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EVALUATION OF ANTIOXIDANT PROPERTY OF EXOPOLYSACCHRIDES OBTAINED FROM BIOFILM PRODUCING BACTERIA

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ABSTRACT

Biofilm formed by various micro-organisms potentially provide a suitable microenvironment for efficient bioremediation processes. High cell density and stress resistance properties of the biofilm environment provide opportunities for efficient metabolism of number of hydrophobic and toxic compounds. Bacterial biofilm formation is often regulated by quorum sensing (QS) which is a population density-based cell–cell communication process via signalling molecules. Biofilm formation is a widespread phenomenon pertaining to microorganisms attaching itself to a surface that is in continuous contact with water. It occurs widely on the ships hulls, implanted medical devices, dentures, eyes due to non-compatible contact lenses, water distribution systems, showers tubes, cooling pipes in nuclear reactors, etc. Biofilms are responsible for loss of billions of dollars to the industries, since they cause fouling of ship hulls, water treatment and distribution systems, causing corrosion of pipes and thereby resulting in contamination. Biofilms are also of great interest in medical context since they result in persistent and chronic infections and also contaminate implanted devices. Environmentally and economically viable solutions are increasingly being investigated to prevent and control the biofilm formation. The current study is to isolate organisms from biofilm sample, identify them and extract the exopolysaccharides produced and testing the EPS for anti-oxidant activity.

KEYWORDS: Biofilm, exopolysaccharide, antioxidant activity.

1. INTRODUCTION

Biofilms are the most prevalent form of microbial life in both natural and processing environments. Biofilms in nature are composed of different microorganisms and include species of bacteria, archaea, yeasts, molds, algae or protozoans.[1] A biofilm may be described as a distinct entity, a multicellular organism comprised of diversely differentiated cells throughout, all with a common goal.[2] It can be defined as a community of microorganisms (bacterial, fungal, algal) attached to a liquid surface interface and enveloped within a matrix of exopolysaccharides and other biological constituents.[3] Costerton et al. (1978)[4] observed that communities of attached bacteria in aquatic systems were found to be encased in a glycocalyx matrix that was found to be polysaccharide in nature and this matrix material was shown to mediate adhesion. Donlan and Costerton (2002)[5] based on results obtained from microscopic observation and direct quantitative recovery techniques estimated that more than 99.9% of the aquatic microbes grow in biofilms on a wide variety of surfaces. Costerton et al. (1987)[6] affirmed that biofilm consists of a single cell and micro-colonies, all embedded in a highly hydrated, predominantly anionic exo-polymers matrix. Characklis and Marshell (1990)[7] went on to describe the other defining aspects of biofilms, such as the characteristics of spatial and temporal heterogenecity and the involvement of inorganic or abiotic substances held together in the biofilm matrix. Once biofilm has formed the bacteria within the biofilm are protected from phagocytosis and antibiotics.[8] Costerton et al. (1995)[9] emphasized that biofilms could adhere to surfaces and interfaces and to each other, including aggregates and floccules and adherent populations within pore spaces of porous media. Therefore a new definition for biofilm must be taking into consideration. They have not only the readily observable characteristic, i.e. cells irreversibly attached to a surface or interface, embedded in a matrix of extracellular polymeric substances which these cells have produced, including the non-cellular or abiotic components, but also other physiological attributes of these organisms, including the characteristics such as altered growth rate and the fact that biofilm organisms transcribe genes that planktonic organisms do not.

2. MATERIALS AND METHODOLOGY

2.1. SAMPLE COLLECTION

The biofilm was collected from a sewage in a locality of Palarivattom, Cochin, Kerala. The collected sample transport to lab in aseptically.

2.2 ISOLATION OF ORGANISM

The organisms from the biofilm sample were isolated by serial dilution method.

2.3 SCREENING

The screening of biofilm was carried out by qualitative and quantitative assay methods.

2.3.1 QUALITATIVE ASSAYS

- **TUBE ASSAY**

This was performed in accordance to **Christensen et al**.[10] This is a qualitative method for biofilm detection. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-weak/none, 2-moderate and 3-high/strong.

- **CONGO RED ASSAY**

Freeman et al.[11] have described a simple qualitative method to detect biofilm production by using Congo Red Agar (CRA) medium. The experiment was performed in triplicate and repeated three times.

2.3.2 QUATITATIVE ASSAY

INTENSITY OF BIOFILM

Crystal violet assay

The isolates were first inoculated on Trypticase Soy Agar (TSA), a growth medium, and incubated overnight at 37°C. Then, 1 colony was added to 5mL of Trypticase Soy Broth (TSB) and grown overnight at 37 °C in shaker at 200 rpm. 50 uL of overnight culture was then added to 5 mL of TSB and an overday culture was grown at 37°C for 2-3 hours in a shaker at 200 rpm. Using a spectrophotometer blanked with TSB, the overday incubation was stopped when the OD600 was between 0.5 and 0.7. Each well of a 96-well tissue culture plate was then inoculated with 200 uL of over day culture at ~0.5 OD600; 6 wells/plate/isolate for 3 repetitions. The plate was then grown statically at 37°C overnight. After, 180 uL of each well of the 96-well plate was aspirated and the plate was washed in large beaker of water for 5 rigorous passes. It was then blotted on paper towels 2-5 times. Next, 200 uL of 0.1% aqueous CV was added to each well and the plate was left to stand on the bench for 30 minutes. 180 uL of each well of the 96-well plate was aspirated again and then the plate was washed in large beaker of water for 5 rigorous passes. It was then blotted on paper towels 2-5 times. To elute the bound CV, 200 uL of 95% ethanol was added to each well and the plate was left to stand on the bench for 30 minutes. Lastly, the lid was removed and the plate is read with a microplate reader at 540 nm.

2.4. ANTIBIOTIC SUSCEPTIBILITY TEST

Antibiotic susceptibility test of biofilm producing bacteria was done on Mueller Hinton agar (Oxoid, UK) using the following antibiotic discs: ampicillin, ciprofloxacin, cotrimoxazole, chloramphenicol, erythromycin, amoxicillin/clavulanic acid, penicillin. Antibiotic susceptibility test was performed by using the disc diffusion technique according to The Clinical and Laboratory Standards Institute (**CLSI**) guidelines.

2.5. SYNTHESIS OF EXOPOLYSACCHARIDES

Isolated bacteria were screened for biofilm formation by using glass rods and lancets as surfaces. The surfaces were washed with acetone, immense in a detergent for 1hr, thoroughly washed out with distilled water and dried for 1hr at 160^0C. Surfaces were separately immersed in a conical flask containing 100 ml of Yeast Malt Glucose media (yeast extract 3g, malt extract 3g , glucose 10g, peptone 5g, distilled water 1000 ml) and inoculated with 12 selected organisms. After 7days at 37^0C incubation, glass rods and lancets were taken out and washed with phosphate buffer solution to remove un-adhered cells. Once again surfaces were transfer to a fresh media which was inoculated with the same amount of culture and incubate for 7 days in order to achieve the biofilm formation.

2.5.1. PRODUCTION OF EPS

The production of exopolysaccharide by method of **Sutherland (1990)[12] and Maziero et al., (1999).[13]** For EPS fermentation, the pure culture of biofilm producers grows in broth at pH5.6, temperature 30±1ºC for7 days. After respective incubation period the biomass separate by filtering the fermentation broth with Whatman filter paper no. 1 and the filtrate mix with 5% TCA (tricarboxylic acid), keep overnight at 4ºC for precipitation of proteins. Next day the filtrate centrifuged at 10,000 rpm for 20 minutes at 4°C to remove the protein precipitate and the filtrate add with 4 volumes of ethanol (filtrate: ethanol = 1:4 v/v), stirred vigorously and keep overnight at 4°C for precipitation of the exopolysaccharide. Next day, the precipitate exopolysaccharide separate by centrifuging the solution at 10,000 rpm for 20 minutes at 4°C. The pellet precipitate of crude EPS store for further use.

2.6. FOURIER TRANSFORM INFRARED (FT-IR) SPECTROSCOPY

FT-IR spectrum of the purified EPS was detected by Fourier transform infrared spectroscopy. FTIR spectra are record on a Thermo Nicolet 6700 instrument in the ranges of 400-4000 cm^{-1}.

2.7. APPLICATION OF EPS

ANTIOXIDANT ACTIVITY

1, 1-Diphenyl-2-picrylhydrazyl free radical scavenging assay (DPPH) carry out according to the following procedure **Piao et al., (2004).[14]** One ml of sample and standard (Ascorbic acid) at various concentrations (10, 50, 100, 150 and 200 µg/ml) add to 3 ml of 0.004%

DPPH in ethanol. Without sample use as control experiment and all these reaction mixture shaken vigorously.These solution mixtures keep in dark for 30 min and optical density measure at 517 nm using spectrophotometer. The % scavenging activity was calculated using the formula:
Percentage of inhibition = Inhibition (%) =

$$\frac{\text{Abs 517 (control)} - \text{Abs 517 (extract)}}{\text{Abs 540(control)}} \times 100$$

3. RESULTS

3.1. SAMPLE COLLECTION

The biofilm collect from the nearby locality. The collected sample transport to lab in aseptically.

3.2. ISOLATION OF ORGANISM

3.2.1. SERIAL DILUTION

The organisms were serially diluted at 10^{-1}, 10^{-3}, 10^{-5}. Highest growth was found in 10^{-1} dilution. Four different colonies (named as Merit Organism 1- MO1, Merit Organism 2- MO2, Merit Organism 3- MO3 and Merit Organism 4- MO4) where identified in all three dilutions.

MO1- green colonies, MO2-pale orange colonies, MO3-white colonies, MO4-yellow colonies

3.3. SCREENING

Screening was done by both qualitative as well as quantitative assays.

3.3.1. QUALITATIVE

- **TUBE ASSAY**

This qualitative method for biofilm detection, showed a stained band on the surface of the test tubes inoculated with MO2 and MO3 indicating these organisms to be biofilm producers.

- **CONGO RED ASSAY**

The Congo red agar plates streaked with the four organisms namely MO1, MO2, MO3 and MO4 showed a black slimy layer in the region streaked with MO2 and MO3.

By this two assays we can conclude that MO2 and MO3 are bio film producers.

3.3.2. QUANTITATIVE

A quantitative assay was conducted to check the intensity of the biofilm produced by MO2 and MO3.

INTENSITY OF BIOFILM BY CRYTAL VIOLET ASSAY

From the TABLE 1, we can understand that both organisms are efficient in biofilm producing with MO3 which has more biofilm producing capacity when compared to MO2.

3.4. ANTIBIOTIC SUSCEPTIBILITY TEST

The antibiotic susceptibility of the two isolates, MO2 and MO3 as performed using 7 antibiotics Ampicillin, Ciprofloxacin, Cotrimoxazole, Chloramphenicol, Erythromycin, Amoxicillin or Clavulanic acid and Penicillin.

From the TABLE 2 we can conclude that MO2 has a high resistance to cotrimoxazole with zone formation of 34mm and the least resistance to erythromycin with a zone of 28mm. In the case of MO3, the highest resistance is to ciprofloxacin with a zone formation of 36mm where as it has least resistance to cotrimoxazole and chloramphenicol with a zone of 27mm in both.

3.5. FOURIER TRANSFORM INFRARED (FT-IR) SPECTROSCOPY

The exopolysaccharides (EPS) was structurally elucidated by subjecting to FT-IR spectroscopy. The FT-IR spectrum report was analyzed and interpreted corresponding to the standard peak values. The peak at 3455.70 showed Hydroxide groups (-OH), 2077.64 showed symmetrical stretching ethylene, 1639.49 showed amide and 1041.54 shower ether from polysaccharides. (TABLE 5, FIG 1).

3.6. ANTIOXIDANT ACTIVITY OF EXOPOLYSACCHRIDES

The antioxidant activity was tested in accordance to the procedure by Piao et al., (2004) [14]. The absorbance of the DPPH radical caused by antioxidant was due to the scavenging of the radical by hydrogen donation. It is visually noticeable as a color change from purple to yellow.
Percentage of inhibition
= Inhibition (%)

$$= \frac{\text{Abs 517(control)} - \text{Abs 517 (extract)}}{\text{Abs 517(control)}} \times 100$$

= 88.16%

The EPS produced shows 88.16% of antioxidant properties. (TABLE 6).

TABLE 1: INTENSITY OF BIOFILM BY CRYTAL VIOLET ASSAY.

ORGANISM	BLANK	SAMPLE (intensity @ 540 nm)
MOG 2	0.00	0.583
MOG 3	0.00	0.798

TABLE 2: ANTIBIOTIC SUSCEPTIBILITY TEST.

ANTIBIOTIC	MOG 2 ZONE OF INHIBITION (mm)	MOG 3 ZONE OF INHIBITION (mm)
CIPROFLOXACIN	32	36
AMOXICILLIN	31	29
CORTIMOXAZOLE	34	27
ERYTHROMYCIN	28	28
PENICILLIN	32	29
AMPICILLIN	34	32
CHLORAMPHENICOL	29	27

TABLE 3: MORPHOLOGICAL IDENTIFICTION.

ORGANISM	GRAM STAINING	MOTILITY	AFB STAINING	ENDOSPORE STAINING
MOG 2	+	-	-	-
MOG 3	+	+	-	+

TABLE 4: BIOCHEMICAL TESTS.

TEST	MOG 2	MOG 3
INDOLE	-	-
METHYL RED	+	-
VOGES PROSKAUER	+	+
CITRATE	+	+
SUCROSE	+	+
GLUCOSE	+	+
LACTOSE	+	-
MALTOSE	+	+
CATALASE	+	+
OXIDASE	-	+
TSI	A/-nil-	A/K
UREASE	+	_

TABLE 5: FT-IR SPECTROSCOPY.

WAVE NUMBER, cm^{-1}	FUNCTIONAL GROUP NAME
3455.70	Hydroxide group
2077.64	Ethylene symmetric stretch
1639.49	Amide
1041.54	Ether from polysaccharides
553.43	-

TABLE 6: ANTIOXIDANT ACTIVITY OF EXOPOLYSACCHRIDES.

TUBE	VOLUME OF METHANOL (ml)	VOLUME OF DPPH (ml)	SAMPLE (ml)	Incubation in darkness for 30 minutes	O.D @ 517 nm
B	4	-	-		0.00
C	1	3	-		1.393
TB	3	-	1		0.195
T	-	3	1		1.281

B- Blank, C- Control, TB- Test blank, T- Test sample (EPS)

FT-IR spectroscopy

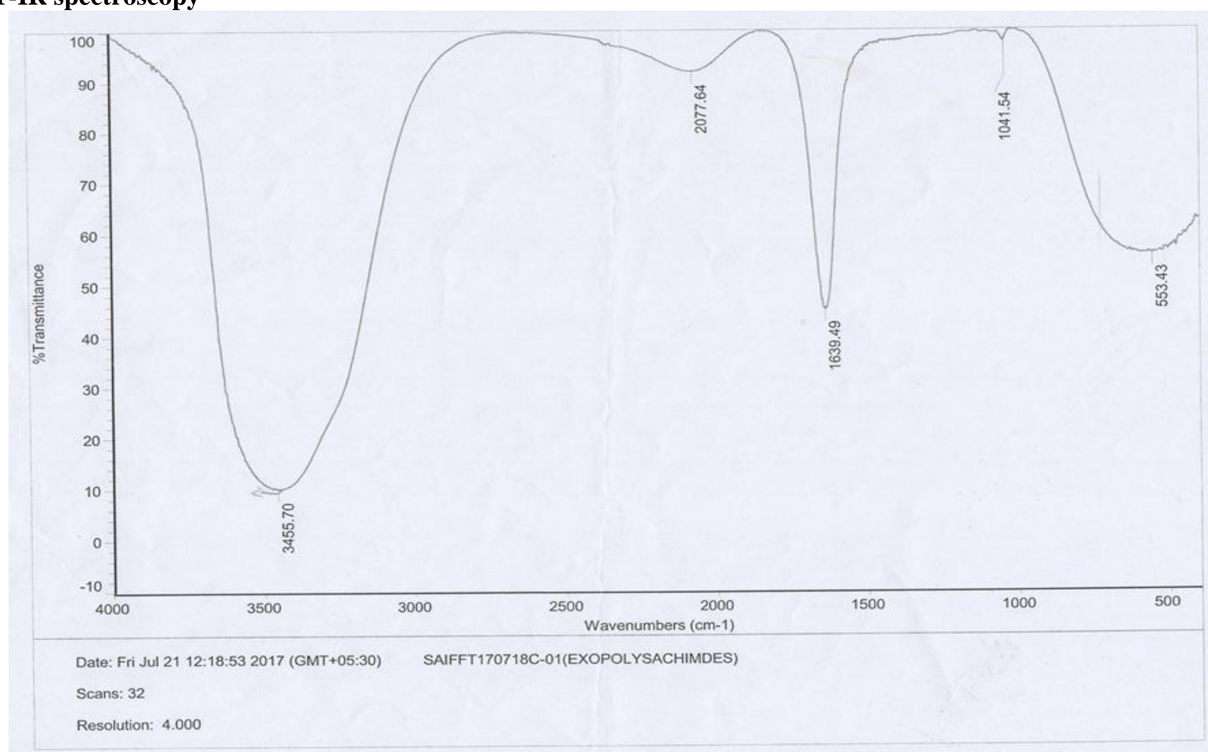

Fig-1.

5. DISCUSSION

Biofilm is an assemblage of the microbial cells that is irreversibly associated with a surface. The EPS matrix plays various roles in the structure and function of different biofilm community. Adhesion to the surface provides considerable advantages such as protection against antimicrobial agents, acquisition if new genetic traits etc. Biofilm formations are a precursor to biofouling which involve the settlement of living plants and animals on the surface that are in continuous contact with water of any type. Microfouling and macrofouling are of two successive stages of fouling process.[15] Microfouling refers to the formation of viscous and syrupy bacteriological layer by adhesive microorganisms whereas macrofouling refers to the attachment of larvae of larger sessile organisms like barnacles, mussels, polychaete worms, hydroids, bryozoans, and seaweeds.[16] One of the most common biofouling sites in the environment is on the hulls of ships, where organisms were often found attached and it has been periodically removed manually during docking of the ships. Biofouling lead to many significant problems like increased fuel requirements, problems related to propulsion system, introduction of invasive species etc. To prevent the attachment of the sessile organism, organometallic compounds were being applied on the underwater hulls of ships and boats. Natural biocides in antifouling paints are also being actively investigated since they are less toxic and environment friendly.[17] Environmental biofilms are complex microbial communities which coexist synergistically with each other.[18] In medical biofilms, it is mostly found that there is only one specific bacterial species affecting the region of the human body whereas the environmental biofilm samples obtained from the aquatic environment usually contain a microbial consortium with more than one bacterial species, thereby forming a complex microbial community. The presence of 132 more than one bacterial species makes it difficult to investigate strategies to inhibit the formation of biofilms in both controlled and natural environments. There are several bacteria reported to be present predominantly in the environmental biofilms like the Gram negative bacteria, *Pseudomonas aeruginosa*, *Escherichia coli*, *Shewanella oneidensis* etc. and Gram positive bacteria, *Bacillus subtilis*, *Bacillus thuringiensis*, *Bacillus cereus* etc. In our study, we have found that the majority of biofilm producing bacteria was from sewage. The biofilm producing organisms MOG2 and MOG3 have been found out to be of the *Staphylococcus* and *Bacillus* spieces respectively. In the TCP method, the number of isolates showing biofilm formation was 2, and non or weak biofilm producers were 2. We have performed the TCP method by addition of 1% glucose in trypticase soy broth. Addition of sugar helps in biofilm formation. This was also reported by studies conducted by Mathur et al.[19] and Bose et al.[20] the Congo Red assay showed perfect correlation with the tube assay. The antioxidant property analysis of the exopolysacchries extracted showed about 88.16% inhibition against the DPPH radicals.

6. SUMMARY

Biofilm formed by various micro-organisms potentially provide a suitable microenvironment for efficient bioremediation processes. High cell density and stress resistance properties of the biofilm environment provide

opportunities for efficient metabolism of number of hydrophobic and toxic compounds. Bacterial biofilm formation is often regulated by quorum sensing (QS) which is a population density-based cell–cell communication process via signaling molecules. Biofilm formation is a widespread phenomenon pertaining to microorganisms attaching itself to a surface that is in continuous contact with water. It occurs widely on the ships hulls, implanted medical devices, dentures, eyes due to non-compatible contact lenses, water distribution systems, showers tubes, cooling pipes in nuclear reactors, etc. Biofilms are responsible for loss of billions of dollars to the industries, since they cause fouling of ship hulls, water treatment and distribution systems, causing corrosion of pipes and thereby resulting in contamination. Biofilms are also of great interest in medical context since they result in persistent and chronic infections and also contaminate implanted devices. Environmentally and economically viable solutions are increasingly being investigated to prevent and control the biofilm formation. The current study is to isolate organisms from biofilm sample, identify them and extract the exopolysaccharides produced and testing the EPS for anti-oxidant activity. From this study it was found that *Staphylococcus species* and *Bacillus species* are effective biofilm producers. The future studies are to apply the exopolysaccharide as encapsulating material such that the coating of tablets or capsules will have antioxidant property preventing a part of the drugs ill effects.

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