ENZYMATIC RECOVERY OF RHODIUM(III) FROM AQUEOUS SOLUTION AND INDUSTRIAL EFFLUENT USING SULPHATE REDUCING BACTERIA: ROLE OF A HYDROGENASE ENZYME

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ABSTRACT

In an attempt to overcome the high maintenance and costs associated with traditional physico-chemical methods, much work is being done on the application of enzymes for the recovery of valuable metals from solutions and industrial effluents. One of the most widely studied enzymatic metal recovery systems uses hydrogenase enzymes, particularly from sulphate reducing bacteria (SRB). While it is known that hydrogenases from SRB mediate the reductive precipitation of metals, the mechanism of enzymatic reduction, however, is not yet fully understood. The main aim of the present study was to investigate the role of a hydrogenase enzyme in the removal of rhodium from both aqueous solution and industrial effluent.

A quantitative analysis of the rate of removal of rhodium(III) by a resting SRB consortium under different initial rhodium and biomass concentrations, pH, temperature, presence and absence of SRB cells and electron donor, was studied. Rhodium speciation was found to be the main factor controlling the rate of removal of rhodium from solution. SRB cells were found to have a higher affinity for anionic rhodium species, as compared to both cationic and neutral species, which become abundant when speciation equilibrium was reached. Consequently, a pH-dependant rate of rhodium removal from solution was observed. The maximum SRB uptake capacity for rhodium was found to be 66 mg rhodium per g of resting SRB biomass. Electron microscopy studies revealed a time-dependant localization and distribution of rhodium precipitates, initially intracellularly and then extracellularly, suggesting the involvement of an enzymatic reductive precipitation process. A hydrogenase enzyme capable of reducing rhodium(III) from solution was isolated and purified by PEG, DEAE-Sephacel anion exchanger and Sephadex G200 gel exclusion. A distinct protein band with a molecular weight of 62kDa was obtained when the hydrogenase containing fractions were subjected to a 10% SDS-PAGE. Characterization studies indicated that the purified hydrogenase had an optimum pH and temperature of 8 and 40°C, respectively. A maximum of 88% of the initial rhodium in solution was removed when the purified hydrogenase was incubated under

hydrogen. Due to the low pH of the industrial effluent (1.31), the enzymatic reduction of rhodium by the purified hydrogenase was greatly retarded. It was apparent that industrial effluent pretreatment was necessary before the application an enzymatic treatment method. In the present study, however, it has been established that SRB are good candidates for the enzymatic recovery of rhodium from both solution and effluent.

Key words: ***Sulphate reducing bacteria, Hydrogenase, Rhodium, Enzymatic reduction.***

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LIST OF ABBREVIATIONS

AAS = Atomic absorption spectrophotometer

ATP = Adenosine triphosphate

ATPase = Adenosine triphosphate synthase

BSA = Bovine serum albumin

C_3 = cytochrome c_3

COD = Chemical oxygen demand

Da = Daltons

DCIP = 2,6-Dichloro-indophenol

DEAE = Diethylaminoethyl

EDTA = Ethylene diamine tetraacetic acid

ETC = electron transfer complex

g = Acceleration due to gravity

H_{ase} = Hydrogenase

k = Kilo

K_M = Michaelis constant

M = Molar

M^{2+} = Divalent metal ions

mg/l = Milligrams per litre

ml = Millilitres

mM = Millimolar

NADH = Nicotinamide adenine dinucleotide

nm = Nanometer

PEG = Polyethylene glycol

PGMs = Platinum group metals

q_{max} = Maximum metal uptake capacity

SDS-PAGE = Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM = Scanning electron microscopy

SRB = Sulphate reducing bacteria

TEM = Transmission electron microscopy

U = Units of enzyme activity (μmol/min)

V_{max} = Maximum velocity

v/v = Volume per volume

w/v = Weight per volume

XRF = X-ray flourescence

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Platinum group metals (PGMs) comprise: platinum, palladium, rhodium, iridium, ruthenium, and osmium. They are currently receiving worldwide attention as they offer the dual attraction of rare, high-value precious metals as well as having major industrial uses due to their unique physical and chemical properties (Kayanuma *et al.,* 2004). Generally PGMs, display a low reactivity and have high boiling and melting points, which makes them resistant to corrosion and heat, respectively (Rao and Reddi, 2000). Consequently, they have important applications as catalysts in car converters, enabling petroleum and other fuels and chemicals to be produced efficiently from crude oil. PGMs also have a permanent lustre, which makes them useful in the manufacture of jewellery (Kendall, 2002). Among the PGMs, rhodium is the most difficult to work with as it is regarded as being chemically stable. Its extreme solubility in aqueous solution makes it rather difficult to precipitate and consequently difficult to extract (Mohammed *et al.,* 1998). For this reason the price of rhodium has increased over the years due to an increased industrial demand, along with an increase in its depletion rate as a non-renewable resource. The natural abundance of rhodium is very low (about 0.1μg per kg of the earth's crust) (Ravindra *et al.,* 2004), consequently, it has become apparently clear that there is an urgent need to reclaim rhodium from secondary sources, in order to be able to meet all its industrial demands.

The ideal metal reclamation process to be adopted should: 1) be flexible to handle changes in the composition of the effluent, 2) simple to minimize the need for highly skilled labour, 3) compatible with existing upstream processes, 4) reliable with the constant supply of effluent and 5) cost effective (Nowottny *et al.,* 1997). Currently existing rhodium extraction and reclamation processes utilize a combination of physical and chemical methods. Such methods, however, result in high operational and maintenance costs and often result in the accumulation of large quantities of sludge that require further treatment. This has prompted further investigations on the application of biological processes, as a cost effective alternative for effective metal recovery purposes. Of all the biological systems investigated; living and non-living, from the smallest to the largest, microorganisms have been found to possess the

ability to accommodate a wide variety of changing environments, mainly brought about by pollutants, both organic and inorganic (Eccles, 1995). One of the most successful biological metal removal systems uses sulphate reducing bacteria (SRB) for metal accumulation. When compared to physical and chemical processes, the SRB metal recovery system is relatively cheap since expensive solvents, reagents or aeration are not necessary. Instead mild growth conditions, such as a neutral pH and moderate temperature are required, and furthermore, there is minimal sludge production (Ohtake and Silver, 1994). The SRB bioremediation system, therefore might as well offer the best solution to the problem of maintaining adequate supplies of rhodium, in the future.

1.2 METAL RECOVERY PROCESSES

1.2.1 Source of metal ions

Metal ions are widely distributed, in low concentrations, within the environment and they differ from other toxic pollutants, because they are non-biodegradable. As a result they tend to accumulate within various tissues, and so become more concentrated throughout the food chain. Natural processes that contribute to the presence of metals within the environment range from the weathering of rocks, to the re-precipitation of metals by wind and water. In addition to natural processes, metal ions are introduced into the environment through interactions between living organisms and metals, as well as the activities of man (Raab and Feldmann, 2003).

The beginning of industrialization and urbanization was manifested by the intensification of industrial activities. The introduction of vehicle exhaust catalysts as well as the use of PGMs for the manufacture of jewellery, as anticancer drugs and in dentistry as alloys, resulted in an increase in their industrial demand. Consequently, considerable quantities of PGMs were released into the environment through these industrial wastewaters. In more developed countries, automotive catalytic converters are the major source of PGMs pollution in the environment. In the absence of catalytic converters, solid or liquid PGMs containing wastes mainly result from mining and mineral processes, and to a lesser extent hospitals and jewellery manufactories (Ravindra *et al.,* 2004). Other traces of heavy metals result from processes such as iron and steel production, mining and mineral processing, the non-ferrous metal industry, pigment manufactures, battery manufacture, the printing and photographic industries and metal working and finishing (electroplating) (Eccles, 1995).

The reserve depletion rate of PGMs, which is used as an indication of the most likely future increase in market prices, has necessitated their recovery from both industrial wastewaters and contaminated soils (Volesky, 2001). The choice of method used for metal recovery not only depends on the metal source characteristics, but also on the applicable technologies (physical, chemical and biological) which are determined by their efficiency in the metal recovery process, operational costs and most importantly the physico-chemical characteristics of the metal to be recovered (Eccles, 1995).

1.2.2 Physico-chemical processes

The depletion of precious metals coupled with the introduction of stricter effluent regulations led to the use of various physical and chemical treatment technologies that were aimed at removing metals from wastewaters. Conventional physical and chemical techniques that have been used include; adsorption, electrochemical, membrane, precipitation and solvent extraction (Table 1) (Eccles, 1995).

Table 1: Characteristics of physical and chemical technologies that have been used for the recovery of heavy metals. Adapted from Eccles (1995).

Technology	Tolerance to pH change	Metal selectivity	Influence of suspended solids	Tolerance to organic molecules	Working level for metal (mg/l)
Adsorption	Limited	Moderate	Fouled	Can be poisoned	<10
Electrochemical	Tolerant	Moderate	Tolerant	Tolerant	>10
Ion exchange	Limited	Selective	Fouled	Can be poisoned	<100
Membrane	Limited	Moderate	Fouled	Intolerant	>10
Hydroxide precipitation	Tolerant	Non selective	Tolerant	Tolerant	>10
Sulphide precipitation	Limited	Limited	Tolerant	Tolerant	>10
Solvent extraction	Limited	Selective	Fouled	Intolerant	>100

Current existing technologies for PGMs extraction and recovery utilize a combination of chemical precipitation, ion exchange and solvent extraction ((Kayanuma, *et al.,* 2004). The suitability of each of the physico-chemical techniques for a particular metal recovery process depends on the pH and toxicity tolerance, influence of suspended solids, metal concentration and ability to recover certain metals (Eccles, 1995). There are limitations, however. The use of membrane technology is limited by the fact that it is tolerant to certain types of organic

molecules and pH values, and the membrane is likely to be degraded in the presence of certain microorganisms. Ion exchange resins, on the other hand are affected by magnesium and calcium ions, and also fouled in the presence of precipitates and organics. Apart from the drawbacks of each method, they are costly as the reactive agents cannot be recovered for re-use in successive treatment cycles (Atkinson *et al.,* 1998). Another disadvantage associated with physical and chemical methods is that they often result in the generation of secondary wastes which present subsequent treatment problems (Veglio and Beolchini, 1997).

1.2.3 Biological processes

Biotechnological approaches that utilize microbial interactions have been used to recover metal ions from metal-bearing effluents. Such approaches have been found to offer practical solutions as they are highly specific, and cost effective alternatives that can be used at a large scale in a range of settings both *in situ* and *ex situ* (Lloyd, 2002). As a result the recovery of metals from various wastewaters using biosorptive and bioaccumulative processes has received significant attention recently. Biosorptive processes are those processes in which metal ions are exchanged for a counter ion attached to the biomass. Biosorption in general involves more than one functional group on the biomass, depending on the pH of the liquid and the chemical characteristics of the metal. Functional groups that have been found to be responsible for the precipitation or non-specific binding of metals on cell surfaces include; carboxyl, sulphonate, phosphate, hydroxyl, amino, imino and imidazole groups (Eccles, 1995). Some of the existing biosorptive and biaccumulative processes that have been successfully used to recover metals from solutions include;

1) THIOPAQ system and the Biomet Mining Corporation biosulphide process - both processes use sulphate reducing bacteria to treat wastewaters, where the metals of interest are precipitated as sulphides. The biological sulphate reduction process occurs in a reactor separate from the metal precipitation to avoid inhibition of SRB growth by heavy metals and to facilitate metal separation and recovery (Jalali and Baldwin, 2000).

2) AMT-BIOCLAIM ^{TM} – this process utilizes a biosorbent (*Bacillus* biomass) to remove metals from dilute solutions (10 to 100 mg/l), and reduce their concentrations to below 1 mg/l. Metals that have been accumulated using this process, include; gold, cadmium and zinc. AMT-BIOCLAIM ^{TM} is non-selective and the metals are stripped using sulphuric acid, sodium hydroxide or complexing agents, and the biosorbent can be regenerated for repeated use (Gupta *et al.,* 2000).

3) AlgaSORB ™ – this process utilizes a non-living algal biomass (*Chlorella vulgaris*) to recover about 99% of metal ions previously in solution. This process is capable of metal ion selectivity, and has been shown to significantly reduce cadmium and mercury ion concentrations in drinking water (Atkinson *et al.,* 1998).

With the successful development of these biosorptive and bioaccumulative processes for metal recovery, the next challenge is to develop an enzymatic process, which will be more effective in metal accumulation. Up to date (2004) the enzymatic reduction process has been studied on a wide range of metals, including; platinum (Rashamuse, 2003), palladium (Lloyd *et al.,* 1998), uranium (Macaskie *et al.,* 2000; Lovley and Phillips, 1992), chromium (Lovley and Phillips, 1994) and technetium (Lloyd *et al.,* 1999).

1.3 MICROBIAL TRANSFORMATION OF METALS

1.3.1 Microbe-metal interactions

Microorganisms play a vital role in determining the availability of metals within the environment, through physico-chemical and biological processes that result in metal transformations (Figure 1.1) (Valls and de Lorenzo, 2002). These processes are essential components of natural biogeochemical cycles for metals and they can be manipulated to ensure adequate supplies of precious metals, while also relieving the environment from heavy metals contamination (White *et al.,* 1997). Microbial processes that aid the removal of metals from a metal-bearing solution affect their solubility, mobility and bioavailability (Raab and Feldmann, 2003). Microbial processes that effect the mobilization of metals include; enzymatic transformations, chelation and protonation, while immobilization of metals occurs through precipitation or crystallization of insoluble organic or inorganic compounds or by sorption, uptake and intracellular sequestration (White *et al.,* 1997).

These processes are exhibited by microorganisms as response mechanisms when exposed to a high metal concentration. There are six established mechanisms, in which microorganisms interact with metals, and these are: exclusion by permeability barrier, intra- and extra-cellular sequestration, active transport efflux pumps, enzymatic detoxification and reduction in the sensitivity of cellular targets to ions. A proper understanding of these microbe-metal interactions can provide an insight into the different processes that can be used to aid the remediation of the environment and wastewaters contaminated with metals and certain organics (Bruins *et al.,* 2000). The type of resistance mechanism that microorganisms exhibit

in response to the presence of metal ions depends on the concentration of the available metal species, for example, through enzymatic transformations, sulphate reducing bacteria can tolerate metal concentrations up to 100μM (Figure 1.2) (Valls and de Lorenzo, 2002).

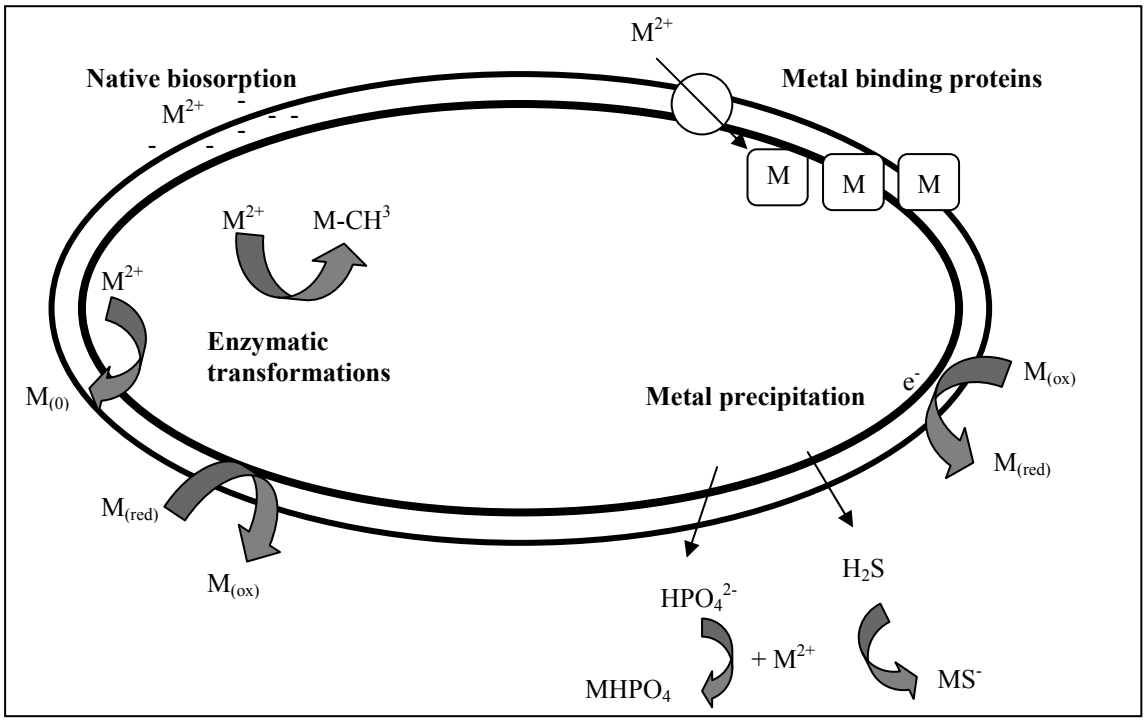

Figure 1.1: Microbial processes within bacterial cell compartments in the presence of metal ions (M^{2+}). Adapted from Valls and de Lorenzo (2002).

METAL CONCENTRATION											
NANO			MICRO			MILLI			MOLAR		
1	10	100	1	10	100	1	10	100	1	10	
o	o	o	o	o	o	o	o	o	o	o	
MECHANISM											
Metallothionein			Export Enzymatic transformations					Precipitation			
ORGANISMS											
Eukaryotes Cyanobacteria			Gram-positives Gram-negatives e.g. *E.coli*, *Alcaligene* sp., *Desulfovibrio* sp.					Acidophilic chemolithotrops e.g. Archaea, *Thiobacillus* sp.			

Figure 1.2: Metal tolerance capacity of microorganisms. Adapted from Valls and de Lorenzo (2002).

1.3.1.1 Metal exclusion by permeability barrier

This mechanism is widespread among microorganisms, particularly bacteria, and it prevents metals from interacting with vital or metal-sensitive cellular components. Metals are barred from entering the cell through one or a more of the following mechanisms: alterations in the cell wall, membrane or envelope of microorganisms. A common example is the exclusion of Cu(II) by *E.coli* through the altered production of the membrane channel protein porin (Rouch *et al.,* 1995). Microorganisms that possess extracellular polysaccharides have the ability to biosorb metals and prevent them from entering essential cell components (Scott and Palmer, 1990).

1.3.1.2 Intracellular and extracellular sequestration

Alternatively metals can be effectively accumulated within or outside the cell as a response mechanism to a high metal concentration. During intracellular sequestration metals are accumulated in the cytoplasm due to the presence of metallothioneins and cysteine-rich proteins, which bind metal ions (Silver *et al.,* 1989). The extracellular accumulation of metals is not a common activity among all microorganisms; it is only common in bacteria and several species of yeast and fungi. These microorganisms excrete glutathione, which has a great affinity for heavy metals (Murata *et al.,* 1985).

1.3.1.3 Active transport pumps

This is the most common metal resistance mechanism exhibited by most microorganisms to keep away metals from essential cell components. Non-nutrient or toxic metals enter the cell through normal nutrient transport systems, but once inside the cell they are immediately exported by chromosomal or plasmid-encoded active transport mechanisms. In addition active transport systems can either be non-ATPase or ATPase-linked, and highly specific for the metal ion they export (Silver *et al.,* 1989).

1.3.1.4 Enzymatic detoxification

The enzymatic detoxification of mercury is the most widely known example of metal resistance mechanism exhibited by most Gram negative (*E.coli, P. aeruginosa, Serratia marcescens* and *Thiobaccilus ferroxidans*) and Gram positive (*Staphylococcus aureus, Bacillus* sp.) bacteria. These species contain a Hg(II) (mer) resistance operon, which both detoxifies and transports and self-regulates the resistance of mercury. The operon encodes for the production of a periplasmic binding protein, which collects any available Hg(II) and then

membrane-associated transport proteins take it to the cytoplasm for detoxification. This Hg(II) (mer) resistance operon also codes for the production of two enzymes. The first one, organomercuric lyase is effective in hydrolyzing the stable mercury-carbon bond and thereby producing a mercury thiolate adduct, which is produced when glutathione is exchanged for the cysteine ligands of the enzyme (Equation 1). The second enzyme, mercuric reductase then reduces the formed mercuric compound [Hg(II)] to metallic mercury [Hg(0)], a process that involves hydride transfer from the electron carrier NADH to flavin (Equation 2) (Misra, 1992).

$$RHgX + H^+ + X^- \longrightarrow RH + HgX_2 \quad \text{.....(1)}$$

$$Hg(SR)_2 + NADPH + H^+ \longrightarrow Hg(0) + NADP^+ + 2RSH \text{.....(2)}$$

1.3.1.5 Reduction in metal sensitivity of cellular targets

Reduction in metal sensitivity of cellular components is an adaptation mechanism developed in some microorganisms due to the presence of toxic metals. Protection is achieved by mutations which result in the alteration of sensitivity of cellular components, or by using alternate pathways in an effort to bypass sensitive components and by also producing metal-resistant components (Rouch *et al.,* 1995).

1.3.2 Metal mobilization

Metal mobilization results from the dissolution of insoluble metals due to oxidation-reductions, and production of mineral or organic acid, as well as changes in pH, which affects the ionic state of the metal (Gazso, 2001).

1.3.2.1 Bioleaching (Enzymatic oxidation)

Enzymatic oxidation is a very useful method of recovering inorganic compounds from solution, especially in cases where the higher oxidation state is less soluble. As a result bioleaching has an important application in the extraction of various metals from their ores, for example, the bioleaching of Cu, Mo, Ni, Pb and Zn from sulphidic ores, and the extraction of uranium from uraninite. Bacteria involved in the bioleaching process include; *Thiobacillus ferroxidans* and *Leptospirillum*, and both are capable of Fe oxidation (Gazso, 2001). Metal solubilization is brought about by the ferric iron in acidic solution, which attacks the ore and dissolves the metal (Ehrlich, 1997).

1.3.2.2 Enzymatic reduction

Microorganisms offer a potentially large gene pool to choose from when searching for enzymes that may be potentially useful for the treatment of metal contaminated wastewaters. These enzymes catalyse several reactions that result in the reduction of metals, from a high valence state to a lower one with or without the help of electron transfer proteins. The enzymatic reduction of metals by facultative and obligate anaerobe microorganisms also has potential applications of *in situ* bioremediation, and has been of much interest recently (Ehrlich, 1997). Different metals have been shown to be enzymatically reduced by a range of microorganisms, for example, the enzymatic reduction of the toxic oxidised form of chromate CrO_4^{2-} to $Cr(OH)_3$ by *Pseudomonas fluorescens* LB300 or *Enterobacter cloace* (Ehrlich, 1997). Among all the microorganisms studied, sulphate reducing bacteria have received much attention because of their ability to enzymatically aid the reductive precipitation of a range of metals including Pt(IV)), Pd(II), Cr(VI), Tc(VI) As(V) and Mo(VI) (Rashamuse, 2003; (Lloyd *et al.,* 1998; Smith and Gadd, 2000; Lloyd *et al.,* 1999; Lovley and Phillips, 1994; Tucker *et al.,* 1998). The enzymatic metal reduction processes carried out by SRB has proved to be one of the most important bioremediation applications for effective recovery of specific metals from metal-contaminated wastewaters (Lloyd *et al.,* 2001).

1.3.2.3 Complexation

Metals in solution may also be adsorbed to cell surfaces through the formation of complexes between the metals and active groups. During complexation, the metal ions bind to a ligand to form a stable complex. Microbial complexing agents range from low molecular weight organic acids and alcohols, to high molecular weight ligands. Toxic metal binding compounds and some bacterial amino acids have been found to have complexing abilities (Gazso, 2001). The microorganism *Pseudomonas syringe* has been found to effect the mobilization of a range of metals, including; calcium, magnesium, cadmium, zinc, copper and mercury solely through complexation (Veglio and Beolchini, 1997).

1.3.2.4 Siderophores

Siderophores are highly specific Fe(III)-chelating compounds that are excreted by microorganisms when they are grown in the absence of iron. In addition to aiding iron assimilation, siderophores also possess the ability to bind other metals such as magnesium, manganese, chromium(III), gallium(III) and radionuclides such as plutonium(IV). Binding groups involved include; catecholate, phenolate or hydroxamate, and play an important role in

the mobilization of metals within the environment (Gazso, 2001). Siderophore-mediated metal solubilization has been used to treat metal-contaminated sandy soils by *Alcaligenes eutrophus* (Gadd, 1988).

1.3.3 Metal immobilization

The immobilization of metals is mainly a result of cellular sequestration and accumulation, or through extracellular precipitation. During immobilization metals are converted to their insoluble form, through metabolism dependent or independent processes. As a result it has been the centre of much research because of its potential application in wastewater treatment (Bruins *et al.,* 2000).

1.3.3.1 Biosorption

Biosorption can be simply defined as the metabolism-independent sorption or uptake of metals and radionuclides from solution onto biomass. Once the threshold limit is reached, however, further metal uptake can result in cell death. Biosorption occurs through cell surface complexation, ion exchange, adsorption, electrostatic and hydrophobic interactions and microprecipitation. Based on these processes and the property of cell biomasses to bind and accumulate metals, biosorption has become an alternative technology to remove metals from dilute aqueous solutions (Veglio and Beolchini, 1997). The surface of cells carry a net negative charge due to the presence of carboxyl, hydroxyl, phosphate and sulphydryl groups, and so during biosorption the metal cations are strongly bound (Lloyd, 2002).

Metal binding appears to be a two-step process, where the first step involves a stoichiometric interaction between the metal and the reactive chemical groups in the cell wall. The second step is an inorganic deposition of the metal on the cell surface. In comparison with other conventional metal biorecovery processes, biosorption kinetics are fairly rapid, usually in the order of seconds or minutes. Previous studies have shown that the majority of metal ions are removed within the first 15 minutes (Atkinson *et al.,* 1998). Different microorganisms have been found to vary in their affinity for different heavy metals and hence differ in their metal binding capacities. For example, it was found that *Desulfovibrio desulfuricans* had more biosorptive capacity for palladium and platinum when compared to *D. fructosivorans* and *D. vulgaris* (de Vargas *et al.,* 2004). The overall amount of metal removed by biosorption is evaluated using isotherm curves derived from equilibrium batch sorption experiments, which are a function of various parameters such as pH, biomass loading, biomass pre-treatments (Sag

et al., 2000). Although biosorption as a metal recovery technique has been suggested to be cheaper and more effective, it has limitations. The low binding capacity of biomass in the presence of certain recalcitrant metals in industrial effluents such as Ni, and failure to effectively remove metals from real industrial effluents due to the presence of organic and inorganic ligands has limited this approach. To overcome such problems, much work has been done on the development of commercial biosorbents that are metal specific (Atkinson *et al.,* 1998). Two commercially available biosorbents include; AlgaSORB™ (*Chlorella* vulgaris) and ATM-BIOCLAIM ™ (*Bacillus* biomass). AlgaSORB™ is capable of decreasing the levels of both cadmium and mercury to below 1mg/l, whereas ATM-BIOCLAIM ™ has been shown to remove maximum amounts of gold, cadmium and zinc from cyanide solutions (Kuyucak and Volesky, 1988).

1.3.3.2 Precipitation

Precipitation of metals by microorganisms may be either metabolism-dependent or independent. During metabolism-dependent precipitation or dissimilatory reduction, the transformation of the metal is unrelated to its intake by the microbial catalyst. Consequently, the reduced metal is precipitated in the extracellular medium, as a defense system (Valls and de Lorenzo, 2002). For example, cadmium removal from solution by *Arthrobacter* and *Pseudomonas* species was determined by detoxification systems that precipitate cadmium on the cell surface (Scott and Palmer, 1990). Another well documented example is the reductive precipitation of soluble U(VI) compounds to insoluble U(IV) hydroxide or carbonate at neutral pH (Phillips *et al.,* 1995). Much research has also been done on SRB, because they can enzymatically mediate the precipitation of heavy metals including; U(VI), Cr(VI), Tc(VI) and As(V) (Lloyd, 2003). During enzymatic precipitation, the adsorbed metal is precipitated at the cell periphery as an insoluble low-valence oxide. Among the SRB it has been suggested that membrane-bound hydrogenases that are either *c*-type cytochromes or associated with such cytochromes catalyze the dissimilatory reduction of metals (Lloyd *et al.,* 1999).

In the case where precipitation is independent of cellular metabolism, it is normally a result of the chemical interaction between the metal and a biogenic ligand on the cell surface (Veglio and Beolchini, 1997). The most widely known natural metal precipitation mechanism is that of sulphide precipitation by SRB. Under anaerobic conditions, SRB produce significant amounts of hydrogen sulphide, and the high environmental sulphide concentrations produced

lead to the precipitation of metal ions as insoluble metal sulphides (Equation 3) (White *et al.*, 1997).

$$M^{2+}_{(soluble)} + S^{2-} \longrightarrow MS_{(insoluble)} \quad \text{.....(3)}$$

In other microorganisms metal precipitation is mediated by the liberation of inorganic compounds, such as; oxalates, phosphates or hydroxides from organic donor molecules (Tucker *et al.,* 1998). A well documented example involves the precipitation of metals by *Citrobacter* sp. This bacterium was found to be tolerant to high concentrations of uranium, nickel, and zirconium through the formation of highly insoluble metal phosphates (Equation 4) (Macaskie *et al.,* 2000).

$$M^{2+}_{(soluble)} + HPO_4^{2-} \longrightarrow MHPO_{4\,(insoluble)} \quad \text{.....(4)}$$

1.3.3.3 Bioaccumulation

Bioaccumulation is an energy dependent process of metal uptake against a concentration gradient. During bioaccumulation metals are transported from the outside of the microbial cell, across the cellular membrane, and into the cell cytoplasm, where the metal is sequestered and therefore immobilized. Examples include the intracellular accumulation of mercury, lead, silver, cadmium and uranium by an energy dependent transport system (Gadd, 1988). The bioaccumulation of metals by intact SRB cells include; Cr(VI) (Michel *et al.,* 2001), platinum (Rashamuse, 2003), Tc(VII) (Lloyd *et al.,* 1999).

1.4 SULPHATE REDUCING BACTERIA

1.4.1 Ecology

SRB are a diverse group of heterotrophic bacteria that require strictly anaerobic conditions, with a redox potential (E_h) of less than -200mV (White *et al.,* 1997). Some strains, however, have been found to also grow in aerobic environments, displaying a high oxygen tolerance and capacity of oxygen respiration in comparison to strains from anoxic sediment layers. In pure cultures oxygen is known to inhibit the sulphate reduction process, however SRB growing in oxic environments consume O_2 by respiration, a mechanism which avoids their exposure to molecular oxygen (Sass *et al.,* 1997). Maximum SRB growth occurs around slightly acidic to slightly alkaline pH (between pH 6-8), with a few exceptional strains that are acid-tolerant and can grow in the pH range 3-4. Most SRB are mesophilic, attaining optimum

growth at a temperature range of 25°C-30°C. Some, however, are able to grow at temperatures below 5°C while spore forming thermophilic species grow at temperatures ranging from 65°C to 85°C. SRB are ubiquitous and active sulphate reduction has been detected in freshwater environments and soils. They have also been detected in polluted environments, spoiled foods, anaerobic purification plants, and sewage plants (Holmer and Storkholm, 2001).

1.4.2 Phylogeny

Based on the rRNA sequence analysis, SRB can be divided into four groups: Gram-negative mesophilic, Gram-positive spore forming, thermophilic bacterial, and the thermophilic archael SRB (Castro *et al.,* 2000). These bacteria display great morphological and physiological diversity; however they all conserve energy by the reduction of sulphate (SO_4^{2-}) to hydrogen sulphide (H_2S) in anoxic environments (Lloyd *et al.,* 2001). To gain a further understanding of the different types of SRB within each group: cell shape, motility, GC content of DNA (number of positions in which the DNA codeword has coordinate G or C), presence of desulfovibrin and cytochromes, optimal temperature, and complete vs. incomplete oxidation of acetate have been used to categorize them. Below is a brief description of the various SRB groups:

1) The Gram-negative mesophilic SRB is mainly dominated by the genera *Desulfobulbus, Desulfomicrobium, Desulfomonas, Desulfovibrio, Desulfobacter, Desulfobacterium, Desulfococcus, Desulfomonile, Desulfonema* and *Desulfosarcina.*

2) The Gram-positive spore forming class include those SRB that are known to form heat-resistant endospores, and so are known to survive long dessication and oxic conditions. So far they comprise of only one family, *Desulfotomaculum*.

3) The thermophilic bacterial SRB are characterized by high optimal growth temperatures when compared to the Gram-negative and Gram-positive spore forming SRB. Two species have been identified within this class,and they are; *Thermodesulfobacterium commune* and *Thermodesulfovibrio yellowstonii.*

4) The archael thermophilic SRB has optimum growth temperatures above 80°C, and to date only two species have been identified; *Archaeoglobus fulgidus* and *A.profundus* (Castro *et al.,* 2000).

1.4.3 Physiology

Sulphate-reducing bacteria (SRB) derive their energy through anaerobic respiration, whereby sulphate and other sulphur compounds are reduced to hydrogen sulphide. SRB are able to use hydrogen as an energy source for sulphate reduction. The main organic carbon or energy substrates utilized by the fastest growing organisms, for example, *Desulfovibrio species*, are low molecular mass organic acids such as lactic or acetic acid, aromatic compounds such as phenols and phenol derivatives and alcohols such as ethanol (Bak and Widdel, 1986). The oxidation of these compounds, however, with the exception of lactate, is incomplete and results in the formation of acetate as an end product. As a result lactate has been identified as the best source of carbon for SRB growth (Postgate, 1984).

When hydrogen is used as an energy substrate, its oxidation is mainly driven by periplasmic hydrogenases, and protons are accumulated in the periplasm, and electrons are transferred to the cytoplasm for sulphate reduction (Louro *et al.,* 1998). When lactate is used as electron donor, however, there is a hydrogen cycling mechanism in which lactate oxidation leads to the cytoplasmic generation of hydrogen. This hydrogen then diffuses to the periplasm where it is reoxidized, thus generating electrons that are transferred across the membrane for sulphate reduction (Odom and Peck, 1981).

The reduction of sulphate occurs through a series of biochemical steps involving an electron transport chain linking dehydrogenases to the terminal reductases (Figure 1.3). Sulphate is transported across the membrane into the cell, and through the action of ATP sulphurylase is combined with ATP to form APS and pyrophosphate which is eventually cleaved to produce inorganic phosphate. This is preceeded by the immediate conversion of APS into sulphite (SO_3^-) with the help of APS reductase, a cytoplasmic enzyme. Finally, a wide range of intermediates are involved in the reduction of sulphite to yield sulphide (Gibson, 1990).

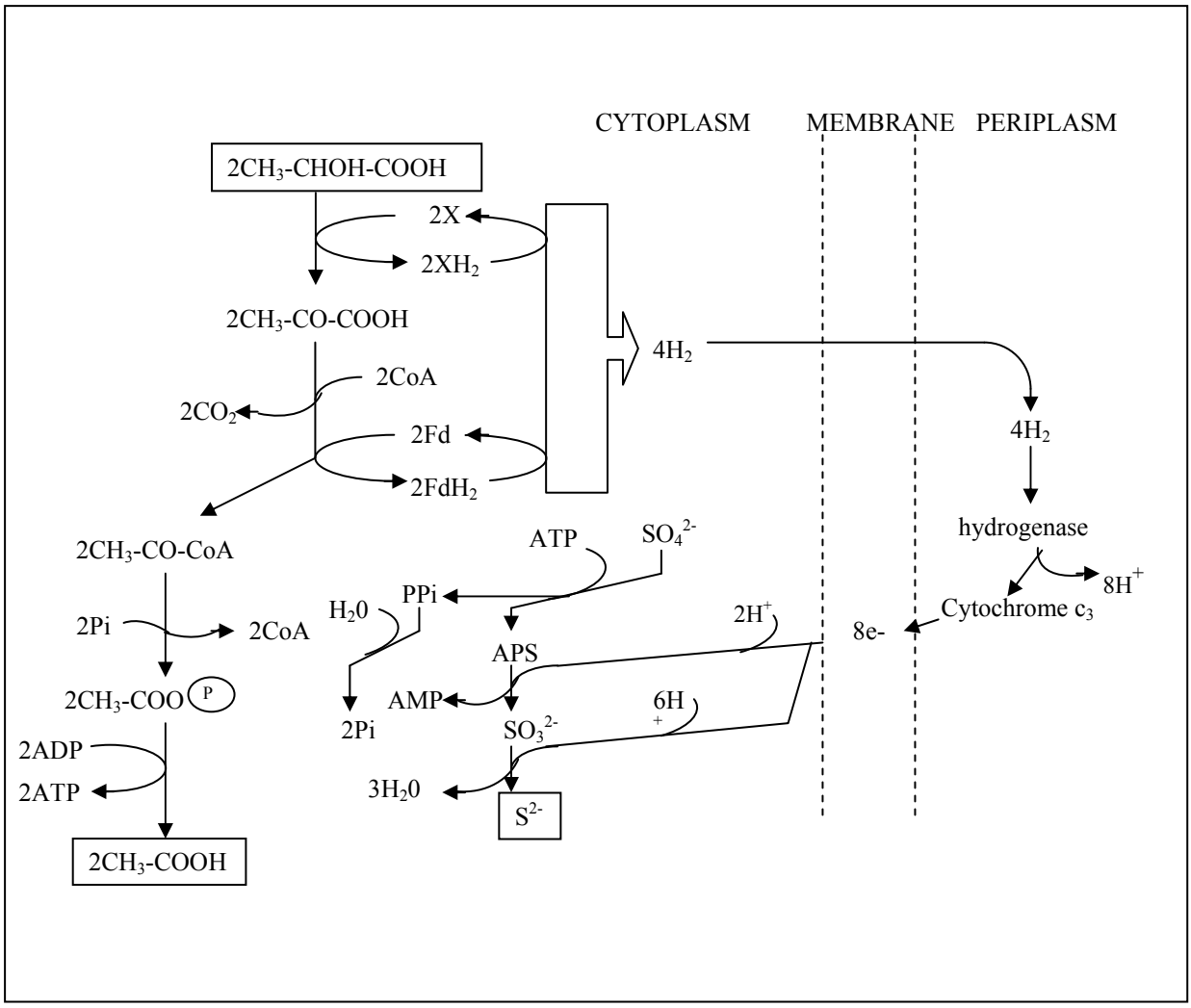

Figure 1.3: Key biochemical pathways of sulphate reducing bacteria for sulphate reduction and sulphide generation. Adapted from Lie *et al.,* (1996).

1.5 METAL REDUCTASE ACTIVITY IN SULPHATE REDUCING BACTERIA

Sulphate reducing bacteria are well known for their involvement in the enzymatic reduction of various metals in both terrestrial and aquatic environments. Generally, metal reductase activity is widespread among SRB; however most metal reduction studies that have been done involve the *Desulfovibrio* genus. So far, the main key enzymes that have been implicated during the reduction of metals are hydrogenases, along with their physiological carrier protein cytochrome c_3. The different types of hydrogenases found within SRB that are involved in the enzymatic reduction of metals are briefly outlined below.

1.5.1 Hydrogenases

Hydrogenases catalyze the reversible oxidation of molecular hydrogen and play a central role in microbial energy metabolism. Three types of hydrogenases, namely: Fe-only, NiFe- and NiFeSe-hydrogenase, have been isolated and characterized within the *Desulfovibrio* genus. Based on the metals present in the active site and subunit, physico-chemical characteristics, amino acid sequences, immunological reactivities, gene structures and catalytic properties two classes dominate: Fe-only and NiFe-hydrogenases. Studies on microbial genome sequences have revealed that Fe-hydrogenases are restricted to bacteria and eucarya, while NiFe-hydrogenases, with one possible exception, seem to be present only in archaea bacteria (Vignais *et al.,* 2001). Both the NiFe- and Fe-hydrogenases contain one or more Fe-S clusters to store and transport electrons. The active sites of both (Fe-only and NiFe-) hydrogenases are characterized by a diatomic π-acceptor ligands CO and CN^- (Figure 1.4a and b) (Armstrong, 2004).

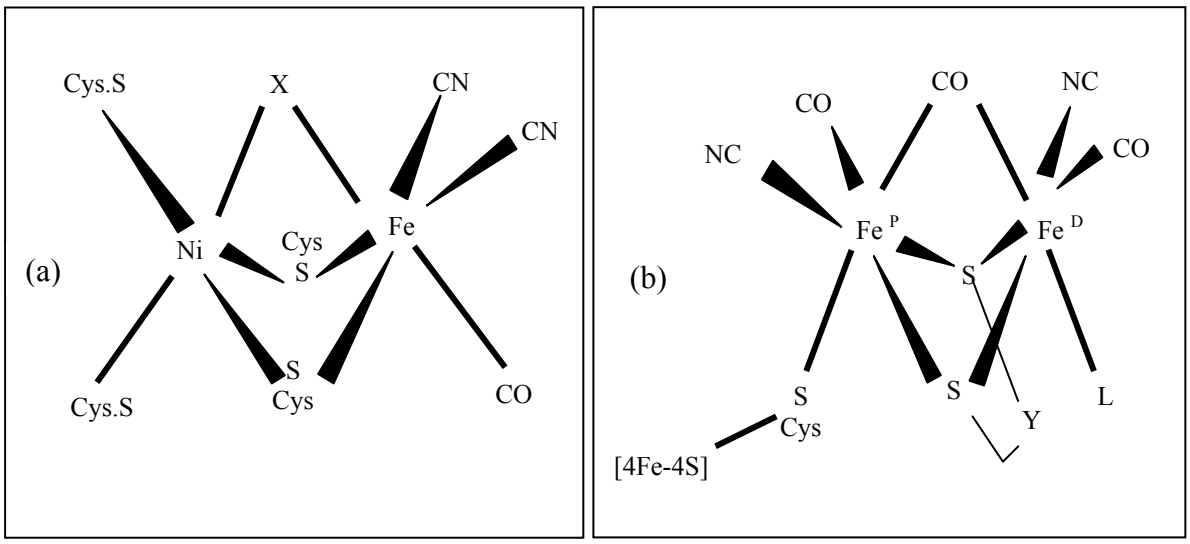

Figure 1.4: The active sites of (a) NiFe hydrogenases and (b) Fe-only hydrogenases. Where X is an additional bridging ligand, which is either an oxo or hydroxo group in the inactive forms Ni-A and Ni-B, and a hydride in the active form Ni-C, L is an exchangeable ligand (H_2O) and Y may be an amino-N atom. Adapted from Armstrong (2004).

NiFe-hydrogenases are present in all known *Desulfovibrio* species. Its catalytic core is a heterodimeric protein, although additional subunits are present in many of these enzymes. The structure around the NiFe-hydrogenase active sites is characterized by two thiolate side chains of cysteinyl residues that are coordinated to a Ni atom, two other thiolate side chains of

cysteinyl residues and one monatomic ligand coordinated to both the Ni and Fe atoms as bridges and three diatomic ligands are linked to the Fe centre. However, there are slight differences between species, for example *Desulfovibrio gigas* enzyme possesses an oxygen species as a monatomic bridge and one CO and two CN groups as diatomic ligands whereas *D.vulgaris* Miyazaki F enzyme has a sulphur species as a monatomic bridge and SO, CO and CN ligands (Türker, 2003).

The catalytic core of the Fe-hydrogenases is a ca. 350-residue domain that accommodates the active site (H-cluster). Fe-hydrogenases are composed of two subunits of 42 and 10 kDa, respectively. The large subunit contains a ferredoxin-like domain with two [4Fe–4S] clusters and a so-called H-cluster constituted of a regular [4Fe–4S] cluster bridged to an active site Fe binuclear centre believed to be involved in the activation of hydrogen. A few monomeric Fe-hydrogenases are about the same size as the H-cluster domain. Many others are monomeric as well, but possess additional domains that contain redox centres, mostly iron–sulphur, and some Fe-hydrogenases are oligomeric. However, not all *Desulfovibrio* species possess the Fe-only hydrogenase (Fauque *et al.,* 1988).

In both enzymes (NiFe- and Fe- hydrogenases), the cleavage of hydrogen is heterolytic, which implies that both catalytic sites should contain both a base and a hydride acceptor. In addition, the hydrogen–proton interconversions in both hydrogenases are also made difficult by oxidative inactivation processes. When compared to their NiFe- counterparts, Fe-only hydrogenases have been found to be more active in proton reduction, while during hydrogen oxidation the activity of NiFe hydrogenases is much higher. Isolated NiFe-hydrogenases were found to catalyze both H_2 evolution and uptake with cytochrome c_3 acting as either electron donors or acceptors, depending on their oxidation state (Armstrong, 2004).

A third and rare class known as the NiFeSe-hydrogenase is believed to exist among the *Desulfovibrio* genus. As opposed to NiFe-hydrogenases, NiFeSe-hydrogenases have one cysteine amino acid replaced by a selenocysteine (Figure 1.5). They also contain equimolar amounts of nickel and selenium, and (4Fe-4S) centres. The functional properties of the NiFe- and NiFeSe-hydrogenases are very similar with respect to the turnover of hydrogen (Fauques *et al.,* 1988).

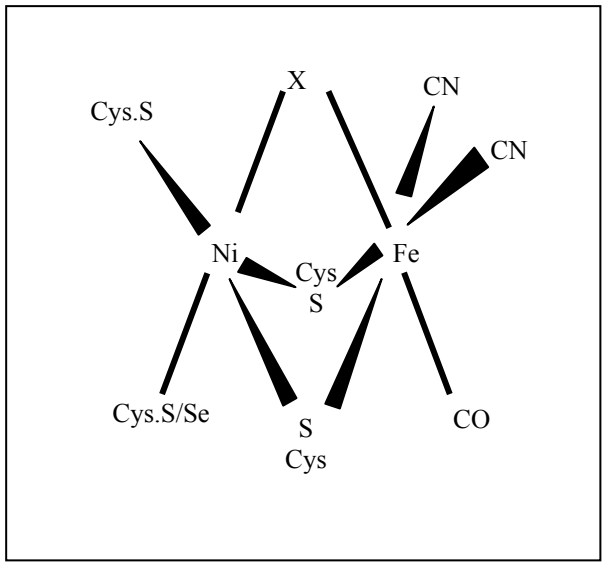

Figure 1.5: Schematic representation of the active centre of NiFeSe-hydrogenases. The Ni and Fe atoms are bridged by two cysteines. In addition there are two terminal cysteine ligands to the Ni, one of which is replaced by a selenocysteine. Adapted from Fauques *et al.* (1998).

1.5.2 Cytochrome c_3

Cytochromes c_3 (M_r 13000) are multiheme low redox potential (-200 to -400mV) periplasmic proteins that are widely distributed among the *Desulfovibrio* species. They contain four hemes covalently bound to the peptide chain with two histidines as axial ligands (Figure 1.6). They present no structural similarities to mitochondrial cytochrome c. Although the sequence identity of cytochrome c_3 among the *Desulfovibrio* species is low, the overall three-dimensional structure and spatial arrangement of the four heme groups is very well conserved (Lojou *et al.,* 1998). *Desulfovibrio* species may contain other soluble multiheme cytochromes including the high molecular weight cytochrome (Hmc). In *D. vulgaris* Hildenborough, it has been reported that the tetraheme cytochrome c_3 mediates the reduction of the high molecular weight cytochromes (Hmc) by the Fe-hydrogenase (Elantak *et al.,* 2003). Cytochrome c_3 functions as a coupling protein to periplasmic hydrogenase and has been involved in a concerted proton-assisted two-electron step (Figure 1.7). Periplasmic hydrogenase provides cytochrome c_3 (C_3), with both energized electrons (e^-) and deenergized protons (H^+) produced in the oxidation of molecular hydrogen (Louro *et al.*, 1997). The functional energy transduction cycle performed by cytochrome c_3 is completed by the donation of deenergized electrons (e^-) to a transmembrane electron transfer complex (ETC) (Rossi *et al.,* 1993) with the subsequent release of energized protons.

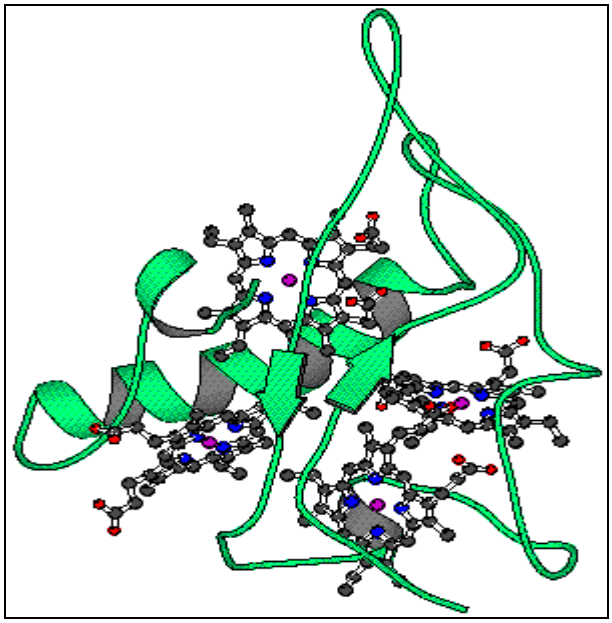

Figure 1.6: Ribbon presentation of the *Desulfovibrio desulfuricans* Norway cytochrome c3 structure (PDB code 2CY3). Adapted from Czjzek *et al.* (1992).

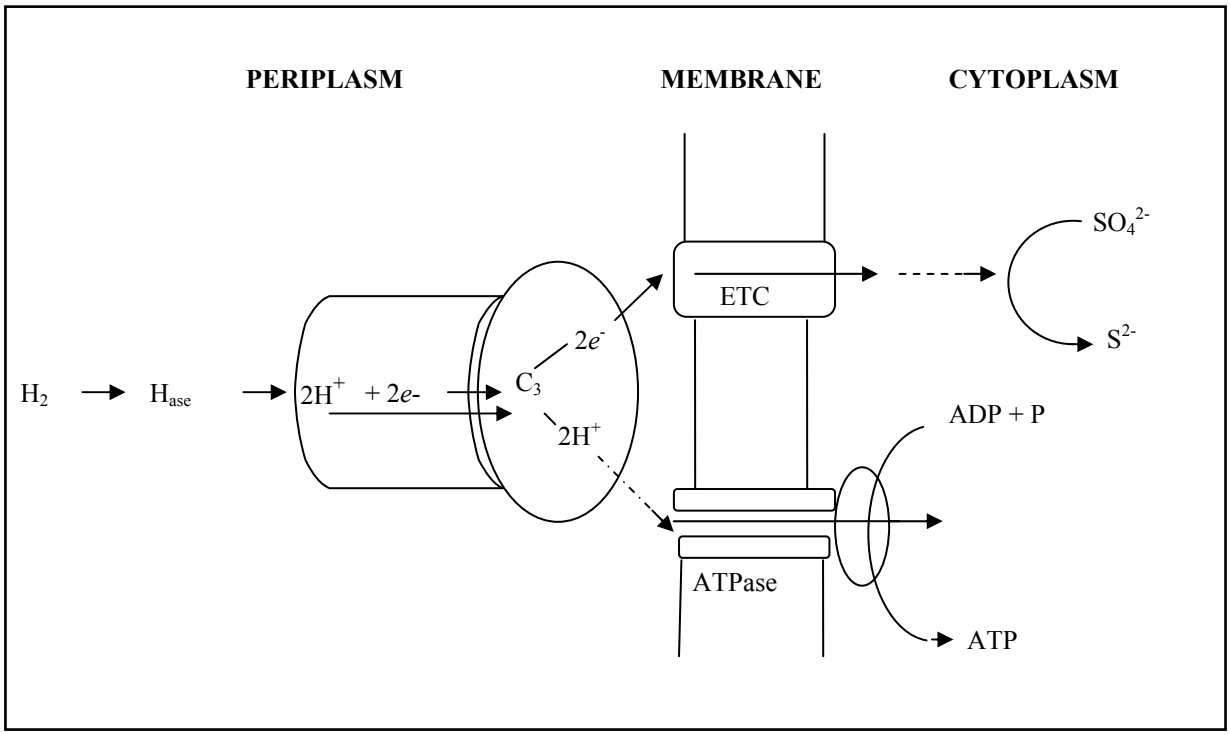

Figure 1.7: Schematic representation of the role of cytochrome c_3 in the bioenergetic system of sulphate reducing bacteria, where H_{ase} = hydrogenase, C_3 = cytochrome c_3, ETC = electron transfer complex. Adapted from Louro *et al*., (1997).

While the electrons (eight electrons required) are used in the reduction of sulphate, the energized protons can be used for the production of ATP by the ATP synthase (ATPase) (Louro *et al.,* 1997). In addition to being natural carrier proteins for periplasmic hydrogenases polyheme c-type cytochromes from *Desulfovibrio* and *Desulfuromonas* bacteria have been found to also function as metal reductases. Electrochemical evidence has been reported on the role of polyheme c-type cytochromes on the catalytic reduction of Fe(III) species (Lojou et al., 1998).

1.6 RHODIUM

1.6.1 Sources of rhodium

PGMs usually occur in native form associated with one or more of the other PGMs, along with gold, iron copper and chromium. The average concentration of PGMs in the lithosphere is very low, estimated to be in the range 0.001-0.005 mg/kg for platinum, 0.015 mg/kg for palladium, 0.0001mg/kg for rhodium and ruthenium, 0.005 mg/kg for osmium and 0.001 mg/kg for iridium (Ravindra *et al.,* 2004). PGMs are currently mined at the Merensky Reef of the Bushveld Complex (South Africa), the Ni-Cu-PGM sulphide deposits of Nroil'sk in the Russian Arctic and placer deposits in the Ural mountains (Russia), Sudbury (Ontario, Canada), the Hartely mine (Zimbabwe), the Stillwater complex (Montana, USA), Northern Territory (Australia) and the Zechstein copper deposit in Poland (Rao and Reddi, 2000). South Africa is also the major supplier of PGMs (85%), with Russia, North America and the rest of the world supplying the rest. South Africa is also the major supplier (79%) of the world's rhodium (Figure 1.8).

1.6.2 Demands and applications of rhodium

Rhodium finds varied applications in many very different fields. The automobile industry is the major consumer (86%) of the total world rhodium supply (Figure 1.9). Rhodium, along with platinum and palladium, is used for reducing levels of carbon monoxide, unburnt hydrocarbons and nitrogen oxides in the exhaust gases (Rao and Reddi, 2000). Part of the remaining rhodium used in the chemical industry as a catalyst for hydrogenation of olefins to alkanes, and the glass industry. The rest is used for other applications such as electrical, jewellery manufacturing and others (Rao and Reddi, 2000). Since the introduction of car converter catalysts in the 1980s, the price of rhodium had increased up to $2264/oz by the year 1992 (Figure 1.10).

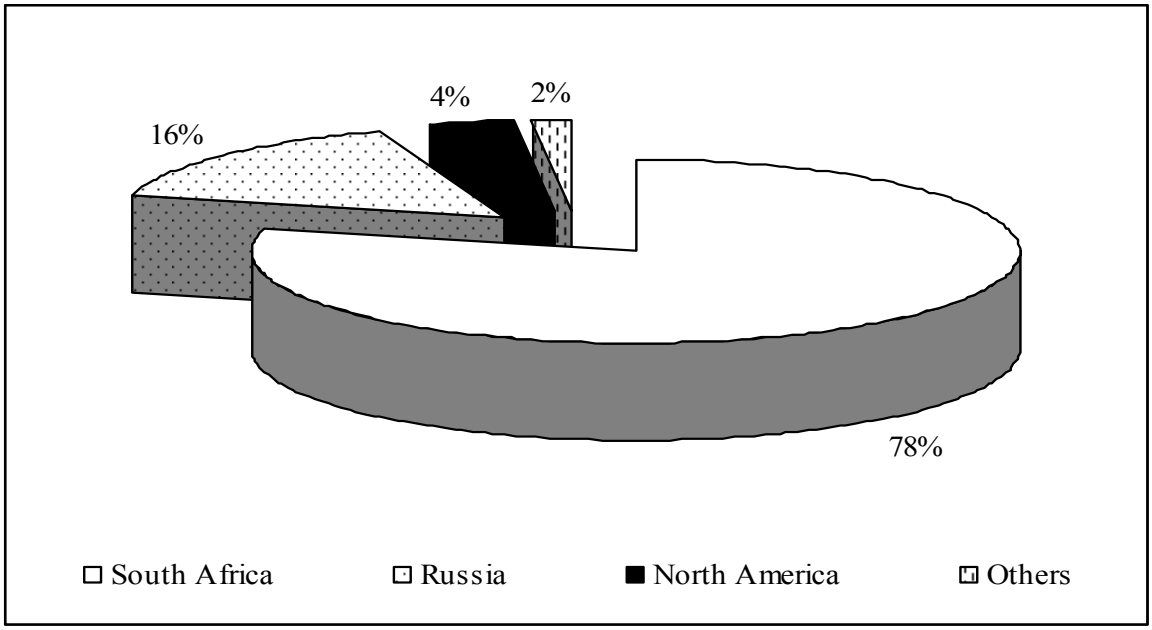

Figure 1.8: Major producers of rhodium. Adapted from Rao and Reddi (2000).

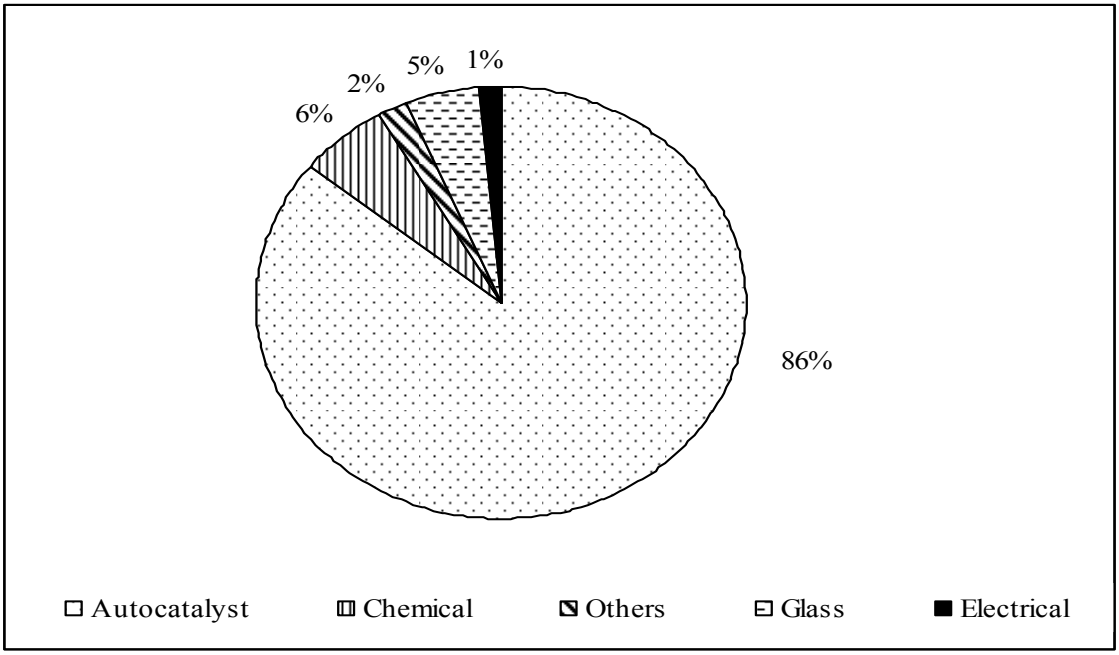

Figure 1.9: Industrial demand of rhodium. Adapted from Rao and Reddi (2000).

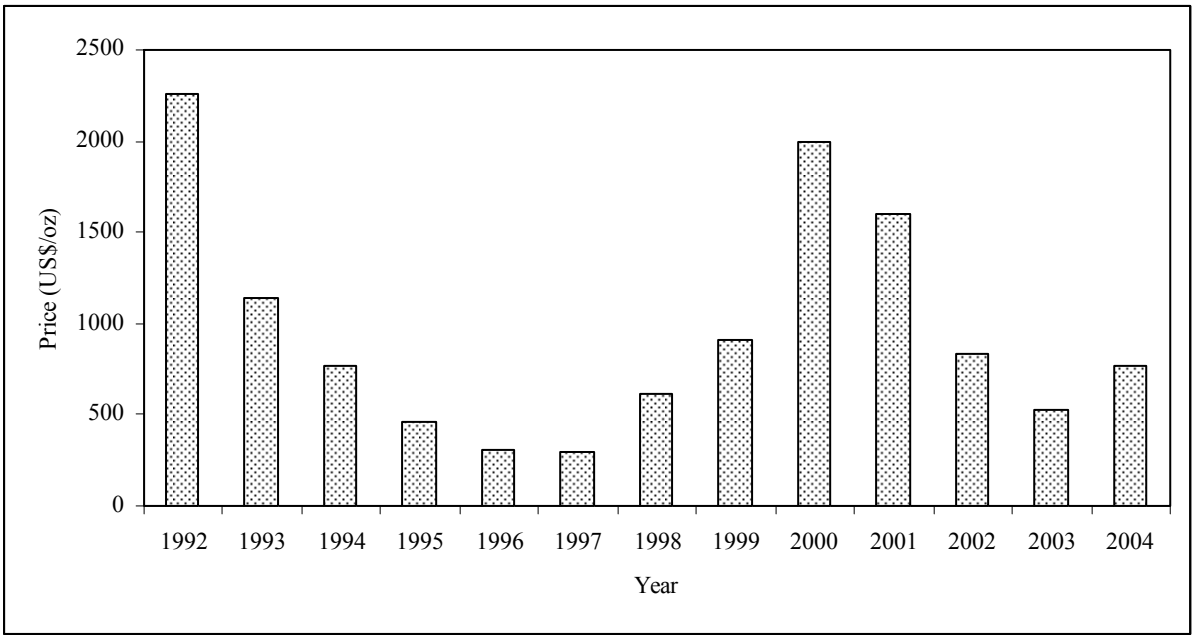

Figure 1.10: Average annual price of rhodium. Adapted from Platinum Today (2004).

1.6.3 Physical and chemical properties

Rhodium belongs to group 9 of the periodic table, has an atomic number of 45 and a molecular weight of 102.9055. It is a silvery white metal, has a high reflectance, ductile, and is hard and durable (Rao and Reddi, 2000). Some of the useful physical and chemical properties of rhodium are listed in Table 2. Rhodium is normally encountered in the oxidation states +1, +2, +3, +4 or +6, with +3 being the most common in chloride solutions (Benguerel *et al.,* 1996). Generally, bond strengths for rhodium-ligand bonds are generally weaker when compared to iridium-ligand bonds. Rhodium is insoluble in all acids, except in aqua regia, where the dissolution process is very slow (Kayanuma *et al.,* 2004). It also dissolves slowly in boiling H_2SO_4 and HBr. Rhodium also dissolves in molten potassium bisulfate ($KHSO_4$), a useful property for its extraction from platinum ores (Rao and Reddi, 2000).

Table 2: Physical and chemical properties of rhodium. Adapted from Rao and Reddi (2000).

Physical/chemical property	
1. Density (Kg/m^3)	12.4
2. Melting point (°C)	1966
3. Boiling point (°C)	3727
4. First ionization potential (eV)	7.7
5.Resistivity (microhm.cm at 20°C)	4.33

One of the important features of the chemistry of rhodium (III) is its tendency to form numerous species when in acidic media. On dilution rhodium (III) tends to be readily hydrolyzed, giving rise to a wider variety (and a varied distribution) of complexed forms. In dilute HCl solutions (0.1M), four differently charged chloro-complexes have been observed; $RhCl_4(H_2O)_2^-$, $RhCl_3(OH)(H_2O)_2^-$, $RhCl_3(H_2O)_3$ and $RhCl_2(H_2O)_4^+$. However, in concentrated HCl (11M), one major Rh chloro-complexes identified as $RhCl_6^{3+}$ and two other chloro-complexes in which one or two of the chloride ligands were probably replaced by water and hydroxyl ion, as a result of hydrolysis were identified. Cationic species of rhodium are predominant in $HClO_4$ and HNO_3, whereas negatively charged rhodium(III) complexes are predominant in H_2SO_4 (Aleksenko *et al.,* 2001). It has been found that the anionic complexes of rhodium are more labile than those of other PGMs, whereas the cationic and neutral complexes are quite inert (Benguerel *et al.,* 1996).

1.6.4 Health risks

For a long time platinum group metals (PGMs) have been thought to be harmless, but recently it has become known that they can be harmful when ingested in high concentrations (Rao and Reddi, 2000). Through anthropogenic emissions, PGMs find their way into freshwater and estuarine sediments, and eventually enter the food chain. The toxicity of PGMs has been investigated among the water hyacinth, and with reference to oxidation states the following trend was observed; Pt^{2+}, $Pd^{2+} > Ru^{3+} \approx Ru^{2+} \approx Ir^{3+} > Pt^{4+} \approx Os^{4+} >> Rh^{3+}$ (Ravindra *et al.*, 2004). In all cases Pt^{2+} has been found to be the most toxic amongst the PGMs. In a study by Schroeder and Mitchener (1971), increased tumour incidences in mice were observed as a result of drinking water contaminated with halogenated palladium and rhodium salts ($PdCl_2$ and $RhCl_3$). Not yet known and still under investigation is whether rhodium accumulated within the environment does pose a threat to human life (Ravindra *et al.,* 2004).

1.6.5 Rhodium recovery process

PGM recovery processes consist of two main stages (Figure 1.11). During the first stage, the PGMs are extracted from the ore concentrate by dissolution in hydrochloric acid applying appropriate oxidants. In the second stage, each of the PGMs are separated and purified through precipitation, solvent extraction and ion exchange. Since rhodium slowly dissolves in aqua regia, a pre-treatment step is required prior to dissolution. Currently, large amounts of energy and strong acids are required for the processing of rhodium, and the wastewaters that result from the process are the main problems faced by the mining industries in the recovery

of rhodium (Kayanuma, *et al.,* 2004). Rhodium is recovered from the raffinate phase by an ion exchange process. Currently traces of platinum group metals present in the barren liquor are recovered using a combination of physical and chemical methods. For example, solvent extraction is commonly used for the recovery of rhodium (III) from hydrochloric acid medium. However, its application is made difficult by the complex nature of the chemistry of rhodium in aqueous solution (Kolekar and Anuse, 2002). Rhodium(III) chloride in HCl solutions forms a number of octahedral rhodium aquo-chloro complexes, due to steric effects (it being difficult to pack three cationic organic molecules around a single atom) and aquation (exchange of a Cl^- ligand for an H_2O ligand), depending on the choride concentration, and to a lesser extent temperature, time after preparation and the pH of the solution (Benguerel *et al.,* 1996).

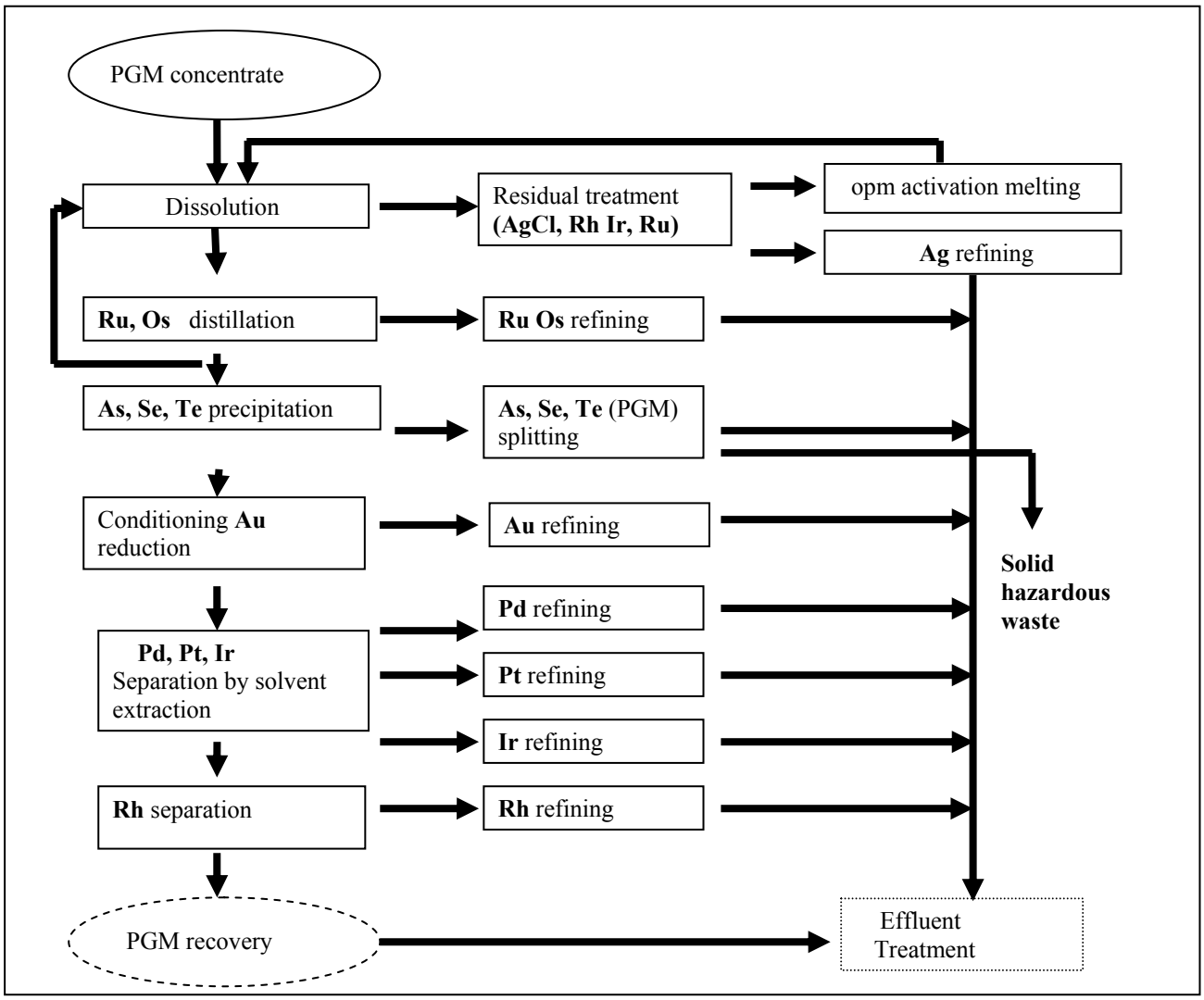

Figure 1.11: Sequential steps during the refining of platinum group metals, their recovery and subsequent effluent treatment. Adapted from Angloplatinum (Pty.) Ltd. (2004).

The species formed range from $Rh(H_2O)_6^{3+}$ to $RhCl_6^{3-}$, but the predominant species of higher concentration are the anionic complexes of the type $[RhCl_{6-n}\ (H_2O)_n]^{n-3}$, where n is 1 or 2. These complexes are labile, with different complexes exhibiting different extractabilities. The existence of a number of species makes the quantitative removal and separation of rhodium salts difficult and careful control of solution conditions is vital. Also, its kinetic behaviour for the formation of extractable species and the different electrostatic strengths of the rhodium-chloro complexes with liquid ion exchangers or oxygen containing solvents make the use of extractants almost impossible to use (Kolekar and Anuse, 2002). The difficulty, high maintenance and operation costs resulting from such processes have led to the investigation of biological methods for effluent treatment for the recovery of PGMs.

1.7 MECHANISMS OF SRB METAL REDUCTION

Enzymatic metal reduction by SRB, particularly *Desulfovibrio* species has been studied in various metals, for example, Cr(VI), Fe(III), Mn(IV)and U(VI) (Lovley, 1993) and Tc(VII) (Lloyd *et al.,* 1999). Similarly PGMs uptake and subsequent reduction by SRB enzymes has been reported with platinum (Rashamuse, 2003) and palladium (Lloyd *et al.,* 1998). Based on these findings, this present study is aimed at establishing useful information on the enzymatic reduction of rhodium(III) to its elemental form, as a cost effective method for metal recovery. Although the precise enzymatic pathway of metal reduction by SRB is not yet fully understood, two possible mechanisms have been proposed in this study. SRB enzymatic reduction of rhodium can occur through a single enzymatic step (direct enzymatic reduction) or through a mediator (electron carrier protein) assisted enzymatic process (Figure 1.12a and b, respectively).

1.7.1 Direct metal enzymatic reduction

Available evidence from various enzymatic metal reduction studies suggests that the direct enzymatic process involves a hydrogenase, an enzyme that catalyses the reversible splitting of hydrogen into protons and electrons. The available metal species then serve as the terminal electron acceptor, and is thereby reduced in the process. This evidence is further supported by the fact that hydrogen has been found to be the most suitable electron donor for effective metal reduction among SRB (Lloyd *et al.,* 1999). Previously, cytochrome c_3 was thought to only function as the main intermediary electron carrier for the reduction of various sulphur oxide electron acceptors in *Desulfovibrio* species (LeGall *et al.,* 1988). Recently, however, it

has also been found to be a useful enzyme in the direct reduction of some metals, for example, Cr(VI) to Cr(III) and U(VI) to U(IV) (Lovley *et al.,* 1993).

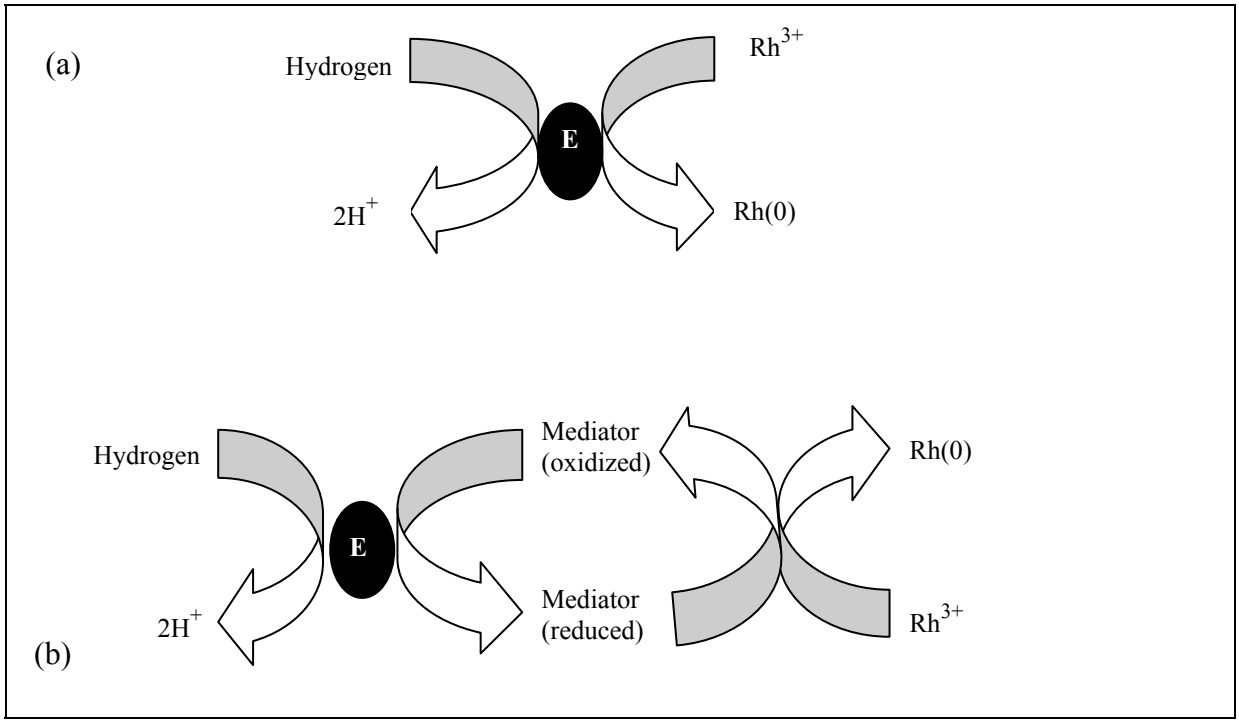

Figure 1.12: Schematic representation of the proposed biochemical steps involved during the enzymatic reduction of rhodium(III) (a) direct enzymatic reduction, and (b) indirect enzymatic reduction (reduction via mediators or electron carriers). **E** = hydrogenase.

1.7.2 Indirect metal reduction

Mediator-assisted enzymatic metal reduction among SRB is achieved by coupling the oxidation of hydrogen to the reduction of a mediator or electron carrier, which then interacts with the metal. During the biocatalysis hydrogen splitting yields two electrons, and these are then transferred, one at a time, to the proximal [4Fe-4S] cluster of the enzyme (hydrogenase) and then to its redox partner (cytochrome c_3) (Fontecilla-Camps *et al.,* 1997), which then interacts with the metal, thereby reducing it. In most of the enzymatic metal reduction by SRB studies reported above, maximum metal recovery was observed when hydrogenase was incubated with its physiological electron carrier cytochrome c_3 (Michel *et al.,* 2001).

1.8 HYPOTHESIS AND OBJECTIVES

1.8.1 Hypothesis

Rhodium can be enzymatically recovered from both aqueous solution and industrial effluent by a hydrogenase enzyme system from sulphate reducing bacteria.

1.8.2 Objectives

(i) To grow a mixed sulphate reducing bacterial culture and monitor the changes in pH, rate of sulphate reduction and sulphide generation during its growth

(ii) To investigate the effect of varying conditions (pH, temperature, SRB biomass concentration, initial rhodium concentration and electron donor effect) on the uptake of rhodium by an intact non-growing SRB consortium.

(iii) To confirm the presence of rhodium precipitates on SRB cells, their effect on SRB cell morphology, their distribution and location within the cell using electron microscopy and X-ray techniques.

(iv) To isolate, purify and characterize a hydrogenase enzyme from the SRB consortium and determine its ability to recover rhodium from solution *in vitro*.

(v) To investigate the recovery of rhodium from industrial effluent by whole SRB cells, soluble extract and a partially purified hydrogenase.

CHAPTER 2

QUANTITATIVE ANALYSIS OF RHODIUM(III) UPTAKE FROM SOLUTION BY WHOLE SULPHATE REDUCING BACTERIA CELLS (Batch studies)

2.1 INTRODUCTION

The high operational and maintenance costs mainly associated with the use of physico-chemical methods of metal recovery have led to the alternative use of biological processes, such as biosorption and bioaccumulation. The accumulation of metals by microorganisms is not novel; it has been there long before mankind as one of the fundamental processes of the geochemical cycle. While biosorption occurs passively, bioaccumulation involves both the biosorptive processes and the active uptake of metals into the cells. During the first stage of bioaccumulation the biomass acts as an exchange resin and eventually the metal is incorporated into the metabolic system. Sulphate reducing bacteria (SRB) have been shown to tolerate high metal concentrations, to decrease sulphate concentration and raise the pH in contaminated environments (Herlihy and Mills, 1985).

Bioaccumulation of metals by SRB has been studied with a range of strains, including *Desulfovibrio, Desulfomicrobium* and *Desulfotomaculum* species, by growing or non-growing, and pure or mixed SRB cultures. The limitation with growing SRB cells is that when the metal concentrations become too high cell death might result and pure cultures can lose their ability to adapt to environmental changes (Postgate, 1984). Hydrogen sulphide produced by a growing SRB culture results in the precipitation of metals as their sulphides (Kaksonen *et al*., 2003). In the absence of hydrogen sulphide, metals are used as terminal electron acceptors, and thereby accumulate within the cell (Tebo, 1998). The ability of microorganisms to accumulate maximum quantities of metals has been shown to be dependent on a number of factors including; biomass type and concentration. This is because different biomass types (dead or alive) contain a number and variety of functional sites on their cell surfaces, and these sites include; carboxyl, imidazole, sulphydryl, amino, phosphate, sulphate, thioether, phenol, carbonyl, amide and hydroxyl groups. As a result, metals are bound to cell surfaces due to interactions between biomass cell surfaces and metals (Darnall *et al.,* 1986).

In addition, the bioaccumulation of metals by microorganisms is also dependent on initial metal concentration, solution pH, ionic strength and temperature. These factors are important because they influence the chemical behaviour of metal ions in solution, and as such also determine the availability of the preferred nature (anion or cation) of metal species (Eccles, 1995). The effect of the above mentioned factors in the amount of rhodium(III) that can be accumulated by a non-growing SRB consortium was investigated. In addition, rhodium kinetics in the presence and absence of SRB cells and electron donors was investigated. Finally, to gain an understanding of the maximum rhodium uptake capacity of SRB cells under specified optimized conditions, an equilibrium sorption isotherm was plotted.

2.2 MATERIALS

Rhodium(III) chloride ($RhCl_3$) (43% assay**)** was supplied by Angloplatinum (Pty) Ltd, Rustenburg (South Africa). Rhodium atomic absorption spectroscopy standard solution was purchased from Fluka Chemie (Buchs, Switzerland). Sulphate reagent and hydrogen sulphide test kits were purchased from Hach Company (Loveland, USA) and Merck (Darmstadt, Germany), respectively. The rest of the reagents, which were of analytical grade were purchased from either Sigma-Aldrich (Steinhem, Germany) or Merck (Darmstadt, Germany). All gases were obtained from Afrox (South Africa). Membrane disc filters (AcetatePlus, supported, plain, 0.45 micron, 25mm) were purchased from Osmonics Inc. (Minnesota, USA). Beckman J2-21 and Eppendorf 5810R centrifuges were used for centrifugation of larger and smaller volumes, respectively. All glassware used for metal uptake experiments was soaked in 30% nitric acid solution for at least 3 hours prior and after use and then rinsed in ddH_2O to eliminate interference by metal ions.

2.3 METHODS

2.3.1 Analytical procedures

In all experiments reported in this chapter, triplicate samples were measured. Sulphate concentration was measured using the Thermospectronic Aquamate (England) and the Spectroquant® Nova 60 (Merck, Germany) was used for sulphide concentrations. Manufacture's protocols were followed for both sulphate and sulphide determination. Protein concentration was determined by the Bradford assay (Bradford, 1976) (Appendix B), and protein absorbance was measured using a PowerWave X (Bio-Tek, Instrumental Inc., Winooski, USA). Sample pH was measured using a Level 1 Inolab pH meter (WTW Ltd, Weilheim, Germany). The concentration of rhodium was determined using a GBC 909 air-

acetylene flame atomic absorption spectrophotometer (GBC Scientific equipment Pty Ltd, Dandenong, Australia) using a rhodium 6mA hollow cathode lamp at 343.5nm.

2.3.2 Growth of sulphate reducing bacteria

2.3.2.1 Growth media and culture conditions

A mixed sulphate reducing bacteria culture previously isolated from an acid mine waste (Grootvlei mine, Gauteng province, South Africa) was grown on a modified Postgate's medium C (Postgate, 1984) prepared as outlined in Appendix A. A stock bioreactor (10L) was filled with modified Postgate medium C, purged with nitrogen gas to achieve an anaerobic environment and then autoclaved for 30 minutes at 121°C prior to inoculation with the starting culture. The stock culture was sub-cultured every 3-4 weeks. For small-scale experiments, 2 litre rubber-sealed reactors were set up from the stock culture. In all experiments, a 10% (v/v) SRB inoculum was used to start new cultures and cultures were incubated at 37°C. To monitor the growth of SRB, pH, sulphate and sulphide concentrations were determined daily over a ten-day period.

2.3.2.2 Preparation of SRB cells for metal uptake experiments

In all experiments performed in this study sulphate reducing bacteria previously grown on modified Postgate medium C were harvested during mid-stationary phase (after 8-9 days) by centrifugation (7000 × g, 15 minutes, 4°C). The resulting SRB pellet was repeatedly washed in deionized water to remove any residual sulphate and sulphide, in order to eliminate any possibility of premature precipitation of rhodium as either its sulphate or sulphide. SRB cells were then suspended in 1ml of Tris-HCl buffer (20mM, pH 7.6) before addition to the metal solution prepared by dissolving a pre-weighed $RhCl_3$ salt in deionized water.

2.3.3 Kinetic experiments on rhodium(III) uptake

2.3.3.1 Effect of initial rhodium concentrations

The effect of initial rhodium concentration on the uptake of rhodium from solution by a resting SRB consortium was studied. Washed SRB cells in Tris-HCl buffer (20mM, pH 7.6, 1 ml) at a biomass density of 5g per litre rhodium chloride solution were challenged with different rhodium concentrations of 43.0, 32.3, 22.0, 10.8 and 4.3mg/l. Hydrogen gas was supplied as an electron donor. The rate of removal of rhodium from solution at timed intervals was monitored using an atomic absorption spectrophotometer.

2.3.3.2 Effect of biomass concentration

The effect of biomass concentration on the uptake of rhodium from solution by a resting SRB consortium was studied at a biomass density range 0.5-5g/l. Washed SRB cells in Tris-HCl buffer (20mM, pH 7.6, 1 ml) at the different biomass densities (0.5-5g/l) were exposed to a rhodium solution (43mg/l) and hydrogen gas was supplied as an electron donor throughout the incubation period. The rate of removal of rhodium from solution was monitored using an atomic absorption spectrophotometer.

2.3.3.3 Effect of pH

The effect of pH on the uptake of rhodium from solution by a resting SRB consortium was studied at pH 2-7. The pH of the rhodium(III) chloride solution (43mg/l) was adjusted with either NaOH or HCl. An unadjusted rhodium(III) chloride solution (43mg/l) at pH 4 served as a control. The solutions were then filtered and analyzed for rhodium. Washed SRB cells (0.5g/l) in Tris-HCl buffer (20mM, pH 7.6, 1 ml) were then added into each of the different pH rhodium solutions. Hydrogen gas was supplied as the sole electron donor. The rate of removal of the remaining (unprecipitated) rhodium was followed at timed intervals using an atomic absorption spectrophotometer.

2.3.3.4 Effect of temperature

The effect of temperature on the uptake of rhodium from solution by a resting SRB consortium was studied at temperature range 0-70°C. Washed SRB cells (0.5g/l) in Tris-HCl buffer (20mM, pH 7.6, 1 ml) were exposed to a rhodium solution (43mg/l) and incubated at the different temperatures. Hydrogen gas was supplied as an electron donor throughout the incubation period. The rate of removal of rhodium from solution at timed intervals was monitored using an atomic absorption spectrophotometer.

2.3.3.5 Effect of lactate, hydrogen and absence of electron donor

The effect of lactate, hydrogen and absence of an electron donor on the uptake of rhodium from solution by a resting SRB consortium was studied. Washed SRB cells (0.5g/l) in Tris-HCl buffer (20mM, pH 7.6, 1 ml) were challenged with 43mg/l rhodium as before. In one flask lactate was supplied as the sole electron donor and in a second flask hydrogen gas was supplied as the sole electron donor throughout the incubation period. In a third flask (control) no electron donor was supplied. In all flasks, the rate of removal of rhodium from solution by SRB cells was monitored at timed intervals using an atomic absorption spectrophotometer.

2.3.3.6 Effect of the presence and absence of SRB cells

The effect of the presence and absence of SRB cells on the uptake of rhodium from solution by a resting SRB consortium was studied. Three different flasks were prepared. To each a rhodium solution at 43mg/l was added. In the first flask live SRB cells (0.5g/l) in Tris-HCl buffer (20mM, pH 7.6, 1 ml) were added, and in the second flask, SRB cells (0.5g/l) in Tris-HCl buffer (20mM, pH 7.6, 1 ml) killed by holding at 121°C for 30 minutes were added. The third flask served as a control (absence of SRB cells) and the rhodium solution (43mg/l) was autoclaved at 121°C for 30 minutes. In all flasks, hydrogen gas was supplied throughout the incubation period and the rate of removal of rhodium was monitored at timed intervals using an atomic absorption spectrophotometer.

2.3.3.7 Sorption isotherm

The maximum rhodium uptake capacity of SRB cells was studied by exposing washed SRB cells (0.5g/l) in Tris-HCl buffer (20mM, pH 7.6, 1 ml) with increasing rhodium concentrations (0-500mg/l). Hydrogen gas was supplied as the sole electron donor throughout the incubation period. The rate of removal of rhodium was monitored at timed intervals using an atomic absorption spectrophotometer. The maximum amount of rhodium uptake (q_{max}) from solution was calculated using Equation 5.

$$q_{max}\ (mg/g) = (Ci - Cf)/X \quad \ldots\ldots (5)$$

Where, q_{max} is the SRB maximum rhodium uptake capacity, expressed as mg rhodium per g SRB biomass, Ci is the initial rhodium concentration (mg/l), Cf is the final rhodium concentration (mg/l), and X is the biomass concentration (g/l).

2.4 RESULTS AND DISCUSSION

2.4.1 Growth of a mixed SRB culture

The rate of sulphate reduction and sulphide generation, as well as the changes in pH during the growth of a mixed sulphate reducing bacteria culture when grown on a lactate-sulphate medium were studied. The highest SRB growth rate, which was indicated by the highest rate of sulphate reduction (450mg/l/day) and sulphide generation (125mg/l/day), was observed between day 7 and 9 (Figure 2.1). Consequently, for all experiments reported in this study, SRB cells were harvested during this period (day 7-9). After day 9, the rate of sulphate

concentration reduction started levelling off, whilst there was an initial decrease, then a slight increase in the generation of sulphide after day 8.

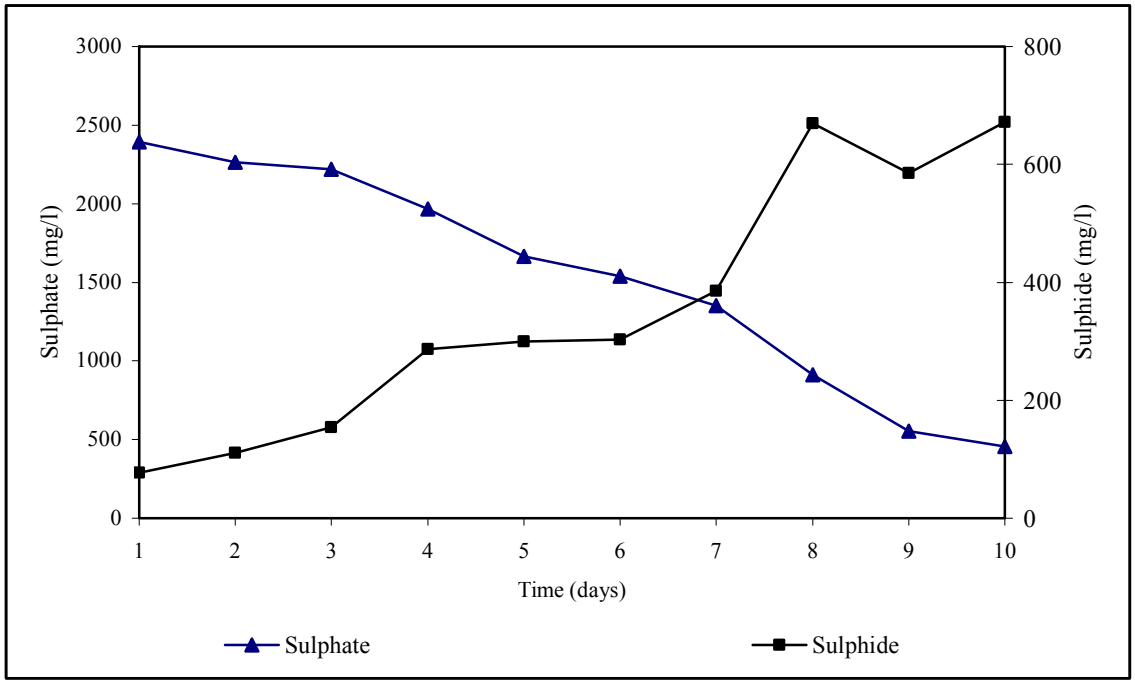

Figure 2.1: Sulphate reduction and sulphide generation during sulphate reducing bacteria growth in a bioreactor. Data are means ± for the triplicate values measured.

The pH was maintained between 7.5 and 7.7 (Figure 2.2), and this may be attributed to the basic chemical and metabolic processes involved in sulphate reduction, particularly electron donor oxidation (White and Gadd, 1996). Under anaerobic conditions, electron donors, such as lactate are oxidised to produce energy in the form of protons. These protons are then used up by SRB for sulphate reduction to sulphide and bicarbonate (Equation 6-8) (Widdel, 1998). When in aqueous solution, the hydrogen sulphide and bicarbonate ions formed during sulphate reduction equilibrate into a mixture of H_2S, S_2^-, CO_2, HCO_3^- and CO_3^-, which help maintain the pH of the solution within a particular range if sufficient sulphate reduction occurs (Herlihy and Mills, 1985).

$2CH_3CHOHCOOH + SO_4^{2-} \longrightarrow 2CH_3COOH + 2HCO_3^- + H_2S$…….. (6)

$CH_3COOH + SO_4^{2-} \longrightarrow 2HCO_3^- + H_2S$…… (7)

$2CH_3CHOHCOOH + 3SO_4^{2-} \longrightarrow 3H_2S + 6HCO_3^-$ (sum reaction)…..…..…….. (8)

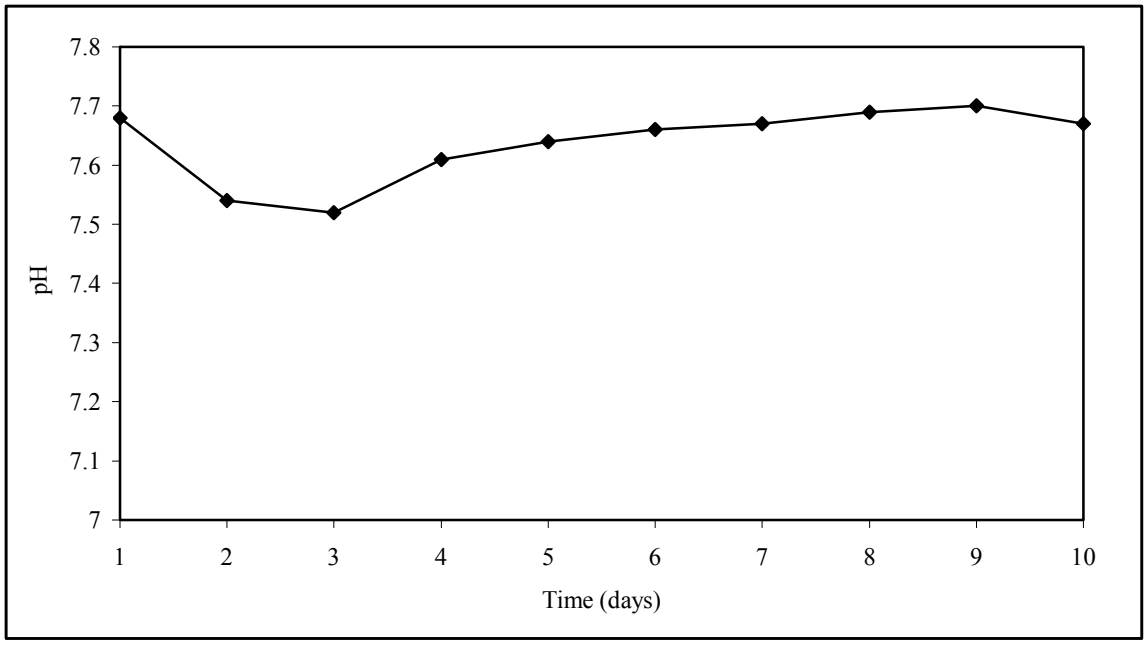

Figure 2.2: Changes in pH during sulphate reducing bacteria growth in a bioreactor.

2.4.2 Effect of initial rhodium concentrations

Results from this experiment showed that major interaction between SRB and rhodium occurred within the first 30 minutes. There was no observed trend in the rate of rhodium removed from solution at the different initial concentrations as 66% rhodium was removed at 22mg/l., 64% at 43mg/l, 44% at 32.3mg/l, 57% at 10.8mg/l and lastly 12% at 4.3mg/l (Figure 2.3). However, at the end of the incubation period, complete rhodium removal was observed at 43mg/l and 4.3mg/l. Consequently, it was difficult to establish the effect of initial rhodium concentration on the rate or amount of rhodium removed by a resting SRB consortium. Contrary to findings in this study, a higher rate of Pt(IV) removal by SRB cells was observed at increased initial platinum concentrations (Rashamuse, 2003). Similarly enhancement in lead, cadmium and zinc sorption was observed with increasing initial metal concentrations (Puranik and Paknikar, 1999). In both cases increased metal uptake at increased initial metal concentrations was attributed to an increase in electrostatic interactions (relative to covalent interactions), involving sites of progressively lower affinity for metal ions (Gadd, 1988). Therefore this experiment only served to establish the fact that the interaction between SRB and rhodium initially occurs at a much faster rate followed by a slower rate. Of all the initial concentrations investigated, 43mg/l as opposed to 4.3mg/l was chosen as the most practical initial rhodium concentration to work with as it showed great potential.

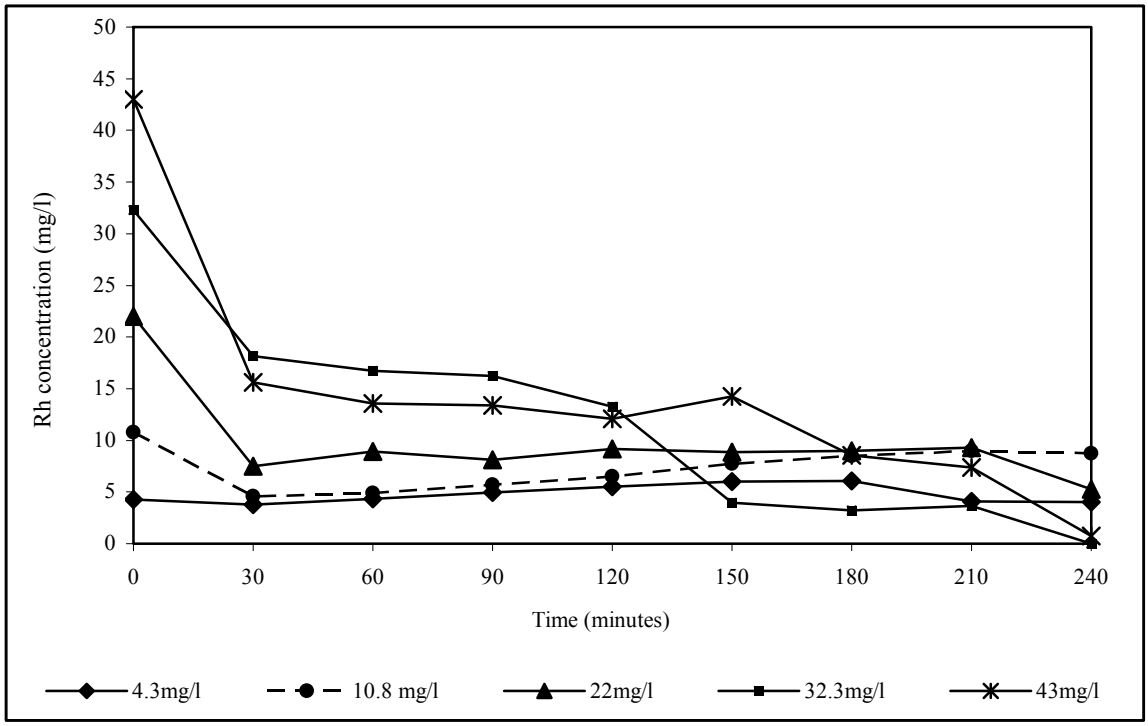

Figure 2.3: Effect of different initial rhodium concentrations on the rate of rhodium removal from solution by a resting SRB consortium (5g/l) with hydrogen as electron donor. Data are means ± for the triplicate values measured.

2.4.3 Effect of biomass concentration

There was no significant difference in the amount of rhodium removed from solution by SRB biomasses in the concentration range 0.5-5g/l (Figure 2.4). In a study by Gadd *et al.* (1988), it was found that increasing the biomass concentration does not necessarily result in remarkably increased metal uptake because larger biomasses have been found to interfere with metal binding sites as they tend to clog together. Lower biomass concentrations can also exhibit higher specific metal uptake capacities due to an decreased ratio of metal-to-biosorbent. For example, in one study decreased sorption of lead, cadmium and zinc uptake was observed with an increase in the biomass concentration of *Citrobacter* Strain MCM B-181 (Puranik and Paknikar, 1999). A similar observation was made in studies on metal sorption of zinc by *Rhizopus arrhizus* (Fourest and Roux, 1992) and sorption of cobalt, copper and iron by *Rhizopus delemars* (Tsekova and Petrov, 2002). However, in this study the rate of removal of rhodium by SRB cells at a biomass concentration range 0.5-5g/l ranged between 72-82% (Figure 2.4). For this reason the metal uptake trend exhibited by low or high biomass concentrations was regarded to be a complicated phenomenon. Different biosorbents, culture media and growth conditions have been shown to have an adverse effect on the growth

characteristics and metal sequestering abilities of microbial cultures (Volesky, 2001). For practical reasons, 0.5g SRB biomass per litre of rhodium solution (43mg/l) was chosen as the least SRB biomass concentration that can be used in this study.

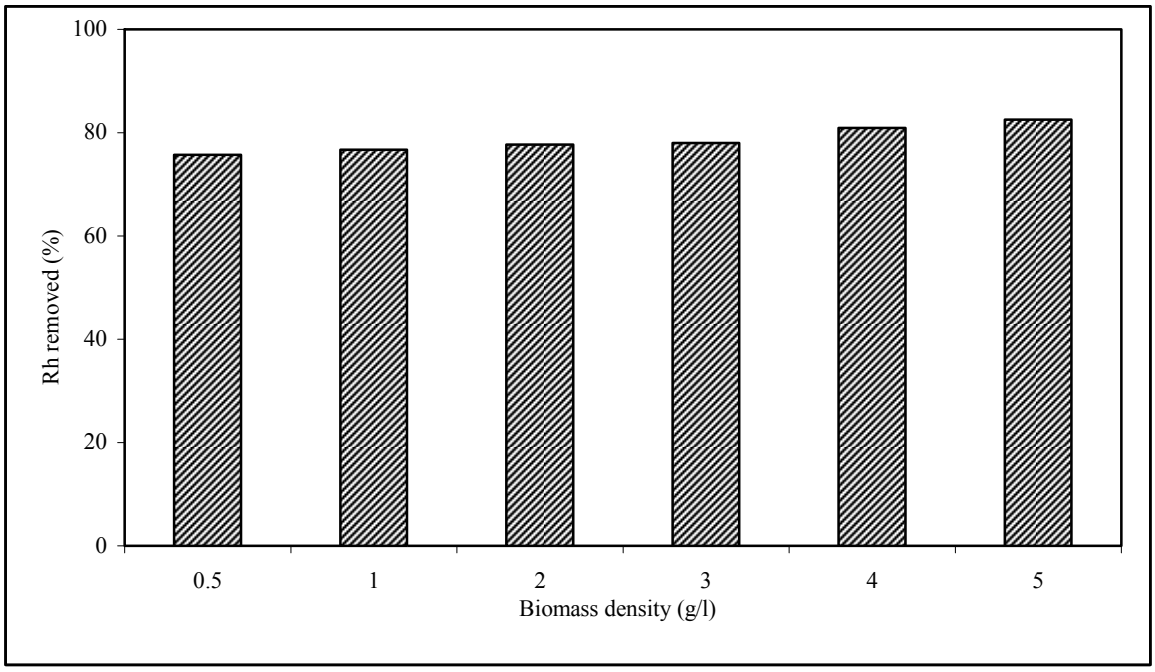

Figure 2.4: Effect of different SRB biomass densities (g/l) on the amount (%) of rhodium removed from solution (43mg/l rhodium) with hydrogen as electron donor.

2.4.4 Effect of pH

In this study pH has been found to have a distinct effect on the amount of rhodium taken up by SRB cells and precipitated (Figure 2.5). It has an effect on the solution chemistry of the metal when in solution, the activity of functional groups on biomass cell surfaces and results in competition of ions within the solution (Ramelow *et al.,* 1992). It was evident that the addition of NaOH and HCl strongly affected the sorption performance and uptake of rhodium, with HCl also affecting the speciation of the metal. In hydrochloric acid media rhodium(III) forms octahedral complexes that presents a complex speciation due to the formation of a variety of aqua-chloro complexes (Equation 9). At sufficiently low pH (pH>3), the resulting aqua-chloro complexes undergo further hydrolysis (Equation 10).

$$RhCl_6^{3-} + nH_2O \longleftrightarrow [RhCl_{6-n}(H_2O)_n]^{(3-n)-} + nCl^- \quad \text{.....(9)}$$

$$[RhCl_{6-n}(H_2O)_n]^{(3-n)-} + H_2O \longleftrightarrow [RhCl_{6-n}(H_2O)_{n-1}OH]^{(4-n)-} + H_3O^+ \quad \text{.....(10)}$$

During the hydrolysis process, at least five species are formed in solution, and these include some chloro- or oxygen-bridged oligomeric species of rhodium(III). Anionic rhodium species, particularly, $RhCl_4(H_2O)_2^-$, have been identified as the main abundant species. However, when equilibrium is reached neutral and cationic species dominate (Sanchez *et al.,* 2002).

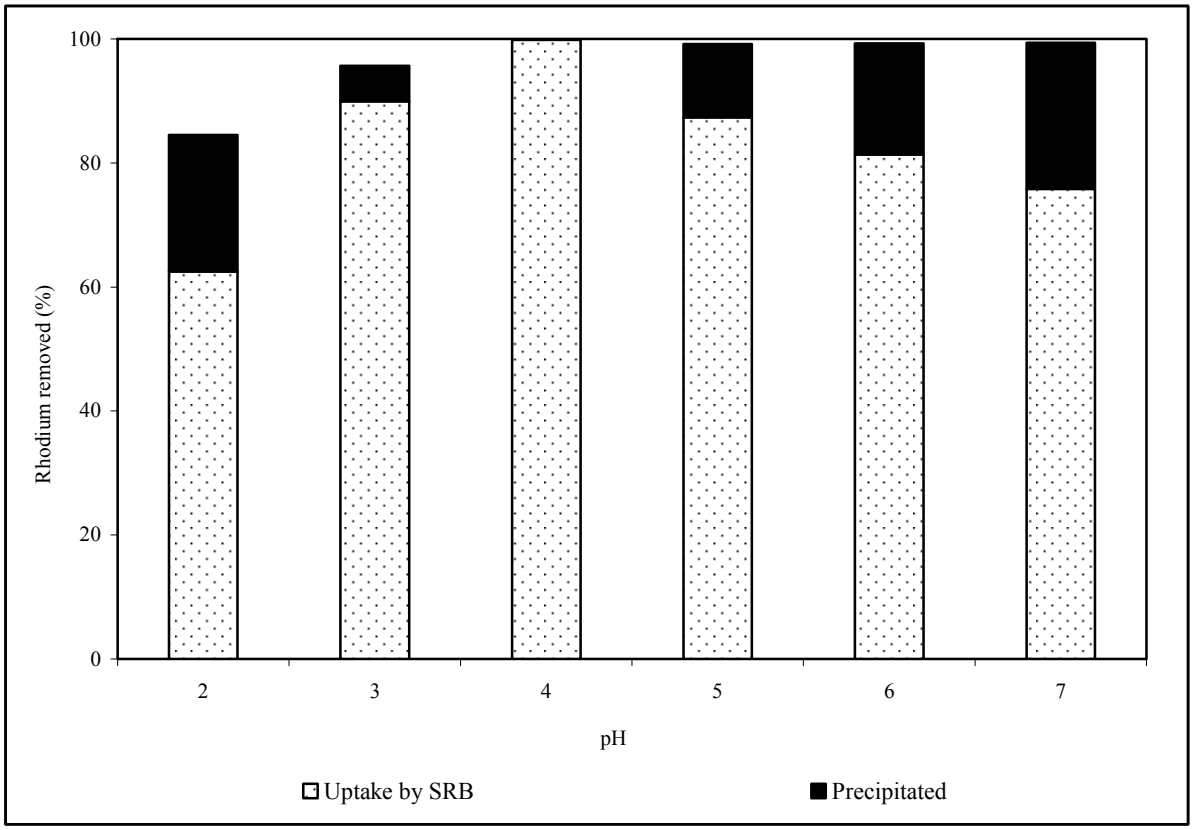

Figure 2.5: Effect of pH on the amount (%) of rhodium precipitated and removed by SRB cells (0.5g/l) with hydrogen as electron donor.

Consequently, the extent of this hydrolysis or speciation process within a solution plays a major role in determining the bioavailability of rhodium(III) in aqueous solutions (Ravindra *et al.,* 2004). In this study, decreased rhodium(III) solubility was observed with increasing and decreasing pH. However, reasons for observed precipitation of rhodium at pH < 3 are not known, since theoretically hydrolysis takes place at pH >3. Apart from its effect on the speciation of rhodium(III), pH is also known to alter the ionic binding properties of biomass cell surfaces. Generally, biomass surfaces exhibit a net positive charge at low pH, and a net

negative charge at high pH (Zouboulis *et al.,* 2004). Essentially this implies that at equilibrium and high pH (pH >3), sufficient removal of rhodium(III) should be observed due to the interaction between the cationic rhodium(III) species and the negatively charged SRB cell surfaces. However, since no hydrolysis occurs at pH <3, only one major rhodium(III) species, identified as $RhCl_6^{3-}$, is present. As a result, uptake by SRB cells is mainly due the interaction between $RhCl_6^{3-}$ and positively charged SRB cells surfaces. The expected results were obtained in this study, because at pH >3 (pH 4-7) a maximum of 98-100% rhodium was removed from solution by a combination of chemical precipitation and biosorption.

At pH <3, however, about 63% of the initial rhodium concentration was removed from solution. Lower removal of rhodium at low pH can be attributed to competition among rhodium(III), hydronium and hydrogen ions for binding sites on cell surfaces (Gadd, 1988). The effect of equilibrium pH onto adsorption performances, as a result of competition between hydrogen and metal ions is widely documented. For example, when an olive pomace was used a specific uptake of 0.065mmol/g and 0.040mmol/g was obtained for copper and cadmium, respectively, at pH 5 while at pH 3, a specific uptake of 0.010mmol/g and 0.014mmol/g, was obtained for copper and cadmium, respectively (Pagnanelli *et al.,* 2003). Similarly, decreased lead, cadmium and zinc uptake by *Citrobacter* Strain MCM B-181 was observed as the pH was decreased to 2 (Puranik and Paknikar, 1999). The dependence of uptake of rhodium(III) on the solution pH strongly suggest that metal biosorption in the present investigation was a result of ionic attraction.

2.4.5 Effect of temperature

Results from this study showed that temperature in the range 0-70°C does not have a significant effect on the uptake of rhodium from solution (Figure 2.6). Temperature is one other factor controlling the speciation of rhodium(III) in solution. When exposed to high temperatures, in the presence or absence of strong reducing agents, such as hydrogen and nitrogen gas, rhodium(III) complexes undergo reduction. Heat treatment results in the formation of different rhodium species, particularly, metal or metal-oxygen species due to thermal decomposition. When rhodium acetate and chloride were heated under hydrogen up to 121°C, about 64.8% and 47%, weight loss of the rhodium salt, respectively, was observed. The observed weight losses were due to the reduction of rhodium(III) to Rh^0 (Fouad *et al.,* 2000). This suggests that the uptake at temperatures exceeding the optimum growth temperature of mesophilic SRB (25-35°C) (Gibson, 1990) was enhanced by the heat

treatment. Thus, the observed high rhodium uptake at the different temperatures can be attributed to the occurrence of sufficient interactions between rhodium species and SRB cell surfaces. Similarly, temperature-independent sorption of lead, cadmium and zinc by *Citrobacter* Strain MCM B-181 (Puranik and Paknikar, 1999), and uranium by *Pseudomonas* sp. (Marques *et al.,* 1991) was observed. In this present investigation, the high uptake of rhodium, regardless of the solution temperature, strongly suggests uptake through biosorption. However, temperature-dependence uptake of platinum (Rashamuse, 2003), uranium and technetium (Lovely and Phillips, 1992) by SRB cells was also observed. Such observations suggest the involvement of a metabolism-dependant metal uptake mechanism and the importance of biosorbent viability for sufficient metal uptake (Hughes and Poole, 1989).

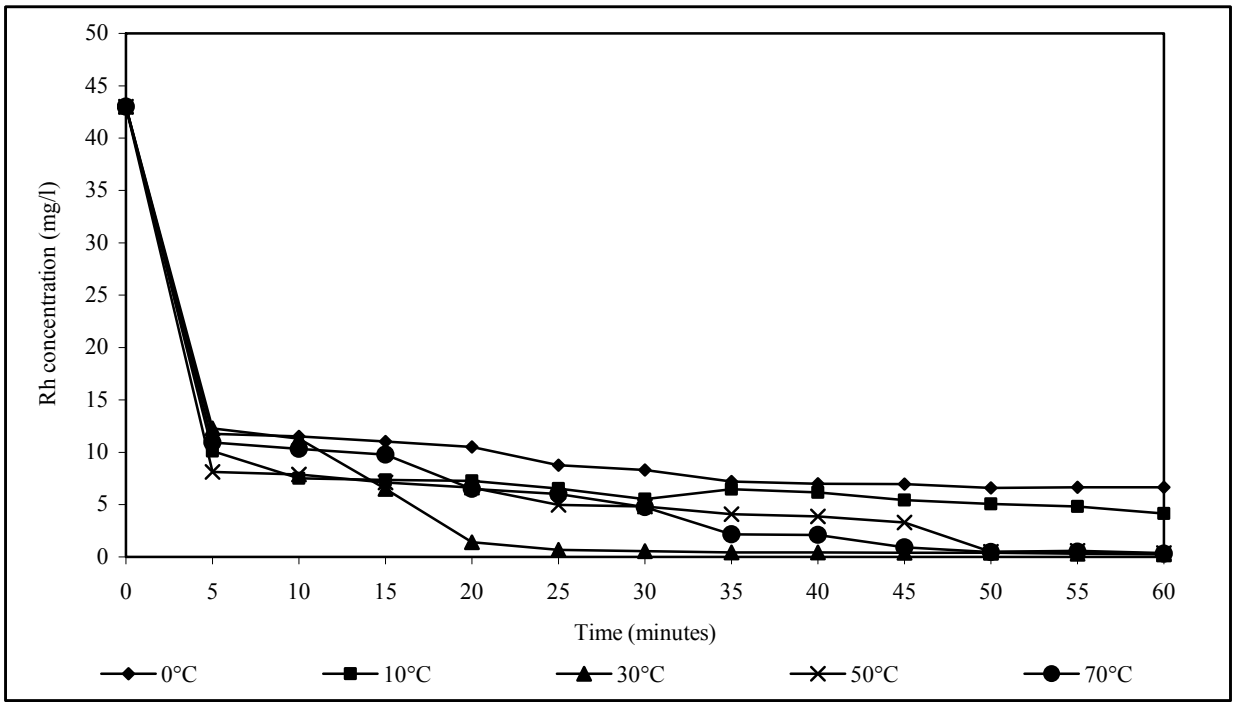

Figure 2.6: Effect of temperature on the rate of rhodium removal from solution by a resting SRB consortium (0.5g/l) with hydrogen as electron donor. Data are means ± for the triplicate values measured.

2.4.6 Effect of lactate, hydrogen and absence of an electron donor

The dependence of rhodium removal from solution by SRB on the availability of specific electron donors was investigated. When an electron donor was absent or lactate was added about 50% of the initial rhodium was removed (Figure 2.7). In the absence of an electron donor, it was interesting to note that removal of rhodium did occur. Previous results obtained

in this chapter strongly suggest that uptake mainly occurred through biosorption. Thus it can be concluded that the observed removal in the absence of an electron donor was through a non-energy consuming mechanism, such as biosorption. The same mechanism of rhodium uptake in the absence of an electron donor (biosorption) was thought to be also responsible for rhodium uptake in the presence of lactate. This is due to the fact that the breakdown of lactate to generate electrons and protons is a very slow and long process (Odom and Peck, 1981). As a result, it is highly possible that at the end of the incubation period (60 minutes) the process was not yet completed, and so there was a deficiency of electrons and protons. Therefore, the involvement of lactate as an electron donor in the uptake of rhodium by SRB cells cannot be guaranteed. These results suggest that if efficient metal uptake within a viable biosorbent system has to occur, a readily oxidized electron donor source, which would result in an instant generation of protons, is vital (Odom and Peck, 1981).

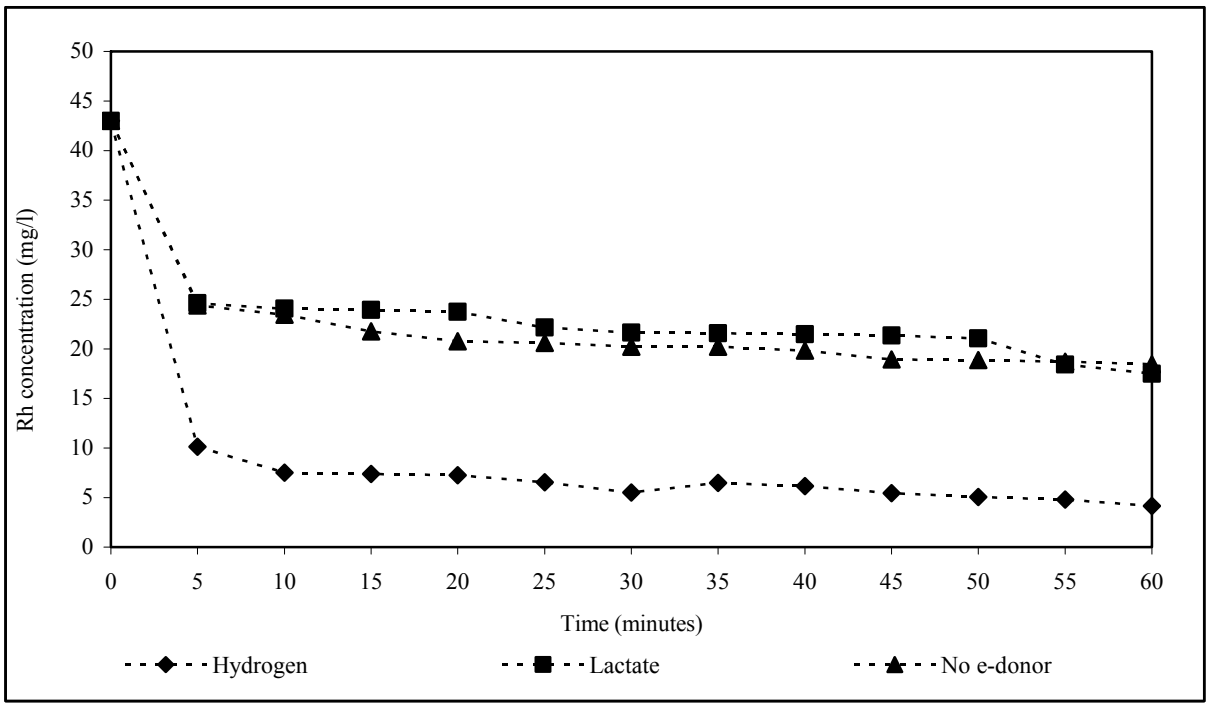

Figure 2.7: Effect of lactate, hydrogen and absence of an electron donor on the rate of rhodium removal from solution by a resting SRB consortium (0.5g/l). Data are means ± for the triplicate values measured.

However, when hydrogen gas was supplied as an electron donor, a maximum of 90% of the initial rhodium was removed. Unlike most electron donor sources, the reversible oxidation of molecular hydrogen, catalysed by a hydrogenase enzyme, is a common activity among SRB.

The oxidation of hydrogen by SRB is a relatively fast and direct process, such that there is a continuous flow of electrons and protons within the system (Louro *et al.,* 1998). As a result, a high metal removal capacity was observed, as there was a constant supply of energy derived from the oxidation of hydrogen. Similarly, increased uptake of platinum (Rashamuse, 2003), Cr(VI), U(VI) and Pd(II) (Lloyd, 2003) by SRB was observed in the presence of hydrogen as an electron donor. Apart from the fact that the observed hydrogenase-dependant uptake of metals exhibited by SRB cells suggests a metabolism-dependant mechanism of metal uptake, the involvement of a hydrogenase enzyme has also been hypothesized (Lloyd *et al.,* 1998).

2.4.7 Effect of the presence and absence of SRB cells

Results from this experiment show the effect of the presence (live and heat-killed) and absence of SRB cells on the rate of removal of rhodium from solution. A 19% reduction of the initial rhodium concentration was observed in the absence of SRB cells, whereas about 51% and 90% rhodium removal was observed in the presence of heat-killed and live SRB cells, respectively (Figure 2.8). The different amounts of rhodium removed in each case have an important implication on the mechanism of rhodium removal from solution. The reduction of metal acetates and chlorides in the presence of strong reducing agents, particularly hydrogen and high temperatures is well documented (Mansour *et al.,* 1990). Rhodium(III) chloride and acetate have been shown to be slightly reduced in the presence of hydrogen gas, a strong reducing agent, at room temperature (25-30°C). However, at 121°C sufficient (64.8%) rhodium reduction was achieved as a result of thermal decomposition. Metal reduction occurs through the formation of acetate or chloride radicals, followed by a decarboxylation and generation of alkyl radicals, of which their by products often are the reduced form of the metal (Fouad *et al.,* 2000). It was evident in this study that high temperatures were necessary for sufficient rhodium reduction in the presence of hydrogen gas.

The uptake of metals by a dead biomass is a common industrial activity, and it is a sole result of biosorption. Biosorption occurs through one or more of the following processes; chemisorption by ion exchange, complexation, co-ordination, chelation, physical adsorption and microprecipitation (Mullen *et al.,* 1989, Volesky, 1990). It follows then that when exposed to a metal solution, SRB cell surfaces function as ion exchange sites, and thus metal uptake through non-specific binding of metal ion species to cell surface associated ligands, extracellular polysaccharides and proteins results (Gazso, 2001). Biosorption occurs up to a point where the SRB cell surface becomes saturated with the metal and no more can be

adsorbed (Volesky, 1990). Biosorption of metals by a non-viable SRB biomass has been observed for Pt(IV) (Rashamuse, 2003), Zn(II) and Cu(II) (Tabak *et al.,* 2000). Similarly, the uptake of uranium, cadmium, zinc, copper and cobalt biosorption by a dead biomass of algae, fungi and yeasts through electrostatic interactions between ions in solution and cell walls has been observed (Kuyucak and Volesky, 1988). However, when a live biomass is used, metal uptake can be a result of both biosorption and bioaccumulation (Ehrlich, 1997). Metals accumulated by live SRB cells include; Pt(IV) (Rashamuse, 2003), Cr(VI) (Lovley and Phillips, 1994), U(VI) (Lovley and Phillips, 1992) and Tc(VII) (Lloyd *et al.,* 1999). Thus it was concluded that a live SRB biomass is the best option if sufficient metal removal has to occur.

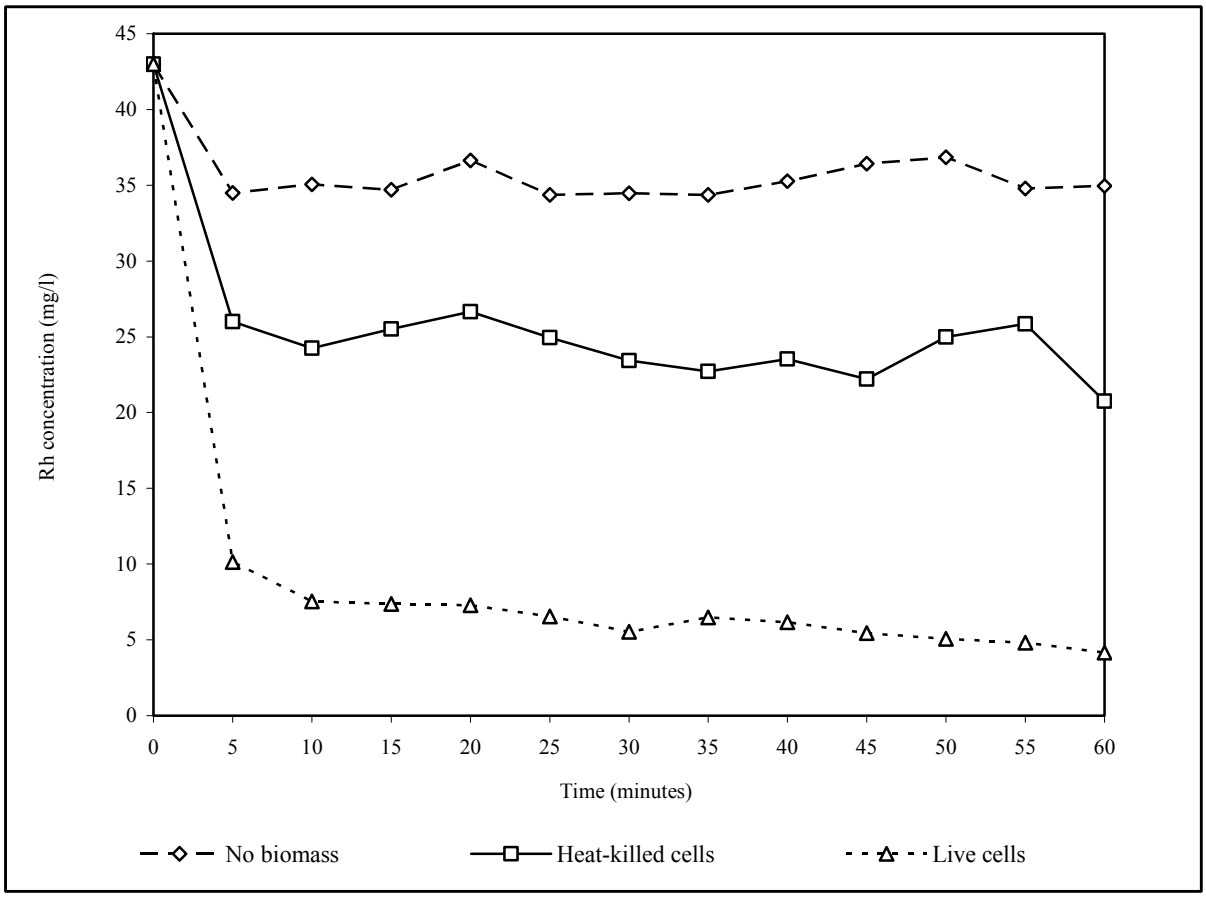

Figure 2.8: Effect of the presence of SRB cells (live and heat-killed) (0.5g/l) and their absence on the rate of rhodium removal from solution with hydrogen as electron donor. Data are means ± for the triplicate values measured.

2.4.8 Sorption isotherm

The maximum biosorption capacity (q_{max}) of SRB for rhodium was determined by fitting the Langmuir model into the obtained data (Figure 2.9). In this study, the maximum rhodium uptake was found to be 66 mg rhodium per g of SRB biomass. This model is based on an assumption that maximum adsorption occurs once the biosorbent becomes saturated with solute molecules, the energy of adsorption is constant and that there is no migration of adsorbate molecules in the surface plane. Several studies have been done on the biosorptive capacities of microorganisms on a range of heavy metals. For example, the maximum removal capacity of Cd(II) and Cr(VI) by live cells of *Bacillus laterosporus* was found to be 84 mg/g and 34 mg/g, respectively (Zouboulis *et al.,* 2004), while a mixed SRB culture was effective in removing a maximum of only 4 mg platinum per g of SRB biomass (Rashamuse, 2003). As shown in a preliminary experiment in this study, the rate of removal of rhodium from solution is controlled by the availability of suitable adsorbable rhodium species. Equilibrium sorption isotherm studies showed that the uptake of rhodium by SRB cells was a chemically equilibrated and saturable mechanism. Equilibrium was a result of both the depletion of adsorbable rhodium species in solution, and the saturation of SRB cells with rhodium over time.

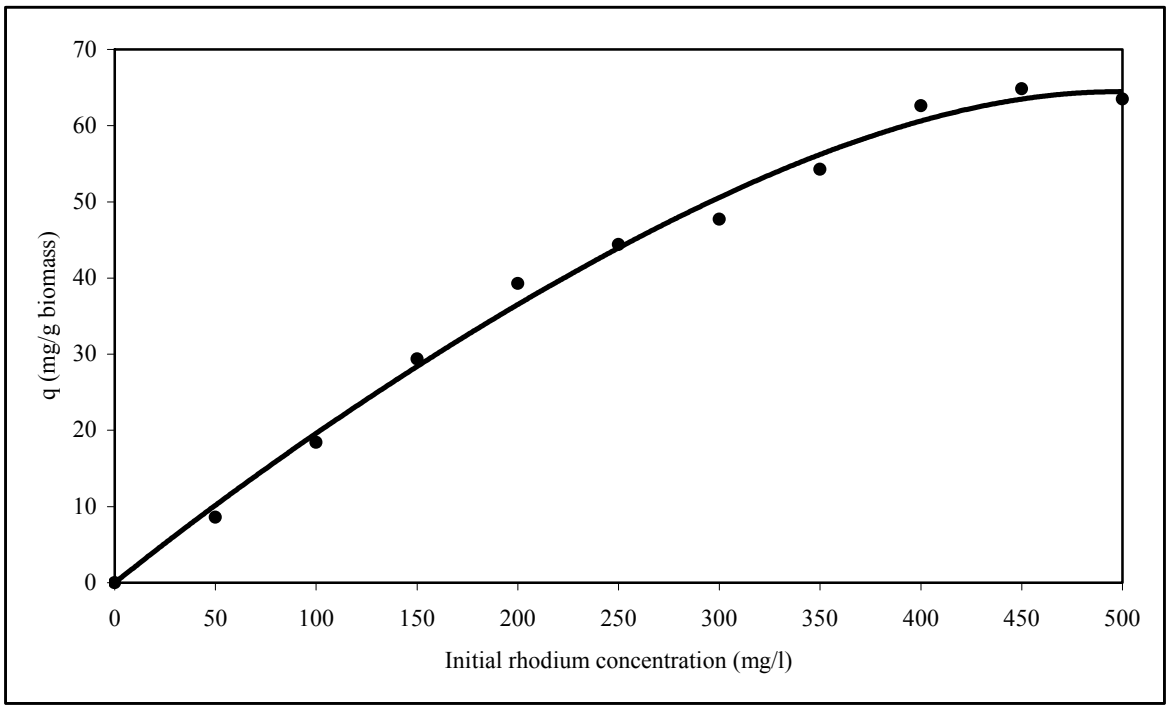

Figure 2.9: An equilibrium sorption isotherm for rhodium removal from solution by a resting SRB consortium (0.5g/l) with hydrogen as electron donor. Data are means ± for the triplicate values measured.

2.5 SUMMARY

The main factor that was found to have an adverse effect on the amount and rate of rhodium removal from solution by a non-growing SRB consortium was the ability of rhodium to form different species at different pH, temperature and concentration conditions (Sanchez *et al.,* 2002). It follows then that in future studies of this nature, preliminary studies on the speciation of rhodium(III) might prove to be useful, since it appears that the types of rhodium species present within a specified experimental condition determine the rate of rhodium removal from solution. There was no observed trend in the uptake of rhodium by SRB cells at different initial rhodium and biomass concentrations. The reason for this is not yet clear. Another difficulty experienced in this chapter was the fact that the removal of rhodium from solution occurred very fast and thus making it impossible to determine the rate of rhodium removal. The sorption performance of a rhodium removal system utilizing a resting SRB consortium showed great potential in the removal of rhodium from an aqueous solution with rhodium concentrations of up to 400mg/l. The uptake of metals by biomass is a complicated phenomenon; while biosorption was identified as one of the uptake mechanisms; sufficient evidence was lacking to identify the involvement of metabolism or energy-dependant uptake mechanisms. The possibility of the involvement of a metabolism-dependant rhodium uptake mechanism, which is manifested by the intracellular accumulation of rhodium in SRB cells, was investigated in the next chapter.

CHAPTER 3

QUALITATIVE ANALYSIS OF RHODIUM PRECIPITATES IN SULPHATE REDUCING BACTERIA CELLS

3.1 INTRODUCTION

The introduction of the electron microscope as a tool for the biologist brought about a complete reappraisal of the micro-anatomy of biological tissues, organisms and cells. With the help of the electron microscope scientists have been able to view any specimen at a magnification of up to 350 000 times (Bozzola and Russell, 1999). More recent developments in electron microscopy have come from biotechnologists, who have used electron microscopy to obtain an understanding of metal-microbe interactions that result in metal reduction. In particular, after the discovery of the highly metal tolerant SRB, the next challenge is to establish an understanding of the metal resistance or tolerance mechanisms that lead to metal reduction. Electron microscopy offers an enormous variety of experimental techniques that aid in the study of cells, and they range from qualitative to quantitative analyses, including those electron microscopy analyses that are capable of detecting *in situ* elements at the cellular level (Nott, 1995). With the help of electron microscopy, it has been established that native SRB cells are relatively small, about 3-5µm long and 0.5-1.2µm wide (Feio *et al.,* 1998), and are of different cell shapes. In particular, the Gram-negative mesophilic SRB (mostly the *Desulfovibrio* genus) are mainly rod-shaped (Castro *et al.,* 2000).

In order to predict the predominant metal uptake and reduction mechanism, changes in the elemental composition, intracellular organization and morphology of the SRB cells after exposure to high metal concentrations are examined. In this chapter, three electron microscopy techniques were used to aid the understanding of rhodium(III) uptake by resting SRB cells. Firstly, an X-ray microanalysis technique with the help of an XRF (X-ray fluorescence) was used for the positive identification of rhodium within SRB cells previously exposed to a rhodium solution. In addition, two other electron microscopy techniques were used; transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The TEM was used for establishing the distribution and localization of rhodium precipitates within SRB cells, while the SEM was used to monitor for any morphological changes in SRB cells induced by rhodium uptake. Sections that follow cover the basic fundamental principles of how the XRF, TEM and SEM work.

3.1.1 X-ray fluorescence (XRF)

XRF is an elemental analysis technique with unique capabilities including highly accurate determinations of elements and a broad elemental survey of the sample composition without standards. Detection limits for XRF are generally in the 10-100 parts per million range for heavy metals, and elements lighter than Na are difficult and almost impossible to detect (Bozzola and Russell, 1999). The main principle behind this technique is that when a specimen is bombarded with energetic photons such as x-rays, vacancies may rise due to the removal of inner orbital electrons. Outer shell electrons then fall into the vacancy left by the displaced inner shell electrons. These transitions are usually accompanied by the emission of light (fluorescence) equivalent to the energy difference between the two states. Since each element has electrons with more or less unique energy levels, the wavelength of light emitted is characteristic of the element. There are generally two types of XRF spectrometers, namely: wavelength dispersive and energy dispersive. Wavelength dispersive system uses a diffraction crystal to focus specific wavelengths onto a detector, whereas energy dispersive spectrometers focus all the emitted x-rays onto an energy analyzing detector. While the energy dispersive spectrometer is faster and less expensive, wavelength dispersive spectrometers are more sensitive and have a higher resolution (Jenkins, 2000).

Thus, the wavelength dispersive spectrometer is the most preferred type of XRF. An x-ray scan of a sample using a wavelength dispersive XRF is produced by rotating the diffraction crystal, which focuses x-ray emission lines of various elements onto the detector. The angle of diffraction (2-theta) is plotted against the detector response. Rhodium, like all other elements has characteristic electron shells referred to as K, L or M, and produces a broad emission band at a diffraction angle range 8-30º (Siegbahn, 1925). The resulting x-ray emission lines are commonly referred to as KA, KB, LA or LB, where K and L refer to the electron shell vacancy being filled, and A and B refer to the source from which the electron originates (A is the nearest and B is from the next shell further out) (Bozzola and Russell, 1999). The spectrum of K-lines is relatively simple as compared to both L- and M-lines, which are complicated by the numerous sublevels. K-spectra consists of six lines (in order of increasing energy) KA2, KA1, KB3, KB1 and two KB2, and most X-ray scans show the K-lines because they require the lowest ionization energy. Thus, in an x-ray scan of a specimen containing rhodium, a Rh(KA1) line at 24.9º represents the x-ray energy emitted when an L electron falls into the K shell, and a Rh(KB) line at 22.1º represents the x-ray emission from an M shell electron moving into a K vacancy (Siegbahn, 1925).

3.1.2 Transmission electron microscopy (TEM)

The transmission electron microscope (TEM) is useful in understanding the arrangement, composition and some of the biochemical functions of cells. TEM replaces visible light (wavelength 500 nm) with a focused beam of electrons (wavelength 0.005 nm), and consequently, the TEM attains a better optimal resolution of images. Thus TEMs can reveal the finest details of internal structure even at atomic level. Magnifications of 350 000 times can be routinely obtained for many specimens. For a specimen to be viewed under the TEM, it must be free from water or other volatile compounds as it has to be stable when exposed to electron beam damage and high vacuum conditions. The TEM has three basic components: illumination (electron gun), imaging (condenser, objective, intermediate and projector lenses), and viewing (fluorescent screen). The condenser lens is used to converge an electron beam into the specimen. Then the beam penetrates the sample and the image produced is magnified by the objective lens. Finally, the projector lens projects the image onto the viewing plane, which might be either a photographic plate, fluorescent screen or the eye (Bozzola and Russell, 1999). The energy of the electrons in the TEM determine the relative degree of penetration of electrons in a specific sample, or alternatively, influence the thickness of material from which useful information can be derived.

3.1.3 Scanning electron microscopy (SEM)

The scanning electron microscope (SEM) is a type of electron microscope that is capable of also producing high resolution images of a sample surface. SEM images have a characteristic 3-dimensional quality and therefore are useful in providing information about the shape, size and arrangement of the particles that are lying on the surface of the sample, and not internal contents of the cell. In a typical SEM, electrons are thermionically emitted from a tungsten or LaB_6 cathode filament towards an anode. The electrical beam, which has an energy ranging from a few keV to 50keV, is focused by two successive condenser lenses into a beam with a very fine spot size (~50nm). The beam then passes through the objective lens, where pairs of scanning coils deflect the beam either linearly or in a certain manner over a rectangular area of the sample surface. As the primary electrons strike the surface they are inelastically scattered by atoms in the sample. Through these scattering events, the primary beam effectively spreads and fills a teardrop-shaped volume extending about 1 μm into the surface. Interactions in this region lead to the subsequent emission of electrons and x-rays which are then detected to produce an image (Bozzola and Russell, 1999).

In order to minimize surface tension, tissues prepared for SEM are usually chemically fixed with glutaraldehyde and then dehydrated in ethanol before being dried. After drying the surface of the tissue is then coated with an electrically conductive layer of gold. Electrons are generated from the thin tungsten wire in the gun of the SEM, and then electricity is passed through the wire and then focused by magnets onto the sample. When the electrons strike the surface coating of the gold, electrons are reflected back off the specimen to a detector. This is transmitted to a television screen where the image is viewed and photographed (Goldstein *et al.,* 1981).

3.2 MATERIALS

Glutaraldehyde [$HCO(CH_2)_3CHO$] (25%) stock reagent, uranyl acetate, lead citrate, ethanol, propylene oxide, araldite, boric acid and all other analytical grade reagents were purchased from either Sigma-Aldrich or Merck (Darmstadt, Germany). Acetateplus 0.45 micron membranes were obtained from Osmonics Inc. (Minnesota, USA). All gases were purchased from Afrox (South Africa).

3.3 METHODS

3.3.1 Growth and preparation of SRB cells for qualitative analysis

SRB cells were grown and cultured, and prepared as previously described Section 2.3.2. Then SRB cells (0.5g/l) were exposed to a rhodium solution (43mg/l) as before.

3.3.2 X-ray fluorescence analysis of rhodium-loaded SRB cells

SRB cells not exposed to a rhodium solution (control) and SRB cells exposed to a rhodium solution for 3 and 9 hours were obtained by filtering through a 0.45 micron membrane. Each of the SRB cell pellets obtained was transferred into a conical flask and was freeze-dried by gently swirling in a bath of liquid nitrogen. Then the frozen SRB pellets were loaded onto a Modulyo Freeze dryer (Edwards High Vacuum Int., England) and left overnight. To avoid metal interference, each of the dried SRB biomasses was ground and pressed onto a boric acid mounting pellet. For metal analysis, the sample was then loaded onto a Philips PW1480 WDXRF spectrometer fitted with a 72-position sample changer and an 80kV rhodium anode x-ray tube, and controlled by Philips X40 computer software.

3.3.3 Distribution and location of rhodium precipitates within SRB cells

A 100µL sample of SRB cells previously challenged with 43mg/L rhodium was fixed overnight in 2.5% glutaraldehyde fixative solution in phosphate buffer (0.1M, pH 7.0). Glutaraldehyde was then decanted off and the pellet was washed twice in phosphate buffer (0.1M, pH 7.0) for 10 minutes each step. The pellet was then dehydrated using a series of ethanol solutions of 30%, 50%, 70%, 80%, 90% and twice in absolute ethanol, with each step lasting for 15 minutes. The pellet was then immersed twice, 15 minutes each in propylene oxide. Propylene oxide was then decanted and the tubes were filled with propylene: resin (Araldite) mixture, 75:25 (left overnight), then a 50:50 left for 4 hours, 25:75 mixture left for 4 hours and lastly a pure resin was used. The mixture with a pure resin was left overnight. Finally the pellet in the pure resin was left for 36 hours at 60ºC to polymerize. Sections (100-150 nm thick) were cut from the polymerized resin block using a microtome and placed onto a carbon coated copper grid. Sections were then stained in aqueous uranyl acetate for 30 minutes, washed twice in deionised water and blot dried in filter paper and further stained in lead citrate for 5 minutes. Sections were viewed using a JEOL 120C X2 TEM at acceleration voltage of 80kV. SRB cells not exposed to a rhodium solution were also treated as above, and served as a control.

3.3.4 Morphological characteristics of rhodium-loaded SRB cells

SRB cells previously challenged with 43mg/l rhodium were obtained by filtering through a 0.45 micron membrane. The membranes containing the rhodium loaded cells were then folded into a quarter, so as to avoid loss of cells during treatment. They were then prepared for scanning electron microscopy (SEM) by first fixing overnight in 2.5 % glutaraldehyde in 0.1M phosphate buffer (pH 7.0) solution. The fixative solution was decanted off and cells were then washed twice in phosphate buffer (0.1M, pH 7.0) for 10 minutes. Thereafter, the cells were dehydrated in a series of ethanol solutions, 30%, 50%, 70%, 80%, 90%, and twice in absolute ethanol, and each step lasted for 15 minutes. The samples were then dried in liquid CO_2 for 2 hours at critical point (31.1º and 73 atmospheres). Pieces of the membranes containing dried cells were cut into small squares and then mounted on stubs with double-sided tape, and then gold-coated for 30 minutes in a Large Desk II Cold Sputter Etch Coater. The prepared samples were then observed under a JEOL-JSM-840 SEM. SRB cells not exposed to a rhodium solution served as a control.

3.4 RESULTS AND DISCUSSION

3.4.1 X-ray fluorescence analysis of SRB cells

XRF spectra for resting SRB cells that were exposed to a rhodium solution (43mg/l) for 3 and 9 hours were obtained. Both were compared with an XRF spectrum for resting SRB cells not exposed to a rhodium solution (control). The obtained spectrum showed that native SRB cells (control) do not contain rhodium (Figure 3.1). Native SRB cells, however, have been found to contain other metals in trace quantities, and these include; nickel, iron, potassium, manganese, magnesium and calcium. These trace metals function as nutrients, catalysts for redox processes, cell wall stabilizers and for maintaining an osmotic balance (Hughes and Poole, 1989; Nies, 1992; Bruins *et al.,* 2000). Some metals, however, are not inherent to SRB cells, but originate from external sources, for example, any P and Cl ions detected originated from buffers and metal solution, respectively. Thus, the observed peaks in native SRB cells (control) could be due to the presence of any of the above mentioned metal ions. When SRB cells were exposed to a rhodium solution for 3 hours, one major rhodium x-ray emission line was observed (Figure 3.2), whereas after 9 hours, two rhodium x-ray emission lines were observed (Figure 3.3).

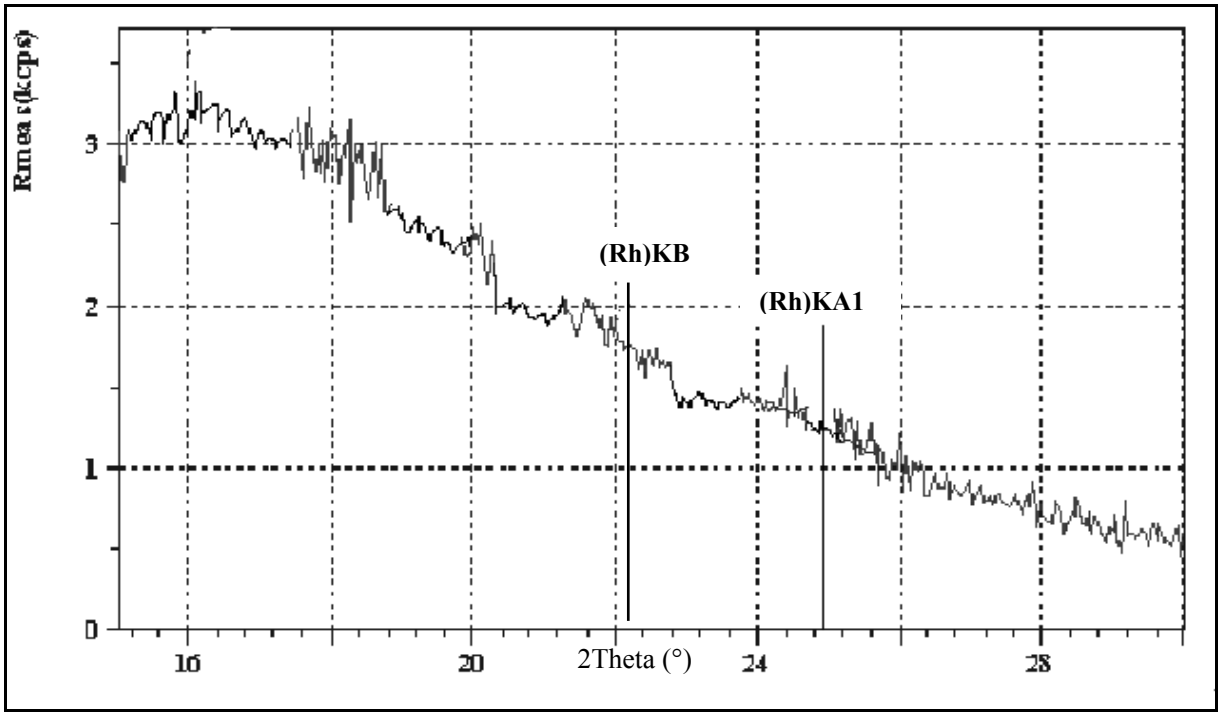

Figure 3.1: Spectrum of K-lines showing the intracellular composition of SRB cells that were not exposed to a rhodium containing solution.

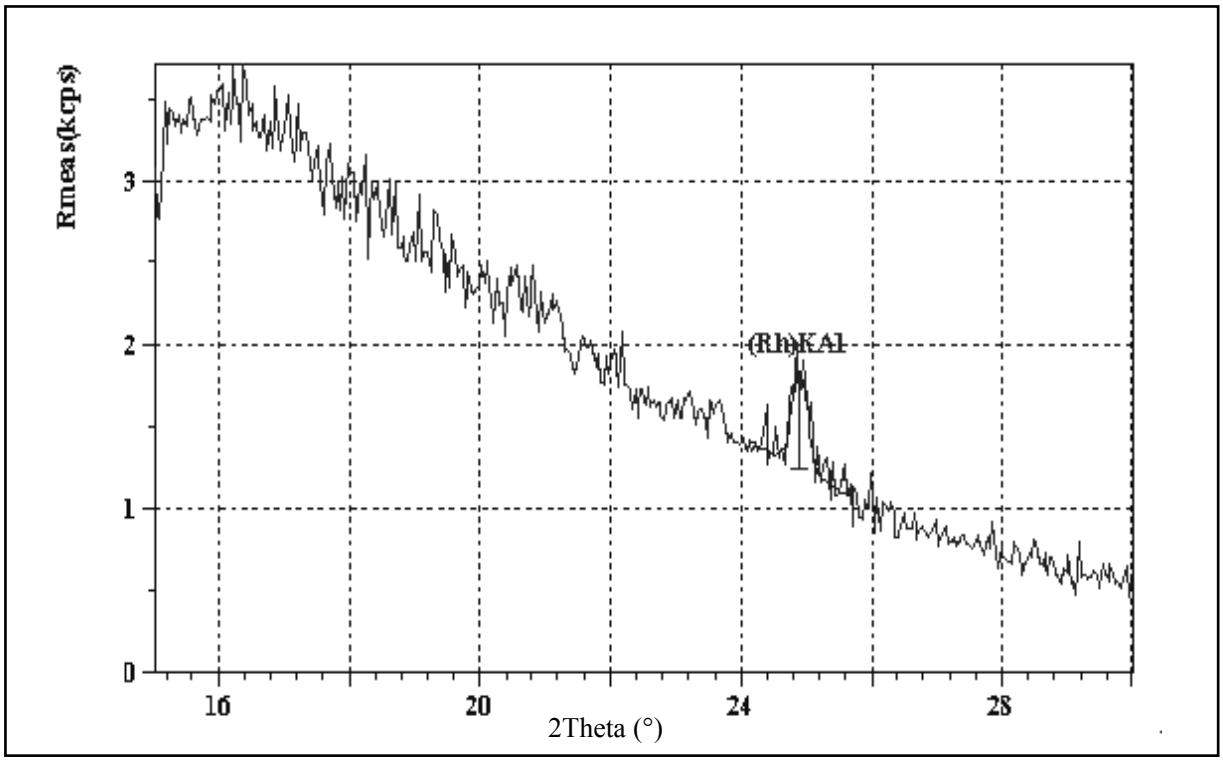

Figure 3.2: Spectrum of K-lines showing the intracellular composition of SRB cells 3 hours after exposure to a rhodium containing solution.

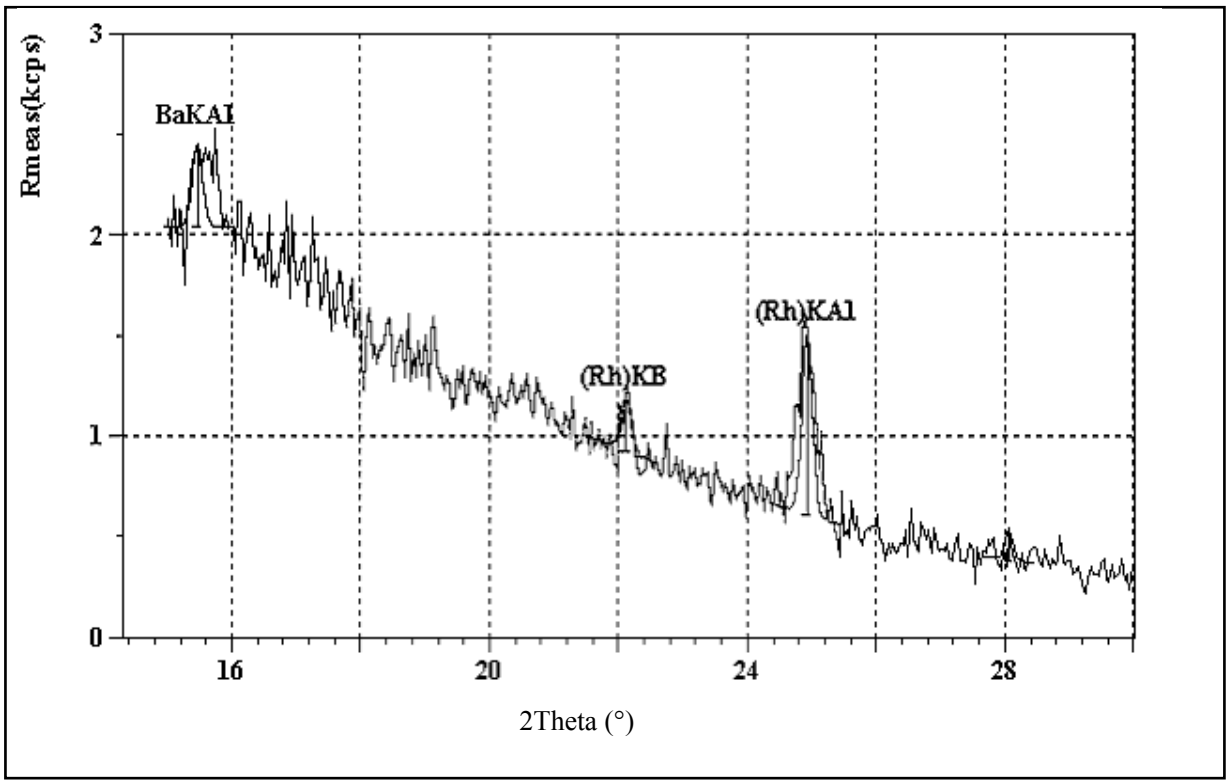

Figure 3.3: Spectrum of K-lines showing the intracellular composition of SRB cells 9 hours after exposure to a rhodium containing solution.

From the obtained spectra of SRB cells 3 and 9 hours after exposure to a rhodium solution, it was evident that rhodium was taken up by SRB cells. The obtained spectra also indicate that Rh(III) was reduced to Rh(0), since the obtained peaks correspond to those for elemental rhodium. This was in accordance with other X-ray microanalysis studies done for the bioreduction of other metals using SRB cells, for example Pd(II) to Pd(0) (Lloyd, 2003) and Pt(IV) to Pt(0) or Pt(II) (Rashamuse, 2003). In other studies metals were not reduced to their elemental forms, for example; the reduction of U(VI) to U(IV) and Cr(VI) to Cr(III) (Lloyd *et al.,* 1998; Lovley and Phillips, 1992; Lovley and Phillips, 1994). Therefore, though in the present study it was apparent that Rh(III) was reduced to Rh(0), the presence of other reduced rhodium species within SRB cells is undoubtedly another possibility that needs further investigation.

3.4.2 Distribution and localization of rhodium precipitates within SRB cells

TEM analyses of thin sections of SRB cells that were exposed to a rhodium solution for 1.5 and 9 hours were obtained. These were compared with thin sections of SRB cells not exposed to a rhodium solution (control). No evidence of localized rhodium precipitates was observed in TEM analysis of native SRB cells (control) (Figure 3.4). Well defined cell organelles and and extrapolymeric substances (EPS) were also observed as light grey attachments at cell peripheries.

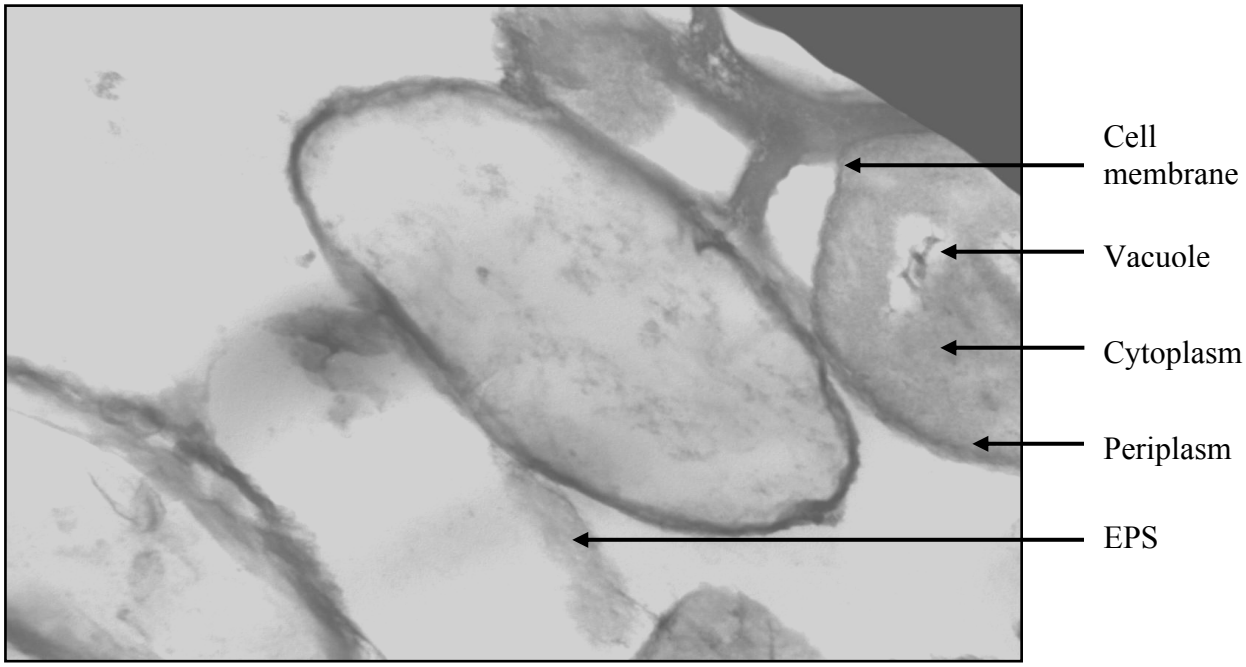

Figure 3.4: Transmission electron micrographs of thin sections of SRB cells before exposure to a rhodium solution. Magnification = 30 000 ×. EPS = extrapolymeric substances.

When SRB cells were exposed to a rhodium solution (43mg/l) for 90 minutes, dark precipitates of granulated rhodium rich areas throughout the cytoplasm and on SRB cell surfaces were observed (Figure 3.5). It was also evident that the presence of rhodium within SRB cells caused an enlargement of the cells, which is manifested by the enlargement of cell vacuoles. Similarly, when SRB cells were exposed to a rhodium solution for 9 hours, rhodium rich deposits were observed, with the majority of these precipitates localized in the EPS (Figure 3.6). Numerous air spaces (vacuoles) were observed in SRB cell interiors, and have been suggested to be a result of an energy-dependent efflux system of metal ions due to their presence in a high concentration (Alm, 2003). For example, in *E.coli* resistance to high copper and zinc concentrations is based on an efflux mechanism by which the metal is transported away from the cell and subsequently accumulated on cell surfaces (Cooksey, 1993; Beard *et al.,* 1997). Similarly the same resistance mechanism was observed in *E.coli* when exposed to 5mM concentrations of PMGs, though rhodium was found to be less toxic as compared to platinum and palladium (Alm, 2003).

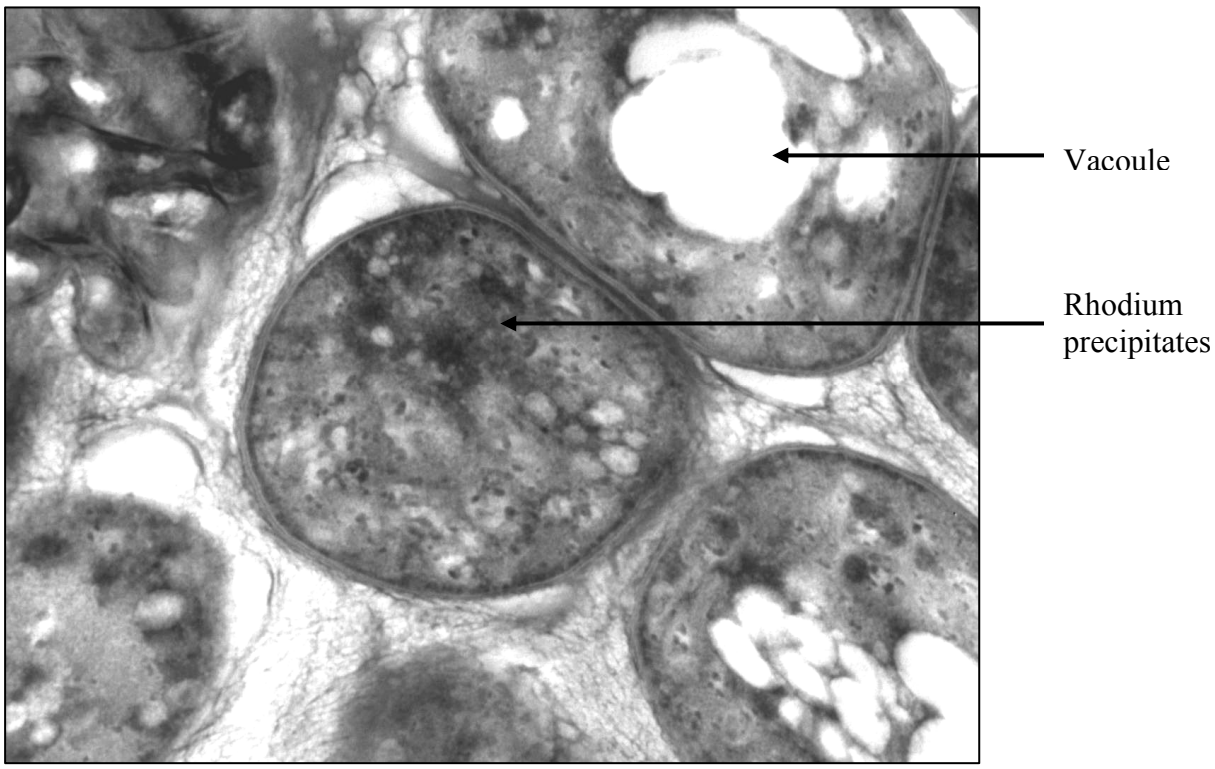

Figure 3.5: Transmission electron micrographs of thin sections of SRB cells 90 minutes after exposure to a rhodium solution. Magnification = 20 000 ×.

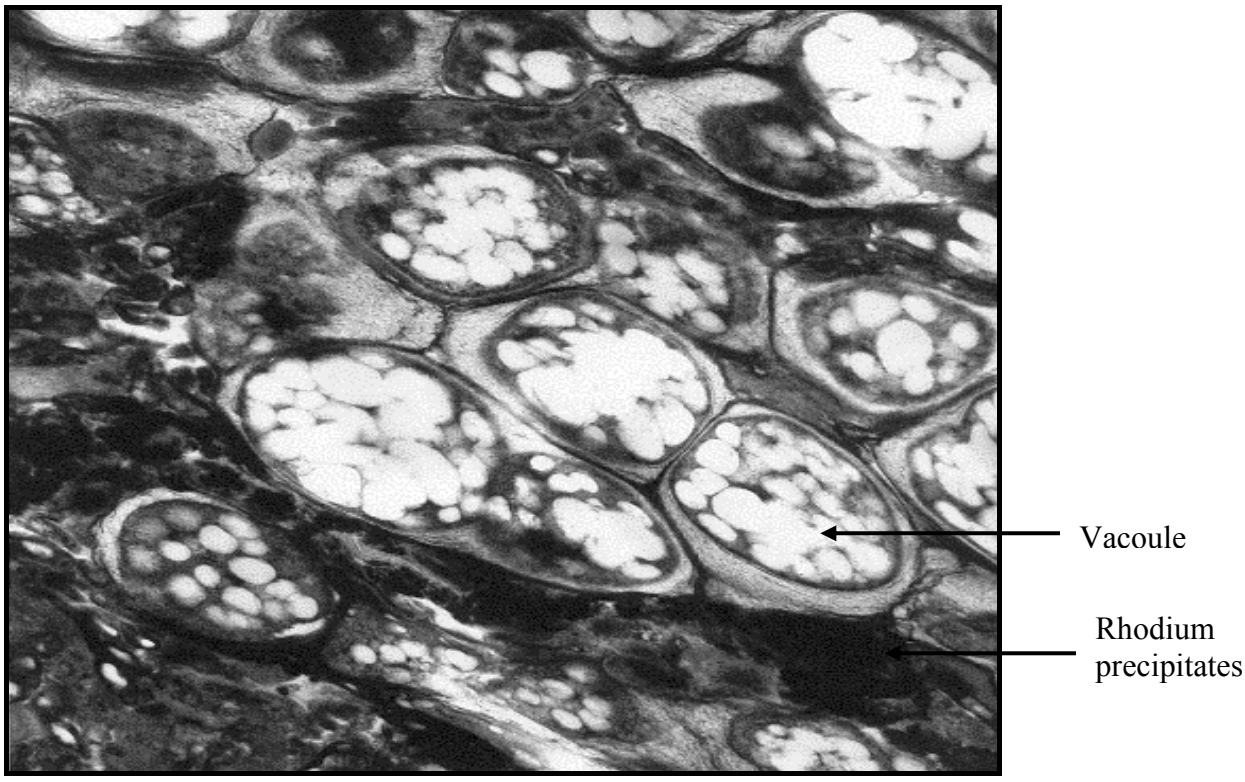

Figure 3.6: Transmission electron micrographs of thin sections of SRB cells 9 hours after exposure to a rhodium solution. Magnification = 8000 ×.

In other studies, SRB cells have been shown to possess a certain degree of metal tolerance and metal uptake continues until equilibrium is reached. Once inside the cells, the metal is subject to enzymatic reduction (Hughes and Poole, 1989). The bioaccumulation and subsequent enzymatic reduction of metals by SRB is a widespread activity, and has been reported for other PGMs such as; platinum (Rashamuse, 2003) and palladium (Lloyd *et al.,* 1998). In both studies, metal-dense areas were observed along the periplasm, strongly suggesting the involvement of an enzymatic reduction process mediated by a periplasmic hydrogenase (Lloyd, 2003). In the present study, however, results obtained indicate that over time SRB cells become more sensitive to rhodium. Consequently, exposing SRB cells for longer periods does not improve overall metal uptake. Sufficient metal uptake occurs during the first 10-30 minutes of exposure, and thereafter a reverse process occurs. When SRB cells are exposed for shorter periods (90 minutes), there is a possibility of an enzymatic reduction process, as precipitates were observed throughout the cytoplasm. This suggests that enzymatic reduction of rhodium could be mediated by either a periplasmic, cytoplasmic or membrane-bound hydrogenase enzyme.

3.4.3 Morphological characteristics of rhodium-loaded SRB cells

SEM micrographs of SRB that were exposed to a rhodium solution (43mg/l) for 3 and 9 hours were obtained. An SEM micrograph of SRB cells not exposed to a rhodium solution (control) was also obtained, and was used to monitor morphological changes induced by the uptake of rhodium. Results obtained showed that native SRB cells occurred in isolation and single, and appeared as thin rods (3-5 μm long and 0.3-0.5 μm wide) (Figure 3.7).

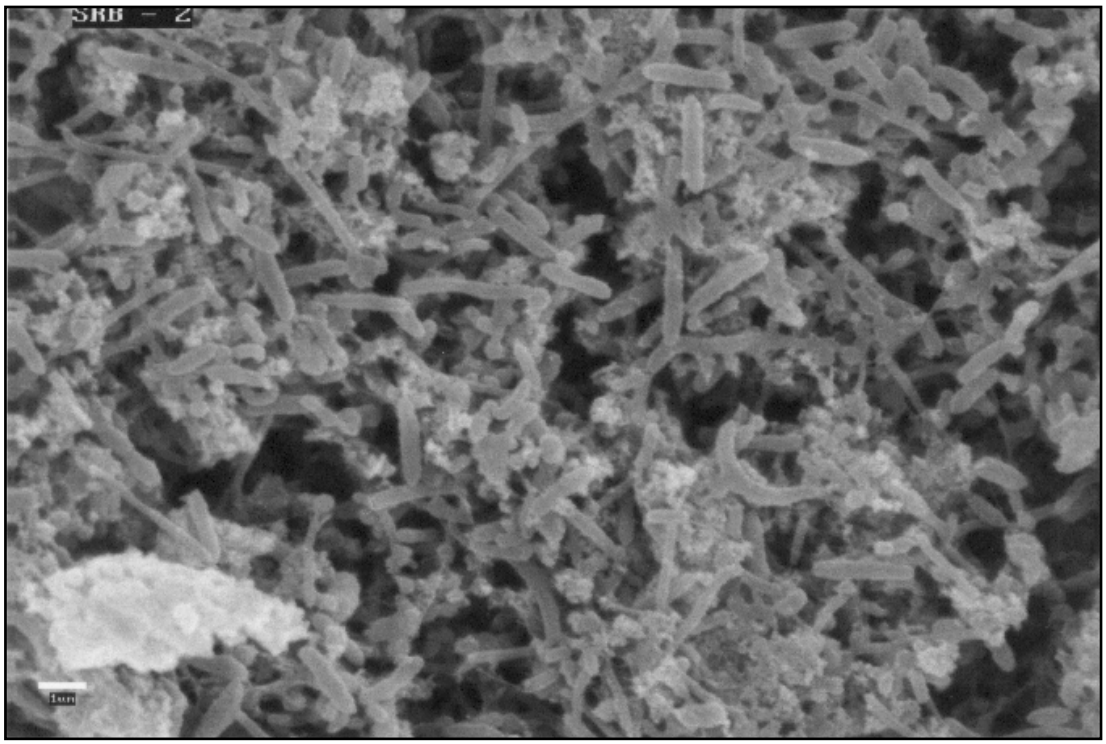

Figure 3.7: SEM micrographs of SRB cells that were not exposed to a rhodium solution. Bar = 1 μm.

Exposing SRB cells to a rhodium solution for 3 hours, however, resulted in the shortening of cell length and broadening of cell diameter (Figure 3.8). These changes were even more evident when the cells were exposed to a rhodium solution for 9 hours (Figure 3.9). In addition, SRB cell surfaces and EPS appeared darker and cell aggregation due to rhodium uptake was also observed. Similarly, shortening of cell length and elongation of diameter has been observed in SRB due to the uptake and subsequent binding of platinum (Rashamuse, 2003), palladium (Lloyd *et al.,* 1998) and uranium (Lovley and Phillips, 1992). In other studies, metal uptake has been shown to have detrimental effects, for example, broken cells of *Citrobacter* sp. Strains N14 and dc5c were observed due to the excessive accumulation of uranium on cell surfaces (Macaskie *et al.,* 2000; Jeong *et al.,* 1997).

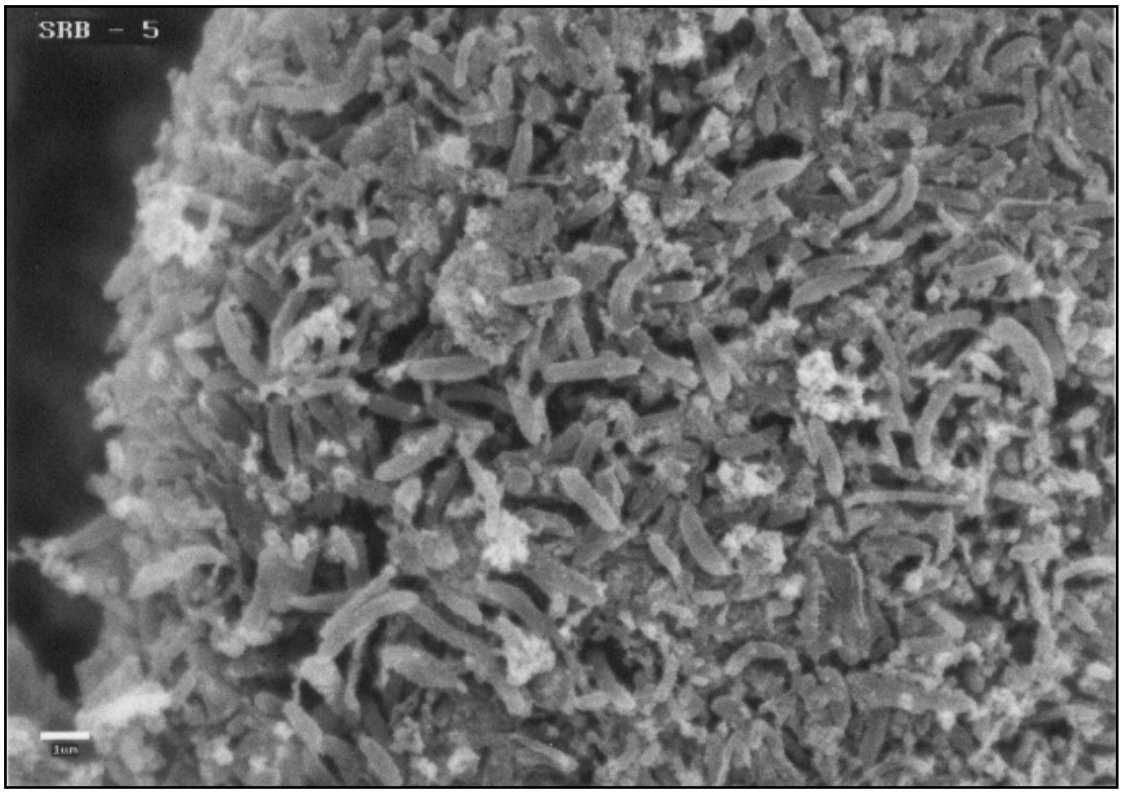

Figure 3.8: SEM micrographs of SRB cells 3 hours after exposure to a rhodium solution. Bar = 1 µm.

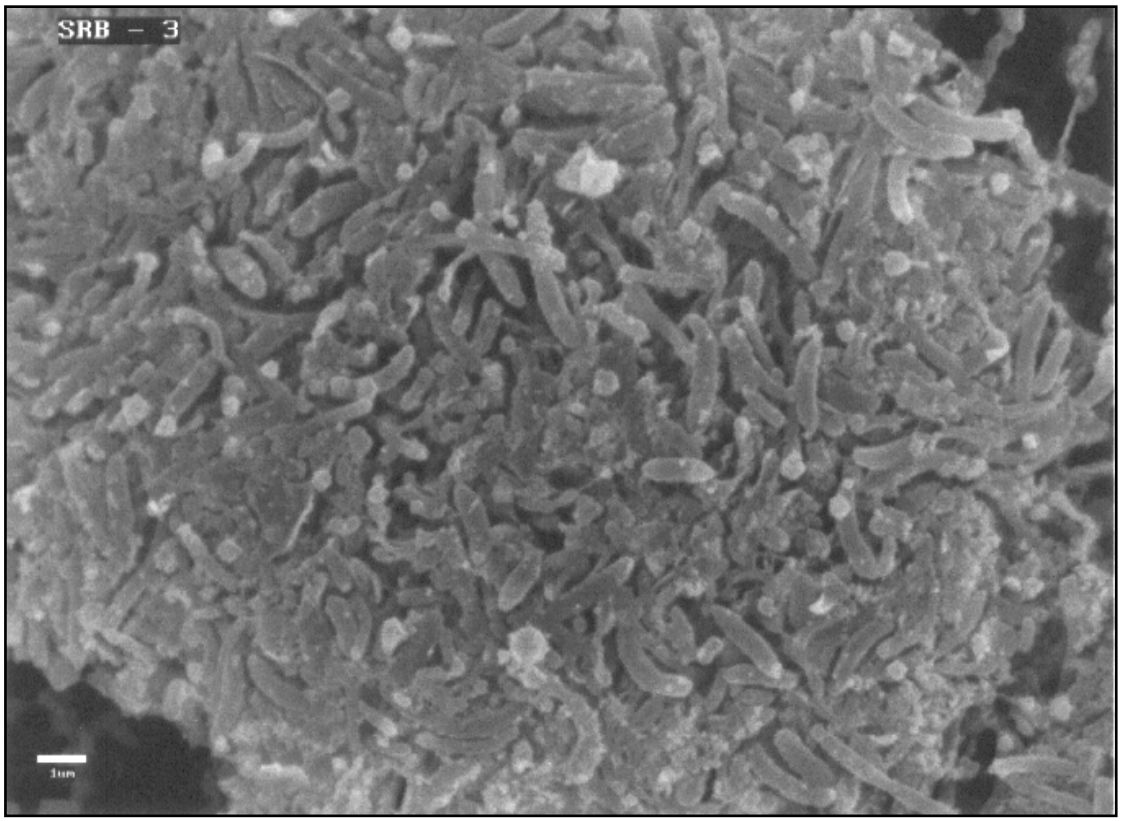

Figure 3.9: SEM micrographs of SRB cells 9 hours after exposure to a rhodium solution. Bar = 1 µm.

There was no visible evidence of lysed SRB cells among the obtained SEM images. This can be attributed the ability of cells to maintain an osmotic balance through a response mechanism when SRB cells are exposed to high metal concentrations (Valls and de Lorenzo, 2002). Results from this section further suggest that when the rhodium concentration within the cells becomes too high, the excess metal is transported out of the cells by an energy-dependant process, hence the accumulation of rhodium on SRB surfaces.

3.5 SUMMARY

Regardless of the sensitivity of SRB cells, the initial response to the presence of rhodium metal ions was metal internalization and subsequent reduction from Rh(III) to Rh(0). In future studies of this nature, it would be worthwhile to investigate the presence of other rhodium species. Changes in the morphology of SRB cells were observed as a result of rhodium uptake. Of much interest was the effect of exposure time to rhodium, where precipitates were observed throughout the cytoplasm after 90 minutes and extracellularly after 9 hours exposure. Due to the complexity of tolerance/resistance mechanism exhibited by SRB during the uptake and subsequent reduction of rhodium, which has been found to vary especially with time of exposure, it was rather difficult to ascertain the involvement of an enzymatic reduction process. In previous studies, however, hydrogenase enzymes in SRB cells were found to mediate the enzymatic reduction of various metals (Rashamuse, 2003; Lloyd *et al.,* 1998; Lovley and Phillips, 1992). Consequently, in the present study, it is possible that the reduction of rhodium(III) to rhodium(0) was mediated by a hydrogenase enzyme. The observed distribution and localization of rhodium precipitates within SRB cells, however, did not provide sufficient evidence on the localization of the hydrogenase enzyme. While in other studies, a periplasmic hydrogenase has been suggested to mediate metal reduction, in this study, the role played by periplasmic, cytoplasmic and membrane-bound hydrogenases in the reduction of rhodium *in vitro* still needs to be investigated.

CHAPTER 4

LOCATION AND EXTRACTION OF HYDROGENASE ENZYME IN SULPHATE REDUCING BACTERIA CELLS

4.1 INTRODUCTION

The first step in enzyme purification is to develop a good enzyme assay that is reliable, rapid, convenient, and readily applicable at all of the purification stages. This is important as an enzyme assay is a good indication of enzyme purity (Willson, 1999). Based on their ability to catalyze certain reactions, hydrogenase activity assays can be categorized into four classes or methods. The first method is based on the evolution of hydrogen gas, where an electron donor (D) is a low potential compound such as cytochrome c_3 or methyl viologen, that is oxidised and results in the liberation of hydrogen gas (Equation 11). The second method is based on the oxidation of H_2, where an electron acceptor (A) such as cytochrome c_3, benzyl viologen or methyl viologen, methylene blue, phenazine methosulfate, potassium ferricyanide or 2, 6-dichloroindophenol (DCIP), is reduced (Equation 12).

$$D_{red} + 2H^+ \rightarrow D_{ox} + H_2 \quad \text{(11)}$$

$$H_2 + A_{ox} \rightarrow 2H^+ + A_{red} \quad \text{(12)}$$

The oxidation and reduction of different electron donors and acceptors, respectively, can be followed spectrophotometrically at a particular wavelength of absorption. This method has been used successfully in the monitoring of hydrogenase activity in various studies. Apart from the above mentioned methods, hydrogenase assays can be based on either deuterium/tritium exchange reactions with H^+, resulting in the conversion of *para* to *ortho* H_2 (spins of protons aligned in opposite directions and parallel to each other, respectively). The use of these last two methods in most studies is limited by the fact tat they often require specialized chambers or instruments. In addition to assessing purity, assays are very useful in the determining the distribution and localization (intra or extracellular) of enzymes. All three hydrogenases (Fe-only, NiFe- and NiFeSe-hydrogenases), that have been characterized from the *Desulfovibrio* genus, have been found to reside in the cells (Peck and LeGall, 1994).

The cellular localization of hydrogenases within the same bacterium, however, is poorly understood. It has been suggested that the localization of hydrogenases among *Desulfovibrio* genus depends on their individual growth conditions, which in turn determine the mechanism of metabolic regulation (Fauque *et al.,* 1988). Generally, most NiFe-hydrogenases from *Desulfovibrio* genus are located in the periplasm and appear to be loosely associated with the membrane. NiFe-hydrogenases from *D. vulgaris* Hildenborough (*Dv*H) and *D. vulgaris* Miyazaki, however, have been found to be tightly bound to the membrane, and thus require detergents for solubilization (Peck and LeGall, 1994). NiFeSe-hydrogenases are located in the membrane facing the cytoplasm, whilst Fe-only hydrogenases can either be located in the periplasm or cytoplasm. (Lissolo *et al.,* 1986). The presence of distinct hydrogenases, with different intracellular localizations implies that appropriate cell lysis techniques should be applied to release all the activity into the aqueous extract (Walker, 2000).

A wide range of cell disruption techniques exist, and they fall into two broad categories: physical disruption and chemical treatment or enzyme lysis. For any disruption process, the suitable method should result in the release of a major proportion of enzyme, with minimal denaturation and contamination (Walker, 2000). Bacterial cells vary from fairly fragile to more resilient thick cell walls. Generally for small scale purposes, vigorous cell disruption techniques such as sonication, bead milling or the French press successfully disrupt bacterial cells (Scopes, 1982). For each cell disruption technique, careful optimization in order for the release of the entire enzyme needs to be developed (Willson, 1999). In this study sulphate reducing bacteria (SRB) cells were satisfactory lysed by sonication. During sonication, micro-scale high pressure sound waves are applied to cells, which results in cell cavitation to cause the emptying of cell contents into the aqueous medium that is normally the suspension buffer. The solubilization of membrane proteins is one of the most important steps in protein purification. Detergent cell lysis is a mild and easy alternative to physical disruption for the liberation of membrane-bound proteins (Minai-Tehrani *et al.,* 2002).

Detergents break the lipid barrier surrounding cells by solubilizing proteins at lipid:lipid, protein:protein and protein:lipid interactions. Detergents, like lipids bind to hydrophobic surfaces. They comprise of a polar hydrophilic head and a non-polar hydrophobic tail and are categorized by the nature of the head group; cationic, anionic, non-ionic or zwitterionic. The use of detergents possessing lipophilic chains which bind to the protein at its hydrophobic end in *lieu* of the normal membrane is very useful in the extraction of membrane bound

enzymes as they are capable of solubilizing membrane located proteins. Commonly used detergents include; sodium cholate, sodium deoxycholate, sodium dodecylsulphate, cetylmethyl ammonium bromide (CTAB), Tween and triton (X-100 and X-450) (Scopes, 1982).

4.2 MATERIALS

Methyl viologen (Gramoxone; Paraquat dichloride; 1,1'-Dimethyl-4,4'-bipyridinium dichloride); Sodium dithionite (Sodium hydrosulphite); Tris(hydroxymethyl)aminomethane GR buffer substance; Hydrochloric acid; Sodium cholate [Cholic acid (3ά, 7 ά, 12 ά-Trihydroxy-5β-cholan-24-oic acid) Sodium salt]; Dialysis tube (cellulose membrane) and other analytical grade reagents were purchased from either Sigma-Aldrich (Steinhem, Germany) or Merck (Darmstadt, Germany). Cell disintegration was performed using a Virsonic-100 sonicator (VirTis, Co. Inc, USA). A Beckman J2-21 centrifuge with a J14 rotor and Eppendorf 5810R centrifuges were used for centrifugation of larger and smaller volumes, respectively. All gases were obtained from Afrox (South Africa).

4.3 METHODS

4.3.1 Analytical procedures

4.3.1.1 Protein concentration

Protein concentration was determined by the Bradford assay (Bradford, 1976) (Appendix B), and protein absorbance was measured using a PowerWave X (Bio-Tek, Instrumental Inc., Winooski, USA).

4.3.1.2 Hydrogenase activity assay

Methyl viologen solution (1mM, 3 ml) in Tris-HCl buffer (20mM, pH 7.6) was placed into a anaerobic cuvette tightly sealed with a rubber stopper. Hydrogen gas was bubbled into the solution through a needle for 15 minutes. A solution of sodium dithionite (100mM, 5µl) in Tris-HCl buffer (20mM, pH 7.6) was then added in order to eliminate any residual oxygen in the cuvette. Before addition into the reaction mixture, the hydrogenase solution was activated by bubbling with hydrogen gas for 20 minutes. The reaction was then initiated by the addition of pre-activated hydrogenase solution either as a supernatant or pellet suspension (100µl) and the kinetics of methyl viologen reduction followed spectrophotometrically at 604nm. One unit of hydrogen evolution activity (U) is defined as 1µmol H_2 evolved per

minute. Absorbance readings were taken using an Ultrospec 100pro spectrophotometer (Amersham Biosciences, Biochrom Ltd, England).

4.3.1.3 Rhodium concentration

The concentration of rhodium was determined using a GBC 909 air-acetylene flame atomic absorption spectrophotometer (GBC Scientific equipment Pty Ltd, Dandenong, Australia) using a rhodium 6mA hollow cathode lamp at 343.5nm.

4.3.2 Location and extraction of hydrogenase enzyme

4.3.2.1 Control

SRB cells (1.5g wet weight) previously grown and cultured as stated before (Section 2.3.2.1) were harvested during mid-stationary phase (after 5-6 days) by centrifugation (7000 × g, 15 minutes, 4°C). The SRB cells were then suspended in Tris-HCl (20mM, pH 7.6, 50ml) buffer and assayed for protein concentration and hydrogenase activity. This sample served as a control.

4.3.2.2 Periplasmic fraction

SRB cells (control) suspended in buffer were then centrifuged (7000 × g, 15 minutes, 4°C) to obtain a cell free supernatant and an SRB cell pellet. Both the pellet and the cell free extract were analysed for protein concentration and hydrogenase activity.

4.3.2.3 Cytoplasmic fraction

The SRB pellet (from the periplasmic fraction) resuspended in Tris-HCl (20mM, pH 7.6, 50ml) buffer was then broken down by sonication at 30-second cycles for 4 minutes at 4°C. This was followed by ultracentrifugation (18 000 × g, 25 minutes, 4°C), then both protein concentration and hydrogenase activity were determined in both the resulting SRB cell debris and cell free extract.

4.3.2.4 Membrane fraction

The SRB cell debris was resuspended in 3% sodium cholate in Tris-HCl (20mM, pH 7.6) buffer (50ml) in order to solubilize the membrane, and release the bound enzyme. The mixture was stirred overnight at 4°C. This was followed by ultracentrifugation (18 000 × g, 20 minutes, 4°C) and the resulting supernatant dialysed overnight against Tris-HCl (20mM,

pH 7.6) buffer. Protein concentration and hydrogenase activity were assayed in both supernatant and pellet.

4.3.3 Enzymatic removal of rhodium (III) by SRB soluble fractions

4.3.3.1 Preparation of SRB soluble fractions

SRB cell soluble extracts containing hydrogenase activity, namely: periplasmic fraction supernatant (S1), cytoplasmic fraction supernatant (S2) and the membrane fraction supernatant (S3) were obtained as described above (Section 4.3.2).

4.3.3.2Removal of rhodium by SRB soluble fractions

Duplicate flasks each containing a 43mg/l rhodium chloride solution were prepared, and to each a 5 ml sample of the three different soluble cell fractions was added. In all flasks hydrogen gas was supplied as an electron donor throughout the incubation period (3 hours). The rate of removal of rhodium from each flask was monitored at timed intervals using an atomic absorption spectrophotometer.

4.4 RESULTS AND DISCUSION

4.4.1 Location of hydrogenase

Enzyme activity obtained in whole SRB cells before treatment of any form was considered to be 100%. In the periplasmic fraction, about 86% and 11% of hydrogenase activity was obtained in the SRB pellet and supernatant, respectively (Figure 4.1), and this process resulted in about 3% loss in enzyme activity (Table 3). From the results obtained it was evident that most hydrogenase activity was resided in the SRB cell. Thus two different extraction methods, sonication and a detergent were employed to release the hydrogenase enzyme.

4.4.2 Extraction of hydrogenase

4.4.2.1 Sonication

Sonication alone was not sufficient for the release of the entire hydrogenase activity entrapped within the SRB cells, as it resulted in the liberation of only 56% hydrogenase activity into the aqueous medium, whilst about 27.5% activity still remained inside the SRB cells (Figure 4.1). The maximum hydrogenase activity that can be released by sonication was investigated over time (Figure 4.2). It was observed that the release of hydrogenase activity from SRB pellet into the aqueous medium (supernatant) increased with an increase in the

sonication time. However, no increase in hydrogenase activity was observed after 4 minutes of sonication, which implied that all the cytoplasm associated hydrogenase activity was released. Thus, throughout the duration of this study, sonication was carried out for 4 minutes in order to release maximum hydrogenase activity. Sonication also resulted in about 16.4% enzyme activity loss (Table 3). Enzyme activity loss during sonication is a common occurrence since this technique employs ultrasonic waves for cell disruption, and might be accompanied by heat generation and subsequent enzyme denaturation (Scopes, 1982).

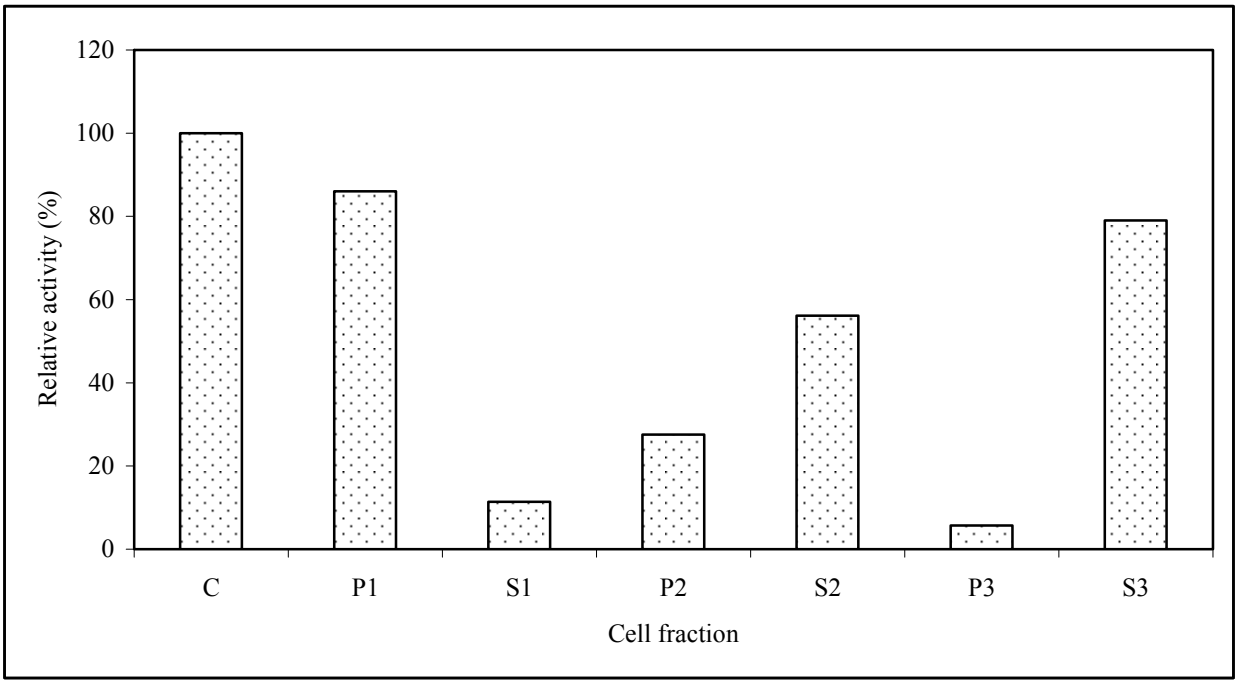

Figure 4.1: Percentage relative hydrogenase activity in the pellet and supernatant in different cell fractions. C = Control, P1 = pellet before sonication, S1 = supernatant before sonication, P2 = pellet after sonication, S2 = supernatant after sonication, P3 = pellet after detergent (3% sodium cholate) and S3 = supernatant after detergent (3% sodium cholate).

Table 3: Relative activity (%) in pellet and supernatant before and after cell treatment.

Treatment	% Relative activity in pellet before treatment	% Relative activity in pellet after treatment	% Relative activity in supernatant after treatment	% Lost activity
Control	100	86.0	11.4	2.6
Sonication	100	27.5	56.1	16.4
Detergent (3% sodium cholate)	100	5.7	79.0	15.3

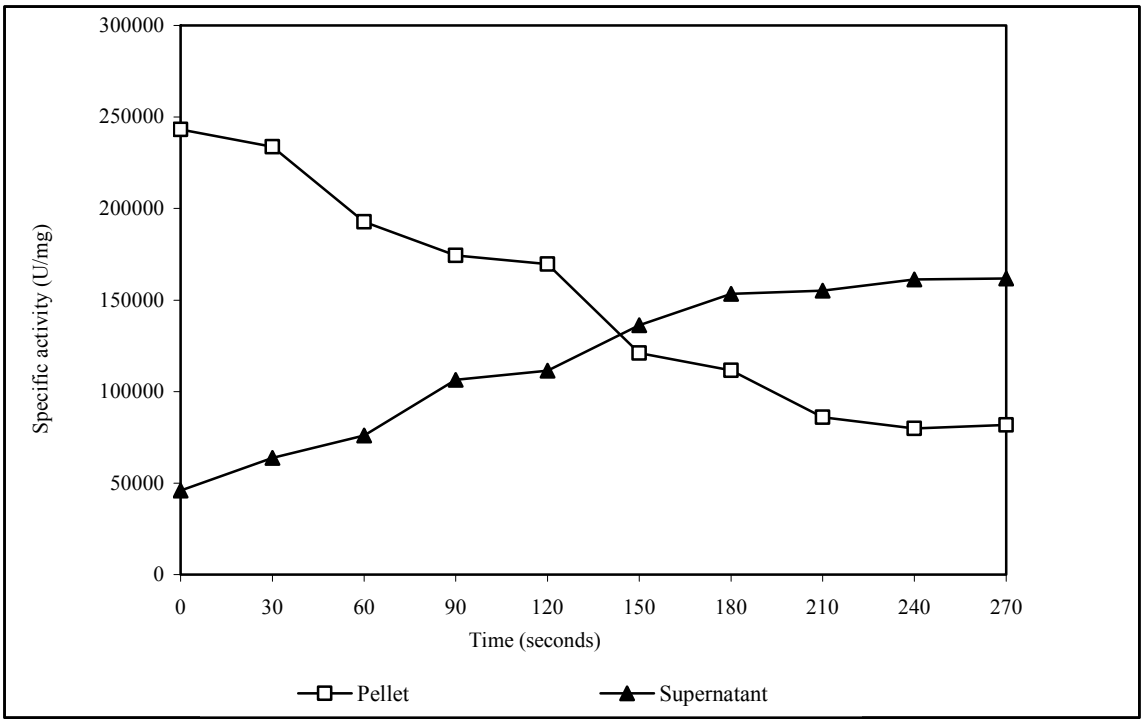

Figure 4.2: Release of hydrogenase activity from sulphate reducing bacteria cells by sonication.

4.4.2.2 Detergent

In this study sodium cholate was used for the extraction of membrane-bound proteins, as opposed to Triton X-100, a non-ionic detergent, because of the unsatisfactory results obtained when it was used for the solubilization of SRB membrane-bound proteins (Rashamuse, 2003). Similarly, detergent could not solubilize proteins from an outer membrane, and respiratory pigments from *Salmonella typhimurium* (Minai-Tehrani *et al.,* 2002). The use of sodium cholate solution for the extraction of membrane-bound hydrogenase enzyme, however, was satisfactory as about 60.8% of the total activity was released (Rashamuse, 2003). In this study, satisfactory results were observed as sodium cholate treatment resulted in the release of about 79% hydrogenase enzyme, while about 5.7% remained within cells (Figure 4.1). This process, however, resulted in a similar loss in enzyme activity (about 15.4%) when compared to sonication, which resulted in about 16.4% activity loss (Table 3).

4.4.3 Enzymatic removal of rhodium by SRB soluble fractions

Previous studies in this study have shown that the predominant mechanism of rhodium uptake when whole SRB cells are used is biosorption and to a lesser extent bioaccumulation. At the end of the incubation period (90 minutes), 53% and 88% of the initial rhodium was

recovered from solution by both the periplasmic and cytoplasmic, and membrane-bound soluble extracts, respectively (Figure 4.3). The periplasmic fraction mainly consists of c-types cytochromes and a periplasmic hydrogenase (Carepo *et al.,* 2002*)*, and numerous authors have suggested that metal reduction is a result of both proteins obtained from this fraction (periplasmic hydrogenase and its carrier cytochrome c_3) (Lloyd, 2003; Rashamuse, 2003; Lloyd *et al.*, 1998; Lovley and Phillips, 1992; Lovley and Phillips, 1994).

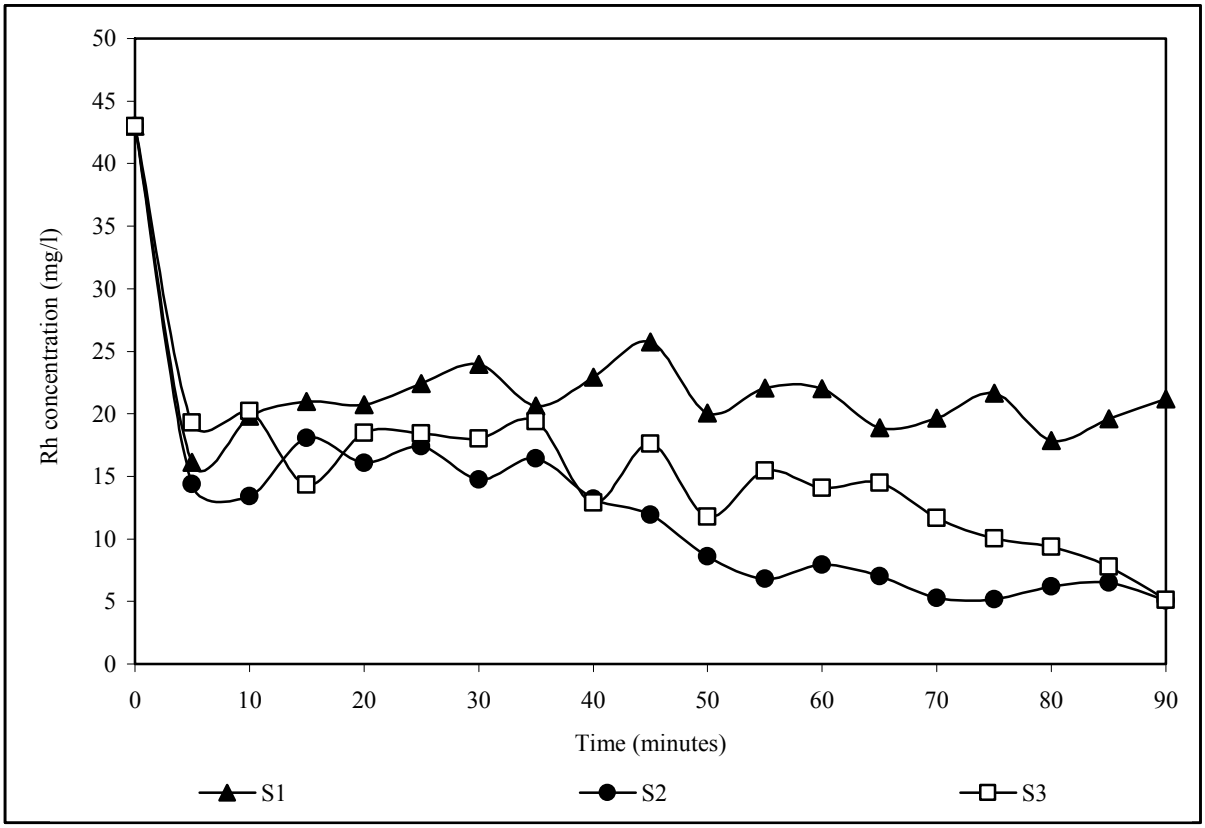

Figure 4.3: Reduction of rhodium by different cell extracts from sulphate reducing bacteria cells. Data shown are means of the triplicate values measured. S1 = supernatant before sonication, S2 = supernatant after sonication and S3 = supernatant after detergent (3% sodium cholate).

Contrary to previous findings, in this study it was found that the periplasmic fraction (S1) resulted in the least rhodium removal (about 53%). This was expected since no dense rhodium precipitates along the periplasm were observed. The high rhodium removal efficiency (88%) observed when cytosolic (S2) and membrane-bound (S3) soluble fractions were used was can be attributed to the fact that hydrogenase enzyme has been found to occur in a higher abundance in these cell compartments (Table 3).

4.5 SUMMARY

In this study, most hydrogenase activity (86%) in SRB cells was found to reside within SRB cells, and it was in accordance with previous studies where hydrogenases have been found to be either freely located in the soluble fraction (periplasm or cytoplasm) or bound to the cellular membrane (Peck and LeGall, 1994; Armstrong, 2004). Methods employed for the liberation of hydrogenases were satisfactory, as 56.1% of cytoplasmic hydrogenase activity was released by sonication, whilst sodium cholate resulted in the release of about 79% of membrane-bound hydrogenase activity. Results from this chapter have confirmed the involvement of hydrogenase enzymes in the recovery of rhodium from solution by SRB. Although abundant hydrogenase activity was found to be in the membrane-bound compartment, it was concluded that cytoplasmic-located were the most effective hydrogenases for rhodium recovery. This conclusion was supported by TEM analysis, which showed dense rhodium precipitates throughout the cytoplasmic space as opposed to the cell membrane when SRB cells after exposure to rhodium for 90 minutes. To gain a further insight on the potential application of cytoplasm-located hydrogenases on the enzymatic recovery of rhodium from both solution and industrial effluent, the next challenge was to establish the physical and chemical properties of a purer form of the enzyme. This is important especially in cases where the enzyme properties do not match the chemical and physical characteristics of the effluent. Thus, effluent pre-treatment measures can be initiated to maximise the potential of the enzyme.

CHAPTER 5

PURIFICATION AND CHARACTERIZATION OF A HYDROGENASE FROM SULPHATE REDUCING BACTERIA

5.1 INTRODUCTION

Proteins are soluble in water because they possess hydrophilic amino acids on their surfaces that attract water molecules and interact with them. This solubility is a function of the ionic strength and pH of the solution. At their isoelectric points the charges of the amino acid group in proteins balance each other, and thus exhibit a neutral charge. If the ionic strength of a solution is either very high or low, the proteins will tend to precipitate at their isoelectric point. Properties that certain enzymes possess become very useful in their purification. The similarities that they have with others are used to separate them from contaminants, and their differences become useful in their separation from one another. Different enzymes are separated based on size, shape, charge, hydrophobicity, solubility and biological activity (Walker, 2000).

5.1.1 Concentration of enzyme extract

As one of the preliminary steps during purification, the enzyme extract is concentrated by precipitation, often with ammonium sulphate or at times by organic solvents, organic polymers such as polyethylene glycol (PEG) or by selective protein denaturation (Scopes, 1994). Besides precipitation, other common methods of extract concentration include; lyophilization (freeze-drying), ultrafiltration, addition of a dry matrix and by dialysis (removal of salts and exchange of buffer) (Harris, 2001). Below is a brief description of the methods investigated in this study.

5.1.1.1 Ammonium sulphate precipitation

The fractionation of proteins by increasing the ionic strength (salting out) is the most common method of concentration by precipitation. Precipitation is achieved by the stepwise addition of pre-weighed amounts of a neutral salt (precipitant) such as ammonium sulphate, into the enzyme extract. Initially, this process precipitates some of the contaminants but then gradually precipitates the desired protein at a high concentration leaving the contaminants in the final supernatant (Willson, 1999).

Salting out is dependant on the hydrophobic nature of the surface of the protein, and in most proteins hydrophobic groups are found in the interior though in some they are on the exterior of the protein. The stepwise addition of ammonium sulphate increases the salt concentration in the system, and thus water is removed from around the protein, eventually exposing the hydrophobic groups. These hydrophobic groups interact with each other and thus result in aggregation, and subsequently fractionation of the protein (Harris, 2001). The ammonium sulphate concentration is usually expressed in percent saturation, and it is assumed that the same amount of ammonium sulphate will be dissolved in both the extract and pure water. The mass of ammonium sulphate (g) added (per litre) to achieve a certain percentage saturation is calculated using Equation 13.

$$g = 533\ (S_2 - S_1)/\ (100 - 0.3S_2) \quad \text{.....(13)}$$

Where S_1 and S_2 are the initial and final percent saturations, respectively.

5.1.1.2 Concentration by freeze drying

Freeze drying involves the removal of water or other solvent from a frozen product by a process called sublimation. Sublimation occurs when a frozen product changes directly to gaseous state without passing through the liquid phase. The result of freeze-drying is a dry powder of enzyme, which is more stable than the aqueous form of the enzyme extract. Freeze-drying is not a selective process; however, thus it results in the concentration of any salt present in the extract. Another major disadvantage with this method is that it requires longer processing times and can result in poor resolution due to the phase change (Harris, 2001).

5.1.1.3 Concentration by PEG

Proteins can also be precipitated out of solution using organic polymers, with the most frequently used polymer being polyethylene glycol (PEG or antifreeze). PEG of molecular weight 4000 or greater is most effective. It is inert and, unlike ammonium sulphate, tends to stabilize proteins. Precipitation by the PEG is one of the simplest and rapid methods of concentrating solutions as it does not require specialized apparatus. The protein solution to be concentrated is poured into a dialysis bag and it is surrounded by a dry matrix of PEG that is allowed to absorb water and other small molecules out of the solution. The protein remains within the dialysis bag as the pores are too small to allow protein to pass through. As the

amount of water within the dialysis bag containing the protein solution decreases it results in protein concentration (Scopes, 1994).

5.1.2 Column Chromatography

5.1.2.1 Ion exchange

Ion exchange chromatography is the most widely used high resolution method for the separation of proteins based on the attraction between oppositely charged particles (Willson, 1999). Ion exchangers are made up of charged groups covalently attached to a support matrix. The suitability of an ion exchange resin for protein purification is governed by the chemical and physical properties of the matrix, which determine the flow characteristics, ion accessibility and stability of the ion exchanger. A matrix with fixed positive charges is called an anion exchanger, and, as the name suggests, it binds anions. Anion exchangers are operated at a pH above the isoelectric point of the protein to be isolated as at this pH the net charge of the protein of interest will have a negative charge, and so will bind strongly to the adsorbent. Cation exchangers operate in the same way; they bear negatively charged groups that reversibly bind positively charged species. Thus cation exchangers are useful in separating proteins at a low pH as the net charge of the protein will be positive. Proteins and polyionic polymers that possess both a negative and positive charge can bind to either an anion or cation exchanger. Since different proteins have different binding affinities, high affinity proteins can be eluted from the column by increasing the salt concentration or changing the pH of the elution buffer (Voet and Voet, 1995).

5.1.2.2 Gel filtration

Gel filtration (also known as size exclusion chromatography) is used to separate molecules based on their size and shape. The solution containing the protein of interest is passed through a column packed with hydrated and sponge-like beads (called a gel), which have pores that span a relatively narrow size range of molecular dimensions. Molecules that are too large to pass through the gel are excluded and so emerge out of the column first. However, smaller molecules within the fractionation range of the gel, penetrate the pores, and so emerge later with the smallest emerging last. Apart from separation based on size, the extent of separation also depends on their shape. Elongated proteins (due to their higher hydration radius) pass through faster than compact globular proteins of the same molecular weight. However, a nucleic acid of the same molecular weight moves rather faster when

compared to both elongated and globular proteins (Willson, 1999). The behaviour of any particular molecule on a gel can be quantitatively characterized as shown in Equation 14.

$$V_t = V_i + V_o \qquad (14)$$

Where V_t is the total bed volume of the column, given by the sum of V_i (the volume occupied by the gel beads) and V_o (the volume of the solvent space surrounding then beads). The sum of the two (V_i and V_o) gives the total bed volume. The elution volume (V_e), is the volume of solvent required to elute the solute from the column, and the V_e of the solute that has a molecular mass higher than the exclusion limit of the gel is also the void volume (V_o) of the column. Thus the behaviour of a solute on any given gel is given by the ratio V_e/V_o (relative elution volume), a quantity that is independent of column size (Voet and Voet, 1995).

5.1.3 Enzyme characterization

Since enzymes find applications in a wide variety of biotechnology fields, it is important that some of their most important properties are known. These properties include stability to temperature change, pH, catalytic properties, size (molecular weight), charge (electrophoretic mobility), and binding partners (substrates and co-factors) (Willson, 1999). With the exception of molecular weight determination, the rest of the characteristics are estimated by performing the enzyme assay at specified conditions for each of the parameters.

5.1.3.1 Electrophoresis

Electrophoresis is the most widely used technique for the analytical separation of proteins. It involves the migration of ions in an electric field. The most common protein characterization method within this technique is SDS-PAGE, which is useful in the determination of the molecular weight of the peptide chain of the protein. Apart from molecular weight determination, SDS-PAGE can also be used to assess the purity of the purified protein. Negatively charged SDS binds to protein molecules, and this causes them to unfold into a rod-like shape of constant charge density per unit mass so that electrophoretic mobility is independent of amino acid composition (Voet and Voet, 1995).

5.2 MATERIALS

Polyethylene glycol (PEG) 20 000 (Merck, Darmstadt, Germany); Ammonium sulphate, DEAE-sephacel (Diethylaminoethyl sephacel), sodium chloride and all other analytical grade reagents were purchased from Sigma-Aldrich (Steinhem, Germany) or Merck (Darmstadt, Germany). Sephadex G200 (Superfine) was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Snakeskin dialysis tubing was purchased from Amersham Biosciences, Pty. Ltd. (Vienna, Austria). A Beckman J2-21 centrifuge with a J20 rotor was used for ultracentrifugation. Hydrogen gas was purchased from Afrox (South Africa).

5.3 METHODS

5.3.1 Analytical procedures

Protein concentration and hydrogenase activity assay were carried out as before (Section 4.3.1.1 and 4.3.1.2, respectively). DEAE-sephacel (pre-swollen) and Sephadex G200 (superfine) were regenerated and equilibrated as described in Appendices C1 and C2, respectively. The purification procedure adopted in this study was one that showed the best results out of several other attempts (results not shown), which proved futile. Both the purity assessment of purified proteins and molecular weight determination was achieved by SDS-PAGE, according to the method by Laemmli (1970), as outlined in Appendix D.

5.3.2 Purification of hydrogenase

5.3.2.1 Preparation of soluble extract

SRB cells were grown and cultured as before (Section 2.3.2.1). SRB cells (10g wet weight) were obtained by centrifugation (7000 × g, 15 minutes, 4ºC). The cells were suspended in 100 ml of 50mM Tris-HCl buffer (pH 7.6) and were broken by means of sonication (30 second cycles, 4 minutes, 4ºC). Broken cells were removed by further centrifugation (10 000 × g, 30 minutes, 4ºC). Any remaining membrane material was removed by further centrifugation of the supernatant (30 000 × g for 40 minutes, 4ºC). Both protein concentration and hydrogenase activity were determined on the clear soluble extract.

5.3.2.2 Concentration of enzyme extract

Of all the extract concentration methods investigated in this study, concentration using PEG was selected because it resulted in minimal loss of hydrogenase activity. The soluble extract (obtained as described in Section 5.3.2.1) was poured into a dialysis bag and was then completely covered with a dry matrix of PEG. The sample was left at 4°C for 2 hours, and

the volume was checked every 30 minutes, until the desired sample volume (15ml) was attained. The protein concentration and hydrogenase activity of the resulting soluble extract concentrate was determined.

5.3.2.3 Ion exchange chromatography

A sample (5ml) of the concentrated soluble extract was carefully loaded onto the DEAE-sephacel column (1.5cm × 20cm) previously equilibrated with 50mM Tris-HCl buffer (pH 7.6). The unbound protein was washed from the column with the same buffer until the absorbance at 280nm of the eluate had reached baseline. The bound protein was then eluted by a stepwise gradient (0-1M) NaCl in Tris-HCl buffer (50mM, pH 7.6) at a flow rate of 1ml/minute. Fractions (5ml) were collected and monitored for both protein and hydrogenase activity. Hydrogenase containing fractions were pooled and dialysed against Tris-HCl buffer (50mM, pH 7.6) to remove the NaCl and then concentrated by PEG as above (Section 5.3.2.2). The purity of the concentrated enzyme was determined by SDS-PAGE.

5.3.2.4 Gel Filtration

From the pooled and concentrated hydrogenase containing fractions from DEAE-sephacel, a 5ml sample was withdrawn and loaded onto a Sephadex G200 column (2.5cm × 15cm). Proteins were then eluted with Tris-HCl buffer (50mM, pH 7.6) at a flow rate of 10ml/hour. Fractions (5ml) were collected and monitored for both protein and hydrogenase activity. Hydrogenase containing fractions were pooled and then concentrated by PEG as before (Section 5.3.2.2), and then assayed for both protein and hydrogenase activity.

5.3.2.5 SDS-PAGE

The molecular weight of the purified protein was estimated using the SDS-PAGE method by Laemmli (1970). Protein samples and molecular weight markers (20µl) of known weights were loaded into wells and then were applied on a 10% SDS-PAGE (Appendix D) at 120V. Gels were stained using a Coomassie brilliant blue R-250 staining solution followed by destaining in a Coomassie Gel destaining solution. The molecular weight of the purified protein was calculated from a graph (Appendix D, Figure II) obtained by plotting the distance migrated versus the log molecular weights.

5.3.3 Characterization of hydrogenase

5.3.3.1 pH profile

The optimum pH for hydrogenase was established by suspending the enzyme extract at the following different pH buffers: 20mM citrate buffer (pH 4-5.5), 20mM phosphate buffer (pH 6-6.5) and 20mM Tris-HCl buffer (pH 7-9). Hydrogenase activity in each of the different pH solutions was carried out as before (Section 4.3.1.2).

5.3.3.2 Temperature profile

The optimum temperature for hydrogenase was established by incubating both the enzyme extract and acceptor solution (1mM methyl viologen) at different temperatures (0-70°C) and at optimum pH (8.0). Hydrogenase activity at each of the different temperatures was carried out as before (Section 4.3.1.2).

5.3.3.3 Thermal stability

The thermal stability of hydrogenase with methyl viologen (1mM) as electron acceptor was determined at both optimum temperature (40°C) and pH (8.0). Hydrogenase activity at time zero served as a control (100% activity). The residual hydrogenase activity was determined at 10 minute-intervals over 150 minutes.

5.3.3.4 Electron acceptor specificity

The activity of hydrogenase in the presence of 4μM cytochrome c (from horse heart) (ε_{550nm} = 7.18$mM^{-1}cm^{-1}$), potassium ferricyanide (ε_{420nm} = 1.04$mM^{-1}cm^{-1}$), 2,6-dichloroindophenol (DCIP) (ε_{600nm} = 20.6$mM^{-1}cm^{-1}$) and methyl viologen (ε_{604nm} = 13.9$mM^{-1}cm^{-1}$), as electron acceptors at optimum pH (8.0) and temperature (40°C) was determined. Hydrogenase activity with methyl viologen and hydrogen as electron acceptor and donor, respectively, served as a control (100% activity).

5.3.3.5 Electron donor specificity

The activity of hydrogenase in the presence of cytochrome c (from horse heart) (ε_{550nm} =7.18$mM^{-1}cm^{-1}$), potassium ferricyanide (ε_{420nm}= 1.04$mM^{-1}cm^{-1}$), 2,6-dichloroindophenol (DCIP) (ε_{600nm} = 20.6$mM^{-1}cm^{-1}$) and methyl viologen (ε_{604nm} = 13.9$mM^{-1}cm^{-1}$), as electron acceptors at optimum pH (8.0) and temperature (40°C) was determined. Hydrogenase activity was carried out as before (Section 4.3.1.2), and either hydrogen gas (control) or 0.5mM NADH was supplied as electron donors.

5.3.3.6 Effect of metal ions

The activity of hydrogenase in the presence of different metal ions at a concentration range 0-10mM was determined. Hydrogenase was incubated at 4°C for 30 minutes with metal ions, including; $CrCl_3.6H_2O$, $ZnCl_2$, $CuSO_4.5H_2O$, $AgNO_3$, $NiCl_2.6H_2O$ and $FeSO_4.7H_2O$. Hydrogenase activity was carried out as before (Section 4.3.1.2) using methyl viologen as an electron acceptor. An assay solution without any metal served as a control (100% activity).

5.3.3.7 Effect of EDTA

The activity of hydrogenase in the presence of a metal chelating agent, ethylenediaminetetraacetic acid (EDTA) at a concentration range 0-10mM was determined. Hydrogenase was incubated with different EDTA concentrations at 4°C for 30 minutes. The residual hydrogenase activity was monitored as before (Section 4.3.1.2) using methyl viologen as electron acceptor. The activity of the control (an assay solution without EDTA) was taken as 100% activity.

5.3.3.8 Kinetic parameters (V_{max} and K_M)

In order to determine the kinetic properties (V_{max} and K_M) of the hydrogenase, kinetic studies using methyl viologen as a substrate electron acceptor at a concentration range 0-15mM were done. Hydrogenase activity was carried out as before (Section 4.3.1.2).

5.4 RESULTS AND DISCUSSION

5.4.1 Purification of hydrogenase

5.4.1.1 Concentration of enzyme extract

The suitability of three methods for the concentration of a soluble enzyme extract prior to purification was investigated (Table 4). An inexplicable result was forthcoming with total protein analyses since more protein was found with subsequent purification steps. This can only be referred as experimental error. Concentration by PEG resulted in complete recovery of enzyme activity, whereas ammonium sulphate or freeze-drying resulted in 35.6% and 59.3% loss in enzyme activity, respectively. The observed activity loss, however, was not expected as ammonium sulphate has been reported elsewhere to stabilize enzymes (Voet and Voet, 1995). On the other hand, the main limitations of freeze-drying is that it requires longer processing times, and the powder formed may contain traces of other reagents, that might affect the activity of the enzyme once in solution (Roe, 2001). Therefore, for all concentration studies reported in this study, PEG was selected as the most suitable method.

Table 4: Methods of extract concentration

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	% Recovery	Enrichment
Soluble extract	30	0.1	19 503	195 030	100	1
1. $(NH_4)_2SO_4$ precipitation	15	0.195	9502.4	48 730	48.7	0.25
2. PEG	15	0.134	20 598.1	153 717	105.6	0.79
3. Freeze-drying	5	0.176	7929.6	45 054	40.7	0.23

5.4.1.2 Column chromatography

After several attempts in hydrogenase purification, the procedure adopted in this study is the one that showed the best results. The summary of hydrogenase purification by PEG, ion exchange and size exclusion chromatography, are shown in Table 5. A soluble extract (30 ml) obtained as described in Section 5.3.2.1, initially containing 0.1mg total protein was concentrated with PEG. After concentration, the volume was reduced to 15ml, and there was an unexpected increase in protein to 0.134mg. This step only served as a concentration process and no significant increase in the enrichment was observed, however the specific activity decreased from the original 195 030 to 153 717 U/mg.

Table 5: Purification table of hydrogenase

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Fold purification
Soluble extract	30	0.1	19 503	195 030	100	1
PEG concentration	15	0.134	20598.1	153 717	105.6	0.79
DEAE-sephacel	10	0.018	17450.6	969 478	89.5	4.97
Sephadex G-200	10	0.009	11 556	1 284 000	59.3	6.6

The concentrated extract was then applied onto a DEAE-Sephacel ion exchange chromatography column, and several protein peaks (measured at 280nm) emerged after the introduction of NaCl, with hydrogenase activity only detected in two major peaks (fractions 45-46 and 76-78) (Figure 5.1). The first hydrogenase peak (45-46) was eluted with 250mM NaCl, whereas the second was eluted with 500mM NaCl salt. When these two peaks (45-46 and 76-78) were pooled together, concentrated and then assayed for protein and hydrogenase

activity, the specific activity increased from 153 717 U/mg to 969 478 U/mg, while the protein concentration decreased from 0.134 to 0.018mg. The decrease in protein concentration can be attributed to the fact that the extract contained a mixture of proteins, most of which were non-hydrogenase as indicated by the several proteins peaks that emerged at 280nm. The ion exchange chromatography step was successful in removing some of the contaminating proteins as the enrichment increased from 0.79 to 4.97. This step, however, resulted in the loss of about 10.5% of the total hydrogenase activity present after PEG concentration step, as the total activity was reduced to 17 450.6 U. Before gel filtration, a sample of the pooled and concentrated fractions exhibiting hydrogenase activity from the DEAE-Sephacel column was set aside for purity analysis using SDS-PAGE. The remainder of the hydrogenase containing fractions (pooled and concentrated) were loaded onto a Sephadex G200 gel filtration column for further purification. During gel filtration, proteins were eluted with a 50mM Tris-HCl buffer (pH 7.6), and one broad hydrogenase exhibiting peak emerged (Figure 5.2). From the graph it can be seen that there were still some contaminating proteins present which were removed during gel filtration.

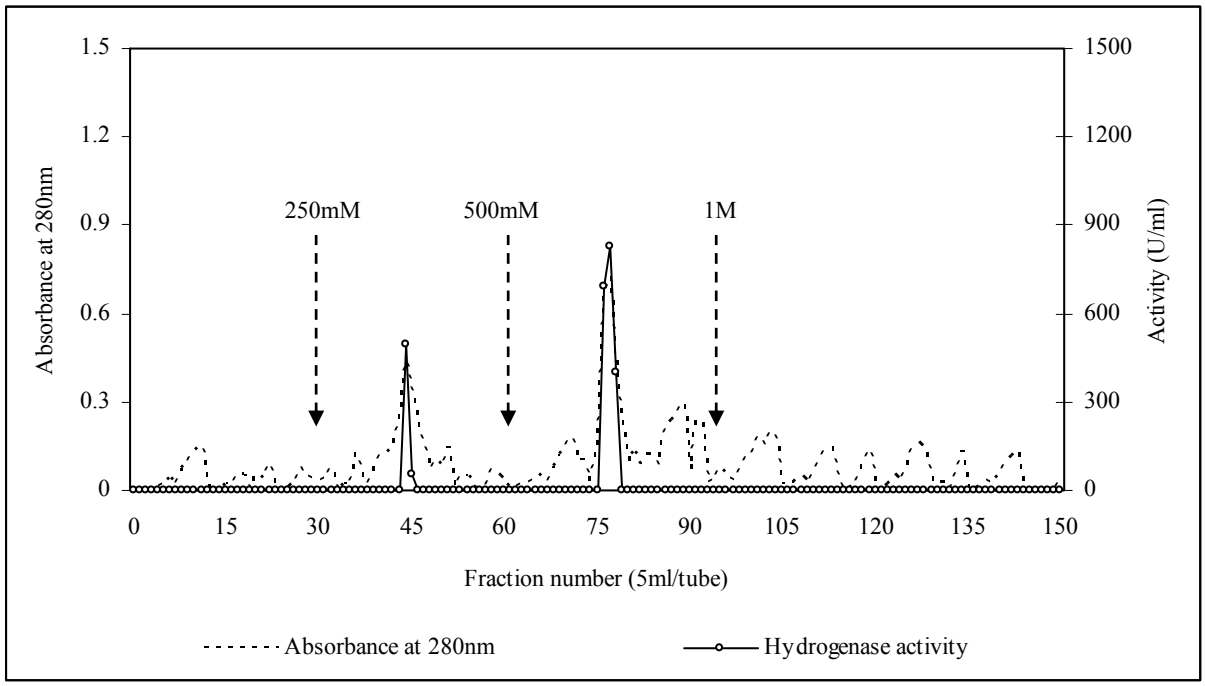

Figure 5.1: DEAE-sephacel ion exchange chromatography of concentrated soluble extract (5ml). Column dimensions = 1.5 cm x 20 cm; flow rate = 1ml/min. Hydrogenase was eluted with a stepwise addition of NaCl (0-1M) in Tris-HCl buffer (50mM, pH 7.6).

Consequently, after the Sephadex G200 gel filtration column the protein concentration dropped from 0.018 to 0.009mg. Meanwhile the specific activity increased from 969 478 to 1 284 000 U/mg, giving an enzyme enrichment of 6.6. A sample of the pooled and concentrated hydrogenase exhibiting fraction from the Sephadex G200 column were then subjected to SDS-PAGE to assess the purity of the enzyme.

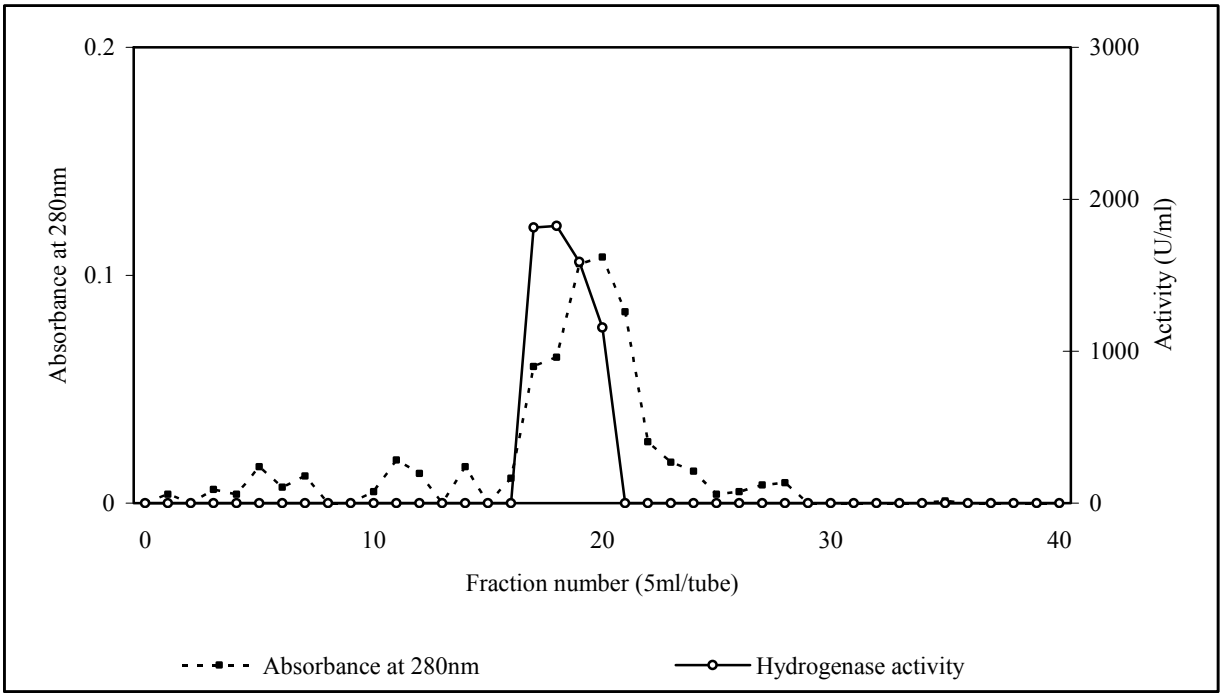

Figure 5.2: Sephadex G200 size exclusion chromatography of pooled and concentrated hydrogenase fractions from DEAE-Sephacel ion exchange column. Column dimensions = 1.5 cm x 25 cm; flow rate = 10ml/hour. Hydrogenase was eluted with 50mM Tris-HCl buffer (pH 7.6).

5.4.1.3 SDS-PAGE

Molecular weight markers, soluble extract concentrate, as well as the pooled and concentrated fraction from the DEAE-Sephacel column were loaded onto a 10% SDS gel (Figure 5.3). Only one band was observed after the ion exchange column chromatography step, and this was attributed to its very low protein concentration. However, this step succeeded in removing most of the contaminants present in the soluble extract. The molecular weight of the most visible protein band thought to be the partially purified hydrogenase was estimated to be about 62kDa. However, due to the low protein concentration attempts to obtain protein bands in the pooled fractions from the Sephadex

G200 gel filtration column stained with both Coomassie brilliant blue R-250 and silver staining proved futile. As a result this procedure remained a partial purification process.

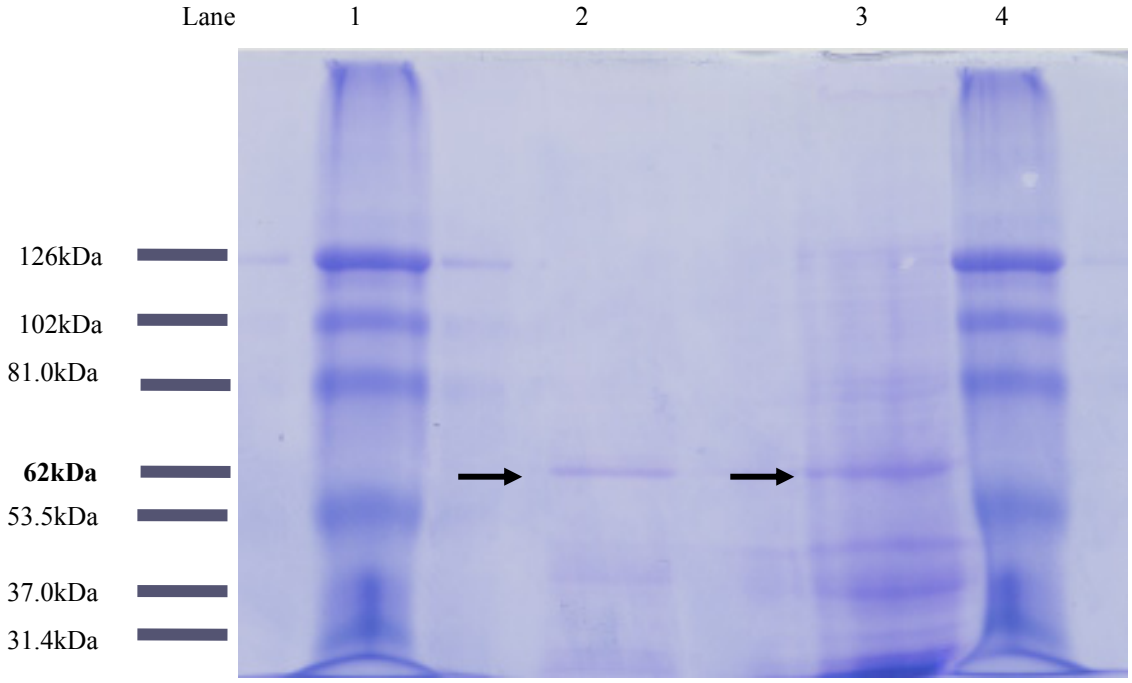

Figure 5.3: A 10% SDS-PAGE analysis of DEAE-sephacel hydrogenase exhibiting fractions. Lane 1 and 4: Molecular weight markers (β-Galactosidase = 126 kDa, Fructose-6-phosphate kinase = 102 kDa, Pyruvate kinase = 81 kDa, Ovalbumin = 53.5 kDa, Lactic dehydrogenase = 37 kDa and Triosephosphate isomerase = 31.4 kDa). Lane 2: Pool of hydrogenase containing fractions from DEAE-sephacel fractions. Lane 3: Soluble extract.

5.4.2 Characterization of hydrogenase

5.4.2.1 pH profile

A long established observation in the study of enzyme kinetics is the way in which catalytic activity is optimized at a certain pH, thus giving rise to a typical bell-shaped curve (Elliot *et al.,* 2002). In this study, the pH profile was determined over a pH range of 4-9, and the hydrogenase was found to be most active at pH 8, which was considered to be 100% (Figure 5.4). This was in accordance with other studies done, where hydrogenase enzymes were found to be active in the pH range 7-8 (Rashamuse, 2003; Bianco *et al.,* 2001). The enzyme displayed a high degree of pH sensitivity, as great activity loss was observed on either side of the pH optimum. This can be explained by the fact that the catalytic activity of enzymes is

dependant on the state of the ionization of the amino acid residues at the active site of the enzyme, such that an enzyme becomes more active at a specific pH (Wilson, 2000).

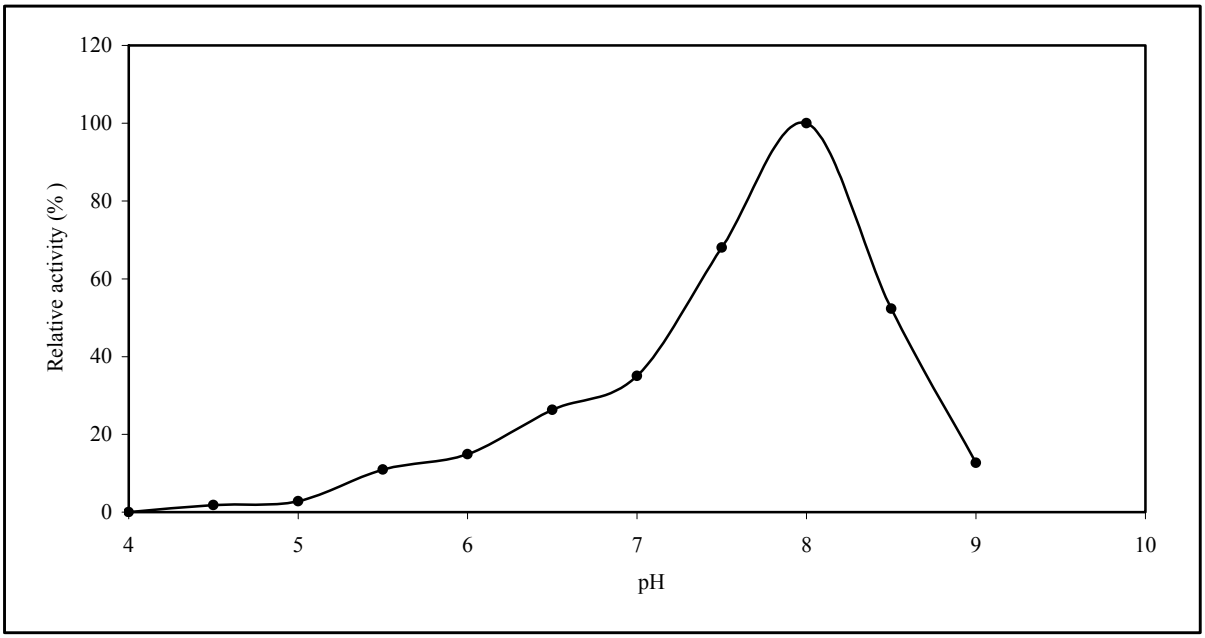

Figure 5.4: Effect of pH on hydrogenase activity (with methyl viologen as electron acceptor). 100% relative activity = 3816.6 μmol/ml/min.

5.4.2.2 Temperature profile

The effect of temperature on the activity of a hydrogenase enzyme was studied over a temperature range 0-70°C. The hydrogenase enzyme was found to have the highest activity at a temperature of 40°C (Figure 5.5), which is within the growth temperature range of mesophilic SRB. The enzyme was found to be completely denatured at temperatures exceeding 55°C, and was gradually inactivated at temperatures below 40°C.

5.4.2.3 Thermal stability

The thermal stability of hydrogenase over time when incubated at both optimum pH (8) and temperature (40°C) was investigated. Minimal activity loss was observed during the first 10 minutes and thereafter, activity steadily decreased with incubation time (Figure 5.6). After 2 hours of incubation, the enzyme had lost all its activity. The lack in thermal stability is a major drawback with most mesophilic enzymes, because apart from pH, activity is influenced by both exposure time and temperature (Roe, 2001).

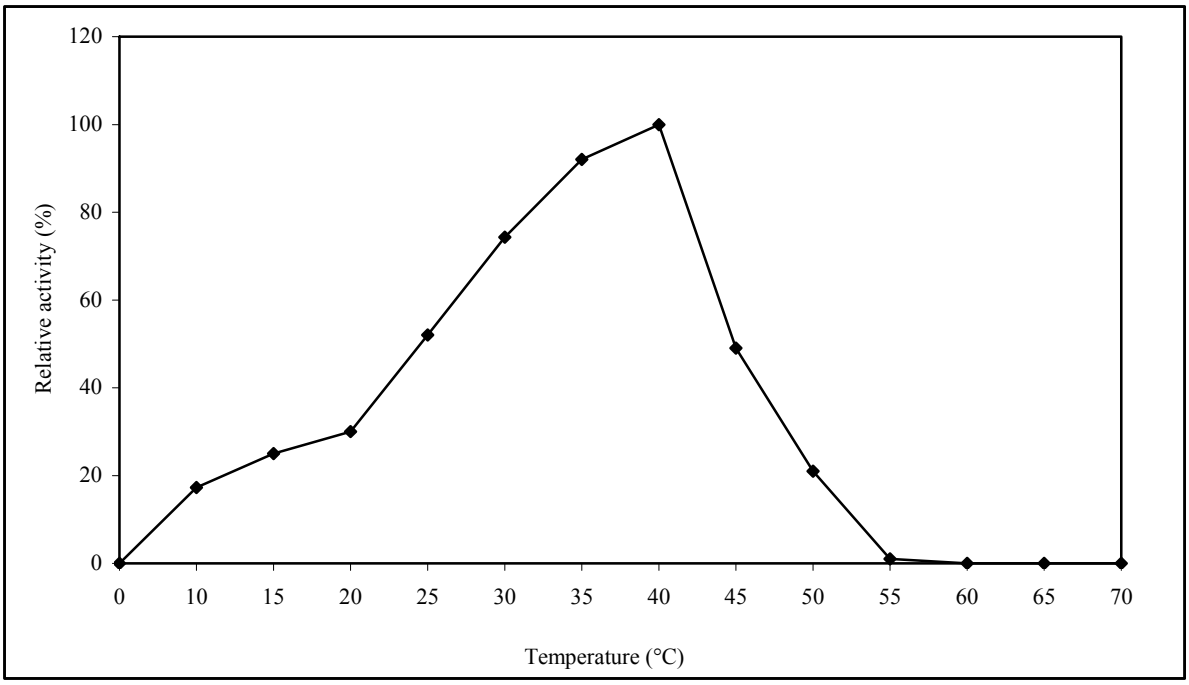

Figure 5.5: Effect of reaction temperature on hydrogenase activity (with methyl viologen as electron acceptor). 100% relative activity = 3816.6 µmol/ml/min.

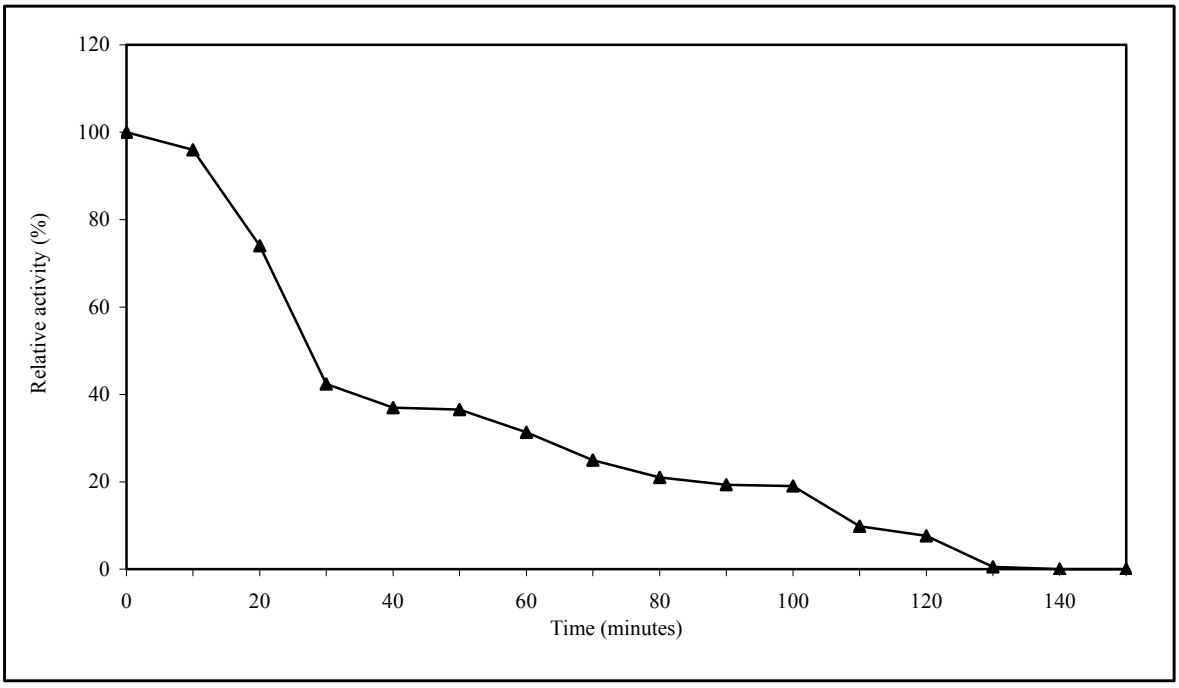

Figure 5.6: Thermal stability profile at 40°C and pH 8.0 of hydrogenase with methyl viologen as electron acceptor. 100% relative activity = 3816.6 µmol/ml/min.

5.4.2.4 Electron acceptor and donor specificity

The specific activity of almost all redox enzymes largely depends on the nature of electron donor and acceptor used (de Lacey *et al.,* 2000). In this study methyl viologen was found to be the most suitable electron acceptor for hydrogenase when hydrogen gas was used as electron donor, and so was considered as 100% (Figure 5.7). Generally, hydrogenases catalyze both hydrogen production and uptake with low-potential electron acceptors and donors (Peck and LeGall, 1994). Consequently, due to its low redox potential (-446 mV) (Table 6), it was not surprising that methyl viologen was found to be the most suitable electron acceptor for the hydrogenase enzyme. Similarly in a study by Rashamuse (2003), methyl viologen was found to be the most suitable electron acceptor for a hydrogenase enzyme. Consequently, the least activity (19.3%) was observed when cytochrome c (from horse heart) was used as an electron acceptor due to its high redox potential. It has been suggested that only low potential multiheme cytochromes (between -195 and -330 mV) are suitable as electron acceptors for hydrogenases (Peck and LeGall, 1994).

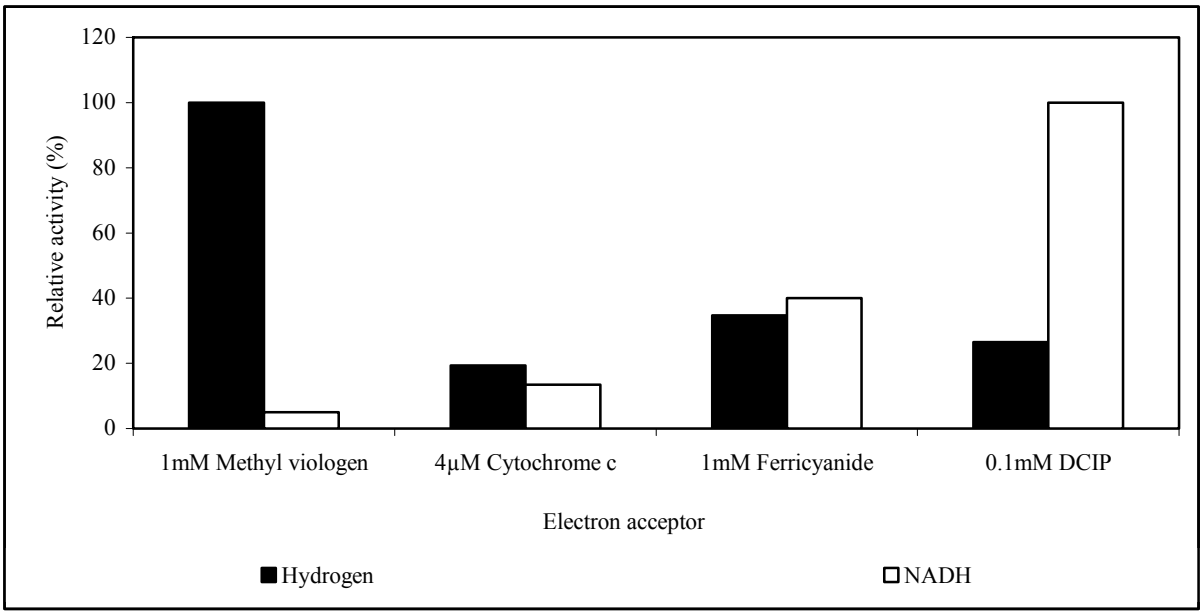

Figure 5.7: Effect of different electron acceptors and donors on the activity of hydrogenase. 100% relative activity = 3816.6 µmol/ml/min, with methyl viologen as electron acceptor under hydrogen, and 100% relative activity = 1606.7 µmol/ml/min when DCIP is the electron acceptor with NADH as electron donor.

Table 6: Characteristics of electron acceptors and donor for hydrogenases.

Electron acceptor/donor	$Em_{7, NHE}$ (mV)	Wavelength (nm)	ε ($mM^{-1}cm^{-1}$)	References
Cytochrome c (from horse heart)	265	550	7.18	This study
Methyl viologen	-446	604	13.9	Peck and LeGall, 1994
DCIP	217	600	20.6	Peck and LeGall, 1994
Potassium ferricyanide	430	420	1.04	Peck and LeGall, 1994
$NADH/NAD^+$	-320	340	6220	Madigan *et al.,* 2003
Hydrogen	**	**	**	

** = unknown

When NADH was used as an electron donor, however, maximum hydrogenase activity (100%) was observed with DCIP as electron acceptor. In this case, maximum hydrogenase activity was not expected (with DCIP as electron acceptor) due to the marked differences between their redox potentials. Consequently, the observed reactions when NADH was used as electron donor were thought to be of a chemical nature as opposed to enzymatic. Several redox enzymes have been found to exhibit interesting current potential dependencies in which an optimum rate occurs at a particular potential, and thereafter the rate decreases even if the thermodynamic driving force is increased. This is because the electrochemical potential reflects the ability of reducing or oxidising equivalents, which are of much relevance in the activity of the enzyme (Elliot *et al.,* 2002). In addition, the observed differences in the activity of the hydrogenase in the presence of different electron acceptors and donors could also be attributed to their extinction coefficients (Table 6).

5.4.2.5 Effect of metal ions

The effect of the presence of different metal ion concentrations on the rate of the biocatalytic hydrogen production by a hydrogenase enzyme was investigated. Metal ions play an integral role in the life processes of microorganisms. Essentially, they function as catalysts for biochemical reactions; they stabilize proteins and bacterial cell walls, and serve in maintaining osmotic balance (Hughes and Poole, 1989). In particular, zinc has been found to stabilize several enzymes and DNA through electrostatic forces (Nies, 1992). In this study, however, only 21% hydrogenase activity was remaining in the presence of 10mM zinc (Figure 5.8). Complete hydrogenase inhibition was observed in the presence of 10mM chromium, whereas in the presence of 10mM silver, only 3.5% relative activity remained.

Contrary to a study by Fitz and Cypionka (1991), complete hydrogenase inhibition by copper was not observed, even with copper concentration up to 10mM. Instead the initial hydrogenase activity was reduced by half (50%) due to the presence of 10mM copper. The reason for these results is not clear, however, it has been suggested that copper plays a vital role during the redox processes (Bruins *et al.,* 2000). The presence of nickel and iron ions, however, resulted in the release of more hydrogenase activity. This was not surprising since hydrogenases contain nickel and iron, and these metals play an indispensable role during redox processes (Peck and LeGall, 1994).

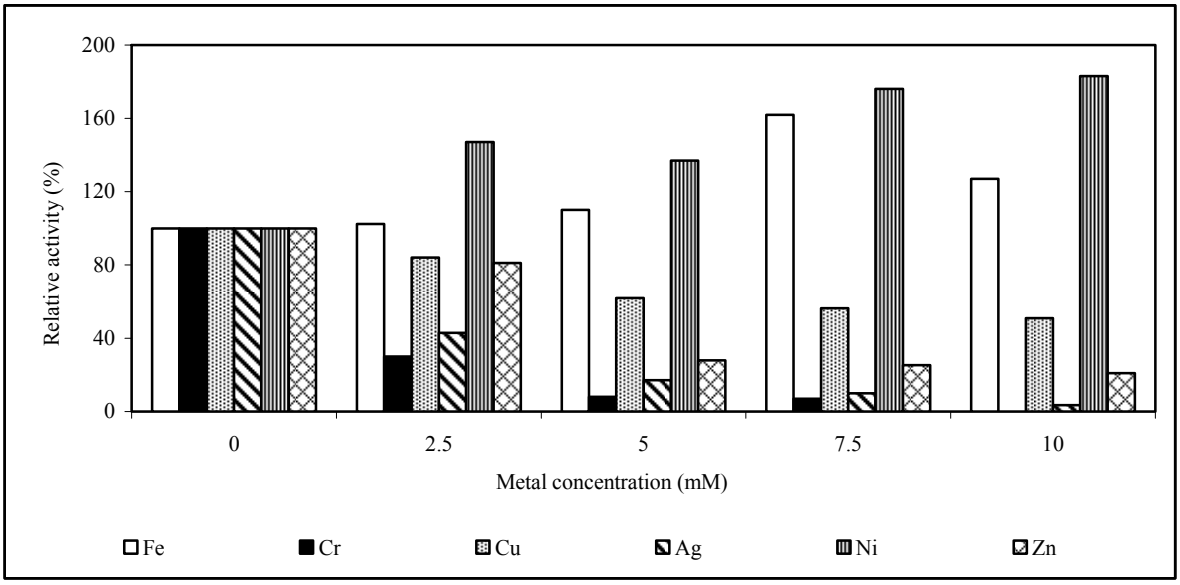

Figure 5.8: Effect of metal ions on hydrogenase activity (with methyl viologen as electron acceptor). 100% relative activity = 3816.6 µmol/ml/min.

5.4.2.6 Effect of EDTA

Hydrogenases are characterized by the presence of metal ions at their catalytic sites, and are referred to as metalloenzymes (Fontecilla-Camps *et al.,* 1997). The dependence of hydrogenase on the presence of metals was investigated by the addition of a chelating agent, EDTA. It was found that increased EDTA concentrations resulted in the liberation of more hydrogenase activity (Figure 5.9). The reason for observed results is not clear. Based on the role of EDTA, it can be suggest that its addition resulted in the chelation of other metal ions present in solution except for those in the redox centres of the hydrogenase enzyme (Peck and LeGall, 1994).

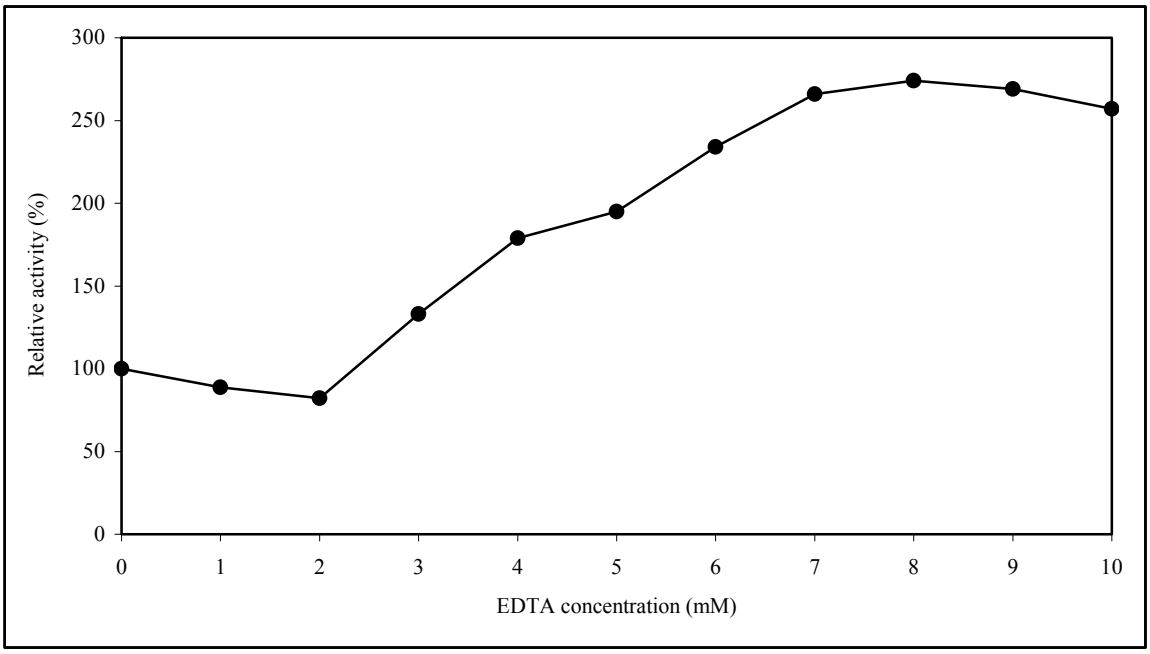

Figure 5.9: Effect of EDTA on hydrogenase activity (with methyl viologen as electron acceptor). 100% relative activity = 3816.6 µmol/ml/min.

5.4.2.7 Kinetic parameters

The dependence of hydrogenase activity on the concentration of methyl viologen as a substrate electron acceptor was investigated. At high methyl viologen concentrations (10mM), maximum hydrogenase activity (V_{max}) of 1111µmol/min/ml was attained (Figure 5.10), while a Michaelis constant (K_M) value of 5.2 mM was recorded. The Michaelis constant (K_M) is a measure of the strength at which a substrate binds to an enzyme, where a large K_M suggests that the enzyme binds substrate weakly. The high V_{max} and low K_M values observed in this study, when compared to a V_{max} and K_M of 45.05 U/ml and 13.19 mM, respectively (Rashamuse, 2003), suggests that the hydrogenase investigated in this study has more affinity for methyl viologen as substrate.

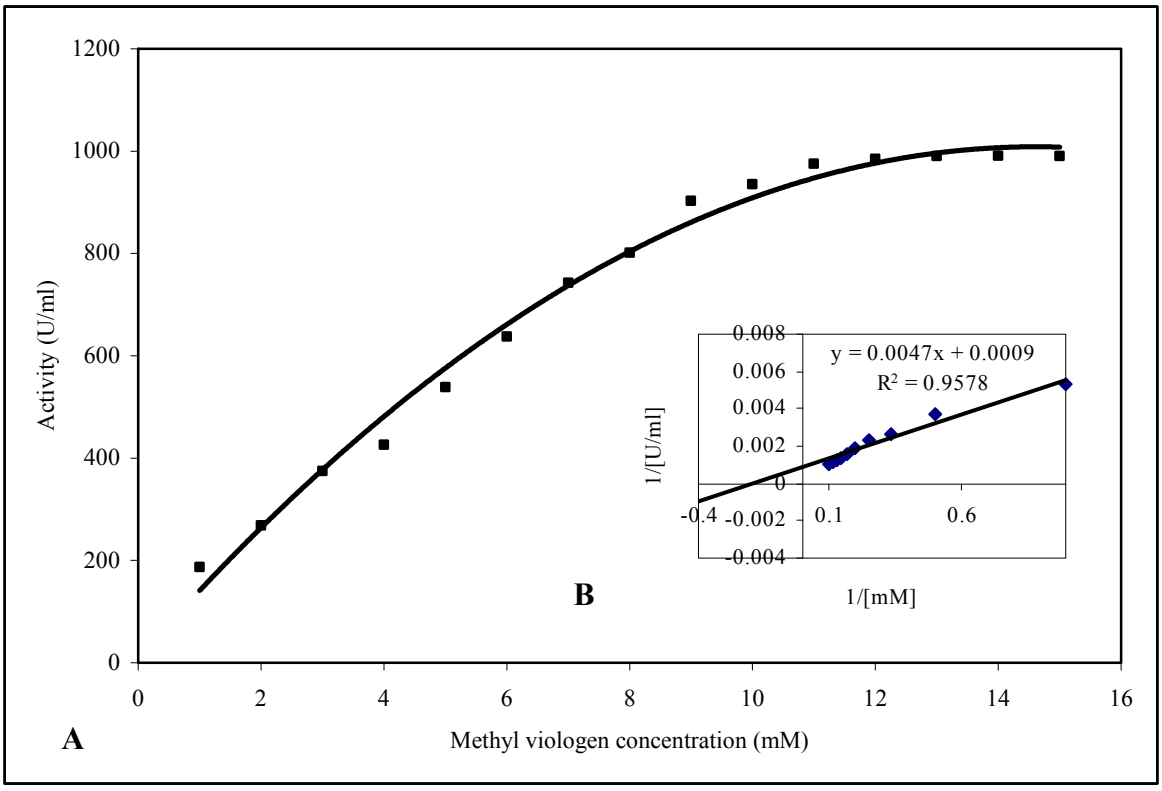

Figure 5.10: Dependence of hydrogenase activity on the concentration of methyl viologen as substrate. (A) Michaelis-Menten plot and (B) Lineweaver-Burk plot (insert).

5.5 SUMMARY

The purified hydrogenase enzyme had an estimated molecular weight of 62kDa. This molecular weight corresponds to that of the larger subunit of most NiFe-hydrogenases of the *Desulfovibrio* genus (Peck and LeGall, 1994). The poor thermal stability of the hydrogenase enzyme presents a major limitation with the use of this enzyme at ambient temperature and longer processing times, and thus it cannot be used for *in situ* bioremediation. However, it can prove useful in shorter industrial process applications aimed at recovering metals from solutions. The hydrogenase enzyme was also found to be sensitive to pH on either side of the optimum (pH 8). This presents a major drawback in the use of this enzyme to recover rhodium from a real industrial effluent, since most industrial effluents from PGM refineries usually have low characteristic pHs (Kolekar and Anuse, 2002). It has also been established that the presence of up 10mM chromium, zinc, silver and to a lesser extent copper concentrations in solution have a detrimental effect on the activity of hydrogenase. Consequently, this makes the enzymatic recovery of rhodium from an industrial effluent containing these metals almost impossible. The addition of increased amounts of EDTA,

however, resulted in increased hydrogenase activity, as metal ions became more available in solution, and this information might be useful in the pre-treatment of biomass for enzyme release. When compared to other documented kinetic parameters of hydrogenases, values obtained in this study (V_{max} = 1111U/ml and K_M = 5.2 mM) showed that the purified hydrogenase had a higher maximum velocity and was more active at low substrate concentrations. The next chapter investigates the possible application of this enzyme in the recovery of rhodium from an industrial effluent.

CHAPTER 6

ENZYMATIC REMOVAL OF RHODIUM(III) FROM SOLUTION AND INDUSTRIAL EFFLUENT USING SULPHATE REDUCING BACTERIA

6.1 INTRODUCTION

The presence of rhodium in wastewaters is mainly a result of man-made activities, such as mining and metallurgical processes, or through the manufacture of electronic components, by-products of electroplating or pigments and paints, or as spent catalysts (Ravindra *et al.,* 2004). Recently, the introduction of strict environmental policies has compelled industries to shift to cleaner production methods, demanding the development of environmentally friendly, low cost and efficient treatment techniques for metal rich effluents (Malik, 2004). Moreover, because rhodiums is a valuable resource for different industrial applications, its recovery and recycling from mine wastewaters assumes even greater significance.

Since its discovery, the microbial reduction of metals has been of much interest since microorganisms can play a crucial role in the recycling of both inorganic and organic species in a range of environmental backgrounds, and if used it may offer the basis for a wide range of innovative biotechnological processes (Lloyd, 2003). Among bacteria, SRB exhibit a number of enzymatic activities that transform metal species through oxidation, reduction, methylation and/or alkylation. These transformations often lead to metal precipitation and immobilization. Precipitation, biosorption and particulate entrapment are some of the best methods that have been fully established so far. However, with time, current research interests have been shifted away from these methods because of the limitations associated with such techniques for metal reclamation. For example, biosorption studies have dominated the literature and subsequently extensive reviews focusing on equilibrium and kinetics of metal biosorption have been done (Volesky, 1990). However, the low binding capacity of biomass in the presence of certain recalcitrant metals in industrial effluents and failure to effectively remove metals from real industrial effluents due to the presence of organic and inorganic ligands has limited this approach. Currently, a novel approach of using enzymes isolated from microorganisms is being explored (Valls and de Lorenzo, 2002).

The enzymatic reduction of metals, particularly by SRB, has been investigated for the reclamation of a range of heavy metals, for example Pd(II) to Pd(0) (Lloyd, 2003), Pt(IV) to Pt(0) or Pt(II) (Rashamuse, 2003), U(VI) to U(IV) and Cr(VI) to Cr(III) (Lloyd *et al.,* 1998; Lovley and Phillips, 1992; Lovley and Phillips, 1994). Although the enzymatic reclamation of metals appears to be more superior over other biological process, it has limitations as well. The fact that enzymes work best at specific optimum conditions is one major problem associated with the use of this technique. This is true especially when metals are to be recovered from wastewaters, which might be of a different pH, temperature and other physico-chemical compositions that tend to inhibit or retard the activity of the redox enzyme. One other controlling factor for redox enzymes (including hydrogenases) is the electrochemical potential, which reflects the availability of reducing or oxidising equivalents. In this case, the presence of other ligands in industrial effluents tends to affect the activity of the enzyme in as far as they tend to affect the electrochemical potential (Elliot *et al.,* 2002). However, with all these disadvantages in mind, the enzymatic reduction of metals is still a technique that is worth exploring. This chapter is aimed at evaluating the potential of this technique in the recovery of rhodium from an industrial effluent.

6.2 MATERIALS

Rhodium sidestream effluent and $RhCl_3$ was supplied by Angloplatinum (Pty.) Ltd, Rustenburg (South Africa). Chlorides test kit, COD, sulphate and sulphide test kits were purchased from Merck (Darmstadt, Germany). All other reagents, which were of analytical grade, were obtained from either Merck (Darmstadt, Germany) or Sigma-Aldrich (Steinhem, Germany). Hydrogen gas was obtained from Afrox (South Africa).

6.3 METHODS

6.3.1 Analytical procedures

Sulphate and COD concentrations were measured using the Thermospectronic Aquamate (England) and the Spectroquant® Nova 60 (Merck, Darmstadt, Germany) was used for both chloride and sulphide concentrations. The Level 1 Inolab pH meter (WTW Ltd, Weilheim, Germany) was used for pH measurement. The concentration of rhodium was determined using a GBC 909 flame atomic absorption spectrophotometer (GBC Scientific equipment Pty Ltd, Dandenong, Australia) using a rhodium 6mA hollow cathode lamp at 343.5nm.

6.3.2 Enzymatic recovery of rhodium from solution

6.3.2.1 Enzymatic recovery of rhodium from solution by SRB soluble extract

Sulphate reducing bacteria cell soluble extract was prepared as before (Section 5.3.2.1), and a 5ml sample was added into a flask containing a rhodium solution (50 mg/l) prepared from a rhodium chloride salt. Hydrogen gas was supplied as the sole electron donor throughout the incubation period. Duplicate samples were removed at timed intervals to monitor the residual rhodium concentration using an atomic absorption spectrophotometer.

6.3.2.2 Enzymatic recovery of rhodium from solution by purified hydrogenase

A 5ml sample of the purified hydrogenase was added into a sealed flask containing a rhodium solution (50 mg/l) prepared from a rhodium chloride salt. Hydrogen gas was supplied as the sole electron donor throughout the incubation period. Duplicate samples were removed at timed intervals to monitor the residual rhodium concentration using an atomic absorption spectrophotometer.

6.3.3 Enzymatic recovery of rhodium from industrial effluent

6.3.3.1 Characterization of industrial effluent

A rhodium containing effluent was obtained from Angloplatinum (Rustenburg, South Africa). The effluent was sampled from the rhodium side-stream at the Precious Metal Refinery (PMR) plant. The pH, rhodium, chloride, sulphate and sulphide concentrations of the effluent were determined.

6.3.3.2 Recovery of rhodium from industrial effluent by whole SRB cells

SRB cells (0.5g/l) in Tris-HCl buffer (20mM, pH 7.6, 1ml) were added into a sealed flask containing rhodium effluent. Hydrogen gas was supplied as electron donor throughout the incubation period, and duplicate samples were removed at timed intervals to monitor the residual rhodium concentration using an atomic absorption spectrophotometer.

6.3.3.3 Recovery of rhodium from industrial effluent by heat-killed SRB cells

SRB cells (0.5g/l) killed by autoclaving (121°C, 30 minutes), were added into a sealed flask containing effluent. Hydrogen gas was supplied as electron donor throughout the incubation period, and duplicate samples were removed at timed intervals to monitor the residual rhodium concentration using an atomic absorption spectrophotometer.

6.3.3.4 Recovery of rhodium from industrial effluent in the absence of SRB cells

Autoclaved (121°C, 30 minutes) rhodium effluent in a sealed flask was sparged with hydrogen gas (as the electron donor) throughout the incubation period. Duplicate samples were removed from each flask at timed intervals and monitored for residual rhodium concentration using an atomic absorption spectrophotometer.

6.3.3.5 Enzymatic recovery of rhodium from industrial effluent by SRB soluble extract

Sulphate reducing bacteria soluble extract was prepared as before (Section 5.3.2.1), and a 5ml sample was added into a flask containing effluent. Hydrogen gas was supplied as the sole electron donor throughout the incubation period. Duplicate samples were removed at timed intervals to monitor the residual rhodium concentration using an atomic absorption spectrophotometer.

6.3.3.6 Enzymatic recovery of rhodium from industrial effluent by purified hydrogenase

A 5ml sample of the purified hydrogenase was added into a sealed flask containing effluent. Hydrogen gas was supplied as the sole electron donor throughout the incubation period. Duplicate samples were removed at timed intervals to monitor the residual rhodium concentration using an atomic absorption spectrophotometer.

6.4 RESULTS AND DISCUSSION

6.4.1 Enzymatic recovery of rhodium from solution

The removal of rhodium from solution by a soluble extract and by a partially purified hydrogenase enzyme was investigated (Figure 6.1). Results obtained showed there was no significant difference in the amount of rhodium removed from solution after 40 minutes of incubation. The soluble extract resulted in a removal of 72%, whereas the partially purified hydrogenase removed 68% rhodium from solution. In a study by Rashamuse (2003), higher platinum removal (46.15%) was observed when purified hydrogenase was used; as compared to 30.8% removed in the presence of a soluble extract. Results obtained in this study, however, were contrary to previous studies. The low rhodium removal efficiency observed with the purified hydrogenase was attributed to the low protein content. In addition, the observed differences in the removal of rhodium can be attributed to the sensitivity of the purified enzyme towards pH away from the optimum (pH 8), since the pH of the prepared rhodium solution was 4.

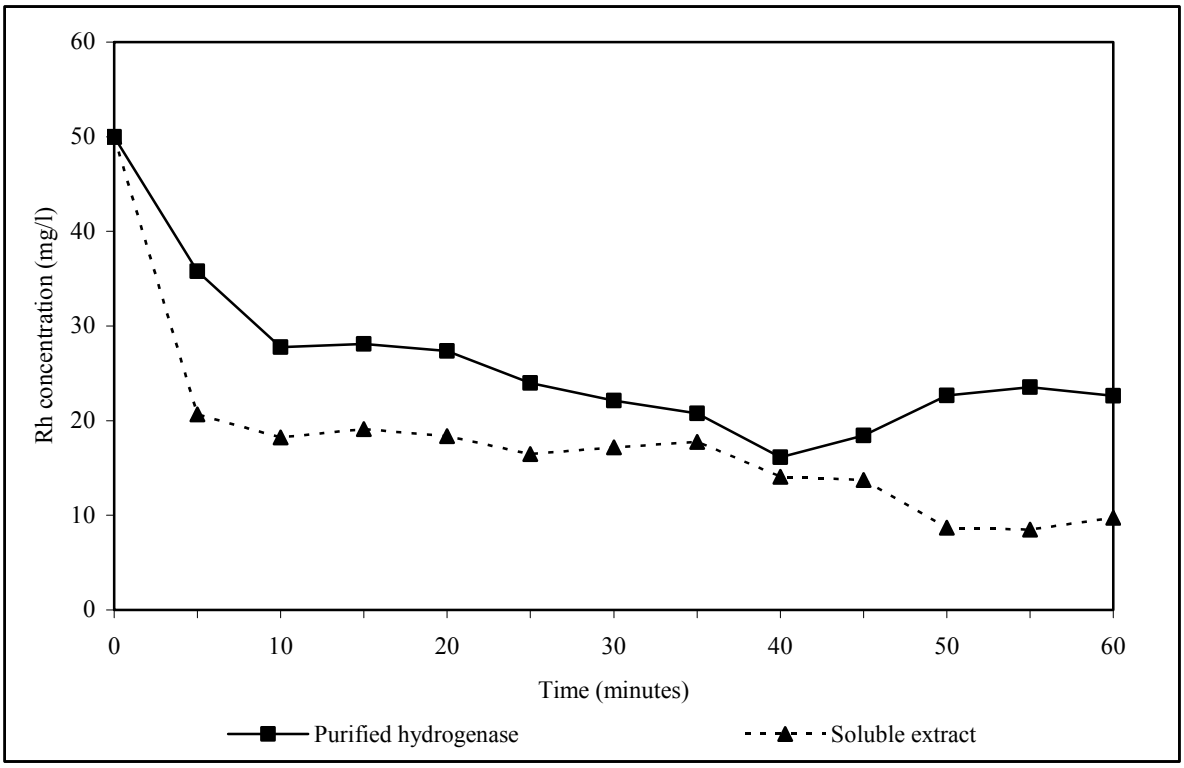

Figure 6.1: Removal of rhodium from solution (pH 4) by sulphate reducing bacteria soluble extract and purified hydrogenase with hydrogen as electron donor.

6.4.2 Enzymatic recovery of rhodium from industrial effluent

6.4.2.1 Characterization of industrial effluent

The effluent was characterized as very low in pH and sulphide concentration, whereas the concentrations of rhodium, chlorides, COD and sulphate were high (Table 7). In addition to these determined parameters, precious metal refinery (PMR) effluents have been found to contain considerable concentrations of other metals, such as Ag, Au, Cu, Ni, Os, Pb and Zn (Rashamuse, 2003). Generally, the presence of these metals in the effluent often poses serious detrimental effects on the use of enzymes for metal removal, mainly due to their role in enzyme inhibition.

Table 7: Characteristics of rhodium side-stream effluent

Rhodium (mg/l)	Sulphate (mg/l)	Sulphide (mg/l)	pH	COD (mg/l)	Chloride (mg/l)
216.2	2615.5	<1	1.31	8800.8	38 220.9

6.4.2.2 Recovery of rhodium from industrial effluent

As noted before, pH is one of the most important factors controlling the activity of the hydrogenase enzyme, as the optimum pH was found to be 8 (Figure 5.4, page 79). Consequently, the low effluent pH (1.31) resulted in the inactivation of the enzyme (both soluble extract and purified hydrogenase) (Figure 6.2). There was no significant difference in the overall amount of rhodium reduced when either the soluble extract or purified hydrogenase was used. Both extracts resulted in a maximum rhodium reduction of 21%, which suggests that enzyme purification in this case is not a necessity. Attempts to increase the effluent pH [with the addition of 1M NaOH (results not shown)], proved futile as complete rhodium precipitation was observed at pH 3.

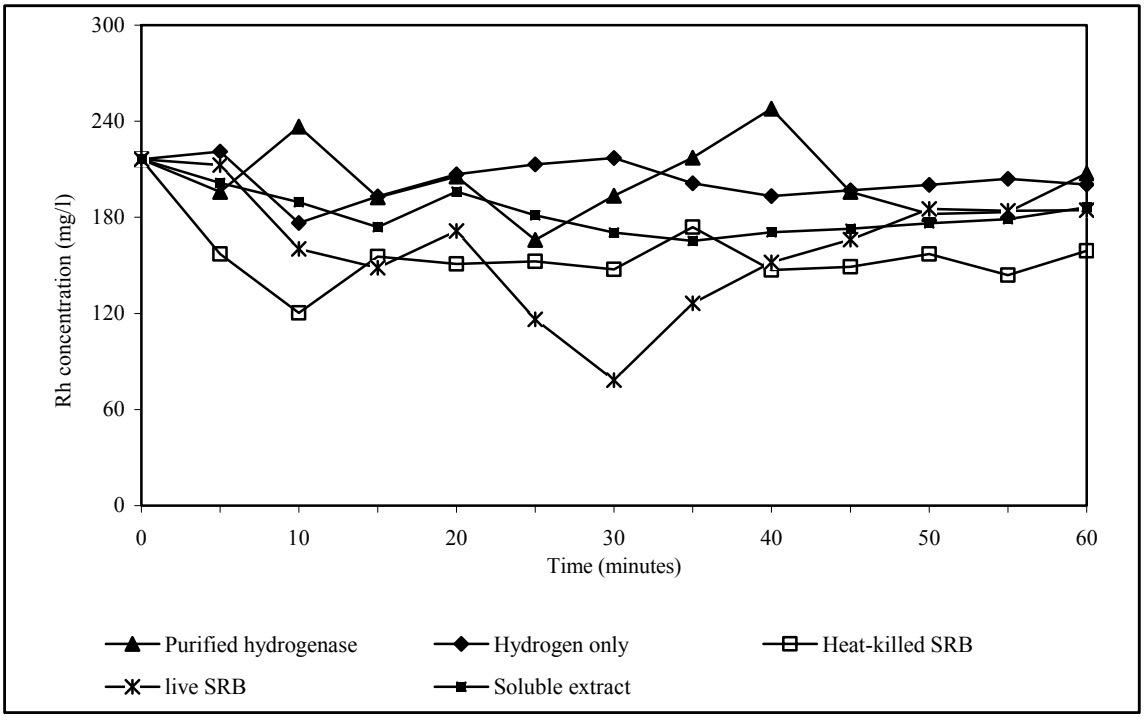

Figure 6.2: Removal of rhodium from industrial effluent (pH 1.31) by sulphate reducing bacteria cells, soluble extract and purified hydrogenase with hydrogen as electron donor.

When whole SRB (live) cells were used, 64% rhodium removal was observed during the first 30 minutes, and thereafter the process was reversed due to a response mechanism within the cells. On the other hand, when heat-killed SRB cells were used, only 44% of the initial rhodium concentration was removed during the first 10 minutes, and after that, no more rhodium was removed. Hydrogen gas alone did not result in major reduction of rhodium, as

about 14% was reduced during the first 10 minutes. The observed pattern in the reduction/removal of rhodium from effluent could be attributed to a number or combination of factors.

6.5 SUMMARY

In this study it was found that the main controlling factor underlying the use of a hydrogenase or soluble extract for the biorecovery of rhodium from an effluent is pH. This was apparent when a hydrogenase and soluble extract were used to recover rhodium from both a rhodium solution and from an industrial effluent. A maximum rhodium recovery of 80% was observed from the pure rhodium solution (pH 4), whereas only 21% was observed from the effluent (pH 1.31) when an SRB cell soluble extract was used. On the other hand, when a partially purified hydrogenase was used, it resulted in a maximum rhodium recovery of 68% in the solution (pH 4) whereas a recovery of 21% was observed in the effluent (pH 1.31). Essentially, the observed differences can be attributed to the different pHs of the two rhodium sources, and in this case the enzymatic reduction process was more applicable with the rhodium solution as compared to the effluent. Apart from the differences in pH, industrial effluents may contain some other organic and inorganic substances that might hinder the activity of hydrogenases. These include metals such as chromium, copper, zinc and silver, which in the present study have been found to inhibit hydrogenase activity. Consequently, the application of enzymes in the bioremediation of real industrial effluents is limited by the fact that enzymes work best within a set of specified optimum conditions. As such, effluent pre-treatment becomes necessary, because the stability of enzymes is of great importance. However, the use of whole SRB cells for the recovery of rhodium from both solution and effluent has potential, as metal recovery ranges from 64-100%. The only major setback with such a technique is exposure time, because if cells are exposed for longer periods (more than 30 minutes), the process might be reversed. All these uptake/reduction process occur best in the presence of hydrogen gas as an electron donor.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

7.1 INTRODUCTION

Biotechnological approaches, particularly methods utilizing the highly metal sorbing SRB have attracted much research lately, because they have been found to succeed in the bioremediation of metal-laden wastewaters. SRB have evolved various measures to respond to heavy-metal stress via a wide range of interactions. Some of these SRB-metal interactions have already been commercially exploited, for example, the THIOPAQ system, which is based on the precipitation of metals as metal sulphides. Other interactions such as, the enzymatic reduction of metals by SRB have been found to have a great potential for metal recovery, however, the mechanism of metal reduction is poorly understood (Lloyd *et al.,* 1998). Based on this, this study was aimed at exploring both the mechanism and potential of hydrogenases from SRB as a cost effective method for the recovery of rhodium from both solution and industrial effluent.

Results from the effect of initial rhodium concentrations on the uptake of rhodium by a resting SRB biomass did not derive any significant information. Therefore, it follows that in future studies careful optimization of the rate of rhodium removal is necessary. To determine the effect of biomass, either a cell count or viability studies should be carried out. However, in this project it was evident that regardless of a change in any of the above mentioned factors, a biphasic (a rapid process, followed by slower energy-dependant process) uptake of rhodium from solution by resting SRB cells was observed. This was in accordance with previous observations in literature (Hughes and Poole, 1989; Volesky, 1990; Lloyd *et al.,* 1998; Macaskie *et al.,* 2000). Apart from the observed mechanism of rhodium ions uptake by SRB biomass, the ability of rhodium to form different species at different pH, temperature, chloride concentration and over time was found to be the main factor controlling the removal of rhodium from solution by SRB cells (Sanchez *et al.,* 2002).

This implies that the amount of rhodium that can be removed by the SRB biomass is dependent on the availability of adsorbable rhodium species, and SRB cells were found to have a higher affinity for anionic rhodium species as compared to the inert cationic and neutral rhodium species. It follows then that in future studies, thorough preliminary studies on rhodium(III) speciation might prove to be useful, since it appears that the types of rhodium species present within a specified experimental condition determine the rate of rhodium removal from solution.

The presence of rhodium on SRB cells previously exposed to a rhodium solution was confirmed by X-ray fluorescence (XRF) analysis. Metallic rhodium (Rh^0) was detected, as one of the major reduced forms of rhodium within the SRB cells. TEM analysis revealed a time-dependant distribution and location of rhodium precipitates on SRB cells. When cells were exposed for 90 minutes, rhodium rich precipitates were observed inside the SRB cells. When cells were exposed for periods up to 9 hours, however, high density rhodium areas were observed both outside SRB cells and on cell peripheries. The observed efflux system of rhodium provided sufficient evidence that an energy-dependant process was involved. Many authors have hypothesized that this energy-dependant transport of metals outside the cell often results in the reduction of the metal (Jeong *et al.,* 1997; Bruins *et al.,* 2000; Macaskie *et al.,* 2000). On the other hand, SEM analysis showed that with longer exposure periods, SRB cell surface darkening and clogging also provided further evidence of the extracellular rhodium accumulation. Results obtained from this section revealed that rhodium(III) was indeed taken up by SRB cells, and eventually reduced to rhodium(0).

To ascertain the involvement an enzymatic reduction process the ability of different hydrogenase-containing SRB cell soluble fractions to recover rhodium from solution was investigated. High rhodium removal efficiency was observed with hydrogenases located in both the cytoplasmic and membrane fractions. For practical reasons, only hydrogenases obtained by sonication were considered for further purification and characterization. The procedure adopted for purification of the cytoplasmic-hydrogenases was not a successful as hoped. Major protein loss was observed at the end of the

purification procedure. Consequently, no protein bands were obtained when pooled fractions from Sephadex G200 size exclusion column were subjected to SDS-PAGE. However, when pooled and concentrated fractions from the DEAE-Sephacel ion exchange column were subjected to a 10% SDS-PAGE analysis, only one band was visible. Characterization studies revealed that the hydrogenase enzyme had an optimum pH and temperature of 8 and 40°C, respectively. However, the enzyme showed poor thermal stability, as all activity was lost after 2 hours of incubation at optimum pH (8) and temperature (40°C). Therefore, much work needs to be done to investigate ways of improving the thermal stability of hydrogenases. However, considering the fact that most metal removal occurs in the first 5-30 minutes, the hydrogenases possess a great potential in the recovery of metals from metal-contaminated solutions. Further studies showed that methyl viologen as an electron acceptor, had a sufficiently low redox potential to mediate the optimal catalysis of hydrogen oxidation by a hydrogenase. A high degree of hydrogenase enzyme inhibition was observed in the presence of increasing concentrations (up to 10mM) of chromium, silver and zinc, and to a lesser extent by copper, which was, perhaps, indirect contradiction to other studies (Fitz and Cypionka, 1991). EDTA concentration up to 7mM resulted in the liberation of more hydrogenase activity. This was attributed to the fact that the addition of EDTA resulted in the chelation of other metals which might hinder hydrogenase activity. This information might serve useful in the pre-treatment of biomass for maximum enzyme release. The K_M value of the purified hydrogenase with methyl viologen and hydrogen as electron acceptor and donor, respectively, was found to be 5.2mM, and had a V_{max} of 1111 μmol/min/ml. It was concluded that the purified hydrogenase was more active at low substrate concentrations.

When known information was used to investigate the potential of SRB for the enzymatic removal of rhodium from a real industrial effluent, unsatisfactory results were obtained. The low rhodium removal efficiency by hydrogenase was attributed to the low pH (1.31) of the industrial effluent. In addition, not all of the rhodium could be removed from solution due to the relatively high initial rhodium concentration (216.2mg/l). Since rhodium speciation does not occur at pH <3, the observed trend in rhodium removal from industrial effluent was mainly due to th efact that the hydrogenase was sensitive to pH. In

this study, the hydrogenase enzyme was found to work best at a pH 8. In future studies, an investigation of effluent pre-treatment methods, and the ability of SRB to recover rhodium from the pre-treated effluent is necessary.

However, regardless of the physico-chemical characteristics of the metal source, the use of viable SRB cells for the recovery of rhodium from both solution and effluent, has a potential as metal recovery ranges from 64-100%. The only major setback with such technique is exposure time, where if cells are exposed for longer periods (more than 30 minutes), the process might be reversed. All these uptake/reduction process occur best in the presence of hydrogen gas as an electron donor. This study serves as an information source on the regimes of metal uptake from solution, as a function of metal speciation. In addition, this study has provided an insight of the role played by hydrogenases in the removal and subsequent enzymatic reduction of metals from both an aqueous solution and a real industrial effluent. However, as more information on this subject of enzymatic reduction of metals by SRB, is revealed, more questions arise. Thus, there is a need for a follow up on this subject to address some of the loopholes created during the course of this study.

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APPENDICES

APPENDIX A: METHOD OF SRB MEDIUM (modified Postgate C) PREPARATION

Solution A: Add 6 ml Sodium lactate (60% solution w/w), 4.5g Na_2SO_4, 1.0g NH_4Cl, 1.0g Yeast extract, 0.5g KH_2PO_4, 0.1g Sodium citrate.$2H_2O$, 0.06g $CaCl_2.6H_2O$ and 0.06g $MgSO_4.7H_2O$ and dissolve all components in 990 ml of distilled/deionized water.

Solution B: Dissolve 1.0g ascorbic acid and 1.0g sodium thioglycholate in 100 ml of distilled/deionized water.

Final preparation of media: The pH of solution A was adjusted to pH 7.5 with 5M NaOH solution. The solutions (A and B) were then autoclaved separately for 15 minutes at 15 psi pressure-121°C. To make 1L of medium, 10 ml solution B was then added to 990ml solution A under a UV hood.

APPENDIX B: Method OF PROTEIN DETERMINATION - BRADFORD ASSAY (Bradford, 1976)

B1: Protein stock solution (2mg/ml)

Dissolve 0.02g of Bovine serum albumin (BSA) in 10 ml of ddH_2O.

B2: Protein standard curve

Triplicate protein concentrations were prepared from the BSA stock solution (2mg/ml) as shown in Table A1. A protein standard curve was generated by reading the absorbance of these different concentrations at 595nm (Figure I). For the test sample, 250µl Bradford reagent was added to 5 µl diluted sample and the absorbance read at 595nm.

Table A1: Preparation of protein standard curve. Data shown are the means of triplicate values measured.

BSA Stock solution (2mg/ml) (µl)	ddH_2O (µl)	Bradford reagent (µl)	Protein concentration (mg/ml)	Absorbance at 595nm
0	5	250	0	0
1	4	250	0.008	0.158
2	3	250	0.016	0.367
3	2	250	0.024	0.523
4	1	250	0.031	0.616
5	0	250	0.039	0.824

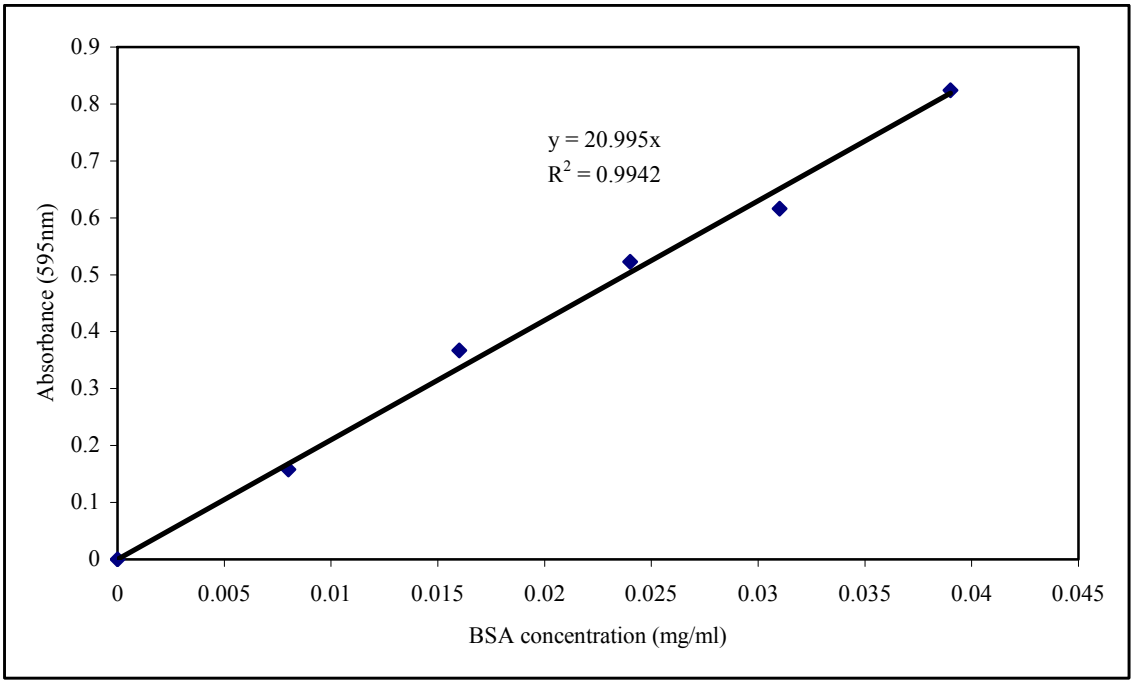

Figure I: Protein standard curve. Points are the means of triplicate values measured.

APPENDIX C: METHODS OF RESIN GENERATION

C1: Preparation of DEAE-sephacel (pre-swollen)

The appropriate volume of resin was poured into a beaker. The ethanol in the resin was removed by washing the resin five times with large volumes of ice-cold deionized water. Lastly, the resin was washed with the starting buffer (50mM Tris-HCl, pH 7.6) two times

and at each time the pH was checked until it was the same as that of the starting buffer (50mM Tris-HCl, pH 7.6). DEAE-sephacel was then packed into the column, and equilibrated with the starting buffer (50mM Tris-HCl, pH 7.6).

C2: Preparation of Sephadex G200

The appropriate amount of dry Sephadex for the required bed volume (20-25 ml swelled per g dry resin) of the column was weighed into a beaker. The resin was then suspended in a large volume of the starting buffer (50mM Tris-HCl, pH 7.6) to equal the total volume of the column and an extra 30% to allow resin to swell. The resin was then incubated at 20ºC for 72 hours to allow swelling. After swelling, fines were removed by gently swirling the swollen resin and then decanting the cloudy supernatant. This process was repeated 10 times. The equilibrating buffer (50mM Tris-HCl, pH 7.6) was added again, this time to make a 75% suspension, and the resin was then packed into the column.

APPENDIX D: SDS-PAGE METHODS (Laemmli, 1970)

D1: Stock solutions

Solution A: Acrylamide stock solution (30% acrylamide, 0.8% bis-acrylamide)

Weigh 29.2g acrylamide and 0.8g bis-acrylamide, and then dissolve in 100ml deionized water and stir until acrylamide powder is completely dissolved.

Solution B: 4x Separating Gel buffer

Add 75ml of 2M Tris-HCl buffer (pH 8.8) and 4ml of 10%SDS and then make up the volume to 100ml with deionized water.

Solution C: 4X Stacking Gel buffer

Add 50ml of 1M Tris-HCl buffer (pH 6.8) and 4ml 10% SDS, and make up the volume to 100ml with deionzed water.

Solution D: 10% Ammonium persulfate solution (APS)

Dissolve 0.5g ammonium persulfate in 5ml deionized water.

Solution E: Electrophoresis buffer

Add together 3g Tris base, 14.4g glycine and 1g SDS. Dissolve all three in 1 litre of deionized water.

Solution F: 5× Sample buffer

Add together 0.6ml 1M Tris-HCl buffer (pH 6.8), 5ml 50% glycerol, 2ml 10% SDS, 0.5ml 2-mercaptoethanol, 1ml 1% bromophenol blue, and make up the volume to 10ml with deionized water.

Solution G: Coomassie blue staining solution

Dissolve 1.0g Coomassie Blue R250 in a mixture of 450ml methanol, 450ml deionized water and 100ml glacial acetic acid.

Solution H: Coomassie Gel Destain solution

Add together 100ml methanol, 100ml glacial acetic acid and 800ml deionized water.

D2: SDS-PAGE procedure

1. Gel preparation

Table A2: Preparation of a 10% SDS-PAGE gel

	10% Separating gel	4% Stacking gel
Solution A	3.3ml	1.3ml
Solution B	2.5ml	2.5ml
Deionized water	4.2ml	6.2ml
10% APS*	50µL	50µL
TEMED	5µL	5µL

* - solution should be prepared fresh daily

2. Sample preparation

Combine 40µL protein sample and 10µL of 5× Sample buffer (Solution F) in an Eppendorf tube. Heat the mixture at 95°C for 2 minutes.

3. Running the gel

The gel was run at a constant voltage of 120V for 60 minutes.

4. Staining procedure

The gels were stained in Coomassie Gel Stain for 5 hours with gentle agitation.

5. Destaining procedure

Gels were destained overnight in Coomassie Gel Destain solution.

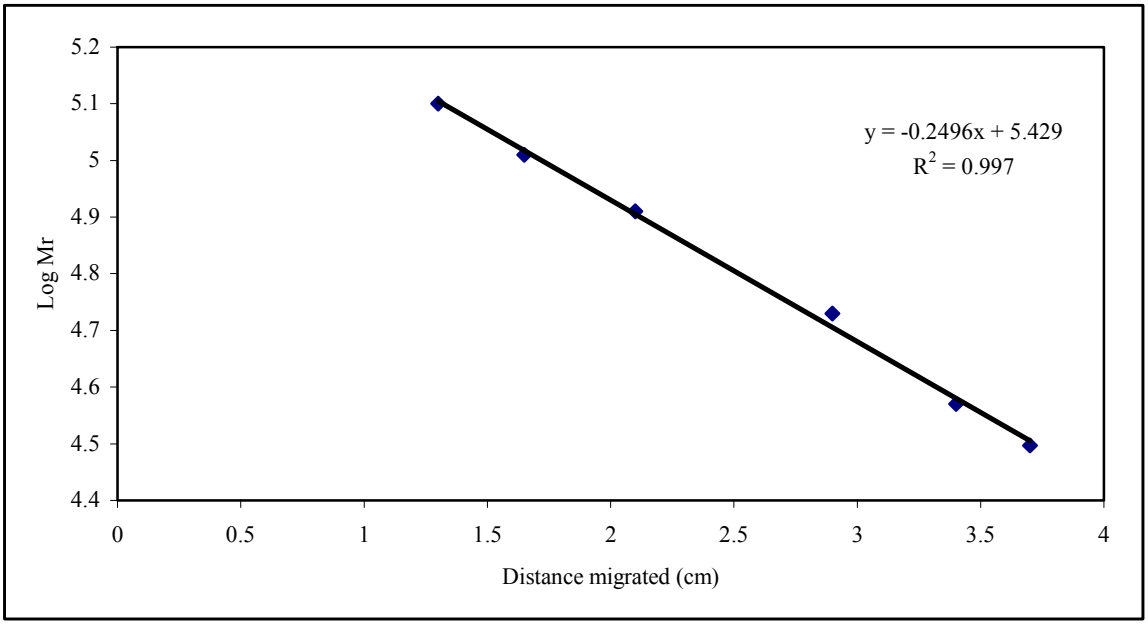

Figure II: Calibration curve of molecular weight (Mr) markers (β-Galactosidase = 126kDa, Fructose-6-phosphate kinase = 102kDa, Pyruvate kinase = 81kDa, Ovalbumin = 53.5kDa, Lactic dehydrogenase = 37kDa and Triosephosphate isomerase = 31.4kDa) versus distance migrated.