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Cr^{6+} Reduction in an Indigenous Mixed Culture of Bacteria in the Presence of As^{3+}

Tony E. Igboamalu, Evans M.N. Chirwa*

Water Utilisation Division, Department of Chemical Engineering, University of Pretoria, Pretoria 0002, South Africa
evans.chirwa@up.ac.za

The indigenous mixed culture of bacteria collected from a Wastewater Treatment Plant in Brits (North West Province, South Africa) biocatalytically reduced Cr^{6+} in the presence of As^{3+}. Treatment of these elements before disposal of wastewater is critical since they are both known to be carcinogenic and mutagenic at very low concentrations. Both compounds are known to be acutely toxic under high exposure conditions (Cr^{6+}>50 mg/L, As^{3+}>20 mg/L). In this study, experiments were conducted to evaluate the rate of Cr^{6+} reduction under anaerobic conditions in the presence of As^{3+}. Near complete Cr^{6+} reduction was achieved under initial Cr^{+6} concentrations up to 70 mg/L under an As^{3+} concentration of 20 mg/L. However, after increasing Cr^{6+} concentrations to 100 mg/L, an inhibitory effect was observed. Further experiments conducted at Cr^{6+} concentration of 70 mg/L and varying As^{3+} concentration from 5-70 mg/L showed that Cr^{6+} reduction rate increased with increasing As^{3+} concentration from 5-40 mg/L. However, 20 % drop in Cr^{6+} reduction efficiency was observed. These results suggest that the rate of Cr^{6+} reduction was hindered by the dual toxicity of the Cr^{6+} and As^{3+} in the system. The observed inhibitory effect was attributed to the toxicity effect of Cr^{6+} and As^{3+}. Initial evaluation of the bacteria using DNA finger printing showed that cells in the mixed culture comprised predominantly of the Gram-positive species: *Staphylococcus sp*., *Enterobacter sp*., and *Bacillus sp.*

1. Introduction

Environmental pollution associated with toxic metal ions (metalloids) is a legacy left by industrial and mining activities. Effluent from gold and antimony mining, textile production, leather tanning, electroplating, paint and pigment manufacturing and wood processing industries contain considerable amounts of toxic metal ions (Marcovecchio et al., 2007). Chromium (Cr) exists in the environment mostly in the hexavalent form (Cr^{6+}) or trivalent form (Cr^{3+}) (Cheunga et al., 2007). In mining processes, it is common to find Cr^{6+} in wastewater together with other metallic co-contaminants such as As^{3+}, Pd^{2+} and Fe^{3+}. Treatment of these elements before disposal of wastewater is critical since most of them are known to be carcinogenic and mutagenic at low concentrations, and acutely toxic at high concentrations (Federal Register, 2004). In addition, inorganic Cr^{6+} and its metallic co-pollutants have been associated with high incidents of skin, lung, bladder and liver cancer (De Flora, 2000).

South Africa is among the top coal, gold, and metal producing nations in the world, accounting for about (70-90) % of the world viable chromite reserve (Bachmann et al., 2010). Improper disposal of Cr^{6+} containing waste from these industries, and its subsequent mobility to groundwater aquifers is a subject of grave health concerns. Contamination of groundwater aquifers has resulted in elevated level of Cr^{6+} and its co-pollutant (As^{3+}). The high level of these compounds in groundwater renders the water unsuitable for potable use. The removal of compounds containing Cr^{6+} and As^{3+} involve redox processes in which Cr^{6+} is reduced to Cr^{3+}, and As^{3+} is oxidised to As^{5+}, followed by precipitation of the metals as hydroxide complexes at medium to high pH values.

Several studies have demonstrated the efficacy of facultative microbes in oxidizing As^{3+} to As^{5+} using nitrate (NO_3^-) or chlorate (ClO_3^-) as terminal electron acceptors, while conserving energy for growth (Aniruddha and Wang, 2010). By exploring the biocatalytic redox reaction taking place in Eq. 4, chromate (CrO_4^-) can be considered a possible alternative oxidant for the reaction.

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$As^{3+} \rightarrow 2\,e^{-} + As^{5+} + Energy$ (1)

$Cr^{6+} + e^{-} \rightarrow Cr^{5+}$ (intermediate product) (2)

$Cr^{5+} + 2\,e^{-} \rightarrow Cr^{3+}$ (3)

Simultaneous redox reaction:

$As^{3+} + Cr^{5+} \rightarrow As^{5+} + Cr^{3+} + Energy$ (- 256 kJ/mol) (4)

Equation above shows the individual stages of As^{3+} oxidation, and step wise reduction of Cr^{6+}. Eq. 2 occurs as a result of the redox cycle in the microbial cell, which generates an intermediate product Cr^{5+} (Cervantes et al*.,* 2001). From biocatalytic redox reactions equation (4), As^{3+} donate $2e^{-}$, and oxidized to As^{5+}, while Cr^{5+} an intermediate product formed, on the other hand accept $2e^{-}$, and reduced to Cr^{3+}. However, the reaction is feasible since only two electrons are required for Cr^{5+} reduction. Secondly, based on bioenergetics consideration, the reaction is feasible as indicated a highly exothermic reaction equation (4), releasing a reasonable amount of energy for cell growth. So far, only few studies have been done on simultaneous Cr^{6+} reduction and As^{3+} oxidation by micro-organism. Bachate et al. (2013) reported simultaneous Cr^{+6} reduction and As^{3+} oxidation by *Bacillus firmus* TE7. Secondly, Wang (2013), reported Cr^{6+} reduction linked to As^{3+} oxidation which was greatly accelerated by the addition of H_2O_2.
In this study, kinetic studies of Cr^{6+} reduction in the presence of As^{3+}, in indigenous mixed culture of bacteria collected from a Wastewater Treatment Plant (Brits North West South Africa), was explored to evaluate the inhibitory effect of As^{3+} on Cr^{6+} reduction, at this stage we did not quantify As^{3+} oxidation, but we assumed that As^{3+} could have served as an electron donor for Cr^{6+} reduction based on equation 4.

2. Material and methods

2.1 Culture and media

Dried sludges samples (S_1, S_2, S_3, S_4 and S_5) from sand drying beds at Brits Wastewater Treatment Work (North West Province, South Africa) was used as inoculum for the mixed culture of bacteria used. The treatment plant is located nearby an abandoned chrome processing facility, which periodically received flows from it and discharged high level of Cr^{6+}. It was reported that Cr^{6+} concentration of the treatment plant's effluent, mixed liquor and dried sludge were 2.45 mg/L, 2.63 mg/L and 25.44 g/m^2 respectively (Molokwane et al.,2008).The sludge samples were cultivated for 24 h in 100 mL of sterile nutrient broth amended with 30 mg/L of Cr^{6+} and 20 mg/L As^{3+}. Bacteria were incubated under shaking at 120 rpm in a rotary Environmental Shaker (Labotech, Gauteng, South Africa). Anaerobic cultures were grown in 100 mL serum bottle purged with nitrogen gas (99 %) for 5 min, closed with silicone rubber and aluminium stoppers. Enriched bacteria strains were isolated (S_1, X_1, S_3, S_4 and S_5) by serial dilution of the cultivated culture after 24 h. All media were autoclave for 5 min at 121 ^{o}C.

2.2 Culture Characterization

Phylogenetic characterization of cells was performed on individual colonies of bacteria from the 7^{th} and 10^{th} tubes in the serial dilution preparation using a method described by (Molokwane et al., 2008). Genomic DNA was extracted from the pure cultures using a DNeasy tissue kit (QIAGEN Ltd, West Sussex, UK) as per 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).

2.3 Cr^{6+} Reduction experiment in the presence of As^{3+}

In a 1 L Erlenmeyer flask containing 400 mL nutrient broth, cultures were grown anaerobically for 24 h. Cells were collected, and centrifuged for 10 min at 6,000 rpm at 4 ^{o}C after 24 h. The supernatant was decanted and the remaining pellet was wash three times in a sterile saline solution (0.85 % NaCL). Anaerobic Cr^{6+} reduction experiments were conducted in 100 mL serum bottles using cells harvested after 24 hours incubation. Anaerobic condition was achieved by purging 99.99 % N_2 gas in the serum bottle containing harvested cell for 10 min to expel any residual oxygen before sealing with silicon stoppers and aluminum seals. Prior inoculating the bottles with harvested cell, 1 mL of the sample was initially withdrawn from the serum bottle to determine the absorbance of Cr^{6+} before introducing the cells in each

serum bottle. In 100 mL basal mineral medium, the cell was re-suspended before adding Cr^{6+} and As^{3+}, to give a desired concentration. Cr^{6+} and As^{3+} stock solution were added to give a final concentrations of (50-500) mg/L and 20 mg/L as well as (5-70) mg/L As^{3+} in a different batch. The experiments were conducted at 30±2 ^{o}C over time at 120 rpm on the orbital shaker (Labotec, Gauteng, South Africa). The samples withdrawn in serum bottles over time were centrifuged using a 2 mL Eppendorf tube at 6,000 rpm for 10 min in a Minispin® Microcentrifuge (Eppendorf, Hambury, Germany) and the supernatant was used for Cr^{6+} reduction analysis.

2.4 Analytical Methods

Cr^{6+} was measured using the UV/Vis spectrophotometer (WPA, light wave II, Labotech, South Africa). The presence of Cr^{6+} in the sample was visualized by the change of the colour after adding DPC (APHA, 2005). However, total Cr on the other hand was determined in the Varian AA-1275 Series Flame Atomic Adsorption Spectrophotometer (AAS) (Varian, Palo Alto, CA (USA)) equipped with a 3 mA and chromium hollow cathode lamp at 359.9 nm wavelength.

3. Results and discussion

3.1 Microbial analysis

Prior to experiment, microbial performance in the dried sludge from Wastewater Treatment Plant (North West Province South Africa) was evaluated for Cr^{6+} reduction in the presence of As^{3+} under anaerobic condition. In various dried sludge samples (S_{s1}, S_{x1}, S_{s3}, S_{s4}, and S_{s5}) was amended Cr^{6+} and As^{3+} concentration of 30 mg/L and 20 mg/L respectively. Results showed a near complete Cr^{6+} reduction within 2 hours of incubation, signifying the capacity of these samples to reduce Cr^{6+} in the presence of As^{3+} figure1. In addition, individual pure isolate (X_1, S_1, S_3, S_4 and S_5) from sludge samples were investigated. The existence of Cr^{6+} reducing bacteria and As^{3+} resistant, were indicated by observed removal rates as shown in the Figure 2. It was observed that highest removal rate was achieved in sample S_3, followed by S_4 and S_1, when compared to X_1, S_2 and S_5 etc. However, high removal efficiency of these isolates was attributed to its acclimatization to Cr^{6+} and As^{3+} toxicity, suggesting that these microbes are resistant to Cr^{6+} and As^{3+} toxicity. Examples of these comparative anaerobes are presented with *Staphylococcus sp.* (S_1, X_1 and S_5), *Enterobacter sp.*, (S_3), *Bacillus sp.* (S_4), as predominant species. Subsequently, individual isolates were mixed in order to make a consortium or mixed culture of Cr^{6+} reducing and As^{3+} resistant anaerobes; after establishing the fact that these isolates can indeed reduce Cr^{6+} in the presence of As^{3+}.

3.2 Individual culture versus reconstitute consortium under anaerobic condition

Figure 3 compares reconstituted consortium culture and selected individual pure isolates at Cr^{6+} and As^{3+} concentration of 50 mg/L and 20 mg/L, respectively. Results showed a near complete Cr^{6+} reduction achieved by reconstituted consortium or mixed culture after 20 h of incubation when compare to other individual isolates. However, this suggests that micro-organism existing as a community possesses significant stability and metabolic capabilities. These findings are in agreement with previous observations; where reconstitute culture outperform individual pure culture (Molokwane et al., 2008). Synergism could be the reason why individual cultures could not perform when compare to mixed culture consortium. A control system was also examined in order to determine the extent of a biotic Cr^{6+} reduction. A biotic control showed that removal of Cr^{6+} in the absence of biomass was negligible within the tested time interval Figure 4.

3.3 Cr^{6+} Reduction in the presence of As^{3+}

Cr^{6+} reduction in the presence of As^{3+} experiment was conducted in a batch essay under varying Cr^{6+} and As^{3+} concentration ranging from (50-500) mgCr^{6+}/L and (5-70) mgAs^{3+}/L using harvested and concentrated cells. The harvested cells used in this experiment were grown under anaerobic condition and been tested for Cr^{6+} reduction as described previously in the experiment. However, the typical Cr^{6+} tolerance concentrations in which cell biological activity ceased were also evaluated.

Result showed that reconstitute consortium or mixed culture achieved near complete Cr^{6+} reduction at initial lower concentration (50-70)mg/L in the presence of 20 mg/L As^{3+} within 48 h of incubation. However, increasing Cr^{6+} concentration up to (100-500) mg/L showed an incomplete Cr^{6+} reduction figure 5. Above 100 mg/L Cr^{6+} concentration, low or negligible reduction rate was observed, suggesting that the typical Cr^{+6} tolerance for this cell occurs at 350 mg/L in presence of 20 mg/L As^{+3}. Furthermore, Cr^{6+} reduction in the presence of As^{3+} concentration ranging from (5-70) mg/L showed an increase in Cr^{6+} reduction rate when As^{3+} concentration was increased from (5-40) mg/L. However, up to 50 mg/L a decrease in Cr^{6+} reduction efficiency was observed Figure 6. These suggests that the observed inhibition effect was as a result of high Cr^{6+} and As^{3+} concentration, which correlate to Cr^{6+} and As^{3+} toxicity to microbes. This

observation is in agreement with previous studies of Cr^{6+} reduction in a batch essay (Molokwane et al., 2008).

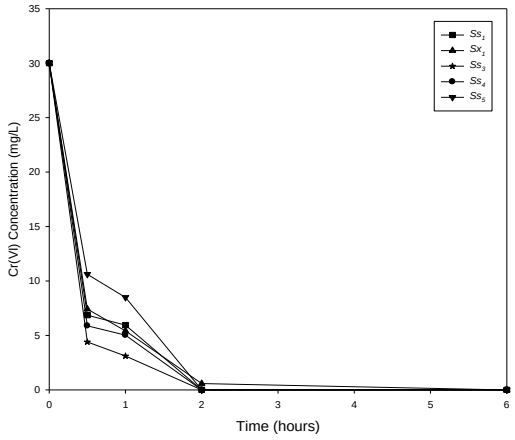

Figure 1: Cr^{6+} reduction of sludge samples In the presence of 20mg/L As^{3+}

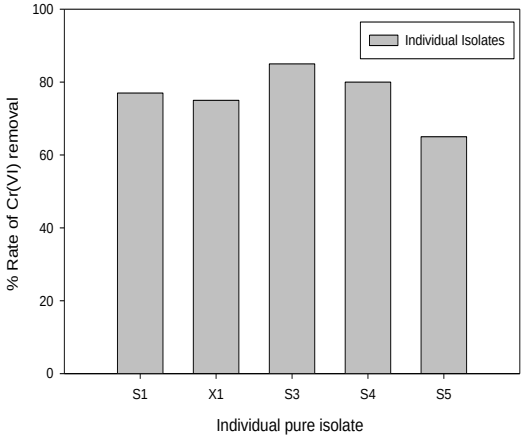

Figure 2: Cr^{6+} reduction efficiency of individual pure isolates.

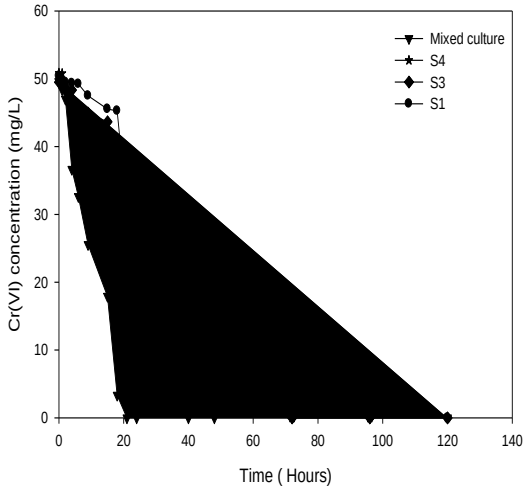

Figure 3: Reconstituted consortium (mixed) culture versus selected individual pure isolates at 50 mg/L

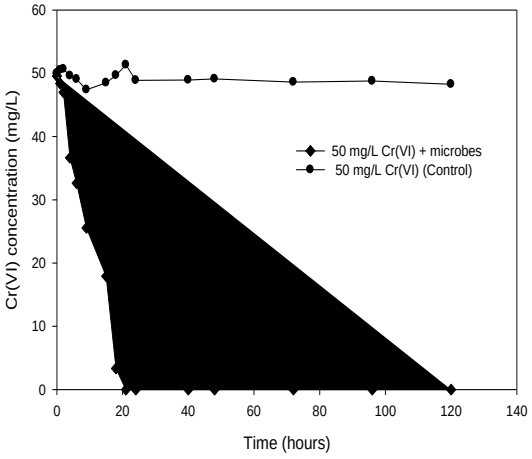

Figure 4: Abiotic Cr^{+6} reduction at 50 mg/L

3.4 Bacterial consortium analysis: 16S rRNA partial sequence analysis

Results of phylogenetic characterization of cell in the mixed culture in the presence of Cr^{6+} and As^{3+} showed 11 possible facultative anaerobes. The isolate numbers are indicated on the genus trees figure 6. The consortium composition obtained in this study differs from the composition initially reported. However, only *Bacilli species* and *Enterobacteria species* present in this investigation are Cr^{6+} reducing bacteria (CRB) initially reported by Molokwane et al., (2008). The difference in microbial composition is attributed to the presence of As^{3+}.

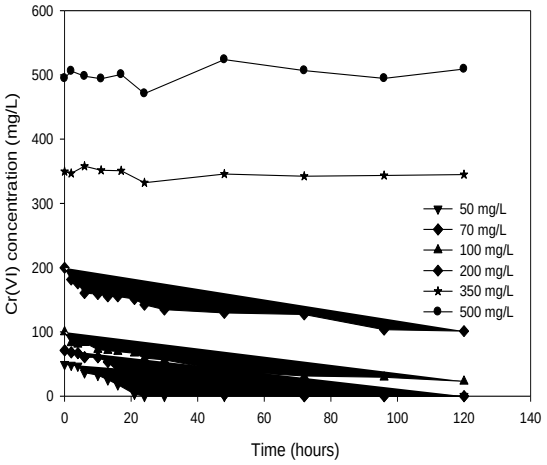

Figure 5: Performance evaluation of reconstitute consortium at different Cr^{6+} concentration

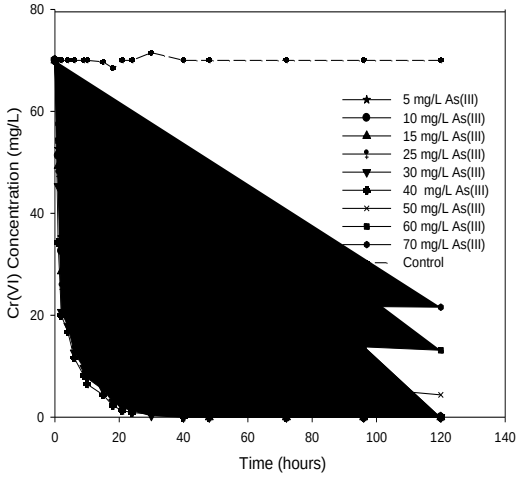

Figure 6: Performance evaluation of reconstitute consortium at different As^{3+} concentration

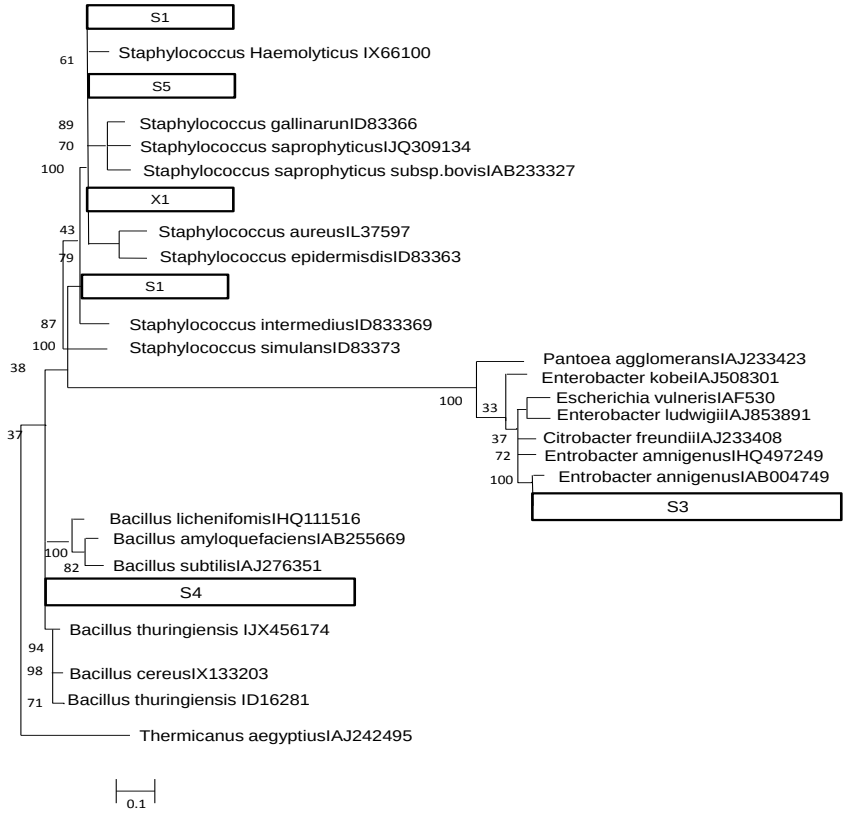

Figure 7: Phylogenetic tree from BLAST search

4. Conclusions

A successful Cr^{6+} reduction in the presence of different As^{3+} concentrations were observed in a mixed culture of facultative anaerobes from Brits North West South Africa. Mixed cultures (predominantly consist of the Gram-positive species: *Staphylococcus sp*., *Enterobacter sp*., and *Bacillus sp.*), reveal high resistant to Cr^{6+} and it co-pollutant As^{3+}, suggesting the capacity of this culture to detoxify Cr^{6+} in the presence of its co-pollutant As^{3+}. However, the tendency of this facultative anaerobes to biocatalytically reduce Cr^{6+} in the

presence of its co-pollutant As^{3+}, afford a potential for simultaneous bioremediation of Cr^{6+} and its co-pollutant As^{3+} in a chromium-arsenic contaminated sites such as underground water. Further studies are required to evaluate the simultaneous Cr^{6+} reduction and As^{3+} oxidation in the mixed culture of facultative anaerobes. However, substantial improvements are necessary to move these approaches from the batch to a continuous scale practice.

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