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Characterization and antifouling activity analysis of extracellular polymeric substances produced by an epibiotic bacterial strain *Kocuria flava* associated with the green macroalga *Ulva lactuca*

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Abstract

Extracellular polymeric substances (EPS) are present externally to the microorganisms and play an important role in attachment and biofilm formation. These polymers possess antibacterial and antifouling activities. In this study, the antifouling activity of EPS produced by an epibiotic bacterium associated with macroalga *Ulva lactuca* was assessed against fouling bacteria and barnacle larvae. Results indicate that the EPS isolated from the epibiotic bacterium inhibits the biofilm formation of the bacteria without much antibacterial activity. Also, the EPS reduced the settlement of barnacle larvae on the hard substrate under laboratory conditions. The epibiotic bacterium was identified as *Kocuria flava* based on 16S rRNA gene sequencing. The EPS was further analysed using Fourier transform infrared (FT-IR), nuclear magnetic resonance (NMR) and X-ray diffraction (XRD) to understand the biochemical composition. NMR analysis revealed the presence of polysaccharides, proteins, acetyl amine and succinyl groups. Scanning electron microscope analysis indicated that the EPS consisted of aggregated and irregular sphere-shaped particles.

Key words: biofouling, antifouling, *Kocuria flava*, exopolymers, antibiofilm activity, Red Sea

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1 Introduction

Many microorganisms are reported to produce extracellular polysaccharides or extracellular polymeric substances (EPS) (Lee et al., 2015; Solmaz et al., 2018). The synthesis of EPS is generally enhanced by environmental stress (Solmaz et al., 2018). Also, during settlement on surfaces, the microorganisms secrete the polymeric substances which enables the attachment process (Costerton et al., 1995) and provides structural framework for the biofilms (Flemming et al., 2007). The EPS secreted by bacteria mainly consists of sugars, proteins, lipids and nucleic acids (Sutherland, 2001; Gu et al., 2017; Powell et al., 2018). Further, the EPS produced by microbial communities play an important role in antimicrobial defence and formation of protective barrier to prevent the desiccation of the cells (Sutherland, 2001; Tian, 2008). The other functions of the EPS include sorption of metals, and organic compounds and enzymatic reactions (Decho, 1990). The EPSs from different microbial sources are widely used in many industrial sectors such as food, textile and pharmaceutical and medical fields (Casillo et al., 2018; Yildiz and Karatas, 2018).

Biofouling is an ongoing problem for marine sectors which incurs economic costs to the shipping, aquaculture and coastal power plants (Schultz et al., 2011; Satheesh et al., 2012). Due to the economic significance associated with this problem, many antifouling measures are currently used by maritime sectors (Satheesh et al., 2016). Previously, antifouling paints containing tributyltin (TBT) as active ingredients were extensively used in all marine installations and maritime sectors. However, TBT is not only affecting the fouling organisms, but also other non-target organisms and the use of TBT based antifouling paints for marine applications was banned by International Maritime Organization in 2008 (Satheesh et al., 2016). Various alternative antifouling formulations are suggested after the TBT restrictions, but most of them are based on herbicides or fungicides (Maréchal and Hellio, 2009). These compounds also pose environmental threats in many parts of the world (Thomas and Brooks, 2010). Natural products mainly from marine organisms are extensively screened for antifouling activities and few successful formulations are developed (Qian et al., 2013; Satheesh et al., 2016). The natural products are generally considered as non-toxic or less toxic and hence can be used as ecofriendly antifoulant (Almeida et al., 2017). Bioprospecting of marine microbes for antifouling compounds is preferred over other marine organisms due to biodiversity concern for getting raw materials for compound extraction (Yang et al., 2007).

The EPS produced by microorganisms are known to have antibiofilm and antifouling properties (Sayem et al., 2011; Rajasree et al., 2014; Viju et al., 2016; Wu et al., 2016). These biopolymers have some unique properties such as biodegradability, nontoxic and absence of secondary pollution product formation (More et al., 2014; Caruso et al., 2018). Due to these properties, microbial EPS may work as an effective environment-friendly antifouling compound. While antifouling activities of marine microorgan-

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isms are studied by many researchers (Wang et al., 2017), the antifouling defence role of EPS produced by the epibiotic bacterial communities received little attention (Camacho-Chab et al., 2016). Hence in this study, the antifouling activities of EPSs produced by an epibiotic bacterium associated with the macroalgae *Ulva lactuca* were assessed to find eco-friendly antifouling compounds from marine microorganisms. Also, the isolated EPS was characterized to understand the biochemical composition. Results obtained in this study will improve our knowledge on the EPS producing bacterial strains associated with marine macroorganisms and provide insights into their biotechnological potentials.

2 Materials and methods

2.1 *Collection of macroalgae and isolation of epibiotic bacteria*

The green macroalgal species *U. lactuca* Linnaeus was collected from the Obhur Creek (21°42.551′N, 39°5.763′E) of the central Red Sea, Saudi Arabia. The alga sample was kept in a sterile container with filtered seawater and brought to the laboratory. In the laboratory, the alga sample was rinsed with filtered (Millipore, 0.47 μm) and sterilized (autoclaved) seawater to remove the debris and other epifauna. After that, the bacterial communities associated with the algal surface were isolated by a cotton swab. The cotton swab was put in filtered and sterilized seawater in a test tube, whirled for 5 min and serially diluted. An aliquot of dilution (10^6 dilutions) was spread on marine agar (difco) plates. The plates were maintained at 37°C for 24–48 h and the colonies developed on the plates were purified by the streak plate method. One yellow coloured bacteria colony was dominant in the culture plate and selected for EPS isolation. The purified colony of this strain was maintained on agar slant at 4°C for further studies.

2.2 *Identification of the bacterial strain*

The genomic DNA was isolated using InstaGene$^{\text{TM}}$ kit according to the protocol supplied by the company. The isolated DNA was amplified in a thermocycler (MJ Research Peltier) using universal 16S rRNA gene primers for bacteria, 27F: AGAGTTTGATCMTGGCTCAG and 1492R: TACGGYTACCTTGTTACGACTT. The polymerase chain reaction (PCR) conditions and sequencing reactions were reported previously (Balqadi et al., 2018). The resulting sequences were aligned and analysed using the basic local alignment search tool of National Center for Biotechnology Information (NCBI BLAST) for the identification of bacteria.

2.3 *Isolation of EPS from the bacterial strain*

The EPS was extracted from the epibiotic bacteria culture according to the methods described by Zhang et al. (2013) with little modifications. In brief, the bacteria strain was cultured in marine nutrient broth (difco) at 25°C for 3 d in a shaker. The EPS was extracted through centrifugation of bacterial culture at 15 000 times gravity at 4°C for 30 min. The supernatant was collected after discarding the pellets. The collected supernatant was filtered using cellulose nitrate filters (0.2 μm). After that, the EPS was precipitated from the filtrate by adding an equal volume of cold ethanol and kept in a refrigerator at 4°C for 48 h. The precipitated crude EPS was collected from the medium and washed with double distilled water and freeze-dried for further characterization and bioassay. Before antifouling assays, the salt content of the EPS was removed by dissolving the EPS in distilled water (double distilled) and dialysed (membrane size 0.1 MDa) against distilled water for 48 h. After dialyzing, the water content of the EPS was removed under vacuum evaporation (rotary evaporator) and lyophilized. This lyophilized EPS was used for antifouling assays against fouling bacteria and barnacle larva. The stock solution of EPS for the experiments was prepared in filtered (0.47 μm) and sterilized seawater at a concentration of 1 mg/mL.

2.4 *Analysis protein and carbohydrate contents of the EPS*

The protein content of the EPS was measured after adding 12% trichloroacetic acid according to the method described by Jiao et al. (2010). The carbohydrate concentration of the EPS was quantified by phenol-sulphuric acid method using glucose as standard (Dubois et al., 1951).

2.5 *Fourier transform infrared spectroscopy (FT-IR) analysis*

There was 3 mg lyophilized EPS sample pressed in KBr pellets and the spectrum was recorded in the wavelength range of 400–4 000 cm^{-1} with a resolution of 4 cm^{-1} using FT-IR spectrometer (Perkin Elmer, USA) (Kavita et al., 2014).

2.6 *^{1}H NMR proton nuclear magnetic resonance analysis*

There was 20 mg lyophilized EPS sample dissolved in 750 μL of deuterium oxide (D_2O) and kept in 5 mm outer diameter NMR tube. The NMR spectrum was recorded at 330 K using a JEOL NMR 500 spectrometer. The ^{1}H NMR spectrum was obtained by recording 1 000 scans with a recycle delay time of 3 s. The recorded chemical shifts of the EPS was expressed in relation to the internal standard, 4, 4-dimethyl-4-silapentane-1-sulfonic acid (Gonzalez-Gil et al., 2015).

2.7 *X-Ray diffraction (XRD) analysis of EPS*

The EPS was analysed by a multipurpose X-ray diffraction (XRD) apparatus (Rigaku Ultima IV XRD) using 2θ ranging from 2° to 80° at 25°C. The operating conditions described by Dogan et al. (2015) was used for the XRD analysis of the EPS. The lyophilized EPS sample from the epibiotic bacterial strain was fixed on a quartz substrate, and the instrument was operated on continuous scan step time of 1 s. The intensity peaks of diffracted X-rays from the sample were recorded, and the d-spacings corresponding to the diffracted X-rays at each θ value were calculated using the Bragg's law:

$$d = \lambda/2\sin\theta, \tag{1}$$

where d is the interplanar distance, λ is the incident wavelength, θ is the scattering angle measured from the incident beam.

The crystallinity index (CI_{XRD}) of the EPS was calculated from the ratio of the crystalline phases of the peak areas to the sum of the crystalline peaks areas and the amorphous profiles (Ricou et al., 2005):

$$\text{CI}_{\text{XRD}} = \Sigma A_{\text{crystal}}/(\Sigma A_{\text{crystal}} + \Sigma A_{\text{amorphous}}). \tag{2}$$

2.8 *Scanning electron microscopy analysis (SEM)*

The surface morphology of EPS was analysed using scanning electron microscopy (VEGA3 TESCAN) with an accelerating voltage of 20 kV and 10 mm working distance. The lyophilised EPS sample was mounted on the metal and gold sputtered prior to the imaging. SEM micrographs were captured at a higher magnification of 500×, 2 000× and 5 000× (Kanamarlapudi and Muddada, 2017).

2.9 *Antifouling activity assays*

2.9.1 *Bacteria growth inhibition assay—turbidity measurement method using spectrophotometer*

Three biofilm-forming bacterial strains, such as *Pseudoalteromonas shioyasakiensis* (NCBI GenBank accession number: KY224086), *Vibrio harveyi* (NCBI GenBank accession number: KY266820) and *Planomicrobium* sp. (NCBI GenBank accession number: KY224087), were used as target microorganisms to test the growth inhibiting and antibiofilm activity of the EPS. These biofilm-forming bacteria were isolated from the nylon nets submerged in the Obhur Creek (Balqadi et al., 2018). The overnight grown biofilm-forming bacteria cultures were adjusted to an optical density of 0.4 at 600 nm for maintaining the equal number of bacteria. About 3 mL of bacterial culture was taken in test tubes and different concentrations (10 μg/mL, 25 μg/mL, 50 μg/mL, 75 μg/mL, 100 μg/mL, 125 μg/mL and 150 μg/mL) of EPS was added. Control tubes (without EPS treatment) were maintained without EPS treatment. The optical density of the bacteria culture was measured immediately after the addition of EPS at 670 nm in a spectrophotometer. The tubes were kept at 29°C in an incubator for 5 h. After 5 h, the optical density of the cultures was measured again. The percentage of bacterial growth inhibition was calculated using the following formula:

$$R = \frac{L - I}{I} \times 100\%, \qquad (3)$$

where R is growth rate, L is final optical density value, I is initial optical density value.

2.9.2 *Antibiofilm activity of the EPS*

The antibiofilm activity of EPS extracted from the bacteria associated with macroalgae was tested against the fouling bacterial strains maintained in the marine laboratory of King Abdulaziz University (KAU) at Obhur. The microtitre plate assay described by Coffey and Anderson (2014) was used for the antibiofilm activity test. In brief, 96 well microtitre plates were filled with overnight grown biofilm bacterial culture (100 μL) and different concentrations (10 μg/mL, 25 μg/mL, 50 μg/mL, 75 μg/mL, 100 μg/mL, 125 μg/mL and 150 μg/mL) of EPS were added to the plates. Controls were maintained without EPS treatment. The microtitre plates were maintained at 37°C for 24 h in dark conditions. After 24 h, the fouling bacterial culture was decanted from the plates and rinsed with sterile water (Millipore filtered and autoclaved). After that, 150 μL of 1% crystal violet was added to each well and kept for 10 min. The stain was removed by inverting the plates and washed with sterile water. Finally, glacial acetic acid (150 μL) was added to the wells and the optical density was measured at 630 nm.

2.9.3 *Barnacle larval settlement assay*

Adults of barnacle *Amphibalanus amphitrite* were collected from the Obhur Creek and maintained in glass tanks in the laboratory. A mixed diet containing *Artemia* nauplii and microalga (*Tetraselmis* sp.) was given to the barnacles. The nauplii released by the adults were collected using a net and light source and transferred to small jars. The nauplii were given microalgal diet (*Tetraselmis* sp.) and maintained in 16 h:8 h light-dark cycle under mild aeration. The nauplii were reared up to cypris (settlement stage) for anti-settlement assays and stage III for toxicity studies. To assess the toxicity of EPS against the barnacle larva, the stage III nauplii were transferred to 6 well plates (10 individuals in each well) containing filtered seawater. After that, the EPS was added to the wells and maintained at 28°C under dark conditions. Four different concentrations of EPS (250 μg/L, 500 μg/L, 750 μg/L and 1 000 μg/L) were used in this study to test the larval toxicity. The control was maintained without EPS treatment. The mortality of nauplii in each well was checked after every 12 h. This experiment was continued up to 96 h (in replicates, n=6) with the exchange of water and EPS in every 24 h.

For the anti-settlement assay, Petri dishes (polystyrene) were used as substratum. The Petri dishes were filled with filtered seawater (5 mL), cyprids (10 in each dish) were transferred to the dish and four different concentrations of EPS (250 μg/L, 500 μg/L, 750 μg/L and 1 000 μg/L) were added. The control dishes were maintained without EPS addition. The Petri dishes were maintained at 28°C under dark conditions. The number of larvae settled on each dish was counted after every 12 h by placing the dish under a stereomicroscope (Leica S6E). This experiment was also continued up to 96 h (in replicates, n=6), and the mean percentage of the settlement for each concentration was calculated.

2.9.4 *Statistical analysis*

One-way ANOVA (P<0.05 was considered as significant) was used to understand the variation in biofilm reduction due to EPS treatment using different concentrations of EPS as a factor. One-way ANOVA was also used to find the difference in survival and settlement of barnacle larvae due to the treatment of EPS. Post-hoc Tukey Test was conducted for the antibiofilm assay and cyprid settlement assay. LC_{50} (the concentration at which 50% of mortality was observed) value for the EPS against barnacle nauplii was calculated using the probit analysis. Also, EC_{50} (effective concentration for 50% of settlement inhibition) value was calculated from the cyprid settlement data using the regression line (Salama et al., 2018).

3 Results and discussion

3.1 *Identification of the bacterium*

The bacterium isolated from the alga *U. lactuca* was identified as *Kocuria flava* based on the phylogenetic analysis. This strain was designated as *K. flava* KAU-MB are the 16S rRNA gene sequence was submitted to NCBI GenBank (accession number: MN381105). The genus *Kocuria* is coming under the class *Actinobacteria*, which is one of the important bacterial groups as nearly 50% of the bioactive metabolites from the microbial origin are reported from this group (Bérdy, 2005). Further, *Kocuria* is considered as a source of novel thiazolyl peptide antibiotic kocurin (Palomo et al., 2013). While many studies highlighted the bioactivity of *Kocuria* strains isolated from different sources (Shiyamala et al., 2014; Bibi et al., 2018; Elbendary et al., 2018), the biotechnological potentials of the EPS are not studied in detail. In a previous study, Mallick et al. (2018) reported that *K. flava* could produce a large amount of EPS under salt stress. Results obtained in this study further widened the bioactivity of the EPS produced by the bacteria *K. flava*.

3.2 *FT-IR analysis*

The EPS isolated from the *K. flava* was completely soluble in seawater under laboratory temperature conditions (28–30°C). Biochemical composition of the EPS isolated from *K. flava* revealed that carbohydrates and proteins were the major components. The total protein and carbohydrate contents of the EPS are 56 μg/mg and 120 μg/mg, respectively. The FT-IR spectrum of the EPS was presented in Fig. 1. The FT-IR spectrum of the EPS revealed the presence of a prominent absorption peak (broad absorption peak) at 3 409 cm^{-1} which indicated the presence of O-H stretching vibration peak (Wang et al., 2018). Peaks were also observed around 2 927 cm^{-1}, 1 629 cm^{-1}, 1 149 cm^{-1} and 615 cm^{-1}. The peak at 2 927 cm^{-1} indicated the presence of the CH_2 group (Wang et al., 2018). The FT-IR peak observed at 1 629 cm^{-1} indicated the presence of alkene group (C=C stretch). The peak observed around 1 149 cm^{-1} revealed the presence of monosaccharides in the EPS (Badireddy et al., 2008; Wang et al., 2018). Specifically, peaks around 1 100 cm^{-1} in FT-IR spectrum of the EPS might indicate the presence of monosaccharides in pyran form (Wang et al., 2018). The peak around 615 cm^{-1} could be assigned to the presence of glycosidic linkage groups between the glycosyl groups (Rani et al., 2017).

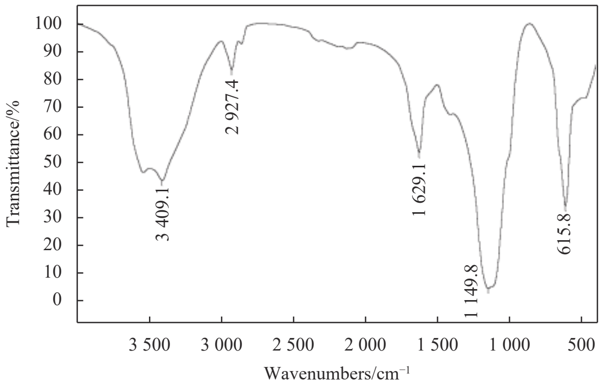

Fig. 1. Fourier transform infrared spectrum of the extracellular polymeric substances isolated from the bacterial strain *K. flava*.

3.3 *¹H NMR analysis*

The ^1H NMR spectrum of exopolysaccharides in DMSO was shown in Fig. 2. The ^1H NMR spectral peaks of EPS were observed at 1.21×10^{-6}, 2.06×10^{-6}, 2.48×10^{-6}, 2.49×10^{-6}, 2.94×10^{-6}, 3.20×10^{-6}, 3.27×10^{-6}, 3.40×10^{-6} – 3.64×10^{-6}, 4.47×10^{-6}. The peaks around 2.48×10^{-6} – 2.49×10^{-6} were attributed to alkyl region. The proton shift at 3.40×10^{-6} – 3.64×10^{-6} revealed the presence of sugar ring resonances. The peak at 4.47×10^{-6} was ascribed to the anomeric proton region. Additionally, the peaks below 3.2×10^{-6} indicated the presence of proteins, acetyl and succinyl groups. The chemical shift at 3.4×10^{-6} was attributed to polysaccharides, proteins, acetyl and succinyl groups (Gonzalez-Gil et al., 2015). The spectral shift at 2.06×10^{-6} and 4.47×10^{-6} showed the presence of N-acetyl glucosamine and N-acetyl lactosamine (Vliegenthart et al., 1981; Liu et al., 2011).

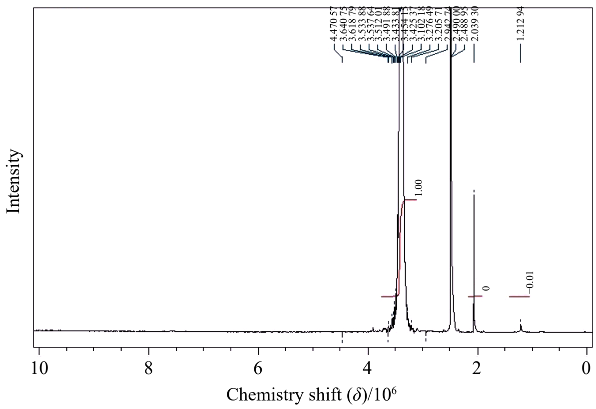

Fig. 2. The proton NMR (^1H NMR) spectrum of the extracellular polymeric substances.

3.4 *XRD analysis of EPS*

The XRD spectrum (Fig. 3) of the EPS with their respective inter-planar spacings (d-spacings) indicated the crystalline nature of exopolymers. The XRD spectral patterns of EPS were attributed to the amorphous characteristics, with a crystalline phase. The crystalline index (CI_{XRD}) of extracted EPS was calculated for the determination of crystalline and amorphous phase, and the values were found as 11% and 89%, respectively. XRD analysis indicated that the EPS produced by the *K. flava* was amorphous as it showed less CI_{XRD}. Previous studies also reported the amorphous structure of bacterial exopolymers (Kavita et al., 2011; Solmaz et al., 2018).

3.5 *SEM analysis of EPS*

The SEM images of EPS clearly described the compact nature of exopolymers (Fig. 4). The exopolymer obtained from *K. flava*

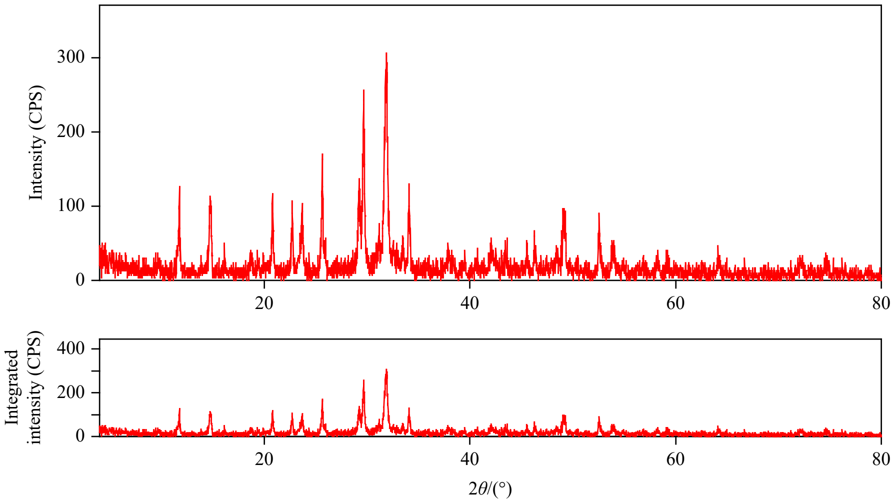

Fig. 3. X-ray diffraction analysis of the extracellular polymeric substances isolated from the bacterium *K. flava*. θ is the scattering angle measured from the incident beam; CPS represents counts per second.

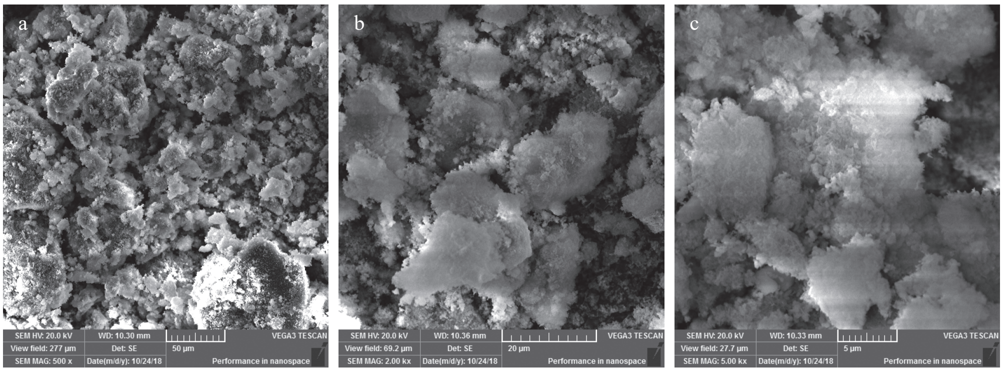

Fig. 4. Scanning electron microscope (SEM) analysis of the extracellular polymeric substances. a. SEM image under 500× magnification; b. SEM image under 2 000× magnification; c. SEM image under 5 000× magnification.

strain KAU-MB consisted of aggregated and irregular sphere-shaped particles. Upon higher magnification (5 000×), the coarse surface with irregular lumps of different size was visible (Fig. 4). Previous studies on the SEM analysis of EPS isolated from the *Lactobacillus* indicated highly compact porous web-like structure (Yadav et al., 2011; Wang et al., 2015). The irregular coarse shaped structure of the EPS of the bacterium *Streptococcus thermophilus* isolated from the milk was also previously reported by Kanamarlapudi and Muddada (2017).

3.6 *Antifouling activity of the EPS*

Results revealed that the EPS isolated from the strain did not affect the growth of the biofilm-forming bacteria significantly (Fig. 5). The growth was considerably reduced on the cultures treated with a higher concentration of EPS (higher than 100 μg/mL) particularly against *V. harveyi* and *Planomicrobium* sp. The EPS of the epibiotic bacterium revealed the biofilm inhibition activity in the microtitre plate assay. Concentration dependent biofilm growth inhibition was observed against all the three biofilm-forming bacteria strains used in this study (Fig. 6). One-way ANOVA indicated a significant variation in the biofilm reduction with different concentrations of EPS against *P. shioyasakiensis* (when F represents F statistic value; df represemts degrees of freedom; F=59.27; df=7, 16; P<0.05), *V. harveyi* (F=38.17; df=7, 16; P<0.05) and *Planomicrobium* sp. (F=7.31; df=7, 16; P<0.05). Post-hoc Tukey Test indicated that *P. shioyasakiensis* culture treated with 10 μg/mL, 25 μg/mL, 50 μg/mL, 75 μg/mL and 100 μg/mL of EPS differed significantly with the control (Table 1). However, higher concentrations (125 μg/mL and 150 μg/mL) of EPS treated *P. shioyasakiensis* did not show significant difference from the control. For *V. harveyi,* significant variation was observed between control and groups treated with 10 μg/mL, 25 μg/mL, 125 μg/mL and 150 μg/mL of EPS. Further, significant reduction in biofilm formation was observed only between the *Planomicrobium* sp. treated with 25 μg/mL and control (Table 1).

The survival of barnacle nauplii was affected by the higher concentrations of the EPS (750 μg/L and 1 000 μg/L). The control and other two treatment groups (250 μg/L and 500 μg/L) showed more than 90% of larval survival after 96 h (Fig. 7). The 96 h LC_{50} value for the EPS was calculated as 1 817.46 μg/L. Moreover, one-way ANOVA indicated a significant difference in the survival of nauplii treated with different concentrations of EPS (F=13.49, df=4, 29, P<0.05). The settlement of cyprid larva of barnacle *A. amphitrite* was considerably reduced after treated with different concentrations of EPS. In the control groups, 91% of cyprids settled on the Petri dishes after 96 h. The cyprids treated with EPS showed a reduction in the settlement with 80%, 55%, 50% and 43% for 250 μg/L, 500 μg/L, 750 μg/L and 1 000 μg/L treatment, respectively (Fig. 7). Results also indicated that the EPS inhibited

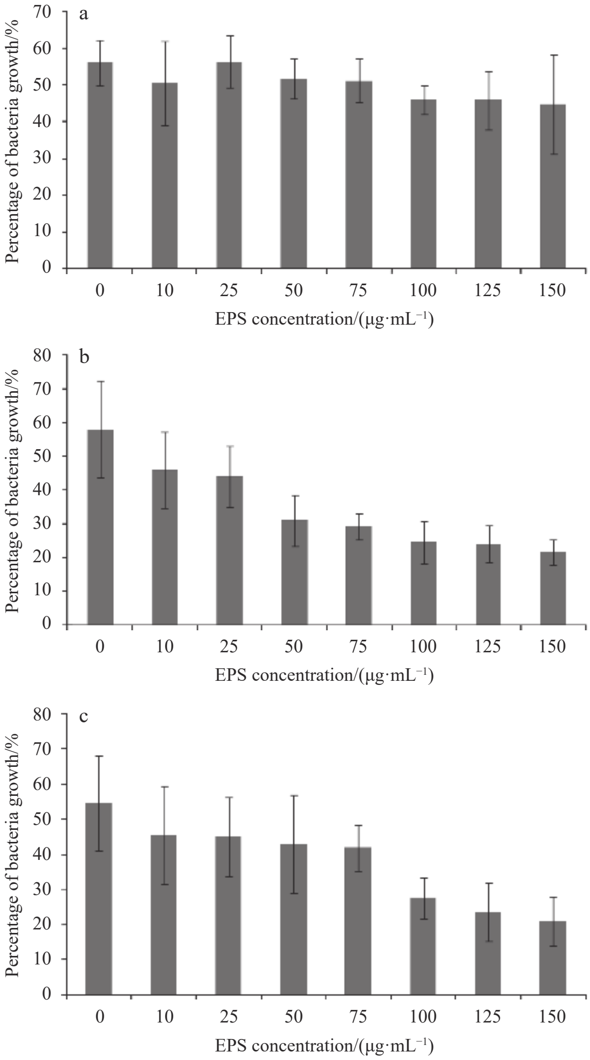

Fig. 5. Bacterial growth inhibiting activity of the extracellular polymeric substances (EPS). The EPS was tested against three biofilm-forming bacteria isolated from the hard substrates. a. Activity against *Pseudoalteromonas shioyasakiensis*; b. activity against *Vibrio harveyi*; c. activity against *Planomicrobium* sp.

the barnacle larval settlement at a moderate EC_{50} value of 748.54 μg/L. One-way ANOVA revealed a significant variation in the settlement of larvae between the different concentrations of EPS (F=25.21, df=4.29, P<0.05). Further, the settlement of cyprids treated with EPS showed significant difference with control groups except those treated with 250 μg/L (post-hoc Tukey Test, Table 2).

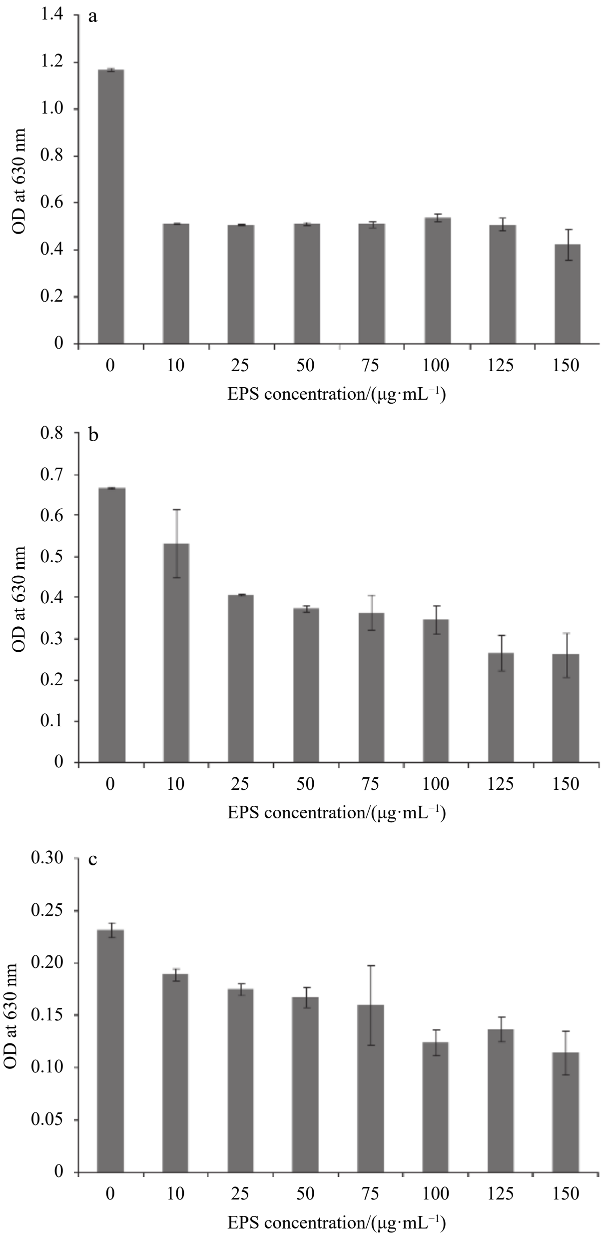

Fig. 6. Antibiofilm activity of the extracellular polymeric substances (EPS) isolated from the epibiotic bacterial strain *K. flava*. a. Activity against *Pseudoalteromonas shioyasakiensis*; b. activity against *Vibrio harveyi*; c. activity against *Planomicrobium* sp. OD represents optical density.

EPSs from microbial sources are gaining importance over other biopolymers due to the applications in different fields (Angelina and Vijayendra, 2015). Considering the significance of biofilm formation in later stages of biofouling (Satheesh et al., 2016), it is important to search compounds which could prevent or control the biofilm formation on the surfaces. The compounds which interfere with the attachment of biofilm-forming bacteria may serve as a potential antifoulant (Camacho-Chab et al., 2016). In this study, the EPS isolated from the strain *K. flava* reduced the biofilm formation by three predominant biofilm-forming bacteria. While the actual mechanism of biofilm reduction needs to be studied, the EPS affected the attachment of bacteria on surfaces. The reduction may be due to the presence of polysaccharides or proteins. For instance, Hwang et al. (2012) reported that the presence of protein in the EPS might have responsible for the inhibition of bacterial adhesion on surfaces. The antibiofilm and antifouling activities of the EPS observed in this study were in parallel with the results of the previous studies (Sayem et al., 2011; Pradeepa et al., 2016; Wu et al., 2016). For instance, the EPS isolated from the biofilm-forming bacterium *Pseudoaltermonas ulvae* showed antibiofilm activity against marine bacteria (Brian-Jaisson et al., 2016). Also, the EPS isolated from the marine bacteria *Vibrio* sp. showed antibiofilm activity without antibacterial activity against different bacterial strains belonging to both Gram-negative and Gram-positive bacteria (Jiang et al., 2011). Another study by Kavita et al. (2014) reported the antibiofilm activity of the EPS isolated from the bacteria *Oceanobacillus iheyensis* against the pathogenic strain *Staphylococcus aureus*.

The barnacle nauplii survival test of this study indicated that the EPS did not affect the barnacle larval survival at low concentration. The survival rate was reduced at higher EPS concentration. However, inhibition of barnacle larval settlement was observed even at low EPS concentrations. The EC_{50} value observed in this study confirmed that the EPS isolated from the bacterium *K. flava* could inhibit the settlement of bacteria and barnacle larvae at low concentrations without causing much toxicity to the fouling organisms. Ideally, a good antifoulant should prevent biofouling growth at low concentration to avoid environmental concerns or toxicity to non-target organisms (Salama et al., 2018). While many natural antifouling compounds were reported from the marine organisms including microbial sources (Qian et al., 2013; Satheesh et al., 2016), most of these compounds are tested under laboratory conditions. A previous field study using the EPS isolated from the cyanobacteria as an auxiliary biocide along with

Table 1. Post-hoc Tukey Test between control and different treatment groups of antibiofilm assay

Treatment group 1	Treatment group 2 EPS concentration/(μg·mL⁻¹)	$P_{Pseudoalteromonas\ shioyasakiensis}$	$P_{Vibrio\ harveyi}$	$P_{Planomicrobium\ \mathrm{sp.}}$
Control	10	0.000 1	0.022 4	0.114 5
Control	25	0.000 1	0.000 1	0.017 1
Control	50	0.000 1	0.203 1	0.092 9
Control	75	0.000 1	1.000 0	0.129 5
Control	100	0.000 2	0.324 2	0.999 4
Control	125	0.448 7	0.009 2	0.999 9
Control	150	0.780 8	0.000 8	0.986 6

Note: EPS, extracelluar polymeric substance. P<0.05 is considered as significant.

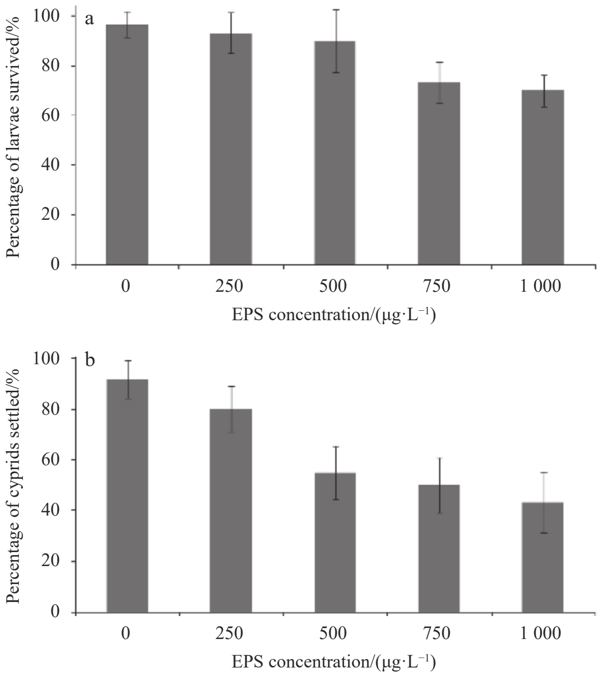

Fig. 7. Antifouling activity of the extracellular polymeric substances (EPS) against barnacle larvae. a. Toxicity of EPS against stage III nauplii; b. anti-settlement activity of EPS against cyprids.

Table 2. Results of post-hoc Tukey Test carried out on the barnacle cyprid settlement between control and different treatment groups

Treatment group 1	Treatment group 2 EPS concentration/(μg·L^{-1})	*P*
Control	250	0.297 7
Control	500	0.000 1
Control	750	0.000 1
Control	1 000	0.000 1

Note: EPS, extracelluar polymeric substances. $P<0.05$ is considered as significant.

copper oxide based paint found promising results which indicate that these biopolymers could be used as potential biocides for the control of biofouling (De Siqueira Melo et al., 2016).

The major advantage of the EPS for antifouling coatings is that they do not have toxic metals or other harmful compounds to the environment (Camacho-Chab et al., 2016). Natural product antifoulants from microorganisms also have some advantages than other marine organisms (Satheesh et al., 2016). Many bacteria may produce good amount EPS under optimum laboratory conditions (Delbarre-Ladrat et al., 2014), and that could negate any product supply constraints for industrial applications. As like other natural products, the use of EPS as an antifouling biocide is not without challenges. Most of the bacterial EPSs are soluble in water (Lembre et al., 2012), and the rate of solubility could affect the stability of the compound for long-term applications. A suitable coating which is biodegradable and controls the release of the biocides for a reasonable period may solve this problem for some extend, but more needs to be investigated under field conditions.

4 Conclusions

To conclude, EPS isolated from the marine epibiotic bacteria strain *K. flava* inhibits the settlement of biofilm-forming bacteria and barnacle larva without antibacterial or antilarval activity at low concentrations. The EPS produced by the *K. flava* was amorphous and consisted of N-acetyl glucosamine and N-acetyl lactosamine, proteins and succinyl groups. While the EPS isolated from the *K. flava* decreases the settlement of bacteria and barnacle larvae, the effect on established biofilms needs to be analysed to use these exopolymers for biofouling control. Previous studies indicated that the biological activities of the EPS were related to the structure and biochemical composition (He et al., 2014). Hence, the structure-dependent antifouling action of exopolysaccharides will also be studied as EPS from many microbial sources showed varying activities against fouling organisms.

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References

Almeida J R, Correia-da-Silva M, Sousa E, et al. 2017. Antifouling potential of nature-inspired sulfated compounds. Scientific Reports, 7: 42424, doi: 10.1038/srep42424

Angelina, Vijayendra S V N. 2015. Microbial biopolymers: the exopolysaccharides. In: Kalia V C, ed. Microbial Factories. New Delhi: Springer, 113–125

Badireddy A R, Korpol B R, Chellam S, et al. 2008. Spectroscopic characterization of extracellular polymeric substances from *Escherichia coli* and *Serratia marcescens*: suppression using sub-inhibitory concentrations of bismuth thiols. Biomacromolecules, 9(11): 3079–3089, doi: 10.1021/bm800600p

Balqadi A A, Salama A J, Satheesh S. 2018. Microfouling development on artificial substrates deployed in the central Red Sea. Oceanologia, 60(2): 219–231, doi: 10.1016/j.oceano.2017.10.006

Bérdy J. 2005. Bioactive microbial metabolites. The Journal of Antibiotics, 58(1): 1–26, doi: 10.1038/ja.2005.1

Bibi F, Naseer M I, Hassan A M, et al. 2018. Diversity and antagonistic potential of bacteria isolated from marine grass *Halodule uninervis*. 3 Biotech, 8(1): 48, doi: 10.1007/s13205-017-1066-1

Brian-Jaisson F, Molmeret M, Fahs A, et al. 2016. Characterization and anti-biofilm activity of extracellular polymeric substances produced by the marine biofilm-forming bacterium *Pseudoalteromonas ulvae* strain TC14. Biofouling, 32(5): 547–560, doi: 10.1080/08927014.2016.1164845

Camacho-Chab J C, Lango-Reynoso F, Castañeda-Chávez M D R, et al. 2016. Implications of extracellular polymeric substance matrices of microbial habitats associated with coastal aquaculture systems. Water, 8(9): 369, doi: 10.3390/w8090369

Caruso C, Rizzo C, Mangano S, et al. 2018. Production and biotechnological potential of extracellular polymeric substances from sponge-associated Antarctic bacteria. Applied and Environmental Microbiology, 84(4): e01624-17, doi: 10.1128/AEM.01624-17

Casillo A, Lanzetta R, Parrilli M, et al. 2018. Exopolysaccharides from marine and marine extremophilic bacteria: structures, properties, ecological roles and applications. Marine Drugs, 16(2): 69, doi: 10.3390/md16020069

Coffey B M, Anderson G G. 2014. Biofilm formation in the 96-well microtiter plate. In: Filloux A, Ramos JL, eds. Pseudomonas Methods and Protocols. New York: Humana Press, 631–641, doi: 10.1007/978-1-4939-0473-0_48

Costerton J W, Lewandowski Z, Caldwell D E, et al. 1995. Microbial biofilms. Annual Review of Microbiology, 49: 711–745, doi: 10.1146/annurev.mi.49.100195.003431

De Siqueira Melo R, Brasil S L D C, De Carvalho L J, et al. 2016. Assessment of the antifouling effect of exopolysaccharides incorporated into copper oxide-based organic paint. International Journal of Electrochemical Science, 11(9): 7750–7763

Decho A W. 1990. Microbial exopolymer secretions in ocean environments: their role(s) in food webs and marine processes. Oceanography and Marine Biology, 28: 73–153

Delbarre-Ladrat C, Sinquin C, Lebellenger L, et al. 2014. Exopolysaccharides produced by marine bacteria and their applications as glycosaminoglycan-like molecules. Frontiers in Chemistry, 2: 85, doi: 10.3389/fchem.2014.00085

Dogan N M, Doganli G A, Dogan G, et al. 2015. Characterization of extracellular polysaccharides (EPS) produced by thermal *Bacillus* and determination of environmental conditions affecting exopolysaccharide production. International Journal of Environmental Research, 9(3): 1107–1116, doi: 10.22059/IJER.2015.998

Dubois M, Gilles K, Hamilton J K, et al. 1951. A colorimetric method for the determination of sugars. Nature, 168(4265): 167, doi: 10.1038/168167a0

Elbendary A A, Hessain A M, El-Hariri M D, et al. 2018. Isolation of antimicrobial producing *Actinobacteria* from soil samples. Saudi Journal of Biological Sciences, 25(1): 44–46, doi: 10.1016/j.sjbs.2017.05.003

Flemming H C, Neu T R, Wozniak D J. 2007. The EPS matrix: the "house of biofilm cells". Journal of Bacteriology, 189(22): 7945–7947, doi: 10.1128/JB.00858-07

Gonzalez-Gil G, Thomas L, Emwas A H, et al. 2015. NMR and MALDI-TOF MS based characterization of exopolysaccharides in anaerobic microbial aggregates from full-scale reactors. Scientific Reports, 5: 14316, doi: 10.1038/srep14316

Gu Di, Jiao Yingchun, Wu Jianan, et al. 2017. Optimization of EPS production and characterization by a halophilic bacterium, *Kocuria rosea* ZJUQH from Chaka Salt Lake with response surface methodology. Molecules, 22(5): 814, doi: 10.3390/molecules22050814

He Jinzhe, Zhang Anqiang, Ru Qiaomei, et al. 2014. Structural characterization of a water-soluble polysaccharide from the fruiting bodies of *Agaricus bisporus*. International Journal of Molecular Sciences, 15(1): 787–797, doi: 10.3390/ijms15010787

Hwang G, Kang S, El-Din M G, et al. 2012. Impact of an extracellular polymeric substance (EPS) precoating on the initial adhesion of *Burkholderia cepacia* and *Pseudomonas aeruginosa*. Biofouling, 28(6): 525–538, doi: 10.1080/08927014.2012.694138

Jiang Peng, Li Jingbao, Han Feng, et al. 2011. Antibiofilm activity of an exopolysaccharide from marine bacterium *Vibrio* sp. QY101. PLoS One, 6(4): e18514, doi: 10.1371/journal.pone.0018514

Jiao Yongqin, Cody G D, Harding A K, et al. 2010. Characterization of extracellular polymeric substances from acidophilic microbial biofilms. Applied and Environmental Microbiology, 76(9): 2916–2922, doi: 10.1128/AEM.02289-09

Kanamarlapudi S L R K, Muddada S. 2017. Characterization of exopolysaccharide produced by *Streptococcus thermophilus* CC30. BioMed Research International, 2017: 4201809, doi: 10.1155/2017/4201809

Kavita K, Mishra A, Jha B. 2011. Isolation and physico-chemical characterisation of extracellular polymeric substances produced by the marine bacterium *Vibrio parahaemolyticus*. Biofouling, 27(3): 309–317, doi: 10.1080/08927014.2011.562605

Kavita K, Singh V K, Mishra A, et al. 2014. Characterisation and antibiofilm activity of extracellular polymeric substances from *Oceanobacillus iheyensis*. Carbohydrate Polymers, 101: 29–35, doi: 10.1016/j.carbpol.2013.08.099

Lee M E, Lee H D, Suh H H. 2015. Production and characterization of extracellular polysaccharide produced by *Pseudomonas* sp. GP32. Journal of Life Science, 25(9): 1027–1035, doi: 10.5352/JLS.2015.25.9.1027

Lembre P, Lorentz C, Di Martino P. 2012. Exopolysaccharides of the biofilm matrix: a complex biophysical world. In: Karunaratne D N, ed. The Complex World of Polysaccharides. Croatia: IntechOpen, 371–392, doi: 10.5772/51213

Liu F C, Su C R, Wu T Y, et al. 2011. Efficient ^{1}H-NMR quantitation and investigation of *N*-acetyl-D-glucosamine (GlcNAc) and *N*,*N*′-diacetylchitobiose (GlcNAc)$_2$ from chitin. International Journal of Molecular Sciences, 12(9): 5828–5843, doi: 10.3390/ijms12095828

Mallick I, Bhattacharyya C, Mukherji S, et al. 2018. Effective rhizoinoculation and biofilm formation by arsenic immobilizing halophilic plant growth promoting bacteria (PGPB) isolated from mangrove rhizosphere: a step towards arsenic rhizoremediation. Science of the Total Environment, 610–611: 1239–1250, doi: 10.1016/j.scitotenv.2017.07.234

Maréchal J P, Hellio C. 2009. Challenges for the development of new non-toxic antifouling solutions. International Journal of Molecular Sciences, 10(11): 4623–4637, doi: 10.3390/ijms10114623

More T T, Yadav J S S, Yan S, et al. 2014. Extracellular polymeric substances of bacteria and their potential environmental applications. Journal of Environmental Management, 144: 1–25, doi: 10.1016/j.jenvman.2014.05.010

Palomo S, González I, De La Cruz M, et al. 2013. Sponge-derived *Kocuria* and *Micrococcus* spp. as sources of the new thiazolyl peptide antibiotic kocurin. Marine Drugs, 11(4): 1071–1086, doi: 10.3390/md11041071

Powell L C, Pritchard M F, Ferguson E L, et al. 2018. Targeted disruption of the extracellular polymeric network of *Pseudomonas aeruginosa* biofilms by alginate oligosaccharides. NPJ Biofilms and Microbiomes, 4: 13, doi: 10.1038/s41522-018-0056-3

Pradeepa, Shetty A D, Matthews K, et al. 2016. Multidrug resistant pathogenic bacterial biofilm inhibition by *Lactobacillus plantarum* exopolysaccharide. Bioactive Carbohydrates and Dietary Fibre, 8(1): 7–14, doi: 10.1016/j.bcdf.2016.06.002

Qian Peiyuan, Chen Lianguo, Xu Ying. 2013. Mini-review: molecular mechanisms of antifouling compounds. Biofouling, 29(4): 381–400, doi: 10.1080/08927014.2013.776546

Rajasree V, Sunjaiy Shankar C V, Satheesh S, et al. 2014. Biofilm inhibitory activity of extracellular polymeric substance produced by *Exiguobacterium* sp. associated with the polychaete *Platynereis dumerilii*. Thalassas, 30(2): 13–19

Rani R P, Anandharaj M, Sabhapathy P, et al. 2017. Physiochemical and biological characterization of novel exopolysaccharide produced by *Bacillus tequilensis* FR9 isolated from chicken. International Journal of Biological Macromolecules, 96: 1–10, doi: 10.1016/j.ijbiomac.2016.11.122

Ricou P, Pinel E, Juhasz N. 2005. Temperature experiments for improved accuracy in the calculation of polyamide-11 crystallinity by X-ray diffraction. In: International Centre for Diffraction Data, ed. Advances in X-ray Analysis, Vol 48. Newtown, Pennsylvania, USA: International Centre for Diffraction Data, 171–175

Salama A J, Satheesh S, Balqadi A A. 2018. Antifouling activities of methanolic extracts of three macroalgal species from the Red Sea. Journal of Applied Phycology, 30(3): 1943–1953, doi: 10.1007/s10811-017-1345-6

Satheesh S, Ba-akdah M A, Al-Sofyani A A. 2016. Natural antifouling compound production by microbes associated with marine macroorganisms—A review. Electronic Journal of Biotechnology, 21: 26–35, doi: 10.1016/j.ejbt.2016.02.002

Satheesh S, Soniamby A R, Shankar C V S, et al. 2012. Antifouling activities of marine bacteria associated with sponge (*Sigmadocia* sp.). Journal of Ocean University of China, 11(3): 354–360, doi: 10.1007/s11802-012-1927-5

Sayem S A, Manzo E, Ciavatta L, et al. 2011. Anti-biofilm activity of an exopolysaccharide from a sponge-associated strain of *Bacillus licheniformis*. Microbial Cell Factories, 10: 74, doi: 10.1186/1475-2859-10-74

Schultz M P, Bendick J A, Holm E R, et al. 2011. Economic impact of biofouling on a naval surface ship. Biofouling, 27(1): 87–98, doi: 10.1080/08927014.2010.542809

Shiyamala D S, Priya P, Sahadevan R. 2014. Pyrrolo [1, 2-A] Pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- and phenol, 2,4-Bis (1,1-dimethy ethyl) novel antibacterial metabolites from a marine *Kocuria* sp. SRS88: optimization and its application in medical cotton gauze cloth against bacterial wound pathogens. International Journal of Pharmaceutical Research and Development, 6(2): 44–55

Solmaz K B, Ozcan Y, Mercan Dogan N, et al. 2018. Characterization and production of extracellular polysaccharides (EPS) by *Bacillus pseudomycoides* U10. Environments, 5(6): 63, doi: 10.3390/environments5060063

Sutherland I W. 2001. Biofilm exopolysaccharides: a strong and sticky framework. Microbiology, 147(1): 3–9, doi: 10.1099/00221287-147-1-3

Thomas K V, Brooks S. 2010. The environmental fate and effects of antifouling paint biocides. Biofouling, 26(1): 73–88, doi: 10.1080/08927010903216564

Tian Yu. 2008. Behaviour of bacterial extracellular polymeric substances from activated sludge: a review. International Journal of Environment and Pollution, 32(1): 78–89, doi: 10.1504/IJEP.2008.016900

Viju N, Satheesh S, Punitha S M J. 2016. Antibiofilm and antifouling activities of extracellular polymeric substances isolated from the bacteria associated with marine gastropod *Turbo* sp. Oceanological and Hydrobiological Studies, 45(1): 11–19, doi: 10.1515/ohs-2016-0002

Vliegenthart J F G, van Halbeek H, Dorland L. 1981. The applicability of 500-mhz high-resolution ^{1}H-NMR spectroscopy for the structure determination of carbohydrates derived from glycoproteins. Pure and Applied Chemistry, 53(1): 45–77, doi: 10.1351/pac198153010045

Wang Chunlei, Fan Qiuping, Zhang Xiaofei, et al. 2018. Isolation, characterization, and pharmaceutical applications of an exopolysaccharide from *Aerococcus Uriaeequi*. Marine Drugs, 16(9): 337, doi: 10.3390/md16090337

Wang Kailing, Wu Zehong, Wang Yu, et al. 2017. Mini-review: antifouling natural products from marine microorganisms and their synthetic analogs. Marine Drugs, 15(9): 266, doi: 10.3390/md15090266

Wang Ji, Zhao Xiao, Tian Zheng, et al. 2015. Characterization of an exopolysaccharide produced by *Lactobacillus plantarum* YW11 isolated from Tibet Kefir. Carbohydrate Polymers, 125: 16–25, doi: 10.1016/j.carbpol.2015.03.003

Wu Shimei, Liu Geihua, Jin Wengyuan, et al. 2016. Antibiofilm and anti-infection of a marine bacterial exopolysaccharide against *Pseudomonas aeruginosa*. Frontiers in Microbiology, 7: 102, doi: 10.3389/fmicb.2016.00102

Yadav V, Prappulla S G, Jha A, et al. 2011. A novel exopolysaccharide from probiotic *Lactobacillus fermentum* CFR 2195: Production, purification and characterization. Biotechnology and Bioengineering, 1(4): 415–421

Yang L H, Miao Li, Lee O O, et al. 2007. Effect of culture conditions on antifouling compound production of a sponge-associated fungus. Applied Microbiology and Biotechnology, 74(6): 1221–1231, doi: 10.1007/s00253-006-0780-0

Yildiz H, Karatas N. 2018. Microbial exopolysaccharides: resources and bioactive properties. Process Biochemistry, 72: 41–46, doi: 10.1016/j.procbio.2018.06.009

Zhang Li, Liu Chunhong, Li Da, et al. 2013. Antioxidant activity of an exopolysaccharide isolated from *Lactobacillus plantarum* C88. International Journal of Biological Macromolecules, 54: 270–275, doi: 10.1016/j.ijbiomac.2012.12.037