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Research Article

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION AND ANTIBIOTIC SUSCEPTIBILITY OF ZINC TOLERANT BACTERIA ISOLATED FROM MINE SOIL OF ZAWAR MINES

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ABSTRACT
Environmental pollution with toxic heavy metals is spreading throughout the world along with the industrial progress. Developing bioremediation processes for soil polluted with such heavy metals is of high importance in the pathway to reduce pollution. For this purpose screening of bacteria having tolerance to heavy metal zinc had been attempted in the present study. A total of 23 zinc tolerant bacteria were isolated from rhizospheric soil of plants growing in tailing dam of Zawar mines. The minimum inhibitory concentration (MIC) of all the 23 isolates against zinc was determined on nutrient agar supplemented with varying concentration of zinc sulphate heptahydrate ranging from 1mg/ml to 31 mg/ml. Two isolates namely HMT16 and HMT7 showed maximum MIC value which was 26 mg/ml and 31mg/ml respectively. They were characterized on the basis of morphological, cultural, biochemical and 16S rRNA sequencing. They were identified as *Pseudomonas aeruginosa* HMT2 and *P. aeruginosa* HMT7. The antibiotic resistance patterns of the two isolates were also studied. Among the fifteen antibiotics used, both the isolates showed resistance for six antibiotics. The fairly high extent of heavy metal tolerance to zinc of these isolates suggests that they can be used in bioremediation of heavy metal contaminated soil.

***Keywords*:** *Pseudomonas Aeruginosa, Zinc Tolerance, Zawar Mines, Antibiotics, MIC*

INTRODUCTION
Heavy metals have been recognized as the deleterious contaminators which have negative effect on the microorganisms of soils. Many metal ions such as zinc, copper etc., are essential as trace elements, but at higher concentrations, they become toxic. Such heavy metals are not easily removable from the environment and are also indestructible, unlike many other pollutants that can be chemically or biologically degraded. Hence, heavy metal constitutes a global environmental hazard. Since metals are increasingly found in microbial habitats due to natural and environmental processes, microbes have evolved several mechanisms to tolerate the presence of heavy metals. Thus, the heavy metal resistance microorganisms have significant role in treatment of the metal contaminated soil.
Zinc is a trace element which is not biologically redox reactive and is thus not used in respiration. However it is important in forming complexes (such as zinc fingers in DNA) and as a component in cellular enzymes (Nies, 1999). Bacterial cells accumulate zinc by a fast, unspecific uptake mechanism (Nies, 1999). Elevated concentrations of Zn exist in soils from management practices including application of sewage sludge or from mining activities, and this may represent a risk to environmental quality and sustainable food production (Li and Christie, 2001)
Pseudomonas species are found ubiquitously in nature and abundantly in water and rhizospheric soils. This genus is found to be tolerant against many heavy metals including zinc. Many workers had reported isolation of bacteria from heavy metal contaminated sites (Bhadra *et al.*, 2007; Raja, 2009; Xie *et al.*, 2010). Malik and Jaiswal (2000) isolated *Pseudomonas* spp. from industrial waste having high level of heavy metals.
Huge amount of zinc is extracted from the sulphide ore found in Zawar mines (Zn-Pb mine) and the waste is dumped in the tailing dam nearby. The residual metal ions (Zn, Pb, Cd, Fe etc.) present in the tailings may cause environmental pollution which may affect the vegetation as well as the population residing in

vicinity of these mines. Therefore, there is a strong need of bioremediation of this polluted area. The aim of the present study was to isolate and characterize zinc tolerant bacteria from Zawar mines which can be used for bioremediation of zinc contaminated sites.

MATERIALS AND METHODS

Study Area

The area in the present study is Zawar mines situated about 45 km away from Udaipur, Rajasthan, India located at 24° 22´north and 73°43´ east.

Sampling

Soil samples were collected from rhizosphere of the plants growing in tailing dam of Zawar mines, Udaipur (India). The samples were kept in sterile zipper plastic bags in refrigerated conditions till transferred to laboratory for further studies.

Isolation of Zinc Tolerant Bacteria

Zinc tolerant bacteria were isolated on nutrient agar supplemented with 1mg/ml of zinc sulphate heptahydrate (Hi-Media Laboratories Pvt. Ltd., India) by standard pour plate method. Plates were incubated at 37°C for 48 h.

Determination of Minimum Inhibitory Concentration (MIC)

For determination of MIC, preliminary isolated zinc tolerant bacteria were grown on nutrient agar plates supplemented with various concentrations of zinc sulphate heptahydrate ($FeSO_4.7H_2O$) ranging from 1mg/ml to 31mg/ml with a difference of 1mg/ml $FeSO_4.7H_2O$. The petri plates were inoculated with the test organism and incubated at 37°C for 48 h.

Characterization of the Isolates

The isolates were characterized on the basis of cultural, morphological, biochemical and molecular analysis. Cultural characterization was based on the colony characteristics while the morphological studies were done by Gram staining. Biochemical characterization was based on showing growth at 42°C, catalase activity, oxidase activity, carbohydrate fermentation, oxidation fermentation test (OF), nitrate reduction, citrate utilization, starch hydrolysis, gelatin hydrolysis, arginine hydrolysis and esculin hydrolysis. The isolates were further tested on differential media like cetrimide agar and King's A and B media.

The molecular characterization of the isolates was done on the basis of 16S rRNA partial sequence analysis. The genomic DNA was extracted by Pospeich and Neumann's method (1995). PCR (Genei TC-3000) amplification reaction was carried out using the genomic DNA of the isolate and bacterial universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'CGGTTACCTTGTTACGACTT-3'). The amplification products were submitted to Bangalore Genei Pvt. Ltd., Bangalore, India for sequencing. The sequenced genomes of the isolates were compared with the available standard sequences of bacterial lineages in the NCBI Genbank using nBLAST.

Antibiotic Resistance

The isolates were tested for antibiotic sensitivity and resistance according to the Kirby-Bauer disc diffusion method (Bauer *et al.*, 1966). A total of fifteen antibiotics namely Ampicillin, Amikacin, Ciprofloxacin, Cefixime, Chloremphenicol, Erythromycin, Gentamicin, Kanamycin, Penicillin, Polymyxin, Rifampacin, Streptomycin, Tetracycline, Trimethoprim and Vancomycin were used. After incubation, the organisms were classified as sensitive or resistant to an antibiotic according to the diameter of inhibition zone given in standard antibiotic disc chart. All the experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Result

A total of 12 soil samples were collected from rhizospheric soil of plants growing in tailing dam of Zawar mines, Udaipur (India). A total of 23 indigenous bacterial strains were recovered on nutrient agar supplemented with 1mg/ml concentration of zinc sulphate heptahydrate by standard pour plate method.

All the 23 isolates were subjected to nutrient agar supplemented with varying concentrations of zinc sulphate heptahydrate for determination of MIC. The MIC values for the 23 isolates ranged between 2mg/ml to 31mg/ml. Among these isolates, two isolates HMT16 and HMT7 showed comparatively high MIC values 26 and 31mg/ml respectively (Table 1).

Therefore, isolate HMT16 and HMT7 were selected for further characterization on the basis of their cultural, morphological, biochemical characteristics and 16S rRNA gene sequencing. Both the isolates were gram- negative and rod shaped. The colonies of the isolates HMT7 and HMT16 appeared red-brown, undulate and flat with irregular edges.

Table 1: MIC of Zinc Tolerant Isolates

S. No.	Name of the Isolate	MIC (mg/ml)
1.	HMT1	12
2.	HMT2	13
3.	HMT3	15
4.	HMT 4	14
5.	HMT5	16
6.	HMT6	11
7	HMT7	31
8	HMT8	2
9	HMT9	4
10	HMT10	2
11	HMT11	21
12	HMT12	10
13	HMT13	11
14	HMT14	9
15	HMT15	9
16	HMT16	26
17	HMT17	8
18	HMT18	7
19	HMT19	18
20	HMT20	10
21	HMT21	7
22	HMT22	14
23	HMT23	3

The isolates gave well defined colonies at 42°C.The isolate gave positive results for catalase and oxidase activity. The isolates were found to be non-fermentative as they do not ferment any of the sugars. Both the isolates showed positive reaction for oxidation and fermentation test. Both of them reduced nitrate, utilized citrate and hydrolysed gelatin and arginine. Both the isolates gave negative results for starch and esculin hydrolysis. Both the isolates were able to grow on cetrimide agar, gave green colour on King's A and fluorescence on King's B media (Table 2).

On the basis of the sequence similarity of the partial 16S rRNA sequence of HMT7 and 16 with available standard sequences of bacterial lineages in NCBI Genbank reference strains, the isolates were identified as *Pseudomonas aeruginosa* HMT7 and *P. aeruginosa* HMT16. Both the isolates showed resistance to six antibiotics namely ampicillin, cefixime, erythromycin, penicillin, trimethoprim and vancomycin (Table 3) out of fifteen used in the study.

Table 2: Biochemical Characterization for the Isolates HMT7 and HMT16

S. No.	Biochemical Test	Isolate HMT7	Isolate HMT16
1.	Growth at 42 °C	+	+
2.	Catalase acitivity	+	+
3.	Oxidase acitivity	+	+
4.	Carbohydrate fermentation (glucose, sucrose, rhamnose, galactose, maltose, mannose, inositol, fructose, cellibiose, lactose)	-	-
5.	Oxidation/fermentation test (using H&L media)	+	+
6.	Nitrate reduction	+	+
7.	Citrate utilization	+	+
8.	Gelatin hydrolysis	+	+
9.	Arginine hydrolysis	+	+
10.	Starch hydrolysis	-	-
11.	Esculin hydrolysis	-	-
12.	Growth on cetrimide agar	+	+
13.	Growth on King's A media (Pyocyanin)	+	+
14.	Growth on King's B media (Florescent)	+	+

(+ positive for reaction ; - negative for reaction)

Table 3: Antibiotic Resistance Pattern of the Two Isolates HMT7 and HMT16

S. No.	Antibiotic	*Pseudomonas Aeruginosa* HMT7	*Pseudomonas Aeruginosa* HMT16
1	Ampicillin	R	R
2	Amikacin	S	S
3	Ciprofloxacin	S	S
4	Cefixime	R	R
5	Chloremphenicol	S	S
6	Erythromycin	R	R
7	Gentamicin	S	S
8	Kanamycin	S	S
9	Penicillin	R	R
10	Polymyxin	S	S
11	Rifampacin	S	S
12	Streptomycin	S	S
13	Tetracycline	S	S
14	Trimethoprim	R	R
15	Vancomycin	R	R

Discussion

The sites that are associated with mining of non-ferrous metals (e.g., Cu, Zn, Pb, or Ag) are characterized by high concentrations of heavy metals in spoil (Smith and Giller, 1992; Singh and Steinnes, 1994). The microbial communities residing the tailing dams of mines are important for their ability to reduce the

toxic effect of heavy metals. The study area in the present study is tailing dam of Zawar mines Udaipur, India. Zawar group of mines is a lead-zinc ore processing plant with a capacity of processing 2000 tons of ore per day. The commonly used ores Mochia and Balaria are composed of different amount of Zn, Pb and Fe ranging from 1 to 7%. The mining waste containing high concentrations of heavy metals such as zinc, iron, lead etc. are dumped in the tailing dam after extraction. Dumping of mining wastes mounts pressure on the ecosystem and consequently causes health hazards to plants animals and humans (Karelova *et al*., 2011).

The aim of the present study was to isolate and characterize potential zinc tolerant bacterial isolates that can be used in bioremediation of polluted soil. In metal-polluted habitats, the frequency of tolerant bacteria increases with an increase of heavy metal concentrations in such habitats (Kuunito *et al*., 2001). In the present study, a total of 23 zinc tolerant bacteria were recovered from 12 rhizospheric soil samples collected from Zawar mines.

The morphological characterization of all the 23 isolates revealed that 22 isolates were gram-negative and only one isolate was gram-positive in nature. Similar finding where gram-negative bacteria are more frequently found to be heavy metal tolerant as compared to gram-positive bacteria was reported previously by several workers (Ellis *et al.,* 2003, Chovanova *et al*., 2004; Karelova *et al*., 2011). The gram-negative bacteria are considered to be more metal tolerant as compared to gram-positive bacteria as two layers of cell membrane and large amount of lipid is found in gram-negative bacterial cell wall. This lipid binds the excessive metal ions enabling them to resist it and grow at higher metal concentration than gram positive bacteria.

All the 23 isolates showed tolerance against zinc with MICs ranging from 2mg/ml to 31mg/ml. A direct comparison of these MIC estimates with those reported by other authors is not conclusive, as the difference in the metal binding capacities of the media used could result in discrepancies in the MIC results. In the present study, more than 25% of the isolated bacteria were found to be tolerant to a high concentration of zinc i.e., above 15mg/ml.

The two isolates *Pseudomonas aeruginosa* HMT7 and P. *aeruginosa* HMT16 showed even better tolerance to heavy metal zinc with MIC 31 mg/ml (107mM of Zn) and 26mg/ml (90mM of Zn) respectively. These results were found to be much better than that of Lee *et al.* (2001) where *P. aeruginosa* strain 06909 exhibited MIC of 11.5 mM zinc, that of Waertz and Mergeay (1997) where, *Alcaligenes eutropus* was reported to exhibit MIC of 20mM zinc and that of Xie *et al* (2010) who reported 35mM MIC against zinc of isolate *Sphingomonas* spp. strain DX-T3-03. Sometimes few contradictory results of isolates exhibiting much higher MIC value of 1.2M for zinc was also reported by Misra *et al.,* (2012).

The mechanisms underlying the toxic effects of chemicals may involve interactions with cell surface receptors, disruption of cell membrane functions and chemical reactions with cellular components or inhibition/competition of enzyme systems (Mariscal *et al.,* 2003).

The high tolerance ability showed by the isolates in the present study may be due to any of the above mentioned mechanisms.

The two isolates HMT7 and HMT16 were identified as *Pseudomonas aeruginosa* on the basis of biochemical and molecular characterization. The two strains *Pseudomonas aeruginosa* HMT7 and HMT16 were found to be resistant against six antibiotics out of fifteen used in the study. The antibiotic resistance and metal tolerance of the two isolates *Pseudomonas aeruginosa* HMT7 and P. *aeruginosa* HMT16 were found to be highly coupled. In previous studies, metal resistance has been reported to hold an association with antibiotic resistance (Verma *et al*., 2001).

Under conditions of metal stress, metal and antibiotic resistance in microorganisms possibly helps them to adopt faster by the spread of resistant factors than by mutation and natural selection (Silver and Misra, 1988).

The application of metal-resistant bacteria for bioremediation offers attractive perspectives (Mergeay *et al*., 2003). The indigenous strains *Pseudomonas aeruginosa* HMT7 and *P. aeruginosa* HMT16 obtained from the mine tailing in this study, owing to their excellent metal tolerance and multiple drug resistant

bacteria, may prove to be good candidates for tailing bioremediation and removal of zinc from zinc dumping sites.

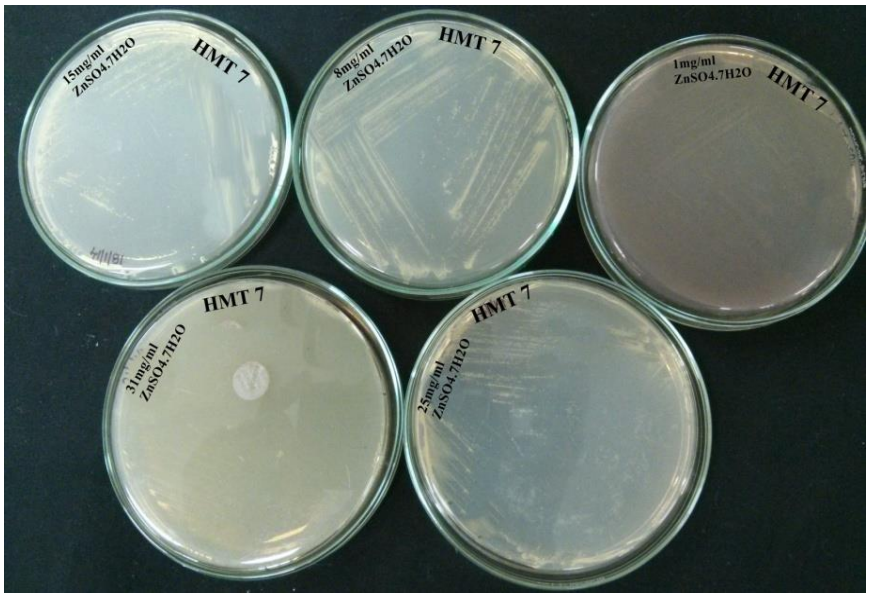

Figure 1: Minimum Inhibitory Concentration of *Pseudomonas Aeruginosa* HMT7 against Zinc on Nutrient Agar Supplemented with Varying Concentrations of Zinc Sulphate Heptahydrate

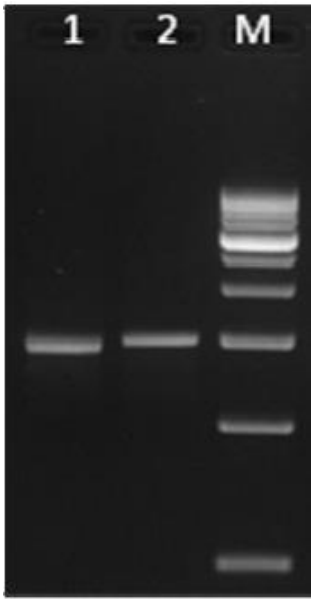

Figure 2: PCR Based Identification of Zinc Tolerant Bacteria using Primer Pair
Lane 1: PCR Amplification of HMT 7
Lane 2: PCR Amplification of HMT16
Lane M: Step Up TM 500bp DNA Ladder

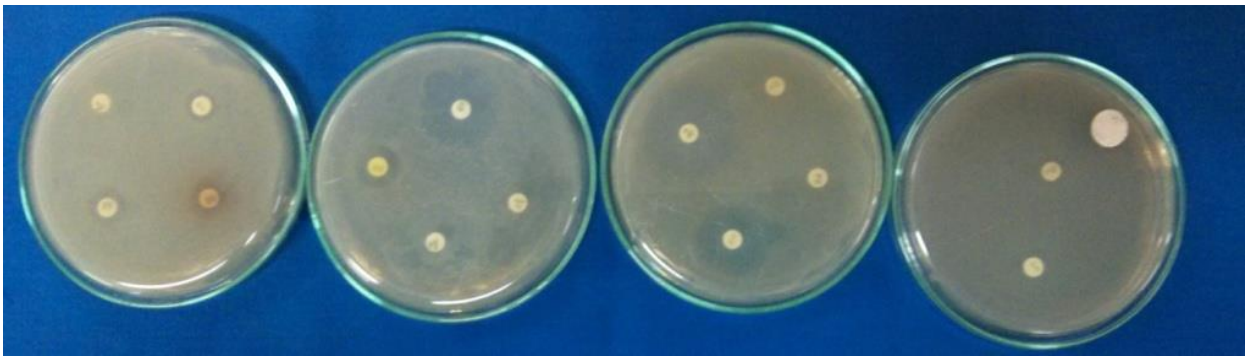

Figure 3: Antibiotic Resistance Pattern of *Pseudomonas Aeruginosa* HMT7 on Nutrient Agar

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