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Isolation and Identification of Nine Dichromate Cr^{+6} resistance Bacteria by Modern Techniques

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Abstract The site of the leather tanning factories in Riyadh industrial area is highly contaminated with Potassium dichromate, and the rate of chromium in the soil samples in this site are 183 mg kg^{-1}, and this exceeds the allowable limits of the Lindsay method or standard (100 mg kg^{-1}), and in the dischargeable wastewater is 3.4 mg L^{-1}, which exceeds the allowable limits of the Jordanian Standard No. 202 f (2 mgL^{-1}) for total chromium. A number of 9 bacteria species which are characterized as dichromate resistant bacteria have been identified. The bacterial isolates have been defined by the16S rRNA method, these are as follows: *Bacillus cereus, Bacillus pumilus, Bacillus licheniformis, Bacillus subtilis, Acinetobacter radioresistens, Acinetobacter venetianus, Ochrobacterum sp, Massilia niabensis* and *Leucobacter chromiireducens*. All bacterial species in this study were shown to have high resistance to potassium dichromate Cr^{6+}, with *Acinetobacter* notherradio resistants leading, followed by *Acinetobacter ventianus*, then comes *Bacillus pumilus* and *B. cereus*.

Keywords Chromium detoxification, chromium resistance bacteria

Introduction

The process of discrimination and assessment of genetic diversity is a major objective for the definition and characterization of bacterial strain. Recent research has shown that the definition and evaluation of many of the microbes on the basis of physiological and morphology variances may be inaccurate because of the great similarity between many of the micro-organisms which leads to erroneous results. In addition, changes in the genetic characteristics continuously occur. The emergence of modern technologies has had a great impact in overcoming these difficulties. The use of randomly amplified polymorphic DNA (RAPD) by the polymerase chain reaction (PCR) [1-2]. The 16S rRNA genes of isolates were amplified by a reverse transcriptase-polymerase chain reaction (RT-PCR) using primers (KAPA Taq m.max, 26(F) Primer, 1492(R) Primer, ddwater). The resulting sequences were compared to known bacteria in the GenBank using a basic BLAST search of the National Center for Biotechnology Information (NCBI, Bethesda,MD). The 16S rRNA is used to established microbial species interrelationships based on proximity of genetic phyla. This method is thus valuable as a tool for studying microbial diversity and interdependence of species in a consortium [3-4]. Environmental pollution problems due to the illegal disposal of dichromate is highly concerned for its high solubility in water and high absorption rate onto animal and plant cells. In addition, soil and groundwater are the most susceptible environments to chromate and dichromate contamination. For example,Cr^{6+} concentrations as low as 10 ppm can reduce plant seed germination, cause root damage, and retard growth, photosynthesis and enzymatic activities in algae. Although Cr^{6+} can be reduced by algae or plants in soil, bacteria are generally considered the most efficient reducers of dichromate [5]. In contrast, trivalent chromium is insoluble and less toxic [6]; however, at high concentration it is too toxic, carcinogenic and teratogenic [7]. Puzon *et al.,* (2002) reported that in order to reduce chromate Cr^{6+}, bacteria must be able to tolerate chromate. Similarly, Coleman (1988) concluded that bacteria, notably Gram positive forms, are significantly tolerant to chromate Cr^{6+} toxicity

even at relatively high concentration of the ion, while Gram-negative chromium reducing bacteria tend to be more sensitive to Cr^{6+}.[8]. In this respect, Kilic *et al.* (2007) proved that reduction of Cr^{6+} was accomplished by other three Gram negative species, namely *Pseudomonas aeruginosa*, *Salmonella eterica*, *Ochrobacterum sp*. Also, Polti *et al*., (2007) reported that some microorganisms including bacteria in the presence or absence of oxygen can reduce the toxic form of chromate Cr^{6+} to trivalent form Cr^{3+} [9-10].

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 [11]. When metals are presented within bacterial cells, there will be changes in function groups where there is a metal binding process taking place on the surface of the cells with certain functional groups. The functional groups involved in the interaction with chromium are ionizable functional groups that included amino, carboxyl, and hydroxyl groups. This is because the amino and carboxyl groups, and nitrogen and oxygen of the peptide bonds are available for coordination bonding with metal ions such as Cr^{6+} [10]. 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 extra-cellularly. Branco *et al.* (2004) and Ramirez *et al*. (2004) showed that most chromate reduction bacteria exhibit resistance to Cr^{6+} even when exposed to contraptions upto 300 mg L^{-1} [12-13].

According to Puzon *et al*. [14] chromate Cr^{6+} present in bacteria can be reduced by either physiological reducing agents, such as Glutathione, which is essential cofactor for many enzymes that require thiol-reducing equivalents, and helps keep redox-sensitive active sites on enzyme in the necessary reduced state. 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 [15]. Abou-Shanab *et al.* (2007) showed that Cr^{6+} resistance and/or Cr^{6+} reduction abilities were found in most of the isolated bacteria in their study in which approximately 54% of the isolated bacteria belonged to the genera *Acinetobacter* and *Ochrobactrum* [16].

The aim of this study is to identify and isolate Cr^{6} resistance bacteria that can absorb and reduce the high levels of dichromate from the discharged water and soil and dry wastes of the leather tanning factories in Riyadh industrial area, Saudi Arabia.

Materials and Methods

The leather tanning factories located at about 16 km from Riyadh city in Saudi Arabia is dispose large amounts of wastewater, sludge and liquid waste, which is estimated at 20-80 cubic meters per ton of any raw leather, *i.e.* almost 8000 cubic meters each month and discharge these wastes in the water of WadiHanifa and closer to some cattle farms, and this may lead to contamination of the underground wells water along the valley. These wastes are contaminated with chromium (Cr), as these factories consume approximately 5- 8 tons of chromium salts (Potassium dichromate) every month.

Determination of chromium concentration in Factories area

Soil dry waste samples and samples of water discharged from the factories were collected from the surface layer at depths ranging from almost 0-30 cm from different locations of the 4 factories area during 2013- 2014 for determination of chromium concentration. The total concentration of chromium in soil samples was determined after digesting the soil with the following acids HF, $HClO_4$, H_2SO_4.The concentration of heavy metals was then determined using plasma ICP- AES /OES (Perkin Elmer, 4300 DV). The total Cr concentrations in soil samples were analyzed as in (Page, 1982), and in wastewater samples [17-18].

Table 1: Chemical analysis of soil, water and dry waste samples

Source	soil	Water	Dry waste
(Cr) Conc. (W: mg/l) (S: mg/kg)	156.92	1.67	21.1

Processes of Isolation of Bacteria Strains

Before identification of the chromium resistant species, a process of enrichment of the bacterial media was carried out, and nutrient broth (100mL) 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 200rpm and 33±37^{o}C.

The spread plate technique-colony-forming units (CFU),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}).

Identification and characterization of Cr-resistant bacteria

The process of discrimination and assessment of genetic diversity is a major objective for the definition and characterization of bacterial strain. The use of randomly amplified polymorphic DNA (RAPD) by the polymerase Chain Reaction (PCR) [1-2].The 16S rRNA method was used. A single colony of each overnight plate was suspended in RNAse containing sterile d.dH_2O (100 µL). Then the suspensions were placed in boiling water for 5 minutes, immediately afterwards it was placed in ice; this procedure breaks down the cells [19]. The suspension was then centrifuged ×8000 g for 10 minutes, the supernatant containing the nucleic acids was kept and to insure purity, the centrifugation was done twice. The supernatant was kept on each occasion. Genomic DNA was extracted from the pure cultures using a DNeasy tissue kit.

The 16S rRNA genes of isolates were amplified by a reverse transcriptase- polymerase chain reaction (RT-PCR) using primers (KAPA Taqm.max, 26(F) Primer, 1492(R) Primer, ddwater).The resulting sequences were compared to known bacteria in the GenBank using a basic BLAST search of the National Center for Biotechnology Information (NCBI, Bethesda, MD). The 16S rRNA is used to established microbial species interrelationships based on proximity of genetic phyla. These isolates were adjoined to existing relatives in a phylogenetic tree in which relationship between these isolates and existing ones were shown where this analysis showed that species shared 95% sequence similarity and had sequence similarities of 94.9-98.1% with respect to species strains of species belonging to the genus all those species.

The Isolated DNA was loaded into wells made in an agarose which is an electrophoresis buffer, and electric field was applied to mobilize the DNA. DNA having negative charge because of its phosphate groups got mobilized towards anode. And based on the molecular weight of the DNA, separation or the movement happened, where the smaller ones move faster and farther than the larger DNA fragments. Ethidium bromide (EtBr) was used as a DNA staining dye which binds to DNA and thus can be visualized under UV light.

Potassium dichromium was determined calorimetrically OD_{540} (Spectronic 1001, Milton Roy Co., Rochester, NY), using the diphenylcarbazide method.

Standard Curve Preparation

To measure hexavalent chromium, the 1,5-diphenylcarbazide reaction was used [20]. A linear Cr^{+6} standard curve (figure 5) was generated by plotting absorbance (540 nm). The standard curve for Cr^{+6} measured demonstrated a high degree of accuracy with R^2 = 99.5 % for a composite data set from predetermined points. This standard curve was then used to determine the Cr^{+6} concentration at OD_{540} using a spectrophotometer. Using the standard curve, results are converted from OD_{540} to ppm. The line equation is of our spectrophotometer is: $x_{ppm} = 0.0122x_{OD} + 0.0255$, and conversion to mM by :$x_{mM} = 0.0027x_{ppm}$.

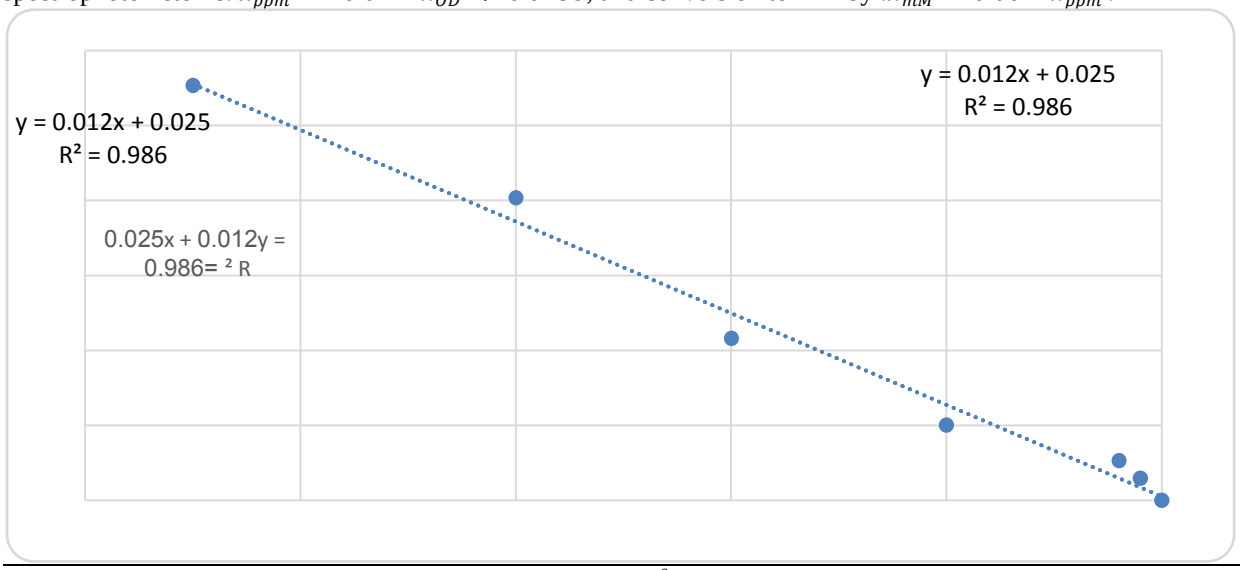

Figure 1: A linearized Cr^{+6} standard curve

Results

The PCR macrogen products obtained from the isolates were sent for sequencing to "Advanced Genetics Technology Center/ University of Kentucky", USA (table 4 & figure 1), and only nine of bacterial species had

been fully defined by PCR analysis. The obtained sequences were compared to bacterial isolates in GenBank using a basic BLAST search of the National Center for Biotechnology Information (NCBI, Bethesda, MD). The bacteria identified are: *Bacillus subtilis, Bacillus licheniformis, Bacillus pumilus, Acinetobacter radio resistens, Acinetobacter venetianus, Massilia niabensis., Ochrobactrum* sp., *Bacillus cereus, Leucobacter chromiireducens.*

Table 2: sample numbers which are used for PCR product

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**
A_{10} w	A_{11} w	A_{12} w	B_2 W	B_8 D	B_9 S	C_8 D	C_{18} w	C_{22} S	Z1 w	Z2 w	Z3a w	Z3b w	Z3c w	Z4 w	Z6 S	Z8a S	Z8b S	Z9a D	Z9b D	Z9e D	Z10 S	Z11 S

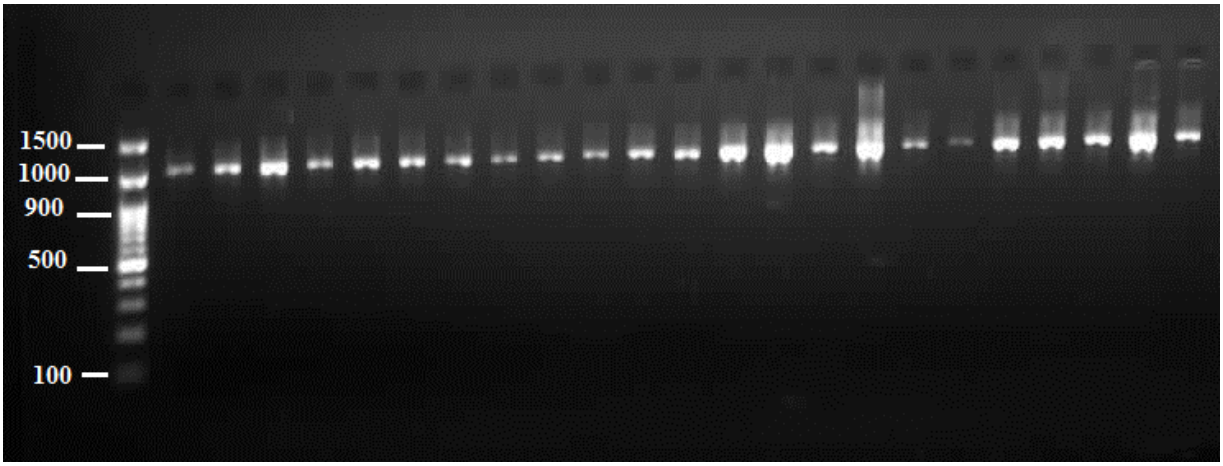

Figure 2: PCR products based on primers specific for 16S rRNA .

The description of the different isolates is as follows:

Bacillus cereus: (Figure 3)

A Gram positive, rod-shaped, facultative anaerobes bacterium and some strains are harmful to humans and cause food borne illness, while other strains produce beneficial robotics for animals. Like other members of the genus *Bacillus*, it can produce protective end spores.

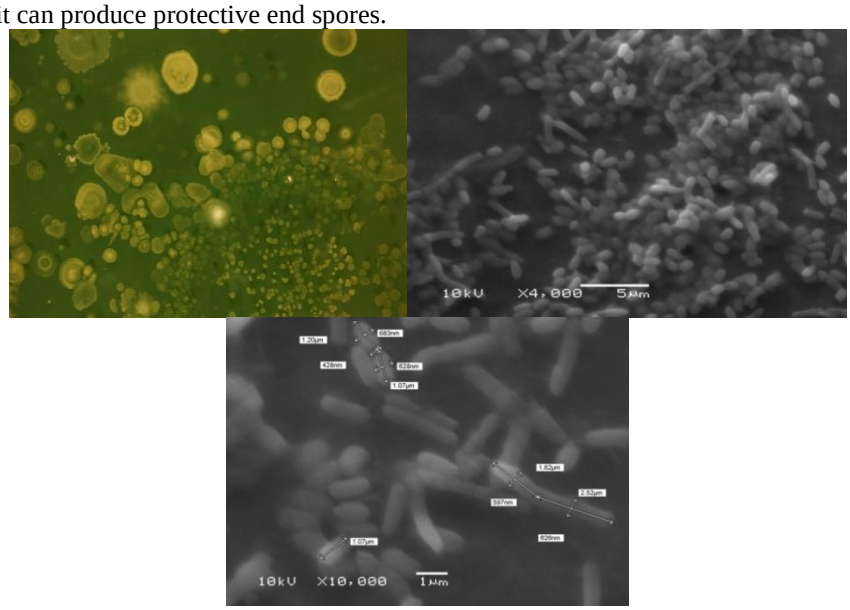

Figure 3: Morphology colony and Scanning Electron microscope image of Bacillus cereus.

***Bacillus pumilus:* (Figure 4)**

A Gram-positive, aerobic, spore-forming species which is commonly found in soil and it generally shows resistance to environmental stresses; including UV light exposure, desiccation and the presence of oxidizing agents, such as hydrogen peroxide. Some strains of *B. pumilus* isolates show high salt tolerance.

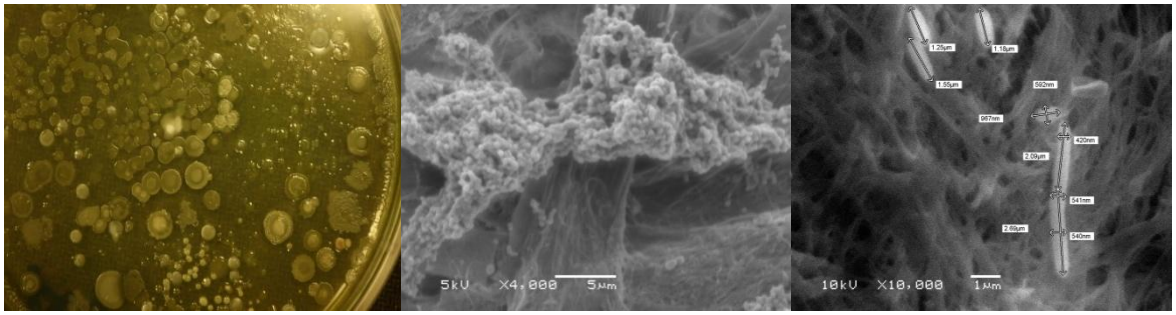

Figure 4: Morphology colonyand Scanning Electron microscope image of Bacillus pumilus.

***Bacillus licheniformis*: (Figure 5)**

It is Gram-positive, aerobic and is commonly found in the soil and bird feathers. It is mesospheric bacterium and its optimal growth temperature is around 30 ^{o}C, although it can survive at much higher temperatures. The optimal temperature for enzyme secretion is 37 ^{o}C. It can exist as spores and resist harsh environments. The bacterium is also well adapted to grow in alkaline conditions.

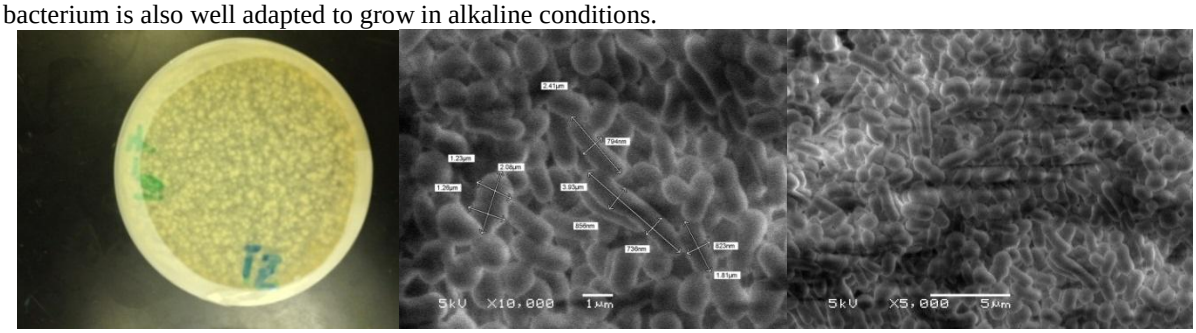

Figure 5: Morphology colony and Electron microscope image of Bacillus licheniforms.

***Bacillus subtilis*: (Figure 6)**

It is Gram positive, catalase, rod-shaped, and an obligate aerobic. It has the ability to form a tough, protective endospore and can tolerate extreme environmental conditions. It is found in soil and is a normal gut commensal in humans.

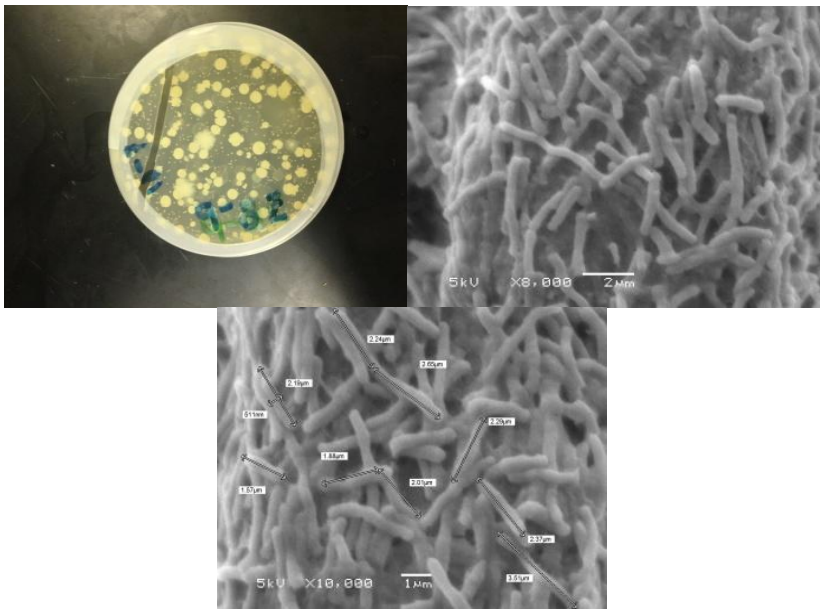

Figure 6: Morphology colony and Electron microscope image of Bacillus subtilis.

***Acinetobacter radioresistens:* (Figure 7)**

It is a Gram negative, aerobic, not spore-forming, non- motile, non-fermentative, pleomorphic and coccobacilli-shaped. Colonies are circular, convex and smooth.

It occurs in microbial communities in a variety of ecosystems such as soil, freshwater, wastewater and solid wastes. Several strains belonging to the genus *Acinetobacter* have been attracting growing interest from the medical, environmental and a biotechnological point of view. Bacteria of this genus are known to be involved in

the biodegradation, leaching and removal of several organic and inorganic man-made hazardous wastes, and can produce important industrial bio-products.

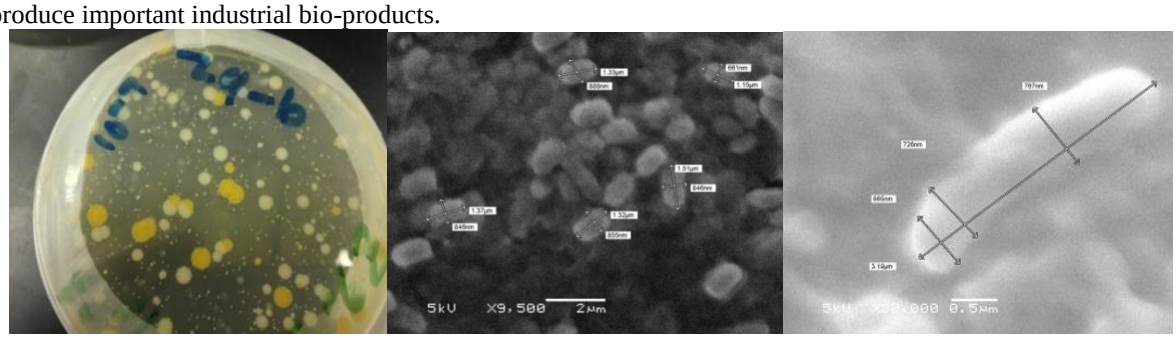

Figure 7: Morphology colony and Electron microscope image of Acinetobacter radioresistens.

Acinetobacter venetianus: **(Figure 8)**

It is Gram negative, aerobic, non spore-forming, non motile, non fermentative, pleomorphic and coccobacilli-shaped. *Acinetobacter venetianus* is a species of bacteria notable for degrading *n*-alkanes. It harbors plasmids carrying sequences similar to the *Pseudomonas oleovorans* alkane hydroxylase gene.

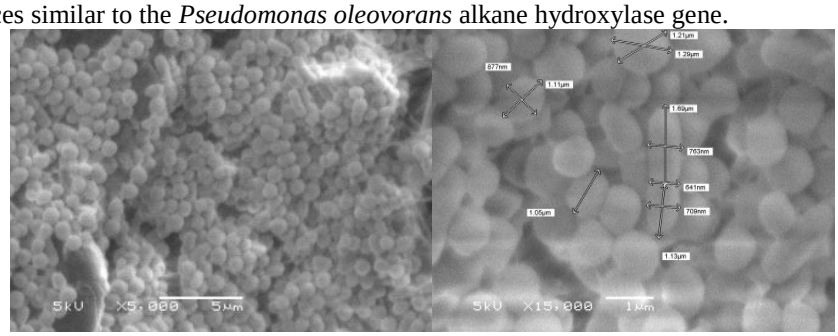

Figure 8: Morphology colony and Electron microscope image of Acinetobacter venetianus.

Ochrobacterum **sp. (Figure 9)**

It is Gram-negative, obligate aerobic, producing short straight or curved rods, non-spore forming and non-fermentative. The genomes of most *Ochrobacterum sp*are complex and often associated with two independent circular chromosomes.

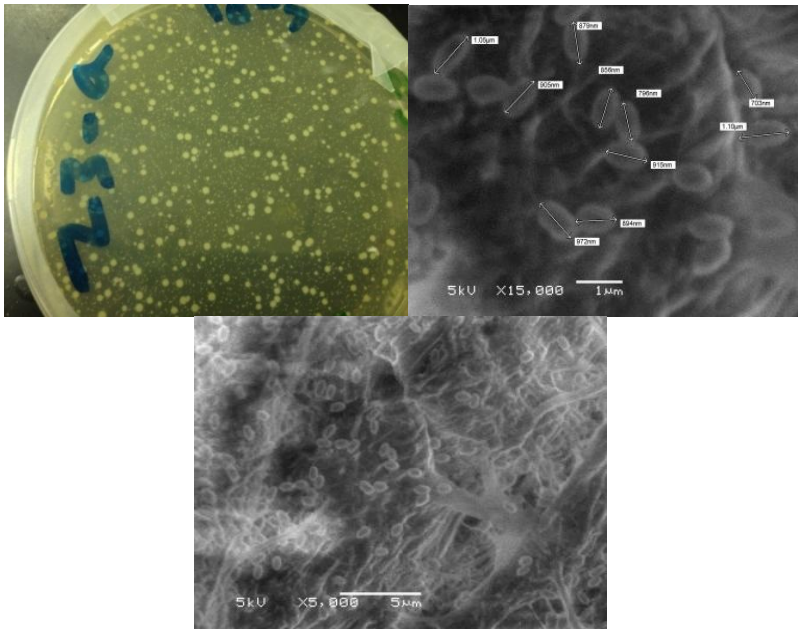

Figure 9: Morphology colony and Electron microscope image of Ochrobacterum sp.

Massilia niabensis: **(Figure 10)**

Massilia niabensis is a gram negative, obligately aerobic, motile rod-shaped bacteria including both animal and plant pathogens, as well as some environmentally important species. It is renowned for being catalase positive as its ability to degrade chlororganic pesticides and polychlorinated biphenyls (PCBs).

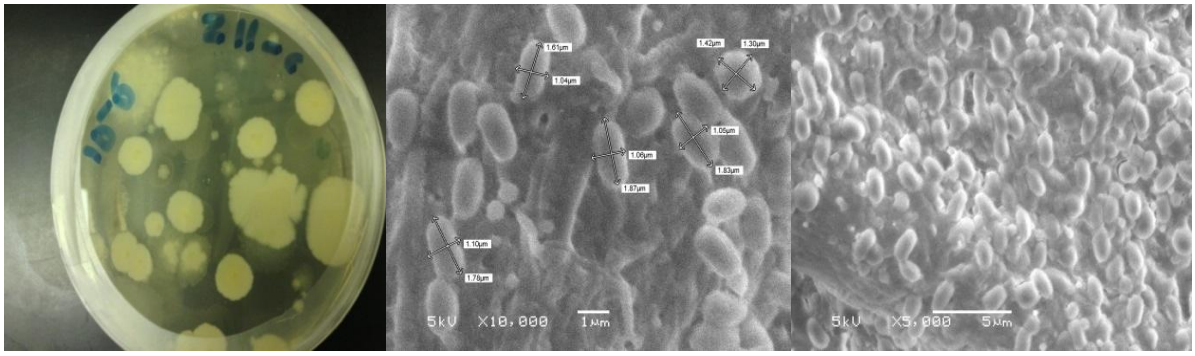

Figure 10: Morphology colony and Electron microscope image of Massilianiabensis.

***Leucobacter chromiireducens*: (Figure 11)**

It is Gram-positive, aerobic, non-motile, non-spore-forming, irregular rod-shaped and produces a yellow-pigmented. It has a DNA G+C content of 69.5 mol%, and possesses 2, 4-diaminobutyric acid in the cell-wall peptidoglycanas well as iso-fatty acids, polar lipids diphosphatidylglycerol and phosphatidylglycerol.

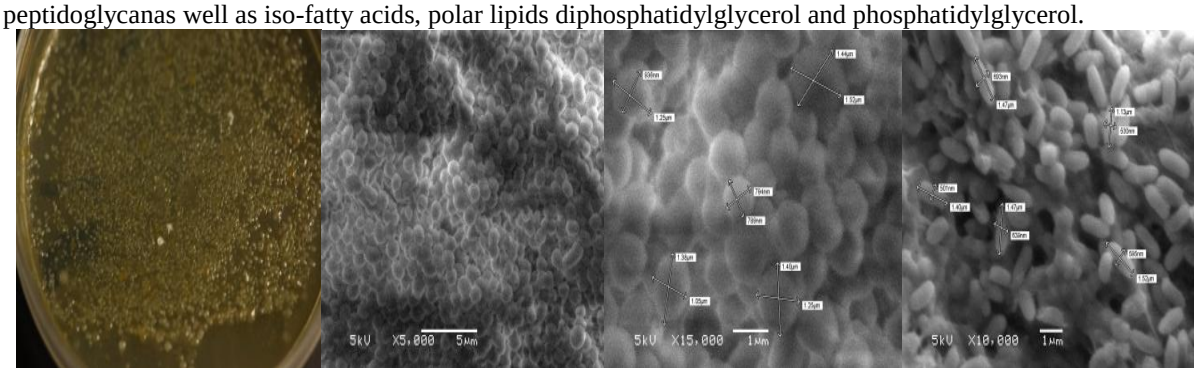

Figure 11: Morphology colony and Electron microscope image of Leucobacter chromiireducens sub sp.

Bacterial Potassium Dichromate Reduction Assays in Minimal Medium

The ability of the 9 bacteria species for detoxification of chromium (Potassium dichromate) or removal of chromium (Cr) from the chromium contaminated area of leather tanning factories was tested and the results are shown in table (3).

Table 3: Concentration of Cr^{6+} OD $_{540}$ at pH =7.5 , 37^{o}C in minimal medium Bacteria cells = 6µl

Species	cont	% 0hr	24 hr	% 24hr	48hr	% 48hr	72hr	% 72hr	96hr	% 96hr
Bacillus cereus	0.26	0	0.21	19	0.20	23	0.14	47	0.13	50
Bacillus pumilus	0.26	0	0.20	23	0.18	31	0.15	42	0.15	42
Bacillus licheniforms	0.26	0	0.22	16	0.21	19	0.15	41	0.15	42
Bacillus subtills	0.26	0	0.21	19	0.20	23	0.14	45	0.14	46
Acinetobacter radioresistens	0.26	0	0.19	23	0.18	32	0.11	58	0.10	62
Acinteobacter ventianus	0.26	0	0.20	22	0.19	25	0.13	50	0.12	54
Ochrobacterumsp	0.26	0	0.22	16	0.21	19	0.16	38	0.16	39
Massilianiabensis	0.26	0	0.23	12	0.22	16	0.17	34	0.17	36
Leucobacter chromiireducens	0.26	0	0.19	22	0.20	23	0.14	46	0.14	46

Three bacteria species can reduce dichromate concentration at percentages more than 0%, *Acinetobacter radioresistens (62%, Acinteobacter ventianus (54%), Bacillus cereus (50%)* after 96 hrs.

Discussion

In the present study, the data of chemical analyses of soil, wastewater, and dry waste (sludge) samples collected from the study areas revealed its contamination with total chromium, which exceeds the permissible limits. The soil type in the study areas is generally "sandy loam" and is considered weak to medium in terms of soil permeability. Ajmal *et al*., [21] investigated the effect of the adsorption of chromium onto the clay sand mixture clay soil and reported its suitability as adsorbent for chromate due to its high cation exchange capacity (CEC) and strong binding capability. Only 23 bacterial isolates out of a total of 60 bacterial isolates were able to grow, and only9 bacterial isolates were highlighted in this study.

Nine bacteria species have been isolated in this study and defined by 16S rRNA method showed good growth at different concentration of chromate Cr^{6+} and had the ability to grow and minimize toxicity of potassium dichromate. These isolates were as follows: *Acinetobacter ventianus*, *Acinetobacter* notherradio resistens,

Ochrobacterum sp, Bacillus cereus, Bacillus pumilus, Bacillu ssubtilis, Bacillus licheniformis, Massilia niabensis, and *Leucobacter chromiireducens.* In other studies conducted by Angle and Cheney, (1989); Lovely (1994) and Francisco *et al.* (2002), *Bacillussp, B. cereus, B. subtilis*, and *P. aeruginosa* can reduce chromate; which corresponds with the isolates of the present study [22-24]. The isolated bacteria showed ability to absorb dichromate from contaminated samples, with *Acinetobactee* notherradio resistens leading, followed by *Acinetobacter ventianus*, then comes *Bacillus pumilus*, and *B. cereus.* 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 extra-cellularly. Branco *et al.*, [12] and Ramirez *et al*., [13] showed that most chromate reduction bacteria exhibit resistance to Cr^{6+} even when exposed to contraptions upto 300 ppm. The results of this study agreed with the findings of Abou-Shanab *et al.* [16] who showed that Cr^{6+} resistance and/or Cr^{6+} reduction abilities were found in most of the isolated bacteria in their study in which approximately 54% of the isolated bacteria belonged to the genera *Acinetobacter* and *Ochrobactrum*.
It is observed that chromium reduction percentage increased with time. Cr^{6+} reduction were obtained with the increase of exposure time, where the reduction was 23% (24 h), 32% (48 h), 58% (72 h) and 62% (96h) for *Acinetobacte* notherradio resistens.

Conclusion

The rate of chromium in the soil samples in the study site were 183 mg/kg and this exceeds the allowable limits of the Lindsay method or standard (100 mg/kg). Also, overall rate of wastewater samples contaminated with chromium in the study site were 3.4 mg L^{-1}, which exceeds the allowable limits of the Jordanian Standard No. (202) of (2 mg/L) for total chromium. The bacterial isolates have been defined by the16S rRNA method, these are as follows: *Bacillus cereus*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus subtilis, Acinetobacter radioresistens*, *Acinetobacter venetianus, Ochrobacterum sp*, *Massilia niabensis*, and *Leucobacter chromiireducens*. All bacterial species in this study were shown to have the ability to grow and reduce potassium dichromate Cr^{6+}, with *Acinetobacter* notherradio resistens leading, followed by *Acinetobacter ventianus, then comes Bacillus pumilus,* and *B. cereus.* Reduction of potassium dichromate Cr^{6+} increases with time up to 96 hrs.

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