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Identification and characterization of a Pb, Cu and antibiotic resistant bacteria from soil of industrial wastewater ground

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Abstract

Industrial effluents consist many pollutant and heavy metals. Bacteria isolated from industrial west water ground may have potential to tolerate heavy metal. In this study we isolate a *Citrobacter sp*. which can resist heavy metal like Cu and Pb. Slurry from industrial west water ground was collected from 22.0663 N, 88.1041 E. The heavy metal content and other parameters of soil were estimated. The bacterial strains were isolated by using nutrient agar plate supplemented with 2 mM copper and lead. Among the strains one was selected for further study on the basis of resistivity against Cu and Pb. The bacteria were identified by 16S rRNA analysis. Extracellular capsules produced by the isolate were precipitated using isopropanol and analysed by Energy-dispersive X-ray spectroscopy. The isolate can tolerate more than 2.25 mM copper and lead. In this study bacteria were identified as *Citrobacter freundii strain NK2*. Presence of copper and lead in extracellular capsule were obtained by Energy-dispersive X-ray spectroscopy analysis. Moreover the bacteria also exhibited multi drug resistance to many antibiotics. Isolated bacteria produce biofilms, which have chelating property. The biofilm might be used as bioabsorbent.

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1. Introduction

Key messages:

Implications for policy makers

This bacteria can be used for bioremediation of the effluents produced at this industrial zone.
The multidrug resistance property of this bacteria is alarming for the region at large.
Hence further research needs to be done in order to make the region eco-friendly.

Implications for public

This research is to make the planet eco-friendly. The chemical remediation process is detrimental to human health which is primarily used in this region. At the same time the multidrug resistance property of this bacteria is alarming. Hence the focal theme of this research is to aware common people of the current scenario of the location of this industrial belt.

Heavy metals are the elements having atomic weight from 63.5 to 200.6 with a specific density of greater than 5 (Fu and Wang, 2011). Some heavy metals belong to essential trace elements; most are toxic to health at high concentrations (Adarsh et al., 2007). The source of heavy metals in environment may be natural like volcanic eruption (MacKenzie and Canil, 2008) or xenobiotic originated like pesticide, paint, human activities like mine tailing, coal combustion and various types of industries (Wuana and Okieimen, 2011). Industrial effluents contain varieties of pollutant like hydrocarbons (Diya'uddeen et al., 2011), toxic compounds and heavy metals like Cd, Cr, Cu, Ni, As, Pb and Zn (Barakat, 2011). Industries are liable to diminish down heavy metal contents in industrial effluents to an acceptable level in wastewater effluents (Dabrowski, 2004). Several methods exist to remove heavy metal contamination from industrial wastewater viz. adsorption (Ngah and Hanafiah, 2008; Mohan and Pittman, 2007) ion exchange, precipitation, membrane filtration, electrodialysis etc. (Fu and Wang, 2011).
Many alternative methods also available for waste water treatment. Plant and microbial biomass efficiently with heavy metal ions may an inexpensive and eco-friendly option for heavy metal remediation (Ahluwalia and Goyal, 2007). Some bacteria can efficiently reduce heavy metal contamination from wastewater by producing extracellular polysaccharide capsule in which metal ions are chelated (Nies, 1999). It is assuming to that microbes isolated from industrial effluent may tolerate heavy metal. In this study we isolated and characterized a bacterial strain from industrial waste water that can resist copper and lead by producing extracellular capsule.

2. Methods

2.1. Sample collection

Soil slurry were collected from the industrial waste water effluent ground of Haldia, West Bengal, India (22.0663 N, 88.1041 E), in a sterilized glass container and aseptically transported to the laboratory in an ice-bucket at approximately 4 ^{0}C to prevent from contamination and allow the sample to stay longer (Dick, 1994).

2.2. Determination of soil property

pH of the aqueous soil extracts (1:2.5, w/v) was measured with a glass electrode by Digital pH Meter DPH500 calibrated by pH 4 and pH 9 standard buffer at room temperature (27^{0}C) in triplicate (Piotrowska-Seget, 2005). Soil salinity was determined in a portable SYSTRONICS Water Analyser 371 instrument. Soil physico-chemical parameter were analysed as per Sharma and Fulekar (2009). Heavy metal content of the soil was estimated as per Yousuf et al. (2015) by Atomic Absorption Spectroscopy.

2.3. Isolation of strain

Heavy metal tolerant organisms were isolated as per Piotrowska-Seget et al. (2005) with modifications. 5 g soil sample was mixed with 45 ml of 1x phosphate buffered saline in Erlenmeyer flasks and shaken at 180 rpm for 30 min. A serial dilatation of 10^{-6} was plated in a nutrient agar plate containing 2 mM of $CuSO_4$ and 2 mM $PbCl_2$ and incubated at 37 ^{0}C for days at inverted position. Eight colonies were selected (for identification named as M1-M8) and determined their Minimum Inhibitory Concentration (MIC) against Cu an Pb.

2.4. Morphological characteristics of the bacteria

Cellular morphologies were determined by optical microscope with 100x magnification by using Gram staining (Moyes et al., 2009) method.

2.5. Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined against Cu and Pb. LB medium with 0.75 mM to 2.75 mM heavy metal were inoculated with 1% bacteria cultures in triplet and incubated in a shaker incubator for 24 hours at 37^0C. Next day bacteria growth was measured in terms of O.D. at 660 nm. The M7 bacteria was selected for final study.

2.6. Scanning electron microscopy

Bacteria were harvested by centrifugation at 3000 X g and washed with PBS for three times, subsequently were fixed with 2.5% glutaraldehyde and 2% formaldehyde solution for 6 hours at 4 ^{0}C. After fixation, the cells were rinsed with PBS several times, centrifuged and re-suspended the pellet in sodium phosphate buffer. The samples were coated with a nanometre-thick layer of gold after mounting them on studs after smearing on glass cover slips and then analysed on a EVO 40 EP Electron microscope at 20 kV AC current, voltage. Images were then taken in 10000x magnification.

2.7. Identification of M7 bacteria

The M7 bacteria was identified as per Jiang et al. (2008). Genomic DNA was isolated from bacteria by alkaline lysis protocol (Choudhuri et al., 2006). PCR amplification of the 16S rDNA gene fragment was done using universal 16S rDNA primers, 27F (5'-GAGTTTGATCACTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3') with an annealing temperature of 50 ^{0}C for 30 second using commercial PCR amplification kit in Eppendorf Master cycler. PCR products were sequenced from Chromous Biotech Pvt. Ltd DNA sequencing services (Bengaluru, India). The sequence was compared using NCBI nucleotide BLAST against the GenBank database (Altschul et al., 1997) to find the genus. Phylogenetic analysis was done by Neighbor Joining method (Liu et al., 1997) using MEGA 6.

2.8. Sensitivity to antibiotic

Agar plate disk diffusion method was performed to find antibiotic sensitivity of the isolate (Bauer et al.,1966). Tests were performed in triplicate with following antibiotic, ampicillin (100 µg/ml), Kanamycin (30 µg/ml), neomycin (30 µg/ml), Chloramphenicol (30g/ml), ciprofloxacin (30g/ml), cifoxitin (50 g/ml), tetracycline (30 g/ml), sulfisoxazole (30 g/ml) with 16 mm disk (Joshi and Modi, 2013).

2.9. Optimum pH and temperature for the growth of the bacteria

To determine the pH optimum, Luria broth medium used for growth of the isolate was adjusted to different pH ranging from 3-11 and was inoculated with 1% inoculum. Overnight grown that 37^0C under shaking condition at 120 rpm, growth was measured in terms of O.D. at 660 nm in spectrophotometer. For determine optimum temperature pH fixed at it optimum value and varied temperature from 8^0C to 50^0C with other condition keeping same (Zhang et al., 2014).

2.10. Bacterial growth phase

Bacterial growth phase was determined by growing bacteria in LB medium at 37 ^{0}C in shaker incubator. Growth are measured in terms of O.D. at 2 hours of time interval until it reached to decline phase.

2.11. Hi-carbo kit test

To order to find the capability of utilization of different carbon source by isolated bacteria, Himedia Hi-Carbo kit test was done as per the instruction on kit. Overnight M7 bacteria cultures diluted serially and plated in nutrient agar plate containing 2mM $CuSO_4$. Plates were kept at 37 ^{0}C for 24 hours in inverted position. Next day picked single colony in 3 ml LB and incubated at 37 ^{0}C under shaking until O.D. at 660 have been reached to 0.4. 30 µl of this culture transferred to each well of Hi-Carbo Part A, B, and C kit. The kits were incubated at 37 ^{0}C for 24 hours. Next day color changed were compared with standard color chart supplied with the kit.

2.12. Isolation of extracellular capsule

Isolation of extracellular capsule was done by isopropanol precipitation method. Bacteria were grown in LB medium with 2mM of $CuSO_4$ for 4 days. Cells were separated from bacterial culture by centrifugation at 8500 X g for 10 min, add equal volume of isopropanol and incubated at -20^0C for 24 hours, followed by centrifugation at 20000 X g for 20 min at 4 ^0C. Pellet was washed twice in deionized water and lyophilized. Extracted material was subjected to Energy-dispersive X-ray spectroscopy (EDX). Same procedure was repeated using $PbNO_3$.

3. Results

3.1. Soil property and heavy metal content in soil

The pH and the salinity of the soil was found 7.42 and 2.52 dS/m respectively. The soil contains 36.3% of moistures, 4% of organic carbon 0.3 g/kg total nitrogen, 145.8 mg/kg phosphate and 111.52 mg/kg potassium. Several heavy metals are presence in the soil. The concentration of Zn, Pb, Cu, Mn and Fe are 627 mg/l, 214 mg/l, 113.8 mg/l, 97.2 mg/l, 467.4 mg/l respectively.

### 3.2. Isolation of strains and basic characteristics of the isolates

Total eight number of colonies were isolated. Basic characteristics of the isolates are given in the Table 1.

Table 1
Basic characteristics of the isolates.

Isolate	Colony shape	Colony color	Colony elevation	Gram stain	Morphology
M1	Round	Yellow	Raised	+	Cocci
M2	Round	Off-white	Raised	+	Rod
M3	Irregular	Pale	Flat	+	Rod
M4	Round	White	Raised	-	Cocci
M5	Irregular	Milky	Convex	+	Rod
M6	Round	Cream	Raised	+	Rod
M7	Round	Cream	Convex	-	Rod
M8	Round	Milky	Flat	-	Cocci

Among of the isolates M7 showed good resistance pattern against Cu and Pb. 2.25 mM concentration of Cu and Pb inhibit 58% and 51% growth of the isolated organism (Fig. 1: 1a,1b)
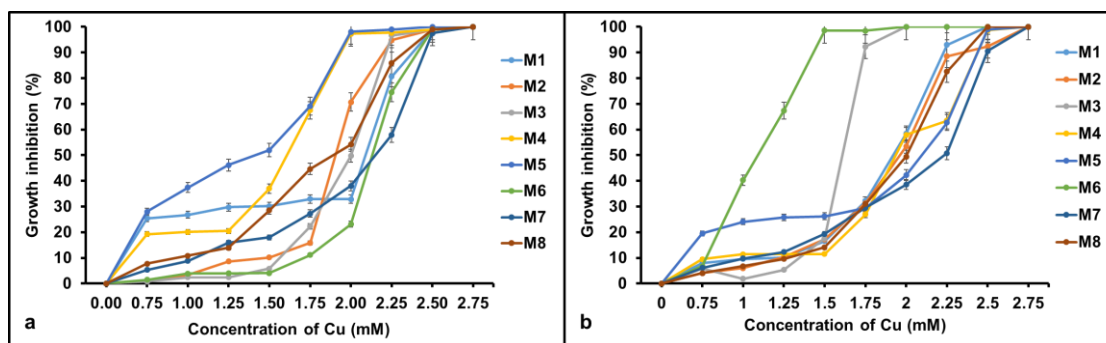
Fig. 1. Heavy metal tolerance pattern for the isolates. 1a, Heavy metal tolerance for Cu and 1b, Heavy metal tolerance for Pb.

3.3. Antibiotic sensitivity test

The bacteria are sensitive to antibiotic like Ampicillin (2.0 cm), and Neomycin (2.1 cm) but resistant to Kanamycin, Chloramphenicol (30 g/ml), ciprofloxacin (30 g/ml), cifoxitin (50 g/ml), tetracycline (30 g/ml), sulfisoxazole (30 g/ml).

3.4. Scanning electron microscopy

Scanning electron microscope image reveals the rod shaped bacteria around 2 μm in size (Fig. 2: 2a, 2b, 2c) and produce extracellular capsules, which were isolated and analysed by EDX.
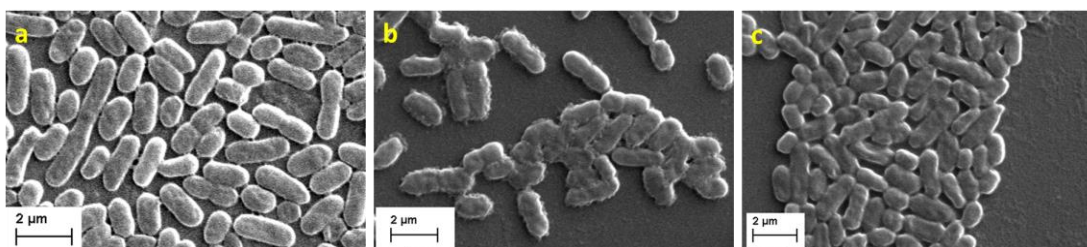

Fig. 2. Scanning electron microscopy image of M7. 1a, without heavy metal. 1b, with 2mM Cu. 1c, with 2mM Pb.

3.5. Identification of M7

16s rRNA study (Fig. 3) indicates the isolated bacteria is belonging to *Citrobater* genus and closely related to *Citrobacter freundii*. The sequence identity with C. freundii is 98%. This bacteria was named as NK2.
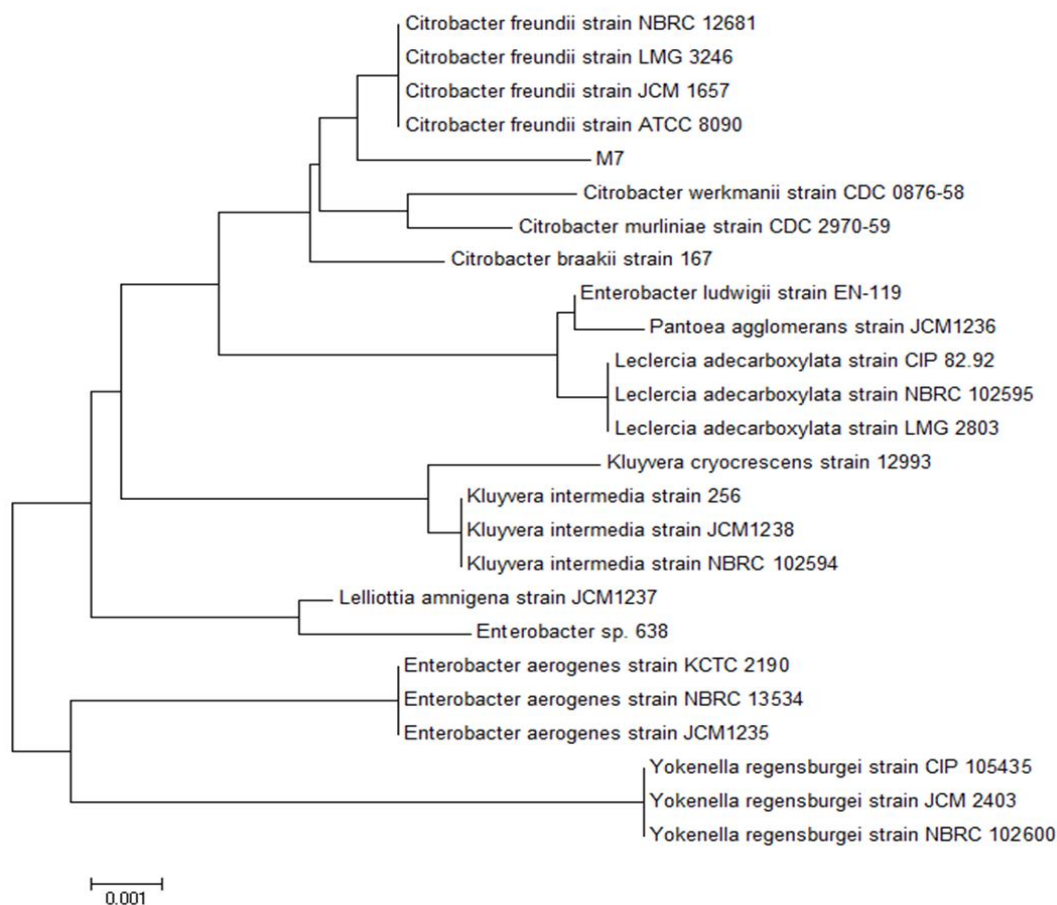

Fig. 3. Phylogenetic analysis by Neighbor-Joining method using MEGA 6.

3.6. General characteristics of the isolates

The bacteria can utilize a wide range of sugar as carbon source (Table 2).

Table 2

Different sugar utilization by the M7 isolate.

Lactose, Maltose, Fructose, Dextrose, Galactose, Trehalose, Sucrose, Glycerol	+++
Insulin	++
Xylose, Raffinose, Dulcitol, Sorbitol, Mannitol, Adonitol, Erythritol, Rhamnose, Cellobinose, Melezytose, D-Arabinose, Sorbose	+
Melibinose, L-Arabinose, Manose, Sodium Gluconate, Salicin, Inositol, Arabitol, A-Methyl-D-Glucoside, A-Methyl-D- Mannoside, Xylitol, Onpg, Esculin, Citrate, Malonate	-

The bacteria can grow in a wide range of pH from 5 to 9.5, and a short range of temperature between 30^0C to 37^0C. Optimum pH and temperature were found 7 and 37 ^{0}C respectively (Fig. 4: 4a, 4b). Fig. 5 shows the bacterial growth phage at optimum pH and temperature.
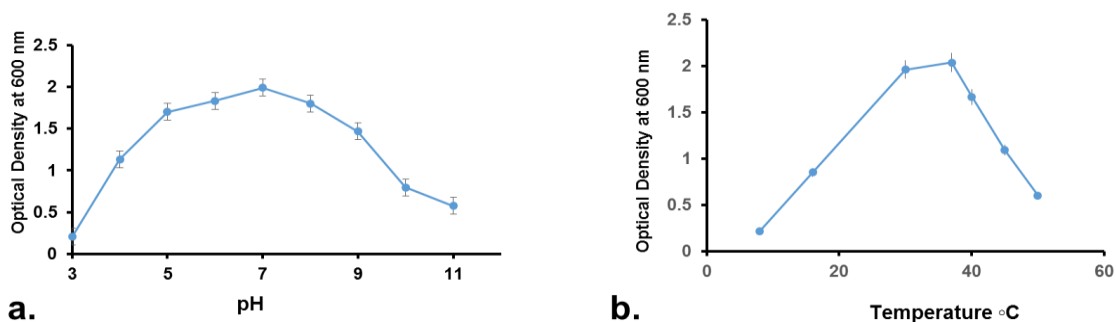

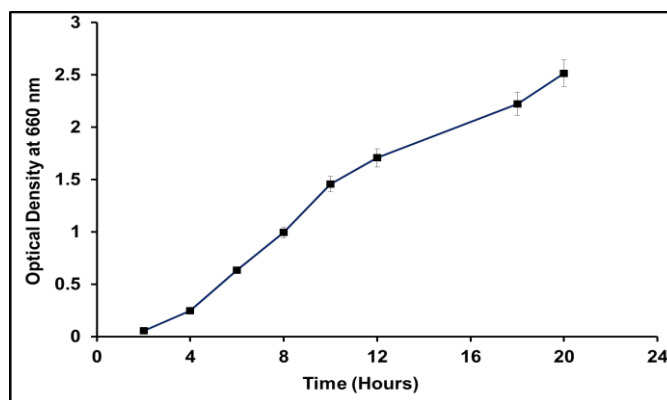**Fig. 4.** 4a, Optimum pH and 4b, optimum temperature of the isolate.

Fig.5. M7 growth curve at optimum pH and temperature.

3.7. Energy-dispersive X-ray spectroscopy (EDX)

Copper and Lead peaks were obtained in EDX spectra of isolated extracellular substances (Fig. 6: 6a, 6b). This indicates the substances have metal binding property.
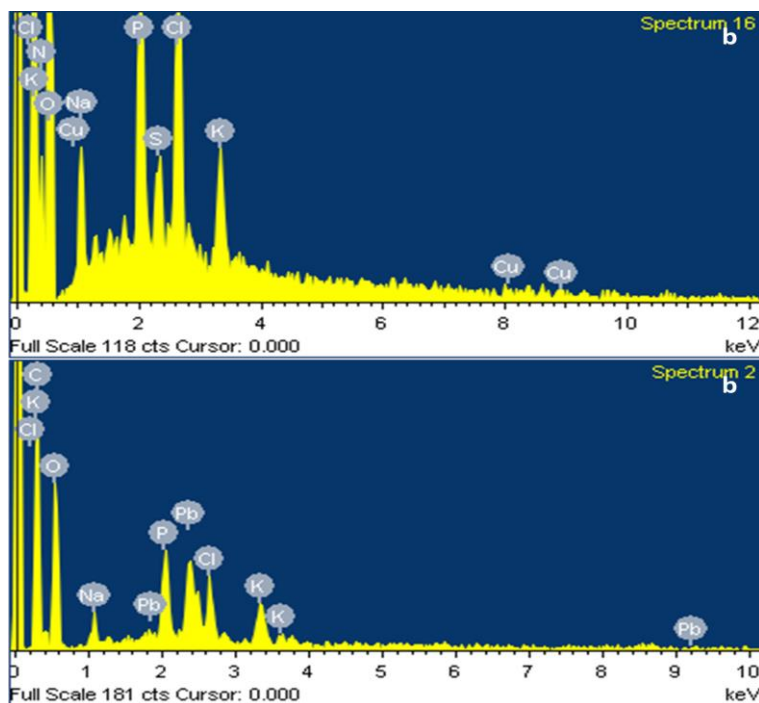
Fig. 6. EDX spectra of exopolysaccharide. 6a, with 2 mM Cu and 6b, 2 mM Pb.

4. Discussion

The isolated bacteria are Gram negative and rod shaped bacteria, can tolerate more than 2.25 mM Copper and Lead. The morphology of the bacteria changed slightly under heavy metal trace condition (Figure 2B and 2C), and bacteria produce extracellular substances. The Energy-dispersive X-ray spectroscopy study reveals these substances have chelating property. The isolated bacteria belong to *Citrobacter* genus and closely related to *Citrobacter freundii.* Sharma and Fulekar in 2009 showed that *Citrobacter freundii* is potential to bioaccumulation of metal ions. Although the nature of the extracellular substances has not been characterized yet, probably it consist OH^- and other negatively charged organic radical, which make it potential to chelate heavy metal ions.

5. Conclusion

Isolated bacteria from industrial west water ground of Haldia, West Bengal, India can tolerate lead and copper. The isolate was identified as *Citrobacter freundii strain NK2* and produce extracellular capsule. For the chelating property, the biofilm might be use as bioabsorbent. In future the extracellular substances will be characterized. In addition, this is the first reported study of antibiotic resistance property of C. freundii collected from industrial waste in India. The emergence of multidrug resistance property of this bacteria in the environment is increasing threat to the society. Hence further study need to be done to determine the basis of this resistance.

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