Antibiofilm activities of extracellular polymeric substances produced by bacterial symbionts of seaweeds

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Received; revised

In this study, 9 bacterial strains were collected from the surface of seaweeds (Sargassum sp., Gracillaria sp. and Ulva sp.) and screened for their antibacterial activity against three biofilm forming bacteria. All the 9 strains inhibited the growth of biofilm-forming bacteria and the best active strains were selected for in vitro and in vivo biofilm control assays. The results showed that the extract of strain S8 controlled the biofilm formation considerably. The toxicity of bacterial extracts was studied by determining the LC50 value against Artemia sp. The LC50 values obtained were found to be 0.061 and 0.105 mg/ml for the extracts of strain S1 and S8 respectively. The best active strain S8 was identified as Pseudomonas taiwanensis based on 16S rRNA gene sequencing. The bioactive EPS was partially purified by TLC and HPLC analysis. Besides, the functional groups present in the EPS was analysed using FT-IR, which showed the presence of alcohol, alkenes, amines, esters and carboxylic acid groups.

[Keywords: Marine natural products; Surface associated bacteria; Extracellular polymeric substance; Antimicrobial activity; Adhesion; Biofilm; Antifouling]

Introduction

The formation of a film like structure by the exopolymers secreted by the surface colonizing bacteria of submerged structures is referred to as a biofilm. Biofilm is generally thought to be a prerequisite for the attachment and metamorphosis of fouling organisms such as macroalgae and invertebrates. Bacteria are the first colonizers on a clean surface and are the major components of biofilms. The motile bacteria form the initial biofilm which provides chemical cues to promote further settlement of macro fouling organisms. Microbial biofilms and marine biofoulers can cause substantial technical and economic problems on man-made surfaces submerged in seawater. Biofouling leads to increased costs particularly in the shipping industry through the use of higher manpower, fuel, material and dry docking time. Hence, maritime industries are using various antifouling measures for controlling fouling on underwater marine structures to minimize economic loss associated with this problem. Among the various coatings, Self-Polishing Copolymer antifouling paints (SPCs) with tributyl tin (TBT) as biocide was found to be the most preferred option to prevent biofouling until recently. However, this most popular antifouling coating turned out to be highly toxic to the environment by causing shell thickening in oyster population and imposex in gastropods. The consequent global ban on Tri butyl tin (TBT) coatings by the International Maritime Organization triggered the search for natural product antifoulants (NPAs)

Natural marine antifouling compounds seem to be the promising alternative to the toxic chemical antifouling coatings. These products are either non-toxic and exhibit sufficient antifouling properties or may be mildly toxic but biodegradable so that it would not be accumulated in the marine environment. Antifouling compounds have been isolated from several marine organisms such as invertebrates, seaweeds and microorganisms. Marine bacteria are of great interest as novel and rich sources of biologically active products and antibiofilm and antifouling activities have been reported in many surface associated marine bacteria. In this study, biofilm inhibitory activity of the surface colonizing marine bacteria associated with seaweeds was analysed through various bioassays.

Materials and methods

Collection and isolation of surface associated bacteria
The seaweed samples were collected from the Muttom coast (Tamil Nadu, west coast of India) and brought to the laboratory in sterile plastic containers along with the seawater. Samples were gently washed with filter-sterilized (Millipore 0.45 µm) seawater to remove loosely attached organisms. The surface colonizing bacteria on the seaweeds were cotton swabbed and placed on Zobell marine agar plates. The plates were incubated at room temperature for 24 h for the development of colonies. Following the incubation, the colonies were isolated and purified by repeatedly streaking on Zobell marine agar plates. The isolated colonies were maintained on Zobell marine agar slants at 4°C for further tests.

**Isolation of extracellular polymeric substance (EPS)**

To isolate the extracellular polymeric substance, a loop full of bacterial culture was inoculated into the flask containing 100 ml Zobell marine broth and incubated for 72 h. Following incubation, the culture broth was centrifuged at 5000 rpm for 15 minutes at 4°C. The cell pellets were discarded and the supernatant was collected and mixed with equal amount of cold absolute ethanol. After one day of incubation the EPS was collected mixed with distilled water and stored at 4°C.

**Antimicrobial activity Assay**

Disc diffusion method was used to access the antimicrobial activity of the EPS. Bacterial EPS (50µl) was loaded on to sterile filter paper disc (6mm, Himedia, India) and was placed in Mueller-Hinton agar (Himedia) plates swabbed with biofilm-forming bacteria such as Galionella sp., Alteromonas sp. and Pseudomonas sp. Simultaneously, the control discs were loaded with ethanol and maintained for cross reference. All the plates were incubated at 37°C for 48 h. Following the incubation period, the zones of inhibition appearing around the discs were measured.

**Preparation of bacterial cell suspension for laboratory bioassays**

The overnight grown culture broth of biofilm-forming bacteria was centrifuged at 5000 rpm for 15 min. The cell pellets obtained after centrifugation were washed with 10ml of PBS and re-suspended in the same buffer to obtain \( \text{OD}_{540} = 0.2 \). This cell suspension was used for further assay.

**Adhesion assay**

The bacterial adhesion assay was carried out in 500 ml glass beakers using microscopic slides (7.5 cm length, 2.5 cm width) as substratum. Five coupons were placed in slanting position in a beaker containing 300 ml of sterile seawater with 0.5 ml of EPS. The whole setup was kept for 24 hours for the formation of conditioning film on glass coupons. Coupons immersed in sterile seawater (without EPS) were used as control. After 24 hours, the coupons were retrieved from the beaker and transferred to another beaker containing 300 ml of sterile seawater along with 3 ml of biofilm bacterial suspension. Three ml of Zobell marine broth was also added to the medium in order to provide the essential nutrients. The coupons were incubated at room temperature for 5 hours. After the incubation period, the coupons were removed, heat fixed and stained with crystal violet for the enumeration of bacterial cells. Numbers of bacteria adhering to the coupons were counted under microscope.

**Biofilm attachment assay**

The effect of EPS was assessed by determining the viable cell counts. The EPS was mixed with varnish at 1:1 ratio. The mixture was coated on the microscopic slides using a brush and allowed to dry in a sterile chamber. Control plates were prepared by coating varnish without adding EPS. These coated slides were placed in a beaker containing 300 ml of sterile sea water along with 3 ml of biofilm bacterial culture. Three ml of Zobell marine broth was added to the medium in order to provide the essential nutrients. This set up was kept at room temperature for 24 hours. Following the incubation period, the coupons were removed from the beaker and the biofilm developed on the coupons were scrapped using a nylon brush and suspended in 1 ml of sterile seawater. This suspension was serially diluted and spread on Zobell marine agar plates. The plates were incubated at room temperature for 24 hours and the colonies were enumerated using microbial colony counter (LAPIZ, Medica).

**Preparation of antifouling coating for biofilm control (Field assay)**

Polyurethane wood polish was used as the binder and the EPS was used as biocide for the coating preparation. The EPS was mixed with wood polish at 1:1 ratio and the mixture
was coated on a fiber glass plate of 10 cm length and 6 cm width using a nylon brush. Wood polish coated fiber glass plates were used as control. These plates were fitted on an iron frame and were immersed in the coastal water at 1 meter depth using sufficient weights and floats. After 10 days of submersion the frame was removed from the coastal water and the biofilm formation on the control and experimental plates was examined.

**Brine shrimp lethality bioassay**

Brine shrimp lethality bioassay was used to understand the toxicity of bioactive metabolites to marine organisms. *Artemia* sp. eggs (OSI, USA) were hatched in a container filled with sterilized, filtered and aerated sea water and illuminated with light source for 48 hours to get the nauplii. Sea water (50 ml) was taken in 100 ml beakers and the bacterial EPS was added in the different concentrations and *Artemia* nauplii (10) were transferred in every beaker having different concentrations of EPS. The control was also run in parallel without the addition of EPS on the beaker containing seawater and *Artemia* nauplii. The beakers were examined every 6 h and the number of surviving nauplii was counted up to 96 hours. The LC$_{50}$ values were calculated using the plot of probit of mortality percentage against log concentration of the extracts using linear regression analysis (MS Excel version 7) and the LC$_{50}$ value was derived from the best-fit line obtained in the graph.

**Partial purification of the crude EPS using Thin-Layer Chromatography analysis and the antimicrobial activity of the EPS obtained from TLC**

The bioactive bacterial EPS was partially purified by thin layer chromatography. The sample was placed on pre coated silica gel plates using benzene, acetic acid and methanol (1:1:3) as mobile phase and iodine crystal was used as the developer. Spots appearing on the thin layer chromatography were scraped and mixed with equal volume of distilled water. This was vortexed for 5 minutes and centrifuged at 5000 rpm for 15 minutes. The supernatant was collected and used for antimicrobial activity assay against biofilm-forming bacteria.

**High Performance Liquid Chromatography analysis**

The TLC purified EPS of strain S8 was used for HPLC analysis. Acetonitrile and water (50:50) were used as the mobile phase and the retention time was fixed 1 ml / min. Retention time is considered to be a characteristic of the analyte.

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

IR spectra were recorded in a SHIMADZU FT-IR system. A small quantity of TLC resolved EPS fraction 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 in a thin layer.

**Identification of bacteria associated with seaweeds**

The isolated strains were initially identified by standard biochemical test and by observing their morphology under microscope. Finally, the strain which showed strong activity was selected for the molecular level identification.

Pure bacterial strain was cultured in marine broth at 37°C, and the total genomic DNA of the strain was extracted using phenol chloroform method. The 16S rDNA was amplified by polymerase chain reaction (PCR) using primers 16S (5’- AGAGTRTGATCMTYGCTWAC-3’) and 16S (5’- CGYTAMCTTWTTACGRCT-3’). The PCR product was sequenced using the same PCR primers and other internal primers to confirm the sequence. The obtained nucleotide 16S rRNA sequence of the bacterial isolate was analyzed using Basic Local Alignment Search Tool (BLAST) and the phylogenetic tree was constructed using 16S rRNA sequence of others obtained from the NCBI data base.
Table 1: Biochemical and physiological characteristics of the bacterial strains used in the present study

<table>
<thead>
<tr>
<th>Biochemical and Physiological Test</th>
<th>Organisms</th>
<th>Strain</th>
<th>Strain</th>
<th>Strain</th>
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<td></td>
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<td>S2</td>
<td>S3</td>
<td>S7</td>
<td>S8</td>
<td></td>
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<tr>
<td>Morphology</td>
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<td>S1</td>
<td>S2</td>
<td>S3</td>
<td>S7</td>
<td>S8</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
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<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Produce</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Voges</td>
<td>Prousker</td>
<td>Citrate</td>
<td>utilization</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
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<tr>
<td>TSI Agar Test</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>(b) Alkaline</td>
<td>(c) Gas</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>Production</td>
<td>(d) H₂S</td>
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<td>Urea hydrolysis</td>
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</tbody>
</table>

Results

Antibacterial activity of EPS

Nine bacterial strains were isolated from the surface of seaweeds (Gracillaria sp., Sargassum sp. and Ulva sp.) and screened against three biofilm-forming bacteria (Gallionella sp., Alteromonas sp. and Pseudomonas sp.). The results are shown in table 2. All the 9 strains showed activity against target bacteria and the zone of inhibition ranged from 8 to 12 mm. The extract of strain S1 showed the highest zone of inhibition as 12 mm against Gallionella sp. (Table-2).

Table 2: Antimicrobial activity of bacterial EPS against biofilm forming bacteria

<table>
<thead>
<tr>
<th>Biofilm bacteria</th>
<th>Seaweed associated bacteria</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S7</th>
<th>S8</th>
<th>S9</th>
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</thead>
<tbody>
<tr>
<td>Alteromonas sp.</td>
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<td>8</td>
<td>1</td>
<td>9</td>
<td>9</td>
<td>8</td>
<td>1</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallionella sp.</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>

Bacterial adhesion assay

The adhesion of bacterial cells to glass slides coated with EPS enriched conditioning film was assessed using Gallionella sp., Alteromonas sp., and Pseudomonas sp. as target organisms. This assay showed that low numbers of bacterial cell were found on the slides treated with the EPS of strain S1 and S8 than the control slides. The number of Alteromonas sp. cells adhering on the control slides (without EPS conditioning) showed a density of 2808.5 ± 16.26 cells cm⁻². The coupons conditioned with the EPS of strain S1 and S8 showed a density of 1705 ± 78.58 and 1745 ± 88.45 cells cm⁻² respectively. The number of Pseudomonas sp. cells adhering to control slide was 2842.5 ± 10.60 cells cm⁻² and showed reduced number of cells on the slides conditioned with the EPS of strain S1 and S8 (1885.5 ± 4.94 and 1847.5 ± 5.44 cells cm⁻² respectively). The number of Gallionella sp. cells adhering to the control slides (without EPS conditioning) was 1997.5 ± 53.03 cells cm⁻² and the coupons conditioned with EPS of strain S1 showed a density of 1760 ± 94.04 cells cm⁻². The number of Pseudomonas sp. cells adhering to control slides was 1985.5 ± 4.94 and 1847.5 ± 88.45 cells cm⁻². The bacteria conditioned with EPS of strain S8 showed a density of 1508.3 ± 75.66 cells cm⁻² (Figure-6).

Table 3: Antimicrobial activity of the partially purified EPS using TLC against biofilm forming bacteria

<table>
<thead>
<tr>
<th>Biofilm bacteria</th>
<th>Partially purified EPS of best active strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>S8</td>
</tr>
<tr>
<td>Alteromonas sp.</td>
<td>9</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>10</td>
</tr>
<tr>
<td>Gallionella sp.</td>
<td>9</td>
</tr>
</tbody>
</table>

Biofilm attachment assay

The anti-adhesion effect of antifouling coat prepared by incorporating bacterial EPS with varnish (synthetic wood polish) was assessed by counting the colony formed on the agar plats. The number of viable Alteromonas sp. cells adhering to the varnish coated coupon was 950 ± 114.55 x 10⁵ CFU/ml. The number of Alteromonas sp. adhesion to the coupon coated with the EPS of strain S1 was 508.5 ± 31.81 x 10⁵ CFU/ml and it showed reduced number of cells on the slides conditioned with the EPS of strain S1 and S8 (1985.5 ± 4.94 and 1847.5 ± 5.44 cells cm⁻² respectively). The number of Gallionella sp. cells adhering to the control slides (without EPS conditioning) was 1997.5 ± 53.03 cells cm⁻² and the coupons conditioned with EPS of strain S1 showed a density of 1760 ± 94.04 cells cm⁻². The number of Pseudomonas sp. cells adhering to the control slides was 1985.5 ± 4.94 and 1847.5 ± 88.45 cells cm⁻². The bacteria conditioned with EPS of strain S8 showed a density of 1508.3 ± 75.66 cells cm⁻² (Figure-6).
respectively. The number of *Gallionella* sp. cells adhering to the coupons coated with varnish was $713 \pm 26.87 \times 10^5$ CFU/ml. The coupons coated with EPS of strain S1 and S8 mixed with varnish showed a density of $701 \pm 10.40 \times 10^5$ and $447 \pm 12.72 \times 10^5$ CFU/ml respectively (Figure-7).

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

In this assay, bacterial extract of strain S1 and S8 were coated along with varnish (synthetic wood polish) on fiber glass plates ($12.5 \times 8.5$ cm). Among the two extracts used, plates coated with the EPS of strain S8 considerably inhibited the settlement of marine microfoulers than the plates coated with the EPS of strain S1 (Figure-1).

**Brine shrimp toxicity assay**

In this study, the toxicity of the EPS of strain S1 and S8 to *Artemia* nauplii was assessed by determining the LC$_{50}$ value using the best fit line slope of the graph. The LC$_{50}$ value of the EPS of strain S1 was 0.061 mg/ml and for S8 it was 0.105 mg/ml (Figure-9a and b).

**Partial purification of EPS and antimicrobial activity of the EPS obtained from TLC**

The bioactive EPS was partially purified by silica gel precoated glass plates. The single spot (Figure-2) appearing on the Thin layer chromatogram was collected and tested for its antibacterial activity. The EPS from TLC also showed activity against biofilm forming bacteria. The results are given in Table 3.

**Analysis of EPS by high performance liquid chromatography (HPLC)**

The EPS of active strain S8 showed two peaks on the HPLC spectrum which were observed at the retention time of 1.407 min and 2.160 min respectively. The heights of the 1st and 2nd peaks were 0.558 and 0.293 mv respectively. The maximum height of the peak 0.558 mv was observed at the retention time of 1.407 min (Figure-3).

**Analysis of functional groups present in the EPS using Fourier transform infrared analysis**

Based on the FT-IR spectrum, there is a possibility for the presence of five functional groups such as alcohol, alkenes, carboxylic acid, esters and amines. A broad O-H stretch between 3600-3200 and a C-O stretch at 1114.78 cm$^{-1}$ indicate the presence of an alcohol. Likewise, a C-H stretch between 3200-3010 and a C=C at between 1680-1600 cm$^{-1}$ indicate the presence of alkenes. Moreover, an O-H peak between 3400-2400, a C=O stretch at 1730-1700 and a C-O stretch between 1320-1210 cm$^{-1}$ indicate the carboxylic acid group. Similarly, a N-H stretch between 3500-3300, a N-H bending at 1680-1580 and a C-N stretch at 1350-1000 cm$^{-1}$ indicate the presence of amines. The C=O stretch at 1740-1715 and a C-O stretch at 1300-1000 cm$^{-1}$ revealed the presence of esters (Figure-4).

**Identification and phylogenetic analysis of isolated strains**

Five most active strains from the 9 bacteria isolated from seaweeds were characterized by standard biochemical methods and morphological observations (Table 1). The most active strain was identified based on 16S rRNA gene sequencing. The sequencing data was analyzed using *n* BLAST programme where this sequence was compared with that of available rRNA genes that have been sequenced so far. The phylogenetic analysis showed 99% similarity with *Pseudomonas taiwanensis* (Figure-5). The nucleotide sequence data have been deposited at GenBank (GenBank, NCBI) and the accession number is KF964670.

**Discussion**

The search for natural antifouling compounds has gained urgency because of the economic loss in the marine environment owing to fouling. Marine bacteria can produce a verity of metabolites which inhibit the settlement of biofoulers and natural products from different marine organisms including bacteria, algae, sponges and higher invertebrates have been screened for AF activities$^{14,15}$. All the 9 strains of bacteria isolated from seaweeds in this study showed inhibitory activity with the zone of inhibition ranging from 8 to 12 mm. Lemos *et al.*$^{26}$ too has previously reported the antimicrobial activity of seaweed associated bacteria against fouling bacteria.

Bacterial biofilm formation is a remarkably complex process entailing a range of molecular and physiological events. It is generally considered to proceed through several steps, including adhesion, microcolony formation, and structural maturation$^{27,28}$. Enumeration of bacteria in biofilm is usually performed by microscopy counting after cell...
labelling or by spreading the cells on agar. In our study, the findings from the adhesion assay demonstrate that the glass surfaces conditioned with the extract of strain S1 and S8 considerably inhibited the adhesion of biofilm-forming bacteria. Likewise, biofilm attachment assay result of the cell viable counting assay also revealed that the number of colonies counted in the control plates were higher than the experimental plates. The EPS of strain S8 which showed strong anti-adhesion properties against biofilm forming bacteria giving hope that this strain may be useful useful to develop antibiofouling coatings.

Environment friendly prevention of initial bacterial microfouling can be achieved by applying the paint with natural antifouling compounds having bacterial repellent activity. A paint used in the marine surface to prevent the initial microfouling, especially bacterial, could also prevent further macrofouling. In this study, isolated bacterial EPS which showed strong activity (strain S1 and S8) against biofilm forming bacteria were incorporated with paint matrix (Synthetic wood polish) for the development of NAPs. The results revealed that the EPS of the strain S8 showed strong repellent activity against marine biofouling organisms in short term field trail (10 days). In a reported study, extracts of the sea pansy _Renilla reniformis_, incorporated with commercially available paint and encapsulated in metallic microtubules was effective in controlling biofouling over short periods in the marine environment.

Brine shrimp lethality bioassay is used for the determination of general toxic property of the bioactive extracts and also it is an efficient, rapid and inexpensive test that requires only a relatively small amount of sample. The bioactive compounds are generally considered to be toxic to the marine environment when the concentration is above 1 mg/L. The toxicity of two bacterial extracts (strain S1 and strain S8) against _Artemia_ nauplii and the LC50 values of 0.061 and 0.105 mg/L respectively show that active fraction contained in the EPS could be toxic to the marine organisms at very low concentrations.

There are a number of techniques for the separation, purification and identification of the bioactive compounds. In the present study,
Fig. 2 - (a) Thin layer chromatography analysis of the EPS of strain S_1 (b) Thin layer chromatography analysis of the EPS of strain S_8. The samples were placed on pre coated silica gel plates using benzene, acetic acid and methanol (1:1:3) as mobile phase.

Fig. 3 - HPLC analysis of the EPS of best active strain S_8. Acetonitrile and water (1:1) was used as solvent system.
Fig. 4 - FT-IR analysis of the EPS of best active strain S8.

Fig. 5 - Identification of the best active strain S8 using the 16S rDNA sequences. The 16S rDNA were aligned and used to construct the neighbor-joining phylogenetic tree and it was identified as *Pseudomonas taiwanensis*. 
Fig. 6 - Influence of bacterial EPS on the adhesion of biofilm-forming bacteria on glass slides after 5 hours of immersion.

Fig. 7 - Influence of antifouling coating (Bacterial EPS incorporated with varnish) on the attachment of biofilm-forming bacteria.
TLC and HPLC techniques were used for the partial purification of the bioactive crude EPS of strain S8. The TLC purified EPS showed two major peaks in HPLC at the retention time of 1.407 and 2.160. In addition, the functional groups present in the EPS were analyzed using FT-IR, which showed the presence of alcohol, alkenes, amines, esters and carboxylic acid groups.

The strain which showed strong activity was identified as *Pseudomonas taiwanensis* with 99% homology using 16S rRNA gene analysis. Previously, several antimicrobial and antifouling compounds have been isolated from *Pseudomonas* sp. For example, in a study Kodani et al. isolated the antialgal compound, harmame (1-methyl-b-carboline) from the bacterium, *Pseudomonas sp.* K44–1. Since the extracts have anti-fouling activity in both *in vitro* and *in vivo* bio-assays, a study like this could be a lead for the development of antifouling compound from marine sources and also an active compound isolated from this extract could be incorporated in paint matrix for the development of natural antifouling paints.

**Reference**


Fig. 8 - Determination of LC50 value of EPS isolated from the isolates against brine shrimp nauplii (*Artemia* sp.). (a) Mortality curve for the EPS of S1 (b) Mortality curve for the EPS of S8.


