THE BIOACCUMULATION OF PLATINUM (IV) FROM AQUEOUS SOLUTION USING SULPHATE REDUCING BACTERIA - ROLE OF A HYDROGENASE ENZYME

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ABSTRACT

The enzymatic reduction of a high-valence form of metals to a low-valence reduced form has been proposed as a strategy to treat water contaminated with a range of metals and radionuclides. Metal reduction by sulphate reducing bacteria (SRB) is carried out either chemically (involving reduction by hydrogen sulphide) or enzymatically (involving redox enzymes such as the hydrogenases). While reduction of metal ions by hydrogen sulphide is well known, the enzymatic mechanism for metal reduction is poorly understood. The aims of this study were to investigate the role of SRB in facilitating platinum removal, and to investigate the role of a hydrogenase in platinum reduction *in vitro*.

In order to avoid precipitation of platinum as platinum sulphide, a resting (non-growing) mixed SRB culture was used. The maximum initial concentration of platinum (IV), which SRB can effectively remove from solution was shown to be 50 $mg.l^{-1}$. Electron donor studies showed high platinum (IV) uptake in the presence of hydrogen, suggesting that platinum (IV) uptake from solution by SRB requires careful optimization with respect to the correct electron donor. Transmission electron microscopy (TEM) and energy dispersive X-ray (EDX) analysis indicated that platinum was being precipitated in the periplasm, a major area of hydrogenase activity in SRB. Purification of the hydrogenase by ammonium sulphate precipitation (65%), Toyopearl-Super Q 650S ion exchange and Sephacryl S-100 size exclusion chromatography revealed that the hydrogenase was monomeric with a molecular weight of 58 KDa, when analyzed by 12% SDS-PAGE. The

purified hydrogenase showed optimal temperature and pH at 35 °C and 7.5 respectively, and a poor thermal stability.

In vitro investigation of platinum reduction by purified hydrogenase from mixed SRB culture showed that hydrogenase reduces platinum only in the presence of hydrogen. Major platinum (IV) reduction was observed when hydrogenase was incubated with cytochrome c_3 (physiological electron carrier *in vivo*) under hydrogen. The same observations were also noted with industrial effluent. Collectively these findings suggest that *in vitro* platinum reduction is mediated by hydrogenase with a concerted action of cytochrome c_3 required to shuttle the electron from hydrogenase.

Keywords: ***Sulphate reducing bacteria, Hydrogenase, Enzymatic reduction of platinum, Bioremediation.***

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LIST OF ABBREVIATIONS

AAS = Atomic absorption spectrophotometer
AMP = Adenosine monophosphate
APS = Adenosine phosphosulphate
ATP = Adenosine triphosphate
ATPase = Adenosine triphosphatase
BSA = Bovine serum albumin
Cl_6K_2Pt (IV) = Potassium hexachloroplatinate (IV)
DCIP = 2, 6-dichloro-indophenol
ddH_2O = Double deionised water
dH_2O = Deionised water
EDX = Energy dispersive X-ray
EPS = Extracellular polymeric substance
ETLP = Electron transfer linked phosphorylation
FDA = Fluorescein diacetate
h = Hour
H_2ase = Hydrogenase
H_2S = Hydrogen sulphide
HSAB = Hard and soft acids and bases
HSO_3^- = Bisulphite
L = Liter
M = Molar
mg = Milligram
$mg.l^{-1}$ = Milligram per liter
Min = Minutes
mM = Milli-Molar
MOPS = Morpholinepropanesufonic acid
MV = Methyl viologen
NADH = Nicotinamide adenosine dinucleotide reduced
nm = Nanometer
OFN = Oxygen free nitrogen
PAGE = Polyacrylamide gel electrophoresis
PDH = Pyruvate dehydrogenase
PEI = Polythyleneimine
PGMs = Platinum group metals
PPi = Pyrophosphate
ppm = Parts per million
q_{max} = Maximum uptake capacity
rpm = Revolutions per minute
rRNA = Ribosomal RNA
SDS = Sodium dodecyl sulphate
Sec = Seconds
SEM = Scanning electron microscopy
SLP = Substrate level phosphorylation
SRB = Sulphate reducing bacteria
TEM = Transmission electron microscopy
U = Units
UG2 = Upper group 2
v/v = Volume per volume
w/v = Weight per volume

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CHAPTER ONE

Literature Review

1.1 General Introduction

The increased use of metal and chemical processing in South Africa has resulted in the generation of large quantities of aqueous toxic effluents that contain high levels of metal ions and pose environmental disposal problems. Furthermore, specific industries producing similar effluents are not localized in their various topographic regions (Bux *et al.*, 1994). The problem of waste containment and recovery therefore becomes more of a diffuse source pollution problem, which increases treatment costs considerably. The presence of metal ions in the environment at concentrations above critical levels stipulated by national regulatory bodies are now considered unacceptable (Bux *et al.*, 1998). Metal ions in various water bodies are of major concern especially in a country like South Africa, which is primarily a water scarce country, with extensive industrialization and ever increasing population density (Asmal, 1998; Conley and Van Niekerk, 2000). Decreasing the toxic metals to environmentally acceptable limits in a cost effective and environmentally friendly manner thus assumes great significance.

It has been established beyond any doubt that dissolved heavy metals escaping into the environment pose a serious health hazard. Unlike other pollutants, metals cannot be chemically or biologically degraded and are ultimately indestructible. Today heavy metals constitute a global environmental hazard (Bulow and Mejare, 2001). Heavy metals comprise an ill-defined group of approximately 65 metallic elements of density greater than 5 $kg.m^3$ (Gadd and Griffiths, 1978). They have diverse physical, chemical and biological properties but generally, all exert toxic effects on living organisms. Although elevated levels of toxic heavy metals occur in some natural locations, on average, their presence is generally low (Gadd, 1990). Most of the naturally occurring concentrations of heavy metals occur in an immobilized form in sediments and ores and are biologically unavailable (Babich and Stotzky, 1978).

To date (2002), with the exponentially increasing industrialization, the need for controlling metal emissions into the environment is even more pronounced. This is best done at the source of emissions, before toxic metals enter the complex ecosystem. The effects of heavy metals on the ecosystem function vary considerably and are of economic and public health significance. To follow the fate of metallic species after they enter the ecosystem becomes very difficult and they start to inflict damage as they move through from one ecological trophic layer into another. They accumulate in living tissues throughout the food chain, which has humans at its apex (Figure.1.1). The resulting health problems demonstrate themselves on acute as well as chronic levels and are reflected in the well being of individuals and in society's spiraling health care costs (Volesky, 2001).

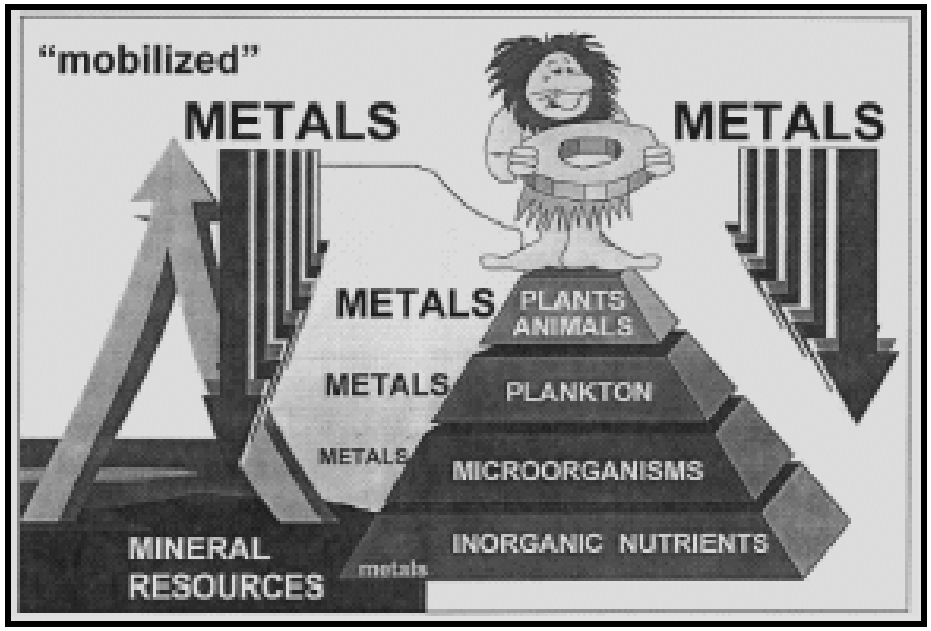


Figure 1.1: The food-chain pyramid receives metal through man's activities. On top of the pyramid is man receiving pre-concentrated metal toxicity (Volesky, 2001).

1.2 Microbial resistance of metals in the environment

Several factors determine the extent of metal resistance in a microorganism; (i) the type and number of mechanisms for metal uptake; (ii) the role each metal plays in normal metabolism (iii) the chromosomes may contain genes that encode proteins that confer resistance (Oehme *et al.,* 2000). Five mechanisms have been postulated to be involved in bacterial resistance to metals, and such mechanisms include: (i) exclusion by a

permeability barrier, (ii) intra- and extracellular sequestration, (iii) active transport efflux pumps, (iv) enzymatic detoxification, and (v) reduction in the sensitivity of cellular targets to metal ions (Silver, 1992; Rouch *et al.,* 1995). Microorganisms can possess one or a combination of several resistance mechanisms and each mechanism relies on inputs of cellular energy. Other mechanisms may exist but examples in nature have not yet been discovered.

1.2.1 Metal exclusion by permeability barrier

Alterations in the cell wall, membrane, or envelope of a microorganism are examples of metal exclusion by a permeability barrier. This mechanism is an attempt by the organisms to protect metal sensitive, essential cellular components. A prominent example is the exclusion of Cu (II) resulting from altered production of the membrane channel protein porin by *Escherichia coli B* (*E. coli*) (Rouch *et al.,* 1995). This is usually a single gene mutation, which decreases the permeability of the membrane to metal ions (Ji and Silver, 1995). Another example is nonspecific binding of metals by the outer membrane or envelope. This offers limited metal protection due to saturation of binding sites (Holye and Beveridge, 1983). Although not fully proven, it is believed that some forms of copper resistance are based on periplasmic binding (Mergeay, 1991; Silver and Ji, 1994). In some species of *Staphylococcus aureus* (*S. aureus*), penicillase plasmids can mediate resistance by changing cell membrane permeability to Cd (II) as well as to other metals. In this case there appears to be conformational changes in the membrane that prevent metal ions from entering. This is actually low-level resistance in the range 0.01 to 0.1 nM of Cd (II) (McEntee *et al.,* 1986).

1.2.2 Sequestration of metals by binding proteins

1.2.2.1 Intracellular sequestration

Intracellular sequestration is the accumulation of metals within the cytoplasm of the cell in order to prevent exposure to essential cellular components. This is a metabolism dependent mechanism, occurring in living cells and may be accompanied by toxic symptoms. This is one of the most important mechanisms by which a cell combats metal exposure and subsequent accumulation. Once inside the cell, metal ions may be

preferably located within specific organelles and/or bound by intracellular low molecular weight metal binding proteins such as metallothioniens in bacteria and phytochelatins in algae. Examples of this form of metal resistance include: intracellular binding of uranium to metallothionein produced in *Synechococcus alengatus* (*S. alengatus*) and cysteine rich proteins in *Pseudomonas aeruginosa* (*P. aeruginosa*), which leads to the formation of dense internal deposits (Rouch *et al.,* 1995; Silver and Phung, 1996). Another example of metal accumulation is that of bacterium *Pseudomonas stutzeri* (AG259) that is capable of producing silver based single crystals in the size range of 1 nm up to 200 nm. These biologically fabricated nanostructures showed a well defined shape and composition. To protect the cell from the toxic consequences of silver, this bacterial strain used the advantage of a detoxification mechanism of precipitation of silver in the periplasmic space and its reduction to elemental silver (Klaus, 1999).

1.2.2.2 Extracellular sequestration

Metal resistance based on extracellular sequestration has been hypothesized only in bacteria, but has been found in several species of yeast and fungi (Joho *et al.,* 1995). It is a metabolism independent mechanism and it involves nonspecific binding of metals to the cell surface. This mechanism includes extracellular binding, whereby cells synthesize and release organic materials that chelate metals to reduce their bioavailability. One of the forms of Ni (II) resistance in yeast is based on this mechanism. *Saccharomyces cerevisiae* (*S. cerevisiae*) reduces absorption of Ni (II) by excreting large amounts of glutathione (Murata *et al.,* 1985). Glutathione binds with great affinity to heavy metals. Yeast carrying the methylglyoxal resistance gene demonstrates the ability to form extracellular metal glutathione complexes in metal rich media. Resistance results when the toxic metal is bound in a complex and cannot enter the cell membrane (Murata *et al.,* 1985).

A similar mechanism exists in Cu (II)-resistant fungi (Murphy and Levy, 1983). These fungi secrete oxalate to form a metal-oxalate complex. Other organisms such as yeast or *Citrobacter sp.* form insoluble complexes of cadmium phosphate to confer resistance (McEntee *et al*., 1986). For example yeast forms a complex during hydrogen sulphide

production while *Citrobacter* use phosphate. A strain of *Klebsiella aerogenes* (*K. aerogenes*) has exhibited the ability to remove Cd (II) ions from the surrounding environment by excreting sulphur to limit metal influx by external precipitation (Scott and Palmer, 1990). Some mercury-resistant mutants of *S. cerevisiae* were found to have a requirement for methionine. Evidence suggested that this compound itself is an efficient chelating agent and was used by yeast to produce a diffusible substance, which acted as a detoxifying agent toward mercurials (Murata *et al.,* 1985).

1.2.3 Active transport of the metal away from the microorganism

Active transport or efflux systems represent the largest category of metal resistance systems. These efflux systems can be either ATPases (e.g. the cadmium and copper ATPases of gram-positive and arsenate ATPase of plasmids of gram-negative bacteria) or chemiosmotic (e.g. the divalent cation efflux system of soil *Alcaligenes* and the arsenate efflux system of the chromosome of gram-positive bacteria and of plasmids in gram positives) and they are linked and highly specific for the cation or anion they export (Silver *et al.,* 1989; Nies and Silver, 1995). These mechanisms can be chromosomal or plasmid-encoded. Much resistance to toxic metals studied so far is encoded on the plasmids (Ji and Silver, 1995), including resistance to Ag^{+}, AsO_4^{3-}, Cd^{2+}, CrO_4^{2-}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Ni^{2+}, Sb^{3+}, and Zn^{2+} (Silver and Phung, 1996). Most commonly encountered type of plasmid encoded resistance function as the efflux ‘pump’ (removing toxic metal ions away from the cytoplasm, that entered the cell through normal nutrient transport systems) which relies on an dependent P-type ATPase.

One of the best characterized resistance efflux systems includes bacterial ATPase efflux mechanism for the essential metal ion Cu (II). The *cop* operon has been studied in *Pseudomonas syringae* and it contains four genes: *copA, copB, copY and copZ,* which encode for structural proteins CopA, CopB, CopY and CopZ. CopD and CopB are the inner and outer membrane proteins respectively, while CopY and CopZ are periplasmic proteins (Figure 1.2). CopY and CopZ are blue copper-binding proteins containing 11 and 1 Cu^{2+} ions respectively. Storage of excess copper in the periplasmic space is considered to protect the cell from toxic copper. How membrane proteins CopB and

CopD are involved in the movement of copper across the outer and inner membrane is not yet understood. However, a mutant operon containing *copD* but not all three of the other genes confers hypersensitivity and hyperaccumulation of intracellular copper, indicating the role for CopD in copper uptake by the cell (Cooksey, 1994; Silver and Phung, 1996).

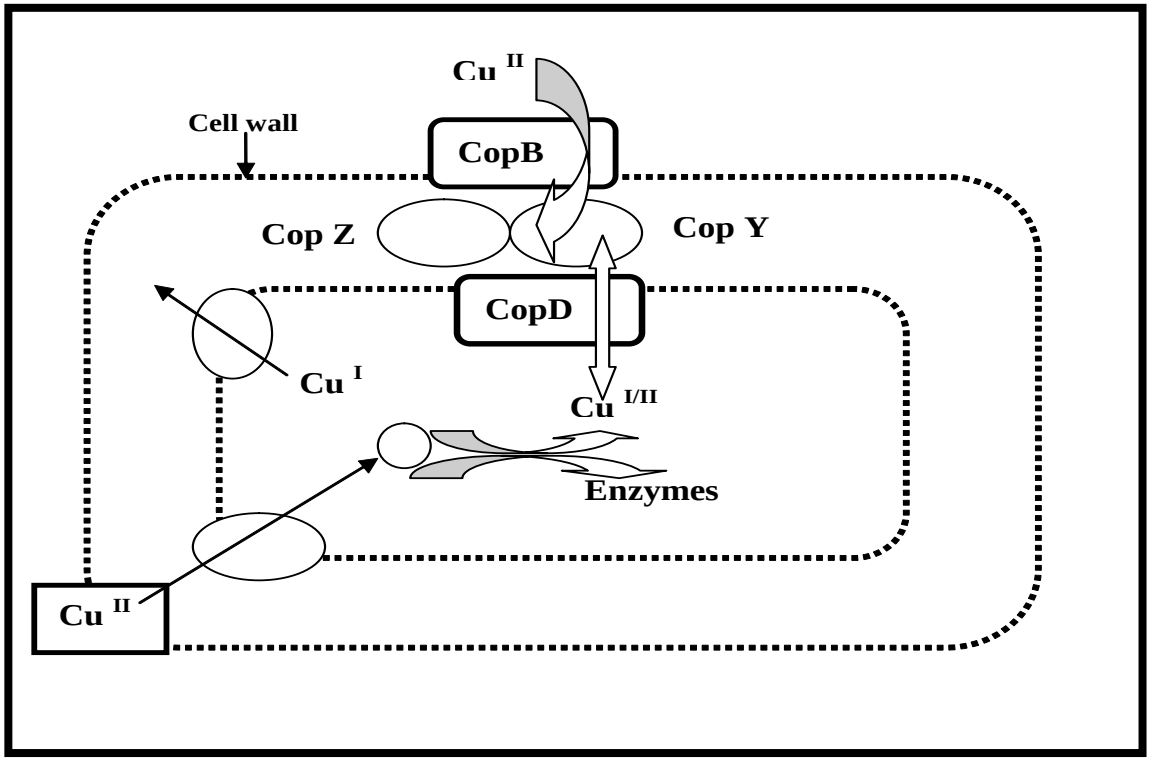


Figure 1.2: Copper transport and resistance in *Pseudomonas syringae* (Cooksey, 1994).

1.2.4 Enzymatic detoxification of metal ions

Mercury resistance is a model example of an enzymatic detoxification system in microorganisms. More is known about this resistance system that any of the others. Both gram-positive (*S. aureus*, *Bacillus sp*.) and gram-negative bacteria (*E. coli*, *P. aeruginosa*, *Serratia mercescens* and *Thiobacillus ferrooxidans*) demonstrate resistance to Hg (II) (Misra, 1992). Mercury is toxic because it binds to and inactivates essential thiols that are part of enzymes and proteins. Some bacteria contain a set of genes that form a Hg (II) (*mer*) resistance operon. This operon not only detoxifies Hg (II) but also self regulates resistance (Misra, 1992; Ji and Silver, 1995). In times of Hg (II) absence the operon codes for a regulatory protein that down regulates transcription. The same set

of genes also encodes the production of a periplasmic metal binding protein and membrane associated transport proteins.

The periplasmic binding protein collects Hg (II) in the surrounding environment and transport proteins take it to the cytoplasm for detoxification. The Hg (II) resistance or *mer* operon codes for production of two enzymes. The first, organomercurial lyase, catalyses the following reaction (Misra, 1992; Weiss *et al*., 1997)

$$RHgX + H^+ + X^- \leftrightarrow RH + HgX_2 \text{ .....1}$$

Mercuric ion reductase catalyses the following reaction (Misra, 1992; Weiss *et al*., 1997)

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

Organomercurial lyase is effective in hydrolyzing the stable mercury-carbon bond. It works without cofactors by binding Hg (II) in the active site with cysteine sulphahydryl residues. The product of these enzymes is a mercury thiolate adduct produced when glutathione is exchanged for the cysteine ligands of the enzyme. This product is then utilized as a substrate for mercuric reductase, which contains the cofactor flavin in each of its subunits (Misra, 1992). Mercuric reductase reduces Hg (II) to Hg (0) (metallic mercury), a process that involves hydride transfer from the electron carrier NADPH to flavin. Metallic mercury is then released to diffuse through the cell membrane and into the surrounding environment.

1.2.5 Reduction in metal sensitivity of cellular targets

Some organisms adapt to the presence of toxic metals by altering the sensitivity of essential cellular components; this provides a degree of natural protection (Rouch *et al*., 1995). Protection is achieved by mutations that decrease sensitivity but do not alter basic function or by increasing production of a particular cellular component to keep ahead of metal inactivation. DNA repair mechanisms also provide limited protection to plasmid and genomic DNA. The microorganism may protect itself by producing metal resistant

components or alternate pathways in an effort to bypass sensitive components. Adaptation has been found in *E. coli*. Upon exposure to Cd (II), unadapted *E. coli* demonstrated considerable DNA damage; however, after subculture the same organisms show resistance (McEntee *et al*., 1986; Mergeay, 1991). The growth lag phase of the organism extended the longer it was exposed to Cd (II). It was postulated that the extended lag phase was initially due to a period of induction of DNA repair mechanisms. Natural resistance can result from normal cellular functions that give organism a basic level of tolerance (Rouch *et al*., 1995). An example is glutathione, which may offer protection to the metal ions like Ag (I), Cu (I, II), Cd (II), and Hg (II) (Ni'bhrianin *et al*., 1983). Glutathione may offer protection from Cu (II) and Fe (II) by suppressing free radicals formation (Rouch *et al*., 1995)

There appear to be differences in the ability of gram-negative and gram-positive bacteria to tolerate certain metal ions (Morozzi *et al*., 1986). Gram-negative bacteria (*E. coli* and *Pseudomonas sp*.) are better than gram-positive bacteria to carry on protein synthesis in the presence of Cd (II). A species of *Pseudomonas* could tolerate 5 to 30 times more Cd (II) in growth media before protein synthesis was reduced by 50% compared to gram-positive *S. aureus, S. faecium, and B. substilis* (Minz *et al*., 1996). On the other hand gram-positive organisms are able to bind 28 to 30 times more Cu (II) when compared to *E. coli* (Beveridge and Fyfe, 1985).

1.3 Conventional metal removal technologies

Municipal treatment plants are not designed and equipped for handling toxic wastes. Metals and their toxicity persist even in sludges and by-product streams of municipal treatment plants. Due to their classification as "toxic substances" they require special handling, disposal methods and sites. Heavy metals need to be removed right at their source in specially designed cost effective 'pre-treatment' steps. These specific treatments need to be cheap because they most often deal with large volumes of effluents. Under both the public and the media pressure, governments introduce progressively stricter regulations with regard to the metal discharge particularly for industrial operations. In past decades several physicochemical and biotechnological treatment

processes have been developed to remove heavy metals from synthetic and industrial effluents.

1.3.1 Physicochemical processes

Physicochemical processes for metal removal are well established (Eccles, 1995; Volesky, 2001). Commonly applied chemical processes for the treatment of industrial effluents are listed in Table 1.1. As the policies on emission standards tighten these currently practiced technologies are becoming progressively inadequate, often creating secondary problems, with metal-bearing sludges, which are extremely difficult to dispose of and are prohibitively expensive (Volesky, 2001).

Table 1.1: Conventional physicochemical metal removal technologies (Volesky, 2001)

Method	Disadvantages	Advantages
Chemical precipitation	Applicable for higher concentrations only, Difficult separation, Resulting in sludges	Simple, Cheap
Electrochemical treatments	For high concentrations only, Expensive	Metal recovery
Reverse Osmosis and Filtration	High pressures, Membrane scaling, Membrane may be pH sensitive	Pure effluent (for recycle)
Ion exchange	Sensitive to particles, Expensive resins	Effective pure effluent metal recovery possible
Adsorption	Not effective at low metal concentrations	Cost effective (conventional sorbent is carbon)

For example, precipitation processes are not readily applicable because of their complex solution chemistry and they produce wastes difficult to treat thus failing to guarantee the metal concentration required by regulatory standards. On the other hand ion exchange and adsorption are very effective but require expensive adsorbent materials, which are subjected to saturation constraints, necessitating sorption desorption cyclic processes during use. In the case of ion exchange, resins currently applied in waste treatment are prone to oxidation attack by chemicals. Solvent extractions are widely used but require substantial plant investment, and in addition to leaving final solutions containing low levels of residual metal, the solvent may be toxic to the environment (Kratochvil and

Volesky, 1998). Electrochemical treatments are feasible, but recovery of the thin metal deposit from the electrode may limit industrial adoption. Reverse osmosis requires high energy cost and membranes have limited tolerance for metal because of their weak resistance to pH.

1.3.2 Biological processes

Microbial-based technologies for metal removal present an economic alternative for today's mining, mineral and waste water treatment industries, at a time when high grade mineral resources are being depleted, energy costs are increasing, and diverse environmental effects becoming more apparent as results of conventional chemical technologies. Biological processes fall into one of two categories: (i) Biosorptive uptake by non growing biomass or (ii) Bioaccumulation by living organism.

1.3.2.1 Biosorption

Biosorption is the process by which metals are sorbed and/or complexed to either living or dead biomass (Volesky and Holan, 1995; Eccles, 1995). It encompasses those physicochemical mechanisms by which metal species are removed from aqueous solution by microbial biomass or products (Gadd, 2000). Biomass, however, unlike mono-functional ion exchange resins, contains a number and variety of functional sites. These sites, contributed by the cell biopolymers include: carboxyl, imidazole, sulphydryl, amino, phosphate, sulphate, thioether, phenol, carbonyl, amide, and hydroxyl moieties (Bedell and Darnall, 1990).

Biosorption of metals is affected by many experimental factors such as pH, ionic strength, biomass concentration, temperature and presence of different metallic ions in solution. The variability of these factors in real wastewaters makes it necessary to know how they influence biosorption performance. As a consequence of these possible multiple interactions the comprehension of biosorption phenomena is very complex and requires a study of both the solution chemistry of metal ions (depending on pH, anions and or ligands in solution) and the mechanisms of metal uptake (ion exchange, complexation and micro-precipitation, etc,) (Volesky and Holan, 1995; Veglio and Beolchin, 1997).

It is apparent that in order to be accepted by the engineering community a new metal biosorption process must be commercially competitive and perform as well as existing technologies. Preliminary cost calculations suggest that biosorption may be economically competitive with ion exchange or chemical precipitation for treating some waste streams (Eccles, 1995; 1999). The advantages of biosorption lie in good performance in metal removal, often comparable with their economic competitors (ion exchangers), and cost effectiveness, making use of algae and raw materials of fermentation and agricultural processes (Volesky and Schiewer, 1999). This aspect plays an important role to improve a zero tolerant waste economic policy especially in the case of the reuse of the biomasses. Another advantage of biosorption is the selectivity shown by some biomasses towards heavy metal even in the presence of other metal ions such as alkaline and alkaline earth metals.

Several biosorption processes are under development or have been developed, patented and introduced for the application in removing metal contaminants from surface and ground waters. Brierley, (1990) described the state of the art of these specific technologies. The most important are the following:

❖AMT BIOCLAIM Process: this employs microorganisms, principally bacteria of the genus *Bacillus*, that have been: (i) treated with strong caustic solution to enhance metal accumulation; (ii) washed with water to remove residual caustic; and (iii) immobilized in extruded beads using polyethyleneimine (PEI) and glutaraldehyde.

❖AlgalSORB^{TM} Process: this process contains non-living algal biomass immobilized in batch or column systems. Columns are slurry-packed with immobilized algal particles, 40-400 mesh size. Selective metal recovery is by treatment with appropriate reagents after which generated biomass retains approximately 90% of the original metal uptake efficiency even after >18 months of regular use. AlgalSORB^{TM} has been successfully used for the removal of Ag, Al, Au, Co, Cu, Cr, Hg, Ni, Pb, Pt, U, and Zn from contaminated effluents and process streams (Bedell and Darnall, 1990).

❖BIO-FIX Process: Biomass, including sphagnum peat moss, algae, yeast, bacteria and /or aquatic flora immobilsed in polysulfone.

❖Immobilized *Rhizopus arrhizus* biomass: a proprietary process involving the immobilsed fungus *Rhizopus arrhizus* has been evaluated for the recovery of uranium from an ore bioleach solution (Tsezos and Volesky, 1982). In this study the non-available biomass showed uranium uptake capacity of over 180 $mg.g^{-1}$ dry weight, which is more than double the capacity of IRA-400, a common anion exchange resin used for accumulation of uranium.

1.3.2.2 Bioaccumulation

Bioaccumulation includes all processes responsible for uptake of metal ions by living cells and includes biosorptive mechanisms, together with intracellular accumulation and bioprecipitation mechanisms (Eccles, 1995). While the use of living organisms is often successful in treatment of toxic organic contaminants, living organisms in conventional biological systems generally have not been useful in the treatment of solutions containing heavy metal ions. Once the metal ion concentration becomes too high the organism's metabolism is disrupted, leading to death. Because of metal toxicity, most of the living cell systems exploited to date have been used for decontamination of the effluents at sublethal metal concentrations. Living organisms have potential only if biomass replenishment is possible. Living cells may be grown as biofilms on inert supports with larger surface area. Support used may be porous (preferred to reduce blocking) or solid (such as glass, metal sheets, plastics, wood etc,). Rotating biological contactors, trickling filters, fixed or fluidized beds and airlift bioreactors could also be used (Gadd and Romme, 1988).

Bioaccumulative metal uptake forms the basis for waste detoxification processes using, for example biological fluidized beds containing continually growing biofilm (Remacle and Houba, 1983). Biofilm based processes have been used in the treatment of industrial flows (Macaskie and Dean, 1989). Notable established examples of large-scale industrial development include the Homestake wastewater treatment process (USA) (Mudder and

Whitlock, 1984) and chromate treatment process developed in the former USSR with large scale plants in Moscow, Ukraine and Kazakhstan (Koren'Kov *et al.*, 1980; Karavaiko *et al.*, 1988).

The Homestake process utilizes cyanide-oxidizing bacteria, which grow in the presence of CN^- and fortuitously sorb the co-contaminating metal components. The plant employs 48 rotating biological contactors (total biofilm surface area 9290 m^2) to treat a flow of 21000 $m^3.day^{-1}$ with 95% removal of input metal such as Ni, Pb, and Zn (Whitlock and Mudder, 1985). The chromate process harnesses microbial chromate reductase activity to the production of Cr^{3+} and precipitation of insoluble $Cr(OH)_3$ onto immobilized biofilm. The continuous process achieves reduction of chromate at an input concentration of 190 $\mu g.ml^{-1}$ at a flow rate of 7200 $m^3.day^{-1}$(Koren'Kov *et al.*, 1980).

1.4 Sulphate Reducing Bacteria (SRB)

Sulphate-reducing bacteria (SRB) constitute a diverse group of heterotrophic prokaryotes that contribute to a variety of essential functions in many anaerobic environments (Peck, Jr., 1993; Postgate, 1984). They are considered as a group because of their common ability to conserve energy by the dissimilatory reduction of sulphate to H_2S in anoxic environments. Since the discovery of microbial sulphate respiration more than 100 years ago (Beijerinck, 1895), SRB are still described as strict anaerobic organisms, although several reports have shown that some of these bacteria, mainly from the *Desulfovibrio* genus, are quite resistant to low levels of oxygen (Cypionka, 2000). Sulphate-reducing bacteria are broadly distributed on earth, and many of them have been isolated in a wide range of anaerobic environments particularly in the anoxic sediments of fresh water (Zudryk *et al*., 2000) and marine systems (Widdel, 1988; Isaksen and Jorgensen, 1996). Recently they have been found in anoxic microenvironments in anaerobic wastewater treatment system (Sahm *et al*., 1999).

1.4.1 Ecology

Sulphate-reducing bacteria play significant roles in nature by virtue of their potential for numerous interactions (Barton and Tomei, 1995). In addition to their obvious importance to sulfur cycle, SRB are important regulators of a variety of processes in wet land soils, including organic matter turnover, biodegradation of chlorinated aromatic pollutants in anaerobic soils and sediments and mercury methylation (Singleton, Jr., 1993). Because of their importance to critical processes in ecosystem functioning and environmental remediation, interest in SRB has increased over the past ten years. Sulphate-reducing bacteria are ecologically important as terminal electron accepting microorganisms in environments where sulphate is present in sufficient amounts (e.g. marine or brackish water) (Buisman *et al*., 2000). The upper limit of their tolerance to sulphate has been reported to be about 2000 $mg.l^{-1}$. They are almost all neutrophiles with maximum growth obtained in the pH range of 6-12. The optimum pH for growth of most of the known SRB lies in the range of 6.5-7.5, while the growth is usually inhibited at a pH lower than 5.5 or higher than 9.0 (Hao *et al*., 1996). In contrast some isolates, however have been reported to grow in the moderate acidic conditions such as marine and surface water, where the pH is in the range of 3-4 (Hedin and Nairin, 1991). These bacteria grow over a broad temperature range from 0-98 ^{o}C, with an optimum temperature for growth at around 30 ^{o}C. Some SRB strains have been isolated from deep aquifers, where the temperatures exceed 60 ^{o}C (Olson *et al.,* 1981).

1.4.2 Classification

Sulphate-reducing bacteria are a complex physiological bacterial group and various properties have been used in traditional classification schemes (Table 1.2). The most important of these properties are cell shape, motility, guanine: cytosine (GC) content of DNA, presence of desulfovirin and cytochromes, optimal temperature, and complete versus incomplete oxidation of acetate. For classification within a particular genus, different electron donors are tested. Analysis of ribosomal RNA (rRNA) sequences has allowed organization of the various SRB species into four distinct groups: Gram-negative mesophilic SRB; Gram-positive spore forming SRB; thermophilic bacterial SRB; and thermophilic archaeal SRB. Thirteen SRB genera are currently recognized with

considerable metabolic activity within different phylogenetic groups (Stackerbrandt *et al.*, 1995; Swofford, 1999; Ogram *et al*., 2000).

Table 1.2: Important characters in the classification of representative sulphate reducing bacteria (Ogram *et al*., 2000).

	Shape	Motility	GC content of DNA (%)	Desulfovirin	Cytochromes	Oxidation of acetate	Growth temp. (°C)
Gram-negative mesophilic SRB							
Desulfobulbus	Lemon to rod	+/-	59-60	-	b,c,c_3	I[a]	25-40
Desulfomicrobium	Ovoid to rod	-/+	52-67	-	b, c	I	25-40
Desulfomonas	Rod	-	66	+	c	I	30-40
Desulfovibrio	Spiral to vibrioid	+	49-66	+/-	c_3, b, c	C[b]	25-40
Desulfobacter	Oval to rod	+/-	44-46	-	b, c	C	20-33
Desufobacterium	Oval to rod	+/-	41-52	-	b, c	C	20-35
Desulfococcus	Spherical to lemon	-/+	46-57	+/-	c_3	C	28-35
Desulfomonile	Rod	-	49	+	b, c	C	37
Desulfonema	Filaments	gliding	35-42	+/-	b, c	C	28-32
Desulfosarcina	Oval rods or coccoid, packages	+/-	51	-	b,c	C	33
Gram-positive spore-forming SRB							
Desulfotomaculum	Straight to curved rods	+	48-52	-	b, c	I/C	Most 25-40 some 40-65
Bacterial thermophilic SRB							
Thermodesulfobacterium	Vibrioid to rod	-/+	30-38	-	c_3, c	I	65-70
Archaeal thermophilic SRB							
Archaeoglobus	Coccoid	+/-	41-46	-	n.r.[c]	I	64-92

[a]I-incomplete; [b]C-complete; [c]n.r., not reported.

Even though SRB are currently only divided into four groups, new divisions could be added as more information on the diversity of SRB in extreme environments becomes available. Jorgensen *et al*. (1992), observed sulphate reducing activity in sediments from the Guaymas Basin in different ranges of temperatures than previously described for the four known groups of SRB. Sulphate reduction between 100 and 110 ^{o}C were reported, temperatures from which no SRB have yet been isolated. These authors postulated that SRB may be present in those extreme thermophilic environments and there may be some hyperthermophilic SRB still to be discovered (Jorgensen *et al*., 1992).

1.4.3 Physiology

Sulphate-reducing bacteria utilize a very wide spectrum of compounds as electron donor and also as carbon or energy sources, ranging from simple low molecular organic compounds (lactate, acetate, propionate, succinate, pyruvate, ethanol, aliphatic acids, sugars, amino acids, indole, nictotinic, etc), to more complicated aromatics (phenol, catechol, p-and m-cresol, benzyl alcohol, etc.) (Postgate, 1984; Widdel, 1988; Hansen, 1993; Gadd and White, 1996a; Kalyuzhnyi *et al*., 1997). Some SRB are able to metabolize hydrogen as electron donor (Van Houten *et al*., 1995). Sulphate-reducing bacteria that are capable of degrading complex high molecular mass aromatic hydrocarbons such as naphthalene and phenanthrene have not been isolated to date. However Coates *et al*. (1996) reported oxidation of polycyclic aromatic hydrocarbons under sulphate reducing conditions. Although SRB were not isolated, incubation of [^{14}C] naphthalene- or phenanthrene-spiked harbour sediments under sulphate reducing conditions resulted in the production of CO_2. Moreover, addition of molybdate, a specific inhibitor of sulphate reduction, resulted in a complete inhibition of $^{14}CO_2$ evolution, suggesting that undescribed SRB may be present (Coates *et al*., 1996).

Besides inorganic sulphur compounds (sulphate, sulphite, thiosulphate, and elemental sulphur) and fumarate (Miller and Wakerley, 1966), various inorganic compounds can serve as electron acceptors. Although named after a single electron acceptor (sulphate), today SRB appear to be the organisms that reduce the greatest number of different electron acceptors. Some SRB carry out dissimilatory reduction of nitrate to nitrite to

ammonia (Widdel and Pfenning, 1982). This process leads to a higher growth yield than sulphate (Seitz and Cypionka, 1986). *Desulfosporosinus orientis* (formerly *Desulfotomaculum orientis* (Stackebrandt *et al*., 1997)), carries out carbonate respiration, using CO_2 as electron acceptor, which is reduced to acetate in a homoacetate fermentation (Klemps *et al*., 1985). Furthermore, *Desulfovibrio* species can reduce Fe (III) (Lovely *et al*., 1993) and even uranium (VI) (Lovely and Philips, 1992a; b) or chromate (Lovely and Philips, 1994). Reduction of the metal ions appears not to be coupled to growth in *Desulfovibrio* species. However, *Desulfotomaculum* species were found to grow with arsenate (Newman *et al*., 1997) or Mn (IV) (Tebo and Obraztsova, 1998) as electron acceptor.

1.5 Bioenergetics of dissimilatory sulphate reduction

Most of the sulphur in all organisms occurs in protein, as a component of amino acids such as cysteine and methionine. The energetically stable and most abundant source for organisms is sulphate (SO_4^{2-}) at the highest oxidation state of (+6) which is reduced to sulphide (S^{2-}) which is then incorporated into amino acids. This process in known as assimilatory sulphate reduction and it is purely a biosynthetic process. In the absence of oxygen the oxidized forms of sulphur are used as electron acceptors for the biological oxidation of the carbon substrates by specialized group of anaerobic bacteria such as SRB. Since this reduction of the organic compound serves for energy conservation, the process is termed dissimilatory sulphate reduction (Peck and LeGall, 1982; Peck and Lissolo, 1988)

Sulphate is a stable non-reactive compound that must first be activated in order to participate in subsequent metabolic reactions such as reduction and sulphate transfer (Schiff and Saidha, 1987). The first step in dissimilatory sulphate reduction involves the transfer of exogenous sulphate across the bacterial membrane into the cell, the process that is catalyzed by the enzyme ATP-sulphurylase. This enzyme catalyzes the formation of a highly activated adenosine phosphosulphate (APS) and pyrophosphate (PPi), which may subsequently be cleaved to yield inorganic phosphate by the enzyme inorganic pyrophosphatase. Adenosine phosphosulphate is rapidly converted to sulphite (SO_3^-) and

adenosine monophosphate (AMP) by the enzyme APS reductase. Sulphite is then protonated through chemical reaction to form bisulphite (HSO_3^-) and reduced to sulphide by the enzyme bisulphite reductase. The pathway of dissimilatory sulphate reduction and generation of sulphide is shown in Figure 1.3 (Peck and Lissolo, 1988; Gibson, 1990; Dahl *et al.*, 1994; Gavel *et al.*, 1998; Sperling *et al.*, 1998).

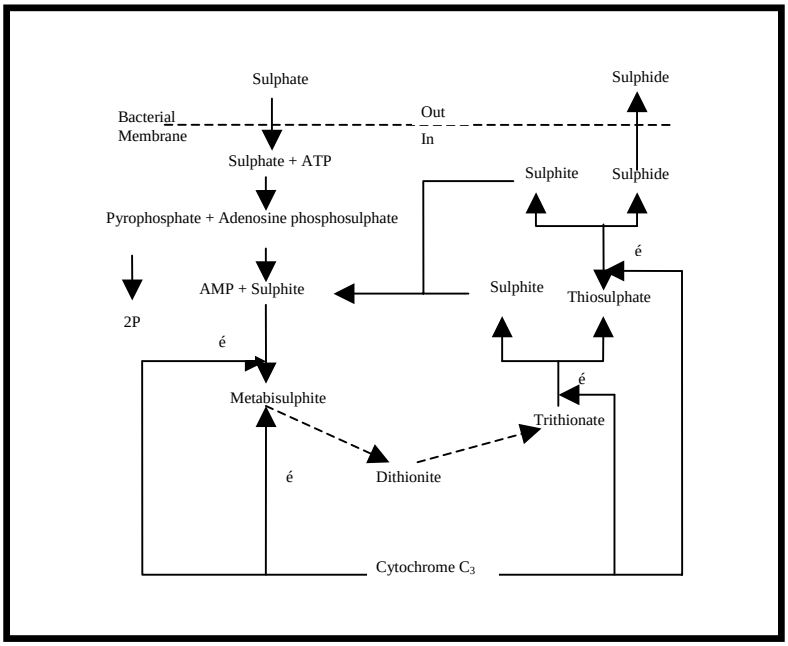


Figure 1.3: The pathway of dissimilatory sulphate reduction and generation of sulphide (e^-=electron) (Gibson, 1990).

A unique characteristic of all dissimilatory sulphate reduction is their ability to couple net production of ATP via electron transfer linked phosphorylation (ETLP) or substrate level phosphorylation (SLP) to the reduction of sulphate depending on the oxidation of organic electron donor (Peck, 1966). However SLP cannot provide the ATP required for sulphate reduction and growth; thus another mechanism of energy conservation must be linked to the oxidation of organic substrate by these bacteria. Hydrogen metabolism plays a central role in the energy-generating mechanisms of various microorganisms. For the genus *Desulfovibrio*, hydrogen cycling with vectorial electron transfer has been proposed as a general energy-coupling mechanism when the cells are grown on organic substrates with sulphate. Evidence suggested that certain *Desulfovibrio* that are known to be able to produce or consume hydrogen can carry out intracellular transfer of molecular hydrogen

between cytoplasmic fermentation and a membrane-bound respiratory chain. This recycling of molecular hydrogen within the same organism bestows an energetic and adaptive advantage on the organism.

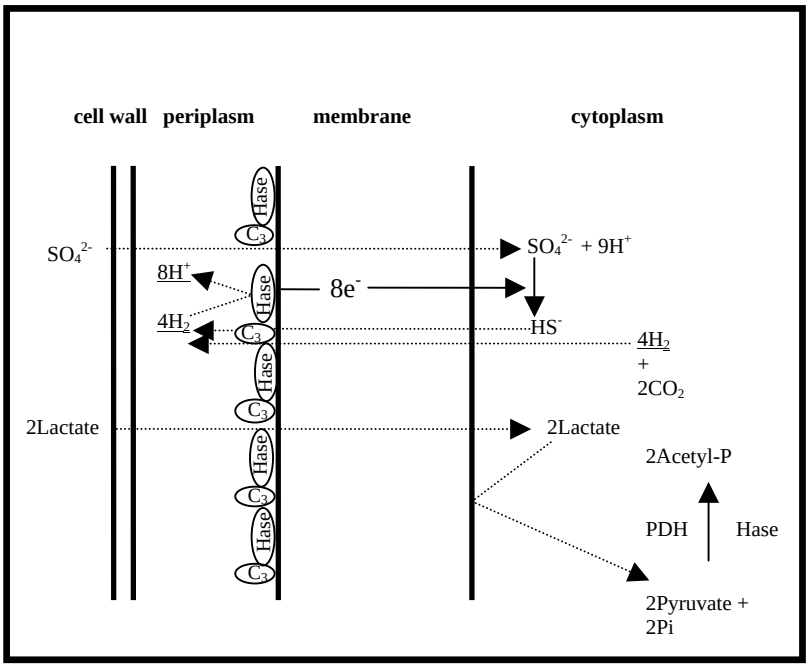


Figure 1.4: Hydrogen cycling in *Desilfovibrio vulgaris* [C_3=Cyrochrome C_3; Hase=Hydrogenase; PDH = pyruvate dehydrogenase] (Odom and Peck, 1984).

Odom and Peck (1984), proposed a scheme of bioenergetics mechanism in SRB which suggests that intracellular hydrogen cycling accounts for energy coupling occurring during the growth on organic substrates (such as lactate) (Figure 1.4). In this mechanism cytoplasmically produced molecular hydrogen from the organic substrate diffuses across the cytoplasmic membrane, followed by oxidation by the periplasmic hydrogenases coupled to vectorial electron transfer across the cytoplasmic membrane, which is used for the reduction of APS and involves the consumption of protons. On the other side of the scheme, lactate enters the cell and is oxidized to pyruvate by lactate dehydrogenases located on the internal aspects of the cytoplasmic membrane or cytoplasm (Ogata *et al*., 1981; Stamps and Hansen, 1982). Electrons from the oxidation are utilized to reduce protons to hydrogen. Pyruvate is then converted to carbon dioxide, acetyl phosphate, and lower potential electrons that are also used to reduce protons to molecular hydrogen. This segment of the scheme requires an internal hydrogenase (Odom and Peck, 1981) and

necessitates that electron transfer leading to the production of hydrogen to sulphate be entirely separate from electron transfer leading to reductases involved in respiratory sulphate reduction. The cytoplasmically produced hydrogen diffuses rapidly across the cytoplasmic membrane and in the presence of electron acceptors such as sulphate, the hydrogen is not lost but is reoxidised to protons and electrons by periplasmic hydrogenase and its cofactor cytochrome c_3 (Amber, 1987). The electrons are transferred across the cytoplasmic membrane leaving protons at the external surface of the membrane. This establishes the proton gradient and the energy that can be utilized for transport and the synthesis of ATP.

1.6 Hydrogenases

Microbial H_2 formation is catalyzed by either nitrogenase or hydrogenase enzymes that can only function under anaerobic conditions (Ghirardi *et al*., 2000). The first hydrogenase was described by Stephenson and Stickland (1931), as a bacterial enzyme that catalyzed the reversible oxidation of hydrogen to protons and electrons according to the following reaction:

$$H_2 \leftrightarrow 2H^+ + 2e^- \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 3$$

Most, if not all, known hydrogenases can catalyze the reaction in either direction *in vitro*, they are usually committed to catalyze either hydrogen uptake or evolution *in vivo*, depending on the demands of the host organism (Vignais *et al*., 2001). The importance of these enzymes in anaerobic life is now legend, for they allow fermentative anaerobes to use protons in water as electron acceptors, producing molecular hydrogen which is an ideal substrate for anaerobic respiration by methanogens, acetogens and SRB.

1.6.1 Classification

The majority of hydrogenases are metalloenzymes, and their metal sites belong to two main categories. One of these consists of the classical [2Fe-2S], [3Fe-4S], and [4FE-4S] iron sulphur clusters. These ubiquitous metal sites shuttle electrons between the H_2 –

activating site and the redox partners of hydrogenases (Beinert, 2000). The second types of metal clusters are the H_2-activating sites, which are idiosyncratic to hydrogenases. They come in two varieties, the [NiFe] and [Fe] active sites (Volbeda *et al.,* 1995; Nicolet *et al.,* 1999). Crystal structures have unveiled the general fold and details of the catalytic sites of several hydrogenases of the [NiFe] (Volbeda *et al.,* 1995, Volbeda *et al.,* 1996; Higuchi *et al.,* 1999) and [Fe] (Adams and Stiefel., 1998; Nicolet *et al.,* 2000) classes. Currently hydrogenases are classified into three classes: the [NiFe]-hydrogenases, the [Fe]-hydrogenases and metal-free hydrogenases. The vast majority of known hydrogenases belong to the first two classes, and over 100 of these enzymes have been characterized genetically and/or biochemically.

1.6.1.1 [NiFe]-Hydrogenases

The first Ni-containing hydrogenases were isolated as αβ heterodimers with small (α) and large (β) subunits (Figure 1.5). The X-ray structure of the [NiFe]-hydrogenases from *Desulfovirio gigas* (Volbeda *et al.,* 1995) and *Desufovibrio vulgaris* (Higuchi *et al*., 1999) showed that the two subunits interact very extensively through a large contact surface and form a globular heterodimer. The bimetallic NiFe center of the active site is located in both subunits and is deeply buried inside the protein. The small subunit contains up to three Fe-S clusters, which conduct electrons between the H_2-activating center and the physiological electron acceptor or donor of hydrogenase (Figure 1.5).

The [4Fe-4S] cluster that is proximal to the active site is essential to H_2 activation in [NiFe]-hydrogenases (Volbeda *et al.,* 1995; Fontecilla-Camps *et al*., 1997). Hydrophobic channels expanding through both subunits have been identified by crystallographic analysis of xenon binding and molecular dynamics simulations of xenon and H_2 diffusion within the interior of the enzyme (Montet *et al.,* 1997). Those channels linking the active site to the surface of the molecule were suggested to facilitate hydrogen gas access to the active site (Fontecilla-Camps *et al.,* 1997). From the comparison of the conserved sequence elements in the nickel binding regions of 16 [NiFe]-hydrogenases, Voordouw (1992), divided these enzymes into five subgroups. Subsequently, Wu and Mandrand (1993) proposed a more elaborated classification which divides these enzymes into four

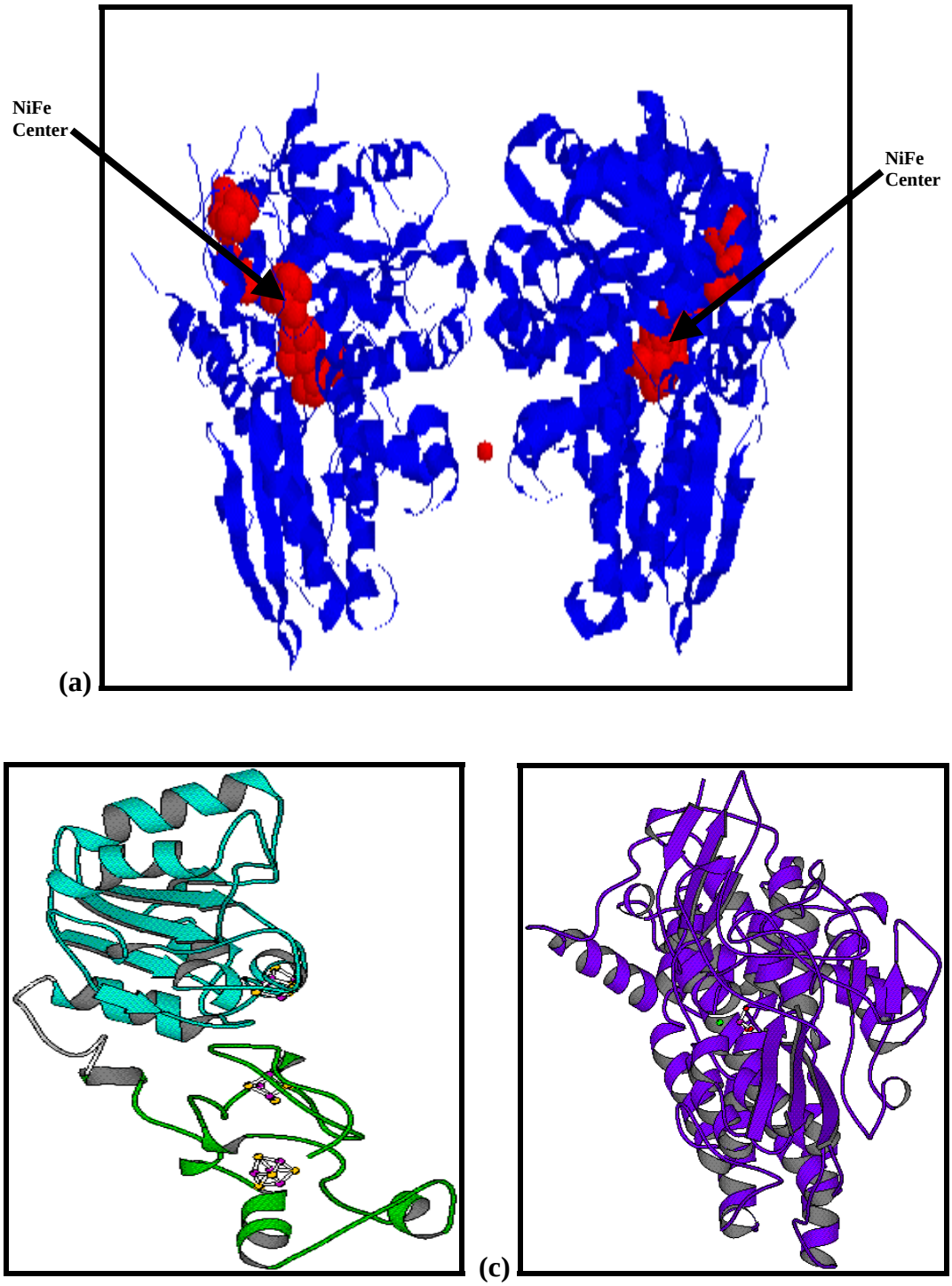


Figure 1.5: (a) Ribbon representation of the *Desulfovibrio gigas* Ni-Fe hydrogenase structure (PDB code 1HFE). Colour codes: NiFe center is displayed in space fill model and is coloured red. (b) The small subunit (α-subunit) only. Colour codes: N-terminal I_S domain = cyan; C-terminal II_S domain = green. (c) The large subunit (β-subunit).

classes based on the amino acids sequence of 30 microbial hydrogenases using FASTA and RDF2 programs: H_2-uptake membrane-bound hydrogenase, H_2-uptake NiFe(Se)-hydrogenases from SRB, anaerobic periplasmic Fe-hydrogenase, and F_{420}, MV-and NAD reducing soluble NiFe(Se)-hydrogenases. Recently Vignais *et al.* (2001) divided these enzymes into four groups: membrane–associated respiratory uptake [NiFe]-hydrogenases, cytoplasmic heterodimeric [NiFe]-hydrogenases, cytoplasmic heteromultimeric reversible [NiFe]-hydrogenases, and the membrane-associated H_2-evolving respiratory [NiFe]-hydrogenases.

1.6.1.2 [Fe]-Hydrogenases

Unlike [NiFe]-hydrogenases, which are composed of at least two subunits, many [Fe]-hydrogenases are monomeric and consist of the catalytic subunit only (Figure 1.6a). However, dimeric, trimeric (Verhagen *et al.,* 1999) and tetrameric enzymes have also been reported (Malki *et al.,* 1995). The catalytic subunits of [Fe]-hydrogenases, in contrast to those of the [NiFe] enzymes, display considerable variations in size. In addition to a conserved domain of approximately 350 residues containing the active site [H-cluster, (Adams, 1990; Nicolet *et al.,* 1999)], they most often comprise additional domains which accommodate Fe-S clusters and which may altogether consist of more than 800 residues.

The first [Fe]-hydrogenase sequences (Meyer and Gagnon, 1991; Horner *et al.,* 2000) revealed modular structures composed of previously known domains and of a unique and very conserved C-terminal domain. The latter was therefore assumed to contain the H-cluster, and was subsequently found to be present in all [Fe]-hydrogenase sequences (Meyer and Gagnon, 1991; Florin *et al.,* 2000). The three dimensional structures have confirmed the modular structure of [Fe]-hydrogenases and revealed the unique protein fold of the H-cluster domain, its high conservation, and its unprecedented metal cluster. The latter consists of a binuclear iron site bound to a [4Fe-4S] cluster by a bridging cysteine ((Figure 1.6b) (Peters *et al.,* 1998; Nicolet *et al.,* 1999)). Totally conserved amino acids include the four cysteine ligands of the active site cavity, as well as those suspected to be involved in the H_2 and H^+ channels connecting the buried active site to the surface of the protein (Peters *et al.,* 1998; Nicolet *et al.,* 2000).

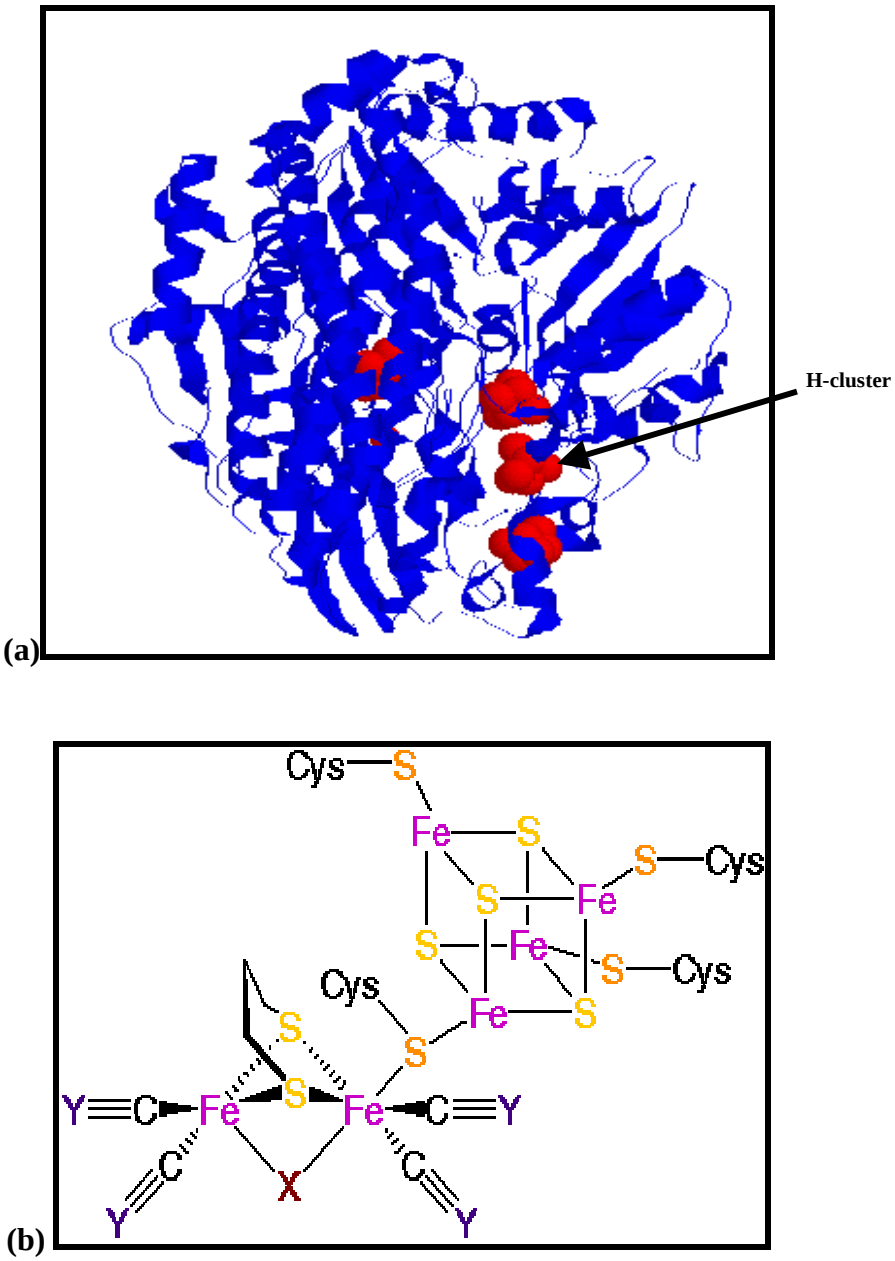


Figure 1.6: (a) Ribbon representation of the *Desulfovibrio gigas* Fe-only hydrogenase structure (PDB code 1H2A). H-cluster is displayed in space fill model and is coloured red. (b) Classical [Fe_4S_4] subcluster in H-cluster bridged via cysteine thiolate to the [Fe_2] (binuclear iron subcluster) of the active domain of Fe-only hydrogenase.

Many [Fe]-hydrogenases possess, in addition to the H-cluster domain, an N-terminal domain homologous to the 2[4Fe-4S] bacterial ferredoxins. Significant examples include the two short (ca. 50 kDa) monomeric *Trichomonas vaginalis* (T. *vaginalis*) hydrogenases (Bui and Johnson, 1996), and the *Megasphaera esldenii* hydrogenases (Atta and Meyer, 2000). Some [Fe]-hydrogenases having the same structural frameworks

are dimeric. The clostridial type [Fe]-hydrogenases (ca. 64 kDa) are significantly larger (Adams, 1990; Meyer and Gagnon, 1991), and contain three domains in addition to the H-domain: starting from the N-terminus, a [2Fe-2S] plant ferredoxin-like domain (Peters *et al.,* 1998; Atta *et al.,* 1998), a unique [4Fe-4S]-containing fold, and a 2[4Fe-4S] domain (Peters *et al.,* 1998). A recently sequenced gene from *T. vaginalis* (Horner *et al.,* 2000), encodes a clostridial-type monomeric hydrogenase of 64 kDa (Payne *et al.,* 1993). The catalytic subunit of the tetrameric NADP-reducing hydrogenase from *D. fructosorovorans* also has the same size and domain composition as clostridial hydrogenases (Malki *et al.,* 1995).

In keeping with their occurrence in very diverse organisms endowed with a wide range of metabolic capabilities, [Fe]-hydrogenases are very versatile with respect to electron donors and acceptors. The small hydrogenases from the green algae *S. obliquus, C. reinhardtii and C. fusca* are devoid of accessory domains (Florin *et al.,* 2000). In these organisms the electrons for H_2 evolution are provided by the fermentative metabolism, fed via the plastaquinone pool into PS I, which in turn reduces the [2Fe-2S] ferredoxin. The latter protein has been shown by inhibition studies to be the physiological electron donor to [Fe]-hydrogenases (Florin *et al.,* 2000). The periplasmic dimeric [Fe]-hydrogenase from *Desufovibrio* reduces cytochrome c_3 (Morelli *et al.,* 2000). The tetrameric cytosolic [Fe]-hydrogenases from *D. fructosovorans* reduces NADP (Malki *et al.,* 1995).

1.6.1.3 Metal free Hydrogenases

Much less is known about this class than the other two. A metal-free hydrogenase has been discovered in some methanogens (Hartmann *et al.,* 1996; Hagemeier *et al.,* 2000). This enzyme is a homodimer encoded by a monocistronic gene (Thauer *et al.,* 1996; Thauer, 1998), and is described as an H_2-forming methylenetetrahydromethanopterin dehydrogenase (Hmd) (EC 1.12.99.4). Hmd catalyses the reversible reduction of N^5, N^{10}-methylenetetrahydromethanopterin (methenyl-H_4MPT^+) with H_2 to N^5, N^{10}-methelynetetrahydromethanopterin (methylene-H_4MPT). The mechanism of H_2 formation has been analyzed using hydrogen isotopes (Klein *et al.,* 1995) and

stereoselective hydride transfer by 2D NMR spectroscopy (Schleucher *et al.*, 1994). Together with the F_{420}–dependent methylenetetrahydromethanopterin dehydrogenase (Mtd), Hmd catalyses the reduction of F_{420} to H_2 (Thauer *et al.*, 1996; Thauer, 1998; Reeve *et al.*, 1997). Both enzymes are induced during growth under conditions of nickel deprivation (Afting *et al.*, 1998). Recently it has been demonstrated that under Ni-limited growth, Hmd synthesis is increased 6-fold in *Methanothermobacter marburgensis* (Afting *et al.*, 2000), while the synthesis of F_{420}–reducing hydrogenase (Frh) is 20-fold lower than in cells grown on nickel replete medium (Adams and Stiefel, 1998). The presence of an organic cofactor in these metals free hydrogenases has recently been reported (Buurman *et al.*, 2000).

1.6.2 Role of hydrogenases in bioreduction of metals

Conventionally metal ions have been removed from effluent streams by increasing the pH of the solution by addition of chemical ligands (such as lime, limestone, sulphide etc.). In contrast enzymatic processes can generate metal-desolubilising ligands on a continuous basis with extracellular or intracellular deposition of metals as a metal-ligand precipitate. This has advantages compared with the addition of ligands directly to the metal solution. Metal reduction usually results in the precipitation of low valence, reduced, forms of metals, and has therefore been proposed as a strategy to treat water contaminated with a range of metals and radionuclides. A mechanism for involvement of hydrogenases in metal reduction has been proposed in SRB (Figure 1.7). Under the right strictly anaerobic conditions, SRB can strip a metallic compound back to a base metal via hydrogenase activities. Protons generated from oxidation of molecular hydrogen by hydrogenases are used for metabolic activities of the cell while the electrons released are channeled away to electron acceptor through electron transport chain. Ordinarily this electron acceptor is sulphate ion, but if these bacteria are presented with certain metal ions, they will use these as the electron dump or sink, leading to the reduction of the base metal, which is then precipitated from solution (Pereira *et al.*, 1996; Macaskie *et al.*, 2001).

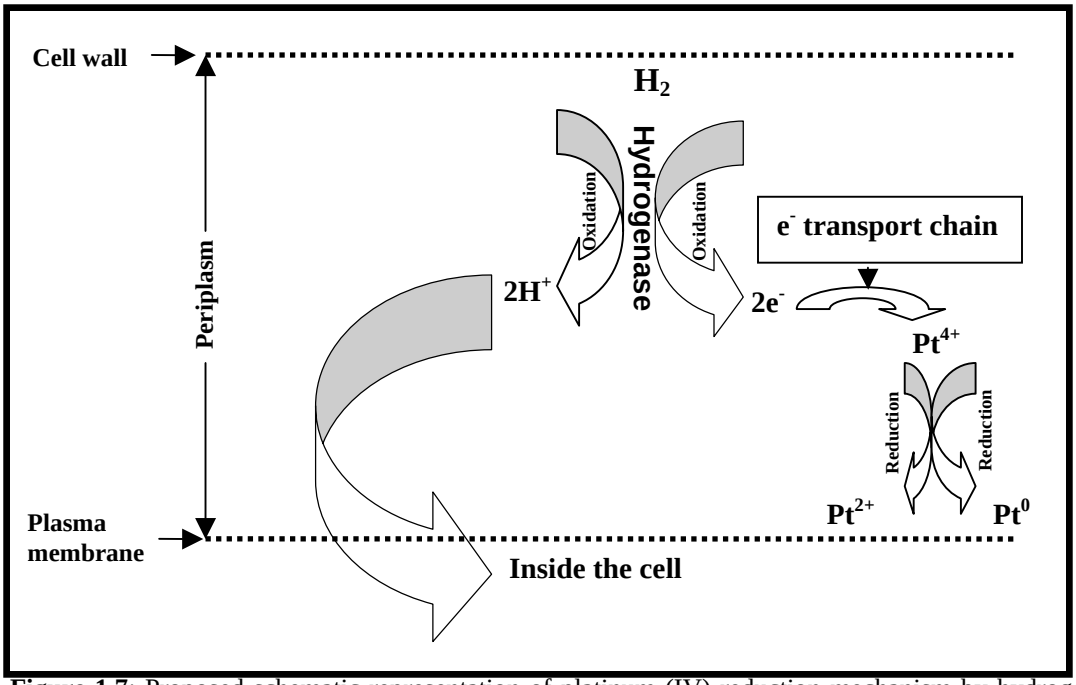


Figure 1.7: Proposed schematic representation of platinum (IV) reduction mechanism by hydrogenase in sulphate reducing bacteria.

1.7 Platinum

Platinum (Pt) together with palladium (Pd), iridium (Ir), rhodium (Rh), ruthenium (Ru) and osmium (Os) collectively constitute a group of elements known as platinum group metals (PGMs) (Cawthorn, 1999a). Platinum group metals occur together in nature although their relative abundance varies. They are amongst the least abundant of all metals on earth and form an important group of increasing usage in technological developing world. Platinum is far the most important and widely used member of the group. It has received world attention as it offers the dual attraction of rare, high value precious metal as well as having major industrial uses (McDonald *et al.*, 1995).

1.7.1 Economic importance

South Africa has more than 80% of the world platinum reserves, and is the largest world producer of PGMs, with current production of the half the world requirements of these metals (Ashwertn *et al.*, 1999). Platinum as ore deposits is restricted to large layered igneous intrusions, of which the Bushveld Complex in South Africa is by far the largest, possibly the most spectacular and certainly the most valuable in hosting the largest platinum reserve. The mineralization is restricted to two stratigraphic layers known as

Merensky Reef and upper group 2 (UG2) chromatite (Cawthorn, 1999a; b; c). On the earth's crust platinum usually occur in native forms associated with one or more of the other PGMs along with gold, silver and nickel (Wagner, 1995; Cawthorn, 1999c). The use of platinum and its alloys in industries is mainly related to its extraordinary catalytic properties (Okafor and Coville, 1999). Large amounts of platinum are used in the chemical industry (manufacturing of sulphuric and nitric acid), the refining of petroleum (reformation of hydrocarbons), and electronics industry (making electric contacts and electrodes) (Brugman, 1971). In medical research, platinum has been the main focus in the treatment therapy for cancer utilizing Cisplatin and related compounds (Neuse, 1999). One of its major industrial uses is in the manufacturing of mobile catalytic converter systems. These converters reduce the amount of exhaust gases of harmful carbon monoxide and oxides of nitrogen into harmless carbon dioxide and nitrogen (Lee, 1991). Because of its aesthetic qualities and a permanent luster, platinum is also used in manufacturing of jewellery which accounts for one third of the platinum market (Collins, 1986).

1.7.2 Chemical and physical properties

Platinum belongs to group VIII of the periodic system, with an atomic number of 78 and a molecular weight of 195.08. It is a relatively soft grey-white metal, which in addition to being exceedingly malleable and ductile, is practically infusible and unoxidesable. Its unique properties, such as a high melting point (1772 ^{o}C), high boiling point (4170 ^{o}C) high density (21.45 kg.m^{-3}), strength (4.3 Mho's scale) and toughness as an alloy, make this element a first and foremost high technology metal. It is irreplaceable in several key industrial processes and hence of great strategic importance (Rao and Reddi, 2000). It is a good conductor of electricity and heat, and has a lower coefficient of expansion than any other metal. Platinum is capable of absorbing large quantities of hydrogen and other gases, the gases thus occluded then becoming more active, and for this reason platinum is used as a catalyst. The principal oxidation states of platinum are (0), (+2) and (+4), with a highest oxidation sate of +6 (Hartley, 1973). Platinum binds to a large number of ligands (ions and neutral molecules), some of which have more than one binding site, to form neutral or charged complexes or salts. The divalent compounds are predominately four

coordinate and square planar; the tetravalent compound six coordinate and octahedral and the zerovalent compounds four coordinate and tetrahedral (Hartley, 1973). Halogen- and nitrogen donor ligands are common, but in the divalent oxidation state platinum readily forms complexes with ligands containing donor atoms from most groups of the periodic table. Several of these chemicals exist as *cis* and *trans* isomers and the geometric arrangement is of great importance in biochemical processes (Kanematsu, 1990; Uno and Morita., 1993).

1.7.3 Toxicity and environmental impact

It is known that the metallic form of this element is inert as far as biological reactions are concerned, but that in contrast some of the compounds such as the hexachloroplatinate and tetrachloroplatinate complexes are highly potent sensitizing substances causing asthma and rhinoconjunctivitis (Merget, 1999). Platinum has been found to be toxic to aquatic life, including the water flea *Daphnia magna* (Lusting, 1997), the freshwater worm *Valriegatus lubriculus* (Veltz *et al.,* 1996) and the marine bacterium *Photobacterium phosphoreum* (Wei and Morrison, 1994). Platinum is also recognized to be toxic to humans (Lindell, 1997) and an increase might affect aquatic ecosystems as well as humans via the food chain (Vaughan and Florence, 1992). In addition, some platinum complexes bind to nitrogen and sulphur in proteins producing a possible reduction in essential enzymatic activity (Wei and Morrison, 1992). The problem created by the use of this car catalyst is that portions of the PGM are released from the catalyst surface and they are spread and accumulated in the environment (Palacios *et al*., 2000). The PGM levels have increased in several environmental compartments as a consequence of automobile catalyst emissions. At present, the focus of concern for ecological and human health is the lack of understanding of PGM transformations and potential bioaccumulation in the environment (Motelica-Heino *et al*., 2001).

1.8 Hypothesis, Aims, and Objectives

1.8.1 Hypothesis

On the basis of broad metal reductase activity via hydrogenase, SRB can reduce platinum enzymatically.

1.8.2 Aims

Aims of this study are to investigate the growth of SRB and their efficacy in removal of platinum from solution and ultimately as a commercial perspective to develop a low cost bioprocess for wastewater treatment, with an intention of producing an integrated biological system that can remove platinum with the minimum number of processing steps. The choice of metal (platinum) was made with regard to its increased industrial uses and recent reports of platinum as a potential environmental pollutant.

1.8.3 Objectives

(i) To optimize growth conditions for SRB, that will support high rate platinum removal.

(ii) To investigate the role of both growing and resting SRB cells in facilitating platinum removal.

(iii) To observe the effect of platinum on cell morphology, its distribution and location within the cell using electron microscopy and energy dispersive X-ray microanalysis techniques.

(vi) To isolate, purify and characterize hydrogenase and investigate its role in platinum reduction *in vitro*.

(v) To test the hypothesis (and/or mechanism) of enzymatic platinum removal from industrial effluent.

CHAPTER TWO

Platinum (IV) Uptake from Solution by a Sulphate Reducing Consortium: Batch Studies

2.1 Introduction

Sulphate-reducing bacteria are economic, environmental and biotechnological important organisms. They have a number of features that make them attractive as a model biotreatment process. For example, several species of SRB have been identified for their ability to reduce inorganic aqueous ions in solution (Tebo and Obraztsova, 1998). Some SRB such as *Desulfovibrio desulfuricans* can couple the oxidation of H_2, or simple organic acids such as lactic acids to metal reduction (e.g. Fe (III), U (VI) (Lovely *et al*., 1993); Cr (VI) (Lovely *et al*., 1991); Tc (VII) (Lloyd *et al.,* 1998a); Mo (VI) (Tucker *et al.,* 1998); and Pd (II) (Lloyd *et al.,* 1998b), in lieu of sulphate. Generally however, they are unable to couple their growth to metal reduction. Similar non-energy conserving metal reductase activities have also been attributed to the closely related organisms *Desulfovibrio vulgaris*. To date (2002), only one SRB can conserve energy through metal reduction-a newly isolated strain of *Desulfomaculum reducens* isolated from a heavy metal contaminated sediments and which is able to grow with Cr (VI), Mn(IV), Fe(III) and U(VI) as electron acceptor (Tebo and Obraztsova, 1998). Models for the SRB reduction of these metals and radionuclides can be used to develop and design treatment systems employing SRB for bioremediation.

Several SRB based technologies have been successfully developed to treat heavy metal contaminated effluents; these include the Paques THIOPAQ system (Barnes *et al.,* 1991, 1992), the NTBC (now Biomet Mining Corporation) biosulphide process (Rowley *et al.,* 1997) and Budel-Dorplein in the Netherlands (Barnes, 1991). The third process uses a 9 m^3 reactor containing an undefined sulphate reducing community, supplemented with ethanol. Metals were removed to sub-parts per billion (ppb) levels and the process was subsequently expanded to commercial scale using an 1800 m^3 concrete bioreactor. In these processes hydrogen sulphide produced by the SRB is used to precipitate metals as

metal sulphides. In the biosulphide process, the biological sulphate reduction occurs in a reactor separate from the metal precipitation with H_2S. This is done to avoid inhibition of SRB growth by heavy metals and to facilitate metal separation and recovery. These studies demonstrate that SRB can be used in stable efficient processes on a large scale. In addition to removing metals as insoluble metal sulphide precipitates, SRB have additional important roles in facilitating metal uptake. It has been demonstrated that SRB themselves facilitate metal uptake by binding metal ions directly in their cell walls (Baldwin and Jalali, 2000). The extracellular polymers in the cell wall of SRB have also been shown to bind metal ions (Geesey and Jang, 1989; Gadd *et al.*, 1999; Gadd and White, 1998; 2000). In addition, membrane uptake of metal ions and enzymatic processes for reduction of metals has also been reported in SRB (Macaskie *et al.*, 1994, 2001; Tebo *et al.*, 2001).

Microbial processes for bioremediation of metal ions from waste streams employ living cells, non-living biomass or biopolymers as biosorbents (Gadd, 1992; Macaskie and Dean, 1990; Volesky and Holan, 1995). The mechanism of metal ion uptake has been found to vary according to the metal ion in question, the nature of biomass and whether the process is metabolically driven or not (Tsezos and Volesky, 1982; Avery and Tobin, 1993). Despite the quite extensive literature available on metal–microbe interactions, few authors have attempted to relate differing mechanisms and/or relative levels of metal uptake or toxicity to the chemical characteristics of the metal under investigation. Some reviews and reports on metal-microbe interactions have considered the Pearson's principle of hard and soft acids and bases (HSAB) as a means of predicting microbial metal uptake behavior (Pearson, 1963; 1968; Gardea-Torresdey *et al.*, 1990; Gadd, 1992; Hughes and Poole, 1997). Very few studies, however have examined the validity of this principle in a biological context. This principle predicts that hard metals bind preferentially to hard ligands, whereas soft metals form more stable bonds with soft ligands (Table 2.1; Pearson, 1963).

Table 2.1: Classification of hard and soft acids (Pearson, 1963; Wood and Wang, 1983)

Hard acceptor	**Intermediate**	**Soft acceptor**
H^+, Na^+, K^+, Be^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Al^{3+}, Cr^{3+}, Co^{3+}, Fe^{3+}, As^{3+}	Fe^{3+}, Co^{2+}, N^{2+},Cu^{2+}, Zn^{2+}, Pb^{2+}	Cu^+, Ag^+, Au^+, Ti^+, Hg^{2+}, Pt^{2+}
Soft donor	**Intermediate**	**Soft donor**
OH^-, F^-, Cl^-, PO^{3-}, SO_4^{2-}, CO_3^{2-}, O^{2-}	Br^-, NO^{2-}, SO_3^{2-}	SH^-, S^{2-}, RS^-, CN^-, SCN^-, CO,

A refinement of this classification for biological systems was described by Nieboer and Richardson, (1980) who considered the Pauling electronegativity, the charge and the ionic radius of the donor or acceptor molecule in determining their relative class A (hard) or class B (soft). Some metals, borderline (also not as intermediate), do not fall clearly into either class and can bind both hard and soft ligands. This principle predicts that hard metals, which are generally nontoxic and often essential micronutrients for microbial growth, bind preferentially oxygen containing (hard) ligands, whereas soft metals which often display great toxicity, form more stable bonds with nitrogen-or sulphur containing (soft) ligands. Thus, no sequence of relative metal affinities for any particular ligand will be universal for all ligands.

During this study a mixed SRB culture was utilized, because a mixed culture offers several advantages over a pure culture for environmental biotechnology. A mixed culture is intrinsically less liable to contamination from other organisms. In addition a mixed culture is able to adapt to minor changes in conditions because it comprises a number of populations with varying optima for culture variables e.g. nutrient concentrations, temperature, redox potential and pH (Gadd and White, 1996a; b; Song *et al.*, 1998). Furthermore in an operational biotreatment scheme, purity of culture in treating large volumes of effluent will be difficult to maintain (Spear *et al.*, 2000, Peyton et al., 2001) and understanding platinum uptake by SRB in a consortium is likely to be far more relevant for an operational condition. The objectives of this section were:

(i) To compare platinum uptake kinetics in the presence viable and non-viable SRB cells.

(ii) To investigate the effect of parameters such as initial platinum concentration, pH and temperature on platinum uptake by SRB.

(iii) To gain understanding of the range of different electron donors that could support high rate platinum uptake.

(iv) To determine maximum platinum uptake capacity by SRB biomass.

(v) To assess the extent to which the interaction of platinum with SRB could be accounted for by the hard and soft acids and bases principle.

2.2 Materials and Methods

2.2.1 Materials

Analytical-grade reagents were used except where stated otherwise. Gases were purchased from Afrox (South Africa); Membrane disc filters were purchased from Osmonics (South Africa); Potassium hexachloroplatinate (IV), [Cl_6K_2Pt (IV)] was purchased from Fluka; Platinum atomic absorption spectroscopy standard solution was purchased from Wirsam Scientific. All other reagents were purchased either from Sigma or Merck (South Africa). All glassware used for all experiments performed were pre-washed in 15% nitric acid to eliminate the interference from other metal ions and rinsed with de-ionized water (dH_2O). For pH adjustment, 1 M hydrochloric acid (HCl) and 1 M sodium hydroxide (NaOH) solutions were used as required. Culture shaking was performed on a Labcon orbital shaker. Centrifugation was achieved using a Beckman J2-21 centrifuge with a JA-20 rotor, or Eppendorf 5810R centrifuge. Absorbances were measured using a Shimadzu UV visible 160A recording spectrophotometer or PowerWave X (Bio-Tek, Instrumental INC, South Africa).

2.2.2 Methods

2.2.2.1 Analysis of metal ions

The concentrations of platinum ions in the solution were analyzed on a GBC 909 atomic absorption spectrophotometer (AAS) [(GBC 909 instrument; GBC scientific Equipment Pty Ltd, Dandenong, Australia)], with air-acetylene flame using platinum 10 mA hollow cathode lamp at 266 nm. The instrument response was periodically checked with known standards. A calibration curve was obtained with a correlation coefficient of 0.98 or greater to ensure quality measurements. The samples were read three times and the mean values and the relative standard deviations were computed.

2.2.2.2 Preparation of platinum stock solution and calibration curve

Platinum stock solution (1000 $mg.l^{-1}$) was prepared by dissolving 1 g of Potassium hexachloroplatinate (IV) in 1000 ml milli-Q water (ddH_2O). This was diluted as required and used in subsequent experiments. Standard platinum concentrations (0, 5, 10, 20, 40, 60 $mg.l^{-1}$) were prepared by making appropriate dilutions of platinum atomic absorption spectroscopy standard solution (1 mg Pt/ ml). A calibration curve of platinum concentration versus absorbance at 266 nm was generated (*Appendix A1*).

2.2.2.3 Source of the organism and culturing conditions

2.2.2.3.1 Culture Source

The organisms employed were a mixed culture of SRB, previously isolated from acid mine drainage (Grootvlei mine, Gauteng province, South Africa). The culture was maintained in a 5 L bioreactor flask containing modified Postgate medium B (*Appendix A2*), under oxygen free N_2:CO_2 (80:20) gas (referred hereafter as OFN), then incubated in the dark at 30 ^oC while shaking at 100 rpm. This culture served as stock throughout and all subsequent cultures were initiated using SRB inoculum 10 % (v/v) of this stock culture. (Descriptions of reactor set up, operational, sampling procedure, and standard anaerobic techniques used to prepare the media and maintenance of the culture used throughout the project are described in Appendix *A3*).

2.2.2.3.2 Sub-culturing and cell suspensions of stock culture

For routine sub-culturing, stock culture was sub-cultured every four weeks. Cells were harvested by centrifugation (5000 x g, 30 min, 25 ^{o}C), resuspended in (morpholinepropanesufonic acid) MOPS-NaOH buffer (20 mM, pH 7.0), then inoculated into a new medium under OFN. Lactate was included as the electron donor in all stock culturing experiments unless otherwise stated. All manipulations of the cells were carried out under OFN.

2.2.2.4 Determination of the biomass concentration

A variety of methods have been attempted to determine the concentration of bacterial cultures, including cell counts, optical density readings at 600 nm, fluorescence assays and dry mass. None of these methods however, proved accurate and reproducible mainly as result of interference from extracellular polymeric substances associated with the cells and the reactions of iron in the media with sulphide. As a result biomass concentration was estimated by weighing wet mass of stationary growth phase cells after centrifugation.

2.2.2.5 Analytical procedures

2.2.2.5.1 Modified turbidimetric method

Sulphate was assayed in triplicate using a modified turbidimetric assay as described by Kolmert *et al*. (2000). 1 ml of sample (diluted if necessary) was added to 1 ml of the conditioning reagent in a test tube and thoroughly mixed. 1 ml of 0.25 M barium chloride solution was added and the solution was vortexed for 60 sec at a constant speed. The absorbance at 420 nm ($A_{420\ nm}$) of the resulting suspension was then determined. A complete reaction mixture excluding sulphate served as a blank. Any sample with a high content of suspended matter was centrifuged (4000 x g, 10 min, 25 ^{o}C), prior to analysis since suspended matter in large amounts interfered with the method (Preparation of a sulphate calibration curve appears in *Appendix A4*).

2.2.2.5.2 Methylene blue method

Concentration of dissolved sulphide was determined in triplicate using the methylene blue assay as described by *Rees et al.* (1971). 5 ml of sample (diluted if necessary) was

added to 500 µl of 20 mM amine sulphuric acid solution and 60 mM $FeCl_3$ solution. The colour was allowed to develop (60 min) and the absorbance was measured at 670 nm ($A_{670\ nm}$). Complete reaction mixture, including dH_2O instead of sulphide, served as a blank. Occasionally Merck hydrogen sulphide Spectroquant® test kits (Merck # 1.14779) were used to determine concentrations of sulphide (Preparation of sulphide calibration curve appears in *Appendix A5*).

2.2.2.5.3 pH analysis

2 ml samples for pH analysis were placed in the test tube and the pH readings were taken using the Level 1 Inolab pH meter (WTW Ltd).

2.2.2.6 The growth of SRB in the batch bioreactor

In order to monitor metabolic activity of SRB during growth, an anaerobic batch bioreactor system containing 1 L of modified Postgate medium B was used. The reactor was inoculated with actively growing SRB stock inoculum (10% v/v), flushed with OFN for 15 min, and then incubated as previously described (Section 2.2.2.3). A sample (10 ml) was immediately taken using a disposable hypodermic syringe, for measurement of initial parameters. Sulphate, sulphide, and pH were monitored daily by the analytical methods previously described (Sections 2.2.2.5.1, 2.2.2.5.2, and 2.2.2.5.3).

2.2.2.7 Platinum uptake kinetics by SRB at different initial platinum concentrations

Cultures for metal uptake experiments were grown in modified Postgate medium C (*Appendix A6*) that contained no amount of iron in addition to citrate. This medium prevents precipitation of the FeS in the culture otherwise formed in large quantities in the modified Postgate medium B. The SRB inoculum (10% v/v) was added anaerobically (under OFN), to five bioreactor flasks containing 1L modified Postgate medium C. The cells were grown as previously described (Section 2.2.2.3), until mid stationary phase (approximately 5-6 days) and sub-cultured repeatedly until negligible iron sulphide precipitate was noted at the end of the growth phase (typically two subcultures).

Platinum uptake by resting SRB cells: Platinum uptake by SRB was conducted under non-growth or resting conditions prepared as follows; during the stationary phase, cells

were harvested by centrifugation (5000 x g, 10 min, 25 ^{o}C), washed twice in minimal volume of ddH_2O, and then resuspended anaerobically in MOPS–NaOH buffer (20 mM, pH 7.0, 100 ml) pre-equilibrated under OFN at a biomass density of 5 g (wet weight).l^{-1} in Erlenmeyer flasks sealed with butyl rubber stoppers. The cultures were further incubated (room temperature, 15 min), while being purged with OFN, to maintain strict anaerobic conditions before the addition of platinum. Cells were then challenged with Pt (IV), at different initial concentrations of 10, 25, 50, 75, and 100 mg.l^{-1}. Hydrogen was supplied as an electron donor (3.95 atm.min^{-1}) throughout the incubation period at the head space of the flasks displacing OFN. Duplicate samples (3 ml) were taken using a sterile disposable hypodermic syringe, at timed intervals, filtered through a reusable Millipore filtering system (25 mm diameter, 0.45 µm cellulose-acetate plus disc filter), and then analyzed for platinum using AAS.

2.2.2.8 Effect of electron donors for platinum uptake by SRB

To determine the optimal electron donors for platinum uptake by SRB, resting stationary SRB cells (pre-grown in lactate as an electron donor) were divided into four 200 ml volumes in 250 ml Erlenmeyer flasks sealed with rubber stoppers, washed, and resuspended under OFN in MOPS buffer (20 mM, pH 7.0, 200 ml) at a biomass density of 5 g (wet weight).l^{-1} in the presence of different electron donors (lactate, ethanol and hydrogen) at a concentration of 10 mM. Hydrogen was supplied as an electron donor (3.95 atm.min^{-1}) throughout the incubation period at the head space of the flasks displacing OFN. A control flask with no electron donor supplied was included as a reference control. To each flask 50 mg.l^{-1} of Pt (IV) was added, and duplicate samples (3 ml) were drawn at regular intervals, filtered and analyzed for platinum.

2.2.2.9 Platinum uptake kinetics with and without bacterial cells

One liter of modified Postgate medium C was inoculated with 10% (v/v) SRB inoculum, and grown as previously described (Section 2.2.2.3). Cells were harvested, washed and resuspended in MOPS–NaOH buffer (20 mM, pH 7.0, 400 ml) at a biomass density of 5 g (wet weight).l^{-1} as previously described (Section 2.2.2.7). Two 200 ml volumes were transferred under OFN to 250 ml Erlenmeyer flasks sealed with butyl rubber stoppers,

one of which was further centrifuged (5000 x g, 10 min, 25 ^{o}C; supernatant discarded), the removed cells were then killed by heat (killed cells control flask). A cell free contro1 flask contained autoclaved (121 ^{o}C, 15 min) medium only.

To heat kill the cells: 250 ml MOPS buffer (20 mM, pH 7.0) was heated to 80 ^{o}C in a water bath and maintained at this temperature for an additional 15 min after the addition of the cells and prior to the addition of Pt (IV). Air was then bubbled through the cell suspension (10 min), followed by purging with OFN (5 min) before platinum was added. In each of the three different flasks, 50 mg.l^{-1} of Pt (IV) was added and then hydrogen was supplied as an electron donor (3.95 atm.min^{-1}) throughout the incubation period at the head space of the flasks, displacing OFN. Duplicate samples (3 ml) were withdrawn at timed intervals, filtered and analyzed for platinum.

2.2.2.10 Uptake of different platinum salts by SRB

Modified Postgate medium C (1L) was inoculated with 10% (v/v) SRB inoculum, grown, harvested, washed and resuspended as described (Section 2.2.2.8). The culture was divided (2 x 200 ml) volumes in 500 ml Erlenmeyer flasks, then incubated (room temperature, 10 min) while being purged with OFN, before the addition of platinum salts. In one flask, cells were challenged with 50 mg.l^{-1} of Pt (II) as $Na_2Pt(II)Cl_4$, whereas in the other flask, cells were challenged with 50 mg.l^{-1} of Pt (IV) as $K_2[PtCl_6]$. Hydrogen was supplied as an electron donor (3.95 atm.min^{-1}) throughout the incubation period at the head space of the flasks, displacing OFN. Triplicate samples (3 ml) were taken at timed intervals, filtered and then analyzed for platinum using AAS.

2.2.2.11 Effect of inorganic ligands on platinum uptake by SRB

Stationary phase culture were harvested by centrifugation (5000 x g, 10 min, 25 ^{o}C), washed twice in minimal volume of ddH_2O, and then the pellet resuspended anaerobically in MOPS–NaOH buffer (20 mM, pH 7.0, 300 ml), pre-equilibrated with OFN at a biomass density of 5g (wet weight).l^{-1}. Resting cell suspensions (50 ml) were transferred into 250 ml Erlenmeyer flasks, sealed with butyl rubber stoppers, and then flushed with OFN (15 min, room temperature) before the addition of ligand and platinum.

Platinum (IV) concentration (50 mg.l^{-1}) and ligand (SO_4^{2-}, SO_3^{2-}, and S^{2-}) concentrations (0.2, 0.4, 0.6, 0.8, 1 mM) were then added to each respective flask. The flasks were maintained (room temperature, 3 h) while shaking at 100 rpm. Hydrogen was supplied as an electron donor (3.95 atm.min^{-1}) over the 3 h incubation period at the head space of the flasks, displacing OFN. Triplicate samples (3ml) were removed, filtered and analyzed for platinum.

2.2.2.12 Equilibrium sorption isotherm

Biomass using 10 % (v/v) inoculum was cultivated in a 5 L flask containing modified Postgate C medium. During mid stationary growth phase 10 x 500 ml volumes of the culture were dispersed in pre-weighed 500 ml Beckman centrifuge tubes under OFN. Cells were collected after centrifugation (5000 x g, 10 min, 25 ^oC). Cell free supernatant was discarded and the tubes containing biomass were reweighed. The difference between the mass of the tube and the tube containing biomass was assumed to be the mass of the SRB biomass. Cells were washed twice in ddH_2O and resuspended in MOPS buffer (20 mM; pH 7.0) in 500 ml Erlenmeyer flasks sealed with butyl rubber stoppers at a biomass density of 5g (wet weight).l^{-1}. Cells were then challenged with initial Pt (IV) concentration ranging from 10-120 mg.l^{-1} and incubated (room temperature, 3 h). Hydrogen was supplied as an electron donor (3.95 atm.min^{-1}) over the 3 h incubation period at the head space of the flasks, displacing OFN. Triplicate samples (3 ml) were then withdrawn from each flask, filtered and then analyzed for platinum. The maximum amount of platinum uptake (q_{max}) from solution by SRB biomass was calculated using general equation 4:

$$q_{max}\,(mg.g^{-1}) = (C_i - C_f)/X \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 4$$

Where q_{max} is the SRB maximum platinum uptake capacity, C_i initial platinum concentration (mg.l^{-1}), C_f is final platinum concentration (mg.l^{-1}), and X is a biomass concentration in g (wet weight).l^{-1}.

2.3 Results and Discussion

2.3.1 Control experiments

2.3.1.1 Platinum (IV) binding by different membrane filters

This experiment was performed in order to determine whether different membrane filters bind platinum during filtration, since the samples to be analyzed on AAS need to be free of particles to avoid interference with analysis. Membrane filters used in this study were nylon, nitrocellulose, cellulose acetate, and cellulose acetate plus. An initial concentration of platinum [13 mg Pt (IV).l^{-1}], was filtered through a Millipore reusable filtering system with a 25 mm diameter, 0.45 µm pore size of each of the four different disc membranes. Filtrates were then analyzed for platinum using AAS. Unfiltered sample served as a reference control. The difference between the initial concentration and the remaining concentration was assumed to be bound to a membrane filter.

Figure 2.1 shows the interaction of platinum (IV) with different membrane filters. Percentage platinum (IV) binding of 54.2%, 28.8%, 15.7% and 5.2% were observed after filtration with nylon, nitrocellulose, cellulose acetate and cellulose acetate plus respectively. These observations show very strong interaction of platinum (IV) with nylon membrane filters. High binding characteristics of the nylon to platinum could be due to the nylon structure which in addition to polymers of aminohexanoic acid, contains free carboxylic and primary amino groups that can easily interact with the complex platinum form ($K_2[Pt(Cl)_6]$), resulting in a large amount of platinum being trapped by these groups. High binding of platinum to nitrocellulose could be due to the interaction of platinum with complex cellulose nitrate and cellulose acetate polymers within the membrane, resulting in low throughput of platinum.

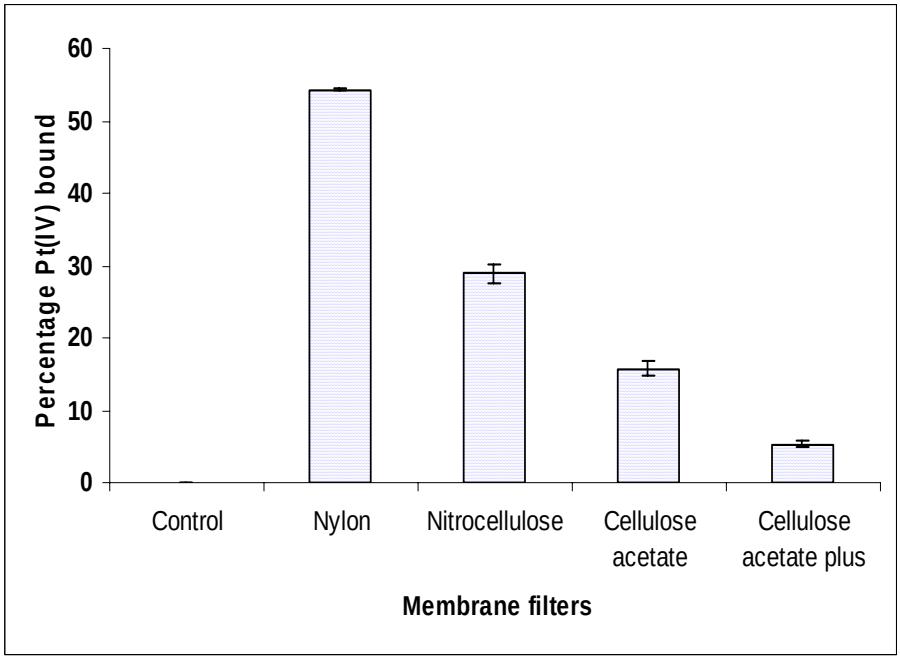


Figure 2.1: Percentage platinum (IV) bound to different membrane filters during filtration. The points are the means ± the standard deviation of triplicate values measured. The experiment was conducted at room temperature and the initial platinum (IV) concentration was 13 $mg.l^{-1}$.

In contrast cellulose acetate plus membranes showed very low binding characteristics (only 5.2%) to platinum (IV) compared to nylon, nitrocellulose and cellulose acetate membranes. Cellulose acetate plus membrane filters are composed of pure cellulose acetate polymers providing them with lowest binding characteristics and high throughput. Because of low binding capacity to platinum (IV), cellulose acetate plus membranes were used for filtering in all experiments and 5.2% binding capacity was accounted for.

2.3.1.2 Effect of pH adjustment on platinum (IV)

To determine the effect of pH on platinum (IV), an initial platinum solution (16 $mg.l^{-1}$), was adjusted to pH (2.0, 3.0, 4.0, 5.0, 6.0, 7.0) using 1 M HCl and 1M NaOH solutions. Unadjusted Pt (IV) solution at pH 2.4 served as a control. The difference between the initial concentration of Pt (IV) and the remaining concentration was assumed to be due to precipitation.

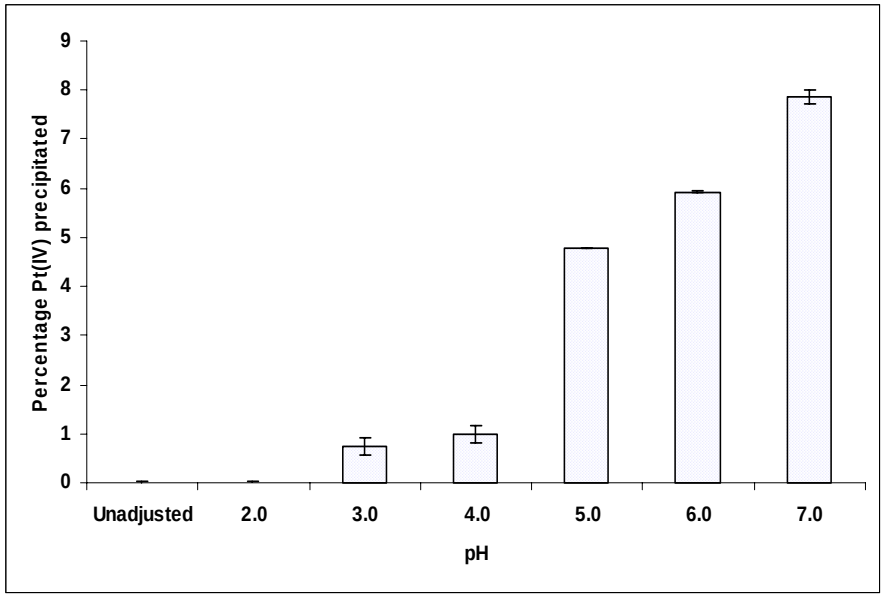


Figure 2.2: Effect of pH adjustment on initial platinum (IV) concentration of 16 mg.l^{-1} in the absence of SRB biomass. The points are the means ± the standard deviation of triplicate values measured. The experiment was conducted at room temperature and initial platinum (IV) concentration was 16 mg.l^{-1} and incubation time was 1h.

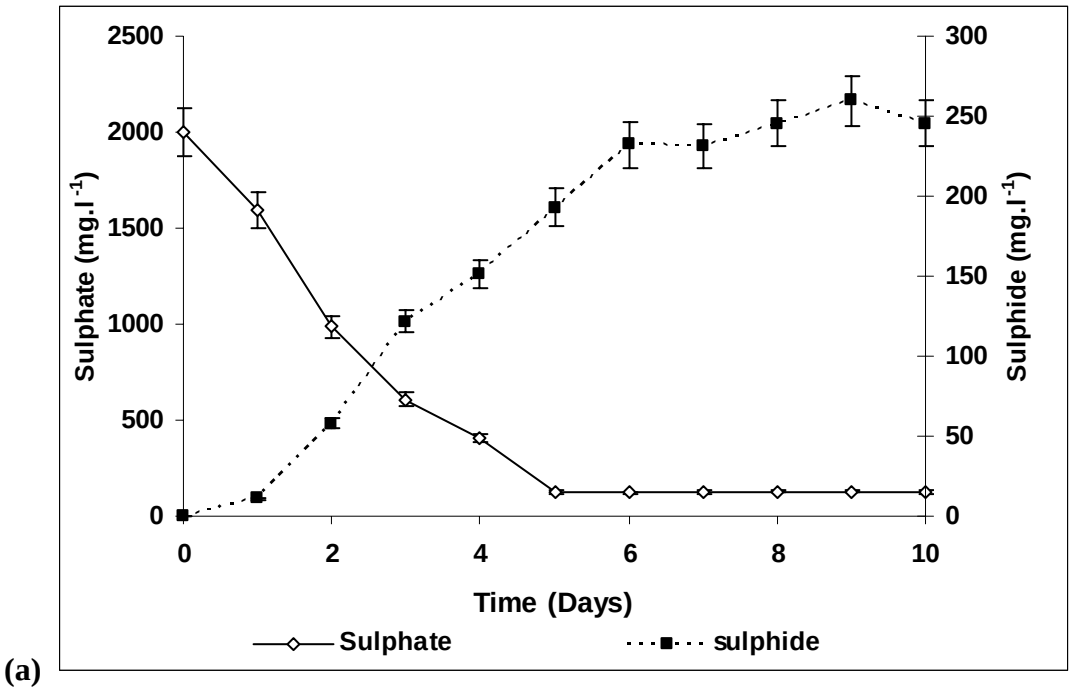
The experiment was performed in order to account for any amount of platinum that could precipitate at pH 7.0, since SRB metabolic activity have been shown to be optimal at this pH. Figure 2.2 demonstrates that the increase in pH of the solution results in increased platinum (IV) precipitation. Significant platinum precipitation occurred at pH 5.0, 6.0 and 7.0, with 4.8%, 5.9% and 7.8% platinum precipitation observed respectively. This is a low level precipitation compared to other metals such as copper (II), lead (II) and gold (III) which have been shown to precipitate in large amounts at pH 7.0 upon addition of alkali (NaOH) to the solution (Greenwood and Earnshaw, 1989; Antunes *et al.*, 2001). This suggests that the interaction of platinum (IV) with alkali solution in the absence of biomass is likely to be both covalent and ionic. According to Pearson (1968), platinum (IV) is a soft acid and is likely to bind to a hard base such as oxygen-donor ligands (OH^-), as a combination of both covalent and ionic interaction. In addition, low platinum precipitation at pH 7.0 as compared to other metals (such as Cu(II), Pb (II) and Au (III)), could be due to the fact that platinum (IV), in this study was provided as a complex compound $K_2[Pt(Cl)_6]$, such compounds will dissolve in water to give a complex ion $Pt(Cl)_6^{4+}$ and two $2K^+$ ions (Greenwood and Earnshaw, 1989; Cotton and Wilkinson, 1998). Because Cl^- ions within the platinum complex ion are securely attached to the

metal atom (Pt) they cannot be precipitated upon addition of NaOH. The free K^+ ions outside the complex however can react with the hydroxide from NaOH, increasing the pH of the solution, while not necessarily precipitating platinum. These principles explain the observations of low platinum precipitation at pH 7.0, where most metals are known to precipitate in large amounts. The amount of platinum precipitated at pH 7.0 was therefore accounted for, in all experiments performed at this pH by subtracting 7.8% from total percentage platinum removed in the presence of SRB biomass.

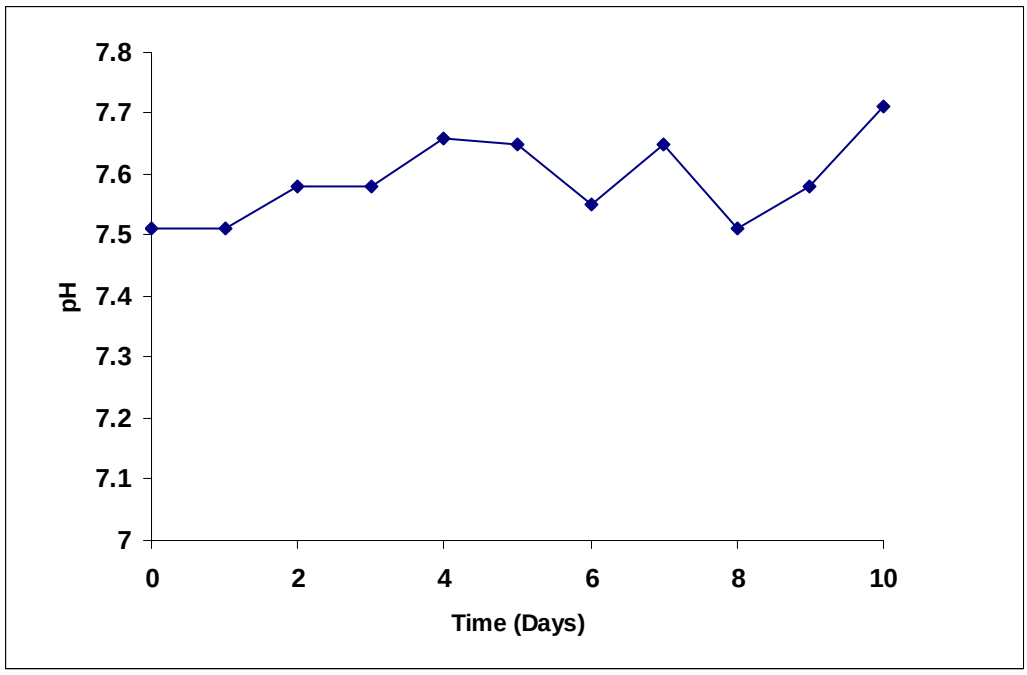
2.3.2 Batch bioreactor experiment

In order to monitor the metabolic activity of SRB, batch culture was carried out over the duration of ten days. Sulphate reduction, sulphide production and pH were monitored daily throughout the growth period. Figure 2.3a demonstrates typical sulphate reduction, sulphide production and pH profiles for SRB growth under anaerobic conditions. A rapid sulphate reduction was observed, with initial sulphate concentration of 2000 $mg.l^{-1}$ reduced to 407 $mg.l^{-1}$ within the first four days, followed by sulphate concentrations leveling off at 127 $mg.l^{-1}$ after day five (Figure 2.3a). This represents optimum sulphate efficiency of about 93.7 %, at a conversion rate of 187.3 $mg.l^{-1}.day^{-1}$. The reason for sulphate concentration leveling off after day 5 at 127 $mg.l^{-1}$ is unclear, but there are possible explanations. Firstly, since sulphate reduction by SRB depends largely on energy source (lactate in this study; equation 5) it could be possible that there was complete utilization of lactate ahead of sulphate resulting in a limitation of the energy source, hence a cessation of sulphate reduction. Secondly, it may have been a result of a large part of the energy source being used for maintenance, therefore resulting in uncoupling of sulphate reduction.

The pH data (Figure 2.3b) show that the culture pH fluctuated well above pH 7.0 throughout the growth time course, with increase in pH from 7.5 ± 0.1 to 7.7 ± 0.0. This observation reflects the dominance of SRB activity in the system. A deviation in pH range to less than 6.5 would be indicative of gross bioreactor failure. The increase in pH in a sulphate reducing system (sulphidogenic system) results from the metabolic processes involved in reduction of sulphate to sulphide.



(a)



(b)

Figure 2.3: Batch bioreactor results of SRB metabolic activity during SRB growth at 30 ^{o}C. (a) Time course of sulphate reduction and concurrent sulphide production by SRB. The points are the means of the three triplicate and the error bars are ± standard deviation of the triplicate values measured. Where error bars cannot be seen is because they are very small. (b) Corresponding pH profile during the time course.

There are two possible mechanisms whereby conversion of sulphate to sulphide can raise the pH: (i) Firstly, H^+ may be involved directly in the reactions where protons are therefore mopped up, as indicated by equation 5 below, when lactate is used as terminal electron donor. (ii) Secondly, the main mechanism of pH increase is probably the conversion of a strong acid, H_2SO_4 (which is effectively completely dissociated in dilute solutions), to a weak acid H_2S (Gadd and White, 1996a; b).

$$CH_3COO^- + SO_4^{2-} + H^+ \longrightarrow H_2S + 2HCO_3^- \quad \ldots\ldots\ldots\ldots\ldots\ldots 5$$

Sulphate reduction also marked increased sulphide production throughout the growth period, stabilizing after day 6 at 232 - 245 $mg.l^{-1}$. These sulphide levels reported in Figure 2.3a are an estimation of total sulphide produced. This is because hydrogen sulphide is in chemical equilibrium with sulphide according to equation 6:

$$H_2S \leftrightarrow HS^- + H^- \leftrightarrow S^{2-} + 2H^+ \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 6$$

The chemical equilibrium between these three sulphide species is strongly dependent on pH. At pH 8.0 most of the total sulphide (TS) is in the HS^- form, while at pH 6.0 most is in the $H_2S_{(g)}$ form (Okabe *et al.,* 1995). Modelling with the MinteqA2 software package indicates that at pH 7.0, 56.8% of the sulphide will exist as HS^- and 43.2% as $H_2S_{(g)}$ (Van Hille, 2001). Therefore it is reasonable to assume that some of the sulphide will escape as gaseous hydrogen sulphide, which could not be accounted for by the analytical technique applied in this study. The $H_2S_{(g)}$ has been found to be the major toxic form of sulphide to SRB themselves because it can pass through the cell membrane (Reis *et al*., 1991). The findings reported here show that the biological sulphate reduction was effective, indicating that SRB were active from a metabolism point of view.

2.3.3 Platinum uptake kinetics by SRB at different initial platinum concentration

Non-growth or resting cell conditions with hydrogen as an electron donor were utilized for platinum uptake throughout the study. These conditions were chosen to separate any platinum uptake from any growth related processes such as possible precipitation of platinum as platinum sulphide (via hydrogen sulphide produced during sulphate reduction). Truex *et al.* (1997) suggested that metal reduction under non-growth conditions might simulate competition for limited substrates in a natural environment. In addition 'the six-day' batch culture was used to investigate the extent of platinum uptake from solution. Six days was chosen since this was the time required for the SRB to reach stationary growth phase as observed by sulphate leveling off (Figure 2.3a). Because removal of platinum from solution by intact SRB cells is likely to involve a number of different processes, such as biosorption to the cell walls, entrapment in extracellular capsules, uptake by membrane transport, micro-precipitation and oxidation-reduction reactions, the term 'uptake' is used in this study to indicate that platinum is removed by one, or more, of these processes and it does not convey any mechanistic information.

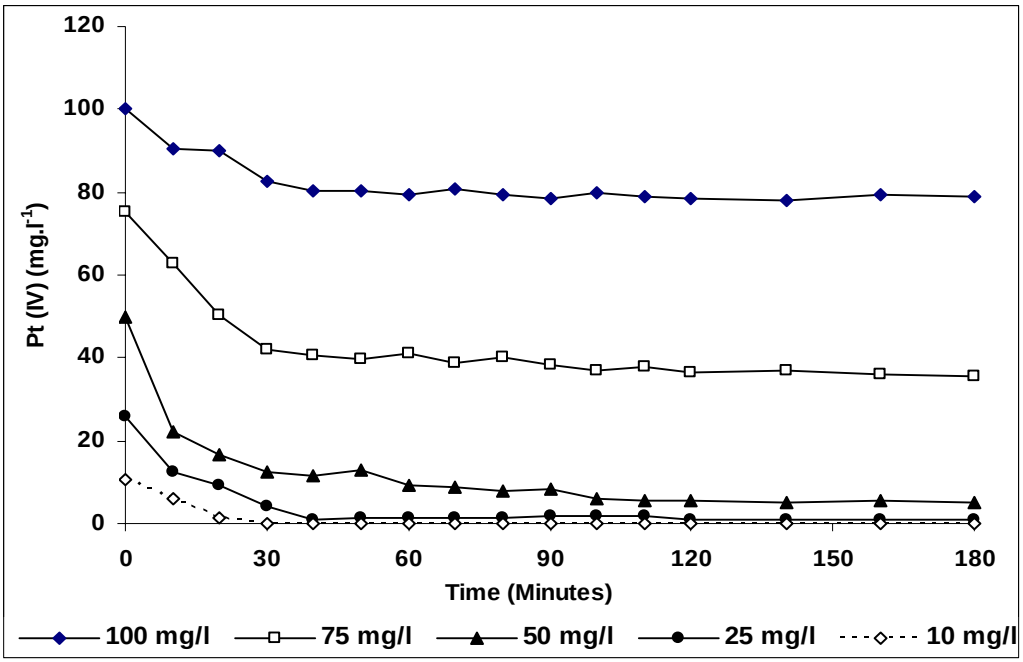


Figure 2.4: Rate of Platinum (IV) uptake from solution by a mixed SRB culture at five different initial platinum (IV) concentrations. The points are the means of duplicate values measured. The following conditions were used: Initial resting SRB cells concentration = 5 g (wet weight).l^{-1}, room temperature, pH 7.0, and hydrogen was included as an electron donor).

Uptake of different initial platinum concentrations by SRB was carried out to determine the platinum concentrations at which SRB could remove platinum effectively. Figure 2.4 shows different initial platinum uptake kinetics by SRB over time. Where initial platinum concentration was less than or equal to 50 $mg.l^{-1}$, the platinum in the solution had dropped to 5 $mg.l^{-1}$ or less after 60 min. In the flasks with initial platinum concentrations of 75 and 100 $mg.l^{-1}$, the platinum in the solution reached a steady state after 40 min at 40 and 80 $mg.l^{-1}$ respectively. As a result 50 $mg.l^{-1}$ was therefore utilized as initial platinum concentration in all subsequent experiments. This concentration was considered to be the maximum sublethal concentration for which SRB can effectively remove platinum and any concentration above this is likely to stimulate loss of bacterial viability.

2.3.4 Effect of electron donors on platinum uptake by SRB

Hydrogen, lactate and ethanol were chosen as the electron donors for platinum uptake because they have previously been shown as the electron donors for metal removal in SRB (Lloyd *et al.,* 1999) and because they are candidate electron donors for stimulating metal uptake in an *in situ* and *ex situ* remediation processes (Wildung *et al*., 2000). Equilibrium percentage platinum removals for different electron donors were 74.9%, 64.7% and 53.1% for H_2, lactate, and ethanol respectively (Figure 2.5). The control flask with no electron donor gave expected results with lowest platinum uptake of 29.9%. High percentage platinum uptake by SRB was much greater when H_2 was provided as the electron donor, compared to lactate and ethanol. This phenomenon is consistent with the findings from related studies, in which the percentage uptake of variety of metals, including Cr (VI), U (VI) and Pd (II) were consistently higher with H_2 as the electron donor (Lovely *et al*., 1991; 1993; Lloyd *et al.,* 1998a; b; Tucker *et al.,* 1998). In addition, metal uptake with H_2 as the preferred electron donor, has been linked to a mechanism involving direct enzymatic uptake catalyzed by a hydrogenase in those studies. A H_2-platinum dependent uptake observed in this study provides the first line of evidence that a hydrogenase could be involved. High hydrogenase activity in mixed SRB culture has been noted before, making them potentially useful catalysts to treat metals-contaminated effluent (Lloyd *et al*., 1998b).

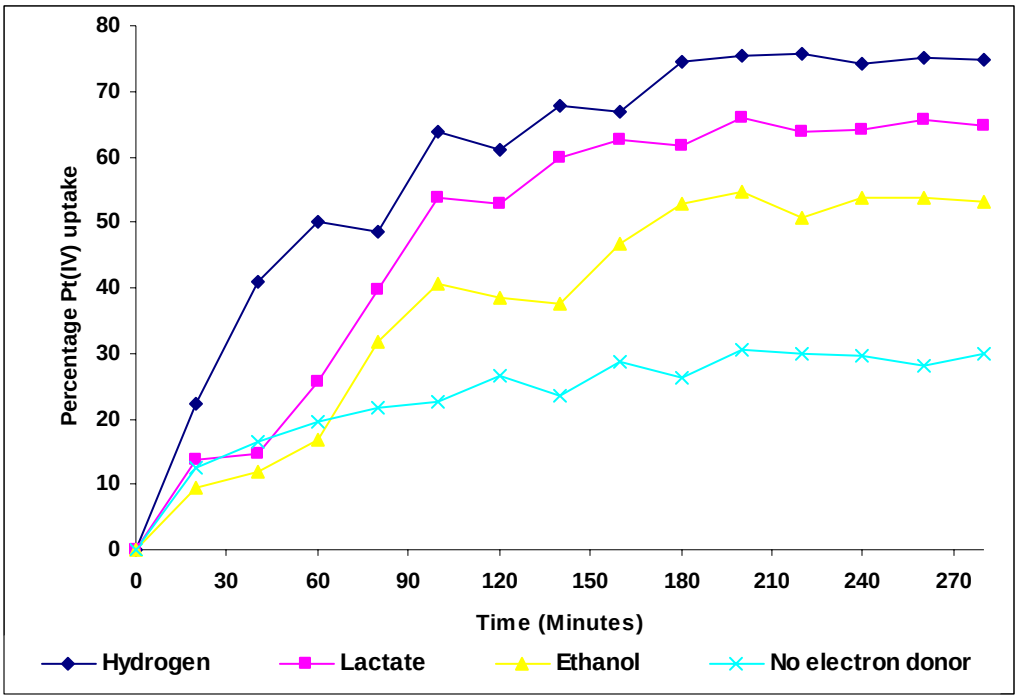


Figure 2.5: Percentage platinum (IV) uptake from solution by a mixed SRB culture under different electron donor conditions. The points are the means of duplicate values measured. (The following conditions were used: Initial resting SRB cells concentration = 5 g (wet weight).l^{-1}, room temperature, pH 7.0, Pt (IV) = 50 mg.l^{-1}).

The reason for high platinum uptake with H_2 as an electron donor, as compared to the organic electron donors (lactate and ethanol) observed in this study is still unclear, but there are several possible explanations. First, H_2 is a relatively small, diffusible molecule that can readily cross the outer membrane and enter the periplasm region, where it can be oxidized by soluble hydrogenases or hydrogenases associated with the cytoplasmic membrane. In contrast, lactate dehydrogenase is typically located in the cytoplasmic membrane or cytoplasm and transport of lactate to these sites may be rate limiting. Also, electrons from this process feed through intermediates such as nicotinamide adenosine dinucleotide reduced (NADH) before entering the electron transport chain, adding additional steps that can slow the overall process. A second possible explanation is that electrons liberated during oxidation of H_2 enter a separate and perhaps less complex electron transport chain than it is used when lactate or ethanol is the electron donor (Wildung *et al.*, 2000).

2.3.5 Platinum uptake kinetics with and without bacterial cells

This experiment was carried out to investigate the role of SRB in facilitating platinum uptake. Platinum uptake by live cells in comparison with heat killed cells and a cell-free controls over time is shown in Figure 2.6. Considerably more platinum was removed from solution in the flask with live cells compared to the control flasks (heat killed cells and cell-free flasks). After 10 min, 48% of the initial platinum concentration (50 $mg.l^{-1}$) was removed in the flask with live cells. An equilibrium uptake of 75% was reached after 90 min. The cell-free control flask showed expected results with only 4% of initial 50 $mg.l^{-1}$ removed after 3 h. In addition, heat killed cells also showed low percentage uptake of platinum, reaching equilibrium at 22% uptake after 3 h. These results strongly suggest that the presence of viable SRB cells facilitates high percentage platinum uptake from solution.

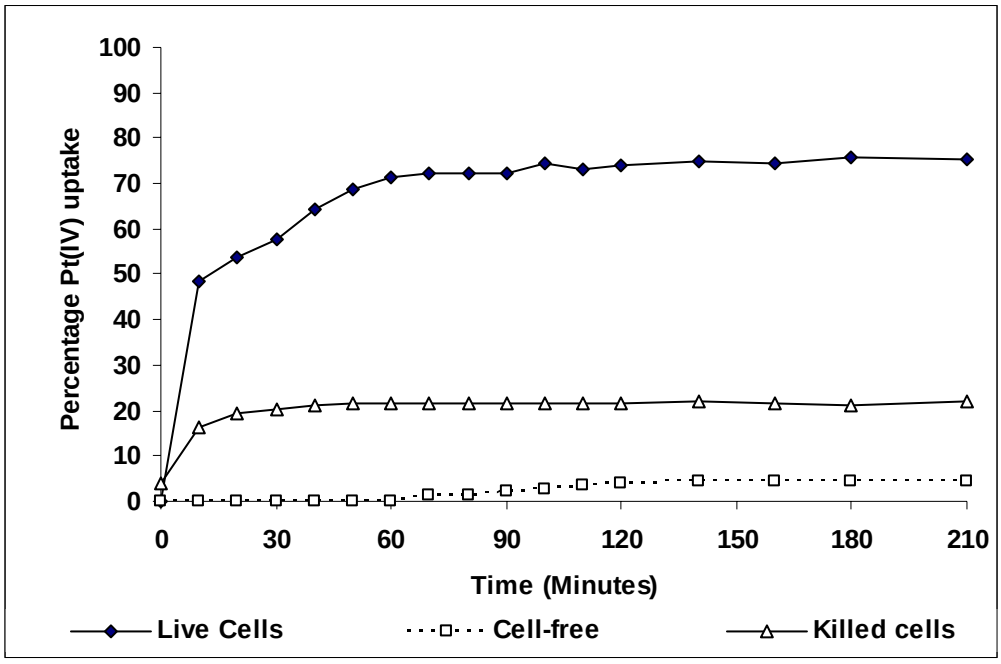


Figure 2.6: Percentage platinum (IV) uptake kinetics from solution with and without the presence of viable sulphate reducing bacteria cells. The points are the means of duplicate values measured. (The following conditions were used: Initial resting SRB cells concentration = 5 g (wet weight).l^{-1}, room temperature, pH 7.0, Pt (IV) = 50 $mg.l^{-1}$ and hydrogen was included as an electron donor).

The interaction of metal with bacterial cells normally results in either an initial rapid metal uptake by cells, which is a process believed to be metabolism-independent involving binding to the cell wall surfaces, or a slower secondary phase which is metabolism-dependent and is a process involving intracellular internalization of the metal into the cell (Hughes and Poole, 1989). Platinum uptake by live SRB cells appeared to be biphasic, in which the rapid phase was followed by the slower secondary phase (Figure 2.6). The rapid platinum uptake by live SRB cells suggests that the platinum binding ligands might be on the cell wall surface. This suggestion is further supported by the observation of platinum uptake by heat killed cells, which indicates non–active platinum uptake by the SRB biomass. This type of mechanism is associated with non-viable biomass (dead cells) in which metal ions either binds directly to functional groups on the bacterial cell walls or to anionic sites associated with extracellular polymeric substances in the cell wall. This kind of phenomenon has also been noted in the studies on biosorption of Zn (II) and Cu (II) by non available biomass of *D. desulfuricans* (Tabak *et al.,* 2000). In contrast the possibility of diffusion and internalization of platinum into the cell is also assumed to be taking place. This assumption is supported by the fact that platinum uptake in the presence of live cells was followed by typical slow secondary metal uptake between 10 and 80 min before equilibrium was reached after 90 min, indicating metabolism dependent platinum uptake mechanism.

2.3.6 Effect of temperature on platinum uptake by SRB

The optimal temperature for platinum uptake by resting SRB cells was assessed by anaerobically incubating a cell suspension with platinum over a range of temperatures (4, 25, 30, 37, 50 and 70 ^{o}C). Figure 2.7 demonstrates temperature dependent platinum (IV) uptake by SRB biomass. Platinum (IV) uptake percentages of 13.6%, 51.6%, 72.6% 68.7% 19.1% and 11.7% were observed at 4, 25, 30, 37, 50, and 70 ^{o}C respectively. The temperature optimum uptake of platinum by SRB at 30 ^{o}C probably reflects the temperature of a natural environment of mesophilic SRB, which has been reported to be between 28-32 ^{o}C (Hao *et al.,* 1996). Temperature dependent platinum (IV) uptake reveals that platinum uptake by SRB could be occurring via metabolism-dependent processes. Hughes and Poole (1989), reported that metabolic dependent metal uptake by

viable bacterial biomass is likely to be influenced largely by temperature changes and the presence of metabolic inhibitors. Previous studies on U (VI) and Tc (VIII) by the sulphate reducing bacterium *Desulfovibrio desulfuricans* also showed strong temperature dependent metal uptake (Lovely and Philips, 1992b; Lloyd *et al*., 1998b). In general, veryfew studies have investigated temperature as a relevant variable in biosorption/bioaccumulation of metals, mainly because adjusting temperature for a bioprocess treatment scheme at an industrial scale requires high energy inputs and as a result is very expensive.

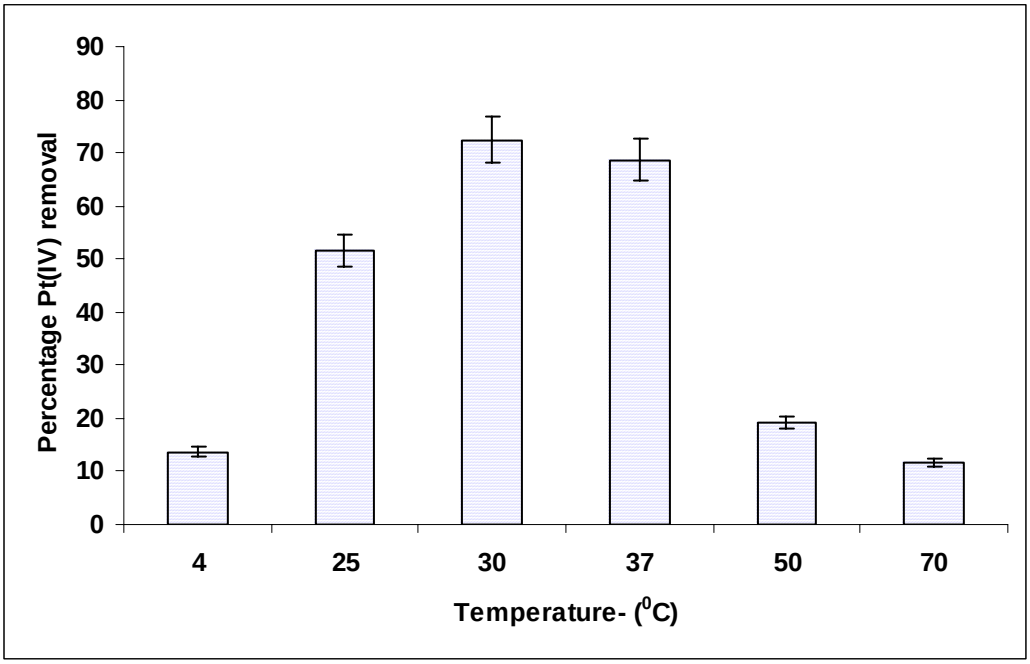


Figure 2.7: Extent of platinum (IV) uptake from solution by a mixed SRB culture at the stated temperatures. The initial platinum (IV) concentration was 50 mg.l^{-1}. Data are means ± the standard deviation for the triplicate values measured. (The following conditions were used: Initial resting SRB cells concentration= 5 g (wet weight).l^{-1}, pH 7.0, incubation time = 3 h, Pt (IV) = 50 mg.l^{-1} and hydrogen was included as an electron donor).

2.3.7 Effect of pH on platinum uptake by SRB

The pH of the solution has been shown to be an important parameter in a biological metal treatment process by SRB (Ballester *et al*., 1999). Platinum (IV) uptake by SRB was evaluated at a range of pH values (3.0, 4.0, 5.0, 6.0, and 7.0). The pH of the platinum solution was adjusted prior to contact with the SRB cells.

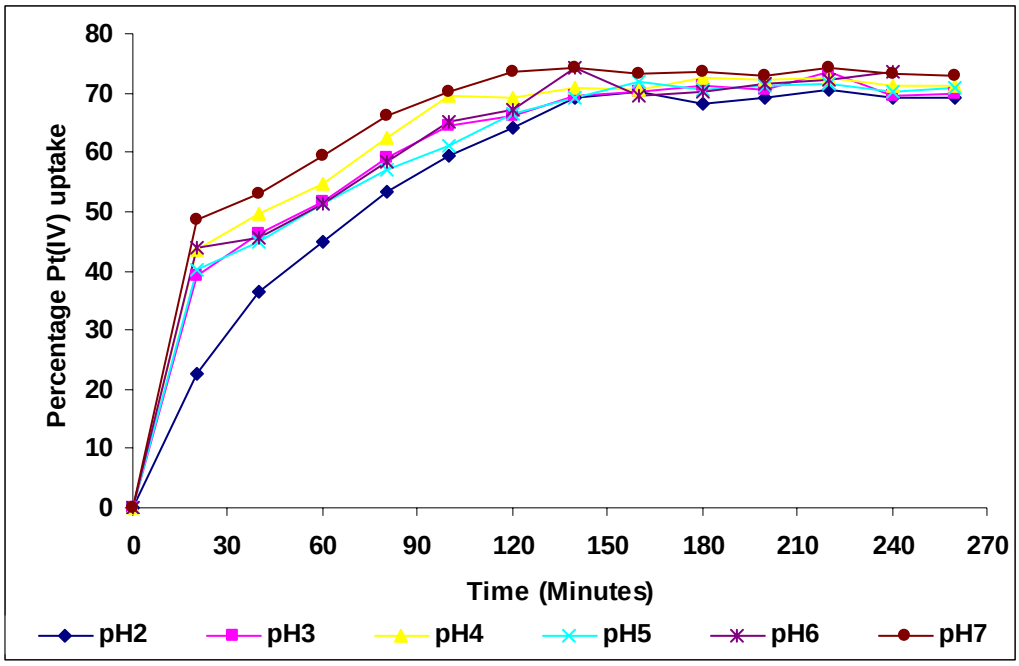


Figure 2.8: Percentage platinum (IV) uptake from solution by a mixed SRB culture under different pH conditions. The points are the means of duplicate values measured. (The following conditions were used: Initial resting SRB cells concentration= 5 g (wet weight).l^{-1}, room temperature, incubation time 3 h, Pt (IV) = 50 mg.l^{-1} and hydrogen was included as an electron donor).

The pH studies showed substantial pH sensitivity in platinum (IV) uptake by SRB at pH 2.0 (Figure 2.8). A significantly high percentage platinum uptake occurred at pH 7.0. The overall trend observed in pH dependent platinum (IV) uptake by SRB suggests that platinum uptake could be occurring through an ion exchange type of mechanism, as it is well established that heavy metals are taken up from solution predominantly by ion exchange (Volesky and Holan, 1995; Tabak *et al*., 2000; Gadd, 2000). This study proposes that pH dependent platinum uptake by SRB could be due to both the various functional groups on the bacterial cell walls and to the chemistry of platinum itself. The functional groups capable of metal sorption are usually basic (e.g. carboxyl, phosphate, amine groups etc), which are deprotonated at high pH values. In the absence of surface effect the pKa of carboxyl range from 3-5, primary amines 9-11 and secondary phosphates from 6-7. As the pH increases, more functional groups are dissociated and become available for ($[PtCl_6]^{2-}$ or $[PtCl_4]^{2-}$) ion uptake due to much less competition from protons (Tabak *et al*., 2000). In the presence of an alkaline solution platinum has been reported to form a range of hydroxide complexes containing halide, as shown by the

following chemical formula: $[PtX_n(OH)_{6-n}]^{2-}$ where X= Cl^-, Br^-, or I^-, thus facilitating platinum precipitation conditions (Greenwood and Earnshaw, 1989). However, control experiments (Section 2.3.1.2) showed that such conditions are unlikely to be a major influence of platinum precipitation.

2.3.8 Uptake of different platinum salts by SRB

Uptake of metal by biomass may in some cases be influenced by the oxidation (valence) states of the metal in the solution. For example, *Asellus aquaticus* accumulated platinum (II) at a lower rate then platinum (IV) (Rauch and Morrison, 1999). The platinum salts selected for this study were Pt (II) and Pt (IV), which were provided in solution as $Na_2Pt(II)Cl_4$ and K_2 $[PtCl_6]$ respectively at initial concentration of 50 $mg.l^{-1}$. Figure 2.9 represents the percentage platinum uptake of the two different platinum salts by SRB after 3 h. A percentage platinum uptake of 66.0% and 66.9% were achieved for Pt (II) and Pt (IV) respectively. These findings do not show significant difference in uptake by SRB, suggesting that platinum uptake by SRB may not depend on the form in which platinum is present in the environment.

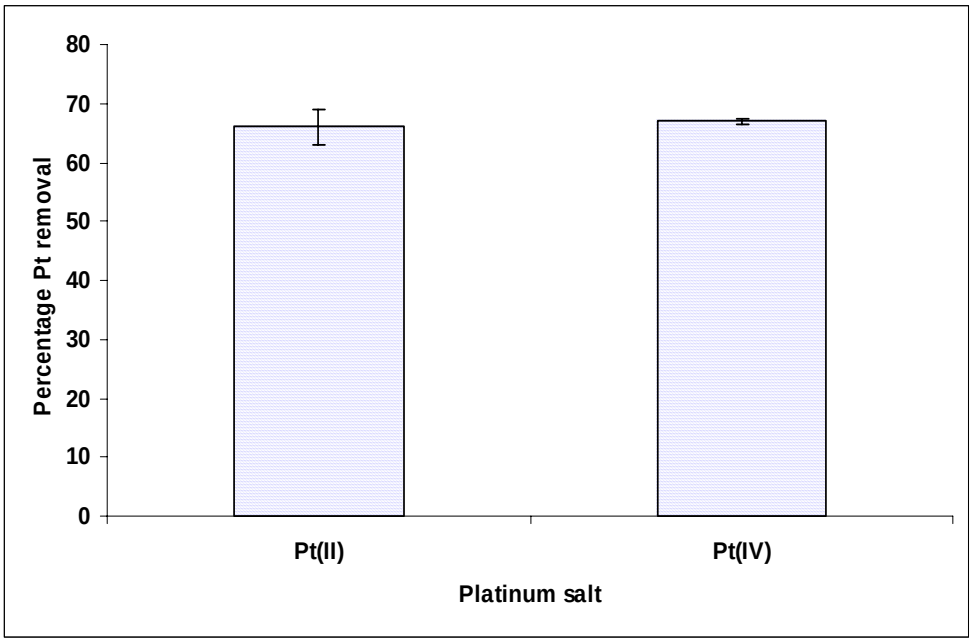


Figure 2.9: Percentage platinum (IV) uptake from solution by a mixed SRB culture with different platinum salts. Data are means ± the standard deviation for the triplicate values measured. The following conditions were used: (Initial resting SRB cells concentration = 5g (wet weight).l^{-1}, room temperature, pH 7.0, incubation period = 3 h, Pt (IV) = 50 $mg.l^{-1}$ and hydrogen was included as an electron donor).

2.3.9 Effect of inorganic ligands on platinum uptake by SRB

The hard, borderline, and soft anions, SO_4^{2-}, SO_3^{2-}, and S^{2-} respectively were supplied in various concentrations (0-1.0 mM) with the appropriate platinum (IV) concentration at 50 $mg.l^{-1}$ in order to assess their influence in platinum (IV) uptake by SRB. The control containing no ligands was also included to distinguish the effect of ligands. Figure 2.10 shows percentage platinum (IV) uptake by SRB of 61%, 62% and 84% in the presence of SO_4^{2-}, SO_3^{2-}, and S^{2-} respectively, at a maximum ligand concentration studied of 1.0 mM. The percentage platinum (IV) uptake of 57% was observed in the control, indicating platinum (IV) uptake activation of 4%, 5% and 27% for SO_4^{2-}, SO_3^{2-}, and S^{2-} respectively. The slight increase in platinum (IV) uptake in the presence of highest SO_4^{2-} concentration studied was not surprising, because according to Pearson's HSAB principle sulphate (hard base) is not expected to bind platinum (soft acid), and as a result avoids forming a complex that cannot be taken up by SRB.

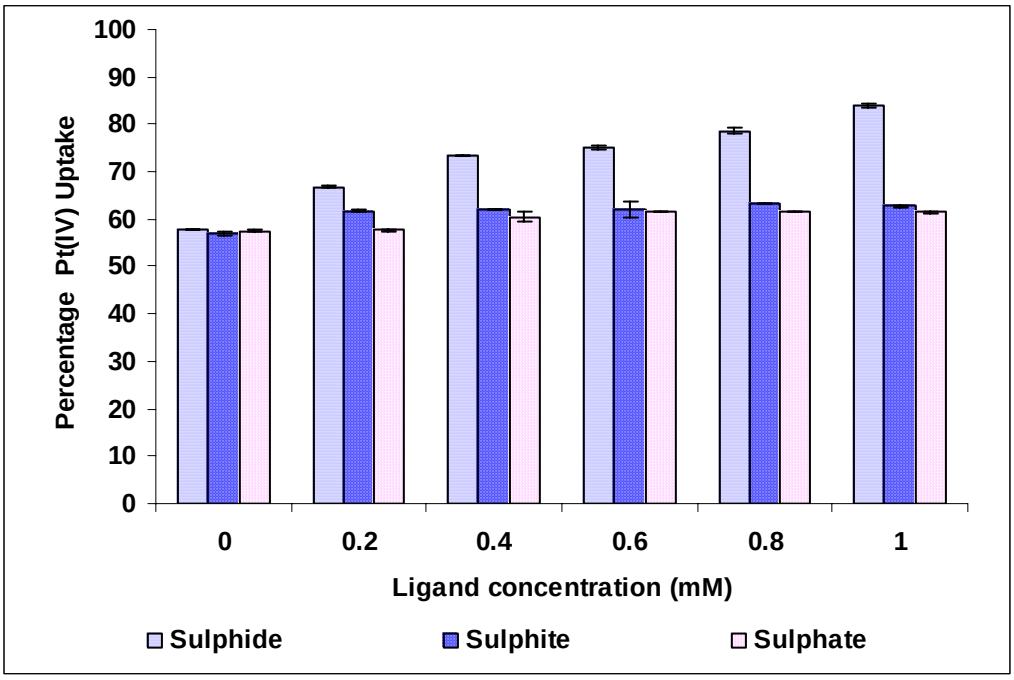


Figure 2.10: Influence of exogenous hard, borderline and soft anions on platinum (IV) uptake from solution by a mixed SRB culture. Data are means ± the standard deviation for the triplicate values measured. The following conditions were used: (Initial resting SRB cells concentration= 5 g (wet weight).l^{-1}, room temperature, pH 7.0, and hydrogen was included as an electron donor).

Likewise low activation of SO_3^{2-} was equally not surprising given the fact that SO_3^{2-} (borderline) may or may not bind platinum depending on the environmental conditions, biomass utilized or solution chemistry. The highest platinum uptake was observed at the highest S^{2-} concentration studied (1.0 mM). These findings were expected, because according to Pearson's principle, S^{2-} will covalently interact with platinum (IV), since they are soft donor and acceptor respectively. The HSAB principle also predicts that bonds formed between hard metals and hard ligands are predominantly ionic, whereas those of soft metal-ligand complexes are more covalent in character. Sulphide is known to precipitate most metals as stable metal sulphides, which can therefore be removed from solution. Precipitation of platinum as platinum sulphide observed in this study suggests that under SRB growing conditions as opposed to the non-growing cell conditions utilized in this section, high platinum uptake will be promoted as a result of platinum sulphide formation. The results presented here indicate that the mechanism of platinum uptake by SRB is related to its softness, although the clarity of such a relationship may be obscured under certain experimental conditions because of the strong concentration dependence.

2.3.10 Equilibrium sorption isotherm

The maximum platinum uptake capacity (q_{max}) was evaluated by plotting the equilibrium sorption isotherm (Figure 2.11). The initial platinum concentration utilized in this experiment ranged from 10-120 $mg.l^{-1}$ at a pH value of 7.0. The q_{max} of the mixed SRB culture for platinum was calculated using a general formula (Equation 4), and it was determined to be 4 $mg.g^{-1}$ (mg platinum/ g wet weight biomass). The value of q_{max} is the measure of the binding capacity of the biomass to the metal of interest. It provides the basis of comparison among potential biosorbents and allows selection of the best biosorbent to be utilized for the uptake of the given metal contaminant. The q_{max} from this study is reported relative to wet weight due to the nature of biomass.

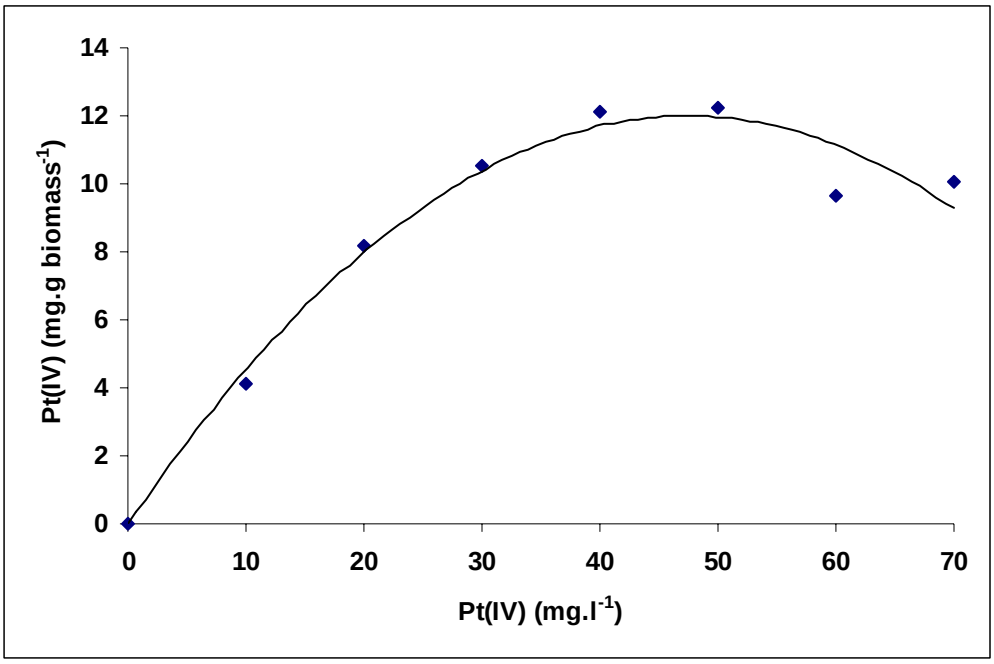


Figure 2.11: An equilibrium sorption isotherm for platinum (IV) uptake from solution by a mixed SRB culture. Data are means ± the standard deviation for the triplicate values measured.

To compare different biosorbents in terms of q_{max}, the experimental conditions should be stated to secure reproducibility of the results and allow a straightforward basis of comparison. Table 2.2 shows the comparison of q_{max} of platinum obtained from different studies to the one obtained in this study. Therefore this study shows that using a mixed SRB as a biosorbent is practical, even though cultivation of SRB requires strictly anoxic conditions. In addition, a high uptake capacity above the 4 $mg.g^{-1}$ stimulated bacterial viability loss is shown by a decrease in the q_{max} with increases in platinum concentration (Figure 2.11).

Table 2.2: Comparison of platinum uptake by different biosorbents

Metal	Biosorbents	q_{max} ($mg.g^{-1}$)	References
Platinum	Chitosan	0.30	Guibal *et al.*, 1999
	Asellus aquaticus	0.51	Rouch and Morrison, 1999
	E.coli (AB264)	3.9×10^{-4}	Beveridge and Koval, 1981
	Pseudomonas fluorescens	2.73×10^{-2}	Krueger *et al.*, 1993
	SRB	4.0	This study

2.4 Summary

The observations in this chapter suggest that the presence of viable SRB cells accelerates platinum uptake from solution. Electron donor studies revealed that platinum reduction from solution requires careful optimization with respect to the correct electron donor. Moreover, hydrogen dependent platinum uptake presents a first link of hydrogenase activity of SRB cells in platinum uptake. This study also demonstrated that platinum uptake by SRB is strongly dependent on both pH and temperature changes, indicating that ubiquitous distribution of SRB under anaerobic environments and diverse metabolic activity of SRB may have important implications for treatments of platinum contaminated waste streams presented at extremes of pH and temperatures. In an attempt to relate the HSAB principle to platinum uptake by SRB in the present of exogenous bases, this study revealed that the formation of platinum complexes with sulphide may facilitate uptake or permeation through the cell surface of SRB. The findings in this study also suggest that platinum (IV) uptake by SRB proceeds via several different mechanisms, both extracellular and intracellular. Evidence of extracellular platinum uptake has been shown by a rapid platinum uptake upon initial contact of platinum with SRB biomass. Two important details have been observed in this study which suggest intracellular platinum uptake. Firstly, high platinum uptake occurred in a narrow Pt (IV) concentration range (0-50 $mg.l^{-1}$). Secondly slow secondary platinum uptake profiles observed also suggest that a platinum internalization across the cell wall could be promoted. Having revealed such observation, it is not enough to explain extracellular and intracellular uptake mechanisms. Combinations of rigorous analytical and microscopy approaches are required to investigate the bioaccumulation of platinum (IV) into SRB cells in order to enhance and understand the mechanism of direct platinum (IV) uptake.

CHAPTER THREE

Characterization of Platinum (IV) Accumulation in Sulphate Reducing Bacteria by Electron Microscopy and Energy Dispersive X-ray Analysis Techniques

3.1 Introduction

All bacteria share several cellular attributes. They are anucleate. They contain one circular chromosome (and frequently one or more plasmids) and many 70S ribosomes. They do not possess an internal cytoskeleton, and their membranes usually do not contain sterols. Several substances are unique to them (e.g peptidoglycan, lipopolysaccharide, poly-β-hydroxybutyrate). Finally they are small (Beveridge, 1989). This last characteristic is so apparent and its consequences often neglected. Bacteria rely entirely on diffusion for their livelihood. As Beveridge *et al*. (1989) put it, "they cannot reach out to grab a food source, nor can they fling away toxic waste product. Since they are small, a low Reynolds number of about 10^{-5} applies to them. They cannot out swim their local aqueous environment and in fact drag it around no matter how fast they swim". They rely on an efficient design to overcome the extracellular diffusion problem this entails. A high surface area to volume ratio is the best design for getting solutes in and out of the cell, so as a result shape becomes very important. Smallness restricts complexity of cytoplasmic organization and precludes the development of cellular compartmentalization. Yet smallness makes intracellular diffusion the most efficient route and compartmentalization not necessary (Beveridge *et al.,* 1997).

Most bacteria possess a cell wall that forms the outermost limit to the cell and that separates the vital protoplast from the external milieu. This wall is of fundamental importance to the bacterium, since it not only contributes cellular shape and form, but it must also provide an inanimate boundary through which the cell perceives the surrounding environment. All substances that enter or exit the bacterium eventually percolate through this barrier (Beveridge and Fyfe, 1985). Like most cell surfaces the bacterial wall is anionic (Beveridge and Koval, 1981).

This characteristic is independent of whether or not the bacterium is gram-positive or gram-negative. Given that bacterial cell walls are anionically charged, it is reasonable to assume that they will interact strongly with metallic ions in dilute solution within the natural water bodies. In some cases surface metal concentrations frequently exceed the stoichiometry expected per reactive chemical site within the cell walls. The sorption of soluble metals can be so great that often visible precipitates can be seen by electron microscopy (Marquis *et al.*, 1976; Doyle *et al*., 1980, Rouch *et al*., 1995; Klaus, 1999).

High interaction of metals with biological cell surfaces is a prerequisite for intracellular accumulation, where metal ions may fulfill essential functions in cellular metabolism or in certain cases, exert toxic effects toward the cells (Hughes and Poole, 1989). Thus, a detailed understanding of the mechanisms involved in metal accumulation can facilitate assessment of the impact of metals in biological systems. Considerable interest in such interactions has focused on microbial metal uptake (Gadd, 1992). This is primarily because of the suitability of microorganisms for studying metal nutrition and toxicity at the cellular level and the important roles of these organisms in biogeochemical cycling and in the biological food chain (Avery and Tobin, 1993), although recent attention has also concentrated on their biotechnological potential in metal removal processes (Macaskie, 1991).

Because of a higher surface area to volume ratio than any other life form and as a strictly physical cellular interface, bacteria have a high capacity of metal uptake from solution and this has an influence on the migration of toxic heavy metal ions in a variety of anthropogenically loaded settings (Erhlich and Brierley, 1990). All biomass contains a significant quantity of metallic constituents and mineralization in living and dead biodebris may contribute to element transport from the hydrosphere into sediments. A considerable amount of work has been done to evaluate the adsorption, accumulation and complexation of various metals. Several early investigations have shown that relatively large quantities of metallic ions are complexed by algae (Laube *et al.,* 1979), bacteria (Strandberg *et al.,* 1981) and fungi (Marquis *et al.,* 1976).

The binding and accumulation of metals by bacteria are measured by methods which are usually destructive to the cells. For example, the amount of metal bound to the bacteria cells can be determined by AAS following hydrolysis of cells in strong acids or by rapidly carbonizing the cells in a graphite furnace attachment (Krueger *et al*., 1993). Because bacteria are so small and their surfaces so thin, researchers have relied on transmission electron microscopy (TEM) coupled with energy dispersive X-ray (EDX) to locate metals in the cells and to establish metal composition and mineral form (Little *et al*., 2002). The electron microscopy methods mentioned above are high-resolution, sophisticated techniques, but they suffer from serious drawbacks. The specimen should be devoid of water. Chemical fixation, dehydration and staining of the cells for TEM sometimes complicates observations of metal-bacteria interactions since estimation of the true nature of metal bound by cells suspended in a natural hydrated medium is not possible (Beveridge *et al.*, 1997). Despite their drawbacks, however, these techniques are still very useful, in establishing location and morphology of metal precipitates within the cell. The aim of this section was to assess the effect of platinum (IV) on viable SRB cells with respect to the sites of deposition of the internalized platinum cations, with the following objectives:

(i) To determine if there is any change in cell morphology after challenging cells with platinum (IV) using scanning electron microscopy (SEM).

(ii) To observe the distribution and location of platinum within the cells using transmission electron microscopy (TEM).

(iii) To confirm platinum (IV) location in the cells using energy dispersive X-ray (EDX) analysis.

(iv) To monitor the effect of platinum (IV) on the integrity of the cells.

3.2 Materials and Methods

3.2.1 Materials

Phosphate buffer (0.1 M pH 7.0); 25% glutaraldehyde, [HCO $(CH_2)_3$ CHO] stock reagent; Ethanol series concentrations (30%, 50%, 70%, 80%, 90%, 100%); Propylene oxide; propylene oxide: embedding medium mixture (75:25; 50:50; 25:75); Embedding gelatin medium; Ultramicrotome, Grids (200 mesh); baskets for critical point drier apparatus, specimen stubs, conductive film aerosol specimen adhesive and double sided tape; gram-stain reagent kit; fluorescent diacetate (Sigma). Centrifugation was achieved by Heraeus Sepatech biofuge 13 Microfuge or Eppendorf 5810 R centrifuge. Fluorescence determination was accomplished using a Perkin-Elmer Fluorescence spectrophotometer 203.

3.2.2 Methods

3.2.2.1 Gram staining preparation of SRB culture

For gram staining preparations, 25 µl of bacterial culture was deposited on a 1 cm^2 glass slide and air dried. The cells were heat fixed by passing over a flame, stained with crystal violet for 60 sec then washed with dH_2O for 2 sec. The cells were flooded with Iodine for 60 sec, rinsed with dH_2O, washed with 95% ethanol for 30 sec followed by a further rinsing with dH_2O. The specimen was flooded with Safranin for 60 sec, washed with dH_2O, blotted and viewed under an Olympus BX50 camera-light microscope attached to an 'Olympus' PM -30 exposure control unit.

3.2.2.2 Platinum accumulation

Sulphate-reducing bacteria inoculum (10% v/v) was cultured in 1 L of modified Postgate medium C for six days at 30 ^oC as previously described (Section 2.2.2.3, Chapter 2). Cells were harvested by centrifugation (4000 x g; 10 min, 25 ^oC; supernatant discarded). The bacterial pellet was resuspended under OFN in MOPS–buffer (20 mM, pH 7.0, 150 ml) at a biomass density of 5g (wet weight).l^{-1} in an Erlenmeyer flask sealed with a rubber stopper. Cells were then challenged with 50 mg.l^{-1} platinum. Hydrogen was supplied as an electron donor (400 KPa.min^{-1}) throughout the incubation period at the

head space of the flasks, displacing OFN. Samples were removed at timed intervals for preparation of electron microscopic experiments. Cells unchallenged with platinum served as a reference control.

3.2.2.3 Scanning electron microscopy (SEM) preparation

Scanning electron microscopy preparation was performed according to the method developed by Cross (2001). Sulphate reducing bacterial cells were collected by filtering 10 ml of culture through cellulose acetate plus membrane filters (25 mm diameter, 0.45 µm pore size) using a Millipore reusable filter unit. Specimens were fixed overnight in 2.5% glutaraldehyde fixative solution prepared from a 25% glutaraldehyde stock reagent by diluting with phosphate buffer (0.1M, 7.0). Glutaraldehyde solution was decanted off and the cells washed twice with phosphate buffer (0.1 M, pH 7.0, 10 min). The cells were then dehydrated using an ethanol series (30%, 50%, 70%, 80%, 90%, 100%, with two changes of 100%; 15 min at each step). Cells were then placed in a critical point dryer for 2 h. The specimen filters were mounted on 12 mm diameter aluminum posts with 12 mm carbon conducting adhesive tabs. The specimens were transferred to a Large Desk II cold Sputter Etch Coater and gold coated for 160 sec at 80 mT pressure at an applied current of 45 mA. Carbon paint was applied from the surface of the gold coating to the aluminum post to insure a conductive path from electrons on the surface to reach ground state. The specimens were examined with a JEOL-JSM-80 scanning electron microscope.

3.2.2.4 Transmission electron microscopy (TEM) and solid state-metal analysis

Transmission electron microscopy preparation was performed according to the method developed by Cross (2001). For electron microscopy and solid state-metal analysis, samples (100 µl) in Eppendorf tubes were harvested (13000 x g, 10 min, 25 ^{o}C) and the supernatant discarded. Bacterial pellets were fixed overnight in a 2.5% glutaraldehyde fixative solution prepared from a 25% glutaraldehyde stock reagent by diluting into phosphate buffer (0.1 M, pH 7.0). Glutaraldehyde solution was decanted off and the cells washed twice with phosphate buffer (0.1 M, pH 7.0, 10 min). Because of interference with heavy metals secondary staining with 1% (OsO_4) was avoided. Cells were then dehydrated using an ethanol series (30%, 50%, 70%, 80%, 90%, 100%, with two changes

of 100%; 15 min at each step). Cells were infiltrated by washing twice in propylene oxide for 15 min. Propylene oxide was decanted off and the tube were refilled with propylene: resin (Araldite) mixture, 75:25, 50:50, and 25:75, 90 min at each step. Samples were transferred into a tube containing 100% pure resin and left to polymerize (36 h; 60 ^{o}C).

Sections (100-150 nm thick) were cut from the polymerized resin block using a microtome and placed onto a carbon coated copper grid. All sectioning and trimming was conducted on a RMC MT-7 Utramicrotome, using glass knives. Sections were then stained in aqueous uranyl acetate for 30 min, washed twice with distilled water and blot dried on filter paper and further stained in lead citrate for 5 min. Sections were viewed using a JEOL 120CX2 Transmission Electron Microscope (TEM) at acceleration voltage of 80 kV.

3.2.2.5 Energy Dispersive X-ray (EDX) microanalysis

The concentration ratios of the metals in the cells were obtained by using the technique of energy dispersive X-ray (EDX) microanalysis in a 120 kV Philips EM420 coupled to a EDAX-DX-4 energy dispersive X-Ray system. Quantification was achieved using the standardless thin foil model, which includes the absorption correction and with a theoretical k factor calculated according to Zaluzec (Williams and Carter, 1996). Only the metallic elements present in the spectrum were used in the quantification and the atomic percentages obtained were then used to calculate the concentration ratios of the various metals present in the SRB cells. Because of interference with heavy metals, secondary staining with 1% (OsO4) was omitted. Due to the higher acceleration voltage used for EDX microanalysis, thicker sections were required. As a result, 250 nm thick sections were cut and collected onto copper grids. The presence of intracellular elements was confirmed through examining the carbon coated section using a Philips EM 420 TEM at an acceleration voltage of 120 kV. The sites of the metal ion deposition, localization and evidence of ion exchange were confirmed from EDX spectra obtained from the EDAX-DX-4 energy dispersive X-ray system. Analysis of the cells was obtained by focusing the electron beam on the area of interest.

3.2.2.6 Influence of platinum (IV) on membrane integrity

Membrane permeability of SRB cells was determined by fluorescence techniques based on a method by Slavik *et al.* (1982). The experiment was conducted in triplicate. Cells were harvested by centrifuging (4000 x g, 10 min, 25 ^{o}C), the supernatant was discarded and the bacterial pellet washed twice in ddH_2O, and then resuspended in MOPS buffer, (20 mM, pH 7.0, 100 ml) at a biomass density of 5 g (wet weight).l^{-1}. Cell suspensions (10 ml) were dispensed in test tubes containing (2% w/v in acetone, 0.2ml) fluorescent diacetate under an OFN atmosphere, sealed with butyl rubber stoppers. Platinum (IV) concentrations (10, 20, 30, 40 and 50 mg.l^{-1}) were injected through the rubber stopper into each respective tube using a hypodermic syringe. The blank sample contained no platinum solution. The suspensions were incubated (room temperature, 1 h), while being purged with hydrogen (400 KPa.min^{-1}, over the period of 1 h), in the head space of the tube, displacing OFN, and then centrifuged (4000 x g, 15 min, 25 ^{o}C) and the supernatant analyzed for fluorescence. Fluorescence intensity was recorded at emission wavelength of 520 nm after excitation at 435 and 490 nm.

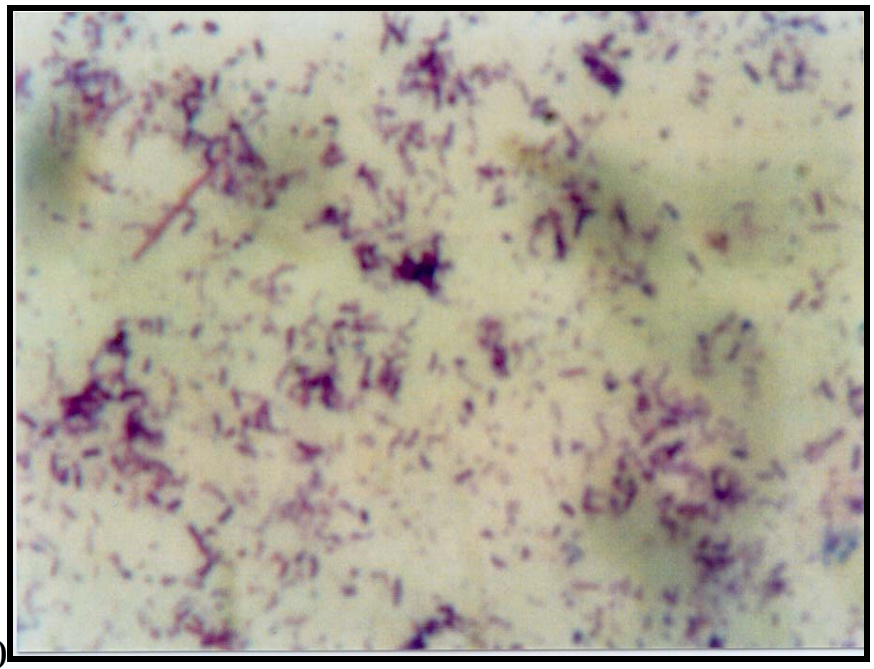
3.3 Results and Discussion

3.3.1 Microscopic analysis of the SRB Consortium

A light microscope preparation of gram stained SRB culture grown in modified Postgate medium C is shown in Figure 3.1a. Based on the gram staining observations the SRB consortium consisted of a range of gram-negative and gram-positive rods. These observations suggest that the consortium is comprised largely of *Desulfovibrio* and *Desulfotomaculum* genera. Previous studies of morphological features of SRB consortia indicated that the genus *Desulfovibrio* stain gram-negative with many of the cells showing rod-like morphology (Postgate, 1984; Widdel, 1988). This suggestion is further supported by physiological studies, which showed that *Desulfovibrio* and *Desulfotomaculum* genera dominate the consortium when grown in the medium with lactate as the sole electron donor (Gibson, 1990).

Scanning electron microscopic observations further confirmed that the SRB consortium is made up of both gram positive and gram negative rod. Scanning electron microscopic analysis of the SRB consortium is shown Figure 3.1b. From metal removal point of view one of the striking features observed from the SEM analysis of the consortium was the formation of extracellular polymeric substances (EPS) (Figure 3.2). It has been reported that bacterial microcolonies often synthesize EPS composed generally of polysaccharides, which often enclose the cells in a gelatinous-like matrix (Ladd and Costerton, 1990). EPS has been described as a trap for dissolved species (Schwartz *et al.*, 1998) and has also been noted for its tendency to avidly bind metals (Toni *et al.*, 1999; Gadd and White, 1998; 2000). It is also known that bacterial colonies can form dense sticky EPS layers which can cause the deposition of dissolved metal species (Beech and Cheing, 1995; Geesey and Jang, 1989). Thus it is suspected that metal biosorption of stationary phase cells of SRB may strongly relate to the production of EPS (e.g polysaccharides). This phenomenon suggests that EPS produced by SRB in this study might be strongly interacting with platinum (IV). However it is important to emphasize that SEM analysis is unsatisfactory to confirm the mechanism for bioaccumulation.

(a)



(b)

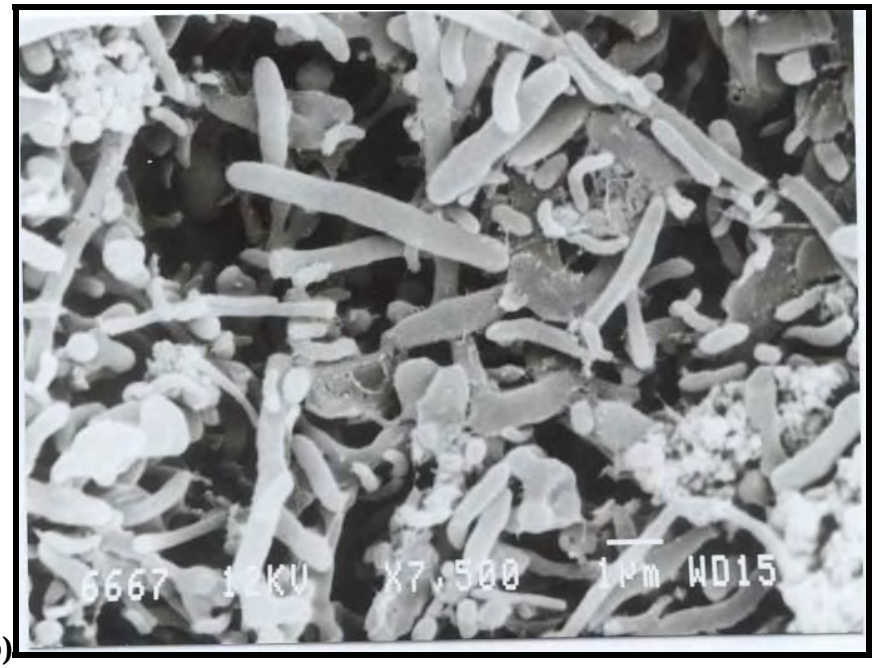


Figure 3.1: **(**a) Light microscope preparation of Gram stained mixed SRB sample, grown for two days in Postgate modified medium C with lactate as an electron donor (400 x magnification). (b) Corresponding scanning electron micrograph of the same sample (7.500 x magnification).

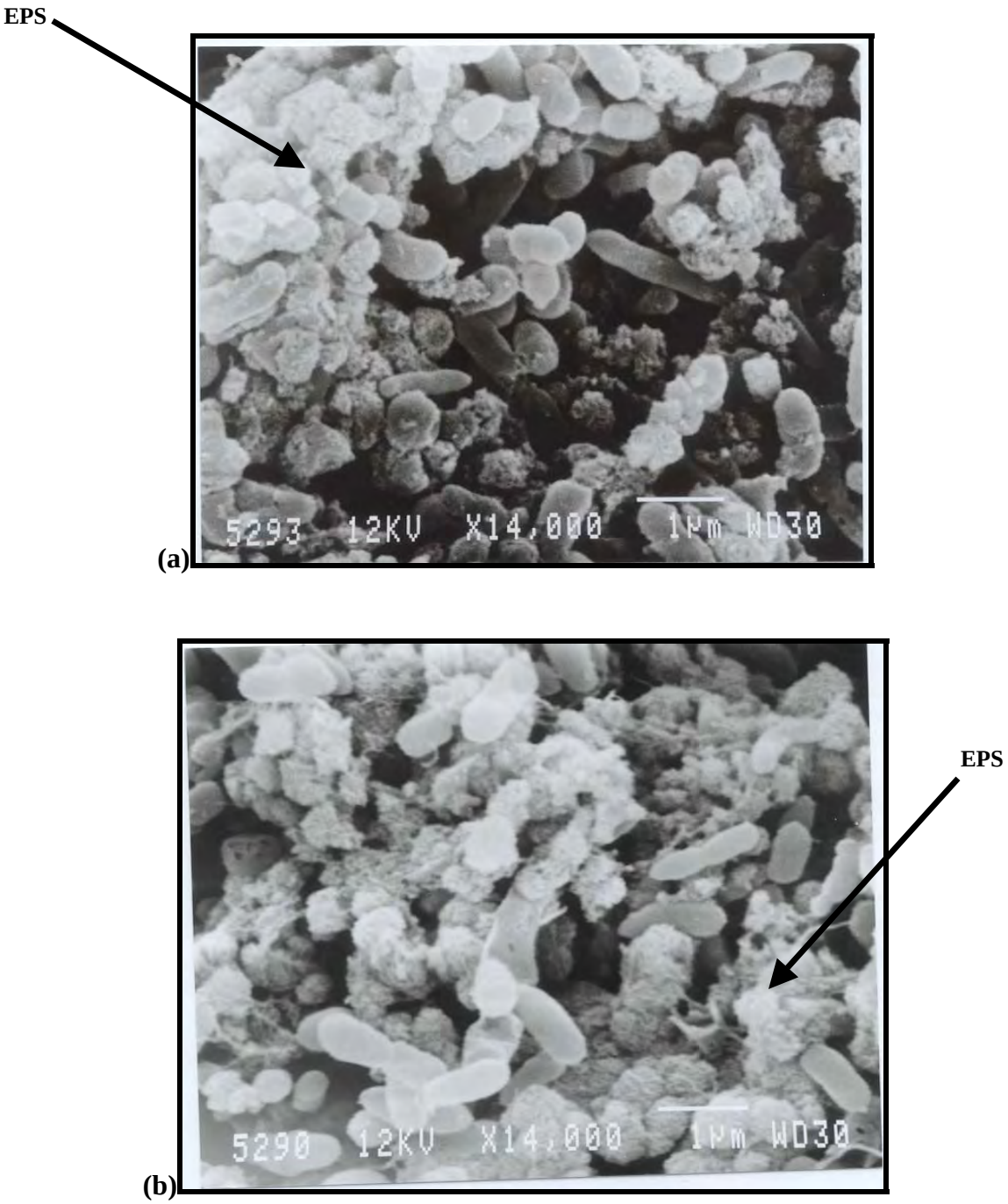


Figure 3.2: Scanning electron micrograph of mixed SRB culture grown in Postgate medium C, with lactate as an electron donor, showing increase in production of extracellular polymeric substances (EPS) (a) at day four and (b) at day six.

3.3.2 Effect of platinum on SRB morphology

It is well documented that non essential metals stimulate some morphological changes in the bacterial cells as a result of biological unavailability of metals (Hughes and Poole, 1989). The biological role of platinum in SRB metabolism has yet to be established. It was therefore assumed that the interaction of platinum (IV) with SRB was likely to create some morphology disruption in the SRB cells. This assumption was made based on the fact that some bacteria accumulate metal by growing in the metal containing solution, mainly because of two reasons: (i) as a defense mechanism against the poisonous effect of metals and (ii) as a result of the side effect of the cell wall chemistry. The electron scanning micrographs of the effect of platinum on SRB morphological changes is shown in Figure 3.3.

The investigations using SEM showed that the presence of platinum did result in morphological cell changes. These cytological investigations provide some details of the mechanism responsible for the platinum (IV) effect on resting SRB cells. As indicated in Figure 3.3b, some changes of the surface structure and cell shape (swelling; labeled (i) and lengthening; labeled (ii) of the cell) were revealed. Both changes can testify to damage of the cell wall structure: apparently such damage can be a result of binding of platinum (IV) on the cell wall, or as a result of increased permeability of the SRB membrane due to platinum (IV) toxicity. The reason for the morphological changes at a high platinum concentrations (50 $mg.l^{-1}$; Figure 3.3b), is presumed to be due to the reaction of platinum (IV) with DNA. As reported by Hughes and Poole (1989), platinum is capable of coordinating covalent interactions with macromolecules. This normally results in several types of DNA lesions and the inhibition of macromolecular synthesis. At a cellular level, it may lead to cell cycle arrest, apoptosis, premature terminal growth arrest or neoplastic transformation (Beveridge *et al.,* 1997). No doubt the cellular locations of platinum precipitate in the cell require a thorough general TEM analysis in further experiments.

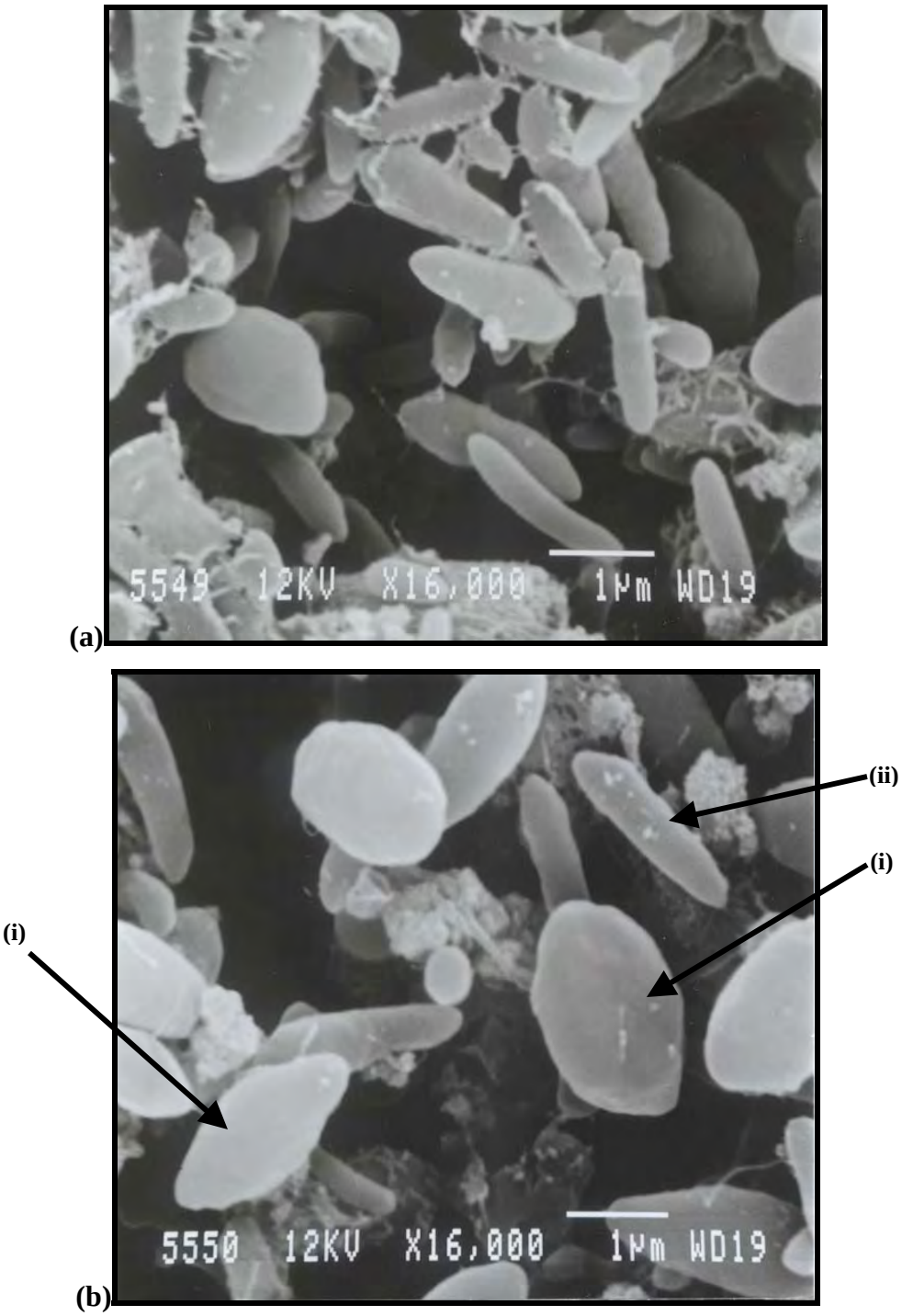
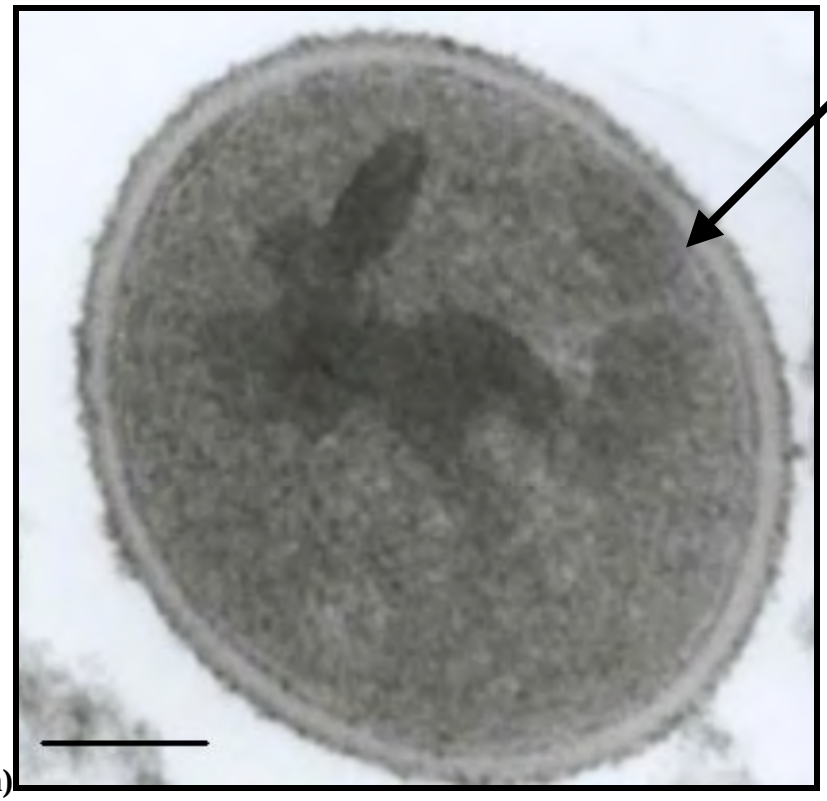


Figure 3.3: Effect of platinum on resting SRB cell morphology (a) Scanning electron micrograph of resting SRB cells before challenged with platinum (Control). (b) Scanning electron micrograph of resting cells after challenged with 50 $mg.l^{-1}$platinum. Incubation time was 3 h.

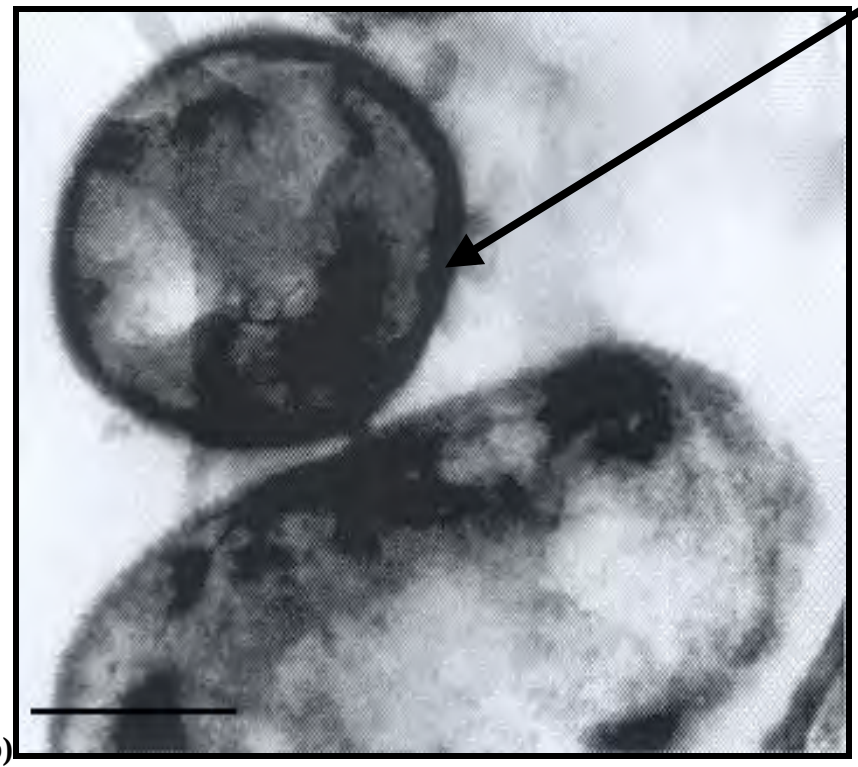
3.3.3 TEM studies on platinum deposition site and EDX microanalysis

In order to establish the site for platinum deposition thin sections of SRB cells that had been incubated in the absence of platinum (Control) and in the presence of platinum (Experimental) were viewed using TEM (Figure 3.4). The control cell, not incubated with platinum, did not show any precipitates within the periplasmic region or in the outer membrane surface (Figure 3.4a). Cells incubated with platinum exhibited electron dense precipitates principally within the periplasmic region (Figure 3.4 b, c, and d). A close examination of the thin section of SRB cell challenged with platinum for 12 h (Figure 3.3 c) revealed that some platinum precipitates were being formed on the outer margin of the membrane surface. This observation suggests that some of the platinum precipitates were being formed as a result of interaction with EPS. This assumption was also supported by the fact that the precipitates were not as dense as those observed within the periplasmic region, presumably because they were more hydrated. The platinum precipitates observed in this study were not surprising based on the fact that bacteria are excellent nucleation sites for mineral formations due to their high surface-to-volume ratio and the electronegative surface functional groups (e.g. carboxyl, phosphoryl, and hydroxyl groups). It is assumed that as platinum (IV) is being reduced to either platinum (II) or platinum (0) it is free to bind stoichiometrically to these sites, and once bound, the cell wall acts as a template for further heterogeneous nucleation precipitates. In addition the presence of clearly visible platinum precipitates observed within the periplasmic region also suggests that a proportion of platinum was reduced, presumably as a formation of platinum complex with soluble organic ligands found on the SRB cell wall.



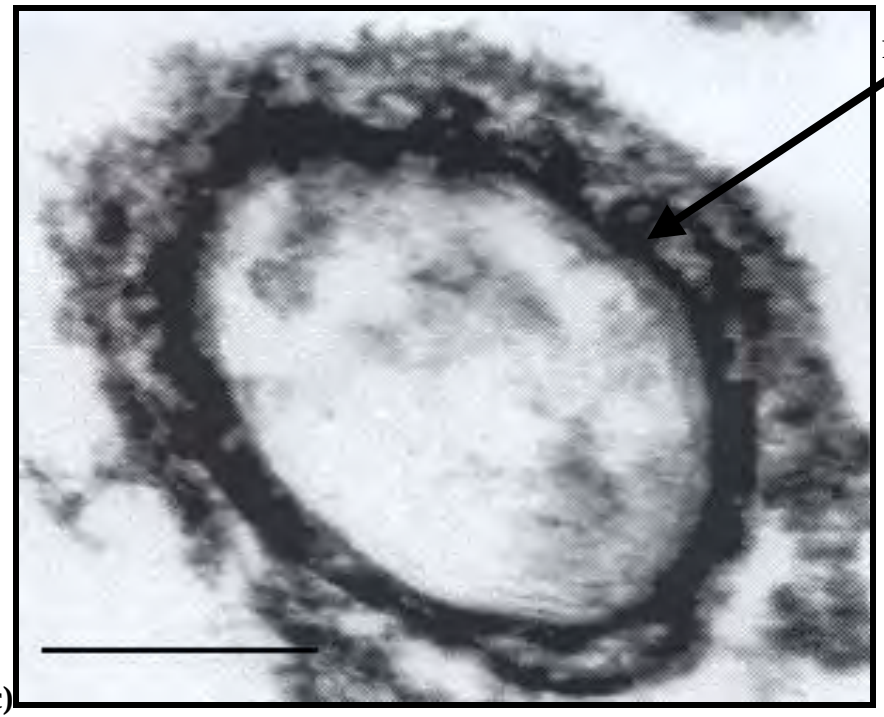
(a)

(Bar = 50 nm)



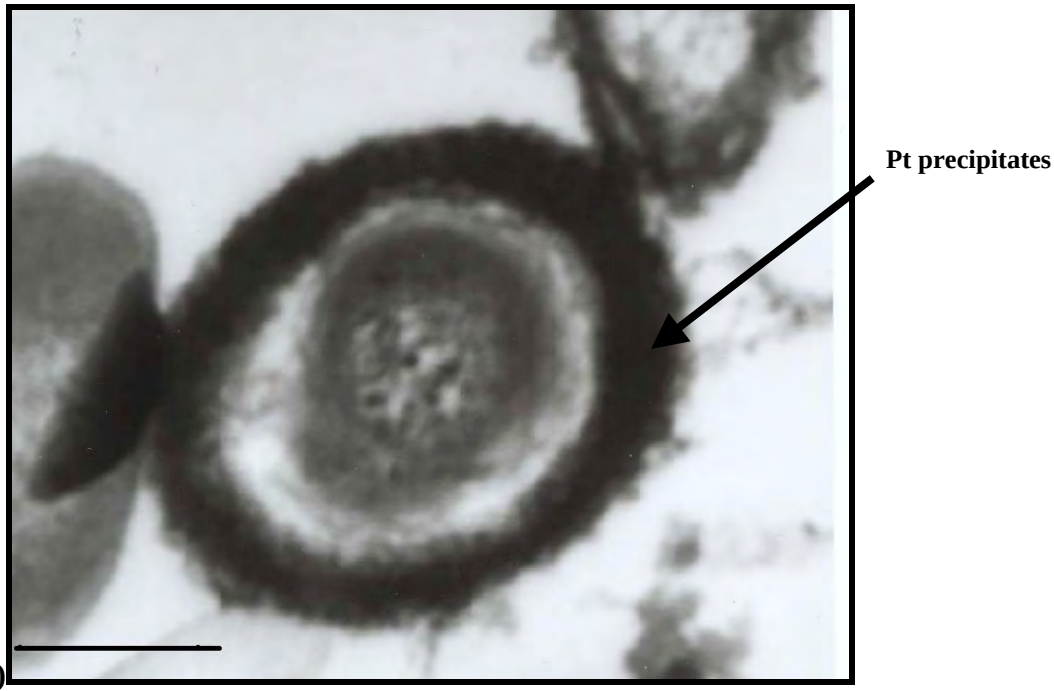
(b)

(Bar = 50 nm)



(c)

(Bar = 50 nm)



(d)

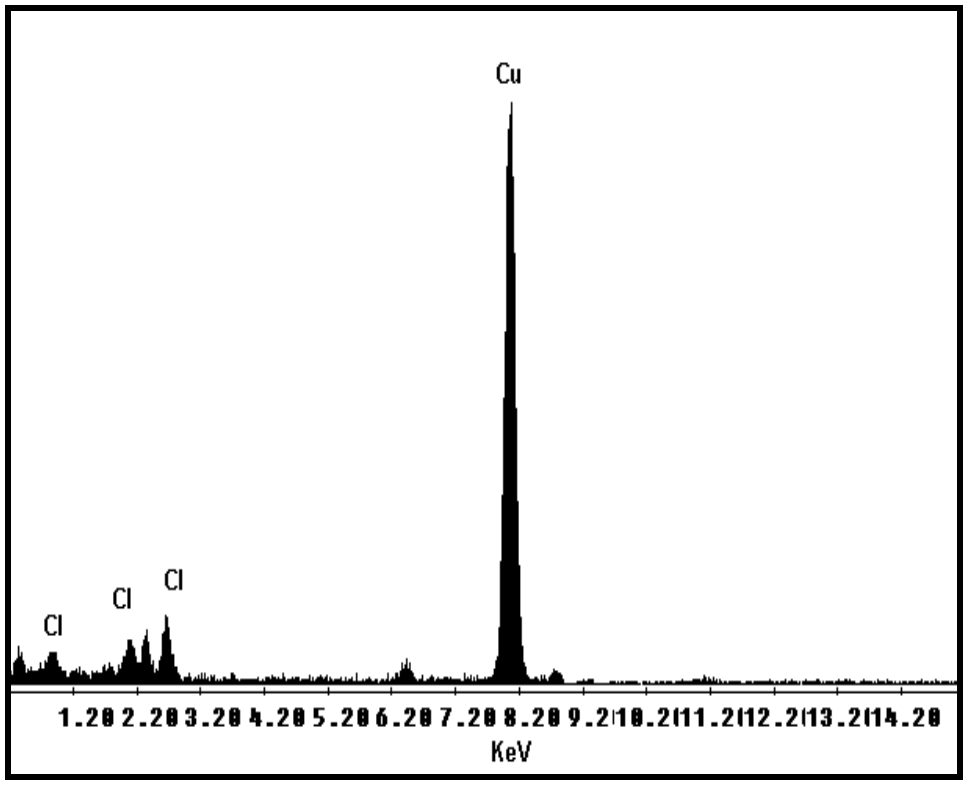
Figure 3.4: Transmission electron micrographs of thin sections of sulphate reducing bacterial cells before and after challenged with 10mg.l^{-1} of platinum: (a) cells unchallenged with platinum (Control), (b) Cells challenged with platinum for 6 h, (c) Cells challenged with platinum for 12 h, (d) Cells challenged with platinum for 18 h. Hydrogen was supplied as the electron donor for platinum reduction. Electron dense precipitates (indicated by the arrows) suspected to be reduced platinum at the periphery of the cells were further analyzed by energy dispersive X-ray diffraction to confirm the presence of the platinum (Bar = 50 nm).

The exact mechanisms of precipitation and mineral formation by bacteria are under investigation and thorough understanding is hampered by the chemical diversity of bacterial surfaces, the extreme smallness of these surfaces, and the variety of mineral or precipitate forms. Two mechanisms for metal depositions by bacteria have been proposed by Beveridge *et al*. (1976) and Macaskie et *al.* (2000). The former proposed a two-step mechanism for bacterial facilitated mineral formation. In the first step the metal ions bind to an active site on the cell wall. In the second step: this metal ion acts as a nucleation site for further metal deposition. Thus the cell wall serves as a nucleation site for metal deposition and subsequent metal mineralization. Macaskie *et al.* (2000) suggested another mechanism of heavy metal deposition for a *Citrobacter* species. For this strain the authors proposed that an envelope-bound phosphatase generates enough HPO_4^{2-} to surface-precipitate large quantities of Cd^{2+} as $CdHPO_4$. If this scheme is correct, then a number of other heavy metals could also be immobilized by a similar mechanism.

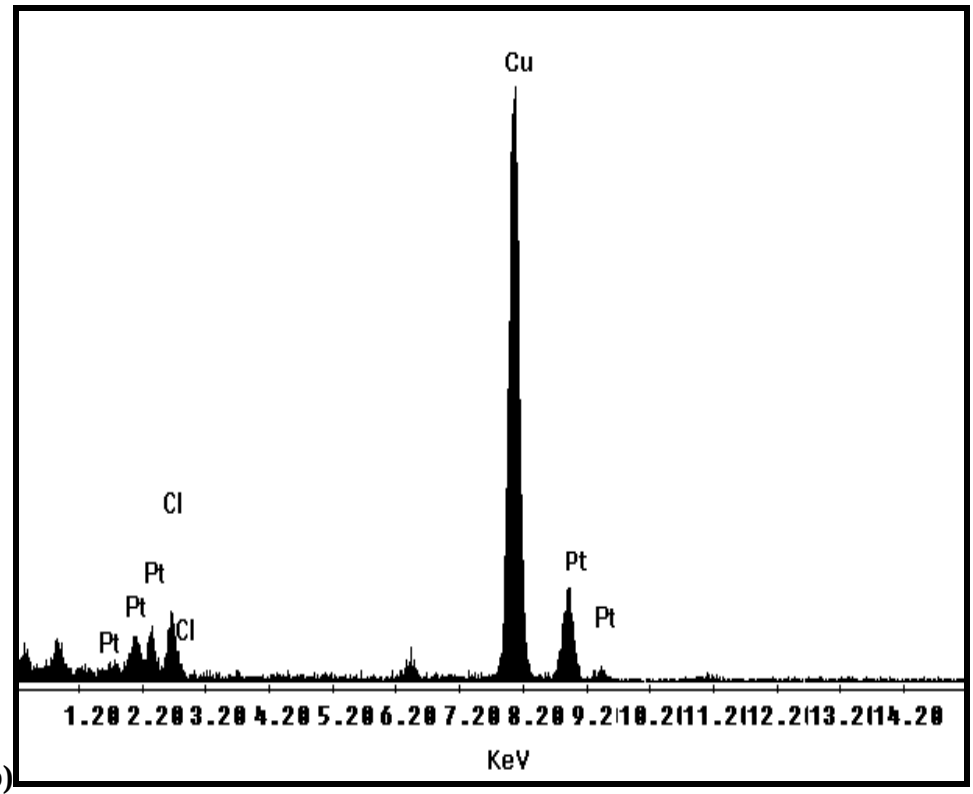
The platinum precipitation formation at the periplasmic region requires an additional comment. The periplasm is a major site of hydrogenase activity in SRB (Peck, 1993), and observations of platinum precipitates at the periplasm are consistent with direct enzymatic reduction of platinum catalyzed by periplasmic hydrogenases. These hydrogenases have recently been identified as oxyanion metal reductase in *Clostridium pasteuriziaum* (Yanke *et al*., 1995), sulphate reducing bacteria (Macaskie *et al*., 2001) and *E. coli* (Lloyd *et al*., 1997a). Previous research into microbe-mediated metal reduction has identified the enzymatic mechanism to be a detoxification, where cells use soluble enzyme to reduce metal, internal or external to the plasma membrane (Bopp, 1983). There are obvious advantages to the bacterium if it transports the enzyme into its microenvironment in the presence of non essential metal (platinum in this case), since the enzyme would act as a detoxifying agent. In addition, since the majority of hydrogenases reside in the periplasm they would further inhibit the entry of platinum into the plasma membrane.

In order to confirm that the electron dense precipitates observed in Figure 3.4 contained platinum, SRB sections were analyzed by EDX. The operation principle of EDX is that as the electron beam of the TEM is scanned across the sample surface, it generates X-ray fluorescence from atoms in its path. The energy of each X-ray photon is characteristic of the element which produced it. The energy dispersive X-ray spectroscopy (EDS) microanalysis system attached to the TEM collects the X-rays, sorts and plots them by energy and automatically identifies and labels the elements responsible for the peaks in this energy distribution. The EDS data are typically compared to the computer generated standards to produce a full analysis of the sample composition.

A prerequisite for the detection of elements by EDX microanalysis was a minimum composition of 1% of the area under analysis. Restrictive criteria (e.g. biological variables), prevented quantitative analysis of the metal concentration within the cells, forcing qualitative interpretation of the results. Analysis of the sections by EDX confirmed that platinum was located in the periplasmic region of the cells (Figure 3.4). Cells not challenged with platinum did not show any platinum peak in the EDX spectrum (Figure 3.5 a). Some elements observed in the spectra originated from external sources e.g. Cu was from the copper grid used to coat the cells, while Cl was provided by metal solution. The close analysis of the spectra (Figure 3.5 b, c, and d) showed a varied response to platinum presumably as a result of different exposure time. While EDX microanalysis is a useful technique for analysis of biological samples it does not provide the oxidation (valence) state of the precipitated reduction products. As a result it is not known at this stage whether platinum (IV) was being reduced to platinum (II) or platinum (0).


Cu
Cl
Cl
Cl
1.20 2.20 3.20 4.20 5.20 6.20 7.20 8.20 9.20 10.20 11.20 12.20 13.20 14.20
KeV

(a)


Cu
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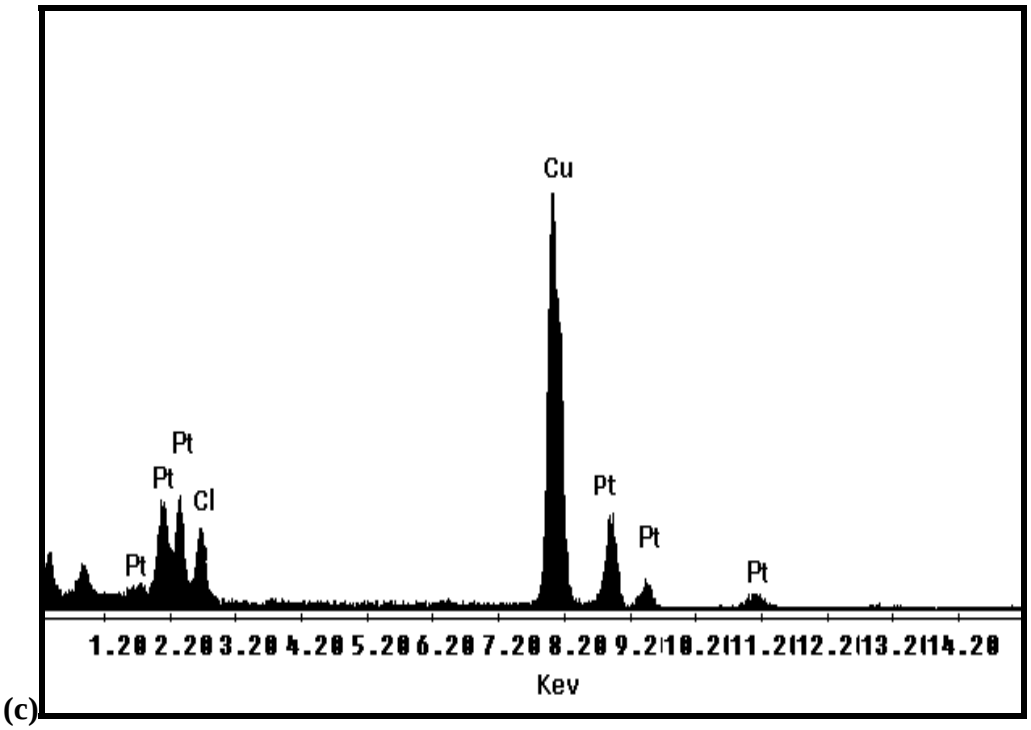
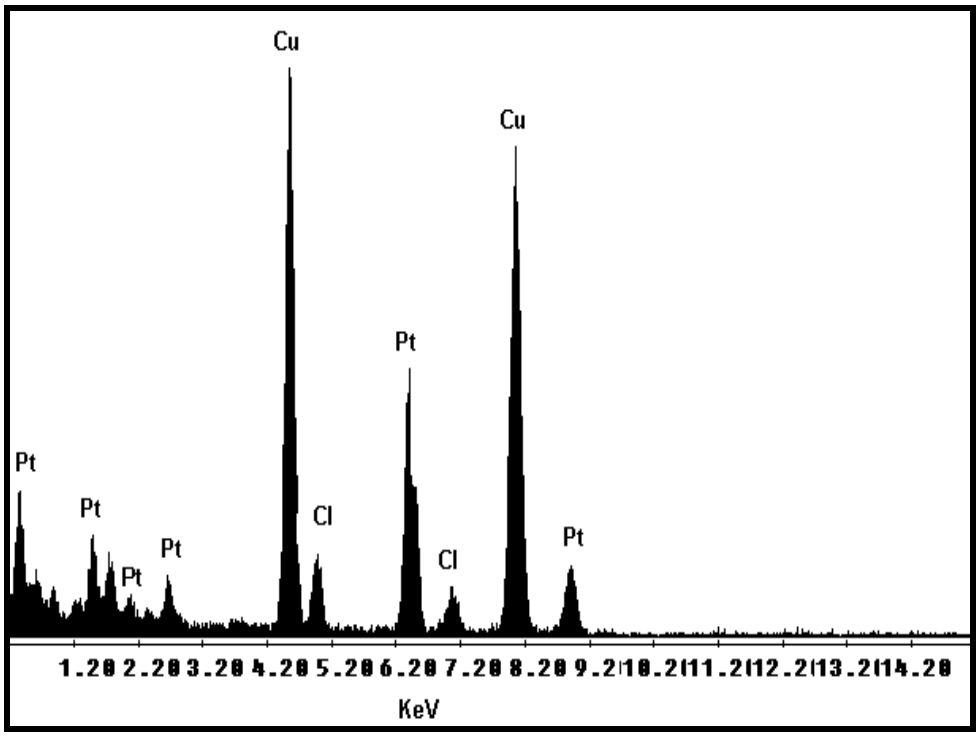
KeV

(b)



(c)



(d)

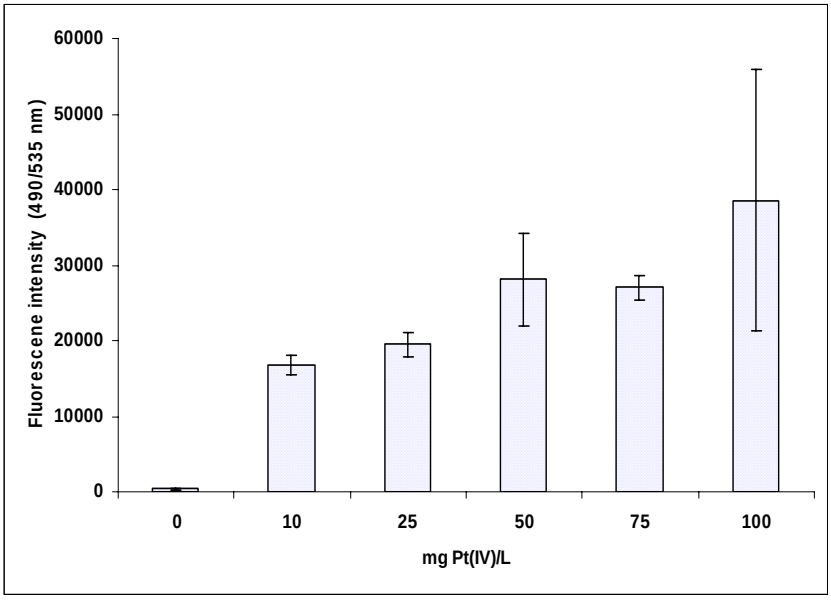
Figure 3.5: Energy dispersive X-ray diffraction analysis spectra of the thin sections of SRB cells showing strong copper and platinum peaks of the black precipitate formed during platinum reduction. (a) Spectrum of thin sections of SRB before challenged by platinum (control), (b) spectrum of thin sections of SRB after challenged by platinum for 6 h (c) for 12 h, and (d) for 18 h. These spectra were analyzed from thin sections showed in Figure 3.4.

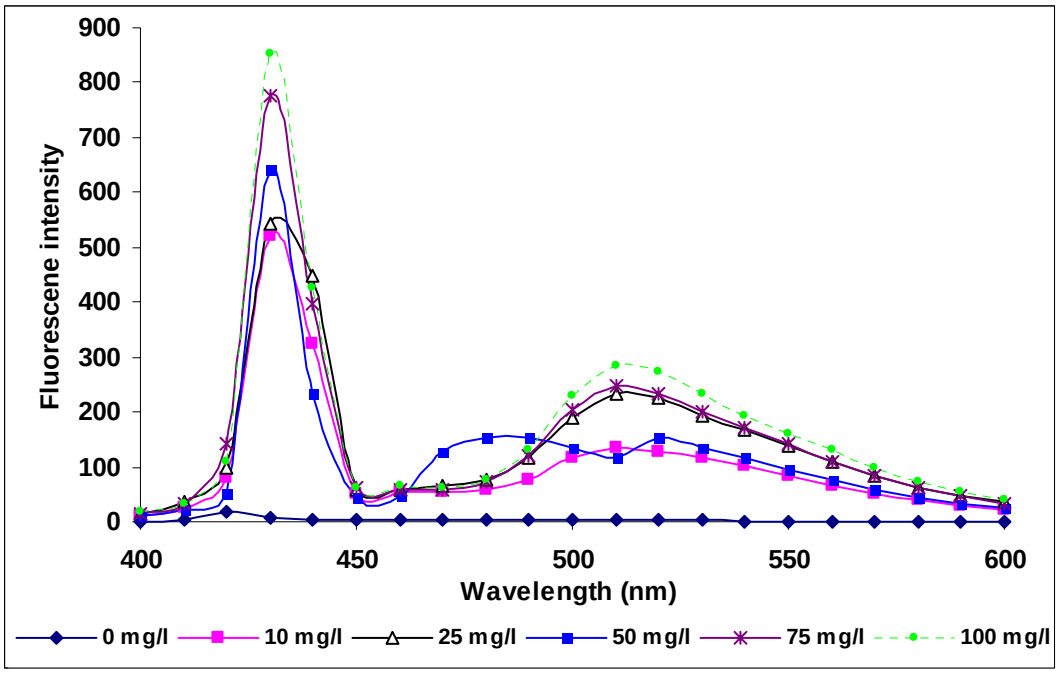
3.3.4 Effect of platinum on membrane permeability

The effect of platinum (IV) on SRB membrane permeability was measured using a fluorescence technique, based on the conversion of non-fluorescent substrate fluorescein diacetate (FDA) into a highly fluorescent product. Fluorescein diacetate is a non-polar, non fluorescent fluorescein analogue which can pass through the cell membrane whereupon intracellular hydrolases cleave off the diacetate group producing the highly fluorescent product fluorescein. An advantage of this conversion is the fact that fluorescein shows very low permeability across the cell plasma membrane, so in these experiments with intact SRB cell plasma membrane, very little fluorescent dye is lost back to the medium. The amount of fluorescein in the media is an indication of the loss of plasma membrane.

Figure 3.6 shows the effect of platinum (IV) on plasma membrane integrity of the SRB cells. The results obtained in Figure 3.6 indicate that the SRB exposure to platinum (IV) caused membrane damage. This is because the increase in platinum (IV) concentrations led to an increase in membrane disruption as marked by an increase in the fluorescence intensity in the solution. These observations provide a link to the electron dense platinum precipitates observed in Figure 3.3. Accordingly this study suggests that platinum (IV) uptake by SRB involves both surface phenomena and diffusion, the latter most likely a result of increased membrane permeability.

(a)





(b)

Figure 3.6: (a) Fluorescence determination of the supernatant of the platinum (IV) exposed cells as an indicator of the effect of platinum on plasma membrane integrity of the SRB cells. (b) Spectra of the supernatant of platinum (IV) challenged cells across the visible region (400-600 nm).

3.4 Summary

It is well documented that bacterial cells are capable of binding large quantities of metallic cations. The role of microbial cells in the fate of metals in the environment has not been thoroughly understood, though it is conceivable that they represent an important component of metal dynamics. This study has demonstrated that SRB consortium is able to interact and accumulate platinum (IV) from aqueous solutions. It appeared, however that SRB have developed a highly complex set of strategies to deal with the presence of platinum, through both direct enzymatic and an indirect non enzymatic processes. Membrane permeability studies revealed induced permeability of the plasma membrane with an increase in platinum concentration. Examination of thin sections of platinum challenged SRB cells by TEM and EDX revealed platinum-rich electron dense opaque deposits at the cell periphery, a major site for hydrogenase activity in SRB. In addition of the requirement of hydrogen as an electron donor for high platinum uptake (Section 2.3.4, Chapter 2), the observations of platinum precipitation in the periplasmic space of the cells provide the second line of evidence on hydrogenase involvement in the reduction process. This study therefore suggests that in the presence of hydrogen, SRB could

produce hydrogenases which catalyze platinum reduction to either Pt (II) or Pt (0), through the electron produced from the oxidation of hydrogen as a mechanism of detoxifying platinum toxicity and further inhibiting platinum from entering the cytoplasm. In addition to precipitation of metal by H_2S and the enzymatic mechanism postulated in this study, it is possible that there are other mechanisms involved in SRB to deal with metal presence. Enzymatic reduction of metals in SRB is poorly understood and the lack of supporting and convincing evidence of hydrogenases' involvement in the process led to a study into the isolation and purification of the hydrogenase enzyme and to get an insight into its platinum reduction mechanism *in vitro*.

CHAPTER FOUR

Extraction and Assay of Hydrogenase from a Sulphate Reducing Consortium

4.1 Introduction

The catalytic process of hydrogenases involves the following steps: (a) an activation (or production) of H_2 at the active center; (b) a transfer of two electrons between the active center and the redox partner of the hydrogenase; and (c) a transfer of two protons between the active center and the medium solvent (De Lacey *et al.,* 2000). During the last six decades, four general methods have been developed for the assay of hydrogenases, based on their ability to catalyze the following reactions (Grahame, 1988; Peck, Jr., and LeGall, 1994):

(i) Evolution of H_2:

$$D_{red} + 2H^+ \rightarrow D_{ox} + H_2 \quad \text{.....7}$$

Where the electron donor D is a low mid point (redox) potential compound, such as cytochrome c_3 or methyl viologen.

(ii) Oxidation of H_2:

$$H_2 + A_{ox} \rightarrow 2H^+ + A_{red} \quad \text{.....8}$$

Where the electron acceptor A may be either a low mid point potential compound (such as cytochrome c_3 or methyl viologen) or a higher reduction potential compound (such as ethylene blue or 2,6-dichloro-indophenol (DCIP)).

(iii) Deuterium or tritium exchange reactions with H^+, in the absence of electron donors or acceptors. The following is an example with deuterium:

$$^{2}H_2 + 2\,^{1}H_2O \rightarrow \,^{1}H_2 + 2\,^{1}H^{2}HO \quad \ldots\ldots 9$$

and

$$^{2}H_2 + \,^{1}H_2O \rightarrow \,^{1}H^{2}H + \,^{1}H^{2}HO \quad \ldots\ldots 10$$

The proportion of these two reactions depends on the type of hydrogenase, and other conditions such as pH.

(iv) Conversion of *para* to *ortho* H_2:

$$_{p}H_2\,(\uparrow\downarrow) + H_2O \rightarrow \,_{o}H_2 + (\uparrow\uparrow) + H_2O \quad \ldots\ldots 11$$

The assays for hydrogenases are also grouped according to the instrumental technique used. (i) Manomeric assay makes use of a Warbug apparatus for manomeric measurement of hydrogen evolution or uptake (Mayhew *et al.,* 1978).(ii) An amperometric assay involves the amperometric determination of hydrogen with a Clark-type hydrogen electrode (Bianco *et al*., 2001).(iii) Mass spectrometric assay (Deuterium exchange reaction) in which the evolution of masses *m/z*= 2($^{1}H_2$), 3($^{1}H^{2}H$), and 4($^{2}H_2$) in equation (9) and (10) is followed (De Lacey *et al.,* 2000). The method is based on the analysis of dissolved gases admitted to a mass spectrometer via a membrane inlet. (iv) Radio assay, in which hydrogen is exchanged with titrated water, based on the analysis of tritium in the gas phase with a gas ionization chamber. For this assay the tritium-labeled hydrogen gas is conveniently produced by the reaction of lithium metal with titrated water (Peck, Jr., and LeGall, 1994). (v) Spectrophotometer assays are based on reduction of electron acceptors with H_2. Besides cytochrome c_3, which has been suggested to be the natural substrate for [NiFe] hydrogenases, some organic dyes (such as methyl viologen, methylene blue, benzyl viologen, DCIP, and ferricyanide) are reduced with H_2 by these enzymes. The reduction of these compounds by H_2 may be followed spectrophotometrically (Peck, Jr., and LeGall, 1994; Lissolo *et al*., 2000). (vi) Another

method is based on gas chromatography analysis of hydrogen evolved by a hydrogenase from a solution of dithionite-reduced methyl viologen (Maura *et al.*, 1996). Of these methods only the principle of spectrophotometric determination of hydrogenase activity will be discussed.

4.1.1 Spectrophotometric assay (with dithionite-reduced methyl viologen)

The principle described here is for the measurement of the rate of reduction of methy1 viologen as shown in equation 12:

$$H_2 + 2MV^{2+} \rightarrow 2H^+ + 2MV^+ \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 12$$

Since the non-activated hydrogenase shows an induction period, it is necessary to add small amounts of dithionite solution to produce an absorbance at 640 nm of 0.1-0.2 before adding the enzyme (Cammack *et al.,* 1986). This serves to remove residual traces of oxygen, and to reduce the hydrogenase at the start of the reaction. Dithionite is a sufficiently strong reducing agent for H_2 production, and it is capable of reducing the metal centers in the enzyme. However, the reduction rate is too slow for a convenient assay of enzyme activity, hence the need for electron transferring mediators such as methyl viologen. A serious drawback of the use of dithionite as a reductant for methyl viologen in this assay is the pH dependence of dithionite to its reduction potential, in contrast to the pH dependence to the oxidized/reduced methyl viologen (MV^{2+}/MV^+) redox potential. As a consequence the concentration of MV^+ in equilibrium with a fixed concentration of dithionite is pH dependent; dithionite is ineffective in the reduction of methyl viologen at pH values below 6.5 (Peck, Jr., and LeGall, 1994). It is very important to note that the reduced form of methyl viologen is readily oxidized in air (auto-oxidation) and so experiments should be carried out under anaerobic conditions.

4.1.2 Cell disintegration and extraction

Most of the proteins and enzymes studied in the early days of protein chemistry were isolated from extracellular fluids. This is probably because extracellular enzymes for the most part are more stable, often as a result of disulphide cross links, and they tend to be

small proteins. Most of the enzymes, however, are found inside the cells, and are often far less stable; disulphides are generally absent because of a more reducing intracellular environment. In order to be able to isolate, purify and characterize enzymes an efficient method of cell disruption, which is not harmful to the enzyme of interest, must be used (Scopes, 1982). The aim of this section was to determine the best extraction method for the hydrogenases.

4.2 Materials and Methods

4.2.1 Materials

Gases were from Afrox (South Africa). Absorbances were measured using a Shimadzu visible 160A spectrophotometer and PowerWave X (Bio-Tek, Instrument INC, South Africa). Cell disruption was performed with a Yeda-press (LINCA Lamon Instrumentations Co., Ltd. Tel-Aviv), and Virsonic-100 sonicator (VirTis, Co, Inc, USA). Centrifugation was achieved using a Beckman, J2-21 centrifuge with a JA-20 rotor. Samples were stirred using a magnetic stirrer (Snijder, South Africa). Detergents, sodium cholate, (Triton X-100 (Polyoxyethylene [9-10] ρ-*t*-ocytl phenol)), and other analytical-grade reagents were purchased either from Sigma or Merck, South Africa.

4.2.2 Methods

4.2.2.1 Culture

The SRB inoculum 10% (v/v) was grown in a 1L modified Postgate medium C incubated in the dark at 30 ^{o}C while gently shaking at 100 rpm, until mid stationary phase (six days) as previously described (Section 2.2.2.3, Chapter 2).

4.2.2.2 Hydrogenase activity assay

Hydrogenase activity was determined spectrophotometrically at 25 ^{o}C with methyl viologen (ε_{604nm} = 13.9 mM^{-1} cm^{-1}) as the electron acceptor and hydrogen gas as the electron donor with Tris-HCl (20 mM, pH 7.8) as the buffer system. This well established procedure is specific for hydrogenase activity (Peck, Jr. and Gest, 1956; Lissolo *et al.*,

2000; De Lacey *et al.,* 2000). The reaction was carried out in a specially designed anaerobic cuvette equipped with side arm for enzyme incubation and a rubber stopper to allow direct injection.

The reaction mixture: Methyl viologen solution (1 mM, 3 ml) in Tris-HCl (20 mM, pH 7.8) buffer was placed into a cuvette. H_2 was bubbled into the solution through the needle in the rubber septum for 15 min at (1 atm. $30ml^{-1}$. min^{-1}). This was followed by the addition of sodium dithionite (100 mM, 5 µl) in Tris-HCl (20 mM, pH 7.8) in order to eliminate any residual oxygen in the cuvette. Finally 100 µl of pre-activated hydrogenase suspension was added and the kinetics of the reduction of methyl viologen was measured at 604 nm. Complete reaction mixture including ddH_2O instead of the enzyme suspension served as a blank. The methyl viologen reduction rate was measured under a N_2:CO_2 (80:20) atmosphere instead of H_2 as a reference control. The baseline activity was subtracted to evaluate the true hydrogenase activity. One unit of activity is defined as the amount of hydrogenase that catalyzes the reduction of 1µmol of methyl viologen per min in the presence of excess hydrogen.

Activation of hydrogenase: H_2 gas (1 atm. $30ml^{-1}$. min^{-1}) was bubbled through a cell free hydrogenase suspension for 20 min before being transferred to the reaction mixture.

4.2.2.3 Protein determination

Protein concentration was determined according to the method of Bradford (1976), using bovine serum albumin (BSA) as a standard. The reaction was carried out as follows: 5 µl of the sample was added to a microtiter plate, followed by the addition of 250 µl Bradford reagent. The absorbances were the determined at 595 nm. Complete reaction mixture containing ddH_2O, instead of reaction sample, served as a blank. The calibration curve is shown in *Appendix B1*.

4.2.2.4 Cell fractionation

4.2.2.4.1 Detergents

The membrane-containing pellet from centrifugation (5000 x g, 15 min, 4 ^{o}C) were suspended in minimal volume of Tris-HCl buffer (20 mM, pH 7.0, 100 ml) containing either sodium cholate (3% w/v) or Triton X-100 (3% v/v). This slurry was stirred gently overnight at 4 ^{o}C, centrifuged (18 000 x g, 20 min, 4 ^{o}C), and both pellet (resuspended in minimal volume of Tris-HCl buffer (20 mM, pH 7.0) and supernatant fractions were extensively dialyzed for 12 h against ddH_2O to remove the detergent, and finally analyzed for protein and hydrogenase activity.

4.2.2.4.2 French Press

The bacterial pellets from centrifugation (5000 x g, 15 min, 4 ^{o}C) were suspended in minimal volume of Tris-HCl (20 mM, pH 7.0, 100 ml) and cells were disrupted by two passages through a Yeda-press at 15 MPa, 4 ^{o}C at a flow rate of one drop per second, followed by centrifugation (18 000 x g, 20 min, 4 ^{o}C). Both pellet and supernatant fractions were assayed for protein and hydrogenase activity.

4.2.2.4.3 Sonication

The bacterial pellet from centrifugation (5000 x g, 15 min, 4 ^{o}C) were suspended in minimal volume of Tris-HCl (20 mM, pH 7.0, 100 ml) and cells were broken by sonication at 4 ^{o}C on ice at 30 sec cycles using a Virsonic-100 sonicator, energy setting 80W, followed by centrifugation (18 000 x g, 20 min, 4 ^{o}C). Both pellet and supernatant were assayed for protein and hydrogenase activity.

4.3 Results and Discussion

The results were expressed as a relative percentage, so as to be able to correlate the activity of hydrogenase obtained by the variety of extraction techniques. Because the majority of activity was in the pellet fraction in all cases before any cell disruption treatment (Figure 4.1, 4.2 and 4.3b), the activity in the raw pellet fraction was therefore considered as 100%.

4.3.1 Detergents

The first attempt to extract the hydrogenase was carried out using detergents, which are used mainly for the extraction and purification of membrane proteins. They are capable of displacing proteins which are tightly bound by the hydrophobic forces within a membrane, firstly by dissolving the membrane, and secondly by replacing the membrane with aliphatic or aromatic chains to form the lipophilic part of the detergent (Hjelmeland and Chrambach, 1984). In order to solubilize membrane bound hydrogenase two detergent treatments were carried out using ionic (sodium cholate) and nonionic (Triton X-100) detergents. Ionic detergents are those detergents that contain charged head groups either positively charged (cationic detergent) or negatively charged (anionic detergents), while nonionic detergents have uncharged hydrophilic head groups (Helenius and Simons, 1975).

Figure 4.1 shows the release of the hydrogenase from SRB cells using sodium cholate and Triton X-100. Treatment with the former resulted in an increase in hydrogenase activity in the supernatant fraction (60.8%) with only 8.5% of the activity remaining in the pellet fraction. The same trend was observed with Triton X-100 where 49.8% of the activity was detected in the supernatant fraction, while the activity in the pellet fraction decreased to 17.0%. Taken together these findings indicate 30.7% and 33.2% loss of activity with sodium cholate and Triton X-100 extraction respectively. This is major loss of activity considering the fact that one-third of total hydrogenase activity cannot be recovered by use of this technique. The cause of the high loss of activity is not clear but there is one possible explanation. Detergents are known to cause decreases in the activity of the enzymes, possibly by chemically reacting with the active sites of the enzymes, thereby changing their conformational integrity (Helenius and Simons, 1975; Helinius *et al*., 1979; Bordier, 1981).

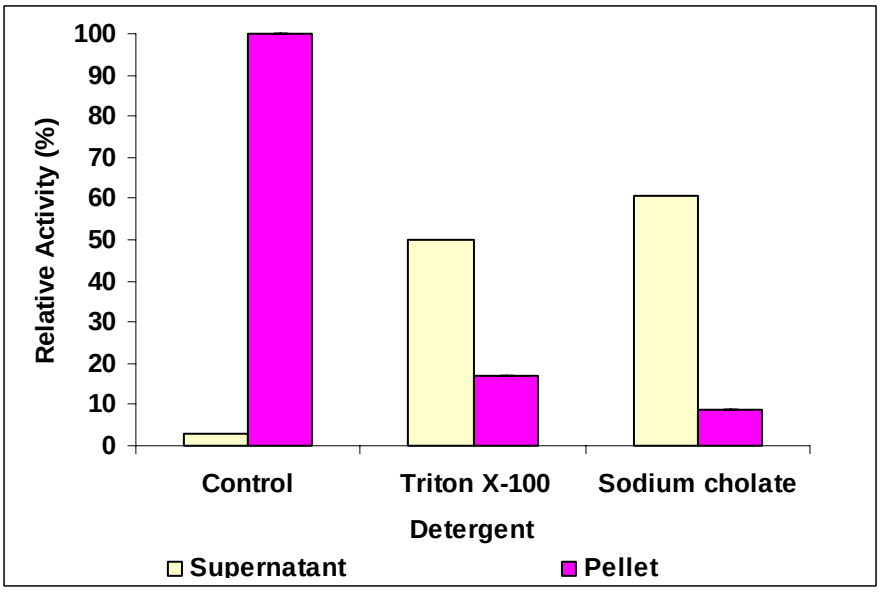


Figure 4.1: The release of hydrogenase activity from a sulphate reducing consortium using detergents. Net enzymatic activity was obtained by subtracting non-enzymatic activity measured in presence of N_2 (control) instead of hydrogen.

4.3.2 The French Pressure cell (French Press)

The French laboratory press is a widely used method for cell disruption (bacterial cells in particular). It uses high pressure, where the internal French pressure and cell pressure increases as the pressure developed by the laboratory press increases. The intracellular pressure increases as well. As the sample is dispensed through the sample outlet tube, the external pressure on the cell walls drops rapidly toward atmospheric pressure. The pressure within the cell drops but not as quickly as the pressure external to the cell. These pressure differentials cause the cell wall membrane to burst, releasing the intracellular contents. The now free cellular artifacts can be collected and separated as required (van Renswoulde and Kempf, 1984).

Figure 4.2 demonstrates the release of hydrogenase from SRB cells by French Press. After the first passage of SRB cell through a laboratory French Press, hydrogenase activity increased to 34.6% in the supernatant fraction, with the major fraction of activity still remaining in the pellet (69.7%). In an attempt to obtain more of the activity released in the supernatant fraction, a second passage was carried out. This time the hydrogenase

activity increased to 70.0% in the supernatant fraction, with concomitant decrease in activity of pellet fraction to 12%.

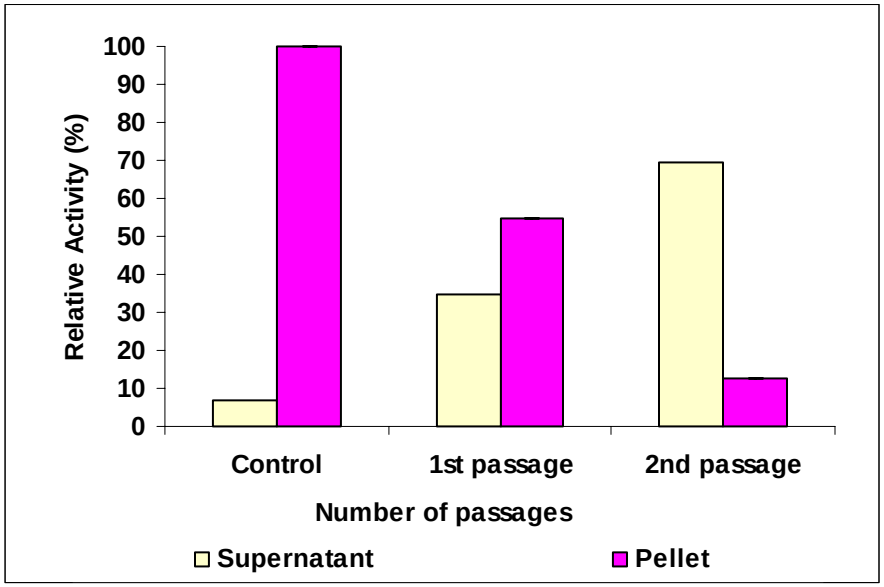


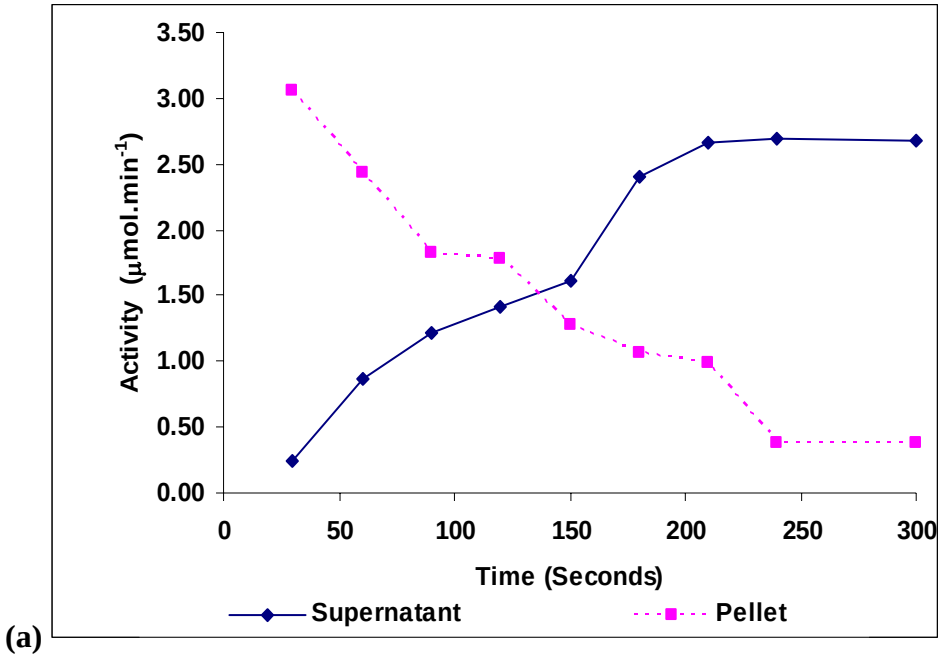
Figure 4.2: The release of hydrogenase activity from a sulphate reducing consortium by French pressure cell (French Press). Net enzymatic activity was obtained by subtracting non-enzymatic activity measured in presence of N_2 (control) instead of hydrogen.

The French Press offers some major advantages, because of the fact that the biochemical integrity of the sample is maintained and the sample is not heated. In addition the system has a pressure range up to 40000 psi which can be manually set to maximize cell disruption and minimize extracted protein degradation.

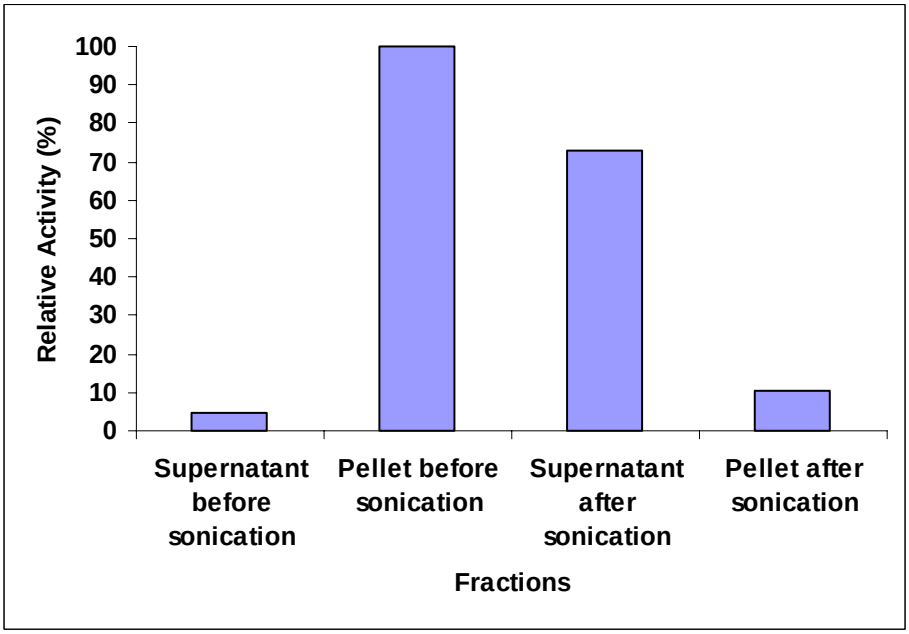
4.3.3 Sonication

Cell lysis by sonication has been widely used for extraction of enzymes. This technique uses high frequency sound that is produced electronically and transported through the metallic tip to an appropriate concentrated cellular suspension, which leads to mechanical shearing of the cell wall. The concept of ultrasonic cell disruption is based on the creation of cavities in a cell suspension by a rapid formation of microbubbles which grow and coalesce until they reach their resonant size, vibrate, and eventually collapse, thereby disrupting the cell. Maximum shearing is needed to achieve maximum lysis, though it

should be below the level where there is formation of foam in the solution, since the solution will aerate, leading to protein denaturation (Bollag and Edelstein, 1991).



(a)



(b)

Figure 4.3: The release of hydrogenase activity from a sulphate reducing consortium using sonication: (a) observed hydrogenase activity with increased sonication time over time, (b) percentage relative activity of the fractions before and after sonication. Net enzymatic activity was obtained by subtracting non-enzymatic activity measured in presence of N_2 (control) instead of hydrogen.

Some major drawbacks with this technique are the generation of heat, free radicals and ions which may cause denaturation as the time of contact increases. This technique however uses an empirical approach in order to determine the best frequency and the time needed for disruption of a particular cell (Harris and Angal, 1994).

Figure 4.3 demonstrates the efficiency of sonication in extraction of the hydrogenase from SRB cells over the sonication time. The sonicated supernatant fraction exhibited an increase in hydrogenase activity with increased sonication time, with concomitant decrease of activity in the pellet fraction. About 73.0% of hydrogenase activity was observed in the sonicated supernatant fraction after 4 min, before starting to level off. The remaining activity in the pellet after sonication was 10.3%, indicating that about 16.7% of the activity was lost by this technique. This is the least activity loss observed so far, in comparison with other disruption techniques.

4.4 Summary

Using a variety of cell disruption techniques, fractionation studies were performed to determine the location of hydrogenases within the SRB consortium, i.e. whether they are extracellular (raw supernatant) or intracellular (within the cell or membrane bound). Very low hydrogenase activity was detected in the extracellular fraction. This lack of extracellular activity was not surprising, because *in situ*, the hydrogenase substrate (molecular hydrogen), is produced in the cytoplasm which then diffuses across the cytoplasmic membrane, to be oxidized by the periplasmic hydrogenase (Cammack, 1995; Happe *et al*., 1997; Nandi and Sengupta, 1998). This indicates that very few hydrogenase activities take place extracellularly. The presence of high hydrogenase activity in the raw pellet (i.e. before any cell disruption treatment) was equally not surprising given the fact that hydrogenase substrate (hydrogen) is a very small molecule which can easily diffuse through the cell wall to the active site of the enzyme without any steric hindrance to the active region of the enzyme (Fontecilla-Camps *et al.,* 1996). This suggests that substrate permeability to the active region of the enzyme may not be such a major problem. Highest hydrogenase activity was obtained in the supernatant fractions after cell disruption in all treatment processes. The increase in hydrogenase activity in these

fractions is believed to be due to the loss of cellular integrity, presumably making substrate more available to cellular hydrogenases and resulting in an increase in catalytic rate. Taken together these findings suggest that the majority of hydrogenases are soluble proteins located intracellularly, principally in the periplasm of the cell, and corroborate the work of previous authors (Fauque *et al*., 1988). Since a little of the activity was observed in the pellet after disruption even with the detergent treatment, it is possible that some of the enzyme is membrane bound. These observations are in accordance with some of the literature showing a diverse localization of hydrogenase in different species. For example, hydrogenases from *D. vulgaris, D. desulfuricans* (NRC 49001) and *D. gigas* are periplasmic (Odom and Peck, Jr. 1984; Hausinger, 1987; Peck, Jr. and LeGall, 1994), while hydrogenases from D. *desulfuricans* (ATCC), and *D. multispirans* are cytoplasmic (Czechowski *et al.*, 1984; Odom and Peck, Jr, 1984). In contrast, hydrogenases from *D. desulfuricans* (Norway 4) and *D. baculalus* are membrane bound (Odom and Peck, Jr. 1984; Lissolo *et al*., 1986; Teixeria *et al.*, 1987).

In order to evaluate the best extraction technique used in this study a comparison was performed (Table 4.1). Because of the highest activity obtained in the soluble form (73%) and minimal loss of activity (10.3%) obtained with sonication, this technique technique was chosen as the best extraction method and utilized in all subsequent cell disruption experiments. As Scopes (1982) perceptively stated, " *A true enzymologist is one who is rarely concerned with where the enzyme comes from, but only want as much as possible of it from the most convenient source"*.

Table 4.1: Summary of hydrogenase activity released by different cell disruption technique

Treatment methods	Activity ($Pellet_A$) (%)	Activity ($Suparnatant_B$) (%)	Activity ($Pellet_B$) (%)	Activity Lost (%)
Detergent: *Sodium cholate*	100	60.8	8.5	30.7
: *Triton X-100*	100	49.8	17.0	33.2
French press	100	69.7	12.8	17.5
Sonication	100	73.0	10.3	16.7

Activity ($Pellet_A$) = Activity in the pellet before disruption; Activity ($Suparnatant_B$) = Activity in the supernatant after treatment; Activity ($Pellet_B$) = remaining activity in the pellet after disruption.

CHAPTER FIVE

Purification and Partial Characterization of Hydrogenases from a Mixed Sulphate Reducing Consortium

5.1 Introduction

Protein purification is one of the most important steps in studying physical and biological properties of the protein. Generally protein purification is governed by three factors, quality, quantity and cost (Wilson and Walker, 1994). Because of the fact that many proteins are denatured by contact with an air-water interface and at low concentrations, a significant fraction of the protein present may be lost by adsorption to surfaces. Hence a protein solution should be handled in a relatively concentrated state throughout the purification procedures. There are, of course, other influences to which a protein may be sensitive, including oxidation of the cysteine residues to form disulphide bonds; heavy metal contaminants which may irreversibly bind to the protein, the salt concentration and polarity of the solution which must be kept within the stability range of protein (Garrett and Grisham, 1999).

Like other metalloenzymes, hydrogenases are extremely sensitive to inactivation by oxygen, high temperature and other environmental factors. These properties are not favorable for potential biotechnological applications. Thus, the purification and characterization of hydrogenase would provide valuable information to optimize the conditions for application of hydrogenase in effluent treatment process. The objectives of this section include:

(i) To purify hydrogenase from a mixed SRB culture.

(ii) To characterize purified hydrogenase with respect to temperature and pH.

(iii) To study the influence of heavy metal ions on hydrogenase activity.

(v) To establish catalytic properties of hydrogenase such as K_m and V_{max}.

5.2 Materials and Methods

5.2.1 Materials

Solid ammonium sulphate; Prestained SDS-PAGE standard markers; Coomassie brilliant Blue R-250 protein staining solution; Coomassie dye protein reagent kit for protein determination and Dialysis tubing (16 mm cut off, 10 KDa) were purchased from Sigma. Sephacryl S-100 was from Pharmacia Fine Chemicals. Toyopearl-Super Q 650S; RediFrac, fraction collector were from Pharmacia Biotetch. Power Pac 300 was obtained from Bio-Rad. Modulyo freeze dryer was from Edwards. Other common biochemical reagents were purchased either from Merck or Sigma. Absorbances were measured using a Shimadzu visible 160A spectrophotometer. Gases were from Afrox. Centrifugation was achieved using a Beckman J2-21 centrifuge with a JA-20, or JA-10 rotors.

5.2.2 Purification methods

5.2.2.1 Culture

Cells were cultured in a large reactor flask (5 L) containing modified Postgate medium C (*Appendix A6*), incubated in the dark at 30 ^{o}C while shaking at 100 rpm, until mid stationary phase was reached (six days) as previously described (Section 2.2.2.3, Chapter 2).

5.2.2.2 Hydrogenase activity and protein determination

Hydrogenase activity was assayed spectrophotometrically, by the evolution of methyl viologen, reduced by sodium dithionite at 25 ^{o}C as previously described (Section 4.2.2.2, Chapter 4). Protein was quantified by the Bradford method, using BSA as a standard as previously described (Section 4.2.2.3, Chapter 4).

5.2.2.3 Purification of hydrogenase

The purification procedure reported here is the one which showed the best results from several attempts made in this study. Purification was carried out at 4 ^{o}C under aerobic conditions except the assay for activity which was carried out anaerobically. Unless otherwise stated, the buffer used was Tris-HCl (20 mM, pH 7.6). A procedure for regeneration and equilibration of the columns is outlined in (*Appendix C1*).

5.2.2.3.1 Preparation of crude homogenate

The bacterial pellet (15 g wet weight) obtained by centrifugation (5000 x g, 15 min, 4 ^{o}C) was suspended in buffer (110 ml) and the cells were broken by sonication (4 ^{o}C, 30 sec cycles) as previously described (Section 4.2.2.4.3, Chapter 4). The lysate was centrifuged (10 000 x g, 15 min, 4 ^{o}C) to remove unbroken cells and debris resulting in crude extract, and the supernatant further centrifuged (18 000 x g, 30 min, 4 ^{o}C) to remove membrane-associated material, generating a soluble extract which was then assayed for protein and hydrogenase activity (this fraction is referred to as SA1).

5.2.2.3.2 Ammonium sulphate precipitation

To the crude soluble extract (SA1), solid ammonium sulphate was slowly added with gentle stirring over the period of 30 min to yield a final 65 % saturation. The mixture was further stirred gently (60 min) and centrifuged (18 000 x g, 30 min; 4 ^{o}C), the supernatant fraction was decanted and the pellet dissolved in Tris-HCl buffer (20 mM, pH 7.6), followed by dialysis overnight against the same buffer and the dialysate analyzed for protein and hydrogenase activity (This fraction is referred to as SA2).

5.2.2.3.3 Toyopearl-Super Q 650S ion exchanger column

Hydrogenase containing fraction (SA2) was applied onto a Toyopearl-Super Q 650S ion exchanger column (2.5 x 25 cm) equilibrated with Tris-HCl buffer (20 mM, pH 7.6). The unbound protein was washed from the column with buffer until the absorbance at A_{280nm} of eluate had reached base line, followed by a stepwise (0-1M in buffer) NaCl-gradient elution, at a flow rate of 1ml.min^{-1}. Fractions (5ml) were collected and monitored for protein at A_{280nm} and hydrogenase activity (This fraction is referred to as SA3).

5.2.2.3.4 Gel filtration on Sephacryl S-100

The Toyopearl-Super Q 650S fraction (SA3) exhibiting hydrogenase activity were pooled together, and then concentrated by freeze drying and the freeze dried sample redissolved in minimal volume of buffer (3 ml). The concentrated fraction was applied onto a Sephacryl S-100 column (1.5 x 20 cm). The column was pre-washed with buffer until A_{280nm} of eluate had reached the baseline and equilibrated with the same buffer before the loading of the sample. The samples were eluted with the same buffer and fractions (5ml) were collected at a flow rate 1ml.min^{-1} and monitored for protein at A_{280nm} and hydrogenase activity. This fraction is referred to as SA4. The fraction exhibiting hydrogenase activity was analyzed by SDS-PAGE.

5.2.2.4 SDS-PAGE analysis

The molecular weight of purified hydrogenase from both purification procedures was determined by SDS-PAGE according to the method of Laemmli (1970). A purified sample (10 µl), together with SDS-PAGE standard molecular weight (26.6 - 116 KDa) markers (10 µl) were electrophoresed on 12% SDS-PAGE at 250 V. The gels were stained with coomassie brilliant blue R-250 staining solution (*Appendix C2*), then destained in methanol-acetic acid-water (1:1:8 v/v/v) destaining solution (*Appendix C2*). The molecular weight of the purified hydrogenase was correlated from calibration curve of log molecular weight versus distance migrated (*Appendix C3*).

5.2.3 Characterization methods

5.2.3.1 Effect of electron acceptors

The hydrogenase assay was performed under the following different electron acceptor conditions: 0.1mM Dichloroindolephenol (ε_{604nm} = 20.6 mM^{-1}.cm^{-1}), 1.0 mM Ferricyanide (ε_{604nm} = 1.04 mM^{-1}.cm^{-1}), 5.0 µM Cytochrome c_3 (ε_{604nm} = 111 mM^{-1}.cm^{-1}) and 1.0 mM Methyl viologen (ε_{604nm} = 13.9 mM^{-1}.cm^{-1}) as a positive control. Electron acceptor (3 ml) in Tris-HCl (20 mM, pH 7.8) buffer was placed into a cuvette; H_2 was bubbled into the solution through a needle inserted through a rubber septum (20 min, 1atm. 30ml^{-1}. min^{-1}). This was followed by the addition of sodium dithionite (5µl, 100 mM) in Tris-HCl (20

mM, pH 7.8), in order to eliminate residual oxygen in the cuvette. Finally, pre-activated hydrogenase suspension (100 µl, 0.11 $mg.l^{-1}$) was added and the kinetic reduction of these redox partners of the hydrogenase was measured at their respective wavelengths. Blanks, control samples and activation of enzyme were prepared as previously described (Section 4.2.2.2, Chapter 4).

5.2.3.2 pH profile

The following buffers were used to establish the pH profile of the purified hydrogenase: sodium acetate (20 mM, pH 4.0-4.5); sodium phosphate (20 mM, pH 6.0-6.5); Tris-HCl (20 mM, pH 7.0-9.0) and carbonate/bicarbonate buffer (20 mM, pH 9.5-11.0). To determine the pH optimum of hydrogenase, electron acceptor solution (3 ml) was prepared in different buffer conditions, bubbled with hydrogen (20 min, 1atm. $30ml^{-1}.min^{-1}$), followed addition of sodium dithionite (5 µl, 100 mM), and the reaction was started by addition of pre-activated hydrogenase solution as previously described (Section 4.2.2.2,Chapter 4).

5.2.3.3 Temperature profile

The temperature optimum of the hydrogenase was determined over a temperature range of 10 to 70 ^{o}C. The reaction mixture was prepared as previously described (Section 4.2.2.2, Chapter 4). The reaction was started by the addition of hydrogenase suspension pre-activated by bubbling with H_2 (20 min, 1atm. $30ml^{-1}.min^{-1}$) at the different temperature ranges before being transferred to the reaction mixture.

5.2.3.4 Thermal stability profile

A stability profile of hydrogenase was performed at optimum pH and temperature. An electron acceptor solution (methyl viologen and cytochrome c_3) was prepared at pH 7.5, see page 106) in 20 mM Tris-HCl buffer, bubbled with H_2 (15 min, 1atm. $30ml^{-1}.min^{-1}$), followed by the addition of sodium dithionite (5 µl, 100 mM), and the reaction was started by addition of hydrogenase suspension pre-activated by bubbling with H_2 (20 min, 1atm.$30ml^{-1}.min^{-1}$) at optimum temperature (35 ^{o}C, see page 107) before being

transferred to the reaction mixture. The hydrogenase activity was then monitored over 1h at 10 min intervals for 1 h.

5.2.3.5 Effect of metal ions

Electron acceptor (3 ml) in Tris-HCl (20 mM, pH 7.8) buffer was bubbled with hydrogen, followed by the addition of sodium dithionite (5 µl, 100 mM) as previously described (4.2.2.2, Chapter 4). The reaction was started by addition of hydrogenase suspension pre-activated by bubbling with H_2 (20 min, 1atm. $30ml^{-1}$. min^{-1}) under different metal concentrations: 0 - 1 mM [Fe (III), Zn (II), Ni (II) and Cu (II)].

5.2.3.6 Electron acceptor dependent hydrogenase activity

The catalytic activity of hydrogenase at different concentrations of electron acceptors was studied. Electron acceptor solutions were prepared at different concentrations (0 - 4.0 mM methyl viologen and 0-14 µM cytochrome c_3) in 20 mM Tris-HCl buffer, bubbled with hydrogen, followed by addition of sodium dithionite (5 µl, 100 mM). The reaction was started by addition of pre-activated hydrogenase suspension (100 µl, 0.11 $mg.l^{-1}$) as previously described (Section 4.2.2.2, Chapter 4).

5.3 Results and Discussion

5.3.1 Purification

5.3.1.1 Hydrogenase purification

The purification of hydrogenases in previous studies has been hampered by their oxygen sensitivity and the need to pre-activate the enzyme before assaying for activity (Henry *et al.,* 1980). In view of these facts it was decided to empirically select a fast purification procedure comprising of minimal steps, while not compromising the purity of the enzyme. A summary of the hydrogenase purification with ammonium sulphate precipitation (65%), Toyopearl-Super Q 650S ion exchange, and Sephacryl S-100 size exclusion chromatography is shown in Table 5.1. In order to precipitate the hydrogenase, the crude homogenate (SAl), with 26.6 mg total protein and total hydrogenase activity of

580.4 U was subjected to ammonium sulphate precipitation (65%) saturation. Ammonium sulphate increased the specific activity from 21.8 to 23.4 U.(mg protein)$^{-1}$ with 88.0% of recovered activity. This precipitation step managed to remove minor fractions of contaminated proteins as judged by a minimal decrease in total protein from 26.6 to 21.8 mg, and a concomitant decrease in total activity from 580.4 to 510.8 U. No significant purification fold occurred in this step as evaluated by an increase in purification factor from 1 to 1.07 fold. In order to avoid salt interference in further purification steps, ammonium sulphate was removed by dialysis. Ammonium sulphate dialysate fraction (SA2) resulted in a decrease in total hydrogenase activity from 510.8 to 480.2 U. The specific activity increased from 23.4 to 29.3 U.(mg protein)$^{-1}$, with a 1.34 fold increase in purity and 82.7% activity recovered (Table 5.1). For further purification, ammonium sulphate dialysate fraction was loaded onto a Toyopearl Super Q 650S ion exchange column. The elution profile of the hydrogenase from Toyopearl-Super Q 650S ion exchange column chromatography is shown (Figure 5.1). One distinct protein peak measured at A_{280nm} was observed (SA3) showing corresponding hydrogenase activity. The hydrogenase was eluted at high concentration of NaCl (0.75M), indicating that the enzyme bound tightly to the resin.

Table 5.1: Purification table of hydrogenase

Step	Volume (ml)	Total Protein (mg)	Total activity (U)	Specific Activity (U.mg^{-1})	Recovery (%)	Purification (Fold)
Crude fraction (SA1)	80	26.6	580.4	21.8	100	1.00
Ammonium sulphate (65%)	75	21.8	510.8	23.4	88.0	1.07
Dialysis (SA2)	66	16.4	480.2	29.3	82.7	1.34
Toyopearl-Super Q 650S (SA3)	50	11.0	401.0	36.5	69.1	1.67
Sephacry1 S-100 (SA4A)	10	1.3	120.1	92.3	20.7	4.23
Sephacryl S-100 (SA4B)	15	1.7	180.6	109.5	31.1	5.02

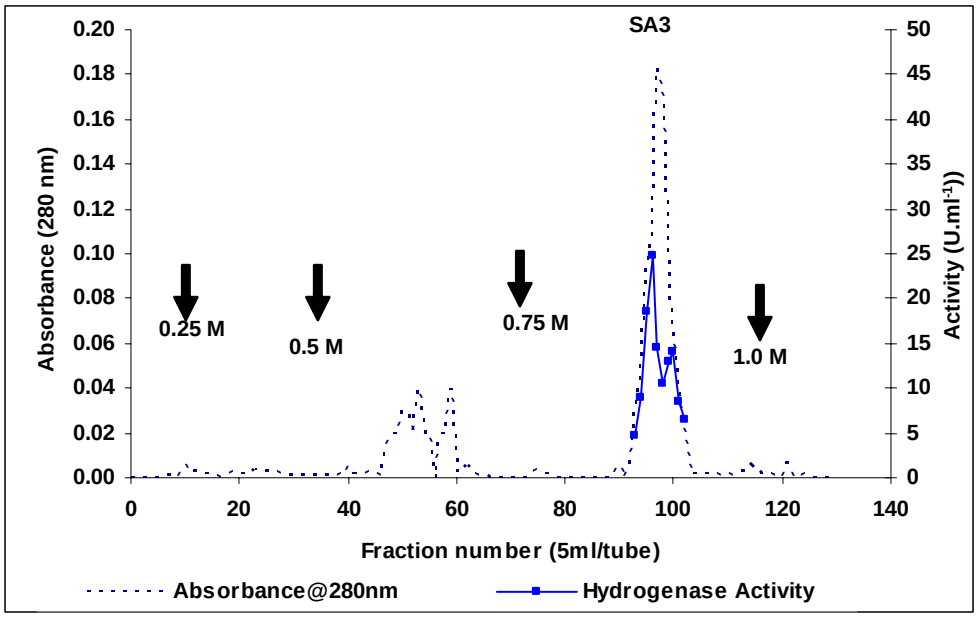


Figure 5.1: Toyopearl-Super Q 650S ion exchange chromatography of ammonium sulphate fraction (SA2). Column dimension: (2.5 x 25cm); Flow rate: 1 ml.min^{-1}. The hydrogenase activity was eluted with stepwise addition of 0-1 M NaCl in Tris-HCl buffer (20 mM; pH 7.6). Arrows indicate stepwise increase in NaCl. SB3 fraction refers to the peak (tube number 93-102) that showed hydrogenase activity which were pooled together for further purification.

The pooled hydrogenase-containing fraction (SA3) showed a decrease in total activity from 480.2 to 401.0 U, and a concomitant total protein decrease from 16.4 to 11.0 mg (Table 5.1). The specific activity of this Toyopearl-Super Q 650S pooled fraction increased to 36.5 U.(mg protein)$^{-1}$, with 69.1% of recovered activity, indicating that this purification step resulted in about 19% loss of activity. The purification factor increased to 1.67 fold indicating a minor improvement in hydrogenase purification (Table 5.1). For further purification the fraction was subjected to size exclusion on Sephacryl S-100.

Pooled fractions containing hydrogenase activity from the Toyopearl-Super Q 650S column were concentrated by freeze drying (3 ml) and showed a specific activity of 63.5. U.(mg protein)$^{-1}$. The exclusion profile of the hydrogenase from Sephacryl S-100 chromatography is shown (Figure 5.2). Two protein peaks were produced (SA4A and SA4B) measured at A_{280nm} each with hydrogenase activity. These two fractions were pooled separately and tested for purity by SDS-PAGE. This separation step led to a drastic decrease in total activity from 401.0 to 120.1 and 180.6 U and total protein from

11.0 mg to 1.3 and 1.7 mg for fraction SA4A and SA4B respectively (Table 5.1). A major improvement in specific activity of the two fractions to 92.3 and 109.5 U.(mg protein)$^{-1}$ for peak SA4A and SA4B respectively was observed. Recovered activity of 20.7% and 31.1% and an increase in purification factor to 4.23 and 5.02 for fraction SA4A and SA4B respectively was obtained. From Table 5.1 it can be seen that Sephacryl S-100 size exclusion chromatography is a most successful step in the purification of the hydrogenase. To confirm its purity and to estimate the molecular size these fractions, together with those from other steps, were analyzed by SDS-PAGE.

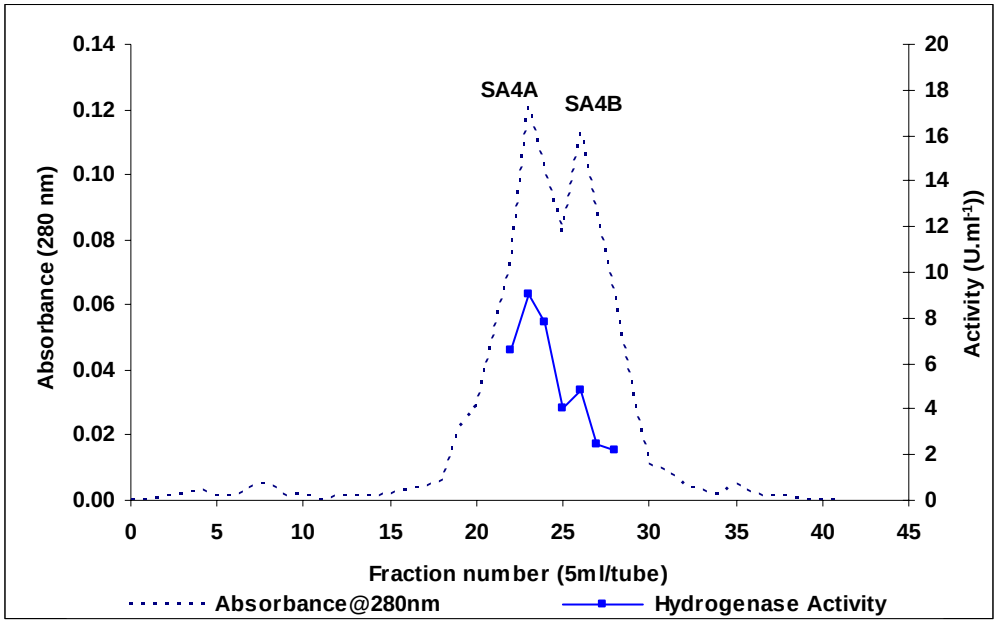


Figure 5.2: Sephacryl S-100 size exclusion chromatography of pooled fractions (SA3). Column dimension: (1.5 x 20 cm); Flow rate: 1 ml.min^{-1}. SA4A and SA4B fractions refer to the peaks (tube number 22-24) and (tube number 25-28) respectively, that showed potential hydrogenase activity which were pooled together for further analysis of molecular weight.

5.3.1.2 Molecular weight determination by SDS-PAGE

The analysis of fractions showing hydrogenase activity at different purification steps by 12% SDS-PAGE gel is shown in Figure 5.3. The hydrogenase appeared to be monomeric, since the results of the SDS-PAGE analysis of the final purification step showed a single distinctive protein band corresponding to a molecular weight of 58 KDa (Figure 5.3, lane 6 and 7). This molecular weight is in the same order of magnitude as that of the hydrogenase from sulphate reducing bacterial origin (Odom and Peck, Jr., 1984). Both

monomeric protein bands observed in Figure 5.3 (lane 6 and 7) may be either NiFe-hydrogenase or Fe-hydrogenase.

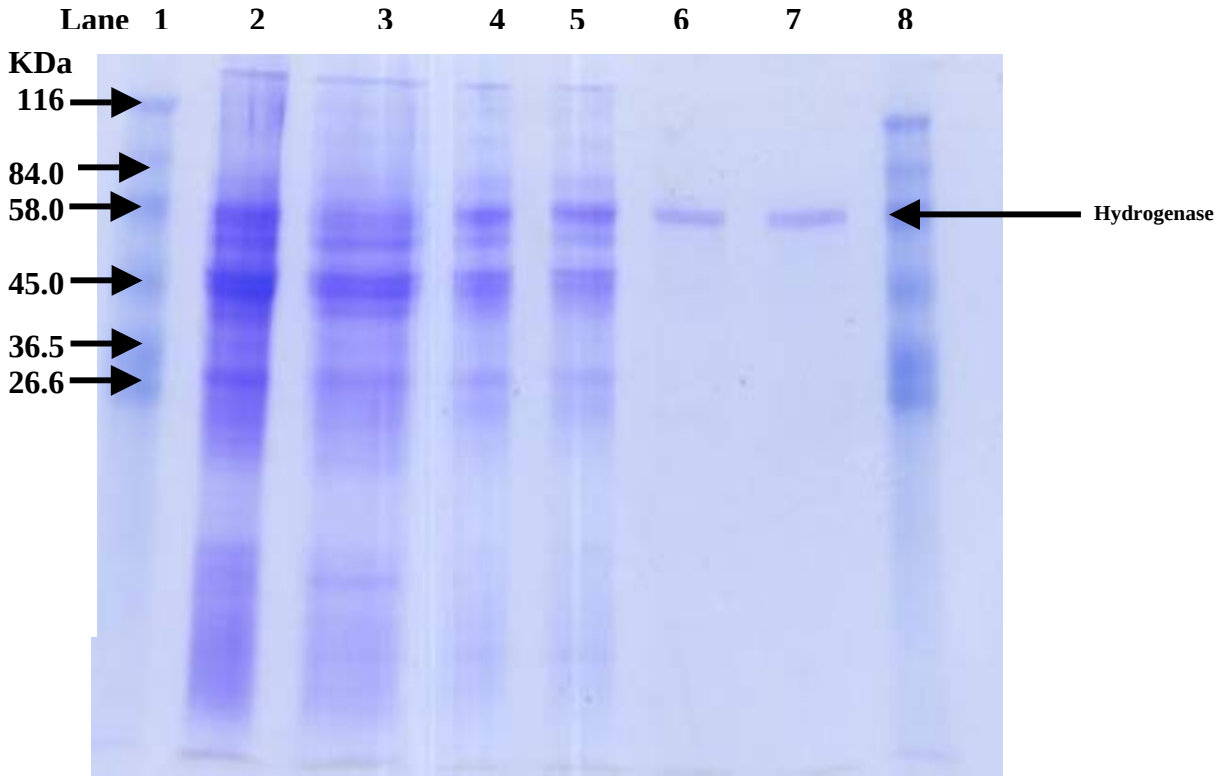


Figure 5.3: 12% SDS-PAGE analysis of purified hydrogenase (second procedure). Lane 1: Sigma prestained molecular weight markers (β-Galactosidase 116 KDa, Fructose-6-phosphate kinase 84 KDa, Pyruvate Kinase, 58 KDa; Oval Albumin, 45 KDa, Lactic Dehydrogenase, 36.5 KDa and Triophosphate Isomerase 26.6KDa). Lane 2: Crude Pellet. Lane 3: Crude supernatant (SB1). Lane 4: Ammonium sulphate dialysate fraction (SB2). Lane 5: Toyopearl-S Q 650S fraction (SB3). Lane 6: Sephacryl S-100 (SA4A). Lane 7: Sephacryl S-100 (SA4B). Lane 8: Sigma pre-stained molecular weight markers.

A possibility of them being a metal-free hydrogenase was ruled out due to the fact that the metal-free hydrogenases reported, unlike the other two classes, are the most oxygen sensitive and all studies reported (including purification and isolation) have been carried out under very strict anaerobic conditions (Hartmann *et al.,* 1996). Secondly, all known metal metal-free hydrogenases isolated to date have only been found in methanogens and none has been found in sulphate reducers (Zirngibl *et al.*, 1990; Hagemeier *et al.,* 2000). Thirdly, all metal-free hydrogenases can only catalyze the reduction of F_{420} as an electron acceptor in the presence of hydrogen, in contrary to the other classes (Thauer *et al.,* 1996; Reeve *et al*., 1997; Thauer, 1998). Although all metal-free hydrogenases are monomeric, they have all been reported to have molecular weights exceeding 60 KDa (Buurman *et*

al., 2000). A positive control could not be included because no hydrogenase is commercially available. The suggestion that the bands in lanes 6 and 7 (Figure 5.3), represent a nickel containing hydrogenase class is supported by comparison of the molecular weight with other nickel-containing hydrogenases which have been purified previously (Table 5.2).

Table 5.2: Properties of hydrogenase from *Desulfovibrio* (Modified from Odom and Peck, Jr., 1984)

Properties	**Fe- H_2ases** ***D.vulgaris*** **(Hildenborough)**	**NiFe- H_2ases** **D. *gigas***	**NiFe H_2ases** ***D.baculatus***	**NiFe -H_2ases** ***D.desulfuricans*** **(NRC 49001)**	**NiFe -H_2ases** ***D. desulfuricans*** **(Norway)**
Cellular localization	periplasmic	periplasmic	periplasmic	Periplasmic/ cytoplasmic	Membrane/ Cytoplasmic/Periplamic
Molecular weight (KDa)	49	89.5	85	52	58
Subunits	1	2 (62 + 26 KDa)	2 (56 + 29)	1	1
Nickel	0	1	1	1	1
Iron atoms	12	12	8	12	6
References	Mayhew *et al.,* 1978; Odom and Peck, Jr. 1984	Hausinger, 1987; Odom and Peck, Jr., 1984	Hausinger, 1987; Grahame, 1988; Odom and Peck, Jr. 1984	Odom and Peck, Jr. 1984; Lissolo *et al.* 1986	Odom and Peck, Jr. 1984; Reider *et al.* 1984; Cammack *et al.* 1986; Teixeira *et al.* 1987

H_2ases = hydrogenases

Periplasmic localized nickel containing hydrogenases from *D. desulfuricans* (NRC 49001) and *D. desulfuricans* (Norway 4), for example, are monomeric with molecular weights of 52 and 58 KDa respectively (Rieder *et al.,* 1984; Lissolo *et al.,* 1986; Cammack *et al.,* 1986; Teixeira *et al.,* 1987). Some nickel-containing hydrogenases are heterodimeric, such as those purified from *D. gigas* and *D. baculatus* which have molecular weights of 89.5 KDa (63 + 26.5 KDa) and 85 KDa (56 + 29 KDa) respectively (Hausinger, 1987). It has been postulated that these monomeric nickel-containing

hydrogenases, might be an active large subunit of the heterodimeric nickel-containing hydrogenases (Grahame, 1988).

It is possible that the hydrogenase purified in the present study could be that of an Fe-only class, based on the fact that the majority of this class are monomeric, consisting of catalytic subunits of a range between 55-57 KDa (Ueno *et al*., 1999; Vignais *et al.,* 2001; Fontecilla-Camps *et al.,* 2002). Periplasmic localized iron containing hydrogenase for example, from *D. vulgaris* (Hildenborough) is monomeric and has a molecular weight of 49 KDa (Mayhew *et al.,* 1978; Odom and Peck, Jr., 1984), while monomeric Fe-only hydrogenase from genetically modified *D. vulgaris* has a molecular weight 57 KDa (Voordouw and Brenner, 1985). The speculation that the purified hydrogenase in the present study can actually be a homodimeric Fe-only hydrogenase and not a nickel containing hydrogenase, as discussed before, remains a reasonable argument. It appears that molecular weights of hydrogenases are species dependent, varying from one organism to another. Since the hydrogenase in the present study was purified from a mixed culture it is impossible to speculate which sulphate reducing species produced this enzyme, because hydrogenases are uniformly distributed in sulphate reducing species. In addition many studies conducted so far on the bioreduction of metals by hydrogenase have not been concerned with the class of hydrogenase involved in the process. Nevertheless the objective of purifying the hydrogenase for further study had been achieved.

5.3.2 Characterization

5.3.2.1 Kinetics with different electron acceptors

Hydrogenases differ significantly in their structural organization, catalytic properties, stability and physiological substrates. Electron transfer between the hydrogenase and its redox partner seems to be a rate limiting step in the hydrogen uptake assay, as the value of the specific activity of the enzyme depends on the nature of electron acceptor used. (De Lacey *et al.,* 2000). *In vitro* hydrogenase had been shown to catalyze the reactions of hydrogen uptake with a number of electron acceptors such as cytochrome c_3 (Mr 13

KDa), potassium ferricyanide, Dichloroindolephenol (DCIP) and methyl viologen (Peck, Jr. and LeGall, 1994).

The hydrogenase activity with these various electron acceptors is shown (Figure 5.4). Methyl viologen reflected the highest activity followed by cytochrome c_3, DCIP and then ferricyanide. Low activity with DCIP and ferricyanide as electron acceptors could be attributed to their high mid points potential DCIP ($E_{\theta'}$ = +0.22 V) and ferricyanide ($E_{\theta'}$ = +0.36 V) (Williams and Wilson, 1979), and so they are not readily reduced or oxidized by hydrogenases. High hydrogenase activity with methyl viologen and cytochrome c_3 was not surprising, because methyl viologen has a low mid point potential ($E_{\theta'}$ = -0.45 V), and reacts readily with cytoplasmic and periplasmic hydrogenases. Methyl viologen is an almost universal electron acceptor for all hydrogenases (Peck, Jr. and Gest 1956).

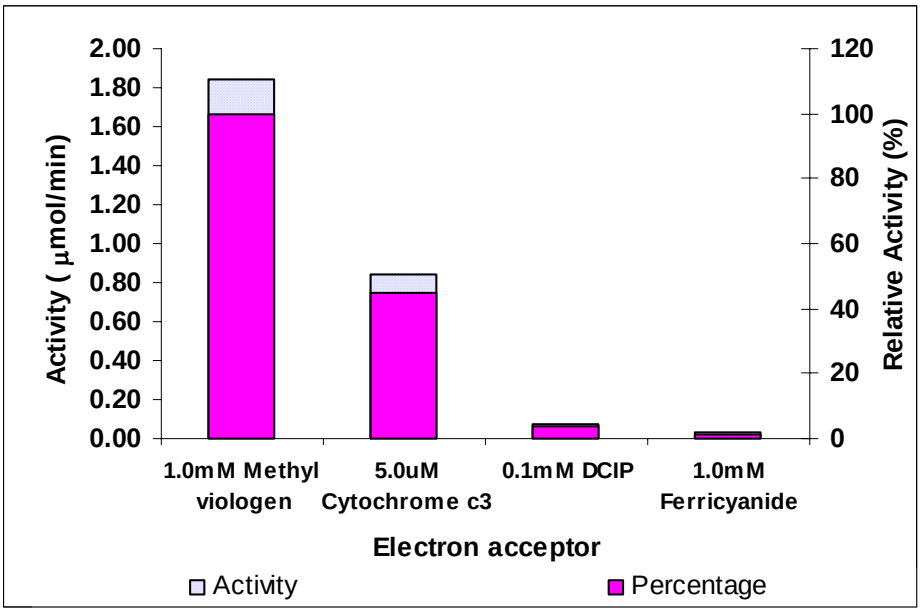


Figure 5.4: Hydrogenase uptake activity with different electron acceptors. (Activity of the hydrogenase with methyl viologen as electron acceptor was considered as 100%). Net enzymatic activity was obtained by subtracting non-enzymatic activity measured in the presence of N_2 (control) instead of hydrogen.

Considerable activity with cytochrome c_3 as the electron acceptor was also expected given the proposed role of cytochrome c_3 *in situ* where it acts as an energy transducing device by capturing both protons and electrons produced by the hydrogenases in SRB (Dolla *et al.*, 2000; Okura *et al.*, 2001). In addition, cytochrome c_3 is a naturally

occurring electron carrier of hydrogenase and it is found among the periplasmic proteins in a ratio of 4.5 cytochrome molecules per hydrogenase molecule (Odom and Peck, Jr., 1984). Consequently in the present study, further characterization was performed using both methyl viologen and cytochrome c_3 as electron acceptors.

5.3.2.2 pH optimum profile

Enzyme-substrate recognition and catalytic events *in situ* are dependent on the pH of the microenvironment. A definite optimum pH for enzyme activity is usually observed, because like other proteins, enzymes possess many ionizable groups, hence the pH dependence may alter the conformation of the enzyme, binding of the substrate, and the active site of the enzyme (Dixon and Webb, 1979).

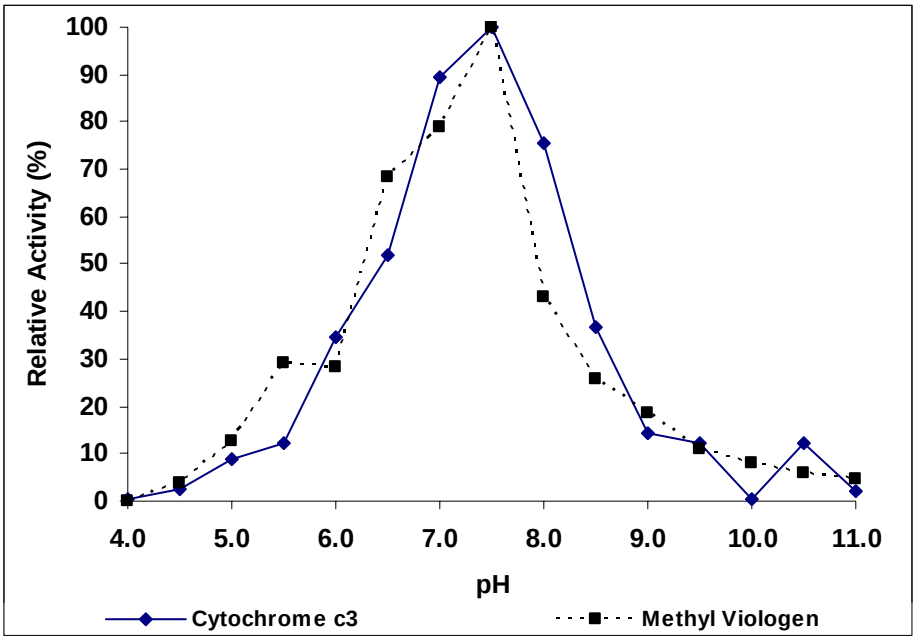


Figure 5.5: Effect of pH on hydrogenase activity. Net enzymatic activity was obtained by subtracting non-enzymatic activity measured in the presence of N_2 (control) instead of hydrogen.

Due to the nature of the hydrogenase catalyzed reactions (reversible redox reaction) in which protons are involved, it was suspected that the pH would have a prominent effect in the catalytic production or consumption of hydrogen *in vitro*. The pH effect on hydrogenase activity was studied in the presence of acetate, Tris-HCl, potassium phosphate, and carbonate buffers (20 mM) in the range from 4.0 to 11.0. An optimum pH

of 7.5 for hydrogenase was found in both cases when using cytochrome c_3 and methyl viologen as the electron acceptor (Figure 5.5). These findings are in accordance with previous studies, in which the optimum pH of the hydrogenases was reported to be in the range of 7.0 to 8.0 (Giudici-Oriticoni *et al*., 1999; Bianco *et al.*, 2001).

5.3.2.3 Temperature profile

Like most chemical reactions, the rate of an enzyme catalysed reaction generally increases with an increase in temperature. Unlike chemical catalysed reactions however, at temperatures above 50 to 60 ^{o}C, most of enzymes show a decline in activity. Two effects are known to be occurring viz. the characteristic increase in the reaction rate with temperature and thermal denaturation of protein structure at elevated temperature. Temperature optimization studies for hydrogenase using cytochrome c_3 and methyl viologen as electron acceptors showed highest activity at 35 ^{o}C (Figure 5.6). These findings are in agreement with those reported elsewhere (Llama *et al.*, 1979; Giudici-Oriticoni *et al.*, 1999). These observations were not surprising given the fact that most known SRB grow optimally between 30-40 ^{o}C, and since hydrogenases are located in the periplasm close to the external environment, such temperature optimum is obvious.

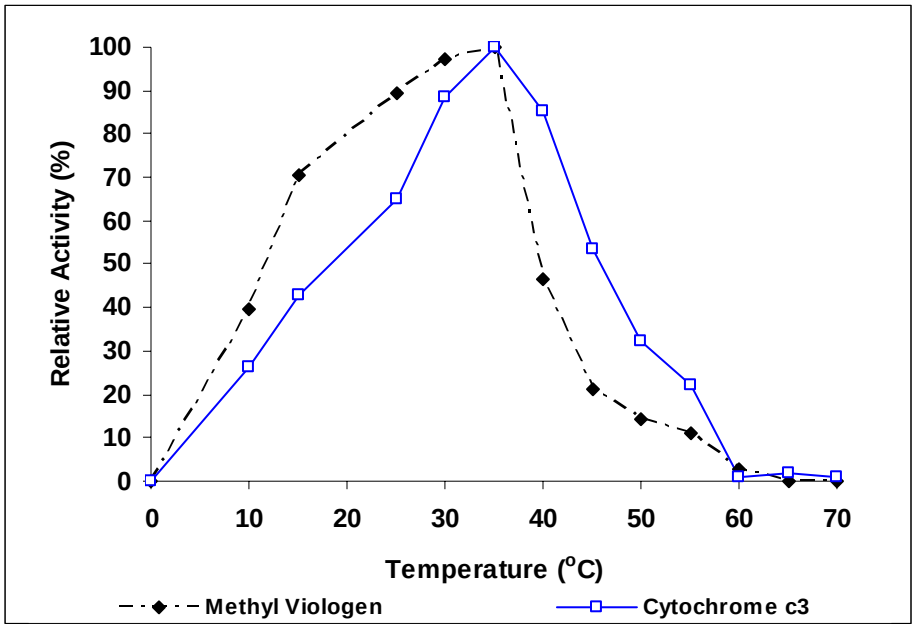


Figure 5.6: Effect of reaction temperature on hydrogenase activity. Net enzymatic activity was obtained by subtracting non-enzymatic activity measured in presence of N_2 (control) instead of hydrogen.

5.3.2.4 Thermal stability

The thermal stability of the hydrogenase was investigated at optimum temperature (35 ^{o}C) and pH (7.5). Hydrogenase showed a relatively poor stability, with only about 34% of activity remaining after 80 min when methyl viologen was used as electron acceptor (Figure 5.7). No activity was observed with cytochrome c_3 as an electron acceptor after 80 min (Figure 5.7). Although the reason for this poor stability of hydrogenase is not very clear, there is a possible explanation. The loss of protective matrices during the isolation and purification of the hydrogenase is believed to result in partial denaturation of the enzyme by heat or change in pH, thereby altering the precise conformation of hydrogenase to catalyze the reaction, resulting in rapid decrease in activity.

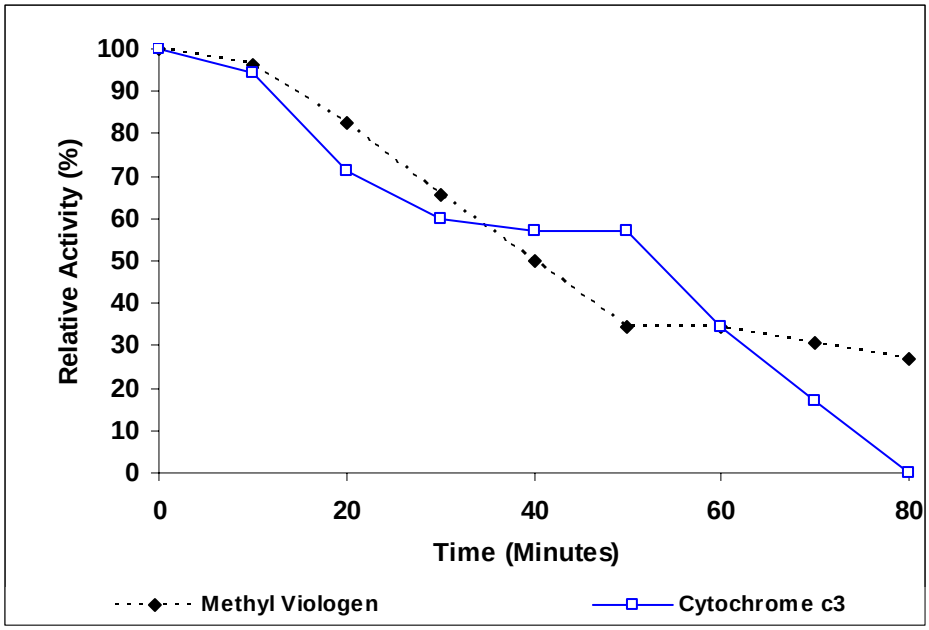


Figure 5.7: Thermal stability profile of the hydrogenase. Net enzymatic activity was obtained by subtracting non-enzymatic activity measured in presence of N_2 (control) instead of hydrogen.

The poor thermal stability of hydrogenase observed in this study presents a grave disadvantage for biotechnological application in metal biotreatment processes, because an ideal biocatalyst should be able to withstand changes in temperature which are often observed in an industrial scale. These findings suggest that immobilization of the hydrogenase will be required, since immobilized enzymes have been shown to demonstrate considerable thermal stability. The immobilization techniques provide the

enzyme with a protective shell, preventing it from altering its conformation in response to changes heat and pH (Chibata *et al*., 1976).

5.3.2.5 Effect of metal ions

Metal ions can stabilize macromolecular structures participate in cross-linking, and affect the binding of small molecules. They may induce conformational changes in enzymes or in other proteins which may themselves enhance or inhibit the enzyme. The acquisition of metals and their insertion into metal dependent enzymes like hydrogenase involve the activity of a multitude of so-called accessory proteins. Of particular interest are those steps in the maturation process which guard the specificity of incorporation. As to the present state of information there are three different check points which metal has to pass to reach its final destination (Bock *et al*., 2001). The first one is the uptake into the cell by transport systems which can be of high affinity and specific type or of low affinity type, normally responsible for the uptake of some other metals. The second one, less well defined biochemically, involves the function of some shuttle system binding the metal after entry and delivering it to the final target, which in step three, by some special folding aid is held in an open conformation amenable for metal insertion. While metal acquisition in enzymes is more or less understood, how the metal exerts its influence on a catalyzed reaction is still not known. Figure 5.8 shows the effect of heavy metal ions on hydrogenase activity. The concentration range was from 0-1mM for all metals.

Ni (II) and Fe (III) up to 1 mM showed no considerable effect on hydrogenase activity (Figure 5.8a). Zn (II) at 1 mM showed reductions in hydrogenase activity of 55.5% with cytochrome c_3 and 77.6% with methyl viologen as the electron acceptor. Cu (II) at 0.8 mM showed a complete inhibition of hydrogenase, with only 5% of activity observed in the presence of methyl viologen as the electron acceptor. The mechanism of hydrogenase inhibition by Zn (II) and Cu (II) is not clear, but there is a possible explanation. These metals bind irreversibly into the nickel or iron binding sites in the hydrogenase active site, resulting in precursors containing Zn (II) or Cu (II) which do not efficiently form a stable substrate-enzyme complex (Bock *et al.,* 2001). In addition, complete inhibition of activity with Cu (II) is in accordance with other previously reported studies in which Cu

(II) has been shown to have the same effect although the concentrations of Cu (II) used in those studies varied from the present study (Fernandez *et al.,* 1989). This observation of inhibition of hydrogenase by Cu (II) establishes unequivocally that the enzyme being characterized is of the hydrogenase family.

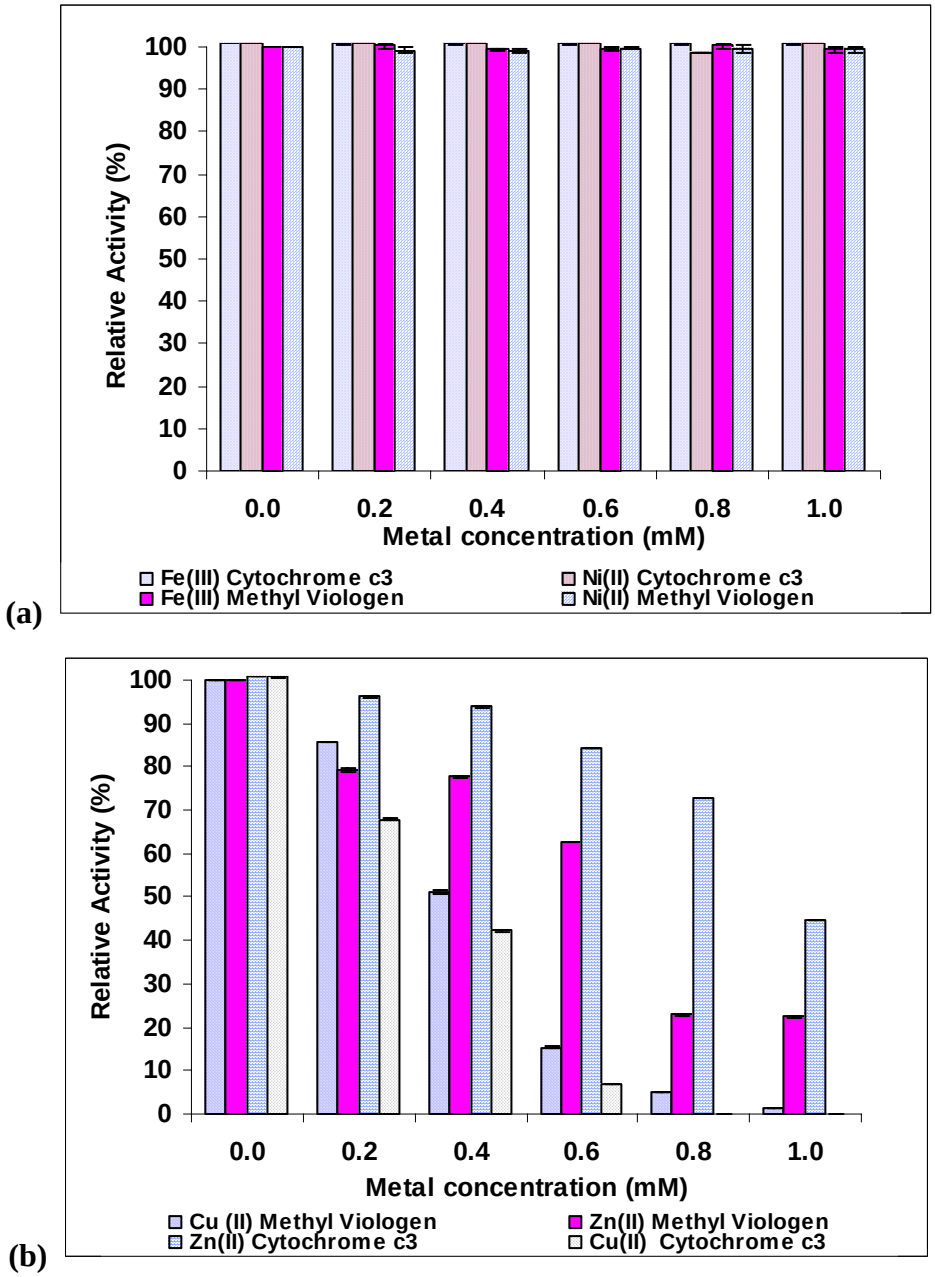


Figure 5.8: Effect of heavy metal ions on hydrogenase activity (a) Fe (III) and Ni (II), and (b) Cu (II) and Zn (II). Net enzymatic activity was obtained by subtracting non-enzymatic activity measured in presence of N_2 (control) instead of hydrogen.

5.3.2.6 Electron acceptor dependent hydrogenase activity

In enzyme catalyzed reaction, the rate of the reaction increase with an increase in substrate concentration. At high substrate concentrations however, the rate tends to level off. This saturation phenomenon occurs because there are a limiting number of catalytic sites of the enzyme (Dixon and Webb, 1979; Page and Williams, 1987). Dependence of the H_2-uptake of hydrogenase on the concentration of the electron acceptor was studied in the presence of cytochrome c_3 (Figure 5.9) and methyl viologen (Figure 5.10). The experimental data was fitted to Michaelis-Menten curves, parameters such as K_m (affinity of enzymes for substrate) and V_{max} (maximum velocity of enzyme at maximum substrate concentration) were determined for both electron acceptors. The K_m values of 7.14 µM and 13.19 mM and V_{max} of 1.35 and 45.05 $U.ml^{-1}$ were obtained with cytochrome c_3 and methyl viologen respectively.

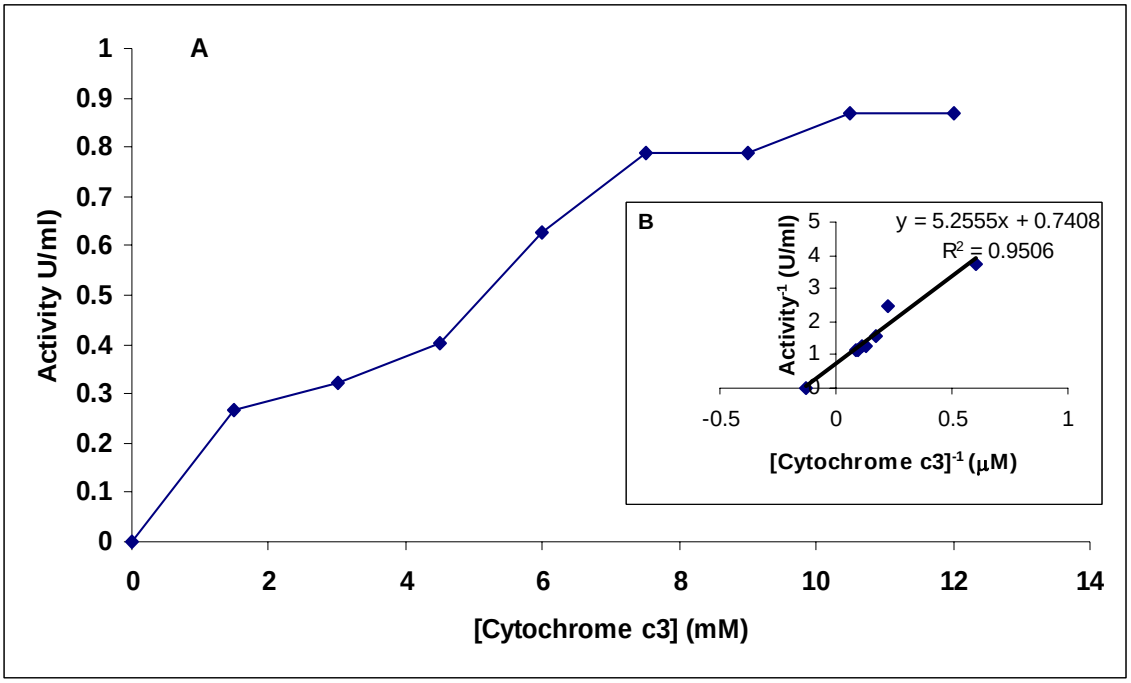


Figure 5.9: Dependence of the H_2-uptake by hydrogenase on the concentration of cytochrome c_3. (A) Michaelis-Menten plot of the initial reaction velocity (V_o) as a function of electron acceptor concentration (cytochrome c_3), and (B) Double-reciprocal (Lineweaver-Burk) plot of $1/V_o$ as a function of 1/[cytochrome c_3].

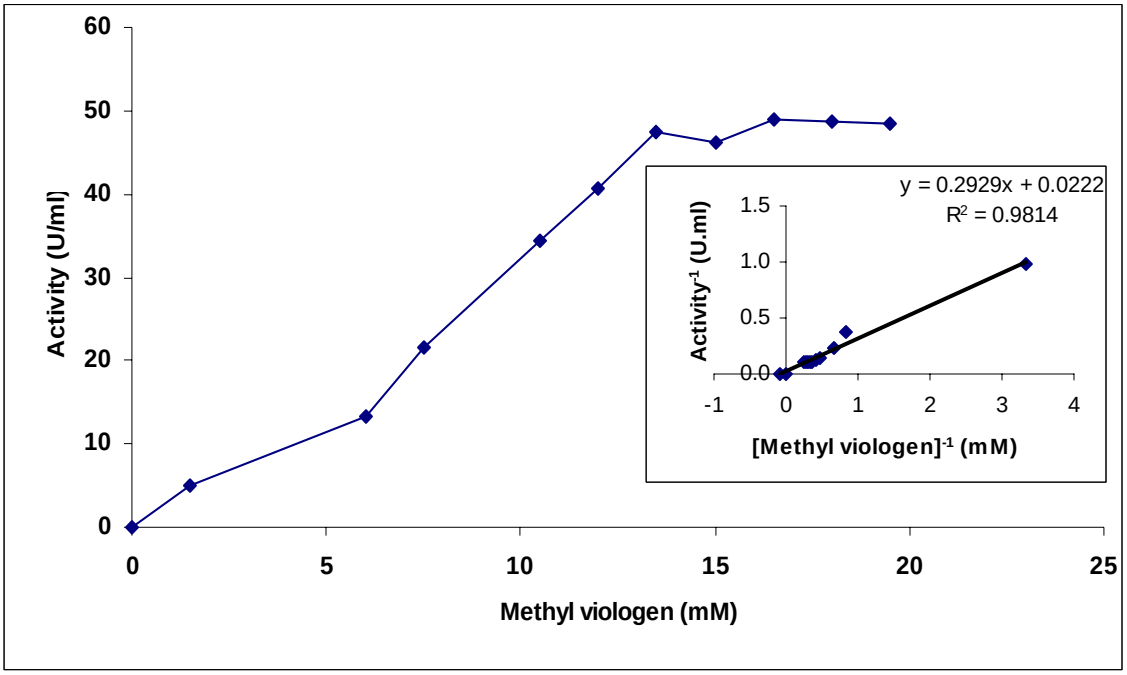


Figure 5.10: Dependence of the H_2-uptake by hydrogenase on the concentration of methyl viologen. (A) Michaelis-Menten plot of the reaction initial velocity (V_o) as a function of electron acceptor concentration (methyl viologen), and (B) Double-reciprocal (Lineweaver- Burk) plot of $1/V_o$ as a function of 1/[methyl viologen].

5.4 Summary

The molecular weight of the purified hydrogenase was determined to be 58 KDa, in the same order of magnitude for other hydrogenases characterized from SRB with a specific activity of 109.5 U.(mg protein)$^{-1}$ was obtained. Hydrogenase was only able to show hydrogen uptake with methyl viologen and cytochrome c_3 as the electron acceptors. The purified hydrogenase showed optimal temperature and pH of 35 ^{o}C and 7.5 respectively and poor thermal stability over time. Fe (II) and Ni (II) had very little effect on hydrogenase activity. In contrast Zn (II) and Cu (II) showed very strong inhibitory effects on hydrogenase. The kinetics parameters Km and V_{max} were determined to be 7.14 µM and 1.35 U.ml^{-1} respectively for reduced cytochrome c_3 and 13.19 mM and 45.05 U.ml^{-1} for methyl viologen. This purified hydrogenase was used further, in order to gain an insight in the role of the hydrogenase in platinum reduction.

CHAPTER SIX

Reduction of Platinum (IV) by Hydrogenase and the Bioremediation of Industrial Effluent Utilizing a Biosulphidogenic System

6.1 Introduction

Microorganisms play important roles in the environmental fate of metals with a multiplicity of mechanisms affecting transformations between soluble and insoluble forms. These mechanisms are integral components of natural biogeochemical cycles and are of potential for both *in situ* and *ex situ* bioremedial treatment processes for liquid wastes. Amongst these mechanisms is enzymatic reduction of metals which have been examined in the context of environmental biotechnology as a means of removal, recovery and detoxification of metal toxicity.

Sulphate reducing bacteria have an important influence on the geochemistry of sedimentary environments and are of great environmental interest as they are involved in the reductive precipitation of highly toxic metals (Lovely and Philips, 1992a). Their enzymatic reduction ability may be a useful tool for the bioremediation of water and waste streams contaminated with metal ions. A few microbial enzymes that use metals as substrates have been purified. These include the intensively studied mercuric reductases which detoxify ionic mercury (Schiering *et al*., 1991) and Cr (VI) reductase of unknown physiological function (Suzuki *et al.*, 1992), as well as Fe (III) reductases that reduce chelated Fe (III) as a part of the process of iron assimilation (Gaines *et al*., 1981). All of these metal reductases however are expressed during aerobic metabolism and their function has little in common with dissimilatory metal reduction under anaerobic conditions.

The enzymatic mechanisms for dissimilatory metal reduction by anaerobic microorganisms are largely unknown. Several studies have revealed the roles of cytochrome c_3 and hydrogenase in metal reduction under anaerobic conditions, particularly in SRB. For example, the enzymatic bioreduction of hexavalent uranium to stable UO_2 by *Desulfovibrio desulfuricans* and *Geobacter metallireducens*, with cytochrome c_3 have been reported (Lovely, 1993; Francis *et al.*, 1994). Involvement of hydrogenase activity in metal reduction by *Micrococcus lactiyticus* was implicated in early work with uranium (Woolfolk and Whiteley, 1962), confirmed for the obligate anaerobe *Clostridium pasteurianum* with selenite used in this case (Yanke *et al*., 1995), and attributed unequivocally to the hydrogenase 3 component of the formate hydrogenlyase complex of *Escherichia coli*, for the reduction of Tc (VII) anaerobically (Lloyd *et al.*, 1997a; b). Use of this facultatively anaerobic organism showed conclusively that Tc reductases (in this case hydrogenase 3) was sited in the cytoplasm and was under the control of the anaerobic switch protein FNR, upregulated upon shifting to anaerobiosis (Lloyd *et al.*, 1997a). Evaluation of bioreductive potential of Pd (II) and Tc (VII) by *Desulfovibrio desulfiricans* and *fructosovorans* respectively via activity of hydrogenase have also been reported (Lloyd *et al*., 1998b; Vermeglio, *et al*., 2001).

To date, the enzyme responsible for the reduction of metals by SRB has not yet been completely characterized. The potential for using SRB in the uptake of platinum (IV) for the removal of platinum from aqueous solution led to further investigation of the enzymatic mechanisms for platinum (IV) reduction with the aim to further optimize this process. The main objectives of this section were:

(i) To establish the role of hydrogenase in platinum reduction *in vitro*.

(ii) To evaluate the potential of platinum reduction from an industrial effluent using a hydrogenase based system.

(iii) To utilize a sulphidogenic (sulphate reducing) system in bioprecipitation of platinum.

6.2 Investigation of platinum (IV) reduction by hydrogenase *in vitro*

6.2.1 Material and Methods

6.2.1.1 Materials

Potassium hexachloroplatinate (IV), [Cl_6K_2Pt (IV)] Wirsam scientific; Cytochrome c_3, Sodium dithionite, BSA from Sigma; Gases were from Afrox; A GBC 909 atomic absorption spectrophotometer (GBC 909 instrument; GBC scientific Equipment Pty Ltd, Dandenong, Australia); Shimadzu UV visible 160A recording spectrophotometer; Platinum containing effluents, named AECO and BMCO were from AngloPlatinum, South Africa.

6.2.1.2 Analytical procedures

Protein concentrations were determined by the Bradford method using BSA as a standard as previously described (Section 4.2.2.3, Chapter 4). Platinum concentration was measured using an atomic absorption spectrophotometer. UV-visible spectra were recorded using the Shimadzu spectrophotometer. The hydrogenase uptake activity was measured spectrophotometrically by monitoring the reduction of methyl viologen (A_{604nm}) at 25 ^oC as previously described (Section 4.2.2.2, Chapter 4).

6.2.1.3 Growth of organisms and preparation of Pt (IV) reduction extracts

Bacteria were grown at 30 ^oC in the dark while shaking at 100 rpm as described previously (Section 2.2.2.3, Chapter 2). Mid stationary phase cells were collected by centrifugation (5000 x g, 20 min, at 4 ^oC), supernatant were kept under OFN (referred to as crude fraction). The bacterial pellet were suspended in minimal volume of Tris-HCl (20 mM, pH 7.0, 100 ml) and cells were broken by sonication (5 min, 30 sec burst) as previously described (Section 4.2.2.4.3, Chapter 4) followed by centrifugation (18 000 x g, 20 min, 4 ^oC). Both fractions: the supernatant (referred to as soluble fraction) and pellet (referred to as membrane fraction) were kept under OFN prior to use.

6.2.1.4 Platinum (IV) reduction by different protein fractions

Protein fractions were as follows: crude, (0.51mg.ml^{-1}); soluble, (1.19 mg.ml^{-1}); membrane-bound, (0.39 mg.ml^{-1}). One 5 ml aliquot in Tris-HCl (0.1 M, pH 7.5) of each fraction was added to test tubes sealed with butyl rubber stoppers under OFN using disposable hypodermic syringes, followed by addition of Pt (IV) solutions to give a final concentration of 0.26 mM. Finally, (10 ml) hydrogen was added to the reaction mixture by injecting through the headspace. Samples were removed at timed intervals and analyzed for platinum. A control mixture was prepared exactly as above, but with N_2:CO_2 bubbled into the system instead of hydrogen.

6.2.1.5 Platinum (IV) reduction activity by column fractions

Fractions referred to here were obtained from the hydrogenase purification procedures (Section 5.2.2.3.3, Chapter 5). The wash fraction (refers to the pooled protein fractions which did not have any retained hydrogenase activity after Toyopearl-Super Q 650S ion exchanger column) and eluted fraction (refers to the fraction that showed hydrogenase activity after eluting with NaCl, referred to previously as SA3) were analyzed for platinum reduction activity. Protein fractions were as follows: wash (0.011mg.ml^{-1}) and eluted (0.22 mg.ml^{-1}). One 5 ml aliquot in Tris-HCl (0.1 M, pH 7.5) of each fraction was added to test tubes sealed with butyl rubber stoppers under OFN using disposable hypodermic syringes, followed by addition of Pt (IV) solutions to give a final concentration of 0.26 mM. Finally, 10 ml hydrogen was added to the reaction mixture by injecting through the headspace. Samples were removed at timed intervals and analyzed for platinum. A control mixture was prepared exactly as above, but with N_2:CO_2 bubbled into the system instead of hydrogen.

6.2.1.6 Investigation of enzymatic platinum (IV) reduction

Additional studies on the reduction on platinum by pure hydrogenase were modeled after those of Lovely and Philips (1994) and Vermeglio *et al.*, (2001). Five reaction mixtures were prepared as follows:

Reaction mixture 1: A 5 ml aliquot of pre-activated hydrogenase solution (0.11 mg protein.ml^{-1}) in Tris-HCl buffer (0.1M, pH 7.5) was added to a test tube sealed with butyl rubber stopper under OFN using a disposable hypodermic syringe, followed by addition of Pt (IV) solutions to give a final concentration of 0.26 mM. Finally, 10 ml hydrogen was added to the reaction mixture by injecting through the headspace. Samples were removed at timed intervals and analyzed for platinum. A Control mixture was prepared exactly as above, but with N_2:CO_2 bubbled into the system instead of hydrogen.

Reaction mixture 2: A 5 ml aliquot of pre-activated hydrogenase solution (0.11 mg protein.ml^{-1}) in Tris-HCl buffer (0.1M, pH 7.5) was added to a test tube containing 5 µM cytochrome c_3 sealed with butyl rubber stopper under OFN using a disposable hypodermic syringe, followed by addition of Pt (IV) solutions to give a final concentration of 0.26 mM. Finally hydrogen (10 ml) was added to the reaction mixture by injecting through the headspace. Samples were removed at timed intervals and analyzed for platinum. A control mixture was prepared exactly as above, but with N_2:CO_2 bubbled into the system instead of hydrogen.

Reaction mixture 3: A 5 ml aliquot of pre-activated hydrogenase solution (0.11 mg protein.ml^{-1}) in Tris-HCl buffer (0.1M, pH 7.5) was added to a test tube containing 0.5 mM Cu^{2+} (an inhibitor of periplasmic hydrogenase) sealed with butyl rubber stopper under OFN using a hypodermic syringe, followed by addition of Pt (IV) solutions to give a final concentration of 0.26 mM. Finally, 10 ml hydrogen was added to the reaction mixture by injecting through the headspace. Samples were removed at timed intervals and analyzed for platinum. A control mixture was prepared exactly as above, but with N_2:CO_2 bubbled into the system instead of hydrogen.

Reaction mixture 4

A 5 ml aliquot of cytochrome c_3 (5 µM) in Tris-HCl buffer (0.1M, pH 7.5) was added to a test tube sealed butyl rubber stopper under OFN, using a hypodermic syringe, followed by addition of Pt (IV) solutions to give a final concentration of 0.26 mM. Finally, 10 ml

N_2:CO_2 was added to the reaction mixture by injecting through the headspace. Samples were removed at timed intervals and analyzed for platinum.

Reaction mixture 5

A 5 ml aliquot of cytochrome c_3 (5 µM) in Tris-HCl buffer (0.1M, pH 7.5) was added to a test tube sealed butyl rubber stopper under OFN, using a hypodermic syringe, followed by addition of Pt (IV) solutions to give a final concentration of 0.26 mM. Finally, 10 ml hydrogen was added to the reaction mixture by injecting through the headspace. Samples were removed at timed intervals and analyzed for platinum. A control mixture was prepared exactly as above, but with N_2:CO_2 bubbled into the system instead of hydrogen.

6.2.2 Results and Discussion

6.2.2.1 Investigation of platinum (IV) reduction by hydrogenase

The term reduction here refers to the decrease in platinum (IV) oxidation states as results of electron generated from oxidation of hydrogen by hydrogenase. In order to exclusively study the contribution of enzymatic platinum (IV) reduction by the sulphate reducing consortium and to provide cellular evidence of the involvement of the hydrogenase in the platinum (IV) reduction process, different protein fractions (crude supernatant, soluble fraction and membrane bound protein fraction) were tested for platinum reduction activity. Platinum (IV) reduction by various protein fractions, with and without hydrogen is shown in Figure 6.1. High platinum (IV) reduction activity was obtained in the soluble protein fraction compared to crude and membrane bound protein fractions in the presence of hydrogen. No platinum (IV) reduction was observed with the same fractions in the absence of hydrogen. Initial platinum (IV) concentration of 0.26 mM was reduced to 0.177, 0.201 and 0.238 mM by soluble, crude and membrane bound protein fractions respectively (Figure 6.1). These findings demonstrate that reduction of platinum *in vitro* is carried out by soluble proteins. Furthermore, the low platinum (IV) reduction activity by membrane bound proteins strongly suggests that the precipitates of reduced forms of platinum *in vitro* do not necessitate the presence of membrane as nucleation sites.

Previous investigations of metal reducing activity by different proteins revealed the same phenomenon. Lovely and Philips (1994), investigating enzymatic-dependent Cr (VI) reducing activity by *Desulfovibrio vulgaris* showed that the soluble protein accounts for all major Cr (VI) reducing activity (86%). Earlier observations of enzymatic reduction of U (VI) by the same sulphate reducing bacterium also showed major U (VI) reducing activity (95%) in the soluble fractions (Lovely, 1993). In both studies, metal reducing activities were shown to be hydrogen dependent, suggesting direct involvement of a hydrogenase in abstracting the electron for metal reduction from hydrogen in those soluble fractions.

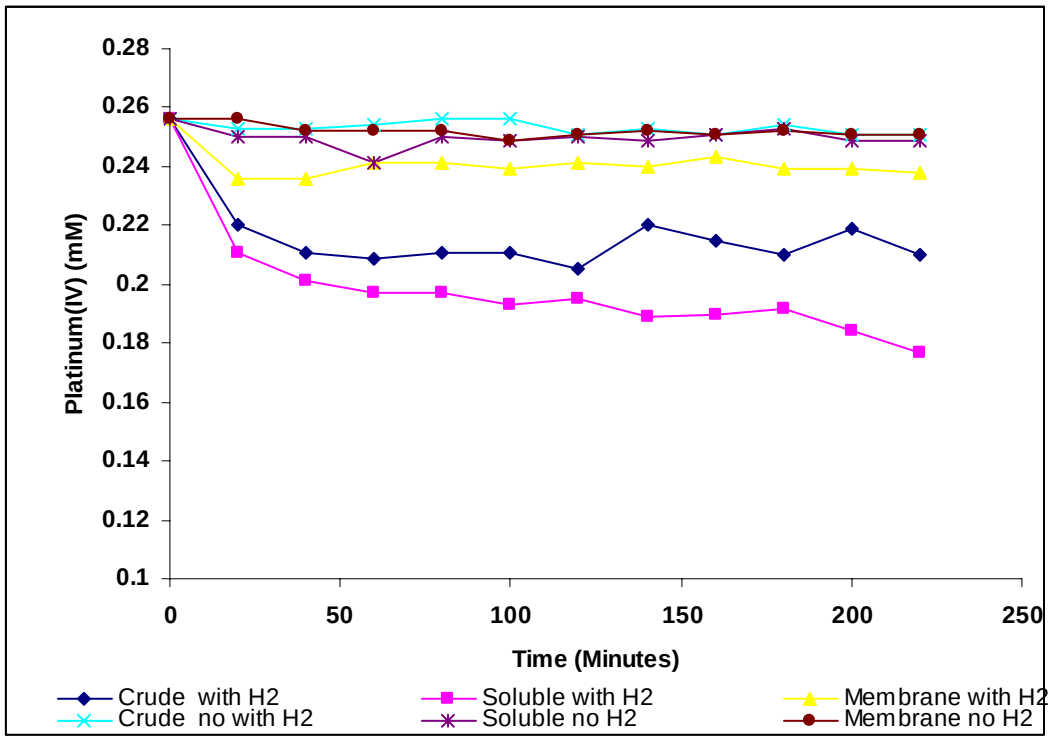


Figure 6.1: Platinum (IV) reduction over time by various protein fractions from sulphate reducing consortium with and without hydrogen.

High reduction activities in those studies compared to low platinum (IV) reducing activity in this study (30.8%) is accountable to the bicarbonate buffer system used in Lovely's studies where it may have acted as complexing agent, minimizing the inhibitory effects of the metals studied. Work by Vermeglio *et al.* (2001) on the reduction of Tc (VII) by *Desulfovibrio fructosovorans* showed that unlike *in vivo*, Tc (VII) reduction by

soluble protein depended mainly on the pH and chemical nature of the buffer used. This indicates that the use of a buffer system that forms a complex with the metal under investigation should be avoided.

Platinum (IV) reduction by various column fractions with and without hydrogen is shown Figure 6.2. When the soluble protein fraction was passed through the Toyopearl-Super Q 650S ionic exchange column, hydrogen-platinum (VI) reducing capacity was lost in the wash fraction, in the presence of hydrogen. However a protein fraction that was eluted from the column with 0.75 M NaCl (eluted fraction) restored hydrogen dependent platinum reducing activity. The initial platinum (IV) concentration of 0.26 mM was reduced to 0.202 mM by the eluted fraction, indicating a percentage reduction of 22.3% (Figure 6.2). No significant platinum reduction activity occurred in the absence of hydrogen, in both the wash and the eluted fractions (Figure 6.2).

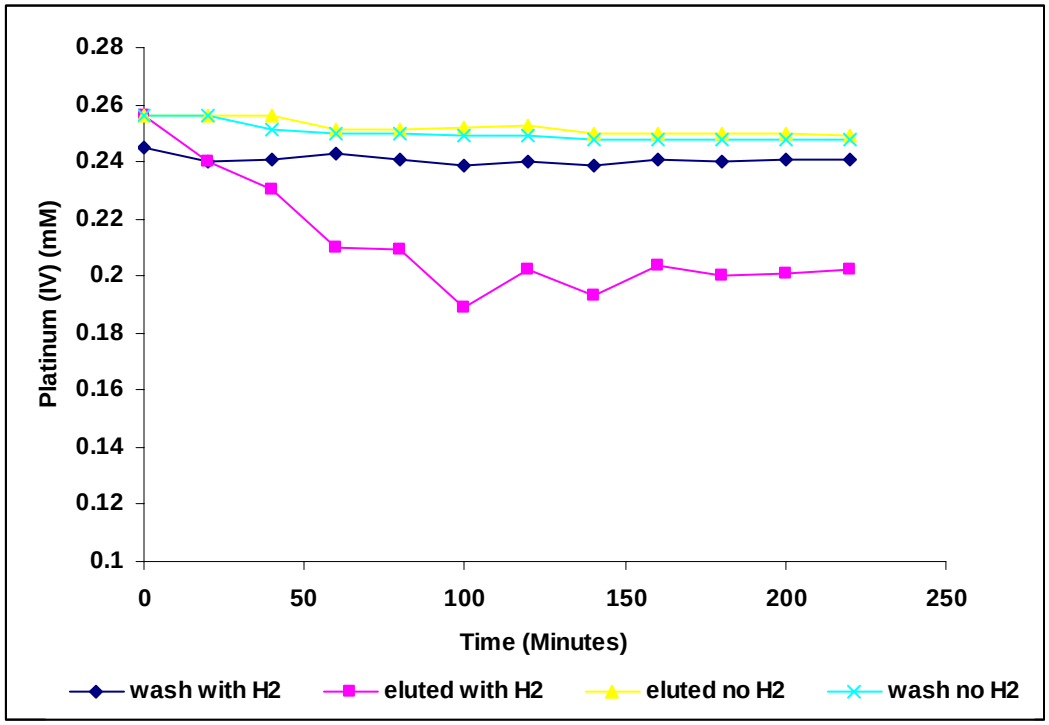


Figure 6.2: Platinum (IV) reduction over time by various protein fractions from sulphate reducing consortium after Toyopearl-Super Q 650S ion exchange column in the presence and absent of hydrogen.

To identify the protein or enzyme involved in platinum (IV) reduction *in vitro*, the involvement of the purified hydrogenase and its physiological electron carrier cytochrome c_3 was tested (Odom and Peck, Jr., 1994). Cytochrome c_3 in the presence of hydrogen, and absence of the hydrogenase showed lowest platinum reduction with the initial platinum concentration (0.26 mM) only reduced to 0.25 mM (Figure 6.3), indicating a percentage platinum reduction of 3.85 % (Figure 6.3). The initial platinum concentration (0.26 mM) was reduced to 0.218 mM in the presence of the hydrogenase, cytochrome c_3 and no hydrogen (Figure 6.3), representing a percentage platinum (IV) reduction of 16.1% (Figure 6.3). The same observations were noted when the hydrogenase in the presence of hydrogen was incubated with $CuCl_2$, a specific inhibitor of the periplasmic hydrogen uptake *in vivo* (Fernandez *et al*., 1989). The hydrogenase in the presence of hydrogen showed significant platinum (IV) reduction, with the initial platinum concentration (0.26 mM) reduced to 0.145 mM (Figure 6.3), indicating a percentage platinum reduction of 46.15% (Figure 6.3). The hydrogenase in the presence of cytochrome c_3 and hydrogen showed highest platinum reduction, with initial platinum (IV) concentration (0.25 mM) reduced to 0.145 mM (Figure 6.3), representing a highest percentage reduction of 57.69%. High platinum reducing activity observed under these conditions is believed to be related to reactivation of the hydrogenase in the presence of cytochrome c_3 (Vermeglio *et al.,* 2001).

The reduction of platinum from effluent by hydrogenase was also studied (Figure 6.4). In the presence of the hydrogenase and industrial effluent, an initial platinum concentration 0f 0.04 mM was reduced to 0.036 mM, which is about 10% platinum reduction (Figure 6.4). In the presence of the hydrogenase, hydrogen and cytochrome c_3, the initial platinum concentration (0.04 mM) was reduced to 0.034 mM (Figure 6.4), indicating a slight increase in platinum reduction to 15% (Figure 6.4). These observations indicate that hydrogenase *in vitro* mediates platinum reduction in SRB, probably as a detoxification mechanism against platinum toxicity. Because of the limited capacity of mass culturing cells, it was difficult to generate enough pure hydrogenase for further studies.

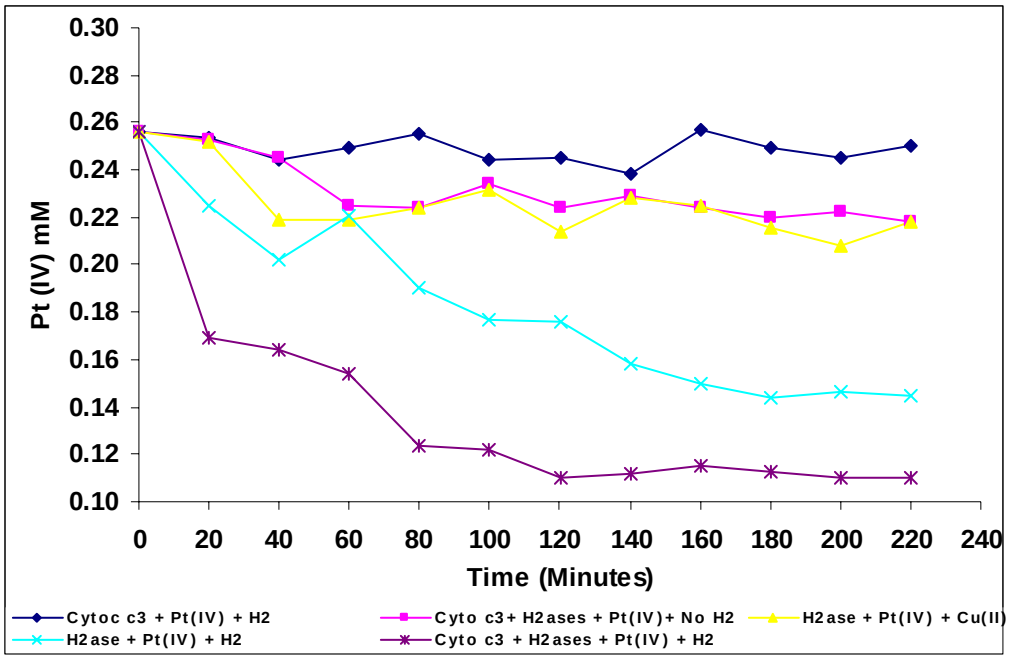


Figure 6.3: Rate of platinum (IV) reduction by electron transfer from oxidation of hydrogen by hydrogenase to platinum (VI) with cytochrome c_3 as an electron carrier.

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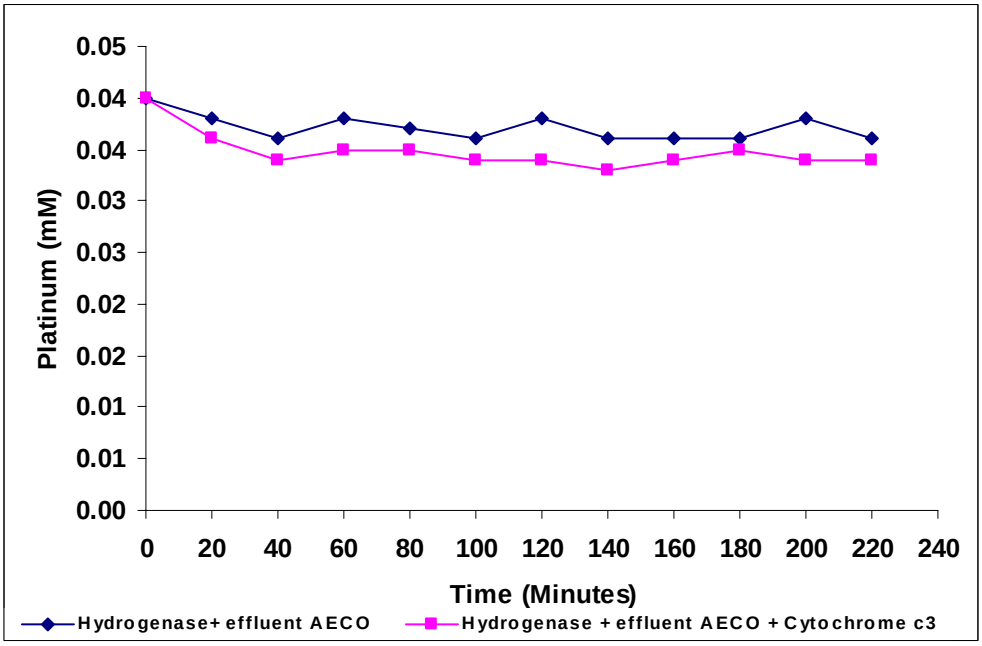


Figure 6.4: Rate of platinum reduction by hydrogenase from AECO effluent.

6.3.2 Physiological and Bioremedial implications

Hydrogenase provides the enzymatic model for anaerobic dissimilatory platinum (IV) reduction. At one time it was considered that metal reduction in SRB occurs only through a non-enzymatic process whereby sulphide produced by dissimilatory sulphate reduction precipitates metals as metal sulphide. However, these results indicate that in addition to platinum (IV) reduction as platinum sulphide, the platinum (IV) reduction is an enzymatically mediated process via a hydrogenase. These findings are in accordance with other reports. For example, a study on U (VI) reduction by *Micrococcus lactilyticus* had shown hydrogenase involvement in U (VI) reduction process (Woolfolk and Whiteley, 1962). More recently a study on metal reduction by SRB has conclusively shown the involvement of hydrogenase in the metal reduction process (Macaskie *et al*., 2001). Work on Tc (VII) reduction by *Desulfovibrio fructosovorans* has provided both genetic and biochemical evidence of the essential role of a hydrogenase in the Tc (VII) reduction process. The role was provided by (i) *in vivo* and *in vitro* inhibition of the activity by Cu (II), (ii) oxygen tolerance of the activity, (iii) the dramatic decrease of Tc (VII) reduction in a mutant lacking a hydrogenase structural gene, and (iv) demonstration of direct reduction by a purified hydrogenase (Vermeglio *et al*., 2001)

Steady loss of platinum (IV) over time in the presence of hydrogenase, cytochrome c_3 and H_2 was not surprising given the proposed role of hydrogen cycling in SRB. In this model, hydrogen generated from organic electron donors via cytoplasmic hydrogenase activity diffuses to the periplasm where electrons are abstracted for sulphate reduction and passed back across the cytoplasmic membrane via cytochromes to sulphate reducing apparatus in the cytoplasm. Protons liberated in the periplasm are also pumped into the cell, thus generating ATP. If this model is correct, platinum (IV) reduction from organic donors such as lactate and acetate could also proceed via hydrogen liberated from an organic substrate. This study therefore proposes that platinum (IV) reduction is via a hydrogenase which abstracts electrons from hydrogen to the platinum metal along with a concerted action of cytochrome c_3 which is required to shuttle the electrons from hydrogenase. If this is true, other PGMs should be reduced by the same mechanism in SRB.

These findings presented here demonstrate that the hydrogenase can be potentially be used for the reduction of platinum (IV). The use of a hydrogenase in treatment of platinum effluent offers several advantages over biogenic sulfide precipitation including the following: (i) there is no need to supplement low-sulphide wastewater streams with added sulphate; (ii) non-growing cells can be used, leading to the generation of a low-biomass waste for disposal; (iii) potentially hazardous toxic H_2S is not generated as a by-product of the process, and indeed other workers have noted technical difficulties in using resting SRB cells to reduce sulphate to H_2S; (iv) the process is potentially environmentally benign if hydrogen is used as a feedstock because there is no additional carbon substrate added to the wastewater; and (v) the reduced platinum (IV) is held within the outer compartments of the cell, potentially spatially separated from oxidizing and chelating agents that may be present in the effluent.

6.3 Bioremediation of industrial effluent utilizing sulphidogenic system

6.3.1 Materials and Methods

6.3.1.1 Materials

Gases were from Afrox; A GBC 909 atomic absorption spectrophotometer (GBC 909 instrument; GBC scientific Equipment Pty Ltd, Dandenong, Australia); Thermospectronic Aquamate and Merck Nova 60 Spectroquant were used to determine the absorbance. Platinum containing effluents: AECO and BMCO were from AngloPlatinum, South Africa.

6.3.1.2 Analytic procedures

Platinum containing effluents: AECO and BMCO were analyzed for platinum, pH, chloride and sulphate. Platinum was analyzed using an atomic absorption spectrophotometer; pH was measured using the Inolab Level 1 pH meter. Because of the acidic nature of the effluent, sulphate was analyzed using a sulphate determination Thermospectronic Aquamate test (kit # M0046f45). Total chloride was determined using

a Merck Nova 60 Spectroquant test (kit # 1.14897.0001) and sulphide was determined using a Merck Nova 60 Spectroquant test (kit # 1.14779.0001).

6.3.1.3 Removal of platinum by resting cells

Bacteria were grown at 30 ^{o}C in the dark while shaking at 100 rpm as previously described (Section 2.2.2.3, Chapter 2). During mid stationary phase, cells were harvested by centrifugation (5000 x g, 10 min, 25 ^{o}C), washed twice with minimal volume of ddH_2O, and then resuspended anaerobically in MOPS–NaOH buffer (20 mM, pH 7.0, 100 ml) pre-equilibrated with OFN at a biomass density of 5 g (wet weight).l^{-1}. The cultures were further incubated at room temperature for 15 min while purging with OFN, to maintain strict anaerobic conditions before the addition of platinum. Hydrogen was applied as an electron donor at the head space of the flask, displacing OFN. Cells were then challenged with effluent containing 7.91 mg platinum.l^{-1}. Duplicate sample (3 ml) were removed, filtered and analyzed for platinum.

6.3.1.4 Removal of platinum by growing cells

Bacteria were grown at 30 ^{o}C in the dark in 1L modified Postgate C medium, while shaking at 100 rpm as previously described (Section 2.2.2.3, Chapter 2). During mid stationary phase, cells were challenged with effluent at a flow rate of 33.3 ml.h^{-1}. Sampling was done at two points labeled A and B (Figure 6.5). Samples were analyzed for sulphide, sulphate, chloride, platinum and pH by analytic method described in section 6.3.1.2.

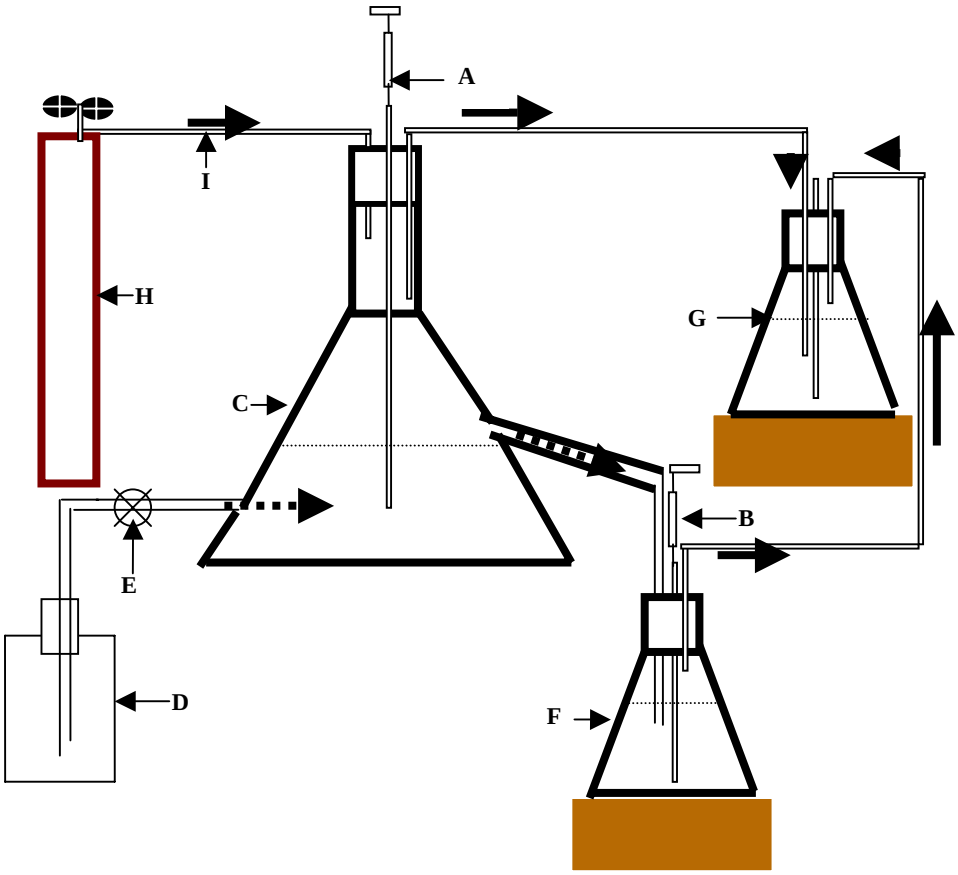


Figure 6.5: Laboratory equipment for continuous operation. (A) First sampling point, (B) Second sampling point, (C) CSTR, (D) Effluent reservoir, (E) Periplasmic pump, (F) Static tank reactor, (G) Zinc acetate solution flask, (H) hydrogen gas cylinder (I) Tygon® tubing, connected to glass tubing into the reactor. Solid arrows indicate the direction of hydrogen gas and/ or H_2S flow within the reactor and dotted arrows indicate the flow of the effluent.

6.3.2 Results and Discussion

6.3.2.1 Effluents characterization

Two platinum contaminated effluents named AECO and BMCO were obtained from Anglo Platinum (South Africa). Both effluents contained relatively high concentrations of other PGMs and base metals. Due to an agreement with the Anglo Platinum company, the characterization of exact concentration of other metals in the effluent can not be disclosed. Table 6.1 however gives an overview of metal concentrations in both effluents.

Table 6.1: Typical metal composition of the effluent ($g.l^{-1}$)

METAL	**AG**	**AS**	**AU**	**BI**	**CU**	**FE**	**IR**	**NI**	**OS**	**PB**
Concentration range	0-1	0-1	1-100	0-1	100-1000	100-1000	1-100	100-1000	1-100	1-100
Metal	**Pd**	**Pt**	**Rh**	**Ru**	**Sb**	**Se**	**Sn**	**Te**	**Zn**	
Concentration range	1-100	0-1	1-100	1-100	1-100	1-100	0-1	0-1	1-100	

The concentrations of sulphate and chloride in the effluents were determined to obtain a better understanding of the status of the effluents. Knowledge of the physicochemical properties of the effluents and dissolved elements present therein, assist in determining the speciation of the metal ions and any precipitation that may result during the course of the experiments. AECO and BMCO effluents contained chloride concentrations of 0.69 and 0.002 mM respectively (Table 6.2), while sulphate concentrations were 169.1 and 751.9 $mg.l^{-1}$ respectively (Table 6.2). Platinum concentrations for both effluents were 7.91 and 0.33 $mg.l^{-1}$ for AECO and BMCO effluent respectively (Table 6.2). The pH of the AECO was 0.38 ± 0.3, and that of BMCO was 0.28 ± 0.2 (Table 6.2).

Table 6.2: Analysis of AECO and BMCO effluent composition

	AECO	**BMCO**
Platinum (mM)	0.69	0.002
Chloride ($g.l^{-1}$)	74	70
Sulphate ($mg.l^{-1}$)	169.1	751.9
pH	0.38 ± 0.3	0.28 ± 0.2

The analyses of the two effluents indicate high concentrations of inorganic pollutants (Cl^-) and oxyanions (SO_4^{2-}). While the behavior of sulphate ions under sulphidogenic system is well understood, the behavior of the chloride ions in sulphidogenic system and the effects on the system operation are unknown. The acidic nature of the effluent poses serious problem since the sulphidogenic (sulphate reducing) system is very sensitive to low pH. Because of low platinum concentration in BMCO effluent further studies were performed with AECO effluent only.

6.3.2.2 Batch platinum removal from effluent

The ability of resting SRB to remove platinum from industrial effluent was also investigated. Platinum removal of 34% was observed after 4 h (Figure 6.6). These results indicate that, regardless of a low pH level of the effluent, resting SRB cells still offer great potential for removal of platinum from industrial effluent. These findings suggest that platinum removal by resting SRB is not strongly dependent on pH of the solution. Previous findings on the removal of commercial platinum from solution (Section 2.3.9, Chapter 2) also showed no significant dependence of platinum uptake on pH. It should be noted however that the platinum concentration in the effluent is 5-fold lower compared to that used for commercial platinum. It is believed that a significant platinum removal in a low level pH effluent is due to the combination of many processes including bioaccumulation, biosorption, and bioprecipitation by biopolymers. Because resting cells were utilized the possibility of platinum removal by precipitation as platinum sulphide is ruled out. It is therefore postulated that under growing SRB conditions platinum removal will increase significantly as a result of sulphide precipitation.

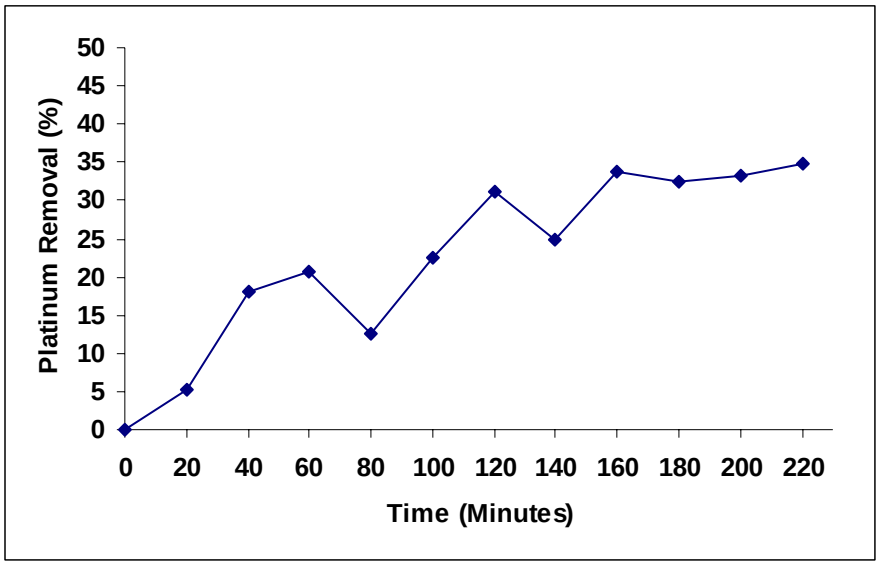


Figure 6.6: Percentage platinum removal from AECO effluent by resting SRB cells.

6.3.2.3 Bioprecipitation of platinum

One of the main objectives of this section was to investigate whether SRB were able to remove platinum from an industrial effluent. This was required in order to demonstrate the principle of SRB platinum reduction outside of controlled conditions. It should be mentioned however that at this point, this study is no longer focused on a specific mechanism for platinum removal from the effluent and as result a term 'removal' will be used refer to all processes involved in platinum removal from effluent by growing SRB. Since the removal was performed by growing SRB biomass it is believed that the majority of platinum removal will be due to precipitation of platinum as platinum sulphide.

For the laboratory removal of platinum, SRB were batch grown over a period of six days. During mid-stationary growth phase, 400 ml of AECO effluent were pumped into the growing SRB at a flow rate of 33.3 $ml.h^{-1}$ and a hydraulic retention time of 12 h. The flow rate was set in such a way that the dilution of effluent concentration with SRB (1L) was taken into account. Over this period the system was continuously flushed with hydrogen gas. Samples were taken at point A and B as shown in Figure 6.5, and were analyzed for platinum, pH, sulphide and sulphate. Chloride analysis was avoided since its behavior under sulphate reduction is not at all understood.

6.3.2.3.1 Platinum removal

Platinum removal of 78% from AECO effluent was achieved from both points (A and B) (Figure 6.7a), High percentage platinum removal is believed to be a consequence of platinum precipitation by SRB activity after the reaction with the hydrogen sulphide produced in the sulphate reduction according to the following reaction.

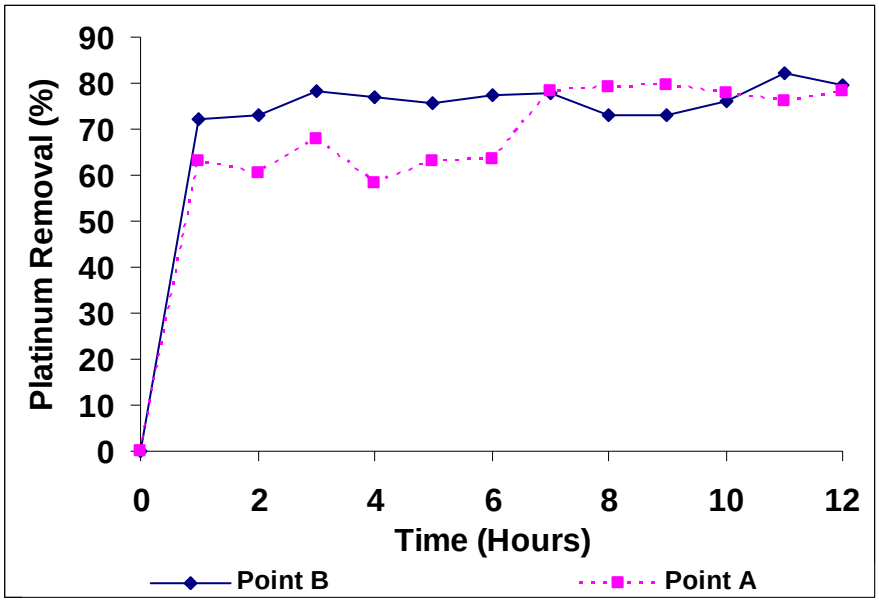
$$H_2S + Pt \rightarrow PtS + 2H^+ \quad \text{.....13}$$

For biological reduction to be totally effective for the removal of platinum, 1 mole of sulphate, after reduction would eliminate 1 mole of platinum as shown in equation 13.

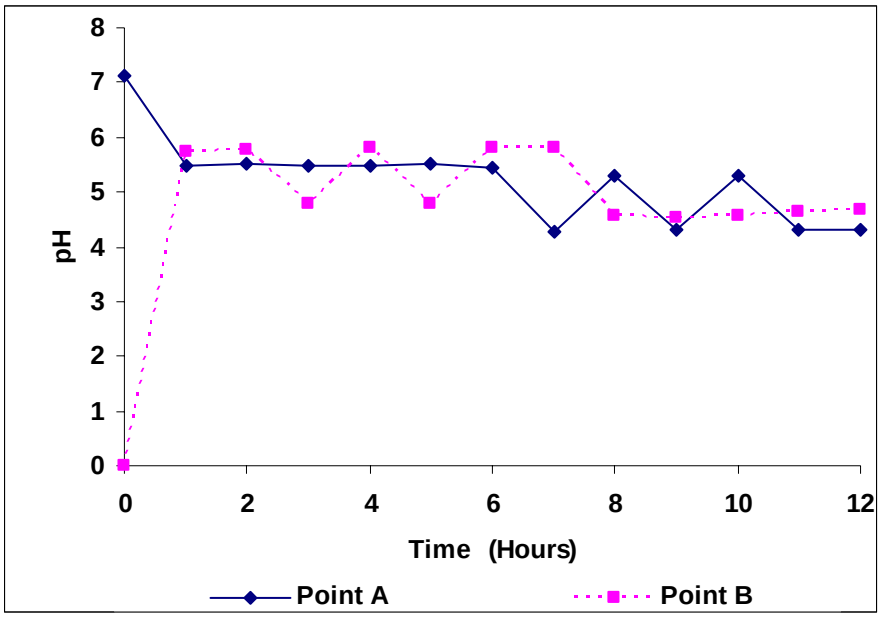
The suggestion of platinum sulphide precipitation was confirmed by a sudden change of culture colour to black on contact with the effluent.

6.3.2.3.2 pH profile

The pH of the culture (point A) decreased from 7.3 ± 0.4 to 5.6 ± 0.1, while the pH of the effluent (point B) increased from 0.38 ± 0.0 to 5.6 ± 0.1 (Figure 6.7b). Such major pH fluctuations were not surprising given the fact of the strong buffering capacity of the sulphidogenic system. The difficulty of growing SRB in acid media has been mentioned often in the literature and it is still a controversial aspect in the possible treatment of acidic effluent (Arnesen and Iversen, 1991; Hao *et al*., 1994). It is believed that low pH inhibits the SRB activity by increasing the solubility of the metallic sulphides formed through the classical reaction of solubilization in the presence of oxidants and acids. In contrast, a recent report on bioremediation of industrial acid mine water by SRB has shown that mixed SRB can be adapted to pH 5.0 without any problems. For efficient treatment of metal contaminated effluent by SRB either *in situ* or *ex situ*, there must be sufficient knowledge of the toxicity of various metals to the SRB populations. Bacterial sulphate reduction will not take place if metals are present at concentrations that are toxic to target organisms.



(a)

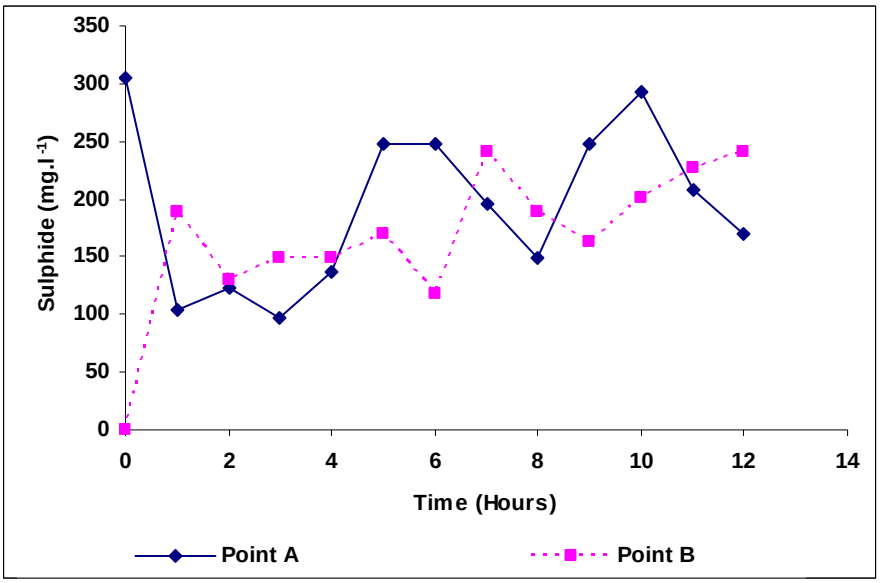


(b)

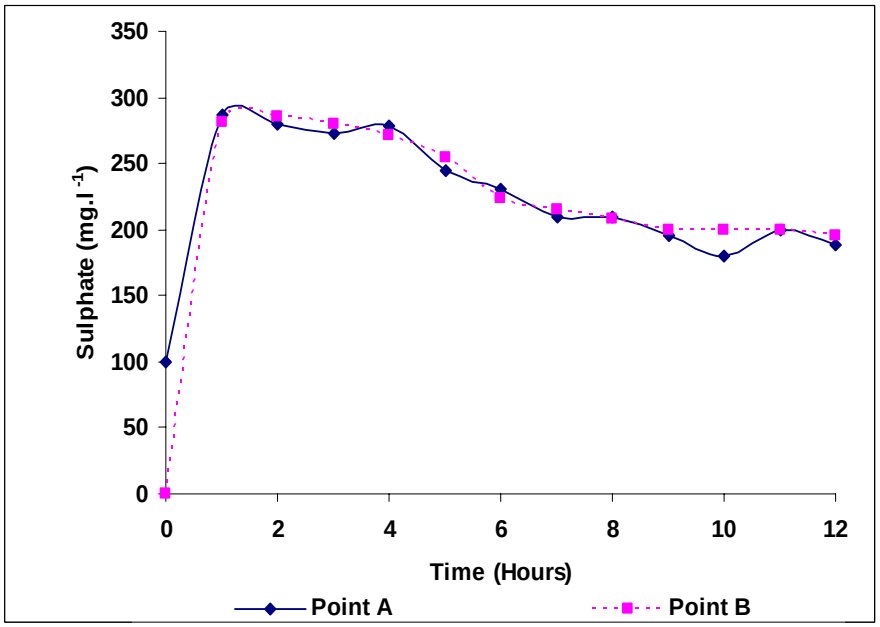
Figure 6.7: Platinum removal under growing SRB conditions (a) percentage platinum removal over the period of 12 h.(b) changes in pH value over 12 h.

6.3.2.3.3 Bacterial sulphate reduction

In order to investigate whether sulphate reduction was occurring over the 12 h treatment process, sulphate and sulphide content were monitored throughout the process. Sulphide concentrations over the treatment process in both points are shown in Figure 6.8a. After the first hour of effluent contact with the culture, sulphide concentration decreases from 300 $mg.l^{-1}$ to 100 $mg.l^{-1}$ (Figure 6.8a). A major decrease in sulphide values is believed to be due to continuous bubbling of the system with hydrogen gas, resulting in some of the sulphide escaping as gaseous hydrogen sulphide. Increased fluctuation in sulphide concentrations after 4 h between 150-250 $mg.l^{-1}$ was observed, indicating that sulphate reduction could be taking place in the presence of hydrogen as an electron donor, although not as effectively as in the presence of lactate as an electron donor. The use of hydrogen as the electron donor for SRB provides a low cost process, with minimum waste disposal in comparison to the use of electron organic electron donor.



(a)



(b)

Figure 6.8: Biological sulphate reduction under continuous treatment of platinum containing effluent. (a) Sulphide generation. (b) Sulphate reduction over the treatment period.

Changes in sulphate concentration over the effluent treatment period are shown (Figure 6.8 b). Before effluent pumping the sulphate concentration in the reactor was 100 $mg.l^{-1}$. Upon addition of the effluent (containing 170 $mg.l^{-1}$ sulphate), sulphate concentration fluctuated around 280 $mg.l^{-1}$ for the first 3 h. A significant decrease in sulphate reduction was observed after 5 h, fluctuating around 180 $mg.l^{-1}$(Figure 6.8b). These results suggest

that sulphate reduction might be taking place after 4 h, in the presence of hydrogen as an electron donor. Observation of sulphate reduction at pH as low as 5.0, indicates that the mixed culture could be adapted to this low pH level without any difficulties. From a future point of view there are two possible limiting factors for bacterial growth in the presence of platinum containing effluent: the sulphate concentration and the carbon source. Concerning the first factor, the concentration of sulphate contained in platinum effluent is very low (170 $mg.l^{-1}$) compared to the sulphate concentration required for effective sulphate reduction (ca. 1500 $mg.l^{-1}$). This indicates that if this system is to be used in a pilot scale additional source of sulphate will be required. The other limiting factor could be the carbon source for the SRB growth. While lactate is an effective carbon source of SRB, supplying lactate at pilot and industrial scale would be impractical and expensive. In addition while hydrogen as an electron donor provides advantage of low biomass generation during sulphate reduction, its utilization in pilot scale will not be cost effective. As a result part of the future work will include the investigation of a cheap carbon source for SRB.

6.4 Summary

Investigation of platinum (IV) reduction *in vitro* by different protein fractions, showed highest platinum reduction activity with soluble fraction only in the presence of hydrogen, suggesting that platinum precipitation *in vitro* do not necessitate the presence of membrane as nucleation sites. Column fractions revealed hydrogen-dependent platinum (IV) reducing activity in the eluted fraction. Investigation of enzymatic platinum (IV) reduction *in vitro*, showed highest hydrogen-dependent platinum (IV) reducing activity in the presence of hydrogenase and its physiological electron carrier, cytochrome c_3. Bioremediation studies utilizing resting SRB cells showed 34% platinum removal from the effluent. Platinum removal over the period of 12 h, by growing SRB cells showed platinum removal of 78%, with the pH of the system fluctuating at around 5.6 ± 0.1. Evidence of sulphate reduction and sulphide generation were observed over the period of 12 h treatment process, regardless of the low pH levels, suggesting that the system could potentially be applied for treatment of platinum containing effluent at continuous basis.

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Appendices

Appendix A: Methods used for P t(IV) uptake from solution by a sulphate reducing Consortium.

A1: Platinum standard curve

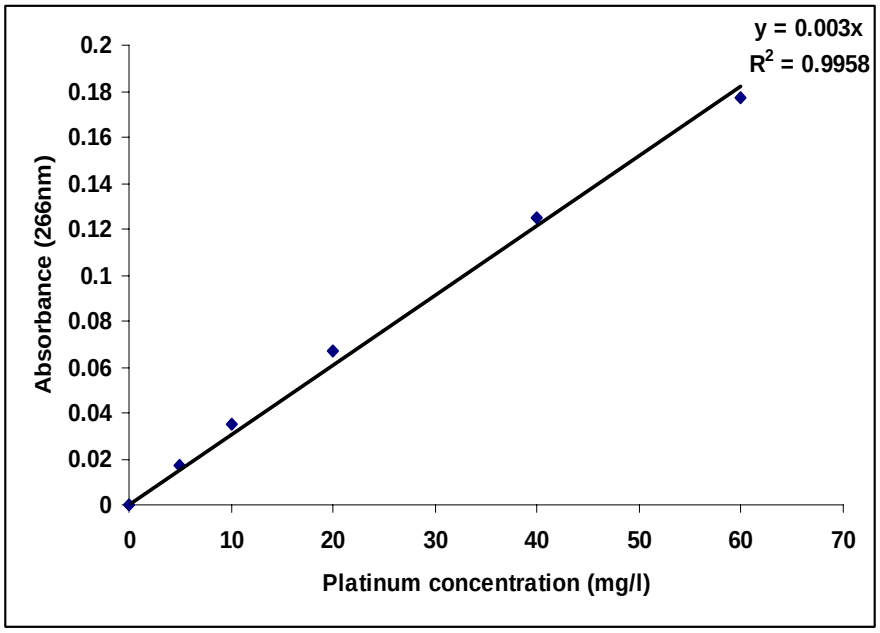

Figure A1: Platinum standard curve at 266 nm.

A2: Modified Postgate medium preparation (modified from Atlas, 1993)

Solution A (g/970ml): Add 1.0 g NH_4Cl, 1.0 g $CaCl_2.2H_2O$, 2.0 g $MgSO_4.7H_2O$, 1.0 yeast extract, 6 ml 60% sodium lactate, make up the volume to 970 ml with ddH_2O and adjust the pH to 8.50 with 1 N NaOH before autoclaving.

Solution B: Dissolve 2.5 g KH_2PO_4 in 100 ml ddH_2O

Solution C: Dissolve 0.1 g of Ascorbic acid and thioglycollate in 100 ml ddH_20

Solution D: Dissolve 0.5 g $FeSO_4$ in 100 ml

To complete the media:

(i) Autoclave solutions **A, B**, **C** and **D** separately at 121^{o}C for 15 min

(ii) Under UV-hood add 10 ml of autoclaved solution **B**, **C**, and **D** to solution **A** and adjust the pH to 7.5 with 1NaOH

A3: Reactor set up Operational and sampling procedure

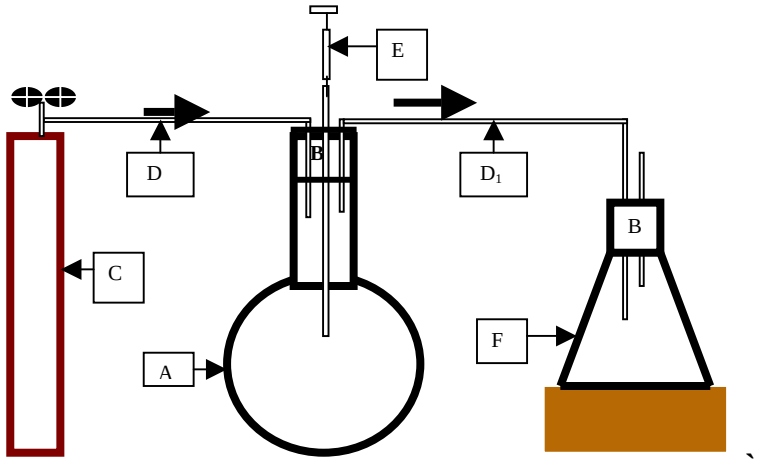

Figure A2: A schematic representation of continuous shaken tank (CSTR) bioreactor used to culture SRB, under anaerobic condition: (A) Bioreactor; (B) butyl rubber stopper; (C) N_2 gas cylinder; (D and D_1) Oxygen impermeable tygon® tubing plastic tubings, connected to glass tubings; (E) sampling syringe; (F) Zinc acetate solution flask.

Operational procedure*:* In order to culture SRB anaerobically a continuous shaken tank bioreactor was set up as shown in fig II. A reactor had a total volume of 5 L (A) as shown in Figure A2, sealed with butyl rubber stopper (B). Samples for subsequent inoculum were collected using disposal syringe (E), connected to glass tubing, going into the reactor. To maintain strict anaerobic conditions during sampling and the start of each run, high rate N_2:CO_2 gas (C) was purged through oxygen impermeable tygon® tubing, connected to glass tubing into the reactor (D). The mixture of N_2:CO_2 gas and H_2S (produced during sulphate reduction) flows out of the reactor through (D_1), while residual H_2S is trapped in (15 % w/v) zinc acetate solution flask (F). The arrows indicate the direction of N_2:CO_2 gas and/ or H_2S flow within the reactor. All the reactor flasks referred to hereafter have the same basic set up as the reactor.

A4: Sulphate standard curve

Conditioning reagent: Dissolve 150 g of NaCl in 1 L of solution containing 100 ml glycerol, 1 M HCl, 200 ml 95% ethanol, 640 ml ddH_20.

0.246 M Barium chloride solution Dissolve 60 g of $BaCl_2.2H_2O$ in 1L ddH_20

Sulphate stock solution: Dissolve 0.153 g of $Na_2SO_4^{2-}$ anhydrous in 250 ml ddH_2O and the standard range (0- 400 mg/l).

Table A1: Preparation of sulphate standard curve

SULPHATE CONCENTRATION ($MG.L^{-1}$)	SULPHATE STOCK SOLUTION (ML)	DDH_2O (ML)
0	0	0
25	0.04	0.96
50	0.08	0.92
100	0.16	0.84
200	0.33	0.67
300	0.50	0.50
400	0.66	0.40

Analysis: Measure 1 ml of a sample, and add 1 ml of conditioning reagent and barium chloride solution and vortex the mixture. Read absorbance immediately at 420 nm.

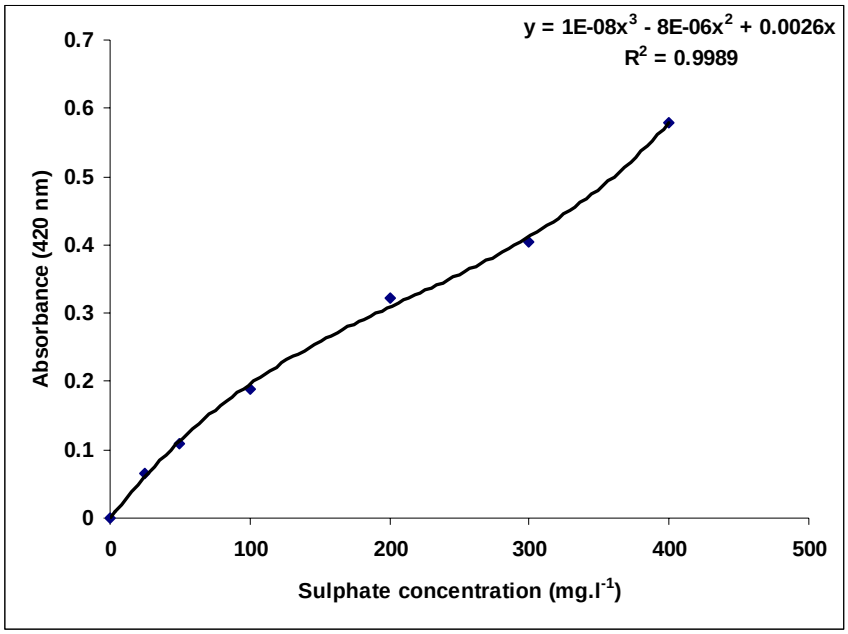

Figure A3: Sulphate standard curve. (Third polynomial equation was solved using MATLAB software)

A5: Preparation of sulphide standard curve

0.02 M Amine-sulphuric acid solution: Dissolve 2 g of N, N-Dimethyl-ρ-Phenyldiamine Dihydrochloride in 500 ml 6 M HCl.

0.06 M $FeCl_2$ Solution: Dissolve 8 g of $FeCl_3.6H_2O$ in 500 ml 6 M HCl.

Sulphide stock solution: Dissolve 0.789 g of $Na_2S.9H_2O$ in 500 ml ddH_2O

Table A2: Preparation of sulphide standard curve

SULPHIDE CONCENTRATION ($MG.L^{-1}$)	SULPHIDE STOCK SOLUTION (ML)	DDH_2O (ML)
0	0	100
0.2	2	98
0.4	4	96
0.6	6	94
0.8	8	92
1	10	90

Analysis:

Measure 5 ml of the sample. Add 0.5 ml each of amine-sulphuric acid and ferric chloride reagents. The colour is allowed to develop for an hour and the absorbance read at 670 nm.

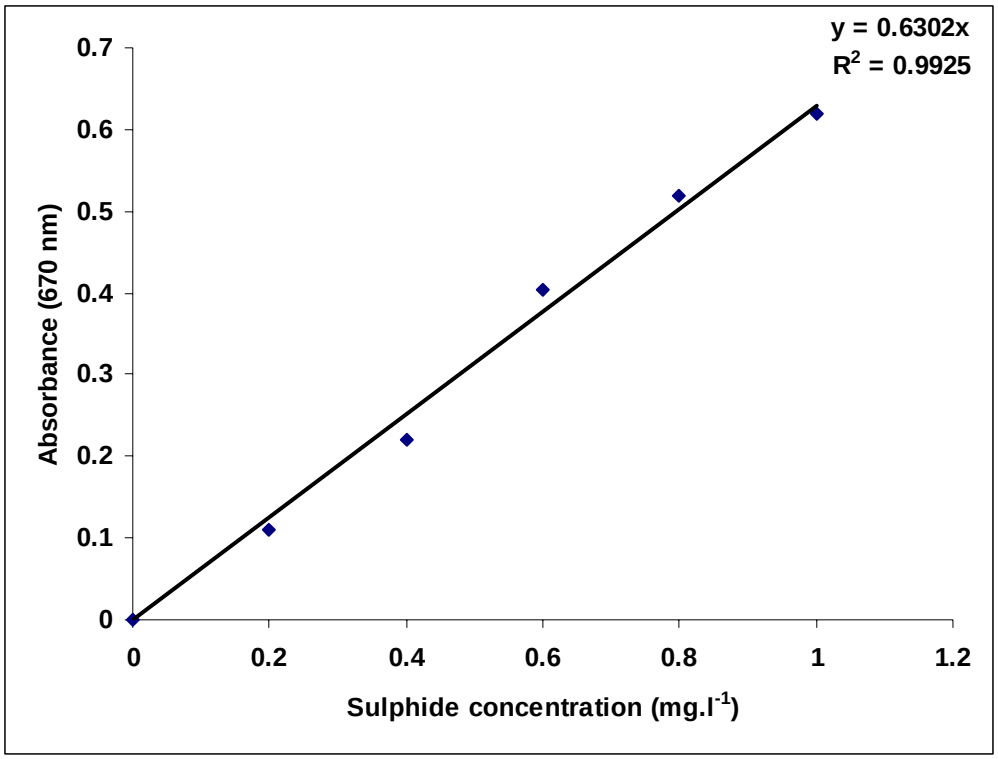

Figure IV: Sulphide standard curve

A6: <u>Modified Postgate medium C preparation (modified from Atlas, 1993)</u>

Modified Postgate C was prepared exactly as medium B described in appendix B, with an exception of solution **D** which was prepared as follows. Dissolve 0.5 g tri-Sodium Citrate in 100 ml.

<u>Appendix B</u>: <u>Method used for protein determination</u>

B1: <u>Protein standard curve</u>

Protein stock solution: Dissolve 0.01g of Bovine serum albumin (BSA) in 10 ml of ddH_2O

Table B1: Preparation of protein standard curve

PROTEIN CONCENTRATION ($MG.ML^{-1}$)	BSA STOCK SOLUTION (ML)	DDH_2O (ML)	BRADFORD REAGENT (ML)
0	0	5	250
0.4	1	4	250
0.8	2	3	250
1.2	3	2	250
1.6	4	1	250
2.0	5	0	250

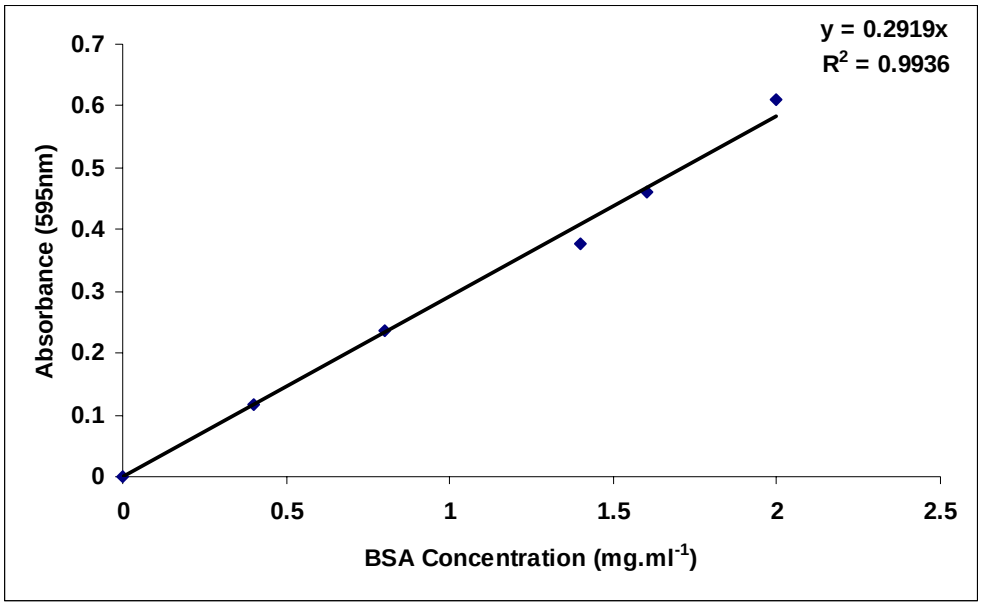

Figure V: Protein standard curve

Appendix C: Methods used for purification and partial characterization of hydrogenases from a mixed sulphate reducing consortium

C1: Regeneration and equilibration of the column

(i)Wash approximately 50 g of resin (1M HCl, 100 ml, 30 min decant)

(ii)Wash the resin three times (ddH_2O, 500 ml, 30 min each step decant)

(iii)Wash the resin (1M NaOH, 100ml, 30min, decant)

(iv) Wash the resin three times (500 ml ddH_2O, 30 min each wash, decant)

(v) Equilibrate with the starting buffer and check pH of the washing buffer until is equal to that of the starting buffer.

C2: SDS-PAGE (Laemmli , 1970) Recipes

Acrymalide/Bis: (30% T, 2.6% C): Dissolve 87.6 g of acrylamide and 2.9 g N'N'-methane-acrylamide in 300 ml. Filter and store at 4 ^oC in the dark (30 days maximum)

0.5% (w/v) Bromophenol blue: Dissolve 10 g bromophenol blue in 90 ml ddH_2O with gentle stirring and bring to 100 ml with ddH_2O

Resolving Gel Buffer: (1.5 M Tris-HCl, pH 8.8) : Dissolve 27.2 Tris base in 80 ml ddH_2O, adjust the pH to 8.8 with 6 M HCl , bring the total volume to 150 ml with ddH_20 and store at 4 ^oC.

Stacking Gel Buffer: (0.5M Tris-HCl, pH 6.8): Dissolve 6.0 g of Tris base in 60 ml ddH_20, adjust the pH to 6.8 with 6 M HCl , bring the total volume to 100 ml with ddH_20 and store at 4 ^oC.

10% Ammonium per sulphate (APS)(Prepared daily): Dissolve 0.1 g of APS in 1.0 ml ddH2O

10% (w/v) SDS: Dissolve 10 g in 90 ml water with gentle stirring and bring to 100 ml with ddH_2O.

Staining solution: Brilliant blue R-staining solution (Sigma, B6529).

Coomassie Gel Destain: Add 100 ml of methanol and glacial acetic acid and make the volume up to 1L with ddH_20

Sample buffer: Store at room temperature

REAGENT	**VOLUME (ML)**
ddH_2O	3.50
0.5M Tris-HCl, pH 6.8	1.25
Glycerol	2.50
10% (w/v) SDS	2 .00
0.5 (w/v) Bromophenol Blue	0.2 0

Add 5µl of β-mercaptoethanol to 950 µl sample buffer prior use. Dilute the sample at least 1:2 with sample buffer and heat at 95 ^oC for 4 min.

10X Electrode (Running) Buffer, pH 8.3:

REAGENT	**QUANTITY**
Tris base	30.3 g
Glycine	144 g
SDS	1.00 g
ddH_2O	1.0 L

Dilute 1:100 ml before use

Making up gels

10% Resolving Gel **4% Stacking Gel**

REAGENT	**VOLUME**	**REAGENT**	**VOLUME**
ddH_2O	4.1 ml	ddH_2O	6.1 ml
Acrylamide/Bis	3.3 ml	Acrylamide/Bis	1.3 ml
Resolving gel buffer	2.5 ml	Stacking gel buffer	2.5 ml
10% (w/v) SDS	0.1 ml	10% (w/v) SDS	0.1 ml
10% (w/v) APS	50 µl	10% (w/v) APS	50 µl
TEMED	5.0 µl	TEMED	10 µl

C3: Molecular weigh determination

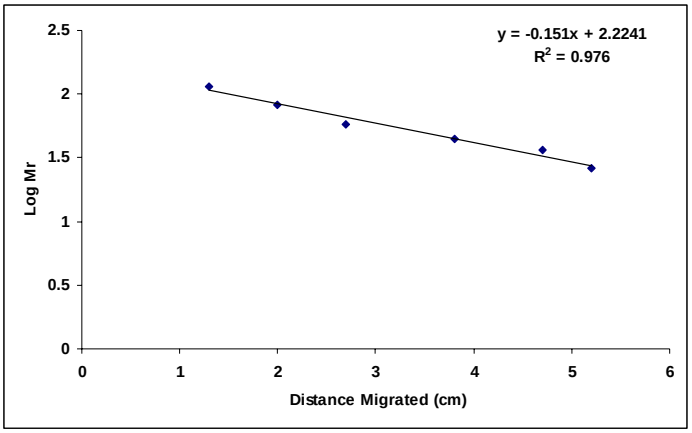

Figure VI: Calibration curve of the log molecular weight (M_r) markers (β-Galactosidase 116 KDa, Fructose-6—phosphate kinase 84KDa, Pyruvate Kinase, 58 KDa; Oval Albumin, 45 KDa, Lactic Dehydrogenase, 36.5 KDa and Triophosphate Isomerase 26,6KDa) versus distance migrated.