

Original Research Article

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Luteimonas aestuarii SA13A as a Novel Chromium Reducing Strain isolated from Tannery Effluent

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ABSTRACT

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A novel bacterium SA13A was isolated from tannery effluent. Its morphology, physiology and 16S rRNA gene sequence were characterized. Bacterium SA13A was found to be gram negative, rod shaped and produces yellowish pigment on nutrient agar plates. The 16S rRNA gene sequence similarity indicated that isolate SA13A is associated with genus *Luteimonas* (99%). This isolate has been found to reduce 100% of hexavalent chromium Cr (VI) (100 mg L^{-1}) 100% in 16 h. Growth conditions were optimized for Cr (VI) reduction. Maximum reduction was observed at a temperature of 37°C and pH 8.0. Additionally, *Luteimonas aestuarii* SA13A showed resistance against various heavy metals like Cr^{+6} , Cr^{+3} , Cu^{+2} , Zn^{+2} , Co^{+2} , Ni^{+2} and Cd^{+2} . Hence, *Luteimonas aestuarii* SA13A could be used as potent Cr (VI) reducing strain as well as significant bioremediator in heavy metal contaminated sites.

Introduction

Industrialization is hallmark of civilization but environmental degradation happening due to this is becoming a matter of great concern. Industrial emissions unfavorably affect the environment, leading to large-scale worldwide destruction of agricultural land and water bodies (Poopal *et al.*, 2009; Alam *et al.*, 2012). Various toxic substances released during industrial processes accumulate in the environment and food chains, thereby upsetting ecosystems and biological processes in many organisms. Chromium (Cr) is one of the heavy metal that is employed widely in numerous industrial processes, including chrome leather tanning, chrome plating, ceramics, dyes, paints and pigments manufacturing, textile processing and metal

finishing (Thacker *et al.*, 2006; Cheung and Gu, 2007; Desai *et al.*, 2008; Masood and Malik, 2011). Leather processing industry is one of the major industries utilizing chromium in the form of chrome liquor or chrome powder. Residual chromium is thus discharged in the form of solid as well as liquid effluent into land and water bodies.

Chromium exists in various oxidation states with valencies ranging from -2 to +6 but the most persistent forms are hexavalent (Cr^{6+}) and trivalent (Cr^{3+}) forms. Hexavalent chromium is the most toxic form, mainly available as oxyanions, whereas trivalent chromium which is hundred-fold less toxic, less soluble, less mobile and is mostly found

as oxides, hydroxides or sulfates (Cervantes *et al.*, 2001; Patra *et al.*, 2010). Hexavalent chromium is a strong oxidizing agent, mutagen and teratogen (Cheung and Gu, 2007; Costa and Klein, 2006). Its oxyanionic form (CrO_4^{2-}) is analogous in structure to sulfate and phosphate ions which readily permeates through bacterial and eukaryotic cells resulting in intracellular reduction finally leading to chromate-induced toxicity (Cheung and Gu, 2007; Patra *et al.*, 2010; Asatiani *et al.*, 2004). The presence of chromate in the environment inhibits most microorganisms, but it also promotes the selection of metal resistant bacteria (Alam *et al.*, 2012; Mondaca *et al.*, 1998).

In Kanpur, about 400 leather processing industries at Jajmau, discharge both liquid as well as solid wastes into canals and rivers with residual chromium. This causes chromium contamination not only in the water bodies but also accumulates in aquatic organisms, lands, vegetables, farm products and crops thereby posing a great threat to environment and human health. The most direct and severe effect comes out with leather-cut wastes, which are conventionally being processed into feed ingredient (as a protein source). This chromium content thus acts as major source for its migration into the food chain.

Chromium does not degrade completely but can be transformed or removed either through adsorption/accumulation or by physico-chemical treatments. Large amount of chemicals as well as energy is required during these processes, making them unsuitable for execution on a large scale (Camargo *et al.*, 2003). Chromium resistant bacteria offer an economical and eco-friendly alternative method for chromate detoxification along with its bioremediation (Patra *et al.*, 2010; Pal *et al.*, 2005). Chromium reduction takes place under both aerobic as well as anaerobic

conditions. Numerous bacteria have been reported for their ability in reducing/transforming Cr^{6+} to Cr^{3+} e.g. *Bacillus* sp., *Escherichia coli*, *Enterobacter cloacae*, *Pseudomonas fluorescens*, *Providencia* sp., *Exiguobacterium* sp. *etc.* (Bopp *et al.*, 1983; Wang *et al.*, 1989; Shen and Wang, 1993; Okeke *et al.*, 2008; Yang *et al.*, 2009).

The present study aimed to isolate the chromate resistant and chromate-reducing bacteria from the chromium landfill where the hexavalent chromium level is quite high, analyze the bacterial chromate reduction efficiency and determine the preferable conditions for bacterial chromate reduction, to provide useful knowledge for the bioremediation of chromate polluted areas.

Materials and Methods

Isolation of bacterial strains and cultural conditions

Samples were collected from outlet of tannery effluent treatment plant situated at Jajmau (Kanpur, Uttar Pradesh) and further processed. Isolation of the bacterial culture was done using enrichment culture technique. Luria Bertani (LB) broth was amended with 10 mg L^{-1} of $\text{K}_2\text{Cr}_2\text{O}_7$ and soil suspension (10%, w/v) was prepared in sterile distilled water, which was incubated at 37°C for 24 h. After 24 h enriched bacterial strains were isolated by plating on LB agar plates amended with 10 to 100 mg L^{-1} of $\text{K}_2\text{Cr}_2\text{O}_7$. Only one isolate (SA13A) was able to grow upto conc. of 100 mg L^{-1} of hexavalent chromium ($\text{Cr}(\text{VI})$), and was used in further studies.

Identification of isolated strain

Identification was done by 16S rRNA analysis using universal primers. The sequences of the primers used for the

amplification were: 16SF (5'-AGAGTTTGATCCTGGCTCAG-3') and 16SR (3'-ACGGCTACCTTGTTACGACTT-5').

Sequence was analyzed at NCBI server (<http://www.ncbi.nlm.nih.gov>) using BLAST (N) program. Phylogenetic tree was constructed by the UPGMA method using the MEGA 5 (Molecular Evolutionary Genetics Analysis) software (v. 5.05) (Tamura *et al.*, 2011). The final sequence was deposited at GenBank.

Evaluation of metal tolerance

The minimum inhibitory concentration (MIC) was determined in LB broth amended with heavy metal salts such as CdCl₂, CuSO₄, CoCl₂, CrCl₃.6H₂O, K₂Cr₂O₇, NiCl₂ and ZnCl₂ at varying concentrations from 50 to 3000 mg L⁻¹ (Aleem *et al.*, 2003; Ansari *et al.*, 2008). The minimum concentration of the metal inhibiting complete growth was taken as the MIC.

Time course of growth and chromium reduction

Growth curve of the bacterial isolate SA13A was determined in LB medium amended with Cr (VI) (100 mg L⁻¹) and without chromium (control). Media was inoculated with 1% exponential phase bacterial culture and incubated at 37 °C with shaking (120 rpm). An aliquot of culture was taken out in sterilized tube at regular intervals (2 h) and absorbance was measured at 600 nm.

Withdrawn samples were centrifuged (10,000 g; 15 min) and supernatant was used to determine residual Cr (VI) concentration using S-diphenylcarbazide (DPC) method (Ilias *et al.*, 2011). The absorbance of the color produced was measured at 540 nm using UV-Visible spectrophotometer (Systronics, 2202).

Cr (VI) reduction at optimized cultural conditions

To characterize the Cr (VI) reduction efficiency, the effect of temperature and pH on growth and Cr (VI) reduction was investigated using LB medium containing 100 mg L⁻¹ of Cr (VI) (as K₂Cr₂O₇). Media were inoculated with 25 µl of overnight bacterial culture. Chromium reduction and growth was studied at various incubation temperatures (20 to 45 °C). For the effect of pH, sterilized culture medium was adjusted to pH ranging from 6.0 to 10.0 and incubated at 37 °C. Growth was measured after 24 h of incubation by taking absorbance at 600 nm. The culture was centrifuged (10,000 g; 15 min) and the supernatant was used to determine the residual Cr (VI) concentration. In order to examine any abiotic Cr (VI) reduction, cell-free controls were used for each Cr (VI) reduction assay.

Statistical Analysis

All the experiments were performed in triplicates. Data are presented as mean ±SD (Standard deviation).

Results and Discussion

Isolation of bacterial strain and cultural conditions

Effluent released from tanneries contains high concentration of chromium and other heavy metals. This heavy metal contamination is responsible for shifts in microbial communities along with the emergence of elevated metal tolerant bacteria (Masood and Malik, 2011; Ansari *et al.*, 2008; Stepanauskas *et al.*, 2005). In search for chromium-resistant microorganisms, a total of 8 Cr resistant bacteria were isolated on plates amended with 10 to 100 mg L⁻¹ of Cr (VI) as K₂Cr₂O₇, Out of which only one strain

(SA13A) was able to tolerate Cr (VI) (Conc. 100 mg L⁻¹). All further studies were done on this strain.

Identification of isolated strain

Amplified DNA fragments were sequenced using Sanger Dideoxy method (Hattori *et al.*, 1986). Forward and reverse sequences of the isolate were joined using DNA Baser (v 3.5.3) which identified the isolate as *Luteimonas aestuarii*. The 16S rRNA gene sequence obtained from the isolate was compared with other bacterial sequences using NCBI mega BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for their pair wise identities. Isolate showed 99% 16S rRNA sequence homology to *Luteimonas* sp. at NCBI database. The 16S rRNA sequence of the isolated bacterium was submitted in the Genbank (NCBI) with an accession number KC540915. Further, consensus sequences were aligned and compared with the available database in NCBI. The phylogenetic tree was constructed using MEGA 5.05 software to determine evolutionary relationships of the isolate. The phylogenetic relationship of the SA13A isolate showed close relatedness with *Luteimonas aestuarii* strain B9 (Figure 1). According to scientific literature, this species of bacteria has never been reported as chromium resistant strain.

Evaluation of metal tolerance

Cr (VI) bearing wastewaters usually contain many other heavy metal cations. Therefore in addition to Cr (VI), resistance to various metallic salts was also determined. The bacterial isolate SA13A possessed multiple metal ion resistance. MIC of various heavy metal salts *i.e.* CdCl₂, CuSO₄, CoCl₂, CrCl₃.6H₂O, K₂Cr₂O₇, NiCl₂ and ZnCl₂ were found to be 200, 800, 400, 1500, 3000, 400 and 600 mg L⁻¹ respectively. The degree of inhibition caused by tested metal cations was:

Cr⁺⁶ > Cr⁺³ > Cu⁺² > Zn⁺² > Co⁺² = Ni⁺² > Cd⁺². SA13A showed maximum tolerance towards Cr (VI). Similar findings have been suggested by Camargo *et al.*, (2003). Various chromium resistant bacteria have been reported that could tolerate Cr (VI) up to concentration of 2000 mg L⁻¹ (Ilias *et al.*, 2011). Also, MIC values of chromium resistant bacteria have been compared and it has been found that different isolates exhibited different level of tolerance (Masood and Malik, 2011; Viti *et al.*, 2003) towards various heavy metals. Tolerance towards other heavy metals provides an additional advantage of the bacteria, as it can perform the preferred activity in the presence of metallic ions also.

Time course of growth and chromium reduction

Time course of growth and Cr (VI) reduction was investigated in presence of Cr (VI) (Conc. 100 mg L⁻¹) in shake flask culture. Growth curves of the bacterial isolate SA13A with and without Cr (VI) were plotted (Figure 2) and compared. Cells grew well in the medium containing 100 mg L⁻¹ Cr (VI), although differences in growth patterns were observed between cells grown in control and those grown in the medium. It has been found that growth of the isolate SA13A is lower in Cr than the control indicating that Cr (VI) has toxic effect on the growth of the cells.

Cr (VI) reduction potential of the isolate SA13A was assessed by DPC method. It was found to be growth associated (Figure 3). Isolate SA13A efficiently reduced 100 mg L⁻¹ of Cr (VI) (38.98 ± 0.62) %, (53.45 ± 0.76) %, (85.78 ± 0.54) % and (99.98 ± 0.56) % after 06, 08, 10 and 16 h respectively. Ilias *et al.*, (2011) reported complete Cr (VI) (20 mg L⁻¹) reduction by *Staphylococcus aureus* and *Pediococcus pentosaceus* in 6 h and 24 h respectively. Similar observations have been

reported by Masood and Malik (2011) or *Bacillus* sp. It has been found to involve in 100% Cr (VI) (100 mg L^{-1}) removal within 48 h. *Bacillus sphaericus* AND303 has been reported to provide Cr (VI) removal (72%) within 24 h (Pal *et al.*, 2005).

Cr (VI) reduction at optimized cultural conditions

Temperature has noteworthy effect on microbial Cr (VI) reduction. Cr (VI) reduction was evaluated at various range of temperature ($20 \text{ }^{\circ}\text{C}$ to $45 \text{ }^{\circ}\text{C}$) and it has been found to be growth associated. The isolate SA13A significantly reduced Cr (VI) (100 mg L^{-1}) at all temperatures but the optimum reduction ($98.98\% \pm 0.29$) was observed at $37 \text{ }^{\circ}\text{C}$. Moreover, it has been found that Cr (VI) reduction increased with increase of temperature up to $37 \text{ }^{\circ}\text{C}$ and thereafter

decreased above $40 \text{ }^{\circ}\text{C}$ (Figure 4). Similar reports have been given by Ilias *et al.* (2011) for *Staphylococcus aureus* and *Pediococcus pentosaceus*. Maximum Cr (VI) reduction was observed between $35 \text{ }^{\circ}\text{C}$ and $40 \text{ }^{\circ}\text{C}$ for both the isolates. Moreover, Cr (VI) reduction by *Bacillus* sp. strain FM1 was also found to be growth associated and optimum reduction (100%) was at $37 \text{ }^{\circ}\text{C}$ (Masood and Malik, 2011). Similarly, maximum Cr (VI) reduction by *Ochrobactrium intermedium* SDCr-5 was reported at $37 \text{ }^{\circ}\text{C}$ (Sultan *et al.*, 2007). As seen in figure 4, the bacterial isolate SA13A possessed the capability of growing and reducing more than 95% Cr (VI) (Conc. 100 mg L^{-1}) over a wide range of pH (6.0 to 8.0) but the optimal Cr (VI) reduction ($100\% \pm 1.09$) was observed at pH 8.0. The optimal pH for Cr (VI) reduction by various bacterial isolates has been reported between 7.0 and 8.0 (Masood and Malik, 2011; Ilias *et al.*, 2011).

Fig.1 Phylogenetic relatedness of *Luteimonas aestuarii* SA13A based on 16S rRNA gene sequence

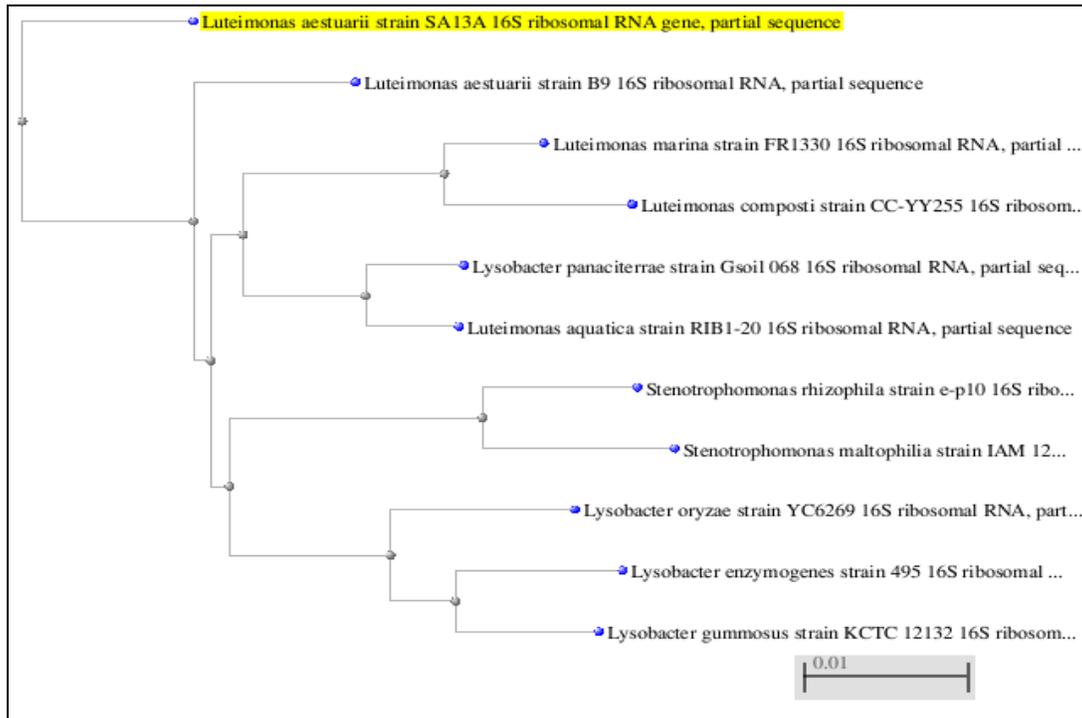


Fig.2 Growth curve of SA13A in absence and presence of Cr (VI) (Conc. 100 mg L⁻¹)

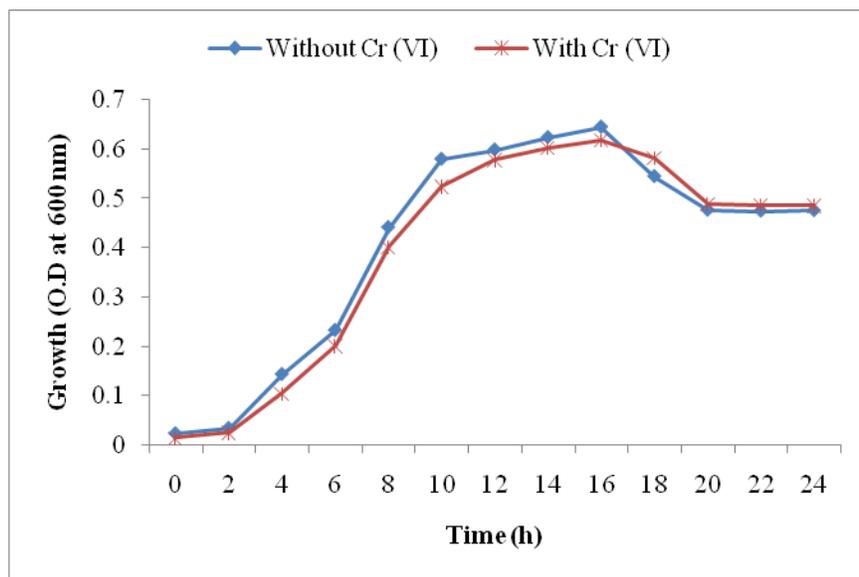


Fig.3 Time course of growth and Cr (VI) (100 mg L⁻¹) reduction by isolate SA13A

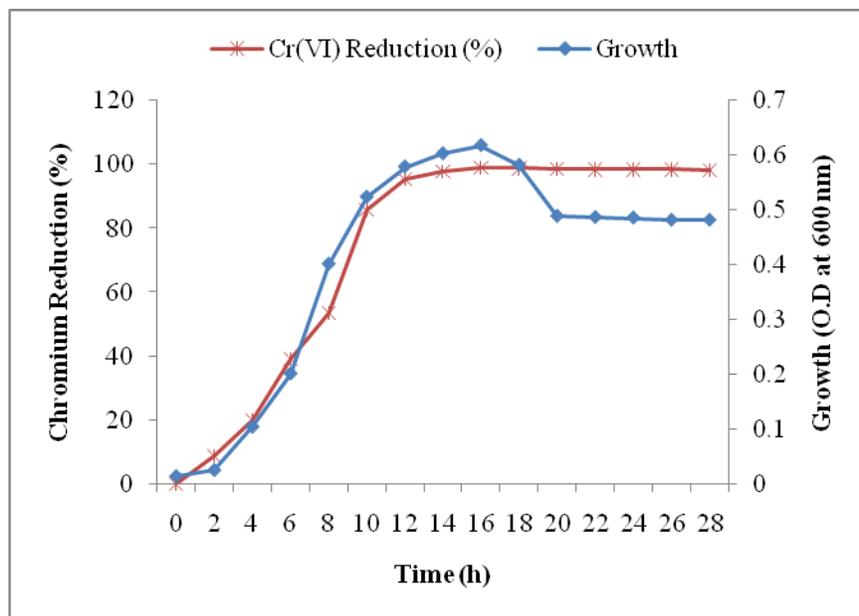


Fig.4 Effect of temperature on Cr (VI) reduction (Conc. 100 mg L⁻¹)

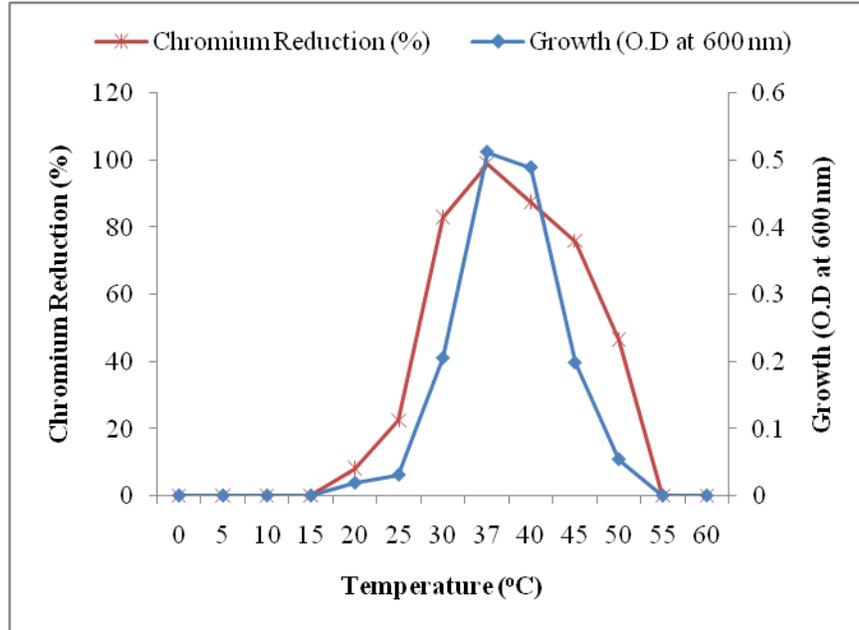
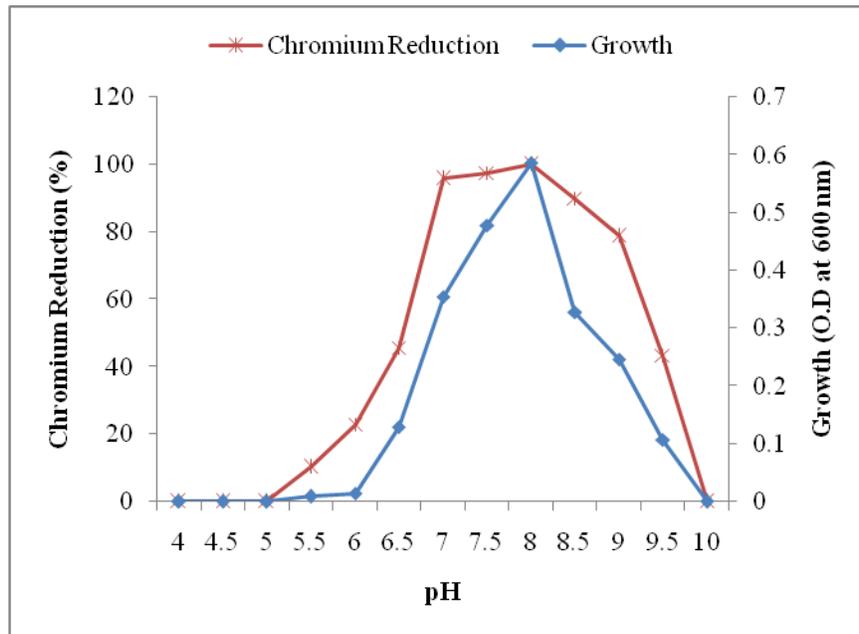


Fig.5 Effect of pH on Cr (VI) reduction (Conc. 100 mg L⁻¹)



But Liu *et al.*, (2006) reported maximum chromate reduction at pH 9.0. The change in optimal pH indicates that pH modification is important for various cultures to attain the maximum Cr (VI) reduction during Cr (VI) detoxification.

In conclusion, industrial workplace is an ideal site for selecting potent metal resistant bacteria since these sites are heavily polluted with heavy metals and other pollutants. Soil contamination *via* heavy metals is often permanent and may inhibit or even eradicate

native microbial community. Normally, it is assumed that the heavy metal exposure leads to the establishment of a tolerant microbial population. A gram negative *Luteimonas* strain SA13A isolated in this study has shown high chromium (100 mg L⁻¹) reducing efficiency (100%, 16 h) which is the first compilation of data in conviction with chromate reduction by *Luteimonas aestuarii*. Moreover, it exhibited resistance against many other heavy metals. Therefore, it may serve as potent novel bacteria for bioremediation applications especially in heavy metal contaminated sites.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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