Detoxification of hexavalent chromium Cr(VI) by *Bacillus laterosporus* and its application in Lebanese waste water

H. Holail, A. Al-Bahadly & Z. Olama
Faculty of Science, Beirut Arab University, Lebanon

Abstract

A novel chromate resistant bacterial strain was isolated and purified and identified as *Bacillus laterosporus* and used *for* Cr(VI) reduction application using living and resting cells. The evaluation of the environmental factors that affect the chromate reduction was investigated using a multifactorial experiment (Placket-Burman statistical design. Free and immobilized cells on natural supports were tested using a bioreactor for the treatment of some chromate loaded waste waters in Lebanon. *Bacillus laterosporus* is considered as a promising and ideal candidate that must be used in knowledge-based on toxic metal remediation system.
Keywords: Bacillus laterosporus, detoxification of Cr(VI), immobilization, optimization.

1 Introduction

The widespread use of chromium compounds by industries has led to large quantities of this element being released to the environment. Only hexavalent chromium Cr(VI) and trivalent chromium Cr(III) are ecologically important because they are the most stable oxidation states of chromium compounds. Being mutagenic, carcinogenic and teratogenic, Cr(VI) is approximately 100-fold more toxic than Cr(III) (Shen and Wang [1]). However, Cr(III) is considered to be relatively innocuous because it is less soluble and does not permeate eukaryotic and prokaryotic and membrane. Serious concerns about the toxicity of Cr(VI) compounds necessitate the recovery and reuse of chromium from industrial wastes and it is essential to convert the unrecovered Cr(VI) to the less toxic form (Francisco *et al*. [2]).

WIT Transactions on Ecology and The Environment, Vol 153,© 2011 WIT Press
www witpress com ISSN 1743-3541 (on-line)
doi:10.2495/WS110211

Biological reduction of Cr(VI) to Cr(III) is a potentially useful mechanism to detoxify Cr pollution and to bioremediate contaminated wastes (Wakatsuki [3]). The Cr(VI)-reducing ability found in some bacteria has raised the possibility of using these microorganisms a biotechnological tool for remediation of chromate-polluted zones (Lovley [4]). The main advantage of using bacterial Cr(VI) reduction is that it does not require high energy input or toxic chemical reagents and the possibility of using native, non-hazardous strain. A part from brief mention to the capacity of some gram-positive bacteria (including a strain of *Bacillus subtilis*) to reduce chromate under anaerobic conditions, only one report was published on the aerobic chromate reduction by *Bacillus subtilis* (Garbisu *et al*. [5]). All studies on chromate reduction have been carried using gram-negative bacteria (mostly, *Pseudomonas* and *Enterobacter* strains).

The objective of the study was to isolate and characterize a chromium resistant gram-positive bacterium from contaminated sites and evaluate its potential for reducing Cr(VI) to Cr(III) under aerobic conditions. Factors affecting chromate reduction also studied. We also aimed to describe a microbiological treatment for tannery effluent that may be suitable for the processing of Cr-contaminated waste.

2 Materials and methods

2.1 Microorganisms

Bacterial strain used throughout this study was isolated from effluent of EKMEKJI Leather Tanning Company south Beirut – Lebanon and was identified as *Bacillus laterosporus* on the basis of the Practical Atlas for Bacterial identification (Roy [6]). Bacteria were maintained either as spore stock, or for a short period of time, on nutrient agar slants.

2.2 Media

All media were prepared with distilled water. Sterilization was carried out by autoclaving at 120ºC for 20 min. when needed, initial pH was adjusted to 7.0 using 1M HCl prior to autoclaving. The media used throughout the work are given in g/l.

Minimal medium (MM): (modified after Wong and Yuen [7]), Lauria broth medium (LB) and sporulation medium.

2.2.1 Isolation of chromate resistant bacteria

The method used for the isolation of Cr-resistant bacteria was reported by Shakoori *et al*. [8].

2.2.2 Selection of chromate resistant spore-formers

Spore formers were then isolated by streaking a loop full from each tube onto a nutrient agar plate and incubated at 37ºC overnight. Selected spore forming bacterial strains were maintained as spore suspensions or on nutrient agar slants for short periods.

2.2.3 Evaluation of environmental factors for bioaccumulation of metal chromate multifactorial experiment

The Plackett-Burman experimental design, a fractional factorial design, (Plackett and Burman [9]) was used in this research to reflect the relative importance of various environmental factors on the activity of bacterial enzymes to for degradation of chromate. In this experiment, for example seven independent variables, a high (+) and low (-) level was tested. All trials were performed in duplicates and the residual Chromate was treated as the responses. The main effect of each variable was determined with the following equation:

$$(\sum Mi+) - (\sum Mi-) / N \quad (1)$$

where Exi is the variable main effect $(\sum Mi+) - (\sum Mi-)$ are the activity percentage in trials where the independent variable (xi) was present in high and low concentrations, respectively, and N is the number of trials divided by 2. A main effect figure with a positive sign indicates that the high concentration of this variable is near to the optimum and a negative sign indicates that the low concentration of this variable is near to the optimum. Using Microsoft Excel, statistical t-values for equal un-paired sample were calculated for determination of variable significance.

Plackett-Burman design was carried out with growing cells in the presence of 250 g/l Cr(VI) and resting cells in the same amount of Cr(VI), in order to determine that the chromate reduction was depended on the cell or any possible compounds excreted by the cell to the surrounding medium, the experiment was repeated with supernatant filtered fraction.

2.2.4 Bioremediation of tannery effluent using continuous bioreactor

The industrial effluent sample taken from EKMEKJI Leather Tanning Company was filtered to remove the suspended particles, and then treated by passing through column containing bacterial cells on sponge, loofa and pumice surfaces as a solid support. The column was about (2 cm in diameter and 20 cm in height). It was washed with 0.1N HCl followed by a 0.1N NaOH solution and finally by deionized water. The waste water was allowed to pass in the column twice and stay in the column for 30 minutes in each run and the samples were taken every 10 minutes with a flow rate of 2ml/10min, then the necessary analyses were carried out.

3 Results and discussion

3.1 Isolation and determination of chromate resistance pattern of the bacterial isolates of Cr(VI)-resistant bacteria

In the present investigation, eleven Cr(VI) resistant bacterial strains were isolated from tannery effluent . Most of the isolates were able to reduce Cr(VI) to Cr(III), but at different levels. Nine of them are gram +ve spore formers that showed a remarkable tolerance to high chromate concentration. Based on

chromate-resistant patterns, isolate number 4 (I4) was selected for its expression of high tolerance to chromate salt and the ability to grow in up to 2200 mg/l Cr(VI), as well as its efficiency to reduce Cr(VI) into Cr(III).

Genus *Bacillus* was reported to establish Cr(VI) reduction under aerobic conditions (Garbisu *et al*. [5] and Ganguli and Tripathi [10]). However, several studies on Cr(VI) reduction under anaerobic conditions were reported in Michel *et al*. [11] (Figure 1).

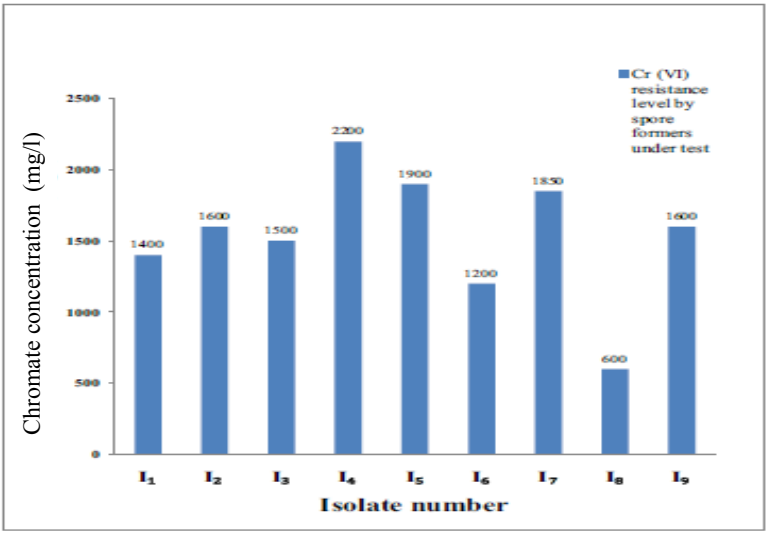
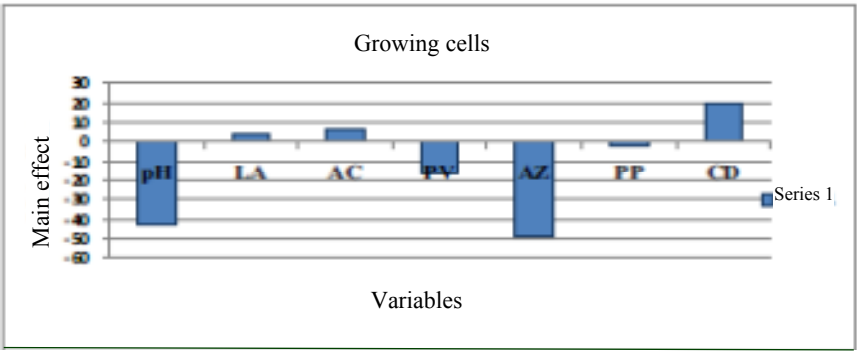


Figure 1: Cr(VI)-resistance pattern of the bacterial isolates (I_1-I_7).

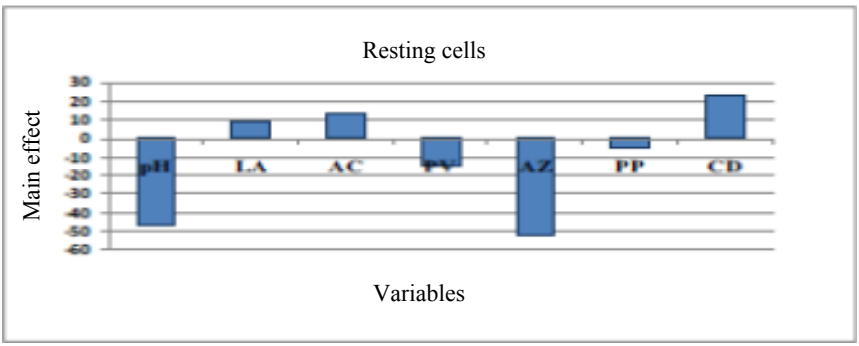
3.2 Evaluation of environmental factors for bioaccumulation of metal chromate multifactorial experiment

The metal uptake process; however, is complex and dependent on the chemistry of the metal ions, specific surface properties of the organisms, cell physiology, and the physicochemical influence of the environment, for example, pH, temperature, and metal concentration. Therefore, optimization experiments which lead to maximum chromate sequestration using growing and resting *Bacillus laterosporus* cells were studied. Factorial experiments were designed using the approach given in standard texts on the design experiments, the seven factors affecting the Cr(VI) reduction were studied using factorial design.

A wide variation was shown in chromate uptake throughout the different trials, according to chromate uptake using growing cells the variation was in the range of (43.57-100 mg/l) with 100% reduction after 16 hrs, while the chromate uptake by the resting cells was in the range of (26.18-100 mg/l) with 100% reduction after 1 hr.



(a)



(b)

Figure 2: Main effect of the environmental factors on Cr(VI) uptake by *Bacillus laterosporus* growing cells (a) and resting cells (b) based on Plackett-Burman design results.

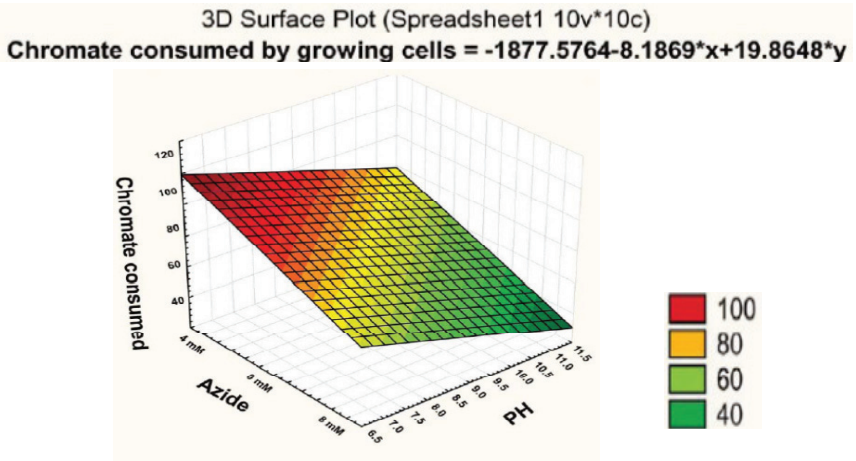
It was revealed that the pH and absence of azide strongly affect the Cr(VI) reduction by *Bacillus laterosporus.*

The biosorption by resting cells was more efficient than bioaccumulation by growing cells, by the monitoring the time that cells taken to reduce 100 mg Cr(VI)/l from the aqueous solution. Complete reduction of 100 mg Cr(VI)/l lasting 1 hr (for resting cells) and 16 hrs (for growing cells). The selected *Bacillus laterosporus* can reduce Cr(VI) from an aqueous solution both through bioaccumulation (on using growing cells), and biosorption (on using resting cells). Biosorption has distinct advantages over conventional methods; the process does not produce chemical sludge. It can be highly selective, more efficient, easy to operate, and hence cost effective for the treatment of large volumes of wastewaters containing low metal concentrations (Puranik and Paknikar [12]). The complete reduction of Cr(VI) by *Bacillus laterosporus* resting and growing cells generally were achieved during one and 16 hours respectively of contact at initial pH 7, 37ºC and 100 mg/l Cr(VI) concentration

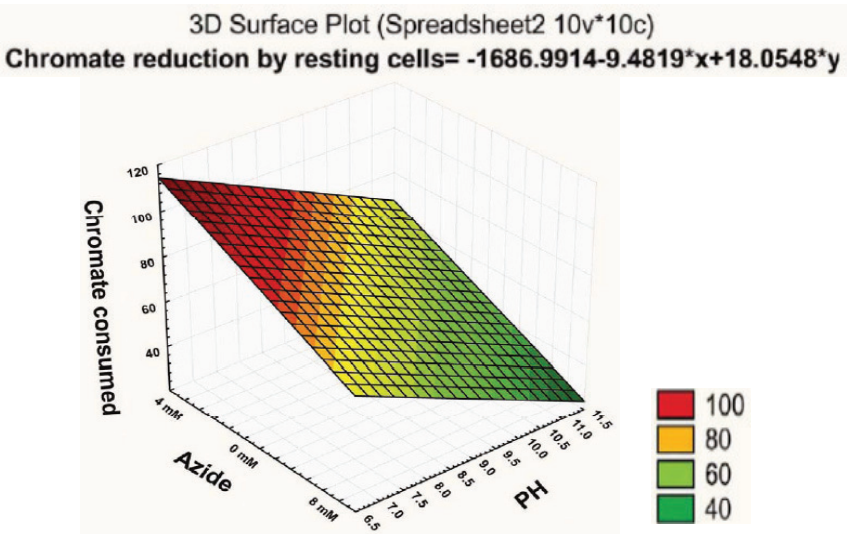
indicating the high efficiency of the resting cells than growing cells in Cr(VI) reduction. Maximum Cr(VI) removal (85%) on using *Bacillus circus* growing cells was observed at 30^{o}C and pH 6.0, during 10 hours of fermentation, when initial chromate concentration was 50 mg/L (Ray *et al*. [13]). pH exerts a controlling effect on chromate removal in the presence of bacteria for metabolic enzymatic Cr(VI) reduction. The experimental results clearly showed a direct correlation between pH and Chromate uptake at pH values above 7. Inhibition of chromate reduction was noticed at pH 11 with little growth recorded. *Bacillus laterosporus* can reduces Cr(VI) in a wide range of pHs (7-11) with maximum Cr(VI) reduction at pH 7.0 . These results are in accordance with that obtained by Liu *et al*. [14].

The present study proved that the reduction rate of Cr(VI) increased rapidly with the increase of electron donors in the range between 4000–6000 mg/l. Maximum reduction of C(VI) was achieved with acetate concentration (4000 mg/l) as an electron donor by *Bacillus laterosporus.* In the present study Cr(VI) reduction increases with the increase of lactate concentration, while concentration of lactate reach 6000 mg/l, the reduction rate enhanced 40% higher than control sample. Therefore, lactate could substantially stimulate the reduction ability of *Bacillus laterosporus* as an efficient electron donor. Pyruvate consider as an organic anions and electron donor which enhance Cr(VI) reduction and cell growth, acetate, lactate, pyruvate and glucose are known electron donors for Cr(VI) reduction by several organisms. On the contrary pyruvate has a negative effect on Cr(VI) reduction by *Bacillus laterosporus*, similar results were observed with *Desulfovibrio vulgaris* cells grown on pyruvate displayed more tolerance to Cr(VI) compared to lactate-grown cells. Klonowska et al. [15] indicated that *Desulfovibrio vulgaris* utilized lactate during Cr(VI) exposure without the reduction of sulfate or production of acetate, and that ascorbate and Pyruvate could protect the cells from Cr(VI)/Cr(III) toxicity.

The presence of azide in culture media has a negative effect on Cr(VI) reduction that inhibited both cell growth and chromate reduction. The present study showed that high level +1 inhibits Cr(VI) reduction from 100% Cr(VI) reduction to 34% Cr(VI) in the presence of 8mM azide, A similar results was found by Garbisu *et al*. [5] who reported that metabolic poisons included sodium azide and sodium cyanide inhibit the aerobic reduction of chromium in *Bacillus subtilis* because of the prevention of de novo protein synthesis and not inhibit the activity of reductase enzymes. In the present study peptone was used as one of the factor that affect Cr(VI) reduction, it was showed that peptone has a negative effect on Cr(VI) reduction, this could be due to the addition of azide (Garbisu *et al*. [5]). The effect of cell density on Cr(VI) uptake by the *Bacillus laterosporus* was carried out using different initial cell densities. Consumption of Cr(VI) increased with the increase of cell density, at low level of cell density Cr(VI) reduction was 39% and 100% at high level, meanwhile, Cr(VI) showed some inhibitory effect on the growth rate. The increased rate of Cr(VI) reduction was proportional to the increase in initial cell concentration. Additionally, Cr(VI) reduction rate generally decreased with time regardless of the initial cell concentration (Figure 3(a) and (b)).



(a)



(b)

Figure 3: (a) and (b) Effect of interaction between Azide and pH on chromate consumption by *Bacillus laterosporus* on growing and resting cells.

At the end of the study of the optimization we got conditions nearer the optimum environmental and physiological conditions for Cr(VI) uptake by *Bacillus laterosporus,* the optimum conditions for growing cells were: lactate, 2000 mg/l; acetate, 6000 mg/l; Pyruvate, 6000 mg/l; peptone, 5 g/l; inoculum size, 5 ml for 16 hrs incubation at pH7 for complete reduction. While the optimum conditions for resting cells were: lactate, 2000 mg/l; acetate, 6000 mg/l; pyruvate, 6000 mg/l; peptone, 5 g/l; Cell density (O.D550 2.2) and the time which taken for complete reduction of Cr(VI) was 1 hrs at pH7 and 37^{o}C.

3.3 Bioremediation of tannery effluent

In a trial to use the bacterium under investigation in bioremediation of wastewater that proved to contain 113.04 mg/l Cr(VI). The wastewater sample was allowed to pass through a column containing living *Bacillus laterosporus* immobilized cells on different solid supports (loofa, pumice and sponge one at a time). It was revealed that pumice is more efficient than other solid support. Moreover, cultures containing adsorbed cells on pumice particles showed the highest Cr(VI) reduction (66 and 100%) during the first and the second application runs (160 minutes), while both cases of loofa and in sponge the reduction rate was (45 and 64.6%) and (52 and 66.9%) respectively during the application runs (160 minutes). These results are in agreement with those obtained by El-Sersy and El-Sharony [16], who proved that highest Cr(VI) uptake was achieved with cultures immobilized on pumice surfaces.

The role of the *Bacillus* species in the detoxification of hexavalent chromium compounds, by reducing Cr(VI) to Cr(III), is very important in the bioremediation process (Clausen [17]). The attempts to remove the environmentally released Chromium compounds with biological methods have been performed especially using phytoremediation techniques, biosorption, bioaccumulation and bioprecipitation (Kratochvil and Volesky [18]), as well as bacterial activated sludge treatment of wastewaters (Figure 4).

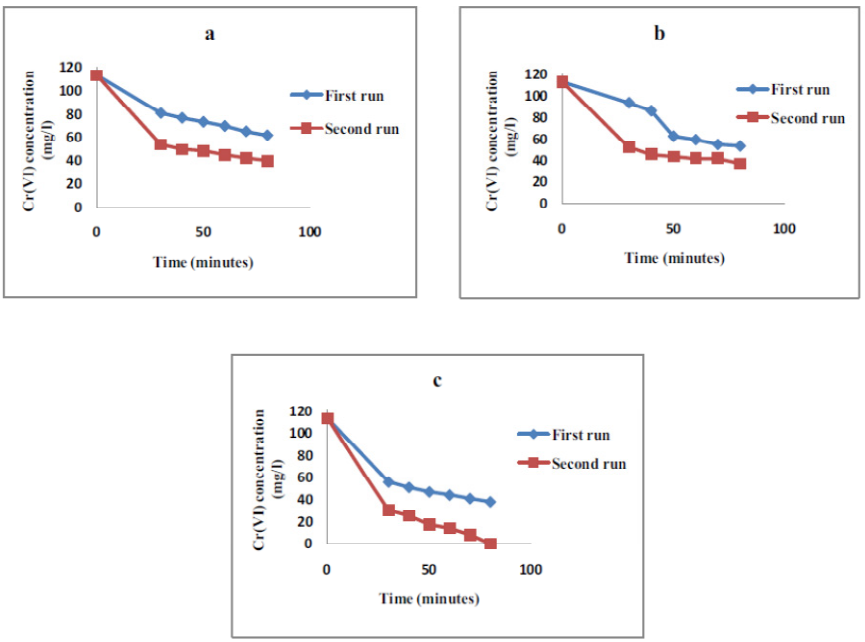


Figure 4: Bioremediation of the collected tannery effluent using immobilized bacterial cells on a-loofa, b-sponge and c-pumice as different solid supports. (Flow rate 2ml/10 minutes.)

4 Conclusion

The results of this study provide a basis for assessing a potential of using a chromate resistant *Bacillus laterosporus* for Cr(VI) reduction application. Overall, several key microbial characters of the bacterium under test namely: high tolerance to Cr(VI), ability to form dormant spores resistant to heat and desiccation, make it easily stored, transported and rapidly germinated into vegetative cells by the addition of simple nutrients and the ability to carry out aerobic chromate reduction under high temperature (22-55ºC) and alkaline pH which characteristic to the tannery effluent. Accordingly, *Bacillus laterosporus* is considered as a promising and ideal candidate that must be used in knowledge-based on toxic metal remediation system.

References

[1] Shen, H. and Wang, Y. Simultaneous chromium reduction and phenol degradation in a coculture of *Escherichia coli* ATCC 33456 and *Pseudomonas putida* DMP-1. Appl. Environ. Mirobiol., 61:2754-2758. 1995.

[2] Francisco, R.; Alpoim, M. and Morais, P. Diversity of chromium resistant and reducing bacteria in a chromium-contaminated activated sludge. Appl. Microbiol., 92: 837-843. 2002.

[3] Wakatsuki, T. Metal oxidoreduction by microbial cells. Ind. Microbiol., 14: 169-177. 1995.

[4] Lovley, D. Dissimilatory metal reduction. Annual Review of Microbiology, 47: 263-290. 1993.

[5] Garbisu C.; Alkorta, I.; Liama, MJ. and Serra, JL. Aerobic chromate reduction by *Bacillus subtilis.* Biodegradation, 9: 133-141. 1998.

[6] Roy, D. Practical Atlas for Bacterial Identification. CRC Press LLC ISBN., 1-56670-392-1. 2000.

[7] Wong, P. and Yuen, P. Decolorization and biodegradation of methyl red by *Klebsiella pneumoniae* RS-13. Water Re., 30: 1736-1744. 1996.

[8] Shakoori, A.; Tahseen, S. and Haq, R. Chromium-tolerance bacteria isolated from industrial effluents and their use in detoxification of hexavalent chromium. Folia Microbiol., 44:50-54. 1999.

[9] Plackett, R. and Burman, J. The design of optimum multifactorial experiments. Biometrika., 33:305-325. 1946.

[10] Ganguli, A. and Tripathi, A. Bioremediation of toxic chromium from electroplating effluent by chromate-reducing *Pseudomonas aeruginosa* A2Chr in two bioreactors. Appl. Microbiol. Biotechnol. 58:416-420. 2002.

[11] Michel, C.; Brugna, M.; Aubert, C.; Bernadac, A. and Bruschi, M. Enzymatic reduction of chromate: comparative studies using sulfate reducing bacteria. Appl. Microbiol. Biotechnol., 55:95-100. 2001.

[12] Puranik, P. and Paknikar, K. Biosorption of lead and zinc from solutions using *Streptoverti cillium cinnamoneum* waste biomass. Biotechnol., 55: 113-124. 1997.

[13] Ray, K.; Tisdale, J.; Dodd, R.; Dauban, P.; Puat, M. and Northup, J. Calindol appositive allosteric modulate or of the human Ca^{+2} receptor activities an extracellular ligand-inding domain-deleted rhodoposinlike seven trans membrane structure in absence Ca^{+2}. Biol. Chem., 280: 37013-37020. 2005.

[14] Liu, YG.; Xu, W.; Zeng, G.; Li, X. and Gao, H. Cr(VI) reduction by *Bacillus* sp. isolated from chromium landfill. Process Biochem., 41:1981-1986. 2006.

[15] Klonowska, A.; Clark, M.; Thiemon, S.; Giles, B. and Wall, D. Hexavalent chromium reduction in *Desulfovibrio vulgaris* Hildenborough causes transitory inhibition of sulfate reduction and cell growth. Appl. Micro. Bio. Technol., 78:1007-1016. 2008.

[16] El-Sery, N. and El-Sharouny, E. Nickel Biosorption by Free and Immobilized Cells of Marine *Bacillus subtilis* N10. Biotech., 6: 316- 321. Environmental Microbiology., 67: 1517-1521. 2007.

[17] Clausen, C. Isolating metal-tolerant bacteria capable of removing copper chromium and arsenic from treated wood. Waste Manage. Res., 18: 264-268. 2000.

[18] Kratochvil, D. and Volesky, B. Advances in the biosorption of heavy metals. TIBTECH, 6: 291-296. 1998.