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# Isolation And Characterization Of Heavy Metal Tolerant Bacteria From Industrial Effluent

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**Abstract**

Heavy metal contamination has recently become one of the most critical environmental problems. Hazardous heavy metal poisoning of the environment is spreading around the world with the advancement of industries. Microorganism-related technologies could offer an alternative or replacement for traditional metal recovery or removal techniques. This research focuses on the investigation of heavy metal-tolerant bacteria isolated from industrial effluent in the CLZS, Chanderiya, Chittorgarh, Rajasthan, and their characterization. Initially, A total of 237 isolates were screened as Zn-tolerant bacteria, and 29 isolates were screened as Pb-tolerant bacteria from an industrial effluent sample. The three isolates were selected for further analysis due to their high level of heavy metal tolerance. The isolates were determined to be *Bacillus subtilis, Pseudomonas sp.*, and *Bacillus flexus* according to biochemical and morphological parameters. The identified isolates show high tolerance to heavy metals like Zn and Pb. They could be effective and useful as a potential bioremediation tool for heavy metals from industrial effluent.

**Keywords**- Heavy Metals, Environment, Industrial Effluent, Bioremediation, Tolerant

## I. Introduction

The increasing population and growing demand for industrial facilities to meet human demands have led to excessive use of available resources and rising air, water, and land pollution. One of the major environmental issues is the contamination of soil and water by heavy metals (Ahirwar *et al*., 2016; Cheng and S., 2003). Due to human activities like mining and the discharge of industrial waste, metals have accumulated in the environment and finally made their way up the food chain, causing serious ecological and health problems (Gupta and Kumar, 2012). The extensive use of industrial effluent for irrigation in most developing nations has the potential to change soil fertility. Gupta and Kumar (2012), Bouwer and H (2002), and Koropatnick *et al*., (1997) all point to the possibility that human health might be impacted by heavy metal deposition in plants due to wastewater irrigation.

While certain heavy metals have no known function and are entirely destructive, others are necessary for life at low concentrations but become toxic at high concentrations. Various heavy metals like As, Ag, Cd, Hg, Sb, and U are among the 17 most important heavy metals that are categorized as highly toxic. In contrast, Fe, Cu, Mo, and Mn are categorized as low toxicity whereas V, Co, W, Cr, Zn, and Ni as average toxicity (Badar *et al*., 2000; Gadd, 1992; Franke *et al*., 2003; Neis, 1999; Pandit *et al*., 2013; Shi *et al*., 2002). According to Ahirwar *et al*., (2019), bioremediation is a regulated spontaneous process that breaks down, degrades, or changes hazardous substances into less hazardous or non-toxic forms via microbial processes. Organic waste can be consumed by microorganisms. Numerous metabolites are produced during this process, which break down complex waste into simpler molecules.

To protect themselves from harmful heavy metals, microbes have evolved a wide range of defense mechanisms (Mustapha and Halimoon, 2015). Heavy metals must be eliminated from soil, water bodies, and wastewater using innovative treatment techniques.
Many microorganisms have been proposed as an affordable alternative to removing heavy metals from water and soil (Ahirwar *et al*., 2016; Waisberg *et al*., 2003). The study's overarching goal was to identify and describe morphologically and biochemically heavy metal-tolerant bacteria that might one day be used as a bioremediation tool to remediate industrial effluents.

## II. Material and methods

### 2.1 Sample Collection

The CLZS wastewater treatment facility in Chanderiya, Chittorgarh, Rajasthan, was sampled for its industrial effluent. The sample was collected in a pre-sterilized, nitric acid-acidified sampling bottle and aseptically transported to the laboratory to avoid contamination and extend its shelf life by being preserved at 4 $^0$c in the refrigerator (Mustapha and Halimoon, 2015).

### 2.2 Analysis of Physico-Chemical Parameters of Industrial Effluent

The various physicochemical parameters, such as pH, Turbidity, Total Dissolved Solids (TDS), Electrical conductivity (EC), Dissolved oxygen (DO), Biological Oxygen Demand (BOD), and Chemical Oxygen Demand (COD) of the effluent sample were determined by standard methods (Chaithra *et al*., 2023).

### 2.3 Analysis of Heavy Metal Concentration in Industrial Effluent

The heavy metals concentration in an effluent sample, including Zn, Mn, Pb, Fe, and Cr, were determined with an Atomic Absorption Spectrophotometer (AAS) (Perkin Elmer Analyst 200) (Bahiru, 2019).

### 2.4 Isolation of Bacterial Strain from Industrial Effluent

Heavy metal tolerant bacteria were isolated on nutrient agar medium supplemented with varying concentrations of Zinc sulfate ($ZnSO_4$) and lead nitrate ($Pb(NO_3)_2$) ranging from 50 ug/ml to 250 ug/ml. The sterility of the nutrient agar medium was maintained by autoclaving at 121$^0$c,15 psi for 15 min. Various concentrations of heavy metals were added to nutrient agar medium and poured into Petri plates. The effluent sample was successively diluted in 9 ml of sterile saline water in 5 test tubes, where 1 ml of the effluent was added to the first test tube to obtain a $10^{-1}$ serial dilution, repeating this until $10^{-5}$. Then, 0.1 ml of the diluted solution was aseptically streaked on the surface of nutrient agar supplemented with various concentrations of heavy metals and maintained at 36-37$^0$C for 48 hours.

Several colonies were obtained, and unique colonies were chosen according to their colony morphology. The isolated colonies were then streaked individually to obtain a pure culture of isolate, it was cultured for 24 hours at 37$^0$c on a nutrient agar medium. Each isolate was examined for its colony shape, color, elevation, size, and texture. Stocks were subsequently prepared with 40% glycerol and stored at 20°C until needed for further analysis (Khanam *et al*., 2024).

### 2.5 Morphological and Biochemical Characterization of Isolated Bacterial Strains

The obtained bacterial strains were characterized using a variety of biochemical assays, cultural traits, and microscopic features. The findings were contrasted with information from the ninth edition of Bergey's Manual of Determinative Bacteriology. The motility test, endospore staining, and gram staining were used for morphological characterization.

Biochemical tests including Oxidase test, Catalase test, Nitrate reduction test, Citrate utilization test, Methyl red & Voges-Proskauer test (MR-VP test), Indole production test, Gelatin hydrolysis test, and $H_2S$ Production (Triple sugar iron (TSI) test), Starch hydrolysis test was performed for biochemical characterization (Abbas *et al*., 2014).

## III. Results and discussion

### 3.1 Physicochemical analysis of effluent sample

Table 1 depicts the physicochemical properties of industrial effluent. The Physico-chemical characterization of industrial effluent revealed that the sample resulted in a foul smell and was pale yellow. **pH** is among the most crucial factors in assessing how aggressive water is. More acidic (lower pH) water is more corrosive (Nigam *et al*., 2015). The pH of the effluent sample was 7.4. The high pH value indicated a slightly alkaline nature of the effluent sample. The alkaline nature of the effluent is due to weak basic salts.

**Turbidity** is a measure of the total suspended solids (TSS), which is measured in Nephelometric unit (NTU). The water's transparency and light-scattering properties are determined by the concentration of the substance (Banunle *et al*., 2018). The turbidity of the effluent sample was 8.9 NTU. **Electrical conductivity (EC)** is a measure of its ability to conduct electricity and indirectly indicates the amount of salt in the water (Parveen *et al*., 2017). The unit of measure is Siemens/meter (S/M). The EC value of the effluent sample was determined to be 32000 us/cm.

**Total dissolved solids (TDS)** refer to all the inorganic salts that are dissolved in water, together with a tiny quantity of organic stuff. The presence of dissolved organic and inorganic contaminants is behind the higher TDS value (Parveen *et al*., 2017). The TDS of the effluent sample was 20800 mg/l.

**Dissolved oxygen (DO):** The dissolved oxygen (DO) of the effluent sample indicates the quantity of oxygen in the water. Numerous factors, including temperature and salinity, influence it: daylight, air pressure, and water turbulence (Banunle *et al*.,2018). The DO of the effluent sample was found to be 3.1 mg/l.

**Biochemical Oxygen Demand (BOD)** is the quantity of dissolved oxygen that aerobic bacteria require to break down organic materials in water. The high concentration of dissolved organic matter resulted in high oxygen consumption and elevated the BOD level. It is a well-established parameter for organic pollutants in surface and wastewater. It shows how many organic molecules are present in water (Benit and Roslin, 2015). The BOD of the effluent sample was 28 mg/l.

**Chemical Oxygen Demand (COD)** measures the amount of oxygen in water required to chemically oxidize the organic materials and inorganic nutrients. 110.31 mg/l of COD was found in the effluent sample.

**Table 1: Physicochemical characteristics of effluent sample.**

| S.No. | Parameter | Result |
|---|---|---|
| 1. | pH | 7.4 |
| 2. | Electrical conductivity (EC) | 32000 us/cm. |
| 3. | Total dissolved solids (TDS) | 20800 mg/l. |
| 4. | Turbidity | 8.9 NTU |
| 5. | Dissolved oxygen (DO) | 3.1 mg/l. |
| 6. | Biological oxygen demand (BOD) | 28 mg/l. |
| 7. | Chemical oxygen demand (COD) | 110.31 mg/l. |

### 3.2 Heavy Metal Estimation

The predominant composition of these pollutants is heavy metal ions derived from mining effluent. Heavy elements such as Zn, Mn, Pb, Fe, and Cr were among those analyzed in the sample. Heavy metal concentration was in the order of Zn>Mn>Fe>Pb>Cr as revealed by the results. The concentration of heavy metals in the effluent sample is presented in Table 2. The highest levels of heavy metals in the effluent sample were related to Zn and the lowest to Cr (Sorsa *et al*., 2015).

**Table 2: Concentration of heavy metals in the wastewater sample.**

| S.No. | Heavy metal | Concentration (PPM) |
|---|---|---|
| 1. | Zinc | 14.10 |
| 2. | Manganese | 3.25 |
| 3. | Lead | 1.0 |
| 4. | Iron | 1.12 |
| 5. | Chromium | 0.25 |

### 3.3 Isolation of heavy metal-tolerant bacteria

Here, we describe and classify microorganisms that can withstand heavy metals found in wastewater from industries. Initially, 237 colonies were screened as Zn tolerant, and 29 colonies as Pb tolerant from a nutrient agar medium supplemented with 50 ug/ml to 250 ug/ml concentrations of heavy metals, respectively. Out of 237 colonies, two isolates of Zn tolerant, namely Zn1 and Zn2, and one isolate of Pb tolerant, namely Pb1, out of 29 colonies, were selected based on the high degree of heavy metals tolerance capability and were used for further investigation (Ahirwar *et al*., 2016). A pure culture of isolates was maintained on a nutrient agar medium for further characterization. Figure 1 displays the pure culture of bacterial colonies that have been isolated.

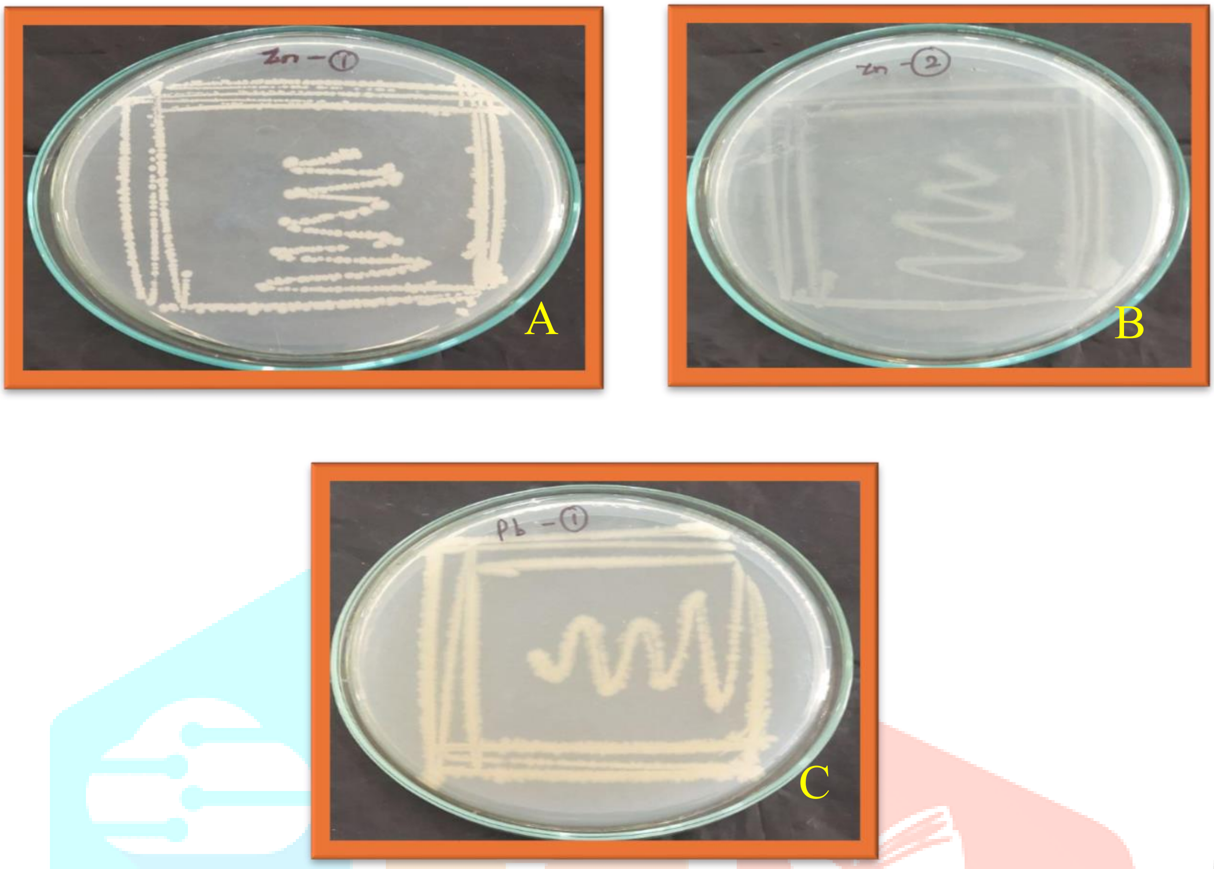


**Figure 1: Pure culture of Isolates. Isolate Zn1 (A) Isolate Zn2 (B) Isolate Pb1 (C)**

### 3.4 Morphological and Biochemical characteristics of Isolated bacterial strains

Isolate Zn1 was brownish and in a small, round form with a flat elevation, and Isolate Zn2 was white in color, moderate, and irregular in form with a flat elevation. Meanwhile, Isolate Pb1 was yellowish in color, moderate, and round in form with flat elevation. After performing gram staining, endospore staining, and a motility test, it was observed that isolates Zn1 and Pb1 were gram-positive and isolate Zn2 was gram-negative. Isolate Zn1 was spore-forming, whereas isolates Zn2 and Pb1 were nonspore-forming. All three isolates were non-motile. Phenotypic study results have been depicted in Tables 3 & 4. Biochemical study shows that all three isolates were positive for nitrate reductase and citrate utilization test and negative for indole production and gelatin hydrolysis. Isolates Zn2 and Pb1 were positive for the oxidase test, whereas Zn1 showed a negative result. For catalase tests, isolates Zn1 and Pb1 were negative, and isolate Zn2 showed a positive result. Isolate Zn1 and Zn2 were negative for methyl red-Voges Proskauer test, whereas isolate Pb1 was positive for methyl red and negative for Voges Proskauer test. Isolate Pb1 fermented all three sugars: glucose, lactose, and sucrose. Isolate Zn2 showed a negative result for sugar fermentation, whereas Isolate Zn1 fermented only glucose. For the starch hydrolysis test, isolates Zn1 and Pb1 were positive, and isolate Zn2 was negative. The biochemical characteristics of isolated bacterial strains have been summarized in Table 5.

**Table 3: Colony Morphological characteristics of isolated bacterial strains**

| Isolate no. | Size | color | Elevation | Form |
|---|---|---|---|---|
| Zn1 | Small | Brownish | Flat | Round |
| Zn2 | Moderate | Whitish | Flat | Irregular |
| Pb1 | Moderate | Yellowish | Flat | Round |

**Table 4: Microscopic characteristics of isolated bacterial strains**

| Isolate no. | Gram' staining | Endospore staining |
|---|---|---|
| Zn1 | Positive | Positive |
| Zn2 | Negative | Negative |
| Pb1 | Positive | Negative |

**Table 5: Biochemical characteristics of bacterial strains that have been isolated**

| Isolate no. /Biochemical characteristics | Zn1 | Zn2 | Pb1 |
|---|---|---|---|
| Oxidase test | Negative | Positive | Positive |
| Catalase test | Negative | Positive | Negative |
| Nitrate reductase test | Positive | Positive | Positive |
| Citrate utilization test | Positive | Positive | Negative |
| Methyl red test | Negative | Negative | Positive |
| Voges Proskauer test | Negative | Negative | Negative |
| Indole production test | Negative | Negative | Negative |
| Gelatin Hydrolysis Test | Negative | Negative | Negative |
| Triple sugar iron test | Alk/A | Alk/Alk | A/A |
| Starch Hydrolysis test | Positive | Negative | Positive |

## V. Conclusion

The ability of some microbial strains to thrive when exposed to heavy metals might be used for wastewater treatment purposes. We set out to find microorganisms that might withstand heavy metals in the industrial effluent of CLZS in Chanderiya, Chittorgarh, Rajasthan. We have isolated and characterized three Zn and Pb-tolerant bacterial strains and identified them based on biochemical characterization. The Zn tolerant isolate Zn1 is identified as a *Bacillus subtilis* and Zn 2 is identified as a *Pseudomonas sp*., whereas isolate Pb1 is *Bacillus flexus*. The results demonstrate that the isolates can withstand the tested heavy metals with relative ease. Therefore, there is hope for the bioremediation of industrial wastewater using these isolates.

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