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REDUCTION OF SELENIUM BY *PSEUDOMONAS STUTZERI* NT-I; GROWTH, REDUCTION AND KINETICS

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

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Declaration

I, **CHARLOTTE ELIZE WESSELS,** hereby declare that the work provided in this dissertation is to the best of my knowledge original (except where cited) and that this work has never been submitted for another degree at this or any other tertiary education institution.

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Signature of candidate

This …….. day of ……. 2017

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Dedication

This dissertation is dedicated to

My family

My husband, who believed in me and encouraged me along the way

My mother and father who supported me and carried me with their prayers

God, above all.

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I would like to express my sincere gratitude to the following persons without whom this dissertation would not have been possible:

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ABSTRACT

Bioremediation of seleniferous water is gaining more momentum, especially when it comes to bacterial reduction of the selenium oxyanions. More and more bacterial strains that are able to reduce selenium are being isolated. These bacteria need to be studied further to determine whether they are suited for industrial application. In this study, the reduction of Se(VI) to Se(0) by *Pseudomonas stutzeri* NT-I was examined using batch experiments with the bacteria suspended in MSM. For the determination of the optimum conditions for the growth of the bacteria, the linearized rate during the exponential phase for different conditions were compared. A pH of 7, temperature of 37 ºC, salinity of 20 $g.L^{-1}$ NaCl and initial concentration of 5 mM selenate were found to be the best at promoting growth.

To determine the optimum conditions for the reduction of selenium, the amount of Se(0) recovered from the plug after 16 hours of incubation was measured. A pH of 8, temperature of 37 ºC and salinity of 5 $g.L^{-1}$ resulted in the most Se(0) recovered.

The kinetics of the reduction of Se(VI) to Se(0) was found to follow the adapted Monod equation. An increase in the initial Se(VI) concentration positively affected the reduction rate indicating that substrate saturation had not yet been reached. One k_{max} could be fitted to each of the two reactions but not one K_s. It was found that K_s decreased with increasing initial selenate concentration. Visually it can be deduced that inhibition starts playing a role in the reduction of selenate at a concentration of 4 mM.

Pseudomonas stutzeri NT-I is an exemplary selenium reducing agent and deserves more attention, not only for industrial application but also in the research world, for further understanding of the complex mechanism behind metal reduction in bacteria.

Keywords: *Pseudomonas stutzeri* NT-I, selenium, bioremediation, aerobic, bacterial growth, metal reduction, reduction kinetics.

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List of Abbreviations

AAS	Atomic adsorption spectrophotometer
APHA	American Public Health Agency
Conc.	Concentration
CS	Carbon source
MSM	Mineral salt medium
pE	Potential electrical
pH	Potential hydrogen
ppm	Parts per million
rpm	Rotation per minute
SSF	Subsurface flow
TSA	Tryptone soy agar
TSB	Tryptone soy broth
US EPA	United States Environmental Protection Agency
UV	Ultraviolet
WHO	World Health Organization

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Symbol nomenclature

t	Time
X	Biomass concentration at time, t ($g.L^{-1}$)
$[Se^{6+}]$	Selenate concentration mM
$[Se^{4+}]$	Selenite concentration mM
$[Se^{0}]$	Concentration of elemental selenium mM
μ	Specific growth rate (h^{-1})
OD_{600}	Absorbance of cell suspension at 600 nm
k_{max64}	Maximum reaction rate for the reduction of selenate to selenite ($mol.(g.min)^{-1}$)
k_{max40}	Maximum reaction rate for the reduction of selenite to elemental selenium ($mol.(g.min)^{-1}$)
K_{s64}	Substrate saturation coefficient for the reduction of selenate to selenite (mM)
K_{s40}	Substrate saturation coefficient for the reduction of selenite to elemental selenium (mM)
K_{I64}	Inhibition constant for the reduction of selenate to selenite (mM)
K_{I40}	Inhibition constant for the reduction of selenite to elemental selenium (mM)

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Subscripts

600	Wavelength of 600 nm
OD_{600}	Optical density at 600 nm
o	Initial
64	Notation for reaction of selenate to selenite
40	Notation for reaction of selenite to elemental selenium
Max	Maximum rate
S	Substrate
4+	Selenite
6+	Selenate
0	Elemental selenium

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CHAPTER 1: INTRODUCTION

1.1. Background

Selenium, first discovered in 1818, is a metalloid and chalcogen. It has multiple oxidation states, beginning with the most reduced state, namely selenate (SeO_4^{2-}, Se(VI)), followed by selenite (SeO_3^{2-}, Se(IV)), elemental selenium (Se^0, Se(0)) and finally selenide (Se^{2-}).

Selenium occurs naturally in soil, but at low concentrations ranging between 0.1 – 2 µg $Se.g^{-1}$ soil, whereas in seleniferous soil the concentration can go up to 320 – 324 µg $Se.g^{-1}$ soil (Girling, 1984).

There are many natural pathways through which selenium is released into the environment and redistributed. These processes range from volcanic activity to wildfires, volatilisation from soils, plants and water bodies, and weathering of rocks (Sandy & DiSante, 2010).

Of the selenium released into the environment, 37.5 – 40.6 % can be ascribed to anthropogenic activities. It is released from the earth's crust by the mining of coal and various metals, oil production, use of agricultural products, as well as during the melting of non-ferrous metals (Lenz & Lens, 2009). Landfill ash disposal that generates toxic leachate poses a risk of groundwater contamination, which leads to the polluting of nearby water bodies (Lemly, 2004). According to different studies, acid mine drainage waters contain selenium at concentrations ranging between 2 $x10^{-4}$ and 6.2 $x10^{-3}$ mM of total selenium (Lenz et al., 2008).

Selenium is mostly present in water as selenate or selenite. This poses a problem, since these two oxyanions are toxic and bio-accumulate readily. In one case it was found the concentration of selenium in the apex predators (birds and humans) in the

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area were 2 000 times higher than in the water (Wu, 2004). Birth defects and reproductive problems among fish and waterfowl are examples of the effects of selenium poisoning. In the human body, selenium poisoning – referred to as selenosis – can have many adverse effects, including respiratory difficulty, gastrointestinal distress and liver damage. It also has a negative impact on the central nervous system (Kenward et al., 2006).

1.2. Problem statement

At the moment, the excessive presence of selenium in water bodies used as sources of potable water poses a problem due to the narrow window between its essentiality and toxicity. For humans, the RDA is 55 $\mu g.day^{-1}$ for women, and 70 $\mu g.day^{-1}$ for men. The tolerable upper intake level for humans is 400 $\mu g.day^{-1}$ (Navarro-Alarcon & Cabrera-Vique, 2008). In terms of toxicity, the LD_{50} for selenite is 2.5 mg.kg body $weight^{-1}$ (rat) (Lenz & Lens, 2009). No information in terms of the LD_{50} for humans could be found.

The upward trend in energy production from coal, along with the burning of fossil fuels contribute to this increase in selenium release. Since the amount of selenium allowed in water bodies is regulated, industrial waste water is in need of effective selenium removal before it can be discharged into local water bodies. The allowed levels of selenium in water bodies is given in chapter 2, section 2.8 for America, South Africa as well as the WHO standard.

Different treatment technologies for the removal of selenium from water are available, including physical, chemical and biological methods. One biological method is biological precipitation which starts with the bacterial reduction of the oxyanions. This method uses the capability of certain bacterial strains to reduce selenate and selenite to elemental selenium. The elemental selenium can then be removed, since the nanoparticles that are formed are insoluble in water. One such bacterial strain is *Pseudomonas stutzeri* NT-I.

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1.3. Purpose and focus of study

The purpose of this study is to further the understanding of *Pseudomonas stutzeri* NT-I. In turn, this will enable the determination of its suitability for the reduction of the selenium oxyanions under the conditions found in industry. The focus of this study is to determine the optimum conditions for growth of this bacteria in terms of pH, temperature, salinity and initial selenate concentration. Secondly, the optimum conditions for reduction of selenate to elemental selenium will be investigated for different pH levels, temperatures and salinities. Thirdly and finally, the kinetics of the reduction reactions will be investigated.

1.4. Scope

All experiments are batch experiments and are performed in aerobic conditions in a temperature controlled rotary shaker. Sodium selenate (Na_2SO_4) is used as the source of selenium, and glucose is the carbon source. Salinity is varied by changing the amount of sodium chloride (NaCl) added to the reaction mixture. The pH of the mineral salt medium is adjusted by varying the ratio of dipotassium hydrogen phosphate to potassium dihydrogen phosphate (KH_2PO_4:K_2HPO_4).

1.5. Structure of document

This document is divided into chapters. Chapter 1 gives the reader an introduction to the study. Chapter 2 reviews the available literature relevant to this study. Chapter 3 covers the materials used and methods applied in this study. Chapter 4 discusses the results from the growth and reduction experiments, as well as the implications of the findings. Chapter 5 gives a discourse into the kinetics governing the reduction reactions. Chapter 6 gives the conclusions and recommendations as proposed by

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the author. Thereafter the references are listed in the bibliography. The appendix contains the program information for one of the simulations that will enable the reader to repeat the work.

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CHAPTER 2: LITERATURE REVIEW

2.1. Introduction

Selenium was discovered in 1818 by Jons Jakob Berzelius (Lenz & Lens, 2009). It is a metalloid with properties intermediate to metals and non-metals and belongs to group VIA (Chalcogens) on the periodic table. In its pure form it is allotropic, which means it exists in more than one form (Frankenberger Jnr. & Benson, 1994 : 94).

Selenium has multiple oxidation states. These range from the most highly reduced state, namely selenide (Se^{2-}, H_2Se), followed by elemental selenium (Se^0), then the oxidised states of selenite (Se^{4+}, SeO_3^{2-}), and finally selenate (Se^{6+}, SeO_4^{2-}).

2.2. Other selenium compounds

Additional selenium compounds common in the environment are dimethylselenide (DMSe); dimethyldiselenide (DMDSe); dimethylselenone; trimethylselenonium; selenous acid; selenium dioxide, and selenic acid, to name a few (Frankenberger Jnr. & Benson, 1994 : 63).

The most reduced form of selenium, namely selenide, is found as part of molecules such as inorganic metalselenides, organic compounds, and the highly toxic and volatile H_2Se, which is analogous to H_2S (Lenz & Lens, 2009). Selenide is present under strongly reducing conditions (Fernandez-Martinez & Charlet, 2009).

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2.3. Impact of selenium pollution on the environment

As mentioned before, selenate and selenite are toxic to different aquatic groups. An example of this was during the mid-1970s, when the oxyanions from ash deposits of a coal-fired power plant leached into Lake Belews in North Carolina. In that water body,19 out of 20 fish species were eradicated as a consequence (Lemly, 2002).

Another incident worth mentioning occurred in the 1980s, when agricultural drainage water, polluted with selenium, had a negative impact on the migratory bird population in the Kesterton Reservoir, California (Staicu, 2015).

More examples of environmental disasters due to overexposure to selenium are discussed in 'Selenium poisoning of fish and wildlife in nature: lessons from twelve real world examples' (Skorupa, 1998 : 56), as well as in Appendix A of the book, 'Ecological Assessment of Selenium in the Aquatic Environment' (Young et al., 2010).

2.4. Concentrations of selenium in different environments

There are many natural pathways in which selenium is released into the environment and redistributed. These processes range from volcanic activity, wildfires, volatilisation from soils, plants and water bodies, and weathering of rocks (Sandy & DiSante, 2010).

Selenium occurs naturally in soil, but at low concentrations ranging between 0.1 and 2 $\mu g.g^{-1}$ soil, whereas in seleniferous soil the concentration can go up to 320 to 324 $\mu g.g^{-1}$ soil (Girling, 1984).

Of the selenium released into the environment, 37.5 to 40.6% can be ascribed to anthropogenic activities. It is released from the earth's crust by the mining of coal, by oil production, through the use of agricultural products, as well as during the melting of non-ferrous metals (Lenz & Lens, 2009). In addition, landfill ash disposal

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generating toxic leachate poses a risk of groundwater contamination (Lemly, 2004). According to different studies, acid mine drainage waters contain selenium at concentrations ranging between $2x10^{-4}$ and $6.2x10^{-3}$ mM (reported as total selenium) (Lenz et al., 2008). Waste water from oil refineries in the San Francisco Bay (USA) contains relatively low concentrations of selenium of about 50-300 $\mu g.L^{-1}$ (Lawson & Macy, 1995). The waste water from a selenium refinery plant in Japan contained an average of 30 $mg.L^{-1}$ selenium, with the majority of the selenium present as selenite (Satoshi et al., 2012).

2.5. Selenium activity in water

Selenium is found in all the above states and in different phases (gas, dissolved in water, minerals) in the environment. In water, the predominant selenium species are selenate and selenite. Selenate and selenite are both highly bio-available and readily bio-accumulate. In natural aquatic settings, selenate and selenite only interact to a small extent with cations such as Ca^{2+} and Mg^{2+}. This limits the removal of these oxyanions by precipitation (Staicu, 2015). They also become more mobile and soluble than other metals as the pH of the water increased (Chapman et al., 2010 : 92).

As mentioned above, selenium bio-accumulates up the food chain. Even low levels of selenium in water can have a devastating effect on apex predators in the area, which have shown selenium levels up to 2 000 times higher than the area's water concentration (Wu, 2004).

Elemental selenium is not soluble in water and thus has a much lower bio-availability than the oxyanions. Elemental selenium's toxicity is also much lower than that of the oxyanions (Lenz & Lens, 2009). Even though elemental selenium is less of a threat to water users, it is still important that the elemental selenium be removed from waste water before it is discharged into water bodies This is due to elemental

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selenium being easily reoxidised to selenate and selenite in water bodies with a high redox potential, such as surface waters (Zhang et al., 2004).

2.6. Selenium applications

A popular end use of selenium as a semiconductor is in thin-film solar cells. Other than this, industrial uses of selenium are the following: a decolouriser in glass manufacturing, a pigment to colour glass and ceramics, and an additive in electrolytic manganese production to increase its efficiency, selenium can also improve machining properties of lead-free alloys and steels. In addition, it is added to fertilizer as it has value as a micro-nutrient (Kavlak & Graedel, 2013).

An emerging use for selenium is in the field of nanotechnology, especially nanowires (Oremland et al., 2004). The capability to form elemental selenium nanoparticles as the end product of the reduction of the selenium oxyanions by different bacteria has peeked the interest of many researchers. This suggests that waste water not only uses selenium reducing bacteria to remove the toxic oxyanions, but also to produce such nanoparticles (Nancharaiah & Lens, 2015).

2.7. Therapeutic dosage and toxicity

Selenium in trace amounts has been identified as an essential element in fish, animals, birds and humans, but not in plants. As mentioned before, there is a narrow gap between therapeutic and toxic dosages. For humans, the RDA is 55 $\mu g.day^{-1}$ for women, and 70 $\mu g.day^{-1}$ for men. The tolerable upper intake level for humans is 400 $\mu g.day^{-1}$ (Navarro-Alarcon & Cabrera-Vique, 2008). The LD_{50} for selenite is 2.5 mg.kg body $weight^{-1}$ (rat). This value must be used with caution, since the toxicity of the different species of selenium differs with three orders of magnitude (Lenz & Lens, 2009).

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Selenosis is the condition the body is in when the concentration of selenium in the body is above the toxic level. The symptoms of selenosis in humans are hair and nail loss, limb numbness, loss of pain sensation in the skin, giddiness and also dizziness (Chen et al., 2014). Other selenosis-related conditions include pulmonary oedema, cirrhosis of the liver (Nelms, 2016), cardiovascular diease, lowered mental alertness, skin discolouration, a garlic odour on the breath (Nancharaiah & Lens, 2015) and eventually, death. This list of adverse effects of selenosis is by no means exhaustive.

On the other side of the spectrum, a deficiency in selenium in humans may cause cardiomyopathy, muscular weakness, pain and a list of other diseases (Dungan & Frankenberger, 1999). Not only is selenium necessary for normal functioning of the body, it also has curative properties with regard to illnesses such as Acquired Immune Deficiency Syndrome (AIDS), Alzheimer's disease, asthma, arthritis, pancreatitis and viral infections (George, 2003).

For animals, a concentration as high as 20 $\mu g.L^{-1}$ is unlikely to have any negative health effects. In the case of animals, birds and fish, a dose higher than 5 to 25 mg Se.kg body $weight^{-1}$ is toxic. Selenosis, in the case of animals, can cause alkali disease and blind staggers.

2.8. Current Legislation

In the US, the National Primary Drinking Water Standard is 50 ppb of total selenium, while the National Fresh Water Quality Standard is 5 ppb of total selenium (USEPA, 1999).

For countries that don't have a legislative framework for drinking water pollutants, the World Health Organization (WHO) provides guidelines for the measure of selenium in water bodies. They propose a value of 40 ppb (WHO, 2011).

Regarding South Africa, there are two documents specifying the maximum selenium concentrations in water bodies. These are the 'South African water quality guidelines

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volume 7: Aquatic ecosystems' (DWAF, 1996a) and 'South African water quality guidelines volume 1: Domestic water use' (DWAF, 1996b). The Target Water Quality Range (TWQR) is a management objective and represents the ideal concentration range. The Chronic Effect Value (CEV) is reserved for special cases where the TWQR is exceeded, while still protecting the environment from acute toxicity effects. Examples of where the CEV is used are:

- In the mixing zone of a river where an effluent stream discharges into the river.
- As an interim value before the remediation of the water body commences or where remediation takes more than one step.

The Acute Effect Value (AEV) is reserved for cases that require immediate attention due to the aquatic environment being under threat. However, this value cannot be used as a requirement and should be avoided. The values given in Table 2-1 and

Table 2-2 have been taken from the above documents. The concentrations specified represent the total selenium in the water.

Table 2-1: Maximum selenium concentrations allowed in aquatic ecosystems.

TWQR and Criteria	Selenium concentration ($\mu g.L^{-1}$)
Target Water Quality Range (TWQR)	*2*
Chronic Effect Value (CEV)	5
Acute Effect Value (AEV)	30

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Table 2-2: Selenium concentration range in water for domestic use*

Selenium Range ($\mu g.L^{-1}$)	Effects
Target Water Quality Range (TWQR) 0–20	No adverse health effects.
20–50	No adverse health effects with short- to medium-term use. With lifelong use, potential danger of selenium accumulation in individuals with a selenium-rich diet, for example a diet very rich in sea foods.
50–100	No danger with short-term use, but a danger of liver toxicity with long-term use, particularly in children.
>100	Danger of selenium toxicity, with liver damage.

*It is recommended that the concentration of selenium in potable water never exceeds 50 $\mu g.L^{-1}$ due to the risk of liver damage, particularly in children.

2.9. Treatment technologies

There are many challenges in the treatment of selenium laden waste water, some of them are (Sandy & DiSante, 2010):

- The selenium being present in different forms.
- Competing cations and anions which decrease removal efficiency.
- The low concentration and high flow rate of the waste water.
- The generation of by-products that need further treatment.
- Re-release from residuals can occur.

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- Removal is limited by the feasible design flow ranges.

Table ES-1 in the final report to the North American Council, titled 'Review of Available Technologies for the Removal of Selenium from Water' (Sandy & DiSante, 2010), gives an exhaustive list of available technologies, together with more information on each technology. Only the technologies discussed in the above-mentioned document that are in full scale operation are given in alphabetical order in Table 2-3.

Table 2-3: List of treatment technologies for selenium removal

Technology	Development stage
ABMet®	Full scale
Constructed wetlands	Full scale
Enhanced evaporation system	Full scale
Evaporation ponds	Full scale
Ferrihydrite adsorption or iron co-precipitation	Full scale
Ferrous hydroxide	Full scale
Ion exchange	Full scale
Mechanical evaporation/crystallisation	Full scale
Passive biochemical reactor	Full scale
Permeable reactive barriers	Full scale
Reverse osmosis	Full scale
Salinity gradient solar pond	Full scale

Below follows a discussion of the most popular treatment technologies.

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2.9.1. Abiotic treatment technologies

Ion Exchange involves exchanging an undesirable dissolved component for a more desirable solute, which is electrostatically attached to an ion exchange material. It is, in most cases, more economical to use reverse osmosis (discussed below) when treating agricultural drainage water and, thereafter, ion exchange as a polishing treatment rather than the primary method.

A resin targeted at removing selenate can be used, but since sulphate and selenate have similar properties, it is difficult to preferentially remove the trace amounts of selenate instead of the higher amounts of sulphate. Appropriate pre-treatment (adjusting pH and pE) may enhance the removal of specific compounds. This process does however produce a salty waste stream (Frankenberger, Jnr. et al., 2004).

There are two membrane processes of interest, namely: **reverse osmosis** (hyperfiltration) and **electrodialysis**. Reverse osmosis removes monovalent and divalent ions from water. The water moves through the membrane due to a pressure difference between the feed water and permeate side. The driving force in electrodialysis is electric potential; it is the charged ions that are transferred through the membrane.

In industry, reverse osmosis tends to be less expensive than electrodialysis. The advantage electrodialysis has above reverse osmosis is that, while reverse osmosis removes a broader range of constituents, electrodialysis only removes charged particles, such as selenium oxyanions (Frankenberger, Jnr. et al., 2004).

Nanofiltration does not require such a high differential pressure over the membrane. Although nanofiltration has a lower salt rejection efficiency, it was designed to have a higher efficiency in removing divalent ions, such as selenate and selenite (Sandy & DiSante, 2010).

Chemical reduction can be applied to reduce selenium oxyanions using different electron donors, two of which are ferrous hydroxides and zero-valent iron. This

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process can lower selenium concentrations to a very low level, and should be considered as a polishing step after primary treatment. With ferrous hydroxide and selenate, the reduction reaction occurs under alkaline conditions and produces ferrous oxides and elemental selenium (Frankenberger, Jnr. et al., 2004).

Selenite and, to a lesser extent, selenate can be removed by different **coagulants**, such as aluminate, ferrous and ferric, to name a few. Coagulation occurs when the surface charge of the ion is neutralised, allowing for the agglomeration of particles to form flocs, which can be removed from water (Moore and Mahmoudkhani, 2011).

2.9.2. Biotic treatment technologies

Biotic processes for the removal of selenium from water include the algal bacterial process; algal bioremediation; volatilisation; biological precipitation and wetlands (Dungan & Frankenberger, 1999).

The Applied Algae Research Group developed a treatment process called **"algal-bacterial selenium removal" (ABSR),** which consists of a series of ponds (Applied Algal Research Group, 1999).

The idea behind ABSR is to grow micro-algae using the nitrate present in the water inside the high-rate ponds**.** The algal biomass then acts as a carbon source for bacteria native to the water to reduce the selenium oxyanions and remaining nitrate to elemental selenium and nitrogen gas in the reduction ponds.

The concentrated selenium will be reduced in the anaerobic zone at the bottom of the reduction ponds which, at 5 to 6 m depth, cannot support invertebrate life forms. Thus, these ponds do not attract waterfowl (Amweg et al., 2003).

Algal bioremediation: A few micro-algae species that have been isolated from evaporation ponds have the ability to transform selenium oxyanions into volatile compounds like alkylselenides, selenomethionine and selenonium ions. While a significant amount of selenium bio-accumulates in the algal biomass (especially in

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proteins), laboratory studies found that a major loss of selenium can be accounted for by volatilisation.

What complicates the matter is that the selenium allocation is different for the various algal species. This poses an ecotoxic threat, since selenium can then transfer in various ways up through the food chain, making it difficult to control (Frankenberger, Jnr. et al., 2004).

Volatilisation is a natural process that forms part of the selenium cycle in the biosphere, and is one of the primary mechanisms through which selenium ions are dissipated out of sediments and water. Methylating micro-organisms, including bacteria, algae and fungi have been isolated from evaporation ponds and sediment. Adding a protein source and pectin stimulates volatilisation up to 300 times the natural rate (Dungan & Frankenberger, 1999).

The main product of volatilisation is DMSe, which has been proven to be non-toxic. More developed plants, including rabbitfoot grass and brass buttons, that possess the capacity to volatilise selenium have been identified (Hansen et al., 1998). The uptake of selenium occurs mainly at the roots of these plants. Brassica species, especially Indian mustard, are also effective at volatilising selenium (Frankenberger, Jnr. et al., 2004).

Biological precipitation is the process where bacteria is used to reduce the selenium oxyanions to elemental selenium (microbial reduction, discussed in more detail under section 2.10), which then precipitates out as agglomerated nanoparticles. A wide range of bacteria that reduce selenium oxyanions have been isolated.

Different reactors were used for this, such as above-ground tanks containing sludge beds, fixed film reactors and fluidised beds (Golder associates, 2009; Envirogen Technologies, 2011; Soda et al., 2011). Different carbon sources were evaluated, such as molasses, ethanol, methanol, acetate, acetylene, citrus peel and manure, to name a few (Karlson & Frankenberger, 1990; Zhang et al., 2008; Soda et al., 2011).

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This process has advantages as well as disadvantages. The advantages are high reduction efficiency, low production of residual solids due to slow growth rate, and eliminating exposure to wildlife and the environment. The disadvantages are the high cost associated with the addition of a carbon source, and the fact that the lowest residual soluble concentration achievable at this stage is 30 ppb, which means a polishing step will most probably be required (Frankenberger, Jnr. et al., 2004).

Constructed wetlands form complex ecosystems where both the physical and biological parameters interact to provide an atypical filter capable of contaminant removal from water. The removal mechanism deployed in constructed wetlands is selenium oxyanion reduction to insoluble elemental selenium. This then deposits in the sediments, bio-accumulates in the plant tissue (phytoremediation) and volatilises into the atmosphere via plants and micro-organisms (Ye et al., 2003). The cost of constructed wetlands is orders of magnitude lower than other treatment processes.

An alternative to the typical wetland design is the constructed subsurface flow wetland (SSF) (Azaizeh et al., 2003). This option is especially effective where the water flow rate is low and the selenium concentration is high. The advantage of this variation of the standard constructed wetlands' design is that the potential for selenium exposure to fish and birds is much lower than in the case of standard constructed wetlands (Frankenberger, Jnr. et al., 2004).

Chemical/physical treatment processes require high investment and operating costs, whereas biological processes have a high footprint and a performance that can be erratic (Staicu, 2015). In most cases it would be best to use a combination of different treatment technologies to achieve the set discharge limits.

2.10. Microbial reduction of selenium

As already mentioned, microbial reduction is the core reaction that leads to the formation of selenium nanoparticles that can be removed through precipitation. Selenate and selenite can be reduced in three main ways: detoxification,

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dissimilatory and assimilatory reduction. Dissimilatory reduction occurs when micro-organisms reduce selenate/selenite to elemental selenium with selenate/selenite acting as the terminal electron acceptor during anaerobic respiration (Nancharaiah & Lens, 2015).

Assimilatory reduction occurs when selenate and selenite enter the cells of a variety of microorganisms via permeasis. Once in the cell, selenate and selenite are reduced to selenide, which is then incorporated into cellular proteins, such as selenomethionine and selenocysteine (Dungan & Frankenberger, 1999). Bacteria that reduce selenium are discussed later on.

Another process that prevents organisms from being poisoned by selenium is methylation. When followed by reduction and volatilisation, it becomes a critical step in the transport of selenium out of terrestrial and aquatic environments contaminated with selenium (Chasteen & Bentley, 2003).

Methylation occurs in water, sediment and soil and is mostly a biotic process. The organisms responsible for the methylation of selenium are bacteria, algae and fungi. Examples of the compounds that are thereby formed are DMSe, DMDSe and DMSeS (Dungan & Frankenberger Jnr, 2001).

2.11. Selenium reducing bacteria

After a review of published articles, 68 bacterial strains were identified as selenium reducers (Wessels, 2015). It is the author's opinion that this list is not exhaustive. Figure 2-1 below lists 45 of those that have been identified thus far, arranged in terms of mode of respiration.

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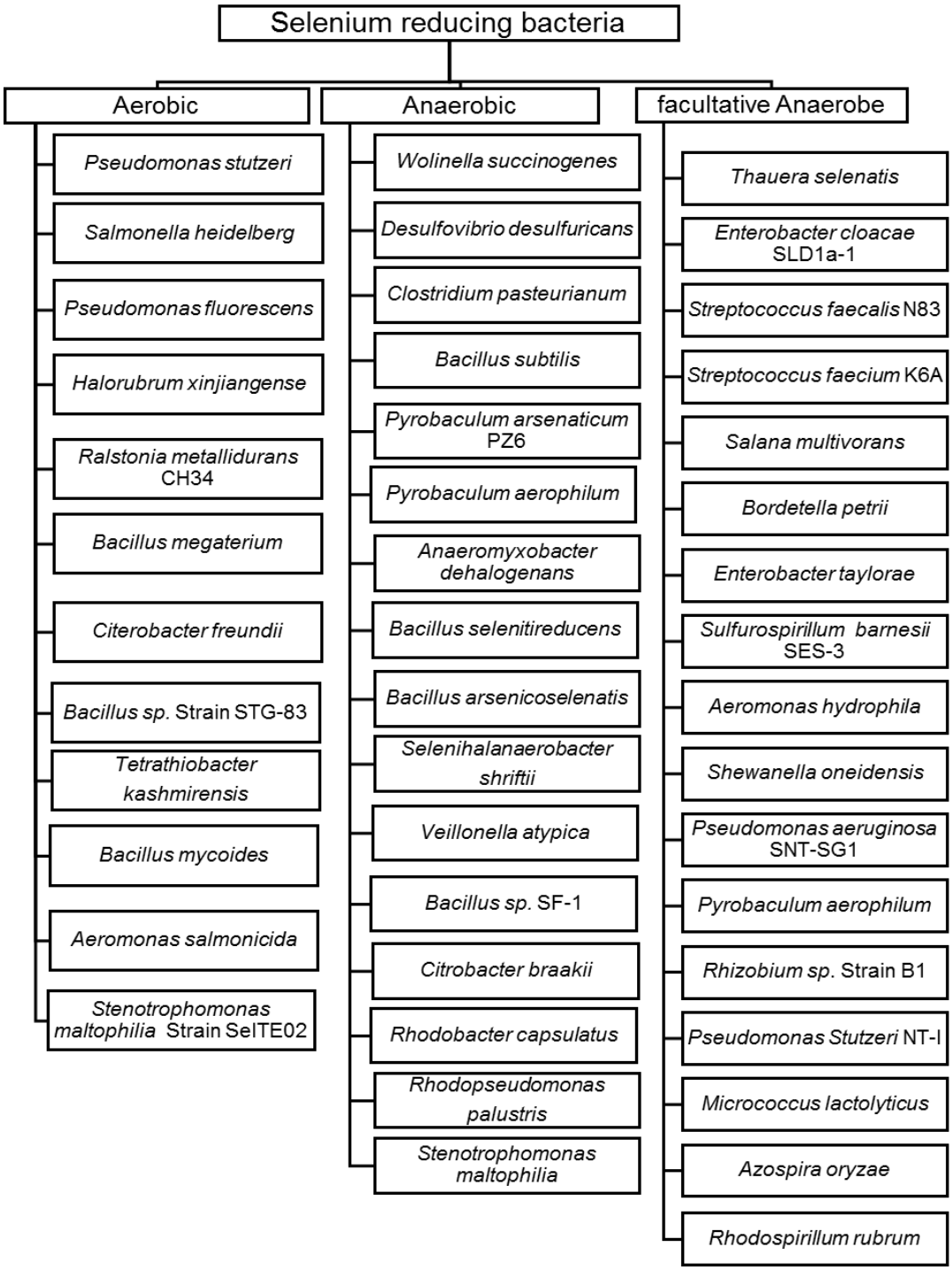

Figure 2-1: List of selenium reducing bacteria according to mode of respiration (Wessels, 2015).

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Most of the identified selenate-reducing bacteria function under anaerobic conditions and, except for a few, can only reduce low concentrations of selenate. Oxygen also limits the capability of these bacteria to reduce selenate, which poses a problem with industrial waste water treatment, due to the presence of oxygen.

Several bacterial strains capable of reducing selenate under aerobic conditions have been isolated by different researchers. A few of them are *Pseudomonas stutzeri* (Lortie et al., 1992); *Enterobacter cloacae* SLD1a-1 (Losi & Frankenberger, 1997); *Stenotrophomonas maltophilia* (Dungan et al., 2003), and *Bacillus sp*. STG-83 (Saudi et al., 2009). Two advantages of aerobic selenate reducing bacteria are firstly, that selenate reduction is not limited by the presence of oxygen and secondly, the aerobic conditions allow for a higher growth rate and thus an increase in cell production.

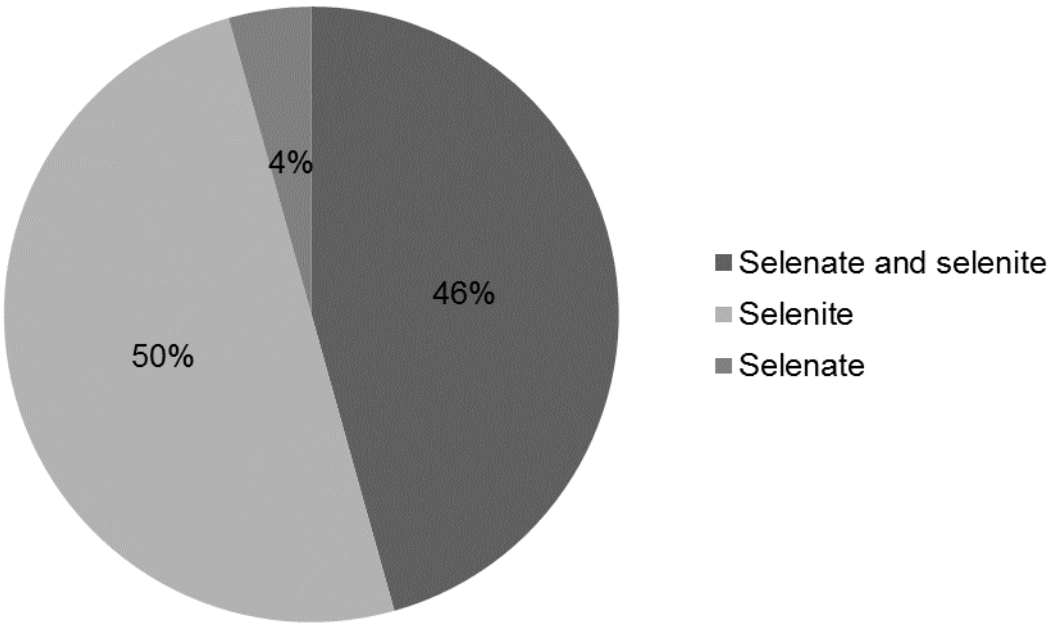

Figure 2-2: List of bacteria given above divided according to oxyanion reduced (Wessels, 2015).

It is evident that in the world of selenium reducing bacteria, there are many types of bacteria using different mechanisms to reduce selenium. The predominant reduction

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mechanism seems to be anaerobic growth/respiration. As can be deduced from Figure 2-2 above, all but two of the bacteria reduce selenite, whereas only about half of those that reduce selenite also reduce selenate. The bacteria that only reduce selenite can only be used when it is co-cultured with a selenate or selenate plus selenite reducing bacteria. This may prove to be a worthwhile option. These two bacteria, each reducing one of the oxyanions at a high rate, but incapable of reducing the other (or reducing it very slowly), can make a very effective team that reduces selenate to elemental selenium at a high rate. To accomplish this though, the operating conditions of the two bacteria need to be almost the same. An example of this would be co-culturing *Bacillus sp*. SF-1 with *Streptococcus faecalis* N83.

The reduction rate varies greatly between the different bacteria and disqualifies some of them from being considered for bioremediation. Table 2-4 below gives an introduction to a few promising selenium reducing micro-organisms.

Table 2-4: Short list of selenium reducing micro-organisms (Dungan & Frankenberger, 1999).

Organism	Description of reaction
Thauera selenatis	Uses selenate and nitrate to grow anaerobically. Grows anaerobically using SeO_4^{2-}. Reduction of selenate occurs via selenate reductase. Nitrate needs to be present for complete reduction to elemental selenium.
Enterobacter cloacae SLD1a-1	Respires selenate and nitrate and reduces selenate to elemental selenium only in the presence of nitrate.
Pseudomonas stutzeri	Reduces selenate and selenite to elemental selenium under aerobic conditions.

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Organism	Description of reaction
Wolinella succinogenes	Adapted cultures reduce selenate and selenite to elemental selenium in anaerobic conditions.
Salmonella heidelberg	Reduces selenite to elemental selenium aerobically.
Desulfovibrio desulfuricans	Reduces selenate and selenite anaerobically to elemental selenium. Both oxyanions are unable to support growth.
Streptococcus faecalis N83 *Streptococcus faecium* K6A	Reduces selenite to elemental selenium under anaerobic conditions.
Clostridium pasteurianum	Reduces selenite by hydrogenase.
Bacillus subtilis *Pseudomonas fluorescens*	Reduces selenite to elemental selenium by a nitrite and sulphite-independent enzyme system.

2.12. *Pseudomonas stutzeri* NT-I

More than one *Pseudomonas* species that can metabolise selenate and selenite have been found. Examples are *P. seleniipraecipitatus* (Hunter & Manter, 2011); *P. moraviensis stanleyae* (Staicu et al., 2015); *P. aeruginosa* (SNT-SG1) (Gupta et al., 2010); *P. stutzeri* (Lortie et al., 1992), and *P. Stutzeri* NT-I (Kuroda et al., 2011).

The genus *Pseudomonas* includes a wide variety of bacterial species. *Pseudomonas* representatives are Gram negative γ Proteobacteria. They are rod-shaped, aerobic, catalase positive and oxidase positive (Krieg, 1984 : 27).

P. stutzeri is better known for denitrification under aerobic conditions, however an isolate of *P. stutzeri* has been found to reduce selenate/selenite aerobically (Lortie et

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al., 1992). This isolate has the same taxonomical features as *P. Stutzeri* NT-I, but these two strains differ in a few aspects. For example, the isolate could not reduce selenate at concentrations above 48.1 mM, nor could it reduce selenate anaerobically (Kuroda et al., 2011).

This study focuses specifically on *Pseudomonas stutzeri* NT-I. The characteristics of the reduction process for this strain are discussed under the Kinetic Model chapter (chapter 5), together with the findings of this study.

2.13. Reduction reaction mechanisms and kinetic modelling

The reduction of selenium oxyanions facilitated by bacteria are based on different mechanisms. Sulphate reducing bacteria have been shown to reduce selenium in anaerobic conditions without it involving growth. There are also phototrophic bacteria that are able to reduce selenate when they are in the stationary phase of growth (Nancharaiah & Lens, 2015). Another two of the mechanisms described in literature are anaerobic respiration (Macy et al., 1989; Lawson & Macy, 1995; Cantafio et al., 1996; Fujita et al., 2002) and detoxification (Lortie et al., 1992; Garbisu et al., 1996; Sarret et al., 2005). Although the detoxification mechanism has been found to be a plausible explanation for the reduction mechanism, it is not as well understood as anaerobic respiration yet.

First order kinetics is often used to model the reduction of the selenium oxyanions to elemental selenium (Yiqiang Zhang et al., 2004, 2008; Yiqiang Zhang & Frankenberger Jnr., 2006; Kuroda et al., 2011). The following equations represent first order kinetics for the batch reduction of selenate to elemental selenium (Yiqiang Zhang et al., 2004):

$$\frac{d[Se^{6+}]}{dt} = -k_{64}[Se^{6+}] \qquad 2\text{-}1$$

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$$\frac{d[Se^{0}]}{dt} = +k_{40}[Se^{4+}] \qquad 2\text{-}2$$

$[Se^{6+}]$ Selenate concentration, mM

$[Se^{4+}]$ Selenite concentration, mM

$[Se^{0}]$ Elemental selenium concentration, mM

k_{64} Reaction rate constant for the reduction of selenate to selenite, (min^{-1})

k_{40} Reaction rate constant for the reduction of selenite to elemental selenium, (min^{-1})

The Monod kinetic model, originally developed to describe bacterial growth kinetics, is applied to the reduction reaction facilitated by *Enterobacter cloacae* SLD1a-1 (Frankenberger Jr. & Dungan, 1998) as well as other bacteria (Lortie et al., 1992; Oremland et al., 1999; Tam et al., 2010). The Monod equation is adapted for selenium oxyanion reduction by adding another variable, namely the biomass. Below follows the resultant equations (Ma et al., 2007).

$$\frac{-dSe^{6+}}{Xdt} = \frac{k_{max64}[Se^{6+}]}{K_{s64} + [Se^{6+}]} \qquad 2\text{-}3$$

$$\frac{-dSe^{4+}}{Xdt} = \frac{k_{max40}[Se^{4+}]}{K_{s40} + [Se^{4+}]} - \frac{k_{max64}[Se^{6+}]}{K_{s64} + [Se^{6+}]} \qquad 2\text{-}4$$

$$\frac{dSe^{0}}{Xdt} = \frac{k_{max40}[Se^{4+}]}{K_{s40} + [Se^{4+}]} \qquad 2\text{-}5$$

k_{max64} Maximum reaction rate for the reduction of selenate to selenite ($mol.(g.min)^{-1}$)

k_{max40} Maximum reaction rate for the reduction of selenite to elemental selenium ($mol.(g.min)^{-1}$)

K_{s64} Substrate saturation coefficient for the reduction of selenate to selenite (mM)

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K_{s40}	Substrate saturation coefficient for the reduction of selenite to elemental selenium (mM)
X	Biomass ($g.L^{-1}$)

The change in biomass concentration (X) can be assumed negligible in cases where the starting biomass concentration is already very high due to the fact that some bacteria will only grow up to a point where the critical population level is reached. Before considering this as the reason for the bacteria reaching stationary phase other possible explanations must be eliminated. The other possible reasons for reaching stationary phase are (Cooke, 1970):

- The system is nutrient limited.
- The carbon source has been depleted.
- The rate at which cells form and die are relatively the same.
- The accumulation of toxic waste products (mainly in the case of anaerobic systems).

Another way to look at it is to consider the cell protein yield per mole of selenate reduced. According to (Liu et al., 2002), the effect of the growth of bacteria on substrate reduction can be neglected if $X_0 >> YS_0$. Where Y presents cell protein yield per mol of substrate reduced.

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CHAPTER 3: MATERIALS AND METHODS

3.1. Chemical Reagents and Standards

All chemicals used were of analytical grade and were obtained from Merck, Johannesburg, except for $CaCl_2$, Na-EDTA, DAN, $NaSeO_4$ and $NaSeO_3$ which were obtained from Sigma Aldrich, Johannesburg.

3.1.1. Physiological saline

The physiological saline (0.85% w/v NaCl) used for washing the cell suspensions after centrifugation was prepared by dissolving 8.5 g of sodium chloride salt in 1 L distilled water and autoclaved at 121 °C at 0.4 MPa for 15 minutes (Hirayama HV-50, Labotec, Johannesburg).

3.1.2. Selenate stock solution

Selenate stock solution with a concentration of 4 mM was made up by adding 755 mg sodium selenate to 1 L of distilled water and autoclaved at 121 °C at 0.4 MPa for 15 minutes.

3.1.3. Selenium stock solution

Selenium stock solution (1000 ppm, 1 mg = 1 mL = 12.66 mmol) in 0.55 N HNO_3 was purchased from Labcon, Johannesburg. The selenium was in the form of SeO_2. This solution was used for the calibration of the ICP-OES and Spectrophotometer.

3.1.4. Glucose stock solution

A glucose stock solution with a concentration of 100 $g.L^{-1}$ was made up using distilled water and autoclaved at 121 °C at 0.4 MPa for 15 minutes. The required

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amount of glucose stock solution to achieve the desired concentration in the medium was pipetted into the MSM just before the commencement of the various experiments.

3.1.5. 2, 3 Diaminonaphtalene reagent solution (DAN)

DAN was prepared by dissolving 0.2 g DAN in 200 mL 0.1 N HCl and shaking it for 5 minutes. Thereafter it was extracted three times with 25 mL portions of cyclohexane and the aqueous phase was retained. It was then filtered into an opaque container (Whatman 42, pore size 2.5 µm) and used immediately.

3.1.6. Hydroxylamine-EDTA reagent solution (HA-EDTA)

A total of 4.5 g of Na_2EDTA was dissolved in 450 mL distilled water. 12.5 g hydroxylamine hydrochloride ($NH_2OH.HCl$) was then added, after which the volume was adjusted to 500 mL with distilled water.

3.2. Growth Media

3.2.1. Mineral salt medium

One litre of Mineral Salt Medium (MSM) was prepared according to the following recipe: 30 mM NH_4Cl, 10 mM K_2HPO_4, 20 mM KH_2PO_4, 0.8 mM Na_2SO_4, and 0.2 mM $MgSO_4$. For experiments where the MSM had to be at pH 6, 8 and 9, the ratio of K_2HPO_4 to KH_2PO_4 was adjusted. 1 mL of a solution containing 50 µM $CaCl_2$, 25 µM $FeSO_4$, 0.1 µM $ZnCl_2$, 0.2 µM $CuCl_2$, 0.1 µM NaBr, 0.05 µM Na_2MoO_2, 0.1 µM $MnCl_2$, 0.1 µM KI, 0.2 µM H_3BO_3, 0.1 µM $CoCl_2$, and 0.1 µM $NiCl_2$ was added to every 1 L of MSM. The MSM was then autoclaved at 121 °C at 0.4 MPa for 15 minutes to ensure sterility.

The MSM, saline and stock solutions were all autoclaved shortly before an experiment was started to further minimise the possibility of contamination.

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3.2.2. Cultivation media

Tryptone Soy Broth (TSB) (Merck, Johannesburg, South Africa) was used for the cultivation of the bacteria used in the washed cell suspensions. The TSB was prepared by dissolving 30 g of powder in 1 L distilled water. The TSB was autoclaved at 121 °C at 0.4 MPa for 15 minutes before use. Tryptone Soy Agar (TSA) (Merck, Johannesburg, South Africa) was used for colony development and was prepared by dissolving 38 g in 1 L distilled water. After it was autoclaved, it was cooled down to +/- 40 °C and then poured into petri dishes and left to solidify, forming TSA plates that were used to cultivate the bacteria from the frozen vials that were prepared according to the procedure outlined below.

3.3. Bacterial Culture

3.3.1. Source

The strain used in this study, *Pseudomonas stutzeri* NT-I, was furnished from the NITE Patent Microorganisms Depository (NMPD) in Chiba Ken, Japan. The strain ID is NITE BP-685. The bacteria was originally isolated from the drainage water of a selenium refinery plant in Hyogo, Japan by Masashi Kuroda and his team (Kuroda et al., 2011).

3.3.2. Cultivation and storage

The received ampoule containing the bacteria was opened by making a cut in the ampoule at the centre of the cotton plug, after disinfecting the cut with 70 % ethanol. Sterile gauze was then wrapped around the ampoule and it was broken at the scored area. Immediately after opening the ampoule, 0.3 mL physiological saline was added and it was mixed until the bacterial deposit dissolved in the saline. The liquid culture was then transferred to 100 mL TSB in an Erlenmeyer flask and capped with cotton wool and foil. It was cultivated for 24 hours at 28 ºC on a rotary shaker at 120 rpm (FSIM-SPO8, Labcon, Johannesburg).

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After the bacteria had been cultivated for 24 hours, the long-term storage process commenced. It was started off by adding 0.2 mL glycerol solution (50% glycerol in distilled water) to twenty 2 mL vials and sterilizing them at 121 ºC at 0.4 MPa for 15 minutes. The next step was to add 0.8 mL of the TSB containing the bacteria to the vials. The vials were closed and placed in the -70 ºC storage chamber.

To revive the bacteria in the vial, splinters of the solid ice was scraped off with a wire loop and streaked onto an agar plate. The stock was not yet completely thawed. The cultures were then left to grow for 24 hours at 37 ºC on the TSA plates whereafter it was stored at 0 ºC.

3.4. Washed Cell Suspension

3.4.1. Reduction rate batch experiments (refer to 3.5.3)

The bacteria was cultivated by taking a loopful of bacterial cells from a TSA plate and inoculating it into 20 mL of TSB medium in a 100 mL serum bottle capped with foil and cotton wool. The serum bottle was then placed in the incubator for 24 hours at 37 ºC. Subsequently 10 mL was transferred again to 100 mL of fresh TSB medium in a 250 mL Erlenmeyer flask and cultivated for a further 8 hours. The cells were harvested by centrifugation (6 000 rpm, 15 minutes, room temperature) and then washed with physiological saline, whereafter it was resuspended in 50 mL of glucose-MSM (amended with 5 $g.L^{-1}$ NaCl) with an OD_{600} above 1.5, to ensure that further growth of the bacteria is marginal. The 50 mL of washed cell suspension was then distributed between three 100 mL serum bottles, each containing 15 mL of washed cell suspension in the end.

3.4.2. Kinetic batch experiments (refer to 3.5.4)

The bacteria was cultivated by taking a loopful of bacterial cells from a TSA plate and inoculating it into 20 mL of TSB medium in a 100 mL serum bottle capped with foil and cotton wool. Whereafter it was placed in the incubator for 24 hours at 37 ºC.

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Subsequently 10 mL was transferred again to 100 mL of fresh TSB in a 250 mL Erlenmeyer flask and cultivated for 8 hours. The cells were harvested by centrifugation (6 000 rpm, 15 minutes, room temperature) and then washed with physiological saline, following which it was resuspended in 30 mL of MSM amended with glucose, containing different amounts of Na_2SeO_4 (0.5, 1, 2 and 4 mM) (pH 8, glucose 10 g.L-1) with a high biomass (between 20 to 40 $g.L^{-1}$ depending on experiment).

3.5. Batch Experiments

3.5.1. Relationship between biomass and OD_{600}

Cells were cultivated following the procedure set out in section 3.4.1, the only difference being that a litre of washed cell suspension was made and 4 samples of 200 mL were taken periodically. The OD_{600} of these samples were measured whereafter the sample was centrifuged. The plug, containing the cells, was resuspended in 10 mL saline and dried in an oven at 70 ºC for 5 days. After it was dried, the weight of the cells were measured. The results are shown in section 4.1. The experiment was conducted in triplicate.

3.5.2. Determination of exponential growth phase constant

The bacteria was cultivated by taking a loopful of bacterial cells from a TSA plate and inoculating it into 20 mL of glucose-MSM in a 100 mL serum bottle that was covered with cotton wool and foil and incubated for 24 hours at 37 ºC. Thereafter 5 mL was transferred to 100 mL of fresh glucose-MSM medium containing 1 mM selenate in a 250 mL Erlenmeyer flask. 3 mL samples were withdrawn with a syringe to measure the OD_{600} every 2 hours for at least 24 hours. These experiments were repeated at different temperatures (20, 30, 37 and 45 ºC, 1 mM selenate, pH 7 and salinity of 5 $g.L^{-1}$), different pHs (6, 7, 8, 1 mM selenate, temperature of 37 ºC and salinity of 5 $g.L^{-1}$ NaCl), different salinities (0, 5, 10, 20, 30 $g.L^{-1}$, 1 mM selenate, temperature of 37 ºC and pH 7) and finally different initial concentrations (1, 2, 5, 10 and 50 mM

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selenate, 37 ºC, pH 7 and salinity 5 g.L^{-1}). All experiments were conducted in triplicate. The specific growth rate was measured by the change in absorbance during the log phase of growth according to the following equation:

$$\mu = \frac{lnOD_{600} - lnOD_{600,t_0}}{t - t_0} \qquad 3\text{-}1$$

μ: exponential growth phase constant, h^{-1}

t = time, h

OD_{600}: absorbance of cell suspension at 600 nm

3.5.3. Characterisation of reduction rate with washed cell suspensions (refer to 3.4.1)

An amount of 5 mL of the 4 mM selenate stock solution, spiked with glucose was added to the serum bottle containing 15 mL washed cell suspension prepared to give a final concentration of 1 mM selenate in the adjusted washed cell suspension (final volume of 20 mL). The time at which the selenate stock solution was added was taken as t_0. The extent of selenate reduction to elemental selenium by *Pseudomonas stutzeri* NT-I was quantified at different temperatures (30, 37 and 45 ˚C; pH 7, salinity 5g.L^{-1}), pH's (6, 7, 8 and 9, 37 ˚C, salinity 5 g.L^{-1}) and salinities (0, 5, 10 and 20 g.L^{-1}; pH 7, 37 ˚C). The percentage of elemental selenium recovered after 16 hours was determined by taking the whole reaction volume and centrifuging it to separate the reaction volume into a plug containing the precipitated elemental selenium nanoparticles and cells, and the supernatant containing the unreacted selenium oxyanions. The elemental selenium present in the plug was determined according to the procedure given in section 3.6.4.

For the experiment conducted to determine the selenium mass balance, 10 mL of the supernatant was made up to 100 mL using distilled water, whereafter it was filtered

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(Whatman 42) and placed in a sample container and analysed using ICP-OES (refer to section 3.6.2).

3.5.4. Kinetic profiling of reduction rate with washed cell suspensions (refer to 3.4.2).

Selenate reduction experiments using washed cell suspensions of *Pseudomonas stutzeri* NT-I were conducted at 4 different initial selenite concentrations (0.5, 1, 2 and 4 mM). The procedure followed for the baseline experiment (0.5 mM Selenate, 37 ºC, pH 8) was as follows: The 100 mL flask containing 30 mL of washed cell suspension was capped with cotton wool and foil whereafter it was incubated at 37°C with continuous shaking on a rotary shaker at 120 rpm. 1 mL samples were taken at different time intervals and each one transferred to a 1.5 mL centrifuge tube. The reaction in the sample was stopped by centrifugation (Eppendorf Minispin Microcentrifuge, 6 000 rpm, room temperature, 15 min). The supernatant from the tube was decanted from the plug and diluted to a final volume of 25 mL with distilled water and filtered (Whatman 42). 5 mL was used to analyse for selenite using the colorimetric method described in section 3.6.3. The rest of the diluted supernatant was analysed for total selenium using ICP-OES. All experiments were conducted in triplicate.

3.6. Analytical Methods

3.6.1. OD_{600} analysis by spectrophotometer

The wavelength at which the spectrophotometer (WPA, Light Wave II, Labotech, South Africa) measured the optical density (OD_{600}) of the cell suspension was set at 600 nm. The blank used to zero the spectrophotometer was MSM containing no bacterial cells.

In order to test whether the linear relationship between cell density and optical density holds at higher values of OD_{600}, samples with a high OD_{600} were diluted and

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the OD_{600} was measured again. This lower value was then multiplied by the number of dilutions and compared to the original higher value. The results are shown in section 4.1.

3.6.2. Total dissolved selenium analysis by ICP-OES

The concentration of total dissolved selenium was determined using Inductively-Coupled Plasma-Optical Emission Spectrometry (ICP-OES) (ARCOS FHS12, Spectro Analytical Instruments, Kleve, Germany). The sensitivity of this method is 10 ppb and the lower detection limit is 10 ppb.

3.6.3. Determination of selenite

The measurement of selenite was carried out using the colorimetric method described in *Standard methods for the examination of water and waste* (APHA, 2012) (3500-Se C Colorimetric method, p 3-93). The analysis for selenite started by taking 5 mL of the diluted sample and adding 1 mL of the HA-EDTA solution in a 15 mL centrifuge tube. The pH was adjusted to 1.5±0.3 with 0.1 N HCl using a pH meter and micropipette. The samples were then stored overnight. The following morning, 2.5 mL of the DAN solution was added, and after closing the tube it was thoroughly mixed by shaking. Thereafter the cap was loosened for venting while the sample was heated in a covered water bath at 50 ºC for 30 min. After it had cooled down sufficiently, 1 mL cyclohexane was added, the centrifuge bottle was capped and it was shaken for 5 min. After the cyclohexane layer had separated from the aqueous layer, the organic layer was transferred to a PMMA cuvette using a micropipette. The absorbance at 480 nm was measured and the concentration was obtained using the linear calibration curve generated during the calibration of the instrument. The sensitivity of this method is 10 ppb, the lower detection limit is 100 ppb and follows Beer's law up to 2 $mg.L^{-1}$ according to the standard methods (APHA, 2012).

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3.6.4. Determination of elemental selenium in plug

For the reduction experiments, the elemental selenium in the plug was determined according to the following procedure. The plug containing the elemental selenium and the cells was resuspended in 20 mL distilled water whereafter 10 mL concentrated HCl and 10 mL concentrated HNO_3 were added, and the volume was made up to 50 mL by adding distilled water. With the addition of the acids, the characteristic colour of the elemental selenium disappeared, thereby confirming that the elemental selenium was oxidised back to the selenium oxyanions. After the volume was made up to 50 mL, 10 mL was taken and placed in a heating block (Lovibond ET108, Germany) at 100 ˚C for 60 minutes. The next step was to make up the 10 mL sample to 50 mL using distilled water and then filtering the sample (Whatman 42), following which it was placed in a sample container and analysed using ICP-OES.

3.7. Quality Assurance

3.7.1. Colorimetric calibration

Prior to selenite analysis after every experiment, the UV/Vis spectrophotometer was calibrated for the DAN method. The calibration curve was prepared using the 1 000 ppm standard with MSM as diluent (0, 0.5, 1, 1.5 and 2 $mg.L^{-1}$). The colorimetric method as described above was then followed. The calibration curve (linear) was generated by plotting absorbance versus the known concentration of selenite (R^2 > 0.99). This curve was then used to calculate the selenite concentration in the samples.

3.7.2. ICP-OES Calibration

Prior to selenium analysis after every experiment, the ICP-OES was calibrated at 196 nm using the 1 000 ppm standard and MSM as diluent (0, 4, 7, 10 and 15 $mg.L^{-1}$). The calibration curve (linear) was generated by plotting emission versus the known

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concentration (R^2 = 0.99). This curve was then used to calculate the selenium concentration in the samples.

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CHAPTER 4: GROWTH AND REDUCTION

4.1. Preliminary studies

Optical density at a wavelength of 600 nm (OD_{600}) was used in this study to measure the bacterial growth of *Pseudomonas stutzeri* NT-I. The relationship between OD_{600} and cell density is linear but only up to a certain point; for most bacteria this is at an OD_{600} of 0.4, thereafter it starts deviating (Widdel, 2007). To evaluate whether this is the case with *Pseudomonas stutzeri* NT-I, dilution tests were conducted. The results given in Table 4-1 show that the linear relationship is still intact at higher values of OD_{600} since the average error is only 6.1 % with a maximum of 9.4 %.

Table 4-1: Dilution test to determine whether the relationship between OD_{600} and cell density stays linear with increasing OD_{600}

#	Original OD_{600}	Diluted OD_{600}	Diluted OD_{600} x 3	Δ in OD_{600} between original and diluted sample	% Difference
1	1,219	0,424	1,272	0,053	4,3
2	1,165	0,412	1,236	0,071	5,9
3	1,141	0,418	1,254	0,113	9,4
4	0,911	0,286	0,858	0,053	6,0
5	0,899	0,292	0,876	0,023	2,6
6	0,909	0,329	0,987	0,078	8,2
Average				0,065	6,1

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Before growth and reduction studies were carried out, the OD_{600} of the bacteria at stationary phase was determined. This was done with no selenium present, since the discoloration due to the red elemental selenium forming when nearing the stationary phase interfered with the accurate determination of the complete growth curve. The OD_{600} at stationary phase was determined as 1.5 as can be seen in Figure 4-1. During experiments where a washed cell suspension was used, the OD_{600} at the beginning of the experiment was measured to ensure it was far above 1.5. It should also be noted that there was no bacterial growth when glucose was absent.

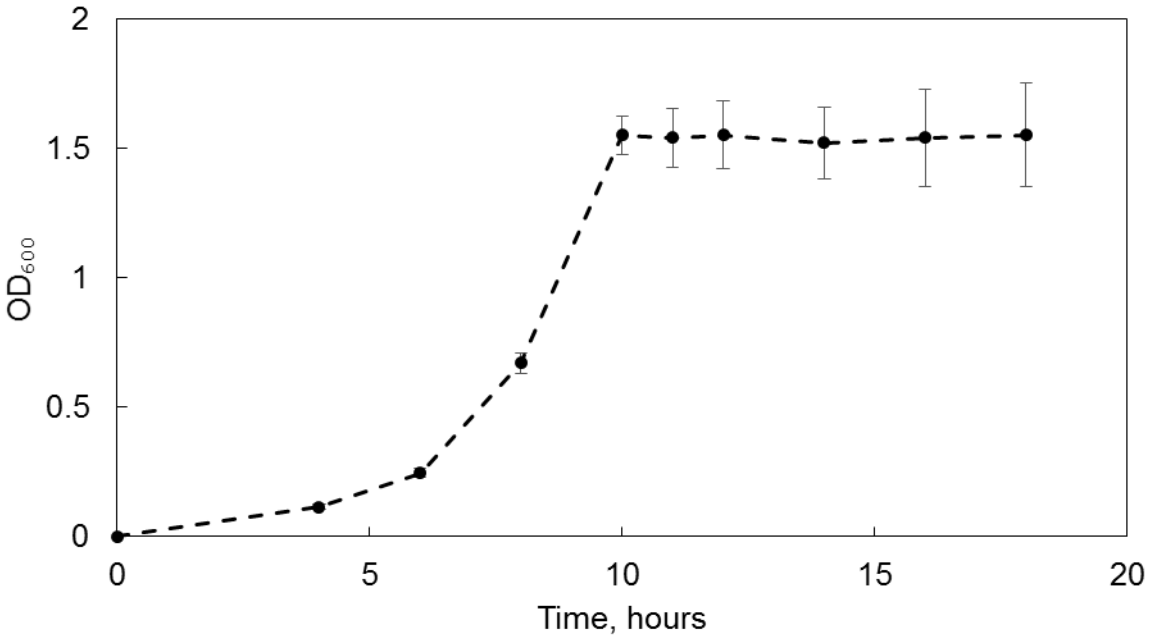

Figure 4-1: The optical density of the cell suspension stabilised at 1.5

The minimum glucose concentration that can be used without glucose being a limiting substrate was also determined by developing the growth curve for different initial glucose concentrations of 1, 2, 4 and 6 $g.L^{-1}$ (MSM with pH 7 was used as

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growth medium). The results showed that for glucose concentrations above 2 g L^{-1}, the OD_{600} was the same when the stationary phase was reached.

The relationship between the OD_{600} and dry biomass (g L^{-1}) was also determined as described in section 3.5.1. From Figure 4-2 it can be seen that at an optical density of 1, the weight of the dry biomass is 0.416 g.L^{-1}.

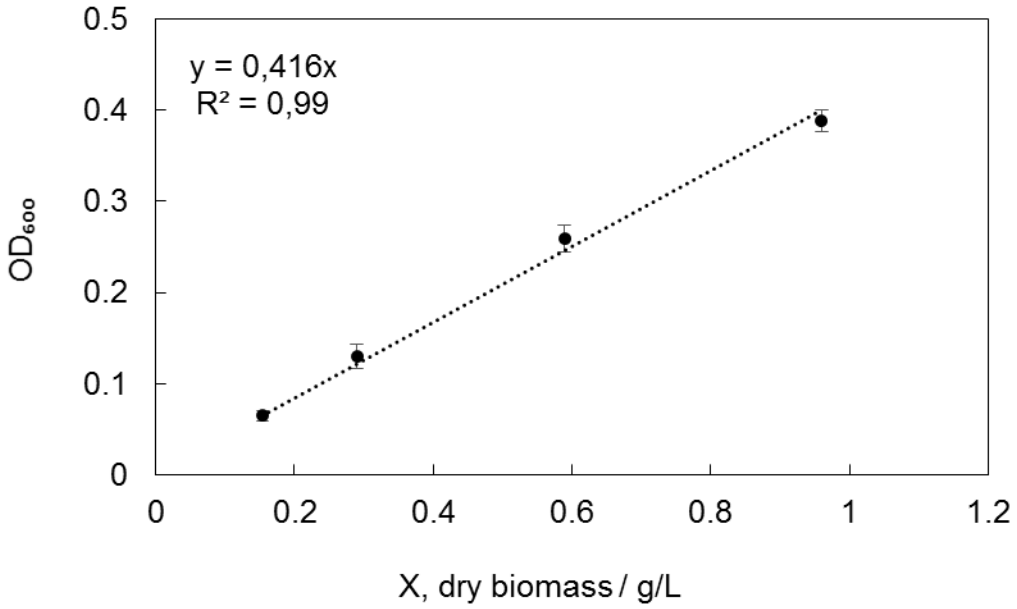

Figure 4-2: The relationship between dry biomass and OD_{600}

4.2. Growth studies

The optimum conditions in terms of pH, salinity, temperature and initial selenite concentration were determined for growth as described in section 3.5.2. It should be noted that the growth rates were determined at specific predetermined values and therefore the optimum values determined cannot be taken as the absolute optimum condition but only as the optimum for the values at which it was measured. The exponential growth phase constants, μ (h^{-1}), for the different experiments were compared. Figure 4-3 show the results of the experiments completed at different temperatures.

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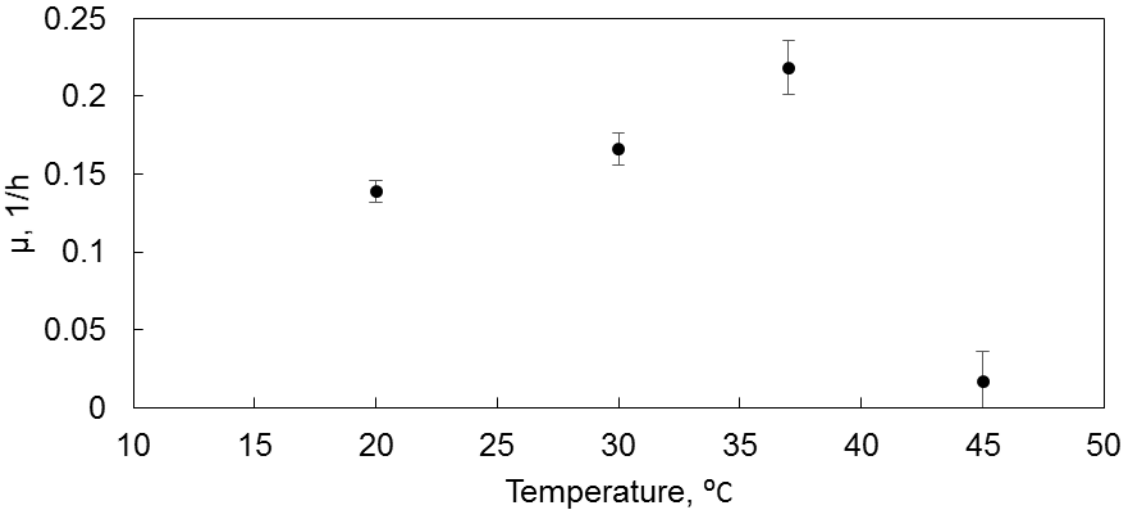

Figure 4-3: Influence of temperature on growth of bacteria

As can be deduced from Figure 4-3, the optimum temperature for growth is 37 °C with an exponential growth rate constant of 0.22 h^{-1}. What can also be seen from the data is that there is a sharp decline in growth rate for temperatures above 37 °C going as low as 0.02 h^{-1} at 45 °C whereas the relative difference in growth rate between 30 and 37 °C is much less. An optimum growth temperature between 35 and 40 °C is common for bacteria. This means that should a consortium of bacteria be amended with this bacteria in an industrial setup where the temperature fluctuate around 37 °C , good growth can still be achieved for both the consortium and this bacteria.

In terms of pH, no growth was observed at a pH of 6; in fact the inoculum died soon after the experiment commenced. The optimum pH was found to be 7 with an exponential growth rate constant of 0.22 h^{-1} as shown in Figure 4-4.

The experiments done at pH 6 and pH 7 was the only experiments done. After a study of the available literature on selenium reducing bacteria was done, a trend was observed in terms of pH at which reduction occurs, the trend indicated that selenium reducing bacteria have a higher affinity for neutral to alkaline environments therefore it was expected that the optimum growth rate would be between a pH of 7 and 8.

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Hence no further experiments were considered. Table 4-2 below lists other bacteria that reduce selenate and their operating pHs.

Table 4-2: Optimum pH for several selenium reducing bacteria.

Bacterium	pH for reduction reaction	Reference
Bacillus sp. SF-1	8	(Fujita et al., 2002)
Pseudomonas stutzeri NT-I	7	(Kuroda et al., 2011)
Pseudomonas stutzeri	7 - 9	(Lortie et al., 1992)
Bacillus beveridgei sp. nov.	8.5	(Baesman et al., 2009)
Thauera selenatis	7	(Macy et al., 1993)
Pseudomonas fluorescens	6.8	(Belzile et al., 2006)
Salmonella heidelberg	8.5	(McCready et al., 1966)
Streptococcus faecalis N83	7.2	(Tilton et al., 1967)
Halorubrum xinjiangense	7 - 8	(Güven et al., 2013)
Salana multivorans	7.5	(Wintzingerode et al., 2001)
Sulfurospirillum barnessi SES-3	7.5	(Stolz et al., 1997)
Bacillus arsenicoselenatis	9.8	(Blum et al., 1998)
Selenihalanaerobacter shriftii	7.2	(Switzer et al., 2001)
Veillonella atypica	7.5	(Pearce et al., 2009)

The decline in growth rate from pH 7 to 8 is not that significant, the implication being that the pH can be increased to a value between 7 and 8 without losing to much growth potential.

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The growth rate was also evaluated for different salinities ranging from 0 to 20 $g.L^{-1}$ NaCl. The optimum growth rate of 0.26 h^{-1} was at a salinity of 20 $g.L^{-1}$ NaCl as can be seen in Figure 4-5.

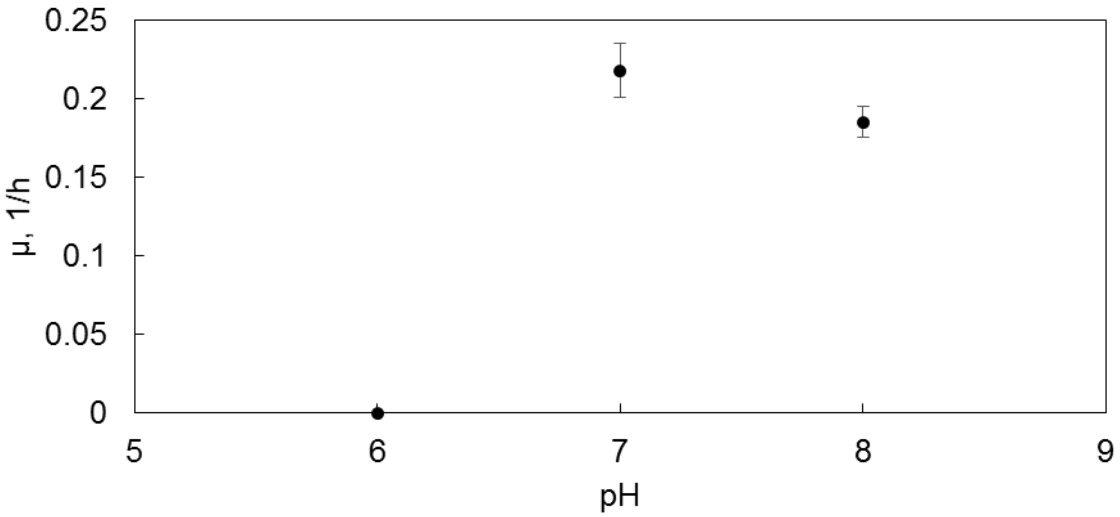

Figure 4-4: Influence of pH on growth of bacteria

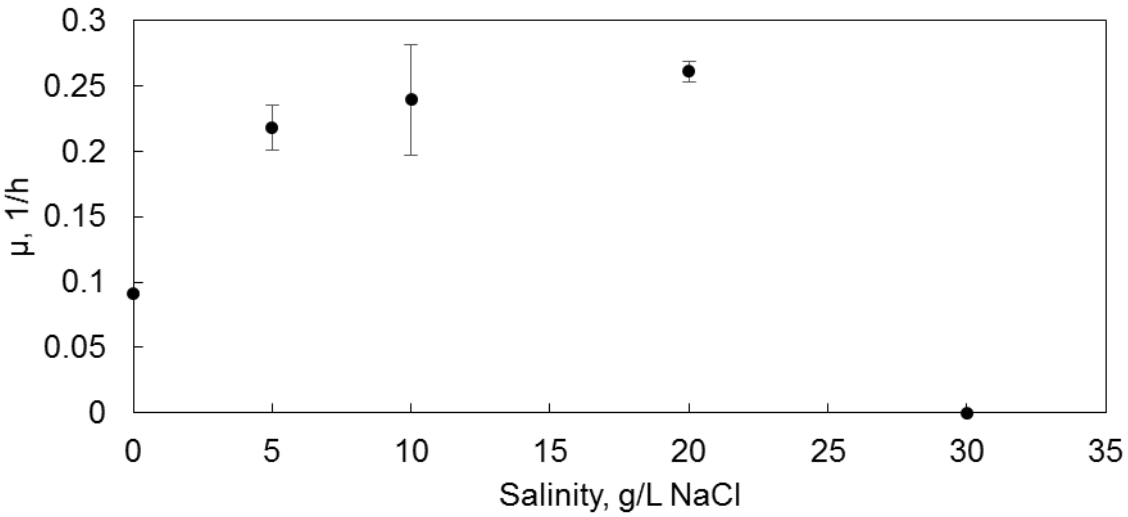

Figure 4-5: Influence of salinity on growth of bacteria

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The impact of initial selenate concentration is shown in Figure 4-6. Optimum growth (0.36 h^{-1}) is observed at a concentration of 5 mM selenate (715 $mg.L^{-1}$) indicating that the selenate is useful for energy and growth. What is notable is that the growth rate for the experiment which contained no selenium is lower than the maximum growth rate.

From this data it can be seen that the optimum combination of conditions for growth is 37 °C, pH 7, salinity 20 $g.L^{-1}$ NaCl and initial selenate concentration of 5 mM. Kuroda et al. (2011) determined the optimum conditions for growth to be at 38 ºC, pH 7 and 10 $g.L^{-1}$ salinity. This compares well with the findings of this study. These results prove *Pseudomonas stutzeri* NT-I to be an aerobic mesophile that is halotolerant and grows at the usual optimum temperature for bacterial growth.

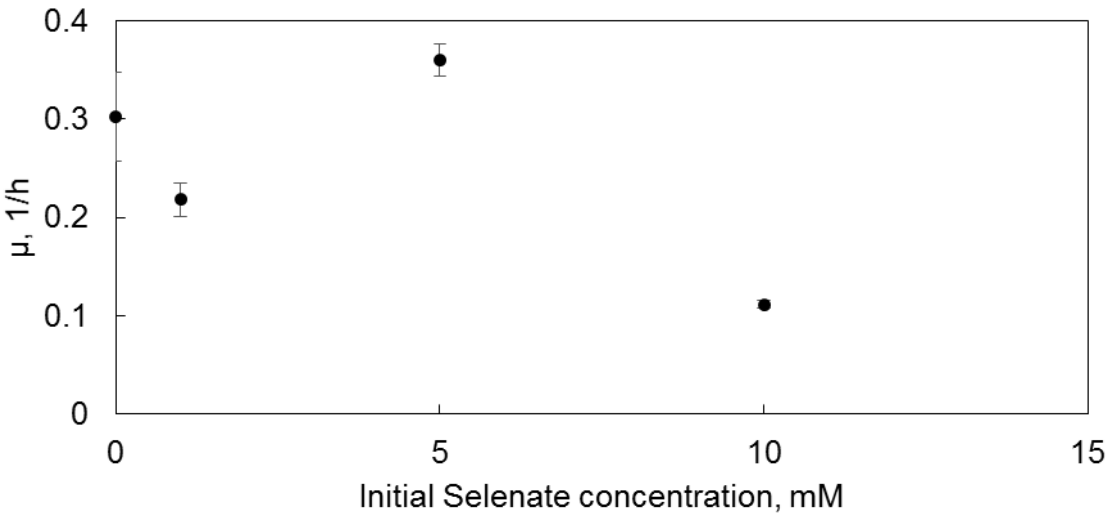

Figure 4-6: Influence of initial selenate concentration on growth rate

4.3. Reduction rate studies

In the study done by Kuroda et al. (2011), it was determined that the bacteria could reduce selenate to selenite within 6 hours and the selenite can then be further reduced to elemental selenium in another 18 hours. This information was used to determine the length of the reduction experiments. However, for the experiments

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done as part of this study it was found that the reduction proceeded at a much faster rate. This may be due to a different carbon source being used (glucose instead of lactate) or the temperature of the experiments being different. In the paper referred to earlier, the temperature of the reduction reaction experiments is not given.

Due to this inconsistency, the amount of elemental selenium recovered from the plug after 16 hours of incubation was taken as the criterion by which the optimum reduction conditions were determined. ICP-OES analysis of the supernatant confirmed that after 16 hours the reduction of both oxyanions were complete except for the experiment conducted at 30 ºC. It is the opinion of the author that this criterion is more appropriate since expulsion of the selenium nanoparticles from the bacterial cells are also an important factor that must be considered when evaluating optimum conditions for operational parameters should this bacteria be used in a bioremediation scheme. Extra reduction experiments were performed, one without glucose, one with a heat-killed washed cell suspension and, lastly, one without any bacterial cells. No reduction was observed in these experiments. This means that the reaction is dependent on glucose as an electron donor and that the reduction occurs strictly due to microbial activity.

Figure 4-7 shows the impact temperature has on the amount of elemental selenium recovered after 16 hours of reaction time with a washed cell suspension of *Pseudomonas stutzeri* NT-I. The temperature at which the experiment was conducted is important since a change in temperature leads to a change in the pK_a value of an enzyme, which in turn impacts the enzyme's ability to reduce the metal anion involved. The optimum temperature, 37 ºC, is the same as that of growth and is within the region of the temperature of industrial wastewater. This adds to its suitability as a reducing agent for selenate in wastewater.

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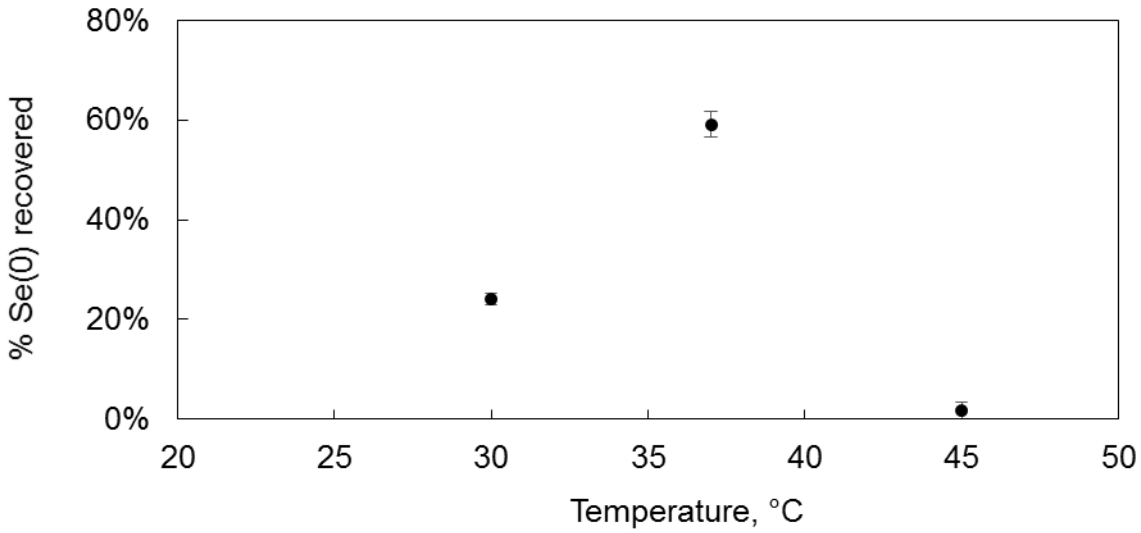

Figure 4-7: Influence of temperature on reduction rate of selenate

The experiment that was done at a temperature of 30 ºC was used to do a mass balance check. The supernatant was analysed on the ICP-OES for total selenium and added to the amount of elemental selenium measured in the plug. After adding 79 mg.L^{-1} of selenium to the reaction mixture, at the end there was 19 mg.L^{-1} in the supernatant and 53.2 mg.L^{-1} in the plug. The difference between what was added and the amount at the end was 6.8 mg.L^{-1} and translates to a 91.4 % recovery of selenium, which is within the acceptable range for mass balances done in microbial systems.

The optimum pH for reduction of selenate is 8, which is different to the optimum pH of 7 for growth. When the pH of the solution is changed it affects the enzymes involved in the reaction by affecting their solubility, activity and stability. Each enzyme has a pK_a value specific to them. Different selenium reducing bacteria operate best at various pH's, which indicates that there are multiple reduction mechanisms involved depending on which bacteria is being used.

As can be seen in Figure 4-8, the change in reduction capacity between pH 7 and pH 8 is not much. This makes the setting up of a reactor where growth and reduction

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occurs at the same time easier in that the pH only needs to be kept between 7 and 8 to still achieve a high reduction rate.

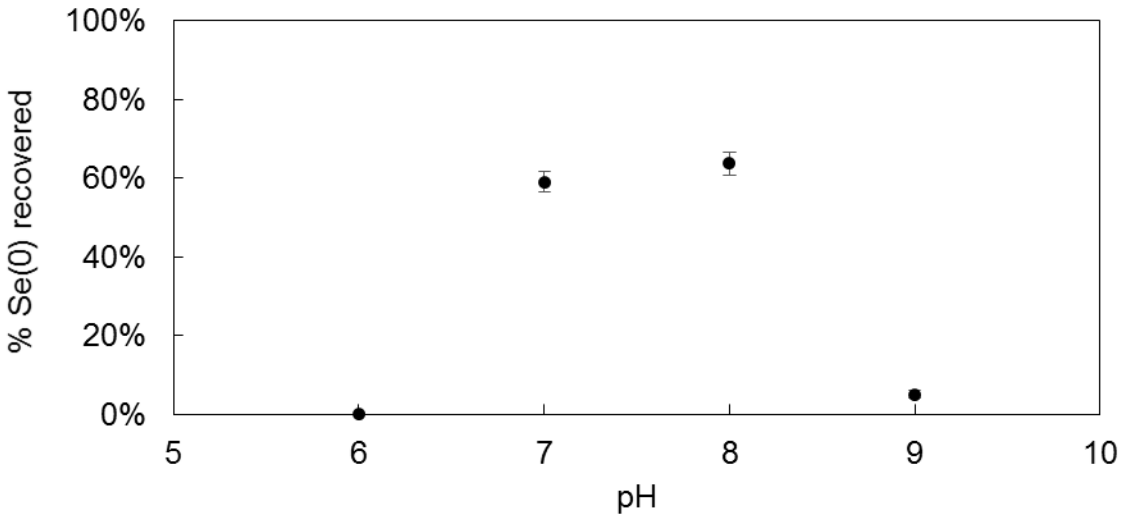

Figure 4-8: Effect of pH on reduction capacity

When looking at Figure 4-9, it can be seen that from a salinity of 5 $g.L^{-1}$ a further increase lowers the reduction capacity. This may be due to the denaturing of the enzymes involved in the reduction pathway. This is the opposite of what is seen with the impact of salinity on growth. A compromise between reduction capacity and growth will have to be made when both are occurring in the same reactor. A salinity of 5 $g.L^{-1}$ is suggested for such instances.

In the other study already mentioned (Kuroda et al., 2011), the optimum conditions for reduction of selenate by *Pseudomonas stutzeri* NT-I was 40 ºC, pH 8 and low salinity. This is once again in close agreement with the findings presented here.

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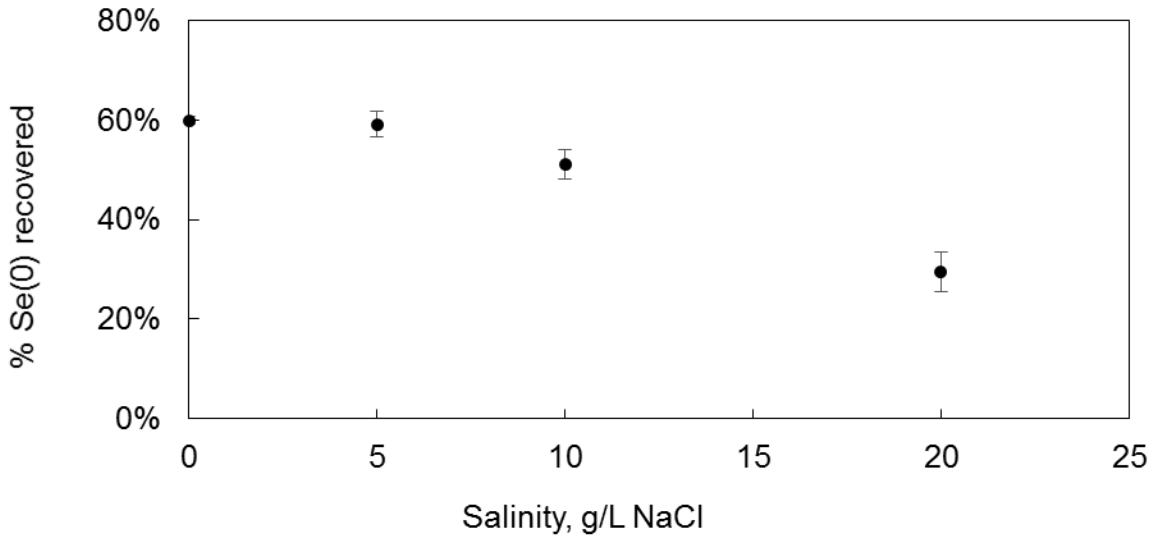

Figure 4-9: Effect of salinity on reduction capacity

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CHAPTER 5: KINETIC MODEL

5.1. Observed reaction rate

The bacteria took 2 hours to completely reduce 2 mM of selenate which translates to a reduction rate of 0.0313 mmol.$(g.h)^{-1}$. When compared to other bacterial strains identified as selenium reducers, *Pseudomonas stutzeri* NT-I reduces the selenate to elemental selenium at a relatively high rate. *E cloacae* SLD1a-1 reduces selenate at a rate of 0.0184 mmol.$(g.h)^{-1}$ (Ma et al., 2007). Pseudomonas fluorescens only reduced 95 % of 0.2 mM selenate after 45 hours (Belzile et al., 2006) and *Desulvibrio desulfuricans* took 25 – 37 hours to reduce 86 % of 1 mM of selenate (Tucker et al., 1998) which is much slower than what was observed in this study.

5.2. Analysis limitations

Total selenium in the supernatant was measured using ICP-OES. The motivation behind this was that the selenate concentration in the supernatant could be calculated then from the difference between the selenite concentration and the total selenium. However when the samples were analysed the amount of selenium in the sample did not go to zero as would be expected if only the selenium oxyanions were present and all the elemental selenium was present as nanoparticles that would be centrifuged out from the supernatant into the plug. This discrepancy can be explained by postulating that the elemental selenium is present in three states. Firstly, it is present as colloidal selenium withinin the cells, the colloidal selenium then form nanoparticle seeds which are transported to the outside of the cell where over time it ripens into nanoparticles that are big enough to centrifuge out with the cells forming the plug. The consequence of this is then that the total selenium measured in the supernatant is not only made up of the selenium oxyanions but also elemental selenium nanoparticle seeds that have been dispelled from the cells but

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have not yet evolved into nanoparticles heavy enough to centrifuge out. Figure 5-1 shows the concentration of selenium in the supernatant over time proving that the elemental selenium is spread between the plug and the supernatant making it very difficult to do a mass balance for the sake of the kinetic modelling. Therefore only the selenite concentration in the supernatant was used.

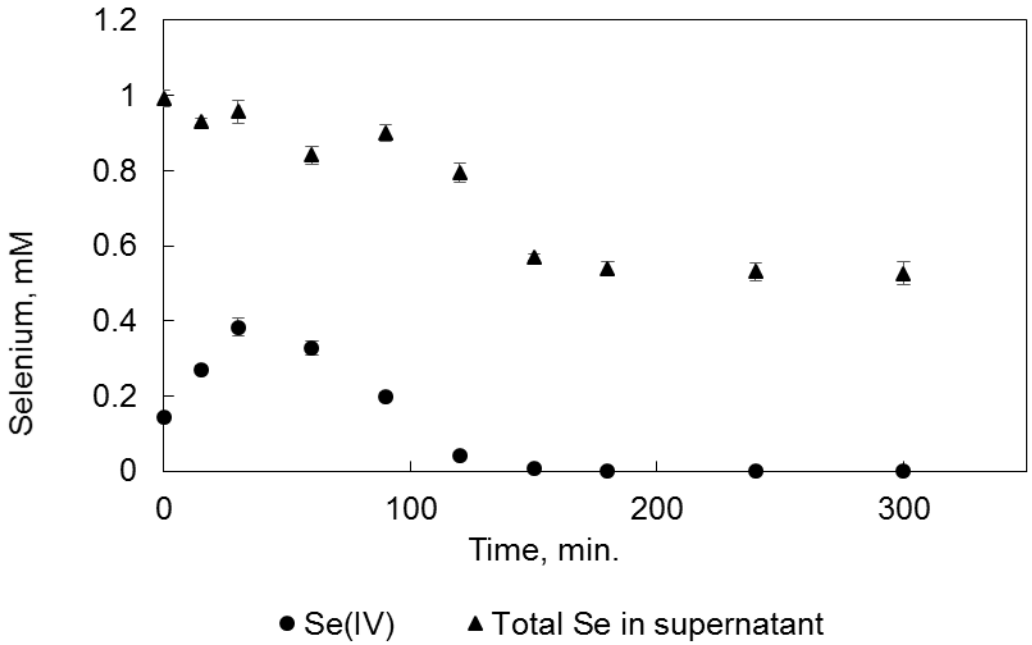

Figure 5-1: Total selenium and selenite present in the supernatant.

5.3. Reduction rate

Figure 5-2 plots how the reduction rate changes with initial selenate concentration used.

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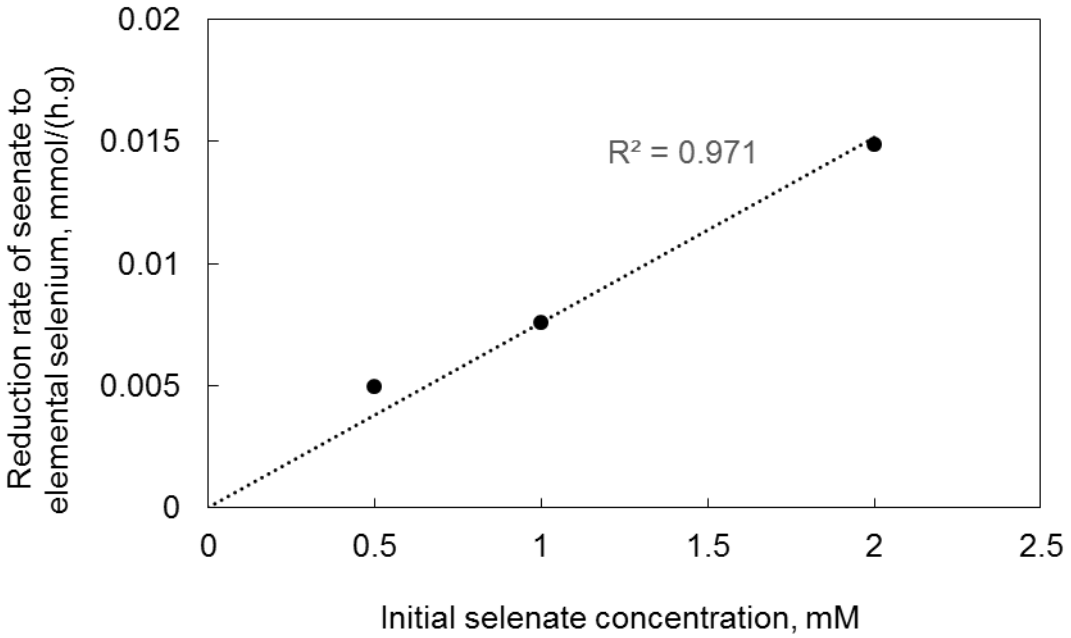

Figure 5-2: Change in reduction rate with initial selenate concentration.

As can be seen from Figure 5-2, the reduction rate increases with initial selenate concentration. This trend where reaction rate increases linearly with increasing initial selenate/selenite concentration have been observed by other researchers as well and is most likely due to substrate saturation not being reached yet. Substrate saturation occurs when all the enzymes available to catalyze the reaction involved is saturated with the substrate (Shuler & Kargi, 1992 : 61).

Starting with Lortie et al. (1992) (Figure 5-3) who found that at an initial selenite concentration of 19 mM, the reduction rate of selenite starts decreasing whereas at concentrations up to 19 mM the reduction rate increases. They suggest that this might be due to the high toxicity of the selenite ion starting to induce cell death. The reduction rate of selenate stabilized at 19 mM suggesting that the concentration of selenate at which substrate saturation is around 19 mM of initial selenate. Data that followed a similar pattern (reaching substrate saturation) would have been ideal for this study but that was not achieved in the concentration span used. The main reason why the concentration span used in this study was chosen was because it

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relates well back to the maximum selenium concentrations found in wastewater streams.

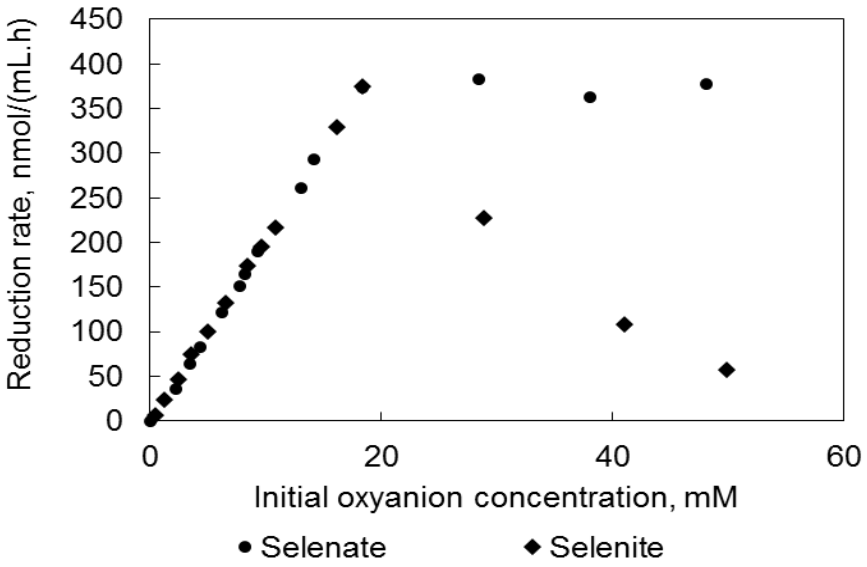

Figure 5-3: Reduction rate for *Pseudomonas stutzeri* (Lortie et al., 1992).

The next research group to publish similar results is that of Ma et al. (2007) who worked with the bacterium Enterobacter cloacae SLD1a-1. They did not reach or exceed the minimum concentration needed for substrate saturation as shown in Figure 5-4. There are more research groups that found a similar trend (Kashiwa et al., 2000; Takada et al., 2008).

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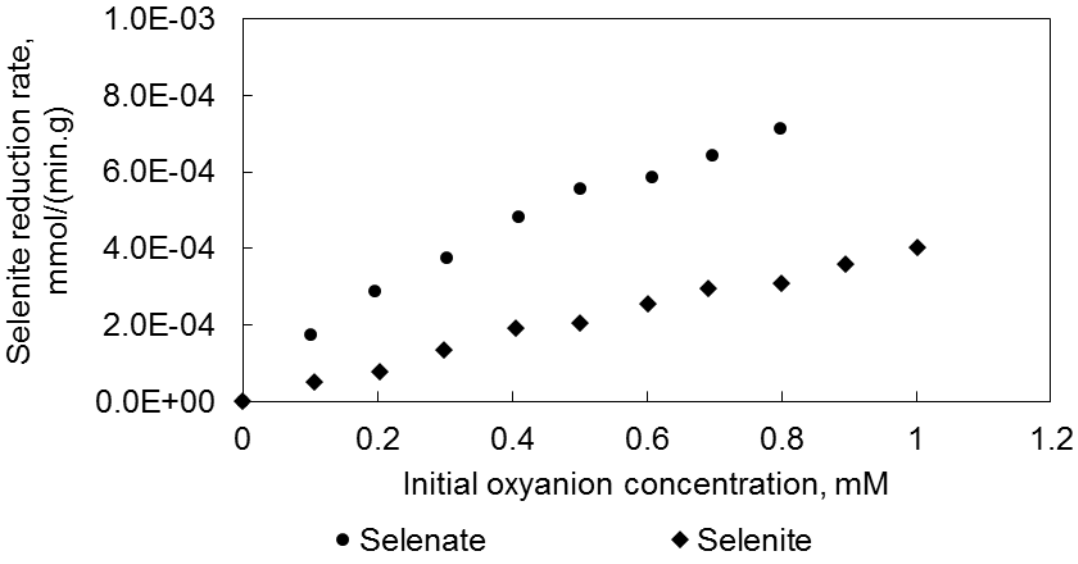

Figure 5-4: Reduction rate for Enterobacter cloacae SLD1-a1 (Ma et al., 2007).

5.4. Kinetic model evaluation

Two kinetic models were considered when parameter estimation was performed, namely a first order rate equation and an adapted Monod equation. The first order rate equation did not fit the data but the Monod equation, adapted for metal reduction did.

Differential equations were used in the software package AQUASIM (Reichert, 1994) to estimate the parameters for the reduction of selenate to selenite and selenite to elemental selenium. AQUASIM estimates the parameter values by minimising the sum of squares of the weighted deviations between calculated results and measured values.

$$\frac{-dSe^{6+}}{Xdt} = \frac{k_{max64}[Se^{6+}]}{K_{s64} + [Se^{6+}]} \qquad 5\text{-}1$$

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$$\frac{-dSe^{4+}}{Xdt} = \frac{k_{max40}[Se^{4+}]}{K_{s40} + [Se^{4+}]} - \frac{k_{max64}[Se^{6+}]}{K_{s64} + [Se^{6+}]} \quad 5\text{-}2$$

$$\frac{dSe^{0}}{Xdt} = \frac{k_{max40}[Se^{4+}]}{K_{s40} + [Se^{4+}]} \quad 5\text{-}3$$

k_{max64} Maximum reaction rate for the reduction of selenate to selenite, mol.(g.min)$^{-1}$

k_{max40} Maximum reaction rate for the reduction of selenite to elemental selenium, mol.(g.min)$^{-1}$

K_{s64} Substrate saturation coefficient for the reduction of selenate to selenite, mM

K_{s40} Substrate saturation coefficient for the reduction of selenite to elemental selenium, mM

X Biomass concentration, g.L^{-1}

It would have been preferable to estimate the values of the substrate saturation constant (K_s) and the maximum reaction velocity (k_{max}) using one of several visual methods like the Lineweaver-Burk plot or the Eadie-Hofstee plot. However, this was not possible because of the concurrent nature of the two reduction reactions.

Since only selenite measurements could be used, the program was set up to fit the parameters of equation 5-2 to the datapoints from three experiments where the initial concentration of selenate differed (0.5, 1 and 2 mM). Figure 5-5 shows the results of this fit and Figure 5-6 shows the predicted concentrations for all three species (selenate, selenite and elemental selenium) for the experiment with an initial concentration of 1 mM.

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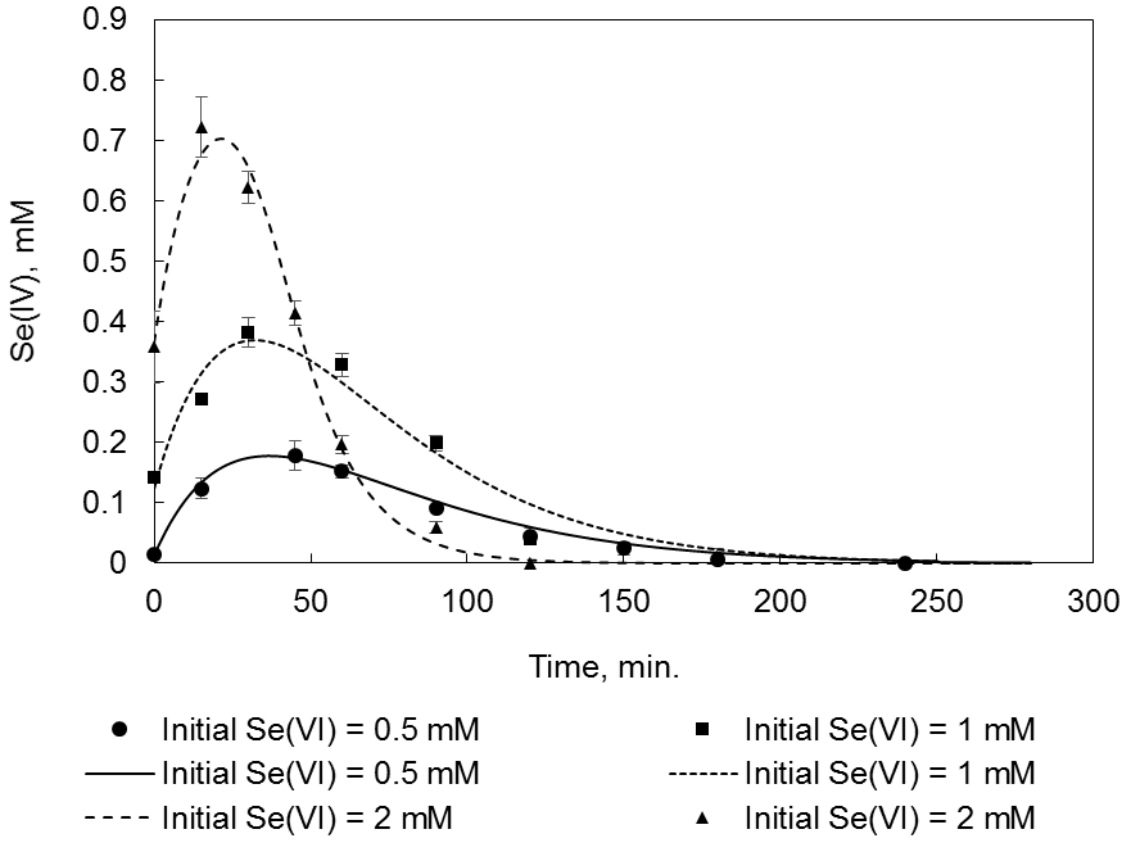

Figure 5-5: Adapted Monod equation fitted to measured selenite data for three experiments

From these fits it can be seen that the assumption that the change in biomass is negligible and that the biomass concentration is directly proportional to the reaction rate holds. Other observations that can be made are that there is no delay time for the reaction of selenate to selenite and that selenite reduction is faster than that of selenate. Ma et al. (2007) as well as Lindblow-Kull et al (1985) also found that selenite was reduced faster than selenate.

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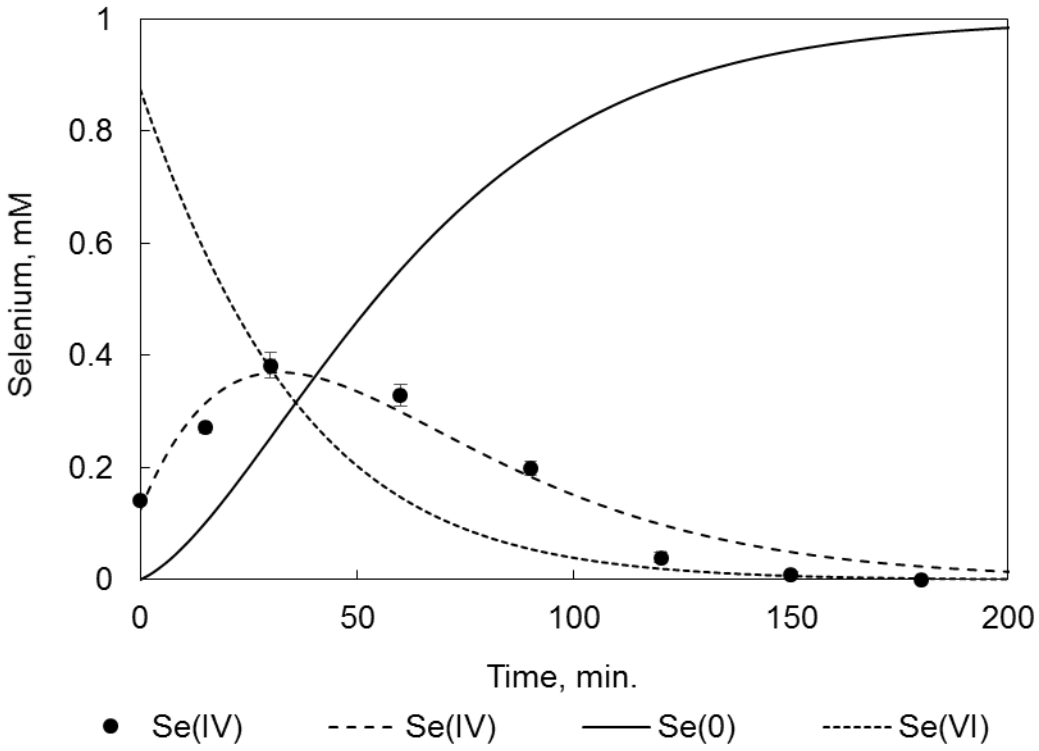

Figure 5-6: Concentration profile for all three species of selenium present in reaction mixture as calculated by AQUASIM

The values of the fitted parameters are given in Table 5-1. The k_{max} values for E. Coli K12 was 0.00681 and 0.00076 mmol.(g.min)$^{-1}$ for selenite and selenate reduction respectively (Lindblow-Kull et al., 1985). The reduction rate for both oxyanions is slower than *Pseudomonas stutzeri* NT-I. The K_S values are higher than that of other selenium reducing bacteria like *Enterobacter cloacae* SLD1-a1 which had K_S values of 3.1 and 0.72 mM for selenate and selenite reduction respectively (Ma et al., 2007).

One k_{max} value for each of the two reactions in all three of the experiments were fitted since the maximum reduction rate for a specific temperature and pH is constant. One K_S value for each of the three initial concentrations could not be fitted and was therefore optimised for each experiment.

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Table 5-1: Parameters for the adapted Monod equation fitted to experiments 1 – 3

#	Initial selenate concentration ($mmol.L^{-1}$)	k_{max64} ($mmol.min^{-1}.g^{-1}$)	k_{max40} ($mmol.min^{-1}.g^{-1}$)	K_{s64} ($mmol.L^{-1}$)	K_{s40} ($mmol.L^{-1}$)	R^2
1	0.5	0.0109	0.0533	12.6	58.7	0.993
2	1	0.0090	0.0575	7	49.8	0.964
3	2	0.0106	0.0547	2.9	18.8	0.997
	Average	0.0102	0.0552			

The K_s value for each of the reaction is also supposed to remain constant like the k_{max}. The fact that one K_s value could not be fitted, but that the unique values fitted decreases almost linearly as initial selenate concentration is increased points to some form of increasing interference with the two reactions.

5.5. Inhibition

When looking at the data from the experiment with an initial concentration of 4 mM (Figure 5-7) it can be seen that some form of inhibition is becoming apparent. One can only speculate what the cause is behind the inhibition based on what other researchers have found.

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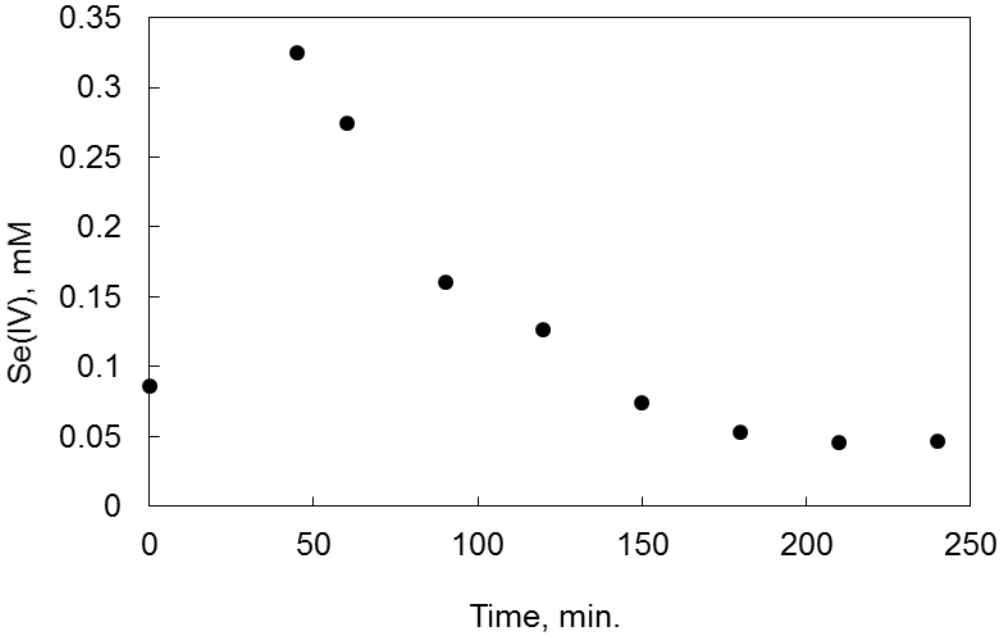

Figure 5-7: Concentration of selenite for experiment with an initial selenate concentration of 4 mM.

Adding a non-competitive inhibition term related to elemental selenium to the adapted Monod equation was considered due to the formation of insoluble seed-nanoparticles of elemental selenium within the cell. This was done with caution since the true K_s value is not known. Upon adding a product inhibition term the fit was not significantly improved. Other inhibition models were also tested for the sake of being thorough but none of them led to an improved fit.

The lower K_s values (implying lower enzyme activity) observed at a high reduction rate alludes to the fact that elemental selenium might be building up in the cell due to a slower transportation than reduction rate at high concentrations. In the case where the reduction rate is slower due to the lower initial selenate concentration, the elemental selenium seed-nanoparticles have time to be transported out of the cell and therefore interfere less with the reduction reaction. It has been shown in another study (Liu et al., 2002) that elemental selenium seed-nanoparticles can physically get in the way of different steps in the reaction chain, for example blocking the redox

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enzyme or interfering with the intermediate electron transfer sites. These seed-nanoparticles can also negatively influence the porosity of the cell membrane. Another possible reason for product inhibition may be that the elemental selenium seed-nanoparticles cause the cell viability to be lowered or physiological state altered.

This possible explanation was not further investigated due to the scope of this study but do warrant further investigation. The interpretation of the data requires fundamental insight into the mechanisms involved, which include mass transfer of glucose (energy source for the detoxification reaction), the selenium oxyanions and the elemental selenium between the reaction sites.

The structure of the cell surface and membrane as well as the participation of different enzymes in the electron transport chain all add to the complexity of the detoxification mechanism. It has been suggested that unique redox enzymes are involved in the reduction of the valence state of metals and metalloids like zinc and selenium to avoid cellular damage and death (Wu et al., 2010). A few research groups have studied this detoxification mechanism (Dungan et al., 2003; Zheng et al., 2014; Lampis et al., 2015) and have found that in some cases the reduction occurs in the periplasm and in other cases in the cytoplasm. But they all require energy in some form.

5.6. Sensitivity analysis

AQUASIM has a built-in function, called sensitivity analysis, which evaluates the impact that each parameter has on the curve generated to fit the data. This function provides a framework that enables the user to evaluate the robustness and accuracy of the applied model (Wang & Sheu, 2000). Figure 5-8 shows the results for the sensitivity analysis done on experiment 3 (initial Se(VI) = 2 mM) using the adapted Monod equation.

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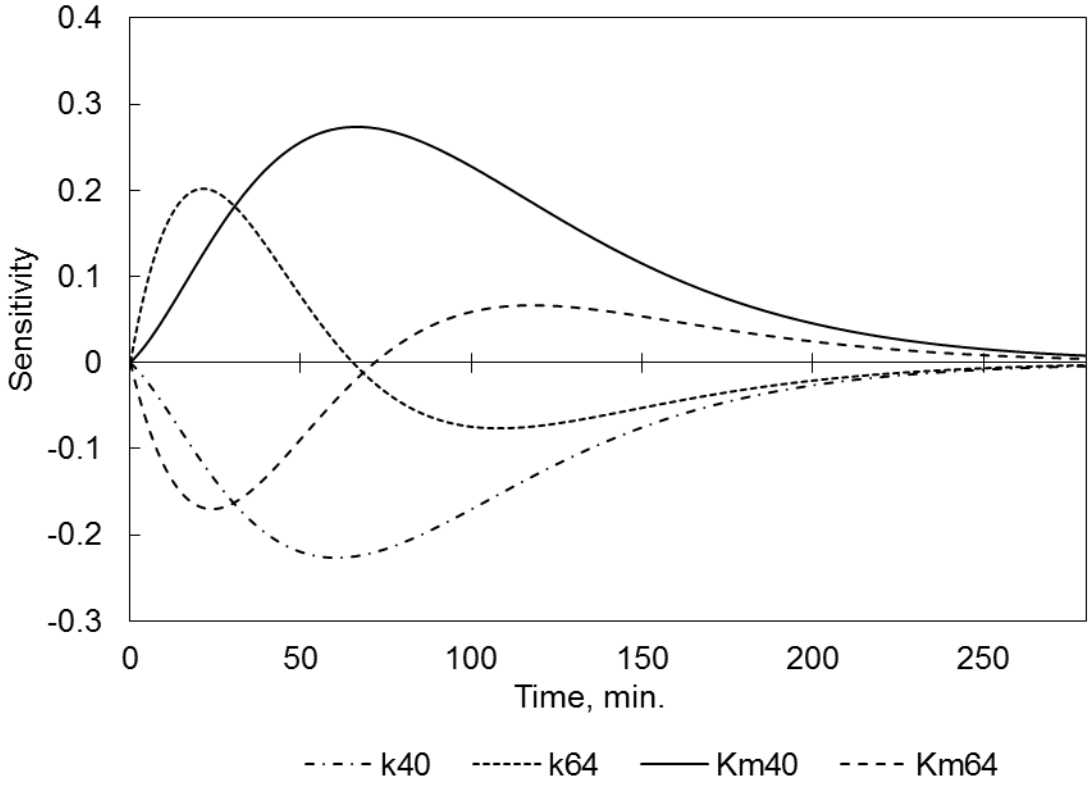

Figure 5-8: Sensitivity analysis results for adapted Monod equation.

From the figure it can be seen that the four parameters all contribute to the goodness of fit of the adapted Monod equation to the macrokinetics.

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CHAPTER 6: CONCUSIONS AND RECOMMENDATIONS

The optimum conditions in terms of pH, salinity, temperature and initial selenite concentration for growth were determined. The exponential growth phase kinetic constants, μ (h^{-1}), for the different experiments were compared. From this data it can be seen that the optimum combination of conditions for growth is 37 °C, pH 7, salinity 20 $g.L^{-1}$ NaCl and initial selenate concentration of 5 mM.

In terms of reduction capacity, measured by determining the amount of elemental selenium in the plug formed during centrifugation of the reaction mixture, the optimum temperature is 37 ºC and the optimum pH is 8. From a salinity of 5 $g.L^{-1}$, a further increase lowers the reduction capacity. The growth and reduction studies show that *Pseudomonas stutzeri* NT-I is a suitable candidate for use in bioremediation of seleniferous industrial waste water. It is therefore recommended that pilot studies be conducted to determine the most effective reactor design for the facilitation of the reduction reactions by this bacterial strain. Augmenting current biological reactors that use a local consortium of bacteria with this bacteria to improve the reduction performance can also be investigated.

When considering the kinetics of the two consecutive reduction reactions, the Monod equation, adapted for metal reduction, is a good representation of the measured selenite concentration over time. The assumption that biomass is constant and directly proportional to the reaction rate holds, as would be expected for a biomass concentration exceeding 2 $g.L^{-1}$. The three experiments shared a k_{max} value for each of the two reduction reactions. One K_s value for each of the two reactions in the three experiments could not be fitted though and was therefore optimised for each experiment. A linear trend, where K_s decreases with increasing initial selenate concentration, was observed. It is postulated that this is due to elemental selenium build-up which interferes with the reduction reaction. Therefore the inhibition model

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considered was non-competitive inhibition (with elemental selenium as the inhibitor). This models could not be fitted to the data due to the inability to determine the true K_S value for the system.

The interpretation of the kinetic modelling should be done with caution since it requires fundamental insight into the mechanisms involved in the reduction process from where the oxyanion comes into contact with the bacteria to where the elemental selenium particle is expelled from the cell. Further investigation into these mechanisms is encouraged since this will enable the design of pilot plants to be based on the understanding of the bacteria's functioning instead of solely empirical data. It will also contribute to the knowledge base of selenium reduction by aerobes. The enzymatic working of the detoxification mechanism involved has not been fully defined yet and will therefore benefit from research focused thereon.

Pseudomonas stutzeri NT-I is an exemplary selenium oxyanion reducing agent and deserves more attention, not only so that it can be used in industry for the bioremediation of industrial waste water, but also in the research world to further our understanding of the complex working of bacteria.

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APPENDIX AQUASIM

AQUASIM Version 2.0 (win/mfc) - Listing of System Definition

****

Variables

****

kmax40: Description: maximum velocity constant for rxn of Se(IV) to Se(0)

Type: Constant Variable

Unit: mmol/(g.min)

Value: 0.00275

---

kmax64: Description: maximum velocity constant for rxn Se(VI) to Se(IV)

Unit: mmol/(g.min)

Value: 0.0011

Ks40: Description: substrate saturation coeff for rxn Se(IV) to Se(0)

Type: Constant Variable

Unit: mM

Value: 3.293

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Ks64: Description: Subtrate saturation coeff for rxn Se(VI) to Se(IV)

Type: Constant Variable

Unit: mM

Value: 0.959

Se0: Description: Elemental selenium

Type: Dyn. Volume State Var.

Unit: mM

Se4: Description: Selenite

Type: Dyn. Volume State Var.

Unit: mM

Se4i: Description: Initial selenite concentration

Type: Constant Variable

Unit: mM

Value: 0.0142

Se4meas: Description: Measured values of selenite, Se(IV)

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Type: Real List Variable

Unit: mM

Argument: Time

0 0.0154

15 0.1233

45 0.1786

60 0.152

90 0.0915

120 0.0446

150 0.0244

180 0.0069

240 0

Se6: Description: Selenate

Type: Dyn. Volume State Var.

Unit: mM

Se6i: Description: Initial Selenate concentration

Type: Formula Variable

Unit: mM

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Expression: 0.5-Se4i

Time: Description: Time

Type: Program Variable

Unit: min

Reference to: Time

X: Description: biomass

Type: Constant Variable

Value: 34

****

Processes

****

rxn0: Description: Formation of elemental selenium, Se(0)

Type: Dynamic Process

Rate: X*kmax40*Se4/(Ks40+Se4)

Stoichiometry:

Variable : Stoichiometric Coefficient

Se0 : 1

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rxn4: Description: Reduction of selenite, Se(IV)

Type: Dynamic Process

Rate: X*kmax40*Se4/(Ks40+Se4)-X*kmax64*Se6/(Ks64+Se6)

Stoichiometry:

Variable : Stoichiometric Coefficient

Se4 : -1

---

rxn6: Description: reduction of selenate, Se(VI)

Type: Dynamic Process

Rate: X*kmax64*Se6/(Ks64+Se6)

Stoichiometry:

Variable : Stoichiometric Coefficient

Se6 : -1

****

Compartments

****

Reactor: Description: mixed batch reactor

Type: Mixed Reactor Compartment

Compartment Index: 0

Active Variables: Se0, Se4, Se6

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Active Processes: rxn0, rxn4, rxn6

Initial Conditions:

Variable(Zone) : Initial Condition

Se4(Bulk Volume) : Se4i

Se6(Bulk Volume) : Se6i

Inflow: 0

Loadings:

Volume: 1

Accuracies:

Rel. Acc. Q: 0.001

Abs. Acc. Q: 0.001

Rel. Acc. V: 0.001

Abs. Acc. V: 0.001

****

Definitions of Calculations

****

calc1

Calculation Number: 0

Initial Time: 0

Initial State: given, made consistent

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Step Size: 1

Num. Steps: 280

Status: active for simulation

active for sensitivity analysis

****

Definitions of Parameter Estimation Calculations

****

fit1

Calculation Number: 0

Initial Time: 0

Initial State: given, made consistent

Status: active

Fit Targets:

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

Se4meas : Se4 (Reactor,Bulk Volume,0)

****

Plot Definitions

****

Plot1: Description: Plot of selenium species over time for initial Se(VI) = 0.5

Abscissa: Time

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Title: Plot 1

Abscissa Label: Time, min

Ordinate Label: Concentration, mM

Curves:

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

Value : Se0 [0,Reactor,Bulk Volume,0]

Value : Se4 [0,Reactor,Bulk Volume,0]

Value : Se6 [0,Reactor,Bulk Volume,0]

Value : Se4meas [0,Reactor,Bulk Volume,0]

****

Calculation Parameters

****

Numerical Parameters: Maximum Int. Step Size: 1

Maximum Integrat. Order: 5

Number of Codiagonals: 1000

Maximum Number of Steps: 1000

Fit Method: secant

****

Calculated States

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****

Calc. Num. Num. States Comments

Range of Times: 0 - 280

Parameters for sensitivity analysis:

kmax40, kmax64, Ks40, Ks64, Se4i, X

****

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