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Reduction of Cr (VI) by *Micrococcus luteus* isolate from Common Effluent Treatment Plants (CETPs)

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A B S T R A C T

The present study was envisaged with the objective to isolate indigenous chromate tolerant bacteria from effluents and their subsequent utilization for chromium uptake or reduction. Samples were collected from two different Common Effluent Treatment Plants (CETPs), located in Ludhiana (sample 1) and Jalandhar (sample 2). In both the samples, chromium was found to be the dominant metal contaminant. A total of 10 morphologically distinct isolates were tested for their tolerance to chromium in terms of minimum inhibitory concentration (MIC) of Cr required for complete inhibition of growth. Four isolates (HM 2, HM 3, HM 15 and HM 16) showed maximum tolerance to chromium. There was no active uptake of Cr in sample 1 but considerable uptake was observed in sample 2. Chromium reduction efficiency was determined by S-diphenyl-carbazide (DPC) method, whereby complete reduction was observed with the standard culture (*Shewanella putrefaciens*) followed by 76.66% by HM 16 and 46.76% by HM 2 after 7 hours of incubation. Molecular characterization of most potent isolate (HM 16) was carried out using 16S rDNA based molecular method.

Introduction

Chromium is one of the most toxic heavy metal used in several industries and is a common industrial pollutant. A large quantity of chromium is discharged into the environment mainly from industrial operations including metal finishing industry, petroleum refinery, leather tanning, iron and steel industries and causes a serious threat to human health (Oladipo *et al.*, 2014; Dixit *et al.*, 2015). The effluents of these industries contain chromium at concentrations ranging from tenths to hundreds of milligrams per liter (Dermou *et al.*, 2005; Boyd 2010, Singh and Prasad 2015). Safe value in water for drinking purposes is 0.05 mg/L and recommended value for discharge is less than 5 mg/L (Directive EPA, USA, 2003; Debabrata *et al.*, 2006).

In nature, chromium mainly exists in two forms, hexavalent Cr (VI) and trivalent Cr (III) form. In the industrial wastes it is primarily present in the hexavalent form as divalent oxyanions, chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$). It is an essential trace metal, but overexposure to Cr (VI) produces

ulceration in the skin, mucous membranes and nasal septum, allergic dermatitis, renal tubular necrosis and increases risks of respiratory tract-cancer (Lu and Yang 1995; Flavio *et al.,* 2004). The hexavalent chromium compounds are comparatively more toxic than those of Cr (III) due to their higher solubility in water, rapid permeability through biological membranes and subsequent interaction with intracellular proteins and nucleic acids (Basu *et al.,* 1997). Hexavalent chromium (Cr (VI)) reduction to trivalent chromium (Cr (III)) could constitute a potential detoxification process that could be achieved via chemical or biological methods. However, chemical reduction requires energy input and large quantities of chemicals and generation of sludge (Srivastava *et al.,* 1986; Hashim *et al.,* 2011; Yao *et al.,* 2012). Biological reduction could, therefore, provide a useful alternative economical process (Congeevaram *et al.,* 2007). The processes by which microorganisms interact with toxic metals enabling their removal and recovery are bioaccumulation, biosorption and enzymatic reduction (Ohtake and Silver 1994; Srinath *et al.,* 2002).

Reduction of Cr (VI) to Cr (III) represents a potentially useful approach for the detoxification of chromate from wastewater and environment (Marsh and McInerney, 2001; Liu *et al.,* 2012; Shah *et al.,* 2014). Biological Cr (VI) detoxification which is more ecofriendly and an economically feasible technology can be a suitable approach (Wang and Xiao 1995; Mclean and Beveridge 2001; Srinath *et al.,* 2002; Elangovan *et al.,* 2006). However, the potential for biological treatment of Cr (VI)-contaminated waste is limited because some microorganisms lose viability in the presence of high concentrations of chromate. Isolating chromate-reducing bacteria from contaminated environments could therefore be useful (Dmitrenko *et al.,* 2003; Balamurugan *et al.,* 2014, Katyal *et al.,* 2015). In *Bacillus* sp. ES29 chromate reducing activity was localized in the cell free extract which utilizes NADH as the sole electron donor (Pal *et al.,* 2005). In some cases, the reduction of Cr (VI) was shown to take place in the extracellular domain due to the excretion of metabolites possessing a chemical reducing power. For example, *Thiobacillus ferrooxidans* was shown to generate sulphite and thiosulfate which reduce Cr (VI) at low pH (Sisti *et al.,* 1996). The Cr (VI) reduction by bacterial cultures (Klaus-Joerger *et al.,* 2001; Francisco *et al.,* 2002; Cheung and Gu 2003; Ilias *et al.,* 2011) has been extensively studied under aerobic and/or anaerobic conditions. This work mainly focuses on the isolation of chromium resistant strains of bacteria for the reduction/uptake of Cr (VI) and their applicability in treatment of metal-rich effluents.

Materials and Methods

Sample collection and preparation

Effluent samples were collected from Punjab Small Industries and Export Cooperation (PSIEC) Leather Complex common effluent treatment plant (CETP), Kapoorthala road, Jalandhar and Ludhiana Electroplaters Association CETP, Focal Point, Ludhiana. Samples were collected in sterile plastic containers in the month of September-October, 2015 and were allowed to settle for 2h. After filtration through Whatmann filter No. 1, samples were tested for their pH, Chemical Oxygen Demand (COD), dissolved oxygen (DO), Biological Oxygen Demand (BOD) using standard methods (APHA 2001).

Heavy metal profile of effluent

The concentration of heavy metals present in both the effluent samples was estimated using Inductively Coupled Argon Plasma-Atomic Emission Spectroscopy (ICAP-AES). One

hundred ml of sample was digested with 5ml of concentrated HNO_3 and suitably diluted for heavy metal analysis by iCAP 6300 (Singh *et al.,* 2015).

Procurement and maintenance of standard culture

The standard culture of *Shewanella putrefaciens* MTCC 8104 was procured from Institute of Microbial Technology (IMTECH), 39A, Sector 39, Chandigarh, India. It was maintained by periodic sub-culturing on Luria Bertani Agar after every 3 weeks.

Isolation and maintenance of bacterial isolates

Isolation of indigenous chromium resistant bacteria was carried out using standard microbiological techniques by which Luria Bertani Agar plates supplemented with 5mg/L concentration of Cr was used. Pure cultures of bacterial colonies were preserved at 4°C as slant cultures for further analysis.

Determination of Minimum Inhibitory Concentration (MIC) of different selected heavy metals

Maximum resistance of the isolates to Cr was evaluated in LB agar plates amended with Cr in concentration ranging from 5ppm to 100ppm. The lowest concentration of heavy metal at which no growth occurred when compared with the control plates was considered as the Minimum Inhibitory Concentration (MIC).

Determination of heavy metal uptake by selected isolates

To determine the ability of selected isolates for heavy metal uptake, attempt was made to grow both the selected isolates on effluent samples collected from Ludhiana (sample 1) and Jalandhar (sample 2). Two-fifty ml of effluent sample was taken in 500 ml volumetric flask, autoclaved at 15 lbs for 20 minutes and was inoculated with 0.2 ml of 12 hour old culture of HM 2 and HM 16 individually. Samples were taken to observe the heavy metal profile after 5 days and 10 days of growth. A set of un-inoculated effluent sample was kept as control. Following set of treatments were used:

Control:- Effluent samples as such without any modification was used as growth medium for both the selected isolates.

Treatment 1:- Effluent samples were supplemented with D-glucose-2.5 g/L, $MgSO_4.7H_2O$-0.5 g/L and KNO_3-0.18 g/L.

Treatment 2:- Effluent samples were modified to adjust their pH at 6.0 because at this pH most of the metals exists in their free ion state.

Treatment 3:- Effluent samples were supplemented as in Treatment 1 and their pH was adjusted to 6.0.

Growth profile of selected isolates w.r.t standard culture

Growth of selected isolates w.r.t standard culture was studied in 250 ml flasks containing 50 ml sterile LB broth. These flasks were inoculated separately with 0.5ml of overnight culture of selected isolates and standard culture and agitated on a rotary shaker at 150 rpm. Growth was monitored by measuring the optical density (O.D) at 600 nm using spectrophotometer at different time interval 0, 1, 2, 3, 4, 5, 6 and 7 h (Camargo *et al.,* 2003).

Chromium reduction efficiency of selected isolates and standard culture

Selected isolates and the standard culture were grown in 50 ml of LB medium with 20 ppm $K_2Cr_2O_7$ at 37°C with orbital shaking (150

rpm). Samples were withdrawn at 1 h interval and centrifuged at 10,000 rpm for 5 min and the supernatants were assayed for residual Cr (VI) concentration by using S-diphenylcarbazide (DPC) method (Barlett and James 1996). Hexavalent chromium was determined colorimetrically. Standard curve was plotted with the different readings obtained by taking absorbance at 540 nm.

Determination of site of chromate reductase activity

The reaction for the chromate reductase activity contained 20mg/L Cr(VI) as $K_2Cr_2O_7$ in 0.5 ml of 100 mM phosphate buffer, held at 37°C in a water bath. Bacteria were grown overnight in 100 ml of LB medium with 20 mg/L $K_2Cr_2O_7$ at 37ºC with orbital shaking (150rpm). Thereafter, cells of each isolate were harvested by centrifugation of 30 ml culture at 5,000 rpm for 10 min. Culture supernatant was collected and the cell pellet was resuspended in 30 ml phosphate buffer (10 mM, pH 7). Cells in an ice bath were disrupted with an ultrasonic probe. Power was applied ten times in 30s pulses with 30s intervals. The sonicate was centrifuged at 16,000 rpm at 4ºC for 20 min. Cell extract supernatant was transferred in a fresh tube and was kept in ice. Cell lysate was also resuspended in 30 ml phosphate buffer (Ilias *et al.,* 2011).

The reaction was initiated by the addition of 0.5 ml each of culture supernatant, cell extract supernatant and cell lysate and residual Cr (VI) concentration was measured after 1 h following the (DPC) method. One unit of enzyme activity was defined as 1 µmol of Cr (VI) reduced/min/ml at 37°C.

Molecular identification of the bacterial isolate by 16S rDNA sequencing

Molecular identification of the bacterial isolate was done through outsourcing by Eurofins Genomics India Pvt Ltd. DNA was isolated and its quality was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by 27F and 1492R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose gel. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7 (Kumar *et al.,* 2016).

Results and Discussion

Physico-chemical and Comparative heavy metal profile of effluents from CETPs

Effluent sample taken from Ludhiana (sample 1) was found to be highly acidic (pH-2.5) with BOD of 17.2 mg/l and COD about 390 mg/l. Whereas, sample 2 (tannery effluent from Jalandhar) was showing pH-9.0 with BOD value 69.8 mg/l and COD of 372 mg/l, indicating far difference in physico-chemical parameters of effluents depending on type of industries these are catering for. Sample 1 was taken from a CETP mainly receiving waste from electroplating industries of Ludhiana and sample 2 was from a CETP handling tannery waste. Dissolved oxygen content of sample 1was 47.8±0.5 and of sample 2 was 90.3±0.3. The heavy metal profile of both the effluent samples was determined by using Inductively Coupled Argon Plasma-Emission Spectroscopy (ICAP) analysis by the method

of Thompson and Walsh (1989). Both the samples were pretreated as suggested by Singh *et al.,* (2015). Sample 1 was analyzed for complete metal profile irrespective of the fact that these metals are of environmental concern or not, whereas, for sample 2 only the heavy metal contaminants of environmental concern was recorded and others are mentioned as N.D (not-determined). The results presented in Table 1 revealed that in sample 1, Cr was found to be the dominant metal contaminant with a concentration of 238 ppm, followed by nickel (92 ppm), copper (18.9 ppm), lead (18.5 ppm) and cadmium (0.3 ppm). In sample 2 also, highest level of Cr (23.2 ppm) followed by lead (20.6 ppm), Ni (6.65 ppm) and Cd (0.32 ppm) was observed.

The level of heavy metal contaminants in sample 1 was higher in comparison to sample 2 and was above permissible limits in both the samples. The concentration of Cr was 2380 times higher than the permissible limit in sample 1. This is in accordance with the earlier study by Verma *et al.,* (2001), who analyzed tannery effluents for the content of the various heavy metals and found that the total chromium (28.96 ppm) and nickel concentrations (1.08 ppm) in the effluent exceeds the permissible limits.

Isolation of heavy metal resistant bacteria

For isolation of chromate tolerant bacteria, the samples were enriched in peptone water and incubated for 18 hours at 37ºC. Then the loopful of sample was streaked as such on Luria Bertani (LB) agar plates amended with $K_2Cr_2O_7$ at concentration of 5ppm. The plates were incubated at 37 ºC for 24-48 hour. Different bacterial isolates were obtained by picking well isolated colony and transferring on LB slant. A total of 21 bacterial isolates were isolated and purified. These isolates were named as HM 1 to HM 21. Similarly, Pandit *et al.,* (2013) isolated 40 isolates from industrial effluent samples which can tolerate 50 ppm of different metal i.e Cu, Cd, Ni and Cr concentrations. Singh *et al.,* (2013) isolated fifty three morphologically different bacterial strains from the treated tannery effluent and eighteen strains were selected for the determination of chromate reduction efficiency on the basis of higher MIC values. Five morphologically different Cr (VI) resistant bacterial strains designated as TUV-K1, TUV-K2, TUV-K3, TUV-K4 and TUV-K5 were isolated from the treated tannery effluent (Vijayananda and Hemapriya 2014).

Determination of Minimum Inhibitory Concentration (MIC) of different selected heavy metals

Minimum Inhibitory Concentration (MIC) was determined by growing cells in LB agar plates amended with different concentrations of Cr in the form of their salt K_2 Cr_2 O_7 . The isolates were grown on increasing concentration (5-120 ppm) of Cr till there is complete inhibition of growth (Table 2). Four isolates i.e HM 2, HM 3, HM 15 and HM 16 showed maximum tolerance (upto 120 ppm) to Cr. In another study, isolation of 53 different species was carried out from the sediment samples collected from Krishna Godavari basin. Of these isolates, 79.24% were found to be resistant to 350 ppm of Mercury (11.53%), 250ppm of Cadmium (3.77%), 700ppm of Chromate (50.94%) and 250 ppm of Zinc (13.20%) (Gunaseelan and Ruban, 2011).

In the study carried out by Alam *et al.*, (2011), a total of 198 bacteria were isolated, 88 from the tannery effluents and 110 from agricultural soil irrigated with the tannery effluents. All isolates were tested for resistance against Cr^{6+}, Cr^{3+}, Ni^{2+}, $Zn^{2+,}$ Cu^{2+}, Cd^{2+} and Hg^{2+}. Maximum bacterial isolates were found to be resistant to Cr^{6+} 178 (89.9%) followed by Cr^{3+} 146 (73.7%), Cd^{2+} 86 (43.4%), Zn^{2+} 83 (41.9%), Ni^{2+} 61 (30.8%) and Cu^{2+} 51

(25.6%). However, most of the isolates were sensitive to Hg^{2+}. Among the isolates from tannery effluents, 97.8% were resistant to Cr^{6+} and 64.8% were resistant to Cr^{3+}.

Determination of Cr uptake by selected isolates

The concentration of Cr in effluent samples was determined by using Inductively Coupled Argon Plasma-Atomic Emission Spectroscopy (ICAP-AES) analysis. Attempt was made to grow both the selected bacterial isolates on the effluent samples and Cr uptake by the isolates was determined by measuring the Cr concentration in the inoculated effluents after 5 days and 10 days of growth. Samples were analyzed for any decrease in Cr concentration by ICAP-AES.

Determination of heavy metal uptake in effluent sample 1 (Ludhiana-CETP)

To determine the Cr uptake, Four set of treatments were used, and Cr uptake in effluent sample as such (Control), effluent sample with supplements (Treatment 1), Effluent sample with pH adjustment (Treatment 2), Effluent sample both with supplements and pH adjustment (Treatment 3) was determined.

Control: Untreated effluent sample 1 was inoculated with the selected isolates i.e. HM 2 (U 2) and HM 16 (U 10) and was observed for microbial growth in terms of increase in optical density w.r.t un-inoculated control U(C). The heavy metal profile was recorded after 5 and 10 days of growth and compared with un-inoculated control U(C) by using ICAP-AES. The results revealed that there was no decrease in concentration of Cr even after 5 and 10 days of incubation (Table 3) indicating that none of the isolate is showing any metal uptake. Though an increase in concentration of metal can be linked to increase availability of metal in soluble form due to acid production accompanied with microbial growth.

Treatment 1: Sample was supplemented with D-glucose-2.5 g/L, $MgSO_4.7H_2O$-0.5 g/L and KNO_3-0.18 g/L and inoculated with selected isolates. Supplemented effluent sample 1 was inoculated with the selected isolates i.e. HM 2 (S 2) and HM 16 (S 16) and was observed for microbial growth in terms of increase in optical density w.r.t un-inoculated control S(C). The heavy metal profile was measured after 5 and 10 days of growth and compared with un-inoculated control S(C) by using ICAP-AES. Table 4 revealed that even after supplementation there was no uptake of heavy metals by selected isolates.

Treatment 2: The pH of the sample was altered to 6 and inoculated with selected isolates. The concentration of Cr was measured after 5 and 10 days by ICAP-AES analysis. The initial concentration in the control was decreased due to precipitation of metal ions whereas no uptake was observed in the inoculated samples (Table 5). From these results, it is clearly shown that the pH in the range between 5.5 and 6 is nearly the same range of pH at which the different metals under investigation exist in free ion state.

Treatment 3: Sample was supplemented as well as the pH of the sample was altered and inoculated with the selected isolates. The concentration of Cr was measured after 5 and 10 days by using ICAP-AES analysis. The results revealed the decrease in concentration in the control due to precipitation of metal ions.

The results of the above four treatments indicated that there was no active uptake of Cr (VI) by any of the selected isolates even after 5 and 10 days of growth in sample 1. Active uptake was not observed even after adjustment

of pH and incorporation of supplements, individually as well as collectively (Table 5 and 6). An increase in metal concentration, after 5 and 10 days of growth, might be correlated to increased acidity of the sample due to acid production from the microbes. Both the isolates were growing in effluent sample at a very low rate with increase in O.D of HM 2 (0.01 to 0.052) and HM 16 (0.03 to 0.062). This indicated that the strains were unable to bioaccumulate heavy metals in initial 5-10 days of active growth in effluent sample 1 containing a very high level of heavy metal contaminants. The concentration of heavy metals was higher than the MIC of selected isolates.

Determination of heavy metal concentration in effluent sample 2 (Jalandhar-CETP)

To determine the Cr uptake, two treatments were used, Cr uptake in effluent sample with pH adjustment to 6 (Treatment 1) and effluent sample with supplements and pH adjustment (Treatment 2) was determined.

Treatment 1: The pH of the sample was altered to 6 and samples were inoculated with selected isolates. The concentration of Cr was measured after 5 and 10 days of growth by ICAP-AES analysis. The result indicated a significant decrease in concentration of Cr (VI) by the selected isolates (Table 7).

Treatment 2: The sample was supplemented with D-glucose-2.5 g/L, $MgSO_4.7H_2O$-0.5 g/L and KNO_3-0.18 g/L as well as the pH of the sample was altered. The concentration was measured after 5 and 10 days by ICAP-AES analysis. From the results, a significant decrease in concentration of Cr was observed even after 5 days of incubation (Table 8).

As indicated in above tables sample 2 (J-CETP) supported good growth of microbes indicating the lower level of heavy metal contaminants. Active growth of isolates co-relate well with their heavy metal uptake revealed (99%) reduction of Cr in sample 2 (J-CETP).

Growth profile of selected isolates w.r.t standard culture in Cr (VI) supplemented media

As revealed in Tables (3-6), both the selected isolates were unable to show any Cr uptake in effluent sample 1, even after supplementation with D-glucose, $MgSO_4$ and KNO_3 and pH adjustment to 6.0. Whereas, both the isolates were showing comparatively higher growth and active Cr uptake in effluent sample 2 (Table 7 and 8). To further determine the ability of selected isolates to show reduction of Cr (VI) to Cr (III), both the isolates were tested for enzyme chromate reductase and compared with a standard strain *Shewanella putrefaciens* (MTCC 8104).

The growth profile of selected isolates with respect to standard culture (*Shewanella putrefaciens*) was determined in Cr (VI) supplemented media (Figure 1). Both the isolates (HM-2 and HM-16) and *Shewanella putrefaciens* (S) were inoculated in a media supplemented with 20 ppm of Cr (VI) and incubated on incubator shaker at 37ºC temp and 100 rpm. Samples were withdrawn at 1 h interval and absorbance was measured at 540 nm. The maximum growth was shown by the standard culture (0.021 to 1.245) followed by the growth of HM 16 and HM 2 after 7 h of incubation (Table 9). Similarly, Singh *et al.*, (2013) measured the growth of chromate tolerant bacterial isolate (*Bacillus cereus*) in anaerobic broth in the presence or absence of Cr (VI) at different concentrations of Cr (VI). The results revealed that the rate of growth of *B. cereus* decreased with the increase in Cr (VI) concentration of the medium and the chromate reduction was directly correlated to the growth of the strain.

Chromium reduction efficiency of selected isolates and standard culture

The chromium reduction efficiency of selected isolates and standard culture was determined by S-diphenylcarbazide (DPC) method as discussed above. The results revealed complete reduction of Cr (VI) by the standard culture followed by 76.66 % reduction by HM 16 and 46.76 % reduction by HM 2 after 7 hours of incubation (Table 10, Figure 2). Camargo *et al.,* (2003) isolated some chromium resistant bacteria that can tolerate or reduce Cr (VI) at concentrations of 1500-2500 mg/L. It has been reported that *Bacillus* sp. ES29 reduced 90% of Cr (VI) added @ 2mg/L to the medium in less than 6 h. Similarly, Ilias *et al.,* (2011) isolated two chromium resistant bacteria (IFR-2 and IFR-3) capable of reducing/transforming Cr (VI) to Cr (III) from tannery effluents. Thacker and Madamwar (2005) have shown that the bacterial isolate DM1 reduces 50 ppm of chromium to 0 ppm in 54 h. They added a second aliquot of chromium which was reduced to 0 ppm in 99 h, and the third aliquot was reduced to 21.8 ppm in 126 hour.

Table.1 Comparative heavy metal profile of effluents from CETPs

S.No	Metals	Sample no. 1	Sample no. 2	Permissible limit
		Concentration of metal (ppm)		
1.	Cadmium (Cd)	0.30	0.32	2.00
2.	**Chromium (Cr)**	**238.0**	**23.2**	**0.10**
3.	Copper (Cu)	18.9	0.0075	3.00
4.	Iron (Fe)	5840	0.023	3.00
5.	Magnesium (Mg)	45	N.D	N.D
6.	Nickel (Ni)	92	6.65	3.00
7.	Lead (Pb)	18.5	20.6	0.1
8.	Zinc (Zn)	266	0.0063	5.0

N.D – Not determined

Table.2 Minimum inhibitory concentration (MIC) of morphologically distinct isolates

Isolate No.	Cr
	concentration in ppm
HM 1	30
HM 2	**120**
HM 3	**100**
HM 5	10
HM 8	30
HM 9	20
HM 12	70
HM 14	30
HM 15	**110**
HM 16	**100**

- indicates no growth

Table.3 Heavy metal concentration in untreated effluent sample (1) determined by using ICAP-AES

	Sample (after 5 days)			(after 10 days)		
Metals	**U(C)**	**U 2**	**U 16**	**U(C)**	**U 2**	**U 16**
	Concentration in ppm					
Cr	**238**	**341**	**298**	**283**	**335**	**314**
Cu	18.9	29.5	25.5	24.4	28.6	27.1
Fe	5840	6940	6975	6650	6560	6930
Mn	46.4	55.8	59.2	56.6	57.1	61.9
Ni	92	102	106	119	137	133
Pb	18.5	24.9	25.7	22.3	25.2	27.1
Zn	266	310	301	293	309	309
Cd	0.30	0.49	0.41	0.39	0.45	0.43

U indicating - Untreated effluent sample

Table.4 Heavy metal concentration in supplemented effluent sample (1) determined by using ICAP-AES

	Samples (after 5 days)			(after 10 days)		
Heavy Metals	**S(C)**	**S 2**	**S 16**	**S(C)**	**S 2**	**S 16**
	Concentration in ppm					
Cr	**332**	**336**	**291**	**270**	**290**	**293**
Cu	28.7	29	25.5	23.5	24.9	25
Fe	7415	7780	6345	6490	6830	6920
Mn	65.8	66.9	60.1	54.1	57	57.7
Ni	138	137	125	117	121	124
Pb	28.1	27.4	27.5	20.7	24.5	27.2
Zn	308	306	300	291	292	299
Cd	0.45	0.46	0.40	0.38	0.39	0.40

S indicating - Supplemented effluent sample

Table.5 Heavy metal concentration in sample (1) with altered pH determined by using ICAP-AES

	Samples (after 5 days)			(after 10 days)		
Heavy Metals	**P(C)**	**P 2**	**P 16**	**P(C)**	**P 2**	**P 16**
	Concentration in ppm					
Cr	**4.4**	**14**	**13**	**13**	**4.8**	**32**
Cu	14.7	18.9	13	13.2	26.9	14.5
Fe	5405	3319	4508	4547	3373	4720
Mn	53.6	53.3	46.5	45.9	49.4	51.4
Ni	96	62	87	86	74	97
Pb	3.1	1.7	6	4.9	1.1	1.3
Zn	2440	154	237	230	183	29
Cd	0.25	0.14	0.20	0.21	0.16	0.21

P indicating – Altered pH

Table.6 Heavy metal concentration in supplemented effluent sample (1) with altered pH determined by using ICAP-AES

	Samples (after 5 days)			(after 10 days)		
Heavy Metals	**SP(C)**	**SP 2**	**SP 16**	**SP(C)**	**SP 2**	**SP 16**
	Concentration in ppm					
Cr	**2.9**	**18**	**16**	**3.8**	**16**	**34**
Cu	9.6	9.4	15.8	14.3	23.3	18
Fe	5295	5305	4307	4616	3029	4460
Mn	57.3	66.6	46.2	50.5	56.5	51.7
Ni	56	51	78	66	74	92
Pb	1.3	1.9	4.1	0.5	2	3.5
Zn	162	139	235	195	178	247
Cd	0.24	0.25	0.19	0.21	0.13	0.20

SP indicating – Supplemented effluent sample with altered pH

Table.7 Heavy metal concentration in sample (2) with altered pH determined by using ICAP-AES

	Samples	(after 5 days)		(after 10 days)	
Heavy Metals	**Control**	**P 2**	**P 16**	**P 2**	**P 16**
	Concentration in ppm				
Cd	0.0323	0.00125	0.0002	0.0018	0.0002
Cr	**23.205**	**0.55095**	**0.0014**	**0.6069**	**0.0034**
Ni	6.6545	2.178	0.0153	1.9955	0.01825
Zn	30.525	4.626	0.01925	4.3285	0.03095
Cu	1.907	0.8234	0.0173	0.6117	0.0191
Fe	N.D	31.07	0.021	26.96	0.0349
Mn	3.033	1.734	0.0948	1.713	0.116
Pb	20.61	1.231	0.0062	0.5985	0.0116

P indicating – Altered pH

Table.8 Heavy metal concentration in supplemented effluent sample (2) with altered pH determined by using ICAP-AES

	Samples	(after 5 days)		(after 10 days)	
Heavy Metal	**Control**	**SP 2**	**SP 16**	**SP 2**	**SP 16**
	Concentration in ppm				
Cd	0.0323	0.00035	0.00025	0.00025	0.00025
Cr	**23.205**	**0.03365**	**0.00435**	**0.0082**	**0.0051**
Ni	**6.6545**	**1.352**	**0.0341**	**0.7155**	**0.0722**
Zn	30.525	1.2365	0.0319	2.969	0.0662
Cu	1.907	0.0252	0.0235	0.0486	0.0203
Fe	N.D	-0.0024	0.0781	0.034	0.0347
Mn	3.033	1.239	0.1263	1.916	0.1563
Pb	20.61	0.0081	-0.0197	0.016	0.0083

SP indicating – Supplemented effluent sample with altered pH

Table.9 Time course of growth of selected isolates and standard culture

	Isolates		
Time	**HM 2**	**HM 16**	**S**
	O.D at 540 nm		
0 hour	0.026	0.012	0.021
1 hour	0.043	0.087	0.082
2 hour	0.044	0.135	0.132
3 hour	0.096	0.256	0.396
4 hour	0.196	0.421	0.859
5 hour	0.382	0.562	1.026
6 hour	0.434	0.643	1.184
7 hour	0.529	0.720	1.245

Table.10 Chromium reduction efficiency of selected isolates and standard culture

O.D at 540 nm						
Time (h)	**HM 2**	**% reduction**	**HM 16**	**% reduction**	**S**	**% reduction**
0	0.402		0.453		0.345	
1	0.395	1.74	0.425	6.18	0.310	10.14
2	0.391	2.73	0.324	28.47	0.246	28.69
3	0.327	18.65	0.216	52.31	-0.004	101.1
4	0.290	27.86	0.174	61.58	-0.036	
5	0.234	41.79	0.121	73.28	-0.300	
6	0.228	43.28	0.118	73.95	-0.196	
7	0.214	46.76	0.106	76.66	-1.034	

Table.11 Determination of chromate reduction activity in different fractions

Fraction	HM 2	HM 16	**S**
Enzyme units µmole of Cr (VI) reduced/min/ml			
Culture supernatant	0.152	0.145	0.150
Cell extract supernatant	0.161	0.163	0.175
Cell lysate	0.141	0.135	0.137

Table.12 Sequence producing significant alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
***Micrococcus luteus* strain INBI-1 16S ribosomal RNA gene, partial sequence**	2713	2713	100%	0	99%	EU438932.1
***Micrococcus luteus* 16S rRNA gene, isolate CV44**	2710	2710	100%	0	99%	AJ717369.1
***Micrococcus luteus* partial 16S rRNA gene, isolate B9**	2708	2708	100%	0	99%	HG941665.1
***Micrococcus yunnanensis* strain N1-7 16S ribosomal RNA gene, partial sequence**	2708	2708	100%	0	99%	JX094178.1
***Micrococcus* sp. MN7-5 16S ribosomal RNA gene, partial sequence**	2708	2708	100%	0	99%	JQ396588.1
***Micrococcus* sp. 3498 16S ribosomal RNA gene, partial sequence**	2706	2706	99%	0	99%	KP345957.1
***Micrococcus* sp. 3451 16S ribosomal RNA gene, partial sequence**	2706	2706	99%	0	99%	KP345947.1
***Micrococcus* sp. PX9_S4 16S ribosomal RNA gene, partial sequence**	2704	2704	99%	0	99%	JF274943.1

Figure.1 Comparative growth profile of selected isolates w.r.t standard culture

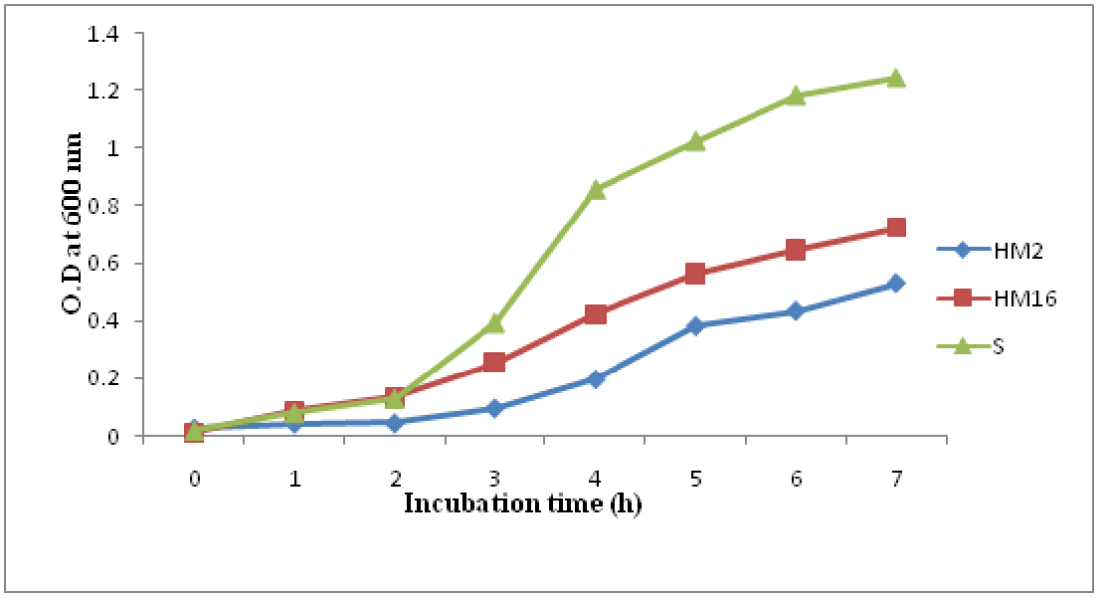


Figure.2 Comparative Cr reduction efficiency of selected isolates w.r.t standard culture

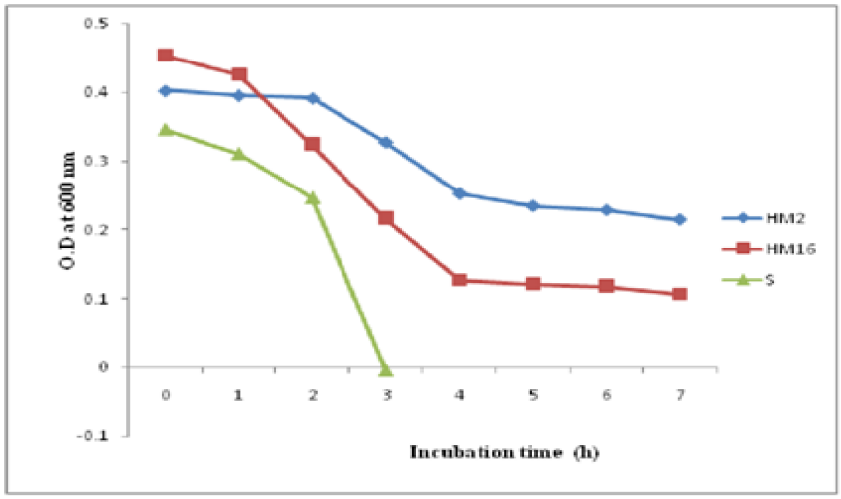


Figure.3 gDNA and 16S PCR amplicon

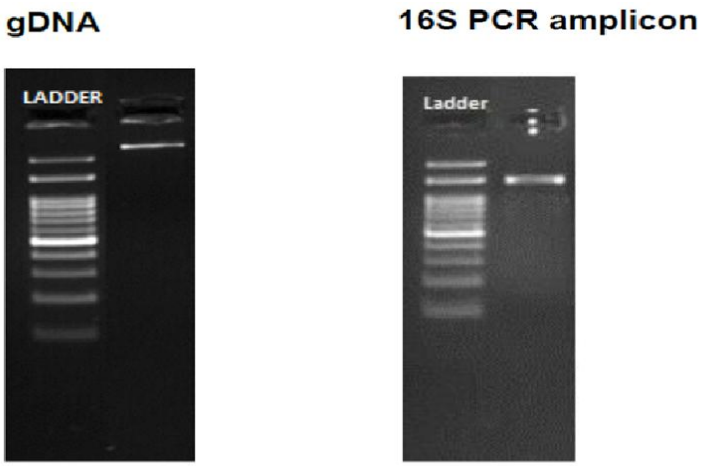
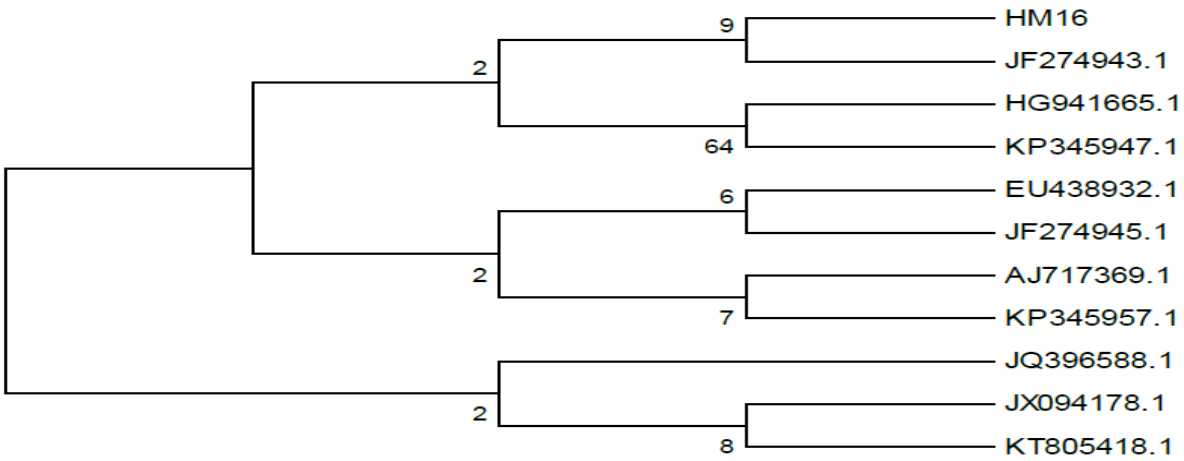


Figure.4 Molecular phylogenetic analysis by maximum likelihood method



In another study, Rehman *et al.,* (2008) found that *Bacillus* sp. had the ability to reduce hexavalent chromium into its trivalent form. These bacteria could reduce 91% of chromium from the medium after 96 hours and were also capable of reducing 84% chromium from the industrial effluents after 144 hours.

Determination of site of Chromate reductase activity

The chromate reductase activity of the selected isolates and standard culture was determined by DPC method. To determine the probable site of chromate reductase enzyme location, the activity was determined in cell culture supernatant, in cell lysate and in cell extract supernatant in both the isolates and standard culture. As revealed in Table 11, maximum chromate reductase activity (HM 2- 0.161 µmole, HM 16- 0.163 µmole and S- 0.175 µmole) were reported in cell extract supernatant in both the isolates and in standard culture. The minimum activity was reported in cell lysate.

Similarly, Ilias *et al.,* (2011) reported the Cr (VI) reduction due to chromate reductase activity detected in the culture supernatant and cell lysate but not at all in cell extract supernatant of both isolates (IFR-2 and IFR-3). Camargo *et al.,* (2003) observed chromate reductase activity in the cell-free extract and soluble fraction but very low activity in the membrane fraction of *Bacillus* sp. ES29. Wang *et al.,* (1990) reported that chromate reductase activity is preferentially associated in the membrane fraction of *E. cloacae* HO1. Batool *et al.,* (2012) have shown the intracellular reduction of Cr (VI) by reductase assay using cell-free extract. Maurya and Verma (2014) also determined the concentration of Cr (VI) in the culture supernatant spectrophotometrically by using S-diphenyl-carbazide method.

Molecular identification of the bacterial isolate by 16S rDNA sequencing

The Genomic DNA was isolated from the pure culture pellets of isolate HM 16 and 16S rDNA fragment was amplified by PCR from the above isolated genomic DNA using 27F and 1492R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose gel. The PCR amplicon was purified by column purification in order to remove contaminants (Figure 3). The sequencing of purified gene segment was done using forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Sequencing and analysis of the 16S rDNA region of the isolated bacterial strain HM 16 revealed that these regions had the highest identity with *Micrococcus luteus*. Phylogenetic relationships were inferred through the alignment and cladistic analysis of homologous nucleotide sequences of known microorganisms (Table 12). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The phylogenetic tree was depicted showing the relationship of this isolate with the genus *Micrococcus* (Figure 4). In this study, results revealed that sample which was labelled as

HM 16 showed high similarity with *Micrococcus luteus* based on nucleotide homology and phylogenetic analysis. Similarly, Pattanapipitpaisal, Brown and Macaskie (2001) isolated chromium tolerant bacterium from tannery effluent and was identified as a *Microbacterium* sp. by 16S rDNA gene sequence homology. Chromium reduction has been reported in several bacterial strains. An indigenous chromium reducing bacterial strain isolated from a tannery water sample, was identified as *Ochrobactrum intermedium*, on the basis of 16S rDNA gene sequencing (Batool *et al.*, 2012). Balamurugan *et al.*, 2014 isolated two strains (CTBI 1 and CTBI 2) having chromium reduction capacity and showed a sequence homology of 95.68% with *Pseudomonas putida* and 95.19% with *Bacillus subtilis*.

In conclusion, the present study revealed the capacity of the bacterial isolates HM 2 and HM 16 (*Micrococcus luteus)*, isolated from effluent samples, to tolerate and grow at different concentrations of Cr (VI) with Cr (VI) reduction ability. The isolates were able to resist upto 100ppm of chromium. To determine the ability of selected isolates to show reduction of Cr (VI) to Cr (III), both the isolates were tested for enzyme chromate reductase and compared with a standard strain *Shewanella putrefaciens*. Whereby, 99% reduction of Cr (VI) by the standard culture followed by 76.66 % reduction by HM 16 and 46.76 % reduction by HM 2 within 7 hours of incubation was observed. Molecular characterization of HM 16 showed high similarity with *Micrococcus luteus* based on nucleotide homology and phylogenetic analysis. This study elucidated the potential of resident microbes of effluents/sludge for heavy metal mainly chromium removal, as these bacterial species were well acclimatized to the ecology of metal contaminated aquifers.

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Conflict of Interest

The authors state that there are no conflicts of interest regarding the publication of this article.

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