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Biological U(VI) Reduction in a Fixed-film Reactor Using Indigenous Culture Under Shock Loading Conditions

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Uranium (VI) contamination in the environment has become a global problem in aquifers, water supplies and other related ecosystems. Bioremediation has gained importance as a feasible and eco-friendly strategy in remediating uranium contaminated environments. This study investigates biological U(VI) removal in a bench-scale, fixed-film bioreactor using a mixed-culture of bacteria sourced from an abandoned uranium mine in Limpopo, South Africa. Preliminary batch kinetic studies showed that these species have the potential to effectively reduce U(VI) in aqueous solutions by means of more than one mechanism. The bench-scale bioreactor was operated as a continuous flow system under a range of influent U(VI) concentrations (75-100 mg/L) without any added external organic carbon source. Near complete U(VI) removal was achieved in a biofilm reactor operated at influent feed concentrations of 75 mg/L and 85 mg/L. The extent of abiotic U(VI) reduction was also evaluated in this study using cell-free reactor. Results showed insignificant U(VI) removal in a cell-free rector, demonstrating the feasibility of the microbial species used in this study in reducing or immobilizing U(VI) in contaminated wastewater. The proposed bioremediation technology in this study could be applicable to end pipe *ex situ* bioremediation. The results from this study demostrated the possibility of combining biological processes with non-biological processes for effective treatment and recovery of uranium from U(VI) contaminated wastewater streams.

1. Introduction

Uranium is the most important energy mineral, which is found in the environment as a consequence of various pathways which includes earth crust layer, anthropogenic activities, nuclear power accidents, nuclear weapon testing, and others. Uranium exist in several oxidation states U(III), U(IV), U(V), and U(VI) of which U(IV) and U(VI) are the most stable in the environment. The highly soluble U(IV) ion always exist as UO_2^{2+} (uranyl), while the sparingly soluble U(IV) exist as UO_2 in aqueous systems. The existence of either of these uranium species in tailings dumps is dependent on the pH and redox conditions at the site. However, keeping uranium in the dump sites as U(IV) is more desirable since U(VI) is highly toxic and has the ability to leach into surrounding water bodies causing water pollution.

Because U(VI) is known to be both toxic and bioaccumulative efforts have been made in developing methods that remediate U(VI)-contaminated wastewater systems (Sivaswamy et al., 2011). The most commonly used technology for treating U(VI) contaminated wastewaters involves utilization of chemical reagents such as organic or inorganic acids to chemically reduce the pollutant. The methods associated with chemical precipitation have been shown to be cost intensive and ineffective particularly at lower metal concentration (Martins et al., 2010).

The use of microbial agents such as bacteria has gained importance as a feasible and eco-friendly strategy in remediating uranium contaminated environments (Ahemad, 2012). Microorganisms have the potential to interact with metals and radionuclides altering their physical and chemical state through different mechanisms such as biosorption, bioaccumulation, bioprecipitation, and bioreduction (Mtimunye and Chirwa, 2014). Biological U(VI) reduction studies have been widely and successfully conducted in batch reactor systems, such as closed and sealed serum bottles (Chabalala and Chirwa, 2010). Although batch studies were observed to be effective in treating U(VI) in aqueous solutions, the results obtained from batch kinetics studies could not be effective in addressing the problem of U(VI) contamination in practical systems that

receives feeds with various concentrations . Processes employing biofilm 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 (Kiranmai et al., 2005).
This study contributes the knowledge on U(VI) reduction under various U(VI) loadings employing a biofilm system which is operated without biostimulation. The biological treatment technology proposed in this study can be employed for treatment of U(VI) contaminated wastewater streams and decontamination of U(VI) contaminated sites as part of pump-and-treat processes. The intergration of bioremediation processes with non-biological processes may be significant for developing well advanced remediation strategies for practical application.

2. Materials and Methods

2.1. Sample collection and characterization

The soil samples were collected from the tailing dumps of the closed uranium mine in Phalaborwa, South Africa. Metallic elements of the soil samples were characterised using Inductively-Coupled Plasma-Mass Spectrometry (ICP-MS) Spectromass 2000 (Spectro Analytical Instruments, Kleve, Germany). Background uranium concentration in the samples was detected at levels as high as 29 mg/kg (72 mg/L) much higher than the values observed in natural soils (0.3-11.7 mg/kg) (UNSCEAR, 1993). Therefore the bacteria in the soil were thus expected to be acclimatized to high uranium exposure conditions.

2.2. Bioreactor set-up

Polyvinyl chloride (PVC) columns (1 m long, 0.1 m internal diameter) were installed in a laboratory with an empty bed volume of approximately 7.8 L. Each column consisted of an influent port, four equally space intermediate sampling ports and the effluent port as shown in Figure 1. The two columns were packed with plastic media, filled with water and then allowed to stand overnight to saturate and stabilize the matrix. The water in the columns was drained to determine the pore volume. Flow rates were also measured and adjusted to establish the hydraulic retention time (HRT) of approximately 24 h in each reactor.

2.3. Start-up culture

Individual pure colonies isolated from the soil sample were characterized in terms of their tolerance to oxygen and uranium toxicity using 16S rRNA genotype fingerprinting analysis as according to Jukes and Cantor (1969). Batch kinetic studies were conducted to evaluate the performance of each isolated species in reducing U(VI) as an individual species. The species that effectively reduced U(VI) up to 75 mg/L as individual species in batch kinetic studies were grown as a mixed-culture and used as a start-up culture in the bioreactor. Table 1 below summarizes the kinetic data of tested pure isolates that were able to effectively reduce and tolerate U(VI) toxicity at 75 mg/L, target uranium concentration at the study site.

Table 1: Summary of the performance of each individual species at U(VI) concentrations of 75 mg/L

Pure Culture	NCBI Blast	Removal Efficiency after operation (%)
Y1	*Kocuria turfanesis*	95.4
Y3	*Arthrobacter creatinolyticus*	88.8
Y5	*Microbacterium aerolatum*	92.5
Y6	*Bacillus licheniformis*	86

2.4. Bioreactor start-up and operation

Two laboratory scale bioreactors R1 and R2 were operated in a continuous up flow mode under reducing conditions. One reactor (R1) was seeded with viable cells solution of 10^8 cells/mL while the other column (R2) was operated as a control (cell-free). The seeding material was pumped at low speed of 0.1 L/h in the biofilm column to allow adherence of the cells to the matrix, the columns were allowed to stand for 24 h without any disturbance. During the experimental run, sterile basal mineral medium (BMM) and U(VI) solution of specific or target concentration ranging from (75-100 mg/L) was continuously prepared in batches of 25 L and simultaneously fed into the two reactors operated under oxygen stressed conditions in the absence of any external organic carbon source. The BMM was prepared as according to Roslev et al. (1998).The pH in the reactors was kept at 6.5±0.5 by $HPO_4^{2-}/H_2PO_4^-$ present in the BMM.

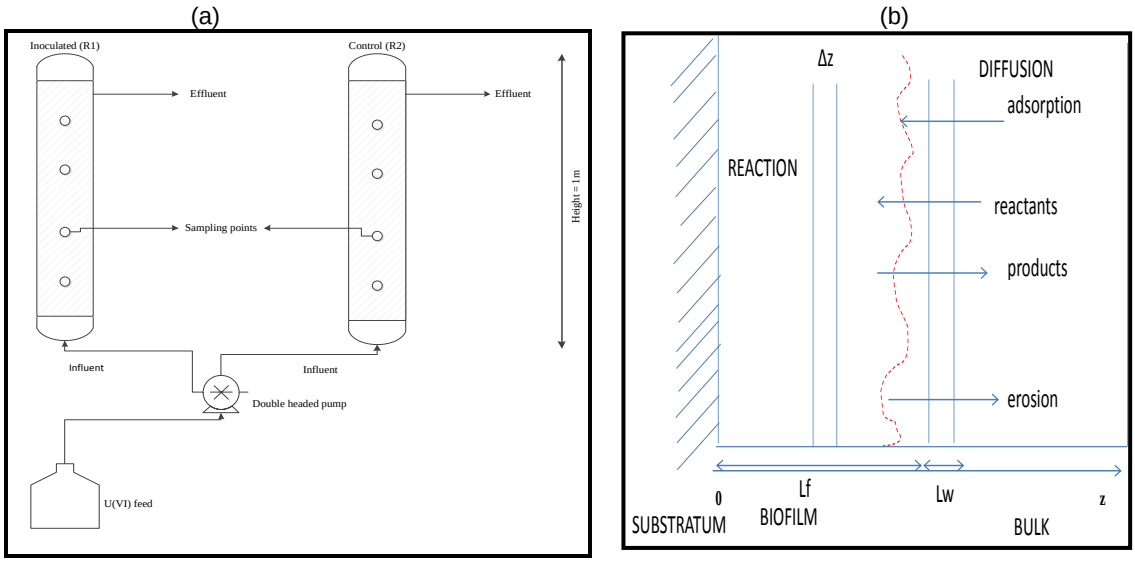

Figure 1: (a) Laboratory set-up of a fixed-media continuous flow reactor (b) Conceptual biofilm model.

2.5. Sampling and analysis

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 [Bhatti et al., 1991]. Measurement of U(VI) was carried out by sampling 2 mL of solution from each sampling port in the reactors using disposable syringes. The withdrawn samples were then centrifuged at 6000 rpm (2820 g) for 10 minutes using Minispin-Microcentrifuge. The centrifuged sample (0.5-1 mL) was then diluted with 0.4 mL of 2.5 % diethylene-triaminepenta acetic acid (DTPA) and then diluted up to mark with BMM in a 10 mL volumetric flask. The homogenous solution was the mixed with 2 mL of complexing reagent (Arsenazo III), allowed to stand for full colour development prior analysis for U(VI) at 651 nm. Samples were taken from the effluent port for the immediate measurement of physical parameters such as oxidation reduction potential (ORP), pH and temperature using ORP/pH probe (pHC101, MTC101, Hach, USA). The experiments were conducted at 30 ± 2 ºC.

2.6. Biomass analysis

A provision was made to analyse the biomass at one end point of the reactor. This was due to the difficulty of sampling the media across the entire reactor which was designed to operate continuously under oxygen deprived conditions. Samples for attached biomass measurement were obtained from an allocated point in the biofilm reactor (R1) using sterile tweezers. The pre-weight sample media was placed into a 9 mL sterile buffered solution (Ringer's solution) solution which was prepared by dissolving 2 Ringer's tables into 1 L distilled water as per manufacture instruction (Merck, Johannesburg, South Africa). The solution was then agitated gently for 30 minutes to dislodge most of the microbes without destroying them. The supernatant was serially diluted up to 10 times dilution factor. From each tube, 0.1 mL of the solution was transferred into the agar plate using a spread method. This was done in triplicate for each dilution to have statistical representivity. The plates were then incubated at 30 ± 2°C and the number of colonies were counted and multiplied by a dilution factor. The bacterial count was reported as colony forming units (CFU) per mL of sample.

Surface morphology of the culture attached to the support material was evaluated before treating U(VI) in a bioreactor and after 99 days of biofilm exposure to various U(VI) concentrations using Scanning electron microscopy (SEM) (Joel, JSM-5800LV). Samples for attached biomass measurement were obtained from the biofilm reactor (R1) using sterile tweezers. The biofilm on the support material was fixed in a 2.5 % glutaradehyde in 0.1 M phosphate buffer (pH 7.0) solution. The fixative solution was decanted off and cells attached to the support material were then washed in a phosphate buffer, prior dehydrating in a series of ethanol solutions (30 %, 50 %, 70 %, 80 %, and 90 %). Samples were dried in liquid CO_2 and the mounted on stubs with double sided tape, coated with gold, and the observed under SEM.

3. Results and Discussion

3.1. Biotic U(VI) reduction profile

U(VI) removal across the fixed-bed reactor was evaluated using data collected from equally spaced longitudinal sampling ports. Figure 2a demonstrate that at the initial U(VI) feed concentration of 75 mg/L, U(VI) removal was notably higher at the first sampling port from the bottom of the reactor (port 1, h= 0.2 m) than in (port 2, h= 0.4 m). Higher U(VI) removal observed in port 1 may be due to (i) the possibility of high accumulation of biomass at the bottom part of the reactor as the cells were inoculated in the reactor in the up-flow mode, and/or (ii) delayed response of the reactor to the feed concentration. The system achieved an average U(VI) removal efficiency of 94 % at the loading treatment of 75 mg/L.

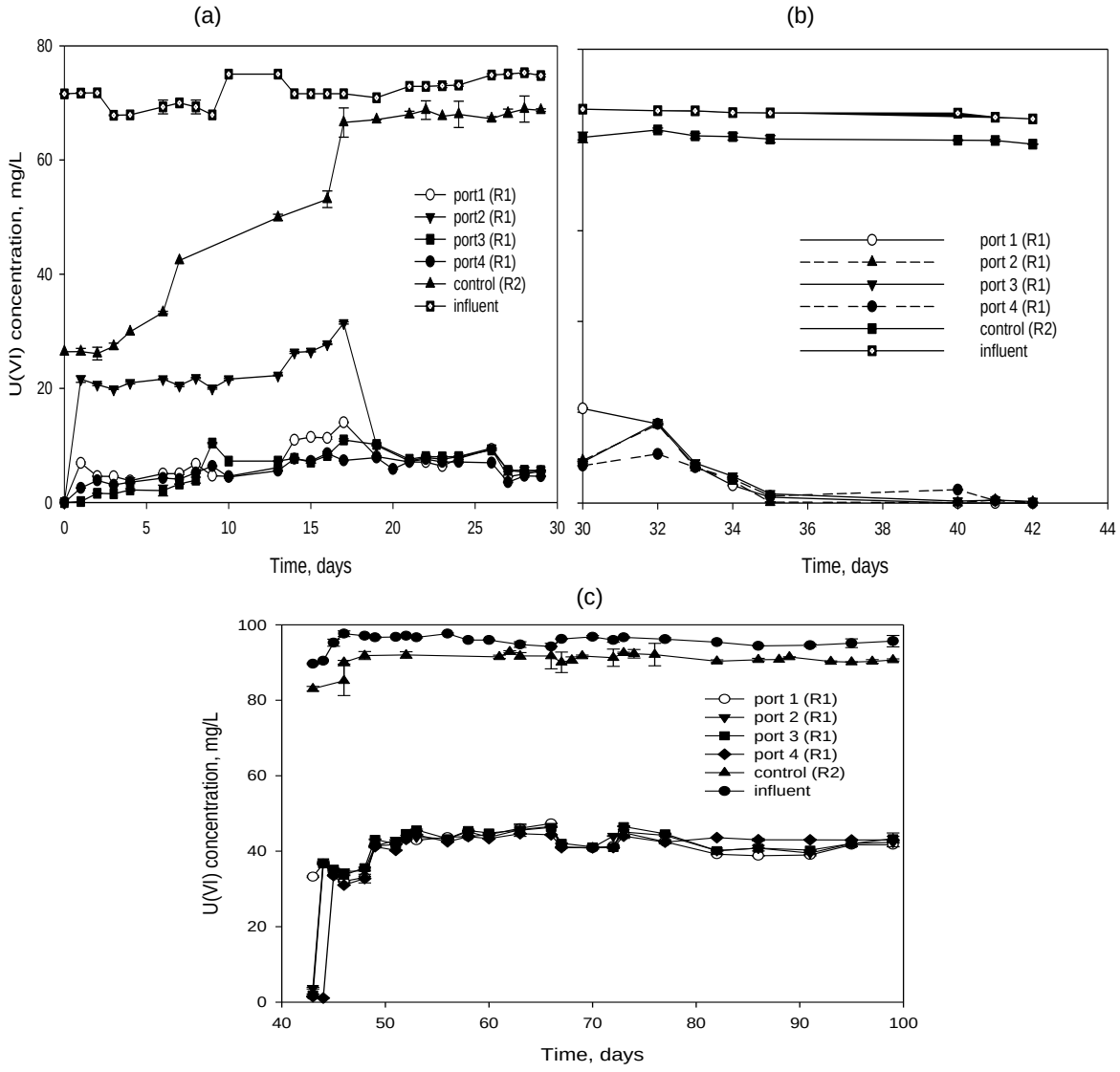

Figure 2: U(VI) removal across the biofilm reactor at (a) 75 mg/L, (b) 85 mg/L, and (c) 100 mg/L.

Operating the biofilm reactor at the higher U(VI) feed concentration of 85 mg/L showed higher rate of U(VI) removal across the column Figure 2b. 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 favorable conditions were sustained. The system achieved an average U(VI) removal efficiency of 98 % at the loading treatment of 85 mg/L.The U(VI) reduction profile observed in Figure 2a and Figure 2b showed a good correlation with the ORP of the solution which indicated reducing conditions in the system (data not shown). Further increase of U(VI) feed concentration up to 100 mg/L resulted to near constant U(VI) removal across the bioreactor Figure 2c. The system achieved an average U(VI) removal efficiency of 60 % at the loading treatment of 100 mg/L.

The decrease rate of U(VI) reduction observed after 42 days of operation was associated with mass transfer resistance across the biofilm layer that resulted from precipitate built-up as U(VI) was reduced.
The insignificant U(VI) removal across the cell-free reactor observed in Figure 2a-c demonstrated the effectiveness of indigenous species isolated in the study in tolerating and reducing high levels of U(VI) in an aqueous solution. The cell-free system achieved an average of 9 % U(VI) removal efficiency at the loading treatment of 100 mg/L. U(VI) removal efficiency achieved in a cell-free system may be attributed to complexation of the uranium species with minerals present in the BMM.

3.2 Biomass yield

The growth curve in Figure 3 shows slight decrease in attached cells population number within the first 7 days of operation which may be attributed to initial exposure of cells to U(VI). The exponential rise observed between 15-42 days of biofilm operation was attributed to cell defence mechanism such as cell acclimation to U(VI) shock loading conditions. The characteristic of flattened growth curve observed after 42 days of operation suggests maximum attainable cell growth which is associated with precipitate built-up onto the matrix which resulted to limited cell-metal interactions and decrease in matrix surface area available for cell growth. Energy-Dispersive X-ray spectroscopy (EDX) analysis showed that the precipitate formed within the biofilm reactor constituents mainly phosphates and uranium (data not shown).
Scanning electron microscopy of the support media before and after operating the bioreactor for 99 days at various U(VI) concentrations is shown in Figure 4. Figure 4a shows the evidence of biofilm formation onto the matrix prior feeding simulated U(VI) containing wastewater into the bioreactor. After operating the column for 99 days in the presence of U(VI), the biofilm formation onto the matrix was not observed (Figure 4b). The unclear visibility of the biofilm after column operation may be associated with the formation of precipitate onto the matrix that accumulated over time.

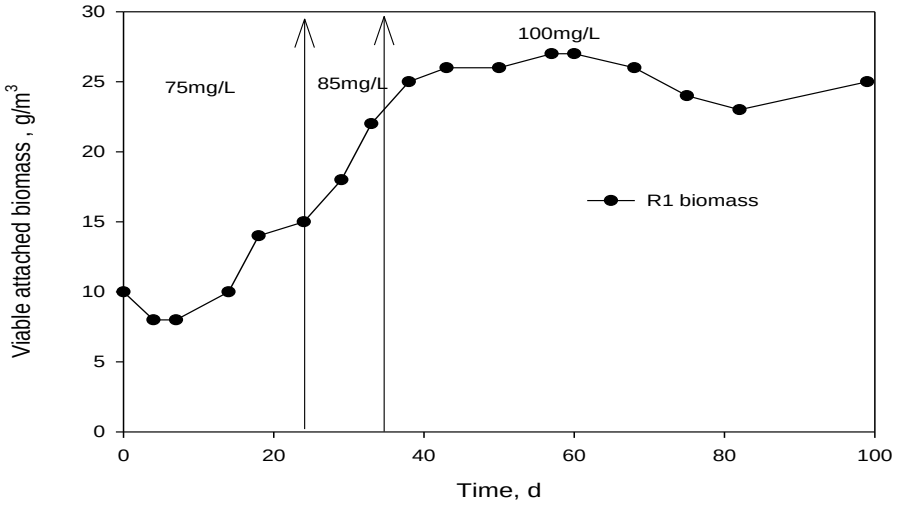

Figure 3: Evaluation of biomass yield within the biofilm reactor.

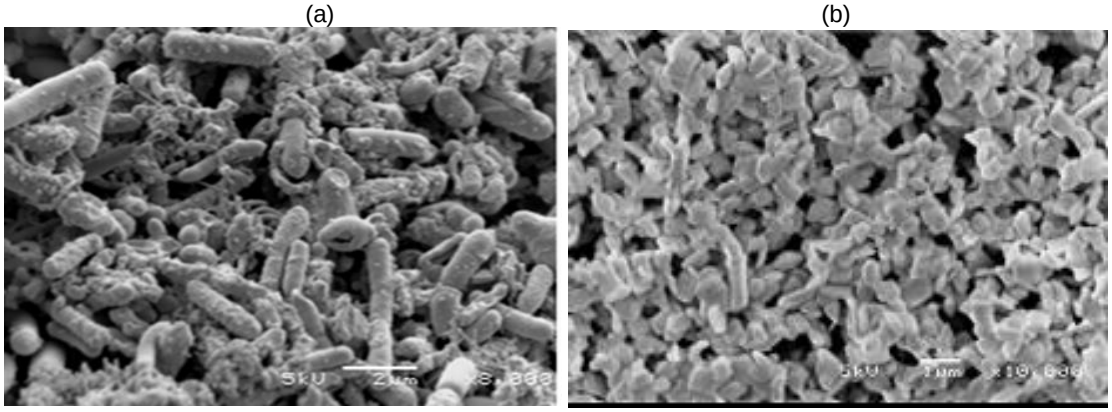

Figure 4: SEM analyses of the column (a) after seeding with viable cell solution (b) after 99 days of continuous exposure with various U(VI) feed concentrations.

4. Conclusions

Successful U(VI) reduction was achieved over the operational period of the bioreactor. Therefore, it can be concluded that the indigenous bacteria grown as a biofilm were able to effectively stabilize U(VI) at various U(VI) feed concentrations without biostimulation. Although, results presented in this study have strong implications of biological U(VI) reduction ex-situ through the use of the bioreactor system, these results could also be effective in optimizing and improving the operation and performance of in situ bioremediation of U(VI) at target site. Further studies are required to understand the interaction of bacteria with other radionuclides that co-exist with uranium in the environment and also to evaluate effect of operating the packed-bed reactor under various HRT's while occasionally backwashing or dislodging the accumulated precipitate from the system.

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