International Journal of Applied Research 2017; 3(7): 878-885

ISSN Print: 2394-7500
ISSN Online: 2394-5869
Impact Factor: 5.2
IJAR 2017; 3(7): 878-885
www.allresearchjournal.com
Received: 17-05-2017
Accepted: 19-06-2017

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A qualitative approach to nickel and lead uptake by heavy metal resistant bacteria *Klebsiella* sp. 10KN

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Abstract
Urbanization, industrialization and other human activities has introduced a number of heavy metal contaminants in the environment. Microorganisms inhabiting such sites are well efficient in developing resistance strategies against toxic heavy metal stress. The present work qualitatively describes uptake of nickel (Ni) and lead (Pb) by epiphytic root bacteria isolated from the rhizosphere of the weedy plant, *Acorus calamus* treated with heavy metals *viz.* Ni and Pb contaminated drainage water. This bacterial strain was designated as 10KN and classified as member of *Klebsiella* sp. on the basis of 16S rRNA gene sequence. The isolated strain showed maximum tolerable concentration (MTC) 1500 mgL^{-1} in Ni and 1400 mgL^{-1} in Pb toxicity. On the basis of residual analysis study using Microwave Plasma Atomic Emission Spectroscopy (MP-AES) it was proved that *Klebsiella* sp. 10KN can tolerate Ni stress upto 76.2% whereas Pb stress upto 74.4% in 48 hrs incubation time. Due to the toxicity of Ni and Pb some morphological alterations occurred which were demonstrated by Scanning Electron Microscopy and *Energy-Dispersive X*-ray spectroscopy (SEM–EDX), Fourier Transform Infrared Spectroscopy (FTIR) and Atomic Force Microscopy (AFM) micrographs of *Klebsiella* sp. 10KN. The ability of heavy metal resistant bacteria *Klebsiella* sp. 10KN to uptake Ni and Pb could be utilized in developing feasible bioremediation processes like biosorption and bioaccumulation technologies against toxic heavy metals contaminated site.

Keywords: Heavy metal uptake, Heavy metal stress, Nickel (Ni), Lead (Pb), Rhizosphere, *Klebsiella* sp

1. Introduction
Rapid industrialization is the principal cause of toxic heavy metal contamination of water bodies (Bhattacharya *et al*. 2015; Sharma and Kansal 2011; Singh and Kumar 2006) [4, 34, 35]. Irrigation with such contaminated water source can cause numerous harmful effects on crops as well as human health (Lin *et al*. 2012) [23]. Like other organic pollutants heavy metals cannot be degraded and so remain in the ecosystem for a long period of time (Eduardo and Helena 2011) [14]. Essential heavy metals such as Co, Cr, Ni, Fe, Mg, Cu and Zn have role in various biochemical activities of an organism (Bruins *et al*. 2000) [16]. However, nonessential heavy metal such as Cd, Hg, Sn and Pb do not possess any biological role and cause severe toxicity to microorganisms even in trace amount (Roane and Pepper 2000; Huang *et al*. 2004) [31, 18].

Lead (Pb) is very hazardous nonessential heavy metal and extremely toxic to organisms (Low *et al*. 2000) [24]. Even a small concentration of Pb exposure can result in damage of central nervous system, headache, skin allergies etc. Whereas, Ni though being an essential heavy metal can cause skin allergies, respiratory tract cancer, lung fibrosis and iatrogenic effects (Clarkson *et al*. 1988; Kasprzak *et al*. 2003) [11, 19]. Conventional physicochemical methods such as ion exchange, filtration, acid leaching was developed for heavy metal remediation (Xio *et al*. 2010) [43]. However most of them are costly, demand high reagent and energy input and produce large amount of toxic sludge (Gola *et al*. 2016) [15].

Microbes have evolved numerous resistance mechanisms (transformation, biosorption, bioaccumulation, precipitation, complexation and oxidation- reduction) to deal with the heavy metal toxicity (Huang *et al*. 1990; Avery and Tobin 1993; Brady and Duncan 1994; Veglio *et al*. 1997; Li *et al*. 2011) [17, 3, 5, 40, 21]. Removal of excess heavy metal from waste water using microbes is environment friendly and economical way to protect aquatic and terrestrial life forms (Prasenjit and Sumathi 2005; Munoz *et al*. 2006) [29, 27].

Number of bacterial species like *Streptoverticillium* sp., *Streptomyces* sp., *Pseudomonas* sp., *Enterobacter* sp., *Corynebacterium* sp. and *Bacillus* sp. are capable to tolerate Pb stress (Chang *et al*. 1997; Puranik and Paknikar 1997; Pardo *et al*. 2003; Choi and Yun 2004; Selatnia *et al*. 2004; Lin and Lai 2006; Lu *et al*. 2006; Tunali *et al*. 2006; Uslu and Tanyol 2006) [7, 30, 28, 8, 33,22, 25, 37, 39]. Other bacterial species like *Moraxella* sp., *Acinetobacter* sp., *Providencia* sp., *Branhamella* sp., *Staphylococcus* sp., *Methylobacterium* sp., *Klebsiella* sp., *Cupriavidus* sp., *Achromobacter* sp. and *Helicobacter* sp. have employed various Ni resistance mechanisms (Alboghobeish *et al*. 2014 and Salvador *et al*. 2007) [1, 32].

Rhizospheric zones of various plants are associated with numerous beneficial micro-organisms such as bacteria and fungi, which help in nitrogen fixation and nutrient/mineral mineralization (Clark 1940) [10]. Use of rhizospheric micro-organism to remediate heavy metal from the soil provides great advantage and will reduce the availability of heavy metal present in soil to the plants.

In view of this, present study was carried out with the micro-organism isolated from the rhizospheric zone of the weedy plant *Acorus calamus*. Using Microwave Plasma Plasma Atomic Emission Spectroscopy (MP-AES), heavy metal uptake efficiency of isolated strain *Klebsiella* sp. 10KN was tested against Ni and Pb. Further, for the qualitative determination of the uptake strategy of Ni and Pb under heavy metal toxicity, Scanning Electron Microscopy and *Energy-dispersive X*-ray spectroscopy (SEM-EDX) were carried out. Cell surface roughness changes caused by the heavy metal toxicity in *Klebsiella sp.* 10KN studied using Atomic Force Microscopy (AFM). Moreover, Fourier Transform Infrared Spectroscopy (FTIR) spectroscopy was performed to determine the involvement of surface functional group in heavy metal microbe interaction. In addition, *Klebsiella* sp. 10KN was also tested against various antibiotics and showed resistance against ampicillin, amoxicillin, cefadroxil, cloxacillin, erythromycin, penicillin and vancomycin. This study describes the concept of metal microbe interactions employed by bacteria under heavy metal stress.

2. Materials and Methods

2.1 Sampling and Enrichment

In the present investigation, samples were collected from Water Technology Centre, Indian Agricultural Research Institute (WTC, IARI) New Delhi, India. Epiphytic root bacteria were isolated from roots of the weedy plant *Acorus calamus* treated with Ni and Pb contaminated drainage water. Obtained roots were kept in phosphate buffer saline (PBS, pH 7, 0.05M) at 150 rpm for 1 hr at 30ºC, for the release of bacteria into buffer saline. Inoculation of experimental flasks was done with 1ml of phosphate buffer sample having released epiphytic root bacteria. In 250 ml Erlenmeyer flasks, the sample were cultured in 50 ml autoclaved Luria broth (gm/L; Tryptone 10, yeast extract 5, NaCl 10, Agar 15; pH 7.2±0.2 at 30 °C) supplemented with the initial concentration of 10 mgL^{-1} of heavy metal (Ni & Pb separately). Heavy metal stock was prepared by dissolving their respective salts $Ni(NO_3)_2.6H_2O$ and $(Pb(NO_3)_2$ in milliQ water. The experimental flasks (LB media + inoculum + Heavy metal) along with Control (LB media + Inoculum without Heavy metal) were kept in BOD (Biological Oxygen Demand, SR® Shellab) incubator for 48 hrs at 150 rpm and at 30±2 ºC. The isolates were streaked on LB agar plate for the purification of distinct bacterial colonies.

2.2 Determination of maximum tolerable concentration

Heavy metal concentrations 10, 30, 50, 100, 200, 300, 400, 500, 700, 1000, 1200, 1500, 1800 mg L^{-1} of Ni and Pb were checked for Maximum Tolerable Concentration (MTC) of strains. MTC is the maximum concentration of heavy metal that allows microbial growth even after 48 hrs of incubation and bacterial growth was determined by microplate absorbance reader (BioRad, Imark™) in terms of $OD_{\lambda 595nm}$. Out of all the strains that were isolated, strain 10KN showed the maximum biomass production and tolerance against Ni and Pb in LB media and thus chosen for further studies.

2.3 Identification of strain 10 KN

The molecular characterization was performed for the identification of isolated strain 10KN. Using universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3') 16S rDNA was amplified. PCR was carried out in a thermal cycler (C1000 BIO RAD, India) with 25µl reaction mixture consisting of 2µl dNTP (200µM), 2.5µl buffer (1X), 2.5µl primer 8F (1mM), 2.5µl primer 1492R (1mM), 0.5µl Taq Polymerase (1.5 U) and 7µl template DNA. Analysis of amplified PCR products was done by 1% (w/v) agarose gel electrophoresis in 0.5x TBE buffer with ethidium bromide (0.5µg/ml) in a gel documentation system (BIORAD Gel documentation system). 16S rDNA fragment was eluted by using clean up kit (Wizard SV Gel and PCR clean-up system, Promega, USA) and then sent to Scigenome Pvt. Ltd., Kerala (India) for sequencing. Comparative analysis of obtained sequences was done with previously published databases using ADVANCED BLAST (Altschul *et al*. 1997) [2], (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Obtained 16S rDNA sequences submitted to GenBank via Bankit and then MEGA software version 6.0 used for phylogenetic analysis.

2.4 To study physiological parameters, biochemical arameters and antibiotic resistance of the isolate

Among physiological parameters pH, temperature and salt (NaCl) hold a great value for bacterial growth. For this investigation, 50 ml nutrient media seeded with 0.2 ml inoculums was used. To determine the optimum pH range, LB media was adjusted to different pH ranging from 5, 6, 6.5, 7, 7.5 and 9 and after incubating for 48 hrs at 30 ºC under shaking condition, growth was measured in terms of $OD_{\lambda 595nm}$. To check the temperature and salt tolerance, incubation was done at the different temperatures (10 ˚C, 20˚C, 30˚C, 40˚C, 50 ˚C) and at different salt concentrations (0%, 0.5%, 1%, 3%, 5%, and 7%) respectively for 48 hrs. KB003 Hi25™ Enterobacteriaceae Identification kits (HiMedia) were used to study the conventional biochemical parameters. For the antibiotic resistance analysis, the isolate was incubated on LB agar plate containing antibiotic disc (HiMedia). After incubation, zones of inhibition were observed and classified as resistant or sensitive.

2.5 Residual analysis of heavy metal uptake using Microwave Plasma Atomic Emission Spectroscopy (MP-AES)

Strain 10KN cultured in 50 ml media seeded with 0.2 ml inoculums and 50mgL^{-1} concentration of each heavy metal,

Ni and Pb separately for 48 hrs. Control was also observed for comparative analysis of microbial growth. Then at every 4th hr, 5 ml culture was taken out from each flask and centrifuged at 6000 rpm for 10 mins. The supernatant was digested in microwave digester (HACH, DRB200) and then analysed for residual heavy metal ions in MP-AES (Agilent 4200 MP AES).

2.6. Tools to study morphological alterations of isolate under heavy metal stress

Morphological investigations were done to check the altered cell surface morphology after heavy metal stress. For the heavy metal loaded samples, the *Klebsiella* sp. 10KN was exposed to Ni and Pb concentrations of 50 mgL^{-1} (pH 7.2) and control (Heavy metal-free). After 48 hr, the grown cultures were spun down by centrifugation (6000 rpm for 10 mins) and then pellet washing was done to remove excess heavy metal from the suspension. After that fixation of obtained pellet of Ni, Pb loaded samples and control were done with 2.5% glutaraldehyde for 30 mins followed by three times washing with PBS. After fixation, the samples were lyophilized (vacuum dried) for further analysis.

2.6.1 Scanning Electron Microscopy and *Energy-dispersive X*-ray spectroscopy (SEM-EDX)

Using cathodic spraying (Polaron Gold), obtained lyophilized samples were gold coated and observed under a scanning microscope (ZEISS EVO-50) under following analytical condition. EHT = 20.0 kV, Signal A = SE1 (Mishra 2013; Tyagi and Malik 2010). For EDX analysis lyophilized samples were carbon coated and observed under the same microscope (ZEISS EVO 50).

2.6.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was used to study the altered spectral feature of the microorganism (Kazy *et al*. 2009) [20] and responsible functional group for metal uptake on *Klebsiella* sp. 10KN. 1 mg fine lyophilised biomass of *Klebsiella* sp. 10KN from control (Heavy metal-free) and heavy metal loaded Ni and Pb (50 mgL^{-1}) samples were mixed uniformly with 100 mg KBr. FTIR spectra were analysed by FTIR spectroscopy (Perkin Elmer One spectrum).

2.6.3 Atomic Force Microscopy (AFM)

AFM micrographs were obtained from control (Heavy Metal-free) and heavy metal loaded *Klebsiella* sp. 10KN culture pellets of Ni and Pb (50 mgL^{-1}). Obtained bacterial pellets were washed with deionised water and then for conditioning, pellets were suspended in 50mM citrate-phosphate buffer (pH 3). Pellets were placed on coverslips for 30 mins, followed by repeated washing with deionised water. Air dried pellets were analysed under AFM (Vecco Metrology, Nanoscope III a) by silicon cantilever with force constant 0.22- 0.77 N/m and tip height 10-12 nm in the contact mode (Das *et al*. 2008) [13] and software version V531r1 used for AFM images.

3. Results and Discussion

3.1. Sampling and Enrichment

Sampling was done from rhizospheric site treated with heavy metal enriched drainage water. Initially, around three hundred Ni and Pb resistant bacteria were obtained. Later on, only four different strains were found to survive under high concentrations of heavy metal, Ni and Pb after successive transfers during enrichment.

3.2. Determination of maximum tolerable concentration

Out of the four, strain 10KN was isolated and characterized on the basis of Maximum Tolerable Concentration up to 1500 mgL^{-1} of Ni and 1400 $mgL^{-1}$of Pb and predominant growth over others (Table 1).

3.3. Identification of strain 10KN

Strain 10KN was designated as *Klebsiella* sp. 10KN on the basis of 16S rDNA sequence analysis and BLAST search. Obtained sequence was submitted to GenBank (accession number KU934222). Using neighbor-joining method a phylogenetic tree was constructed (Figure 1).

3.4. To study physiological parameters, biochemical parameters and antibiotic resistance of the isolate

Metal tolerance capability of microbes can be influenced by the environmental conditions such as pH, temperature and salt (NaCl) concentration (Mishra and Malik 2013). *Klebsiella* sp. 10KN was able to grow at pH range from 6 to 8 (Table 2), however it showed maximum biomass growth at pH 7. Growth of strain 10KN was observed from 20˚C to 40˚C temperature (Table 2) but the maximum growth was observed at 30˚C (optimum). Similar to pH and temperature, salinity is also an important parameter on which microbial growth depends. *Klebsiella* sp. 10KN was also found to tolerate a wide range of salinity from 0% to 7% NaCl concentration (Table 2). While studying biochemical parameter, strain 10KN showed positive response towards ONPG (β galactosidase activity), urease activity, nitrate reduction, VP (Voges Proskauer's) test, esculin hydrolysis, sugar fermentations and utilization of lysine, citrate, malonate (Table 2). The strain also showed resistance against antibiotics such as ampicillin, amoxicillin, cefadroxil, cloxacillin, erythromycin, penicillin and vancomycin (Table 2). Tolerance of strain against various physiological factors (Mishra and Malik 2013) determines its ability to grow under toxic and fluctuating environment of contaminated sites.

3.5. Residual analysis of heavy metal uptake using MP-AES

MP-AES analysis showed that the concentration of Ni and Pb metal ion decreased in supernatant after 48 hrs (Table 3). Here, growth curve of strain 10KN has shown that in 24 hrs of incubation, there was around 60-65% reduction (Figure 2) of heavy metal in culture media along with an increase in biomass ($OD_{\lambda 595nm}$) as 0.67 OD in Ni loaded culture and 0.634 OD in Pb loaded culture. Increased biomass in the inoculated flasks was an indicator of tolerance of heavy metal stress by *Klebsiella* sp. 10KN for its growth. Furthermore, after 48 hrs this reduction was 76.2% in case of Ni stress whereas in Pb stress it was around 72.4%. Heavy metal uptake was negligible after 36 hrs because further growth of microorganisms was affected. A previous study revealed that exposure of bacterial strain to metal leads to deviation of its energy from growth to other functions like resistance to metal toxicity (Hernandz and Dorian 2012) [16]. Increase in biomass along with increase in stress (Munoz *et al*. 2006) [27] presents it as a good candidate for feasible bioremediation processes of heavy metal from contaminated sites.

3.6. Tools to study morphological alterations of isolate under heavy metal stress

3.6.1. Scanning Electron Microscopy and *Energy-dispersive X*-ray spectroscopy (SEM-EDX)

The SEM micrographs clearly indicate morphological changes and heavy metal deposition on the *Klebsiella* sp. 10KN grown in heavy metal loaded condition (Figure 3, B & C). Bacterial cells appeared intact, clear with smooth surface in control (Figure 3, A) condition whereas in the presence of Ni, cells appeared dense, distorted and adhered to each other due to the toxic effects of Ni. Adherence and physical disintegration of the bacterial cells shows reduction in total surface area exposure towards heavy metal toxicity. Pb loaded cells appeared dense, tightly packed and with Pb deposition on cell surface. Heavy metal deposition on cellular surface describes the adsorption phenomenon of remediation. Similar morphological alterations were observed with various bacterial species (Das *et al*. 2008) [13] under heavy metal stress. From the above result it was observed that bacterial cell wall composition and surface properties (Gola *et al*., 2016) [15] plays an important role in survival under metal stress.

Additionally, the EDX analysis of the bacterium established the presence elemental content on the microbial biomass (Figure 4). The EDX micrograph of control (Heavy metal-free) biomass showed only prominent peaks of alkali and alkaline earth metal indicating the presence of these elements in the bacterial biomass. Further in Ni and Pb loaded samples new peaks of particular heavy metal along with the earlier peaks of the alkali and alkaline earth metal were appeared in the biomass. The heavy metal peaks in the EDX monograph clearly indicate the presence of the particular heavy metal (Ni and Pb) on the biomass of strain 10KN.

3.6.2. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra for control, Ni and Pb containing biomass were obtained in the range of 650-4000 cm^{-1} to demonstrate the surface functional groups involved in Ni and Pb binding on the bacterial biomass (Figure 5). Obtained spectra display the absorption peaks of the control biomass and shift in this absorption peak bands in heavy metal containing biomass. Assignments and analysis of distinctive peaks in this investigation were based on earlier published data (Xiaolong *et al*. 2016; Choudhary and Sar 2011; Kazy *et al*. 2009) [42, 9, 20].

The spectra for control biomass shows prominent peaks at 3310.3 cm^{-1} (representing H bonded –OH group, -C≡C-H: C-H stretching of alkynes and N-H stretching of 1º and 2º amine and amides); 2922.7 cm^{-1} and 2853 cm^{-1}(C-H stretch of alkanes); 1745 cm^{-1}(C=O stretch of carbonyls and carboxylic acids); 1632.8 cm^{-1} (N-H bend of 1º amines); 1546.6 cm^{-1} (N-O asymmetric stretch of nitro compounds); 1456.6 cm^{-1}(C-C stretch of aromatics and C-H bend of alkanes) and 1370 cm^{-1} (C-H rock of alkanes); 1310 cm^{-1} (Nitro compounds and C-N stretch of aromatic amines); 1246.6 cm^{-1}(C-O, C-H and C-N stretch); 1146.6 $cm^{-1}$and 1031.2 cm^{-1} (C-O stretch of alcohols, carboxylic acids, esters and ethers and C-N stretch of aliphatic amines). However as compared to control, spectra of Ni loaded biomass shift was seen in the peak representing 3298.8 cm^{-1} (H bonded –OH group, -C≡C-H: C-H stretching of alkynes and N-H stretching of 1º and 2º amine and amides). Peak corresponding N-O asymmetric stretching has been completely masked by peak 1634.5 cm^{-1} (N-H bend of 1º amines). In similar manner, FTIR analysis also revealed the mechanism of Cr (VI) removal by *Termitomyces clypeatus* (Das and Guha 2009). Further, a shift was seen in Pb loaded biomass at peak 1416.6 cm^{-1} (C-C stretch of aromatics amines without any C-H bend) whereas in control C-H bend was present. Next shift was observed in C-N stretch of aliphatic amines at peak 1243.3 cm^{-1} from aromatic amines and nitro compounds. From observed data it can be concluded that hydroxyl functional group, aliphatic amines, aromatic amines and nitro compounds plays an important role in bacterial interaction with Ni and Pb.

3.6.3. Atomic Force Microscopy (AFM)

AFM is an ideal technique for investigating changes in cell surface roughness. RMS (Root mean square) analysis of control and heavy metal loaded (Ni and Pb, 50 mgL^{-1}) biomass indicated the impact of heavy metal on bacterial cells. RMS or cell surface roughness value increased under heavy metal stress Ni (6.205 nm) and Pb (4.115 nm), as compared to control (1.609 nm). The arithmetic average roughness Ra, also used to determine change in cell surface morphology. It was observed that, Ra value also increased under heavy metal stress Ni (4.528 nm) and Pb (2.699 nm), in compared to control (0.843 nm). Similar increase in RMS and Ra values in presence of Uranium and thorium was observed with *Pseudomonas* sp. (Kazy *et al*. 2009) [20]. Moreover, similar altered cell morphology (Wang *et al*. 2007) [41] in presence of Ni was observed for *E.coli*. From earlier reports it was found that outer cell membrane composition (lipopolysaccharides, phospholipids, and lipoproteins) of gram-negative bacteria also helps in their survival under heavy metal toxicity (Suriya *et al*. 2013) [36]. Interaction of heavy metals with surface proteins and lipopolysaccharides possibly leads to cell roughness. Another reason to altered cell morphology might be due to biosorption of heavy metal on cell surface.

Table 1: Determination of maximum tolerable concentration

Strain name	Maximum tolerance against Ni (mgL^{1})	Maximum tolerance against Pb (mgL^{1})
Klebsiella sp. 10KN	1500	1400

Table 2: To study physiological parameters, biochemical parameters and antibiotic resistance of the isolate

Physiological Parameters	Klebsiella sp. 10KN	Biochemical Parameters	Klebsiella sp. 10KN
Growth at pH		ONPG	+
		Lysine utilization	+
		Ornithine utilization	-
5	-	Urease	+
6	+	Phenylalanine deaminase	-
6.5	+++	Nitrate reduction	+
7	++++	H_2S production	-
7.5	+++	Citrate utilization	+
8	+	Voges Proskauer's	+
9	-	Methyl Red	-
Growth on NaCl (Salt %)		Indole	-
		Malonate utilization	+
0	++	Esculin Hydrolysis	+
0.5	+++	Arabinose	-
1	+++	Xylose	+
3	+++	Adonitol	+
5	+++	Rhamnose	+
7	++	Cellobiose	+
Temperature (ºC) Profile		Melibiose	+
		Saccharose	+
10	-	Raffinose	+
20	++	Trehalose	+
30	+++	Glucose	+
40	++	Lactose	+
50	-	Oxidase	-

Antibiotics	Concentration	Resistivity (R)/ Sensitivity (S)
Amikacin	30 mcg	S
Ampicillin	10 mcg	R
Amoxicillin	10 mcg	R
Cefadroxil	30 mcg	R
Cefoperazone	75 mcg	S
Ceftazidime	30 mcg	S
Ceftriaxone	30 mcg	S
Chloramphenicol	30 mcg	S
Ciprofloxacin	5 mcg	S
Cloxacillin	1 mcg	R
Co-Trimoxazole	25 mcg	S
Erythromycin	15 mcg	R
Gentamycin	10 mcg	S
Nalidixic acid	10 mcg	S
Netillin	10 mcg	S
Nitrofurantoin	300 mcg	S
Norfloxacin	10 mcg	S
Penicillin	10 units	R
Tobramycin	10 mcg	S
Vancomycin	30 mcg	R

Table 3: Residual analysis of heavy metal uptake using MP-AES.

Heavy Metal	Heavy Metal conc. in supernatant at 0 hr (mg L^{-1})	Heavy Metal conc. in supernatant after 48 hr (mg L^{-1})	Heavy Metal uptake	Percentage (%) of Heavy Metal uptake
Klebsiella sp.10KN +Nickel(Ni)	50	11.9	38.1	76.2
Klebsiella sp. 10KN +Lead(Pb)	50	13.3	36.7	72.4

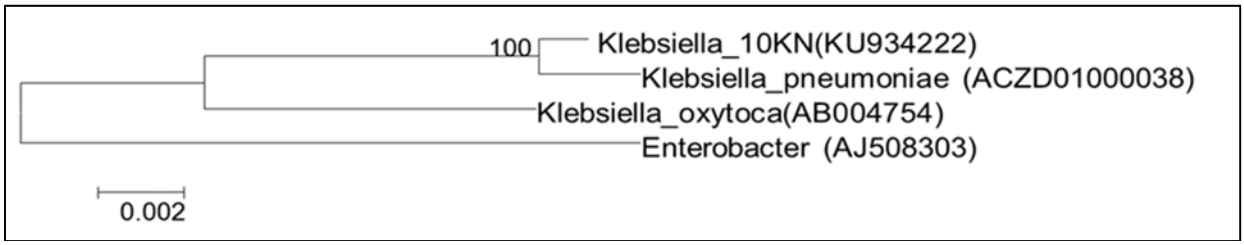

Fig 1: Phylogenetic tree of *Klebsiella* sp. strain 10KN derived from neighbor joining method. (Scale bar 0.002 substitution per site)

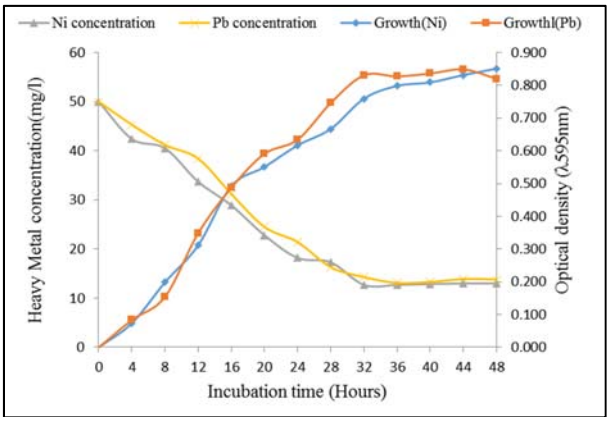

Fig 2: Growth rate of *Klebsiella* sp. 10KN and reduction in Heavy Metal (Ni and Pb) concentration.

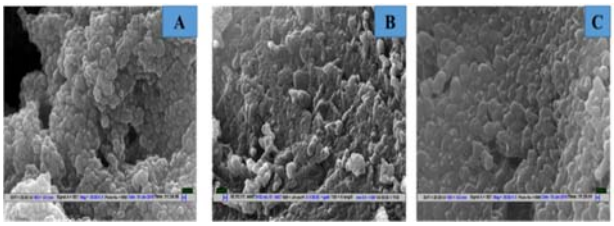

Fig 3: SEM micrograph of *Klebsiella* sp. 10KN (A) In control; (B) at 50 mgL^{-1} of Ni; (C) at 50 mgL^{-1} of Pb (magnified to 20,000 X).

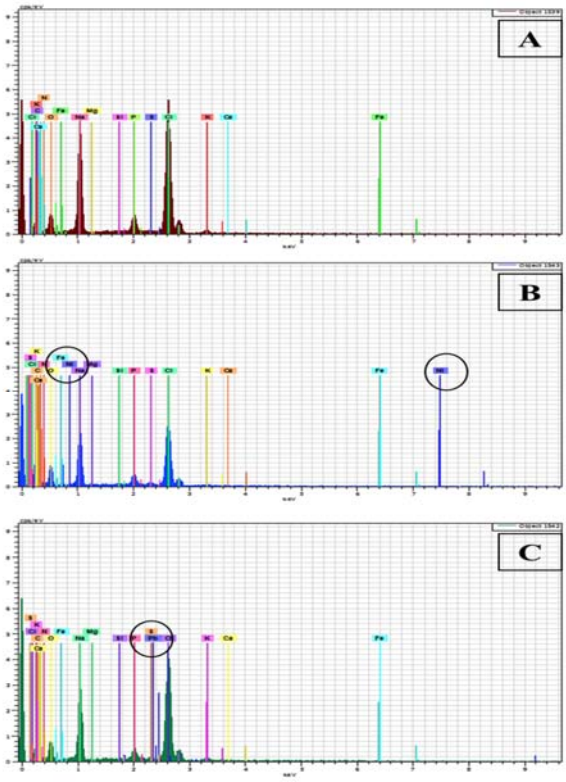

Fig 4: EDX graph of *Klebsiella* sp. 10KN (A) Control; (B) at 50 mgL^{-1} of Ni; (C) at 50 mgL^{-1} of Pb.

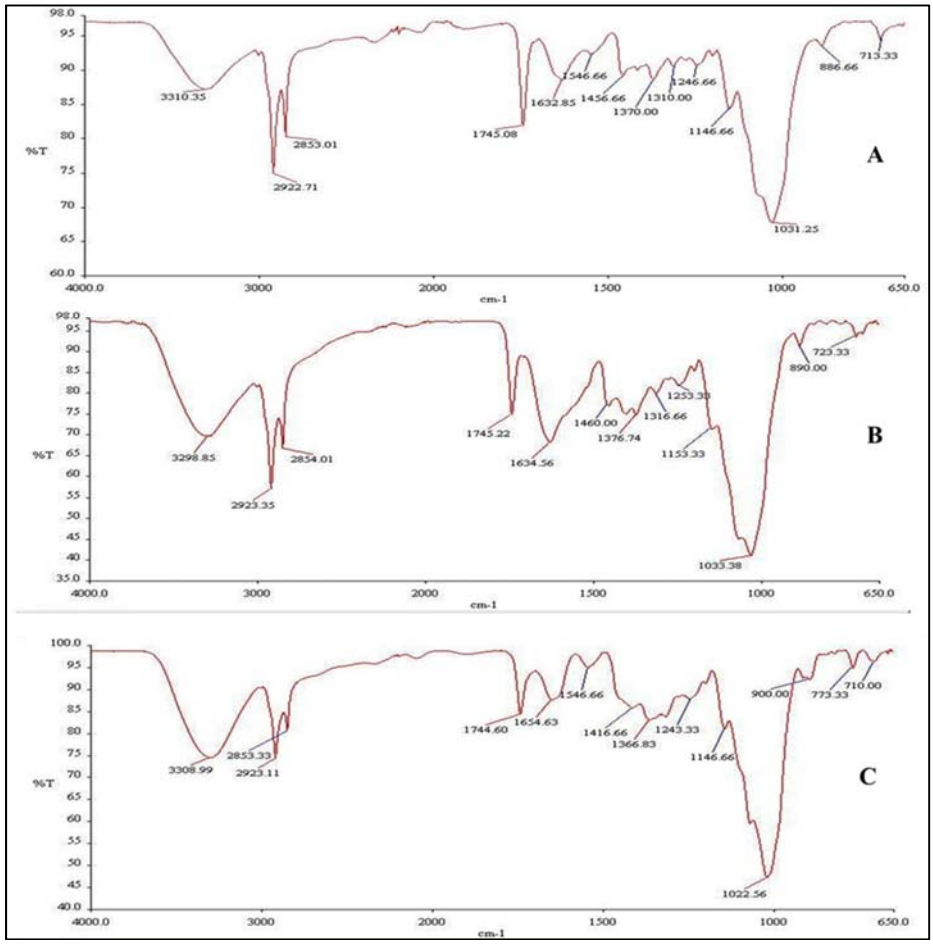

Fig 5: FTIR graph of *Klebsiella* sp. 10KN (A) In control; (B) at 50 mgL^{-1} of Ni; (C) at 50 mgL^{-1} of Pb**.**

Fig 6: Values of various parameter obtained by AFM graph of *Klebsiella* sp. 10KN (A) In control; (B) at 50 mgL^{-1} of Ni; (C) at 50 mgL^{-1} of Pb.

4. Conclusion

The present study describes the heavy metal resistance Ni and Pb, by gram negative bacteria *Klebsiella* sp. 10KN isolated from heavy metal treated rhizospherice zones. Various physiological factors (pH, Temperature, Salt), biochemical parameter and antibiogram were also studied to check its growth under diverse environment. From MP-AES analysis, it can be concluded that strain *Klebsiella* sp. 10KN possessed the high level of Ni (1500 mgL^{-1}) and Pb (1400 mgL^{-1}) tolerance and great efficiency of Ni (76.2%) and Pb (72.4%) uptake. The morphological alterations were observed due to heavy metal toxicity were clearly demonstrated by various tools like SEM-EDX, FTIR and AFM. This overall report strengthens the concept of survival strategies employed by bacteria under fluctuating environment of physiological factors and heavy metal stress. Present investigations on uptake of Ni and Pb could be helpful in removal, recovery and remediation strategy of heavy metals from the contaminated site using active biomass of strain *Klebsiella* sp. 10KN.

Acknowledgements

Authors gratefully acknowledge financial assistance provided by ICAR, (NFBSFARA/WQ-3032/2013-14) under the project, "Bioremediation of contaminants in polluted sites: Use of weedy plants". The first author is thankful to University Grants Commision (UGC) for providing the fellowship as JRF/SRF.

References

1. Alboghobeish H, Tahmourespour A, Doudi M. The study of Nickel Resistant Bacteria (NiRB) isolated from wastewaters polluted with different industrial sources. J Environ Health Sci Eng. 2014; 12:44-50. doi: 10.1186/2052-336X-12-44
2. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W *et al*. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids 1997; 25:3389-402. doi: 10.1093/nar/25.17.3389.
3. Avery SV, Tobin JM. Mechanism of adsorption of hard and soft metal ions to *Saccharomyces cerevisiae* and influence of hard and soft anions. Appl Environ Microbiol. 1993; 59:2851-2856. doi: PMC182376
4. Bhattacharya A, Dey P, Gola D, Mishra A, Malik A, Patel N. Assessment of Yamuna and associated drains used for irrigation in rural and peri-urban settings of Delhi NCR. Environ Monit Assess. 2015; 187:1-13. doi: 10.1007/s10661-014-4146-2
5. Brady D, Duncan JR. Chemical and enzymatic extraction of heavy metal binding polymers from isolated cell walls of *Sccharomyces cerevisiae*. Biotechnol Bioeng. 1994; 44:297-302. doi: 10.1002/bit.260440307
6. Bruins MR, Kapil S, Oehme FW. Microbial resistance to metals in the environment. Ecotoxicol. Environ Safety 2000; 45:198-207. doi: 10.1006/eesa.1994.1860
7. Chang JS, Law R, Chang CC. Biosorption of lead, copper and cadmium by biomass of *Pseudomonas aeruginosa* PU21. Water Res. 1997; 31:1651-1658. doi: 10.1016/S0043-1354(97)00008-0
8. Choi SB, Yun YS. Lead biosorption by waste biomass of *Corynebacterium glutamicum* generated from lysine fermentation process. Biotechnol Lett. 2004; 26:331-336. doi: 10.1023/B:BILE.0000015453.20708.fc
9. Choudhary S, Sar P. Uranium biomineralization by a metal resistant *Pseudomonas aeruginosa* strain isolated from contaminated mine waste. J Hazard Mater 2011; 186:336-343. doi: 10.1016/j.jhazmat.2010.11.004
10. Clark FE. Notes on types of Bacteria associated with plant roots. Trans Kans Acad Sci. 1940; 43:75-84. doi: 10.2307/3625499
11. Clarkson TW, Friberg L, Nordberg GF, Sager PR. Biological Monitoring of Toxic Metals; Plenum. Press, New York, 1988, 265-282. doi:10.1007/978-1-4613-0961-1
12. Das SK, Guha AK. Biosorption of hexavalent chromium by *Termitomyces clypeatus* biomass: Kinetics and transmission electron microscopic study. J Hazard Mater 2009; 167:685-691. doi: 10.1016/j.jhazmat.2009.01.037
13. Das SK, Mukherjee M, Guha AK. Interaction of chromium with resistant strain *Aspergillus versicolor*: Investigation with atomic force microscopy and other physical studies. Langmuir 2008; 24:8643-8650. doi:10.1021/la800958u
14. Eduardo VS, Helena MVMS. Bioremediation of industrial effluents containing heavy metalsusing brewing cells of *Saccharomyces cerevisiae* as a green technology: a review". Environ Sci Pollut Res 2011; 19:1066-1083. doi: 10.1007/s11356-011-0671-5

15. Gola D, Dey P, Bhattacharya A, Mishra A, Malik A, Namburath M *et al*. Multiple heavy metal removal using an entomopathogenic fungi *Beauveria bassiana*. Bioresour Technol 2016; 218:388-396. doi:10.1016/j.biortech.2016.06.096
16. Hernandz B, Dorian A. Zinc and lead biosorption by *Delftia tsuruhatensis*: a bacterial strain resistant to metals isolated from mine tailings. J water Resource Prot. 2012; 4:1-11. doi:10.4236/jwarp.2012.44023
17. Huang C, Huang C, Morehart AL. The removal of copper from dilute aqueous solutions by *Saccharomyces cerevisiae*. Water Res. 1990; 24:433-439. doi: 10.1016/0043-1354(90)90225-U
18. Huang XD, El-Alwai Y, Penrose DM, Glick BR, Greenberg BM. A multiprocess phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated soil. Environ Pollut 2004; 130:154-162. doi: 10.1016/j.envpol.2003.09.031
19. Kasprzak KS, Sunderman FW, Salnikow K. Nickel carcinogenesis. Mutat Res 2003; 533:67-97. doi: 10.1016/j.mrfmmm.2003.08.021
20. Kazy SK, D'Souza SF, Sar P. Uranium and thorium sequestration by a *Pseudomonas* sp.: Mechanism and chemical characterization. J Hazard Mater. 2009; 163:65-72. doi:10.1016/j.jhazmat.2008.06.076
21. Li L, Hu Q, Zeng JH, Qi HY, Zhuang GQ. Resistance and biosorption mechanism of silver ions by *Bacillus cereus* biomass. J Environ Sci. 2011; 23:108-111. doi:10.1016/S1001-0742(10)60380-4
22. Lin CC, Lai YT. Adsorption and recovery of lead (II) from aqueous solutions by immobilized *Pseudomonas aeruginosa* PU21 beads. J Hazard Mater. 2006; 137:99-105. doi:10.1016/j.jhazmat. 2006.02.071
23. Lin J, Fu C, Zhang X, Xie K, Yu Z. Heavy metal contamination in the water-level fluctuating zone of the Yangtze River within Wanzhou Section, China. Biol Trace Elem Res. 2012; 145:268-272. doi:10.1007/s12011-011- 9179-6
24. Low KS, Lee CK, Liew SC. Sorption of cadmium and lead from aqueous solution by spent grain. Process Biochem. 2000; 36:59-64. doi:10.1016/S0032-9592(00)00177-1
25. Lu WB, Shi JJ, Wang CH, Chang JS. Biosorption of lead, copper and cadmium by an indigenous isolate *Enterobacter* sp. J1 possessing high heavy-metal resistance. J Hazard Mater 2006; 134:80-86. doi:10.1016/j.jhazmat.2005.10.036
26. Mishra A, Malik A. Recent Advances in Microbial Metal Bioaccumulation. Crit Rev Environ Sci Technol. 2013; 43:1162-1222. doi: 10.1080/10934529.2011.627044
27. Munoz RA, Munoz MT, Terrazas E, Guieysse B, Mattisasson B. Sequential removal of heavy metals ions and organic pollutants using an algal-bacterial consortium. Chemosphere. 2006; 63:903-991. doi:10.1016/j.chemosphere.2005.09.062
28. Pardo R, Herguedas M, Barrado E, Vega M. Biosorption of cadmium, copper, lead and zinc by inactive biomass of *Pseudomonas putida*. Anal Bioanal Chem. 2003; 376:26-32. doi:10.1007/s00216-003-1843-z
29. Prasenjit B, Sumathi S. Uptake of chromium by *Aspergillus foetidus*. J Mater Cycle Waste. 2005; 7:88-92. doi: 10.1007/s10163-005-0131-8
30. Puranik PR, Paknikar KM. Biosorption of lead and zinc from solutions using *Streptoverticillium cinnamoneum* waste biomass. J Biotechnol. 1997; 55:113-124. doi:10.1016/S0168-1656(97)00067-9
31. Roane TM, Pepper IL. Microbial responses to environmentally toxic cadmium. Microb Ecol 2000; 38:358-364. doi: 10.1007/s002489901001
32. Salvador M, Carolina G, Jose E. Novel nickel resistance genes from the rhizosphere metagenome of plants adapted to acid mine drainage. Appl Environ Microbiol. 2007; 73:6001-6011. doi:10.1128/AEM.00048-07
33. Selatnia A, Boukazoula A, Kechid N, Bakhti MZ, Chergui A, Kerchich Y. Biosorption of lead (II) from aqueous solution by a bacterial dead *Streptomyces rimosus* biomass. Biochem Eng J. 2004; 19:127-35. doi:10.1016/j.bej.2003.12.007
34. Sharma D, Kansal A. Water quality analysis of River Yamuna using water quality index in national capital territory, India (2000-2009). Appl Water Sci. 2011; 1:147-157. doi: 10.1007/s13201-011-0011-4
35. Singh S, Kumar M. Heavy metal load of soil, water and vegetables in peri-urban. Delhi. Environ. Monit Assess. 2006; 120:79-91. doi:10.1007/s10661-005-9050-3
36. Suriya J, Bharathiraja S, Rajasekaran R. Biosorption of heavy metals by biomass of *Enterobacter cloacae* isolated from metal polluted soils. Int J Chem Tech Res. 2013; 5:1329-1338. doi:
37. Tunali S, Çabuk, Akar T. Removal of lead and copper ions from aqueous solutions by bacterial strain isolated from soil. Chem Eng J. 2006; 115:203-211. doi:10.1016/j.cej.2005.09.023
38. Tyagi AK, Malik A. In situ SEM, TEM and AFM studies of the antimicrobial activity of lemon grass oil in liquid and vapour phase against *Candida albicans*. Micron. 2010; 41:797-805. doi:10.1016/j.micron.2010.05.007
39. Uslu G, Tanyol M. Equilibrium and thermodynamic parameters of single and binary mixture biosorption of lead (II) and copper (II) ions onto *Pseudomonas putida*: Effect of temperature. J Hazard Mater. 2006; 135:87-93. doi:10.1016/j.jhazmat.2005.11.029
40. Veglio F, Beolchini F, Gasbarro A. Biosorption of toxic heavy metals: an equilibrium study using free cells of *Arthrobacter* sp. Process Biochem. 1997; 32:99-105. doi:10.1016/S0032-9592(96)00047-7
41. Wang J, He S, X L, Gu N. Transmission electron microscopy and atomic force microscopy characterization of nickel deposition on bacterial cells. Chin Sci Bull. 2007; 52:2919-2924. doi:10.1007/s11434-007-0390-y
42. Xiaolong L, Congcong D, Jiali L, Liang D, Qun S, Jijun Y *et al*. Bioaccumulation characterization of uranium by a novel *Streptomyces sporoverrucosus* dwc-3. J Environ Sci. 2016; 41:162-171. doi:10.1016/j.jes.2015.06.007
43. Xio X, Luo SL, Zeng GM, Wei WZ, Wan Y, Chen L *et al*. Biosorption of cadmium by endophytic fungus (EF) *Microsphaeropsis*sp. LSE10 isolated from cadmium hyperaccumulator *Solanum nigrum*. L. Bioresour Technol. 2010; 101:1668-1674. doi:10.1016/j.biortech.2009.09.083