Detoxification of potassium dichromate (Cr^{6+}) by nine isolated bacteria species as affected by incubation periods

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Abstract— Nine dichromate resistant bacteria were isolated: *Bacillus cereus*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus subtilis*, *Acinetobacter radioresistens*, *Acinetobacter venetianus*, *Ochrobacterum* sp, *Massilia niabensis*, and *Leucobacter chromiireducens*. All of the 9 isolated bacteria species absorbed and reduced Potassium dichromate Cr^{6+} from the growth medium amended by dichromate Cr^{6+} at concentration of 100 ppm. The Potassium dichromate Cr^{6+} absorption from the growth medium by bacteria species increased with increase in incubation period from 0 to 24, 48, 72 and 96 h. The highest dichromate reduction was attained by *Acinetobacter radioresistens*, with 62% *Acinteobacter ventianus*, with 54% and *Bacillus cereus* with 50%. The remaining Potassium dichromate Cr^{6+} in the growth media for all species decreased gradually with time from zero to 24, 48, 72 and 96 h.

Keywords—Detoxification, Potassium dichromate (Cr^{6+}), chromium resistant bacteria

1 INTRODUCTION

It is known that bacteria usually have the ability to accommodate a variety of pollutants such as heavy metals. They cannot destroy metals; instead they can influence the metal-mobility in the environment by modifying their chemical and/or physical characteristics. In addition, bioremediation can also play an increasing role in concentrating metals (including chromium), both to avoid toxicity and to recover metals for reuse (Yilmaz, 2003). Bacteria that survive and adapt in polluted soils depend on intrinsic biochemical and structural properties, physiological, and/or genetic adaptation including morphological changes of cells, as well as environmental modifications to metal speciation (Abou Shanab et al. 2007).

Generally, there are number of other ways by which bacteria can reduce the impact of free-metal toxicants. This may include: metal-binding and chelation to various components of the media; the formation of complexes; and of particular importance, the sorption or chelation of metals to unspecified organic compounds found in most growth media. Heavy metals and metalloids can be involved in a series of complex chemical biological interactions. The most important factors which affect their mobility are pH, sorbent nature, presence and concentration of organic and inorganic ligands, including humic and folic acids, root exudates and nutrients (Gadd, 2008).

Generally, mechanism of dichromate entry within bacterial cells and reduction include that potassium dichromate Cr^{6+} first enters the cells through the cellular membrane and reduced to Cr^{3+} in the cytoplasm, but $Cr^{3+}$is impermeable to biological membranes. Hence, Cr^{3+} generated inside the cell binds to protein and interacts with nucleic acids; Cr^{3+} is then free to bind to ionic sites and once bound, will act as a template for further heterogeneous nucleation and crystal growth (Daulton et al., 2001). Heavy metals (such as chromium salts) are soluble and have a high capacity for movement; these are the most damaging because they can cause contamination of the groundwater and also become accessible to plants where they can accumulated and magnified and then enter the food chain, finally reaching humans (Wu, L., 2004). Heavy metals are important man-made pollutants usually originate from various industrial wastes, especially leather tanning; this being considered one of the most important hazardous waste worldwide (Doble and Kumar, 2005). Studies indicated the possibility of using bacteria (e.g. *Pseudomonads, Aeromonads, Providencia sp*) to detoxifying polluted environments. For example, the last named species can grow at high concentrations of hexavalent chromium, ranging between (100 - 400 mg L-1, 37°C, pH 7), (Thacker et al., 2006; Srivastava et al., 2007; Congeevaram, et al., 2007). Although soluble chromate reductases have been reported from numerous bacteria, only a few have been purified and characterized.

The followings are some example of facultative anaerobes bacteria which reduce Cr^{6+} include: P. dechromaticans; P. chromatophila; Aeromonas dechromatica; Microbacterium sp. MP30; Geobacter metallireducens; Shewanella putrefaciens MR-1; Pantoea agglomerans SP1; Agrobacterium radiobacter EPS-916 and a consortium capable of simultaneously reducing $Cr^{6+}$and degrading benzoate (Pattanapipitpaisal et al 2001; Myers et al 2000). Other examples of bacteria reducing Cr^{6+} which are compatible with this study include: *B. cereus; B. subtilis, P. aeruginosa; P. ambigua; P. flourescens; E. coli; Achromobacter Eurydice; Micrococcus roseus; Enterobacter cloacae*; *Desulfovibrio desulfuricansand De. Vulgaris* (Lovley, 1994). The immobilization of cells in the agarose-alginate gel was found to slightly enhance Cr^{6+} reduction activity in *P.aeruginosa* A2Chr .However as the thickness of the biomass layer increases, mass transport will inevitably reduce the rate of detoxification (Ganguli and Tripathi, 2002).

In this study 9 dichromate resistance bacteria isolated from the leather tanning factories at El-Riyadh industrial area were used in Detoxification of dichromate (Cr^{6+}) concentrations.

2 Materials and Methods

2.1 Preparation of Bacteria Isolates

From the highly Potassium dichromate (Cr^{6+}) contaminated soil, solid wastes and discharged water of the leather tanning factories in Riyadh industrial area nine bacteria isolates : *Bacillus cereus*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus subtilis*, *Acinetobacter radioresistens*, Acinetobacter venetianus, *Ochrobacterum* sp, *Massilia niabensis*, and *Leucobacter chromiireducens* were isolated using the following chemicals, Potassium dichromate ($K2Cr_2O_7$ 99% purity) - Sulfuric acid (H_2SO_4 99% purity)- 1,5 diphenyl carbazide (99% purity)- Luria Bertani (LB) broth and Luria Bertani agar, (LB) agar, and also Nutrient Broth- agar- Plate count (PC) agar, sodium phosphate, potassium phosphate, sodium chloride (NaCl) and ammonium chloride.

2.2 Preparation of Cr^{6+} Stock Solutions

A chromate Cr^{6+} ($K_2Cr_2O_7$) stock solution was made by dissolving (2.827 g) of $K_2Cr_2O_7$ in 1L of distilled water to make a solution of 1000 mg Cr^{6+} per liter; and the Cr^{6+} standard solutions were prepared from this Cr^{6+} stock solutions by dilution with distilled water according to the following equation: (V x C='V x 'C) to give the desired final Cr^{6+}concentrations (50- 500 ppm). The total Cr concentrations in soil samples were analyzed as in (Page, 1982), and in wastewater samples according to (Hossner, 1991).

Before identification of the chromium resistant species, a process of enrichment of the bacterial media was carried out, and nutrient broth (100 mL) was mixed with all samples and with 50 ppm of Cr^{6+} from a potassium dichromate solution ($K_2Cr_2O_7$), and concentrations of Cr^{6+} were used to insure the selectivity of the medium and isolation of chromium-tolerant bacteria only. After inoculation; samples were incubated on a rotary incubator, where incubation lasted for 48 hours at 200 rpm and 33 ± 37°C.

The spread plate technique- colony-forming units (CFUs), was used to determine the number of viable cells on LB agar and nutrient agar plates supplemented with Cr^{6+} concentrations ranging from (zero, 50, 100, 150, 200, 300, 400 mg L^{-1} (Moore and Dowhan, 2002; Bertani, 2004).

2.3 Preparation of Minimal Media 20% LB Preparation Protocol

This was prepared taking into account the optimum culturing condition of the isolated bacteria species, where they could grow best and yield the highest reduction rates, and to identify the resistivity of the bacteria to toxic chromium and characterise the environment in which the bacteria can be grown. This experiment focused on starving bacteria using nutrient-poor media. Minimal media with 20% L.B was used for culturing; pH was set to 7, 6 and 8 in other experiment using Tris-HCL buffer for preparation of the reagents.

Potassium dichromium was determined calorimetrically OD540 according to (Spectronic 1001, Milton Roy Co., Rochester, NY), using the diphenylcarbazide method.

2.4 Standard Curve Preparation

To measure hexavalent chromium, the1, 5-diphenylcarbazide reaction was used (Pflaum and Howick, 1956). A linear Cr^{6+} standard curve (figure 1) was generated by plotting absorbance (at 540 nm). The standard curve for Cr^{6+} measured demonstrated a high degree of accuracy with R2 = 99.5 % for a composite data set from predetermined points. This standard curve was then used to determine the Cr^{6+} concentration at OD540 using a spectrophotometer. Using the standard curve, results are converted from OD540 to ppm.

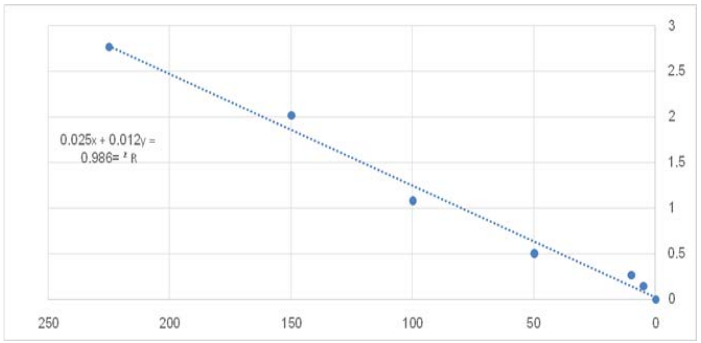


Figure (1): A linearized Cr^{6+} standard curve

3 RESULTS

Bacterial Potassium Dichromate Reduction Assays in Minimal Medium

The results in (tables 1 & 2 and fig.2-3) showed that the bacteria species reduction rate of concentration of potassium dichromate Cr^{6+} at 100 ppm which was recorded by the spectrophotometer OD540 at pH 7.5, ± 37 °C, continued increase with increase in incubation period up to 96 h. Generally the 9 isolated bacteria species started reducing concentration of Potassium dichromate Cr^{6+} from the medium and Cr^{6+} reduction increased with time from 22% up to 24 h, to 26% up to 48 h, and reached 46% at 72 and 96 h. The *Acinetobacter radiresistens* recorded an average percentage reduction up to (58 - 62%) in minimal media through 72- 96 h, as the highest rate of reduction compared to all tested bacteria species. In addition, all bacterial species recorded continuance increase in dichromate concentration absorption with increase in incubation period from < 24 up to 96 h. Figures (4-12) illustrate the fractions of Cr^{6+} remained after reduction assay at 100 ppm in the minimal media through the periods 0, 24, 48, 72 and 96 h; the fractions of Cr^{6+} remained in case of the different species are as follows: *Bacillus cereus* (50%), *Bacillus pumilus* (58%), *Bacillus licheniformis* (58%), *Bacillus subtilis* (54%), *Acinetobacter radioresistens* (38%), *Acinetobacter venetianus* (56%), *Ochrobacterum sp* (62%), *Massilia niabensis* (64%), and *Leucobacter chromiireducens* (54%), and there are continuance reduction in concentration with increase in time for all bacteria species.

Table 1: Dichromate reduction by bacterial cells in minimal medium at 100ppm

Time period	24 h	48 h	72 h	96 h
Overall rate of growth reduction in minimal medium at 100 ppm	22 %	26 %	46%	46%

4 DISCUSSION

The 9 isolated bacteria species succeeded in adsorbing Potassium dichromate Cr^{6+} from the growth medium, and the percentage of removed dichromate start increasing with increase in incubation period up to 96 h. Only 3 bacteria species succeeded in reducing dichromate to levels more than 50%, and these are *Acinetobacter radioresistens*, with 62% Acinteobacter ventianus, with 54% and *Bacillus cereus* with 50%. The species with the lowest dichromate removal is *Massilia niabensis* which was able to remove up to 36% after 96 h. However, it is unclear whether cellular uptake of toxic Cr^{6+} occurs with reduction localized to the cytoplasm or periplasm, and/or electrons are transferred outside the cells to reduce chromium extracellularly. Branco et al. (2004) and Ramirez et al. (2004) showed that most chromate reduction bacteria exhibit resistance to Cr^{6+} even when exposed to concentrations up to 300 ppm.

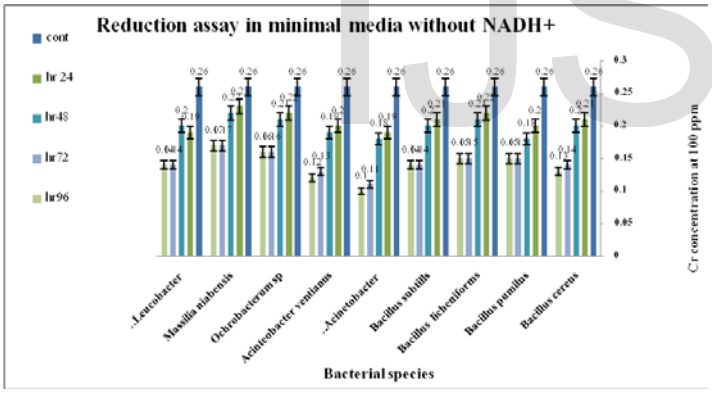


Figure (2):Overall rate of dichromate reduction in minimal medium at 100ppm

Generally, mechanism of dichromate entry within bacterial cells and reduction include that potassium dichromate Cr^{6+} first enters the cells through the cellular membrane and reduced to Cr^{3+} in the cytoplasm, but $Cr^{3+}$is impermeable to biological membranes. Hence, Cr^{3+} generated inside the cell binds to protein and interacts with nucleic acids; Cr^{3+} is then free to bind to ionic sites and once bound, will act as a template for further heterogeneous nucleation and crystal growth as was mentioned by (Daulton et al., 2001). Chromium penetrates into the cells and accumulates within the cells as precipitates, and this could be due to precipitation of Cr^{3+} in the form of hydroxyl and carboxyl group (McLean and Beveridge, 2001).

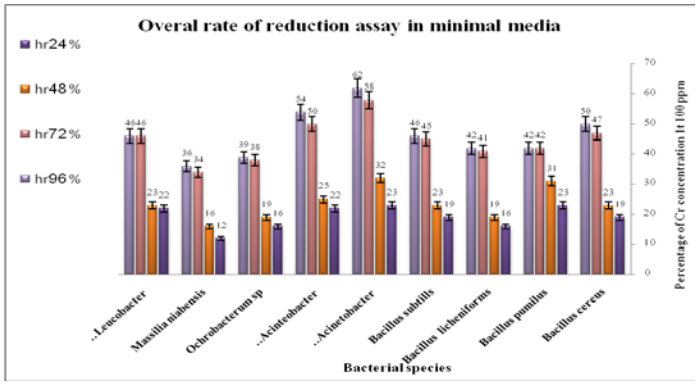


Figure (3): Overall rate of potassium dichromate reduction assay in 100 ppm medium by bacterial cells

Figure (4): Fraction of Cr^{6+} remained after reduction assay at 100 ppm in minimal media by *B. cereus*

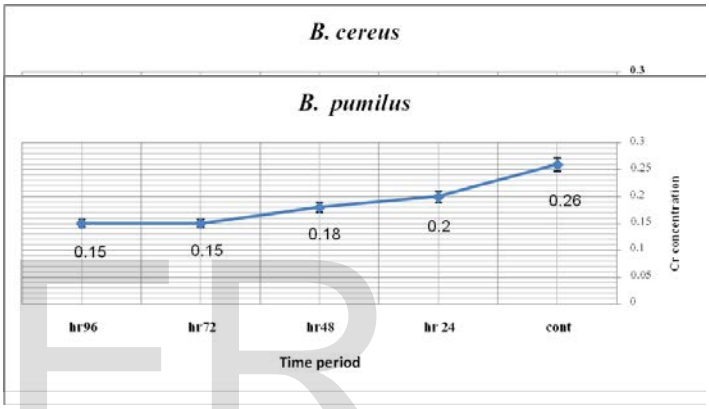
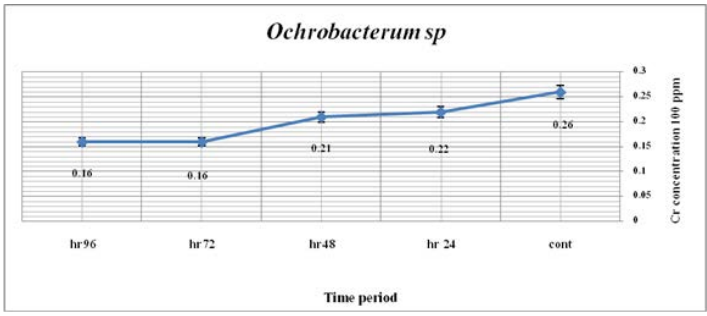


Figure (5): Fraction of Cr^{6+} remained after reduction assay at 100 ppm in minimal media by *B. pumilus*

Bishnoi, et al. (2004) concluded that after 96h, removal efficiency of Cr^{6+} by Pseudomonas putidabecame remained constant because adsorption and desorption becomes equal to each other. They also found that most of the hexavalent chromium (90%) absorbed by Pseudomonas putida from the industrial effluent was during 96 hours of incubation period, and increased with the increase of concentrations. Laxman and More, 2002 & Singh et al., 2013 said that metal biosorption increased with increase in incubation period as long as binding sites are available and reach its capacity or equilibrium after 96 h.



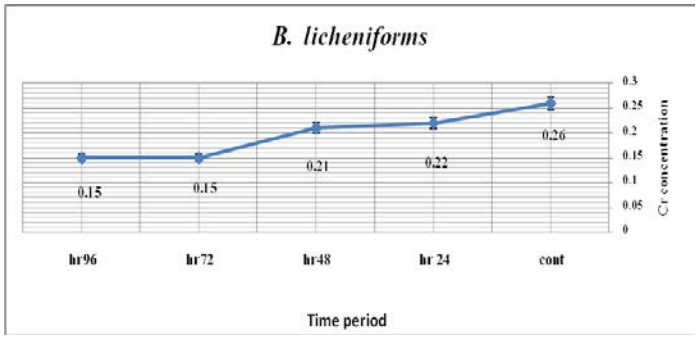


Figure (6): Fraction of Cr^{6+} remained after reduction assay when 100 ppm in minimal media by *B. licheniforms*

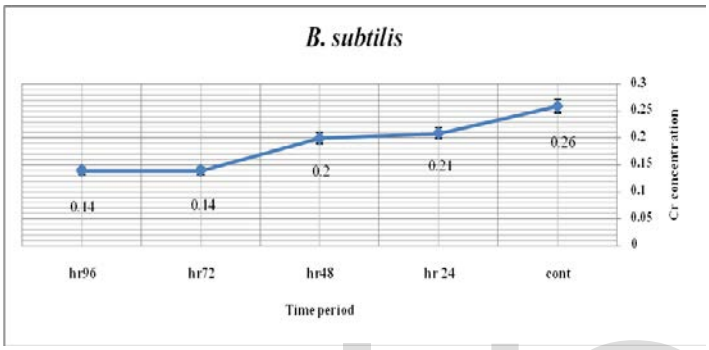


Figure (7): Fraction of Cr^{6+} remained after reduction assay at 100 ppm in minimal media by *B. subtilis*

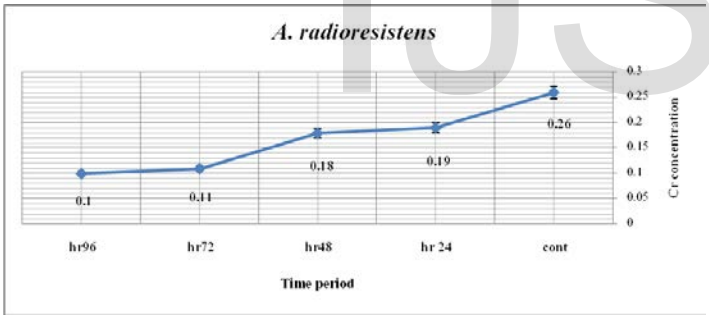


Figure (8): Fraction of Cr^{6+} remained after reduction assay at 100 ppm in minimal media by *A. radioresistence*

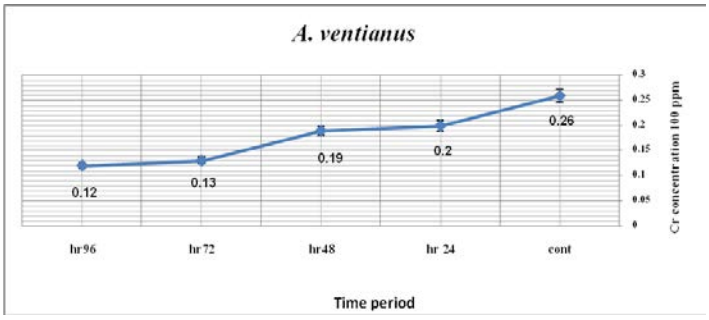


Figure (9): Fraction of Cr^{6+} remained after reduction assay at 100 ppm in minimal media by *A. ventianus*

Figure (10): Fraction of Cr^{6+} remained after reduction assay at 100 ppm in minimal medium by *Ochrabacterium* sp.

Figure (11): Fraction of Cr^{6+} remained after reduction assay at 100ppm in minimal media by *M. niabensis*

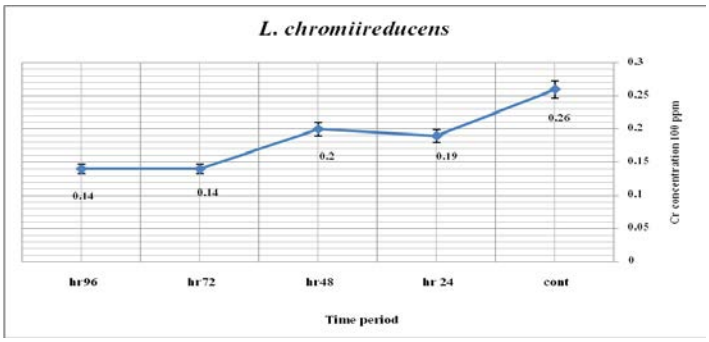


Figure (12): Fraction of Cr^{6+} remained after reduction assay at 100 ppm in minimal media by *L. chromiireducens*

Reduction of Cr^{6+} has been reported by bacteria belonging to diverse genera such as Enterobacter, Bacillus, Ochrobacterium, Escherichia, Pseudomonas, Arthrobacter, Streptomyces, Providencia, Exiguobaterium and Leucobacter (Cheung and Gu, 2007; Sarangi and Krishnan, 2008). Chromate Cr^{6+} reduction by bacteria can be chemical or enzymatic; enzymatic reduction of Cr^{6+} involves soluble and membrane bound reductases (Donati et al., 2003). Although soluble chromate reductases have been reported from numerous bacteria, only a few have been purified and characterized. Sequence homology studies indicated that chromate reductase activity is probably not the primary function of these enzymes. Therefore, it appears that chromium reductase activity is actually a secondary function associated with different primary catalytic functions (Opperman et al., 2007).

CONCLUSION

All of the 9 isolated bacteria species absorbed and reduced Potassium dichromate Cr^{6+} from the growth medium. The Potassium dichromate Cr^{6+} absorption from the growth medium by bacteria species increased with increase in incubation period from 0, 24, 48, 72 to 96 h. It was found that overall proportion for total reduction to all bacterial species at 100 ppm in LB media in this study was approximately 64% after 96 h, and 44% taking into account the percentage of each species. The fractions of Cr^{6+} remained in case of the different species are as follows: *Bacillus cereus* (50%), *Bacillus pumilus* (58%), *Bacillus licheniformis* (58%), *Bacillus subtilis* (54%), *Acinetobacter radioresistens* (38%), Acinetobacter venetianus (56%), *Ochrobacterum* sp (62%), *Massilia niabensis* (64%), and *Leucobacter chromiireducens* (54%), and there are continuance reduction in concentration with increase in time for all bacteria species.

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