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Isolation, identification and characterization of heavy metal resistant bacteria from industrial affected soil

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Abstract
With the active spread and development of the industries, heavy metals, such as cadmium, nickel, lead, chromium, arsenic etc, the well-known components of industrial effluents, are directly or indirectly discharged into the environment and consequently pollute the ecosystem. The presence of these heavy metals in the ecosystem has been a subject of great concern due to their non-biodegradable nature, toxicity and long biological half-lives hindering their removal from biological tissues. This investigation is focused on isolation, identification and characterization of heavy metal resistant bacteria from iron industry soil. The soil samples were collected from ISA Steel Private Limited Company in Gulaothi, (Bulandshahr). Nine bacterial strains were isolated from the soil and characterized on the basis of morphological, physiological and biochemical characteristics. Minimum inhibitory concentration (MIC) of the isolates was studied by which Fe, Pb, Cu, Zn, and Co tolerance was found highest in *Proteus mirabilis* Strain 3, (61.97%); *Serratia marcescens* Strain 4, (99.56%) and *Escherichia coli* Strain 8, (94.66%) respectively in bacterial isolates grown in nutrient media and supplemented with heavy metal. The identified heavy metal resistant bacteria could be effective and useful for the bioremediation of heavy metal contaminated soil.

Keywords: Heavy metal tolerance, industrial soil, bacterial resistance, bioremediation

1. Introduction
Determination of heavy metals concentration in soil is widely used to evaluate soil pollution. The metal exists in soil because of atmospheric deposition and mineral weathering originating from anthropogenic and natural sources [1]. Metal plating, mining by product, coal-based waste, chemical, industrial, nuclear and pesticide waste, gasoline and mineral leaching are some of the major sources of these metals [2, 3, 4]. Higher concentration of some essential metals such as zinc and copper are toxic, other metals including lead and cadmium do not play any known physiological role and are in fact toxic to cells. Lead reacts with the sulphydryl groups of protein and inhibits their function. Many technologies are currently used to clean up heavy metal contaminated soils. The mostly used once are soil removal and land filling, stabilization/solidification, physico-chemical extraction, soil washing, flushing and phytoremediation. None of these techniques are completely accepted as best treatment option. Bioremediation is one of the promising technologies used to detoxify the harmful form of metals to its non harmful form in soil matrix. Owing to various natural processes & urbanization, high proportions of heavy metals are commonly found in microbial habitats. Microorganisms are omnipresent and are found to be involved in different biological processes of life. Presence of higher concentrations of metals, force these organisms to adjust themselves with different biological mechanisms so that they can cope with High heavy metal conditions [5]. Many studies have reported that indigenous microbes are capable of tolerating high metal concentrations and may play a pivotal role in the restoration of contaminated soil [6, 7].

The present study aims to isolate and identify indigenous bacterial strains from heavy metal contaminated soil to determine their tolerance to copper, Zinc, iron, lead and Cobalt. The physiological and biochemical features were used to characterize the strains.

2. Material and Methods
2.1 Sample collection
Soil sample were collected from the site of ISA Steel Private Limited Company, Gulaothi. Soil sample were collected in sterilized polyethylene bottle using sterilized spatula and were immediately transported to the laboratoryin icebox to ensure minimal biological activity.

2.2 Isolation of bacteria
The microbial strains were isolated from the collected soil samples by serial dilution technique.

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Selective isolation of bacterial spp. was performed by spreading the samples on Nutrient agar media.

2.3 Identification and characterization of bacteria from soil sample

Individual distinct colonies were further undergone repeated sub-culturing. Selected colonies were grown on nutrient agar media (Himedia, India). The bacteria isolates were subjected for the morphological, microscopic and biochemical identification tests such as oxidase, catalase, indole production, methyl red, Voges Proskeur, Urease, Mannitol and Sucrose Test.

2.4 Determination of MIC

Maximum resistance of the selected isolates against increasing concentrations of heavy metals on Nutrient agar plates was evaluated until the strains unable to grow colonies on the agar plates. Nutrient Agar plates supplemented with heavy metal at the following concentration: $Cu(NO_3)_2$ - 1.0mM, 1.5mM,2.0mM, 2.5mM, 3.0mM, $FeSO_4$ – 2.0mM, 2,5mM, 3.0mM, 3.5mM, 4.0mM, 4.5mM, 5.0mM, 5.5mM, 6.0mM, 6.5mM, $Pb(NO_3)_2$ – 2.0mM, 2.5mM, 3.0mM, 3.5mM, 4.0mM, 4.5mM, 5.0mM, $Zn(NO_3)_2$ – 1.0mM, 1.5mM, 2.0mM, 2.5mM, 3.0mM, $CoCl_2$ – 0.5mM, 1.0mM, 1.5mM were prepared in distilled water and sterilized by

autoclaving at 121^0C for 15 min. Based on the evaluation minimum inhibitory concentration (MIC) was determined at 37^0C for 5 days.

2.5 Evaluation of heavy metal tolerance

Tolerance of selected bacterial strains to various heavy metals was determined by agar dilution method. Freshly grown agar cultures of these isolates were inoculated aseptically on nutrient agar plates supplemented individually with heavy metals. The metal salts used were $Cu(NO_3)_2$, $FeSO_4$, $Pb(NO_3)$, $Zn(NO_3)_2$, $CoCl_2$.

3. Results
3.1 Isolation of heavy metal resistant bacteria
In the present study we identified and characterized heavy metals resistant bacteria isolated from industry affected soil. 110 colonies were screened from initial level of heavymetal supplemented nutrient agar medium. 9 isolates were selected based on high degree of heavy metals resistances were used for further studies.

Morphological characteristics: The morphological characteristics of strains are shown in Table 1. The isolated strains were of varying shape and morphology.

Table 1: Morphological characteristics of isolate bacterial strains.

Strain No.	Gram Stain	Shape	Morphology
1	Negative (-)	Cocci & chained	Flattened
2	Negative (-)	Rod	Upraised
3	Negative (-)	Rod & cluster	Flattened
4	Negative (-)	Rod & cluster	Little bit upraised
5	Negative (-)	Diplococci & separated	Flattened
6	Negative (-)	Cocci & chained	Little bit upraised
7	Negative (-)	Cocci & strep	Flattened
8	Negative (-)	Spiral & chained	Flattened
9	Negative (-)	Rod & Staphy	Upraised

Biochemical characteristics: Table 2 shows biochemical characteristics of bacterial strains.

Table 2: Biochemical characteristics of bacterial strains.

Biochemical Test	1	2	3	4	5	6	7	8	9	
Catalase Test	+	+	+	+	+	+	+	+	+	
Indole Test	+	+	-	-	-	-	-		+	
MR Test	-	-	-	-	-	-	-	-	-	
VP Test	-	+	-	+	+	+	+	+	+	
Citrate Test	-	+	+	+	+	-	-	-	+	
Oxidase Test	-	+	+	+	+	+	+	+	+	
Urease Test	+	-	+	-	-	-	-	-	-	
Mannitol Test	-	+	-	+	+	+	+	+	+	
Sucrose Test	-	-	-	-	-	-	-	+	-	
Lactose Test	+	+	+	+	-	-	+	-	+	

Following strains were identified according to Bergey's manual of systemic bacteriology:
Strain 1 - *Morganella morganii* (75.65%)
Strain 2 - *Serratia marcescens* (99.59%)
Strain 3 - *Proteus mirabilis* (61.97%)
Strain 4 - *Serratia marcescens* (99.56%)
Strain 5 - *Serratia marcescens* (99.45%)
Strain 6 - *Escherichia coli* (99.92%)
Strain 7 - *Serratia marcescens* (99.56%)
Strain 8 - *Escherichia coli* (94.66%)
Strain 9 - *Serratia marcescens* (99.87%)

3.2 Heavy metals resistance by isolated bacterial strains
Table 3-7 shows heavy metal resistance of the nine strains, isolates 1,5 and 8 showed resistance till $2.0mMCu(NO_3)_2$ Concentration, remaining were resistant till 2.5mM $Cu(NO_3)_2$ Concentration. Strain 4 and 8 showed resistance till 6nM $FeSO_4$ Concentration. Strains 4, 6, 7, 8, 9 showed resistance till 4.5mM $Pb(NO_3)_2$ Concentration. Strains 2, 5 showed resistance till 2.5nM $Zn(NO_3)_2$ Concentration. Strains 3, 4, 6, 7, 8, 9 showed resistance till 1.0nM $CoCl_2$ Concentration. Results clearly indicate that isolates have prominent metal resistance capability.

Table 3: Growth of isolates at different Cu $(NO_3)_2$ concentration.

S. No	Cu$(NO_3)_2$ Concentration	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6	Strain 7	Strain 8	Strain 9
1.	1.5mM	+++	+++	+++	+++	+++	+++	+++	+++	+++
2.	2.0mM	+	++	++	++	+	++	++	+	++
3.	2.5mM	-	+	+	+	-	+	+	-	+
4.	3.0mM	-	-	-	-	-	-	-	-	-

(-) No Growth, (+) Slight Growth, (++) Moderate Growth, (+++) Vigorous Growth

Table 4: Growth of isolates at different FeSO$_4$ concentration.

S. No	FeSO$_4$ Concentration	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6	Strain 7	Strain 8	Strain 9
1.	5.0mM	+++	+++	+++	+++	+++	+++	+++	+++	+++
2.	5.5mM	+	++	++	+++	++	++	++	+++	++
3.	6.0mM	-	+	+	++	+	+	+	++	+
4.	6.5mM	-	-	-	+	-	-	-	+	-
5.	7.0mM	-	-	-	-	-	-	-	-	-

(-) No Growth, (+) Slight Growth, (++) Moderate Growth, (+++) Vigorous Growth

Table 5: Growth of isolates at different Pb $(NO_3)_2$ concentration.

S. No	Pb$(NO_3)_2$ Concentration	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6	Strain 7	Strain 8	Strain 9
1.	3.0mM	+++	+++	+++	+++	+++	+++	+++	+++	+++
2.	3.5mM	+	++	+	++	++	++	++	++	++
3.	4.0mM	-	+	-	++	+	++	++	++	++
4.	4.5mM	-	-	-	+	-	+	+	+	+
5.	5.0mM	-	-	-	-	-	-	-	-	-

(-) No Growth, (+) Slight Growth, (++) Moderate Growth, (+++) Vigorous Growth

Table 6: Growth of isolates at different Zn $(NO_3)_2$ concentration.

S. No	Zn$(NO_3)_2$ Concentration	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6	Strain 7	Strain 8	Strain 9
1.	1.5mM	+	+++	++	++	+++	++	++	++	++
2.	2.0mM	-	++	+	+	++	+	+	+	+
3.	2.5mM	-	+	-	-	+	-	-	-	-
4.	3.0mM	-	-	-	-	-	-	-	-	-

(-) No Growth, (+) Slight Growth, (++) Moderate Growth, (+++) Vigorous Growth

Table 7: Growth of isolates at different CoCl$_2$ concentration.

S. No	CoCl$_2$ Concentration	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6	Strain 7	Strain 8	Strain 9
1.	0.5mM	+	+	++	++	+	++	++	++	++
2.	1.0mM	-	-	+	+	-	+	+	+	+
3.	1.5mM	-	-	-	-	-	-	-	-	-

(-) No Growth, (+) Slight Growth, (++) Moderate Growth

4. Discussion

The bacterial strains isolated from the soil of ISA Steel Private Limited Company, Gulaothi, (Bulandshahr). From this sample, nine (9) bacterial isolates have been selected. Among them Strain 1,6 & 7 are gram negative and cocci shaped while the Strain 2,3,4 & 9 are gram negative and rod shaped. Except Strain 5 & 8 are gram negative but they are diplococci and spiral separately. These isolated strains are capable to reduce the extricable metals from the contaminated soil. The leaching of metals and hydrocarbons to the environment can be reduced by these isolates. This study has revealed the MIC of isolates, which will help in bioremediation process. The isolates strains are resistant to the heavy metals, which are toxic to the air, water and soils with a subsequent hazardous impact on both ecological and human health. This study determined that these strains are resistant to the heavy metals i.e., Fe, Cu, Pb, Zn & Co. Minimum inhibitory concentration (MIC) of the isolates was studied by which Fe, Pb, Cu, Zn, and Co tolerance was found highest in *Proteus mirabilis* Strain 3, (61.97%); *Serratia marcescens* Strain 4, (99.56%) and *Escherichia coli* Strain 8, (94.66%) respectively in bacterial isolates grown in nutrient media and supplemented with heavy metal.

5. Conclusion

The process of bioremediation depends on various natural factors such as soil type, pH, temperature, nutrient, amendments and oxygen. Bacteria develop heavy-metal resistance mostly for their survivals. Metal resistant bacteria can be utilized in bioremediation of metal contaminated environments. However, we need a better understanding of the microbial tolerance mechanisms in order to reduce the overall effect of toxic heavy metals in the environment. In the present study high degree of heavy metals resistance associated with multiple heavy metals was detected in industrial affected soil bacteria. Their uniqueness and characteristics could be used as potential bioremediation agents to remove the heavy metals from the environment. Further studies are necessary to evaluate the heavy metal removal abilities of those isolates.

6. References

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