ISOLATION AND IDENTIFICATION OF HEAVY METAL RESISTANT BACTERIA BASED ON 16S rRNA SEQUENCING FROM BARITE MINES OF KADAPA DISTRICT ANDHRA PRADESH.

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ABSTRACT

This present study focuses on isolation and screening of a indigenous heavy metal resistant bacteria strain from Agricultural and Mining soils N:14^{0}03'07.4" E 79.18'.01.0" ,N ; 14.01 32.5" E 79 ^{0} 19'12.7"of Barites mines Mangampet YSR Kadapa District Andhra Pradesh, Individual colonies of bacteria which varied in shape and colour were picked up and purified by streaking on Nutrient agar, four bacteria were screened for heavy metal resistance succeeding isolation from respective soils they were exposed to different concentrations of Chromium, Lead, Copper, Nickel, Zinc from 0 to 8000mg/l. conclusively a strain outstood in tolerating 8000mg/l of Lead acetate besides growing in other metal solutions for over 300mg/l, k2cr2o7 ,200mg/l Nicl2,Cuso4 300mg/l 300mg/l Zncl2. Hence the strain which could tolerate 8000 mg/l of lead acetate felt the need of identification and was identified based on 16S rRNA sequencing as klebsiella pneumoniae with Accession number KJ 194599.

Keywords: Klebsiella pneumoniae, Heavy metal resistant, Barites mining area, YSR Kadapa District.

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INTRODUCTION

Heavy metals is a general collective term to the group of metals and metalloids with atomic density greater than 4000 kg m-3 or 5 times more than water however some of them act as essential micro nutrients for living beings but when it exceeds in concentrations they can be harmful. In the environment the heavy metals continue to exist for a longer period and are stable than organic contaminants and are non- biodegradable The environmental pollution by heavy metals arrives from anthropogenic sources such as smelters, mining, power stations and the application of pesticides containing metal fertilizer and sewage sludge and the reckless disposal of wastes by various industries they can become mobile in soils depending on soil pH and their speciation So a fraction of the total mass can leach to aquifer or can become bio available to living organisms heavy metals can stagnate in biological systems and ultimately be introduced into food web via different mechanisms them.[15-19] Microbes which are continuously exposed to heavy metal stress have adapting mechanisms to the metal

contaminants, have a multiple ways to carry on with high concentrations of heavy metals and often are specific to one or a few metals [20-24] Indigenous organisms have adapted to novel environments but have also thrived under them.[4-5]They possess a variety of mechanisms to deal with higher concentrations of heavy metals because they have developed mechanisms to tolerate heavy metals through efflux, complexation, or reduction of metal ions or to use them as terminal electron acceptors in Anaerobic respiration.[7]Microorganism able to survive well in higher concentrations of heavy metals are of great interest as bioremediation agents because they can achieve different transformation and immobilization processes Specifically they bio accumulate based on the incorporation of metals inside the living biomass or bio sorption in which metal ions are adsorbed at the cellular surface by different mechanisms[6] Metal accumulative bioprocess generally falls into one of two categories, absorptive(passive)uptake by nonliving, non growing biomass or biomass products and bioaccumulation by living cell [31-36] and the complex structure of microorganisms implies that there are many ways for the metal to be taken up by the microbial cell according to the dependence on the cell's metabolism[1]Many microorganisms have developed chromosomally or extra chromosomally controlled detoxification mechanisms to overcome the detrimental effects of heavy metals[3]The detoxifying ability of these resistant microorganisms can be applied for bioremediation of heavy metals in wastewater. Effluents having heavy metals can be treated with these microorganisms by the processes like bio- sorption, bioaccumulation and bio precipitation this study aimed to isolate and identify heavy metal resistant bacteria tolerating higher concentrations of heavy metal from Barite Agricultural and mining area situated at Mangampet YSR kadapa district Andhra Pradesh and its application like above.

ISOLATION OF BACTERIA FROM SAMPLES

Study area and sampling

Soil samples were collected from Barite Mining and Agricultural area Mangampet Y.S.R District, Samples were collected from the depth of approximately 15 cm after which they were placed in sterilized polyethylene bags using a sterilized spatula, All samples were stored at 4 ºC until analysis.[24]

CHEMICALS AND MEDIA

Stock solutions of heavy metal (1000 mg L-1) were prepared in distilled water the elemental salts employed were lead acetate Pb(C2H3O2)2,Potassium dichromate(k2cr2o7) Nickel chloride(Nicl2),Zinc chloride(Zincl2),copper sulphate (Cuso4),(from.DDH, Mumbai India) Nutrient agar was used for Isolation of bacteria. [35, 42]

ISOLATION OF BACTERIA

Soil samples were passed through a sieve (2 mm) to remove large pieces of debris and vegetation. The bacteria were originally isolated by plating dilutions of soils in distilled Water on Nutrient agar and then incubated at 30 °C for 48 h. Individual colonies of bacteria which varied in shape and colour were picked up and purified by streaking on nutrient agar.

The bacterial isolates were maintained on nutrient agar at 4°C and recultured every four weeks. All isolated, selected bacteria were purified and investigated for further work. [45]

SCREENING OF HEAVY METAL RESISTANT BACTERIA

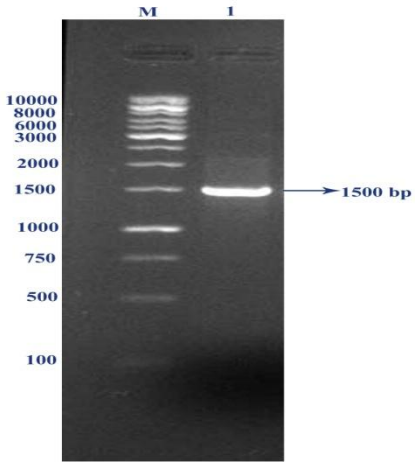
The four isolated strains were screened for resistance using the Agar diffusion method. Agar plates containing 20 ml nutrient agar medium supplemented with different concentrations from (0 to 8000mg/l) lead acetate Pb(C2H3O2) potassium dichromate(k2cr207) and Nickel chloride(Nicl2), zinc chloride (Zincl2),copper sulphate(Cuso4) were inoculated with the purified bacteria and incubated at 30 ºC for 48h[35]. An Isolate showed high resistance to lead which is at concentration of 8000mg/ l besides 300mg/l k2cr2o7 , 200mg/l Nicl2 , Cuso4 300mg/l,300mg/l Zncl2. and was therefore identified based on its 16S rRNA gene of selected isolate.[45,42]

Resistance of bacterial isolates to different heavy metals and concentrations

Elements	Conc./mg/l	X2	X3	X4
Lead	50	+	+	+
	100	-	-	+
	200	-	-	+
	300	-	-	+
	40 to 8000mg/l	_	_	+
Chromium	50	_	_	+
	100	_	_	+
	200	_	_	+
	300	_	_	+
	400 to 8000mg/l	_	_	_
Zinc	50	+	+	+
	100	+	+	+
	200	_	_	+
	300	_	_	+
	400 to 8000mg/l	_	_	_
Nickel	50	_	_	+
	100	_	_	+
	200	_	_	+
	300	_	_	_
	400 to 8000mg/l	_	_	_
Copper	50	_	_	+
	100	+	_	+
	200	+	+	+
	300	_	_	+
	400 to 8000mg/l	_	_	_

ANALYSIS OF 16S rRNA GENES WAS CONDUCTED AS

The method described by Ausubel et al(1994)[2]was slightly modified and used for Genomic DNA isolation PCR was performed for the amplification of 16S region, Amplification was performed using the following Amplification conditions Amplification Step1. 94^0Cfor5min [Initialdenaturation], : 94^0C for 1 min [Denaturation] Step 3: 55^0C for 1 min (annealing) 40 cycles**,** Step 4: 72^0C for 1 min (Elongation) Step 5: 72^0C for 10 min (final extension) DNA oligomers used in this experiment were Forward 16sF: 5'AGAGTTGATCCTGGCTCAG-3'Reverse16sR:5'-CAAGGCATCCACCGT3'Theforward primer was complementary to the upstream of 16S rDNA The PCR products were visualized on an UV illuminator (UV Tech, France). The images of gels were further analysed by a Image J computer programme.



SEQUENCING

The sequencing reactions were performed in a SEQ4×4 Personal Sequencing System (MWG Biotech, Bangalore). The sequences obtained were then evaluated in GenBank using BLAST programme and accession numbers were obtained.

RESULTS AND DISCUSSION

In this study different heavy metals which are known to be toxic viz, chromium, lead, were used. Presence of heavy metal tolerant bacterium in a particular environment may be an indication that such area is affected by heavy metals, Such an area may endorse heavy metal resistant organisms **[**11] Bacteria are primarily the first ones to be affected by discharges of heavy metals into the environment resulting in an increase of heavy metal resistant bacteria in these environments [9, 20 21,10] Isolation of bacteria from metal polluted environment would represent an appropriate practice to select metal resistant strains that could be used for heavy metal removal an bioremediation purposes[12] Microorganisms undergo selection pressures in the presence of toxic compounds and develop resistance1[13]In the current study in Barite mining area Bacteria varied in colour and shape were picked for screening of heavy metals from isolated bacteria from the soils of respective places mentioned above The purified isolates were tested for their resistance to heavy metals like Nickel, Chromium, Zinc, Lead, Copper, using the Agar diffusion method on Nutrient agar medium**.**[35**]**The degree of heavy metals resistance to the highest concentration in the nutrient agar media was evaluated

based on the ability of the isolated bacteria to grow on the subsequent higher concentrations Among the bacterial isolates klebsiella pneumoniae (KJ 194599) tolerated k2cr2o7 300mg/l Nicl2 200mg/l, Cuso4 300mg/l , Zncl2 300mg/l lead at the maximum 8000 mg/ L The difference in the toxicity toward the bacterial isolates could be explained by the conditions of bacterial isolation and the nature and physiological characteristics of each bacterial isolate [35**]** In high concentrations, heavy metal ions react to form toxic compounds in cells , [22**].**It must first enter the cell. Because some heavy metals are necessary for enzymatic functions and bacterial growth, uptake mechanisms exists that allow for the entrance of metal ions into the cell. There are two general uptake systems one is quick and unspecific driven by a chemiosmotic gradient across the cell membrane and thus requiring no ATP and the other is slower and more substrate-specific, driven by energy from ATP hydrolysis. While the first mechanism is more energy efficient it results in an influx of a wider variety of heavy metals and when these metals are present in high concentrations, they are more likely to have toxic effects once inside the cell [23] To survive under metal-stressed conditions bacteria have evolved several types of mechanisms to tolerate the uptake of heavy metal ions. These mechanisms include the efflux of metal ions outside the cell Accumulation and complexation of the metal ions inside the cell, and reduction of the heavy metal ions to a less toxic state [22], whereas in bacteria, various peptides consisting of metal-binding amino acids (mainly histidine and cysteine residues have been studied for enhanced heavy metal accumulation by bacteria. various peptides consisting of metal-binding amino acids mainly histidine andcysteine residues have been studied for enhanced heavy metal accumulation by bacteria Novel metal binding peptides might offer a higher affinity, higher metal-binding capacity and/or specificity and selectivity, for a target metal ion than known metal-binding proteins [33]The most important of these groups are Carbonyl (ketone), Carboxyl, Sulfhydryl (thiol), Sulfonate,Thioether,Amine,Secondaryamine,Amide,Imine,Imidazole,Phosphonate,Phosphodiester[39] Duxbury, (1986) have opined that the tolerance to heavy metals is seemed to be remarkable in Gram Negative bacteria only.[44] Thus the organisms isolated and identified from a heavy metal polluted environment developed the mechanisms to survive a highly toxic environment. This study thus highlights the presence of bacteria which survives in a highly heavy metal polluted environment.These isolates are of interest for molecular characterization of mechanisms for resistance to multiple metals and hold promise for bioremediation of toxic heavy metals, including in environments that are contaminated by several metals

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