# AN INTEGRATED APPROACH FOR SAFE REMOVAL OF CHROMIUM (VI) BY *BREVIBACTERIUM* SP.

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**ABSTRACT:** Chromium (VI) tolerant *Brevibacterium* sp. AKR2 was isolated from the tannery effluent polluted site and consequently screened for chromate removal potential and exopolymer production. This strain showed stable growth with white-gray colonies on LB-agar plates supplemented with chromate (1500mg/L). Strain AKR2 was taxonomically identified by 16S rRNA sequencing as *Brevibacterium* sp. (MN932133). Presumptive biochemical tests were also performed. This strain was found to have multiple heavy metals and antibiotic tolerance patterns. Additionally, the tolerance profile of *Brevibacterium* sp. against Cr(VI) (2-30mg/mL) was investigated. Cr(VI) reduction ability at 1000 and 1500mg/L of $K_2CrO_4$ highlighted that the strain AKR2 reduced 91 and 86% of chromate oxyanions, respectively, after 24h of incubation. Enhanced exopolymer i.e. 1.275mg/mL dry weight was observed by growing in LB-broth medium under chromate stress conditions 1500mg/L as compared to EPS yield under non-stress 0.15mg/mL. Hence, the current investigation suggests the possible use of chromate resistant *Brevibacterium* sp. as an emerging tool for remediating the sites polluted with elevated levels of Cr(VI).

**Keywords:** Chromium (VI), *Brevibacterium* sp., exopolysaccharide production, multiple heavy metal resistance, Cr (VI) reduction.

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## INTRODUCTION

Rapid industrial development and population growth have created severe ecological contamination that is greatly affecting human and animal lives (He *et al.,* 2020). The inadequate treatment processes of industrial effluents has caused the accumulation of toxic chemical compounds in the environment (Vendruscolo *et al.,* 2017). These include various heavy metals for example chromium, lead, cadmium, mercury and selenium, which are among the most significant and pervasive environmental contaminants. These contaminants are not degraded biologically, rather accumulate in living organisms (Modoi *et al.,* 2014; Zhao *et al.,* 2016). Heavy metals have the strong potential to react with proteins and inactivate several enzymes (He *et al.,* 2020). Elevated level of heavy metal pollutants have been recorded in the environment leading to severe health problems (Joshi 2018). Chromium (Cr) pollution is linked with chemical, metallurgy, electroplating, paper making and leather tanning (Jobby *et al.,* 2018). Cr is stable in trivalent and hexavalent valence states. The former is innocuous and precipitates in the solution at pH greater than 5 (Mohapatra *et al.,* 2017). Continual exposure of Cr (VI) can lead to carcinogenesis, mutagenesis and teratogenesis (Ali *et al.,* 2016). It readily dissolves in water and generally exists in the form of $CrO_4^{-2}$ and $Cr_2O_7^{-2}$. The toxicity of Cr (VI) is 1000 times more than Cr (III), even so, it is an indispensable component for various human metabolic processes (Raman *et al.,* 2018; He *et al.,* 2020). Untreated industrial wastes containing high amounts of Cr released in to environment creates an alarming situation for environmental safety (Alam and Ahmad, 2011; Mohapatra *et al.,* 2017). US Environmental Protection Agency has listed Cr in top 20 hazardous chemicals to humans. The intracellular formation of highly unstable Cr (V/IV) reactive oxygen species makes chromate ions harmful to living organisms (Mala *et al.,* 2015).

Physiochemical, chemical, electrical and biological approaches have been applied to attain the removal of Cr (VI) ions (Habibul *et al.,* 2016). Conventional treatment processes include precipitation, ion exchange, solvent extraction and membrane filtration (Witek-Krowiak *et al.,* 2011). These techniques are inefficient in terms of cost, high energy consumption, incomplete metal removal and production of toxic byproducts. Thus, producing an efficient methodology for dealing with the problem of metal pollution is a big challenge nowadays. Currently, microbes are being exploited for removal of metallic oxyanions, since they are very operative in terms of environmental safety and protection, and metal recovery (Devi *et al.,* 2012). Thus, biotic techniques for detoxifying Cr (VI) are inexpensive, environmental friendly, nontoxic and free from the production of lethal side products (Coetzee *et al.,* 2018).

Bioremediation involves the removal of chromate ions through microbes with an additional mechanism i.e. chromate resistance. Microbes particularly bacteria establish various survival mechanisms such as DNA methylation, uptake through adsorption, metal ions efflux and bio-transfer of metal ions directly or indirectly to cope with high concentrations of toxic metal ions. Previous studies described a variety of bacteria exhibiting Cr (VI) remediation such as, *P. aeruginosa*, *Bacillus subtilis*, *Bacillus circulans* strain MN1, *Acinetobacter*, *Micrococcus*, *Aeromonas* and *Saccharomyces cerevisiae* etc. (Saxena and Bharagava, 2015). This work proposes to investigate the Cr (VI) removal potential and EPS production ability of highly chromate tolerant indigenous bacteria isolated from local tannery waste.

## MATERIALS AND METHODS

**Isolation of indigenous chromate tolerant bacteria:** The sample was collected from a tannery effluent contaminated site in District Kasur, Punjab. Luria-Bertani (LB) agar medium supplemented with $K_2CrO_4$ (1500 mg/L) was employed to isolate chromate resistant bacteria by serial dilution approach and plates were observed after 48h of incubation at 37◦C. Chromium (VI) resistant strain AKR2 was selected and purified by quadrant streak and preserved at 4◦C for further investigation.

**Physio-chemical characteristics of strain AKR2:** Chromate resistant strain AKR2 was identified on the basis of cell morphology and biochemical examinations. Biochemically strain was characterized by catalase, oxidase, motility, DNase, citrate, starch and gelatin hydrolysis, urease, MR-VP, indole and TSI tests.

**Taxonomic identification of strain AKR2:** Strain AKR2 was sent to Macrogen Inc. (Seoul, Korea) for 16S rRNA sequencing to taxonomically identify it using primers 518F (CCAGCAGCCGCGGTAATACG) and 800F (TACCAGGGTATCTAATCC). MEGA-X was used for phylogenetic analysis and expression of the tree by the neighbor joining method considering the two main domains archaea and eubacteria for bootstrap analysis (Katoh and Standley, 2013).

**Tolerance capacity to chromium (VI)** Cr (VI) tolerance capacity of strain AKR2 was investigated at a stress of 2-30 mg/mL Cr (VI). For this, LB agar plates supplemented with respective initial chromate concentrations were used and strain AKR2 was streaked and kept in an incubator for 24-48h at 37°C. After incubation, the bacterial growth was recorded for chromate resistance.

**Tolerance capacity to other heavy metals and antibiotics:** To determine the resistance capacity of strain AKR2 to multiple metal ions such as $Ni^{+2}$, $Co^{+2}$, $Zn^{+2}$, $Pb^{+2}$, $Cu^{+2}$ and $Hg^{+2}$, LB agar plates were prepared and supplemented with a range (100-1600 mg/L) of selected heavy metals and incubated for 24-48h at 37°C. After incubation, the plates were assessed by observing the growth on plates for multiple heavy metal resistance capacity. Antibiotic tolerance patterns for ampicillin, streptomycin and chloramphenicol (10-40 mg/L) were also observed in the LB agar medium.

**Chromium (VI) reduction ability of strain AKR2:** To estimate the chromate reduction ability of strain AKR2, DeLeo and Ehrlich medium (tryptone 10, yeast extract 5, NaCl 5, citric acid 1, $Na_2HPO_4$ 6.9 g/L) was prepared. Three sets of 100 mL flasks were prepared (in triplicates), one set was supplemented with 1000 mg/L, second set with 1500 mg/L of Cr (VI) and the third set was as control to analyze the chromate reduction in abiotic condition. All the sets of flasks were inoculated with bacterial isolate (strain AKR2) except control and placed in shaking incubator (120 rpm) at 37°C for 24h. Samples (1mL) were collected aseptically and centrifuged at 14,000 rpm for 5 min and residual chromium (VI) was determined in supernatant by diphenyl carbazide method (Faisal and Hasnain, 2004).

**Estimation of exopolymer production:** To study the variations caused by Cr (VI) stress, EPS was estimated quantitatively. EPS was extracted from the method described in Batool *et al.* (2015) with some alterations. Overnight culture of strain AKR2 was inoculated in 250 mL flasks containing 100 mL LB-broth supplemented with and without 1000 mg/L and 1500 mg/L Cr (VI) stress. All the flasks were prepared in triplicates and kept at 37°C for 5 days (150 rpm shaking). After incubation time, cells were centrifuged at 12,000 rpm for 10 min. Supernatant was treated with double volume of ice chilled absolute ethanol and left overnight at 4◦C. Treated samples were centrifuged to get precipitated EPS and dried.

**Statistical analysis:** All the experiments were performed in triplicates and the standard error of the mean was calculated in Ms. Excel 2013.

## RESULTS AND DISCUSSION

Rapidly increasing urbanization and deforestation has caused the accumulation of hazardous pollutants in the environment which has led to various health problems related to living organisms. The contamination of water and soil by chromium (VI) compounds is an emerging issue nowadays. Therefore, remediation of Cr (VI) contaminated areas with microorganisms is gaining more attention for the safe removal of chromate. Living organisms for example microbes are viable, long term and sustainable option for

treating chromium contamination because they are efficient and noninvasive. Microbes play a critical role in regulating the biogeochemical behavior of Cr in environmental contamination (Xia *et al.,* 2019). The Cr polluted areas can be ameliorated by removing Cr (VI) ions through bacteria. This study investigated the chromate removal and exopolymer (EPS) potential of Cr (VI) resistant strain AKR2.

Among the various strains isolated at 1500 mg/L chromate stress (data not shown), strain AKR2 was selected that showed good growth under Cr (VI) stress (1500 mg/L). The bacterial isolate (AKR2) was Gram-positive short club shaped rod with small size colonies of white-gray color and smooth appearance. The bacterial strain was non–spore former, catalase and oxidase positive (Table 1). Bacterial tolerance to chromium (VI) has been studied in several genera (Faisal and Hasnain, 2004). Numerous studies have described the importance of gram-positive bacteria which cope chromate stress by ameliorating Cr (VI) into Cr (III) (Patra *et al.,* 2010; Marzan *et al.,* 2017). Taxonomically, strain AKR2 showed 99% homology with *Brevibacterium* sp. by 16S rRNA sequencing. The DNA sequences were submitted to GenBank of NCBI with accession number MN932133. Phylogenetic tree was constructed and illustrated in Figure 1. Consequently, the bacterium was identified as *Brevibacterium* sp. Faisal and Hasnain (2004) also described the isolation of indigenous chromate resistant bacterial strain *Brevibacterium* CrT-13 from tannery effluents.

Strain AKR2 indicated that it can grow up-to 30 mg/mL of chromate stress. Previous studies described the highest MIC values of gram-positive bacteria such as, 2.5 mg/L, 40 mg/mL and 80 mg/mL (Cheng and Li, 2009; Sultan and Hasnain, 2005; Shakoori *et al.,* 2000). A group of scientists evaluated the chromium (VI) tolerance levels of chromate resistant *Micrococcus* sp. exhibiting resistance of 8 mg/mL Cr (VI) concentration. These findings suggested the application of these highly Cr resistant bacteria for decontamination of Cr (VI) polluted areas (Congeevaram *et al.,* 2007). Multiple heavy metal resistance profiling was also explored because during industrial procedures Cr (VI) wastewater also contains a wide range of other heavy metallic cations, developing tolerance in the indigenous flora to these heavy metals (Masood and Malik, 2011). Strain AKR2 showed tolerance capacity towards antibiotics and multiple heavy metals (Table 2). The maximum level of inhibition instigated by tested heavy metallic cations on strain AKR2 was $Ni^{+2}>Cu^{+2}>Pb^{+2}>Zn^{+2}>Co^{+2}>Hg^{+2}$.

Bacteria develop various tolerance mechanisms that assist them to cope with the environmental toxins (Joutey *et al.,* 2014; Batool *et al.,* 2017). It is not likely to associate various tolerance levels reported in literature by different microorganisms growing under variable optimized conditions. Nevertheless, the present and cited studies suggested the high resistivity of gram-positive bacteria towards chromate. The effect of variable chromate concentrations on strain AKR2 was assessed in liquid cultures for Cr(VI) removal. The response of strain AKR2 towards Cr (VI) stress varied greatly. Cr (VI) removal potential was determined at an initial concentration of 1000 and 1500 mg/L. Chromate tolerant strain AKR2 exhibited maximum Cr (VI) removal of 91%, and 86% at 1000 and 1500 mg/L ($K_2CrO_4$), respectively (Figure 2). Resistance towards chromate and other metal ions greatly enhanced the significance of these bacteria for reclamation of heavy metal polluted areas. Despite the fact that Cr (VI) resistant bacteria persist in toxic chromate concentration but also play a significant role in its removal which is an important aspect and qualifying attribute towards bioremediation.

Various researchers have analyzed the possible role of bacterial exudates (EPS) in chromium oxyanion reduction as a reclamation strategy (Dogan *et al.,* 2011). Other studies also demonstrated the production of EPS upon exposure to toxic compounds such as Cr (VI). Wani *et al.* (2019) described the significant role of EPS by giving a supplementary advantage of the continuous bacterial growth as an adaptation to metal stress environment. Keeping this in view, EPS production by strain AKR2 was estimated. This strain was able to produce 0.15 mg/mL exopolymer dry weight in LB medium without any metal contaminant stress, however, EPS content was increased i.e. 0.902 and 1.275 mg/mL when medium was supplemented with 1000 and 1500 mg/L chromium (Figure 3).

Oves and his co-researchers shown that exopolymer production by *A. xylosoxidans*, was considerably increased when the media was supplied with an increasing concentration of heavy metals. EPS content was increased to 29.5% when media was supplemented with 0 to 600 µg/mL $Zn^{+2}$ concentration (Oves *et al.,* 2019). A previous investigation of EPS content produced by Cr (VI) tolerant *Micrococcus* sp., *Ochrobactrum* sp. and *P. aeruginosa* showed 0.38 g/L, 0.26 g/L and 0.45 g/L respectively, under chromate stress of 100 mg/L (Kılıç and Dönmez, 2008). With increasing temperature, chromate tolerant *Micrococcus* sp. exhibited highest tolerance to chromate and EPS production. In a study, *Bacillus subtilus* PAW3 showed substantial quality of EPS production under chromate stress. EPS aid in binding of metal ions and do not allow it to enter into the cell while reducing it by chromate reductases (Wani *et al.,* 2019). Bacterial exudates comprised of various functional groups such as, carboxylic acid –COOH assessable for oxyanion complex formation such as Cr (III). These reactions between bacterial EPS and Cr (III) initiates a prominent impact on chromium bioavailability, solubility and sorption/transport behavior in subsurface systems. Generally, in bacteria the Cr (III)-EPS complex may affect the reduction of chromate in two ways i.e.

protecting the cells and enzymes responsible for chromate removal (Dogan *et al.*, 2011).

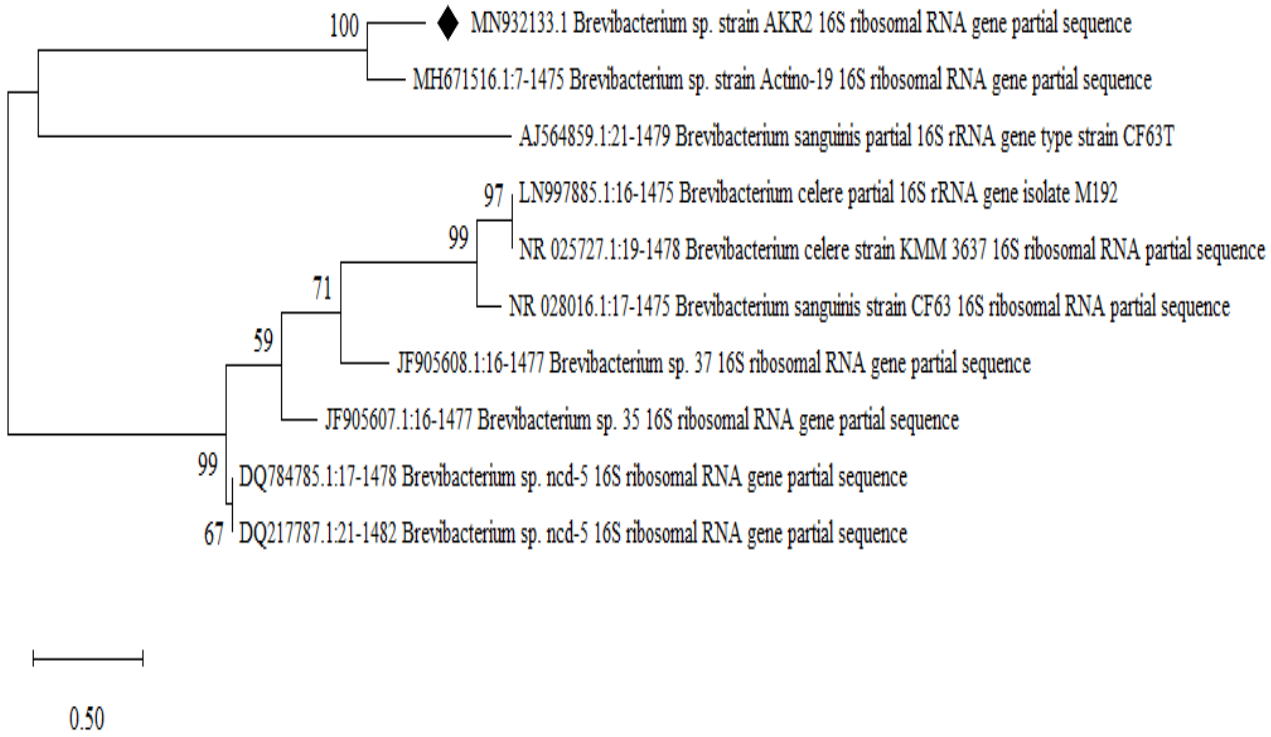


**Figure 1 Phylogenetic analysis of Cr (VI) tolerant strain AKR2 by Neighbor joining method**

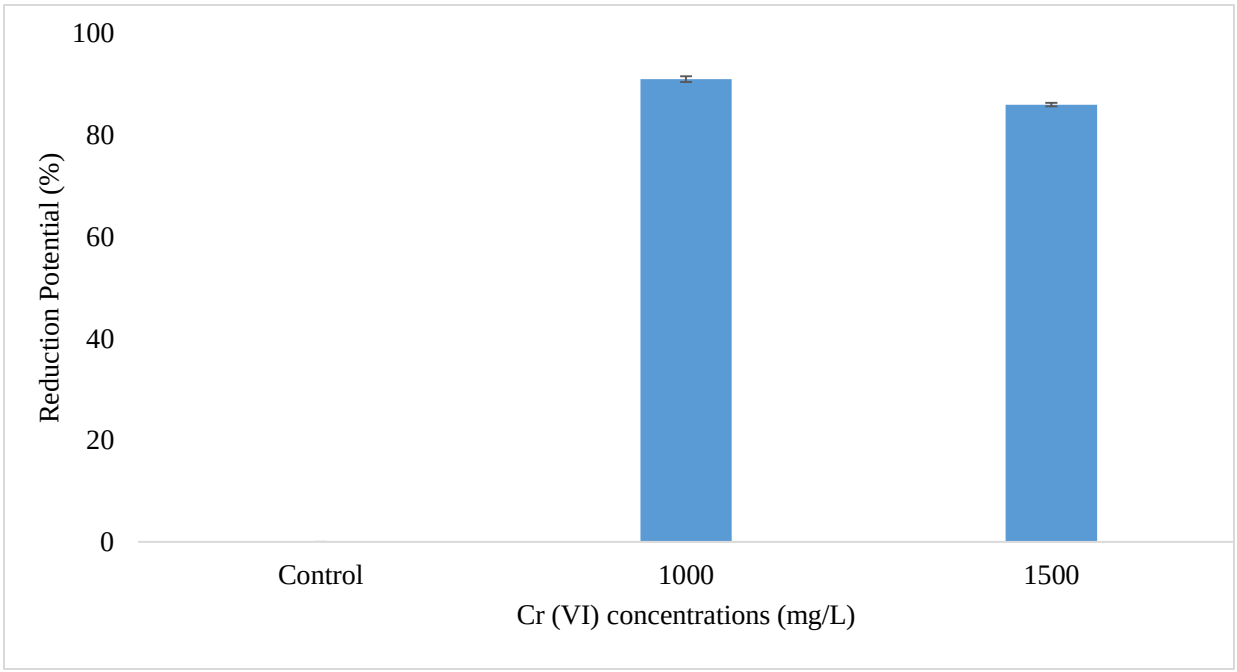


**Figure 2 Cr (VI) removal potential of strain AKR2 at various chromate concentrations**

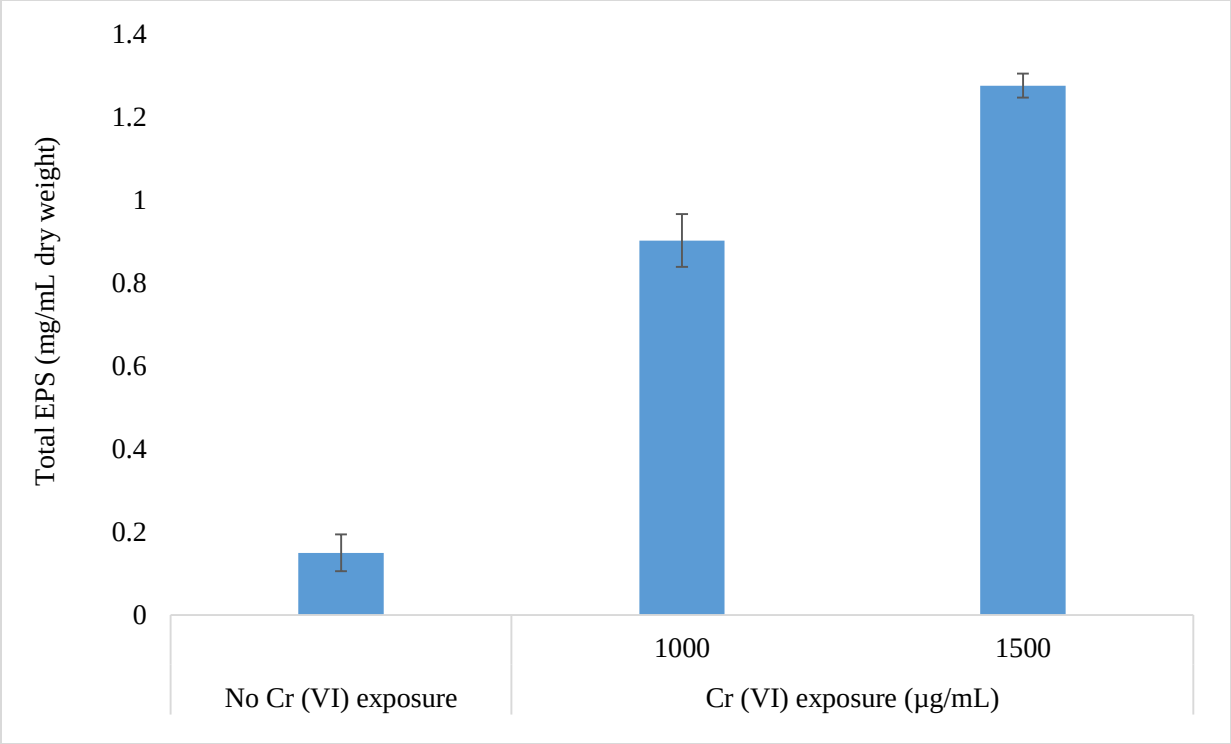


**Figure 3 Total exopolymer produced by Cr (VI) resistant bacterial strain AKR2 at different chromate stress**

**Table 1. Morphological and biochemical characteristics of strain AKR2.**

| **Morphological characteristics** | **Bacterial strain AKR2** |
|---|---|
| Colony size | Small |
| Shape | Round |
| Margins | Circular |
| Appearance | Smooth |
| Color | White Gray |
| Texture | Smooth |
| **Biochemical tests** | |
| Catalase | + |
| Oxidase | + |
| Motility | - |
| DNase | + |
| Citrate | + |
| Starch hydrolysis | - |
| Gelatin hydrolysis | + |
| Urease | - |
| MR | - |
| VP | - |
| TSI | - |
| Indole | - |

Positive =+; negative=-

**Table 2. Tolerance pattern of strain AKR2 for (A) multiple heavy metals and (B) antibiotics.**

| (A) Heavy metals | MIC (mg/L) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 20 | 50 | 100 | 200 | 300 | 500 | 800 | 1000 | 1300 | 1500 | 1600 | 1700 |
| $CoCl_2$ | + | + | + | - | - | - | - | - | - | - | - | - |
| $NiCl_2$ | + | + | + | + | + | + | + | + | + | + | - | - |
| $ZnCl_2$ | + | + | + | + | - | - | - | - | - | - | - | - |
| $PbCl_2$ | + | + | + | + | + | + | + | - | - | - | - | - |
| $CuSO_4$ | + | + | + | + | + | + | + | + | + | + | - | - |
| $HgCl_2$ | + | - | - | - | - | - | - | - | - | - | - | - |

| (B) Antibiotics | MIC (mg/L) | | | | |
|---|---|---|---|---|---|
| | 10 | 20 | 30 | 40 | 50 |
| Ampicillin | + | + | + | - | - |
| Streptomycin | + | + | - | - | - |
| Chloramphenicol | + | + | + | - | - |

Positive =+; negative=-

**Conclusion:** Chromium (VI) tolerant indigenous strain *Brevibacterium* sp. AKR2 showed high resistance to chromium (30mg/mL) and other heavy metals. The strain was proficient to remove 91 and 86% of chromate oxyanions at 1000 and 1500mg/L of $K_2CrO_4$ respectively, after 24h of incubation. Enhanced exopolymer i.e. 1.275mg/mL dry weight was observed by growing in LB-broth medium under chromate stress conditions 1500mg/L as compared to non-stress 0.15mg/mL. Bioreduction of Cr (VI) to Cr (III) along with EPS production is a promising technique for the safe removal of chromate from tannery effluents. Hence, the current study highlights the significance of chromate removal by indigenous bacteria as an alternative environmental tool for green chemistry.

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