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BIOFILM INHIBITORY ACTIVITY OF EXTRACELLULAR POLYMERIC SUBSTANCE PRODUCED BY *Exiguobacterium* sp. ASSOCIATED WITH THE POLYCHAETE *Platynereis dumerilii*

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ABSTRACT

The extracellular polymeric substance (EPS) produced by an epibiotic bacterium isolated from the surface of the polychaete worm *Platynereis dumerilii* was assessed for its inhibitory activity against two biofilm forming bacterial strains. The polychaete *Platynereis dumerilii* was collected from the coastal waters and the bacterial communities associated with the surface was isolated using traditional culture method. The EPS of the bacterial strain strongly inhibited the growth of target bacteria in disc diffusion assay. The adhesion assay showed that the number of cells adhered on the control slides were higher than that the number of cells found on the slides coated with EPS. The bacterial strain was identified as *Exiguobacterium* sp. by 16S rRNA gene sequencing. Thin layer chromatography analysis showed the presence of a single active compound in the EPS with the Rf value of 1.47 cm. The HPLC spectrum of the EPS showed two prominent peaks. Results indicate that epibiotic bacterial communities associated with polychaetes would serves as a potential source for the biofilm inhibitory compounds.

Key words: Biofouling; bioactive compounds; adhesion assay; antifouling; epibiotic microbes; 16S rRNA .

RESUMEN

Se evaluó la actividad inhibitoria sobre dos cepas bacterianas formadoras de biopelículas de la sustancia polimérica extracelular (EPS) producida por una bacteria epibiótica aislada a partir de la superficie del gusano poliqueto *Platynereis dumerilii.* Se recogió el poliqueto *Platynereis dumerilii* en aguas costeras y se aislaron mediante cultivo tradicional las comunidades bacterianas asociadas a su superficie. Los EPS de las cepas bacteriana inhibieron fuertemente el crecimiento de bacterias diana en el ensayo de difusión en disco. El ensayo de adhesión demostró que el número de células adheridas en los portaobjetos de control fueron superiores a que el número de células que se encuentran en los portaobjetos recubiertos con EPS. La cepa bacteriana se identificó como *Exiguobacterium sp*. Mediante la secuenciación genética de rRNA 16S. El análisis por cromatografía en capa fina mostró la presencia de un único compuesto activo en la EPS con valor de Rf = 1,47 cm. El espectro de HPLC de la EPS mostró dos picos prominentes. Los resultados sugieren que las comunidades bacterianas epibióticas asociadas a poliquetos podrían ser una fuente potencial de compuestos antiincrustantes.

Palabras clave: Bioincrustación; compuestos bioactivos; ensayo de adhesión; antincrustantes; microbios epibióticos; 16S rRNA

Figure 1:
Antimicrobial activity of extracellular polymeric substance against Alteromonas sp. *(EPS loaded discs were placed in agar plates in triplicate, C=control).*

Figure 2:
Antimicrobial activity of extracellular polymeric substance against Galionella sp. *(EPS loaded discs were placed in agar plates in triplicate, C=control).*

INTRODUCTION

The adhesion of microbes on surfaces leads to the development of a complex layer called "biofilms". The development of biofilms on hard surfaces submerged in the aquatic medium is the primary step during the biofouling process (Maki 1999). The economic impacts of the biofouling are well known as it affects shipping, offshore oil mining, coastal power plants, mariculture, naval applications and marine constructions. Toxic chemicals used to control the biofouling on hard surfaces submerged in the marine waters leads to pollution and health hazard problems. Bioactive metabolites isolated from certain marine organisms can be used as environmentally acceptable antifouling agents (Clare, 1996). Incorporation of naturally repellent products into antifouling paints has been tried by some researchers (Armstrong *et al*. 2000; Peppiatt *et al*. 2000). Some marine organisms such as corals, algae, sponges, and ascidians have been shown to produce antifouling substances which in nature maintain them free from undesirable encrusting organisms (Dobretsov and Qian, 2002; Harder *et al*. 2003). The bacteria associated with marine invertebrates are reported to have played a key role in the antifouling activity expressed by the hosts. The antifouling activity of bacteria associated with marine invertebrates and seaweeds have been studied by Boyd *et al*. (1999), Egan *et al*. (2000), Dobretsov and Qian (2002), Burgess *et al*. (2003) and Hari *et al*. (2007) Satheesh *et al*. (2012). In the present study, an attempt has been made to screen the biofilm inhibitory activity of extracellular polymeric substances produced by the bacteria associated with polychaetes. Exopolysaccharides or extracellular polymeric substances (EPS) is a term first used by Sutherland (1982) to describe the high molecular weight carbohydrate polymers produced by many marine bacteria. The extracellular polymeric substance produced by many microbes can serve as anti-bacterial components (Gauthier and Flatau, 1986), control bacterial attachment (Fletcher and Floodgate, 1973) and benefit the survival of both the host and other organisms that live in the vicinity of the producer strain (Vincent *et al*. 1994). The major objective of this study was to screen the biofilm inhibitory activity of bacteria associated with polychaetes. A study of this kind will improve our understanding on the bioactive potential of bacteria associated with the polychaetes in marine waters.

MATERIALS AND METHODS

Sampling of polychaetes and isolation of bacteria

Polychaete *Platynereis dumerilii* samples were collected from the surface of the seaweed *Sargassum* sp. inhabiting rocky shores of Kanyakumari coastal waters (West Coast of India). For the collection of the polychaete, a portion of the seaweed *Sargassum* sp. was detached from the substratum and kept in an icebox with sterile seawater. The collected samples were brought to the laboratory for the isolation of associated bacteria. In the laboratory, the polychaete samples were removed from the seaweed by gentle agitation and collected in a jar with seawater. The samples were rinsed with sterile seawater to remove the loosely attached organism and the surface was swabbed with a sterile nylon brush. The bacterial film swabbed

Figure 3:
Thin layer chromatography separation of extracellular polymeric substance produced by Exiguobacterium sp. (triplicate samples were loaded on silica gel plates).

Table 1:
Biochemical and physiological characteristics of the bacterial strain isolated from the surface of Platynereis dumerilii.

S.No	Biochemical test	Result
1	Gram staining	+
2	Morphology	rod
3	Motility	+
4	Gelatin hydrolysis	−
5	Starch hydrolysis	−
6	Catalase activity	+
7	Oxidase activity	+
8	Nitrate reduction	+
9	Citrate utilization	+
10	Indole production	−
11	MR – reaction	−
12	VP –reaction	+
13	Glucose	+
14	Fructose	−
15	Mannitol	+
16	Sorbitol	−
17	Sucrose	−
18	Urease	−
19	TSI test	+
20	H_2S Production	+
21	Gas Production	+

using the brush was dispersed in 1ml filter sterilized seawater (Millipore, 0.45µm). This bacterial suspension was serially diluted and appropriate dilutions were poured on Zobell marine agar (Hi Media, India) plates. The plates were incubated at room temperature for 48 hours and the developed colonies were purified by repeatedly streaking on Zobell marine agar plates. The permanent cultures were maintained in Zobell marine agar slants at 4° C. The isolated bacterial colonies were characterized based on Bergey's manual of determinative bacteriology. The bacterial colonies were initially screened for the antimicrobial activity against biofilm bacterial strains.

Isolation of Extracellular Polymeric substance (EPS)

The bacterial strain which showed antimicrobial activity was inoculated into Zobell marine broth (Hi Media, India) and incubated at 37°C for 3 days. After incubation, the broth was centrifuged at 5000 rpm for 15 minutes. After centrifugation, the supernatant was collected and the pellet was removed. This supernatant was mixed with equal volume of ethanol and kept for 24 hours at room temperature. After 24 hours, the precipitate found in the bottom of the tube was collected and stored at 4° C. The precipitated EPS was filtered through a membrane filter (Millipore, 0.45µm) and diluted with known volume of distilled water.

Antimicrobial assay

The EPS of the marine bacterium was screened for antimicrobial activity using *Gallionella* sp. and *Alteromonas* sp. as test organisms. These organisms were isolated from the biofilm developed on hard surfaces submerged at coastal waters. Antimicrobial activity was assessed by disc diffusion assay. The EPS (20 µl) was loaded on the sterile disc (6 mm, Hi Media, India) and allowed to saturate. The discs were placed onto the agar surface containing the test microorganisms (*Gallionella* sp. and *Alteromonas* sp.). The plates were incubated at room temperature for a period of 24 hours. After the incubation, inhibition zones if any around the paper discs were measured.

Identification of bacterial strain

The bacterial colony which showed strong antimicrobial activity was inoculated in Zobell marine broth and incubated for about 24 h at room temperature. After incubation, the culture was used for DNA extraction as described in Satheesh *et al*. (2012). The 16S rDNA was amplified from the extracted genomic DNA using

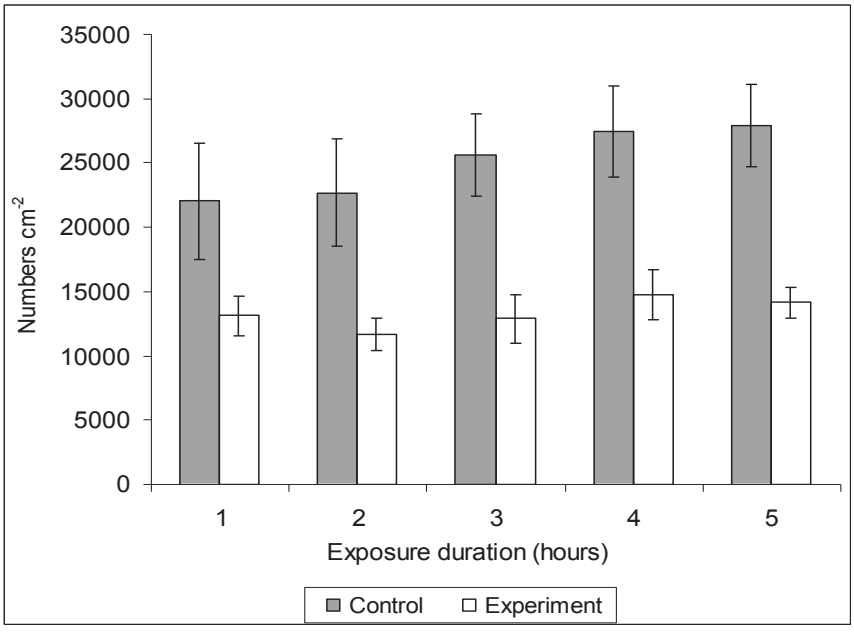

Figure 4:

Results of the adhesion assay using Alteromonas sp. on glass surface conditioned with extracellular polymeric substance (experiment) and control (mean ±standard deviation).

universal 16S rRNA primer The PCR product was sequenced and the sequences were analyzed with sequences in the NCBI database using BLAST and the species level was identified as the nearest phylogenetic neighbor method.

Bacterial adhesion assay

The biofilm bacterial culture broth (*Gallionella* sp. and *Alteromonas* sp.) was centrifuged at 5000 rpm for 15 minutes. The cell pellets obtained after centrifugation was washed with phosphate buffer saline and resuspended in the same buffer to obtain a $O.D_{540}$=0.2. This bacterial cell suspension was used for the adhesion assay. Three millilitres of biofilm bacterial cell suspension was treated with 1ml EPS isolated from the bacterial strain associated with *Platynereis dumerilii*. The biofilm cell suspension was incubated for 24 hours at room temperature. After incubation, 3 ml of bacterial suspension (treated with EPS) was added to 500 ml beaker having 300 ml of sterile seawater. Three millilitres Zobell marine broth was also added to the medium in order to provide nutrition. Five glass slides (microscopic slides, 7.5 x 2.5 cm) were placed inside the beakers in slanting position. The beakers were incubated at room temperature in a sterile chamber. The slides were removed after 1, 2, 3, 4 and 5 hours of incubation (1 slide in each hour). Slides were then air dried, heat fixed and stained with crystal violet. Number of bacteria adhered to the slides were counted under a microscope. The experiment was replicated (n = 6) and the mean values were considered. The experiment carried out using the bacterial cells without EPS treatment was considered as control. Student's 't' test was used to evaluate the effect of extracellular polymeric substances on the adhesion of bacterial cells.

Analysis of extracellular polymeric substance by thin layer chromatography and HPLC

The EPS was characterized by thin layer chromatography. The EPS was loaded on silica gel plates and kept in a TLC chamber for the separation of active compounds. Benzene, acetic acid and methanol (1:1:3) were used as solvent system for the thin layer chromatography.

The distinct spots observed on the thin-layer chromatogram were scraped using a scalpel and kept in separate vials. The compounds were recovered from the silica gel by adding known volume of distilled water and centrifuged at 5000 rpm for 15 minutes. The supernatant was collected and stored at 4°C. The TLC resolved fraction of the EPS was analysed in a HPLC system using acetonitrile and water (50:50) as mobile phase at a flow rate of 1ml min^{-1}.

RESULTS

The biochemical and physiological characteristics of the bacterial strain isolated from the surface of

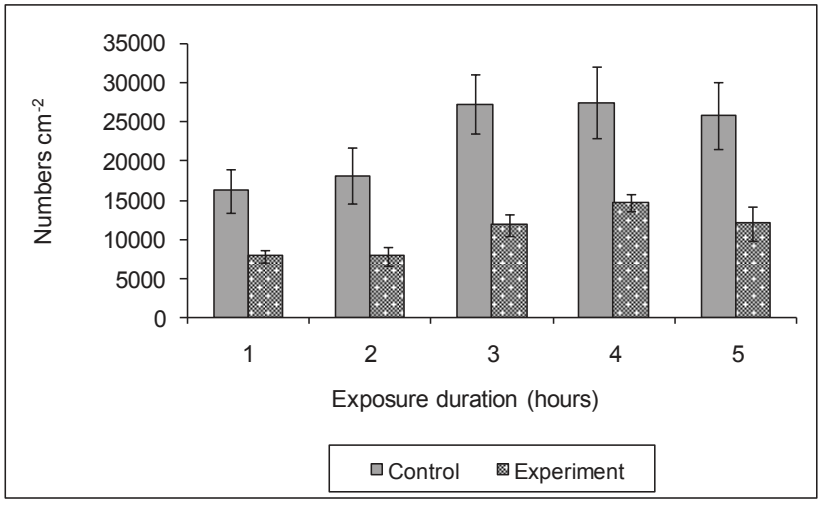

Figure 5:

Adhesion of Gallionella sp. on glass surface conditioned with extracellular polymeric substance (experiment) and control (mean ±standard deviation).

Platynereis dumerilii are given in table 1. The strain was Gram-positive rod and showed motility. It also showed positive results for oxidase activity, catalase activity and nitrate reduction. The bacterial strain was closely related to the species *Exiguobacterium* sp. (99% similarity) based on 16S rRNA sequencing (NCBI GenBank accession number: HM851458). The EPS isolated from the *Exiguobacterium* sp. showed an inhibition zone of 8 mm against *Alteromonas* sp. (Fig. 1) and 10 mm against *Gallionella* sp. (Fig. 2). The EPS was loaded on silica gel plates for the analysis of active compounds. Thin layer chromatogram showed the presence of a single spot in the EPS with the Rf value of 1.47 cm (Fig. 3).

The adhesion assay showed that the extracellular polymeric substance inhibits the adhesion of biofilm bacterial strains on glass surface. The number of *Alteromonas* sp. on the coupons conditioned with the EPS was 13104 cm^{-2} after 1h. The number of cells attached on the slides without EPS was 22048 cm^{-2} after 1h (Fig. 4). After 5 hours of glass coupon submersion, the number of *Alteromonas* sp. cells found on the control coupons was 27872 cm^{-2}. The number of *Alteromonas* sp. cells adhered on the coupons conditioned with EPS was 14144 cm^{-2} after 5 hours. Student's 't' test showed a significant variation in the adhesion of bacterial cells on control and EPS treated coupons ($t=14.09$; $d.f.=4$; $P<0.05$).

The adhesion assay also showed considerable variation in the settlement of *Gallionella* sp. on control and EPS treated coupons. The number of *Gallionella* sp. cells found on the coupons conditioned with EPS was 7904 cm^{-2} after 1 h. On the control coupons, number of *Gallionella* sp. cells adhered was 16224 cm^{-2} after 1 h (Fig. 5). After 5 hours, 12064 cm^{-2} cells were found on the coupons treated with EPS whereas on the control coupons 25792 cm^{-2} were observed. Statistical analysis (Student's 't' test) showed significant variation in the adhesion of *Gallionella* sp. on coupons treated with EPS and control ($t=9.57$; $d.f=4$; $P<0.05$). The HPLC spectrum of the EPS showed two distinct peaks at the retention time of 1.12 and 2.129 min. and four more minor peaks (Fig. 6).

DISCUSSION

Marine benthic organisms are frequently colonized by microbes from the surrounding environment (Mitchell and Chet, 1975; Rublee *et al.* 1980; Ducklow, 1990; Walls *et al.* 1993). The present study indicates that bacteria associated with polychaetes could be used as a potential source for the biofilm inhibitory compounds. The development of biofilm and fouling communities is a multiple event with numerous interactions taking place between fouling organisms colonizing the surface. The first colonizers on any newly exposed surface in marine waters are bacteria and they have been found to affect the subsequent settlement of larval forms of invertebrates. Hence, the adhesion assay using bacteria isolated from the biofilms may be useful for the formulation of natural product based antifouling agents.

In recent years, many studies have been conducted in which diverse substances from different marine animals as well as from their symbionts (e.g., bacteria, zooxanthellae) have been isolated as antifoulants (Dobretsov and Qian, 2002; Burgess *et al.* 2003; Dobretsov *et al.* 2007; Hari *et al.* 2007). Most of the previous studies are related to the bacterial community associated with marine sponges and

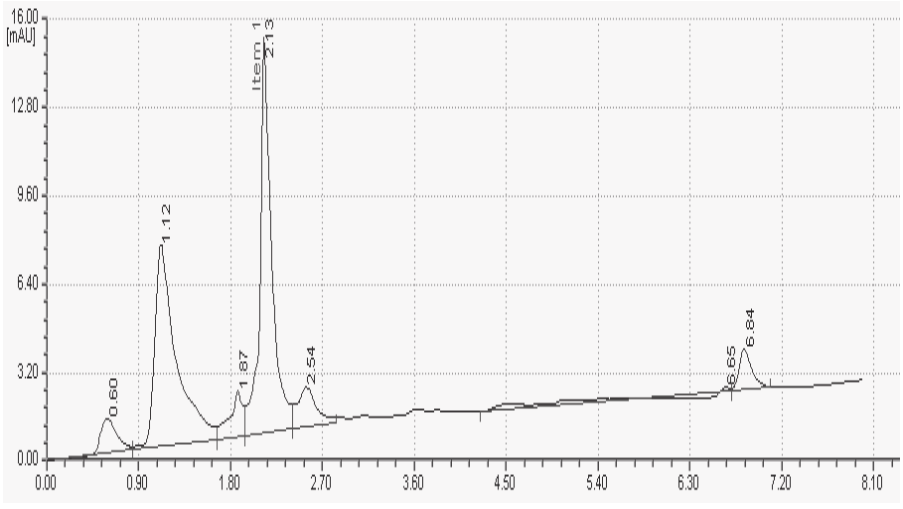

Figure 6:

HPLC spectrum of extracellular polymeric substance isolated from the Exiguobacterium sp.

The HPLC spectrum was obtained using acetonitrile and water (1:1) at the flow rate of 1 ml min^{-1}.

corals (Gnamambal *et al*. 2005; Anand *et al*. 2006). This study expands our knowledge on the ability of bacteria associated with benthic polychaetes to synthesize inhibitory compounds against biofilm development. Bacteria produce a wide range of extracellular polymeric substance composed of polysaccharides, protein, nucleic acids and lipids. Most EPS produced by marine bacteria are heteropolysaccharides consists of three of four different monosaccharides arranged in groups of ten or less to form repeating units (Decho, 1990). EPS can be part of the capsular material that closely surrounds the bacterial cell or released into the surrounding environment as dispersed slime with no obvious association to any one particular cell (Sutherland, 1982; Decho, 1990). In the natural environment, EPS production seems to be essential for survival since most bacteria occur in microbial aggregates whose structural and functional integrity is based on the presence of matrix of extra cellular polymeric substances (Wingender, 1999). Results of the present study showed that EPS produced by *Exiguobacterium* sp. has inhibitory activity against the biofilm forming bacterial strains. This was evidenced from the disc assay and adhesion assay. Some earlier studies have shown that compounds released by the bacteria isolated from the marine organisms repel other bacteria (Boyd *et al*. 1999; Burgess *et al*. 2003). For example, biofilms of the bacteria *Pseudoalteromonas tunicata* isolated from the surface of ascidian showed antifouling activity against larvae of *Balanus amphitrite* and *Ciona intestinalis* (James *et al*. 1996; Holmstrom and Kjelleberg, 1999).

Thin-layer chromatography analysis indicated a single compound in the EPS isolated from the bacterial strain. Analysis of EPS by HPLC showed two prominent peaks indicating the need of further purification of the active compound. In conclusion, results indicate that polychaetes harbour a variety of microbes with antibacterial activity. Since traditional culture method can reveal only a small portion of microbial population in the environmental samples, application of molecular techniques may provide more insights in to the diversity of bacteria associated with polychaetes. Further studies on the chemical characterization of the EPS and evaluation of inhibitory activity against invertebrate larval settlement may improve our knowledge on the antifouling activity of epibiotic microbes associated with polychaetes.

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