CHEMICAL ENGINEERING TRANSACTIONS
VOL. 88, 2021
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Petar S. Varbanov, Yee Van Fan, Jiří J. Klemeš

ISBN 978-88-95608-86-0; **ISSN** 2283-9216
DOI: 10.3303/CET2188019

Bioreduction of Cr(VI) Using Bacterial Consortia Isolated from a Municipal Wastewater Sludge Experiencing Cr(VI) Loading from an Abandoned Chrome Foundry

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Due to the threat posed by Cr(VI) on humans and aquatic wildlife, the removal of this metal must be applied effectively and without causing an impact on the environment. Reduction of Cr(VI) using microorganisms has been proposed as an alternative technique for remediation of both soils and groundwater, owing to its environmental friendliness and low-cost. Extensive work on Cr(VI) reduction by microorganisms has been reported, however, most of these scholars have paid attention to pure cultures. In this study, a natural consortium of microbial cultures from a municipal wastewater sludge site was used to investigate the effect of initial Cr(VI) concentration and pH on the Cr(VI) removal by the consortia. The results show that the optimised bacterial consortium was resistant to greater than 400 mg/L Cr(VI) concentration with 50 mg/L being completely removed within 4 h of incubation. The consortia exhibited considerable Cr(VI) removal efficiency in the pH range from 2 to 11, with 100% removal being achieved at a pH value of 7. This study demonstrated that bacteria the bacterial consortium from municipal wastewater sludge could be used for Cr(VI) remediation. Thus, bioremediation is a viable, environmentally friendly technology for applications in hexavalent chromium contaminated site.

1. Introduction

In recent decades, hexavalent chromium has been extensively used in many industrial applications such as textile dyeing, pigments, electroplating, tanned leather, wood preservation, and other industries (Mishra and Bharagava, 2016). The overuse of hexavalent chromium (Cr(VI)) by these industries has resulted in the production of large amounts of solid and liquid waste containing Cr(VI). Improper storage and disposal of Cr(VI) containing waste has led to an immense amount of Cr(VI) flowing into the ecosystem and caused serious environmental pollution (Tan et al., 2020). Cr(VI) is highly mobile, soluble and bioavailable in the environment, it is the most toxic among chromium species, and is a known carcinogen. In contrast, trivalent chromium (Cr(III)); is immobile, less stable in water and is an essential dietary element (Mbonambi and Chirwa, 2019). Cr(VI) is also internationally recognised for its toxicity as it is listed as a priority environmental pollutant by the U.S EPA (Zheng et al., 2019). Cr(VI) containing waste treatment is the most important step before discharging them into the environment. Therefore, reducing Cr(VI) to Cr(III) is beneficial in eliminating the toxicity of Cr(VI) of wastewaters.

To reduce the adverse effects of Cr(VI) on human health and the environment, suitable remedial methods and remediation interventions are required. Conventional physical and chemical methods for removal of Cr(VI) from wastewater or groundwater have been developed, and these include adsorption, chemical or electrochemical reduction to Cr(III) and subsequent precipitation or electrokinetics (Peng et al., 2019). Although these technologies can decrease the adverse metal impact, their major drawbacks include the production of toxic waste sludge, high-energy demands or inefficient removal (Seh-Bardan et al., 2012). Therefore, the search for innovative, cheaper, environmentally friendly and more effective techniques have become important for the removal of toxic Cr(VI) ions from polluted areas.

Paper Received: 29 June 2021; Revised: 7 August 2021; Accepted: 5 October 2021
Please cite this article as: Kholisa B., Chirwa E.M., 2021, Bioreduction of Cr(VI) Using Bacterial Consortia Isolated from a Municipal Wastewater Sludge Experiencing Cr(VI) Loading from an Abandoned Chrome Foundry, Chemical Engineering Transactions, 88, 115-120 DOI:10.3303/CET2188019

In recent years, researchers have increasingly shown interest in using biological systems as an alternative technique to detoxify and reduce Cr(VI) from the environment. The removal of Cr(VI) using biotechnologies is considered to have great potential, and it is an environmental-friendly strategy for remediation of Cr(VI), owing to its low-cost, robustness and suitability. The first work on Cr(VI) reduction using microorganisms was reported in the late 1970s, Romanenko & Koren'Kov (1977) isolated Pseudomonas strain that can reduce Cr(VI) under anaerobic conditions. Since then, a variety of bacteria strains have been isolated and reported their Cr(VI) reducing capabilities; these include *Pseudomonas* putida (Mbonambi et al., 2019), *Bacillus* sp. (Shim et al., 2016), *Achromobacter* sp. (Rao et al., 2017). Different environments such as tannery effluents, waste disposal sites, chromate mines, abandoned mine sites and industrial landfills have been sought after to isolate these potential bacterial strains for Cr(VI) bioremediation. The extent and rate of reduction of Cr(VI) differ, as do the mechanisms. They depend on a number of factors, including the source from which the species is isolated and the characteristics of their growth (Banerjee et al., 2019).
Though there has been extensive work on Cr(VI) reduction by microorganisms, most of these scholars have paid attention to pure cultures of microorganisms. In this study, consortia of bacteria culture from a municipal wastewater sludge site was used to investigate the effect of initial Cr(VI) concentration and pH on the Cr(VI) removal by the consortia.

2. Methods and materials

2.1 Source of Bacterium Consortia

The natural bacteria consortia were obtained from sludge collected at the Brits Wastewater Treatment Works (North West Province, South Africa). An abandoned sodium dichromate processing facility was reported to discharge high levels of Cr(VI) periodically into the sewage treatment works. The chrome processing facility was commissioned as early as 1996. Three sludge samples were collected, namely primary sludge, secondary sludge in the mixed liquor reactor and sludge cake after undergoing a belt filter dewatering process in the treatment plant. Although the nearby chrome foundry periodically discharges high levels of Cr(VI) to the treatment plant, at the time of collecting the samples for this study did not correspond with the discharging times.

2.2 Cr(VI) stock solution

A 1,000 mg/L Cr(VI) concentration stock solution was prepared by dissolving 3.73 g of 99 % pure K_2CrO_4 (analytical grade) in 1,000 mL deionised water. The 1,000 mg/L Cr(VI) stock solution was used as sources of Cr(VI).

2.3 Preparation of culture medium

i) 25 g of Luria Broth (LB) powder containing 40 %wt peptone, 20 %wt yeast extract, and 40 %wt sodium chloride was added in 1,000 mL of distilled water for bacterial growth medium.
ii) Mineral salt medium (MSM) consisted of 2.12 g K_2HPO_4, 2.12 g KH_2PO_4, 2 g NaCl, 1 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 4 g KNO_3 and 5 g glucose as a carbon source in 1,000 mL of distilled water (Jeyasingh and Philip, 2005).
iii) 40 g LB agar powder with a composition of 37.5 %wt agar, 25 %wt peptone, 12.5 %wt yeast extract, and 25 %wt sodium chloride was added in 1,000 mL of distilled water for colony development.
The pH was adjusted to 7 ± 0.2 by using HCl or NaOH. All mediums in this study were autoclaved at 121 °C for 15 min cooled to room temperature before use except for LB agar which was cooled to 40 °C. All chemicals and solvents were analytical grade and required no further purification.

2.4 DPC solution

Diphenyl carbazide (Merck, South Africa) solution was prepared for Cr(VI) analysis by dissolving 0.5 g of 1,5 diphenyl carbazide in 100 mL of HPCL grade acetone and was stored in a brown bottle covered with a foil.

2.5 Cr(VI) tolerance

Three different environments were identified as possible sources of Cr(VI) reducing cultures at Brits wastewater treatment plant: (1) dried primary sludge (Sludge (I)), (2) secondary sludge in the mixed liquor reactor (Sludge (II)) and, (3) dried sludge after undergoing belt filter dewatering process (Sludge (III)). The consortia bacterial cultures were screened for Cr(VI) reduction on the basis of their reduction performance under a free-oxygen

environment. 1 g of sludge sample was added to a 250 mL conical flask containing 100 mL of LB broth and incubated for 24 h at 37 °C by agitation at 120 rpm using a Labcon SPL-MP 15 Lateral Shaker (Labcon Laboratory Services, South Africa). The conical flask was closed with cotton to allow oxygen flow while preventing contaminants from entering the flask. Then, after 24 h of incubation 1 mL of this was transferred into a fresh 100 mL LB broth supplemented with 100 mg/L of Cr(VI). The fresh LB broth was incubated for 24 h under the same conditions. This method was replicated by gradually increasing the Cr(VI) concentration in the growth medium up to 500 mg/L.

2.6 Cr(VI) reduction assay

Reduction of Cr(VI) by freshly grown cells of the bacterial consortia was determined in MSM. The experiments were conducted in a 250ml Erlenmeyer flask containing 100 mL MSM supplemented with 50 – 400 mg/L Cr(VI) concentration. The cells were harvested after 24 h incubation and washed thrice by centrifugation with 0.85% NaCl sterile solution and finally resuspended in the MSM. Flasks were inoculated with cells concentrated to a 5:1 ratio before adding Cr(VI), and the flasks were covered with cotton to allow oxygen while preventing microorganisms from entering. The flasks were incubated at 37 °C and under constant shaking of 120 rpm. All experiments were conducted in duplicate. 1 mL samples were taken at time intervals, determined by the observed rate of Cr(VI) removal. The samples were centrifuged at 6500 rpm for 10 min in a Hermle 2323 centrifuge (Hermle Laboratories, Wehigen, Germany) to remove suspended cells before analysis.

2.7 Effect of pH on Cr(VI) Reduction

The effect of different pH on Cr(VI) reduction was studied, ranging from 2 to 11. The experiments were conducted at 50 mg/L initial Cr(VI) concentration. The pH was adjusted by using HCl or NaOH.

2.8 Microbial characterisation

Phylogenetic characterisation of cells was performed on individual colonies of bacteria grown aerobically from sludge sample. LB agar was used for colony development. Agar plates were inoculated with 1 mL samples, and the colonies were sub-cultured using differential techniques (exhibited colours and morphologies) and incubated at 37 °C for 24 h. In preparation for the 16S rRNA sequence identification, the colonies were first classified based on morphology. Genomic DNA was extracted from the pure cultures using a DNeasy tissue kit (QIAGEN Ltd, West Sussex, UK) as per the manufacturer's instructions. The 16S rRNA genes of isolates were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers pA and pH1 (Primer pA corresponds to position 8 - 27; Primer pH to position 1541-1522 of the 16S gene). An internal primer pD was used for sequencing (corresponding to position 519 - 536 of the 16S gene). The resulting sequences were matched to known bacteria in the GenBank using a basic BLAST search of the National Centre for Biotechnology Information (NCBI, Bethesda, MD).

2.9 Determination method

1 mL samples were withdrawn periodically, then centrifuge at 6,500 rpm for 10 min. 0.2 mL of the sample was added in a 10 mL volumetric flask, followed by 1 mL of 1 N H_2SO_4 and the addition of distilled water up to the 10 mL mark. 0.2 mL of DPC solution was finally added to produce a purple colour. Cr(VI) was measured by UV-Vis spectrophotometer (WPA, Light Wave II, Labotech, South Africa) operated at a wavelength of 540 nm.

3. Results and Discussion

3.1 Cr(VI) tolerance

Mixed bacterial cultures from three locations of the wastewater treatment plant were screened based on their Cr(VI) reduction capacity. The samples exhibited Cr(VI) reducing bacteria in them, and this was indicated by excellent Cr(VI) reduction potential, as shown in Figure 1. All the sludge samples completely reduce Cr(VI) at 100 mg/L initial concentration. However, as the Cr(VI) concentration increases, the reduction capacity of the cultures decreases, with only about 6 % reduction occurred at 500 mg/L due to the inhibition effect of Cr(VI) on the culture. The dried sludge cultures from the belt filter press [sludge(III)] showed to have a higher reduction capacity than the two other sludges. This was attributed to better acclimation and longer exposure to Cr(VI) than the cultures from primary and activated sludge and sludge(III) consortia were chosen for further studies.

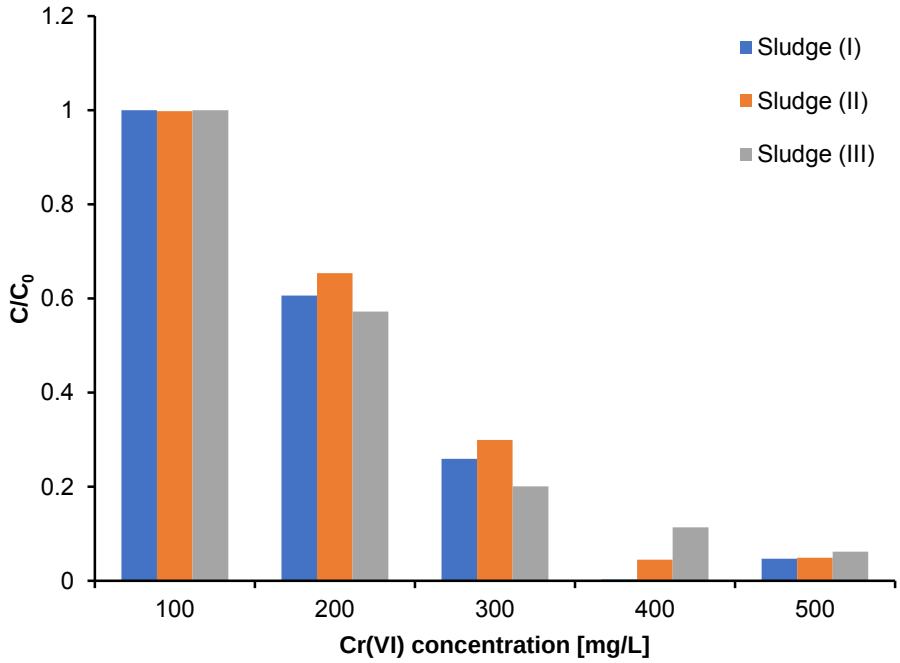

Figure 1: Cr(VI) reduction in consortia cultures from different sources (primary sludge, activated sludge, and dry sludge) under varying initial Cr(VI) concentration incubated for 24 h.

3.2 Effect of Initial Cr(VI) Concentration

The effect of initial Cr(VI) concentration on microbial Cr(VI) reduction was studied using Sludge (III) bacterial consortia with varying initial Cr(VI) concentrations of 50 – 400 mg/L under aerobic conditions, and the results are shown in Figure 2.

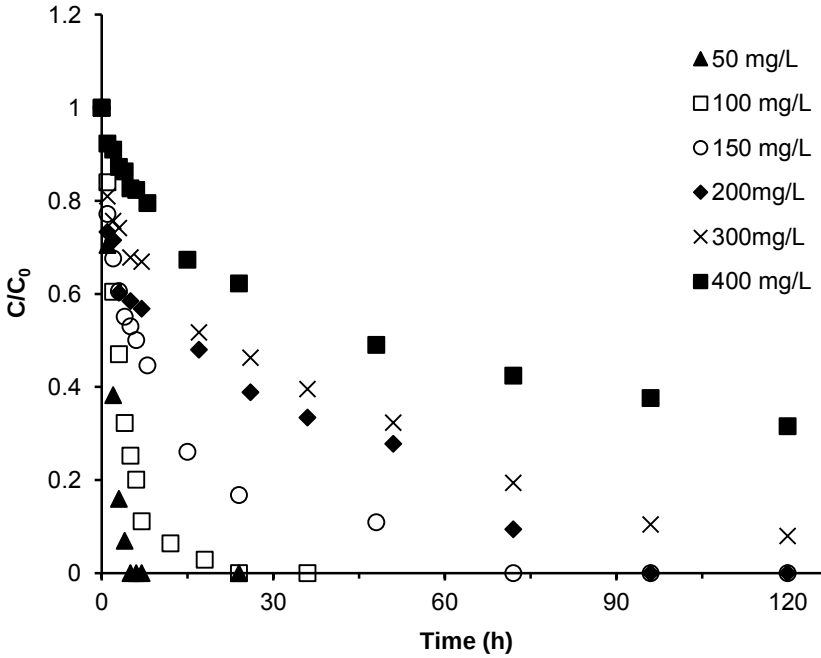

Figure 2: Kinetics of Cr(VI) reduction by Sludge (III) bacterial consortium under varying initial Cr(VI) concentration

It can be seen that Cr(VI) reduction rate decreased with increasing initial Cr(VI) concentrations. 50 mg/L initial Cr(VI) concentration was completely reduced in 5 h. Higher Cr(VI) concentrations of 100 mg/L, 150 mg/L and

200 mg/L took 18 h, 72 h, 96 h for complete reduction. Complete Cr(VI) reduction was not obtained above 200 mg/L initial Cr(VI) concentration. However, when the experiments were terminated after 120 h, up to 92 % of Cr(VI) was removed at the initial concentration of 300 mg/L, and 70 % Cr(VI) reduction was observed at 400 mg/L initial concentration. These microorganisms are able to remediate Cr(VI) even at higher concentrations though it takes a long time. This shows that the bacterial consortium was able to withstand Cr(VI) toxicity at a high initial concentration without much adverse effect. Given the fact that in situ bioremediation is being considered, the observation in this study is more significant in this regard. Cr(VI) usually co-exist with other heavy metals in groundwater and may influence Cr(VI) removal by microorganisms. However, Tan et al. (2020) observed no influence on the Cr(VI) removal in the presence of Fe^{3+}, Ni^{2+}, Zn^{2+}, and Pb^{2+} at 15 mg/L.

3.3 Effect of pH

In most biological systems, pH plays a significant role. Many studies have demonstrated that the optimum pH in biological systems is in the range of 6 - 8 (Dey et al., 2014). The effect of pH on Cr(VI) reduction was carried out in the range of pH 2 - 11 at 50 mg/L Cr(VI) concentration under aerobic conditions. The results are illustrated in Figure 3. The Cr(VI) reduction activity was enhanced as the pH increased from 2 to 7. At neutral pH, complete Cr(VI) reduction was observed within 5 h. A further increase in pH from 8 to 11 resulted in decreasing the Cr(VI) reduction. These results indicate that the optimum pH for Cr(VI) reduction using wastewater sludge bacteria consortium is 7. Wani et al. (2019) have reported that a bacterial species *Pseudomonas sp.* MAI4 showed significant Cr(VI) reduction at pH 7.

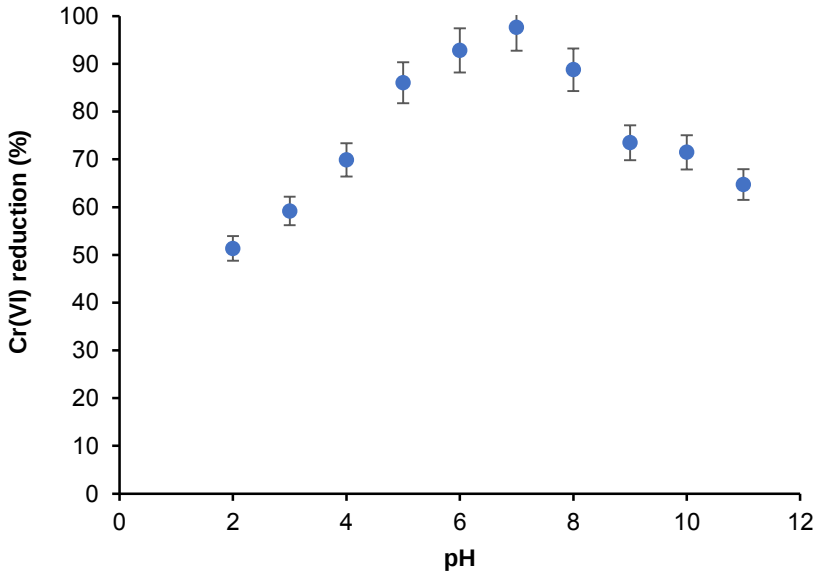

Figure 3: Effect of pH on Cr(VI) reduction

3.4 Microbial characterization

Sludge (III) bacteria consortium was chosen for characterization due to its high performance. Culture purification and 16S rRNA sequencing were performed at the Department of Microbiology, the University of Pretoria, where the identification was made to identify bacterial communities present after the sludge had been exposed to 75 mg/L Cr(VI). The results are presented in Table 1, and the predominant species under aerobic conditions were the Bacillus groups – Bacillus cereus ATCC 10987, B. cereus 213 16S, Bacillus thuringiensis (serovar finitimus), Bacillus mycoides – and two Microbacterium group – Microbacterium foliorum and Microbacterium sp. S15-M4 at 99 % identity index.

4. Conclusion

The current study gives attention to the capability of a mixed population of microbial culture isolated from sludge coming from a wastewater treatment plant that receives high loads of hexavalent chromium from an abandoned chrome foundry to biotransform Cr(VI). Amongst the bacterial consortia isolated from three different sections of

the wastewater treatment plant, the bacteria consortia (Sludge(III)), which was isolated from dried sludge from the belt filter dewatering process, showed high Cr(VI) reduction potential. Batch experiments were carried out to study the effect of Cr(VI) initial concentration on Cr(VI) reduction by bacteria consortium.

Table 1: Sludge Cr(VI)-Reducing Bacteria strain characterisation using 16S rRNA (Molokwane et al., 2008)

Pure Isolates	Blast results	ID index
Y1	Bacillus cereus strain 213 16S, Bacillus thuringiensis 16S	99
Y2	Bacillus sp. ZZ2 16S, B. cereus ATCC 10987, B. thuringiensis str. Al Hakam	99
Y3	Bacillus sp. 32-661 16s, B. cereus strain 16S	
Y4	Bacillus mycoides strain BGSC 6A13 16S, B. thuringiensis serovar finitimus strain BGSC 4B2 16S	99
Y5	Microbacterium sp. S15-M4, Microbacterium foliorum	99

It was found that the bacteria consortium was able to completely transform Cr(VI) up to 200 mg/L initial Cr(VI) concentration. This shows that the bacterial consortium was able to withstand Cr(VI) toxicity at a high initial concentration without much adverse effect. The initial pH value is found to affect the Cr(VI) reduction rate of the bacteria consortium greatly, and the optimum pH is 7. The study shows that the bacteria consortium from a wastewater treatment plant could be effectively used as a bioremediation technique for the mitigation of treating high Cr(VI) from chrome foundry.

Acknowledgments

The research was funded through the National Research Foundation (NRF) Incentive Funding for Rated Researchers, Grant No. IFR2010042900080 awarded to Prof Evans MN Chirwa of the University of Pretoria. Buyisile Kholisa would like to acknowledge the National Research Foundation (NRF) for granting him the Doctoral Scholarship, Grant No. MND190613447392

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