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ISOLATION AND CHARACTERIZATION OF METALLIC RESISTANT BACTERIA FROM HEAVY METAL POLLUTED SOIL

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Abstract

The recent expansion of human and industrial activity, including mining, smelting, and synthetic compound creation has led to the increase in the amount of the heavy metals released into the soil, water and atmosphere. The heavy metals include Cu, Mg, Al, Ni, Cd, Zn, Li, Hg etc. Screenings of the soil and water resources are conducted regularly to prevent the overconsumption of heavy metals. Many countries have regulatory guidelines for heavy metals presence and exposure. The heavy metal polluted soil contains some microorganisms that exhibit tolerance to the metals which in turn helps in the reduction of heavy metals toxicity accumulated in the soil. Isolation, Screening and Characterization are involved to determine the level of resistance of the bacteria. Here, out of several isolates only one isolate *Bacillus safensis* (accession no: OL456244) has showed increased resistance to ($Al_2(So_4)_3$) and $MgSo_4$. Consequently, this microbial isolate can be potentially used in bioremediation of heavy metal polluted environment.

Key Words: Heavy metals, resistance, *Bacillus safensis*, atomic absorption spectroscopy, (Al2(So4)3), $MgSo_4$

Introduction

"In every walk with the nature, one receives far more than he gives" quotes the American Environmentalist, John Muir. Day by day the pollution is increasing drastically, one such pollution that risks human is the land pollution. The heavy metals from several electroplating industries pollutes the soil and causes thread to several beneficial microorganisms which in turn affects plant growth and also the texture of the soil which in some cases affects the human health also. Heavy metals are defined as metallic elements that have a relatively high density compared to water. With this supposition the heaviness and toxicity are inter-related, heavy metals also include metalloids, such as arsenic, that are able to induce toxicity at low level of exposure. In recent years, there has been an increasing ecological and global public health concern associated with environmental contamination by these metals(Bradl H, 2005).

Heavy metals are considered one of the major sources of soil pollution. Heavy metal pollution of the soil is caused by various metals, especially Cu, Ni, Mg, Cd, Zn, Cr, Al and Pb (Karaca A*et al*., 2010). Heavy metals exert toxic effects on soil microorganism hence results in the change of the diversity, population size and overall activity of the soil microbial communities(Kalamdhad Ajay S *et al*., 2011). The metal plant uptake from soils at high concentrations may result in a great health risk considering food-chain implications. Utilization of heavy metals by plants and subsequent accumulations along with the food chain is a potential threat to human health. (Nascentes C.C *et al*., 2006).The adverse effects of heavy metals on soil biological and biochemical properties

are known clearly. The soil properties i.e., organic matter, clay contents and pH have major influences on the extent of the effects of metals on biological and biochemical properties (Percivalc H.J *etal*.,1999). Heavy metals accidentally affect soil enzymatic activities by transporting the microbial community which synthesizes the enzymes. Heavy metals exhibit toxic effects towards soil biota by affecting key microbial processes, decrease in the number of farmer's friend i.e., the earthworms and decrease the activity of soil microorganisms(Bing P etal., 2009).

Heavy metals are classified as i)Bound to reducible phases ii)exchangeable iii)Bound to organic matter and sulphides(Huiping Wang*et al*., 2010). Microorganisms continued existence in polluted soils depends on intrinsic biochemical and structural properties, genetic and physiological adaptation including morphological changes of cells, as well as ecological modifications of metal speciation. To endure under metal-stressed circumstances, bacteria have evolved up to a several types of adjustment to stand with the uptake of heavy metal ions. These mechanisms include the i) efflux of metal ions outside the cell, ii) bioaccumulation of the metal ions inside the cell, iii) the decreasing concentration of the heavy metal ions (Joshi B. H. et.al.,2013).

Adaptation of bacteria to heavy metals is attributed to a variety of chromosomal, transposon, and plasmid mediated resistance systems(Kapil, S et al., 2000). The incidence of plasmid-bearing strains is more in polluted places than in the unpolluted area (Jaiswal, R *et al*.,2000).Heavy metal contamination in soils is one of the serious environmental problems in the world, causing significant risks to ecosystems and public health. Accordingly, for environmental conservation and human health, the development of a remediation strategy for metal-contaminated soils is urgent**(**Whiting, S.N*et al*., 2001).Therefore, the application of heavy metal-resistant and heavy metal-solubilizing microorganisms is a promising outlook for increasing heavy metal bioavailability in heavy metal amended soils. Soil bacteria-assisted phytoremediation has been reported (Usmani, S *et al*., 2006 Dell'Amico, *et al*., 2008).

Many technologies are currently being used to clean up heavy metal contaminated soils. The most commonly used technologies are soil removal and land filling, stabilization/solidification, physio-chemical extraction, soil washing, flushing and phytoremediation. None of the above-mentioned techniques are completely accepted as best treatment option till date. Bioremediation is one of the most promising technologies used to detoxify the harmful form of metals to its non-harmful form in soil matrix(Habi S *et.al*., 2009).

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(Lian MF *et al*.,2009). 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 (Carrasco JA*et al*., 2005)

Materials and Methods

Collection of Samples

The samples were collected from the electroplating industries in and around Coimbatore with the clean and sterile zip lock plastic bags. The industries were selected based on the amount of sedimentation of the heavy metals according to the product produced in the particular industries. The samples were collected at the point of source by pit drilling for about 3-20 cm depth below the top soil. The samples were then immediately transported to the laboratory. It is stored at 4℃.

Sample 1 Sample 2 Sample 3 Sample 4

Fig.1. Samples collected from various Electroplating industries

1. Detection of Concentration of Heavy Metals present in the soil

Atomic absorption spectroscopy and atomic emission spectroscopy is a Spectro analytical course of action for the quantitative determination of chemical elements using the absorption of optical radiation by free atoms in the gaseous state. Atomic Absorption Spectroscopy (AAS) is based on the absorption of light by the metallic ions. In analytical chemistry the technique is used for the determining the concentration of the particular element when the sample to be analysed. AAS can be used to find out over 70 different elements in a solution, or directly in solid samples via electrothermal vaporization. The concentration of Heavy metals in the soil were detected using the Atomic Absorption Spectroscopy (AAS). The aqua regia digestion method was considered to be effective for the measurement of "total trace elements" in the soil. This method gives the complete recovery of Cd, Mg, Cu, Pb, Cr, Al and Zn while it is to provide partial recovery of Ag, Ni and Ba. This method consists of treating the soil sample with a 3:1 mixture of hydrochloric acid (Hcl) and nitric acid HNO_3. The nitric acid demolishes organic matter and oxidizes sulphide material. It reacts with the concentrated hydrochloric acid to generate aqua regia.

$$3HCl + HNO_3 \rightarrow 2H_2O + NOCl + Cl_2$$

In this current study, AAS was performed for heavy metals such as Aluminium and Magnesium for all the four samples as the point of source where the sample collection carried out was rich in these heavy metals and the results were recorded.

2. EVALUATION OF HEAVY METAL TOLERANCE

2.1 Primary Screening of Heavy metal resistant bacteria

For the selective screening of heavy metal resistant bacteria, the heavy metals (Aluminium, Magnesium) at different concentrations of about 100µg/ml to 1mg/ml were incorporated in LB agar plates. These plates were used to screen the tolerance by the standard spread plate method observed at 37°C. The Plates were observed after 24-48 hours of incubation. Colonies that showed increased resistance were selected, picked, sub cultured and then preserved on different plates for further studies.

2.2Aluminium metal tolerance

Aluminium is a chemical element with the atomic number 13. Aluminium has the lower density than that of the other common metalsat approximately one-third of the steel. Aluminium visually resembles silver both in colour and the ability to reflect light. Nutrient agar medium was prepared separately and autoclaved. Aluminium metal supplement was prepared separately according to the required concentration from 1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml and 5mg/ml. Plating was performed for the isolates and the results were recorded after 24-48 hours of incubation at 37°C.

2.3Magnesium metal tolerance

Magnesium is the chemical element Nutrient with the atomic number 12. Most commonly added to molten iron and steel to remove sulphur.Nutrient agar medium was prepared separately and autoclaved. Magnesium metal supplement was prepared separately according to the required concentration from0.5mg/ml, 1mg/ml, 1.5mg/ml, 2mg/ml and 2.5mg/ml. The results were obtained after 24-48 hours of incubation at 37°C.

2.4Determination of pH

The Optimum pH for the growth of the heavy metal resistant bacteria is determined by the alterations in the normal pH of the nutrient agar medium. For adjusting the pH, 1N NaOH was used to rise the pH to base and 1N HCl to lower to pH to base.

3.Morphological and Physiological Analysis

3.1 Grams Staining

The primary analysis in the identification of the morphological structure of any bacteria was Gram's staining. It was performed to distinguish microbial isolates into the two distinct groups. It is differentiated by only one colony that showed higher resistance when compared to other isolates. So, the characterization studies were done only for that isolate S2, 10^{-7} col 2.

3.2 Biochemical Analysis

The biochemical characterization of the isolate was done for the identification ofmicrobes on the basis of different biochemical activities (IMVIC test, catalase test, carbohydrate fermentation test and urease test).

4.Molecular Analysis

The 16s rDNA sequencing was carried out to study the bacterial taxonomy and phylogeny. It starts with the

- Isolation of DNA,
- checking the quality of DNA,
- PCR amplification of the isolated DNA and
- 16s rDNA Sequencing(Sanger Sequencing)

The Sequence analysis was done using BLAST and the Phylogenetic tree was also constructed.

5. Results

1. Detection of Concentration of Heavy Metals present in the soil

According to the AAS report, higher the concentration gives higher the amount of heavy metals present in the soil. So, here Sample 2 (Steel Scrap Industry) showed higher concentration of Aluminium and Magnesium when compared with the other three samples.

Heavy metals	Sample 1 (Mg/Kg)	Sample 2 (Mg/Kg)	Sample 3 (Mg/Kg)	Sample 4 (Mg/Kg)
Aluminium (Al)	2907	3278	2467	1264
Magnesium (Mg)	4647	6308	3901	1339

Table 1: Table showing determination of heavy metals using AAS

2. EVALUATION OF HEAVY METAL TOLERANCE

2.1 Primary Screening of Heavy metal resistant bacteria

Out of 10 isolates, the colonies of Sample 2 such as 10^{-5} col1 and 10^{-6} col 1 and 10^{-7} col 2 were taken for analysis. Out of which 10^{-5} col1 and 10^{-6}col1 showed tolerance only below 1mg/ml. while, 10^{-7} col 2showed increased tolerance and were subjected for further analysis.

10^{-5} col-1

10^{-6} col -1

10^{-7} col -2

Fig 2. Plates showing initial screening of heavy metals

2.2 Aluminium metal tolerance

Control

$(Al_2(SO_4)_3)$ – 1mg/ml

$(Al_2(SO_4)_3)$ – 2 mg/ml

$(Al_2(SO_4)_3)$ – 3 mg/ml, 4mg/ml and 5mg/ml

Fig 3. Plates showing Aluminium metal tolerance

The heavy metal tolerance for $(Al_2(SO_4)_3)$ was obtained up to 2 mg/ml concentration of the metal supplement. The growth was not observed on the plates having the higher concentration such as 3 mg/ml, 4mg/ml and 5mg/ml as it could not tolerate the higher concentrations.

2.2Magnesium metal tolerance

Control MgSo4 0.5 mg/ml MgSo4 1 mg/ml

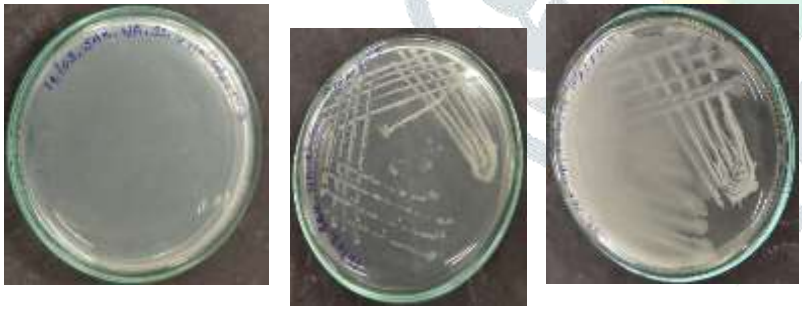
MgSo4 1.5 mg/ml MgSo4 2 mg/ml MgSo4 2.5 mg/ml

Fig 4. Plates showing Magnesium metal tolerance

The heavy metal tolerance for ($Al_2(SO_4)_3$) was obtained up to 2.5 mg/ml concentration of the metal supplement. This showed increased concentration of heavy metal tolerance by the obtained microbial isolate.

2.3 Determination of pH

pH5 **pH 6** **pH7**



pH8 **pH9** **pH10**

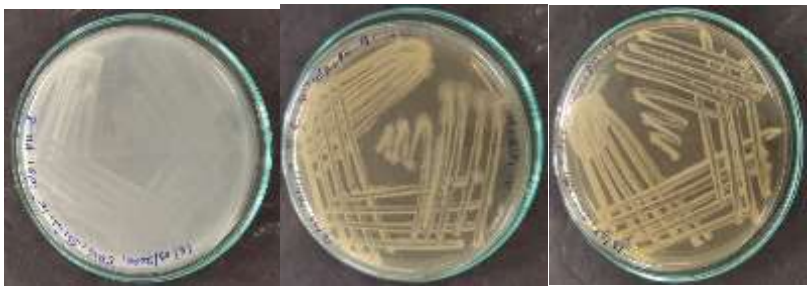


Fig 5. Plates showing optimum pH

There was no observation of microbial growth on the plate with pH 5. It also showed proper growth in pH 6. Whereas in pH7,8, 9 and 10 colour change of the media was observed. It was noted that the optimal growth of the microbe was in the plate where the pH was adjusted to 6.

3. Morphological and Physiological Analysis

3.1 Grams Staining

Grams staining of microbial isolate 10^{-7} col 2 was performed and the isolate was found to be gram positive cocci.

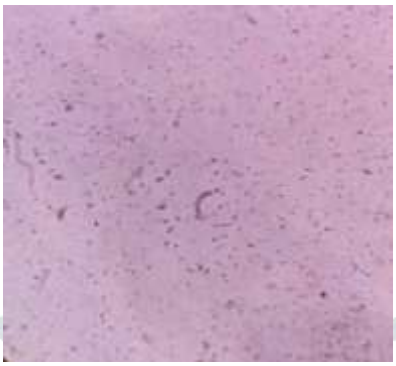


Fig 6. Microscopic Observation of Gram's Staining

3.2 Biochemical Analysis

The following table shows the results of Biochemical analysis of the selected isolate.

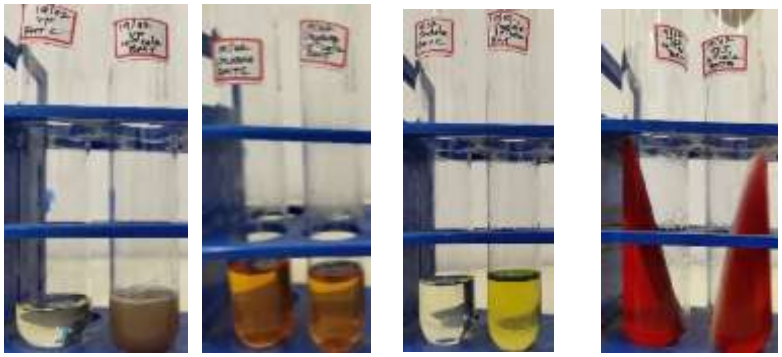
Colony	Indole	MR	VP	TSI	Citrate utilization	Catalase	Carbohydrate fermentation	Urease
10^{-7} col 2	-	+	-	-	+	+	-	-

1.Citrate Utilization

2. Catalase

3. Carbohydrate fermentation

4.MR



5. VP **6. Urease** **7. Indole** **8. TSI**

Fig 7. Tubes showing results of biochemical analysis

4. Molecular Analysis

The 16S rDNA sequence of the obtained microbial isolate was

GCTCAGGCGAACGCTGGCGGCGTGCCTAATCCTGCAAGTCGAGCGGACAGAAGGGAGCTTGCTCCCGGATGTTAGCGGCGGACGGG
TGAGTAAC

The sequence was run in nBLAST and the phylogenetic tree was constructed

Fig 8: Phylogenetic tree of obtained microbial isolate

Conclusion:

The heavy metal pollution is the greatest risk of land pollution that is masking the environment. Thus, the isolation, screening and characterization of the heavy metal tolerance were carried out in this study. The soil samples were collected from the electroplating industries and were serially diluted and plating was carried out in order to visualize the colonies and observe it. After considering the Atomic Absorption Spectroscopy (AAS) report, the samples that showed higher concentration of the heavy metals were used for further analysis. Gram's staining was performed for all the isolates to determine the morphology. Out of 10 isolates only 3 isolates 10^{-5} col-1 and 10^{-6} col -1 and 10^{-7} col -2 were subjected to further analysis, as they were morphologically distinct. Out of which only one (10^{-7} col 2) showed the increased resistance to the heavy metals like Aluminum and Magnesium.

This isolate was then subjected to physiological and molecular analysis. The physiological analysis involved the biochemical activities of the microbial isolate. After the molecular analysis i.e., 16s rRNA sequencing, it is found that the isolate was *Bacillus safensis* strain submitted to GenBank with the **accession number OL456244**. BLAST (Basic Local Alignment Search Tool) is an algorithm and program are used in order to compare the biological sequence information of the obtained molecular isolate.Phylogenetic relationship of the species was analysed with other closely related other bacterial species present in GenBank.

Bioremediation is the process of using the biological compound in order to treat the environment. The obtained isolate *Bacillus safensis* can be further used in the Bioremediation process to rescue the polluted environment. The isolate will be mixed with the polluted soil, where the adsorption or the reduction process takes

place which in turn reduces the amount of heavy metal contamination of the soil. This can be determined by the soil analysis and also through the salt estimation process.

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