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REDUCTION OF URANIUM-(VI) UNDER MICROAEROBIC CONDITIONS USING AN INDIGENOUS MINE CONSORTIUM

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A dissertation submitted in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE: APPLIED SCIENCE
WATER UTILIZATION ENGINEERING

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In the Faculty of Engineering and Built Environment
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Pretoria

July 2011

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Declaration

I, **SIMPHIWE CHABALALA,** hereby declare that all the work provided in this dissertation is to the best of my knowledge original (except where cited) and that neither the whole work nor any part of it has been, or is to be submitted for another degree at this or any other University or tertiary education institution or examining body.

SIGNATURE: ……

DATE: ……

Dedication

This dissertation is dedicated to

My family

This document is solid proof of their love, support and faith in me, I am eternally grateful.

Acknowledgements

The following persons are gratefully acknowledged for their assistance during the course of the study:

- GOD, without whom I am nothing.
- Professor E.M.N. Chirwa, without him, I would never have gained exposure to the exciting and dynamic field of water research.
- Professor S.N. Venter from the Department of Microbiology for his assistance with the characterization of bacterial isolates.
- Colleagues and Laboratory Technicians for your friendship and support. I have learned so much from all of you and greatly enjoyed working with you.

I wish to express my appreciation to the following organizations that made this dissertation possible:

- SANHARP Bursary Programme and,
- National Research Foundation (NRF) for financial support.

ABSTRACT

The utilisation of fossil fuels for energy worldwide depletes the natural reserves and at the same time releases billions of tonnes of carbon dioxide and other greenhouse gases into the atmosphere. In order to reverse the negative effects of this accumulation, i.e., global warming and climatic changes, countries around the world are now considering nuclear energy and other cleaner sources of energy as a substitute to the burning of fossil fuels. The deployment of the later technology has progressed slowly due to lack of public support. The general public and environmental lobbyists worry about the discharge of radioactive waste from nuclear power generation and accidents that have occurred in the nuclear power industry in the recent past. One of pollutants of concern is uranium which is discharged from the nuclear generation processes as the highly toxic uranium-6, (U(VI)). U(VI) coming from the reactors is radioactive as well as highly toxic to aquatic life forms.

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. 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 reducing/removing the pollutants have been used as they preserve the complex interrelationships that exist between species in the source.

The results of this study demonstrate the potential of microbial U(VI) reduction as a possible replacement technology for physical/chemical processes currently in use in the nuclear industry. A detailed analysis of the biological reduction of uranium-(VI) was conducted and the following were the main findings of the study: (1) Background uranium concentration in soil from the mine was determined to be 168 mg/kg, a very high value compared to the typical concentration of uranium in natural soils; (2) Among six bacteria species isolated from a uranium mine in Limpopo, South Africa, three anaerobic species – *Pantoea sp.*, *Enterobacter sp*. and *Pseudomonas stutzeri* – reduced U(VI) to U(VI) and facilitated the removal of the uranium species from solution.

Based on batch studies and cell disruption studies, the laws governing microbial U(VI) reduction were determined and the kinetic parameters for U(VI) reduction were determined. The cultures in this study reduced uranium-U(VI) at a rate better than rates found in literature for other microorganisms. Reduction rates reported in this paper can be used to assess the applicability of bioreduction for uranium removal processes.

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List of Abbreviations

APHA	American public health agency
BMM	Basal Mineral Medium
CISB	Committee on *in situ* bioremediation
CFU	Colony forming units
DMRB	Dissimilatory metal-reducing bacteria
DNA	Deoxyribonucleic acid
HLW	High level waste
HRT	Hydraulic retention time
IAEA	International Atomic Energy Agency
ICP-OES	Inductively-coupled plasma atomic emission spectrometry
ICRP	International Atomic Energy Agency
ILW	Intermediate level waste
LPS	Lipopolysaccharide
LLW	Low level waste
LMW	Low molecular Weight
MOX	Mixed oxide
MQ	Menaquinone
MSM	Mineral salt medium
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal Ribonucleic acid
RT-PCR	Reverse transcriptase- Polymerase chain reaction
TOC	Total Organic Carbon
TEM	Transmission electron microscope
TRU	Transuranic
UNSCEAR	United Nations Scientific Committee on the Effects of Atomic Radiation
US EPA	United States Environmental Protection Agency
UV	Ultraviolet
WHO	World Health Organization

Symbol Nomenclature

U concentration of Uranium-(VI)

t time

X density of active bacterial cells at time t (mg/L)

k_u specific rate of U(VI) reduction (mg/mg/h)

K_u half velocity constant of uranium-(VI) (mg/L)

U_0 initial concentration of U(VI) (mg/L)

X_o initial cells density of U(VI)-reducing strains (mg/L)

T_c maximum U(VI) reduction capacity coefficient (mg/mg)

$T_c X_o$ reduction capacity of cells (mg/L)

r^2 coefficients of regression

E Uranium-(VI) reductase

E^* enzyme–U(VI) complex

r overall rate of reduction (mg/L/h)

k_1, k_2 and k_3 reaction rate constants in the directions indicated by arrows

°C degrees Celsius

CHAPTER 1

INTRODUCTION

1.1 Background

The discharge of radionuclides such as uranium from contaminated sites and their subsequent mobility in the environment is a subject of paramount concern to the public. Among all elements currently in use in the energy industry worldwide, uranium is the most abundant. The highest radiation hazard from uranium occurs when uranium compounds are ingested or inhaled since all uranium isotopes – uranium-238 (U-238) accounting for 99.275%, U-235 - 0.720% and traces of U-234 - 0.005% – mainly emit alpha particles that have little penetrating ability (WNA, 2007). Natural sources of radioactive uranium are present in the Earth's crust at concentrations ranging from 1.8 ppm to 4 ppm. Uranium-containing wastes are also produced at various steps of the nuclear fuel cycle, and vary considerably from low level radioactive effluents produced during uranium mining to intensely radioactive levels in nuclear power plant, spent fuel, and liquid wastes (Merroun *et al*., 2008).

Unfortunately, the anthropogenic use of uranium for nuclear research, fuel production, and weapons manufacturing is so far the main contributor of radioactive uranium in the environment (Wall and Krumholz, 2006). Other anthropogenic processes such as the burning of coal and the manufacture and application of phosphate fertilizers also contribute significant amounts of radioactive uranium (Markich, 2002).

1.2 Environmental and Health Concern

At the exposure levels typically associated with the handling and processing of uranium, the primary radiation health effect of concern is an increased probability of the exposed individual developing cancer during their lifetime. The probability of developing radiation-induced cancer increases with increasing uranium uptake (UNSCEAR, 1999). The largest

proportion of uranium is generated in the form of oxides: U_3O_8, UO_2 and UO_3 (Harley *et al*., 1999). These three uranium oxides are relatively insoluble, dissolving only slowly in body fluids (weeks for UO_3 to years for U_3O_8 and UO_2). Uranium can enter the body in the form of any of these oxides. In the body fluids, uranium is dissolved as a uranyl ion (UO_2^{2+}) - an ionic form that may react with biological molecules (Lin *et al*., 1993; McLean, 1995). The uranyl ion is readily absorbed into the blood. It may also be accumulated and retained in body tissues and organs. Once absorbed, uranium forms soluble complexes with bicarbonate, citrate, or proteins (Stevens *et al*., 1980; Cooper *et al*., 1982). In individuals exposed to soluble or moderately soluble compounds such as uranyl fluoride (UO_2F_2) or uranium tetrafluoride (UF_4), uranium enters the bloodstream and reaches the kidney and other internal organs. In this case, direct chemical toxicity is the primary cause of health problems.

1.3 Treatment Options

As an alternative treatment method, biological treatment methods could potentially be used in removing metal/radionuclide pollutants from dilute solutions (Lovley *et al*., 1992). Biological treatment processes may work better in place of physical chemical methods especially when treatment should be conducted under natural pH and temperature conditions. Biological processes can be deployed as either *in situ* or *ex situ* methods. Biological methods employ mostly microbial mixed cultures to simulate their function in natural environments (Nancharaiah *et al*., 2006).

Currently, there are four mechanisms suggested by several researchers for metal removal in a medium, namely; (a) biosorption, (b) bioaccumulation, (c) precipitation by reaction with inorganic ligands such as phosphate, and (d) microbial reduction of soluble metal to insoluble metal (Nancharaiah *et al*., 2006). The fourth process has been observed in Fe(III)-reducing

and sulphate-reducing bacteria (Khijniak *et al*., 2005). 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) co-metabolically, i.e. no energy is attained from the reduction process (Khijniak *et al*., 2005).

1.4 Scope and Objective

The primary objective of this research was to identify indigenous cultures of U(VI) reducing bacteria from the local environment. Among six bacteria species isolated from a uranium mine in Limpopo, South Africa, three anaerobic species – *Pantoea agglomerans*, *Enterobacter cloacae* and *Pseudomonas stutzeri* – reduced U(VI) to U(IV) and facilitated the removal of the uranium species from solution. Preliminary studies suggest that uranium reduction occurs under anaerobic conditions in most cases. The pure cultures mentioned above showed a high reduction rate at pH 5 to 6. U(VI) reduction was investigated further in a completely-mixed, continuous-flow reactor under anaerobic conditions with repetitive U(VI) loadings, starting with a feed concentration of 5 mg U(VI)/L.

1.5. Items Studied

Activities undertaken to achieve the main objective of this study include:

- Isolation and purification of microorganisms for use in further experiments.
- Characterization of microorganisms in order to identify and classify the microorganisms involved in the reduction of uranium (VI).
- Investigation of the reduction potential of microorganisms that reduce uranium (VI) to uranium (IV) using the consortium in a batch system to establish kinetic parameters for use in reactor scale-up,

- Analysis and characterization of the activity of cytosolic and outer membrane proteins in reducing U(VI), and
- Investigation of the enzymatic process of reduction of U(VI) in the isolated cultures.

To be of practical relevance for industrial application, stimulated indigenous microorganisms may be used for metal removal with the advantage of longer term operation. The results presented here have strong implications of *ex situ* biological reduction of U(VI) through the use of bioreactor systems. Kinetic modelling of uranium reduction and cumulative removal studies could help us to better predict the performance of the cultures.

CHAPTER 2

LITERATURE STUDY

2.1 Uranium and its Uses

Uranium, a compound emitted in radioactive form in nuclear waste is the heaviest of all naturally-occurring elements. Like other elements, uranium occurs in slightly differing forms known as isotopes. Natural uranium as found in the Earth's crust is a mixture of three isotopes: uranium-238 (U-238), accounting for 99.275%, U-235 - 0.720% and traces of U-234 - 0.005% (Katz, 1952 and Winde, 2010). Like all radioactive isotopes, U-238 decays but decays very slowly. Its half-life is the same as the age of the Earth, making it barely radioactive. Uranium-238 has a specific radioactivity of 12.4 kBq/g, and U-235 80 kBq/g, but the smaller amount of U-234 is very active (231 MBq/g). On average the specific activity of natural uranium is 25 kBq/g. During its decay process it generates 0.1 watts/tonne. This is enough to warm the Earth's mantle (IAEA, 1989).

Soon after its discovery, uranium was used to colour glass (from as early as 79 AD). After that time, old uranium deposits were mined to obtain its decay product, radium. Radium was used in luminous paint, particularly on the dials of watches and aircraft instruments, and in medicine for the treatment of disease. For many years from the 1940s, almost all of the uranium that was mined was used in the production of nuclear weapons, but this came to an end in the 1970s. Today, the only significant use for uranium is as fuel in nuclear reactors, mostly for electricity generation. Uranium-235 is the only naturally-occurring material which can sustain a fission chain reaction – releasing large amounts of energy (OECD/IEA, 2009). While nuclear power is the major use of uranium, the waste heat from the reactors can be used in other industrial processes. Nuclear reactors have also been used for marine propulsion

(mostly naval submarines and aircraft carriers). And nuclear reactors are important for making medical and scientific research radioisotopes (Uijt de Haag et al., 2000).

2.2 From Uranium Ore to Reactor Fuel

The end product of the mining and milling stages is the uranium oxide concentrate $(U_3O_8)^{2+}$. Before uranium can be used in a reactor for electricity generation, however, it must undergo a series of processes to produce a useable fuel. The uranium oxide is thus converted into a gas, uranium hexafluoride (UF_6), which is easy to enrich. During uranium enrichment, the U^{235} concentration is increased and the U^{238} isotope is decreased in uranium as the U^{235} isotope is fissionable (Soudek *et al*., 2006).

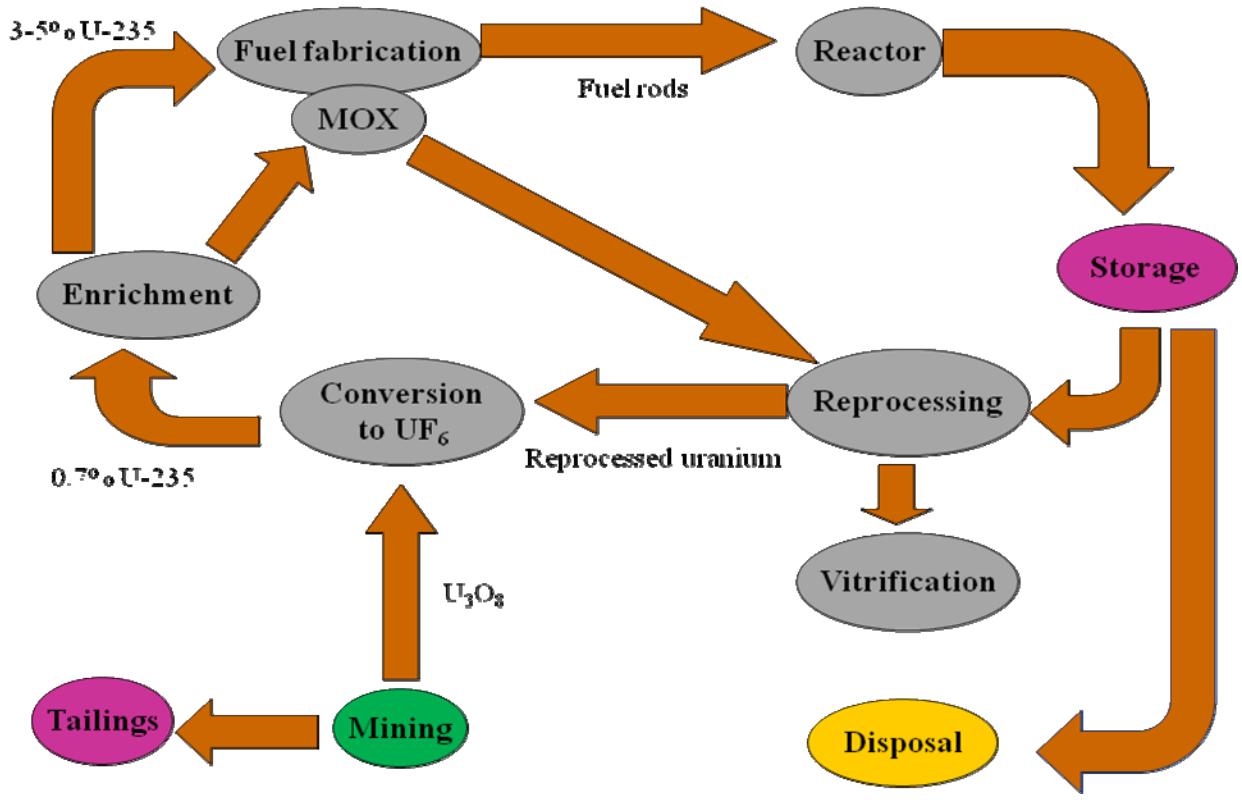


Figure 2.1 A range of activities associated with the production of electricity from nuclear reactions are referred to collectively as the nuclear fuel cycle. The nuclear fuel cycle starts with the mining of uranium and ends with the disposal of nuclear waste. Mixed Oxide (MOX) (WNA, 2009).

After enrichment, the UF_6 gas is converted to uranium dioxide (UO_2) which is packaged into fuel pellets. These are then placed inside thin metal tubes which are assembled in bundles to become the fuel elements for the core of the reactor. Thereafter, used reactor fuel is removed and stored, either to be reprocessed or disposed of underground (Soudek *et al*., 2006).

2.3 Radioactive Wastes

2.3.1 Origin of Radioactive Waste

Activities involving the use of radionuclides and nuclear power generation result in generation of radioactive waste. The activities in all steps of the nuclear fuel cycle including mining, processing and power generation generate radioactive waste in different amounts and radiation levels. Waste can also be generated outside the nuclear activities by large-scale processing of raw materials containing naturally occurring radionuclides including phosphate ore processing and gas or oil exploration. The waste can be solid, liquid or gaseous. Radioactive waste can be discharged as items ranging from spent radioactive sources, pumps, to ion exchange resins, or as liquids and sludge. Equally broad is the range of isotopic half-lives found in the radioactive waste (IAEA, 1981).

2.3.2 Classification of Waste

Radioactive waste is classified into four (4) types, namely: Transuranic waste (TRU), high level waste (HLW), intermediate level waste (ILW), and low level waste (LLW) and transuranic (TRU) (IAEA, 1981). High level waste includes highly radioactive liquid containing mainly fission products as well as some actinides generated from the use of uranium fuel in a nuclear reactor core. It also includes any other waste with radioactivity levels intense enough to generate significant quantities of heat by the radioactive decay

process and lastly spent reactor fuel. Waste in this category accounts for over 95 % of the total radioactivity produced in the process of nuclear electricity generation.

ILW is waste which requires shielding but needs little or no provision for heat dissipation during its handling and transportation. It typically consists of resins, chemicals sludges and metal fuel cladding, as well as contaminated materials from reactor decommissioning. It may be solidified in concrete or bitumen for disposal. This type of waste is generally short-lived waste (from reactors) and is buried in shallow repositories while long-lived waste (from fuel reprocessing) is buried deep underground (Chirwa, 2010).

Waste that does not require shielding during normal handling and transportation because of its low radionuclide content is LLW. It consists of paper, rags, tools, clothing, and filters, etc, mostly from hospitals, industry and the nuclear fuel cycle, that are compacted or incinerated to reduce their volume before shallow land burial (IAEA, 1981).

2.3.3 Transuranic Waste (TRU)

Transuranic (TRU) waste is waste that is contaminated with alpha-emitting transuranium radionuclides with half-lives shorter than 20 years, and concentrations more than 100nCi/g but not including HLW. In the U.S. it arises from weapons production, and is comprised of clothing, tools, rags, residues, debris and other such items contaminated with smaller amounts of radioactive elements mostly plutonium. The elements in this type of waste have atomic numbers larger than uranium (beyond uranium) hence the name transuranic. It cannot be disposed of as either low level or intermediate level waste because of the relatively short half-lives of the elements it contains. TRU does not have the high radioactivity of high level waste or its heat generation. This type of waste is currently disposed of deep underground (Chirwa, 2010).

2.4 Potential Health Effects of Exposure to Uranium

2.4.1 Chemical Toxicity

The target organ for soluble and moderately soluble compounds such as (UO_2F_2) will normally be the kidneys. For insoluble compounds such as uranium dioxide (UO_2) the target will normally be the lungs. The major chemical effect associated with exposure to uranium and its compounds is kidney poisoning. Radiation poisoning of kidneys is common in patients exposed to uranium dust through inhalation or consumption of food containing uranium particles, which then enters the bloodstream. Once in the bloodstream, all forms of uranium are filtered by the kidneys, where they cause damage to the kidney cells. Very high uranium intake ranging from (50 - 150 mg) can cause acute kidney failure and death. At lower intakes levels (25 – 40 mg), damage is detected by the presence of protein and dead cells in the urine, but there are no other symptoms (Bleise et al., 2003).

2.4.2 Compound Solubility and Route of Exposure

The extent of damage from exposure to a uranium compound depends on the solubility of the compound and route of exposure. More soluble uranium compounds will enter the blood faster than insoluble compounds, and will thus reach the kidneys earlier. The target organ for soluble and moderately soluble compounds such as UO_2F_2 will normally be the kidneys. For insoluble compounds such as uranyl dioxide (UO_2) the target will normally be the lungs. In most cases, the contaminant does not leave the organism for the rest of its lifespan. The main concern from exposure to insoluble forms of uranium is increased cancer risk from the internal exposure to radioactivity (ICRP, 1990).

2.5 Effects of Uranium on the Environment

Natural uranium is a weakly radioactive element that is also categorized as a heavy metal with a chemotoxic potential (Burkart, 1988, 1991). Uranium compounds that are formed when uranium reacts with other elements and substances dissolve in water to some extent. The solubility of a uranium compound determines its mobility in the environment, as well as its toxicity (Winde, 2010).

In water, most of the uranium exists as dissolved uranium derived mostly from rocks and soil. Uranium in air exists as dust which may later settle on land and surface water and later accumulate in plants (U.S. EPA, 1991). Generally, the levels of uranium in drinking water are very low. Because of its soluble nature, uranium is not likely to accumulate in fish or vegetables and uranium-6 and be detectable in urine and faeces of animals consuming plants from contaminated land (Ribera *et al*., 1996 and Winde, 2010).

Uranium is found in soils at low to moderate concentrations that range from 0.7 - 11 ppb. Anthropogenic uranium mainly comes from industrial and mining activities. For example, phosphate fertilizers are typically produced involving uranium rich materials (up to 15 ppb). Occasionally, high levels of uranium are detected in root vegetables such as radishes. This is not necessarily a concern as the concentrations which vary between 5 – 60 ppb are below the no impact level concentrations (300 µg/kg to 11.7 mg/kg) prescribed for water and soil. Erosion of tailings from mines and mills may also cause larger amounts of uranium to be released into the environment (Merkel and Hasche-Berger, 2006).

2.6. Conventional Methods for Treating Uranium-Bearing Waste

2.6.1. Membranes

The application of membranes is becoming extremely important in the chemical, nuclear, biotechnology and pharmaceutical industries. Conventional membrane systems are used in treating uranium, i.e.: nanofiltration, ultrafiltration, microfiltration and reverse osmosis. Microfiltration and ultrafiltration are often the pre-treatment step for reverse osmosis (Raff and Wilken, 1999). These systems are quite dependable and possess significant processing capability which makes them viable for liquid radioactive waste processing. Lately, the economic viability of these processes has improved due to the decline in cost of membranes. The common limitation of membrane processes is the generation of considerable quantities of radioactive solid waste in the brine (Pabby and Sastre, 2008).

Uranium and uranium complexes are very heavy, which allows the reverse osmosis process to work in the 95-99 percent rejection range. Reverse osmosis does have limitations around process efficiency and may suffer high maintenance and operational costs due to the effects of mineral deposition on the membrane. There may also be difficulty dealing with high rejection waste volumes from a waste management standpoint (Sastri and Ashbrook, 1976).

In the ion exchange method, uranium is adsorbed onto various ion exchange resins. Disadvantages of this method are; (i) the ion exchange material has a limited capacity for adsorption, (ii) other metals and ions are adsorbed which limits the effectiveness and lifetime of the ion exchange material for uranium, (iii) uranium held in complexes may not be extracted with ion exchange materials, (iv) removal of the uranium from the ion exchange material with high concentrations of salt or acid produce a highly corrosive uranium-containing waste and the use of such extractants is expensive, and (v) ion exchange resins

poorly extract uranium from water when uranium is in low concentrations. A further limitation is due to high cost of most ion exchange resins available today (Ging-Ho et al., 1989).

2.6.2. Reductive Precipitation

A potential remediation method is to use elemental iron (Fe^0) to remove dissolved uranium. As a transition metal, iron serves as both a catalyst and an electron source in which it immobilizes soluble trace element contaminants by surface reduction. Besides its low cost, the secondary and tertiary reaction products, such as Fe(II) and Fe(III) compounds, impose no harm to the environment (Qui *et al*., 2000). The reaction mechanism is still unknown. However, studies have focused primarily on bulk chemical analysis and as a consequence. Very little is known about the basic surface chemical reactions and therefore the system will require additional research before implementing it in the environment. Uranium removal by reaction with iron may occur via adsorption onto iron corrosion products, and by reduction to less soluble valence states by reactions with elemental iron (Farrell *et al*., 2005). According to Farrell *et al*, uranium adsorption is highly dependent on pH and the concentration and speciation of the background electrolyte solution.

2.7. Biological Treatment Alternatives

Biological treatment systems have various applications including remediation of contaminated sites such as water, soils, sludge, and waste streams (Boopath, 2006). With the appropriate microbe or combination of microbes, toxic organic compounds can generally be mineralised completely to CO_2 and H_2O. On the other hand, toxic metals cannot be degraded, but they can be transformed into less mobile valency states (Gadd, 2004). Microorganisms

use one of the following four processes to immobilize metals and radionuclides, viz:- biosorption, bioaccumulation, bioprecipitation and bioreduction (Nancharaiah *et al*., 2006).

Biosorption is a technique in which the uranium-bearing water is brought into contact with living or resting cells of bacteria, fungi, yeast, algae, or other forms of biological material that possess abundant functional groups on their surfaces. Uranyl species react with these sites through passive, physical-chemical mechanisms. Microbial enzymatic activity is not directly involved although polymers secreted by many metabolizing microbes also immobilize metals (Francis, 1998). *Pseudomonas aeruginosa* strain CSU, a non-genetically engineered bacterial strain known to bind dissolved hexavalent uranium (UO_2^{2+}) was characterized with respective to its sorptive activity (equilibrium and dynamics) (Hu *et al*., 1996). Living, heat-killed, permeable, and unreconstituted lyophilized cells were all capable of binding uranium. U(VI) removal by biomass was comparable to removal by commercial cation-exchange resins, particularly in the presence of dissolved transition metals as it adsorbed a large amount of uranium (Michael *et al*., 1996). Michael *et al* also found that the binding by the biomass was pH dependant.

Desorption and recovery of the biosorbed radionuclides is easy. Radionuclide-binding to cell surfaces and polymers is a promising technology for remediating contaminated waters. However, the effectiveness of biosorbants, for example, fungal or bacterial biomass, is affected by poor selectivity against competing ions and saturation at high radionuclide concentrations (Ashley and Roach, 1990). Other limitations of the biosorption methods include the complexation of the metal with carbonates resulting in slower adsorption rates. Although it is possible to regenerate some of the biomass by extracting the uranium with salt solutions or acids, this extraction is expensive and can result in a large volume of corrosive

uranium containing waste. And lastly, biosorption poorly extracts uranium when it is present at low concentrations (Lloyd *et al*., 2002).

Bioaccumulation is an active process whereby metals are taken up into living cells and sequestered intracellularly by complexation with specific metal-binding components or by precipitation. All classes of microorganisms have the capability to accumulate metals intracellularly by an energy-dependent transport system. Localizing the metal within the cells permits its accumulation from bulk solution, although the metals cannot be easily desorbed and recovered (Macaskie et al., 1992). A major drawback associated with the use of active uptake systems is the requirement of metabolically active cells. This may prohibit their use in the treatment of highly toxic waste. Regardless, this approach is promising as a means of remediating fission products from dilute waste streams. The process involves uptake of U(VI) via the K^+-transport system (Tsuruta, 2006).

Biocrystallization, also known as bioprecipitation or biomineralization, is the generation of metal precipitates and minerals by bacterial metabolism. Bacteria interact very strongly with metal ions and immobilize and concentrate them, eventually generating minerals. Microbial biofilms bind significant quantities of metallic ions naturally, and also function as templates for the precipitation of insoluble mineral phases. The biochemistry of the interactions of metal ions with bacterial cell walls, extracellular biopolymers, and microfossil formations in immobilizing toxic metals has been extensively studied (Appukuttan, 2006). In 2000, Macaskie *et al* also investigated *Citrobacter sp*. accumulation of uranyl ion (UO_2^{2+}) via precipitation with phosphate ligand liberated by phosphatase activity. This yielded a novel approach for monitoring the cell-surface-associated changes using a transmission electron microscope. Using this method Macaskie et al., (2000) illucidated that metal deposition occurs via an initial nucleation with phosphate groups localized within the lipopolysaccharide

(LPS). Accumulation of metal phosphate within the LPS was suggested to prevent fouling of the cell surface by the accumulated precipitate and localization of phosphatase exocellularly was consistent with its possible functions in homeostasis and metal resistance (Macaskie, *et al*., 2000).

Although this process involves an enzymatic reaction, it does not involve uranium directly. Disadvantages of this method are similar to those of biosorption. Firstly, the process is hindered by the presence of carbonate and it precipitates metals, other than uranium, that form an insoluble phosphate complex on the cell surface. Secondly, the amount of uranium that can be sorbed onto the cell surface is limited (Gadd, 1992).

Reduction, this involves the transformation of an element from a higher valency state to a lower valency state. Reduction of element may facilitate precipitation or volatilization (Lovley, 1991). Reduction of hexavalent uranium to the tetravalent state has been observed in axenic cultures of iron-reducing, fermentative, and sulfate-reducing bacteria and in cell-free extracts bacteria such as *Micrococcus lactilyticus*. U(VI) reduction in microbial species is extremely diverse as shown by a wide variety of U(VI) reducing species in Table 2.1.

Table 2.1 U(VI) reducing bacteria, their source and preferred environmental conditions.

Bacterium	**Source of Culture**	**Environmental Conditions**	**References**
Anaeromyxobacter dehalogenans strain 2CP-C	Stream sediment, Lansing, MI	Anaerobic, reduces 2-chlorophenol	Sanford et al., 2004
Cellulomonas flaigena ATCC 482	Sugar cane field	Aerobic or facultatively anaerobic, uses glucose and others	Sani et al., 2002
Cellulomonas sp. WS01	Subsurface sediment	Aerobic or facultatively anaerobic, uses glucose and others	Sani et al., 2002
Cellulomonas sp. WS18	Subsurface sediment	Aerobic or facultatively anaerobic, uses glucose and others	Sani et al., 2002
Cellulomonas sp. ES5	Subsurface sediment	Aerobic or facultatively anaerobic, uses glucose and others	Sani et al., 2002
Clostridium sp.	Mine pit water	Anaerobic, uses citric acid and glucose	Francis et al., 1994
Clostridium sphenoides ATCC 19403	Mine pit water	Anaerobic, uses citric acid and glucose	Francis et al., 2004
Deinococcus radiodurans R1	Irradiated ground pork and beef	Aerobic, uses non-fermentable carbon sources	Frederickson et al., 2000
Desulfomicrobium norvegicum DSM 765	Sediment core	Anaerobic, uses acetate and others	Lovley et al., 1993
Desulfotomaculum reducens	Saltwater, California, USA	Anaerobic, uses butyrate and lactate	Tebo et al., 1998
Desulfosporosinus orientis DSM 765	Groundwater in uranium mine	Anaerobic, uses lactate, glycerol and others	Suzuki et al., 2004
Desulfosporosinus spp. P3	Wastewater trickling filter	Anaerobic, uses ascorbic acid, lactate and others	Suzuki et al., 2004
Desulfovibrio baarsii DSM 2075	Ditch mud, Germany	Anaerobic, uses butyrate, ethanol and others	Lovley et al., 1993
Desulfovibrio desulfuricans ATCC 29577	Tar-sand mixture in waterlogged clay, UK	Anaerobic, uses acetate and lactate	Lovley et al., 1992
Desulfovibrio desulfuricans strain G20	Soured oil reservoir, Alaska	Anaerobic, uses lactate, aceatate, glucose and others	Payne et al., 2002
Desulfovibrio sp. UFZ B 490	Uranium dump, Saxony, Germany	Anaerobic, uses pyruvate, fumarate, succinate and ethanol	Pietzsch et al., 2003
Desulfovibrio sulfodismutans DSM 3696	Freshwater mud	Anaerobic, uses acetate and bicarbonate	Lovley et al., 1993
Desulfovibrio vulgaris Hildenborough	Wealden clay, England	Anaerobic, uses lactate	Lovley et al., 1993
Geobacter metallireducens GS-15	Sediment, Potomac River, Maryland, USA	Anaerobic, uses phenol, acetate, formate and others	Lovley et al., 1991
Geobacter sulfurreducens	Surface sediments, Norman, OK	Anaerobic, uses fumarate and acetate	Jeon et al., 2004
Pseudomonas putida	Uranium mill tailing sites	Anaerobic, pyruvate, glucose and others	Barton et al., 1996

Table 2.1 U(VI) reducing bacteria, their source and preferred environmental conditions.

Bacterium	**Source of Culture**	**Environmental Conditions**	**References**
Pseudomonas sp.	Sludge and wastewater treatment systems	Anaerobic, uses pyruvate, glucose and others	Barton et al., 1996
Pseudomonas sp. CRB5	Chromate containing sewage	Anaerobic, uses lactate and others	McLean and Beveridge, 2001
Pyrobaculum islandicum	Icelandic geothermal power plant	Anaerobic, uses iron, thiosulfate and elemental sulfur	Kashefi and Lovley, 2000
Salmonella subterranea sp. nov. strain FRC1	Uranium-contaminated sediment, Tennesee	Aerobic, uses citrate, acetate and others	Shelobolina et al., 2004
Shewanella alga BrY	Sediment from estuary, New Hampshire	Facultative anaerobic, uses insoluble mineral oxides	Caccavo et al., 1992
Shewanella oneidensis MR-1	Sediment, Oneida Lake, New York, USA	Anaerobic, uses lactate and others	Lovley et al., 1991
Shewanella putrefaciens strain 200	Oil pipeline, Alberta, Canada	Anaerobic, uses formate, lactate and others	Blakeney et al., 2000
Veillonella alcalescens	Human saliva	Anaerobic, uses lactic acid	Woolfolk and Whiteley, 1962
Thermoanaerobacter sp.	Geothermal spring	Anaerobic, uses glucose, pyruvate, peptone and others	Roh et al., 2002
Thermus scotoductus	Hot tap water, Selfoss, Iceland	Aerobic, uses acetate and other organic compounds	Kieft *et al*., 1999
Thermoterrabacterium ferrireducens	Hot springs in Yellowstone NP, USA	Anaerobic, uses glycerol and citrate	Khijniak *et al*., 2005

The main advantage of biotransformation is that it is not limited by saturation as long as the product is continuously removed from the system (e.g., via precipitation or advection). Indeed, because many radionuclides are both redox active and less soluble when reduced, direct enzymatic reduction (bioreduction) shows potential for controlling the solubility and mobility of target radionuclide (Runde, 2000).

To overcome physiological problems associated with metal and radionuclide solubility, dissimilatory metal-reducing bacteria are postulated to employ a variety of novel respiratory strategies not found in other gram-negative bacteria which utilise a range of compounds, e.g. O_2, NO_3^-, SO_4^{2-}, and CO_2 as electron sinks (Di Christina *et al*., 2000). From the latter, the novel respiratory strategies are suggested including: 1) direct enzymatic reduction at the outer membrane, 2) electron shuttling pathways and 3) metal solubilisation by exogenous or bacterially-produced organic ligands followed by reduction of soluble organic-metal compounds.

2.8 Enzymatic mechanisms of radionuclide reduction by *Geobacter* and *Shewanella*

The mechanisms by which Fe(III)-reducing bacteria transfer electrons to insoluble Fe(III) oxides during anaerobic growth have been extensively studied in *Geobacter* and *Shewanella* species (Lloyd *et al*., 2002). In both organisms, an electron transfer chain containing *c*-type cytochromes is thought to pass through the periplasm and terminate at the outer membrane, facilitating electron transfer to the extracellular solid phase substrate (Gaspard *et al*., 1998; Lloyd *et al*., 2002). Given that U(VI) is reported to precipitate outside the cell in *Geobacter* and *Desulfovibrio* species, it was hypothesized that a similar electron transfer pathway terminating at the cell surface may be important in U(VI) reduction by *Geobacter sulfurreducens*.

2.8.1 *Shewanella* Reductase(s)

To date, only four strains have been reported to gain sufficient energy from U(VI) respiration to support growth: *S. putrefaciens*, *G. metallireducens*, *Desulfotomaculum reducens*, and *Thermoterrabacterium ferrireducens* (Kennedy *et al*., 2004; Shelobolina *et al*., 2004, Lovley and Phillips, 1992). In order to harness energy for metal reduction, these organisms might also be those functioning to reduce U(VI). Early work with *S. putrefaciens* showed that cells limited for Fe were unable to use Fe(III) as a terminal electron acceptor (Beliaev and Saffarini, 1998). These cells also lost their orange colour and this indicated a major decrease in *c*-type cytochrome content (Obuekwe and Westlake, 1982). The interpretation of these observations was that cytochromes were involved in the transfer of electrons to the terminal electron acceptor or were the terminal reductases. Subsequently, various cytochromes of *Shewanella* were shown to localize in the periplasm and with either the cytoplasmic or the outer membrane (Myers and Myers, 1992). Further mutant studies have implicated other proteins and cytochromes to be involved in metal reduction and a model for electron transfer was proposed, shown on Figure 2.2.

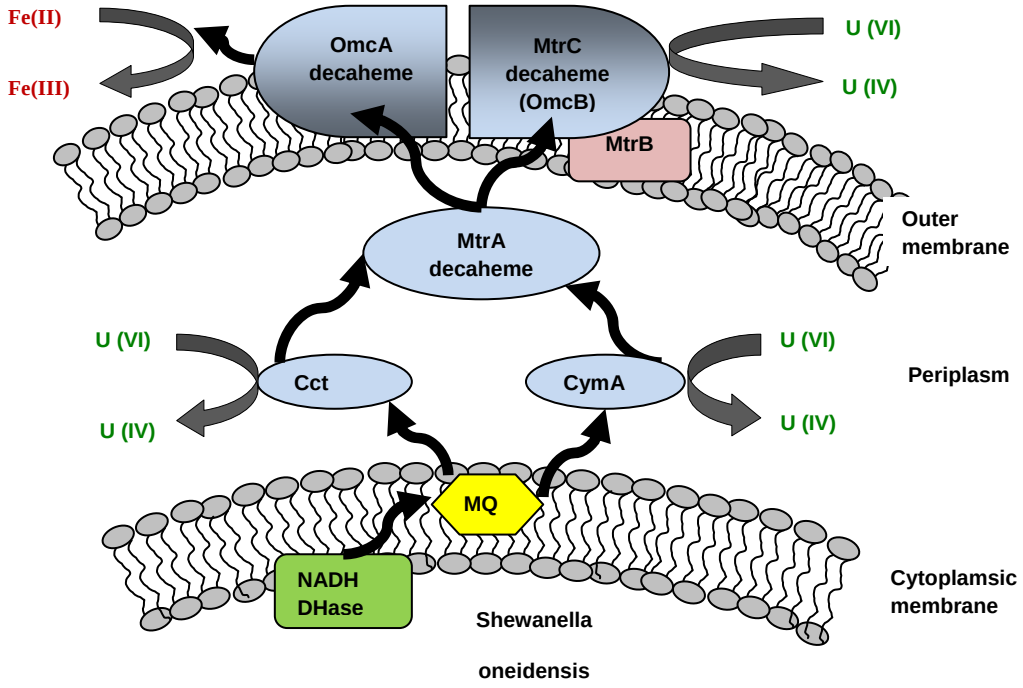


Figure 2.2 An adapted model by Wall and Krumholz shows possible electron transport pathways for U(VI) reduction, emphasizing the possibility of reduction at multiple sites in the periplasm and outer membrane. MQ, menaquinone; CymA, tetraheme membrane-bound cytochrome; Cct, tetraheme periplasmic cytochrome; OmcA, decaheme outer membrane cytochrome; MtrA, decaheme periplasmic cytochrome; MtrB, outer membrane structural protein; MtrC (OmcB), decaheme outer membrane cytochrome (Wall, J.D. and Krumholz, L.R., 2006).

Mutation of the *S. putrefaciens* 200 enzyme, tetraheme *c*-type cytochrome (SO3980), implicated the nitrite reductase in U(VI) reduction because of the simultaneous loss of U(VI) and NO_2^- reduction in the absence of this reductase (Wade and DiChristina, 2000). Transposon mutagenesis of *S. putrefaciens* identified a decaheme outer membrane *c*-type cytochrome, MtrA, as necessary for Fe(III) and Mn(IV) reduction (Beliaev and Saffarini, 1998).

The function of these electron carriers for U(VI) reduction was only recently evaluated as a part of the analysis of global transcriptional responses to U(VI) (Bencheikh-Latmani *et al.*, 2005). Genome sequencing of *S. oneidensis* MR-1 revealed the presence of 42 putative *c*-type

cytochromes (Beliaev *et al*., 2005 and Heidelberg *et al*., 2002). Global transcript analysis of these cytochrome genes during growth on different metal and non-metal electron acceptors (but not uranium or chromium) showed only one cytochrome, SO3300, to be significantly increased in expression during metal reduction (Beliaev *et al*., 2005). In contrast, when these cells were incubated under non-growing conditions with 0.1 mM U(VI) or Cr(VI) present, of the 32 genes that increased (threefold) in both cultures, 12 were cytochromes, but SO3300 was not among them (Bencheikh-Latmani *et al*., 2005).

Several proteins including one involved in menaquinone biosynthesis (MenC), an outer membrane protein (MtrB), a periplasmic decaheme cytochrome (MtrA), an outer membrane decaheme cytochrome (MtrC, also named OmcB), and a tetraheme cytochrome (CymA) anchored in the cytoplasmic membrane were all shown to be needed for optimal U(VI) reduction (Wall and Krumholz, 2006).

Also of vital importance was the observation that the mutants lacking one or more of these electron transfer components were all still capable of U(VI) reduction with lactate as electron donor. Thus multiple pathways for electron delivery to U(VI) are available in *Shewanella*. Comparison of UO_2(s) deposition by o*mcA* or *mtrC* mutants lacking outer membrane decaheme *c*-type cytochromes showed accumulation predominantly in the periplasm versus the deposition of uraninite external to wild-type cells (Kennedy *et al*., 2004). This result is consistent with the observation that U(VI) reduction is not eliminated by any of the single mutants analyzed and supports the hypothesis that uranium reductases are likely non-specific, low potential electron donors present in both the periplasm and the outer membrane. It remains to be determined whether the mutants altered for U(VI) reduction are similarly

affected in their ability to use U(VI) as terminal electron acceptors for growth (Wall and Krumholz, 2006).

2.9 Cellular Location of UO_2 Precipitates

Because of the insoluble nature of U(IV) oxide, the site of deposition should give an indication of the location of the reductase. Many researchers using transmission electron microscopy (TEM) images have observed that dissimilatory metal-reducing bacteria (DMRB) that reduced U(VI) and have confirmed uraninite both outside of the cells and accumulated in the periplasm of gram-negative DMRB (Wall and Krumholz, 2006).

Remarkably, for the gram-positive bacterium *Desulfosporosinus*, uraninite was found in a similar location, concentrated in the region between the cytoplasmic membrane and the cell wall (Suzuki *et al*., 2004). These results would point to a uranium reductase on the periplasmic (outer) face of the cytoplasmic membrane or in the periplasm itself. Uraninite deposits within the cytoplasm of a pseudomonad and *D. desulfuricans* strain G20 have also been observed (McLean et al., 2001, Sani *et al*., 2004). The pseudomonad isolated from a site formerly used for treating wood for preservation removed U(VI) from solution under aerobic or anaerobic conditions. When TEM thin sections of those cells were examined, U(IV) was found inside as well as concentrated at the envelope. As uranium has no biological function and is toxic, the observation of its precipitation in the cytoplasm was surprising (McLean and Beveridge, 2001) speculated that the polyphosphate granules present in the pseudomonad might protect the cell by forming strong complexes with uranium, thus impounding it in the cytoplasm.

The internal deposition of uraninite observed in *D. desulfuricans* G20 occurred in cells that were grown in a medium intended to limit heavy metal precipitation and maximize toxicity (Sani *et al*., 2004). In order to prevent the formation of strong complexes, the medium had no specifically added carbonate or phosphate. Amendments such as these could also alter the physiology of the bacterium, stimulating uptake systems that might allow access of the toxic metal to the cytoplasm. Cytoplasmic deposition of U(IV) has not been reported from other studies with *Desulfovibrio* (Barton *et al*., 1996, Lovley and Phillips, 1992, Lovley *et al*., 1993), and future studies on the effects of nutritional stresses on U(VI) reduction may prove interesting.

With the exception of these unusual reports of cytoplasmic uraninite, the localized precipitation of insoluble U(IV) in the periplasm and outside of both gram-negative and gram-positive cells suggests that U(VI) complexes do not generally have access to intracellular enzymes. Therefore, the best candidates for reductases would be electron-carrier proteins or enzymes exposed to the outside of the cytoplasmic membrane, within the periplasm, and/or in the outer membrane (Wall and Krumholz, 2006).

2.10 *In situ* Immobilization of Uranium

Groundwater contamination by uranium is of major concern especially since the anthropogenic uranium is discharged mostly in its highly soluble and mobile oxyionic forms. Techniques for removing uranium from the groundwater rely on less effective pump-and-treat methods or simple groundwater flushing to lower *in situ* metal concentrations to be below acceptable limits (Anderson *et al*., 2003).

In situ immobilization of uranium takes advantage of the redox nature of uranium and has been suggested to be a potential strategy to remove uranium from groundwater. Reduction of U(VI) to U(IV) within aquifers could precipitate uranium, preventing further down gradient spread of groundwater contamination (Ginder-Vogel *et al*., 2006).

Experiments were conducted at a field site in Rifle, Colorado to determine if results from the laboratory sediment incubation could be extrapolated to *in situ* uranium bioremediation in a contaminated aquifer (Wu *et al*., 2006). As in the laboratory incubations, acetate addition stimulates the growth of *Geobacter* species and effective removal of U(VI) from the groundwater *in situ*. However, further optimization is required in order to promote long term growth and activity of *Geobacter* species because the sulfate-reducing microorganisms that became predominant with continued acetate injection appeared to be less effective at U(VI) reduction. Also, the work showed that hydraulic control could be achieved by a nested recirculation system and conditioning of the subsurface is useful for remediation, even in highly complex and contaminated aquifers (Wu *et al*., 2006).

2.10.1 Limitations for *In Situ* Bioremediation

The long-term stability of biologically reduced uranium will be determined by the complex interplay of soil and sediment mineralogy, aqueous geochemistry, microbial activity, and potential U(IV) oxidants (Ginder-Vogel *et al*., 2004). Many of these factors have been studied under laboratory conditions. However, the impact of these factors on uranium cycling in natural, subsurface environments is still poorly understood (Ginder-Vogel *et al*., 2004). So far, three types of limitations have restricted the use of bioremediation to rid the environment of contaminants in water and contributed to the inadequate understanding of how microbes behave in the field (CISB, National academy Press, Washington D.C., 1993). Firstly, the uptake and metabolism of the contaminants sometimes stop at concentrations above standards

that are normally required, therefore, research on bio-augmentation and direct control of the cell's genetic capability and/or regulation is very active today and may lead to methods to overcome such microbiological limitations. Secondly, it is the difficulty in supplying the microbes with stimulating material such as carbon sources and gasses, and thirdly, problems with making sure that sufficient contact between the microbes and the contaminant is made so as to increase bioremediation efficiency. As a result, only a few of the numerous microbial processes that could be used in bioremediation are applied in practice (CISB, National academy Press, Washington D.C., 1993).

2.11 Summary

The cleanup of uranium-contaminated groundwater and soils by methods such as pump-and-treat and excavation can be costly and disruptive to ecosystems whereas microbial reduction of uranium offers a cost-effective and friendlier alternative (Martinez et al., 2007). Microorganisms play important roles in the environmental fate of toxic metals with a wealth of physical-chemical and biological mechanisms effecting transformations between soluble and insoluble phases. Although the biotechnological potential of most of these processes has only been explored at laboratory scale, it is still a viable means for remediating the environment.

In the year 2009, Francis and Matin showed that the obligately anaerobic, spore-forming Clostridia are involved in reductive precipitation of uranium in the subsurface environments. This fermentative bacteria reduced uranium with the excess of electrons generated during fermentation of organic materials, and the research addressed the need for detailed studies of the enzymatic mechanisms for reduction by fermentative micro-organisms (Francis and Matin, 2009). In addition to that, they attempted to determine the role of hydrogenases in uranium reduction, purify the enzymes involved, determine whether reduction is a one or two

electron transfer reaction and also reveal the genetic control of the enzymes and cellular factors involved in uranium reduction.

Further research by Francis in the same year addressed the need to understand the principal mechanisms in which micro-organisms alter radionuclide-organic complexes, and the resultant impacts on radionuclide solubility and stability under anaerobic conditions. The work elucidated the mechanisms of biotransformation and the fate of uranium complexed with organic and inorganic ligands under anaerobic conditions. With this they were challenged to identify which factors regulate the bioreduction of complexed uranium as well as to enhance the reductive precipitation and stabilization of soluble uranium organic and inorganic complexes. All research conducted was multidisciplinary collaborative involving bimolecular science, biochemistry microbiology and electrochemistry to achieve the objectives they set out to accomplish.

Shelobolina et al., (2004) studied the effect of deleting outer membrane c-type cytochrome genes on the U(VI) reduction capacity of *Geobacter sulfurreducens* in 2007, and they found that the deficient mutant's ability to reduce U(VI) was lower than that of the wild-type strain. This research provided new evidence for extracellular uranium reduction, but does not rule out the possibility of periplasmic U(VI) reduction. Additional studies are required to clarify the pathways leading to U(VI) reduction in *G. sulfurreducens*.

CHAPTER 3

EXPERIMENTAL METHODS

3.1 Materials

3.1.1 Microorganisms

The cultures were isolated in our laboratory from soil samples obtained from an abandoned uranium mine in Phalaborwa, Limpopo. Batch cultures (120 mL) were used to determine initial values of U(VI) reduction kinetic parameters for all pure cultures.

Cells for batch cultures inoculation were cultivated in Nutrient Broth (Merck, Johannesburg, SA) and maintained on Nutrient Agar (Merck). The Nutrient Broth and Nutrient Agar were prepared by dissolving 31 g/L and 16 g/L respectively, of powder in 1L distilled water, followed by autoclaving in the Tomin TM-323 autoclave (Durawell Co. Limited, Taiwan) for 15 minutes at 121°C at 115 kg/cm^2. The agar was cooled to 50°C before dispensing into Petri dishes. The pure cultures were incubated overnight at a temperature of 30°C with incubation at 120 rpm in a Rotary Environmental Shaker (Labotec, Gauteng, SA). The cells were harvested by centrifugation in the Hermle Z 323 centrifuge (Memingen, Germany) at 5000 x g and 4°C and washed three times in sterile 0.85 % NaCl solution.

3.1.2 Reagents

Arsenazo III reagent: was prepared by dissolving 0.07 g (1,8-dihydroxynaphthalene-3,6 disulphonic acid-2,7-bis[(azo-2)-phenylarsonic acid]) in 24.8 mL of 70% Perchloric acid ($HClO_4$) (Merck, SA) and then filled the volumetric flask up to 2 L with distilled water. The solution was kept at 4°C until further use.

3.2. Culture Characterisation

3.2.1 Purification of Indigenous Bacteria

In preparation for the 16S rRNA sequence identification, the bacterial cultures were purified by performing serial dilution to obtain individual colonies. The diluted culture samples from the 7^{th} to 10^{th} tube were then plated out onto nutrient agar plates and incubated for 24 h at 30 °C. Six different morphologies were identified from the cultures, of which four were facultative anaerobes and two were aerobes. These were then individually streaked on nutrient agar plates followed by incubation at 30 °C for another 24 h. This process was repeated twice in order to obtain the desired pure cultures.

3.2.2 16S rRNA Fingerprinting

A 16S rRNA 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 pH1. The primer pA corresponds to position 8-27 and primer pH1 corresponds to position 1541-1522 of the 16S gene (Coenye et al., 1999). The PCR products were then sent to Inqaba Biotech sequencing facility for sequencing where an internal primer pD was used. Primer pD corresponds to position 519-536 of the 16S gene. The 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 gene library (Chabalala and Chirwa, 2010).

3.3 Growth Medium

Pure culture batch studies were conducted in basal mineral medium (BMM) which 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 $NiCl_2$, supplemented with glucose as a carbon source.

3.4 Experimental Batches

3.4.1 Sample Collection and Preparation

A 0.5 mL sample 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. Samples were analyzed in duplicate to minimize random errors and a non-biological control was employed.

3.4.2 Determination of Mine Soil Mineral Composition

The mineral composition of the soil was quantitatively measured using the Optima 7300DV ICP-OES System (PerkinElmer, Massachusetts, USA). A gram of soil was mixed with 500 mL of distilled water and incubated overnight at a temperature of 30°C with incubation at 120 rpm in a Rotary Environmental Shaker (Labotec, Gauteng, SA). The supernatant thereof, was used to determine the mineral composition according to the methods described in *Standard methods for the Examination of Water and Wastewater* (APHA, 1992).

3.5 Analytical methods

3.5.1 Instrument Calibration

A uranyl nitrate stock solution (1004 mg/L) obtained from Sigma-Aldrich, St. Louis, MO) was used as the primary analytical standard. A multi-point calibration curve was constructed for standards of known concentration to quantify unknown samples. The instrument response was linear up to a concentration of 15 mg/L. Six standards used namely; 0, 1, 3, 6, 9, 12, 15 mg/L were prepared by appropriately diluting the uranyl nitrate stock solution with distilled deionized water. Standards were analyzed by mixing 2 mL of standard solution with 2 mL of complexing agent described earlier.

3.5.2 U(VI) Determination by Colorimetric Method

A UV/vis spectrophotometer (WPA Lightwave II, Biochrom, Cambridge, England) was used to measure uranium in all samples. This instrument measures the level of hexavalent uranium (oxidized state of uranium – U^{6+}) in the sample. The absorbance of each sample was measured using light with a wavelength of 651nm. Arsenazo III (Sigma-Aldrich, St. Louis, Missouri, USA) (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 (Chabalala and Chirwa, 2010a).

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 which was then measured. 0.5 mL of unfiltered sample (well shaken) was added to 2.5 mL of 2N nitric acid and diluted with 2 mL of BMM (1:10 dilution) and then centrifuged and 1 mL of this oxidized, diluted sample was mixed with 2 mL of Arsenazo III and then analyzed.

3.5.3 Precision and Accuracy

The accuracy and precision of the method was determined by measuring the concentration of standard uranium solutions in the range of 0.02 mg/L to 1 mg/L 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 whereas, the deviation decreased to zero when the concentration was decreased to 0.02 mg/L. This method proved to be reliable and accurate and is useful in routine analysis of uranium at mg/L 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.

3.6 Continuous Flow Bioreactor

The continuous flow reactor was constructed from a 10 L flat-bottomed flask (Figure 3-1) and to minimize adsorption of uranium, the reactor was made of glass, and recirculation and feeding lines were made of silicone tubing (Masterflex, Cole-Palmer Inst. Co., Niles, Illinois). The reactor, connecting tubing and filters were autoclaved at 121°C for 15 minutes then assembled under a laminar biosafety hood. A rubber stopper was used to plug the

opening at the top of the reactor to achieve and maintain anaerobic conditions, and four holes were made in it to fit four glass tubes. Glass tube (1) was for nitrogen gas inlet, (2) was an outlet for used medium to keep volume of the reactor at 8 L, (3) was an inlet for feed (basal mineral medium supplemented with glucose and U(VI)) and (4) was a sampling port.

During the experiment, sterile basal mineral medium and a U(VI) solution were fed simultaneously using pre-calibrated double-headed peristaltic pump (Masterflex, Cole-Palmer Inst. Co., Niles, Illinois). The reactor was placed on a AM4 multiple heating magnetic stirrer (Velp Scientifica, Labex Pty Ltd, South Africa) and a sterile magnetic stirrer bar was inserted into the reactor to achieve completely mixed conditions and operated at 35°C.

The continuous reactor was inoculated with, continuous flow reactor comprised of *Pseudomonas stutzeri*, *Pantoea agglomerans* and *Enterobacter cloacae*. The reactor was operated under influent feed rates of 400 -500 mL/day resulting in HRT of 24 hours. Liquid volume was maintained at 8 L (80% of total volume). The nitrogen supply installed on the air inlet and nitrogen (N_2) was purged into the reactor every 24 hours to ensure that the reactor remains anaerobic.

3.6.1 Reactor Start-up

To start-up the reactor, 80 mL of overnight grown mixed culture of *P. stutzeri*, *P. agglomerans*, and *E. cloacae* (10 % (v/v)) was charged directly into the feed port at the top of the reactor. The reactor was then operated under the influent uranium-(VI) concentration of 5 mg/L and 24 hours HRT for the first 5 days. Different influent uranium-(VI) concentrations of 10, 20, 50, 100 mg/L were tested, for 5 days at each concentration, to determine optimum uranium-(VI) loading for the experiment.

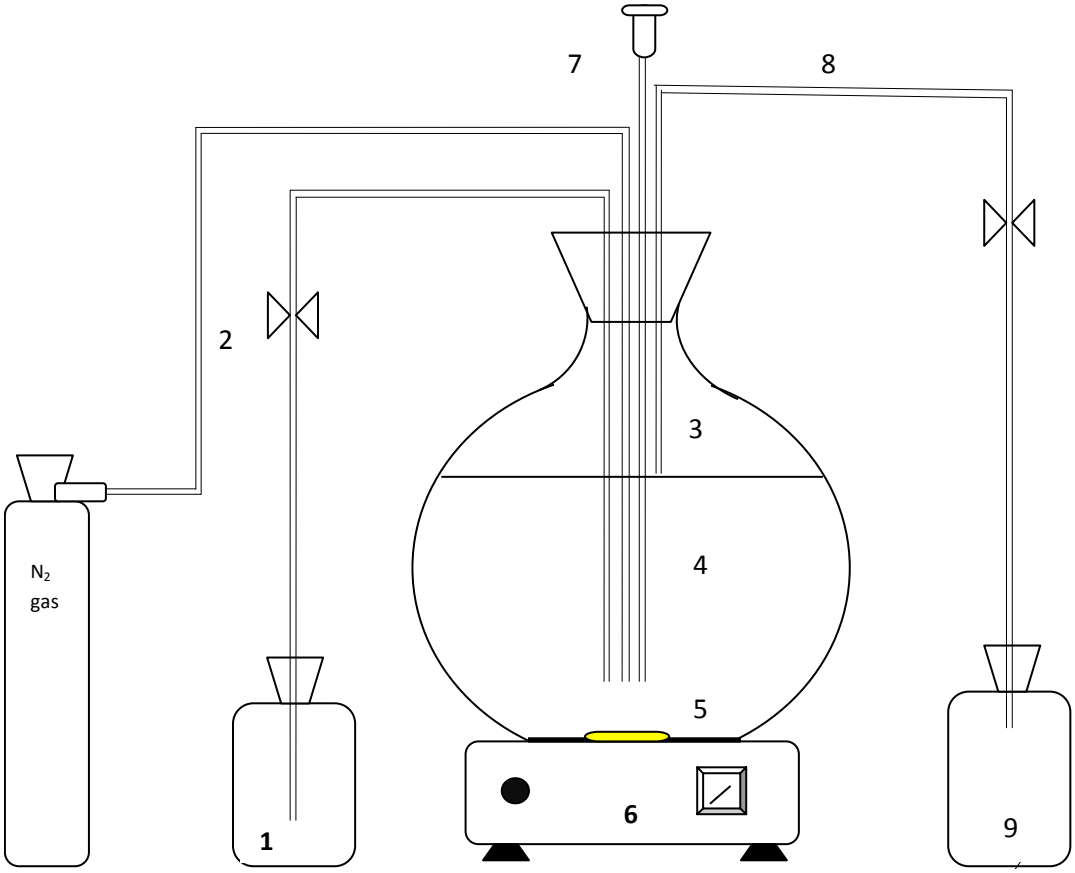


Figure 3.1 Schematic diagram of the laboratory scale anaerobic continuous bioreactor (Nitrogen gas, 1= Feed, 2= Peristaltic pump, 3= Glass tubing, 4= Bioreactor 10 L, 5= Stirrer bar, 6= Magnetic stirrer/ Heating block, 7= Sampling port, 8= Silicon tubing, 9= Waste bottle).

3.7 Total Organic Carbon

Total organic carbon (TOC) was measured using Total Organic Carbon Analyzer (Model TOC-VWP, Shimadzu Corporation, Kyoto, Japan) after acidifying a 40 mL of sample with one drop of concentrated orthophosphoric acid (Merck, SA). 0.5 mL of the sawdust solution was dissolved in basal mineral medium and filled up to 1 L with ultrapure water to make a 0.5 % solution. Standards were prepared from different dilutions of a potassium hydrogen

phthalate stock solution. The potassium hydrogen phthalate stock solution was prepared by dissolving 2.1254 g (anhydrous) (Merck, SA) in 1 L of ultrapure water which is equivalent to 1000 mg/L. TOC was determined with a precision of ±0.5 mg carbon/L in all samples.

3.8 Electron Donor Variation Study

Three different carbon sources were chosen for this study; Ethanol (Merck, SA), Sodium Acetate (Merck, SA) and Organic Carbon source in the form of Sawdust from a wood workshop. 0.5 % solutions of these electron donors were made in basal mineral medium and used for reduction experiments.

3.9 Biomass Analysis

3.9.1 Viable Suspended Cells

Viable mixed culture cells were determined using the spread plate method on Nutrient Broth (NB). The standard spread plate method and colony counts were performed as described in Section 9215C of the *Standard methods for the Examination of Water and Wastewater* (APHA, 1992). Samples for the analysis of viable suspended cell concentration were withdrawn from the batches at daily intervals. 1 mL samples were serially diluted in 0.9 mL sterile 0.85% NaCl solution. 1 mL of each dilution was then pipetted onto agar (100 mm size) followed by spreading. Colony forming units were counted manually after incubation for 24 hours at 30°C. The colonies were creamy-white, shiny white and pale yellow, a representation of the mixed culture. Plates with 100-300 colonies were selected for a more representative CFU count for each sample.

3.9.2 Total Suspended Cells

Bacterial cells were incubated at 35°C in Nutrient Broth and harvested after 24 hours by centrifuging at 6000 rpm for 10 minutes. The cells were then washed three times in 0.85 %

NaCl solution and then resuspended in 10 mL 0.85 % NaCl solution. 1 mL of each cell suspension was pipette into eppendorf tubes and then centrifuged at 10000 rpm for 10 minutes. The supernatant was discarded and the pellet was weighed to determine the initial wet weight per mL of solution. Thereafter, batch experiments were carried out in uranium concentrations of 50 mg/L and 200 mg/L, and the same procedure was followed to determine the wet weight per mL of solution. For verification, manual counting was performed through the use of a Petroff-Hausser counting chamber (Hausser Scientific, Horsham Pa, USA) employing a Carl Zeiss Axioskop II phase microscope (Zeiss, Germany). 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 400X. Each manual count was performed with a freshly cleaned and loaded chamber.

3.10 Scanning Electron Microscopy

Transmission electron microscopy of bacterial cells was performed following the methodology by Mathews (1986) and Hayat (1981). Metal free (control) and metal loaded bacterial cells were concentrated by centrifugation and then fixed in 2.5% glutaraldehyde in the sodium-phosphate buffer (0.075 M, pH 7.4) for 2 h. Thereafter, the material was washed three times with the same buffer followed by fixing in 0.5 % osmium tetraoxide stain for 2 hours. Cells were dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, 100%, 100%, 100%), infiltrated with 50% Quetol epoxy resin and embedded in pure Quetol epoxy resin for 3 hours (Glauert, 1975). Cells were then polymerised at 60°C for 39 hours and unstained ultrathin sections were cut by a Reichert Ultracut E Ultramicrotome (Reichart, Germany). The sections were loaded in carbon coated copper grid and soaked in Reynolds' lead citrate for 2 minutes and then rinsed in water. The copper coated samples were then

examined in a JEOL-JSM-840 transmission electron microscope at 100 kV. The mixed culture not exposed to U(VI) served as a control.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Elemental Characteristics of Soil

Samples were taken from 100 m below the surface as well as on the surface of the mine. The samples consisted of medium and coarse grained soil. Mineral composition of the soil was quantitatively measured by inductively-coupled plasma atomic emission spectrometry (ICP-OES). Results are given in Table 4.1. The concentration of uranium in the soil was 168.1 mg/kg. According to the **Error! Bookmark not defined.** 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. Other predominant elements in the soil included calcium, iron, and magnesium (Mg), which detected 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 spectrometric analysis of U(VI) (Chabalala and Chirwa, 2010b).

4.2 Growth Study

The growth curve shows a very steep decline in population numbers which can be attributed to initial exposure to uranium. Two-log kills of cells occurred during the first 1 hour of exposure to U(VI). The population stabilised between 1 and 12 hours. During the time 1-12 hours, cells are assumed to be making a complete complement of enzymes for synthesis of essential nutrients not present in the medium.

Table 4.1 Mineral composition of mine soil collected from a closed uranium mine.

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
Potassium	K	673.0
Magnesium	Mg	40478.0
Manganese	Mn	472.0
Sodium	Na	2399.0
Nickel	Ni	40.1
Chromium	Cr	16.6
Cadmium	Cd	6.4
Selenium	Se	28.0
Zinc	Zn	41.5
Vanadium	V	20.4
Cobalt	Co	7.0
Lead	Pb	9.3
Molybdenum	Mo	1.3
Silver	Ag	0.2
Bismuth	Bi	10.8
Gallium	Ga	12.6
Lithium	Li	1.3
Strontium	Sr	269.9
Arsenic	As	5.7
Titanium	Ti	76.5
Barium	Ba	176.4
Beryllium	Be	0.3

Cell population entered a log growth phase between 12 and 18 hours followed by the stationery phase after hour 18. When viewed under the microscope, a large number of Gram positive cocci were observed for the first 12 hrs, and after 15 hrs only rods were observed, indicating a change in community. The cell count in the controls increased until it reached a pseudo-equilibrium concentration for the remainder of the experiment (Figure 4.1).

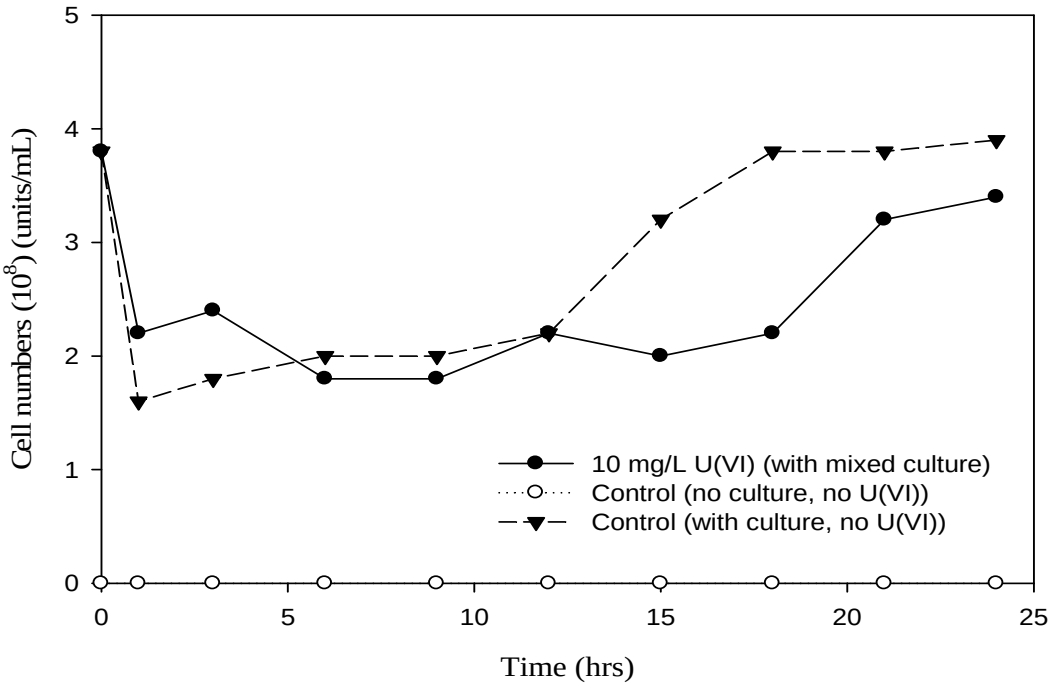


Figure 4.1 Growth curve of bacteria exposed to10 mg/L U(VI) at 25- 30°C.

4.3 Culture Characterization

4.3.1 Gram Staining

Morphological observations showed a dense population of Gram-negative Bacilli, Streptobacilli and Gram-positive cocci under anaerobic conditions (Figure 4.2a). In the aerobic experiment, the culture was predominated by Gram-negative Bacilli with sparse populations of *Streptobacilli* and Gram-positive *cocci* (Figure 4.2b). All

bacterial types presented here are characteristic of bacterial communities found in the soil.

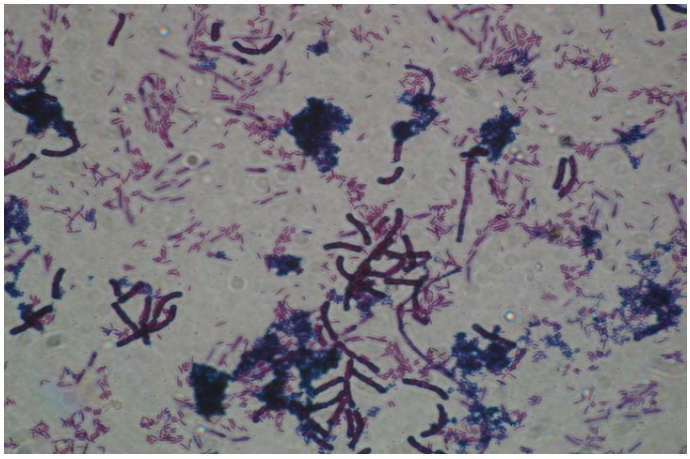


Figure 4.2.a Optical micrographs of anaerobically grown consortium taken under the Zeiss Axioskop II microscope (Carl-Zeiss, Oberkochen, Germany).

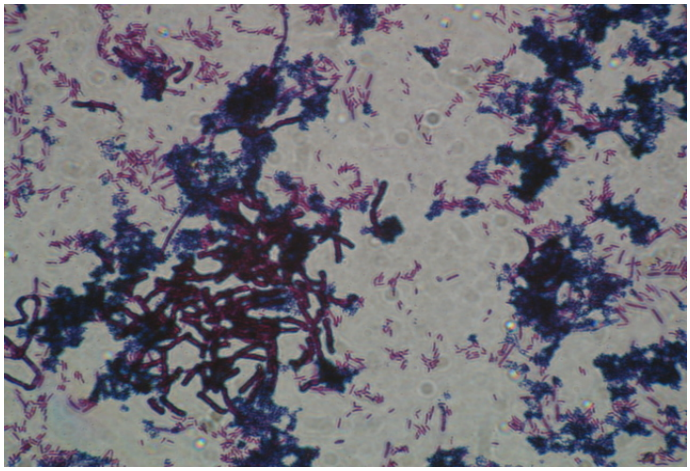


Figure 4.2.b Optical micrograph of aerobically grown consortium taken under the Zeiss Axioskop II microscope.

4.3.2 16SrRNA Secondary Identification by Sequencing for Classification

After purifying and sequencing the rRNA genes from the mine soil bacteria, a total of 6 bacterial species were found. The rRNA sequences were isolated from bacteria 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 4.2 and 4.3.

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).

Table 4.2 Characterisation of uranium-reducing facultative anaerobic bacteria isolated from the closed uranium mine.

Sample name	Blast result	Max ID (%)	Further down on list (same max ID)
B 1-1A	*Pseudomonas stutzeri*	98	Other *Pseudomonas* spp
B 1-1B	*Pantoea sp*	98	*Pantoea agglomerans*, Enterobacteriaceae
A 3 -2	*Klebsiella pneumoniae*	98	Other *Klebsiella* and uncultured
A 2-1	*Enterobacter sp.*	98	*Enterobacter cloacae* & others
A 2-2	*Enterobacter sp.*	97	*Enterobacter cloacae* & others

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). 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) (Table 4.2).

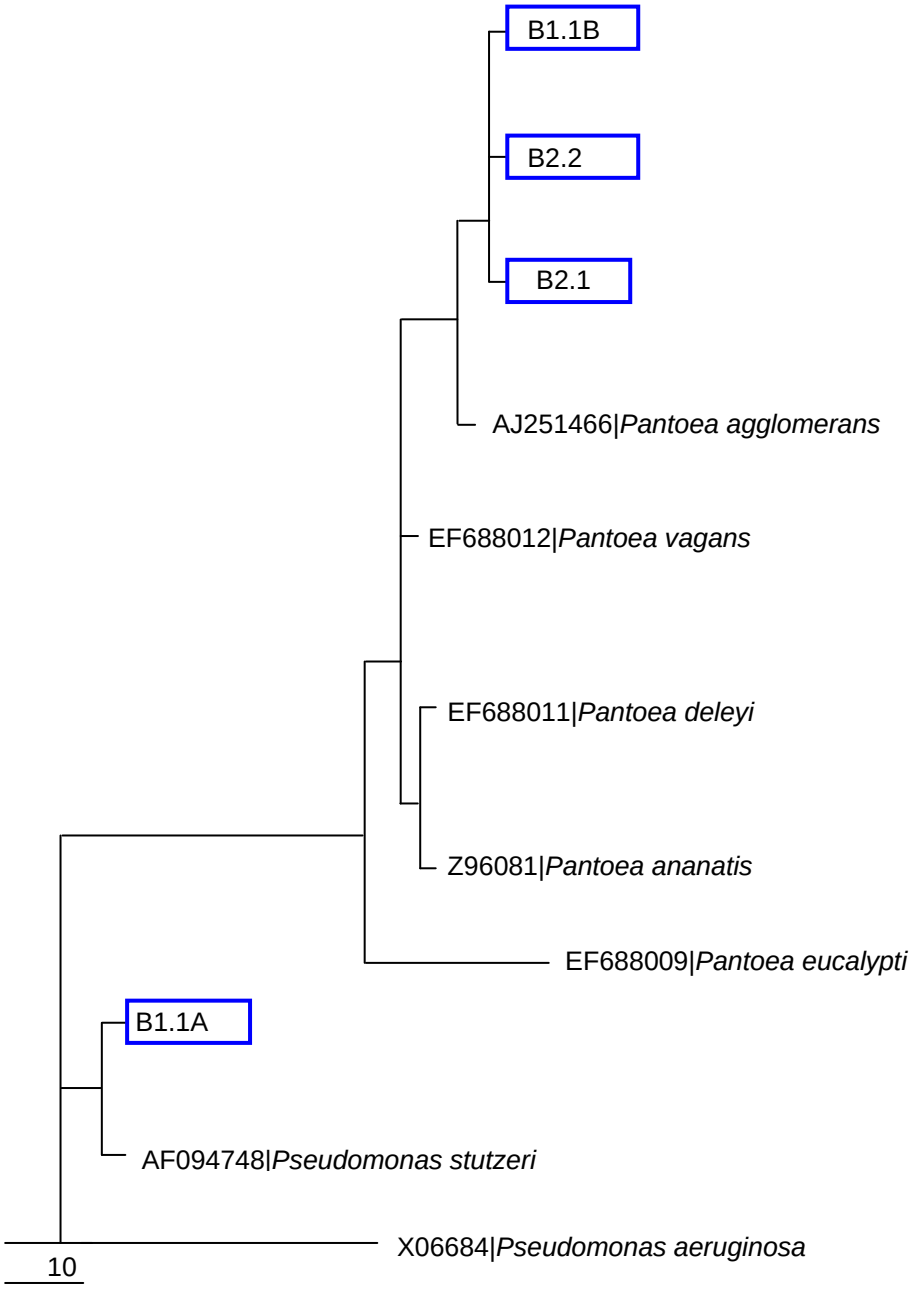


Figure 4.3 Phylogenetic analysis of the dominant cultivable bacteria (Gram-negative) present in the soil sample. The phylogenetic tree is based on 16S rRNA gene sequences for the bacteria. The length of each branch is proportional to the estimated number of substitutions per position. The distance between two species is obtained by

summing the lengths of the connecting horizontal branches using the scale at the bottom.

Table 4.3 Characterisation of aerobic uranium-reducing bacteria isolated from the closed uranium mine.

Sample name	Blast result	Max ID (%)	Further down on list (same max ID)
A 1-1	*Acinetobacter schindleri*	99	Other *Acinetobacter* spp
A 3-2	*Bacillus circulans*	98	Other *Bacillus* and uncultured
A 3-1	*Acinetobacter schindleri*	99	Endosymbiont of *Sphenophorus levis*
A 3-4	*Acinetobacter schindleri*	99	Other *Acinetobacter spp.*

Bacillus species, an aerobic species is known to be resistant to U(VI) toxicity and removes soluble U(VI) by precipitation (Lovley *et al*.; 2004). Other species observed are known to oxidise U(VI) to U(IV), this could need to be eliminated from the culture. An example is the *Klebsiella sp*. Anaerobic enzymatic U(IV) oxidation at near-neutral pH conditions has been observed also in pure cultures of nitrate grown, but not Fe(III)-grown, cells of and nitrate reducing *Klebsiella* sp. (Merroun and Selenska-Pobell, 2008).

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; Vrionis *et al*., 2005).

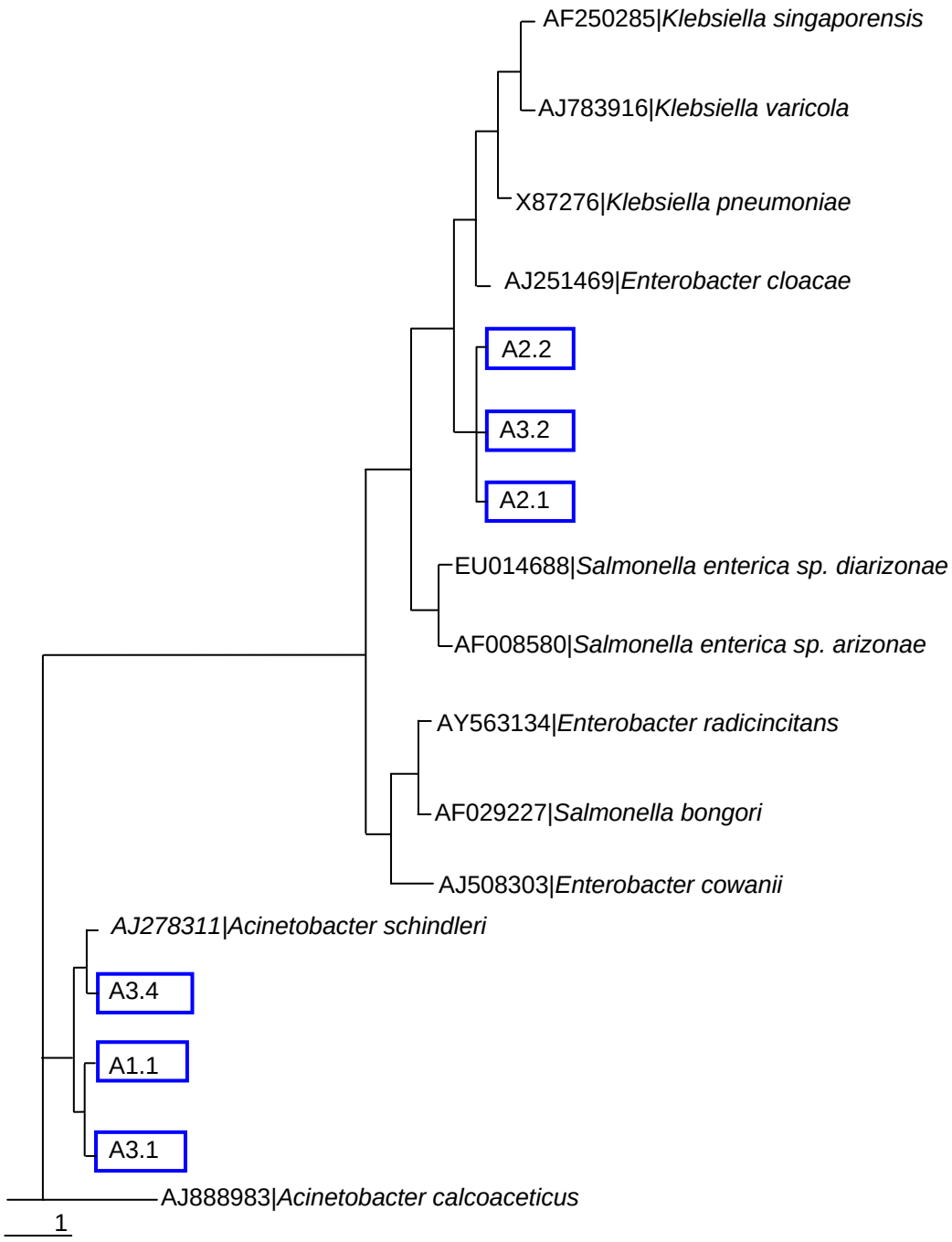


Figure 4.4 Phylogenetic analysis of the dominant cultivable bacteria (Gram-Negative) present in the soil sample. The phylogenetic tree is based on 16S rRNA gene sequences for the bacteria. The length of each branch is proportional to the estimated number of substitutions per position. The distance between two species is obtained by summing the lengths of the connecting horizontal branches using the scale at the bottom.

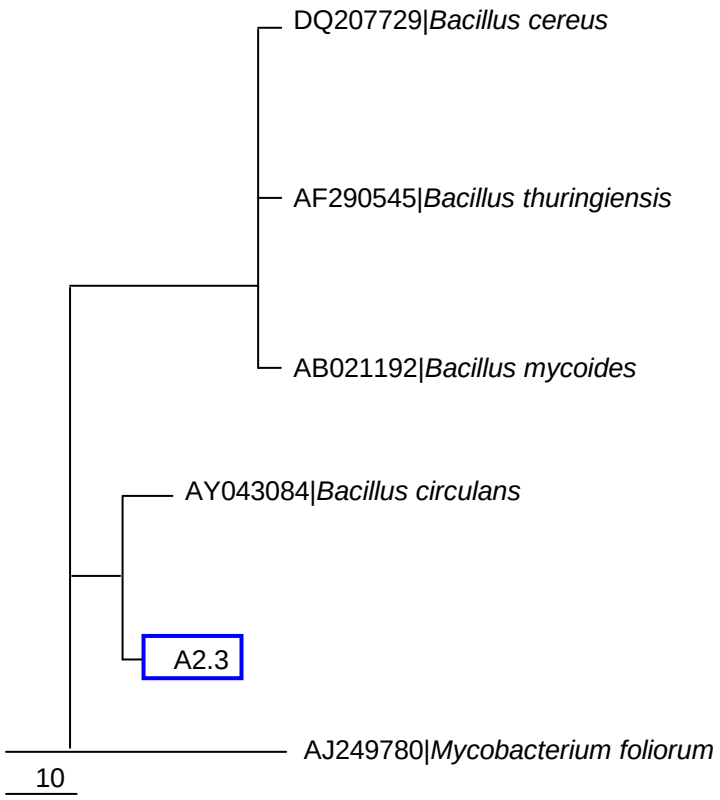


Figure 4.5 Phylogenetic analysis of the dominant cultivable bacteria (Gram-Positive) present in the soil sample. The phylogenetic tree is based on 16S rRNA gene sequences for the bacteria. The length of each branch is proportional to the estimated number of substitutions per position. The distance between two species is obtained by summing the lengths of the connecting horizontal branches using the scale at the bottom.

4.4 Removal of Uranium at Low Concentrations (10 mg/L)

Uranium(VI) removal was observed in both the aerobic and anaerobically grown cultures. 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. Overall, the anaerobic cultures performed better with the highest performance observed in the culture supplemented with the BMM. The aerobic cultures, both with and without BMM, did not perform as well. Although the percent removals are reasonably high (78% without BMM and 64% with BMM) the actual amount removed cumulatively is very low due to the low initial values recorded. 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. However, this finding is not conclusive since the method used in this study could only directly quantify total U. Thus the U(VI) removal is implied as the conversion to less soluble species that were removed by centrifugation and/or adsorption and complexation with cell surface structures.

The data on the U(VI) removal and varying growth conditions is summarised in Table 4.4. The data in the table shows that U(VI) removal percentage was highest in the anaerobic culture with BMM (96 % removal). The observed removal was attributed to biological transformation to a less soluble and adsorbable species such as uranium-4 (IV). On the other hand, the anaerobic culture without BMM performed poorly. The removal in the aerobic culture differed in that, the one without BMM performing better than the one with BMM.

It is also shown in Table 4.4 that the U(VI) recovery in anaerobic cultures was higher than in aerobic cultures. This indicates the removal of uranium species through abiotic processes under aerobic conditions. Possible mechanisms include complexation with

hydroxylated species and precipitation with other oxidised metal forms. This problem will later be remedied by using higher concentrations of uranium in the experiments to offset the effects of interference from other metallic species in solution (Chabalala and Chirwa, 2010).

Table 4.4 Summary of performance data under added U(VI) of 10 mg/L incubated for 24 hours under anaerobic and aerobic conditions listed with increasing efficiency.

Batch No.	Experimental Conditions	U(VI) Removal (%)	Actual U(VI) Removal mg/L	U(VI) Recovery (%)
1	Anaerobic no BMM*	89	4.99	56
2	Aerobic no BMM	78	1.46	18
3	Anaerobic + BMM	96	4.60	48
4	Aerobic + BMM	64	1.57	26

* The best performing batch based on total uranium removed.

4.5 U(VI) Removal at Moderate Concentrations (30-75 mg/L)

Initial U(VI) concentrations were varied in batch studies under anaerobic conditions. *Pseudomonas stutzeri*, a denitrifying bacteria, showed a gradual increase in the rate of uranium-6 removal at 50% of added U(VI) as the concentrations increased. The percentage removal of 100% was achieved at 24 hours for all the different concentrations as shown on Figure 4.6 and 4.7.

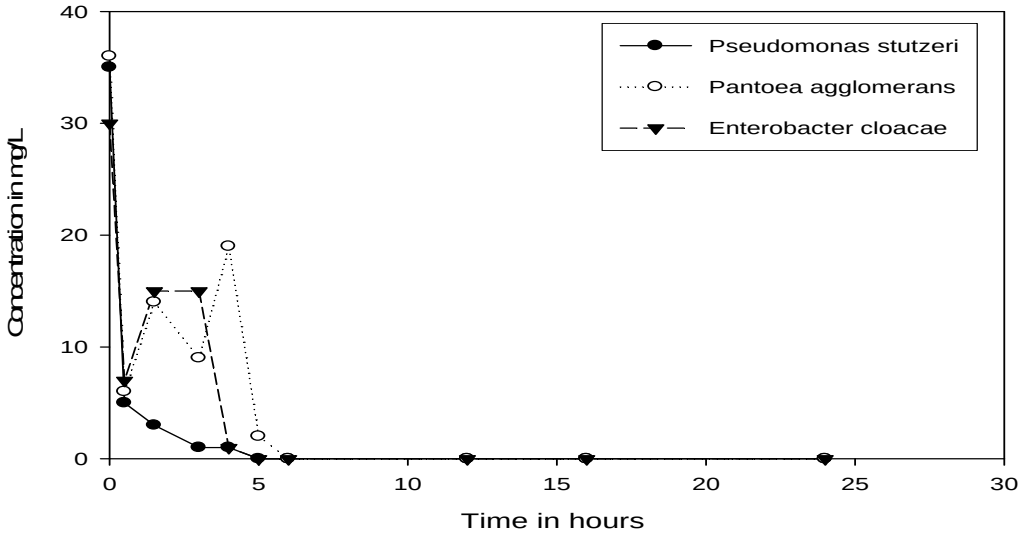


Figure 4.6 Uranium(VI) removal for the three pure cultures of bacteria *Pseudomonas stutzeri*, *Pantoea agglomerans* and *Enterobacter cloacae* under an initial concentration of 30 mg/L.

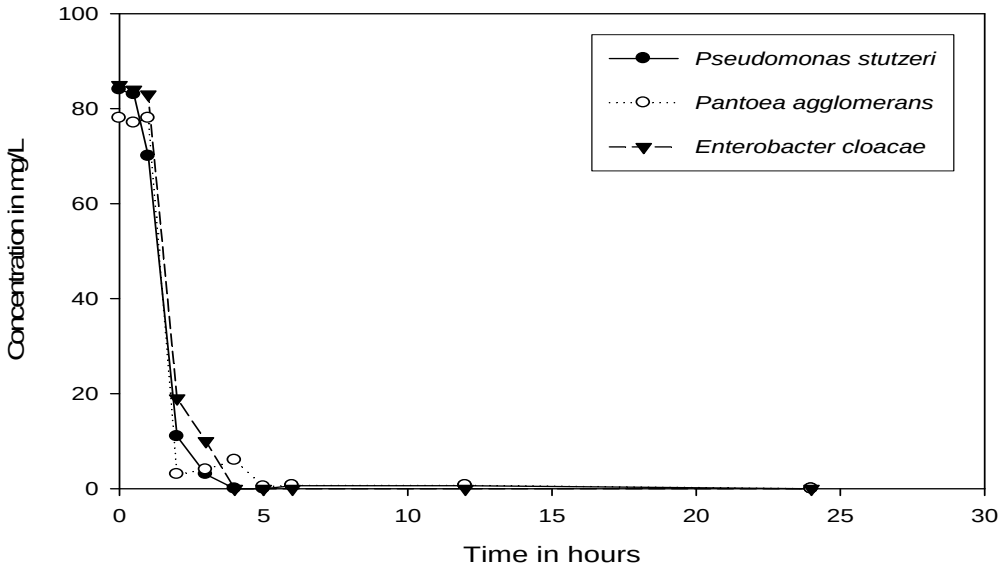


Figure 4.7 Uranium(VI) removal for the three pure cultures of bacteria *Pseudomonas stutzeri*, *Pantoea agglomerans* and *Enterobacter cloacae* under an initial concentration of 75 mg/L.

The strain of *Enterobacter sp.* proved to be the least efficient U(VI) remover among the cultures. It exhibited a lowest removal at all the concentrations (30 mg/L, 75 mg/L and 100 mg/L) and had a low percentage recovery of total uranium.

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 75 mg/L as well as 100 mg/L. Generally for all species, the removal of metal was very fast initially, and equilibrium was attained within 24 hours at pH of 5 to 6.

Percentage recovery of total uranium was low for the higher concentrations (75, 100 mg/L) and very high for the lowest concentration (30 mg/L) as shown on Table 4.5. The rate of uranim-6 removal at 75% of added U(VI) was not conclusive. It was high for the concentrations (30 mg/L and 100 mg/L) and low for the concentration (75 mg/L).

Table 4.5 Kinetic data for the moderate concentrations of uranium U(VI); (35,75mg/L).

Species	Initial concentration in mg/L	Rate of removal at 50% (mg/L per hour)	Rate of removal at 75% (mg/L per hour)	% Removal at 24 hrs	Total Uranium at the end of the experiment	% Percentage Recovery
Pseudomonas stutzeri	30	17	54	100	19	63
	75	20	22	100	22	29
Pantoea sp.	30	16	54	100	15	50
	75	20	42	100	20	27
Enterobacter sp.	30	9	46	100	10	33
	75	16	37	100	14	19

Pantoea sp. displayed a steady increase in rate of removal at 50% of added U(VI) as the concentration increased. Rate of removal at 75% of added U(VI) was also not convincing. This culture showed 100% removal at the end of 24 hours for all three concentrations. Total uranium recovery was highest for the lowest concentration (30 mg/L).

4.6 U(VI) Reduction at High Concentrations (100 -800 mg/L)

Initial U(VI) concentrations were varied in batch studies under anaerobic conditions. *Enterobacter sp.* showed a steady increase in the rate of uranium-6 removal at 50% of added U(VI) as the concentrations increased as shown on Figure 4.6. The percentage removal of 85-100% was achieved at 24 hours for all the different concentrations shown on Table 4.6. Thus, the highest removal efficiency for *Enterobacter sp.* was observed in batches started at 200 and 400 mg/L U(VI). The *Enterobacter* culture 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 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). And, BLAST and similarity analyses in literature indicated that some known U(VI)-reducers have a 96.3% match to *Enterobacter cloacae* (Lovley *et al*.; 2004).

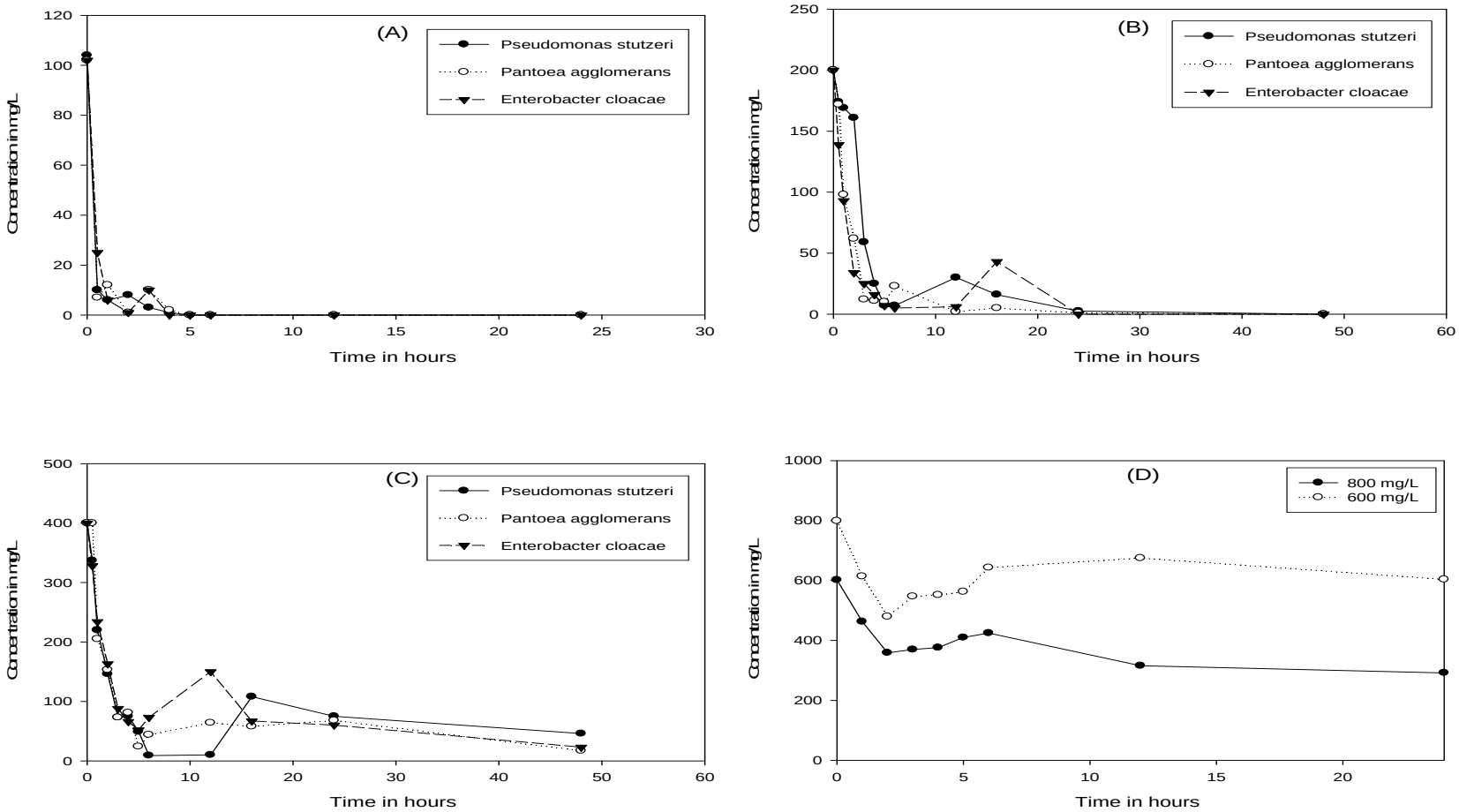


Figure 4.8 Uranium(VI) removal for the three pure cultures of bacteria *Pseudomonas stutzeri*, *Pantoea agglomerans* and *Enterobacter cloacae* under varying concentrations; A: 100 mg/L B: 200 mg/L C: 400 mg/L and D: 600 and 800 mg/L.

Table 4.6 Kinetic data for varying concentrations of uranium U(VI). (1) *Pseudomonas sp*., (2) *Pantoea sp*., (3) *Enterobacter sp*., and (4) Mixed culture.

Species	Initial conc. in mg/L	Removal Rate at 50% (mg/L/hr)	% Removal at 24hrs	Final Total U recovery %
1	100	50	100	32
	200	33	99	87
	400	100	81	84
2	100	50	100	36
	200	57	99.5	98
	400	111	83	71
3	100	37	100	30
	200	63	100	91
	400	198	85	89
4	600	24	51	100
	800	6	24	100

Percentage recovery of total uranium in all cultures was low for the 100 mg/L U(VI) and 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, although *Pseudomonas sp*. and *Enterobacter sp*. displayed inconsistencies during the period 10 to 20 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) had only managed to remove 89% of 400 mg/L U(VI), *Enterobacter sp.* 94% and the best performing specie was *Pantoea sp.* at 96% (Chabalala and Chirwa, 2010b).

The mixed culture achieved up to 51% removal at 600 mg/L after incubation for 24 hours; and a further decrease of removal to 24% for the higher concentration tested (800 mg/L). Furthermore, the rate of removal at 50% was limited but the percentage recovery of total uranium was at 100% for both 600 and 800 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 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.

4.7 Fate of U(VI) in the Batch Experiments

The TEM approach was used to visualize sites of preferred mineral formation as shown on Figure 4.9. Because of the insoluble nature of U(IV) oxide, the site of its deposition in or outside the cells should give an indication of the location of reductase. Reduction of U^{6+} produces black uraninite nanocrystals precipitated outside the cells. Some nanocrystals are associated with outer membranes of the cells as revealed from cross-sections of these metabolically–active bacteria. In this instance, heavy extracellular accumulation of U(IV) on the cell surface of the mixed culture. The distribution of biogenic UO_2 is consistent with the current understanding of electron transfer mechanisms in DMRB, where the metal reductase activity is associated with the cell membrane, periplasm and outer membrane. Due to low solubility of reduced U(IV), uraninite probably precipitated at or near the actual site of reduction (Beyenal et al., 2004).

Previous TEM analysis indicated that the precipitated uraninite has been located in the periplasm and outside of both Gram-negative and Gram-positive bacterial cells (Lovley and

Phillips, 1992; Xu et al., 2000; Liu and Fang, 2002) suggesting that U(VI) complexes do not generally have access to intracellular enzymes (Wall and Krumholz, 2006). The cytoplasmic uraninite deposit localization has been reported in few studies including those on *Pseudomonas sp.* and *D. desulfuricans* strain G20 (McLean and Beveridge, 2001; Sani et al., 2004). An intracellular localization of uraninite precipitated by the cells of *Desulfovibrio äspöensis*, a sulphate-reducing bacterium isolated in the vicinity of the Äspö Hard Rock Laboratory in Sweden, was observed also using TEM analysis. The mechanisms of intracellular uraninite precipitation are still not understood but one can speculate on the process of diffusion of reduced uranium nanoparticles from the periplasm to the cytoplasm (Xu et al., 2000).

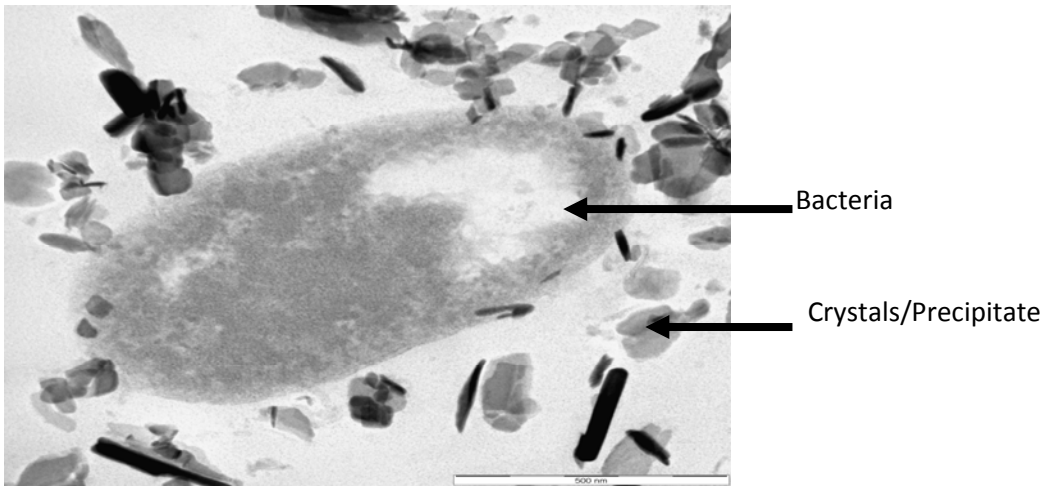


Figure 4.9 Transmission electron micrograph of thin sections of the mixed culture of P. *agglomerans*, E. *cloacae* and P. *stutzeri* — treated with uranium. The arrows indicate the presence of U in the uranium deposits as demonstrated by EDX analysis (data not shown) and bacterial cells 1:500 nm.

4.8 Viable Biomass Analysis at High U(VI) Concentrations (200mg/L)

The biomass count decreased sharply for the *Pseudomonas* sp. and *Enterobacter* sp. and *Pantoea* sp. decreased gradually initially due to changes in environment while the bacteria

adapt to their new environment. Within 3 hours, only 2% of *Pseudomonas* biomass was still viable,and thereafter kept decreasing until the end of the 24 hours of incubation where it increased by a small amount. Also, *Enterobacter* sp. had 16% of biomass that was viable after 3 hours and thereafter displayed the same behaviour as *Pseudomonas* sp. 83% of biomass was viable in Panotea sp. after 3 hours but by 6 hours it had reached zero and stayed that way for the next 6 hours and then increased very slowly thereafter. The significant decrease in viable biomass in all cultures throughout the experiment showed that the bacteria were not actively growing and were using just surviving at this relatively high concentration of uranium. The exponential death rate correlates with the results obtained for uranium removal, and it can therefore be postulated that removal occurs while the cells are metabolically active because after 5 hours, growth is actually negative for all species and becomes positive after 12 hours.

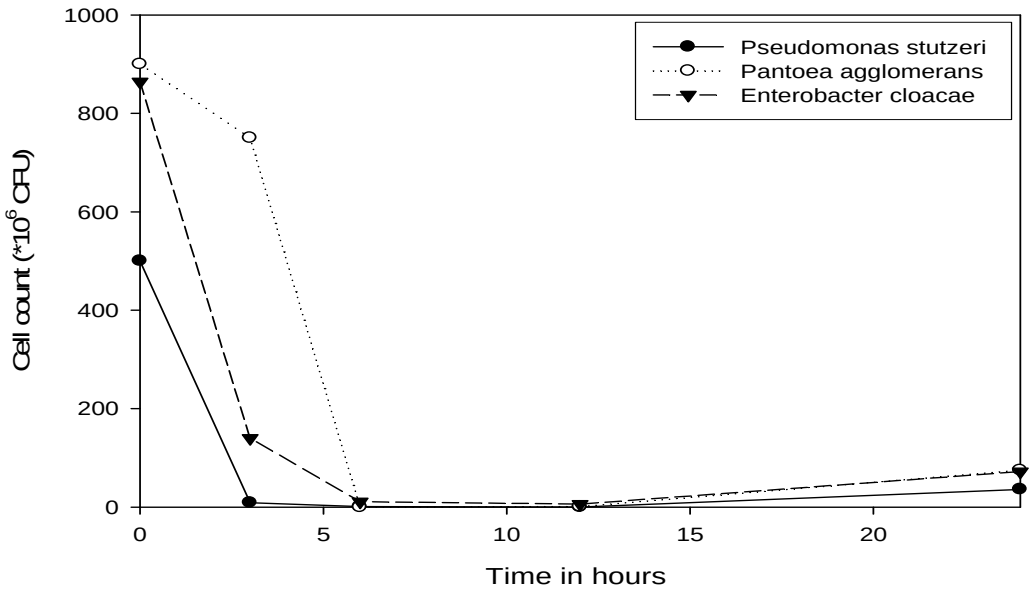


Figure 4.10 Viable biomass analysis for the three cultures *Pseudomonas stutzeri*, *Pantoea agglomerans* and *Enterobacter cloacae* under varying concentrations at 200 mg/L.

4.9 Influence of Different Electron Donors on Biological Uranium U(VI) Reduction

Reduction and immobilization of microbially-reduced U(VI) is of great concern for *in situ* uranium bioremediation. This study investigated the effect of carbon sources; Sodium Acetate (NaAc), Ethanol (EtoH) and Sawdust (Organic carbon source) on uranium removal all at 5 g/L for each experiment.

4.9.1 Influence of Different Carbon Sources on the Rate of U(VI) reduction by *Enterobacter cloacae*

In all the U(VI) concentrations (35, 75, 100, 200, 400 mg/L), this culture removed uranium(VI) the fastest. At 30 mg/L U(VI) the batch with NaAc as the carbon source removed U(VI) at the fastest rate and at 75 mg/L, the batch with EtoH as the carbon source removed the fastest. At higher concentrations, all the batches of different carbon sources performed well and achieved high removal rates. The total U(VI) concentrations were also quite high for the higher concentrations (100 – 200 mg/L) but low for the lower concentrations (35 and 75 mg/L). At 50 % and 75 % of added U(VI), rates of removal were remarkably high for all carbon sources and all concentrations.

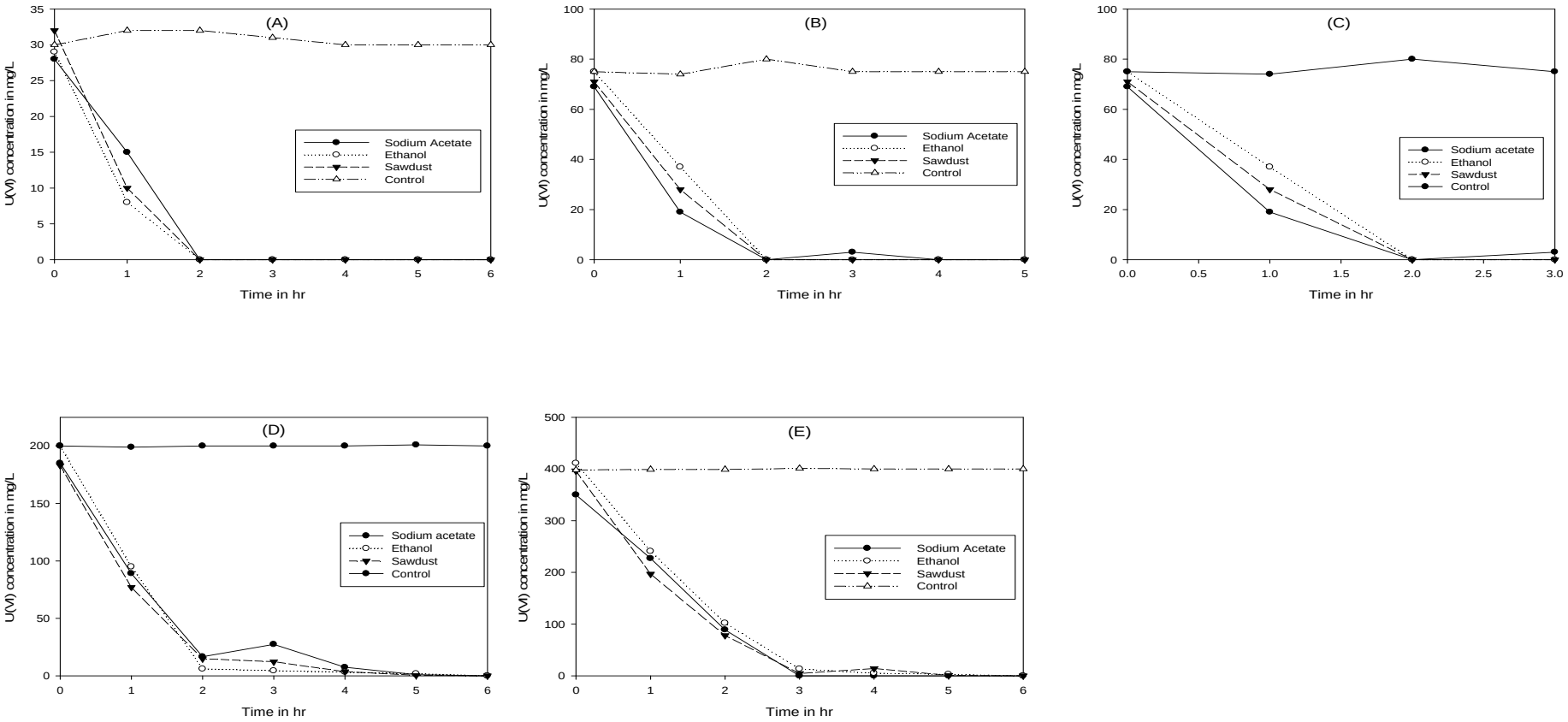


Figure 4.11 *Enterobacter sp.* under varied carbon sources, A: 30 mg/L, B: 75 mg/L, C: 100 mg/L, D: 200 mg/L, E: 400 mg/L.

4.9.2 Influence of Different Carbon Sources on the rate of U(VI) Reduction by *Pantoea agglomerans*

For concentrations; 30, 75, 100, 200 mg/L, sawdust was the carbon source in which the batches achieved 100 % removal of U(VI) after 3–4 hours. At 35 mg/L, after 2 hours the concentrations of NaAc and EtOH batches increased dramatically to initial concentrations and thereafter recovered and decreased to zero after 3 hours. At 400 mg/L, the total U(VI) at the end of the experiment was about 50 % of the initial concentration and the rate of removal was remarkably high as this culture was removing at a rate of approximately 300 mg/L/h in the first hour. EtOH was the carbon source that allowed the culture to remove U(VI) at the fastest rate of all the other carbon sources.

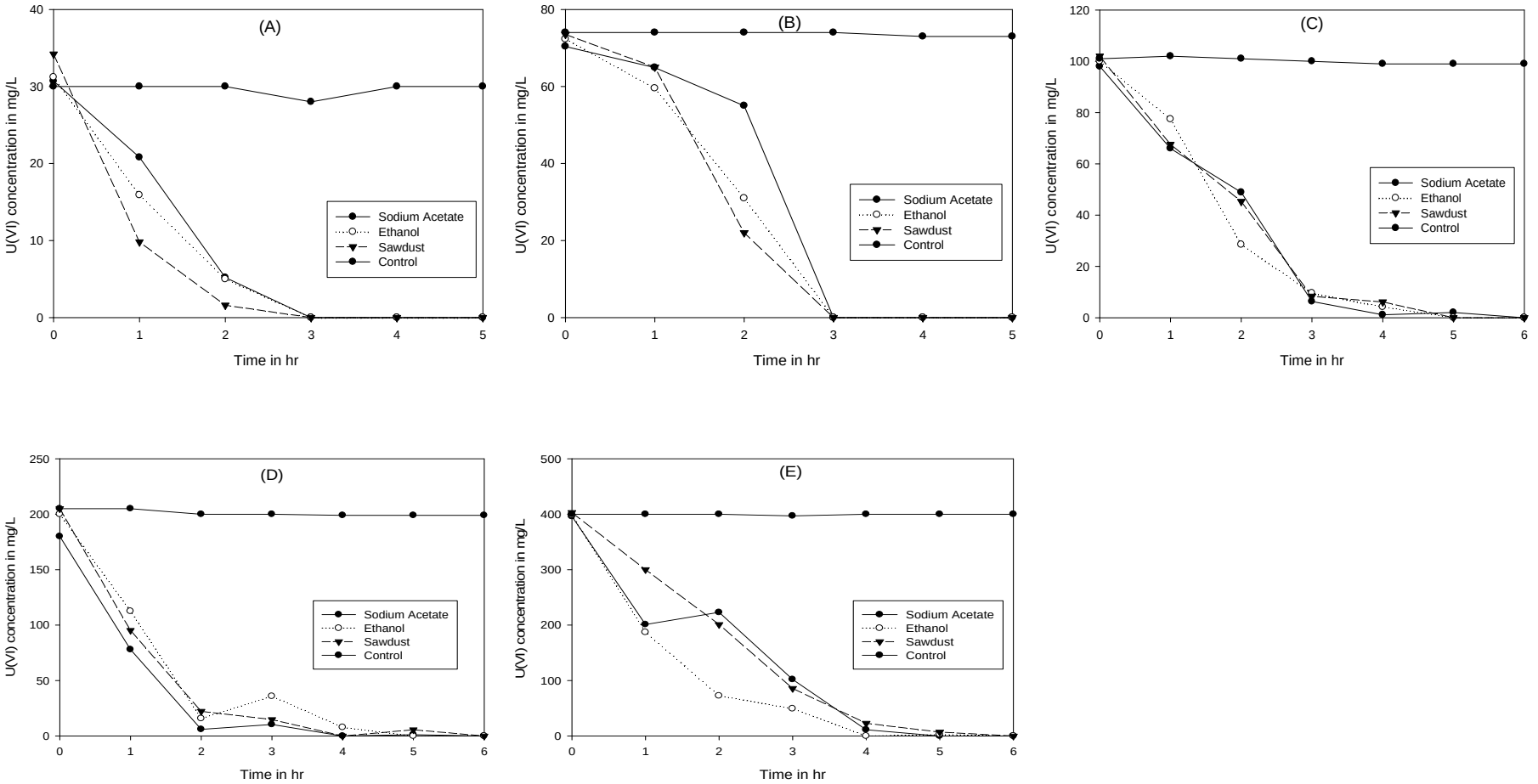


Figure 4.12 *Pantoea sp.* under varied carbon sources, A: 30 mg/L, B: 75 mg/L, C: 100 mg/L, D: 200 mg/L, E: 400 mg/L.

4.9.3 Influence of Different Carbon Sources on the Rate of U(VI) Reduction by *Pseudomonas stutzeri*

At 50 % of added U(VI), the rate of removal was highest in Sawdust at 23 mg/L/h, also the total uranium recovery was highest at the end of the experiment. This culture removed the slowest in medium containing NaAc. In all the carbon sources, at the end of 3 hours all the U(VI) had been removed. In the lower concentrations (35 – 75 mg/L), the batch containing Sawdust as a carbon source performed well and at a fast rate compared to the other carbon sources. Total U(VI) at the end of the experiments for the 30 mg/L batch were quite good.

At every data point, the batch grown in EtOH as a carbon source removed 400 mg/L U(VI) at a faster rate. 50% of added was removed after the hour. The U(VI) recovery rate was also very high for all carbon sources. At 75 % of added U(VI), the rate of removal for the 200 mg/L batch was almost 100 mg/L/h. The rate of reduction of 100 mg/L was slow compared to the higher concentrations 200 – 400 mg/L. Total uranium concentrations went down by as much as 50 % and more and remained there until the end of the experiment.

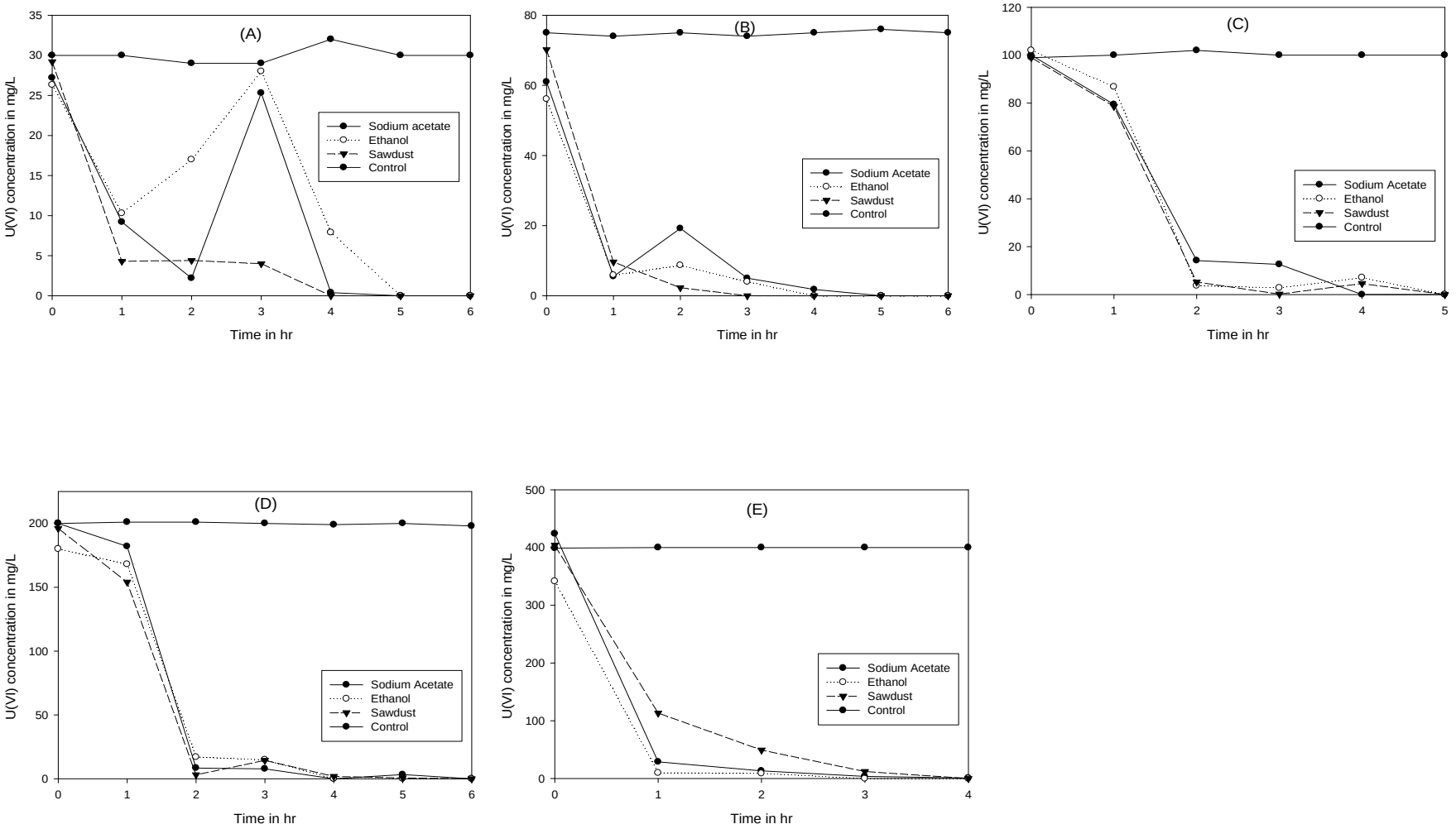


Figure 4.13 *Pseudomonas sp.* under varied carbon sources. A: 30 mg/L, B: 75 mg/L, C: 100 mg/L, D: 200 mg/L, E: 400 mg/L.

4.9.4 Influence of Different Carbon Sources on Rate of U(VI) Reduction by the Mixed Culture

4.9.4.1 At Low U(VI) Concentrations

At 50 % of added U(VI), the rate of removal for sawdust was at 30 mg/L/h which was the highest compared to that of EtOH and NaAc, also Sawdust reached zero after 3 hours of incubation as shown in Figure 4.14. At 75 % of added U(VI), the mixed culture was removing the fastest in Sawdust carbon source, at 42 mg/L/h, closely followed by NaAc and EtOH. Total U(VI) values were varying from 40 - 78 %, after an hour, the experiments carried out in NaAc had a uranium recovery of 78 %, after 3 hours, a recovery of 67 % and lastly after 6 hours, a recovery of 52 %, there was a definite decrease in total uranium recovery, whereas the experiments carried out in sawdust there was an increase in total uranium recovery. EtOH on the other hand, in the first hour total uranium concentration had been half the amount initially began with, then wnet up to 73 % and finally after the experiment, it went back to 50 %.

The culture grown in medium with sawdust as an electron donor removed all the U(VI) within 4 hours of incubation, the other two carbon sources only reached zero after 5 hours of incubation. Recovery of uranium at the end of the experiment was very low in NaAc and EtOH, and moderate in Sawdust. By the end of the experiment all the carbon sources total uranium concentrations were at their lowest between 20 – 26 %.

At the beginning of the experiments, the total uranium concentrations were at around 60 % and after the experiment, it went down drastically except for the NaAc experiment which ended at 100 % of added U(VI). Sawdust and EtOH carbon sources removed U(VI) levels to zero within the first hour, but after that the data was scattered until they reached zero after 5

hours of incubation. There was a definite trend in terms of carbon sources but in the lower concentrations (35 – 75 mg/L) Sawdust seems to be the carbon source that allows for U(VI) concentrations to reach zero first. For the higher concentrations (200 – 400 mg/L), NaAc is the carbon source where U(VI) levels reached zero first

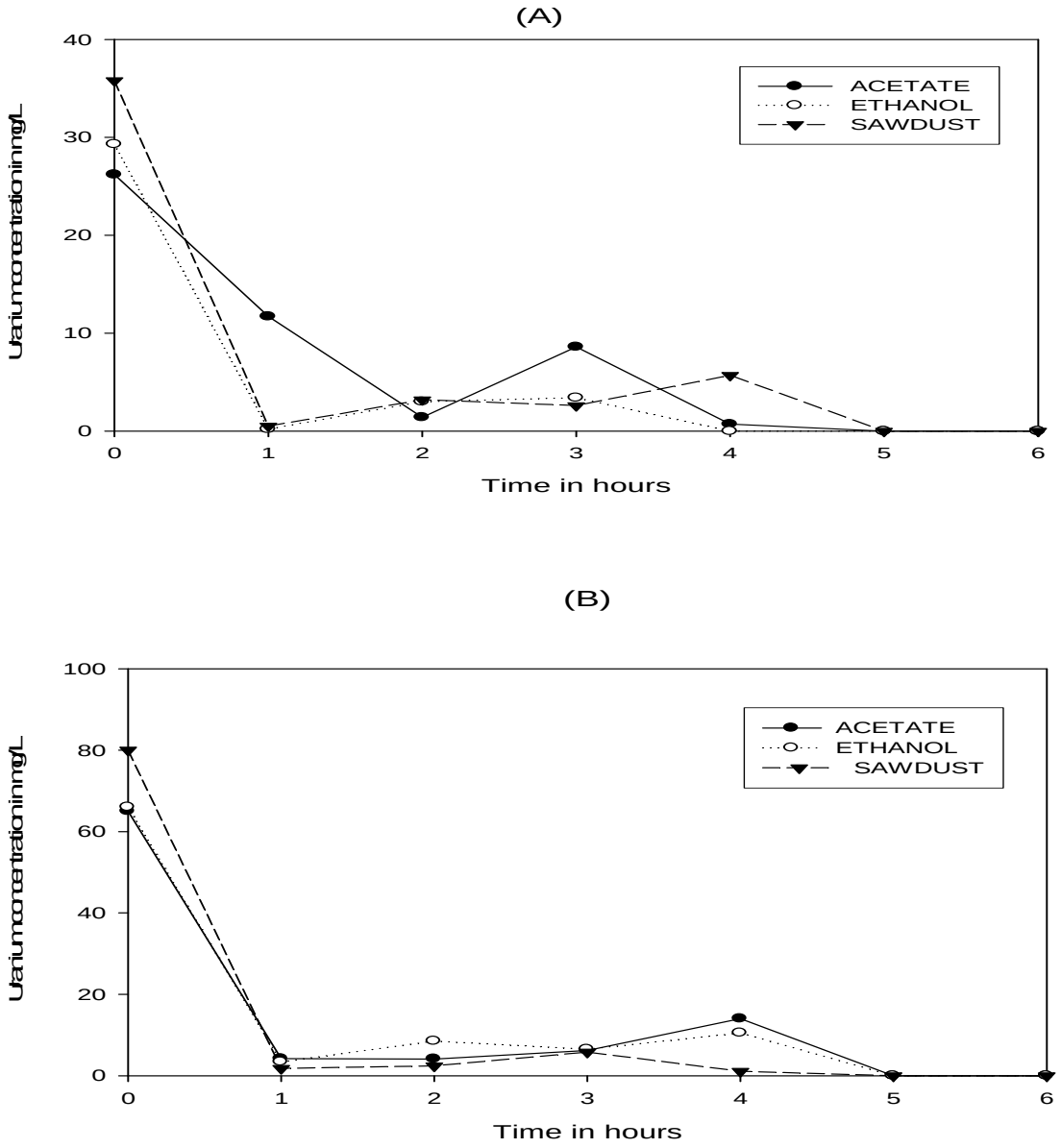
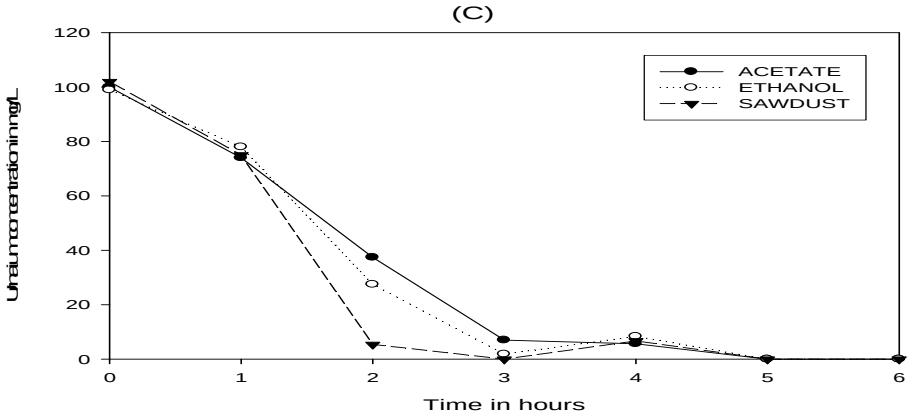


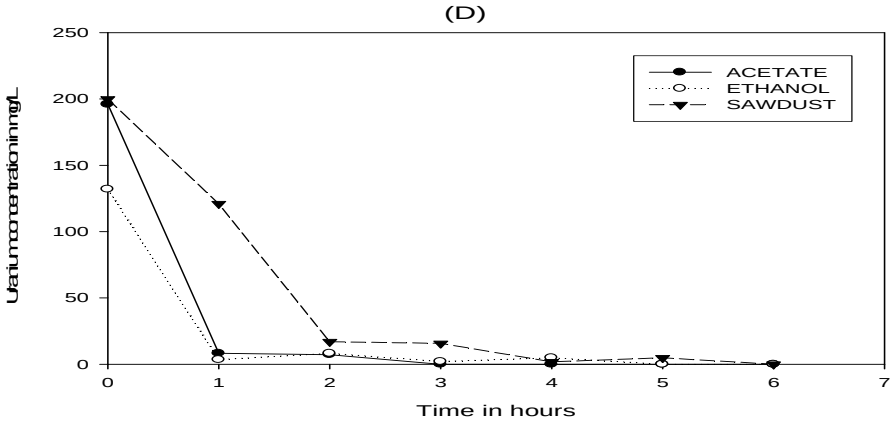
Figure 4.14. Mixed culture under varied carbon sources, A: 30 mg/L, B: 75 mg/L.

4.9.4.2 At High U(VI) Concentrations

At 400 mg/L of U(VI), mixed culture removed approximately 93% of added uranium within the first hour for all carbon sources, moreover, 84-99% of U(VI) was also recovered at the end of the experiment for all carbon sources. It has to be noted that the uranium concentration of the batch culture grown in NaAc, reached zero first although the U(VI) rised again after 3 hours only to go back to zero after 5 hours. The rate of reduction (mg/L/h) at 50 % of added U(VI) for NaAc and Sawdust was very high at 285 mg/L/h, EtOH was just under that at 253 mg/L/h. Looking at the very high concentrations of total U(VI), we can conclude that recovery rates are very good and that the values are remaining relatively constant shows that U(VI) is being reduced to another form and when treated with HNO_3 all these forms are oxidized to U(VI).

U(VI) recovery rates ranging from 75 - 100 % for all carbon sources were recorded. The mixed culture with EtOH and NaAc as electron donors, reached zero first within the first 3 hours of incubation, the experiment carried with Sawdust only reached zero after 4 hours of incubation. The highest rate of reduction was observed in NaAc followed by EtOH then sawdust and shown in Figure 4.15.





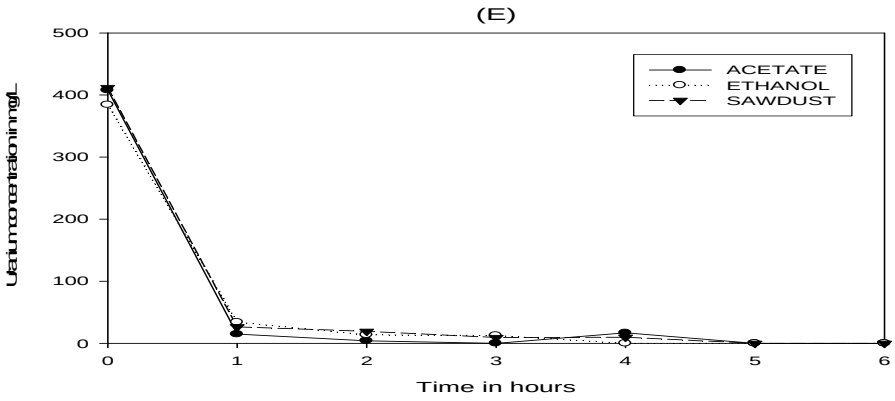


Figure 4.15. Mixed culture under varied carbon sources, C: 100 mg/L, D: 200 mg/L, E: 400 mg/L.

4.10 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) removal were performed with repetitive U(VI) loadings. 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 removed 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 removing U(VI) 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 removal 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).

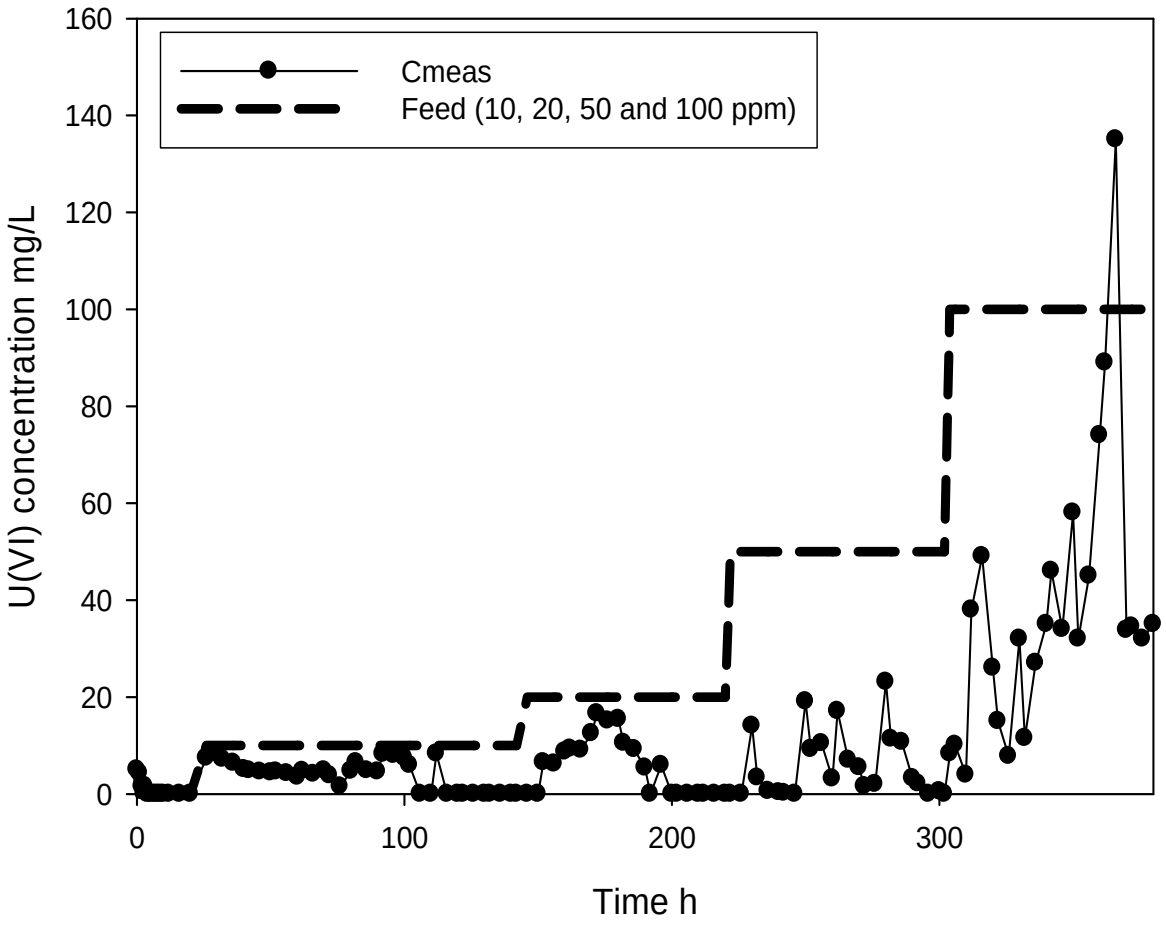


Figure 4.16 Continuous flow reactor performance for U(VI) removal by mixed culture; Pseudomonas sp., Pantoea sp. and Enterobacter sp. The concentration of U(VI) in feed was 10, 20, 50 and 100 mg/L respectively.

4.11 Summary

U(VI) reduction under heterogenous carbon source (sawdust) was higher because of the high carbon content for the micro-organisms to utilize. But also, it is not as good as glucose for this purpose as shown by the lower value of 32.5 mg carbon/L of medium, compared with

1.8 g carbon /L glucose. Nevertheless, sawdust as a carbon source is a viable alternative as it is available in abundance and is cheap to obtain. Ethanol was expected to increase the rate of reduction of the cultures as it provides both the carbon source and the electron donor in the forms of ethanol, acetate (metabolic intermediate), and methanol (an impurity in industrial ethanol) but it performed poorly (Cardenas et al., 2008). In this study, the low reduction rates recorded for acetate can be attributed to the fact that the U(VI) may have been unavailable for reduction due to U(VI)-acetate complexation and/or poor growth of anaerobic microorganisms capable of degrading acetate (Duff et al., 1999).

The results show that there is an interaction between the U(VI) and cultures because U(VI) concentration decreased when they were exposed to U(VI). And that interaction can be regarded as reduction because U(VI) was converted to another form, presumably U(IV), the total uranium at the end of the experiments verify that a chemical change occurred. All carbon sources tested promoted consortium activity and stimulated the reduction and immobilization of aqueous uranium by the indigenous microbial community. It has been reported that the particular electron donor chosen affects not only the rate of uranium removal from solution, but also the extent of U^{6+} conversion to U^{4+}.

CHAPTER 5

URANIUM(VI) REDUCTION KINETICS

U(VI) reduction data obtained with three bacterial species; *Pseudomonas sp., Pantoea sp*., and *Enterobacter sp.* were analyzed using an enzyme-based model. The model was developed by incorporating enzyme kinetics and a U(VI) reduction capacity to illustrate toxic effects of U(VI). The reduction capacity indicates the maximum amount of U(VI) that a batch culture can reduce and the loss of U(VI) reduction capacity in bacterial cultures may be attributed to toxic effects of U(VI). This model was originally used by Wang and Shen (1997) to describe Cr(VI) reduction in *E. coli* 33456 and Chirwa and Wang (2004) to describe Cr(VI) reduction in *Bacillus sp.* The model described a similar pattern of U(VI) reduction in the different bacterial species.

A thorough understanding of the underlying kinetic processes of U(VI) transformation in bacteria is required in order to design and operate U(VI) bioremediation systems. Biological reactions are identified and modelled based on enzyme kinetics and the kinetic parameters are determined through batch studies. In this study, optimum values of kinetic parameters were estimated using a computer programme for Simulation of Aquatic Systems (AQUASIM 2.0)

5.1 Kinetic Model Development

In living bacterial cells, metal reduction, particularly that of Cr(VI), is linked to cellular metabolic processes as illustrated in earlier by Wang *et al*. (1989), Ohtake *et al*. (1990), Shen and Wang (1993) and others. Shen and Wang (1993) demonstrated that Cr(VI) was reduced by NADH as a sole electron donor. They assumed the reaction was catalyzed by the transmembrane bound NADH-dehydrogenase, while others such as Lovley and Phillips

(1994) showed that transmembrane electron carriers such as cytochrome c_3 was responsible for reduction.

The biochemical process of metal reduction (Cr(VI) was assumed to be that electron donors from the cytosol transfer electrons to the transmembrane electron carriers and protons pumps, which in turn transfer the electrons onto Cr(VI) directly, or mediated through a membrane associated reductase (Bopp and Ehrich, 1988; Ishibashi *et al*., 1990). We assume that the same biochemical process applies to U(VI) reduction.

To simplify the model, they suggested that a single representative enzyme reduced U(VI) as a result of overall U(VI) biochemical reduction process by bacterial cultures. The U(VI) reduction equation was thus written as follows:

$$U(VI) + E \underset{k_2}{\overset{k_1}{\longleftrightarrow}} E*U(VI) \xrightarrow{k_3} E + U(IV) \tag{5.1}$$

Where: E is a *U(VI)* reductase

$E*U(VI)$ is a transitional enzyme-U(VI) complex

k_1, k_2, k_3 are reaction rate constants in the directions indicated by the arrows

If the U(VI) concentration is represented by U and the enzyme-U(VI) complex by $E*$ *U(VI)*, and assuming that $E*$ forms and disappears spontaneously, the rate of U(VI) reduction is equal to the formation of the U(IV) formation. The overall rate of the reaction can be represented by:

$$r = -\frac{dU}{dt} = \frac{dU(IV)}{dt} k_3 \cdot E* \tag{5.2}$$

The formation of $E*$ can be described by:

$$-\frac{dE^*}{dt} = k_1(E - E^*)(U) - k_2(E^*) - k_3(E^*) \quad (5.3)$$

In the above equation, steady-state conditions can be assumed to prevail, as long as E^* is formed and destroyed spontaneously so that $d(E^*)/\ d(t) = 0$. The mass balance represented by Equation 5.3 can thus be written as:

$$0 = k_1(E - E^*)(U) - k_2(E^*) - k_3(E^*) \quad (5.4)$$

After rearranging Equation 5.4, E^* can be expressed as:

$$E^* = \frac{U \cdot E}{U + \frac{k_2 + k_3}{k_1}} \quad (5.5)$$

Thus, the U(VI) reduction rate in Equation 5.2 becomes:

$$r = -\frac{dU}{dt} = \frac{k_3 \cdot U \cdot E}{U + \frac{k_2 + k_3}{k_1}} \quad (5.6)$$

In this equation, k_1, k_2 and k_3 are constants, the groups of constants in Equation 6 can be replaced by meaningful symbols from enzyme kinetics as follows: $(k_2 + k_3)/ k_1$ can be replaced by the half velocity concentration K_u (mg/L), and k_3 can be replaced by the maximum specific U(VI) reduction rate coefficient k_u (mg/mg/h) such that:

$$r = -\frac{dU}{dt} = \frac{k_u \cdot U \cdot E}{U + K_u} \quad (5.7)$$

For any amount of cells X, the amount of enzyme produced will be proportional to the cell concentration such that the enzyme E can be replaced by the total cell biomass term X, if cells are harvested during the log growth phase. This gives a Monod type equation:

$$r = -\frac{dU}{dt} = \frac{k_u \cdot U \cdot X}{U + K_u} \qquad (5.8)$$

Where: U is the U(VI) concentration at time t (mg/L)

k_u is the maximum specific U(VI) reduction rate coefficient ($mg/mg/h$)

K_u is the half velocity constant (mg/L)

X is the concentration of viable cells at time t (mg/L), and

t is time (t)

A similar expression was derived previously by other researchers for Cr(VI) reduction in batch systems (Shen and Wang, 1994; Mazierski, 1995; Schmieman et al., 1998; Guha et al., 2001; Li et al., 2006; Shashidhar, 2007).

In this model, the only unknowns are K_u and k_u, and we can assume a stationary phase with respect to X as the experiment is carried out under high biomass concentrations. The high biomass concentrations lead to the assumption that X is constant, $X = Xo$. To determine kinetic parameters, the analytical solution of Equation 5.8 was used and expressed as a function of time as shown below:

$$t = -\frac{K_u}{X_o \cdot k_u} \ln\left(\frac{U_o}{U}\right) + \frac{1}{X_o + k_u}(U - U_o) \qquad (5.9)$$

where X_O = initial biomass concentration (mg/L) and U_O = initial U(VI) concentration (mg/L).

The parameters were estimated by optimization of Equation 5.9 against batch experimental data using AQUASIM 2.0.

To model the system, the reaction scheme, rate equations and kinetic constants for the processes taking place in the batch reactor were chosen from published models on enzymatic (UVI) reduction. Shen and Wang (1997) demonstrated that the rate of U(VI) reduction by

enzymes can be expressed as the Monod equation (5.10) below when enzyme activity is the predominant mechanism of U(VI) reduction in bacterial cells:

$$-\frac{dU}{dt}=\frac{k_u \cdot U}{K_u + U}\cdot X \tag{5.10}$$

where U (mg/L) is the concentration of U(VI) at time t (h); X (mg cells/L) is the density of active bacterial cells at time t; k_u (mg U(VI)/mg cells/h) is the specific rate of U(VI) reduction; and K_u (mg U(VI)/L) is the half velocity constant. However, the active cell concentration, X, may be assumed to decrease in proportion to the amount of U(VI) reduced due to the toxicity of U(VI):

$$X = X_o - \frac{U_o - U}{T_c} \tag{5.11}$$

where U_0 (mg/L) is the initial concentration of U(VI); X_o (mg cells/L) is the initial cells density of U(VI)-reducing strains; and T_c (mg U(VI)/mg cell) is the maximum U(VI) reduction capacity of cells. Substituting Equation (5.11) into Equation (5.10) yields the following equation:

$$-\frac{dU}{dt}=\frac{k_u \cdot U}{K_u + U}\left(X_o - \frac{U_o - U}{T_c}\right) \tag{5.12}$$

U(VI) reduction data obtained with the pure cultures and the mixed culture were analyzed using Equation (5.12). AQUASIM simulations were performed for best fits of Equation (5.12) to U(VI) versus time curves to search the parameter values of k_u, K_u and T_c. Trial values of parameters were initially random, and the Equation (5.12) was solved by minimizing the sum of squares of difference between the dependent variable t, in the model

and experimental data. All parameters were searched within the prescribed range of constraints based on prior knowledge of the most likely range for biological systems.

5.2 Model Calibration

The model was calibrated using data from the batch reactor which was operated for 48 hours at a time under micro-aerobic redox conditions. Samples were collected at 1 hour intervals and analyzed for U(VI) concentration using the Arsenazo-III method.

5.3 Model Simulation and Parameter Optimization

Kinetics parameters were initialized by guessed values and values from batch studies found in literature for U(VI) reduction, followed by optimization. To ascertain that the values obtained using the mathematical model were reliable, upper and lower constraints were set for each parameter to allow the omission of nonsensical or invalid parameter values. Whenever optimization converged at/or very close to a constraint, the constraint was relaxed until the constraint did not force the model. The procedure was repeated until unique values lying away from the constraints but between set limits were found for each parameters (Chirwa and Wang, 2004).

5.4 Results and Discussion

5.4.1 Modelling U(VI) Reduction by *Enterobacter cloacae*

The model equation (3) also describes U(VI) reduction by *Enterobacter* sp. very well. Parameters k_u, K_u and T_c listed in Table 5.1 were obtained with curves of initial concentrations 100 mg/L. Good fits between model simulation and experimental data were noted for all data sets with initial concentrations ranging from 30 mg/L to 400 mg/L as shown on Fig. 5.1. The U(VI) reduction capacity, T_c, was again evident in the batch *E. cloacae*

culture, as reduction increased with biomass concentration. Thus, reduction capacity concept used in the kinetic expression is consistent with experimental data obtained with *Enterobacter* sp.

Table 5.1 Kinetic parameters for U(VI) reduction in *Enterobacter cloacae.*

Concentration (mg U(VI)/L)	K_u **(mg U(VI)/L)**	k_u **(mg U(VI)/mg cell/h)**	T_c **(mgU(VI)/mg cell)**	***Xo* (mg cells/L)**	χ^2
30	79.9	0.26	0.97	293.9	248
75	79.7	0.25	0.97	379.8	1587.8
100	79.8	0.25	0.9	1504.3	114.6
200	79.9	0.25	0.9	694.7	2371
400	79.9	0.26	0.98	670.6	36318.8

In 1993, Yamamoto *et al*., noted that this culture reduced Cr(VI) with an increased rate while cell density decreased progressively during Cr(VI) reduction over initial Cr(VI) concentrations ranging from 0.46 to 0.85 mM. Similar behaviour is seen here with U(VI) reduction and it may be attributed to toxic effects of the U(VI) on the bacterial cells. The reduction capacity of this culture was up to 294 mg/L and this may suggest the energy yields from U(VI) reduction may not be conserved as biochemical energy for cell growth under the micro-aerobic conditions. This is shown on the model which assumes continuous reduction of active cells during U(VI) reduction and is also supported by the absence of association between U(VI) reduction and cell growth. U(VI) reduction may occur as a result of co-metabolism.

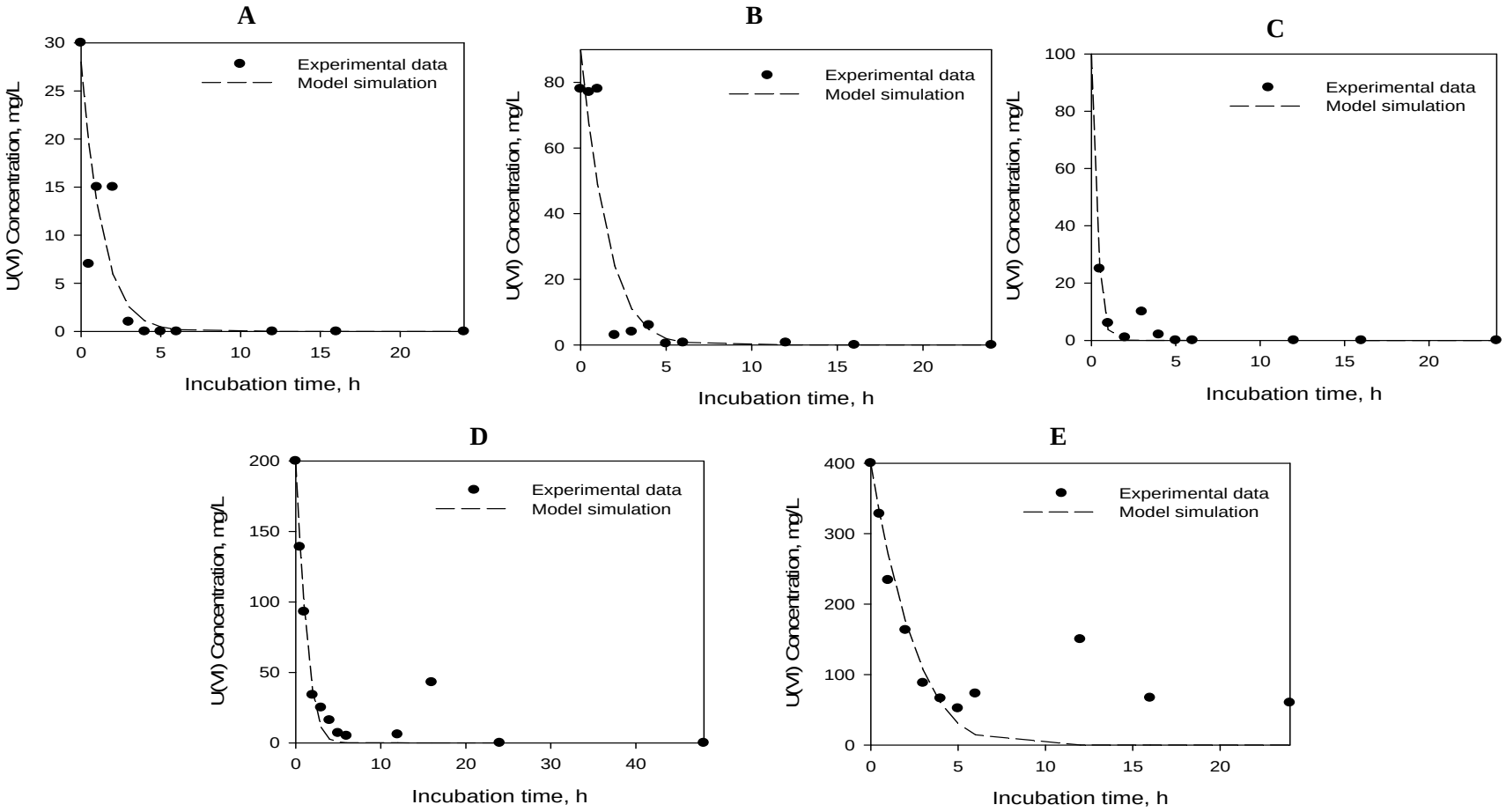


Figure 5.1 U(VI) reduction in batch cultures of *Enterobacter sp.* for concentrations ranging from 30 to 400 mg/L.

5.4.2 Modelling U(VI) Reduction by *Pantoea agglomerans*

A very high reduction capacity was observed in the *Pantoea* sp., and this capacity varied significantly with the initial U(VI) concentration (Fig. 5.2). The reduction capacity was limited with regard to initial biomass concentration. However, the loss of U(VI) reduction capacity cannot be attributed to termination of cell metabolism but rather to a decrease in active biomass over time due to its exponential decay during U(VI) reduction.

Table 5.2 Kinetic parameters for U(VI) reduction in *Pantoea agglomerans*.

Concentration (mg U(VI)/L)	K_u **(mg U(VI)/L)**	k_u **(mg U(VI)/mg cell/h)**	T_c **(mgU(VI)/mg cell)**	***Xo*** **(mg cells/L)**	$\chi 2$
30	99.7	0.39	0.4	210	491
75	99.6	0.31	0.49	340	2037.8
100	99.3	0.3	0.4	2400	246.5
200	98.6	0.3	0.49	663.5	1360
400	98	0.39	0.4	919.3	14078

The best fit for this culture was observed at uranium concentration 200 mg/L, the k_u, K_u and T_c values obtained for the 75 mg/L data set were used to simulate all the other concentrations. The fits were very good at the higher concentrations; 200 and 400 mg/L, but were not very good at lower concentrations; 30 and 75 mg/L. Compared to *Pseudomonas sp*. this culture's experimental data and model predictions were dissimilar. This shows that U(VI) reduction did not cease at any initial concentration as the T_cX_o value increased with an increase in initial U(VI) concentration thus the reduction capacity concept used in the kinetic expression in consistent with experimental data obtained with this culture. Additionally, χ^2 is stable until 200 mg/L, therefore at 400 mg/L was inaccurate

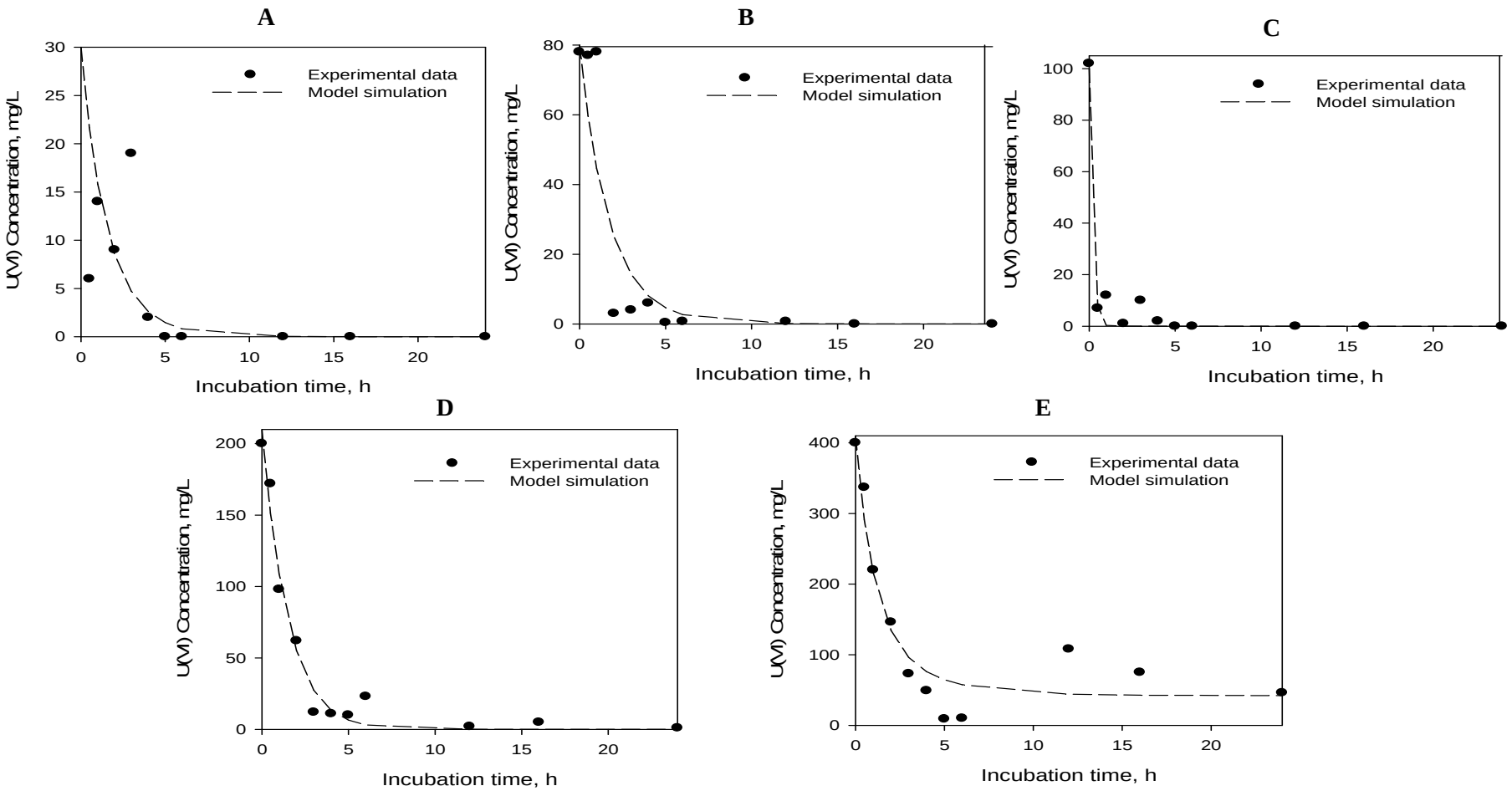


Figure 5.2 U(VI) reduction in batch cultures of *Pantoea sp.* for concentrations ranging from 30 to 400 mg/L.

5.4.3 Modelling U(VI) Reduction by *Pseudomonas stutzeri*

Figure 5.3 shows the model fits and experimental data of U(VI) versus time in batch cultures of *Pseudomonas* sp. at high initial U(VI) concentrations; 200 and 400 mg/L. The data set that was used to estimate parameters k_u, K_u and T_c was the initial U(VI) concentration 200 mg/L as shown on Table 5.3.

Using the obtained parameter values, the model simulated U(VI) reduction well at high concentrations only as shown in Figure 5.3. The reduction capacity T_c remained stable and was much lower than that of the other two species examined under the same conditions. Also, a loss of accuracy with increasing initial U(VI) concentration as illustrated by an increase in χ^2.

Table 5.3 Kinetic parameters for U(VI) reduction in *Pseudomonas stutzeri*.

Concentration (mg U(VI)/L)	K_u (mg U(VI)/L)	k_u (mg U(VI)/mg cell/h)	T_c (mgU(VI)/mg cell)	Xo (mg cells/L)	χ^2
30	99.4	0.01	0.08	42837.7	7.6
75	99	0.01	0.079	9288.1	1105.6
100	99.8	0.01	0.08	65243.3	104.8
200	99.6	0.01	0.089	12212.8	2776.1
400	99.8	0.01	0.08	19673.7	21835.9

Pseudomonas sp. viable cells decreased very slowly from an initial concentration of 12.9 mg cell/L to 0.05 mg cell/L during U(VI) reduction. Despite such significant cell death, the rate of U(VI) reduction in this culture increased accordingly even after 3 h incubation and eventually approaching zero. In addition, the model only requires the input of the initial

biomass and describes the experimental data very well without the need for further consideration of biomass growth or death.

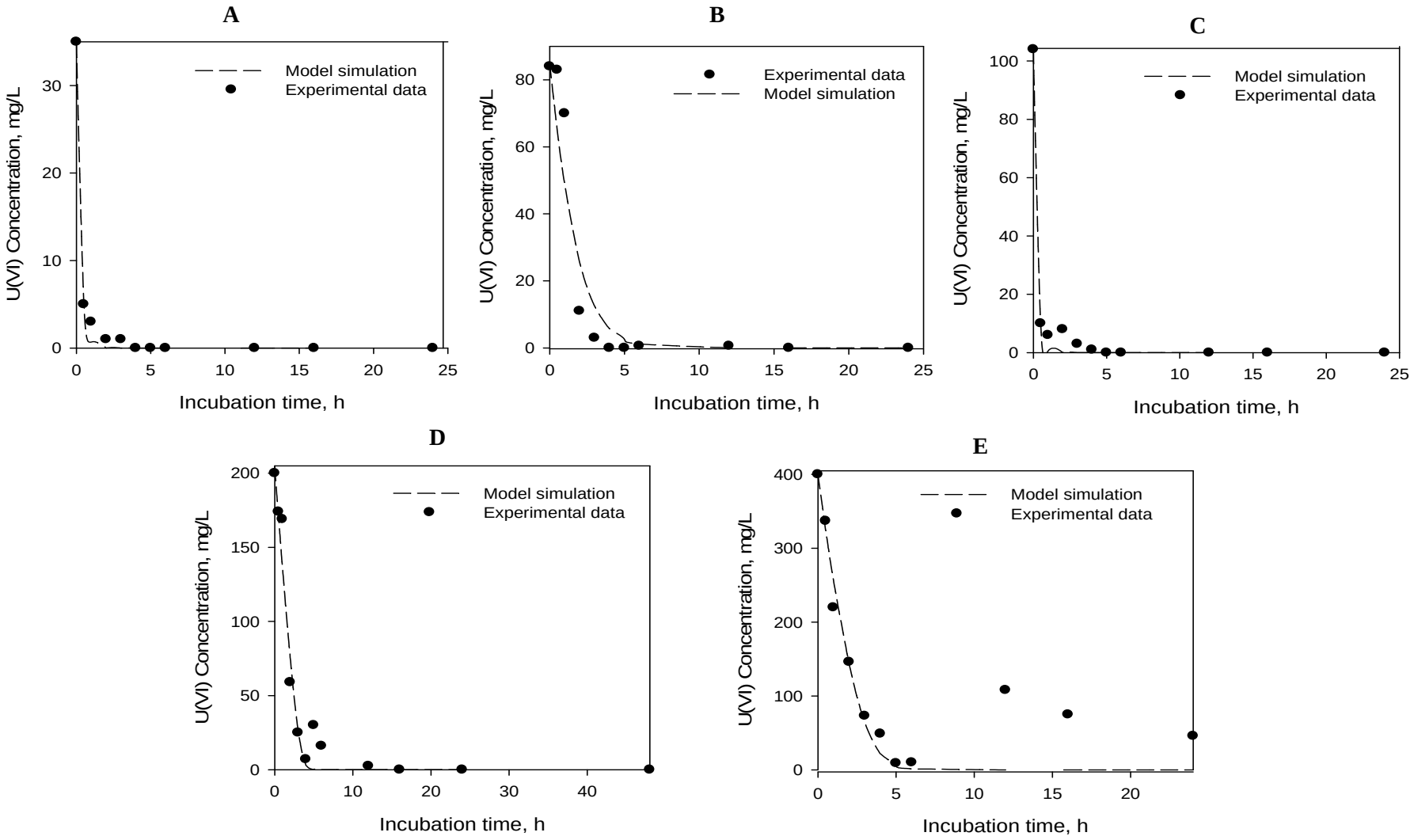


Figure 5.3 U(VI) reduction in batch cultures of *Pseudomonas sp.* for concentrations ranging from 30 to 400 mg/L.

The reduction capacity varied significantly with the initial U(VI) concentration. The removal capacity increased with increasing initial concentration. All the values observed from experimental data (ranging from 35-320 mg/L) fell within the limits of the model and it was found that the higher the X_o, the higher the reduction capacity of the microorganism.

5.4.4 U(VI) Reduction by the Consortium

The reduction capacity of the mixed culture was up to 234 mg/L with an initial maximum biomass of 11.94 mg cells/mL at an initial U(VI) concentration of 200 mg/L. The infinite reduction capacity of U(VI) reduction was further demonstrated in this culture as a rise in biomass concentration resulted in a rise in reduction capacity $T_C X_O$ in all experiments. In addition, the model only requires the input of the initial biomass and describes the experimental data very well without the need for further consideration of subsequent cell growth/death. Therefore the model equation is appropriate to describe the toxic effects of U(VI) on U(VI) reduction. An inverse relationship was observed between the cell concentrations and U(VI) concentration. Statistical comparisons were made between the experimentally determined data and the best fit model defined by the three constants by using coefficients of determination, r^2. In 90% of the experiments, r^2 was 0.91 or better. The comparable kinetic values for both the mixed and pure cell cultures for the reduction of a range of U(VI) concentrations are similar as seen in Table 5.4.

Table 5.4 Kinetic parameters for U(VI) reduction in the consortium consisting of *P. stutzeri, P. agglomerans* and *E. cloacae.*

Concentration (mg U(VI)/L)	K_u (mg U(VI)/L)	k_u (mg U(VI)/mg cell/h)	T_c (mgU(VI)/mg cell)	*Xo* (mg cells/L)	$\chi 2$
30	7.9	0.025	0.04	1689.8	256.6
75	8.1	0.02	0.049	2174.1	1114.5
100	8	0.02	0.043	11048.8	150
200	8	0.02	0.039	4919.8	5081.7
400	8	0.028	0.043	8536.7	4768.9
600	8	0.025	0.04	5785	67419.2
800	8	0.025	0.04	6112.1	29636.8

Using the obtained parameter values the model simulated U(VI) reduction very well for the upper limit concentration 100, 200, 400 mg/L as shown in Fig. 5.4. The majority of uranium reduction occurs in the first 5 hours of incubation and the model captures that information. Cultures assumed to be in exponential phase at the time of transfer. Other studies by Spear (1999) showed a lag time for uranium removal because of a slower initial uranium removal rate. They proposed a model based on a modified Monod non-growth model that includes a rate-limiting reactant term. The model was fit to experimental data for uranium concentration 100 mg/L fitting the data well with R^2 of 0.9 and yielded values for k_u, K_u and T_c, these values were kept constant to simulate the other concentrations. The predicted model of concentrations higher or lower than that did not quite fit the experimental data.

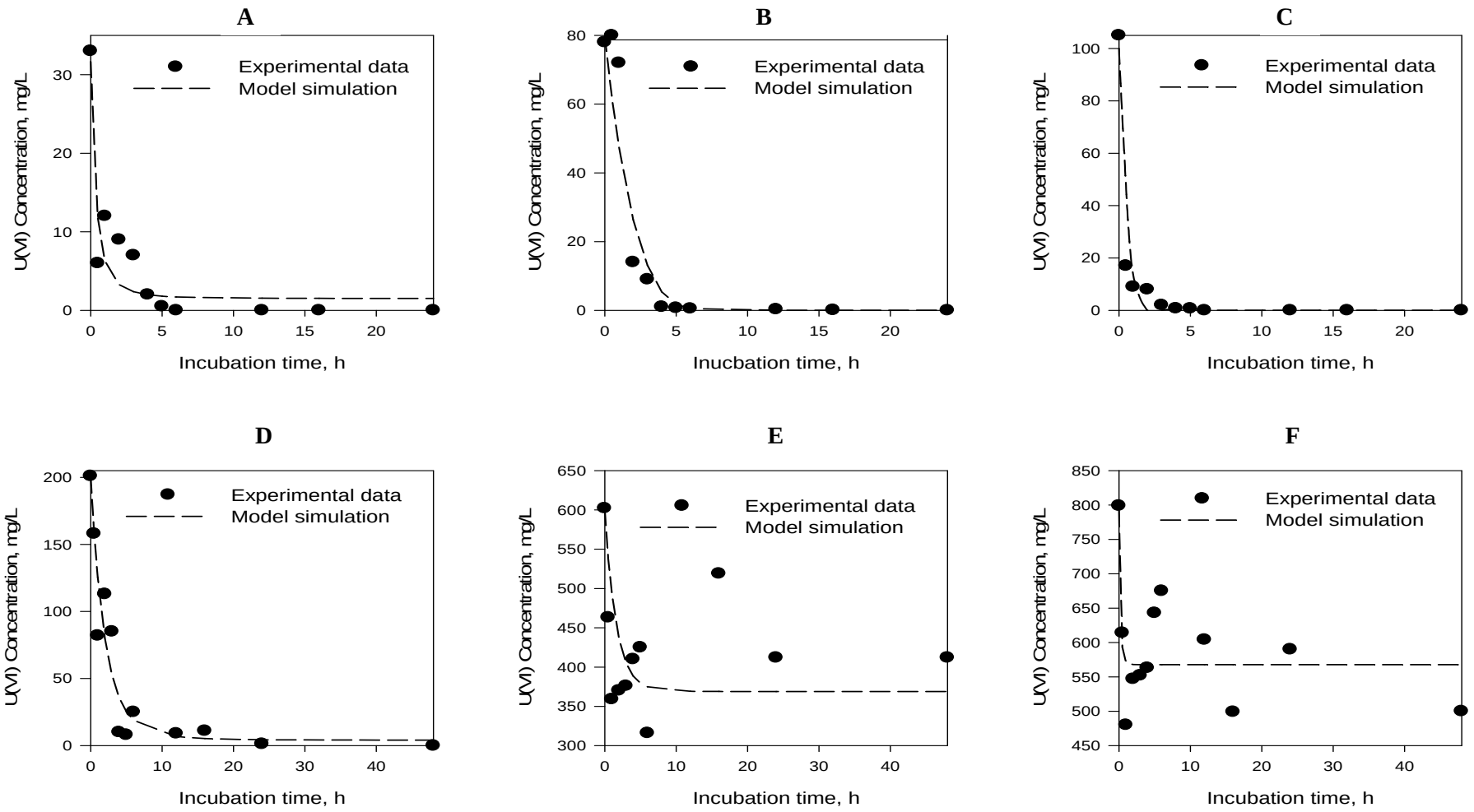


Figure 5.4 U(VI) reduction in batch cultures of *the consortium.* for concentrations ranging from 30 to 800 mg/L.

5.5 Discussion

Currently, the model does not fit certain ranges of data. The parameters are not constant and the model is not fitting, therefore further investigation is required. Moreover, the parameters have to be optimized further and constraints be studied properly. The modelling work is ongoing so as to address these concerns.

Despite the limitations, we expect that the kinetic expressions and parameters obtained from this study will prove useful for engineering applications. They can be incorporated into reactive transport models used for the design and operation of remediation systems. Because these cultures reduce uranium at a rate comparable to or better than rates found in literature for other microorganisms, reduction rates reported in this paper can be used to assess the applicability of bioreduction for uranium removal processes. Lovely and Phillips (1992) showed that *Desulfovibrio desulfuricans* could reduce an initial 1 mM U(VI) down to 0.1 mM in 3 to 4 hours.

It is believed that biological activities are the main mechanisms for U(VI) reduction in these cultures. Such enzymatic reduction of U(VI) was described very well by the developed model. The model's ability to analyze U(VI) reduction can be extended to other strains that are known to reduce U(VI). The model demonstrates a vital characteristics of microbial U(VI) reduction, that is, the rate and extent of U(VI) in batch cultures are dependent upon the reduction capacity, which is regulated by the initial cell density regardless of subsequent growth or death. During U(VI) reduction, a significant decrease in cell density were observed in the pure cultures as well as the mixed culture. For the observed cell density decrease during U(VI) reduction, the reduction capacity in batch cultures may have been caused by termination of metabolic activity due to U(VI) toxicity.

5.6 Implications of this Research

The kinetic parameters developed here could be used to design U(VI) reducing bioreactors such as sequencing batch reactors, submerged packed columns, or continuously stirred tank reactors. However, there is a concern about the separation of cells and precipitate and this may need to be addressed in further studies. Also, temperature is a major influencing factor, as bench-scale was operated at 30±5°C and cultures performed well but further kinetic analysis at temperatures less than 20 °C is needed if this research is to be applied in the environment, as mine water and groundwater may have temperatures in the 5-15 °C range.

One may also need to expose the pure cultures and as well as the consortium for long periods of time so that motility of cells can be observed before, during and after exposure to U(VI) and to also build a resistant to the toxic effects of U(VI). This phenomena has been published by Lovley and Phillips where no toxicity at concentrations as high as 5 mM U(VI) was observed. Additional studies can make use of the consortium in naturally occurring U(VI) containing waters to monitor to what extent U(VI) removal is possible (Spear, 1999).

The parameters determined using the second-order Monod model allow for the thorough evaluation of the bioreduction of U(VI) by three separate cell cultures as well as the consortium, herein, which may be applied to SRB cultures as well as other cultures and/or processes demonstrating similar behaviour when exposed to U(VI).

Table 5.5 Parameter values after optimization.

Parameter symbol	Optimized value	Parameter constraints	Units
Pseudomonas sp. Ku	99.8	1 - 100	mg/L
Pseudomonas sp. ku	0.01	0.002 – 0.2	mg/mg/h
Pseudomonas sp. Tc	0.08	0.05 - 1	mg/mg
Pseudomonas sp. Xini	19673.7	0 -100000	mg/L
Pseudomonas sp. Uini	400	300 - 400	mg/L
Pantoea sp. Ku	99.7	0 - 100	mg/L
Pantoea sp. ku	0.31	0.3 – 0.4	mg/mg/h
Pantoea sp. Tc	0.49	0.01 – 0.5	mg/mg
Pantoea sp. Xini	340	0 - 100000	mg/L
Pantoea sp. Uini	79	75 - 80	mg/L
Enterbacter sp. Ku	79.7	1 - 100	mg/L
Enterbacter sp. ku	0.25	0.2 - 0.4	mg/mg/h
Enterbacter sp. Tc	0.97	0.001 - 1	mg/mg
Enterbacter sp. Xini	379	0 - 100000	mg/L
Enterbacter sp. Uini	89.7	79 - 90	mg/L
Mixed culture Ku	8	1 - 10	mg/L
Mixed culture ku	0.02	0.01 – 0.09	mg/mg/h
Mixed culture Tc	0.039	0.01 – 0.05	mg/mg

Mixed culture Xini	4919.8	0 – 100000	mg/L
Mixed culture Uini	200	200 - 210	mg/L

5.7 Summary

The kinetics of U(VI) in batch cultures of several bacterial species may be adequately described by an enzyme-based mathematical model. Model analysis of U(VI) reduction data indicates that each U(VI)-reducing species may possess a very high U(VI) reduction capacity, which is not influenced by cell death during U(VI) reduction which may be influenced by the toxic and mutagenic effects of U(VI).

AQUASIM is able to simulate the reduction of U(VI) in a batch reactor, and is adequate in modelling such a system. However, the model underestimates U(VI) reduction in the batch reactor since it does not include pH, carbon source and temperature effects. The model is useful for predicting trends only. Results of the abovementioned experimental work would allow for the development of a more predictive model, and allow for accurate prediction of the overall performance of the reactor thereby allowing for integration and optimisation of the continuous flow reactor operations. Although rate equations and kinetic constants are available in literature, investigation of certain critical processes is required before a more accurate model can be developed.

CHAPTER 6

CONCLUSION

The rate of bioreduction is at its highest at the beginning of the experiment when uranium U(VI) concentration is also at its highest. The results should indicate that another form i.e.UO_2 is produced at the same rate. Also, the rate of reduction did not remain constant over time due to a suspected decrease in the metabolic activity of the pure cultures as well as the mixed culture, as the results of the viable cell count have shown. In the environment, where pump and treat processes are used to bioremediate, there is likely to be constant concentrations, the data collected from this experiment can be more appropriately applied to the optimization of bacterial uranium reduction on a bench scale. Significant research will have to be carried out in order to scale up uranium bioreduction to industrial levels.

Microorganisms play important roles in the environmental fate of toxic with an array of physico-chemical and biological mechanisms effecting transformations between soluble and insoluble phases. Although the biotechnological potential of most of these processes has only been explored at laboratory scale, some mechanisms, notably bioleaching, biosorption and precipitation, have been employed at a commercial scale. Continued exploitation of other biological processes will undoubtedly depend on a number of scientific, economic and political factors. Finally, it should be emphasised that this area of research also provides understanding of the biogeochemistry of metal cycling in the environment and the fundamental role of microorganisms in affecting metal mobility and transfer between different biotic and abiotic locations.

Further development is necessary with respect to both technical and biological aspects. This includes increasing the rate of bio-reduction and the tolerance of the microorganisms to

U(VI). Genetic improvement of uranium reduction bacteria, whether by mutation and selection or by genetic engineering, will bring results more rapidly than conventional processes.

Further investigation is warranted not only for the purposes of uranium reduction, but also to provide insight into the mechanisms of bioreduction of heavy metals in general. It is also important to understand how bacteria can interact with other radionuclides as environmental contamination is never really limited to a single element, many radionuclides can be present simultaneously. It might be useful to understand how the 3 pure cultures; *Pseudomonas stutzeri*, *Pantoea agglomerans* and *Enterobacter cloacae*, as well as the consortium will behave in the presence of several other radionuclides. Immobilization of these other contaminants would be an advantage in the environment as it limits its transport. The rate of reduction and speciation of other elements by these bacteria could be examined. The studies could have the potential to allow us to make informed decisions about how to remove radionuclide contaminants from the environment.

Uranium and other radionuclides are of particular importance because of current contamination as well as the continued burial of a considerable amount of nuclear waste underground. It is important to understand the impact that will have on the environment in the near future and in the long term. The efforts described here emphasize that the study of interactions between bacteria and uranium can be applied to environment remediation programmes as well as to improve existing ones.

RECOMMENDATIONS

Much is still not known about the mechanisms of bioreduction i.e. the biochemical reactions and pathways involved and whether the mechanisms are the same across all metal-reducing

bacteria. Kinetic modelling of uranium reduction and cumulative removal studies (substrate and biomass concentration as well as environmental conditions, pH and oxidation-reduction potential measurements for the continuous reactor process) should help us to better predict and model how uranium will behave in situ. In addition to that, to some extent differentiate between discrete reduction mechanisms, as well as provide a basis for optimizing bacterial reduction processes in vitro. Also, it would be more relevant if the cytosolic and outer membrane proteins that are involved in uranium reduction are identified and characterized so as to better understand the biochemical processes that are occurring. There is a need for further investigation of the underlying U(VI) reduction mechanisms at the cellular level. The model used in this study was adapted from other models, therefore there is a need to evaluate a simultaneous fit of different datasets using other computer programs. Moreover, the parameters are not constant and also have not been optimized yet hence the poor fit of the model. Lastly, constraints would need to be studied properly, χ^2 variability would have to be limited and a proper investigation should be done in the first 5 – 6 hours of the batch experiments.

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Internet Resources

136. www.world-nuclear.org/info/reactors.html.

137. www.iaea.org/archives.

APPENDIX A

AQUASIM Version 2.0

****

Variables

****

Ku: Description: coefficient

Type: Constant Variable

Unit: mg/L

Value: 79.971762

Standard Deviation: 1e-015

Minimum: 78

Maximum: 80

Sensitivity Analysis: active

Parameter Estimation: active

---

ku: Description: maximum specific reduction rate

Type: Constant Variable

Unit: mg/L*mg

Value: 0.26008543

Standard Deviation: 1e-015

Minimum: 0.25

Maximum: 0.3

Sensitivity Analysis: active

Parameter Estimation: active

---

mu: Description:

Type: Constant Variable

Unit: 1/h

Value: 0.14

Standard Deviation: 0.1

Minimum: -0.0001

Maximum: 1

Sensitivity Analysis: inactive

Parameter Estimation: inactive

---

t: Description: time

Type: Program Variable

Unit: h

Reference to: Time

Tc: Description: removal capacity of cells

Type: Constant Variable

Unit: mg/mg

Value: 0.97

Standard Deviation: 1e-015

Minimum: 0.9

Maximum: 1

Sensitivity Analysis: active

Parameter Estimation: active

U: Description: Uranium conc

Type: Dyn. Volume State Var.

Unit: mg/L

Relative Accuracy: 1e-006

Absolute Accuracy: 1e-006

Uini: Description: initial uranium concentration

Type: Constant Variable

Unit: mg/L

Value: 28.024645

Standard Deviation: 1e-010

Minimum: 28

Maximum: 35

Sensitivity Analysis: inactive

Parameter Estimation: active

Umeas: Description: Uranium measured

Type: Real List Variable

Unit: mg/L

Argument: t

Standard Deviations: global

Rel. Stand. Deviat.: 0

Abs. Stand. Deviat.: 1

Minimum: 0

Maximum: 1e+009

Interpolation Method: linear interpolation

Sensitivity Analysis: inactive

Real Data Pairs (11 pairs):

0	30
0.5	7
1	15
2	15
3	1
4	0
5	0
6	0

12 0

16 0

24 0

X: Description: biomass

Type: Formula Variable

Unit: mg

Expression: Xini-((Uini-U)/Tc)

Xini: Description: Initial biomass concentration

Type: Constant Variable

Unit: mg/mL

Value: 293.94317

Standard Deviation: 1e-015

Minimum: 0

Maximum: 100000

Sensitivity Analysis: inactive

Parameter Estimation: active

Xmeas: Description: Measured biomass

Type: Real List Variable

Unit: mg

Argument: t

Standard Deviations: global

Rel. Stand. Deviat.: 0

Abs. Stand. Deviat.: 1

Minimum: 0

Maximum: 1e+009

Interpolation Method: linear interpolation

Sensitivity Analysis: inactive

Real Data Pairs (11 pairs):

0	14.6
0.5	13.2
1	12.9
2	9.8
3	8.9
4	7.5
5	7.2
6	7
12	7.1
16	7
24	7

****

Processes

****

reduction: Description: Uranium reduction

Type: Dynamic Process

Rate: (ku*X*U)/(Ku+U)

Stoichiometry:

Variable : Stoichiometric Coefficient

U : -1

****

Compartments

****

reactor: Description: batch reactor

Type: Mixed Reactor Compartment

Compartment Index: 0

Active Variables: U, X

Active Processes: reduction

Initial Conditions:

Variable(Zone) : Initial Condition

U(Bulk Volume) : Uini

X(Bulk Volume) : Xini

Inflow: 0

Loadings:

Volume: 1

Accuracies:

Rel. Acc. Q: 0.001

Abs. Acc. Q: 0.001

Rel. Acc. V: 0.001

Abs. Acc. V: 0.001

****

Definitions of Calculations

****

calc1: Description: uranium reduction

Calculation Number: 0

Initial Time: 0

Initial State: given, made consistent

Step Size: 0.1

Num. Steps: 1000

Status: active for simulation

active for sensitivity analysis

****

Definitions of Parameter Estimation Calculations

****

fit1: Description:

Calculation Number: 0

Initial Time: 0

Initial State: given, made consistent

Status: active

Fit Targets:

Data : Variable (Compartment,Zone,Time/Space)

Umeas : U (reactor,Bulk Volume,0)

****

Plot Definitions

****

concentration: Description: uranium concentration

Abscissa: Time

Title: Concentration of U(VI)

Abscissa Label: Time in hours

Ordinate Label: Concentration in mg/L

Curves:

Type : Variable [CalcNum,Comp.,Zone,Time/Space]

Value : U [0,reactor,Bulk Volume,0]

Value : Umeas [0,reactor,Bulk Volume,0]

---

sens: Description: sens_30

Abscissa: Time

Title: sens_30

Abscissa Label: time

Ordinate Label: variables

Curves:

Type : Variable [CalcNum,Comp.,Zone,Time/Space]

SensAbsRel : U(Ku) [0,reactor,Bulk Volume,0]

SensAbsRel : U(ku) [0,reactor,Bulk Volume,0]

SensAbsRel : U(Tc) [0,reactor,Bulk Volume,0]

****

Calculation Parameters

****

Numerical Parameters: Maximum Int. Step Size: 1

Maximum Integrat. Order: 5

Number of Codiagonals: 1000

Maximum Number of Steps: 1000

Fit Method: simplex

Max. Number of Iterat.: 100

****

Calculated States

****

Calc. Num. Num. States Comments

0 1001 Range of Times: 0 - 100

****