Vanadium reduction by bacterial isolates from South African mines

by

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This dissertation is dedicated to my mother, brother and sister who supported me through the good and the not so good times, and in loving memory of my father who believed in a higher education for his children, yet never had the opportunity to see me graduate.

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ABBREVIATIONS

°C	Degrees Celsius
6-PGDH	6-Phosphogluconate dehydrogenase
A	Absorbance
A	An entrophy constant
ADPV	Adenosine diphosphate vanadate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BLAST	Basic Local Alignment Search Tool
bp	Base pair
Br-PADAP	4-(5-Brom-2-pyridylazo)-N,N-diaethyl-3-hydroxyanilin
BSA	Bovine serum albumin
Cells/ml	Cells per millilitre
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-propanesulfonate
CM	Carboxylmethyl
cm	Centimeter
CMC	Critical micelle concentration
DDT	Dichlorodiphenyltrichloroethane
DER736	Diglycidyl ether of polypropylene glycol
DMAE	(S1) dimethylaminoethanol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNS	Dioksie ribonukleinsuur
dNTP	Nucleotides
E	Reduction potential
EDTA	Ethylene diaminetetraacetic acid
EHF	Expand High Fidelity
g	Gram
g/l	Gram per liter
G6PDH	Glucose-6-Phosphate dehydrogenase
GDPV	Guanosine diphosphate vanadate
Glc	Glucose
GlcNAc	*N*-acetylglucosamine

GTP	Guanosine triphosphate
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
IPTG	Isopropylthiogalactoside
K	Kelvin
kb	Kilobase
kDa	Kilodalton
K_m	Michaelis constant
kV	Kilovolt
L	Ligand
LB	Luria Broth
LExEn	Life in extreme environments
M	Molar
mg/l	Milligram per liter
mg/ml	Milligram per millilitre
ml	Milliliter
mm	Millimeter
mM	Millimolar
Mr	Relative molecular mass
MurNAc	*N*-acetylmuramic acid
MWCO	Molecular weight cut-off
μ	Specific growth rate
μg	Microgram
μg/ml	Microgram per milliliter
μl	Microliter
μl/ml	Microliter per milliliter
μm	Micrometer
μM	Micromolars
μMax	Maximum specific growth rate
N	Normal
Na_2-EDTA	Sodium ethylene diaminetetraacetic acid
NADH	Reduced nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NADPV	Nicotinamide adenine dinucleotide phosphate vanadate

NADV	Nicotinamide adenine dinucleotide vanadate
NH_4VO_3	Ammonium metavanadate
nm	Nanometer
NMWL	Nominal molecular weight limit
NSA	Nonenyl succinic anhydrate
OD	Optical density
Omc	Outer membrane cytochrome
OsO_4	Osmium tetraoxide
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulphonyl fluoride
ppm	Parts per million
R	Universal gas constant
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction fragment length polymorphisms
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SEM	Scanning electron microscopy
SOB	Tryptone, yeast extract, sodium chloride, potassium chloride, magnesium chloride and magnesium sulphate
STET	Sucrose, Triton X-100, Ethylene diaminetetraacetic acid and 2-Amino-2-(hydroxymethyl)-1,3-propandiol chloride
T	Absolute temperature
TAE	2-Amino-2-(hydroxymethyl)-1,3-propandiol, ethylene diamine tetraacetic acid, glacial acetic acid
TB	Piperazine-1,4-bis(2-ethanesulphonic acid), manganese chloride, calcium chloride and potassium chloride
TE	2-Amino-2-(hydroxymethyl)-1,3-propandiol, ethylene diamine tetraacetic acid
TEM	Transmission electron microscopy
Tris-HCl	2-Amino-2-(hydroxymethyl)-1,3-propandiol, hydrochloric acid
TYG	Tryptone, yeast extract and glucose
U/ml	Activity expressed in Units per millilitre
UF	Ultra-filtration
units/µl	Units per microliter

UV	Ultraviolet
V	Vanadium
V	Volts
V/cm	Volts per centimeter
V/cm	Volts per centimetre
v/v	Volume per volume
V_2O_5	Vanadium pentoxide
Vanabins	Vanadium-binding proteins
VCD	Vinylcyclohexene
V_{max}	Maximum velocity
V_{min}	Minimum velocity
VO_2	Vanadium (IV) oxide
w/v	Weight per volume
w/w	Weigh per weight
x g	Acceleration due to gravity
X-gal	5-Bromo-4-Chloro-3Indolyl-BD-Galactopyranside

CHAPTER 1

Literature review

1.1 Introduction

Most of the 110 chemical elements in the periodic table are metals. Generally speaking, a metal is a material with high reflectivity and conductivity that can usually be deformed plastically. A metal reflects light like a mirror unless the surface has been corroded (Hillert, 1997).

Most people use the term "metal" to refer to materials which exhibit the metallic properties mentioned above. The term metal also refers, however, to the metallic elements even when these are combined with other elements to form non-metallic compounds such as salts and oxides. Metals will display different characteristics depending on temperature, among other factors. For example, tin may exhibit non-metallic characteristics under certain conditions, while it can also behave like a metal under a different set of conditions. Metals can combine in almost any proportion, offering a vast range of alloys which generally show all the characteristics of a metal and are therefore regarded as metals. Alloys can be shaped by casting, machining and plastic forming. They can also be varied by heat treatment to exhibit advantageous mechanical properties (elasticity, strength, etc.). The metallic elements are often divided into light metals and heavy metals (Hillert, 1997).

Heavy metals are defined as chemical elements with a specific gravity that is at least five times the specific gravity of water, with the specific gravity of water being one at 4°C. Thus, the transition elements from vanadium to the half-metal arsenic can be referred to as heavy metals. Of the 90 naturally occurring elements, 21 are non-metals, 16 are light metals and the remaining 53 (with arsenic included) are heavy metals (Weast, 1984).

1.2 Microbial interaction with metals

Most heavy metals are transition elements with incomplete filled d-orbitals. These d-orbitals provide heavy-metal cations with the ability to form complex compounds which may or may not be redox-active. Thus, heavy-metal cations play an important role as trace elements in sophisticated biochemical reactions. Even though some heavy metals are essential trace elements, at high concentrations most can be toxic to all branches of life, including microbes, by forming complex compounds within the cell (Nies, 1999). Heavy-metal cations, such as Hg^{2+}, Cd^{2+} and Ag^{+}, are so toxic complex-formers that they are too dangerous for any biological function.

Because of locally elevated concentrations of heavy metals as a result of various commercial and industrial processes, microbes have evolved several mechanisms to tolerate the presence of heavy metals, i.e. efflux, complexation, or reduction of metal ions both assimilatory and dissimilatory. Because the intake and subsequent efflux of heavy-metal ions by microbes usually includes a redox reaction involving the metal, bacteria that are resistant to, and grow on metals also play an important role in the biogeochemical cycling of those metal ions. This is an important implication of microbial heavy metal tolerance because the oxidation state of a heavy metal relates to the solubility and toxicity of the metal itself (Ahmann *et al.,* 1994; Ehrlich, 1997; Spain, 2003).

The way microbes interact with metals depends in part on whether the organisms are prokaryotic or eukaryotic. Both types of microbes have the ability to bind metal ions present in the external environment at the cell surface or to transport them into the cell for various intracellular functions. On the other hand, only the prokaryotes (eubacteria and archaea) include organisms that are able to oxidize Mn (II), Fe (II), Co (II), Cu (I), AsO^{-}_{2}, Se or SeO_3^{2-}, or reduce Mn (IV), Fe (III), Co (III), AsO_4^{2-}, SeO_4^{2-} or SeO_3^{2-} on a large scale and conserve energy in these reactions. Some microbes may reduce metal ions such as Hg^{2+} or Ag^{+} to Hg^{0} and Ag^{0} respectively, but derive no energy in the process. Some prokaryotes and eukaryotes may form metabolic products, such as acids or ligands that dissolve base metals contained in minerals, such as Fe, Cu, Zn, Ni and Co. Other reactions may form anions, such as sulfide or carbonate, that precipitate dissolved metal ions (Ehrlich, 1997; Lovely, 1993).

Microorganisms which are able to use metals as terminal electron acceptors, or reduce metals as a detoxification mechanism have an important influence on the geochemistry of the surrounding environment, i.e. aquatic sediment, submerged soils or terrestrial subsurface. Furthermore, it is becoming increasingly apparent that microbial metal reduction may be manipulated to aid in the remediation of environments and waste streams contaminated with metals and certain organics (Lovely, 1993).

Of late vanadium has had an increased use in modern life, especially in industry as additives to steel and as catalysts in the production of a variety of products (Broderick, 1977; Cruywagen *et al*., 1981). Due to the increased use of vanadium, vanadium contamination is becoming a real concern. Vanadium is a highly toxic heavy metal (Roschin, 1967) and it is therefore necessary to give attention to its fate as well as possible means to control the level of its toxicity.

1.3 History on Vanadium

Vanadium was discovered twice! The first time by Andres Manuel del Rio in 1801 when he analyzed a sample of brown lead ore from Zimapan, Hidalgo, Mexico and concluded that it contained a new metal similar to uranium and chromium. He called the new substance panchromium, as the colors found were similar to those shown by chromium. He later changed the name to erythronium after he noticed that most of the salts turned red after they had been heated. Upon further study, however, he decided that his new discovery was an impure basic lead chromate, and H.V. Collet-Descotils, a French professor, erroneously confirmed this assumption in 1805 (Chasteen, 1983; Weeks and Leicester, 1968).

The element was rediscovered in 1830 by the Swedish chemist Nils Sefstrom who recognized that certain Swedish iron ores when smelted were more ductile and must contain an additional element that he identified and gave its present name - Vanadium. The beauty of the new compound led to the element‘s name. It comes from the word “Vanadis.” Vanadis is the goddess of beauty in Scandinavian mythology (Chasteen, 1983; Weeks and Leicester, 1968).

1.4 Occurrence in nature

Vanadium is a naturally occurring multivalent transition metal. It occurs in nature as a white-to-grey metal and is often found as crystals. Vanadium is found in about 65 different minerals among which camotite, roscoelite, vanadinite and patronite are important sources of the metal. Vanadium is also found in phosphate rocks and certain iron ores. Vanadium does not occur in highly concentrated forms, although it is as abundant in the earth's crust as zinc and nickel, thus vanadium is seldom found in deposits rich enough to be mined economically for vanadium alone (Broderick, 1977; Cotton and Wilkinson, 1988; Duke, 1969; Spectrum, 2004). General conditions under which vanadium can be precipitated and concentrated locally are:

1. By reactions with hydroxides of aluminium or ferric iron. Vanadium concentration in some bauxites and in some residual and sedimentary iron ores could be enriched by this process;
2. By reaction with cations of heavy metals, such as lead, copper and zinc. The epigenetic vanadate minerals in the oxidized zones of base-metal deposits are formed in this way;
3. By reduction in the presence of organic material. This process could form epigenetic deposits such as those found in sandstone on the Colorado-Plateau, if the vanadium-bearing solutions are moving through the rocks. If, however, the solutions are surface waters, the result could be deposits of synergetic metal-organic compounds and sulphides of vanadium and other metals in sediments such as carbon-acetous shales (Broderick, 1977).

Vanadium also has the ability to combine with many different elements to form numerous and frequently complicated compounds. This is due to its variable valences, where the pentavalent (5+) form is the most stable and typical (Broderick, 1977). Table 1.1 lists examples of some of these compounds.

Table 1.1. Some of the numerous and frequently found vanadium compounds (Table complied from Web elements, 2004)

Compounds	Examples		Compounds	Examples	
Hydrides	VH	Vanadium (I) hydride	**Iodides**	VI_2	Vanadium (II) iodide
	V_2H	Vanadium hydride		VI_3	Vanadium (III) iodide
				VI_4	Vanadium (IV) iodide
Fluorides	VF_2	Vanadium (II) fluoride			
	VF_3	Vanadium (III) fluoride	**Oxides**	VO	Vanadium (II) oxide
	VF_4	Vanadium (IV) fluoride		VO_2	Vanadium (IV) oxide
	VF_5	Vanadium (V) fluoride		V_2O_3	Vanadium (III) oxide
				V_2O_5	Vanadium (V) oxide
Chlorides	VCl_2	Vanadium (II) chloride		V_3O_5	Vanadium oxide
	VCl_3	Vanadium (III) chloride			
	VCl_4	Vanadium (IV) chloride	**Sulfides**	VS_2	Vanadium (IV) sulphide
				V_2S_3	Vanadium (III) sulphide
Bromides	VBr_2	Vanadium (II) bromide			
	VBr_3	Vanadium (III) bromide	**Selenides**	VSe_2	Vanadium (IV) selenide
	VBr_4	Vanadium (IV) bromide			
			Nitrides	VN	Vanadium (III) nitride

1.5 Inorganic chemistry and characteristics of vanadium

The chemistry of vanadium is characterized by multiple oxidation states (Figure 1.1). These states are 1-, 2-, 2+, 3+, 4+ and 5+, with the latter four being the most common forms. The redox chemistry of this metal plays an important role in its biochemistry and of the four common oxidation states, only V (III), V (IV) and V (V) are important biologically, with V (II) being too reducing to exist in any known organism. The best known example of the occurrence of V (III) is in the vanadocytes of the blood of tunicates; otherwise, vanadium is largely found in the 4+ and 5+ oxidation states, both of which are readily accessible under physiological conditions (Chasteen, 1983; Michibata *et al*., 2003).

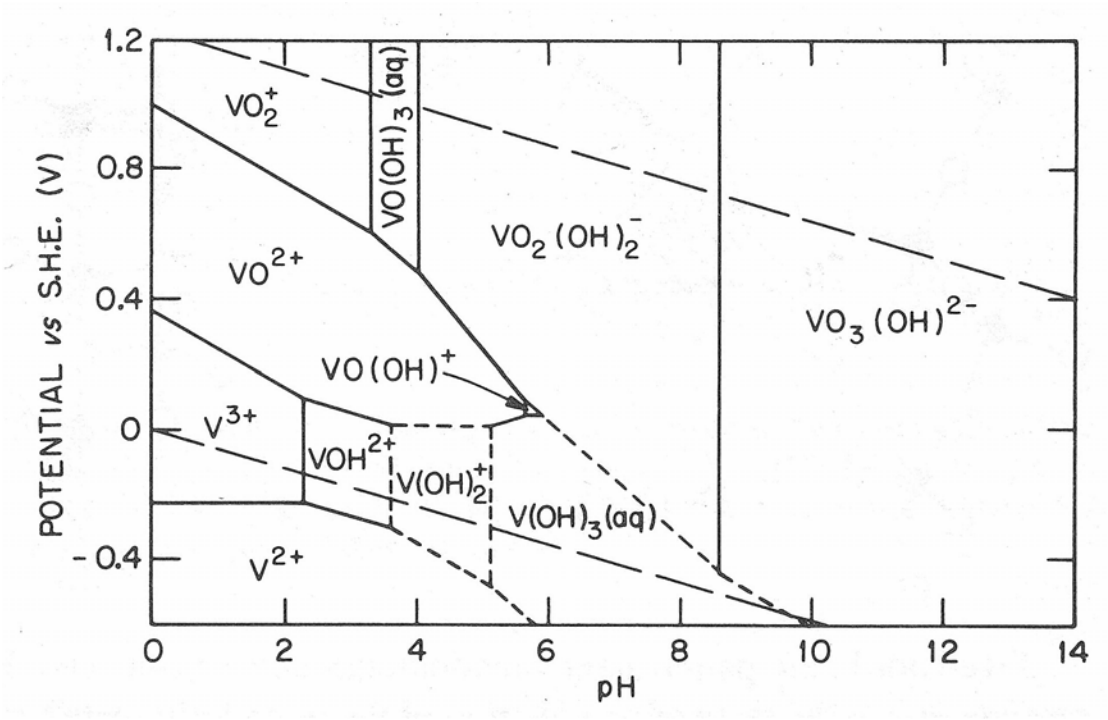

Figure 1.1. Reduction potential, E, (reference to the standard hydrogen electrode) versus pH for various species of vanadium. Boundary lines correspond to E, pH values where the species in adjacent regions are present in equal concentrations. The short dashed lines indicate uncertainty in the location of the boundary. The upper and lower dashed lines correspond to the upper and lower limits of stability in water. Standard reduction potentials are given by the intersections of "horizontal" lines with the abscissa pH = 0. The half reactions are $O_2 + 4H^+ + 4e = 2H_2O$, $E° = 1.23V$; $VO_2^+ + 2H^+ + e = VO^{2+} + H_2O$, $E° = 1.0V$; $VO^{2+} + 2H^+ + e = V^{3+} + H_2O$, $E° = 0.36V$; $2H^+ + 2e = H_2$, $E° = 0.0V$; and $V^{3+} + e = V^{2+}$, $E° = -0.24V$. V^{2+} is therefore a strong reductant. Air oxidation of VO^{2+} presumably proceeds the reaction $4VO^{2+} + O_2 + 2H_2O = 4VO_2^+ + 4H^+$, $E° = 0.23V$ which is favored at higher pH. Not all known species are represented on this diagram. Reproduced form Baes and Mesmer, (1976).

Under acidic conditions, the predominant vanadium species are V^{3+}, VO^{2+} and *cis*-VO_2^+. For simplicity, coordinated water molecules have been omitted from these formulas. As the pH is raised, hydrolysis takes place and a number of monomeric and oligomeric species are formed, only some of which are shown in Figure 1.1. Because of the multiple equilibria involved and the tendency for equilibrium to be attained slowly, the elucidation of all of the species present and their respective formation constants is a difficult task (Chasteen, 1983).

Diverse biological processes can be affected by vanadium due to its ability to exist in two oxidation states at neutral pH. In the physiological pH range 6 to 8 when the

total vanadium concentration is less than 10mM, the species present in appreciable amounts are the vanadates (5+) $H_2VO_4^-$, HVO_4^{2-}, $HV_2O_7^{3-}$ and $V_3O_9^{3-}$ in which the metal is tetrahedrally coordinated. Vanadate resembles phosphate (HPO_4^{2-}), and can consequently take its place in phosphate-metabolizing enzymes (Baysse *et al*., 2000; Chasteen, 1983). In aqueous solution, the chemistry of tetravalent vanadium is centered around the VO^{2+} ion. This ion forms strong complexes with a diversity of ligands and is known to bind to numerous proteins (Chasteen, 1983).

1.6 Commercial and industrial uses

Small quantities of vanadium salts were used during the 19th century for making ink, and for coloring fabrics, leather, glass and pottery (Broderick, 1977). Other minor functions of vanadium compounds include their use as color modifiers in mercury-vapor lamps, driers in paints and varnishes, corrosion inhibitors in flue-gas scrubbers and as components in photographic developers.

Presently vanadium is mostly produced to be used industrially. The main use of vanadium is as an alloying ingredient in steel, but it also plays an important role as a catalyst in certain chemical reactions. Vanadium is usually added in the form of ferrovanadium, a vanadium-iron alloy. Vanadium is used in producing steels that are rust resistant and used in the manufacture of high-speed tools. About 80% of the vanadium currently produced is used as ferrovanadium or as a steel additive. Vanadium (added in amounts between 0.1 and 5.0 percent) has two effects upon steel: it refines the grain of the steel matrix and with the carbon present it forms carbides. Thus, vanadium steel is especially strong and hard, with improved resistance to shock. Vanadium foil is used as a bonding agent in cladding titanium to steel and generally by the aerospace industry in titanium alloys. Medical implants often contain vanadium alloys, which contribute to their long life (Broderick, 1977; Duke, 1969).

Vanadium-containing catalysts are used in several oxidation reactions such as the manufacture of phthalic anhydride and sulphuric acid, as well as in the production of pesticides. Vanadium pentoxide (V_2O_5) is used as a catalyst in the

manufacturing of sulphuric acid (H_2SO_4) through the contact process (Cruywagen *et al*., 1981; Physchem, 2002). Millions of tons of sulphuric acid are made every year by the contact process, which converts raw sulphur, oxygen and water to sulphuric acid. The contact process consists of six steps, where the first step is to melt sulphur and burn it in the presence of oxygen to produce sulphur dioxide, SO_2. This is followed by passing the SO_2 gas through a precipitator to remove dust and other impurities which may interfere with the catalyst, the SO_2 is washed with water and dried. After passing through a heating chamber, the SO_2, which is still mixed with air, is passed through a reactor. There, using vanadium pentoxide as catalyst, the SO_2 is converted to sulphur trioxide, SO_3.

$$2\,SO_2 + O_2 \xrightarrow{V_2O_5} 2\,SO_3$$

Finally, the SO_3 is absorbed in concentrated sulphuric acid, giving the so-called oleum or pyrosulphuric acid. This is then diluted with water to give about 98% pure H_2SO_4.

$$SO_3 + H_2SO_4 \longrightarrow H_2S_2O_7$$

Vanadium is also used in the Sulfolane process (Figure 1.2), where V (V) is used to convert hydrogen sulphide, H_2S, to elemental sulphur (Janse van Vuuren, 1996). Maleic anhydride, which is a chemical used to make polyester resins and fibreglass, is manufactured by using vanadium as a catalyst.

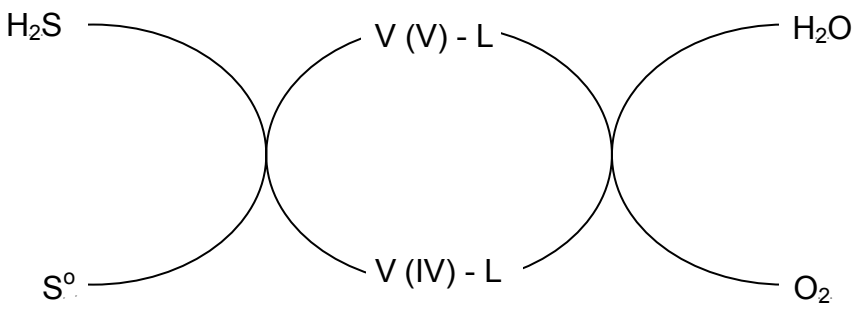

Figure 1.2. Schematic presentation of the sulfolane process (Adapted from Janse van Vuuren, 1996).

1.7 Isolation and preparation

Vanadium occurs primarily as a by-product or co-product during the extraction of other compounds such as iron, titanium, phosphate or petroleum. Pure vanadium is difficult to obtain as it tends to be readily contaminated by other elements. Until about 1960, vanadium from domestic ores was recovered initially in the form of fused oxide containing 86 to 92 percent V_2O_5, or as air-dried oxide containing 83 to 86 percent V_2O_5. The air-dried oxide was converted to ammonium metavanadate, NH_4VO_3, which in turn was used to make pure vanadium oxide or other vanadium chemicals. Process improvements and innovations after 1960 made it possible to produce 98 to 99 percent fused oxide and pure ammonium metavanadate directly from ores (Broderick, 1977; Duke, 1969).

Vanadium is commercially produced from several different sources. Primary vanadium production is derived from mining vanadiferous ores. The ore is crushed, ground, screened and passed through magnetic separators to produce a concentrated magnetite. The magnetite is mixed with a sodium salt such as sodium chloride, sodium carbonate or sodium oxylate. This charge is then roasted at about 850°C to convert the oxides to sodium metavanadate, which can be leached in hot water. After various processes (such as desilication), which depend on the specific impurities contained in the ore-body, the vanadium is precipitated as ammonium metavanadate. After filtration, the precipitate is calcined to produce V_2O_5 of purity greater than 99.8 percent (Cotton and Wilkinson, 1988). Secondary vanadium production comes from by-product of iron and steel manufacturing.

1.8 Toxicity

The toxicity of vanadium depends on its physico-chemical state; particularly on its valence state and solubility. Based on acute toxicity, pentavalent NH_4VO_3 has been reported to be more than twice as toxic as trivalent VCl_3 and more than 6 times as toxic as divalent VI_2. Pentavalent V_2O_5 has been reported to be more than 5 times as toxic as trivalent V_2O_3 (Roschin, 1967). This indicates that the toxicity is related to the valence of the vanadium compound (increasing with increasing valence). Vanadium is toxic both as a cation and as an anion.

Occupational exposure to vanadium containing dusts is encountered in the mining of vanadium-bearing ores. Most of the vanadium-bearing ores in the United States comes from Arkansas, Colorado and Idaho while other sources include South Africa, Chile and the USSR. In mining, exposure to vanadium-containing dust can occur near the production sites of numerous vanadium compounds. The toxic effects of vanadium in industry have occurred mainly through inhalation, and possibly, though to a lesser extent, ingestion of the pentoxide, sulfate or mixed vanadium dust (Spectrum, 2004).

The mechanisms by which vanadate exerts a toxic effect on living organisms are not completely understood. This is principally due to the variety of intracellular targets of the metal and to the changes in the chemical form and oxidation states that vanadate can undergo, both in the external environment and intracellularly (Mannazzu *et al*., 2000).

The toxicity of heavy metals on living systems is largely mediated by their ability to form coordination complexes and clusters with important cellular targets, such as phosphates, purines, pteridines, porphyrins, cysteinyl and histidyl side chains of proteins. Moreover, heavy-metal ions are able to cause oxidative damage either directly, through their redox cycling activities that produce the extremely reactive OH radical, or indirectly, by depleting free radical scavengers such as glutathione and protein-bound sulphydryl groups (Liochev and Fridovich, 1987; Mannazzu *et al*., 2000).

Vanadate, the pentavalent state of vanadium, is known to inhibit many enzymes which form covalent, phosphoryl-enzyme intermediates as part of the enzyme reaction mechanism, particularly plasma membrane Na^+, K^+ ATPases. This inhibition is caused by competition between vanadate and phosphate for enzyme binding (vanadium is more stable than phosphate, thus has a lower activation energy, thus higher affinity for binding). Competitive inhibition is observed between vanadate and phosphate due to vanadate's close structural similarity to phosphate. (Cantley *et al.,* 1977; Cantley *et al.,* 1978; Henderson *et al*., 1989a; Henderson *et al.,* 1989b).

1.8.1 Mechanism of Na^+, K^+ ATPases inhibition

Na^+, K^+ ATPases has both a high and low affinity ATP binding site. ATP is bound in both sites (Figure 1.3). Sodium is bound to enzyme and the ATP in high affinity site is hydrolyzed. Mg^{2+} binds to stabilize the phosphate, the enzyme flips and the sodium is released. The high affinity site is now the low affinity site, thus phosphate:Mg^{2+} is bound in the low affinity site. Potassium binds and phosphate:Mg^{2+} is released and ATP can now bind in the low affinity site. Potassium is released and sodium binds. The enzyme can now repeat the cycle. However vanadium can bind at the low affinity site and thus cause enzyme inhibition by binding more strongly than phosphate, thus causing partial competitive inhibition (Cantley *et al.,* 1978).

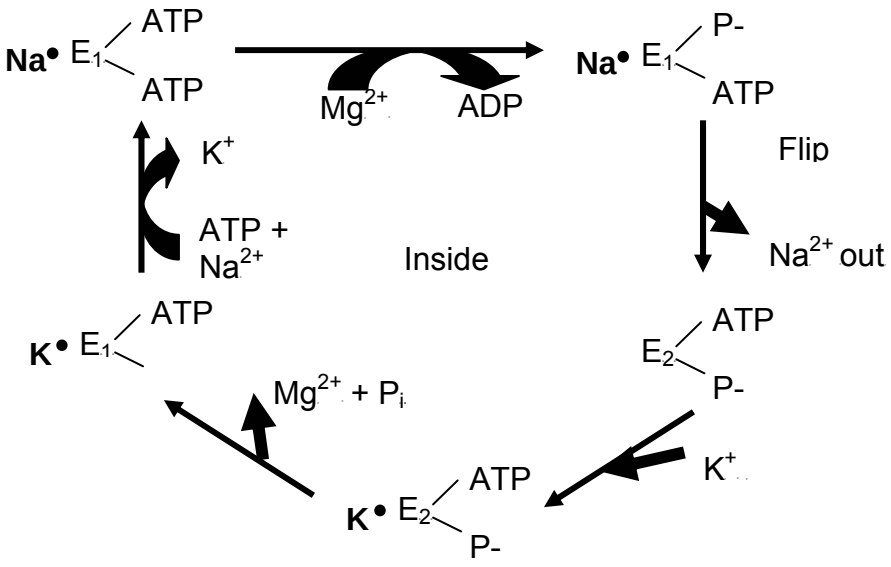

Figure 1.3. Schematic diagram of the mechanism of Na^+, K^+ ATPases inhibition by vanadate (Adapted from Cantley *et al.,* 1987).

1.9 Resistance mechanisms

Up to six mechanisms have been recognized by which bacteria can exert resistance against toxic metals, including metal exclusion by permeability barrier, intracellular or extracellular sequestration, detoxification and active transport of the metal out of the cell.

Microorganisms respond to the presence of potentially toxic metal ions through several intrinsic mechanisms to regulate intracellular concentrations of the metal ions:

1. Exopolysaccharide production;
2. Detoxification through specific efflux systems;
3. Metal sequestration by specific mineral ion binding components;
4. Tight coupling between membrane transport metal efflux proteins and ATPases;
5. Enzymatic transformation converting more toxic to less toxic or less available metal ion species (Holden and Adams, 2003; Nies, 1999; Silver and Phung, 1996).

1.9.1 Vanadium resistance

1.9.1.1 Efflux

Nothing is known about the mechanisms of resistance against vanadyl- or vanadate ions. In their study, Hernández *et al.* (1998) described that *Escherichia hermannii* cells grown in the presence of vanadium accumulate the metal and show the induction of a 45kDa outer-membrane protein, which could be indicative of the induction of an efflux system porin. This study is a first attempt to elucidate the mechanisms of bacterial resistance against vanadium. It is known that active efflux plays an important role in the development of resistance against some toxic metals. Some important efflux systems include the efflux of zinc (Beard *et al*., 1997; Nies, 1999), arsenic (Nies and Silver, 1995; Silver and Phung, 1996) and copper (Cooksey, 1994). In a study done by Aendekerk *et al.* (2002) it was found that an efflux pump, MexGHI-OpmD, was needed for resistance to vanadium in the bacterium *Pseudomonas aeruginosa*. Other than the work done by Aendekerk *et al.* (2002) and Hernández *et al.* (1998) little to no information is available on how vanadium resistance in bacteria is exerted.

1.9.1.2 Biological reduction

Vanadium becomes toxic when present above micromolar concentrations intracellularly, although some species of marine tunicates can accumulate extremely high concentrations of this metal (up to 1 M). This metal accumulation occurs in specialized cells called vanadocytes. Studies on *Neurospora crassa* and erythrocytes demonstrate that vanadate enters the cells through the phosphate transport system. Once inside the cell, vanadate is likely to be reduced to vanadyl by glutathione, catechol and other cellular components (Bowman, 1983; Macara *et al.,* 1980). Vanadate can also substitute for organic phosphate in key molecules of oxidoreductive and energy metabolism, reacting either with NAD^+ to give NADV (an analogue of NADP), or with some diphosphate nucleotides to give ADPV and GDPV [analogues of ATP and GTP] (Mannazzu *et al.,* 1997).

Phosphate uptake studies in different strains of the dimorphic pathogenic yeast *Candida albicans* were undertaken by Mahanty *et al.* (1991) to show that this yeast actively transported phosphate with apparent K_m in the range of 90 - 170µM. Vanadate resistant mutants of *Candida albicans* showed a 20 - 70% reduction in the rate of phosphate uptake in high phosphate medium and phosphate uptake pumps exhibited an increased K_m and reduced V_{max}. These mutants, therefore, developed resistance to elevated concentrations of vanadate by modifying the rate of entry of vanadate.

Ortiz-Bernad *et al.* (2004) showed that when *Geobacter metallireducens* was inoculated into media with vanadium (V) as sole electron acceptor, vanadium (V) was reduced to vanadium (IV) under anaerobic conditions. Also Antipov *et al.* (2000) isolated *Pseudomonas isachenkovii* from an ascidium worm that can accumulate vanadium ions in their blood cells up to a concentration of 350mM, which exceeds the vanadium ion concentration in seawater by ~10^7. This bacterium is highly resistant to toxic vanadium and under anaerobic conditions can reduce vanadate to the IV and III oxidation states by utilizing vanadate as the final electron acceptor.

Electron microscopic studies of *Pseudomonas isachenkovii* cells showed that cultivation of the bacterium on vanadate-containing medium (in contrast to nitrate-

containing media) resulted in the formation of a large number of vanadium-accumulating swells on the surface of the membranes of the cell wall (Figure 1.4). During the course of bacterial growth and vanadate reduction, these containers seem to separate, and as a result, vanadium-binding-protein (vanabins) accumulate in the culture medium. The synthesis of vanadium-binding proteins by the cells of the vanadate-reducing bacterium, followed by excretion of the protein-vanadate complex in the culture medium, appears to be physiologically important. For the bacterium, it is a way of vanadate detoxification; whereas the ascidians in which the bacteria live go on to accumulate the intermediate tetravalent vanadium that is subsequently reduced to the trivalent state in blood vanadocytes. Possibly, there are symbiotic relations between the ascidians and the vanadate-reducing bacteria living in them. The ascidians afford a constant flow of seawater from which the bacteria accumulate vanadium; they also provide microaerophilic or even anaerobic growth conditions necessary for vanadate dissimilation. In turn, the bacteria reduce vanadium to the tetravalent state and accumulate it on the protein described above (Antipov *et al.,* 2000).

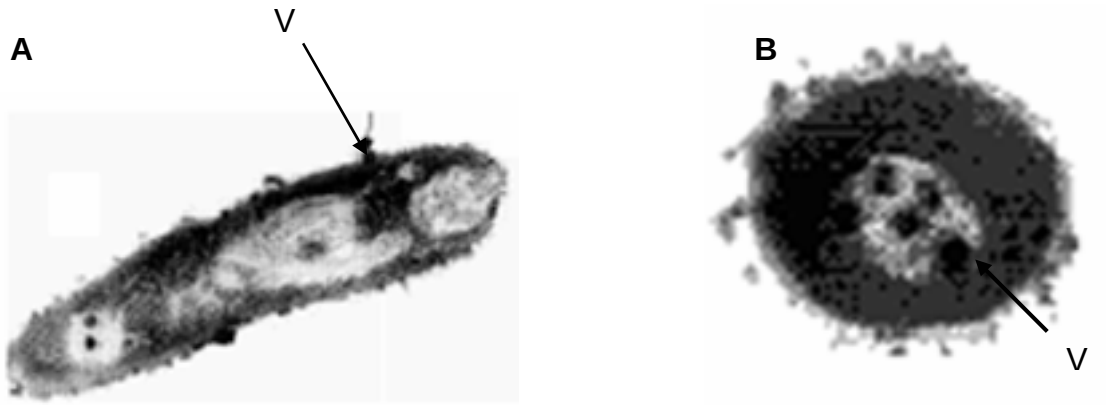

Figure 1.4. Thin sections of *Pseudomonas isachenkovii* cells: (A) lengthwise, (B) cross-section (Taken from Antipov *et al.,* 2000).

Many candidates for the reduction of vanadium in ascidian blood cells have been proposed: tunichromes, a class of hydroxy-Dopa containing tripeptides, glutathione, H_2S, NADPH, dithiothreitol and other thiols such as cysteine. The third enzyme in the pentose-phosphate pathway, 6-Phosphogluconate dehydrogenase (6-PGDH), as well as the first enzyme in the pentose phosphate pathway, Glucose-6-phosphate

dehydrogenase (G6PDH), is localized in the cytoplasm of vanadocytes. These enzymes produce 2 moles of NADPH in the pentose phosphate pathway. It has been reported that V (V) stimulates the oxidation of NADPH; specifically, when V (V) is reduced to V (IV) in the presence of NADPH *in vitro*. These observations suggest that NADPH conjugates the reduction of V (V) to V (IV) in the vanadocytes of ascidians (Figure 1.5). From this it can be suggested that vanadium in seawater is incorporated into the interior of vanadocytes by vanabins (vanadium-binding-proteins) and reduced to the IV oxidation state with NADPH produced by the pentose phosphate pathway. The V (IV) bound with vanabin is transferred to an unknown protein on the vacuolar membrane by means of a metal-transporter, where V (IV) is further reduced to the 3+ oxidation state by unknown reductant(s) (Michibata *et al*., 2003).

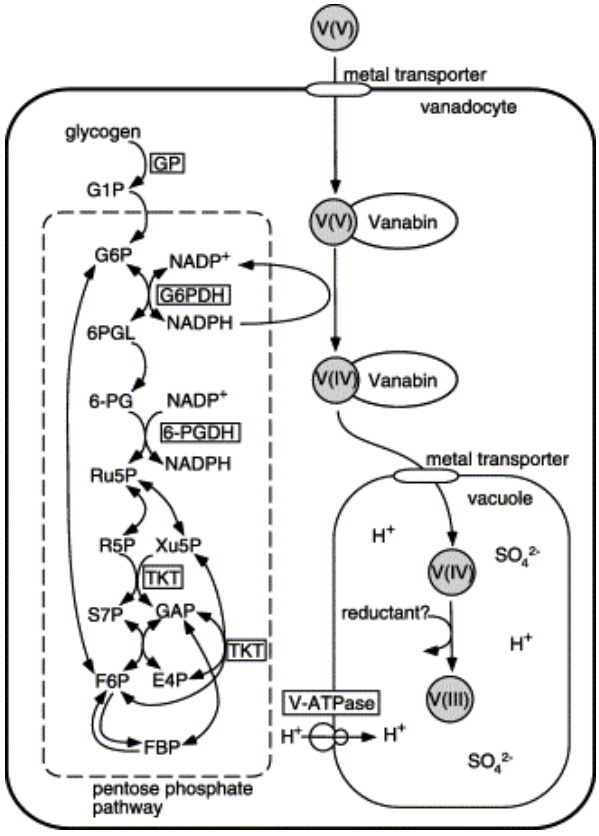

Figure 1.5. A schematic representation of the pathway of vanadium accumulation and mechanism of vanadium reduction (Taken from Michibata *et al.,* 2003).

Studies performed by Shi and Dalal (1991) showed that flavoenzymes such as glutathione reductase, lipoyl dehydrogenase and ferredoxin-$NADP^+$ can reduce

vanadium (V) to vanadium (IV) in the presence of NADPH by functioning as a one-electron vanadium-(V)-reductase (Figure 1.6). The oxidation of NAD(P)H by vanadate plus any source of O_2^- or by vanadyl plus any source of H_2O_2 could contribute significantly to the toxicity of vanadate (Liochev and Fridovich, 1987).

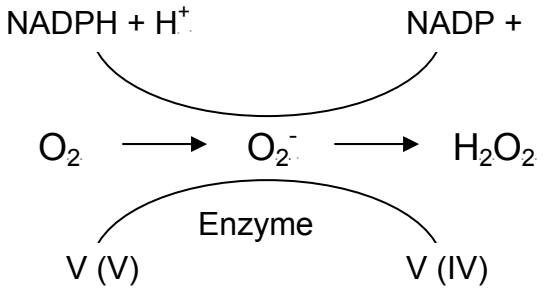

Figure 1.6. Formation of V (IV) by a flavoenzyme.

1.9.1.3 Compartmentalization

Vanadium resistance in the yeast *Hansenula polymorpha* affects vacuolation and phosphate metabolism. Mannazzu *et al*. (1997) showed that *Hansenula polymorpha* grown on vanadate-containing medium correlated with various physiological and ultrastructural modifications. These include: (i) the presence of high amounts of polyphosphates that are mainly localized in the vacuole, (ii) an increase in cell vacuolation, and (iii) the appearance of cytoplasmic vesicles and an increase in cristae at the level of the plasma membrane.

The observed increase in cell vacuolation, together with the high amount of polyphosphates and their vacuolar localization, suggests that once inside the cell, metal ions could be compartmentalized to the vacuole, as suggested by Davies *et al*. (1992) in plant cells, and trapped by polyphosphates. Such a compartmentalization process could be an effective detoxification mechanism which may precede the extrusion of the accumulated metals from the cell (Mannazzu *et al.,* 1997).

It has been established that polyphosphates can act as metal sequestering agents in some organisms. Studies done on *Klebsiella aerogenes* have shown that accumulation of polyphosphates may correlate with heavy metal detoxification. On

the other hand, in *Escherichia coli* the ability to hydrolyze polyphosphates seems to be more important for heavy metal tolerance than the intracellular polyphosphate concentration (Keasling and Hupf, 1996).

1.10 Remediation

The quality of life on Earth is linked inextricably to the overall quality of the environment, and the waste products resulting from human activities have always been a serious problem. One such problem is contaminated soils which are generally the result from past industrial activities when awareness of the health and environmental effects connected with the production, use and disposal of hazardous substances were less well recognized than they are today (Leung, 2004; RIMS, 1999). It is now widely recognized that contaminated soil is a potential threat to human health, and its continual discovery over recent years has led to international efforts to remedy many of these sites, either as a response to the risk of adverse health or environmental effects caused by contamination or to enable the site to be redeveloped for use (RIMS, 1999; Vidali, 2001).

The conventional remediation technologies used for *in situ* and *ex situ* remediation include soil flushing or washing, chemical reduction/oxidation, incineration, excavation and retrieval, landfill and disposal (Prasad and De Oliveira-Freitas, 1999). These methods have some drawbacks. In the landfill method, the contaminant is simply moved elsewhere and may create significant risks in the excavation, handling, and transport of hazardous material. Additionally, it is very difficult and increasingly expensive to find new landfill sites for the final disposal of the material. Also, methods such as incineration may increase the exposure to contaminants for both workers at the site and nearby residents. In general the conventional remediation technologies are expensive and destructive (Vidali, 2001).

Bioremediation is an alternative to traditional remediation technologies, where bioremediation offers the possibility to transform or degrade contaminants into non-hazardous or less hazardous chemicals using natural biological activity (Leung, 2004; NABIR, 2003; Vidali, 2001). As such, it uses relatively low-cost and low-technology techniques, which generally have a higher public acceptance and can

often be carried out on the site. It will not always be suitable, however, as the range of contaminants on which it is effective is limited, the time scales involved are relatively long, and the residual contaminant levels achievable may not always be acceptable (Vidali, 2001).

1.11 Bioremediation

By definition, bioremediation is the use of living organisms, primarily microorganisms, to degrade environmental contaminants into less toxic forms. Bioremediation uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment (NABIR, 2003; Vidali, 2001). The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. There are three classifications of bioremediation: (i) biotransformation, which is the alteration of contaminant molecules into less or non-hazardous molecules; (ii) biodegradation, which is the breakdown of organic substances in smaller organic or inorganic molecules; and (iii) mineralization, which is the complete biodegradation of organic materials into inorganic constituents such as CO_2 or H_2O (Leung, 2004).

For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products. As bioremediation can be effective only where environmental conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate (Vidali, 2001). Microorganisms already living in contaminated environments are often well adapted to survival in the presence of existing contaminants and to the environmental parameters. These indigenous microbes tend to utilize the nutrients and electron acceptors that are available *in situ*, provided that liquid water is present (NABIR, 2003), and this then results in a lesser degree of manipulation of the environmental parameters.

Like other technologies, bioremediation has its limitations. Some contaminants, such as chlorinated organic or highly aromatic hydrocarbons, are resistant to microbial attack. They are degraded either slowly or not at all, hence it is not easy to predict

the rates of clean-up for a bioremediation exercise; there are no rules to predict if a contaminant can be degraded. Bioremediation techniques are typically more economical than traditional methods such as incineration, and some pollutants can be treated on site, thus reducing exposure risks for clean-up personnel, or potentially wider exposure as a result of transportation accidents. Since bioremediation is based on natural attenuation, the public considers it more acceptable than other technologies (Leung, 2004; Vidali, 2001). Most bioremediation systems are operated under aerobic conditions, but under anaerobic conditions microbial organisms may be coerced into degrading otherwise recalcitrant molecules.

1.11.1 Bioremediation strategies

Different bioremediation techniques are employed depending on the degree of saturation and aeration of an area. *In situ* techniques are defined as those that are applied to soil and groundwater at the contaminated site with minimal disturbance. *Ex situ* techniques are those that are applied to soil and groundwater at an alternate site where the contaminant has been removed via excavation (soil) or pumping (water). Bioaugmentation techniques involve the addition of microorganisms with the ability to degrade pollutants to the contaminated sites (Leung, 2004; Vidali, 2001).

1.12 Microbial interaction with metals – a possible solution to metal contaminated sites

1.12.1 Chromium

Microbial reduction of chromium (VI) is attractive for several reasons. Microbes reduce chromium under either aerobic or anaerobic conditions (Garbisu *et al.,* 1998; Lovley and Phillips, 1994; Wang and Shen, 1995; Wang *et al.,* 1989). The reason that some microbes have developed a capacity for chromium (VI) reduction has not yet been adequately explained. It has been suggested that the reduction may be a mechanism for chromate resistance, or that chromium (IV) reduction may just be a fortuitous reaction carried out by enzymes that have other physiological substrates. It has also been suggested that chromium (VI) reduction may provide energy for a few microbes (Ramasamy *et al.,* 2000).

Russian researchers first proposed the use of chromium (VI)-reducing bacterial isolates in the removal of chromates from industrial effluents. Since then, various reduction parameters have been evaluated, which accelerated chromium (VI)-reducing capabilities, for a diverse group of microorganisms with the prospect of developing commercially viable bioremediation techniques exploiting these organisms. Bioreactors have been used which essentially consist of a reduction and removal phase: Chromium (VI)-reducing bacteria are immobilized on inert matrices within the reactor, chromate-contaminated effluent is pumped into the reactor and supplemented with various carbon sources and nutrient additives, followed by a settling or filtration phase to remove chromium (III) precipitates (Ramasamy *et al.,* 2000). Another application of direct reduction was demonstrated using anaerobic chromate-reducing bacteria. Cultures were contained within dialysis tubing and submerged in contaminated water. Chromate diffusing into the tubes was reduced and precipitated and thus unable to diffuse out. Laboratory studies using this system showed that 90% of chromium was removed from the wastewater (Ramasamy, 1997).

1.12.2 Arsenic

Arsenic is an ubiquitous element, and its presence in soil is due to both natural and anthropogenic inputs. The properties of some arsenic compounds have been known and used for thousands of years (Wise *et al.,* 2000). In soils, arsenic is present in organic and inorganic species that differ in their toxicities and mobilities. Arsenic (III) is the stable oxidation state in reduced soils. It is more toxic, soluble and mobile than arsenic (V). As_2O_3 is one of the most common arsenic compounds in anthropogenically contaminated soils. Arsenic (V) is the most common oxidation state in aerobic conditions and its geochemistry is similar to that of phosphate in soils (Deuel and Swoboda, 1972).

Biotechnologies that exploit microbes, which are capable of mediating a variety of reactions to protect themselves from toxic pollutants or to use the contaminants as substrates to obtain energy, led to a more advanced state of development for remediation of organic compounds. Microbial methylation of arsenic has been known for a long time and is common to both bacteria and fungi. Bacterial

methylation seems to be favored by anaerobic conditions and may be employed only in *ex situ* bioreactor systems (McBride and Wolfe, 1971). Fungal methylation seems to be important in the volatilization of arsenic compounds used in agriculture (Cullen *et al.,* 1984).

In 1994 Ahmann *et al.* isolated a bacterium (MIT-13), from arsenic-contaminated sediments, which was able to reduce arsenic (V) to arsenic (III) by dissimilatory reduction. Disappearance of arsenic (V) was associated with arsenic (III) appearance and an increase in biomass. In the presence of 2mM lactate, cells were able to reduce 10mM arsenic (V) within four days.

1.13 Conclusions

Since some environments (volcanic soils, deep-sea vents) naturally contain high concentrations of toxic metals, microbes have been exposed to such materials long before mankind began increasing local concentrations through industrial activity. From literature it is clear that there exist microorganisms which are capable of interacting with metals and in some cases to render these metals less toxic, either through reduction/oxidation reactions, or by making these metals less bio-available through precipitation. Microorganisms can enzymatically reduce a variety of metals in metabolic processes that are not related to metal assimilation. Some microorganisms can conserve energy to support growth by coupling the oxidation of simple organic acids and alcohols or hydrogen to the reduction of certain metals (Lovley, 1993; Williams and Silver, 1984).

Enzymatic detoxifications of heavy-metal ions are not only of interest from a biogeochemical perspective, but also prove to be of value in the control of metal pollution. Microorganisms that use metals as terminal electron acceptors, or reduce metals as a detoxification mechanism play an important role in the cycling of both organic and inorganic species in a variety of environments, including aquatic sediments, submerged soils and terrestrial subsurface. It is also becoming increasingly apparent that microbial metal reduction may be manipulated to aid in the remediation of environments and waste streams contaminated with metals and certain organics. Research on metal reduction is being driven forward by both the

need to understand the fundamental basis of biogeochemical cycles of several key elements, and also by the possibility of harnessing reduction activities for a range of biotechnological applications. The processes include the bioremediation of metal-contaminated sites, as well as metal recovery in combination with the formation of novel biocatalysts (Lloyd, 2003; Lovley, 1993).

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CHAPTER 2

Introduction to present study

2.1 Introduction

Metals have played a crucial role in the development of human civilizations. Metals like gold and copper were used since early times to make useful and desirable objects. The large-scale production of iron and steel, due to the development of the blast furnace, ushered in the Industrial Revolution. Metals are not just a part of mankind's long industrial heritage, but metals are also finding an increasing use in areas as diverse as medicine, electronics, catalysis and the generation of nuclear power. Given mankind's long and intimate association with metals, and the continued dependence on these important natural resources, it is not surprising that their use can lead to significant environmental problems that need to be addressed (Lloyd, 2002).

Vanadium is a heavy metal which has an increasing and wide variety of uses, especially in modern times. An increased use of vanadium has led to an increased risk of exposure and environmental contamination (Broderick, 1977; Cruywagen *et al*., 1981). Vanadium is toxic to most forms of life as it is an inhibitor of phosphate associated enzymes; due to the structural similarity of phosphate to vanadate.

Biotechnological approaches that exploit microbial activities may offer practical solutions to metal contaminated environments. Bioaugmentation is the process of introducing microorganisms into an environment. Although microorganisms cannot destroy metals they can alter their chemical properties via a surprising array of mechanisms, some of which can be used to treat metal contamination (Leung, 2004; Lloyd, 2002; Vidali, 2001).

During December 2001 and January 2002 samples were collected from the Evander gold mine by students attending the 2001 Research Experience for Undergraduates program and from an Antimony mine by members of the Extreme Biochemistry

Group (University of the Free State). Bacteria obtained through enumeration were screened for a variety of functions.

In this study the bacteria obtained through enumeration were screened for vanadium reducing capabilities, and the vanadium reducing ability of one of the bacteria characterized. The suitability of this bacterium as a bioremediation tool was assessed.

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CHAPTER 3

Vanadium reduction by bacteria isolated from South African mines

3.1 Introduction

The industrial revolution of recent times has resulted in large scale pollution of the biosphere. The primary source of this pollution are mining and smelting industries, municipal wastes, fertilizers and sewage sludge. Toxic metal contamination of soil poses a major environmental and human health concern, which is still in need of an effective and affordable technological solution (Wise *et al.,* 2000).

Vanadium contamination is a real problem in South Africa. Vanadium contamination of communal grazing lands in the North West Province occurred after a vanadium dam in the region of the grazing land collapsed, which resulted in the death of several cattle (McCrindle *et al.,* 2001). Also, in September 2003 Vametco was sued by its workers, whom had shown symptoms associated with vanadium poising, after being exposed to large quantities of vanadium while working at the company's open cast mines just outside Brits (SABC News, 2003).

Cleanup of contaminated sites in the recent past have been mediated through microbial interactions with the contaminants. These microorganisms have the ability to render the contaminants less toxic and in some cases even resulted in the precipitation of the contaminant making it less bio-available (Ahmann *et al.,* 1994; Ramasamy, 1997; Silver and Phung, 1996; Williams and Silver, 1984).

The aim of this study, therefore, became to obtain a microorganism capable of reducing vanadate to a less toxic state when present in moderate to high concentrations, and to find the optimum growth conditions for said microorganism.

3.2 Materials and methods

3.2.1 Materials and chemicals

Analytical reagent grade chemicals were obtained from commercial sources and were used without any further purification. Unless mentioned otherwise, all reagents were purchased from Sigma or Merck. Tryptone was obtained from Biolab. Yeast extract was from Difco.

3.2.2 Microorganisms

Mesophilic bacteria were isolated from samples obtained from South African mines by enumeration in TYG-medium (5g/l Tryptone powder, 3g/l yeast extract and 1g/l glucose) at 37°C for 24 hours. The samples from the Evander gold mine were collected by students attending the December 2001 Research Experience for Undergraduates program, while the Antimony mine samples were collected by members of the Extreme Biochemistry Group (University of the Free State) in January 2002. Table 3.1 lists the locations where the bacteria were isolated.

The pure cultures were sustained aerobically at 4°C on TYG-plates containing bacteriological agar at 1.6% (w/v). Fresh TYG-plates of the cultures were prepared every two months. For liquid cell growth a pre-culture was standardized. A loop of cells were inoculated into 50ml TYG-medium and grown at 37°C to an OD_{600nm} of 1.5 (approximately 4 – 6 hours). A 10% inoculum was used for the main culture grown in 500ml shake flasks containing a final volume of 100ml.

3.2.3 Screening for vanadium tolerance and reduction

Preliminary screening was done by subjecting the sixteen bacterial samples (Table 3.1) to varying concentrations (0.1 to 20mM) of ammonium metavanadate (NH_4VO_3). The bacteria which indicated tolerance or reactivity at high NH_4VO_3 concentrations were passaged on TYG-plates until pure cultures were obtained and numbered accordingly. The now pure cultures (Table 3.2) were subjected to another round of screening on TYG-agar plates containing 20mM NH_4VO_3. Since metal reduction

usually occurs under oxygen limited conditions (Carpentier, 2003; Lloyd, 2003; Lovley, 1993; Wang *et al.,* 1989), the bacteria were grown anaerobically. This was performed by making stab cultures in screw cap test-tubes containing 5ml TYG-agar medium supplemented with 20mM NH_4VO_3. These experiments were repeated under micro-aerophilic conditions. Micro-aerophilic conditions were simulated by making TYG-agar slants, supplemented with 20mM NH_4VO_3, in screw cap test-tubes, but the isolates were allowed to grow on the slant surface

Table 3.1. The locations at which the bacteria were isolated

Isolate	Source
1	Evander 221 BH 12/17/02
2	Evander Gr1 BF
3	Evander V 211
4	Evander Gr1 BF 5
5	Evander Gr1 FW
6	Evander 221BM 12/17/02
7	Evander 221BF 12/17/02 G2
8	Antimony Site 14
9	Antimony Site 15
10	Antimony Site 9
11	Antimony Site 17
12	Antimony Site 10.1
13	Antimony Site 10.2
14	Antimony Site 17.2
15	Antimony Site 16
16	Antimony Site 17.3

.

Table 3.2. Pure bacterial cultures obtained from vanadium tolerant isolates.

Isolate	Figures 3.8 and 3.9	Source
1.1	A	Evander 221 BH 12/17/02
1.2	B	Evander 221 BH 12/17/02
2.1	C	Evander Gr1 BF
4.1a	D	Evander Gr1 BF 5
4.2a	E	Evander Gr1 BF 5
4.2b	F	Evander Gr1 BF 5
6.1	G	Evander 221BM 12/17/02
6.2	H	Evander 221BM 12/17/02
7	I	Evander 221BF 12/17/02 G2
13.1	J	Antimony Site 17
13.2a	K	Antimony Site 10
13.2b1	L	Antimony Site 10
13.2b2	M	Antimony Site 10
14.2	N	Antimony Site 17

3.2.4 Whole cell reductions

Whole cell reduction experiments and all further experiments were performed using the oxide form of pentavalent vanadium (V_2O_5) rather than the salt (NH_4VO_3), as the reproducibility of the assay described in section 3.2.5.1 is not as consistent when using the salt as it is when using the oxide. However, reduction occurs when either the oxide or the salt is used, as a blue color which is indicative of vanadium (IV) is present in the culture medium.

To separate the resistant bacteria from the bacteria which were able to reduce pentavalent vanadium, the selected bacteria were subjected to whole cell reduction experiments under anaerobic conditions (Carpentier *et al.,* 2003). The bacteria were grown in 500ml shake flasks containing 100ml TYG-medium at 37°C for 12 hours in the presence of 2mM vanadium pentoxide (V_2O_5). The cells were harvested by centrifugation at 5000 x g for 10 minutes and washed three times with sodium hydrogen carbonate buffer, pH 7.0. The cells were resuspended in the same buffer in a 1:5 w/v ratio and flushed with nitrogen gas. The isolates were supplemented with an electron donor (10mM pyruvic acid) and electron acceptor (5mM V_2O_5) and incubated at 37°C. Samples were withdrawn at zero, two and four hours and assayed for vanadium reduction, as described in section 3.2.5.1.

Aerobic whole cell reductions were also preformed. Cells were washed and resuspended (1:5 w/v ratio) in 20mM HEPES buffer, pH 7.0. Pyruvic acid was used as electron donor to a final concentration of 10mM, while V_2O_5 was added as electron acceptor to a final concentration of 5mM. The isolates were incubated at 37°C and samples were withdrawn at zero, two and four hours and assayed for vanadium reduction, as described in section 3.2.5.1. The appropriate blank rates were monitored. All experiments from the point onwards were conducted using HEPES buffer, pH 7.0. HEPES is a biological buffer and is thus more suitable than sodium bicarbonate buffers for buffering in the physiological pH range of 7.2 - 7.6 (Freshney, 2000). Also in comparison to the inorganic sodium bicarbonate buffering system, the HEPES buffering system may be used with or without a CO_2 blanket.

3.2.5 Assays

3.2.5.1 Pentavalent vanadium determination

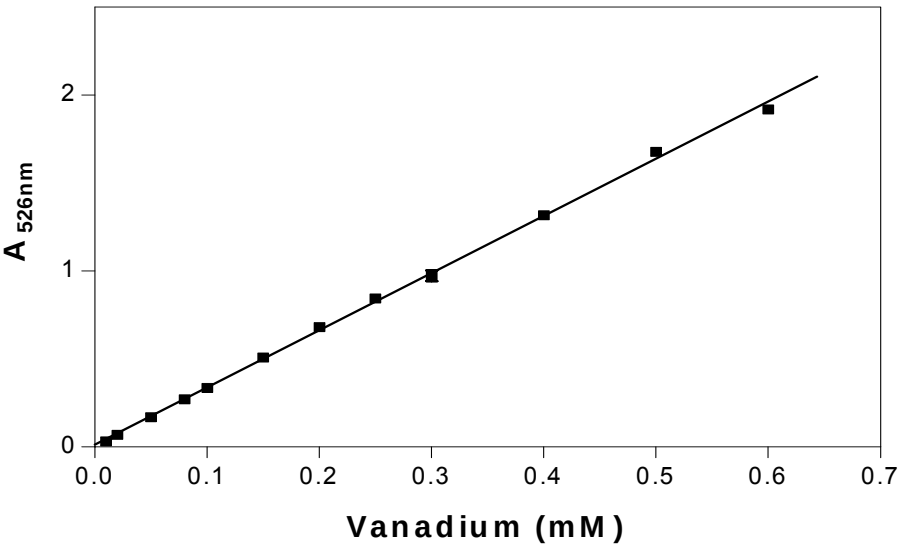

Figure 3.1. Standard curve for vanadate concentration. Error bars indicate standard deviations, but are smaller than symbols used.

Pentavalent vanadium concentrations were determined using a modified version of the method of Mohamed and El-Shahat (2000). Seven hundred and fifty micro-liters of the assay mixture were added to 500µl 10M ortho-phosphoric acid and 1250µl redistilled water. The mixture was vortexed briefly and color formation produced by

adding 125µl 20mM perphenazine. Absorbance was read at 526nm and triplicate values were used to construct a standard curve (Figure 3.1).

3.2.5.2 Tetravalent vanadium determination

Tetravalent vanadium concentrations were determined using a modified version of the method of Costa *et al.* (1998). Eight hundred micro-liters of sample were added to 100µl 100µg/ml ferric iron, 500µl 0.05% Br-PADAP (4-(5-Brom-2-pyridylazo)-N,N-diaethyl-3-hydroxyanilin) and 250µl acetate buffer, pH 5.75. The mixture was vortexed for two minutes after which 100µl 0.1% (w/v) EDTA was added and made up to a final volume of 2.5ml with redistilled water. The absorbance was read at 748nm and triplicate values were used to construct a standard curve (Figure 3.2).

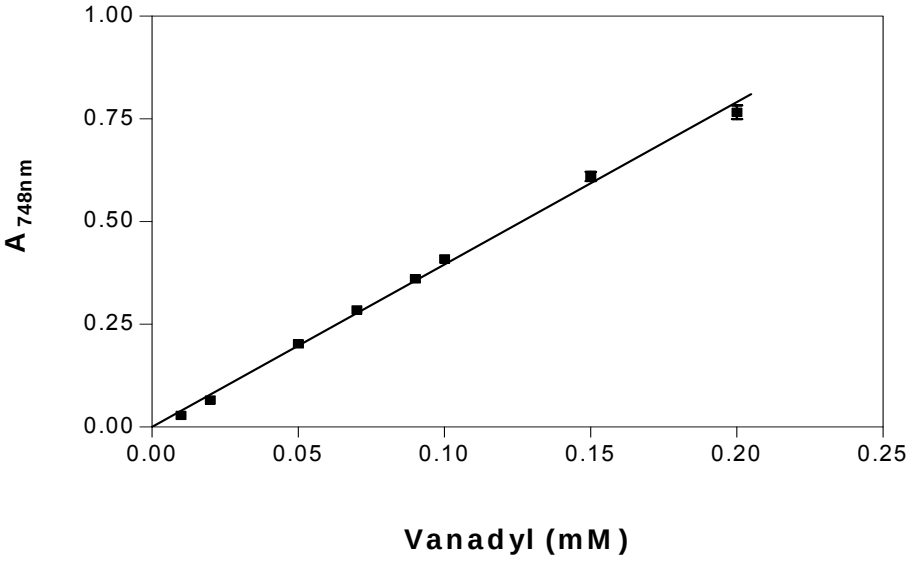

Figure 3.2. Standard curve for vanadyl concentration. Error bars indicate standard deviations, but are smaller than symbols used.

3.2.5.3 Vanadium (IV) oxide (VO_2) interference

The degree of interference of VO_2 on the pentavalent vanadium assay was determined by adding VO_2 to a final concentration of 1.5mM to the pentavalent vanadium dilution series used to construct the standard curve. This was done in such a manner that the final V (V) concentration for the samples with and without

VO_2 was the same for each of the respective points on the standard curve (Figure 3.3).

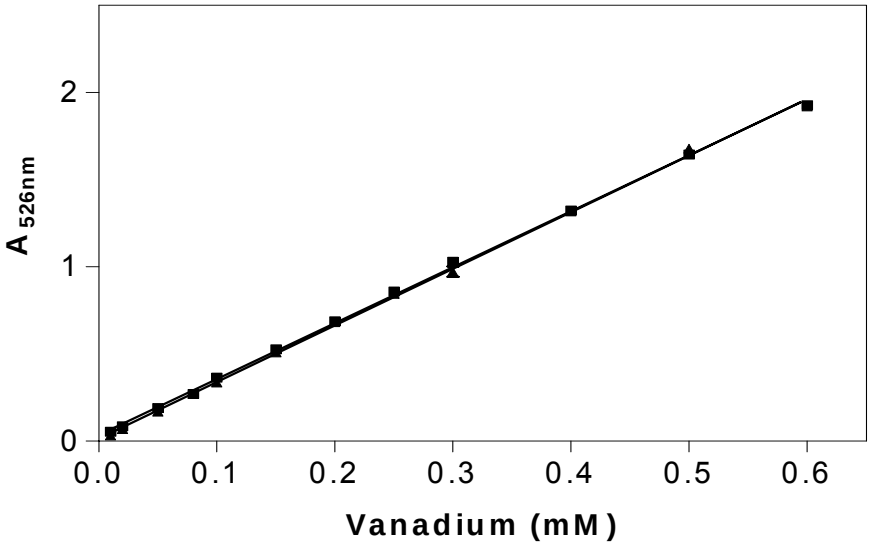

Figure 3.3. Interference of vanadium (IV) oxide (■) on the standard curve for pentavalent vanadium (▲) concentration. Error bars indicate standard deviations.

3.2.5.4 Vanadium pentoxide interference

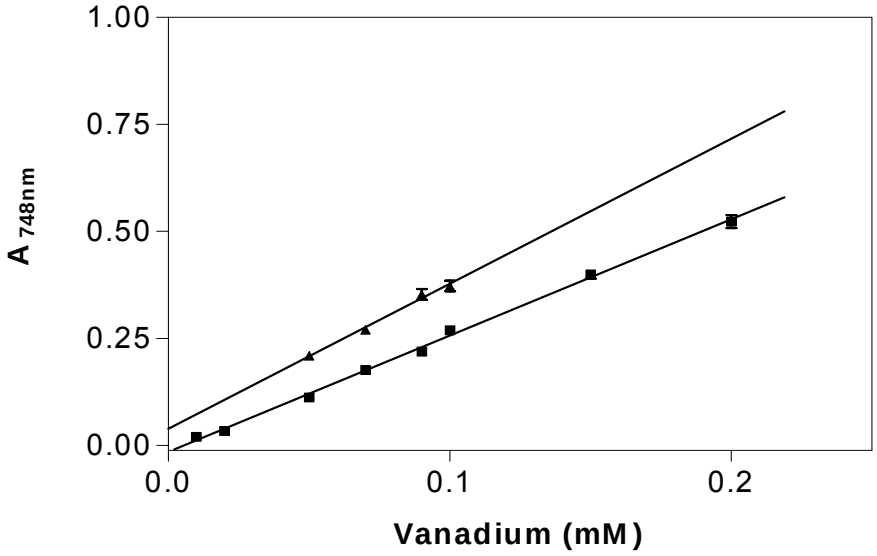

Figure 3.4. Interference of vanadium pentoxide (■) on the standard curve for vanadium (IV) concentration (▲). Error bars indicate standard deviations.

The degree of interference of V_2O_5 on the vanadium (IV) assay was also assessed. This was done in the same manner as described in section 3.2.5.3, but V_2O_5 was added to the V (IV) dilution series to a final concentration of 0.833mM (Figure 3.4).

Vanadium reduction activity was calculated by measuring the rate of substrate [vanadium (V)] depletion. Substrate depletion was followed because vanadium (V) interfered with the vanadium (IV) assay, but the product [vanadium (IV)] did not interfere with the vanadium (V) assay. As V (IV) did not interfere with the vanadium (V) assay there was no need for oxidation of the samples to determine total vanadium concentrations and the subsequent calculations to determine the amount of vanadate reduced. Vanadate reductase activity is therefore defined as: the micromolar amount of vanadium (V) reduced per minute per milliliter enzyme.

3.2.5.5 Assay for vanadium

One milliliter cell suspension, unless otherwise stated, was flushed with nitrogen and incubated at 37°C for 30 minutes along with a final concentration of 5mM V_2O_5 (electron acceptor) and 10mM electron donor, except in the cases were NADH was used as electron donor. NADH was added to a final concentration of 1mM. The vanadium concentration was determined as described in section 3.2.5.1.

3.2.6 Identification of bacterial isolate

The following experiments were performed using isolate 6.2 which exhibited the highest vanadate reducing capabilities under both aerobic and anaerobic conditions.

3.2.6.1 Light microscopy

A 24 hour old culture of isolate 6.2 was used in the preparation of gram staining slides (Lagasse *et al.,* 2001). A drop of culture was placed onto an alcohol cleaned microscope slide, heat fixed and left to cool at room temperature for a few seconds. Crystal violet stain was added over the fixed culture and rinsed off with water after 60 seconds. This was followed by staining with iodine for 60 seconds. The cells were washed to remove excess iodine and covered with ethanol for 30 seconds. This was

followed by another wash step and counter-stained with safranin for 30 seconds. After removal of excess safranin by washing, the slide was dried by blotting it with tissue paper. A Nikon light microscope attached to a camera was used to obtain the results for the gram stain.

3.2.6.2 Electron Microscopy

Cells were grown for 4 hours in the presence of 2mM V_2O_5 and in the absence thereof under aerobic conditions. The cells were harvested and washed with 0.1M sodium phosphate buffer, pH 7.0, and fixed overnight in a 3% glutaraldehyde solution, prepared in sodium phosphate buffer, pH 7.0. Cell recovery was done by centrifugation and washing twice with sodium phosphate buffer, pH 7.0. A 0.5% OsO_4 (osmium tetraoxide) solution was used for a fixation time of between 30 minutes and 2 hours, until the cells turned dark in color. The OsO_4 was decanted and the cells repeatedly washed with sodium phosphate buffer, pH 7.0 (Van Wyk and Wingfield, 1991).

3.2.6.2.1 Scanning Electron Microscopy

The fixated cells were dehydrated with a series of 50-90% ethanol for 30 minutes followed by a 100% ethanol step for 1 hour followed by another 100% ethanol step left overnight. The cells were transferred to the critical point drier specimen holder, which was flushed four times with liquid nitrogen for between 5 and 10 minutes at a time. The temperature was increased and samples dried. This was followed by mounting of the specimens onto SEM examination stubs using epoxy glue. Once the glue had fully set (2 to 24 hours) the specimens were transferred to a sputter coating apparatus, where the specimens were coated with a layer of gold.

3.2.6.2.2 Transmission Electron Microscopy

The fixated cells were mixed with molten 1% agar and placed drop-wise on clean microscope slides. Once the droplets were solidified it was cut into small pieces and dehydrated with a series of 50-90% ethanol for 30 minutes followed by a 100% ethanol step for 1 hour followed by another 100% ethanol step left overnight.

Epoxy resin was prepared freshly at the onset of the final dehydration step. All but approximately 1ml of the remaining 100% ethanol was decanted and to this 1ml of epoxy was added. The mixture was stirred well and allowed to impregnate the samples for up to 8 hours. The 1:1 mixture (epoxy: ethanol) was then removed and replaced by 1ml epoxy and this step repeated again overnight. The samples were then placed in a desiccator to remove all traces of the ethanol.

Epoxy was prepared by weighing (not volumetric measuring) of the following components, 23g VCD (vinylcyclohexene dioxide), 62g NSA (nonenyl succinic anhydride), 14g DER736 (diglycidyl ether of polypropylene glycol) and 1g DMAE [(S1) dimethylaminoethanol] added together with constant stirring.

The moulds were dried in an embedding oven overnight. Samples and epoxy were transferred to the pre-dried and pre-heated moulds and allowed to polymerize at 70ºC for eight hours. Two days after polymerization the embedded material was sectioned by ultra-microtome, yielding sections of approximately 0.2µm.

Sections were mounted on copper grids and stained using drops of stain solutions placed on a wax layer in a Petri dish. The uranium stain is prepared in the dark to prevent photo-oxidation (using a bell jar). The first step is a 20 minute stain with 6% (saturated aqueous solution) uranyl acetate, triple rinsing in three beakers with distilled water. The second stain was a 10 minute stain with lead citrate with the same rinsing procedure with fresh water. The addition of NaOH pellets in the Petri dish where staining procedure took place prevented the formation of lead-carbonate crystals contaminants.

The lead citrate stain was prepared as follows: 0.665g lead nitrate was dissolved in 15ml water, 0.880g sodium citrate was added, which immediately formed a milky-white precipitate. This was swirled continuously for 60 seconds (not shaking to prevent the introduction of oxygen). The solution was allowed to settle for 30 minutes, stirred intermittently and the precipitate cleared with the introduction of 4ml 1N NaOH solution.

Electron micrographs were taken with a Joel 6400 WINSEM (Japan) and a Philips CM 100 (The Netherlands) TEM (Van Wyk and Wingfield, 1991).

3.2.6.3 Genomic DNA isolation

Genomic DNA from isolate 6.2 was prepared from a 5ml TYG culture grown overnight at 37°C. Two milliliters of the cells suspension were harvested by centrifugation for 2 minutes using a bench top micro-centrifuge. One milliliter of DNAzol (Gibco, BRL) was added and vortexed for 30 – 60 seconds to lyse the cells. Cell debris was pelleted by centrifugation at room temperature for 10 minutes at 14 000 x g. The supernatant was transferred to a clean tube and the genomic DNA precipitated with 500µl cold 100% ethanol. The DNA was recovered by centrifugation (20 minutes, 4°C, 14 000 x g) and the resulting pellet was washed with 70% (v/v) ethanol. The sample was again centrifuged (10 minutes, 4°C, 14 000 x g), after which the ethanol was aspirated and the pellet dried under vacuum in a SpeedVac (Savant, USA). The pellet containing the isolated DNA was dissolved in 50µl TE buffer [10mM Tris-HCl (pH 7.8) and 1mM EDTA (pH 8.0)] containing 5mg/ml RNase and stored at -20°C for further use (Ausubel *et al.,* 1990; Cox, 1968).

All PCR and DNA products were electrophoresed and assessed on a 1% agarose gel containing 0.4 µg/ml ethidium bromide. The agarose gels were prepared and electrophoresed in TAE buffer [0.1M Tris-HCl, 0.05M EDTA (pH 8.0) and 0.1mM glacial acetic acid] at 5.6V/cm for 60 minutes. DNA was visualized under a high radiation UV source, while DNA to be isolated from agarose gels for further studies was visualized using a low radiation UV source, and was isolated using the High Pure PCR Purification Kit (Roche). Nucleic acid concentrations were determined using the Eppendorf BioPhotometer (Eppendorf, AG).

3.2.6.4 16S rDNA

3.2.6.4.1 PCR

Polymerase chain reactions (PCR's) were carried out, unless other wise stated, using the Expand High Fidelity (EHF) PCR system (Roche Molecular Biochemicals)

according to the manufacturer's recommendations. The reaction mixture consisted of: 10µl of the 5 x EHF buffer containing 15mM $MgCl_2$, 300nM each of the forward and reverse primers, 200µM dNTP's, 0.5µg template DNA, 2.6 units of EHF enzyme mix, filled to a final volume of 50µl using sterile redistilled water.

Thermal cycling was performed using an Eppendorf Mastercycler Temperature Gradient Personal with the following cycling program, unless otherwise stated, initial denaturation of 5 minutes at 94°C, 35 cycles of denaturation (95°C for 30 seconds), annealing (52°C for 1 minute) and elongation (72°C for 90 seconds). After 35 cycles, a final elongation step of 10 minutes at 72°C was performed to complete synthesis of the amplified product.

The 16S rDNA region of isolate 6.2 was amplified from the genomic DNA using universal 16S rDNA bacterial primers (27F and 1492R). The purified PCR product was ligated overnight into pGem-T easy plasmid vector according to manufacturer's instructions. Transformations and plasmid extractions were preformed as described in section 3.2.6.4.2.2. The 16S rDNA fragment was sequenced as described in section 3.2.6.4.3 by using primers specific for the SP6 and T7 promoter regions of the pGem-T easy vector.

The pGem-T easy vector containing the 16S rDNA fragment was digested with *Eco*RI restriction enzyme and electrophoresed. The resulting bands were cleaned using a High Pure PCR product purification kit (Roche), according to manufacturer's instructions. The cleaned products were ligated overnight into pUC 18 vector which have been digested with *Eco*RI and dephosphorilated with alkaline dephosphorylase. Transformations and plasmid extractions were performed as described in section 3.2.6.4.2.2. The inserts were sequenced as described in section 3.2.6.4.3 by using primers specific for the M13 region of the pUC 18 vector.

3.2.6.4.2 Cloning

3.2.6.4.2.1 Competent cells

Competent *Escherichia coli* cells were prepared according to the method by Hanahan (1983). Flasks containing 100ml SOB media (20g/l tryptone; 2.5g/ yeast extract; 5.0g/l NaCl; 0.19g/l KCl) supplemented with 1ml Mg^{2+} were inoculated with 1ml of an overnight culture and grown at 18°C until an OD_{600} of 0.6 was reached. Cells were incubated on ice for 30 minutes and collected by centrifugation at 3 000 x g for 10 minutes at 4°C. Cells were resuspended in 80ml TB buffer (10mM HEPES; 15mM $CaCl_2.6H_2O$; 250mM KCl; 55mM $MnCl_2$) and incubated on ice for 15 minutes, the cells were collected by centrifugation at 3 000 x g for 10 minutes at 4°C. Cells were resuspended in 20ml TB-buffer and DMSO (Dimethyl sulfoxide) was added to a final concentration of 7%. This was then incubated on ice for 10 minutes; cells were aliquoted and snap freezed in liquid nitrogen. Cells were then stored at -70°C.

3.2.6.4.2.2 Ligation and transformation

Ligation and transformation were preformed according to manufacturer's instructions (pGem-T Easy kit, Promega). Cleaned PCR products were added to 5µl ligation buffer, 0.5µl plasmid vector and 1µl T4 DNA ligase (3 Weiss units/µl). The mixture was made up to a final volume of 10µl with sterile distilled water and incubated overnight at 4°C.

Two and a half microliters of the ligation mixture were added to 50µl of competent *E. coli* Strain JM 109 and left on ice for 30 minutes. This was followed by an incubation step at 42°C for 40 seconds and cooled down on ice for 2 minutes. Eight hundred micro-liters of LB-media supplemented with 50µl 2M Mg^{2+} and 100µl 1M glucose was added to the cell suspension and incubated at 37°C for 1 hour. Cells were pelleted by centrifugation at 4 000 x g for 1 minute and 800µl of supernatant was removed. Cells were resuspended in remaining supernatant and plated onto LB plates supplemented with ampicillin (60mg/l), IPTG [isopropylthio-β-D-galactoside (10mg/l)] and X-gal [5-bromo-4-chloro-3-indolyl-β-D-galactoside (40mg/l)]. Plates were incubated at 37°C for 16 hours. Single, white colonies were selected and inoculated

into 5ml LB-media supplemented with ampicillin (10µl/ml) and grown for 16 hours with shaking at 37°C followed by DNA mini-preparations.

DNA mini-preparations were performed using the lysis by boiling method (Sambrook *et al.*, 1989). Cells were grown overnight at 37°C and the cells harvested by centrifugation. Cells were resuspended in 350µl STET buffer (0.1M NaCl, 10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0 and 5% Triton X-100) and to this 25µl lysozyme (10mM) was added. Samples were boiled for 40 seconds and the bacterial lysate collected by centrifugation at 12 000 x g for 10 minutes at room temperature. The bacterial debris was removed with a sterile tooth pick and the supernatant mixed with 300µl chloroform to precipitate any proteins present. This was again centrifuged at 12 000 x g for 10 minutes. The aqueous phase was transferred to a new tube; 40µl sodium acetate (2.5mM, pH 5.2) and 420µl propan-2-ol were added and incubated at room temperature for 5 minutes. Nucleic acids were recovered by centrifugation at 12 000 x g for 5 minutes at 4°C. The supernatant was removed and 1ml 70% ethanol was added. The mixture was centrifuged at 12 000 x g for minutes at 4°C. The supernatant was removed and the samples dried under vacuum in a SpeedVac (Savant, USA). Screening for the correct recombinant plasmid was performed by restriction analysis using *Eco*RI restriction enzyme, separated by electrophoreses as described in section 3.2.6.3. DNA fragments obtained from this restriction digest were purified using the High Pure PCR Purification Kit (Roche) and eluted in 50µl elution buffer (10mM Tris-HCl, pH 8.5).

3.2.6.4.3 Sequencing

The purified PCR products were used in sequencing reactions to determine the nucleotide composition. Sequencing was preformed by using the ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit V. 3.0 or 3.1 (Applied Biosystems, USA) according to the manufacturer's instructions. Approximately 30 – 50% of each sequencing reaction was loaded onto a 4% acrylamide gel, separated at 1.6kV and data collected on an ABI Prism® 377 DNA Sequencer (Perkin Elmer Biosystems, USA). The data was analyzed using Sequencing Analysis V. 3.3. Sequences were assembled using AutoAssembler V. 1.0. Reverse-

complementation and alignments were done using DNAssist V. 2.0. Analyzed sequences were used to search the Genbank Database (NCBI, 2004).

3.2.6.5 Alternative classical identification tools

3.2.6.5.1 API

An API 20E Identification system for *Enterobacteriaceae* and other gram-negative rods (BioMérieux, Marcy l'Etoile, France) was used according to manufacturer's instructions. The conversion of the numerical profile obtained was preformed by the North-West University.

3.2.6.5.2 Biolog

The pure 6.2 isolate was grown for 24 hours at 37°C on TYG-agar plates. These plates were analyzed at the Food Science section of the Department Biotechnology of the University of the Free State.

3.2.7 Culturing of isolate 6.2

3.2.7.1 Monitoring growth

Biomass production was followed by measuring the optical density of the culture at 600nm. The OD readings were then converted to g/l biomass produced by making use of a standard curve constructed with OD versus biomass (Figure 3.5). Vanadate reduction during growth was followed as described in section 3.2.5.5.

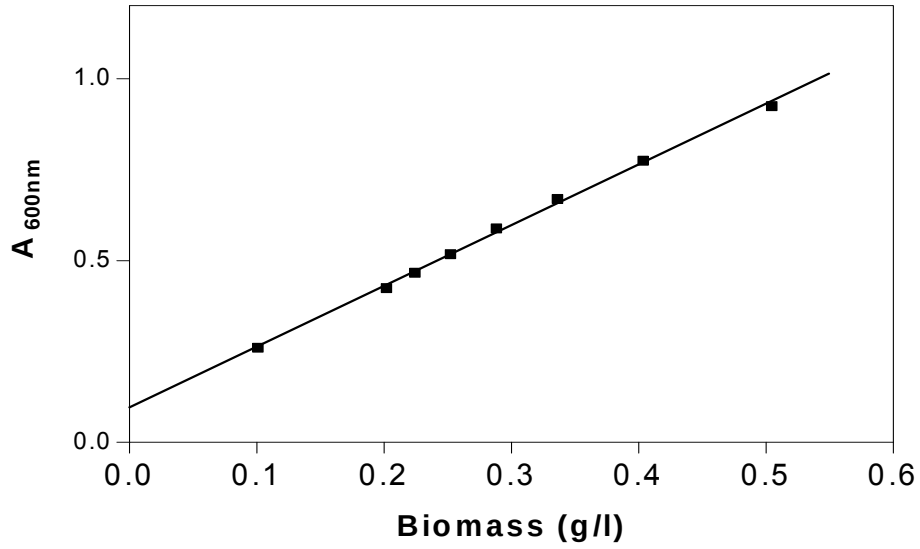

Figure 3.5. Standard curve for determining biomass.

3.2.7.2 Optimum temperature

Optimum temperature for growth was determined by using a temperature gradient incubator (Scientific industries, U.S.A.), consisting of a solid aluminum bar heated at one end and cooled at the other to produce a stable temperature gradient. The bacterial isolates were cultured in L-shaped test tubes made of optically selected glass and the growth was monitored directly by turbidity using a Photolab S6 Photometer (WTW, Weilheim, Germany). These tubes fit snugly into thirty sample wells across the created temperature gradient, and were capped by metal test tube caps. Aeration and agitation was provided by a rocking motion through an arc of approximately 30° at 50 oscillations per minute.

The temperature gradient incubator temperature limits were set at 25 – 55°C and allowed to equilibrate for up to three days with appropriate temperature measurements taken every 12 hours until the gradient remained constant. At the onset of every experiment the culture tubes, containing 10ml of sterile TYG-media, were placed in the temperature gradient incubator overnight to allow equilibration prior to inoculation (Du Preez, 1978).

The Arrhenius equation was used to obtain a linear relationship between growth rate and temperature where μ was the specific growth rate, *A* an entrophy constant, E_a the activation energy, R the universal gas constant (8.314J/mol/K) and T the absolute temperature in K.

$$\mu = Ae^{-(Ea/RT)} \qquad (1)$$

On taking natural logarithms, equation 1 becomes

$$\ln \mu = \ln A - (E_a/R).(1/T) \qquad (2)$$

The value of E_a was thus derived from the slope of the plot of ln μ as a function of 1/T.

3.2.7.3 Optimum pH

The pH of TYG-media was adjusted prior to sterilization. After sterilization a flask of each pH was sacrificed to measure pH change during sterilization. The flasks were inoculated and grown at 37°C, with samples taken periodically to follow biomass production. A pH range of 4 – 9 was tested.

3.3 Results and discussions

3.3.1 Tolerance and reduction screening

3.3.1.1 Aerobic conditions

Nickel and vanadium resistant studies on environmental isolates of *Escherichia hermannii* and *Enterobacter cloacae* showed metal tolerance by the bacterial colonies on agar plates (Hernández *et al*., 1998). A dark coloration of the bacterial colony was considered to be a preliminary marker for metal accumulation. The dark color was considered to be possible reduction and precipitation of the metal, while the formation of a clear halo around the colonies was considered to be possible sequestration of the metal.

Preliminary vanadium tolerance of the sixteen isolates (Table 3.1) was measured as described in section 3.2.3. High concentrations (up to 20mM) of NH_4VO_3 were used to select resistant isolates. Colonies displayed remarkable growth irrespective of concentrations discussed in literature (Bredberg *et al.,* 2004; Ortiz-Bernad *et al.,* 2004; Willsky *et al.,* 1984). It is well known that different valence states of vanadium display different colors, with vanadium (V) being yellow, vanadium (IV) blue and vanadium (III) black (Brownlee, 1960). It was, therefore, especially encouraging to see discoloration of colonies 1 and 14 on the 20mM NH_4VO_3 containing Petri-dish, as well as discoloration of colony 1 on the 10mM NH_4VO_3 containing Petri-dish (Figure 3.6), because this could suggest possible vanadate reduction or precipitation.

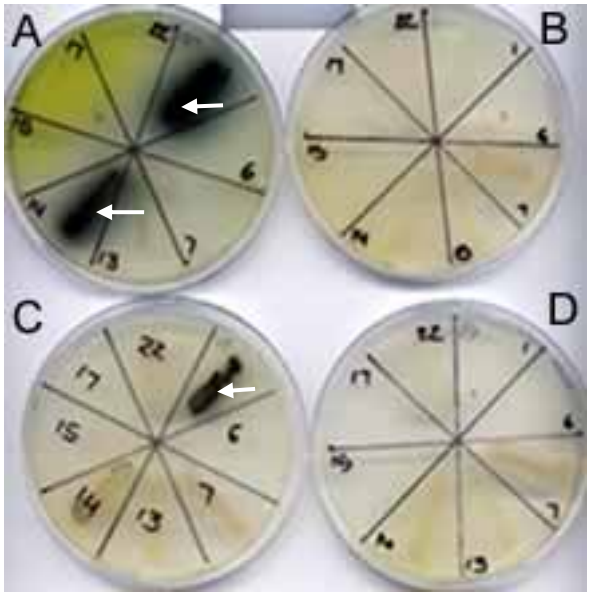

Figure 3.6. Preliminary screening of mine soil samples on NH_4VO_3 containing TYG – agar plates to identify resistant isolates. A) 20mM, B) 1mM, C) 10mM and D) 5mM.

From the sixteen isolates only eight were able to grow at moderate to high NH_4VO_3 concentrations. These eight isolates were purified and eighteen pure cultures were retrieved. These were then again screened on NH_4VO_3 containing plates. Only fourteen of the pure cultures displayed tolerance similar to that of the original samples (Figure 3.7).

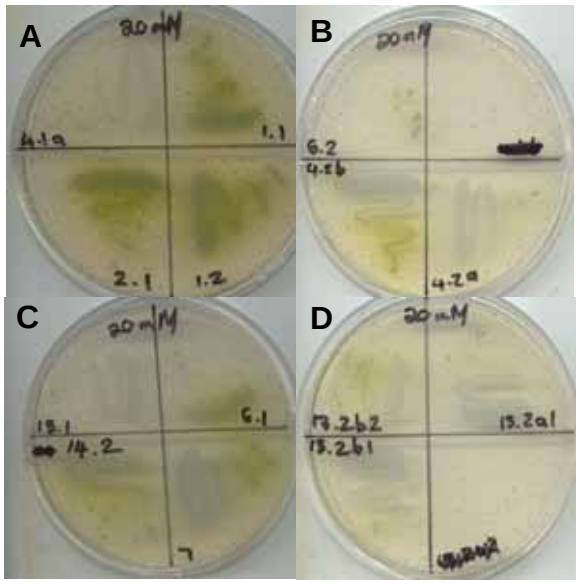

Figure 3.7. Pure bacterial cultures displaying NH_4VO_3 tolerance up to a concentration of 20mM. A is representative of isolates 1.1; 1.2; 2.1 and 4.1a. B is of isolates 4.2a; 4.2b and 6.2. While C is of 13.2a; 13.2b1 and 13.2b2 and D of isolates 6.1; 7; 14.2 and 13.1.

3.3.1.2 Growth under micro-aerophilic and anaerobic conditions

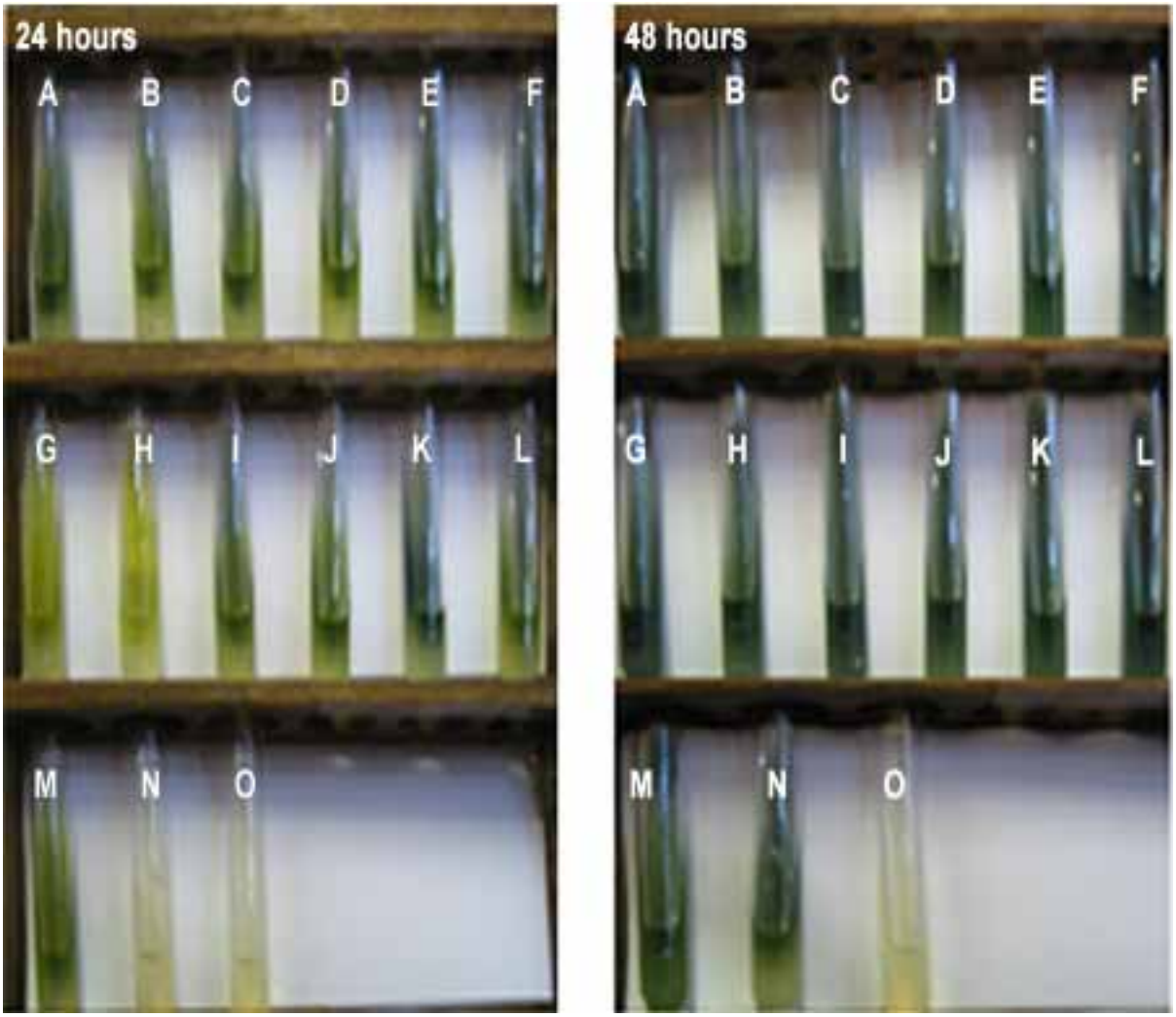

Figure 3.8. Tolerant pure cultures grown on 20mM ammonium metavanadate TYG-agar slants under micro-aerophilic conditions after 24 and 48 hours. A – N represents the individual pure bacterial cultures (Table 3.2), while O is a non-inoculated control.

From literature it is known that most metals are reduced under oxygen limited conditions (Carpentier, 2003; Lloyd, 2003; Lovley, 1993; Wang *et al.,* 1989). Thus, the fourteen tolerant pure cultures were grown under both micro-aerophilic and anaerobic conditions as described in section 3.2.3. Under micro-aerophilic conditions growth could be observed along with a color change (yellow to greenish - blue) (Figure 3.8). This color change might be indicative of vanadate reduction, as a blue color is indicative of vanadyl (Brownlee, 1960) while a green color is indicative of a mixture of the 4+ and 5+ valence states. The color change was more prominent

after 48 hours than after 24 hours suggesting an adaptation period for possible enzyme activation.

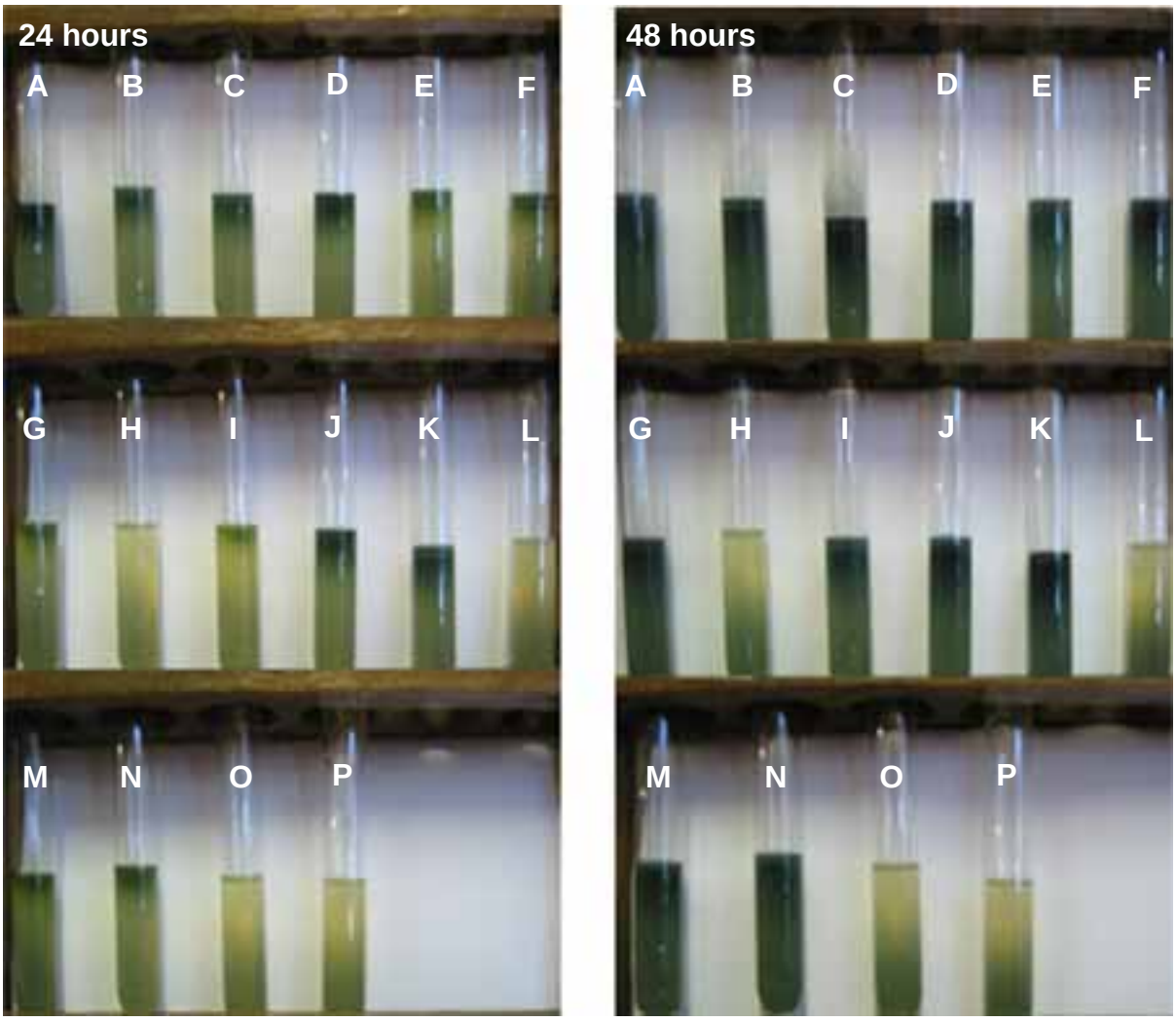

Figure 3.9. Tolerant pure cultures grown as stab cultures in 20mM ammonium metavanadate TYG-agar slants under anaerobic conditions after 24 and 48 hours. A – N represent the individual pure bacterial cultures (Table 3.2), while O and P are non-inoculated controls.

The anaerobic growth experiments also indicated that possibly all of the fourteen cultures with the exception of cultures 6.2 and 13.2b1 could reduce vanadate as a color change from yellow to bluish-black occurred (Figure 3.9). Metal reduction is usually associated with oxygen limited conditions (as simulated by micro-aerophilic and anaerobic growth) and this initial data was very encouraging especially as Figure 3.7 indicate that not all isolates were able to reduce vanadium during the preliminary screening that was performed aerobically.

3.3.1.3 Whole cell reduction

Antipov *et al*., (2000) purified and studied vanadium-binding proteins excreted by the vanadate-reducing bacterium, *Pseudomonas isachenkovii.* They also found that the bacterium was able to reduce vanadate to the 4+ and 3+ oxidation states under anaerobic conditions, and that the bacterium could utilize vanadate as the final electron acceptor by anaerobic respiration. This was also found to be the case when *Geobacter metallireducens* was grown anaerobically with only vanadium (V) as electron acceptor and acetate as electron donor (Ortiz-Bernad *et al.,* 2004).

The fourteen bacterial cultures exhibiting growth in the presence of 20mM NH_4VO_3 were subjected to whole cell reduction experiments as described in section 3.2.4. The cells were harvested and incubated under anaerobic conditions in 20mM bicarbonate buffer, pH 7.0 containing 5mM V_2O_5 as electron acceptor and 10mM pyruvic acid as electron donor (Carpentier *et al*., 2003). The depletion of vanadate was monitored as the V_2O_5 interfered with the product formation [V(IV)] assay. This was shown in section 3.2.5.4.

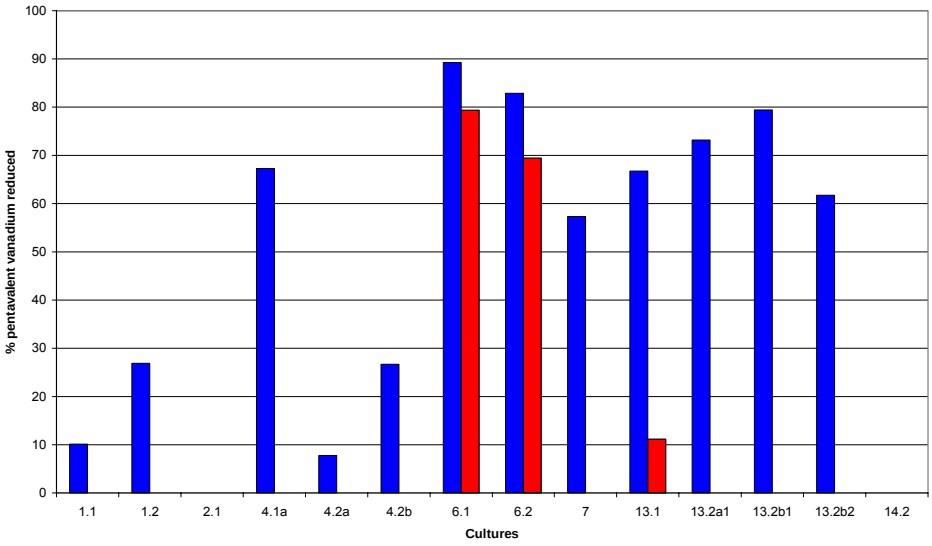

Figure 3.10. Percentage vanadate reduced under non-growth, anaerobic conditions in 20mM sodium bicarbonate buffer, pH 7.0 (■), as well as under non-growth, aerobic conditions in 20mM HEPES, pH 7.0 (■).

All the cultures, except 2.1 and 14.2 were able to reduce V_2O_5. (Figure 3.10). Cultures 6.1 and 6.2 had the highest reduction rate (between 80 and 90% vanadate conversion), followed closely by culture 13.2b1 (approaching 80% conversion). These results obtained differed from those obtained in the initial screening (sections 3.3.1.1 and 3.3.1.2), as no color change was apparent under anaerobic conditions (section 3.3.1.2) for culture 6.2, but in actual fact this culture had the highest reduction rate. It would therefore seem that the preliminary screening is a good indication of resistance to vanadium, but can be erroneous in predicting possible reduction.

To verify that the environmental conditions are important in the reduction of vanadium and that vanadium reduction is enhanced under anaerobic conditions the seven bacterial cultures with the highest reduction rate under anaerobic conditions were subjected to whole cell reduction experiments as described in section 3.2.4. The cells were harvested and whole cell reduction experiments performed under aerobic conditions in 20mM HEPES buffer, pH 7.0.

Most of the seven isolates were unable to reduce V_2O_5 under aerobic conditions, again confirming that the reduction observed was most probably dissimilatory in nature. However, cultures 6.1, 6.2 and 13.1 showed reduction even under aerobic conditions (Figure 3.10), opening a window of either reduction associated with a known protein adapted to reduce vanadium (fortuitous reactions) or by an enzyme(s) specific for vanadate reduction under aerobic conditions.

From the screening results it is clear that under aerobic conditions most of the isolated bacterial cultures can only tolerate vanadate, while under anaerobic conditions vanadate reduction is possible. This could be indicative of two separate mechanisms, one which confers resistance under aerobic conditions and another which confers reduction capabilities under anaerobic conditions.

From all these cultures the most promising one, chosen on the basis of growth on increasing concentrations of NH_4VO_3, as well as possible reduction as discussed in sections 3.3.1.1, 3.3.1.2 and 3.3.1.3, was isolate 6.2. **Isolate 6.2 was therefore used in all further experiments.**

3.3.2 Identification of selected isolate

3.3.2.1 Genomic DNA

Genomic DNA from isolate 6.2 was obtained as described in section 3.2.6.3. After electrophoresis it could be deduced that the genomic DNA was intact (Figure not shown).

3.3.2.2 PCR amplification and sequence analysis of 16S rDNA

The PCR amplification was performed as described in section 3.2.6.4.1 using universal bacterial primers (27F and 1492R). Figure 3.11 shows the expected (±1500bp) 16S rDNA PCR amplification product obtained from the genomic DNA of isolate 6.2.

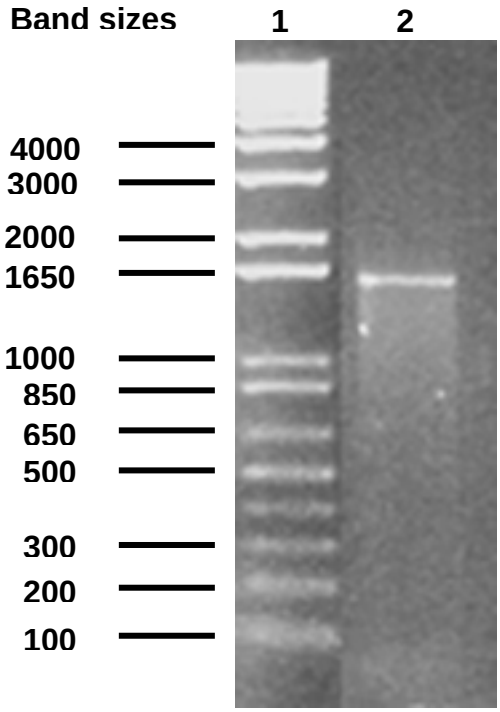

Figure 3.11. Gel electrophoresis of PCR amplification product of 16S rDNA of isolate 6.2. In the first lane the 1kb plus marker was loaded and in the second lane the PCR amplification product.

The 16S rDNA product was cloned into pGEM-T Easy® cloning vector and competent *Escherichia coli* (Strain JM-109) were transformed. The Minipreps were preformed according to section 3.2.6.4.2.2 to isolate proliferated plasmid. This product was subjected to RFLP with *Eco*RI as described in section 3.2.6.4.2.2. Restriction digestion with *Eco*RI enzyme produced two bands (approximately 850 and 650bp, indicated A and B respectively) and the pGEM-T Easy® vector (Figure 3.12). This indicated the presence of an *Eco*RI restriction site in the 16S rDNA fragment.

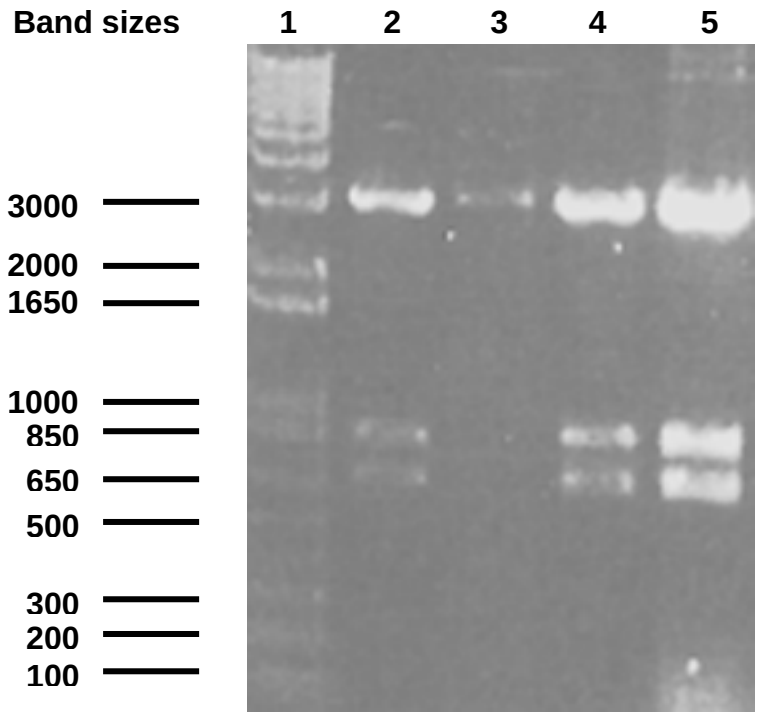

Figure 3.12. Gel electrophoresis of *Eco*RI digest of 16S rDNA insert from pGem-T Easy vector. Lane 1 shows the 1kb plus marker and lanes 2 through 5 the resulting products.

The PCR product was subjected to sequencing as discussed in section 3.2.6.4.3. Sequence analysis of the approximately 1500bp 16S rDNA insert ligated into a pGEM-T Easy® cloning vector gave a 455bp (primer T7) and a 560bp (primer Sp6) sequence. These were subjected to a BLAST (Basic Local Alignment Search Tool) search (NCBI, 2004) where none of the sequences obtained gave a definitive result.

The two resulting bands obtained from the digest of the 16S rDNA fragment ligated into pGem-T easy® cloning vector with *Eco*RI were purified from the agarose gel (Section 3.2.6.3) and cloned into the pUC 18 vector which have been digested with *Eco*RI and dephosphorilated with alkaline dephosphorylase. After transformation Minipreps were performed according to section 2.3.6.4.2.2 to isolate the proliferated plasmid. Restriction digest with *Eco*RI enzyme produced one band [approximately 650 (A) or 850bp (B)] and the pUC 18 vector (Figure 3.13).

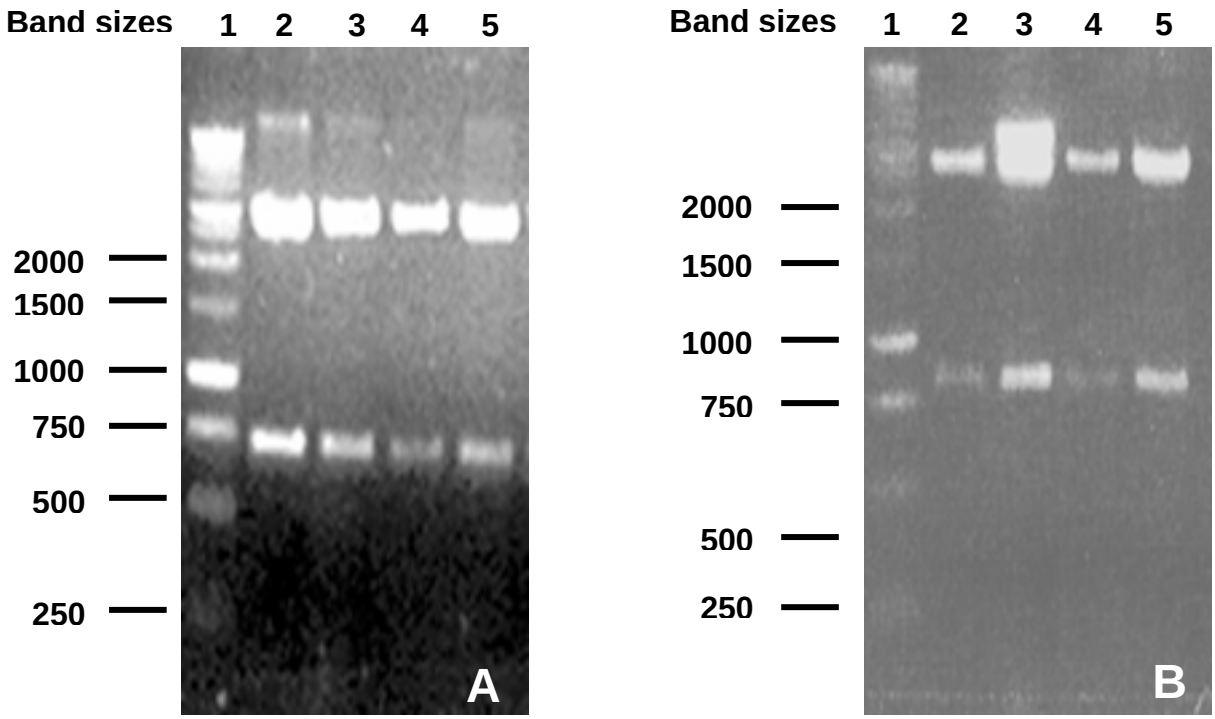

Figure 3.13. Gel electrophoresis of *Eco*RI digest of the 650 (A) and 850bp (B) inserts from pUC 18vector. Lane 1 in both A and B shows the 1kb marker and lanes 2 through 5 the resulting products

Sequence analysis of the approximately 650 and 850bp inserts ligated into a pUC 18 vector and the subsequent BLAST search still resulted in an undefinitive answer. A nested primer (F2'→ 5'-ACT CCT ACG GGA GGC AGC-3') was used to obtain the complete ±1500bp sequence of the 16s rDNA product of isolate 6.2. All the sequences were aligned and edited using ChromasPro V. 1.2 and DNAssist V. 2.0. The complete sequence was subjected to a BLAST search where it revealed a 98% identity with the *Enterobacter cloacae* partial 16S rDNA sequence (Figure 3.14).

```
E. cloacae      1                                   TGAACGCTGGCGGCAGGCCTAACA 24
16S rDNA of 6.2 1    AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACA 46

E. cloacae      25   CATGCAAGTCGAACGGTAGCACAGAGAGCTTGCTCTCGGGTGACGA 70
16S rDNA of 6.2 47   CATGCAAGTCGAACGGTAGCACAGAGAGCTTGCTCTCGGGTGACGA 92

E. cloacae      71   GTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGG 116
16S rDNA of 6.2 93   GTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGG 138

E. cloacae      117  GATAACTACTGGAAACGGTAGCTAATACCGCATAAYGTCGCAAGAC 162
16S rDNA of 6.2 139  GATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGAC 184

E. cloacae      163  CAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGAT 208
16S rDNA of 6.2 185  CAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGAT 230

E. cloacae      209  GGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCC 254
16S rDNA of 6.2 231  GGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCC 276

E. cloacae      255  CTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACG 300
16S rDNA of 6.2 277  CTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACG 322

E. cloacae      301  GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG 346
16S rDNA of 6.2 323  GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG 368

E. cloacae      347  GCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCG 392
16S rDNA of 6.2 369  GCGCAAGCCTGATGCA-CCATGCCGCGTGTATGAAGAAG-CCTTCG 412

E. cloacae      393  GGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAA 438
16S rDNA of 6.2 413  GGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAA 458

E. cloacae      439  CCGCAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCG 484
16S rDNA of 6.2 459  CCACAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCG 504

E. cloacae      485  TGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAAT 530
16S rDNA of 6.2 505  TGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAAT 550

E. cloacae      531  TACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGA 576
16S rDNA of 6.2 551  TACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGA 596

E. cloacae      577  AATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTG 622
16S rDNA of 6.2 597  AATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTG 642

E. cloacae      623  GAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGC 668
16S rDNA of 6.2 643  GAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGC 688

E. cloacae      669  GTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACA 714
16S rDNA of 6.2 689  GTAGAGATCTGGAGGAATACCGGTGGCGAAG-CGGCCCCCTGGACA 733

E. cloacae      715  AAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAG 760
16S rDNA of 6.2 734  AAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAG 779

E. cloacae      761  ATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGT 806
16S rDNA of 6.2 780  ATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGT 825

E. cloacae      807  GCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCC 852
16S rDNA of 6.2 826  GCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCC 871

E. cloacae      853  TGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGG 898
16S rDNA of 6.2 872  TGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGG 917

E. cloacae      899  CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAA 944
16S rDNA of 6.2 918  CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAA 963

E. cloacae      945  GAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGAT 990
16S rDNA of 6.2 964  GAACCTTACCTGGTCTTGACATCCACAGAACTTAGCAGAGATGCTT 1009

E. cloacae      991  TGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCA 1036
16S rDNA of 6.2 1010 TGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCA 1055
```

Figure 3.14. Sequence alignments of *Enterobacter cloacae* and 16S rDNA PCR fragments from isolate 6.2 obtained by sequencing. Alignments performed with DNAssist V. 2.0.

3.3.2.3 API tests

The test wells (Figure 3.15) were inoculated with cell culture suspended in sterile distilled water and incubated at 37°C for 18 hours. The results were obtained by following the manufacturer's instructions and a numerical profile of 3305 573 was obtained. The North-West University used this numerical profile to identify the bacteria. The highest similarity obtained was with *Enterobacter cloacae* (95%).

Figure 3.15. Results obtained from the API strip gave a 95% similarity to *Enterobacter cloacae*.

3.3.2.4 Biolog

Biolog experiments were preformed as described in section 3.2.6.5.2; the highest similarity was found with *Enterobacter cloacae* (94%).

All the identification tools described in sections 3.2.6.3 through 3.2.6.5.2 gave indications that isolate 6.2, which was isolated from the Evander mine site, belongs to the genus *Enterobacter,* the specie identification is not 100% positive and for the purpose of this study the isolate was designated as *Enterobacter sp.*EV-SA01. Microbial taxonomic work could reveal that this isolate is a unique organism indigenous to South Africa and its specific environments. Another organism which is unique to South Africa and isolated as part of the LExEn (Life In Extreme Environments) program is *Thermus scotoductus* (Kieft *et al.,* 1999). This bacterium can reduce a number of compounds as electron acceptors through the dissimilatory pathway. These compounds include Fe (III), Mn (IV), Co (III)-EDTA, Cr (VI) and U (VI).

3.3.3 Microscopy

3.3.3.1 Light microscopy

Gram staining preformed according to section 3.2.6.1 indicated that isolate 6.2 is a gram negative rod-shaped bacterium (Figure 3.16).

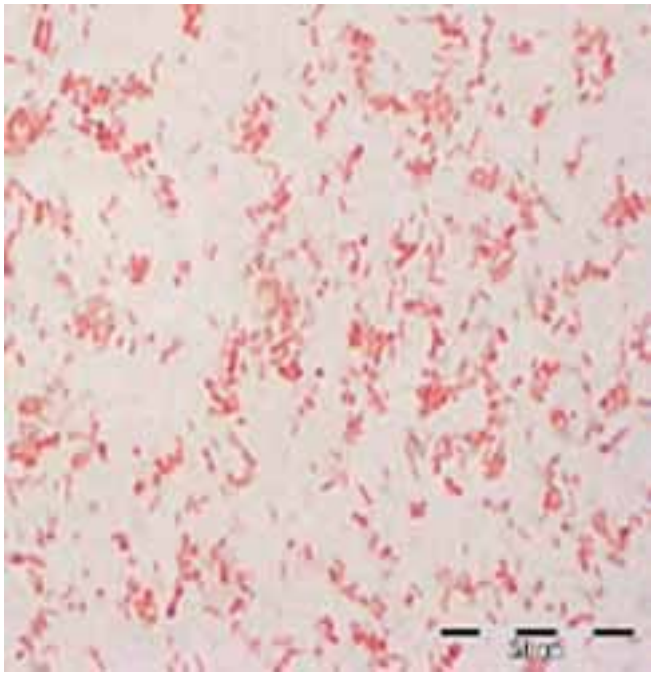

Figure 3.16. Micrograph of isolate 6.2 treated with gram staining solution indicates a gram negative, rod-shaped bacterium.

3.3.3.2 Scanning and transmission electron microscopy

Scanning electron micrographs of cells grown in the presence and absence of 2mM vanadate reveals that when cells are grown in vanadate containing media, there is an increase in cell thickness and also a degree of deformity (Figure 3.17 and Figure 3.18). This deformity is already visible at 2mM concentrations and can be the cause of the decrease in biomass production at higher vanadate concentrations (Section 3.3.5). A probable explanation is that the degree of deformity is so great that it hinders cell growth and replication.

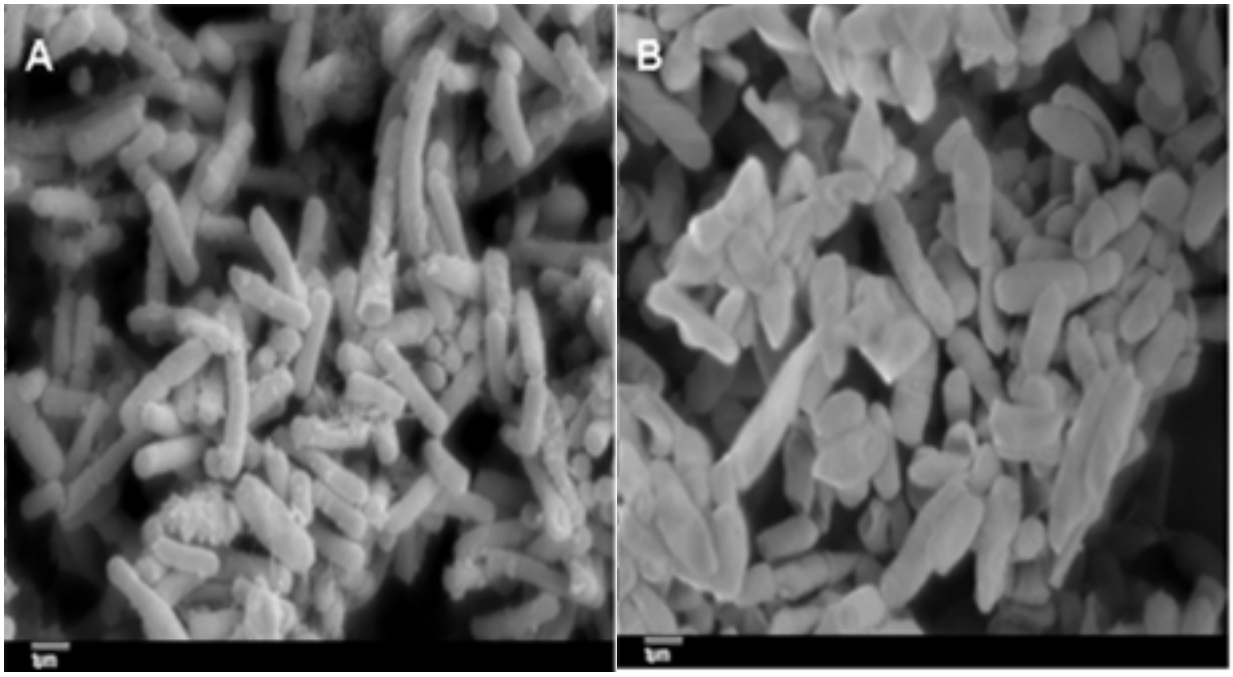

Figure 3.17. Scanning electron micrographs of cells grown in the absence of vanadate (A), and in the presence of 2mM vanadate (B).

In 1997, Fukuda and Yamase found that vanadate and vanadyl compounds had antibacterial activity against *Streptococcus pneumoniae.* Micrographs published by these authors showed that when the bacterium was grown in vanadium concentrations less than the minimum inhibition concentration, it resulted in an elongation of the cells due to the interference of cell division. However upon sub-culturing of these elongated cells in vanadium-free media, they recovered the normal morphology for growth.

The transmission electron micrograph of the cells of isolate 6.2 grown in the presence of vanadate indicates a precipitation inside the cells (Figure 3.18B). This precipitate is indicative of a heavy metal (Personal communication with P.W.J van Wyk). However, further investigation on this precipitate will elucidate whether it is merely sequestration of the metal or sequestration in conjunction with reduction. Studies done by Bisconti *et al*. (1997) on vanadate sensitive strains of *Saccharomyces cerevisiae* found evidence that vanadium accumulated as vanadate, while in a vanadate-resistant strain accumulation of vanadium was as vanadium (IV), implying reduction in conjunction with sequestration.

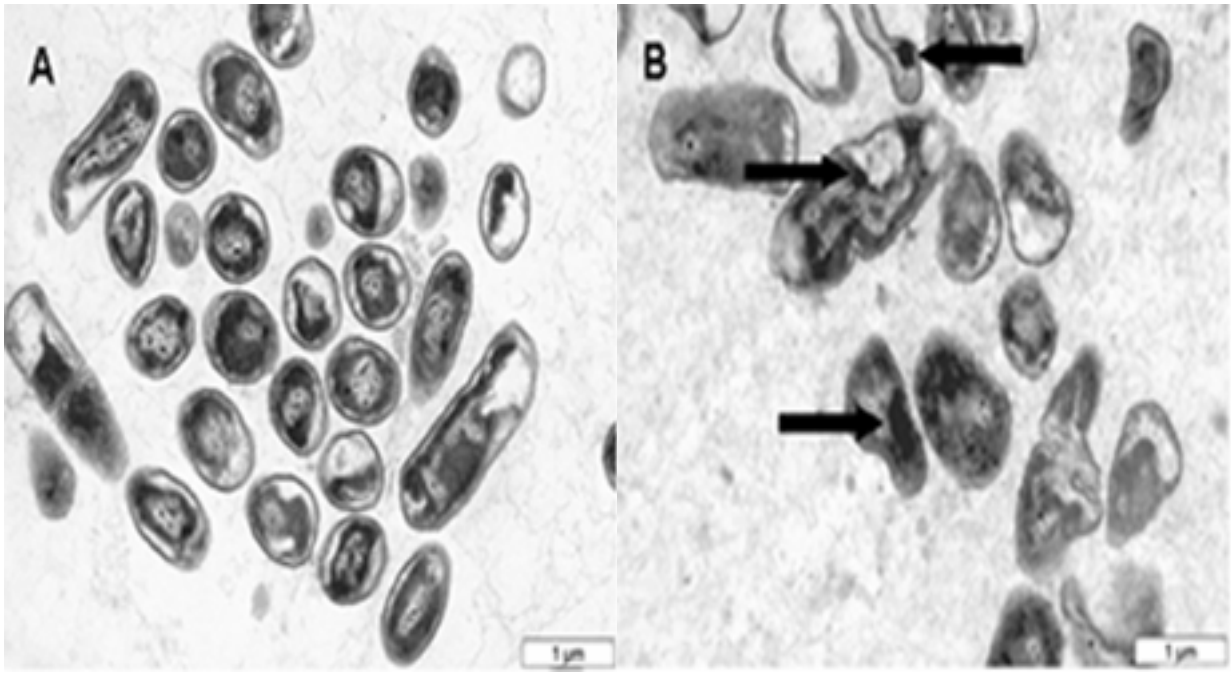

Figure 3.18. Transmission electron micrographs of cells grown in the absence of vanadate (A), and in the presence of 2mM vanadate (B).

Anaerobic experiments done by Carpentier *et al*. (2003) on *Shewanella oneidensis* concluded that *Shewanella oneidensis* can reduce vanadate to vanadyl where this reduction results in a granular precipitate containing predominantly vanadyl. Electron microscopic studies done on *Pseudomonas isachenkovii* cells grown anaerobically in vanadium-containing media showed vanadium accumulation in swells formed on the membranes of the cell wall, with separation of the swells from the cells upon further growth (Antipov *et al*., 2000).

3.3.4 Culturing conditions for bacterial isolate 6.2

The effective bioremediation of contaminated sites is dependant on environmental parameters, which include temperature and pH (Alexander, 1994; Ehrlich, 1997). Thus the next step, in the flow of the experiments, was to determine the optimum growth conditions for isolate 6.2.

3.3.4.1 Optimum temperature

To determine the effect of temperature on the growth rate a temperature profile (Figure 3.19) was constructed using 256 data points obtained from three experiments. The profile indicates that the optimum growth temperature ranges from 37 to 39°C. Above 44°C and below 12°C there is a cessation of growth. The maximal growth rates obtained in the temperature gradient incubator (μMax = 1.601 h^{-1}) compared very well with the batch cultivations in shake flasks (μMax = 1.594h^{-1}).

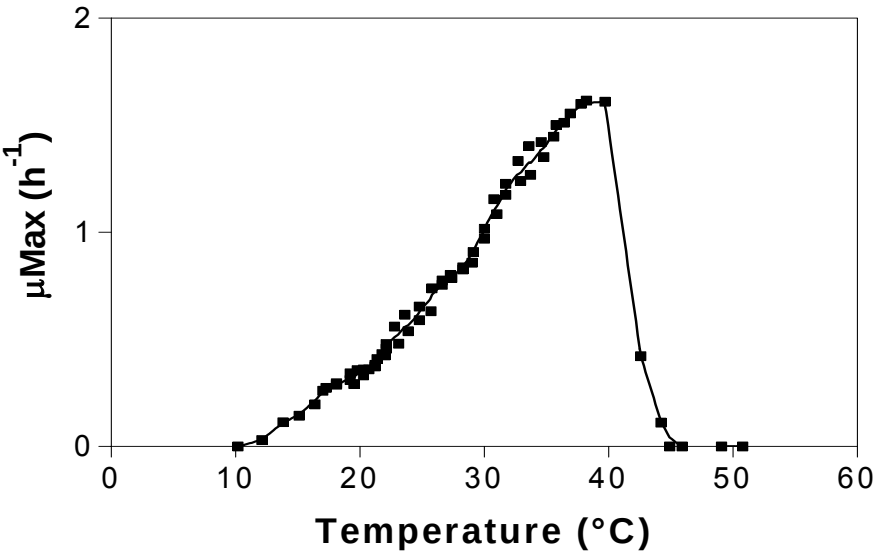

Figure 3.19. The effect of temperature on the maximum specific growth rate of *Enterobacter sp.*EV-SA01. Cultivation was done in a temperature gradient incubator.

An Arrhenius transformation of the temperature profile is shown in Figure 3.20. The activation energy values (E_a), determined by linear regression of the slopes of the regions denoted as A-B and B-C, are shown in Table 3.3. As is evident from Figure 3.20, the specific growth rate increased with an increase in temperature to point A. At point B a distinct inflection point was discerned, which resulted in a 1.19-fold increase in the E_a values when the temperature decrease below 18°C.

Table 3.3. The correlation coefficient and activation energy values obtained from the Arrhenius model for the growth of *Enterobacter sp.*EV-SA01 at different temperatures.

Interval Fig. 3.	Temperature range (°C)	Activation energy (kJ/mol)	Correlation coefficient	Arrhenius equation
A to B	10 to 24	73.529	0.9874	$M = (29.12)e^{-8844/T}$
B to C	25 to 39	61.6566	0.9125	$\mu = (110.3)e^{-32390/T}$

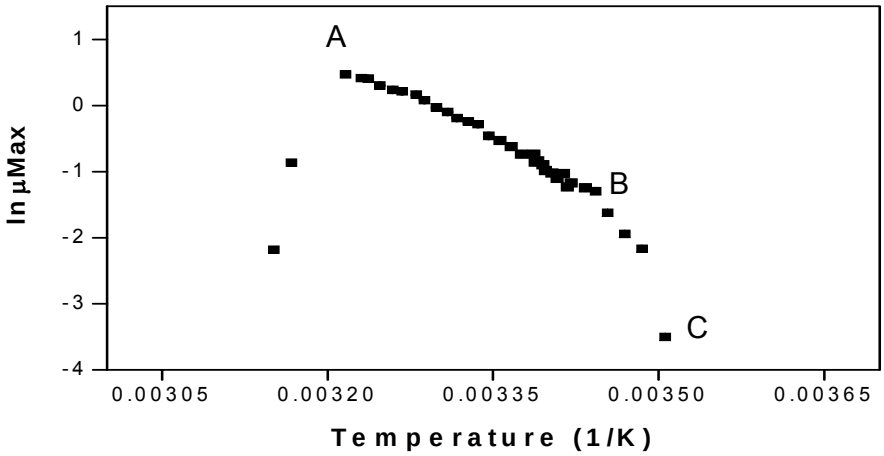

Figure 3.20. Arrhenius plot of maximum specific growth rate of *Enterobacter sp.*EV-SA01 at different temperatures. Plot was constructed using data from three experiments.

The Arrhenius plot is a useful model to show the linear relationship between the growth rate and the cultivation temperature. The value of activation energy (E_a) used in the Arrhenius transformation gives an indication of the rate at which a reaction will proceed with an increase in temperature (Stanbury and Whitaker, 1984). Since the growth rate of a culture can be equated to a reaction rate, the E_a value can thus be used to predict the effect of temperature on the growth rate over a temperature range (Pirt, 1975). In other words, the E_a value provides an indication of the sensitivity of the culture to a change in temperature.

The distinct infection point discerned in the Arrhenius profile of *Enterobacter sp.*EV-SA01 (Figure 3.20) supported the findings of Mohr and Krawiec (1980) who investigated the influence of temperature on different psychrophilic, mesophilic and thermophilic bacteria. According to these authors, an inflection point in the Arrhenius

plot is usually associated with bacteria with an optimum growth temperature above 37°C. In contradiction to these findings, Reichardt and Morita (1982) found that the Arrhenius plots of microorganisms with lower optimum temperatures might also exhibit inflection points.

3.3.4.2 Optimum pH

The influence of pH on the maximum specific growth rate (μMax) was determined by batch cultivations in shake flasks. The maximal specific growth rate was calculated from the linear regression preformed on the exponential phase of growth. A symmetrical pH profile (Figure 3.21) was obtained in the pH range tested with the highest μMax value of 1.537h^{-1} recorded at pH 7.0. The μMax values at the lower and the higher extremes of the range were 0.29 h^{-1} and 0.933h^{-1}, respectively.

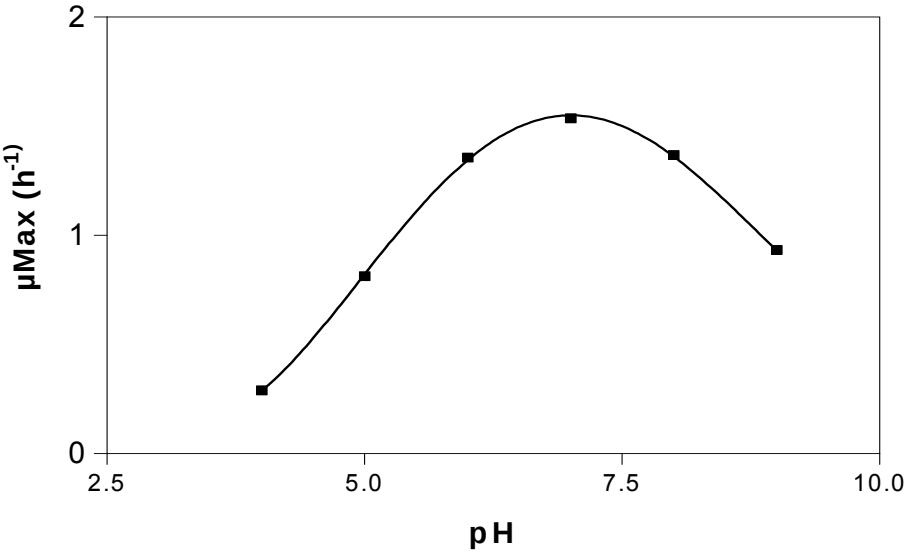

Figure 3.21. Effect of pH on the maximum specific growth rate of *Enterobacter sp.*EV-SA01. Cultivations were done in shake flasks with the pH adjusted prior to sterilization.

3.3.5 Growth monitoring

Experiments done by Bredberg *et al*. (2004) showed that *Acidithiobacillus ferrooxidans* was able to grow in high vanadium concentrations and that growth was not inhibited by vanadium pentoxide or by vanadium (IV). On the other hand growth

of *A. thiooxidans* was inhibited by vanadium pentoxide when more than 0.15mM was added to the growth medium. These results indicate that vanadium reducing bacteria can do so at different maximum concentrations. Yeast metabolism studies in the presence of orthovanadate showed that the growth of *Saccharomyces cerevisiae* was severely inhibited upon the addition of 5mM vanadate (Willsky *et al*., 1984). Also found was that the vanadate becomes cell associated which is then converted to vanadyl. Vanadyl is subsequently release into the culture media.

To determine the maximum vanadate concentration which can be tolerated by *Enterobacter sp.*EV-SA01, the bacterium was grown in different vanadate concentrations. Growth was monitored over time to determine the influence of toxicity on the growth rate. Figure 3.22 shows that there is no significant difference in the initial growth rate at low concentrations of vanadate, but at higher vanadate concentrations (exceeding 2mM) the stationary phase is reached much faster. Up to a concentration of 2mM there are no significant differences in the growth rate of the bacteria, thus maximum vanadate concentration without affecting biomass production is 2mM.

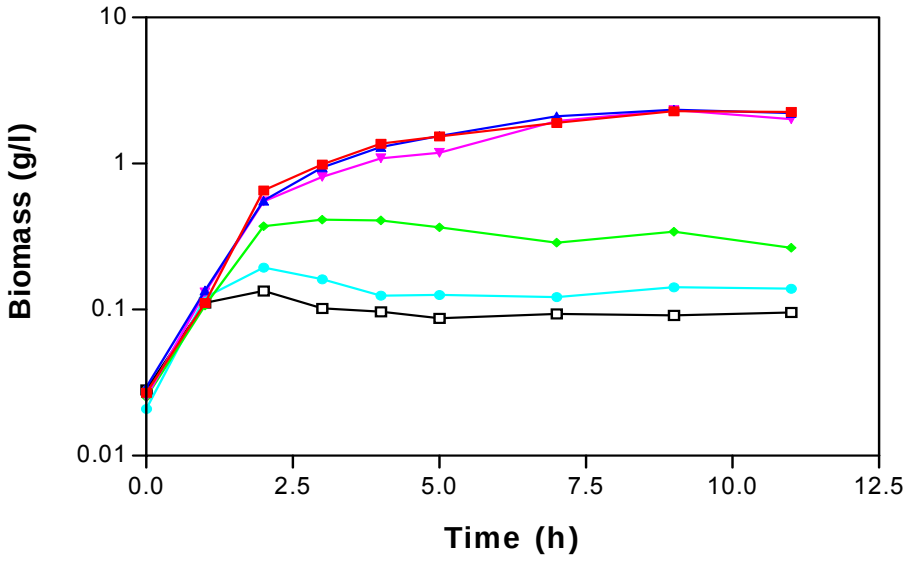

Figure 3.22. Growth of *Enterobacter sp.*EV-SA01 over time in different concentrations vanadate. No vanadate (━), 1mM (━), 2mM (━), 3mM (━), 4mM (━) and 5mM (━).

During this study, samples were withdrawn periodically and assayed for reduction activity according to section 3.2.5.5. When grown on 2mM vanadate it was found that maximum enzyme activity was found after four hours of growth which coincides with the early stationary phase. Upon reaching the mid to late stationary phase most, if not all of the activity have disappeared (Figure 3.24). Growth in the absence of vanadate gave the same results; the only variation is that the cells grown on vanadate had a higher activity (results not shown). These results again point to dissimilatory metal reduction, but complicates the question whether this vanadate reductase is inducible or indeed that the vanadate is a competitive substrate for other metal reductases.

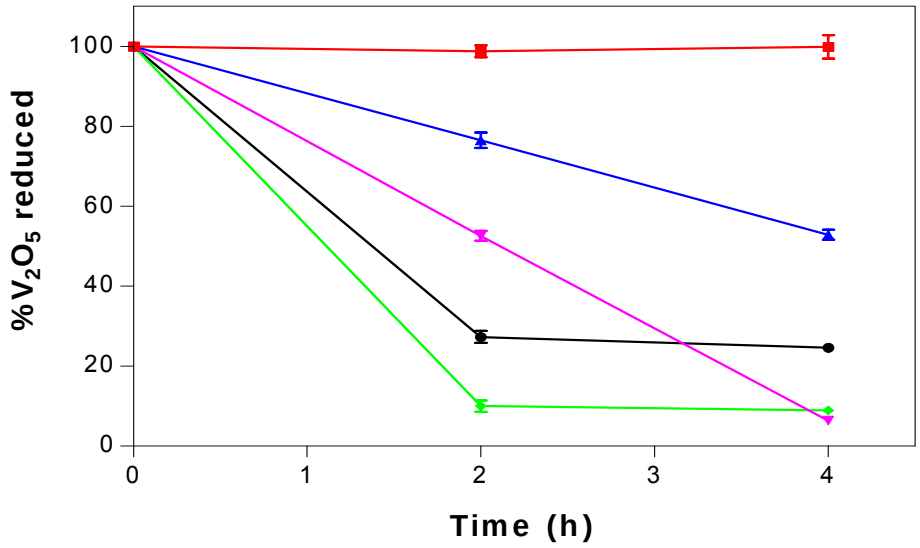

Figure 3.23. This graph shows the difference between cells grown in the presence and absence of vanadium pentoxide. 6.2 grown in vanadate ( ), 6.2 grown without vanadate ( ), 13.1 grown in vanadate ( ), 13.1 grown without vanadate ( ) and the blank rate ( ).

To assess these theories the previous isolates 6.2 and 13.1 were used to verify the effect of the presence of vanadate during growth had on the reduction rates. The activity measurements were taken when samples were withdrawn periodically during growth, but the cells were washed and assayed under anaerobic conditions in 20mM HEPES, pH 7.0. Isolates 6.2 and 13.1 were grown in the presence and absence of 2mM V_2O_5. Figure 3.23 indicates that in the case of isolate 6.2 there is no significant difference in the initial reduction rate. However, isolate 13.1 grown in the presence of V_2O_5 had a higher initial reduction rate (slope) and also reduces more vanadium

over time. Thus the proteins responsible for vanadate reduction need not to be induced, but it has a higher activity when grown in the presence of vanadate. It seems as if the vanadate reducing proteins are thus constitutively expressed as found by Carpentier *et al*. (2003).

The maximum vanadate concentration which can be tolerated during growth in liquid cultures was very low in comparison to the concentration tolerated on the plates. This can be due to the fact that in plates, dispersion of the nutrient and consequently the vanadate is limited to the adjacent regions, which will result in a limited amount reaching the cells growing on the plates. On the other hand in liquid cultures the cells are in constant contact with the nutrients and the vanadate, resulting in an increase exposure to vanadate, this may be the reason why the cells are able to tolerate higher vanadate concentrations when growing on the plates. Another possible reason might be that the vanadium oxide is more toxic than the vanadium salt.

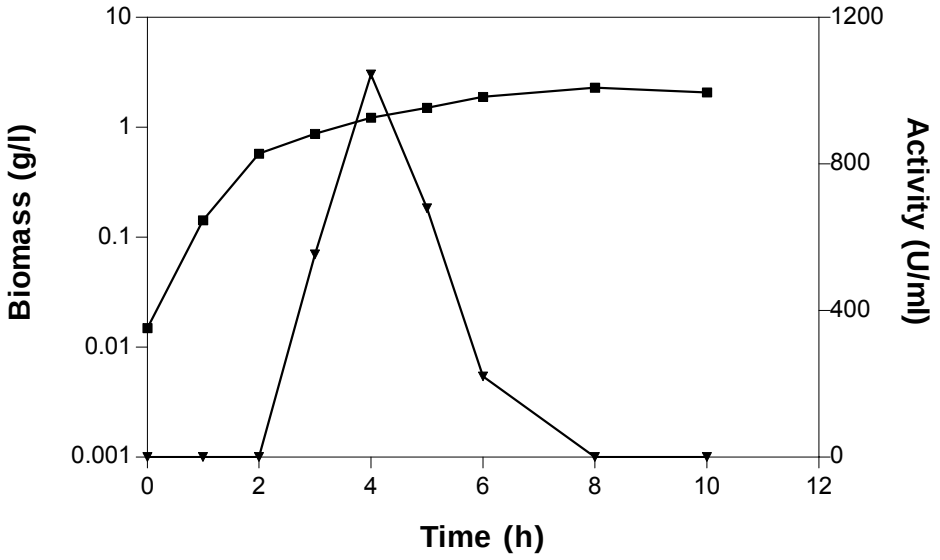

Figure 3.24. Optimum enzyme production during growth on 2mM vanadate. Biomass (■) and activity (▼).

The vanadate reducing capability of cells in different growth phases was determined by harvesting cells at different stages of growth and assessing their ability to reduce vanadate under non-growth, anaerobic conditions as described in section 3.2.5.5. It was found that maximum vanadate reduction ability was reached after 4 hours,

which corresponds to the early stationary growth phase (Figure 3.24). After 4 hours the cells rapidly loses the vanadate reducing ability with no activity remaining at 8 hours and beyond.

Vanadate depletion during growth was followed and correlated to enzyme activity. Figure 3.25 indicates that the vanadate concentration only starts to decrease once the cells reached the early stationery phase. This correlates well with the optimum enzyme activity (Figure 3.24). Even though Figure 3.23 shows that the proteins involved in vanadate reduction need not be induced or activated it seems that the production of these proteins is growth phase dependant.

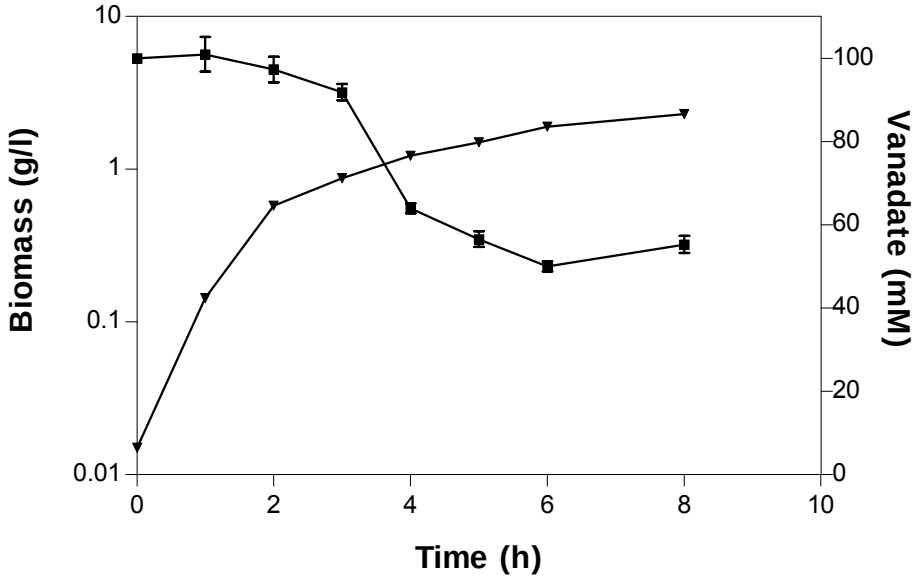

Figure 3.25. Vanadate reduction by cells during growth on 2mM vanadate. Cells (▼) and Vanadate (■).

3.3.6 Conclusions on growth and reduction

It was found that not all the mine sample isolates were resistant to vanadium and that all the resistant isolates were not able to reduce vanadate. Preliminary screening was a good indicator for resistance but not a quantitative indicator of reduction. It was also found that the isolates able to reduce vanadium did so much more efficiently under anaerobic conditions than under aerobic conditions. It would also seem that the protein(s) responsible for vanadium reduction is constitutively

expressed and this activity can be enhanced when the isolate is grown in the presence of vanadate. Maximum activity was associated with the early stationary growth phase, with the optimum growth conditions at neutral pH and 37 to 39°C. Vanadate at high concentrations has an inhibitory effect on biomass production in liquid cultures, but the organism would most probably be able to tolerate high concentrations in the environment since the pallet screening was done at much higher concentrations.

From the results obtained during this study, it is clear that this bacterium, *Enterobacter sp.*EV-SA01, can reduce vanadate during growth as well as under non-growth conditions. Future research will elucidate whether the protein(s) responsible for the reduction of vanadate is specific in its function or if is merely an existing protein, or set of proteins, adaptable to dispose of vanadate. It will also be important to determine the optimal conditions for its function.

3.4 References

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CHAPTER 4

In situ bioremediation of vanadate

4.1 Introduction

Mankind's careless utilization of the earth's resources has resulted in the unfavorable alteration of our surroundings. Contamination of the earth as a by-product of human interference is liable to cause hazards to human health, harm to living resources or ecological systems, damage to structures and amenities, or interference with legitimate uses of the environment. Contamination by especially metals has a negative impact on the environment, through impairing ground- and surface water quality. The presence of these contaminants is frequently due to inadequate disposal methods as well as leaks and spills.

A practice often utilized to remove hazardous waste is to landfill the contaminant in soil. Alternative methods are bioremediation or bioaugmentation which offer relatively inexpensive methods in comparison to the mechanical methods (Pearce *et al.,* 1995). Bioremediation and bioaugmentation are efficient solutions to remove toxic chemicals from the contaminated soil. It is biological techniques which harnesses and enhances the natural ability of microorganisms to degrade hazardous chemicals into innocuous forms. In South Africa bioremediation techniques have been used to treat a number of contaminated sites arising from industrial activities, service stations, vehicle accident spills and bulk storage facilities. Despite many opportunities for its use, bioremediation has not been utilized to its full potential in South Africa (Leung, 2004; Pearce *et al*., 1995; Vidali, 2001). Full scale bioremediation in South Africa has had the most application within the petrochemical industry.

Vanadium contamination of groundwater resulting from industrial, mining or natural sources can be an environmental concern. Vanadium in the 5+ oxidation state is more toxic and bio-available than vanadium in the 3+ and 4+ oxidation states. Reduction of V (V) to a lower oxidation state is thus a potentially useful process for

the remediation of vanadium contaminated environments. Several microorganisms have been identified which can enzymatically reduce vanadate to a lower oxidation state (Antipov *et al.,* 2000; Bisconti *et al.,* 1997; Bredberg *et al.,* 2004; Carpentier *et al.,* 2003; Ortiz-Barnad *et al.,* 2004), however, little have been done to localize and characterize the responsible protein(s). Thus an organism with the ability to tolerate high concentrations of vanadate and which is able to reduce vanadate to a lower oxidation state is a potential candidate for bioremediation or bioaugmentation studies.

From Chapter 3 we were encouraged by the ability of *Enterobacter sp.*EV-SA01 to tolerate high concentrations of vanadate as well as the ability to reduced vanadate to a lower oxidation state. Since we now had an organism with the required properties we set out to define the remediation parameters. This part of the study was done to characterize the vanadium-reducing ability of *Enterobacter sp.*EV-SA01, as well as to identify and characterize the protein(s) directly responsible for vanadate reduction. Also evaluated in this part of the study is the organism's potential for *in situ* vanadate reduction.

4.2 Materials and methods

4.2.1 Microorganism

The bacterium was cultivated as described in section 3.2.2 with the following adaptations. A final concentration of 2mM V_2O_5 was added to all liquid cultures followed by a 10% inoculum using the pre-culture.

4.2.2 Assay for vanadium reduction and determination of vanadium concentration

The assay for vanadium reduction was preformed as described in section 3.2.5.5.
The concentration of the pentavalent form of vanadium was determined as described in section 3.2.5.1.

4.2.3 Enzyme localization studies

Culturing of the isolate was preformed as described in section 4.2.1. The cells were harvested through centrifugation, washed three times with 20mM HEPES buffer, pH 7.0, and resuspended in a 1:20 (w/v) ratio in the same buffer. The sample was prepared for whole cell reduction experiments as described in section 4.2.2.

The culture supernatant was concentrated using an Amicon stirred cell (model 8050) fitted with a membrane (Osmonics Ultra Sep Disc, UF 10 000 NMWC). The sample was filtered under high pressure and concentrated 10 times. The concentrated culture supernatant was assayed for vanadate reduction activity under anaerobic conditions in the same manner as for the whole cell reduction as described in section 4.2.2.

4.2.4 Fractionation studies

The supporting skeleton of the bacterial cell wall consists of a regular polymer, the peptidoglycan, murein. This macromolecule is a heteropolymer made up of chains of alternating molecules of *N*-acetylglucosamine (GlcNAc) and its lactic acid ester, *N*-acetyl muramic acid (MurNAc) linked by 1,4-β-glycosidic bonds. These heteropolymers are straight chains without branching and form the backbone of murein. In gram-negative bacteria the murein network consists as a single layer. Attached to the outer surface of the murein skeleton are large quantities of lipoproteins, lipopolysaccharides and other lipids. This lipopolysaccharide layer appears to require calcium ions to maintain its stability; removal of the calcium ions by treatments with EDTA liberates the polysaccharides which make the murein layer accessible to degradation by the enzyme lysozyme. Lysozyme is an acetyl muramidase, it attacks the glycosidic bonds between the C-1 atom of *N*-acetylmuramate and the C-4 atom of *N*-acetylglucosamine. Lysozyme thus degrades murein to GlcNAc-MurNAc (Schlegel, 1997). The first step thus in most fractionation methods is the removal of the cell wall by treatment with lysozyme in the presence of EDTA.

4.2.4.1 Fractionation by Freezing

The protein minipreps under native conditions protocol obtained from QIAGEN was used as is to fractionate the cells (QIAGEN, 2000).

One gram cells were suspended in 20ml 20mM $NaHCO_3$ buffer, pH 7.0 and let to freeze at -80°C. After freezing the cells were thawed on ice for 30 minutes which was followed by another incubation step on ice for 30 minutes in the presence of 1mg/ml lysozyme. The cells were lysed by gentle vortexing and the supernatant (soluble proteins) separated from the cellular debris (membrane) by centrifugation (10 minutes; 4000 x g; 4°C).

4.2.4.2 Fractionation by sonification

Subcellular fractions were prepared as described by Kaufmann and Lovley (2001). Cells were resuspended in a 1:20 (w/v) ratio in 20mM HEPES buffer, pH 7.0, containing 25% (w/v) sucrose. To accomplish cell wall lysis, lysozyme (1mg/ml) was added to cell suspension and stirred for 20 minutes. Na_2-EDTA was added to a final concentration of 5mM and stirred for another 15 minutes. Finally $MgCl_2$ was added to a final concentration of 13mM and the suspension was stirred for another 15 minutes. Separation of the spheroplasts from the periplasmic fractions was obtained by centrifugation at 20 000 x g for 30 minutes. Spheroplasts were resuspended in 20mM HEPES buffer, pH 7.0.

To obtain the membrane and cytoplasmic (soluble) fractions, a protocol adapted from Gaspard *et al*. (1998) was used. A few crystals of DNase were added to the EDTA-lysozyme-treated cell suspension, and the cells were broken by ultrasonic treatment. Sonification consisted of cycles of 30 seconds burst with 30 seconds resting time. This was done at 50% output and done six times using a sonifier (Branson Sonic Power Cell Disruptor B-30) in an ice-water bath. The suspension was then centrifuged for 10 minutes at 4000 x g to remove the cellular debris (pellet). The supernatant was subjected to ultra-centrifugation at 100 000 x g for 90 minutes at 4°C to separate the cytoplasmic proteins (supernatant) and the membranes (pellet). The pellet was resuspended in a 1:5 (w/v) ratio in 20mM HEPES, pH 7.0.

All the fractions obtained were assayed for vanadate reducing activity under anaerobic conditions as described in section 4.2.2.

4.2.4.3 Cell disruption by using glass beads

Spheroplasts were obtained by the using the fractionation method described in section 4.3.4.2. The spheroplasts were broken mechanically by using glass beads (450µm in diameter). Glass beads were added to the spheroplasts in a 1:2 (w/w) ratio and vortexed for 1 minute followed by incubation on ice for 30 seconds. This was done 10 times. Glass beads were removed and washed several times with 20mM HEPES buffer, pH 7.0, through centrifugation (1000 x g; 5 minutes, 4°C). The supernatant after each wash step was pooled and separated into the cytoplasmic and membrane fractions by ultracentrifugation as described in section 4.3.4.2. All fractions were assayed for vanadate reducing activity under anaerobic conditions as described in section 4.2.2.

4.2.5 Dissociation of membrane proteins

As the greater part of the activity was found to be cell associated different solubilization methods were tested for release of membrane proteins.

4.2.5.1 Solubilization of proteins by using either potassium chloride or B-per® Reagent

Peripheral proteins were released form the spheroplasts obtained from treatment of cells with lysozyme as described in section 4.2.4.2. The spheroplasts were stirred at 4°C for an hour in a high-ionic-strength salt buffer [100mM Tris-HCl, 0.1mM DDT, 0.5M KCl (pH 7.6)] (Gaspard *et al*., 1998). The spheroplast mixture was separated into the fraction containing the released proteins (supernatant) and the fraction containing the membrane bound proteins (debris) through centrifugation at 5000 x g for 30 minutes. Both the resulting supernatant and debris were assayed for vanadate reducing activity under anaerobic conditions as described in section 4.2.2.

The soluble proteins were released from the spheroplasts obtained from treatment of cells with lysozyme as described in section 4.2.4.2. The spheroplasts were treated with B-per® Reagent (Pierce, USA) according to manufacturers instructions. The spheroplast mixture was separated into the fraction containing the soluble proteins (supernatant) and the fraction containing the membrane bound proteins (debris) through centrifugation at 5000 x g for 30 minutes. Both the supernatant and the debris were subjected to dialysis. Dialysis was necessary to change the buffer from Tris-HCl to HEPES as the Tris-HCl buffer of the B-Per solution interferes with optimum vanadate reducing activity (Carpentier *et al.,* 2003). Both the dialyzed supernatant and dialyzed debris were assayed for vanadate reducing activity under anaerobic conditions as described in section 4.2.2.

4.2.5.2 Release of membrane bound proteins using detergents

Spheroplasts obtained by fractionation as described in section 4.2.4.2 were resuspended in a 1:20 (w/v) ratio in buffer (150mM NaCl, 5% Glycerol, 20mM HEPES buffer, pH 7.0) and incubated in the desired detergent at 4°C for 1 hour with occasional mild shaking. Three different detergents (Table 4.1) were used in the solubility experiments. After treatment with the detergents, the mixtures were centrifuged at 100 000 x g for 90 minutes and both the supernatant and debris was assayed for vanadate reducing activity under anaerobic conditions as described in section 4.2.2. The detergents were removed from the supernatant fractions by using a detergent removal resin (Sigma) according to the manufacturer's instructions.

Table 4.1. CMC values of the chosen detergents

Detergent	CMC (mM)
CHAPS	6 to 10
Deoxycholic acid	1.5
Triton X-100	0.25

The concentration ranges for each detergent was chosen based on the critical micelle concentration (cmc) of the detergent. The concentrations chosen were above and below that of the cmc value. Table 4.1 gives the cmc values for each of the chosen detergents. The first detergent used was CHAPS, which is a non-

denaturing zwitter-ionic detergent. Different concentrations ranging from 4 to 12mM, increasing in 2mM increments were used in the experiments. The second detergent used was Triton X-100, which is a non-ionic detergent. Concentrations ranging from 0.025 to 2.5mM were used in the experiments. The last detergent tested was deoxycholic acid, which is an anionic detergent. This detergent was used in a concentration range from 0.4 to 40mM.

4.2.6 Protein characterization in whole cells

When trying to solubilize the protein(s) responsible for vanadate reduction the activity could not be recovered, therefore all further experiments were done on whole cells.

4.2.6.1 Optimum pH

A 20mM buffer "cocktail" was prepared using equimolar amounts of HEPES, Bis-Tris-HCl propane and glycine. Optimum pH for activity with whole cells was determined over a pH range of 6 to 9 by adjusting the pH with NaOH or HCl. Assays were done in triplicate together with a blank rate at each pH. The rest of the assay procedure was the same as described in section 4.2.2.

4.2.6.2 Optimum temperature

A temperature range of 20-65°C was tested. The assay mixture was equilibrated at the specific temperature. Assays were done in triplicate as described in section 4.2.2 with the appropriate blank rates at each temperature. The temperature with the highest activity was taken as 100% (Figure 4.13).

4.2.6.3 Electron donor specificity

Electron donors were tested for specificity of the enzyme, which included glucose, sodium lactate, fumaric acid, pyruvic acid, citric acid, acetate, succinate and sodium benzoate. As many electron donors have a natural buffering capacity, the pH of the electron donor stock solutions was adjusted to 7.0 to ensure no variations in the reaction pH. The final concentration of the electron donors in the reaction mixtures

was 10mM. Electron donors were tested under both aerobic and anaerobic conditions as described in section 4.2.2 with the appropriate blank rates for each electron donor.

4.2.6.4 Metabolic inhibitors

Cells were prepared for anaerobic whole cell reductions as described in section 4.2.2, with sodium lactate as electron donor. To these mixtures sodium malonate, sodium cyanide and sodium fluoroacetate was added to a final concentration of 0.1, 1, 5 and 10mM. The rate of vanadate reduction was determined as described in section 4.2.2 with the appropriate blank rates for each metabolic inhibitor.

4.2.6.5 Kinetic characterization

The effect of substrate concentration on the vanadate-reducing activity of whole cells under non-growth conditions was evaluated by subjecting the cells to varying vanadate concentrations. The concentrations of vanadate used ranged from 1 to 40mM and during these evaluations the NADH concentration was kept constant at 1mM. Similar procedures were followed for evaluation of the electron donor concentrations and in this case the vanadate concentration was kept constant at 5mM. Assays were performed as described in section 4.2.2.

4.2.7 *In situ* reduction of vanadate

4.2.7.1 Determination of biofilm biomass constituents

A standard curve for protein concentration (Figure 4.1) and a standard curve for carbohydrate concentration (Figure 4.2) were constructed to obtain the measured protein and carbohydrate concentrations of the cells respectively.

4.2.7.1.1 Protein determination of cells

Protein concentrations were determined using the bicinchoninic acid (BCA) method (Smith *et al*., 1985). The Pierce BCA protein assay reagent is a highly sensitive

reagent for the spectrophotometric determination of protein concentration. The commercially available kit from Pierce was used according to the manufacturer's instructions. A standard curve relating protein concentration to absorbance at 562nm was constructed (Figure 4.1). A set of protein standards was prepared with bovine serum albumin (BSA). Fifty microliters of each standard / unknown sample was pipetted into a test tube (50µl of diluent was used for blanks); 1ml of working reagent was added to each tube and vortexed. Tubes were incubated at 60°C for an hour and the absorbance was read at 562nm.

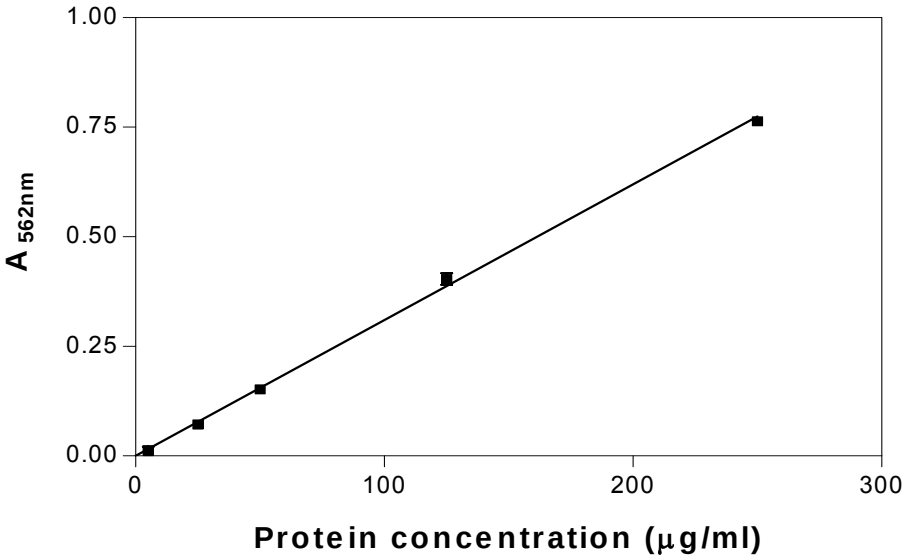

Figure 4.1. Standard curve for the BCA-protein assay with BSA as the protein standard for the test tube protocol. Error bars indicate standard deviations, but are smaller than symbols used.

4.2.7.1.2 Carbohydrate determination of cells

Carbohydrate content was determined by using a phenol/sulphuric acid assay (Dubois *et al.,* 1956). Half a milliliter of a 5% (v/v) two times distilled phenol solution was added to 0.5ml cell suspension. This was followed by the addition of 2.5ml of the acid solution and left for an hour at room temperature. The optical density was determined at 490nm and the carbohydrate concentration was determined by using a standard curve constructed with glucose (Figure 4.2).

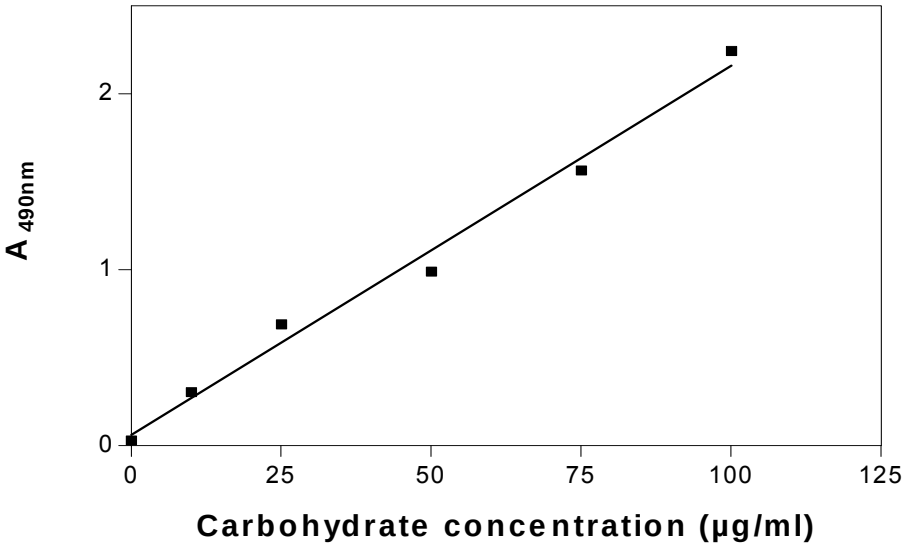

Figure 4.2. Standard curve for carbohydrate determination.

4.2.7.2 Cell counts

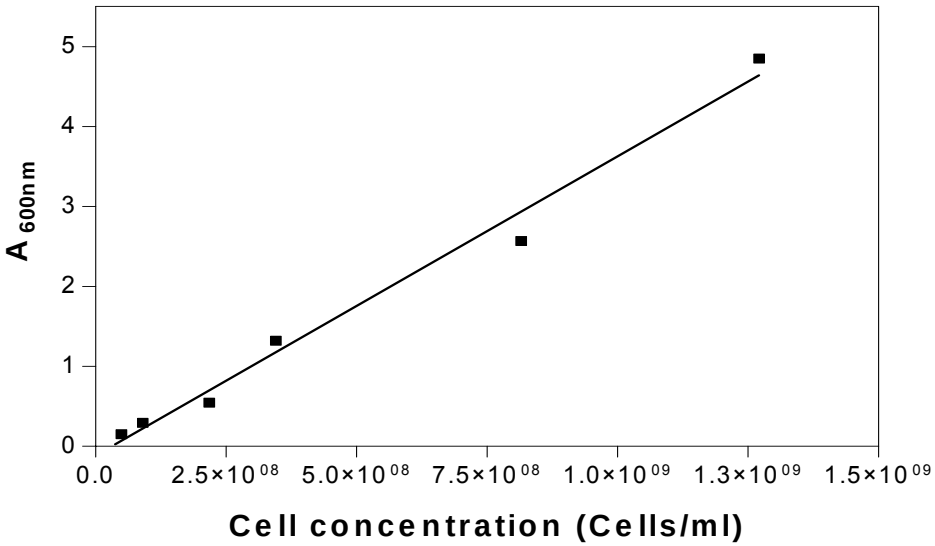

Figure 4.3. Standard curve for the optical density (600nm) against cell concentration (cells/ml).

Cells were grown and harvested as described in sections 4.2.1 and 4.2.3. The cells were resuspended in a quarter of the original volume using artificial groundwater [$Ca(NO_3)_2.4H_2O$, 0.07g/l; $MgSO_4.7H_2O$, 0.06g/l; $NaHCO_3$, 0.06g/l; $CaCl_2.2H_2O$, 0.029g/l; $CaSO_4.2H_2O$, 0.025g/l; KNO_3, 0.01g/l and NaH_2PO_4, 0.0004g/l] (Fuller et al., 2000). Appropriate dilutions were made and the optical density at 600nm for

each dilution was measured. Each dilution was subjected to counting the cells with Petroff-Hauser counting chamber using Thoma scaling. A standard curve of optical density (600nm) against cell concentration (cells/ml) was constructed to determine the OD value which corresponded to 1 x 108 cells/ml (Figure 4.3).

4.2.7.3 Cell adhesion trails

Percentage adhesion was performed according to an adapted method obtained from DeFlaun *et al.* (1999). This assay will reveal the capacity of the cells to adhere to surfaces over a specific time period and therefore be indicative of retention and properties of the cells. The adhesive bacteria will be retained inside the column, while the bacteria with reduced retention tend to pass through. The relative proportion of retained versus injected bacteria is defined as percentage adhesion.

Figure 4.4. Grain size of the sand used in the adhesion trails.

Four 20ml glass pipettes were cleaned with chromic acid and baked at 180°C for 3 hours. Each pipette was packed with 25 grams of sand [grain size between 1000µm and 1500µm (Figure 4.4)]. The columns were washed several times with distilled water and baked again for 3 hours at 180°C. Each column was injected from the bottom with 12ml 1 x 10^8 cells/ml, and left for 1, 2, 4 and 6 hours respectively. At the set time the liquid from a column was harvested and cell counts were preformed as described in section 4.2.7.2. Washed out cells was expressed as a percentage of initial load. Cell adhesion trails were also done in 20ml plastic syringes packed up to

the 20ml mark with sand. Each column was saturated with 1 x 10^8 cells/ml cell suspension and left for 3 hours, where after the liquid from the columns were harvested and cell counts were preformed as described in section 4.2.7.2.

4.2.7.4 Construction of column experiment

The basic column set up is displayed in Figure 4.5. The column was placed in an upright position. The side with the 4 inlets was placed at the bottom.

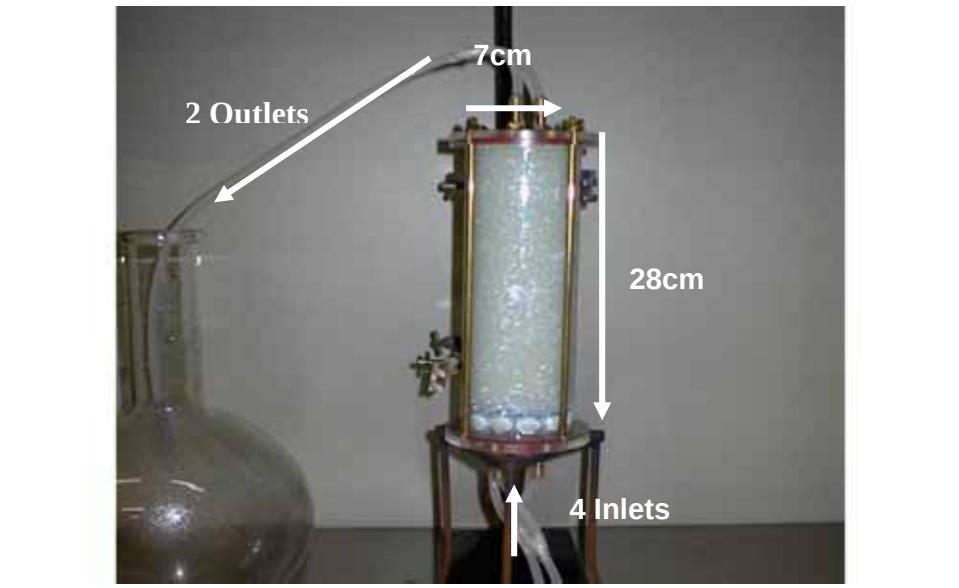

Figure 4.5. Column dimensions and setup.

4.2.7.5 Packing of the columns

A fine (material net) mesh was placed at the bottom of the column to prevent the sand from flowing into or blocking the tubes. One layer of glass beads (2mm in diameter) was placed at the bottom of the column (approximately 1cm high - 100g). Then the sand was placed on top of the glass beads and the column was filled to about 2cm from the top. Here another layer of glass beads were placed before the addition of another fine mesh. The column was washed with two pore volumes 70% (v/v) ethanol to sterilize the column. This was followed by washing with sterile

artificial groundwater for 24 hours to remove any traces of ethanol and to compact the sand in the column.

4.2.7.6 Hydraulic conductivity

The hydraulic conductivity of the sand column was calculated by making use of Darcy's Law (Wolf *et al.,* 1991). Darcy's law states that the rate of flow through a porous medium is proportional to the loss of head, and inversely proportional to the length of the flow path.

$$Q = KiA$$

$$K = \frac{Q}{iA}$$

$$K = \frac{Q}{\frac{(H2 - H1) \times A}{l}}$$

i =	Gradient
A =	Cross-sectional area normal to flow direction
Q =	Volume rate of flow
H2 - H1 =	Head loss
ℓ =	Cross-sectional through flow area
K =	Hydraulic conductivity

The hydraulic head in the measuring cylinder was kept constant by pumping artificial groundwater out of the reservoir into a measuring cylinder. The water flows through the column by connecting the inlet of the column to the measuring cylinder. The flow through was determined at the outlet side by measuring the amount of water collected over one minute intervals. The tests were done a few times to get the average hydraulic conductivity of the sand.

4.2.7.7 Tracer tests

The movement of a conservative tracer (Sodium bromide, i.e. a non-reactive, non-sorbing solute) through a sand column was monitored. For the purpose of the tracer test, 1.29g sodium bromide was dissolved in 1 liter artificial groundwater to represent the conservative tracer stock solution (1000ppm). After saturation of the sand column with artificial groundwater the inlet was connected to the bromide solution. A 100ml of a 100ppm sodium bromide tracer was injected into the column. The tracer was washed through the column with artificial groundwater at a rate of 10ml per 5 minutes. The bromide concentration at the outlet side of the column was measured using a bromide combination electrode (Orion, USA). The concentration decrease was measured until it reached the initial background value, or until the concentration leveled out at a lower value. Figure 4.6 shows the experimental setup. A breakthrough curve to show the movement of a tracer through the system was plotted (Figure 4.13).

The tracer test was repeated with a 1 x 10^8 cells/ml suspension to obtain the breakthrough curve for the bacterial isolate. This was done by measuring the optical density (600nm) instead of the bromide concentration at the outlet side of the column.

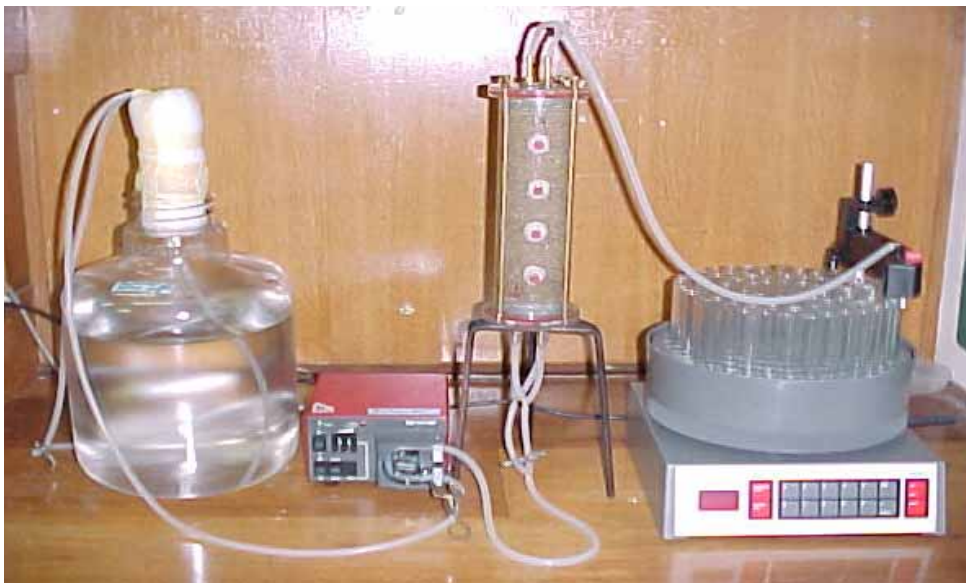

Figure 4.6. Tracer and bacterial test setup.

4.2.7.8 Loading of the column

One pore volume (420ml) cell suspension (1 x 10^8 cells/ml in artificial groundwater) was used to load the column. The column was left for 3 hours to allow for cell adhesion, this was followed by pumping in one pore volume TYG-medium and left for 24 hours. Hydraulic conductivity was tested as described in section 4.2.7.6. The column was washed with artificial groundwater and reloaded with cells and left for 3 hours to allow for cell adhesion. This was followed by pumping in one pore volume TYG to allow for cell growth (24 hours). Artificial groundwater supplemented with 5mM V_2O_5 and 10mM sodium lactate was pumped through the column at a rate of 0.45ml per minute. Vanadate reduction was followed by measuring the vanadate concentration, as described in section 3.2.5.1, at the side ports and in the effluent over time, while the bacterial transport was followed by doing cells count of the samples obtained from the side ports and the effluent as described in section 4.2.7.2.

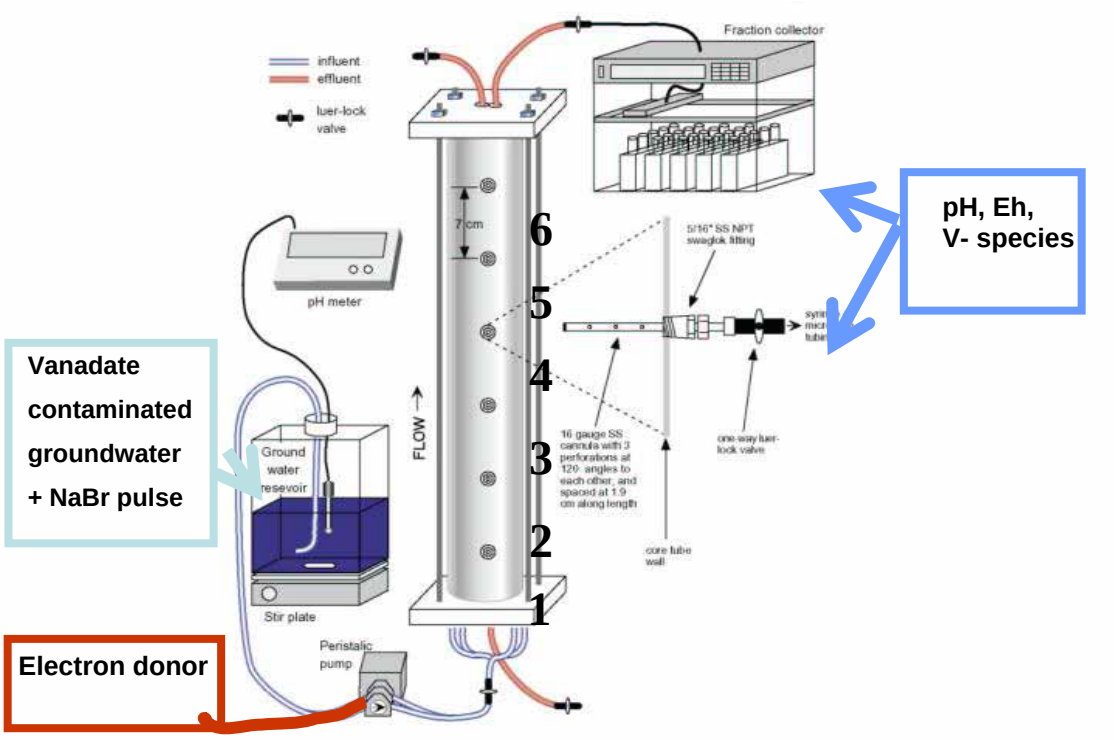

Figure 4.7. Laboratory setup for the study of in situ reduction of vanadium (Adapted from DeFlaun *et al.,* 2001).

4.3 Results and discussions

4.3.1 Enzyme localization studies

After harvesting the cells and concentrating the supernatant as described in section 4.2.3, both the culture supernatant as well as the whole cells were assayed for vanadate reducing capabilities as described in section 4.2.2. This was done to determine whether the protein(s) responsible for vanadate reductions is extra-cellular or intra-cellular. It was found that the culture supernatant had no vanadate reducing capabilities, while the whole cells were able to reduced 50 – 90% of the vanadate present over a 4 hour period.

4.3.2 Whole cell disruption experiments

As the vanadate reducing activity was cell associated, the cells needed to be disrupted to elucidate which protein(s) contributed to vanadate reduction.

4.3.2.1 Fractionation by freezing

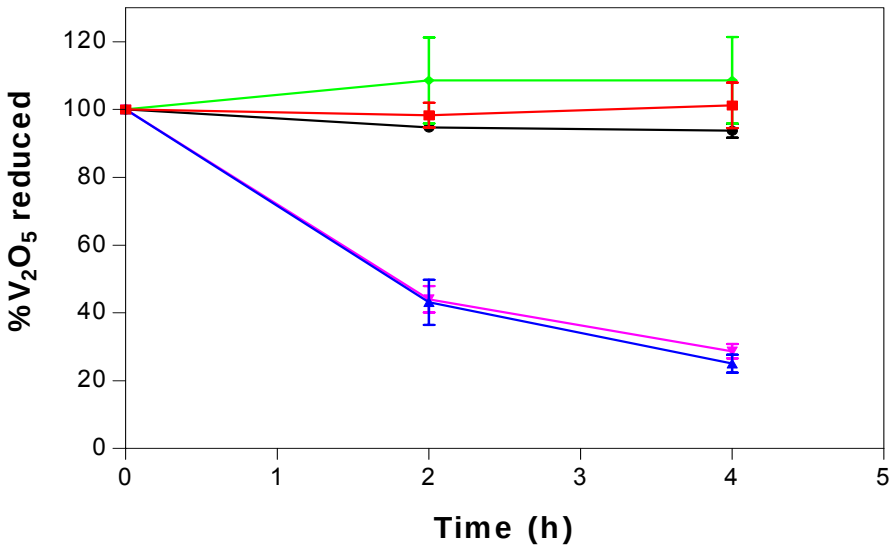

Figure 4.8. Vanadate reduction over time by fractions obtained using the protein minipreps under native conditions protocol (QIAGEN, 2000). Blank rate (▬), whole cells (▬), frozen cells (▬), cytoplasm (▬) and membranes (▬).

When using the QIAGEN (2000) assay as described in section 4.2.4.1 to fractionate cells, it was found that neither the supernatant nor the membrane fractions retained any activity compared to the whole cells and frozen cells which had converted about 70% of the vanadate in the give experimental time frame (Figure 4.8). The fact that the cells retained activity after being frozen is a very noteworthy observation as it supplies a method of storing the cells without affecting the enzyme's ability of reducing vanadate. Since the activity was lost upon disruption of the cells using this protocol, other methods were investigated.

4.3.2.2 Fractionation by sonification

The fractions obtained by using the methods of Gaspard *et al.* (1998) and Kaufmann and Lovley (2001) showed that the activity is localized in the spheroplasts (Figure 4.9) and not in the periplasmic region. The periplasmic fraction had no activity while the spheroplast portrayed about the same initial reduction rate as the whole cells suspended in a sucrose solution.

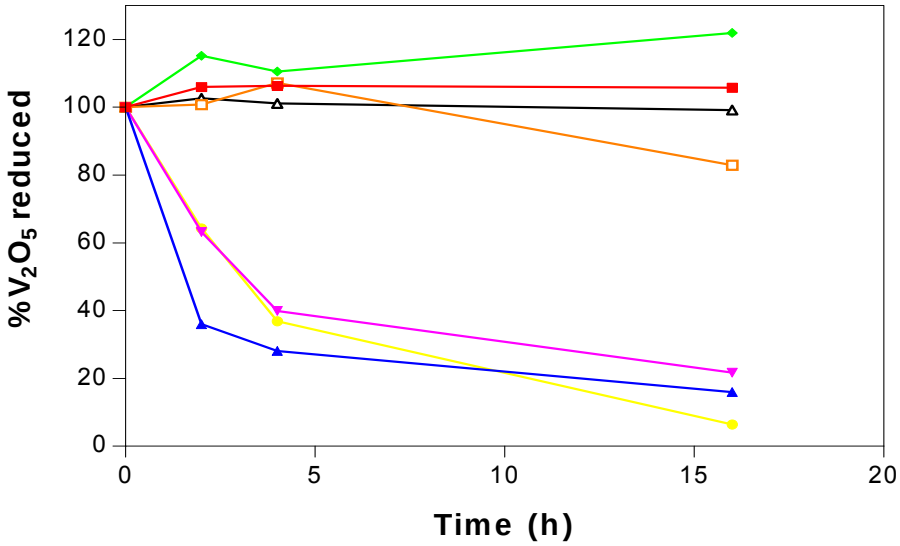

Figure 4.9. Vanadate reduction over time by fractions obtained using the protocols supplied by Kaufmann and Lovley (2001) and Gaspard *et al.* (1998). Blank rate (▬), whole cells (▬), whole cells in sucrose (▬), spheroplasts (▬), cytoplasm (▬) and membranes (▬).

Further fractionation of the spheroplasts as described in section 4.2.4.2 was done to obtain the membrane and cytoplasmic fractions. After fractionation the vanadate

reducing activity disappears. It seemed that sonification was a very harsh method and that the protein(s) responsible for the reducing activity is destroyed in the fractionation process. A less harsh mechanical method was used next to determine if the desired protein(s) are membrane associated or localized in the cytoplasmic region.

4.3.2.3 Cell disruption by using glass beads

The cells were disrupted as described in section 4.2.4.3 and the obtained fractions were assayed for vanadate reducing activity as described in section 4.2.2. In comparison to the disruption by sonification method, the disruption of cells using glass beads gave some indication to the location of the protein(s) responsible for vanadate reduction. It was found that 18% of the activity is located in the cytoplasm while 41% of the activity is located in the membranes (Figure 4.10). The activity obtained when assaying the spheroplasts was taken as 100% activity. There is thus a 32% loss of activity that can not be accounted for. As it seemed that the majority of the activity is membrane associated the following experiments concentrated on the dissociation of the membrane associated protein.

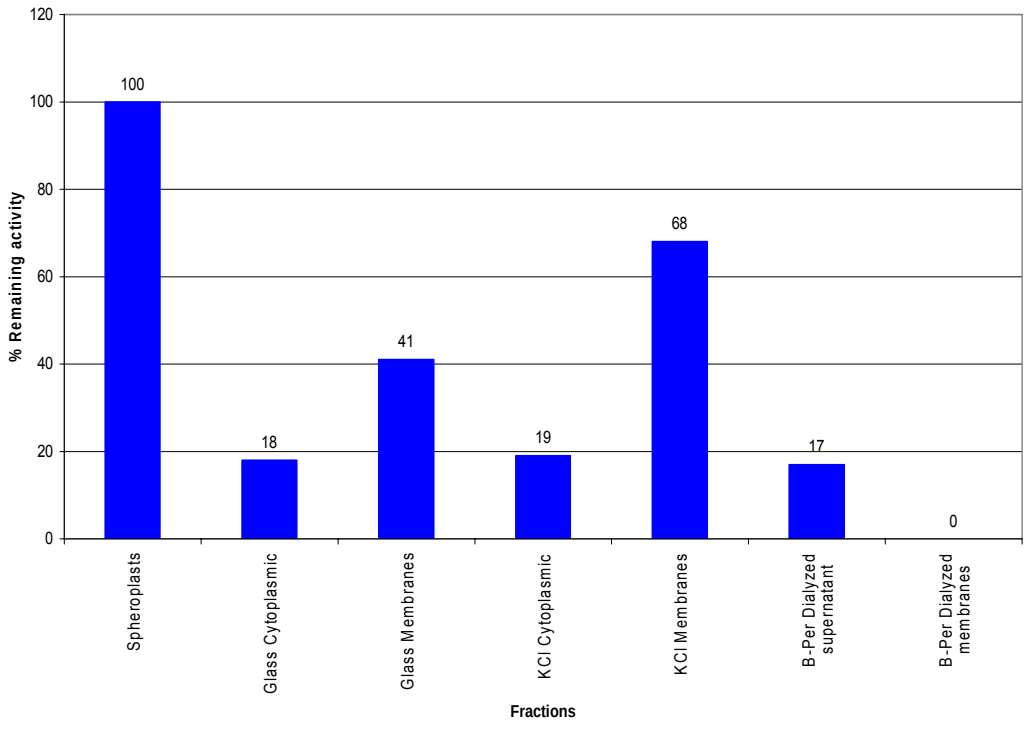

Figure 4.10. Comparison of the remaining activity obtained when disrupting the cells by using glass beads and dissociating the proteins using B-Per and KCl.

4.3.3 Dissociation of membrane proteins

4.3.3.1 Solubilization of proteins by using either potassium chloride or B-Per® Reagent

Treatment of the spheroplasts with a high-ionic-strength buffer as described in section 4.2.5.1 resulted in the release of 19% of the activity while 68% of the activity remained membrane associated (Figure 4.10). Thirteen percent of the activity was unaccountable for. The activity obtained when assaying the spheroplasts was taken as 100%. These results indicate that the protein(s) of interest are not peripheral proteins (Gaspard *et al.,* 1998) but the majority of these protein(s) are membrane associated.

B-per treatment of spheroplasts as described in section 4.2.5.1 resulted in the release of 17% of the activity with the rest of the activity being unaccountable for (Figure 4.10). The activity obtained when assaying the spheroplasts was taken as 100% activity. These results indicate that the proteins can be released from the membranes. The function of B-Per® reagent is to release soluble proteins from the cells; therefore the results obtained are not surprising.

4.3.3.2 Release of membrane bound proteins using detergents

In section 4.3.3.1 the results indicated that the protein(s) responsible for vanadate reduction can be released from the membranes. Several different detergents were tested to assist in the optimum release of the sought after protein(s).

Results obtained from the treatment of the spheroplasts with the different detergents as described in section 4.2.5.2 showed that only Triton X-100 (1.25mM) and deoxycholic acid (20 and 40mM) were able to release the activity from the membranes. Blank rates were run simultaneously with the experiments to ensure that the detergents did not affect or add to the activity. These detergents were only able to release the activity at concentrations exceeding the cmc as micelles are formed. Triton X-100 was only effective at a concentration 5 times higher than its cmc, while deoxycholic acid was most effective at a concentration 26 times higher

than its cmc, although at concentrations 13 times higher than the cmc, release were measured (Figure 4.11). The 1.25mM Triton X-100 extraction recovered 28% of the activity but after removal of the detergent the protein lost its activity, most probably due to structural changes (Figure 4.11). On the other hand treatment with 40mM deoxycholic acid resulted in a recovery of all the activity even slight activation was observed, however, after removal of the detergent all the activity was lost.

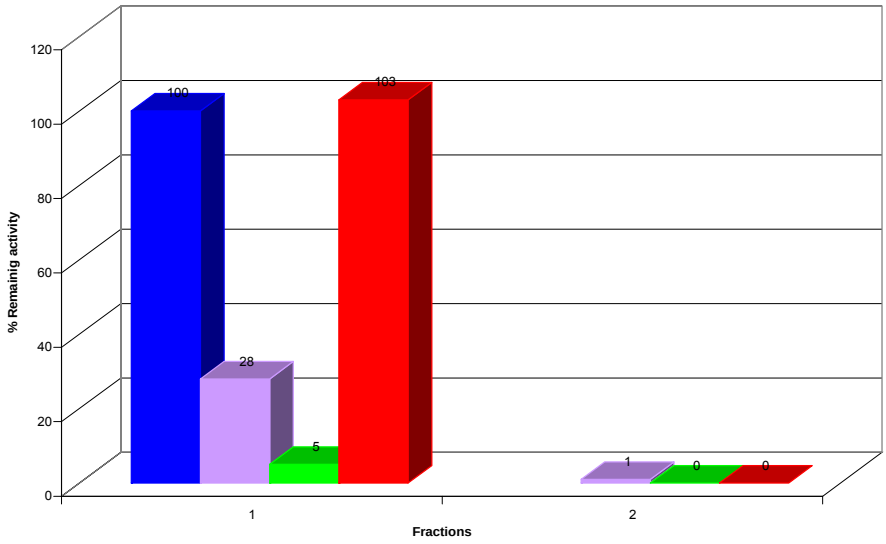

Figure 4.11. Comparison of the activities obtained after treatment with different detergents before (1) and after (2) removal of the detergent. Spheroplasts ( ), 1.25mM Triton X-100 ( ), 20mM Deoxycholic acid ( ), 40mM Deoxycholic acid ( ).

These results indicate that the protein(s) responsible for vanadate reduction is dependant on its environment, in other words it needs the membrane and its structure to function. In 2004 Myers *et al.* found that V (V) reduction in *Shewanella oneidensis* was mediated by an electron transport chain, which includes cytoplasmic membrane components (menaquinone and the tetraheme cytochrome CymA) and the outer membrane cytochrome OmcB. A partial role for the outer membrane cytochrome OmcA was also evident; in all probability this reduction could mimic the reported data.

4.3.4 Protein characterization in whole cells

The enhanced loss of activity during the fractionation and the detergent removal procedures hindered further purification steps. The “reductase” activity was explored further using whole cells. All characterization was preformed under anaerobic conditions as the activity was enhanced under anaerobic conditions as discussed in section 3.3.1.3.

4.3.4.1 Optimum pH

The optimum pH was determined over a pH range of 6 to 9 as described in section 4.2.6.1. The pH that showed maximum activity was taken as 100%. Optimal pH was determined to be 7 (Figure 4.12). All further experiments were conducted at neutral pH, which corresponds to the optimum pH for growth. All work done by Carpentier *et al.* (2003) on V (V) reduction was done at pH 7.0. Work done by Opperman and Van Heerden (2005) on chromate reduction in *Thermus scotoductus* indicated that the reduction was optimum at pH 7.5, while optimum pH for growth of the bacteria was 6.5 (Kieft *et al.,* 1999).

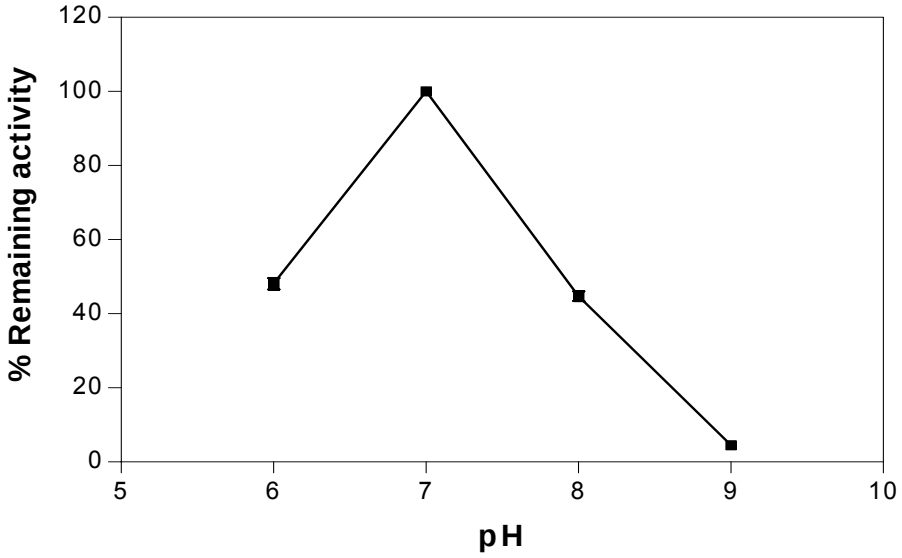

Figure 4.12. pH profile depicting optimum pH for activity in whole cells under non-growth, anaerobic conditions.

4.3.4.2 Optimum temperature

The optimum temperature was determined as described in section 4.2.6.2. The temperature that showed maximum activity was taken as 100%. The activity increased up to 45°C and then declined as the temperature was increased further (Figure 4.13). The optimum temperature for whole cell reduction under anaerobic conditions was thus established to be at 45°C, which is 5°C above the optimum growth temperature. A similar temperature difference was observed by Vadas *et al.* (1999) when characterizing a ferric reductase obtained from *Archaeoglobus fulgidus*. Chromate reductases characterization by Ishibashi *et al.* (1990), Park *et al.* (2000), and Suzuki *et al.* (1992) found that the optimum temperature for enzyme activity was higher under non-growth conditions. According to Calcott (1981) it is not unusual for even essential enzymes to exhibit maximum activity *in vitro* under conditions different from the optimal growth conditions of an organism.

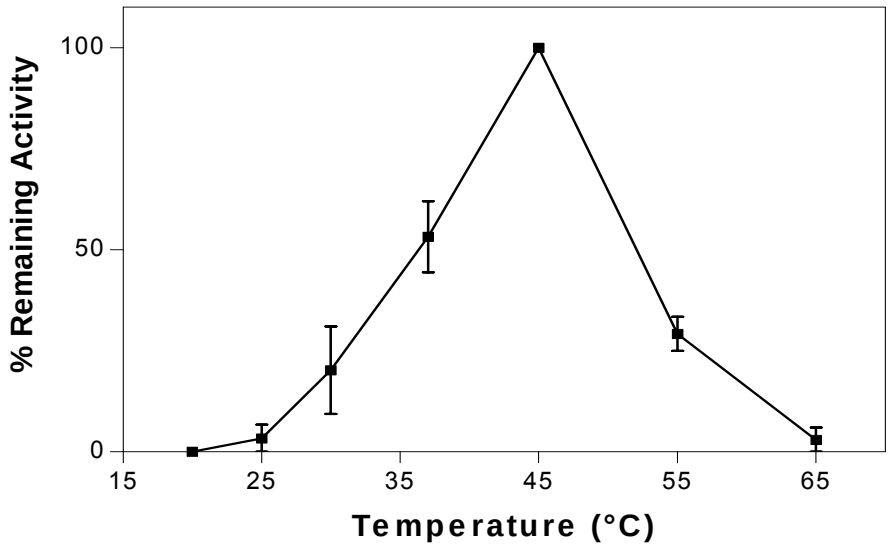

Figure 4.13. Temperature profile depicting optimum temperature for activity in whole cells under non-growth conditions. Error bars indicate standard deviations.

4.3.4.3 Electron donor specificity

Different electron donors were tested as described in section 4.2.6.3. Cells obtained from cultures grown in the presence of 2mM vanadate had a higher activity than the cells obtained from culture grown without vanadate, independent of which electron

donor used. The reduction of V (V) was supported by a variety of organic electron donors (Table 4.2), with lactate being the most efficient under anaerobic conditions. This was also observed by Carpentier *et al.* (2003) when vanadium reduction by *Shewanella oneidensis* was studied.

In section 3.3.1.3 it was elucidated that vanadate can be reduced by *Enterobacter sp.*EV-SA01 (culture 6.2) under aerobic, non-growth conditions and here it was found that glucose was the preferred electron donor under these conditions. Irrespective of which electron donor used, reduction of vanadate under anaerobic conditions was preferred; therefore the reduction rates were higher under anaerobic conditions (Table 4.2). Possibly vanadate reduction is dependant on the redox potential. This data corresponds to data obtained in section 3.3.1.3.

Table 4.2. Summary of activities obtained by using different electron donors under both aerobic and anaerobic conditions.

	Activity (U/ml)			Activity (U/ml)	
Electron donors	**Aerobic**	**Anaerobic**	**Electron donors**	**Aerobic**	**Anaerobic**
Glucose	1554	1158	Acetate	138	623
Lactate	830	2076	Benzoate	0	862
Succinate	654	1686	Fumaric acid	38	875
Pyruvic acid	522	1781	Citric acid	994	0

4.3.4.4 Metabolic inhibitors

The metabolic inhibitors are inhibitors of energy generation via respiration (Komori *et al.,* 1989). Cells were incubated with the different metabolic inhibitors as described in section 4.2.6.4. No significant inhibition of the vanadate reducing activity was observed upon the addition of the metabolic inhibitors. This data indicates that vanadate does not serve as terminal electron acceptor in *Enterobacter sp.*EV-SA01. Vanadium reduction by *Shewanella oneidensis* indicated that V (V) reduction does not support anaerobic growth (Myers *et al.,* 2004), unlike the results obtained by Ortiz-Bernad *et al.* (2004) when vanadium reduction by *Geobacter metallireducens* was studied.

4.3.4.5 Kinetic studies

Vanadate reduction by *Enterobacter sp.*EV-SA01 does not confer to normal Michaelis Menton kinetics. The data was fitted to the following equation representing an uphill dose response curve.

$$v_0 = V_{\min} + \frac{V_{\max} - V_{\min}}{(1+10^{((\log EC_{50} - [S])XH})}$$

This model illustrates that co-operative behavior exists and that there is a basal level of activity which was probably not completely catered for. Although the model gives a realistic estimate of the EC_{50} interpretation of the model, in terms of mechanism, interpretations must be done with care as the vanadate reduction observed is the sum of a number of processes including passage of the ion across the membrane, which possibly happens through the phosphate carrier, the reduction process as well as the transport of the reduced metal out of the cell. Clearly further work needs to be done to elucidate the mechanism of reduction. Figure 4.14 illustrates the data and the line describing the best fitted model, while table 4.3 gives the values obtained for the different parameters.

Table 4.3. The values obtained for the different parameters when using an equation representing an uphill dose response curve.

Parameter	Value	Std error
V_{min}	417	239
V_{max}	4682	339
EC_{50}	5.44mM	1.07mM
H	4.214	1.265

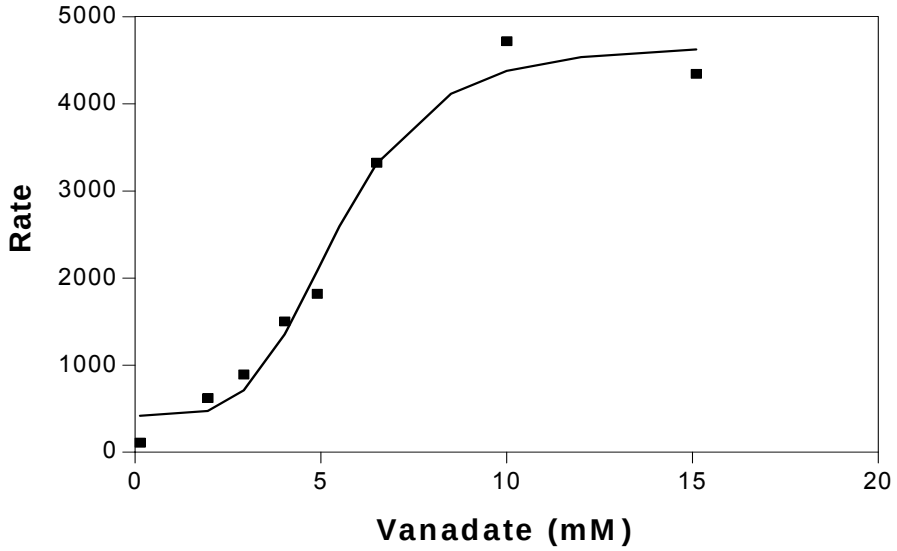

Figure 4.14. The graph depicts the fit of the enzyme kinetic data with vanadate as substrate. The fit was done using an equation representing an uphill dose response curve.

The NADH followed saturation kinetics and it was found that the enzyme had a high affinity for the substrate as it had a low k_m value of approximately 0.0071mM. The V_{max} value was approximately 2183 (Figure 4.15). The activity increased proportionally to the NADH concentration with vanadate reduction rate approaching a maximum at higher concentrations.

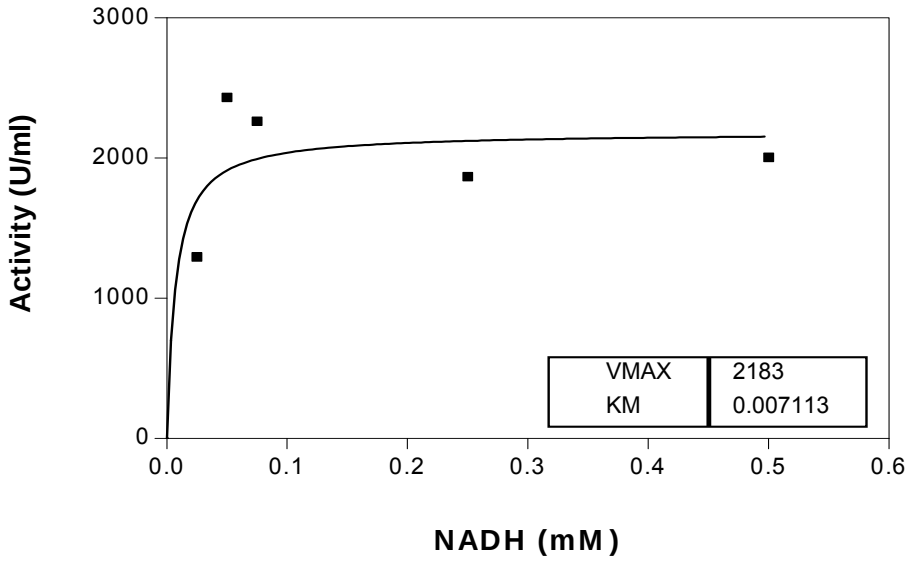

Figure 4.15. The graph depicts the substrate saturation of NADH in the presence of V (V) with whole cells under non-growth, anaerobic conditions.

4.3.5 Determination of parameters for *in situ* experiments

Bacterial transport in porous media is of great significance to bioremediation of contaminants in the subsurface environments. Several bacteria are capable to transform toxic metals and radionuclides into more or less mobile species (Ahmann *et al.,* 1994; Antipov *et al.,* 2000; Deuel and Swoboda, 1972; Lloyd, 2003; Lovley, 1993) resulting in a less toxic environment. Cost-effective bioremediation of subsurface contaminants most likely depends on bioaugmentation (Dong *et al.,* 1999; Dong *et al.,* 2002). For effective bioaugmentation it is important for bacteria to reach the zone of contamination. Despite the availability of bacterial strains competent to remove contaminants, field application of bioaugmentation is not common (Dong *et al.,* 1999).

Bacterial transport through the subsurface is partially dependant on its adhesion capabilities. Adhesion can be affected by any number of factors, including, formation of capsules or slime layers, covalent or ionic bond formation, van der Waals forces and hydrophobic interactions (Ward and Berkeley, 1980; Tadros, 1980). However, upon bacterial adhesion to an interface, the physiological processes may induce changes in the bacterial surface for example the composition of adsorbed polymers can change (Fletcher *et al.,* 1980), these change may include the visualization of polysaccharides which were not visible before adhesion (Ward and Berkeley, 1980). The transmission electron micrographs (Figures 3.18) of *Enterobacter sp.*EV-SA01 indicated a relatively low cell to volume ratio of exopolysaccharides; this could be due to the extensive dehydration steps in the preparation of the cells for the micrographs (Section 3.2.6.2.2). The carbohydrate : protein ratio obtained as described in section 4.2.7.1 was only 1:8, but despite the apparent lack of exopolysaccharides and the low carbohydrate : protein ratio the percentage adhesion results obtained from experiments preformed as described in section 4.2.7.3 were very encouraging. The percentage adhesion when using the glass pipettes had maximum adherence (35% adhesion) at 4 hours with no additional adherence beyond 4 hours (Figure 4.16). However, when adhesion studies were conducted in the plastic syringes about 75% of the cells adhered in 3 hours, this difference in adhesion percentage could be caused by the difference in cell to surface ratio. The cell to surface ratio in the syringes is larger than the cell to

surface ratio in the pipettes; therefore the percentage adhesion in the syringes is greater.

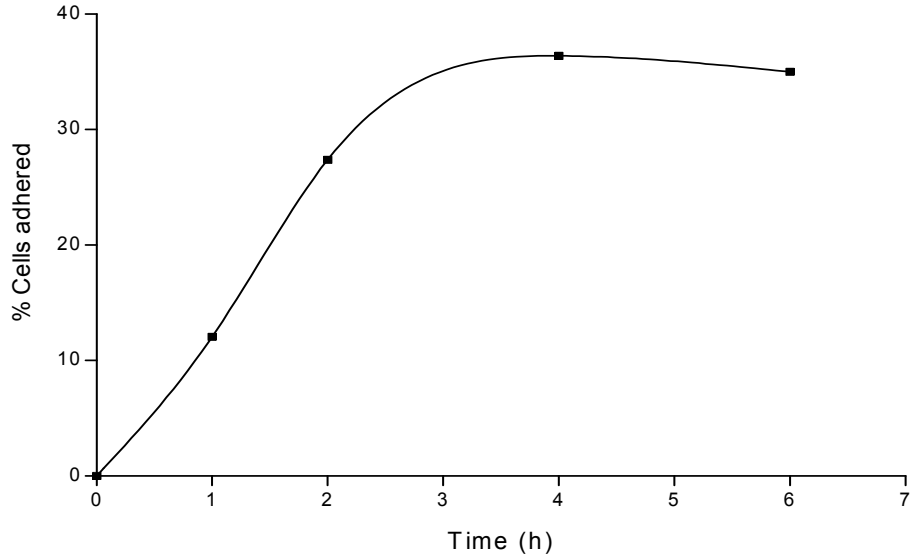

Figure 4.16. Cell adhesion trails over time in 20ml glass pipettes.

Barrier formation was determined by measuring the hydraulic conductivity as described in section 4.2.7.6. There was no decrease in the hydraulic conductivity thus no clogging of the column occurred. This was favorable because although the cells adhere the soil particles the contaminants pumped into the column can still move through the column to reach the bacteria throughout the column.

As mentioned previously bacterial transport is of importance in bioremediation of contaminated sites, this is because bioremediation processes require that an effective concentration of bacteria be deployed so that the microorganism introduced is capable of reaching and traversing through the contaminated area (Fuller *et al.,* 2000). The tracer test was preformed as described in section 4.2.7.7. The bromide eluted from the column as a single peak, with maximum concentration occurring after one pore volume of artificial groundwater had passed through the column (Figure 4.17). The cells exhibited a very similar profile, with maximum cell concentration also occurring after one pore volume. The bacterial breakthrough had no significant tailing and eluted in a sharp peak. Bacterial breakthrough is controlled by flow rate, grain size, grain surface-cell interactions and preferential flow paths through macropores (Fuller *et al.,* 2000). The results obtained suggest that both the bromide

and the cells follow similar flow paths since their respective breakthrough curve were very similar (Figure 4.17), thus if the bacteria was used in remediation of groundwater the bacteria could be transported as well as survive in the flow.

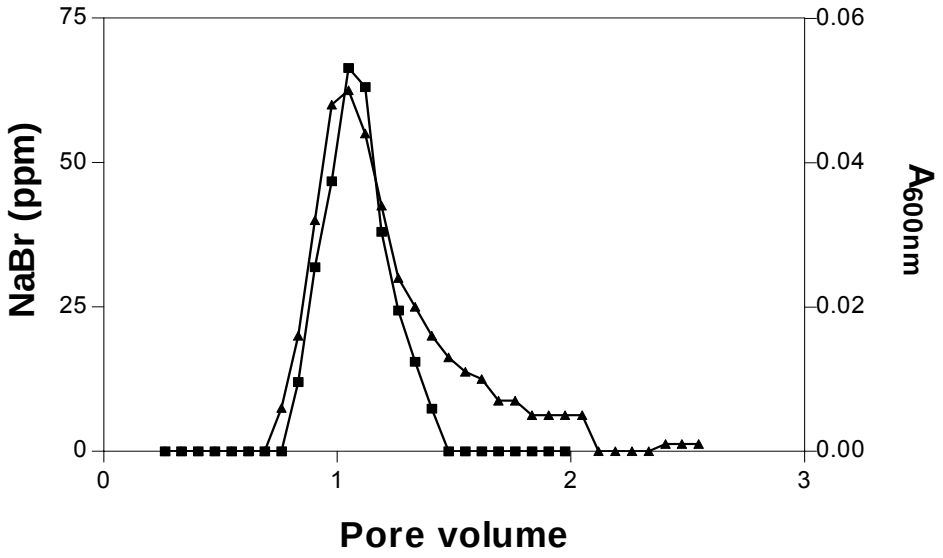

Figure 4.17. Breakthrough curves obtained for the sodium bromide tracer (■) and the injected bacteria (▲).

4.3.6 *In situ* reduction of vanadate

The results obtained in section 4.3.5 showed that *Enterobacter sp.*EV-SA01 were able to adhere to the sand particles yet did not form a dense slime layer. Although the bacteria had adhesion capabilities it is important to know whether the bacteria in the column still have the ability to reduce vanadate and that the viability status of the bacteria is satisfactory.

The column was loaded as described in section 4.2.7.8 and vanadate reduction was monitored over time by determining vanadate concentration at each of the side ports as well as at the effluent as described in section 3.2.5.1. Cell viability was tested by plate spreading of the samples obtained from the side ports on TYG-agar plates and incubating it at 37°C overnight. It was found that the cell viability rate was satisfactory as the TYG-agar plates were overgrown by the bacteria.

The vanadate reducing ability of *Enterobacter sp.*EV-SA01 was not lost during the *in situ* experiments as vanadate was still reduced. Vanadate reduction could be

followed visibly by following the color change from yellow to bluish-black (Figures 4.18 through 4.20). After 13 hours (approximately one pore volume) there was a 94% decrease in vanadate concentration at the fourth side port, 58% decrease at the third side port, 25% at the second side port and 14% at the first side port. This clearly indicates that the longer the retention time in the column and contact with the bacteria the more vanadate will be reduced.

Cell counts were done on the side port samples and the effluent as described in section 4.2.7.2. Initially the effluent contained no cells but as the vanadate mixture was pumped through the column cells began to accumulate in the effluent. Simultaneously the cell concentration in the first side port began to decrease with an increase of cell concentration in the following ports. This was observed until no cells could be detected in the first, second and third side ports. After 24 hours (approximately two pore volumes) the vanadate reduction rate started to decrease. The decrease in vanadate reduction rate corresponded to the loss of cells. From these results it is obvious that vanadate reduction *in situ* is dependant on the presence of bacteria. The subsequent decrease in reduction capabilities was therefore concluded to be a result of cell loss through out the column.

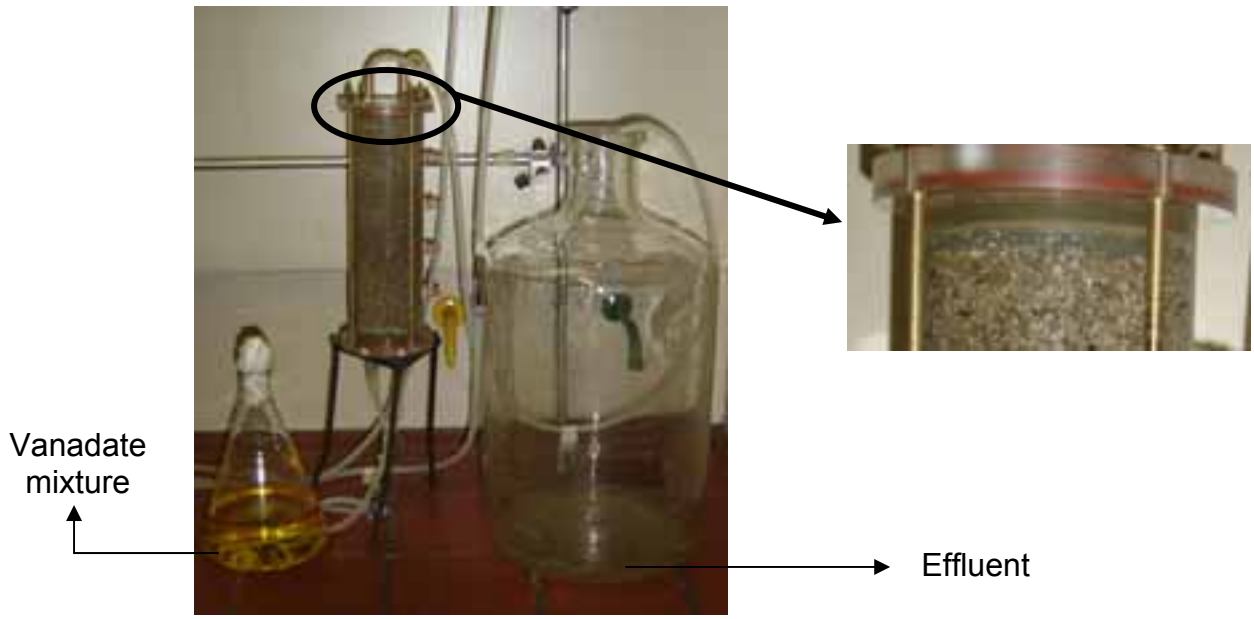

Figure 4.18. Monitoring of vanadate reduction after 6.5 hours.

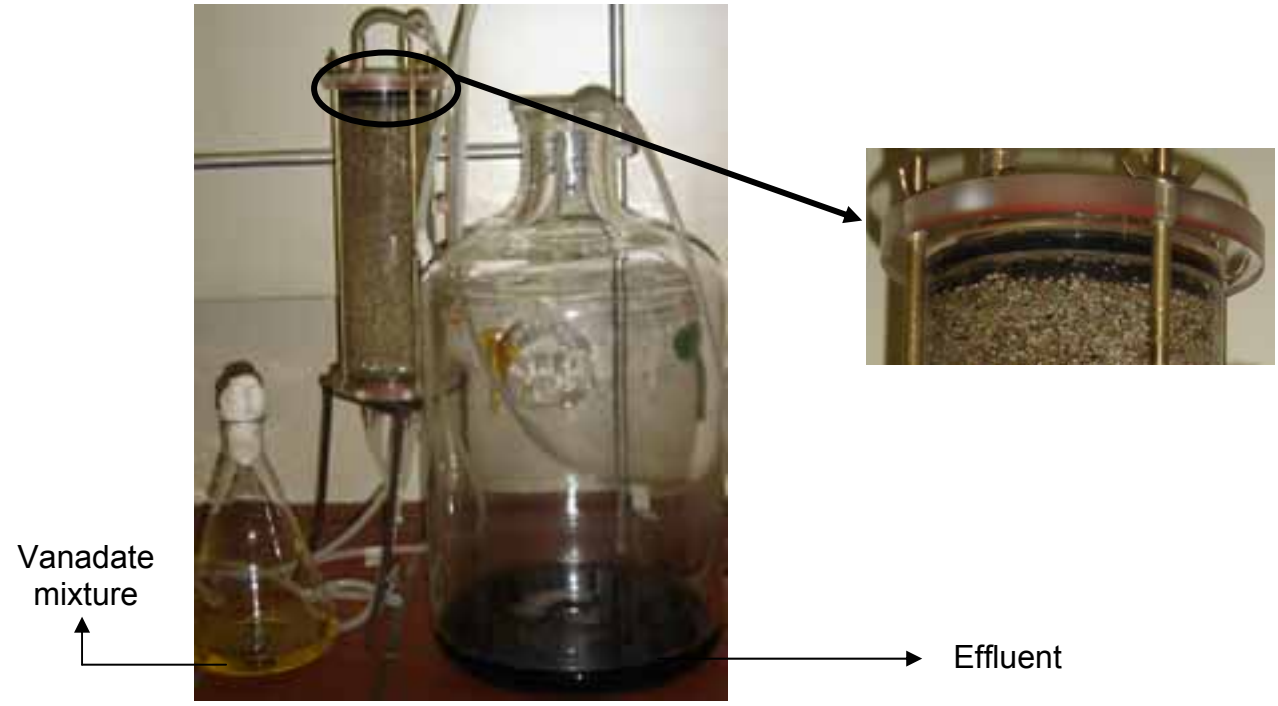

Figure 4.19. Monitoring of vanadate reduction after 24 hours.

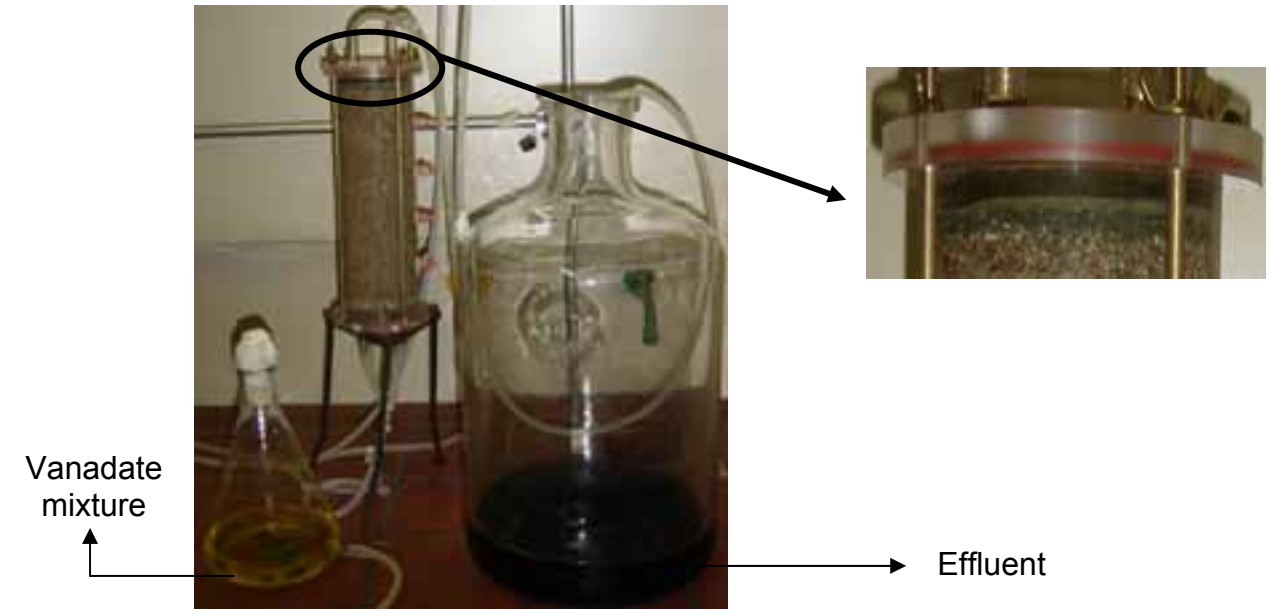

Figure 4.20. Monitoring of vanadate reduction after 49 hours.

4.3.7 Conclusions

In this chapter it was found that the protein(s) responsible for the reduction of vanadate is membrane associated and that the activity can be released from the membranes by using detergents. However, a combination of the disruption methods and the release of the protein(s) with the help of detergents resulted in a major loss of the vanadate reducing activity. This complication led to the characterization of the vanadate reducing activity using whole cells. In the whole cells the activity was optimum at 45°C and neutral pH. The activity was enhanced under anaerobic conditions with lactate being the preferred electron donor. It was also noted that vanadate does not act as finale electron acceptor as the metabolic inhibitors had no effect on the activity rate.

*Enterobacter sp.*EV-SA01 had the ability to adhere to the soil particles in the column but did not form an impenetrable barrier. The cells in the column were viable and still possessed the ability to reduce vanadate. The injected bacteria followed the same flow path as the tracer and the *in situ* vanadate reduction experiments gave very promising results; it showed that the longer the contact time between the bacteria and the contaminant, the greater the reduction. The reduction rate decreased after prolonged flow of artificial groundwater as this resulted in the elution of cells from the column.

4.4 References

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SUMMARY

In 1996, members of the Princeton group isolated a thermophilic Fe (III) reducing bacterium from a South African gold mine. Further collections at other mines confirmed that the mining environment harbors distinctive microbial populations, which may have novel applications. The purpose of this study was then to screen bacterial mine isolates for metal reducing capabilities. The bacteria where screened for their resistance to vanadium. Of the group of bacteria studied, one isolate showed high vanadium reducing capability. This unique characteristic was further studied, as well as it's suitability as a bioremediation tool.

The bacterial isolates were screened for vanadate resistance under aerobic, anaerobic and micro-aerophilic conditions. The tolerant isolates were then subjected to whole cell reduction under both aerobic and anaerobic conditions. It was found that not all of the tolerant isolates could reduce vanadate, and that vanadate reduction was enhanced under anaerobic conditions. The isolate which had the highest reduction rate under both aerobic and anaerobic conditions were selected for further experiments. The isolate was identified as belonging to the genus *Enterobacter* by using 16S rDNA sequencing. Sequencing results was confirmed using both the API 20E and the Biolog system. The isolate was designated as *Enterobacter sp.*EV-SA01, where EV refers to the place of isolation namely Evander gold mine. The maximum vanadate reduction by this microorganism during growth was associated with the early stationary phases, while the optimum conditions for growth were a neutral pH and a temperature between 37 to 39°C.

Experiments to elucidate the protein(s) involved with vanadate reduction showed that the majority of the activity was associated with the membranes. The protein(s) responsible for the activity could be released from the membranes by treatment with detergents, but further characterization of the vanadate reducing activity was done using whole cells. The protein(s) showed optimum activity at pH 7 which corresponded with the optimum pH for growth, while optimum temperature was slightly higher at 45°C. A variety of electron donors could be utilized by the bacteria under both aerobic and anaerobic conditions, but the vanadate reducing ability was

much higher under anaerobic conditions irrespective of which electron donor was used, with sodium lactate being the preferred electron donor. The *in situ* reduction experiments showed that the bacteria will be suitable as a bioremediation tool as it was able to survive *in situ* and reduce the vanadate present.

This research has shown new aspects of vanadium reduction, the results can be explored in further studies to refine the bioremediation application of this bacterium. Also, it should be determined whether vanadium reduction in this microorganism is simply a detoxification process or if it has a dissimilatory role. A means to purify the vanadium reducing protein(s) and subsequent characterization should also be explored further.

OPSOMMINIG

In 1996, het lede van die Princeton groep ‘n termofiliese Fe (III) reduserende bakterium geïsoleer uit ‘n Suid Afrikaanse goud myn. Verdere isolasies uit ander myne het bevestig dat myn-omgewings ryk is aan unieke mikrobiese populasies, wat mag beskik oor unieke toepassings. Die doel van hierdie studie was om die bakteriese myn isolate te toets vir metaal reduserende eienskappe. Van die groep bakterieë wat ondersoek is, het een isolaat oor die hoë vanadium reduserende vermoë beskik. Hierdie unieke eienskap is verder ondersoek asook die moontlikheid om hierdie isolaat te gebruik in bioremediëring toepassings.

Die bakteriese isolate is getoets vir vanadium toleransie onder aërobiese, anaerobiese en mikro-aerofiliese toestande. Die tolerante isolate is onderwerp aan heel-sel reduksie onder beide aërobiese en anaerobiese toestande. Daar is gevind dat nie alle tolerante isolate oor die vermoë beskik het om vanadaat te reduseer nie, en dat vanadium reduksie verhoog was onder anaerobiese toestande. Die isolaat wat vanadaat die beste gereduseer het onder beide aërobiese en anaerobiese toestande, is gekies vir verder eksperimente. Op grond van 16S rDNS is die isolaat geïdentifiseer as deel van die *Enterobacter* genus. Die 16S rDNS resultate is bevestig deur gebruik te maak van beide die API 20E asook die Biolog sisteem. Hiervolgens is die bakterium as *Enterobacter sp.* EV-SA01 gedoop waar EV die plek van isolasie aandui naamlik Evander goudmyn. Die maksimum vanadaat reduksie deur hierdie mikroorganisme tydens aktiewe groei was geassosieer met die vroeë stasionêre fase, terwyl die optimum toestande vir groei voorgekom het by ‘n neutrale pH en temperatuur tussen 37 en 39°C.

Eksperimente om die proteïen(e) betrokke by vanadaat reduksie te identifiseer het gewys dat die grootste deel van die aktiwiteit membraan gebonde is. Die betrokke ensieme met gepaardgaande aktiwiteit kon los gemaak word vanaf die membrane deur dié te behandel met oplosmiddels, terwyl verdere karakterisering van die vanadaat reduserende aktiwiteit gedoen is deur gebruik te maak van die heel-selle. Optimale aktiwiteit van die vanadaat reduserende proteïen(e) vind plaas by pH 7, wat ooreenstem met die optimum pH tydens groei. ‘n Temperatuur van 45°C is

nodig vir optimale ensiem funksie wat effens hoër is as die optimale groei temperatuur. ’n Verskeidenheid elektron skenkers kan deur die bakterium gebruik word onder beide aërobiese en anaerobiese toestande. Die vanadaat reduserende vermoë van die bakterium is verhoog onder anaerobiese kondisies, ongeag watter van die elektron skenkers gebruik is. Van al die elektron skenkers wat getoets was het dit geblyk dat natrium laktaat die verkose elektron skenker vir vanadaat reduksie is. Die *in situ* reduksie eksperimente het getoon dat die bakterium geskik is vir bioremediëring toepassings omdat dit in staat was om *in situ* te oorleef en ook beskikbare vanadaat te reduseer.

Hierdie navorsing het nuwe aspekte getoon van vanadium reduksie en die resultate wat verkry is kan gebruik word in verdere studies om die bioremediëring toepassings van hierdie bakterium te verfyn. Verder is dit ook belangrik om te bepaal of die vermoë van hierdie bakterium om vanadaat te reduseer ‘n detoksifiserings proses is, of is dit deel van ‘n proses om energie te genereer? ’n Metode om vanadium reduserende proteïen(e) te suiwer behoort ook verdere aandag te geniet.