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# Occurrence of Multiple Plasmids in Metal Resistant Bacterial Population Isolated from Coal Fly Ash Contaminated Soil

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**Heavy metals pollution in the environment due to coal fly ash generated from thermal power stations posses significant global problem and necessitate definitive remediation measurement. Due to its excessive use and dumping, at high concentrations, they become toxic to both human and microbes. Bacteria have evolved mechanisms to adapt in heavy metals contaminated environments and thus represent a potential source for bioremediation processes. In the present study soil samples were collected from the vicinity around selected thermal power stations of Chhattisgarh and Maharashtra state in India. Ten heavy metals, Cr, Cu, Ni, Co, Cd, Mn, Pb, Hg, Fe and Zn were tested. The statistical analysis of test samples revealed considerably high levels of Cd, Pb and Hg. A total of 49 strains were isolated from soils from different sampling locations and 19 strains were found tolerant for Cd, Pb and Hg. The maximum tolerable concentrations (MTCs) of Cd, Pb and Hg for each isolate were determined. Observed maximum MTCs were 800 ppm for Cd, 900 ppm for Pb and 900 ppm for Hg. Plasmids of sizes approx 12, 1.3 and 0.9 kb were detected in most bacterial strains with resistance to Cd, Pb and Hg.**

**Key words:** Heavy metals; Metal Resistant Bacteria; Plasmids; 16SrDNA; Coal fly ash.

Industrial development is increasing globally and exploitation of natural resources such as coal in energy sectors has become the major issue regarding the heavy metal contamination. Dependence on coal as an energy source in thermal power plants resulted in vast quantities of coal residues known as fly ash (FA). FA generation has been considered as a matter of concern all over the world. In India, more than 100 million ton of FA is generated annually from coal-based thermal power plants[1]. The production of FA (including both fly ash and bottom ash) may likely to exceed 140 million ton per annum by 2020 [2]. The major FA producing countries were USA followed by China and India and their production was estimated to be 129, 125 and 105 million tonnes at the end of 2002 respectively [3] but now the scenario has changed and India became the major FA producing country till the end of 2014 [4].

The coal fly ash contains significant amounts of toxic metals such as As, Ba, Hg, Cr, Ni, V, Pb, Zn and Se [5, 6]. The coal fly ash occupies much space in the premises of industrial plants and is mixed with water to discharge into fly ash settling ponds or landfills. Large quantities of coal fly ashes lead to the contamination of environmental component as a major source of inorganic pollution.

Metal pollution in soil environment affects the organisms such as bacteria. Heavy metals are acutely toxic to microbes but still there are metal resistant bacteria present in the natural

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environment. Metal tolerant bacterial populations often represented by several Gram positives belonging to *Bacillus*, *Arthrobacter* and C*orynebacterium* as well as Gram negatives as *Pseudomonas*, *Alcaligenes*, *Ralstonia* and *Burkholderia* [7,8,9]. Microorganisms, including bacteria possess a variety of mechanisms to deal with high concentrations of heavy metals and often are specific to one or a few metals [10]. These mechanisms may be encoded by chromosomal genes; however, most of the time resistance associated with plasmids[11].

The plasmid can be the source of resistance genes for cloning purpose which have potential use in biotechnology such as the manufacture of biosensors and bioremediation processes[12]. Some bacterial strains are studied for their resistant capabilities for different metals with the ability of detoxify and resist the metals to remediate the contaminate soils [13]. There are very few studies showing the relationship between these species heavy metal resistance patterns and the presence of plasmid. Therefore, from environmental point of view, isolation should focus on multiple heavy-metal resistance bacteria.

The main objective of the present study is to determine the presence of plasmid in heavy metals resistant bacteria isolated from contaminated soil around the vicinity of thermal power plants, evaluate the predominant contaminating metals by statistical tools and investigation of maximum tolerable metal concentration (MTC) of predominating metals for bacteria. Identification of species was performed using 16SrDNA analyses.

## MATERIALS AND METHODS

### Sampling locations

Soil samples were collected from different locations from selected thermal power plant (TPP) sites at NTPC, Korba, NTPC Sipat in Chhattisgarh and TPP Koradi and TPP Akola in Maharashtra, India. Samples were collected in sterile polythene bags and stored at 4 °C for further study. Control soil was also collected far from the industrial zone.

### Heavy metal analysis

The levels of selected ten heavy metals Cd, Pb, Zn, Cr, Cu, Ni, Fe, Hg, Mn and Co in soil samples were analyzed in triplicates with an atomic absorption spectrophotometer (AAS). Mercury analysis was done by direct mercury analyzer DMA - 80 (milestone Inc.). Element analyses were carried out by using the standard method described by AOAC, 1999 [14]. The blank was prepared in the same manner as the samples. The values of the elements and blank obtained from the AAS were calculated, and values were expressed as mg/Kg of dry mass.

### Determination of predominant metals

Enrichment of metal concentration above baseline concentrations was calculated using the method proposed by Muller (1969)[15], termed as geo-accumulation index ($I_{geo}$) and given by:

$$I_{geo} = \log [C_{m:\ Sample} / (1.5 \times C_{m:\ Background})] \quad \ldots (1)$$

The factor 1.5 minimizes the effect of possible variations in the background values, $C_{m:\ Background}$, which may be attributed to lithogenic variations in soils (Figure 1). The $I_{geo}$ classes with respect to soil quality are provided in Table 1.

### Preparation of soil-born bacterial suspension

A 10 g portion (wet weight) of the soil was mixed in a sterile 250 ml Erlenmeyer flask with 90 ml of a 0.85% (w/v) salt solution and incubated at 30°C in a shaker incubator at 90 rpm for 2 hours. The suspensions obtained were filtered through Whatman no-1 filter paper (Merck, Germany) under sterile conditions, and these filtered bacterial suspensions were used for further work.

### Isolation of bacteria

Subsamples (1.0 ml) withdrawn from the soil-borne bacterial suspension were serially diluted (in range: $10^{-1}$–$10^{-6}$) and each dilution was plated in triplicate on nutrient agar (Himedia, India). The pH of the final medium was adjusted to 7.2. 50 mg/l of actinomycin D was added to the medium to preclude the growth of fungi [16] .The plates were incubated aerobically at 37°C for either 24–48 h. Independently growing colonies were randomly selected (on the basis of morphology) for further analysis.

### Determination of Maximum Tolerance Concentration (MTC)

For testing heavy metal resistance, the predominant metals Cd, Hg and Pb used as $CdCl_2$, $HgCl_2$ and $Pb(CH_3COO)_2.3H_2O$ were added to sterilized Luria agar medium in concentrations varying from 50 ppm to 1000 ppm. Plates were then spot inoculated and incubated at 37 °C for 24- 48 hours. The MTC of heavy metals was designated as the highest concentration of heavy

metal that allowed. Investigation of heavy metal resistance in some bacterial strains isolated from industrial soils growth after 2 days. All of the experiments were replicated thrice. Different tolerable concentrations of metals in bacteria are shown in Fig. 2.

**Plasmid isolation and electrophoresis**

Plasmid DNAs were isolated from the bacteria by Plasmid isolation kit (3BBlackBio, India). The isolated plasmids were characterized by agarose gel electrophoresis according to the standard procedure of Sambrook and Russell (2001)[17]. Agarose gel electrophoresis was performed through horizontal slab gel of 0.7% agarose submerged in TAE buffer at 100 V for 1 hour. The molecular sizes of the plasmids were determined by comparison with DNA ladder (EcoRI-Hind III double digest) on a gel documentation system (Alpha Imager).Fig. 3.

**DNA extraction and amplification of 16S rRNA by polymerase chain reaction (PCR) and analysis of the PCR products**

Bacterial DNA was extracted manually using alkaline lysis method [17] from soil isolates. The resulting high-molecular-weight DNA was stored at ≤ 20°C and was used as a template in appropriate PCR experiments. The 16S rRNA fragment were amplified with bacterial universal primers[18] 27F (forward 5' GAGTTTGATCACTGGCTCAG 3') and 1492R (reverse 5' TACGGCTACC TTGTTACGACTT 3') .Each reaction mixture contained 5 µl of template DNA, 2.5 µl of 10X Mg-free Taq buffer (Fermentas, Canada), 2 µl of 2 mmol/l $MgCl_2$, 2.5 µl of 2 mmol/l of each dNTP (Fermentas, Canada), 0.5 µmol of each primer and 1.25U of Taq DNA polymerase (Fermentas, Canada). PCR conditions were as follows: Initial denaturation at 94°C for 3 min followed by amplification in which the following conditions were used: denaturation at 94°C for 40 s, annealing at 60°C for 30s and elongation for 1 min at 72°C for 35 cycles. Final extension was given at 72°C for 10 mins and PCR product was stored at -20°C for further analysis. The amplified fragments of 1500 bp were visualized by running 5µl of the PCR products on 1.5% agarose gels containing ethidium bromide (Fig. 4). A 1 kb DNA ladder (Fermentas, Canada) was used as a size marker. Amplified DNA i.e., of 16S rRNA fragment, were sequenced in both directions in ABI Genetic Analyzer 3500 using ABI Big Dye™ Terminator Cycle sequencing kit by Chromous Biotech Pvt. Ltd, Bangalore, Karnataka, India with raw data files returned for analysis. Sequences obtained were compared to the non-redundant nucleotide database in GenBank using BLAST (Basic Local Alignment Search Tool) algorithm.

## RESULTS

**Metal analysis of industrial soils**

AAS was used for determination of the heavy metal contents of the contaminated soils (data not shown). Soil from a different geographical region was used as the control. Fig. 1 gives a clear picture of the moderate to high contamination of soils with fly ash by a variety of heavy metals. The concentrations of Cd, Pb and Hg in the soils were considerably higher as compared with the values of the control soil by Geoaccumulation Index study ($I_{geo}$). The values and contamination level of $I_{geo}$ is given in Table 1.

**Maximum tolerance concentration (MTC)**

**Cadmium toxicity**

The resistance to Cd was found up to the range of 800 ppm of $CdCl_2$ by 8 species out of 49 species isolated from all locations. *Pseudomonas spp*. Strains showed a maximum frequency of

**Table 1**. The $I_{geo}$ classes with respect to soil quality (Muller, 1969)

| $I_{geo}$ Value | $I_{geo}$ Class | Designation of Soil Quality |
|---|---|---|
| >5 | 6 | Extremely contaminated |
| 4 – 5 | 5 | Strongly to extremely contaminated |
| 3 – 4 | 4 | Strongly contaminated |
| 2 – 3 | 3 | Moderately to strongly contaminated |
| 1 – 2 | 2 | Moderately contaminated |
| 0 – 1 | 1 | Uncontaminated to moderately contaminated |
| 0 | 0 | Uncontaminated |

resistance i.e. 4 strains followed by two *Bacillus spp*., one *Klebsiella pneumoniae* and one *Acromobacter spp*. (Fig. 2).

**Lead toxicity**

The resistance to Pb was found to be in the range of 900 ppm of Pb $(CH_3COO).3H_2O$, opposite to cadmium *Bacillus spp*. Strains showed higher frequency of resistance followed by *Pseudomonas spp*.

**Mercury toxicity**

The most abundant heavy metal in the soils of Maharashtra state locations was Mercury, as compared with the values of the control soil (Fig. 1). The resistance was found to be in the range of 900 ppm of $HgCl_2$, and more variant of bacterial species were found resistant to mercury such as *Pseudomonas spp.* followed by *Bacillus spp., Aeromonas spp., Achromobacter spp. and Staphylococcus spp.*

**Plasmid profiles of bacterial strains**

To determine the plasmid contents in bacteria from the soil samples, plasmid DNAs were isolated from all the nineteen species of bacteria that showed the highest MTC values for Pb, Cd and Hg. According to the electrophoresis separation approximately 12, 1.3 and 0.9 kb size

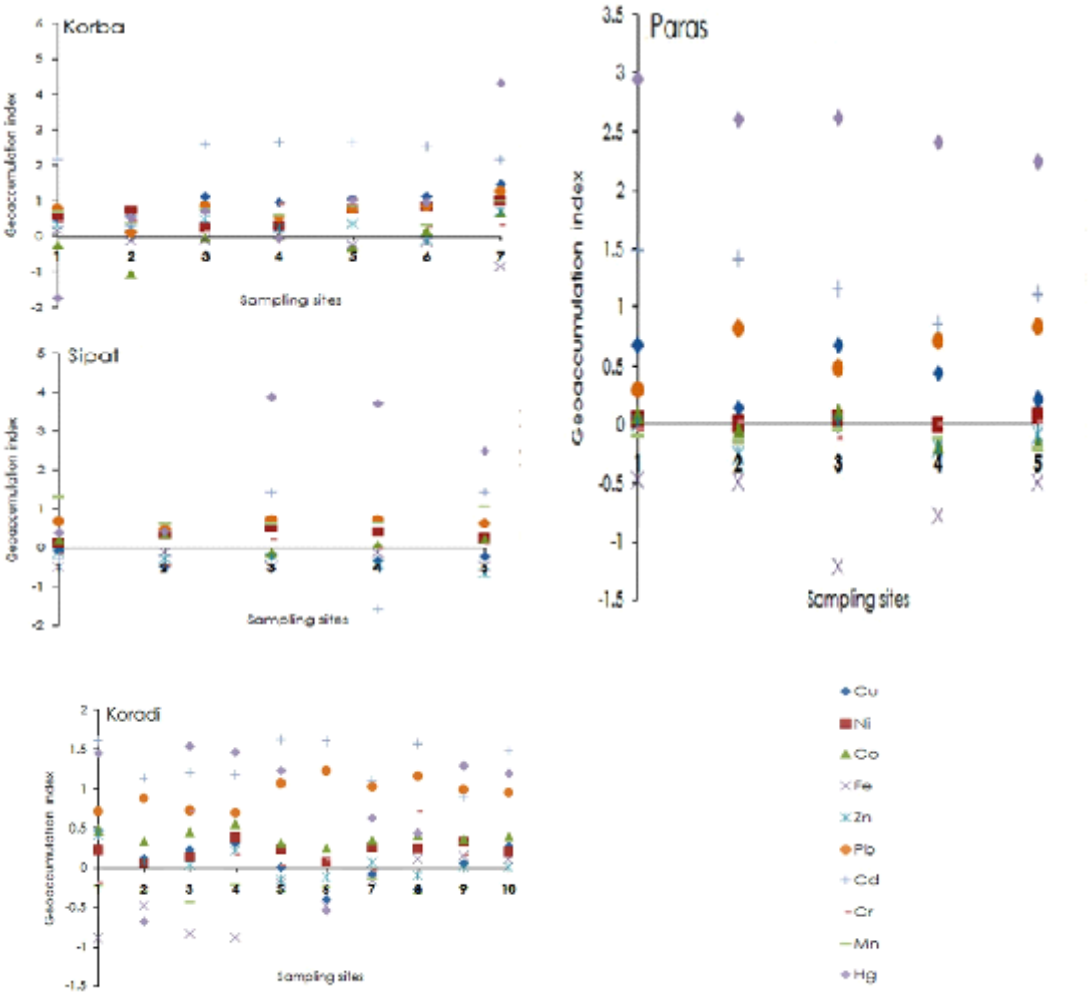


**Fig. 1.** (A): $I_{geo}$ index map showing enrichment of heavy metals in soil samples around thermal power station Korba and Sipat (C.G) (b) $I_{geo}$ index map showing enrichment of heavy metals in soil samples around thermal power station, Koradi (M.S) (c): $I_{geo}$ index map showing enrichment of heavy metals in soil samples around thermal power station Paras, Akola (M.S) (d): Symbols used for metals in $I_{geo}$ index mapping

**Table 2.** GenBank accession number and identical species name of isolates

| S.No. | GenBank Accession Number | Name of species | Percent Identification |
|---|---|---|---|
| 1 | KP238211 | *Pseudomonas plecoglossicida* | 99% |
| 2 | KP238212 | *Pseudomonas spp.* | 100% |
| 3 | KP238213 | *Bacillus firmus* | 99% |
| 4 | KP238214 | *Pseudomonas stutzeri* | 100% |
| 5 | KP238215 | *Bacillus aerophilus* | 100% |
| 6 | KP238216 | *Aeromonas hydrophila* | 99% |
| 7 | KP238217 | *Pseudomonas otitidis* | 100% |
| 8 | KP238218 | *Bacillus mojavensis* | 100% |
| 9 | KP238219 | *Achromobacter xylosoxidans* | 99% |
| 10 | KP238220 | *Pseudomonas aeruginosa* | 100% |
| 11 | KP238221 | *Pseudomonas putida* | 100% |
| 12 | KP238222 | *Pseudomonas monteilii* | 100% |
| 13 | KP238223 | *Bacillus firmus* | 99% |
| 14 | KP238224 | *Staphylococcus arlettae* | 99% |
| 15 | KP238225 | *Klebsiella pneumoniae* | 99% |
| 16 | KP238226 | *Bacillus aquimaris* | 99% |
| 17 | KP238227 | *Pseudomonas spp.* | 99% |
| 18 | KP238228 | *Pseudomonas spp.* | 99% |
| 19 | KP238229 | *Pseudomonas spp.* | 99% |

plasmids were detected in most of the *Pseudomonas spp*. Strains and 12 kb size of plasmids were detected in *Bacillus spp*. (Fig. 3).

**Bacterial Identification by 16S rDNA analysis**

PCR amplification of the 16S rDNA gene produced fragments of approximately 1500 base pairs in size (Figure 4). Full length sequence of the 16 S rDNA gene was deduced for 19 isolates showing tolerance to high concentration of Pb, Cd and Hg. Sequences were aligned and the closest match was detected using BLAST. Identification of the strains by 16S rDNA gene sequence revealed that the strains belonged to *Pseudomonas spp., Bacillus spp., Aeromonas spp., Klebsiella spp. and Acromobacter spp*. (Table 2). Nucleotide sequences generated in this study have been

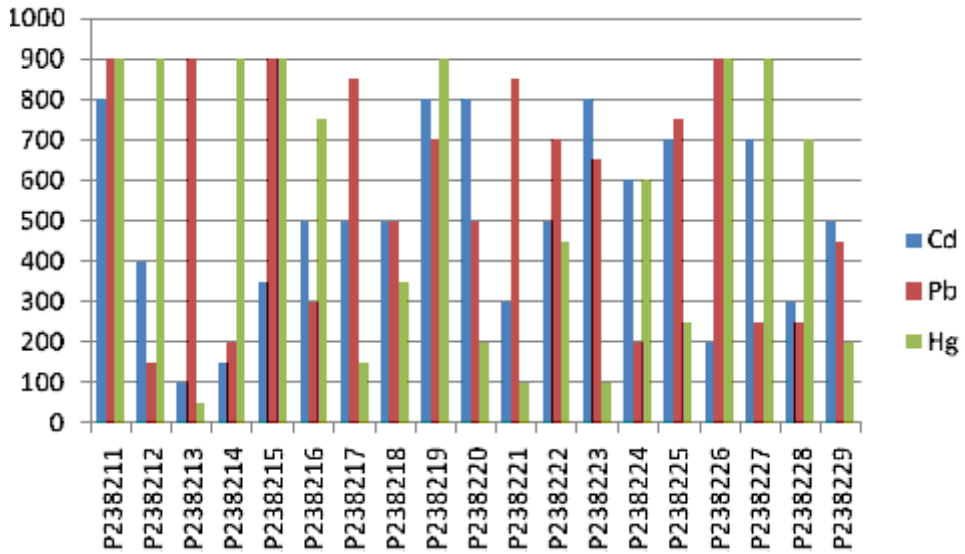


**Fig. 2.** Maximum tolerance concentration of isolates for Cd, Pb and Hg

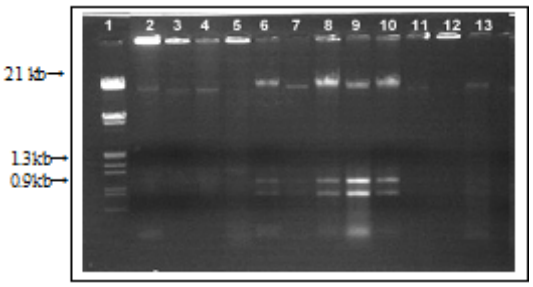


**Fig. 3.** Plasmid isolation from metal tolerant isolates. Lane 1-13: (1: Marker EcoRI hind III; 2: KP238225; 3: KP238222; 3: KP238217; 4: KP238228; 5: KP238219; 6: KP238221; 7: KP238213; 8: KP238227; 9: KP238214; 10: KP238220; 11: KP238218: 13: KP238211)

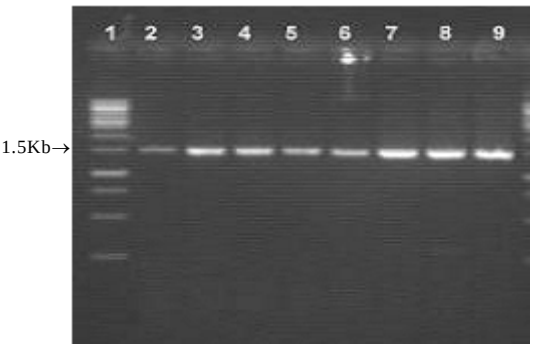


**Fig. 4.** 16S rRNA gel picture of some metal resistant isolates. Lane 1-8: (1: Marker 1KB; 2: KP238211; 3: KP238212; 4: KP238213; 5: KP238214; 6: KP238215; 7: KP238216; 8: KP238217; 9: KP238218)

deposited with the GenBank databases under the accession numbers (KP238211 to KP238229).

## DISCUSSION

Our findings indicated that considerably high levels of Pb, Cd and Hg were present in the soils near thermal power plants as compared with the levels of control soil. Studies have shown that some metals such as Zn, Cu, Ni and Cr are essential or beneficial micronutrients for plants, animals and microorganisms, whereas others, such as Cd, Hg and Pb have no known biological or physiological functions. However, all these metals could be toxic at certain concentrations[19, 20]. In some studies when exposed to moderate heavy metal concentrations, soil microorganisms were found to be very sensitive [21].

The pure isolated strains obtained from the polluted locations were studied. Different concentrations of each of three metal solutions were prepared, the minimum concentration of each metal added was 50 ppm and the concentration was gradually increased till MTC was achieved. Some of the strains were able to grow on higher concentration of all three metals. This varying response of tested bacteria might be due to variation in resistance mechanisms[22]. Resistance of heavy metals might be chromosomal or plasmid mediated [23].

Bacteria found in heavy metal enriched environment have adapted various resistance mechanisms. Heavy metals resistant genes are often carried by plasmids and make bacteria able to utilize metals for detoxification and removal of heavy metals from contaminated environment[24]. In this study we found a variety of bacterial population showed resistance to metals concentration in which maximum belongs to the genus *Pseudomonas* followed by *Bacillus, Staphylococcus Aeromonas, Achromobacter* and *Klebsiella*.

In earlier studies resistance to heavy metals is observed in a wide variety of bacteria, especially in gram negative bacteria [25], such as *Pseudomonas, Alcaligenes, Ralstonia* and *Burkholderia* [26, 27, 28, 29, 30]. Numerous studies have reported on the heavy metal resistance of *Pseudomonas aeruginosa* obtained from different heavy metal polluted environments[8, 31, 32, 33].

In the present study, we also evidenced the tolerance of the strains that belongs to non-Pseudomonas genus such as *Bacillus, Klebsiella, Staphylococcus, Aeromonas and Ahcromobacter* species to various concentrations of Pb, Cd and Hg. Various *Bacillus* species have also been investigated which are heavy metals resistant in some research work in which they reported *Bacillus aquamaris, Bacillus cereus, Bacillus subtilis* as a potential strains for heavy metal resistance[19, 34, 35].

The results of this study showed a higher plasmid incidence in the isolated bacteria (Fig. 3). These isolated bacteria demonstrate that resistance to heavy metals by genes present on

their plasmids suggests the exertion of selective pressure on such bacteria through contamination with heavy metals in their environment. Heavy metal resistance genes are often found on plasmids and transposons [36, 37].

## CONCLUSION

The presence of bacteria capable of tolerating heavy metals from soil samples from fly ash contaminated sites around the vicinity of selected thermal power plants investigated. Bacteria that resist high levels of heavy metals were isolated in pure cultures. All were identified to belong to the different genera such as *Pseudomonas, Bacillus, Aeromonas, Achromobacter, and Staphylococcus*. In summary our results revealed that the 19 isolates showed remarkable tolerance against heavy metals, could be potential agents for the development of a soil inoculants applicable in bioremediation of heavy metals polluted agricultural and industrial sites. The genetic capacity of bacteria can be exploited for the remediation of heavy metal pollution. Genetic improvement may help to develop the field of existing methodologies to decontamination processes. Considering the wide range of multiple-metal resistant at high concentrations by bacterial isolates of present study, these isolates could help in the effective bioremediation of heavy metal contaminated sites.

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## REFERENCES

1. Jala, S., Goyal, D. Fly ash as a soil ameliorant for improving crop production—a review. *Bioresour Technol*, 2006; **97**:1136–1147.
2. Pandey, V., Abhilash, P., Upadhyay, R., Tewari, D. Application of fly ash on the growth performance and translocation of toxic heavy metals within *Cajanus cajan* L.: Implication for safe utilization of fly ash for agricultural production. *J Hazard Mat*, 2009; **166**: 255–259.
3. Hynes, R. Reclamation and revegetation of fly ash disposal sites – Challenges and research needs. *J Environ manage*, 2009; **90**: 43-53.
4. Dwivedi, A., Jain, M.K. Fly ash- waste management and overview: A Review. *Recent Resear Scienc Tech*, 2014; **6**(1): 30-35.
5. Adriano, D. C., Page, A. L., Elseewi, A. A., Chang A. C., Straughan, I. Utilization and disposal of fly ash and other coal residues in terrestrial ecosystems: a, review., *Journal of Environmental Quality*, 1980; **9**: 333–344.
6. Mattigod, S. V., Rai, D., Eary, L. E., and Ainsworth, C. C. Geochemical factors controlling the mobilization of inorganic constituents from fossil fuel combustion residues: review of the major elements. *Journal Environmental Quality*, 1980; 19:188–201.
7. Ellis, R., Morgan, P., Weightman, A., Fry, J. Cultivation dependent approaches for determining bacterial diversity in heavy-metal-contaminated soil. *Appl Environ Microbiol*, 2003; **69**: 3223–3230.
8. Akinbowale, O., Peng, H., Grant, P., Barton, M. Antibiotic and heavy metal resistance in motile aero monads and pseudomonad's from rainbow trout (*Oncorhynchus mykiss*) farms in Australia. *Int. J Antimicrob Agents*, 2007; **30**: 177–182.
9. Singh, S., Tripathi, V., Jain, R., Vikram, S., Garg, S. An antibiotic, heavy metal resistant and halo tolerant Bacillus cereus SIU1 and its thermo alkaline protease. *Microb Cell Fact*, 2010; 9: 59.
10. Bruins, M., Kapll, S., Oetme, F. Microbial resistance in the environment. *Ecotoxicol Environ Safety*, 2000; **45**:198–207.
11. Wuertz, S., Mergeay, M: The impact of heavy metals on soil microbial communities and their activities. In: J.D. Elsas, J.T. Trevors, and E.M.H. Wellinngton (Ed). Modern soil microbiology, Marcel Dekker, New York, NY, USA, 1997; p 607-639.
12. Collard, J., Corbisier, P., Diels, L., Dong, Q., Jeanthon, C., Mergeay, M., Taghavi, S., Vander Lelie, D., Wilmotte, A., Wuertz, S. Plasmids for heavy metal resistance in *Alcaligenes eutrophus* CH34: Mechanism and applications. *FEMS Microbiol Rev*, 1994; **14** (4): 405-414.
13. Sevgi, E., Coral, G., Gizir, A., Sangun, M. Investigation of heavy metal resistance in some bacterial strains isolated from industrial soils. *Turk J Biol*, 2010; **34**: 423-431.
14. AOAC. AOAC official methods of analysis. 15th

ed. Association of Official Analytical Chemists, Arlington, Virginia, 1999; pp. 84–85.
15. Muller, G. Index of geo accumulation in sediments of the Rhine River. *Geol J*, 1969; **2**(3):108–118.
16. Hamaki, T., Suzuki, M., Fudou, R., Jojima, Y., Kajiura, T., Tabuchi, A., Sen, K., Shibai H., Isolation of novel bacteria and actinomycetes using soil-extract agar medium. *J Biosci Bioeng*, 2005; **99**: 485–492.
17. Sambrook, J., Russell, D. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York. 2001.
18. Byers, H.K., Stackebrandt, E., Hayward, C., Blackall, L.L .Molecular investigation of a microbial mat associated with the great artesian basin. *FEMS Microbiol Ecol*, 1998; **25**: 391–403.
19. Gadd, G. Metals and microorganisms: a problem of definition. *FEMS Microbiol Lett*, 1992; **100**: 197 204.
20. Singh, Y., Ramteke, P., Tripathy, A., Shukla, P. Isolation and Characterization of *Bacillus* resistant to multiple heavy metals. *Int J Curr Microbio Appl Sci*, 2013; **2**(11): 525-530.
21. Giller, K., Witter, E., McGrath, S. Toxicity of heavy metals to microorganisms and microbial process in agricultural soils: a review. *Soil Biol Biochem*, 1998; **30**: 1389- 1414.
22. Abou Zeid, A., Hassanein, A., Hedayat, S., Fahd, G. Biosorption of some heavy metal ions using bacterial species isolated from agriculture waste water drains. *Egypt J Appl Sci Res*, 2009; **5**(4): 372-383.
23. Gupta, A., Phung, L., Chakravarty, L., Silver, S. Mercury resistance in Bacillus cereus RC607: Transcriptional organization and two new open reading frames. *J Bacteriol*, 1999; **181**:7080-7086.
24. Ahmed, N., Nawaz, A., Badar, U. Screening of copper tolerant bacterial species and their potential to remove copper from the environment. *Bull Environ Contamin Toxicol*, 2005; **74**: 219–226.
25. Poole, K. Outer membranes and efflux: The Path to Multidrug Resistance in Gram- Negative Bacteria. *Curr Pharm Biotechnol*, 2002; **3**:77-98.
26. Malik, A., Jaiswal, R. Metal resistance in *Pseudomonas* strains isolated from soil treated with industrial wastewater. *World J Microbiol Biotechnol*, 2000; **16**: 177-182.
27. Kozdro´j, J., van Elsas J. Structural diversity of microorganisms in chemically perturbed soil assessed by molecular and cytochemical approaches. *J Microbiol Methods*, 2001; **43**: 197-212.
28. Ünaldi, M., Korkmaz, H., Arikan, B., Coral, G. Plasmid-Encoded heavy metal resistance in *Pseudomonas* sp. *Bull Environ Contam Toxicol*, 2003; **71**: 1145-1150.
29. Zolgharnein, H., Azmi, M., Saad M., Mutalib, A. Mohamed C. Detection of plasmids in heavy metal resistance bacteria isolated from the Persian Gulf and enclosed industrial areas. *Iran. J Biotechnol*, 2007; **5**: 232-239.
30. Singh, V., Chauhan, P., Kanta, R., Dhewa, T., Kumar, V. Isolation and characterization of Pseudomonas resistant to heavy metals contaminants. *Inter J Pharm Sci Rev Res*, 2010; **3**(2):164-167.
31. Filali, B., Taoufik, J., Zeroual, Y., Dzairi, F., Talbi, M., Blaghen, M. Wastewater bacterial isolates resistant to heavy metals and antibiotics. *Curr Microbiol*, 2000; **41**: 151-156.
32. Raja, C., Selvam, G. Plasmid profile and curing analysis of *Pseudomonas aeruginosa* as metal resistant. *Int J Environ Sci Tech*, 2009; **6**(2): 259-266.
33. Raja, C., Anbazhagan, K., Selvam, G. Isolation and characterization of metal-resistant *Pseudomonas aeruginosa* strain. *World J Microbiol Biotech*, 2006; **22**: 577-585.
34. Hookoom, M., Puchooa, D. Isolation and Identification of Heavy Metals Tolerant Bacteria from Industrial and Agricultural Areas in Mauritius. *Curr Res Microbiol Biotechnol*, 2013; **1**(3): 119-123.
35. Zhang, Q., Achal, V., Xiang, W., Wang, D. Identification of Heavy Metal Resistant Bacteria Isolated from Yangtze River, *China. Int. J Agric Biol*, 2014; **16**: 619-623.
36. Chu, L., Mukhopadhyay, D., Mongri, Y., Kum, S., Misra, T. Regulation of the *Staphylococcus aureus* plasmid PI258 mercury: resistance operon. *Bacteriol*, 1992; **174**: 7044-7047.
37. Silver, S. Plasmid determined metal resistance mechanisms range and overview. *Plasmid*, 1992; **27**: 1-3.