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Chromium (VI) reduction in activated sludge bacteria exposed to high chromium loading: Brits culture (South Africa)

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ARTICLE INFO

Article history:
Received 23 April 2008
Received in revised form
28 July 2008
Accepted 30 July 2008
Published online ■

Keywords:
Indigenous cultures
Cr(VI) reduction
Culture characterisation
Biocatalysis
Bacillus sp.
Microbacterium sp.

ABSTRACT

A mixed-culture of bacteria collected from a wastewater treatment plant in Brits, North-West Province (South Africa) biocatalytically reduced Cr(VI) at much higher concentrations than previously observed in cultures isolated in North America. Cr(VI) reduction rate up to 8 times higher than the rate in previous cultures was achieved by the Brits culture under aerobic conditions. Near complete Cr(VI) reduction was observed in batches under initial concentrations up to 200 mg Cr(VI)/L after incubation for 65 h in aerobic cultures. Under anaerobic conditions up to 150 mg Cr(VI)/L was completely removed after incubating for 130–155 h. In the previous cultures, complete removal was only achieved in cultures at an initial Cr(VI) concentration lower than 30 mg/L after incubation for 96–110 h. Consortium cultures were characterised using 16S rRNA partial sequence analysis. Results showed that the Gram-positive *Bacillus* genera predominated under aerobic conditions with a small composition of the Gram-negative *Microbacterium* sp. More biodiversity was observed in anaerobic cultures with the marked appearance of *Enterococcus*, *Arthrobacter*, *Paenibacillus* and *Oceanobacillus* species. Experiments run on purified individual species did not achieve the same level of Cr(VI) reduction as observed in the original consortium from sludge indicating possible existence of interspecies interactions necessary for optimum Cr(VI) reduction. All Cr(VI) reduced was accounted for as Cr(III) with a small error range (2–6%).

1. Introduction

Hexavalent chromium [Cr(VI)] compounds are used in a wide variety of commercial processes and unregulated disposal of chromium containing effluents has led to the contamination of soil, aquatic sediments, and surface and groundwater environments. Chromium, a steel-grey, lustrous, hard and brittle metal, occurs in nature in the bound form that constitutes 0.1–0.3 mg/kg of the Earth's crust. It has several oxidation states ranging from (−II) to (+VI), the trivalent and hexavalent states being the most stable. A maximum acceptable concentration of 50 μg/L for Cr(VI) in drinking water has been established on the basis of health considerations (Kiilunen, 1994). In some American states, the exposure limit for Cr(VI) is as low as 15 μg/L for humans and 10 μg/L for aquatic organisms (Levitskaia et al., 2008) which is below the detection limit for most low cost colorimetric methods. Cr(VI) concentrations above the allowable limit cause cancer in humans and aquatic fauna, and is acutely toxic at much higher concentrations (U.S.EPA, 1978; Federal Register, 2004).

Cr(VI) is discharged into the environment through anthropogenic activities such as chromite ore processing,

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doi:10.1016/j.watres.2008.07.040

Nomenclatures

C	Cr(VI) concentration at time t (ML^{-3})
C_o	initial Cr(VI) concentration (ML^{-3})
R_c	Cr(VI) reduction capacity (MM^{-1})
X	viable cell concentration at time t (ML^{-3})
X_o	initial viable cell concentration (ML^{-3})

electroplating, corrosion control, wood preservation and leather-tanning processes, among others (Chuan and Liu, 1996; Palmer and Wittbrodt, 1991; Lawson, 1997). In most Cr(VI) contaminated sites in South Africa, the problem is exacerbated by the existence of abandoned and closed mining or processing operations. Current methods of environmental remediation of Cr(VI) include the pump-and-treat method in which chemical processes that involve the adjustment of pH using strong acids and bases are utilised in the treatment of Cr(VI). Chemical processes often generate other harmful by-products that require further treatment (Patterson, 1985). Biological processes offer a cleaner cost effective alternative that can be carried out under a natural pH range (6.8–7.2).

Microbial Cr(VI) reduction was first reported in the late 1970s when Romanenko and Koren'kov (1977) observed Cr(VI) reduction capability in *Pseudomonas* spp. grown under anaerobic conditions. Since then, several researchers have isolated new microorganisms that catalyse Cr(VI) reduction under varying conditions (Ackerley et al., 2004; Chirwa and Wang, 1997a; Ohtake et al., 1990; Ganguli and Tripathi, 2002; Suzuki et al., 1992; Ramírez-Ramírez et al., 2004; Shen and Wang, 1993; Baldi et al., 1990). Other researchers have also observed Cr(VI) reduction in consortium cultures isolated from the environment (Chirwa and Wang, 2000; Stasinakis et al., 2004; Dermou et al., 2005; Chen and Gu, 2005; Chang and Kim, 2007). Cr(VI) reduction has been demonstrated to be cometabolic (not participating in energy conservation) in certain species of bacteria, but is predominantly dissimilatory/respiratory under anaerobic conditions (Ishibashi et al., 1990). In the latter process, Cr(VI) serves as a terminal electron acceptor in the membrane electron-transport respiratory pathway, a process resulting in energy conservation for growth and cell maintenance (Horitsu et al., 1987; Lovley and Phillips, 1994).

Cr(VI) reduction by microorganisms often results in consumption of large amounts of proton as reducing equivalents which results in the elevation of the background pH. The increased pH facilitates the precipitation of the reduced chromium as chromium hydroxide, $Cr(OH)_3(s)$ as shown in Eqs. (1) and (2) below (Brock and Madigan, 1991; Zakaria et al., 2007):

$$CrO_4^{2-} + 8H^+ \xrightarrow{CRB} Cr^{3+} + 4H_2O \xrightarrow{\text{neutral pH}} Cr(OH)_3(s) + 3H + H_2O \quad (1)$$

$$3CH_3COO^- + 4HCrO_4^- + 4CrO_4^{2-} + 33H^+ \rightarrow 8Cr^{3+} + 6HCO_3^- + 20H_2O \quad (2)$$

Eq. (1) illustrates the general biological Cr(VI) reduction by Cr(VI) reducing bacteria (CRB) reconstructed from redox half reactions whereas Eq. (2) illustrates a typical reaction under anaerobic conditions using acetic acid as a carbon source and electron donor. Other fatty acid by-products of hydrolysis can also serve as electron donors for Cr(VI) reduction (Viamajala et al., 2006).

In this study, a high performing mixed-culture of bacteria was isolated from dried sludge at a wastewater treatment plant in Brits (SA). The culture achieved reduction rates three to 8 times higher than those observed in cultures studied elsewhere (Ohtake et al., 1990; Shen and Wang, 1994a; Chirwa and Wang, 1997a,b). In order to determine the reason for the observed exceptionally high Cr(VI) reduction rates, the cultures were purified and characterised to determine the species composition. The research is part of an effort to develop the bioremediation process for treatment of Cr(VI) contaminated sites in South Africa. Since 1940, South Africa has produced 72% of the world's chrome ore, the majority of which is mined in the North Eastern region of the country formally known as Transvaal (U.S.EPA, 2001; Mintek, 2004).

2. Materials and methods

2.1. Culture and media

2.1.1. Source of microorganisms

The mixed-culture of bacteria was obtained from dried sludge collected from sand drying beds at the Brits Wastewater Treatment Works (NW). The treatment plant receives periodic flows from a nearby abandoned sodium dichromate (SDC) processing facility reported to discharge high levels of Cr(VI) in the sewerage works. The chrome processing facility was commissioned as early as 1996. The measured Cr(VI) concentration in the influent and mixed liquor from the treatment plant was 2.45 and 2.63 mg/L, respectively, and the Cr(VI) content in dried sludge was 25.44 g/m^3 at the time of sampling. Higher values of the reduced form of total Cr were expected in the mixed liquor and dry sludge due to the presence of Cr(VI) reducing bacteria. High Cr(VI) loadings from nearby chrome foundries are periodically discharged to the treatment plant, but the times at which microbial samples were collected for this study did not coincide with the discharge events. The sludge cultures were cultivated for 4 days at $30 \pm 1\,^{\circ}C$ in 100 mL of sterile Luria-Bettani (LB) broth containing varied concentrations of Cr(VI). Aerobic cultures were grown in 1 L Erlenmeyer flasks covered with cotton plugs, in suspension by agitation at 120 rpm using a Labcon SPL-MP 15 Lateral Shaker (Labcon Laboratory Services, South Africa). Anaerobic cultures were grown in 100 mL serum bottles, sealed after purging for 5–10 min with 99% pure nitrogen gas. All media were autoclaved for 15 min at 121 °C and cooled to room temperature before use. Agar used for colony development was cooled to 40 °C before use.

2.1.2. Culture isolation

Pure cultures were prepared by depositing 1 mL of a serially diluted sample on LB agar followed by incubation at 30 °C to develop separate identifiable colonies. Individual colonies were transferred using a heat-sterilised wire loop into 100 mL sterile LB broth spiked with 75 mg Cr(VI)/L. The cells were allowed to grow; colonies were grown again from serially diluted samples. Loop-fulls from individual colonies were used to inoculate fresh media containing 150 mg Cr(VI)/L.

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Cultures from the third isolation were used in the detailed Cr(VI) reduction rate analysis. Cr(VI) reducing colonies were selected by observing complete Cr(VI) reduction after incubation for 72 h. The selected colonies were stored at 4 °C in test tube slant cultures or agar-plate streaks.

2.1.3. *Culture characterisation*

Phylogenetic characterisation of cells was performed on individual colonies of bacteria from the 7th to 10th tube in the serial dilution preparation. LB and Plate Count (PC) agar was used for colony development. In preparation for the 16S rRNA sequence identification, the colonies were first classified based on morphology. Seven different morphologies were identified for the aerobic cultures (19 morphologies for the aerobic cultures). These were streaked on nutrient agar followed by incubation at 37 °C for 18 h.

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) (Coenye et al., 1999). 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).

2.2. *Cr(VI) reduction experiments*

2.2.1. *Abiotic controls*

Killed culture cells and azide exposed cultures were used to determine the extent of abiotic Cr(VI) reduction in the batch experiments. Overnight grown cells were heat-killed by autoclaving at 121 °C for 30 min. Another set of overnight grown cells was subjected to azide toxicity by incubating the cells in a broth consisting of 0.1% azide solution using sodium azide (NaN_3) (Ginestet et al., 1998).

2.2.2. *Aerobic culture experiments*

Aerobic Cr(VI) reduction experiments were conducted in 100 mL Erlenmeyer flasks using cells harvested after 24 h incubation, concentrated by a ratio of 4:1, resulting in an average viable cell concentration of $5.2 \pm 2.1 \times 10^9$ 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 50–600 mg/L was added and the solution was incubated under shaking at 30 °C. Experimental units consisted of the different initial concentrations and all experiments were conducted in duplicate. Samples (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.

2.2.3. *Anaerobic culture experiments*

Anaerobic Cr(VI) reduction experiments were conducted in 100 mL serum bottles using cells harvested after 24 h incubation under anaerobic conditions. The cells were transferred under an anaerobic glove bag purged with 99.99% N_2 gas. The cells were concentrated to a 4:1 ratio, and washed twice in a sterile solution of 0.085% NaCl before adding Cr(VI). The bottles were purged with nitrogen gas (99.99%) for 10 min to expel any residual oxygen before sealing with silicon stoppers and aluminium seals. After sealing, the cultures were incubated at 30 ± 1 °C for 7 days. Samples (1 mL) were withdrawn using a sterile syringe at time intervals determined by the observed rate of Cr(VI) removal. The samples were centrifuged at 2820*g* for 10 min in a Hermle 2323 centrifuge (Hermle Laboratories) to remove suspended cells before analysis. Headspace gases were sampled by syringe and analysed by gas chromatography.

2.2.4. *Cell free extracts and membrane fragments*

Pure cultures isolated in this study were grown in 500 mL for 24 h in sterile LB broth. The cells were then harvested by centrifugation at 2820*g* for 10 min. Pellets formed at the bottom of the centrifuge tubes were washed 2 times with sterile 0.85% NaCl solution. The washed pellets were re-suspended at 2–3 g wet weight per 10 mL sterile 0.85% NaCl. Cells were disrupted by a 3 mm diameter microtip mounted to the Model VCX 500 Sonics VibraCell (Sonics & Materials, Inc., Newtown, CT). The tubes containing concentrated cells were placed inside an ice container to avoid overheating during sonication. The tip was cleaned with ethanol and dried thoroughly before use. The cells were sonicated in four cycles of 15 min with 5 min rests between cycles.

The disrupted cells were centrifuged at 11,300*g* for 20 min to extract the membrane fraction pellet from the disrupted cell mixture. The pellet was re-suspended into a 100 mg Cr(VI)/L batch. The supernatant poured out from the centrifuge bottle was filled to 100 mL and Cr(VI) was added to prepare the second experimental batch of 100 mg Cr(VI)/L to evaluate Cr(VI) reduction by the cytoplasmic component of the cells.

2.3. *Analytical methods*

2.3.1. *Cr(VI) and total Cr*

Cr(VI) was measured using a UV/vis spectrophotometer (WPA, Light Wave II, Labotech, South Africa). The measurement was carried out at a wavelength of 540 nm (10 mm light path) after acidification of 0.2 mL samples with 1 N H_2SO_4 and reaction with 1,5-diphenyl carbazide to produce a purple colour (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.

2.3.2. *Dry weight of biomass*

LB broth (5 mL) containing grown cells was withdrawn by sterile pipette after 24 h of incubation at 30 °C and filtered

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through a washed, dried and weighed sintered glass (tare weight). The sintered glass and wet biomass was dried in the oven at 105 °C, cooled in a desiccator and weighed. The drying, cooling and weighing was carried out until a constant dry weight was obtained. The dry weight of the biomass in 5 mL was calculated as the difference in weight between that of the sintered glass plus biomass and that of the empty sintered glass. The dry weight of the biomass per liter was obtained by extrapolation from the 5 mL volume.

2.3.3. *Viable biomass*

Viable cells were determined using the pour plate method using heterotrophic (pour) plate method and colony counts as described in the *Standard Methods for the Examination of Water and Wastewater* (APHA, 2005), with the colonies grown on Luria-Bettani (LB) and Plate Count (PC) agar. Samples for the analysis of viable suspended cell concentration were withdrawn from experimental batches at 6–12 h intervals. Samples (1 mL) were serially diluted in 0.9 mL sterile 0.85% NaCl solution. Each dilution (1 mL) was then added to agar plates (100 cm × 15 cm size) followed by thorough mixing with approximately 10 mL of liquid agar at 46 °C. Colonies were counted after 24 h incubation and the bacterial count was reported as colony forming units (CFU) per mL of sample. The CFU count was converted to mass concentration by measuring dry weight of cells with a known CFU count during the log growth phase when over 95% of the cells were expected to be viable. A conversion factor of 1.833×10^{-10} mg/cell was determined (with $R^2 = 0.997$). The inactivated mass concentration of viable cells was used to determine the Cr(VI) reduction capacity of the cells (R_c).

2.3.4. *Cr(VI) reduction capacity*

The Cr(VI) reduction capacity of the cells was determined as the amount of Cr(VI) reduced per amount of viable cells inactivated during incubation (Shen and Wang, 1994b):

$$R_c = \frac{C_o - C}{X_o - X} \quad (3)$$

where R_c = Cr(VI) reduction capacity (mg Cr(VI) removed/mg cells inactivated), C_o = initial Cr(VI) concentration (mg/L), C = Cr(VI) concentration at a time of incubation t, X_o = initial viable cell concentration (mg/L), and X = viable cell concentration (mg/L) at any time t. A viable cell conversion factor of 1.833×10^{-10} mg/cell was used to convert cell count (CFU) to the mass concentration.

3. Results and discussion

3.1. *Preliminary studies*

3.1.1. *Cr(VI) reducing bacteria*

A survey was conducted which involved collection and testing cultures from four different sources for Cr(VI) reduction, i.e., soil from a contaminated site, influent to sewage treatment plant, activated sludge tanks (mixed liquor), and dry sludge from sand drying beds. The bacteria from the above sources was incubated for 96 h in LB broth at initial concentrations of 20, 50, 100, 150, 200, 300, 400 and 600 mg Cr(VI)/L under aerobic conditions (Fig. 1). Existence of Cr(VI) reducing bacteria in the samples was indicated by observed removal rates as shown in the figure. 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. This was attributed to better acclimation and selection for Cr(VI) reducing species in the sludge due to exposure to higher Cr(VI) concentrations and longer exposure in the sludge zone than in the mixed liquor. The culture isolated from soil yielded the lowest Cr(VI) removal rate. Very low to insignificant Cr(VI) reduction was observed at very high initial Cr(VI) concentration of 600 mg/L except for the dry sludge culture where 18.7% was removed after incubation for 96 h. This was attributed to the inhibition effect of Cr(VI) on the culture.

3.1.2. *Test for abiotic Cr(VI) reduction*

Abiotic Cr(VI) reduction activity was evaluated by conducting experiments at 100 mg Cr(VI)/L with heat-killed and azide inhibited cultures (Fig. 2). A live cell culture control showed best performance with near complete Cr(VI) removal at 22.5 h. There was significant decrease in Cr(VI) reduction activity due to heat inactivation of the cells. Only 30% Cr(VI) removal was observed in heat-killed cultures after incubation for 22.5 h, a much lower removal value than that observed in the live consortium. The 30% removal may be due to Cr(VI) reductase released into the medium from heat-lysed cells and regrowth of cells that escaped destruction by heat. An azide inhibited culture indicated partial inactivation of cells with an observed Cr(VI) reduction potential of the oxygen stressed culture. Approximately 50% Cr(VI) was removed in the azide inhibited cultures.

3.2. *Cr(VI) reduction under aerobic conditions*

Experimentation under varying initial Cr(VI) concentration of 50–400 mg/L in media with harvested and concentrated cells showed that the culture achieved complete Cr(VI) removal in

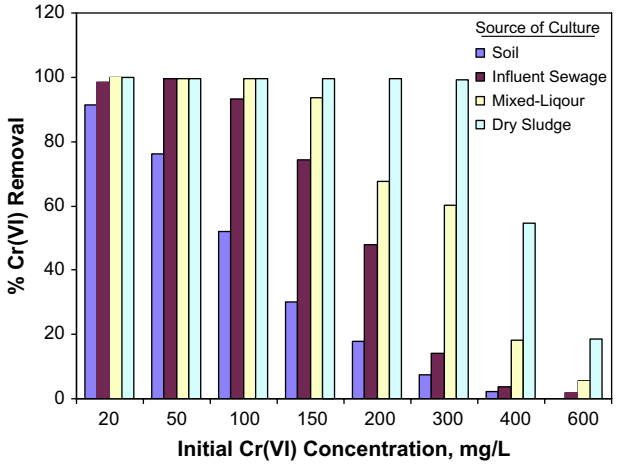

Fig. 1 – Cr(VI) reduction in cultures from different sources (soil, influent stream, mixed liquor, and dry sludge) after incubation for 96 h under varying initial Cr(VI) concentration and growing cells (inoculated with 5×10^4 CFU/mL before incubation).

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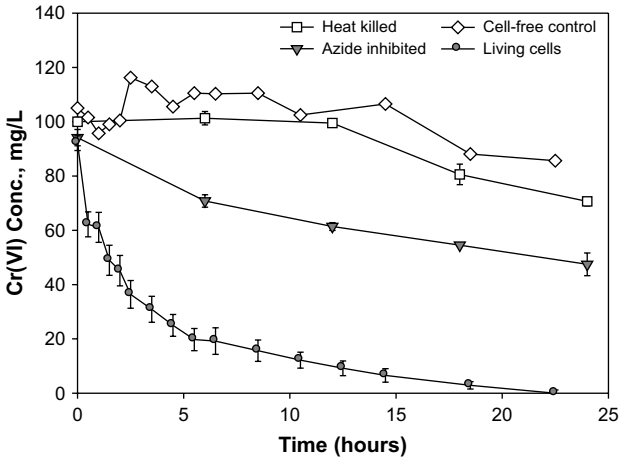

Fig. 2 – Evaluation of abiotic Cr(VI) reduction in heat-killed and azide inhibited cells (inoculated with 5×10^4 CFU/mL before incubation).

batches under initial concentration up to 200 mg/L in less than 64.3 h (2.7 days) (Fig. 3). Up to 94% of Cr(VI) was removed at the initial concentration of 300 mg/L after incubation for 110 h. Very little Cr(VI) was reduced at the highest concentration tested (400 mg/L). The loss of the capability to reduce Cr(VI) in cells under very high Cr(VI) loadings was directly correlated to the loss of cell viability. Viable cell concentration in the 400 mg/L batches decreased from $5.2 \pm 2.0 \times 10^9$ to $4.8 \pm 1.5 \times 10^5$ cells/mL after 22.5 h incubation, a kill rate of 4.1 log, whereas the kill rate at lower concentration of 100 mg/L was only 1.2 log ($6.1 \pm 1.8 \times 10^9$–$3.81 \pm 1.5 \times 10^8$ cells/mL). This was in agreement with earlier observations in previous studies where Cr(VI) reducing cells were irreversibly inactivated in batch cultures when the initial concentration exceeded a certain limiting concentration (Wang and Shen, 1997). These results demonstrate that Cr(VI) reduction is significantly affected by the Cr(VI) toxicity. Based on the highest concentration completely removed, i.e., 200 mg/L batch, the R_c value of 0.21 mg Cr(VI) reduced/mg cells deactivated was determined. This value is much higher than the values previously reported in literature (Shen and Wang, 1994b; Nkhalambayausi-Chirwa and Wang, 2005).

3.2.1. *Fate of Cr(VI) [mass balance]*

Biotransformation of Cr(VI) to Cr(III) was validated by a mass balance analysis on Cr species during incubation. Since Cr(VI) was the only added form of Cr, the total Cr measured using the AAS at the end of the experiment was expected to be equal to the amount of Cr(VI) added. The added Cr(VI) in all batches was accounted for as total Cr at the end of the experiment with measurement errors within the ±5% range. Only one value (at 100 mg/L, error = +8%) exceeded the acceptable error range of ±5%.

3.3. *Cr(VI) reduction in an anaerobic mixed-culture*

Cr(VI) reduction under anaerobic conditions was investigated due to its relevance to certain applications such as bioremediation of sediment zones and groundwater environments. The experiment under anaerobic conditions was conducted over a lower concentration range (50–300 mg Cr(VI)/L) since slower growth was observed in the anaerobic cultures. The rate of Cr(VI) reduction was generally slower in the anaerobic cultures. Complete Cr(VI) reduction occurred in cultures with a lower initial Cr(VI) concentration of 150 mg/L after a longer incubation period (155 h) than in aerobic cultures (Fig. 4). These results indicate that the Cr(VI) reduction mechanism in the cells is either coupled to the metabolic processes of the culture or different species in the culture with different growth requirements are responsible for Cr(VI) reduction. Characteristic anaerobic gases (CO_2 and H_2O vapour) accumulated in the headspace of the serum culture bottles. The amount of gas produced (determined by partial pressure) was lower at higher initial Cr(VI) concentration showing that gas

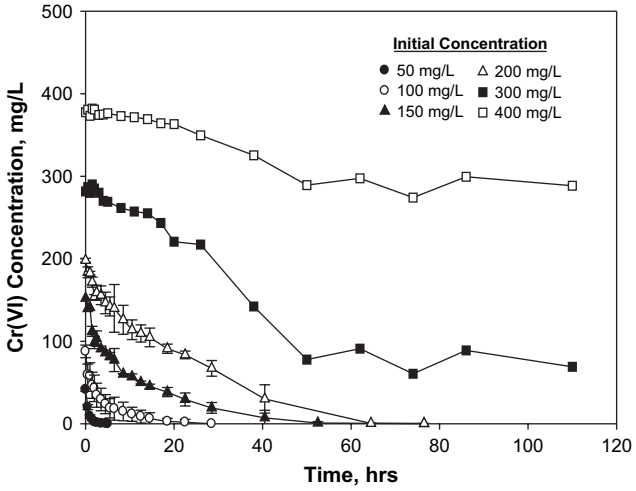

Fig. 3 – Aerobic culture experiment of Cr(VI) reduction in consortium from dried sludge grown at initial Cr(VI) concentrations ranging from 50 to 600 mg/L (resting cells: $5.2 \pm 2.1 \times 10^9$ CFU/mL).

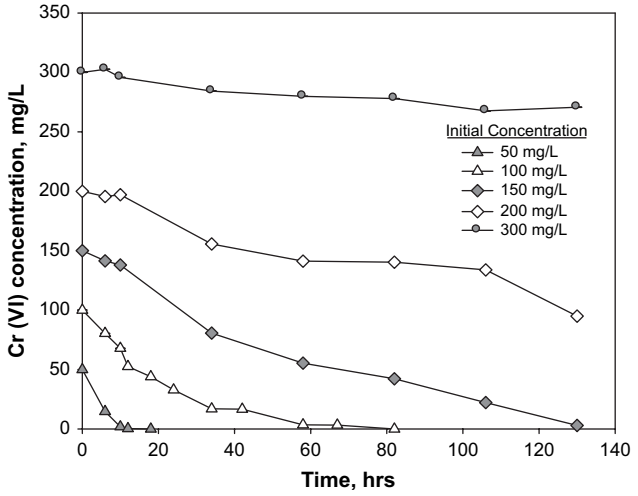

Fig. 4 – Anaerobic culture experiment of Cr(VI) reduction in consortium from dried sludge grown at initial Cr(VI) concentrations ranging from 50 to 300 mg/L (resting cells: $1.58 \pm 1.8 \times 10^9$ CFU/mL).

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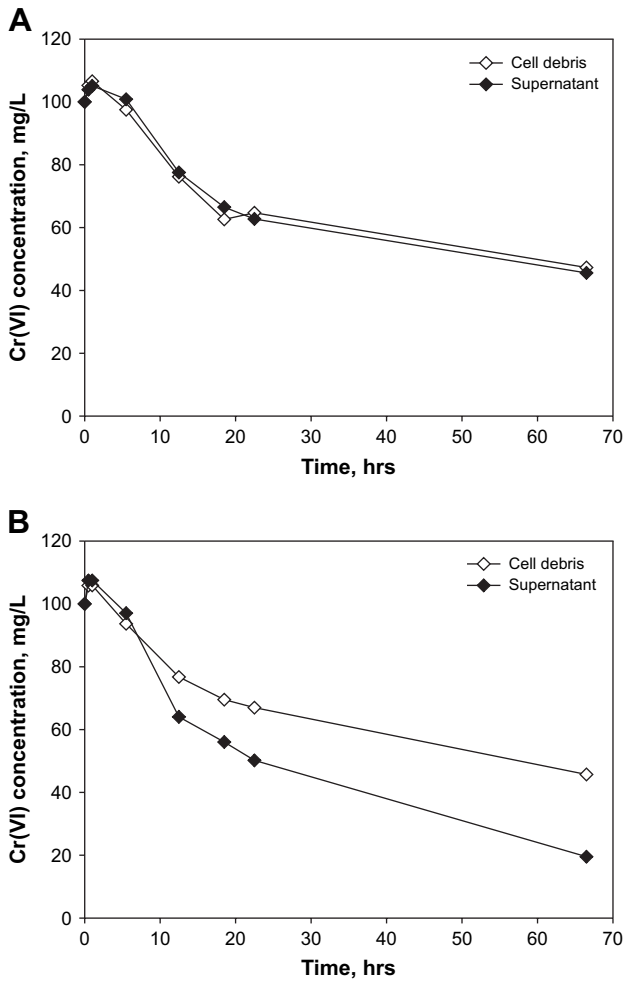

Fig. 5 – Cr(VI) reduction in disrupted (A) aerobically grown cells and (B) anaerobically grown cells showing the higher Cr(VI) reduction rate in the cytosolic component (supernatant) than in the membrane fraction (pellet) after centrifugation of disrupted cells at 11,300*g* for 24 min.

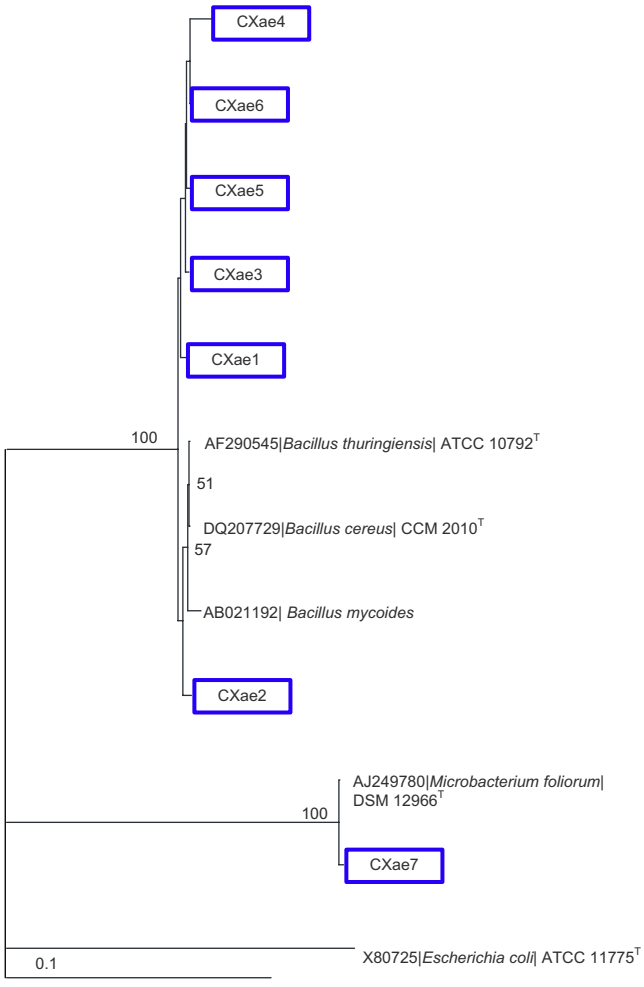

Fig. 6 – Phylogenetic tree of species from Brits dry sludge reflecting microbial diversity under aerobic conditions.

production was an essential component of the metabolic process and that it was inhibited by Cr(VI). For example, the anaerobic gas (CO_2) composition at an initial concentration of 50 mg/L N_2-purged reactors [88(±2)% CO_2, 6(±3)% H_2O, and 4.3(±2)% N_2] was much higher than the composition at 300 mg/L [15.2(±3)% CO_2, 2(±3)% H_2O, and 78(±1)% N_2].

For the anaerobic batch experiments, Cr(VI) reduction was incomplete at 200 mg/L initial Cr(VI) concentrations after incubation for 130 h (only 50% reduced). This was a much lower performance compared to the observed under the same concentration in aerobic cultures where 99.7% removal was achieved after 96 h. The lower Cr(VI) removal rates observed under anaerobic conditions were accompanied by lower Cr(VI) reduction capacity of the cells ($R_c = 0.011427$ g Cr(VI) reduced/g cells inactivated at 150 mg/L and 0.051697 g Cr(VI) reduced/g

Table 1 – Partial sequencing of aerobic CRB isolated from Brits dry sludge grown in solution containing 100 mg/L Cr(VI)

Pure culture	Partial 16S ID[a]	% Identity
X1	*Bacillus cereus* strain 213 16S, *Bacillus thuringiensis* 16S	99
X2	*Bacillus* sp. ZZ2 16s, *B. cereus* ATCC 10987, *B. thuringiensis* str. Al Hakam	99
X3	*Bacillus* sp. 32-661 16s, *B. cereus* strain 16S	99
X4	*Bacillus mycoides* strain BGSC 6A13 16S, *B. thuringiensis serovar finitimus* strain BGSC 4B2 16S	99
X5	*B. mycoides* strain BGSC 6A13 16S, *B. thuringiensis serovar finitimus* strain BGSC 4B2 16S	99
X6	*B. mycoides* strain BGSC 6A13 16S, *B. thuringiensis serovar finitimus* strain BGSC 4B2 16S	99
X7	*Microbacterium* sp. S15-M4, *Microbacterium foliorum*	99

a S ID = 16 Svedburg rRNA *I*dentity of partial sequences (16 Svedburg unit ribosomal Ribo-Nucleic-Acid *I*dentity).

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cells inactivated at 200 mg/L). The R_c value under anaerobic conditions was thus an order of magnitude lower than the value obtained from aerobically grown cultures from the same source.

3.4. Cr(VI) reduction pathway

Cells were disrupted by ultrasonication to release the cytosol into the solution. This was done to allow direct access to the intracellular enzymes without the limitation of mass transport through the cellular membranes of the bacteria. This also meant that the cells would be killed in the process. The Cr(VI) reduction experiments were conducted on the supernatant and cell fragments collected as a pellet after centrifugation at 11,300*g* for 20 min and re-suspended in medium for Cr(VI) reduction experiment. Results for the disrupted aerobic and anaerobic cultures are shown in Fig. 5.

The difference in the Cr(VI) reduction rate between the cytosolic component and the membrane fraction in the aerobic culture was insignificant (Fig. 5a). However, in the anaerobic culture, a higher removal rate was observed in the supernatant than in the membrane fraction – comparatively, the cytosolic component achieved 50% removal in 24 h, a much higher level than the observed 20–25% in the membrane fraction. Cell disruption was most effective in anaerobic cultures which resulted in a higher Cr(VI) reduction rate in the cytosolic component as evidenced by the higher Cr(VI) reduction in the diluted supernatant (Fig. 5b). It was later observed that, under aerobic conditions, cells were predominantly Gram-positive Bacilli. The Gram-positive cells are protected by a thick peptidoglycan cell wall expected to be difficult to disrupt.

It was interesting to note that the percentage removal rate in the cytosolic component of the crushed anaerobic culture was similar to the azide inhibited culture experiment whereby up to 50% removal was achieved in 22.5 h. This may be due to the fact that enzymes that were produced during the cell cultivation process were still available for Cr(VI) reduction after the cell growth inhibitor was added. In heat-killed culture, enzymes were expected to be denatured and inactivated at high temperatures (between 50 and 80 °C), thus no Cr(VI) reduction activity was observed under these conditions.

3.5. Culture characterisation

3.5.1. Aerobic cultures

The natural consortium sampled from dried sludge from the Wastewater Treatment Works at Brits (NW) produced the higher Cr(VI) reduction rate. For this reason, the culture from the dry sludge was chosen for characterisation. Culture purification and 16S rRNA sequencing were performed at the Department of Microbiology, University of Pretoria where the identification was done; at 99%, results indicated the predominance of four aerobe phenotypes.

Partial sequences of 16S rRNA matched 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 (Table 1). *A phylogenetic tree was constructed for the species from purified cultures grown under aerobic conditions based on a basic BLAST search of rRNA sequences in the NCBI database* (Fig. 6).

3.6. Anaerobic culture

Anaerobic bacteria was isolated from dry sludge following the same procedure described for aerobic cultures, modified by maintaining anaerobic conditions by purging reactors with

Table 2 – Characteristics of pure cultures and nearest matches based on the BLAST analysis of 16S rRNA partial sequences

	Pure culture	Colour on plates	Blast result	% Identity
Chromium(VI) 100 mg/L				
1	X1	Light brown/cream	Could not subculture/amplify	
2	X2	Off-white	*Enterococcus avium*, *Enterococcus pseudoavium*	99
3	X3	Cream	Uncultured bacterium clone Y2, *Acinetobacter* sp. ANT9054	97
Chromium(VI) 150 mg/L				
4	X4	Coral	Could not subculture/amplify	
5	X5	Yellow	Could not subculture/amplify	
6	X6a	Yellow	*Arthrobacter* sp. Sphe3, uncultured soil bacterium clone TA12	93,94
7	X6b		*Arthrobacter* sp. AK-1	99
8	X7	Cream & yellow rings	*Bacillus drentensis*, *B. drentensis*	96, 97
9	X8	Light brown	Could not subculture/amplify	
10	X9	Light brown	Could not subculture/amplify	
11	X10	Light brown	*Oceanobacillus* sp. JPLAk1, *Virgibacillus necropolis*	99,98
12	X11	Off-white	*Enterococcus faecium* strain R0026, *Rumen bacterium* R4-4	99
13	X12	Coral	*Paenibacillus pabuli*, *Paenibacillus xylanilyticus* strain XIL14	99
Chromium(VI) 200 mg/L				
14	X13	Yellow	Could not subculture/amplify	
15	X14	Orange	Could not subculture/amplify	
16	X15	Cream	[*Brevibacterium*] *frigoritolerans*, *Bacillus* sp. R21S	99
17	X16	Yellow	Could not subculture/amplify	
18	X17	Cream	Uncultured bacterium, *Bacillus* sp. BS19	93

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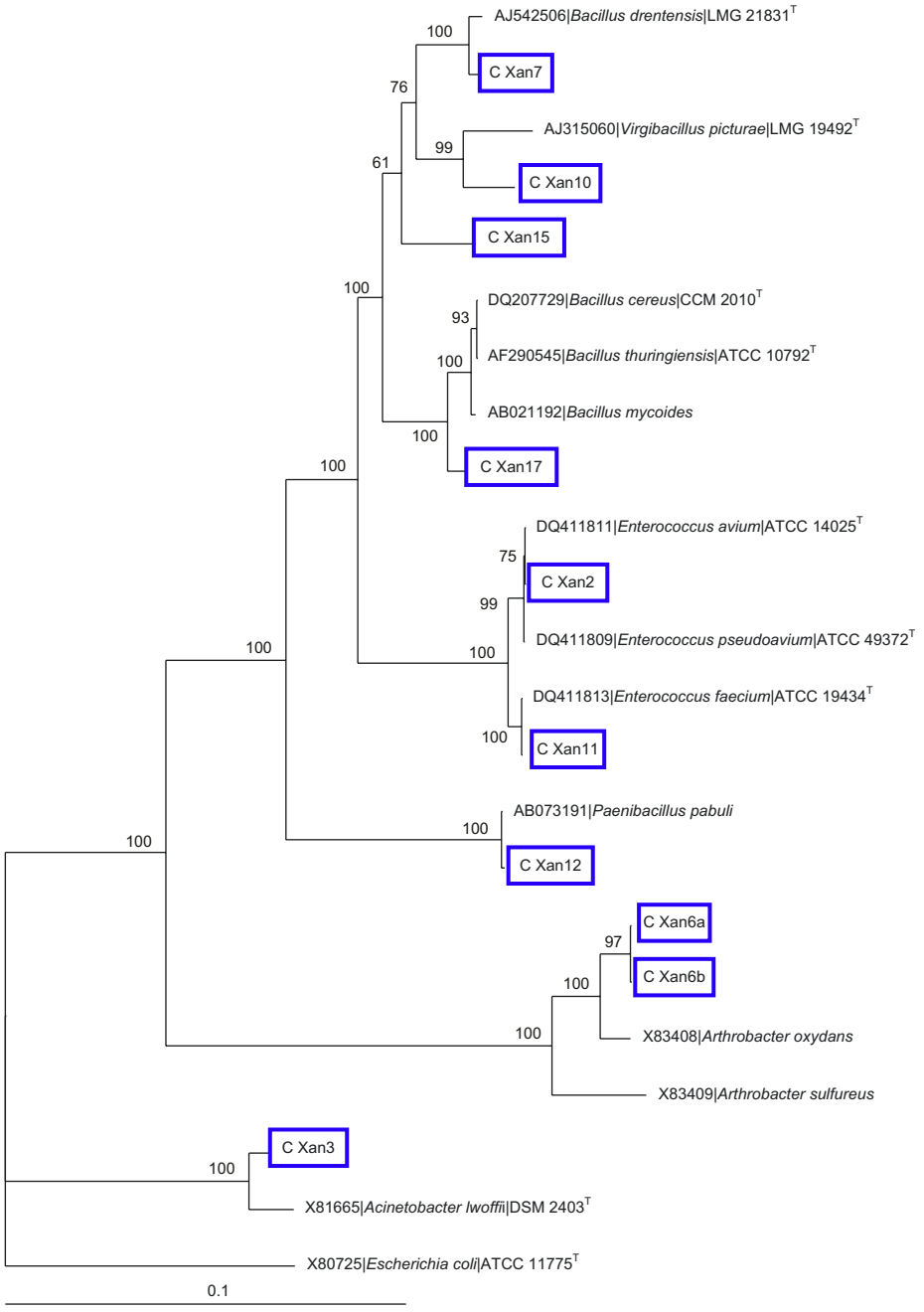

Fig. 7 – Phylogenetic tree of species from Brits dry sludge reflecting microbial diversity under anaerobic conditions.

nitrogen and sealing in serum bottles. All transfers were conducted in an anaerobic glove bag purged with nitrogen. The cultures were isolated under 100, 150 and 200 mg Cr(VI)/L. Eighteen different morphologies were identified from anaerobic cultures (Table 2). Some of the bacteria were unculturable but produced a fingerprint during 16S rRNA analysis. Some were cultured but were marked as unidentified. Only 11 colonies from the anaerobic cultures were partially identified and seven colonies could not be amplified for partial gene sequencing.

Results indicated the predominance of eighteen anaerobic phenotypes. Partial sequences of 16S rRNA matched the seven *Bacillus* groups – *Bacillus drentensis*, *Bacillus* sp. *BS19*, *Bacillus* sp. *R21S*, *Oceanobacillus* sp. *JPLAk1*, *Paenibacillus pabuli*, *Paenibacillus xylanilyticus strain XIL14*, *Virgibacillus necropolis;* eight *Microbacterium* groups – *Acinetobacter* sp. *ANT9054*, *Arthrobacter* sp. *AK-1*, *Arthrobacter* sp. *Sphe3*, [*Brevibacterium*] *frigoritolerans*, *Rumen bacterium* R4-4, three uncultured *bacterium* groups – uncultured *bacterium clone Y2*, Uncultured soil *bacterium clone TA12*, and three *Enterococci* – *Enterococcus avium*, and *Enterococcus faecium strain R0026*, *Enterococcus pseudoavium* (Table 2).

A phylogenetic tree was also constructed for the anaerobic cultures using data generated through the BLAST search (Fig. 7). The anaerobic data showed a wider microbial diversity

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Table 3 – Comparison of Cr(VI) reduction between the indigenous culture and cultures isolated earlier

Culture type	Initial Cr(VI) concentration, mg/L	Culture Conditions		% Removal at 24 h	% Removal at 48 h
		Temperature, °C, O_2 requirement	Viable cell concentration, cells/mL		
Brits culture[a]	100	30 ± 1.0, Aerobic	5.20×10^9	97.9	100.0
Brits culture[b]	99	30 ± 1.0, Anaerobic	1.58×10^9	67.1	89.9
Pseudomonas fluorescens LB300[c]	90	30 ± 0.5, Aerobic	1.00×10^{10}	22.2	31.1
Bacillus sp.[d]	94	30 ± 0.5, Aerobic	9.80×10^9	7.5	20.2
Escherichia coli ATCC 33456[e]	120	30 ± 0.5, Anaerobic/micro-aerobic	9.00×10^9	7.5	16.7

a This study, grown aerobically.
b This study, grown anaerobically.
c Chirwa and Wang, 1997b.
d Chirwa and Wang, 1997a.
e Wang and Shen, 1997.

probably due to the partially anaerobic conditions in the aeration tanks at the Wastewater Treatment Plant from which the bacteria was originally collected.

3.7. *Performance evaluation*

3.7.1. *Comparison with previous isolates*

The dried sludge cultures from the Wastewater Treatment Works at Brits (NW) reduced Cr(VI) at higher concentrations and at a higher rate than known Cr(VI) reducing cultures including the pure cultures of *Bacillus* sp. [isolated from a Cr(VI) contaminated site in Newark (New Jersey) (Chirwa and Wang, 1997a)], *Pseudomonas fluorescens* LB300 [originally isolated from soil (Chirwa and Wang, 1997b)], and *Escherichia coli* ATCC 33456 [purchased (Wang and Shen, 1997)]. Comparison of Cr(VI) removal at 48 h incubation for 90–120 mg Cr(VI)/L cultures shows Cr(VI) removal rate in indigenous sludge culture approximately 3, 8, and 8 times higher than values observed in *P. fluorescens* LB300 (Chirwa and Wang, 1997b; Bopp and Ehrlich, 1988), *Bacillus* sp. (Chirwa and Wang, 1997a) and *E. coli* ATCC 33456 (Wang and Shen, 1997), respectively (Table 3).

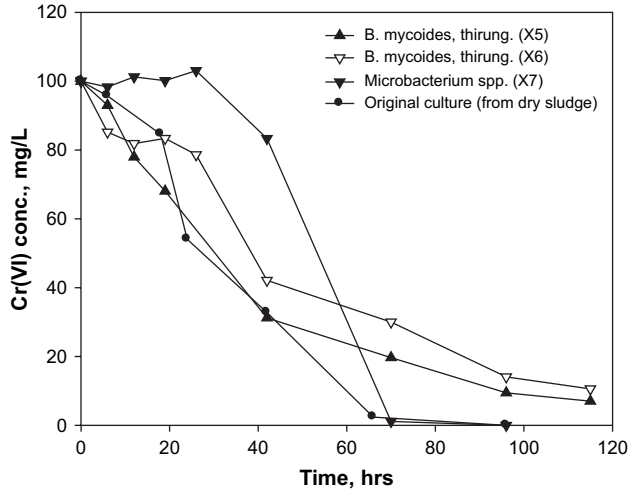

Fig. 8 – Cr(VI) reduction in individual isolates under aerobic conditions (resting cells: 3.3 ± 3.1 × 10^8 CFU/mL).

3.7.2. *Purified cultures versus consortium*

Cr(VI) reduction in pure cultures (X1–X7) was compared with the Cr(VI) reduction in the original consortium culture from the dried sludge. An example of the comparative data analysis is shown in Fig. 8 with the *B. mycoides/thuringiensis* (X5 and X6) culture and the *Microbacterium* sp. The preliminary analysis showed that the performance of different species matched that of the consortium culture at different times during incubation. For example, Cr(VI) reduction rate in the culture X5 was approximately equivalent to that of the consortium culture during the first 40 h of incubation after which, the removal rate was significantly slower. On the other hand, the

Table 4 – Comparison Cr(VI) reduction between the indigenous natural consortium culture, its isolated species and cultures isolated earlier

Culture type	Initial Cr(VI) concentration, mg/L	% Removal after 24 h
Natural Brit's consortium[a]	100	100
X1	100	56.8
X2	100	61.9
X3	100	59.7
X4	100	64.4
X5	100	69.98
X6	100	67.7
X7	100	38.1
X1 + X2 + X3 + X4[b]	100	91.8
X5 + X6[b]	100	84.6
X1 + X2 + X3 + X4 + X5 + X6 + X7[b] (All seven species)	100	94.3
Pseudomonas fluorescens LB300[c]	90	22.2
Bacillus sp.[c]	94	7.5
Escherichia coli ATCC 33456[d]	120	7.5

a Natural consortium from Brits.
b Recombination of isolates.
c Chirwa and Wang, 1997a,b.
d Wang and Shen, 1997.

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Cr(VI) removal in the *Microbacterium* culture (X7) was equivalent to the consortium culture removal after 75 h incubation. This shows that a fast performing culture may be more susceptible to Cr(VI) toxicity. Such a culture may act earlier in the presence of other more resilient slower performing cultures. This further indicates the importance of synergism for optimal performance of the culture as the main reason why individual cultures could not perform as well as the original mixed-culture consortium.

3.7.3. *Performance validation – reconstituted consortium*

Further analysis was conducted using individual isolates to determine the Cr(VI) reduction rate in purified cultures. The data in Table 4 confirm that no species acting alone achieved the same level of Cr(VI) reduction rate as the original consortium. Additionally, reconstituted cultures, e.g., X1 + X2 + X3 + X4, performed better than individual cultures. For example, X5 and X6 acting alone achieved 70.0 and 67.7% Cr(VI) removal in 24 h, but when grown together as a mixed-culture, they achieved 84.6% with the same time of incubation (24 h).

In the same table (Table 4), the fully reconstituted consortium from individual pure cultures (X1 + X2 + X3 + X4 + X5 + X6 + X7) showed 94.3 % Cr(VI) removal after 24 h and complete removal after 28.5 h (not shown). This indicates that some synergistic process occurred that resulted in higher performance of the mixed-culture. This also validates the capability of the CRB from the dried sludge.

One experiment conducted at an initial Cr(VI) concentration of 100 mg/L showed that addition of pure cultures X5 and X6 to the mixed-culture containing X1, X2, X3, and X4 improved the culture performance by 7.2% (i.e., 84.2–91.8 %) after incubation for 22.5 h. These experiments led us to the assumption that synergistic processes occurred between species that resulted in the higher performance of combined cultures.

4. Conclusion

The culture isolated from dried activated sludge from aeration tanks at the Water Treatment Works in Brits reduced Cr(VI) at higher concentrations and shorter incubation times than known cultures studied previously in 1994 and 1997. Characterisation using 16S rRNA fingerprinting yielded seven identifiable potential Cr(VI) reducing species in aerobic cultures. The independent species in the aerobic cultures were predominantly Gram-positive Bacilli. The individual colonies from anaerobic cultures were predominantly Gram-negative. Further characterisation under anaerobic conditions showed a more diverse culture with 18 species identified. The original consortium and a consortium reconstituted from individual isolates performed better than any of the species acting alone, suggesting possible existence of interspecies interactions necessary for optimum Cr(VI) removal in the original culture.

Acknowledgements

The research was funded by the National Research Foundation (NRF) of South Africa through the NRF Focus Areas Grant No. FA2006031900007 awarded to Prof Evans M.N. Chirwa of the University of Pretoria.

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