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Isolation and identification of heavy metal resistant bacteria from petroleum soil of Loni, Ahmednagar

Neha A. Gurave, Vrishali V. Korde, Snehal S. Dhas and Mahesh Disale

Department of Biotechnology, P.V.P. College of Arts, Science & Commerce, Loni, Maharasthra, India

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ABSTRACT

Increasing bioremediation processes for soils contaminated by heavy metals is one of the severe concern. For the same, broad range of screening procedures for the bacteria having resistance to heavy metals such as lead, nickel, cobalt, chromium, copper, manganese and zinc was attempted. The current study deals with isolation and identification of heavy metal resistant bacteria from petroleum soil collected from petrol pump of Loni, Ahmednagar, Maharashtra. A strain were characterized on the basis of its morphological and biochemical screening tests. It was identified as Bacillus thuringiensis by 16s rRNA sequencing. The strains showed diverse metabolic pattern of carbon sources and other growth factors. They also show tolerance to other heavy metals,such as copper, lead and nickel. antibiotic resistance pattern of the isolate was studied. This study supplied potential bacterial materials for bioremediation in the future.

Keywords: Bioremediation, Heavy metal resistance, metabolic patterns, growth factors, *Bacillus thuringiensis*.

INTRODUCTION

Contamination of the heavy metal is major environmental problem because of their toxicity. Heavy metals are threat to human life and environment. Much research has been conducted on heavy metals contamination in soil from various sources such as Industrial waste, Automobile emission and agricultural practices. Metals are classified into three classes on the basis of their biological function and effects: (1) The essential metals with known Biological function, (Na, K, Mg, Fe, Co, Ni, Cu, Zn) (2) The toxic metals (Ag, cd, Sn, Au, Hg, Ti, Pb, Al Ge, As, Sb and Se)(3) The non essential non toxic metals with no known biological effects (Rb, Cs, Sr).Heavy metals are classified as i)Bound to reducible phases ii)exchangeable iii)Bound to organic matter and sulphides. Excessive levels of Heavy metals like Zinc, Cadmium, Copper, Lead, Nickel and mercury are considered as mainly toxic pollutants [6]. Lead(Pb) a major pollutant that is found in atmosphere is greatly toxic to human, animals, plants and microorganisms [7].Heavy metals contaminate the environment by gathering in the food chain and remains in nature. Each heavy metal has its sole toxicity. Copper and Zinc can improve microbial growth at low concentrations but suppresses growth at elevated concentrations [8]. 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 efflux of metal ions outside the cell, bioaccumulation of the metal ions inside the cell, and the decreasing concentration of the heavy metal ions.[3].Bacteria have modified to heavy metals

through a range of plasmid-mediated resistance systems [4].In several studies it has been observed that the heavy metal sensitivity or resistance of bacteria isolated from diverse habitat and its mechanisms to adapt the toxic metal during exposure .Some microorganisms have the resistance against the heavy metal and they can grow in the environment having rich heavy metal concentration**.** The occurrence of emergence of resistant bacteria to specific heavy metals can be associated with increasing loads of metals in the environment. As a result heavy metal resistant bacteria might be used as organic monitors of environmental contamination. Bioremediation may be employed in order to attack specific contaminants such as pesticides, hydrogenated carbons that are broken down using various techniques including the addition of fertilizer to assist the decomposition of contaminant by bacteria. Bioremediation can be considered as major useful technique for environmental cleanup and ecological repair. The objective of the present study is to isolate and identify the bacteria from Petroleum Soil of Loni, Ahemadnagar, Maharashtra. The bacteria were biochemically and genetically identified and their potential to resist the heavy metals such as Zinc, Silver, Mercury and Copper was determined.

MATERIALS AND METHODS

Sample Collection:
Soil samples were collected from the petrol pump of Loni District- Ahmednagar. Samplings was carried out from soil surface and at a depth of approximately 20cm were taken in sterilized polyethylene bags and stored at 4 ºC until examination.

Isolation and Identification of Bacteria
The soil sample was serially diluted upto 10-6 dilutions and plated on Nutrient Agar and Minimal Agar plates. One ml of diluted sample was spread onto the surface of Nutrient Agar medium and minimal agar medium and incubated at 37ºC for 24 h. Single colony was picked spread on the Nutrient Agar plates and subcultured in nutrient broth and again cultured on plates in order to obtain pure cultures. Pure bacterial strain were obtained after successive transfer of a single colony on Nutrient Agar plates and incubated for 24 h at 37ºC temperature. Morphological characters were determined by Gram's Staining and motility was determined by hanging drop method .

Biochemical Characterization:
Biochemical screening was done according to Bergey's Manual of Systematic Bacteriology [9]. By performing tests such as IMViC ,Sugar utilization,H2S production ,Urease production, Catalase detection, Starch Hydrolysis test, Antibiotic sensitivity tests etc.

Physiological Characterization:
Physiological characteristics were screened by supplying wide range of growth temperature10°C-50°C and 4-12 pH range for growth, tolerance uoto 5% NaCl concentrations were tested. To examine the ability of isolate to resist heavy metals, Spot inoculation was done on nutrient agar plate provided with different concentrations (0.5, 1.0, 3.0 and 5.0 mM) of heavy metals (silver in silver nitrate, Mercury in Mercurric II chloride, Zinc in Zinc sulphate and Copper in Copper II sulphate) Lead in Leadacetate and incubated at 37°C for 24 hours and cell growth were observed.

Molecular Characterization
1.DNA Extraction:
1. Bacterial Genomic DNA was isolated using the InstaGeneTM Matrix Genomic DNA isolation kit – As per the kit instruction below procedure followed.
2. An isolated bacterial colony was picked and suspend in 1ml of sterile water in a micro centrifuge tube.
3. Centrifuge it for 1 minute at 10,000–12,000 rpm to remove the supernatant.
4. Add 200 µl of Insta Gene matrix to the pellet and incubate at 56 °C for 15 minutes.
5. Vortex at high speed for 10 seconds and place the tube in a 100 °C in heat block or boiling water bath for 8 minutes.
6. Finally, vortex the content at high speed for 10 seconds and Spin at 10,000–12,000 rpm for 2 minutes.
7. In result, 20µl of the supernatant was used per 50 µl PCR reaction.
PCR Protocol:
Using below 16S rRNA Universal primers gene fragment was amplified using MJ Research Peltier Thermal Cycler.

2 Primer Details :-

Primer Name	Sequence Details	Number of Base
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTTACGACTT	22

Add 1µL of template DNA in 20 µL of PCR reaction solution. Use 27F/1492R primers used for bacteria, and then PCR reaction performed with below conditions

Initial Denaturation:94℃ for 2 min and then 35 amplification cycles at 94℃ for 45 sec, for 60 sec, and 72℃ for 60 sec. Final Extension at 72℃ for 10 min.

DNA fragments are amplified about 1,400bp incase of bacteria. Include a positive control (*E.coli* genomic DNA) and a negative control in the PCR.

3 Purification of PCR products
Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the 518F/800R primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

4. Sequencing protocol
Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Sequencing Primer Details :-

Primer Name	Sequence Details	Number of Base
518F	CCAGCAGCCGCGGTAATACG	20
800R	TACCAGGGTATCTAATCC	18

Sequence data was aligned and analyzed for Identifying the Sample.

5. Bioinformatics protocol:
1. The 16s r RNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.
2. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007).Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.

3. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering.

RESULTS AND DISCUSSION

A single strain was isolated from the sample collected from the petroleum soil of Loni, Ahmednagar. The density of microorganism was found to be 13 x 10-4 cfu/ml, pure culture was obtained in Nutrient Broth and they were screened for Gram reaction, motility detection and biochemical characterization. Results of morphological characterization were observed as Gram Positive, Rod Shaped and Motile. Biochemical Characterization were Indole production Positive, Methyl Red Positive, Citrate Utilization Positive, Starch hydrolysis test Negative, Catalase Positive, Gelatin Liquefaction Negative, Urease Positive, Carbohydrate utilization test Glucose- Positive, Sorbitol-Negative, Sucrose- Negative, Rhamnose- Negative, Manitol-Negative. Physiological characteristics were screened by providing wide range of tempreture range 20-40°C 0 and 4-12 pH range results are tabulated in

Table.1.Isolate was resistant to the following antibiotics: Chloramphenicol (5-50 µg/ml), streptomycin (5 - 250 µg/ml) and ampicillin (5-1500 µg/ml).Isolate show tolerance with Silver Nitrate (Ag) at 0.5mM,1mM,3mM concentrations and Mercuric Chloride tolerance was observed at 3mM, 5mM while media containing Zinc and Copper showed no tolerance . Sequence analysis of the 16S rRNA gene has been measured fast and precise technique to recognize the phylogenetic position of bacteria. Full-length 16S rDNA of strain were sequenced and used to create phylogenetic development tree. We found that strain in the branch of Bacillus sp. and it had 99% similarity to *Bacillus Thuringiensis*.

Table 1

Parameters	
1.Physiological Characteristics	
Temperature (°C)	
15°C	+
20°C	+
25°C	+
30°C	+
35°C	++
40°C	++
45°C	-
50°C	-
Growth at pH	
4	-
6	-
7	++
7.5	++
10	+
12	-
Antibiotic Resistance	
Chloramphenicol	50 µg/ml
Streptomycin	250 µg/ml
Ampicillin	1500 µg/ml

Fig-1 Growth on Nutrient Agar Plate

Fig-2 Growtth on Minimal Agar plate

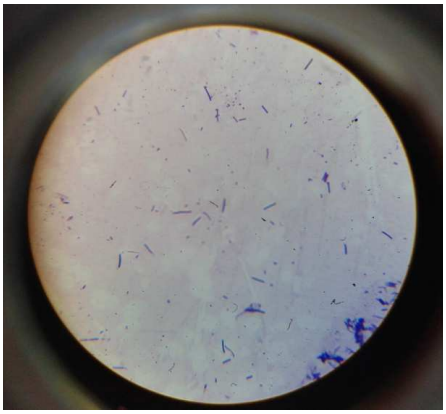

Fig-3 Gram's Staining

CONCLUSION

The presence of bacteria capable of tolerating heavy metals from soil samples from heavily contaminated petroleum sites was investigated. Bacteria that resist high levels of heavy metals were isolated in pure cultures. In summary our results that isolate strain characterized with remarkable tolerance against heavy metals, could be potential agents for the development of a soil inoculants applicable in bioaugmentation of heavy metals polluted sites. The genetic modification of bacteria can be opened new horizons of bioremediation in heavy metal pollution. Genetic upgrading may help to expand the field of accessible methodologies to refining processes. Regardless, the genome sequencing of microorganisms and the use of proteomics, genomics, metabolomics, can offers insights into some metabolic pathways in order to discover genes, proteins, metabolites that could be involved in heavy metal tolerance.

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