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Remediation of lead (Pb) by a novel *Klebsiella sp.* isolated from tannery effluent of Ranipet, Vellore district

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Lead is found to be one of the most toxic heavy metal according to American public health association (APHA). Vellore district is one of the most polluted sites in the world. It is more common for lead poisoning to build up slowly over time. Over time, even low levels of lead exposure can harm a child's mental development. Therefore new resources for the removal of lead are the need of the hour. Soil and effluent samples were obtained from common effluent treatment plant; Ranipet, Vellore district. The concentration of heavy metal was also assessed in the collected samples and then isolated lead tolerant bacteria over lead containing mineral salt medium. The isolated desired bacteria was also tested for their ability to remediate other heavy metals like chromium (Cr), iron (Fe), zinc (Zn), cadmium (Cd) which are present in the tannery effluent. The one with good bioremediation activity was further characterized by sequencing 16S rRNA gene and it was found to be a novel species of *Klebsiella* genus.

Key words: Lead tolerant bacteria, *Klebsiella*, heavy metal remediation.

INTRODUCTION

Microorganisms have been used in various biological treatment processes for metals remediation (Thompson et al., 1987; Macaskie et al., 1989; Smith et al., 1994). The microbe-based technologies provide not only an alternative to conventional methods but are also are environmentally safe (Gadd, 1992). They can affect the metal ions directly or indirectly (pH changes, bioaccumulation, or biosorption) (Al-Shahwani et al., 1984; Beveridge et al., 1992; Vesper et al., 1996). Microorganisms may be used as pure as well as mixed cultures for the effective detoxification process (Smith et al., 1994), which also works over a wide pH range and temperature conditions; is cheaper, and also minimizes waste created by the detoxification process (Churchill et al., 1995). This interaction includes both bioaccumulation and biosorption. Microorganisms can remove Pb through biosorption (Tsezos, 1985), and bioaccumulation (Flemming et al., 1990; Thompson et al., 1987), formation of an exopolymer (Pradhan et al., 1995), or as biomethylate Pb (Gadd, 1992). Few heavy metals are

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Abbreviations: APHA, American Public Health Association; **CETP,** common effluent treatment plant; **ICP-OES,** inductively coupled plasma optical emission spectrometry; **MIC,** minimum inhibitory concentration; **MSM,** mineral salt medium; **MTCC,** microbial type culture collection; **TSI,** triple sugar iron agar.

important for proper functioning of biological systems but their deficiency or excess could lead to a number of disorders (Ward, 1995). The European Union has specified a level of 300 mg/kg for sewage sludge and 120 mg/kg for soil. In general, it is harder to characterize national standards for soil than for air and water. In addition to differences between nations, many nations have standards that vary according to geographic region, and soil type (for example, pH or organic material content). Moreover standards for cleaning contaminated soil are generally less stringent than are normative standards for uncontaminated soils.

These heavy metals have a harmful effect on humans and the environment as a whole. Heavy metal pollution has arisen due to rapid urbanization, evolution of metal based industries and other developmental activities. Some of the heavy metal acts as harmful environmental pollutants. The sources of these metals include metal plating, mining by-product, pesticide wastes, chemical wastes, coal based wastes, industrial wastes, gasoline, nuclear wastes and mineral leaching (Zayed et al., 2003; Mohan et al., 2006).

Tannery industries are one of the oldest industries in the world. In old days, tanning was performed to fulfill the demands of leather footwear, drums and musical instruments. But recently, due to the increasing demand of leather and its products, tanning industry has become commercially large. The effluent of Tanning industries are known to have higher concentration of heavy metals (Rao et al., 1982). Ranipet in Vellore district is one of the lead polluted places in the country, as several Tannery industries are located in these regions (Sundar et al., 2011). Lead being the least studied heavy metal present in the tannery waste made us choose this topic for our research.

Cadmium occurs in high concentration in the wastes from electroplating, paints, dyes, chrome tanning, paper industries among others (Malarkodi et al., 2007). Lead is also a toxic element and is obtained from industrial wastewater such as printing, dyeing and oil refineries. Hence, the need for a biological method of removal of heavy metals has become essential. The objective of this study was to isolate and characterize these heavy metal sorbing bacteria from tannery sludge.

MATERIALS AND METHODS

Sampling

The tannery effluent and effluent contaminated soil were collected from a common effluent treatment plant (CETP) at Ranipet, Vellore dist. Tamil Nadu, India. The tannery effluent was collected in sterile bottles. The effluent contaminated soil was also collected in a sterile polythene bags from nearby canals. The samples were transported to the laboratory on the same day itself. The microbiological analysis (fungi, bacteria and actinomycetes) of the samples were carried out on reaching the laboratory, as per the American public health association (APHA) (Clesceri et al., 2005). Inductively coupled plasma-optical emission spectrometry (ICP-OES) tests for tannery sludge were carried out to obtain the presence of heavy metal within the effluent.

Isolation of lead tolerant bacteria

The tannery effluent and effluent contaminated soil were serially diluted and then spread plate technique was performed on a mineral salt medium (MSM) media containing lead acetate at the concentration of 500 mg/L. The MSM media consists of (in g/L) Na_2HPO_4, 4.0; KH_2PO_4, 1.5; NH_4Cl 1.0; $MgSO4•7H_2O$, 0.2; $C_6H_8O_7FeNH_3$, 0.05; along with modified Hoagland trace element solution (in g/3.6 L) BH_3, 11.0; $MnCl_2•4H_2O$, 7.0; $AlCl_3$, 1.0; $CoCl_2$, 1.0; $CuCl_2$, 1.0; KI, 1.0; $NiCl_2$, 1.0; $ZnCl_2$, 1.0; $BaCl_2$, 0.5; KBr, 0.5; LiCl, 0.5; Na_2MoO_4, 0.5; $SeCl_4$, 0.5; $SnCl_2•2H_2O$, 0.5; $NaVO_3•H_2O$, 0.1; pH 7.0). After incubation, morphologically different colonies were isolated and purified by repeated streaking on agar plates. The isolates were then stored in agar slants for further studies. The strain with higher lead bioremediation was selected for further biochemical (Table 2) and molecular characterization.

Phenotypic characterization

The bacterial cultures were grouped to various genera based on their morphological and biochemical characters as given in Bergey's Manual of Determinative Bacteriology. The bacterial isolates were morphologically characterized by colony morphology, staining techniques like Gram staining and capsule staining. Motility of the organism was tested by hanging drop test. The bacterial isolates were then characterized by various biochemical tests including triple sugar iron agar (TSI), Indole, methyl red, Voges Proskauer, citrate utilization and catalase tests. The tests were carried out on MSM media (Himedia pvt. Ltd). For the comparison of the best lead degrading strain with the standard strains, type cultures were obtained from MTCC, Chandigarh, India.

Minimum inhibitory concentration (MIC) Test

It is the lowest concentration of an antimicrobial agent that inhibits the visible growth of any microorganism after an overnight incubation. In this case, we used lead acetate of concentrations 500, 750, 1000, 1250 and 1500 mg/L, which was added to the MSM broth, stored for 24 h at 25°C, along with the inoculated microorganism (Waranusantigul et al., 2011).

Removal of Pb at different pH

The bacterium was tested for the activity of bioremediation of lead at pH range of 3 to 8. The cells were inoculated into six 100 mL MSM broth containing lead acetate at the concentration of 1250 mg/L with the pH adjusted to 3 to 8 at progression of pH 1 in each of the six broths, respectively. The lead acetate concentration was chosen to be 1250 mg/L as this was the highest concentration at which VITKAS-2 showed growth. The pH was adjusted using sodium hydroxide and acetic acid. The setup was stored for 24 h at 25°C and shaken at 150 rpm. To confirm that the Pb had been taken up by the bacteria, the MSM culture supernatant was subjected to atomic absorption spectrophotometer mentioned by Al-Momani (2007). Briefly, 25 mL of MSM broth medium was supplemented with lead acetate, the bacterial isolates were inoculated and incubated at 37°C for 24 h and shaken at 150 rpm. After the incubation period, the bacterial cultures were centrifuged at 5000 rpm for 10 min, and from the supernatant, 5 mL aliquot was taken for AAS analysis. The differences between the initial and the

Table 1. Inductively coupled plasma optical emission spectroscopy (ICP-OES) results.

Analyte	Mean concentration (mg/L)
Aluminium (Al)	329.0
Boron (B)	313.0
Cadmium (Cd)	13.60
Cobalt (Co)	56.80
Chromium (Cr)	786.0
Copper (Cu)	16.90
Iron (Fe)	283.9
Magnesium (Mg)	451.7
Manganese (Mn)	74.80
Nickel (Ni)	18.90
Lead (Pb)	558.0
Zinc (Zn)	13.8

Table 2. Results of biochemical characterization.

Test	VITKAS-2	*K. pneumoniae*	*K. aerogenes*
Capsule	+	+	+
Catalase Test	+	+	+
Motility	-	-	-
TSI	A/K	K/K	K/K
Indole	-	-	-
Methyl Red	+	-	-
Voges Proskauer	-	+	+
Citrate Utilization	+	+	+
Gas from Lactose	-	-	-
Dextrose	+	+	+
Lactose	+	+	+
Malonate	-	+	+
L-Sorbose	-	+	-

* A/A, Acidic slant and acidic butt; A/K, acidic slant and alkaline butt; K/A, alkaline slant and acidic butt; K/K, alkaline slant and alkaline butt.

final concentrations indicated the amount of heavy metals uptake by the bacterial isolates.

Molecular characterization and phylogenetic analysis

The isolate showing best heavy metal bioremediation property was further characterized by sequencing 16S rRNA gene sequence. The 27F (5' - AGA GTT TGA TCC TGG CTC AG - 3') and 1492R (5' - GGT TAC CTT GTT ACG ACT T - 3') universal bacterialprimers were used to amplify the 16S rRNA gene from the genomic DNA of the isolate. The 16S rDNA molecule was sequenced using an automated DNA sequencer (ABI Prism 310 Genetic Analyzer, Tokyo, Japan). The FASTA sequence of the VITKAS-2 was aligned along with various type culture FASTA sequences belonging to the family Enterobacteriaceae. These aligned sequences were used for construction of phylogenetic tree using MEGA v5.04 package (Tamura et al., 2011).

RESULTS AND DISCUSSION

The effluent was tested for the heavy metal composition by the ICP-OES test, which clearly showed the presence of heavy metals like lead, iron, cadmium, chromium, nickel and zinc, in toxic concentrations (Table 1).

Isolation of lead tolerant bacteria

The growth of bacterial isolates obtained in MSM media supplemented with lead acetate, showed that these isolates were lead tolerant. From the gram staining techniques, the isolates were found to be gram negative rods. After morphological characterization, the colonies

Table 3. Minimum inhibitory concentration of lead tolerant isolate.

Concentration of lead acetate (mg/L)	VITKAS-1	VITKAS-2	VITKAS-3
500	+	+	+
750	+	+	-
1000	-	+	-
1250	-	+	-
1500	-	-	-

+, Growth of colonies; -, no growth of colonies.

Table 4. Effect of different pH values on remediation of lead by VITKAS-2.

pH value	Lead concentration (mg/L)	
	Initial	Final
3.0	1250	1181 ± 2.08
4.0	1250	993 ± 2.65
5.0	1250	765 ± 3.15
6.0	1250	840 ± 4.01
7.0	1250	891 ± 2.50
8.0	1250	1028 ± 5.00

were observed to be non-motile, irregular, raised and smooth in nature.

Minimum inhibitory concentration (MIC) test

The growth of bacteria, VITKAS-2, was seen in all the tubes from 500 to 1250 mg/L of lead acetate containing MSM broth, except at 1500 mg/L of the same (Table 3) whereas, both, VITKAS-1 and VITKAS-3 were found to have lower tolerance level to the higher concentrations of lead acetate compared to VITKAS-2. From the results obtained, we can safely assume that bacteria growing in lead acetate medium can absorbed/accumulate lead present in the medium. We know that some bacteria are able to immobilize the Pb into various less toxic forms. Therefore the lead can be actively removed from the soil, leading to effective reduction of soil pollution.

Bioremediation of Pb at different pH

The VITKAS-2, was then checked in different pH levels for the variation in bioremediation activity, since the pH can be a crucial parameter in the bioremediation of these heavy metals (Sundar et al., 2011; Tamura et al., 2011; Thompson and Watling, 1987; Tsezos, 1985; Vesper et al, 1996; Waranusantigul et al., 2011). VITKAS-2 shows highest removal of lead at pH 5 (Table 4). Compared to *K. pneumonia* (Ward, 1995) at pH 5 (17%), VITKAS-2 reduced 48% of lead (Figure 1). Though, *K. pneumonia* showed highest activity at pH 4 in their study, the influence of acidic pH cannot be ignored.

Characterization and phylogenetic analysis

The 16S rRNA gene sequence results suggested that the culture VITKAS-2 belongs to genus *Klebsiella*, a member of the family Enterobacteriaceae. The nearest neighbour VITKAS-2 was *K.* pneumoniae DSM 30104, Acc. No. X87277 with a similarity of 93.75%. Since, the bacteria with 16S ribosomal RNAs (rRNAs) that are ≤98.7% identical to their nearest neighbor, are always members of different species (Stackebrandt et al., 2006), as such strong differences in rRNA correlate with <70% DNA-DNA similarity. Therefore, *Klebsiella* sp. VITKAS-2 is a potential candidate for novel species. The 16S rRNA gene FASTA sequence has been submitted in GenBank with the Acc. No. JQ398846. The phylogenetic tree further supports the divergence of VITKAS-2 from the *Klebsiella* pneumonia type strain (Figure 2).

Conclusion

Use of a novel *Klebsiella* sp. growing on lead acetate medium was obtained and can serve as a cost effective means of bioremediation of heavy metals present in tannery sludge (Waranusantigul et al., 2011). This can serve as a potent and safe method to curb soil and water pollution from tannery sludge, without producing any

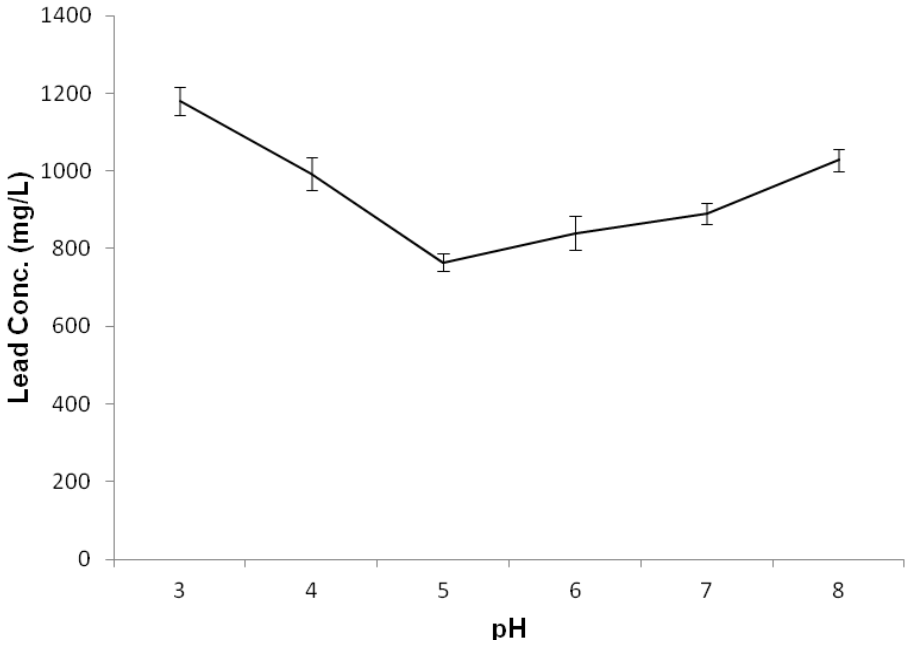

Figure 1. Effect of different pH values on remediation of lead by VITKAS-2.

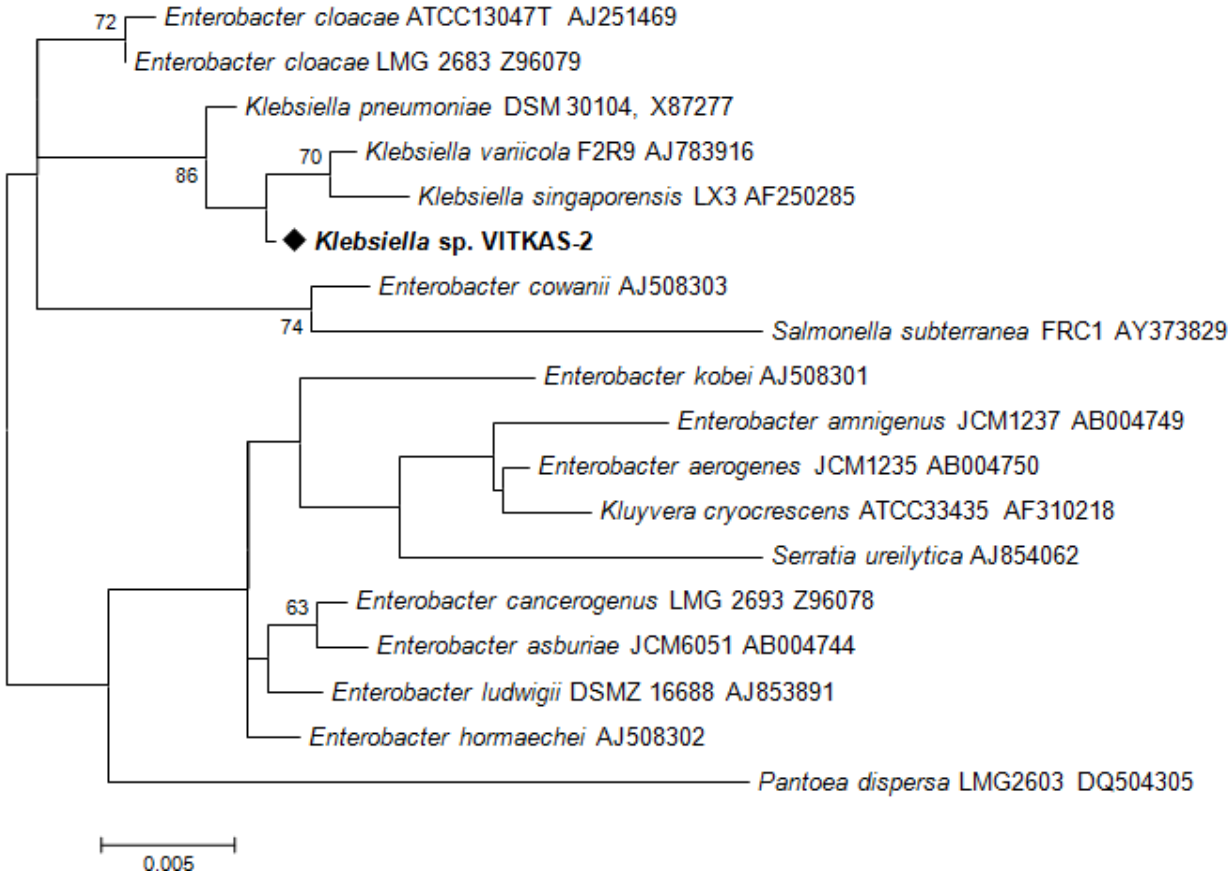

Figure 2. Neighbour joining method based phylogenetic tree for VITKAS-2. Phylogenetic tree based on 16S rDNA gene sequence of VITKAS-2 with various type culture sequences belonging to the family *Enterobacteriaceae*. The numbers at the nodes indicate the levels of the bootstrap support based on a neighbour-joining analysis of 1,000 re-sampled data sets. The bootstrap values below 50% were not indicated. Bar 0.005 substitutions per site.

effective by-products which in turn may lead to soil and water contamination.

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