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Isolation Screening and Transformation of Cadmium and Lead by Potential Microorganisms in Wastewater of Patna

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Abstract : *Heavy metal pollution is a worldwide problem, with cadmium (Cd) and lead (Pb) being the most common pollutants. They are more commonly referred to as "most problematic heavy metals" and "toxic heavy metals" (THMs). One of the primary topics in remediation research is the treatment of heavy metals through a range of biological processes, where heavy metal-microbe interaction techniques are valued for their cost-effective and environmentally beneficial solutions. Pseudomonas aeruginosa, a unique microbe, was successfully isolated, identified, and characterized. The strain has shown promising resistance to heavy metals like Cadmium and Lead, but further research is needed to understand the genes and/or molecular pathways that cause their tolerance to various heavy metals.*

Keywords: *Bioremediation, Pseudomonas aeruginosa, Cadmium, Lead.*

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Introduction:

Heavy metal contamination in the environment is becoming an increasingly serious issue, causing widespread worry owing to its negative consequences. Because of the rapidly developing agricultural, metal, and pharmaceutical sectors, as well as poor waste management, fertilizers, and pesticides, these inorganic pollutants are widely spread in the environment via water and soil. Concerns have been raised about their possible consequences on human health and the environment (MathuMitha et al., 2021). Water is a crucial supply for humans since water is required for numerous aspects of life, including drinking, healthcare, agriculture, economics, and industry. However, millions of people worldwide face a dearth of safe drinking water (Jadaa and Mohammed, 2023). Soil organic matter (SOM) is an important component of soil that serves as the foundation for soil health and fertility. It is an intricate combination of decomposed plant and animal products, microbes, and the material they synthesize in the soil. It has a vital role in numerous soil processes depending on soil structure, cycling, and water retention, among other things (Hussain et

al., 2023). Heavy metal poisoning is seen as a major concern, with several health problems linked to it. They can operate as pseudo-elements in the body at times, but they can also be related to metabolic processes (Bhat et al., 2019). Heavy metals and contaminants found in water are tenacious in the environment and have been linked to mutagenic, teratogenic, and carcinogenic effects. These pollutants are not impacted by biological degradation, but they frequently persist in the environment for lengthy periods, moving from one oxidation or organic complex state to another. As a result, chemical contamination of bodies of water is a severe hazard to the ecology (Obinnaa and Ebere, 2019). Toxic metals are a major cause of soil pollution. Toxic metal poisoning in agriculture (copper, cobalt, nickel, cadmium, zinc, chromium, and lead) disrupts soil function, limits plant development, and can harm food and human health. Heavy metals affect soil organisms by altering population size, species composition, and physical structure. The density and activity of bacteria and enzymes in the soil alter as a result of different types and levels of heavy metal contamination, which is a clear sign of soil biology. Toxic metals are neither used nor biodegraded (Fatima and Singh, 2023).

Bioremediation is an innovative and promising approach for recovering and reducing heavy metals in water and damaged soils. Heavy metal bioremediation relies heavily on microorganisms (Verma and Kuila, 2019). Cadmium (Cd) contamination of soil and food crops is a common environmental problem caused by uncontrolled industry, unsustainable urbanization, and intensive agricultural techniques. Cadmium is a dangerous element that threatens soil quality, food safety, and human health. Cadmium enters the body by taking in polluted water or air, as well as contaminated food. Increased cadmiumconcentrations in the human body cause a range of issues, including renal failure; hence, the kidneys, liver, and genitals retain most of the cadmium. Cadmium is also a carcinogen that harms the bones, respiratory system, and brain (Mahdi et al., 2021). Lead (Pb) is the second most harmful metal, naturally occurring in very minuscule amounts but mostly generated due to human-made industries, automobiles, batteries, and so on, resulting in lead pollution in people and their environments. Pb is found in both organic and inorganic forms; both are equally dangerous; however, organic forms are particularly destructive to biotic organisms, whilst inorganic forms are usually found in soil, dust particles, old paint, and other such materials. Because of all this pollution, mine workers and people living nearby suffer from a variety of respiratory diseases because of dust particle inhalation in the long term. Humans are now at significant risk due to soil pollution produced by heavy metals from mine sites, which make their way into houses through edible water and dust. Pb is known to cause cancer in humans. Pb is primarily consumed and absorbed by humans (Raj and Das, 2023). In aqueous solutions, *Pseudomonas aeruginosa* has a high capacity for Cd and Pb adsorption. Live *Pseudomonas* BC15 cells, on the other hand, were capable of biosorbing Cd as well as other metals such as Pb in a medium, and *P. aeruginosa* isolated from active sludge was also reported to efficiently remove 94.7% Cd from the solution in 60 minutes (Chellaiah, 2018).

Requirements:

Beakers, Glass rod, measuring cylinder, Conical flask, Glass slides, Test tubes, Spreader, Glass vials, Glass funnel, Petri plates, Micropipette, Spirit lamp, Spatula, Scissors, Inoculating loop, Measuring cylinder, Test tube stand, Dropper, Micropipette tips, Wash bottle, Autoclave machine, Microwave oven, Laminar Air Flow, Incubator, Shaker incubator, Vortex, Microscope, weighing balance, UV-visible spectrophotometer, Centrifuge, AAS, Colony counter, Agar, Distilled water, NaCl, Peptone, Beef extract, Yeast extract, Cadmium chloride, Lead nitrate, Hydrogen peroxide, Tetramethyl-p-phenylenediamine dihydrochloride, Magnesium sulphate, Ammonium hydrogen phosphate, Dipotassium phosphate, Sodium nitrate, Potassium hydroxide, Alpha naphthol, Urea, Bromothymol blue, Phenol red, Methyl red, Safranin, Crystal violet, Gram's iodine, Ethanol, Staining bucket, Wastewater sample, Cotton, Tissue paper, and Whatman filter paper.

Methodology:

1. **Sample collection:** The wastewater was collected from the sewage pipes of Patna Women's College on 9-8- 2023 at 12:15 pm. These samples were taken in a sterile conical flask.
2. **Isolation of *Pseudomonas aeruginosa* from wastewater of Patna Women's College:** Wastewater was collected from the sewage pipes of the college and then serial dilution of 10^{-4} and 10^{-5} was prepared using

normal saline. The dilution was poured on LB media and then incubated for 24 hours at 37^0C using a bacteriological incubator.

3. **Staining:** Gram staining consists of three steps: staining with a water-soluble dye called crystal violet, decolourization, and counterstaining, which is often done with safranin. The following steps were included in the procedure: (a) Take a clean glass slide and make a thin smear of the bacterial colony with the help of an inoculating loop. Heat fixed the slide by carefully passing the slide through a Bunsen burner three times. (b) Added the primary stain (crystal violet) to the slide and left for 1 minute. Rinsed the slide with a gentle stream of distilled water. (c) Added Gram's iodine for 1 minute, this is a mordant or an agent that fixes the crystal violet to the bacterial cell wall. (d) Rinsed slide with ethanol dropwise. The alcohol will decolourize the sample if it is Gram-negative, removing the crystal violet. However, if the alcohol remains on the sample for too long, it may also decolourize Gram-positive cells. (e) Added the secondary stain, safranin, to the slide and left for 1 minute. Washed with a gentle stream of distilled water.

4. **Screening of *Pseudomonas aeruginosa* by biochemical testing**: A series of biochemical tests were performed to confirm *P. aeruginosa*.Catalase, oxidase, citrate, and MRVP tests were performed.

   (a) Catalase test: Catalase is an enzyme synthesized by some microorganisms. This enzyme defends bacteria against hydrogen peroxide (H_2O_2), which can harm and kill them. Catalase is an enzyme that converts hydrogen peroxide into liquid water (H_2O) and oxygen gas (O_2). As a result, if catalase is very active owing to a high concentration of hydrogen peroxide, the quick creation of oxygen gas (O_2) would result in bubbles. As a harmful consequence of aerobic metabolism, bacteria create hydrogen peroxide (H_2O_2). Toxic hydrogen peroxide can induce intracellular damage, including DNA, lipid, and protein damage. Cells generate enzymes that break down H_2O_2 and other related substances to eliminate them, such as catalase. It consists of the following steps: (a) Take a clean glass slide and a bottle of hydrogen peroxide. (b) Using a sterilized inoculating loop, make a thin smear of bacteria onto the dry slide. (c) Placed a drop of hydrogen peroxide on the smear. (d) Observed the bubbles.

   (b) Oxidase test: The oxidase test identifies the existence of a cytochrome oxidase system, which catalyzes electron transfer between electron donors in bacteria and a redox dye, tetramethyl-p-phenylene-diamine. The dye has been reduced to a dark purple colour. This test can help identify *Pseudomonas, Neisseria, Alcaligens, Aeromonas, Campylobacter, Vibrio, Brucella* and *Pasteurella*, which all generate the enzyme cytochrome oxidase. The dry filter paper approach was employed since the oxidase reagent is unstable and must be newly produced for use. The steps were as follows: (a) A strip of Whatman's No. 1 filter paper was soaked in a freshly prepared 1% solution of tetramethyl-phenylene-diamine dihydrochloride. The strips are freeze-dried and stored in a dark bottle tightly sealed with a screw cap. (b) A strip was placed in a petri dish and moistened with distilled water. The colony to be tested was picked up with a platinum loop and smeared over the moist area. (c) A positive reaction was indicated by an intense deep-purple type, appearing within5-10 seconds.

   (c) Citrate test: The Citrate test was conducted in the medium of Simmon's citrate agar using thefollowing steps: (a) Made a Simmon's Citrate Agar media slant and streaked with the bacterial colony with the help of inoculating loop. (b) Observed for the colour change after incubation of 24 hours.

   (d) MRVP test : The MR-VP test is a combination of two distinct assays that employ a single broth medium containing glucose to evaluate the many forms of

glucose fermentation that a bacterial species may perform. Some bacterial species may ferment glucose via the mixed acid fermentation pathway (as identified by the MR test or methyl red test), whilst others may ferment glucose via the butanediol fermentation pathway (as indicated by the VP test or Voges-Proskauer test). Testing the kind of fermentation pathway is important for characterizing and identifying bacteria since the type of fermentation performed varies by bacterial species (or strain). Metabolic responses are reliant on the enzymes that a species (or strain) possesses, which are reliant on the genes that a species (or strain) contains in its DNA.

As a result, studying the different sorts of metabolisms that different species have given insight into variances in their genetics (their DNA). It consists of the following steps: (a) Make an MR-VP broth tube. (b) Inoculated the MR-VP broth with bacterial colonies aseptically. (c) Incubate the inoculated tube at 37^0C for 24 hrs. (d) Methyl red test: (i) Add 10 drops of methyl red reagent to the “MR” tube. (ii) Examine the colour of the medium. (iii) Record the results. (e) Voges-Proskauer test: (i) First add 15 drops of Barritt’s A reagent (alpha-naphthol) to the "VP" tube. (ii) Added 5 drops of Barritt’s B reagent (40% KOH) to the “VP” tube. (iii) Hold the test tube from the glass and mix the tube well by flicking it with yourfingers. (iv) Let the test tube sit undisturbed in a test tube rack for 20 minutes. (v) Examine the colour of the top layer of liquid and record the results.

5. **Obtaining pure culture of *P. aeruginosa:*** Slants were prepared using Malachite Green Agar Media, and streaking was done under aseptic conditions inside laminar airflow. The slants were then placed in the bacteriological incubator, which was set at 37°C for 24 hours.

6. **Biotransformation experiment:** This experiment employed malachite green broth added with various amounts of Cd (5,4,3,2, and 1 µg/L) and Pb (14,12,10,8, and 6 µg/L). The concentrations of the two most frequent heavy metal contaminants were additionally supplemented with malachite green broth, lead and cadmium. The broth that did not contain the heavy metal served as the control. The broth was autoclaved for 15 minutes at 121°C and allowed to cool. In both the heavy metal enriched and control broths, *Pseudomonas aeruginosa* was inoculated. It was then incubated for a day at 37°C. The remediation capacity of *P. aeruginosa* was assessed by monitoring biomass growth using an ultraviolet (UV) spectrophotometer set at 600 nm. An atomic absorption spectrophotometer (AAS) was used to measure the amount of heavy metal left in the broth. The biotransformation occurred exactly as described.

Sample	Conc. (mg/l)	%RSD	Mean Abs.
Cadmium sample	0.005	0.45	0.0022
Lead sample	0.045	0.23	0.0042

Results:

Isolation of potential microorganisms : Bacterial colonies were grown well in Luria Bertani (LB) medium at 37ºC under ambient aeration. Colonial Morphology on LB plates appear as large (3-5mm) and small (2-1.5mm) yellow-green, irregularly round, Mucoid colonies with butyrous centres after 24 hours incubated at 37°C. They may be *P. aeruginosa*.

Staining of potential microorganisms: Both strain A and strain B are Gram-negative and rod-shaped like *P. aeruginosa*.

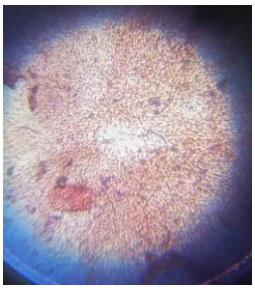

Fig. 1. Gram-negative bacteria

Screening of potential microorganisms

S. No.	Biochemical test	Result
1.	Catalase	Positive
2.	Oxidase	Positive
3.	MR	Negative
4.	VP	Negative
5.	Citrate	Positive

After doing biochemical tests, we confirmed that both strain A and strain B are *Pseudomonas aeruginosa*.

Fig. 2. Catalase test

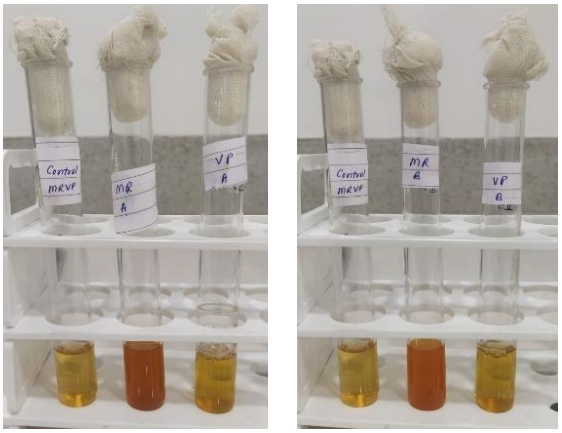

Fig. 3. MR / VP test

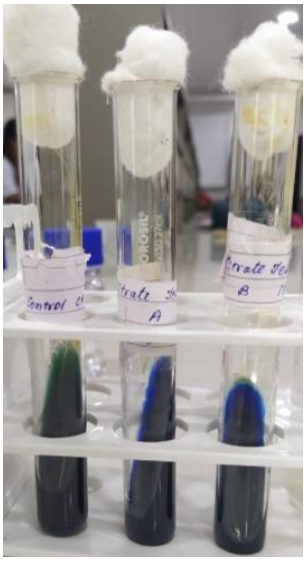

Fig. 4. Citrate test

Pure culture preparation on malachite green agar media: Pure culture of both strain A and strain B of *Pseudomonas aeruginosa* was obtained on malachite green agar media.

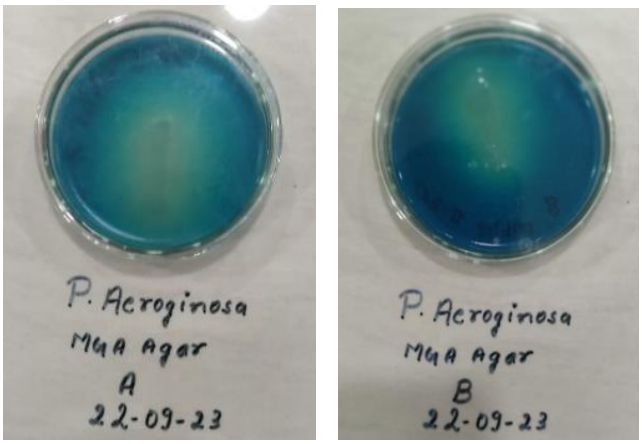

Fig. 5. Pure culture of *P.aeruginosa*

Biotransformation experiment results:

- The bioremediation capabilities of two strains i.e. strain A and strain B of indigenous gram-negative bacteria *P. aeruginosa* isolated from sewage wastewater of Patna Women's College were evaluated using Cd and Pb supplemented medium.
- UV visible spectrophotometric analysis shows the effect of selected bacteria isolates of *P. aeruginosa* on heavy metal remediation.

STRAIN- A (Concentration in µg/L of Cd in 5 ml MGA)	OD
5	3.76
4	3.80
3	3.90
2	4.00
1	4.10

STRAIN- B (Concentration in µg/L of Cd in 5 ml MGA)	OD
5	4.12
4	4.13
3	4.15
2	4.18
1	4.19

STRAIN- A (Concentration in µg/L of Pb in5 ml MGA)	OD
14	2.90
12	3.10
10	3.13
08	3.15
06	3.17

STRAIN- B (Concentration in µg/L of Pb in5 ml MGA)	OD
14	2.80
12	2.87
10	2.90
08	3.00
06	3.10

- UV-visible spectrophotometer results of two samples whose concentrations were made by the most frequently found pollution in the rivers of India.

Heavy metal concentration in strain A (Concentration in µg/L in 5 ml MGA)	OD
Lead =10	3.10
Cadmium=3	4.00

- Result of AAS analysis of two samples whose concentrations were made by the most frequently found pollution in the rivers of India. A process of "digestion" was carried outbefore the analysis. It involved mixing an acid to the media supplemented with heavy metal.

Heavy metal	Heavy metal concentration (Concentration in µg/L in 5 ml MGA)	Conc. (mg/l)	%RSD	Mean Abs.
Cadmium	3	0.005	0.45	0.0022
Lead	10	0.045	0.23	0.0042

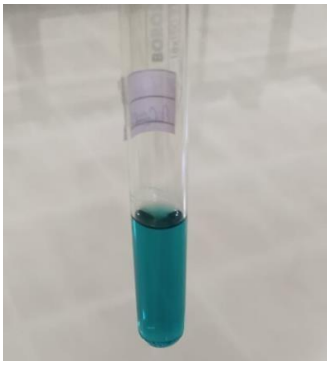

Fig. 6. Before inoculation with *P. aeruginosa*

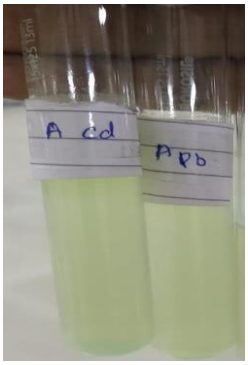

Fig. 7. After inoculation with *P. aeruginosa*

- The results of the present investigation showed consistent data in favour of aims and objectives. These results confirmed that varying levels of heavy metal concentration affect the level of uptake of heavy metal by bacterial species *Pseudomonas aeruginosa.*

Discussion:

Microbial analysis of wastewater from Patna, exposed to potential contamination with heavy metals, revealed the presence of many *Pseudomonas* isolates with great potential for bioremediation of cadmium and lead, which have not been previously reported from this region. We found some potential for bioactivity through biochemical assays. One of the isolates identified in this water corresponds to *Pseudomonas aeruginosa*, a species commonly associated with the natural environment in hospitals, indicating that it is still considered an infectious disease (Ochoa et al., 2013). Well, it can easily adapt to a harsh environment. Two strains of the indigenous Gram-negative bacterium *Pseudomonas aeruginosa*, type A and type B, were used in this study. *P. aeruginosa spp.* was isolated from wastewater in Patna and analyzed using a culture medium supplemented with cadmium and lead.

Recent studies have focused on understanding the microbial diversity in wastewater and exploring the unique capabilities of microorganisms to sequester and transform Cd and Pb. A study of the microbial community in wastewater revealed a great diversity of bacteria and fungi with the potential to survive and interact with increased Cd and Pb concentrations (Manorma et al., 2023). It is important to note that, the diversity of microorganisms is closely linked to redox activity and the presence of heavy metals, such as phosphorus or nitrogen in the environment (Wang et al., 2020). Recent research has shown that certain bacterial species, including *Pseudomonas* and *Bacillus*, can tolerate higher concentrations of Cd and Pb (Malik and Garg, 2024). The study utilized advanced techniques to analyze the genetic basis of metal resistance in these isolates, providing insights into the mechanisms underlying their survival in metal-contaminated environments. The screening process not only unveiled the capacity of these microorganisms to endure high metal concentrations but also shed light on potential genetics associated with metal resistance. Understanding the transformation

mechanisms employed by microorganisms is crucial for developing effective bioremediation strategies. This enzymatic biotransformation represents a promising avenue for reducing the environmental impact of heavy metal contamination, offering a sustainable approach to detoxifying wastewater. The environmental implications of the isolation, screening, and transformation of Cd and Pb by microorganisms in wastewater are substantial. Heavy metal pollution poses significant risks to ecosystems and human health, making the development of efficient and environmentally friendly remediation strategies imperative. Bioremediation, utilizing the natural abilities of microorganisms, emerges as a viable solution. Recent studies have highlighted the potential for scaling up these bioremediation techniques in wastewater treatment plants in Patna, showcasing the practical application of these findings in real-world scenarios (Yadav and Sharma, 2023).

In conclusion, the isolation, screening, and transformation of Cd and Pb by potential microorganisms in wastewater represent a cutting-edge and promising field of research in environmental microbiology. The latest studies underscore the diverse microbial resources present in the region, their capacity to resist heavy metal stress, and the potential for enzymatic biotransformation of toxic ions. When grown in MGA media, temperature, and duration, *P. aeruginosa* (strain A) grows faster than strain B and can degrade lead more effectively than cadmium. This demonstrates that it develops well at high lead concentrations. As global concerns about water pollution continue to rise, these findings contribute not only to the scientific understanding of microbial-metal interactions but also offer practical solutions for mitigating heavy metal pollution in urban water systems, with direct implications for wastewater treatment and environmental management in Patna and beyond.

Conclusion:

This study evidenced the presence of the *Pseudomonas* genus has high biotechnological potential for lead and cadmium biotransformation, from water sources that obtain industrial waste and are potentially contaminated with heavy metals. It was proven that there is a potential for the *Pseudomonas* genus because the cell remains viable in high metal concentrations.

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