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ANTIFOULING POTENTIAL OF *PSEUDOMONAS AEUROGINOSA* ISOLATED FROM MARINE WATER

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Abstract–In the marine environment, most solid surfaces are covered by microbial biofilms, mainly composed of bacteria and diatoms. Biofouling is a major problem in marine sector which needs high economy for control and cleaning processes. Marine micro-organisms are an important source of bioactive metabolites and they synthesize ecofriendly antifoulants that inhibit the adhesion of organisms. To investigate the antifouling activity, the marine bacterial strains were isolated from marine water samples collected from Colachel harbour. Totally seven bacterial strains were isolated and subjected to biofouling and antifouling assays and among that S18 strain showed remarkable antifouling activity. The extracts of *Pseudomonas aeuroginosa* was screened for antimicrobial and antimicrofouling assays. The results showed that the extract inhibited the growth and attachment of biofilm forming bacterial strains S7 and S11. Antifouling activities are mainly due to the presence of bioactive metabolites present in the *Pseudomonas aeuroginosa*. By the 16S rDNA screening the highly potent S18 strain was identified as *Pseudomonas aeuroginosa.* The current study revealed that the *Pseudomonas aeuroginosa* could produce potent antifoulant which can be used to control biofouling in marine environment.

INTRODUCTION

Marine biofouling is a complex assemblage of micro and macro-organisms on artificial structures comprising micro-colonies that are attached to a surface and shielded themselves to the extracellular matrix of polysaccharide, protein and nucleic acids. Marine biofouling causes huge economic losses and serious problems to maritime industries Qian *et al*., (2021). It is the settlement and development of unwanted aquatic species on natural and artificial surface. Biofouling increases the hydrodynamic drag and decreases the maneuverability in naval and merchant ships and cause more than $200 billion loss/annum Chambers *et al*., (2006).

Biofilm formation is generally described as a temporal process involving a succession of different stages: first, a reversible attachment of micro-organisms to conditioned surfaces, then an irreversible attachment and finally biofilm maturation through synergistic and/or competitive interactions between the different microbial communities (Dang and Lovell, 2000). In the marine environment, biofilms can also alter the properties of colonized metallic structures through the phenomenon of microbiologically influenced corrosion, particularly due to the intense metabolic activity of some bacteria Lanneluc *et al*., (2015). This phenomenon causes great damage to maritime infrastructure, resulting in serious economic losses. The costs related to the fouling of ship hulls also have an important economic impact due to increased fuel consumption and maintenance Schultz *et al*., (2011).

Commercial antifoulants containing Tributyltin (TBT) paints have adverse effect on marine ecosystem but due to the environmental concern, application of TBT for marine applications was banned from January 1, 2008 by, the International Maritime Organization and Marine Environment Protection Committee Yebra *et al*., (2004). Many substitutes for TBT were introduced such as Irgarol1051 and Diuron, have been found to be harmful to many non-target organisms. One of the

organotin compounds, 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.

Natural compounds such as 10-β-formamidokalihinol-A and kalihinol A, polymeric 3-alkylpyridinium salts, terpens and pyrrole-imidazole alkaloids, succinic acid, taurine acid substituted bromopyrrole alkaloids and dibromophakellin derivatives, 3-phenyl-2-propenoic acid, 2- hydroxymyristic acid and cis-9-oleic acid are used as biocides Yang *et al*., (2007). Diketopiperazines were identified from a deep-sea bacterium, *Streptomyces fungicidicus*, showed promising antifouling activity Li *et al.,* (2013). These compounds should target adhesive properties without affecting bacterial viability in order to avoid the appearance of resistant mutants Papa *et al*., (2013). Some natural compounds, such as extracts from shellfish periostracum, exhibit strong antifouling property which can be commercialized Cahill *et al*., (2013). In this context an attempt has been made to identify the potent antifouling microbe from the marine environment as the large diversity of marine bacteria constitutes a vast potential reservoir for novel molecules to control biofilm formation.

MATERIALS AND METHODS

Collection of Samples

The marine water samples were collected from Colachel, Tamil Nadu, India. The samples were collected according to the standard microbiological procedures and brought to the laboratory in sterile polythene bags for further laboratory analysis (Cappuccino and Sherman, 2014).

Isolation and Characterization of fouling microorganisms

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 were spread on sterile Zobell's Marine Agar 2216 (Himedia, Mumbai) and incubated at 37 °C for 24-48 h. After enumeration, morphologically different bacterial colonies were selected, purified by repeated streaking on Zobell marine agar plates and it was maintained on Zobell marine agar slants at 4 °C for further analysis Saravanan *et al*., (2008).

All the bacterial isolates were tested for adherence property by inoculating them into sterile seawater containing glass cover slip in the beaker. After 6 hrs, 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 considered as biofoulants and those bacterial isolates which failed to adhere on the cover-slip are considered as antifoulants. The antifoulant strains were selected for further characterization (Abdi-Ali and Peeters, 2008).

Characterization and identification of antifouling bacteria

Phenotypic characteristics such as micro morphology (gram staining, motility), cultural and biochemical characterization of the selected isolates were studied using standard protocol from the Bergey's manual of Determinative Bacteriology, 9th edition (2000).

Preliminary Screening for antifouling activity

The antifouling activity of antifouling strains were preliminarily screened by cross streaking method Mounyr *et al.,* (2016). A single line streak of bacterial strain was made on the surface of the Zobell Marine agar medium separately and incubated overnight. After observing ribbon like growth on the surface of the plates the biofouling bacterial strains were streaked at right angles to the original streak and incubated at 37 °C for 24-48 hr.

Secondary Screening for antifouling activity

Antifouling activity of selected strains was carried out using agar disc diffusion method. About 0.25 mg of crude extract was impregnated on filter paper disc (5 mm diameter) and placed on Muller Hinton agar plates inoculated with selected biofouling bacterial isolates (S7 and S11) from biofouling samples and *Staphylococcus* and *Bacillus sp.* All the plates were incubated at 37 °C for 24 hrs and observed for Zone of inhibition.

Anti macrofouling assay

Anti-settlement assay

Twenty brown mussels, *Perna indica* were collected from seashore and were checked for byssal thread formation and attachment to glass beaker by anti-settlement assay described by Murugan and Ramasamy (2003). Different concentrations (0.2-1.4 ml) of cell free supernatant of bacterial isolates were then incubated at room temperature with mild aeration for 24h. The mortality of the mussels at

various concentrations was observed and lethal concentration (LC50) and (LC 90) was determined by probit analysis.

Naupliar toxicity assay

For the toxicity studies, static bioassay was performed under the laboratory conditions described by Ortega-Morales *et al*., (2008). Twenty newly hatched nauplii were introduced in small test tube containing 10ml of brine solution and crude cell free supernatant of antifouling bacterial isolates at various concentrations (0.2-1.4 ml) was added diluted and maintained at room temperature for 24h under the light. After 24h of exposure, the mortality of larvae was noted and the lethal concentration LC50 and LC90 values were determined through probit analysis using IBM SPPSS Version.24.

Barnacle cytotoxicity assay

Adult barnacle, Am*phibalanus amphitrite* were used for this toxicity study. Different concentrations of cell free supernatant of (01-1.1ml) were prepared (based on the serial dilution method) into 24-well polystyrene plates, containing 20 larvae. The plates were kept in dark at 25 °C. After 24 hrs of exposure, the toxicity of the extract was monitored based on the number of larval death and the LC50 and LC90 value was calculated based on Probit analysis with 95% of confidence interval Darya *et al*., (2020).

Sequencing using 16S rDNA for Microbial identification

The S18 bacterial strain DNA was isolated based on (Doyle and Doyle 1987) and subjected to 16SrDNA amplification using 16S rDNA gene, with universal primers 27F(52-AGAGTTTGATCCTGGCTCAG-32) and 1492R (52-TACGGYTACCTTGTTACGACTT-32). Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7.

RESULTS

Isolation, characterization and identification of antifouling bacteria

In the present study the antifoulant strains were isolated from the collected marine water. Totally 2.5×10^4cfu/ml of morphologically different colonies were observed and among them seven isolates were selected and named as S1, S3, S7, S9, S11, S13 and S18. Based on the Bergey's manual of Determinative Bacteriology (2000) the isolated strains namely S1, S3, S7, S9, S11, S13 and S18 were identified as *Pseudomonas aeruginosa, Mycobacterium sp, Staphylococcus sp, Shigella sp, Neisseria sp, Bacillus sp* and *Lactobacillus sp*. respectively.

Screening and identification of antifouling activity using assays

In the present study, the adherence property of antifouling strains was screened. The bacterial isolates S3 and S18 strain do not showed adherence property in cover-slip assay and agar disc diffusion assay. The bacterial isolate S1, S7, S9, S11 and S13 showed attachment on the surface of cover-slip also which failed to produce zone of inhibition and are considered as biofouling strains. Among the five biofouling bacterial isolates, S7 and S11 showed maximum biofouling activity. In the antifouling screening the CFS of S18 strain showed maximum inhibition against the two fouling bacterial isolates (S7, S11) and pathogenic bacterial strains S*taphylococcus* and *Bacillus* and hence S18 was considered as potential antifouling strain. Antimicrobial activity of antifouling bacteria against biofoulers were shown in Table 1.

Antimicrofouling assays

Anti-settlement assay of S18 against *Perna indica*

The cell free extract (CFS) of bacterial strain S18, significantly inhibited the byssal thread production and attachment of mussel *P. indica.* The LC50 value was 0.658 which indicates better inhibition activity against attachment of mussel since the LC50 values are below 0.9 mg/ml and the LC90 value of S18 is 1.847 which also showed better activity. The LC50 and LC90 values are statistically proven to be significant (<0.005); their chi-square value is 5.929 and degree of freedom 5 and the probit transformed response is y=0.06+2.92*x and R2 is 0.0848.

Naupliar toxicity of S18 against *Artemia salina*

Anticrustacean assay is one of the most reliable and inexpensive bioassays to test the toxicity of the extract. In the present study S18 bacterial strains showed LC50 value of 0.699 and LC90 value of 1.968 which showed better inhibition. The chi-square value ranges from 2.555 and the degree of freedom

Table 1. Antimicrobial activity of antifouling bacterial strains against biofoulers

Sl. No.	Biofouling isolates	Inhibition Zone of Antifouling isolates (cm)	
		Mycobacterium (S3)	*Pseudomonas aeroginosa* (S18)
1	*Lactobacillus* (S7)	1.2 ± 0.05	1.4 ± 0.1
2	*Shigella* (S11)	0.9 ± 0.1	1.6 ± 0.05
3	*Staphylococcus sp*	0.6 ± 0.15	1.7 ± 0.1
4	*Bacillus sp*	0.3 ± 0.1	1.2 ± 0.05

* Each mean± SD values based on triplicates

values is 3 and the values were statistically proven to be significant since P <0.005. The probit transformed responses of potent S18 bacterial strain is y= 0.48+2.81*x and there R2 linear value is 0.932.

Anti- Barnacle assay of S18 against *Amphibalanus amphitrite*

As shown in Table 2, S18 bacterial strains showed LC50 value of 0.747 and LC90 value of 1.663 using Probit analysis. The chi-square value is 4.356 and the degree of freedom is 5 and the values were statistically proven to be significant since P <0.005. The probit transformed responses of potent S18 bacterial strain is y= 0.54+3.56*x and there R^2 linear value is 0.910. Figure 1 shows the probit analysis of S18 bacterial strain against antimicrofouling assays

Identification of antifouling strain using 16s Rdna sequencing and Phylogenetic analysis

The potent bacterial isolate S18 subjected for 16S rDNA sequencing showed high similarity with *Pseudomonas aeruginosa* based on nucleotide homology and phylogenetic analysis.

Figure. The evolutionary history was inferred by using the Maximum Likelihood method based on Kimura (1980). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed Felsenstein (1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate

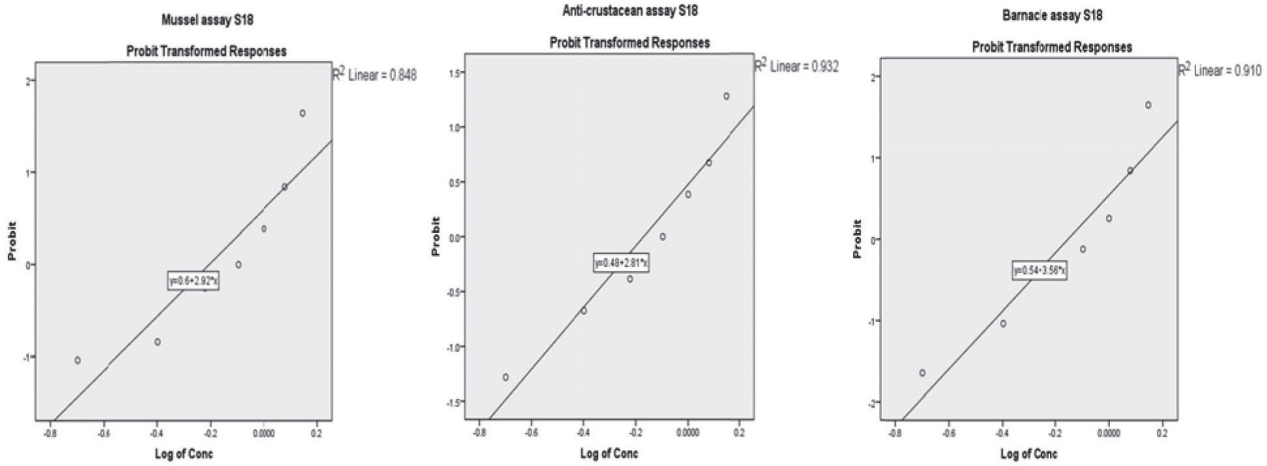
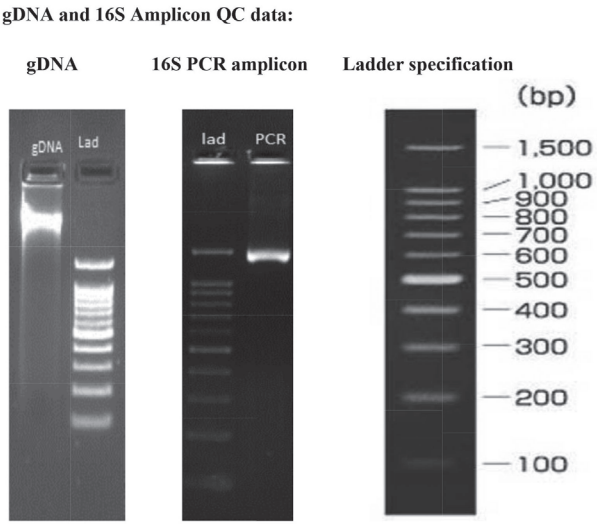


Fig. 1. Probit analysis of S18 bacterial strain against antimicrofouling assays

Table 2. Antimicrofouling assays of CFS of S18 strain

Antifouling assays of S18	LC -50 (Fiducial limit)	LC-90 (Fiducial limit)	Chi-Square	Degree of freedom	Significance
Anti-settlement assay	0.658 (0.535- 0.795)	1.847 (1.383-3.132)	5.929	5	0.003
Naupliar toxicity assay	0.699 (0.570-0.847)	1.968 (1.458-3.453)	2.555	3	0.001
Barnacle toxicity assay	0.747 (0.634 -0.872)	1.663 (1.326-2.472)	4.356	5	0.00



trees in which the associated taxa clustered together in the bootstrap test (1000 replicates were shown next to the branches Felsenstein (1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1434 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 Kumar *et al*., (2016).

DISCUSSION

Marine ecosystem is highly productive and biologically diverse habitats. Because of richness in carbon and other nutrient contents, the marine ecosystem harbours diversified microbial communities. Marine derived secondary metabolites may constitute one of the most promising alternatives to the toxic and harmful synthetic antifoulants. The synthetic antifoulants may hazardous to marine ecosystem and cause lot of environmental pollutions. So, there is a need for the extensive research for the continuous exploration of marine organisms for novel antifoulants. The present study mainly focuses on isolation and identification of potential antifouling bacteria against marine biofouling organisms.

In the present study totally seven bacterial strains were isolated from marine water samples collected from colachel harbour. Among the isolated strains S3 and S18 exhibited antifouling property and the remaining five bacterial strains S1, S7, S9, S11 and S13 showed biofouling property, while S7 and S11 are strong biofouling microbial isolates. Dhanasekaran *et al*., (2009) isolated 11 isolates from three ships from Royapuram harbour, Chennai, Tamil Nadu, India. Among the 11 isolates, DR4 showed maximum biofouling activity in the microtiter plate assay with a significant optical density of 0.596. Viju *et al*., (2014) isolated 9 bacterial strain from the sea weed and screened for their antibacterial activity against three biofilm forming bacteria and all the 9 strains were inhibited the growth of biofilm forming bacteria. In the current study the bacterial isolate S1, S7, S9, S11 and S13 showed attachment in the surface of cover-slip and those bacterial strains are regarded as biofoulers and the remaining S3 and S18 failed to attach on coverslip and regarded as antifouling bacterial strain.

In the present study *P. aeruginosa* exhibited strong inhibitory activity against S7 and S11 biofouling bacterial isolates and pathogenic bacterial strains *Staphylococcus* and *Bacillus* sp. The maximum zone of inhibition was noted as 1.6 ± 0.05cm against S11 biofouling bacterial strain. Ulyana Kharchenko *et al*., (2012) stated that, antimicrobial activity of *Pseudomonas aeruginosa* 1242 strain inhibit the growth of 19 cultures out of 23 and exhibit glycosidase, glycanase, proteolytic, and amylase activities. The combination of the high anti-microbial and enzymatic activity of the *P. aeruginosa* 1242 strain has proven to be the metabolites incorporated in an epoxy matrix signicantly reduces adhesion and settlement in both micro- and macrofoulers so it is justied to use as commercial coatings.

In the present study, the bacterial strain S18 showed remarkable antimicrobial activity and showed positive response to antifouling assays. The antifouling assays such as anti-settlement assay, naupliar toxicity and barnacle toxicity assays were performed in CFS of S18 bacterial strain at different concentration. The result of the assays showed remarkable antifouling activities with significant LC50 and LC90 values ($P<0.005$). This Phenomenon is mainly due to the production of antimicrobial proteins and antibiotics as well as chemical compounds synthesized by secondary metabolism pathways. The degree of lethality is directly proportional to the concentrations of the extract.

Lopes *et al.,* (2010) tested the cytotoxicity of crude methanolic extract of *Nodularia* sp. (LEGE 06071) against nauplii of *A. salina* and recorded LC50 value of 17.81µg/ml. In the present study, CFS of S18 antifouling bacterial strains showed LC50 value of 0.699 and LC90 value of 1.968 which showed better inhibition against *Artemia nauplii*. In general, lethality of an extract mainly depends on the cytotoxic property of the extract.

Pseudomonas aeruginosa is a ubiquitous gram-negative rod-shaped environmental microbe which are widely used as a source of various enzymes, as well as in soil remediation and water purication processes. The potent antifouling strain S18 was subjected to 16S rDNA sequencing and it was identified as *Pseudomonas aeruginosa* which showed nearly 20% similarity to NR114471.1 and 19% similarity to NR113599.1. Ulyana Kharchenko *et al.*, (2012) isolated *Pseudomonas aeruginosa* 1242 strain from the fouling microflora of brass plate Marine Corrosion Station of the Maritime Branch of the Russian-Vietnamese Tropical Research and Technology Centre in Nha Trang Bay, Vietnam, in January 2009.

CONCLUSION

Marine microbes are taxonomically diverse and unique, which makes them as potential source for discovery of novel bioactive molecules. Biofilm-forming microorganisms have the ability to modify settlement surfaces, making them rougher, which reduces significantly the activity and efficacy of antifouling paints. Marine microbes are being explored as potential sources for the production of environmentally friendly antifouling metabolites. In the present study, totally seven bacterial strains were isolated from marine water samples. The bacterial strain S1, S7, S9, S11 and S13 showed attachment on the surface of cover-slip and regarded as biofouling in which S7 and S11 are strong biofouling microbial isolates. The remaining S3 and S18 strains failed to attach on coverslip and regarded as antifouling bacterial strain. Among the S3 and S18 antifouling strains S18 exhibited maximum zone of inhibition of 1.6±0.05cm against S11 biofouling bacterial strain which strengthen its antifouling property. The present study reported that LC50 and LC90 values of antifouling assay such as anti-settlement, naupliar toxicity and barnacle toxicity assays performed in cell free supernatant of S18 bacterial strain exhibited potent antifouling activity and were proven to be statistically significant, since P<0.005. Furthermore, the S18 bacterial strain is identified as *Pseudomonas aeruginosa through* 16S rDNA sequencing. The present study concluded that *Pseudomonas aeruginosa* is a potent antifouling microbial source.

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