Abstract

Heavy metals are well reported to cause several diseases such as respiratory complications, emphysema, renal failure, bone disorders, immune suppression and various types of cancers in human beings. Industrial activities such as mining operations, metallurgical, electroplating, tanneries, battery and chemical manufacturing etc. are main sources of heavy metal contamination in the environment and have deleterious effect on the biodiversity and ecosystem. Microbes particularly bacteria in the metal contaminated areas have evolved and developed resistance against the metal stress; but the knowledge on the molecular mechanism of heavy metal detoxification is limited. The understanding on the biochemical and molecular mechanism of metal detoxification might be exploited for bioremediation, bio-mining or biosensor applications. The present study focuses on the mechanistic study of heavy metal detoxification in metal resistant bacterial strains isolated from the nearby/adjoining areas of mining sites of East Singhbhum district of Jharkhand (India).

In the present investigation, forty two (42) bacterial isolates were found resistant to different heavy metals (Cd, Cr & Ni) and among them eight (08) strains were resistant to all the 3 metals. The identified strains were subjected to the morphological and biochemical test. Based on the distinct biochemical characteristics five (05) strains were selected and were identified as *Bacillus* (two strains), *Pseudomonas, Lactobacillus* and *Staphylococcus* strains using Biolog microbial identification system. Two strains *Pseudomonas* sp. (JHCO2018) and *Bacillus* sp. (JHNI40215) were found to grow in presence of all the metals (Cd, Cr & Ni) and were identified as *Pseudomonas aeruginosa* RV3 (accession no: JX313019) and *Bacillus cereus* NI40215 (accession no: KT072743) using 16S rDNA sequence which showed 99% similarity with the corresponding sequences of *Pseudomonas* and *Bacillus* sp. respectively. The optimization of the growth conditions showed that the optimal temperature and pH for both the strains *Pseudomonas aeruginosa*

RV3 and *Bacillus cereus* NI40215 were 37 °C and pH 7. The average MIC value for all the metals was found as 2.46 and 2.16 respectively. Both the bacterial strains exhibited significant decrease in growth rates in presence of heavy metals (Cd, Cr, Ni) which might be due to oxidative stress induced by these metals.

Reduced glutathione and antioxidant enzyme (i.e. SOD, CAT, GPx and GR) play an important role in neutralizing ROS and protects the cellular system from metal toxicity. In the present study dose dependent decrease in GSH levels were observed in both the bacterial strains (RV3 & NI40215) in response to heavy metals (Cd, Cr & Ni). The depletion of GSH in the present situation might be due to significant increase in the lipid peroxide level on exposure to the metals in both the strains and could be responsible for the decreased growth rate. SOD and CAT are front line antioxidant enzymes which neutralizes the superoxide and H_2O_2 respectively. In the present investigation the metal resistant bacterial strains (RV3 & NI40215) showed significant increase in the SOD and CAT activity in response to the metals (Cd, Cr & Ni) at 6 h and 12 h respectively. Also GPx and GR are important GSH-related enzymes having an important role in GSH/GSSG maintenance which is crucial for cellular redox status and were found to increase significantly in dose dependent manner in response to metal exposure.

Biophysical approaches were used to study the metal-microbe interaction in the metal resistant bacterial strains RV3 and NI40215. SEM image analysis exhibited morphological changes i.e. increase in the volume but decrease in surface/volume ratio were observed particularly at the highest dose of the metal treatment in both the bacterial strains (RV3 & NI40215). This relative reduction in cell surface/volume ratio is considered as effective mechanism adopted by the cells to lower the toxic effects of environmental stress by decreasing the attachable/exposed surface. EDX analysis showed the presence of metals (Cd, Cr & Ni) on the surface of both the bacterial strains

and their interaction with surface functional groups were studied by FTIR. FTIR analysis exhibited complex interactions of metals (Cd, Cr & Ni) within the wide range of functional groups such as –OH, aliphatic C-H, secondary amines, carboxyl, carbonyl, phosphate and phosphate diesters present on the bacterial biomass of the strains RV3 and NI40215. TEM analysis was carried out to further investigate the intracellular localization of absorbed metals within the bacteria *Pseudomonas aeruginosa* RV3. The electron micrograph of the metal loaded bacterial cells showed metal deposition in both the membrane and cytoplasm but preferentially in the periplasmic region and cell wall which is represented by relatively darker and opaque region in the electron micrograph. AAS analysis showed increased levels of metals in dose dependent manner in the dry biomass of both the bacterial strains (RV3 and NI40215). Bio-sorption/accumulation of metal in the dry biomass were found to be greater for Cd followed by Ni and Cr. ICP-OES analysis of metal loaded bacterial dry biomass confirms the similar trend of dose dependent increase in the metal biosorption/accumulation in both the metal resistant bacterial strains even though the amount of metal detected were much higher as compared to AAS data which might be due to the difference in the sensitivity of the instruments.

The SEM, FTIR and TEM analysis confirmed the interaction and localization of metals within the bacterial strains. To study the molecular level interaction proteomic approach was used to identify differentially expressed proteins and their role in metal detoxification in *Pseudomonas aeruginosa* RV3. The bacterial strain RV3 responded to metals (Cd & Cr) by up-regulating proteins involved in protein repair, ATP synthesis, protein biosynthesis, maintenance of cell shape/cell envelop, transportation, Cd-Zn-Co resistance and arginine synthesis. The protein expression data of the metal resistant RV3 strain in response to Cd exhibited the induction of lipoyl synthase (EC 2.8.1.8) which catalyzes the synthesis of lipoic acid through lipoylation which is essential for

the functioning of several important enzymes such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched chain ketoacid dehydrogenases, glycine cleavage enzymes etc. involved in oxidative metabolism. Recently lipoic acid has received attention for its role as a biological antioxidant and has been reported to form stable complex with transition metals. The present investigation showed the induction of lipoyl synthase in response to Cd in the metal resistant RV3 strain and suggested the possible role of lipoic acid in metal detoxification, so the *lipA* gene was PCR amplified and cloned in InsTA cloning vector. The cloned *lipA* gene was sequenced and the length of gene was found to be 972 base pairs, further phylogenetic analysis exhibited 99% similarity with the *lipA* gene of *Pseudomonas aeruginosa*. Further the PDB BLAST result showed that the translated LipA enzyme have a 47% structural similarity with Chain B, crystal structure of *Thermosynechococcus elongatus* Lipoyl Synthase 2 Complexed with Mta and Dtt [*Thermosynechococcus elongatus* BP-1]. Hence the 3D structure was predicted using I- TASSER and from the predicted secondary structure, the 5 best models were taken for model refinement using ModRefiner to refine the geometrical errors of the predicted structures. Based on the ANOLEA assessment analysis and the Ramachandran plot, **model-4** having an energy value of -1204 and 299 (93.1%) residues present in favored region with only 5 (1.6%) residues in the outlier region is proposed as the most stable structure.