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Lanosterol expressed bio-fouling inhibition on Gulf of Mannar coast, India

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ABSTRACT

The present study aims to investigate the antifouling efficacy of lanosterol when lanosterol coated PVC sheets were exposed into natural sea water (Tuticorin coastal water, Gulf of Mannar, India) up to one year. The average fouling load on the lanosterol coated PVC coupons were 0.16 kg/m^2 (10 μg), 0.095 kg/m^2 (100 μg) and 0.02 kg/m^2 (1000 μg) recorded from July 2013 to June 2014. Interestingly, up to one year of exposure, there was no significant growth/fouling organisms on lanosterol coated coupons, whereas drastic fouling was observed in control. Thus, the bactericidal chemical compounds could be used as substitutes for anti-fouling compounds owing to their eco-friendly nature. Lanosterol exhibits anti-biofilm activity and anti-quorum sensing activity against dominated micro-fouling bacterial groups and their receptor proteins like *N*-acylhomoserinelactone and oligopeptide.

Results from this study shows early stage micro-fouling bacterial colonies produce allelopathic substance like bacteriocin or antimicrobials which control the biofouling.

1. Introduction

The natural process of biofouling causes serious economic consequences to the marine engineered structures [1,2]. Chemical antifouling paints containing copper and tributyltin (TBT) have proven detrimental effects on the environment [3,4]. The prevailing marine biofouling menace and the added deficit of antifouling compounds have intensified the exploration for novel eco-friendly antifouling agents of biological origin. Such analysis calls for the development of eco-friendlier antifouling compounds. On a relative note, marine bacteria reflect as a rich source of anti-biofilm compounds namely quorum sensing signal molecules, surfactants, enzymes and polysaccharides that function as regulating biofilm architecture [5,6]. These bio-molecules often exhibit broad-spectrum biofilm inhibiting or biofilm detaching activity, when tested against biofilm cultured *in vitro* [7–9]. Qi et al. [10] studied antibacterial and anti-larval compounds from marine bacterium *Pseudomonas rhizosphaerae*. Nithya et al. [11] described the inhibitory effect of marine bacterium *Bacillus pumilus* on biofilm formations. Antifouling compounds namely, 2-hydroxymyristic acid (HMA) and *cis*-9-oleic acid (COA), were isolated from a chloroform extract of the marine pelagic bacterium, *Shewanella oneidensis* SCH0402 [12]. The microfouling prevention was tested by using natural product Kojic acid reveals decreasing biofilm densities [30]. Marine biofilm influences to enhance or inhibition of macrofouling organisms through allelopathic chemical substances that might be used to develop as novel antibiofilm or antifouling composite preparation. In the present study, antifouling compound lanosterol identified from the rare marine biofilm bacterium *Kocuria rosea* strain VBSKNY-01 (Ac. No: KC505190), was incorporated into vinyl resin paint and evaluated for its antifouling property in natural seawater.

2. Materials and methods

2.1. Biofilm development

In this study, Polyvinyl chloride (PVC) sheets were cut in to thicknesses of 12″ × 12″dimension and degreased the sheets using acetone. These sheets were mounted on a wooden rack with the total size of 75″ × 15″ using brass bolt and nut. The rack was kept at < 2 m depth from the mean surface seawater below in the coastal water at ten different stations (Kanyakumari, Mandapam, Rameshwaram, Thondi, Nagapatinum, Cuddalore, Pitchavaram, Pondicherry, Kovalam,

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Chennai (Kasimedu)) along the Tamil Nadu coast, India. Biofilm samples were collected at different intervals: 30 min, 1, 2, 4, 5 and 6 h, respectively. Zobell Marine medium (Hi-Media) was used to count the total number of heterotrophic bacteria in the biofilm using spread plate method. Besides, the average bacterial counts of the replicates were recorded simultaneously. Identification of isolated marine biofilm bacteria was done by phenotypic methods and by 16S rRNA gene sequencing method. Finally, the obtained sequence results were compared with the known bacterial sequences available in the NCBI database using the bioinformatics tool BLAST [13,14].

2.2. Extraction and purification of bioactive secondary metabolites

For the biosynthesis of the secondary metabolites from *K. rosea*, three different culture broths namely Zobell Marine broth, LB medium and Nutrient broth (Hi-media) was used. All three cultures were inoculated with 1 mL of *K. rosea* culture having a 0.1 OD value at 600 nm. The culture media was maintained for 5 days at 28 °C in an orbital shaker (150 rpm) [12]. The secondary metabolites were separated from the culture broth by centrifugation using cooling centrifuge (8000 rpm at 4 °C for 20 min). Then the extraction was carried out by cold steep method using ethanol (99% HPLC grade) at −10 °C. This ethanol extract was concentrated under reduced pressure and subjected to partial purification on silica gel column chromatography (60–120 mesh size 3 × 60 cm, Merk) with *n*-hexane-ethyl acetate (4:6) solvent system as a mobile phase.

2.3. Chemical characterization

The FT-IR spectra (Instrument Model RXI) of *n*-hexane-ethyl acetate (4:6) column fraction extracted from *K. rosea* was pelleted and recorded for functional group identification using KBr. The FTIR spectrum was scanned at the wavelength range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}. The partially purified *n*-hexane-ethyl acetate (4:6) column fraction were separated and analyzed using GC–MS (Model: DSQ, DB 35-MS capillary standard non-polar column, temperature range between 50 °C and 260 °C with an increment of 10 °C/min, Helium, flow: 1.0 mL/min, volume 1 μL). The resultant spectrum was verified via compatibility analysis of the mass spectra of peaks that was presented by Wiley and National Institute Standard and Technology (NIST) library's mass spectral database [5].

2.4. Quorum sensing inhibition

To analyze the quorum sensing inhibition, *N*-acylhomoserinelactone (AHL) and Oligopeptides (OPPA) were retrieved from PDB with the help of ID 2WYC and the hydrophobicity was confirmed by the ProtScale tool employing Kyte and Doolittle method. The identified biofilm bacterial QS proteins such as AHL (*Vibrio* sp.) and OPPA (*Bacillus* sp.) was retrieved from protein data bank (www.rcbs.org/pdb) to use as target receptor site. Moleculor docking study was carried out with the help of Argus Lab software 4.0.1.version to explore the ligand (lanosterol) interactions within biofilm QS proteins [15,16].

2.5. In vitro anti-biofilm activity

The primary screening of antagonistic activity was done by agar diffusion method against micro-fouling bacteria. *In vitro* antibiofilm activity, the overnight cultures of early stage micro-fouling biofilm bacterial groups were diluted to about 1 × 10^{-6} CFU/mL with fresh LB sterile medium (Hi-Media) and were added to the 96-well micro-titer plate containing various concentrations *viz* 10, 20, 40, 60, 80 and 100 μL of column fraction (4:6) and placed on orbital shaker at 150 rpm and without column fraction served as negative control. After 24 h of incubation the biofilm formation was measured using UV–vis absorption spectra (iMark Microplate Reader – Model: S/N 13562) at 595 nm [17].

2.6. Preparation of coating formulation and field evaluation

Vinyl resin was a used as a binder and polyamide were used as a hardener for the preparation of coating using test compound lanosterol (w/v) (CAS No.79-63-0) with a concentration of 10,100, and 1000 μg as antifoulant to a thickness of 75 μm [5,12]. All the coated coupons (size 75″ × 50″ × 2 mm) were allowed to air dry for a week and mounted on a wooden rack and exposed in a natural seawater at the depth of < 2 m below the mean sea level from the CSIR-CECRI's offshore Platform at Tuticorin (08°46′25.15″N lat., 78°11′16.05″E long) Tamil Nadu, India.

3. Results and discussion

3.1. Micro-fouling bacteria

The isolated total biofilm bacterial population density was relatively higher in Kanyakumari coastal biofilm sample (81 ± 1.5/CFU × 10^{-5}/m^2) and lower in Thondi (61 ± 1.5/CFU × 10^{-5}/m^2). The total population density shows no significant variations among the different study locations (Fig. 1). However, the samples collected at 4th, 5th and 6th h, depicted a relatively higher population density at Kanyakumari and lower population density at Thondi. Phenotypic results reveal that Gram-negative genera dominated by Gram-positive genera. The identified genera *Vibrio*, *Pseudomonas*, *Flavobacterium* and *Aeromonas* belonged to Gram-negative groups while the genera identified under Gram-positive groups included *Bacillus*, *Micrococci* and *Kocuria*. The molecular confirmation using 16S rRNA sequence was done for *V. alginolyticus* strain VMB-01 (A.No.: JQ260828), *B. badius* strain VBSCH-01 (A.No.: KF995734), *K. rosea* strain VBSKNY-01 (A.No.: KC505190), *K. flava* strain VBSNAG-01 (A.No.: KF995733) and *E. cloacae* strain VBS-D (A.No.: JX424421). Followed by 16S rRNA sequence analysis, the phylogenetic tree was constructed (Fig. 2). The compositions of micro-fouling bacterial communities in biofilm samples collected during 30 min ~6 h period exhibited marked variations among the locations. Generally, Gram negative bacterial cell wall has more lipo

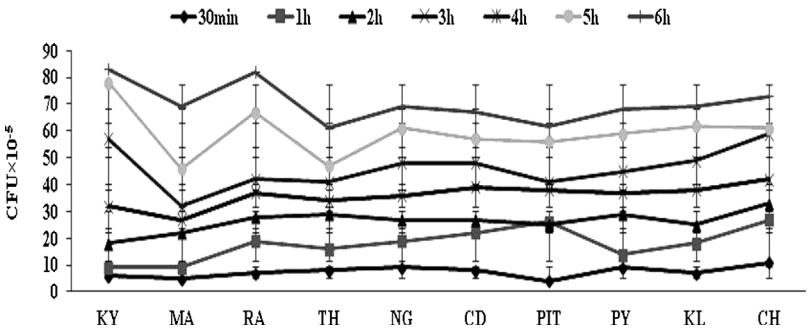

Fig. 1. Total heterotrophic bacterial population densities.