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Bioremediation of Lead by Indigenous Bacteria Isolated from an Idol Immersion Site (Lead remediation by *Pseudomonas*)

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ABSTRACT

Heavy metal contamination particularly lead (Pb) is a fast-emerging environmental issue around the globe with special concern in the areas where idol immersion is a major activity. The current study was undertaken to isolate and characterize a lead-resistant bacteria from Prempura Ghat, Bhopal, being one of the major idol immersion sites. From the surface, eleven bacterial colonies were isolated, out of which two demonstrated tolerance up to a significant level with lead concentration. The most tolerant isolate was Pseudomonas sp. designated as PM9 which had a resistance level up to 500 ppm for lead. This bacterium was further grown on lead stress in minimal salt medium over 120 hours. The growth was still maintained with a higher lead concentration. After 72 hours, atomic absorption spectroscopy examination revealed that Pseudomonas had eliminated 61.30 ± 1.0% of the lead, suggesting that it might be used for bioremediation. Investigations into the bacterium's antioxidant defence mechanisms revealed that exposure to lead increased SOD and CAT activity, indicating the bacterium's capacity to cope with oxidative stress. These results reveal Pseudomonas as an excellent candidate for the bioremediation of lead-contaminated water bodies, particularly those impacted by idol immersion episodes.

Keywords: *Idol immersion, Bioremediation, Pseudomonas, Atomic absorption spectroscopy*

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INTRODUCTION

Water pollution is one of the fundamental environmental problems of a deteriorating balanced ecosystem (Liosis *et al*., 2021; Ratnasari, 2023). Several factors disrupt the natural balance in aquatic ecosystems, including the disposal of industrial waste (Wu *et al*., 2020) and the discharge of household sewage (Chen *et al*., 2019). In India, a major contributor to pollution is linked to religious rituals (Bambharoliya *et al*., 2019; Gharat & Shaikh, 2021), especially during festivals that are an integral part of the country's rich cultural heritage. One of the major contributing factors to rising pollution level of water bodies is the immersion of idols every year (Bengani *et al*., 2020; Patel *et al*., 2021). The environmental concerns related to idol immersion stem from the materials used in their creation, which consist of both non-biodegradable and biodegradable substances such as plastics, thermocols, paints, plaster of Paris, cloth, clay, paper, bamboo and wood (Sangani & Manoj, 2017; Pathan, 2022; Roy *et al*., 2022).

These idols are often decorated with bright pigments and various embellishments (Joshi *et al*., 2017). The paints used for decoration frequently contain heavy metals like white lead, red lead, cadmium sulfide, chrome yellow, lithopone, barium chromate and vermillion which lead to increased heavy metal concentrations in water after immersion (Lokhande, 2019; Mondal & Sinha; 2020; Sagarika & Manas, 2022; Warsi *et al*., 2024).

Out of the various forms of pollution that menace the environment, heavy metals are notorious for their toxicity and rank among the most widespread environmental contaminants (Dixit *et al*., 2015; Bharagava & Mishra, 2018; Cabral Pinto *et al*., 2020; Kumar *et al*., 2021; Prasad *et al*., 2021). Among these, lead (Pb) is particularly dangerous due to its high toxicity (Sorrentino *et al*., 2018). Lead-containing paints are widely acknowledged as a significant emerging source of lead contamination (O'Connor *et al*., 2018; Ranjbar *et al*., 2023). Traditionally, paint manufacturers have added lead to paints for their protective qualities, which enhance durability (Gilbert & Weiss, 2006), boost colour vibrancy (Greenway & Gerstenberger, 2010), and improve adhesion to surfaces (Lin *et al*., 2009).

Lead exposure poses serious health risks, particularly affecting the haematological, renal, and central nervous systems, which can lead to significant medical complications (Kalia & Flora, 2005; Lanphear *et al*., 2018; Bloor *et al*., 2021). Acute toxicity typically results from occupational or accidental exposure to high lead levels, while chronic toxicity is more prevalent, occurring when blood lead levels reach 40–60 µg dL^{-1} (Flora *et al*., 2012). Chronic lead poisoning has historically been linked to various complications, including physiological, neurological, and reproductive disorders, as well as conditions like hypertension, baldness, and anaemia (Nakhaee *et al*; 2018; Wang *et al*., 2020; Obeng-Gyasi *et al*., 2021). It can also lead to dementia, cognitive impairments (as lead mimics calcium and

selectively blocks voltage-dependent calcium channels at low concentrations), and even death (Wani *et al*., 2015; Ortega *et al*., 2020; Zhou *et al*., 2020).

In light of the public health threats posed by lead pollution, the Environmental Protection Agency (EPA) has prioritized lead in its regulatory efforts, designating 20–26 October as National Lead Poisoning Prevention Week (NLPPW). To promote protective measures for human health, particularly for children, and to increase awareness of the dangers of lead exposure, this project brings together individuals, organisations, businesses, and state and local governments (Brink *et al*., 2020; Shoushtarian & Negahban-Azar, 2020; Yew, 2020). Despite efforts to reduce lead contamination using conventional physical and chemical techniques, such as ion exchangeprecipitation (Gambhir *et al*., 2012), and chemical oxidation/reduction (Garcia-Segura *et al*., 2018), these approaches have frequently shown themselves to be unsustainable. They can be inefficient, costly, energy-intensive, and may generate secondary waste (Aryal & Liakopoulou-Kyriakides, 2015). As a result, there is a growing focus on bioremediation techniques, which provide a more sustainable and environmentally friendly approach. Bioremediation utilizes biological systems to clean up pollution in a cost-effective way while avoiding the creation of secondary pollutants (Ali *et al*., 2023; Kuppan *et al*., 2024). Among the various biological agents, bacteria have proven to be highly effective for lead bioremediation due to their impressive versatility and adaptability (Jarosławiecka & Piotrowska-Seget, 2014; Yuan *et al*., 2021). These microorganisms have developed specialized mechanisms that allow them to survive and thrive in environments contaminated with lead, all without hindering their growth or metabolic functions (Ustiatik *et al*., 2022; Narayanan & Ma, 2023). Several bacterial species, including *Bacillus thuringiensis* (Uqab *et al*., 2020), *Bacillus cereus* (Idrees *et al*., 2023), *Bacillus pumilus* (Sayyadi *et al*., 2017), and the psychrotrophic strain *Pseudomonas* sp. I3 (Li *et al*., 2017), have shown significant resistance to lead, highlighting their potential in bioremediation efforts.

MATERIALS AND METHODS

Sample collection and isolation of bacterial strains

Water samples were collected from Prempura Ghat, one of the main idol immersion sites of Bhopal. Considering how many idols were submerged in October, the collection was done at random. On LB agar (pH ±7.0) plates, water samples were spread out and incubated for 24 hours at 37°C. on order to screen for lead-tolerant bacterial isolates, eleven phenotypically different colonies were chosen based on their morphology and colour. These colonies were subsequently streaked on LB agar medium containing 100 ppm of lead nitrate (Pb (NO3)2). Only two pure colonies exhibited Pb(II) tolerance out of the eleven strains that were chosen (Chatterjee *et al*., 2012).

Minimum inhibitory concentration

The minimum inhibitory concentration of the two lead tolerant isolates were checked in LB broth media (pH 7.0) supplemented with increasing concentration of lead (100–1500 ppm). The bacterial strain PM 9 showing the highest tolerance level was selected for further studies (Elahi & Rehman, 2019).

Identification of the lead-tolerant bacteria

Primary characterization of the bacterial isolate PM9 was done by colony morphology and microscopic characterization. Different biochemical tests (catalase, oxidase, MR, VP, indole, citrate utilization, urease production, cetrimide test and gelatin hydrolyis) were carried out according to Mondal *et al*. (2010).

Growth in minimal salt medium

50 ml of M9 media was taken in a 250 ml Erlenmeyer flask and inoculated with bacterial isolate PM9 from LB agar plates subjected to 50 ppm-500 ppm Pb $(NO_3)_2$ stress. The flasks were incubated at 37ºC in a shaking incubator (120 rpm). The OD was taken every 24 h for up to 120 hr at 600nm in a UV-Vis spectrophotometer (Shimadzu UV 1900 I series) (Kalita & Joshi, 2017).

Quantification of metal removal by atomic absorption spectroscopy

To evaluate the heavy metal removal capacity, the bacterial isolate PM9 was cultured in LB broth (pH 7.0) supplemented with 500 ppm of lead nitrate. The removal of heavy metals from the culture medium was assessed using a modified method based on Ahemad and Malik (2011). 5 ml of culture broth was collected at 24-hour intervals over a period of 3 days. The supernatant was obtained by centrifuging the samples at 5000×g for 10 minutes, followed by the addition of 2 ml of concentrated HNO3 and heating at 70°C until the solution became clear. The concentration of metals in the supernatant was analyzed using atomic absorption spectroscopy (ECIL Model- 4141). A control was established using LB broth containing the bacterial culture without lead. The % of heavy metal utilized by the bacteria was calculated using the formula according to Kaczorek (2012);

$$\frac{\text{Initial concentration of lead} - \text{Residual concentration of lead}}{\text{Initial lead concentration}} \times 100 \quad (1)$$

Antioxidant profile of the bacterial strain

To monitor oxidative stress, bacterial isolate PM9 was cultured in LB broth with lead at a concentration of 500 ppm. After 24 hours of incubation, the bacterial cells were harvested using cooling centrifugation (8000×g for 10 minutes), washed twice with 50 mM phosphate buffer (pH 7.2), and then resuspended in the same buffer in a 2 ml Eppendorf tube. The cytosolic content was obtained by disrupting the bacterial cells through ultrasonication in an ice bath (30-second pulse followed by 30 seconds of cooling, for a total of 3 minutes). The supernatant was collected by centrifugation (8000×g for 10 minutes) at 4 °C and stored at −80 °C for further analysis.

Superoxide dismutase activity

SOD activity was determined using the method of Ewing and Janero (1995). A 25 µl aliquot of supernatant was mixed with 20

µl of reaction buffer (50 mM phosphate buffer, 0.1 mM EDTA, 98 µM NADH, and 62 µM NBT, pH 7.4). The reaction was initiated by adding 20 µl of an initiating reagent (50 mM phosphate buffer and 33 µM PMS in 0.1 mM EDTA, pH 7.4). Absorbance was measured at 560 nm using a spectrophotometer

Catalase activity

Catalase activity in the bacterial supernatant was measured using a slightly modified version of the Aebi method (1984). A 20 µl supernatant sample was combined with 980 µl of H_2O_2 buffer, and absorbance at 240 nm was monitored for 60 seconds at 15-second intervals using a spectrophotometer. Activity was calculated based on the decrease in H_2O_2 absorbance at 240 nm.

Statistical analysis

Version 20.0 of SPSS software was used for all statistical analyses. Results are presented as the standard error of the mean, with statistical significance set at $p < 0.05$.

RESULTS AND DISCUSSION

Isolation and screening of lead-resistant bacteria

The isolation of lead-resistant bacteria from the idol immersion site at Prempura Ghat, Bhopal, resulted in 11 distinct bacterial colonies. Among these, only two isolates showed tolerance to 100 ppm of lead nitrate in the LB agar medium. The selection process, which focused on variations in colony morphology and color, indicates a diverse microbial community in the water body affected by heavy metal contamination during idol immersion.

Minimum inhibitory concentration (MIC) determination

The MIC assays showed that isolate PM9 had the highest tolerance, surviving lead nitrate concentrations up to 500 ppm. The resistance of isolate PM9 to thrive in such high levels of lead highlights its potential for use in bioremediation, especially in areas that are heavily contaminated with lead.

Bacterial identification

The morphological, biochemical, and microscopic identification of the lead-resistant isolate PM9 confirmed its affiliation with the genus *Pseudomonas* **(Table 1)**.

Table 1. Bacterial characterization of the bacterial isolate PM9

S. No	Characteristics	Reaction
1	Gram Staining	-
2	Shape	Rod
3	Motility	+
4	Catalase	+
5	Oxidase	+
6	MR	-
8	VP	-
9	OF (Oxidative/Fermentative)	Oxidative
10	Indole	-
11	Citrate	+
12	Urease	-
13	Cetrimide test	+
14	Gelatin hydrolysis	-

Growth in minimal salt medium

The growth profile of *Pseudomonas* in M9 minimal salt medium with lead concentrations ranging from 50 to 500 ppm showed considerable turbidity over 120 hours **(Figure 1)**. This indicates that the bacteria can grow effectively even in nutrient-limited conditions while tolerating high levels of lead. It suggests that PM9 could be a promising candidate for bioremediation in environments with low nutrients, which are often found in polluted areas. The bacteria likely cope with metal stress through several mechanisms, such as creating an extracellular barrier, actively transporting metal ions out of the cell, sequestering metal ions both inside and outside the cell, and reducing metal ions (Ndeddy & Babalola, 2017).

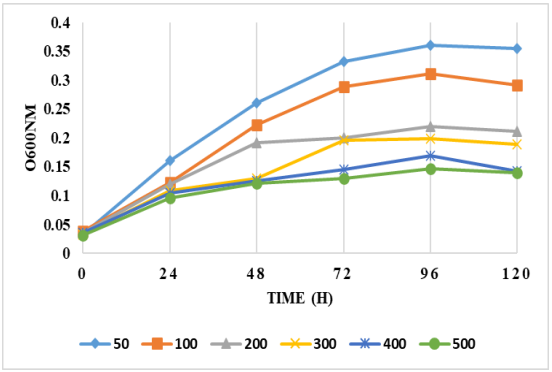

Figure 1. Growth profile of *Pseudomonas* at different concentrations of lead (50 ppm -500 ppm)

Lead removal efficiency by atomic absorption spectroscopy

Lead removal by *Pseudomonas* was measured using AAS at 24-hour intervals over a period of 72 hours **(Figure 2)**. After 24 hours, the lead concentration in the medium decreased from 500 ppm to 380 ± 5 ppm, indicating a removal rate of 24.5 ± 1.2%. By the 48-hour mark, 47.45 ± 1.5% of the lead had been removed, with the concentration dropping to 250 ± 4 ppm. Finally, at 72 hours, 61.30 ± 1.0% of the lead was removed, resulting in a remaining concentration of 200 ± 3 ppm in the medium.

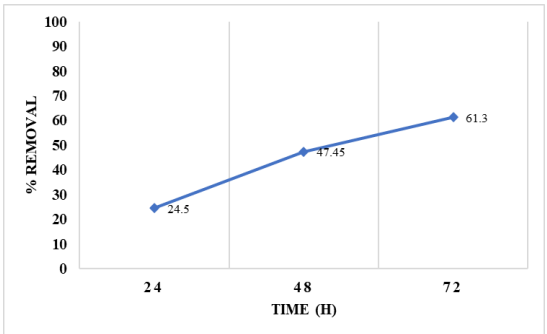

Figure 2. % lead removal by *Pseudomonas* as determined by AAS analysis

These findings show that *Pseudomonas* has a strong ability to bioremediate lead, with a significant reduction observed within the first 72 hours. The consistent decrease in lead concentration suggests that *Pseudomonas* probably uses both bioaccumulation and biosorption methods to remove lead from the environment. The high efficiency of removal by 72 hours highlights the potential use of this bacterium in cleaning up lead-contaminated water bodies, particularly those affected by idol immersions.

Antioxidant profile: Superoxide dismutase and catalase activities

Following a 72-hour exposure to 500 ppm lead, the anti-oxidative stress activities of SOD and CAT in Pseudomonas were observed. From 18.5 ± 0.8 U/mg protein at 24 hours to 38.9 ± 1.3 U/mg protein at 72 hours, the SOD activity rose dramatically **(Figure 3)**. The fact that SOD activity increased so gradually indicates that Pseudomonas was controlling the superoxide radicals generated under lead stress as it expanded. The bacterium's defensive systems were effectively neutralising oxidative stress, limiting damage to the cellular components, as indicated by the sharp increase in SOD activity between 48 and 72 hours. Similarly, the bacterial cell successfully eliminated hydrogen peroxide, one of the products of oxidative stress, as evidenced by the rise in CAT activity from 32.2 ± 1.0 U/mg protein at 24 hours to 56.4 ± 1.5 U/mg protein at 72 hours **(Figure 3)**.

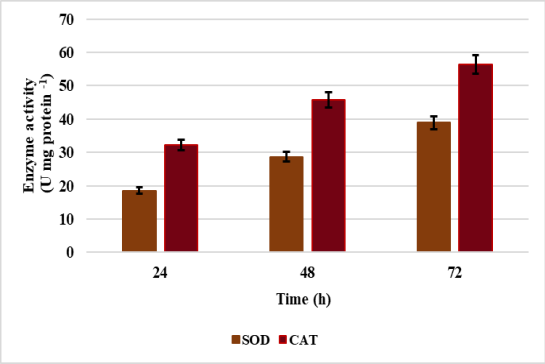

Figure 3. Enzymes (SOD & CAT) activity exhibited by *Pseudomonas* upon Pb exposure. The data are presented as mean ± SEM, with vertical bars representing three replicates (n=3).

Currently, heavy metals contamination is a global problem due to their high toxicity and persistence in the environment which further affects the fragile ecosystem and human health (Briffa *et al*., 2020; Uchimiya *et al*., 2020). Lead is one such heavy metal classified by the WHO as one of the 10 chemical products that induces severe complications. Thus, several studies have demonstrated the presence of microorganisms from the contaminated sites which are heavy metal resistant as potential candidates for metal bioremediation (Mo *et al*., 2020; Sun *et al*., 2021). These resistant microorganisms exhibit adaptive mechanisms for resisting heavy metal toxicity and thrive in harsh conditions. Hence, studying native isolates from such areas is crucial.

In the current investigation, bacterial samples were isolated from idol immersion site of Bhopal, where high concentrations of lead (Pb) are present due to the paint used on the idols. The bacterial isolates were subjected to Pb $(NO_3)_2$ stress. The bacterial strains that exhibited growth under lead-induced stress were subjected to MIC. Isolate PM9 demonstrated MIC at 500 ppm which was further characterized and identified as *Pseudomonas*. The genus *Pseudomonas* is a dominant member of the *Gammaproteobacteria* class in both wastewater and sediment environments. Several studies have documented the bioremediation potential of *Pseudomonas* from residual water as well as hot spring water (Kalit & Joshi, 2017; Kumari & Das, 2019; Vélez *et al*., 2021; Tasleem *et al*., 2023).

It was observed from this study that *Pseudomonas* showed no cellular growth at concentrations exceeding 500 ppm after 120 hours of exposure, indicating the high toxicity of lead at these levels. Various studies have demonstrated the ability of *Pseudomonas* spp. to resist lead concentrations up to 0.05 mg mL^{-1} (Malik & Aleem, 2011) and 0.5 mg mL^{-1} (Gabr *et al*., 2008). Based on the present findings, the gram-negative isolate PM9 exhibited high growth potential and metal adsorption capacity in minimal salt medium (MSM) supplemented with lead, with atomic absorption spectroscopy revealing a lead removal efficiency of 61.30 ± 1.0% at 72 hours. *Pseudomonas* species are capable of withstanding heavy metal stress through various mechanisms, such as active extrusion of metal ions and cellular processes that reduce metal ions to less toxic forms (Fakhar *et al*., 2020; Balíková *et al*., 2022). Additionally, biofilm produced by *Pseudomonas aeruginosa* has been shown to resist copper, lead, and zinc ions (Jasu & Ray, 2021; Priyadarshanee & Das, 2021; Mehra *et al*., 2024). Similarly, biosorption ability of inactive freeze-dried *Pseudomonas putida* biomass removed 80% of lead within 10 minutes of contact (Pardo *et al*., 2003).

In heavy metal-resistant organisms, stress conditions trigger the adaptive responses in microorganisms to oxidative stress, leading to increased activity of detoxifying enzymes such SOD and CAT (Balali-Mood *et al*., 2021). The induction of superoxide dismutase (SOD) activity is recognized as an initial defense mechanism against the formation of toxic oxygen species (Liu *et al*., 2022). SOD catalyzes the dismutation of superoxide radicals into hydrogen peroxide (H_2O_2) and oxygen, with the resulting H_2O_2 further broken down by catalase (CAT) into water and oxygen (Younus, 2018; Zheng *et al*., 2023). Therefore, SOD and CAT are essential components of the antioxidant defense system in nearly all cells. Alterations in the activity of these and other antioxidant enzymes are often used as indirect indicators of oxidative stress (Morano *et al*., 2012; Trist *et al*., 2021). Our findings align with those of Behera *et al*. (2014), who suggested that heavy metal-induced stress, such as cadmium (Cd), increases SOD activity in bacteria *Bacillus cereus* as an important adaptive mechanism under metal stress. Similarly, a significant increase in SOD activity was observed in *Bacillus subtilis* and *Bacillus thuringiensis* in response to arsenic (As) toxicity (Xie *et al*., 2014). The enhanced antioxidant capacity of bacterial cells is closely linked to metal tolerance, with SOD being a key enzyme in the antioxidant defense system (Behera *et al*., 2014). Supporting our experimental data, David *et al*. (2016) also reported that arsenic and cadmium exposure increased SOD and CAT activity in *Bacillus* sp. MDPMK-02.

CONCLUSION

The *Pseudomonas* isolate PM9 showed significant tolerance to lead even in a nutrient-deprived condition, which makes it a good candidate for bioremediation. The strain not only can grow

in the presence of high lead concentrations but also appears to possess mechanisms that help it sequester and expel metals and carry out certain enzymatic processes to aid in the abatement of heavy metal pollution. These results prove the usefulness of *Pseudomonas* PM9 in environmentally safe bioremediation interventions in places subjected to industrial or human activities.

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