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HEXAVALENT CHROMIUM REDUCTION POTENTIAL OF CHROMIUM RESISTANT TANNERY EFFLUENT BACTERIA AND THEIR CONSORTIA

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Abstract:-

Seventeen bacterial isolates from tannery effluent was tested for their ability to resist chromium (Cr) and reduce hexavalent chromium (Cr (VI)). Reduction study was carried out by individual isolates and also by their consortia. All the isolates could resist 25 mg L^{-1} chromium. Some isolate was found to resist >90 mg L^{-1} chromium. It was evident that isolates those were more resistant to chromium were not necessarily reduce more chromium. Some of these isolates were identified by their 16S rRNA gene sequencing. In general tested isolates showed more reduction potential when Cr added at the starting point of culture propagation than Cr addition after passing exponential phase. Micrococcus sp. showed maximum Cr (VI) reduction ability in both condition. For construction of consortia bacteria were chosen according to their antagonistic and synergistic property. Those isolates exhibiting antagonistic activity to other isolates were excluded from consortia preparation. Two types of consortia were prepared by mixing well grown isolates in equal proportion. Type 1 consortia that was prepared by mixing six most chromium reducing bacteria showed lesser chromium reduction ability than type 2 consortia which was prepared by mixing isolates able to reduce >1500 µg Cr(VI) g^{-1} dry cell biomass. It has been observed that, although isolate Micrococcus sp. individually reduce more chromium, consortia including this isolate did not performed well. Other combination of isolates including Micrococcus sp. might be able to reduce more chromium and could be studied further.

Keywords:- *Tannery effluent bacteria; Chromium resistant; Hexavalent chromium reduction; Bacterial consortia*

INTRODUCTION

Chromium contamination in the environment poses significant threat to human health and ecosystem because of its toxic effects (Dermou et al., 2005). Once they enter the food chain, large concentration of heavy metals may accumulate in human body and can cause serious health disorder (Babel and Kurniawan, 2004). Major source of chromium contamination in the environment are paints and pigments, steel production, wood preservation, leather industry, electro plating (Ganguli and Tripathi, 2002) and mining industry (Mangaiyarkarasi et al., 2011). Cr (VI) is more toxic compared to Cr (III) and it is mutagenic and carcinogenic in nature (Leive, 1965). The United States Environmental Protection Agency (US EPA) had considered chromium as one of the most threats to humans. The permissible limit of hexavalent chromium in drinking water is 0.05 mg L^{-1} (Samuel et al., 2012). Hence chromium removal or reduction from chromium contaminated sites is more essential. Several physical methods are available to remove chromium but all are facing one or several short comings (Prigione et al., 2009). Existing processes are costly and generate secondary waste (Montanher et al., 2005). Compared to physico-chemical methods microbe-base approaches are gaining importance due its cost effectiveness and eco-friendliness. It is evident that microorganism interact with chromium in various ways such as bio-adsorption, bio-precipitation, change of redox state and bioaccumulation (Barakat 2011). Some bacteria could convert Cr (VI) to Cr(III) which enable us to separate chromium from contaminated site. Studies show indigenous bacteria from chromium contaminated sites have enhanced potential to reduce chromium (Megharaj, 2003; Viti et al., 2003). In-situ remediation relies on microbial community as a whole rather than single isolate as contaminated environment already inhabited by several microbes (Viti et al., 2003). Current investigation emphasized on use of natural attenuation to combat hazardous effect of contaminants (Dogan et al., 2011). Therefore, it is better to utilize bacterial consortia made up of indigenous isolates to reduce Cr (VI) instead of single isolate (Samuel et al., 2012).

The major objective of present study was to build bacterial consortia for efficient Cr (VI) reduction by Cr (VI) resistant tannery isolates. To fulfil the objective individual isolates were tested for their chromium resistant and reduction potential. In consortia bacteria must synergistically perform chromium reduction. Therefore antagonistic and synergistic interactions among the bacteria were tested. Consortia were built by taking synergistic, maximum Cr (VI) tolerance and reduction potential bacteria.

Materials and Methods

Bacteria and growth medium

Bacteria isolated from tannery waste were considered for this study. All bacteria were routinely maintained in mineral salt medium (MSM) with the following composition (g L^{-1}), NaCl-4.68; NH_4Cl-1.07; KCl-1.49; Na_2SO_4-0.43; $MgCl_2$, $6H_2O$-0.2; $CaCl_2$, $2H_2O$-0.03) supplemented with glycerol phosphate (2 mM), dextrose (0.05%), yeast extracts (0.1%) at 30°C. Aliquot of full grown culture of each isolates was preserved in -20°C with 15% glycerol.

Microbial tolerance of Cr (VI)

The Cr (VI) stock solution (1000 mg L^{-1}) was prepared by dissolving $K_2Cr_2O_7$ in sterilized distilled water, passes through 0.22 µm membrane syringe filter and was used for different experiment. The maximum tolerable concentrations (MTC) of the bacteria were determined by allowing their growth on the surface of agar medium (MSM), amended with different concentration of Cr (VI). Initially, the following concentration (mg L^{-1}) was used: 0, 10, 30, 50, 70, and 90. Bacteria that were able to grow in any of the selected concentration have been further tested in higher concentration of Cr. The maximum concentration of Cr (VI) in the medium which supported the growth of the isolates was taken as the MTC.

Antagonistic and synergistic assay

To know if any of the selected isolates inhibited the growth of other or not, bacteria were allowed to grow together taking two of them at a time. Full grown culture of each isolate was prepared in MSM liquid medium supplemented with 2 mg L^{-1} chromium following incubation at 30°C and 150 rpm shaking. Two strategies were followed; (i) inoculating two bacteria at same time, 100 µl of one culture broth was spread onto the surface of the agar plate supplemented with 2 mg/L Cr (VI), and a loop full culture of another isolates were streaked on the plate, (ii) inoculating one bacteria when other in full grown stage, a loop full culture of one isolate was streaked into middle of the minimal agar plate containing Cr (VI) and allowed to grow for 72 hrs. Then loop full cultures of each isolate were streaked beside of the previously grown isolate. All plates were incubated at 30ºC for 72 hrs. The zone of inhibition between isolates shows antagonistic effect and absence of zone of inhibition between isolates shows synergistic effect, due to lack competitive inhibition.

Chromium (VI) reduction assay

Chromium reduction ability of the isolates or spent medium or cell lysate was studied following the modification of protocol of (Ilias et al., 2011) with spectrophotometric technique using standard S-diphenylcarbazide (DPC). 100 ml DPC reagent was prepared by mixing two solutions, solution 1 (24 ml of 85% H_3PO_4 in 56 ml distilled water) and solution 2 (76 mg DPC in 20 ml 95% ethanol) and kept in dark at 4ºC. Chromium reduction assay was carried out by adding 125 µl of DPC reagent to 1 ml of test samples, and the solution was mixed gently and kept at room temperature for 20 min. The absorbance of the treatment was measured by spectrophotometer (Thermo Scientific; Evolution 201 UV Visible Spectrophotometer) at 540 nm wavelength.

Sample for Cr(VI) assay was prepared in following two ways: (i) bacteria were allowed to grow in 10 ml minimal broth without chromium (initiated by 1% inoculum from overnight culture (V/V)). Cr (VI) was added in each culture (final

concentration 2 mg L^{-1}) when it reaches to visible growth and incubated for several hours. Cr (VI) in each treatment was monitored by withdrawing 1 ml culture followed by centrifugation in 10,000 rpm for 10 min. The supernatant was collected for Cr (VI) assay. (ii) Bacteria were allowed to grow overnight in 10 ml of minimal broth containing 2 mg/L Cr (VI). Cr (VI) was monitored in the treatments as mentioned above. Reduction of chromium by the biomass was calculated from a metal biomass balance, yielding: q (mg metal g^{-1} dry biomass) =V (C_i –C_f)/m, where V is the sample volume (L), C_i and C_f are the initial and the final metal concentrations (mg L^{-1}) in the supernatant, respectively and m is the amount of dry biomass (g) (Nedelkova et al., 2007). Biomass dry weight was determined by drying the cell pellets at 70°C for 72 h until the weight became constant.

Construction of Consortium

Consortia were developed by mixing bacteria based on their antagonistic and synergistic property and Cr (VI) reduction potential. Mixed bacterial culture was used to reduce Cr (VI) where chromium was added at the commencement of growth.

Molecular characterization of the bacteria

Bacterial isolates were grown in MSM broth to exponential phase. The cells were harvested by centrifugation and DNA was extracted using DNeasy tissue kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. After measuring the concentration by nanodrop 2000 spectrophotometer (Thermo Scientific) the isolated DNA was used for polymerase chain reaction (PCR) amplification of 16S rDNA using 27F & 1492R primer following standard protocol (Islam and Sar, 2011). Each 50 µl PCR reaction mixture contained the following (final concentration): 1.5 mM $MgCl_2$, 200 µM of each dNTPs, 10 pmol of each primer, 1× PCR buffer, 1.5 U Taq DNA polymerase (New England Biolab, MA, USA) and 15 ng of genomic DNA. The following temperature regime was applied using Applied Biosystem thermo-cycler, 95°C for 5 min followed by 30 cycles of denaturation 95°C for 30 sec, annealing at 58°C for 45 sec, extension at 72°C for 45 sec and final extension at 72°C for 7 min. After PCR reaction the product length was verified using 1% agarose gel electrophoresis. PCR amplified DNA were directly sequenced from commercial vendor.

Phylogenetic analysis

Phylogenetic affiliation of the bacteria was determined from the sequence data using nucleotide BLAST program of NCBI.

Result and discussion

Microbial tolerance of Cr (VI)

Total seventeen bacterial isolates were tested for their tolerance to hexavalent chromium.

Maximum tolerance concentration of Cr (VI) for each isolates was determined and presented in Table 1. In general all the isolates showed MTC value above mgL^{-1}. Isolates 2A, 4A, 4B and 4E showed lowest MTC 25 mg L^{-1}, while isolates 1.4, 2.1, 2.3 and 2.4 showed highest MTC value 90 mg L^{-1}. Among the tested isolates 2.2, 3.2, 3.1 showed fare MTC value 30 mg L^{-1}. Some isolates could tolerate pretty good concentrations of Cr (VI) with MTC value 50 (1.1, 3.3 and 3.4) and 70 mgL^{-1} (1.2, 1.3 and 4D) (Table1).

Table 1: Maximum tolerance concentration of Cr (VI) for the isolates.

Isolates	MTC value of Cr(VI) (mg L^{-1})
2A,4A,4B,4E	25
2.2, 3.2, 3.1	30
1.1, 3.3, 3.4	50
1.2, 1.3, 4D	70
1.4, 2.1, 2.3, 2.4	90

Antagonistic and synergistic assay

To achieve a higher reduction rate of Cr (VI), consortia were developed based on antagonistic and synergistic assay. In this assay all isolates are tested for their inhibitory function. Some isolates showed antagonistic effect for other isolates. 2A isolate was inhibited the growth of 4A, 4E and 4D isolate antagonist of all other isolates, showed a high zone of inhibition (Fig 1A). Rest of other isolates was showed synergistic effect due to lack zone of inhibition (Fig 1B),

(Table 2).

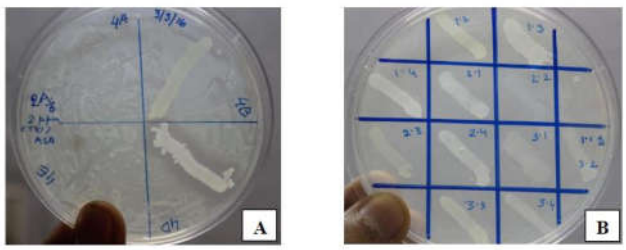

Fig 1. Isolates 4D showed high antagonistic effect on other bacteria (A). Isolates were synergistic to each other; where 1.1 isolates was spread onto the agar and other isolates were streaked (B).

Table 2: Synergistic and antagonistic property of the bacterial isolates. ('A' denotes antagonistic behaviour while 'S' synergistic behaviour)

	2A	4A	4B	4D	4E	1.1	1.2	1.3	1.4	2.1	2.2	2.3	2.4	3.1	3.2	3.3	3.4
2A		A	S	A	A	S	S	S	S	S	S	S	S	S	S	S	S
4A			S	A	S	S	S	S	S	S	S	S	S	S	S	S	S
4B				S	S	S	S	S	S	S	S	S	S	S	S	S	S
4D					A	A	A	A	A	A	A	A	A	A	A	A	A
4E						S	S	S	S	S	S	S	S	S	S	S	S
1.1							S	S	S	S	S	S	S	S	S	S	S
1.2								S	S	S	S	S	S	S	S	S	S
1.3									S	S	S	S	S	S	S	S	S
1.4										S	S	S	S	S	S	S	S
2.1											S	S	S	S	S	S	S
2.2												S	S	S	S	S	S
2.3													S	S	S	S	S
2.4														S	S	S	S
3.1															S	S	S
3.2																S	S
3.3																	S
3.4																	

Cr (VI) reduction by the bacterial isolates

Cr (VI) reduction by the isolates was tested by adding Cr (VI) in the medium at the time of inoculation. Reduction of Cr (VI) was allowed with the growth of isolates up 60 hr and 84 hr. The result is expressed µg Cr (VI) g^{-1} dry cell biomass and presented in (Fig 2). It was noticed that all the test organisms showed significant Cr (VI) reduction potential. In general all isolates could reduce more than 2000 µg Cr (VI) g^{-1} dry cell biomass. Isolates 1.1, 3.1 and 4E showed higher reduction potential. Among the isolates, 4E can reduce maximum amount of Cr (VI) (~8000 µg g^{1} dry biomass). Increasing the incubation time, very little increase of Cr (VI) reduction was noticed. Cr (VI) reduction was also tested by adding Cr when cells of isolates were in log phase (Fig 3). In this situation very little Cr (VI) reduction was observed except isolate 4E. Isolate 4E could reduce ~6000 µg Cr (VI) g^{-1} dry biomass in 60 hr and increasing the incubation time the reduction increased to 10, 000 µg g^{-1} dry biomass in 84 hr.

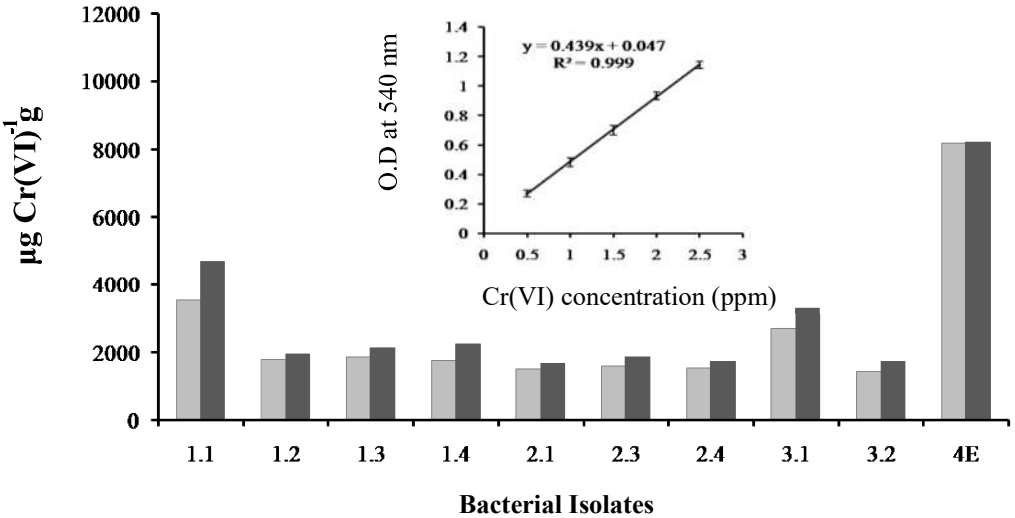

Fig 2. Chromium reduction by isolates when chromium was added at the commencement of growth. Standard curve obtained during Cr (VI) estimation (insight).

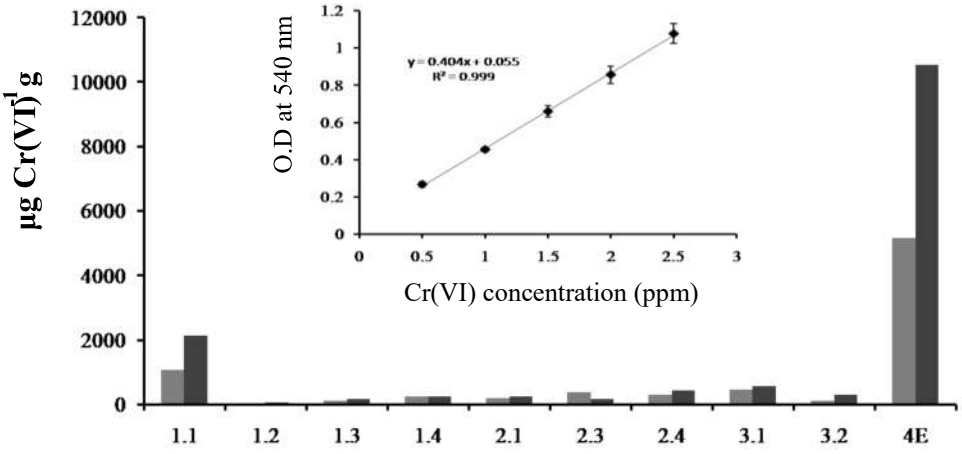

Fig 3. Chromium reduction by isolates where chromium was added after reaching the exponential growth phase. Standard curve obtained during Cr (VI) estimation (insight).

Cr (VI) reduction by the consortia

Two consortia were prepared one by mixing isolates that showed higher Cr (VI) reduction potential, the type 1consortium and by mixing all the synergistic isolates irrespective of their Cr (VI) reduction potential, the type 2 consortium. Each consortium was tested for chromium reduction. Average result of duplicate experiment is presented in Fig 4. Consortium 1 and 2 reduce nearly 2500 and 3000 µg Cr (VI) g^{-1} dry cell biomass in 60 hr and 84 hr, respectively. Increasing the incubation time, although consortium 1 reduces higher amount of Cr (VI), consortia 2 did not show further reduction.

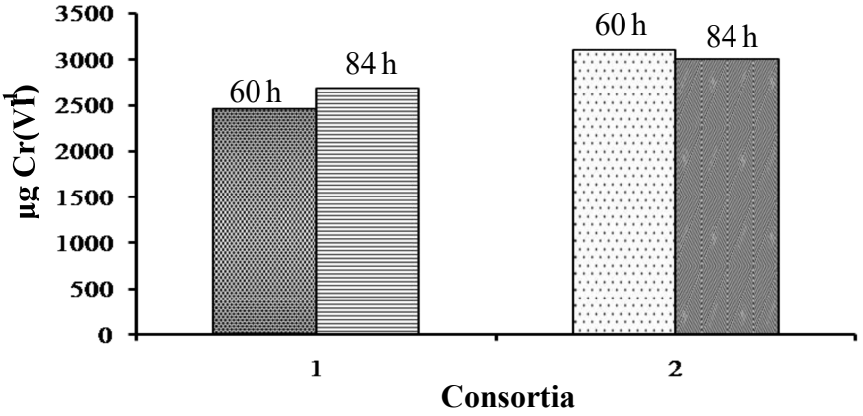

Fig 4. Chromium reduction by consortia, (1) combination of six isolates (1.2, 1.3, 1.4, 2.3, 3.1, 4E), (2) Combination of ten isolates (1.1, 1.2, 1.3, 1.4, 2.1, 2.3, 2.4, 3.1, 3.2, 4E). Composition of each consortium is described in the text.

Characterization of the bacteria

Six bacterial isolates were identified based on their 16S rRNA gene similarity in NCBI data base. Nearest BLAST match of 16S rRNA gene from each isolate is presented in Table 3**.** Isolates 1.1 showed maximam identity to *Homoserinibacter gongjuensis.* Isolates 1.3 was found to affiliated with *Ochrobactrum* sp. Y6. Isolate 2.3 was identified as *Pseudomonas*. Isolates 3.1 and 3.2 showed maximum identity to *Rhizobium.* Isolate that showed maximum chromium reduction ability was identified as Micrococcaceae bacterium.

Table 3. Nearest BLAST match of 16S rRNA gene sequence of the isolates

Isolate	Nearest BLAST match (Accession No)	% identity
1.1	*Homoserinibacter gongjuensis* 5GH 26-15 (NR_134058.1)	99
1.3	*Ochrobactrum* sp. Y6 (KM396913.1)	99
2.3	*Pseudomonas* sp. LT1 (AJ007005.1)	99
3.1	*Rhizobium* sp. KD2009-36 (FN645726.1)	100
3.2	*Rhizobium* sp. HGR13 (GQ483459.1)	100
4E	Micrococcaceae bacterium SAP61_2 (JN872520.1)	99

Conclusion

All tannery isolates showed significant Cr (VI) resistant ability. Ten out of seventeen isolates reduce significant amount of chromium Cr (VI). Isolate 4E which identified as *Micrococcus* sp. showed maximum Cr (VI) reduction ability. Isolate 4D was found antagonistic to all other isolates. Prepared consortia in study did not perform well. Different combination of isolates including 4E in the preparation of consortia for better reduction is suggested.

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