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Plasmid curing and protein profiling of heavy metal tolerating bacterial isolates

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ABSTRACT

Heavy metal tolerating bacterial isolates- AD1, AD2, AD3, AD4, AD5, AD6 and AD7 were isolated from garden soil, industrial waste water and textile effluent samples from the local area of Kalyan, Dist Thane, MS. The isolates were observed to tolerate high levels of mercury, cadmium, arsenic and lead and were seen to be resistant to a wide range of antibiotics. Plasmid isolation was carried out using Alkaline lysis method. The size of the isolated plasmid DNA was approximately 15-26 kb. Plasmid curing was carried out by Ethidium Bromide and 2 % Sodium Dodecyl Sulphate. Lead, cadmium and arsenic resistance gene was found to be present on the chromosomal DNA rather than the plasmid DNA whereas, mercury genes were found to be present on the plasmid. Curing result showed the loss of antibiotic and heavy metal resistance property from the isolated strain and confirms a relationship between antibiotic and heavy metal resistance with plasmid. The whole cell protein samples from the isolates treated with different concentrations of lead, cadmium, arsenic and mercury were isolated, electrophoresed on SDS-PAGE and the protein profile was studied. A significant change in the banding pattern was observed.

Key words: Protein profile, Antibiotic resistance, Plasmid curing, heavy metals, SDS-PAGE.

INTRODUCTION

Heavy metal contamination is spread worldwide. The environmental pollution by heavy metals comes from anthropogenic sources such as smelters, mining, power stations and the application of pesticides containing metal, fertilizer and sewage sludge. Many of these heavy metals like Zn, Cu, Co, Ni, Mn and Fe are known as essential “trace elements” and are necessary for living organisms [1] because at a certain concentration levels, these elements participate in some enzyme activities.

Microorganisms are ubiquitous in nature and are involved in almost all biological processes of life. Due to urbanization and natural processes, heavy metals have been found in increasing proportions in microbial habitats. Metals are known to play a major role either directly or indirectly in almost all metabolic processes, growth and development of microorganisms [2, 3]. However, increasing concentrations of metals beyond tolerance levels have forced these organisms to adapt to various biological mechanisms to cope with this condition.

At high concentrations, the toxic effects of these metals are revealed. These heavy metals can block functional groups of important molecules and transport channels for required nutrient ions, damaged cell membranes and DNA structure, and alter enzyme specificity leading to disruption of the cellular functions [4]. Hence, microorganisms have evolved metal resistance strategies, including exclusion by permeability barrier, cellular sequestration, enzymatic transformation, reduction of metal to less toxic forms, and efflux of the metal ions from the cell [5-7].

This may be due to a variety of chromosomal-, transposon-, and plasmid-mediated resistance systems [8]. This increasing heavy metal tolerance has yet another repercussion in the environment as it may contribute to the maintenance of antibiotic resistance genes by increasing the selective pressure of the environment. The presence of

multiple metal and antibiotic resistance property in the bacterial population poses a potential threat towards human and environmental health. There is concern that metal contamination functions as a selective agent in the proliferation of antibiotic resistance [9]. Curing experiments have suggested that metal tolerance and antibiotic resistance are based on certain plasmid derived genes [10].

Regulations of cellular processes following exposure to metal ions at both transcriptional and translational levels have been reported [11-14]. However, the molecular mechanisms and underlying responses of cells against various metal ions are not yet completely understood. Stress conditions induce a variety of responses inside bacterial cells which may result in the change of various proteins in the cell.

The present study aims to determine variations in protein expression in response to heavy metal stress. The bacterial isolates were subjected to plasmid curing experiments to determine the likelihood of plasmid-borne resistance pattern and relationship between heavy metals and antibiotic resistance genes.

MATERIALS AND METHODS

Material:
Cadmium Sulphate, Lead Acetate, Sodium Arsenate were obtained from Loba Chemie, India; Mercuric Chloride was procured from Molychem Chemicals, India. The media components, biochemicals and antibiotic discs were obtained from Hi-Media, India. All other chemicals used were of analytical grade. All the chemicals were made in distilled water.

Isolation of metal tolerant microorganism:
The metal tolerant bacteria were isolated from garden soil, industrial waste waters and textile effluent samples from the local area in Kalyan. Enrichment of the samples was carried out using sterile nutrient broth containing 25 ppm of heavy metal salts (Cadmium Sulphate, Lead Acetate, Sodium Arsenate and Mercuric Chloride) and incubated at 28 ± 2˚C for 7 days. Growth was isolated on sterile nutrient agar plates. The colony characters, Gram staining and biochemical characteristics of the isolates were studied. The isolates were identified using various biochemical tests according to Bergey's Manual of Systematic Bacteriology (Vol I and II). Standard Biochemical tests included Indole, Methyl red, Vogues Prousker and Citrate Test; Triple Sugar Iron slant test and sugar fermentation containing 1% sugar solutions of sucrose, glucose, lactose, xylose, maltose and mannitol with Andrade's indicator. The isolates were further identified using 16 S rRNA carried out at NCCS, Pune.

Antibiotic and heavy metal Tolerance:
The bacterial isolates were tested for their sensitivity to different antibiotics by means of Kirby –Bauer Disc diffusion method [15, 16]. The following antibiotics from Hi-Media, India, were used: Gentamycin (G), Ampicillin (A), Vancomycin (Va), Sulphafurazole (Sf), Chloramphenicol (C), Tetracycline (T), Streptomycin (S), Penicillin (P), Erythromycin(E), Ciprofloxacin (Cf), Aztreonam (AT), Mecillinam (MEC), Trimethoprim (TR), Doxycycline hydrochloride (Do) and Carbenicillin (CB)

Metal tolerance (MIC) was examined on sterile nutrient agar plates in which varying concentrations of heavy metal salts *viz* Cadmium Sulphate, Lead Acetate, Sodium Arsenate and Mercuric Chloride, were incorporated. The isolates were spot inoculated using sterile cotton swabs and the plates were incubated at 37˚ C for 24 hours

Isolation of plasmid:
Pure cultures of the isolates were grown overnight in 25 ml of sterile Luria-Bertani broth (Hi-Media) and cell pellet was harvested by centrifugation at 6000 rpm for 10 min at 16˚C. The cell plasmids were isolated using Birnboim and Doly's Alkaline Lysis method (Miniprep method) [17, 18]. The extracted plasmid was suspended in 20 µL of TE buffer and was further analyzed by 1% Agarose gel electrophoresis [19, 20].

Plasmid curing by various chemicals:
The 24 hrs old cultures of the isolates were grown in sterile nutrient broth containing various chemical agents *viz*, Ethidium Bromide (100 mcg/ ml) and 2% SDS [21, 22]. The tubes were incubated at 37˚C for 24 hrs. After the incubation the isolates were reinoculated in sterile nutrient broth and incubated further for 24 hrs.

The cured isolates were checked for their heavy metal tolerating capacity and antibiotic sensitivity.

Protein estimation on exposure to heavy metal salts:
Bacterial cultures were grown in sterile nutrient broth containing heavy metal salts – Mercuric Chloride (10 to 250 ppm) / Cadmium Sulphate (10 to 250 ppm) /Lead Acetate (10 to 250 ppm) / Sodium Arsenate (10 to 250 ppm) along

with plain nutrient broth control containing no metal solutions added to the medium. 3 ml of the broth culture was removed from each flask and centrifuged at 10,000 rpm at 4˚C for 5-10 min. The supernatant was discarded and 1 ml lysis buffer (General lysis buffer: 50mM Tris HCl;100 mM NaCl;1mM Tween 20;5% Gycerol;1mM EDTA) was added to the pellet. The pellets were sonicated for a total of 2 minutes (4 times for 30 sec each time with a gap of 30 sec between successive sonications). The sonicated sample was centrifuged at 5000 rpm at 4˚C for 2 min. 200 µL of this supernatant was incubated with chilled 1600 µL acetone for 30 min at 4˚C. The precipitated protein was dried. 100 µL PBS was added to this precipitated protein for bringing it into solution. The protein estimation was carried out using Folin Lowry's method (1951) using std. BSA having a concentration of 100 mcg/ml [23, 24].

Whole cell protein extraction Protein profiling by SDS- PAGE:

The isolates were grown overnight in sterile nutrient broth containing 250 ppm of heavy metal salts *viz*, Cadmium Sulphate, Lead Acetate, Sodium Arsenate and Mercuric Chloride-at 37˚C. The cells were harvested by centrifugation at 13,000 rpm for 10 mins. The bacterial cell pellet was washed with phosphate buffer (pH 7.0) to remove the traces of remaining media and again centrifuged at 1000 rpm for 10 mins. The cell pellet obtained was mixed with 1 ml of 2X Sample Buffer (0.5% SDS; 1.25 % β mercaptoethanol; 0.03%Bromophenol Blue; 2.5% glycerol; 15mM Tris Cl; pH-6.8) and incubated in a boiling water bath for 30 mins. This was further used for protein profiling using SDS-PAGE. 18 µL of the protein sample was taken and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) containing 2.5 % stacking and 12.5 % of resolving gels and separated based on Laemeli discontinuous buffer system (Harlow and Lane 1988). After electrophoresis on a vertical slab unit under a constant voltage of 100 V for 3 hrs, the gels were stained with Coomassie brilliant blue R-250 (Himedia). A large protein marker calibration kit (Fermentas, Life Sciences) was used to estimate the molecular weight of protein bands [25].

RESULTS AND DISCUSSION

The enrichment and isolation process yielded seven morphologically different bacterial isolates which were further tested for colony characters and various biochemical characteristics (Table 1; Table 2). The isolates were further identified using 16S rRNA (NCCS, Pune) (Table 3).

Table 1: Colony Character of the bacterial isolates

Characters	AD1	AD2	AD3	AD4	AD5	AD6	AD7
Size	3 mm	1 mm	4 mm	1 mm	2 mm	1 mm	2mm
Shape	Circular	Circular	Circular	Circular	Irregular	Circular	Circular
Colour	Dull white, yellow pigment	Dull white	Dull white	Colourless	Dull white	Dull white	Colourless
Elevation	Flat	Low convex	Flat	Flat	Flat	Convex	Flat
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Opacity	Translucent	Translucent	Translucent	Transparent	Opaque	Opaque	Translucent
Consistency	Butyrous	Butyrous	Butyrous	Butyrous	Dry	Butyrous	Butyrous
Mobility	Motile	Motile	Motile	Motile	Motile	Motile	Motile
Gram nature	Gram negative cocobacilli	Gram negative cocobacilli	Gram positive bacilli	Gram negative cocobacilli	Gram positive bacilli	Gram positive bacilli	Gram negative cocobacilli

Table 2: List of biochemical characteristics for the bacterial isolates

Colony No	In	MR	VP	Cit	TSI				Sugar fermentations						Oxi	Cat
					Butt	Slant	H_2S	Gas	Glu	Xyl	Lact	Malt	Man	Sucr		
AD1	+	-	+	+	Acidic	Alkaline	-	+	A	A+G	A+G	A+G	A+G	A+G	+	+
AD2	+	+	-	+	Alkaline	Acidic	-	+	A	-	A+G	A+G	A+G	A+G	+	+
AD3	+	+	-	-	Acidic	Acidic	-	+	A	A+G	A+G	A+G	A+G	-	-	+
AD4	-	+	-	+	Acidic	Alkaline	-	-	A+G	A+G	A+G	A+G	A+G	A+G	+	+
AD5	-	+	-	+	Acidic	acidic	-	-	A+G	-	A	A	A	A	+	+
AD6	-	-	+	-	Acidic	Acidic	-	-	A+G	-	A+G	A+G	A+G	A+G	_	+
AD7	-	+	-	+	Acidic	Alkaline	-	-	A+G	A+G	A+G	A+G	A+G	A+G	+	+

In-Indole; MR- Methyl Red; VP- Vogues Prousker; Cit- Citrate; TSI- Triple Sugar Iron; Glu- Glucose; Xyl- Xylose; Lact- Lactose; Malt-Maltose; Man- Mannitol; Sucr- Scurose; Oxi-oxidase; Cat-catalyas; A - Acidic; G-Gas; '+'- Positive; '-'- Negative

Table 3: Identified isolates by 16 S r RNA (NCCS, Pune)

Sr. No.	Isolate No.	Name of the organism
1.	AD1	*Klebsiella pneumoniae subsp. rhinoscleromatis* ATCC 13884(T)
2.	AD2	*Paracoccus chinensis* KS-11(T) (Acc. No. EU660389)
3.	AD3	*Planococcus rifietoensis* (T); M8 (Acc. No. AJ493659)
4.	AD4	*Pseudomonas aeruginosa (Acc. No.* PAL06)
5.	AD5	*Bacillus megaterium*
6.	AD6	*Brevibacillus choshinensis strain DSM 8552* (Acc. No.NR_040980.1)
7.	AD7	*Pseudomonas aeruginosa (Acc. No.* DQ464061)

The Antibiotic sensitivity testing (AST) was carried out using ready to- use antibiotic discs and checked using Kirby Bauer's Disc Diffusion method (Table 4). The isolates were found to resistant to most of the antibiotics used.

Table 4: Antibiotic Sensitivity testing using Disc Diffusion method (Kirby Bauer)

Antibiotic	P	E	SF	G	C	VA	A	S	T	CF	MEC	AT	DO	TR	CB
AD1	R	I	S	I	S	S	R	S	I	S	S	S	S	S	S
AD2	R	I	R	I	S	S	R	S	R	S	S	R	I	R	S
AD3	R	I	R	I	S	S	R	S	R	S	R	S	R	R	S
AD4	R	R	R	I	R	R	R	R	R	S	S	S	R	R	S
AD5	R	S	R	I	S	S	S	R	I	S	S	I	S	S	S
AD6	R	S	S	S	S	S	R	S	I	S	S	S	S	R	S
AD7	R	R	R	S	R	R	R	R	R	S	S	S	R	S	S

According to Kirby Bauer's Chart.; R- Resistant; S- Sensitive; I- Intermediate

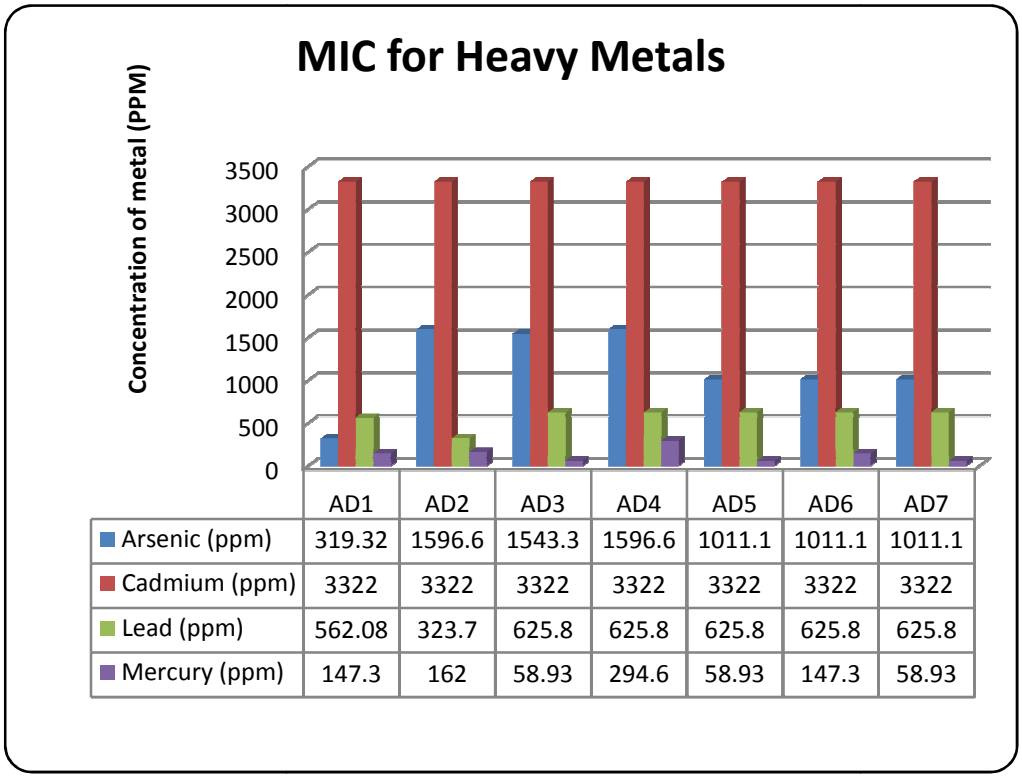

Figure 1: Minimum Inhibitory Concentration (MIC) for heavy metals

The heavy metal tolerance was checked by subjecting the isolates to varying concentrations of metal salts. It was seen that *Pseudomonas aeruginosa* (AD4) could tolerate 294.60 ppm of mercury, 1596.60 ppm of arsenic and 625.80 ppm of lead. The concentration of cadmium tolerated by all the organisms is same *i.e* 3322 ppms (Figure 1).
Plasmid isolation was carried out using Birnboim and Doly's Alkaline Lysis method (Miniprep method) (Figure 2). The detection of the isolated plasmid was carried out using 1% Agarose gel electrophoresis using Ethidium bromide for plasmid visualisation. The plasmid DNA had a molecular weight of ~26 kD.

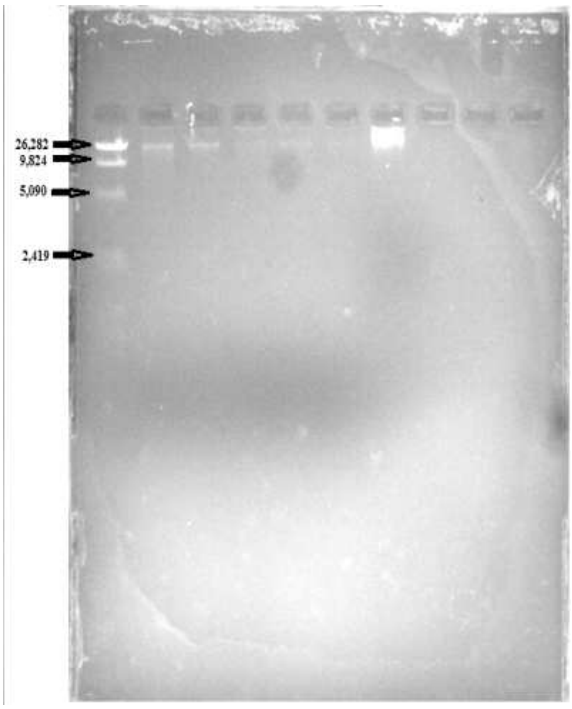

Figure 2: Plasmid isolation by Miniprep (Alkaline lysis) method
1) λ digest DNA, 2) AD1, 2) AD2, 3) AD3, 4) AD5, 5) AD6, 6) AD7, 7) AD4

24 hr old cultures of the isolates was subjected to plasmid curing using Ethidium bromide (100 mcg/ ml) and 2% SDS for 24 hrs. After the curing, the isolates were subjected to antibiotic sensitivity testing and heavy metal tolerance. After comparing the results of the AST and the heavy metal tolerance studies (performed before the plasmid curing), it was seen that Ethidium bromide was a much efficient agent for curing the plasmid (Figure 3). In most of the isolates, except AD4 and AD7, the isolates lost the capacity to grow on nutrient agar plate containing mercury, while the isolates were able to grow on the other nutrient agar plates containing cadmium, arsenic and lead. This indicated that in these isolates the 'mer' genes were present on the plasmid while the rest may be present on the chromosome. AD4 and AD7 were seen to gown on all the heavy metal supplemented plates indicating that the genes for heavy metal tolerance may be present on the chromosomal DNA only.

Table 5: Metal tolerance after growth in Ethidium bromide (100mcg/ml)

Colony No	Hg (25ppm)	As (100 ppm)	Cd (100 ppm)	Pb (100 ppm)	Nutrient Agar
AD1	-	-	+	+	+
AD2	-	-	-	-	+
AD3	-	-	+	+	+
AD4	+	+	+	+	+
AD5	-	+	+	+	+
AD6	-	+	+	+	+
AD7	+	+	+	+	+

Table 6: Metal tolerance after growth in 2% SDS

Colony No	Hg (25ppm)	As (100 ppm)	Cd (100 ppm)	Pb (100 ppm)	Nutrient Agar
AD1	-	+	+	+	+
AD2	-	+	+	+	+
AD3	-	+	+	+	+
AD4	+	+	+	+	+
AD5	-	+	-	+	+
AD6	-	-	+	+	+
AD7	+	+	+	+	+

Antibiotic sensitivity testing showed that the isolates treated with Ethidium bromide lost the capacity to resist certain antibiotics. The isolates treated with 2%SDS, showed no significant change in their tolerance and antibiotic sensitivity pattern, indicating that 2% SDS could not be used as a plasmid curing agent. Higher concentrations of

SDS can be further checked. After incubation in Ethidum bromide and 2% SDS, the isolates were gown on sterile nutrient agar plate to check their viability (Table5; Table 6; Table 7; Table 8).

Table 7: Antibiotic sensitivity testing after growth in Ethidium bromide (100mcg/ml)

Antibiotic	P	A	T	E	S	TR	AT
AD1	R	I	I	S	NA	NA	NA
AD2	R	R	I	S	NA	NA	NA
AD3	R	I	R	S	NA	NA	NA
AD4	R	R	R	I	NA	NA	NA
AD5	R	NA	S	NA	S	NA	S
AD6	R	I	S	NA	NA	I	NA
AD7	R	R	R	R	NA	NA	NA

R: Resistant; S: Sensitive; I: Intermediate; NA: Not Applicable

Table 8: Antibiotic sensitivity testing after growth in 2%SDS

Antibiotic	P	A	T	E	S	TR	AT
AD1	R	R	R	R	NA	NA	NA
AD2	R	R	R	S	NA	NA	NA
AD3	R	R	R	R	NA	NA	NA
AD4	R	R	R	R	NA	NA	NA
AD5	R	NA	R	NA	R	NA	S
AD6	R	R	R	NA	NA	R	NA
AD7	R	R	R	R	NA	NA	NA

R: Resistant; S: Sensitive; I: Intermediate; NA: Not Applicable

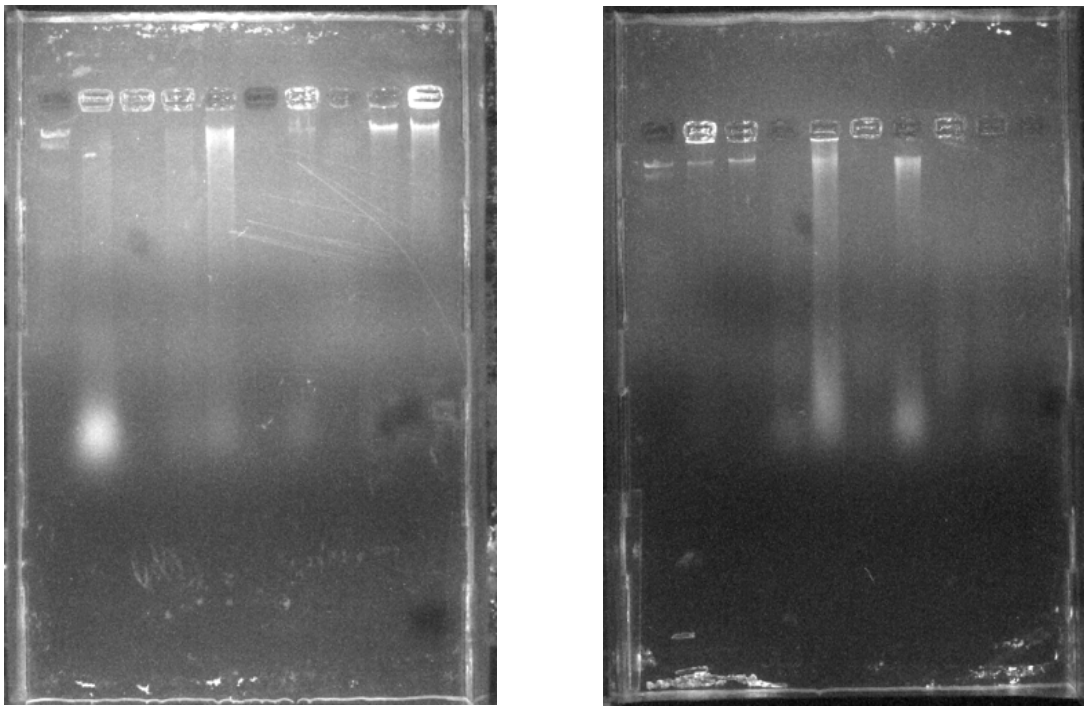

Figure 3: Plasmid isolation after curing with 2%SDS and Ethidium bromide (EB)

Gel I: 1) λ digest DNA, 2) EB treated AD1, 3) EB treated AD2, 4) EB treated AD3, 5) EB treated AD4, 6) EB treated AD5, 7) EB treated AD 6, 8) EB treated AD7, 9) SDS treated AD1, 10) SDS treated AD2 Gel II: 1) λ digest DNA, 2) SDS treated AD3, 3) SDS treated AD4, 4) SDS treated AD5, 5) SDS treated AD6, 6) BLANK, 7) SDS treated AD7

Pseudomonas aeruginosa (AD 4) was exposed to increasing concentration to heavy metal salts. The protein was extracted and was estimated using Folin Lowry's method. The results showed that variations were seen in protein concentrations in case of the isolate exposed to 10 -250 ppm heavy metal salts of cadmium, mercury, arsenic and lead (Figures 4 and 5). There was an increase in the total protein concentration in the medium in the presence of each heavy metal salt though the amount of protein did not increase with further increasing concentration of heavy metal salt. This state was seen in response to all the heavy metal salts indicating that the proteins/ enzymes required for the uptake of heavy metals within the cell remained constant even if the cells are growing in increasing concentrations of heavy metal salts.

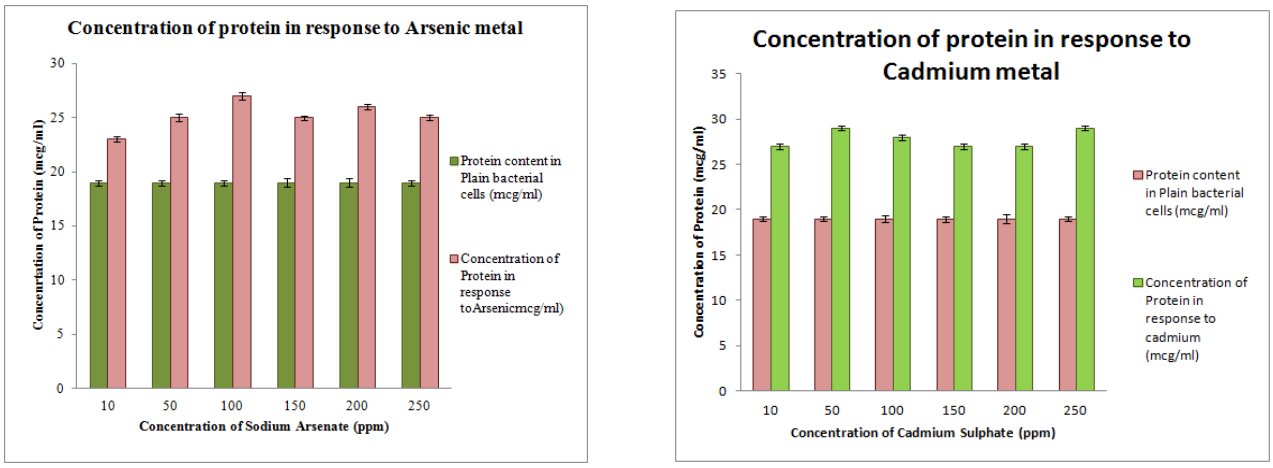

Figure 4: Concentration of protein in response to arsenic and cadmium

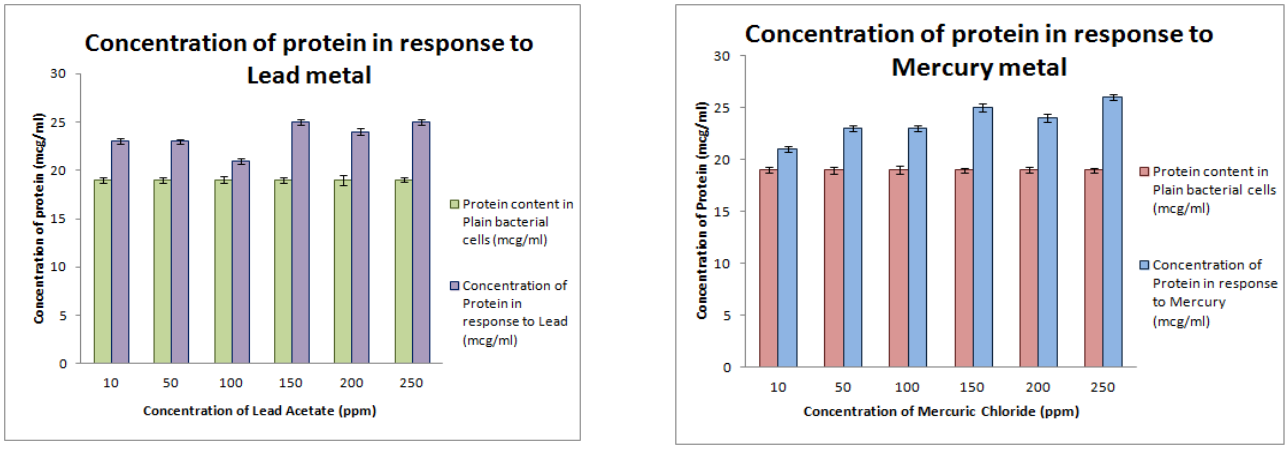

Figure 5: Concentration of protein in response to lead and mercury

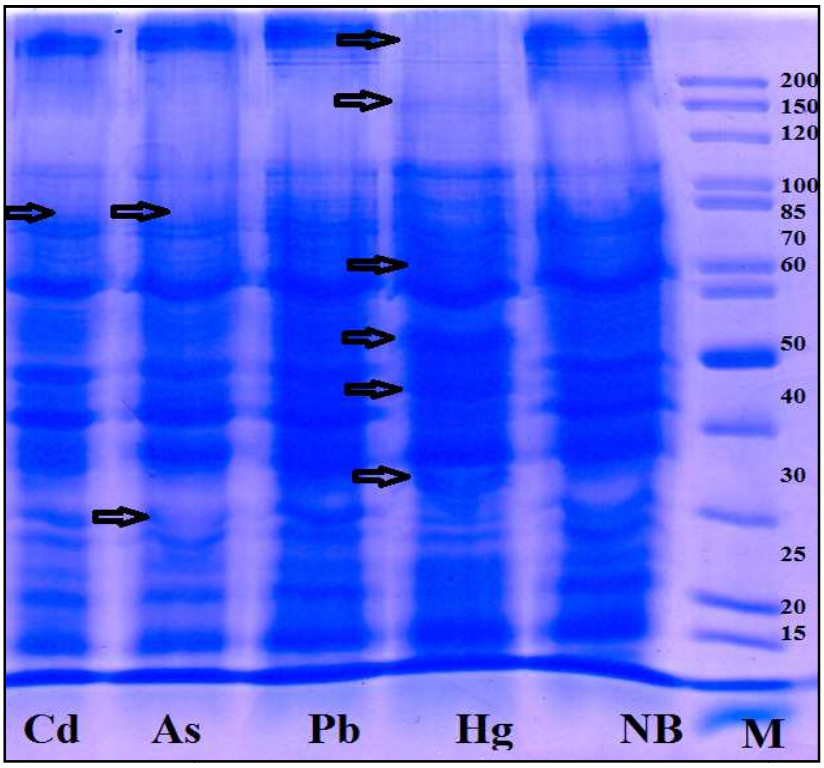

Figure 6: SDS PAGE of proteins from *P. aeruginosa* grown in different metals

The protein analysis was carried out for *Pseudomonas aeruginosa* (AD4) using SDS PAGE analysis. The cells were grown in heavy metal salt solution and treated with 2X sample buffer and heated in a boiling water bath for half hour. The gel was stained using Commassie brilliant blue solution. The inducible response of metal stress (Hg, Cd, As and Pb) of *Pseudomonas aeruginosa* (AD4) was studied. The electrophoresis analysis in 12.5% SDS-PAGE of whole cells lysate protein are summarised in Figure 6.

On exposure of *Pseudomonas aeruginosa* to 250 ppm of $HgCl_2$, there were certain protein bands which were up-regulated and certain bands were lost. The thickening of a protein band indicates that the protein has increased its concentration in the cell in presence of the heavy metal salt. Protein bands of molecular weight < 200 kD, 60kD and 25 kD were absent while bands having molecular weight of 150 kD, 70 kD, 50 kD, 30 kD were seen. In cells exposed to cadmium and arsenic, 100 kD band was absent which was seen in the other lanes. A 30 kD band was absent in lane for arsenic. The gel showed certain bands which were thickened indicating more of that particular protein. 15kD protein band was seen to be more thickened in the Hg lane indicating more of that protein been produced in the cell. It was obvious that some sets of proteins were induced under 250 ppm of heavy metal salts (Hg, Cd, As and Pb). This group of proteins characterised with high intensity and may be responsible for Hg, Cd, As and Pb reduction. A more distinct pattern was seen when the cells were grown in 250 ppm of $HgCl_2$.

CONCLUSION

In the present study, we have isolated 7 heavy metal resistant strains having the potential to bioremediate or reduce toxic heavy metals- mercury, arsenic, lead and cadmium. The tolerance to these heavy metals was found to be both chromosomal as well as plasmid mediated in the isolates. The total protein estimation revealed that there was an increase in the protein concentration in presence of heavy metals but the amount remained constant even in the presence of higher concentration of heavy metal. SDS-PAGE showed induction of some sets of proteins under heavy metal stress especially mercury. Probably, the identification of protein sequence will reveal the intimate role that each protein plays in the cell.

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