CHAPTER 3

MATERIALS AND METHODS

3. Materials and Methods

3.1. Materials

3.1.1. Consumables

Consumables like petriplates, polystyrene tubes (15 & 50 ml), microfuge tubes (1.5 & 2 ml), PCR tubes, micropipette tubes (10, 200 & 1000 µl) were procured from Tarson, India; Erlenmeyer flasks (100, 250 & 500 ml) and glass tubes from Riveria, India. Autoclavable bags and paraffin roll were purchased from Himedia lab, India.

3.1.2. Chemicals and Media

For microbiology work different media reagents such as peptone, beef extract, yeast extract, agar, NaCl for nutrient media and Luria Bertini media, were used for regular bacterial cultures. For Minimum salt medium, Na_2HPO_4, KH_2PO_4, $MgSO_4$, NH_4 $(CH_3COO)_3$ Fe and $Ca(NO_3)_2.4H_2O$ was used. Heavy metals used to give metal stress conditions to bacterial cultures were Cd $(NO_3)_2.4H_2O$, $K_2Cr_2O_7$ and $NiCl_2.6H_2O$ purchased from Merck, India. For different buffer preparation and other reagents, Tris HCl, NaOH, Na_2CO_3, K_2HPO_4, NaH_2PO_4, KCl etc, solvents like ethanol, methanol and acetone, acids like HCl, H_2SO_4, HNO_3, glacial acetic acid etc were used. For enzyme assays some of the important chemicals used were 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), oxidized GSH (GSSG), pyrogallol, 2,6-dichlorophenol-indophenol (DCPIP), potassium ferricyanide, triton X-100, ethylenediamine tetraacetic acid (EDTA), bovine serum albumin (BSA), sodium pyruvate, thiobarbituric acid (TBA), reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The rest of the chemicals utilized were obtained from local firms (India) and were of

highest purity grade. For proteomic analysis, chemicals used were Urea, CHAPS, Bromophenol blue, DTT, acrylamide, bisacrylamide, SDS, APS, TEMED, glycine, glycerol, iodoacetamide, coomassie brilliant blue, BSA, agarose, trypsin, acetonitrile, NH_4HCO_3 and protein ladder (Sigma, Merck, and SRL, India). Molecular grade reagents such as protein inhibitor mix, IPG buffer, Immobiline dry strip cover fluid, IPG strip holder cleaning solution, 2D clean up kit was purchased from GE Life Science. For molecular biology work, genomic DNA isolation kit, gel extraction kit, TA cloning kit, restriction enzymes, PCR master mix, nuclease free water, Taq polymerase, DNA ladder etc were purchased from Fermentas and Qiagen, USA and Thermo Scientific, UK.

3.2. Methods

3.2.1. Sample collection

The area for collection of soil samples was nearby the adjoining areas of mining sites of East Singhbhum District, Jharkhand, India (22.39 °N 86.22 °E). The area was chosen based on its richness in different mineral ores. Sterile plastic bottles were used for storing collected soil samples.

3.2.2 Isolation and Screening of metal resistant strains

For selective isolation of heavy metal resistant bacteria, bacterial colonies were first isolated from soil samples by serial dilution method grown on Nutrient agar plates at 37 °C for 24 h to 48 h incorporated with heavy metals like Cd, Cr, Ni, Pb, Co etc separately. After the incubation, plates were observed for any kind of growth on the media. The isolated distinct colonies were repeatedly subcultured on the same media for isolation of pure colonies. Finally the isolated resistant strains were enriched by cautiously increasing the concentration of the metal ions until the desired concentration was attained that can be resisted by the strains.

3.2.3. Identification of metal resistant strains

Three approaches were implied for taxonomic bacterial identification. First on the basis of their morphological and biochemical characteristics, followed by biolog system and finally by molecular approach (16S rDNA sequencing).

3.2.3.1 Biochemical test

For identification of initially isolated metal resistant strains, biochemical tests were performed taking Bergey's Manual of Systematic Bacteriology [201] as a reference. Different biochemical tests performed are as follows: i) Grams staining; ii) Cellulase; iii) Amylase; iv) Casein hydrolysis; v) Triple Sugar Iron agar test ; vi) Carbohydrate fermentation; vii) H_2S production; viii) Nitrate reduction; ix) Lipid hydrolysis; x) Catalase; xi) Indole; xii) Methyl red; xiii) Citrate; xiv) Gelatinase.

3.2.3.2. Biolog microbial identification system

Five most highly resistant strains were again identified by Biolog microbial identification system. The phenomenon of Biolog OmniLog Identification system [Biolog (Biolog, Hayward, CA)], is based on utilization or oxidation of a panel of 95 carbon sources. Tetrazolium violet is incorporated in each of the substrates contained in a 96-well microtiter plate. After incubation of the strains it leads to reduction of the dye and results in a purple color, indicating utilization of the carbon source. Depending on the organism category, the microplates are incubated at 30 °C or 35 °C for 4 to 24 h. A unique biochemical pattern or 'fingerprint' data are generated that are analysed, compared to a database (GN or GP Omnilog Biolog, Hayward, CA), and identification is generated [202].

3.2.3.3. Identification of bacteria by 16S rDNA sequencing

For identification by molecular approach, bacterial cells were sent to GCC Biotech, India. The protocol followed was as follows where genomic DNA of the tested two most metal resistant bacterial strains RV3 and NI40215 was prepared using pure cultures grown from a single colony. Full length 16S rDNA gene was PCR amplified using 100 ng genomic DNA as template with 20 pmol of bacteria specific primers 27 f and 1492 r or 1525 r. 35 PCR cycles was set initially from 96 °C for 1min, 96 °C for 10 s, 55 °C for 30 s, 50 °C for 5s and 60 °C for 4 min for amplification. The amplified fragment was separated by electrophoresis on 1 % agarose gel, and the expected 1.5 kb full length 16S rDNA gene was eluted by gel extraction kit (Qiagen, Germany) and then purified fragment was used for the sequencing template. Sequencing was carried out as described by Sanger et al., 1977, in ABI 3500 XL Genetic Analyzer (Applied Biosystem, Singapore) [57, 203].

3.2.3.4. Phylogenetic analysis

Sequence similarity was analyzed via BLAST search in Genbank. Multisequence alignment was done using ClustalW 1.6 program at (http:/www.ebi.ac.uk/clustalw).

The phylogenetic tree was constructed using aligned sequences by the neighbor joining algorithm using CLC sequence viewer software. Boot strap percentage (1000 boot strap replications) was used to test the robustness of phylogenetic relationships within the tree [204].

3.2.3.5. Nucleotide sequence accession numbers

The nucleotide sequences of 16S rRNA gene of metal resistant bacterial strains identified as *Pseudomonas aeruginosa* RV3 and *Bacillus cereus*

NI40215 have been deposited in GenBank and an accession number for both the strains was assigned [57].

3.2.4. Determination of optimal growth of *Pseudomonas aeruginosa* RV3 and *Bacillus cereus* NI40215

The optimal growth conditions of the two metal resistant strains RV3 and NI40215 was checked taking temperature (28, 37, 45 °C) and pH (5, 6, 7, 8 and 9) as two parameters. The cultures were grown for 48 h under shaking conditions at 37 °C (Orbitex, Scigenic Biotech, India) and O.D was taken at 600 nm (UV-10, Thermo Scientific, UK) at definite intervals of time [205].

3.2.5. Comparative study of Antibiotic sensitivity for bacterial strains RV3 and NI40215

A comparative study of antibiotic sensitivity test was performed by growing the cultures of two strains in agar media having different antibiotics. Here a total of 29 antibiotics present in different hexastrips in each culture plates was used for the test. The antibiotic hexastrips were placed on freshly prepared lawns of the bacterial strains RV3 and NI40215 on agar plates and incubated at 37 °C for 24 h. The diameter of the inhibition zone was measured, and the bacteria were classified as resistant (R), intermediate (I) and susceptible (S). Following antibiotics present in hexastrips were tested on bacterial strains for the study: Ampicillin (10 µg), Amoxyclav (30 µg), Cefotaxime (30 µg), Co-Trimoxazole (25 µg), Gentamicin (10 µg), Tobramycin (10 µg), Ceftazidime (30 µg), Ciprofloxacin (5 µg), Amikacin (30 µg), Nitrofurantoin (300 µg), Netillin (30 µg), Nalidixic acid (30 µg), Streptomycin (10 µg), Sulphatriad (300 µg), Tetracycline (25 µg), Cephalothin (30 µg), Clindamycin (2 µg), Erythromycin (15 µg), Oxacillin (1 µg), Vancomycin (30 µg), Ciprofloxacin (5 µg), Fosfomycin (200 µg), Nitrofurantoin (300 µg) Norfloxacin (10 µg), Penicillin G (10 units), Gentamicin (10 µg), Levoflaxin (5 µg), Ceftriaxome

(30 µg), Chloramphenicol (30 µg), Rifampicin (5 µg) and Kanamycin (5 µg) [60].

3.2.6. Effect of heavy metals on bacterial growth of identified bacterial strains RV3 and NI40215, and determination of MIC Value

To study the effect of heavy metals on bacterial growth, both the strains RV3 and NI40215 were grown in nutrient broth containing heavy metals (Cd, Cr and Ni) at two concentrations (0.5 & 2 mM) for 48 h incubated at 37 °C, 160 rpm. Initially 1% of inoculum was inoculated from mid log phased stock culture and the growth was monitored as absorbance at 600 nm using a UV-VIS spectrophotometer (Thermo Scientific, UK) at definite intervals of time. For each set of experiment cultures were taken in triplicates along with the control (without treated with metal ions).

To find out MIC value of the two strains for the three heavy metals (Cd, Cr and Ni), the cultures were grown in agar plates with gradual increasing concentration of metal ions at 37 °C. The lowest concentration at which cultures were unable to grow is designated as the minimal inhibitory concentration of the cells. MIC value was evaluated when the isolates failed to grow on agar plates even after 10 days of incubation [59].

3.2.7. Effect of Heavy metals on cellular damage: antioxidant enzyme system in *P. aeruginosa* RV3 & *B. cereus* NI40215

Experimental set up was designed where both the strains were cultured in presence of heavy metals (Cd, Cr and Ni) separately taking two concentrations (0.5 & 2 mM) along with the control (without treatment of metals) at 37 °C, 160 rpm and cultures were withdrawn at 6 and 12 h. Cells were pelleted down by centrifugation (4500 rpm, 15 min at 4 °C), washed thrice in PBS buffer and then sonicated (Labsonic M, Sartorius, Germany) for 10 s pulse thrice maintained in ice. Sonicated cells were centrifuged (10000 rpm, 30 min at 4

°C) and the supernatant obtained was used for the enzyme assays. Cultures for all the assays were taken in triplicates.

3.2.7.1. Reduced glutathione

The level of reduced GSH was estimated as an acid soluble non-protein sulfhydryl (-SH) group by the method of Moron et al. (1979). Homogenates were immediately precipitated with trichloroacetic acid (5%) and the precipitate was removed after centrifugation. Free -SH groups were assayed by reacting the supernatant with 0.6 mM DTNB prepared in 0.2 M sodium phosphate buffer (pH 8.0) and absorbance was measured at 412 nm using a UV-10 spectrometer (Thermo Scientific, UK). Reduced glutathione (GSH) was used as a standard to calculate mM of –SH content/g cell [206].

3.2.7.2. Lipid peroxidation

Lipid peroxidation in supernatant, isolated from sonicated bacterial cells, was estimated spectrophotometricaly by thiobarbituric acid reactive substances (TBARS) method, as described by Varshney and Kale (1990) and is expressed in terms of Malondialdehyde (MDA) formed per mg protein. In brief, supernatant was mixed with 0.15 M Tris KCl buffer to which 30% TCA was added. Then 52 mM TBA was added and placed in a water bath for 45 min at 80°C, cooled in ice and centrifuged at room temperature for 10 min at 3,000 rpm. The absorbance of the clear supernatant was measured against blank of distilled water at 538.1 nm in spectrophotometer.

The amount of MDA formed in a sample was estimated according to the equation

$$\text{nmoles of MDA} = V \times O.D/0.152$$

Where, V is final volume of test solution and O.D is optical density [207]

3.2.7.3. Superoxide dismutase

The activity of superoxide dismutase was assayed as described by Marklund and Marklund (1974), which involves inhibition of pyrogallol auto-oxidation at pH 8.0. A single unit of enzyme was defined as the quantity of superoxide dismutase required to produce half-maximal (50%) inhibition of auto-oxidation. The principle behind is that it catalyzes the dismutation of superoxide radical to yield H_2O_2 and oxygen.

Supernatant treated with Triton X-100 (1%) on ice for 30 min and was added to assay (1ml) which contained 0.05 M sodium phosphate buffer (pH 8.0), 0.1 mM EDTA, 0.27 mM pyrogallol and absorbance was measured for 5 min at 420 nm. Stock solution of pyrogallol was made in 10 ml HCl. [208].

3.2.7.4. Catalase

Catalase activity was performed by the method of Aebi (1984), which is based on the principle where it catalyzes the decomposition of H_2O_2 to give oxygen and water. The supernatant was treated with ethanol (10 ml/ml) for 30 min on ice. Then Triton X-100 (1%) was added and again kept for 30 min on ice. The treated supernatant was added to the assay mixture which contained 0.05 M sodium phosphate buffer (pH 7.0), 10 mM H_2O_2 and decrease in absorbance was measured at 240 nm. The activity was calculated using extinction coefficient 0.04 $nmole^{-1}cm^{-1}$. One unit of catalse activity was defined as amount of enzyme required to decompose one mole of H_2O_2 per min [209, 210].

3.2.7.5. Glutathione peroxidase

Glutathione peroxidase GP_x (EC1.11.1.9) activity was measured by the coupled assay method as described by Paglia and Valentine (1967). Briefly, 1 ml of the reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0)

containing 1 mM EDTA, 0.24 U/ml yeast glutathione reductase, 0.3 mM glutathione (reduced), 0.2 mM NADPH, 0.2 mM H_2O_2 and cytosol sample. Reaction was initiated by adding NADPH and its oxidation was monitored at 340 nm by observing the decrease in OD/min for 3 min. One unit of enzyme activity has been defined as nmoles of NADPH consumed /min based on an extinction coefficient of 6.22 mM^{-1} cm^{-1}[211].

3.2.7.6. Glutathione reductase

The activity of glutathione reductase GR (EC 1.6.4.2) was determined by the procedure as described by Carlberg and Mannervick (1985) [212], measuring the rate of oxidation of NADPH at 340 nm. Briefly, 1 ml reaction mixture contained 0.125 M sodium phosphate buffer (pH 7.0) containing 0.2 mM EDTA, 1 mM oxidized glutathione (GSSG) and 0.2 mM NADPH. The reaction was started by adding cytosol to the reaction mixture and the enzyme activity was measured indirectly by monitoring the oxidation of NADPH following decrease in OD/min for minimum 3 min at 340 nm. One unit enzyme activity has been defined as nmoles NADPH consumed/min/mg protein based on an extinction coefficient of 6.22 mM^{-1} cm^{-1} [115].

3.2.7.7. Protein Estimation:

Total protein content of the bacterial cells was determined following the method of Lowry et al. (1951) using Bovine serum albumin as standard, at 660nm. Proteins react with Folin-ciocalteau reagent to give a colored complex and the color so formed is due to the reaction of the phosphomolybdate with tyrosine and tryptophan. Alkaline solution (50 ml of 2 % Na_2CO_3 in 0.1 N NaOH + 1 ml of 0.5 % $CuSO_4$ in 1 % Na^+K^+ tartarate) was prepared freshly when required. This alkaline solution (5ml) was added to the 1 ml of test solution, which contains BSA or other protein. It was mixed thoroughly and allowed to stand at RT for 10 min. Subsequently 1N Folin's reagent (0.5 ml)

was added and allowed to stand for another 30 min. The absorbance was measured at 660 nm against the reference blank. The protein content of each sample was evaluated from the standard curve with BSA and was expressed in mg ml^{-1} [213]

3.2.7.8. Statistical analysis:

All the results were presented as Mean ± S.D. Statistical analysis was performed using ANOVA following Mann-Whitney *U*-test. A value of $p<0.001$, $p<0.01$ and $p<0.05$ were considered to indicate a significant difference between groups.

3.2.8. Metal uptake assay

Metal treatment (Cd, Cr & Ni; 0.1 & 0.5 mM each) was given to both the strains RV3 and NI40215 at early log phase and grown for another 6 h and 12 h separately at 37 °C and 160 rpm. The bacterial cells were harvested at given interval of time by centrifugation at 10,000 rpm for 10 min at 4 °C. Cell pellets were lyophilized (Lyohahn 112N-G, Hahntech, Germany) and weighed. Dried pellets were treated with 10 ml 1 M HCl for 15 h, and then finally sonicated for 45 s at 4 °C. Sonicated cells were centrifuged (10,000 rpm, 10 min) and the supernatant obtained was diluted with 5 ml of 10 % HNO_3. Concentration of metal ions in the supernatant was determined using Atomic absorption spetrophometer (AAS-ICE 3500, Thermo Scientific, UK) and then finally with inductively coupled plasma optical emission spectrometer (ICP OES) (Optima 2100 DV, Perkin Elmer, Singapore). Standard solutions of individual metal ions were prepared for calibration of the instrument and for experimentation all the samples were taken in triplicates [60].

3.2.9. Biophysical analysis for metal microbe interaction

For all the biophysical analysis, cells of both the strains RV3 and NI40215 were grown in nutrient broth up to mid log phase (OD 0.6) and then with the respective metal ions (Cd, Cr and Ni) individually at 37 °C for 48 h at 160 rpm. For each experiment two concentrations of metal ions (0.5 & 2 mM) treated cells along with a control (metal free cells) were taken. Standard protocols were followed for sample preparation for all the instrumental analysis.

3.2.9.1. Cell morphology

3.2.9.1.1. Scanning electron microscopy (SEM)

The 48 h control and heavy metal (Cd, Cr and Ni; 0.5 & 2 mM) treated cultures of strain RV3 and NI40215 were centrifuged at 4500 rpm for 15 min at 4 °C. The pellet formed was washed twice with 1ml of PBS buffer. The cells were treated with 3 ml of 2.5% glutaraldehyde in PBS buffer for 3 h and dehydrated with graded ethanol (30%, 50%, 70%, 90% and 100%). The cells were then placed on the cover slip and fixed by simple air drying. The specimens were mounted on aluminum stubs with double sided adhesive carbon tape, allowed to dry for 3 h, and then coated with gold palladium with an ion sputter. The segments were examined under a SEM (JEOL, JSM-6390LV, Singapore) at 20 k and magnification of 20,000 x [60].

3.2.9.1.2. Morphometric analysis.

From SEM micrographs of normal and stressed bacterial cells, cell volume (*V*) and surface area (*A*) was calculated by the following equations:

$V\ (\mu m^3) = \pi r^2 h$ (1)

$A\ (\mu m^2) = \pi r^2 + \pi r h$ (2)

Where *r* and *h* are radius and length of the cell in µm (Neumann et al., 2005). . Mean cell dimensions of the test cultures of the strain RV3 and NI40215 were measured. [60, 214].

3.2.9.2. Energy Dispersive X-ray analysis (EDX)

Sample preparation for EDX analysis is similar to the SEM, and the analysis of metal free (control) cells and metal treated cells was done using an micro analytical system attached with JEOL, JSM-6390LV Scanning Electron Microscope [60].

3.2.9.3. Fourier Transform Infrared spectroscopy (FTIR)

To obtain a qualitative and preliminary analysis of the main functional groups present on the cell wall and its contents, an infrared (IR) analysis in solid phase was performed on lyophilized cells in a potassium bromide (KBr) disc using FTIR spectrophotometer (Spectrum-100, Perkin Elmer, Singapore). The IR obtained at 4000-400 cm^{-1} range was used in examining the cells before and after metal treatment (Cd, Cr & Ni at two concentrations 0.5 mM & 2 mM for 48 h at 37 °C, 160 rpm) [57, 215].

3.2.9.4. Transmission Electron Microscopy (TEM)

For TEM analysis, both control and metal treated cells were isolated from the culture broth by centrifugation at 3000 rpm for 15 min at 4 °C. Then the cells were washed twice with sodium cacodylate buffer (0.1 M, pH 7.2) followed by fixation in 2.5% glutaryldehyde for 2 h at 4 °C and then post fixed with 1% osmium tetraoxide for 1h. Cells are dehydrated through a graded ethanol series, treated with propylene oxide and embedded in epoxy resin. Unstained ultrathin sections were cut with the help of a ultra cut E, Ultramicrotome, loaded in formvar carbon coated copper grid and then examined in a JEOL

JEM 2100 (JEOL, Singapore) Transmission Electron Microscope at 100 Kv [57, 216].

3.2.10. Identification of differentially induced proteins in response to heavy metals (Cd & Cr) by 2DGE & MALDI TOF MS

3.2.10.1. Sample preparation for 2-DGE

Most efficient strain RV3 isolated after screening process were grown in minimal salt medium (MSM) containing heavy metal in a chemostat for 10 days at 30 °C, 160 rpm. The composition of MSM (g/l, pH7) was: $Na_2HPO_4.2H_2O$, 7.8 g; KH_2PO_4, 6.8 g; $MgSO_4$, 0.2 g; $NH_4(CH3COO)_3Fe$, 0.05 g; $Ca(NO_3)_2.4H_2O$, 0.05 g; $NaNO_3$, 0.085 g (Thakur et al. 2001). Viability of the cells was checked by growing on nutrient agar plates. The bacterial cells were harvested from 150 ml MSM broth by centrifuging at 6000 x g at 4 °C for 15 min. The pellets were washed twice with PBS buffer and resuspended in lysis buffer (2 ml) containing Urea, CHAPS, Pharmalyte and protease inhibitor cocktail. Cells were then sonicated for 2 cycles 10 s each (in ice) followed by centrifugation at 14000 x g for 15 min maintaining the temperature at 4 °C. Protein content in the supernatant was determined by lowry method using bovine serum albumin as standard (Lowry et al. 1951). Taking 100 µl of protein sample, it is cleaned with 2D clean up kit (G. E Health Care, USA). [117,118, 119]

3.2.10.2. Isoelectric focusing

Isoelectric focusing (IEF) (Ettan IPGphor 3, GE Healthcare, USA) was carried out using 13 cm Immobiline pH gradient strips (IPG), pH 3-10, (G. E, Healthcare, USA). Protein sample mixed with rehydration buffer and loaded on the IPG strips in the IEF tray were passively rehydrated for overnight (approx.16 h). For the second dimension electrophoresis, IPG strips were

equilibrated for 15 min each in buffer. The proteins were subsequently separated on 12% SDS PAGE (SE 600 Ruby, G.E. Healthcare, USA). The gels were stained with 0.1% Coomassie Brilliant Blue R-250 overnight followed by distaining step with distilled water, methanol and glacial acetic acids (45:45:1) [117,118, 120].

3.2.10.3. Sample preparation for In-gel digestion of Coomassie stained gels

3.2.10.3.1. Excision of protein spots from polyacrylamide gels

The stained gels were first washed with miliQ water twice for 10 min each followed by excision of protein spot of interest. Gel pieces were washed with acetonitrile and 100 mM NH_4HCO_3 solution (v/v), and the step is repeated till the pieces turns colorless. Then enough acetonitrile is added to cover-up the gel particles which led the gel plugs to shrink and stick together. After removing the acetonitrile, gel particles are dried down in a vacuum centrifuge.

3.2.10.3.2 Reduction and alkylation

The gel particles are swelled in freshly prepared 10 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3 solution and incubated for 45 min at 56 °C in water bath. After chilling the tubes at room temperature excess liquid is removed and equal volume of freshly prepared 55 mM iodoacetamide (IAA) in 100 mM NH_4HCO_3 was added. Then incubated for 30 min at room temperature in the dark followed by washing the gel particles with 100 mM NH_4HCO_3 and acetonitrile (1:1), one or two change each for 15 min per change. Enough acetonitrile was added to cover the gel particles. After the gel pieces have shrunken, acetonitrile was removed and gel particles were dried down in a vacuum centrifuge.

3.2.10.3.3. In gel digestion

10 µl of freshly prepared trypsin solution (10-20 ng/µl in 25 mM NH_4HCO_3) was added to cover the excised gel spots and incubated at 37 °C for 30 min. Enough 25mM NH_4HCO_3 solution (approx. 25 µl) was added to keep the gels wet and incubated overnight at 37 °C.

3.2.10.4. Extraction of peptides for Mass spectrometric analysis and protein identification

Supernatant was collected by centrifugation (10,000 rpm, 4 °C) and stored. 10 µl of 1% trifluro acetic acid (TFA) and 10 µl of acetonitrile was added again to the gel and sonicated for 20 min at RT. Fresh supernatant obtained by centrifugation were mixed with previous supernatant. Supernatant was evaporated by a speed vac and again resuspended in 8 µl of 0.1% TFA and 2 µl of acetonitrile. An aliquot of peptides from each spot was mixed with MALDI matrix and analyzed on MALDI-TOF MS (AB SCIEX TOF/TOF 5800, Applied Biosystem, Foster City, CA). External calibration was performed using calibration standard immediately before data acquisition. Protein homologs were identified using an in-house mascot database search engine using NCBInr database [117,118, 120].

3.2.11. Cloning and characterization of *lipA* gene induced in response to heavy metals and prediction of it protein structure

3.2.11.1. Chromosomal DNA isolation and purification

Chromosomal DNA of bacterial isolates was isolated and purified either by using alkaline lysis method or by Genomic DNA extraction kit (Fermentas, USA). Genomic DNA extraction by alkaline lysis method was done as described by Ausubel et al. 1995 [217]. The cell pellet was obtained from 5ml of overnight culture by centrifugation at 10,000 rpm for 10 min at 4°C (5430

R, Eppendorf, Germany). The cell pellet was then re-suspended in a 0.8 ml of solution-I (Appendix-I) and to this mixture; 160µl of lysozyme (10 mg /ml) was added and incubated at room temperature (24℃) for 20 min. Subsequently, 44.5 µl of 10% (w/v) SDS solution was added to the reaction mixture and re-incubated for 10 min at 50 ℃. Thereafter, 53.3 µl of RNase A (10 mg /ml) was added and incubated at 37 ℃ for 90 min. This was followed by addition of 45.3 µl of Na-EDTA (0.1 M, pH 8.0) and re-incubated at 50 ℃ for 10 min. To in-activate the nuclease as well as to digest the proteins of the reaction mixture, 26.6 µl of proteinase K (5.0 mg/ml stock) was added to it and incubated at 50 ℃ for 16 h. Then equal volume of saturated phenol (saturated with 0.1M Tris-HCl, pH 8.0) was added to the reaction mixture and mixed thoroughly.

The mixture was then centrifuged at 10,000 rpm for 10 min and the upper (aqueous) phase was aspirated into sterile microfuge tube. Then 700 µl of (1:1) phenol and chloroform-isoamylalcohol (24:1) was mixed to the reaction mixture. After centrifugation at 10,000 rpm for 10 min, the upper phase was transferred into a sterile microfuge tube, and then equal volume of chloroform-isoamylalcohol (24:1) was added and spun for another 10 min at 10,000 rpm. The upper phase was then transferred to a sterile microfuge tube and one tenth volume of 3 M Na-acetate, pH 7.0 solution was added. Subsequently, DNA was precipitated by adding two volumes of ice-cold absolute ethanol and DNA was recovered by centrifugation. After removal of alcohol, DNA was re-suspended in 10 mM Tris HCl-1mM EDTA buffer (pH 8.0) at a final concentration of 1 µg/ml and was stored at 4 ℃ until it was further used.

Bacterial genomic DNA isolation using GeneJET genomic DNA purification kit (Fermentas, USA) was performed by harvesting 5 ml of overnight culture in a microfuge tube by centrifugation for 10 min at 5,000 rpm. This was followed by suspension of pellets in 180 µl of lysis buffer and further

incubation for 30 min at 37 °C. Later, 200 µl of Lysis solution and 20 µl of proteinase K were added and mixed uniformly. The reaction mixture was then incubated at 50 °C for 30 min and after the period of incubation it was cooled and 20 µl of RNase A solution was added followed by vortexing. This was then incubated for 10 min at room temperature and 400 µl of 50% ethanol was added and mixed by vortexing. The clear lysates were then transformed into a GeneJET™ Genomic DNA purification column, which was in turn inserted into a collection tube. The column was then centrifuged for 1 min at 6,000 rpm and the flow-through was discarded. Subsequently, the column was placed into a new 2 ml collection tube to which 500 µl of wash buffer-I (Ferments, USA) was added and after centrifugation at 8,000 rpm for 1 min, the flow-through was discarded. Consequently, 500 µl of wash buffer-II was added to the purification column and it was centrifuged at 12,000 rpm for 3 min. The flow-through so obtained was discarded and column was placed into sterile 1.5 ml microfuge tube. To elute the genomic DNA from the purification column, 200 µl of elution buffer was added to the center of the column and after incubation for 2 min at room temperature, the DNA was collected in a fresh microfuge tube by centrifugation at 10,000 rpm for 1 min. The purity of the preparation was then determined by taking optical density of the eluted DNA at 260 and 280 nm.

3.2.11.2. Primer designing

A set of primer was designed using the software Primer 3 to amplify the *Lip*A gene from the *P. aeruginosa* RV3. Primer was designed in such a way that it can amplify a fragment of 1 kb with a restriction site of BamHI inside the amplified product.

Primer name	Primer sequence	No. of bp
Lip IF	GGAATTCGTGGAGAAGTCCGGAGAAGCCA	29
Lip IR	CCCAAGCTTCTCAGCCGATCTTGTTGCCGTGC	32

3.2.11.3. PCR amplification

After designing the gene specific primer, the respective gene was PCR amplified. The reaction was set up in 15 µl with buffer 1.5 µl, $MgCl_2$ 1.5 µl, dNTP 1.2 µl, Taq polymerase 0.2 µl, Primer 1 µl each forward, reverse and volume was make up to 15 µl with DNAse, RNAse free water. PCR amplification consisting of total 35 cycles: initial heating at 94 ℃ – 5 min, denaturation at 94 ℃ – 1 min, annealing at 50 ℃ – 30 s and extension at 72 ℃ – 2 min and final extension at 72 ℃ – 10 min was set in PCR thermal cycler (Applied Biosystem, USA).

After amplification the PCR product was seperated in 0.8 % agarose gel along with a 1 kb ladder (Fermentas, USA) and the expected 1 kb band in the gel was observed under UV illuminator

3.2.11.4. Ligation of PCR product into cloning vector

The eluted DNA fragment was separated in 0.8 % gel to confirm the elution of the exact band. After gel extraction ligation was set up with the InsTA cloning vector and *lipA* amplified gene product. Ligation is initiated by setting up a reaction mixture containing following components in a microfuge tube kept on ice: 10 µl of ligase buffer (10X), 1µl of gel purified PCR product (50-100 ng), 1µl of cloning vector (50 ng/µl), 1µl of T4 DNA Ligase (5 units/µl) and final volume was adjusted up to 20 µl with the nuclease free sterile water. The reaction mixture was then vortexed for a short period, centrifuged for 10 s and subsequently incubated at 16 ℃ for overnight.

3.2.11.5. Competent cell preparations

DH5α/BL21a competent cells were prepared by calcium chloride method. In brief, a single bacterial colony was inoculated into 5ml of SOB broth (Appendix-I) and the cells were allowed to grow for overnight at 37 °C with shaking at 250 rpm. On the subsequent day, 100 ml of SOB medium was inoculated with the 1 ml of saturated overnight culture and inoculated media was allowed to grow at the above conditions until OD reached 0.4 at 600 nm (usually 2-3 h). Thereafter, the cells were chilled on ice for 10 min and suspensions were spun down at 5,000 rpm for 5 min at 4 °C. After centrifugation steps, cell pellets were re-suspended in half the original volume of ice cold 100 mM $MgCl_2$ and further incubated on ice for another 15min. After the incubation step, cell suspensions were spun down at 5,000 rpm for 5 min at 4 °C and pellets were collected, which was further re-dissolved in half the original volume of ice cold 100 mM $CaCl_2$ solution. This was followed by re-incubation of cell suspension on ice bath for another 15 min and cell pellets were harvested by spinning down at 5,000 rpm for 5 min at 4 °C. The pellets so obtained were re-suspended in 1ml of ice cold solution of 100 mM $CaCl_2$ containing 20% glycerol and subsequently aliquots of 100 µl were prepared in pre-chilled microfuge tubes and stored at -70 °C until further use.

3.2.11.6. Transformation of competent cells by heat shock method

For transformation experiments, competent cell aliquots were taken out from -70 °C freezer and allowed to thaw on ice for 10 min. Then the cells were suspended evenly by mixing. Successively, 2 µl of ligation product was added to the cells and were mixed gently. After the incubation of reaction tube on ice for 30 min, the cells were given heat shock for 90 sec at 42 °C in a water bath, followed by keeping the cells on ice for another 2 min. This was followed by addition of 600 µl of LB media to each tube and then the tubes were incubated

at 37 °C for 1 h. After the incubation period, about 350 µl of transformed cells were plated on the premade LB agar plates containing kanamycin (30 µg/ml) / ampicillin (100 µg/ml) antibiotics depending upon vector used. Cells were spread evenly on the plates, and then incubated in upside down position for 12-16 h at 37 °C in a static incubator.

3.2.11.7. Blue white selection of recombinant clones

For screening of recombinant clones on transformation plates, blue/white selection procedure was followed. Before plating of transformed cells to a LBA plates, 40 µl X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) stock solution (20 mg/ml) and 40 µl IPTG (isopropyl-b-D-thiogalactopyranoside) stock solution (100 mM) were spread evenly on the surface of the plate. The plates were then left for drying for few minutes under laminar hood and successively the transformed cells were plated on this X-gal, IPTG, and antibiotics containing LBA plates.

3.2.11.8. Plasmid isolation

To investigate successful ligation of the PCR product, recombinant plasmids need to be isolated from the recombinant clones (white colonies from blue-white selection). Therefore, recombinant plasmids were isolated by following alkaline lysis method as described by the miniprep plasmid isolation kit (Fermentas, USA). Briefly, 5 ml of overnight culture cells harboring plasmids were grown in appropriate antibiotics [kanamycin (30 µg/ml) /ampicillin (100 µg/ml)] and were harvested by centrifugation at 8,000 rpm for 5 min. Cell pellets were then re-suspended by vortexing into 250 µl of RNase containing suspension buffer (P1), this was followed by mixing of cell suspension in 250 µl of lysis buffer (P2) by gently inverting the tubes for 4-6 times. Then the reaction mixture was neutralized by the addition and mixing of the 350 µl of neutralization buffer (P3) by inverting tubes for 4-6 times. This was followed

by centrifugation at 13,000 rpm for 10 min and subsequent transfer of supernatant into spin column and further centrifugation at same speed for 30 s. After centrifugation, flow-through was discarded and the column was washed twice with the 0.75 ml of washing buffer (Fermentas, USA) by centrifuging at 13,000 rpm for 30 s. To remove any residual wash buffer, spin column was centrifuged again at 13,000 rpm for 30 s. After the washing steps, DNA was eluted from the column in a clean microfuge tube (1.5 ml) by adding 50 µl of elution buffer (Fermentas, USA) to the center of the column. After incubating the spin column for 1 min it was eventually centrifuged at 13,000 rpm for 1 min.

3.2.11.9. Confirmation of clones by colony PCR

After ligation transformation was done in DH5α competent cell and plated on LBA + Amp plate. The white colonies appeared on the plate were taken and colony PCR was performed to find out the positive transformants. The positive colonies were stored for further use.

3.2.11.10. Clone confirmation by restriction digestion

From the positive clone, plasmids were isolated, and both single and double restriction digestion was performed with the EcoR1, and latter with the EcoRI and HindIII that releases the insert out from the clone as they are present in the either side of the insert. The expected band of 4 kb (single restriction digestion), and 1 & 3 kb band (by double restriction digestion) was separated by electrophoresis and observed in 0.8% gel

3.2.11.11. Sequencing, phylogenetic analysis and stucture prediction

Sequencing of recombinant plasmid as well as cloned fragment of *lipA* was accomplished to ascertain successful ligation of PCR product by chain termination method using plasmid specific and also with gene specific primers

(as mentioned above) by ABI 3500 XL Genetic Analyzer (Applied Biosystem, Singapore) [203]. Sequences so obtained were then blast searched using NCBI database for checking the completeness of the coding sequence followed by construction of a phylogenetic tree using CLC sequence viewer. Boot strap percentages (1000 boot strap replications) were used to test the robustness of phylogenetic relationships within the tree [204].

The gene sequence was deposited in the NCBI GenBank Database and an accession ID was assigned. Further the gene sequence was translated into its corresponding amino acid sequence and the three dimensional structure of the amino acid sequence was predicted using I-TASSER server [218].