Removal of Uranium(VI) under Aerobic and Anaerobic Conditions using an Indigenous Mine Consortium

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ABSTRACT

Biological uranium removal was investigated using bacteria sourced from a uranium mine in Limpopo, South Africa. Background uranium concentration in the soil from the mine was determined to be 168.1 mg/kg using the ICP-OES calibrated against the uranium atomic absorption standard solution. Thus the bacteria isolated from the site were expected to be resistant to uranium-6 [U(VI)] toxicity. Preliminary studies suggest that uranium reduction occurs under anaerobic conditions in most cases. U(VI) reduction by obligate aerobes isolated from the soil consortium was poor. The pure cultures mentioned above 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. However, *Enterobacter sp.* proved to be the most efficient U(VI) reducer among the cultures with the highest U(VI) reduction rate observed at high concentrations, 200 mg/L and 400 mg/L. A high percentage of uranium recovery occurred at these concentrations. The results demonstrate the potential of microbial U(VI) reduction as an alternative technology to currently used physical/chemical processes for treatment and recovery of uranium in the nuclear industry.

Keywords: Uranium(VI) reduction, biosorption, indigenous culture, high-level waste bioremediation.

1. INTRODUCTION

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, 2008). 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 U(VI) as an electron acceptor thereby reducing the highly mobile and toxic U(VI) to U(IV) which is less toxic and easier to remove from solution by precipitation (Lovley *et al*., 1992). Much research has been dedicated to exploring the mechanism of metal reduction in bacteria. However, the reaction kinetics that would help illucidate the underlying processes have not been researched sufficiently. 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 *et al*., 2002).

Biological treatment of metal pollutants is viewed as an environmentally friendly alternative to conventional physical/chemical treatment methods, especially in dilute solutions where physical/chemical methods may not be effective (Lovley *et al*., 1992). Microbial processes may be applied both as *in situ* and/or *ex situ* processes. Microbial consortia, consisting of several species of microorganisms in the form of bioflocs for removing/degrading the pollutants have been used as they preserve the complex interrelationships that exist between species in source (Nancharaiah *et al*., 2006). So

far, there are four suggested mechanisms by which bacteria may immobilize metals or radionuclides namely; (a) biosorption, (b) bioaccumulation, (c) precipitation by reaction with inorganic ligands such as phosphate and (d) microbial reduction of soluble metal ions to the insoluble elemental forms (Nancharaiah *et al*., 2006). The fourth process has been observed in Fe(III)-reducing and sulphate-reducing bacteria (Khijniak *et al*., 2005). For example, some of the metal reduction activities in bacteria have been shown to be cometabolic (with no energy derived from the reaction) whereas some reduction processes contribute metabolic energy to cell growth and maintenance (Chirwa and Wang, 2000). Mesophilic representatives of the genera *Geobacter*, *Shewanella*, and *Desulfotomaculum* are also known 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).

A fundamental understanding of mechanisms of microbial transformation of uranium under a variety of environmental conditions will be valuable in developing appropriate remediation and waste management strategies as well as predicting the microbial impacts on the long-term stewardship of contaminated sites. The aim of this study is to utilize indigenous cultures of bacteria from the local environment to biologically reduce U(VI)–U(IV) and the objective was to investigate the ability of the three pure cultures; *Pseudomonas* sp., *Enterobacter* sp. and *Pantoea* sp. as well as mixed culture to reduce U(VI) at various concentrations.

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 analyzed by ICP-OES (Perkin-Elmer, Massachusetts, USA) against the uranium atomic absorption standard solution (Sigma-Aldrich, St. Louis, Missouri, USA).

2.2. Isolation of Indigenous Bacteria

A mixed culture of bacteria was cultivated by suspending 1 g of soil obtained from an abandoned uranium mine (Phalaborwa, Limpopo) in 100 mL of sterile basal mineral medium (BMM). This was amended with glucose as a sole supplied carbon source. The BMM was prepared by adding (in 1 L deionized water): 10 mM NH_4Cl, 30 mM Na_2HPO_4, 20 mM KH_2PO_4, 0.8 mM Na_2SO_4, 0.2 mM $MgSO_4$, 50 µM $CaCl_2$, 25 µM $FeSO_4$, 0.1 µM $ZnCl_2$, 0.2 µM $CuCl_2$, 0.1 µM NaBr, 0.05 µM Na_2MoO_2, 0.1 µM $MnCl_2$, 0.1 µM KI, 0.2 µM H_3BO_3, 0.1 µM $CoCl_2$, and 0.1 µM $NiCl_2$. The media was autoclaved at 121°C and 2 atm for 15 min before inoculating with the soil culture. Bacterial cultures were isolated from cultures grown aerobically and anaerobically at 25-30°C under shaking at 120 rpm in a Rotary Environmental Shaker (Labotec, Gauteng, South Africa). Anaerobic cultures were grown in cotton plugged Erlenmeyer flasks whereas anaerobic cultures were grown in 100 mL serum bottles bubbled with pure N_2 gas (99% pure grade) and sealed with silicon rubber tubes and aluminium seals. All chemicals were analytical reagent grade and deionized water was used in all experiments.

2.3. Biomass analysis

Morphological differentiation of Gram-positive and Gram-negative bacteria was used as the first step towards determining the microbial composition of the cultures before and after exposure to U(VI). The Gram staining procedure was carried out for the aerobic and anaerobic cultures to identify the isolates based on the chemical and physical properties of their cell walls.

2.4. Culture Characterisation

A 16S rDNA fingerprinting method was used to obtain DNA sequences of pure isolated cultures. Genomic DNA was extracted from the pure cultures using a DNeasy tissue kit (QIAGEN Ltd, West Sussex, UK) as per manufacturer's instructions. 16S rRNA genes of the isolates were then amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers pA and pH^1; the pA primer corresponds to position 8-27 and the pH^1 primer corresponds to position 1541-1522 of the 16S gene. The PCR products were then sent to Inqaba Biotech sequencing facility for sequencing where an internal primer (pD) was used. The pD primer corresponds to position 519-536 of the 16S gene. Sequence relationships to known bacteria were determined by searching known sequences in GenBank using a basic BLAST search of the National Center for Biotechnology Information (NCBI).

2.5. Sample collection and Preparation

For uranium analysis, 0.5 mL of the homogenous solution was collected using a syringe and then centrifuged using a Minispin® Microcentrifuge (Eppendorf, Hamburg, Germany). The 0.5 mL sample was then diluted with 4.5 mL of BMM (1:10 dilution), mixed with 2 mL of complexing reagent and analyzed for U^{6+}

immediately. For microbial analysis, manual counting was performed through the use of a Petroff–Hausser counting chamber employing dark-field microscopy. A 1:100 dilution of bacterial cultures were prepared using distilled water. Diluted culture samples were then loaded individually into the counting chamber and enumerated under the dark-field microscope at a magnification of 400×. Each manual count was performed with a freshly cleaned and loaded chamber.

2.6. U(VI) Reduction experiments

Single colonies of each of the pure cultures; *Pseudomonas* sp., *Pantoea* sp. and *Enterobacter* sp. were picked from a nutrient agar plate then inoculated in nutrient broth and grown for 24 h. At the end of 24 h, the broth (10% v/v of batch volume) was then centrifuged and the pellet was washed three times with 0.85% NaCl solution and then used for the anaerobic experiments. The batch reduction experiments were carried out in Mineral Salt Medium (MSM) supplemented with glucose purged with N_2 for 5 min each in 100 mL serum bottles. Uranium solutions of different concentrations (30–400 mg/L) were prepared in Basal Mineral Medium. These bottles were incubated at 30 °C for a predetermined time interval at 120 rpm on the orbital shaker (Labotec, Gauteng, South Africa). After reduction, the solution was centrifuged at 10,000 rpm for 10 min. A syringe was used to withdraw sample at the following time intervals; 0.25, 0.5, 1, 2, 3, 4, 5, 6, 12, 24, 48 h. Uranium-6 remaining in the supernatant was measured. The amount of metal reduced was taken to be the difference between the initial metal concentration and measured concentration at any time. Metal concentration in the solution was estimated using an ultraviolet–visible (UV/vis) spectrophotometer (WPA Lightwave II, Biochrom, Cambridge, England). To assess the extent of chemical reaction, the set of experiments were carried out under anaerobic and sterile conditions.

2.7. Analytical methods

An UV/vis spectrophotometer (WPA Lightwave II, Biochrom, Cambridge, England) was used to measure uranium and its speciation in all samples. The concentration of hexavalent uranium U(VI) in the sample complexed to a chromatogen was measured by absorbance of light with a wavelength of 651 nm. Arsenazo III (Sigma–Aldrich, St. Louis, MO) (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 uranium(VI) detection. The oxidized fraction of uranium was measured from a sample (0.5 mL) of the homogenous solution collected using a syringe and then centrifuged using a Minispin® Microcentrifuge (Eppendorf, Hamburg, Germany). The 0.5 mL sample was then diluted with 4.5 mL of BMM (1:10 dilution), mixed with 2 mL of complexing reagent and analyzed for U(VI) immediately at a wavelength of 651 nm against a reagent blank. Total uranium level in each sample (U(IV) and U(VI)) was determined by oxidizing an unfiltered sample with nitric acid prior to uranium measurement. This treatment converted U(IV) in the sample to U(VI) which was then measured colorimetrically as described above. The accuracy and precision of the method was determined by measuring the concentration of standard uranium solutions in the range of 0.02–1 mg L^{-1} after appropriate dilution. The results showed that recovery of uranium was quantitative with good precision (92–100%). The percentage deviation was found to be at a maximum (0.4%) at dilution 0.5 mg L^{-1} whereas, the deviation decreased to zero when the concentration was decreased to 0.02 mg L^{-1}. This method proved to be reliable and accurate and is useful in routine analysis of uranium at mg L^{-1} level in other solutions and materials. From literature, it was observed that anionic concentrations greater than 70-fold and

cationic concentrations greater than 50-fold excess over the uranium concentration decreased the normal absorbance of the uranium–arsenazo-III complex (Khan et al., 2006). The limit of detection for the UV/vis spectrophotometer was determined to be 0.02 mg L^{-1}.

3. RESULTS AND DISCUSSION

3.1. Elemental Characteristics of Soil

Samples were taken from below the surface as well as on the surface of the mine. The samples consist of medium and coarse grained soil. Quantitative mineral composition of the soil was obtained by inductively-coupled plasma atomic emission spectrometry (ICP-OES). Results are given in Table 1. The concentration of uranium in the soil is 168.1 mg/kg. According to the United Nations Scientific Committee on the Effects of Atomic Radiation the typical concentration of uranium in natural soils lies in the range 0.30-11.7 mg/kg of soil (UNSCEAR, 1993). Therefore the observed value of 168.1 mg/kg was much higher than normal. The bacteria in the soil was thus expected to be acclimated to high uranium exposure conditions.

Table 1. Mineral composition of mine soil.

Element	**Symbol**	**Mass concentration (mg/kg)**
Uranium	U	168.1
Aluminium	Al	1763.0
Calcium	Ca	124168.0
Phosphorus	P	30686.0
Sulphur	S	1469.0
Copper	Cu	2964.0
Iron	Fe	24536.0
Magnesium	Mg	40478.0

Element	Symbol	Mass concentration (mg/kg)
Manganese	Mn	472.0
Sodium	Na	2399.0

Other predominant elements in the soil include calcium, iron, and magnesium (Mg), which occurred at 2 to 3 orders of magnitude higher than the uranium concentrations. The impact of these other elements on the experiment could be in the form of additional toxicity to the bacteria and interference in spectrophotometric analysis of U(VI).

3.2. Preliminary culture characterisation

Morphological observations showed a dense population of Gram-negative bacilli, streptobacilli and Gram-positive cocci under anaerobic conditions (Fig. 1a). In the aerobic experiment, the culture was predominated by Gram-negative bacilli with sparse populations of streptobacilli and Gram-positive cocci (Fig. 1b). All bacterial types presented here are characteristic of bacterial communities found in the soil.

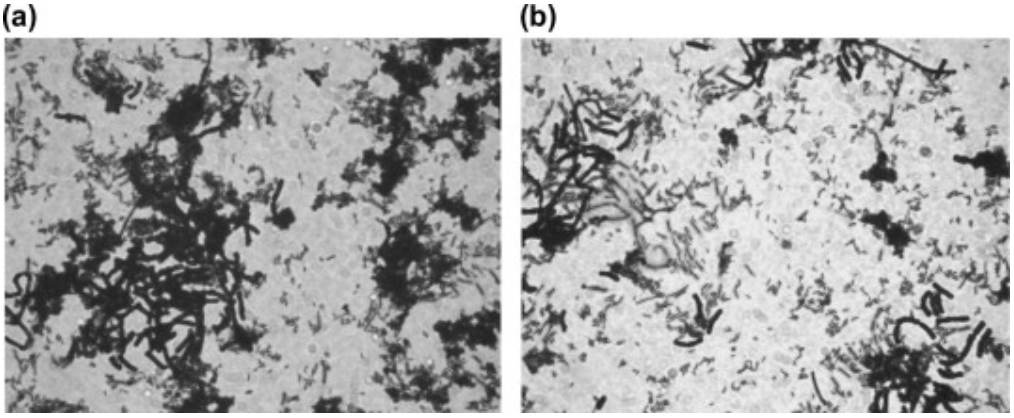

Fig. 1. (a) Optical micrographs of anaerobically grown consortium and (b) optical micrograph of aerobically grown consortium taken under the Zeiss Axioskop II microscope (Carl-Zeiss, Oberkochen, Germany).

3.3. Microbial Analysis

After purifying and sequencing the rRNA genes from the mine soil bacteria, a total of six bacterial species were found, four facultative anaerobes and two aerobic species. The rRNA sequences were isolated from bacteria isolated from preliminary experiments (10 mg/L batches) with some resistance to U(VI) toxicity and were thus candidate species for U(VI) reduction. The results of the culture characterisation are shown in [Table 2] and [Table 3].

Table 2. Characterisation of uranium-reducing facultative anaerobic bacteria isolated from the mine.

Sample name	**Blast result**	**Max ID (%)**	**Further down on list (same max ID)**
B1–1A	*Pseudomonas stutzeri*	98	Other *Pseudomonas spp*.
B1–1B	*Pantoea* sp.	98	*Pantoea agglomerans,* Enterobacteriaceae
A3–2	*Klebsiella pneumoniae*	98	Other *Klebsiella* and uncultured
A2–1	*Enterobacter* sp.	98	*Enterobacter cloacae* and others

Table 3. Characterisation of aerobic uranium-reducing bacteria isolated from the mine.

Sample name	**Blast result**	**Max ID (%)**	**Further down on list (same max ID)**
A1–1	*Acinetobacter schindleri*	99	Other *Acinetobacter spp*.
A2–3	*Bacillus circulans*	98	Other *Bacillus* and uncultured

The facultative anaerobic bacteria from the mine soil showed a wide biodiversity of species. *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 facultatively 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). Another predominant species observed from the soil cultures is *Pseudomonas stutzeri* as shown in Table 2. Cytoplasmic uraninite deposit localization has been reported in a few studies on *Pseudomonas* sp. including *Pseudomonas stutzeri* using TEM analysis. Furthermore, *P. stutzeri* a denitrifying bacteria 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). Other species observed are known to oxidize U(VI)–U(IV) like *Klebsiella* sp. and it was therefore not used in further experiments. Anaerobic enzymatic U(VI) reduction at near-neutral pH conditions has been observed also in pure cultures of nitrate reducing *Klebsiella* sp. (Merroun and Selenska-Pobell, 2008). 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* (Lovely et al., 2004).

Bacillus species, an aerobic species is known to be resistant to U(VI) toxicity and removes soluble U(VI) by precipitation (Lovely et al., 2004). *Acinetobacter schindleri* has not been recorded in literature as being able to reduce any metal.

These findings support the hypothesis that U(VI) reduction in bacteria may be a dissimilatory respiratory process using U(VI) as the terminal electronic acceptor (Holmes et al., 2002; Lloyd, 1995; (Lovley and Phillips, 1992), (N'Guessan et al., 2008) and (Vrionis et al., 2005)). In such cases, the cell conserves energy during the electron transport to U(VI) and the presence of oxygen and other competing electron

acceptors inhibits this process. There is still a possibility that other removal mechanisms are involved such as adsorptive processes on the cell surface or uptake within the cell (Nancharaiah et al., 2006). The later processes are subjects of further investigation in this research.

3.4. Preliminary experiment

Preliminary analysis of U(VI) removal in 10 mg/L U(VI) batches grown under both aerobic and anaerobic conditions showed significant U(VI) removal from solution as summarised in Table 4. The presence of basal mineral medium (BMM) in solution caused a slight change in initial concentration probably due to chemical reaction and complexation with some of the mineral components in solution. Other than this, the presence of BMM did not significantly impact the rate at which U(VI) was removed. The anaerobic cultures performed better overall in the culture supplemented with the BMM. The aerobic cultures, both with and without BMM, did not perform well.

Table 4. Summary of performance data under added U(VI) of 10 mg/L incubated for 24 h under anaerobic and aerobic conditions (listed with increasing efficiency).

Batch no.	**Experimental conditions**	**U(VI) removal (%)**	**Actual U(VI) removal (mg/L)**
1	Anaerobic no BMM[a]	89	4.99
2	Aerobic no BMM	78	1.46
3	Anaerobic + BMM	96	4.60
4	Aerobic + BMM	64	1.57

[a] The best performing batch based on total uranium removed.

The high removal rate under anaerobic conditions may be indicative of a U(VI) reduction mechanism with U(VI) serving as a terminal electron acceptor. In such a case, the presence of oxygen could provide an alternative pathway drawing away electrons for the U(VI) reduction pathway (Finneran et al., 2002).

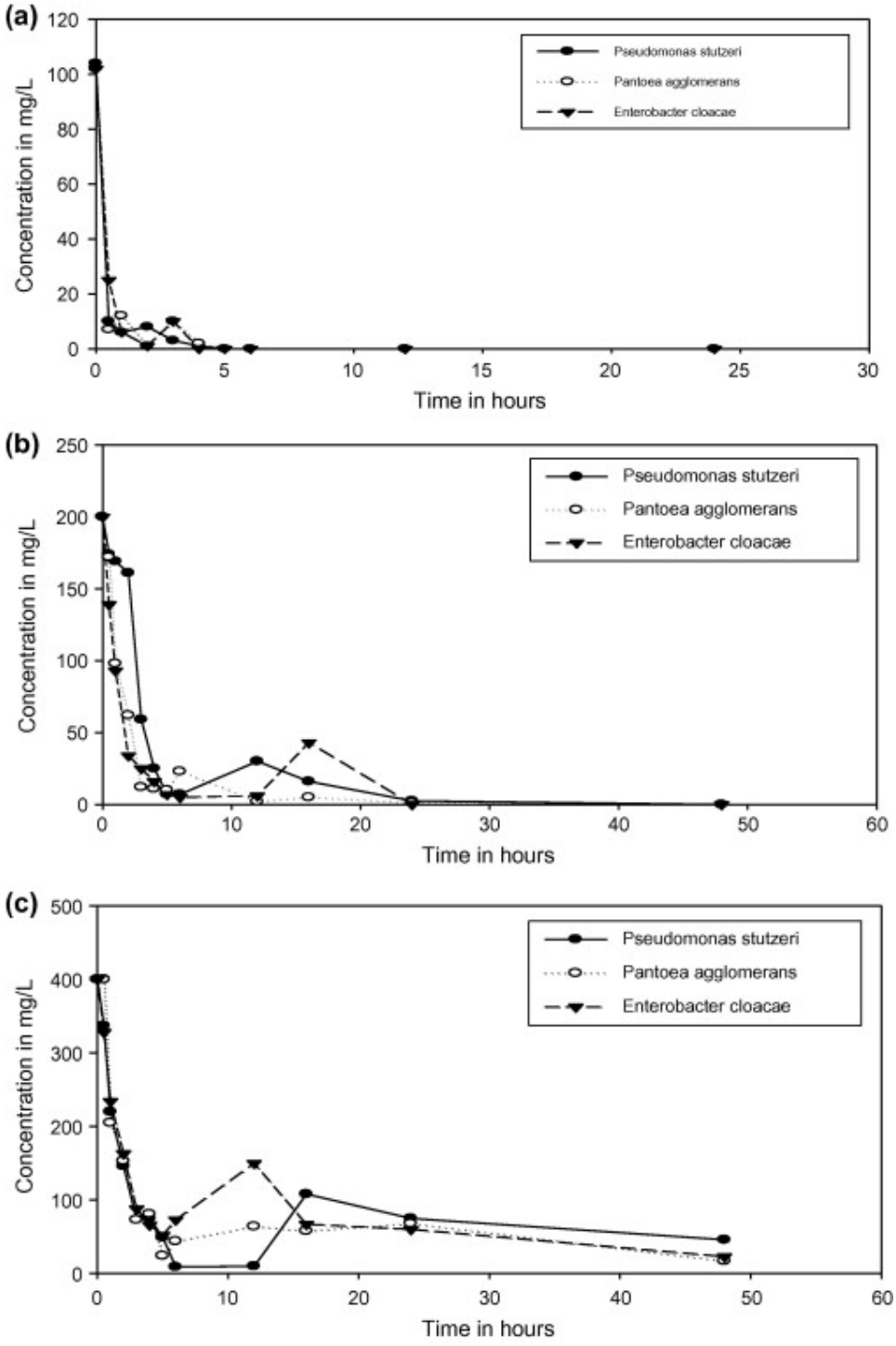

Fig. 2. Uranium(VI) reduction for the three pure cultures of bacteria *Pseudomonas stutzeri, Pantoea agglomerans* and *Enterobacter cloacae* under varying concentrations: (a) 100 mg/L, (b) 200 mg/L and (c) 400 mg/L.

3.5. U(VI) Reduction under Varying Initial Concentrations

U(VI) removal performance under varying initial concentration showed 85–100% removal of U(VI) after 24 h under the tested conditions (Table 5). 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 h for all three concentrations as shown in Fig. 2.

The percentage removal of 100% was achieved at 24 h for the lower concentrations for *Pseudomonas* sp. and *Pantoea* sp. Using the rate of removal at 50% added U(VI), overall *Pseudomonas* sp. performed best at 30 mg/L, *Pseudomonas* sp. and *Pantoea* sp. performed well for 100 mg/L. At 200 mg/L, all the species had removed all the uranium by 24 h (Fig. 2).

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 (Lovley and Phillips, 1992), and equilibrium was attained within 24 h at pH of 5–6 compared to the 1 mM U(VI) removed over 4 h by *Desulfovibrio desulficans* suspended in bicarbonate buffer with lactate as the electron donor.

Table 5. Kinetic data for varying concentrations of U(VI).

Pure culture species	**Initial concentration (mg/L)**	**Removal rate at 50% (mg/L/h)**	**U(VI) Removal at 24 h (%)**
Pseudomonas stutzeri	30	17	100
	75	20	100
	100	50	99
	200	33	81
	400	100	100
Pantoea sp.	30	16	100
	75	20	100
	100	50	99.5
	200	57	83
	400	111	100
Enterobacter sp.	30	9	100
	75	16	100
	100	37	100
	200	63	100
	400	198	85

4. CONCLUSION

The three pure cultures namely; *Pantoea* sp., *Enterobacter* sp. and *Pseudomonas stutzeri* showed potential to reduce U(VI) under anaerobic conditions with a pH ranging from 5 to 6. The results obtained prove the acceptable potential of the three cultures for reduction of uranium from solution. Further studies are needed to increase the bioreduction capacities of biomass and develop appropriate technologies applicable in the treatment of nuclear industry waste waters.

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