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Determination of lead biosorption efficacy of soilborne *Achromobacter* sp. through atomic absorption spectrophotometer (AAS) furnace

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Abstract

Rapid industrialization has been posed overall environment in jeopardy by discharging huge amount of industrial contaminants, mainly heavy metals into soil and water bodies. Among various heavy metals, lead is a major pollutant and it is necessary to eliminate from environment for minimizing the risk of uptake by plants, animal and human. In view of this, lead contaminated soil sample was collected from industrial area and inoculated in mineral salt (MS) medium supplemented with lead at different concentrations for 5 days for the isolation of bacterial strain. The optimal culture condition of the bacterium was at pH 7.0 and temperature 30°C. Morphological and biochemical properties indicated that the bacterium was gram negative, rod shaped, motile, citrate utilizing, catalase positive and showed negative result for methyl red, urease, starch hydrolysis, mannitol salt agar and lactose fermentation test. Isolated bacterial strain was identified as *Achromobacter* sp. with 92% homogenity through molecular identification. Isolated *Achromobacter* sp. was multidrug resistant and showed resistance pattern against cefuroxime, nalidixic acid, cefotaxime, chloramphenicol. Moreover, MIC of gentamycin against the isolated bacterium was determined at 12.5 µg/ml concentration. Lead toxicity was also evaluated against *Artemia salina* and LC_{50} value was 2.769± 0.018 µg/ml after 24 hour of exposure which exhibited higher toxicity of lead. Finally, lead biosorption efficiency of the *Achromobacter* sp. was determined through AAS furnace and highest degradation rate (90%) was observed at 100 µg/ml concentration at day 7 of exposure. Therefore, the study concluded that *Achromobacter* sp. proved to be a potential biosorbent for the removal of lead from contaminated soil.

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Introduction

Technological advancements that led to improved life standards, has raised new challenges with respect to environmental safety, as unrestrained industrialization and urbanization without proper emission controls and pollution abatement have put human lives at risk (Bennet *et al.*, 2003). In developing countries, a need for economic growth that generally relies on agricultural and industrial development has by passed environmental protection guidelines to a greater extent (Ikhuoria *et al.*, 2000; Sahu and Arora 2008). Rapid increase of population and the increased demand for industrial establishments to meet human needs have created problems such as over exploitation of available resources, increased pollution taking place on land, air and water environment. Heavy metals are significant environmental pollutants and their toxicity is a problem of increasing significance for ecological, evolutionary, nutritional and environmental reasons (Jaishankar *et al.*, 2014; Nagajyoti *et al.*, 2010). Human activities such as mining operations, fuel combustion, application of agricultural chemicals and discharge of industrial waste, have resulted in accumulation of heavy metals in the environment (Fakayode, 2005). Heavy metal pollution of soil and wastewater is a significant environmental constraint for the survival of all living beings. Soil pollution results in a massive change in the physical, chemical and biological characteristics of the soil which not only curtails the area of cultivable land but also contaminate overall food chain of ecology.

Among various heavy metals, lead (Pb) is one of the most abundant natural substances and its toxicity is a particularly insidious hazard with the potential to cause irreversible health effects (Karrari *et al.*, 2012). The brain is the most sensitive organ to lead exposure (Cleveland *et al.*, 2008). Acute lead poisoning results in a well characterized syndrome manifested in adults by colic, anemia, headache, fatigue, gumline and peripheral neuropathy (Singh *et al.*, 2010). It has also adverse effects on oxidative stress (Roy *et al.*, 2015). The Institute for Health Metrics and Evaluation (IHME) estimated that in 2016 lead exposure accounted for 540000 deaths worldwide due to long-term effects on health (IHME, 2016).

However, the remedy of this massive cry meets with the demand in nature itself. With regard to removal of heavy metals, bioremediation is an advantageous technique which is comparatively safer and cheaper (Volesky, 1994).

Microorganisms are omnipresent and are found to be involved in one or other form in different biological processes of life. Owing to natural processes and urbanization, heavy metals are commonly found in microbial habitats in high proportions. Many reports have revealed that indigenous microbes are capable of tolerating high metal concentrations in one way or another and may play a pivotal role in the restoration of contaminated soil (Carrasco *et al.*, 2005; Ge *et al.*, 2009). It has been reported that microorganisms become adapted to these environments by acquisition of specific resistance systems (Dopson *et al.*, 2003). Hence, the present study was designed for the isolation and characterization of lead detoxifying bacterial strain and to observe growth behavior in different levels of lead concentration through Atomic Absorption Spectrophotometer in order to use the bacterial strain for detoxification in an integrated bioremediation system.

Materials and methods

Sample collection, isolation and optimization of growth characteristics of lead detoxifying bacterium

Soil sample was collected from industrial area of Rajshahi silk factory, Sopura, Rajshahi. Microorganism capable of detoxifying lead was isolated from enrichment cultures by plating out on agar solidified mineral salt (MS) medium supplemented with different concentrations of lead (100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml and 600 µg/ml). The plates were then incubated at 37°C for 5 days. The single colonies from these plates were sub cultured onto replicate plates and colonies from these eventually transferred into MS medium containing different lead concentrations. Pure strains were maintained by weekly passage in

liquid mineral salts (MS) medium containing lead and also by weekly subculture onto the same medium solidified with 3% agar.

Temperature and pH influenced on the bacterial growth. For the effect of pH, culture medium was adjusted to pH 5, 6, 7, 8. Incubation temperature varied at 25°C, 30°C and 35°C. Bacterial cell density of liquid cultures were determined by measuring optical density at 660 nm using double beam spectrophotometer (ANALYTIK JENA AG, SPOKOL 1500/1, GERMANY).

Morphological and biochemical tests

Several morphological (gram staining and motility test) and biochemical (methyl red, catalase, simmons citrate, lactose fermentation, urea hydrolysis, starch hydrolysis, mannitol salt, TSI) tests were done for the specific identification and characterization of isolated bacterium.

Molecular identification of isolated bacterium

16S rRNA gene sequencing is considered as the most reliable tools for identification of microorganism species (Shah *et al.,* 2012). At first, the chromosomal DNA was extracted from the bacterial culture and the extracted DNA was used as template for PCR to amplify 16S rRNA gene. 27F 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492R 5′-GGTTACCTTGTTACGACTT-3′ were used as forward and reverse primer, respectively. After PCR amplification, the gel was run and desired band was found. Next the PCR product was purified and the concentration of purified product was measured using the Nano Drop ND-2000 Spectrophotometer and diluted with sterile water to 50 ng/ml before samples were sequenced. Sequencing was done from both forward and reverse end using either 8F or 1492R primers and carried out in ABI Prisom 310 machine, subsequently we assembled the sequence blast against the nucleotides database in NCBI. 16S rRNA gene sequences of selected bacterial isolate was compared with other reference sequences as available in the NCBI database using the Basic Local Alignment Search Tool (BLAST) algorithm.

Antibiotic sensitivity test and determination of minimum inhibitory concentration (MIC)

Antibiotic sensitivity test was performed with Disc Diffusion method (Bauer, 1966). The isolated bacterial strain was grown overnight in nutrient broths through shaker at 30°C temperature and 120 rpm for the antibiotic sensitivity test. Nutrient agar plates were dried at 30°C. The overnight grown LB culture (O.D. = 0.5) was poured onto nutrient plate and dried. Antibiotic discs were placed centrally on the respective plates and incubated overnight at 30°C. After overnight incubation the zone was observed on the plate and measured with the help of mm scale. Ten commercially available antibiotic discs were used including cefuroxime (30 µg), nalidixic acid (30 µg), vancomycin (30 µg), amoxicillin (10 µg), gentamycin(10 µg), ciprofloxacin(5 µg)ampicillin(10 µg), ceftazidime (30 µg), cefotaxime (5 µg) and chloramphenicol (10 µg).

Minimum inhibitory concentration of gentamycin against isolated bacterium was determined through tube dilution method which is the standard method for determining levels of resistance to an antibiotic.

Determination of toxic effect of lead against Artemia salina

Brine shrimp lethality bioassay is considered as a useful tool for preliminary assessment of toxicity (Solís *et. al.,* 1993) and it has been used for the detection of toxicity of heavy metals (Martínez *et.al.,* 1998), pesticides (Barahona and Sánchez-Fortún, 1999) etc. Brine shrimp eggs were hatched in simulated seawater to get nauplii. In each of the five vials, 10 ml simulated sea-water was taken, containing 10 brine shrimp nauplii. Specific volumes of each sample were transferred from the stock solution of lead with the help of a micropipette to the respective vials to get final concentrations of 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml,100 µg/ml of lead in the culture. For each concentration, one vial containing 10 ml simulated sea-water and 10 shrimp nauplii was used as positive control group. It was used to verify the validity of the test. Survivors were counted after 24 hours. These data were processed in

a simple program for probit analysis to estimate LC_{50} value with 95% confidence intervals. This represents the concentration of the chemical that produces death in half of the test subjects after a certain exposure period.

Determination of lead biosorption by Atomic Absorption Spectrophotometer (AAS) furnace

The atomic absorption spectrometry uses absorption of light of intrinsic wavelengths by atoms and absorption rate is determined by the atomic density. At first isolated bacterial strain was cultured with MS medium in five flasks, each flask contain 99 ml MS medium,1 ml bacterial liquid culture.100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml concentrations of lead was added respectively into five conical flasks in aseptic condition. 2 ml sample from flask was collected in centrifuge tube and this tube was marked for control sample.

After every two days, 2 ml sample was collected from every flask and stored at 4°C for further experiment. After 7 days these samples were centrifuged at 12000 rpm for 5 minutes. Supernatants were transferred in new tubes and cell free supernatants were filtered through 0.45 µm nylon membranes prior to analysis. Finally, lead biosorption efficiency of the isolate was determined through Atomic Absorption Spectrophotometer (Model: AA- 6800) in lead supplemented MS medium.

Statistical analysis

We detected the concentrations of Pb through ASS furnace for five different doses with three replications each on day 0, 3, 5, 7, respectively. On the basis of average values for each doses, we calculated the percentage of lead biosorption through following equation.

$$\text{Percentage (\%) of biosorption} = \frac{\text{Average ASS furnace value at initial day- Average ASS furnace value at last day}}{\text{Average ASS furnace value at initial day}} \times 100$$

Results

Isolation and optimization of growth characteristics of isolated bacterium

Lead detoxifying bacterium was isolated by plating from the old bacterial suspension of the liquid medium onto mineral salt agar containing different concentrations of lead (100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml and 600µg/ml). No growth was found at 600 µg/ml and more than that concentration of the substrate.

Table 1. Morphological and biochemical tests result of isolated bacterium.

Tests	Result
Motility	Motile
Gram staining	Gram negative, rod shaped
Catalase	Positive
Simmons citrate	Positive
Triple sugar iron (TSI)	Positive
Methyl red	Negative
Urease	Negative
Lactose fermentation	Negative
Mannitol salt	Negative
Starch agar	Negative

In the current experiment, different pH ranging from 5 to 8 and different temperature viz. 25°C, 30°C, 35°C was tried. Our result showed that the optimum pH for the growth of our bacterial isolate was 7.0 and extreme pH 5.0 restricted for the bacterial growth (Fig.1.). Our experiments also illustrated that 30°C was the perfect temperature for the optimum growth of the isolated bacterium and extreme temperature 25°C restricted for the bacterial growth (Fig.2.).

Table 2. Antibiotic sensitivity test result of isolated bacterium (*Achromobacter* sp.).

Antibiotics	Zone of inhibition (mm)	Resistant pattern
Cefuroxime	6 mm	Resistant
Nalidixic acid	8 mm	Resistant
Ampicillin	13 mm	Intermediate resistant
Vancomycin	17 mm	Susceptible
Amoxicillin	13 mm	Intermediate resistant
Ceftazidime	12 mm	Intermediate resistant
Gentamycin	20 mm	Susceptible
Cefotaxime	6 mm	Resistant
Ciprofloxacin	23 mm	Susceptible
Chloramphenicol	9 mm	Resistant

Note: Resistant=<10 mm; Intermediate =10-15 mm; Susceptible=>15 mm.

Morphological and biochemical characterization of isolated bacterium

Morphological test indicated that isolated bacterium was motile, gram negative and rod shaped (Table 1.). In biochemical tests, isolate showed positive results for catalase, simmons citrate test with the ability to ferment only glucose in TSI test but it was lactose non- fermenting and negative result was also found for methyl red, urease, mannitol salt, starch agar test (Table 1.).

Table 3. Minimum inhibitory concentration of gentamycin against the isolated bacteria.

Test Organism	Growth response at different concentrations								
Lead detoxifying *Achromobacter* sp.	Gentamycin (µg/ml)								
	100-	50-	25-	12.5-	6.25+	3.125+	1.56+	0.78+	0.39+

Note: The '+' sign indicate the growth of the microorganisms while '-'sign indicate no growth.

Molecular identification of isolated bacterium

After the 16S rRNA gene sequencing and editing of sequences it was checked with the 16S rRNA gene sequences of other organisms that had already been submitted to Gene bank database. From the gene bank, several sequences are found with significant identity and isolated bacterium produced significant 92% identity with *Achromobacter* sp. (Accession no. LC433627). PCR band of Chromosomal DNA was found in the agarose gel after illumination with the UV light which was shown in Fig. 3.

Table 4. Determination of LC_{50} value of lead against *Artemia salina* after 24 and 48 hours of exposure.

Tested sample	Exposure (hours)	LC_{50} value	95% confidence limits		Regression equation	χ^2 value (3df)
			Upper	Lower		
Lead	24	2.768667	5.688329	1.347587	Y = 4.408405 + 1.337631 X	1.449404
	48	-	-	-	-	-

Antibiotic sensitivity test and determination of minimum inhibitory concentration (MIC)

Multidrug resistant bacterium was isolated in our present study which showed resistance pattern against cefuroxime, nalidixic acid, cefotaxime and chloramphenicol (Table.2. and Fig. 4.) On the otherhand, *Achromobacter* sp. was susceptible to three antibiotics (vancomycin, gentamycin, ciprofloxacin) and intermediate resistant to remaining antibiotics out of ten antibiotics tested (Table.2. and Fig. 4.).

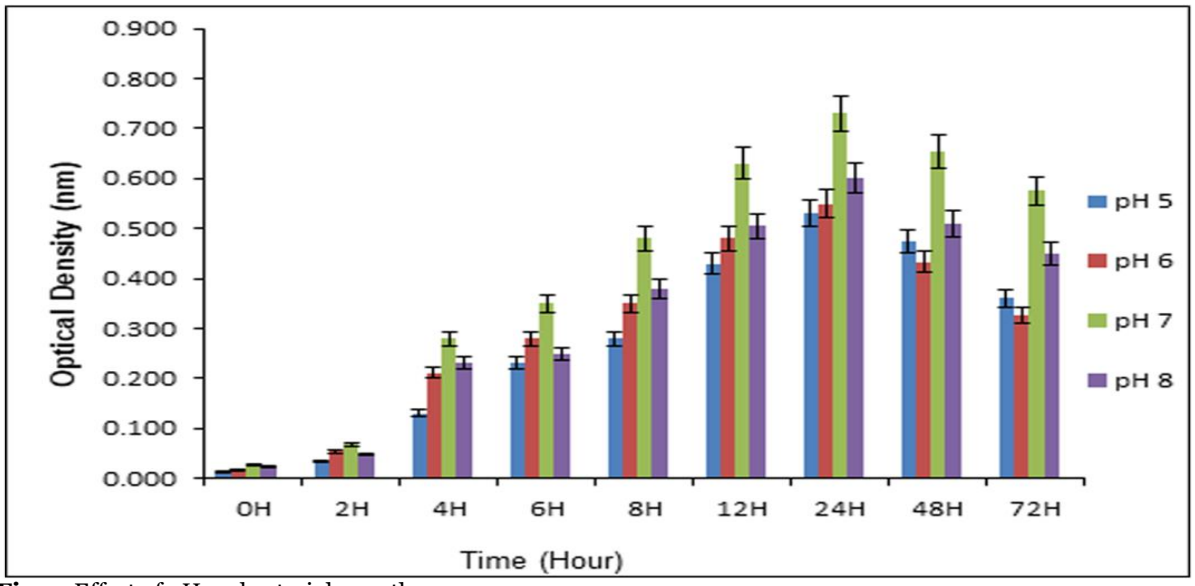

Fig. 1. Effect of pH on bacterial growth.

The MIC value of gentamycin against isolated bacterium was 12.5 µg/ml (Table.3.).

Determination of toxic effect of lead against Artemia salina

In our present study, LC_{50} value of lead was evaluated against *Artemia salina* at different concentrations (20, 40, 60, 80 and 100 µg/ml) through probit mortality software. We calculated dead number of *A. salina* after 24 hour and 48 hour.

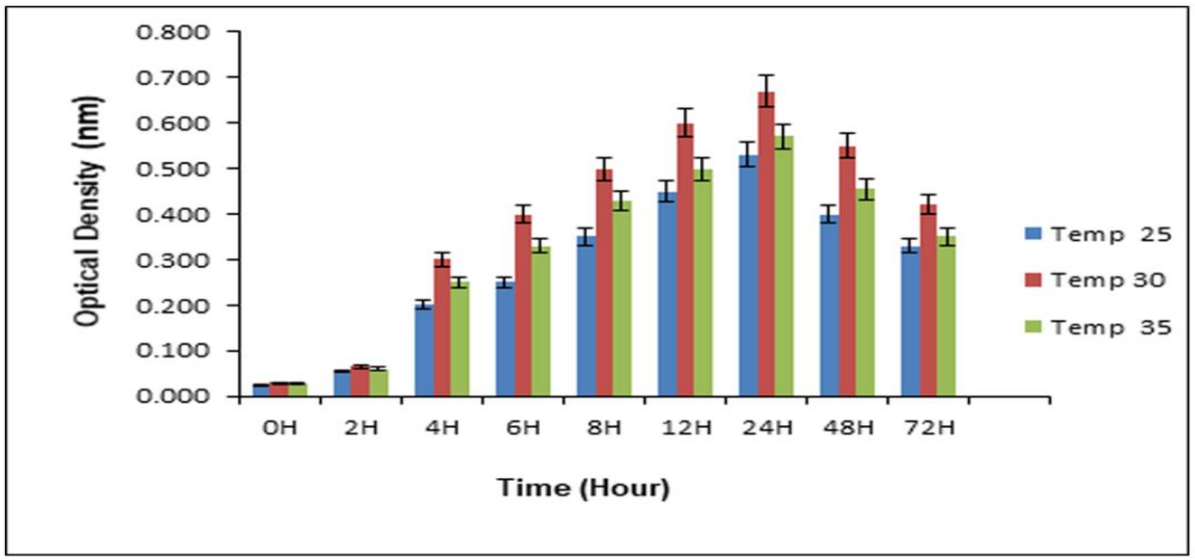

Fig. 2. Effect of temperature on bacterial growth.

The LC_{50} value for the tested lead sample was 2.769± 0.018 µg/ml and the regression equation was Y = 4.408405+1.337631X while the 95% confidence limits were 1.348 to 5.688 µg/ml for 24 hours of exposure (Table 4.). After 48 hour, no live *A. salina* was found (Table 4.).

Determination of lead biosorption rate by AAS furnace

Lead biosorption rate was investigated under laboratory condition through AAS furnace. The result revealed that after 3 days, *Achromobacter* sp. had lead removal efficiency of 60%, 56%, 51%, 45% and 40% at 100, 200, 300, 400 and 500 µg/ml concentrations of lead, respectively (Table 5. and Fig. 5.). While the rates were 76%, 70%, 63%, 55% and 48% at 100, 200, 300, 400 and 500 µg/ml concentrations, respectively after 5 days

(Table 5. and Fig. 5.). Maximum biosorption rates were found 90%, 82%, 73%, 65% and 60% at different concentrations of lead after 7 days (Table 5. and Fig. 5.).

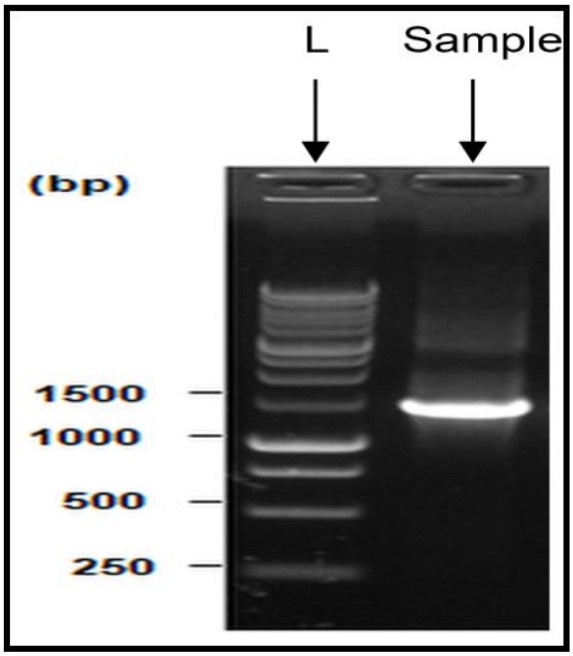

Fig. 3. Band of PCR products from isolated bacterial sample. Here,"L" indicates 1kb DNA ladder (Marker).

Discussion

In our present investigation, lead uptaking bacterium was isolated from contaminated soil and cultured on MS agar solidified medium supplemented with various concentrations of lead by using streak plate and pour plate methods. Isolated bacterium was characterized by several morphological, physiological and biochemical tests. Morphological tests showed that isolated bacterium was motile, gram negative and rod shaped. Biochemical tests indicated that isolated bacterium was catalase positive, citrate utilizing, glucose fermenting in TSI test while it was methyl red negative, lactose and mannitol salt non-fermenting, unable to hydrolyze starch, urea. The optimal growth condition of isolated bacterium was found to be at pH 7.0 and temperature 30°C. Bergeys's Mannual of Determinative Bacteriology (Seventh Edition) indicated similar characteristics of the genus *Achromobacter*. So, it was authenticated that morphological, biochemical and physiological outputs of the isolate had a resemblance with the referred data. Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique (Clarridge *et al.,* 2004). Our sequencing result of the 16S rRNA gene revealed that the bacterial isolate was *Achromobacter* sp. Shamsa and Sikandar, 2016 isolated *Achromobacter* sp. from soil which provided confirmation about our finding.

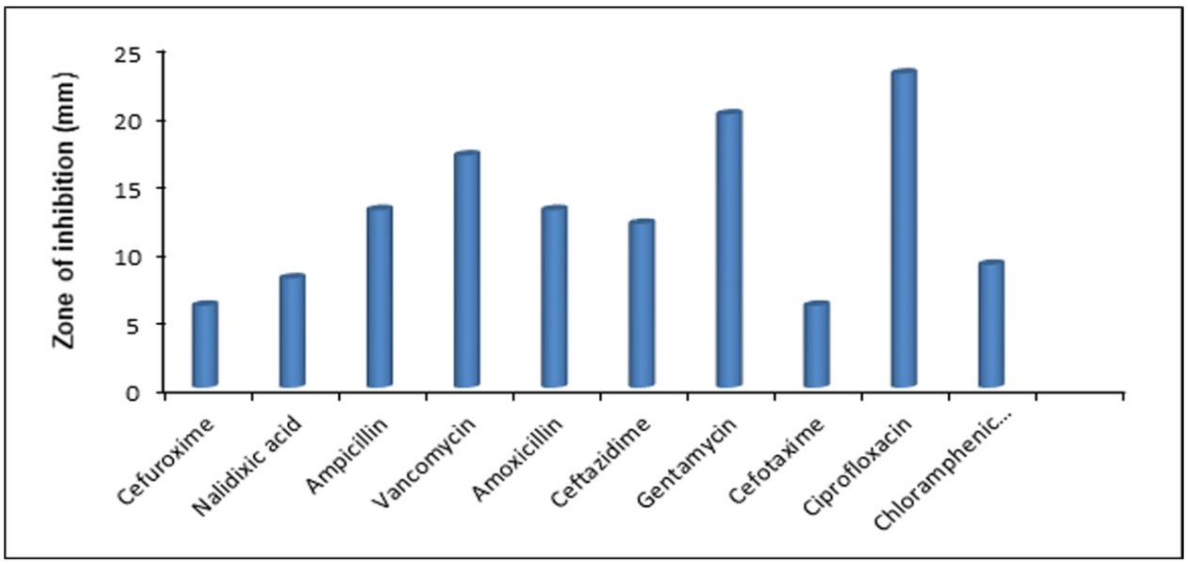

Fig. 4. Antibiotic sensitivity pattern of *Achromobacter* sp.

Edwards *et al.*, 2017 reported the prevalence and outcomes of *Achromobacter* sp. infections in human. Hence, it created a concern for us to control it in case of accidental human pathogenicity. So, antibiotic sensitivity of the isolated bacterium against ten different antibiotics was checked in our present study. The result showed that isolated bacterium was resistant against four antibiotics (cefuroxime,

nalidixic acid, cefotaxime and chloramphenicol) as it was multidrug resistant and susceptible to three antibiotics (vancomycin, gentamycin, ciprofloxacin). Eghomwanre *et al*, 2016 isolated multidrug resistant *S. aureus*, *E. coli and P. aeroginosa* bacteria from contaminated soils.

In their study, fourteen antibiotics were used and isolates exhibited resistance against septrin, chloramphenicol and augmentin while all of them were susceptible to ciprofloxacin and gentamycin.

It was notable that multidrug resistant *Achromobacter* sp. was also isolated from indoor and outdoor environments (Sachiko *et al.*, 2017). So, it can be said that our results had similarity with previous findings. Moreover, the MIC value of gentamycin against isolated bacterium was 12.5 µg/ml.

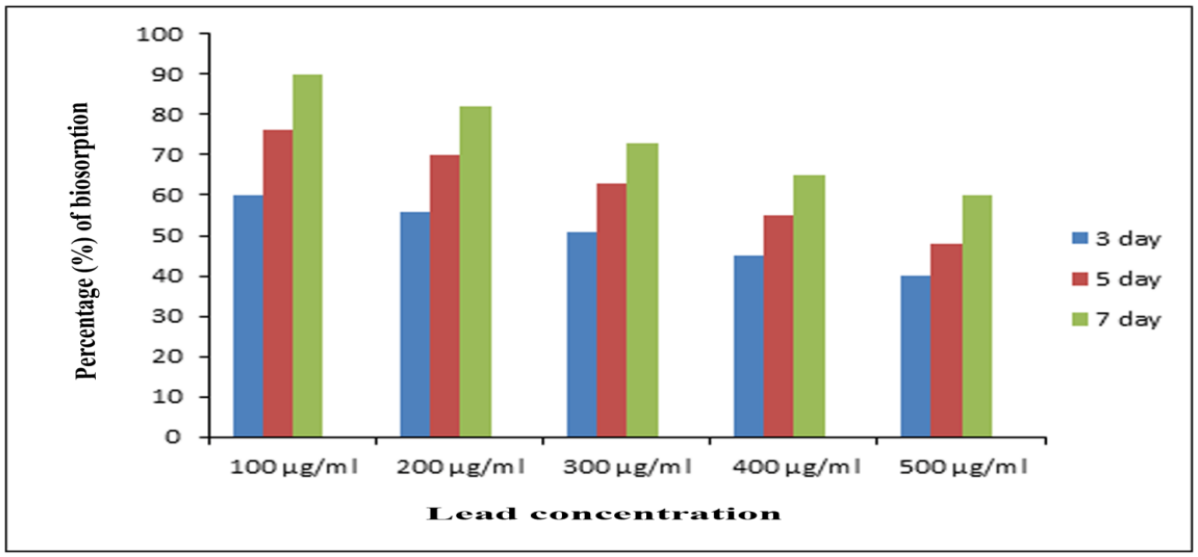

Fig. 5. Lead biosorption efficiency (%) of *Achromobacter* sp.

Toxicity of lead was previously reported against *Artemia salina* with LC_{50} value of 1.7 ± 0.011 µg/ml and 1.4 ± 0.015 µg/ml after 24 hour and 48 hour of exposure, respectively (Gajbhiye and Hirota, 1990).

In our study, LC_{50} value of lead was 2.769± 0.018 µg/ml after 24 hour of exposure against *Artemia salina* which was similar to the referenced data but no survival was found after 48 hour of exposure which indicated higher toxicity of lead against aquatic organism.

Abbas *et al.*, 2016 showed lead biosorption capability (59%) of *Pseudomanas* sp., within 7 days at a concentration of 1000 µg/ml through AAS furnace. Bandela *et al.*, 2016 also investigated Nickel removal efficiency (58%) of *Achromobacter* sp. within 3 days at a concentration of 2000 µg/ml. In our study, the ability of *Achromobacter* sp. to decrease different concentrations of lead was scrutinized after 3 days, 5 days and 7 days, respectively. The percentage of lead biosorption by *Achromobacter* sp. strain was increasing from the beginning. In contrast, significant levels of Pb concentrations were decreased after treating with *Achromobacter* sp. From the result it was clear that *Achromobacter* sp. utilized lead as a carbon source from the MS liquid medium which was confirmed by AAS furnace experiment and highest degradation rate was found 90% at 100 µg/ml concentration of lead after 7 days. Thus, the isolated *Achromobacter* sp. showed remarkable biosorption efficacy of the toxic lead from the MS medium.

Conclusion

The present study deals with isolation, identification and characterization of lead uptaking bacterium from industrial affected soil. The isolated *Achromobacter* sp. was multidrug resistant as well as potential bio-

agent (90% biosorption efficiency) to treat lead contaminated sites for bioremediation and secure the soil quality, food and health of mankind.

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