



Antibiofilm activity of symbiotic *Bacillus* species associated with marine gastropods

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Abstract

Purpose: Generally, symbiotic marine bacteria are renowned for the synthesis of compounds with bioactive properties, and this has been documented in many previous studies. Therefore, the present study was aimed to isolate novel bacterial symbionts of gastropods that have the ability to synthesize bioactive compounds. These bioactive compounds could be used effectively as antibiofilm agents in order to overcome the problems associated with biofilm.

Methods: The bacteria associated with the surface of marine gastropods were isolated and characterized. Following this, the bacterial metabolites were extracted and their antibiofilm effect was evaluated on biofilm-forming bacteria on artificial substrates. Moreover, the biofilm-forming bacterium *Alteromonas* sp. was treated with the extracts of symbiotic bacteria in order to evaluate the influence of extracts over the synthesis of extracellular polymeric substance (EPS). Besides, the biologically active chemical constituents of the extracts were separated using thin-layer chromatography and subjected to gas chromatography and mass spectrometry (GC-MS) analysis for characterization.

Results: Three bacterial strains belonging to the species *Bacillus firmus*, *Bacillus cereus* and *Bacillus subtilis* were identified from the bacterial community associated with the gastropods. The antibiofilm assays revealed that the extract of three symbiotic bacteria significantly ($p < 0.05$) reduced the biofilm formation by the marine bacterium *Alteromonas* sp. on artificial materials. Also, the EPS synthesis by *Alteromonas* sp. was significantly inhibited due to symbiotic bacterial extract treatment. The chemical composition of the bioactive fraction isolated from the symbiotic bacteria extract revealed that most of the detected compounds were belonging to aromatic acid, fatty acid and carboxylic acid.

Conclusion: The results of this study clearly revealed that the bacteria belonging to the above listed *Bacillus* species can be considered as a promising source of natural antibiofilm agents.

Keywords: Biofilm, Symbiotic bacteria, *Bacillus* species, Marine natural products, Antibacterial activity, Antibiofilm activity

Introduction

Microorganisms generally occur in the environment as a community known as biofilm able to grow on abiotic and biotic surfaces. Most of the microorganisms found in the environment prefer biofilm mode of life, as biofilm enhances the survival and metabolism of microbes during adverse conditions (Ikuma et al., 2013). Biofilm can exist on all kinds of surfaces such as plastics, metal, glass, soil particles, wood, medical implants, food products and tissues. Biofilm is a serious problem in many fields such as medical industries, food industries and

paper industries (Campoccia et al., 2006; Chen et al., 2007; Lopez et al., 2010; Francolini and Donelli, 2010; Hoiby et al., 2011). Moreover, biofilm has a drastic impact on the marine industries, where biofilm accelerates the corrosion and facilitates the rapid development of biofouling (accumulation of macrofoulers) on the surface of underwater objects developed by human beings (Little et al., 2008, Cao et al., 2011). Notably, the annual economic loss caused by biofilm in the marine industries has been estimated as 2.5 trillion dollars (Koch et al., 2016), while the economic loss due to biofilm in the health care sector is about 77.7 billion dollars (Khelissa et al., 2017).

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The chemical antifouling biocides were widely used to overcome the problems connected with biofilm, but the consistent usage led to the development of resistance among the biofilm-forming microbes and also these biocides were found to be toxic to the environment (Simoes and Vieira, 2009). In this circumstance, for controlling the biofilms on structures submerged in marine waters, environmentally friendly antibiofilm molecules were considered as highly valuable and alternative to the chemical antibiofilm agents (Donlan, 2009; Estrella et al., 2009). The bioactive molecules derived from the marine organisms are non-toxic or less toxic and biodegradable, and also the negative (toxic) effects may be less compared to the chemical antifouling compounds currently in practice (Satheesh et al., 2016). The antibiofilm properties of natural products obtained from various biological sources including marine bacterial symbionts have been well recorded (Papa et al., 2015; Camesi et al., 2016; Jun et al., 2018). For instance, the polysaccharide (A101) synthesized by the symbiotic marine bacterium *Vibrio* sp. has been reported to inhibit or disrupt the biofilm formation of pathogenic bacteria (Jiang et al., 2011). Many previous studies have also proved that the bacterial symbionts of marine macroorganisms have the ability to synthesize a high proportion of compounds that have biological activities (Viju et al., 2016; Alvarado et al., 2018). In the current research, the antibiofilm activity of the bacterial (*Bacillus* species) symbionts of marine gastropods was assessed against biofilm-forming bacteria.

Materials and methods

Gastropod collection and isolation of their bacterial symbionts

Gastropod samples were collected from three stations such as Thengapattanam (Arabian sea–South-west coast, 8° 29' N and 77° 32' E), Colachel (Arabian sea–South-west coast, 8° 17' N and 77° 26' E) and Kanyakumari (Gulf of Mannar–South-east coast, 8° 26' N and 77° 19' E) along the intertidal region of Kanyakumari coast, India. The commonly available gastropod species such as *Babylonia*, *Busycon*, *Collisella*, *Conus*, *Murex*, *Snar*, *Turbo* and *Turritella* were collected and brought to the laboratory. The bacterial symbionts of gastropods were scraped off using a sterile nylon brush and suspended in 1 ml filter-sterilized seawater. Following this, the agar spread plate method was used for the cultivation of symbiotic bacteria. For this, the bacterial suspension was serially diluted and spread around the Zobell marine agar (ZMA) plate (HIMEDIA, India) and the plate was then put away in a sterile chamber (at room temperature for 24 h) for the cultivation of bacterial colonies. Subsequently, the cultivated bacterial colonies were isolated individually using the quadrant streak plate method described by Viju et al. (2018). The isolated bacterial

colonies were classified based on their morphological characters and reserved on ZMA slant for further studies.

Screening of bioactive bacteria

The agar over-layer method explained by Anand et al. (2006) was used to analyse the synthesis of biologically active substances by bacterial symbionts of gastropods. For this, bacterial colonies were allowed to grow on the ZMA plate at room temperature for 24 h. Following this, a soft agar medium was composed by suspending 0.6% of agar in Zobell marine broth (ZMB). This soft agar was then added with 10% of target bacterium *Alteromonas* sp. (biofilm-forming bacterium isolated by Satheesh et al. (2012)) and mixed properly. After that, the medium was poured on the symbiotic bacteria grown on the ZMA plate and incubated at room temperature. Twenty-four hours later, the antagonistic effect was analysed by observing the inhibition zone that appeared around the colonies of symbiotic bacteria. The selected bioactive bacteria were tentatively identified up to the genus level using the data obtained from the morphological and biochemical analysis (Delfan et al., 2012).

Isolation of bioactive bacteria

The bioactive bacteria were grown in the marine bacterial culture medium (ZMB) for a period of 5 days at room temperature (28 ± 1). After that, the bacterial cell pellet was separated from the culture medium by centrifugation and soaked in methanol for 72 h and the metabolites were extracted using the method explained by Viju et al. (2018). Following this, the antibacterial activity of the extract of symbiotic bacteria (ESB) was tested against biofilm-forming bacterium *Alteromonas* sp. using the agar disc diffusion method. In brief, a 6-mm sterile disc (HIMEDIA) was loaded with the ESB (50 mg of extract was dissolved in 2.5 ml of DMSO), while a control disc was prepared by loading DMSO in place of ESB. Subsequently, the overnight culture of the target biofilm-forming bacterium *Alteromonas* sp. was applied on the surface of Muller–Hinton agar plate. The plate was kept in an incubator at normal room temperature, and the antibacterial potential of the ESBs was assessed with the zone that appeared around the discs after 24 h of incubation (Shankar et al., 2015).

Identification of bioactive bacterial symbionts

The 16S rRNA gene of the symbiotic bioactive bacteria was isolated by the phenol–chloroform method (Satheesh et al. 2012). In brief, the cell pellet of the overnight culture of symbiotic bacteria was obtained by centrifugation (700 r min^{-1} for 10 min). The cell pellet was suspended in sucrose TE buffer (400 μl) and added with the lysozyme at a concentration of 8 mg/ml. An hour later, the enzyme proteinase K was added up and put

away in an incubator (at 55 °C) for 24 h. After incubation, the lysate solution was added up with an equal volume of phenol–chloroform (1:1) solution and mixed properly. This solution was then centrifuged (10000 r min⁻¹ for 10 min), and the collected supernatant was added with chloroform–isoamylalcohol (24:1) and mixed up properly. After that, an adequate amount of ethanol was added and kept away for the precipitation of 16S rRNA. The precipitate (rRNA) was isolated and amplified in the PCR using the universal primers, 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). The PCR reaction steps such as denaturation (at 95 °C for 30 s), annealing (at 52 °C for 30 s) and extension (at 60 °C for 4 min) were carried out until the production of an adequate number of nucleotides (bps). The obtained sequence was analysed using the Basic Local Alignment Search Tool (BLAST), and their similarity was compared with the other bacterial sequences available in the National Center for Biotechnology Information (NCBI) (Viju et al., 2017). The sequence data of all the three symbiotic bacteria (*B. firmus*, *B. cereus* and *B. subtilis*) were deposited in GenBank, and their accession numbers have been assigned by NCBI. Subsequently, the evolutionary phylogenetic tree of the three bacteria was constructed along with the other 17 bacterial sequences belonging to *Bacillus* sp. obtained from NCBI using neighbour-joining method. The evolutionary distances were computed using the Tamura–Nei method with 2000 bootstrap replicates, and the analysis was carried out in MEGA 7 (Kumar et al., 2016).

Preparation of biofilm bacterial cell suspension for laboratory bioassay

To evaluate the antibiofilm effect of ESBs, bacterial cell suspension (BCS) was prepared with the target bacterium *Alteromonas* sp. For this, the cell pellet of the overnight culture of *Alteromonas* sp. was isolated by centrifugation (5000 rpm/15 min) and washed with phosphate buffer saline (PBS). A portion of the pellet was suspended in PBS, and its final concentration (optical density) was made up to 0.2 at 540 nm using a spectrophotometer.

Antibiofilm assays of ESB

Antibiofilm effect of ESB on hydrophobic surface

The microtiter plate was used as the hydrophobic surface to analyse the antibiofilm effect of ESB using the method explained by Ghaima et al. (2013). Two hundred microlitres of BCS and 20 µl of ESB were taken in a microtiter plate (TARSONS) and mixed properly with a micropipette. At the same time, 20 µl of DMSO was used in place of ESB for the preparation of control well and the plate was placed in an incubator at room temperature. Following 24 h of incubation, the liquid

content of the well along with unattached bacteria was discarded and the well was washed with PBS (pH 7.4). Subsequently, the bacteria (biofilm) attached to the surface were dyed with crystal violet (0.1%) for a minute. Finally, the density of the biofilm developed on the well was evaluated with the ELISA reader at a wavelength of 570 nm.

Antibiofilm effect of ESB on hydrophilic surface

The glass test tube was used as the hydrophilic surface to analyse the antibiofilm effect of ESB (Ghaima et al., 2013). In brief, the tubes (Borosil: 15 × 125 mm) containing sterilized ZMB medium (3 ml) were added with 3 ml of BCS and 0.5 ml ESB (0.5 ml of DMSO was added in control tube). Subsequently, the content of the tube was mixed properly and the tubes were placed in a shaker at room temperature. Following 72 h of incubation, the content of the tubes was thrown out and flooded with PBS in order to clear up the unattached bacteria. Then the tubes were air-dried and the biofilm formed on the surface was dyed with crystal violet for a minute. And then, the unbounded dye was removed with PBS and the dye absorbed by the biofilm was retrieved with acetic acid and its density was evaluated using a spectrophotometer at 595 nm.

Influence of ESB on the synthesis of EPS by biofilm-forming bacterium

The influence of ESB on the synthesis of EPS was calculated by estimating the carbohydrate and protein concentration of the extracellular polymeric substance. A test tube containing 3 ml of BCS of *Alteromonas* sp. was treated with 0.5 ml of ESB by keeping at shaker set up at room temperature. Following 24 h of incubation, the EPS synthesized by *Alteromonas* sp. on the ZMB medium was isolated using the method outlined by Viju et al. (2018). Subsequently, the carbohydrate and protein concentration of the isolated EPS was analysed by the procedures proposed by Dubois et al. (1956) and Lowry et al. (1951) respectively. The glucose was used as the standard for carbohydrate estimation, while bovine serum was used as the standard for protein estimation.

Chromatography analysis and screening of bioactive fraction

The thin-layer chromatography analysis explained by Viju et al. (2019) was used for the isolation of the bioactive fractions present in the crude extracts of symbiotic bacteria. A drop of extracts (ESB) was run through the stationary phase prepared with silica gel G with the aid of the mobile phase prepared by mixing methanol (2%), ethyl acetate (3%) and chloroform (5%). Subsequently, the fractions embedded in the silica gel were visualized by iodine and scraped off and mixed with

methanol for 14 min. This mixture was then centrifuged and a fraction (liquid phase) was separated. The fractions were then evaporated and tested for bioactivity using antimicrobial susceptibility test described earlier in the antibacterial assay.

GC-MS analysis

The organic compounds present in the bioactive fractions isolated from the extract of symbiotic bacteria (*B. firmus*, *B. cereus* and *B. subtilis*) were analysed in gas chromatography (GC Clarus 500 Perkin Elmer) assembled with a capillary column. The temperature of the injection port was maintained at 250 °C, and the sample was injected to the column using the mobile phase helium at 1 ml/min flow rate. The column oven temperature was started off at 110 °C for 2 min and then turned up gradually by programming at 10 °C/min and ended up at 280 °C/9 min (an isothermal process). Subsequently, the mass spectral data were taken and compared with the mass spectral data available in the NIST library (Casuga et al. 2016).

Data analysis

Student's *T* test was performed to analyse the biofilm inhibition of the extracts of symbiotic bacteria against *Alteromonas* sp. in different assays. The variation in the production of EPS (carbohydrate and protein) between control and ESB-treated biofilm-forming bacterium was analysed by one-way ANOVA. These statistical analyses were carried out using MS Excel 7 program.

Results

Bacterial symbionts of marine gastropods

The number of bacterial symbionts of gastropods isolated during this study was 65. Among them, 22 bacteria were the symbionts of gastropods which were collected from the Thengapattanam coast, followed by 29 bacteria that were the symbionts of the gastropods collected from the Colachel coast and 14 bacteria that were the symbionts of the gastropods collected from the Kanyakumari coast.

Antagonistic effect of bacterial symbionts of gastropods

Twenty-eight bacterial symbionts of gastropods were found to produce the compounds with bioactive potential against *Alteromonas* sp., and the zone of inhibition observed was lying between 1 and 2.5 mm. Based on the inhibition zone (antagonistic activity) that appeared around the bacterial colony, 15 broad active strains (TPM7, TPM9, TPM10, TPM13, TPM21, CAL9, CAL10, CAL11, CAL16, CAL20, KKI3, KKI4, KKI8, KKI11 and KKI12) were selected for further studies (Table 1). The tentative characterization (based on biochemical characters) result of these 15 bacterial strains revealed that 5 of them were belonging to the genus

Pseudomonas and 4 strains were belonging to *Bacillus*. Also, 3 strains from the genus *Alteromonas* and 2 strains from *Flavobacterium* were recorded. The remaining 1 strain was belonging to the genus *Aeromonas* (Table 2).

Antibacterial activity of ESB

The result of antibacterial assay revealed that 9 out of 15 extracts of symbiotic bacteria showed inhibitory activity against *Alteromonas* sp. (biofilm bacterium) and the observed zones of inhibition were between 7 and 13 mm. Among them, the extract of three bacteria TPM7, CAL9 and KKI12 overpowered the others and the observed zone of inhibition was 13 ± 1 , 12.33 ± 1.15 and 11 ± 0.0 mm respectively (Table 3).

Identification of bioactive bacteria

The 16S rRNA bacterial identification revealed that all the three potent bacteria (TPM7, CAL9 and KKI12) were belonging to the genus *Bacillus* and the constructed phylogenetic tree is given in Fig. 1. The bacterium TPM7 showed 99% similarity with the nucleotides of *Bacillus firmus* available in the NCBI GenBank. Similarly, the bacterium CAL9 showed 98% similarity with *Bacillus cereus* and the bacterium KKI12 showed 99% similarity with *Bacillus subtilis* in the phylogenetic analysis. The 16S rRNA sequences of all the three bacterial strains (*B. firmus*—KX669624; *B. cereus*—KX669625; and *B. subtilis*—KX685340) were submitted to GenBank (NCBI).

Antibiofilm effect of ESB on hydrophobic surface

The microtiter assay revealed the antibiofilm potential of the extracts of symbiotic bacteria against *Alteromonas* sp. The observed OD value of the biofilm developed by *Alteromonas* sp. on the control (without any treatment) microtiter plate well was 0.341 ± 0.01 , whereas this biofilm development (OD value) was significantly decreased to 0.224 ± 0.02 ($P < 0.004$), 0.253 ± 0.01 ($P < 0.012$) and 0.244 ± 0.025 ($P < 0.004$) in the wells treated with the extracts isolated from the bacteria *B. firmus*, *B. cereus* and *B. subtilis* respectively (Fig. 2).

Antibiofilm effect of ESB on hydrophilic surface

The extracts of symbiotic bacteria displayed strong biofilm inhibition, and the variation in biofilm formation between control and bacterial extract-treated tubes can be seen in Fig. 3. The tube which contained control *Alteromonas* sp. showed the biofilm formation of 0.320 ± 0.01 , and this was significantly reduced to 0.237 ± 0.012 ($P < 0.009$), 0.238 ± 0.032 ($P < 0.04$) and 0.255 ± 0.018 ($P < 0.02$) when the test tubes were treated with the extracts of the symbiotic bacterium *B. firmus*, *B. cereus* and *B. subtilis* respectively.

Table 1 Antagonistic activity of bacterial symbionts of molluscans collected from Kanyakumari coastal waters

S. no	Name of the source (gastropods)	Name (code) of the strains	Zone of inhibition (mm)
1	<i>Murex</i> sp.	TPM7	2
2	<i>Murex</i> sp.	TPM9	2.5
3	<i>Murex</i> sp.	TPM10	1
4	<i>Babylonia</i> sp.	TPM13	2.5
5	<i>Pinctada</i> sp.	TPM21	2
6	<i>Turritella</i> sp.	CAL9	2.5
7	<i>Turritella</i> sp.	CAL10	1
8	<i>Perna</i> sp.	CAL11	1.5
9	<i>Pinctada</i> sp.	CAL16	2.5
10	<i>Sepia</i> sp.	CAL20	2
11	<i>Busycon</i> sp.	KKI3	2.5
12	<i>Busycon</i> sp.	KKI4	2.5
13	<i>Turbo</i> sp.	KKI8	2
14	<i>Conus</i> sp.	KKI11	1.5
15	<i>Conus</i> sp.	KKI12	2.5

Influence of ESB on the synthesis of EPS by biofilm bacterium

The extract treatment significantly inhibited the EPS (carbohydrate and protein) synthesis by biofilm bacterium *Alteromonas* sp. The carbohydrate content (0.352 ± 0.02 mg/g) of EPS synthesized by *Alteromonas* sp.

grown without any treatment was significantly (one-way ANOVA: $F = 22.12, P < 0.0003$) reduced to $0.246 \pm 0.02, 0.231 \pm 0.01$ and 0.224 ± 0.02 mg/g on the bacterial culture treated with the extracts of *B. firmus*, *B. cereus* and *B. subtilis* respectively (Fig. 4a). Likewise, the protein content (0.282 ± 0.01 mg/g) of EPS synthesized

Table 2 Characterization of bacterial strains using the data obtained from biochemical and morphological analysis

S. no	Biochemical and physiological test	Symbiotic bacterial strains															
		TPM7	TPM 9	TPM10	TPM13	TPM21	CAL9	CAL10	CAL11	CAL16	CAL20	KKI3	KKI4	KKI8	KKI11	KKI12	
1	Gram staining	+	-	-	-	-	+	-	+	-	-	-	-	-	-	+	
2	Morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	
3	Motility	-	+	+	+	+	-	+	-	+	+	-	+	+	-	-	
4	Indole production	+	-	+	-	-	+	-	+	-	+	-	-	+	-	+	
5	Methyl red	-	+	-	+	+	-	+	-	+	+	-	+	-	-	-	
6	Voges-Proskauer	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	
7	Citrate utilization	+	-	+	-	-	+	-	+	-	+	+	-	+	+	+	
8	Starch hydrolysis	+	+	-	+	+	+	+	+	+	+	-	+	-	-	+	
9	Urea hydrolysis	+	-	-	-	-	+	-	+	+	-	+	-	-	+	+	
10	TSI	Alkaline	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+
		Acid	+	-	-	+	+	-	+	+	-	-	+	+	-	+	+
		Gas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		H ₂ S	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
11	Catalase	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	
12	Oxidase	-	-	+	+	+	-	-	-	+	+	+	-	+	+	-	
13	Nitrate reduction	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	
14	Casein hydrolysis	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	
15	Gelatin hydrolysis	-	+	-	-	-	-	-	-	-	-	+	+	-	+	-	
16	BMDB	Bac.	Pse.	Pse.	Alt.	Alt.	Bac.	Pse.	Bac.	Alt.	Aer.	Fla.	Pse.	Pse.	Fla.	Bac.	

BMDB Bergey's Manual of Determinative Bacteriology, Bac. Bacillus sp., Pse. Pseudomonas sp., Alt. Alteromonas sp., Aer. Aeromonas sp., Fla. Flavobacterium sp.

Table 3 Antibacterial activity of bacterial extracts isolated from the gastropod-associated bacteria

S. no	Symbiotic bacteria	Zone of inhibition (mm) against <i>Alteromonas</i> sp.
1	TPM7 (<i>Bacillus</i> sp.)	13 ± 1.0
2	TPM9 (<i>Pseudomonas</i> sp.)	–
3	TPM10 (<i>Pseudomonas</i> sp.)	11.66 ± 1.15
4	TPM13 (<i>Alteromonas</i> sp.)	8.66 ± 0.57
5	TPM21 (<i>Alteromonas</i> sp.)	–
6	CAL9 (<i>Bacillus</i> sp.)	12.33 ± 1.1
7	CAL10 (<i>Pseudomonas</i> sp.)	–
8	CAL11 (<i>Bacillus</i> sp.)	–
9	CAL16 (<i>Alteromonas</i> sp.)	–
10	CAL20 (<i>Aeromonas</i> sp.)	11.66 ± 0.57
11	KKI3 (<i>Flavobacterium</i> sp.)	11.33 ± 1.15
12	KKI4 (<i>Pseudomonas</i> sp.)	11 ± 0.0
13	KKI8 (<i>Pseudomonas</i> sp.)	9 ± 1.0
14	KKI11 (<i>Flavobacterium</i> sp.)	–
15	KKI12 (<i>Bacillus</i> sp.)	12.66 ± 0.5

by the control *Alteromonas* sp. was significantly (one-way ANOVA: $F = 4.98$, $P < 0.03$) reduced to 0.199 ± 0.04 , 0.182 ± 0.02 and 0.181 ± 0.05 mg/g when the culture was treated with the extracts of *B. firmus*, *B. cereus* and *B. subtilis* respectively (Fig. 4b).

Chromatography analysis and screening of bioactive fractions

The TLC chromatogram of the extract of *B. firmus* developed three (1, 2 and 3) distinct fractions on the thin-layer chromatogram. Of the three, fraction 3 was found to possess bioactive molecules (Fig. 5a and Table 4). Similarly, the TLC chromatogram of the extract of the bacterium *B. cereus* displayed three (1, 2 and 3) distinct spots and fraction 2 was identified as the bioactive fraction (Fig. 5b and Table 4). Whereas the extract produced by the bacterium *B. subtilis* developed only two fractions on the thin-layer chromatogram, fraction 2 was found to possess antibiofilm agents (Fig. 5c).

GC-MS analysis

GC-MS chromatogram of the fraction of *B. firmus* that exhibited an antibiofilm activity showed the presence of compounds like 2-ethylacridine, indolizine and anthranilic acid (Fig. 6a and Table 5), whereas components such as 3-methoxypropionic acid, 3-(dimethylamino) propanamide, phenyltoloxamine, cis-3-octadecenoic acid, octadecenoic acid, N-allyl-N,N-dimethylamine and 3-piperidinol were detected in the fraction of *B. cereus* that displayed bioactivity (Fig. 6b and Table 5). Likewise, components such as 2-acetylbenzoic acid, trans-1-chloropropene, methional, isobutane, 1-buten-3-yne and orthosilicic acid were found in the fraction of *B. subtilis* which showed an antibiofilm activity (Fig. 6c and Table 5).

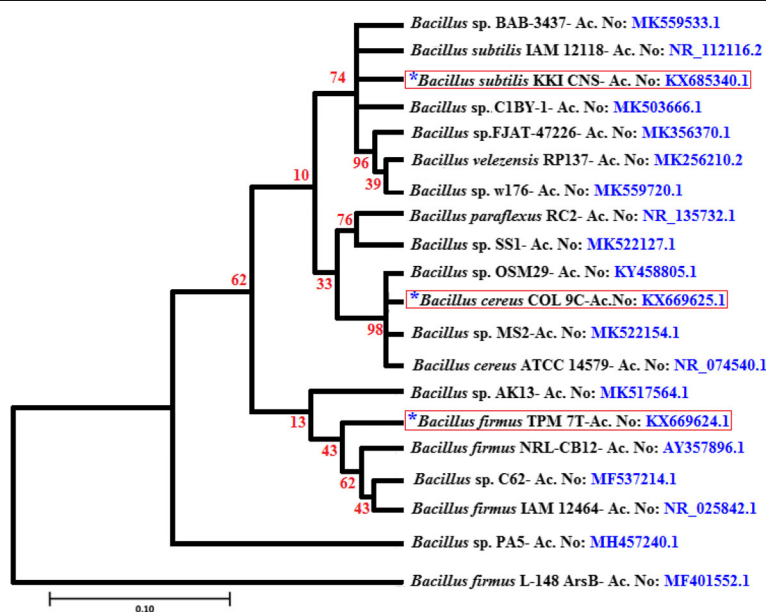


Fig. 1 The phylogenetic analysis of symbiotic bacteria *Bacillus* sp. The sequence of three symbiotic bacteria (indicated in the figure with an asterisk) was aligned together with 17 bacterial sequences obtained from NCBI. The phylogenetic tree was constructed based on the neighbour-joining method using MEGA7 software

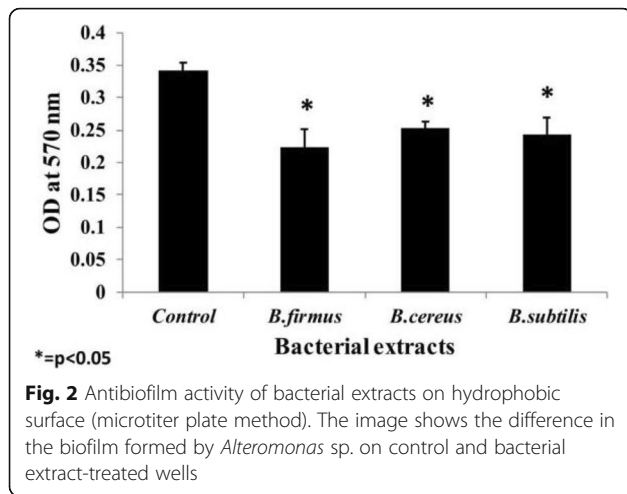


Fig. 2 Antibiofilm activity of bacterial extracts on hydrophobic surface (microtiter plate method). The image shows the difference in the biofilm formed by *Alteromonas* sp. on control and bacterial extract-treated wells

Discussion

Biofilm formation on a substrate is initiated by microbial adhesion, and the rate of adhesion significantly varies based on the characteristics of the surfaces (Gomes and Mergulhao, 2017). Typically, the rate of microbial adhesion on hydrophobic (plastic-microtiter plate) surfaces is higher than that on hydrophilic (glass test tube) surfaces (Bendinger et al., 1993). Therefore, in this study, the antibiofilm effect of the extracts of symbiotic bacteria

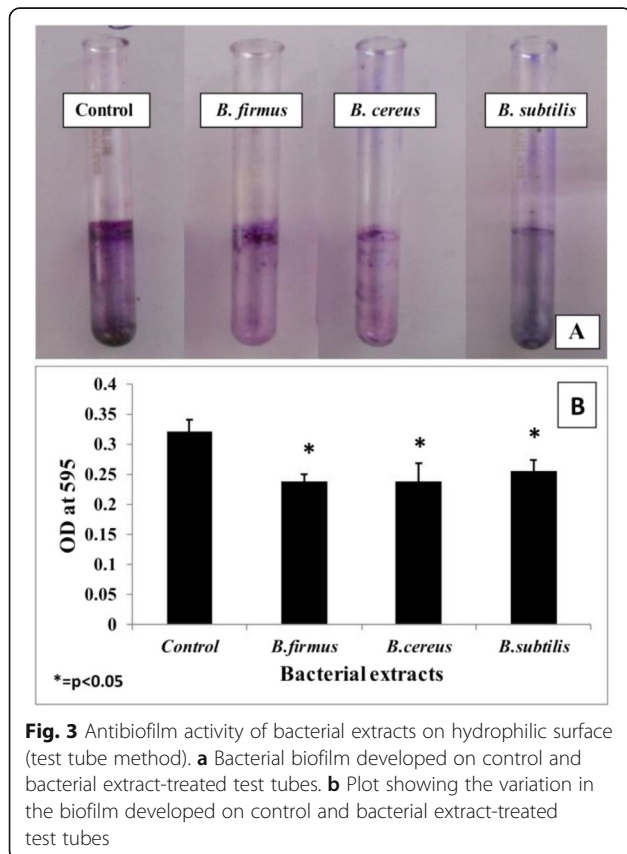


Fig. 3 Antibiofilm activity of bacterial extracts on hydrophilic surface (test tube method). **a** Bacterial biofilm developed on control and bacterial extract-treated test tubes. **b** Plot showing the variation in the biofilm developed on control and bacterial extract-treated test tubes

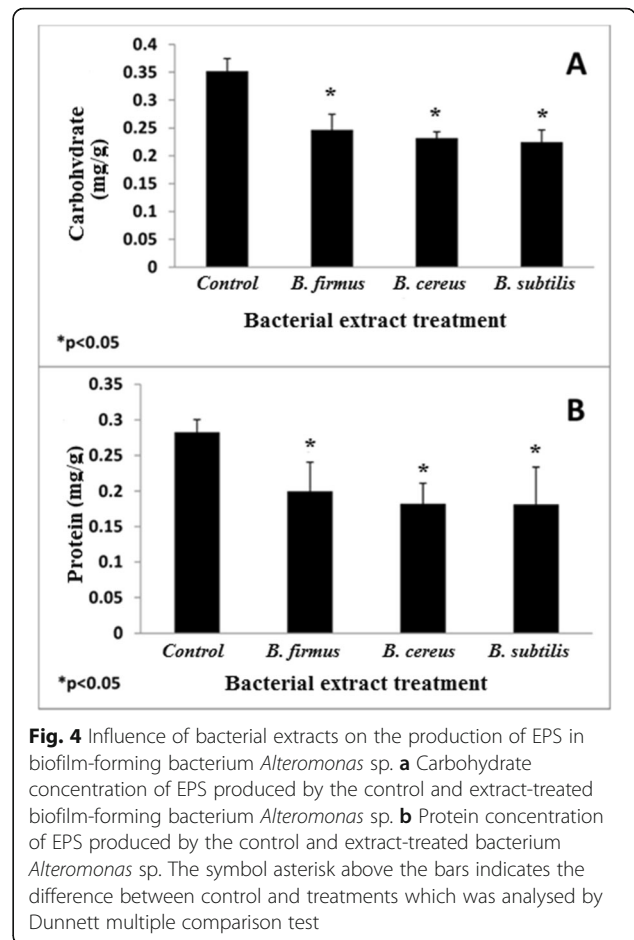


Fig. 4 Influence of bacterial extracts on the production of EPS in biofilm-forming bacterium *Alteromonas* sp. **a** Carbohydrate concentration of EPS produced by the control and extract-treated biofilm-forming bacterium *Alteromonas* sp. **b** Protein concentration of EPS produced by the control and extract-treated bacterium *Alteromonas* sp. The symbol asterisk above the bars indicates the difference between control and treatments which was analysed by Dunnett multiple comparison test

was tested on both hydrophobic and hydrophilic surfaces. The result revealed that the bacterial extracts significantly reduced the biofilm formation on both hydrophobic and hydrophilic surfaces. There are similar studies that used hydrophobic and hydrophilic surfaces to evaluate the antibiofilm effect of natural products. For instance, Karwacki et al. (2013) analysed the antibiofilm effect of bacterial extracts using a microtiter plate (hydrophobic surface) and found 5 out of 12 bacterial extracts considerably reduced the biofilm formation of *S. aureus*, whereas Mathur et al. (2013) reported the antibiofilm activity of the plant oils isolated from garlic, neem, clove, eucalyptus and tulsi in hydrophilic (glass) surface. However, the extracts of symbiotic bacteria (*B. firmus*, *B. cereus* and *B. subtilis*) significantly inhibited the attachment of biofilm-forming bacteria on both hydrophobic and hydrophilic surfaces.

It has been reported that bioactive compounds obtained from living organisms could prevent the microbial adhesion by repulsive action (Kelly et al., 2003). Further antibiofilm mechanisms such as inhibition of quorum sensing pathway, inhibition of regulated stringent response, inhibition of cell division, dispersion of extracellular polymeric

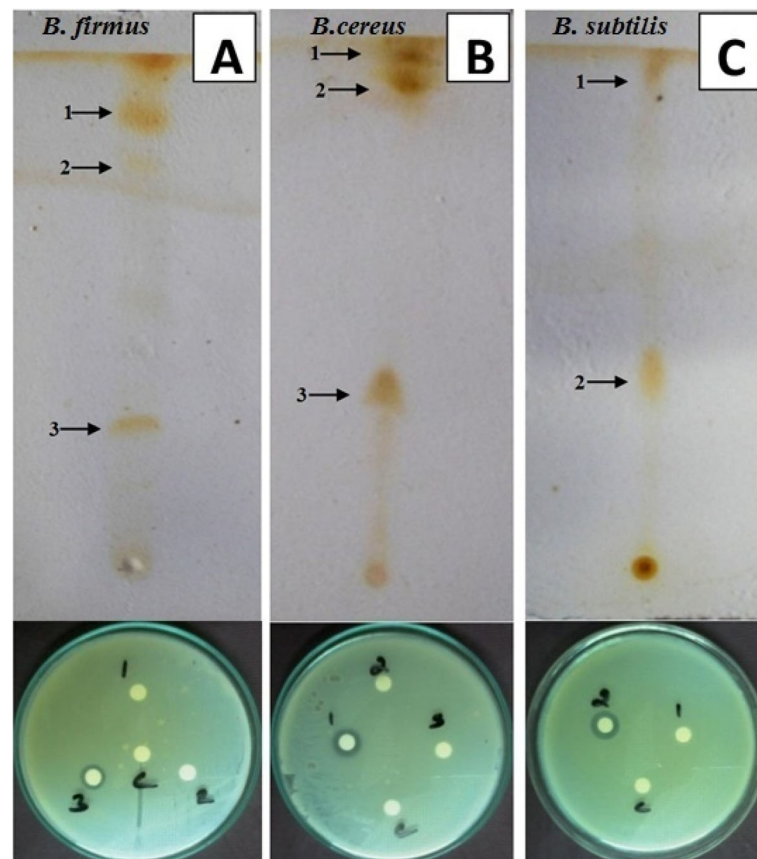


Fig. 5 Isolation of bioactive fractions from the bacterial crude extract. **a** Thin-layer chromatogram and the bioactivity of the fractions isolated from *B. firmus*. **b** Thin-layer chromatogram and the bioactivity of the fractions isolated from *B. cereus*. **c** Thin-layer chromatogram and the bioactivity of the fractions isolated from *B. subtilis*

substances, disaggregation of LPS, inhibition of macromolecule synthesis and inhibition of curli biosynthesis have also been reported (Park et al., 2011; Fuente-Nunez et al., 2014; Roy et al., 2018). For instance, Hentzer et al. (2002) reported the QS mode of the antibiofilm activity of the halogenated furanones isolated from the seaweed *Delisea pulchra*. Contrarily, some authors have revealed the

biofilm dispersal mode of antibiofilm effect of natural products (Rendueles et al., 2013; Karwacki et al., 2013).

Notably, in the current study, the extracts of symbiotic bacteria (*B. firmus*, *B. cereus* and *B. subtilis*) substantially reduced the EPS production in biofilm-forming bacterium *Alteromonas* sp. The reduction in the EPS production would certainly inhibit the biofilm formation, due

Table 4 Rf values of the TLC obtained fractions isolated from the symbiotic bacterial extracts and their bioactivity

S. no	Bacterial extracts	TLC obtained Fractions	Rf value (cm)	Bioactivity (mm)
1.	<i>B. firmus</i> (TPM7)	Fraction 1	0.89	–
		Fraction 2	0.79	–
		Fraction 3	0.25	10
2.	<i>B. cereus</i> (CAL9)	Fraction 1	0.88	–
		Fraction 2	0.84	–
		Fraction 3	0.35	8
3.	<i>B. subtilis</i> (KKI12)	Fraction 1	0.94	–
		Fraction 2	0.38	12

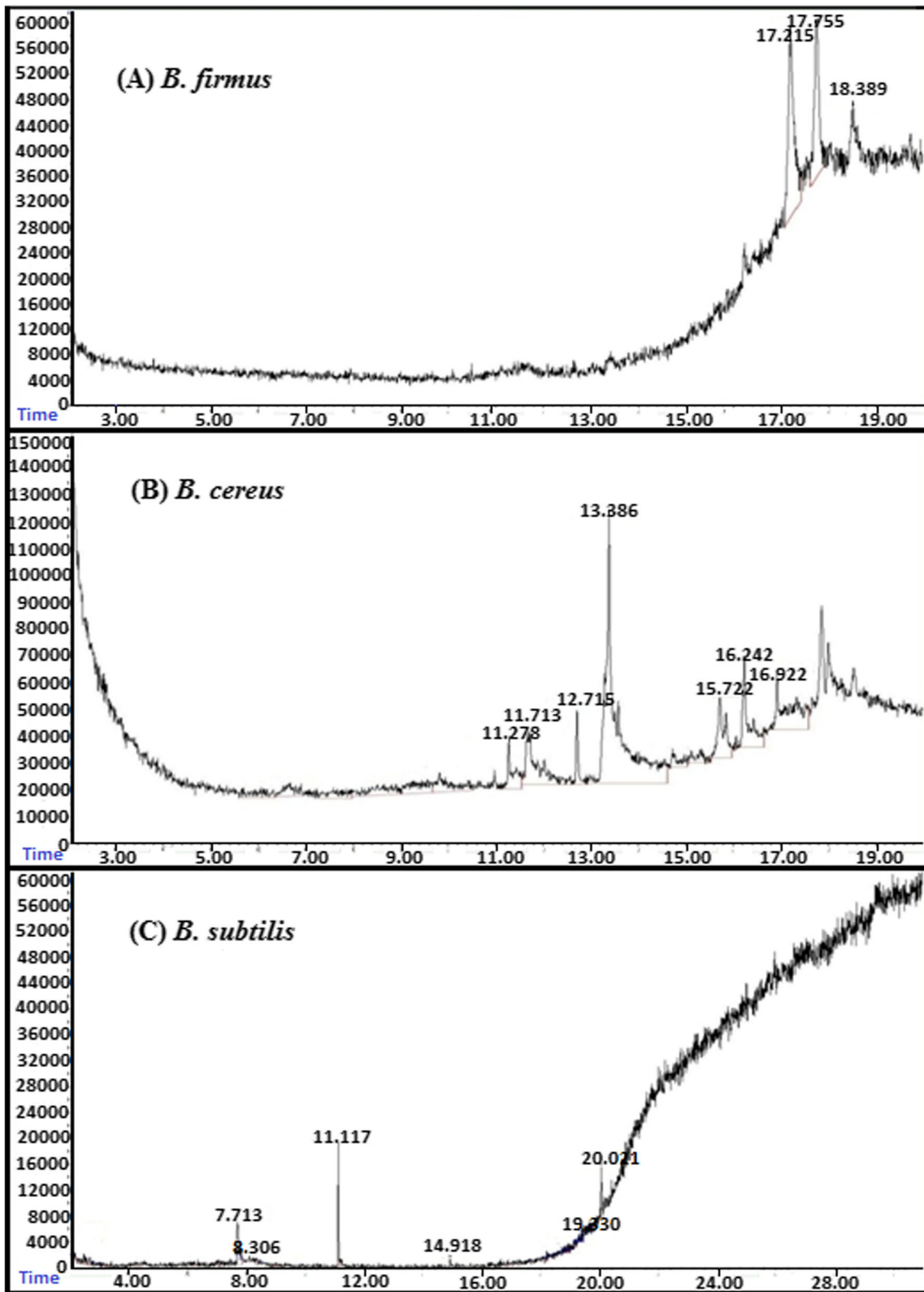


Fig. 6 GC-MS spectrum of the bioactive fractions. **a** Fraction isolated from the extract of the symbiotic bacterium *B. firmus*. **b** Fraction isolated from the extract of the symbiotic bacterium *B. cereus*. **c** Fraction isolated from the extract of the symbiotic bacterium *B. subtilis*

Table 5 Compounds present in the bioactive fractions isolated from the symbiotic bacteria *B. firmus*, *B. cereus* and *B. subtilis*

S. no	Retention time (min)	Name of the compound	Bacterial source
1	17.215	2-Ethylacridine	<i>B. firmus</i>
2	17.755	Indolizine	<i>B. firmus</i>
3	18.389	Anthranilic acid	<i>B. firmus</i>
4	11.278	3-Methoxypropionic acid	<i>B. cereus</i>
5	11.713	3-(Dimethylamino)propanamide	<i>B. cereus</i>
6	12.715	Phenyltoloxamine	<i>B. cereus</i>
7	13.386	Cis-3-Octadecenoic acid	<i>B. cereus</i>
8	15.722	Octadecenoic acid	<i>B. cereus</i>
9	16.242	N-Allyl-N,N-dimethylamine	<i>B. cereus</i>
10	16.922	3-Piperidinol	<i>B. cereus</i>
11	7.713	Trans-1-Chloropropene	<i>B. subtilis</i>
12	8.306	Methional	<i>B. subtilis</i>
13	11.117	2-Acetylbenzoic acid	<i>B. subtilis</i>
14	14.918	Isobutane	<i>B. subtilis</i>
15	19.330	1-Buten-3-yne	<i>B. subtilis</i>
16	20.071	Orthosilicic acid	<i>B. subtilis</i>

to the fact that the polymer component of EPS forms a highly hydrated matrix that keeps the cells together and strongly interacts with the substratum (Pal and Paul, 2013). Furthermore, it has been reported that bacterial strains which are not in a position to produce EPS are not able to form biofilm on the surfaces (Watnick and Kolter, 1999). Supportively, Olofsson et al. (2003) evidenced the biofilm inhibition of N-acetyl-L-cysteine (NAC) against some bacteria (*Acinetobacter*, *Klebsiella*, *Pseudomonas* and *Bacillus*) which were not able to produce EPS due to the bacteriostatic effect of NAC. Some similar studies have also reported the antibiofilm effect of NAC against the bacteria *Staphylococcus*, *Streptococcus*, *Haemophilus* and *Moraxella* species (Zheng et al., 1999). NAC is an antibacterial agent with bacteriostatic mode of action and used in some medical treatments (Riise et al., 2000). According to the previous reports and the result of the present study, it is believed that the antibiofilm effect of the extracts produced by *Bacillus* sp. might be bacteriostatic mode of action. The bacteriostatic mode of biofilm inhibition by the compounds isolated from the natural sources has been documented (Trentin et al., 2013).

Generally, the synthesis of bioactive compounds by the bacteria *Bacillus* species has frequently been documented; some of the reported species are *B. amyloliquefaciens*, *B. cereus*, *B. laterosporus*, *B. licheniformis*, *B. silvestris* and *B. subtilis* (Mondol et al., 2013; Santhi et al., 2017). These compounds (polyketides, peptides and fatty acids) have shown antimicrobial, antifungal, antibiofilm, antifouling, anticancer, antialgal and bioinsecticidal activities (Baruzzi et al., 2011; Hamdache

et al., 2011; Ben Khedher et al., 2011). Notably, the antibiofilm effect of bacteria belonging to *Bacillus* sp. isolated from the marine environment has been reported extensively (Sayem et al., 2011; Satheesh et al., 2012). For instance, in an investigation, Coasta et al. (2018) isolated a bacterium *Bacillus* sp. P34 responsible for the synthesis of the peptide P34 that displayed a strong antibiofilm activity against *Staphylococcus* sp.

In the current study, the compounds present in the bioactive fractions isolated from the symbiotic *Bacillus* sp. were belonging to aromatic acids, fatty acids and carboxylic acids. The production of some of these compounds by the bacteria has already been documented (Gad et al., 2016; Zhang et al., 2017). Wagner-Dobler et al. (2002) isolated an antibacterial anthranilic acid (yellow tryptanthrin) from the North Sea bacterium (*Flexibacteria*). Sharad et al. (2016) reported the production of two fatty acid derivatives such as octadecenoic acid and cis-3-octadecenoic acid by the marine bacterium *Alcaligenes faecalis* that displayed an antibiofilm activity. Besides bacteria, the bioactive fractions of fungi, plants and animals have been reported to possess some of the compounds that were detected in the bioactive fraction of symbiotic bacteria *Bacillus* sp. Teponno et al. (2017) reported the production of anthranilic acid derivatives that displayed antibacterial activity by the fungus *Dendrothyrium variisporum*. The presence of the compound 2-acetylbenzoic acid was found in the bioactive extract obtained from the sea cucumber *Halothuria atra* (Dhinakaran and Lipton, 2014), while Foo et al. (2015) found the presence of 2-ethylacridine in the fraction of *Piper betle* leaves that displayed antimicrobial activity.

However, the antibiofilm mode of action of the compounds produced by the symbiotic bacteria (*B. firmus*, *B. cereus* and *B. subtilis*) is unknown. Further purification and spectroscopy analysis like nuclear magnetic resonance (NMR) could reveal the responsible antibiofilm agents produced by the symbiotic bacteria *Bacillus* sp.

Hundreds of compounds with antibiofilm properties have been isolated, identified and documented from natural sources such as plants, animals and microbes. The compounds isolated from the natural sources can be applied in the environment in the form of antibiofilm coatings. For the development of antibiofilm coatings, a particular amount of compound is required that can be obtained from renewable resources like microbes. The marine environment has vast number of microorganisms, and these microbes could produce high amount of biologically significant metabolites, especially those that are associated with marine macroorganisms. Therefore, these novel symbiotic microbes could be used as the resources for the production of antibiofilm compounds.

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Authors' contributions

SS and SMJP designed the study. NV performed the experiments. SS and SMJP analysed the data. NV and SS wrote the paper. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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