



Received on 30 September, 2015; received in revised form, 15 December, 2015; accepted, 05 January, 2016; published 01 March, 2016

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF HEAVY METAL RESISTANT BACTERIA FROM SOIL OF AN IRON INDUSTRY, HARYANA (INDIA).

Richa Gupta, Tarun Kumar and Amita Mittal*

Department of Biotechnology Engineering, University Institute of Engineering & Technology, Kurukshetra University, Kurukshetra, Haryana, 136119, India.

Key words:

Manganese, *Rhizobium halophytocola*, Antibiotic resistance, 16S rDNA, Industrial Soil.

Correspondence to Author:

Amita Mittal

Assistant Professor
Department of Biotechnology
Engineering, University Institute of
Engineering and Technology,
Kurukshetra University, Kurukshetra-
136119 (Haryana), India.


E-mail: amitakuk@gmail.com

ABSTRACT: Metallic contaminants in the soil are hazardous and their accumulation in plants and water may be dangerous. Microorganisms have properties to adsorb, absorb and accumulate heavy metals. Moreover, they play the role of problem solver by resolving key problems associated with different kinds of metals. The study was undertaken to isolate and identify heavy metal resistant bacteria in soil around iron industries of Sonapat district in Haryana (India). Eight isolates were selected on the basis of heavy metal tolerance and antibiotic resistance in the secondary screening. Out of eight, one of the strain was authentically identified as *Rhizobium halophytocola* strain KT327204.1 (RT7) on the basis of morphological, biochemical, 16S rDNA gene sequencing and phylogeny analysis. The minimal inhibitory concentration (MIC) of isolate against Mn (7mM), Ni (1mM), Pb (3mM) and Fe (1mM) was determined in solid media. The identified isolate showed optimum growth at 37°C, 7.0pH and at NaCl concentration of 1% (w/v).

INTRODUCTION: Heavy metal is a prevalent pollutant in the soil, water and air. Metal plating, coal based waste, mining by product, chemical, industrial, nuclear and pesticide waste, mineral leaching and gasoline are considered as some of the major sources of these metals^{1, 2, 3}. Manganese is one metal which is hard to find as the free metal. On the contrary, it is present in over 100 minerals including various oxides, carbonates, sulphides, phosphates, silicates and borates^{4, 5}. Manganese naturally occur where water will remain in close contact with rock over a long period of time for instance deeper wells.

The metal exists in soil as an outcome of atmospheric deposition and mineral weathering originating from anthropogenic and natural sources^{6, 7}. In soil, oxidation states of manganese are found in three forms: Mn (II), Mn (III) and Mn (IV). Mn (II) is only form, which is found in stable form in the soil solution whereas the other two forms found stable in solid phase of soil⁸. The metal mobility in soil is found to be very sensitive towards some of the soil conditions like wetness, acidity, biological activity^{8, 9}. Lead is another such metal which is toxic in nature and is obtained from sources like printing, oil refineries, dyeing, etc. 90% of the pure manganese produced is consumed by the steel industry as an alloying constituent and deoxidizing and desulfurizing additive. It is also often alloyed with other metals such as aluminium and copper to reduce corrosion^{4, 10, 11}.

The common uses of Manganese dioxide is in the chemical manufacturing, leather and glass

<p>QUICK RESPONSE CODE</p> 	<p>DOI: 10.13040/IJPSR.0975-8232.7(3).1308-13</p> <hr/> <p>Article can be accessed online on: www.ijpsr.com</p>
<p>DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.7(3).1308-13</p>	

manufacturing in the leather and textile industry and in the production of dry-cell batteries. $MnSO_4$ is applied as a fertilizer, livestock nutritional supplement, in glazes and varnishes, and in ceramics¹⁰. The metal is also used for the normal development of the body of mammals. Out of several functions performed by the metal, 20 identified functions are related with proteins and enzymes¹². Mn contributes to maintain healthy nerves and immune system and helps in blood sugar regulation^{13, 14, 15}. The toxicity of the metal has been reported over and over again through dietary and occupational (e.g. welder, miner) overexposure. It has been notified that its toxicity causes harm to the central nervous system, liver, heart, etc¹⁵.

Relatively high doses of manganese affect DNA replication and causes mutations in microorganism and mammalian cells. In the case of mammalian cells, the metal exposure even leads to chromosome aberrations & DNA damage. Large amounts of manganese affect fertility in mammals and are toxic to the embryo and foetus. The metal also affects fertility in mammals and causes harmful effect to foetus and embryo. It is due to this reason only pregnant women are advised to restrict their exposure to the metal at the work place¹⁰.

Microorganisms are omnipresent and are found to be involved in one or other form in different biological processes of life. Owing to natural processes & urbanization, heavy metals are commonly found in microbial habitats in high proportions. Due to higher concentrations of metals, these organisms are forced to adjust themselves with different biological mechanisms so that they can cope with High heavy metal conditions¹⁶. Many reports have revealed that indigenous microbes are capable of tolerating high metal concentrations in one way or another and may play a pivotal role in the restoration of contaminated soil^{17, 18}.

The present study aims to isolate and identify indigenous bacterial strains from heavy metal contaminated soil to determine their tolerance to manganese, iron, lead and nickel. The physiological and biochemical features were used to characterize the strains.

Methodology:

Direct Isolation of heavy metal resistant bacteria by plating: The soil samples were collected in and around iron industries of Sonipat district, Haryana, India. The samples were collected in sterile plastic container and transported to laboratory for bacteriological analysis. A 10-fold dilutions of fresh soil (1g) were made in distilled water and 0.1 ml from each of these dilutions was spread on triplicate NA (Hi Media, India) supplemented with each metal (.1mM) one time by the standard spread plate method. Plates were incubated at 37°C for 5 days. Colonies with different morphological appearance were selected from these culture plates and culture grow at a given concentration were subsequently transferred to the next concentration. Isolates were subcultured repeatedly on the same media for purification and stored in glycerol stock solution at -20°C.

Determination of MIC:

Maximum resistance of the selected isolates against increasing concentrations of heavy metals on Nutrient agar plates was evaluated until the strains unable to grow colonies on the agar plates. Nutrient Agar plates supplemented with heavy metal at the following concentration: $MnSO_4 \cdot H_2O$, 0.1, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 mM; $C_4H_6O_4Pb \cdot 3H_2O$, 0.1, 0.5, 1.0, 2.0, 3.0 mM; $FeSO_4 \cdot 7H_2O$, 0.1, 0.5, 1.0 mM; $NiSO_4$ 0.1, 0.5, 1.0 mM, were prepared in distilled water and sterilized by autoclaving at 121°C for 15 min. Based on the evaluation minimum inhibitory concentration (MIC) was determined at 37°C for 5 days^{19, 20}.

Determination of antibiotic resistance:

Resistance to antibiotics was determined by Disc Plate Diffusion method on NA plates. Inhibition zone was noted after 24h incubation. Strain was considered susceptible when the inhibition zone was 12 mm or more in diameter. All the tests were performed in triplicate. The following antibiotics were tested: Chloramphenicol (50µg/d), Neomycin (30µg/d), Ciprofloxacin (5µg/d), Amoxicillin (10µg/d), Ampicillin (10µg/d), Chlorotetracycline (30µg/d), Ceftazimide (30µg/d) and Kanamycin (5µg/d) (µg/d- microgram/disc)

Determination of Morphological, Physiological and Biochemical characteristics: The shape and color of the colonies were examined under the microscope after Gram staining. The optimal growth conditions with reference to pH, temperature and salt tolerance were determined²¹. Utilization of different carbon sources such as sugars, organic acids and amino acids were analysed using Hi 25TM Enterobacteriaceae Kit (Hi Media, India). Plates were prepared and inoculated according to Hi Media manufacturer's directions. Microplates were covered and incubated at 35⁰C²². Morphological and physiological tests were performed following the protocol given in Bergey's Manual of Determinative Bacteriology²³.

16SrDNA gene amplification:

Genomic DNA was isolated and was amplified by using the universal bacterial 16S rDNA primers, 27F(5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3'). PCR was performed with a 50 µl reaction mixture containing 1 µl of DNA template, 0.5 µl of each primer at a concentration of 5mM, 5 µl MgCl₂/Buffer and 5 µl dNTP's at a concentration of 10mM, 0.5 µl of Taq polymerase. PCR was used to incubate reactions through an initial denaturation at 94⁰C for 1min, annealing at 52⁰C for 1min, extension at 72⁰C for 1min and final extension at 72⁰C for 5min. PCR was carried out in a Thermal cycler (BioRad, India). PCR products were analyzed by 0.8% (w/v) agarose gel electrophoresis

in 1XTAE buffer with ethidium bromide (0.5µg/ml).

Nucleotide sequencing and Phylogeny:

The PCR products with size of about 1.5 Kb were pooled and purified using PCR-DNA & Gel Band Purification Kit (GE Health Care, India) according to the manufacturer's instructions. Concentration of purified PCR product was determined by using Nanodrop Spectrophotometer (Thermo Fisher Scientific Inc., India). The 16S-DNA was sent for sequencing (BioLinkk, India). Sequences were matched with previously published bacterial 16SrDNA sequences in the NCBI databases using BLAST (<http://www.ncbi.nlm.gov/BLAST>). Based on the scoring index the most similar sequences were aligned by using Clustal X software. Further phylogenetic tree was built. 16S rDNA sequences have been deposited in Gen Bank.

RESULTS & DISCUSSION:

Isolation by plating: After incubation at 37⁰C for 3-5 days, colonies formed on each plate. The images of colonies of bacterial strain RT7, on plates have been shown in **Fig. 1**. The shape of the colonies was circular with white color and smooth surface. They could grow well on solid medium supplemented with manganese up to the maximum 7 mM concentration after being incubated for 48 hours. It also tolerated lead, nickel and iron up to 3, 1 and 1 mM respectively on solid medium as shown in **Table 1**.

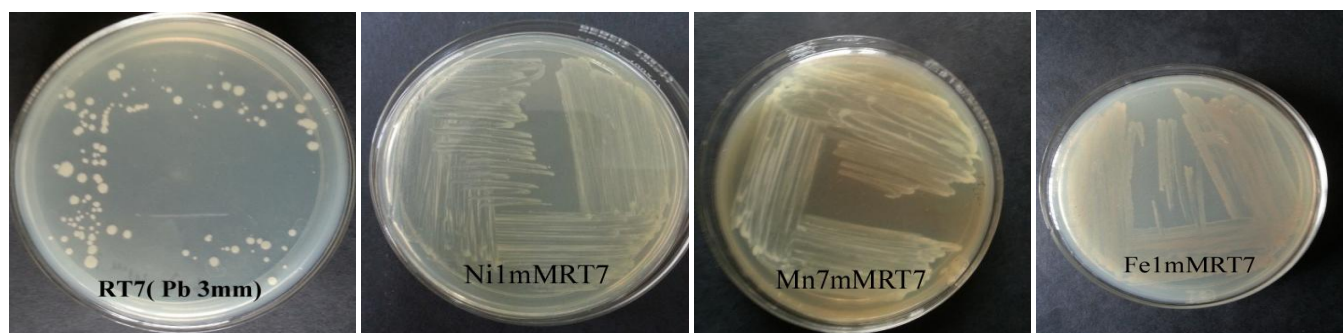


FIG. 1: SHOWING IMAGES OF BACTERIAL STRAINS GROWN IN PRESENCE OF DIFFERENT METAL CONCENTRATION.

TABLE 1: SHOWING MIC OF BACTERIAL STRAIN RT7.

Heavy Metal	MIC (mM)
Manganese (Mn)	7
Lead (Pb)	3
Iron (Fe)	1
Nickel (Ni)	1

Morphological, Physiological and Biochemical characteristics: The morphological, physiological and biochemical characteristics of strain RT7 are shown in **Table 2**. RT7 is a gram –ve bacterium and rod in shape. The strain had a wide growth temperature ranging from 20⁰-40⁰C and the optimal

growth temperature was 37⁰C; the pH range was from 6-9 and the optimal pH was found 7; NaCl tolerance was upto 2%. RT7 was susceptible to following antibiotics: Chloramphenicol, Neomycin, Ciprofloxacin, Amoxicillin, Ampicillin, Chlorotetracycline, Ceftazimide.

TABLE 2: SHOWING BIOCHEMICAL, MORPHOLOGICAL, PHYSIOLOGICAL CHARACTERISTICS AND EFFECT OF ANTIBIOTICS ON THE ISOLATED SOIL BACTERIA

Morphological Tests		Antibiotic	Diameter of Zone of Inhibition (mm)
Colony Configuration	Circular	Chloramphenicol	35 (S)
Cell shape	Rod	Neomycin	21 (S)
Colony Elevation	Flat	Ciprofloxacin	40 (S)
Colony Margin	Entire	Amoxicillin	40 (S)
Colony Surface	Smooth	Ampicillin	24 (S)
Colony Colour	Cream	Kanamycin	0 (R)
Opacity	Opaque	Chlorotetracycline	27 (S)
Gram's reaction	-ve	Ceftazimide	40 (S)
Biochemical Tests		Physiological Tests	
ONPG	+	Growth at 20 ⁰ C Temp.	+
Lysine utilization	-	30 ⁰ C	++
Ornithin utilization	-	37 ⁰ C	+++
Urease detection	-	40 ⁰ C	+
Phenylalanine Deamination	+	50 ⁰ C	-
Nitrate reduction	-	NaCl 1%	+++
H ₂ S production	-	2%	+
Citrate utilization	-	3%	-
Voges proskauer's	-	pH 4	-
Methyl Red	-	5	-
Indole Test	-	6	++
Malonate utilization	-	7	+++
Esculin hydrolysis	-	8	+
Arabinose, Xylose, Adonitol	+	9	+
Rhamnose	-	10	-
Cellobiose, Melibiose, Saccharose,			
Raffinose, Trehalose	+		
Glucose, Lactose	+		
Oxidase Test	+		

Note: (+, scant growth/positive); (++, moderate growth) (+++, abundant growth); (-, no growth/negative); (R-Resistant); (S-Susceptible)

Phylogenetic analysis of 16S rDNA:

The 16S rDNA of bacterial strain RT7 was amplified with primers 27F and 1492R. The PCR amplification products were detected by 0.8% agarose gel electrophoresis in ultraviolet (UV) light. The length of fragment is about 1.5 Kb/1500 bp with a concentration of 38.1 ng/ul (P-1.37) of purified PCR product. Comparative analysis of the sequences with already available database showed that the strain was closer to the genus *Rhizobium*

species. The highest sequence similarity of soil bacteria RT7 was with *Rhizobium halophytocola* (97% similarity to *Rhizobium halophytocola* YC6881). Phylogeny based on Clustal X clearly indicates that RT7 is a strain of *Rhizobium halophytocola*. The 16S rDNA sequences were submitted in the GenBank under the accession number KT327204.1. Constructed phylogenetic tree has been presented in **Fig. 2**.

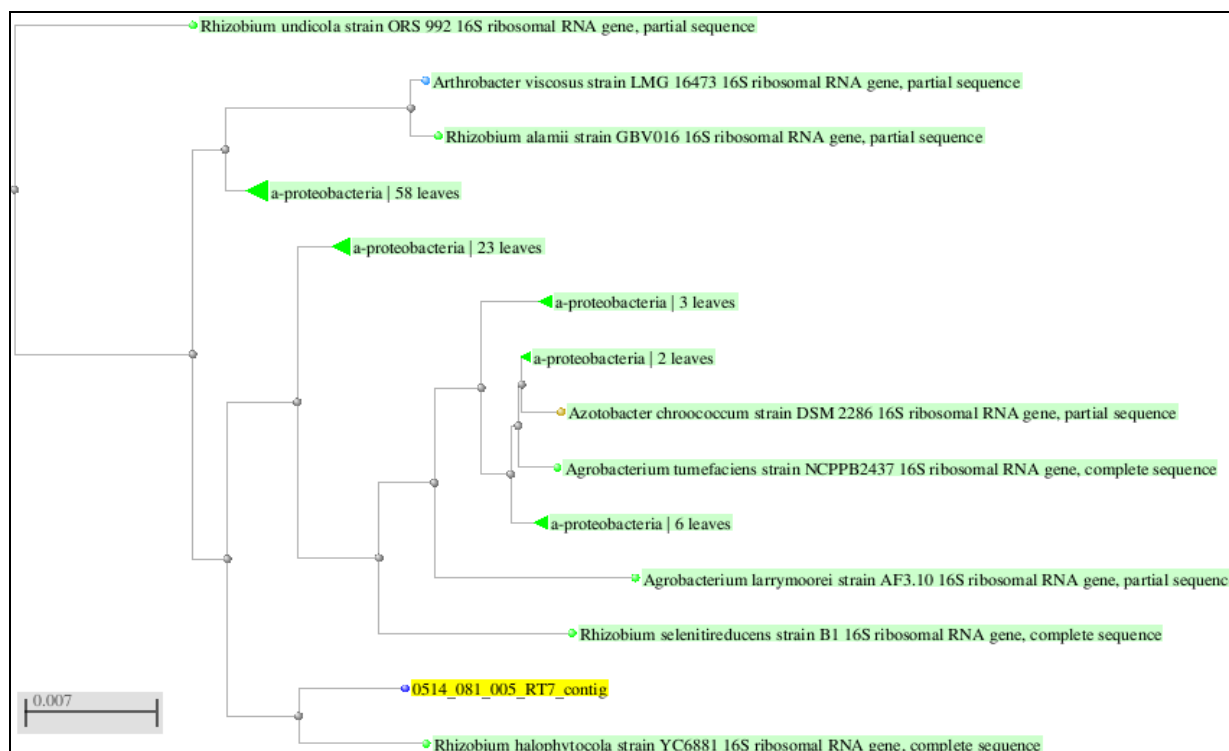


FIG. 2: PHYLOGENETIC TREE CONSTRUCTED FROM 16S rDNA GENE SEQUENCES DETERMINED FROM GENOMIC DNA EXTRACTED FROM BACTERIAL ISOLATE RT7.

In the present study, bacterial strain RT7 has shown resistance against toxic metal manganese up to 7 mM isolated from Industrial soil which was identified by morphological, biochemical and 16S rDNA sequence analysis as *Rhizobium halophytocola*. RT7 has shown multiple tolerance to other heavy metals. In contrast, Mn resistance was observed only up to 6 mM by *Bacillus circulans* which was studied against $MnSO_4$ ²⁴. In this study, soil bacteria was resistant to Pb up to the maximum concentration of 3 mM in Nutrient medium. On the contrary, isolates were resistant against Pb at the concentration of 0.6 mM and 2.5 mM respectively in minimal medium²⁵. Similarly other bacterial strains were resistant to Pb and Cd with MIC 2 mM and 0.5 mM respectively²⁶.

In most of the studies, it has been found that the metal resistance and antibiotic resistance²⁷ are closely associated. RT7 was found resistant to Kanamycin which can be used as an important tool for genetic engineering. RT7 has shown growth at temperature ranging from 20°C to 40°C, on media incorporated upto 2% salt concentration and at pH ranging from 6 to 9. They could be used for bioremediation at different contaminated sites having different temperature, salinity and pH ranges. Their physiological as well as biochemical

characteristics were examined. The utilization of various carbon sources by bacteria was also analyzed which showed their diversity of metabolic patterns.

CONCLUSION: In our study, strain RT7 isolated from the long term heavy metal polluted site near an Iron industry could be considered as "metallophile" as it was found tolerant to heavy metal concentrations well beyond the minimal inhibitory concentration of E.coli based model^{28, 29}. RT7 has shown multiple tolerance to heavy metals. Bacteria developed various resistance mechanisms when they were exposed to environment containing heavy metals especially manganese. This property of multiple heavy metal resistance of RT7 can be beneficial for detoxification and removal of heavy metals from polluted environment.

ACKNOWLEDGEMENT: The authors are grateful to Director of UIET, Kurukshetra University, Kurukshetra, India, for providing instruments and facilities for this research. We would like to thank Council of Scientific and Industrial Research (CSIR), India for their financial support.

REFERENCES:

1. Schwartz C, Gerard E, Perronnet K and Morel J L: Measurement of in situ phytoextraction of zinc by spontaneous metallophytes growing on a former smelter site. *Science of the Total Environment* 2001; 279:215–221.
2. Passariello B, Giuliano V, Quaresima S, Barbaro, Caroli S, Forte G, Garelli G and Iavicoli I: Evaluation of the environmental contamination at an abandoned mining site. *Microchemical Journal* 2002; 73:245–250.
3. Bell FG, Bullock SET, Halbich TFJ and Lindsay P: Environmental impacts associated with an abandoned mine in the Witbank Coalfield, South Africa. *International Journal of Coal Geology* 2001; 45:195–216.
4. Gerber GB, Leonard A and Hantson P: Carcinogenicity, mutagenicity and teratogenicity of manganese compounds. *Critical Reviews in Oncology/Hematology* 2002; 42:25–34.
5. Howe P, Malcolm H and Dobson S: Manganese and Its Compounds, Environmental Aspects, World Health Organization, Geneva 2004; 63.
6. Hookoom M & Puchooa D: Isolation and identification of heavy metals tolerant bacteria from industrial and agricultural areas in Mauritius. *Current Res. in Microbiol. & Biotech.* 2013; 1(3):119-123.
7. Schroeder WH, Dobson M and Kane DM: Toxic trace elements associated with airborne particulate matter: a review. *Journal of the Air Pollution Control Association* 1987; 37:1267-1285.
8. McBride MB: Environmental chemistry of soils. Oxford University Press, Edition 1, 1994; 416.
9. Kabata PA and Pendias H: Trace elements in soils and plants. CRC Press, USA, Edition 3, 2001; 331.
10. Baetty R: The elements: Manganese, Marshal Cavendish Corp., New York 2004; 32.
11. Ryenold CV, Alarcon MA, Lopez H, Serrana D and Valero VP: Total and dialyzable levels of manganese from duplicate meals and influence of other nutrients: Estimation of daily dietary intake. *Food Chemistry* 2008; 109:113-121.
12. Crossgrove J and Zheng GW: Manganese toxicity upon overexposure. *NMR Biomedicine* 2004; 17:544-553.
13. Michalkel B: Manganese speciation using capillary electrophoresis-ICP-mass spectrometry. *Journal of Chromatography* 2004; 1050:69-76.
14. Midwood AJ: Stable Isotope Methods for Estimating the Labile Metal Content of Soils. *Phytoremediation, Methods and Reviews*, Neil Willey, Bristol, University of the West of England 2007; 149-159.
15. Takeda A: Manganese action in brain function: Review. *Brain Research Reviews* 2003; 41:79-87.
16. Habi S and H Daba: Plasmid incidence, antibiotic and metal resistance among *enterobacteriaceae* isolated from Algerian streams. *Pak. J. Biol.* 2009; 12: 1474-1482.
17. Ge HW, Lian MF, Wen FZ, Yun YF, Jian FY and Ming T: Isolation and characterization of the heavy metal resistant bacteria CCNWR533-2 isolated from root nodule of *Lespedeza cuneata* in gold mine tailings in China. *J. Hazard Mater.* 2009; 162: 50-56.
18. Carrasco JA, Armario P, Pajuelo E and Burgos A: Isolation and characterization of symbiotically effective *Rhizobium* resistant to arsenic and heavy metals after the toxic spill at the Aznalcollar pyrite mine. *Soil Biol. Biochem.* 2005; 37:1131-1140.
19. Thakre NA and Shanware AS: Promising Biological Indicator of Heavy Metal Pollution: Bioluminescent Bacterial Strains Isolated and Characterized from Marine Niches of Goa, India. *Indian J. Microbiol.* 2015; 55(3):327-332.
20. Raja E and Selvam GS: Plasmid profile and curing analysis of *Pseudomonas aeruginosa* as metal resistant. *Int. J. Environ. Sci. Tech.* 2009; 6 (2): 259-266.
21. Xie X, Fu J, Wang H and Liu J: Heavy metal resistance by two bacteria strains isolated from a copper mine tailing in China. *African Journal of Biotech.* 2010; 9(26):4056-4066.
22. Shahzad F, Shafee M, Abbas F, Babar S, Tariq MM and Ahmad Z: Isolation and biochemical characterization of *Rhizobium meliloti* from root nodule of Alfalfa (*Medicago sativa*). *The Journal of Animal & Plant Sciences* 2012; 22(2):522-524.
23. Bergey DH and Breed RS: Bergey's Manual of Determinative Bacteriology 1994; 1:416-417.
24. Gayathamma K and Pavani KV: Bioaccumulation of manganese by *Bacillus circulans* from aqueous solutions. *Journal of Environmental Research and Development* 2011; 5(4):933-937.
25. Roane TM: Lead resistance in two isolates from heavy metal contaminated soils. *Microbiol. Ecology* 1999; 37:218-224.
26. Kermanshahi RK, Ghazifard A and Tavakoli A: Identification of bacteria resistant to heavy metals in the soils of Isfahan province. *Iranian Journal of Science & Technology* 2007; 31:7.
27. Verma T, Srinath T, Gadpayle RU, Ramtake PW, Hans RK and Garg SK: Chromate tolerant bacteria isolated from tannery effluent. *Bioresource Technology* 2001; 78:31-35.
28. Nies DH: Microbial heavy metal resistance. *Microbiol. Technol.* 1999; 51:730-750.
29. Nies DH and Silver S: Molecular microbiology of heavy metals. Springer-Verlag, Berlin 1999; 4:407-420.
30. Shahzad F, Shafee M, Abbas F, Babar S, Tariq MM and Ahmad Z: Isolation and biochemical characterization of *Rhizobium meliloti* from root nodule of Alfalfa (*Medicago sativa*). *The Journal of Animal & Plant Sciences* 2012; 22(2):522-524.

How to cite this article:

Gupta R, Kumar T and Mittal A: Isolation, Identification and Characterization of Heavy Metal Resistant Bacteria from Soil of An Iron Industry, Haryana (India). *Int J Pharm Sci Res* 2016; 7(3): 1308-1313. doi: 10.13040/IJPSR.0975-8232.7(3): 1308-1313.

All © 2013 are reserved by International Journal of Pharmaceutical Sciences and Research. This Journal licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License.

This article can be downloaded to **ANDROID OS** based mobile. Scan QR Code using Code/Bar Scanner from your mobile. (Scanners are available on Google Playstore)