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Studies on Metal Microbe Interaction of Three Bacterial Isolates From East Calcutta Wetland

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Abstract: The present paper put forth the isolation and complete characterization of three bacterial isolates from different sites of East Calcutta Wetland (ECW). These isolates show tolerance to heavy metals like Ag, Al, Cu, Cr, Co, Ni, Pb and were found to be efficient metal accumulators as evident from Energy Dispersive X Ray Fluorescence (EDXRF) analysis and Transmission Electron Microscopy (TEM). Beyond intracellular accumulation, these isolates also show metal deposition in form of particles inside the cells which can further open up the area of microbe fabricated nanoparticle generation. The heavy metal accumulation in the cell can trigger different response mechanism such as change in cell morphology which was observed by scanning electron microscopy (SEM). The metal uptake property of these isolates can be applied for the heavy metal removal and recovery from industrial effluents.

Key words: East Calcutta wetland, EDXRF, TEM, SEM, nanoparticle

INTRODUCTION

Microorganisms play an important role in the environmental fate of toxic metals and radionucleotides with a multiplicity of mechanisms effecting transformation between soluble and insoluble forms[1]. Although some heavy metals are essential trace elements, most can be, at high concentrations, toxic to all branches of life, including microbes, by forming complex compounds within the cell. Because heavy metals are increasingly found in microbial habitats due to natural and environmental processes, microbes have evolved several mechanisms to tolerate the presence of heavy metals. These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of metal ions inside the cell and reduction of the heavy metal ions to a less toxic state. Because the intake and subsequent efflux of heavy metal ions by microbes usually includes a redox reaction involving the metal, bacteria that are resistant to and grow on metals also play an important role in the biogeochemical cycling of those metal ions. This is an important implication of microbial heavy metal tolerance because the oxidation state of a heavy metal relates to the solubility and toxicity of the metal itself[2].

The metal accumulating capacity of microorganisms can be exploited to remove, concentrate and recover metals from mine tailings and industrial effluents[3]. While metals cannot be broken down into non-toxic components like organic compounds, bioremediation can be used to stabilize, extract, or reduce the toxicity of soil and groundwater contaminated by acid mine drainage[4]. Several reports of aerobic bacteria accumulating metals like Ag, Co, Cu, Cr, Ni.[5,6,7,8,9] are available. Essential elements, e.g, K, Ca, Mn, Mg, Cu, Zn, Fe, Co, and those with no essential biological function, e.g. Cs, Cd, Pb, Al, Sn, Hg, can be accumulated by microorganisms by non specific physiochemical interactions as well as specific mechanisms of sequestration and transport[10].

Presently metal accumulating bacteria have shown potential for material science. Biomimetics is the area of research dealing with material science and engineering through biology. Bacteria are involved as workers in the living factory and a plethora of novel nanostructured particles with unexpected properties are produced in the living factory which have applications in biomedical sciences, optics, magnetics, mechanics, catalysis and energy science. These biological materials can be used in their native form directly extracted from

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the living systems, or they can be processed after extraction and modified to their desired form[11].

Earlier studies indicate that strains of *Bacillus subtilis* is able to reduce Au^{3+} ions to produce octahedral gold nanoparticles[12]. Similarily *Klebsiella pneumoniae* have been reported to overcome cadmium toxicity through the biotransformation of cadmium ions into photo active, nanometer sized CdS particles deposited on the cell surface[13].

East Calcutta Wetland is low lying area of about 12500 hectares on the eastern region of Calcutta. It is acting as a natural sewage treatment plant to the city and side by side generates product like paddy, vegetables and fish utilizing the sewage[14,15,16,17]. It receives effluents from domestic activities, industries, tanneries, battery manufacturing units as well as health sectors. The purification of the waste products is mainly based on microbial activity. The hot and humid climate all throughout the year favors this site to act as an incubator for diverse group of microbes[18]. Thus the site was selected to explore wide variety of microbes which can be applicable in biotechnology and bioremediation.

The present paper reports the isolation and characterization of heavy metal accumulating bacteria using chemically defined medium.

MATERIALS AND METHODS

Isolation of strain: Water and soil samples from different sites of East Calcutta Wetland such as Captain Bheri and Charak Danga Bheri and Active Solid Dumping Ground were collected for isolation of the microbes. Bheris are shallow flat bottom waste water fed fisheries which purifies the soluble waste and in turn uses the water for pisciculture and irrigation. On the other hand the treated effluent from the bottom of the water body is used as fertile soil for the cultivable lands[19]. Two different lines of screening were applied for the isolation of the microbes. Samples were spread on milk medium (10% double toned milk, 0.3% yeast extract, 1.5% agar) to screen for protease secreting bacteria. With an aim to look for oil degrading microbes, the soil sample was 10 fold diluted and spread on Carbon Minimal Salt medium (CMS)containing K_2HPO_4 2.2 g L^{-1}, KH_2PO_4 0.73 g L^{-1}, $(NH_4)_2SO_4$ 1g L^{-1}, NaCl 30 g L^{-1}, $MgSO_4$ 0.2 g L^{-1}, Oil 15 ml L^{-1} in distilled water with 1.5% agar. Cultures were grown overnight in an incubator-shaker at 30°C and 150 rpm. Further cultivation and maintenance of pure isolates was done in Luria Bertani (LB) broth containing tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 5 g L^{-1} in double distilled water. Liquid cultures were incubated at 37°C with shaking (150 rpm). Agar plates were incubated in the dark at 37°C. Isolated strains were stored at-80°C in culture medium containing 70% (v/v) glycerol.

Characterization of Isolates

Morphological characterization: The cellular morphology was determined by bright field microscopy of a Gram stained preparation at 100X magnification on a Zeiss Axiostar Plus microscope. The detailed surface features were confirmed by Electron Microscopy.

Biochemical characterization: Optimum pH of the isolates were determined. Biochemical characterization was performed for the following enzymatic assays: protease, lipase, DNase, catalase and oxidase. Commercially available ready made media were used for detecting the presence of enzymes like DNAse and lipase (Media No M482 for DNase, M157 for lipase from HiMedia Laboratories Pvt Limited). From the overnight cultures in LB medium, streaking was done on the respective medium and plates were incubated at 37°C for overnight. The results were assessed as per manufacturer's instructions.

Oxidase test: Isolated single colonies from plate were picked up using a tooth pick and were gently scratched on the oxidase disks (DD 018, HiMedia Laboratories Pvt Limited). Oxidase disks acts as an electron donator to cytochrome oxidase. If the bacteria oxidize the disk (remove electrons) the disk will turn purple (obsevation noted within 60 secs), indicating a positive test.

Catalase test: On isolated single colonies, 1% H_2O_2 (Merck chemicals) was dropped using a glass capillary tube and the appearance of effervescence demonstrates the presence of enzyme. The enzyme catalyses the breakdown of hydrogem peroxide (H_2O_2) with the release of free oxygen. The evolution of gas causes bubbles to form and is indicative of a positive test.

DNase test: DNase agar with Toluidine blue (Himedia FD05) added as a supplement was used for the detection of the enzyme DNase. The isolates were streaked on these plates and left overnight at their respective optimum temperature. DNase positive organisms produce enzyme which hydrolyzes DNA present in the medium. Toluidine blue present as an indicator forms a complex with hydrolysed DNA to produce bright zones surrounding colonies on a royal blue background.

Lipase assay: Tributyrin agar with tributyrin oil (Himedia FD08) added as a supplement was the differential medium used for the detection of lipase. The isolates were streaked on these plates and left overnight at their respective optimum temperature. The plates were observed for growth and production of clearing zone around the growth. In this case the plate contains tributyrin oil which forms an opaque suspension in the agar. When an organism produces lipase and breaks down the tributyrin, a clear halo surrounds the areas where the lipase-producing organism has grown.

Protease assay: Protease test was done in milk medium containing 10% double toned milk, 0.3% yeast extract, 1.5% agar. The milk contains caesin which is broken down by protease into lower subunits. The cells producing protease would give a clear transparent zone around them in an otherwise opaque milk media.

Growth Profiles: From an overnight confluent culture of a single colony, 1% inoculum was given in LB medium in a shake flask and the growth was monitored at regular intervals by measuring the optical density at 660nm in a spectrophotometer (Beckman Coulter DV - 530, UV Vis spectrophotometer)

Antibiotic sensitivity test: The Muller Hinton Agar (MHA) medium (Media No M 147 HiMed) was prepared and commercially available antibiotic disks from HiMedia were used. The strains were grown upto mid log phase, diluted upto 10^{-2} times, spread on MHA plates and antibiotic discs were placed. The plates were incubated overnight at 37°C and observed for zone of inhibition. The 18 different antibiotic disks used were gentamycin (10 µg), neomycin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), vancomycin (30 µg), ampicillin (10 µg), polymyxin B (100 units), ciprofloxacillin (5 µg), norfloxacin (10 µg), trimethoprim (30 µg), doxycycline (30 µg), tetracycine (30 µg), chloramphenicol (30 µg), rifampicin (15 µg),doxcycline hydrochloride(30µg), cloxacillin(10µg), trimethoprim (30µg) and metronidazole(4µg).The sensitivity and resistance profile was determined based on the diameter of the zone of inhibition and evaluation done according to National Committee for Clinical Laboratory Standard's (NCCLS) chart provided with the antibiotic kits by Himedia.

Molecular characterization: Genomic DNA was isolated from enriched culture according to modified alkaline lysis protocol as reported by Ray Chaudhuri *et al*.[20] with minor modifications. The modifications were in the process of lysis where 40 µg µg mL^{-1} proteinase K and 20 µg mL^{-1} lysozyme was added as well as polyethylene glycol and sodium chloride aided precipitation of the cell lysate was avoided. PCR amplification of the 16S rDNA gene fragment was done using universal 16S rDNA primers followed by sequencing of the gene fragment using ABI 16SrRNA sequencing Kit as per the manufacturers protocol. The sequence obtained was subjected to nucleotide nucleotide BLAST. The novel sequences were subjected to GenBank.. Phylogenetic analysis was done by neighbor joining method.

Heavy metal tolerance: Metal salts of $Al(NO_3)_3.9H_2O$, $CuSO_4.5H_2O$, $AgNO_3$, $Pb(NO_3)$, $NiCl_2.6H_2O$, $HgCl_2$,CrO_3 , $CoCl.6H_2O$ and $CdCl_3$ were used for tolerance study. Stock solution of each metal was prepared on basis of molar concentration as per formulae M = Moles of solute/Volume of solution. In addition to $AgNO_3$, AgCl was also used in form of supersaturated stock solution. From an overnight grown culture of a single colony 1% (v/v) was transferred to 3 ml of media supplemented with each metal separately. The initial metal concentration was taken as 1mM. The tolerance was measured on the basis of growth observed (turbidimetry method) within 12 to 48 hours. If growth was observed, innoculum was added to the media with increasing concentration of the metal (1mm, 2mm, 10mm, 100mm and so on). This step was repeated for all the metals in each strain till Minimum Inhibitory Concentration (MIC) was obtained as visualized by cessation of growth.

Quantification of metal accumulation: The concentrations of the metals within the cell were measured by Energy Dispersive X Ray Fluorescence Technique (EDXRF). A Jordan Valley EX-3600 EDXRF system was used in the present study. This consists of a X-ray generator (X-ray tube (Rh anode) and its power unit), a Si(Li) detector (with resolution of 143eV at 5.9keV) for the detection of the emitted characteristic X-rays. Elements from Na-U can be detected using this system. The X-rays from the sample reach the detector through a thin (0.00125mm) Be window. The concentrations were measured in microgram per gram. This system consists of an automated sample chamber where 10 samples can be mounted at a time. The quantitative analysis is carried out on line by the inbuilt ExWin software[16].

After growth in highest metal concentration, cells were harvested by centrifugation and washed with 0.1N HCl thrice in order to remove all the particles adsorbed to the cell surface. This step is necessary to avoid the

possibility of bioadsorption so that only the accumulated metal can be quantified. Post washing, the cell pellet was resuspended with Phophate Buffer Saline (PBS). The cell suspension was vaccum filtered. The filter paper containing the cell sample was dried and subjected to element analysis by EDXRF.

Transmission electron microscopy: In order to confirm the intracellular accumulation and detect the exact location of metal inside the cell Transmission electron microscopy (TEM) of the cells was done. Both in control and treated cells, post growth, cells at a density of 10^8-10^9 cells mL^{-1} were harvested. The cell pellet was washed with PBS thrice followed by resuspension in 1 ml of fixative solution (2.5% gluteraldehyde and 2% formaldehyde) and kept for 6 hours at 4°C. Post fixation, the fixative solution was removed by centrifugation and the cell pellet was washed with PBS five times, finally resuspended in 1 ml of sodium phosphate buffer and was analyzed by TEM. Microscopic imaging of thin sections of cells were performed in a transmission electron microscope model number FEI Philips Morgagni 268D at 100kV acc. Voltage.

Scanning electron microscopy: To determine the effect of metal on the surface feature of the cell, the metal treated as well as untreated cells were analyzed by scanning electron microscope. SEM images have a characteristic three-dimensional appearance and are useful for judging the surface structure of the sample. The sample was fixed in the same way as done for TEM. The samples were then coated with a nanometer-thick layer of gold after mounting them on studs after smearing on glass cover slips and then analyzed by SEM. Scanning Electron microscopy was performed at 30 kV Acc. voltage on a SEM model number LEO 435 VP. Images were taken in 6000x and 9000x magnification.

RESULTS AND DISCUSSION

Morphological and biochemical characterization of the isolates: Three bacterial isolates namely C3, BW- and ADG were isolated from different sites of ECW. The code name of the isolates were assigned according to the site of their isolation, C3 from Charakdanga Bheri, BW- was non protease producing bacteria from water of Captain Bheri and ADG was from active solid dumping ground associated with ECW. Though the primary screening was carried out on milk medium and CMS medium with an aim to isolate protease secreting and oil degrading bacteria respectively, the subsequent characterization of the isolates shows that they don't secrete extracellular protease and lipase thereby indicating that these media are not specific for the growth of only those groups of microbes. Since they are

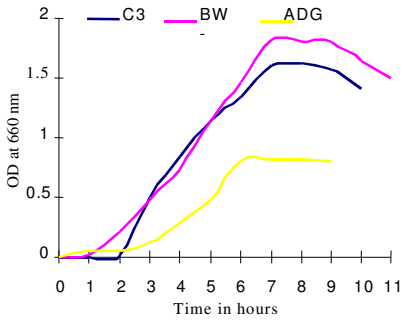

Fig. 1: Growth curve of the three isolates obtained by plotting the Optical Density at 660nm on the Y axis and the time in hours on the X axis. The experiments were

present in ECW, it is expected that they would be exposed to heavy metal. Thus their response to metal was tested.

The preliminary characterization of these isolates were done on the basis of their morphology and gram nature. Isolate C3 was observed to be Gm-ve short bacilli , BW- as Gm-ve cocci while ADG was Gm +ve bacilli. The optimum pH for growth was found to be 7.5 in case of all three isolates. Isolate ADG was found to tolerate a wide range of pH from 4 to 12. This property can enable the application of this bacteria under in situ condition to treat tailing effluents from mines, acid mine drainage etc.

The growth profile of all the three isolates is demonstrated in Fig. 1. All the isolates follow a sigmoid pattern of growth. ADG has a prolonged lag phase up to 3 hrs followed by log phase of about 3 hrs with mid log phase at about 5 hrs of growth. Isolate C3 and BW- reach log phase after 2-3 hours of growth and enter a stationary phase at around 7 to 8 hours.

Biochemical characterization was done in terms of presence of enzymes like DNase, catalase, oxidase, lipase and protease. All of the three isolates were catalase positive. The presence of catalase provides a defense mechanism against the reactive oxygen species and thus ability to exist in aerobic conditions and thereby facilitates the metabolic nature for their efficiency. Out of the three, only ADG was found to be oxidase positive in nature. On the other hand none of the strains were found to produce extracellular enzymes like DNase, protease and lipase.

The antibiotic profile of the isolates (Table 1) indicate the sensitivity of the strains to different antibiotics. All the isolates are resistant to vancomycin,

Table 1: Antibiotic sensitivity profile of the three isolates from East Calcutta Wetland. S stands for Sensitive, I for intermediate sensitivity and R stands for resistance. The isolates are listed in 1st Row while the antibiotics are listed in the 1st column

Isolates→ Antibiotic↓	C3	BW-	ADG
Neomycin	R	R	I
Gentamicin	R	S	S
Cefotaxime	I	I	R
Ceftazidime	S	S	R
Vancomycin	R	R	R
Ampicillin	R	S	R
Polymyxin B	R	R	R
Ciprofloxacillin	I	R	S
Norfloxacillin	S	R	S
Trimethoprim	R	R	R
Doxycycline	R	S	S
Tetracycline	R	R	S
Chloramphenicol	R	R	S
Rifampicin	R	R	I
Doxcycline hydrochloride	R	S	S
Cloxacillin	R	R	R
Metronidazole	R	R	R

polymyxin B and trimethoprim, They show either resistance or intermediate action to cefotaxime and rifampicin. Several reports indicate a correlation between antibiotic resistance and metal tolerance[21]. In certain cases metal tolerance mechanism contribute to the increase in antibiotic resistance, the occurrence of this phenomenon can be attributed to the clustering of these genes in the same plasmid. Thus attempts would be made to look for presence of plasmid in these strains.

Molecular characterization of isolates: The 16SrDNA sequences obtained for the isolates were subjected to BLAST N. Isolate C3 showed 100% similarity to *Escherichia coli* O157H7 , BW- showed 99.76% similarity to *Escherichia coli* W3110ATCC and ADG with 96.2 % similarity with *Brucella melitensis* . The two isolates found to be novel were submitted to GenBank and are available under the Accession No. EU006698 (BW-) and EU326525 (ADG). The phylogenetic position were predicted according to the neighbor joining method (Fig 2).

Metal tolerance: All the strains were found to grow in presence of heavy metals like Ni, Cu, Ag, Al, Fe, Cr, Pb, the MIC for each metal is depicted in Table 2 . The growth in presence of metals can be due to two conditions, metabolism dependent metal uptake inside the cell or because of energy independent process of biosorption to the membrane. The 0.1N HCl wash of the cells post growth in presence of metal ensures removal of all metals sticking to the cell surface so as to confirm that only internal accumulation of the metal is detected through EDXRF analysis. The relative accumulations of different metals as obtained from

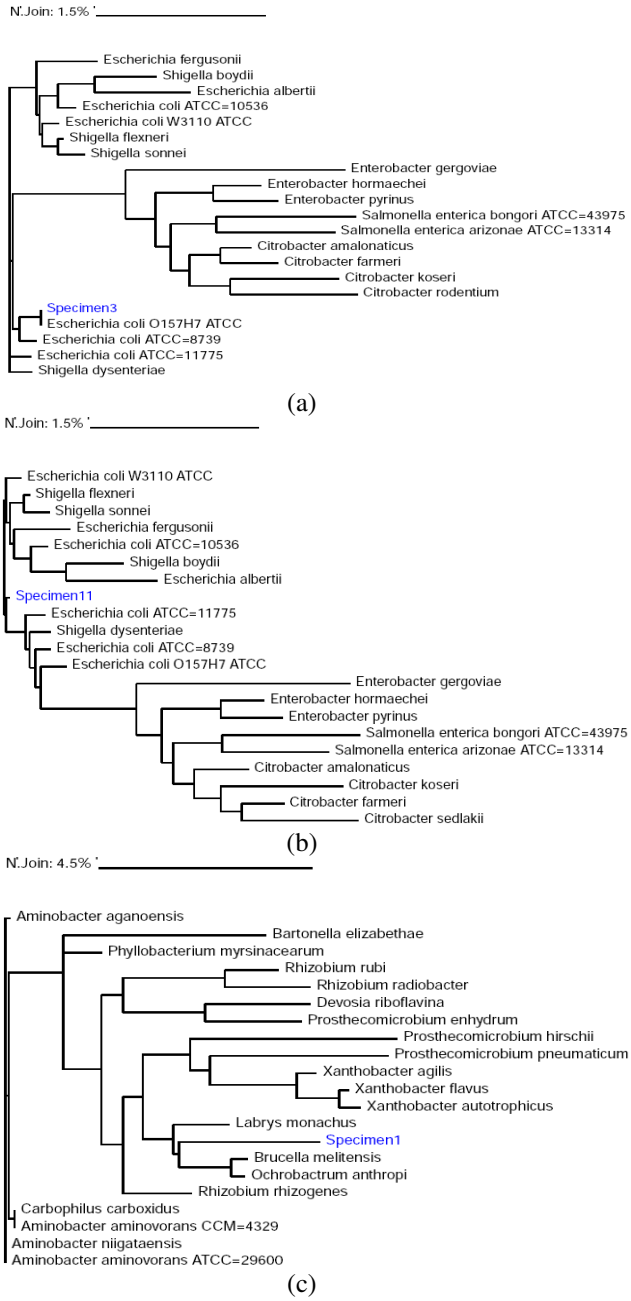

Fig. 2: Tree of 16S rRNA based phylogenetic alanysis of isolate C3 constructed using neighbour joining method. C3 is indicated as specimen3 in the tree, 2b: phylogenetic tree for isolate BW- constructed in similar manner. BW- is indicated as specimen 11 in the tree, 2c: phylogenetic tree for isolate ADG. The isolate has been indicated as specimen 1 in the tree. The % difference is indicated in the scale placed before each tree

Table 2: Minimum inhibitory Concentration (MIC) in mM of different metals obtained for the three isolates from East Calcutta Wetland. For C3 and BW-, $Ag(NO)_3$ was used where as for ADG supersaturated solution of AgCl was used since growth was inhibited in presence of $Ag(NO)_3$. "-" stands for no growth at the lowest concentration tested.

Strains	Ni	Cu	Ag	Al	Cr	Co	Pb	Hg	Cd
C3	12	5	3	15	5	5	5	0.2	2
BW-	8	5	3	15	5	3	5	0.1	0.2
ADG	4	5	400µl	4	2	3	6	-	-

Table 3: Table indicating the number and size range of nanoparticles found inside bacterial cells of isolates BW- and C3 as observed in Transmission Electron Micrographs

	Isolate			
	BW-		C3	
Metal	Max. No of nanoparticles / bacterial cell	Size range of particle in µm	Max No of nanoparticles/ bacterial cell	Size range of particle in µm
Cu	5	0.023- 0.050		
Pb	7	0.023-0.050	6	0.030-0.031
Hg	12	0.011-0.043		
Cr	3	0.020-0.034	2	0.035-0.065
Cd	25	0.015-0.025	32	0.012-0.024
Ni			7	0.028-0.072

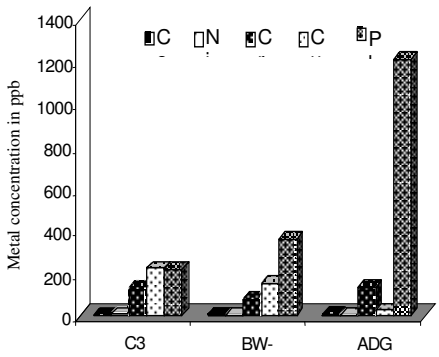

Fig. 3: Graph representing relative accumulation of metals inside the cells in ppb as determined from EDXRF analysis. Isolate ADG shows immense accumulation of Pb as compared to C3 and BW-

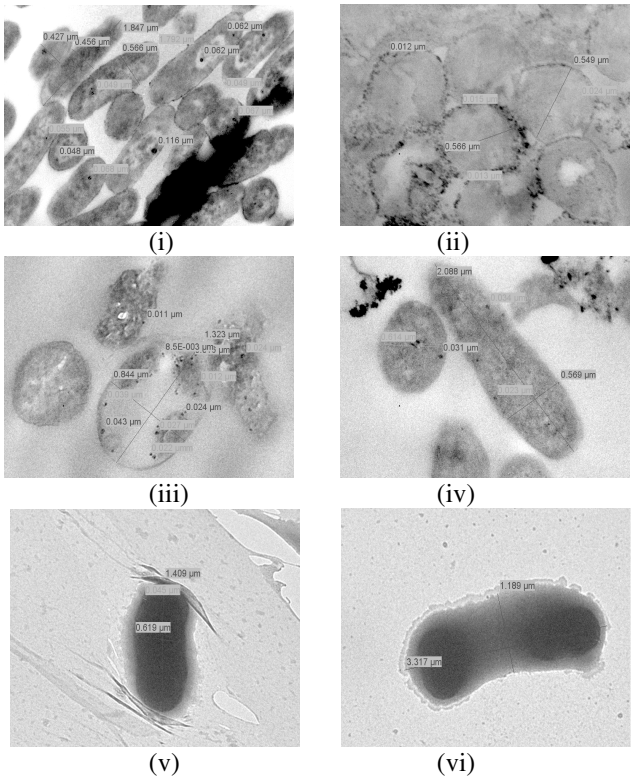

Fig 4: Transmission Electron Micrographs depicting the accumulation of metal inside the bacterial cell on metal treatment. i: Isolate C3 treated with Cr salt, ii: C3 treated with Cd salt, iii: isolate BW- treated with Pb salt, iv: BW- isolate treated with Hg salt, v: isolate ADG treated with Cu salt and vi: ADG treated with Ag salt

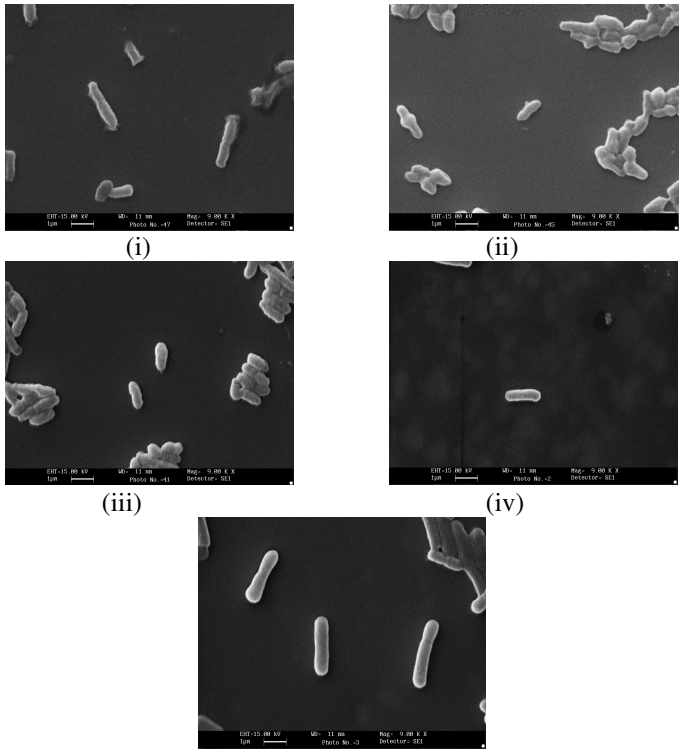

Fig. 5: Scanning Electron Micrographs depicting the effect of metal on the cell morphology and dimensions of isolates ADG and C3 in absence and presence of metal treatment at a magnification of 9000X. i: Isolate ADG under untreated condition, ii: isolate ADG on treatment with Cu salt, iii: isolate ADG upon treatment with AgCl, iv: Isolate C3 in absence of metal treatment and v: isolate C3 upon treatment with Cu salt

EDXRF analysis is depicted in Fig. 3. Maximum accumulation is found for Pb followed by Cr in case of all the isolates. These metals are commonly emitted from industries involved in electroplating, dye, battery manufacturing, organic chemical production, tannery etc. Thus employing these microbes having an excellent efficiency in uptaking these toxic metal opens up the possibility to decontaminate as well as recover and recycle these metals.

In order to locate the region of intracellular accumulation of metal, treated cells were subjected to transmission electron microscopy. The TEM results indicates throughout accumulation of metals in ADG (Fig 4v and vi) while particle formation in C3 and BW- (Fig 4i and iii). There is differential accumulation in ADG (Fig 4v and vi) and localized deposition in C3 as well as BW-(Fig 4ii-vi). C3 and BW- showed formation of nanoparticle inside the cell, the number and size range of the particles were observed to vary from metal to metal. (Table 3).

The metal uptake in cells can induce different response mechanisms in the cell. These can be the induction of molecules like siderophores, metallothioneins accompanied by specific changes in the cell morphology. The effect of metals on cell morphology was demonstrated through scanning electron microscopy results. Distinct changes in cell size and surface features were observed . In case of C3, the EDXRF results indicate higher accumulation of Cu in comparision to that of ADG (Fig. 3). In accordance to this the SEM image shows increase in surface area of C3 cells treated with Cu as compared to untreated cells (Fig. 5iv and v). This can be interpreted as a possible strategy of the cell to accumulate more metals. In contrast SEM image of ADG with Cu treatment clearly shows shrinkage of cells along with development of thick wooly coat around the cell which can be the secreted exopolysaccharide. This alteration in surface features may be explained as a negative reponse of ADG against further uptake of metal by decreasing the

area of contact with the metal. (Fig 5i, ii). The extent of shrinkage can vary with different metals observed in case of ADG treated with Cu and Ag (Fig 5ii, iii). These depict two distinct mode of responses to metal stress.

CONCLUSION

The entire study can head towards an application mode, main utilization being in bioremediation of heavy metals. Further study can proceed towards designing of a bioremedial package constituting either of single pure culture or as a consortium with microbes in varying proportion, keeping an objective of maximum removal with minimum retention time. One of the main application can be in economical recovery of metals like Ag from ores and minerals, photographic plate development effluent. On the other hand the intracellular accumulation can lead to further investigation of the molecular mechanisms underlying the phenomenon. Exploring into the differential expression of genes in response to the stress can give a picture of the entire mechanism.

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