Chapter 3

Monitor microbial heavy metal resistance and predict their possible mode of action in environment cleaning

3.1. Background study:

As a result of abrupt industrialization and other human activities like unplanned cropping and chemical manure application, the deposition of different heavy metals in the environment has increased drastically day by day. Previous studies reported that the untreated wastewater discharged from different industrial outlets for a long while can affect the water bodies adversely with heavy metals and other hazardous chemicals [Wuana and Okieimen, 2011]. In this manner, the wastewater bodies get contaminated with different heavy metals like chromium, copper, zinc, manganese, iron [Devi, 2011]. The heavy metal can enter the human system through skin penetration, food chain or inhalation and cause different serious health issues [Kapahi and Sachdeva,2019]. Non-biodegradable heavy metals can persist within the environment as well as tissues of the plant, animal or human beings and magnified along with each trophic level. That's why remove hazardous heavy metals from the environment become a great challenge for us. The traditional methods like ion exchange technique, precipitation, electrodialysis can be convenient for environmental metal removal but these processes are time and energy-consuming, pH-dependent, non-specific and costly [Aziz et al. 2008]. Careful disposal should require for conventional contaminated slur development [Gunatilake, 2015] and these methods also can't remove the metal in low concentrations [Gray, 1999]. The cost-effective eco-friendly alternative approach of heavy metal removal by bio-agents can be more useful to eliminate different environmental heavy metal contaminants. Microbes are behaving as a potent metal biodegrading agent by removing metallic contaminants or converting them to less hazardous ones. Microbial metal conversion is dependent on environmental factors like pH, temperature, presence or absence of other metal ions, acids, moisture content etc. [Gadd, 2010].

Bioremediation is the mechanism by which microbes degrade the environmental hazardous metal contaminants or converted them to less-hazardous, bio-available organic substances which help in the growth and bio-mass formation of the other residual flora and fauna. Microbes can change the oxidation state of metal by redox reaction and transform the metal from a mobile state to an immobile state or vice-versa. As hexavalent chromium (Cr^{6+}) is converted to less hazardous trivalent chromium (Cr^{3+}) by microbial activity and the trivalent state become immobile because of its oxide or hydroxide forms [Singh et al. 2015].

Bacterial cells bio-absorbed the metal through the cell wall before the transformation. Before bioremediation, bacterial exposure to the contaminant is required for inducing a bacterial

enzymatic system but a minimum amount of contaminant can initiate enzymatic reaction [Adenipekun, 2012]. The metal adherence depends on the nature of (i.e., positivity) heavy metal and bacterial cell membrane. Heavy metals like chromium, cadmium, lead, zinc and copper can rapidly remove from the environment by bio-accumulation [Ozer and Ozer, 2003].

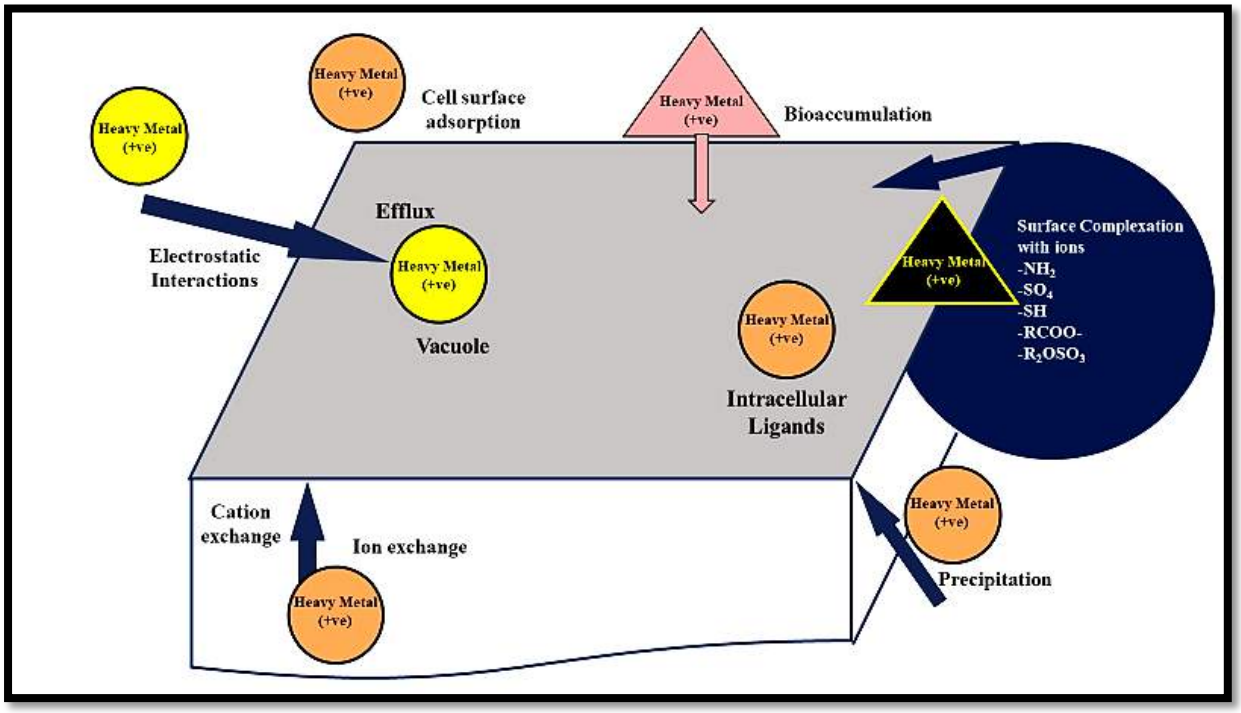


Figure 3.1.1: Different bacterial heavy metal uptake mechanisms [Fabricated according to Bernard et al. (2018) based on the findings of Yang et al. (2015) and Ayangbenro and Babalola (2017)]

Different chemical constituents of bacteria cell wall like poly-N-acetylglucosamine (NAG), N-acetylmuramic acid (NAM), the anionic group-containing peptidoglycan (in Gram-positive bacteria), teichoic acid (in Gram-positive bacteria), peptidoglycan (in Gram-negative bacteria), lipopolysaccharide (in Gram-negative bacteria), phospholipids (in gram-negative bacteria) enhance the metal-binding capability of bacteria [Sherbet, 1978]. Different functional groups present on the bacterial cell wall like hydroxyl group, carboxyl group, phosphate group, amine group are the main cause of this kind of metallic interactions [Doyle et al. 1980]. As per example, the carboxylic acid group of the bacterial cell wall can interact with cadmium ion [Yee and Fein, 2001], whereas the amino group of microbial cell wall play a potent role in chromium removal by creating Van Der Waals interaction and forming a chelate with chromium ion [Kang et al. 2007]. Kang et al. (2007) also stated that the outer

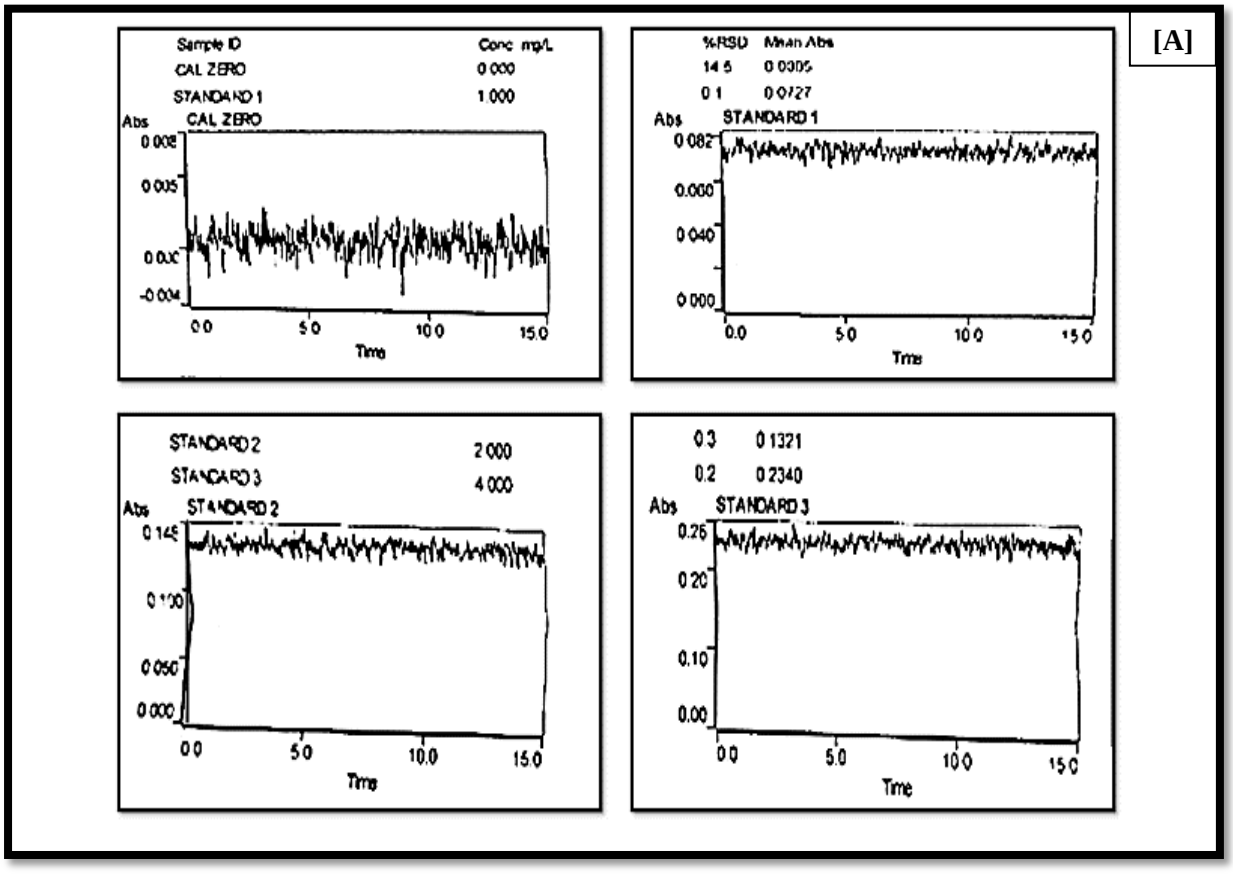
surface of bacterium *Pseudomonas aeruginosa* bio-absorbed hexavalent chromium employing adsorptive and reductive reactions. Different metal removing microbial strains like copper and arsenic removing *Bacillus*, *Micrococcus*, *Geobacter*, cadmium, nickel, chromium, copper, lead and zinc removing *Pseudomonas* had identified previously [Tunali, 2006; Dey et al. 2016; Zouboulis et al. 2004; Rani et al. 2010; Lee et al. 2012; He et al. 2019; Chellaiah et al. 2018; Lopez et al. 2000; Pardo et al. 2003]. These heavy metals removing bacteria uptakes environmental heavy metal through different strategies like physical adsorption, membrane transport, bio-absorption, ion change [Banerjee et al. 2018; Bernard et al. 2018] and utilize the heavy metals to fulfil their physiological needs. The heavy metals or other environmental chemical contaminants are behaving as the growth stimulator to the bioremediating microbes because they provide microbial food and energy through different metabolic pathways [Speight, 2018].

3.2. Methodology:

3.2.1. Estimation of metal within the soil and bacterial sample:

Properly air-dried slurry sewage soil samples were lyophilized using a freeze drier and ground into fine particles. Approximately 150 mg (120 mg in case of bacterial cell pellet) of the finely ground homogeneous sample was placed on the face of the Palletizer and the film of the pallet disc was removed. The pallet discs were inserted within the spectrometer with the help of the sample holder and attaching with scotch tape to determine the heavy metal content by EDXRF (Energy Dispersive X-ray Fluorescence) technique [Sudarshan et al. 2011] in triplicate.

One gm of each air-dried and finely pulverized soil and bacterial (cell pellet) samples were digested with 5 ml concentrated HNO_3, 0.5 ml of concentrated H_2SO_4 and 50 ml distilled water and heated on the hot plate to form a muddy texture. In wet digestion of microbial culture filtrates, HCl and HNO_3 were used in a 3:1 ratio. Finally, the sample was got ready for metal estimation by Atomic Absorption Spectrophotometer (APHA 23rd Ed., 3030E) model Variant AA140, by boiling the acid digested mixture for five minutes after addition of 50 ml distilled water and 1 ml concentrated HNO_3.



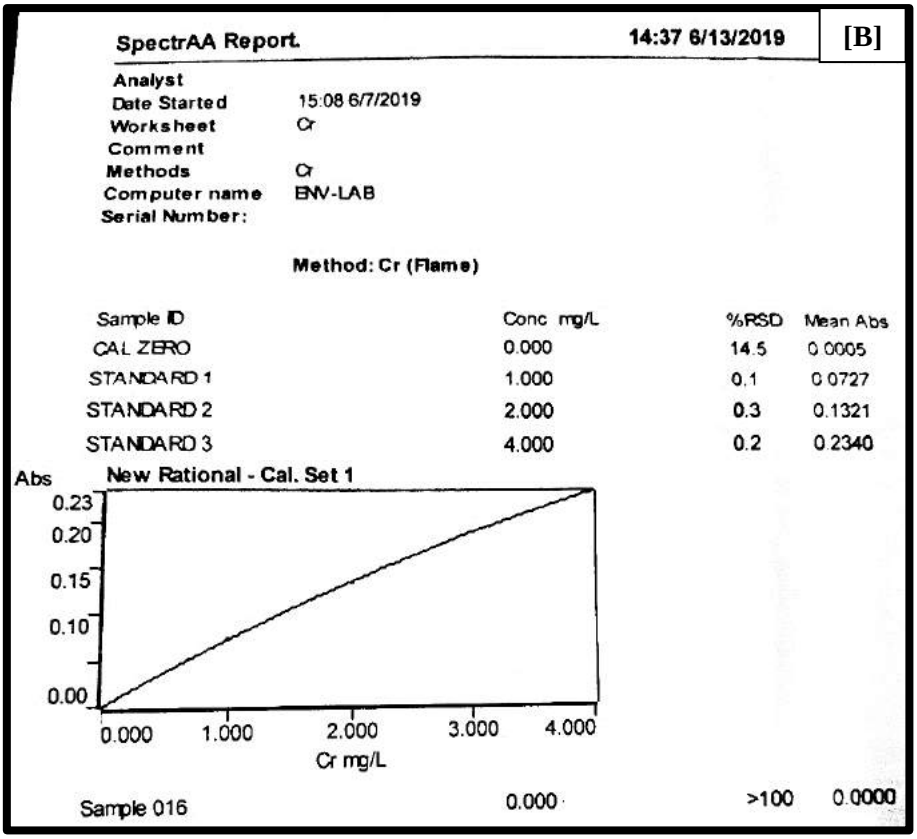


Figure 3.2.1: [A] The atomic absorption spectrometer was calibrated with different known concentrations of chromium containing samples considered as 'standard'. [B] The used standard samples helped to create the calibration curve to determine the chromium concentration in unknown samples

3.2.2. Measure bacterial heavy metal resistance by the agar cup technique:

The resistance of isolated soil bacteria against trivalent (Cr^{3+}) and hexavalent (Cr^{6+}) chromium, cadmium (Cd) and cobalt (Co) was measured individually and also in consortia implementing agar cup technique [Cooper 1955]. The salts of respective heavy metals were used at different concentrations (100mM, 50mM, 10mM, 10µM, 10 nM) to investigate and compare the inhibition zone. In this method, each inoculated plate was bored with the sterile cork-borer (8 mm diameter) for creating wells of equal width and depth into which metal salt solutions were poured, and the zone of inhibition was subsequently measured.

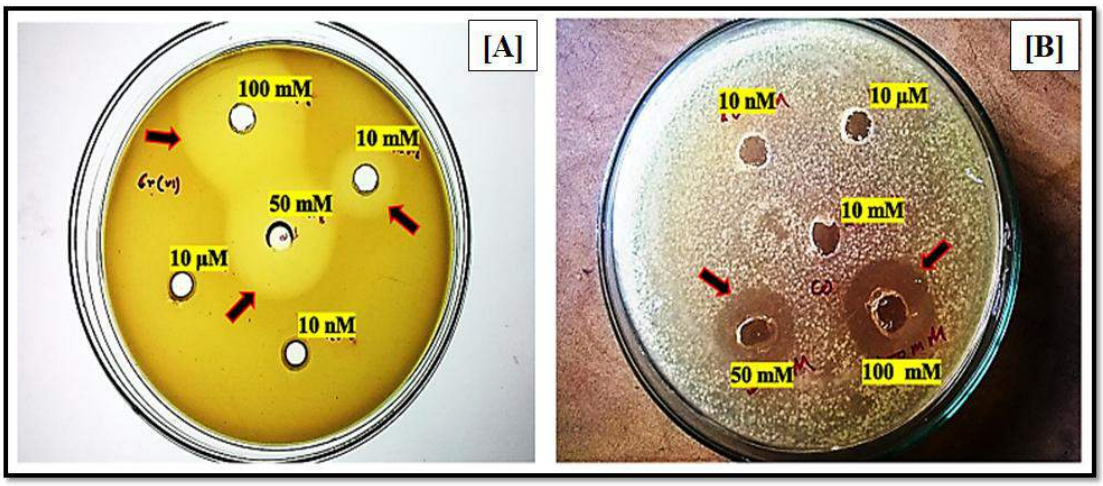


Figure 3.2.2: The two of the selected microbial isolates showed [A] hexavalent chromium [Cr(VI)] and [B] cobalt (Co) resistance by generating the inhibition zones; [A] Cr (VI) and [B] Co salts were applied within the wells at mentioned concentrations (The arrows indicate zones of inhibition)

3.2.3. Determination of MIC against chromium resistant bacteria:

For the determination of the Minimum Inhibitory Concentration (MIC), the microbes were inoculated in gradually increased concentrations of metal supplemented LB broth media till their growth was inhibited and bacterial MIC was determined by measuring turbidity at 595 nm [Yilmaz, 2003].

3.2.4. Estimation of hexavalent chromium:

The presence of hexavalent chromium was confirmed by the diphenyl carbazole spectrophotometry method (APHA 23rd Ed.) in which 1 ml 10 µg - 100 µg Cr^{6+} containing sample was diluted to 100 ml with distilled water, 0.2 ml H_3PO_4 was added to it and adjusted the pH to <6. Then 2 ml diphenyl carbazole solution was added and read the absorbance of the developed pink colour at 540 nm FT-IR spectra after 10 minutes of incubation (Sanchez-Hachair and Hofmann, 2018) against the blank reference. 50mg/ml solution of Cr^{6+} [containing 141.45 mg of potassium dichromate ($K_2Cr_2O_7$)] in one-liter distilled water was used as standard solution (APHA, 22nd Ed., 2012).

3.2.5. Detection of hexavalent chromium and confirmation of chromium reduction within cell along with simultaneous bacterial growth:

The percentage of Cr^{6+} reduction within bacterial the cell pellet with relative growth of bacterial cell was assessed simultaneously following the protocol of Dey *et al* (2014). To

carry out this experiment, the Cr^{6+} supplemented media was cultured with 10% inoculum and cell number and viability was measured along with Cr^{6+} content after definite time intervals.

To confirm bacterial metal absorption and retention, chromium supplemented media were inoculated with bacteria and maintain batch culture. After specific time intervals, the microbial cells were collected from the metal supplemented batches by centrifugation, and residual $Cr^{3+}$and Cr^{6+} concentrations of filtrate and properly washed lysed cell pellets were monitored using diphenyl carbazole spectrophotometry method (APHA 23rd Ed.,2017) and NaOH and NH_4OH test accordingly; remaining pellets were reintroduced into metal-free LB broth for three more times (for 24 hours each) and residual metal content of the cell-free culture filtrate and the pellet was measured batch to batch after specific time intervals.

The presence of trivalent chromium within the bacterial cell pellet was confirmed finally by applying NaOH and NH_4OH to the lysed cell pallet.

3.2.6. Bacterial cell lysis method:

The presence of trivalent and hexavalent chromium was measured within the lysed cells. To lyse bacterial cells, first cells pellets were collected through centrifugation at 5000 rpm for 10 minutes and resuspended pellets were treated in cold with lysozyme for 30 minutes. The cold treated cell pellet was reintubated at 30^oC for 15 minutes. Lysis buffer was then added to sonicated viscus cell pellet in presence of ice and centrifuged in cold at 35,000 rpm for 30 minutes to collect the lysed cell debris.

3.2.7. Detection of chromium reduction in presence of growth stimulators:

Microbial growth stimulators like sodium azide and 2,4 dinitrophenol (DNP) were applied at 10 mM Cr^{6+} and 40 mM Cr^{6+} supplemented media with equimolar concentrations to find the rate of microbial chromium reduction in the presence of these simulators.

3.2.8. Profiling bacterial growth in the presence and absence of heavy metals:

The hourly bacterial growth pattern was monitored in trivalent and hexavalent chromium supplemented and heavy metal-free LB broth to obtain the growth curves. The growth of bacterial culture was measured in terms of optical density at 595 nm.

3.2.9. Observe the effect of chromium on bacterial cell wall by Scanning Electron Microscope (SEM):

The cell biomass was isolated by centrifuging the mid-log phase cells and cell pellets were properly washed and fixed with 3% (v/v) glutaraldehyde and then washed thrice with the 0.1(M) PBS. Following sequential dehydration, the samples were applied on the cover-slip, dried, and SEM micrographs were observed after platinum coating using Zeiss Evo-MA version 10.

3.2.10. Prediction of Bacterial chromium accumulation and deposition site by Transmission Electron Microscope (TEM):

The cells were pelleted down from 10 mM hexavalent chromium supplemented media, were properly washed and fixed using fixative [3% (v/v) glutaraldehyde in 0.1 M PBS] and subsequently washed thrice with the 0.1 M saline phosphate buffer (PBS). The cell pellets were stained with 1% osmium tetraoxide, and 2% UranyLess (Electron Microscopy Sciences) [Zakaria et al. 2007]. After washing and properly removing the formed artefacts with sterile millipore water, sequentially dehydrated samples were applied to the copper grid, dried, and viewed under TEM (JEOL JEM 2100 with EELS).

3.2.11. Optimization of bacterial metal absorbance by batch chromium absorption and removal assay:

To conduct the experiment, 20 ml of 10 mM and 40 mM hexavalent chromium supplemented media were inoculated with 10% bacterial batch culture. After specific time intervals, the microbial cell was collected from the metal supplemented batches and was centrifuged at 5000 rpm for 10 minutes, and residual metal concentration of filtrate and properly washed (with sterile millipore water) cell pellet was monitored using EDXRF spectrometer and AAS.
To estimate the bacterial metal retention efficiency, washed the cell pellets properly with sterile millipore water and reintroduced the washed pellets into metal-free LB broth three more times (for 24 hours) and residual metal content of the cell-free culture filtrate and the pellet was measured batch to batch after specific time intervals.

3.2.12. Statistical analysis:

Each experiment was repeated in triplicate. The data were standardized using one-way ANOVA and two-tailed t-test. Significant differences were compared using a $P \leq 0.05$ significance level (and $p < 0.01$ high significance level) and r value between +1 and -1 using Microsoft Excel and SPSS software.

3.2.13: Composition of used buffer:

3.2.13.1: 10X Phosphate Buffered Saline (PBS)

Ingredients	Amount
Sodium chloride	80.00 g/liter
Potassium chloride	2.00 g/liter
Di-sodium hydrogen phosphate	14.40 g/liter
Potassium hydrogen phosphate	2.40 g/liter
Final pH (25^{o}C)	7.4±0.2

3.2.13.2: 10X Cell lysis buffer:

Ingredients	Amount
Tris-HCl	25 mM (pH 8±0.5)
Sodium chloride	150 mM
Imidazole	10 mM
Triton	1%
DTT	5 mM
PMSF	1 mM
Lysozyme	100 µg/ml
Glycerol	10%
DNase	50 µg/ml

3.3 Results:

3.3.1. Result of soil heavy metal profiling:

Different heavy metals such as chromium, zinc, lead, iron, nickel, arsenic, lead were detected in sewage soil by Energy Dispersive X-Ray Fluorescence (EDXRF) spectrometer and AAS (Atomic Absorption Spectroscopy). The chromium content was alarmingly high in the soil of East Kolkata Wetland, being 20864 ± 0.28 ppm, whereas that of the soils collected from Circular Canal and Kestopur Khal was also much greater than the normal limit (14-70 ppm according to Health assessment document for chromium, 1984; WHO Air Quality Guidelines – 2nd Ed., 2000), being 187.1 ± 0.41 and 187.1 ± 0.15 ppm, respectively. The concentration of soil chromium also varied greatly among the different sewage regions (Table 3.3.1).

Table 3.3.1: Result of soil heavy metal profiling by Energy Dispersive X-Ray Fluorescence (EDXRF) spectrometer:

Regions	Chromium (ppm)	Zinc (ppm)	Lead (ppm)	Iron (ppm)	Arsenic (ppm)	Nickel (ppm)
Circular Canal	187.1 ± 0.41	238.91±0.67	31.22±0.84	52308.37±14.99	7.003±0.015	61.22±0.89
East Kolkata Wetland	20864 ± 0.28	546.83±0.79	111.73±0.21	44664.38±3.08	7.17±0.02	56.19±0.67
Kestopur Khal	187.1 ± 0.15	381±0.01	41.23±0.9	58365.65±6.47	7.02±0.02	62.69±0.91

3.3.2. Bacterial heavy metal tolerance profile:

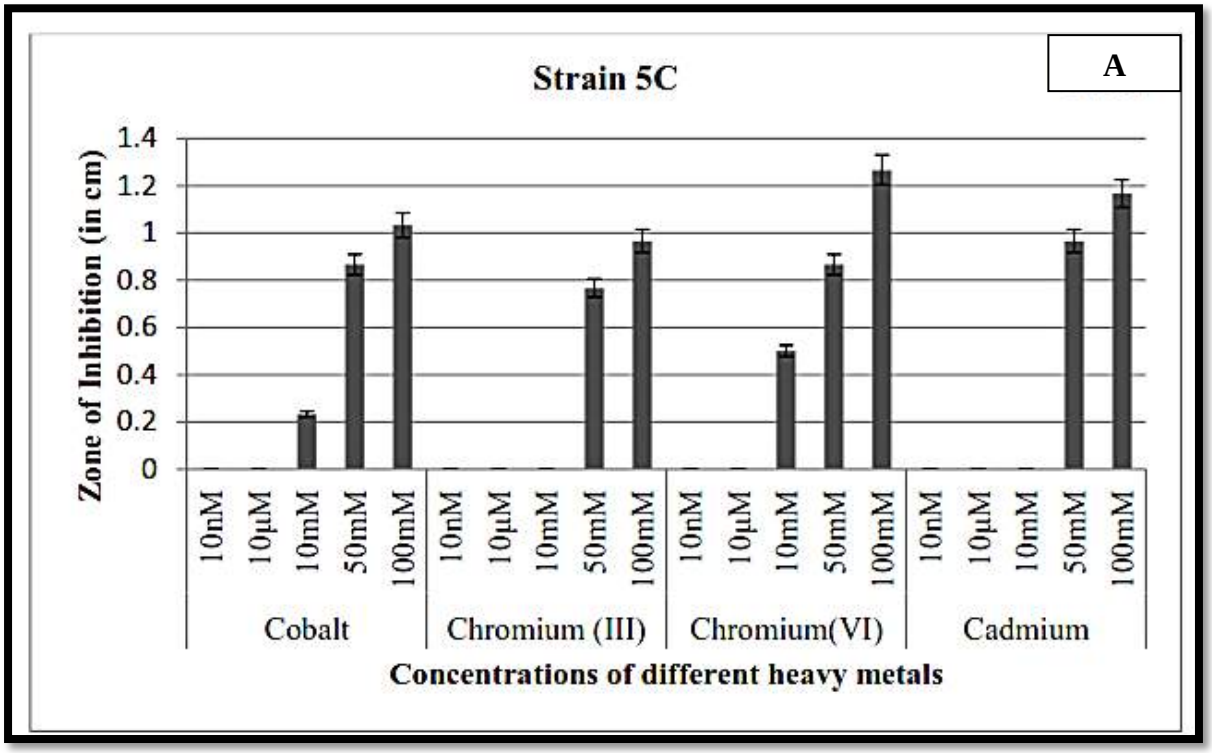
The results of the agar cup technique showed that the majority of the soil microbes isolated from the bank of three wastelands were resistant up to 10 mM of cadmium and cobalt, but most of them were total resistant (i.e., no zone of inhibition) up to 50 mM hexavalent chromium and 100 mM trivalent chromium respectively (Figure 3.3.1; Table 3.3.2). The trend of the microbial zone of inhibition patterns indicated that the microbes isolated from Kestopur Khal, East Kolkata Wetland and Circular Canal showed resistance against 100 mM trivalent chromium and 10 mM - 50 mM hexavalent chromium (Figure 3.3.1; Table 3.3.2).

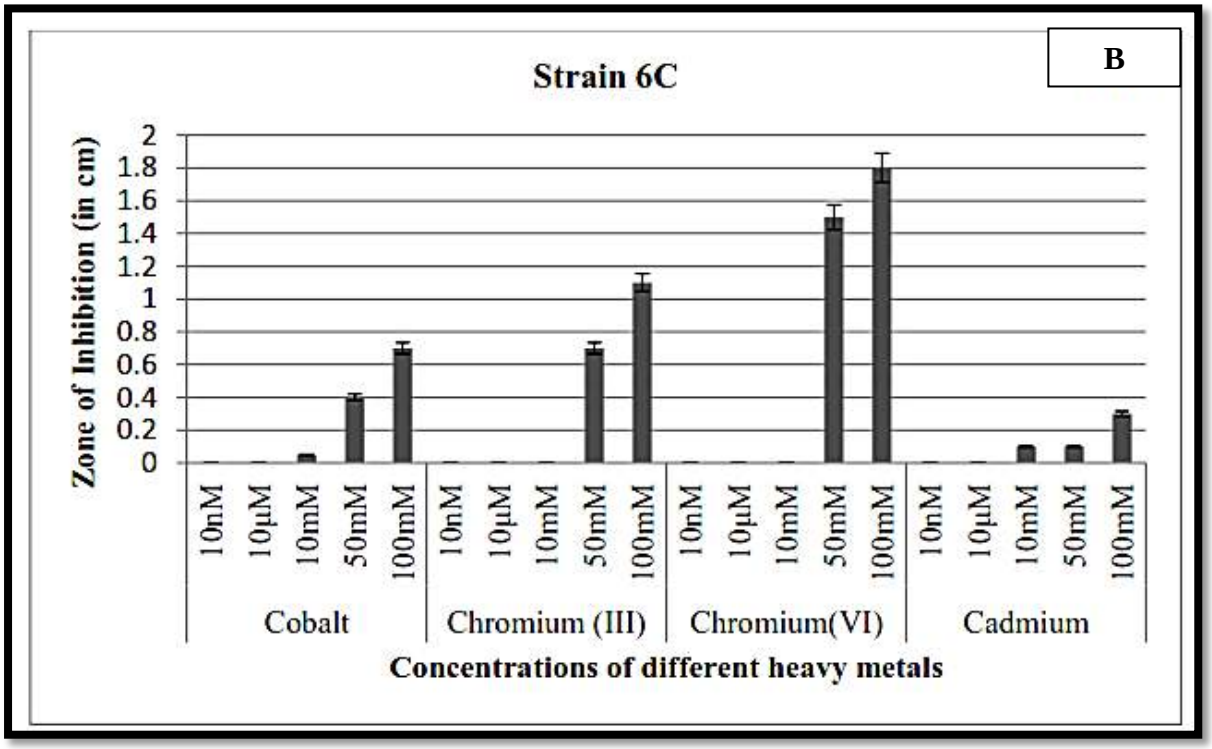
Fifteen isolates namely 5C, 6C, 7C, 8C, 12C, 1E, 2E, 3E, 4E, 7E, 5K, 6K, 7K, 25K and 26K were shortlisted based on the statistically significant data about their resistance patterns against four selected heavy metals viz. Cr (III), Cr (IV), Co, Cd and antibiotic sensitivity. As most of the isolates showed chromium resistance, and soil chromium concentration was also alarmingly high in different sewage sites of Kolkata (Table 3.3.1) [the permissible limit is 14-70 ppm according to Health assessment document for chromium, 1984; WHO air quality guidelines – 2nd Ed., 2000, and WHO drinking water quality guidelines – 3rd Ed., 2008], the chromium resistant isolates were taken in consideration and their chromium accumulation, retention and reduction capability was investigated with proper statistical significance.

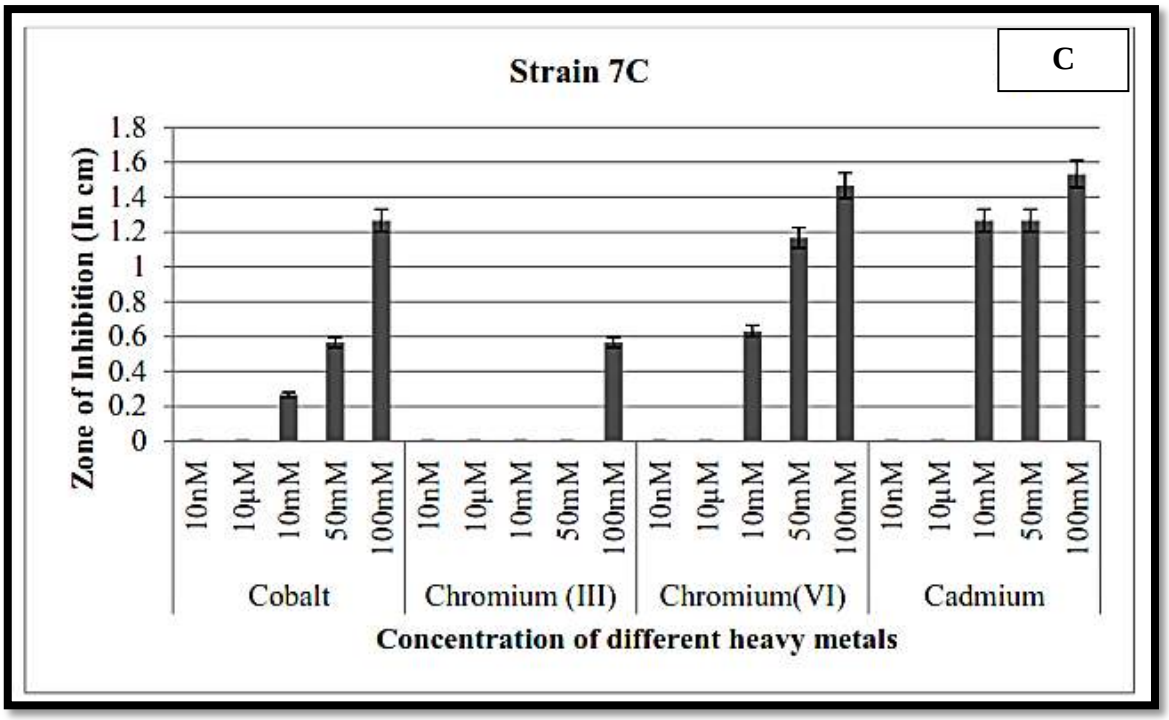
Table 3.3.2: Bacterial heavy metal sensitivity (measured by calculating the zone of inhibition):

Strain	Diameter of zone of inhibition against applied heavy metal (in cm)																			
	Cr(III)					Cr(VI)					Cd					Co				
	10 nM	10 µM	10 mM	50 mM	100 mM	10 nM	10 µM	10 mM	50 mM	100 mM	10 nM	10 µM	10 mM	50 mM	100 mM	10 nM	10 µM	10 mM	50 mM	100 mM
5C	0	0	0	0.8± 0.05	1± 0.05	0	0	0.5± 0.05	0.9± 0.05	1.3± 0.05	0	0	0	1± 0.05	1.2± 0.05	0	0	0.2± 0.05	0.9± 0.05	1± 0.05
6C	0	0	0	0.7± 0.05	1.1± 0.06	0	0	0	1.5± 0.06	1.8± 0.06	0	0	0.1± 0.05	0.1± 0.05	0.3± 0.05	0	0	0.05± 0.013	0.4± 0.06	0.7± 0.05
7C	0	0	0	0	0.6± 0.06	0	0	0.6± 0.05	1.2± 0.06	1.5± 0.02	0	0	1.3± 0.05	1.3± 0.05	1.5± 0.06	0	0	0.3± 0.06	0.6± 0.05	1.3± 0.06
8C	0	0	0	0	0.6± 0.06	0	0	0	0.6± 0.05	1.1± 0.05	0	0	0	0.8± 0.06	1.3± 0.05	0	0	0.15± 0.09	0.3± 0.05	1± 0.05
12C	0	0	0	0	0	0	0	0	0.3± 0.06	0.7± 0.06	0	0	0	0.7± 0.06	1.1± 0.06	0	0	0	0.6± 0.07	0.7± 0.06
1E	0	0	0	0	0	0	0	0	0.02± 0.002	0.53± 0.05	0	0	1.06± 0.05	1.33± 0.5	1.8± 0.1	0	0	0	0.53± 0.05	0.76± 0.05
2E	0	0	0	0	0	0	0	0	1.26± 0.06	1.6± 0.06	0	0	1.06± 0.06	1.46± 0.06	1.6± 0.06	0	0	0	0.36± 0.06	0.86± 0.06
3E	0	0	0	0	0	0	0	0	0.83± 0.06	1.46± 0.06	0	0	1.06± 0.06	1.43± 0.06	1.66± 0.06	0	0	0	0.53± 0.06	0.86± 0.06
4E	0	0	0	0	0	0	0	0	0.18± 0.05	0.29± 0.05	0	0	0.96± 0.05	1.13± 0.05	1.5± 0.05	0	0	0	0.53± 0.05	0.82± 0.05
7E	0	0	0	0	0	0	0	0	0.21± 0.06	0.55± 0.06	0	0	0	1.06± 0.06	1.18± 0.06	0	0	1.06	1.42± 0.06	1.62± 0.06
5K	0	0	0	0	0	0	0	0	0.56± 0.06	1.3± 0.06	0	0	0.66± 0.06	0.96± 0.06	1.13± 0.06	0	0	0	0.26± 0.06	0.46± 0.06
6K	0	0	0	0	0	0	0	0	0.56± 0.05	1.3± 0.05	0	0.36± 0.05	0.4± 0.05	0.53± 0.05	1.16± 0.05	0	0	0	0	0.16± 0.05
7K	0	0	0	0	0	0	0	0	0.1± 0.06	0.7± 0.06	0	0	0	0	0	0	0	0	0.26± 0.05	0.46± 0.06
25K	0	0	0	0	0	0	0	0	0.13± 0.05	0.16± 0.05	0	0.03± 0.02	0.1± 0.05	0.3± 0.05	0.8± 0.05	0	0	0	0.33± 0.05	0.86± 0.05
26K	0	0	0	0	0	0	0	0.43± 0.05	1± 0.05	2.5± 0.05	0	0	1.06± 0.05	1.26± 0.05	1.53± 0.05	0	0	0	0	0

I. Bacteria Isolated from Circular Canal



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Strain 7C
Zone of Inhibition (In cm)
1.8
1.6
1.4
1.2
1
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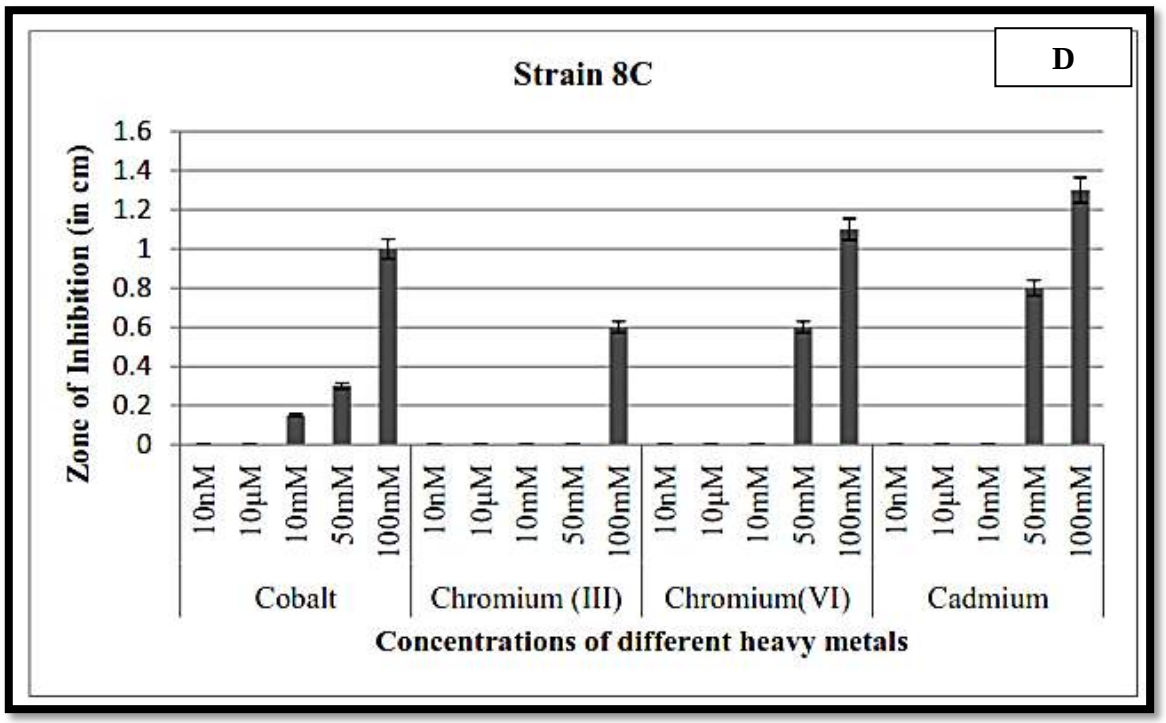
0.4
0.2
0
10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM
Cobalt
Chromium (III)
Chromium(VI)
Cadmium
Concentration of different heavy metals


D
Strain 8C
Zone of Inhibition (in cm)
1.6
1.4
1.2
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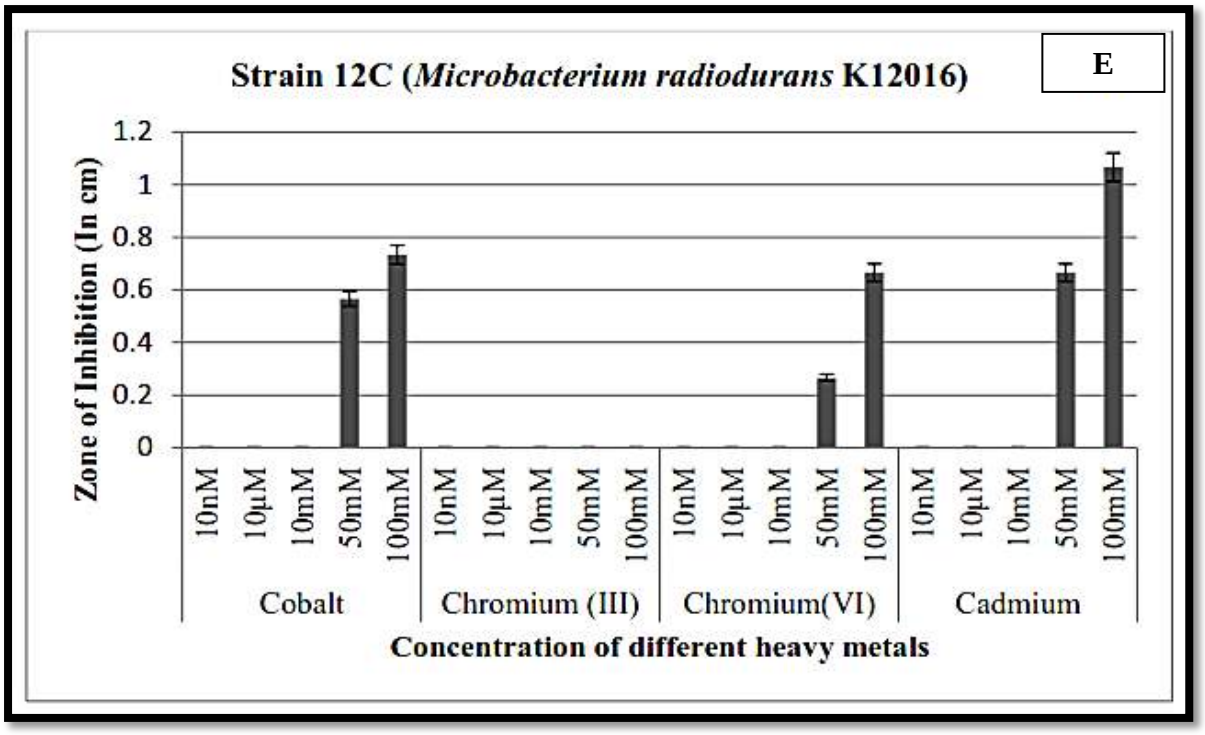
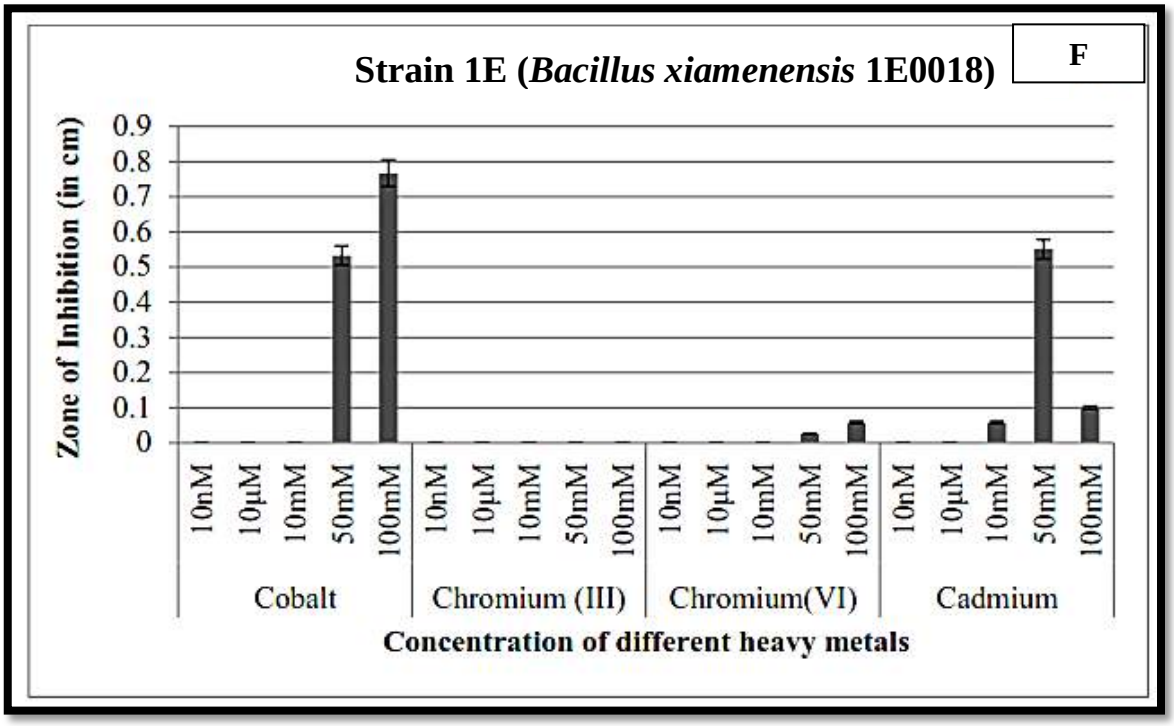
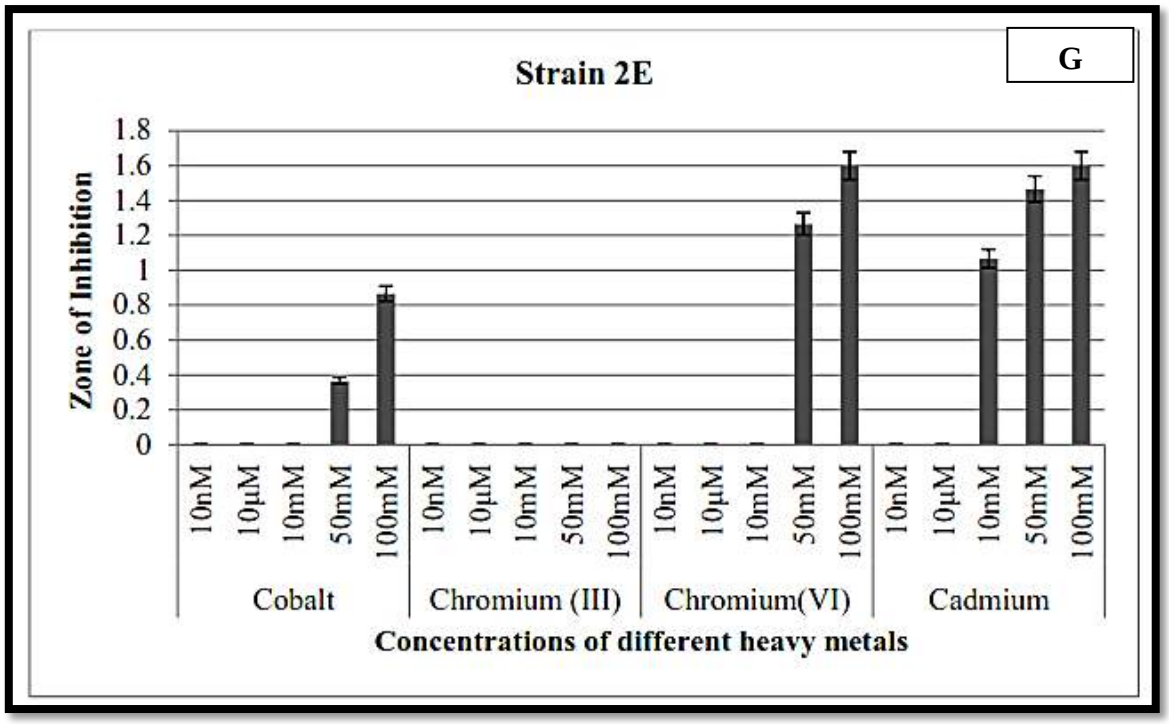
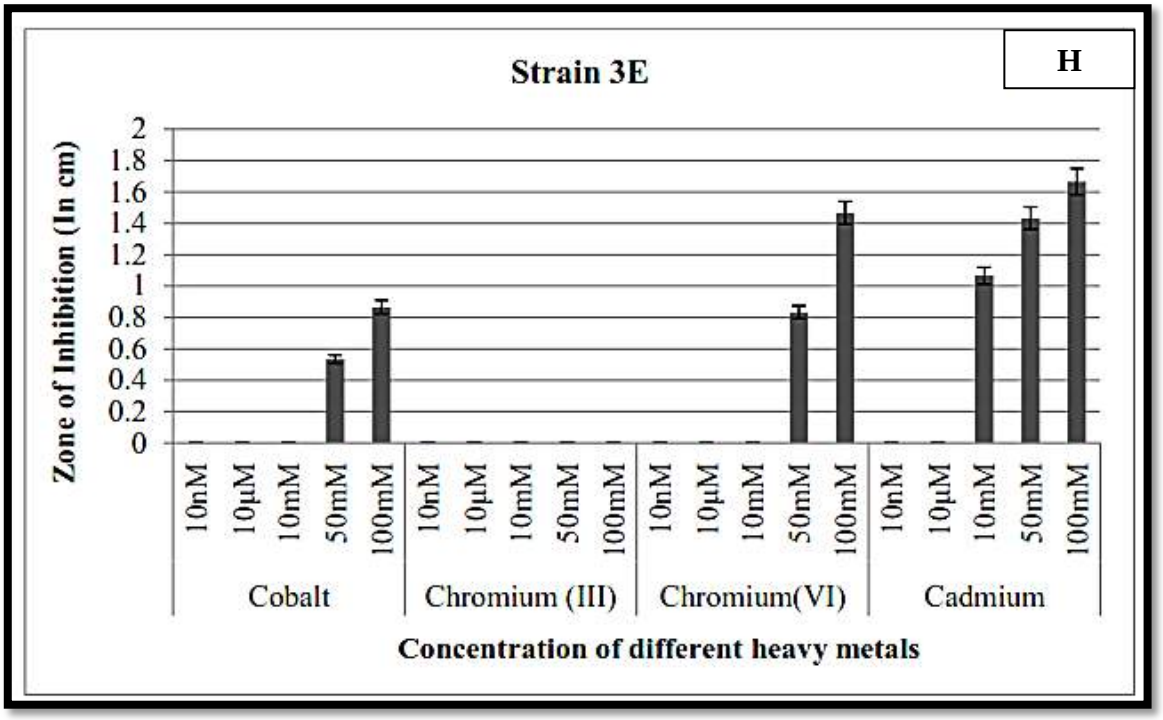
10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM
Cobalt
Chromium (III)
Chromium(VI)
Cadmium
Concentrations of different heavy metals



II. Bacteria isolated from East Kolkata Wetland




G
Strain 2E
Zone of Inhibition
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10nM
10µM
10mM
50mM
100mM
Cobalt
Chromium (III)
Chromium(VI)
Cadmium
Concentrations of different heavy metals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Strain 3E
Zone of Inhibition (In cm)
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10nM
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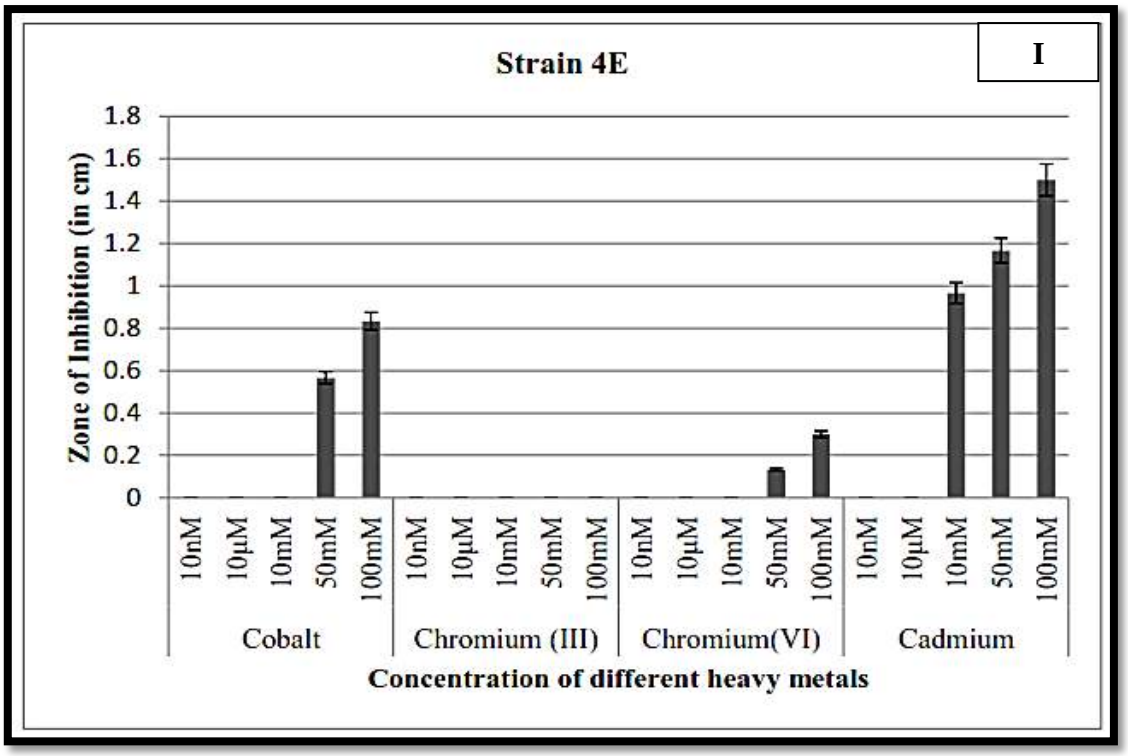
50mM
100mM
Cobalt
Chromium (III)
Chromium(VI)
Cadmium
Concentration of different heavy metals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Strain 4E
Zone of Inhibition (in cm)
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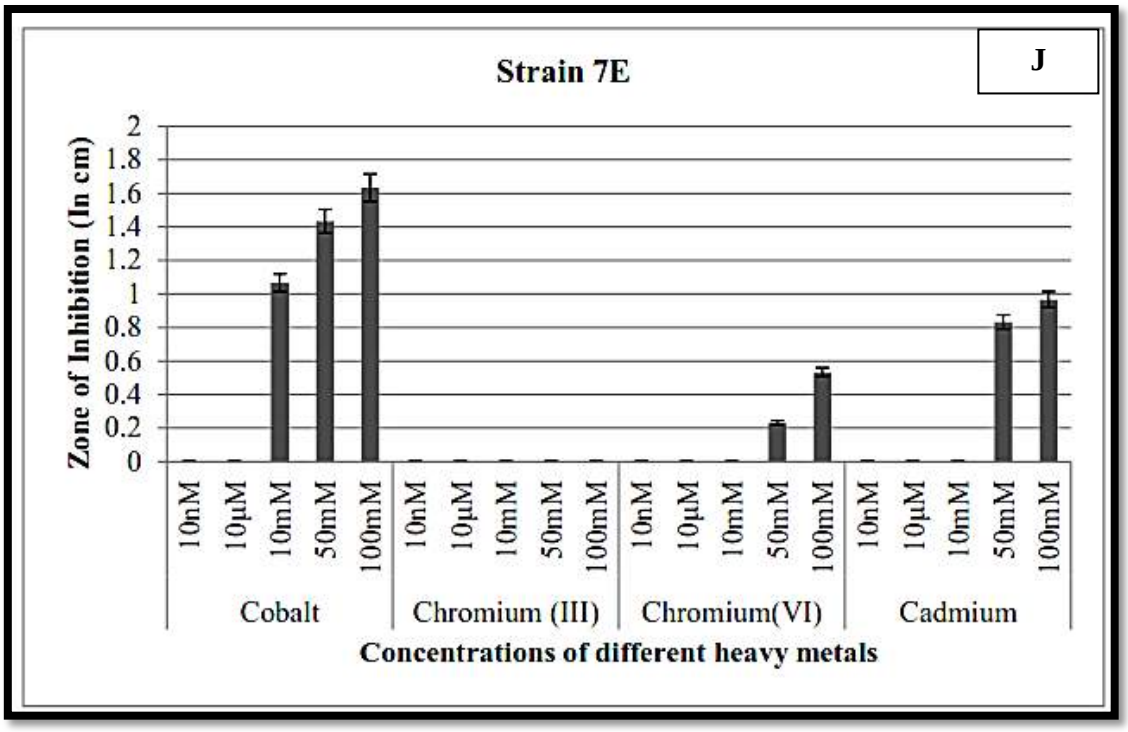
Cobalt
Chromium (III)
Chromium(VI)
Cadmium
Concentration of different heavy metals


J
Strain 7E
Zone of Inhibition (In cm)
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0.6
0.4
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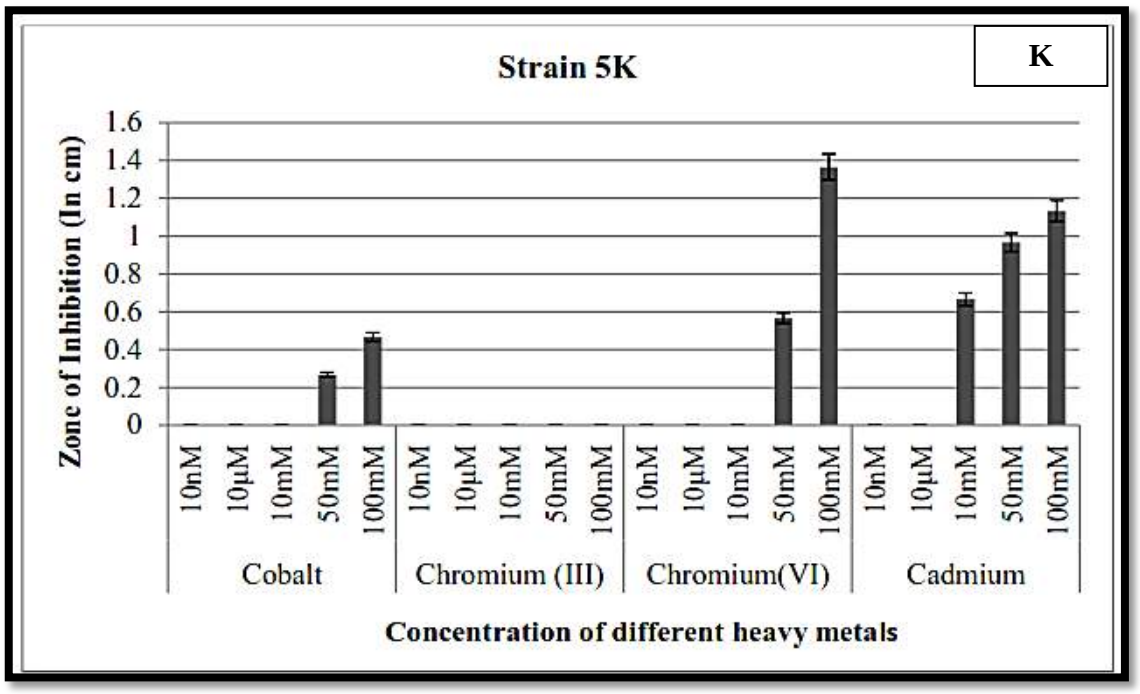
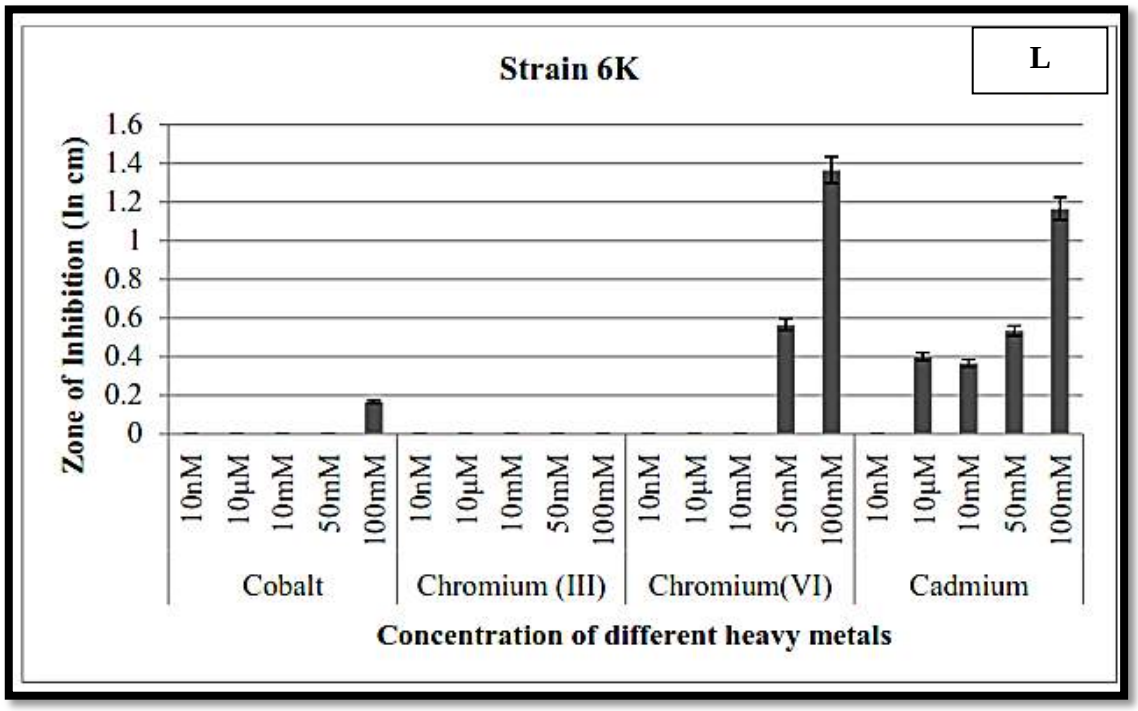
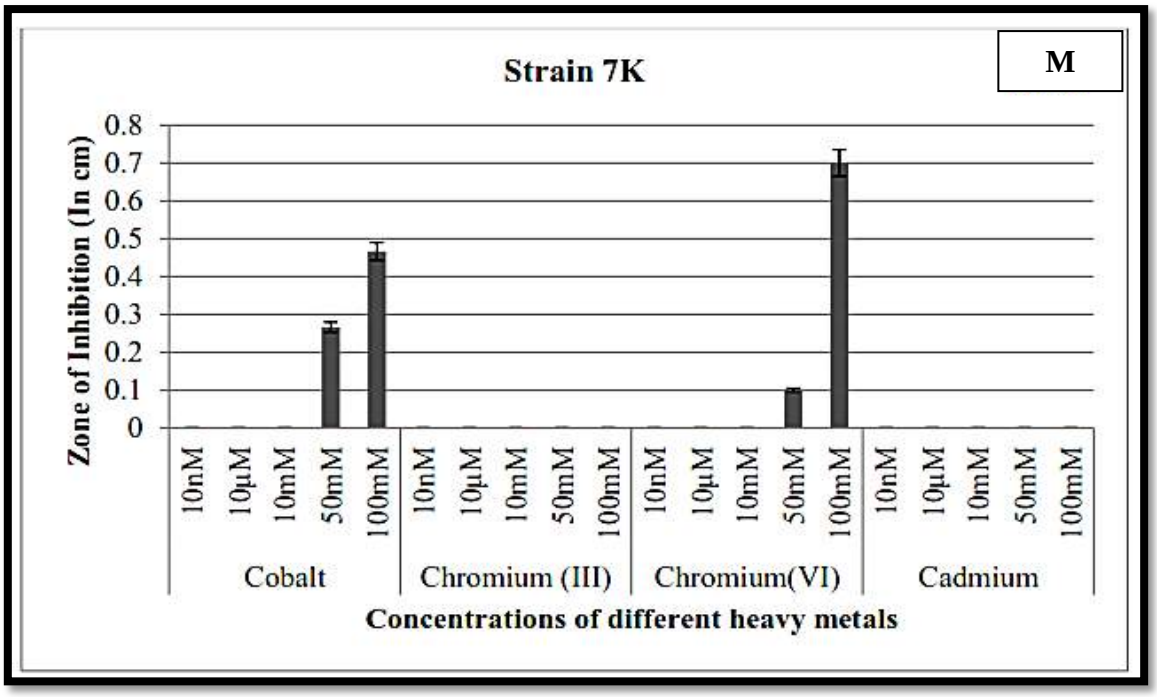
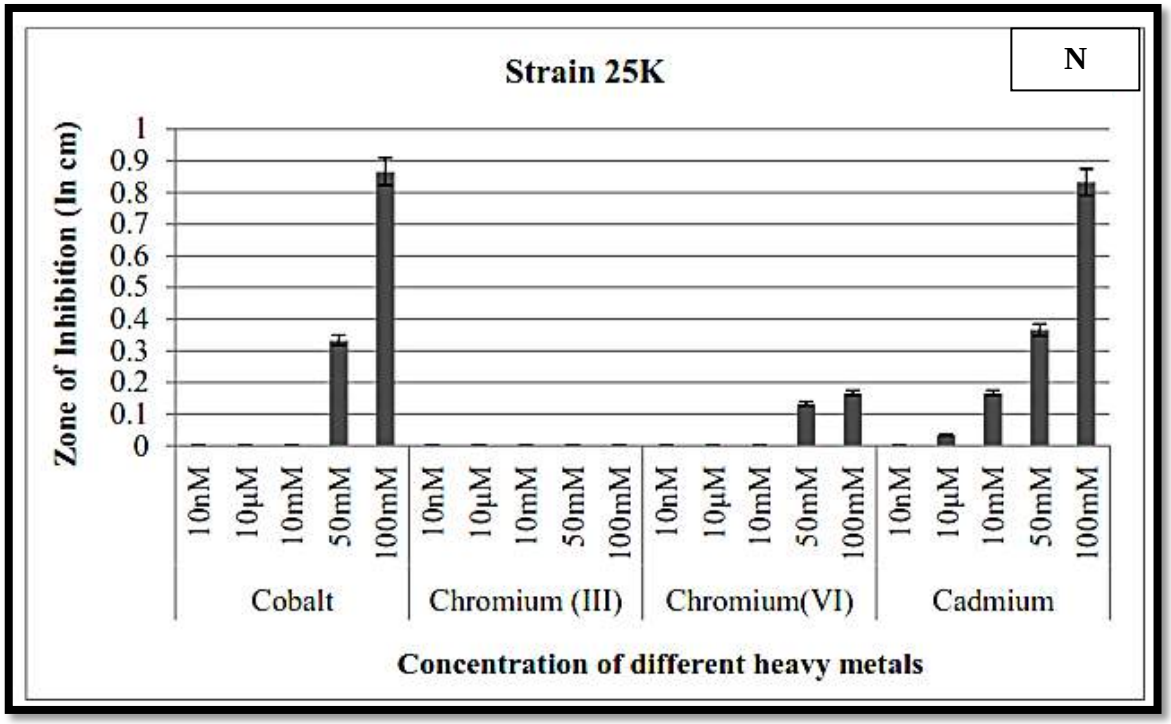
10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM
Cobalt
Chromium (III)
Chromium(VI)
Cadmium
Concentrations of different heavy metals

III. Bacteria isolated from Kestopur Khal






M
Strain 7K
Zone of Inhibition (In cm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM
Cobalt
Chromium (III)
Chromium(VI)
Cadmium
Concentrations of different heavy metals


N
Strain 25K
Zone of Inhibition (In cm)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM 10nM 10µM 10mM 50mM 100mM
Cobalt
Chromium (III)
Chromium(VI)
Cadmium
Concentration of different heavy metals

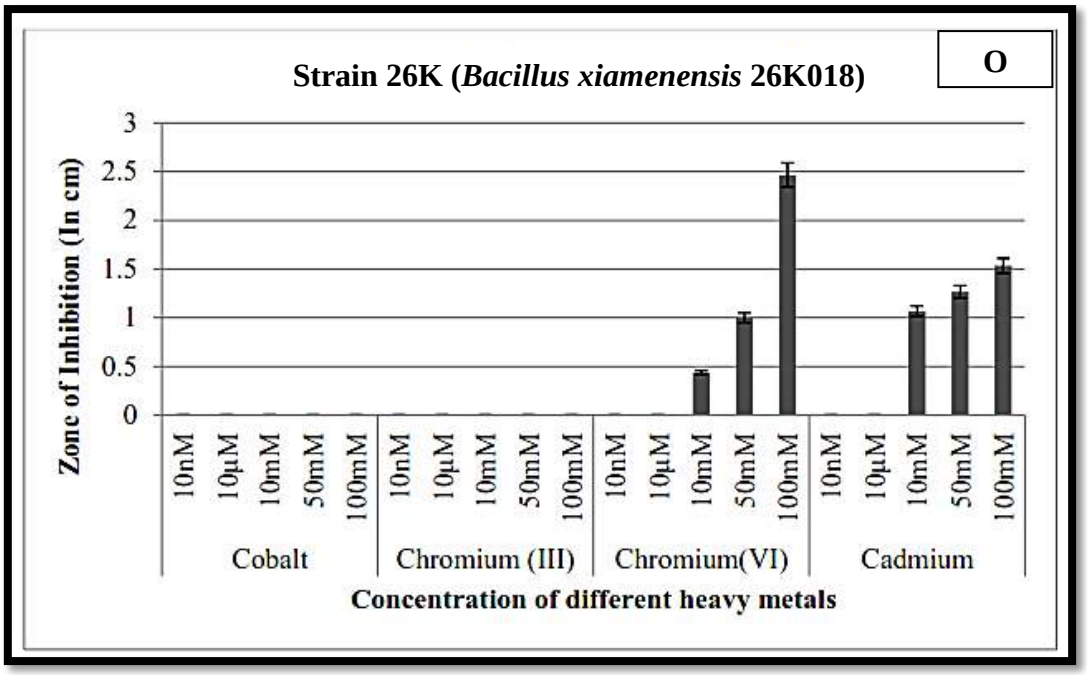


Figure 3.3.1 (A-O): Degree of heavy metal sensitivity (in terms of zone of inhibition) of soil microbes as measured by the agar cup method (all the values were obtained by calculating the bacterial zone of inhibition in triplicate)

Interestingly, isolate 12C (later identified to be as *Microbacterium radiodurans* K12016), isolated from the Circular canal, shown total resistance against trivalent chromium (Cr^{3+}) till the maximum used concentration (100 mM), both individually and in consortia with other susceptible strains (Figure 3.3.2). The Minimal Inhibitory Concentration (MIC) value for hexavalent chromium against *Microbacterium radiodurans* K12016 was 42±0.1 mM. It can therefore be inferred that the chromium resistant *Microbacterium radiodurans* K12016 can be a potent candidate for chromium bioremediation. Two microbial strains (later identified to be as *Bacillus xiamenensis* 1E0018 and *Bacillus xiamenensis* 26K018 respectively) isolated from East Kolkata Wetland and Kestopur Khal (denoted as 1E and 26K primarily) showed resistance up to 100 mM trivalent chromium. The MIC of hexavalent chromium against *Bacillus xiamenensis* 1E0018 and *Bacillus xiamenensis* 26K018 was 20±0.19 mM and 10.5±0.1 mM respectively. In spite of low MIC, *Bacillus xiamenensis* strain 1E0018 and 26K018 could resist 100 mM hexavalent chromium in consortia with other susceptible strains.

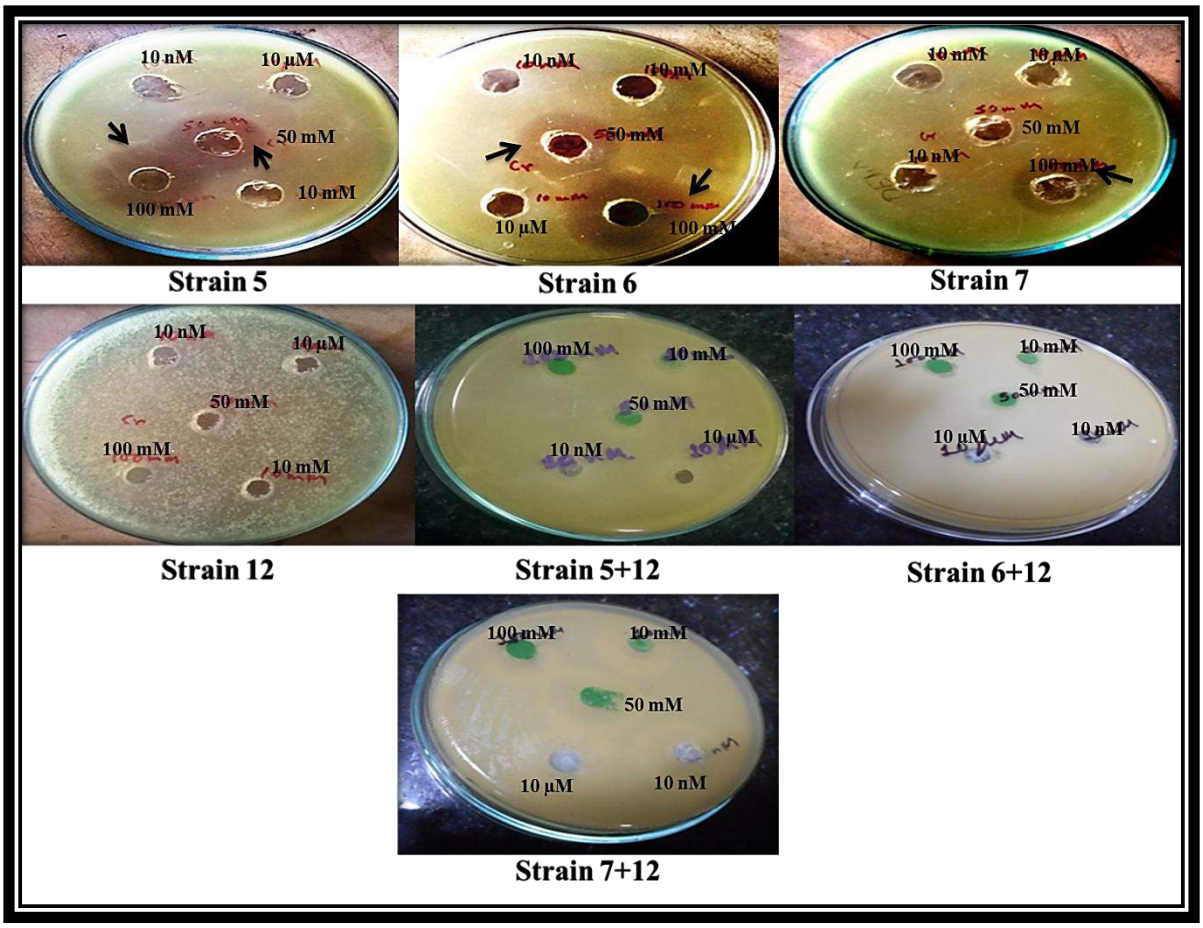
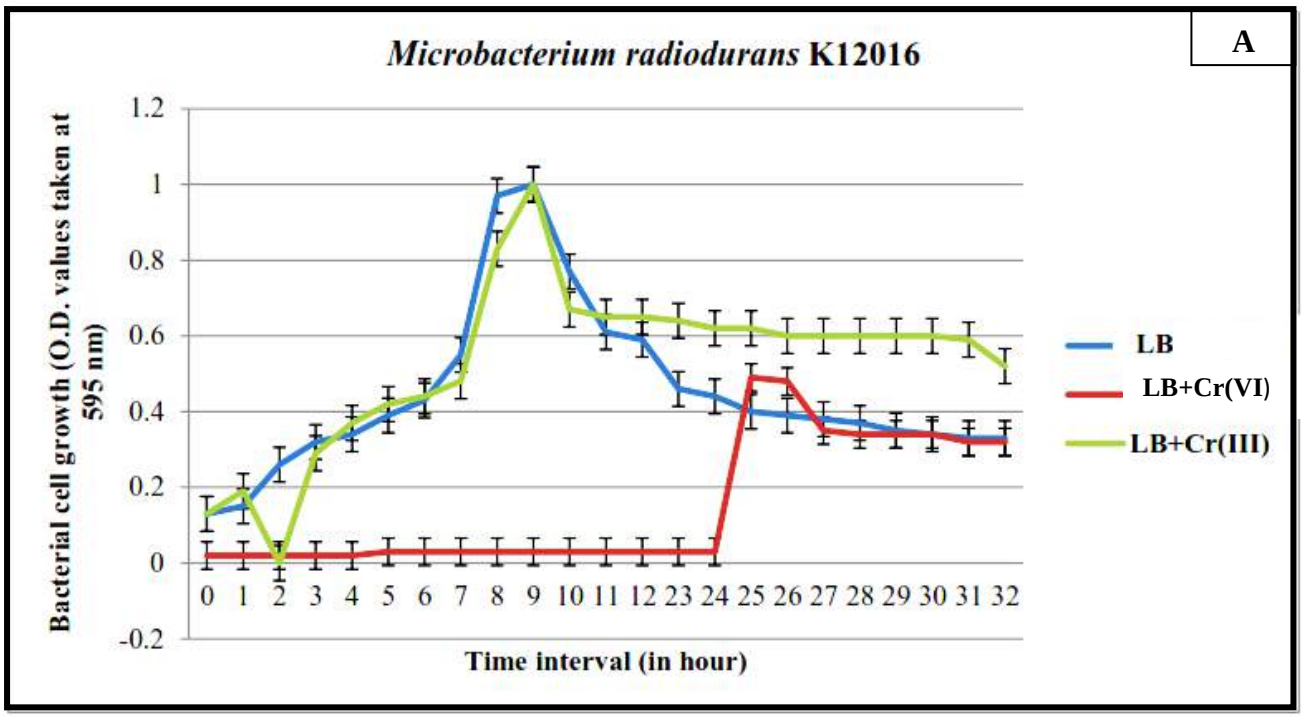
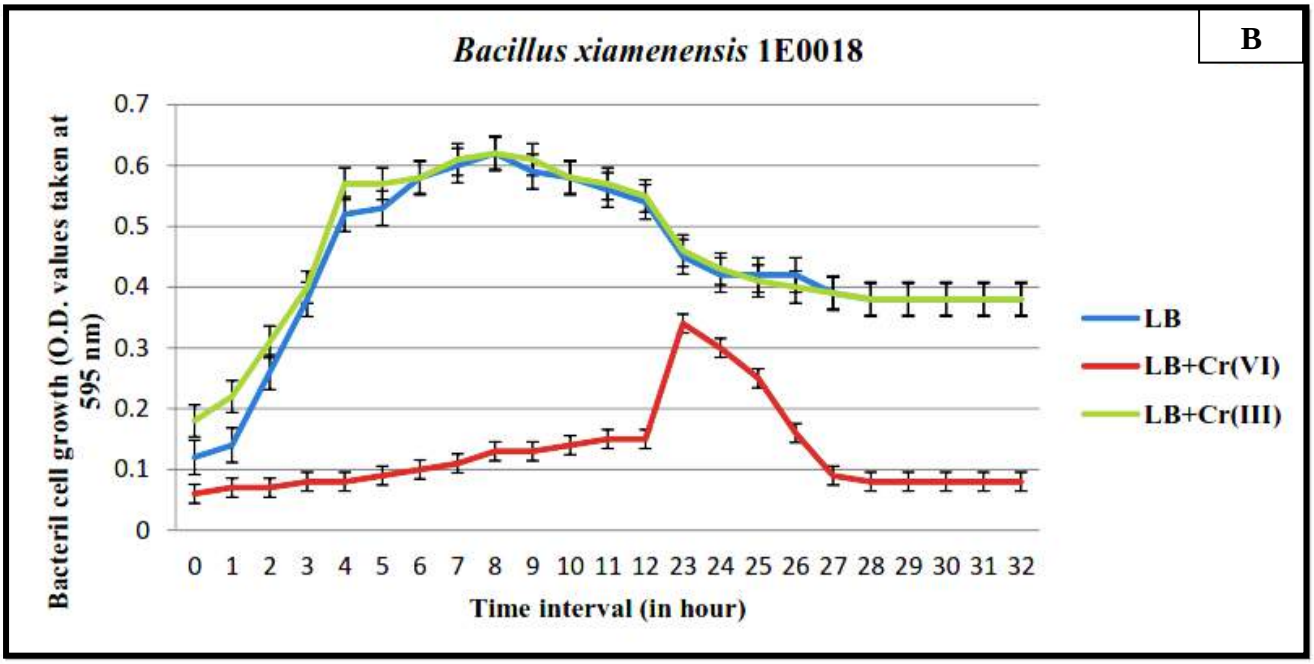


Figure 3.3.2: Chromium resistance pattern of microbial isolates 5C, 6C, 7C and 12C (*Microbacterium radiodurans* K12016) respectively, where 12C [identified as *Microbacterium radiodurans* K12016] showed total chromium resistances till the maximum used concentration (100mM). Consortia of strain 12C with susceptible strains 5C, 6C and 7C showed total resistance against 100 mM chromium (The black arrows indicate zones of inhibition)

3.3.3. Microbial growth pattern in presence and absence of chromium:

The result of growth profiling in the presence and absence of chromium showed that *Microbacterium radiodurans* K12016 exhibited an extended preparatory phase in order to possibly survive in the presence of hexavalent chromium (Figure 3.3.3 A), thus supporting the observation by Akbarpour Nesheli and coworkers in 2018, which stated that a higher concentration of hexavalent chromium affects the bacterial growth curve by lengthening the lag phase. *Bacillus xiamenensis* 1E0018 and *Bacillus Xiamenensis* 26K018 showed a reduced growth rate in the presence of hexavalent chromium (Figure 3.3.3 B and 3.3.3 C). However, the bacteria showed a normal growth pattern in the presence of trivalent chromium as observed in the metal-free environment.


A
Microbacterium radiodurans K12016
Bacterial cell growth (O.D. values taken at 595 nm)
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0 1 2 3 4 5 6 7 8 9 10 11 12 23 24 25 26 27 28 29 30 31 32
Time interval (in hour)
LB
LB+Cr(VI)
LB+Cr(III)


B
Bacillus xiamenensis 1E0018
Bacteril cell growth (O.D. values taken at 595 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6 7 8 9 10 11 12 23 24 25 26 27 28 29 30 31 32
Time interval (in hour)
LB
LB+Cr(VI)
LB+Cr(III)

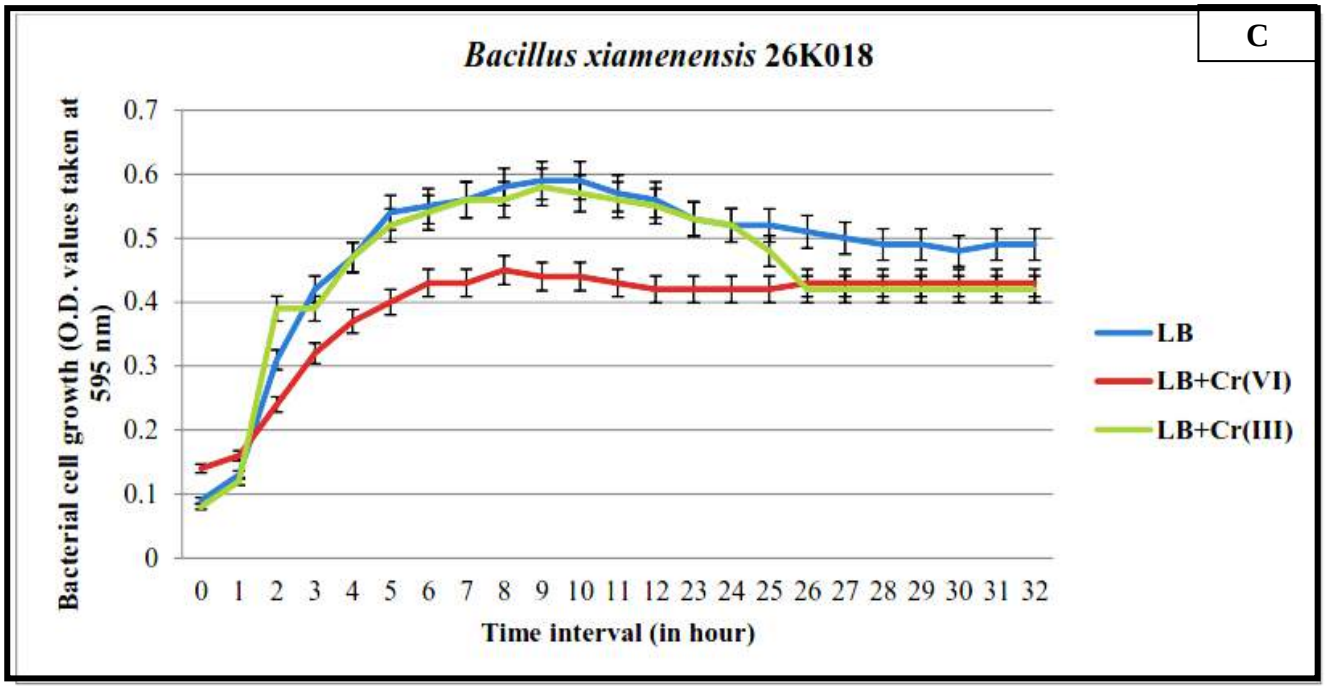
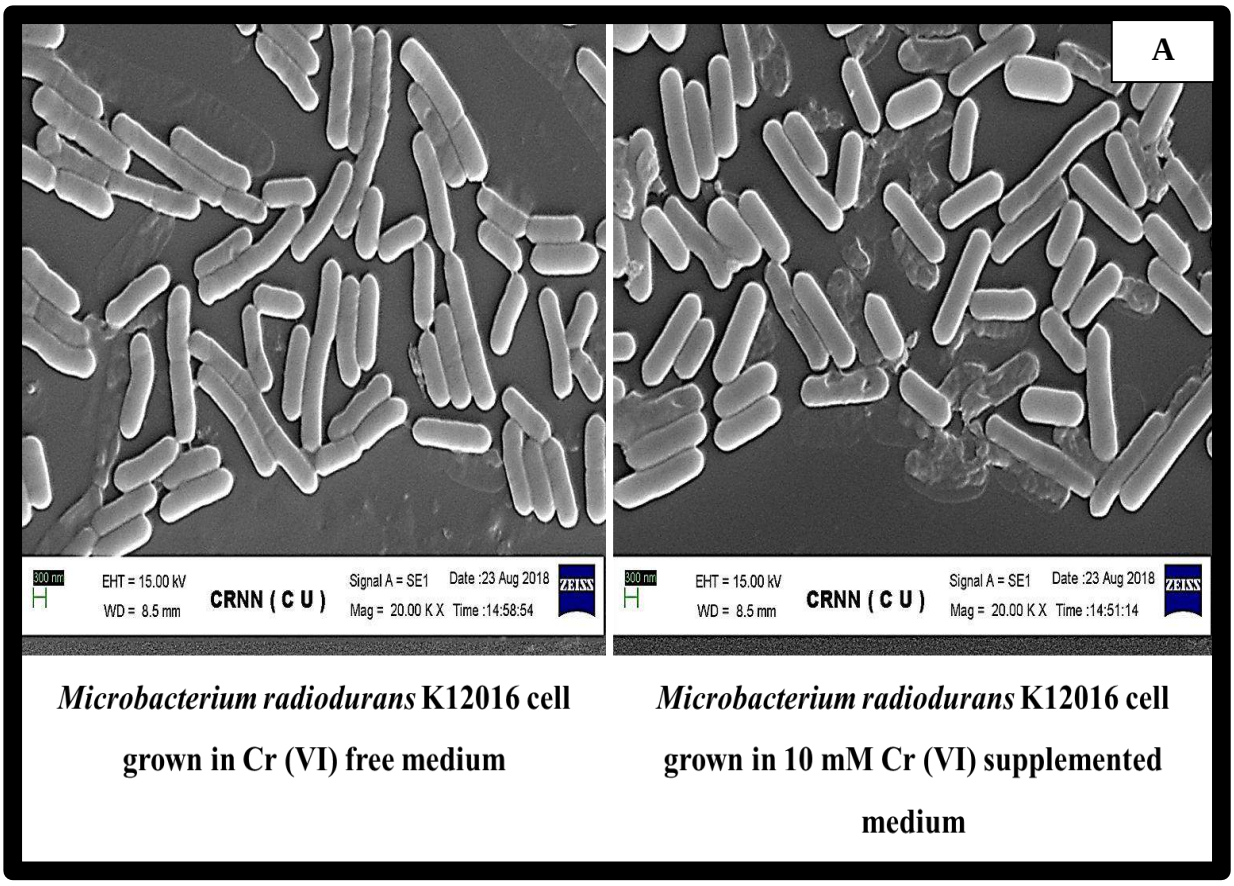


Figure 3.3.3 (A-C): The growth curve of [A] *Microbacterium radiodurans* K12016; [B] *Bacillus xiamenensis* 1E0018; [C] *Bacillus xiamenensis* 26K018 in presence of hexavalent and trivalent chromium and without chromium

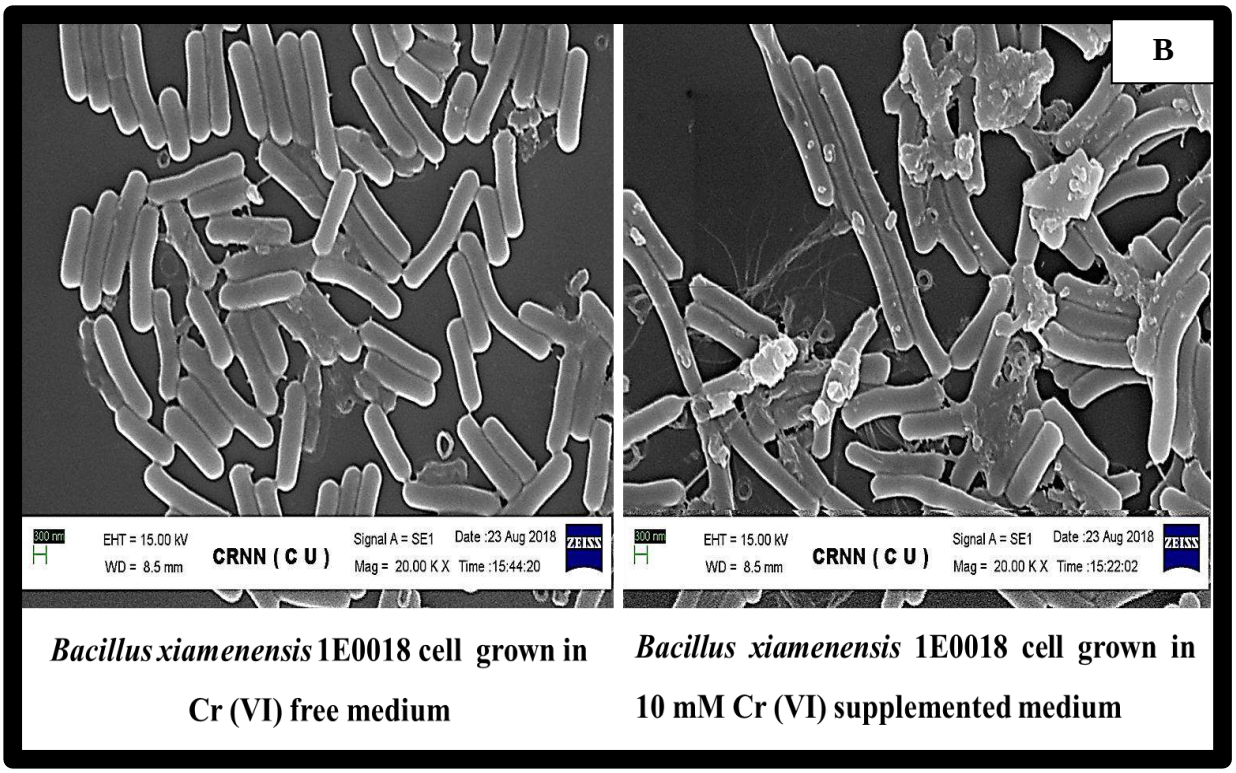
3.3.4. Bacterial viability check through SEM micrograph:

The SEM micrographs showed that 10mM hexavalent chromium-treated cells remained healthy and viable (as the cell surface was found to be unaffected; Figure 3.3.4 A, 3.3.4 B, 3.3.4 C) and became elongated in the presence of chromium to combat chromium-induced stress in comparison to that of the cells grown in chromium-free medium (Figure 3.3.4 A, 3.3.4 B and 3.3.4 C; Table 3.3.3). But the 40 mM hexavelent chromium treated cells could not survive because of chromium stress (Figure 3.3.4 D). The results of MIC value measurement also supported the above-mentioned findings which revealed that 50 mM hexavalent chromium is toxic enough for most of the chromium tolerant microbial isolates. The cells of *Microbacterium radiodurans* K12016 didn't change much morphologically in presence of 10 mM Cr^{6+}. But the cells grown in 40 mM Cr^{6+} supplemented media which was close to their MIC value (42±0.1 mM), showed distorted cell walls due to cell damage.



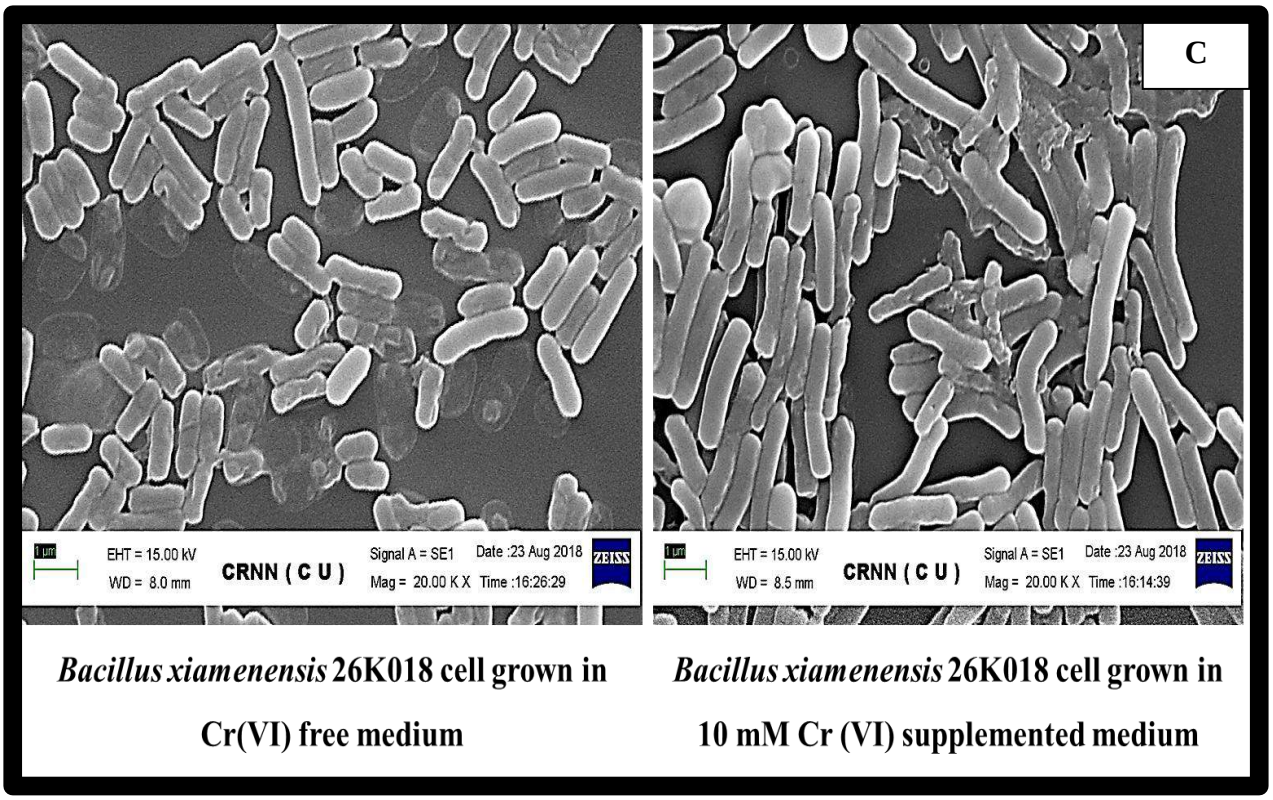
Microbacterium radiodurans **K12016 cell grown in Cr (VI) free medium**

Microbacterium radiodurans **K12016 cell grown in 10 mM Cr (VI) supplemented medium**



Bacillus xiamenensis **1E0018 cell grown in Cr (VI) free medium**

Bacillus xiamenensis **1E0018 cell grown in 10 mM Cr (VI) supplemented medium**



Bacillus xiamenensis 26K018 cell grown in Cr(VI) free medium

Bacillus xiamenensis 26K018 cell grown in 10 mM Cr (VI) supplemented medium

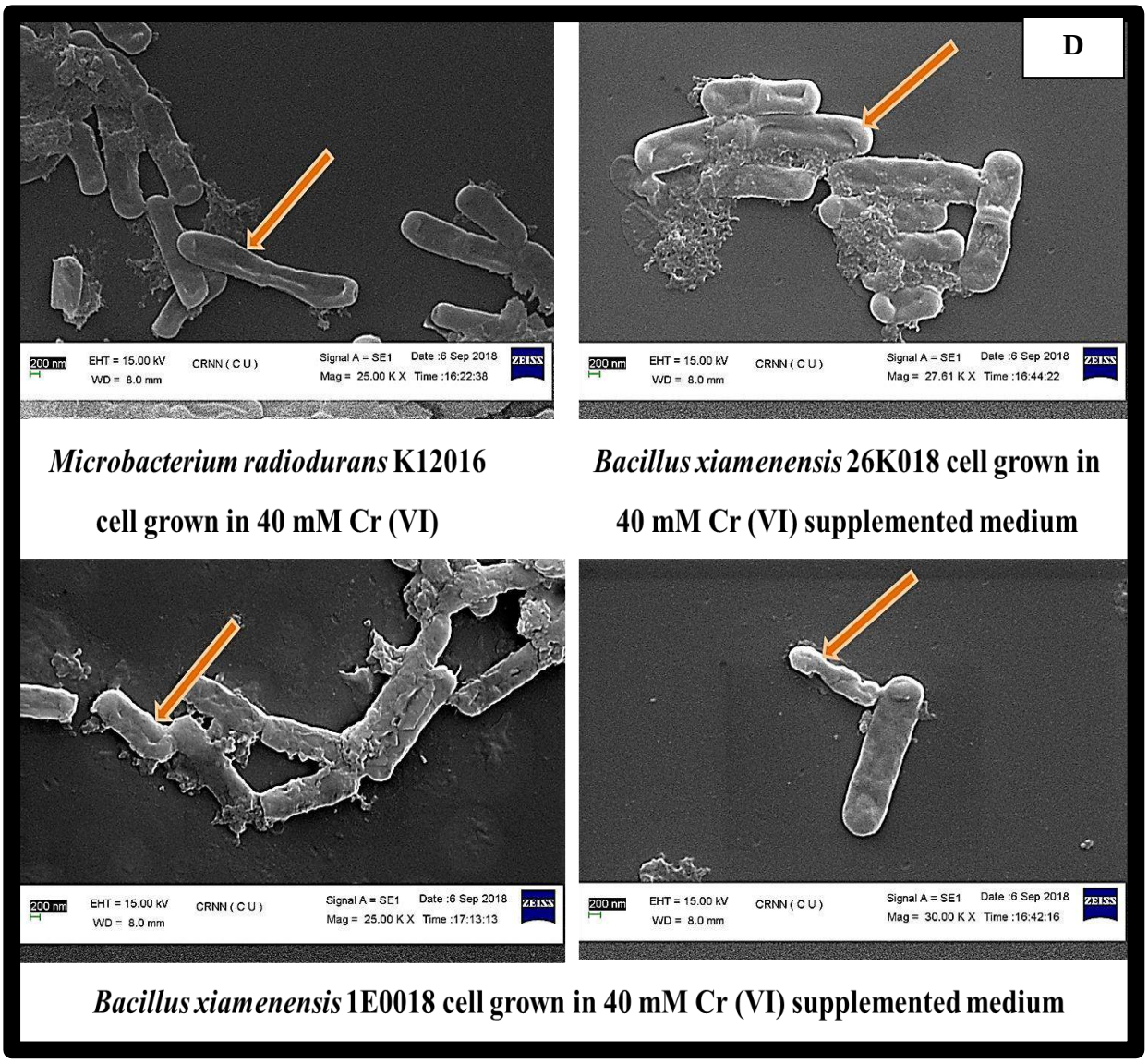


Figure 3.3.4 (A-D): The SEM micrographs (10,000X magnified); Both untreated and 10 mM Cr^{6+} treated cell of [A] *Microbacterium radiodurans* K12016, [B] *Bacillus xiamenensis* 1E0018, [C] *Bacillus xiamenensis* 26K018 remained unaffected and viable; [D] The 40 mM Cr^{6+} treated bacterial cells became distorted and the arrows point out the cell surface distortion due to chromium stress

Table 3.3.3: Comparison of length of 10 mM Cr^{6+} and untreated bacterial cell observed under SEM (measured using ImageJ):

Microbial strain	Length of cell grown without metal (nm)	Length of cell treated with 10 mM Cr^{6+} (nm)	P value (calculated by two tailed t-test)	Number of cells studied
Microbacterium radiodurans K12016	910±0.5	1500±0.05	0.0071	10
Bacillus xiamenensis 1E0018	1342±0.6	3180±1.1	0.0006	10
Bacillus xiamenensis 26K018	1270±0.7	2950±2.9	0.0002	10

3.3.5. Chromium removal strategies implemented by isolated soil microbes:

The chromium removal efficiencies of *Microbacterium radiodurans* K12016, *Bacillus xiamenensis* 1E0018 and *Bacillus xiamenensis* 26K018 were investigated against carcinogenic hexavalent chromium.

Microbacterium radiodurans K12016 cultured in 51.9 ppm (10 mM) and 259.5 ppm (50 mM) hexavalent chromium supplemented medium, absorbed 35.73 ± 0.21ppm and 86.7±0.6 ppm metal, respectively, after 24 hours of incubation. The 1E0018 and 26K018 strains of *Bacillus xiamenensis,* absorbed 40.61±0.86 ppm and 41.53±1.27 ppm chromium from the 51.9 ppm (10 mM) metal supplemented media after 24 hours of incubation. All the data were calculated through four subsequent batch cultures (24 hours each). (Table 3.3.4).

The percentage of removal and amount of metal absorption per unit of bio absorbent was calculated using formulae:

a) Percentage of removal = (Initial concentration - Final concentration/Initial concentration) × 100

b) Metal absorption per unit of bio absorbent = (Initial concentration - Final concentration/ Mass of bio absorbent) × Concentration of metal solution

Table 3.3.4: Bacterial Cr(VI) absorbance and absorbed metal removal:

Microbial isolates	Concentration of Cr in growth media before culture (ppm)	Cr available in growth media (ppm) after 24 hours of microbial growth (ppm)	Cr absorbed by microbe from growth media (ppm)	Cr released back by microbe to metal free media (ppm)
***Microbacterium radiodurans* K12016**	51.9	15.73 ± 0.19	35.73 ± 0.21	0.000129±0.20
***Microbacterium radiodurans* K12016**	259.5	173.7±0.53	86.7±0.6	0.000325 ± 0.39
***Bacillus xiamenensis* 1E0018**	51.9	11.28±0.7	40.61±0.86	<0.00002 (negligible)
***Bacillus xiamenensis* 26K018**	51.9	9.96±1.9	41.53±1.27	0.00048 ± 0.25

The results from Table 3.3.5 stated that a considerable amount of chromium from the surrounding (culture media) had accumulated in the bacteria and released back a negligible amount of accumulated chromium in the metal-free media (Table 3.3.6). These findings indicate that the isolated microbes can be used as well as bioremediating agents in future because of their chromium absorbance and nominal release of the absorbed chromium from bacteria.

Table 3.3.5: Chromium absorption (i.e., removal from surrounding media) from per mg of bacterial biomass:

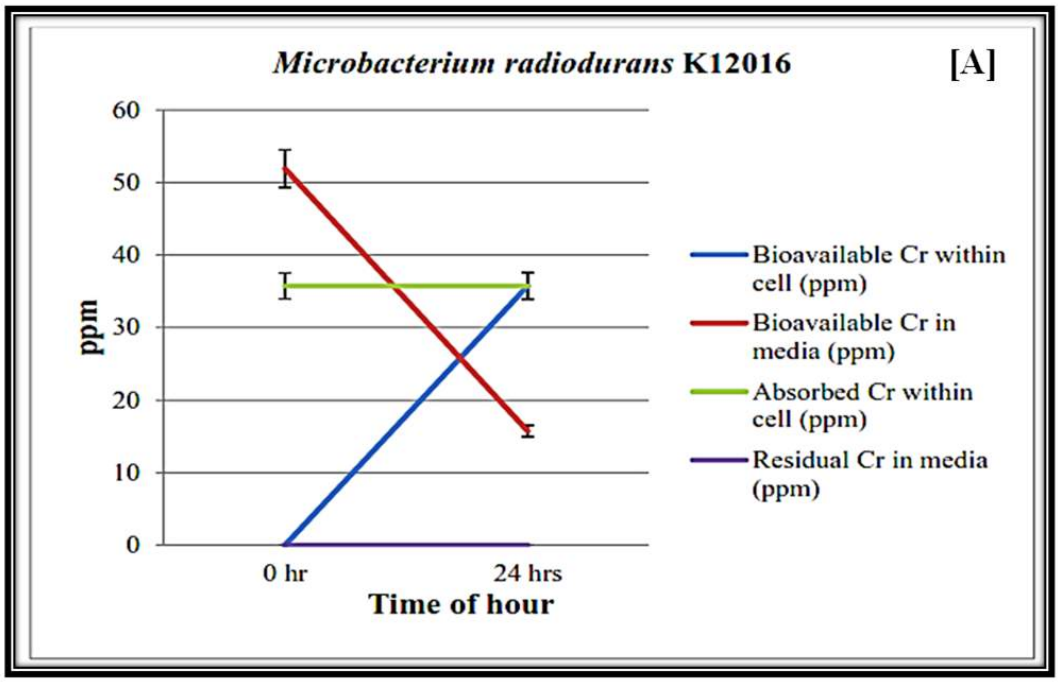
***Microbacterium radiodurans* K12016 (ppm/mg/hr)**		***Bacillus xiamenensis* 1E0018 (ppm/mg/hr)**	***Bacillus xiamenensis* 26K018 (ppm/mg/hr)**
Grown in 10 mM Cr supplemented media	***Grown in 40 mM Cr supplemented media***	***Grown in 10 mM Cr supplemented media***	***Grown in 10 mM Cr supplemented media***
1.37±0.78	**4.59±0.97**	**12.9±0.92**	**1.86±0.09**

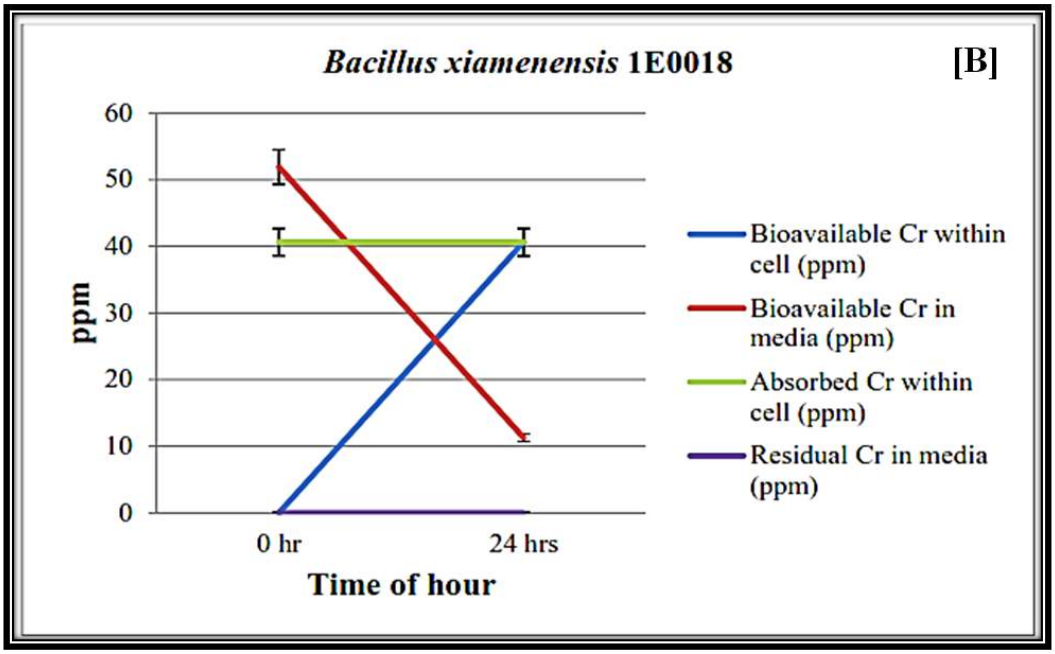
Table 3.3.6: Percentage of chromium released in metal free media from chromium accumulated bacterial biomass (in 24 hours):

***Microbacterium radiodurans* K12016 (Percent)**		***Bacillus xiamenensis* 1E0018 (Percent)**	***Bacillus xiamenensis* 26K018 (Percent)**
Grown in 10 mM Cr supplemented media	***Grown in 40 mM Cr supplemented media***	***Grown in 10 mM Cr supplemented media***	***Grown in 10 mM Cr supplemented media***
0.45±0.1	**0.72±0.002**	**Negligible**	**0.053±0.05**

The bacterial cell released back only a negligible amount of the absorbed heavy metal to the metal-free media after three serial batch cultures. *Microbacterium radiodurans* K12016, *Bacillus xiamenensis* 1E0018 and *Bacillus xiamenensis* 26K018 cell absorbed the metal from hexavalent chromium supplemented media, released only 0.000325 ± 0.39 mg/l, <0.00002 mg/ml (negligible) and 0.00048 ± 0.25 mg/l of chromium to the metal-free media respectively (Table 3.3.4).

Bioavailable chromium present in the media was decreased after 24 hours for *Microbacterium radiodurans* K12016, *Bacillus xiamenensis* 1E0018 and *Bacillus xiamenensis* 26K018 bacterial cells whereas the bioavailable chromium contents within these bacterial cells were increased after 24 hours of incubation. The bacterial accumulated chromium was retained within the cell and no considerable amount of chromium was released out in the metal free-media (Figure 3.3.5).


Microbacterium radiodurans K12016
[A]
60
50
40
30
20
10
0
ppm
0 hr
24 hrs
Time of hour
Bioavailable Cr within cell (ppm)
Bioavailable Cr in media (ppm)
Absorbed Cr within cell (ppm)
Residual Cr in media (ppm)


Bacillus xiamenensis 1E0018
[B]
60
50
40
30
20
10
0
ppm
0 hr
24 hrs
Time of hour
Bioavailable Cr within cell (ppm)
Bioavailable Cr in media (ppm)
Absorbed Cr within cell (ppm)
Residual Cr in media (ppm)

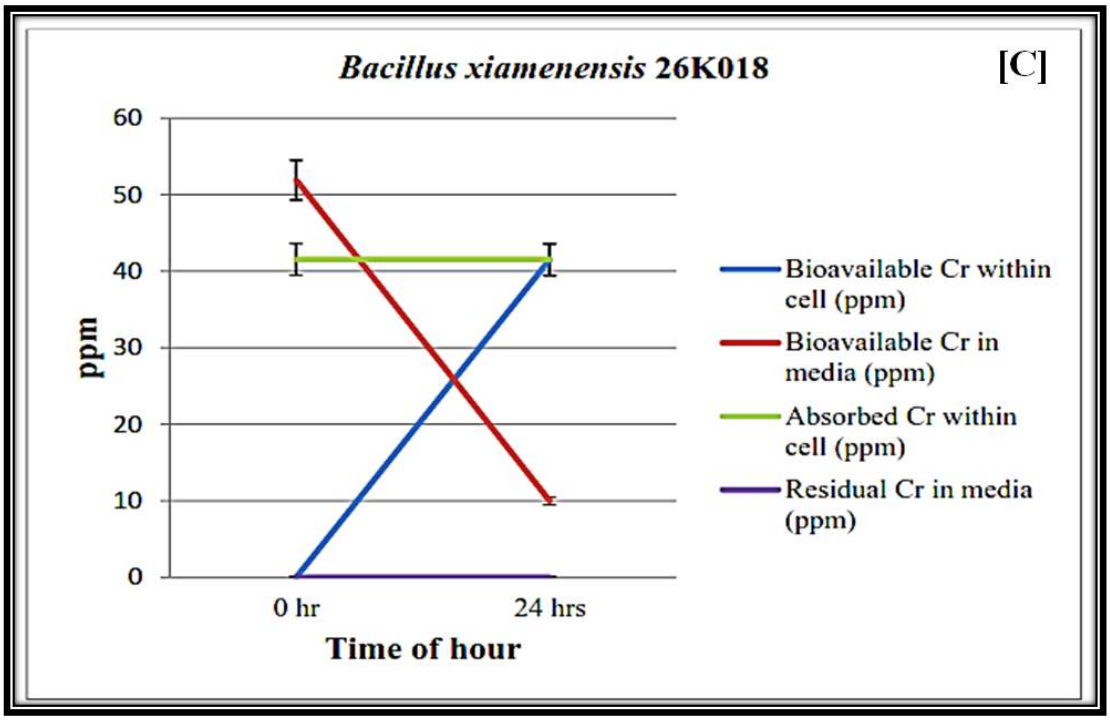
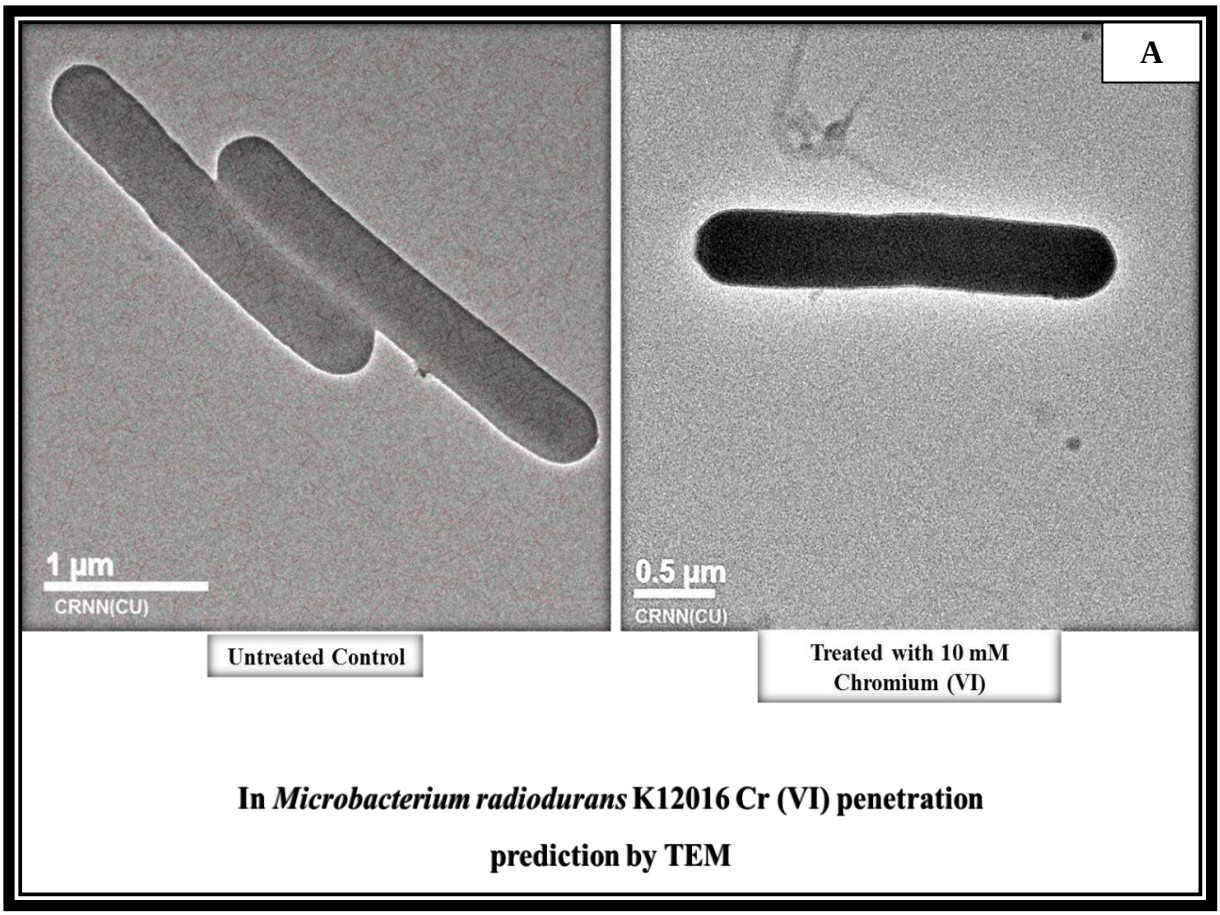


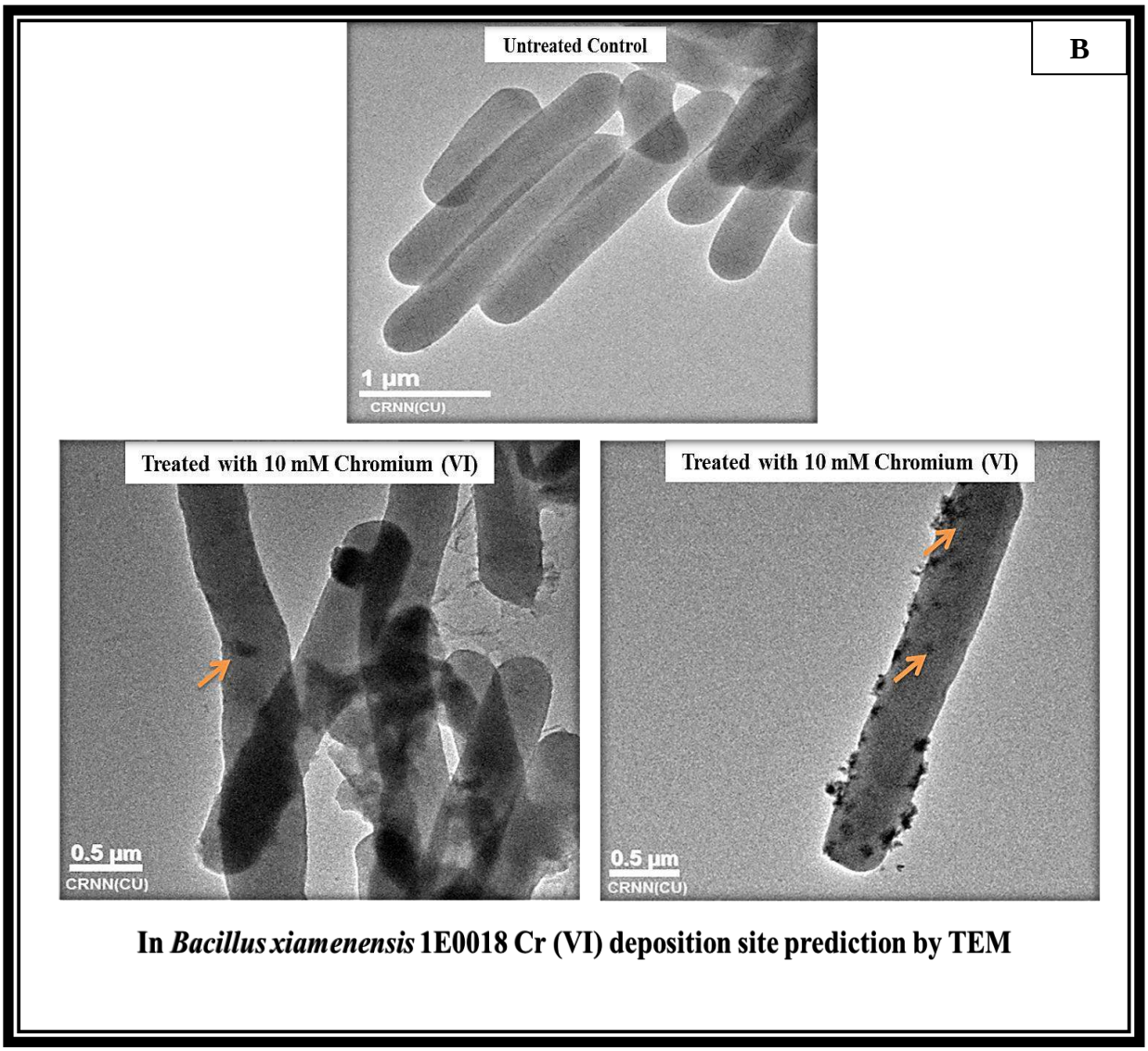
Figure 3.3.5 (A-C): Comparison between the chromium absorption and removal efficiency of selected microbial isolates after 24-hour incubation

3.3.6. Chromium deposition site prediction through TEM micrograph:

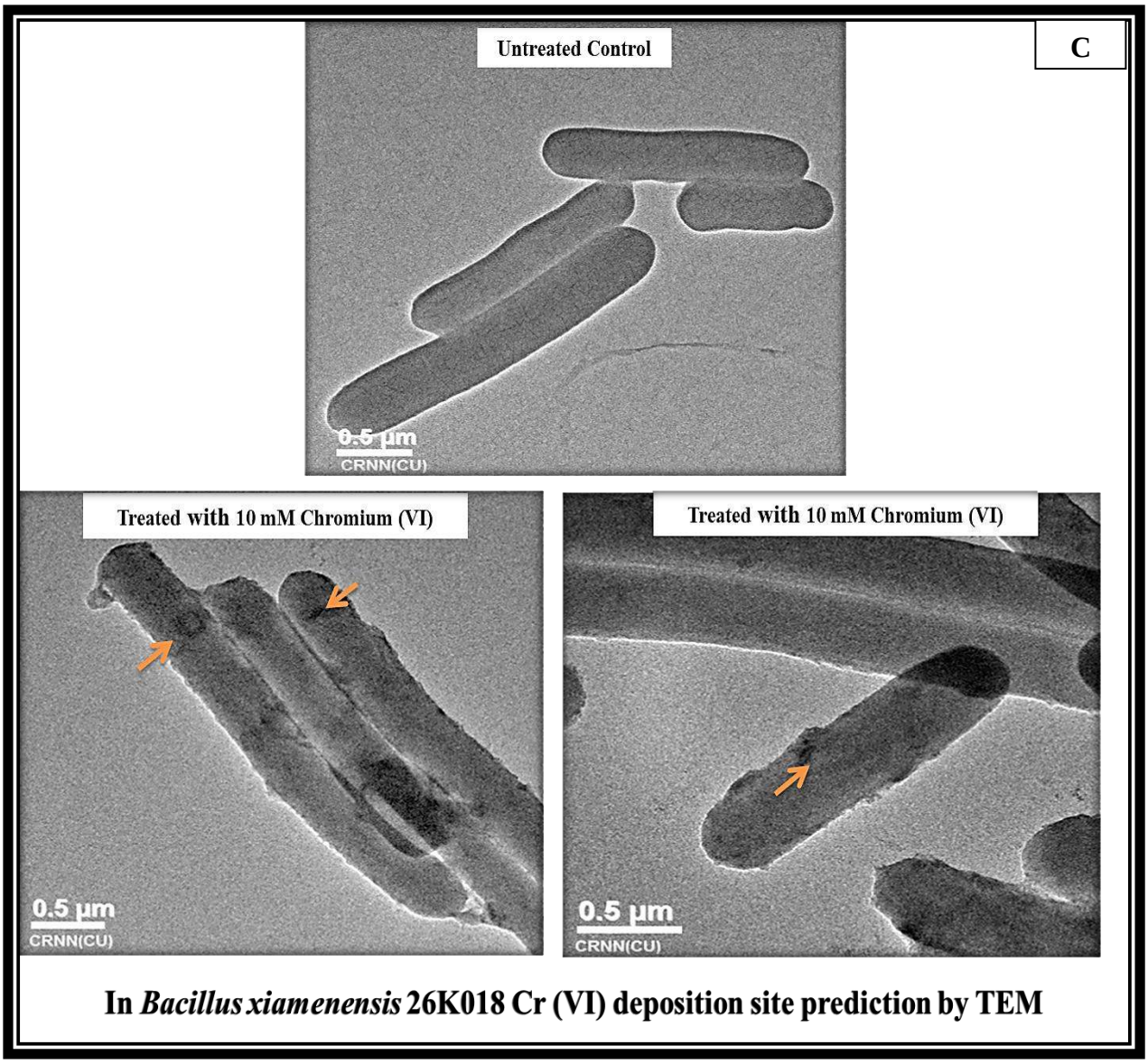
Chromium penetration and deposition within the chromium treated microbial cell was predicted by observing the TEM micrographs (T.M. Roane, 1999). The TEM micrographs showed dark patches only in the presence of heavy metals. The UranyLess stained control cells which remained in chromium free environment, didn't show any dark patches, but the cell treated with chromium, showed dark patches. An extensive chromium penetration was observed within the cells of *Microbacterium radiodurans* K12016 which may be formed due to positive staining by chromium (Figure3.3.6 A). Dark patches were observed inside the chromium treated cell of *Bacillus xiamenensis* 1E0018 (Figure 3.3.6 B) and *Bacillus xiamenensis* 26K018 [Figure 3.3.6 C] but no patches were found in UranyLess stained control cells (grown in chromium free media) which may be the indication of chromium deposition inside cell. Dark patches were also observed at the cell surface of *Bacillus xiamenensis* due to the presence of chromium.



In *Microbacterium radiodurans* K12016 Cr (VI) penetration prediction by TEM



In *Bacillus xiamenensis* 1E0018 Cr (VI) deposition site prediction by TEM



In *Bacillus xiamenensis* 26K018 Cr (VI) deposition site prediction by TEM

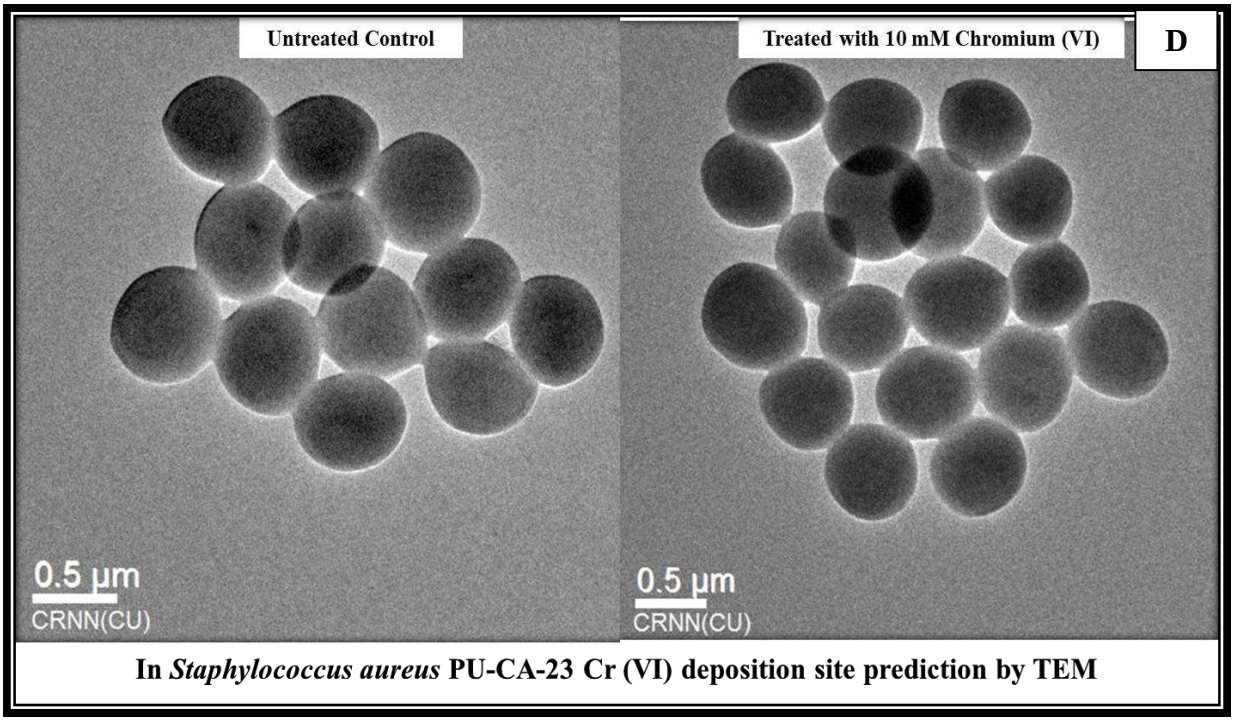


Figure 3.3.6 (A-C): Prediction of chromium penetration and deposition within 24 hours treated bacterial cells by TEM micrographs. The arrows indicate chromium deposition sites. Figure 3.3.6 D: The Cr(VI) treated cells of *Staphylococcus aureus* PU-CA-23 used as negative control where the bacteria were not influenced by hexavalent chromium and no metal deposition was found within cells

3.3.7. Study chromium reduction within microbial cell:

NaOH and NH_4OH were applied to the lysed bacterial cell to confirm the presence of trivalent chromium (Figure 3.3.7). Gray-green precipitate was formed after application of NaOH to the lysed cell, was readily soluble in excess alkali. But the precipitate formed after applying NH_4OH, didn't dissolve by adding excess ammonia. Residual hexavalent chromium content was also measured by the diphenyl carbazole method.

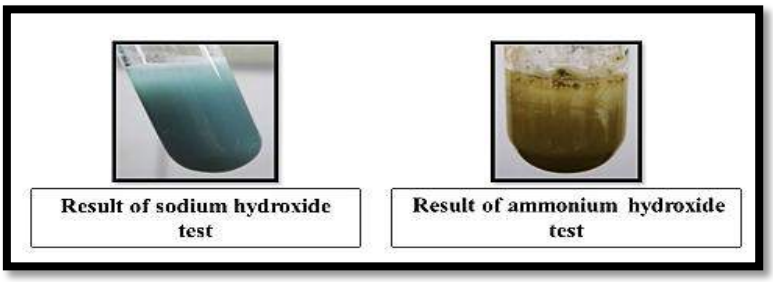


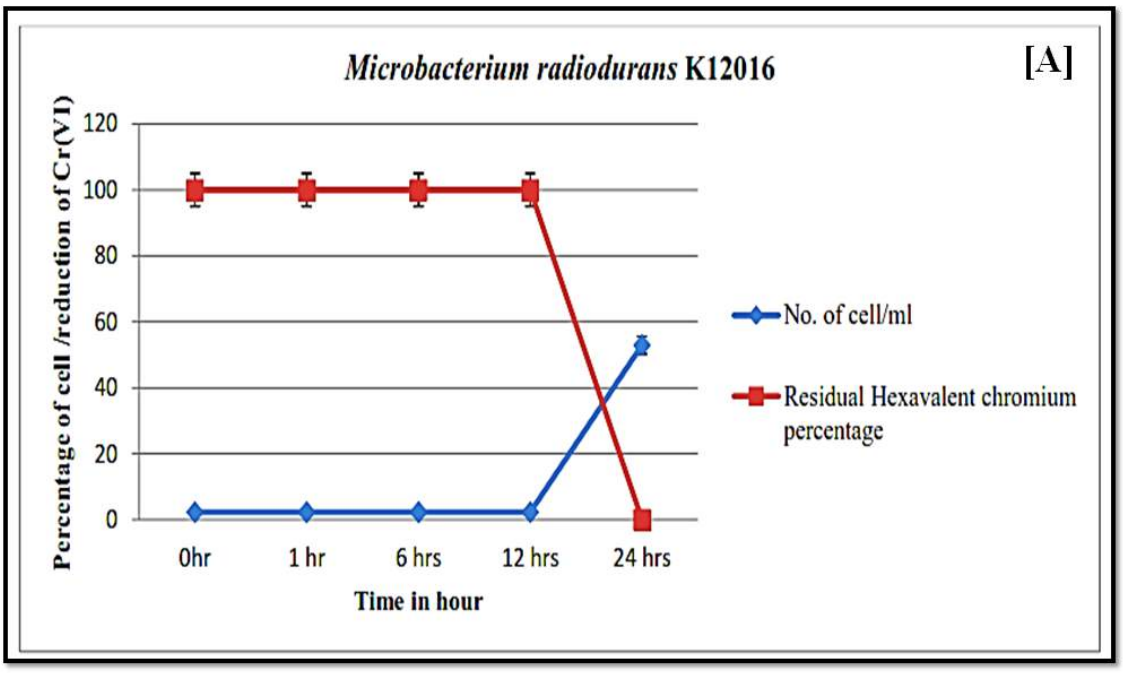
Figure 3.3.7: The results of sodium hydroxide test and ammonia hydroxide test confirmed the presence of trivalent chromium (Cr^{3+})

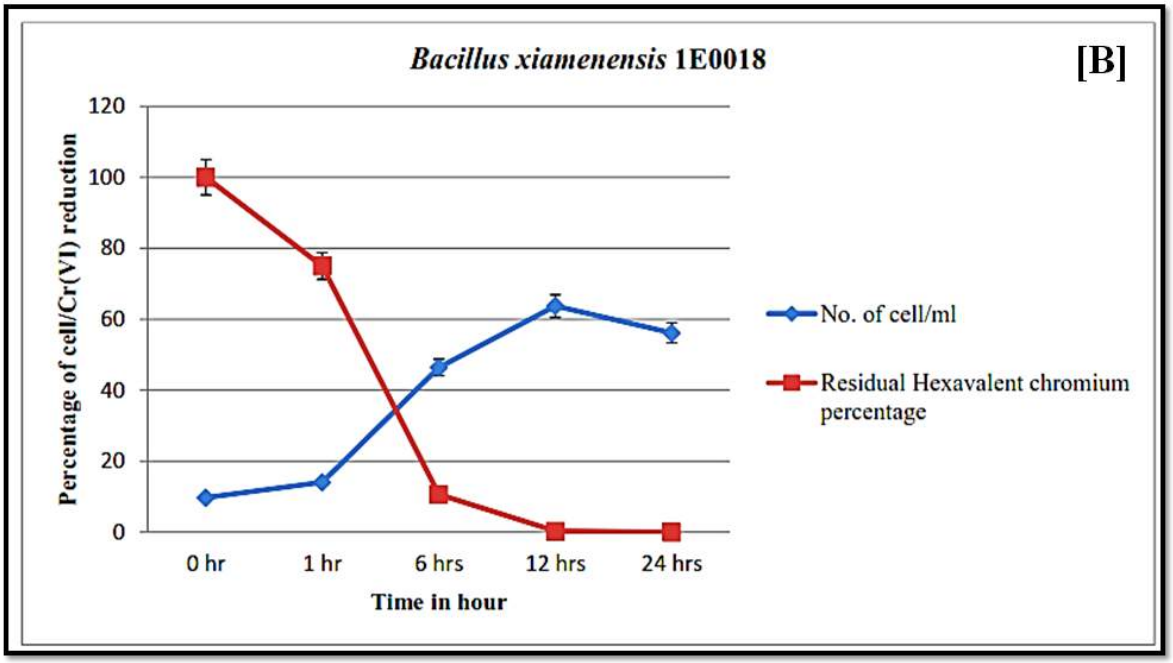
In hexavalent chromium supplemented media, Cr(VI) was reduced gradually during bacterial growth (Figure 3.3.8, Figure 3.3.9). The microbes of our interest completely reduced 10mM Cr(VI) within 24 hours. *Microbacterium radiodurans* K12016 reduced 65±0.01% of 40 mM Cr (VI) in 24 hours and 100% Cr (VI) in 48 hours respectively.

Percentage of hexavalent chromium reduction was calculated using the formula:

Percentage of hexavalent chromium reduction = [(Absorbance of control - absorbance of chromium treated cell sample)/absorbance of chromium treated cell sample] ×100.

In this experiment, cell-free hexavalent chromium supplemented media was considered as the control.





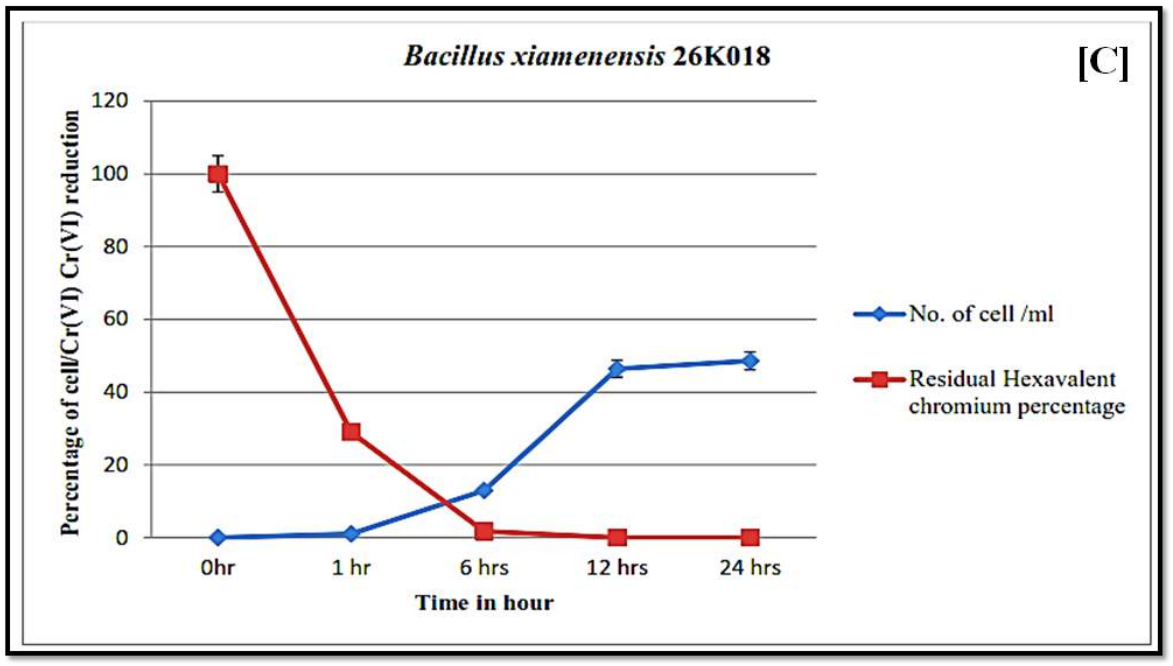


Figure 3.3.8 (A-C): Hexavalent chromium reduction in viable bacterial cell and the simultaneous growth of bacteria cultured at 10 mM chromium supplemented media at the specific time course

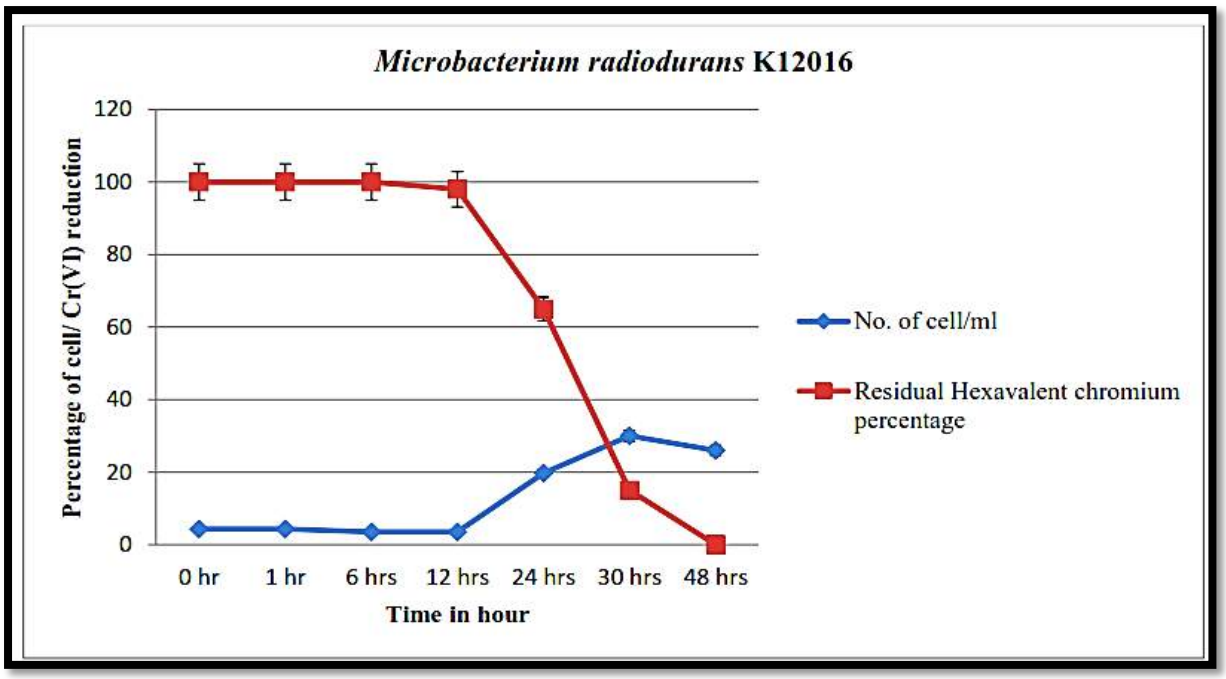


Figure 3.3.9: Hexavalent chromium reduction in viable bacterial cell and the simultaneous growth of bacteria cultured at 40 mM chromium supplemented media at the specific time course

3.3.8. Study chromium reduction in presence of different microbial growth stimulators:

When we observed the rate of reduction of hexavalent chromium in presence of microbial growth inhibitor sodium azide and growth stimulator DNP, we find that DNP initially accelerated the rate of chromium reduction in *Microbacterium radiodurans* K12016 in comparison to control and sodium azide decelerated it. In the case of the *Bacillus xiamenensis* strains (1E0018 and 26K018), DNP didn't show any considerable change in the rate of chromium reduction (Figure 3.3.10).

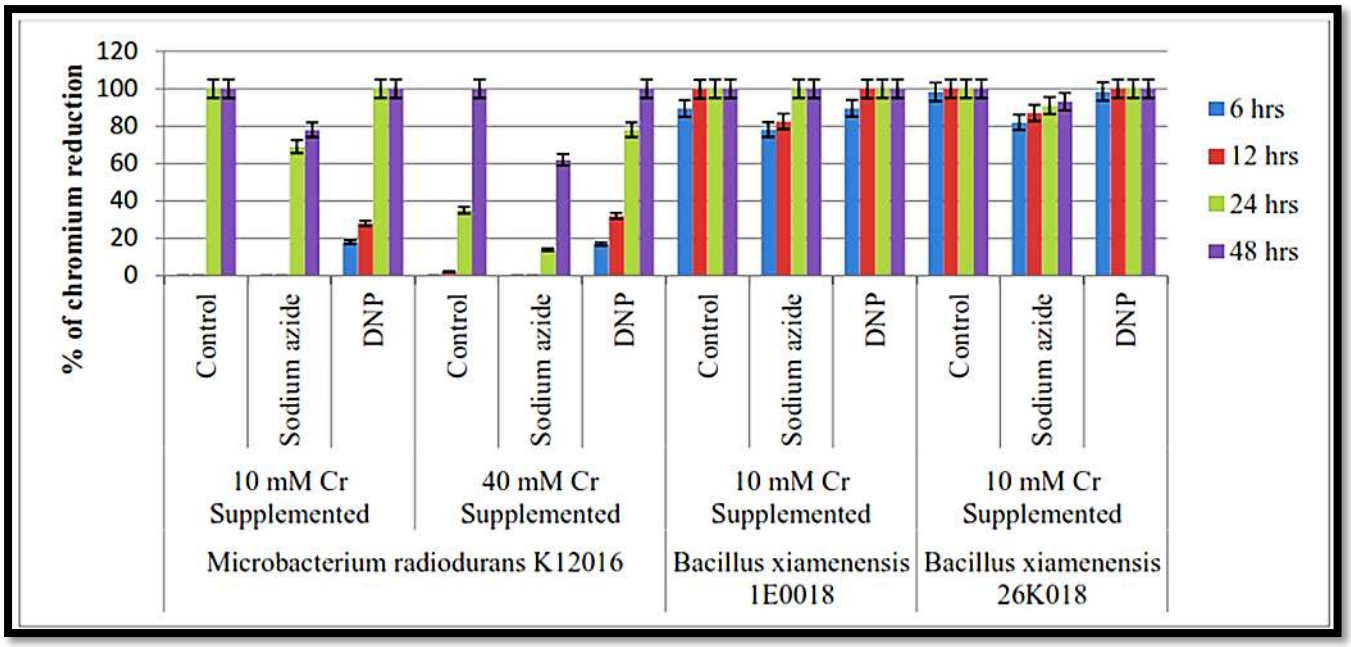


Figure 3.3.10: Influence of microbial stimulator DNP and inhibitor sodium azide on chromate reduction in Cr^{6+} supplemented media

3.4 Discussion:

The acquired inhibition zone of agar cup technique, obtained MIC values and SEM micrographs stated that majority of the microbes isolated from sewage sites of Kolkata, are total resistant up to 100mM trivalent (Cr^{3+}) and 10mM-50mM hexavalent chromium (Cr^{6+}). Because of this reason, we had chosen chromium for further experiments. Another reason behind choosing chromium over other metals was its huge concentration variability among East Kolkata Wetland and other two sewage regions [20864 ± 0.28 ppm at East Kolkata Wetland, whereas at Circular Canal and Kestopur Khal 187.1 ± 0.41 ppm and 187.1 ± 0.15 ppm respectively]. We wanted to investigate if any considerable change had taken place in bacterial nature due to huge chromium concentration variability. The environmental heavy metal removal efficiency of the isolated microbial strains and the strategies they implied for metal removal were also investigated. Three strains [12C (*Microbacterium radiodurans* K12016), 1E (*Bacillus xiamenensis* 1E0018) and 26K (*Bacillus Xiamenensis* 26K018)] amongst the fifteen selected chromium resistant microbial isolates showed substantial tolerance against both hexavalent (Cr^{6+}) and trivalent (Cr^{3+}) chromium (Table 3.3.2; Figure 3.3.1). These three strains were selected to observe their probable mechanism of action against hexavalent chromium (Cr^{6+}) by implementing many add-on investigation techniques.

Gram-negative cell walls containing bacteria possessed an overall negative charge due to the presence of phospholipids, lipopolysaccharides and peptidoglycan which enhance their binding efficiency with positively charged hexavalent chromium [Sherbet, 1978]. In the present investigation, the SEM micrographs showed the presence of chromium patches at the cellular surface. The bacterial chromium absorption and removal capability and TEM micrographs inferred that the bacteria cultured in hexavalent chromium-supplemented media intake hexavalent chromium and retained it within their cell. However, the diphenyl carbazole spectrophotometry [APHA 23rd Ed., 2017] experiment confirmed that there was no trace of hexavalent chromium within the hexavalent chromium treated bacterial cell pellet. This finding predicted that the toxic hexavalent chromium might convert to less hazardous trivalent chromium by the isolated soil bacteria. The sodium chloride and ammonium hydroxide test confirmed the presence of trivalent chromium [Cr(III)] within bacterial cells cultured in hexavalent chromium [Cr(VI)] supplemented media. It was also observed that the hexavalent chromium reduced along with simultaneous bacterial growth. So, in this stage, our concern was to investigate chromium reduction linked microbial metabolic pathway and study the significance of chromium reduction in bacterial growth and survival.

We observed the rate of chromium reduction in presence of microbial growth stimulator 2,4 dinitrophenol (DNP) and microbial growth inhibitor sodium azide. Previous studies stated that respiratory and microbial growth inhibitor sodium azide inhibits reduction by nullifying the activity of enzyme enolase, cytochrome oxidase, ATPase [Dey et al. 2014; Shen and Wang, 1993] and uncoupler 2,4 dinitrophenol (DNP) possesses a potent role in the acceleration of respiratory chain-linked electron transport by increasing membrane proton permeability of mitochondrial inner membrane or microbial cellular membrane and stimulates aerobic respiration and chromium reduction simultaneously [Shen and Wang, 1993; Montague et al. 2001; Dey et al. 2014; Geisler, 2019]. Our investigation also showed similar trends. The present study affirms that in DNP treated cells of *Microbacterium radiodurans* K12016, the rate of chromium reduction got accelerated in comparison to control (Figure 3.3.10) whereas, sodium azide decelerated the chromium reduction rate. This finding revealed the promoting effect of DNP in chromium reduction probably comes from its acceleratory role in respiratory chain-linked electron transport which increased membrane proton permeability and stimulates respiration rate through metal (here chromium) reduction.