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Research Article

ISOLATION AND IDENTIFICATION OF ANTIFOULANT PRODUCING *KYTOCOCCUS SEDENTARIUS* BY 16S rRNA SEQUENCE AND ITS ROLE IN BIOFOULING ACTIVITY

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ABSTRACT

Development of microbial biofilms on the surfaces of manmade structures in the marine environment cause serious problems for the navies and for marine industries around the world. Marine microbes are promising potential sources of non toxic antifouling compounds as they can produce substances that inhibit not only the attachment of microorganism but also the settlement of invertebrates and macro algal spores. In order to evaluate the antifouling activity two bacterial strains were isolated from ship scrapping. Among that M5 bacterial strain showed remarkable antifouling activity. To investigate the antifouling secondary metabolite from M5 GC-MS analysis were performed and two compounds were identified such as Octadecanoic acid, methyl ester and 1R-(1à,3à,4á,5à)]-1-(Hydroxymethyl)-5-[(diisopropoxyphosphinyl)methyl]c.
In addition M5 strain was subjected to morphological, physiological and genomic identification via, 16S rRNA gene sequencing and it was identified as *Kytococcus sedentarius.* This work suggested that *Kytococcus sedentarius* M5 could produce potent antifoulant compound which is responsible for antifouling activity.

INTRODUCTION

Biofouling is a process of colonizing micro and macro-foulers in the aquatic environment, that affects almost every economic sector from shipping to medicine, causing billions of dollars in damage and disruptions annually and the formation of a biofilm, can be generated by numerous species of microorganisms. But are primarily the creation of bacterial microcolonies that have attached to a surface and shielded themselves in an extracellular matrix of polysaccharide, protein, and nucleic acids (Qian *et al.,* 2007; Beech *et al.,* 2004; Costerton *et al.*, 1995).

The biofouling growth on a substratum in the aquatic environment is a complex and dynamic process, which once established are extremely difficult to eradicate creating extensive problems in marine technology. A long-standing problem for naval and merchant ships has been the increased hydrodynamic drag and decreased maneuverability that is caused by biofouling of ship hulls, which increases fuel consumption and voyage costs (Chambers *et al.,* 2006).

Due to the economic significance of the problem in the marine waters, various control strategies are adopted by the marine sectors. Release of environment-friendly antifouling compounds from an antifouling coating at the minimum effective release rate protects the marine structures succumbing to this destruction fouling.

Tributyltin (TBT) containing antifouling paints were widely used in the commercial vessel to control biofouling (Hu *et al.,* 2006). However use of TBT caused environmental problems as it is more toxic to non-target marine organisms (Alzieu *et al.,* 1986; Hoch *et al.,* 2001). Due to the environmental concern over the use of TBT, the International Maritime Organization and Marine Environment Protection Committee banned the application of TBT for marine applications from January 1, 2008 (Qi *et al.,* 2008). The substitutes of TBT, such as Irgarol 1051 and Diuron, have been found to be harmful to many non-target organisms. One of the organotin compound, Tributyltin oxide (TBTO) is still used as a biocide, and has been used as reference or positive control for the evaluation of antifouling activity of new natural compounds. The other common biocides are 10-β-formamidokalihinol-A and kalihinol A (Yang

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et al., 2006), polymeric 3-alkylpyridinium salts, terpens and pyrrole-imidazole alkaloids, succinic acid (Yang *et al.,* 2007), taurine acid substituted bromopyrrole alkaloids and di-bromophakellin derivatives, 3-phenyl-2-propenoic acid, 2-hydroxymyristic acid and cis-9-oleic acid.

While many marine natural products have proven to be good non-toxic antifouling agents, they are difficult to generate for large-scale uses, such as antifouling coatings. An ideal natural antifouling compound will act different ways on the target organisms. Generally, the compound should prevent the formation of biofilms.

In this context an attempt has been made to identify the microorganisms present in the marine biofilm its efficacy, and identification, purification and structure elucidation of antifouling compound from highly potent antifouling strain. Followed by this analysis 16S rRNA study was also carried to identify the highly potent antifouling strain.

MATERIALS AND METHODS

Sample collection

The biofilm samples were scraped from different ships anchored at the Vizhinjam Harbour, Thiruvananthapuram, Kerala were brought to the laboratory in sterile polythene bags for further investigation.

Isolation and identification of bacteria

The collected sample was subjected to vigorous vortexing for 5 min and serially diluted using sterilized seawater. A volume of 100 µl of the diluents was spread on sterile Zobell's Marine Agar 2216 (Himedia, Mumbai). The plates were incubated at 37°C for 24-48 h. After incubation, the selected bacterial colonies were isolated and subcultured in nutrient agar medium for further investigation (Saravanan *et al.,* 2008).

Morphological and Biochemical characterization of bacteria

The isolated colonies were identified through microbiological and biochemical tests such as Gram staining, Motility test, Hydrolysis of starch, lipid and gelatin, Indole production, Methyl red test, Voges Prauskauer, Citrate utilization, Urease test, Nitrate reduction test, TSI agar, Catalase test and Oxidase test, based on the Bergey's manual of Determinative Bacteriology, Ninth edition (2000).

Screening of biofouling activity by coverslip method

All the bacterial isolates were tested for adherence property by inoculating them into sterile nutrient broth containing glass cover slips in the test tubes. After 24 h, the cover slips were removed and stained with 0.4% crystal violet to check the adherence of bacteria. Bacterial isolates which form a slimy layer on the cover slips were selected for further characterization (Bhosale *et al.,* 2002).

Antifouling bioassay

The antifouling activity of bacterial culture was done by cross streaking method. A volume of 100 µl of the biofouling bacterial culture was spread on sterile nutrient agar and the antifouling bacterial culture was cross streaked into it. The plates were incubated at 37°C for 24-48 h (Subramani Ramesh *et al*., 2009., Mounyr Balouiri *et al.,* 2016).

Extraction of Extracellular polymeric substances (EPS) and determination of biomolecules

For the extracton of EPS, 2 ml bacterial aliquot was added to sterile centrifuge tubes. The contents were homogenized for about 30 seconds and spun at 3500 xg for 5 minutes at 4°C. The supernatants were transferred to sterile centrifuge tubes and further spun at 9000 xg for 30 minutes, 4°C. Pellets were resuspended in 2 ml distilled water, freeze dried and dissolved in Phosphate and Tris-Maleate buffers. Dissolved pellets were filtered through 0.8/0.2 µm filters. Filtrates were assayed for EPS composition and enzyme activity (Phyllis Molobela *et al.,* 2010).

Assay of Lipase

Extraction of lipase

Lipase is extracted from the production medium after desired incubation time (48 h) by centrifugation at 10000 xg for 30 min in a refrigerated centrifuge. The resulting supernatant contained extracellular lipase. The pellet was also collected and total cell biomass was calculated. The cell pellet was stored at -20^{o}C for further use. To release the intracellular lipase, 0.2 g of harvested cells were suspended in 1 ml of lysis buffer (0.05 M phosphate buffer, pH 7) and subjected to five rounds of cell disruption (1 min) with the help of sonicator (MSE Manor) at 15 KHz for recovery of maximum enzyme. The sonicated cell suspension was centrifuged (15000 x g for 30 min) and cell free extract (intracellular lipase) was collected.

Lipase assay

The activity of free lipase was determined spectrophotometrically using p-nitrophenyl palmitate (p-NPP) as substrate according to the method of Nawani *et al*.,(2006) with some modifications. The reaction mixture containing 0.3 mL of 0.05 M phosphate buffer (pH 8.0), 0.1 mL of 0.8 mM p-NPP and 0.1 mL of lipase was incubated at 37 °C for 10 min. The reaction was then terminated by adding 1 mL ethanol. A control was run simultaneously, which contained the same contents but the reaction was terminated prior to addition of the enzyme. Absorbance of the resulting yellow colored product was measured at 410 nm in a spectrophotometer. One International Unit (IU) of lipase activity was defined as the amount of enzyme catalyzing the release of 1 µmol of p-nitrophenol per min from p-NPP under the standard assay conditions (Karadzic *et al*., 2006).

Extraction of antifouling compounds

The bacterial isolate of *Kytococcus sedentarius* was grown at 28°C at 150 rev/min for 5 days in marine broth. The culture was centrifuged at 8,000 rev/min at 4°C for 20 minutes. The supernatant was thoroughly extracted three times with an equal volume of chloroform. All of the extracts were pooled, and the solvent was removed at low pressure at 45°C until the extract was completely dry (Hari Datta Bhattarai *et al.,* 2007).

Purification and identification of antifouling compounds

The extract (2.14 g) was subjected to column chromatography over silica gel and eluted with an n-hexane/ethyl acetate combination with increasing polarity (0-100%). Fractions of 100 ml were collected. The major bioactive fractions of n-hexane-ethyl acetate were repeatedly run over the silica gel.

From these fractions compounds were separated and subjected to GC-MS (Hari Datta Bhattarai *et al.,* 2007).

GC-MS Analysis

GC-MS analysis was carried out on a THERMO MS DSQ instrument which includes THERMO GC- TRACE ULTRA VER: 5.0. The column used was DB 35-MS Capillary standard non-polar column measuring 30 m × 0.25 mm with a film thickness of 0.25 µm composed of 95% Dimethyl polysiloxane. The carrier gas used was Helium at a flow rate of 1.0 ml/min. 1 µl sample injection volume was utilized. The inlet temperature was maintained as 250°C. The oven temperature was programmed initially at 80°C for 4 min, then an increase to 250°C. Total run time was 35.92 min. GC-MS was analyzed using electron impact ionization at 70 eV and data was evaluated using total ion count (TIC) for compound identification and quantification. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS library. Measurement of peak areas and data processing were carried out by Turbo-MassOCPTVS-Demo SPL software.

Identification of bacterial strain

Isolation of genomic DNA from microbes

The pure bacterial colony was inoculated in Luria Bertani broth. 1 ml of the culture was transferred to sterile micro centrifuge tubes and the cells were harvested by centrifugation at 12000 rpm for 10 minutes at room temperature. Supernatent was discarded and the pellet was resuspended in 1 ml of 0.85% (w/v) NaCl solution and centrifuged as above. Discarded the supernatant and added 600 µl lysis buffer along with 7 µl of proteinase-K. Vortexed the mixture and incubated at 65°C for 1 h. Added equal volume of chloroform: isoamyl alcohol (24:1) with gentle mixing by inverting the tubes for 2-5 minutes. Centrifuged the samples for 15 minutes 12000 rpm at room temperature. Aqueous phase was collected in another microcentrifuge tube without disturbing the interface and lower phase. Repeated the steps of chloroform: isoamyl alcohol extraction. Again the aqueous phase was collected and added 50 µl volume of 3 m Sodium Acetate (PH 5.2) followed by equal quantity of ice cold isopropanol, so that the DNA gets precipitated and centrifuged it again at room temperature for 5 minutes at 12000 rpm.

The supernatant was discarded and rinsed the pellet twice with 70% ethanol, followed by maintaining the tubes for 1 hour in vaccum desiccators. The desiccated DNA samples was completely resuspended in 50 µl of DNA dissolving buffer (TE buffer) and stored at -20°C (Kutchma *et al.,* 1998).

16S rRNA Sequence analysis

Amplification of 18S rRNA gene - ITS region, was performed by using the following primer pairs. Forward (18S ITS For), 5/-AGAGTTTGATCCTGGCCTCAG-3 and reverse (18S - ITS Rev), 5 - CAAGGCATCCACCGT - 3/, 18S rDNA V3, forward 5/- CCTAGGGGAGG CAGCAG - 3 and 16S rDNA V3, reverse, 5/- ARRACCGCGCTGCTGC-3/. The forward 5'-CCTACGGGAGGCAGCAG-3' and reverse, 5'-ATTACCGCGG CTGCTGG-3', primers used occupied positions 341-358 and 518-534, respectively of the V3 region in the 16S ribosomal DNA of *Escherichia coli* . The primers specify about 200 bp of the PCR products. The V3 primer pair was used for ease of sequencing of the gene, using the variable region 3 (V3), for the genetic identification of the isolates. Sequencing of the purified 18S rDNA DNA products was performed using the sequencing unit of the University of Nottingham; a 373 DNA sequence (Perkin-Elmer Applied Biosystems) was used with the Taq Dye Deoxy terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems). (Bulut *et al.,* 2005)

RESULTS AND DISCUSSION

In the present study, the biofilm samples were collected from different ships anchored at the Vizhinjam Harbour. In this work, twelve different bacterial species have been isolated from biofilm scrapings and named as M1, M2, M3, M4, M5, M6, M7, M8, J1, J2, J3 and J4.

Morphological and Biochemical characterization of bacteria

The different parameters namely morphological and biochemical characters were used for characterization and identification of bacterial isolates. The results are tabulated in table 1.

Biofouling efficacy of bacterial isolates by coverslip method

The isolated marine bacteria are primarily screened for the biofouling efficacy.

Table 1 Morphological and biochemical characterization of bacterial isolates

Biochemical test	M1	M2	M3	M4	M5	M6	M7	M8	J1	J2	J3	J4
Gram staining(+/-)	-	+	-	+	+	+	-	-	-ve	-	+	+
Motility	+	+	+	+	-	+	-	-	-	+	-	+
Indole test	-	-	-	-	+	+	-	-	-	-	-	-
Methyl red test	-	-	-	-	-	-	-	-	-	-	-	-
Voges Proskauer test	-	+	+	-	-	-	+	+	+	+	+	+
Citrate utilization test	+	+	+	+	+	++	+	+	+	+	+	+
Triple sugar iron test	-	-	-	+	-	+	-	-	-	-	-	-
Catalase test	+	+	+	-	+	-	+	+	+	+	-	+
Oxidase test	-	-	-	-	-	-	-	-	-	-	-	-
Urease test	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate test	+	+	+	+	-	+	+	+	-	+	+	+
Starch hydrolysis	-	+	-	-	-	-	+	+	+	-	+	+
Lipid hydrolysis	-	-	-	+	+	-	-	-	-	-	-	-
Gelatin hydrolysis	+	+	-	+	+	-	+	-	+	-	+	+

The isolates M1, M2, M3, M4, M5, M6, M7 and M8 showed significant repellant activity against other bacteria and these isolates were considered as antifoulants. The rest of the isolates J1, J2, J3 and J4 , which not shown the repellant activity, taken as biofouling strains. After the characterization, the bacterial isolates M1, M2, M3, M4, M5, M6, M7, M8, J1, J2, J3 and J4 were identified as *Pseudomonas aeruginosa, Bacillus sp., Serratia sp., Clostridium sp., Kytococcus sp., Bacillus sp., Klebsiella sp., Vibrio sp., Klebsiella sp., Serratia, Streptococcus pyrogenes, and Bacillus subtilis* respectively.

Antibiofouling Assay

Sl.no	Inhibition zone of Antifouling isolates (cm)	Biofouling isolates			
		***Klebsiella sp.* (J1)**	***Serratia* (J2)**	***Streptococcus pyrogenes* (J3)**	***Bacillus subtilis.* (J4)**
1	*P. aeruginosa*(M1)	-	-	4	1
2	*Bacillus sp.*(M2)	0.5	-	-	-
3	*Serratia sp.* (M3)	-	-	-	2.5
4	*Clostridium sp.*(M4)	-	-	-	-
5	*K. sedentarius* (M5)	0.5	1	3	5
6	*Bacillus sp.* (M6)	-	-	-	-
7	*Klebsiella sp.* (M7)	-	-	-	0.5
8	*Vibrio sp.* (M8)	-	-	-	-

Note: '-'Represents no activity.

In the antifouling bioassay method, antifouling strains tested against biofoulants (Table 2). The identified bacterial strain M5 showed highest activity against other biofouling bacterial strains. So the M5 strain is subjected to the estimation of protein and carbohydrate concentration in the EPS.

Estimation of carbohydrate and protein concentration in the EPS of Kytococcus sedentarius

In the estimation of protein and carbohydrate concentration in the EPS, the M5 strain have 246 µg/ml of protein and 2µg/ml carbohydrate concentration respectively. Phyllis Molobela *et al.,* (2010) have reported that the EPS of *Pseudomonas fluorescence* biofilm grown in fed medium had a higher protein and carbohydrate concentration than in the unfed biofilm EPS and the protein concentration were found to be dominant rather than carbohydrate concentration. The EPS components of the biofilms differ in quantity, structure and nature depending on the microorganisms within the biofilm.

Lipase assay

In lipase assay M5 showned the highest enzyme activity of 9.1 unit/ml. The M7 showned 9 unit/ml enzyme activity. The least enzyme activity was shown by M2, which have 2.5 unit/ml enzyme activity. Due to the highest enzyme production the M5 strain is subjected to antifoulant production (Fig. 1).

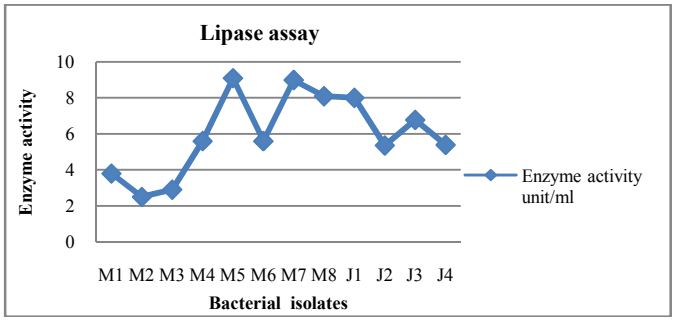

Fig 1 Lipase assay

Leroy *et al.,* (2007) also found the protease, Savinase to be more effective for the prevention of adhesion and detachment of a *Pseudoalteromonas* sp. D14 biofilm than xylanase, amylase, cellulase and lipase. Ledder *et al.,* (2008) also found protease to be effective for the removal of *A. naeslundii* and *F. nucleatum* biofilm.

Identification of Antifouling Compounds

In the present study, highly potent bacterial isolate was used for compound identification. GC-MS technique was used for compound identification. GC-MS revealed the presence of two different compounds with varying abundance at retention time.

The two compounds are Octadecanoic acid, methyl ester (CAS) and [1R-(1à,3à,4á,5à)]-1-(Hydroxymethyl)-5-[(diisopropoxyphosphinyl)methyl]c (Fig.2). Octadecanoic acid (relative abundance of 57.1) is a stearic acid which have antifouling potential.

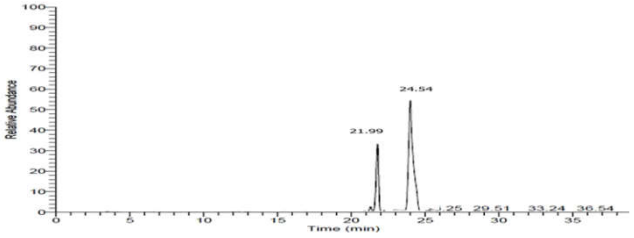

Fig 2 GC-MS chromatogram of *Kytococcus sedentarius*

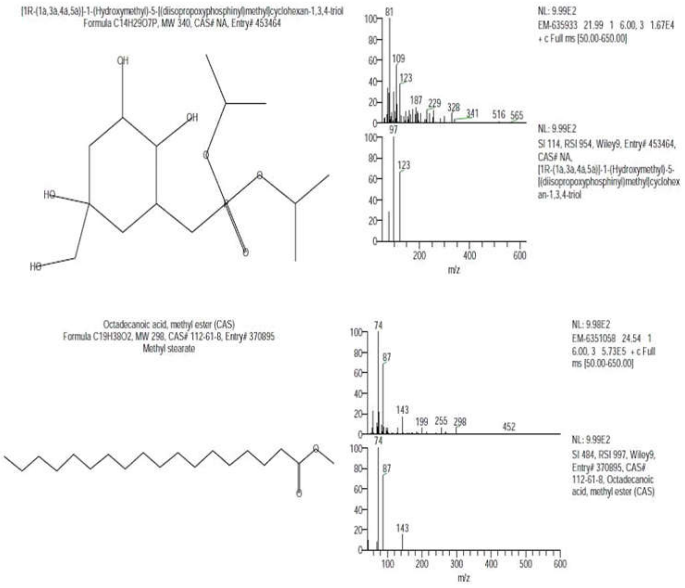

Fig 3 Compounds obtained in GC-MS analysis of the purified extract of *K. sedentarius*

Gao *et al*., reported that the octadecanoic acid isolated from biofilm samples showed potential antifouling activity. It showed significant antibacterial, antialgal and anti larval activity (Gao *et al.,* 2014). GC-MS profile of *L. brandenii* has suggested that the purified fraction is primarily composed of octadecadienoic acid (49.75%) followed by "n- Hexadecanoic acid" (14.24%) which could have functional role in the chemical defense against fouling organisms (Aseer Manilal *et al.,* 2010).

16S rRNA Sequence Analysis

Based on 16S rRNA Sequence Analysis of similarity coefficient the M5 strain showed similarity with *Kytococcus sedentarius* strain.

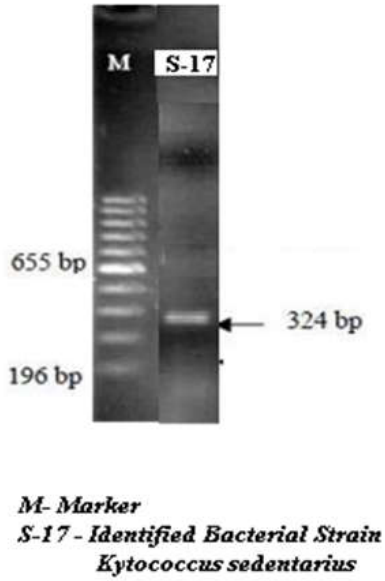

Fig 4 Ethidium Bromide stained gel of PCR Amplification products of the sample bacterial strain

Electropherogram

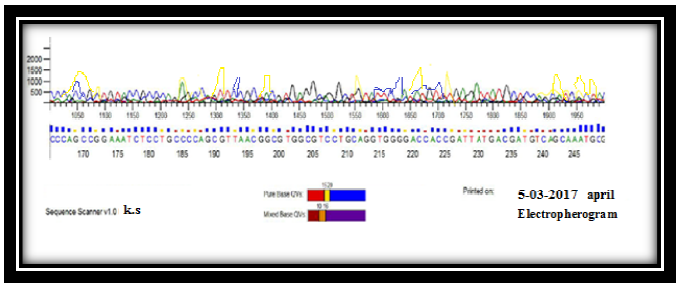

Fig 5 Electropherogram of Kytococcus sedentarius

Graphic View of the sample organism

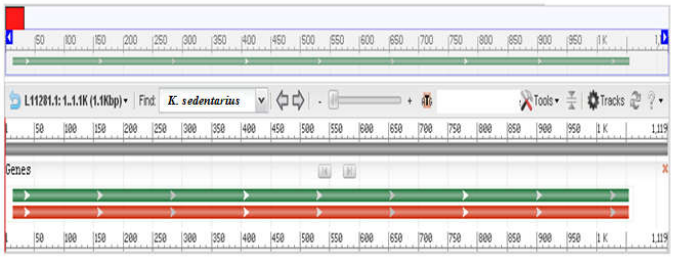

Fig 6 Graphical view of *Kytococcus sedentarius.*

The sequential data of 16S rRNA gene from highly potent bacteria was obtained and the data from digitalized chromatogram (figure 5) as well as the graphical view (figure 6) revealed that the highly potent organism for antifouling compound production as *Kytococcus sedentarius.*

CONCLUSION

An ideal natural product antifouling compound will act different ways on the target organisms. Generally, the compound should prevent the formation of biofilms. The main mechanism of antibiofilm activities of microbial strains includes antibiotic activity and anti-adhesion property and affects the extracellular polymers production which is essential for biofilm formation. In this study, the bacteria *K. sedentarius*, which were isolated from marine biofilms, were found to produce potential antifouling compounds. These compounds were identified by GC-MS as octadecanoic acid, methyl ester (CAS), and [1R-(1à,3à,4á,5à)]-1-(Hydroxymethyl)-5-[(diisopropoxyphosphinyl)methyl]c. These are either chemically synthesized or in the form of crude extracts, would have high application potential as a source of useful compounds for antifouling technology. From this work it is concluded that the bacterial strains present in the biofilm scraping are a good source for the production of antifouling compound. The presence of octadecanoic acid in *K. sedentarius* indicated that it act as a good vector for the prevention of biofouling.

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