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Biological Uranium (VI) reduction by three facultative pure cultures from a soil consortium, a performance evaluation

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Aerobic isolates from a mine waste in Limpopo, South Africa reduced U(VI) to U(VI) and facilitated the removal of the uranium species from solution. Purified cultures showed a high reduction rate at pH 5 to 6. The initial U(VI) reduction rate determined at the 50% point was highest in the *Pseudomonas sp*. at 30 mg/L. *Enterobacter sp*. outperformed the other two species at 200 mg/L and 400 mg/L. Rapid reduction was observed in all cultures during the first 4-6 hours of incubation with equilibrium conditions obtained only after incubation for 24 hours. Preliminary studies suggest that uranium reduction occurs under anaerobic conditions. The U(VI) reduction was investigated further in a completely-mixed, continuous-flow under anaerobic conditions. Up to 60% removal was observed under an influent U(VI) feed concentration of 100 mg/L while operating under anaerobic conditions. The investigation under anaerobic CSTR conditions is still under way.

1. Introduction

Uranium in the environment occurs primarily as 3 of its 17 known isotopes, 238U (99.27%), 235U (0.72%), and 234U (0.005%). All are radioactive; however, it is the chemical toxicity that is of greatest ecological risk (Markich, 2002). Being the forty-ninth most abundant element in the Earth's crust, uranium is not uncommon. Unfortunately, the anthropogenic use of uranium for nuclear research, fuel production, and weapons manufacturing has resulted in extensive environmental contamination. Additional contamination has resulted from trace amounts of uranium being released from the combustion of coal as well as from the manufacture and application of phosphate fertilizers (Markich, 2002).

Uranium contamination of the environment from the mining and milling operations and nuclear waste disposal is a well-known global problem. Natural attenuation processes such as bacterial reductive/precipitation and immobilization of soluble uranium are gaining much interest (Francis and Dodge, 2003). For example, dissimilatory metal-reducing microorganisms have been investigated for their capability to selectively remove uranium from aqueous solutions (Lovley *et al*., 1992). These bacteria can use

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U(VI) as an electron acceptor thereby reducing soluble U(VI) to insoluble U(IV) (Lovley *et al*., 1992).

Biological processes have been proposaed as an alternative to physical-chemical treatment methods for removing metal-radionuclides from dilute solutions (Lovley *et al*., 1992). Furthermore, they biological methods can be employed *in situ* or *ex situ* methods. As one of the common processes, microbial reduction of soluble metals has been employed as first step towards removal of the metal (Khijniak *et al*., 2005). For example, mesophilic representatives of the genera *Geobacter*, *Shewanella*, and *Desulfotomaculum* have been shown to couple U(VI) reduction to growth, whereas *Desulfovibrio* species reduce U(VI) but do not attain energy to support growth from the process (Khijniak *et al*., 2005).

In the future, microbial U(VI) reduction may be engineered for the recovery of uranium and other heavy metals from spent nuclear fuel. Metal removal or recovery will help alleviate the toxic metal pollution problem in the environment (Kovacova and Sturdik, 2002).

2. Materials and Methods

2.1 Microbial source and characterization

Bacterial cultures were isolated from soil cultures grown anaerobically at 25-30°C under shaking at 120 rpm in a Rotary Environmental Shaker (Labotec, Gauteng, South Africa). The cultures were transferred to 100 mL serum bottles, purged with pure N_2 gas (99% pure grade) and then sealed with silicon rubber tubes and aluminium seals. Fully developed cultures were analysed using the 16S rDNA fingerprinting method as described by Chabalala and Chirwa (2009).

2.2 U(VI) reduction experiments

Pure cultures were grown in 100 mL nutrient broth in 250 mL Erlenmeyer flasks for 24 hours and then harvested by centrifugation at 6000 rpm (g) for 10 minutes in a Minispin® Microcentrifuge (Eppendorf, Hamburg, Germany). The pellet was washed 3 times with 0.85 % NaCl solution and then use for the experiments. A 10 % (v/v) of culture was used as inoculum and transferred into 100 mL serum bottles containing U(VI) prepared in BMM. The batches were purged for 5 minutes with 99.9% pure N_2. The batches were then incubated at 30°C under U(VI) concentrations ranging from 30 mg/L to 400 mg/L with agitation by shaking at 120 rpm on the orbital shaker (Labotec). Samples for spectrophotometric analysis were withdrawn at predetermined intervals. Cells were removed by centrifugation at 10000 rpm for 10 minutes in the Minispin® Microcentrifuge (Eppendorf).

2.3 Analytical methods

The oxidized fraction of uranium was measured from a sample (0.5 mL) of the homogenous solution was collected using a syringe and then centrifuged using a Minispin® Microcentrifuge (Eppendorf). The 0.5 mL sample was then diluted with 4.5 mL of BMM (1:10 dilution), mixed with 2 mL of complexing reagent, Arsenazo III (Sigma-Aldrich, St. Louis, MO) and analyzed for U^{6+} immediately using a UV/vis

spectrophotometer (WPA Lightwave II, Biochrom, Cambridge, England) using light with a wavelength of 651 nm (Chabalala and Chirwa, 2009).

3. Results and Discussion

3.1 U(VI) reduction under varying initial concentrations

U(VI) removal performance under varying initial concentration showed 85-100% removal of U(VI) after 24 hours under the tested conditions (Table 1). The highest removal efficiency was observed in batches between 200 and 400 mg/L initial U(VI) concentration. The *Enterobacter sp.* registered the highest uranium recovery percentage among the three isolates. *Pantoea sp*. and *Enterobacter sp*. displayed a gradual increase in rate of removal at 50% of added U(VI) as the concentration increased. Both cultures showed a high percentage removal at the end of 24 hours for all three concentrations. *Pantoea agglomerans,* a member of the family *Enterobacteriaceae* within the gamma subdivision of the *Proteobacteria,* has extensive metabolic capabilities under anaerobic conditions. It is a facultative anaerobic Fe(III) reducer capable of growing via the dissimilatory reduction of Fe(III), Mn(IV), and the toxic metal Cr(VI) (Tebo *et al*., 2000). And, BLAST and similarity analyses in literature indicated that some known U(VI)-reducing bacteria are 96.3% similar to the Gram-negative, facultative anaerobe *Enterobacter cloacae* (Lovley *et al*.; 2004).

Percentage recovery of total uranium in all cultures was very high for the higher concentrations (200- 400 mg/L). At 200 mg/L, all the species had removed all the uranium by 24 hours. By 48 hours, *Pseudomonas sp.,* a denitrifying bacteria that can use U(VI) as an electron acceptor and have been used to catalyze reduction of U(VI) in the presence of H_2 (Merroun and Selenska-Pobell, 2008) only managed to remove 89% of 400 mg/L U(VI), *Enterobacter sp.* 94% and the best performing specie was *Pantoea sp.* at 96%.

Table1 Kinetic data for varying concentrations of U(VI).

Pure Culture Species	**Initial concentration mg/L**	**Removal Rate at 50% mg/L/hr**	**U(VI) Removal at 24 hrs %**	**End Experiment Total U mg/L**	**Final Total U Recovery %**
Pseudomonas stutzeri	30	17	100	19	63
	200	33	81	174	87
	400	100	100	336	84
Pantoea sp.	30	16	100	15	50
	200	57	83	196	98
	400	111	100	283	71
Enterobacter sp.	30	9	100	10	33
	200	63	100	182	91
	400	198	85	355	89

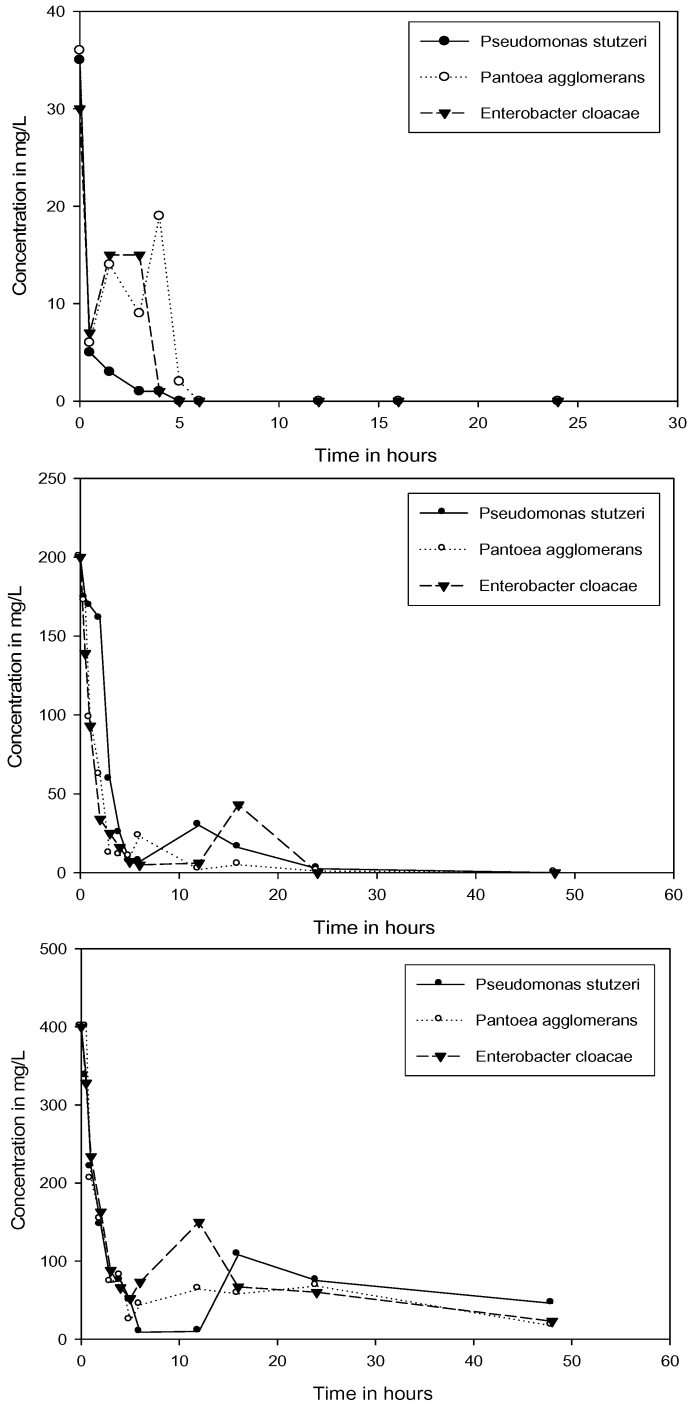

Figure 1. *Uranium(VI) reduction for the three pure cultures of bacteria Pseudomonas stutzeri, Pantoea agglomerans and Enterobacter cloacae under varying concentrations; A: 30 mg/L B: 200 mg/L C: 400 mg/L.*

Using the rate of removal at 50% added U(VI), overall all the cultures performed well at 400 mg/L. Generally for all species, the rate of removal/reduction of metal was very fast compared to those found in literature, and equilibrium was attained within 24 hours at pH of 5 to 6 compared to the 1mM U(VI) removed over 4 hours by *Desulfovibrio desulficans* suspended in bicarbonate buffer with lactate as the electron donor.

3.2 Continuous flow reactor performance

To quantify the U(VI)-reduction capacity of the mixed culture; *Pseudomonas sp. Pantoea sp*. and *Enterobacter sp*., continuous flow reactor experiments for U(VI) reduction were performed with repetitive U(VI) loadings as shown on figure 2. U(VI) was added to the MSM medium containing the mixed culture described above (with an initial concentration of U(VI) of 5 mg/L) and fed to the reactor. The figure below shows the removal of U(VI) through this continuous flow reactor process. In the presence of glucose, it can be seen that the mixed culture in the bioreactor reduced U(VI) steadily within the first 5 hours until the concentration approached zero. Results indicate that after loading the system with a higher concentration (10 mg/L), recovery only occurred after 90 hours. After the feed concentration was raised to 20 mg/L, the system did not react very quickly, only after 24 hours did the concentration peak at 15 mg/L, and thereafter, recovered after 20 hours. With the addition of 50 mg/L, only 23 mg/L was recorded 58 hours after addition. This shows that the culture kept on reducing steadily even when the concentrations were increased. When the feed concentration was increased to 100 mg/L, the system reacted almost immediately and a higher concentration was recorded, as the concentration rose, the rate of reduction became slower, until it reached a high of 135 mg/L and thereafter recovered within 14 hours and remained at 35 mg/L for the rest of the experiment. It was also observed that black precipitates associated with the culture were present after the continuous addition of U(VI). These precipitates were likely composed of reduced uranium U(IV).

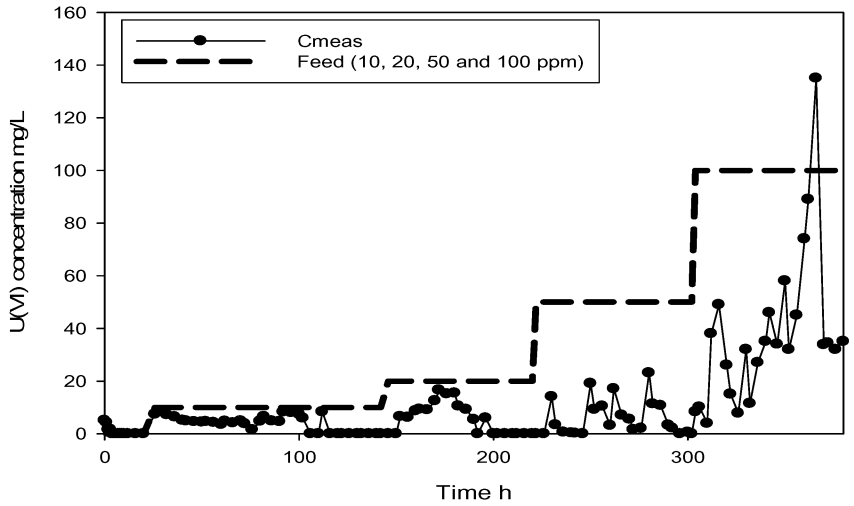

Figure 2. Continuous flow reactor performance for U(VI) reduction by mixed culture; Pseudomonas sp., Pantoea sp. and Enterobacter sp. The concentration of U(VI) in feed was 10, 20, 50 and 100 mg/L respectively.

4. Conclusion

The three pure cultures namely; *Pantoea sp*., *Enterobacter sp*. and *Pseudomonas stutzeri* showed potential to remove U(VI) from solution under anaerobic conditions with a pH ranging from 5 to 6. The mechanism used by these bacteria is not verified. The results suggest the extracellular enzymatic reduction of U(VI) to U(IV) may be the critical step in the removal of U(VI) from solution. The removal rate is metabolically linked with higher removal rates under favourable growth conditions for the three cultures. The total uranium concentrations indicate that there is an additional mechanism by which the three species reduce uranim-6. In two of the cultures, there was a high rate of removal at 50% added uranium as the concentration of uranium increased. To be of practical relevance for in situ biological reduction of metals, stimulated indigenous micro-organisms should remove metals including uranium for extended periods of time and the results presented here have strong implications of in situ biological reduction of U(VI) through the use of the bioreactor system.

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