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Biocontrol of Biofilm Produced by *Pseudomonas aeruginosa* Using Extremely Halophilic Marine Bacteria Isolated from Malvan Region

Mamata P Abhyankar[1], Tanaji B Sawant[1], Arif A Mulani[2], Geetanjali S Patil[4], Suryakant S Wadkar[3] and Avinash A Raut[4]*

[1]*Department of Microbiology, Yashwantrao Chavan College of Science Karad, Vidyanagar, Karad, Maharashtra, India*

[2]*Department of Microbiology, Dr. Patangrao Kadam Mahavidyalaya, Sangalwadi, Sangli, Maharashtra, India*

[3]*Co-ordinator, Department of Biotechnology, Smt. Kasturbai Walchand College, Sangli, Maharashtra, India*

[4]*Department of Biotechnology, Smt. Kasturbai Walchand College, Sangli, Maharashtra, India*

***Corresponding Author:** Dr. Avinash Anand Raut, Assistant Professor, Department of Biotechnology, Smt. Kasturbai Walchand College, Sangli, Maharashtra, India.

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Abstract

New antibacterial drugs are needed to society due to increase in infections and incidence. Fortunately, marine microorganisms are potential source of novel pharmaceutical compounds. During our study we found that marine bacteria from coastal region of Malvan produced compounds that inhibited bacterial growth or biofilm formation. Pure bacterial cultures were obtained from marine sediment, sand, marine water and coastal soil samples from Malvan and these extracts were subjected to inhibition assays of bacterial growth and biofilm formation by *Pseudomonas aeruginosa*.

Keywords: *Marine Bacteria; Halophiles; Extremophiles; Biofilm; Antibiofilm Agents*

Abbreviations

NaCl: Sodium Chloride; NTYE: NaCl Tryptone Yeast Extract; MSCRAMMS: Microbial Surface Adhesions Recognize Adhesive Matrix Molecules; EPS: Extracellular Polymeric Substances

Introduction

Halophiles are salt-loving organisms which live in hypersaline environments. They constitute mainly prokaryotic and eukaryotic microorganisms who balance the osmotic pressure of the environment and resist the denaturing effects of salts. Halophiles are slightly, moderately or extremely halophilic, as per the requirement for NaCl. Halophiles are distributed all over the world in hypersaline environments, out of which many are found in natural hypersaline brines as well as in coastal and even deep sea locations. Hence they are novel in biotechnological point of view. Although salts are required for all life forms, halophiles are distinguished by their requirement of hypersaline conditions for growth. They may be classified according to their salt requirement: slight halophiles grow optimally at 2 - 5% NaCl; moderate halophiles grow optimally at 5 - 20% NaCl and extreme halophiles grow optimally above 20 - 30% NaCl. Halotolerant organisms can grow both in high salinity and in the absence of a high concentration of salts. Many halophiles and halotolerant microorganisms can tolerate wide range of salt concentrations. Though the oceans are the largest saline body of water, hypersaline environments are generally defined as those containing salt concentrations in excess of sea water (3.5% total dissolved salts). Many hypersaline bodies derive from the evaporation of sea water and are called thalassic. A great diversity of microbial life is observed in thalassic brine from marine salinity

up to about 3 - 3.5 mol/L NaCl, at which point only a few extreme halophiles can grow, e.g. *Halobacterium, Dunaliella* and a few bacterial species. Halophilic microorganisms are found in salt waters lakes, brines, ponds and saline soil [1].

As antibiotics derived from bacteria are evolving antibiotic resistance via genetic mutations, existing drugs are becoming less effective and creating a public health crisis [2]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a virulent has developed multi-drug resistance [3]. Marine microorganisms produce antagonistic compounds which inhibit the growth of human pathogenic bacteria [4]. Studies suggest that marine actinobacteria produce secondary metabolites that are structurally distinct from those produced by terrestrial bacteria. Marine bacteria such as Proteobacteria have not been explored as sources of new antibacterial compounds and hence may prove to produce novel antimicrobial drugs [5].

The formation of biofilm which is initiated by the adhesion of microbial cell to surfaces, is the dominant mode of microbial life in nature, and has several advantages of increased nutrient supply, better protection against environment stress factors i.e. desiccation, pH shift and U.V. light and enhanced rates of horizontal gene transfer increases both gene spread and genetic variation [6]. This behaviour is observed in most members of the domain Bacteria, Archaea which include hyperthermophilic, acidophilic and methanogenic members [7]. Bacteria assemble in biofilms in enriched nutrient environments on the surface due to the equilibrium of bacteria, electrostatic and hydrophobic interactions, and van der Waals forces [8,9]. Exopolysaccharides and exogenous substances produced by bacteria make the association and adhesion irreversible. In prosthetic devices such as indwelling catheters and endo-tracheal tubes, bacterial biofilm formation is quite common. Biofilm formation takes place in various steps such as attachment to a surface followed by formation of microbial surface adhesions recognize adhesive matrix molecules (MSCRAMMS) and aggregation, attachment to each other and production of extra polymeric substances (EPS) that interact with host-derived components such as platelets to form a strong biofilm. Extracellular polymeric substances (EPS) such as extracellular genomic DNA and glycoproteins play important roles in microbial biofilms, including initial surface adhesion, biofilm stability and cellular interactions [10-12].

Pseudomonas is a gram negative, aerobic rod bacteria belonging to bacterial family Pseudomonadaceae. Genus *Pseudomonas* is free living bacterium, commonly found in soil and water. It is found on the surfaces of plant and also found on the surface of animals and has very simple nutritional requirements. It is often observed growing in distilled water, which gives an evidence of its nutritional needs.

The formation of biofilms has led to several beneficial and detrimental effects on bacteria and organisms, including humans. *Pseudomonas aeruginosa* forms a biofilm in the airway of patients with cystic fibrosis and aggravates nearby cells and cause mortality [13]. Behavioral changes between planktonic bacteria, bacteria in biofilms and genetically encoded antibiotic resistance have resulted in low antibiotic susceptibility of *P. aeruginosa,* leading to difficulty in controlling biofilms [14]. *Streptococcus mutans* causes biofilm-based infections which leads to dental caries and gingivitis. Biofilms formed on heart valves, pacemakers, and catheters are a serious threat.

There is a great need to discover anti-biofilm compounds as it is difficult to control biofilms and very few biofilm inhibiting compounds are known to the scientific society [15]. Biofilms are more resistant to antibiotics than planktonic cells because bacteria adhered to a surface are encased in a thick extracellular matrix that impedes antibiotic penetration and biofilm-associated bacteria behave differently than planktonic bacteria through quorum sensing.

The heterogeneous mixture of bacteria and nutrients from the environment in the biofilm matrix make it likely multiple resistance mechanisms exist [16]. Compounds that act as non-lethal inhibitors of biofilm formation are expected to pose less selective pressure for evolution of antibacterial resistance as compared to compounds that are lethal to bacteria. It is important to find compounds that control biofilms as inhibition may thwart the detrimental impacts of biofilm-associated bacteria on the health of humans. Thus, we set out to explore the hypothesis that marine bacteria from costal region of Malvan produce compounds that may inhibit bacterial growth/survival and/or biofilm formation.

Biofilm production is considered as a marker of clinically relevant infection. Previous observations have confirmed that biofilms are not only resistant to antibiotics but a variety of disinfectants which emphasizes that their characterization is an important aspect of infec-

tion control. Furthermore, the information on the capacity of a clinical isolate to produce biofilm would help a clinician to evaluate the measure of its virulence and devise an appropriate treatment plan for the patient. While there are many techniques available for biofilm study, it is imperative that standardized techniques should be developed. A variety of methods have been standardized in various laboratories, each having their own merits. These methods include tissue culture plate (TCP), tube method (TM), Congo red agar method (CRA), bioluminescent assay, light or fluorescent microscopic examination, air-liquid interface coverslip assay, and scanning electron microscopy (SEM) [17].

Marine microorganisms may also produce biologically active natural products that are not lethal but instead modulate bacterial behaviors, such as biofilm formation. Biofilm production is very important in case of industrial and domestic waste water treatment methods like Trickling filters modified lagoons. Bacteria produce secondary metabolites that play an important role in drug discovery and design. There is a critical need for new drugs to treat biofilm-based infections.

Materials and Methods

Collection of sample

The marine sediment, sand, marine water and costal soil samples were collected from 10 cm in depth near sea shore in sterile plastic bottles from coastal region of Malvan which represented an unpolluted marine environment.

Enrichment of microorganisms

Enrichment of microorganisms from each sample in NTYE (NaCl Tryptone Yeast Extract) broth (with 20% crude NaCl) for 30 days by shaking at 500 rpm.

Isolation and purification of microorganisms

Isolation was done by using streak plate technique on NTYE agar (with 20% crude NaCl) plate, incubation 10 days at room temperature. Purification was done by streak plate technique on NTYE agar (with 20% crude NaCl).

Study of biofilm production

Isolated colonies were transferred to 25 ML broth medium with foam stopper in jumbo tubes and 1 gm of Amberlight resin was added to the 25 ml ML broth with cultures and incubated with shaking (500 rpm) at 30°C for 6 days.

Liquid-interface coverslip assay (Modified)

Biofilm adhered to coverslips were visualized under light microscope in this assay. Isolates were inoculated in 5 ml of Tryptic Soy Broth and incubated till they reached stationary phase. The watch glasses were filled with 2 ml medium. Sterile glass coverslips were inserted into each watch glass to achieve a 45° angle relative to the bottom of the watch glass so that the meniscus of the medium was at the center of the coverslip. Petri plates were kept in the incubator at 35°C for a period of 18 hours. Isolates appeared on coverslips were stained by using 0.1% crystal violet for 10 minutes. Excess dye was removed by gentle water wash treatment and coverslips were air dried. Bacteria which appeared on air-liquid interface on each coverslip were observed under 100x objective of a light microscope [18,19].

Extraction of secondary metabolites

Isolated colonies were transferred to 25 ml broth medium with foam stopper in test tubes and 1 gm of Amberlight resin was added to the 25 ml broth with cultures and incubated with shaking (500 rpm) at 30°C for 6 days. After incubation liquid media and resins were separated by filtering through 6 layers of muslin cloth and washed it with distilled water. Methanol was added to the traces of liquid extract in 10 - 15 ml amount and were transferred to traced vials. The left solvent was evaporated. The organic compound mixtures were stored

at refrigeration temperatures. Bacterial and chemical extract was solubilized using dimethyl sulfoxide solution. Culture of *Pseudomonas sp.* was inoculated in Tryptic soy broth in plastic vials for biofilm formation [11].

Biofilm inhibition assay

The biofilm inhibition assay for *Pseudomonas sp.* was studied by two methods - Liquid Interface coverslip assay (modified) and Tube method.

Liquid interface coverslip assay (modified)

After biofilm formation chemically extracted compounds from each isolate were added in vials and allowed for reaction for 1 day. Inhibition of biofilm was checked liquid interface coverslip assay and by Tube method.

Tube method

Isolates were subjected for biofilm production by modified method of Christensen. Two milliliters of trypticase-soy broth (Hi-Media make) was inoculated with loopful of suspension of microorganisms from overnight culture plates and incubated for 48 hours at 37°C. The contents were decanted and dried at room temperature. Then the tubes were stained with 4% crystal violet. Each tube was rotated gently for uniform staining and the contents were gently decanted. The test tubes were placed inverted to drain all the liquid and were then observed for biofilm formation. When a visible film lined the wall at the bottom of the tubes the test was considered positive. Formation of ring at the liquid interface was not considered as biofilm formation. The results were recorded visually as follows: 0-absent, 1-weak, 2-moderate, 3-strong.

Results and Discussion

Collection of sample

Total 4 different samples were collected i.e. marine water, marine sediment, sea sand and soil, all samples were brought from coastal region of Malvan. Samples were labeled appropriately and were transported to the laboratory for processing. They were stored at a temperature between 6℃ to 10℃ until further use.

Enrichment of microorganisms from sample

The microorganisms were enriched from all samples using selected enrichment media NTYE broth (with 20% crude NaCl) for 30 days by shaking at 500 rpm

Isolation of microorganism

Total 10 isolates were obtained from all types of marine samples. All the isolates were coded accordingly and results are cited in table 1.

Sample	Isolate obtained (NTYE broth with 20% crude NaCl)		
Sand	Sa1	Sa2	Sa3
Sediment	SE1	SE2	SE3
Soil	SO1	SO2	SO3
Sea Water			SW3

Table 1: *Coding of isolates.*

Philicity of the isolates towards NaCl

Philicity of Isolates towards various concentration of NaCl is studied and cited in table 2.

Isolate code No.	Salt concentration (%)			
	20%	17%	15%	13%
Sa1	+	+	+	+
Sa2	+	+	+	+
Sa3	+	+	+	+
SE1	+	+	+	+
SE2	+	+	+	+
SE3	+	+	+	+
SO1	+	+	+	+
SO2	+	+	+	+
SO3	+	+	+	+
SW3	+	+	+	+

Table 2: *Philicity of Isolates towards various concentration of NaCl.*

From above table results, it is seen that, all the isolates showed growth in NTYE broth with salt concentration 20%, 18%. All the isolates showed moderate growth at 15% salt concentration except isolates SE3 and SW3. Only isolate SO2 showed growth with 13% salt concentration.

Study of biofilm formation ability of isolates: Biofilm formation ability of isolates was studied by inoculating each isolate in ML broth with Amberlight resins in it. After shaking and incubation all the isolates were found to produce biofilm and secondary metabolites.

Study of biofilm formation of isolates

Tube method

Length of biofilm produced by Isolates on inner surface of tube and optical density of Crystal violet stain adsorbed by biofilm and extraction of secondary metabolites are cited in table 3.

Isolate Code	Length of biofilm (mm)	O.D. at 530 nm					
Sa1	3	1.69	+	Sa1	3	1.69	+
Sa2	3.2	2.11	+	Sa2	3.2	2.11	+
Sa3	3.6	2.54	+	Sa3	3.6	2.54	+
SE1	5	4.62	+	SE1	5	4.62	+
SE2	2.7	1.22	+	SE2	2.7	1.22	+
SE3	3	1.70	+	SE3	3	1.70	+
SO1	2	1.53	+	SO1	2	1.53	+
SO2	5	4.60	+	SO2	5	4.60	+
SO3	6	4.96	+	SO3	6	4.96	+
SW3	4.1	2.82	+	SW3	4.1	2.82	+

Table 3: *Length of biofilm produced by isolates on inner surface of tube and optical density of crystal violet stain adsorbed by biofilm and extraction of secondary metabolites.*

+ = Positive; - = Negative.

All the isolates form biofilms on inner surface of tube were within range of 2 mm to 6 mm in length. Optical density of crystal violet stain adsorbed by biofilm were within range of 1 - 5 at 530 nm. All isolates were observed for production of secondary metabolites.

Biofilm inhibition assay

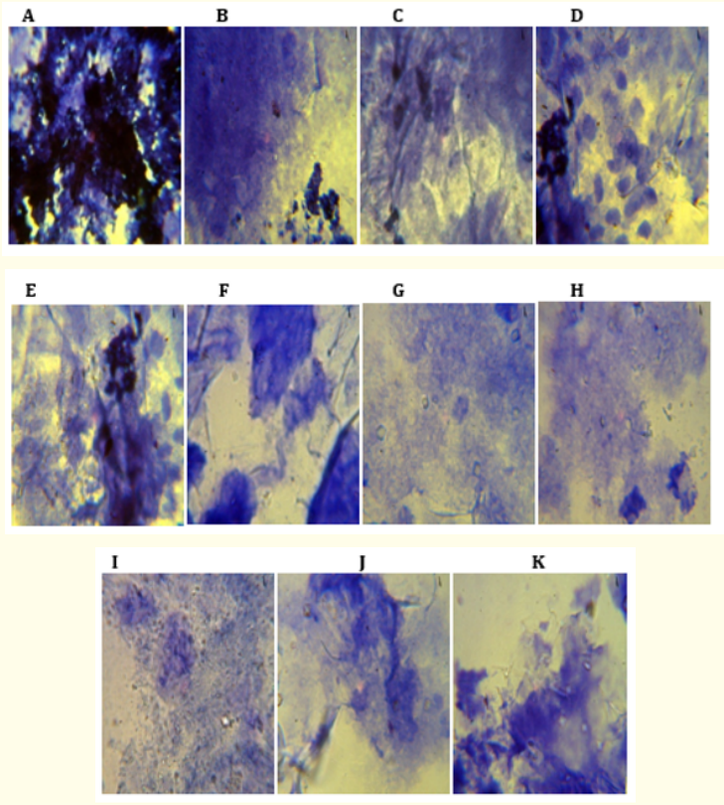

Figure 1: *Compound light microscopy: Detection of biofilm structures of P. aeruginosa attached to coverslip for biofilm inhibition assay. (A) control, (B) ExtSa1, (C) ExtSa2, (D) ExtSa3, (E) EXtSE1, (F) ExtSE2, (G) ExtSE3, (H) ExtSO1, (I) ExtSO2, (J) ExtSO3, (K) ExtSW3 [Ext- extracts code used against P. aeruginosa biofilm].*

All the extracts were observed for inhibition of *P. aeruginosa* biofilm showing more or less inhibition. Maximum inhibition was shown by extract ExtSE3 against *P*. aeruginosa biofilm.

Tube method

Length of biofilm produced by *P. aeruginosa* on inner surface of tube and optical density of Crystal violet stain adsorbed by biofilm after addition of inhibitor molecules extracted from isolate are cited in table 4.

Extract Code	Length of biofilm (mm) *(P. aeruginosa)*	O.D. of inhibition assay at 530 nm	Extract Code	Length of biofilm (mm) *(P. aeruginosa)*	O.D. of inhibition assay at 530 nm	Extract Code	Length of biofilm (mm) *(P. aeruginosa)*
Control	4	1.56	Control	4	1.56	Control	4
Ext Sa1	4.1	1.46	Ext Sa1	4.1	1.46	Ext Sa1	4.1
Ext Sa2	3.7	1.34	Ext Sa2	3.7	1.34	Ext Sa2	3.7
Ext Sa3	4	1.40	Ext Sa3	4	1.40	Ext Sa3	4
Ext SE1	3.8	1.38	Ext SE1	3.8	1.38	Ext SE1	3.8
Ext SE2	4	1.32	Ext SE2	4	1.32	Ext SE2	4
Ext SE3	4	1.30	Ext SE3	4	1.30	Ext SE3	4

Table 4: *TM: length of biofilm produced by P. aeruginosa on inner surface of tube and optical density of crystal violet stain adsorbed by biofilm after addition of inhibitor molecules extracted from isolates.*

P. aeruginosa formed biofilms on inner surface of tube were within range of 3.7 to 4.1 mm in length. Optical density of crystal violet stain adsorbed by biofilm were within range of 1 - 1.56 at 530 nm. All the extracts were observed for inhibition of *P. aeruginosa* biofilm showing more or less inhibition. Maximum inhibitions of *P. aeruginosa* biofilm was shown by extract ExtSE3.

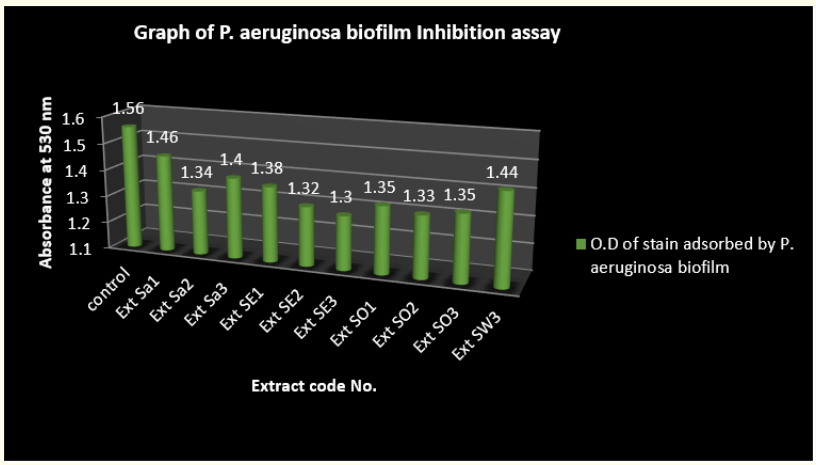

Figure 2: *Graph of P. aeruginosa biofilm inhibition assay. A maximum inhibition of P. aeruginosa biofilm was shown by extract ExtSE3.*

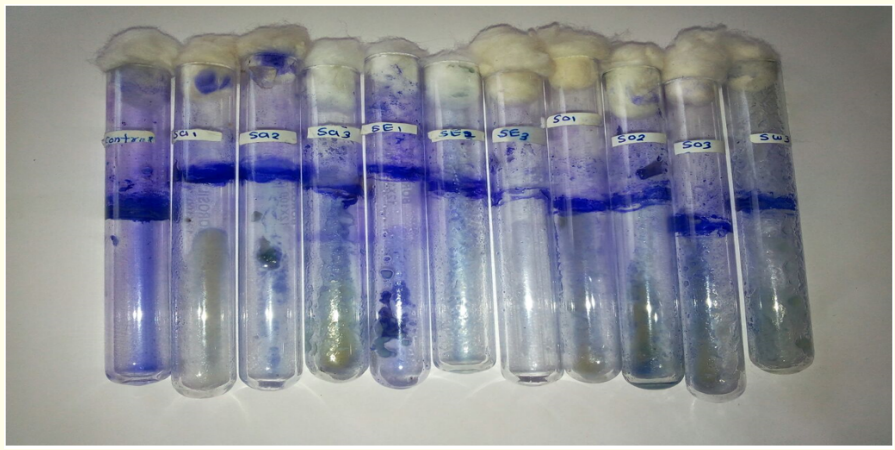

Figure 3: *Pseudomonas biofilm inhibition assay by tube method.*

Coastal region of Malvan is rich in culturable marine microorganisms which can also produce biofilm. A total of 10 bacterial strains were isolated from coastal waters, sediment, sand and soil of Coastal region of Malvan using the 2 media employed in the current study. All 10 isolated strains were subjected to chemical extraction of secondary metabolites and resulting extracts were evaluated for inhibition of growth of human pathogen gram negative *P. aeruginosa*. All chemical extracts showed inhibition of biofilm in *P. aeruginosa* assays. Out of 10 extracts, extract ExtSE3 showed significant inhibition of *P. aeruginosa* biofilm.

Conclusion

A total of 10 bacterial isolates having biofilm inhibition activity were obtained from 4 marine samples of sand, water, sediment and soil from coastal region of Malvan out of which seven marine strains were found to produce compounds reducing *Pseudomonas sp.* to form biofilms. All the isolates were found to produce biofilm and secondary metabolites as biofilm inhibitor molecules against *P. aeruginosa*

biofilm. Therefore, biofilm of all isolates can be used for treatment of waste water and extracts of secondary metabolites can be used as inhibitor against bacterial biofilms but extract obtained from isolate ExtSE3 was found to be most potent for biocontrol of biofilm according to biofilm inhibition assay.

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Conflicts of Interest

There are no conflicts of interest by any of the authors mentioned above.

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