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Isolation and Characterization of Biofilm Producing Bacteria from Water Treatment Tank

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Abstract

Biofilm is a community of bacteria and other microorganisms, which provides various advantages like nutrition, protection from host defences, etc. to name a fewHowever, biofilm is the main cause of the increase in antibiotic resistant microorganisms. Development of biofilms occurring on the inner surface of storage vessels offers a suitable medium for the growth of microorganisms and consequently contributes to the deterioration of treated drinking water quality. Biofilm formers secrete enzymes into the extracellular space to hydrolyze macromolecules into constituents that can be imported for microbial nutrition. The present study aims at the screening, isolation and characterization of biofilm producing bacteria from water treatment tanks using tube test, plate assay and ELISA technique. The 12 predominant isolated bacterial colonies were further studied for the presence of extracellular enzymes which are responsible for their growth and biofilm formation. Differential plate assay techniques were used for the qualitative detection of exoenzymes. Seven out of twelve bacterial isolates showed positive results for biofilm formation in tube assay and furthermore, was confirmed by ELISA plate assay. MTT assay was also performed to check for the cell viability. The potential strains were identified using the standard morphological and biochemical tests. From the obtained results it can be concluded that the exoenzyme activity is not solely mediated by community composition, but possibly influenced by bacterial communication, which is known to regulate such pathways in many bacteria.

Keywords: Biofilm; exoenzyme activity; ELISA technique; water treatment tank

Introduction

Bacteria predominantly remain in the self-produced polymeric matrix, adherent to inert and living surfaces. This microenvironment community of bacteria is known as biofilm and is composed of many other microorganisms. The studies have revealed that there are many bacterial species that form biofilm. According to Costerton *et al.* [1] the “bacterial strain” growing in a biofilm on a surface are generally more resistant to many antimicrobial agents than the one growing in a free-swimming state. Lawrence *et al.* [2] revealed that the bacteria in most biofilms are embedded in extracellular polymeric substance, these exocellular polymers comprise the capsular… polysaccharides which form a cohesive layer or capsule covalently linked to the cell surface, and the exopolysaccharides (EPS), which form a slime layer loosely attached to the cell surface or secreted into the environment. During the process of colonization on particular surfaces, bacteria produce extracellular polymeric substances, which “construct” with “forms the backbone of” bacteria produce extracellular polymeric substances, which forms the backbone of the biofilm matrix [3]

The colonization of bacteria on abiotic materials such as metal surfaces, suspended particles or on biotic surfaces is thought to be one of the microbial survival strategies as it provides microorganisms with various important advantages such as increased access to nutrients, shelter from predation, protection against antibiotics, toxins and maintenance of extracellular enzyme activities [4,5]. The biofilm formation process is dependent on type of microorganism and various ..environmental conditions like pH, ionic strength and temperature. Previously mentioned parameters are also considered as important factors in altering the physicochemical.. properties of bacterial surface and the microbial adhesion to the surface [6-8].

Biofilms usually develop on all surfaces in contact with non-sterile water [9]. Pathogens, even present below detection limit in water, can accidentally attach to biofilms which then can act as their environmental reservoir and represent a potential source of water contamination. It has to be emphasized that bacterial numbers from the water phase do not indicate the quantity of biofilms nor their location. There are very high chances of health risk if humans are exposed to the contaminated water. Infection can occur by ingestion of contaminated water, inhalation of aerosols containing pathogens or contact of skin, mucous membranes, eyes and ears [10]. Metabolic products such as hydrogen sulfide and nitrite or endotoxins also impact the hygienic quality of water. Its aesthetic quality can be impaired by discoloration or turbidity. In some cases, biofilms support the trophic food chain, leading to occurrence and growth of protozoa and eventually invertebrate animals. Biofilm borne pathogens can considerably contribute to water associated nosocomial infection, particularly in the water systems of hospitals and other healthcare facilities rendering them critically unhygienic [11-13]. Reports suggests that about 95% of the biofilm forming bacterial strains isolated from a drinking water system are located at its surfaces while only 5% are found in the water phase [14] used for quality control [14].

In this study, bacteria were isolated, screened and characterized from the water treatment tank using tube test, plate assay and ELISA (Enzyme Linked Immunosorbent Assay) technique. Differential plate assay techniques were used for the qualitative detection of exoenzymes.

Materials and Methods

Sample collection

Sediment samples were collected from water treatment tank in which the biofilm production was observed and was aseptically

brought in Microbial Biotechnology Laboratory, School of Biosciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India.

Isolation of biofilm forming bacteria

The biofilm forming bacteria were isolated using serial dilution and spread plate method on nutrient agar (NA) plates. 100 µL of 10^{-4} ad 10^{-6} dilutions were spread plated on sterilized NA plates for the isolation of bacteria from sediment samples. The inoculated plates were incubated at 37 ± 2°C for 24 h. Morphologically different bacterial strains were selected and maintained on NA plate at 4°C until further analysis.

Screening of biofilm formers

Primary screening: Test tube method was used for the primary screening of biofilm formers. Pure bacterial strains were allowed to grow in 10 mL of Luria Bertani (LB) media in test tubes. The inoculated tubes were incubated at 37°C for 24 h. After the incubation period, contents of the tubes were gently tapped and removed. Furthermore, the tubes were washed with Phosphate Buffer Saline (PBS) at pH 7.3 and were allowed to dry at room temperature. The dried tubes were then stained using 0.1% (w/v) crystal violet (cv). Excess stain was removed by washing the tubes with distilled water. Biofilm formation in tubes was further confirmed by observing the presence of visible film on the wall and bottom of the tube.

Secondary screening: Confirmatory tests for the screening of biofilm formers were performed using the 96 well microtiter plate technique (ELISA) [15]. The wells were individually inoculated with100 µL of LB broth (containing biofilm formers) and sterile water. Sterilised media was usedas blanks. The plates were incubated at 37± 2°C for 24 h to 72 h and analysis was made at 4, 8, 12, and 24 h. The viability of the isolates was checked in LB broth and were screened for the formation of biofilm using tube assay following Christensen et al. [16]. Biofilm biomass was assessed using a protocol described by Mowat *et al.* [17]. Briefly, at each time interval (i.e., 4, 8, 12, and 24 h), the spent culture medium was removed from each well and the adherent cells were washed with phosphate-buffered saline (PBS; 0.1 M, pH 7.2). These were air dried and 200 µL of 0.5% (w/v) CV solution was added for 30 min. The solution was then removed until excess stain was removed. The biofilms were destained by adding 200 µL of 95% ethanol to each well. The ethanol was gently pipetted to completely solubilize the CV for 1 min, the ethanol was transferred to a clean 96 well microtiter plate, and the was read using a microtiter plate reader. The OD values are proportional to the quantity of biofilm biomass, which comprises of extracellular polymeric material (the greater the quantity of biological material, the higher the level of staining and absorbance). The following formula was used to interpret the ELISA plate results:

$$SBF=AB-CW$$

Where, SBF: Specific biofilm formation, AB: OD at 570 nm of the attached and the stained cells, CW: OD at 570 nm of the control wells with only broth.

Biofilm formation was considered strong, moderate, weak or negative if the value of SBF was > 3, in between 0.2 and 0.299, in between 0.1-0.199 or < 0.1 respectively.

MTT Assay

MTT(3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) is a yellow soluble tetrazolium salt that is converted into an insoluble purple crystal by metabolically active cells [18]. MTT solution (5 mg/mL) was prepared in sterile PBS at room temperature and stored at 4°C in a dark, screw-cap container. At each time point, 0.2 mL of MTT solution was added to each well and the 96 well plates were incubated at 37°C for 3 h. After this period, the supernatant was discarded. and 0.2 mL dimethyl sulfoxide (DMSO) was added to each well to solubilize the MTT, which had been cleaved into an insoluble purple formazan through the metabolism of the live cells. Biofilm development was assayed by loading 0.2 mL of the solubilized MTT into a flat-bottom, 96-well polyvinyl chloride microtiter plate, and absorbance measured at OD_{570} nm using a microtiter plate reader.

Enzyme assay

Plate assay method was used to test enzyme activity for protease, cellulase, chitinase, pectinase and lipase enzymes on substrate agar plates [20, 21].

a. **Proteolytic activity:** Casein agar medium which was supplemented with green indicator dye (BCG agar medium) was used to determine the proteolytic activity of the obtained microorganisms. The composition (g/L) of the media was: peptic digest of animal tissue 5, yeast extract 1.5, sodium chloride 5, beef extract 1.5, casein 10, bromocresol green 0.0015%, agar 15, distilled water 1 L). The plates were inoculated and incubated at 37°C for 24-48 h. The proteolytic activity was observed by formation of clear halos around the colonies after incubation.

b. **Cellulose activity:** Carboxymethyl cellulose (CMC) agar medium was used to determine the cellulase activity of the obtained microorganisms. The composition (g/L) of media was as follows: CMC 10, KH_2PO_4 1, K_2HPO_4 1, $MgSO_4$ 0.2, $FeCl_3$ 0.05, $CaCl_2$ 0.02, NH_4NO_3 1, yeast extract 5, agar 20, distilled water 1 L. The plates were inoculated and incubated at 37°C for 24-48 h. After incubation, the plates were flooded with 0.3% congo red solution for 20 min. The plates were then destained using NaCl solution. Cellulase activity was observed by clear halos obtained around the colonies after destaining.

c. **Pectinase activity:** Vincent agar medium was used to determine the pectinase activity. The composition (g/L) of the media was: pectin 10, K_2HPO_4 0.5, KH_2PO_4 1, $MgSO_4$ 0.25, NH_4NO_3 2, KCl 0.5, $CaCl_2$ 0.1, KNO_3 0.6, yeast extract 1, $NaNO_3$ 2, agar 20, distilled water 1 L. T The plates were inoculated and incubated at 37°C for 24-48 h. Thereafter, the plates were flooded with iodine solution (Iodine 1g, KI 5g/ 330 mL of distilled water) for 15 min after incubation. The stain was drained off and clear halos obtained after destaining indicated the presence of pectinase activity

d. **Amylase activity:** Starch agar medium was used to determine amylase activity. The composition (g/L) of the media was as follows: meat extract 3, starch 2, peptic digest of animal tissue 5, agar 15, distilled water 1 L. The plates were inoculated and incubated at 37°C for 24-48 h. Thereafter, the plates were flooded with iodine solution. The stain was drained off and amylase activity was affirmed by presence of clear halos around

the colonies after destaining.

e. **Lipase activity:** Tributyrin agar medium was used to determined lipase activity. The composition (g/L) of the medium is as follows: glycerol tributyrin 10, peptic digest of animal tissue 5, yeast extract 3, agar 20 and distilled water 1 L. The plates were inoculated and incubated at 37°C for 24-48 h. Lipase activity was indicated by presence of clear halos around the colonies after incubation.

f. **Chitinase activity:** Chitin agar medium was used to determine chitinase activity. The composition (g/L) of the media was as follows: chitin 10, NaCl 1, KH_2PO_4 3, NH_4Cl 1, agar 20 and distilled water 1 L. The plates were inoculated and incubated at 37°C for 24-48 h. Thereafter, the plates were flooded with iodine solution. The stain was drained off and chitinase activity was confirmed by presence of clear haloes around the colonies.

g. ***Relative enzyme activity (RA)*** Fresh samples were used for enzyme assays to ensure that the enzyme activity was maximal [19]. Each replicate was examined for the presence of a clear zone around the colony, and the diameters of the colony and of the clear zone (activity zone) were measured. The measurement was repeated in two mutually orthogonal dimensions, and the mean value calculated. The 'relative enzyme activity' (RA) was calculated using the following formula:

Relative enzyme activity= (Clear zone diameter-Colony diameter) (Colony diameter)Remove bold from formula (Colony diameter)

Isolates exhibiting an RA of greater than 1.0 was classified as having a 'significant activity'.

Identification of biofilm formers

The biofilm formers were characterized using Grams' staining techniques, morphological analysis and biochemical tests.

Results and Discussion

Isolation of bacterial isolates

Twelve well defined, morphologically different pure bacterial strains were isolated on NA plates via spread-plate technique from sediment samples collected from wastewater treatment plants. These strains were designated as MB1-MB12 (Figure 1). Furthermore, these strains were maintained at 4°C until further analysis.

Screening of biofilm formers

Bacterial strains MB1-MB12 were screened for the production of biofilm. Out of the 12 isolates, 7 bacterial strains were observed to develop biofilm in tube assay after the incubation period of 24 h (Table 1). The results have been pictorially represented in Figure 2. The results were further confirmed using the secondary screening assay. The results of the ELISA plate assay are represented in table 2. It was found that all bacterial strains were strong biofilm producers, with SBF value greater than 0.5. Strain MB4 was observed to be the maximum biofilm producer with SBF value of 0.898.

MTT assay

Biofilm formation by the potential bacterial strains grown in different culture media over 24 h was characterized by MTT assay.

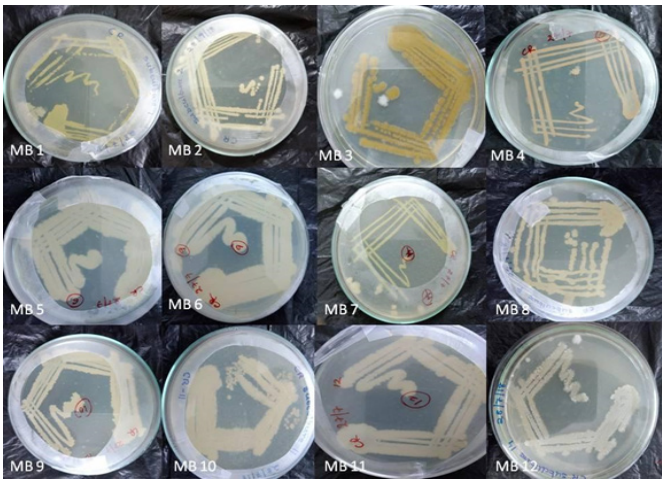

Figure 1: MB1- MB12 bacterial isolates from water treatment tank isolated on NA

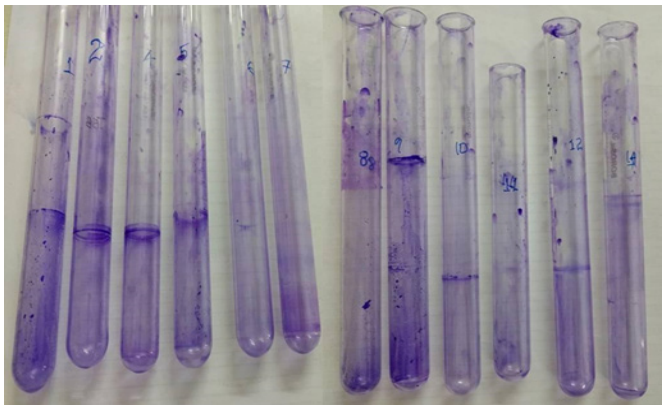

Figure 2: Stained tubes showing biofilm attachment

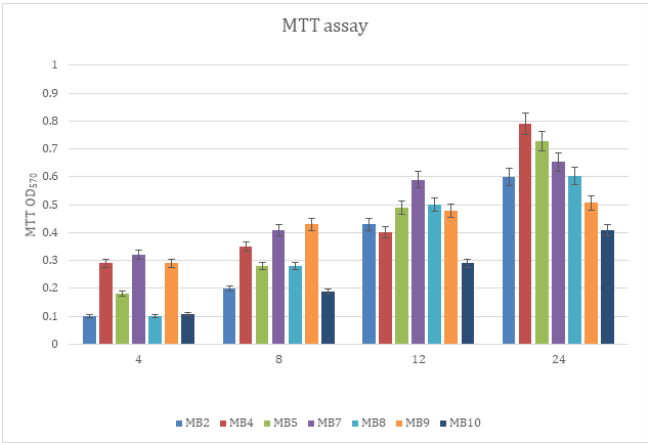

Figure 3: MTT assay for bacterial biofilm formers

Table 1 Tube assay activity Remove bold headings

Bacterial isolates	Tube assay Activity
MB2	++
MB4	+++
MB5	+
MB7	+
MB8	++
MB9	++
MB10	+

Table 2: ELISA plate result interpretation after 24 h

Isolate	FORMULA AND INTERPRETATION	
	AB-CW	Remark
MB2	0.698	Strong
MB4	0.898	Strong
MB5	0.728	Strong
MB7	0.654	Strong
MB8	0.603	Strong
MB9	0.507	Strong
MB10	0.409	Strong

Table 3: Enzymatic activity of bacterial isolates

Isolates	Enzymes					
	Xylanase	Chitinase	Amylase	Pectinase	Lipase	Protease
MB2	Positive	Positive	Positive	Positive	Positive	Positive
MB4	Positive	Positive	Positive	Positive	Positive	Positive
MB5	Positive	Negative	Positive	Negative	Positive	Positive
MB7	Negative	Positive	Positive	Positive	Positive	Negative
MB8	Positive	Positive	Positive	Positive	Positive	Positive
MB9	Positive	Positive	Positive	Positive	Positive	Positive
MB10	Positive	Positive	Positive	Positive	Positive	Negative
MB11	Positive	Positive	Positive	Positive	Positive	Negative
MB12	Negative	Negative	Positive	Negative	Positive	Negative

Table 4: Morphological identification of bacterial isolates

Isolates	Form	Elevation	Pigmentation	Margin	Opacity	Surface
MB2	Irregular	Flat	Yellow	Entire	Opaque	Smooth ,glistening
MB4	Irregular	Flat	Off-white	Undulated	Opaque	Rough ,wrinkled
MB5	Irregular	Raised	Orange	Entire	Opaque	Smooth ,moist ,slimy
MB7	Irregular	Flat	Pink	Entire	Opaque	Smooth
MB8	Regular	Flat	Off-white	Undulated	Opaque	Smooth ,glistening ,moist
MB9	Regular	Flat	Light yellow	Entire	Opaque	Smooth ,glistening ,moist
MB10	Irregular	Flat	Off-white	Undulated	Opaque	Rough ,wrinkled

Table 5: Biochemical profile for bacterial isolates

Test	MB2	MB4	MB5	MB7	MB8	MB9	MB10
Identification	Staphylococcus sp.	Enterococcus sp.	Escherichia coli	Shigella sp.	Proteus sp.	Pseudomonas sp.	Staphylococcus sp.
Gram's reaction	+ cocci	+ cocci	- rod	- rods	- rods	- rods	+ cocci
Indole	-	-	+	+	-	-	-
Motility	+	+	+	-	+	+	+
Arrangement	pairs ,tetrads	pairs /chains	single	single	single	single	pairs ,tetrads
MR	-	-	+	+	+	-	-
VP	-	+	-	-	-	-	-
Citrate utilization	+	-	-	-	-	+	+
H_2S production	-	-	-	-	+	-	-
Urease activity	+	-	-	-	+	-	+
Lactose	+	-	+	-	-	-	+
Sucrose	+	-	-	-	-	-	+
Glucose	+	+	+	+	+	-	+
Mannitol	+	+	+	+	-	+	+
Starch hydrolysis	-	+	-	-	-	-	-
Gelatin liquefaction	-	+	-	-	+	+	-
Catalase activity	+	-	-	+	-	+	+
Oxidase activity	-	-	-	-	-	+	-
Nitrate reduction	+	-	+	+	+	-	+

Table 6: TSI test

Isolates	Gas	Fermentation	Source	H_2S Production
Control	Negative	Yellow/Yellow	No source	negative
MB1	Negative	Red/Red	No G/L/S fermentation	negative
MB2	Negative	Red/Yellow	Glucose fermentation	negative
MB4	Negative	Red/Yellow	Glucose fermentation	negative
MB5	Negative	Red/Yellow	Glucose fermentation	negative
MB6	Negative	Red/Yellow	Glucose fermentation	negative
MB7	Negative	Yellow/Yellow	G/L/S fermentation	negative
MB8	Negative	Red/Yellow	Glucose fermentation	negative
MB9	Negative	Red/Red	No G/L/S fermentation	positive
MB10	Negative	Red/Red	No G/L/S fermentation	negative
MB11	Negative	Yellow/Yellow	G/L/S fermentation	positive
MB12	Negative	Red/Red	No G/L/S fermentation	negative

*Key: G: Glucose; L: Lactose; S:Sucrose

Table 7: Carbohydrate fermentation test

Isolates	Dextrose	Galactose	Fructose	Maltose	Mannose	Xylose
MB1	+	+	-	+	-	+
MB2	+	+	+	+	+	+
MB4	+	+	-	+	+	+
MB5	+	+	-	+	+	+
MB6	+	+	+	+	+	+
MB7	+	+	+	+	+	+
MB8	+	+	+	+	+	+
MB9	+	+	+	+	+	+
MB10	+	+	+	+	+	+
MB11	+	+	+	+	+	+
MB12	++	+	+	+	+	+
MB14	-	+	+	+	+	+

*Key: +:Positive; -:Negative

Figure 3 shows the mean value of absorbance level for MTT staining (viable cells). In the current study, MTT values did not correlate with biofilm biomass assessed by CV, mainly in older biofilms, that is, 24 h aged. Older biofilms showed a higher EPS production, which may interfere in the assessment of MTT to cell. Thus, MTT may not be the best method to evaluate cell viability in mature biofilms.

Enzyme assay

The potential bacterial isolates were further screened for the exoenzyme production. The results are tabulated in table 3. It was observed that all the 7 potential strains were capable of producing exoenzymes amylase and lipase. Previous reports suggests that exoenzymes help in adherence to the solid surfaces, thereby developing biofilm.

Identification of bacterial isolates

The bacterial isolates were identified using morphological analysis and biochemical tests. The results are represented in table 4 – table 7.

Conclusion

Several bacteria were isolated from the sediment sample in contact with the biofilm affected area and analyzed. Twelve predominant bacterial colonies were selected for the further study. Seven out of the twelve bacterial isolates (MB2, MB4, MB5, MB7, MB8, MB 9 and MB10) produced maximum biofilm. These isolates were partially identified by studying the colony characteristics, staining and biochemical tests. Comparing with standard results in Bergy's Manual for Systematic Bacteriology, the isolates mainly belonged to group 18 and group 20 in the manual consisting of *Staphylococcus* sp., *Enterococcus* sp., *Escherichia coli, Shigella* sp., *Proteus* sp., and *Pseudomonas* sp. Enzyme activity for five enzymes were tested by plate assay method and only MB 2, MB 4, MB 8 and MB 9 showed positive results for all the five enzymes. From the obtained results it can be concluded that the exoenzyme activity is not solely mediated by community composition, but possibly influenced by bacterial communication, which is known to regulate such pathways in many bacteria. Furthermore, the results presented here show that the bacterial isolates are able to form biofilms under the applied conditions.

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Conflict of Interest

The authors have no conflict of interest to declare.

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