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Isolation and Characterization of lead (PB) tolerant bacterial isolate and its potential for bioremediation of contaminated soil

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Abstract
High concentrations of non-essential heavy metals (arsenic, cadmium, and lead) in soils represent a threat to the environment, food safety, human and animal health. Microbial bioremediation has emerged as a promising strategy to reduce the concentration of heavy metals in the environment due to the demonstrated ability of microorganisms, especially bacteria, to sequester and transform these compounds. Present study deals with isolation and characterization of lead resistant bacteria isolated from heavy metal contaminated soil followed by the study of lead tolerance and bioremediation. Minimum inhibitory concentration (MIC) was shown against the lead acetate at different levels. The higher MIC was observed at a concentration of 1200µg/ml of lead acetate. The present study reveals that the selected lead resistant bacterial isolate A4 shows high lead resistance and might be possible to use in bioremediation.

Keywords: Heavy metals, lead, bioremediation, bacteria, soil contamination

1. Introduction
Heavy metals can accumulate in soils due to emissions from rapidly expanding industrial areas, high-metal waste disposal, leaded gasoline and paints, coal combustion residues, petrochemical spillage, animal manures, sewage sludge, pesticides, wastewater irrigation, and atmospheric deposition [1, 2]. Heavy metals are an ill-defined set of inorganic chemicals, with lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), and nickel (Ni) being the most frequently discovered at contaminated locations [3]. Soils are the primary sink for heavy metals released into the environment by the above-mentioned anthropogenic activities, and unlike organic contaminants, which are oxidized to carbon (IV) oxide by microbial action, most metals do not undergo microbial or chemical degradation [4], and their total concentration in soils persists for a longer time [5]. However, changes in their chemical forms (speciation) and bioavailability may occur. The biodegradation of organic pollutants can be significantly hindered by the presence of toxic metals in soil [6]. Through direct ingestion or interaction with contaminated soil, the food chain, and drinking contaminated ground water, heavy metal contamination of soil may pose risks and hazards to the ecosystem and humans [7, 8, 9]. In vertebrates and invertebrates, heavy metal poisoning causes oxidative stress and disrupts protein folding and physiological function [10, 11]. While some heavy metals, such as Cu, Ni, Cr, and Zn, are required as micronutrients for organism's growth and development, others, such as Cd, Hg, and Pb, have no physiological action for cells. Furthermore, increased amounts of these hazardous substances above threshold levels have a significant negative impact on microbial communities and their critical functions [12].
Lead is a well-known xenobiotic heavy metal that is employed in a variety of sectors, including automobiles, paint, ceramics, and plastics [13]. Humans have become vulnerable targets for its exposure as a result of its extensive use. There has never been a level of lead that has been shown to be safe or helpful to living beings. Many organs, including the neurological system, renal system, hematopoietic system, reproductive system, and cardiovascular system, are affected by lead exposure. When it comes to lead-induced poisoning, the nervous system is the most vulnerable target [14, 15]. Lead poisoning can result in convulsions, a lack of coordination, delirium, and paralysis, all of which can be fatal. The production of oxidative stress, which occurs as a result of an imbalance between pro-oxidant and anti-oxidant ratios, is the main mechanism of lead-induced damage. Protein oxidation, lipid peroxidation, and nucleic acid peroxidation occur as a result of this imbalance, putting a cell vulnerable to cell

death [16]. The other way of action of lead poisoning is the ionic mechanism. Lead mimics and replaces other monovalent and bivalent ions such as Na^+, Ca^{2+}, and Mg^{2+} in this process, obstructing various biological functions such as intracellular signaling, cell adhesion, protein folding, and ionic transportation [15]. Many natural anti-oxidants, such as vitamins (B, C, and E), flavonoids, and herbal flavonoids, have been used to treat lead poisoning [17].

For the remediation of heavy metals present in water and soil, conventional procedures such as ion exchange, chemical precipitation, reverse osmosis, bio-piles, bio-slurries, and landfilling are used [18]. They do, however, have the primary disadvantage of being expensive due to the need for specialized infrastructure. Furthermore, they produce hazardous sludge that pollutes the environment and may not completely remove the metals [19]. Bioremediation is an environmentally beneficial and long-term technique that can be far more successful and efficient at removing heavy metals from various parts of the environment. The goal of this technique is to clean up the environment while preserving the natural biological processes that occur in it [20]. Bioremediation, according to Ayangbenro and Babalola [21], is a procedure that uses microbes, green plants, or enzymes to clean polluted places and restore their health. In comparison to traditional methods, this methodology is highly recommended since it produces significantly superior outcomes by utilizing low-cost and cost-effective inputs. As a result, bioremediation can be viewed as cost-effective, environment friendly, and more prominent solution to the difficulties caused by the use of transition metals [22]. *Serratia marcescens* and *Proteus mirabilis* have been identified as bacteria that can tolerate lead [23]. *Bacillus strains* resist to lead acetate at 1200mg/L and shows slight changes in cell shape as well as a reduction in cell size. *Proteus sp*. strains have been reported to be resistant to lead acetate at 1600 mg/L [24]. The purpose of this paper is to isolate indigenous potential microbial strains for heavy metal degradation and to evaluate the degradation capabilities of the isolated strain.

2. Material and Methods

2.1 Collection of soil sample

Soil samples were collected from disposal sites of industrial areas contaminated with various heavy metals. The soil samples were taken out from 6-10 inches below the surface from 4-6 different spots and mixed to form a composite soil sample. The soil samples were collected using a shovel, trowel, and spade. Soil samples were dried in laboratory and then crushed and homogenized using pestle and mortar, and then stored at 4°C for further analysis.

2.2 Characterization of soil

The collected soil sample were analyzed for their physicochemical properties such as pH, electrical conductivity (EC), soil moisture and organic matter.

2.3 Determination of Pb

Heavy metal (Pb) was extracted from the soil using an acid digestion process. 1.5 gram of dry soil was treated with 24 ml of aqua regia, which contained a 3:1 mixture of hydrochloric and nitric acids. The acid-treated soil was heated for two hours on a hot plate at 100°C, or until the solution turned translucent. The digested soil contents were cooled, filtered, and the filtrate was diluted with double distilled water until it reached a volume of 50 ml. The Pb content of the diluted filtrate was then determined using an Atomic Absorption Spectrophotometer (AAS).

2.4 Lead stock solution and media

Stock solution of 1000 mg/L of lead were prepared by liquefying stoichiometric amount of lead acetate Pb $(C_2H_3O_2)_2$ in distilled water. Nutrient agar (NA) and Nutrient Broth (NB) medium were used to grow and isolate the microorganisms.

2.5 Enrichment and Isolation of heavy metal resistant bacteria

The materials were adequately mixed and enriched for heavy metal, such as lead, by incubating 10 g of soil in 90 ml of NB medium with 50µg/ml of lead-acetate for 3 days at 37 °C. Supernatants were plated on NA medium containing 50µg/ml lead acetate using the spread-plate method at 10^{-3} to 10^{-6} dilution, then incubated at 37 °C for 24- 48 hours. Pure colonies were selected after incubation, and pure isolates were obtained.

2.6 Screening of heavy metal utilizing bacterial strains

Screening for most efficient heavy metal (Pb) utilizing bacterial culture by taking optical density at 600nm (OD600) and observing the lambda max for highest absorbance using UV-vis spectrophotometer (Virion Bio 50) at intervals of 24 hours for 5 days. The experiment was conducted in triplicates.

2.7 Minimum Inhibitory Concentration (MIC)

For the determination of MIC, heavy metal resistant bacterial isolates were grown on NA plates with gradually increasing the concentration of the lead acetate. The initial concentration used was 200µg/ml. Each subsequent transfer to NA plates with increasing the metal concentration by 1200µg/ml until the isolate failed to grow. The concentration above which bacterial isolates failed to grow was considered as threshold level of metal resistance. Bacterial isolates that were able to grow on NA plates supplemented with 1200µg/ml Pb were finally selected for further studies. The bacterial strain A4 was selected for further experiments on the basis of its faster growth rate and survival capability.

2.8 Biochemical and Morphological Characterization

Bacterial strain A4 was then streaked on nutrient agar and morphological characteristics such as margin, cell shape, size, elevation, surface, and Gram's staining were recorded after 24 hours of incubation. For biochemical characterization, catalase, gelatin liquefaction, casein hydrolysis, starch, and IMVIC tests were performed.

2.9 Determination of Optical density (OD600)

In the presence of Pb enriched nutrient broth, the chosen strain grew best at 28±2 ºC. The addition of nutrients promotes microbial growth. In this experiment, sodium nitrate, urea, and peptone were used as growth substrates to check the augmentation of bacterial growth in nutrient broth in the presence of heavy metal.

2.10 Pb removal capacity of the bacterial strain

The bacterial isolates were grown in nutrient broth at the optimum temperature and pH for the selected strain. The experiment was carried out in a 250ml flask containing 100ml broth. The broth was supplemented with lead acetate and

inoculated with 4ml of overnight culture. The cultures were incubated for 24, 48 and 72h. After incubation the broth centrifuged for 30 minutes at 10000 rpm. Both the pellet and the supernatant were digested separately with a mixture of acids (three parts hydrochloric acid and one-part nitric acid) before being tested for heavy metals. AAS was used to determine the concentration of Pb.

3. Result and Discussion

3.1 Soil characteristics

Soil characteristics and concentration of Pb of the collected soil are presented in Table 1. Because of the high pH, the physico-chemical characteristics imply that the location is inappropriate for plant growth. The soil is more alkaline, and the soil sample also has a low water content.

Table 1: Soil characteristics of heavy metal contaminated site

S. No.	Soil characteristics	Units
1	pH	8.75
2	Electrical Conductivity (µs/ppm)	87.0
3	Moisture Content (%)	7.79
4	Organic matter (%)	0.65
5	Lead (ppm)	12.54

3.2 Classification of the bacterial strain

After isolation, 46 pure cultures were obtained from enriched soil by continual streaking and restreaking. Optical Density (OD) at 600nm at 24-hour intervals for 5 days was used to screen for the 10 most efficient Pb degrading bacterial cultures, which were subsequently evaluated for growth in varied Pb concentrations. The strain A4 developed the fastest on NA plates enriched with 1200µg/ml of Pb was chosen for further experiments among all of the bacterial isolates obtained.

3.3 Biochemical analysis

In aerobic microorganism, catalase catalyzes the breakdown of hydrogen peroxide into water and oxygen [25]. The detection of bubbles was a favorable result of the experiment, showing that the bacterial strain A4 may produce catalase. The casein hydrolysis test reveals that the microbe produces proteinase (Caseinase), a proteolytic exo-enzyme that digests casein, and the gelatin test reveals that the microbe produces gelatinase, an enzyme that liquefies gelatin, both of which are negative for bacterium A4. If the bacteria can solubilize starch, particularly around their growth zones, while the rest of the plate contains non-hydrolyzed starch [26]. There is a clear zone surrounding the line of growth after adding iodine solution, showing that the microbe has hydrolyzed starch. The bacterium A4 provides negative results in the starch test because it is unable to hydrolyze starch. The IMViC test was also carried out using Hi-Media test kits.

Table 2: Morphological characteristics of bacterium A4

S. No.	Feature	Type
1	Margin	Irregular
2	Cell morphology	Rods
3	Size	Small
4	Elevation	Convex
5	Surface	Smooth
6	Gram's stain	Positive

Table 3: IMVic test using KB001 Kit

S. No.	Test name	Result
1	Indole	+
2	Methyl red	+
3	Voges Proskauer	-
4	Citrate	-
5	Glucose	-
6	Adonitol	-
7	Arabinose	-
8	Lactose	+
9	Sorbitol	-
10	Mannitol	-
11	Rhamnose	-
12	Sucrose	+

3.4 Variation in growth pattern of selected bacterial isolate in nutrient broth and in presence of various nitrogen sources

The OD600 value was obtained at definite intervals using UV-vis spectrophotometer to monitor the bacterial growth pattern as shown in figure 1. Addition of all the growth substrates to nutrient broth media at 28±2 °C for 48 hours, resulted in the augmentation of growth pattern of the bacterial isolate as observed in figure 2.

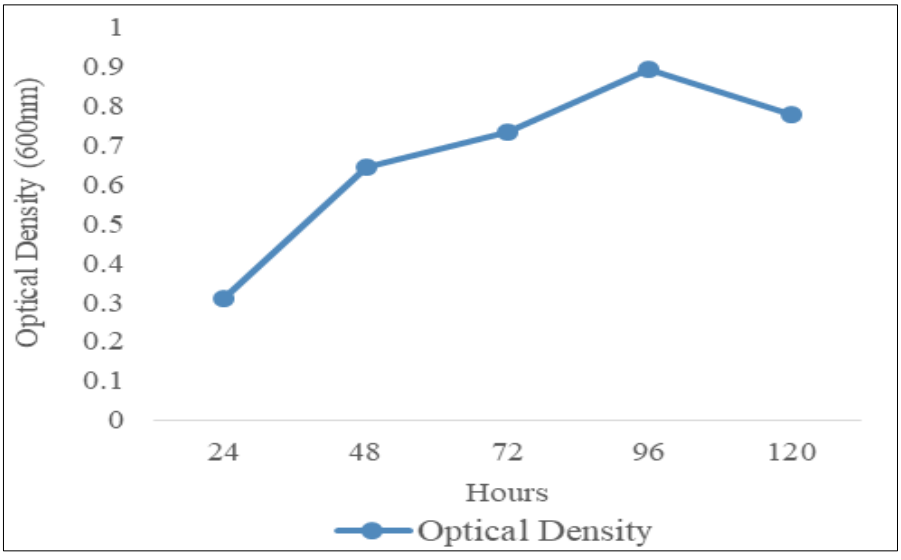

Fig 1: Growth of bacterial isolate A4 in 50µg/ml Lead acetate

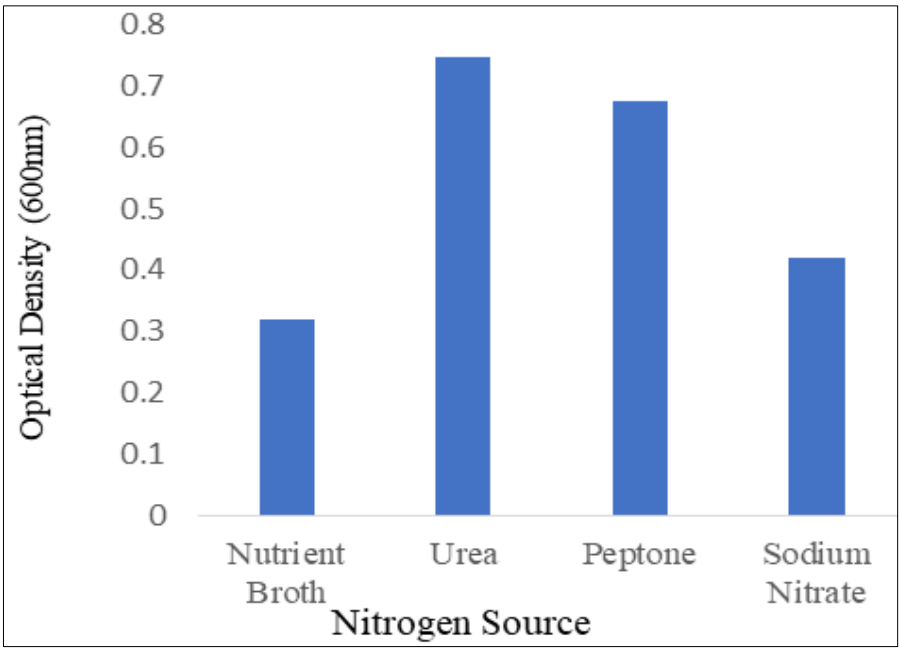

Fig 2: Growth of bacterial isolate A4 in various nitrogen sources

3.5 Heavy metal removal capacity by the selected bacterial strain A4

The lead removal capacity of the selected bacterial isolate was studied in different concentrations (200, 400, 600, 800, 1000 and 1200 µg/ml) of heavy metal. The removal rate in 24, 48, and 72 hours were presented in figure 3. The concentration of Pb was assessed by AAS.

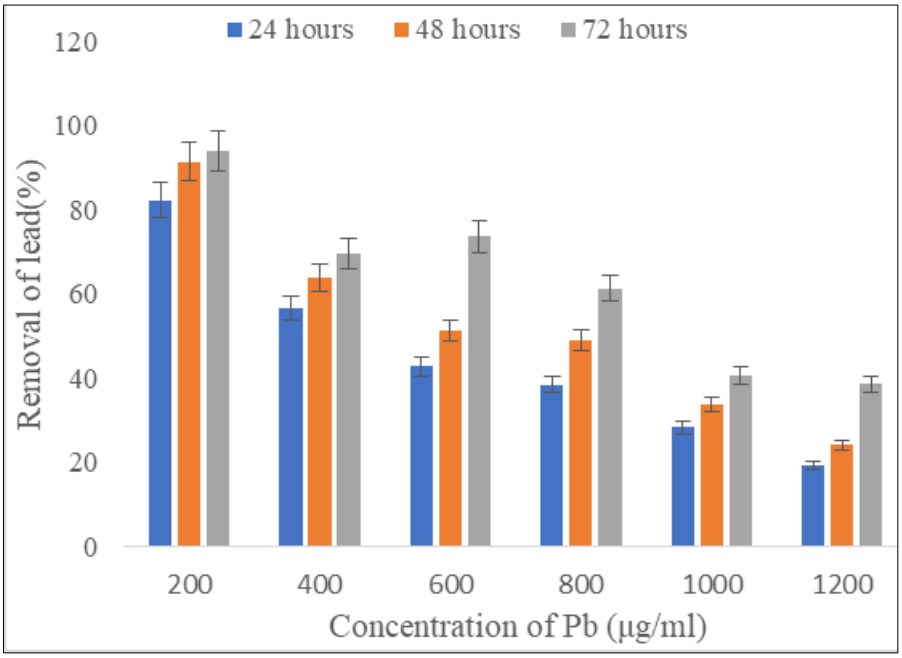

Fig 3: Removal of lead (%) by bacterium A4

The lead removal rate was 82.3%, 91.5% and 94.1% within 24, 48 and 72 hours respectively at 200 µg/ml lead concentration. Lead concentration was reduced by 38.5%, 40.7%, 61.3%, 73.6%, 69.6% and 94.1% in 72 hours from medium containing 1200, 1000, 800, 600, 400, and 200µg/ml lead respectively.

4. Conclusion

In the present work heavy metal (lead) utilizing bacterial strains was successfully isolated and characterized. This study has characterized bacterial isolate A4 as lead degrading strain. The soil enrichment culture technique used in the study suggests that the soil has rich habitat for potential heavy metal degrading microbial communities. The growth pattern of this bacterial isolate was found to be increased in the presence of several nitrogen substrates. The biodegradation process was confirmed by the AAS results, which showed a decrease in lead concentration.

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