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ORIGINAL ARTICLE

Molecular characterization and bioactivity profile of the tropical sponge-associated bacterium *Shewanella algae* VCDB

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Abstract The pigmented, rod-shaped, Gram-negative, motile bacteria isolated from marine sponge *Callyspongia diffusa* exhibiting bioactivity was characterized as *Shewanella algae* (GenBank: KC623651). The 16S rRNA gene sequence-based phylogenetic analysis showed its similarity with the member of *Shewanella* and placed in a separate cluster with the recognized bacteria *S. algae* (PSB-05 FJ86678) with which it showed 99.0 % sequence similarity. Growth of the strain was optimum at temperature 30 °C, pH 8.0 in the presence of 2.0–4.0 % of NaCl. High antibiotic activity against microbes such as *Escherichia coli* (MTCC 40), *S. typhii* (MTCC 98), *P. vulgaris* (MTCC 426), *V. fluvialis*, *V. anguillarum*, *E. cloacae*, and *L. lactis* was recorded. The growth of fungal pathogens such as *Aspergillus niger*, *Aspergillus fumigatus*, *Saccharomyces cerevisiae*, and *Colletotrichum gloeosporioides* was effectively controlled.

Keywords Sponge bacteria · *Callyspongia diffusa* · *Shewanella algae* · Molecular characterization · Antimicrobial activity

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Introduction

In marine sponges, increased evidences indicated the involvement of associated microorganisms for the production of secondary metabolite (Proksch et al. 2002; Chelossi et al. 2007; Flemer et al. 2012). The functional interaction between associated microbes and host sponge is considered as essential in the bioprospecting sphere (Selvin et al. 2010). Among the microbes, the bacterial community in the sponge differs significantly from seawater and sediments in density and diversity. The sponge-associated bacteria could be viewed as a highly potential source for the production of antibiotic compounds (Waters et al. 2010; Graca et al. 2013). The recovery of culturable bacterial strains with different bioactivity profiles shows that the sponge tissues could be rich sources for isolating new strains of bacteria with potential capabilities of producing novel bioactive secondary metabolites (Xiong et al. 2013). Molecular and metagenomic approaches opened up new scientific possibilities, which also revealed the metabolic pathways involved in the production of natural compound by the diverse unculturable symbiotic bacteria. The marine sponge *Callyspongisa diffusa* has been investigated for isolating novel bacterial strains. Bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Vibrio parahaemolyticus*, and *Vibrio cholera* have been reported to be associated with *C. diffusa* (Boobathy et al. 2009). An in-depth knowledge about the *C. diffusa*-associated bacteria and their bioactivity profile is necessary to promote their bioprospecting avenues. Considering this, the marine sponge *C. diffusa* was chosen to retrieve maximum number of bacteria for exploring their bioactivity potential in a sponge-free environment. The results of isolation and characterization of a potential strain of bacteria viz., *Shewanella algae,*

and its characteristics together with bioactive potential are presented in this paper.

Materials and methods

Collection and preparation of extract from *Callyspongia diffusa*

Specimens of *C. diffusa* were collected by SCUBA during September 2011 from southwest coast of India at depth ranging from 6 to 7 m off Vizhinjam (8°22′45°N: 76°59′29°E), at a distance of about 1.5 km from the shore. The sponge specimens were found attached to submerged rocks. Immediately upon collection, the sponge specimens were transferred to new polythene covers in situ to minimize the external contaminants and transient bacteria. The aqueous extract of the sponge was prepared by squeezing the sand-free specimen in sterile seawater. The resultant solution was filtered using Whatman filter paper (No. 1) and stored at 4.0 °C in refrigerator for further experiments.

Isolation of sponge-associated bacteria

The surface particles and microbes of freshly collected *C. diffusa* were removed by rinsing with sterile seawater and immediately transferred to sterile container. From the surface-sterilized sponge, required quantity of sponge tissue was excised using sterile scalpel and then homogenized. The homogenate was diluted in sterile saline, and appropriate dilutions were spread on Zobell Marine Agar (ZMA-HiMedia) and plates were incubated at 28.0 ± 2.0 °C. The colonies of bacteria developed on the agar plates were enumerated to determine the CFU/g. Seawater sample from the site of collection was also examined to enumerate the ambient bacterial load.

Biochemical characterization of the isolate

The morphology and biochemical characteristics of the sponge isolates viz., Gram staining, motility, utilization of carbon sources, H_2S production, and MR–VP test, were carried out as per Smibert and Krieg (1994).

Screening of bacterial isolates from sponge tissue for antimicrobial activity

The bacterial isolates obtained from the sponge were grown individually in Zobell Marine Broth (ZMB) for 48 h. The cell-free culture media of each isolate was prepared by centrifuging the growth medium and filtering of the supernatant using 0.2-µm filters. Sterile discs (6 mm) loaded with 15 µl cell-free culture extracts of the individual bacterial isolates associated with *C. diffusa* were placed on Muller-Hinton agar plates seeded with human and fish pathogenic bacteria. The presence or absence of inhibitory activity against the indicator organisms was determined after incubating the agar plates for 24 h at 37 °C and measuring the zone of inhibition.

The antifungal activity of bacterial strains isolated from sponge tissue against a few food-borne fungi such as *Aspergillus niger*, *Aspergillus fumigatus*, *Saccharomyces cerevisiae,* and plant pathogens *Sclerotia rolfsii*, *Phytophthora palmivora*, *Phytophthora colocasiae*, *and Colletotrichum gloeosporioides* was evaluated. The activity was determined by dual-well agar diffusion technique. For testing antifungal activity, malt extract agar (MEA) medium was used as given by Mushtaq et al. (2010). After solidification of the media, 6.0-mm wells were cut on the agar surface and each well in a plate was loaded with 30 µl of 24-h sponge-isolated bacterial broth culture; similarly, the cell-free supernatant (30 µl) of the same sponge bacterial strains was also added. ZMB served as the control. A circular piece of 4.0 mm diameter from 7-day-old fungal cultures were cut and removed by cork borer, and was stabbed on agar previously loaded with bacterial culture and its cell-free supernatant. The observations with respect to the inhibition of fungal growth were observed after 5 days of incubation.

Determination of growth and antibacterial activity pattern at different conditions

Considering the preliminary results of bioactivity, the potential isolate VCDB was grown in ZMB and response with respect to maximum growth and metabolite production was recorded at varied temperature and pH. For determining the optimum temperature, the inoculated broth was incubated at varying temperatures from 20 to 50 °C at 5 °C intervals. The pH optima was determined by varying the initial pH of the ZMB from 5.0 to 10.0 and inoculated with the isolate VCDB. The samples were removed after every 12 h to determine the growth OD (660 nm) and antibacterial activity.

Molecular characterization and phylogenetic analysis of the isolate VCDB

The isolate VCDB, which showed significant antimicrobial activity, was characterized using 16S rRNA gene sequencing. The DNA of the 12-h culture was purified by HiPurA bacterial DNA isolation and purification kit (HiMedia, India) and amplified by PCR using master mix kit (Chromous, India). The primers and the methodology for the sequencing were adapted from Kamke et al. (2010).

The similarity and homology of the 16S rRNA gene sequence was compared with existing sequences available in the data bank of NCBI using BLAST search. The DNA sequence was aligned, and phylogenetic tree was constructed by Mega 5.05 using neighbor-joining method, and the sequence was deposited in GenBank.

Results

Identification of *Callyspongia diffusa* and characteristics of bacterial isolates

The sponge was identified as *C. diffusa* based on spicules morphology and further confirmed by Dr. P. A. Thomas, Sponge Taxonomist (Fig. 1a, b). From the randomly chosen sponge tissue, seven morphologically and culturally distinct bacterial strains were isolated and designated—VCDB, VCDA, VCDW, VCDI, VCDY, VCDP, and VCDPS; the cultural and biochemical characteristics of the isolates are given in Tables 1 and 2. The total number of bacterial colonies from the sponge interior was recorded to be 50.0 % higher than the bacterial load from the ambient seawater. The black-pigmented VCDB isolate that proved highly potential by further observations was not recorded from the seawater, and the CFU of VCDB constituted 12.5 % of total microbial load of 16×10^4 CFU/g.

Screening of bacterial isolates from sponge tissue for antimicrobial activity

The results of the cell-free supernatant of associated bacteria showed that all the bacterial isolates except VCDI showed a significant antibacterial activity against human and fish pathogens (Table 3). The VCDB isolate showed mild activity against *V. harveyi*, *V. anguillarum, L. lactis*, *P. vulgaris* MTCC 426, and *E. coli* MTCC 40 with zone of inhibition of 10 mm. Significant activity was exhibited against *S. typhi* MTCC 92 (11 mm), *V. fluvialis* (14 mm), and *E. cloacae* (11 mm). The isolate VCDA exhibited inhibitory activity against *E. cloacae* (12 mm) *V. fluvialis,* and *V. anguillarum* (11 mm) and mild activity of 7 mm against *V. harveyi* and 9 mm against *P. vulgaris.*

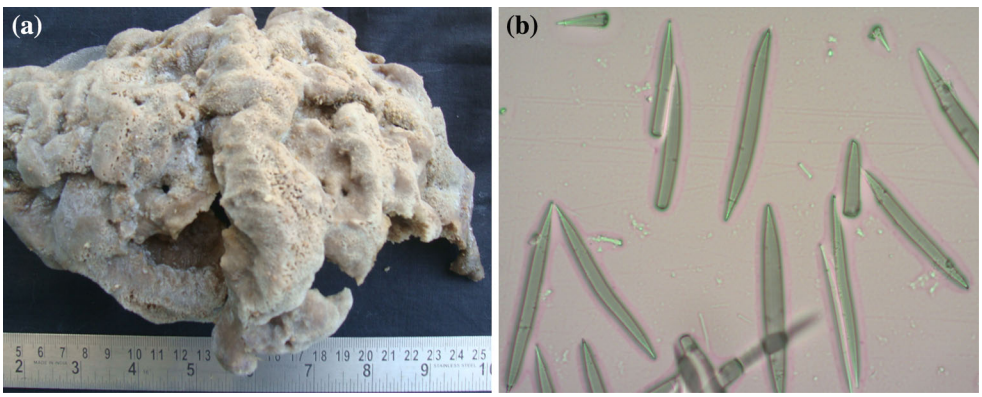

Fig. 1 Morphological characters of *Callyspongia diffusa* (**a**) and shape of spicules (×400) (**b**)

Table 1 Cultural characteristics of sponge-associated bacteria from *Callyspongia diffusa*

Isolates	Cultural characters of sponge-associated bacteria				
	Colony shape	Color	Form	Gram staining and shape	Motility
VCDB	Round	Creamy white with black pigmentation	Convex, mucoid with black pigmentation	− rod	+
VCDA	Spreading	Creamy white	Rhizoid	+ rod	+
VCDW	Round	White	Mucoid	+ rod	−
VCDI	Round	White	Flattened	+ cocci	−
VCDY	Round	Yellow	Raised, pin-headed	− cocci	−
VCDP	Round	Pink	Small concentric ring	+ rod	−
VCDPS	Spreading	Pink	Rhizoid	+ rod	−

Table 2 Biochemical characteristics of the sponge isolates

Biochemical test	VCDB	VCDA	VCDI	VCDW	VCDY	VCDP	VCDPS
Glucose	−	+	−	−	+	−	−
Indole	+	−	−	−	−	−	−
Methyl red	−	−	−	+	+	+	−
Vogues Proskauer	−	−	−	+	+	+	−
Citrate	−	−	−	+	−	−	+
H_2S	+	−	−	−	−	−	−
Starch hydrolysis	−	−	−	−	+	−	−
Catalase	+	+	−	+	−	−	−
Oxidase	+	−	+	+	−	−	+

Table 3 Antimicrobial activity of *Callyspongia diffusa*-associated bacterial isolates

Test pathogens	VCDB	VCDA	VCDW	VCDI	VCDY	VCDP	VCDPS
E. coli	10	10	9	0	9	9	9
S. typhi	11	0	0	0	0	0	0
P. vulgaris	10	9	0	0	0	0	0
V. harveyi	10	7	0	0	0	0	7
V. vulnificus	0	11	0	0	0	0	8
V. fluvialis	14	11	7	0	0	0	0
V. anguillarum	10	11	0	0	7	8	0
E. cloacae	11	12	13	0	0	0	0
L. lactis	10	0	0	0	0	7	0

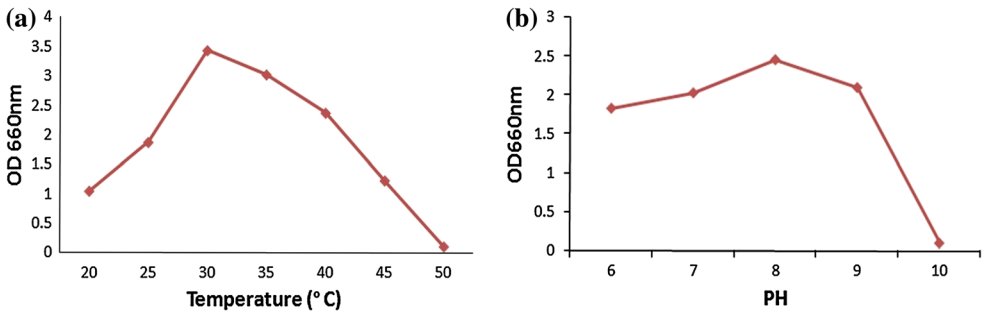

Fig. 2 Effect of temperature (**a**) and pH (**b**) on the growth of bacterial isolate VCDB

Antifungal potential of isolates from sponge tissue

The experimental results on antifungal activity of bacterial strains from *C. diffusa* and its aqueous extract showed that only VCDB and its cell-free supernatant possessed effective biocontrol potential against *A. niger*, *A. fumigatus*, *S. cerevisiae,* and *C. gloeosporioides*.

Determination of growth and metabolite production at different conditions

The growth (OD 660 nm) of the strain VCDB increased drastically between 12 and 48 h after incubation, and a maximum growth was recorded at exponential phase after 48 h of incubation (Fig. 2a, b). The sponge isolate VCDB showed its pigmentation at the late log phase of 24 h and maximized at 48 h. The antibacterial activity increased after 48 h and noted maximum when incubated at 30.0 °C with an initial media pH of 8.0.

Molecular characterization of the VCDB isolate

The BLAST search of the 16S rRNA gene sequence of the isolate VCDB showed a lineage with *S. algae* with 99.0 % similarity to representative strains of genus *Shewanella* (Fig. 3). The gene sequence was deposited in GenBank with accession number KC623651.

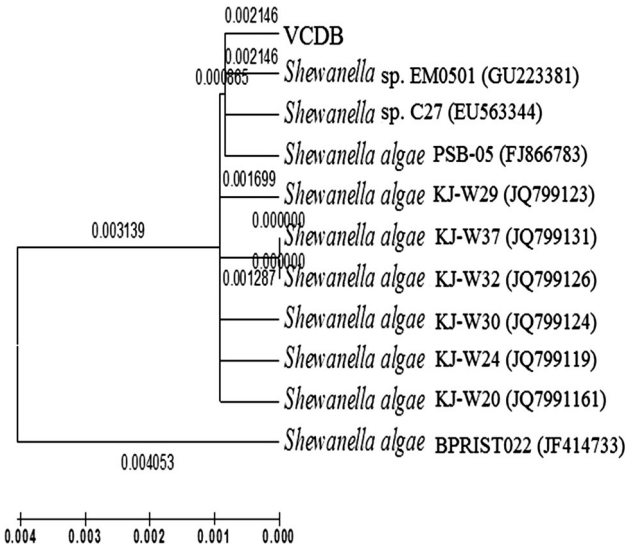

Fig. 3 Phylogenetic tree of strain VCDB drawn in Mega 5.05 using neighbor-joining method showing maximum sequence homology with *S. algae*

Discussion

Several studies have indicated that sponge–microbe interactions are important to produce potential bioactive metabolites in sponge (Selvin et al. 2009; Prabha et al. 2010; Thomas et al. 2010). The results of the present investigation revealed that the tropical sponge *C. diffusa* yielded the bacterial isolate *S. algae* VCDB KC623651 with potential antibacterial activity. Earlier, Hentschel et al. (2001) gave strong evidences about the presence of taxonomically diverse bacteria within mesohyl of the sponge *Aplysina,* which created an environment favoring the production of defense compounds and antimicrobials. Penesyan et al. (2011) identified an antibacterial compound from sponge-associated bacteria *Pseudovibrio* D323.

The bacterial isolate *S. algae* obtained from the tropical sponge *C. diffusa* was characterized as Gram-negative, rod-shaped, motile, aerobic, catalase, and oxidase-positive strain-producing H_2S with black pigmentation at the late exponential phase. Similar observation for differentiating *S. algae* by the production of Pyomelanin at the late exponential phase was reported by Turick et al. (2008). Optimal growth of VCDB strain occurred at temperature 30 °C, pH 8.0 in the presence of 2.0–4.0 % of NaCl. The strain also showed a high antimicrobial activity in these optimized conditions. Yang et al. (2006) described a similar psychrophilic *Shewanella spongiae* from a marine sponge living at 20 m water depth of East Sea (Sea of Japan) with optimum growth at temperature 40 °C in pH level of 6.0–6.5 in the presence of 2.5 % of NaCl. The phylogenetic analysis revealed their type strain HJ037 shared phyletic line with *S. algae* and *Shewanella amazonensis*. Strains of *Shewanella* isolated from aquatic environments and sediments were described by Bowman et al. (1997), Bozal et al. (2002), and Satomi et al. (2006). The bacteria of this genus have attracted great attention due to their diverse respiratory capacities, illustrated by their ability to utilize a wide range of terminal electron acceptors, including oxygen, nitrate, metals, and sulfur compounds (Kostka et al. 2002), and to degrade pollutants such as chlorinated solvents (Petrovskis et al. 1994), petroleum (Semple and Westlake 1987), and RDX (1,3,5-trinitroperhydro-1,3,5-triazine) (Zhao et al. 2004). Lee et al. (2006) isolated *Shewanella iricinae* sp. nov. from marine sponge *Ircinia dendroides* associated with *Posidonia* sea grass from Mediterranean Sea that showed 95.0 % sequence similarity with the recognized bacterial strain *S. algae* (UST040317). Chelossi et al. (2007) isolated 461 bacterial strains from sponge *Chondrilla nucula*, among them 60 strains were reported to be potential. The study also inferred CHC2 strain isolated from *C. nucula* and was sharing 89.0 % similarity to the Gram-negative *S. algae* as well as to uncultured bacterial clone with antibacterial activities toward *S. aureus*, *P. atlantica,* and *P. elongata*. The VCDB strain KC 623651 isolated from *C. diffusa* showed 99.0 % similarity with *S. algae* on molecular analysis exhibited high antimicrobial activity against human and fish pathogens, *E. coli* (MTCC 40) *E. cloacae*, *P. vulgaris* (MTCC 426), *S. typhii* (MTCC 98*),* and *Vibribo* sps. The vibriostatic efficacy of *S. algae* isolated from *Penaeus monodon* against *V. parahaemolyticus* and *V. alginolyticus* was statistically proved by Shakibazadeh et al. (2008). A highly potential bioactivity by VCDB against fish pathogens like *E. cloaceace* and vibrios such as *V. fluvialis* and *V. anguillarum* mostly isolated as pathogenic strains from marine ornamental fishes as well as shrimp aquaculture was observed in this study.

Sponge associates with antifungal activity was reported by Li Zheng et al. (2005) in his study on marine sponge *Hymeniacidon perleve*-associated bacterium NJ6-31, which inhibited the growth of *S. cerevisia* ACCC 2.1882. The antifungal property exerted by *S. algae* KC623651 and its exocellular products against plant fungal pathogen *Collectotrichum gloeosporioides* and food-borne fungus *A. niger, A. fumigatus,* and *S. cerevisiae* indicate the possibility of using *S. algae* and its exocellular products as a biocontrol agents against fungal pathogens. Wang (2006) explored the relationship between microbial diversity and host specificity of marine sponge–bacteria associations in *Cymbastela concentrica*, *Callyspongia,* and *Stylino* sp. by 16S rDNA sequencing of excised DGGE bands. Three distinct types of bacteria with specific characteristics like "specialists" found on only host species, "sponge associates" found on multiple hosts but not in seawater, and

"generalists" found from multiple hosts and seawater. The molecular study on VCDB strain from *C. diffusa* showed 99.0 % similarity with *S. algae* isolated from mangroves. Thus, the present *S. algae* KC623651 could be considered as a true "sponge associates" as the same strain could not be isolated from seawater collected along with the sponge sample from same vicinity of the Vizhinjam coast. Interestingly, this species constituted about 12.5 % of the total retrievable bacterial species recorded.

Microbial associates of sponges gained significance only when a remarkable similarity was found between the compounds isolated predominantly from sponges and those found in terrestrial of entirely different taxa (Perry et al. 1998). The rapid development of pharmaceutical markets and technological development has increased the demand for the production of novel products from sponges toward human health concerns (Yung et al. 2011). At the same time, due considerations are to given to limit and regulate the mass collection of sponges from their natural habitat as suggested by Sipkema et al. (2011). In this context, the sponge-associated bacteria gain much relevance and importance making the least dependence from natural collection and consequent destructions. The findings could encourage the development of antimicrobial metabolites from *S. algae* KC623651 alone, originally isolated from the sponge *C. diffusa* and least further dependence on host collection from marine habitat.

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