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Isolation of Chromium Resistant Bacteria from Contaminated Soil and Its Performance Evaluation for Hexavalent Chromium Removal

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Abstract*: The present study deals with the metal tolerance as well as metal resistance characteristic in bacteria isolated from mostly tannery effluent contaminated soil. Seven chromium resistant bacteria (CrRB 1-7) were isolated for this study in LB medium amended with 100 mg/L filter sterilized hexavalent chromium (Cr(VI)) solution. The minimum inhibitory concentrations (MIC) of these gram positive and gram negative bacteria were found in the range of 350 – 450 mg/L of Cr(VI) concentration. Cr(VI) removal potential of these seven isolates were also studied. One isolate CrRB 1 possessed the ability to remove 98.4% Cr(VI) within 24h in LB medium. The effect of hexavalent chromium on their biomass growth potential were further observed. For CrRB1 least differences were found in doubling time calculated from growth curve in presence and absence of Cr(VI). CrRB 1 was then further studied in real life wastewater i.e. tannery effluent to evaluate its performance for removal of Cr(VI).The favorable outcomes encourages for the use of this isolate in green bioremediation technology for tannery industry. The subsequent reactor studies requires to reveal its performance for in-situ application.

Keywords: Bioremediation, Chromium, Growth potential, Tannery effluent.

I. INTRODUCTION

Heavy metal contamination in environment has gradually become a challenge for life on earth. Anthropogenic activities like mining, processing and applications of heavy metals have enlarged the problem of metal pollution to the environment over the past few decades [1]. Consequently, developing technologies for remediation of heavy metals from the contaminated environment has become a challenging task before the scientists and engineers. With time, the disposal of solid and (or) liquid waste comprising heavy metals from various industrial processes has been extensively discussed and studied. Subsequently, legislation for the environmental protection has gradually become more stringent throughout the globe demanding improved scientific understanding supported by continuous technological invention [2], [3], [4].

Chromium is one of the most extensively used metals in various industries like steel manufacturing, metal processing, electroplating, leather tanning, dyes and pigments, wood preservation etc. [5]. However, chromium is also a toxic element and thereby being treated as a hazardous contaminant [6]. Hexavalent chromium (Cr(VI)) is the one of the most mobile, oxidized, reactive and toxic metals present in the environment. Being mutagenic and carcinogenic in nature it causes oxidative damage [7]. According to USEPA, chromium is reckoned to be one of the 17 chemicals causing greatest hazard to humans [8]. As per World Health Organization, the maximum allowable limit for total chromium in safe potable water is 0.05 mg/L [9] and this safety threshold is being frequently violated due to extensive usage and discharge of chromium derivatives into the ecosystem.

Developing a detailed understanding of microbe-metal interactions within the environment has attained considerable importance during the last two decades [10], [11], [12]. While various aspects of these interactions have been studied, it has emerged that microorganisms can play a vital role in the remediation of contaminated water, sediments and soil [13], [14]. Microorganisms can affect parameters like solubility, toxicity of metals and in situ remediation of contaminated substrate. On the contrary, heavy metals also effect the microbial population by troubling their growth potential, morphology, biochemical properties to finally result in reduction in biomass and diversity [15]. Microorganisms have developed numerous resistant mechanisms to cope with the selective pressure of the stress induced by heavy metals in the growth environment. A wide number of chromium resistant bacteria, both aerobes and anaerobes, were isolated from water and soil contaminated with chromium [16]. However, efficacy of microbial treatment of real life Cr(VI) contaminated wastewater is to some extent limited as some microorganisms fail to sustain high concentration of chromate [17], [18]. Isolating microbial strains showing high tolerance to chromium and removal efficiency in both synthetic as well as real life wastewater can be useful to alter or remove environmental contaminants by direct or indirect means from contaminated sites.

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The present study is aimed at the isolation of few chromium resistant bacteria from contaminated soil and their characterization on the basis of their morphology, minimum inhibitory concentration, gram staining and growth kinetic study under the stress of chromium.
The extent of chromium removal by the selected strain in synthetic culture medium and primary treated tannery effluent was also aimed to assess its overall performance.

II. MATERIALS AND METHODS

A. Sample Site Description

Untreated wastewater from different industrial units including tanneries, electroplating industries, battery industries etc. and municipal sewage of Calcutta City flow down through a web of canals into the East Calcutta Wetlands. The bank of such wastewater carrying canal was selected as the sample site for the reason that bank soil was continuously fed with high metal contaminants.

B. Sampling

Bank soil samples from three different points of the selected sampling site at East Calcutta Wetland were collected under aseptic condition in sterilized glass containers. Samples were used for microbial analysis and therefore carried to the laboratory in refrigerated condition.

C. Media Preparation

In the present study Luria Bertani (LB) agar medium (g/L): (Tryptone (Merck India, AR Grade) 10.0; Yeast extract (Himedia, AR Grade) 5.0; NaCl (Merck India, AR Grade) 10.0; Agar (SRL India, AR Grade) 1.5%; Sugar (SRL India, AR Grade) 0.10) was used for growth of bacteria. Cr(VI) was supplemented to the sterile molten LB medium. A filter sterilized (0.22 µm Whatman filter) solution of $K_2Cr_2O_7$ (Merck India, AR Grade) was used as the source of hexavalent chromium. Deionized water (DI) (18 MΩ) from a Millipore water purification unit (RiOsDI 3) was used for this purpose and in all the subsequent experiments during the entire course of this study.

D. Isolation of Chrome Resistant Bacteria (CrRB)

Isolation of Cr(VI) resistant bacteria was performed from soil following the method described by Das et al.(2012) [19] with slight modification. The soil suspension was serially diluted in dilution technique method to achieve a dilution of 10-5 and then plated initially on LB-Agar (pH 7.0) medium. After incubation of these agar plates at 37ºC for 96 h. colonies obtained on plates were hand-picked and again plated on LB agar plates added with 100 mg/L Cr(VI) as $K_2Cr_2O_7$. Strains which showed resistance towards chromium were picked up and purified till single colony is obtained.

E. MIC Assay for Chromium Resistant Isolates

Minimum inhibitory concentration (MIC) of Chromium Resistant Bacteria (CrRB) was determined as the lowest concentration of hexavalent chromium which can completely prevent the bacterial growth [20]. A fresh overnight culture (OD600=0.86) was inoculated in 5 ml of LB medium amended with various concentration of Cr(VI).A fresh 5 ml tube without chromium was monitored as control. All tubes were incubated at 37ºC with horizontal orbital shaking at 140 rpm in an orbital motion shaker-incubator. The growth of biomass was expressed in terms of optical density of the medium by absorbance at λ600 against sterile LB blank (UV-VIS Spectrophotometer, Lambda 25 Perkin Elmer).

F. Gram Staining of Bacteria and Morphological Study

Cells were cultured in LB medium overnight and then examined microscopically under (1000X) after gram staining following the method of Bailey and Scott (1996) [21]. Morphology of bacterium was inspected under normal visual examination.

G. Growth Curve

The impact of metal stress on growth potential of bacteria was observed by assessment of growth curve. The bacterial growth was observed in LB medium with Cr(VI) in medium. A control i.e. without Cr(VI) in medium was carried out concurrently The experimentation was continued until the stationary phase was reached. Sample was collected at an interval of 15 min or 30 min or 1 h depending on type of bacteria. Growth of biomass was evaluated by measuring absorbance at λ600 against a sterile LB blank sample. Absorbance obtained at different intervals were plotted on X axis against the time plotted along Y axis. This graph presents the growth of the biomass under stress of Chromium.

H. Determination of Cr(VI) removal efficiency in cultured medium

The chromium removal efficiency of the selected bacteria was determined by measuring the residual hexavalent chromium content in supernatant of the growth medium after 24 hrs. Incubation of the bacteria was performed at 150 rpm, 37ºC and for 24 h in LB medium with 100 mg/L Cr(VI) as $K_2Cr_2O_7$. Cr(VI) was determined spectrophotometrically (Lambda 25 Perkin Elmer) by diphenyl carbazide method [22]. The Cr(VI) content in supernatant was determined by adding 0.2 ml ortho-phosphoric acid and 0.2 ml of acetone solution of 1,5- diphenyl carbazide. The absorbance was measured at 540 nm after incubation of sample for 10 mins at room temperature.

I. Determination of Cr(VI) removal efficiency in real life wastewater

The chromium removal efficiency of the selected bacterium was also observed in real life wastewater. For this purpose, primary treated tannery effluent from CETP of Kolkata Leather Complex was collected at the point of its entry to secondary treatment unit and taken to the laboratory. Wastewater sample was collected in 5.0 L PVC containers. Temperature and pH were checked at the time of sampling and characterized. Characterization of the tannery wastewater sample was performed on the basis of following parameters viz. COD, TDS, TSS, BOD5 at 20°C, chloride, phosphate, nitrate, carbonate hardness, total hardness, acidity, alkalinity, sulphides, iron and total chromium as per the standard methods prescribed by APHA, 1998 [22].

Table- I: Composition of Nutrients and Trace Elements

Nutrients	Volume to be added in 1000mL
K_2HPO_4	60.0 mg
KH_2PO_4	40.0 mg
KNO_3	72.0 mg
Trace elements	**Volume to be added in 1000mL**
$MgSO_4.7H_2O$	500.0 mg
$FeCl_3.6H_2O$	710.0 mg
$ZnSO_4.7H_2O$	0.1mg
$CuSO_4.5H_2O$	0.1mg
$MnCl_2.2H_2O$	8.0 mg
$(NH_4)_6Mo_7O_{24}$	0.11mg
$CaCl_2.2H_2O$	100.0 mg
$CoCl_2.6H_2O$	200.0 mg
$Al_2(SO_4)_3.16H_2O$	55.0 mg
H_3BO_3	150
EDTA	100

The bacterium was first acclimatized with primary treated tannery effluent. Since the concentration of hexavalent chromium in the collected tannery effluent samples did not match with the range of required concentration Cr(VI) of the present study, hexavalent chromium was spiked additionally into the broth as a calculated aliquot dosage from a filter sterilized (0.22 µm) $K_2Cr_2O_7$ solution. The concentration of Cr(VI) was gradually varied in the previously acclimatized bacterial system from 0 to 50 mg/L as $K_2Cr_2O_7$. pH value in the range of 7.2-8.0 was adjusted by adding sodium carbonate buffer solution. In this study, the only source of the organic carbon was the organic substrate present in the tannery effluent [14]. Nutrients and trace elements were added to the broth in the recommended dosages as given in Table- I. Chromium removal efficiency was assessed by measuring the residual chromium content in supernatant and hexavalent chromium content was determined by standard diphenyl carbazide method.

III. RESULTS AND DISCUSSIONS

A. Chromium resistant bacterial isolates

The detection of metal tolerant bacteria in a particular given environment can be an indication that such area has been contaminated by heavy metals. Isolation of metal resistant bacteria from the environment contaminated with metal is deemed to be an appropriate practice for the selection of the most effective metal resistant strain capable of remediating heavy metal [23]. In the present study, seven bacterial strains were isolated showing high Cr resistance with different colony morphology from soil. Strains were named as chromium resistant bacteria (CrRB) 1-7. Few microbes exhibited higher growth on solid medium compared to liquid medium due to greater diffusivity of metal in liquid. Availability and complex formation of metals in liquid medium were different from those observed in solid medium. This observation is in agreement with [24].

B. MIC of Bacteria

Over the years, numerous chromium-resistant bacteria have been isolated and each species vary in point of degree of resistance. This variation in properties is due to the inherent capability of the specific strains to resist the toxicity of chromium. MIC of seven isolates were examined in LB medium (Table –II). MICs of four isolates i.e. CrRB 1, CrRB 3, CrRB 5 and CrRB 6 were obtained as 450 mg/L of Cr(VI) as $K_2Cr_2O_7$. CrRB 2 and CrRB 4 showed MIC as 400 mg/L of Cr(VI) as $K_2Cr_2O_7$. Only CrRB 7 showed MIC around 350 mg/L of Cr(VI) as $K_2Cr_2O_7$. Many researchers have been able to isolate species of bacteria with high Cr(VI) resistance but these microorganisms failed to remediate Cr(VI) at high concentrations (equal to their MIC) [25]. Therefore, an appropriate strategy is to be adopted for selection of potential bacterial strains which can be employed in remediating Cr(VI)-contaminated environments. This strategy should be preferably based on the growth capability of a strain in presence of high levels of chromium as well as their respective chromium reduction efficiency [26].

C. Colony Morphology Study of Cr(VI) Resistant Bacterial Strains

In Table- III, a summary of the study of colony morphology of all the seven bacterial strains have been presented. Gram staining of these strains revealed that five out of the seven strains were gram positive. It is also suggested from different studies that Bacillus is the predominant genus in case of gram-positive bacteria [14]. Colony of the isolated bacteria observed to be usually small to medium in size and round. With the exception of CrRB 2 and CrRB 3, other strains were observed to be in paired or long chain or clustered arrangement. The surface of the cream coloured colony of CrRB 1 was rough and convex in elevation, while the surface of colonies for CrRB-2, 3, 5, 6 were smooth, however the elevation and colour were different for these colonies. The colony of CrRB 4 appeared to have wrinkles on the surface and umbonate. The colony of CrRB 7 was examined to be flat and glossy on the surface and dull in colour. None of the bacterial strains have any observed pigmentation.

Table –II: MIC and Cr(VI) Removal Potential of Seven Isolates

Bacterial Sample Name	MIC (mg/L of Cr(VI) conc.	% Cr(VI) removal
CrRB 1	450	98.4
CrRB 2	400	90.45
CrRB 3	450	90.32
CrRB 4	400	92.5
CrRB 5	450	95
CrRB 6	450	93.4
CrRB 7	350	88

Table – III: Morphological Characteristics of Chromium Resistant Isolates

Strain Name	Gram Reaction	Colony Shape/ Size	Cell Arrangement	Surface	Colony Elevation	Colony Colour	Pigment -ation
CrRB 1	Gram +ve, Rod	Round, Medium	Single, paired, long chain	Rough	Convex	Cream	-
CrRB 2	Gram +ve, Rod	Irregular, small	Single	Smooth	Flat	Pale	-
CrRB 3	Gram +ve, Rod	Round, very Small	Single	Smooth	Flat	Pale	-
CrRB 4	Gram - ve, Circular	Circle, very Small	Single, paired	Wrinkled	Umbonate	White	-
CrRB 5	Gram +ve, Rod	Round, Medium	Single, long chain	Smooth	Convex	Cream	-
CrRB 6	Gram -ve, Rod	Round, Small	Single, long chain	Smooth	Raised	White	-
CrRB 7	Gram +ve, Circular	Circle, Medium	Single, Clustered	Glossy	Flat	Dull	-

D. Cr(VI) Removal Efficiency

Cr(VI) removal efficiency of the isolates was observed at 100 mg/L of Cr(VI) concentration after 24 h in LB medium. Seven strains showed Cr(VI) removal efficiency in the range of 88 – 98.5% (Table –II). Masood and Malik (2011) [27] reported that 100% removal of 100 mg/L of Cr(VI) by Bacillus sp. can be achieved within 48 h. Kathiravan et al.(2010) [28] also reported that 95.2% removal of 100 mg/L of Cr(VI) by *Bacillus sp*. can be achieved within 78 h in a minimal salt medium. Mary Mangaiyarkarasi et al., (2011) [29] reported that within 144 h, *Bacillus sp* removed 71 % of 100 mg/L of Cr(VI). In this study, the seven isolates were further studied to check their growth responses in presence of chromium.

E. Growth Curve of Chromium Resistant Strains

The effect of chromium on the microbial response to substrate was carried out in LB medium maintaining a metal concentration of 100 mg/L in solution. Seven isolates exhibited different growth patterns in presence of chromium over an experimental time period of 3 to 16 hrs until the growth of bacteria reached stationary phase. The microbial response to substrate in absence of chromium was also recorded for seven isolates as control to get a details comparison in biomass growth kinetics. The comparative growth curves for the seven isolates (CrRB 1-7) in the presence of chromium are shown in the Fig 1-7. CrRB 1, CrRB 2, CrRB 5 and CrRB 7 exhibited least changes in growth response due the presence of chromium. The major effect of chromium stress on these four bacteria was observed at the end of the log phase and as a result these four isolates moved towards the stationary phase earlier than the control. However, the growth curve pattern suggested that these bacteria exhibited a strong tolerance towards chromium stress. Growth potential of the rest three bacterial isolates i.e. CrRB 3, CrRB 4 and CrRB 6 were highly inhibited by chromium. A lower value of optical density indicated that the biomass growth of these bacterial isolates was highly affected due to presence of chromium in the growth medium. When the tolerance of chromium was compared for all isolates, it was evident that CrRB 1, CrRB 2, CrRB 5 and CRrRB 7 were highly chromium resistant whereas rest three were highly chromium sensitive. Other kinetic parameters like doubling time and growth rate yielded more specific observation for CrRB 1-7. Therefore, growth rate and doubling time were calculated using these sigmoidal growth curves and are tabulated in Table- IV. The doubling time value increased for CrRB 3, CrRB 4 and CrRB 6 when exposed to chromium as they were sensitive to this heavy metal. CrRB 7 also showed increased value in doubling time. While, CrRB 2and CrRB 5 showed significant decrease in doubling time on treatment with chromium. However, CrRB 1 showed least change in doubling time when exposed to Cr(VI) among all the seven strains and the value was lowest for these strains. This indicates that CrRB 1 may have high resistivity towards the chromium contaminated environment and also there is no interference by chromium in the metabolism of the bacteria. CrRB 1 also exhibited maximum removal efficiency of chromium and therefore was selected for further study.

Table- IV: Doubling Time for Seven Bacterial Isolate Samples treated with and without Chromium

Bacterial Sample Name	Doubling Time (h) for sample without Cr(VI)	Doubling Time (h) for sample with Cr(VI)
CrRB 1	1.25	1.25
CrRB 2	3	2.8
CrRB 3	3	5.5
CrRB 4	2	4.9
CrRB 5	1.9	1.2
CrRB 6	2	5
CrRB 7	3.7	6

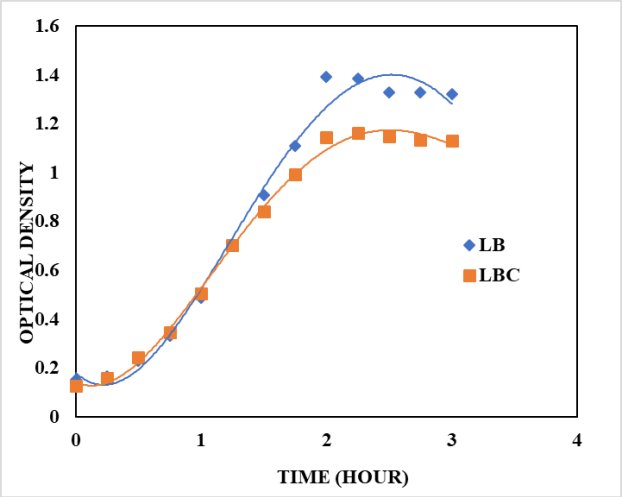


Fig- 1: Growth pattern of CrRB 1 with (LBC) and without (LB) Cr(VI)

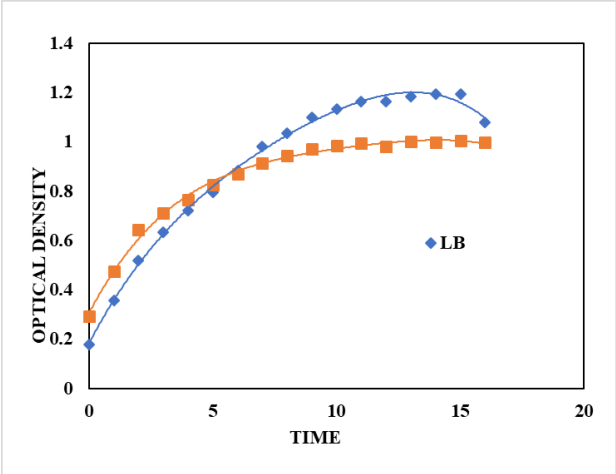


Fig- 2: Growth pattern of CrRB 2 with (LBC) and without (LB) Cr(VI)

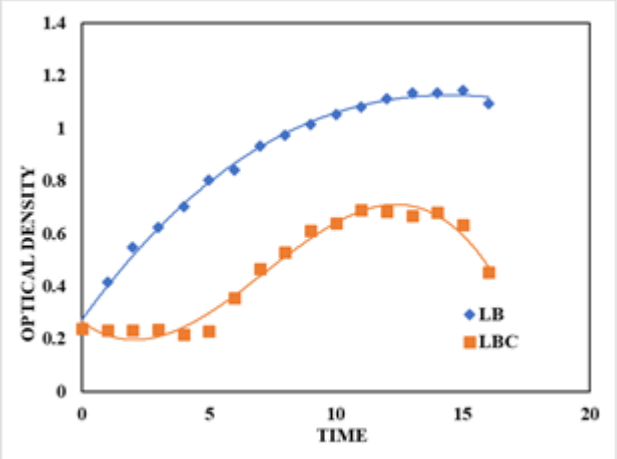


Fig- 3: Growth pattern of CrRB 3 with (LBC) and without (LB) Cr(VI)

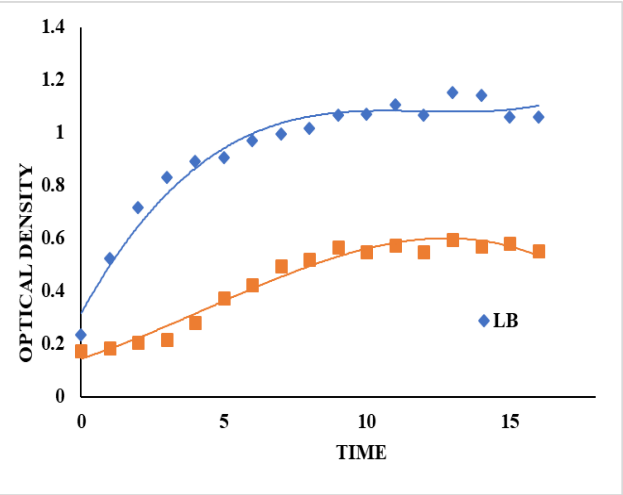


Fig- 4: Growth pattern of CrRB 4 with (LBC) and without (LB) Cr(VI)

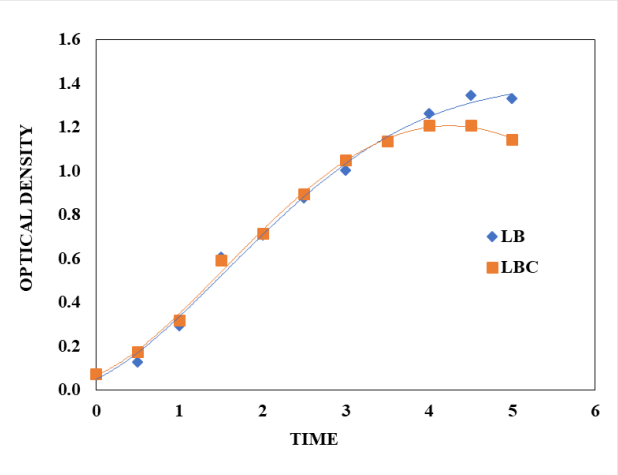


Fig- 5: Growth pattern of CrRB 5 with (LBC) and without (LB) Cr(VI)

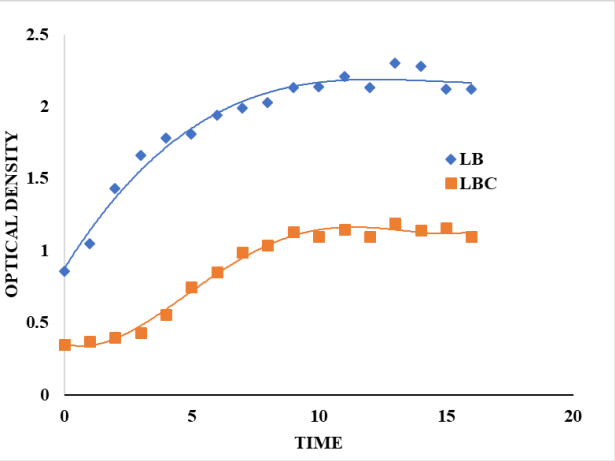


Fig- 6: Growth pattern of CrRB 6 with (LBC) and without (LB) Cr(VI)

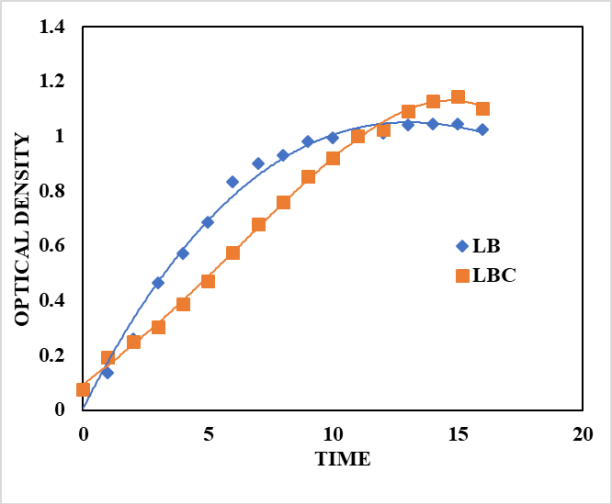


Fig- 7: Growth pattern of CrRB 7 with (LBC) and without (LB) Cr(VI)

F. Characterization of Primary Treated Tannery Effluent

The composite tannery effluent sample, after primary treatment was collected from the inlet box prior to the secondary treatment plant of the CETP of Calcutta Leather Complex. This wastewater sample was characterized in the laboratory in terms of the parameters mentioned in the Table-V.

The average BOD5: COD ratio obtained was 0.323 for primary treated composite tannery effluent. Tannery effluent characteristically contains a composite mixture of both inorganic salts and soluble organic substances, hence the dissolved solid content in primary treated tannery effluent was observed to be in the higher side. Leather processing units generally use a significant amount of alkaline salts which cause the high alkalinity and hardness count in wastewater. The total hardness and carbonate hardness were determined as 880 mg/L and 430.4 mg/L respectively. The alkalinity was recorded as 11.4 mmol/L.

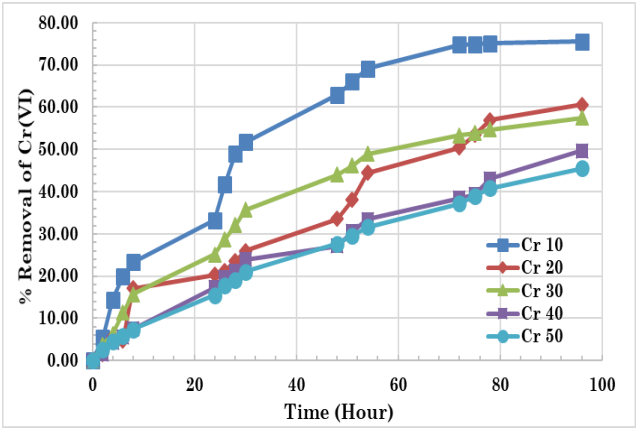
G. Chromium removal profile of CrRB 1 in real life wastewater

Performance evaluation for chromium removal in real life wastewater was carried out for primary treated tannery wastewater with variable Cr(VI) concentration. Experiments were carried out at fixed soluble chemical oxygen demand (SCOD) and dilution factor of 2.0. Cr(VI) concentration was varied from 10 – 50 mg/L while the SCOD was kept constant at around 1100 mg/L. Experiments were conducted for a reaction period of 96 h at 28 ± 2 °C i.e. ambient temperature. The chromium removal profile is presented in Fig- 8. Results showed that the microbial pure culture was able to reduce chromium in the composite tannery effluent. The maximum removal efficiency was obtained as 75.52% for Cr(VI) = 10 mg/L and minimum 45.5% for Cr(VI) = 50 mg/L. A gradual decrease in removal efficiency was noted with the increase in chromium concentration. Similar observation was also documented by other researchers [30]. CrRB 1 can reduce 98.4% of 100 mg/L Cr(VI) in synthetic culture medium at a temperature of 37°C. The results obtained in this study may be attributed to the prevalence of low temperature during the experiments. Furthermore, larger time was required for removal of equal amount of chromium from real life wastewater, which can be caused by other interferences present in tannery wastewater. The results showed interesting characteristics from biotechnology stand point with regard to future remediation processes using CrRB 1.

Table- V: Characterization of the Primary Treated Composite Tannery Effluent

Parameters	Value
Odour	Obnoxious
pH	6.9 ± 0.152
Temperature (˚C)	28.6 ± 0.75
DO (mg/L)	Below detectable limit
COD (mg/L)	2680 ±168.6
BOD5 at 20°C (mg/L)	866.7±101.5
Total Dissolved Solid (TDS) (mg/L)	6420 ± 340.8
Total Suspended Solid (TSS) (mg/L)	100 ± 23.5
Chloride(mg/L)	6150 ± 563.7
Phosphate(mg/L)	0
Nitrate(mg/L)	51 ± 2.4
Carbonate Hardness(mg/L)	430.4 ± 58.9
Total Hardness(mg/L)	880 ± 65.2
Acidity (mmol/L)	1.8 ± 0.88
Alkalinity (mmol/L)	11.4 ± 0.95
Sulphides(mg/L)	19.163 ± 1.53
Iron(mg/L)	2.24 ± .077
Total Chromium(µg/L)	28.32 ± 1.66

Fig- 8: Variation in % chromium removal with time at ambient temperature of 28 °C



IV. CONCLUSION

Microbes have competence to survive in the highly polluted environment with heavy metals within their metabolic system. They can be utilized effectively to clean up metal-contaminated sites. Bacteria exposed to heavy metal contaminated environment are capable of getting adapted to this stress by virtue of numerous resistance mechanisms. Under the purview of the present study discussed about seven chromium resistant isolates from soil samples.

Both gram positive and gram-negative bacteria were found resistant towards chromium and able to remove this heavy metal from medium. CrRB 3, CrRB 4 and CrRb 6 were identified as relatively more sensitive to chromium as compared to other four isolates. Thus these three isolates have greater potential to act as a biosensor to identify the toxicity of chromium present in any environment. Growth potential of CrRB 1, CrRB 2, CrRB5 and CrRB7 were found least inhibited due to the presence of chromium in culture medium. The chromium removal efficiency of CrRB 1 was further studied with real life wastewater. Primary treated tannery effluent was used for this purpose and the removal efficiency was recorded around 45.5% at 28°C ambient temperature. The resistance mechanism in bacteria towards heavy metals are either adsorption or absorption and bioreduction. Bioreduction of metal is attributed by the presence of plasmid or reductase enzyme acted aerobically or anaerobically. Activity of reductase enzyme is highly influenced by temperature. The resistance mechanism of CrRB 1 therefore may be explained as an enzyme-mediated process. This isolate has lot of potential to be developed as a viable and sustainable alternative for existing Cr(VI) removal technologies from wastewater and thus demands extensive studies in this regard.

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