Bacterial Driven Strategies to Decontaminate Toxic Heavy Metals from Mithi River Using Bioremediation Process

Sanction No. MMR-ENV/SOC/1-2015/118/2015

Project Report

Submitted to

Mumbai Metropolitan Region – Environment Improvement Society (MMR-EIS)

By

Dr. BHUPENDRA PUSHKAR

Department of biotechnology

University of Mumbai

Content

Sr. No.	Content	Page No.
1	Objective of the project	12
2	Target of the project	12
3	Appointment of the research staff (JRF)	13
4	Literature survey on the project topic	13
5	Procurement of the requirements	13
6	Collection of water samples of Mithi River	14
7	Physico-chemical analysis of Mithi River water	16
8	Isolation of heavy metal resistant bacteria	17
9	Determination of MIC of isolated bacteria	18
10	Salinity tolerance analysis	19
11	pH effect study	20
12	Morphological and Biochemical Characterization of heavy metal resistant isolates	26
13	Molecular identification of the heavy metal resistant isolates	34
14	Heavy metal scale-up study of the isolated bacteria	36
15	Development of bacterial consortium	53
16	Bacterial cell surface analysis	58
17	Exopolysachharide, FTIR and Scanning Electron Microscopy analysis	58
18	Bio-sorption study of the heavy metal	65
19	Heavy metal reduction study by bacteria	67
20	Proteomics analysis of Heavy metal resistant isolates	68
21	Genomics analysis of Heavy metal resistant isolates	72
22	Development of the biomat using chemical polymers	74
23	Development of biomat using different biological material and Heavy metal analysis	78
24	Simulation of Mithi River condition in lab and Heavy metal removal using biomat	89

25	Project extension work	91
26	Compilation of data and closure report	106
27	Other work done	106
28	Conclusion	106
29	Deliverable	107
30	References	107

List of tables

Table 1	Water samples collected from the various sites along the Mithi River.
Table 2	Mithi River water analyzed for various physic-chemical parameters.
Table 3	MIC of the chromium and mercury resistant isolates against respective heavy metal.
Table 4	Salinity tolerance by the chromium resistant bacterial isolates.
Table 5	Salinity tolerance by the mercury resistant bacterial isolates.
Table 6	Effect of pH (5, 7, 9 and 7.4) on growth of CrS2b.
Table 7	Effect of pH (5, 7, 9 and 7.4) on the growth of CrS2c.
Table 8	Effect of pH (5, 7, 9 and 7.4) on the growth of CrS2d.
Table 9	Change in the pH of the growth medium of CrS2b at different initial pH.
Table 10	Change in the pH of the growth medium of CrS2c at different initial pH.
Table 11	Change in the pH of the growth medium of CrS2d at different initial pH.
Table 12	Gram's nature of Chromium resistant bacterial isolates.
Table 13	Gram's nature of Mercury resistant bacterial isolates.
Table 14	Biochemical analysis of the chromium resistant isolated bacteria.
Table 15	Biochemical analysis of the chromium resistant isolated bacteria.
Table 16	Biochemical analysis of the chromium resistant isolated bacteria.
Table 17	Biochemical analysis of the mercury resistant isolates.
Table 18	Biochemical analysis of the mercury resistant isolates.
Table 19	PCR reaction mixture.
Table 20	Parameters used for PCR reaction of 16S rDNA.
Table 21	NCBI accession number of the 16S rDNA of mercury and chromium resistant bacteria
Table 22	Growth of CrS2b in presence and absence of different concentration of chromium during scale up.
Table 23	Growth of CrS2c in presence and absence of different concentration of chromium during scale up.
Table 24	Growth of CrS2d in presence and absence of different concentration of chromium during scale up.
Table 25	Growth of WHg3a in presence and absence of different concentration of mercury during scale up.
Table 26	Growth of WHg3b in presence and absence of different concentration of mercury

	during scale up.
Table 27	Growth of HgKN3 in presence and absence of different concentration of mercury during scale up.
Table 28	Development of consortium using CrS2b, CrS2c and CrS2d grown in different combination in presence of 100 ppm of chromium.
Table 29	Chromium removal by the chromium resistant bacteria individually.
Table 30	Chromium removal by the chromium resistant bacterial consortium.
Table 31	Development of consortium using WHg3a, HgKN3, and WHg3b grown in different combination in presence of 50 ppm of mercury.
Table 32	Removal of the mercury by the mercury resistant bacteria individually different time interval.
Table 33	Percentage removal of the mercury by various consortium of the bacteria a different time interval.
Table 34	Concentration of unknown protein samples.
Table 35	Details of the bacterial protein identified from chromium resistant bacteria.
Table 36	List of the gene detected for heavy metal remediation using PCR amplification.
Table 37	Chromium removal by the bacterial consortium immobilized on coir and coir without bacteria.
Table 38	Chromium removal by the bacterial consortium immobilized on coir and coir without bacteria.
Table 39	Chromium removal by the bacterial consortium immobilized on orange peel and orange peel without bacteria.
Table 40	Chromium removal by the bacterial consortium immobilized on corn cob and corn cob without bacteria.
Table 41	Chromium removal by the bacterial consortium immobilized on saw dust and saw dust without bacteria.
Table 42	CFU of the difference carrier material used to prepare the biomat of bacterial consortium.
Table 43	Comparison of different material used for biomat preparation in term of bacteria immobilized and chromium removed.
Table 44	Chromium removal by the Biomat prepared using coconut coir in the Mithi River flowing model.
Table 45	Chromium removed by biomat after different treatment cycle from the Mithi River water in the river system.
Table 46	Effect of NaCl on EPS production by bacteria.

Table 47	Effect of biomass on bacterial attachment.
Table 48	Effect of incubation time on bacterial attachment.
Table 49	Comparison of bacteria attached to the biomaterial by previous and present method.
Table 50	Cr removal by coir in River model. Water sample were taken from the starting point of the river model for chromium analysis.
Table 51	Cr removal by coir in River model. Water samples were taken from the middle point of the river model for chromium analysis.
Table 52	Cr removal by coir in River model. Water samples were taken from the end point of the river model for chromium analysis.
Table 53	Cr removal by coir in River model. Water samples were taken after mixing the total water has flown through coir of the river model for chromium analysis.
Table 54	Cr removal by coir immobilized with bacterial consortium in River model. Water samples were taken from the starting point of the river model for chromium analysis.
Table 55	Cr removal by coir immobilized with bacterial consortium in River model. Water samples were taken from the middle point of the river model for chromium analysis.
Table 56	Cr removal by coir immobilized with bacterial consortium in River model. Water samples were taken from the end point of the river model for chromium analysis.
Table 57	Cr removal by coir immobilized with bacterial consortium in River model. Water sample was taken after mixing the total water has flown through coir of the river model for chromium analysis.
Table 58	Effect of NaCl on EPS production by bacteria
Table 59	Effect of biomass on bacterial attachment.
Table 60	Effect of incubation time on bacterial attachment.
Table 61	Cr removal by coir in River model.
Table 62	Cr removal by coir immobilized with chromium bioremediating bacteria in River model.

List of figures

Figure 1	a) Sample collection from Taximens Colony stretch and current status of Mithi River at Taximens Colony.
Figure 2	a) Sample collection from Bandra Kurla Complex stretch of Mithi River and current status of Mithi River at Bandra Kurla Complex stretch.
Figure 3	a) Sample collection from Kapadia Nagar stretch and current status of Mithi River at Kapadia Nagar.
Figure 4	a) Sample collection from Kalanagar stretch of Mithi River and current status of Mithi

	River at Kalanagar stretch.
Figure 5	Growth of bacteria from the Mithi River water sample (with a dilution of a-dilution 10-2; b- dilution 10-3; c-dilution 10-4) on nutrient agar plates containing Chromium.
Figure 6	Growth curve of the CrS2b, CrS2c and CrS2d at different pH (5, 7 and 9) in presence of 100 ppm of chromium. Control was maintained without chromium at pH 7.4.
Figure 7	Graphical representation of the change in pH of medium of CrS2b, CrS2c and CrS2d bacteria in presence of 100 ppm of chromium.
Figure 8	Gram's nature of the isolates (a) Gram positive cocci of CrS1b, (b) Gram negative bacilli of HgKN-5, (c) Gram negative coccobacilli of CrS3a and (d) Gram negative cocci of HgS4b.
Figure 9	Agarose gel image of the 16S rDNA amplified product of chromium resistant bacteria.
Figure 10	Agarose gel image of the 16S rDNA amplified product of mercury resistant bacteria.
Figure 11	Graphical representation of the growth and chromium removed by CrS2b at different concentration of chromium during scale up.
Figure 12	Graphical representation of the growth and chromium removed by CrS2c at different concentration of chromium during scale up.
Figure 13	Graphical representation of the growth and chromium removed by CrS2d at different concentration of chromium during scale up.
Figure 14	Percentage reduction in the chromium by isolated bacteria.
Figure 15	Graphical representation of the growth and chromium removed by WHg3a at different concentration of mercury during scale up.
Figure 16	Graphical representation of the growth and chromium removed by WHg3b at different concentration of mercury during scale up.
Figure 17	Graphical representation of the growth and chromium removed by WHKN3 at different concentration of mercury during scale up.
Figure 18	Percentage reduction in the mercury by isolated bacteria.
Figure 19	Growth curve of the bacteria grown in different combination.
Figure 20	Growth curve of the mercury resistant bacteria grown in different combination.
Figure 21	Comparison of the Hg removal by individual and different consortium of the Hg resistant bacteria.
Figure 22	Graphical presentation of the Hg removal by bacterial consortium. Hg removed by different combinations of Hg resistant bacteria. Set A (WHg3a+WHg3b), Set B (WHg3a+HgKN3), Set C (WHg3b+HgKN3) and Set D (WHg3a+WHg3b+HgKN3).
Figure 23	Exopolysaccharide secretion by CrS1a, CrS1b, CrS2a, CrS2b and CrS2c (pg/cell) in the presence (Red line) and absence (Black line) of the chromium.

Figure 24	FTIR spectrum of the CrS1a isolate in the presence (black) and absence (red) of chromium.
Figure 25	FTIR spectrum of the CrS1b isolate in the presence (black) and absence (red) of chromium.
Figure 26	FTIR spectrum of the CrS2a isolate in the presence (black) and absence (red) of chromium.
Figure 27	FTIR spectrum of the CrS2b isolate in the presence (black) and absence (red) of chromium.
Figure 28	FTIR spectrum of the CrS2c isolate in the presence (black) and absence (red) of chromium.
Figure 29	FTIR spectrum of the HgKN1 isolate in the presence (black) and absence (red) of mercury.
Figure 30	Scanning electron microgram of the HgKN1 bacterial sample grown in the absence (A) and in the presence (B) of 100 ppm of mercury.
Figure 31	Chromium removal by Chromium resistant isolate at varying concentration of chromium.
Figure 32	Chromium removal by Chromium resistant isolate at varying pH of chromium.
Figure 33	Cr reduction by CrS2b at varying time interval (black- 10 ppm, red- 50 ppm, blue- 100 ppm and green- 200 ppm).
Figure 34	Effect of Cr concentration (5 ppm and 200 ppm) on the reduction capability of bacteria CrS2c and CrS2d.
Figure 35	The standard plot of the BSA protein for extrapolation of unknown protein concentration.
Figure 36	Bacterial protein separated on SDS-PAGE.
Figure 37	Chromatogram of the various proteins analysed on the LC-MS.
Figure 38	Agarose Gel electrophoresis of the PCR amplified DNA of the heavy metal resistant gene.
Figure 39	Chromium removal by the alginate bead immobilized with Cr bacterial consortium and without bacterial consortium.
Figure 40	Chromium removal by the alginate and PVA bead immobilized with Cr bacterial consortium and without bacterial consortium.
Figure 41	Chromium removal by the alginate and AC bead immobilized with Cr bacterial consortium and without bacterial consortium.
Figure 42	Chromium removal by the alginate, PVA and AC bead immobilized with Cr bacterial consortium and without bacterial consortium.
Figure 43	Chromium removal by the alginate and PVA bead immobilized with Cr bacterial

	consortium and without bacterial consortium.
Figure 44	Chromium removal by the GAC immobilized with Cr bacterial consortium and without bacterial consortium.
Figure 45	SEM analysis of the coconut coir without bacteria (a) and immobilized with bacteria (b).
Figure 46	Graphical representation of the percentage chromium removal by coir with bacterial consortium and without bacterial consortium.
Figure 47	SEM image of Cr consortium immobilized on the coconut coir using another method (b) and control without immobilized bacteria (a).
Figure 48	Graphical representation of the percentage chromium removal by coir with bacterial consortium and without bacterial consortium.
Figure 49	SEM analysis of the orange peel powder without bacteria (a) and immobilized with bacteria (b).
Figure 50	Graphical representation of the percentage chromium removal by orange peel with bacterial consortium and without bacterial consortium.
Figure 51	SEM analysis of the corn cob without bacteria (a) and immobilized with bacteria (b).
Figure 52	Graphical representation of the percentage chromium removal by corn cob with bacterial consortium and without bacterial consortium.
Figure 53	SEM analysis of the saw dust without bacteria (a) and immobilized with bacteria (b).
Figure 54	Graphical representation of the percentage chromium removal by saw dust with bacterial consortium and without bacterial consortium.
Figure 55	Graphical representation of the chromium removal by different biological waste immobilized with bacterial consortium.
Figure 56	Isolated colonies obtained after plating the bacterial consortium on nutrient agar.
Figure 57	Mithi River water flowing system.
Figure 58	Percentage chromium removed by coconut coir biomat applied to the Mithi River flowing system in the lab.
Figure 59	Image of biomat placed in the river model system for chromium treatment.
Figure 60	Graphical presentation of chromium removal at starting by coir in river model. (a) show the Cr^{6+-} reduction and (b) show total chromium (Cr^{6+} and Cr^{3+}).
Figure 61	Graphical presentation of chromium removal at middle point by coir in river model. (a) show the Cr^{6+-} reduction and (b) show total chromium (Cr^{6+} and Cr^{3+}).
Figure 62	Graphical presentation of chromium removal at end point by coir in river model. (a) show the Cr^{6+-} reduction and (b) show total chromium (Cr^{6+} and Cr^{3+}).

Figure 63	Graphical presentation of chromium removal from total water by coir in river model. (a) show the Cr^{6+-} reduction and (b) show total chromium (Cr^{6+} and Cr^{3+}).
Figure 64	Graphical presentation of chromium removal at starting point by coir immobilized with bacteria in river model. (a) show the Cr^{6+-} reduction and (b) show total chromium (Cr^{6+} and Cr^{3+}).
Figure 65	Graphical presentation of chromium removal at middle point by coir immobilized with bacteria in river model. (a) show the Cr^{6+-} reduction and (b) show total chromium (Cr^{6+} and Cr^{3+}).
Figure 66	Graphical presentation of chromium removal at end point by coir immobilized with bacteria in river model. (a) show the Cr^{6+-} reduction and (b) show total chromium (Cr^{6+} and Cr^{3+}).
Figure 67	Graphical presentation of chromium removal from total water by coir immobilized with bacteria in river model. (a) show the Cr6+ reduction and (b) show total chromium (Cr6+ and Cr3+).
Figure 68	Chromium removal (%) by coir and coir immobilized with bacteria. Water samples were taken from staring point of river model (AAS data).
Figure 69	Chromium removal (%) by coir and coir immobilized with bacteria. Water samples were taken from middle point of river model (AAS data).
Figure 70	Chromium removal (%) by coir and coir immobilized with bacteria. Water samples were taken from end point of river model (AAS data).
Figure 71	Comparison of percentage chromium removal by coir and coir immobilized with chromium resistant consortium. Chromium quantification was done using AAS (Cr^{3+} and Cr^{6+}).
Figure 72	Image of biomat placed in the river model system for chromium treatment.
Figure 73	Removal of chromium in different oxidation (Cr^{6+}, Cr^{6+} plus Cr^{3+} and Cr^{3+}) using coir. Sample collected at various time points and at various positions along the river model.
Figure 74	Removal of chromium in different oxidation (Cr^{6+}, Cr^{6+} plus Cr^{3+} and Cr^{3+}) using coir immobilized with bacteria. Sample collected at various time points and at various positions along the river model.
Figure 75	Chromium removal (%) by coir and coir immobilized with bacteria. Samples taken from staring point of river model (AAS data).
Figure 76	Chromium removal (%) by coir and coir immobilized with bacteria. Samples taken from mid point of river model (AAS data).
Figure 77	Chromium removal (%) by coir and coir immobilized with bacteria. Samples taken from end point of river model (AAS data).
Figure 78	Chromium (Cr^{6+}) removal using coir and coir immobilized with bacteria in Mithi river

	model. Chromium quantification was done using DPC assay (Cr^{6+}).
Figure 79	Comparison of percentage chromium removal by coir and coir immobilized with chromium resistant consortium. Chromium quantification was done using AAS (Cr^{3+} and Cr^{6+}).

Abbreviations

Cr- chromium

Hg- mercury

ppm- part per million

ppt- part per thousand

μg/ml- microgram per millilitre

ml- millilitre

KΩ- Kiloohms

PCR- polymerase chain reaction

PSU- Practical Salinity Unit

NCBI- National Centre for Biotechnology Information

CFU- Colony Forming Unit

MIC- Minimal Inhibitory Concentration

EPS- Exopolysaccharide

Objective of the Project

- To remove the concentration of heavy metals by analysing the genomics and proteomics of potential microorganism / bacterial consortium from Mithi River having high concentration of toxic heavy metals because of the dumping of industrial waste and garbage. The proponent intends to perform bioremediation of heavy metals by scale up process technique using potential microorganisms / bacteria isolated from heavy metal contaminated sites.
- The proponent would also develop a biomat by absorbing the microbial consortia on immobilizing agents such as bagasse, activated charcoal and coconut husk and conduct absorption test in the laboratory on the same.

Targets of the project

(a) Manpower Recruitment (b) Procurement of chemicals, recruitments and literature survey (c) Collection of samples at varying time periods, isolation and purification (d) Identification of bacteria against the selected metals having high concentration
(a) Development of microbial consortia against the selected heavy metals (b) Bioremediation by scale up process using isolated bacteria
(a) Genomic studies (b) Proteomic studies
(a) Study the effect of salinity on the potential identified microbial consortia (b) Simulating bioremediation experiments using microbial consortia in the lab by replicating conditions of Mithi River (c) Development of Biomat for bioremediation of the selected heavy metals (b) Compilation of data and closure of report

A. Experimental pre-work

1. JRF Appointment

A research staff (JRF) was appointed on the project as per the rules of University of Mumbai. Applications for the said post were invited via advertisement on the Mumbai University website for selection of the most potential candidate as Junior Research Fellow (JRF). All the received applications were scrutinized for screening of the eligible candidates for interview process. An Interview committee comprises of three members Dr. Pradeep Verma (Subject Expert), Dr. Gangadhar Meshram (Vice Chancellor's Nominee) and Dr Bhupendra Pushkar (Head of the Biotechnology Department and PI). The interview was conducted on 28th January, 2016 as per the availability and convenience of the members of the interview committee.

2. Literature Survey

Exhaustive literature review was done on the bioremediation of heavy metals especially chromium and mercury. Various aspects of bioremediation of heavy metals such as isolation of wild type heavy metal resistant bacteria, development of consortium, mechanism involved by bacteria in bioremediation of heavy metals, genomics and proteomics analysis of the heavy metal resistant bacteria were reviewed. On the basis of the literature review experiments were designed. The present status of the research on bioremediation of heavy metals using bacteria was reviewed to thoroughly understand the topic.

3. Procurement of Requirements

All the chemicals, glassware, miscellaneous and the other basic materials required for the experimental work were procured before starting the experimental work. The above required materials were purchased from the dealers who have rate contract with the University of the Mumbai.

All the chemicals, glassware, miscellaneous and the other basic materials required for the experimental work were procured before starting the experimental work. All the requirements were procured as per the rule stipulated by the Mumbai University.

Chemicals procured- Nutrient agar, nutrient broth, sodium chloride, Gram's iodine, Gram's crystal violet, Gram's safranin, Glycerol, Eosin Methylene Blue agar, macConkey's agar, Cetrimide agar, DNA ladder, PCR master mix, Primers, Nuclease free water, etc.

Glassware- Conical flask (100, 250 & 500 ml), side arm flask, culture tubes, petri plates, reagent bottles, beakers, etc.

Plastic ware- Centrifuge tubes, beakers, cryo box, cryo vials, PCR rack, PCR tubes, etc.

B. Experimental Work of the Project

1. Collection of water samples from Mithi River

Water samples were collected at different time period from different points from the Mithi River. The first water sampling was done in the month of February 2016 and second water sampling was done in the month of the April 2016. The sites for water sample collection included the high salinity and low salinity zones of Mithi River. The sites for water sample collection were selected on the basis of the level of the industrial effluent discharged. The sites of water collection for **high salinity zones** were **Kalanagar, Western Express Highway** and the sites for collection of water for **low salinity zones** were **Bandra Kurla Complex, Taximens Colony, Kranti Nagar, Kapadia Nagar.**

Water samples were collected from various sites of the Mithi River. The integrated water sampling procedure was employed to collect the water as river is flowing system. Area integrated water sampling was performed by taking the water sample from 2-3 points at single site and then mixing the sample and labeling it as a final sample. From the above mixture of water, 500 ml water was used for the experiment. The water was taken into a sterile glass bottle and covered with silver foil to avoid any photo reaction and was further stored in the cold condition in the refrigerator at 4°C. In the similar manner, water samples were collected from Western Express Highway, Bandra Kurla Complex, Taximens Colony, Kranti Nagar, Kapadia Nagar (table 1) and were stored in cold condition in the refrigerator at 4°C.

Table 1: Water samples collected from the various sites along the Mithi River.

Sr. No.	Site of sample collection	Salinity zone	Time of sample collection
1	Western express highway	High salinity	April
2	Kalanagar	High salinity	February
3	BKC	Low salinity	April
4	Taximens Colony	Low salinity	February
5	Kapadia Nagar	Low salinity	April
6	Kranti Nagar	Low salinity	February

Figure 1: a) Sample collection from Taximens Colony stretch and current status of Mithi River at Taximens Colony.

Figure 2: a) Sample collection from Bandra Kurla Complex stretch of Mithi River and current status of Mithi River at Bandra Kurla Complex stretch.

Figure 3: a) Sample collection from Kapadia Nagar stretch and current status of Mithi River at Kapadia Nagar.

Figure 4: a) Sample collection from Kalanagar stretch of Mithi River and current status of Mithi River at Kalanagar stretch.

2. Mithi River water Physico-chemical analysis

Water samples were collected from various sites of the Mithi River. The integrated water sampling procedure was employed to collect the water as river is flowing system. Area integrated water sampling was performed by taking the sample from 2-3 points of single site and then mixing the sample and labeling it as a final sample. The samples were stored in ice pack and brought to the laboratory at earliest. The samples were stored at 4°C to avoid any changes in the sample. Various parameters were analyzed as mentioned in Table 2.

Mithi River water is highly polluted with various kind of pollutant released from the industries and residential area. These pollutants affect the physico-chemical parameters of the fresh water. Any changes in these parameters are the indication of level of pollution. Various parameters of the Mithi River water were found above the standard critical range as mentioned in Table 2. This information can be used in planning various strategies for removal of pollutants from Mithi River.

Table 2: Mithi River water analyzed for various physic-chemical parameters.

Parameters	WE highway	BKC	Krantinagar	Safeed Phool	Std. value
pH	7.506	7.901	7.929	7.834	6.5-8.5
Conductivity	23 ms	6.96 ms	1.002 ms	0.676 ms	0.750 mS
TDS	3.85 ppt	3.47 ppt	0.493 ppt	0.334 ppt	< 0.5ppt
Salinity	14.14 PSU	3.72 PSU	0.47 PSU	0.33 PSU	0.5 PSU
Resistivity	71 Ω	144 Ω	1.04 kΩ	1.46 kΩ	200 Ω
COD	333.33 mg/l	363.63 mg/l	422.24 mg/l	409.26 mg/l	< 250 mg/ml
BOD	120 mg/l	60 mg/l	270 mg/l	198.76 mg/l	< 30.0 mg/l

Note- Red indicate value higher then stipulated limit.

3. Isolation of heavy metal resistance bacteria

The samples were further processed for isolation of the chromium and mercury resistant bacteria. Water samples were filtered through Whatman filter paper no. 1 to remove the any suspended solids. The water samples were diluted to 10^{-4} dilution to decrease the load of bacteria in the water samples so as to get the isolated colonies of bacteria when grown on the nutrient agar plates. The above diluted water samples were spread plated on nutrient agar plates containing chromium at a concentration of the 100, 150 and 200 ppm for the isolation of chromium resistant bacteria. In the similar manner the nutrient agar plates containing mercury at a concentration of the 50, 100 and 150 ppm was used for the isolation of mercury resistant bacteria. The culture conditions used for growth of all the bacteria was 37°C of temperature for 24 hours duration. Bacterial colonies showing distinct morphology were selected from the nutrient agar plates containing Chromium and Mercury respectively (Figure 5). Bacterial colonies resistant to Chromium and Mercury were further purified to get the single type of bacterial isolate.

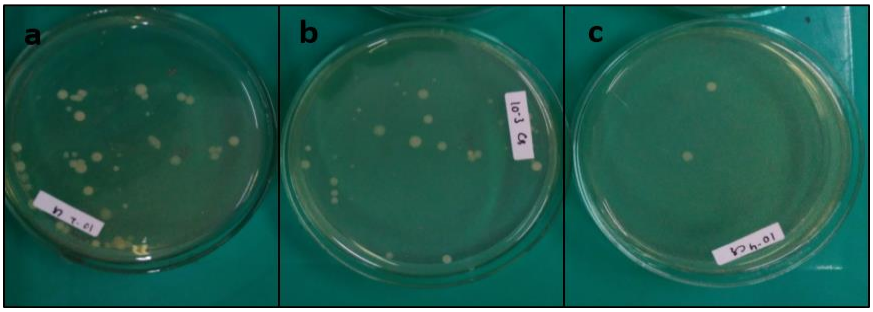


Figure 5: Growth of bacteria from the Mithi River water sample (with a dilution of a-dilution 10^{-2}; b- dilution 10^{-3}; c-dilution 10^{-4}) on nutrient agar plates containing Chromium.

Purification of the heavy metal resistant isolates- Single colony of the bacteria on the nutrient agar plate containing chromium was inoculated in the nutrient broth containing 200 ppm of chromium and incubated at 37°C for 24 hours. The loop-full of above grown culture in broth was inoculated on the nutrient agar plate containing 200 ppm of chromium for further purification of bacteria. Again a single colony from the above nutrient agar plate was inoculated in nutrient broth containing chromium at 200 ppm and incubated at 37°C for 24 hours and the loop-full of the culture was again used for the isolation on nutrient agar plate containing 200 ppm of Chromium. The above step was repeated 3-4 times to get the single purified bacterial colony. All the selected bacterial isolates resistant to chromium were purified by same process as mentioned above. The selected bacterial isolates resistant to mercury were also purified in the similar manner using nutrient agar plate with 100 ppm mercury and nutrient broth with 100 ppm mercury. 15 chromium resistant bacterial isolates and 12 mercury resistant bacterial isolates were selected for further processing.

Glycerol stock preparation – The above purified bacterial isolates resistant to chromium and mercury were further stored in glycerol at -20°C. A single purified colony was selected and was inoculated in nutrient broth containing the respective heavy metals and incubated at 37°C for 24 hours. Glycerol stock was prepared by using log phase culture of the bacteria growing in the nutrient broth containing the heavy metals. 25% glycerol stock was prepared by adding 750 µl of

the log phase culture and 250 µl of the double autoclaved 100% glycerol. The glycerol stocks for each of the bacterial isolates were prepared in triplicates and were stored at -20°C. 15 bacterial isolates resistant to chromium and 12 bacterial isolates resistant to mercury were stored in glycerol at -20°C for further study.

4. Determination Minimal Inhibitory Concentration (MIC)

MIC was checked to determine the maximum tolerance limit of the isolated bacteria towards the respective heavy metals i.e. Chromium and Mercury. MIC was performed using tube method in which the isolate was inoculated in the tubes containing nutrient broth with heavy metal with concentration ranging from 100 to 1000 ppm at interval of 100 ppm (i.e. 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 ppm) for Chromium and for Mercury the range was 50 to 800 ppm at interval of 100 ppm (i.e. 50, 100, 200, 300, 400, 500, 600, 700, 800 ppm). All the tubes were incubated on the shaker incubator at 150 rpm at 37°C for 24 hours. The growth was monitored after 24 hours to determine the MIC.

Out of 15 Chromium resistant bacterial isolates, 3 isolates have shown MIC of 700 ppm, 3 isolates of 600 ppm MIC, 4 isolates of 500 ppm MIC, 3 isolates of 300 ppm and 2 isolates of 200 ppm MIC towards Chromium. Out of 12 mercury resistant isolates, 8 isolates have shown MIC of 700 ppm, 1 of 500 ppm, 2 of 100 ppm and only 1 isolate shown MIC of 50 ppm towards Mercury (table 3).

Table 3: MIC of the chromium and mercury resistant isolates against respective heavy metal.

Sr. No.	Heavy metal	No. of resistant isolates	MIC (ppm)
1	Chromium	3	700
2	Chromium	3	600
3	Chromium	4	500
4	Chromium	3	300
5	Chromium	2	200
6	Mercury	8	700
7	Mercury	1	500
8	Mercury	2	100
9	Mercury	1	50

The selection of the isolates for further study was done on the basis of the MIC. The isolates with high MIC towards Chromium and Mercury were selected as the potential bacterial isolate for bioremediation of the respective heavy metals.

5. Salinity tolerance analysis

Determination of salinity tolerance of bacterial isolates from Mithi River is of importance as the river is flowing system and possesses low salinity at upper stretch of river and a high salinity at estuaries. The bacterial isolates selected for the bioremediation of heavy metals should able to survive in the wide range of salinity of the Mithi River. The salinity tolerance was determined by growing the 15 Chromium resistant bacterial isolates and 12 Mercury resistant isolates in nutrient broth having different salt (NaCl) concentration.

Salinity tolerance of the bacterial isolates was studied in the range of 5, 10, 15, 20, 25, 30 and 35 ppt (parts per thousand) of NaCl. Bacterial isolates stored in glycerol stock were revived and used for the determination of their tolerance to various salt concentrations. The growth conditions used for determination of salinity of all bacterial isolates was 37°C of temperature and 24 hrs of growth period. Chromium and mercury resistant bacteria were found to grow at the salt concentration in range from 5 ppt to 35 ppt.

The growth pattern of the bacterial isolates in various salt concentrations indicates their tolerance to wide range of salt concentration (Table 4 and 5). This result confirm that the selected heavy metal resistant isolates are suitable for their application for bioremediation of heavy metal from Mithi River which have both lower as well as higher salinity zone.

Table 4: Salinity tolerance by the chromium resistant bacterial isolates.

Isolate	5 (ppt)	10 (ppt)	15 (ppt)	20 (ppt)	25 (ppt)	30 (ppt)	35 (ppt)
CrS1a	++	++	++	++	++	++	++
CrS1b	++	++	++	+	-	-	-
CrS1c	++	++	++	++	++	++	+
CrS2a	++	++	++	++	++	++	++
CrS2b	++	++	++	++	++	++	++
CrS2c	++	++	++	++	++	++	++
CrS2d	++	++	++	++	++	+	+
CrS2e	++	++	++	++	+	+	+
CrS3a	++	++	++	++	+	+	+
CrS4a	++	++	++	++	+	+	+
CrS4b	++	++	++	++	++	+	+
CrS4c	++	++	++	++	++	+	+
CrS4d	++	++	++	++	+	+	+

CrT-2	++	++	++	++	+	-	-
CrT-3	++	++	++	++	+	+	+

Note: ++ intense growth, + growth and – no growth.

Table 5: Salinity tolerance by the mercury resistant bacterial isolates.

Isolate	5 (ppt)	10 (ppt)	15 (ppt)	20 (ppt)	25 (ppt)	30 (ppt)	35 (ppt)
HgK-3a	++	++	++	++	++	++	++
HgK-3b	++	++	++	++	++	++	++
HgB-1	++	++	++	++	++	++	+
HgB-2	++	++	++	++	++	++	+
HgB-3	++	++	++	++	++	++	+
HgKN-1	++	++	++	++	++	++	++
HgKN-2	++	++	++	++	++	++	++
HgKN-3	++	++	++	++	++	++	+
HgKN-4	++	++	++	++	++	++	++
HgKN-5	++	++	++	++	++	++	+
HgS4a	++	++	++	++	++	++	+
HgS4b	++	++	++	++	++	++	+
HgS4c	++	++	++	++	++	++	+
HgK-3	++	++	++	++	++	+	+
HgB-1	++	++	++	++	++	++	+
HgB-2	++	++	++	++	++	++	+

6. **Effect of pH-** The effect of pH on bacterial isolates was studied. The pH has very important role in the bacterial growth as well as for heavy metal remediation. Three pH values were included in the study ie, acidic pH 5, neutral pH 7 and alkaline pH 9. The pH study was conducted in presence of heavy metal. A control was maintained without heavy metal. The growth of the isolated bacteria in different pH is given in the Table 6, 7 and 8. The change in the pH of the medium during the bacterial growth was also determined (Table 9, 10 and 11).

Table 6: Effect of pH (5, 7, 9 and 7.4) on growth of CrS2b.

Growth of Crs2b isolate				
Time	Acidic (pH5)	Neutral (pH7)	Alkaline (pH9)	Control (pH7.4)
	100 ppm of Cr			Without Cr
0hr	0.124	0.122	0.139	0.096
30 min	0.164	0.173	0.152	0.155
60 min	0.201	0.216	0.211	0.212
90 min	0.251	0.28	0.272	0.306
120 min	0.373	0.37	0.356	0.454
150 min	0.474	0.444	0.421	0.581
180 min	0.51	0.464	0.448	0.589
210 min	0.591	0.535	0.497	0.688
240 min	0.704	0.574	0.62	0.743
270 min	0.664	0.613	0.548	0.906
300 min	0.685	0.644	0.573	0.919
330 min	0.694	0.644	0.558	0.822
360 min	0.712	0.664	0.581	0.834
24 hrs	0.764	1.116	0.587	0.913

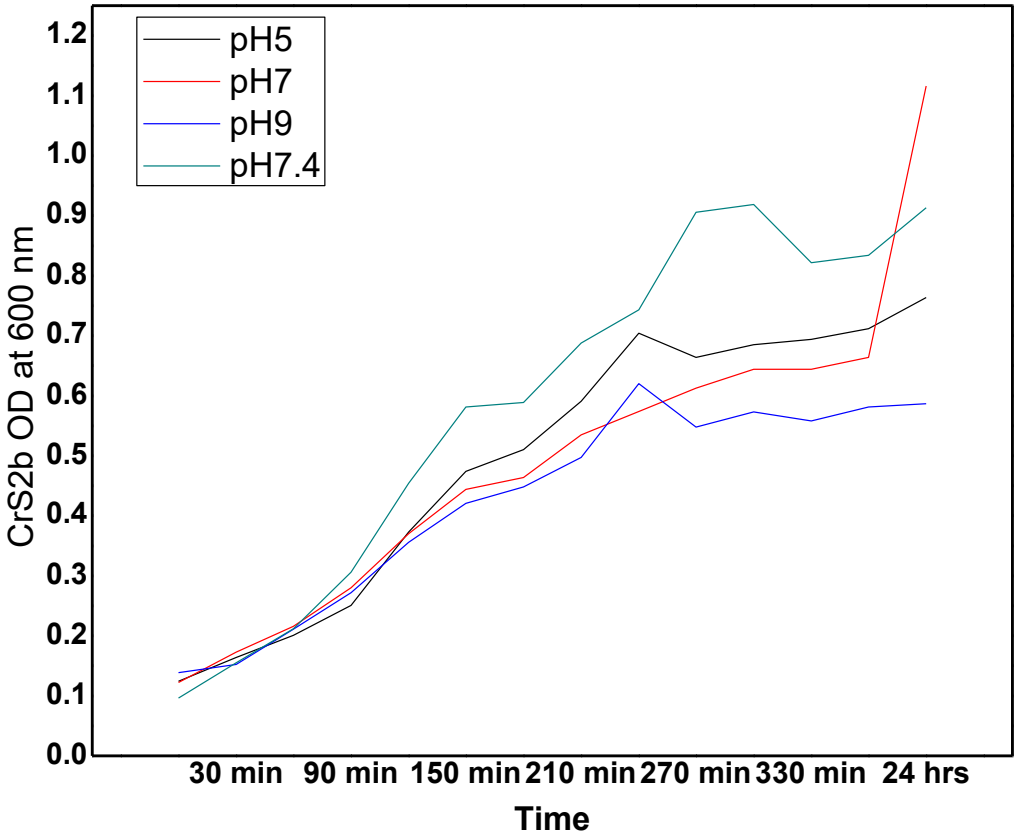
Table 7: Effect of pH (5, 7, 9 and 7.4) on the growth of CrS2c.

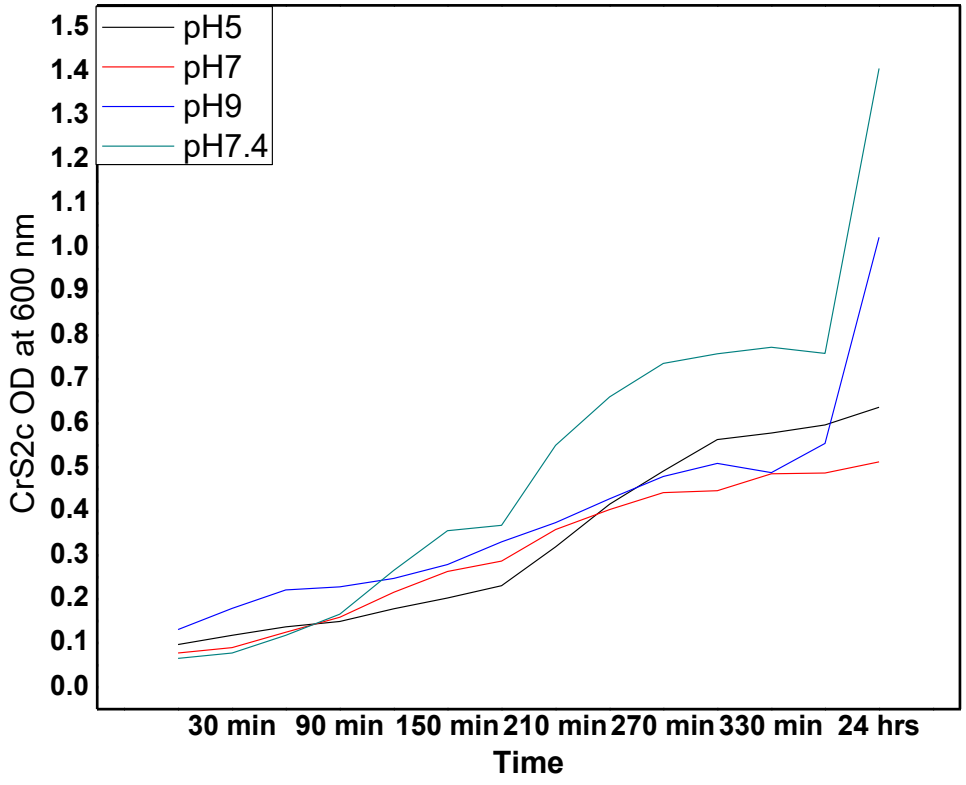
Growth of Crs2c isolate				
Time	Acidic (pH5)	Neutral (pH7)	Alkaline (pH9)	Control (pH7.4)
	100 ppm of Cr			Without Cr
0hr	0.097	0.078	0.131	0.065
30 min	0.118	0.09	0.179	0.078
60 min	0.137	0.125	0.221	0.118
90 min	0.149	0.159	0.228	0.166
120 min	0.178	0.216	0.247	0.266
150 min	0.203	0.263	0.279	0.356
180 min	0.231	0.287	0.33	0.368

210 min	0.319	0.358	0.374	0.55
240 min	0.416	0.404	0.428	0.66
270 min	0.491	0.442	0.479	0.736
300 min	0.563	0.447	0.509	0.758
330 min	0.578	0.485	0.488	0.773
360 min	0.596	0.487	0.554	0.759
24 hrs	0.636	0.512	1.023	1.407

Table 8: Effect of pH (5, 7, 9 and 7.4) on the growth of CrS2d.

Growth of Crs2d isolate				
Time	Acidic (pH5)	Neutral (pH7)	Alkaline (pH9)	Control (pH7.4)
	100 ppm of Cr			Without Cr
0hr	0.135	0.133	0.127	0.082
30 min	0.154	0.156	0.147	0.112
60 min	0.175	0.182	0.18	0.179
90 min	0.201	0.233	0.209	0.208
120 min	0.218	0.264	0.226	0.246
150 min	0.3	0.343	0.283	0.373
180 min	0.41	0.45	0.383	0.584
210 min	0.533	0.533	0.451	0.719
240 min	0.58	0.603	0.475	0.752
270 min	0.64	0.688	0.518	0.818
300 min	0.678	0.731	0.542	0.821
330 min	0.694	0.772	0.56	0.823
360 min	0.722	0.781	0.567	0.809
24 hrs	0.677	0.632	0.482	0.67


pH5
pH7
pH9
pH7.4
CrS2b OD at 600 nm
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
30 min
90 min
150 min
210 min
270 min
330 min
24 hrs
Time


pH5
pH7
pH9
pH7.4
CrS2c OD at 600 nm
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
30 min
90 min
150 min
210 min
270 min
330 min
24 hrs
Time

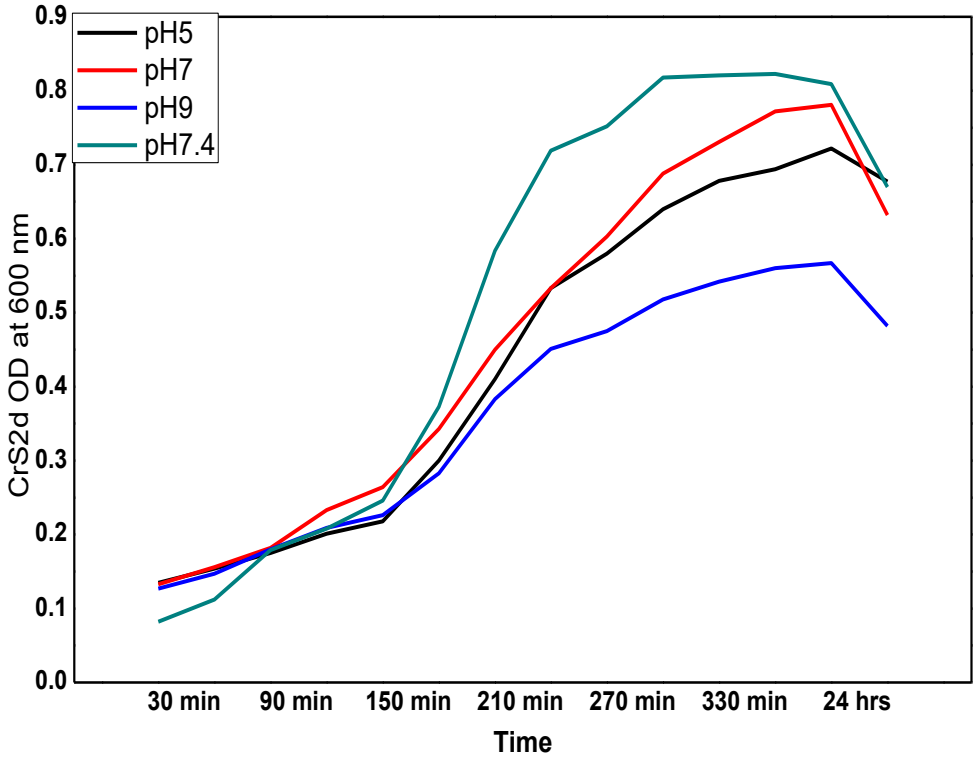


Figure 6: Growth curve of the CrS2b, CrS2c and CrS2d at different pH (5, 7 and 9) in presence of 100 ppm of chromium. Control was maintained without chromium at pH 7.4.

Table 9: Change in the pH of the growth medium of CrS2b at different initial pH.

CrS2b study of pH				
Time	Acidic	Neutral	Alkaline	Control
0 hr	5	7	9	7.4
3hrs	7.195	7.683	8.147	7.613
6hrs	7.621	7.899	8.275	7.971
24hrs	7.886	8.32	8.492	8.21

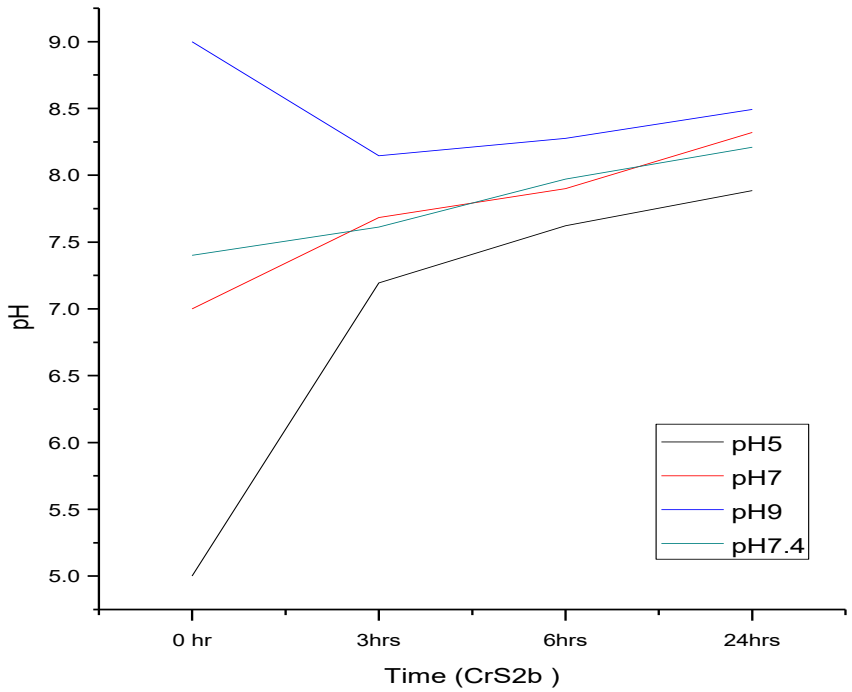
Table 10: Change in the pH of the growth medium of CrS2c at different initial pH.

Crs2c study of pH				
Time	Acidic	Neutral	Alkaline	Control
0 hr	5	7	9	7.4
3hrs	6.661	7.791	8.111	7.461

6hrs	7.417	7.836	8.163	7.887
24hrs	7.922	8.163	8.436	8.31

Table 11: Change in the pH of the growth medium of CrS2d at different initial pH.

Crs2d study of pH				
Time	Acidic	Neutral	Alkaline	Control
0 hr	5	7	9	7.4
3hrs	7.844	7.844	8.293	8.654
6hrs	8.084	8.084	8.391	8.213
24hrs	8.066	8.367	8.665	8.335



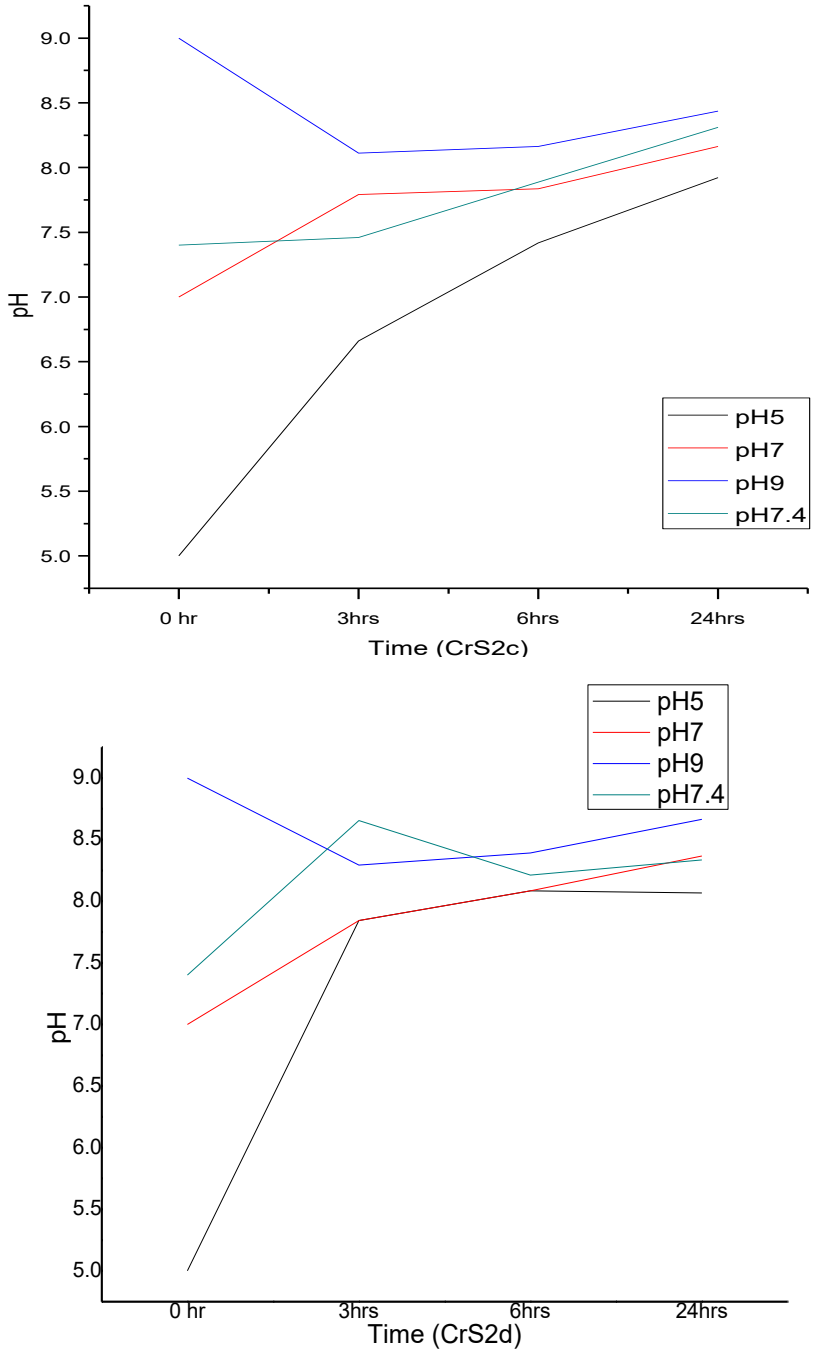


Figure 7: Graphical representation of the change in pH of medium of CrS2b, CrS2c and CrS2d bacteria in presence of 100 ppm of chromium.

7. Morphological and Biochemical Characterization

Characterization of the Chromium and Mercury resistant bacterial isolates were carried out using Gram staining and Biochemical analysis. Previously prepared glycerol stocks of the Chromium and Mercury resistant bacterial isolates were revived for the gram staining and biochemical analysis. Gram's nature of 15 chromium resistant bacterial isolates and 12 mercury resistant bacterial isolates were determined using Gram staining method. Both Gram positive and Gram negative bacteria resistant to chromium and mercury were found. Chromium resistant bacteria include six

gram negative coccobacilli, five gram negative bacilli while 4 gram positive cocci (table 12). All 12 mercury resistant bacteria isolates were gram negative in nature indicating that gram negative bacteria are more tolerant to mercury. Five mercury resistant bacteria were gram negative coccobacilli, six gram negative bacilli, one gram negative cocci (table 13).

Table 12: Gram's nature of Chromium resistant bacterial isolates.

Sr. No.	Isolate	Gram's nature	Resistant against
1	CrS1a	Gram's positive cocci	Chromium
2	CrS1b	Gram's positive cocci	Chromium
3	CrS1c	Gram's negative coccobacilli	Chromium
4	CrS2a	Gram's negative bacilli	Chromium
5	CrS2b	Gram's negative coccobacilli	Chromium
6	CrS2c	Gram's negative coccobacilli	Chromium
7	CrS2d	Gram's positive cocci	Chromium
8	CrS2e	Gram's negative bacilli	Chromium
9	CrS3a	Gram's negative coccobacilli	Chromium
10	CrS4a	Gram's positive cocci	Chromium
11	CrS4b	Gram's negative bacilli	Chromium
12	CrS4c	Gram's negative bacilli	Chromium
13	CrS4d	Gram's negative bacilli	Chromium
14	CrT-2	Gram's negative coccobacilli	Chromium
15	CrT-3	Gram's negative coccobacilli	Chromium

Table 13: Gram's nature of Mercury resistant bacterial isolates.

Sr. No.	Isolate	Gram's nature	Resistant against
1	HgK-3	Gram's negative coccobacilli	Mercury
2	HgB-1	Gram's negative coccobacilli	Mercury
3	HgB-2	Gram's negative coccobacilli	Mercury
4	HgB-3	Gram's negative coccobacilli	Mercury

5	HgKN-1	Gram's negative bacilli	Mercury
6	HgKN-2	Gram's negative bacilli	Mercury
7	HgKN-3	Gram's negative bacilli	Mercury
8	HgKN-4	Gram's negative coccobacilli	Mercury
9	HgKN-5	Gram's negative bacilli	Mercury
10	HgS4a	Gram's negative bacilli	Mercury
11	HgS4b	Gram's negative cocci	Mercury
12	HgS4c	Gram's negative bacilli	Mercury

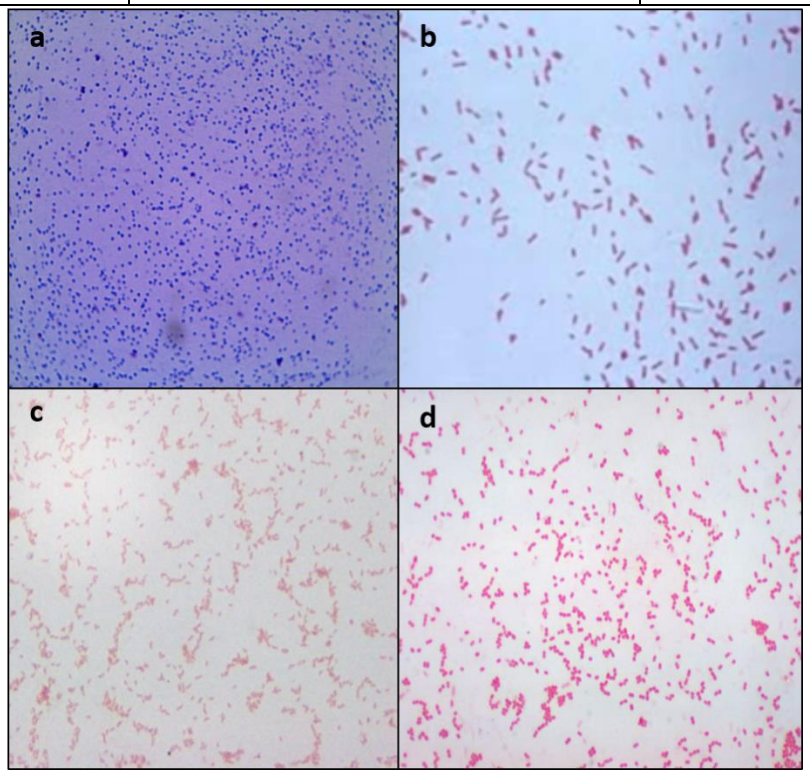


Figure 8: Gram's nature of the isolates (a) Gram positive cocci of CrS1b, (b) Gram negative bacilli of HgKN-5, (c) Gram negative coccobacilli of CrS3a and (d) Gram negative cocci of HgS4b.

The biochemical analysis of chromium and mercury resistant bacterial isolates were performed to determine their probable identity. Isolates were tested to determine their capability to ferment various sugars like glucose, sucrose, lactose, maltose and xylose fermentation. Other biochemical tests included were Indole, Methyl red, Voges–Proskauer and Citrate tests (IMViC test), Gelatinase, Catalase, Motility, Triple sugar iron tests. Chromium and mercury resistant isolates were plated on to the selective and differential media to determine their probable taxonomical family. The results of the biochemical analysis if given in the table 14 to 18.

Table 14: Biochemical analysis of the Chromium resistant bacterial isolates.

Sr. No.	Test	CrS1a	CrS1b	CrS1c	CrS2a	CrS2b
1	Glucose	No growth	Ferment	Ferment	Non-fermentor, GP	Non-fermentor, GP
2	Lactose	Faint, NGP	No growth	No growth	Ferment	Non-fermentor
3	Xylose	No growth	Ferment	Ferment	Ferment+Gas	Non-fermentor
4	Maltose	No growth	Ferment	Ferment	Ferment+Gas	Non-fermentor
5	Sucrose	No growth	No growth	No growth	Ferment+Gas	Non-fermentor
6	Indole	Negative	Negative	Negative	Negative	Negative
7	VP	Negative	Negative	Negative	Negative	Negative
8	Methyl red	Positive	Positive	Positive	Negative	Negative
9	Citrate	Negative	Negative	Negative	Positive	Positive
10	Gelatinase	No Liquification	No Liquification	No Liquification	No Liquification	No Liquification
11	TSI	Yellow/Yellow	Yellow/Yellow	Yellow/Yellow	Yellow/Yellow+Gas	Red/Red
12	Catalase	No Effervescence	No Effervescenc	No Effervescence	Effervescence	No Effervescence
13	Motility	Non motile	Motile	Motile	Motile	Motile
14	MacConkey	No growth	No growth	Pink colonies	Pink, mucoid colony	No growth
15	Cetrimide	No growth	No growth	No growth	No growth	No growth
16	EMB	No growth	No growth	No growth	Purple, mucoid colony	Purple centered colony
17	MSA	White colony	White colony	No growth	No growth	No growth
18	Starch agar	No growth	No growth	No growth	No growth	No growth

Table 15: Biochemical analysis of the Chromium resistant bacterial isolates.

Sr. No.	Test	CrS2c	CrS2d	CrS2e	CrS3a	CrS4a
1	Glucose	Non-fermentor, GP	Ferment+Gas	Non-fermentor	Ferment	No growth
2	Lactose	Non-fermentor	Non-fermentor+Gas	Ferment	Non-fermentor	Ferment
3	Xylose	Non-fermentor	Ferment+Gas	Non-fermentor+Gas	Non-fermentor	Non-fermentor
4	Maltose	Non-fermentor	Non-fermentor+Gas	Ferment+Gas	Non-fermentor	Ferment+Gas
5	Sucrose	Non-fermentor	Ferment+Gas	Ferment+Gas	Non-fermentor	Non-fermentor+Gas
6	Indole	Negative	Negative	Negative	Negative	Negative
7	VP	Negative	Negative	Negative	Negative	Negative
8	Methyl red	Negative	Positive	Negative	Positive	Negative
9	Citrate	Negative	Positive	Positive	Negative	Positive
10	Gelatinase	No Liquification	No Liquification	No Liquification	No Liquification	No Liquification
11	TSI	Red/Red	Yellow/Yellow+ Gas	Red/Yellow	Yellow/Yellow	Red/Red
12	Catalase	No Effervescence	Effervescence	Effervescence	Negative	Effervescence
13	Motility	Motile	Non motile	Non motile	Non motile	Non motile
14	MacConkey	No growth	No growth	Pink, mucoid colony	Pink colony	Pink, mucoid colony
15	Cetrimide	No growth	No growth	No growth	No growth	No growth
16	EMB	Purple centered colony	No growth	Pink, mucoid colony	Growth	Pink, mucoid colony
17	MSA	No growth	White colony	No growth	No growth	No growth
18	Starch agar	No growth	No growth	No growth	No growth	No growth

Table 16: Biochemical analysis of the Chromium resistant bacterial isolates.

Sr. No.	Test	CrS4b	CrS4c	Crs4d	CrT-2	CrT-3
1	Glucose	Non-fermentor	Non-fermentor	Non-fermentor	Ferment+gas	Non fermentor - gas
2	Lactose	Non-fermentor	Ferment	Ferment	Non-fermentor	Ferment gas production
3	Xylose	Ferment	Ferment+Gas	Ferment+Gas	Ferment+gas	No fermentation - gas
4	Maltose	Ferment	Ferment+Gas	Ferment+Gas	Non-fermentor	Non-fermentor
5	Sucrose	Non-fermentor+Gas	Non-fermentor+Gas	Non-fermentor+Gas	Non-fermentor	Non-fermentor
6	Indole	Negative	Negative	Negative	Negative	Negative
7	VP	Negative	Negative	Negative	Negative	Negative
8	Methyl red	Negative	Negative	Negative	Negative	Negative
9	Citrate	Positive	Positive	Negative	Negative	Negative
10	Gelatinase	No Liquification	No Liquification	No Liquification	Negative	Negative
11	TSI	Red/Red+Gas	Red/Yellow+Gas	No change	Red/Yellow	Yellow/Yellow
12	Catalase	Effervescence	Effervescence	No Effervescence	Positive	Negative
13	Motility	Non motile	Non motile	Non motile	Non motile	Non motile
14	MacConkey	Pink, mucoid colony	Pink, mucoid colony	Pink, mucoid colony	Growth	Positive
15	Cetrimide	No growth	No growth	No growth	No growth	Negative
16	EMB	Pink, mucoid colony	Pink, mucoid colony	Pink, mucoid colony	Purple colonies	Negative
17	MSA	No growth	No growth	No growth	No growth	Negative
18	Starch agar	No growth	No growth	No growth	No growth	Negative

Table 17: Biochemical analysis of the Mercury resistant bacterial isolates.

Sr. No.	Test	HgK-3	HgB-1	HgB-2	HgB-3	HgKN1	HgKN2
1	Glucose	Ferment	Negative	Negative	Ferment	Non Ferment	Non Ferment
2	Lactose	Negative	Negative	Negative	Negative	Ferment+gas	Ferment+gas
3	Xylose	Negative	Negative	Negative	Negative	Ferment+gas	Ferment+gas
4	Maltose	Ferment	Negative	Negative	Ferment	Ferment+gas	Ferment+gas
5	Sucrose	Ferment	Negative	Negative	Ferment	Ferment+gas	Ferment+gas
6	Indole	Negative	Negative	Negative	Negative	Negative	Negative
7	VP	Negative	Negative	Negative	Negative	Negative	Negative
8	Methyl red	Negative	Negative	Negative	Negative	Positive	Positive
9	Citrate	Positive	Negative	Positive	Negative	Positive	Positive
10	Gelatinase	Negative	Negative	Negative	Negative	No Liquification	No Liquification
11	TSI	Red/yellow bubble at bottom	Red/red	Red/red	Red/yellow	yellow/yellow+ gas	yellow/yellow+ gas
12	Catalase	No effervescence	No effervescence	No effervescence	No effervescence	Effervescence	Effervescence
13	Motility	Non motile	Motile	Non motile	Motile	Motile	Motile
14	MacConkey	Pink colonies	No growth	No growth	No growth	mucoid white colony	mucoid white colony
15	Cetrimide	No growth	No growth	No growth	No growth	No growth	No growth
16	EMB	Purple colonies	Small pink colonies	Small pink colonies	No growth	purple mucoid colony	purple mucoid colony
17	MSA	No growth	No growth	No growth	No growth	No growth	No growth
18	Starch agar	No growth	No growth	No growth	No growth	No growth	No growth

Table 18: Biochemical analysis of the Mercury resistant bacterial isolates.

Sr. No.	Test	HgKN3	HgKN4	HgKN5	HgS4a	HgS4b	HgS4c
1	Glucose	Non Ferment	Non Ferment	Ferment	Non Ferment	Non Ferment	Non Ferment
2	Lactose	Non Ferment+gas	Ferment+gas	Ferment	Non Ferment	Non Ferment	Non Ferment
3	Xylose	Non Ferment	Non Ferment	Non Ferment	Non Ferment	Non Ferment	Non Ferment
4	Maltose	Non Ferment+gas	Ferment+gas	Non Ferment	Non Ferment	Non Ferment	Non Ferment
5	Sucrose	Ferment+gas	Ferment+gas	Ferment	Non Ferment	Non Ferment	Non Ferment
6	Indole	Negative	Negative	Negative	Negative	Negative	Negative
7	VP	Negative	Negative	Negative	Negative	Negative	Negative
8	Methyl red	Negative	Negative	Negative	Negative	Negative	Negative
9	Citrate	Positive	Positive	Positive	Positive	Positive	Positive
10	Gelatinase	No Liquification	No Liquification	No Liquification	No Liquification	No Liquification	No Liquification
11	TSI	yellow/yellow+gas	yellow/yellow+gas	yellow/yellow+gas	Red/Red	Red/Red	Red/Red
12	Catalase	Effervescence	Effervescence	Effervescence	Effervescence	Effervescence	Effervescence
13	Motility	Motile	Motile	Non motile	Non motile	Non motile	Non motile
14	MacConkey	creamish,mucoid colony	creamish,mucoid colony	Pink colonies	pin point, white colony	No growth	No growth
15	Cetrimide	No growth	No growth	No growth	No growth	No growth	No growth
16	EMB	purple mucoid colony	purple mucoid colony	Purple colonies	Black centered,white colony	No growth	Black centered, white colony
17	MSA	No growth	No growth	No growth	No growth	No growth	No growth
18	Starch agar	No growth	No growth	No growth	No growth	No growth	No growth

Note: VP- voges–Proskauer, TSI- triple sugar iron, EMB- Eosin methylene blue agar and MSA- mannitol salt agar

8. Molecular identification of heavy metal resistant bacteria

Molecular identification of the bacteria was done by analyzing the conserved sequences of 16S rDNA. Eight isolates of chromium and mercury resistant bacteria were selected for 16S rDNA sequencing on the basis of MIC against the heavy metal.

Genomic DNA was extracted from a single isolated colony of the above Chromium and Mercury resistant bacterial isolates. Genomic DNA was extracted by suspending a single isolated colony to sterile the nuclease free water and then boiled for 10 mins to lyse the bacterial cell. The supernatant after centrifugation was used as template DNA for PCR amplification of 16S rDNA.

PCR amplification of the 16S rDNA gene of the above heavy metal resistant bacterial isolates were done by using the universal primers 347F-5'- GGAGGCAGCAGTAAGGAAT -3' 803R5'- CTACCGGGGTATCTAATCC -3' (Wu, Jing, et al, 2014). The optimization of the annealing temperature was performed by setting a gradient PCR at different annealing temperature for efficient binding of the universal primer to the template DNA. PCR reaction and the PCR cycle used for amplification of the desire gene were as shown in table 19 and 20.

Table 19: PCR reaction mixture.

PCR Reaction Mixture	
CONTENT	**VOLUME(µl)**
Nuclease free water	15.2
PCR Master ready mix (containing Buffer, dNTP mix, MgCl2 and Taq DNA Polymerase) -2X.	20.0
Forward primer-10pmol/µl	1.6
Reverse primer-10pmol/µl	1.6
DNA Template	1.6
TOTAL VOLUME	**40**

Table 20: Parameters used for PCR reaction of 16S rDNA.

PCR Cycling Parameters			
Step	**Temperature**	**Time**	**No. of Cycles**
Initial denaturation	94°C	5mins	1
Denaturation	94°C	30 secs	35
Primer annealing	50°C	35secs	35

Extension	72°C	45 secs	35
Final extension	72°C	10 mins	1
Cooling	4°C	59mins	1

On completion of PCR reaction, the PCR amplified DNA was analyzed on the 1% agarose gel containing ethidium bromide to visualize DNA. Figure 9 and 10 shows the DNA band of 500 bp amplified using universal primer for 16S rDNA gene.

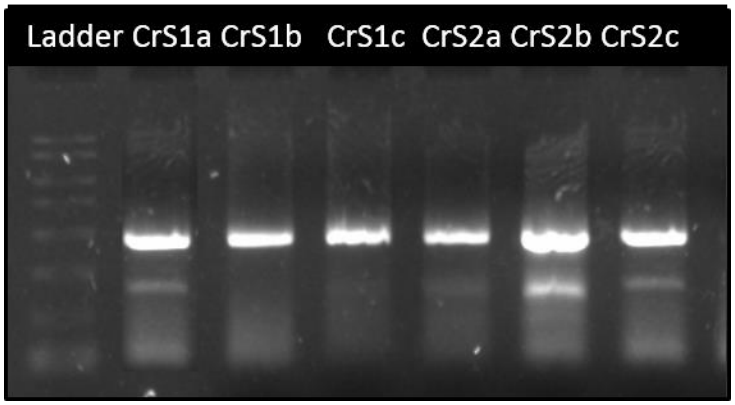


Figure 9: Agarose gel image of the 16S rDNA amplified product of chromium resistant bacteria.

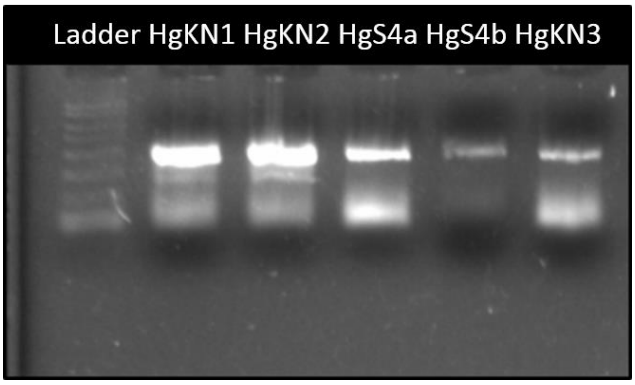


Figure 10: Agarose gel image of the 16S rDNA amplified product of mercury resistant bacteria.

The PCR amplified DNA were sequenced from Scigenome lab. Identification was confirmed by performing the BLAST of the 16S rDNA sequence of the respective bacterial isolates. The isolate CrS1a was found to be *Bacillus safensis* strain YNB154, CrS1b was found to be *Pediococcus acidilactici*strain KTNA3010M, CrS2a was found to be S*almonella enterica* strain 12B, CrS2b was identified as Bacterium NXSXRC8, CrS2c was found to be Enterobacter sp. enrichment culture clone HSL59 and HgKN1 was found to be *Klebsiella pneumoniae* strain FY2 by performing the nucleotide BLAST. The bacteria was selected which was giving maximum similarity score and sequence covered for alignment.

The 16S rDNA sequence of eight mercury resistant and eight chromium bacteria were submitted to NCBI Genebank database (table 21).

Table 21: NCBI accession number of the 16S rDNA of Mercury and Chromium resistant bacteria.

Sr. No.	Heavy metal	Isolate code	Name of bacteria	NCBI accession no. (*)
1	Mercury	WHg3a	*Bacillus thuringiensis* strain RGN1.2	KX832953.1
2	Mercury	WHg3b	*Bacillus sp.* strain CSB_B078	KX832954.1
3	Mercury	HgKN1	*Klebsiella pneumoniae* strain FY2	KX832957.1
4	Mercury	HgKN2	*Klebsiella pneumoniae* isolate 23	KX832948.1
5	Mercury	HgKN3	*Enterobacter sp.* strain Amic_7	KX832949.1
6	Mercury	HgKN4	*Enterobacter sp.* strain 08	KX832950.1
7	Mercury	HgS4a	*Acinetobacter seohaensis* strain S34	KX832951.1
8	Mercury	HgS4b	*Acinetobacter sp.* 815B5_12ER2A	KX832952.1
9	Chromium	CrS1a	*Bacillus safensis* strain YNB154	KX832955.1
10	Chromium	CrS1b	*Pediococcus acidilactici* strain KTNA3010M	KX501224.1
11	Chromium	CrS1c	*Pediococcus acidilactici* strain TUB/2013/3-5	KX832945.1
12	Chromium	CrS2a	*Salmonella enterica* strain 12B	KX501225.1
13	Chromium	CrS2b	*Enterobacter sp.* enrichment culture	KX832956.1
14	Chromium	CrS2c	*Enterobacter sp.* enrichment cuture clone HSL59	KX501226.1
15	Chromium	CrS2d	*Acinetobacter junii* strain B2w	KX832946.1
16	Chromium	CrS3a	*Pediococcus claussenii* strain TMW 2.54	KX832947.1

* https://www.ncbi.nlm.nih.gov/nucleotide/

9. Scale up of Heavy Metal resistance bacterial isolates

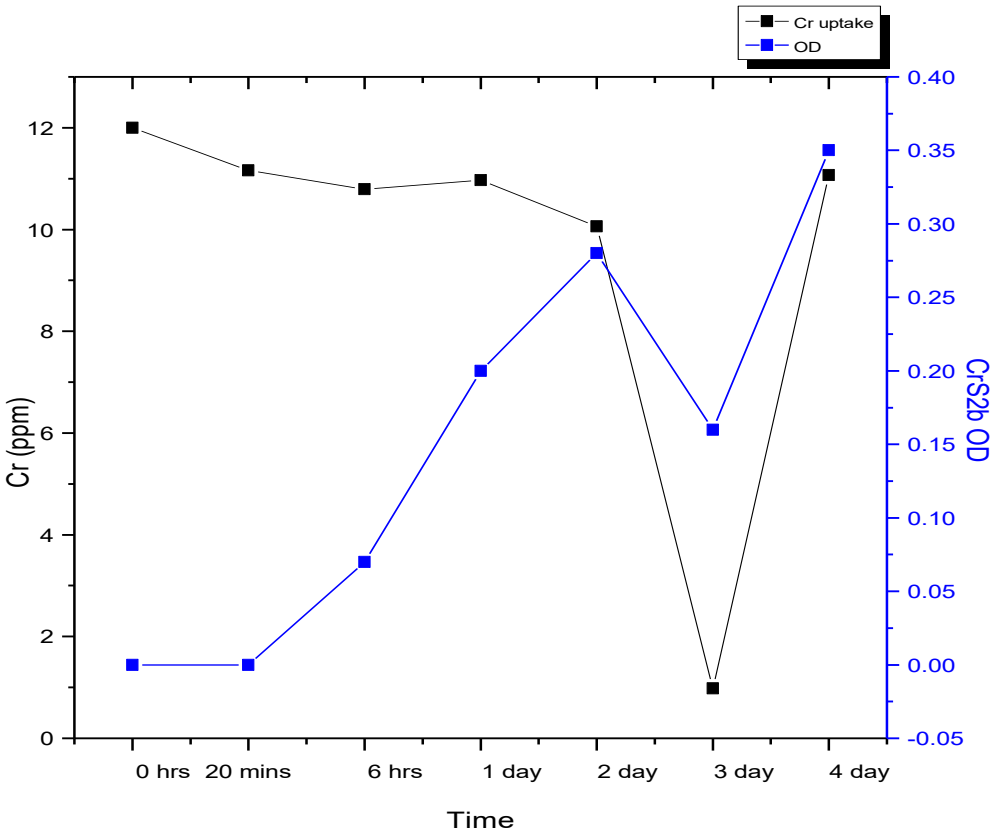
9.1 Scale up of Chromium resistant bacteria isolated from Mithi River

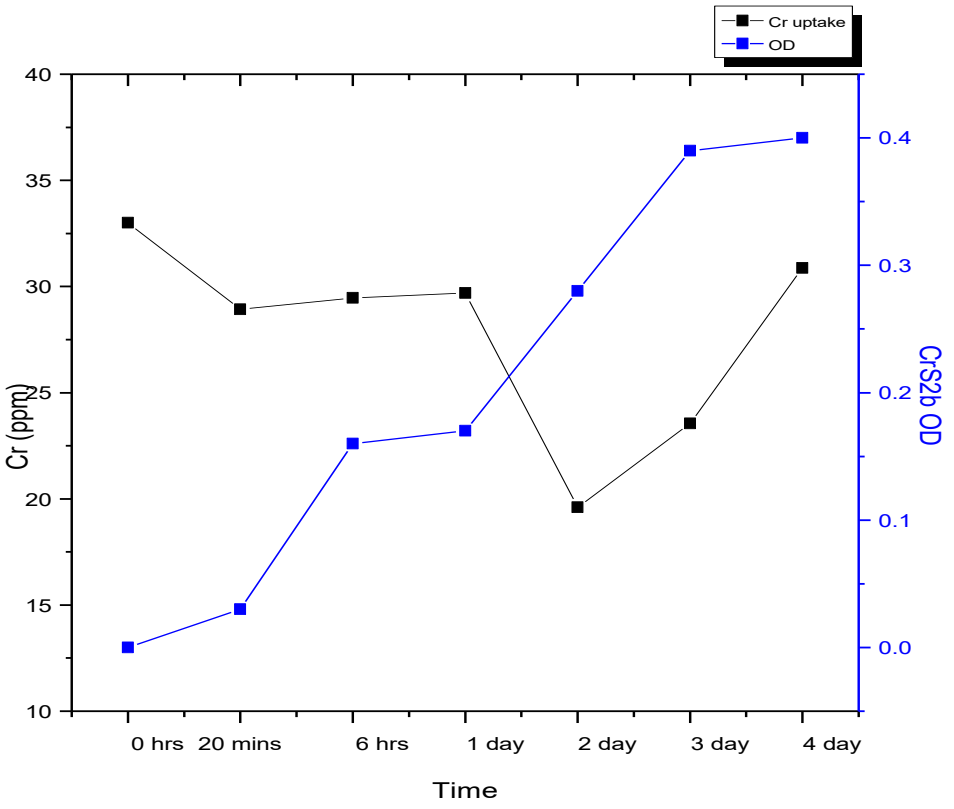
The scale up was performed with the chromium and mercury resistant bacterial isolates. Chromium resistant bacterial isolates were scaled up by growing the bacterial isolates in the increasing concentration of the heavy metal. The scaling up of the bacteria helped in acclimatising the bacteria to a higher concentration of heavy metals. The observation of the scale up analysis of the chromium resistant isolate CrS2b, CrS2c and CrS2d is given in the table 22, 23 and 24.

Bacterial growth and metal uptake were analysed at different time intervals. The heavy metal was quantified using Atomic Absorption Spectroscopy (Perkin Elmer) at Department of Chemistry, University of Mumbai.

Table 22: Growth of CrS2b in presence and absence of different concentration of chromium during scale up.

Bacterial Isolate	Optical density	20 mins	6 hrs	1 day	2 days	3 days	4 days
CrS2b (12 ppm)	+ve control	0	0.03	0.19	0.25	0.15	0.35
	Test	0	0.07	0.2	0.28	0.16	0.35
	Metal conc (ppm)	11.16	10.79	10.97	10.06	0.98	11.07
CrS2b (33 ppm)	+ve control	0.01	0.15	0.2	0.39	0.4	0.41
	Test	0.03	0.16	0.17	0.28	0.39	0.4
	Metal conc (ppm)	28.92	29.47	29.69	19.6	23.55	30.88
CrS2b (65 ppm)	+ve control	0	0.19	0.17	0.17	0.17	0.17
	Test	0.01	0.19	0.19	0.17	0.18	0.17
	Metal conc (ppm)	55.26	61.88	61.94	64.94	64.34	42.9
CrS2b (100 ppm)	+ve control	0.01	0.16	0.18	0.19	0.19	0.19
	Test	0.01	0.14	0.17	0.17	0.16	0.18
	Metal conc (ppm)	77.9	84.225	82.375	83.275	85.225	88.675
CrS2b (200 ppm)	+ve control	0.01	0.11	0.12	0.12	0.13	-
	Test	0	0.06	0.1	0.1	0.11	-
CrS2b (400 ppm)	+ve control	0	0.09	0.14	0.13	0.12	-
	Test	0	0	0.07	0.08	0.09	-


Cr uptake
OD
Cr (ppm)
CrS2b OD
Time
0 hrs
20 mins
6 hrs
1 day
2 day
3 day
4 day


Cr uptake
OD
Cr (ppm)
CrS2b OD
Time
0 hrs
20 mins
6 hrs
1 day
2 day
3 day
4 day

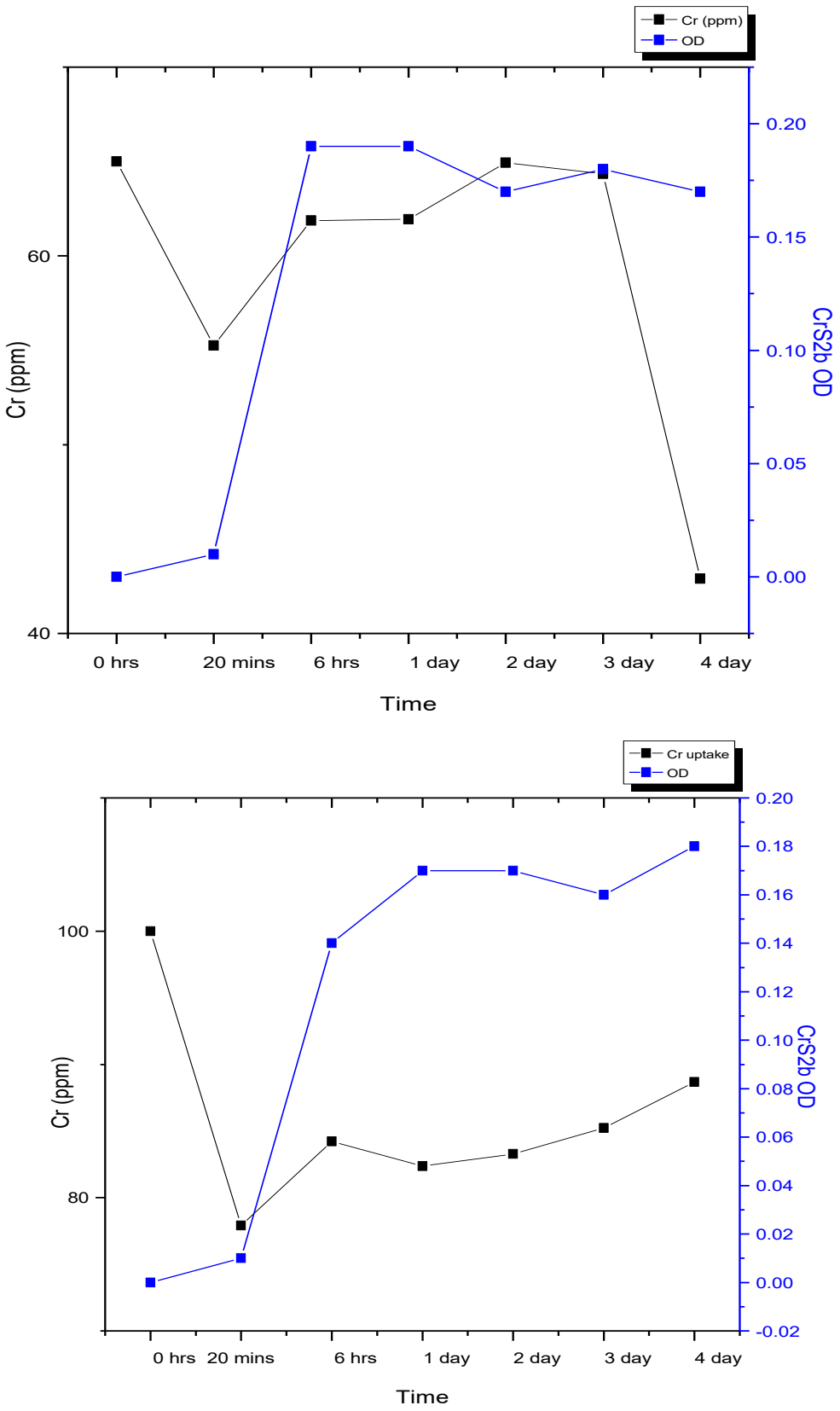
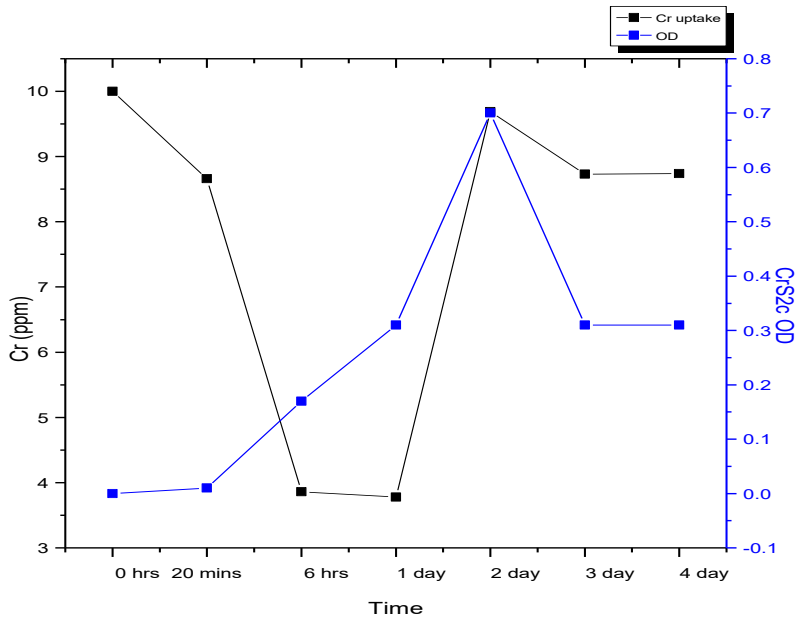
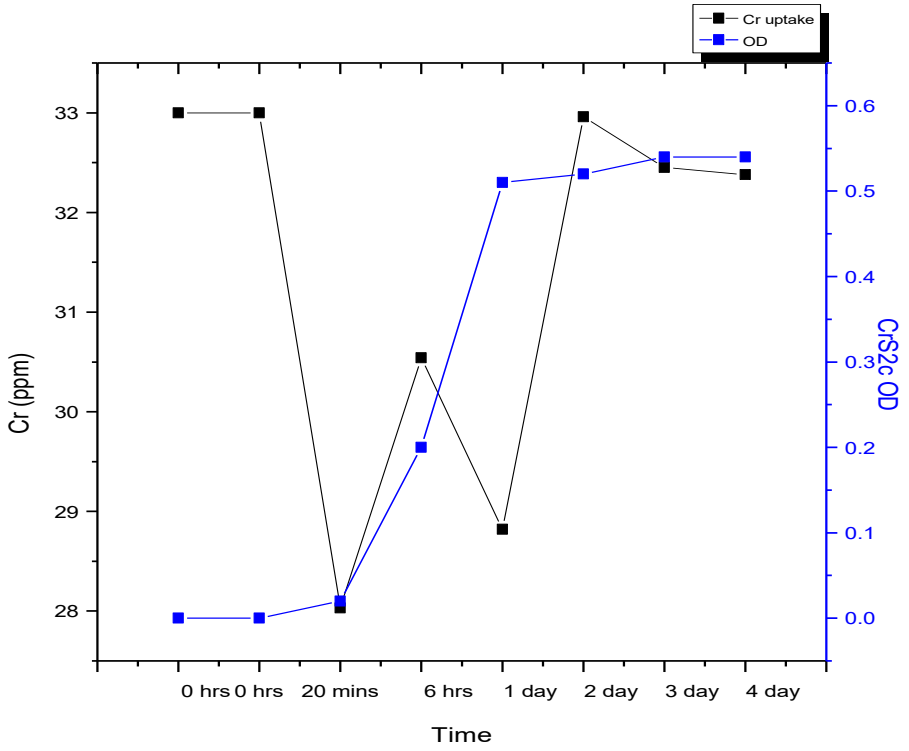


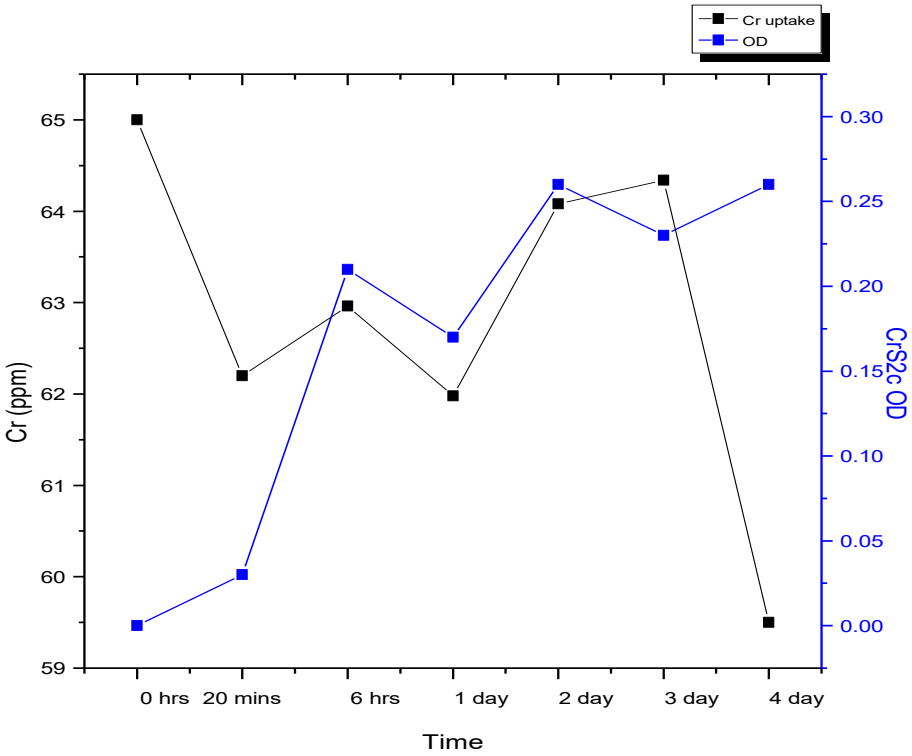
Figure 11: Graphical representation of the growth and chromium removed by CrS2b at different concentration of chromium during scale up.

Table 23: Growth of CrS2c in presence and absence of different concentration of Chromium during scale up.

Bacterial Isolate	Optical density	20 mins	6 hrs	1 day	2 days	3 days	4 days
CrS2c (12 ppm)	+ve control	0	0.09	0.2	0.26	0.16	0.39
	Test	0	0.09	0.2	0.27	0.16	0.16
	Metal conc (ppm)	8.66	3.86	3.78	9.69	8.73	8.74
CrS2c (33 ppm)	+ve control	0.01	0.11	0.43	0.52	0.53	0.54
	Test	0.02	0.2	0.51	0.52	0.54	0.54
	Metal conc (ppm)	28.03	30.54	28.82	32.96	32.45	32.38
CrS2c (65 ppm)	+ve control	0	0.19	0.16	0.17	0.17	0.17
	Test	0.03	0.21	0.17	0.26	0.23	0.26
	Metal conc (ppm)	62.2	62.96	61.98	64.08	64.34	59.5
CrS2c (100 ppm)	+ve control	0	0.16	0.18	0.18	0.15	0.17
	Test	0	0.16	0.17	0.16	0.14	0.17
	Metal conc (ppm)	82.025	87.375	82.1	92.1	98.175	95.425
CrS2c (200 ppm)	+ve control	0	0.09	0.11	0.11	0.11	-
	Test	0	0.08	0.11	0.1	0.1	-
CrS2c (300 ppm)	+ve control	0.011	0.853	1.28	1.233	1.215	-
	Test	0.02	0.097	0.588	0.516	1.071	-




Cr uptake
OD
Cr (ppm)
CrS2c OD
Time
0 hrs
0 hrs
20 mins
6 hrs
1 day
2 day
3 day
4 day


Cr uptake
OD
Cr (ppm)
CrS2c OD
Time
0 hrs
20 mins
6 hrs
1 day
2 day
3 day
4 day

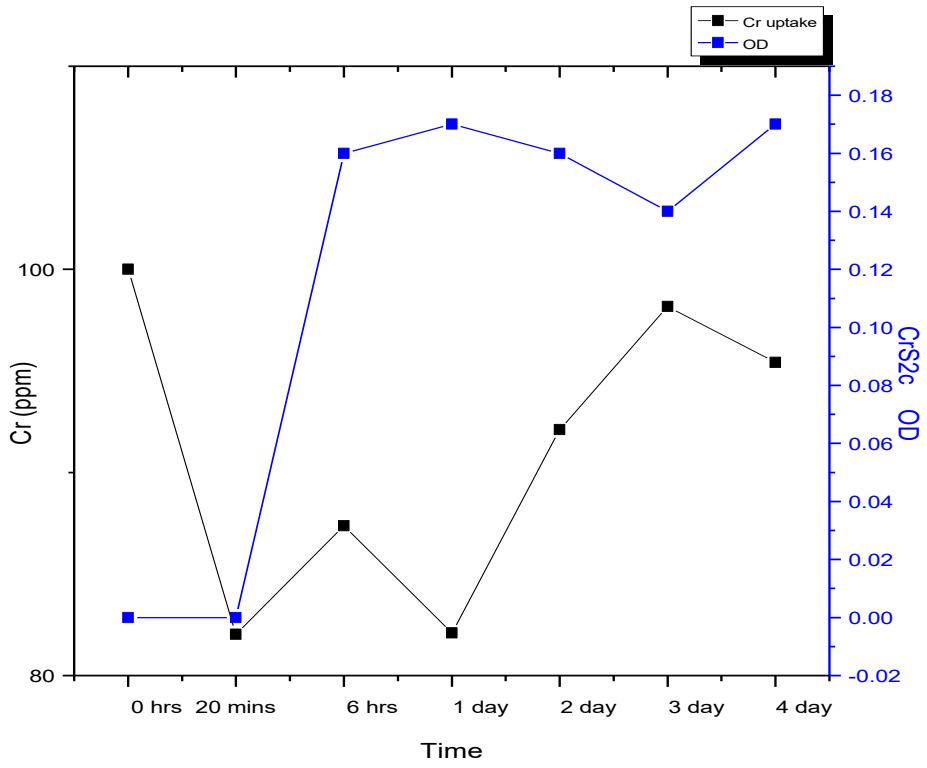
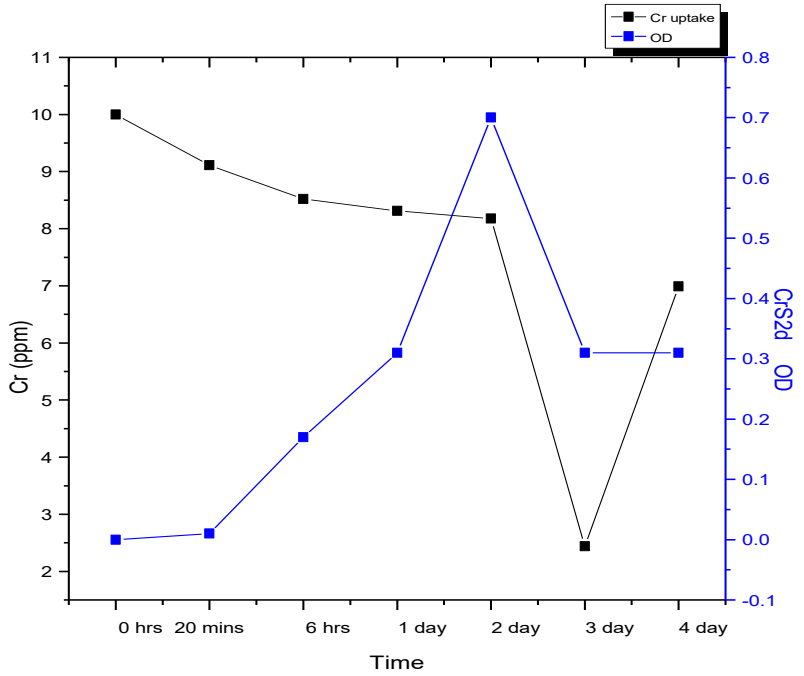


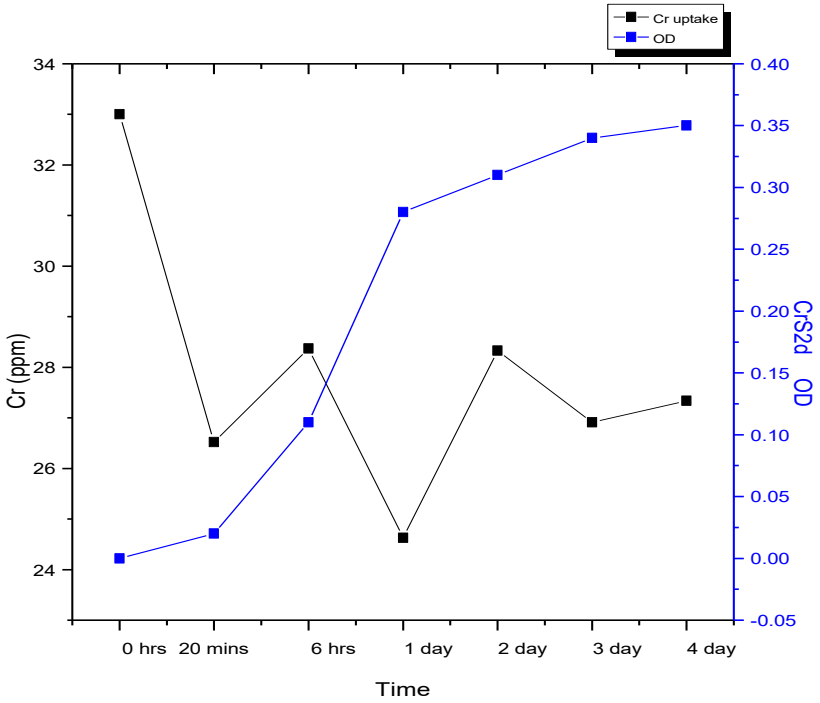
Figure 12: Graphical representation of the growth and chromium removed by CrS2c at different concentration of chromium during scale up.

Table 24: Growth of CrS2d in presence and absence of different concentration of chromium during scale up.

Bacterial Isolate	Optical density	20 mins	6 hrs	1 day	2 days	3 days	4 days
CrS2d (12 ppm)	+ve control	0.01	0.2	0.35	0.69	0.3	0.3
	Test	0.01	0.17	0.31	0.7	0.31	0.31
	Metal conc (ppm)	9.11	8.52	8.31	8.18	2.44	6.99
CrS2d (33 ppm)	+ve control	0.01	0.17	0.36	0.44	0.47	0.46
	Test	0.02	0.11	0.28	0.31	0.34	0.35
	Metal conc (ppm)	26.52	28.37	24.63	28.33	26.91	27.34
CrS2d (50 ppm)	+ve control	0	0.2	0.18	0.18	0.18	0.17
	Test	0.01	0.21	0.17	0.17	0.17	0.16
	Metal conc (ppm)	45	44.2	42.84	43.74	44.46	43.08
CrS2d (100 ppm)	+ve control	0.02	0.16	0.19	0.19	0.19	0.18
	Test	0.02	0.15	0.18	0.16	0.16	0.18

	Metal conc (ppm)	83.225	84.75	85.625	90.9	90.825	92.45
CrS2d (200 ppm)	+ve control	0	0.1	0.13	0.13	0.13	-
	Test	0.01	0.04	0.11	0.11	0.11	-





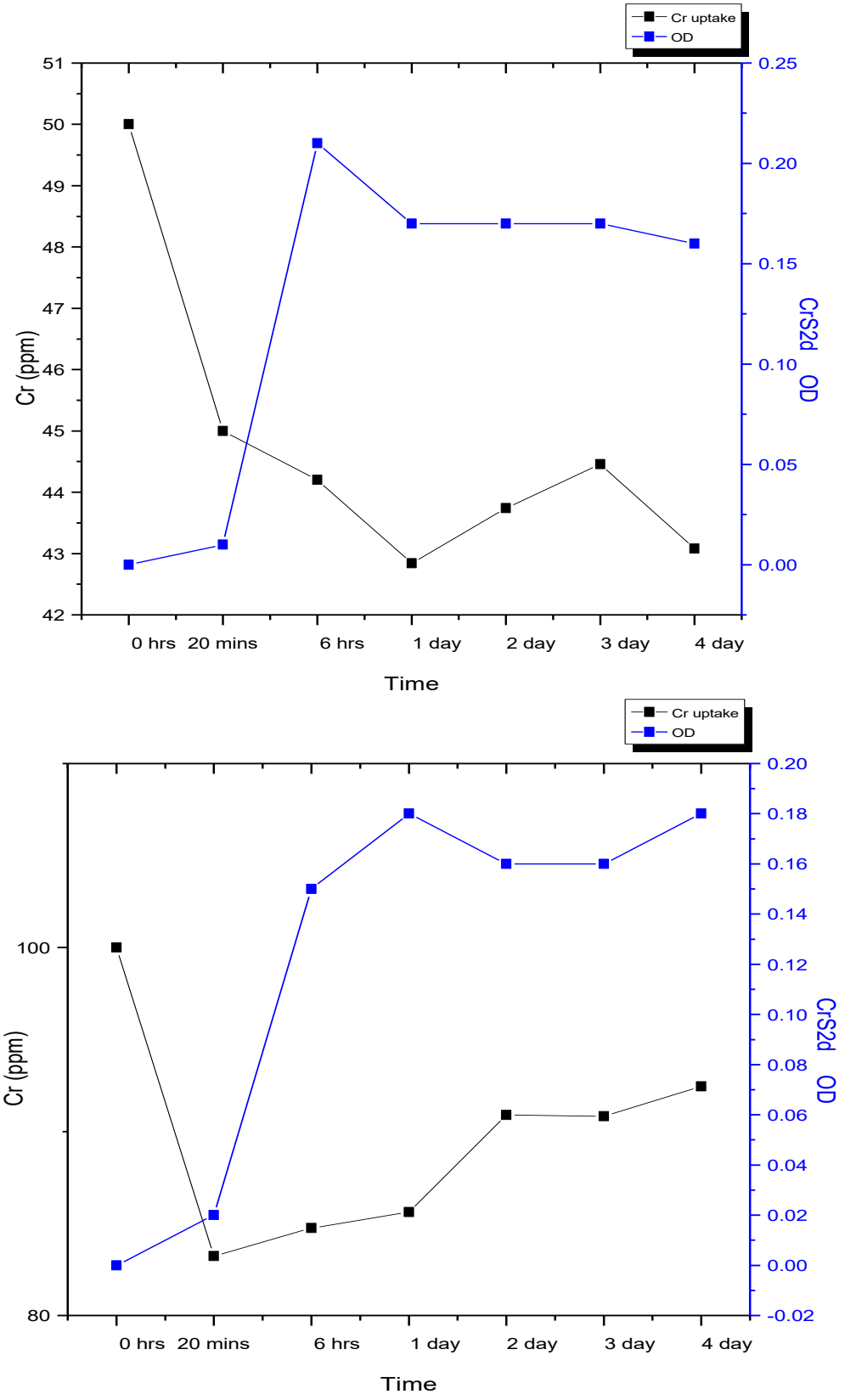


Figure 13: Graphical representation of the growth and chromium removed by CrS2d at different concentration of chromium during scale up.

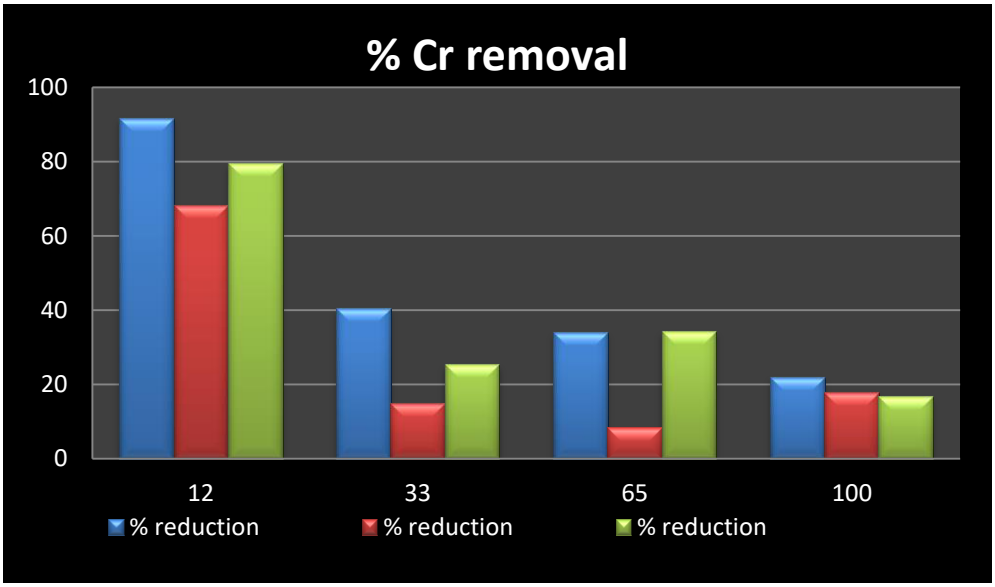


Figure 14: Percentage reduction in the chromium by isolated bacteria (blue- CrS2b, red- CrS2c and green- CrS2d).

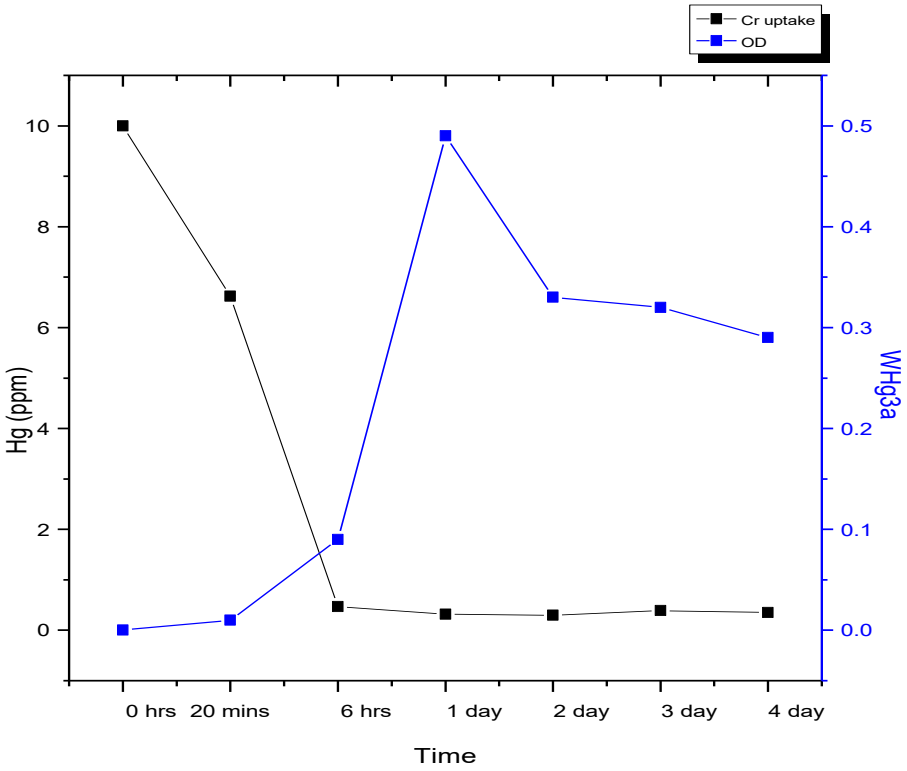
9.2 Scale up of Mercury resistant bacteria isolated from Mithi River

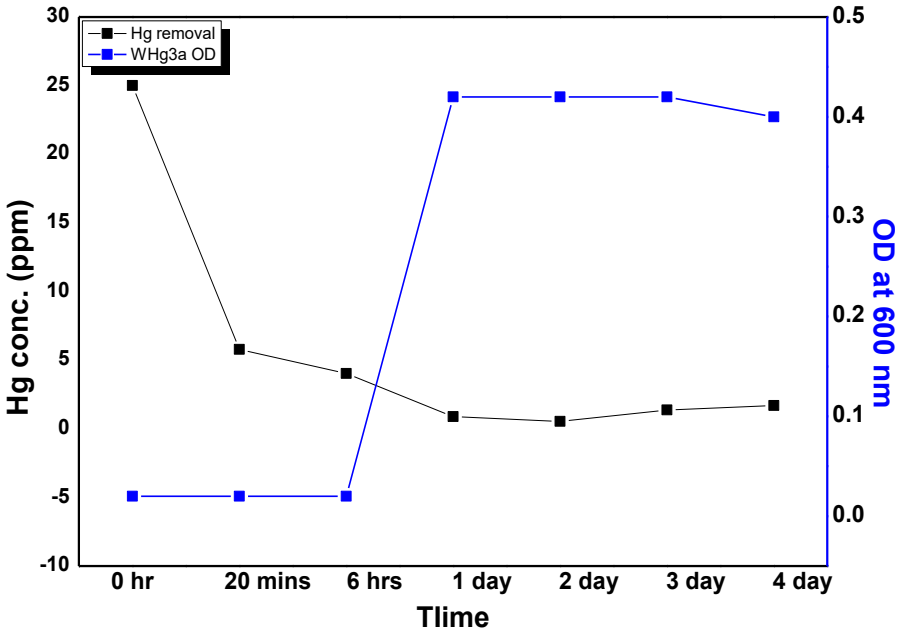
Bacteria isolated from Mithi River against mercury were scaled up. Bacteria were serially grown in presence of increasing concentration of mercury. The observation of the scale up analysis of the mercury resistant isolate WHg3a, WHg3b and HgKN3 is given in the table 25, 26 and 27. The heavy metal was quantified using cold vapour Atomic Absorption Spectroscopy at Analytical Chemistry Division, BARC.

Table 25: Growth of WHg3a in presence and absence of different concentration of mercury during scale up.

Bacterial Isolate	Optical density	20 mins	6 hrs	1 day	2 days	3 days	4 days
WHg3a (10 ppm)	+ve control	0.02	0.21	0.49	0.3	0.3	0.28
	Test	0.01	0.09	0.49	0.33	0.32	0.29
	Metal conc (ppm)	6.62	0.47	0.32	0.3	0.39	0.35
WHg3a (33 ppm)	+ve control	0.03	0.18	0.37	0.42	0.45	0.48
	Test	0.02	0.02	0.42	0.42	0.42	0.4
	Metal conc (ppm)	5.79	4.02	0.88	0.54	1.36	1.7
WHg3a (65 ppm)	+ve control	0	0.2	0.5	0.48	0.47	0.46
	Test	0	0	0	0.52	0.5	0.49

	Metal conc (ppm)	31.03	33.71	29.1	3.07	4.39	5.39
WHg3a (100 ppm)	+ve control	0	0.21	0.4	0.45	0.48	0.31
	Test	0	0	0.01	0.2	0.29	0.32
	Metal conc (ppm)	35.44	33.97	32.55	27.35	21.34	17.64





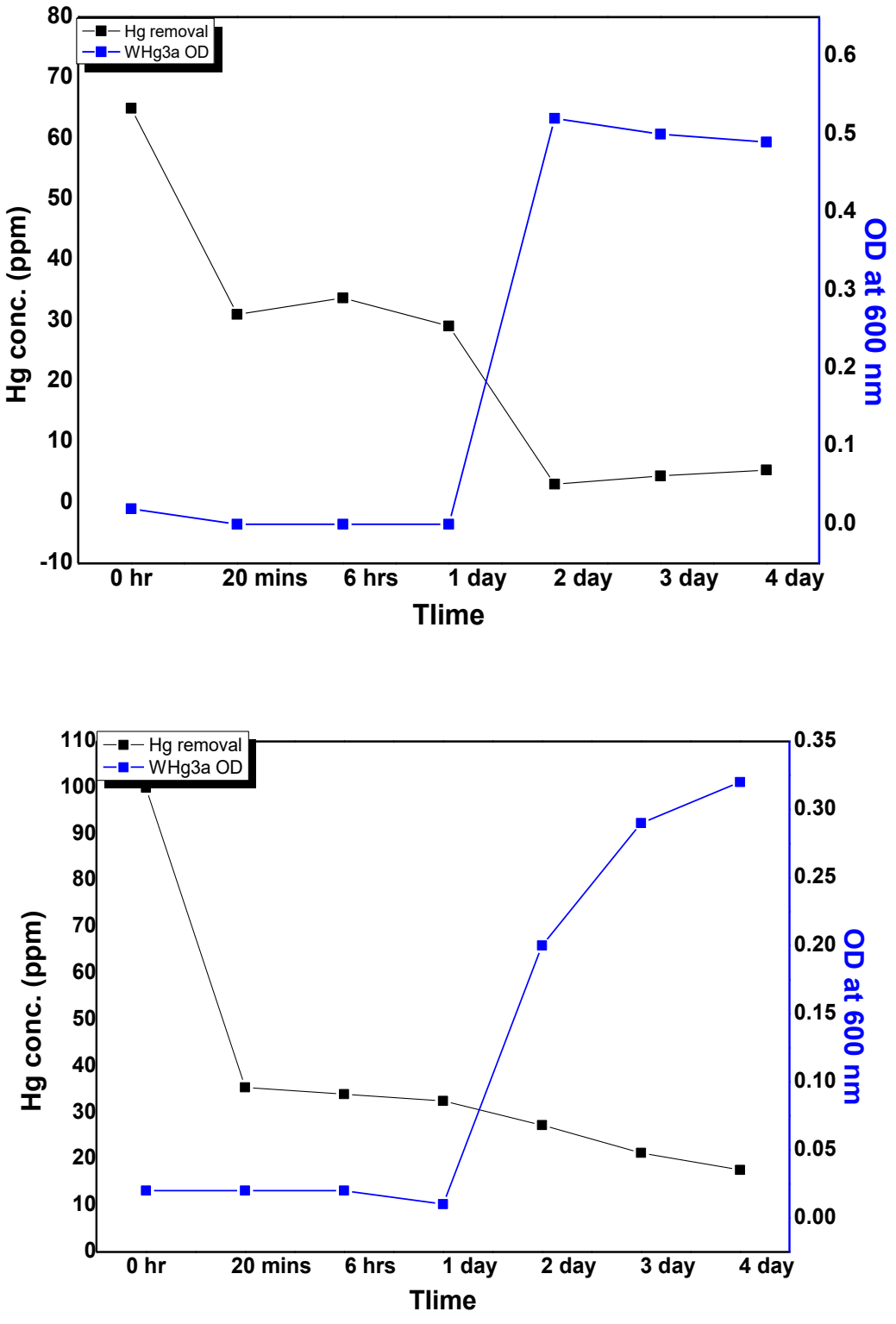
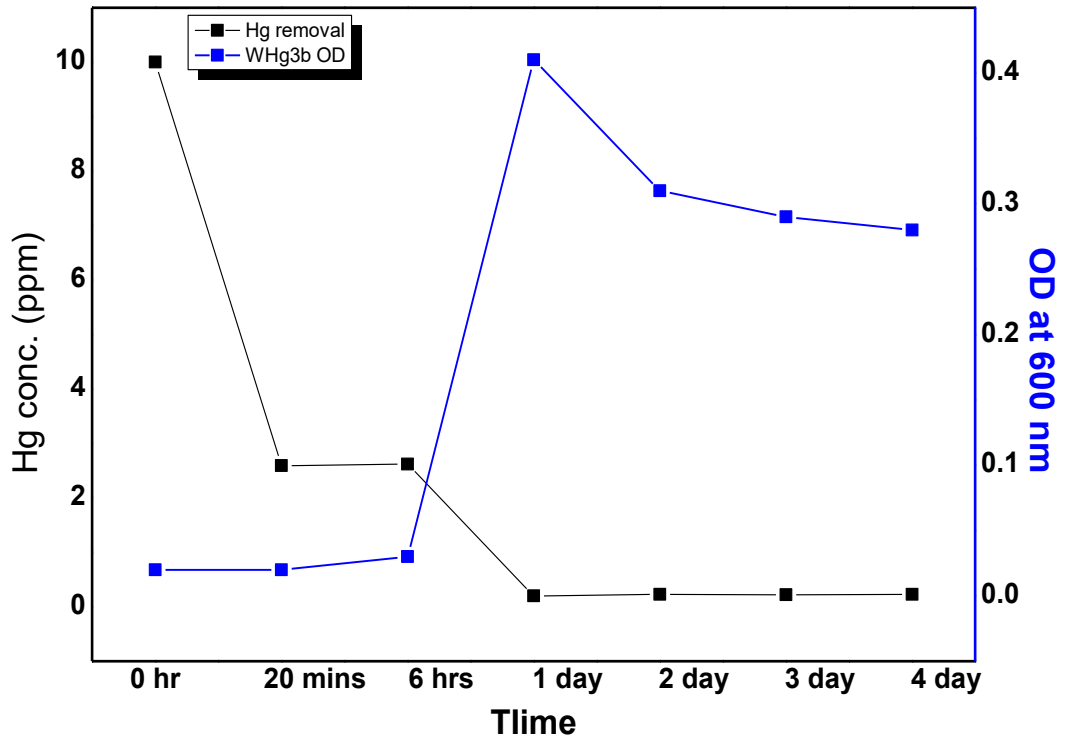
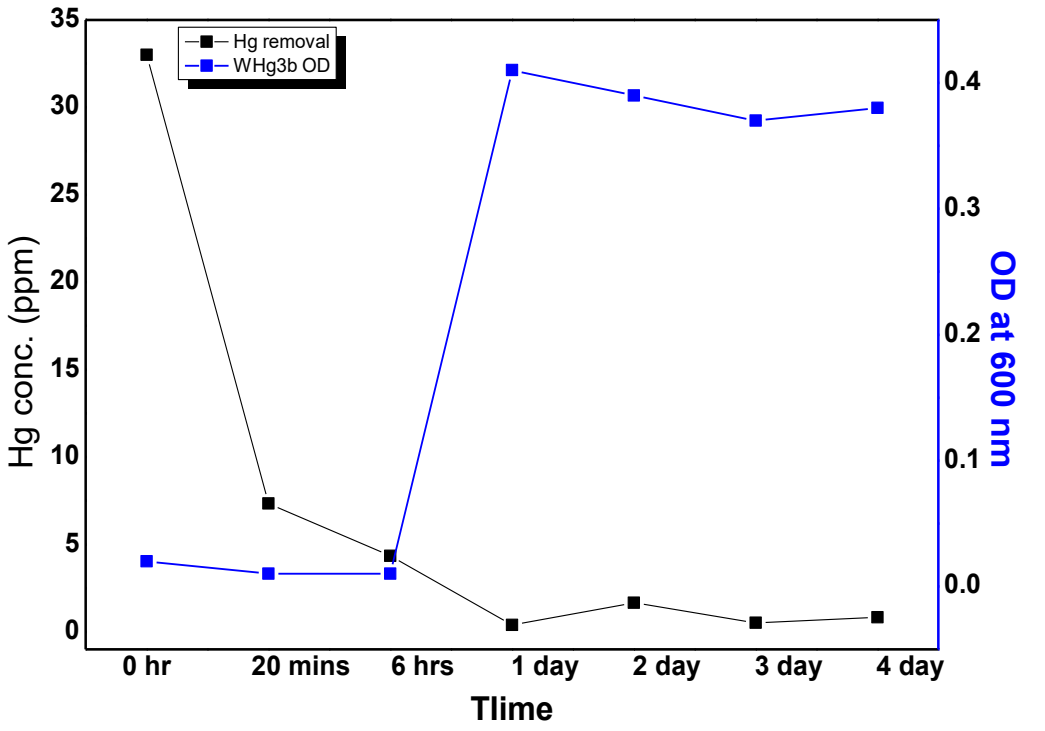


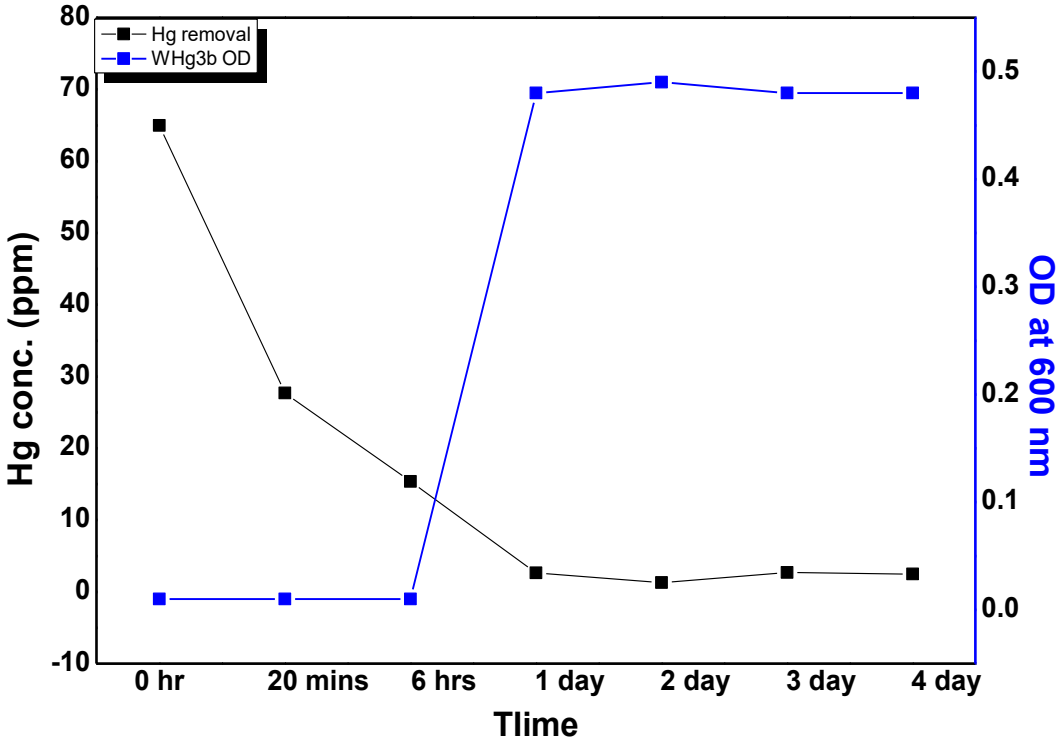
Figure 15: Graphical representation of the growth and chromium removed by WHg3a at different concentration of mercury during scale up.

Table 26: Growth of WHg3b in presence and absence of different concentration of mercury during scale up.

Bacterial Isolate	Optical density	20 mins	6 hrs	1 day	2 days	3 days	4 days
WHg3b (10 ppm)	+ve control	0	0.23	0.48	0.33	0.31	0.29
	Test	0	0.03	0.41	0.31	0.29	0.28
	Metal conc (ppm)	2.56	2.62	0.2	0.23	0.22	0.23
WHg3b (33 ppm)	+ve control	0.01	0.23	0.29	0.49	0.51	0.51
	Test	0	0.01	0.41	0.39	0.37	0.38
	Metal conc (ppm)	7.33	4.35	0.4	1.67	0.520	0.83
WHg3b (65 ppm)	+ve control	0	0.25	0.49	0.46	0.48	0.46
	Test	0.01	0.01	0.01	0.48	0.49	0.48
	Metal conc (ppm)	21.67	15.39	2.61	1.27	2.68	2.5
WHg3b (100 ppm)	+ve control	0.02	0.19	0.45	0.48	0,5	0.32
	Test	0.01	0.01	0.01	0.18	0.23	0.37
	Metal conc (ppm)	47.6	43.1	29.42	20.36	18.67	13.25




Hg removal
WHg3b OD
Hg conc. (ppm)
OD at 600 nm
0 hr
20 mins
6 hrs
1 day
2 day
3 day
4 day
Tlime


Hg removal
WHg3b OD
Hg conc. (ppm)
OD at 600 nm
0 hr
20 mins
6 hrs
1 day
2 day
3 day
4 day
Tlime

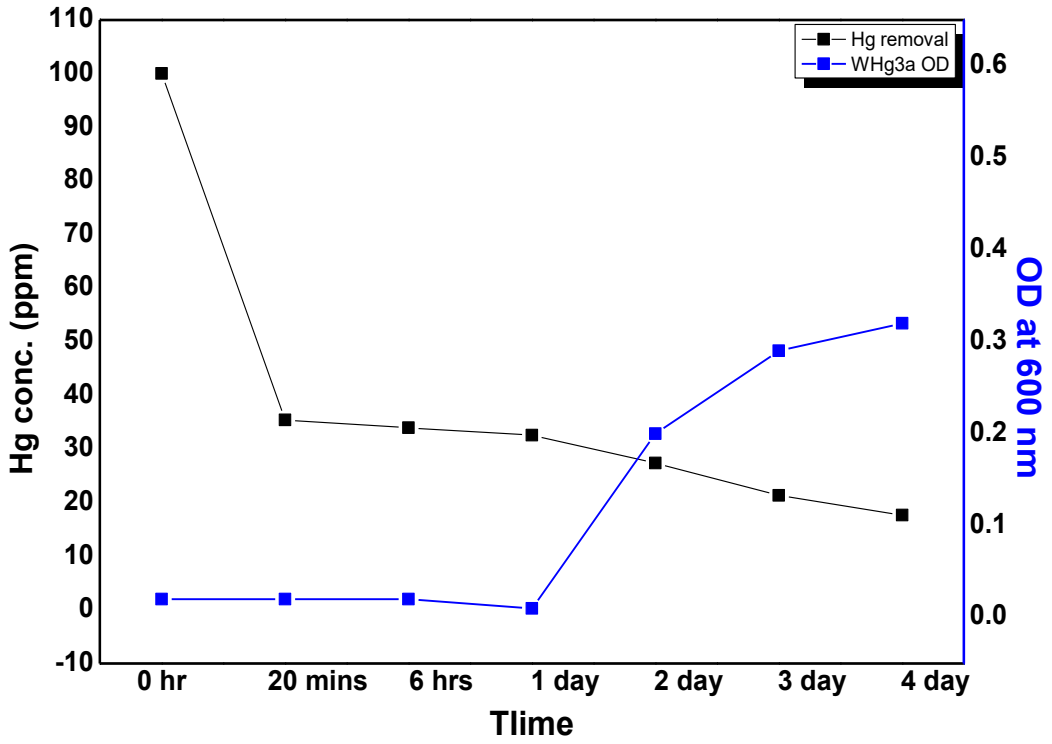
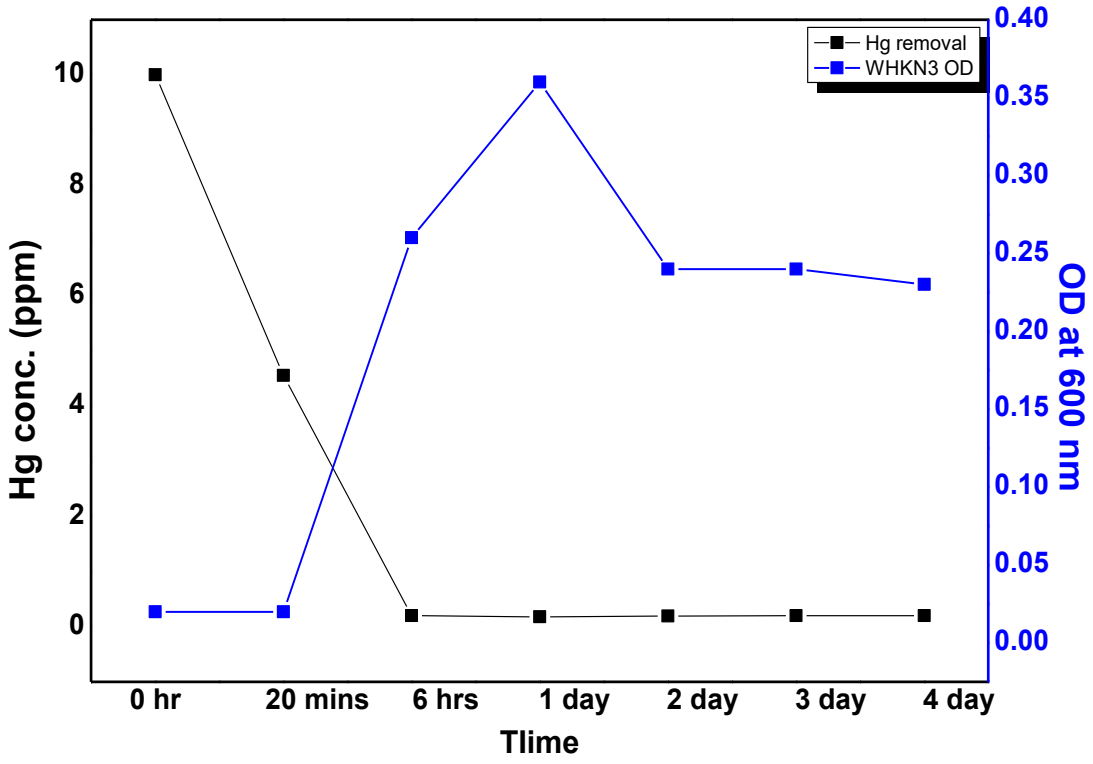
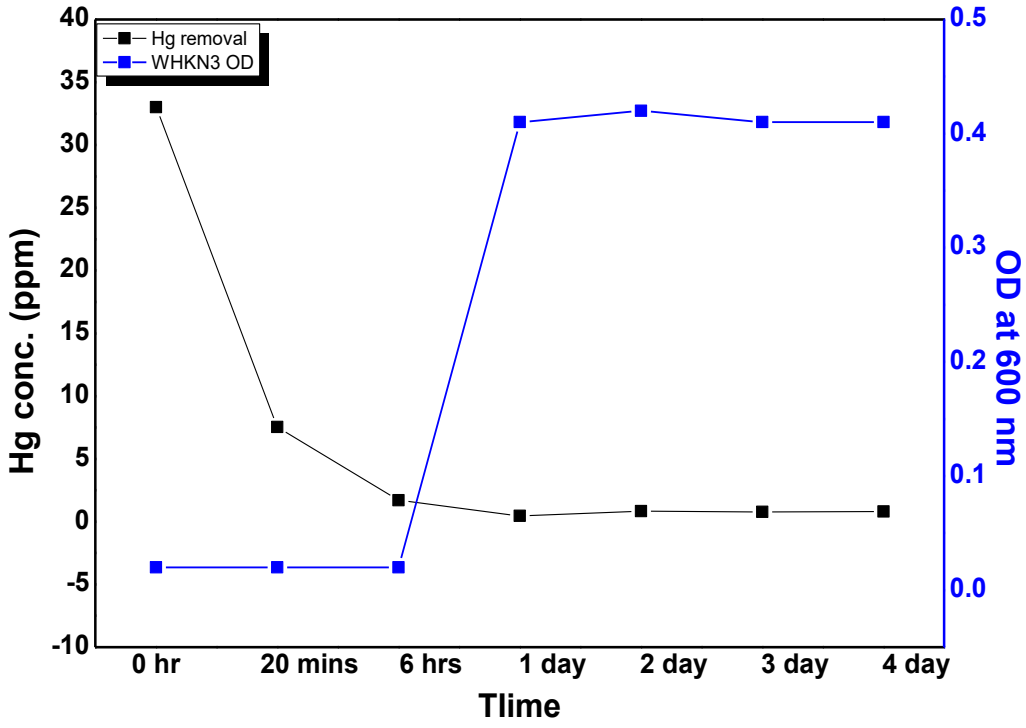


Figure 16: Graphical representation of the growth and chromium removed by WHg3b at different concentration of mercury during scale up.

Table 27: Growth of HgKN3 in presence and absence of different concentration of mercury during scale up.

Bacterial Isolate	Optical density	20 mins	6 hrs	1 day	2 days	3 days	4 days
HgKN3 (10 ppm)	+ve control	0	0.27	0.37	0.23	0.23	0.22
	Test	0	0.26	0.36	0.24	0.24	0.23
	Metal conc (ppm)	4.55	0.2	0.18	0.19	0.2	0.2
HgKN3 (33 ppm)	+ve control	0	0.18	0.24	0.41	0.37	0.48
	Test	0	0	0.41	0.42	0.41	0.41
	Metal conc (ppm)	7.54	1.7	0.46	0.88	0.77	0.79
HgKN3 (65 ppm)	+ve control	0	0.32	0.35	0.49	0.48	0.48
	Test	0	0	0.34	0.41	0.42	0.42
	Metal conc (ppm)	29	28.97	9.91	5.23	7.22	6.14
HgKN3 (100 ppm)	+ve control	0.01	0.28	0.35	0.37	0.38	0.26
	Test	0.02	0.01	0.39	0.45	0.5	0.3
	Metal conc (ppm)	43.62	31.36	4.93	7.95	4.19	4.06


Hg removal
WHKN3 OD
Hg conc. (ppm)
OD at 600 nm
Tlime
0 hr
20 mins
6 hrs
1 day
2 day
3 day
4 day
0
2
4
6
8
10
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40


Hg removal
WHKN3 OD
Hg conc. (ppm)
OD at 600 nm
Tlime
0 hr
20 mins
6 hrs
1 day
2 day
3 day
4 day
-10
-5
0
5
10
15
20
25
30
35
40
0.0
0.1
0.2
0.3
0.4
0.5

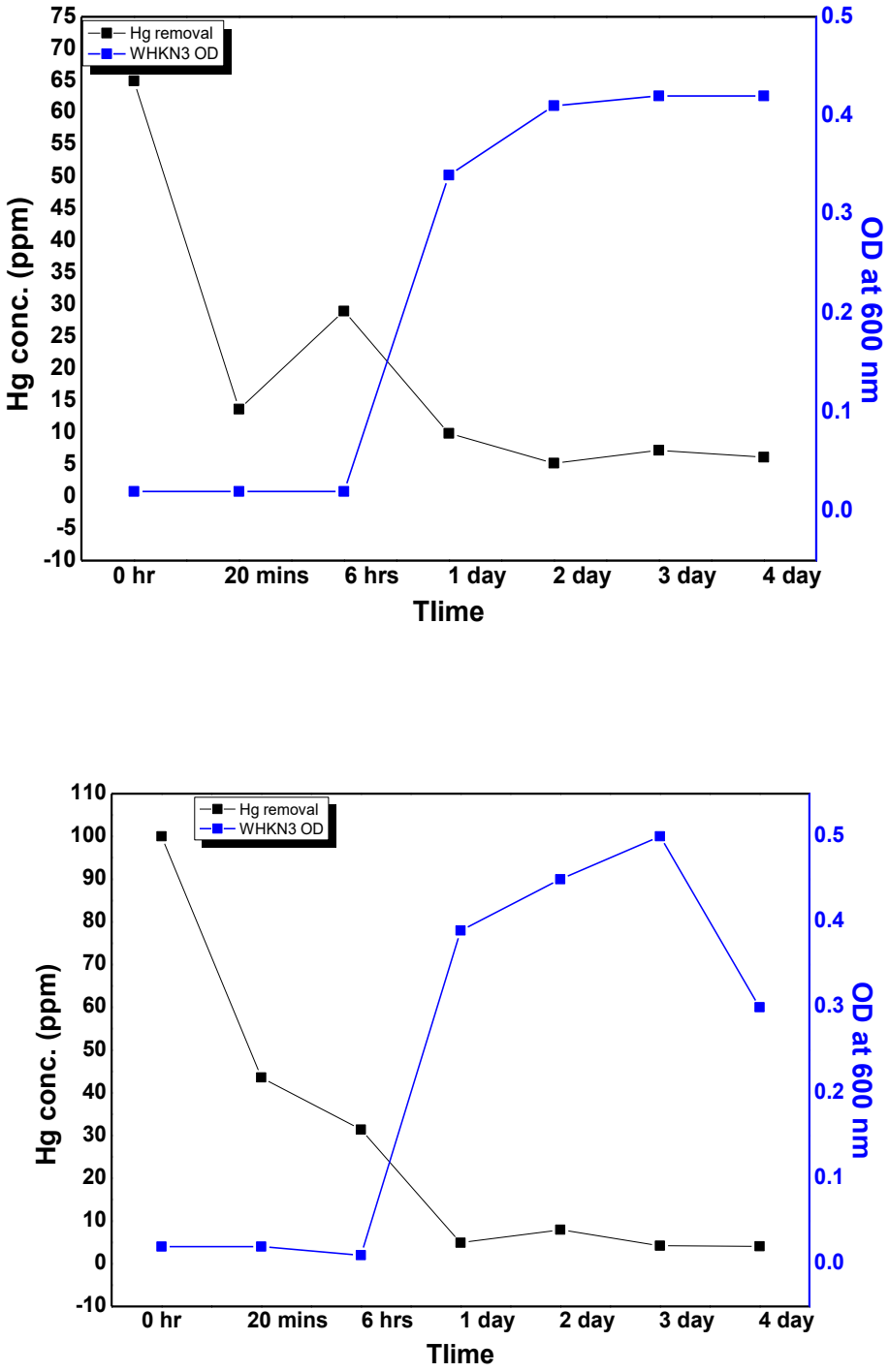


Figure 17: Graphical representation of the growth and chromium removed by WHKN3 at different concentration of mercury during scale up.

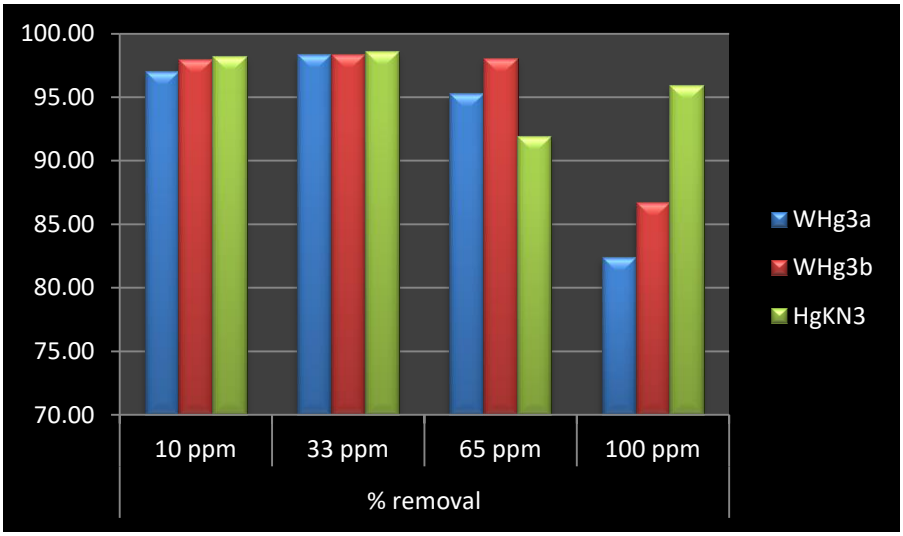


Figure 18: Percentage reduction in the mercury by isolated bacteria.

10. Development of Heavy Metal Resistance Consortium

10.1 Bacterial Consortium against Chromium

Bacterial consortium was developed using isolated bacteria against chromium. Bacterial isolates scaled up for chromium was grown in different combination to determine their synergistic effect. Out of seven isolated bacteria, three potential bacteria were grown together. The three bacteria i.e. CrS2b, CrS2c and CrS2d could grow more efficiently together (Table 28). The CrS2b, CrS2c and CrS2d bacteria isolates can work together by supporting the growth of each other. The chromium removal by the chromium resistant isolates individually (Table 29) as well as by the consortium was performed (Table 30).

Table 28: Development of consortium using CrS2b, CrS2c and CrS2d grown in different combination in presence of 100 ppm of chromium.

Growth curve	Organism (100 ppm of Cr)			
Time (min)	Crs2b+Crs2c	Crs2c+Crs2d	Crs2b+Crs2d	Crs2b+Crs2c+Crs2d
0 hr	0.089	0.069	0.084	0.086
30 min	0.133	0.128	0.124	0.158
60 min	0.217	0.155	0.188	0.2
90 min	0.299	0.233	0.245	0.316
120 min	0.423	0.348	0.399	0.456
150 min	0.56	0.498	0.534	0.626
180 min	0.585	0.579	0.601	0.743
210 min	0.749	0.691	0.707	0.787

240 min	0.813	0.766	0.764	0.856
270 min	0.859	0.825	0.823	0.889
300 min	0.867	0.84	0.843	0.897
330 min	0.883	0.843	0.844	0.905
360 min	0.893	0.849	0.847	0.91
390 min	0.898	0.843	0.848	0.911
24 hrs	0.715	0.712	0.778	0.84

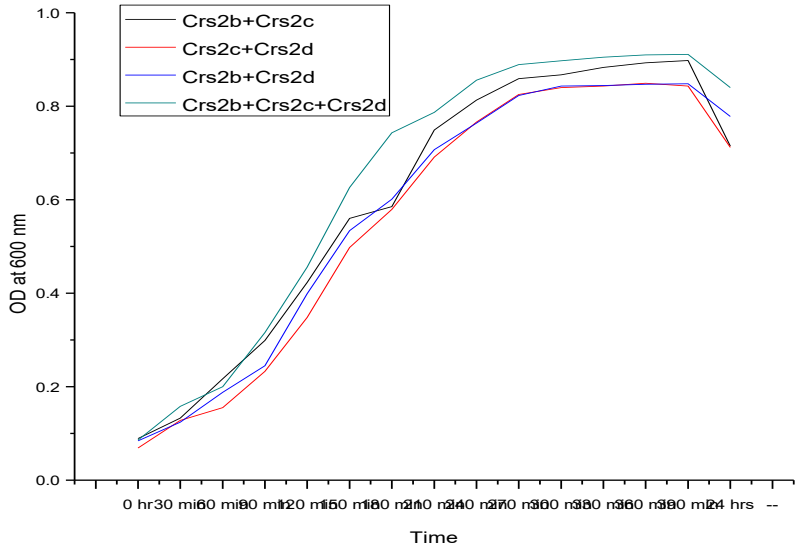


Figure 19: Growth curve of the bacteria grown in different combination.

Table 29: Chromium removal by the Chromium resistant bacteria individually.

Sr. No.	Time	CrS2b		CrS2c		CrS2d	
		Cr removal	% removal	Cr removal	% removal	Cr removal	% removal
1	0 hrs	43.525	0	43.45	0	43.225	0
2	5 mins	39.375	9.53475	44.15	-1.611	32.45	24.9277
3	6 hrs	38.75	10.97071	26.05	40.046	35.16	18.65818
4	1 day	35.725	17.92074	32.5	25.201	36.625	15.26894
5	2 days	35.1	19.35669	33.78	22.255	34.675	19.78022
6	3 days	15.465	64.4687	29.35	32.451	31.8	26.43146

Table 30: Chromium removal by the chromium resistant bacterial consortium.

Sr. No.	Time	Cr removal by Consortium	% removal
1	Cr Sol	44.475	0
2	5 mins	44.325	0.3372681
3	6 hrs	40.65	8.6003373
4	1 day	26.025	41.48398
5	2 days	14.8725	66.559865
6	3 days	0	100

10.2 Bacterial consortium against Mercury

Bacterial consortium against mercury was developed by growing the potential mercury resistant isolated bacteria in different combination to check their synergistic action. Growth of three mercury resistant bacteria in different combination is determined and found as given in the table 31.

Table 31: Development of consortium using WHg3a, HgKN3, and WHg3b grown in different combination in presence of 50 ppm of mercury.

Time (min)	culture(O.D at 600nm) 50 ppm			
	WHg3a+HgKN3	WHg3a+WHg3b	WHg3b+HgKN3	WHg3a+HgKN3+WHg3b
0	0.043	0.047	0.044	0.055
30	0.06	0.058	0.056	0.058
60	0.069	0.055	0.065	0.067
90	0.078	0.052	0.077	0.078
120	0.109	0.048	0.119	0.1
150	0.173	0.049	0.184	0.155
180	0.367	0.052	0.378	0.308
210	0.564	0.041	0.586	0.557
240	0.676	0.045	0.68	0.69
270	0.756	0.046	0.755	0.802
300	0.841	0.049	0.835	0.855
330	0.906	0.051	0.893	0.93

360	0.979	0.054	0.972	0.983
24hrs	1.359	1.487	1.097	1.379

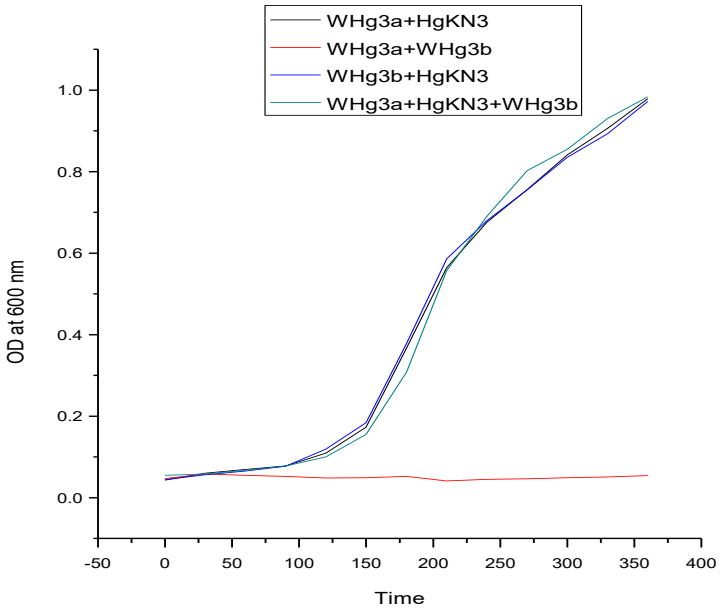


Figure 20: Growth curve of the mercury resistant bacteria grown in different combination.

Bacterial consortium is combination of two or more bacteria which work synergistically to perform any function. The bacterial consortium was prepared to determine their effectiveness for heavy metal remediation. Four set of the bacteria was grown in the different combination in presence of mercury. The mercury removal was determined at different time interval. The mercury was quantified using Cold Vapor Atomic Absorption Spectroscopy (AAS) ContrAA 300 Analytik jena at ACD, BARC, Mumbai.

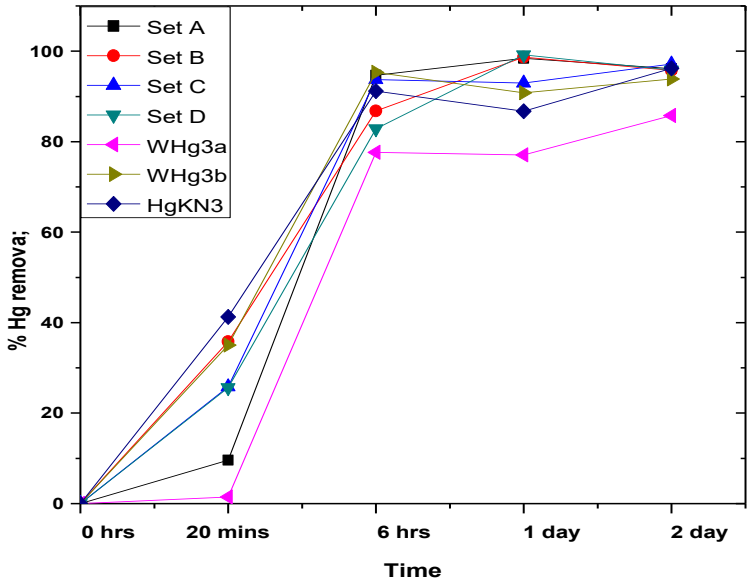


Figure 21: Comparison of the Hg removal by individual and different consortium of the Hg resistant bacteria.

The mercury removal efficiency of the consortium was compared with the mercury removal efficiency of the individual bacterium (table 32) by growing the bacteria under similar conditions as for the consortium (table 33). It was observed that the bacteria in the consortium could remove higher amount of the mercury as compared to the individual bacterium. The bacteria work together to nullify toxic effect of the mercury on their growth. Conversion of mercury to nontoxic form or compartmentalization of mercury does not negatively impact the bacterial growth.

Table 32: Removal of the mercury by the mercury resistant bacteria individually different time interval.

Sr. No.	Time	% removal of the Mercury		
		WHg3a	WHg3b	HgKN3
1	0 hr	0.000	0.000	0.000
2	20 mins	9.596	35.779	25.806
3	6 hrs	94.649	86.769	93.715
4	1 day	98.423	98.684	92.941
5	2 days	96.117	95.742	97.139

Table 33: Percentage removal of the mercury by various consortium of the bacteria a different time interval.

Sr. No.	Time	% removal of mercury			
		Set A (WHg3a+WHg3b)	Set B (WHg3a+HgKN3)	Set C (WHg3b+ HgKN3)	Set D (WHg3a+ WHg3b+ HgKN3)
1	0 hr	0.000	0.000	0.000	0 0.000
2	20 mins	9.596	35.779	25.806	25.614
3	6 hrs	94.649	86.769	93.715	82.854
4	1 day	98.423	98.684	92.941	99.167
5	2 days	96.117	95.742	97.139	95.822

All combination of the bacteria could remove high amount of the mercury but set D comprising of WHg3a, WHg3b and HgKN3 could remove 99.17% mercury in one day.

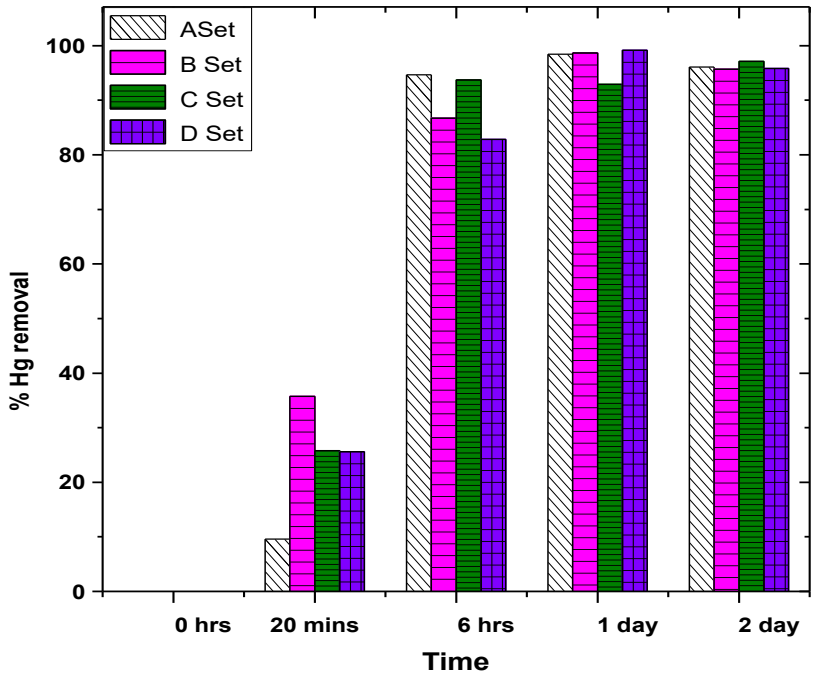


Figure 22: Graphical presentation of the Hg removal by bacterial consortium. Hg removed by different combinations of Hg resistant bacteria. Set A (WHg3a+WHg3b), Set B (WHg3a+HgKN3), Set C (WHg3b+HgKN3) and Set D (WHg3a+WHg3b+HgKN3).

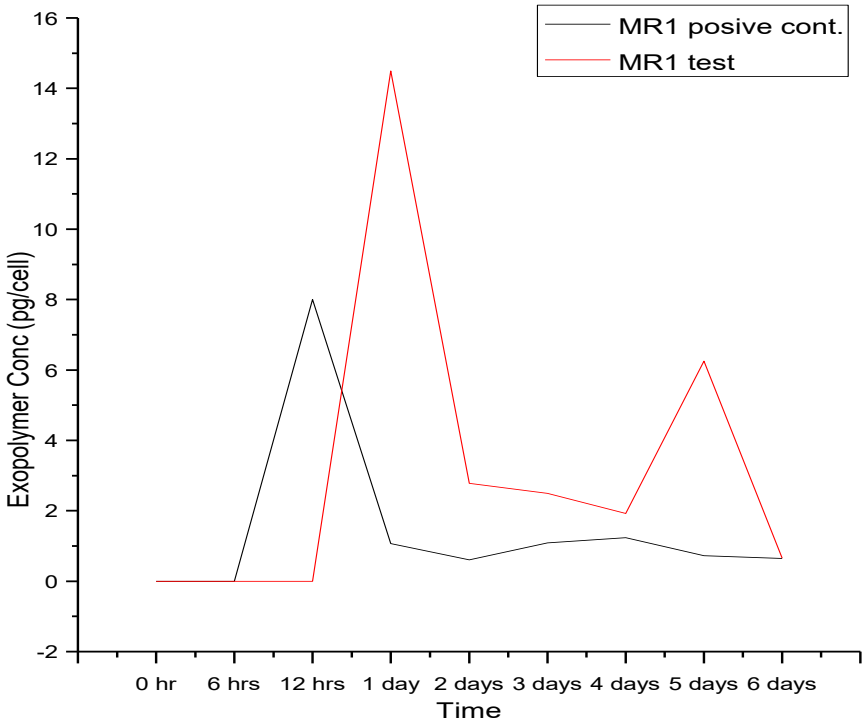
Bacterium could individually remove only 95% of mercury while bacteria in the consortium could remove 99% of mercury. The consortium was found to work effectively together for the bioremediation of the mercury.

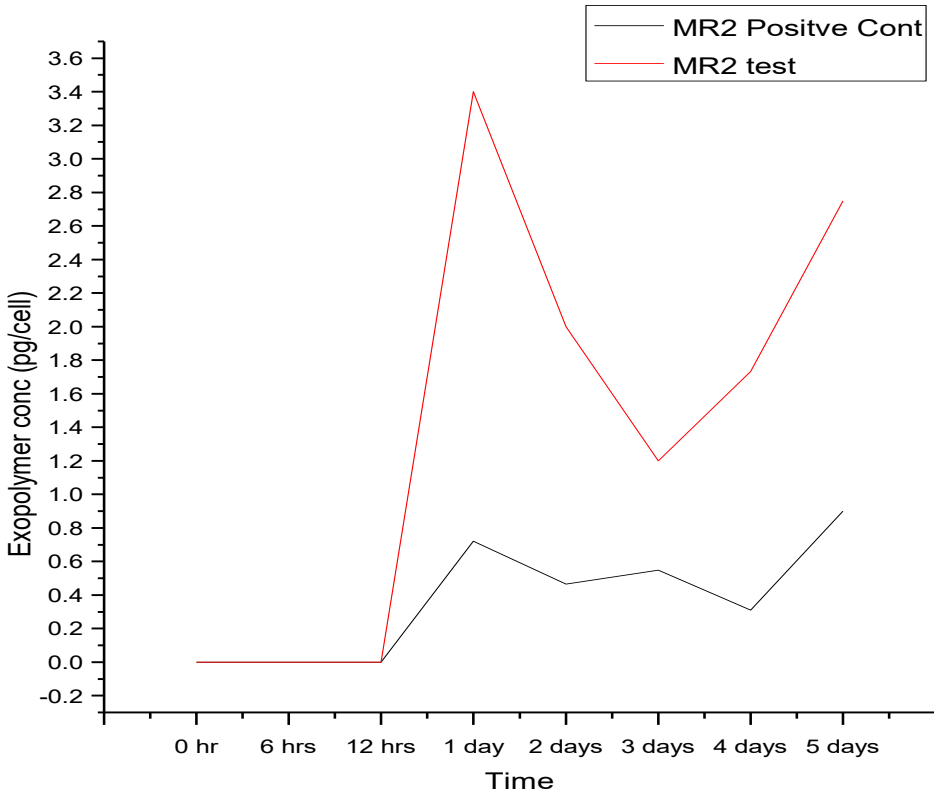
11. Bacterial cell surface study

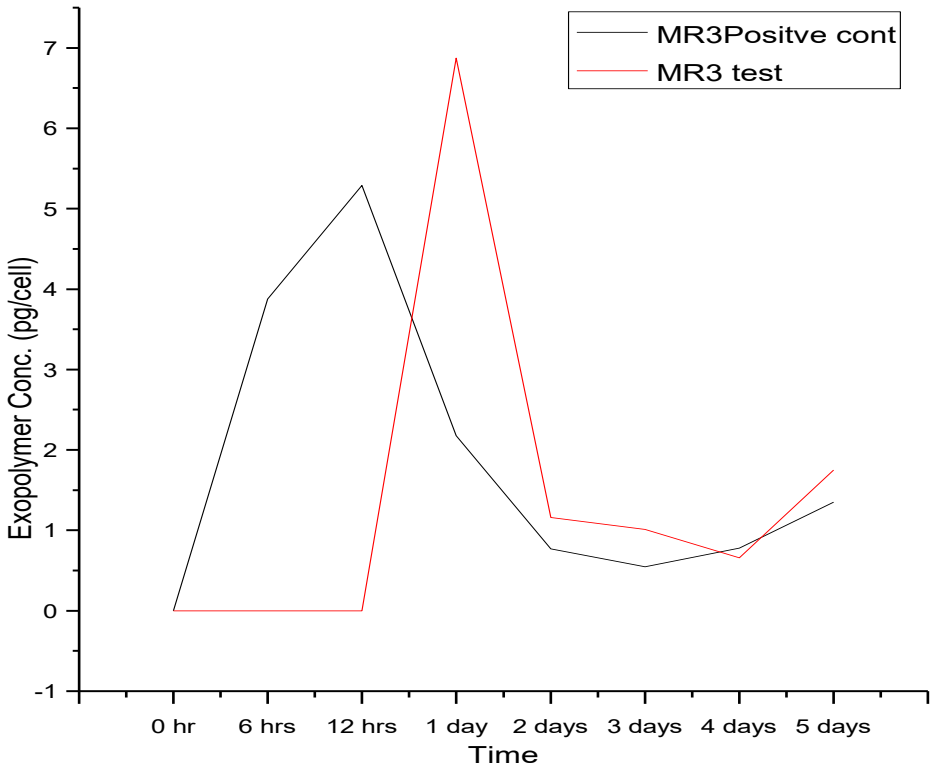
Bacterial cell surface study was performed to understand the initial interaction of heavy metals with bacteria. The bacterial cell surface is known to play very important role in the environmental stress tolerance. The fate of interaction between bacteria and heavy metal depend majorly on the cell surface properties of the bacteria. As the bacteria possess different kind of surface molecules which play a crucial role in interaction with the heavy metals. Bacterial surface provides the cationic and anionic sites which act as a nucleation site for attachment of the heavy metals.

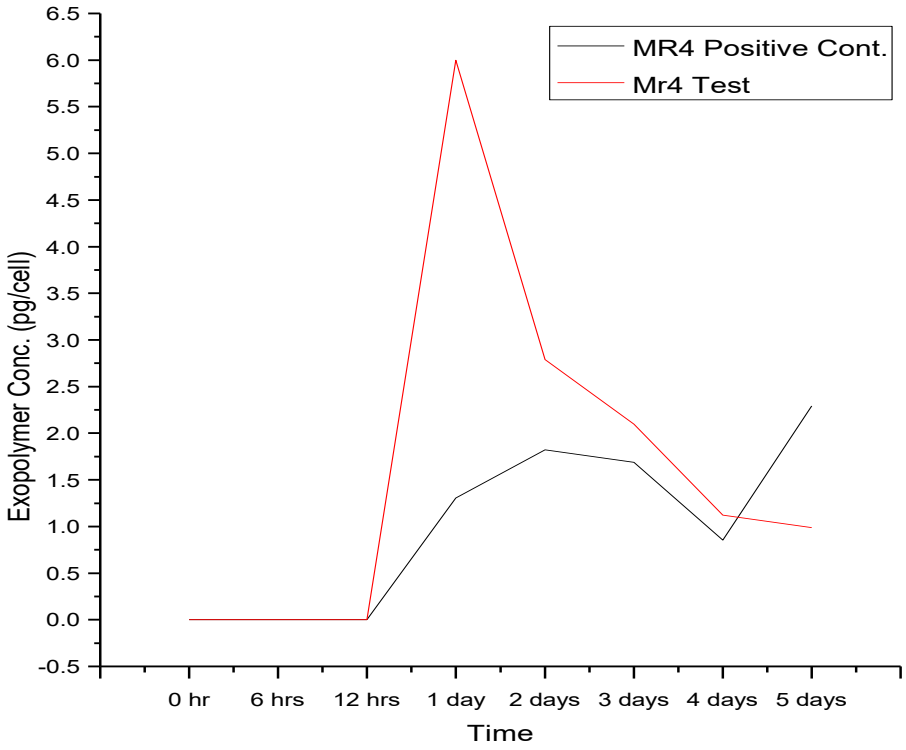
11.1 Exopolysaccharide analysis- The exopolysaccharide secretion by the isolated bacteria in response to heavy metal was studied. The exopolysaccharide was extracted and quantified from bacterial isolate treated with and without heavy metal. The exopolysaccharide was quantified using phenol/sulphuric method.

The exopolysaccharide secreted by the heavy metal resistant isolates was quantified at a different time interval of the bacterial growth. The exopolysaccharide was quantified using phenol/sulphuric method and extrapolated from the standard graph prepared by using glucose as standard. The exopolysaccharide secreted by per cell was calculated from the number of cell present at the time of the exopolysaccharide quantification. The change in the quantity of the exopolysaccharide was the major difference noted between the heavy metal treated and untreated sample. The quantity of the exopolysaccharide secreted per cell in presence of the heavy metal was higher as compared to the bacterial isolates grown in the absence of the chromium.


MR1 posive cont.
MR1 test
Exopolymer Conc (pg/cell)
16
14
12
10
8
6
4
2
0
-2
0 hr
6 hrs
12 hrs
1 day
2 days
3 days
4 days
5 days
6 days
Time


MR2 Positve Cont
MR2 test
Exopolymer conc (pg/cell)
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0 hr
6 hrs
12 hrs
1 day
2 days
3 days
4 days
5 days
Time


MR3Positve cont
MR3 test
Exopolymer Conc. (pg/cell)
7
6
5
4
3
2
1
0
-1
0 hr
6 hrs
12 hrs
1 day
2 days
3 days
4 days
5 days
Time


MR4 Positive Cont.
Mr4 Test
Exopolymer Conc. (pg/cell)
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0 hr
6 hrs
12 hrs
1 day
2 days
3 days
4 days
5 days
Time

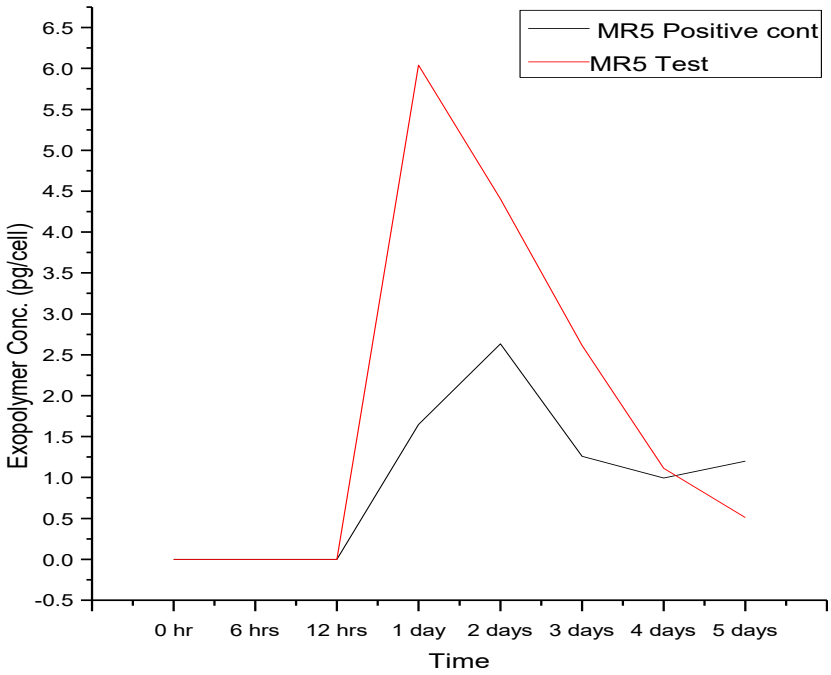


Figure 23: Exopolysaccharide secretion by CrS1a (MR1), CrS1b (MR2), CrS2a (MR3), CrS2b (MR4) and CrS2c (MR5) (pg/cell) in the presence (Red line) and absence (Black line) of the chromium.

11.2 FTIR analysis- Fourier Transform Infra-Red Spectroscopy (FTIR) spectroscopy is a highly sensitive technique for detecting changes in the functional groups. It was performed in order to identify the functional groups available on the cell surface for heavy metals interaction. Microbial components, such as lipids and proteins can also be subjected to FTIR. This shift in the peak positions, bandwidth and the intensity of the bands all give valuable structural and functional information. FTIR was performed on Perkin Elmer Spectrum Version 10.03.07 at the Department of Chemistry, University of Mumbai. The dry powder was directly subjected to the analysis and analysis was done using a probe. The FTIR spectrum was recorded in the range of 4000-400 cm^{-1}.

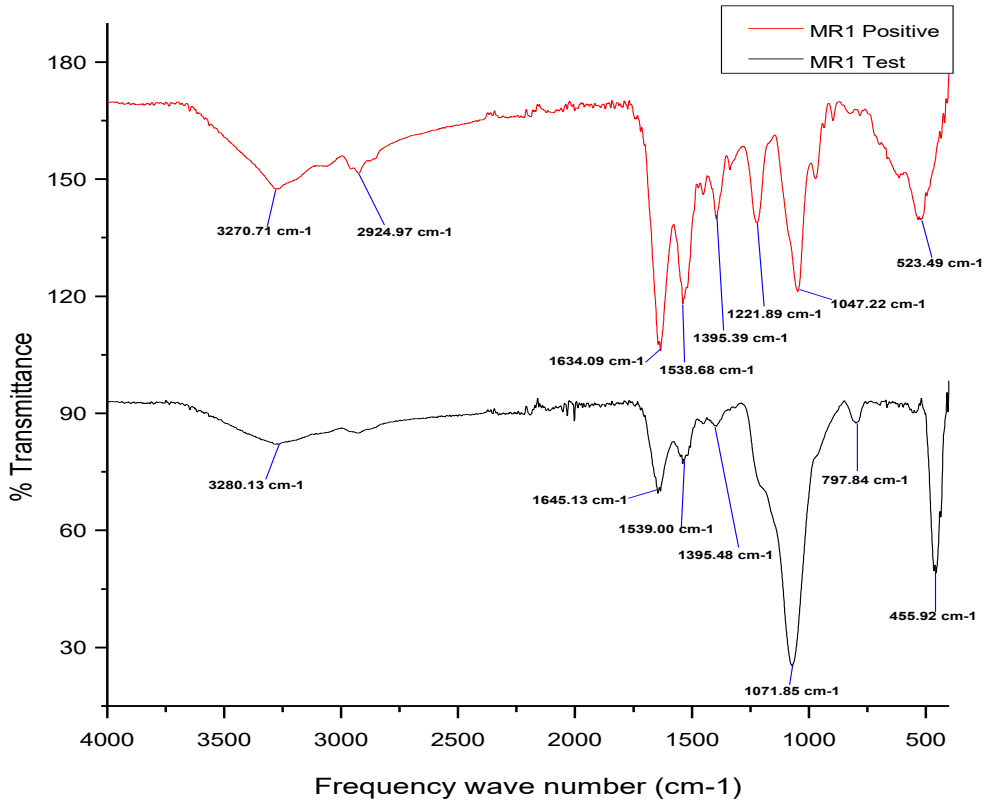


Figure 24: FTIR spectrum of the CrS1a isolate in the presence (black) and absence (red) of chromium.

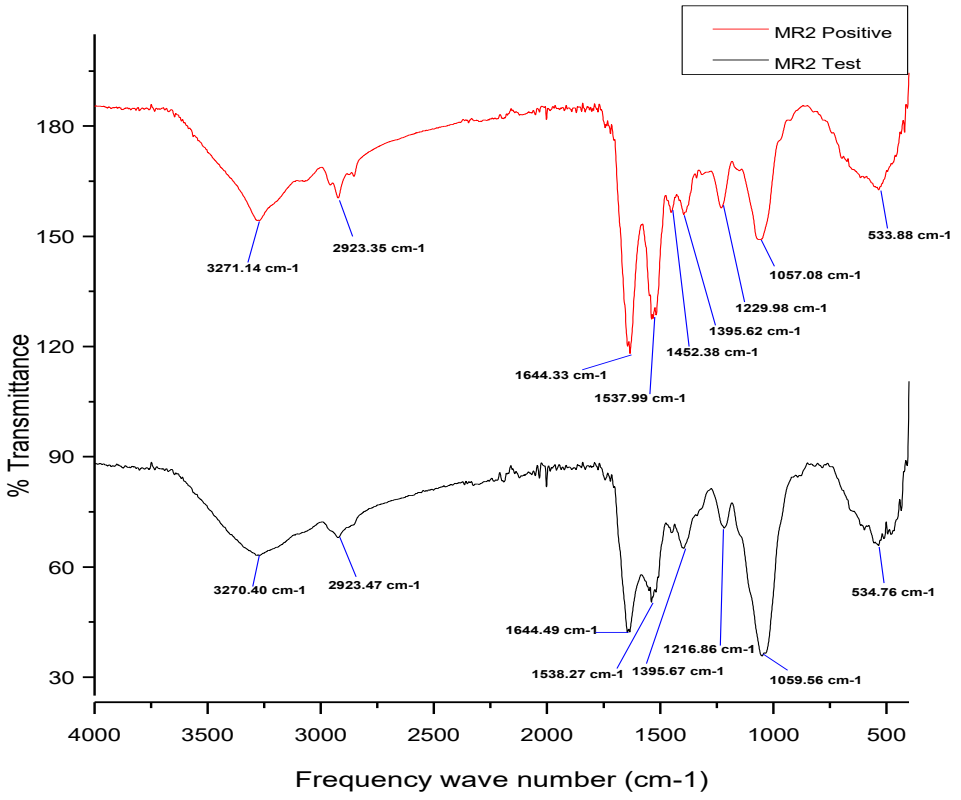


Figure 25: FTIR spectrum of the CrS1b isolate in the presence (black) and absence (red) of chromium.

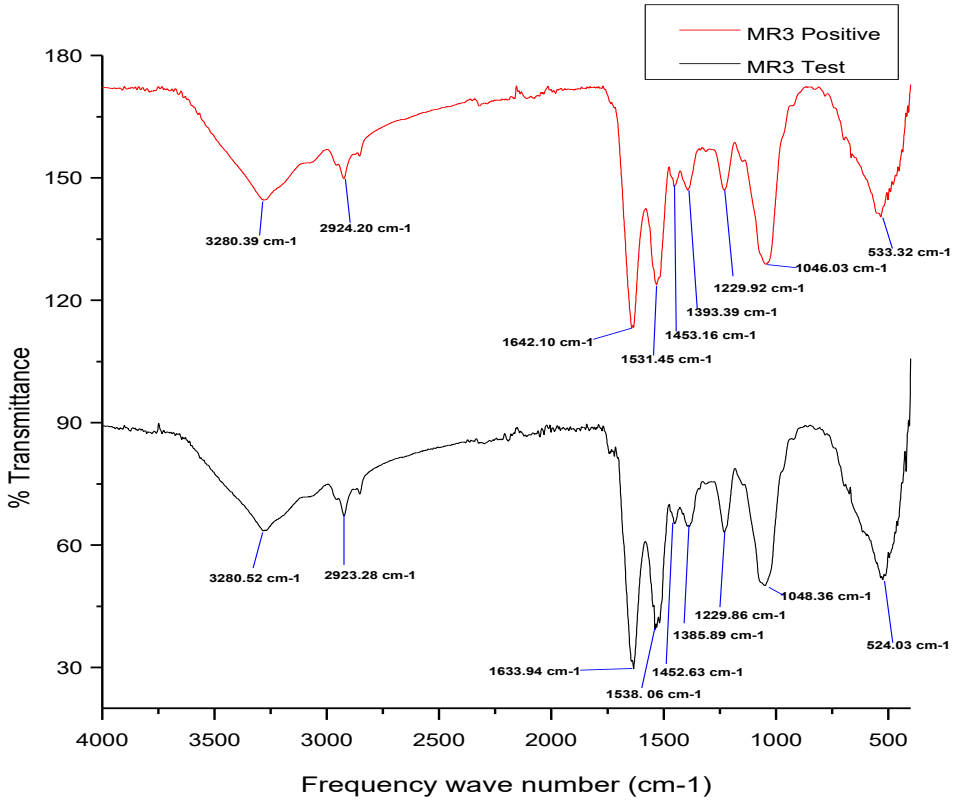


Figure 26: FTIR spectrum of the CrS2a isolate in the presence (black) and absence (red) of chromium.

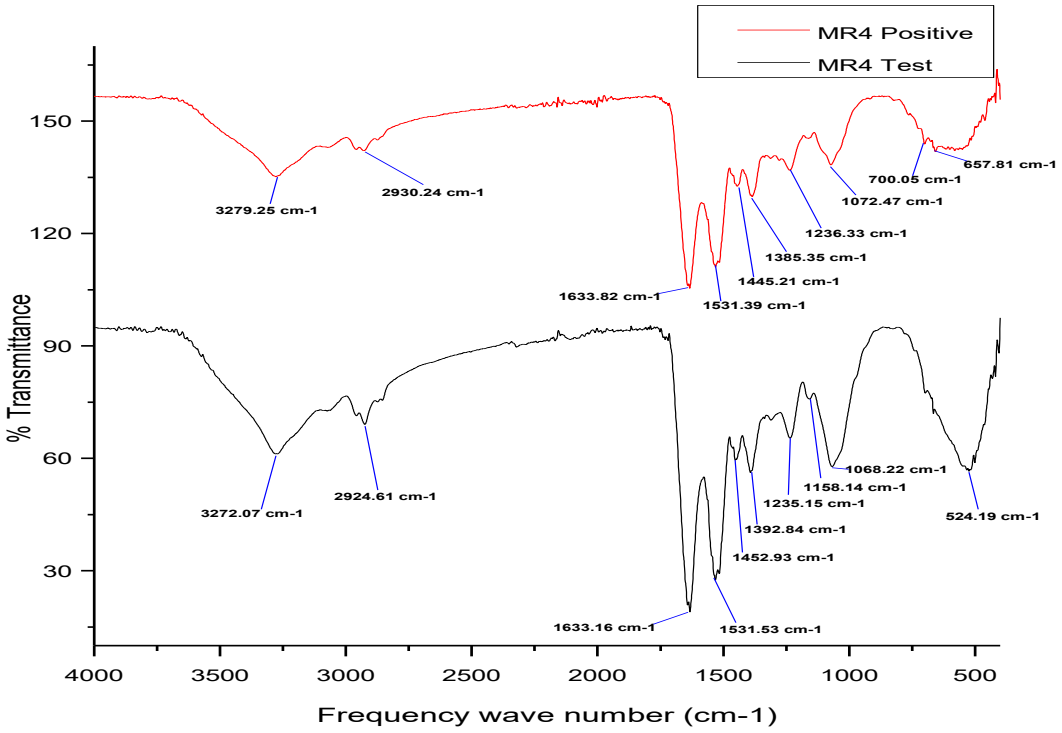


Figure 27: FTIR spectrum of the CrS2b isolate in the presence (black) and absence (red) of chromium.

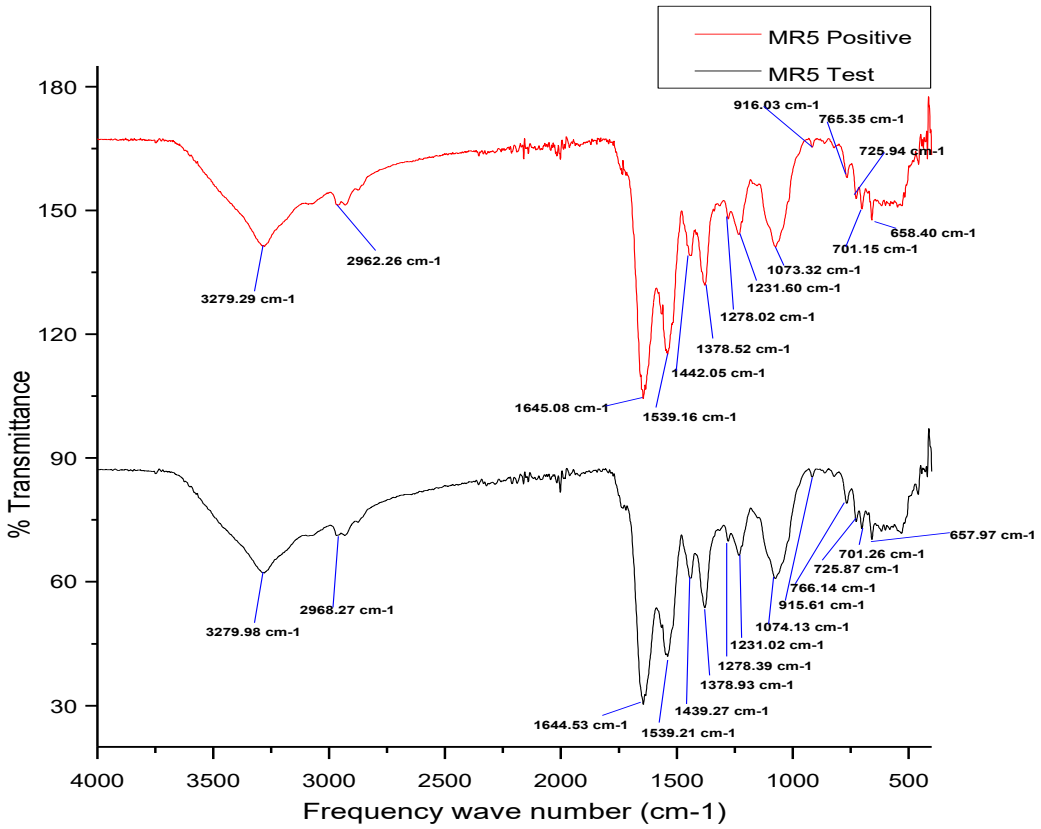


Figure 28: FTIR spectrum of the CrS2c isolate in the presence (black) and absence (red) of chromium.

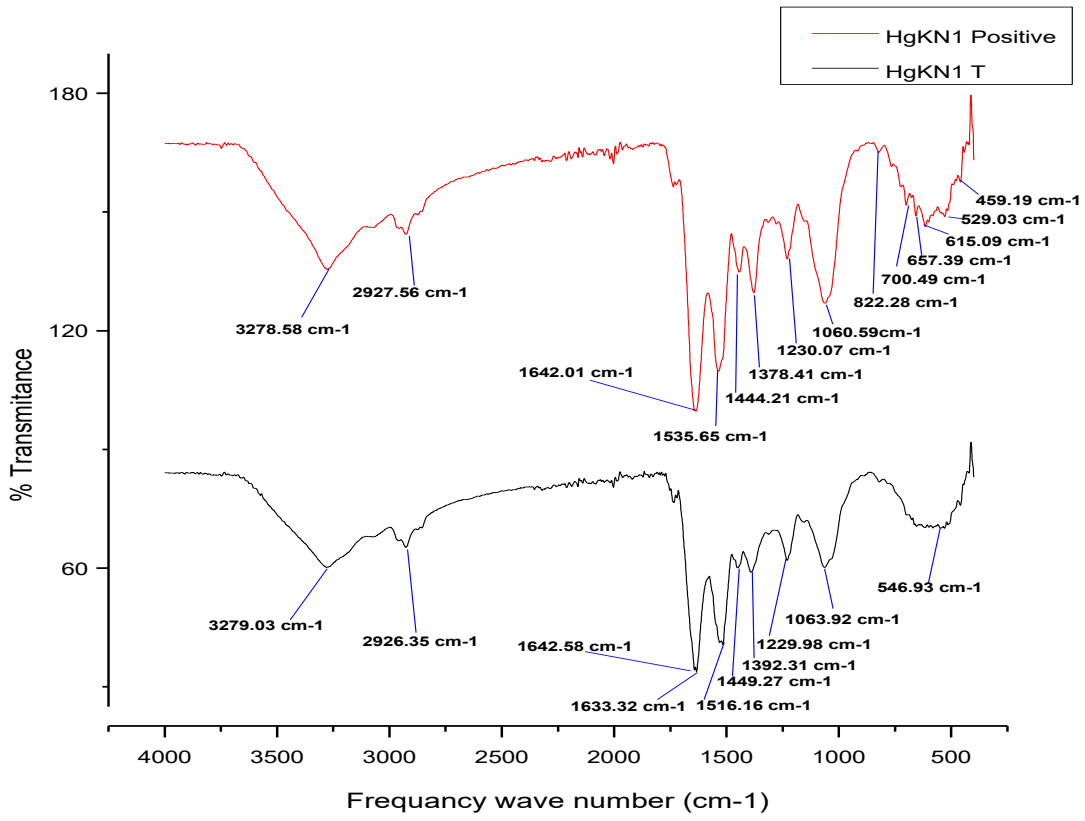


Figure 29: FTIR spectrum of the HgKN1 isolate in the presence (black) and absence (red) of mercury.

11.3 Scanning Electron Microscopy analysis- Heavy metals lead to various changes in the bacterial cell surface which can be analysed through different methods. The change in the morphology of the bacteria due to heavy metals can be traced with scanning electron microscopic analysis. The SEM imaging was performed at NCNN, University of Mumbai, using FEI INSPECT F50 SEM.

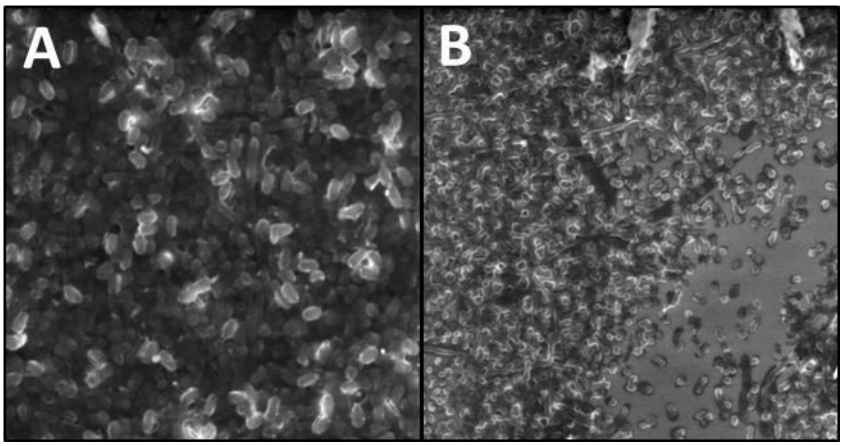


Figure 30: Scanning electron micrograph of the HgKN1 bacterial sample grown in the absence (A) and in the presence (B) of 100 ppm of mercury.

A- Bacteria without heavy metal are more segregated.

B- Bacteria with heavy metal are seen in clumps with secretions on the surface. The morphology was seen to be changed in the presence of heavy metals. The bacteria were smaller in size due to heavy metals

The difference in the morphology of the bacteria after exposing to heavy metal was observed under SEM analysis. The surface of bacteria could be the probable site for chromium accumulation when grown in the presence of chromium. The SEM analysis indicates that the bacteria inhibit the influx of metal in the bacterial cell. This mechanism may be used by the microorganism at the initial stages when exposed to the heavy metal. Some morphological and physiological changes in bacteria have been observed when exposed to metals. The production of exopolymers or biopolymers is sometimes related to the cell's defence mechanisms as it immobilises toxic heavy metal ions thus inhibiting them from entering the cell.

12. Biosorption study of the Chromium by Cr resistant isolates

Biosorption is the ability of biological materials to accumulate heavy metals from waste water through metabolically mediated or physico-chemical pathways of uptake. Bacteria utilize this mechanism to detoxify the heavy metals. Biosorption is either metabolism independent, such as physical or chemical sorption onto the microbial cell walls, or metabolism associated, such as transport, internal compartmentalization and extracellular precipitation by metabolites. In addition, an important aspect of biosorption is that it can be carried out either with metabolically active or inactive cells. Bacteria may protect themselves from toxic substances in the environment by transforming toxic compounds through oxidation, reduction or methylation into more volatile, less toxic or readily precipitating forms. Biosorption is a property of certain types of inactive, non-living microbial biomass to bind and concentrate heavy metals from even very dilute aqueous solution.

Biomass exhibits this property, acting just as chemical substance and as an ion exchange of biological origin. It is particularly the cell wall structure of bacteria, which was found responsible for this phenomenon. Such metal can be desorbed easily from the biosorbent altering the pH. This helps in the recovery of the heavy metal and further their reuse.

Biosorption of the chromium by the isolated bacteria was studied in order to find out their biosorption capability. The biomass of the bacteria was exposed to the chromium solution and the uptake of the chromium by the bacteria was determined. The chromium was quantified using the Atomic Absorption Spectroscopy (AAS) of Shimadzu at Department of Chemistry, University of Mumbai.

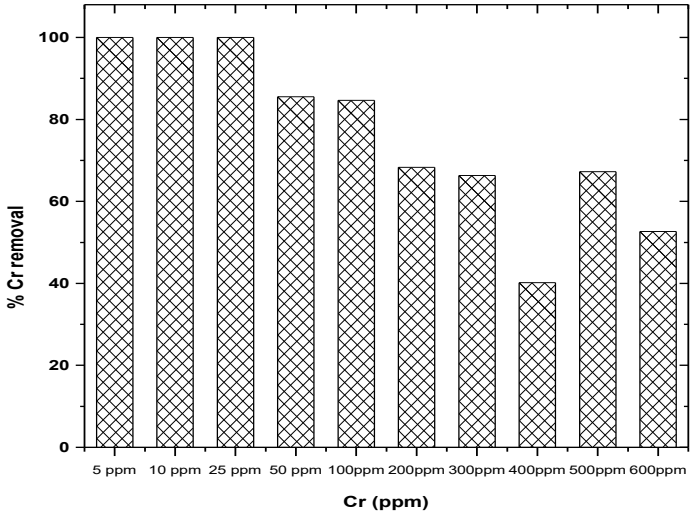


Figure 31: Chromium removal by Chromium resistant isolate at varying concentration of chromium.

The chromium removal was studied at varying initial concentration of the chromium. The removal of the chromium was determined after 1 hour. The results showed that the bacteria could remove 100% of chromium till 25 ppm. As the concentration of the chromium increased the removal efficiency of the bacteria decreased as presented in Figure 32.

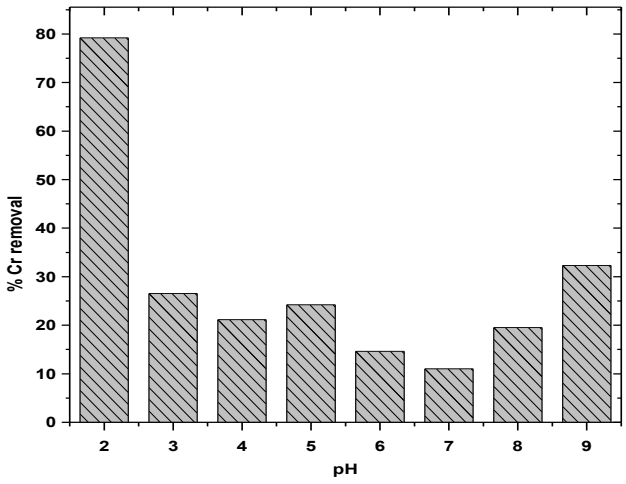


Figure 32: Chromium removal by Chromium resistant isolate at varying pH of chromium.

The biosorption was studied at different pH in order to determine the optimum pH for the chromium biosorption by the bacteria. The pH range was studied from 2 to 9. As presented in the Figure 32, pH 2 was found to be highly effective in the chromium removal activity of the bacteria via biosorption process.

13. Detection of heavy metal reduction by bacterial isolates

Heavy metals are less toxic in their reduced state. Bacteria possess the enzyme which can actively reduce the heavy metal into lower oxidation state. The reduction of the heavy metal by the bacteria was studied using biochemical analysis.

Bacteria possess the heavy metal reduction enzymes which reduces the Cr(VI) into Cr(III). Cr(IV) is highly soluble and in turn highly toxic in nature. The chromium in 3^+ oxidative state is less soluble and 1000 times less toxic. The chromium reduction was detected using S-diphenylcarbazide (DPC) dye which can react only with hexavalent chromium and form a pink colour complex. The concentration of reduced Cr(VI) was estimated from the standard plot prepared from the known concentration of Cr(VI).

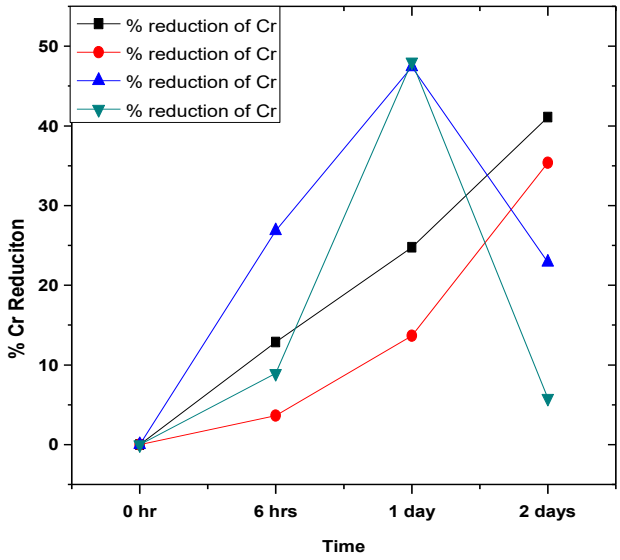


Figure 33: Cr reduction by CrS2b at varying time interval (black- 10 ppm, red- 50 ppm, blue- 100 ppm and green- 200 ppm).

The reduction assay confirms the heavy metal reduction efficiency of the bacteria. The bacteria have shown different pattern of chromium reduction at lower and higher concentration of the chromium ie 10, 50, 100 and 200 ppm of chromium. The bacteria have reduced 50% of the Cr(VI) to Cr(III). The reduction was higher at high concentration of the chromium. While a gradual increase in the reduction of the Cr(VI) was observed at the lower concentration of the chromium.

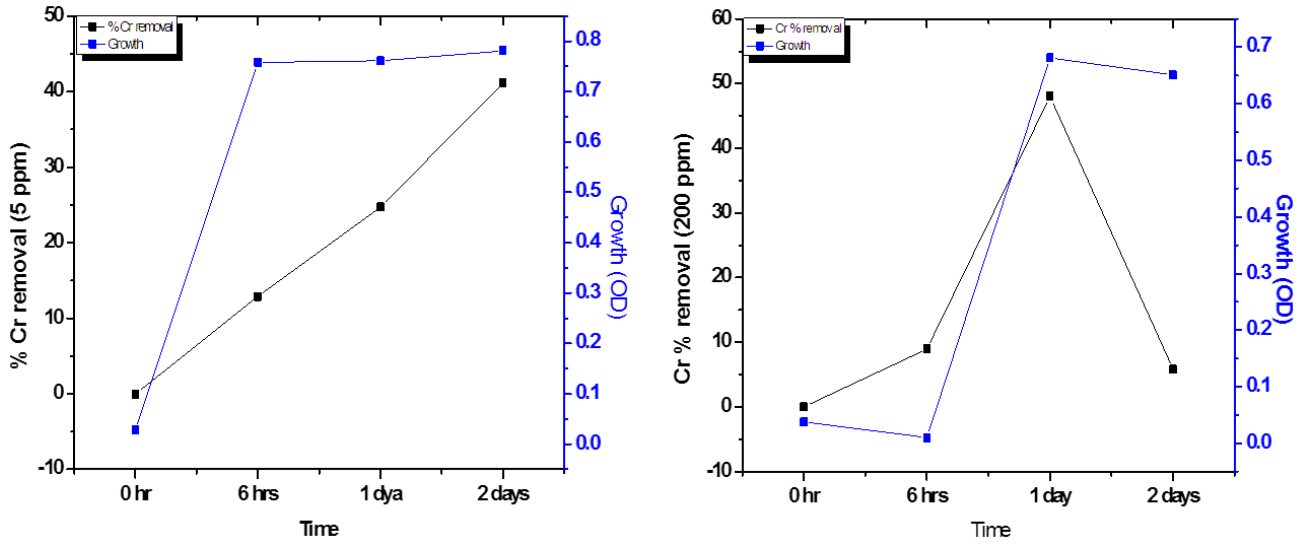


Figure 34: Effect of Cr concentration (5 ppm and 200 ppm) on the reduction capability of bacteria CrS2c and CrS2d.

14. Proteomics of the Heavy Metal Remediating Bacteria

a. Protein extraction

Proteomics study on the heavy metal resistant bacteria was performed in order to determine the proteins involved in the heavy metal remediation. The bacteria isolate was grown in the presence of the chromium till the bacteria attain its full growth. The growth of the bacteria was recorded by measuring the optical density at 600 nm. Whole bacterial protein from the heavy metal treated and untreated bacteria was extracted using the Bugbuster protein extraction reagent from merck. The protein was separated from the rest of the cell debris by centrifugation. The whole bacterial protein was stored in the -20°C for further analysis.

b. Separation of Proteins

The extracted protein was quantified using Folin Lowry method of protein estimation using BSA (Bovine Serum Albumin) as a standard protein. Absorbance was read using Thermo Scientific MultiscanGo, spectrophotometer at 600 nm.

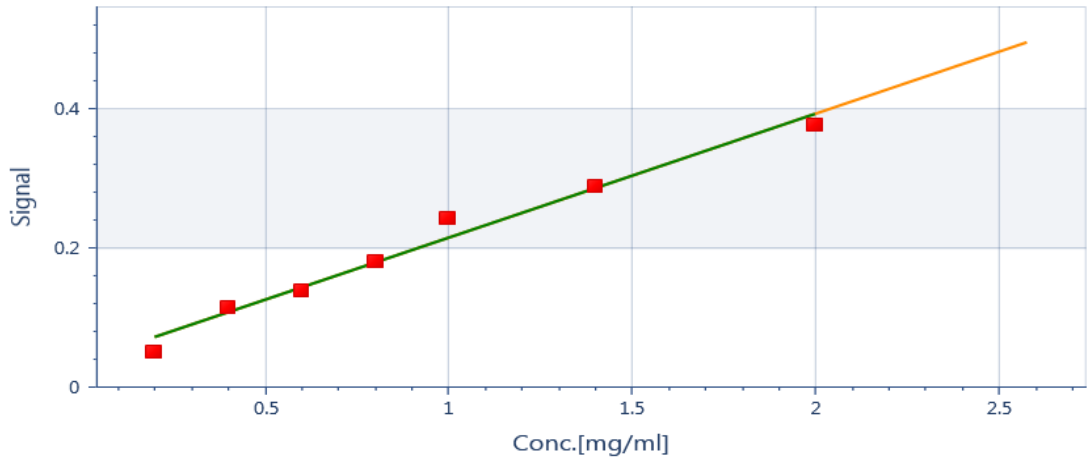


Figure 35: The standard plot of the BSA protein for extrapolation of unknown protein concentration.

Table 34: Concentration of unknown protein samples.

Sr. No.	Sample	Conc. of protein sample (ug/ul)
1	CrS2d +ve control	4.174
2	CrS2d 10 ppm Cr	4.906
3	CrS2d 100 ppm Cr	5.156
4	CrS2d 200 ppm Cr	4.242

The extracted proteins were separated on SDS-PAGE. The treated sample was compared to the positive control. A known molecular weight protein marker was loaded on the gel to determine the molecular weight of the unknown protein separated on the polyacrylamide gel. The protein profile of the heavy metal treated bacteria on the SDS-PAGE was compared with the untreated bacterial protein profile.

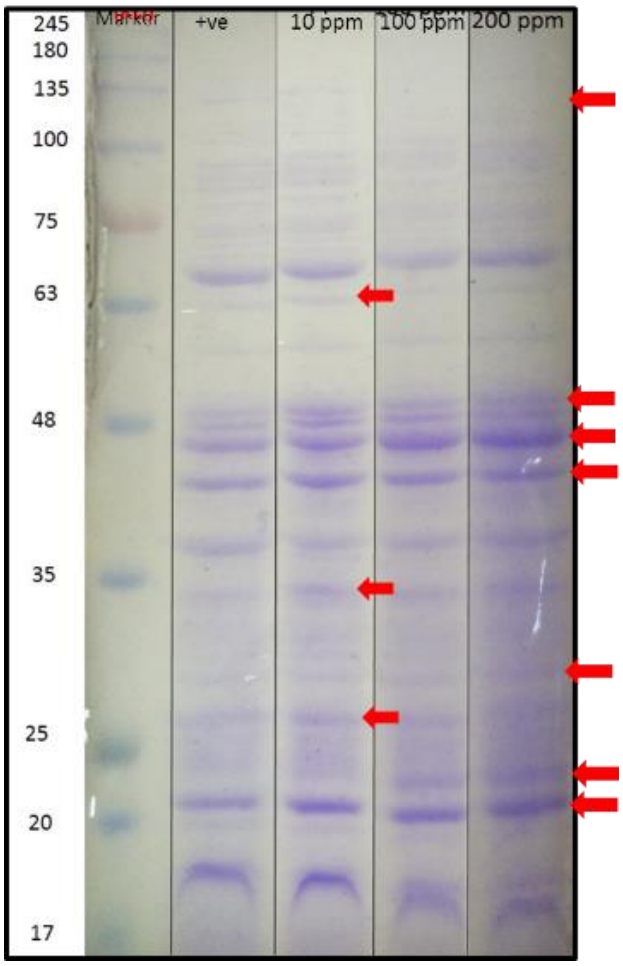


Figure 36: Bacterial protein separated on SDS-PAGE.

Keys:-

Lane 1- Known molecular weight protein marker

Lane 2- Positive control of crS2b

Lane 3- CrS2b isolate treated with 10 ppm Cr

Lane 4- CrS2b isolate treated with 100 ppm Cr

Lane 5- CrS2b isolate treated with 200 ppm C

c. Identification of the Protein involved in Chromium Remediation using LC-MS

The newly expressed, over and under expressed proteins were identified using LC-MS analysis. The NCBI database was searched for protein identification using the Experimental fragment masses as input via Mascot program (Matrix Science, London, UK). The Gene ontology (GO) analysis was also performed to characterize these identified proteins based on their cellular location, molecular functions and biological processes.

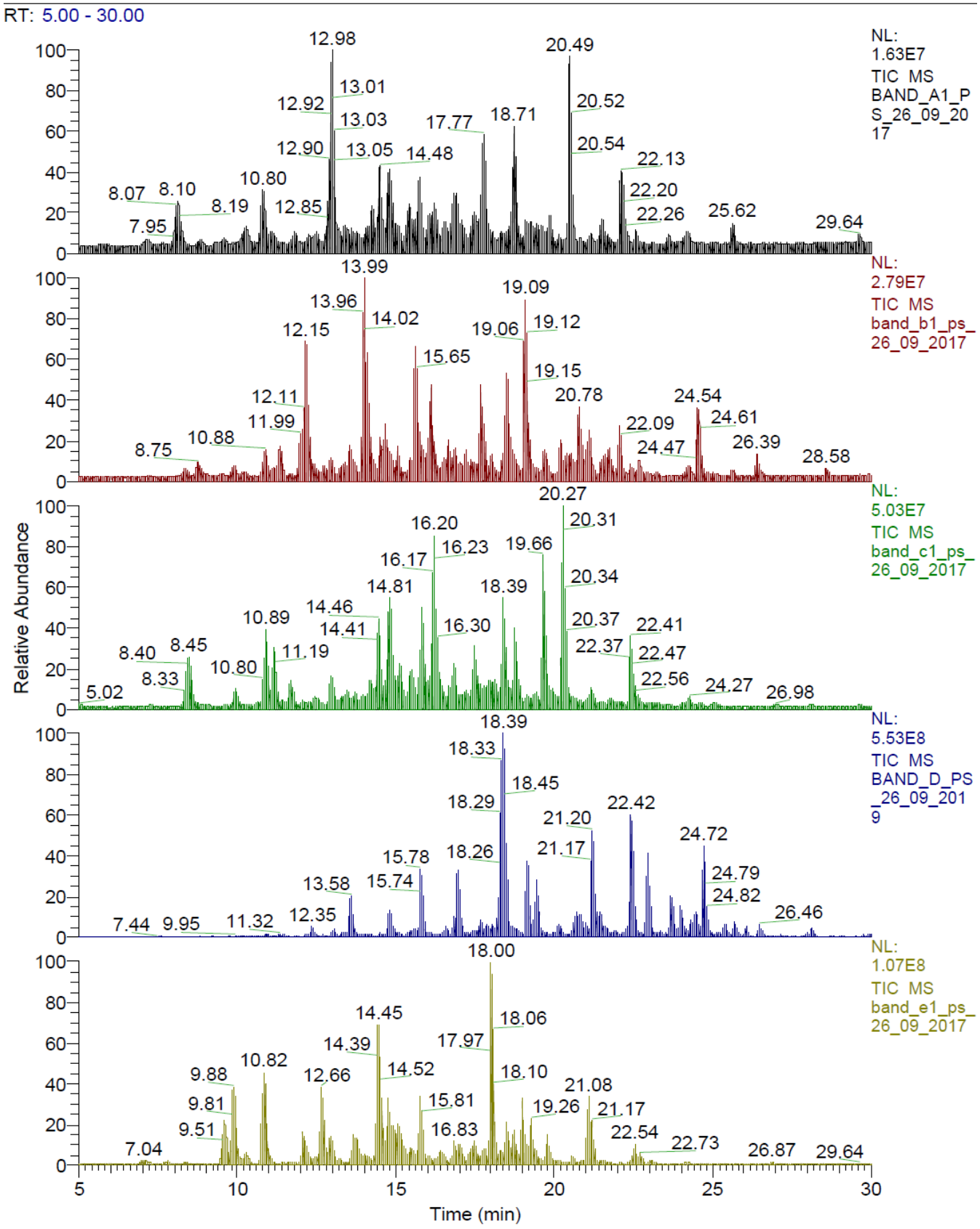


Figure 37: Chromatogram of the various proteins analysed on the LC-MS.

Table 35: Details of the bacterial protein identified from chromium resistant bacteria.

Sr. No.	Band	Molecular Weight (kD)	Name of protein	Category	Accession No.	Function	Expression	% coverage
1	B	51.21	Periplasmic serine endoprotease DegP	envelope stress responses	U7GA41	• Involved in metabolic process • Degradation of misfolded proteins. • Envelope stress response protein. • Help chaperone in folding the other protein and help the bacteria to overcome the heavy metal stress.	Up-regulated	1.46
2	D	44.014	Lysophospholipase	envelope stress responses	S7XXJ8	• Triglyceride lipase activity • Oxidative stress tolerance • Oil degrading • Degrading of organophosphorus (OP) esters	Up-regulated	41.46
3	E	35.407	Malate dehydrogenase	Energy metabolism	A0A1Z9YWA4	• produce reducing agent oxaloacetate and NADH+ • oxaloacetate and oxalate, permit the microbe to expulse heavy metal as precipitate and continue to proliferate • reducing equivalent for lipid biosynthesis	Up-regulated	7.60
4	F	31.76	LuxR family transcriptional regulator		A0A0R0RRI4	• quorum sensing and biofilm formation • DNA binding and transcription factor activity	Up-regulated	10.52
5	G	18.86	Uncharacterized protein		A0A217ECM7	• integral component of membrane • Transmembrane helix	Up-regulated	16.35
6	H	23.707	Peroxidase		A0A1T1GRS9	• peroxidase activity, • maintain cell redox environment and homeostasis • Oxidoreduction	Up-regulated	19.47
7	I	26.102	3-oxoacyl-[acyl-carrier-protein] reductase		A0A217EF91	• 3-oxoacyl-[acyl-carrier-protein] reductase (NADPH) activity • NAD binding • fatty acid biosynthetic process	Up-regulated	3.26

15. Genomics of the Heavy Metal Remediating Bacteria

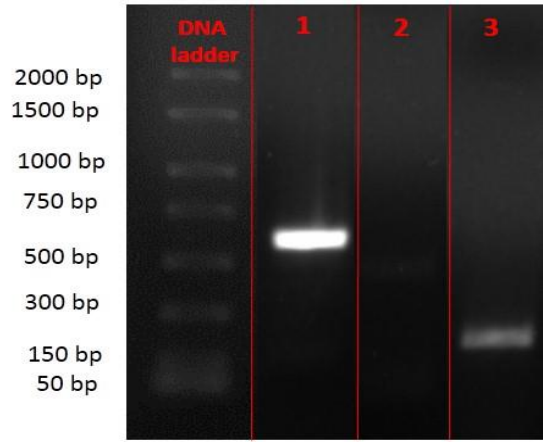
The genes responsible for heavy metal accumulation/resistance in bacteria have been identified. Primer sequence for the selected genes has been designed and ordered. The total DNA of the heavy metal resistance/accumulating bacteria has been isolated. Forward and reverse primer of selected gene has been included in PCR master mix to amplify the selected gene. PCR has been performed by following standard protocol. The genes amplified have been resolved via gel electrophoresis. Presence of gene in the bacteria indicated its role in heavy metal resistance/accumulation.

Table 36: List of the gene detected for heavy metal remediation using PCR amplification.

Sr. No.	Gene	Name of gene	Primers
1	Chromate reduction gene	ChRF	Forward primer 5’ -TCACGCCGGAATATAACTAC-3’ Reverse primer 5’ -CGTACCCTGATCAATCACTT-3’
2	Nitroreductase gene	EcNfsA1	Forward primer 5’ -GTAGGATCCACGCCAACCATTGAAC-3’ Reverse primer 5’ -ACTGAATTCTTAGCGCGTCGCCCAAC-3’
3	Reduction gene	yieF	Forward primer 5’ -GGAGGAAACATCATGAAGGTA-3’ Reverse primer 5’ -ATGCAGAAGTAGCATCTTTCC-3’

The partial amplification of chromate reductase gene, amplified the 268 bp fragment. The results confirm the role of chromate reductase gene activation and further its expression lead to the chromate reduction by Enterobacter sp. enrichment culture. The yieF gene was found to be present in the Enterobacter sp. enrichment culture. The yieF gene was amplified to 626 bp DNA fragment. There was amplification of observed nitroreductase gene. The nitroreductase gene was not present in the chromium resistant bacteria.

The PCR amplified product was run on the 0.8% agarose gel electrophoresis as given in the figure 38.



Key: 1- PCR product of yieF gene

2- PCR product of EcNfsA1

3- PCR product of ChRF gene

Figure 38: Agarose Gel electrophoresis of the PCR amplified DNA of the heavy metal resistant gene.

16. Development of Biomat for bioremediation of the selected heavy metals

A. Biomat Preparation using chemical polymer

The bioremediation of the heavy metals by bacterial consortium was simulated in the lab by replicating the Mithi River Condition. Mithi Water was used to study the remediation of heavy metal. Different chemical polymers were used to entrap the bacterial consortium. The reaction was set up by mixing the matrix with the water and kept on the shaking condition at 120 rpm at room temperature. The water sample was withdrawn from the reaction set up at interval of 1 hour till 24 hour. The water samples were stored in the 0°C till analysis. The sample was analysed for chromium using S-diphenylcarbazide (DPC) spectrophotometric assay.

16.1 Immobilization of the consortia on alginate bead

Chromium resistant bacterial consortia was grown overnight in the nutrient broth at 37^{o} C on shaker at 120 rpm. The biomass was centrifuged and separated from the liquid media. 5 gms of the wet biomass was added to 4% sodium alginate solution and mixed well. The alginate beads were prepared by dropping the sodium alginate containing bacteria to the 2% calcium chloride solution. The blank alginate beads were prepared in the same way but not containing bacteria. The beads were left in the calcium chloride solution overnight for hardening. After hardening the beads were washed with the distilled water and stored at 4^{o} C for further study.

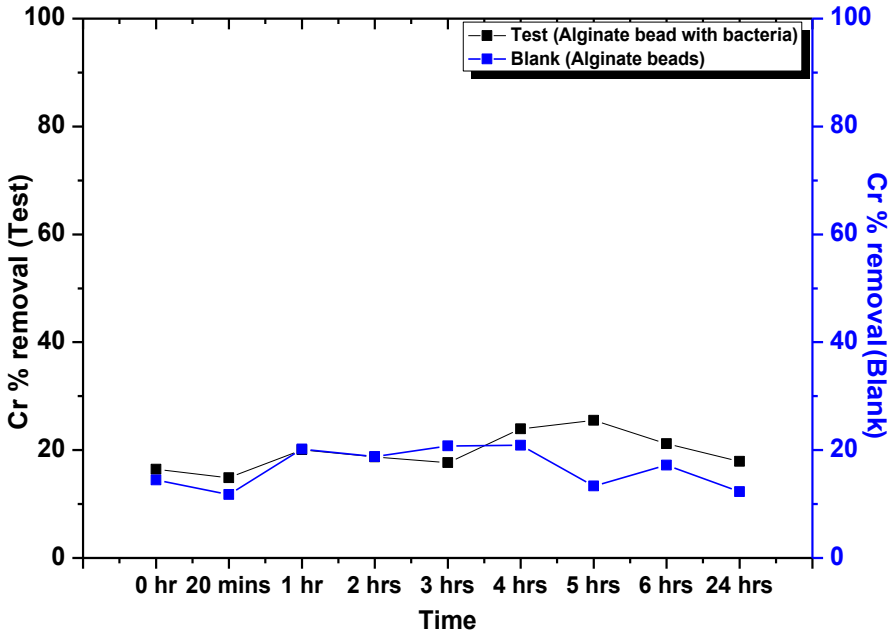


Figure 39: Chromium removal by the alginate bead immobilized with Cr bacterial consortium and without bacterial consortium.

16.2 Immobilization of the consortia on alginate and polyvinyl alcohol (PVA) bead

Chromium resistant bacterial consortia was grown overnight in the nutrient broth at 37^{o} C on shaker at 120 rpm. The biomass was centrifuged at 8000 rpm for 10 mins at 4^{o} C and separated

from the liquid media. 4 % sodium alginate and 2 % PVA solution was prepared and heated at 80^{o} C on magnetic stirrer to achieve homogeneous mixing. 5 gms of the wet biomass was added to sodium alginate and PVA solution and mixed well gently. The alginate PVA beads were prepared by dropping the mixer containing bacteria to the 2:1 solution of 2% calcium chloride solution and saturated boric acid. The blank alginate PVA beads were prepared in the same way but not containing bacteria. The beads were left in the calcium chloride and boric acid solution overnight for hardening. After hardening the beads were washed with the distilled water and stored at 4^{o} C for further study.

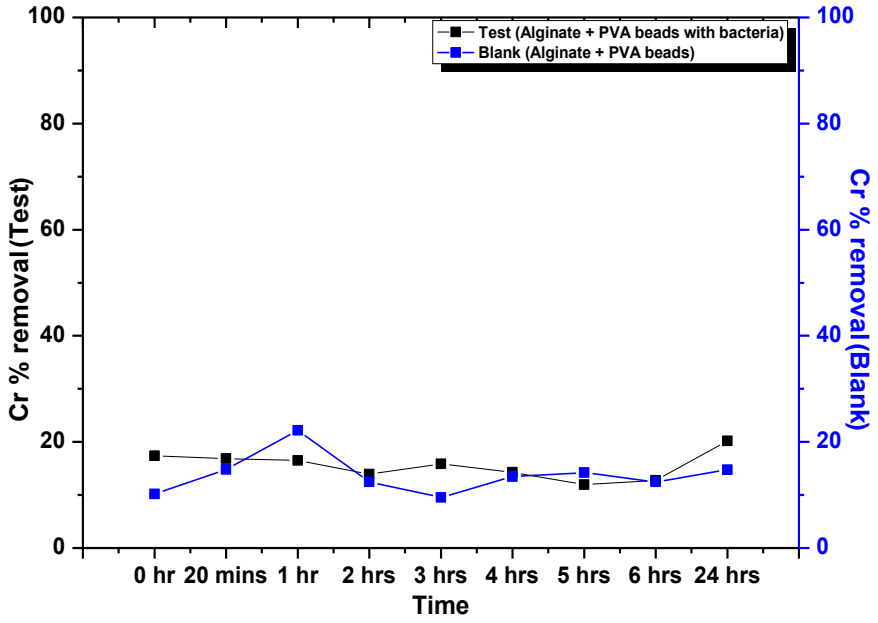


Figure 40: Chromium removal by the alginate and PVA bead immobilized with Cr bacterial consortium and without bacterial consortium.

16.3 Immobilization of the consortia on alginate and activated carbon (AC) bead

Chromium resistant bacterial consortia was grown overnight in the nutrient broth at 37^{o} C on shaker at 120 rpm. The biomass was centrifuged at 8000 rpm for 10 mins at 4^{o} C and separated from the liquid media. 4 % sodium alginate solution was prepared and 1% of activated carbon powder was added to sodium alginate solution and mixed well. 5 gms of the wet biomass was added to sodium alginate and AC solution and mixed well gently. The alginate AC beads were prepared by dropping the mixer containing bacteria to the 2% calcium chloride solution. The blank alginate AC beads were prepared in the same way but not contacting bacteria. The beads were left in the calcium chloride overnight for hardening. After hardening the beads were washed with the distilled water and stored at 4^{o} C for further study.

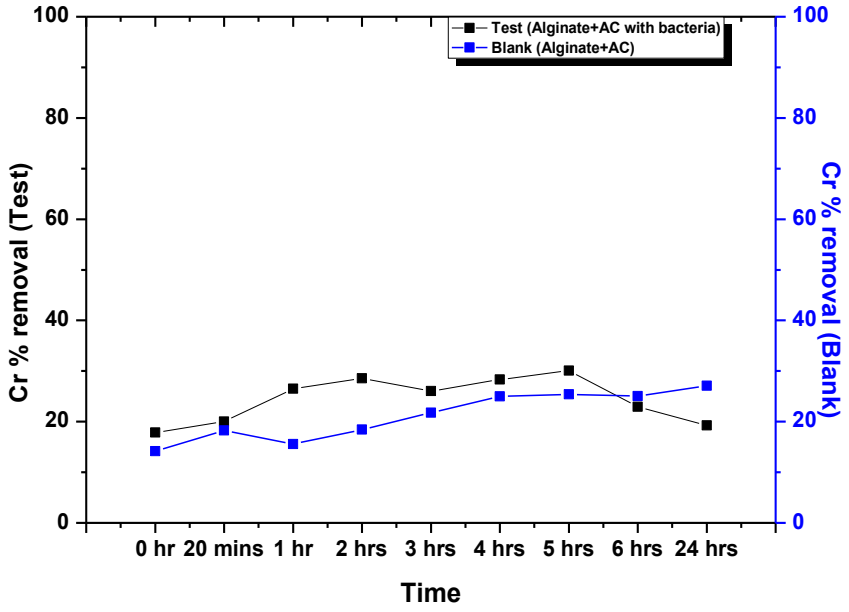


Figure 41: Chromium removal by the alginate and AC bead immobilized with Cr bacterial consortium and without bacterial consortium.

16.4 Immobilization of the consortia on alginate, polyvinyl alcohol and activated carbon bead

Chromium resistant bacterial consortia was grown overnight in the nutrient broth at 37^{o} C on shaker at 120 rpm. The biomass was centrifuged at 8000 rpm for 10 mins at 4^{o} C and separated from the liquid media. 2% sodium alginate and polyvinyl alcohol solution was prepared and 1% of activated carbon powder was added to sodium alginate solution and mixed well. 5 gms of the wet biomass was added to sodium alginate and AC solution and mixed well gently. The alginate AC beads were prepared by dropping the mixer containing bacteria to the 2:1 solution of 2% calcium chloride solution and saturated boric acid. The blank alginate, PVC and AC beads were prepared in the same way but not containing bacteria. The beads were left in the calcium chloride boric acid overnight for hardening. After hardening the beads were washed with the distilled water and stored at 4^{o} C for further study.

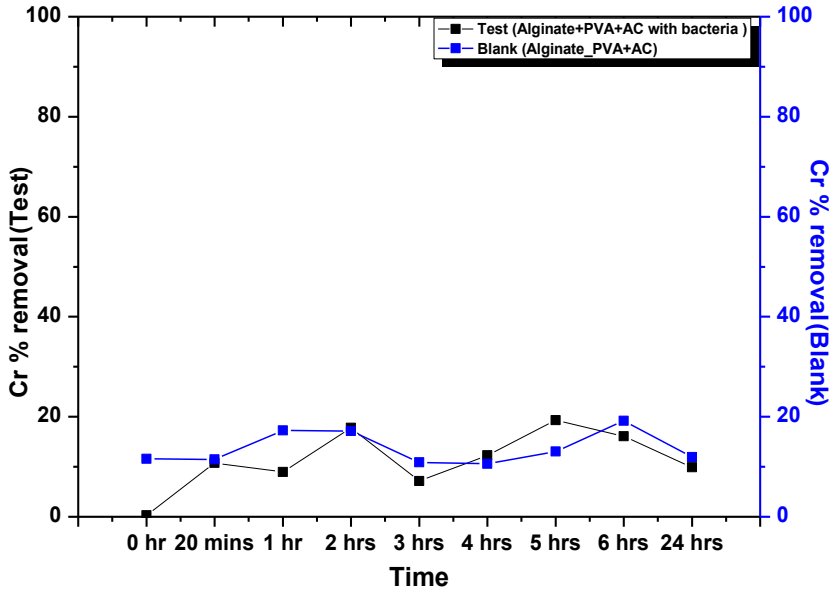


Figure 42: Chromium removal by the alginate, PVA and AC bead immobilized with Cr bacterial consortium and without bacterial consortium.

16.5 Immobilization of the consortia on polyurathen foam

Chromium resistant bacterial consortia was grown overnight in the nutrient broth containing 1 g of polyurathen foam (pieces) at 37^{o} C on shaker at 120 rpm. After incubation period the polyurathen foam immobilized with bacterial consortia was washed with sterile saline. A control was prepared in the similar manner without the bacterial consortia.

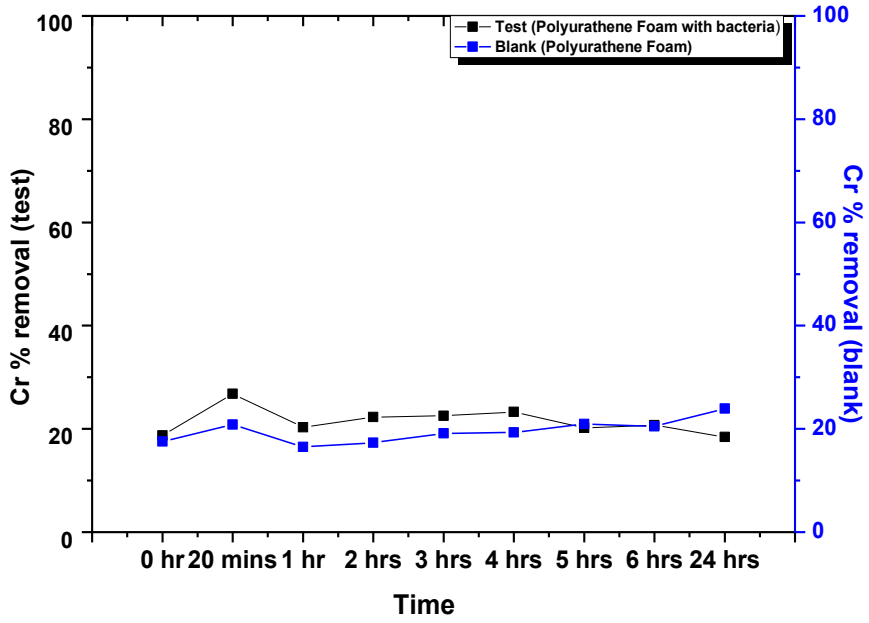


Figure 43: Chromium removal by the alginate and PVA bead immobilized with Cr bacterial consortium and without bacterial consortium.

16.6 Immobilization of the consortia on granulated activated carbon (GAC).

GAC was used for bacterial immobilization. Chromium resistant bacterial consortia was grown overnight in the nutrient broth containing 1 g of GAC at 37^{o} C on shaker at 120 rpm. After incubation period the GAC immobilized with bacterial consortia was washed with sterile saline. A control was prepared in the similar manner without the bacterial consortia.

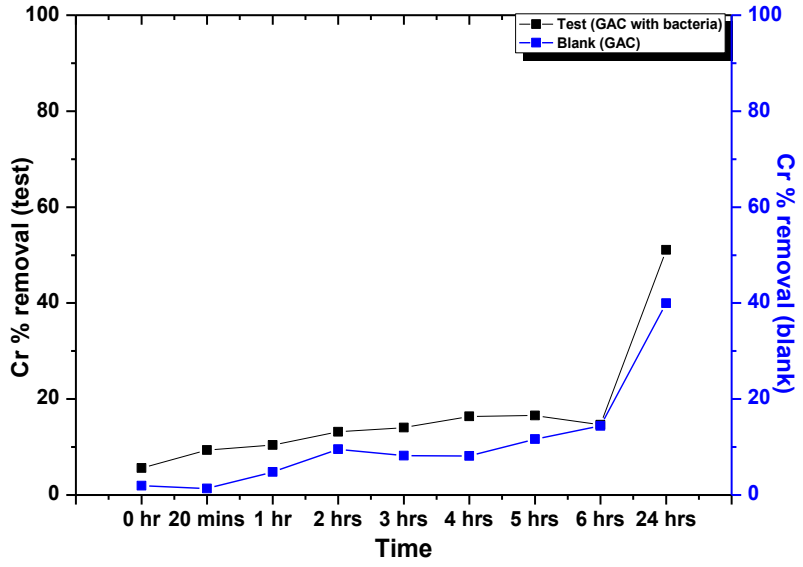


Figure 44: Chromium removal by the GAC immobilized with Cr bacterial consortium and without bacterial consortium.

B. Biomat preparation from Biological waste

Biomat was prepared using different biological waste material available in the market. These waste materials are generated in huge quantity and can be utilized for the biomat preparation. It will also reduce the cost from real time application point of view.

We have provided the reference of the previous study done on the similar line as a validation of the scientific planning of the work and data presentation.

*All the bioremediation experiments were conducted in Mithi River water.

a) Biomat of coconut coir

The Biomat was developed by immobilizing the bacterial consortium on the surface of the coconut coir as performed by Lin et al, 2014 and Liu et al, 2015 with slight modification. Coconut coir was procured from the market as waste material. The coconut coir was washed thrice with distilled water and dried in oven at 100°C. The dried coir was cut and sieved through 1 mm size sieve to get uniform size fibre. The flask containing 100 ml of nutrient medium with 1 gm of coir inoculated with chromium bacterial consortium and incubated at 37°C for 24 hrs in order to achieve bacterial immobilization of coir (Nunal et al, 2014).

To normalise the biofilm formation based on the surface area of the biomat CFU counting and Scanning Electron Microscope (SEM) analysis was employed.

The bacterial attachment to the biocarrier was confirmed by Colony Forming Unit (CFU) counting (Hrenovic et al,2009) and also by visual examination of bacterial attached biocarrier under Scanning Electron Microscopy (SEM)(Figure. 45)(Gupta et at, 2015).

The CFU was found to be 2.865x 10^11 cells per gram of the carrier. The removal study was conducted using Mithi River water at initial concentration of 10 ppm (table 37).

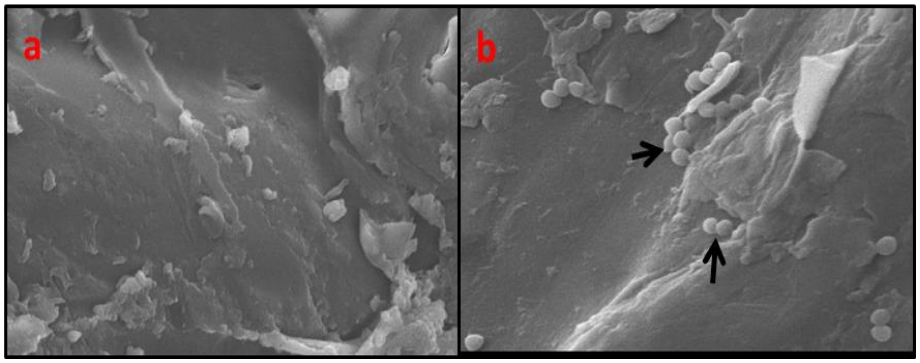


Figure 45: SEM analysis of the coconut coir without bacteria (a) and immobilized with bacteria (b).

Table 37: Chromium removal by the bacterial consortium immobilized on coir and coir without bacteria.

Sr. No.	Time (hrs)	Cr removal by Immobilized Coir (ppm)	Percent Cr removal	Cr removal by Coir without bacteria (ppm)	Percent Cr removal
1	0	9.762	0	9.762	0
2	0.083	8.45	13.42	8.087	17.15
3	1	7.97	18.35	8.037	17.67
4	2	7.76	20.49	7.9985	18.06
5	3	7.63	21.83	7.7261	20.85
6	4	7.22	26.00	7.896	19.11
7	5	7.39	24.21	7.975	18.30
8	6	7.596	22.18	8.111	16.91
9	7	7.287	25.35	8.0192	17.85
10	8	7.34	24.74	7.283	25.39
11	24	6.00	38.43	6.7095	31.269
12	48	5.63	42.27	5.424	44.437
13	72	5.07	48.06	5.237	46.35
14	96	4.336	55.58	4.6227	52.64
15	120	3.751	61.57	3.857	60.48

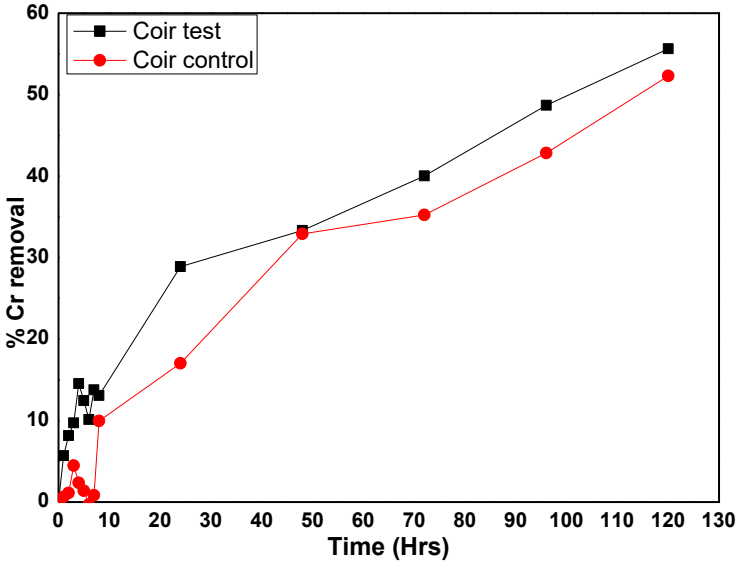


Figure 46: Graphical representation of the percentage chromium removal by coir with bacterial consortium and without bacterial consortium.

Since the chromium removal by the biomat of coir show less efficiency. Another method for bacterial immobilized was tried to get maximum attachment. Another method of immobilization was tried in order to increase the bacterial attachment to the biomaterial. The immobilization was performed as reported by Obuekwe and Esraa in 2001 with little modification. The log phase biomass of the chromium bacterial consortium was collected and mixed with the MSM medium (minimal salt medium) and incubated with 1 gm of the carrier material at 37°C for 3 days. The CFU attached to the coir was found to be 4.54x10^12 per gram of the coir. The bacterial attachment on the surface is analysed under SEM (Figure. 47).

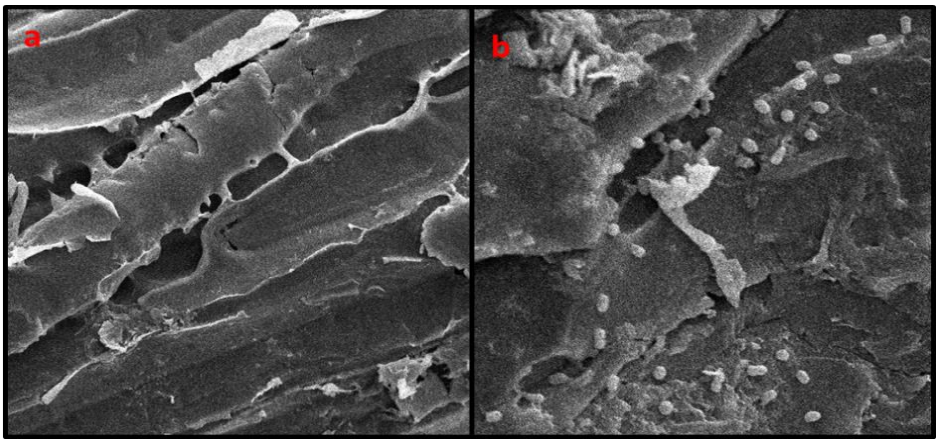


Figure 47: SEM image of Cr consortium immobilized on the coconut coir using another method (b) and control without immobilized bacteria (a).

Chromium removal by the consortium immobilized coir was performed in Mithi River water. The removal of chromium using immobilized bacterial consortium is given in table 38.

Table 38: Chromium removal by the bacterial consortium immobilized on coir and coir without bacteria.

Sr. No.	Time (hrs)	Cr removal by Immobilized Coir (ppm)	Percent Cr removal	Cr removal by Coil without bacteria (ppm)	Percent Cr removal
1	0	9.056	0	9.056	0
2	0.083	6.348	29.90	6.120	32.42
3	1	6.424	29.063	5.436	39.97
4	2	5.580	38.38	6.856	24.29
5	3	4.988	44.92	5.552	38.69
6	4	5.316	41.29	5.520	39.04
7	5	4.804	46.95	5.580	38.38
8	6	4.136	54.32	6.052	33.17
9	7	4.060	55.16	5.692	37.14
10	8	3.840	57.59	4.840	46.55
11	24	0.641	92.91	2.594	71.36
12	48	0.185	97.95	0.555	93.87
13	72	0.097	98.93	0.428	95.26
14	96	0.026	99.71	0.406	95.52
15	120	0.000	100	0.399	95.59

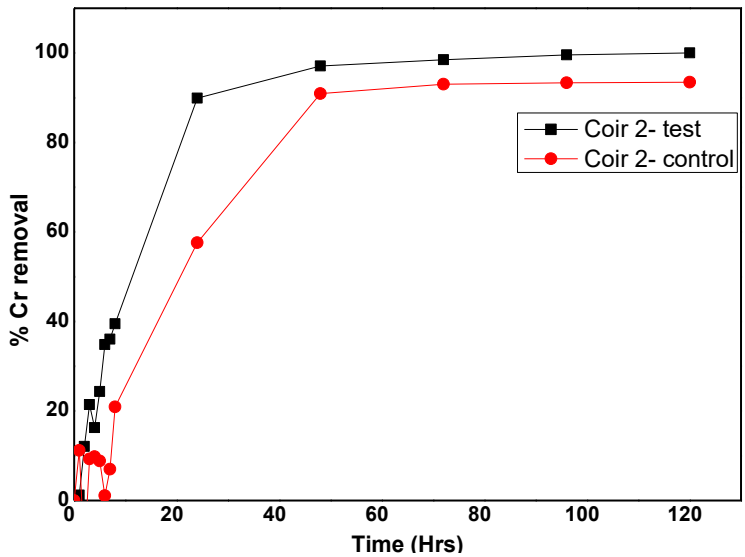


Figure 48: Graphical representation of the percentage chromium removal by coir with bacterial consortium and without bacterial consortium.

b) Biomat of orange peel

Orange peel powder was used for biomat preparation. Orange peel was collected from the market as waste product. Orange peel was washed thrice with the distilled water and dried in the oven at 70°C until completely dried. The powder was prepared in the electrical grinder. The powder obtained was sieved through the 1 mm size sieve to obtain uniform sized particle. This orange peel powder was mixed with the log phase bacterial consortium in MSM and incubated for three days in order to get maximum attachment of the bacteria. The CFU on the orange peel was found to be 7.98x10^12 per gram of the orange peel. Figure 49 shows the attached bacteria on the surface of the orange peel. The removal of the immobilized orange peel powder and orange peel powder without bacteria is given in the table 39.

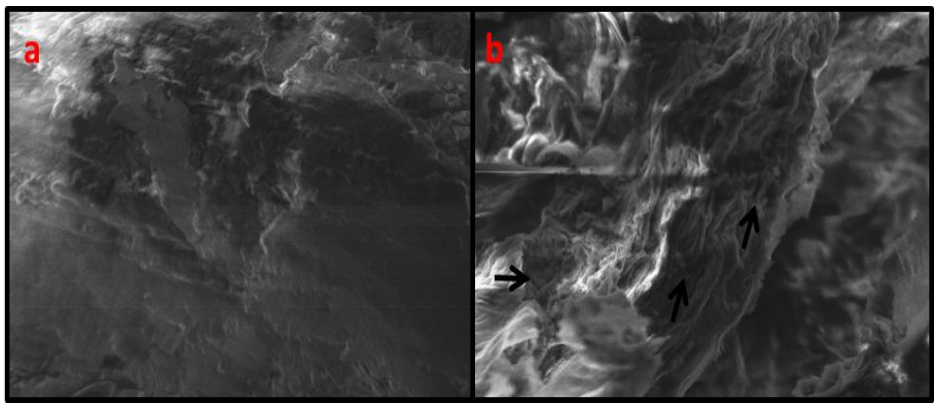


Figure 49: SEM analysis of the orange peel powder without bacteria (a) and immobilized with bacteria (b).

Table 39: Chromium removal by the bacterial consortium immobilized on orange peel and orange peel without bacteria.

Sr. No.	Time (hrs)	Cr removal by Immobilized orange peel powder (ppm)	Percent Cr removal	Cr removal by orange peel powder without bacteria (ppm)	Percent Cr removal
1	0	8.652	0	8.652	0
2	0.083	3.953	54.30	5.824	32.68
3	1	3.909	54.82	5.956	31.16
4	2	3.640	57.92	5.384	37.77
5	3	3.352	61.25	5.668	34.48
6	4	3.330	61.51	5.804	32.91
7	5	2.996	65.36	5.896	31.85
8	6	2.940	66.01	5.888	31.94
9	7	2.924	66.19	5.872	32.13
10	8	2.135	75.32	5.089	41.18

11	24	1.308	84.88	4.540	47.52
12	48	0.627	92.75	2.103	75.69
13	72	0.262	96.96	1.692	80.43
14	96	0.179	97.92	1.237	85.70
15	120	0.118	98.63	1.036	88.02

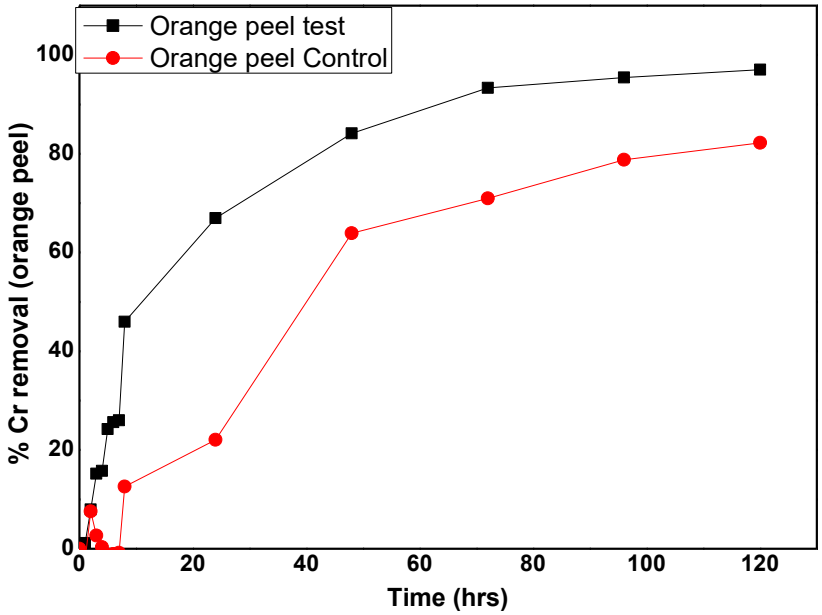


Figure 50: Graphical representation of the percentage chromium removal by orange peel with bacterial consortium and without bacterial consortium.

c) Biomat of corn cob

Corn cob is another waste product which is produced in the large quantity. Corn cob was used for biomat preparation. Corn cob was washed thrice with the distilled water and dried in the oven at 70°C until completely dried. The powder was prepared in the electrical grinder. The powder obtained was sieved through the 1 mm size sieve to obtain uniform sized particle (Ejaz et al, 2018, Paliwal et al 2015). This corn cob powder was mixed with the log phase bacterial consortium in MSM and incubated for three days in order to get maximum attachment of the bacteria.

The CFU count of the bacteria attached to the corn cob was found to be 1.6x10^13 per gram of the corn cob. Figure 51 shows the bacteria attached to the corn cob. The chromium removal by the corn cob with and without bacteria is given in the table 40.

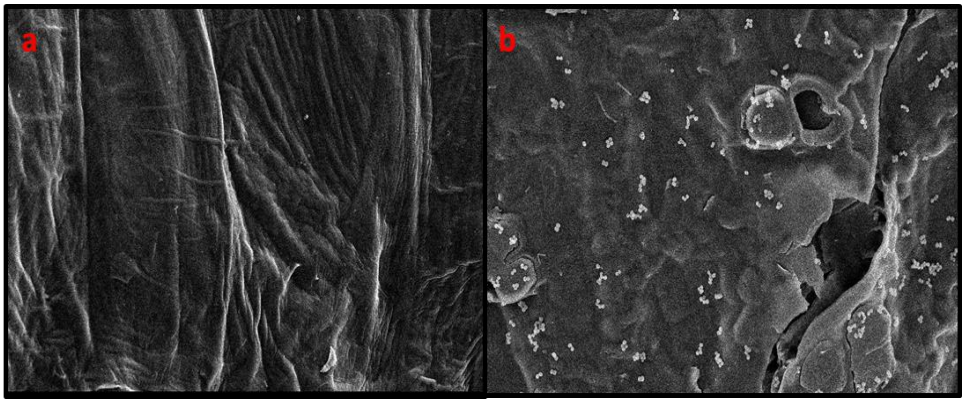


Figure 51: SEM analysis of the corn cob without bacteria (a) and immobilized with bacteria (b).

Table 40: Chromium removal by the bacterial consortium immobilized on corn cob and corn cob without bacteria.

Sr. No.	Time (hrs)	Cr removal by immobilized corn cob (ppm)	Percent Cr removal	Cr removal by corn cob without bacteria (ppm)	Percent Cr removal
1	0	8.652	0	8.652	0
2	0.083	6.648	23.16	7.336	15.21
3	1	6.544	24.36	6.888	20.38
4	2	6.480	25.10	6.884	20.43
5	3	6.208	28.24	7.200	16.78
6	4	6.272	27.50	7.152	17.33
7	5	6.188	28.47	7.192	16.87
8	6	6.100	29.49	7.240	16.31
9	7	6.104	29.44	6.764	21.82
10	8	5.730	33.77	6.253	27.72
11	24	4.504	47.94	6.056	30.00
12	48	3.580	58.62	5.855	32.33
13	72	2.859	66.95	4.708	45.58
14	96	2.616	69.76	4.376	49.42
15	120	2.146	75.19	4.080	52.84

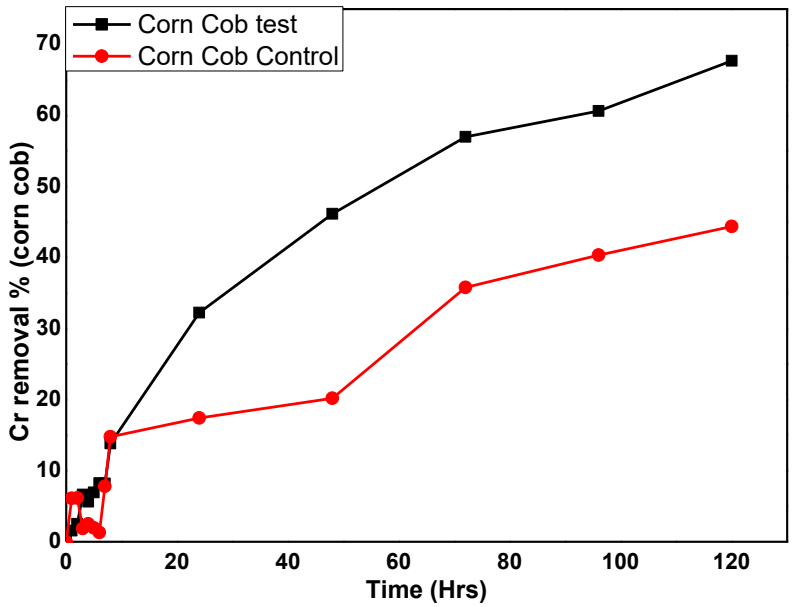


Figure 52: Graphical representation of the percentage chromium removal by corn cob with bacterial consortium and without bacterial consortium.

d) Biomat of the saw dust

Saw dust is large cellulosic waste generated from wood industry. Saw dust powder was collected from the local furniture shop (Hazaimeh, et al, 2014, Li et al 2016). The powder was washed thrice with the distilled water and dried in the oven at 70°C until completely dried. Uniform size particles were obtained by sieving the powder through the 1 mm size. This saw dust powder was mixed with the log phase bacterial consortium in MSM and incubated for three days in order to get maximum attachment of the bacteria.

The CFU count of the bacteria attached to the one gram of the saw dust was found to be $8.040x10^{12}$. The chromium removal by the biomat prepared by the saw dust with and without bacteria is given in table 41.

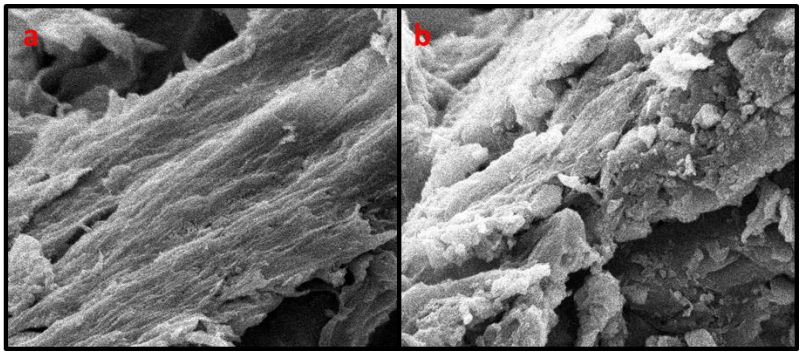


Figure 53: SEM analysis of the saw dust without bacteria (a) and immobilized with bacteria (b).

Table 41: Chromium removal by the bacterial consortium immobilized on saw dust and saw dust without bacteria.

Sr. No.	Time (hrs)	Cr removal by Immobilized saw dust (ppm)	Percentage Cr removal	Cr removal by saw dust without bacteria (ppm)	Percentage Cr removal
1	0	9.056	0	9.056	0
2	0.083	6.500	28.22	6.124	32.37
3	1	6.464	28.62	6.628	26.81
4	2	6.152	32.06	6.356	29.81
5	3	5.700	37.05	6.160	31.97
6	4	5.536	38.86	5.932	34.49
7	5	5.060	44.12	5.892	34.93
8	6	4.728	47.79	5.880	35.07
9	7	4.704	48.05	5.696	37.10
10	8	4.576	49.46	5.512	39.13
11	24	2.873	68.27	4.280	52.73
12	48	1.740	80.79	2.763	69.49
13	72	0.972	89.27	1.689	81.34
14	96	0.550	93.92	1.620	82.10
15	120	0.338	96.27	1.532	83.08

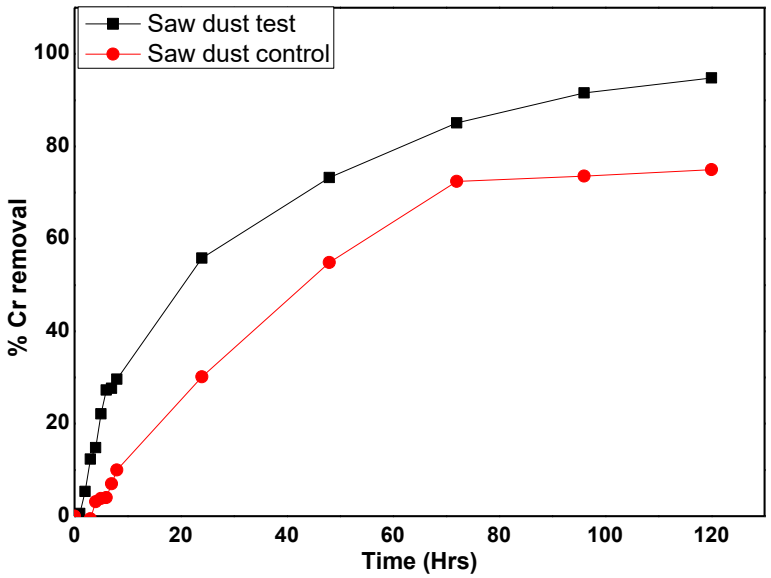


Figure 54: Graphical representation of the percentage chromium removal by saw dust with bacterial consortium and without bacterial consortium.

Table 42: CFU of the difference carrier material used to prepare the biomat of bacterial consortium.

Sr. No	Carrier	CFU/ml	CFU/g
1	Corn cob	3.337x10^11	1.6x10^13
2	Saw dust	1.6x10^11	8.040x10^12
3	Orange peel	1.94x10^11	7.98x10^12
4	Coir second method	9.08x 10^10	4.54x10^12
5	Coir	5.73x10^9	2.865x 10^11

Comparison of the chromium removal by biomat prepared using different biological waste material. Figure 55 shows the efficiency of the different material for chromium removal from the Mithi River water. Biomat prepared using the coconut coir is found to be best it could remove maximum amount of chromium in very short span of time as compare to other material. Feng et al, (2011), Ratan et al, (2016) and Podder et al (2016) have reported the heavy metal removal by biological waste and found similar kind of removal pattern as found by us.

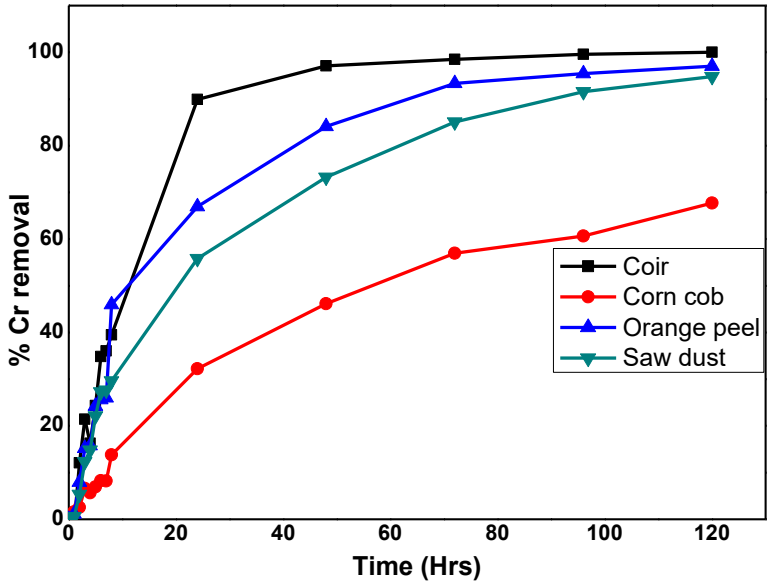


Figure 55: Graphical representation of the chromium removal by different biological waste immobilized with bacterial consortium.

Table 43: Comparison of different material used for biomat preparation in term of bacteria immobilized and chromium removed.

Material	Coconut coir		Orange peel		Saw dust		Corn cob	
CFU/g	4.54x10^12		7.98x10^12		8.040x10^12		1.6x10^13	
Removal	**Test**	**Control**	**Test**	**Control**	**Test**	**Control**	**Test**	**Control**
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.083	29.903	32.420	54.309	32.686	28.224	32.376	23.162	15.210
1	29.064	39.973	54.822	31.160	28.622	26.811	24.364	20.388
2	38.383	24.293	57.929	37.772	32.067	29.814	25.104	20.435
3	44.920	38.693	61.258	34.489	37.058	31.979	28.248	16.782
4	41.299	39.046	61.512	32.917	38.869	34.496	27.508	17.337
5	46.952	38.383	65.368	31.854	44.125	34.938	28.479	16.875
6	54.329	33.171	66.019	31.946	47.792	35.071	29.496	16.320
7	55.168	37.147	66.200	32.131	48.057	37.102	29.450	21.822
8	57.597	46.555	75.324	41.181	49.470	39.134	33.773	27.728
24	92.920	71.360	84.887	47.527	68.277	52.739	47.943	30.005
48	97.958	93.874	92.755	75.691	80.791	69.492	58.627	32.330
72	98.933	95.269	96.966	80.439	89.271	81.347	66.958	45.585
96	99.716	95.521	97.926	85.709	93.927	82.107	69.764	49.422
120	100.00	95.598	98.633	88.024	96.272	83.083	75.196	52.843

The bacterial consortium plated on nutrient plate. The isolated colonies with different morphology indicate that all the three type of the bacteria grow together (Figure. 56).

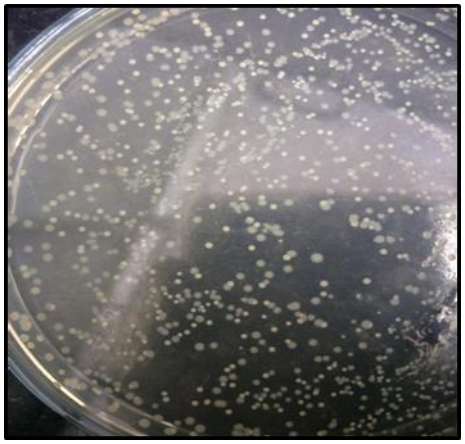
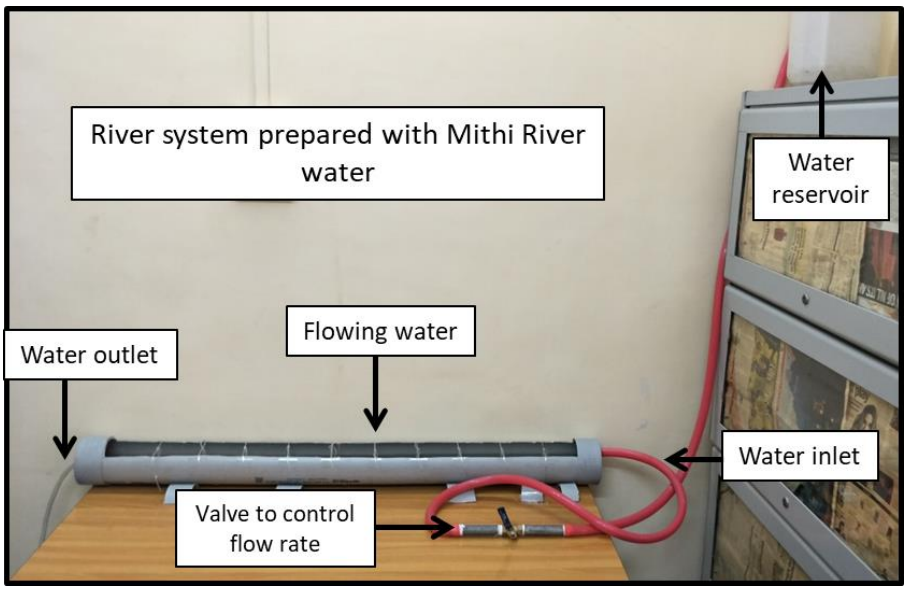


Figure 56: Isolated colonies obtained after plating the bacterial consortium on nutrient agar.

17. Bioremediation of the Heavy Metals by Simulating Mithi River conditions

Bioremediation of the heavy metal was carried out by replicating the Mithi River model in the lab. The system was prepared to replicate the condition of Mithi River in the lab. Water flowing system as river was prepared and Mithi River water was used in order to completely replicate the conditions of the Mithi River. A flowing system like river was prepared in the PVC pipe (4 inches diameter x 48 inches in length). The inlet and outlet was made by creating holes at the end of the pipe. The Mithi River water was flown in through the inlet pipe from a reservoir and the water flown was collected in the collecting chamber. Mithi River water was used heavy metal bioremediation experiment. Water flowing rate was 0.1562 litres/feet. Twenty grams of the total biomat was used for treating 15 litres of Mithi River water containing chromium. Ten pack of biomat was prepared each containing two grams of biomat material immobilized with the chromium resistant bacterial consortium.



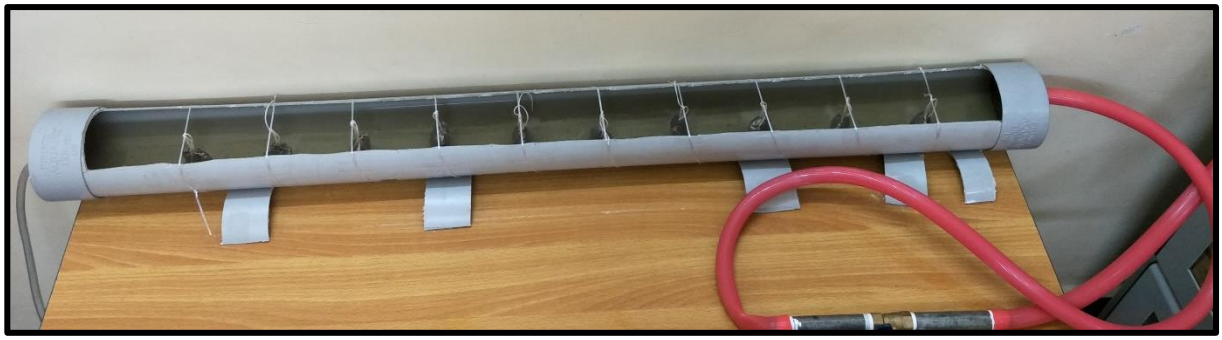


Figure 57: Mithi River water flowing system.

Initially the chromium analysis was done at interval of 1 hour of the water taken from the river system and water coming out of the river system after treatment. Table 44 show the chromium removal while water was flowing through the system.

Table 44: Chromium removal by the Biomat prepared using coconut coir in the Mithi River flowing model.

Time	Cr in water (ppm) taken from water system	% Cr removal	Cr in water (ppm) taken from water coming out of system	% Cr removal
5 mins	6.58	0.15	NA	
1 hr	6.49	1.51	6.055	8.11
2 hrs	6.29	4.55	6.605	-0.22
3 hrs	6.31	4.24	6.345	3.71
4 hrs	6.2	5.91	6.13	6.98
5 hrs	6.065	7.96	6.35	3.64
6 hrs	5.305	19.49	5.865	11.00
7 hrs	6.06	8.04	6.485	1.59
8 hrs	6.335	3.86	5.805	11.91
9 hrs	6.275	4.77997	6.34	3.79
24 hrs	5.875	10.84977	6.78	-2.88
25 hrs	5.76	12.59484	6.1	7.43

Chromium removal study was continued for another 6 cycles. Same 15 litres of Mithi River water which was used for initial treatment was repeatedly used for chromium treatment for next cycle. Same water was collected and flown through the same biomat to determine the time required for complete chromium removal as well as to find the efficiency of the biomat for its repeated use.

Total 15 litres of water was flown through the 20 g biomat material in 10 packs in the river system. The water was flown at the rate of 0.625 litres per hour.

Table 45: Chromium removal from the Mithi River water in the river system by biomat at 6 treatment cycles of 24 hrs each

Time	Solution taken from river system (ppm)	% Cr removal
First treatment cycle	5.37	18.51
Second treatment cycle	2.17	66.94
Third treatment cycle	1.80	72.67
Fourth treatment cycle	1.73	73.61
Fifth treatment cycle	1.57	76.04
Sixth treatment cycle	1.47	77.57

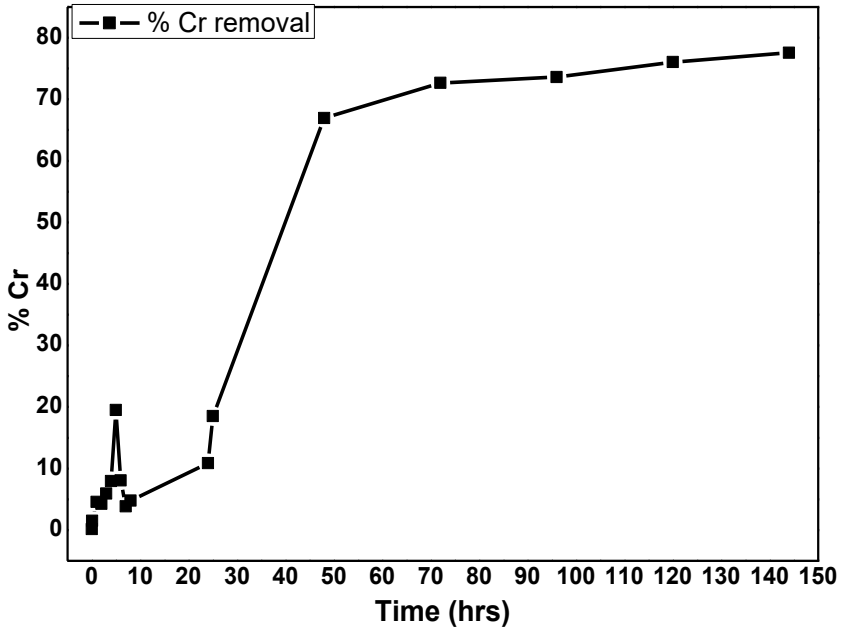


Figure 58: Percentage chromium removed by coconut coir biomat with immobilized bacteria applied to the Mithi River flowing system in the lab.

18. Project Extension work

The number of attached bacteria to the biomaterial has very crucial role in the chromium removal. The above experiment show lesser difference in chromium removal between coir and coir immobilized with bacteria. In order to improve the chromium removal by coir immobilized with

bacteria, the number of attached bacteria was increase by standardizing the method of chromium resistant bacteria immobilization on coir as well as on corn cob.

A) Increasing the number of immobilized bacteria

On the basis of available literature factor which affect the bacterial immobilization was found out and studied to increase the attachment. Exopolysaccharide (EPS) production by bacteria, initial bacterial cell biomass and time period for immobilization were few important factor which was consider for enhancing the attachment. These three parameters were standardized individually keeping the other factors constant.

1) Effect of NaCl concentration on exopolysaccharide (EPS) production by Cr bioremediating bacterial consortium

High concentration of salt in the bacterial medium stimulates bacteria for EPS production. EPS has major role in biofilm formation and attachment of bacterial to substratum. Effect of NaCl was studied by growing the bacterial consortium in Minimal Salt Medium (MSM) at different concentration of NaCl salt for overnight. The EPS produced in response to different NaCl concentration was determined using alcian blue assay. Alcian blue is dye which binds with EPS and the dye can be separated from culture by centrifugation. The unbound dye remains in the supernatant and give high absorbance. The growth of the bacterial consortium and absorbance of supernatant after reaction of dye with EPS is given in the table 46.

Table 46: Effect of NaCl on EPS production by bacteria.

Conc. (NaCl) %	Control MSM (0.05%)	0.5%	1 %	2%	3%
Growth of bacterial consortium In terms of OD at 600 nm	1.448	1.334	1.332	1.229	1.218
Alcian blue assay Absorbance at 570 nm	0.127	0.086	0.066	0.063	0.0695

The optical density (OD) of the culture indicates minor effect of NaCl on the growth of bacteria. The absorbance of the supernatant after reaction with alcian blue was decreased up to 50%. This observation was due to reaction of alcian blue with EPS. High the secreted EPS more the dye will bind to it and lead to decrease in absorbance.

The above data indicate that bacterial consortium produce more EPS in presence of 2% of salt in the medium. On the basis of above data further immobilization was carried out in 2% of salt concentration.

2) Study of effect of initial cell number of immobilization

Initial cell number for immobilization on biomaterial is important factor for maximum attachment of the bacteria. Various quantities of bacteria were achieved by collecting the bacterial biomass from various volume of growth culture (nutrient broth). Different quantity of the initial biomass was tested to check the attachment. The various quantity of bacterial biomass was transferred to flask containing MSM (2% NaCl) and coir under sterile condition. These flasks were incubated for 3 days in shaking condition. After incubation was coir was collected of each flask washed twice with sterile saline and resuspended in 50 ml of saline and subjected to

vigorous shaking for 2-3 hours. The supernatant was diluted appropriately and spread plated to determine the bacterial count. The immobilized bacterial in different initial biomass is given in table 47. It was found that as the initial biomass was increased the number of attached bacteria was also increased.

Table 47: Effect of biomass on bacterial attachment.

Sr. No.	Biomass used for immobilization	Immobilized bacterial CFU/g of coir
1	200 ml of grown biomass/ g of coir	$8.25x10^{11}$
2	300 ml of grown biomass/ g of coir	$6x10^{12}$
3	400 ml of grown biomass/ g of coir	$4.7x10^{13}$
4	500 ml of grown biomass/ g of coir	$1x10^{15}$

Maximum attachment was observed at biomass collected from 500 ml of culture medium. For further experiment biomass from 500 ml culture is used per gram of coir for immobilization.

3) Study the effect of incubation time on immobilization

To achieve the maximum attachment of bacteria on coir, various time period of incubation of bacterial biomass was coir was studied. It was found that maximum attachment was observed at 2 days incubation period (table 48). As the time was increase the CFU immobilized per gram of coir.

Table 48: Effect of incubation time on bacterial attachment.

Sr. No.	Biomass used for immobilization	Immobilized bacterial CFU/g of coir
1	1 day incubation with coir	$6.1x\ 10^{15}$
2	2 day incubation with coir	$6.7x\ 10^{15}$
3	3 day incubation with coir	$1.7x10^{15}$
4	4 day incubation with coir	$3.1x\ 10^{15}$

Maximum immobilization was observed at 2 day incubation. Therefore, immobilization of the coir with chromium consortium was done 2 days.

The above attachment experiments have shown that the maximum attachment was observed at 500 ml of grown bacterial culture was incubated in MSM containing 2% of salt with coir. These standardized conditions were also tested with corn cob as attachment material. These attachment conditions were found to be suitable for corn cob also.

Table 49: Comparison of bacteria attached to the biomaterial by previous and present method.

Sr. No.	Biomaterial	Immobilized bacteria CFU/g of material using previous method	Immobilized bacteria CFU/g of material using new standardized method
1	Coir	4.45×10^{12}	6.7×10^{15}
2	Corn cob	1.6×10^{13}	1.03×10^{15}

B) Chromium removal Study

1) Chromium removal study was first performed at lab scale in the flask with 50 ml of Mithi River water with coir and coir with immobilized bacteria before doing the experiment at in river model system.
2) Changes in the Mithi river model (as suggested by the committee)
   - A mat was prepared on metal wire frame to hold the biomaterial. The mat was put in cross section so that water passed through the mat. This change avoided the cris-cross flow of water.
   - Baffles were placed before the biomat location in the river model. Baffle was used to avoid the flow of water only to the upper layer. Baffle insures mixing of the water and maximum exposure water to biomat.

Figure 59: Image of biomat and baffles placed in the river model system for chromium treatment.

3) Chromium removal study was continued for another 6 cycles. Same 15 litres of Mithi River water which was used for initial treatment was repeatedly used for chromium treatment for next cycle. Same water was collected and flown through the same biomat to determine the time required for complete chromium removal as well as to find the efficiency of the biomat for its repeated use. Total 15 litres of water was flown through the 20 g biomat material in 5 mats each containing 4 gram of biomaterial in the river system. The water was flown at the rate of 0.625 litres per hour. The initial concentration of chromium was 4 ppm (highest reported in literature).

Table 50: Cr removal by coir in River model. Water sample were taken from the starting point of the river model for chromium analysis.

Time	**Cr quantification by DPC (Cr^{6+})**		**Cr quantification by AAS (Cr^{6+} & Cr^{3+})**		**(Cr^{3+}) (ppm)**
	Cr^{6+} conc. (ppm)	**% removal**	**Cr^{6+} and Cr^{3+} conc. (ppm)**	**% removal**	
0 hrs	1.301	0	3.243	0.000	1.942
6 hrs start	1.148	11.760	2.262	30.250	1.114
12 hrs start	1.265	2.767	1.584	51.156	0.319
1 day start	0.971	25.304	0.621	80.851	-0.351
2 day start	0.571	56.080	0.594	81.684	0.023
3 day start	0.346	73.344	0.432	86.679	0.085
4 day start	0.0	100	0.063	98.057	0.063
5 day start	0.0	100	0.351	89.177	0.351
6 day start	0.0	100	0.666	79.463	0.666

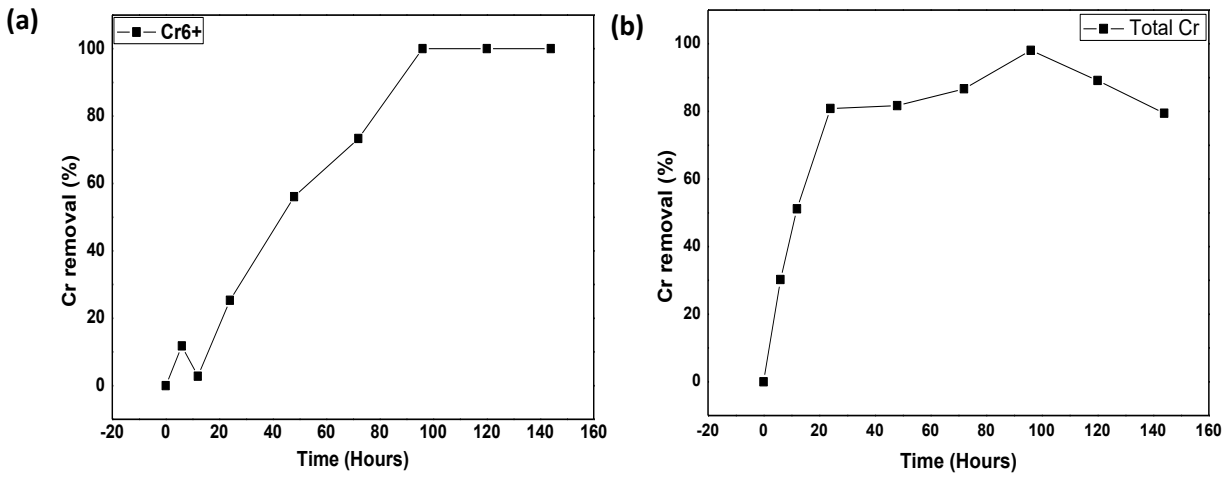


Figure 60: Graphical presentation of chromium removal at starting by coir in river model. (a) show the Cr^{6+-} reduction and (b) show total chromium (Cr^{6+} and Cr^{3+}).

Table 51: Cr removal by coir in River model. Water samples were taken from the middle point of the river model for chromium analysis.

Time	**Cr quantification by DPC (Cr^{6+})**		**Cr quantification by AAS (Cr^{6+} & Cr^{3+})**		**(Cr^{3+}) (ppm)**
	Cr^{6+} conc. (ppm)	**% removal**	**Cr^{6+} and Cr^{3+} conc. (ppm)**	**% removal**	
0 hrs	1.301	0	3.243	0.000	1.942
6 hrs mid	0.866	33.382	3.582	-10.453	2.715
12 hrs mid	0.978	24.819	1.089	66.420	0.111
1 day mid	0.969	25.488	0.756	76.688	-0.213
2 day mid	0.234	81.983	0.792	75.578	0.558
3 day mid	0.215	83.459	0.297	90.842	0.082
4 day mid	0.0	100	0.128	96.053	0.128
5 day mid	0.0	100	1.035	68.085	1.035
6 day mid	0.0	100	0.756	76.688	0.756

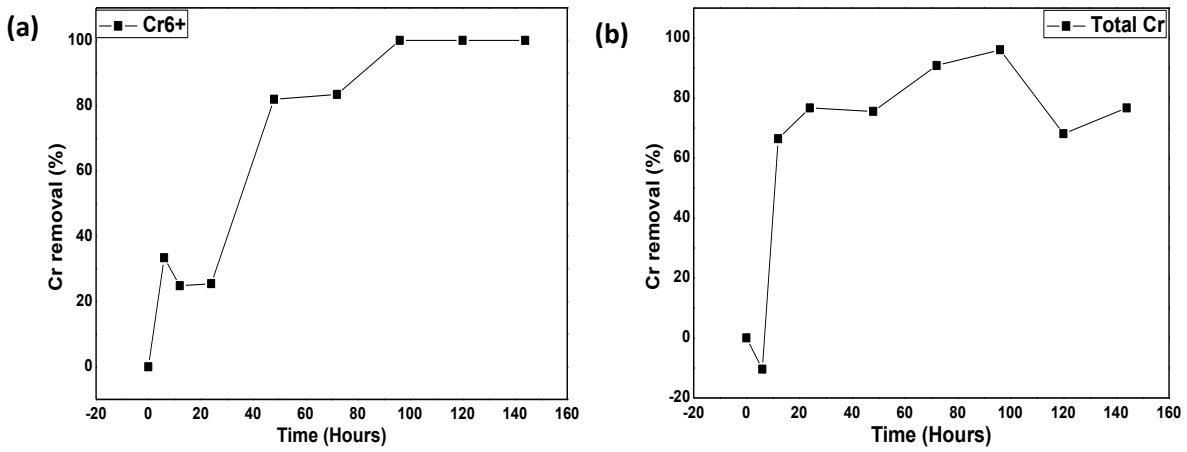


Figure 61: Graphical presentation of chromium removal at middle point by coir in river model. (a) show the Cr^{6+-} reduction and (b) show total chromium (Cr^{6+} and Cr^{3+}).

Table 52: Cr removal by coir in River model. Water samples were taken from the end point of the river model for chromium analysis.

Time	Cr quantification by DPC (Cr^{6+})		Cr quantification by AAS (Cr^{6+} & Cr^{3+})		(Cr^{3+}) (ppm)
	Cr^{6+} conc. (ppm)	% removal	Cr^{6+} and Cr^{3+} conc. (ppm)	% removal	
0 hrs	1.301	0	3.243	0.000	1.942
6 hrs end	0.736	43.420	1.368	57.817	0.632
12 hrs end	0.908	30.138	0.99	69.473	0.081
1 day end	0.532	59.070	0.333	89.732	-0.200
2 day end	0.050	96.151	0.252	92.229	0.202
3 day end	0.375	71.168	0.837	74.191	0.462
4 day end	0.0	100	0.162	95.005	0.162
5 day end	0.0	100	0.594	81.684	0.594
6 day end	0.0	100	1.039	67.962	1.039

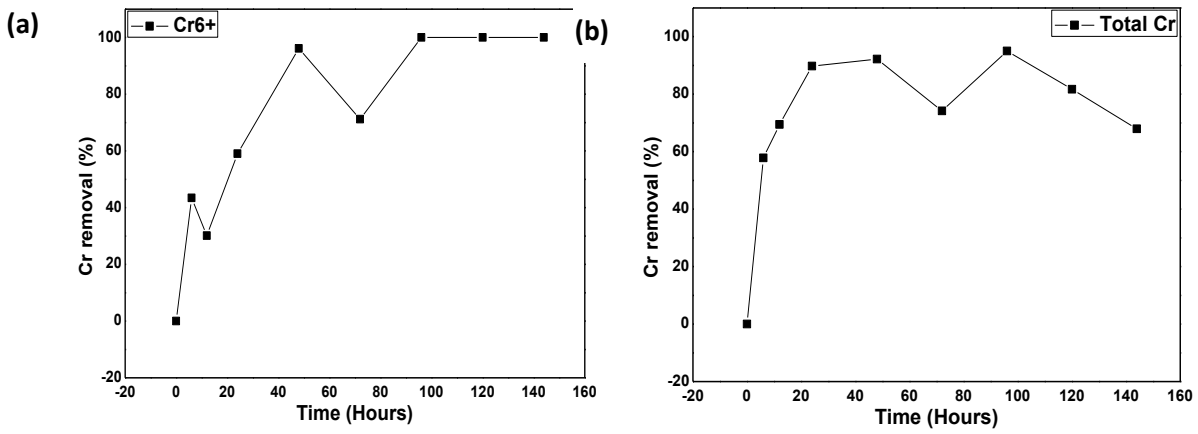


Figure 62: Graphical presentation of chromium removal at end point by coir in river model. (a) show the Cr^{6+-} reduction and (b) show total chromium (Cr^{6+} and Cr^{3+}).

Table 53: Cr removal by coir in River model. Water samples were taken after mixing the total water has flown through coir of the river model for chromium analysis.

Time	Cr quantification by DPC (Cr^{6+})		Cr quantification by AAS (Cr^{6+} & Cr^{3+})		(Cr^{3+}) (ppm)
	Cr^{6+} conc. (ppm)	% removal	Cr^{6+} and Cr^{3+} conc. (ppm)	% removal	
0 hrs	1.301	0	3.243	0.000	1.942
6 hrs total	0.890	31.537	2.052	36.725	1.161
12 hrs total	1.200	7.763	0.963	70.305	-0.237
1 day total	0.654	49.693	0.72	77.798	0.066
2 day total	0.080	93.826	0.49	84.891	0.410
3 day total	0.272	79.032	1.62	50.046	1.347
4 day total	0.0	100	1.107	65.865	1.107
5 day total	0.0	100	1.278	60.592	1.278
6 day total	0.0	100	1.242	61.702	1.242

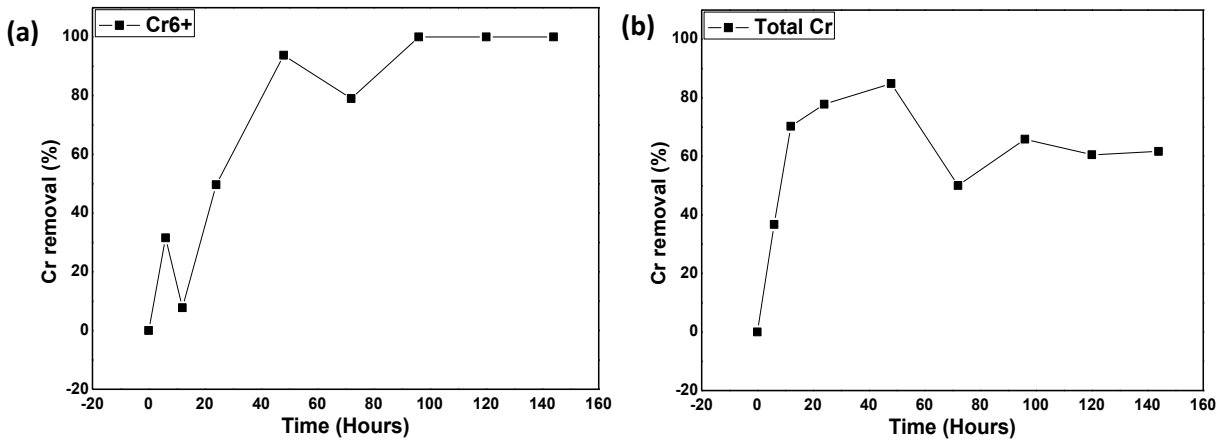


Figure 63: Graphical presentation of chromium removal from total water by coir in river model. (a) show the Cr^{6+} reduction and (b) show total chromium (Cr^{6+} and Cr^{3+}).

The chromium removal was studied using coir immobilized with chromium resistant bacterial consortium. All the conditions were maintained as the control experiment (chromium removal using coir) except the biomaterial used was coir immobilized with bacteria. The experiment was run for 6 cycle of treatment. Water sample was withdrawn from various points of river model system and also at various time points.

Table 54: Cr removal by coir immobilized with bacterial consortium in River model. Water samples were taken from the starting point of the river model for chromium analysis.

Time	Cr quantification by DPC (Cr^{6+})		Cr quantification by AAS (Cr^{6+} & Cr^{3+})		(Cr^{3+}) (ppm)
	Cr^{6+} conc. (ppm)	% removal	Cr^{6+} and Cr^{3+} conc. (ppm)	% removal	
0 hrs	1.66	0.00	5.58	0.00	3.91
6 hrs start	1.60	3.67	3.10	44.48	1.50
12 hrs start	1.35	18.53	4.25	23.80	2.90
1 day start	1.64	1.56	3.12	44.05	1.48
2 day start	0.04	97.68	2.26	59.54	2.22
3 day start	0.00	100.00	0.29	94.84	0.29
4 day start	0.00	100.00	0.02	99.57	0.02
5 day start	0.00	100.00	0.00	100.00	0.00
6 day start	0.00	100.00	0.00	100.00	0.00

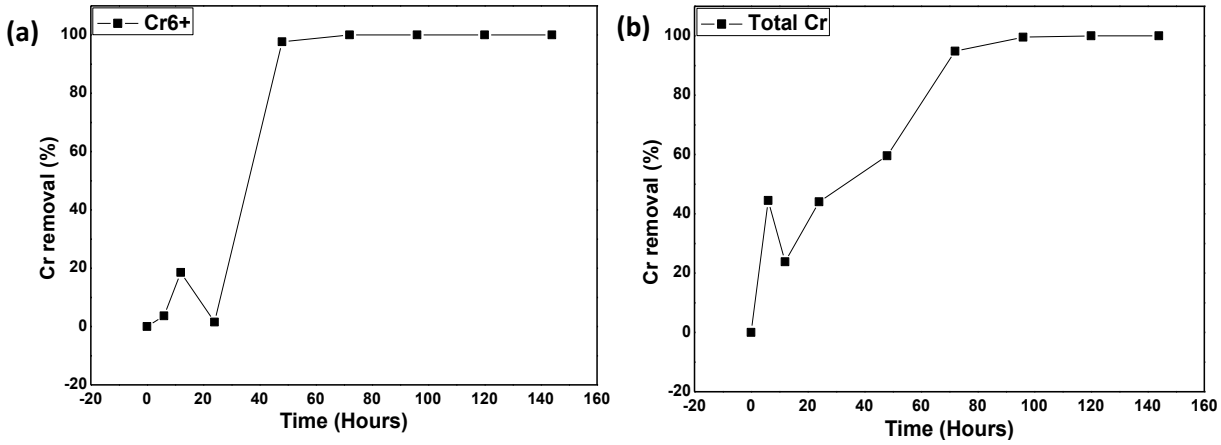


Figure 64: Graphical presentation of chromium removal at starting point by coir immobilized with bacteria in river model. (a) show the Cr^{6+-} reduction and (b) show total chromium (Cr^{6+} and Cr^{3+}).

Table 55: Cr removal by coir immobilized with bacterial consortium in River model. Water samples were taken from the middle point of the river model for chromium analysis.

Time	Cr quantification by DPC (Cr^{6+})		Cr quantification by AAS (Cr^{6+} & Cr^{3+})		(Cr^{3+}) (ppm)
	Cr^{6+} conc. (ppm)	% removal	Cr^{6+} and Cr^{3+} conc. (ppm)	% removal	
0 hrs	1.66	0.00	5.58	0.00	3.91
6 hrs mid	0.88	47.29	4.51	19.08	3.64
12 hrs mid	1.27	23.89	4.04	27.56	2.77
1 day mid	1.67	-0.60	2.66	52.37	0.98
2 day mid	0.00	100.00	1.99	64.28	1.99
3 day mid	0.00	100.00	0.59	89.38	0.59
4 day mid	0.00	100.00	0.17	96.99	0.17
5 day mid	0.00	100.00	0.05	99.14	0.05
6 day mid	0.00	100.00	0.00	100.00	0.00

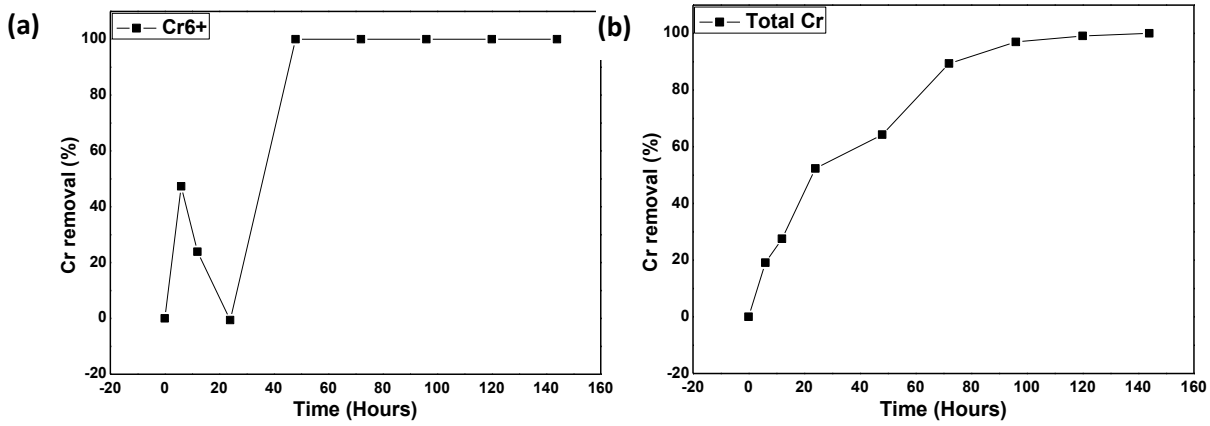


Figure 65: Graphical presentation of chromium removal at middle point by coir immobilized with bacteria in river model. (a) show the Cr^{6+-} reduction and (b) show total chromium (Cr^{6+} and Cr^{3+}).

Table 56: Cr removal by coir immobilized with bacterial consortium in River model. Water samples were taken from the end point of the river model for chromium analysis.

Time	Cr quantification by DPC (Cr^{6+})		Cr quantification by AAS (Cr^{6+} & Cr^{3+})		(Cr^{3+}) (ppm)
	Cr^{6+} conc. (ppm)	% removal	Cr^{6+} and Cr^{3+} conc. (ppm)	% removal	
0 hrs	1.66	0.00	5.58	0.00	3.91
6 hrs end	0.84	49.69	4.02	27.98	3.18
12 hrs end	1.13	32.31	3.87	30.52	2.75
1 day end	0.42	74.80	3.90	29.99	3.49
2 day end	0.00	100.00	1.58	71.74	1.58
3 day end	0.00	100.00	0.35	93.69	0.35
4 day end	0.00	100.00	0.03	99.43	0.03
5 day end	0.00	100.00	0.00	100.00	0.00
6 day end	0.00	100.00	0.00	100.00	0.00

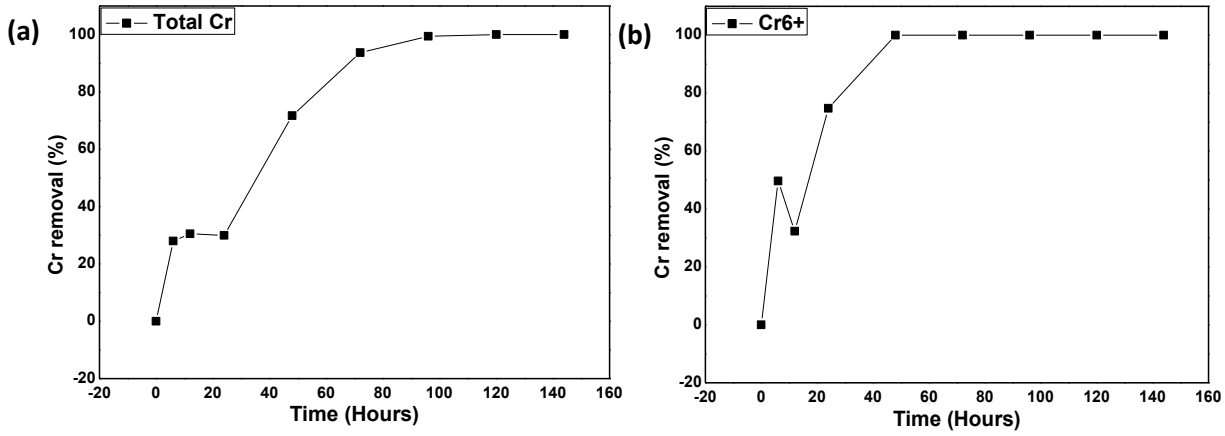


Figure 66: Graphical presentation of chromium removal at end point by coir immobilized with bacteria in river model. (a) show the Cr^{6+-} reduction and (b) show total chromium (Cr^{6+} and Cr^{3+}).

Table 57: Cr removal by coir immobilized with bacterial consortium in River model. Water sample was taken after mixing the total water has flown through coir of the river model for chromium analysis.

Time	**Cr quantification by DPC (Cr^{6+})**		**Cr quantification by AAS (Cr^{6+} & Cr^{3+})**		**(Cr^{3+}) (ppm)**
	Cr^{6+} conc. (ppm)	**% removal**	**Cr^{6+} and Cr^{3+} conc. (ppm)**	**% removal**	
0 hrs	1.66	0.00	5.58	0.00	3.91
6 hrs total	0.92	44.92	4.29	23.01	3.38
12 hrs total	1.45	12.76	3.36	39.69	1.91
1 day total	0.90	45.98	4.07	26.97	3.17
2 day total	0.00	100.00	2.02	63.85	2.02
3 day total	0.00	100.00	0.74	86.66	0.74
4 day total	0.00	100.00	0.23	95.84	0.23
5 day total	0.00	100.00	0.00	100.00	0.00
6 day total	0.00	100.00	0.00	100.00	0.00

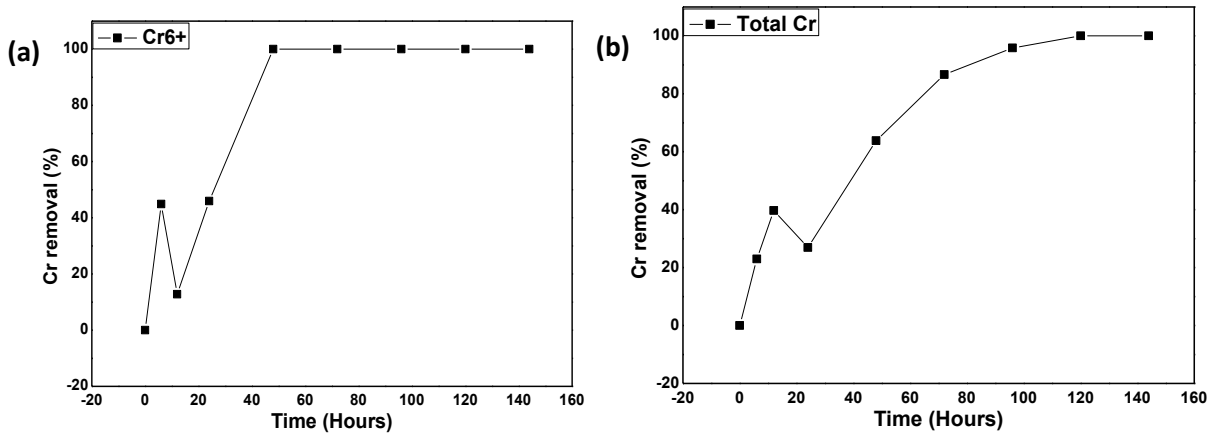


Figure 67: Graphical presentation of chromium removal from total water by coir immobilized with bacteria in river model. (a) show the Cr^{6+-} reduction and (b) show total chromium (Cr^{6+} and Cr^{3+}).

Comparison of the chromium removal pattern using coir (control) and coir immobilized with bacterial consortium. Various graphs below show the comparative removal of chromium along the different point of river model system.

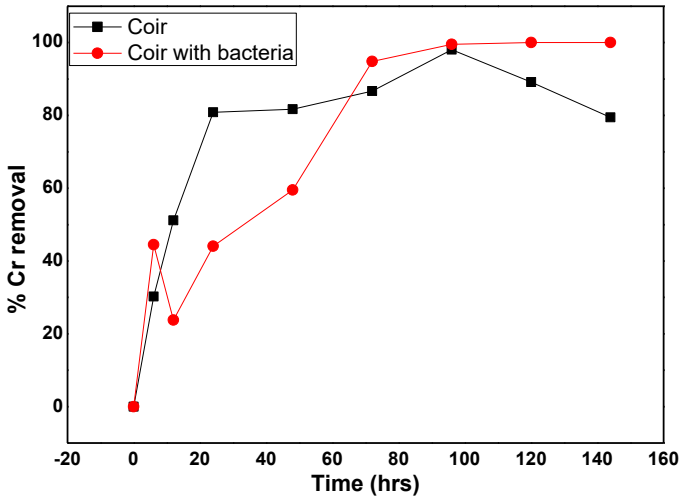


Figure 68: Chromium removal (%) by coir and coir immobilized with bacteria. Water samples were taken from staring point of river model (AAS data).

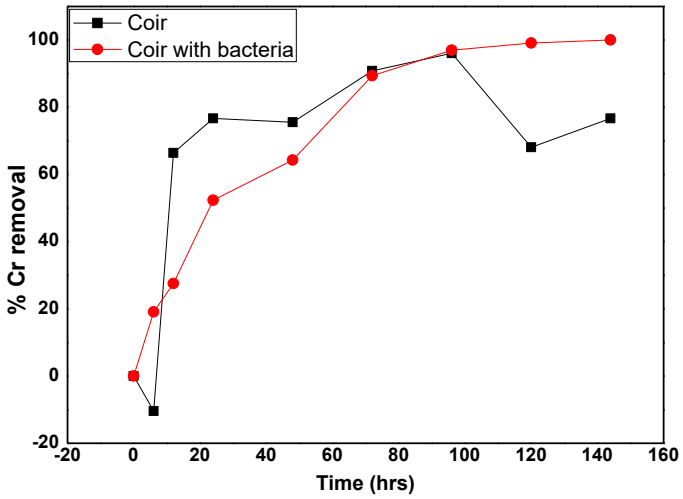


Figure 69: Chromium removal (%) by coir and coir immobilized with bacteria. Water samples were taken from middle point of river model (AAS data).

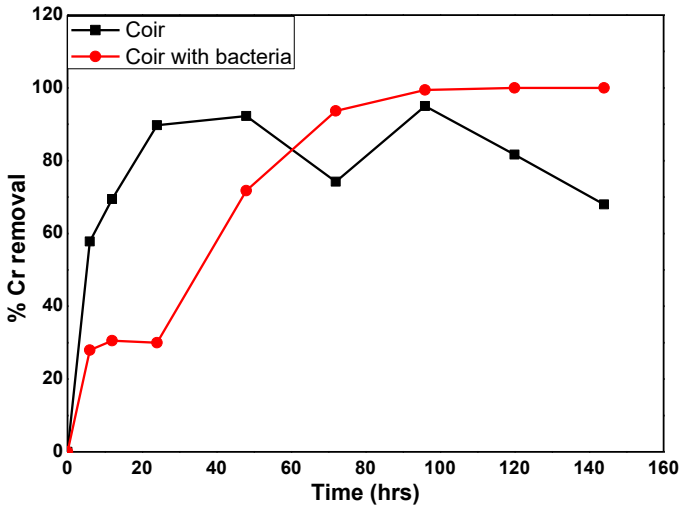


Figure 70: Chromium removal (%) by coir and coir immobilized with bacteria. Water samples were taken from end point of river model (AAS data).

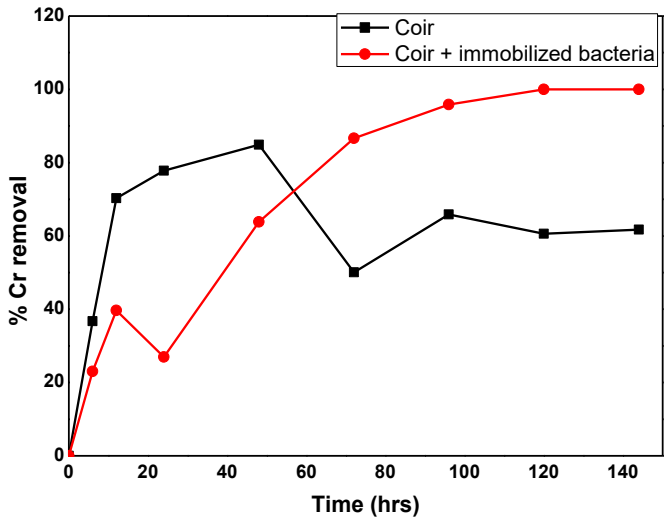


Figure 71: Comparison of percentage chromium removal by coir and coir immobilized with chromium resistant consortium. Chromium quantification was done using AAS (Cr^{3+} and Cr^{6+}).

The above graph (figure 71) is comparison of chromium removal by coconut coir (black line) and coconut coir immobilized with chromium resistant bacterial consortium. The pattern of total chromium removal by coir and immobilized coir is quite different. The coir show sharp increase in the removal within 12 hour exposure. The coir has chromium adsorption property. Therefore, chromium coir has shown the initial adsorption of chromium which start desorption after 48 hrs (2 days) of exposure. While, the chromium removal pattern of immobilized coir shows initial little increase in percentage removal then after that the percentage removal decreases a bit and then it showed steady increase in percentage removal of chromium. Coir immobilized with chromium resistant bacterial consortium show 100% removal of total chromium. Previous study of chromium removal using coir immobilized with bacterial had showed 77.57% chromium removal at sixth cycle of treatment of Mithi River water in river model system. With the increased number of bacteria on coir can completely bioremediate total chromium from the Mithi River water.

These experiments show that the biomat prepared using coir and chromium resistant bacteria can be applied on field for chromium removal.

i) As river water contains numerous other chemicals and pollutants which can interfere with the chromium removal efficiency of biomat. Biomat prepared in the present study was tested for its efficiency in Mithi River water itself without adding any other nutrient to support the bacteria. The bacterial immobilized bacteria can withstand the other chemicals and pollutant present in the Mithi River water and still can retain its efficiency.

ii) The biomat can stably take up the chromium without desorbing it back to the surrounding. As the heavy metal cannot be degraded, it can be either converted to less toxic form or sequester in, making it unavailable for imparting its toxic effect. Biomat stably take-up the chromium and making it unavailable for reaction.

19. Compilation of data and closure report

All the results of the project work were compiled. The data of the project work was presented in different manners like graphs, tables, Figures etc. at each stage of the project to MMR-EIS. The data obtained in the project work were submitted to various journals for publication. MMR-EIS is acknowledged in all the submitted research articles for providing the financial support. The copy of the research articles will be given to MMR-EIS upon publication.

20. Other work done related to project

Study the resistance of the isolated bacteria to other toxic heavy metals

Bacteria were isolated against other toxic heavy metals like arsenic, cadmium and lead. These bacteria were also studied for their bioremediation potential and possible application.

21. Conclusion

Through this project work, it is confirmed that the condition of Mithi River is very critical and required urgent attention for the possible treatment in order to restore the river. Various physico-chemical parameter have showed to be higher which confirm the contamination of the Mithi River water. Isolation work indicated that the Mithi River harbour numerous chromium and mercury resistant bacteria. The high number of heavy metal resistant bacteria in the river may be due to constant exposure of river microbiota to these heavy metal.

The indigenous bacterial from Mithi River was isolated and tested for their application for bioremediation of heavy metals from the Mithi River. The bacteria could remove the heavy metal very efficiently. The bioremediation was carried out at pilot scale by simulating the Mithi River conditions. Biomat was prepared by immobilizing the heavy metal resistant bacterial consortium on the various matrix. Coconut coir was found to be most effective matrix for bacterial immobilization. The pilot scale study for chromium removal with Mithi river water was conducted. This study showed the biomat could completely reduce the chromium to nontoxic form in 2 days and completely remove total chromium in period of 5 days from the Mithi river water.

22. Deliverable

Paper published

1) Pushkar, B., Sevak, P., & Sounderajan, S. (2019). Assessment of the bioremediation efficacy of the mercury resistant bacterium isolated from the Mithi River. *Water Science and Technology: Water Supply*, *19*(1), 191-199.
2) Pushkar, B., Sevak, P., & Singh, A. (2019). Bioremediation treatment process through mercury-resistant bacteria isolated from Mithi river. *Applied Water Science*, *9*(4), 117.

Paper to be published

1) Study on chromium bioremediation using Acinetobacter isolated from Mithi River.
2) Isolation and characterization of heavy metal resistant bacteria from Mithi River.

Patent

We will be filing the patent of the biomat prepared by unique combination of bacterial consortium isolated form Mithi River and coir as a matrix. MMR-EIS will be one of the patentee.

23. References

1. Wu, Jing, et al. "Comparison of DNA extraction methods for human oral microbiome research." *British Journal of Medicine and Medical Research*4.10 (2014): 1980
2. Lin, Meng, et al. "Use of bacteria-immobilized cotton fibers to absorb and degrade crude oil." *International Biodeterioration & Biodegradation* 88 (2014): 8-12.
3. Liu, Jie, et al. "Sugarcane bagasse as support for immobilization of Bacillus pumilus HZ-2 and its use in bioremediation of mesotrione-contaminated soils." *Applied microbiology and biotechnology* 99.24 (2015): 10839-10851.
4. Hrenovic, Jasna, TomislavIvankovic, and DarkoTibljas. "The effect of mineral carrier composition on phosphate-accumulating bacteria immobilization." *Journal of hazardous materials* 166.2-3 (2009): 1377-1382.
5. Gupta, Ankur, and ChandrajitBalomajumder. "Simultaneous removal of Cr (VI) and phenol from binary solution using Bacillus sp. immobilized onto tea waste biomass." *Journal of Water Process Engineering* 6 (2015): 1-10.
6. Obuekwe, C. O., and Esraa M. Al-Muttawa. "Self-immobilized bacterial cultures with potential for application as ready-to-use seeds for petroleum bioremediation." *Biotechnology Letters*23.13 (2001): 1025-1032.
7. Ejaz, Uroosa, Asia Ahmed, and Muhammad Sohail. "Statistical optimization of immobilization of yeast cells on corncob for pectinase production." *Biocatalysis and Agricultural Biotechnology* 14 (2018): 450-456.
8. Paliwal, Rashmi, ShivaniUniyal, and J. P. N. Rai. "Evaluating the potential of immobilized bacterial consortium for black liquor biodegradation." *Environmental Science and Pollution Research* 22.9 (2015): 6842-6853.
9. Hazaimeh, Mohammad, et al. "Enhanced crude oil hydrocarbon degradation by self-immobilized bacterial consortium culture on sawdust and oil palm empty fruit bunch." *Annals of microbiology* 64.4 (2014): 1769-1777.
10. Li, Jinghua, et al. "Bioremediation of petroleum-contaminated acid soil by a constructed bacterial consortium immobilized on sawdust: influences of multiple factors." *Water, Air, & Soil Pollution* 227.12 (2016): 444.
11. Nunal, Sharon N., et al. "Bioremediation of heavily oil-polluted seawater by a bacterial consortium immobilized in cocopeat and rice hull powder." *Biocontrol science* 19.1 (2014): 11-22.
12. Feng, Ningchuan, et al. "Biosorption of heavy metals from aqueous solutions by chemically modified orange peel." *Journal of hazardous materials* 185.1 (2011): 49-54.

13. Ratan, S., et al. "The Removal of Nickel from Waste Water by Modified Coconut Coir Pith." *Chem Sci J* 7.136 (2016): 10-60.
14. Podder, M. S., and C. B. Majumder. "Corynebacterium glutamicum MTCC 2745 immobilized on granular activated carbon/MnFe2O4 composite: A novel biosorbent for removal of As (III) and As (V) ions." *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* 168 (2016): 159-179.
15. Guo, Hanjun, et al. "Bioremediation of heavy metals by growing hyperaccumulaor endophytic bacterium Bacillus sp. L14." *Bioresource technology* 101.22 (2010): 8599-8605.
16. Gao, Yang, and Jun Xia. "Chromium contamination accident in China: viewing environment policy of China." *Environmental Science and Technology-Columbus* 45.20 (2011): 8605.
17. Dash, H.R. and Das, S., 2015. Bioremediation of inorganic mercury through volatilization and biosorption by transgenic Bacillus cereus BW-03 (p PW-05). *International Biodeterioration& Biodegradation*, *103*, 179-185.
18. Kotaś, J., and Z. Stasicka. "Chromium occurrence in the environment and methods of its speciation." *Environmental pollution* 107.3 (2000): 263-283.
19. Schedule VI inserted by Rule 2(d) of the Environment (Protection) Second Amendment Rules, 1993 notified vide G.S.R. 422(E) dated 19.05.1993, published in the Gazette No. 174 dated 19.05.1993.
20. Barceloux, Donald G., and Donald Barceloux. "Chromium." *Journal of Toxicology: Clinical Toxicology* 37.2 (1999): 173-194.
21. Singare, Pravin U. "Studies on polycyclic aromatic hydrocarbons in surface sediments of Mithi River near Mumbai, India: Assessment of sources, toxicity risk and biological impact." *Marine pollution bulletin* 101.1 (2015): 232-242.
22. Singare, Pravin U., Ravindra M. Mishra, and Manisha P. Trivedi. "Heavy metal pollution in Mithi River of Mumbai." *Frontiers in Science* 2.3 (2012): 28-36.
23. Giri, Sindhushree, Hirak R. Dash, and Surajit Das. "Mercury resistant bacterial population and characterization of Bacillus sp., isolated from sediment of solid waste discharged point of steel industry." *National Academy Science Letters* 37.3 (2014): 237-243.
24. Nagarsekar, Aarti S., and Umesh B. Kakde. "A bacteriological study of Mithi River water in Mumbai metropolis." *International Journal of Researches in Biosciences, Agriculture & Technology.* 2 (2014): 362-374.
25. Singare, Pravin U., Ravindra M. Mishra, and Manisha P. Trivedi. "Sediment contamination due to toxic heavy metals in Mithi River of Mumbai." *Advances in Analytical Chemistry* 2.3 (2012): 14-24.
26. Sinha, A. and Khare, S.K., 2012. Mercury bioremediation by mercury accumulating Enterobacter sp. cells and its alginate immobilized application. *Biodegradation*, *23*(1), 25-34.
27. Sinha, A., Pant, K.K. and Khare, S.K., 2012. Studies on mercury bioremediation by alginate immobilized mercury tolerant Bacillus cereus cells. *International Biodeterioration& Biodegradation*, *71*, 1-8.
28. Mirzaei, N., Kafilzadeh, F. and Kargar, M., 2008. Isolation and identification of mercury resistant bacteria from Kor River, Iran. *J BiolSci*, *8*(5), 935-939.

29. Guo, H., Luo, S., Chen, L., Xiao, X., Xi, Q., Wei, W., Zeng, G., Liu, C., Wan, Y., Chen, J. and He, Y., 2010. Bioremediation of heavy metals by growing hyperaccumulaor endophytic bacterium Bacillus sp. L14. *Bioresource technology*, *101*(22), 8599-8605.
30. Jan, A.T., Azam, M., Choi, I., Ali, A. and Haq, Q.M.R., 2016. Analysis for the presence of determinants involved in the transport of mercury across bacterial membrane from polluted water bodies of India. *brazilian journal of microbiology*, *47*(1), 55-62.
31. Banerjee, G., Pandey, S., Ray, A.K. and Kumar, R., 2015. Bioremediation of heavy metals by a novel bacterial strain Enterobacter cloacae and its antioxidant enzyme activity, flocculant production, and protein expression in presence of lead, cadmium, and nickel. *Water, Air, & Soil Pollution*, *226*(4), 91.
32. Chang, S., Wei, F., Yang, Y., Wang, A., Jin, Z., Li, J., He, Y. and Shu, H., 2015. Engineering tobacco to remove mercury from polluted soil. *Applied biochemistry and biotechnology*, *175*(8), pp.3813-3827.