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EXPLORATION, ISOLATION, PHENOTYPICAL, & BIOCHEMICAL CHARACTERIZATION OF INDIGENOUS METALLOTOLERANT BACTERIAL ISOLATES FROM HATTAR INDUSTRIAL ESTATE, HARIPUR: TOWARDS ENVIRONMENTAL REMEDIATION AND SUSTAINABLE INDUSTRIAL PRACTICES

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Abstract

This study addresses heavy metal contamination in Hattar Industrial Estate, Haripur, Pakistan, emphasizing the global issue of environmental pollution. The research focuses on isolating and characterizing metallotolerant bacterial strains from the contaminated soil, aiming to contribute to sustainable industrial practices and environmental health. Soil samples from Hattar Industrial Estate were collected and metallotolerant bacteria were isolated using a serial dilution method. two strains (Strain A, Strain B) were selected for further analysis based on their resistance to heavy metals. Morphological and biochemical characterization, including catalase, citrate, indole, urease, coagulase, and oxidase tests, were performed to understand the strains' traits and metabolic capabilities. The isolated strains, identified as Kingella sp., Listeria sp., demonstrated distinct morphological characteristics. Biochemical tests revealed variations in catalase, citrate utilization, indole production, urease activity, coagulase reaction, and oxidase activity among the strains. The results indicated the strains' potential for biological remediation, with specific resistance to heavy metals. The study underscores the importance of bioremediation as an eco-friendly approach to mitigate heavy metal contamination. The isolated bacterial strains, resistant to various heavy metals, hold promise for sustainable remediation strategies. The research advocates for the application of these strains in

addressing heavy metal pollution, contributing to environmental sustainability, and promoting eco-friendly practices in industrial areas.

Keywords: Metallotolerant bacteria, Heavy metal contamination, Bioremediation, Environmental health, Sustainable industrial practices.

INTRODUCTION

Nine million premature deaths in 2015 were attributed to pollution, a global problem that raises the risk of disease and mortality. This is more than three times the total number of deaths from AIDS, TB, and dengue fever combined (1, 2). Environmental contamination is more common in middle- and low-income countries because of poverty, a lack of legislation, ignorance, and other reasons include daily exposure and fast-paced lifestyles (3). Without realizing it, people contaminate the environment by disposing of chemical waste improperly, burning brush, and deforestation. Population density increases the influence of the environment (4). Environmental pollution is still a worldwide problem due to its interconnection, which is fueled by a number of factors including population growth, industrialization, urbanization, exploration and mining, and transboundary movement (5).

A class of metals and metalloids having an atomic density of more than or equal to 4,000 kg/m3 are known as heavy metals (6). Heavy metals such as cadmium, copper, zinc, and arsenic are dangerous to humans and plants when their concentrations exceed allowable limits, while lead, mercury, and arsenic are not necessary (7). Heavy metal pollution in fertilizers, sewage sludge, industrial effluent, and soil has a negative impact on food production, quality, and health. Plants have the ability to collect metals, slow down growth, and generate less biomass (8). The level of oxidation of the metal determines how poisonous the soil is; heavier metals like Cr (VI) are dangerous. The pH, soil composition, and soil structure all have a significant impact on metal adsorption, desorption, and complexation(9).

Acidic soils contain more mobile and harmful heavy metals, with different species having different levels of toxicity. Hyper accumulators are capable of storing large amounts of metals(10), have barrier mechanisms that protect them from the harm caused by the pressure caused by heavy metals, despite the fact that the length and intensity of exposure as well as other environmental factors increase the People who are exposed to high levels of heavy metals may suffer from a variety of diseases, such as osteoporosis, gastrointestinal and renal toxicity, cardiovascular problems, tumors, hepatitis, and melancholy (11-13). Exposure to heavy metals during infancy, youth, and adolescence can lead to memory problems and developmental problems. Water supplies are contaminated by industrial activity, which creates hazardous issues and interferes with natural benefits (7). Both naturally occurring and artificially manufactured heavy metals are hazardous to human health and aquatic life. Because of their higher bioavailability, excessive creation, transfer, and industrial discharge, they end up accumulating in soil and water (14).

Heavy metals in soil and marine environments pose risks to human health and the food chain, which emphasizes the need for practical methods to remove, recover, and stabilize these contaminants (15). Heavy metal pollution in ecosystems threatens human health in developing nations, affecting aquatic life and causing toxic effects on plants, animals, and people (16). Heavy metals, released into the environment, pose a threat to human health and organisms, as they can interfere with biomolecules like proteins and nucleic acids (17).

Enhancing human health and environmental sustainability, bioremediation is a low-tech, ecologically safe method that uses naturally existing biological activity to break down and neutralize contaminants (18). In the bioremediation process, which removes contaminants from polluted settings, microorganisms are employed. These microorganisms have the ability to degrade and use poisons in

their metabolic processes. The microbial population, surrounding environment, and tolerance to heavy metals are some of the factors that affect how effective bioremediation is (19).

Hattar Industrial Estate Haripur

The 34.4 square kilometer Hattar Industrial Estate in Haripur is home to over 2000 small and medium-sized businesses that manufacture a wide range of products, including textiles, leather goods, chemicals, detergents, iron, steel, vegetable oils, drinks, and culinary items (20). With the hope of using the isolated native heavy metal-tolerant bacterial strains from the Hattar Industrial Estate in biological remediation efforts to detoxify polluted soil, the study hopes to do just that. This strategy is regarded as a sustainable way to deal with environmental risks as it is economical, efficient, and ecologically benign.

MATERIAL AND METHODS

Isolation and collection of samples

Soil samples from Hattar Industrial Estate in Haripur were used to extract indigenous metallotolerant bacteria using a serial dilution approach. The resulting colonies were transferred to starch agar plates for further analysis after being cleaned and grown on nutrient agar plates. The isolates were maintained at 4°C until further investigation.

Identification of Metallotolerant Bacterial Isolates

Morphological characterization

Using a variety of growth medium, including Nutrient Agar, MacConkey Agar, tryptic soya Agar, Eosin methylene blue, and Luria broth, the study looked at the morphology of a specific bacterial strain(21).

Gram Staining

The basis of gram staining is the bacterial cell wall's capacity to hold onto the crystal violet dye following solvent treatment. Gram-positive bacteria absorb the dye, but the solvent breaks down the lipid layer, leaving the germs colorless (9).

Gram reagent was prepared using crystal violet, safranin counterstain, acetone, and Lugol iodine. To create isolates, pure culture was spread out on a slide, and then acetone, crystal violet, Lugol's Iodine, and safranin were added. The mixture was then dried, stained, and examined under a microscope.

Biochemical Characterization

Numerous biochemical tests, such as those using catalase, coagulase, citrate agar, indole, Kovac's oxidase, and urea, were conducted on the bacterial strain. outlined in brief below.

Catalase Test

The test determines if catalase, an enzyme that converts hydrogen peroxide into oxygen and water, is present by inducing bubbles of oxygen in a small inoculum (22). The production of catalase was investigated by adding H2O2 (3 percent v/v.) to a bacterial culture, which exhibited free oxygen gas bubbles. A single colony was put on a sanitized slide and smeared with hydrogen peroxide.

Coagulase Test

The protein known as coagulase, which resembles an enzyme, transforms fibrinogen into fibrin, resulting in a plasma clot. Two types of coagulases are produced by Staphylococcus aureus: bound and free. Cells clump together as a result of the bound coagulase's instant interaction with fibrinogen when combined with plasma. Plasma coagulase-reacting factor (CRP), when activated by free coagulase, combines with fibrinogen to form fibrin clots. Attach a physiological saline drop to a slide, then combine one solution with either human or rabbit plasma to test for coagulase clumping. After

diluting the plasma with physiological saline, mark test tubes T, P, and N. After an hour, check the tubes for clotting by keeping them incubated at 35–37 degrees Celsius.

Citrate Agar Test

Citrate agar measures how well an organism uses its energy by using the citrate-permease enzyme to convert citrate to pyruvate. On this media, bacteria proliferate, suggesting that the Krebs cycle uses citrate. The breakdown of ammonium salts raises alkalinity and pH over 7.6, which turns the green bromothymol blue indicator blue (23). Apply a light inoculum to the slant of a colony, allow it to incubate aerobically at 35–37 degrees Celsius for 24 hours, and see if the colony's color changes from green to blue.

Indole Test

The indole test determines the ability of an organism to produce indole from the breakdown of the amino acid tryptophan (23). Tryptophanase hydrolyzes tryptophan to potentially yield three products: ammonium ion, pyruvate, and indole. The process of detecting indole entails an acidic chemical interaction between indole and Kovac's reagent. A rosin dole red dye that is formed by para-dimethyl amino benzaldehyde precipitates and rises to the medium's surface. Adequate tryptophan is necessary for an appropriate indole test medium. After allowing the organism to incubate in the broth, add Kovac's reagent and check the liquid.

Kovac's Oxidase Test

Cytochrome c, a molecule identified by the oxidase test in bacteria, oxidizes the purple pigment tetramethyl-p-phenylene-diamine. This enzyme is produced by cyanochrome-containing organisms. In the test, fresh bacterial culture is employed. Use one percent Kovac reagent on filter paper. In 30 to 60 seconds, the results are displayed.

Urease Test

When amino acids are decarboxylated to form urea, which alkalizes the medium, ammonia and CO2 are produced. Phenol red's hue shift signifies a pH change. For urease-positive species, the medium turns pink in 24 hours; for weakly positive species, it takes many days; and for negative species, there is no color change at all.

RESULTS

Isolation and Identification

Two bacterial strains, strains A and B, were identified from soil polluted with heavy metals at the Hattar Industrial Estate in Haripur for the current study. A preliminary screening approach for heavy metal resistance capabilities revealed that all of the collected samples grew satisfactorily under conditions of heavy metal-containing growth. Serial dilutions of all samples reveal two (2) different isolates from the population of heavy metal-resistant bacteria based on their appearance.

Morphological characterization

The bacterial isolates were then morphologically evaluated using different media, revealing that Kingella sp. (strain B) does not grow on MacConkey, Listeria sp. (strain A) did. (Table 1).

Table 1: Characterization of the morphology of isolated bacteria

Bacterial Species	MacConkey Agar	EMB Agar	Nutrient Agar	Tryptic Soya Agar	LB Medium
Listeria sp.	No Growth	No Growth	Growth	Growth	Growth
Kingella sp.	No Growth	No Growth	No Growth	Growth	Growth

Gram staining

When bacteria in a sample react with the stain, the bacteria can remain purple or change color to pink or red. Staining with purple indicates that the bacteria are Gram-positive. Bacteria are considered Gram-negative if they change color to pink or red. The findings section shows that two isolates, Strain A (Listeria sp.), was determined to be gram-positive cocci, while the other two, Strain B (Kingella sp.), was determined to be gram-negative rods bacteria (Figure 1).

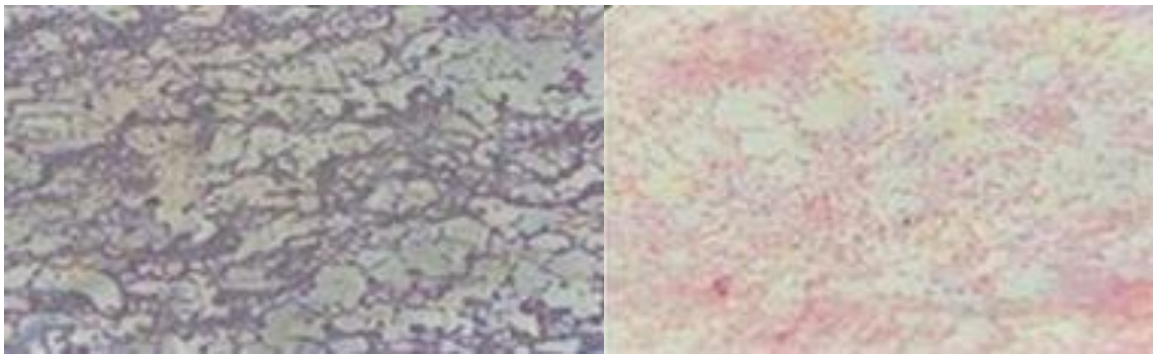

Figure 1: *microscopy of isolated bacteria (Strain A-Gram Positive, Strain B- Gram Negative)*

Biochemical characterization

On biochemical tests, the bacterial isolates were recognized using following common biochemical assays.

Table 2: *Biochemical characterization of bacterial isolates*

Test	**Strain A (**Listeria sp.)	**Strain B (**Kingella sp.)
	+	-
CATALASE	+	-
CITRATE	-	-
INDOLE	-	-
UREASE	-	-
COAGULASE	+	+
OXIDASE		

Catalase Test

Positive catalase test result is exhibited by the test microorganism producing gas bubbles on a glass slide following treatment with a few drops of 3 percent H2O2. The catalase test yielded results for bacterial isolates that varied from strain A positive to strain B Negative. (Table 2, Figure 2).

Figure 2: *Catalase test results for strain A and B bacterial isolates*

Citrate Agar Test

The medium turns royal blue when inoculated with Salmonella typhimurium in Citrate agar. The citrate test came out positive. The medium stays green when Escherichia coli is injected into Citrate

agar. In other words, the citrate test came up empty. In terms of bacterial isolates, the Citrate Agar Test yielded strain A positive, and strain B negative results (Table 2).

Indole Test

After a few seconds of introducing the reagents, red color will form in the reagent layer on the top of the agar deep, indicating a positive indole test. Persistence of yellowish or slightly cloudy reagent layer indicates that the culture is indole negative. Bacterial isolates tested negative with an Indole test at strain A and strain B concentrations (Table 2, Figure 3).

Figure 3: *Bacterial isolates were tested using the Indole method.*

Urease Test

Urease production is marked by a slanting, sometimes butt-extending, brilliant pink (fuchsia) color. Keep in mind that a favorable reaction is indicated by any level of pink. The hydrolysis of proteins in the medium can provide a false-positive result if the incubation time is too long. For bacterial isolates, the Urease Test yielded negative findings (Table 2, Figure 4).

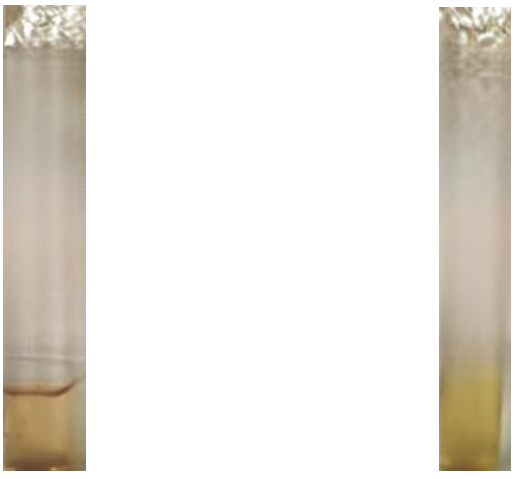

Figure 4: *Bacterial isolates undergone urease testing.*

Coagulase Test

When the bacterial cells agglutinate after plasma is injected, it is considered a positive test. When no clumps form, it means the test was negative. For bacterial isolates, the Coagulase test yielded negative findings for both strains (Table 2, Figure 5).

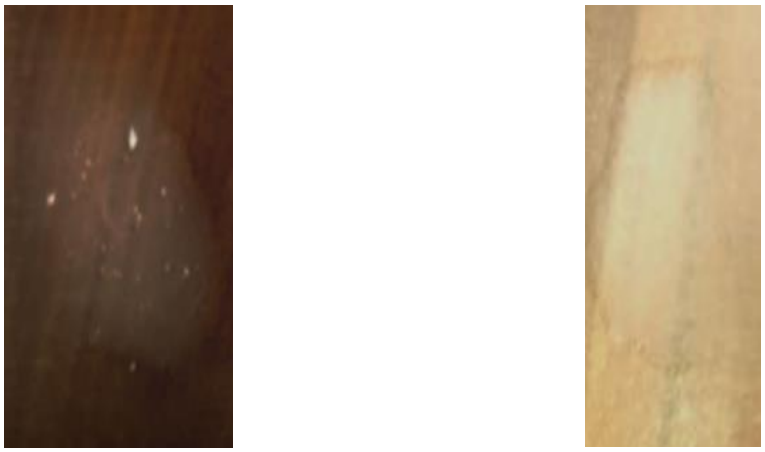

Figure 5: *Coagulase Test for all the bacterial isolates*

Oxidase Test

Cytochrome oxide, a hallmark of saprophytic bacteria, is at the heart of Kovac's oxidase test. If the purple color developed between 30 and 60 seconds, the bacterium passed the test. Two oxidase positive isolates were used in our study. Both bacterial isolates tested positive (Table 2, Figure 6).

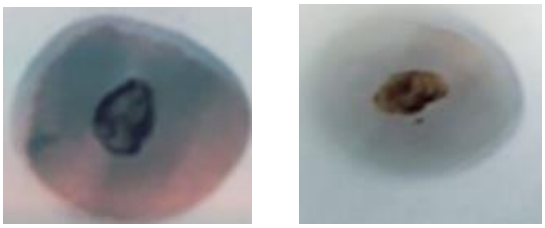

Figure 6: *Oxidase Test for both bacterial isolates*

DISCUSSION

The Hattar Industrial Estate (HIE) is part of the industrial landscape in Hattar, Haripur. It is located inside the district and covers an area of 34.4 square kilometers. About 2000 different small and medium-sized industrial units are housed on this estate, which helps to produce a wide range of commodities, including leather, textiles, chemicals, detergents, iron, steel, vegetable oils, drinks, and culinary items (20).

When heavy metals are inadvertently discharged into soil or water, or when they are exposed to elevated quantities of these metals, they can cause significant harm to humans, plants, and animals. This is because heavy metals cannot be changed into non-toxic forms and have long-term effects on the flora and wildlife. Even at extremely low concentrations, many of them—such as lead, nickel, zinc, chromium, copper, selenium, silver, mercury, cadmium, arsenic, and others—are not only cytotoxic but also carcinogenic and mutagenic. There are serious health risks associated with exposure to toxic metals (24).

The goal of this effort is to isolate heavy metal-tolerant local strains of bacteria for use in biological remediation, the most cost-effective, environmentally safe, and efficient way to remove heavy metals from the atmosphere. Knowing the harmful effects of heavy metals, this is done with the goal of detoxifying the contaminated soil. Isolation of bacteria resistant to heavy metals. This work aims to isolate and characterize heavy metal-resistant bacteria from industrially contaminated soil. Out of the 110 colonies that were screened from the first batch of nutrient agar medium enhanced with heavy metals, 19 isolates were selected from the soil for additional screening, and two strains were ultimately selected for additional research due to their high level of heavy metal tolerance. Two bacterial strains, designated as strains A and B, were found in heavy metal-contaminated soil at Hattar Industrial Estate, Haripur, during the current study.

Following a preliminary heavy metal resistance screening process, all samples collected showed positive development in heavy metal-containing growth conditions. Serial dilutions of all samples, based solely on appearance, identify two (2) distinct isolates from the heavy metal-resistant bacterial population.

Listeria sp. (strain A) and kingella sp. (strain B) did not grow on MacConkey. These findings were made after morphological characterization of the bacterial isolates on various media. As per Bergey's Manual of Determinative Bacteriology, several scientists conducted research in this vein (25, 26).

Gram staining revealed the isolate (strain B) to be gram-negative cocci while the isolate (strain A) was classified as gram-positive rod bacteria, as the findings section illustrates. Alike investigation was undertaken by Marzan et al. (2017) were identified to be gram-positive bacteria and the other (S4) to be gram-negative bacteria by the identification of peptidoglycan, which is a thick coating present on bacteria (Burke and Pister, 1986). Many researchers have reported a range of bacterial species that are resistant to arsenic (27, 28). Another study found that the strains Proteus vulgaris (MR1), Pseudomonas fluorescence (SS4), and Pseudomonas fluorescence (SS5) were Gram-negative, rod-shaped motile bacteria, while Bacillus cereus (MR2) and Bacillus decolorationis (MR3) were Gram-positive, rod-shaped motile bacteria (29).

As part of the biochemical characterization process, strains A and B of the isolates were found to be positive for Oxidase, positive for Catalase, positive for Citrate, negative for Urease, negative for Indole, negative for Urease, negative for Coagulase, and negative for Oxidase. Similar research was carried out by Baker (27), Micrococcus sp. may be identified from other gram-positive bacteria by their oxidase positivity, in contrast to most Staphylococcus sp., which are usually oxidase-negative (30). S4 (Micrococcus sp.) was found to be oxidase positive in their investigation, and it could be Micrococcus luteus because it developed yellow to brown colonies on growth media rather than the red colony of Micrococcus roseus. Gram staining, oxidase testing, and carbohydrate utilization studies revealed that S1 has characteristics with Gemella sp., which is gram positive, oxidase negative, and uses all carbs (31).

CONCLUSION

Our investigation successfully identified and characterized Metallotolerant bacterial species from the Hattar Industrial Estate, which is a significant advancement in the fight against heavy metal pollution. The unique traits of Listeria and Kingella species, along with their resistance to certain heavy metals, demonstrate their potential for effective biological remediation. The results show that it is feasible to use these strains in sustainable methods to detoxify contaminated soil, improving environmental quality and sustainable industrial processes. By using these native strains, we can minimize the negative environmental consequences of industrial activities while addressing the problems associated with heavy metal pollution.

Authors Contributions

Ayisha Bibi: Data collection, Data curation, Editing, Writing the first draft, supervision; **Syed Zeeshan Haider**: Study design, Data analysis, Editing; **Aamna Shah**: Data analysis, Data curation, Editing; **Sadia Ikram & Saima Inam**: Data analysis, Editing. **Attiq Ullah**, **Abdul Ghafar Khan & Sumaira Noor**: Study design, Data analysis; **Sania Javed**: Data collection, Data curation.

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