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Investigating Heavy Metal Stress On Microbial Populations: Adaptations And Growth Responses

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Abstract: Heavy metal contamination in soil ecosystems poses a significant environmental and microbiological concern due to the persistent and toxic nature of metals such as Zinc and Cadmium. This study evaluated the microbial response to heavy metal-induced stress by isolating soil bacteria from industrially impacted areas in Bangalore, India. Using standard microbiological techniques, the isolates were cultured on various media and were exposed to various concentrations of Zn and Cd to evaluate their tolerance profiles. Disc diffusion assays were performed to determine zones of inhibition, revealing concentration-dependent sensitivity. Zinc showed moderate antimicrobial effects, with partial bacterial growth observed at lower concentrations, while cadmium exhibited strong toxicity and completely inhibited microbial growth in most cases. Subculturing in broth media further confirmed these observations, with visible growth in Zinc-supplemented Nutrient Broth but complete inhibition in Luria Broth, potentially due to metal-nutrient interactions causing precipitation. To biochemically characterize the resistant strains, a series of tests including Gram staining, Eosin Methylene Blue screening, and the IMViC test series are done, that enable to distinguishing between different bacterial types and understanding their adaptive metabolic traits under heavy metal stress. The findings suggest that certain environmental bacteria possess physiological and biochemical mechanisms that enable them to tolerate and survive in metal-contaminated environments. This study provides a foundational understanding for the application of such microbes in bioremediation and environmental monitoring. Future studies should focus on molecular characterization are recommended to explore genetic mechanisms of metal resistance.

Key words: Heavy metal stress; Cadmium; Zinc; Disc diffusion assay; Bioremediation potential

I. Introduction

Heavy metals are naturally occurring toxic metals with a density greater than 5 g/cm³ that can accumulate in natural ecosystems [4]. Due to their non-biodegradable nature and chemical stability, these metals persist in the environment for long periods, bioaccumulate through the food chain, and pose long-term ecological and health risks. Among the most common environmental heavy metals are cadmium (Cd) and zinc (Zn), which can be toxic even at low concentrations and may interfere with key biological processes in microorganisms [17], [5].

The major sources of heavy metal contamination are anthropogenic activities, including mining, smelting, industrial manufacturing, and agricultural practices [1]. Once released, these metals contaminate soils, sediments, and water bodies, including groundwater, and are extremely difficult to remove [15]. Accumulation of heavy metals in these ecosystems imposes chemical stress on microbial communities, which are essential for biogeochemical cycling, organic matter decomposition, and nutrient transformation [15]. Microorganisms play a key role in maintaining ecosystem stability by recycling nutrients such as carbon, nitrogen, and phosphorus [19]. However, elevated concentrations of heavy metals disturb microbial homeostasis, leading to a decline in microbial diversity and functionality. Metals may interfere with enzyme activity, damage DNA and cell membranes, or generate oxidative stress through reactive oxygen species

(ROS). Prolonged exposure to heavy metals exerts selective pressure on microbial communities, leading to the evolution of resistance mechanisms [9].

These adaptations enable certain microbes not only to survive but also to thrive in metal-contaminated environments. Heavy metal–resistant microbes may serve as bioindicators of environmental pollution and ecosystem health. Studying microbial adaptation mechanisms provides insights into fundamental evolutionary processes and stress responses. The rapid generation times and high mutation rates of microorganisms make them excellent model systems for understanding adaptation to environmental stressors [19]. Furthermore, understanding these mechanisms is crucial for developing bioremediation strategies, where metal-resistant microbes can be harnessed to detoxify polluted environments [2], [3], [6], [8], [12], [16], [18].

Heavy metals have become a major global concern due to their adverse effects, and they enter the environment mainly through anthropogenic activities such as mining, smelting, industrial manufacturing, and agricultural practices [1]. Heavy metals pose a serious threat to human health, primarily through their entry into the food chain via plant uptake. The accumulation of toxic metals in edible plant tissues can result in chronic health effects, including neurological, renal, and carcinogenic outcomes.

To address this environmental and public health concern, several remediation strategies ranging from physical and chemical methods to biological approaches have been developed. Key factors such as soil pH, organic matter content, redox conditions, and clay minerals play a crucial role in determining the bioavailability of metals to plants and microorganisms. Microbial activity can significantly change the solubility and chemical form of heavy metals, thereby affecting their mobility and persistence in the environment [2]. Further studies have demonstrated that microbes are actively involved in processes such as mineral dissolution and precipitation [3].

Heavy metal contamination can seriously affect soil microbial communities, often reducing their diversity and disrupting their ecological functions. Metals can attach to proteins in microbial cells, block key enzymes, and cause oxidative stress, which damages cells and suppresses growth. Essential microbial processes such as organic matter decomposition, nutrient cycling, and enzyme production are also negatively impacted. Healthy microbial communities are vital for soil fertility and plant productivity. Differential sensitivity between bacterial and fungal communities under heavy metal stress has been reported, highlighting the importance of taxonomic specificity in contamination assessment [7].

Microorganisms have evolved diverse strategies to tolerate heavy metal-rich environments, including efflux pumps, enzymatic transformation, intracellular sequestration, and biofilm formation. Early studies highlighted the complexity and ecological importance of these mechanisms [9]. More recent work elaborated on microbial surface properties and metabolic pathways that contribute to detoxification and resistance [16]. Resistance genes are frequently located on plasmids or transposons, enabling horizontal transfer within microbial communities and raising ecological as well as clinical concerns.

Long-term exposure to heavy metals has been shown to drive stable community adaptations and altered interaction networks, ultimately enhancing microbial community resilience [10]. The remediation potential of plant-associated microbiomes has also been recognized, particularly in soils contaminated with heavy metals [8]. Other studies demonstrated that microorganisms employ strategies such as biosorption, bioaccumulation, biotransformation, and bioleaching to survive in polluted soils, while also modifying rhizosphere interactions that influence soil properties, plant health, and overall agricultural productivity [12].

This research aims to investigate the adaptive responses of diverse microbial communities exposed to heavy metal stress, with a focus on population dynamics, metabolic changes, and resistance mechanisms. Such insights are essential for improving our understanding of microbial ecology in contaminated ecosystems and for designing sustainable remediation technologies.

II. Materials and Methodology

2.1 Materials:

Table.1: Materials and equipment used in the study

Category	Materials/Equipment
Culture Media	Nutrient Agar (NA), Nutrient Broth (NB), Luria Bertani Agar (LBA), Luria Broth (LB), Potato Dextrose Agar (PDA), (Hi Media) Eosin Methylene Blue (EMB) Agar, Simmon's Citrate Agar, Indole broth, MR-VP broth
Heavy Metals	Zinc Sulphate ($ZnSO_4$), Cadmium Chloride ($CdCl_2$)
Staining Reagents	Crystal violet, Gram's iodine, Safranin, Lacto-Phenol Cotton Blue
Biochemical Reagents	Kovac's reagent (Indole), Barritt's reagents A & B (VP), Citrate indicator

2.2 Methodology

2.2.1 Sampling

For this study the soil samples were collected from various places within the Pennya industrial area, Bangalore(sample-1) latitude of 13.0085°N and a longitude of 77.4996°E. They were particularly collected from the places where there was a visible sign of industrial activity and waste disposal known for the elevated concentration level of Zinc and Cadmium. At each location three subsamples were collected by digging 15 cm depth using sterile spatula and samples were collected in clean glass jar.

The samples are delivered to the laboratory under controlled refrigerated conditions. Similarly (Sample 2) Soil sample obtained from Aspen Steel Pvt. Ltd., located in Medahalli, Bangalore 13.0135°N latitude and 77.7310°E longitude. All samples were properly labeled, transported under aseptic conditions, and preserved at 4°C until further processing and within 24 hours serial dilution is done.

2.2.2 Primary isolation of microorganisms

Culture media was prepared-Luria-Bertani Agar (LBA), Nutrient Agar (NA) and Potato Dextrose Agar (PDA). Samples were inoculated by pour plate method and incubated under appropriate conditions. Colony growth was observed on NA and PDA plates, and selected colonies were sub cultured into Nutrient Broth (NB) for further incubation at 37°C.

2.2.3 Heavy Metal Exposure

Plates of NA, LBA, PDA, and LB were inoculated using samples from the primary culture plates. Heavy metal agar plates were prepared with specific concentration 50 ppm of Zinc (Zn) and Cadmium (Cd) inoculated for preliminary tolerance screening.

2.2.4Selective Media Screening, Gram Staining, and Biochemical Characterization of Heavy Metal-Resistant Bacterial Isolates

Fresh serial dilutions of the soil samples were sub cultured on NA plates and in NB. Eosin Methylene Blue (EMB) agar was prepared and inoculated to isolate potential Gram-negative enteric bacteria. Gram staining was performed for different isolated cultures and heavy metal plates. Colony characteristics were observed. Biochemical tests were done for them. Indole broth, Nutrient broth and Simmon's citrate was prepared and the culture is inoculated into the test tubes and incubated. Later tested using the reagents.

2.2.5 Heavy Metal Stock, Disc Preparation, and Assay

Stock solutions of Zn and Cd were prepared. These were inoculated into LB, NB, and distilled water to observe microbial survivability and resistance. Heavy metal discs were prepared from stock solution using Whatman filter paperNo.1. Filter paper was carefully cut into small, round discs and soaked overnight in different heavy metal stock solutions, including zinc sulphate and cadmium chloride. This allowed the discs to absorb the metal ions thoroughly. The next day, they were placed in a laminar airflow chamber and left to dry completely under sterile conditions. Discs were prepared using a 10 mg/mL stock solution. Discs labelled as 5, 10, and 15 correspond to 50 µg, 100 µg, and 150 µg of heavy metal per disc. These metal-infused discs were then ready to be used for testing their effects on microbial growth. and these metal discs were placed

in agar plates with inoculated culture and incubated for 24 hours and observe for the zone of inhibition (Selvi et al., 2012; Hossain & Anwar, 2012).

2.2.6 Subculturing with Metal Stock

Vials containing NB and LB were supplemented with varying concentrations of heavy metal stock solutions. Subculturing was carried out to monitor microbial growth response and tolerance patterns. The broth was supplemented with varying concentrations of each heavy metal 2.5; 5; and 7.5 ppm to study bacterial growth across a gradient of metal exposure. The inoculated vials were incubated at 37°C for 24–72 hour

III. RESULTS AND DISCUSSION

3.2.1 Sampling was done and serial dilution was done up to 9 dilutions for both the sample1 and 2 and used for the primary isolation and for the later process.

3.2.2. Primary isolation of Microorganisms

The morphology of microbial colonies on NA and LBA plates was observed to evaluate diversity and responses to stress at various dilutions. For Sample 1 grown on NA, colonies at higher dilutions (10^{-3} and 10^{-2}) were predominantly circular, creamy, and opaque with smooth, moist textures, indicating active, uniform growth. At a dilution of 10^{-1}, NA Sample 1 displayed irregular, lobate colonies with a raised to flat elevation. These colonies were mucoid and sticky in texture, showing creamy, opaque appearances, with some colonies exhibiting slight translucence. As the dilution increased to 10^{-2}, the colonies became circular in shape with a raised elevation, maintaining a creamy texture and off-white (opaque) coloration. At 10^{-3} dilution, colonies were circular and spread out, featuring a smooth, even, and moist texture, with creamy to off-white and opaque pigmentation.

In contrast, **NA Sample 2** at 10^{-1} dilution showed circular colonies with a raised elevation and smooth, opaque texture. These colonies were yellowish white in colour. At 10^{-2}, the elevation shifted to convex, while maintaining a circular shape and smooth texture; the colour appeared whitish grey. At 10^{-3} dilution, the colonies remained circular with a convex elevation, but became smoother and more evenly spread, exhibiting creamy or white coloration.

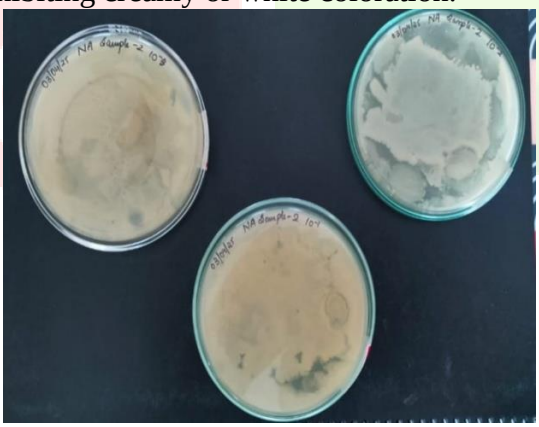

Fig.1 The culture grown in the nutrient agar from the sample 1

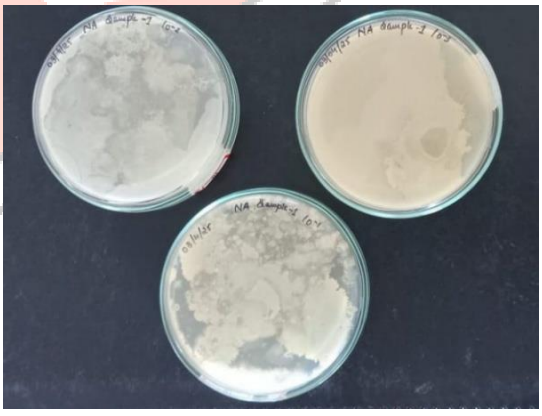

Fig.2 The culture grown in the nutrient agar from the sample 12

Colony characteristics of LBA sample -2

On LBA, colonies from Sample 2 displayed more diverse morphologies. At 10^{-1} dilution, small pinpoint colonies with yellow, orange, and brown pigmentation were observed. These colonies had smooth or slightly slimy textures with flowerlike or lobed margins. At higher dilutions (10^{-3} and 10^{-4}), colony size ranged from pinpoint to large, with colour shifts to orange, whole

Fig.3: Cultures grown in LBA from the sample -2

3.2.3 Heavy Metal Exposure

Some selected bacterial colonies from Sample 1 and Sample 2 were sub-cultured on fresh Nutrient Agar (NA) plates to obtain pure and isolated colonies. The sub-cultured colonies have got their original morphology, indicating stable and viable isolates suitable for further testing.

In the secondary phase, purified isolates were exposed to varying concentrations of Zinc (Zn) and Cadmium (Cd) on NA plates. In Sample 1, bacterial growth was observed on Zinc supplemented plates at all tested concentrations 50 ppm, 100 ppm, and 150 ppm, although colony size and density decreased with increasing concentrations indicating moderate Zn tolerance. However, no growth was observed on any Cadmium-supplemented plates, suggesting high sensitivity to Cd. Sample 2 showed no bacterial growth on either Zinc- or Cadmium-supplemented plate at any concentration, indicating complete growth inhibition and low tolerance to both metals. Heavy metal agar metals of Zn were subculture on the PDA. The zone of clearance in the zinc supplemented media /agar indicates that the microorganisms isolated from soil indicating their ability to tolerate or they adapted.

Table-2: The colony morphology of isolates from Sample 1 and Sample 2 grown on Zn and cd heavy metal agar(10^{-3})

Heavy Metal Agar (10^{-3})	Sample	Shape	Size	Colour	Texture	Edge	Growth/No growth
$ZnSO_4$	Sample 1	Rough circular	One third agar plate	Dark brown, pigmented	Cottony like	Irregular	Growth
	Sample 2	–	–	–	–	–	No growth
$CdCl_2$	Sample 1	–	–	–	–	–	No growth
	Sample 2	–	–	–	–	–	No growth

Table-3: The colony morphology of isolates from Sample 1 Znso4 on the PDA (10^{-3}).

colonies	Shape	Colour	Texture	Margin
Centre colony (PDA)	Circular, irregular and elongated	Light brown or tan	Mucoid and hard	Smooth
Edge colony (PDA)	Circular and slightly irregular	Milky yellow to beige	--	Entire smooth and continuous

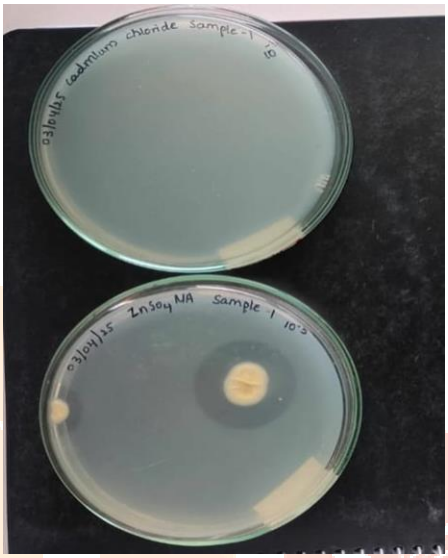

Fig.4: Colonies formed in the Znso4 heavy metal agar and cadmium chloride of sample-1

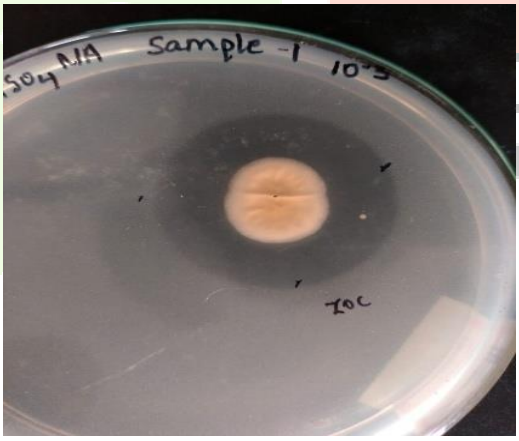

Fig.5: The zone of clearance on Znso4 heavy metal from the sample-1

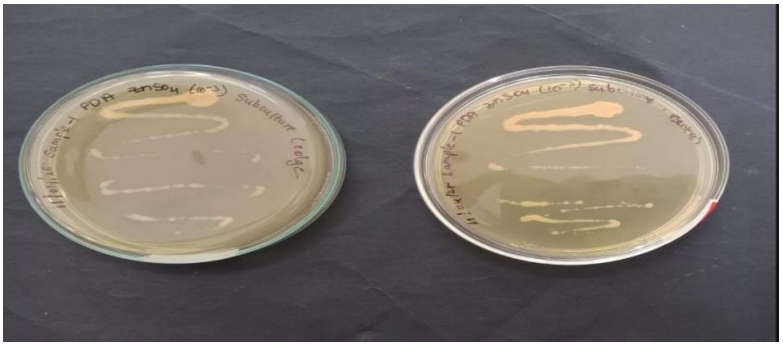

Fig.6: Subcultures of Znso4 heavy metal agar culture in Potato Dextrose Agar

3.2.4 Selective Media Screening, Gram Staining, and Biochemical Characterization of Heavy Metal-Resistant Bacterial Isolates

No growth was seen, which means the bacteria were either Gram-positive or couldn't break down lactose, so they are not part of the common Gram-negative bacteria found in EMB. Purified bacterial isolates from heavy metal-supplemented media were subjected to Gram staining to determine their Gram cell nature. The results indicated that the isolates comprised both Gram-positive and Gram-negative bacteria. It has a mix of cocci (spherical) and rod-shaped (bacilli) bacteria in both Gram groups. This morphological and staining diversity shows that there is a heterogeneous bacterial population capable of adapting to heavy metal stress conditions. Even the biochemical tests are conducted for the isolated cultures.

Table.4: Biochemical test for Zinc resistant and cadmium resistant isolate

Biochemical Test	Zinc-Resistant Isolate	Cadmium-Resistant Isolate
Indole Test	– (Negative)	– (Negative)
Methyl Red (MR) Test	+ (Positive)	+ (Positive)
Voges–Proskauer (VP)	– (Negative)	– (Negative)
Citrate Utilization	+ (Positive)	+ (Positive)

Both isolates exhibited the biochemical pattern (–, +, –, +) in IMViC tests. This doesn't match the common patterns of bacteria like Escherichia coli (+, +, –, –) or Enterobacter (+, +, –, –). The negative result on EMB agar, along with Gram staining evidence showing both Gram-positive and Gram-negative bacteria, suggests that these isolates are likely non-enteric, environmental bacteria. The pattern indicates that the isolates possess acid-forming metabolism (MR^+) and can utilize citrate as a sole carbon source, but do not produce indole or acetoin. This supports the idea that these strains are adapted to nutrient-variable or stress-inducing environments, such as heavy metal-contaminated soils.

3.2.5 Disc diffusion Assay for Heavy Metal Tolerance

In the disc diffusion assay distinct concentration inhibition patterns were observed across Zinc Sulphate and Cadmium Chloride. For Zinc Sulphate ($ZnSO_4$), bacteria isolated from sample1 exhibited moderate tolerance, with small zones of inhibition at 50 µg/disc, which progressively increased at 100 µg/disc and 150 µg/disc concentrations, indicating that bacterial sensitivity intensified with higher zinc levels. In contrast, isolates from sample-2 showed high sensitivity to Zinc Sulphate, with large and clear inhibition zones even at the lowest concentration, which further expanded with increasing concentrations, confirming significant susceptibility. The most striking results were observed with Cadmium chloride, where both samples exhibited complete sensitivity, with inhibition zones around all cadmium discs. The bacterial growth was entirely suppressed in the Cadmium-treated plates, indicating Cadmium's strong bacteriostatic effect across all tested concentrations.

Overall, the results revealed that Cadmium was the most toxic heavy metal to the bacterial isolates. Zinc sulphate ($ZnSO_4$) demonstrated moderate activity, with zones of inhibition ranging from 5.3 mm to 9.0 mm across both sample-1 and sample-2, indicating partial tolerance at lower concentrations and increasing sensitivity at higher concentrations. The inhibition zones slightly fluctuated, suggesting that Zinc, while inhibitory, allows some bacterial adaptability, possibly due to its biological role as a trace element. In contrast, Cadmium Chloride ($CdCl_2$) exhibited the most substantial inhibitory effect signifying a pronounced bacteriostatic effect and low microbial tolerance. Another Cadmium-exposed sample displayed a decreasing trend in inhibition at higher concentrations. Overall, Cadmium demonstrated the highest toxicity confirming

the differential tolerance of bacteria to heavy metal stress and their potential adaptation to sub-lethal concentrations.

Table.5 Zone of inhibition of heavy metals

Heavy Metal Treatment	Mean ZOI (mm)	SD (mm)	Minimum (mm)	Maximum (mm)
$ZnSO_4$ (S-1, Rep1)	8.43	0.51	7.9	8.9
$ZnSO_4$ (S-1, Rep2)	6.73	0.51	6.2	7.2
$ZnSO_4$ (S-2, Rep1)	6.30	1.00	5.3	7.3
$ZnSO_4$ (S-2, Rep2)	6.83	0.40	6.4	7.2
$CdCl_2$ (Sample-1)	11.96	2.21	9.7	14.2
$CdCl_2$ (Sample-2)	38.50	0.17	38.3	38.7

ZOI = Zone of Inhibition. Values are expressed as mean ± standard deviation (n = 3 discs per treatment).

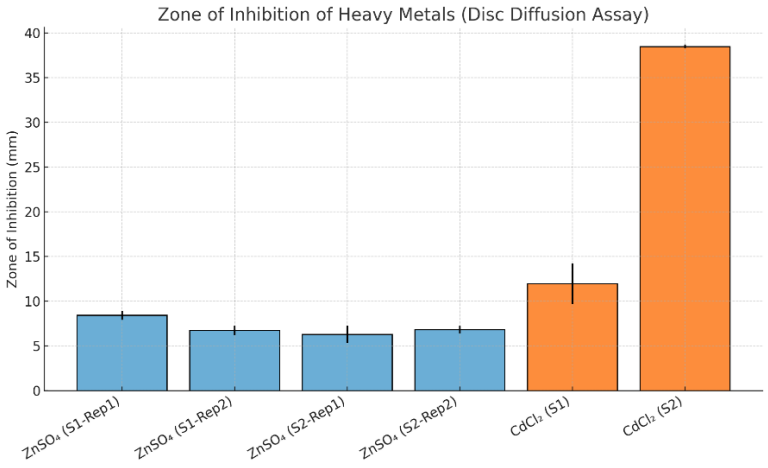

Fig.7: -Zone of inhibition for different heavy metal treatments

3.2.6 Sub-cultured in Vials containing Nutrient broth and Luria Broth

Bacterial isolates were successfully sub-cultured in Nutrient Broth (NB) and Luria Broth (LB) supplemented with Zinc Sulphate ($ZnSO_4$) and Cadmium Chloride ($CdCl_2$), at concentrations of 50 ppm, 60 ppm, 70 ppm, 80 ppm, and 90 ppm. In Nutrient broth, bacterial growth was observed across all concentrations for Zinc with reduced turbidity at higher concentrations, indicating partial tolerance and a concentration-dependent inhibitory effect. No visible growth was noted in Nutrient Broth supplemented with Cadmium, suggesting strong toxicity even at lower concentrations.

In contrast, no bacterial growth was observed in any of the Luria Broth (LB) vials across all heavy metals and concentrations. Instead, precipitate was observed forming at the bottom of the LB tubes, indicating chemical interactions or salt precipitation, which may have contributed to complete growth inhibition in LB.

3.2.7 Integrated discussion

This study provides valuable insights into how bacterial populations isolated from industrial soils respond to exposure to two heavy metals zinc ($ZnSO_4$) and cadmium ($CdCl_2$). The results showed that cadmium chloride ($CdCl_2$) was significantly more toxic to soil bacteria than zinc sulphate ($ZnSO_4$), consistent with earlier studies that cadmium disrupts essential cellular components, including DNA and enzymes. Saxena et al. (2022) and Hossain & Anwar (2012)]. In contrast, zinc sulphate showed milder antimicrobial

effects, and some bacterial tolerance was evident particularly in Sample 1 (Nies 1999). The variation in zinc tolerance between Sample 1 and Sample 2 shows the adaptive responses Qiao et al. (2017). The clearance zones confirm that microorganisms possess zinc-resistance or detoxification mechanisms (such as efflux, chelation, or biosorption). This demonstrates successful isolation of zinc-tolerant bacteria, suggesting their potential in bioremediation of zinc-contaminated environments and providing a model to study microbial adaptation under abiotic stress**.**

In agreement with Gadd (2010), it shows that cadmium severely inhibits microbial growth, possibly by interfering with DNA replication and enhancing ROS generation. Lack of growth on EMB agar suggests that metal-tolerant bacteria were not Gram-negative enteric while gram staining confirmed a diverse bacterial community, including both Gram-positive and Gram-negative forms, supporting the broader microbial diversity (Kirillova et al. 2017). The pigment production and mucoid colonies observed on PD suggest stress-related adaptations including biofilm formation (Gadd 2010).

IV. Conclusion

Overall, the study highlights metal-specific microbial tolerance, with Zinc showing limited compatibility and Cadmium being strongly inhibitory. These findings support the potential of using metal-tolerant microbes in bioremediation and environmental monitoring. Future studies should focus on molecular characterization of resistant strains for identification of genes and regulatory pathways (via PCR and whole-genome sequencing), biochemical assays and bioaccumulation capacity. Field trials, biofilm formation studies, and long-term adaptability assessments are essential to evaluate the real-world viability of these microbes. Together, the integration of molecular biology, environmental microbiology, and applied biotechnology will not only advance scientific understanding but also contribute significantly to sustainable environmental remediation and pollution management.

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