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Isolation and Identification of Heavy Metals Tolerant Bacteria from Industrial and Agricultural Areas in Kerala



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Research Paper :

INTRODUCTION :

"Heavy metal" is a general collective term, which applies to the group of metals and metalloids with atomic density greater than 4000 kg m^{-3}, or 5 times more than water (Garbarino *et al*., 1995). Although some of them act as essential micronutrient for living beings, at higher concentration they can lead to severe poisoning (Kavamura and Espisoto, 2010). Elevated levels of heavy metals not only decrease soil microbial activity and crop production, but also threaten human health through the food chain (Mclaughline *et al*, 1999). Heavy metals are generally more persistent and stable than organic contaminants such as pesticides or petroleum byproducts and are non-biodegradable (Lasat, 2002).

The most important features that distinguish heavy metals from other toxic pollutants are their non-biodegradability and propensity in living materials (Orha and Buyukgungor, 1993). Heavy metals have been reported to inhibit bacterial growth, as indicated by an extension of growth rate and generation time (Mahapatra and Banarjee, 1996). Heavy metals can damage the cell membranes, alter enzymes specificity, disrupt cellular functions and damage the structure of the DNA. Toxicity of these heavy metals occurs through the displacement of essential metals from their native binding sites or through ligand interactions.

Industrial development results in the generation of industrial effluents, and if untreated results in water, sediment and soil pollution (Fakayode, 2005). Metal processing, finishing and plating are the main sources of metal wastes. Since soil is one of the most important environments for microbes and is easily exposed to many pollutants, evaluating the effects of pollutants on the microbial population is very valuable. Since the resistance of bacteria depends not only on their type and environment, but also on physical factors such as the type and concentration of the substances, the microorganisms

with significant resistance can reveal the condition of their environment (Javadi and Kargaran, 1997; Mullen *et al*., 1989).

Heavy metal pollution of soil and wastewater is a significant environmental problem (Cheng, 2003). Use of industrial wastewater for irrigation is a common practice in most of the third world countries which could alter the fertility of soil (Bouwer, 2002). Moreover, accumulation of heavy metals in vegetation due to irrigation with waste water could affect human health (Koropatnick and Zalups, 1997). Heavy metals including cadmium, lead, chromium, mercury, copper and nickel, which contaminate the soils, ground water, sediments and surface water are extremely toxic to both the biological and ecological systems. These heavy metals are released due to the discharge of effluent into the environment by a large number of processes such as electroplating, leather tanning, wood preservation, pulp processing, steel manufacturing, etc., and the concentration levels of these heavy metals varies widely in the environment. Heavy metals pose a critical concern to human health and environmental issues due to their occurrance as a contaminant, low solubility and due to the carcinogenic and mutagenic potential (Alloway, 1995; Diels *et al*., 2002).

Conventional physicochemical techniques for metal remediation such as filtration, acid leaching, electrochemical processes or ion exchange are expensive and may not be very effective (Dixon, 1996). Microbes have developed mechanisms to tolerate the metals through efflux, complexation, or reduction of metal ions or to use them as terminal electron acceptors in anaerobic respiration (Haferburg and Kothe, 2010). Most mechanism reported involves the efflux of metal ions outside the cell. The genes for tolerance mechanisms have been found on both chromosomes and plasmids. Bacteria that are resistant to and grow on metals play an important role in the biogeochemical cycling of these metal ion (Meghraj and Dhaneswar, 2013).

The present paper deals with the isolation and characterization of heavy metal tolerant bacteria from industrial effluents and agricultural areas in and around Kochi, Kerala and determination of Minimum Inhibitory Concentration (MIC) and antibiotic resistance of the heavy metal resistant bacterial isolates. The characterization of strains were done by both biochemical methods and molecular techniques using 16SrDNA primers.

MATERIALS AND METHODS

Isolation of bacteria from samples

The areas for sample collection was identified based on the need, diversity, and extend of pollutants produced by various industries located in Kerala.

A serial dilution of samples (1g in the case of fresh soil & 1ml in case of water sample) was made using sterile distilled water until a dilution of 10^{-6} was obtained. 0.1ml of 10^{-4}, 10^{-5}, and 10^{-6} dilutions were inoculated on nutrient agar and incubated at 37°C for 24h. Pure culture was isolated and sub cultured twice in the medium at 37°C.

Screening of heavy metal tolerance of the isolated strains in nutrient agar media

To examine the heavy metal tolerance of the isolated strains, cells of overnight grown cultures were inoculated on nutrient agar plates supplemented with different concentrations of heavy metals (silver in silver nitrates, lead in lead acetate, mercury in mercuric chloride, zinc in zinc sulfates and copper in copper sulfates. After the incubation period, the plates were observed for any kind of growth on the media. The isolated and distinct colonies on these selective media were subcultured repeatedly on the same media for purification.

Biochemical tests

The following biochemical tests were performed for the identification of isolated species-starch hydrolysis, indole test, methyl red test, Voges-Prausker test, citrate utilization test, growth at 7%, 8% and 10% concentration of sodium chloride (NaCl), Gram's staining and growth at 55°C. The pure culture was identified on the basis of their morphology and biochemical characters

according to Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) and further by molecular analysis.

Determination of minimum inhibitory concentration (MIC) of heavy metals

Heavy metals of different concentrations were added into the media after autoclaving. Overnight grown cultures of heavy metal resistant bacteria were inoculated into nutrient agar plates supplemented with different concentrations of (0.5, 1.0, 3.0, 5.0mM) heavy metals (silver in silver nitrate, lead in lead acetate, mercury in mercuric chloride, zinc in zinc sulfate and copper in copper sulfate). The culture growing on the last concentration was transferred to nutrient broth. The broth after 24 hours of incubation was spread plated on to next higher concentration. The procedure was repeated until the isolates were unable to grow on the heavy metal incorporated media and MIC value was determined.

Antibiotic sensitivity test

Standard suspensions of rapidly growing bacteria were inoculated on to the surface of Muller-Hinton agar plates. Filter paper disc containing specific concentration of antimicrobial agents were presented on to the surface and incubated at 37°C overnight (18-24hours) (Table 1). Zone of inhibition of growth around each disc was measured and susceptibility was measured.

Amplification of 16S rDNA genes by polymerase chain reaction (PCR)

Genomic DNA was isolated according to the method of Janarthanan and Vincent, 2007.The 16S rDNA genes were amplified with bacterial universal primers specific for eubacterial 16S rDNA gene (Forward, AGTTTGATCATGGCTCAG) and (Reverse, TTACCGCGGCTGGCA) primers (Hookoom and Puchooa, 2013). The PCR (25 µl)contained 0.25 µl of each forward and reverse primer,2.5 µl of 10X Taq buffer (stock 10mM), 0.315 µl(10mM) of each deoxynucleotide (ddATP, ddGTP, ddCTP and ddTTP), 0.48 units of *Taq* DNA polymerase(3units/µl) and 2.5 µl DNA. Amplification of DNA was performed in a thermal cycler (Eppendorf) using the following conditions : denaturation at 94° C for 3 min, 30 cycles of denaturation at 95° C for 1 min, annealing at 61°C for 1min, extension at 72°C for 1 min, and a final extension

step at 72° C for 10 min. The PCR product obtained from DNA extracted from the samples were first analyzed by electrophoresis in 1.5% agarose gel , stained with ethidium bromide and was visualized and photographed under gel documentation system (Vilber Lourmat Gel Documentation Systems, France).

Nucleotide sequencing and Alignment

Automated sequencing of the positive samples was performed. The nucleotide sequence of the two samples were compared with the sequences available in the nucleotide databases using BLAST tool (www.ncbi.nlm.nih.gov/blast) provided by NCBI. Nucleotide-nucleotide sequence comparison was done using Blast X.

RESULTS AND DISCUSSION

Isolation and screening of bacteria from samples

Six samples were collected from industrial and agricultural areas in and around Koch, India. The samples were grown in nutrient agar medium and 275 isolates were observed. The heavy metal tolerance of the strains was analyzed by their ability to grow in media incorporated with various concentrations of heavy metals. About six samples showed significant growth on heavy metal incorporated media from which two samples with profuse and characteristic growth pattern were selected for further analysis. The capacity of isolated strains to detoxify heavy metals at different concentrations (0.5mm to 5mM) were tested. These studies resulted in the selection of two most efficient strains for heavy metal detoxification.

Biochemical identification

The strains were characterized by biochemical tests according to the Bergy's manual of Determinative Bacteriology showed the presence of *Bacillus megaterium* and E.coli in the sample. Further, the samples were subjected to molecular analysis for confirmations.

Minimum Inhibitory Concentration (MIC) of heavy metals

MIC value of heavy metal is the particular concentration of heavy metal above which microorganisms are not able to tolerate the heavy metals. The

two organisms were found to cope up with higher concentrations of metals like zinc sulfate, copper sulfate and lead acetate. However they were found to be less effective in the bioremediation of heavy metals like silver nitrate and mercuric chloride (Table 2).

Antibiotic sensitivity test

Antibiotic resistance genes and heavy metal resistance genes are usually seen closely associated within the plasmids and can be used for further genetic manipulation studies of the resistance traits present. This result of the study gives us details of antibiotic resistance traits present in the bacteria. Resistance gene distribution pattern among the isolated strains were analysed by Kirby Bauer Disc Diffusion Method. From the antibiotic sensitivity test *Bacillus megaterium* was found to be resistant to penicillin and *E.coli* was found to be resistant to antibiotics such as penicillin, cefalexin and erythromycin (Table 3).

Molecular characterization

The samples were amplified with forward and reverse primer specific for 16S rDNA which generated specific amplicons 502 and 499bp respectively (Figure 1, 2). Then the nucleotide sequences of the two samples were compared with the sequences available in the nucleotide databases using the Basic Local Alignment Search Tool (BLAST). The results further confirmed the identities of the strains. The sequence of amplicons were then compared using BLAST software which showed 97% similarity of sample 1 for *Bacillus megaterium* and 96% similarity of Sample 2 for *E.coli*. Thus the study identified two efficient strains of bacteria with potent heavy metal bioremediation capacity which can be used for bioremediation of environment.

CONCLUSION

In the present study we have isolated two efficient bacterial strains with potential heavy metal bioremediation capacity from industrial and agricultural areas of Kerala. By biochemical analysis the strains were found to be *Bacillus megaterium* and *E.coli* respectively. The two organisms were found to be able to grow in the presence of higher concentrations of heavy

metals like zinc sulfate, copper sulfate and lead acetate as obtained from MIC studies. Since anibiotic resistance genes and heavy metal resistance genes are closely associated within the plasmid, antibiotic resistance can be used as a selective marker for further gene manipulation studies. From the antibiotic sensitivity test both the samples were found to be resistant to antibiotics such as penicillin, cefalexin and erythromycin. The identification of these strains were further confirmed by 16S rDNA sequence analysis. For this DNA samples were isolated from these two strains and 16S rDNA sequences were amplified by specific primers using PCR. The sequence of amplicons were then compared using BLAST software which showed 97% similarity of sample 1 for *Bacillus megaterium* and 96% similarity of Sample 2 for *E.coli*. Thus the study identified two efficient strains of bacteria with potent heavy metal bioremediation capacity which can be used for bioremediation of environment.

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TABLES

Antibiotics	Concentration
Rifampicin	30 mcg
Chloramphenicol	30 mcg
Erythromycin	10 mcg
Tetracycline	30 mcg
Kanamycin	30 mcg
Nalidixic acid	30 mcg
Doxycycline hydrochloride	5 mcg
Cefalexin	30 mcg
Penicillin	10 units

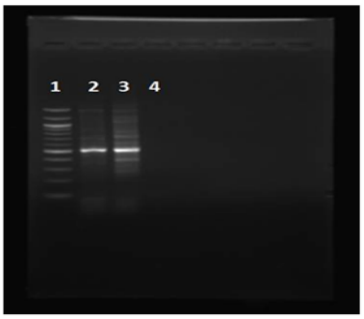
Table: 1 Concentration of the antibiotics selected for the antibiotic sensitivity assay.

Sample	Lead acetate	Silver nitrate	Zinc sulphate	Copper sulphate	Mercuric chloride
S1	2mM	0.5mM	5mM	3mM	0.5mM
S2	2mM	0.5mM	5mM	5mM	0.5mM

Table: 2 MIC values of S1 and S2 samples

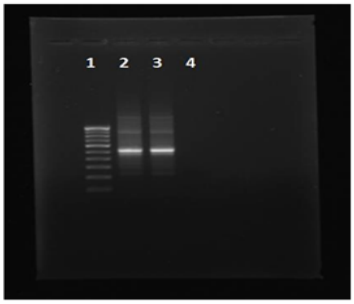
Sample	Antibiotics used in the study								
	Penicillin	Chloramphenicol	Rifampicin	kanamycin	Nalidixic acid	Tetracycline	Cefalexin	Erythromycin	Doxycycline hydrochloride
S1	Resistant (13mm)	Sensitive (25mm)	Sensitive (25mm)	Sensitive (22mm)	Sensitive (20mm)	Sensitive (30mm)	Sensitive (20mm)	Sensitive (31mm)	Sensitive (30mm)
S2	Resistant	Sensitive (29mm)	Sensitive (15mm)	Sensitive (30mm)	Sensitive (25mm)	Sensitive (27mm)	Resistant	Resistant (12mm)	Sensitive (18mm)

Table: 3 Antibiotic sensitivity test and inhibitory zone size of S1 and S2 samples



Lane 1- 100 b p marker
Lane 2- Positive control
Lane 3- S1 sample
Lane 4- Negative control

Figure: 1 PCR confirmation of S1 sample using 16S rDNA specific primers



Lane 1- 100 b p marker
Lane 2- Positive control
Lane 3- S2 sample
Lane 4- Negative control

Figure: 2 PCR confirmation of S2 sample using 16S rDNA specific primers

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