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Antifouling activities of extracellular polymeric substances produced by marine bacteria associated with the gastropod (*Babylonia* sp.)

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Abstract

Bacteria associated with surfaces have been frequently cited as a potential source for the isolation of bioactive metabolites. In this study, bacteria associated with marine gastropod, *Babylonia* sp. were isolated and screened for antibacterial activity against biofilm-forming bacteria. The antibiofilm and antifouling effect of the selected surface- associated bacterial strains were examined under *in vitro* and *in vivo* conditions. Results showed that the extracellular polymeric substances (EPS) of the bacterial strain CML associated with gastropod species considerably reduced the adhesion of biofilm-forming bacteria on glass coupons. Besides, the antifouling coat prepared by incorporating of this EPS into polyurethane varnish prevented the settlement of biofoulers on test substratum submerged in marine waters. The functional groups present in the EPS were analyzed using FT-IR. The bacterium responsible for the production of the bioactive EPS was identified as *Bacillus subtilis* subsp. by 16S rRNA gene sequencing. More detailed characterization of the identified bioactive EPS could lead to the isolation of a novel natural antifouling product.

Introduction

Biofouling continues to be one of the important problems in the maritime sector, which causes severe problems on the surfaces of the marine living organisms and submerged structures (Almedia *et al*. 2017). Although, highly effective biocides are used as coatings for avoiding the settlement of organisms, TBT (Tributyltin) like biocides has created environmental problems to non-target organisms (Thomas and Brooks 2010). There is a growing need for the effective and eco-friendly antifoulants for marine applications particularly after the ban of TBT based paints; therefore research interests on natural antifoulants has been increased (Satheesh *et al*. 2016).

The marine environment is a rich source of biological and chemical diversity, and also an exceptional reservoir for unique bioactive compounds (Fenical 1993). Therefore, the marine organisms have been placed into focus discovering novel bioactive compounds (Clare 1996). A number of bioactive metabolites have been isolated from marine sources (Kijjoa and Sawangwong 2004; Xu *et al*. 2010). Notably, marine bacteria have been the source for the isolation of many such compounds in medicine,

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industry and agriculture (Bowman 2007). The bacteria associated with marine algae and other organisms are reported to produce a higher percentage of bioactive compounds comparing to free floating bacteria (Zheng *et al.* 2005). Hence, a consequent attention has been paid on marine bacteria associated with the surface of invertebrates for the isolation of novel antifouling compounds (Satheesh *et al.* 2012; Viju *et al.* 2017). The production of compounds such as Giffinisterone B, Lobocompacto and Dicitrinin A etc. by the symbiotic bacteria has been reported (Cho 2012; Cho and Kim 2012). Therefore, the aim of the present study was to identify novel antifouling compound from the bacteria living at the surface of marine molluscs. For the screening, fouling bacteria isolated from the acrylic panel submerged in the Kudankulam coastal water (Satheesh *et al.* 2012) were used as the target. It has been reported that the biofilm formed by such bacteria could act as a cue for the settlement of following macrofouling organisms (Lau *et al.* 2003). Therefore preventing formation of this initial biofilm (microfouling) would also prevent the subsequent macrofouling (Armstrong *et al.* 2000). With this reasoning the selected fouling bacteria have been used for the preliminary screening of extracellular polymeric substances (EPS) for antifouling property. Such study might contribute to knowledge about biofouling and antifouling activities expressed by surface associated bacteria.

Experimental

Collection and isolation of surface associated bacteria

The marine gastropod *Babylonia* sp. collected from Colachel coast (West coast of India) were brought to the laboratory in a sterile plastic container with seawater. The bacteria associated with the gastropod were isolated according to the method described by Viju *et al.* (2016). In brief, the loosely attached organisms on the surface of gastropod were removed by washing with sterile seawater. Following this, the bacteria associated with the surface of gastropod were scraped out using a nylon brush and suspended in 1 mL of filter-sterilized seawater. This suspension was serially diluted and spread on agar plate prepared with Zobell marine agar (ZMA). These plates were then incubated for 24 h at 37 °C for the growth of bacterial colonies. The colonies grown were isolated based on the morphology and maintained on slants at 4 °C for further studies.

Isolation of extracellular polymeric substance

The bacterial colonies were inoculated into a beaker containing a 500 mL of Zobell marine broth (ZMB) and placed in a shaker at 37 °C for 72 h of incubation. After that, the culture broth was centrifuged (at 5,000 rpm for 15 min 4 °C) and the supernatant was collected. The collected supernatant was added with adequate amount of ethanol (1 : 1 ratio) and kept in a safe place (overnight) for the precipitation of EPS. The collected precipitate (EPS) was filtered using a membrane filter and diluted in double distilled water. This suspension was then dialyzed using a tubular dialysis membrane (8 kDa) and the obtained fraction was used for further studies (Rajasree *et al.* 2012).

Antimicrobial Assay

Agar disc diffusion method described by Bauer *et al.* (1966) was used to test the antimicrobial activity of the EPS. For this, the sterile disc (6 mm, Himedia, India) was loaded with 50 µL of bacterial EPS and placed on Mueller-Hinton agar plates swabbed with target (biofilm-forming) bacteria such as *Gallionella* sp., *Alteromonas* sp. and *Pseudomonas* sp. These bacteria were isolated from the artificial surfaces immersed in the seawater (Satheesh *et al.* 2012). Control discs loaded with distilled water were also maintained. All the plates were incubated at 37 °C for 48 h. Following incubation, the inhibition zone appeared around the discs were measured. The presented data represent the average of two measured values.

Preparation of bacterial cell suspension for laboratory assays

The biofilm-forming bacteria were grown at 37 °C for overnight in Zobell marine broth. This broth

was then centrifuged at 5,000 rpm for 15 min and the cell pellets obtained were washed with phosphate-buffered saline (10 mL PBS). Following this, the washed pellet was re-suspended in the same buffer in order to obtain OD_{540} = 0.2 and used for the further assay (Jain *et al.* 2007).

Adhesion assay

The bacterial adhesion assay was performed according to the procedure outlined by Rajasree *et al.* (2014). Briefly, the microscopic slides (7.5×2.5 cm) were treated with EPS by placing them in beaker (500 mL) containing seawater (300 mL) and 0.5 mL of EPS. After 24 hours, the slides treated with EPS were taken out and placed into another beaker which contained 300 mL of sterile seawater, 3 mL of ZMB and 3 mL of biofilm-forming bacterial suspension. Following 5 h of incubation, the microscopic slides were retrieved and the bacteria adhered on the slides were stained with crystal violet. The stained bacterial cells were then enumerated under a microscope. The variation between the bacterial cells adhered on the control and EPS treated slides were statistically analysed using Student's *t*-test (Microsoft Excel program).

Influence of surface-associated bacterial extract (EPS) on the EPS production of biofilm-forming bacteria

Five hundred microliter of EPS was taken in a test tube containing 3 mL of biofilm forming bacterial suspension and 3 mL of Zobell Marine broth was added in order to provide the nutrients for bacterial growth. Control tubes were prepared without the addition of bacterial EPS and all the tubes were incubated at 37 °C for 24 h. After that, the culture broth was centrifuged at 5,000 rpm for 5 min at 4 °C and the supernatant was collected and filtered through Whatman No.1 filter paper (0.47 µm). The filtered supernatant was added with equal volume of cold absolute ethanol and allowed for the precipitation of EPS by keeping at room temperature for overnight. The precipitated EPS was isolated and the carbohydrate (Dubois *et al.* 1956) and protein (Lowry *et al.* 1951) content of the EPS were estimated (Viju *et al.* 2013). The obtained data were subjected to Student's *t*-test (Microsoft Excel program) in order to analysis the significant influence of gastropod associated bacterial EPS on the production of carbohydrate and protein by biofilm-forming bacteria.

Preparation of EPS mixed coating for biofilm control (Field assay)

An insoluble matrix antifouling coating was prepared in order to study the antifouling activity of bacterial EPS in the coastal waters. In brief, the commercially available binder polyurethane varnish (Berger) was mixed with the EPS (biocide) at 1 : 1 ratio. This mixture (coating) was coated on fiber glass plates (10 cm×6 cm) using a nylon brush. The varnish coated fiber glass plate was used as a control for the comparison of results. All these plates were fitted on to an iron frame and immersed in to the coastal water for a period of 15 days. After immersion, the plates were taken out and the settlement of biofoulers on the plates was analysed (Viju *et al.* 2014).

Partial purification of the bioactive EPS using Thin-Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC)

A drop of bacterial EPS was placed on a silica gel-G pre-coated glass plate and placed in a TLC chamber containing the mobile phase prepared by mixing benzene, acetic acid and methanol in 1 : 1 : 3 ratio. The movement of the mobile phase on the TLC plate was watched carefully and the plate was taken out once the mobile phase reached the other end of the TLC plate. Following this, the plate was air dried and placed in a glass chamber containing iodine crystal for the visualization of EPS. The spot developed on the TLC plate was scraped off and mixed with adequate amount of distilled water. This mixture was then centrifuged at 5,000 rpm for 15 min and the supernatant was collected. This supernatant was then concentrated and subjected to HPLC analysis. In HPLC, acetonitrile and water (1 : 1) were used as the solvent system and the flow rate was fixed as 1 $mL.min^{-1}$ (Satheesh *et al.* 2012).

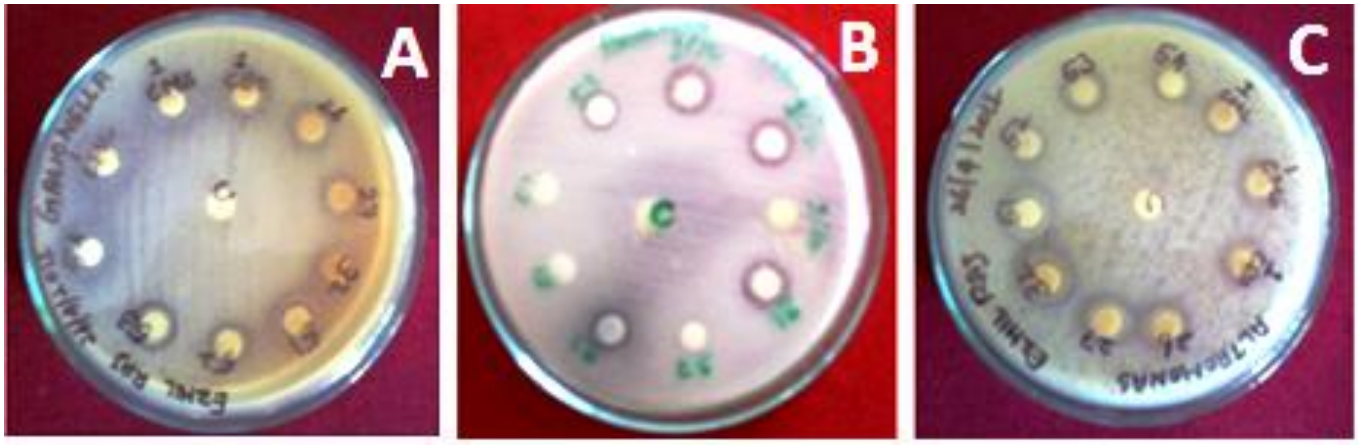

Fig. 1. Antibacterial activity of Extracellular Polymeric Substances from different bacterial strains colonizing the surface of *Babylonia* sp. Activities were tested against biofilm-forming *Pseudomonas* sp. (**A**) *Gallionella* sp. (**B**) and *Alteromonas* sp. (**C**). EPS (50 µL) were loaded on sterile discs on the agar plate swabbed with target bacteria. The inhibitory activity was observed after 24 h of incubation at room temperature.

Analysis of the functional groups present in the EPS using Fourier Transform Infrared (FT-IR) spectroscopy

The FT-IR spectrometer was used to identify the functional groups present in the EPS. A small quantity of TLC purified EPS was placed on the face of a highly polished KBr salt plate and another KBr plate was positioned on the top to spread the compound as a thin layer. The frequencies were measured as wave numbers over the range of 400 to 4,000 cm^{-1} (Viju *et al.* 2017).

Identification of bacteria

The bacterial identification was performed according to the procedure described by Satheesh *et al.* (2012). Briefly, the 16S rRNA of the 24 h old bacterial culture was extracted using phenol chloroform method. The 16S rRNA was amplified by polymerase chain reaction (PCR) using universal primers 16S (F-5`-AGAGTTTGATCCTGGCTCAG-3`) and 16S (R-5`- GGTTACCTTGTTACGACTT-3`). The PCR reaction steps such as denaturation, annealing and extension were maintained at 95 °C, 52 °C and 60 °C for the time duration of 30 sec, 30 sec and 4 min, respectively. The whole procedure was repeated in order to get adequate number of nucleotides (bps). Following this, the obtained PCR product was purified and sequenced using the same PCR primers and other internal primers (F-5´ACAAACAACGTGAAACGTCAA 3´ and R-5´-AAACGAAACACGGAAACTT 3´) to confirm the sequence. The obtained sequence of the bacterial isolate was analyzed using Basic Local Alignment Search Tool (BLAST) and the phylogenetic tree was constructed by comparing the sequences available in NCBI database.

Results

Antimicrobial activity of EPS

Bacteria were isolated from the surface of *Babylonia* sp. and single colonies were picked and cultivated individually. The EPS was isolated from a total of 21 bacterial strains and tested for antibacterial activity against three biofilm-forming bacterial strains *Alteromonas* sp., *Gallionella* sp. and *Pseudomonas* sp., respectively. The assay revealed the growth inhibitory activity of EPS isolated from 10 bacterial isolates against biofilm-forming bacteria (Fig. 1). Notably, the EPS produced by the strains CMS and CML showed maximum zone of inhibition against all the tested bacteria reproducibly in 2 independent tests (Table 1). The former strain CMS was selected and used for further analyses.

Bacterial adhesion assay

The antifouling effect of EPS was first tested in laboratory conditions using glass plates. The EPS of CMS strain considerably reduced the

Table 1. Antibacterial activity of the Extracellular Polymeric Substances (EPS) isolated from the bacterial clones associated with the surface of *Babylonia* sp.

Biofilm-forming bacteria	Zone of growth inhibition [mm]									
	CMS	CML	CBO	26	27	32	51	52	53	54
***Alteromonas* sp.**	12	11	10	10	8	11	9	7	11	10
***Gallionella* sp.**	13	13	10	9	9	8	8	8	9	11
***Pseudomonas* sp.**	12	12	-	9	-	7	-	-	7	-

biofilm bacterial density on the microscopic slides; the number of *Alteromonas* sp. cells adhered to glass surface was significantly reduced upon treatment with EPS (Student's *t*-test, *t* stat = 2.29, $P < 0.041$). Similar restriction of bacterial adhesion was achieved for *Gallionella* sp. (Student's *t*-test, *t* stat = 1.66, $P < 0.085$) and also for the *Pseudomonas* sp. cell density (Student's *t*-test, *t* stat = 3.59, $P < 0.011$) (Fig. 2).

Influence of surface associated bacteria on EPS production in biofilm-forming bacteria

The antifouling agents can have direct effect on biofilm-forming bacteria by means of their growth rate (Fig. 2) or metabolic performance. Therefore, we analysed some metabolic products of previous experiment (Fig. 2). The gastropod associated bacterium CMS considerably reduced the carbohydrate and protein concentration of EPS produced by biofilm-forming bacteria. The carbohydrate concentration of EPS produced by *Alteromonas* sp. culture was significantly reduced (Student's *t*-test, *t* stat = 3.18, $P < 0.016$) after treated with the EPS of the strain CMS. Similarly, there was a significant reduction in the concentration of carbohydrate produced by *Gallionella* sp. culture treated with the EPS of the strain CMS (Student's *t*-test, *t* stat = 14.56, $P < 6.46E^{-05}$). The carbohydrate concentration of EPS produced by the *Pseudomonas* sp. significantly reduced when treated with the EPS of the strain CMS (Student's *t*-test, *t* stat = 2.42, $P < 0.036$). In the same way, the EPS of gastropod associated bacterium CMS substantially reduced the protein content of EPS produced by biofilm-forming bacteria *Alteromonas* sp., *Gallionella* sp. and *Pseudomonas* sp.; the obtained results were statistically significant (Student's *t*-test, *t* stat = 3.10, $P < 0.018$; *t* stat = 2.5, $P < 0.033$ and *t* stat = 4.87, $P < 0.004$, respectively) (Fig. 3).

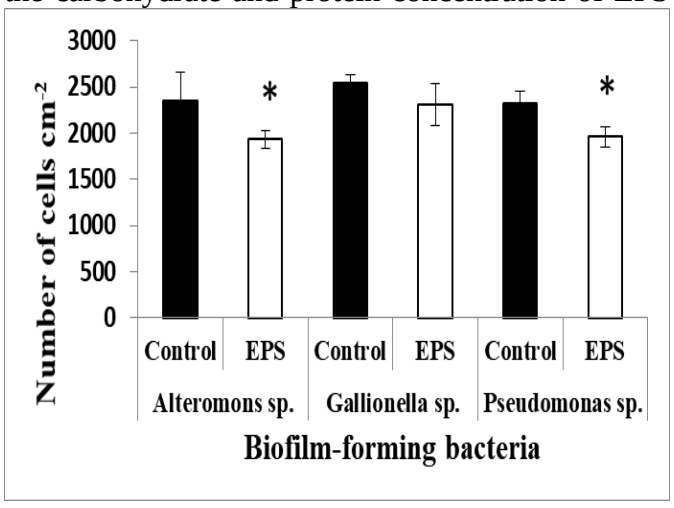

Fig. 2. Influence of surface – associated bacterial EPS on the adhesion of biofilm-forming bacteria. EPS treated glass slides were placed in a beaker containing biofilm bacteria and the slides were retrieved and stained with crystal violet. The number of cells adhered was counted under a microscope. Data presented are average ± standard deviation of three replicates. * indicates significance at $P < 0.05$.

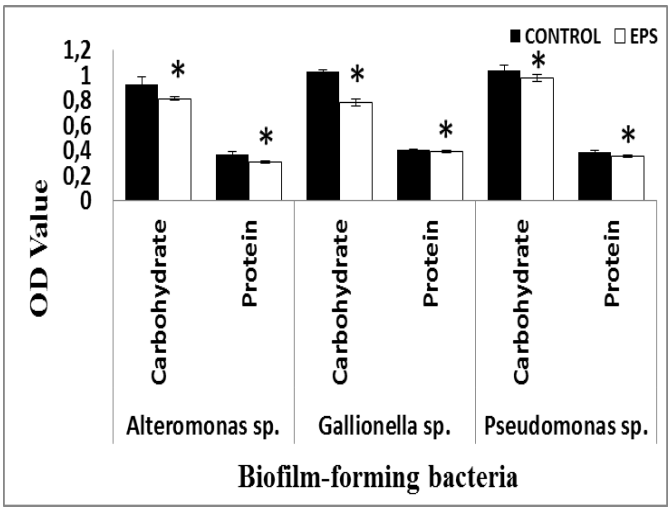

Fig. 3: Carbohydrate and protein concentrations in the EPS produced by biofilm-forming bacteria after treated with the EPS from bacteria associated with the gastropod *Babylonia* sp. Data presented are average ± standard deviation of three replicates. Symbol * above the bars indicate the significant differences at $P < 0.05$.

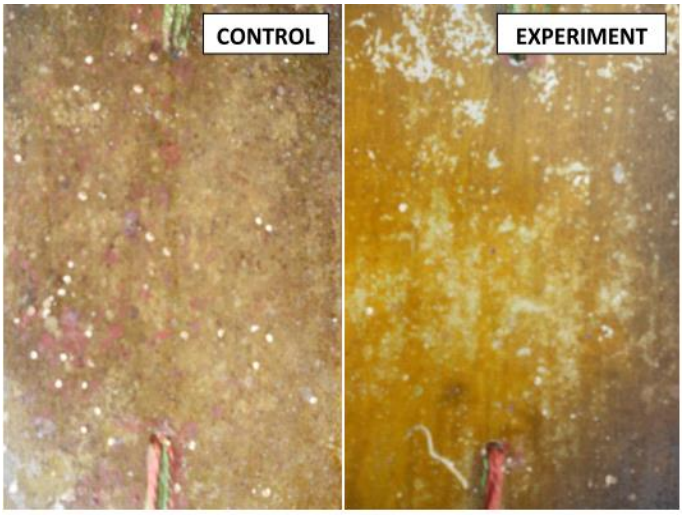

Fig. 4. Antifouling effect of EPS: Control plate was coated with polyurethane varnish and experimental plate was coated with polyurethane varnish and EPS. Both plates were submerged in to the coastal water for a period of 15 days. The growth of biofouling in the plates was observed and it was found that the (experiment) plate coated with EPS considerably reduced the settlement of biofoulers. The control plate coated with polyurethane varnish was completely covered by the biofouling growth.

Preparation of EPS mixed coating for biofilm control (Field assay)

Since laboratory results not necessarily reflect the possible scenarios in a natural (and often much more complex) environment, we tested the antifouling efficiency of antifouling coating prepared with EPS in the coastal water against marine biofoulers using fiber plate as substratum. The fiber plate coated with the EPS of strain CMS considerably inhibited the settlement of marine biofoulers for a short period of 15 days (Fig. 4).

Partial purification of the bioactive EPS using TLC

Thin-layer chromatography was used to separate and partly purify the studied EPS substance from the CMS bacteria. The resulting chromatogram showed a single spot with the *Rf* value of 0.80 cm (Fig. 5). After elution of the compounds into water

Fig. 5. Thin layer chromatography analysis of the EPS isolated from the strain CMS. The sample was placed on silica gel coated plates and the solvent system used was benzene, acetic acid and methanol (1 : 1 : 3).

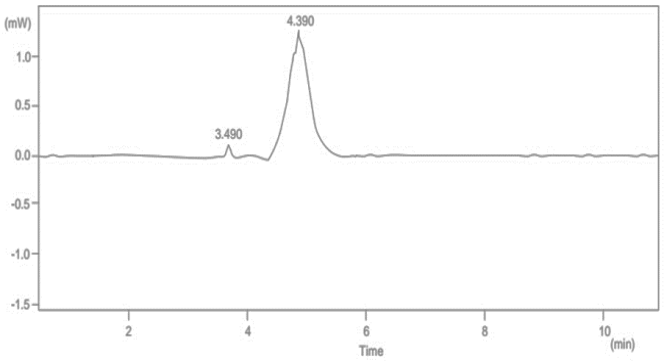

Fig. 6. HPLC spectrum of the EPS fraction resolved through thin-layer chromatography analysis. HPLC analysis was performed using acetonitrile and water (1 : 1) as the solvent system.

and subsequent analysis by HPLC, the corresponding spectrum showed two major peaks at the retention time of 3.490 and 4.390 min respectively. The heights of the peaks were 0.504 and 1.266 mv, respectively (Fig. 6)

Fourier transform infrared (FT-IR) analysis

The EPS may contain the functional groups such as alcohol, carboxylic acid and esters, therefore FT-IR analysis of eluted EPS was performed. The obtained spectrum showed an O-H stretch at 3600 – 3200 and a C-O stretch between 300 - 1000 cm^{-1} indicating the presence of alcohol.

Table 2: Details of the peaks obtained from the HPLC spectrum of EPS isolated from the bacterial strain CMS (*Bacillus subtilis* subsp.).

Peaks	R. Time [min]	Area [mV]	Height [mV]	Area [%]	Height [%]	WOS [min]
1	3.490	193.330	0.504	87.0	28.5	3.76
2	4.390	28.999	1.266	13.0	77.5	0.26
	Total	222.330	1.770	100.0	100.0	

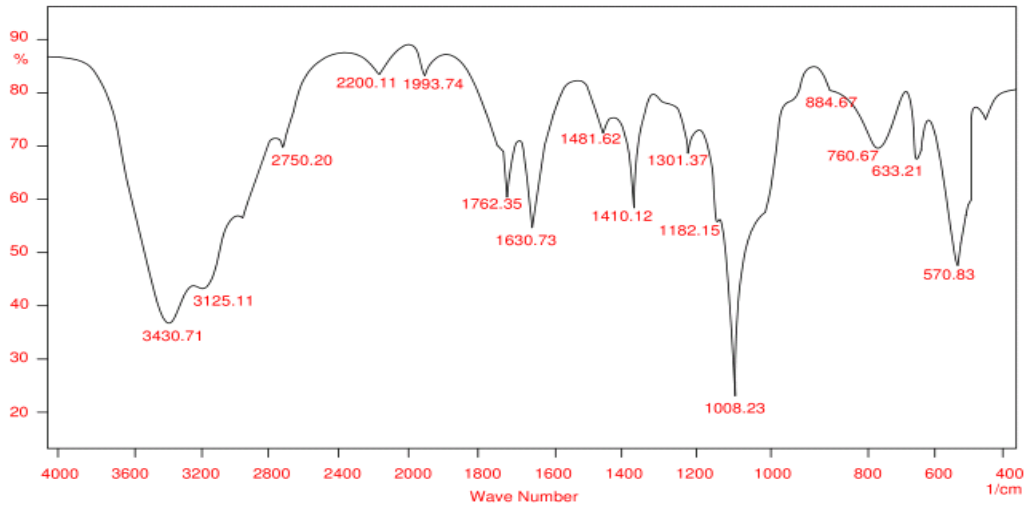

Fig. 7. FT-IR analysis of the EPS isolated from the strain CMS (*Bacillus subtilis* ssp.).

Likewise, a C=O between 1,730 – 1,700, an O-H stretch between 3,400 – 2,400 and a C–O stretch at 1,320 – 1,210 cm^{-1} indicate the presence of carboxylic acid. Besides, a C=O stretch at between 1,740 – 1,715 and a C–O stretch at 1,300 – 100 cm^{-1} indicates the presence of esters. (Fig. 7). It has been reported that the broad peak appeared between 3,600 – 3,200 could indicate the presence of saccharides (Iyer *et al.* 2005) while the peaks appearing between 1,000 – 1,300 could be due to the presence of polysaccharides (Biswas *et al.* 2015).

Identification and phylogenetic analysis of the strain CMS

The gram positive bacterium (CMS) associated with the surface of gastropod was identified by sequencing the 16S rRNA gene. The sequence data of the bacterium CMS was related to the database of *Bacillus subitilis* subsp. available in NCBI with 99 % homology.

Discussion

This study showed that bacteria associated with gastropods possess antagonistic activities against the biofilm-forming bacteria. Ten out of 21 isolates inhibited the growth of biofilm-forming bacteria on the agar plates. It has been reported that bacteria associated with the surface of marine organisms are more pronounced reservoir of bioactive compounds than the bacteria obtained from other sources (Burgess *et al.* 1999). Zheng *et al.* (2005) also confirmed that at least 20 % of bacteria associated with marine invertebrates were likely to produce antibacterial compounds. There are plenty of studies which demonstrate the bioactivity of bacteria associated with marine invertebrates (Shankar *et al.* 2015). For instance, Punitha *et al.* (2013) reported the bioactivity of bacteria associated with the surface of marine gastropod.

Microorganisms naturally have the tendency to attach to surfaces which are considered as one of the initial steps leading to biofilm formation (Costerton *et al*. 1987; Simoes *et al*. 2010). Hence, preventing initial adhesion process of bacteria would disrupt subsequent biofilm formation. Our adhesion assay results showed that the adhesion of biofilm-bacteria on the glass surface was considerably reduced by the EPS isolated from the bacterium CMS (*Bacillus subtilis* subsp.). Generally, bacterial metabolites are reported to possess anti-adhesion or anti-attachment activity against the biofilm-forming bacterial strains. For example, the inhibitory activity of polysaccharide produced by a bacterial species was previously reported by Valle *et al.* (2006). Similarly, biofilm inhibitory activities of EPS produced by surface-associated marine bacteria have been demonstrated by Rajasree *et al.* (2012, 2014) and Viju *et al.* (2016).

Following adhesion, bacteria produce extracellular polymeric substances which are essential for cell-to-cell adhesion (Azedero and Oliveira 2000) cell aggregation (Burdman *et al.* 2000) and biofilm formation (Decho 2000). The EPS is mainly composed of polysaccharides (Fletcher and Floodgate 1973; Costerton *et al*. 1985) and proteins

(Danielsson *et al*. 1977). Hence, to prevent or reduce the biofilm formation on surfaces, it is necessary to screen the natural products targeting the EPS production of bacteria involving in biofilm formation. In this study, EPS isolated from the bacteria associated with the gastropod affected the carbohydrate and protein concentration of the EPS produced by the biofilm-forming bacteria. This observation showed that the metabolite present in the surface-associated bacterial EPS has the potential to reduce the bacterial adhesion on the surface. This observation correlated with a previous study by Satheesh *et al*. (2012) in which the authors reported that the sponge-associated bacterial extract significantly reduced the carbohydrate and protein content of the EPS produced by the biofilm-forming bacteria.

Incorporation of bacterial inhibitory compounds into a polymer matrix is a model system representing a method to discover eco-friendly antifouling coating (Hellio and Yebra 2009). The active compounds could be incorporated into the matrix at a concentration higher than those in the natural environment without altering the physical characteristics of the settlement surface (Henrikson and Pawlik 1995). Previously, Armstrong *et al.* (2000) incorporated the sponge extract into a paint matrix and it was active against barnacle larvae. Similarly, in our study the EPS of strain CMS (*Bacillus subtilis* subsp.) was incorporated into synthetic wood polish (Varnish) and tested for antifouling activity in the coastal waters. Many previous studies have also reported the antifouling performance of coatings developed with the crude bacterial extracts (Satheesh *et al.* 2012; Viju *et al.* 2014, 2017). However, these studies including the present study were conducted for a short period of field trials. Further long-term field studies using the coatings developed with natural products may provide sufficient information on the performance of these compounds in marine waters

Generally, chromatography methods are used for the separation and purification of the organic compounds (Iyyeparaj *et al.* 2014). In this study, TLC and HPLC methods were used for the purification of the EPS. The HPLC chromatogram revealed the presence of minor and major peaks at retention time of 3.490 and 4.390, respectively. These peaks likely correspond to the polysaccharide units (sugars) such as arabinose (3.63), maltose or galactose (4.50 – 5.00). The presence of these sugars (polysaccharide) has been reported in the HPLC spectrum of the exopolysaccharide produced by *Micromonospora* sp. along with respective standards (Ledezma *et al.* 2016). Moreover, the FT-IR spectrum showed the presence of polysaccharides in the purified EPS with the full stretch appeared between 1,000 – 1,300 (Biswas *et al.* 2015). It has been reported that polysaccharides are the major components of EPS (Vu *et al.* 2009) which include homo-polysaccharides (α-D-glucan, *β*-D-glucans, fructans and polygalactan) and heteropolysaccharides (D-glucose, D-galactose, L-rhamnose, N-acetylglucosamine and glucuronic acid) (Ruas-Madiedo *et al.* 2002). The production of polysaccharides by wide variety of bacteria have been reported (Guo *et al.* 2010), which is based on the physiochemical factors such as pH, temperature, and medium composition (Heumann *et al.* 1994). Saccharides are considered as potential antifouling agents (Almedia *et al.* 2017) since their hydrophilic groups have the ability to form hydrogel when they react with water (Piehler *et al.* 1999).

The antifouling activity of hydrogels has been studied extensively and reported ideal antifouling agents (Rasmussen *et al.* 2002). For instance, in a study Katsuyama *et al.* (2002) reported the antifouling activity of polyelectrolyte hydrogels against the spores of seaweed. It is believed that the softness of the hydrogels could be the reason for their antifouling properties, as surface roughness plays major role in biofouling (Petronis *et al.* 2000). However, the exact antifouling mechanism of hydrogels is unclear.

The bacterium responsible for the production of EPS which showed promising results was identified as *Bacillus subtilis* subsp. with 99 % homology (with the sequences available in NCBI GenBank). The genus *Bacillus* is widely distributed in the marine environment and produces a variety of useful products (Arias *et al*. 2003). The species *Bacillus subtilis* is known to produce compounds with biotechnological and biopharmaceutical applications (Stain 2005). For instance, Satheesh *et al.* (2012) reported the antifouling activity of *Bacillus* sp. isolated from the surface of sponge

(*Sigmadocia* sp.). Similarly, Rajasree *et al.* (2012) studied the antifouling activity of *Bacillus* sp. isolated from the surface of seaweed *Sargassum wightii*.

Conclusions

The present study demonstrated the antifouling activity of EPS produced by the marine bacterium *Bacillus subtilis* subsp. in laboratory as well field conditions. Further characterization and confirming the lack of toxicity of the responsible compound in the EPS would promote the isolation of novel eco-friendly antifouling agent from the marine bacterium *Bacillus subtilis* subsp. Moreover, selecting a suitable binder (matrix) for its incorporation to the antifouling coat, as well as long-term field trials of the applied coating would provide further required knowledge for the development of an effective eco-friendly antifouling coating based on marine bacterial metabolites.

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References

Almedia JR, Da-Silva MC, Sousa E, Antunes J, Pinto M, Vasconcelos V, Cunha I (2017) Antifouling potential of Nature inspired sulfated compounds. Sci. Rep. 7: 42424.

Arias P, Dankere C, Liu P, Pilkauskas P (2003) The world banana economy: 1985-2002 FAO. Commodity studies 1 FAO Rome.

Armstrong E, Boyd KG, Burgess JG (2000) Prevention of marine biofouling using natural compounds from marine organisms. Biotechnol. Ann. Rev. 6: 221-241.

Azeredo J, Oliveira R (2000) The role of exopolymer in the attachment of paucimobilis. Biofouling 1: 59-67.

Bauer AW, Perry DM, Kirby WMM (1959) Single disc antibiotic sensitivity testing of *Staphylococci*. AMA Arch. Int. Med. 104: 208-216.

Biswas J, Ganguly J, Paul AK (2015) Partial characterization of an extracellular polysaccharide produced by the moderately halophilic bacterium *Halomonas xianhensis* SUR308. Biofouling 9-10: 735-744.

Bowman JP (2007) Bioactive compound synthetic capacity and ecological significance of marine bacterial genus *Pseudoalteromonas*. Mar. Drugs. 5: 220-241.

Burdman S, Jurkevitch E, Soria-Diaz ME, Gil Serrano AM, Okon Y (2000) Extracellular polysaccharide composition of *Azospirillum brasilense* and its relation with cell aggregation. FEMS Microbiol. Lett. 189: 259-264.

Burgess JG, Jordan EM, Bregu M, Mearns-Spragg A, Boyd KG (1999) Microbial antagonism: a neglected avenue of natural products research. J. Biotechnol. 70: 27-32.

Cho JY (2012) Antifouling steroids isolated from red alga epiphyte filamentous bacterium *Leucothrix mucor*. Fish. Sci. 78: 683-689.

Cho JY, Kim MS (2012) Induction of antifouling diterpene production by *Streptomyces cinnabarinus* PK209 in co-culture with marine-derived *Alteromonas* sp. KNS-16. Biosci. Biotechnol. Biochem. 76: 1849-1854.

Clare AS (1996) Marine natural product antifoulants: Status and potential. Biofouling. 9: 211-229.

Costerton JN, Marrie TJ, Cheng KJ (1985) Phenomena of bacterial adhesion. *In* Savage DS, Flectcher MS (Eds.), Bacterial adhesion, Plenum Press, New York, USA, pp. 3-43.

Costerson JW, Cheng KJ, Geesey GG, Ladd TIM, Nickel JC, Dasgupta M, Marie TJ (1987) Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. 41: 435-464.

Danielsson A, Norkrans B, Bjornsson A (1977) On bacterial adhesion ± the effect of certain enzymes on adhered cells of a marine *Pseudomonas* sp. Bot. Mar. 20: 13-17.

Decho AW (2000) Microbial biofilms in an intertidal system: an overview. Cont. Shelf Res. 20: 1257-1273.

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colometric method for determination of sugars and related substances, Anal. Chem. 28: 350-256.

Fenical AW (1993) Chemical studies of bacteria: developing a new resource. Chem. Rev. 93: 1673-1683.

Fletcher M, Floodgate GD (1973) Electron Microscopic Demonstration Of Acidic Polysaccharide involved in adhesion of a marine bacterium to solid surfaces. J. Gen. Microbiol.74: 325-334.

Guo S, Mao W, Han Y, Zhang X, Yang C, Chen Y, Chen Y, Xu J. Li H, Qi X, Xu J (2010) structural characteristic and antioxidant activities of extracellular polysaccharide produced by marine bacterium *Edwardsiella tarda*. Biores. Technol. 101: 4729 -4732.

Hellio C, Yebra D (2009) Advances in marine antifouling coatings and technologies, Woodhead Publishing Ltd., Cambridge, UK, 762 p.

Henrikson A, Pawlik JRA (1995) A new method of assaying extracts of marine organisms for antifouling properties. J. Exp. Mar. Biol. Ecol. 194: 157-165.

Heumann D, Barras C, Severin A, Glauser MP, Tomasz. A (1994) Gram positive cell walls stimulate synthesis of tumor necrosis factor alpha and interleukin-6 by human monocytes. Infect. Immun. 62: 2715-2721.

Iyapparaj P, Revathi P, Ramasubburayan R, Prajash S, Palavesam A, Immanuel G, Anantharaman P, Sautreau A, Hellio C (2014) Antifouling and toxic properties of the bioactive extracts from the seagrasses *Syringodium isoetifolium* and *Cymodocea serrulata*. Ecotox. Environ. Safe 03: 54-60.

Iyer A, Mody K, Jha B (2005) Characterization of an extracellular polysaccharide produced by marine *Enterobacter cloacae*. Indian J. Exp. Biol. 43: 467-471.

Jain A, Nishad KK, Narayan B (2007) Effect of DNP on the cell surface properties of marine bacteria and its implication for adhesion to surfaces. Biofouling 23: 171-177.

Katsuyama Y, Matsumoto J, Okada T, Ohtsuka Y, Chen L, Okado H, Okamura, Y (2002) Regulations of synaptotagmin gene expression during ascidian embryogenesis. Dev. Biol. 244: 293-304.

Kijjoa A, Sawangwong P (2004) Drugs and cosmetics from the sea. Mar. Drugs. 2: 73-82.

Lau SCK, Harden T, Qian PY (2003) Induction of larval settlement in the serpulid polychaete *Hydroides elegans* (Haswell): Role of bacterial extracellular polymers. Biofouling 19: 197-204.

Ledezma OEV, Méndez HIP, Manjarrez LAM, Cano ETQ, Alvarez NM, López-Luna A (2016) Characterization of extracellular polymeric substances (EPS) produced by marine *Micromonospora* sp. J. Chem. Pharma. Res. 8: 442-451.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Petronis S, Berntsson K, Gold J, Gatenholm P (2000) Design and microstructuring of PDMS surfaces for improved marine biofouling resistance. J. Biomat. Sci. 11: 1051-1072.

Piehler J, Brecht A, Hehl K, Gauglitz G (1999) Protein interactions in covalently attached dextran layers. Colloids Surf. B: Biointerfaces. 13: 325-336.

Punitha SMJ, Viju N, Sharminvini S, Shanker CVS, Micgel Babu M, Citarasu T (2014) Antibacterial activity of bacteria associated with of gastropod. Int. J. Pure Appl. Microbiol. 7: 2879-2884.

Rajasree V, Satheesh S, Vincent SGP (2012) Antifouling activity of a marine epibiotic bacterium from the seaweed *Sargassum wightii*. Thalassas 28: 37-43.

Rajasree V, Shankar CVS, Satheesh S, Punitha SMJ (2014) Biofilm inhibitory activity of extracellular polymeric substance produced by *Exiguobacterium* sp. associated with the polychaete *Platynereis dumerilii.* Thalassas 30: 13-19.

Rasmussen TL, Bäckström D, Heinemeier J, Klitgaard-Kristensen, D, Knutz, PC, Kuijpers A, Lassen S, Thomsen E, Troelstra SR, Van Weering TCE (2002) The Faeroe – Shetland Gateway: Late Quaternary water mass exchange between the Nordic seas and the northeastern Atlantic. Mar. Geol. 188: 165-192.

Ruas-Madiedo P, Hugenholtz J, Zoon P (2002) An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. Int. Dairy 12: 163-171.

Satheesh S, Soniambi A, Shankar CVS, Punitha SMJ (2012) Antifouling Activities of Marine Bacteria Associated with Sponge (*Sigmadocia* sp.). J. Ocean Univ. China. 11: 354-360.

Satheesh S, Ba-Akdah MA, Al-Sofyani AA (2016) Natural antifouling compound production by microbes associated with marine macroorganisms – A review. Elect. J. Biotechnol. 21: 26-35.

Shankar CVS, Satheesh S, Viju N, Punitha SMJ (2015) Antibacterial and biofilm inhibitory activities of bacteria associated with polychaetes. J. Coast. Life. Med. 3: 495-502.

Simones SM, Blankenship JT, Weitz O, Farrell DL, Tamada M, Fernandez-Gonzales R, Zallen JA (2010) Rho-Kinase directs bazooka/Par-3 planar polarity during *Drosophila* axis elongation. Develop. Cell. 19: 377-388.

Stein T (2005) *Bacillus subtilis* antibiotics: structures, synthesis, and specific functions. Mol. Microbiol. 56: 845-857.

Thomas KV, Brooks S (2010) The environmental fate and effects of antifouling paint biocides. Biofouling 26: 73-88.

Valle J, Da Re S, Henry N, Fontaine T, Balestrino D, Latour-Lambert P, Ghigo JM (2006) Broad spectrum biofilm inhibition by a secreted bacterial polysaccharide. Proc. Natl Acad. Sci U. S.A. 103: 12558-12563.

Viju N, Satheesh S, Vincent SGP (2013) Antibiofilm activity of coconut (*Cocos nucifera* Linn.) husk fibre extract, Saudi J. Biol. Sci. 20: 85-91.

Viju N, Anitha A, Vini SS, Shankar CVS, Satheesh S, Punitha SMJ (2014) Antibiofilm activities of extracellular polymeric substances produced by bacterial symbionts of seaweeds. Ind. J. Geo- Mar. Sci. 43: 2136-2146.

Viju N, Satheesh S, Punitha SMJ (2016) Antibiofilm and antifouling activities of extracellular polymeric substances isolated from the bacteria associated with marine gastropod *Turbo* sp. Oceanol. Hydrobiol. St. 45: 11-19.

Viju N, Satheesh S, Punitha SMJ (2017) Antifouling activities of antagonistic marine bacterium *Pseudomonas putida* Associated with an Octopus. Proc. Natl. Acad. Sci., India, Sect. B. Biol. Sci. 87: 1113-1124.

Vu B, Chen M, Crawford RJ, Ivanova AP (2009) Bacterial extracellular polysaccharides involved in biofilm formation. Molecules 14: 2535-2554.

Xu Y, He H, Schultz S, Liu X, Fusetani N, Xiong H, Xiao X, Qian PY (2010) Potent antifouling compounds produced by marine *Streptomyces*. Bioresour. Technol. 10: 1331-1336.

Zheng L, Han X, Chen H, Lin W, Yan LX (2005) Marine bacteria associated with marine macroorganisms: the potential antimicrobial resources. Ann. Microbiol. 55: 119-124.