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KOCURIA SPECIES IDENTIFIED AS LEAD RESISTANT BACTERIA ISOLATED FROM WATER BODIES OF UDAIPUR REGION

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ABSTRACT

Lead is a highly toxic heavy metal that gives toxic effects to humans and environment. It shows higher toxicity in children. The present study was carried out for isolation and identification of lead resistant bacteria that can be further utilized for bioremediation of lead. A potential lead resistant strain GVG2 and GVP8 was isolated from the contaminated sites of water bodies of Udaipur region *viz*. Udaisagar lake and Gadwa pond that was found to be resistant against lead with MIC 1200 mg/l and 1300 mg/l, respectively. 16S rDNA sequence analysis showed that GVG2 and GVP8 isolates matched best with genus of *Kocuria* and was identified as *Kocuria sediminis* (*Kocuria* sp. GVG2) and *Kocuria marina* (*Kocuria* sp. GVP8) with permanent accession number KY859844 and KY859852.

KEY WORDS : Kocuria sp., Heavy metal resistant bacteria, Lead resistant bacteria, Bioremediation, Minimum Inhibitory concentration (MIC), 16S rDNA sequencing.

INTRODUCTION

Heavy metal pollution of soil and wastewater is a significant environmental problem (Cheng, 2003). Wastewaters from the industries and sewage sludge applications have permanent toxic effects to human and the environment (Rehman *et al*., 2008). It is well established that domestic sewage and industrial effluents that fall into natural water bodies change the water quality and lead to eutrophication. Heavy metals are difficult to remove from the environment (Ren *et al*., 2009). Lead is hazardous to children even in very low concentration and causes mental retardation. Chemical methods such as precipitation, evaporation, electroplating, ion-exchange etc. have been widely used to remove metal ions from industrial waste water but these methods are ineffective or expensive and have several disadvantages such as unpredictable metal ion removal, high reagent requirement, generation of toxic sludge etc. There are a number of biological materials that can be used to remove metals from waste water, such as molds, yeasts, bacteria, and seaweeds (Vieira and Volesky, 2000 and Waisberg *et al*., 2003). The ability of micro-organisms is recognized for environmental management, and microbes have superseded the conventional techniques of remediation. This ability of microbial stains to grow in the presence of heavy metals would be helpful in the waste water treatment where microorganisms are directly involved in the decomposition of organic matter in biological processes for waste water treatment (Munoz *et al*., 2006 and Prasenjit, 2005) In this study two water bodies Udaisagar lake and Gadwa pond belonging to Berach river system, Udaipur region were selected to isolate and characterize lead tolerant bacteria. The morphological, biochemical and molecular characterization using 16S rDNA sequencing of isolates have been done to identify the metal tolerant isolates. These isolates can be used to remove toxic lead from industrial effluents.

MATERIALS AND METHODS

A. Sample collection

Samples of water were collected from heavy metal

contaminated sites of Udaisagar lake and Gadwa pond, Udaipur, Rajasthan, India and kept in sterile bottles under refrigeration to ensure minimal biological activity until processing in the laboratory.

Isolation of lead resistant bacteria

Lead tolerant bacteria were isolated on nutrient agar supplemented with 100 mg/l concentration of lead acetate trihydrate by the standard pour plate method. Plates were incubated at 37 °C for 24-48 hours.

Morphological and Biochemical characterization of lead resistant bacteria

Characterization of isolates was done by studying morphological (Gram staining and shape) and biochemical characteristics (catalase activity, oxidase activity, acid production from glucose, citrate utilization, nitrate reduction). The tests were used to identify the isolates according to Bergey's Manual of Systematics Bacteriology (Claus and Berkeley, 1986).

Determination of Minimum Inhibitory Concentration (MIC) of lead for isolates

The MIC was defined as the lowest concentration of the heavy metal that inhibits the visible growth of the organisms. After the preliminary isolation of the lead tolerant bacteria, the minimum inhibitory concentration (MIC) of lead was determined by agar plate dilution method as adopted by Malik and Jaiswal, (2000). The metal lead was used in different concentration ranging from 1000 mg/l to 2000mg/l. Stock solutions of the metal salt (lead acetate) 4g/ 100 ml were prepared in sterile water to obtain final concentrations of 600 to 1500 mg/l lead. The petri plates were inoculated with 10 µl of an overnight broth culture and incubated at 37°C for 24-72 hours.

Isolation of genomic DNA

Genomic DNA from all the isolates was extracted with some modification in phenol chloroform method. Bacteria were cultured overnight in nutrient broth and 1.5 ml of culture was harvested in TE buffer (pH 8). Lysis was initiated by the addition of lysozyme (4 mg/ml). After incubation at 37 °C for 30 minutes, 40 µl of 10% sodium dodecyl sulphate (SDS) was added and the tubes were incubated at 45 °C for 60 minutes. Cell debris and protein were separated with phenol and chloroform extraction and DNA was precipitated by adding sodium acetate (3M) and twice volumes of absolute ethanol. Tubes were incubated at -20°C for 12 hours. After incubation, tubes were centrifuged and the pellet was washed with 70% ethanol and air dried. The pellet was dissolved in 100 µl of TE buffer and stored at -20 °C for further use.

16S rDNA amplification

The 16s rRNA gene of bacteria was amplified using 16s rRNA gene sequence as forward primer (5'AGAGTTTGATCCTGGCTCAG-3') and as reverse primer (5' AAGGAGGTGATCCAGCCGCA 3'). The PCR conditions were standardized as follows. Initial denaturation at 94 °C for 5 minutes, 35 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 45 sec and extension at 72 °C for 1 minutes and the final extension at 72 °C for 10 minutes. The resulting amplified products were run on 1.5% agarose gel and visualized by using gel documentation system (Gel Doc XR+, Bio Rad).

Sequence analysis

Amplified 16S rDNA products were sequenced from Bioinovations, Mumbai. Sequencing was determined by the dideoxy chain termination method. The closest relatives of 16S rDNA sequences were determined by a search of the GenBank DNA database using the BLAST algorithm. Homology comparisons were performed using the Basic Local Alignment Search Tool (BLAST), online at the National Centre for Biotechnology Information (NCBI) homepage (www.ncbi.nlm.nih.gov). Identities of isolates were determined based on the highest score.

RESULTS

Isolation of lead resistant bacteria

A total 11 indigenous bacterial strains were recovered from water samples on nutrient agar supplemented with 100 mg/l concentration of lead acetate trihydrate by the standard pour plate method. Further all of these isolates were grown on 500 mg/mL concentration of lead. Out of them, two strains GVG2 and GVP8 were selected that showed good growth at this concentration.

Morphological and Biochemical characterization

Two selected isolates were characterized by detecting their cultural, morphological and biochemical characteristics (Table 1). The investigation results indicated that both the isolates were gram-positive, cocci shaped bacteria and GVP8

Table 1. Morphological and Biochemical characterization of lead resistant isolates.

Characteristics	Metal tolerant Isolates	
	GVG2	GVP8
Morphology(Colony colour and Shape)	Orange, small circular	Yellow, circular
Cell Shape	cocci	Cocci in group
Gram Reaction	+	+
Catalase	-	+
Oxidase	-	-
Citrate Utilization	-	-
Nitrate Reduction	-	-
Glucose Fermentation	-	+

+ = Positive, - = Negative

gave positive reaction for catalase activity and glucose fermentation. Both the isolates gave negative reaction for oxidase activity, nitrate and citrate utilization test. The above results obtained for morphological and biochemical characteristics were further matched with Bergey's Manual of Systematics Bacteriology (Claus and Berkeley, 1986).

Minimum Inhibitory Concentration of lead ions

The MIC of lead was determined using increasing concentrations of lead (1000 to 2000 mg/l). The growth of the isolate GVG2 and isolate GVP8 were observed after 72 hours of incubation in the nutrient agar medium up to 1200 and 1300 mg/l concentration of lead. The result showed in Table 2 revealed that MIC of lead for isolates GVG2 and GVP8 was 1200 and 1300 mg/l respectively. Isolate GVP8 showed maximum value of MIC. GVG2 and GVP8 didn't show any growth on higher concentration of lead (1300 mg/l and 1400 mg/l respectively).

Molecular identification of lead resistant bacteria

Genomic DNA was isolated and visualized on 1.0 percent agarose gel. For the identification of isolates at species level the 16S rDNA amplification was done using PCR technique. Amplified products were run in 1.5 percent agarose gel and visualized using gel documentation system. The amplified products of isolates were sequenced and the obtained sequences were compared with the available gene sequences at NCBI website by using BLASTn. Sequences submitted to GenBank, USA (Table 3) showing more than 95% similarity with the GenBank sequences.

Table 2. Minimum Inhibitory Concentration (MIC) values of lead for two lead tolerant isolates

Isolate	MIC (mg/l)
GVG2	1200
GVP8	1300

DISCUSSION

Heavy metal tolerant bacteria have been isolated from soil, industrial effluents, fresh water bodies, rivers, waste water and sewage (Mgbemena *et al*., 2012; Pandit *et al*., 2013; Narasimhulu *et al*., 2010; Murthy *et al*., 2012). In this study lead tolerant bacteria were isolated from polluted sites of water bodies named as Udaisagar lake and Gadwa pond of Berach river system, Udaipur.

A total of 11 bacterial isolates were recovered on nutrient agar supplemented with 100 mg/l concentration of lead acetate. Out of them 2 isolates were selected on the basis of high level of lead resistance (up to 500 mg/l). Morphological characterization of isolates showed these isolates as Gram-positive species. *Kocuria* isolates GVG2 and GVP8 showed high level of Minimum inhibitory concentration values 1200 and 1300 mg/l respectively. Maximum MIC of lead for the isolates was found upto 1300 mg/l. MIC value of lead for

Table 3. Accession numbers generated by GenBank, NCBI, USA for submitted nucleotide sequences of lead tolerant isolates.

S.No.	Isolate	Accession number	Name of identified strain
1	GVG2	KY859844	*Kocuria sediminis*
2	GVP8	KY859852	*Kocuria marina*

Bacillus cereus was reported at 1000 mg/l concentration and MIC of zinc was reported at 10mM for *Pseudomonas aeruginosa* (by Murthy *et al.*, 2012 and Bhojiya and Joshi, 2012). Molecular Identification of metal tolerant bacteria have been done using 16S rDNA sequencing technique, this technique was also used in various studies for the identification of metal tolerant isolates at species level (Chatterjee *et al.*, 2012; Pandit *et al.*, 2013). In this study *Kocuria* spp. was identified as lead tolerant bacteria. *Kocuria sediminis* sp. nov., type strain FCS-11(T) isolated for the first time from a marine sediment sample taken from Kochi fort area, Kerala, India by Bala *et al.*, 2012. Similarly *Kocuria* sp. have been identified as arsenic resistant bacteria by Ponce *et al.*, (2016) and and as copper resistant bacteria by Hansda *et al.*, (2017).

The industrial effluents and waste water are rich source of heavy metals and bacteria reside in them must be tolerant to heavy metals. The isolation of lead tolerant bacteria from polluted sites of Udaisagar lake and Gadwa Pond and their molecular characterization using 16S rDNA sequencing up to species level lead to the application of these isolates for the removal of lead from waste water. The results of the study suggest that *Kocuria* species can be useful for the bioremediation of lead contaminated industrial effluents and waste waters.

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