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Isolation of a lead tolerant novel bacterial species, *Achromobacte*r sp. TL-3: Assessment of bioflocculant activity

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Lead is one of the four heavy metals that has a profound damaging effects on human health. In the recent past there has been an increasing global concern for development of sustainable bioremediation technologies for detoxification of lead contaminant. Present investigation highlights for lead biosorption by a newly isolated novel bacterial species; *Achromobacter* sp. TL-3 strain, isolated from activated sludge samples contaminated with heavy metals (collected from oil refinery, Assam, North-East India). For isolation of lead tolerant bacteria, sludge samples were enriched into Luria Broth medium supplemented separately with a range of lead nitrate; 250, 500, 750, 1000, 1250 and 1500 ppm respectively. The bacterial consortium that could tolerate 1500 ppm of lead nitrate was selected further for purification of lead tolerant bacterial isolates. Purified lead tolerant bacterial isolates were then eventually inoculated into production medium supplemented with ethanol and glycerol as carbon and energy source to investigate for bioflocculant production. Bioflocculant production was estimated by monitoring the potential of lead tolerant bacterial isolate to flocculate Kaolin clay in presence of 1% $CaCl_2$. Compared to other isolates, TL-3 isolate demonstrated for maximum bioflocculant activity of 95% and thus was identified based on 16S rRNA gene sequence analysis. TL3 isolate revealed maximum homology (98%) with *Achromobacter* sp. and thus designated as *Achromobacter* sp. TL-3. Bioflocculant activity of TL-3 isolate was correlated with the change in *p*H and growth. *Achromobacter* sp. TL-3 has significant potential for lead biosorption and can be effectively employed for detoxification of lead contaminated waste effluents/waste waters.

Keywords: *Achoromobacter*, Bioflocculant, Biosorption, Kaolin clay, Pb, Tolerance

Heavy metal bioaccumulation in the environment has become a major concern owing to their toxicity and detrimental impact on human health. Moreover, in the recent past due to the heavy metal contamination, the prospective ecological hazards and damage to flora and fauna are rising at an alarming rate in the environment[1,2]. Many industries discharge large amount of waste water into the environment that are contaminated with heavy metals. Heavy metals even in trace amount are toxic to the environment and human health[3].

Among heavy metals, lead (II) is as one of the most toxic heavy metals as it is associated with many health hazards[4]. Lead is one of the major pollutants found in water, soil and air having no biological function[5-7]. It has been reported that lead impacts in decreasing the efficiency of the ATPase pump as well as protein kinase activity and negatively affects the concentration of various essential cations such as Na, K and Ca, which are required to maintain the concentration gradient of cells. Another adverse effect of lead is the increase in formation of inclusion bodies in the cell. These inclusion bodies in turn translocate metals into the nuclei of the cells and thus alter the gene expression[8]. Lead contamination and its infilteration in the biological community is known to be toxic to human health and cause mental retardation in children[9].

Several conventional methods have been employed for the treatment of such heavy metals. Some of these methods include ion exchange, reverse osmosis, adsorption, solvent extraction and chemical precipitation[10]. However, the conventional methods employ a number of non-regenerable materials which in turn increases the cost of these processes and are not environmental friendly as these processes generate substantial secondary waste[3]. In contrast, biosorption or adsorption by biological sources has

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become a preferred alternative for heavy metal treatment as these processes are cost-effective and environmental friendly.

The process of bioflocculation or biosorption is a complex one. It is the adsorption of any compound by biological materials through metabolically dependent or independent uptake mechanisms[11]. The main principle of biosorption is based on the metal binding abilities of biological material[13]. It has been observed that a number of functional groups such as carboxyl, sulphydryl, amino and hydroxyl, present in bacteria aid in the adsorption of the metal ions from the wastes[14,15]. Over the time microorganisms are known to have developed resistance to toxic heavy metals by mechanisms such as enzymatic detoxification of the metal to a less toxic form and reduction in metal sensitivity of cellular targets, intracellular sequestration of the metal by protein binding[16-18], extracellular sequestration by exopolysaccharides and cell surface biosorption by negatively charged groups. Intracellular sequestration and homeostasis in bacteria is known to be induced by small cystein rich metal induced and metal binding proteins designated as metallothioneins[19-23].

The biosorption capacity of microbes is governed by factors such as the physico-chemical properties of the heavy metal in question and the biomass characteristic of the biological material. Besides these, *p*H, presence of other ions and charged compounds have also profound effect on the biosorption capacity of the microbes[24]. Some microbial biomass has potential to tolerate and retain substantial quantities of heavy metal ions[25]. Tolerance to heavy metals is postulated to be due to stress induced conditions of these microbes. Microorganisms, due to their minute size have a very high surface area to volume ratio. This provides with a comparatively larger contact area for interaction with their macro-environment[13].

Tapping any of these biological mechanisms has significant potential for development of environmental friendly and cost effective process for heavy metal decontamination. Present investigation highlights on screening and isolation of microbes having good potential for lead tolerance including investigations on their bioflocculation production potential for bioflocculant assisted biosorption of lead.

Materials and Methods

Sample collection and analysis—Activated sludge and effluent treatment water samples were collected from effluent treatment plants of oil refining sector, from three refineries in Assam; Guwahati Refinery, Numaligarh Refinery and Bongaigaon Refinery. Activated sludge and effluent samples were analysed for the presence of 15 metals; (Zn, Co, Cr, Cd, Ca, Mg, Mn, Cu, Pb, Ni, Cr, Fe, Al, V, Na and K) by Atomic Absorption Spectroscopy (AAS-7000, Shimadzu, Japan). Metal analysis results on observation demonstrated that the activated sludge samples demonstrated comparatively higher concentrations of heavy metal contaminants and thus were selected for further experimental investigation. For convenience these samples were designated as GB4 (Guwahati Refinery Activated Sludge Sample), NB3 (Numaligarh Refinery Activated Sludge Sample) and BB3 (Bongaigaon Refinery Activated Sludge Sample), based on the source of sample collection site. All these samples were diluted in sterile distilled water and used for further enrichment studies for isolation of lead tolerant microbes.

Metal stock solution—For isolation of lead tolerant microbes, the activated sludge samples were enriched into lead supplemented nutrient medium. Lead was used in the form of lead nitrate. Stock solution of lead nitrate was prepared to achieve the final concentration of 10,000 ppm.

Isolation of the bacterial strains, production medium preparation and growth conditions—Activated sludge samples of each refinery demonstrated for comparatively higher concentration of lead contaminant and thus were selected for enrichment of lead tolerant microbes. Luria Broth (LB) medium was used as growth medium. Enriched samples were incubated at 37 °C, at 150 rpm till substantial microbial growth was observed. Enrichment was repeated for three generation. Screening for lead tolerant bacteria was carried out by inoculating the enriched culture in LB media supplemented separately with a range of lead concentrations (in the form of lead nitrate); 250, 500, 750, 1000 and 1500 ppm. Following three enrichment cycles, the cultures were purified by plating on lead supplemented LB and Agar (LA) medium and incubated at 37 °C, overnight. Isolated colonies were purified and characterised on the basis of gram staining and colony morphology. All the purified isolates were stored at 4 °C for future experimental use. The purified lead tolerant isolates were investigated for bioflocculant production potential by employing kaolin clay assay study. For

this, the lead tolerant isolates were grown in production media[18] (0.5% yeast extract, 0.5% peptone, 2% ethanol, 1% glycerol, 0.05% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.2% NaCl and 0.2% $CaCO_3$), for 5 days at 37 °C, 150 rpm. Subsequently, lead tolerant isolates were investigated for bioflocculant production in presence of 1500 ppm of lead nitrate. The isolate showing maximum bioflocculant activity, was selected for identification based on 16S rRNA gene sequence analysis and change in *p*H profile analysis with respect to biflocculant activity.

Bioflocculation activity Assay—The bioflocculation production potential of the lead tolerating bacterial isolates for removing heavy metals (flocculation activity), was assessed by following the protocol as mentioned previously with little modifications[21,26,27]. Kaolin Clay was used as the suspended solid for monitoring the bioflocculant activity. The cell free culture supernatant (1.5 mL of culture was centrifuged at 6000 rpm for 30 min)[28] was used as crude bioflocculant. Bacterial supernatant (1 mL) was added to 9 mL of kaolin suspension (5g/L) in the presence of 3 mL 1% $CaCl_2$. The mixture was vortexed for 30 sec and then left undisturbed for 5 min. The OD (optical density) of the clarifying solution was measured using a UV visible spectrophotometer (Shimadzu UV-2450, Singapore) at 550 nm. A control experiment was set up using the same protocol, except that the crude bioflocculant was replaced with un-inoculated culture media. The flocculating activity was calculated by following formula:

$$\textit{Flocculating}\ \ \% = \frac{A - B}{A} \times 100$$

where, A is the optical density of the control experiment at 550 nm and B is the optical density of the sample experiment.

Morphological characterization, 16S rRNA gene sequencing and phylogenetic analysis—Gram staining was performed as per the manufacturer's instructions using a gram staining kit, (Hi-Media, India). Morphological examination was performed using a bright field microscope (Olympus, Japan). RNA free genomic DNA was isolated by employing kit (Real Genomics, RBC, India). Presence of genomic DNA was confirmed by 0.8% agarose gel electrophoresis. 16S rRNA gene amplification of the RNA free genomic DNA was amplified by PCR in a thermal cycler (Eppendorf, Germany) by employing the universal specific forward and reverse oligonucleotide PCR primers (Sigma, USA); 27F (5′-AGATTGATCMTGGCTAGGGA-3) and 1492R (5′- TACGGYTACCTTGTTACGCTT-3′), as described earlier[29]. PCR reaction mixture (total volume 50 µL) consisted of 2.0 µL of forward and reverse primer each (20 pmol μL^{-1}), 1.0 µL of dNTP (10 mM each), 2.0 µL of template (11 ng μL^{-1}), 5.0 µL 10X Buffer, 0.6 µL BSA (10 mg mL^{-1}), 0.8 µL Taq polymerase (5 unit μL^{-1}) and 36.6 µL molecular grade water. The temperature profile used for PCR amplification was as follow; initial denaturation at 95 °C for 10 min followed by 30 cycles of denaturation at 95 °C for 1 min; annealing at 56 °C for 1 min and extension at 72 °C for 1 min 30 sec; final extension at 72 °C for 10 min. The PCR products were analysed by electrophoresis on 1% agarose gel. PCR amplified 16S rRNA gene product was purified using gel extraction kit (Real Genomics, RBC, India). The purified PCR amplified DNA was processed for 16S rRNA gene sequence analysis (Macrogen, Korea).

16S rRNA gene sequence 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. Closely related sequences were retrieved and aligned using Clustal W program. Phylogenetic tree was constructed from the evolutionary distance matrix calculated through the neighbor-joining method[30]. Neighbor-joining analysis was performed with the program MEGA 5. Confidence in the resultant tree topology was evaluated by re-sampling 100 bootstrap trees[31,32].

Analytical methods—The lead tolerant bacterial isolate showing maximum bioflocculation activity was subjected to growth profile studies and were correlated with the change in *p*H and Kaolin Activity as mentioned above[26]. For this 1% of the bacterial isolate was inoculated in production media as mentioned previously and incubated at 37 °C at 150 rpm for 5 days. The samples were collected at every 24 h under aseptic conditions. The optical density of the culture was measured at 600 nm using a UV visible spectrophotomer (Shimadzu UV-2450, Singapore). Only the media (without inoculum) was used as blank. Test sample was analyzed (after calibration of the *p*H meter) for determination of *p*H profile with respect to growth and bioflocculation activity to correlate the bioflocculation activity with the growth profile

and change in *p*H. Lead concentrations were determined by using Atomic Absorbance Spectrometer (AES Laboratories (P) Ltd., Noida).

Nucleotide sequence accession numbers—The 16S rRNA gene sequence of selected lead tolerant bacterial isolate was deposited in the NCBI/EMBL nucleotide sequence database under the accession number; KC285780.

Results

Sample characteristics and morphological characterization of lead tolerant isolates—Activated sludge samples from each refinery were chosen for isolation and screening of lead tolerant microbes, based on the presence of comparatively higher lead contaminants in these samples (Table 1). GB4 activated sludge samples were dark brown in colour that had some visible suspended solids, whereas NB3 and BB3 activated sludge samples were semi solid and black in colour.

After multiple cycles of enrichment, purification and screening, a total of eight lead tolerant isolates (tolerant to 1500 ppm of lead nitrate) were isolated. These isolates were designated as; TL-1, TL-2, TL-3, TL-4, TL-5, TL-6, TL-7 and TL-8, respectively. These isolates had potential to tolerate as high as 1500 ppm of lead nitrate and demonstrated substantial growth at this concentration.

Morphological characterization of the lead tolerant isolates was carried out on the basis of gram staining and colony morphology. Microscopic observation of these isolates revealed that the cells were gram negative and rod shaped. TL-1 isolate was convex and dirty brown glossy in colour with entire margin and demonstrated its optimum growth after 24 h incubation; TL-2 isolate had flat dirty brown colour with entire margin and its optimum growth was observed after 24 h incubation; TL-3 isolate was dark brown glossy colour and comparatively slow to grow (optimum growth was observed after 36 h of incubation); TL-4 isolate had raised dirty white colonies and could grow fast (optimum growth observed after 24 h incubation); TL-5 isolate was found to have flat dull white colonies with undulate margin and its growth was observed after 48 h of incubation; TL-6 isolate was very small dull white grew well after 24 h incubation; TL-7 isolate was found to and its growth was observed after 48 h of incubation; TL-6 isolate was very small dull white grew well after 24 h incubation; TL-7 isolate was found to have colony characteristics same as that of TL-5 (hence this isolate was not considered for further investigations); TL-8 isolate (re designated as TL-7) was very small, convex in shape, pale white glossy in colour and its growth and revealed optimum growth after 24 h incubation.

Table 1—Concentration of lead contamination in activated sludge and effluent samples collected from oil refinery (Guwahati, Assam, North-East India)

S.No.	Sample ID	Lead concentration (ppm)
1	GB 1(Treated effluent)	0.01
2	GB 2(Untreated effluent)	0.01
3	GB 3(Chemical sludge)	0.03
4	GB 4 (Activated Sludge)	0.45
5	NB 1(Treated effluent)	0.01
6	NB 2(Untreated effluent)	0.01
7	NB 3 (Activated Sludge)	30.86
8	BB 1(Treated effluent)	0.01
9	BB 2(Untreated effluent)	6.97
10	BB 3 (Activated Sludge)	28.86

Bioflocculation activity of lead tolerant isolates—The purified cultures were inoculated in standard production media for monitoring their bioflocculant production potential. Flocculation activity of the purified isolates was assayed by analysing their ability to flocculate Kaolin clay (Fig.1). Bioflocculation activity was estimated in terms of percentage activity (Fig.1)

Three out of seven bacterial isolates(TL-1, TL-3 and TL-4) revealed high flocculation activity (Fig.1), whereas the others demonstrated comparatively lower flocculation activity. Maximum bioflocculation activity (95%) was recorded for TL-3 lead tolerant isolate. Other isolates revealed bioflocculation activity in the following order; TL-4> TL-1> TL-5> TL-6> TL-7> TL-2, showing 92.6, 91.4, 78.2, 73.6, 48.1 and 47% bioflocculation activity, respectively. Since TL-3 isolate demonstrated maximum bioflocculation activity (95%), it was selected further for identification based on 16S rRNA gene sequence analysis and other analytical experimental investigations.

Identification and phylogenetic analysis of lead tolerant TL-3 bacterial isolate based on 16S rRNA gene sequence analysis—Based on multiple sequence alignment performed by Clustal W and phylogenetic analysis (using Mega 5) of the 1000 bp length 16S rRNA gene sequence (Fig. 2) of TL-3 isolate demonstrated maximum homology of 98% with the genus *Achromobacter* and therefore was identified as *Achromobacter sp.* However, the isolate did not show

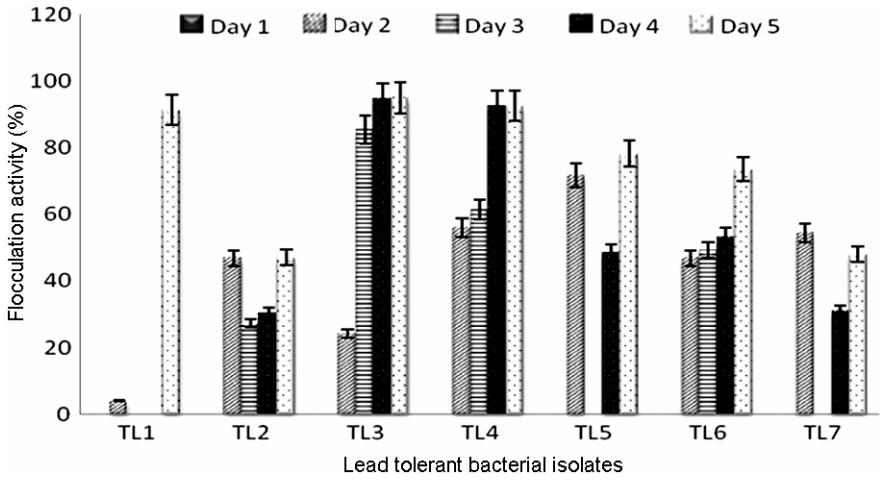

Fig. 1—Bioflocculation activity assay of the lead tolerant bacterial isolates.

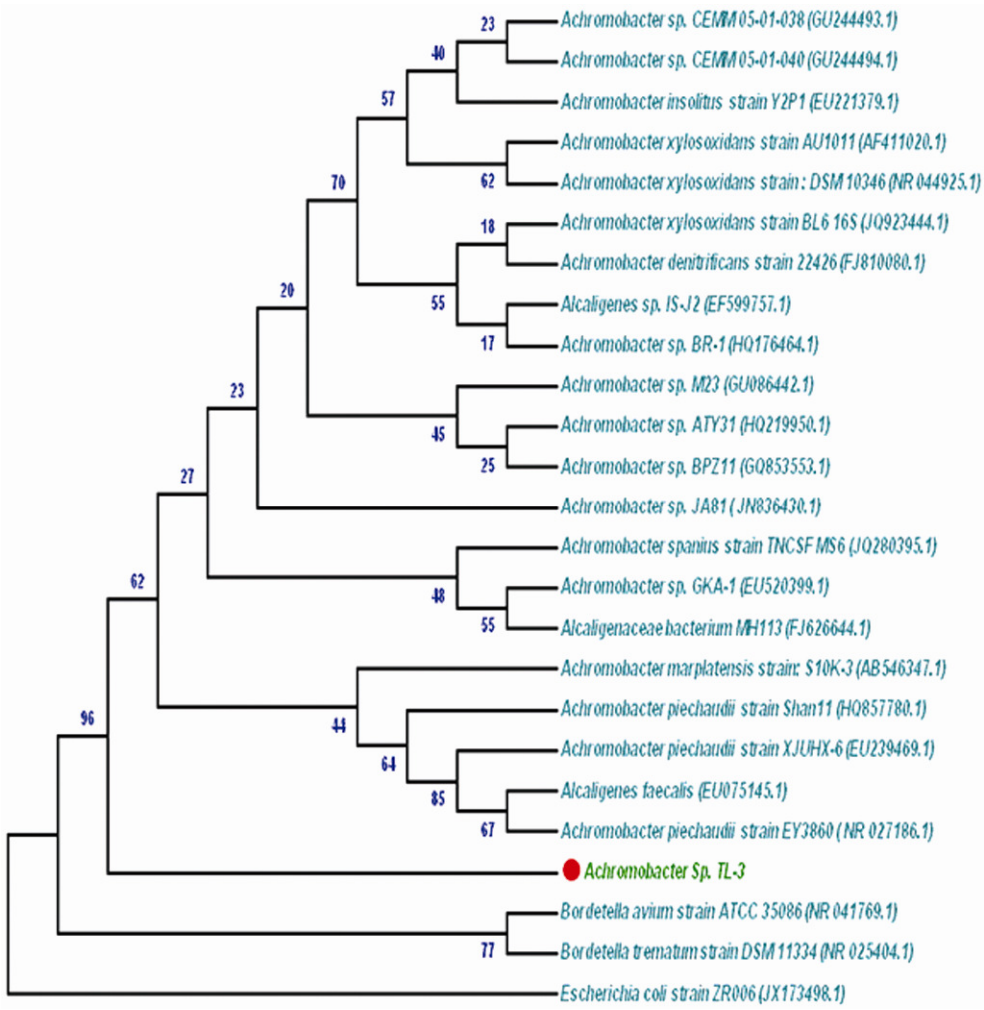

Fig. 2—Neighbor-joining tree showing the phylogenetic position of lead tolerant bacterial isolate, *Achromobacte*r sp TL-3, identified based on the 16S rDNA sequences. The numbers at the nodes indicate the level of bootstrap support based on the neighbor-joining analysis of 100 replicates. The phylogenetic tree represents only the topology. Accession numbers are given in parenthesis

any significant homology with specific species of the genus. Accordingly the lead tolerant bacterial isolate TL-3 was designated as *Achromobacter sp. TL-3.*

Bioflocculation activity of Achromobacter sp TL-3 with respect to its growth and change in final pH—Achromobacter sp TL-3 was further investigated for its growth profile and change in final *p*H (Fig. 3). Growth profile and final *p*H change of TL-3 isolate were compared with its bioflocculant activity. Experimental investigations demonstrated for sharp rise in bioflocculation activity of TL-3 on the third day, when the bacterial isolate entered its early stationary phase (Fig. 3). Eventually the flocculating activity of TL-3 isolate reached a maximum of 95% in its late stationary phase, on the 5th day.

With increase in bioflocculation activity of TL-3 isolate over the period of five days (Fig. 3), the culture *p*H dropped down gradually from 7 to 5.2 (Fig. 3).

Discussion

In recent years, heavy metal pollution of the environment has become one of the major global concerns. This is mainly due to rapid development of industries especially of mining, metallurgy, surface finishing, iron and steel, electroplating, metal surface treating industries including oil sector activities. Considerable amount of heavy metals are being released by these industries which are discharged in to the environment, causing a serious threat to human health. Heavy metal decontamination of aquatic and terrestrial environment has become global challenge. Lead is one of the most extensive environmental contaminant. Presently several chemical methods are being employed for treatment of heavy metal contaminated aqueous waste. However, these processes

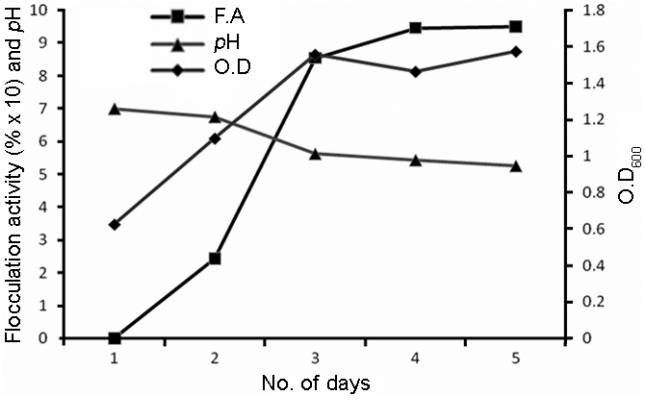

Fig. 3—Bioflocculation activity of lead tolerant *Achromobacter* sp TL-3, with respect to its growth and change in final *p*H

are expensive and not environmentally benign as they release substantial amount of sludge that requires treatment prior to discharge. In contrast, biosorption of heavy metals by microbes is cost effective and environmental friendly. Biosorption is a mechanism that involves adsorption of metal by biological mechanism by certain microbes. These microbes have various mechanisms that render them resistance, which could be of different orders[13]. Reports concerning lead resistant microbes are scarce and require extensive investigation. Hence, attempts were made to isolate robust microbe(s) having potential for resistance to lead.

Enrichment of activated sludge samples of oil refineries led to the isolation of bacteria having significant potential for tolerance to higher lead concentrations, as high as 1500 ppm lead nitrate. Metal stock solution was prepared by using nitrate salt of the metal considered, (lead). Formation water has a high concentration of sulphates and nitrates which revealed that the microbes present in these samples could able to grow in the presence of sulphates and nitrates[33]. A total of eight isolates demonstrated for tolerance to 1500 ppm of lead nitrate. Lead tolerance capability of these isolates is significantly higher than the lead tolerance potential of *Pseudomonas* sp. CH_8 microbes as reported previously by Lin and Harichand[26]. *Pseudomonas* sp. CH_8 could tolerate 1000 ppm lead[26].

Only few reports are reported for bioflocculant assisted biosorption of lead by microbes. *Pseudomonas* sp. CH_8 has been reported for having capability for bioflocculant assisted biosorption of lead[26]. In order to investigate for bioflocculant production potential, all the lead tolerant bacterial isolates were monitored for their bioflocculation activity by inoculating in standard production media supplemented with 1500 ppm of lead nitrate, as reported previously[26]. All these isolates demonstrated a certain level of bioflocculation activity. These results indicated that optimization of growth conditions might be required to yield higher levels of bioflocculation activity. The bioflocculating activity was determined with the use of kaolin clay as the suspended particles in presence of Pb^{2+} (1500 ppm). The results on observation revealed that the bacterial isolate, TL-3, could demonstrate maximum (95%) bioflocculation activity. Bioflocculation activity of TL-3 isolate could most probably be involved in biosorption of lead, as no bioflocculation activity was observed in the control medium. Based on 16S rRNA

gene sequence analysis, TL-3 was identified as *Achromobacter* sp. TL-3. Lead tolerance and bioflocculation activity of *Achromobacter* sp. TL-3 isolate were significantly higher than the previously reported bioflocculation activity of lead tolerant bacterial isolate[26]. Till date, this is the first report that highlights for capability of a *Achromobacter* sp. to produce bioflocculant in presence of lead, one of the extensive environmental contaminant. Lead biosorption results revealed that *Achromobacter* sp. TL-3 could remove up to 80% of lead. Lead removal potential of TL-3 is close to the lead removal potential of *Pseudomonas* sp. CH8[16]. These results demonstrate that *Achromobacter* sp. TL-3 has significant lead removal capacity and this activity can further be enhanced by optimizing its process parameters.

Further investigation on growth profile and change in *p*H with respect to bioflocculant activity revealed that maximal activity of TL-3 was observed in the late stationary phase. This indicates that the bioflocculant being produced by this microbe is probably not involved in any other regulatory function and could be a secondary metabolite produced, during its growth[34]. The process of lead bioflocculation in *Achromobacter* sp. TL-3, is most probably a passive process and not metabolically regulated[12]. Initial *p*H of the production media is reported to be a very important factor in the bioflocculation process[3,14] and determines the electric charge on the cells, which in turn plays role in the biosorption characteristic of the bacterial isolate[28]. *p*H affects the presence of charged groups on the cell which then binds to the heavy metal ions[35] and impacts on the physico-chemistry and hydrolysis of the metal[36,37]. With increase in bioflocculation activity, final *p*H of the TL-3 culture dropped down gradually from 7 to 5.6 after 36 h and remained constant thereafter. This result is in consistence with the finding by Yuyan *et al.*[38] and Dermlim *et al*[39]. Downfall of *p*H could be attributed either to the production of acidic metabolites released during lead biosorption by the *Achromobacter* sp. TL-3 isolate or to the acidic nature of bioflocculant produced by *Achromobacter* sp.TL-3 isolate[39].

This is the first report that highlighted for isolation of a novel sp. of *Achromobacter* genus, which has high lead biosorption potential. These studies also reveal that lead biosorption by *Achromobacter* sp. TL-3, most probably be assisted by bioflocculant produced by this microbe in presence of lead. Further studies on optimization of process parameters are in progress for enhancement of bioflocculant activity of *Achromobacter* sp. TL-3, that would aid in increasing its lead biosorption potential.

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