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Isolation and Identification of Marine Biofilm Bacteria on Cement Panels at Chennai Port, India

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Abstract: Morphological and biochemical analyses of bacterial strains were carried out in the biofilm formed on the concrete cement panels suspended in Dr. Ambedkar dock at Chennai port. Chemical constituents (macromolecules) absorbed in the biofilm were carbohydrates, proteins, calcium, magnesium, nitrite, nitrate, phosphate, total suspended solids, and a total viable count. Hydrological characteristics such as pH, salinity, temperature, dissolved oxygen, nitrate, nitrite, and phosphate were analyzed during the study period. Bacterial colonization in the biofilm sample was investigated by five different media Zobell marine agar (ZMA), blood agar (BA), chocolate agar (CA), nutrient agar (NA) and MacConkey agar (MAC). From the biofilm sample, a total of 16 types of bacterial strains were isolated. The majority of the strains are motile, gram-positive bacilli. Biofilm-forming bacteria were found by Congo red agar (CRA) media. Among the 16 bacterial strains, eleven types of bacterial strains produce biofilms.

Keywords: Biofilm, Bacterial colonization, Gram positive, Biochemical constituents

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Introduction

The marine environment serves as a habitat for a variety of unusual microorganisms, which create biologically active substances to adapt to specific environmental conditions. The first step in the biofouling process on submerged objects in the marine environment is the production of biofilms (Maki, 1999). The biofilm microorganisms have grown and connected to biotic and abiotic surfaces such as manmade surfaces, rocks, particle compounds, and organism surfaces (Flemming and Wuertz, 2019). Biofilms often begin with the adhesion of bacterial cells that change the surface's physicochemical characteristics and influence the adherence of subsequent colonisers such as algae, cyanobacteria, and protists (Wetzel, 2001; Dang and Lovell, 2016). Bacterial cells

adhering to surfaces, releasing extracellular polymers, and attracting a variety of micro-organisms to the substrate.

Bacterial cells and the extracellular polymeric matrix (EPM), in which the cells are embedded, make up the complex three-dimensionally structured community known as a biofilm. The majority of matrix components are carbohydrates. More specifically, the bacterial extracellular polymeric matrix is made up of lipids (1%-40%), proteins (1%-60%), nucleic acids (1%-10%), and polysaccharides (40%-95%) (Flemming *et al*., 2001). Exo-polysaccharides make up the majority of the biofilm matrix among these biopolymers. The EPS matrix which surrounds the biofilm, shield the cells from dehydration (Czaczyk and Myszka, 2007). It aids in shielding members of the biofilm community from environmental challenges (Flemming, 1998; Liu *et al*., 2004). Bacteria are protected by an extracellular polymeric matrix that aids in-- (i) trapping nutrients and toxic substances, (ii) stabilising extracellular enzyme activity by buffering pH and salinity fluctuations, (iii) quorum sensing, (iv) exchanging genetic information, (v) providing physical anchorage, and (vi) protecting against predators (de Carvalho and Fernandes, 2010; Decho and Gutierrez, 2017).

Bacteria and diatoms predominate in marine biofilms (Railkin, 2003). In order to attach themselves to the substrate, bacteria and other colonising microorganisms encourage further growth and the recruitment and settlement of macro-organisms (Chambers *et al*., 2006), leading to biofouling. Bacteria identified in biofilms may influence the mature biofilm form and function because they are the first to colonise a surface (Dang *et al*., 2008; Dang and Lovell, 2016).

Biofilms are frequently utilised in environmental monitoring studies because of the buildup of nutrients and contaminants as well as their distinctive reaction to changes in water quality (Gold *et al*., 2002; Mages *et al*., 2004). The biogeochemical cycles and dynamics of coastal ecosystems are significantly influenced by biofilms (Schorer and Eisele, 1997). Lower chordates and larval forms of sessile invertebrates settle as a result of biofilm growth. Biofilm can be a key mediator in the settling and metamorphosis of invertebrate larvae (Maki *et al*., 1988).

The behavior of marine biofilms can be studied by creating biofilms on artificial surfaces, which allows for the observation of initial microbial attachment, community succession, and microbe-host interactions (Pedersen, 1990; Chung, 2010; Zhang, 2014; Lee, 2014). The composition of marine microbial biofilms is generally different from the surrounding seawater, according to assessments of artificial surface supported biofilms, and can be modified by both location (Lee, 2014) and substratum type (Pedersen, 1990; Chung, 2010; Zhang, 2014). In addition, environmental factors including pH, dissolved oxygen, light, and nutrient availability have a significant impact on how marine biofilm micro-biomes are structured (Orland *et al*., 2019). The purpose of the current study was to examine the biochemical analysis of the biofilm, the hydrology of the marine water, and the types of bacteria participating in the production of biofilm on artificial concrete cement panel.

Materials and Methods

This study was conducted at Dr. Ambedkar Dock region of Chennai port (Fig. 1). Chennai port has 24 berths, three docks with draughts ranging from 8.5 to 16.5 metres, and they are named Bharathi, Jawahar, and Dr. Ambedkar docks. The panels were properly cleaned, dried, and rinsed with 70% alcohol prior to immersion (Rao, 2003). Artificial concrete cement panel (21cm x 21cm x 1 cm) bound with a 2 m nylon rope was suspended at a depth of 1 m in the Chennai port water on March 2019.

Sample collection:

After being exposed for seven days, a concrete cement panel was removed and brought to the lab in a box filled with filtered seawater. Using a sterile nylon brush, the biofilm was scraped. The removed material was then mixed with 100 ml of filtered, sterilized seawater and kept at -20 °C. One

Fig. 1: Location of Dr. Ambedkar Dock in Chennai port.

portion of this biofilm suspension was used to analyse the microfouling bacterial community, and the other portion was used for chemical constituent analyses of biofilm.

Hydrological parameters:

During the study period, hydrological characteristics of marine waters, including pH, temperature, salinity, dissolved oxygen, nitrite, nitrate, and phosphate were estimated by standard methods of APHA (2005). Triplicates of each analysis were carried out. Standard deviation was used to express the data as mean.

Chemical constituents of biofilm:

The total amount of suspended solids, carbohydrates, proteins, nitrites, nitrates, phosphates, calcium, magnesium, chlorophyll a and the total number of viable counts in biofilms growing on the concrete-cement panel were measured. To measure the above components, the resulting biofilm suspension was brought to a defined volume and filtered through a GF/C membrane filter (0.45, Whatman).

Based on the sample and the methodology described by Parsons *et al*. (1984), the total suspended solids in the biofilm were estimated. Total Viable Count (TVC) was calculated from the biofilm after serial dilution. In a sterile petridish, 20 ml of the material was placed on the surface of Zobell marine agar (ZMA) and incubated at 28ºC for 24 h (Buckand Cleverdon, 1960; Moat and Foster, 1995). Viable colony counts were obtained by visually counting them. According to Lowry *et al*. (1951) and Dubois *et al*. (1956), the total protein and carbohydrate contents of the biofilm were calculated using the phenol-sulphuric acid method with glucose as the standard and Lowry's methods using crystalline bovine serum albumin as the standard, respectively. The techniques described by Venugopalan and Paulpandian (1989) were applied for the quantification of nitrate, nitrite, phosphate, calcium and magnesium.

Isolation and identification of biofilm bacteria:

Serial dilutions of the biofilm slimes formed on the concrete cement panel were prepared, and the relevant dilutions were then applied to nutrient agar, Zobell marine agar, MacConkey agar, blood agar, and chocolate agar. The agar plates were incubate at 37ºC for 24 h. Following a 24-h incubation period, clear colonies were examined and characterised based on Bergey's manual of determinative bacteriology (Schaal, 1985, Sakazaki *et al*., 1986; Boon *et al*., 2001; Garrity *et al*., 2001). In order to purify the bacteria, bacterial colonies were chosen based on colonial morphology and repeatedly streaked onto the plates. The long-term cultures were kept alive on 4°C Zobell marine agar slants.

Standard biochemical tests were used to characterise purified typical bacterial colonies. Smears from the pure cultured bacterial strains were prepared on transparent slides, stained with gram stain, and examined for their morphology. Morphological characteristics of bacterial strains

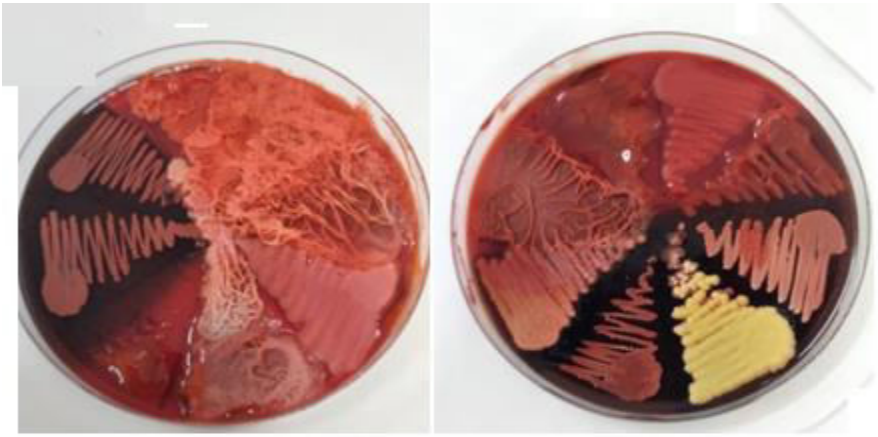

Fig. 2: Biofilm formation (black colonies) in Congo Red Agar plates.

Table 1: Hydrological parameters of Chennai port water during March 2019

S. No.	Parameters	Mean ±SD
1	pH	7.76 ± 0.25
2	Salinity (ppm)	34.65 ± 1.45
3	Temperature (ºC)	24.66 ± 1.52
4	Dissolved oxygen (mg/l)	5.1 ± 0.98
5	Nitrate (mg/l)	23.46 ± 1.90
6	Nitrite (mg/l)	0.23 ± 0.03
7	Phosphate (mg/l)	0.72 ± 0.24

were observed under a microscope for 100 X oil immersion to identify the bacterial strains up to genus level based on the standard biochemical tests like gram staining, colony colour, motility, elevation, colony shape, and cell shape.

Detection of biofilm formation:

Biofilm formation was determined for all isolates using the Congo red agar technique (CRA) (Fig. 2), as reported by Freeman *et al*. (1989) and Jain and Agarwal (2009). CRA plates were then infected with test organisms and incubated aerobically at 37 °C for 24 h. Biofilm formation was indicated by black colonies with a dry crystalline quality, whereas no-biofilm formation was recognised by red or pink crystalline colonies.

Results

The water samples that were collected for this study seemed to be odorless and colorless. Table 1 shows the physical and chemical parameters for the Chennai port water during the study period of March 2019. The temperature was 24.66^{o}C, and the mean pH value was 7.76. The mean value of dissolved oxygen was found to be 5.1 mg/l, and the average salinity was 34.65 ppm. Nitrate, nitrite, and phosphate were found to have mean concentrations of 23.46 mg/l, 0.23 mg/l, and 0.72 mg/l in the water sample, respectively.

Table 2 shows the biochemical components of a seven day old biofilm sample per 100 ml. There were 23.18 g of protein and 1.22 g of carbohydrate, respectively. The nutritional amounts of nitrite, nitrate, and phosphate were 54.5 ppm, 120.5 ppm, and 204.5 ppm, respectively. Chlorophyll A was discovered to be present in the biofilm. 145.6 mg of magnesium and 204.5 mg of calcium were found. The total suspended solids content was 1657.8 ppm and the

Table 2: Biochemical constituents of biofilm sample

S. No.	Chemical parameters	Values
1	Carbohydrate	1.22 g
2	Protein	23.18 g
3	Nitrite	54.5 ppm
4	Nitrate	120.5 ppm
5	Phosphate	204.5 ppm
6	Calcium	204.5 mg
7	Magnesium	145.6 mg
8	Total suspended solids	1657.8 ppm
9	Total viable count	55 cfu/ml

Table 3: Morphological characteristics of the bacterial strains

Bacterial strains	Morphological tests						
	Gram Stain	Morphology	Motility	Elevation	Colour	Colony shape	Cell shape
S1	+	Bacilli	-	Flat	White	Irregular	Chains
S2	+	Bacilli	+	Flat	White	Filamentous	Short rod
S3	+	Bacilli	+	Flat	White	Irregular	Chains
S4	+	Bacilli	-	Flat	White	Translucent	Curved chains
S5	+	Bacilli	-	Opaque	White	Irregular	Chains
S6	+	Bacilli	+	Opaque	Pink	Irregular	Chains
S7	+	Bacilli	-	Convex	White	Translucent	Curved chains
S8	+	Bacilli	+	Flat	Greyish white	Irregular	Short rod
S9	+	Bacilli	+	Flat	Grey white	Filamentous	Short rod
S10	+	Bacilli	+	Flat	Grey	Irregular	Short rod
S11	+	Cocci	-	Convex	White	Translucent	Tetrad
S12	+	Cocci	-	Convex	White	Translucent	Tetrad
S13	+	Bacilli	-	Flat	Brownish	Translucent	Tetrad
S14	+	Bacilli	+	Flat	White	Translucent	Long chain
S15	+	Bacilli	+	Flat	White	Translucent	Shot rod
S16	+	Bacilli	+	Flat	White	Translucent	Long chain

total viable count was 55 cfu/ml.

Morphological and Biochemical analysis of biofilm bacteria:

Morphological and biochemical features of the marine biofilm bacteria are shown in Tables 3 and 4. The majority of the strains were bacilli and motile, and all of the strains were gram-positive. Overall, five isolates were discovered on the nutritional agar and zobell agar mediums, while two isolates were discovered on the blood agar, MacConkey agar, and chocolate agar media. At 6% NaCl, every isolate demonstrated good growth, confirming their marine origin. A total of 16 isolated strains, designated S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14, S15, and S16, were isolated from the biofilm sample. S10 and S11, two of these strains were cocci, lacked motility. Eleven different species of bacteria that create biofilms were found among the 16 bacterial isolates such as S2, S3, S4, S6, S7, S8, S9 S11, S12,

Table 4: Biochemical characterization of bacterial strains

Biochemical Tests	Bacterial strains															
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MR	-	-	-	-	-	-	-	+	-	-	-	-	+	_	+	-
VP	-	-	-	-	+	+	+	-	+	-	+	-	+	-	+	+
Oxidase	+	+	-	+	+	-	-	+	+	-	-	-	+	+	+	-
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Congo Red	-	+	+	+	-	+	+	+	+	_	+	+	-	+	_	+
Citrate	-	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-
TSI GAS	K/A	K/A	K/A	A/A	K/A	K/A	A/A	A/A	A/A	A/A	K/A	A/A	A/A	A/A	A/A	K/A
Nitrate	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
MMM	F	F	F	F	F	F	F	NF	NF	NF	F	NF	NF	F	F	F

S14, and S16. S1, S2, S3, S4, S5, S7, S8, S9, S10, S13, S14, S15, and S16 were Firmicutes, Bacilli family, and S6, S11, and S12 were Bacillota, Staphylococcaceae family.

Discussion

The physical and chemical water quality of Chennai port remained steady during the investigation and offering a good environment for study on the production of bacterial biofilm. The concentration of protein and carbohydrates rises with prolonged exposure (D'souza and Bhosle, 2003). Satheesh and Wesley (2010) discovered that within the first 24 h of biofilm formation on acrylic coupons, the concentration of protein and carbohydrate were 0.41 mg, and 0.28 mg, respectively. In this study seven-day biofilm investigation showed protein and carbohydrate concentration (100 ml), 1.22 g and 23.18 g, respectively. It indicates that the exposure duration raises higher values of concentration.

Rice *et al*. (2000) and D'souza *et al*. (2005) reported that bacterial populations produce more proteins and fewer carbohydrates in the early stages of biofilm growth. Similarly, in this study, more protein and fewer carbohydrates were found.

Nitrite, nitrate, and phosphate are only a few of the nutrients that the bacteria in the biofilm are able to acquire (Sutherland, 2001). The biofilm sample had higher levels of nitrate, nitrite, and phosphate than the marine water. The types of bacteria in the biofilm vary depending on the availability of nutrients, oxygen, and water pH (Gilbert *et al*., 1990).

Divalent cations, such as Ca^{2+} and Mg^{2+}, increase the stability of biofilm. Ca^{2+} plays a key role in the establishment of biofilms (Koerstgens *et al*., 2001; Lattner *et al*., 2003). In the current investigation, the calcium concentration (204.5 mg/l) was higher than the magnesium content (145.6 mg/l). In addition to being involved in bacterial adherence to substrates, calcium is essential for the formation of bacterial biofilms (Craven and Williams, 1998; *Waligora et al*., 1999). The wide range of bacterial adherence is supported by total suspended solids and total viable counts.

According to Sravankumar *et al*. (2014) the marine biofilm on the iron panels along the Visakhapatnam coast contained ten different types of bacteria. Bacillus sp. was the most frequent and pervasive of these ten species. The recent study indicates that the biofilm in the water at Chennai's port is made up of 16 different bacterial species. Three of these bacterial types were Staphylococcaceae family, and 13 of them were Bacilli family. It was shown that the Bacilli species

dominated the 16 different bacterial strain types examined in this investigation.

At Ennore harbour, marine bacteria from the ship's hull were extracted, cultivated, and 16 strains were identified by Inbakandan *et al*. (2010). In comparison to the high GC, Gram-positive bacteria (18.75%), G-Proteobacteria (12.5%), CFB group bacteria (6.25%), and Enterobacteria (6.25%), the 16 strains that belonged to the representatives of the Firmicutes were prevalent (56.25%). Similarly, 16 different bacterial strains were detected in this study, but all were Gram-positive and Firmicutes predominated.

Conclusion

Bacteria are the most varied and significant microorganisms on marine surfaces, and the shape, dynamics, and role of established biofilm communities may be determined by early invaders. Hydrological parameters of Chennai port water and chemical elements of a seven-day biofilm aid in the establishment of microfouling communities on an artificial concrete cement panel. The seven-day biofilm sample enclosed 16 marine bacterial isolates. These specific inter-population interactions determine the composition, organization, and roles of surface-associated microbial communities.

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