).

Fasta Format Sequence

TATTTATTTTATTCCCCCCTCAGATTGAACGCTGCGGCAGGCCTACAC
ATGCAAGTCGAACGGTAACAGGAAGCAGCTTGCTTCTTTGGCTGACG
AGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGG
GATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACC
AAAGGGGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATG
GGATTAGCTTGTTGGTGGGGTAACGGCTCCCCCAAGGCGACGATCCCC
TAGCTGGTCTGAGAGGATGACCAGCCCACACTGGAACTGAGACACGG
TCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGC
GCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGT
TGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTT
GCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCA
GCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGG
GCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCC
GGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGT
AGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGGAAT
ACCGGTGGCGAAGGCGGCCCCTGGACGAAGACTGACGTCAGGGCGAA
TGCTCGCCCGAGATCTG

Hentschel *et al*., (2002) studied the technique to isolate bacteria with antimicrobial activities from the marine sponges *Aplysina aerophoba* and *Aplysina cavernicola*. 27 isolates were obtained, and were subdivided into eight phylogenetically different clusters based on comparative sequence analysis of their 16S rDNA genes. The sponge isolates were affiliated with the low (*Bacillus*) and high G+C Gram-positive bacteria (*Arthobacter, Micrococcus*), as well as the K-Proteobacteria (unknown isolate) and Q-Proteobacteria (Vibrio, Pseudoalteromonas). **Song *et al*., (2005)** studied that sequence analysis of the 16S rRNA gene represents a highly accurate and versatile method for bacterial classification and identification, even when the species in question is notoriously difficult to identify by phenotypic means. In the study, they evaluated the utility of 16S rRNA gene sequencing as a means for identifying clinically important *Bacteroides* species**. Jirge *et al*., (2010)** reviewed that bioactive compounds from marine flora and fauna have extensive past and present use in the treatment of many diseases and serve as compounds of interest both in their natural form and as templates for synthetic modifications.

Several molecules isolated from various marine organisms (microorganisms, algae, fungi, invertebrates, and vertebrates) are currently under study at an advanced stage of clinical trials, some of them have already been marketed as drugs. These studies give an overview of current trends in screening and the activity analysis of metabolites from marine resources. Although the marine resources have been somewhat limited to date, selected bioactive from marine flora and fauna have already been published.

Sponge-associated bacteria that occur within the sponge surface are of great interest to search for their secondary metabolite-producing property. Isolation and screening for secondary metabolite-producing bacteria in marine ecosystems have been strongly neglected until now in comparison with the invertebrates. With the increased occurrence of multidrug resistant human pathogens, the search for novel antibiotics has gained new urgency. Many clinically relevant microbes have developed resistances resulting from the exposure to sublethal concentrations of antibiotics. Marine sponges have been recognized as an important and untapped resource for novel bioactive compound. The chemical compound of marine organism are less well known than those of their terrestrial counterparts however, in the last decade several bioactive compound have been isolated from marine bacteria and are new resources for the development of medically useful compound. Sponge-microbial associations are understood to be very specific in the production of particular bioactive compounds. However, the mutual mechanism between host and the microbial associate, in compound production is not well understood. The easiest and best way for commercial production of these compounds are either by culturing the host and/or the associated microbe under controlled conditions. But, the ability of the symbiont

to produce the compound consistently for several generation in culture media has to be tested and standardized **(Thomas *et al.*, 2010)**. Previous work has demonstrated a phylogenetically diverse array of bacterial groups present in this sponge: representatives of *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Cytophaga* /*Flavobacteria*, the *Deinococcus* group, low-G+C-content Gram-positive bacteria, *Actinobacteria*, and *Planctomycetales* were identified by means of a genetic approach. Among these, though representing only 3 to 20% of the sponge-associated bacterial community, *Actinobacteria* are the most promising bacterial group regarding secondary metabolite production (**Schneemann *et al.*, 2010**). In another recent study, sponge sample of *Dysidea granulosa (D. granulosa)* was source to potential bacteria LB3 identified as *Enterobacter* sp TTAG. LB3 strain was selected as potential producer of secondary metabolites and crude extract implied for MIC of LB3 have confirmed with lowest concentration of 5.0 mg/mL in broth medium influence of crude extract on growth inhibitory activity after 5 h of incubation period and completed the inhibitory activity at 15 h (**Gopi *et al.*, 2012**). Phylogenetic analysis for bacterial diversity of breadcrumb sponge, *Halichondria panicea* pointed specific Alphaproteobacteria of the Roseobacter group, which was predominant in most sponge 'tissue' samples. *H. panicea* harbored a specific Roseobacter population with varying bacterial co-populations occurring seasonally or on a small-scale geographically, sometimes even dominating the bacterial community (**Wichels *et al.*, 2006**). In spite of much successful isolation of cultures, most symbiotic microorganisms are difficult to isolate and cultivate (**Schmidt *et al.*, 1991**). Only less than 0.1% of the total bacterial community is amenable to culture. To overcome the problems of culture based isolation, symbiotic microorganisms can be identified directly using culture-independent methods such as molecular taxonomy. Phylogenetic analysis of 16S rRNA genes give data on microbial diversity and the phylogenetic position of each symbiotic microorganism (**Webster *et al.*, 2001**). These potentially novel isolates can be a useful resource for screening for bioactive natural products (**Lee *et al.*, 2001**).

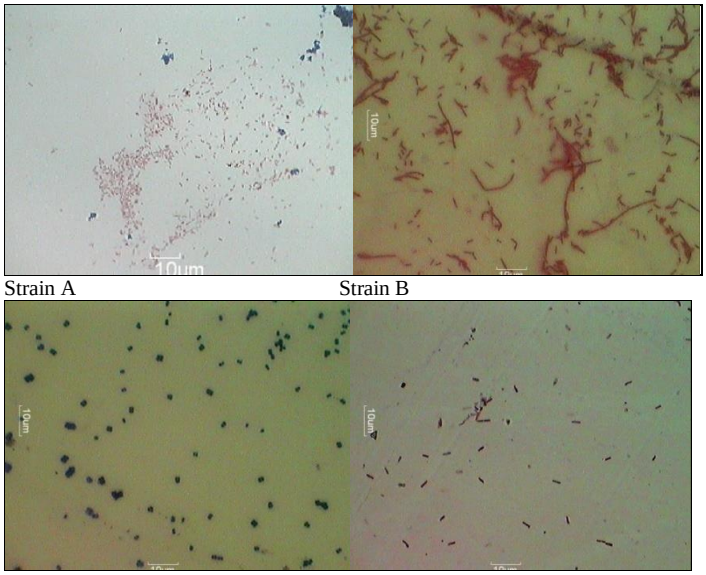

Figure 1 Images of gram staining (Magnification 100x)

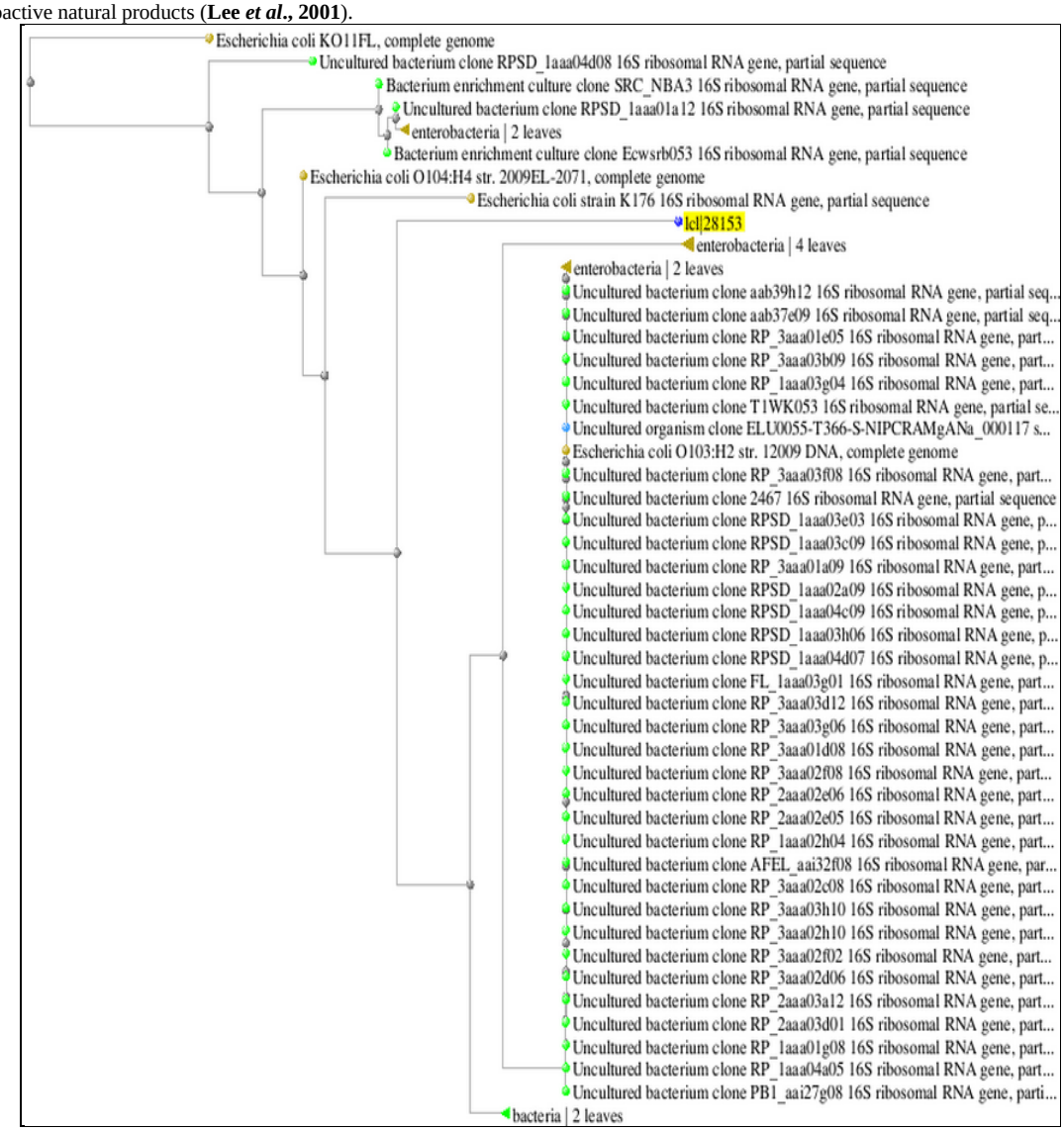

Figure 2 Cluster analyses showing the similarity of activity pattern of lcl28153, sponge isolate with *Escherichia* and *Enterobacteria* and uncultured bacterium

Table 1 Results of Gram staining

Test	*Halichondria glabrata*	*Cliona lobata*		*Spirastrella pachyspira*
Strain	A	B	C	D
Gram staining	-	-	+	+
Shape of bacteria	Bacilli	Cocco-Bacilli	Diplo-Cocci	Bacilli

Table 2 Results of Biochemical tests

Test	*Halichondria glabrata*	*Cliona lobata*		*Spirastrella pachyspira*
Strain	A	B	C	D
Motility test	+	+ +	-	+
Indole test	-	-	-	-
Gelatin hydrolysis test	-	+	-	-
Urease test	-	-	-	-
Starch hydrolysis test	+	-	-	-
Triple sugar ion Butt	-	-	-	-
test Stab	-	-	+	+
Catalase test	-	-	-	-
Growth at 60^0C	-	-	-	-
Growth at 45^0C	+	-	-	+
Growth on 7% NaCl	+	-	-	+

Table 3.a Microorganisms result for Strain A (Gram negative)

Name	Possibilities (%)	Accuracy (%)
Bacillus cibi	98	18
Bacillus carboniphilus	88	18
Bacillus farraginis/ B. fordii/ B. fortis	89	18
Bacillus korlensis	89	18

Table 3.b Microorganisms result for Strain B (Gram negative)

Name	Possibilities (%)	Accuracy (%)
Pseudomonas guinea	75	28
Pseudomonas fusovaginae	85	25
Stenotrophomonas maltophilic	70	28
Comamonas testosterone	68	28

Table 3.c Microorganisms result for Strain C (Gram positive)

Name	Possibilities (%)	Accuracy (%)
Staphylococcus arlettae	99	16
Staphylococcus microti	77	18
Staphylococcus succinus subsp. Succinus	77	16
Staphylococcus succinus subsp. casei	77	18

Table 3.d Microorganisms result for Strain D (Gram positive)

Name	Possibilities (%)	Accuracy (%)
Bacillus nealsonii	96	30
Bacillus licheniformic	81	30
Bacillus siamensis	81	30
Paenbacillus massilionsis	92	26

Table 4 Results of antibacterial activity of Strain A supernatant (after 48 hours)

Microorganisms	Volume of supernatant			
	40 µL (cm)	50 µL (cm)	60 µL (cm)	70 µL (cm)
P. vulgaris	1.1	1.2	1.5	1.7
S. typhi	1.3	1.4	1.4	1.5
B. subtilis	1.1	1.3	1.3	1.4
E.coli	0.9	1.0	1.1	1.1

Zone of Inhibition of Ampicillin disc was found to be 2.7 cm

Table 5 Results of Minimum Inhibitory volume (after 48 hours)

Microorganisms	I(cm)	II(cm)	III(cm)	Average(cm)	Std. Deviation
P. vulgaris	1.9	1.8	2.0	1.90	0.10
S. typhi	1.9	1.7	2.2	1.87	0.29
B. subtilis	1.4	1.5	1.5	1.47	0.06
E.coli	1.2	1.2	1.1	1.17	0.06

CONCLUSION

The present study made an attempt to find out the bioactive potential of sponge associated microorganisms. It indicated one among four marine bacteria associated with collected sponges, growth inhibition against indicator microorganism (Table 5). This offers the possibility to use sponge bacteria as the source of antibacterial compounds for controlling the pathogenic bacteria such as *P. vulgaris*, *S. typhi*, *E. coli* etc. The strain A with antimicrobial activity was associated with sponge *Halichondria glabrata* belonging to family Halichondriidae. In the same attempt we have been able to isolate four different strains here. We isolated four bacteria from the sponges, out of which one caught our attention due to its antimicrobial activity. Although these bacteria were not yet identified to the species level, morphological and biochemical characteristics indicate they belonged to the genus *Bacillus.* From the phylogenetic and clustar analysis, it showed very similar phylogenies with bacteria of class of *Escherichia* and *Enterobacteria*. Furthermore investigation is required to understand the exact nature of the compounds produced by the strain. Results of this study indicate the potential of the sponge associated microorganism for production antimicrobial compounds that can be useful for many applications.

Acknowledgments: We are grateful to SVKM'S NMIMS, Mumbai for supporting and funding this work.

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