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Microbial Cr(VI) Reduction by Indigenous Activated Sludge Bacilli and Pure Culture of *Escherichia coli* ATCC 33456

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Cr(VI) reducing microbial species *Bacillus thuringiensis*, *Bacillus cereus* and *Bacillus mycoides* were isolated from dried sludge collected from sand drying beds at a local wastewater treatment plant in Brits (South Africa). The plant received high periodic loadings of Cr(VI) contaminated effluent from an abandoned chrome processing foundry within the chrome mining town of Brits. Other Cr(VI) producing organisms in the sludge sample were identified as *Microbacterium foliorum* and *Lycinobacillus sphaerococcus*. Among the five confirmed Cr(VI) reducers, *B. thuringiensis* was determined to have the highest performance in reducing Cr(VI) to Cr(III). The Cr(VI) reducing capability of organisms isolated at the site has not diminished over a 15 year period (2006-2021). In the current study, the performance and pathway structures responsible for Cr(VI) reduction are analysed and compared with the activity of a metabolically versatile *Escherichia coli* ATCC 33456. The observations from this study showcase advanced enzymatic Cr(VI) reductase structures in *B. thuringiensis* never before observed in other microbial species. Blocking of electron carrier enzymes suggested the involvement of dissolved thioredoxin in the cytosol and bulk media as possible biocatalysis activators for Cr(VI) reduction in resting cells.

1. Introduction

Reduction of Cr(VI) to Cr(III) in the environment is beneficial to ecosystems since Cr(VI) is highly toxic and mobile in aquatic systems, whereas Cr(III) is about 1000 times less toxic than Cr(VI) and less mobile, readily forms insoluble precipitates and (Chattopadhyay et al., 2010). Additionally, Cr(III) is a nutritional cofactor in the metabolism of carbohydrates in mammals. Similar metal reduction reactions have been used lately in reducing uranium-6 (U(VI)) to the less mobile tetravalent form [U(IV)] for possible application in areas around nuclear waste repositories (Mtimunye and Chirwa, 2019).

Current studies show that biological Cr(VI) reduction is mostly limited by the inhibitory effect of Cr(VI) to the organisms. In certain groups of bacteria, the Cr(VI) reduction capability may be transferred across species and such a possibility was demonstrated by Bopp and Ehrlich (1988) whereby Cr(VI) reduction genes were transferred on plasmids across different serotypes of *Pseudomonas fluorescens*.

In 1992-1993, Shen and Wang (1993) evaluated Cr(VI) reduction activity in novel *Escherichia coli* species formerly known as B1. *E. coli* B1. *E. coli* B1 was designated ATCC 33456 and was demonstrated to function very well in multi-pollutant environments. For instance, *E. coli* B1 (ATCC 33456) was demonstrated to grow on metabolites formed during degradation of aromatic compounds while reducing Cr(VI) to Cr(III) in the process. It was however observed that Cr(VI) reduction activity by *E. coli* ATCC 33456 was limited by inactivation of the cells by Cr(VI) suggesting inhibition of metabolic factors in the cells.

In later years, various species of Cr(VI) reducing bacteria have been isolated from different sites around the world showing that the Cr(VI) reducing capability of microorganisms is ubiquitous in nature (Ahmad et al., 2021). This article articulates the underlying electron pathway differences in G(+ve) bacteria grown under micro-aerobic

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conditions (DO ≥ 2.0 mg /L) and G(−ve) bacteria grown under micro-aerobic and facultative conditions (DO ≤ 0.5 mg /L). The paper elucidates the alternative pathways for the first time based on experimental evidence.
The reaction rate used in this study was derived earlier based on the assumption that each Cr(VI) reducing living cell in the reactor produces a certain amount of Cr(VI)-reductase (***E'***) and that deactivating the cell deprives the system of that particular amount of Cr(VI)-reductase. Assuming that the remaining active enzyme ***E*** is proportional to the amount of live cells in the system, i.e., $\boldsymbol{E} \propto X_a$, then the reaction rate mediated by the enzymes could be considered as being directly proportional to the reaction rate catalysed by cells. Based on this assumption, Shen and Wang (1994) derived the Cr(VI) reduction rate law of a closed batch system, represented by the specific Cr(VI) reducing activity of living cells in the system:

$$-\frac{dC}{dt} = \frac{k_{mc} \cdot C}{K_c + C}\left(X_o - \frac{C_o - C}{R_c} \right) \tag{1}$$

where C = Cr(VI) concentration (ML^{-3}) at any time t (T), k_{mc} = maximum specific Cr(VI) reduction rate coefficient (T^{-1}), K_c = half velocity constant (ML^{-3}), and the term $(C_o\text{-}C)/R_c$ represents cells killed due to exposure to Cr(VI) such that the term in brackets represents the concentration of active cells available at time t:

$$X_a = X_o - \frac{C_o - C}{R_c} \tag{2}$$

where X_a = viable cell concentration (mg/L) at any time t, X_o = initial viable cell concentration (mg/L) at time zero, C_o = initial Cr(VI) concentration (mg/L), and R_c = finite Cr(VI) reduction capacity (g Cr(VI) reduced/g of cells deactivated).

2. Materials and Methods

2.1 Culture Isolation and Identification

Aerobic cultures were cultivated from colony picks in sterilized media in cotton plugged vessels whereas anaerobic cultures were grown in vessels purged with 99.9 % pure N_2 gas and sealed with rubber stopper at 30±1 ºC. Genomic DNA was extracted from the biomass grown from pure colonies following the protocol described for the Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI, USA). 16S rRNA genes were then amplified by using a 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 under the following reaction conditions: 1 min at 94 ºC, 30 cycles of 30 s at 94 ºC, 1 min at 50 ºC and 2 min at 72 ºC, and a final extension step of 10 min at 72 ºC). An internal primer pD was used for sequencing (corresponding to position 519-536 of the 16S gene). PCR fragments were then cloned into pGEM-T-easy (Promega) [Promega Wizard® Genomic DNA Purification Kit (Version 12/2010)]. For microbial cultures sourced from dried sludge, the 16S rRNA gene sequences of the strains were aligned with reference sequences the genetic sequence of Escherichia coli ATCC 11775 as an Out Group. Pairwise evolutionary distances based on an unambiguous stretch of 1274 bp were computed using the method developed by Jukes and Cantor (1969). The DNA sequence for each pure colony was then uploaded to the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI). A phylogenetic tree was constructed from the identified 16S rRNA sequences using the neighbour-joining method in the MEGA Version 6 software (Tamura et al., 2013).

2.2 Aerobic Batch Experimental Setup

Micro-aerobic Cr(VI) reduction experiments were conducted in 100 mL Erlenmeyer flasks using cells harvested after 24 hours incubation by washing three times and resuspending in fresh media to a concentrated ratio of 4:1. The viable cell concentration was determined to be $5.2 \pm 2.1 \times 10^9$ cells/mL. The cells were washed twice by centrifugation and re-suspension in a sterile solution of 0.085 % NaCl before adding Cr(VI). The batches were covered with cotton plugs during incubation to allow aeration while filtering away microorganisms from the air. Cr(VI) concentration in the range of 50 to 600 mg/L was added and the solution was incubated under shaking at ±30 ºC. Experimental units consisted of the different initial concentrations and all experiments were conducted in duplicates. Samples of 1 mL were withdrawn at time intervals determined by the observed rate of Cr(VI) removal. The samples were centrifuged at 2820 g (6000 rpm, 7 cm rotor radius) for 10 min in a Hermle 2323 centrifuge (Hermle Laboratories, Wehigen, Germany) to remove suspended cells before analysis. Dissolved oxygen in the flasks was determined to average 0.45±0.70 mg O_2/L, conditions which were determined to support some aerobic and some anaerobic cultures. The Bacilli species thus far identified using standard agar

media method were determined to be facultative. Heat killed culture cells and azide exposed cultures were used to determine the extent of abiotic Cr(VI) reduction in the batch experiments (Chirwa, 2005). Overnight grown cells were heat-killed by autoclaving at 121 ºC for 30 min.

2.3 Cr(VI) and Total Cr Determination

Cr(VI) was measured using a UV/VIS spectrophotometer (WPA, Light Wave II, Labotech, South Africa) at a wavelength of 540 nm (10 mm light path) in acidified samples treated with 1,5-diphenyl carbazide (APHA, 2005). Total Cr was measured at a wavelength of 359.9 nm using a Varian AA – 1275 Series Flame Atomic Adsorption Spectrophotometer (AAS) (Varian, Palo Alto, CA (USA)) equipped with a 3 mA chromium hollow cathode lamp. Before analysis using the AAS, 10 mL samples were acidified with 1 mL 1 N H_2SO_4 to dissolve chromium hydroxide precipitates and to extract adsorbed Cr(VI). Cr(III) was determined as the difference between total Cr and Cr(VI) concentration.

3. Results and discussion

3.1 Indigenous Culture Isolation and Testing

The indigenous microbial cultures were collected from four different sources, i.e.: Cr(VI) contaminated soil, sewage, activated sludge tanks (mixed liquor), and dry sludge. The bacteria from the above sources was incubated for 96 hours in LB broth at initial concentrations of 20–600 mg Cr(VI)/L under aerobic conditions (Table 1). Existence of Cr(VI) reducing bacteria in the samples was indicated by observed removal rates as shown in the table. The highest removal rate was observed in the culture from dried sludge with near complete Cr(VI) removal observed in batches up to 300 mg Cr(VI)/L. The effect of Cr(VI) toxicity inhibition on culture performance was evident at high Cr(VI) exposure. A culture of preserved *E. coli* ATCC 33456 purchased from the American Type Culture Collection was introduced in the experimental matrix as a control.

Table 1: Percentage Cr(VI reduction (%) in cultures from different sources after 96 h (4 d) of incubation at different initial Cr(VI) concentration.

Sources of CRB	**Initial Cr(VI) concentration (mg/L)**						
	20	**50**	**100**	**150**	**200**	**300**	**600**
Dried sludge cultures	100	100	100	100	100	99.2	18.7
Mixed liquor cultures	100	100	100	93.6	67.6	60.3	11.5
Sewage cultures	100	100	92.5	74.2	55.5	14.1	4.8
Soil cultures	91.5	76.0	58.3	29.9	22.0	7.45	0

Based on this initial screening, individual colonies were grown from the Dried Sludge and Mixed Liquor cultures which were further characterized using 16S rRNA gene sequencing and species identification using the Premega genetotype identification method described in Figure 1 below.

Figure 1 shows a graphical representation (phylogenetic tree) constructed from the BLAST search results of 16S rRNA DNA from a Cr(VI) reducing consortium culture isolated from a wastewater treatment plant that was continuously exposed to sodium di-chromate pollution in Brits (South Africa). The predominant Cr(VI) reducing group consisting of *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus mycoides* earlier detected by Molokwane and Chirwa (2009) was detected under both aerobic and anaerobic conditions and was further verified to be the predominant Cr(VI) reducing component from the sludge culture. From further analysis of samples collected 15 years later by Kholisa et al. (2021), the critical reactive components have remained the same.

Two G(+ve) variants of CXan17 and a G(−ve) *Microbacterium* spp. showed the highest potential as candidate Cr(VI) reducers. Pure cultures of these isolates were cultured in LB broth and Cr(VI) reduction of the pure cultures were compared to the performance of the original consortium sludge culture and a purchased proved Cr(VI) reducer, *E. coli* ATCC 33456.

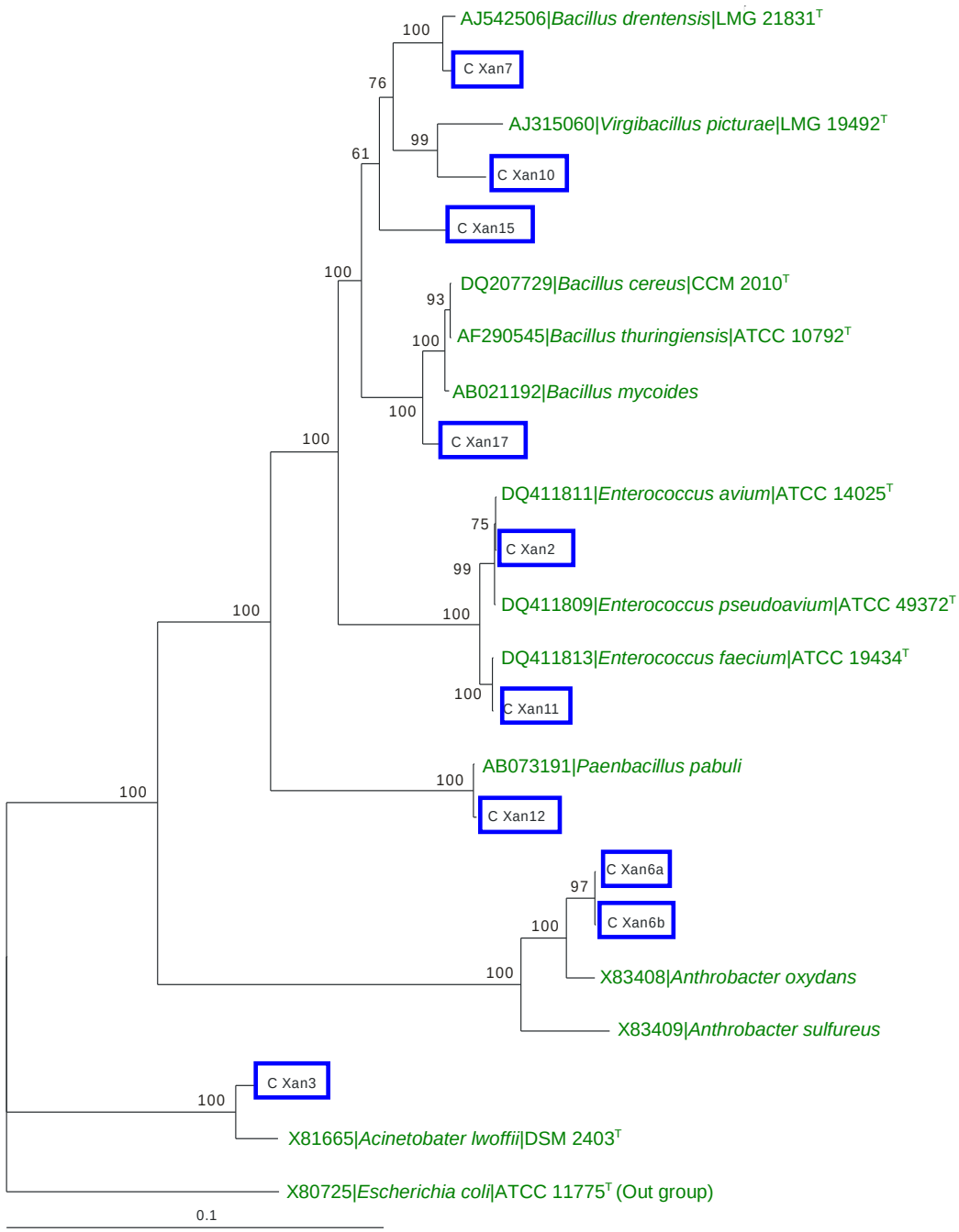

Figure 1: Phylogenetic tree of species from Brits Dry Sludge reflecting microbial diversity under anaerobic conditions with apparent predominance of the Gram-positive species – Bacillus cereus, Bacillus thuringiensis and Bacillus mycoides as the prominent Cr(VI reducing group.

3.2 Comparative Cr(VI) Reduction by G(+ve) and G(–ve) Bacteria

The results in Figure 2 show that cell physiology contributed to the extent and mode of biotransformation in the bacterial culture. Figure 2 suggests that there was a delayed response in Cr(VI) reducing activity in G(–ve) cells compared to the trend in G(+ve) cells. It was postulated that this difference could be due to mass transport effects imposed by the outer membrane of the G(–ve) cell and the fact that the reductase containing peptidoglycan layer in much thinner in G(–ve) cells than in G(+ve) cells. Both G(–ve) cells acquired an optimum Cr(VI) reduction capability at around 38 hours, Cr(VI) reduction occurred rapidly until the concentration in the

liquid medium was near neutral with respect to Cr(VI). This observation was validated by the observed trend in the purchased G(–ve) pure culture control of *E. coli* ATCC 33456 which followed a similar trend as the G(–ve) *Microbacterium spp*. (Figure 2a).

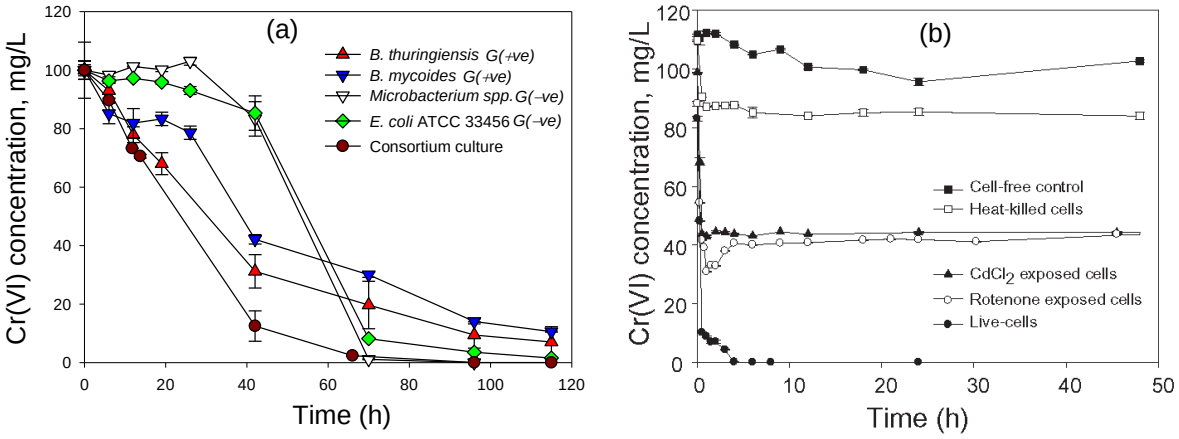

Figure 2: Comparative Cr(VI) reduction in mixed cultures under micro-aerobic conditions (a) comparison between G(+ve) and G(–ve) species, (b) effect of blocking enzymes on Cr(VI) reduction in a consortium culture of chromium (VI) reducing bacteria.

3.3 Pathway Analysis by Blocking Metabolic Pathway Enzymes

Enzyme co-factors which form part of the central metabolic mechanism of living cells are involved in the reduction of metals. In the current study, deactivating of thioredoxin activity by $CdCl_2$ resulted in termination of the enzymatic Cr(VI) reduction in mixed cultures of bacteria (Figure 2b). Additionally, metallic species in both active and resting cells were located predominantly on the surface of the cell. This suggests that thioredoxin either influenced external factors responsible for Cr(VI) reduction or a component of thioredoxin itself is excreted into the periplasm of the Cr(VI) reducing cells. Similar trends were observed in cells exposed to the NADH inhibitor (rotenone). Inhibitory effects of Cr(VI) reduction by the thioredoxin inhibitor, $CdCl_2$, after 4–6 h demonstrated thioredoxin is directly or indirectly involved in the Cr(VI) reduction in live cultures.

3.4 Impact of NADH-Dehydrogenase Inhibitors

NADH-dehydrogenase serves as a gateway into the electron transport respiratory (ETR) system. The objective of this component of the study was to determine whether Cr(VI) reduction is associated with the ETR system. Results in (Figure 2b) showed that, in the presence of $C_{23}H_{22}O_6$ (rotenone) immediate Cr(VI) reduction was observed within the first 6 h of incubation. The initial removal of Cr(VI) from solution was attributed to physical-chemical and biosorptive processes occurring during the first few hours of incubation.

3.5 Distribution of Chromium and Reduced Cr Species in Cells

To establish the distribution and localization of chromium deposits in the cells, thin section of cells exposed to Cr(VI) were viewed using Transfer electron microscopy (TEM). TEM of Cr(VI)-loaded cells revealed a dark electron opaque region extracellularly (Figures 3a & b), suggesting that the metal reductase activity in the isolated species is associated with the periplasm and outer cell membrane. The observed accumulation of precipitates on the surface of cells imply surface reaction. However, further analyses are under way to finally present a definite model for Cr(VI) reduction in living cells.

Further analysis of the deposited elements was achieved with SEM-EDX at higher beam energy exposure. High resolution micrographs of biomass from a Cr(VI) loaded culture showed $CrOH_3$ precipitates on cell surfaces (Figure 3b) is compared to the clean surfaces observed under sterile culture conditions. It was observed that the constituent percentage of Cr (78.2 %) calculated as a percentage of the sum of all observed peak areas at a higher energy beam strength was relatively higher than that of other associated elements identified in the precipitate, i.e., (w/w) Ca (0.2 %), Cd (0.5 %), Cu (8.8 %), Au (3.3 %), and P (8.4 %). The reductive potential of Cr(VI) was verified by the low Cr content (0.25 %) in precipitates on cell surfaces in heat sterilized cultures (data not shown).

(a) (b)

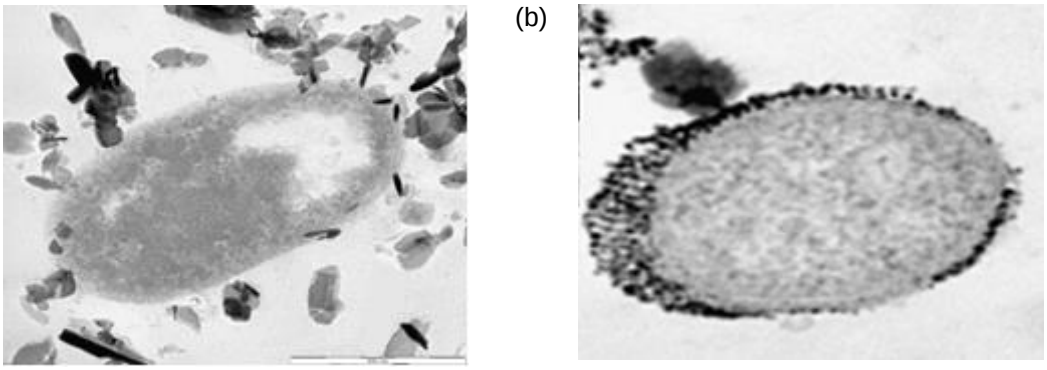

Figure 3: Cell surface deposit of reduced Cr species visualized by TEM at (a) 5 h and (b) 24 h, showing accumulation of Cr(III) hydroxide precipitates under 50 mg/L live culture batches.

4. Conclusion

The *Bacillus thuringiensis, Bacillus cereus and Bacillus mycoides* cultures isolated from Cr(VI) contaminated sludge from a sewage treatment plant in Brits (South Africa) reduced Cr(VI) at rapid rates more than 10 times faster than the rate observed in a Cr(VI) reducing *Escherichia coli* ATCC 33456 isolated from contaminated from New Jersey (USA). TEM and SEM-EDX results showed accumulation of reduced Cr species on the surface on the bacterial cells. The location of the majority of Cr species in cells suggest Cr(VI) reductase transport is the limiting factor in Cr(VI) reduction rate. Results show that initial fast reduction in *B. thuringiensis*, *B. cereus and B. mycoides* cultures is a result of fast deposition of Cr(VI)-reductase at cell surfaces than in the dual membrane G(–ve) *E. coli* ATCC 33456 species.

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