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Biological U(VI) Reduction in a Fixed-bed Bioreactor Using Radiotoxic Tolerant Mixed-Culture of Bacteria

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Nuclear energy has been proposed as an alternative energy source in the bid to reduce carbon emissions that results from fossil fuel. Hexavalent uranium [U(VI)] is the most abandoned radioactive waste discharged from nuclear fuel processing, electricity generation power plants, radioisotope manufacturing plants. Improper disposal or storage of U(VI) containing waste may poses a threat to aquatic systems and related ecological systems. Treatment of U(VI) containing effluents from these industrial activities requires the reduction of highly mobile and radiotoxic U(VI) to tetravalent U(IV) which readily forms the hydroxide precipitate [$U(OH)_4(s)$] under neutral pH conditions. Processes employing biofilm and fixed-film systems for treatment of wastewater are considered more robust than planktonic culture systems in the presence of high toxicity, and therefore, are preferred in the treatment of toxic liquid waste. In this study U(VI) reduction is investigated using a fixed-film reactor inoculated with U(VI) reducing bacteria isolated from tailing dump soil collected from an abandoned uranium mine in South Africa. The fixed-film bioreactor was operated as a continuous flow reactor under oxygen stressed conditions without the addition of external organic carbon source. The results from this study showed that the fixed-bed bioreactor was able to achieve >90 % U(VI) removal efficiency at U(VI) concentration of up to 85 mg/L, concentration which is higher than that observed at the study site. This demonstrates the feasibility of the fixed-bed bioreactor in continuously removing U(VI) in aqueous solutions without the need of introducing external organic carbon source and without re-seeding the bioreactor. The bioremediation technology proposed in this study significantly demonstrated the effectiveness of the biofilm processes in treating process effluent streams and U(VI) contaminated sites as part of the pump-and treat. Fundamental understanding of uranium-bacteria interactions taking place in a complex biofilm structure under such conditions would be effective in developing an appropriate radioactive waste treatment system for the subsurface bioremediation process.

1. Introduction

Uranium is the most important energy mineral, which is found in the environment as a consequence of various pathways. Uranium and other toxic metals are present in the environment in various forms, such as oxides, organic or inorganic complexes and naturally occurring metal (Mtimunye and Chirwa, 2013). Uranium is a very reactive and therefore rarely exists in the environment in its elemental form. The oxygen bound uranium species that occur in the environment are uraninite (UO_2), uranium trioxide (UO_3), and triuranium octaoxide also known as pitchblende (U_3O_8) (Stefaniak et al., 2009). Triuranium octaoxide (U_3O_8) is the most thermodynamically stable uranium oxide species in the environment which is used for various commercial purposes.

Improper disposal of highly soluble and mobile U(VI) containing waste from various nuclear and industrial activities has led to increased outputs of hazardous uranium to the environment. Because U(VI) is known to be both radiotoxic and bioaccumulative, the release of U(VI) containing waste to the environment is a subject of intense public concern which has prompted researchers to investigate radioactive elements in the environment. The treatment of U(VI) containing waste using physico-chemical methods has been shown to be expensive, ineffective, and also generate secondary waste which is difficult to dispose. Biological methods have been proposed to improve or substitute the conventional physico-chemical methods for the remediation

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of U(VI) contaminated environments. Strategies suggested for the removal of metals and radionuclides using biological methods include biosorption, bioaccumulation, bioprecipitation, and bioreduction (Nedelkova, 2007; Chabalala and Chirwa, 2010).

Treatment of U(VI) containing wastewater using biological processes have been widely and successfully conducted in batch reactor systems under anaerobic conditions (Reed et al., 2007, Chabalala and Chirwa, 2010). Although batch studies were observed to be effective in treating U(VI) containing wastewater, treatment of large volumes of U(VI) containing wastewater from various industrial processes using batch systems could be very difficult.

This study evaluates the feasibility of a continuous flow fixed-film bioreactor in reducing U(VI) in contaminated wastewater. Biofilm systems have been observed to provide better treatment efficiency of wastewater streams due the high volumetric density of microorganisms accumulated in the presence of large surface area inducing biofilm resistance to environmental changes (Rittman and McCarthy, 2001; Kermani et al., 2008). The results from this study demostrated the potential of hetorogenous nature of the biofilm proesss in treatment and recovery of uranium from wastewater streams. The intergration of this bioremediation processes with other technolgies may be significant for developing well advanced remediation strategies for practical application.

2. Materials and Methods

2.1 Elemental analysis of soil

Uranium contaminated soil was collected from a closed uranium mine in Limpopo, South Africa. The sample was analysed using Inductively-Coupled Plasma–Mass Spectrometry (ICP-MS) (Spectro Arcos, FHS12, Boschstroisse, Germany) against the uranium atomic absorption standard solution (Sigma–Aldrich, St.97 Louis, Missouri, USA).

2.2 Culture and Media

U(VI) reducing bacteria was isolated from soil samples collected from tailing dumps of an abandoned uranium mine in Limpopo, South Africa. Background uranium concentration in the original samples was detected at levels as high as 29 mg/kg. Bacteria cultures were isolated from the soil samples using the enrichment culture technique. To isolate U(VI) tolerant species, a gram (1 g) of soil sample was added to 100 mL of sterile basal mineral medium (BMM) which was prepared by adding: 10 mM NH_4Cl, 30 mM Na_2HPO_4, 20 mM KH_2PO_4, 0.8 mM $Na2SO_4$, 0.2 mM $MgSO_4$, 50 µM $CaCl_2$, 0.1 µM $ZnCl_2$, 0.2 µM CuCl2, 0.1 µM NaBr, 0.05 µM Na_2MoO_2, 0.1 µM MnCl2, 0.1 µM KI, 0.2 µM H_3BO_3, 0.1 µM $CoCl_2$, and 0.1 µM $NiCl_2$ into 1 L of distilled water as according to Roslev et al. (1998). The medium was amended with D-glucose (5 g/L) as sole added carbon and energy source and 75 mg/L of U(VI). The inoculum was grown under anaerobic conditions for 24 h at 30±2 °C in 100 mL serum bottles purged with nitrogen gas (99.9 % N_2) and sealed with rubber stoppers and aluminium seals. After 24 h enriched bacterial strains were isolated by serial dilution. The isolated species were identified using 16S rRNA gene sequencing method as according to Jukes and Cantor (1969). 16S rRNA gene analysis showed phylotypes related to *Bacilli*, *Microbacterieceae*, *Arthrobacteriae*, and *Acinetobater* groups as shown in Table 1.

Table 1: Characterization of U(VI) reducing species using 16S rRNA

Pure Culture	NCBI Blast	Accession number	% Identity
Y1	*Kocuria turfanesis*	DQ531634	99 Actinomycetes from Micrococcaceae
Y3	*Arthrobacter creatinolyticus*	D88211	93
Y5	*Microbacterium aerolatum*	AJ309929	100
Y6	*Bacillus licheniformis*	CP000002	100
Y7	*Bacillus altitudinis*	AF234854	100
Y8	*Anthrobacter sulfonivorans*	AF235091	100
Y9	*Acinetobacter baumanii*	X81660	100
Y10	*Chryseobacterium indoltheticum*	AY468448	100
Y11	*Bacillus pumilus*	AJ831841	100

2.3 Reactor setup

Two packed-bed reactors constructed from Plexiglas (PVC glass) tubes (1 m long, 0.1 m internal diameter) were used for this study. One reactor was used as a control while the other reactor was operated as a biofilm reactor. The reactors consisted of influent and effluent ports and four equally spaced intermediate sampling ports with bed heights of (0.2 m, 0.4 m, 0.6 m, and 0.8 m). The columns were packed with plastic media. One column was operated as a biotic reactor (R1) while the other column was operated as a control (abiotic reactor) (R2). The packing material in R1 served as contact medium for the attachment of U(VI) reducing species. Provision was made for biomass analysis through sealable holes on PVC caps placed on the top end of each column. The packed columns were installed vertically in a room with temperature set at 30±2 °C (Figure 1). The pore volume which represents the total reaction volume was determined from the difference between the weight of the saturated column with packing material and the weight of a dry fully packed column using the density of water occupying the pore spaces. The reactors were operated in an up flow mode using peristaltic pump.

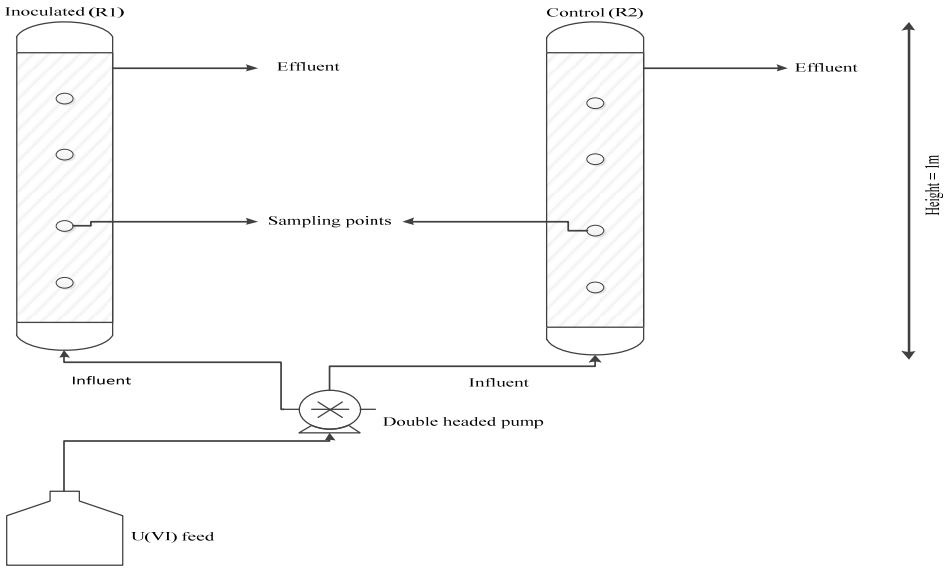

Figure 1: Continuous-flow system laboratory se

2.4 Start-up and operating of biofilm reactor

The potential of each isolated culture (Table 1) in tolerating and reducing U(VI) as individual species was evaluated in batch reactors. Preliminary batch studies showed that the species (Y1, Y3, Y5, and Y6) were able to reduce up >85 % of U(VI) at 75 mg/L as individual pure isolates within 48 hours of operation (Results not shown). These species were further used as stat-up culture for continuous-flow studies. The species were initially grown as a mixed-culture in a batch reactor supplemented with nutrient broth under anaerobic conditions at 30±2 ºC. The above batch of viable cells was harvested at log growth phase with a viable concentration of approximately 10^8 cells/mL. The harvested cells were re-suspended in sterile BMM amended with D-glucose as a carbon and energy source and then inoculated into the fixed-bed reactor in batch mode with 100 % recirculation using a pre-calibrated peristaltic pump. The reactor was operated in the batch mode until biofilm was visible on the surface of the reactor packings. Biological activity in the biofilm reactor was also confirmed by the gas bubbles observed in the seal pot connected to the reactor. Inductively-Coupled Plasma–Mass Spectrometry (ICP-MS) analysis of the leachate revealed varying levels of major elements such as aluminium, calcium, magnesium, iron, potassium, phosphorus, sodium and toxic metals such as manganese, zinc, cadmium, nickel, boron, and uranium. The leachate contained (29 mg/kg ≈ 72 mg/L) of uranium. It was observed from this analysis that most the components present in the soil leachate are also present in BMM in amounts above the limit concentrations for microbial growth. Therefore, a 10 %v/v dilution of BMM stock solution was used to simulate U(VI) leachate water for continuous flow studies. The simulated U(VI) containing water with various initial U(VI) concentration was simultaneously fed in each fixed-bed reactor from the bottom inlet through a peristaltic pump to maintain a hydraulic retention time (HRT) of approximately 24 h. The pH in the reactors was kept at 6.5±0.5 by HPO_4^{2-}/$H2PO_4^-$ present in the BMM. The experiments were conducted

under oxygen stressed conditions without re-seeding and without the addition of external organic carbon source. Each experiment was allowed to continue until the operation approaches a stable state condition with regards to effluent U(VI) concentration.

2.5 Data generation

Samples were collected across the reactor at four sampling ports and analysed for pH, biomass activity, U(VI) and total uranium concentration.

2.5.1 Determination of U(VI) and total uranium

U(VI) reduction rate was determined by measuring the decrease in U(VI) in the solution using UV/Vis spectrophotometer (WPA, Light Wave II, and Labotech, South Africa). Arsenazo III (1, 8-dihydroxynaphthalene-3, 6 disulphonic acid-2, 7-bis [(azo-2)-phenylarsonic acid]), a non-specific chromogenic reagent, was selected as the complexing agent for facilitating U(VI) detection. Measurement of U(VI) was carried out by sampling 2 mL of solution from each sampling port in the reactor using disposable syringes. The withdrawn samples were then centrifuged at 6000 rpm (2820 g) for 10 minutes using Minispin-Microcentrifuge (Eppendorf, Hamburg, Germany). The centrifuged sample was then analysed in UV/ vis spectrophotometer at the wavelength of 651 nm using Arsenazo III method (Bhatti et. al., 1991).
For measurement of total uranium (Total U) sample was withdrawn from the reactor and digested with 2 M HNO_3. The digested sample was then allowed to stand for about 5 minutes to achieve complete reaction before analysis. Total uranium was then measured using Inductively-Coupled Plasma–Mass Spectrometry (ICP-MS) (Spectro Arcos, FHS12, Boschstroisse, Germany) which was previously calibrated against the uranium atomic adsorption standard solution following the method previously developed by Chabalala and Chirwa (2010).

2.5.2 Biomass Analysis

Samples for viable cell analysis were extracted from the biofilm column using sterile tweezers. The sampled packings were initially weighed and then placed into a 9 mL sterile buffered Ringer's solution which was prepared by dissolving 2 Ringer's tablets into 1 L distilled water as per manufacture instruction (Merck, Johannesburg, South Africa). The packing material was washed thoroughly by agitation in 0.85 % NaCl solution for three cycles until adequate detachment of attached biomass from the packing media was achieved. The washing solution was then serially diluted and 1.0 mL of contents of each test tube was platted on Luria-Bettani agar to determine cell count. The plates were then incubated for 48 h at 30±2 °C. The number of colonies were then counted and multiplied by a dilution factor. The bacterial count was reported as colony forming units (CFU) per mL of sample. A conversion factor of 1.766×10^{-10} mg/cell was determined (with R^2 = 0.998) using the method previously derived by Molokwane et al. (2008).

3. Results and Discussion

3.1 Performance of the continuous flow reactors

U(VI) removal efficiency of 94 % at the loading treatment of 75 mg/L was achieved in the biofilm reactor within 29 d of operation. Operating the biofilm reactor at high loading rate of 85 mg/L showed improved U(VI) removal rate with the overall removal efficiency of up to 98 % within 13 d of operation (Figure 2a). The improved U(VI) removal rates observed at the loading treatment of 85 mg/L may be attributed to the improvement of the biofilm system over time when certain favourable conditions were sustained. Abiotic (cell-free) reactor showed insignificant U(VI) removal efficiency of <10 % in both tested concentrations, suggesting the potential of isolated species in tolerating and reducing U(VI).
Total uranium which was measured at the end of each experimental run was determined as the sum of U(VI) and U(IV) in the effluent. The results in Figure 2b show high total uranium (Total U) and U(IV) concentration in the effluent when compared to effluent U(VI) concentration, suggesting biological U(VI) reduction to U(IV) in the biofilm reactor. Correlation between U(VI) removal efficiency and effluent U(IV) concentration at the end of each experimental run was evaluated (results not shown). Results showed negative correlation between U(VI) removal efficiency and U(IV) concentration in the effluent, implying that the effluent U(IV) concentration was decreasing with increasing U(VI) removal efficiency. Lower effluent U(IV) concentration achieved at the end of each experimental run, suggest that most of the reduced uranium species were trapped on the packing media and therefore could not be detected in the final effluent. Results in Table 2 show the overall performance of the biotic and biotic reactor.

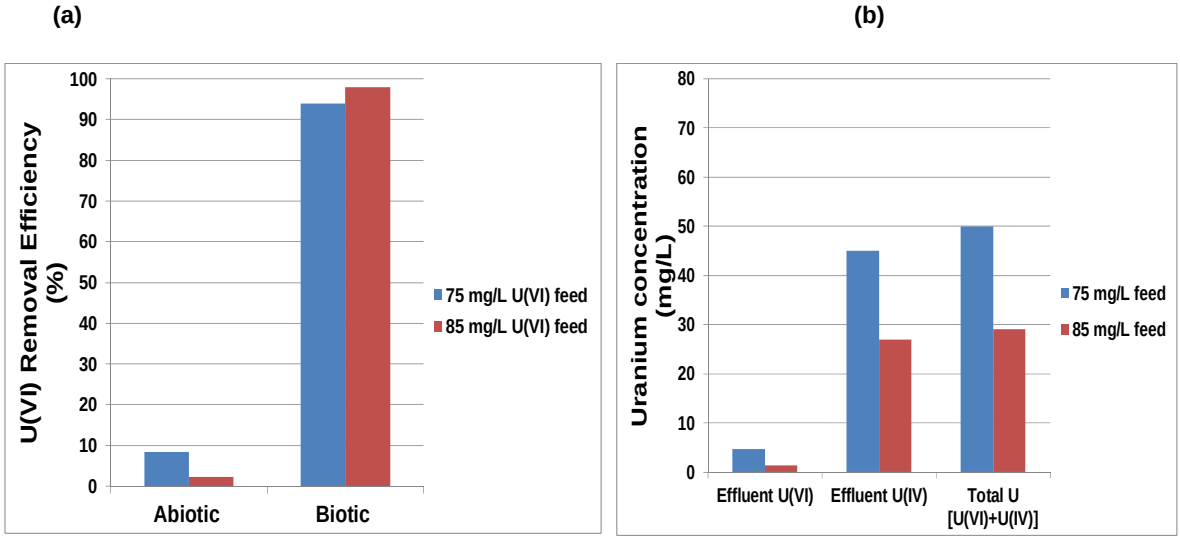

Figure 1: (a) U(VI) removal efficiency at the initial U(VI) concentration of 75 mg/L and 85 mg/Lin a abiotic (cell-free) and biotic and abiotic reactor, (b) Uranium balance [U(VI), U(IV) and total uranium] in the effluent of the biofilm reactor after each experimental run.

Table 2: Performance of the biofilm reactor after each experimental run

Influent U(VI) concentration (mg/L)	Duration (days)	Average Effluent U(VI) (mg/L)	Average Effluent U(IV) (mg/L)	Average U(VI) Removal Efficiency (%)	Total Uranium (Total U) (mg/L)
75	29	4.6	45	94	50.8
85	13	1.4	27	98	29.04

3.2 Biomass Yield

The results in Table 3 showed that the activity of cells in the bioreactor is dependent on the cell-metal interactions within the system. Table 3 shows high microbial activity at high U(VI) removal efficiency . The high microbial activity observed over time when the biofilm reactor was operated at various U(VI) loading rates, demonstrated the effectiveness of the species isolated in this study in biocatalytically reducing U(VI) under anaerobic conditions at near neutral pH.

Table 3: Microbial activity within the biofilm reactor

Influent U(VI) concentration (mg/L)	Initial biomass (cells/mL)	Biomass after operation (cells/mL)	Average U(VI) Removal (%)
75	$40x10^7$	$43x10^7$	94
85	$43x10^7$	$48x10^7$	98

4. Conclusions

Continuous-flow bioreactor systems have the potential of treating U(VI) contaminated wastewater at much higher volumes. The results from this study showed the ability of the fixed-bed biofilm reactor in sustaining high U(VI) concentrations of up to 85 mg/L, much higher than that observed at the study site. The treatment of U(VI) contaminated water at various concentrations in the biofilm reactor without re-seeding the reactor demonstrated the robustness of the biofilm reactor in treating high volumes of wastewater contaminated with

toxic metals. The reduced uranium precipitate observed on the biofilm matrix over time demonstrated the potential of uranium recovery on the packing media, through various techniques. The results from this study could be effective in designing a pilot scale reactor that could effectively improve the performance of the biofilm reactor in continuously reducing U(VI) from process water effluent streams.

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