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Heavy Metal Resistant Bacteria Isolation from Koodankulam Coast: *Bacillus* sp. and *Pseudomonas* sp. as Potential Agents for Bioremediation

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Abstract

The present study investigates the heavy metal resistant bacteria were isolated from offshore sea soil of Koodankulam coast, Gulf of Mannar, South East coast of India., Tamil Nadu, India. Bacterial communities in the soil were screened by morphological, biochemical and plasmid analysis and were identified as Bacillus sp., and Pseudomonas sp. High concentrations of toxic metals negatively affect bacterial growth, and therefore, the minimum inhibitory concentration of isolated bacteria was determined against Pb, Hg and Zn by agar dilution technique. 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 the Bacillus sp. and Pseudomonas sp. has showed increased resistance to Pb, Hg and Zn. Consequently, this microbial isolate can be potentially used in bioremediation of heavy metal polluted environment.

Keywords: *Minimum inhibitory concentration, Heavy metal, Bioremediation and resistance Bacillus sp., Pseudomonas sp., Plasmid analysis.*

1. Introduction

Accumulation of heavy metal has been arising in soil and water due to the heavy discharge of industrial waste to the environment which is result of growth of industrialization and extraction of natural resources. Since these hazardous heavy metals and toxic chemicals can't be broken down to nontoxic forms, is the major cause that the world is facing today as it not only contaminates the soil, groundwater, sediments, surface water and air but also have long lasting effects on the ecosystem. (Asha and Sandeep, 2013) in their recent study emphasis on the new technologies for the remediation of the natural resources by destructing the pollutants instead of using conventional methods as there is chance of the potential to enter the food chain. It is found scientifically that metals can cause serious health issues if present in body as they disturb our normal functioning, explained by study of (Suranjana and Manas, 2009). But some of these metals when present in low concentration are beneficial like Arsenic, Copper, Iron and Nickel etc. High concentration leads to toxicity which are carcinogenic and mutagenic in nature according to study by Salem et al. (2000).

There have been a lot of researches carried on heavy metal contamination in the sea from last 10 years due to the major factors like mercury problems and the analytical techniques development. Marine environment is a natural resource of heavy metals (Fe, Cu, Zn, Co, Mn, Mo, Se, Ni, Sn) which are biologically essential. Like metallo proteins or metal-protein complexes they occur in enzymes and respirator as metallo proteins or metal protein complexes they occur in enzymes and respiratory pigments, for example, and may have structural role in polychaete jaws. As a result, studies on metal contamination tells the problem of metal present in natural levels and those which are enriched from anthropogenic sources and may, since metals are considerably toxic, produce unwanted effects.

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. 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 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. The objective of the present study is to isolate and identify the bacteria from Koodankulam coastal area. The bacteria were morphologically and their potential to resist the heavy metals like mercury, lead and zinc were determined.

2. Materials And Methods

Sample collection

The samples were collected around the area of offshore sea soil of Koodankulam coast, Gulf of Mannar, Tamil Nadu, South East coast of India, with the clean and sterile zip lock plastic bags. 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°C.

Isolation and identification of bacteria

Isolation and quantitative computation of bacteria from soil samples were performed by serial dilution technique (Harrigan, 2014). One hundred microliters of samples was spread onto nutrient agar plates and incubated at 37 °C for 24 h.

Assessment of bacterial tolerance to toxic metals

Bacterial tolerance to lead (Pb), mercury (Hg) and zinc (Zn) were determined by agar dilution method (Abou Shanab, 2007) which were added in the form of lead acetate, mercury choloride, zinc sulphate respectively. The initial concentration of these metal salts in nutrient plates was 1mg/1mL, and bacterial growth was observed by streaking on respective plates. Metal concentration was progressively increased by 2-3 mg/mL on a fresh agar plate, and the MIC was noted when the isolates failed to grow on respective plates. The experiment was conducted separately for Pb, Zn and Hg taking five replicates at each concentration.

Morphological and Physiological Analysis

Grams Staining and capsule staining

The primary analysis in the identification of the morphological structure of any bacteria was Gram's staining, Capsule staining.

Biochemical Analysis

The biochemical characterization of the isolate was done for the identification of microbes on the basis of different biochemical activities (indole production, methyl red, citrate, catalase, oxidase, Utilization of carbohydrates) (Bergey et al., 1974; Williams and Wilkins, 1994).

Plasmid DNA extraction

Plasmid DNA extractions of heavy metal resistant bacteria were done according to the alkaline lysis method (Sambrook and Russel, 2001). Then visualized it on gel electrophoresis at 1% agarose gel and compared with marker DNA (1kbp sharp DNA Marker- RBC Bioscience) to assess plasmid DNA.

3. Results and Discussion

Identification of metal-resistant bacteria

Visual observation of growth in heavy metal (Lead, Mercury, Zinc) separately supplemented (1000 µg/mL) LB medium after 24 h of incubation indicated that the collected sample consists of heavy metal degrading bacteria, as they can grow there by degrading heavy metals. Total ten bacterial isolates were isolated, among them five (S1, S2, S3, S4 and S5) were selected for further study (Figure 1 and 2).

Relative heavy metals consumption rate on bacterial growth

Thousand µg/mL amount of heavy metals (Pb, Hg and Zn separately) were supplemented in LB broth medium for each isolate, where each heavy metal concentration was gradually increased from1000 to 3000 µg/mL. The cultures were incubated for 24 h and bacterial resistance against each heavy metal. Then two potential isolates (S2 and S5) have been selected for conducting further MIC and characterization and identification tests (Figure 2).

Assessment of MIC against each heavy metal

Minimum inhibitory concentration (MIC) for each heavy metal was examined ranging from 1000 to 3000 µg/mL. It was found that all isolates exhibited resistance to heavy metals. MIC of heavy metals showed highest tolerance to Pb and Zn, by the selected isolates S2 and S5 (Table 1).

Characterization and identification

Top two potential heavy metal degrading isolates (S2, and S5) were characterized based on their cultural, morphological and biochemical characteristics (Table 1). Depending on gram staining, S2 was identified as gram-positive and S5 as gram negative bacteria. Capsule staining S2 as capsule negative and S5 was identified as capsule positive (Figure 3). Compared with standard description of Bergey's Manual of determinative bacteriology 9th edition (Bergey et al., 1974; Williams and Wilkins, 1994), the isolates were provisionally identified up to genus level as Bacillus sp. (S2); Pseudomonas sp.(S5) are consistent with past field studies (Claus and Berkeley, 1986).

Plasmid DNA extraction

Bacillus sp. (Fig. 4, Lane 2) and *Pseudomonas sp.* (Fig. 4, Lane 3)

Table: 1 Morphological and biochemical characteristic, carbohydrate utilization test, heavy metal resistance capacity and MIC of bacterial isolates (Barrow and Feltham, 1993; Claus and Berkeley, 1986).

Bacterial isolates	S2	S5
Morphological characteristics		
Colony colour	White, Milky yellowish	Green
Gram nature	Positive	Negative
Capsule staining	-	+
Motility	+	+
Cell shape	rod	rod
Biochemical test results		
Oxidase	-	+
Catalase	-	+
Indole	-	-
Methyl-Red	-	-
Citrate	+	-
Utilization of carbohydrate		
Glucose	+	+
Sucrose	+	-
Maltose	+	_
Xylose	-	+
Lactose	+	_
Resistance capacity		
Pb	++++++	++++++
Hg	+++	+++
Zn	++++++	++++++
MIC µg /ml		
Pb	1500	1880
Hg	700	650
Zn	1800	1600
Identified Bacteria	Bacillus sp.	Pseudomonas sp.

Figure 1 Culture plates (A) with and (B) without heavy metal incorporated media.

A

B

Figure 2 Pure cultures of five (S1, S2, S3, S4, S5) bacterial isolates.

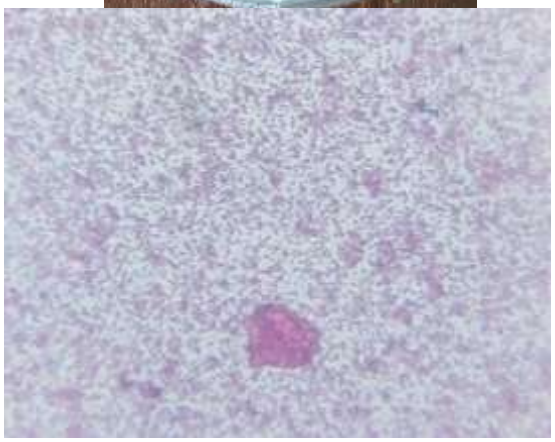
S1

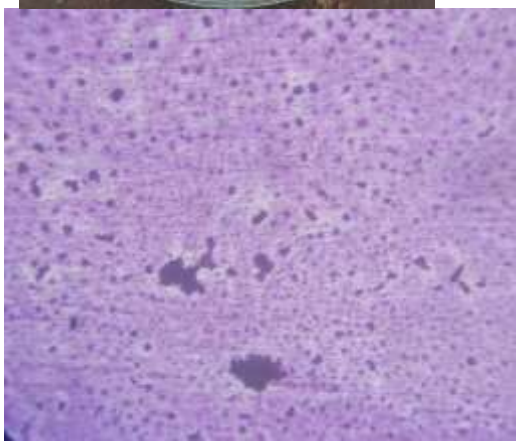
S2

S3

S4 **S5**

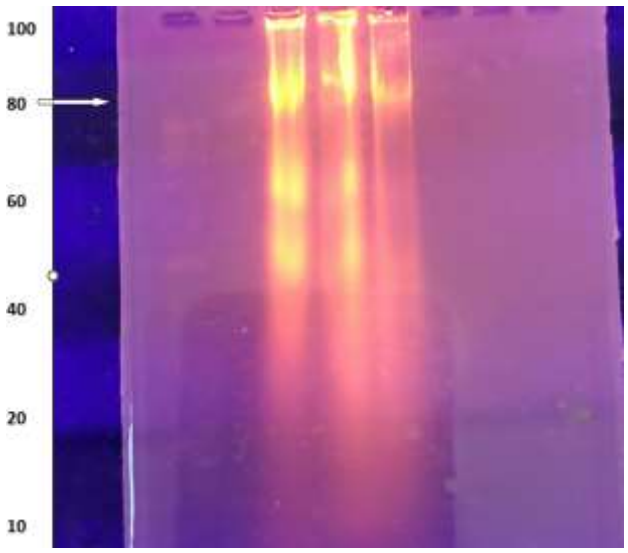
Figure 3 Staining of bacterial isolates





Gram's stain **Capsule stain**

Figure 4 Lane 1 show ladder DNA, 2 and 3 show plasmid of *Bacillus* and plasmid of *Pseudomonas* sp.



Lane 1 Lane 2 Lane 3

Heavy metals in our environment cause a devastating effect; no effective remediation technique has still been taken in hand. Bioremediation is cost-effective, safe and eco-friendly; can be virtually restored a solution to its pure condition (Liu et al., 1997). So, the major focus of this study is to isolate and identify the major bio remediating bacterial agents that help the natural recovery of Koodankulam surroundings. Preliminary screening of the collected samples for heavy metal resistance ability showed that sample was positively grown utilizing heavy metal (Pb, Hg and Zn) in their culture media. Serial dilutions of all samples yield five (2) distinct isolates from the heavy metal resistant bacterial population based on their morphology. The bacterial isolates were then characterized by morphological, biochemical tests. Identification of bacterial isolates were done according to Bergey's Manual of Determinative Bacteriology (Barrow and Feltham, 1993; Bergey et al., 1974). Depending on gram staining, S2 was identified as gram-positive and S5 as gram negative bacteria by detecting peptidoglycan which is present in a thick layer in bacteria (Burke and Pister, 1986). Micrococcus sp. are oxidase-positive, which can be used to distinguish them from other gram positive bacteria like most Staphylococcus sp., which are generally oxidase-negative (Thelwell et al., 1998).

In our study, S5 (Pseudomonas sp.) was identified as oxidase positive. Gram staining, oxidase tests and carbohydrate utilization studies showed similarities of S2 with Bacillus sp., which is gram positive, oxidase negative and utilizes all carbohydrates except xylose (Nucifora et al., 1989). The heavy metal resistance capacity approached two bacterial isolates Bacillus sp. and Pseudomonas sp. are highly resistant to Pb and Zn and less resistant to Hg. Upon above experiments, the resistance level Pb > Zn > Hg showed for Bacillus sp. and Pseudomonas sp. It was also reported that the tolerant levels of heavy metal for sewage bacteria Pseudomonas aeruginosa, Acinetobacter resistance, Proteus vulgaris were shown to be Pb > Cd > Cr (Powers and Latt, 1977). Minimum inhibitory concentration (MIC) is the lowest concentration at which the isolate is completely suppressed (as demonstrated by the absence of visible bacterial growth) is recorded. In this study order of MICs for the isolates S2 and S5 was found to be Pb > Zn > Hg. Bacillus sp. is resistant against Hg with MIC of 700 µg/mL, Pb with MIC of 1500 µg/mL has been recently shown by Ashour et al. (2011). Pseudomonas sp. was characterized in our study with MIC against Pb 1880 µg/mL, Hg 650 µg/mL as well as Zn 1600 µg/mL. Janda (2006) demonstrated that 13 bacteria are resistant to heavy metals (Zn, Pb, Cr, Cd); where Micrococcus luteus was found to be the most multiple heavy metals resistant. Recent study shows heavy metal resistance capacity either plasmid mediated or chromosomal DNA mediated (Virender et al., 2010). For determination of genetic basis for metal resistance, plasmid profiling is important. Plasmid DNA extraction of two bacterial isolates having biodegradation capacity was assessed to understand whether their heavy-metal resistance capacity is plasmid DNA or chromosomal DNA mediated. In our study, two strains Bacillus sp. and Pseudomonas sp. showed plasmid DNA, Probably the high degrading capacity of Bacillus sp. and Pseudomonas sp. can be the reason for their plasmid retaining ability (Ghosh et al., 1997). In bacteria, the heavy metal resistant genes are located either on the bacterial chromosome or in the plasmids or on both (Nies and Brown, 1997).

According to Malik (2004), Cd and Cr resistant genes are present in plasmid DNA but Pb resistance gene is located on chromosomal DNA of Enterobacteria. In this way, chromosomal gene might be responsible for this kind of lower degrading capability but more usually conferring resistance are located on plasmid (Woertz and Mergeny, 1997). Although this fundamental study will support for

plasmid curing, transformation and evaluation of heavy metal resistance can pave way for the genetic basis of heavy metal resistant mechanism. Plasmid mediated heavy metal resistance is important for further transformation study, which will render any heavy metal sensitive bacteria (recipient) into being heavy metal resistant bacteria (Mergeay et al., 2003; Vaijiheh and Naser, 2003). Further study of the effects of different supplements and conditions in their growth is needed to identify their efficiency as bioremediation agents, where optimization of pH, temperature, and incubation time can influence metal resistance capacity (Shivakumar et al., 2014). Bioremediation is the process of using the biological compound in order to treat the environment. The obtained isolates Bacillus sp. and Pseudomonas sp. 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.

4. Conclusion

In conclusion, bacterial isolation against heavy metals is a promising and crucial area of study with numerous environmental and industrial applications. Bacteria have evolved various mechanisms to resist and detoxify heavy metals, making them valuable tools in mitigating heavy metal pollution. The isolation and screening of metal-resistant bacteria, along with advances in genetic engineering and synthetic biology, provide opportunities for developing effective bioremediation strategies. However, challenges such as ensuring specificity, maintaining long-term viability, and addressing ethical and safety concerns must be carefully considered when employing bacteria for heavy metal remediation. Continued research in this field offers the potential for discovering new bacterial mechanisms, optimizing bioremediation techniques, and integrating bacterial solutions into broader sustainable practices. Overall, the isolation of heavy metal-resistant bacteria represents a promising avenue for addressing the significant environmental issue of heavy metal contamination and offers the potential to create a cleaner and more sustainable future.

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