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Protein Profiling and Antioxident Enzyme Activity of Cadmium and Lead Tolerant *Kocuria sp*. BRI 36

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Abstract

Cadmium and lead have been recognized to be highly toxic heavy metals often contaminating the ecosystem and harmful to living organisms living in such polluted environment. *Kocuria* species BRI 36 has an ability to survive at high concentrations of such heavy metals. The present study deals with protein profiling of BRI 36 and its response to metal stress in terms of Reactive Oxygen Species enzymes. Cadmium treated cells exhibited shrinkage when exposed to heavy metals (50 ppm) as observed with the help of Field emission scanning electron microscopy. Protein estimation experiments revealed increase in extracellular proteins with increase in metal concentration. Protein expression profiles were deduced from sodium dodecyl sulphate-polyacryamide gel electrophoresis. The results exhibited over-expression of certain proteins with increase in metal concentration as compared to control. Antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) activities were observed to increase up to 20 ppm metal concentration. Malondialdehyde (MDA), a measure of cellular oxidative stress level, decreased with increase in metal concentration due to stress induced cellular response.

Keywords: *Kocuria* sp., Cadmium-tolerance, Lead-tolerance, Antioxidant enzymes, Protein expression, Antioxidant activity

INTRODUCTION

Metal pollution is serious concern worldwide due to increase in concentration of heavy metals present in the environment. Heavy metals like cadmium and lead are used in variety of industries like electroplating, plastic manufacturing etc. resulting in terrestrial and aquatic environmental contamination. Along with evidences of adverse effects on the marine animals, birds, fishes and plants, it has also known to have harmful effects on human body with increased concentrations.

Cadmium and lead are not known to have any biological functions in bacteria and may be toxic at even small concentrations (Bruins et al., 2000); however, they are taken up by the Mn^{2+} uptake system or Mg transport system. The heavy metals transported into the cytoplasm seem to inhibit the DNA replication, and make the DNA more susceptible to nucleolytic attack, resulting in single-strand DNA breaks. Therefore, these heavy metals cause serious damage during the growth of bacteria present in polluted environments (Jaroslawiecka and Piotrowska-Seget, 2014).

Exposure of heavy metal ions affects cellular process in bacteria at both transcriptional and translational levels have been reported previously (Easton et al., 2006). However, the molecular mechanisms and underlying responses of cells against various heavy 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.

Heavy metal creates different types of physiological stress in bacteria and produce Reactive Oxygen Species (ROS) like O_2^-, OH^-, and H_2O_2 that causes increase in oxidative stress (Choudhary et al. 2013). ROS are partially reduced oxygen derivatives produced by cell metabolism such as the superoxide anion, the hydroxyl free-radical and hydrogen peroxide that are more reactive than oxygen itself. High concentrations of ROS cause damage to different cell macromolecules. For instance, proteins with sulphydryl groups are oxidized in response to stress, forming sulphonic derivatives in an irreversible process (Herrero et al., 2008). In normal physiological condition, ROS level remains low due to activity of certain protective enzymes like, catalase, superoxide dismutase, and glutathione reductase (Pandey et al. 2013). However, with increase in oxidative stress the concentration of protective enzymes that converts ROS into oxygen and water is known to increase. It helps to maintain cellular integrity. Superoxide dismutase (SOD), a metaloenzyme is found to convert toxic superoxide into oxygen and less toxic hydrogen peroxide which is further acted upon by heme-containing catalase (Abassi et al., 1998).

Considering this, the present work investigated to response of *Kocuria* sp. BRI 36 to physiological stress in terms of protein expression and the levels of antioxidant enzymes. The metal resistant characteristic was found to be plasmid independent (Mulik and Bhadekar, 2017b) and removal of calcium and lead was observed mainly by accumulation (Mulik and Bhadekar, 2017a).

MATERIALS AND METHODS

Organism:

The halotolerant *Kocuria* sp. BRI 36was used in this work. The organism was grown in Mineral Salt Medium (MSM) at 25±2°C for 48 h with shaking at 120 rpm (Pote et.al. 2014). It was further used for inoculation in all the experiments at 10 % concentration. The organism grown under similar conditions without metal served as control in all the experiments.

Chemicals and Reagent

All chemicals used were of analytical grade. The media components were purchased from Hi Media Laboratories Pvt. Ltd. (Mumbai, India). The stock solutions of cadmium and lead at concentration of 1000 ppm were purchased from Sigma-Aldrich.

Field emission scanning electron microscopy (FESEM)

Effect of heavy metal accumulation on cell morphology was examined with the help of FE-SEM. BRI 36 was grown in MSM supplemented with $Cd^{2+}$and Pb^{2+} each at 50 ppm concentration. At the end of 48h of incubation, the samples were removed, centrifuged at 8000 rpm for 15 min. Cell pellet was used for smear preparation on glass slide as described by Ishii et al., (2004). The samples were then coated with chromium by using a sputter apparatus (Quarum) and observed under NOVA NANOSEM/450 (FEI) operating at 10kV.

Protein estimation

Kocuria sp. BRI 36 was grown in MSM amended with different concentrations of cadmium and lead (10 ppm to 50 ppm for each) at 30˚C, 120 rpm for 48 h. Biomass was then separated by centrifugation at 8000 rpm for 15 min. The protein extraction was carried out by adding general lysis buffer (50mM tris HCl; 100 mM NaCl; 1mM tween 20; 20.5% gycerol; 1mM EDTA) in the pellet and pellet was sonicated for 15 min (30 s each time with a gap of 30 s between successive sonication). The sonicated samples were centrifuged for 15 min at 8000 rpm. The cell free broth was used for intracellular protein estimation by Folin Lowry's method (1951) using BSA as a standard (Ayudhya et al., 2009; Durve et al 2013).

Polyacryamide gel electrophoresis (PAGE)

The isolate was grown in MSM as mentioned earlier. The cells were harvested by centrifugation at 8000 rpm for 15 min. 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 min. The cell pellet obtained was mixed with 1 ml of 2X gel loading buffer (0.5% SDS; 1.25 % β- mercaptoethanol; 0.03% bromophenol blue; 2.5% glycerol; 15 mM tris Cl; pH-6.8) and incubated in a boiling water bath for 30 min. This was further used for protein profiling using sodium dodecyl sulphate -polyacrylamide gel electrophoresis (SDS-PAGE) containing 18 µL of the protein sample was taken and subjected to SDS-PAGE consisting of 2.5 % stacking and 10 % resolving gels (Lamelle). After electrophoresis on a vertical slab unit (Biorad) under a constant voltage of 100 V for 90 min, the gels were stained with coomassie brilliant blue R-250. A protein marker (10 to 250 kD) (Biorad) was used to estimate the molecular weight of protein band.

Antioxident activity

In order to determine the oxidative stress on bacterial cells, BRI 36 was grown in presence of cadmium and lead at different concentration (10 ppm to 50 ppm) individually. The cells were harvested by centrifugation at 8000 rpm for 15 min. The cell lysate for enzyme assay was obtained by general lysis buffer as mentioned above and cell free broth was used for further study. The activity of catalase, malondialdehyde and superoxide dismutase were determined as mentioned below.

Catalase activity

Catalase activity in the bacterial cell free broth was measured by the method described by Aebi (1984) with slight modification. In brief, 4 µl of supernatant was mixed in 96 µl of H_2O_2 buffer and absorption was measured at 240 nm using spectrophotometer for 60 s at 15s interval. Activity was calculated by decrease in absorbance of H_2O_2 at 240 nm.

Malondialdehyde level (MDA)

MDA production was measured according to the method of Draper and Hadley (1990). Here, 1 ml of sample was carefully mixed with 2 ml of TBA (thiobarbituric acid) reagent (20 % thiobarbituric acid, 0.5 % TBA, and 2.5 N HCl), heated for 20 min in boiling water bath, cooled, and supernatant was collected by centrifugation (5000 rpm, 10 min) at 4 °C. Absorbance of the collected supernatant was measured at 532 nm. MDA equivalent content in the sample was calculated using an extinction coefficient of $1.56\times10^{5}\ M^{-1}\ cm^{-1}$.

Superoxide Dismutase Activity (SOD)

SOD activity was assayed by the method of Ewing and Janero (1995). 25 µl of supernatant was taken in a microtiter well and mixed with 20 µl of reaction buffer (50 mM phosphate buffer, 0.1 mM EDTA, 98 µM NADH, and 62 µM NBT, pH 7.4). Reaction was initiated by adding 20 µl of an initiating reagent (50 mM phosphate buffer and 33 µM PMS in 0.1 mM EDTA, pH 7.4). Absorbance was measured at 560 nm using the Epoch microplate spectrophotometer (Biotek, USA).

RESULTS AND DISCUSSION

Bacteria have developed different resistance mechanisms to cope up with the negative effects of these metal ions (Khan et al. 2016). *Kocuria* sp. BRI 36 has an ability to remove high concentration of cadmium from aqueous solution by accumulation. (Mulik and Bhadekar, 2017a). Morphological changes in BRI 36 under stress conditions were observed by FESEM (Fig. 1).

Metal affected cells were found to be shrunken as compared to the control cells. Cell deformation due to membrane alterations in presence of heavy metals may be attributed to their effects on membrane proteins that are required to maintain cell shape. Earlier reports showed a strong effect of heavy metals on the organization of actin and tubulin, the

main eukaryotic cytoskeleton components which function as cellular scafolds to maintain cell shape (Eun et al., 2009; Liu et al., 2009; Olabarrieta et al., 2001).

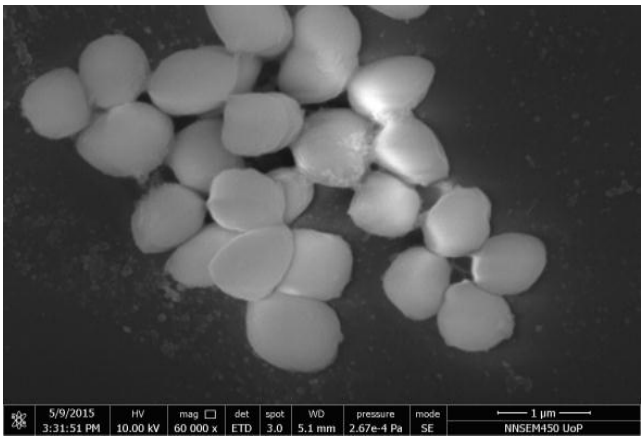


Figure 1a. Cell morphology of *Kocuria* sp. BRI 36

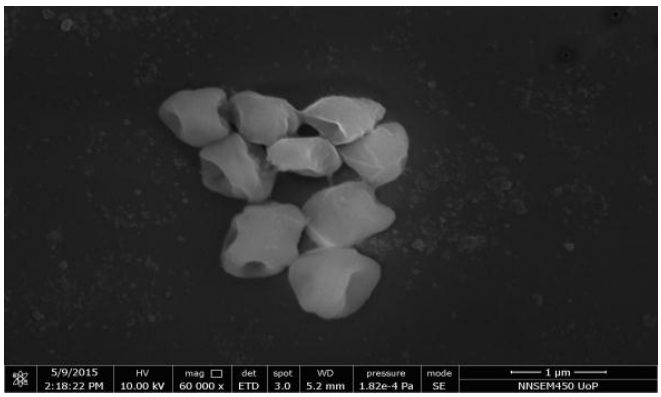


Figure 1b. Cell morphology of *Kocuria* sp. BRI 36 under cadmium stress

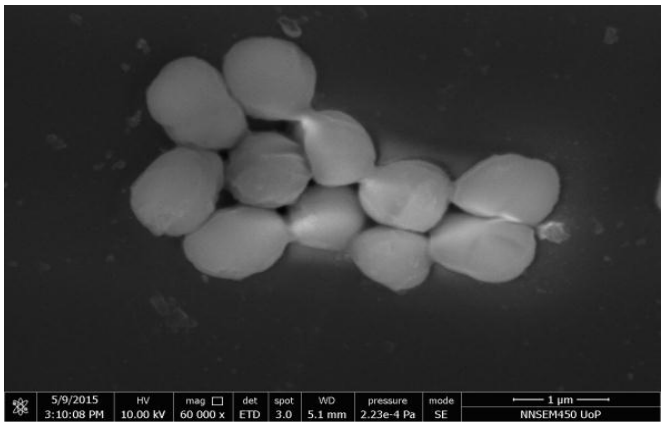


Figure 1c. Cell morphology of *Kocuria* sp. BRI 36 under lead stress

On the contrary effect of lead on cell morphology was almost negligible (Figure 1c).

Cell response to metal stress in terms of proteins produced was analysed by measuring total intracellular proteins and comparing with that of control. Increase in protein concentration was observed with increase in metal concentration upto 50 ppm for both lead and cadmium affected cells (Figure 2). Higher protein content in rhizobacterial strains isolated from metal contaminated soil as compared to rhizobacteria from normal soil had been reported by Deepthi et al., (2014).

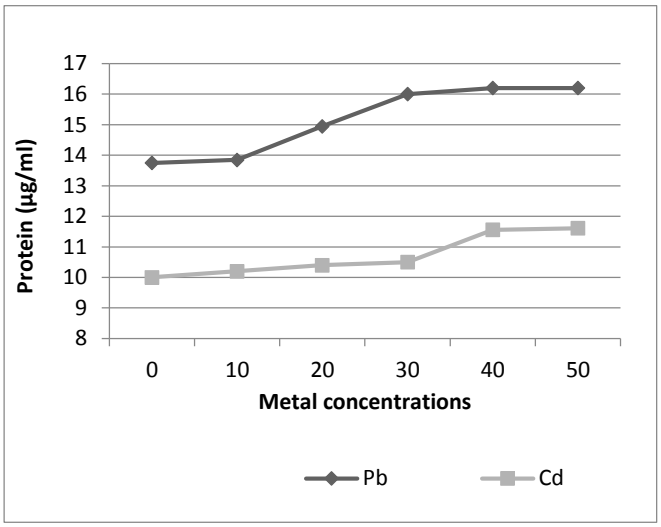


Figure 2: Protein estimation under cadmium and lead stress

Further, the results of protein profiling indicated over expression of certain proteins in metal affected cells. Both cadmium and lead exposed cells showed increase in concentration of 10 kD protein with increase in metal concentration (Figure 3). These may be metal induced, low molecular weight thiol containing metal binding proteins and are involved in sequestration of metal ions (Khan et al., 2016). Another significant observation is increase in concentration of protein with Mr between 50 - 60 kD in cadmium affected cells. Khan et al., (2016) observed increase in 60 kD protein concentration with increase in concentration of cadmium from 1 mM to 10 mM in *S. enterica*. The cells under lead stress showed presence of proteins of approximate molecular weight 80 kD. Its concentration was found to increase with increase in lead concentration. It was absent in the cells grown in presence of cadmium. Thus protein profiles of both cadmium and lead exposed cells appeared different. Similar observation was earlier reported by Banerjee (2015) during their studies on *Enterobacter cloacae* B1. However, protein expression pattern showing such interesting results need further research to understand the detailed mechanism of these stress protein expression and their role in metal tolerance.

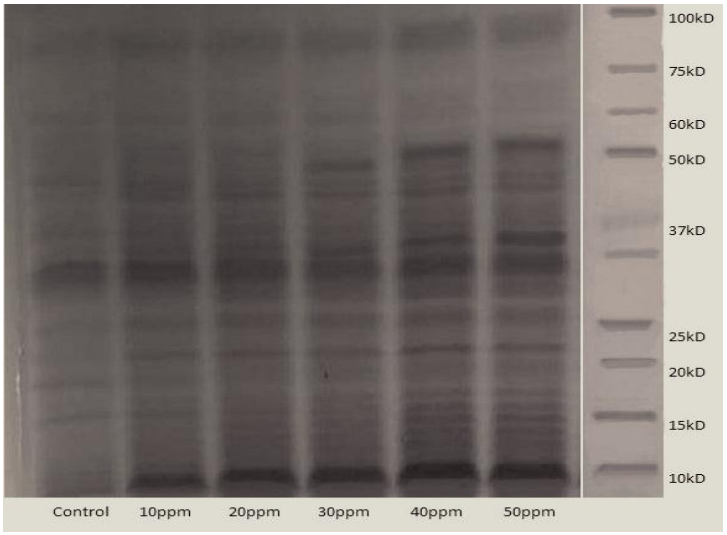


Figure 3.a: Protein profiling under cadmium stress with different Cd^{2+} concentrations

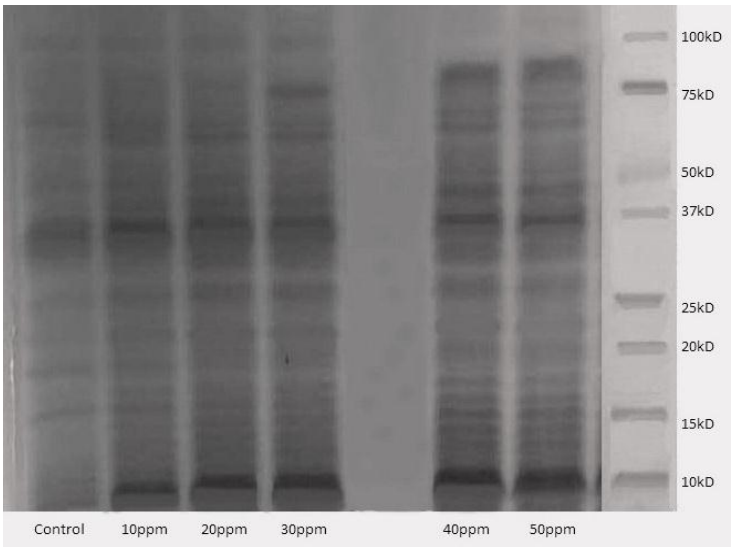
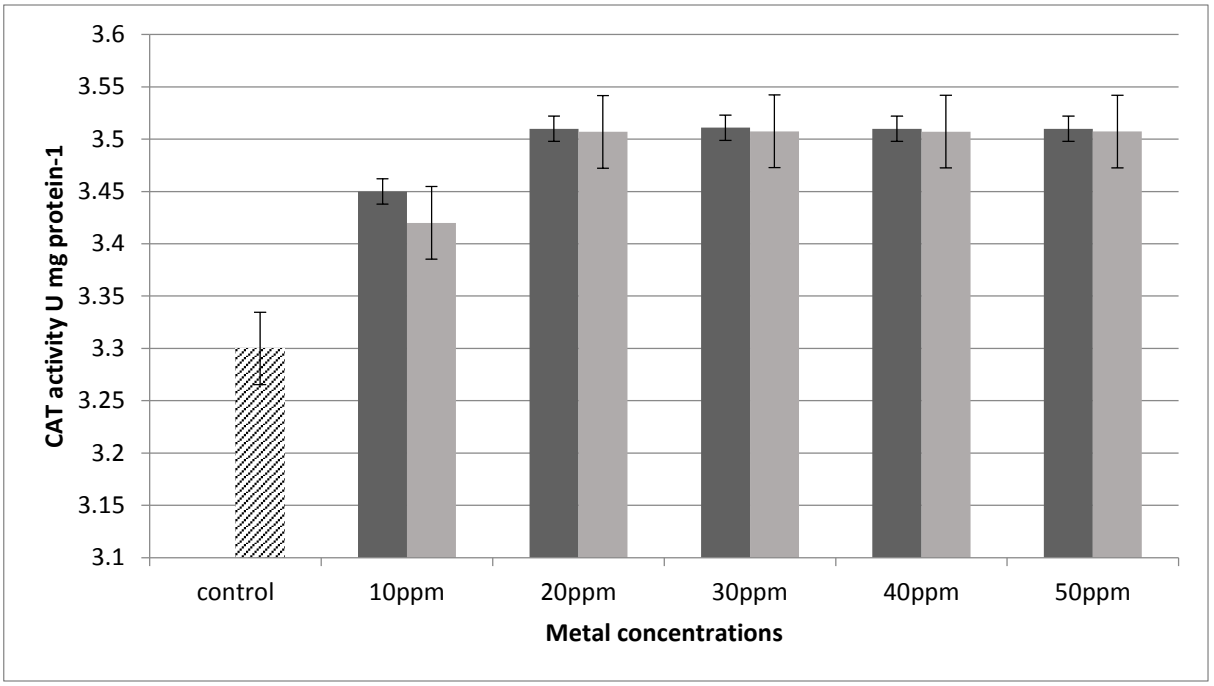


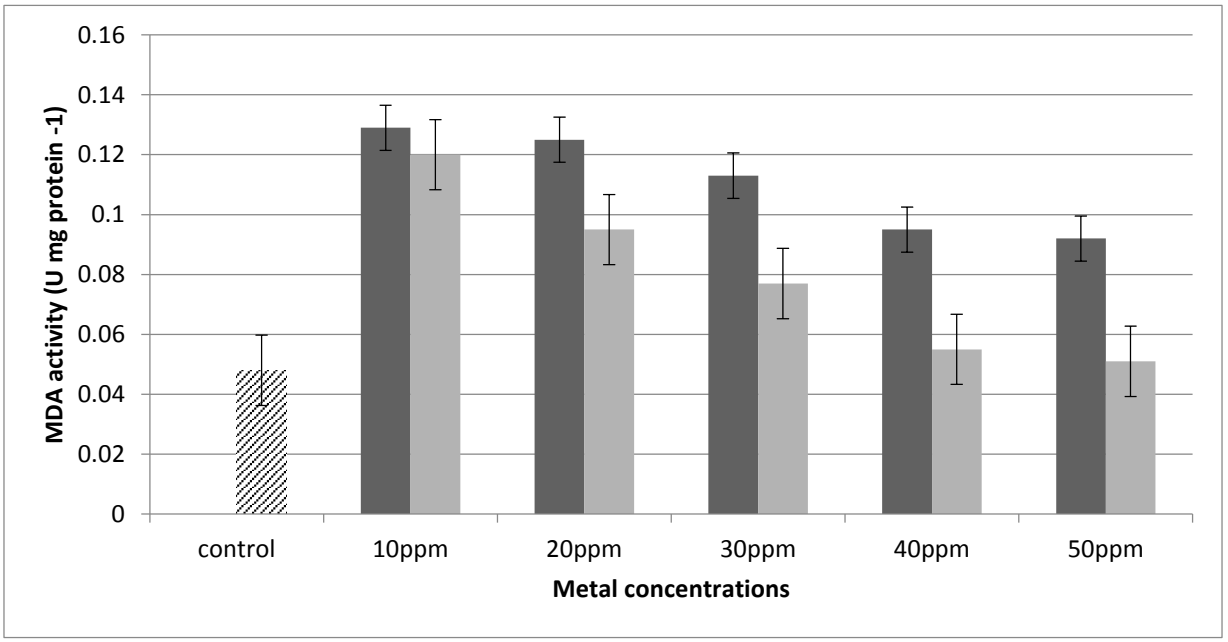
Figure 3.b: Protein profiling under cadmium stress with different Pb^{2+} concentrations

Antioxidant enzymes activities in presence of cadmium and lead at different concentrations were observed in this study and are presented in Fig.4. A significant ($p<0.05$) higher catalase (CAT) activity (fig.4a) was observed with increase in the cadmium concentration up to 20 ppm. Similar results were observed in superoxide dismutase (SOD) (fig. 4c) activity with significantly ($p<0.05$) higher levels with increase in cadmium and lead concentration. Both the antioxidant enzyme activity were seen to be higher as compared to the control. However, a slight decrease ($p> 0.05$) in Malondialdehyde (MDA) level was observed as compared to control (fig. 4b).

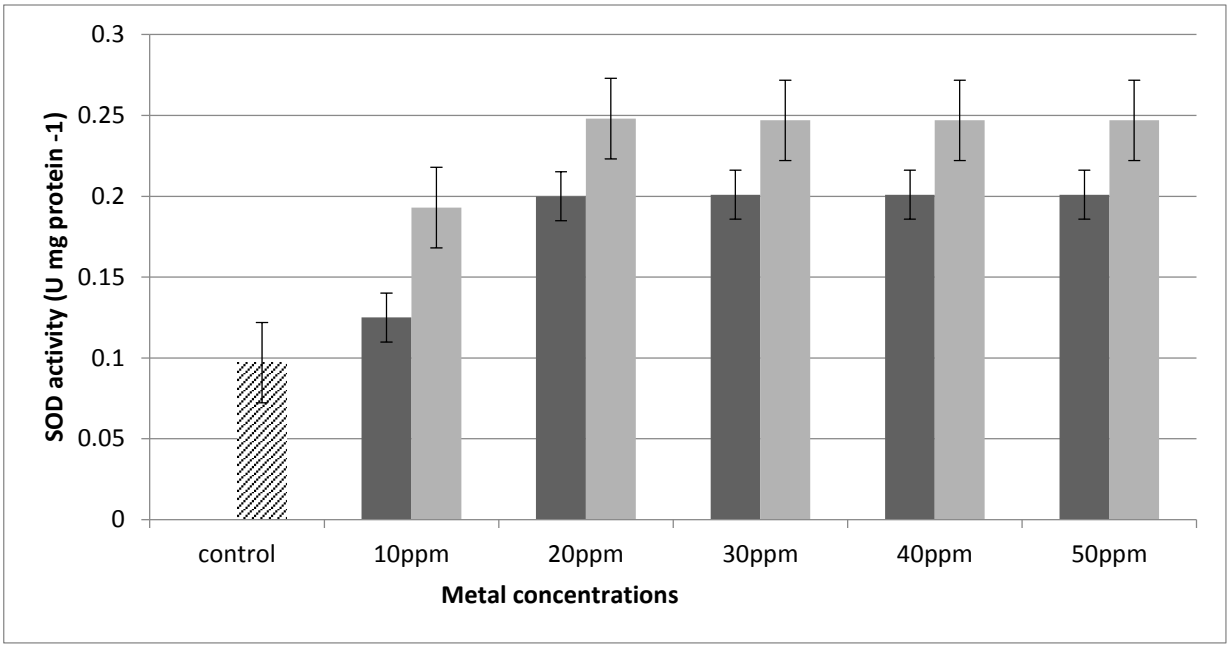
Oxidative stress, caused by an imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses, is an unavoidable aspect of such aerobic living organisms (Sevcikova et al., 2011). ROS reacting with bio-molecules leads to lipid peroxidation, protein denaturation and DNA mutations, hence proving to be lethal for the cell (Morsy et al., 2012). To reduce such oxidative stresses, the bacterial cell produces different types of antioxidant enzymes like CAT and SOD. Our results indicating increase in ROS scavenging enzymes CAT and SOD with increase in metal concentration denoted their protective function against heavy metal oxidative stress. MDA, a polar molecule of small molecular mass, is used widely as an indicator of oxidative stress due to its procedure simplicity (Suran et al., 2013). As depicted in Figure 4b MDA level increased at 10 ppm followed by gradual decrease with increase in metal concentration proving ROS responsive enzyme system (Kweicien et al., 2014). Earlier, similar records have been reported wherein *Enterobacter cloacae* B1 showed significant increase in CAT and SOD activity in presence of cadmium while reduced MDA activity was observed as compared to control (Banerjee, 2015).


3.6
3.55
3.5
3.45
3.4
3.35
3.3
3.25
3.2
3.15
3.1
CAT activity U mg protein-1
control
10ppm
20ppm
30ppm
40ppm
50ppm
Metal concentrations

(a)


0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
MDA activity (U mg protein -1)
control
10ppm
20ppm
30ppm
40ppm
50ppm
Metal concentrations

(b)



(c)

Figure 4: Antioxidant enzyme activity exhibited by *Kocuria sp.* BRI 36 upon exposure of cadmium and lead at different concentrations; a) catalase (CAT) activity; b) Malondialdehyde (MDA) level c) superoxide dismutase (SOD) activity. Data were presented as mean±SEM in vertical bars, n=3 replicate. Data were analyzed using one-way ANOVA.

CONCLUSION

Kocuria sp. BRI 36 has significant potential for removal of lead and cadmium by accumulation. The cells showed deformed morphology when exposed to cadmium. Intracellular protein concentration was found to be higher in the metal treated cells as compared to control. Polyacrylamide gel electrophoresis revealed distinct protein profile for lead and cadmium exposed cells. As expected MDA levels were found to decrease in cells grown in presence of metals whereas SOD and CAT activities increased as compared to control since it is a ROS scavenging cellular response. Purification and molecular characterization of stress induced proteins might be helpful to understand further the mechanism of heavy metal remediation by *Kocuria* sp. BRI36.

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DISCLOSURE STATEMENT

No conflict of interest was reported by the authors.

REFERENCES

[1] Abassi, N. A., Kushad, M. M. and Endress, A. G., 1998. Active oxygen-scavenging enzymes activities in developing apple flowers and fruits. Sci. Horti. 74(3), pp. 183-194.

[2] Banerjee, Goutam., 2015. Bioremediation of Heavy Metals by a Novel Bacterial Strain Enterobacter cloacae and Its Antioxidant Enzyme Activity, Flocculant Production, and Protein Expression in Presence of Lead, Cadmium, and Nickel. Water Air and Soil Pollution. 226. 91-99. 10.1007/s11270-015-2359-9.

[3] Bruins, M. R., Kapil, S. & Oehme, F. W., 2000, Microbial resistance to metals in the environment. Ecotoxicol Environ Saf 45, pp. 198-207.

[4] Choudhury, S., Panda, P., Sahoo, L., and Panda, S. K., 2013. Reactive oxygen species signaling in plants under abiotic stress.Â Plant Signal. Behav. 8:e23681. doi: 10.4161/psb.23681

[5] Deepthi, M.S , Reena. T , and Deepu, M.S, 2014, In vitro study on the effect of heavy metals on PGPR microbes from two different soils and their growth efficiency on Oryza sativa (L.). JBiopest 7(1), pp. 64-72.

[6] Durve A, Naphade S, Bhot M, Varghese J, Chandra N., 2013. Plasmid curing and protein profiling of heavy metal tolerating bacterial isolates. Arch Appl Sci Res 5, pp. 46-54

[7] Easton, J.A., P., Thompson, and M.W., Crowder, 2006. "Time-dependent translational response of E. coli to excess Zn(II)", Journal of biomolecular techniques 17, pp. 303-307

[8] Eun, S.O., Youn, H.S. and Lee, Y. , 2000, Lead disturbs microtubule organization in the root meristem of Zea mays. Physiologia plantarum, 110, pp. 357-365.

[9] Herrero M. Antonia ,Jennifer M. Kremsner, and, andÂ C. Oliver Kappe, 2008. The Journal of Organic Chemistry: 73 (1), pp. 36-47 DOI: 10.1021/jo7022697

[10] Ishii, Shun'ichi & Koki, Jun & Unno, Hajime & Hori, Katsutoshi., 2004. Two Morphological Types of Cell Appendages on a Strongly Adhesive Bacterium, Acinetobacter sp. Strain Tol 5. Applied and environmental microbiology. 70, pp. 5026-9.

[11] Jaroslawiecka, A. and Piotrowska-Seget, Z., 2014, Lead resistance in micro-organisms.Â Microbiology, 160(Pt_1), pp.12-25.

[12] Khan et al., 2016a, "Cadmium Resistance and Uptake by Bacterium,Â Salmonella EntericaÂ 43C, Isolated from Industrial Effluent". AMB Express 6, pp.54

[13] Kwiecien S1,Â Jasnos K,Â Magierowski M,Â Sliwowski Z,Â Pajdo R,Â Brzozowski B,Â Mach T,Â Wojcik D,Â Brzozowski T., 2014, Lipid peroxidation, reactive oxygen species and antioxidative factors in the pathogenesis of gastric mucosal lesions and mechanism of protection against oxidative stress - induced gastric injury. Journal of Physiology and Pharmacology: an official journal of the Polish physiological Society, vol. 65 (5),pp. 612-622.

[14] Laemelli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227(5259), pp. 680-685.

[15] Liu, D., Xue, P., Meng, Q., Zou, J. and Gu, J., 2009, Pb/Cu effects on the organization of microtubule cytoskeleton in interphase and mitotic cells of Allium sativum L. Plant cell reports, 28, pp. 695-702.

[16] Lowry O H, Rosebrough N J, Farr A L & Randall R J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, pp. 265.

[17] M. Sevcikova, H. Modra, and A. Slaninova Z, 2011, Svobodova metals as a cause of oxidative stress in fish: A review, Veterinarni Medicina, vol. 56, no. 11, pp. 537-546.

[18] Morsy A. , K. H. A. Salama, H. A. Kamel, and M. M. F. Mansour, 2012,Effect of heavy metals on plasma membrane lipids and antioxidant enzymes of zygophyllum species, Eurasia J. Biosci, vol. 6, pp. 1-10.

[19] Mulik Anuradha andÂ Rama Bhadekar, 2017a. Heavy metal removal by bacterial isolates from Antarctic oceanic region. Int J Pharm Bio Sci. ;Suppl 8, pp. 535-43

[20] Mulik Anuradha andÂ Rama Bhadekar, 2017b. co-relation between heavy metal resistance and antibiotic susceptibility in halotolerant bacteria isolated from the antarctic oceanic region, Int J Pharm Bio Sci. ;Suppl 8 (4), pp. 302-306

[21] Olabarrieta, I., L'Azou, B., Yuric, J., Cambar, J. and Cajaraville, M.P., 2001, In vitro effects of cadmium on two different animal cell models. Toxicology in vitro, 15, pp. 511-517

[22] Pandey et al. , 2013.Â Frataxin directly stimulates mitochondrial cysteine desulfurase by exposing substrate-binding sites, and a mutant Fe-S cluster scaffold protein with frataxin-bypassing ability acts similarly.Â J Biol Chem288(52), pp.36773-86

[23] P Isarankura-Na-Ayudhya; C Isarankura-Na-Ayudhya; L Treeratanapaiboon; K Kasikun; V Prachayasittikul, 2009, European Journal of Scientific Research, 25(4), pp. 679-688.

[24] Pote, W., Tagwireyi, D., Chinyanga, H.M., Musara, C., Nyandoro, G., Chifamba, J., Nkomozepi, P., 2014. Cardiovascular effects of Boophone disticha aqueous ethanolic extract on early maternally separated BALB/c mice. Journal of Ethnopharmacology 148, pp.379-385.

[25] Sevcikova M, Modra H, Slaninova A, Svobodova Z., 2011. Metals as a cause of oxidative stress in fish: a review.Â Vet Med.;56: pp.537-546.

[26] Suran J., M. Prisc, D. Rasic, E. Srebocan, and A. P. Crnic, 2013, Malondialdehyde and heavy metal concentrations in tissues of wild boar (sus scrofa L.) from central Croatia, Journal of Environmental Science and Health, Part B, vol. 48, pp. 147-152.