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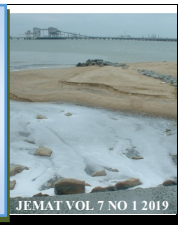

Assessing Bioaccumulation Potentiality of Lead- and Chromium-Tolerant Bacterial Strain from Tannery Effluents of Bangladesh through Atomic Absorption Spectrophotometer

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ABSTRACT

Rapid growth of industries and directly discharge of hazardous toxic effluents particularly heavy metals into water streams creating alarming circumstances for entire ecosystem. With regard to removal of heavy metals, bioaccumulation is an advantageous technique which is comparatively safer and cheaper. In this context, attempt was made to evaluate the bioaccumulation potentiality of heavy metal removing microbial flora from tannery effluents of Bangladesh. Effluent sample was collected from the drainage pipes of leather industrial zone at Hazaribagh, Dhaka, Bangladesh with proper precautions and isolation was done on mineral salt medium supplimented with different concentrations of lead and chromium separately. Antibiotic resistance pattern was checked through disc diffusion method and efficacy was determined using Atomic Absorption Spectrophotometer (AAS) furnace. Isolated bacterial colony was gram negative, rod shaped, motile, white colored morphologically. The optimum culture condition for *Achromobacter* sp. was found at temperature 35°C, pH 7.6 and observed as a halo-tolerant strain that could utilize upto 10% salt. Molecular identification revealed that isolated bacterium was *Achromobacter* sp. (85% similarity) which was also similar with the results found in morphological, biochemical and physiological tests. Isolated *Achromobacter* sp. was multi-drug resistant (MDR) which was confirmed against penicillin, cefuroxime, cefixime and cefotaxime and LC_{50} value was evaluated through probit mortality software against *Artemia salina* at the concentration of 93.01±0.2 µl/ml. However, isolate did not show antagonistic effect against Rhizobacteria. Highest detoxification rate of chromium and lead were 96% and 93%, respectively at 100 µg/ml concentration at day 9 of exposure. From the present study, it was clear that isolated *Achromobacter* sp. had remarkable biosorption efficacy of the toxic lead and chromium from tannery effluents.

INTRODUCTION

Heavy metals are naturally occurring elements and common inorganic pollutants in water [1]. They are highly allocated over the earth's crust [2] and stand for one of the most toxic environmental contaminants [3]. Their bioaccumulation in the environment has posed a major drawback owing to their toxicity and detrimental impact on human health and other species. They are recognized as powerful inhibitors of biodegradation activities, generally cannot be degraded and are ultimately everlasting for a long time. Unlike many other pollutants, heavy metals are difficult to remove from the environment [4]. Among various metals, lead and chromium are considered to be the potential toxic and carcinogenic that persists in to the soil for a long time and cause serious pollution to the water bodies also. These are directly or indirectly involved in all aspects of growth, metabolism and differentiation of the biota [5]. Cell adhesion, intra- and inter-cellular signaling, protein folding, maturation, apoptosis, ionic transportation, enzyme regulation, and release of neurotransmitters are the most vital biological processes in human systems. The ionic mechanism of lead toxicity affects these biological systems adversely [6].

Acute lead poisoning results in a well characterized syndrome manifested in adults by colic, anemia, headache, fatigue, gumline and peripheral neuropathy [7]. Cr (VI) potential

risk has also reported in humans. Workers who have been occupationally exposed to Cr (VI) containing compounds may have a risk to suffer from respiratory cancers [8] as well as abnormal peripheral lymphocytes and lipid peroxidation [9] products in urine. Genotoxic effects such as chromosomal abnormalities and DNA strand breaks are also the consequence of Cr adulteration [10].

In Bangladesh, tanning industries have been increasing during the last decade and playing a significant role in the country's economy. However, every day, the tanneries collectively discharge and dump 22,000 cubic liters of toxic waste (viz. lead, cadmium, chromium, mercury, zinc, nickel, copper etc.) without any treatment into the Buriganga river in Dhaka city [11]. The direct discharge of these wastes has been contaminated the ground and surface water with high concentrations of chromium, cadmium, arsenic, and lead [12]. Consequently, all living beings and ecosystem are in jeopardy. Therefore, bioremediation of heavy metal is an urgent need to completely resolve this alarming consequence of heavy metal contamination.

Microbial degradation is usually considered as an eco-friendly and cost-effective alternative compare to conventional physical and chemical methods as microorganisms are the most diverse group on earth [13]. Microorganisms have acquired a variety of astonishing mechanisms for adaptation to the presence of toxic heavy metals [14] such as metal biosorption capacity [15]. The organics present in effluents are utilized as nutrients for the microorganisms specially bacteria which in turn reduce them and leading to the removal of heavy metals from the environment [16]. Therefore, the present study was planned on the isolation and characterization of lead and chromium tolerant bacterial strain to observe its growth behavior in different levels of metal concentrations in order to use it for detoxification in an integrated bioremediation system.

MATERIALS AND METHODS

Sample collection, isolation of Pb and Cr tolerant bacterial strain

The waste water sample was collected from the discharge and drainage pipes of leather industrial zone at Hazaribagh, Dhaka, Bangladesh, during January 2016. The collected tannery effluent was filtered by filter paper in a beaker and mixed bacterial cultures were grown in Mineral Salt (MS) medium containing different concentrations of lead and chromium. 5 ml of the tannery effluents were added to 100 ml MS medium (K_2HPO_4 2.00 gm/L;$(NH_4)_2$ $SO_4$0.50 gm/L; $KH_2PO_4$0.02 gm/L; $MgSO_4$0.05 gm/L; Trace elements 1.00 gm/L;

Trace elements components: $FeSO_4.4H_2O$ 400 mg/L; $MnSO_4.4H_2O$ 400 mg/L; $ZnSO_4.7H_2O$200 mg/L; $CuSO_4.7H_2O$ 40 mg/L; $CoCl.6H_2O$ 40 mg/L; KI300 mg/L; $Na_2MoO_4.2H_2O$ 50 mg/L) containing lead as lead nitrate [$Pb(NO_3)_2$] and chromium as chromium nitrate [$Cr(NO_3)_3$] at different concentrations (100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml, 600 µg/ml) for enrichment, selection and incubated for 48-72 hour at 37°C with shaking. Then the bacterial isolate was screened on nutrient agar (NA) plates supplemented with different concentrations of each metal one time by the standard pour plate method. Plates were incubated at 37°C for 24 hour and the single colonies from these plates were sub cultured into MS liquid medium and used for further study.

Morphological, physiological and biochemical features of isolated strain

Isolated bacterium was characterized by several morphological (Gram staining and motility test) and biochemical (Methyl red, catalase, MacConkey, starch agar, mannitol salt agar, TSI, simmon citrate agar and urea agar) tests. The physiological characterization was done at 660 nm wavelength using spectrophotometer (Analytik Jena, Germany) to optimize temperature, pH and growth response at different carbon sources. The goal of the optimization was to create most favorable growth condition for the isolated bacterial strain.

PCR amplification and sequencing of 16S rRNA genes

The genomic DNA of the isolated lead and chromium tolerant bacterium was extracted using Tiangen DNA extraction kit. Therefore, to ensure accurate results, nucleic acid samples were purified prior to measurement. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA. The 16S rRNA genes were amplified by PCR using 16S rRNA specific primer (Sigma, USA) forward primer 27 F 5′- AGA GTT TGA TCM TGG CTC AG -3′ and reverse primer 1492R 5′-GGTTACCTTGTTACGACTT-3′ [17].

The PCR reactions were carried out in thermal cycler (Applied Biosystem 9700, USA) using following amplification conditions: an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and the final extension at 72°C for 10 min. The PCR products were purified by the Freeze 'N Squeeze DNA gel extraction spin columns and were sequenced on both strands on genetic analyzer (Prism 310, USA). The sequences were then edited by bioinformatics software Chromas. The homology of the 16S rRNA gene sequences was checked with the 16S rRNA gene sequences of other organisms using the BLASTN (http://www.ncbi.nih.gov/BLAST) algorithm.

Cytotoxicity, salt tolerance ability and antagonistic test of the isolated strain

Brine shrimp lethality bioassay is considered as a useful tool for preliminary assessment of toxicity [18]. Brine shrimp eggs were hatched in simulated sea water to get nauplii. In each of the eight vials, 10 ml simulated sea water was taken, containing 10 brine shrimp nauplii. For test sample, 10 ml of bacterial culture were taken in 10 Eppendorf tubes and were centrifuged at 7000 rpm for 10 min. Supernatants were collected which was further used as test sample and specific volumes of test sample was transferred with the help of a micropipette to the respective vials to get, final concentrations of 25 µl, 50 µl, 75 µl, 100 µl, 125 µl, 150 µl, 175 µl and 200 µl, respectively. For each concentration, one vial containing 10 ml simulated sea water and 10 shrimp nauplii were 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} values 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.
Salt tolerant ability of the isolated strain was determined by optimizing the growth rate of the isolate using (5-30)% NaCl in LB medium. Again in our experiment, we tested the antagonistic ability of the isolate against *Acetobactor* sp., *Pseudomonas* sp., *Brevibacillus* sp., *Xanthomonas campestris, Rhizobium* for *Vigna mungo* (RVM) and *Rhizobium* for *Cicer arietinum* (RCA) at a dose of 50 µl/disc through disk diffusion method.

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

The antibiotic sensitivity test of the isolated bacterium was done against cefuroxime, cefixime, cefotaxime, azithromycin, penicillin, amoxicillin, ceftazidime, gentamycin, ciprofloxacin through Kirby-Bauer [19] disk diffusion susceptibility test protocol and MIC was detected against gentamycin and amoxicillin through tube dilution methods [20].

Determination of growth of *Achromobacter* sp. in presence of lead and chromium

At first, the colony of bacterium was cultured in MS broth medium without metals at 37°C with160 rpm for 24 hours. 10 ml of the medium was centrifuged and bacterial suspension was prepared. In order to measure the growth of bacterium, 3ml of the bacterial suspension was transferred into each of the flask containing 20 ml of MS broth containing 100, 200, 300, 400, 500 and 600 µg/ml concentration of $[Pb(NO_3)_2]$ and $[Cr(NO_3)_3]$ solution. An extra sample without the addition of metals was prepared as control. Samples were incubated at 37°C for 9 days. The optimum density of the suspension was monitored during 24 hour of interval. The measurement of bacterial growth (optical density) was achieved at 660 nm wavelength using spectrophotometer.

Determination of lead and chromium tolerant rate by Atomic Absorption Spectrophotometer furnace (AAS furnace)

The atomic absorption spectrometry uses absorption of light of intrinsic wavelengths by atoms and absorption rate is determined by the atomic density. Firstly, isolated bacterial strain was cultured with MS medium in six flasks, each flask contains 99 ml MS medium and 1 ml bacterial liquid culture. 100, 200, 300, 400, 500 and 600 µg/ml concentrations of both metal, Pb and Cr were added, respectively into six conical flasks in aseptic condition. 2 ml sample from each flask was collected in centrifuge tubes and marked this tube for control sample. After every two days, 2ml sample was collected from every flask and store them at 4°C for further experiment. After 9 days, these samples were centrifuged at 12000 rpm for 5 minutes. Then, the supernatant was transferred in new tubes. Cell free supernatants were filtered through 0.45 µm nylon membranes prior to analysis. Finally, lead and chromium tolerant efficiency was determined through Atomic Absorption Spectrophotometer (Model: AA-6800).

RESULTS

Isolation and optimization of Pb and Cr tolerant bacterial strain

Lead and chromium tolerant bacterium were isolated by plating from the old bacterial suspension of the liquid medium into an agar solidified MS medium supplemented with 100 to 600 µg/ml concentrations of both metal and no growth were found at 600 µg/ml and more than concentration of the substrate. Isolated bacterium revealed maximum growth at pH 7.6 (**Fig. 1**) and in 35°C temperature (**Fig. 2**). The data was taken by measuring the OD at 660 nm after 24 hours of incubation.

Different carbon sources were used to know the ability of the strain to use the source for their growth and among used carbon sources isolated bacterium showed optimum growth performance on peptone (Fig. 3).

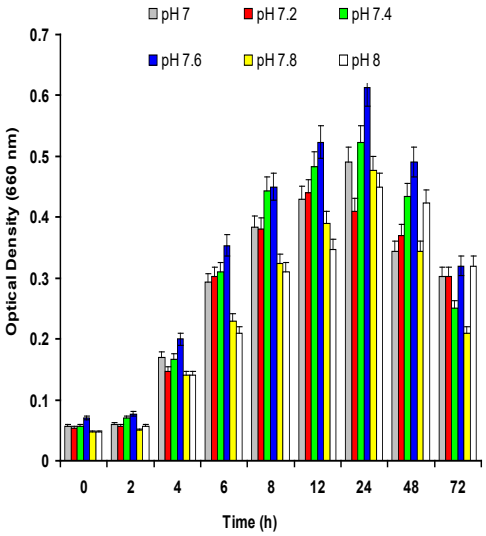

Fig. 1. Optimization of pH of the isolated bacterium.

Morphological and biochemical results

Morphological and biochemical characteristics indicated that the isolate was motile, gram negative, rod shaped bacterium, lactose fermenting and gas non-producing while methyl red, urease, starch hydrolysis, mannitol salt, citrate and catalase tests were positive.

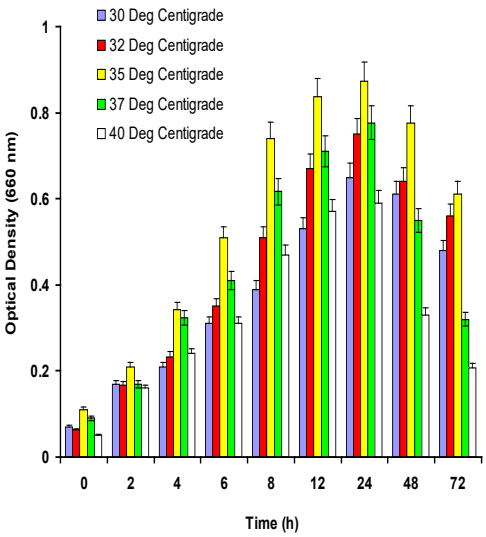

Fig. 2. Optimization of temperature supporting growth.

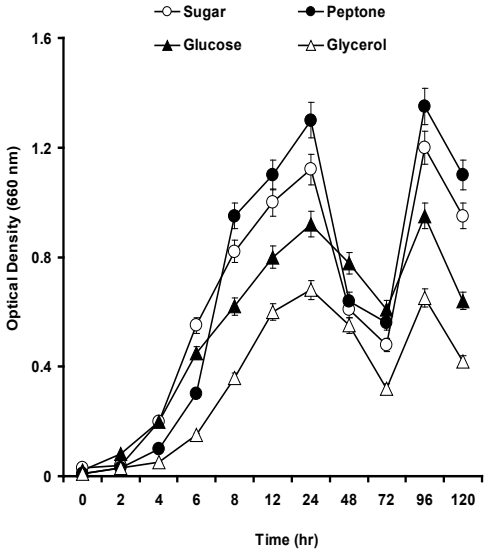

Fig. 3. Optimization of carbon sources utilization of the isolate.

16S rRNA sequencing result

After, the gene sequencing and editing of sequence it was checked with the 16S rRNA gene sequences of other organisms that had already been submitted to NCBI Gene bank database using the BLASTN (http://www.ncbi.nih.gov/BLAST) algorithm. From the Gene bank, several sequences were found for the isolate with significant identity and isolated bacterium showed 85% identity with *Achromobacter* sp. DNA quantification analysis and PCR band of isolated bacterium was shown in **Fig. 4** and **Fig. 5**, respectively.

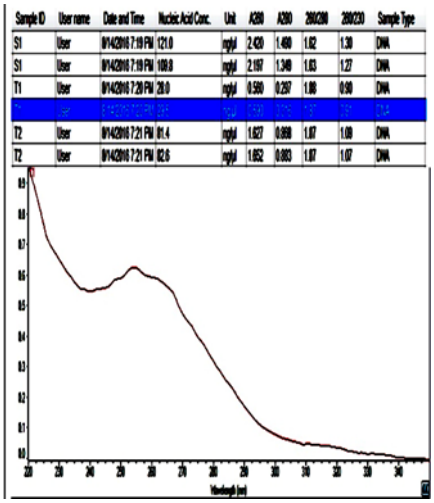

Fig. 4. DNA quantification analysis of the isolate.

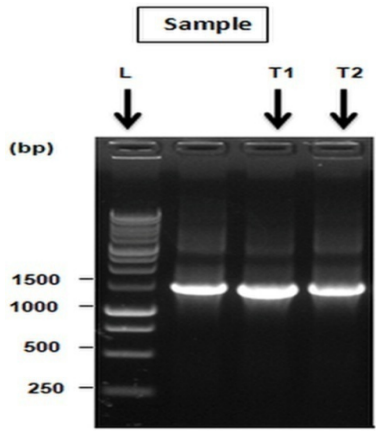

Fig. 5. 16S rRNA gene profiling of isolated bacterial species (T1) using 27F & 1492R primers. L denotes 1kb DNA ladder (marker)

Cytotoxicity, salinity and antagonistic test results

For *Achromobacter* sp., the LC_{50} value obtained from brine shrimp lethality bioassay was 93.01±0.21 µl/ml while the 95% confidence limits were 56.46 to 153.23 µl /ml and regression equation was Y =2.015x+1.034 after 48 hours of exposure. Probit mortality rate of isolated bacterium was shown in **Fig. 6**.

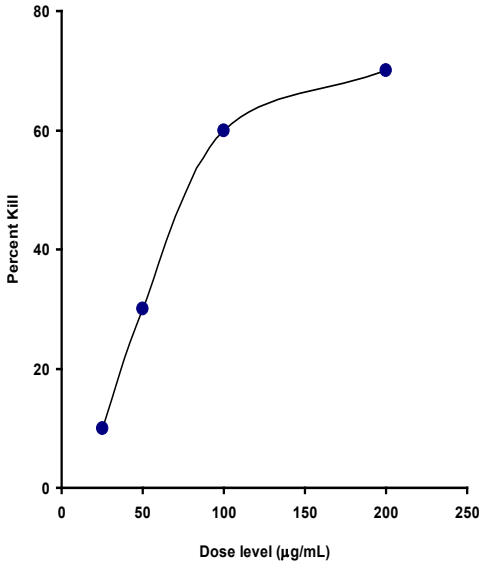

Fig. 6. Probit mortality rate of *Achromobacter* sp. against brine shrimp nauplii after 48 hours of exposure

The isolated *Achromobacter* sp. showed optimum growth (O.D.=0.688) after 18 hour in 10% NaCl supplemented LB medium (**Fig. 7**).

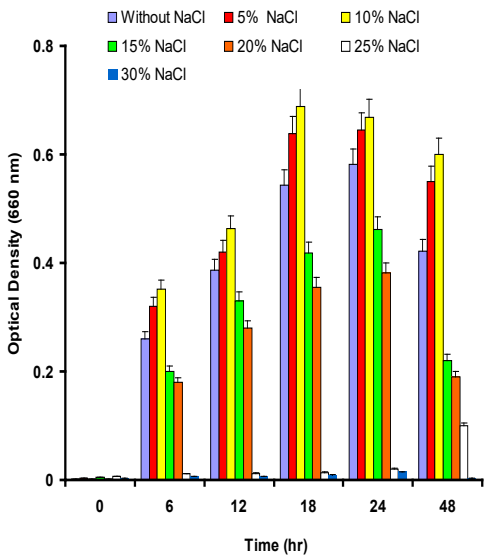

Fig. 7. Optimum growth rate of *Achromobacter* sp. in media with different NaCl concentrations.

In the current study, isolated *Achromobacter* sp. showed antagonistic effect against *Pseudomonas* sp., *Bravibacillus* sp. and *Acetobactor* sp. Among them *Pseudomonas* sp. showed intermediately resistant against the isolate. Fortunately, no antagonistic effect was found against *Rhizobium* for *Cicer arietinum* (RCA) and *Rhizobium* for *Vigna mungo* (RVM) and *Xanthomonas campestris.*

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

The result showed that, isolated bacterium was resistant against penicillin, cefuroxime, cefixime and cefotaxime. But susceptible to gentamycin and ciprofloxacin while intermediate resistant to remaining antibiotics (**Table 1**).

Table 1. Antibiotic sensitivity test used for the detection of the resistance pattern of isolated *Achromobacter* sp.

Name of Antibiotic	Zone of inhibition (mm)	Resistant pattern
Penicillin	9 mm	Resistant
Amoxicillin	15 mm	Intermediate resistant
Gentamycin	18 mm	Susceptible
Tetracycline	12 mm	Intermediate resistant
Ciprofloxacin	28 mm	Susceptible
Cefuroxime	6 mm	Resistant
Cefixime	6 mm	Resistant
Cefotaxime	8 mm	Resistant

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

The minimum inhibitory concentrations of the isolate were 12.5 µg/ml and 100 µg/ml against gentamycin and amoxicillin, respectively.

Growth measurements of *Achromobacter* sp. in presence of lead and chromium

The growth of *Achromobacter* strain in MS medium containing lead and chromium was shown in **Fig. 8** and **Fig. 9**. The growth of *Achromobacter* sp. strain was sharply decreased in the presence of increased metal concentrations in culture. The maximum OD in culture containing 100, 200, 300, 400, 500 and 600 µg/ml of lead were 0.115, 0.095, 0.078, 0.073, 0.060, 0.037 (**Table 2**) and for chromium were 0.127, 0.111, 0.088, 0.080, 0.071, 0.053, respectively, at day 9 of exposure (**Table 3**).

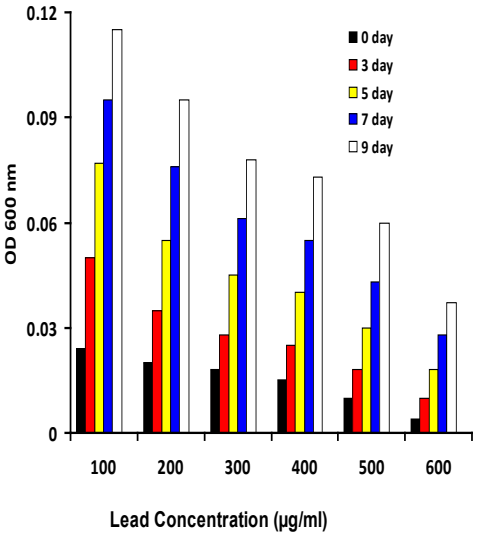

Fig. 8. Growth of *Achromobacter* sp. strain in different concentrations of lead

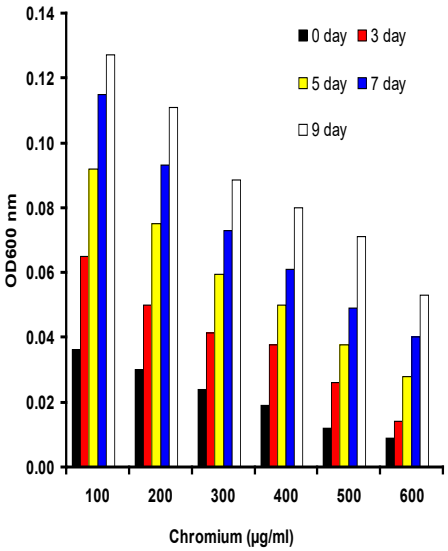

Fig. 9. Growth of *Achromobacter* sp. strain in different concentrations of chromium

Determination of lead and chromium tolerant rate by Atomic Absorption Spectrophotometer furnace (AAS) furnace

Lead and chromium tolerant rate were investigated under laboratory condition through AAS furnace. From the result it was clear that significant levels of Pb (**Fig. 10**) and Cr (**Fig. 11**) concentrations were decreased after treating with *Achromobacter* sp. On the other hand, the percentage of lead and chromium biosorption by *Achromobacter* sp. strain was increasing from the beginning. The result confirmed that after 9 days, *Achromobacter* sp. had lead removal efficiency of 93%, 91%, 83%, 76%, 65% and 48% at 100, 200, 300, 400, 500 and 600 µg/ml concentrations of lead, respectively (Table 4). While the rates were 96%, 94%, 87%, 81%, 70% and 55% at 100, 200, 300, 400, 500 and 600 µg/ml concentrations of chromium, respectively. Comparison of detoxification rate was also shown in **Fig. 12**.

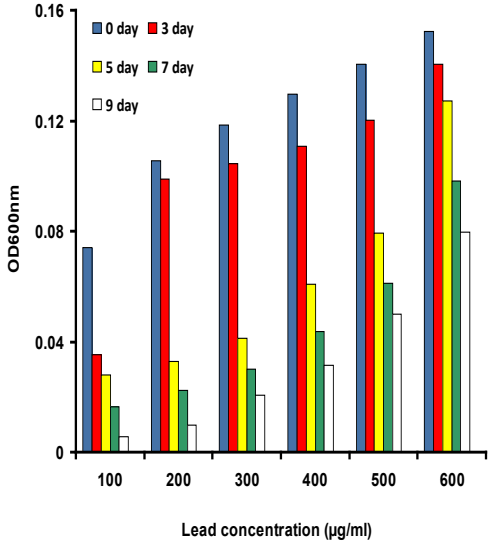

Fig. 10. Lead concentration from 0 to 9 day after treated with *Achromobacter* sp.

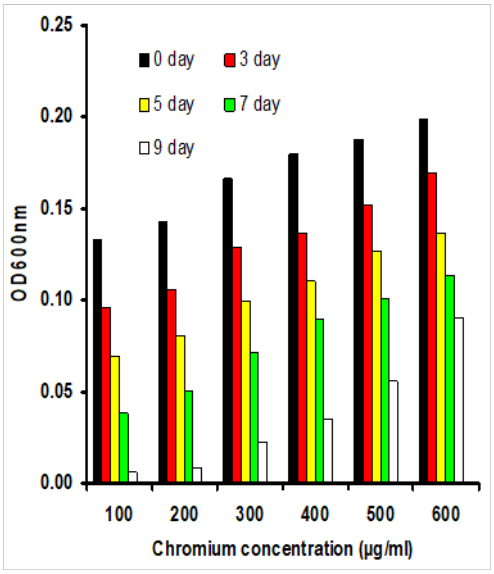

Fig. 11. Chromium concentration from 0 to 9 day after treated with *Achromobacter* sp.

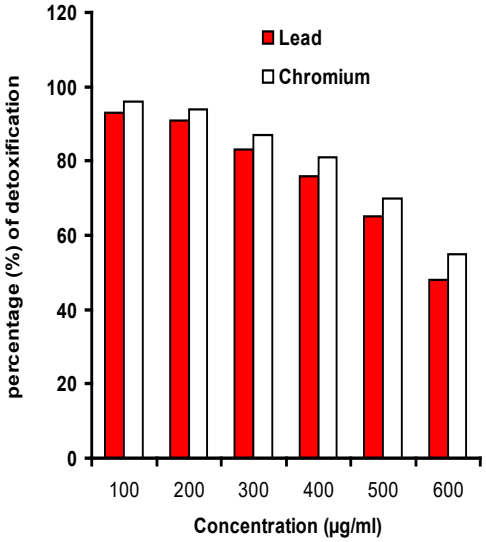

Fig. 12. Comparison of the detoxification rate of lead and chromium by *Achromobacter* sp.

DISCUSSION

Metal resistant microorganisms play an important role in the bioremediation of heavy metal contaminated soils and water [21]. Many bacterial species isolated from industrial zone had been shown to develop resistance to heavy metals [22, 23]. In present investigation, lead and chromium tolerant bacterial strain was isolated from tannery effluents and cultured on MS agar solidified medium supplemented with metals (lead and chromium) by using streak plate and pour plate methods. Morphological and biochemical tests indicated that isolated bacterium was fairly round shaped, gram-negative, motile, methyl red, citrate and catalase positive, lactose and mannitol fermenting and in TSI test, the isolate showed negative result for hydrogen sulphide. Isolated bacterium revealed maximum growth at pH 7.6, temperature 35°C and showed highest growth in case of peptone among four different carbon sources. Bergeys's Mannual of Determinative Bacteriology [24] indicated similar features of the genus *Achromobacter*. So, it was confirmed that morphological, biochemical and physiological outputs of the isolate had a similarity with the referred data.

Comparison of the bacterial 16S rRNA gene sequence has appeared as a preferred genetic technique [25]. In the present investigation, sequencing result of 16S rRNA gene confirmed that the bacterial isolate showed 85% significant alignments with *Achromobacter* sp. *Achromobacter* was proposed by Yabuuchi and Yano [26] as a genus and included in the family *Alcaligenaceae.*

It was reported from several studies that there was an interrelationship between heavy metal and antibiotic resistance in bacteria [27]. *Achromobacter* sp. was also identified as opportunistic human pathogens from previous study [28]. Hence, resistance pattern of the isolate was checked to control sudden pathogenic catastrophe. Mgbemena et al [29] found that heavy metal resistant bacteria isolates; *Pseudomonas* sp., *Aeromonas* sp., *Bacillus* sp. *Micrococcus* sp., *Proteus* sp. and *E. coli* exhibited high tolerance to gentamycin (77.7%), rifampicin (66%), and ofloxacin (57.3%).

It was noteworthy that multi drug resistance *Achromobacter* sp. was isolated from outdoor and indoor environments [30]. In our study, isolated *Achromobacter* sp. was susceptible to gentamycin and ciprofloxacin while it was resistant against penicillin, cefuroxime, cefixime and cefotaxime. Our result had similarity with previous data. Again, the MIC value of gentamycin and amoxicillin against the isolated strain was found 12.5 µg/ml and 100 µg/ml, respectively.

LC_{50} value of different fungal toxins such as gliotoxin, ochratoxin A was found 3.5 µg/ml, 10.1 µg/ml, respectively after 16 hour against *Artemia salina* [31]. In our investigation, LC_{50} result of *Achromobacter* sp. was evaluated and the value was 93.01±0.21 µl/ml after 48 hours. From the above data, it was clear that the bacterial isolate had a little cytotoxic effect against *A. salina* compared to the referred data. In addition, *Achromobacter* sp. showed 10% salt tolerant ability and it could be considered as a high salt tolerant bacterium because most of the halophilic bacteria have a strict Na demand [32]. In antagonistic study, *Achromobacter* sp. showed antagonistic effect against all three pathogenic bacteria at a dose of 50 µl/disc but no antagonistic effect was found against Rhizobacteria such as *Rhizobium* for *Cicer arietinum* (RCA) and *Rhizobium* for *Vigna mungo* (RVM). So, the isolated *Achromobacter* sp. would not be risky for any Rhizobacteria at field levels in future application.

Pradyut et al. [33] published article on isolation and identification of heavy metal (lead, zinc and copper) resistant bacteria from oil field soil. In their study, three strains were identified as *Klebsellia* sp., *Staphylococcus* sp., and *Bacillus* sp. Among all, *Klebsellia* sp. exhibited highest resistance to lead and zinc at concentration of 800 µg/ml. Marzan et al. [34] also identified naturally occurring bacteria capable of reducing and detoxifying heavy metals (Chromium, Cadmium and Lead) from tannery effluent. Three isolates were identified up to genus level based on their morphological, cultural, physiological and biochemical characteristics as *Gemella* sp., *Micrococcus* sp. and *Hafnia* sp. Among them *Gemella* sp. and *Micrococcus* sp. showed resistance to lead (Pb), chromium (Cr) and cadmium (Cd). Bandela et al. [35] also reported Nickel (Ni) tolerant ability of *Achromobacter* sp.

In our present study, six different concentrations of lead and chromium were used (100, 200, 300, 400, 500 and 600 µg/ml). But the highest bacterial growths (O.D.) were 0.115 and 0.127 at 100 µg/ml for lead and chromium, respectively after 9 days. Gradual increase of the bacterial growth was observed from day 0 to day 9, which confirmed that isolated *Achromobacter* sp. utilized these metal salts as their carbon sources from MS liquid medium. This was the indication of the possible detoxification of lead and chromium by the isolated *Achromobacter* strain and detoxification rate of the metals were determined by using AAS furnace which showed significant changes in the values of the lead and chromium efficacy. Highest detoxification rates were 91% of lead and 96% of chromium at 100 µg/ml concentration. Thus, *Achromobacter* sp. proved to be a potential biosorbent for the removal of lead and chromium from tannery effluents.

CONCLUSION

The overall finding suggests that using of native isolate for tolerant of heavy metals is promising treatment option for the removal of liquid wastes from contaminated soil or wastewater as biodegradation observed only in the presence of acclimated microorganism under aerobic conditions. The isolated bacterial strain, *Achromobacter* sp. showed significant growth in the presence of different concentrations of lead and chromium and can be used to detoxify lead and chromium at field level by the process of bioremediation.

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