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Isolation and Characterization of Heavy Metals-Resistant Bacterial Strain from Industrial Effluents of Metal Processing Industries

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KEYWORDS

Bacteria, Heavy Metal Resistance, Industrial effluents, Toxicity, Culture medium.

ABSTRACT:

Most Industries release their effluents in natural reservoirs after necessary treatment. In due course of time, heavy metal contaminants are accumulated in natural reservoirs and contaminate the natural ecosystem. Heavy metals like Ni, Al, Fe, Cr, Zn, Cu, Cd, and Pb are released in natural reservoirs from industrial effluents. It accumulates and pollutes the sail and water ecosystem at large. Heavy metals released from industrial wastes and combustion of non-renewable fuels contaminate the underground water resources and various horizons of soil, which causes deleterious ill effects on human health. The second most prevalent source of heavy metal contamination includes industrial waste, metal corrosion, effluents of metal processing industries, and mining practices, which infiltrate the underground water and soil. In the current research, five small metal processing industrial sites were identified for the isolation and characterization of the heavy metal-resistance bacterial strain. Contaminated soil and water samples were collected from the wastewater disposal site of the industries. After the identification of the heavy metals in water and soil samples of the contaminated areas of the waste disposal sites were taken to isolate heavy metal resistance strains of microbial flora. To isolate the heavy metal-resistant bacterial strain, the culture medium was fortified with optimum concentration of heavy metal salts. After the isolation and biochemical characterization of isolated bacterial strains, a variety of bacteria with unidentified strains were obtained. A series of known bacterial strains were identified that survive in the heavy metal enriched environment. To identify the species and strain of the bacteria, molecular characterization was subjected to study. The purpose of the present study is to isolate the bacterial strain of heavy metal resistance and to apply it for the purpose of heavy metal bioremediation. After the validation and optimization of bacterial mass culture production, it may be used for the reclamation of soil and contaminated drinking water.

1. Introduction

Industrial effluent includes a variety of contaminants having toxic and health hazardous effects on natural resources and human health. Most of the industrial effluents are utilized for the purpose of field irrigation after their treatment as per the industrial waste treatment guideline. After the treatment of industrial wastewater, most of the insoluble contaminants are separated but soluble contaminants may be present in the treated wastewater. The treated wastewater utilized for field irrigation causes deleterious toxic and health hazardous effects on the natural reservoir and ultimately affects plants, animals, and human beings. The soil contamination by the release of toxic heavy metals in underground water or soil is absorbed by the crops, as well as rendering land inappropriate for plant development. The usage of contaminated water regularly for irrigation purposes, diminishes the soil's ability to hold heavy metals. Toxic heavy metal exposure reduces

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microbial community and activity in the soil, consequently reducing soil quality and crop development.

Heavy metals are divided into 3 categories: transition metal, metalloid, and basic metal. The metallic group has qualities that are less than 20 atomic masses. The toxicity of metalloids and light metals varies depending on metal oxidation state and concentrations. Certain heavy metals are trace elements at some oxidation states but become exceedingly dangerous at other oxidation states. As a result, not all heavy metals are hazardous to living species. Several heavy metals are important components at lower concentrations but become harmful as concentration increases. Nevertheless, heavy metals like Hg and Pb, are very dangerous even at low concentrations [1]–[4]. heavy metals, for instance, become poisonous in dissolved form, influencing the physiologic condition of organisms. Whenever heavy metals are present in the state of cations, they may attach to protein as well as other biomolecules, causing toxicity. As a result, non-essential heavy metal build-up in organism tissue was exploited as a bio-indicators of environment contamination [5]–[7].

Several industrialized countries have major environment concerns and difficulties in regulating heavy metal contamination, as well as health-related problems, with very limited effectiveness in preventing heavy metal contamination in the environment. This is a significant issue in developing nations. Consumption usage of mining, industrial wastes, heavy metals, and the use of fossil fuels all contribute to heavy metal build-up in the food chain, water, sediments, and soil. To reduce pollution of heavy metal, an efficient monitoring system that controls industrial wastewater pollutants prior to their being discharged into the environment is required. Heavy metal toxicity has expanded fast in many regions of the globe as a result of industrialization and increasing pollution discharge into aquatic and terrestrial habitats, posing concerns to human health. Heavy metal contamination has been linked to the contamination of domesticated animals, plants, and people when ingested. Numerous research found that heavy metal contamination was a major contributor to decreased populations in various ecosystems. Heavy metals are well known to cause chronic illnesses when they concentrate abundantly in organisms.

Bioremediation:

Micro-organisms are an important part of elemental bio-geochemical cycles of metal transformations between insoluble and soluble species, they are ubiquitously present everywhere in nature. Several researches have evidenced that interaction between the microbes and metals can either be beneficial for the environment. Geoffrey Michael Gadd [8] studied about the significant role of microbes in nature and stated about various negative as well as positive effect of microbes in nature. Bioremediation is known as the method to convert or remove the harmful contamination (heavy metals) into less hazardous material. Bioremediation is applicable on both water and soil media through the ex-situ and in-situ techniques. In-Situ bioremediation is defined as the removal or conversion of contaminants on the site while the ex-situ bioremediation is defined as the removal or conversion of contaminants away from the site of contamination.

There is currently insufficient technology to de-toxify heavy metal pollution. The goals of this study are to identification and isolation of heavy metal-resistant & antibiotic-resistant bacteria from polluted specimens (sewage and soils) obtained from several industrial regions in Moradabad, with the goal of determining their function in heavy metal removal. This presentation will also provide an introduction to microbial bioremediation of heavy metals in polluted effluents.

2. Literature Review

Heavy metals have an anatomic number > 20, a density > 6 g/cm3, and a molecular weight > 53 [9]. Heavy metal pollution is a severe concern nowadays [10]. These types of metals are incorporated in the surroundings by metal smelting, metalliferous mining, metallurgical industry operations, disposal of waste, metal corrosions, and so on. The discharge of effluents containing heavy metals like Ni, Al, Fe, Cr, Zn, Cu, Cd, and Pb, stresses the environment, posing potential threats to vegetation, wildlife, ocean animals, and people [11], [12]. Micro - organisms have a variety of methods for dealing with heavy metal stress, involving extracellular and intracellular sequestration, enzymatic detoxification, efflux pumps, active transport, barrier blockage of permeability, and decreased sensitivity of cellular targets to metal ions [13]–[15]. These systems aid in the detoxification or removal of metals from the environment [16], [17]. Metal tolerance refers to an organism's capacity to endure or collect metals in high quantities deprived of dying [18]. Metal exposures also promotes the formation of tolerable microbial population, that are frequently depicted by Gram-positive bacteria from the genera Corynebacterium, Arthrobacter, and Bacillus, as well as Gram-negative bacteria from the genera Burkholderia, Ralstonia, Alcaligenes, and Pseudomonas [19].

Philp and Atlas [20] in their study discussed various methods of in situ and ex situ approaches for the bioremediation. They stated that the in-situ techniques

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include bioventing, bio-stimulation, bio-attenuation and bio-sparging, and the ex-situ techniques included bioreactor, bio-pile, windrow and land farming. In-situ approach for the bioremediation does not disturbs the structure of the soil, excavation or removal of soil. In this techniques, microbial flora is utilized to for the bioremediation [21]. V de Lorenzo [22] introduced the term Environmental Galenics in their study which means the in-situ application of the engineered microorganisms in the deteriorated environment to reduce the contamination and efficiently recover such ecosystems. Kha Mong *et al.* [23] discussed about the various engineered microbes that are applicable in the contaminated environment to remove or convert the heavy metals and reduce the contamination.

This study aims to identify and isolate heavy metal resistant bacteria from different contaminated localities at Moradabad, Uttar Pradesh, India. In addition, we also aimed to study the co-resistance to different antibiotics to determine the resistance mechanism as well as possible applications in bioremediation processes.

3. Methodology

Design

Contaminated soil and water samples are collected from various dumping sites from the areas located in different parts of Moradabad region. These are five current sites, from where soil samples are collected and for the further analysis of heavy metals and for isolation of the microorganism in that particular area. The digestion method was adopted for the extraction of heavy metals in the sample (Figure 1).

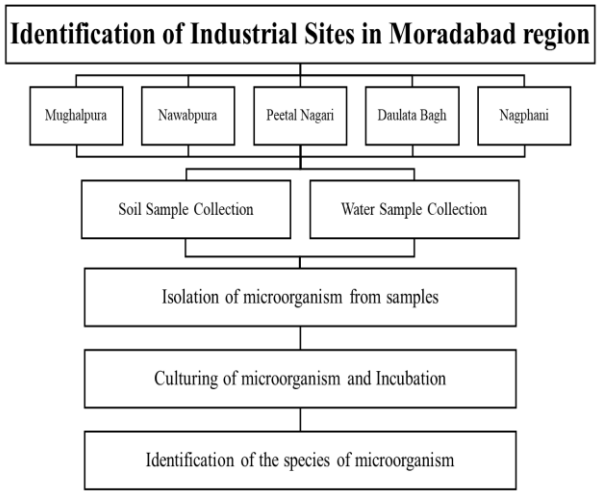

Figure 1. Illustrating the Flow of the Research Conducted to Identify the Different Bacterial Strains in the Small Industrial Regions Of Moradabad

Samples:

Collection of samples is done from the surveyed sites; basically, five polluted sites has been identified for primary study. These all sites are all the small industrial sites situated in the Moradabad Region namely Mughalpura, Nawabpura, Peetal Nagari, Daulata Bagh, and Nagphani. From these 5 sites 11 soil samples has been collected for the further digestion process for the identification and isolation of the heavy metal tolerant strains (Table 1).

Soil samples (0.5g) were digested at 80oC with 15ml of concentrated $HClO_4$, H_2SO_4, and HNO_3 in a 1:1:5 ratio until a translucent mixture was formed. The solution was cooled, filtered using Whatman number 42filter paper and then diluted to 50 ml with deionized water. The filtrate was placed at RT (Room Temperature) for further analysis of heavy metals. Basically, heavy metals Cu, Zn, Pb, Cr, Fe, Ni, Cd were analysed.

Table 1. Representing the Name of the Small Industrial Sites in the Moradabad Region, India

Site	Name of Sites	Samples
1.	Mughalpura	S1 (1)
		S2 (2)
		S3 (3)
2.	Nawabpura	S1 (4)
		S2 (5)
		S3 (6)
3.	Peetal Nagari	S1 (7)
		S2 (8)
		S3 (9)
4.	Daulata Bagh	S1 (10)
5.	Nagphani	S1 (11)

Instruments:

- Serial Dilution Technique

The serial dilution technique is employed to isolate microorganism colonies from soil samples. The preparation of serial dilutions is a technique of diluting a stock solution in which the concentrations decline by the same amount in each succeeding phase. As shown in Figure 2, Test tube A has 10 times dilution or 10^{-1} dilution of the stock solutions. Test tube B has 100 times dilution 10^{-2} dilution, test tube C has 1000 times dilution that is 10^{-3}, test tube D has 10000 times dilution that is 10^{-4}, test tube E has 100000 times dilution that is 10^{-5} and test tube F has 1000000 times dilution that is 10^{-6} [24].

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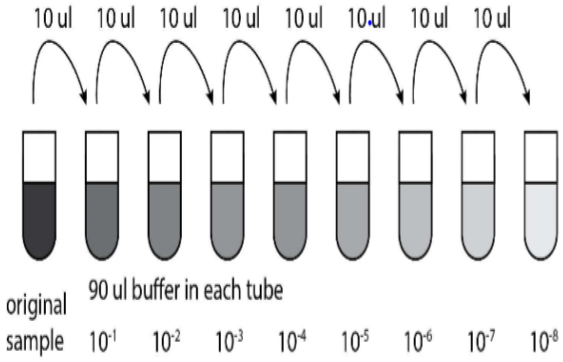

Figure 2. Representing the Serial Dilution Technique Employed in Current Research

- Gram Staining:

Gram staining is a methodology which is utilized for the classification of the different bacteria into gram-positive and gram-negative bacteria. This technique was developed in 1884 by a Danish bacteriologist named Hans Christian Gram. In this techniques the classification of the bacteria is done on the basis of their cell walls [25].

- Endospore Staining:

Endospore Staining is used to identify the presence of the endospores in the bacteria species [26]. Endospores are the assemblies in the bacteria, that helps them to survive in the extreme temperatures and conditions [27].

- Catalase Test:

Catalase enzyme is found in every organism that intake the oxygen. These are responsible for catalysing the hydrogen peroxide to oxygen and water [28]. For the identification of the bacteria, bubble formation is reported when the isolate is added to the hydrogen peroxide. Bubble formation represents the positive results [29].

- Glucose Test:

Zhenxiang et al. [30] identified the bacillus subtilis with the help of glucose test. Same methodology was utilized in this study for the identification of the bacterial species. The glucose broth which contained the glucose, gelatin peptone, and beef extract. Phenol red indicator was introduced in the broth, yellow colour indicated that the pH is decreased and the result is positive, no colour changes indicated that no acid is produced and the result is negative.

- Mannitol Test:

Mannitol test utilized to test the ability of the bacteria to survive in the 7% concentration of salt and ferment mannitol. In current study media was selective. Md Atiqur Rahman et al. [31] utilized the similar biochemical test to identify the bacteria in the fresh guava.

Data Collection:

Soil Samples:

As shown in the below Figure 3, Initially the nutrient agar plates were prepared for the isolation of microbial colonies.

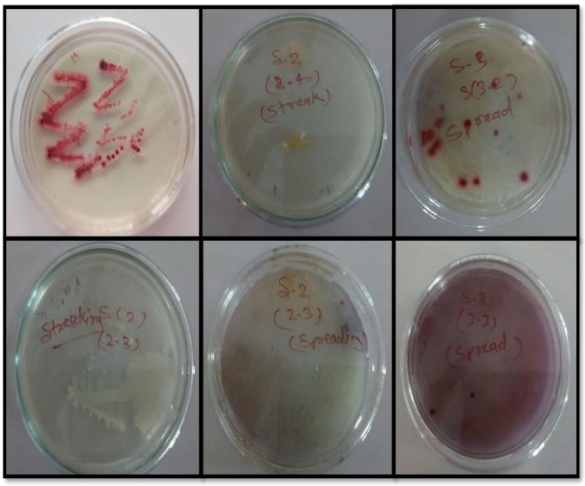

Figure 3. Representing the Streaked Nutrient Agar plates for the isolation of microbial colonies

Water Samples:

The number of colonies in the culture's media plates inoculated by serial dilutions of the specimen will decrease as the dilution factor increases, so plate number 1 will have the most colonies and plate number 6 will have the fewest colonies (GA-1, GA-2, and RG-1, RG-2, and RG-3), which will be distributed more or less sparsely throughout the entire plate. Choose a distinct, well-established colony and make a note of its features, such as colour, shape, size, appearance, elevation, and pigmentation, among others. To get the pure culture of the bacterial cells for future research, these colonies might be streaked, or transferred, to the new medium plates (Figure 4). The streak plates are seen in Figure 5. This approach gives the most practical way to get pure culture and distinct colonies. The standard procedure was used for the streak plating technique. Figure 6 illustrates how Gram-positive bacteria stain blue/purple and Gram-negative bacteria stain pink/red. The endospore staining findings are shown in Figure 7. The endospores will be green in colour and the vegetative cells will be pink or red.

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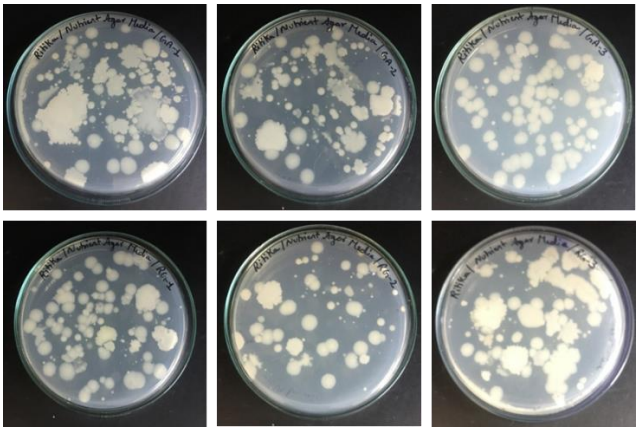

Figure 4. Representing Colonies in the Cultures Media Plates Inoculated by the Serial Dilutions of the Specimens

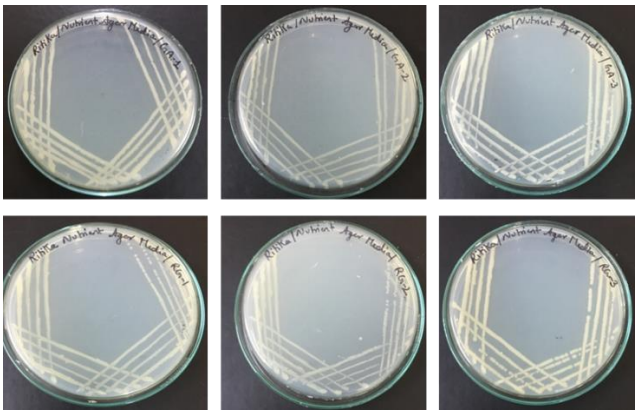

Figure 5. Representing the Streaked Plates of GA-1, GA-2, GA-3, RG-1, RG-2, and RG-3

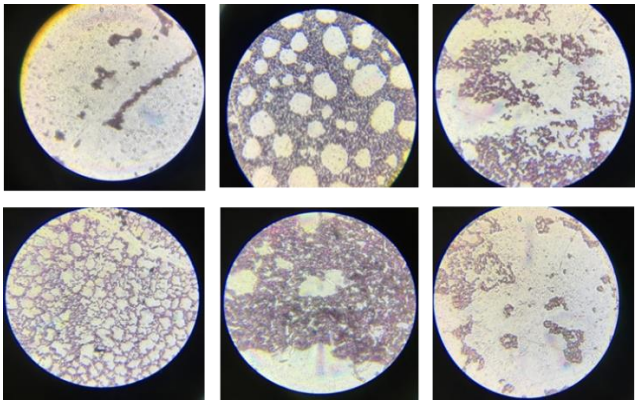

Figure 6. Representing the Gram Staining of GA-1, GA-2, GA-3, RG-1, RG-2, and RG-3

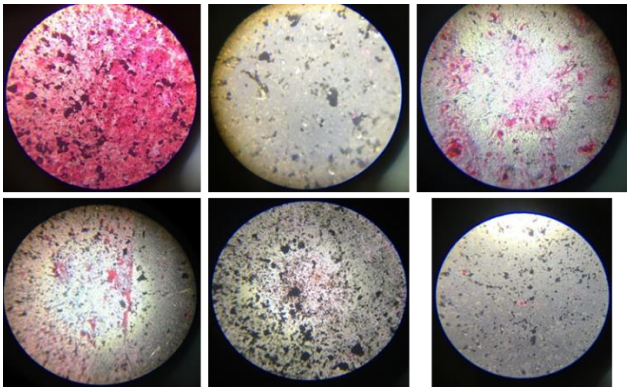

Figure 7. Representing the Endospore Staining of GA-1, GA-2, GA-3, RG-1, RG-2, and RG-3

Data Analysis:

Biochemical test for Soil Samples:

- Glucose Test:

Figure 8 is representing the Glucose fermentation test of bacterial strain pink colony which showed negative result that means not fermented glucose.

Figure 8. Representing the result of Glucose fermentation test of bacterial strain pink colony.

- Mannitol Test:

As shown in Figure 9, results of Mannitol test of bacterial strain MS-1 2.4, MS-3 2.2, MS-2 2.2, P-3 2.1 came negative.

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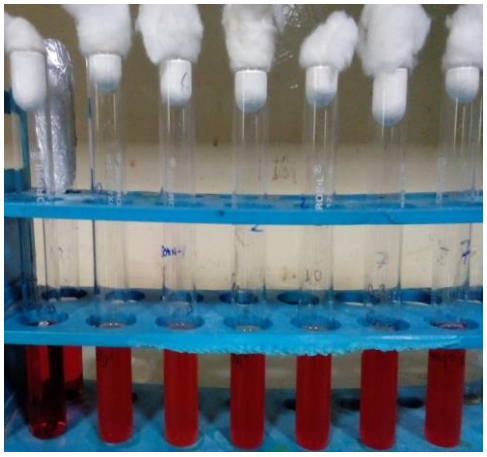

Figure 9. Representing the result of Mannitol test of bacterial strain MS-1 2.4, MS-3 2.2, MS-2 2.2, P-3 2.1.

Biochemical test for Water Samples:

- Mannitol Test:

In the Mannitol test, if the liquid inside the tube turns yellow after incubation. The yellow colour indicates that the decrease in the pH, which is due to the carbohydrates or sugar's fermentation and acid is produced Then it indicates that the outcome is positive. Though if the medium-filled tube stays red, this means the bacteria are unable to ferment the specific carbohydrate resource in the medium. It means that the result is negative. As shown in Figure 10, RG-1 and RG-2 came positive while all others are negative.

Figure 10. Representing the Mannitol Test of GA-1, GA-2, GA-3, RG-1, RG-2, and RG-3

- Glucose Test:

Same as the Mannitol test, if the liquid inside the tube turns yellow after incubation. The yellow colour indicates that the decrease in the pH, which is due to the carbohydrates or sugar's fermentation and acid is produced. Then it represents that the result is positive result for the glucose test. Though if the medium-filled tube stays red, this means the bacteria are unable to ferment the specific carbohydrate resource in the medium. It means that the result is negative for the glucose test. As shown in Figure 11, all results came positive for the glucose test.

Figure 11. Representing the Glucose Test of GA-1, GA-2, GA-3, RG-1, RG-2, and RG-3

- Catalase Test:

Positive outcomes in the catalase test are shown by an instantaneous effervescence (bubbles creation), whereas negative reactions are indicated by a lack of bubbles which can be interpreted as there is absence of the catalase enzyme which can hydrolyze the hydrogen peroxide. As shown in Figure 12, all results came positive for the Catalase test.

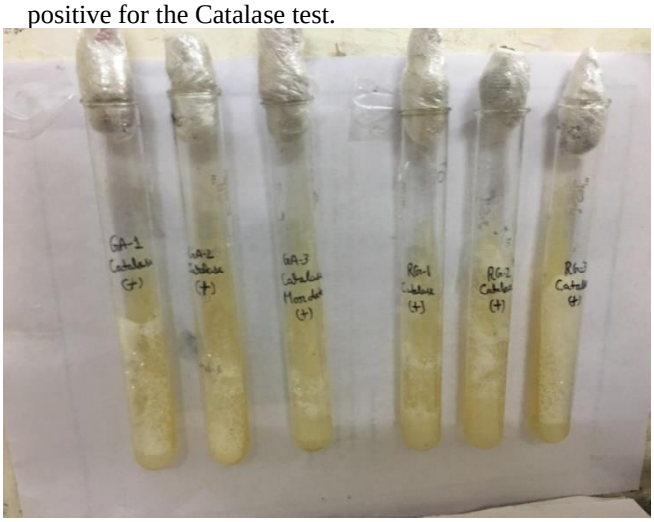

Figure 12. Representing the Catalase Test of GA-1, GA-2, GA-3, RG-1, RG-2, and RG-3

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4. Results And Discussion

Identification test of Soil Samples for bacterial strains:

Identification of bacteria done in soil samples in which bacterial strain MS-1 2.4, MS-3 2.2, MS-2 2.2, P-3 2.1 & pink colony were isolated and stain MS-2 2.1 were not isolate due to lack of chemicals (Table 2). Identification of bacteria done in water samples in which all bacterial strains were identifies as shown in Table 3.

Table 2. Representing the Results of Different Test of Soil Samples for Isolate Identification

Strain	Gram Staining	Endospore Test	Catalase Test	Glucose Test	Mannitol Test	Identified Bacteria
MS-1 2.4	Positive	Positive	Positive	NA	Negative	*B. cereus*
MS-3 2.2	Positive	Positive	Positive	NA	Negative	*B. cereus*
MS-2 2.2	Positive	Positive	Positive	NA	Negative	*B. cereus*
MS-2 2.1	Negative	NA	NA	Negative	NA	----
P-3 2.1	Positive	Positive	Positive	NA	Negative	*B. cereus*
Pink colony	Negative (spherical)	NA	NA	Negative	NA	*Neisseria flavescens*

Table 3. Representing the Results of Different Test of Water Samples for Isolate Identification

Strain	Result of Gram staining	Result of Endospore staining	Result of Glucose Test	Result of Mannitol Test	Result of Catalase Test	Identified Bacteria
GA-1	Gram Positive	Vegetative	Negative	Positive	Positive	*B.subtilis*
GA-2	Gram Positive	Non-Vegetative	Negative	Positive	Positive	*S.aureus*
GA-3	Gram Positive	Non-Vegetative	Negative	Positive	Positive	*B.anthracis*
RG-1	Gram Positive	Non-Vegetative	Positive	Moderate Positive	Positive	*B.cerecis*
RG-2	Gram Positive	Vegetative	Negative	Positive	Positive	*C.tetani*
RG-3	Gram Positive	Vegetative	Moderate positive	Positive	Positive	*G.viginalis*

5. Conclusion

The successful execution of the proposed research will led to the isolation of bacteria which are able to survive at different ppm concentration of heavy metal. The results shows the impact practically in addition to expanding our knowledge of the metabolic variety of bacteria. Bio informational study of specific proteins responsible for the bioremediation as well as effect on human health, will be utilized for the screening of disease-causing protein and will helps to recognize the specific causative agent of the disease which effects the human health.

The identification of an entirely new class of microorganisms will make it possible to address their function in remediation, which will eventually enhance the design and functionality of biotechnologies that may be utilised for remediation purposes. A possible agent for bioremediation of heavy metal contamination might be metal-resistant bacteria. As a result, the bacterial isolates acquired from the current investigation may be effectively used biotechnologically for the bioremediation of ecosystems that have been polluted with metal.

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