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Bioremediation of hexavalent chromium (VI) by a soil borne bacterium, *Enterobacter cloacae* B2-DHA

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Abstract

Chromium and chromium containing compounds are discharged into the nature as waste from anthropogenic activities, such as industries, agriculture, forest farming, mining and metallurgy. Continued disposal of these compounds to the environment leads to development of various lethal diseases in both humans and animals. In this paper, we report a soil borne bacterium, B2-DHA that can be used as a vehicle to effectively remove chromium from the contaminated sources. B2-DHA is resistant to chromium with a MIC value of 1000 µg/mL potassium chromate. The bacterium has been identified as a Gram negative, *Enterobacter cloacae* based on biochemical characteristics and 16S rRNA gene analysis. TOF-SIMS and ICP-MS analyses confirmed intracellular accumulation of chromium and thus its removal from the contaminated liquid medium. Chromium accumulation in cells was 320 µg/g of cells dry biomass after 120 h exposure and thus it reduced the chromium concentration in the liquid medium by as much as 81%. Environmental scanning electron micrograph revealed the effect of metals on cellular morphology of the isolates. Altogether, our results indicate that B2-DHA has the potential to reduce chromium significantly to safe levels from the contaminated environments and suggest the potential use of this bacterium in reducing human exposure to chromium, hence avoiding poisoning.

Key Words: Bioremediation; Chromium; *Enterobacter cloacae*; Human Health; Tannery Effluents; Soil Borne Bacterium

Introduction

There are high chromium contaminations spreading through soil and water by industrial activities, in particular, the use of chrome liquor in leather processing. In addition to leather processing, chromium is widely used in wood preservation, steel production, chromium/electroplating, metal processing, alloy formation, textiles, ceramics and thermonuclear weapons manufacturing, among others.^[1-3] Also, several agronomic practices including the use of organic biomass, like sewage sludge or fertilizers based on leather that contain varying degrees of chromium, are the contributors to environment contamination. For example, there are about 185 leather processing industries in Bangladesh discharging solid and liquid wastes without proper treatment directly into the environments including river and natural canals.^[4] Chromium is one of the major sources of environmental pollution and is well known for its toxic, carcinogenic, and mutagenic effects on humans and other living organisms, hence chromium is classified as a priority pollutant.^[5] Trace amount of chromium is an essential element in the diet, because it regulates the glucose metabolism in the human body.^[6] Chromium is commonly present in the soil in two oxidized forms: (i) trivalent chromium Cr(III) and (ii) hexavalent chromium Cr(VI). Cr(III) is less mobile, hence less toxic^[7] whereas Cr(VI) is a soluble oxidizing agent that is easily reduced intracellularly to Cr^{5+} and reacts with nucleic acids and other cellular components to create carcinogenic and mutagenic effects in biological systems.^[8] Therefore, bioremediation of Cr(VI) is essential to protect human health and the environment.

Many metal pollutants including chromium cannot be degraded like organic contaminants. To date, many conventional physicochemical methods have been developed for removing toxic CrO_4^{2-} such as ion exchange, electrochemical treatment, evaporation, reverse osmosis, precipitation, and adsorption on activated coal. Nevertheless, most of these methods are

inefficient and very expensive especially when the contamination levels are very low.^[8-9] Alternatively, various cost effective and eco-friendly biological approaches have been considered for bioremediation.^[10] To that effect, a number of microorganisms have been reported to resist Cr(VI) by periplasmic biosorption, intracellular bioaccumulation, and/or biotransformation to a less toxic speciation state through direct enzymatic reaction, including *Pseudomonas* - sp.^[11] *Enterobacter aerogenes*,^[12] *Enterobacter cloacae*,^[13-16] *Microbacterium*,^[17] *Desulfovibrio*,^[18] *Escherichia coli*,^[19] *Shewanella alga*,^[20] *Bacillus* sp.^[8] and several other bacterial species.^[21] Although, most of these microbes have been isolated from tannery sludge, industrial sewage, evaporation ponds, or discharged water, or were purchased from culture collections, the availability of high Cr(VI)-reducing organisms is an essential prerequisite for the efficient bioremediation of chromate-containing industrial waste water.

Over the past decade we have collected water and soil samples from many regions of South Asia, particularly Bangladesh and India, where metal including chromium, arsenic contaminated ground water is frequently used for both consumption and for irrigation of cultivated crops.^[22-24]

The purpose of this study was to identify, isolate and characterize naturally occurring bacterial strain(s) that can grow on chromium contaminated soil or water and can also accumulate chromium in the cells thus reducing the level of this toxic metal in the contaminated source to a safe level. Until today, we have collected several hundred bacterial strains isolated from the chromium contaminated environment. Among this collection, we have recently identified and characterized a soil-borne bacterium B2-DHA that can survive and grow on medium containing up to 1000 µg/mL potassium chromate and thereby reducing chromium contents in the contaminated source by 81%.

In this study we have used several modern techniques to localize chromium in the bacterial cells qualitatively and quantitatively. Dynamic time of flight secondary ion mass spectrometry (TOF-SIMS) imaging and depth profiling^[25] have been employed to follow the distribution of chromium ion and its products within the cells.^[26] Inductively coupled plasma - mass spectrometry (ICP-MS), the fastest growing trace element technique has been used to measure the amount of chromium in the dry bacterial cells. The concentration of chromium in the cell free broth has been measured by using the inductively coupled plasma - atomic emission spectroscopy (ICP-AES).

We also report the results obtained from morphological, biochemical, and 16S rRNA characterization of the bacterium. Investigation of this strain to be utilized as a potential candidate for eliminating or significantly reducing chromium level in the contaminated source has been made. Hence, the results obtained in this investigation provide us with useful knowledge for the microbial bioremediation of chromium pollution.

Materials and Methods

Collection of soil samples

Soil samples were collected from the landfills of leather manufacturing tannery industries in Bangladesh. These are located in the Hazaribagh tannery areas, a very close vicinity of the capital city Dhaka, where the tannery wastes have been disposed for many years. The soil surface at 0-15 cm in depth was collected, retained in plastic bags and kept at 4°C until further analysis including bacterial isolation.

Analysis of soil samples

The pH of soil samples was determined by shaking 5 g of soil in 50 mL of distilled water for one hour by using pH meter (pH meter 2210 Hanna Instruments, Carrollton, TX, USA). To determine the concentrations of different metals such as chromium, arsenic, sodium, potassium, chloride, phosphate, and nitrate, 5 g of the soil sample was air dried and mixed with 2 mL of HNO₃ (65 %, Merck, Darmstadt, Germany) and 6 mL of HCl (37 %, Merck, Darmstadt, Germany). The mixture was heated at 70°C for one hour, and then diluted with 10 mL of deionized water. The acid-digested solution was filtered to remove residual particulates. Concentration of chromium, arsenic, sodium and potassium was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES).^[27] However, concentrations of chloride and phosphate were determined by spectrophotometric analysis,^[28] whereas nitrate was measured in a solution with KCl followed by flow injection analysis (FIA).^[29] All samples were digested based on heat block digestion and analyzed by Eurofins Environment Testing Sweden AB (Lidköping, Sweden).

Chemicals and bacterial enrichment

Chemicals used in this experiment were analytical standard grade (Merck and Sigma-Aldrich). All solutions were prepared with autoclaved double deionized water (ddH₂O), sterilized by syringe filtration (0.2 µm pore-size) and stored at 4°C in the dark until further use. The media for bacterial culture were autoclaved at 120°C for 15 min.

For isolation of chromium resistant bacteria, the soil sample was serially diluted in sterile distilled water and plated aerobically in the modified Luria-Bertani (LB) medium containing

peptone 10 g/L, sodium chloride 5 g/L, yeast extracts 5 g/L, D (+) glucose 1 g/L (Merck, Darmstadt, Germany) supplemented with 50, 100, 150 or 200 µg/mL potassium chromate K_2CrO_4 (Sigma). Following incubation of these plates at 37°C for 48 h, several morphologically different colonies were picked randomly and streak-purified at least twice on the same medium for isolation of the single colonies. A number of colonies showing resistance to chromium were selected for further screening. Subsequently, only one strain B2-DHA was used for further studies because it demonstrated the best growth in presence of and resistance to chromium. Purified single colonies were inoculated in LB broth, cultured for 24 to 48 h and stored in 25% glycerol at -80°C for further analyses.

Effects of pH and temperature

The influences of pH and temperature on bacterial growth were assessed using LB medium. Autoclaved culture media at pH 5.0, 6.0, 7.0, 8.0 or 9.0 were used to test the effect of pH on bacterial growth. The inoculum of B2-DHA strain was 1% of the total volume of culture and incubated under continuous shaking at 180 rpm for 24 h. The bacterial growth was measured every 2 h using optical density measurement at 600 nm by Genesys 20 visible spectrophotometer (Thermoscientific, Madison, WI, USA). For evaluation of the effect of temperature on bacterial growth, the cultures were incubated at 25, 30, 37 or 40°C. Similarly, the inoculum of B2-DHA strain was prepared from the logarithmic-phase cultures in LB broth without supplementation of chromium and incubated for 24 h with shaking at 180 rpm. The growth of bacterial cells was evaluated by measuring optical density of medium at regular intervals of 2 h at 600 nm. All experiments were carried out in triplicates. For the rest of this study, the strain B2-DHA was grown on media with pH 7 and culture temperature at 37°C due to the optimum growth at these conditions.

Minimum inhibitory concentration (MIC) of chromate and other heavy metals

The MIC of chromium for B2-DHA isolate was determined as reported by Mergeay.^[30] Minimal salt broth^[31] supplemented with different concentrations (600 µg/mL, 800 µg/mL, 1000 µg/mL and 1200 µg/mL) of Cr(VI) was used to determine the MIC for chromium. One percent inoculum of B2-DHA was added to the media in 50 mL falcon tubes. Bacteria cultured in minimal medium without Cr(VI) were used as controls. All tubes were incubated at 37°C with shaking at 180 rpm for 48-96 h. Optical density measurements for estimation of cell growth were carried out at 600 nm by using Genesys 20 visible spectrophotometer. Besides potassium chromate, the MIC of the bacterial isolate was checked for other heavy metals like sodium arsenate (Na₂HAsO₄), ferric chloride (FeCl₃), manganese chloride (MnCl₂), zinc chloride (ZnCl₂), nickel chloride (NiCl₂) and silver nitrate (AgNO₃) at different concentrations.

Morphological and biochemical analyses

The colony morphology of the purified bacterial strains grown on medium containing potassium chromate was evaluated. Gram-staining and cellular morphology of the strain (data not shown) were studied based on both light- and electron microscopy.^[32] Negative staining was performed to determine the production of a polysaccharide capsule by bacterial strains. A bacterial colony was mixed up in a drop of India ink on a slide and successively observed with the light microscope. Biochemical tests including motility, mannitol test, indole production, H₂S production, catalase test, oxidase test, urease test, carbohydrate (glucose, lactose, sucrose and rhamnose) fermentation, citrate utilization, hydrolysis of casein etc. were

performed to facilitate the identification of the bacterium following Bergey's Manual of Systematic Bacteriology. ^[33] The morphological analysis of strain B2-DHA was carried out by the Environmental Scanning Electron Microscope (ESEM) (Model: FEI-Quanta 200) with an attached X-ray energy dispersive system (EDS). Bacterial culture was inoculated in a nutrient broth supplemented with 100 µg/mL of Cr(VI) kept at 37°C on rotator shaker at 150 rpm. Control incubations were carried out under the same conditions in the absence of Cr(VI). After 48 h of incubation 30 mL from each was centrifuged at 5000 rpm for 15 min. to remove excess of liquid media. Pellet obtained was washed twice with sterile deionized water and further pellet was washed twice with Phosphate Buffered Saline (PBS). The cells were chemically fixed at 4°C for 18 h with 2% glutaraldehyde: formaldehyde in 1 mL PBS (1:2 glutaraldehyde: formaldehyde) in a dark conditions. After 18 h of incubation each sample was centrifuged at 5000 rpm for 15 min. Dehydration of pellet carried out by series of alcohol treatment (40, 60, 80, 90, and 100%) for 5 min respectively. 10 µL of sample was taken on 1mm X 1mm slide. Slides transferred to desiccator for moisture absorption. Samples processed were taken for ESEM-EDS analysis at IIT, Powai, Mumbai, India.

16S rRNA sequencing

Genomic DNA was isolated using DNeasy Blood and Tissue Kit (Qiagen, Cat. No. 69504) as per manufacturer. For PCR amplification of the nearly full-length 16S rRNA domain, *Bacteria* specific universal primers 8F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-TACGGTTACCTTGTTACGACTT-3) were used in PCR reaction mixtures containing deoxynucleotide triphosphates (dNTPs) at 50 µmol each, primers at 2.5 pmol each, 1.5 U Taq DNA polymerase in 1X buffer, 50 ng of DNA template, 2.5 mM MgCl₂ in a 50 µL reaction. The thermal cycling program included initial denaturation at 95°C for 5 min, followed by 34

cycles of denaturation at 95°C for 45 sec, primer annealing at 55°C for 1 min and primer extension at 72°C for 1.5 min. This was followed by a final extension step at 72°C for 10 min and the samples were cooled to 4°C. The amplified PCR products were visualized by agarose-gel electrophoresis and the DNA fragment was purified using QIAquick Gel Extraction Kit (Qiagen). DNA sequencing reaction of PCR amplicon was carried out using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer with same primers: 8F and 1492R. A similarity search for the nucleotide sequence of 16S rRNA of the test isolate was carried out using the NCBI nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/>). The 16S rRNA sequence was submitted to NCBI GenBank for registration.

Evolutionary relationship of taxa

The evolutionary history was revealed using the Neighbor-Joining method.^[34] The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was shown next to the branches.^[35] The evolutionary distances were computed using the Kimura 2-parameter method^[36] and these were in the units of the number of base substitutions per site. The analysis involved 23 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. This means that fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1227 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.^[37]

Analysis of chromium contents in liquid medium by ICP-AES

The capacity of B2-DHA strain to decrease the chromium contents in the liquid medium was determined by the inductively coupled plasma atomic emission spectroscopy (ICP-AES).^[27] The strain B2-DHA was grown in 50 mL LB medium supplemented with 100 µg/mL of chromium and incubated at 37°C with shaking at 180 rpm for five days. The B2-DHA cells grown similarly but without potassium chromate were used as controls. Five sets of experiments were performed with three replicates along with controls. The cell free medium was collected by centrifugation (10000 rpm for 10 min) using a Sorvall rotor (Sorvall Super T21, USA). The cell free broth was filtered through 0.2 µm filter and acidified to pH 2.0 with 30% suprapure nitric acid (Merck, Germany). The acidified cell free broth was analyzed for total chromium content by using ICP-AES.^[27]

Analysis of chromium contents in the bacterial cells by ICP-MS

The bioaccumulation of chromium was determined in the B2-DHA strain grown in 50 mL LB medium supplemented with 100 µg/mL of chromium and incubated at 37°C for five days. After incubation, samples were collected and centrifuged at 10000 rpm for 10 min at Sorvall rotor (Sorvall Super T21, USA). The pellet was washed with 0.9 % saline twice and air-dried until a constant dry weight was achieved. The entire contents of the flask were harvested and the dry weight of the cells was recorded. Cells were digested with 30% suprapure nitric acid (Merck, Germany) according to ratio of 7.5 mL nitric acid per g dry biomass using microwave digestion. The samples were brought to a constant volume prior to determination of chromium contents. Measurement of chromium was also carried out similarly in the control experiments using media containing chromium but not exposed to B2-DHA and in media devoid of chromium but treated with B2-DHA. All analyses were carried out after filtration of the cell

digest through 0.2 μm filter. The chromium present in the dried pellets was determined by the inductively coupled plasma mass spectroscopy (ICP -MS).^[38]

Confirmation of chromium ions in the bacterial cells by TOF-SIMS

Bacterial samples were grown in liquid medium supplied without or with 100 $\mu\text{g/mL}$ chromium to perform the TOF-SIMS ion imaging analyses. This technique uses a focused, primary ion beam to bombard a solid sample in ultrahigh vacuum, producing secondary ions from the sample surface. Cells were collected by repeated (3 times) centrifugation at 10000 rpm for 15 minutes in a micro centrifuge and washing with autoclaved deionized distilled water. Cells were then deposited and spread out on microscopic slides and analyzed using TOF-SIMS Version 6.1 instrument (ION-TOF, GmbH, Münster, Germany) equipped with a 30 keV Bi_3^+ LMIG analysis gun^[39-40] with a $512 \times 512 \mu\text{m}$ raster. Electron bombardment (20 eV) was used to minimize charge built-up at the surface. Depth profiling of the chromium ions inside the cells was performed by using a 0.5 keV Cs^+ sputter gun. Depth profiling and imaging were performed in the burst mode (analyse 30 scans, sputter 0.20 s, pause 6.0 s, to a total of approx. 250 s of sputtering). Desorbed secondary ions were accelerated to 2 keV, mass analysed in the flight tube, and post-accelerated to 10 keV before detection. The Bi_3 -LMIG was set in the high current bunched mode (negative polarity, analysis area $47 \times 47 \mu\text{m}$, mass resolution $m/\Delta m$: 6000; focus of the ion beam: 150 nm)^[41] with a target current of 0.15 pA while Cs ions were used for sputtering, with a current of 5 nA and with a $250 \times 250 \mu\text{m}$ raster.

All image analyses were performed using the ION-TOF Surface Lab software (Version 6.1, ION-TOF, GmbH, Münster, Germany) except for image resizing, for publication purposes, which was done in Adobe Photoshop CS-2 (Adobe Systems Incorporated, San Jose, CA).

Each ion image was normalized to the intensity of the brightest pixel ranging color values of 0 (zero intensity) to 256 (brightest intensity). All other intensities are assigned accordingly using a linear relationship.

Statistical analyses

Statistical analyses were performed using standard statistical package Microcal (TM) Origin 6.0 version (<http://www.microcal.com/>). For the examined parameters one- and two-factor variance analyses using the independent system were done. The zero or alternative hypotheses were accepted on the basis of the F test at $p=0.05$ or $p=0.01$ and marked as */ or **/, respectively. The significance of differentiation in mean values for individual properties was checked using the least significant difference (LSD) test. All analyses were performed in triplicate and the results are presented as mean value with standard deviation.

Results and Discussion

Isolation of toxic-metal-resistant bacteria from soils contaminated with tannery effluents – soil characteristics

Soils containing heavy metals are potential sources for identifying toxic-metal-resistant bacteria. In this study, we have isolated chromium resistant bacterial strains from soil samples collected from the landfills comprised of tannery effluents discharged from leather manufacturing industries located in the Hazaribagh area in Bangladesh. The pH of the soil samples was recorded to be neutral, 7.1 (± 0.3). Distribution of soil metals was found to be as follows: (i) 15.4 (± 0.06) μg chromium/g soil d.wt., (ii) 7.52 (± 0.02) μg arsenic/g soil d. wt.,

(iii) 4.1 (± 0.30) μg chloride/g soil d.wt., (iv) 6.4 (± 0.04) μg sodium/g soil d.wt., (v) 4.4 (± 0.05) μg potassium/g soil d.wt (vi) 0.85 (± 0.03) μg phosphate/g d.wt., and (vii) 0.32 (± 0.02) μg nitrate/g soil d.wt.

Characterization and identification of the soil borne B2-DHA

Characterization of the strain B2-DHA was performed on the basis of colonial morphology, cellular morphology and Gram staining properties. The colonial morphology of the strain appeared to be circular and convex, whereas cellular shape of the strain was found to be cocci-like and the isolate was Gram negative and motile in character. Biochemical characterization of the isolate was performed in terms of carbohydrate utilization such as the strain showed positive in glucose, lactose, sucrose fermentation. On the other hand the strain showed negative in indole-, urea-, capsule- and hydrogen sulfide production. Moreover, B2-DHA was positive in catalase test and negative in oxidase test. The data revealed that B2-DHA belongs to the genus *Enterobacteria*. The BLASTN search of the 16S rRNA gene sequence (1227 bp) of the isolate (accession number KF920746) showed 97% similarity with that of *Enterobacteria cloacae* (Fig. 1) confirming further that the B2-DHA strain belongs to the genus *Enterobacteria*. This is consistent with previously reported studies on chromate reduction by *Enterobacter cloacae*.^[13-16, 42-44]

Effect of temperature and pH on bacterial growth

Both temperature and pH in combination play a major role in growth and metal accumulation capacities of the bacterial strains.^[45-46] Although the B2-DHA isolate was able to grow at 20, 30, 37 and 45°C but the optimum growth (estimated based on optical density) was observed at

37°C (data not shown). Similarly, the isolate could grow at pH ranging from 5 to 9 but the optimum growth was found at pH 7.0 (data not shown). Thus, all subsequent experiments were conducted at 37°C in media with pH 7.0. These data are consistent with previously reported^[13] optimum pH and temperature for *Enterobacter cloacae* ranging from 7.0-8.0 and 30-37°C respectively. Wang et al.^[43] reported that reduction of Cr(VI) by *E. cloacae* occurred at pH 6.5 to 8.5 and it was strongly inhibited at pH 5.0. In fact, this strain depicted poor growth at temperature below 25°C and at pH of 5 or less compare to that of a faster growth at temperature ranging between at 28 and 40°C and resistance at pH up to 9. In addition, Cr(VI) could be desorbed from soil at a faster rate at elevated pH values.^[47]

Minimum inhibitory concentration (MIC) of chromate

The strain *E. cloacae* B2-DHA exhibited a MIC higher than 1000 µg/mL of K₂CrO₄ which is an indication that this strain is resistant to very high concentrations of chromate than that reported previously by Komori et al,^[13] Yamamoto et al,^[14] Clark,^[15] Rege et al,^[16] and Cervantes et al.^[44] While the resistance parameter is not absolute; it is correlated to the growth medium. The MIC obtained in rich media is usually two to five times higher than those obtained in TRIS minimal medium.^[30] This is due to that heavy metals can complex with certain components in the media, especially organic substances and phosphate. Thus, it is important to employ an appropriate strategy to select potential bacterial strains for remediation of Cr(VI) from contaminated environments. This can not only be based on the capability of a strain to grow in the presence of high levels of chromate, but it must also include the test of chromate reduction. It is also important to bear in mind that the strain is able to catalyze the reduction of Cr(VI) into the much less toxic and mobile Cr(III), since the chromate resistance and the chromate reduction may be unrelated processes.^[48]

Resistance of B2-DHA to chromium

Chromium resistance of B2-DHA was estimated based on bacterial growth detected by optical density with or without exposure to 1000 µg/mL potassium chromate. The results indicated that after 48 h of incubation the bacterial growth was highest in the control medium whereas the bacterial growth in medium containing chromium was gradually increasing and after 120 h of incubation the growth was highest (Fig. 2a).

To verify if the B2-DHA strain was indeed resistant to chromium, we performed a viable cell count experiments where the cells were grown first in liquid medium containing 1000 µg/mL chromium for up to 120 h and then transferred onto solid medium fortified with or without chromium. Samples were taken every 24 h and the colony forming units (CFU) were counted. The results showed that the number of cells on the plates containing no chromium increased rapidly up to 72 h (approximately 2.56×10^{10} CFU) and then decreased drastically (approximately 1.9×10^9 CFU) up to 96 h of growth (Fig. 2b). The number of CFU in the chromium free plates decreased because of the stationary phase of cell cultures after 96 hours when the medium is lack of adequate nutrient required for survival of bacterial cells.^[49] However, when the cells were plated on chromium containing medium, the cell count increased gradually up to 120 h (approximately 2.49×10^{10} CFU), confirming that this strain was highly resistant to chromium. The higher cell counts on Luria agar without chromium could be because of the reversible dormancy of the cells due to removal of the stress factor (chromium) from the growth medium. These results suggest that the bacterium was able to grow well in presence of chromium.

Resistance of B2-DHA to other metals

To verify that the B2-DHA isolate was also resistant to other toxic metals, the growth of this bacterium was monitored in presence of metals like $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$, FeCl_3 , MnCl_2 , ZnCl_2 , NiCl_2 and AgNO_3 at different concentrations in LB broth. The MIC of these metals was found to be 15 g/L for $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$, 500 mg/L for FeCl_3 , 400 mg/L for MnCl_2 , 350 mg/L for ZnCl_2 , 260 mg/L for NiCl_2 and 85 mg/L for AgNO_3 .

ICP –MS and ICP-AES Analysis of Cr(VI)

To confirm if the B2-DHA strain can indeed accumulate chromium inside the cells as performed by inductively coupled plasma mass spectroscopy (ICP -MS). This analysis revealed that the amount of chromium inside the bacterial cells after 120 h of exposure to 100 $\mu\text{g/mL}$ chromium resulted in 320 $\mu\text{g/g}$ d.wt. of bacterial biomass (Fig. 3a). If this is the case, theoretically equal amounts of chromium should be decreased from the growth medium exposed to the B2-DHA isolate. We verified this by performing inductively coupled plasma atomic emission spectroscopy (ICP-AES) and found that the chromium concentration in the cell free growth medium, after 120 h exposure, decreased from 100 $\mu\text{g/mL}$ to 19 $\mu\text{g/mL}$ (81%) (Fig. 3b). In control samples (medium without exposure to B2-DHA), we did not observe any temporal change in the concentration of chromium. The rates of chromate reduction by the B2-DHA isolate were comparable to those of other chromate-resistant bacterial strains as previously reported by many researchers^[21, 50-52] who demonstrated that several chromium-reducing bacteria with biotransformation potential and can reduce the different amount of chromium in the medium. Wang and Xiao^[50] observed that, the rate of

Cr(VI) reduction by *Bacillus* sp. Increased with initial Cr(VI) concentrations ranging from 20 to 70 mg/L and decreased at higher concentrations. Thacker et al.^[21] reported a Gram negative strain of *Brucella* sp., which has a potential to decrease chromium in the contaminated sources. These authors demonstrated that increasing concentrations of Cr(VI) in the medium lowered the bacterial growth. But the decrease in the growth rate could not be correlated directly with the amount of Cr(VI) reduced. Resistance to high concentration of Cr(VI) and high ability for reduction of this toxic metal make the strain a suitable candidate for bioremediation. Desai et al.^[52] reported three efficient Cr(VI) reducing bacterial strains, *Bacillus cereus*, *Bacillus fusiformis* and *Bacillus sphaericus*, isolated from Cr(VI) polluted landfills and characterized for *in vitro* Cr(VI) reduction. These researchers showed that the suspended cultures of all *Bacillus* sp. exhibited more than 85% reduction when exposed to 1000 μ M Cr(VI). Megharaj et al.^[51] reported two bacterial strains, *Arthrobacter* sp. and a *Bacillus* sp., isolated from soils contaminated a longer term with tannery wastes and their resistance abilities to hexavalent chromium [Cr(VI)] as well as their reducing capabilities of Cr(VI) to Cr(III), a detoxification process in cell suspensions and cell extracts. Overall results of these experiments indicated that *Arthrobacter* sp. could reduce chromate to 60% after 72 h when Cr(VI) concentration is 50 μ g/mL in the medium, whereas *Bacillus* sp. was able to reduce chromate 60% after 72 h when grown in presence of 20 μ g Cr(VI) /mL in the medium. At higher concentration of Cr(VI) *Arthrobacter* sp. was distinctly superior to the *Bacillus* sp. in terms of their Cr(VI)-reducing ability and resistance to Cr(VI). However, both bacteria can reduce 100% chromium from the medium by 72 h when the chromium concentration is very less ranging from 5 to 10 μ g/mL. Our data similar to those previously reported suggest that the reduction of chromium concentration in the growth medium treated with B2-DHA is due to the biological activity of this bacterium.

TOF-SIMS analyses of chromium

To further verify the presence of chromium inside the cells or absorption to the outside we performed ion imaging analyses by TOF-SIMS. Ion imaging analyses by TOF-SIMS based on time of flight-secondary ion mass spectrometry was performed to determine whether the bacterium B2-DHA absorbs and/or accumulates chromium inside the cells. This analysis relies on the use of a pulsed ion beam to ionize surface molecules that later can be studied by a mass spectrometer. With the help of ion imaging we have found that B2-DHA cells, when exposed to chromium, uptake and accumulate the different forms of chromium inside the cells, such as chromium oxide (CrO^-) and chromium dioxide (CrO_2^-) (Fig. 4b-c). In addition, the total protein signals in the cells are also detected by TOF-SIMS (Fig. 4a). On the other hand, the ion imaging analyses by TOF-SIMS of the control B2-DHA cells grown in absence of chromium had no detectable chromium ions (CrO^- or CrO_2^-) (Fig. 4d). Surprisingly, the intensity of chromium ion (CrO^-) inside the cells was much lower than the intensity of other chromium ion (CrO_2^-). These results show that the bacterium, B2-DHA, prefers to accumulate chromium inside the cells.

To further confirm the distribution of chromium ions inside the cells we have performed depth profiling analyses by TOF-SIMS. This analysis, also known as “sputtering”, relies on gradual measurement of chromium at the different depth of the bacterial cell layers. Sputtering was done up to 1250 seconds and these results demonstrated that the intensity counts for chromium ions (CrO^-) were approximately $10^{3.5}$ and the intensity counts for chromium ions (CrO_2^-) were approximately $10^{2.8}$ (Fig. 4e). Furthermore, sputtering was made up to 1000 seconds for the control sample and the results indicated that the intensity levels remained very low throughout the sputtering time (from 0-1000 seconds) which can be

attributed to the background activity of chromium (Fig. 4f). The intensity counts of signal protein are much higher in the control sample than that observed in chromium exposed sample (Fig. 4e-f). By combining the pulsed ion beam with another ion beam in direct current (DC) mode, depth profiles were obtained as a result of consecutive removal of surface layers. The depth profiling further confirmed the results obtained by ion imaging indicating comparatively higher amounts of chromium inside the cells. Importantly, as our isolate B2-DHA exhibited a very high resistance to chromium, its oxidation/reduction characteristics are therefore significantly beneficial for remediation of chromium from the contaminated water and/or soils in the affected regions. Hence, TOF-SIMS can be used for obtaining both ion images as well as chemical information on the distribution of chromium ions from the surface and downwards into the sample. Rahman et al.^[24] practiced same technique to profile arsenics in bacterial cells. Appropriate application of this bacterium may maintain the biogeochemical cycles of chromium in the nature.

ESEM-EDS analysis

Morphologic changes in bacteria cultured in medium with (100 µg/mL) or without chromium (control) were investigated using environmental scanning electron microscopic (ESEM) with an attached X-ray energy dispersive system (EDS). ESEM images were taken at 12000X magnification. The results are presented in Figure 5. Results indicated that B2-DHA control bacteria exhibited a typical cocci shape whereas the chromium-treated bacteria formed long chains in compared to the untreated cells (Fig. 5a-b). Cell aggregation and surface modification besides increasing irregularity of cell morphology took place in case of cells suspended in chromium solution. The long chain like structures represent mode of response to metal stress. These changes in morphological structure might be a possible strategy for cells

to accumulate metals inside the cells indicating that the strain B2-DHA could accumulate chromium and contribute to bioremediation of this toxic metal. The EDS spectrum recorded in the examined region of the cells confirmed the presence of chromium ions on cell surface with respect to control (Fig. 5c-d). The signals for C, N, and O may originate from biomolecules present on the surface of biomass. The ESEM-EDS of the cells grown in presence of chromium clearly revealed that the elongation of cells and cell aggregation due to the metal stress support the membrane transport process.^[53] In growing cells, biofilm formation or aggregation is a common phenomenon under stress conditions; however, this is unusual for non-growing live cells. Rahman et al.^[24] reported a significant elongation of bacteria when studying the effects of arsenate on the *Lysinibacillus sphaericus*. Also Desale et al.^[54] reported morphological changes of bacteria when studying the effects of nickel on *Lysinibacillus sp.* Several other researchers have shown the bacterial elongation in presence of toxic heavy metals.^[55-56]

Conclusions

In this paper we report that the *Enterobacter cloacae* strain B2-DHA isolated from a landfill containing effluents disposed from leather manufacturing tannery industries has potentials for decreasing chromium concentration to a safe level in the contaminated environment. In a longer perspective, several hundred millions of people worldwide can avoid many lethal diseases caused by chronic chromium poisoning. Consequently, our findings will contribute to a significant positive impact on the socio-economic status of the people particularly in the developing world. The continuation of this research is vital not only for people in the affected area but also for populations in other countries that have credible health concerns as a consequence of chromium contaminated water and foods.

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References

- [1]. Ortegell, J.W.; Staren, E.D.; Faber, L.P.; Warren, W.H.; Braun, D.P. Modulation of tumor-infiltrating lymphocyte cytolytic activity against human non-small cell lung cancer. *Lung Cancer*. **2002**, *36*, 17-25.
- [2]. Viti, C.; Pace, A.; Giovannetti, L. Characterization of Cr(VI)-resistant bacteria isolated from chromium-contaminated soil by tannery activity. *Curr. Microbiol.* **2003**, *46*, 1–5.
- [3]. Chourey, K.; Thompson, M.R.; Morrell-Falvey, J.; VerBerkmoes, N.C.; Brown, S.D.; Shah, M.; Zhou, J.; Doktycz, M.; Hettich, R.L.; Thompson, D.K. Global molecular and morphological effects of 24-hour chromium(vi) exposure on *Shewanella oneidensis* MR-1. *Appl. Environ. Microbiol.* **2006**, *72*, 6331–6344.
- [4]. The Guardian, Sunday 7 July 2013 (collected on 25th Nov. 2013 at <http://www.theguardian.com/world/2013/jul/07/bangladesh-tanneries-human-rights-violations>).
- [5]. Avudainayagam, S.; Megharaj, M.; Owens, G.; Kookana, R.S.; Chittleborough, D.; Naidu, R. Chemistry of chromium in soils with emphasis on tannery waste sites. *Rev. Environ. Contam. Toxicol.* **2003**, *178*, 53–91.

- [6]. Offenbacher, E. G.; Pi-Sunyer, F. X. Chromium in Human Nutrition. Annual Review of Nutrition. **1988**, 8, 543-563.
- [7]. Jeyasingh, J.; Philip, L. Bioremediation of chromium contaminated soil: optimization of operating parameters under laboratory conditions. J. Hazard. Mater. **2005**, 118, 113-120.
- [8]. Camargo, F.A.O.; Bento, F.M.; Okeke, B.C.; Frankenberger, W.T. Chromate reduction by chromium-resistant bacteria isolated from soils contaminated with dichromate. J. Environ. Qual. **2003**, 32, 1228–1233.
- [9]. Zafar, S.; Aqil, F.; Ahmad, I. Metal tolerance and biosorption potential of filamentous fungi isolated from metal contaminated agricultural soil Bioresour. Technol. **2007**, 98, 2557–2561.
- [10]. Congeevaram, S.; Dhanarani, S.; Park, J.; Dexilin, M.; Thamaraiselvi, K. Biosorption of chromium and nickel by heavy metal resistant fungal and bacterial isolates. J. Hazard. Mater. **2007**, 146, 270–277.
- [11]. Alvarez, A.H.; Moreno-Sanchez, R.; Cervantes, C. Chromate efflux by means of the chrA chromate resistance protein from *Pseudomonas aeruginosa*. J. Bacteriol. **1999**, 181, 7398–7400.
- [12]. Panda, J.; Sarkar, P. Bioremediation of chromium by novel strains Enterobacter aerogenes T2 and Acinetobacter sp. PD 12 S2. Environ. Sci. Pollut. Res. Int. **2012**, 19, 1809-1817.
- [13]. Komori, K.; Rivas, A.; Toda, K.; Ohtake, H. A method for removal of toxic chromium using dialysis-sac cultures of a chromate reducing strain of Enterobacter cloacae. Appl. Microbiol. Biotechnol. **1990**, 33, 117-119.
- [14]. Yamamoto, K.; Kato, J.; Yamo, T.; Ohtake, J. Kinetics and modeling of hexavalent chromium reduction in Enterobacter cloacae. Biotechnol. Bioeng. **1993**, 41, 129–133.

- [15]. Clark, D.P. Chromate reductase activity of *Enterobacter aerogenes* is induced by nitrite. FEMS Microbiol. Lett. **1994**, *122*, 233–238.
- [16]. Rege, M.A.; Peterson, J.N.; Johnstone, D.L.; Turick, C.E.; Yonge, D.R.; Apel, W.A. Bacterial reduction of hexavalent chromium by *Enterobacter cloacae* strain HO1 grown on sucrose. Biotechnol. Lett. **1997**, *19*, 691–694.
- [17]. Pattanapitpaisal, P.; Brown, N.L.; Macaskie, L.E. Chromate reduction and 16S rRNA identification of bacteria isolated from a Cr(VI)-contaminated site. Appl. Microbiol. Biotechnol. **2001**, *57*, 257–261.
- [18]. Michel, C.; Brugma, M.; Aubert, C.; Bernadac, A.; Bruschi, M. Enzymatic reduction of chromate: Comparative studies using sulfate-reducing bacteria. Appl. Microbiol. Biotechnol. **2001**, *55*, 95–100.
- [19]. Shen, H.; Wang, Y. Characterization of enzymatic reduction of hexavalent chromium by *Escherichia coli* ATCC 33456. Appl. Environ. Microbiol. **1993**, *59*, 3771–3777.
- [20]. Guha, H.; Jayachandran, K.; Maurrasse, F. Kinetics of chromium (VI) reduction by a type strain *Shewanella alga* under different growth conditions. Environ. Pollut. **2001**, *115*, 209–218.
- [21]. Thacker, U.; Parikh, R.; Shouche, Y.; Madamwar, D. Reduction of chromate by cell-free extract of *Brucella* sp. isolated from Cr(VI) contaminated sites. Bioresour. Technol. **2007**, *98*, 1541–1547.
- [22]. de Crécy E, Metzgar D, Allen C, Pénicaud M, Lyons B, Hansen CJ, de Crécy-Lagard V (2007) Development of a novel continuous culture device for experimental evolution of bacterial populations. Appl Microbiol Biotechnol *77*: 489–496
- [23]. de Crécy E, Jaronski S, Lyons B, Lyouns TJ, Keyhani NO (2009) Directed evolution of a filamentous fungus for thermo tolerance. BMC Biotechnol *9*: 74

- [24]. Rahman, A.; Nahar, N.; Nawani, N.N.; Jass, J.; Desale, P.; Kapadnis, B.P.; Hossain, K.; Saha, A.K.; Ghosh, S., Olsson, B.; Mandal, A. Isolation of a *Lysinibacillus* strain B1-CDA showing potentials for arsenic bioremediation. *J. Environ. Sci. and Health, Part A*. **2014**, *49*, 1349–1360.
- [25]. Vickerman, J.C.; Briggs, D. TOF-SIMS surface analysis by mass spectrometry. IM Publications and SurfaceSpectra Limited, West Sussex, 2001.
- [26]. Norrman, K.; Krebs, F.C. Lifetimes of organic photovoltaics: Using TOF-SIMS and $^{18}\text{O}^2$ isotopic labelling to characterise chemical degradation mechanisms. *Sol. Energy Mater. & Sol. Cells*. **2006**, *90*, 213-227.
- [27]. Stefánsson, A.; Gunnarsson, I.; Giroud, N. New methods for the direct determination of dissolved inorganic, organic and total carbon in natural waters by Reagent-FreeTM Ion Chromatography and inductively coupled plasma atomic emission spectrometry. *Analytica Chimica Acta*. **2007**, *582*, 69–74.
- [28]. Schwedt, G. The Essential Guide to Analytical Chemistry. (Brooks Haderlie, trans.). Chichester, NY: Wiley. (Original Work Published 1943). **1997**, 16-17.
- [29]. Xu, W.; Sandford, R.; Worsfold, P.; Carlton, A.; Hanrahan, G. Flow Injection Techniques in Aquatic Environmental Analysis: Recent Applications and Technological Advances. *Critical Reviews in Analytical Chemistry*. **2005**, *35*, 237-246.
- [30]. Mergeay, M. Heavy metal resistances in microbial ecosystems. In: Akkermans ADL, an Elsas JD, de Bruijn FJ (eds) *Molecular microbial ecology manual*. Dordrecht: Kluwer Academic Publishers, 1995, 6.1.7/1–6.1.7/17
- [31]. Villegas-Torres, M.F.; Bedoya-Reina, O.C.; Salazar, C.; Vives-Florez, M.J.; Dussan, J. Horizontal *arsC* gene transfer among microorganisms isolated from arsenic polluted soil. *Int. Biodeterior Biodegradation*. **2011**, *65*, 147–152.

- [32]. Eichorst, S.A.; Breznak, J.A.; Schmidt, T.M. Isolation and characterization of soil bacteria that define *Terriglobus* gen. nov., in the Phylum Acidobacteria. *Appl. Environ. Microbiol.* **2007**, *73*, 2708–2717.
- [33]. Krieg, N.R. *Bergey's manual of systematic bacteriology volume one*. Garrity, G.; Boone, D.R.; Castenholz, R.W. (Eds.) Williams and Wilkins, 1984; 722.
- [34]. Saitou, N., Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. & Evol.* **1987**, *4*, 406-425.
- [35]. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **1985**, *39*, 783-791.
- [36]. Kimura, M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111-120.
- [37]. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. & Evol.* **2013**, *30*, 2725-2729.
- [38]. Ammann, A.A. Inductively coupled plasma mass spectrometry (ICP MS): a versatile tool. *J. Mass Spectrom.* **2007**, *42*, 419–427.
- [39]. Kollmer, F. Cluster primary ion bombardment of organic materials. *Appl. Surface Sci.* **2004**, *231-232*, 153-158.
- [40]. Touboul, D.; Kollmer, F.; Niehuis, E.; Brunelle, A., Laprevote, O. Improvement of biological time-of-flight-secondary ion mass spectrometry imaging with a bismuth cluster ion source. *J. Am. Chem. Soc.* **2005**, *127*, 1608-1618.
- [41]. Sodhi, R.N.S. Time-of-flight secondary ion mass spectrometry (TOF-SIMS): versatility in chemical and imaging surface analysis. *Analyst.* **2004**, *129*, 483-487.
- [42]. Wang, P.C.; Mori, T.; Komori, K.; Sasatsu, M.; Toda, K.; Ohtake, H. Isolation and characterization of an *Enterobacter cloacae* strain that reduces hexavalent chromium under anaerobic conditions. *Appl. & Environ. Microbiol.* **1989**, *55*, 1665-1669.

- [43]. Wang, P.C.; Mori, T.; Toda, K.; Ohtake, H. Membrane associated chromate reductase activity from *Enterobacter cloacae*. J. Bacteriol. **1990**, *172*, 1670–1672.
- [44]. Cervantes, C.; Campos-Garcia, J.; Devars, S.; Gutiérrez-Corona, F.; Loza-Tavera, H.; Torres Guzmán, J.C.; Moreno-Sánchez, R. Interactions of chromium with microorganisms and plants. FEMS Microbiol. Rev. **2001**, *25*, 333–347.
- [45]. Bhakoo, M.; Herbert, R.A. The effects of temperature on the fatty acid and phospholipid composition of four obligately psychrophilic *Vibro spp.* Arch. Microbiol. **1979**, *121*, 121–127.
- [46]. Doenmez, G.; Aksu, Z. Bioaccumulation of copper (II) and Nickel (II) by the non-adapted and adapted growing *Candida sp.* Water Res. **2001**, *35*, 1425–1434.
- [47]. Wang, C.H.; Huang, C.P.; Sanders, P.F. Transport of Cr(VI) in soils contaminated with chromite ore processing residue (COPR). Pract. Period. Hazard. Toxic Radioact. Waste Manage. **2002**, *6*, 6–13.
- [48]. Bopp, L.H.; Ehrlich, H.L. Chromate resistance and reduction in *Pseudomonas fluorescens* strain LB300. Arch. Microbiol. **1988**, *150*, 426–431.
- [49]. Novick, A. Growth of Bacteria. *Annual Review of Microbiology*. **1955**, *9*, 97–110. DOI: 10.1146/annurev.mi.09.100155.000525
- [50]. Wang, Y.T.; Xiao, C. Factors affecting hexavalent chromium reduction in pure cultures of bacteria. Water Res. **1995**, *29*, 2467–2474.
- [51]. Megharaj, M.; Avudainayagam, S.; Naidu, R. Toxicity of hexavalent chromium and its reduction by bacteria isolated from soil contaminated with tannery waste. Curr. Microbiol. **2003**, *47*, 51–54.
- [52]. Desai, C.; Jain, K.; Madamwar, D. Evaluation of in vitro Cr(VI) reduction potential in cytosolic extracts of three indigenous *Bacillus sp.* isolated from Cr(VI) polluted industrial landfill. Bioresour. Technol. **2008**, *99*, 6059–6069.

- [53]. Vijayakumar, G.; Tamilarasan, R.; Dharmendra, M. K. Removal of Cd²⁺ ions from aqueous solution using live and dead *Bacillus subtilis*. *Eng.Res.Bull.* **2011**, *15*, 18–24.
- [54]. Desale P., Kashyap D., Nawani N., Nahar N., Rahman A., Kapadnis B. and Mandal A. Biosorption of Nickel by *Lysinibacillus* sp. BA2 native to bauxite mine. *Ecotoxicology and Environmental Safety*. **2014**, *107*. 260-268.
- [55]. Nepple, B.B.; Flynn, I.; Bachofen. Morphological changes in phototrophic bacteria induced by metalloid oxyanions. *Microbiol. Res.* **1999**, *154*, 191–198.
- [56]. Banerjee, S.; Datta, S.; Chattyopadhyay, D.; Sarkar, P. Arsenic accumulating and transforming bacteria isolated from contaminated soil for potential use in bioremediation. *J. Env. Sci. and Health, Part A.* **2011**, *46*, 1736–1747.

Figure legends

Fig. 1 Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship of the isolated strain B2-DHA in comparison with species belonging to the *Enterobacter* sp. The analysis included data from 1227 positions in the final dataset. The bootstrap values (expressed as percentages of 500 replicates) above 50 % are shown at the branch points.

Fig. 2 Study of chromium resistance of B2-DHA by measuring optical density and colony forming units (CFU). a, represents optical density obtained with cells exposed to chromium and without any exposure to chromium (control). Error bars denote standard error of mean. $P > 0.05$ (Two tailed t-test), not significant. b, represents cell counts. The number of CFU of bacterial cells, exposed to chromium and without exposure to chromium (control). Error bars denote standard error of mean. $*P \leq 0.05$ (two-tailed t-test), significant.

Fig. 3 Estimation of Cr(VI) concentration. a, amount of chromium absorbed by the bacterial cells. Error bars denote standard error of mean. $*P \leq 0.05$ (one-tailed t-test), significant. b, reduction of chromium by *Enterobacter* sp. B2-DHA in growth medium. Error bars denote standard error of mean. $**P \leq 0.01$ (one-tailed t-test), significant.

Fig. 4 Ion imaging and depth profiling of chromium species inside the bacterial cells by using TOF-SIMS. a, total protein signals. b, chromium oxide. c, chromium dioxide. d, bacterium was grown in absence of Cr(VI) and considered as control. The colored scale represents the intensity of ion imaging. e, depth profiling of bacterial cells grown in presence of chromium. Blue, red and black colours represent protein signals, chromium (II) oxide and chromium dioxide, respectively. f. depth profiling of bacterial cells grown in absence of chromium

(control). Blue color represents protein signals, whereas red and black colours stand for background activity of chromium.

Fig. 5 Effect of metals on cellular morphology of the isolates studied by environmental scanning electron micrograph (ESEM). a, strain B2-DHA in absence of chromium, magnification 12000X. b, strain B2-DHA in presence of 100 µg/mL chromium, magnification 12000X. c, corresponding X-ray energy dispersive system (EDS) spectra in absence of Cr(VI) and d, EDS spectra in presence of Cr(VI).

Figures

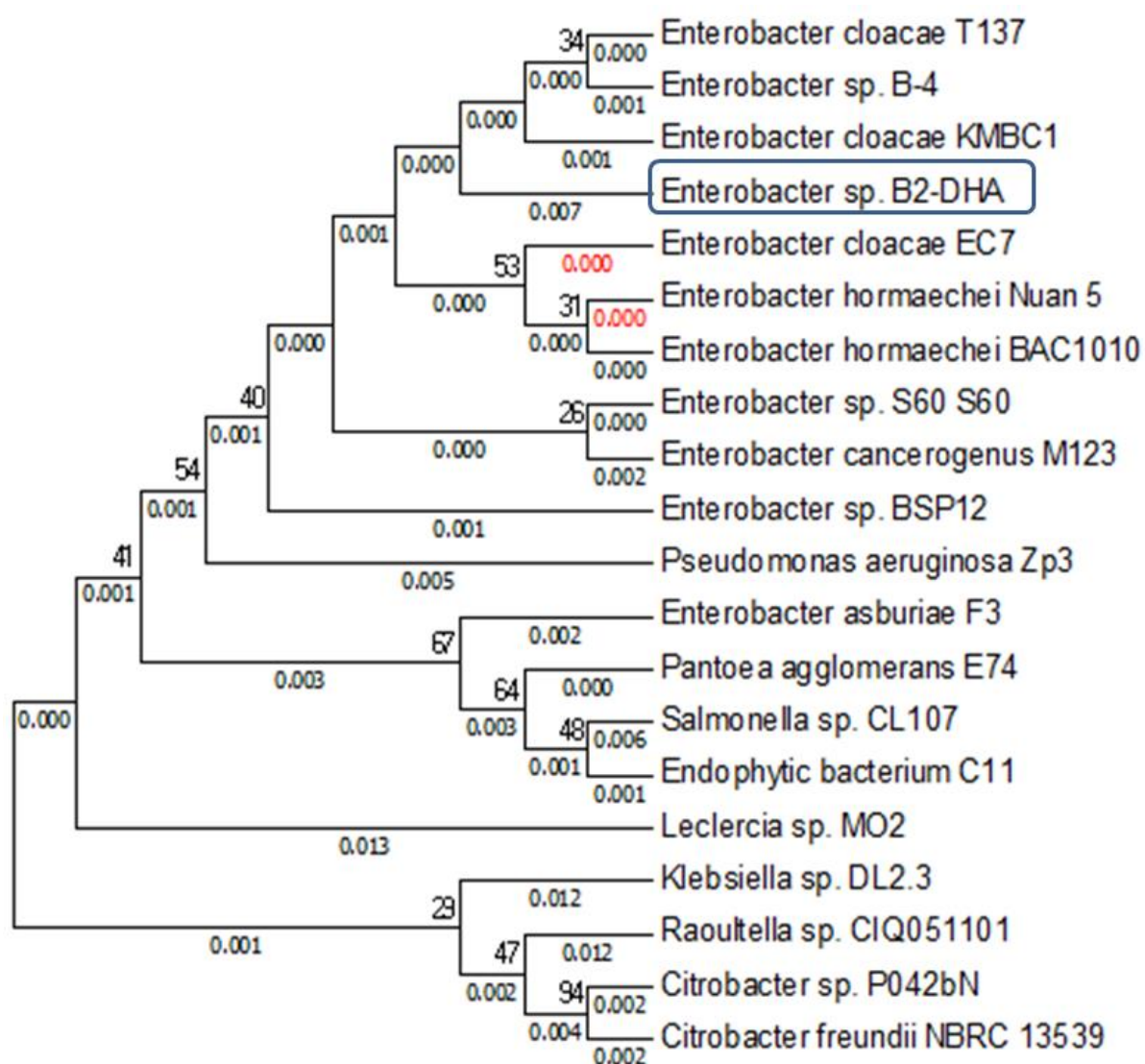


Figure 1

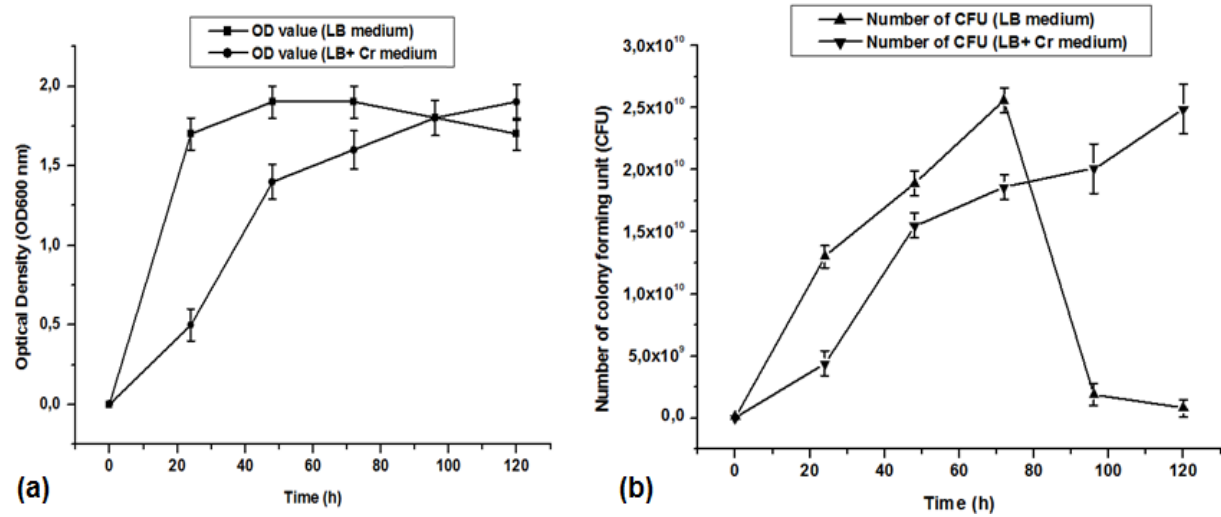


Figure 2

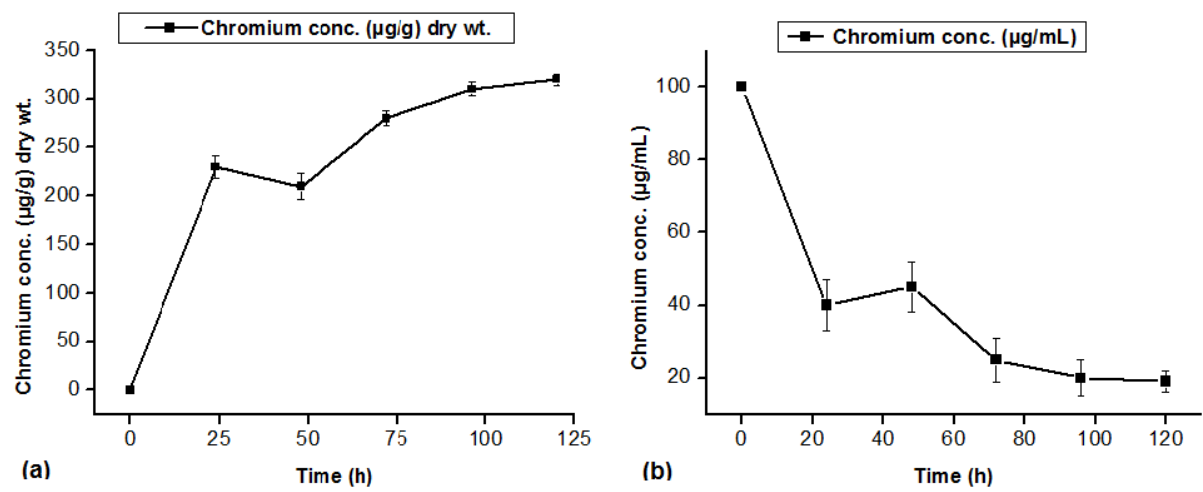


Figure 3

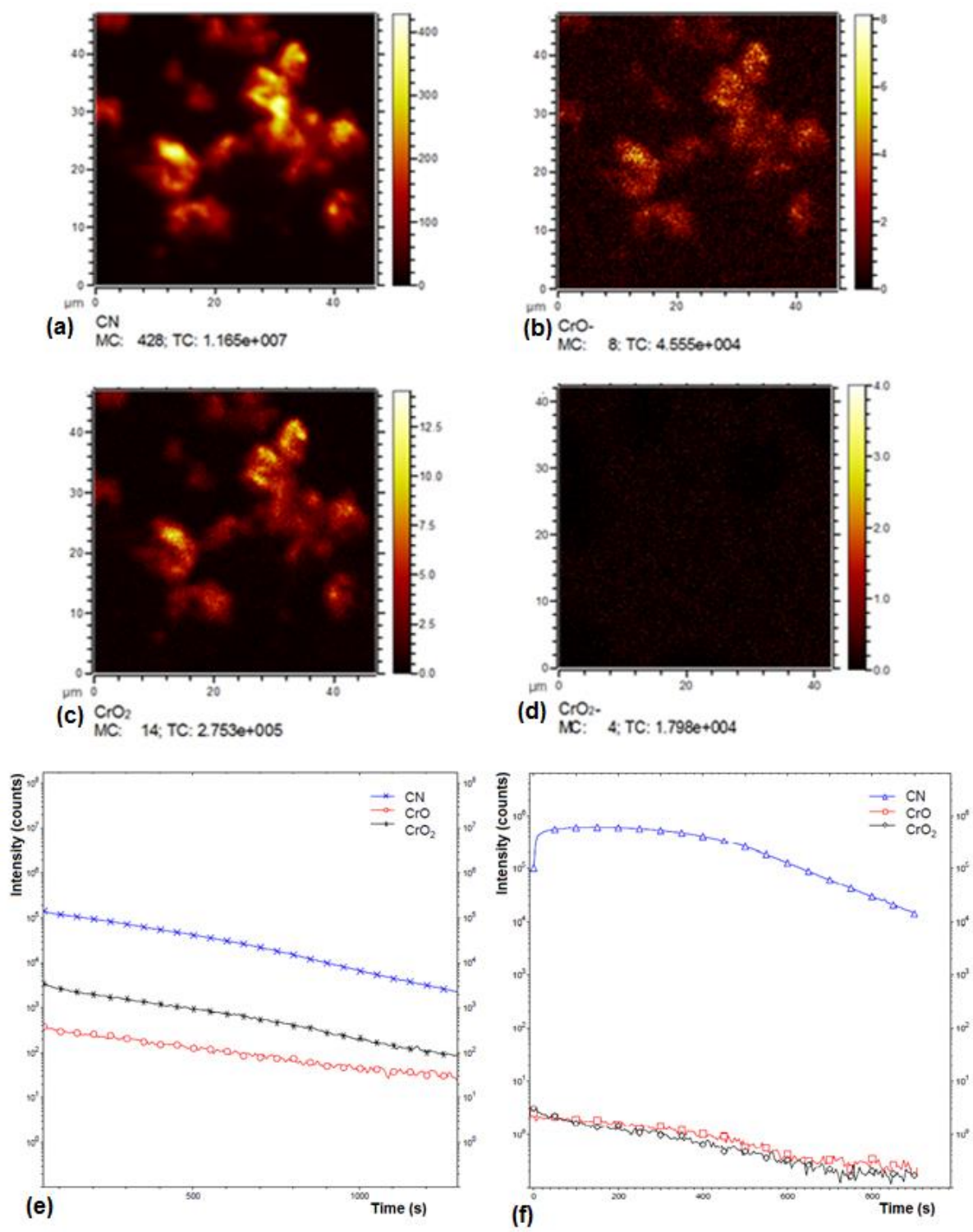


Figure 4

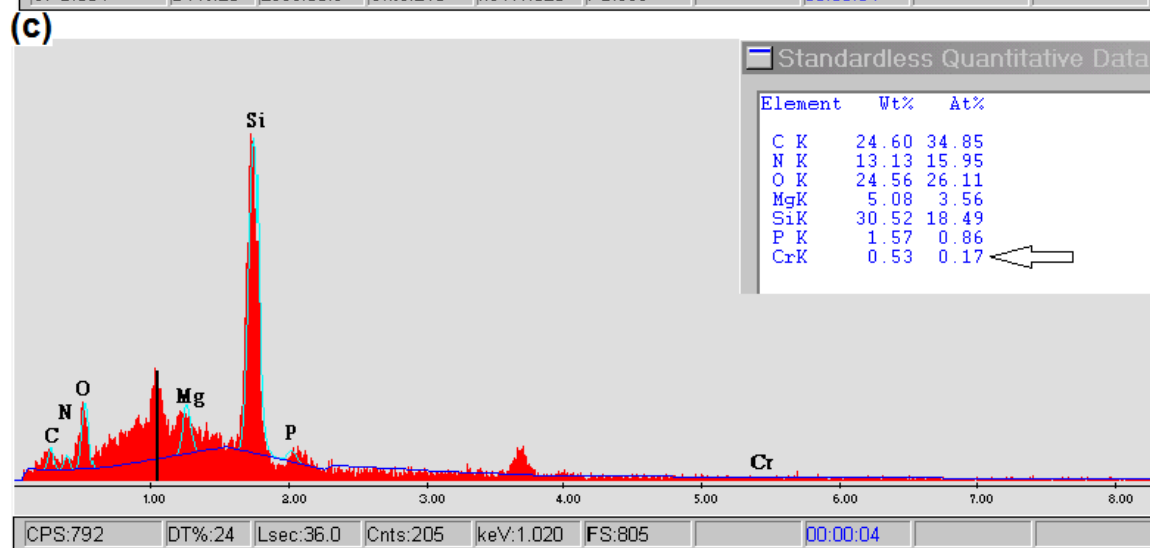
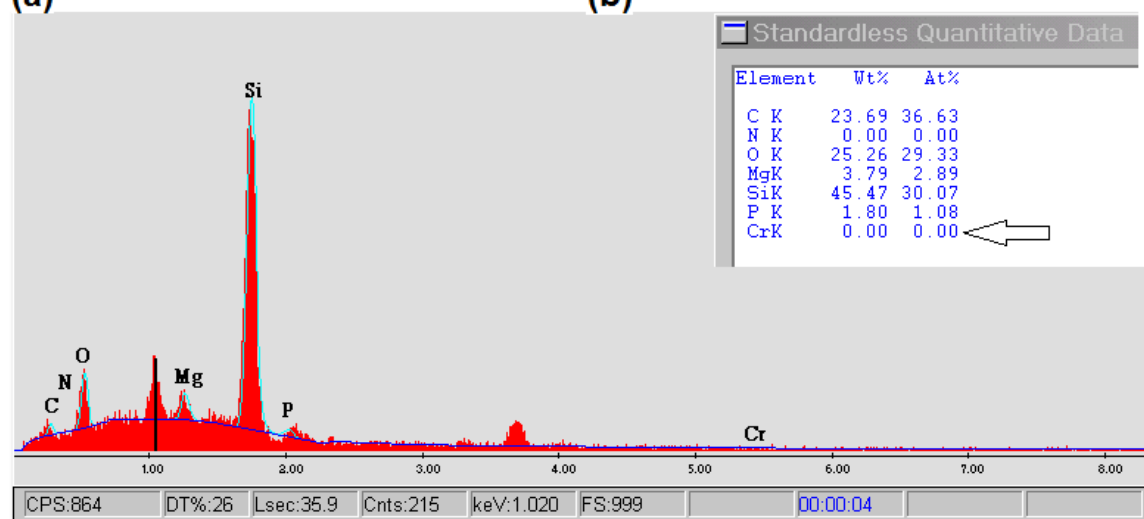
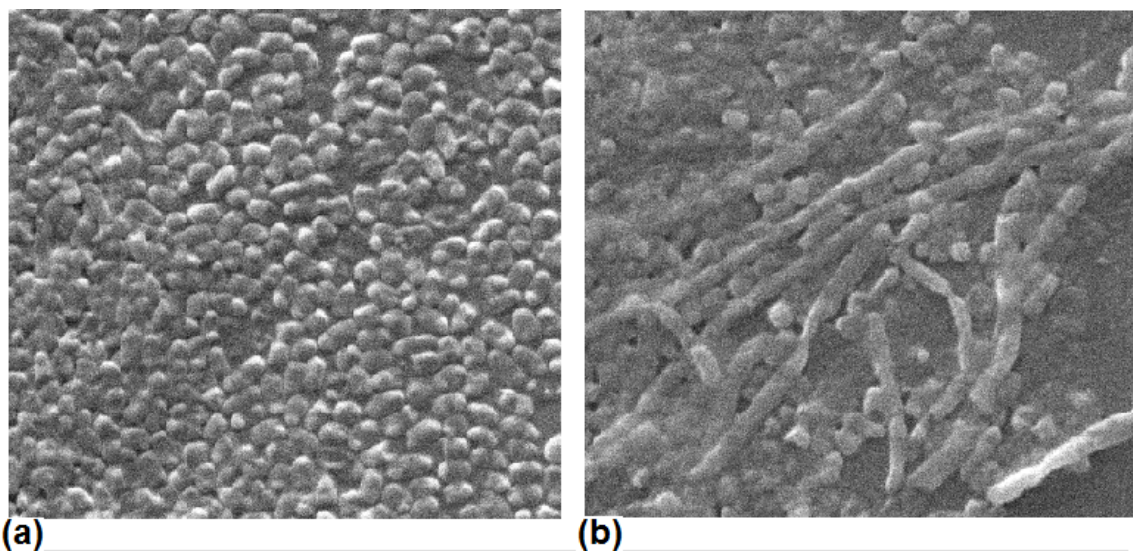


Figure 5