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CHARACTERIZATION OF EXOPOLYSACCHARIDES AND BIOFILM PRODUCING BACTERIAL STRAINS ISOLATED FROM MANGROVE SEDIMENTS OF NIJAMPATNAM

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ABSTRACT

A total of ten bacterial isolates were used for this study and the soil samples were collected from mangrove areas of Nijampatnam, Andhra Pradesh. Isolation procedures were carried out by using nutrient agar medium (NAM). Marine water was used for the biofilm forming bacterial isolation. A biofilm forming device was made by using PVC pipes with sterile glass slides. Ten morphologically different bacteria were isolated from the scrapping of glass slides from the device after a month period of time. Morphological characterization was done by all these isolates. The present ten isolates are Pseudomonas, Staphylococcus, Micrococci, Klebsiella sp.1 Klebsiella sp.2, E. coli, Enterobacter, Proteus sp.1, Proteus sp.2, and Citrobacter are morphologically different. Maximum colony size 8 mm was recorded in Enterobacter and Proteus sp.1. The efficiency of EPS and biofilm production was checked for 24 to 96 hours of incubation period. After 72hours of incubation maximum exopolysacharide (96 mg/100ml) and Biofilm (98 mg/100ml) production was recorded in Enterobacter. Biofilms have the ability to bear a high concentration of antimicrobial agents. Bacterial biofilm may play a role in the pathogenesis of disease has led to an increased focus on identifying diseases that may be biofilm-related.

KEYWORDS: Biofilms, Exopolysaccharide, Mangrove soils, Nutrient Agar Medium.

INTRODUCTION

Mangrove sediments provide essential habitat for thousands of microbial species. Microorganisms reside in biofilms i.e. surface attached aggregates embedded in a matrix of extracellular polymeric substance. Microbial cells generally contain various polysaccharide structures contributing to their shape and gelatinous nature. These EPS are produced mainly

during the log phase of bacterial growth and slime EPS produced during the stationary phase accordingly (Plante and Shriver. 1998). The biofilm matrix consists of polysaccharides structural proteins enzymes, environmental challenges and allows long term colonisation and spatial organisation with microorganisms. Flemming *et al*., (2016).

A biofilm may also be considered a mucilaginous gel like material, which is a complex polymer that contains many times its dry weight in water. Biofilms can attach to a surface such as a tooth or rock, and may include a single species or a diverse group of microorganisms. Subpopulations of cells within the biofilm differentiate to perform various activities for motility, matrix production, and sporulation, supporting the overall success of the biofilm. The biofilm bacteria can share nutrients and are sheltered from harmful factors in the environment, such as desiccation, antibiotics, and a host body's immune system. A biofilm usually begins to form when a free-swimming bacterium attaches to a surface (Lewis, 2001).

Exopolysaccharides (EPS) are most important in the attachment of bacteria to substrata and thus development of biofilms (Costerton *et al*., 1987). Further Exopolysaccharides are excreted from multiple bacterial species, which make biofilms a good source for screening exopolysaccharides producing bacteria was also reported by (Davey and O'Toole, 2000). Majority of the Biofilms can be controlled by either preventing EPS matrix secretion by the bacteria or treating the established biofilm with cleaning products (Zhao *et al*., 2017).

Biofilms can contain many different types of microorganism, i.e. algae, archaea, bacteria, protozoa and fungi. Each group performs specialized metabolic functions. However, some organisms will form single-species films under certain conditions (Hall-Stoodley *et al*., 2004; Aggarwal *et al*., 2016). Biofilms have been found to be involved in a wide variety of microbial infections in the body, by one estimate 80% of all infections. Infectious processes in which biofilms have been implicated include common problems such as bacterial vaginosis, urinary tract infections, catheter infections, middle-ear infections, formation of dental plaque, gingivitis, coating contact lenses and less common but more lethal processes such as endocarditis, infections in cystic fibrosis, and infections of permanent indwelling devices such as joint prostheses, heart valves, and intervertebral disc (Rogers, 2008; Imamura *et al*., 2008; Capoor, 2017; Lewis, 2001).

However there were limited reports on biofilm and exopolysacharide production and characterization from mangrove sediments. The present research mainly focussed on exopolysaccharide and biofilm production by using these bacteria *i.e. Pseudomonas, Staphylococcus*, *Micrococci*, *Klebsiella* sp.1 *Klebsiella* sp.2, *E. coli*, *Enterobacter*, *Proteus* sp.1, *Proteus* sp.2, *and Citrobacter* isolated from mangrove sediments of Nijampatnam, Andhra Pradesh.

MATERIALS AND METHODS

Isolation of bacteria

One gram representative mangrove soil sample was suspended in 10 ml of sterile distilled water and shaken thoroughly for 10 minutes. The microorganisms were isolated from collected samples by the serial dilution plate technique using Nutrient Agar Medium (NAM). Serial dilutions up to 10^{-5} of each sample were prepared by using sterilized water (Sneath, 1986). Sample dilutions were plated (in triplicates) on NAM and incubated at 35^0 C for 24 to 48 h. Pure Colonies were picked and maintained on NAM slants at 4^0 C and further assessed for production and characterization of EPS and Biofilm.

Tube method

A loop full of bacteria were inoculated into Trypticase soy broth supplemented with 1% glucose and incubated for 24 hours at 37°C. Tubes were decanted and washed with PBS (pH 7.3) and dried according to Mathur, (2006). Dried tubes were stained with crystal violet (0.1%). Excess stain was removed, and tubes were washed with by using deionized water. Tubes were then dried in an inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Based on the intensity of the color formed, they were characterized as moderately positive and strongly positive (Mathur, 2006).

Biofilm formation

Biofilm development was studied with polyvinyl chloride (PVC) pipe which was 9 cm as length and 6 cm in width. The glass slide (25 mm in width, 75 mm in height and 1mm in thickness) were used for the biofilm formation. The glass slides were pre cleaned with 1N HCL and treated with sodium hypochlorite solution of 10 mg/100ml for 48 hours and rinsed with sterile distilled water before the experimental setup. Then the slides were placed in PVC

chamber at 21^{0}C and covered with aluminium foil. Marine water was pumped at flow velocity of 10 to 20 drops/minute in the PVC chambers were used in this work. Twenty slides were placed in the PVC chamber with regular distance for the formation of biofilm (Hong *et al*., 2002).

EPS Production

EPS extraction was performed by solvent extraction method with slight modifications (Djordjevic *et al*., 1986). The bacterial isolates were inoculated into Erlenmeyer flasks (250 ml) containing 100 ml YEM broth supplemented with 1% Mannitol. The flasks were incubated at room temperature on a orbital shaker at 200 rpm for 72 h. After incubation the broth was centrifuged at 3000 X g and the culture supernatant was mixed with 2 volumes of ethanol. The crude polysaccharide precipitate was collected by centrifugation at 3000Xg for minutes. The EPS was washed with distilled water and ethanol alternately. The precipitate was freezing-dried and re dissolved in 100 ml of deionised water. Dissolved EPS was mixed with 80% ethanol at a ratio of 1:3 to remove any impurities.

RESULTS AND DISCUSSION

Isolation of bacteria from mangrove soils

Soil samples were collected from mangrove sediments, and used for isolation of bacterial isolates. One gram of soil was suspended in sterile distilled water and mixed thoroughly for one hour at room temperature before using for isolation studies. Sterile 1 % nutrient agar plates were prepared and incubated at 4^{o}C for 15 min to solidify the agar solution. The plates were kept at room temperature and spread with 0.1 ml of soil solution under sterile conditions. These plates were incubated at 30^{o}C in an incubator. After 24 hrs of incubation, the plates were checked for bacterial growth for further studies.

RESULTS AND DISCUSSION

From the results the three strains Pseudomonas, Klebsiella sp.1and Klebsiella sp.2 showed gram positive reaction on gram staining test. The shape of the colonies varies from round to irregular from all the strains studied. The colony sizes were ranged from 4 to 8 mm was recorded. The maximum colony size 8 mm was observed in Enterobacter and proteus sp.1. The colors of the colonies are light yellow, orange and pink was recorded. The texture of the ten strains also studied by smooth to rough in nature was observed. Enterobacter and proteus sp.1 was showed white colonies on agar plates (Table-1).

Experiment was done by the Production of EPS and Biofilm for all the ten bacterial strains. Among them three strains Pseudomonas, Klebsiella and Enterobacter produced maximum EPS and biofilm after 24 of incubation. From the results it is evident that the initial production of EPS and biofilm are a quick response in the plates (Table-2).

EPS and Biofilm production was observed at 48 hours of incubation periods. Of the 10 there are five bacterial strains i.e. Pseudomonas, Klebsiella sp.1, Klebsiella sp.2, Enterobacter and Proteus sp. showed maximum EPS and Biofilm production. Among them Enterobacter and Proteus sp. Showed maximum EPS 92 mg/100 ml and Biofilm 98 mg/100ml respectively (Table-3).

The incubation period was increased and gradually the EPS and Biofilm production was observed by all these five strains (Table-4). EPS and Biofilm production was increased with increasing incubation period up to 72 hours. The bacterial strain Enterobacter showed maximum EPS (96 mg/100ml) and Biofilm production (98mg/100ml).

Further the incubation period was increased up to 96 hours for all these five strains. There were no changes on EPS and Biofilm productions (Table-5). Maximum EPS and Biofilm production was recorded at 72 hours of incubation period (G. Kranthi kumar and Raghu ram 2014). This is the optimum incubation period for all these bacterial strains i.e. Pseudomonas, Klebsiella sp.1, Klebsiella sp.2, Enterobacter and Proteus sp. (G. Kranthi kumar and Raghu ram 2016). Further studies like characterization, factors affecting the growth of EPS, Biofilm production and purification studies were based on these results.

Table 1: Morphological Characterization of bacterial isolates.

Isolate no	Gram's test	Shape of the colony	Colony size (mm) after 48 h	Color of the colony	Texture
1	Gram positive	Round	4	Pink	Smooth
2	Gram negative	Irregular	6	Orange	Smooth
3	Gram negative	Irregular	4	Light yellow	Smooth
4	Gram negative	Round	6	Orange	Rough
5	Gram positive	Round	4	White	Rough
6	Gram negative	Round	6	Light yellow	Rough
7	Gram negative	Irregular	8	White	Smooth
8	Gram negative	Irregular	8	White	Smooth
9	Gram negative	Irregular	6	Light yellow	Rough
10	Gram negative	Round	6	Light yellow	Rough

*Each data is an average of three replicates

Table 2: EPS & Biofilm formation of bacteria after 24 hours of incubation.

Isolate no	Exopolysaccharide production (mg/100ml)	Biofilm production (mg/100ml)
1	56	65
2	Nil	Nil
3	Nil	Nil
4	Nil	Nil
5	82	94
6	Nil	Nil
7	88	96
8	Nil	Nil
9	Nil	Nil
10	Nil	Nil

*Each data is an average of three replicates

Table 3: EPS & Biofilm formation of bacteria after 48 hours of incubation.

Isolate no	Exopolysaccharide production (mg/100ml)	Biofilm production (mg/100ml)
1	75	82
2	Nil	Nil
3	Nil	Nil
4	84	90
5	90	98
6	Nil	Nil
7	92	96
8	92	94
9	Nil	Nil
10	Nil	Nil

*Each data is an average of three replicates

Table 4: EPS & Biofilm formation of bacteria after 72 hours of incubation.

Isolate no	Exopolysaccharide production (mg/100ml)	Biofilm production (mg/100ml)
1	75	82
2	Nil	Nil
3	Nil	Nil
4	84	90
5	90	98
6	Nil	Nil
7	96	98
8	92	98
9	Nil	Nil
10	Nil	Nil

*Each data is an average of three replicates

Table 5: EPS & Biofilm formation of bacteria after 72 hours of incubation.

Isolate no	Exopolysaccharide production (mg/100ml)	Biofilm production (mg/100ml)
1	75	82
2	Nil	Nil
3	Nil	Nil
4	84	90
5	90	98
6	Nil	Nil
7	96	98
8	92	98
9	Nil	Nil
10	Nil	Nil

*Each data is an average of three replicates

CONCLUSION

It is an evident that the marine isolates like *Pseudomonas*, *Klebsiella* sp.1, *Klebsiella* sp.2, *Enterobacter* and *Proteus* sp. were produced maximum EPS and Biofilm after 48 hours of incubation period. The tube method and CRA was used in this study are significant for the identification of EPS and Biofilms. According to my results further studies are needed to evaluate the potential of the biofilm exopolysaccharides for industrial application.

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