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Biological reduction of hexavalent chromium and mechanism analysis of detoxification by *enterobacter* sp. HT1 isolated from tannery effluents, Mongolia

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Abstract: *Enterobacter* sp. HT1, Cr (VI) resistant bacterial strain was isolated from the wastewater sample of the tannery in Mongolia. Batch experiments on hexavalent chromium removal was carried out at 10, 20, and 30 mg/L of Cr (VI) added as potassium dichromate ($K_2Cr_2O_7$), at pH 7 and temperature of 30 °C using pure culture of *Enterobacter* sp. HT1 as inoculum. The isolated HT1 is capable of reduction nearly 100% of Cr (VI) resulting in the decrease of Cr (VI) from 10 to 0.2 mg/L within 20 hours. When the concentration of Cr (VI) increased to 20 and 30mg/L, almost complete reduction of Cr (VI) could achieve after 72 and 96 hours, respectively.

Keywords: Indigenous bacteria; hexavalent chromium; wastewater; tannery industry

INTRODUCTION

Chromium is belonged to toxic heavy metals and very dangerous chemical form on biological systems as it can induce mutagenic, carcinogenic and teratogenic effects and it is able to induce oxidative stress in cells, damaging its DNA and it has been classified as a human carcinogen [1, 2, 3]. In addition, the microbial diversity where Cr (VI) is prevalently accumulated is detected to be much less than the uncontaminated soil, which is an indication of devastating negative impact of Cr (VI) to the microbial diversity [4]. All these evidences prove that Cr (VI) does have the fetal impact not only to human, but also to the living organisms in the surrounding environment. Bioremediation has been developed from the laboratory to a fully commercialized technology over the last 30 years in many industrialized countries. Discovery of microorganisms capable of reducing Cr (VI) to Cr (III) have significant potential in development of in situ or on-site bioremediation strategies. Chromium resistant microorganisms are responsible for the biological reduction of soluble and toxic Cr (VI) into the insoluble and less toxic Cr (III), and its consequent precipitation, an effective method for detoxification of Cr (VI) contaminated sites, suggests a potential application in bioremediation [5]. A wide variety of micoorganisms such as bacteria, yeast, algae, protozoa, and fungi are found in waters receiving industrial effluents.

In recent years, environmental pollution issue has been one of the global priorities for countries. In view of this, eco-friendly technologies are encouraged in the practice of industries in order to alleviate or mitigate environmental pollution. Additionally, some appealing environmentally friendly technologies have been practiced in the remedial measures for polluted environment. Scientists have continued to make great efforts further in search of advancement. One of such eco-friendly technologies is bioremediation [6].

The environment in Mongolia has been exposed to various pollutants originated from human activities. Therefore, the government and ministries have committed to search for feasible green technologies to preserve the environment. Accordingly, some recommendations and resolutions have been made on the proper treatment of wastewaters from tanneries and operational procedures to comply with industrial technologies. Biological wastewater treatment is regarded as a proper treatment method for industrial effluents, which can be one of the eco-friendly technologies. From the status of tannery wastewater treatment in Mongolia, it's obvious that some additional treatment is prerequisite for dealing with the wide range of toxic chemicals in untreated tannery wastewaters and their effects on the

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environment. Hence, the objectives of my research work were to isolate the indigenous bacterial strains resistant to hexavalent chromium from wastewater of tanneries and then search for the feasibility of conducting lab scale degradation through the biological reduction of hexavalent chromium by the isolated strains. In addition, the possible isolation and detection of biodegrading indigenous bacterial strain from the wastewater of tanneries could reveal the further potential of bioremediation. The introduction of bioremediation would be considered as more cost-effective and time-efficient.

EXPERMENTAL

Enrichment cultures and isolation of the bacterial strain: Tannery effluent samples were collected in sterile tubes from collection tank of Khargia Treatment Company. Some physicochemical parameters of effluent pH, heavy metals, COD, BOD, etc were measured. Enrichment culture method was mainly used for the isolation of chromium resistant bacteria. Enrichment cultures were grown in flask using a complex YP-EG medium, containing 10.0 g of tryptone, 5.0 g of yeast extract, 5.0 g of sodium chloride, and 1.0 g of D-glucose per liter of distilled water, in the presence of 100 mg of Cr (VI), and inoculated with 0.5 mL of effluent samples, by using an incubator shaker at 28°C for 24 hours. After 24 hours from flask, a 100 µL aliquot was spread on Petri dishes containing the complex solid medium in the presence of the same initial concentration of Cr (VI) and then incubated at 28°C for 48 hours. Cultures were purified by isolating single colonies grown on solid medium containing 2% (w/v) agar.

Microbial identification: Genomic DNA was isolated from the overnight culture by DNeasy Blood and Tissue kit, QIAGEN. The 16S rRNA gene was amplified by PCR using two universal 16S rRNA primers (27F; ACAGTTTGATCCTGGCTCAC, 1492R; GGTTAGCTTGTTACGAGTT). The PCR products were purified by QIAquick purification kit, QIAGEN. Sequencing of the purified 16S rRNA genes was performed using a BigDye Terminator cycle sequencing ready reaction kit as recommended by the manufacturer. The almost full 16S sequence was compared at the prokaryotic small subunit rDNA on the FASTA search system on the DDBJ website (http://www.ddbj.nig.ac.jp). The sequences of the isolates was aligned with the sequences which all-significant similarity GenBank entries. Multiple alignments of the sequences were performed by the CLUSTAL_X [7] and edited manually for proper alignment. Phylogenetic trees were constructed by the neighbor-joining method and NJPlot was used to display and analyze the trees. Bootstrap analysis (1000 replicates) was used to test the topology of the neighbor-joining method data. The trees were unrooted.

Chromium and biomass determination: The bacterial strains were pre-cultured overnight in LB broth and the active culture was inoculated into flasks containing 100 mL of LB broth supplemented with 10, 20, and 30 mg/L of Cr (VI) and incubated at 30°C and 100 rpm. Controls consisted of LB broth added with 10, 20 and 30 mg/L of Cr (VI) without bacteria. At different time intervals, 1-10 mL of each sample was collected by centrifugation at 10,000 rpm for 5 minutes. The supernatant was analyzed for residual Cr (VI) using 1,5- diphenylcarbazide (DPC) method. DPC solution was prepared by dissolving 0.25 g of 1,5-diphenylcarbazide in 50 mL acetone and stored in a brown bottle. The reaction mixture was set up in 10mL tube as follows: 0.1 mL sample volume was made to 10mL distilled water and then added 0.1mL of 2N H_2SO_4 and 0.4 mL of DPC. Measurements were made at 540 nm using UV spectrophotometer. The standard curve was plotted using 0.02-1 mg/L Cr (VI) prepared from Cr (VI) stock solution. At the same time optical density of the cultures at 600 nm was monitored to indicate the biomass of bacteria.

RESULTS AND DISCUSSION

Tannery effluent samples were collected in sterile tubes from the collection tank of Khargia Treatment Company during February and September 2013, respectively. Some physicochemical parameters of effluent pH, heavy metals, COD, BOD, etc were measured (data not shown). The pH of the wastewater sample was in the range of 9-9.5. This indicated that the Mongolian tannery effluents were weakly alkaline. The chemical indicators between the sampling periods varied, demonstrating that the effluent quality is dependent upon the seasonality of tanneries. Even though approximately 30 tanneries are currently available, their operation does not take place during the same period, taking an example, some tanneries even have had no operation. Such phenomena would justify why Cr (VI) was prevalent September.

Identification of bacterial strains by 16S rRNA, phylogenetic tree **:** Starting from 1980, the gene sequence of 16S rRNA that is more stable to the taxonomy of prokaryotic has been widely used in the practice and further the application enabled to conduct comparative analysis of gene sequence on public domains at the level of bacteria genera and species. In addition, this method allows identifying cultured bacteria as well as the uncultured bacteria. The sequence obtained were edited manually and compared to nucleotied database provided by Data bank of Japan (DDBJ) using the BLAST. The results revealed that HT1 and HT3 belonged to the genus *Enterobacter* and HT2 and HT4 could be grouped into the genus *Serratia.* Levels of 16S rRNA gene sequence similarity between the strains isolated in this study and the type strains of *Enterobacter* species and

Table 1. Phylogenetic analysis of 16S rRNA gene sequence

	Number of strains	Genus and species	16S rRNA similarity	Number of nucleotids
1	HT1	Enterobacter aerogenes B14	99.3%	1528
2	HT2	Serratia proteamaculans 568	99.2%	1498
3	HT3	Enterobacter ludwigii M162B	100%	1520
4	HT4	Serratia liquefaciens ATCC 27592	99.4%	1517

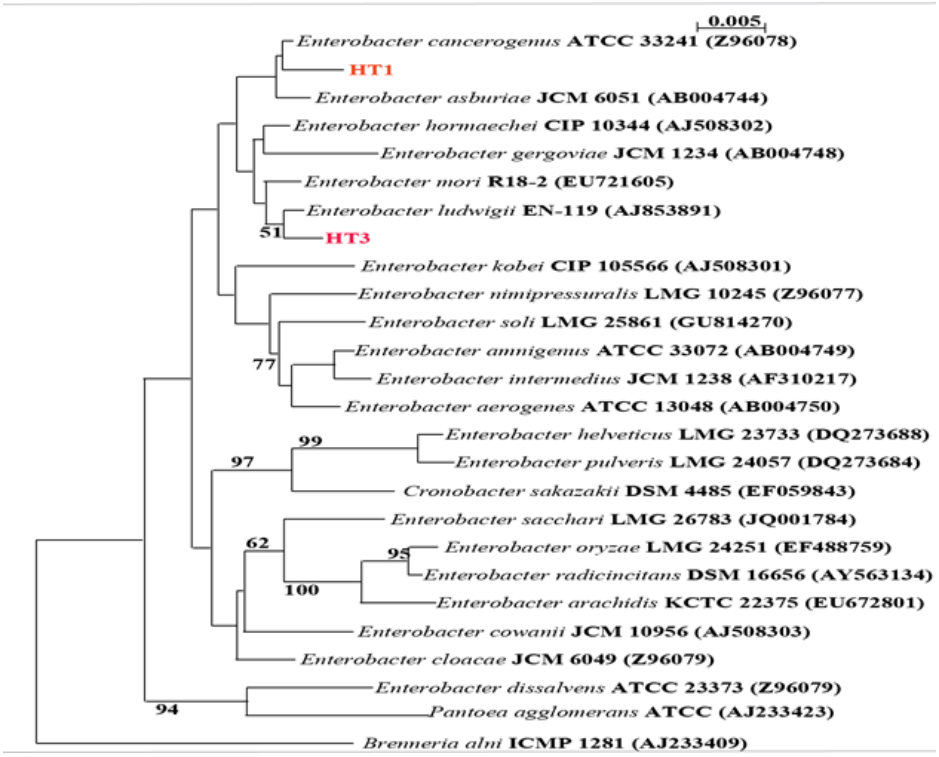

Fig. 1. Phylogenetic tree of HT1 and HT3.

Serratia species were in the range of 99 - 100% (Table 1).

The phylogenetic tree constructed from full length 16S rRNA gene sequences of the bacterial isolates is shown in Figure 1.

Phylogenetic analysis based on 16S rRNA gene sequence comparison showed that strain HT1 was most close to *Enterobacter aerogenes* (99%) and strain HT3 was most close to *Enterobacter ludwigii* (99%). For constructing phylogenetic tree and analyzing the phylogenetic relationship analysis between the isolates and the species of *Enterobacter* genus, sequences of type strains of all validly published up to present 25 species were used. *Brenneria alni* was used as the out-group. Phylogenetic analysis showed that strain HT1 clustered with *Enterobacter cancerogenus* and *Enterobacter asburiae* while HT3 fell in to cluster with *Enterobacter ludwigii* and *Enterobacter mori*. Some researchers have identified bacterial strains isolated from tannery effluent, which have capability of reducing Cr (VI), based on 16S rRNA gene sequence; and the most dominating genera were *Pseudomonas, Stenothropmonas, Enterobacter,* and *Halomonas.* Moreover, some other researcher demonstrated that some strains of genus of *Serratia* are capable of reducing Cr (VI) as well [8]. From the result, our strains belonged to the bacterial genera, namely, Enterobacter and Serratia, which have members capable to reduce Cr (VI).

Cr (VI) reduction and biomass growth at different chromium concentrations: The tolerance to various Cr (VI) concentrations was tested for the 4 strains, and they all showed tolerance in variable degrees. Among them strain HT1was most resistant to Cr (VI) and thus the HT1 strain was selected for the further study. The strain HT1 was tested for the chromate tolerance at different concentrations (1-500 mg/L) in liquid LB medium and it was capable of tolerating Cr (VI) of 1-500 mg/L, however, its well growth was observed at the concentration of 1-100 mg/L. It should not be assumed that each strain of bacteria

resistant to Cr (VI) has the capability of reducing Cr (VI) and then the following experiments were carried out to test whether the culture was capable of reducing Cr (VI) or not. For determination of the capability of Cr (VI) reduction, 10, 20, and 30 mg/L of Cr (VI) were selected. The reason was that the culture growth was normal when the concentration was at 10 mg/L, however at 20, and 30 mg/L of Cr (VI), inhibition effect was noticed at the initial 12 hours. The difference in growth of *Enterobacter* sp. HT1 was compared between without Cr (VI) and with 10, 20, and 30 mg/L of Cr (VI) through the measurement of optical density (Fig. 2)

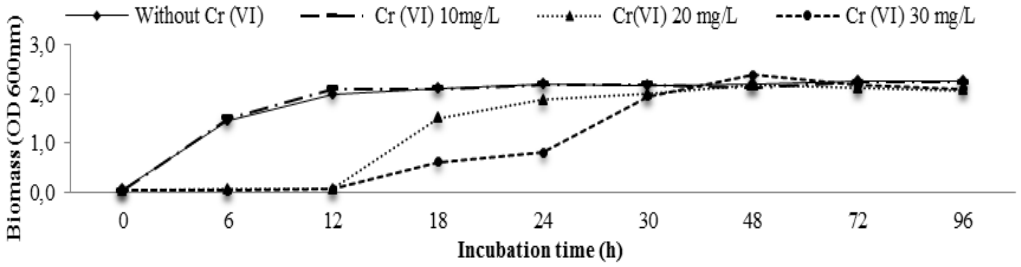

Fig. 2. Biomass growth of *Enterobacter* sp. HT 1 (indicated by OD value) in LB broth with and without Cr (VI).

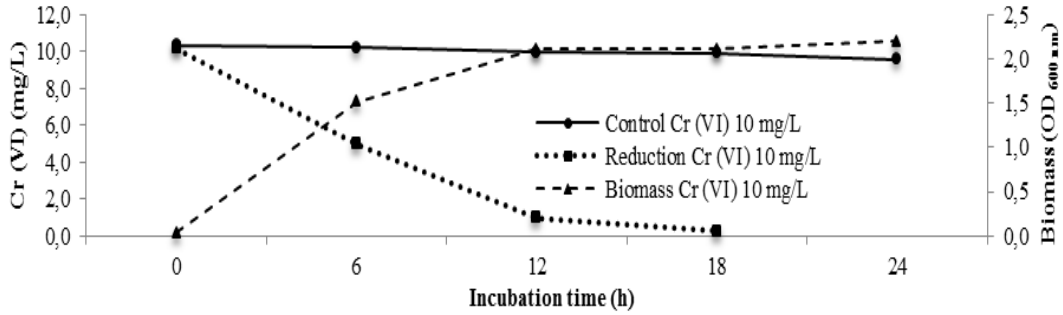

Fig. 3. Reduction capacity of *Enterobacter* sp. HT1 at 10 mg /L of Cr (VI)

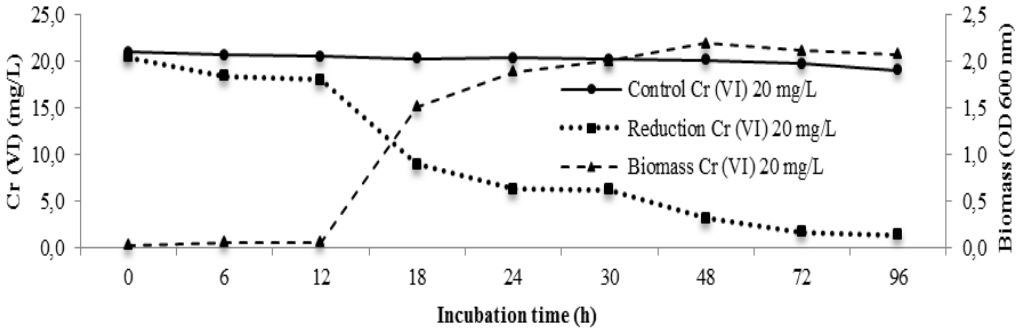

Fig. 4. Reduction capacity of *Enterobacter* sp. HT1 at 20 mg/L of Cr (VI)

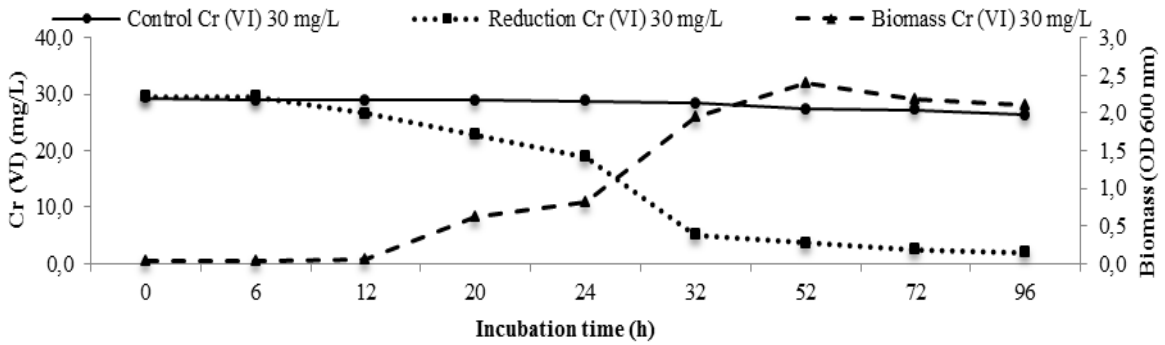

Fig. 5. Reduction capacity of *Enterobacter* sp. HT1 at 30 mg/L of Cr (VI)

From the figure, the bacteria immediately achieved an exponential phase at 10 mg/L of Cr (VI) with almost no lag phase and reached stationary phase in 12 hours. In case of Cr (VI) concentration at 20 or 30 mg/ L, it shifted to the exponential phase after the lag phase ended 12 hours. These findings exhibit that as the Cr (VI) concentration increases, the cell requires adaptation period.

Figures 3, 4, and 5 present how the reduction of Cr (VI) is dependent upon the biomass of *Enterobacter* sp. HT1, and as a control the medium with Cr (VI) at 10, 20, or 30 mg/L was taken as control. The slight decrease in amount of Cr (VI) in the control medium can clearly be observed from the figures 3, 4, and 5. That is considered to be due to the reaction of soluble Cr (VI) with other chemicals in the medium. The reduction of Cr (VI) was immediately observed at 10 mg/L Cr (VI) and the residual Cr (VI) was 0.2 mg/L when the cell growth reached to the exponential phase and its reducing rate was 0.54 mg/L/h. Lag phase was extended while the concentration of Cr (VI) was at 20 and 30mg/L, however the slight reduction occurred. For instance, at 20 mg/L the residual Cr (VI) was 18.04 mg/L and at 30 mg/L it was 26.58 mg/L within 12 hours. The amount of Cr (VI) rapidly decreased when the cell growth reached the exponential phase, however the reduction of Cr (VI) at the stationary phase was decreased resulting in not complete reduction of Cr (VI). For instance, 1.7 mg/L was remained after 72 hours for initial 20 mg/L of Cr (VI) while 1.9 mg/L was remained for 96 hours reaction when initial Cr (VI) was 30 mg/L. The reduction rate was 0.26 mg/L/h at 20 mg/L and 0.29 mg/L/h at 30 mg/L of Cr (VI). Chromium resistant strains of *Enterobacter* genus have been isolated from tannery effluents by several groups. For instance, the *Enterobacter cloacae* and *Enterobacter aerogenis* are currently reported to be capable of Cr (VI) reduction [8]. In terms of *Enterobacter cloacae* SUKCrD1 strain, the resistance concentration of Cr (VI) was 10-1000 mg/L, though as the Cr (VI) concentration increased, the well growth of culture was inhibited [9]. However, the resistance capability to Cr (VI) varies on each strain, even they belong to the same species. For instance, *Enterobacter cloacae* IAM 1624 strain compared with *Enterobacter cloacae* HO1 strain is relatively less resistant to Cr (VI) [10]. During the present investigation *Enterobacter* sp. HT1 was found to be highly tolerating Cr (VI) of 1-500 mg/L, however, its well growth was observed at the concentration of 1-100 mg/L. First research work for the reducing toxic Cr (VI) to less toxic Cr (III) was reported for *Pseudomonas* spp., and characterization of bacteria capable of reducing the Cr (VI) was reported successively in 1970s [11; 12]. Since then many microorganisms have been reported to reduce the highly soluble and toxic Cr (VI) to the less soluble and less toxic Cr (III) such as Pseudomonas *putida* [13], *Enterobacter aerogenes* and *Acinetobacter* sp. [5], *Arthrobater* [14] and *Bacillus* sp. [15]. In the present study Enterobacter sp. HT1 could reduce Cr (VI) (10 mg/L) almost 100% from the medium within 20 hours. While the concentration of Cr (VI) increased from 20 mg/L to 30mg/L, the optimum time of removing Cr (VI) was extended from 72 to 96 hours, respectively. Compared to other species of *Enterobacter* in literature published, *Enterobacter aerogenes* T2 could remediate almost all the 8-16 mg/L of Cr (VI) ions from a synthetic medium within 26.05h. In terms of *Enterobacter clocae*, the Cr (VI) concentration of 5 mg/L was completely reduced within 24 hours while the complete reduction for the increased concentration of 10 mg/L required 4 days [16]. All these comparisons demonstrate the excellent capacity of Cr (VI) reduction for *Enterobacter* sp. HT1.

CONCLUSION

Hexavalent chromium is a highly toxic pollutant introduced into natural water due to the discharge of industrial wastewater, mainly from tannery industry. Microorganisms with the ability to tolerate and reduce Cr (VI) can be used for detoxification of environment contaminated with Cr (VI). Hence, this study was aimed to examine the presence of indigenous bacterial strains in the Cr (VI) containing tannery effluent and furthermore to screen the reduction capability of Cr (VI) by those bacterial strains. Based on the experimental results, the following main conclusions could be arrived at:

1. Hexavalent chromium resistant indigenous bacterial strains were isolated from the tannery effluents, Mongolia.
2. Two strains belonged to the genus of *Enterobacter* and two strains belonged to the genus of *Serratia*.
3. The strain identified as *Enterobacter* sp. HT1 had the highest capacity of reduction Cr (VI).

The influence of various operating conditions such as initial pH of the medium, temperature, and initial concentration of Cr (VI), on Cr (VI) degradation helps to optimize the maximum growth condition of bacterial strains and then to estimate the hydraulic resilience time of the strains in the reactor for a completely detoxification of the water. In addition, the isolated bacteria from the wastewater of tanneries, highly resistant to Cr (VI) and capable of Cr (VI) reduction, proved the feasibility of isolating culturable microbes from the contaminated site. As mentioned in the objectives, tremendous efforts have been made to launch green technology to eliminate the environmental pollution issues in Mongolia. Accordingly, the prospective introduction of biological degradation of Cr (VI) would be considered as the largest part of the green technology.

REFERENCES

1. Kotas J., and Stasicka Z. (2000). Chromium occurrence in the environment and methods of its speciation. *Environmental Pollution,* **107**, 263-283.
2. Reynolds M.F., Peterson-Roth E.C., and Bespalov I.A., (2009). Rapid DNA double-stand breaks resulting from processing of Cr-DNA cross-links by both MutS dimers. *Cancer research,* **69**, 1071-1079.
3. USEPA. (1998). Toxicological review of hexavalent chromium. http://www.epa.gov/iris/ toxreviews/0144tr.pdf .
4. Zhang K., and Li F. (2011). Isolation and characeterization of chromium-resistant bacterium Serratia sp. Cr-10 from a chromate-conaminated site. *Applied Micorbiology and Biotechnology,* **90**, 1163-1169.
5. Panda J., and Sarkar P. (2012). Bioremediation of chromium by novel strains *Enterobacter aerogenes* T2 and *Acinetobacter* sp. PD 12 S2. *Environmental Science and Pollution Research,* **19**, 1809-1817.
6. Dhal B., Thatoi H.D., Das N.N., and Pandey B.D. (2013). Chemical and micorbial remediation of hexavalent chromium from contaminated soil and mining/metallurgical solid waste. *Journal of Hazardous Materials,* **250**, 271-291.
7. Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F., and Higgins D.G. (1997). The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research,* **25**, 4876–4882.
8. Zhou J., Xia B., Treves D.S., Wu L.Y., Marsh T.L., O'Neill R.V., Palumbo A.V., and Tiedje J.M. (2002). Spatial and resource factors influencing high microbial diversity in soil. *Applied and Environmental Microbiology,* **68**, 326-334.
9. Harish R., Samuel J., Mishra R., Chandrasekaran N., and Mukherjee A. (2012). Bi-reduction of Cr (VI) by exoplysaccharides (EPS) from indigenous bacterial species of Sukinda chromite mine, India. *Biodegradation,* **23**, 487-496.
10. Wang P.C., Mori T., Komori K., Sasatsu M., Toda K., Ohtake H. (1989). Isolation and Characterization of an (2006) strain that reduces hexavalent chromium under anaerobic conditions. Applied and *Environmental Microbiology*, **55** (7), 1665-1669.
11. Romanenko V.I., and Koren'kov V.N. (1977). A pure culture of bacteria utilizing chromates and bichromates as hydrogen acceptors in growth under anaerobic conditions. *Microbiologiya,* **46**, 414-417.
12. Lebedeva E.V., and Lyalikova N.N., Reduction of chrocoite by *Pseudomonas chromatophila* sp. nov. *Microbiologya,* **48**, 517-522.
13. Ishibashi Y., Cervantes C., and Silver S. (1990). Chromium reduction in *Pseudomonas putida. Applied and Environmental Microbiology,* **56**, 2268-2270.
14. Megharaj M., Avudainayagam S., and Naidu R. (2003). Toxicity of Hexavalent Chromium and its reduction by bacteria isolated from soil contaminated with tannery waste. *Current Microbilogy*, **47**, 51-54.
15. Elangovan R., Abhipsa S., Rohit B., Ligy P., and Chandraraj K. (2006). Reduction of Cr (VI) by *Bacillus* sp. *Biotechnology Letters,* **28**, 247-252.
16. Rege M.A., Petersen J.N., Johnstone D.L., Turick E.C., Yonge D.R., and Apel W,A. (1997). Bacterial reduction of hexavalent chromium by *Enterobacter cloacae* strain HO1 grown on sucrose. *Biotechnology Letters,* **19**, 691-694.