Lead Tolerant *Chryseobacterium indologenes* HMT 47 showing Plant Growth Promotion in Maize

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Abstract

The study of metal-tolerant bacteria is important for transformation of toxic metal ions into less toxic compounds and bioremediation of contaminated environments. The present investigation was implemented to search for high efficient bacterial strain in bio-remediating the toxic influence of lead nitrate (PbN_2O_6) on maize plantlets grown in soil supplemented with lead nitrate. Seventeen strains were isolated on nutrient agar supplemented with 0.1mM lead nitrate from the rhizosphere of different plants (Tridax, Calotropis and Acacia) growing in polluted soil of tailing dam in Zawar, Udaipur, India. The minimal inhibitory concentration of lead tolerant bacterial isolates ranged from 200 to 1000 µg/ml. Isolate HMT 47 showed highest MIC value (1000 µg/ml) and was further characterized by morphological, cultural, biochemical and molecular characterization.

It was identified as Chryseobacterium indologenes HMT 47. The bioaccumulation of Pb by the strain was evidenced by transmission electron microscopy. A pot experiment under Pb stress conditions was performed using Zea mays L. as a test crop. Pb toxicity reduced various plant growth parameters; however, bioremediation of Pb contaminated soil with Chryseobacterium indolegenes HMT 47 significantly increased maize plantlet shoot, root length, fresh and dry weight of shoot and root compared to the uninoculated treatments.

Keywords: *Chryseobacterium indolegenes* HMT 47, lead tolerance, bioremediation, bioaccumulation, plant growth parameters.

Introduction

Some heavy metals such as zinc (Zn), iron (Fe), cadmium (Cd), copper (Cu) are recognized as trace elements that are essential for metabolic activity. On the other hand, excess intake of these elements leady to toxicity. Besides these, lead is also considered as an environmental pollutant that is found in soil, water and air and is hazardous waste and highly toxic to human, animals, plants and microbes[17]**.** Plant growth is negatively affected by the heavy metal stress as a consequence the plant cells may die[1,18,22]. The pollutants enter the environment from industrial activities such as production of batteries, pigments, metal smelting and manufacture of lead arsenate insecticides or through natural processes like soil erosion, volcanic emission[8,26]. The risk of lead poisoning through the food chain rises as the soil lead level rises above the concentration of 300 ppm[6].

Despite the high toxicity of lead, some microorganisms have developed various mechanisms that enable them to withstand toxicity by binding of lead ions by cellular wall components, exopolysaccharides and metallothioneins, chelation by siderophores, biosorption, precipitation as oxides and phosphates and cellular exclusion by efflux systems[13,22,26]. To date, a variety of lead-tolerant bacteria, including *Bacillus megaterium, Bacillus cereus, Pseudomonas marginalis, Pseudomonas vesicularis and Streptomyces* sp., have been isolated from lead-contaminated sites[4,12,15,16,25].

Several workers have reported use of metal-tolerant/resistant bacteria from contaminated sites that could be used for biosorption, bioprecipitation, chelation for biomineralization of toxic metals in mine tailings and the establishment of bioremediation techniques[3,9,14,20]. Metal tolerant bacteria surviving in these habitats could be explored for their potential application in restoration of contaminated sites. The lead-zinc ore processing plant of Zawar group of mines (Udaipur district) is processing large amount ores. The commonly used ores from Mochia and Balariamines of Zawarare composed of different amounts of lead, zinc and iron ranging from 1 to 7%.

Significant quantities of cadmium and silver are also present in them. After processing of different ores, large amount of waste (90%) is produced which is dumped in the tailing dams. The waste contains 0.05-0.1 % lead, 0.16-0.3% zinc, 3-5% iron, 3-4% sulphur, 13-14% calcium, 8-9% magnesium and 31-33% acid insolubles. The finer particles from the tailing dams are carried away by the wind during the dry season and the atmosphere becomes polluted with dust. The waste present in the tailing dams is continuously increasing the heavy metal (Zn, Pb, Cd, Fe) content of the soil in nearby area resulting in barren lands that is expanding nearby. Considering the above-mentioned facts, the present investigation was designed to study the isolation and identification of lead tolerant bacteria from polluted soil of tailing dam of Zawar, Udaipur for their possible use in bioremediation.

Material and Methods

Soil sampling: Soil samples were collected from rhizospere of *Tridax, Calotropis and Acacia* plants growing in tailing dam of zinc smelter situated at Zawar (24° 21' N; 73° 41'E)

Udaipur (India). Collection of these samples was done in sterilized zipper polythenes.

Isolation and analysis of lead tolerance in bacteria: The lead tolerant bacteria were isolated on nutrient agar supplemented with 0.1mM of lead nitrate (PbN_2O_6). To test bacterial tolerance to lead ions (Pb^{2+}) in solid media, the minimum inhibitory concentration (MIC) was determined. Briefly, the bacterial isolates were inoculated onto nutrient agar plates supplemented with increasing concentrations of lead nitrate 100 µg/ml to 1200 µg/ml with a difference of 100 µg/ml and incubated at 37 °C for 24 to 48 h to obtain visible bacterial colonies. The lowest concentration of metal salt that prevented growth on the plates, was recorded as the MIC[19].

Phenotypic and molecular characterization of lead-tolerant isolates: Morphological and biochemical characterizations of lead tolerant strains were based on colony characteristics, Gram staining, Scanning electron microscopic analysis and biochemical properties such as catalase test, oxidase test, oxidation fermentation (o/f) test, nitrate reduction, citrate utilization, gelatin hydrolysis, arginine hydrolysis and starch hydrolysis. For carbohydrate, fermentation test was performed using different sugar discs namely dextrose, sucrose, galactose, maltose, mannose, inositol, fructose, cellibiose, lactose and rhamnose. The identifcation was established according to the Bergey's Manual of Determinative Bacteriology[11]. Molecular identifcation of the isolate was done using 16S rDNA amplifcation and sequencing.

The universal primers for 16S rDNA viz. 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'TACGGYTACCTTGTTACGACTT-3') designed by Weisburg et al[29] based on 16S rRNA gene sequence of bacteria were used to amplify the genomic DNA. The amplified products were submitted to Bangalore Genei Pvt. Ltd., Bangalore (India) for sequencing. The partial sequences of 16S rRNA were compared with available standard sequences of bacterial lineages in the NCBI Genbank using Basic local alignment search tool (BLASTn) to identify the nearest taxa.

TEM analysis for accumulation of lead: The isolate was inoculated in nutrient broth supplemented with appropriate concentration of lead. Incubation was done at 37ºC for 48 h. The nutrient broth containing growth was then centrifuged at 3000 rpm for 15 min. The pellet of bacterial cells was resuspended in MQ water in microfuge tube and then was submitted to SICART, Vallabh Vidhyanagar, Anand (Gujarat) for Transmission electron microscopy (TEM) analysis to detect bioaccumulation of lead.

Plant growth promotion experiment on maize by lead tolerant isolate under lead stress: To determine the effect of Pb on maize seedlings with and without the treatment of lead tolerant isolate, plant growth promotion experiment in complete randomized design (CRD) with three treatments was conducted. The treatments are as follows: T1 (control, uncontaminated soil), T2 (500 mg Pb/kg amended soil), T3 (500 mg Pb/kg amended soil +bacterial isolate. The pots were filled with sterile soil (1.5 Kg/pot) and left for 2 weeks for metal stabilization. Seeds of *Zea mays* var. Godrej Himadri were surface-sterilized by immersing them in 0.1% sodium hypochlorite solution for 10 min and then washed three times with double distilled water.

Surface sterilized seeds were then soaked in bacterial suspension (OD 0.45 at 620 nm), approximately 30 min prior to planting, while for T1 and T2 treatment seeds were soaked in sterile biological saline. The experiment was conducted in triplicate and 5 seeds were sown in each pot. Seedling emergence was recorded daily. The potted plants were cultivated with open-field conditions. After 45 days, the plants were carefully removed from the pots and the root surface was thoroughly cleaned with distilled water and blot dried. Different growth parameters like average shoot and root length, fresh and dry weight were analyzed. Stress tolerance indices for different growth parameters were calculated[2].

Statistical analysis: All the experiments were conducted in triplicate and the data is presented as mean± standard deviation. Statistical analysis was carried out using a statistical package GraphPad prism. Comparison between mean values in different treatments was carried through the Student test (t-test) with confidence levels of 5% or $P \leq 0.05$ being considered significant.

Results

Isolation and MIC of lead tolerant bacteria: 17 isolates were recovered during the isolation procedure on nutrient agar supplemented with 0.1 mM of lead nitrate (PbN_2O_6) from rhizosphere of plants growing in tailing dam of zinc smelter situated at Zawar. All the isolates showed moderate to high tolerance to lead. MIC for isolates ranged between 200 µg/ml to 1000 µg/ml. The highest MIC 1000 µg/ml exhibited isolate HMT 47 which was selected to carry out further studies.

Characterization of lead tolerant isolate: The colonies of isolate HMT 47 were medium sized, light-yellow colored, circular, entire and raised. It was Gram-negative rod shaped organism. Scanning electron micrograph of isolate HMT 47 is presented in fig. 1. The isolate gave positive reaction for catalase test, oxidase test, nitrate reduction, citrate utilization, gelatin hydrolysis and starch hydrolysis which showed negative reaction for arginine hydrolysis. Oxidation fermentation reaction was alkaline. Isolate HMT 7 gave negative reaction for the fermentation of ten common sugars namely dextrose, sucrose, galactose, maltose, mannose, inositol, fructose, cellibiose, lactose and rhamnose which suggested that the isolate was non-fermentative. The 16S rRNA sequence of the isolated DNA was amplified using universal primers, 27 f and 1492 r in PCR and an amplified

product of 1.5Kb was obtained (Fig. 2). Isolate HMT 47 showed 98% sequence similarity to *Chryseobacterium indologenes* strain S8, therefore it was identified and named as *Chryseobacterium indologenes* HMT 47.

Bioaccumulation of lead by *Chryseobacterium indologenes* HMT 47: TEM analysis revealed the electron dense grains in the cytosol and towards the cell envelope which confirmed the bioaccumulation of lead by the cells of lead tolerant *Chryseobacterium indologenes* HMT 47. The transmission electron micrograph is presented in fig. 3.

Promotion of maize plant growth by *Chryseobacterium indologenes* HMT 47 under stress: The alleviation of phytotoxicity of lead by *Chryseobacterium indologenes* HMT 47 on the growth of maize (*Zea mays* var. Godrej Himadri) was studied. The study was conducted based on three treatments as T0 (control, uncontaminated soil), TZ1 (1mg Pb/Kg amended soil), TZ2 (1mg Pb/Kg amended soil inoculated with *Chryseobacterium indologenes* HMT 47). The pot experiment data were recorded under Pb stress condition after 45 days of germination and summarized in table 1. In uninoculated control TZ1 (1mg Pb/Kg amended soil), the overall plant growth was significantly decreased due to Pb stress compared to control plantlets T0 (without any stress). The stress tolerance indices for different growth parameters shoot length, root length, shoot and root dry and fresh weight observed were 0.38, 0.39, 0.26, 0.54, 0.60 and 0.53 respectively. Higher plant growth was observed in maize plantlets treated with *Chryseobacterium indologenes* HMT 47 (TZ2) compared to uninoculated control. The stress tolerance indices for different growth parameters shoot length, root length, shoot and root dry and fresh weight observed were 1.51, 1.75, 1.58, 0.73, 1.27 and 1.11 respectively. The lead tolerant *Chryseobacterium indologenes* HMT 47 significantly influenced the observed parameters and contributed to plant growth under heavy metal stress conditions.

Discussion

Lead contamination is environmental hazard that leads to pollute environment and affects human health adversely causing serious threats to the society. In recent years, researchers have started to explore the use of bacteria for bioremediation of metal-contaminated sites and providing nanotechnological based ecofriendly solution to the problem. The present study was conducted to isolate and screen lead tolerant bacteria and their use in plant growth promotion under lead stress. In earlier studies, species of *Staphylococcus* and *Bacillus* genus have been frequently reported as lead-tolerant bacteria[5,12,15,16,20,28]. The present study revealed a novel Gram-negative bacterium *Chryseobacterium indologenes* HMT 47 having significantly high lead tolerance ability. This species has not been yet reported for its lead tolerance potential.

According to Ferris et al[7], the gram-negative bacteria are considered to be more metal tolerant as compared to Gram-positive bacteria as the two layers of cell membrane and large amount of lipid are found in Gram-negative bacterial cell wall. This lipid binds the excessive heavy metal (zinc, lead) ions enabling them to resist it and grow at higher metal concentration than Gram-positive bacteria. This may be possible reason for finding *Chryseobacterium indologenes* HMT 47 in the present study which efficiently tolerated lead.

The present finding was supported by the earlier workers[23,24] and also confirmed about the ability of alleviating heavy metal stress by microbes. The improved growth of maize plantlet under Pb stress conditions in the present study was due to the reduced accumulation and uptake of Pb in the maize plantlet.

Table 1
The effect of *Chryseobacterium indologenes* HMT 47 on growth and biomass of maize seedling under Pb stress conditions (1mg Pb/Kg amended soil). Data are recorded after 45 days of germination; data is presented as means of 3 replicates ± SD (standard deviation).

Treatment	Shoot length (cm)	Shoot length Stress Tolerance Index	Root length (cm)	Root length Stress Tolerance Index	Shoot Fresh weight (g)	Shoot Fresh weight Stress Tolerance Index	Root Fresh weight (g)	Root Fresh weight Stress Tolerance Index	Shoot Dry weight (g)	Shoot Dry weight Stress Tolerance Index	Root Dry weight (g)	Root Dry weight Stress Tolerance Index
T0	38.7 ± 1.49	-	17.4 ±0.82	-	2.67 ±0.21	-	1.71 ±0.04	-	0.25 ±0.01	-	0.17 ±0.01	-
TZ1	14.6 ± 1.02	0.38	6.8 ±0.26	0.39	0.69 ±0.11	0.26	0.92 ±0.01	0.54	0.15 ±0.01	0.60	0.09 ±0.01	0.53
TZ2	22.08 ± 1.63	1.51	11.9 ± 0.64	1.75	1.09 ±0.30	1.58	0.67 ±0.02	0.73	0.19 ±0.01	1.27	0.10 ±0.01	1.11

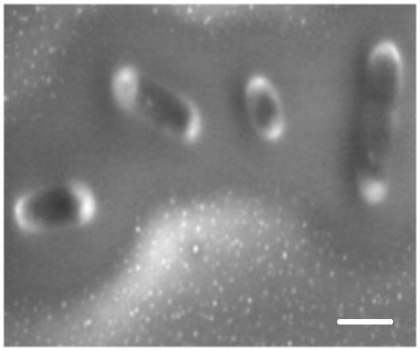

Figure 1: Scanning electron microscopic image of lead tolerant isolate HMT 47 showing rod shaped cells (Bar=1.5 µm)

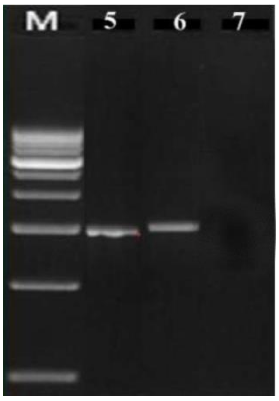

Figure 2: PCR amplicons of 16S rRNA genes in lead tolerant bacterial isolate. Lane M: StepUpTM 500bp DNA ladder, Lane 5: Isolate HMT 47, Lane 6: positive control Lane 7: negative control

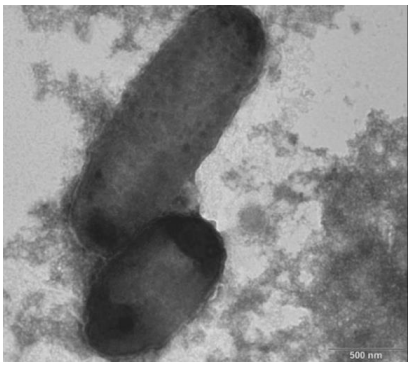

Figure 3: TEM analysis of *Chryseobacterium indologenes* HMT 47 cells grown for 24h in nutrient broth with lead nitrate (900 µg/ml) showing bioaccumulation of lead within the cell (Bar=500 nm)

Inoculation of *Chryseobacterium indologenes* HMT 47 led to the reduced Pb toxicity. This could happen due to the reduced bioavailability and bioaccumulation of Pb by the bacterial isolate. Similar findings of metal tolerant rhizobacteria inoculation were found very effective upon inoculation in different crops and also conferred metal tolerance[10].

Conclusion

The overall study shows that *Chryseobacterium indologenes* HMT 47 has high capacity to tolerate Pb. The application of bacterial isolate significantly improved the growth in maize plantlet under Pb stress. Bioaccumulation of heavy metals inside the strain minimized the heavy metal bioavailability in the rhizosphere. This strain could help in the bioremediation of heavy metal contaminated agricultural field for better growth and yield of the crop.

The genetic capacity of the strain could be exploited for the effective remediation of heavy metal polluted sites. The inoculation of *Chryseobacterium indologenes* HMT 47 as a biotechnological tool for reducing the Pb toxicity will help in understanding various adaptive processes which are poorly understood till date.

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