A STUDY OF ANTIBIOTIC RESISTANCE AND HEAVY METAL TOLERANCE BY EXTRACELLULAR POLYMERIC SUBSTANCE (EPS) PRODUCING ISOLATES FROM MANGROVE SOIL

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Abstract: Extracellular polymeric substance (EPS) producing bacteria were isolated from rhizosphere soil of *Avicennia marina* from Thane creek. EPS plays a pivotal role in the formation of biofilms of bacteria and biofilms are known to enhance heavy metal tolerance of some bacteria. The isolates were studied and compared for their tolerance to heavy metal ions (Cu^{+2}, Zn^{+2}, Cr^{+6}, Cd^{+2}) as well as resistance to ten antibiotics. Of these , two EPS producing isolates which tolerated maximum concentration of heavy metals were also found to be resistant to 80 % of the antibiotics. One of the selected isolates was less tolerant to heavy metals and was resistant to only 20% of the antibiotics tested. Alkaline lysis method revealed the presence of plasmid in the resistant isolates while sensitive isolate was devoid of the plasmid, despite the production of profuse EPS.

Keywords: Heavy metal ion, EPS, Mangrove, Rhizosphere, Antibiotic, Plasmid.

Introduction: Heavy metal ions like Fe^{+2}, Cu^{+2}, Zn^{+2} are important constituents of biological systems. At extremely low concentrations, they function as micronutrients acting as inorganic cofactors for various enzymes [1]. They play a crucial role in cellular and molecular processes.

Heavy metal ions are present naturally in fresh water through the processes of geochemical weathering and microbial leaching [2]. However they become hazardous pollutants due to human activity. Thane creek, located app. 28 kms north of Mumbai harbour, acts as a sink for various municipal wastes and industrial discharges of organic and inorganic pollutants which can get deposited in the mangrove soil [3]. Property of exopolymeric substance (EPS) formation is ubiquitous among soil micro organisms. It is a survival strategy for the organisms. It protects the cell against desiccation and gives resistance towards harmful agents like metal cations, by forming complexes with the negatively charged components of EPS matrix [4],[5]. Polysaccharides are the fundamental structural elements of EPS matrix that give mechanical strength to biofilms.[6]

In the present study EPS producing bacteria were isolated from rhizosphere soil of *Avicennia marina* from Thane creek mangrove. The isolates were studied for their tolerance towards four heavy metals. They were further tested for antibiotic resistance. Plasmid isolation was carried out to explore its role in heavy metal and antibiotic resistance [1].

Materials and Methods:

Rhizosphere soil samples around pneumatophores of *Avicennia marina*(co ordinates - N 19° 00' 22'', E 72° 53' 03'') were collected in sterile petri dishes.

In order to determine the bacterial cell density, the soil sample was processed for viable count by surface spread method using sterile nutrient agar plates [7]. Ten fold serial dilutions were performed. CFU/gm of soil was recorded. On the

basis of consistency feature, three mucoid isolates were processed for: i) Extraction of EPS, ii) Heavy metal tolerance, iii) Antibiotic resistance and iv) Plasmid isolation.

For extraction of EPS one ml of overnight broth culture of respective isolate was inoculated in 100 ml of sterile Nutrient broth and incubated on shaker (at 100 rpm) at room temperature for five days. EPS was precipitated from the cell free broth by chilled ethanol precipitation method.

Dry EPs was weighed and Molisch test was performed to detect presence of carbohydrate.

The selected isolates were tested for their heavy metal tolerance.

Copper, Zinc, Chromium and Cadmium were the heavy metals included in the study.

Each isolate was evaluated for its heavy metal tolerance by employing sterile Nutrient broth in a tube assay. The medium was supplemented with a range of concentrations from 20 to 300 ppm of heavy metal ions. Stock solutions of metal ions (2000 ppm of $Cu^{2+,}$ Zn^{2+}, Cr^{6+}, Cd^{2+}) were prepared by dissolving respective salts ($CuSO_4$ $5H_2O$, $ZnSO_4$ $7H_2O$, $K_2Cr_2O_7$, $CdCl_2$ H_2O) in deionized water [8]. Working solutions of each metal ion were prepared in sterile nutrient broth prepared in deionized water.

In all three isolates were evaluated for antibacterial susceptibility test. Of these, two isolates with high metal tolerance were selected. Third isolate although with low metal tolerance but profuse mucoid consistency was included in the study.

Antibacterial susceptibility test was carried out for each of the three isolates using ten antibiotics by disc diffusion technique. Broad spectrum antibiotics like Tetracycline and narrow spectrum antibiotic like Penicillin were incorporated in the study. Following antibiotics were used: Ampicillin 10 mcg, Bacitracin 30 mcg, Chloramphenicol 10 mcg, Methicillin 5 mcg, Nalidixic acid 30 mcg, Nitrofurantoin 300 mcg, Penicillin 10 mcg, Polymyxin B 300 u, Streptomycin 10 mcg, Tetracycline 30 mcg.

Plasmid isolation was carried out using alkaline lysis method [9].

Requirements:

1) Solution 1:

a) 50Mm glucose (stock 1M glucose add 2.5ml)

b) 25mM Tris Hcl (pH 8) (stock 1M Tris add 1.25ml)

c) 10Mm EDTA (pH 8) (Stock solution 0.5 M add 200µl)

2) Solution 2: (100ml)

a) 0.2N NaOH (from 10 N stock)

b) 1%SDS diluted from 10%SDS.

3) Solution 3:

a) 5M Potassium acetate (60ml)

b) Glacial acetic acid (11.5ml)

c) Distilled water (28.5ml)

4) 100% ethanol (100ml)

5) 70% ethanol (100ml)

6) TE buffer pH 8

Harvesting: Transfer a single colony into 2ml St LB broth containing appropriate antibiotics. Incubate the culture overnight at 37°C with rigorous shaking. Pour 1.5ml of culture into eppendorf tube. Centrifuge at 12000rpm for 30secs at 4°C.Remove the supernatant and again add 1.5ml of culture into eppendorf tube .Centrifuge at 12000g for 30 seconds at 4°C. Discard the supernatant.

Alkaline Lysis: Resuspend the bacterial pellet in 100µL of ice cold solution 1 by vigourous shaking. Add 200 µL of freshly prepared solution 2. Mix the contents by inverting the tube rapidly 5 times. Do not vortex. Keep the tube on ice. Add 150µL of ice cold solution 3. Keep the tubes on ice for 3-5 mins. Centrifuge at 12000 rpm for 5 mins at 4°C and transfer the supernatant to fresh

tube. Precipitate ds DNA with 2 volumes of ethanol at room temperature. Mix by vortexing. Allow the mixture to stand for 2 mins at room temperature. Centrifuge at 10000 rpm for 5 mins at 4°C.

Remove the supernatant. Stand the tube in inverted position on a paper to allow fluid to drain away. Rinse the pellet on ds DNA with 1 ml of 70% ethanol and allow the pellet of Nucleic acid to dry for overnight. Redissolve the nucleic acid in 50µL of TE pH8. Add RNase A (20 µg/ml) and incubate for 2 hrs. store at -20°C.

Agarose gel electrophoresis:

Requirements:

1% Agarose solution -50ml

Running buffer 1X Tris Acetate EDTA Buffer pH 8

a) Tris base - 242g

b) Glacial Acetic acid - 57.1 ml

c) Distilled water - 842.9 ml

Prepare 50 ml of 1% agarose solution in 1X TAE. Weigh 0.5 g of agarose and dissolve in 50ml distilled water. Boil the agarose until it is completely dissolved. Cool the agarose solution to around 40°C and add 5µl of 5mg/ml stock of ethidium bromide and mix properly. Take a clean dry casting plate and seal it on both sides or put it in a casting plate holder and place the combs in the casting plate ensuring the teeth of the comb do not touch the glass plate. Pour the agarose mixture into the tray containing comb. After complete solidification of the agarose remove the seal from either side of the tray and take it out of the casting plate holder without disturbing the gel.

Remove the comb and transfer the gel plate to the electrophoresis tank such that the wells formed are towards the cathode. Pour 1X TAE buffer into the tank till the gel is completely submerged .Connect the electrodes to the power supply. Load the DNA samples with the help of micropipettes into the wells. Load 1KB ladder in one of the wells to determine the fragment size of unknown DNA.

After loading switch on the power pack and adjust the voltage to 50 or 100v.Monitor the running of the fast running tracking dye (Bromophenol Blue).Turn off the power supply when the tracking dye has reached near the opposite edge of the gel. Remove the gel and place it under UV transilluminator and observe for presence of orange fluorescent bands.

Refer to Figure No.1.

Results and discussion:

The bacterial count from rhizosphere soil of *Avicennia marina* was found to be 8.7 x 10^6 CFU/g.

The high bacterial count showed proliferation of bacteria in response to the aerobic conditions prevailing on these breathing roots.

Three glistening and mucoid colonies observed on sterile nutrient agar plates were selected for further work. On Gram staining isolates i1 and i2 were found to be Gram positive rods while isolate i3 was found to be a Gram negative rod.

Table No 1. EPS extraction and Molisch test.

Isolate	mg/100ml medium	Molisch test
i1	30	+
i2	60	+
i3	38	+

Extraction of EPS was done for all the isolates and the results are given in table no 1.

Isolate i2 was found to have maximum mucoid consistency with its EPS content found to be 60mg/100ml of the medium, whereas isolates i1 and i3 were found to have comparatively less

mucoid consistency. This correlates well with their EPS content of 30mg/100ml of medium and 38mg/100 ml of medium of respectively.

Heavy metal tolerance of the three isolates was carried out and the results are given in table no 2.

Table No. 2.Heavy Metal Tolerance (ppm) of the soil isolates.

Isolate	Cu^{2+} ppm	Zn^{2+} ppm	Cr^{6+} ppm	Cd^{2+} ppm
i1	100	160	100	200
i2	60	20	40	Less than 20
i3	180	200	140	220

Isolate i1 showed maximum tolerance towards Cadmium, followed by Zinc. However it could tolerate only 100 ppm of Copper and Chromium. Although isolate i2 showed highly mucoid consistency it was sensitive to less than 20 ppm of Cadmium. It could tolerate only 60 ppm of Copper. Isolate i3 on the other hand could tolerate 220 ppm of Cadmium and 200 ppm of Zinc. The isolates i1 and i3 exhibited maximum tolerance towards all four heavy metals. Isolate i2 showed least tolerance towards all the four heavy metals.

The above three isolates were evaluated for the antibacterial susceptibility test. By referring to standard Kirby Bauer chart following inferences were recorded in Table no.3

Table No 3.Antibacterial Susceptibility Test of the isolates.

Antibacterial	i1	i2	i3
Ampicillin	R	S	R
Bacitracin	R	R	R
Chloramphenicol	S	S	S
Methicillin	R	S	R
Nalidixic Acid	R	S	R
Nitrofurantoin	R	S	R
Penicillin	R	S	R
Polymyxin B	R	R	R
Streptomycin	S	S	S
Tetracycline	S	S	R

R: Resistant ; S: Sensitive

Thus it was found that:The isolate i1 was resistant to 70 % of the antibiotics. The isolate i3 was resistant to 80 % of the antibiotics. The isolate i2 was resistant to only 20 % of the antibiotics. Isolate i1 and i3 were multidrug resistant organisms.

The summary of the results obtained with reference to isolates i1, i2 and i3 is depicted in Table No. 4.

Table No 4. Comparison of heavy metal tolerance, antibiotic resistance and EPS production of the isolates.

Isolate	Cu^{+2} ppm	Zn^{+2} ppm	Cr^{+6} ppm	Cd^{+2} ppm	Antibiotic Resistance (%)	EPS mg/100 ml medium
i1	100	160	100	200	70	30
i2	60	20	40	0	20	60
i3	180	200	140	220	80	38

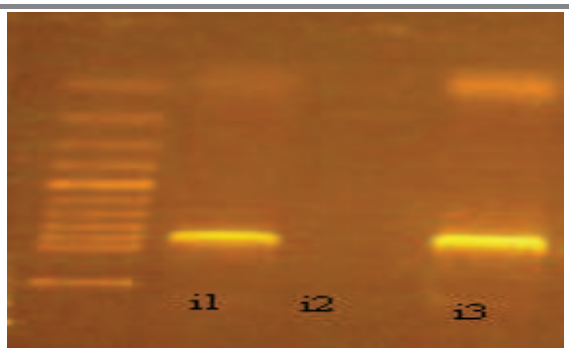

Figure No 1. Plasmid isolation by alkaline lysis method.

Plasmid was isolated from isolate i1 and isolate i3.

Isolate i2 was devoid of plasmid.

The three isolates exhibited variable response towards Cu+2, Zn+2, Cr+6 and Cd+2

Isolate i2 produced abundant EPS (60 mg/100 ml of medium) as compared to isolate i1 (30 mg/100ml of medium) and isolate i3 (38 mg/ 100ml of medium). However the profuse EPS producer isolate i2 was least tolerant to all the four heavy metals as compared to Isolate i1 and isolate i3. Thus isolate i2 was found to tolerate only 60 ppm of Cu+2, 20 ppm of Zn+2, 40 ppm of Cr+6 and was totally inhibited by even the lowest concentration- 20 ppm of Cd+2. The same isolate was found to be resistant to only 20 % of the antibiotics.

Isolate i3 produced 37 % less EPS than isolate i2. But it exhibited maximum tolerance towards all four heavy metals. Isolate i3 tolerated 180 ppm of Cr+2, 200 ppm of Zn+2, 140 ppm of Cr+6 and 220 ppm of Cd+2. Isolate i3 was found to be resistant to 80 % of the antibiotics tested. It was a multi drug resistant organism.

Isolate i1 exhibited an intermediate response to heavy metals. It tolerated 100 ppm of Cu+2, 160 ppm of Zn+2, 100 ppm of Cr+6 and 200 ppm of Cd+2, and was resistant to 70 % of the antibiotics tested. It was also a multi drug resistant organism.

EPS plays an important role in formation of biofilms and thus are known to enhance tolerance of organisms for heavy metals [10].

In the present study it was found that the isolate which produced maximum EPS (Isolate i2) was least resistant to heavy metals and antibiotics. A positive relationship between the presence of plasmid and resistance to heavy metals as well as antibiotics by the same isolates (i1 & i3) was observed.

Aminov and Mackie indicate that the genes for heavy metal tolerance may be present on the same plasmid that harbors genes for antibiotic resistance [11]. Our results are in concurrence with the suggestion of Aminov and Mackie.

Thus the resistance of isolate i1 and isolate i3 to heavy metals and antibiotics may be governed by plasmid. It needs to be confirmed.

According to Baker-Austin et al metal pollutants may act as co-selection agents for antibiotic resistance in bacteria [12]. Hence resistance towards heavy metals will also lead to resistance against antibiotics.

With rapid industrialization and discharge of effluents containing heavy metals in Thane creek like environments, the antibiotic resistance of bacterial populations will always be maintained and remain a serious threat to human health.

Conclusion: The three bacterial isolates from

rhizosphere soil of Avicennia marina displayed variable resistance to heavy metals and antibiotics.

Study revealed that profuse EPS produced by isolate i2 did not enhance its heavy metal tolerance and resistance to antibiotics. Metal tolerance and antibiotic resistance of isolate i1 and isolate i3 was plasmid mediated.

References:

1. O. Goniyu, Oyetibo, O. Mathew. " Bacteria with duel resistance to elevated concentrations of heavy metals and antibiotics in Nigerian contaminated systems ", Environmental monitoring and assessment. Vol.168, pp. 305 -314, 2010.
2. J. Eugenia, E.Olguin, G. Sonchez , E. Hemandez , " Environmental biotechnology and cleaner bioprocesses". Philadelphia. Pub-Tailor and Francis 2003.
3. R.P. Athalye, Gokhale K.S. “Macrobenthos from mudflats of Thane creek, Maharashtra.” Journal of bombay natural history society. Vol.95, pp. 258-266, 1998
4. A.Pal, A.Paul, “Microbial extra-cellular sub central elements in heavy metal bioremediation”, Indian Journal of Microbiology Vol. 48, No.1, pp.49-60, 2008.
5. S. Kamaluddin, K. Ramaswamy, “Rhizoremediation of metals: harnessing microbial communities.” Indian Journal of Microbiology, Vol.48, No.1,pp.80-88, 2008
6. J. Wingender et al. " Microbial Extracellular polymeric Substances " @ Springer-Verlag Berlin Heidelberg,. Chap 1 1999, pp.11
7. A. Agte, Bonde S. and K. Kamaran, " Proceedings of symposium of significance of mangrove", Pune, pp.52-55, 1991.
8. E. Ezaka , C.U. Anyanwu, " Chromium tolerance of bacterial strains isolated from sewage oxidation ditch". International Journal of Environmental sciences. Vol. 1, No.7, pp. 1725-1733, 2011.
9. Ehrt, Sabine, and Dirk Schnappinger. "Isolation of plasmids from E. coli by alkaline lysis." *E. coli Plasmid Vectors*. Humana Press, pp.75-78, 2003.
10. M. Miller, M.B. Bassler, B.L. “Quorum sensing in bacteria”, Annual Reviews in Microbiology, Vol.55, No.1, pp.165-199, 2001.
11. R.I. Aminov, and Mackie R.I., “Evolution and ecology of antibiotic resistance genes.” FEMS Microbiology Letters. Vol. 271, No.2, pp.147-161, 2007.
12. Robert S. Boyd. “Heavy metal pollutants and chemical ecology: exploring new frontiers”, Journal of Chemical Ecology, Vol.36, No.1, pp.46-58, 2010.

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