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A study of chemolithoautotrophic bacterial rhodanese, and its potential contribution to cyanide wastage during cyanidation of biooxidized concentrates

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Abstract

Refractory gold-bearing metal-sulfide ores and concentrates can be successfully and economically treated by biooxidation prior to cyanidation. However, one of the major drawbacks of the biooxidation process is the excessive consumption of cyanide during the gold dissolution process. The largest proportion of cyanide wastage is attributed to thiocyanate formation. Thiocyanate can be formed by spontaneous chemical reactions between reactive sulfur species and cyanide. The enzyme rhodanese (thiosulfate: cyanide sulfurtransferase EC 2.8.1.1) is also able to catalyze the formation of thiocyanate using thiosulfate and cyanide as substrates. Therefore, the most relevant members of the microbial consortium responsible for biooxidation of gold-bearing ores or concentrates were investigated to determine whether they were able to contribute to thiocyanate formation by means of a enzyme reaction mechanism indicative of rhodanese. Together with a *Thiobacillus caldus* strain (*T. caldus* MNG), isolated from a biooxidation pilot plant, the sulfur-oxidizers *Thiobacillus ferrooxidans* ATCC 33020 (also able to oxidize iron) and *Thiobacillus thiooxidans* ATCC 19377 demonstrated rhodanese activity. However, the obligate iron-oxidizer *Leptospirillum ferrooxidans* DSM 2705 had no detectable rhodanese activity. High levels of rhodanese activity were detected from the mixed microbial population of a biooxidation pilot plant, which appeared to be dominated by *T. caldus* MNG. This *T. caldus* strain was initially identified in the biooxidation pilot plant using the PCR based 16S rDNA profiling technique of Rawlings (1995), and the identification confirmed by 16S rDNA sequencing. Therefore, *T. caldus* MNG is considered the major contributor to the rhodanese activity of the biooxidation pilot plant mixed culture. Within the range of sulfur substrates tested, the rhodanese enzyme of *T. caldus* MNG behaved exclusively as a thiosulfate: cyanide sulfurtransferase, and the rhodanese activity of *T. caldus* MNG appeared to be dependent on the physiological state of the cell during batch growth. An attempt to isolate a rhodanese gene from *T. caldus* MNG by Southern hybridization, using the *Azotobacter vinelandii rhdA* gene as a probe, was unsuccessful.

On balance of the information available from this study, and reported elsewhere, rhodanese activity probably does not contribute as much to thiocyanate formation in the cyanidation plant as does the spontaneous chemical formation of thiocyanate.

Abbreviations

A	adenosine
A	alanine
Å	Ångström units
Ala	alanine
ADP	adenosine 5'-diphosphate
Amp	ampicillin
Amp^R	ampicillin resistance
AMP	adenosine 5'-phosphate
APS	adenosine 5'-phosphosulfate
Arg	arginine
Asp	aspartic acid
ATCC	American Type Culture Collection
ATP	adenosine 5'-triphosphate
bp	base pairs
C	carbon
C	cysteine
C	cytosine
cAMP	cyclic adenosine 3',5'-monophosphate
C-terminal	carboxyl-terminus
CsCl	cesium chloride
CSPD®	disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.$1^{3,7}$]decan}-4-yl) phenyl phosphate
Cys	cysteine
°C	degrees Celsius
D	aspartic acid
DANTS	5-dimethyl-amino-1-napthalene-thiosulfonate
DIG	dioxigenin-11-dUTP
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DSM	Deutsche Sammlung von Mikroorganismen
dUTP	deoxyuradine triphosphate
E	glutamic acid
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
F	phenylalanine
Fe-S	iron-sulfur

G	guanine
G	glycine
G+C	guanine: cytosine ratio
Glu	glutamic acid
Gly	glycine
H	histidine
His	histidine
hr	hours
I	inosine
I	isoleucine
Ile	isoleucine
K	1000rpm
K	lysine
kb	kilobase pair(s) or 1000bp
KAc	potassium acetate
kDa	kilodaltons
kW	kilowatt
L	leucine
l	litre
Leu	leucine
Lys	lysine
M	methionine
Met	methionine
mg	milligram
min	minute(s)
ml	millilitres
mM	millimolar
mmol	millimole
mol%	molar percentage
MST	mercaptopyruvate sulfurtransferase
N	asparagine
N	normal
NAD	nicotinamide-adenine dinucleotide
NADH	reduced form of NADH
NCBI	National Center for Biotechnology Information
N-terminal	amino-terminus
nm	nanometers

P	proline
PCR	polymerase chain reaction
Phe	phenylalanine
P_i	inorganic phosphate
pKa	acid dissociation constant
Pro	proline
PVC	polyvinyl chloride
Q	glutamine
R	arginine
$-rCO_2$	carbon dioxide utilization rate
$-rO_2$	oxygen utilization rate
rDNA	ribosomal deoxyribonucleic acid
rpm	revolutions per minute
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RDP	Ribosomal Database Project
S	sedimentation coefficient
S	serine
s	second(s)
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
Ser	serine
SET	sucrose EDTA buffer
T	threonine
T	thymine
TE	Tris EDTA buffer
TCA	trichloroacetic acid
Thr	threonine
Tris	Tris (hydroxymethyl) aminomethane
Trp	tryptophan
Try	tyrosine
µg	microgram
µl	microlitres
µM	micromolar
µm	micrometer
µmol	micromole
UCT	University of Cape Town

V	valine
V	volts
v/v	volume/volume
Val	valine
W	tryptophan
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-galactoside
Y	tyrosine

Chapter One

Literature review

Contents

1.1) Introduction

In the past gold was recovered from ore by amalgamation with mercury. However, for the last 100 years cyanide has been preferentially used. Although a simple and universal process in the mining industry, cyanidation has its limitations. As the gold has to be accessible to the cyanide solution, it is only economically viable to use ore grinding as a pretreatment process with what is termed 'free-milling ore' – ore that allows for efficient gold recovery by direct cyanidation (Morin, 1995). However, gold occurs commonly in association with base metals which do not respond well to cyanidation, even after grinding the ore to fine particles. Such ores are said to be refractory. In refractory ores the gold is locked up in a matrix of metallic sulfides, mainly pyrite (FeS_2) and arsenopyrite (FeAsS). These refractory ores were in the past simply discarded with tailings from the milling process (Morin, 1995).

Until recently, only two physical processes were successful as pretreatment processes for refractory gold-bearing metal-sulfide ores prior to cyanidation (Morin, 1995):

The most common process for dealing with refractory ores or concentrates is roasting. This pyrometallurgical process is a two-stage process in which the ore is subjected to intense temperature (700°C) under oxygen deficient conditions. This pyro-physical method alters the ore lattice producing a calcine that consists mainly of magnetite (Fe_3O_4). The magnetite is then transformed into porous hematite (Fe_2O_3) which allows for gold recovery by cyanidation of the calcine (Morin, 1995).

Pressure-leaching is also used for gold recovery from refractory ores and concentrates. This hydrometallurgical process oxidizes the ore in an aqueous acidic medium under high pressure and an oxygen enriched atmosphere to convert sulfides to sulfates (Rawlings and Silver, 1995). The ore is then baked at high temperature in the presence of oxygen for the complete conversion of elemental sulfur. This process is said to result in a high degree of gold liberation (Morin, 1995).

Ultimately, the ore from both pyrometallurgical and hydrometallurgical processes is subjected to cyanidation for the dissolution of the gold particles from the ore matrix. Without a pretreatment process of refractory ores or concentrates only 40-50% of the gold is said to be recovered by cyanidation (Rawlings and Silver, 1995).

However, the above mentioned pyrometallurgical and hydrometallurgical processes cause the environmentally unacceptable release of volatile toxic substances such as sulfur dioxide, arsenic and lead (Torma and Oolman, 1992). This has meant that these pretreatment methods are becoming increasingly unpopular for dealing with refractory ores (Brierley, 1982; Morin, 1995).

Microbial biooxidation of gold-bearing metal-sulfide ores and concentrates in continuous-flow bioreactors has become a technically and economically viable alternative. A biohydrometallurgical process patented as the Biox® process was developed in the 1980's by Gencor S.A. Ltd., South Africa (now Billiton) for the pretreatment of refractory gold-bearing arsenopyrite ores. This was followed by a biohydrometallurgical process, developed and commercialized by the company BacTech, which operates at temperatures above that of the Biox® process. The BacTech process is currently in use treating gold-bearing concentrates at the Youanmi mine in Western Australia (Miller, 1997). Both the Biox® and BacTech processes are carried out in highly aerated stirred-tank bioreactors. Alternative biohydrometallurgical processes for the pretreatment of gold-bearing metal-sulfide ores and concentrates are the heap reactor technology and Geobiotics processes. These two processes may be used as alternatives to stirred-tank processes as, due to their mineralogical characteristics, certain ores may not be amenable to flotation-concentration. Biooxidation heap reactor technology is used by the Newmont Gold Company at its Gold Quarry mine in Carlin, Nevada, USA. Geobiotics Inc. has built a demonstration plant in South Dakota, USA, based on the use of heaps of support rock coated with an ore concentrate (Brierley, 1997; Whitlock, 1997).

1.2) Biox® process design

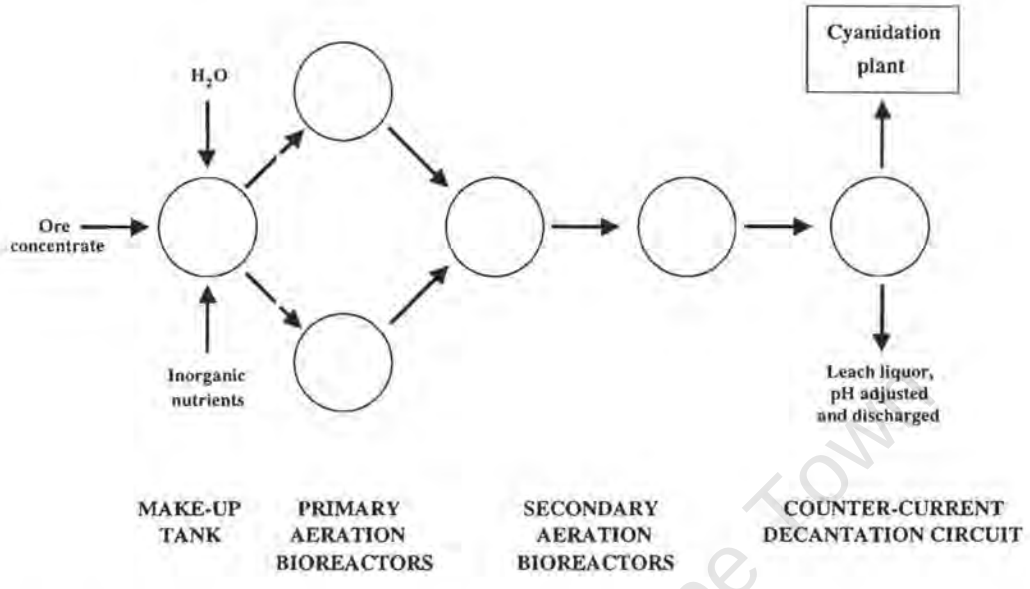

Fig 1.1: Flow chart of the Biox® process. Reproduced from Rawlings and Silver (1995).

Prior to entry into the Biox® plant (Fig 1.1), the refractory gold-bearing ore is milled to a fine powder and a gold-bearing concentrate prepared by flotation. The feed concentrate then enters the make-up tank of the Biox® plant where inorganic nutrients are added in the form of inexpensive fertilizer-grade ammonium sulfate and potassium phosphate (Rawlings, 1998). The solids content in the slurry feed entering the primary aeration bioreactors is typically 18-20% by mass (Dew *et al.*, 1997).

Depending on the mineral biooxidation rate achieved, the residence time of the pulp in the biooxidation bioreactors is four days (Dew *et al.*, 1997). Biox® plants are typically configured into two stages. In the first stage the primary aeration bioreactors act in parallel. This is to allow for a two day pulp retention time, whereas in the secondary aeration bioreactors it is 0.67 days (Fig.1.1). The longer pulp retention time in the primary aeration bioreactors allows for the establishment of a stable microbial population, microbial attachment to the concentrate, and prevents washout (Dew *at al.*, 1997).

The slurry then passes to the counter-current decantation circuit where the slurry is washed for removal of reactive iron species. This enhances gold recovery during cyanidation. The oxidized ore is then transferred to the cyanidation plant for final gold recovery. The liquor from the decantation circuit enters the neutralization plant where it is pH adjusted to pH 7.0-8.0. This pH adjustment causes the precipitation of iron and arsenic which allows for safe disposal of the waste on a tailings dam (Dew *et al.*, 1997).

1.3) The biomining bacteria

The temperature and pH range at which the Biox® process operates provides optimum growth conditions for the mesophilic bacteria responsible for the biooxidation of refractory ores. Dependent on the ore to be oxidized, the operating temperature of the Biox® process is between 35 and 45°C (Brierley, 1997), and the pH range is 1.2 to 2.0 (Dew *et al.*, 1997). The operating conditions of the Fairview Biox® plant, South Africa, is reportedly 41°C and pH 1.6 (Dew *et al.*, 1997).

The most important Biox® bacteria are a mixed population of highly motile Gram-negative acidophilic chemolithoautotrophic bacteria (Dew *et al.*, 1997; Rawlings and Silver, 1995). These organisms are capable of growth in what is described as an extreme environment (Rawlings, 1997). The predominant chemolithoautotrophic bacteria in the Biox® bioreactors have been reported to be *Thiobacillus ferrooxidans, Thiobacillus thiooxidans, Thiobacillus caldus* and *Leptospirillum ferrooxidans* (Rawlings, 1997; Rawlings, 1998).

These bacteria are highly adaptable to the acidic, inorganic environment in which biooxidation occurs (Rawlings, 1997). This environment is very nutrient poor, but these obligate autotrophs are able to meet their energy requirements by utilizing an inorganic energy source and air. Air provides carbon (CO_2), nitrogen (N_2) and the final electron acceptor, oxygen (O_2). Carbon, in the form of CO_2, is fixed by the Calvin reductive pentose phosphate cycle (Holuigue *et al.*, 1987; Rawlings, 1997). Atmospheric ammonia,

which is highly soluble in acidic solutions, could possibly provide sufficient nitrogen for growth although in practice a small quantity of ammonium sulfate is added (Rawlings, 1997). Water and ore provide trace elements in the form of impurities (Rawlings, 1998). The ore, being composed mainly of iron and sulfur, provides the inorganic energy source. The thiobacilli oxidize reduced inorganic sulfur compounds for energy acquisition, whereas *L. ferrooxidans* can only oxidize ferrous iron. *T. ferrooxidans* is, however, able to utilize both ferrous iron and reduced inorganic sulfur compounds as its energy source (Brierley, 1982; Rawlings and Silver, 1995).

During the complete oxidation of pyrite only one electron is derived from the ferrous iron component of the ore, while 14 electrons are derived from the inorganic sulfur moiety. Therefore, reduced inorganic sulfur is far more bioenergetic than ferrous iron. It has been shown that *T. ferrooxidans* yields a higher biomass per mol of electrons during batch growth in an inorganic sulfur medium than in a ferrous iron medium (Pronk *et al.*, 1990). It would therefore be expected, and a view long held, that *T. ferrooxidans* was the dominant bacterial species in the biooxidation tanks. This assumption has been revised as quantitative bacterial enumeration techniques have shown that in the Fairview and Sao Bento Biox® bioreactors *L. ferrooxidans* predominates. In the Sao Bento Biox® bioreactors 48% of the microbial population was composed of *L. ferrooxidans*, 34% *T. thiooxidans*, and 10% *T. ferrooxidans* (Dew *et al.*, 1997).

T. ferrooxidans is approximately 15-fold more sensitive to end product inhibition by ferric iron, following ferrous iron oxidation, than *L. ferrooxidans* (Norris *et al.*, 1988; Rawlings, 1997). This would suggest that in the Biox® bioreactors where the quantity of ferric iron is high, *L. ferrooxidans* would predominate due to its selective advantage over *T. ferrooxidans*. This hypothesis is consistent with the quantitative enumeration of bacteria in the Biox® bioreactors and studies showing the presence of *L. ferrooxidans* rather than *T. ferrooxidans* (Helle and Onken, 1988; Rawlings, 1997).

Furthermore, it has been determined that as the slurry moves through the cascade of bioreactors in a Biox® plant the proportion of sulfur-oxidizing species increases with a concomitant decrease in *L. ferrooxidans*. This is explained by the decrease in oxidizable iron and an increase in the quantity of reduced inorganic sulfur compounds (Dew *et al.*, 1997).

Although the most significant increase in number has been attributed to *T. thiooxidans* (Dew *et al.*, 1997), it has been suggested that it is in fact the phylogenetically related *T. caldus* which shows a proportionate increase. *T. caldus* is an obligate sulfur-oxidizing bacterium which has an optimum growth temperature at 45°C and is able to rapidly oxidize sulfur in the temperature range of 35 to 50°C (Hallberg and Lindström, 1994). *T. thiooxidans*, however, has a temperature tolerance range of only 20 to 35°C (Schnell, 1997; Rawlings, 1998). As noted by Norris (1997), most commercial biooxidation bioreactors operate at 40°C which is the temperature threshold of the mesophilic *T. ferrooxidans* and *T. thiooxidans*. The thermophilic bacteria can exceed the growth of the mesophilic species at this temperature; one of these moderate thermophiles being *T. caldus* (Norris, 1997).

Harrison (1982) in his study of the genomic comparison between *T. ferrooxidans* and *T. thiooxidans* found that a strain designated *T. thiooxidans* DSM 612 had a G+C content vastly different to the 51-53 mol% (Holt *et al.*, 1994) reported for *T. thiooxidans*. This DSM 612 strain had a G+C content of 62 mol% which led Harrison (1982) to suggest, after disproving possible contamination, that this was a new species. It is interesting to note that Norris *et al.* (1986) initially determined the G+C content of strain BC13 (now recognised as a strain of *T. caldus*) to be 61.7 mol% before correction of this value to 63.1 mol% (Hallberg and Lindström, 1994).

Goebel and Stackebrandt (1994) in their qualitative study to determine if strain or species selection occurred when mixed microbial populations are transferred from natural sites to commercial batch or continuous bioreactors noted a *T. thiooxidans*-like isolate that grew

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at 45°C unlike the *T. thiooxidans* isolates that grew at 28°C. Hallberg and Lindström (1994) noted that the strain C-SH12 isolated by Goebel and Stackebrandt (1994) from a 40°C continuous bioreactor showed very high 16S rDNA sequence similarity to their *T. caldus* strain BC13. The C-SH12 isolate had a 3.3% 16S rDNA sequence divergence from *T. thiooxidans* ATCC 19377; the type strain (Goebel and Stackebrandt, 1994). It should also be noted that Goebel and Stackebrandt (1994) reported a 97.6% sequence similarity between the isolate C-SH12 and *T. thiooxidans* DSM 612.

By slot immunobinding, Amaro *et al.* (1994) showed that *T. caldus* accounts for a significant percentage of the microbial population found in the leachate of bioreactors operating at elevated temperatures. This suggests that the identification of *T. thiooxidans* in bioreactors, or rather the proportion of the mixed population that comprises this species, has been overestimated. However, García and Jerez (1995) found by an immunological analysis of a biooxidized ore that the distribution of ferrous iron-oxidizing and the sulfur-oxidizing bacteria was different in the leachate to that of the ore-attached population. This emphasizes the need for caution when attempting to quantify the proportion of various bacterial species in bioreactors.

1.4) Biooxidation economics and the problem of cyanide wastage

A new industrial process can only be successful if it competes economically with established technologies. In comparison to physico-chemical methods, the biooxidation process has the lowest capital costs (Rawlings and Silver, 1995). Roasting is said to have the lowest operating costs, but the biooxidation process requires inexpensive equipment and fewer skilled operators. However, roasting and pressure-leaching have a greater energy requirement in the form of heat and pressure compared to the biooxidation processes (Rawlings and Woods, 1995). Hydrometallurgical processes usually result in higher gold recoveries than pyrometallurgical processes and their environmental impact both in gaseous output and effluent is satisfactory (Morin, 1995). An important advantage of the biooxidation process is the ease with which it can handle pyritic and

high arsenic content ores without creating the pollution problems associated with pyrometallurgical processes (Torma and Oolman, 1992). Therefore, the biooxidation process is economically competitive.

Following the pretreatment of refractory gold-bearing ores the solids are separated from the aqueous medium by filtration or settling. The solid residue then enters the cyanidation plant (Fig 1.1) where it is flushed with cyanide (Morin, 1995). The attraction of the cyanidation process lies in its simplicity. Cyanide binds to the gold forming a highly stable chemical gold-cyanide complex (equation 1.1) which allows for the rapid dissolution of gold from the ore at ambient temperature (Adamson, 1973; Morin, 1995):

$$2Au + 4NaCN + O_2 + 2H_2O \rightarrow 2NaAu(CN)_2 + 2NaOH + H_2O_2 \qquad (1.1)$$

The dissolved gold is extracted from solution by either adsorption to activated carbon, absorption to ion-exchange resins, or by cementation (precipitation). The coarse product known as doré is then refined to its economically important metal (Morin, 1995).

A major problem that may occur during cyanidation is excessive cyanide consumption. Although this occurs for both pyrometallurgical and hydrometallurgical pretreated ores, cyanide wastage during cyanidation of biooxidation treated ores is greatly increased and is, therefore, a major drawback of the biooxidation process (Hackl and Jones, 1997; Rawlings, 1998).

Cyanide consumption data published for operational biooxidation plants show consumption quantities of 7.5 kg cyanide/ton of solids at the Youanmi mine in Western Australia (BacTech process), 15 kg cyanide/ton solids at the Ashanti mine in Ghana and 30kg cyanide/ton solids at the Wiluna mine in Australia (Biox® processes). These values are excessive if one compares typical cyanide consumption values for a pressure-leaching plant; approximately 1-2 kg cyanide/ton solids (Hackl and Jones, 1997). In this regard pressure-leaching has a significant economic advantage over biooxidation pretreatment.

Thus a detailed understanding of the mechanisms of cyanide consumption is required if a solution is to be found to improve the competitiveness of the biooxidation process (Hackl and Jones, 1997).

A large number of factors appear to contribute to cyanide consumption. These include: the formation of metallo-cyanides, particularly ferric cyanide ($Fe(CN)_6^{3-}$); the formation of iron and arsenic precipitates during biooxidation; and the sulfide content of the ore being oxidized. The sulfide content of the ore determines the quantity and nature of the sulfur species present in the bioresidue (Morin, 1995; Hackl and Jones, 1997). These cyanicides and reactive sulfur species can complex with cyanide thereby depleting the cyanide available for gold dissolution (Morin, 1995).

Hackl (1989) was the first to suggest that the production of thiocyanate (SCN^-) may contribute to the wastage of cyanide. It was later determined that thiocyanate formation was accountable for the largest proportion of cyanide consumption (Hackl and Jones, 1997). Thiocyanate formation was attributed to the presence of reactive sulfur species in the bioresidue. This can be directly related to the sulfide content of particular ores. Arsenopyrite, for example, when oxidized by microorganisms can release significant amounts of elemental sulfur (Morin, 1995). This elemental sulfur is proposed to exist in a polymeric form rather than its thermodynamically stable orthorhombic (S_8) form. The polymeric sulfur is considered to be very cyanide reactive which would account for consumption quantities of 100kg cyanide/ton of solids being recorded (Komnitsas and Pooley, 1990; Morin, 1995; Hackl and Jones, 1997).

Elemental sulfur (S^o) reacts with cyanide in a spontaneous chemical reaction to form thiocyanate (equation 1.2) (Hackl and Jones, 1997).

$$S^o + CN^- \rightarrow SCN^- \qquad (1.2)$$

It is possible that the bacteria themselves contribute to cyanide wastage by producing a hydrophobic 'biological' elemental sulfur (S^0) (Hazeu *et al.*, 1988). Hazeu *et al.* (1988) found sulfur globules embedded in the periplasm and outer membrane wall of *T. ferrooxidans*. These sulfur globules are thought to be formed by the combination of higher polythionates with elemental sulfur forming a reactive elemental sulfur (Steudel *et al.*, 1987; Hazeu *et al.*, 1988). Colloidal sulfur particles formed by the aggregation of elemental sulfur atoms released during biooxidation have also been observed in the exopolymer layer surrounding *T. ferrooxidans* cells (Rojas *et al.*, 1995). These sources of elemental sulfur are thought to act as sulfur reserves that are used when the available sulfur has been depleted (Schippers *et al.*, 1996; Rojas *et al.*, 1995). However, during cyanidation this 'biological' elemental sulfur may provide the substrate for spontaneous thiocyanate formation.

Bacterial leaching of metal-sulfides occurs via an indirect mechanism that gives rise to thiosulfate (Sand *et al.*, 1995). Thiosulfate is considered a key intermediate in microbial sulfur metabolism, and it is well known that thiosulfate readily reacts with cyanide forming thiocyanate (equation 1.3) (Hackl and Jones, 1997).

$$S_2O_3^{2-} + CN^- \rightarrow SCN^- + SO_3^{2-} \qquad (1.3)$$

Therefore, the proportion that thiocyanate contributes to cyanide wastage is argued to be a consequence of the production of intermediate sulfur species resulting from biooxidation (Hackl and Jones, 1997).

1.5) Microbial biooxidation of gold-bearing metal-sulfide ores

Sand *et al.* (1995) argued that at the pH at which biooxidation occurs, pH <3.5, ferric iron hexahydrate is the primary oxidant of pyrite. The first sulfur intermediate formed following the attack on the ore by ferric iron hexahydrate is thiosulfate ($S_2O_3^{2-}$) (equation 1.4).

$$FeS_2 + 6Fe(H_2O)_6^{3+} + 3H_2O \rightarrow Fe^{2+} + S_2O_3^{2-} + 6Fe(H_2O)_6^{2+} + 6H^+ \quad (1.4)$$

Schippers *et al.* (1996) continued with this argument but pointed out that biomining bacteria such as *T. ferrooxidans* and *L. ferrooxidans* have an exopolymer layer containing a considerable load of iron (III) ions which acts as a reaction compartment allowing the bacteria to initiate the indirect attack on the ore surface. Due to the presence of iron (III) ions in the exopolymer layer, and the instability of thiosulfate in acidic solutions, thiosulfate is rapidly oxidized to tetrathionate ($S_4O_6^{2-}$); a chemical reaction which proceeds via an intermediate iron (III)-thiosulfate complex (equation 1.5) (Schippers *et al.*, 1996).

$$2Fe^{3+} + 2S_2O_3^{2-} \leftrightarrow 2FeS_2O_3^{+} \rightarrow 2Fe^{2+} + S_4O_6^{2-} \quad (1.5)$$

As this reaction (equation 1.5) proceeds faster than the dissolution of pyrite, thiosulfate is thought to be barely detectable in biooxidation solutions.

The tetrathionate product (equation 1.5) is then hydrolyzed, in the presence of pyrite, to highly reactive disulfane-monosulfonic acid and sulfate (equation 1.6).

$$S_4O_6^{2-} + H_2O \rightarrow HS_3O_3^{-} + SO_4^{2-} + H^+ \quad (1.6)$$

From this disulfane-monosulfonic acid several reactions are postulated to give rise to elemental sulfur, thiosulfate, trithionate, pentathionate and sulfite (Schippers *et al.*, 1996); as indicated in the following equations (1.7-1.10).

$$S_3O_3^{2-} + S_2O_3^{2-} + 0.5O_2 + 2H^+ \rightarrow S_5O_6^{2-} + H_2O \quad (1.7)$$

$$S_3O_3^{2-} + S_4O_6^{2-} \rightarrow S_2O_3^{2-} + S_5O_6^{2-} \quad (1.8)$$

$$S_3O_3^{2-} + 1.5O_2 \rightarrow S_3O_6^{2-} \quad (1.9)$$

$$4S_3O_3^{2-} \rightarrow S_8 + 4SO_3^{2-} \quad (1.10)$$

Thiosulfate produced by reaction 1.8 may enter the cycle again (Schippers *et al.*, 1996). Thiosulfate is unstable below pH 4 and rapidly decomposes to elemental sulfur and sulfite (Roy and Trudinger, 1970; Pronk *et al.*, 1990). This may account for why elemental sulfur is believed to be the first sulfur intermediate in pyrite biooxidation (Sand *et al.*, 1995).

The biomining bacteria are able to metabolize the iron (II) ions and various sulfur species produced by the above chemical reactions (equations 1.5-1.10) (Schippers *et al.*, 1996). Enzymatic oxidation of these reduced inorganic sulfur compounds by the acidophilic chemolithoautotrophic bacteria generates electrons that drive the electron transport chain for the production of metabolic energy.

Many of these enzymatic oxidation reactions result in the regeneration of various sulfur species which has led Pronk *et al.* (1990) to propose a hypothetical cyclic pathway for the oxidation of sulfur oxy-anions (Fig 1.2). This pathway has been called the 'S_4 intermediate', or S4I, pathway which is said by Kelly *et al.* (1997) to be characteristic of obligate chemolithoautotrophic bacteria such as *T. ferrooxidans* and *T. thiooxidans*. The S4I pathway considers thiosulfate as the initiating sulfur species in chemolithoautotrophic sulfur metabolism. Microbial metabolic regeneration of reactive sulfur species in this

cyclical pathway suggests that the bacteria may contribute to the production of reactive sulfur substrates for the spontaneous chemical formation of thiocyanate.

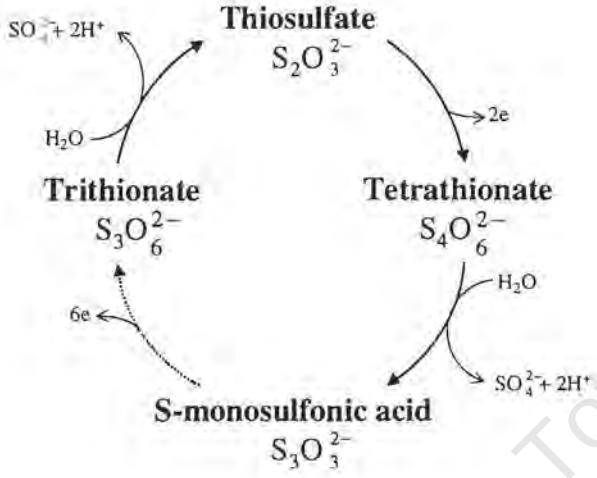


Fig.1.2: Hypothetical pathway for the microbial oxidation of sulfur compounds. Solid lines represent oxidation reactions demonstrated in thiobacilli species. Reproduced from Pronk *et al.* (1990).

It has been shown with *T. ferrooxidans, T. thiooxidans* and *T. caldus* that the initial step in thiosulfate metabolism is its oxidation to tetrathionate (Fig.1.2) (Pronk *et al.*, 1990; Hallberg *et al.*, 1996) by a thiosulfate oxidizing enzyme referred to by Kelly *et al.* (1997) as tetrathionate synthase. Tetrathionate synthase oxidatively condenses two thiosulfate anions to form tetrathionate (Kelly *et al.*, 1997). It appears that the tetrathionate synthase enzyme of *T. caldus* is located in the periplasm (Hallberg *et al.*, 1996), and an earlier study of the tetrathionate synthase enzyme of *T. ferrooxidans* suggested that the enzyme acted "extra-cytoplasmically"; a suggestion based on the finding that the pH optimum of this enzyme was pH 4.5. It was noted that below pH 4.5 enzyme activity could not be determined because of the instability of thiosulfate (Silver and Lundgren, 1968; Pronk *et al.*, 1990).

A tetrathionate hydrolase is then proposed to catalyse the hydrolysis of tetrathionate to S_3-sulfane monosulfonic acid (Pronk *et al.*,1990). The enzyme is active at low pH which according to Pronk *et al.* (1990) is in agreement with the observation that intermediary

sulfur formation occurs between the inner and outer membranes of *T. ferrooxidans*. Tano *et al.* (1996) purified a tetrathionate decomposing enzyme from *T. thiooxidans*. This enzyme catalyzed the decomposition of tetrathionate to thiosulfate and sulfate at pH 3.0-3.5 suggesting that tetrathionate is decomposed outside of the plasma membrane. Hallberg *et al.* (1996) noted that tetrathionate is probably transported across the cellular membrane before oxidation. This is based on the observation that metabolism of tetrathionate occurred in the cytoplasm of *T. caldus*, or at least on the inner surface of the cell membrane. Tano *et al.* (1996) suggested that the enzyme is a metallo-enzyme as the activity of the enzyme increased markedly in the presence of cupric ions. The enzyme activity also reportedly increased 100-fold in the presence of sulfate ions. Tano *et al.* (1996) proposed the tetrathionate decomposition reaction as follows:

$$4S_4O_6^{2-} + 5H_2O \rightarrow 7S_2O_3^{2-} + 2SO_4^{2-} + 10H^+ \qquad (1.11)$$

Tetrathionate is relatively stable in acidic environments and oxidation of tetrathionate leads to the formation of long chain polythionates and elemental sulfur (Pronk *et al.*, 1990). Steudel *et al.* (1987) attempted a detailed analysis of the intermediary sulfur formed during oxidation of tetrathionate by *T. ferrooxidans*. These authors found that the intermediary sulfur consisted mainly of long-chain polythionates and orthorhombic sulfur. The oxidation of tetrathionate is thought to result in the formation of sulfane-monosulfonic acids (HS_nSO_3H). The formation of polythionates can be explained through the oxidative condensation of two sulfane-monosulfonic acids (Pronk *et al.*, 1990). Sulfane-monosulfonic acids are important intermediates as polysulfur compounds can be rapidly and reversibly synthesized, degraded, and transported across bacterial membranes only as these highly reactive and readily soluble substances in a strongly acidic medium (Steudel *et al.*, 1987). Sulfane-monosulfonic acids are highly reactive compounds that undergo spontaneous elongation reactions leading to the formation of important polythionates such as tri-, penta-, hexathionate (Wentzien *et al.*, 1994; Sand *et al.*, 1995) and polythionates of up to S_{13} (Steudel *et al.*, 1987).

The elongation reactions of the sulfane-monosulfonic acids was proposed by Pronk *et al.* (1990) to ultimately yield elemental sulfur and sulfite through the following set of reactions:

Tetrathionate hydrolysis to sulfane-monosulfonic acid and sulfate:

$$S_4O_6^{2-} + H_2O \rightarrow HS_2SO_3^- + HSO_4^- \quad (1.12)$$

Elongation of sulfane-monosulfonic acids and formation of sulfite:

$$2HS_2SO_3^- \leftrightarrow HS_4SO_3^- + HSO_3^- \quad (1.13)$$

$$2HS_4SO_3^- \leftrightarrow HS_8SO_3^- + HSO_3^- \quad (1.14)$$

Formation of elemental sulfur and sulfite:

$$HS_8SO_3^- \leftrightarrow S_8 + HSO_3^- \quad (1.15)$$

Of particular interest is the formation of trithionate ($S_3O_6^{2-}$), a polythionate that has been reported in the oxidation of tetrathionate by *T. ferrooxidans* and *T. thiooxidans*. Trithionate is proposed to be formed by the biological oxidation of S_3-sulfane monosulfonic acid (Sinha and Walden, 1966; Pronk *et al.*, 1990). Okuzumi (1966) has reported the hydrolysis of trithionate by *T. thiooxidans* to yield thiosulfate (Pronk *et al.*, 1990). This suggests metabolic cycling between thiosulfate and tetrathionate in the sulfur oxidation pathways of chemolithoautotrophic bacteria, which would account for the observed formation of thiosulfate following tetrathionate decomposition by *T. thiooxidans* in the work of Tano *et al.* (1996).

Oxidation of elemental sulfur, usually in its orthorhombic form (S_8), by the thiobacilli has been shown to occur at pH values above 4.0. With intact cells of *T. ferrooxidans* the rate of elemental sulfur oxidation is greatly enhanced in the presence of sulfate ions. This sulfate ion effect is very specific (Pronk *et al.*, 1990). Its significance to the reported sulfate requirement of the tetrathionate decomposing enzyme of *T. thiooxidans* (Tano *et al.*, 1996) and *T. ferrooxidans* (Kuenen *et al.*, 1993; Tano *et al.*, 1996), and the oxidation of elemental sulfur is undetermined.

At pH 5.0 the oxidation of elemental sulfur by *T. ferrooxidans* yields significant amounts of sulfite. However, at lower pH values this accumulation of sulfite does not occur (Pronk *et al.*, 1990). Low rates of thiosulfate formation from elemental sulfur has been observed with cell-free extracts of *T. thiooxidans*, but this reaction required a glutathione dependent enzyme which exhibited a pH optimum of 7.5 and produces sulfite as an initial product. A non-enzymatic condensation of sulfite and elemental sulfur ultimately forms thiosulfate (Suzuki, 1974).

Sulfite is an essential intermediate in the proposed pathways for the oxidation of reduced sulfur compounds by thiobacilli. At pH 3 cell suspensions of *T. ferrooxidans* exhibit significant rates of sulfite oxidation. Oxidation of sulfite by this microbe can be coupled to the phosphorylation of ADP (Pronk *et al.*, 1990). Intact cells of *T. thiooxidans*, however, exhibit very low levels of sulfite oxidation at pH 3, and only when the pH is increased to pH 6.0 can significant rates of sulfite oxidation be observed (Pronk *et al.*, 1990). This conflicting pH optimum for the enzymic oxidation of sulfite suggests that sulfite oxidation may be either a periplasmic or cytoplasmic reaction (Pronk *et al.*, 1990). Hallberg *et al.* (1996) were able to show, through the use of an oxidation uncoupler, that *T. caldus* sulfite oxidation occurs in the cytoplasm. However, a sulfite oxidase purified from *T. ferrooxidans* AP19-3 was located in the plasma membrane (Sugio *et al.*, 1992).

At the enzyme level, two mechanisms have been proposed for the biological oxidation of sulfite. In the first mechanism it is proposed that sulfite is oxidized directly to sulfate by a sulfite: cytochrome *c* oxidoreductase (sulfite oxidase). The sulfite oxidase which has been purified from *T. ferrooxidans* oxidizes sulfite to sulfate with cytochrome *c* as electron acceptor (Suzuki, 1974; Pronk *et al.*, 1990). In the model of *T. caldus* sulfite oxidation proposed by Hallberg *et al.* (1996), the ubiquinone-cytochrome *b* complex is the electron acceptor. However, the sulfite: ferric ion oxidoreductase purified from *T. ferrooxidans* AP19-3 catalyses the oxidation of sulfite ion using Fe^{3+} as an electron acceptor (Sugio *et al.*, 1992). This sulfite oxidase does not use cytochrome *c* as an

electron acceptor and its products are SO_4^{2-} and Fe^{2+}. The reduced Fe^{2+} is thought to be oxidized by iron oxidase to supply energy for cell growth (Sugio *et al.*, 1992).

In the second mechanism (Fig 1.3) it is suggested that sulfite may undergo an oxidative condensation with adenosine 5'-monophosphate (AMP) to form adenosine 5'-phosphosulfate (APS). Substrate level phosphorylation is achieved when sulfate is ultimately liberated from APS by ADP sulfurylase with the concomitant formation of ADP and subsequent generation of ATP by adenylate kinase (Suzuki, 1974; Pronk *et al.*, 1990).

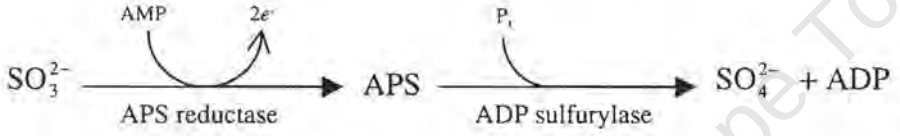


Fig 1.3: Diagram of the adenosine 5'-phosphosulfate (APS) pathway. Note that a substrate level phosphorylation occurs via the enzyme ADP sulfurylase. Reproduced from Brock and Madigan (1991).

The presence of an APS reductase has been reported for *T. thiooxidans* (Peck, 1961; Pronk *et al.*, 1990). Suzuki (1974) suggested that APS reductase with a cytochrome *c* as electron acceptor should be able to conserve energy through oxidative phosphorylation as well as substrate level phosphorylation. The substrate level phosphorylation mechanism involving APS yields half a molecule of ATP for each molecule of sulfite oxidized. If one assumes that generated electrons from the APS pathway are fed into the electron transport chain, they may contribute to the formation of a proton-motive force for oxidative phosphorylation (Pronk *et al.*, 1990). However, AMP-independent oxidation of sulfite by cell free extracts of *T. thiooxidans* has also been reported (Pronk *et al.*, 1990). Pronk *et al.* (1990) note that data on sulfite oxidation by the acidophilic thiobacilli is sparse and can be conflicting. Furthermore, there is little unity in the catabolic mechanisms employed by different sulfur-oxidizing chemolithoautotrophic bacteria. Therefore, until more information is obtained it is not possible to construct a definite

scheme for the oxidation of reduced inorganic sulfur compounds in the acidophilic thiobacilli.

A substantial amount of thiosulfate and tetrathionate may be located in the cytoplasm of certain thiobacilli. The intracellular pH of the bacteria is considered to be pH 6.5 (Cox *et al.*, 1979); a pH at which both sulfur species are stable (Roy and Trudinger, 1970). It has been suggested that during the shift in pH from the acidic biooxidation environment (pH 1.2) to that of the cyanidation plant (≈pH 10) lysis of the cells may result (Lawson, 1997) which would release these sulfur species making them accessible to cyanide.

It would appear that a range of sulfur species produced by both metabolic and chemical reactions are probably present in the bioresidue during cyanidation. Should these oxy-sulfur species described above persist, they could readily consume cyanide during cyanidation of the ore or concentrate (Hackl and Jones, 1997).

1.6) Microbial wastage of cyanide during cyanidation

The idea of exclusive chemical consumption of cyanide by biooxidation pretreated ore may be naïve when one considers that aerobic organisms are particularly sensitive to cyanide toxicity.

Although the chemolithoautotrophic bacteria responsible for gold-bearing ore biooxidation are aerobes, they require oxygen as an electron acceptor to generate ferric iron. These bacteria use either ferrous iron or reduced sulfur compounds as electron donors to the electron transport chain thereby maintaining a transmembrane pH gradient from which ATP is generated (Rawlings and Kusano, 1994). The cytochrome oxidase enzymes of the electron transport chain are particularly susceptible to cyanide (Knowles, 1976). Cyanide toxicity is attributed to its tight chelation of di- and tri-valent metals of the cytochrome oxidase prosthetic groups. Cyanide is not a specific inhibitor of cytochrome oxidase as it also inhibits a wide range of enzymes, many of which are

hemoproteins or other metal-containing oxidases or oxygenases (Knowles, 1976). Cyanide also reacts with keto compounds to form cyanohydrin derivatives of enzyme substrates, with Schiff-base intermediates during enzymic reactions to form stable nitrile derivatives, and it can reduce thiol groups (Knowles, 1976; Knowles, 1988).

Since aerobically respiring bacteria are sensitive to cyanide, microbial wastage of cyanide via a possible cyanide detoxification pathway must be considered and the implications of such a pathway determined in terms of the microbial contribution to cyanide consumption during cyanidation.

The use of excess cyanide to counteract this inactivation of cyanide is unsatisfactory economically and environmentally. Therefore, an understanding of microbial cyanide resistance is of economic importance in the understanding of why ore pretreated by biooxidation is associated with excessive cyanide consumption.

An ubiquitous enzyme called rhodanese has been implicated in the wastage of cyanide during cyanidation (Lawson, 1995). This thiosulfate: cyanide sulfurtransferase transfers the sulfane-sulfur of thiosulfate to cyanide forming thiocyanate. Therefore, if there is a direct microbial contribution to the wastage of cyanide the rhodanese enzyme must be considered as a likely candidate (Lawson, 1995).

1.7) Rhodanese (thiosulfate: cyanide sulfurtransferase EC 2.8.1.1)

As a result of a search for the "active principle" (enzyme) in animal tissues known to convert toxic cyanide to less toxic thiocyanate, Lang reported in 1933 the discovery of rhodanese in rat liver (Westley, 1981; Nagahara *et al.*, 1995). This thiosulfate: cyanide sulfurtransferase (EC 2.8.1.1) is able to convert cyanide to thiocyanate utilizing a sulfur-donor substrate (Westley, 1973). Lang (1933) demonstrated rhodanese activity in a wide variety of animal tissues and in *Escherichia coli* (Knowles, 1976). Sorbö (1953) isolated and crystallized the first rhodanese isoenzyme from bovine liver, with the rat liver

rhodanese being purified only in 1979 by Wasylewski and co-workers (Nagahara *et al.*, 1995). Much of the initial work on rhodanese and the establishment of its catalytic mechanism was done on bovine liver rhodanese. Comparative studies on bovine kidney rhodanese revealed no differences (Westley, 1973).

The first formal rhodanese reaction mechanism was an irreversible Theorell-Chance mechanism. Initially, it was assumed that a disulfide bond was present in the enzyme active site. The mechanism involved the cleavage of the disulfide by thiosulfate to form a sulfenyl thiosulfate. An enzyme-substrate complex is formed, which is then cleaved by cyanide, eliminating sulfite, forming an organic thiocyanate. The thiocyanate is subsequently decomposed by displacement of thiocyanate to regenerate the disulfide active site (Westley, 1973).

Szczepkowski (1961) proposed an alternate mechanism whereby a trisulfide enzymic intermediate is formed. This mechanism was proposed when two chemical weaknesses of the Theorell-Chance mechanism could not be solved. Firstly, when rhodanese was treated with thiosulfate in the absence of cyanide, the predicted formation of the sulfhydryl group could not be demonstrated. Secondly, typical sulfenyl thiosulfates are not cyanolysed with elimination of sulfite (Westley, 1973).

Sörbo (1962) later reported that rhodanese contained no disulfide bond, thereby supposedly negating both mechanisms (Westley, 1973). Spectroscopic studies, however, have demonstrated that rhodanese undergoes conformational changes upon acceptance and release of a sulfenyl sulfur (Ogata *et al.*, 1989). When isolated in the presence of substrate the native rhodanese contains four free sulfhydryl groups but no disulfide bonds (Ogata *et al.*, 1989), and all four sulfhydryl groups are in a reduced state (Miller *et al.*, 1991). If rhodanese is isolated free of substrate sulfur it undergoes facile oxidation resulting in the formation of an intramolecular disulfide bridge. It is this oxidized form of rhodanese which can accept a negatively charged sulfide-sulfur from a donor substrate. This results in concomitant reduction of the enzymic disulfide bridge and formation of

the catalytically active enzyme-persulfide intermediate (Ogata and Volini, 1990). Therefore, in the course of sulfane-sulfur transfer, rhodanese cycles between two stable catalytic intermediates (Miller *et al.*, 1991).

The double-displacement (Ping-Pong) mechanism of Szczepkowski (1961) is still considered to be likely. Direct demonstration of a sulfur-substituted enzymic intermediate and its reactions with appropriate thiophilic acceptor substrates was proven utilizing polargraphic techniques (Green and Westley, 1962), ^{35}S-radiolabeled thiosulfate (Westley and Nakamoto, 1962), and spectrophotometric two-substrate kinetic analysis (Volini and Westley, 1966). The results obtained indicated the operation of a formal double-displacement mechanism (Westley, 1973).

Sulfurtransferase enzymes generally show low substrate specificity (Pagani *et al.*, 1991). The wide range of reactions catalyzed by rhodanese defines the enzyme as either a thiosulfate: cyanide sulfurtransferase, or as a thiosulfate: dithiol sulfurtransferase (Colnaghi *et al.*, 1996). Sulfane-sulfur donors include: thiosulfate, thiosulfonates, persulfides, polythionates, ethanesulfonate and thiotaurine, while thiophilic acceptors include cyanide, sulfite, organic sulfinates, thiols, dithiols, dithionite and lipoate.

The double displacement catalytic mechanism of rhodanese (Fig 1.4) is now accepted as involving the concomitant electrophilic and nucleophilic attack on the sulfur-sulfur bond of a sulfane-sulfur anion. This yields a rhodanese-sulfur complex in which the sulfur atom is covalently attached to a single cysteine residue by a persulfide linkage (Ploegman *et al.*, 1978). Following the establishment of the enzyme-substrate complex the first product is discharged. The sulfane-sulfur is then transferred from the sulfur-substituted enzyme intermediate to a thiophilic acceptor and the free enzyme is regenerated (Westley, 1973; Ploegman *et al.*, 1978).

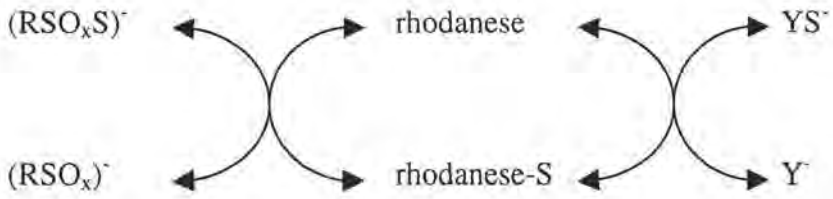


Fig 1.4: The double displacement mechanism of rhodanese. $(RSO_xS)^-$ is the sulfane-sulfur anion, where R can be an organic moiety, and Y^- is the unrelated thiophilic anion. Reproduced from Westley (1973).

Many of the above rhodanese catalyzed reactions are readily reversible. However, the rhodanese catalytic reaction involving cyanide as the thiophilic acceptor (equation 1.15) is atypical as the reaction results in the irreversible formation of thiocyanate (SCN^-) and sulfite (Westley, 1973).

$$S_2O_3^{2-} + CN^- \rightarrow SCN^- + SO_3^{2-} \qquad (1.15)$$

1.8) The covalent and tertiary structure of rhodanese

Prior to the extensive investigation of the covalent and tertiary structure of bovine liver rhodanese by Ploegman *et al.* (1978) the size and number of polypeptides in rhodanese had been the subject of much debate. A molecular weight of 37.5 kDa was initially reported for the enzyme and it was thought to be a dimer (Ploegman *et al.*, 1978). It has since been determined that bovine liver rhodanese is a single polypeptide chain of 293 amino acids with a calculated molecular weight of 32.9 kDa (Ploegman *et al.*, 1978).

Eukaryotic rhodanese is encoded in the nucleus, translated in the cytosol and then imported into the mitochondrial matrix (Miller *et al.*, 1991). The first 30 amino acids of rhodanese resemble an import signal, and an α-helix formed by residues 11 to 22 conforms to the requirements of a functional mitochondrial targeting sequence. Although the import signal sequence is usually cleaved in the mitochondrial matrix, the amino-terminus of rhodanese is not further cleaved, except for the removal of the initiator methionine (Miller *et al.*, 1991). The rhodanese nucleotide sequence was found to

encode three amino acids, Gly-Lys-Ala, at the carboxyl terminus that were not found by protein sequencing. Thus, the carboxyl terminus of rhodanese is post-translationally processed in the mitochondria. This carboxyl terminus processing would account for the two major electrophoretic variants of rhodanese commonly isolated from mammalian cells. As many as four electrophoretic variants have been observed (Miller *et al.*, 1991).

Rhodanese is described as being ellipsoid, of dimensions 60 X 50 X 40 Å, and consisting of two globular domains of nearly equal size and conformation (Fig 1.5) connected by a proline-rich loop of an extended chain (Ploegman *et al.*, 1978; Russell *et al.*, 1978). The two independent domains are held together by extensive hydrophobic interactions (Luo and Horowitz, 1994).

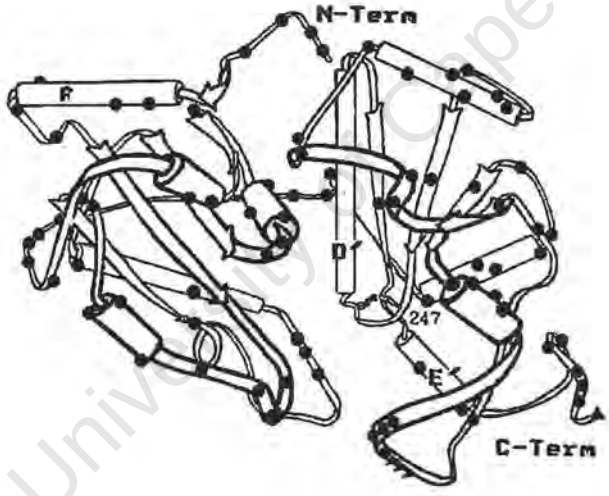


Fig 1.5: Secondary structure representation of bovine liver rhodanese. Reproduced from Miller *et al.* (1991).

The N-terminal domain (I) comprises residues 1-142, while the C-terminal domain (II) extends from residues 159-293 (Fig 1.6). The connecting loop of the two domains is composed of 15 residues (Ploegman *et al.*, 1978). Each domain of rhodanese contains a central five stranded parallel β-sheet with two α-helices located on one side of the sheet and three on the other. There are no anti-parallel β-strands in the rhodanese secondary structure. In each domain, two of the fives helices connect and run anti-parallel to the

neighbouring strands of the sheet forming two β-α-β units. The two β-α-β units in each domain are thought to provide potential hinge sites allowing for the structural flexibility demonstrated by rhodanese (Cerletti, 1986). The distribution of amino acids in rhodanese is: 37% α-helices, 15% parallel β-structures, and 24% turns (Ploegman *et al.*, 1978).

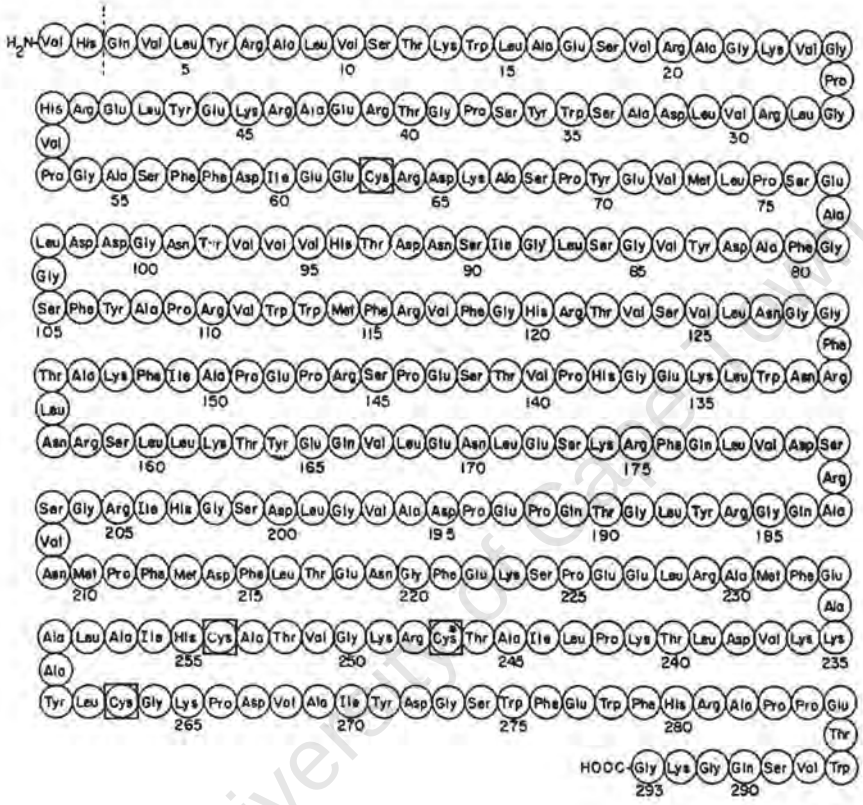


Fig 1.6: The covalent structure of bovine liver rhodanese. The positions of the four cysteine residues are indicated by boxes, and the active site Cys-247 is starred. Reproduced from Ploegman *et al.* (1978).

The active site of rhodanese is located in a depression between domains I and II and spans from residues 170 to 266 (Ploegman *et al.*, 1978; Nagahara *et al.*, 1995). The bound substrate sulfur atom forms a persulfide bond with residue Cys-247 which is located at the bottom of a pocket in a turn between a β-strand and an α-helix (Ploegman *et al.*, 1978; Russell *et al.*, 1978). The geometry of residues 247-249 can align in a cooperative way in the α-helix which is catalytically crucial since the initial nucleophilic attack on the sulfur donor is favoured by electrostatic influences that lower the pK_a of Cys-247 to make

it a better nucleophile (Luo and Horowitz, 1994). Side chains from both domains form the walls of the pocket which in turn create a hydrophobic and a hydrophilic region (Ploegman *et al.*, 1978). The hydrophobic wall of the active site is contributed by the side-chains of Trp-35, Phe-106 and Tyr-107 found in the N-terminal domain (I), while domain II contributes to this hydrophobic wall through residues Phe-212 and Val-251. However, all the residues of the hydrophilic wall, which includes Cys-247, Arg-186 and Lys-249, are derived from the C-terminal domain (II) (Russell *et al.*, 1978). Therefore, the two domains contribute asymmetrically to the active site (Ploegman *et al.*, 1978).

Arg-186 and Lys-249 are part of the cationic component of the active site and are close enough to Cys-247 to participate in binding the sulfur substrate in the correct orientation. Arg-186 and Lys-249 are located at the entrance of the pocket of the active center and the substrate binding site (Nagahara *et al.*, 1995). Lys-249 is essential for thiosulfate binding as it is thought that the electrostatic interaction between the negative charge of the oxygen atom of thiosulfate and the positive charge of the Lys residue may be important in transferring the outer sulfur atom of thiosulfate to form the persulfide intermediate (Nagahara *et al.*, 1995). Arg-186 and Lys-249 are thought to act in concert to bind thiosulfate in the correct orientation (Luo and Horowitz, 1994) allowing the positive charges of these two amino acids to polarize the highly polarizable S-S bond in the sulfur substrate, rendering the sulfane-sulfur even more susceptible to thiophilic attack by Cys-247 (Ploegman *et al.*, 1978; Russell *et al.*, 1978). Arg-248 is thought to stabilize the persulfide intermediate (Nagahara and Nishino, 1996), while Lys-249 is particularly critical for substrate selectivity and protein stability (Luo and Horowitz, 1994).

Therefore, with thiosulfate as the sulfane sulfur-containing anion, rhodanese reacts with thiosulfate to yield a sulfur-substituted enzyme-substrate complex in which the sulfane-sulfur is covalently attached to Cys-247. Sulfite is then discharged, followed by the enzyme-bound sulfur which is transferred to a thiophilic acceptor such as cyanide. Thiocyanate is thus formed and the free enzyme regenerated (Ploegman *et al.*, 1978). This is a classic double-displacement mechanism.

It was initially thought that a divalent metal ion participated in the catalytic mechanism, but this has been difficult to demonstrate and it is thought that bovine liver rhodanese is not a metallo-enzyme. There are, however, three water molecules in the structure that are bound to Arg-186. One of these bridges to Thr-252 and the other to Lys-249. Thiosulfate binding displaces the water between Arg-186 and Thr-252 suggesting that water may be involved in catalysis (Luo and Horowitz, 1994).

There is an extensive salt bridge network in rhodanese. All the six residues creating these salt bridges are in the vicinity of the active site (Ploegman *et al.*, 1978). The two independent domains of rhodanese are orientated with respect to each other by two of these salt bridges. The salt bridges between Glu-71 and Arg-248, and Arg-110 and Asp-272 span across the domain interface (Luo and Horowitz, 1994).

The folding of the two domains of bovine liver rhodanese is very similar, showing symmetry between, as well as within, each domain. It was, therefore, surmised that the amino acid sequence of each domain would show extensive identity, but this was not to be found (Ploegman *et al.*, 1978). Atypically, the rhodanese enzyme assumes equivalent tertiary structures but with differing amino acid sequences in each domain. Ploegman *et al.* (1978) proposed that the two domains may have arisen out of gene duplication and divergence, resulting in altered amino acid sequences but preserving their conformational similarity. However, Russell *et al.* (1978) questioned what selective pressure would have led to similar conformation patterns of the two domains, despite considerable changes in covalent structure. These authors suggested that domain I may have possessed an active site that has only recently changed; perhaps by the loss of a cysteine residue. This would mean that the N-terminal domain's (I) structural similarity to the C-terminal domain (II) would only be a reflection of its ancestral function.

1.9) The functional role of rhodanese

Although the rhodanese enzyme is well characterized and it has been used extensively in studies on protein folding, mitochondrial import and chaperonin assisted folding (Luo and Horowitz, 1994), to date the physiological role of the enzyme is still not well understood (Nagahara and Nishino, 1996). It was initially thought that the physiological role of rhodanese was the detoxification of cyanide due to its *in vitro* activity of cyanide conversion to thiocyanate (Knowles, 1976; Miller *et al.*, 1991). Discoveries of rhodanese in the mitochondria of mammalian cells, and that cyanide-inhibited cytochrome oxidase is reactivated by the addition of rhodanese and thiosulfate, appeared to confirm this proposal (Knowles, 1976).

In cyanophoric plants which release cyanide by enzymic cleavage of cyanogenic glucosides, rhodanese may indeed act as a detoxifying enzyme (Cerletti, 1986). If ingested by mammals, however, these cyanogenic materials are not metabolized by intestinal enzymes to cyanide. Rather, cyanide liberation is probably the result of intestinal flora (Cerletti, 1986). However, it has been recognised by many authors that the quantity of cyanide *in vivo*, or synthesized in biological systems, cannot account for the ubiquity or kinetic rate of rhodanese activity observed (Finazzi Argò *et al.*, 1971; Ogata *et al.*, 1989).

A second detoxifying role proposed for rhodanese was the disposal of highly neurotoxic free sulfide. This sulfide is produced by cystathionase (cysteine desulfhydrase) catabolism of cysteine. The sulfide released from cysteine is thought to be used by rhodanese to form thiosulfate (Cerletti, 1986). However, the weakness of this proposal is the inefficient integration of these two enzymes into a detoxification mechanism, as cystathionase is confined to the cytosol while rhodanese is found exclusively in mitochondria (Westley, 1973). A more plausible argument for the functional role of rhodanese, and for which the evidence is mounting, is that rhodanese plays a pivotal role

in the activation of enzymes of oxidative metabolism through the transfer of sulfane-sulfur atoms to non-heme iron proteins.

Rhodanese has been postulated to participate in the biological mechanism by which the prosthetic group in an iron-sulfur protein is formed (Pagani *et al.*, 1982). Finazzi Argò *et al.* (1971) suggested that the non-protein component of conjugated proteins probably comes from outside the mitochondrial and chloroplastic matrix. In terms of iron-sulfur proteins it seems unlikely that inorganic sulfide is the source of 'labile' sulfide *in vivo*. Rather, there are three sulfurtransferases which, in the presence of their substrates and a thiol reducing agent, can catalyse the conversion of sulfur to sulfide. These enzymes are β-mercaptopyruvate sulfurtransferase, rhodanese, and thiosulfate reductase (Ogata and Volini, 1990). Thiosulfate, which is freely diffusible into and readily metabolized in mitochondria, may be the source of 'labile' sulfide (Finazzi Argò *et al.*, 1971). Rhodanese has been considered as a carrier protein that transfers 'labile' sulfide to non-heme iron proteins of the electron transport chain (Finazzi Argò *et al.*, 1971). Thus a possible role for rhodanese in the formation or reconstitution of iron-sulfur proteins was suggested by its apparent ability to transfer sulfur from the sulfane oxidation state in thiosulfate to iron-sulfur proteins where it is inserted as sulfide (Pagani and Galante, 1983).

This argument stems out of reports that rhodanese is able to protect the 'labile' sulfide of iron-sulfur proteins by the reversible insertion of sulfide into Fe-S clusters of non-heme iron proteins. It was found that there was a direct relation between sulfide insertion and enzyme reactivation of NADH dehydrogenase (Pagani and Galante, 1983), xanthine oxidase (Nishino *et al.* 1983) and succinate dehydrogenase (Bonomi *et al.*, 1977). Rhodanese was also able to reconstitute ferredoxin apoprotein to active ferredoxin by transferring the sulfane-sulfur from thiosulfate to the 2Fe-2S cluster of spinach ferredoxin and the two 4Fe-4S clusters of ferredoxin purified from *Clostridium pasteurianum* (Pagani *et al.*, 1982). The model proposed for rhodanese-assisted Fe-S cluster formation and insertion (Fig 1.7) suggests that dihydrolipoate, a known acceptor of sulfide from

rhodanese, is the participating reducing agent as it is able to complex with ferric iron and thus may serve as the iron carrier for Fe-S cluster formation (Cerletti, 1986). Notably, when rhodanese and thiosulfate substituted for inorganic sulfide the rate of cluster formation increased 3-4 fold (Cerletti, 1986).

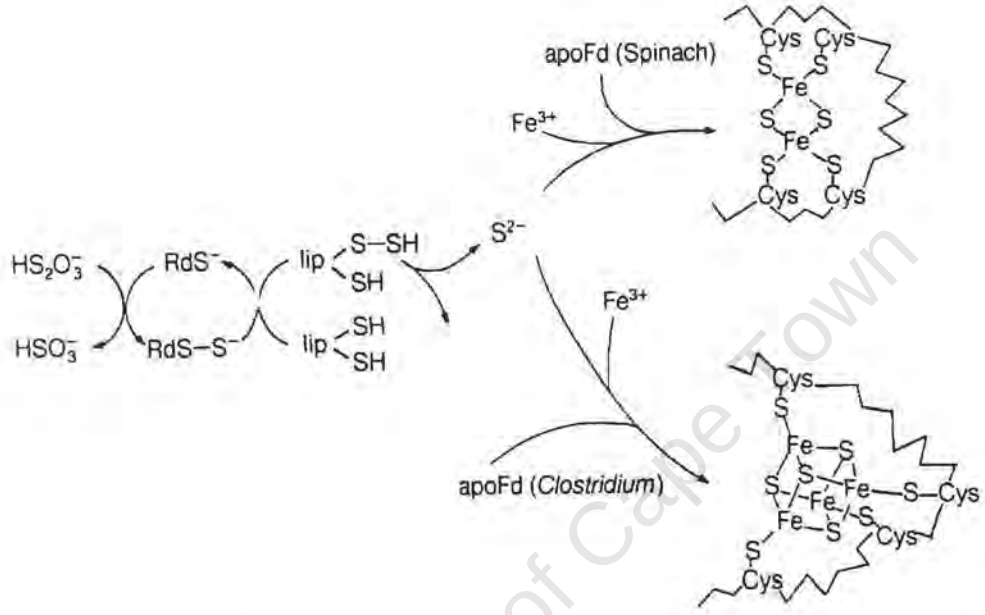


Fig 1.7: Rhodanese assisted Fe-S cluster formation of spinach and *Clostridium* ferredoxin. Rhodanese initiates the process by supplying sulfur to dihydrolipoic acid or dihydrolipoamide. Reproduced from Cerletti (1986).

It was observed, in work using succinate dehydrogenase as the thiophilic acceptor, that in the presence of thiosulfate there is little or no transfer of its sulfane-sulfur to this enzyme. However, if rhodanese was incorporated into the system, sulfide was inserted and rhodanese was fully reloaded by thiosulfate (Bonomi *et al.*, 1977). In all cases, sulfide insertion required the presence of rhodanese and was specific for iron-sulfur proteins. No binding was observed with proteins which have no iron-sulfur clusters in their native state (Pagani *et al.*, 1982).

These findings suggest that rhodanese functions as a protein sulfurase regenerating the 2Fe-2S and 4Fe-4S redox centers of iron-sulfur proteins (Ogata *et al.*, 1989; Pagani *et al.*,

1993). In doing so, rhodanese may be functionally linked to the electron transport chain in mitochondria and chloroplasts (Finazzi Argò *et al.*, 1971).

The ability of iron-sulfur proteins to act as substrate thiophilic acceptors of rhodanese catalyzed reactions has led Ogata *et al.* (1989) to propose a more plausible argument for the functional role of rhodanese. These authors suggest that rhodanese in mitochondria serves as a converter enzyme which directly alters the rate of the electron transport chain activity by reversible sulfuration of the respiratory chain iron-sulfur proteins. This in turn will regulate ATP production in the mitochondria. By acting in a bicyclic cascade system rhodanese participates in a mechanism by which mitochondria can recognize and meet changing energy demands (Ogata *et al.*, 1989).

The discovery that bovine mitochondrial rhodanese alternates between a phosphorylated and dephosphorylated state (the probable site of the phosphoryl group being Ser-124) led Ogata *et al.* (1989) to propose a model in which rhodanese participates in direct interactions with key iron-sulfur proteins of the electron transport chain. The model proposes that dephosphorhodanese is phosphorylated by ATP in a reaction mediated by a cAMP-independent rhodanese kinase, an enzyme which has been identified in liver mitochondrial extracts (Ogata *et al.*, 1989). Phosphorylation of dephosphorhodanese results in an immediate conformational transition which brings the side-chain of Cys-247 into close proximity with one of the four cysteine sulfhydryl groups found in the enzyme (Russell *et al.*, 1978; Ogata *et al.*, 1989). This results in the formation of an intramolecular disulfide bridge, which renders the phosphorhodanese inactive to sulfane donor substrates, but able to extract 'labile' sulfide from non-heme iron-sulfur proteins of the electron transport chain. This causes a down-regulation of the rate of electron transport and thus a decrease in ATP production during periods of low energy requirement (Ogata *et al.*, 1989).

The 'labile' sulfide extracted by the phosphorhodanese reduces the disulfide bridge formed by the phosphorylation event, giving rise to an enzymic persulfide. This

persulfide sulfur is transferred to either cysteine or another sulfur acceptor. The phosphorhodanese can either undergo oxidation to accept more 'labile' sulfide to maintain the down-regulation of ATP synthesis, or it is dephosphorylated by a protein phosphatase (Ogata *et al.*, 1989). If dephosphorhodanese is formed, it accepts a sulfur atom from a sulfane-sulfur donor substrate and transfers the sulfur atom to an iron-sulfur center reconstituting non-heme iron proteins of the electron transport chain. This consequently increases the rate of electron transport, thereby up-regulating ATP synthesis (Ogata *et al.*, 1989)

It is the 'labile' sulfide extracted by the phosphorhodanese which rhodanese uses to modulate the rate of electron transport by the direct activation or inhibition of non-heme iron proteins. The model assumes that rhodanese is acted upon by a signal pathway that involves a protein kinase and phosphatase. The bicyclic rhodanese cascade may be the terminal phase of pathways by which hormones and neurotransmitters signal changes in ATP production and oxygen consumption (Ogata and Volini, 1990).

Supporting this model was the finding that bovine liver mitochondrial rhodanese is bound to the mitochondrial membrane as a component of multiprotein complexes that form iron-sulfur centers *in situ* (Ogata and Volini, 1990). Miller *et al.* (1991) in their investigation of rhodanese post-translational modification suggested that the cleavage of three carboxyl terminus amino acids might assist in maintaining the close association of rhodanese with these centers at the matrix membrane. The duplication of the tripeptide at the carboxyl terminus might act as a retention signal for localization within the matrix (Miller *et al.*, 1991).

Kelly *et al.* (1997), however, reiterated the question of whether rhodanese does play a role in sulfur metabolism as the enzymology involved in the conversion of the central sulfane atom of polythionates to sulfate in sulfur oxidizing microbes is not yet resolved. Rhodanese may thus be considered as one of the enzymes necessary for the 'sulfane pool' (Cerletti, 1986).

The discovery of rhodanese in a wide range of eubacteria, but more importantly in the thiobacilli, suggested that in these microbes, at least, rhodanese plays a role in sulfur metabolism (Knowles, 1976).

Silver and Lundgren (1968) proposed that rhodanese may be directly involved in sulfur metabolism in the thiobacilli where its thiosulfate hydrolysing activity yields sulfur and sulfite. Rhodanese activity has been detected in, and the enzyme purified from, *T. ferrooxidans* (Tabita *et al*, 1969). However, Pronk *et al.* (1990) felt that this role was unlikely due to the near-quantitative formation of tetrathionate from thiosulfate oxidation observed in *T. ferrooxidans*.

The possible physiological role of rhodanese as a cyanide and inorganic sulfide detoxifying enzyme, and now as an iron-sulfur protein sulfurase suggests a multiplicity of roles for this sulfurtransferase. However, what must be stressed is that the rhodanese mediated transfer of sulfane-sulfur of thiosulfate to cyanide has only been demonstrated *in vitro*. Likewise, the reconstitution of iron-sulfur apoproteins has only been demonstrated *in vitro* (Miller *et al.*, 1991). Two different functions, and even an argument that rhodanese is a biochemical relic (Schievelbein *et al.*, 1969; Knowles, 1976; Cerletti, 1986), has left the physiological role of rhodanese still open to debate.

1.10) Comparison of rhodanese and rhodanese-like enzymes

The discovery of rhodanese in both eukaryotes and prokaryotes has led to investigations into the structural and possible evolutionary relationships among the sulfurtransferase enzyme family (Knowles 1988).

Structural comparisons between the active site of rhodanese and cytosolic mercaptopyruvate sulfurtransferase (MST) suggested that these two enzymes are evolutionarily related (Nagahara *et al.*, 1995). Although these two enzymes were considered to be different due to their substrate specificity, it was found that MST could

transfer a sulfur ion from thiosulfate or mercaptopyruvate to the molybdenum ligand of xanthine oxidase. Therefore, the apparent rhodanese activity of MST lead to investigations into the relatedness of the two enzymes. It was found that the critical Cys-247 and substrate binding Arg-186 in rhodanese were conserved in MST. However, Arg-248 and Lys-249 found in the active site of rhodanese were replaced by Gly and Ser, respectively, in MST (Nagahara *et al.*, 1995).

Site-directed mutagenesis studies using cloned rat liver rhodanese were undertaken in an attempt to convert rhodanese to rat liver MST. Amino acid exchange experiments showed that replacement of Arg-248 of rat rhodanese with Gly did not affect rhodanese activity; that replacement of Lys-249 with Ser decreased rhodanese activity but did not effect MST activity; and replacement of Lys-249 with Ala completely eliminated rhodanese activity highlighting the importance of this residue in binding thiosulfate. Double replacement of Arg-248 and Lys-249 with Gly and Ser, respectively, decreased rhodanese and increased MST activity (Nagahara *et al.*, 1995). This demonstrated that Arg-248 and Lys-249 were critical residues in determining MST or rhodanese activity. The partial conversion of rhodanese to MST by replacement of only two amino acids in the active site, together with a 66% sequence identity strongly suggests that these two enzymes are evolutionarily related members of the same enzyme family (Nagahara *et al.*, 1995). Subsequently, Nagahara and Nishino (1996) attempted to convert rat liver MST to rhodanese by site-directed mutagenesis of the essential amino acids in the active site. The replacement of the Cys-247 residue with Ser completely abolished both MST and rhodanese activity indicating that Cys-247 of MST was the catalytic site at which the persulfide link is formed. The substitution of Gly-248 and Ser-249 of MST with Arg and Lys, respectively, resulted in an increase in the ratio of rhodanese to MST activity (Nagahara and Nishino, 1996). Arg-186 is conserved, like Cys-247, between the rhodanese and MST sequences. Replacement of MST Arg-186 with Gly resulted in decreased rhodanese and MST activity confirming that this amino acid is the essential residue for binding thiosulfate and mercaptopyruvate, respectively (Nagahara and Nishino, 1996). Arg-96 is unique to MST and it facilitates the catalysis of

mercaptopyruvate, but hinders that of thiosulfate. Possibly the positively charged side chain of Arg-196 interacts with the carboxyl group of mercaptopyruvate and assists in polarizing the substrate (Nagahara and Nishino, 1996). Thus, in these experiments, MST was partially converted to rhodanese, again confirming the evolutionary relatedness of rhodanese to MST.

Rhodanese activity has been detected in many prokaryote species. Using a technique for the rapid detection of intracellular rhodanese, Vandenbergh *et al.* (1979) detected rhodanese in *Bacillus subtilis, B. megaterium,* various *Pseudomonas aeruginosa* isolates, *Acinetobacter calcoaceticus* subspecies, and strains of *Shigella*. Rhodanese has also been detected in many thiobacilli. Some of the prokaryote rhodanese enzymes have been purified and enzymes characterized.

Alexander and Volini (1987) purified an enzyme from *Escherichia coli* that displayed rhodanese activity. This enzyme had a radius of 17 Å and a molecular weight of 14 kDa; approximately half that of bovine rhodanese. Like the bovine rhodanese, this *E. coli* rhodanese was phosphorylated by protein kinase, and inactivated by autoxidation. The active enzyme could be reactivated by cysteine which is thought to be the result of its conversion from the inert dimeric form to the active monomer (Alexander and Volini, 1987). This *E. coli* rhodanese also demonstrated the double displacement (Ping-Pong) mechanism of bovine liver rhodanese by forming a binary Michaelis-Menten complex which transfers bound sulfenyl sulfide to a thiophilic acceptor following discharge of sulfide (Alexander and Volini, 1987).

This *E. coli* rhodanese was able to catalyze the formation of iron-sulfur centers at a rate that was three-fold higher than that of thiocyanate formation at physiological pH (Alexander and Volini, 1987). However, this enzyme had a catalytic activity that was 40% lower than that of bovine rhodanese. It was presumed by these authors that the *E. coli* rhodanese has a single domain structure which would be unable to be conformationally mobile like bovine rhodanese. Due to its large double domain structure,

bovine rhodanese is able to undergo a conformational change upon formation of the enzyme-substrate complex. This domain movement, relative to each other, results in distortion of the persulfide bond aiding bond cleavage (Alexander and Volini, 1987). Thus, the single domain structure of this *E. coli* rhodanese would account for its lower enzyme activity (Alexander and Volini, 1987).

Two rhodaneses have been identified in *E. coli*. One is localized in the periplasmic space and the other within the cytoplasmic membrane (Alexander and Volini, 1987). The proximal location of the periplasmically located *E. coli* rhodanese to the aerobic respiratory chain and its observed ability to catalyse iron-sulfur centers adds weight to the protein sulfurase proposal put forward by Ogata *et al.* (1989). Further evidence has shown that the *E. coli* rhodanese is regulated by the cAMP-cAMP receptor protein system, subject to catabolite repression, and has an observable maximal specific activity under limiting oxygen conditions, as do the *E. coli* respiratory chain enzymes, succinate dehydrogenase, NADH oxidase and cytochrome a_2 (Alexander and Volini, 1987).

An enzyme displaying rhodanese-like activity was cloned by Hama *et al.* (1994) from *E. coli*. The product from the designated *sseA* gene showed 50 and 35% nucleotide and amino acid identity to rat rhodanese. It consisted of 238 amino acid residues and had a calculated molecular weight of 26.1 kDa (Hama *et al.*, 1994). However, Nagahara and Nishino (1996) suggested that this enzyme was in fact an *E. coli* MST as it had higher nucleotide and amino acid sequence identity to rat MST. Nagahara and Nishino (1996) postulated that the SseA protein was in fact an evolutionary prototype of the two families of enzymes.

Overexpression of the SseA protein resulted in the retardation of isoleucine biosynthesis in *E. coli* (Hama *et al.*, 1994). Cysteine is synthesized from serine and is known to inhibit isoleucine biosynthesis. Therefore, an explanation given was that the SseA protein elevated the levels of cysteine synthesis thereby inhibiting isoleucine synthesis (Hama *et al.*, 1994). It is interesting to note that the addition of cysteine to autoxidized *E.*

coli rhodanese can partially or completely reactivate the enzyme (Alexander and Volini, 1987).

A gene with 50.7% amino acid similarity to bovine liver rhodanese was cloned from *Saccharopolyspora erythraea*, and its chromosomal copy inactivated by a recombination event involving disruption of the gene with an apramycin marker (Donadio *et al.*, 1990). This resulted in a *S. erythraea* cysteine auxotroph. However, this auxotroph and wild-type *S. erythraea* both demonstrated equivalent levels of rhodanese activity. In an attempt to explain this, the authors suggested that the gene coded for a different enzyme that was evolutionarily related to rhodanese and possibly had a similar function (Donadio *et al.*, 1990). The finding of two rhodanese forms in *E. coli* (Alexander and Volini, 1987) seems to strengthen this argument, but no additional bands were identified in genomic digests of *S. erythraea* when this gene was used as a probe in Southern hybridization (Donadio *et al.*, 1990).

This *S. erythraea* cysteine auxotroph was unable to grow on minimal media unless supplemented with cysteine or methionine. If the minimal media contained thiosulfate, but not sulfate or sulfite, the auxotroph was able to grow (Donadio *et al.*, 1990). Considering that rhodanese had been demonstrated in other systems to catalyse the reversible transfer of sulfane-sulfur between sulfite and thiosulfate it is suggested by the authors that this rhodanese-like CysA protein assisted in the cysteine biosynthesis pathway of *S. erythraea* (Donadio *et al.*, 1990).

A prominent 33 kDa polypeptide was found to accumulate to high levels in the *Synechococcus* sp. PCC 7942 when the cyanobacterium experienced sulfur-limited growth (Laudenbach *et al.*, 1991). This polypeptide was localized to the periplasmic space and encoded by a gene with a deduced amino acid sequence showing 21% similarity to the rhodanese-like protein of *S. erythraea* (Donadio *et al.*,1990) and 26% similarity to bovine liver rhodanese. In comparison to bovine liver rhodanese, the active

site Cys-247 was conserved, and this polypeptide was of similar size (Laudenbach *et al.*, 1991).

Similarly to the study on *S. erythraea* (Donadio *et al.*,1990), the *rhdA* gene of *Synechococcus* sp. PCC 7942 was interrupted by integration of a spectinomycin resistance marker. As this rhodanese-like enzyme was located adjacent to the genes encoding the sulfate permease the authors wanted to study the role of this enzyme in sulfur metabolism (Laudenbach *et al.*, 1991). While the *rhdA* mutant no longer expressed the RhdA protein under sulfur-limited growth conditions it still exhibited basal levels of rhodanese activity. Like the CysA protein of *S. erythraea* (Donadio *et al.*,1990), the 33 kDa protein of *Synechococcus* sp. PCC 7942 exhibited nominal rhodanese activity (Laudenbach *et al.*, 1991). However, elimination of the 33 kDa protein by mutagenesis had resulted in the limitation of the number of inorganic sulfur sources that the cyanobacterium could grow on (Laudenbach *et al.*, 1991). This lead the authors to suggest that the rhodanese activity of this polypeptide may not represent its primary function and that this non-specific enzyme may simply enhance the transport of inorganic sulfur into the cell (Laudenbach *et al.*, 1991).

Contrarily, the expression of the *rhdA* gene found in *Azotobacter vinelandii* was not controlled by the external sulfur source on which the bacterium was cultured (Colnaghi *et al.*, 1996). This rhodanese enzyme displayed typical rhodanese activity as it was capable of using only thiosulfate as a donor substrate (Colnaghi *et al.*, 1996). Although this 30 kDa enzyme was of similar size to bovine liver rhodanese it had low amino acid sequence similarity to bovine liver rhodanese. Significantly, the prokaryotic rhodanese-like proteins described above show low sequence similarity even between themselves (Colnaghi *et al.*, 1996). Amino acid sequence analysis of the *A. vinelandii* RhdA protein revealed that the active site Cys residue of bovine liver rhodanese is conserved, but that basic residues thought to be catalytically important are not (Colnaghi *et al.*, 1996). Residual rhodanese activity in *A. vinelandii rhdA* mutants suggested that this bacterium may also contain more than one enzyme with rhodanese activity (Colnaghi *et al.*, 1996).

A prokaryote sulfane sulfurtransferase of 17 kDa was purified from *Acinetobacter calcoaceticus lwoffi* which had a predominance of α-helices in its secondary structure, unlike bovine rhodanese (Aird *et al.*, 1987a; Aird and Horowitz, 1990). Due to the size of the bacterial sulfurtransferase it is unlikely to have the same conformational mobility as bovine rhodanese, a point made by Alexander and Volini (1987) for *E. coli* rhodanese of similar size. The bacterial sulfane sulfurtransferase has only one cysteine residue, as opposed to four in bovine rhodanese, and thus cannot form intramolecular disulfides or disulfide bonded aggregates (Aird and Horowitz, 1990).

This prokaryote sulfane sulfurtransferase has the unique property among known prokaryote rhodaneses of varying its formal mechanism with respect to its acceptor substrate (Aird *et al.*, 1987a). With inorganic thiosulfate as the sulfane-sulfur donor substrate and cyanide as the thiophilic acceptor the enzyme displayed a double displacement mechanism like that of mammalian rhodanese. In contrast, with a thiol as the acceptor substrate the reaction proceeded by a single displacement mechanism reminiscent of the sulfurtransferase thiosulfate reductase (Aird *et al.*, 1987a). Thiosulfate reductase (EC 2.8.1.3), however, is unable to transfer the sulfane-sulfur of thiosulfate to cyanide (Westley, 1973). When dithiothreitol is the acceptor substrate the sulfane sulfurtransferase cycles through both the single and double displacement mechanisms (Aird *et al.*, 1987a). When cyanide was used as an acceptor substrate the sulfurtransferase demonstrated substrate inhibition by thiosulfate, and sulfite was a non-competitive inhibitor with respect to thiosulfate. The enzyme displayed enzyme inhibition by competing non-substrate anions, as sulfate was a competitive inhibitor of the sulfurtransferase when thiosulfate was used as a substrate (Aird *et al.*, 1987a).

A 35 kDa rhodanese monomer had been previously purified from an *A. calcoaceticus* strain that demonstrated constitutive expression of this enzyme (Vandenbergh and Berk, 1980). Unlike the sulfurtransferase purified from *A. calcoaceticus* by Aird *et al.* (1987a), this enzyme demonstrated strict thiosulfate specificity. The rhodanese was sensitive to

the thiol-group alkylators *N*-ethylmaleimide and iodoacetate, and was strongly inhibited by sulfite. The reducing agents cysteine-HCl, reduced glutathione and mercaptoethanol enhanced enzyme activity (Vandenbergh and Berk, 1980).

In photosynthetic bacteria rhodanese has been detected in *Rhodopseudomonas spheroides, R. palustris, Rhodospirillum rubrum* and *Chromobacterium violaceum* (Yoch and Lindstrom, 1971). In *C. violaceum* it was found that the expression of rhodanese only occurred after cyanogenesis (Rodgers and Knowles, 1978). It is known that *C. violaceum* is able to condense cyanide with cysteine forming β-cyanoalanine which is then metabolized to aspartate (Rodgers and Knowles, 1978; Padmaja and Balagopal, 1985). However, Rodgers and Knowles (1978) did not speculate as to the possible role of rhodanese except to note that rhodanese, β-cyanoalanine synthase and γ-cyano-α-aminobutyric acid synthase expression increased with the onset of stationary phase. However, in a later study by Rodgers (1982) the author noted that the three enzymes thought to be active in cyanide assimilation for *C. violaceum* were only demonstrated *in vitro* and that *in vivo* the accumulation of only β-cyanoalanine suggested that β-cyanoalanine synthase was the only cyanide-utilizing enzyme operating in a cyanide assimilation pathway. Despite this, Padmaja and Balagopal (1985) felt that rhodanese in fungi may be used in cyanide assimilation, as some fungi utilize cyanide in the synthesis of alanine, glutamine, and asparagine.

Rhodanese in *P. aeruginosa* was localized to the periplasmic space (Ryan *et al.*, 1979). Unlike *C. violaceum*, *P. aeruginosa* constitutively expressed rhodanese regardless of the source of inorganic sulfur provided for growth (Schook and Berk, 1978).

The only reference to extracellular rhodanese comes from the fungus *Rhizopus oryzae* for which 78% of the rhodanese activity was detected extracellularly (Ray *et al.*, 1990). This fungus was able to oxidize thiosulfate and elemental sulfur to sulfate which the authors felt implicated rhodanese in sulfur oxidation (Ray *et al.*, 1990). It was found that cyanide was an efficient inducer of rhodanese although the reaction products sulfite and

thiocyanate inhibited rhodanese (Ray *et al.*, 1990). Sulfite was also a strong inhibitor of a *Streptomyces* sp. rhodanese when the organism was grown in thiosulfate supplemented media (Oi and Yamamoto, 1977). High concentrations of thiosulfate repressed growth of the *Streptomyces* sp. although the presence of thiosulfate induced rhodanese expression six fold. Cyanide and cysteine did not appear to inhibit rhodanese activity (Oi and Yamamoto, 1977), but neither were tested as inducers of rhodanese expression.

Enhancement of rhodanese activity by the addition of cyanide to media has been reported for *R. palustris* and *Tniobacillus denitrificans* (Padmaja and Balagopal, 1985). *R. palustris* doubled its rhodanese activity (Yoch and Lindstrom, 1971), and *T. denitrificans* increased its rhodanese activity three-fold (Bowen *et al.*, 1965) in cyanide supplemented media.

The *T. denitrificans* rhodanese has an apparent molecular weight of 38 kDa (Bowen *et al.*, 1965). A smaller enzymatically active sub-unit of 9 kDa which could be further hydrolyzed in mercaptoethanol to a 2 kDa and a 7 kDa fragment was also purified (Bowen *et al.*, 1965). This may indicate the presence of two isoenzymes in *T. denitrificans*, as reported for *E. coli* (Alexander and Volini, 1987). The 38 kDa rhodanese monomer exhibited sulfite, iodoacetamide and *N*-ethylmaleimide inhibition (Bowen *et al.*, 1965). Therefore, the enzyme was susceptible to end product inhibition, while the thiol-group alkylators demonstrated that thiol groups were present in the active site (Bowen *et al.*, 1965). These thiol-group alkylators also inactivated the rhodanese enzyme purified from *T. ferrooxidans* (Tabita *et al.*, 1969). Although the authors did not stipulate which were used, the rhodanese enzyme of the sulfate-reducing bacterium *Desulfotomaculum nigrificans* was not inhibited by thiol group alkylators but it did show slight sulfite inhibition (Burton and Akagi, 1971). No rhodanese inhibition studies were undertaken for the facultative autotroph *T. acidophilus* although it demonstrated a marked increase in rhodanese activity for cells cultivated in a sulfur medium compared with those cultivated in a glucose medium (Guay and Silver, 1975).

The detectable levels of rhodanese activity in cell free extracts of *Thiobacillus novellus* (Charles and Suzuki, 1966) and *Thiobacillus intermedius* (Charles, 1969) suggested to these authors that in th obacilli sulfur metabolism rhodanese initiated the cleavage of thiosulfate yielding sulfite. Unlike the cysteine inhibitory effect demonstrated by Hama *et al.* (1994) in *E. coli*, cysteine had no effect on elemental sulfur oxidation by cell free extracts of *T. novellus* (Charles and Suzuki, 1966). Cysteine did decrease the rate of sulfur oxidation in *T. neapolitanus* by 20%, a bacterium known to express rhodanese (Taylor, 1968).

When dihydrolipoic acid and dihydrolipoamide where used as acceptor substrates for *Paracoccus versutus* (*Thiobacillus versutus*, which was previously *Thiobacillus* A2), the cells catalyzed the cleavage of thiosulfate producing α-lipoate and lipoamide, respectively (Silver and Kelly, 1976). In this reaction the persulfides of dihydrolipoate and dihydrolipoamide were formed as intermediates (Silver and Kelly, 1976). The formation of sulfite and organic persulfides (e.g. dihydrolipoate persulfide) as end products of thiosulfate cleavage was attributed to the presence of rhodanese in *P. versutus* (Silver and Kelly, 1976).

1.11) Rhodanese in biomining

Rhodanese activity has been demonstrated during experimental biooxidation of metal-sulfide ores (Cwalina *et al.*, 1988). In an attempt to establish criteria for the biooxidation suitability of *T. ferrooxidans* on metal-sulfide ores, certain enzymes involved in inorganic sulfur metabolism, particularly sulfite oxidase, thiosulfate oxidase and rhodanese, were considered (Cwalina *et al.*, 1988).

T. ferrooxidans F26-77 biooxidation of pyrite (FeS_2), covellite (CuS), chalcopyrite ($CuFeS_2$) and sphalerite (ZnS) was undertaken and the activities of these enzymes determined during biooxidation (Cwalina *et al.*, 1988; Cwalina *et al.*, 1990a; Cwalina *et al.*, 1990b). In all cases the enzyme activity of rhodanese increased during the course of

ore biooxidation (Cwalina *et al.*, 1988; Cwalina *et al.*, 1990a; Cwalina *et al.*, 1990b). However, it was observed that the increase in the studied enzymes did not accelerate the rate of zinc bioextraction from sphalerite (Cwalina *et al.*, 1990b). Thus, the authors of these studies recognised that enzyme activity was not an appropriate criterion for determining culture suitability for ore biooxidation. Rather, it was argued that this increase of enzyme activity should be attributed to strain adaptation and acclimitization to a particular metal-sulfide ore, and that this resulted in enzyme induction (Cwalina *et al.*, 1990a).

1.12) Conclusion

The wastage of cyanide during the cyanidation of refractory metal-sulfide ores pretreated by biooxidation is considered one of the major disadvantages of this biohydrometallurgical process. Although the formation of reactive sulfur species resulting from the oxidation of these ores by the resident mixed population of chemolithoautotrophic bacteria can contribute to non-enzymic cyanide wastage, the direct involvement of enzymic cyanide wastage must be considered.

The production of thiocyanate is thought to account for the largest proportion of cyanide wastage. Although thiocyanate is formed by spontaneous chemical reactions of reactive sulfur species with cyanide, the *in vitro* activity of rhodanese defines this enzyme as a thiosulfate: cyanide sulfurtransferase. Therefore, rhodanese may contribute to cyanide wastage through the transfer of sulfenyl sulfur to cyanide, forming thiocyanate.

The ubiquitous existence of rhodanese, and its detected activity in *T. ferrooxidans*, suggests that this enzyme is present in some, if not all, the chemolithoautotrophic bacteria responsible for refractory ore biooxidation.

In spite of being defined as a thiosulfate: cyanide sulfurtransferase, rhodanese has demonstrated diverse catalytic abilities. To date, the physiological role of rhodanese is

still unclear and thus Knowles' (1973) definition as a general sulfane sulfurtransferase is probably the most descriptive.

Colnaghi *et al.* (1996) elaborated on this description by arguing that the seemingly diverse functions in prokaryote rhodanese-like enzymes and the low sequence similarity of these polypeptides suggests that the rhodanese structure "may present an effective solution to general 'labile' sulfide transfer that occurs in more than one metabolic context". From the evidence presented, it seems possible that genetically and physiologically distinct rhodanese-like enzymes may coexist in the same cell. An extensive sequence comparison of prokaryote rhodaneses with bovine liver rhodanese by Colnaghi *et al.* (1996) resulted in the finding that through the introduction of gaps in the sequence of six surface loops present in one domain, but absent from the other, the N-terminal and C-domains aligned according to the conformational duplication observed in bovine liver rhodanese. This suggests that the rhodanese structure was originally generated by gene duplication (Ploegman *et al.*, 1978; Colnaghi *et al.*, 1996). The different molecular weights of purified rhodanese and the demonstrated mercaptopyruvate sulfurtransferase activity of human rhodanese suggests that the rhodaneses originate from a common ancestor (Nagahara *et al.*, 1995). The functionally diverse rhodaneses described for *E. coli* (Hama *et al.*, 1994), *S. erythraea* (Donadio *et al.*, 1990), *Synechococcus* sp. PCC 7942 (Laudenbach *et al.*, 1991), and *A. vinelandii* (Colnaghi *et al.*, 1996) implicates a common ancestry from which evolved divergent families of enzymes (Colnaghi *et al.*, 1996).

1.13) Aims of this project

The main aim of this project was to identify the potential contribution to cyanide wastage by the predominant chemolithoautotrophic bacteria in the biooxidation process. The significant accumulation of thiocyanate during cyanidation, and the possibility that these microbes utilize a thiosulfate: cyanide sulfurtransferase to contribute to the formation of thiocyanate initiated the investigation into the potential rhodanese contribution to the wastage of cyanide during cyanidation. The following approach was undertaken.

The rhodanese activity of the microbial consortium present in a biooxidation pilot plant, and the rhodanese activity of pure cultures of relevant biomining chemolithoautotrophic bacteria, was determined. An attempt was made at defining the microbial population in the biooxidation pilot plant using a polymerase chain reaction (PCR) based restriction enzyme analysis technique. It was envisaged that together with the determined pure culture rhodanese activities of the relevant biomining bacteria, the PCR technique would assist in identifying which of the bacteria were responsible for rhodanese production.

A second aim was to attempt to isolate the gene(s) for rhodanese from the chemolithoautotrophic bacterium that had the highest enzyme activity in pure culture. Since there is no direct selection for rhodanese activity in a bacterium like *E. coli*, a Southern hybridization approach was adopted. It was hoped that although taxonomically distant to *A. vinelandii*, the *rhdA* gene encoding the rhodanese enzyme in these microbes would yield a signal when genomic digests of the pure and biooxidation cultures were subjected to Southern hybridization using a probe constructed from the *A. vinelandii rhdA* gene.

Chapter Two

The rhodanese activity of biomining bacteria

Contents

2.1) Introduction

Rhodanese is commonly assayed using the method established by Sörbo (1953). This method is a colourimetric assay based on the spectrophotometric determination of the end product thiocyanate which is formed by the enzyme catalyzed reaction of thiosulfate with cyanide (Singleton and Smith, 1988). Two products are formed by the enzyme reaction with these substrates, namely thiocyanate and sulfite (equation 2.1).

$$S_2O_3^{2-} + CN^- \rightarrow SCN^- + SO_3^{2-} \qquad (2.1)$$

Conventional rhodanese assays colourimetrically measure either thiocyanate or sulfite formation (Singleton and Smith, 1988). For example, both Vandenbergh *et al.* (1979) and Lu *et al.* (1983) developed an ethylenediaminetetraacetic acid (EDTA) and lysozyme cell lysis protocol for the rapid detection of intracellular rhodanese using the formation of thiocyanate as an indicator of rhodanese. Guilbault *et al.* (1971), however, used sulfite as an indictor of rhodanese in non-denaturing polyacrylamide gels. The reaction of sulfite with $CaCl_2$ yields a white precipitate, $CaSO_3$, which made the *in situ* visualization of rhodanese possible. In a non-conventional protocol for detection of rhodanese the intrinsic fluorescence of 5-dimethyl-amino-1-naphthalene thiosulfonate (DANTS) is used as an indicator (Aird *et al.*, 1987b). DANTS replaces thiosulfate in the conventional assay. Enzymatic cleavage of the sulfane-sulfur of DANTS by rhodanese releases the quenched fluorogen allowing for the visual detection of rhodanese in non-denaturing polyacrylamide gels (Aird *et al.*, 1987b).

The method developed by Sörbo (1953) for the detection of thiocyanate is still the most commonly used method for the detection of rhodanese, although the assay protocol has undergone slight modifications. One of the initial problems with the assay was the formation of an interfering blue complex formed upon addition of the ferric nitrate colour reagent to the reaction mixture. This blue complex was attributed to the formation of an iron-thiosulfate complex. This complex was subsequently prevented by the addition of formaldehyde to the assay protocol (Sörbo, 1963). Formaldehyde also has the added

feature of terminating the reaction by stripping the reaction mixture of the cyanide through the formation of cyanohydrins (Singleton and Smith, 1988). Another problem with the assay is the instability of the ferric-thiocyanate colour. The colour instability was attributed to the reaction between the nitric acid component of the colour reagent and thiocyanate (Nor and Tabatabai, 1975). This instability is promoted in the presence of ferric irons and light (Nor and Tabatabai, 1975). These factors were taken into account for the colourimetric assays performed in this study.

Possibly the most important consideration for the rhodanese assay is the potential for non-biological interference (Westley, 1973). Singleton and Smith (1988) established that during the standard (conventional) rhodanese assay three thiocyanate contributing reactions can occur. The first of these is the reaction catalyzed by the enzyme (equation 2.1). The second is the non-enzymic reaction between thiosulfate and cyanide yielding thiocyanate and sulfite. In other words, the spontaneous chemical reaction of equation 2.1. Lastly, the formation of thiocyanate may occur by the spontaneous cyanolysis of elemental sulfur forming thiocyanate (equation 2.2).

$$S^{\circ} + CN^{-} \rightarrow SCN^{-} \qquad (2.2)$$

Singleton and Smith (1988) felt that if these reactions were not taken into account results obtained should be deemed questionable. Secondly, assay protocols that attempted to account for these reactions usually involved the addition of formaldehyde to the reaction mixture prior to the enzyme suspension and cyanide. This was done under the assumption that the formaldehyde would denature the enzyme suspension, and this would enable the spontaneous chemical formation of thiocyanate to be assayed. Such an assay control was shown by Singleton and Smith (1988) to be inappropriate as formaldehyde rapidly removes cyanide from the reaction mixture. Thus, any non-biological reactions would be unable to proceed. Rather, the enzyme suspension should be boiled to denature the enzyme, and formaldehyde used exclusively to terminate the assay (Singleton and Smith, 1988).

It was due to these assay problems, and the solutions offered, that the rhodanese assay protocol of Singleton and Smith (1988) was followed throughout the current work presented. Assay modifications and optimization were investigated for the use of this assay to determine the presence of rhodanese in the biomining bacteria.

2.2) Materials and Methods

2.2.1) Bacterial strains and growth: *T. ferrooxidans* ATCC 33020 and *T. thiooxidans* ATCC 19377 were batch cultured in 800ml tetrathionate media at 30°C with constant shaking. *T. ferrooxidans* ATCC 33020 was grown in 5mM tetrathionate media pH 1.6, while *T. thiooxidans* ATCC 19377 was grown in 2.5mM tetrathionate media pH 2.0. The tetrathionate media used throughout this study is described in detail in Appendix A(i). *L. ferrooxidans* DSM 2705 was cultured in 800ml 9K iron media pH 1.6 at 30°C with constant shaking. The 9K iron media is described in detail in Appendix A(ii). Strains used in this study are described in Appendix B.

The biooxidation pilot plant (Dept. of Chemical Engineering, University of Cape Town [UCT]) was inoculated with a natural population of mesophiles obtained from the Biox® bioreactors at the Fairview mine (South Africa). The culture was maintained in a 23 litre fed-batch culture on a pyrite-arsenopyrite concentrate obtained from the Fairview mine at 40±1°C and pH 1.65±0.2. The concentrate sample contained 7% As, 26% S and 24% Fe, and 86% of the material was finer than 75µm. The mineral analysis of the concentrate was 16% FeAsS and 41% FeS_2 (A. Breed, per. comm.). The bioreactor was agitated by a pitched (45°C) six-bladed turbine impeller rotating at 570rpm. The impeller was driven by a 0.37kW Flender Himmel motor. The polyvinyl chloride (PVC) 230mm diameter bioreactor had four baffles aligned at 90°, and separate internal heating and cooling coils. The temperature control system consisted of an Electrolink LM35 temperature sensor and a Hitech Micro Systems temperature controller. The pH was determined by a Foxboro® 871PH electrode and a Foxboro® Electrochemical analyzer was used to control H_2SO_4 and NaOH addition to maintain the pH. Acid and base addition was achieved through separate circuits. Aeration was achieved using compressed air and the feed rate was maintained by a rotameter. Air was sparged below the impeller for maximum gas dispersion. A YSI Model 5739 dissolved oxygen probe was used to maintain a dissolved oxygen concentration of ±6mg.l^{-1}. A Prominent® gamma/40216 diaphragm metering pump controlled the nutrient feed rate. The nutrient feed consisted of (g.l^{-1}) $(NH_4)_2SO_4$, 1.83; $(NH_4)_2HPO_4$, 1.11; and K_2SO_4, 0.53. As the bioreactor was also being operated by

the Gold Fields Minerals Bioprocessing Laboratory (Dept. of Chemical Engineering, University of Cape Town) bioreactor conditions were subject to change.

2.2.2) Harvesting bacteria: Cells from the pure cultures were obtained by high speed centrifugation and resuspended in 3ml acidified water pH 1.8. Cells were washed three times in acidified water by a process of low and high speed centrifugation to remove extraneous reactive sulfur species or jarosite; depending on the species harvested. Finally, the cell pellet was resuspended in the rhodanese assay buffer pH 7.5. The biooxidation pilot plant (UCT) mixed culture was harvested by collection of washout after the addition of an appropriate volume of mineral feed. The dense particulate matter was allowed to settle and the resulting supernatant was then centrifuged at 3K for 10 minutes to pellet the finer particles from the liquor. High speed centrifugation was then used to pellet the microbial population in the resulting supernatant. The cell pellet was resuspended in acidified water pH 1.8 and subjected to the same washing process as described for the pure cultures. Finally, the washed cell pellet was resuspended in the rhodanese assay buffer pH 7.5.

2.2.3) Rhodanese assay: The rhodanese fraction consisted of either whole unlysed cells or a crude enzyme extract. A Virsonic (Virtis) sonicator was used to prepare the crude enzyme extract. Cells were resuspended in 1.5ml assay buffer and subjected to pulse cycles of 60 seconds with 30 second cooling intervals for a total of 4 minutes sonication time. Sonication was performed on ethanol cooled ice to prevent heat denaturation of the soluble extract. The extract was then centrifuged in a refrigerated microcentrifuge at 12K for 30 minutes and the supernatant stored on ice prior to use in the assay.

To standardize the assay, total protein was determined using the Bio-Rad protein assay reagent and assay protocol (Bio-Rad Laboratories).

The rhodanese fraction (182µl) was added to a reaction mixture containing 91µl 67mM K_2HPO_4 + 20% NaCl pH 7.5 (assay buffer) and 9lµl 100mM $Na_2S_2O_3$ + 20% NaCl. Following a 10 minute preincubation at 30°C, 91µl of pre-warmed 100mM KCN + 20%

NaCl was aliquoted intc the reaction mixture yielding a total volume of 455µl. This reaction mixture was then incubated for a further 30 minutes prior to reaction termination by the addition of 45µl 37% formaldehyde. The reaction mixture was then centrifuged for 5 minutes at 14K to remove spectrophotometric interfering protein. After transfer of the supernatant to a new Eppendorf tube, colour development was achieved by the addition of 500µl of the ferric nitrate colour reagent (15%, w/v, $Fe(NO_3)_3$ in 1N HNO_3). The absorbance of the 1ml final volume was determined at 460nm in a Beckman DU®-64 spectrophotometer. Rnodanese specific activity is defined as the formation of 1 µmol SCN^- per minute per mg total protein (µmol $SCN^-.min^{-1}.mg\ protein^{-1}$).

All substrates and buffers were made up in double distilled water (Milli^{TM}-Q) as metal ions can accelerate the decomposition of thiosulfate (Roy and Trudinger, 1970).

The assay blank was prepared with all the above constituents except that assay buffer replaced the rhodanese fraction. To account for extraneous sulfane-sulfur anions introduced by the rhodanese fraction a control was included. This control comprised a boiled rhodanese fractior which was added to the assay substrates. To achieve complete inactivation of the rhodanese fraction the sample was boiled for 40 minutes in a water bath.

2.2.4) Scanning electron microscopy (SEM): Cells were harvested and resuspended in SET buffer (Appendix A). The bacteria were fixed in 2.5% glutaraldehyde and incubated overnight at 4°C. The fixed cells were resuspended in 1% osmium tetroxide and SET buffer, and then dehydrated in a graded series (30-99%) of ethanol. The bacteria were attached to SEM stubs using an unspecified commercially available glue mixed with graphite. The bacteria were photographed using a Leica Stereoscan S440 electron microscope. All cultures investigated in this study were prepared by and photographed with assistance from the Electron Microscope Unit (University of Cape Town).

2.3) Results

Given the number of potential problems associated with the rhodanese assay it was felt that assay optimization would yield rhodanese specific activity results of greater accuracy.

2.3.1) Rhodanese assay optimization

The mechanism of thiosulfate degradation in acid pH is not well understood, and at alkaline pH the ionic strength of the solution determines the decomposition products (Roy and Trudinger, 1970). Therefore, it was preferable to determine the pH at which thiosulfate in the assay was most stable. Minimal thiosulfate decomposition would allow for accurate determination of non-biological thiocyanate formation, thereby improving the quantitative sensitivity of the assay. Assays were performed across a pH range of pH 5.0-10.0, without a biological fraction, to determine the spontaneous formation of thiocyanate (Fig 2.1).

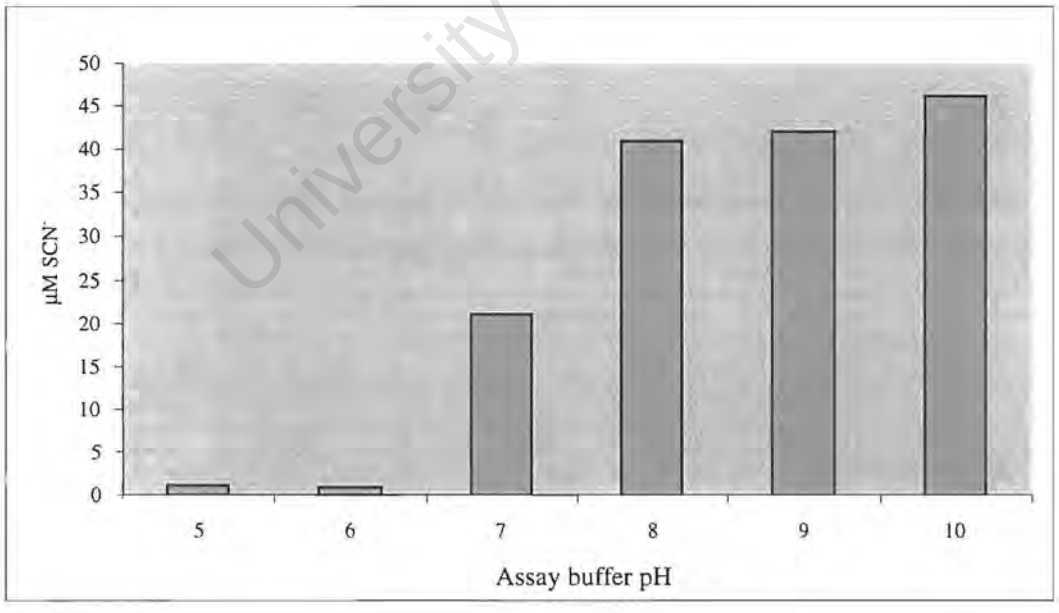


Fig 2.1: Spontaneous thiocyanate formation at different assay pH. Assays were performed in triplicate and the standard deviation found to be minimal. Standard deviation error bars fall within the line at the top of the columns and are therefore not depicted on the graph.

The end product thiocyanate was found to be stable across this pH range and linear with increasing concentration (data not shown).

As the spontaneous chemical formation of thiocyanate increased above pH 7.0 (Fig 2.1), and since in weakly acidic solution thiosulfate is known to be converted almost entirely to sulfur and bisulfite (Roy and Trudinger, 1970), which would account for the very low thiocyanate formation at pH 5.0 and 6.0, it was decided that the assay would be buffered at pH 7.5. Thiosulfate is stable at neutral pH (Roy and Trudinger, 1970 and Budavari, 1989), and the ability of *T. ferrooxidans* to maintain its intracellular pH at a value close to neutral over an external pH range of 1.0 to 8.0 (Cox *et al.*, 1979) meant that the assay for rhodanese at pH 7.5 would be reasonable. This assay pH was also chosen on the basis that the rhodanese enzyme purified from *T. ferrooxidans* (Tabita *et al.*, 1969), one of the biomining bacteria to be considered in this study, showed a broad pH optimum between pH 7.5-9.0.

A problem encountered in the initial assays was the large deviation between assays. It was considered that the method of rhodanese inactivation by boiling the rhodanese sample in a water bath for 30 minutes may not completely denature the biological sample. If a control for determining the spontaneous formation of thiocyanate contained a rhodanese sample that was not completely inactivated this would affect the specific activity result. This problem was particularly prevalent for whole cell assays. Possible denaturing agents such as trichloroacetic acid (TCA) and nitric acid were considered for the rapid denaturation of the rhodanese sample used as the control. These denaturing reagents rely on a change of solution ionic strength to denature the sample.

However, a denatured acidic rhodanese sample would require a pH adjustment for its use in the assay; for reasons discussed above. As direct pH adjustment was prone to titration inaccuracy and buffer dialysis was too time consuming, this method of denaturation was determined to be unfeasible. Alkaline denaturing agents such as NaOH could also not be used. Apart from the problems associated with direct pH adjustment to the pH of the assay, hydroxyl ions compound the problem. Hydroxyl ions are nucleophilic and their

The mean specific activity standard deviation for all cultures was large, but as Table 2.1 shows, the variation between assay samples was considerably larger than the variation within triplicate assays of the same sample.

Table 2.1: Rhodanese specific activity for whole cell and crude enzyme extract assays showing the standard deviation within and between assays. The specific activities of *T. ferrooxidans* ATCC 33020 (**T.f**), *T. thiooxidans* ATCC 19377 (**T.t**), *L. ferrooxidans* DSM 2705 (**L.f**) and the biooxidation pilot plant (UCT) (**Biox**) whole cell and crude enzyme extracts are presented.

	Whole cell rhodanese assay (μmol $SCN^-.min^{-1}.mg\ protein^{-1}$)			**Crude enzyme extract assay (μmol $SCN^-.min^{-1}.mg\ protein^{-1}$)**		
	No. of assays	**Specific activity**	**Mean**	**No. of assays**	**Specific activity**	**Mean**
	1	1.71 ±1.05		1	6.44 ±0.2	
T.f	2	1.66 ±0.41	2.68 ±1.73	2	8.73 ±0.2	8.21±1.57
	3	4.68 ±0.21		3	9.45 ±0.17	
	1	5.84 ±0.21		1	5.98 ±0.14	
T.t	2	8.02 ±0.2	6.93 ±1.54	2	2.89 ±0.12	6.41±3.75
				3	10.36 ±0.22	
L.f		Not detected			Not detected	
	1	5.13 ±0.08		1	14.27 ±0.36	
	2	1.95 ±0.1		2	27.82 ±0.15	
Biox	3	0.82 ±0.05	1.68 ±1.8	3	14.3 ±0.12	14.68 ±9.63
	4	0.46 ±0.12		4	21.96 ±0.19	
	5	0.29 ±0.01		5	23.47 ±0.69	
	6	1.41 ±0.09		6	19.94 ±0.27	
				7	18.66 ±0.18	
				8	24.67 ±0.66	
				9	1.09 ±0.21	
				10	3.69 ±0.22	
				11	2.6 ±0.02	
				12	3.65 ±0.1	

In general, whole cell assays were less consistent than crude enzyme extract assays. This was possibly due to variable cell lysis occurring on neutralization of the samples prior to assay.

reaction at physiological pH and temperature with any S_8 present in the biological sample would possibly yield sulfide and thiosulfate (Roy and Trudinger, 1970). To account for this would further complicate the assay. Therefore, no substitute for the time consuming boiling of the biological sample could be found. Empirical inactivation studies (data not shown) led to the period of boiling being extended to 40 minutes to ensure effective denaturation of rhodanese in the fraction to be used as the control.

2.3.2) Rhodanese specific activities of the chemolithotrophic bacteria

The rhodanese specific activities for the pure cultures of some of the predominant chemolithoautotrophic bacteria reported to be present in biooxidation bioreactors, and the rhodanese specific activity of the mixed population in a biooxidation pilot plant (UCT), are graphically represented in Fig 2.2. The rhodanese specific activity for each culture was determined as a mean of assays performed for both whole cells and lysed crude enzyme extracts (Table 2.1).

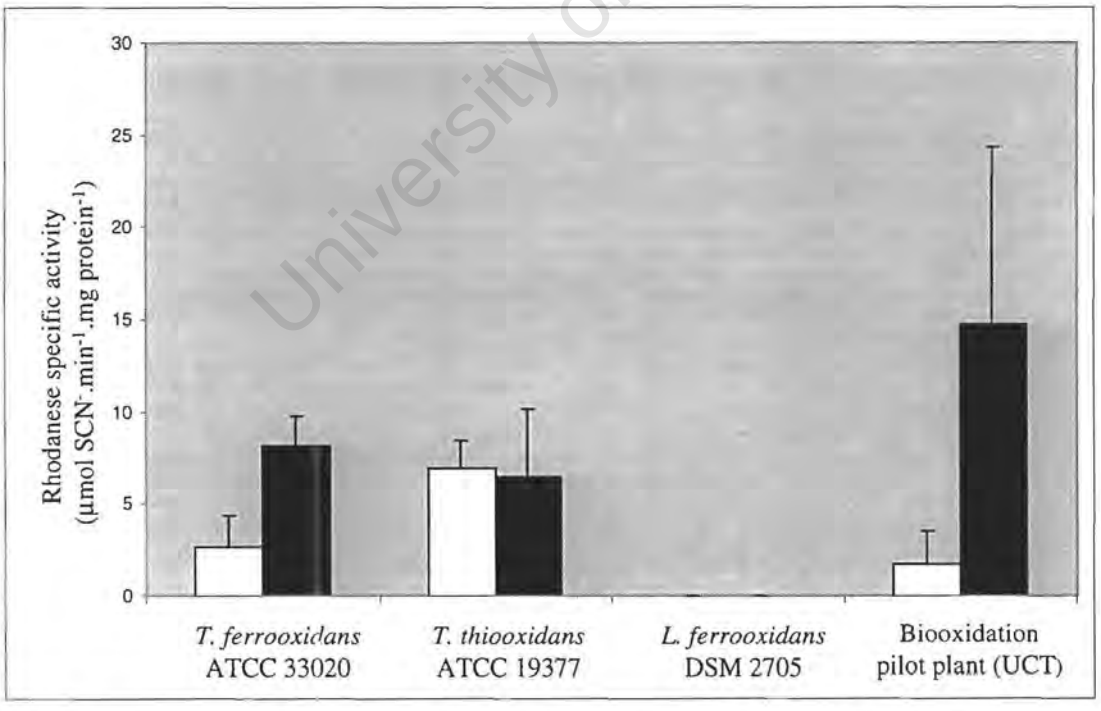


Fig 2.2: Rhodanese specific activities of chemolithoautotroph pure cultures and the mixed culture from the biooxidation pilot plant (UCT). Error bars for (□) whole cell and (■) crude enzyme extract specific activities represent the standard deviation between assays.

To determine whether this assay variation was a consequence of the growth substrate, the sulfur- and iron-oxidizing chemolithoautotroph, *T. ferrooxidans* ATCC 33020, was cultured in tetrathionate and 9K iron media (Fig 2.3).

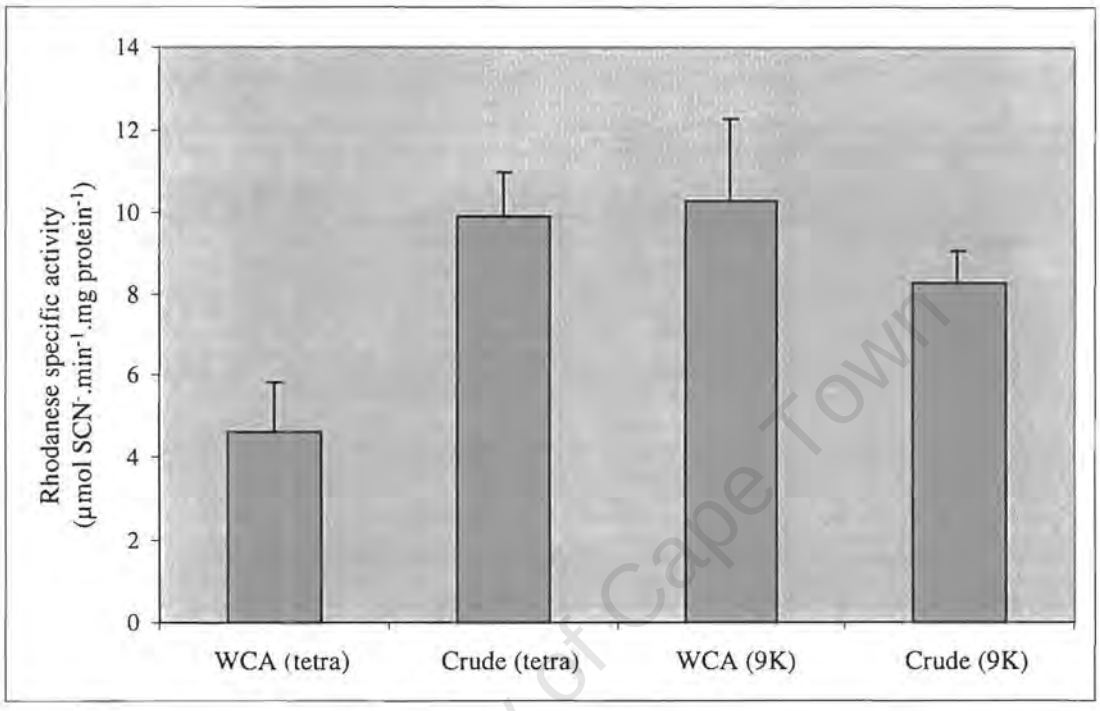


Fig 2.3: Rhodanese specific activity determined for whole cells (**WCA**) and crude enzyme extracts (**Crude**) of *T. ferrooxidans* ATCC 33020 cultured in 5mM tetrathionate media pH 1.6 (**tetra**) and in 9K iron media pH 1.6 (**9K**). Error bars represent the standard deviation between assays.

Rhodanese appeared to be expressed in *T. ferrooxidans* ATCC 33020 regardless of the substrate in which it was cultured. It therefore appeared to be expressed constitutively. The fairly large variation in specific activity between assays was still observed regardless of the growth substrate (Fig 2.3).

An attempt was made at determining whether rhodanese was exported from the cell by harvesting the cells from the growth media and assaying the media for rhodanese activity. Exported protein in the growth medium was concentrated with 90% ammonium sulfate, dialysed against assay buffer and assayed. No rhodanese activity was detected but this was expected as at such a low pH (media pH after growth was ± pH 2.0) any active enzyme would most likely have been denatured.

2.3.3) SEM micrographs of the bacterial cultures

Scanning electron micrographs of the pure cultures and the biooxidation pilot plant (UCT) culture used in this study are presented in Fig 2.4 to Fig 2.7.

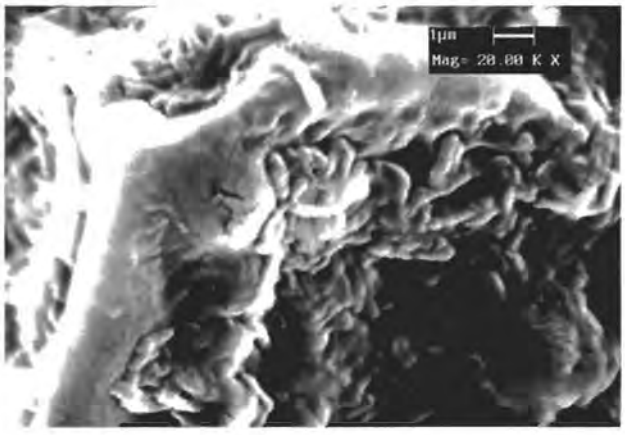


Fig 2.4: SEM of *T. ferrooxidans* ATCC 33020.

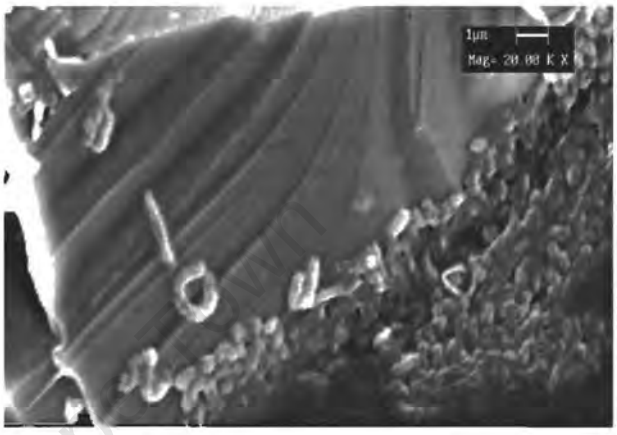


Fig 2.5: SEM of *T. thiooxidans* ATCC 19377.

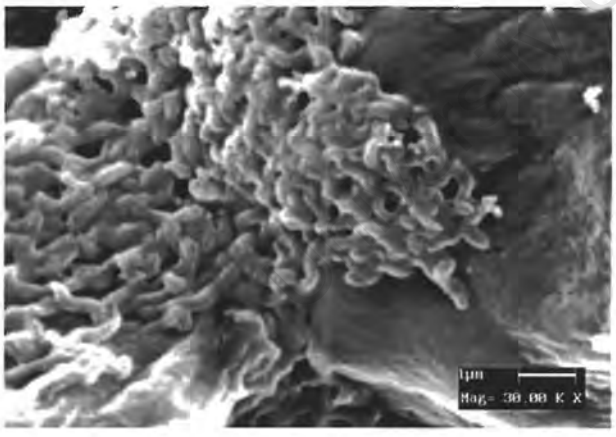


Fig 2.6: SEM of *L. ferrooxidans* DSM 2705.

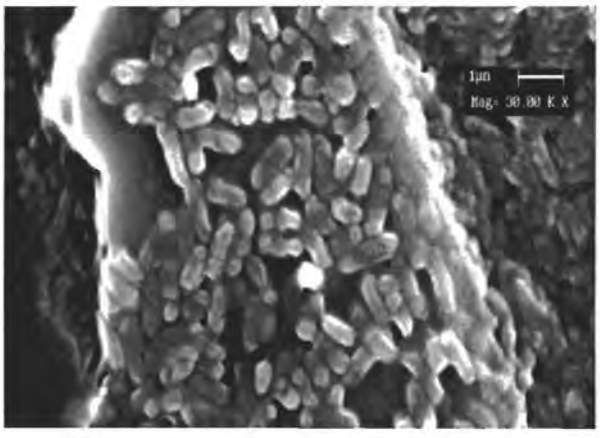


Fig 2.7: SEM of Biooxidation plant (UCT) mixed culture.

2.4) Discussion

To enable the quantitative determination of each strain's rhodanese specific activity an assay pH was sought at which thiosulfate degradation was limited. The demonstrated instability of thiosulfate (the rhodanese substrate) at acid and alkaline pH values (Fig 2.1) limited the assay to a pH range near neutrality. An assay pH compromise of pH 7.5 was chosen for two reasons. Firstly, as a result of the degradation of thiosulfate at weakly acidic pH, low spontaneous thiocyanate formation occurred at values below pH 6.0. Conversely, marked spontaneous chemical formation of thiocyanate was found at pH 8.0 (Fig 2.1). Secondly, the integrity of cells in whole cell assays should not have been affected at pH 7.5 if the ability of *T. ferrooxidans* to counteract external alkaline conditions (Cox *et al.*, 1979) can be extrapolated for the bacteria considered in this study.

Representatives of the biooxidation bacteria were grown in pure culture to determine if they possessed any rhodanese activity. Only the sulfur-oxidizing bacteria demonstrated rhodanese activity, while the iron-oxidizing *L. ferrooxidans* DSM 2705 was unable to convert cyanide to thiocyanate (Fig 2.2). Crude enzyme extracts prepared from the natural mixed population of bacteria harvested from the biooxidation pilot plant (UCT) exhibited a mean level of rhodanese higher than that of the pure cultures (Fig 2.2 and Table 2.1). However, the standard deviation between biooxidation pilot plant (UCT) and pure culture assays indicated that these results were too variable to draw a comparison between pure culture and biooxidation bioreactor rhodanese activities.

The large standard deviation between rhodanese assays of both pure cultures and the biooxidation bioreactor was of concern. Many factors may have contributed to this large deviation. Singleton and Smith (1988) reported difficulties with the standard rhodanese assay and were the first to demonstrate the contribution of non-enzymatic thiocyanate formation in the rhodanese assay. As the accumulation of reactive sulfur species during growth of *T. ferrooxidans* (Hazeu *et al.*, 1988) has been demonstrated, the biological sample would most certainly introduce reactive sulfur species into the rhodanese assay. These reactive sulfur species would contribute to the non-enzymatic formation of

thiocyanate over and above the assay substrate-catalyzed spontaneous formation of thiocyanate. The assay protocol of Singleton and Smith (1988) uses a denatured biological control to account for the non-enzymatic formation of thiocyanate, and it was for this reason that the protocol of these authors was used. The spontaneous formation of thiocyanate was observed to contribute between 30-60% of the total thiocyanate formed in a rhodanese assay reaction (see Appendix C). Despite this, the assay itself appeared to be fairly reliable as the standard deviation within triplicate assays of the same sample was generally low (Table 2.1). However, the standard deviation between assays was large.

The potentially weakest component of the rhodanese assay is the denatured biological control. Should this biological control not be completely denatured then both non-enzymatic and residual rhodanese activity would result in the thiocyanate produced in the control being attributed to spontaneous chemical formation of thiocyanate. The control would therefore not be a true representation of the non-enzymatic formation of thiocyanate. This would influence the enzymatic determination of thiocyanate formation in the non-denatured biological sample. As no substitute for heat denaturation of the assay control could be found (see section 2.3.1) it was possible that incomplete denaturation of the assay control influenced the deviation between assays. However, boiling a rhodanese fraction for 40 minutes appeared to be sufficient to achieve complete inactivation of the rhodanese enzyme.

Another possible contributing factor to the variation between assays is inhibition of rhodanese by substrate, non-substrate anions, and end-products. Sulfur oxidation by chemolithotrophic bacteria produces thiosulfate, sulfite and sulfate. If these sulfur species are present in excess in the assay sample they may influence the catalytic ability of the enzyme by means of competitive and non-competitive inhibition. Such inhibition has been described previously for rhodanese enzymes (see section 1.10). The end-product thiocyanate has also been demonstrated to inhibit rhodanese (Ray *et al.*, 1990). As the accumulation of reactive sulfur species appears to be correlated to the age of the culture and degree of substrate oxidation (Rojas *et al.*, 1995), the growth phase at which the culture is harvested for the assay may determine the contribution of these sulfur

species to the molar concentration of thiocyanate. For instance, the reaction of elemental sulfur with cyanide results from the nucleophilic attack by cyanide on the S_8 ring and is known to be rapid (Roy and Trudinger, 1970; Hackl, 1989). Therefore, within the assay period, the reaction shown in equation 2.2 may contribute significantly to the thiocyanate inhibition of rhodanese. Since another end-product of the rhodanese catalyzed reaction with cyanide is sulfite (equation 2.1), it is possible that in the presence of elemental sulfur the reaction of S_8 with sulfite (equation 2.3) could form thiosulfate (Roy and Trudinger, 1970).

$$S_8 + 8SO_3^{2-} \rightarrow 8S_2O_3^{2-} \qquad (2.3)$$

This argument, however would not hold for *T. ferrooxidans* cultured in iron-media.

The extraneous thiosulfate formed could contribute to the inhibition of rhodanese by either spontaneous chemical formation of thiocyanate (equation 2.1) or by substrate inhibition. This substrate inhibition effect was observed for the sulfane sulfurtransferase purified from *A. calcoaceticus lwoffi* (Aird *et al.*, 1987a). The varying degree to which these rhodanese inhibiting anions are present in the rhodanese assay may be a function of the growth phase at which the culture is harvested. Growth phase may therefore influence the catalytic activity of rhodanese, which could contribute to the large standard deviation between assays.

Therefore, the problems associated with the specific activity standard deviations could be attributed to such diverse factors as the physiological state of the cell, substrate and end-product inhibition of rhodanese, and the incomplete denaturation of the assay control. With so many factors influencing the assay this could have contributed significantly to the deviation observed between assays. The influence of accumulation of reactive sulfur species and the physiological state of the cell on rhodanese assays will be further addressed in chapter three.

Finally, considering the comparatively large standard deviation between assays of the biooxidation pilot plant (Table 2.1), the impact of changing species dominance during concentrate biooxidation may have compounded the problem of deviation between assays. The rhodanese assay results for the biooxidation pilot plant (UCT) listed in Table 2.1 were obtained under different operating conditions. These different operating conditions could have caused a change in the bacterial species population dominance in the bioreactor. For instance, iron substrate limitation in the concentrate after prolonged oxidation of the concentrate could lead to a shift in population dominance that would have resulted in an increased biomass of sulfur-oxidizers (see section 1.3). As the prominent iron-oxidizer in bioreactors, represented by *L. ferrooxidans* DSM 2705, had no observable rhodanese activity (Fig 2.2), an increase in the relative proportion of sulfur-oxidizers in the biomass would result in higher rhodanese activities being determined for the biooxidation pilot plant (UCT) sample. Therefore, shifting species dominance caused by the oxidation state of the pyrite-arsenopyrite concentrate could have influenced biooxidation pilot plant (UCT) rhodanese assays which would have exacerbated the problem of deviation between assays.

Therefore, the only conclusion that could be derived from Table 2.1 was that *T. ferrooxidans* ATCC 33020 and *T. thiooxidans* ATCC 19377 demonstrated the ability to convert cyanide to thiocyanate through an *in vitro* catalyzed reaction indicative of the rhodanese enzyme. The above arguments may also explain the variation in rhodanese specific activity for *T. ferrooxidans* ATCC 33020 grown in iron and tetrathionate media (Fig 2.3). What could not be determined was whether the growth media directly influenced the level of rhodanese activity, although the constitutive expression of rhodanese in *T. ferrooxidans* ATCC 33020 was demonstrated (Fig 2.3).

Should the physiological state of the cell affect rhodanese activity, or the oxidation state of the Biox® concentrate influence the species dominance, these factors could be used to improve the economics of the process. It might be possible to optimize the conditions within the bioreactors to allow complete gold recovery and also to minimize the wastage of cyanide during cyanidation.

Chapter Three

Detection of *Thiobacillus caldus* and rhodanese activity of the bacterium in a biooxidation pilot plant

Contents

3.1) Introduction

The difficulty in isolating, identifying and enumerating individual species and strains of microorganisms in environmental samples which display large microbial diversity yet common metabolic requirements is one of the major obstacles encountered in the study of microbial ecology. However, the identification and enumeration of microbes in biooxidation processes is of importance as an ability to define the role and interaction of these species will allow the intimate link between microbial physiology and metal-sulfide ore biooxidation to be understood (Goebel and Stackebrandt, 1994). The importance of determining population composition and dynamics is done under the pretext that strains best suited for a particular kind of mineral biooxidation will be selected (Harrison, 1982). Consequently, such knowledge could translate into process optimization. For example, biooxidation at high acid concentrations increases the rate of recovery from copper-sulfide ores and prevents ferric iron precipitation, yet a compromise of pH 1.3 was made to allow for the growth of *T. ferrooxidans* as it was considered one of the more relevant bacteria in biomining. However, it has been found by direct molecular analysis of DNA prepared from bioreactors that significant bacterial growth can occur at pH values below pH 1.0, and that meaningful shifts in population composition will occur under these highly acidic conditions (Pizarro *et al.*, 1996; Vásquez and Espejo, 1997).

The introduction of molecular techniques to the study of microbial ecology is rapidly replacing the more laborious and frequently problematic techniques of plating and microscopic enumeration. Plating is possibly the most highly selective isolation and enumeration technique, and it is prone to bias. It is now recognised that only a minor fraction of the overall diversity of microbes in environmental samples are accounted for by plating as not all community members are guaranteed to be culturable (Polz and Cavanaugh, 1997; Wood *et al.*, 1998). Apart from the tedious and nonspecific nature of microscopic enumeration, microscopy is inadequate for at least two reasons. It is unable to discriminate between populations of morphologically similar bacteria, and it is unable to discriminate between metabolically active and dormant or dead microorganisms (Amaro *et al.*, 1994; Stoner *et al.*, 1996).

Dot (DIMA) and slot (SIMA) immunobinding assays were developed to overcome the problems of conventional techniques. Although these immunoassays were able to detect *T. caldus* and the thermophilic archaeon *Sulfolobus acidocaldarius* in biooxidation samples containing as few as 10^3-10^4 cells, it suffered from the inability to distinguish between viable and non-viable cells (Amaro *et al.*, 1994). Immunoassays do, however, provide a convenient means of monitoring specific bacteria within industrial bioreactors as it is a technique that is fast, sensitive and provides identification and enumeration results simultaneously. However, the identified multiple serotypes of *T. ferrooxidans* and *T. caldus* (Hallberg and Lindström, 1996) presents yet another problem for microbial ecologists; the need for a barrage of antisera to recognize all microbes present in the population. This infers that the microorganism(s) must already have been isolated and be available in pure culture. DNA-DNA and DNA-RNA hybridization analysis of population diversity is hampered by the similar need to develop a panel of oligonucleotide probes that can only be constructed using available sequence data (Wood *et al.*, 1998). Goebel and Stackebrandt (1994) cautioned against the development of 16S rRNA oligonucleotide probes specific for the iron and sulfur-oxidizing phenotypes because of the polyphyletic nature of these groups as shown by Lane *et al.* (1992). Thus, using these techniques uncultured microbes and their contribution to the microbial consortium will continue to go unrecognized.

A high-resolution denaturing gradient gel electrophoresis assay was developed to overcome the problem of distinguishing between metabolically active and inactive cells in a biomining environment. The basis of the assay is the electrophoretic separation of 5S rRNA species extracted from the biomass, and identification of microbes based on the unique migration behaviour of each 5S rRNA species (Stoner *et al.*, 1996). This assay, however, is also compromised by its reliance on known community members as assay controls. Despite this being a non-selective direct molecular method for the characterization and mor itoring of the predominant, metabolically active members of the bioreactor microbial consortium De Wulf-Durand *et al.* (1997) considered this method to be too insensitive and laborious due to the complicated experimental procedure.

The application of polymerase chain reaction (PCR) techniques for the analysis of rDNA sequences has revolutionized the study of microbial ecology and presented potential solutions to some of the problems described above. The amplification by PCR of 16S rRNA genes from microbial communities is an alternative method to investigate the species present in a consortium. This provides a means to determine the number of different populations and the degree of dominance of individual populations (Polz and Cavanaugh, 1997), but is also subject to certain inaccuracies.

Although PCR provides a means of exploring the microbial diversity in a consortium it cannot yield reliable quantitative information on the relative dominance of populations (Polz and Cavanaugh, 1997). This is attributed to the inadequately understood influence of PCR selection. PCR selection refers to the potential for preferential amplification of certain sequences during multi-template PCR. PCR selection is considered the major force driving unequal amplification of templates. It results in altered template to product ratios which affects the quantitative nature of PCR. PCR selection describes all mechanisms that favour the amplification of certain templates in multi-template samples due to intrinsic properties of the genes targeted, their flanking sequences, or of their overall genome. Important contributors to PCR selection include preferential template denaturation due to low G+C content, different binding energies of degenerate primers, differential accessibility of rRNA genes within genomes, and bias associated with variations in 16S rRNA gene copy numbers across species. Another factor that must be considered is the lysis efficacy of all bacteria in the consortium during extraction of DNA for PCR (Rawlings, 1995; Polz and Cavanaugh, 1998).

Various approaches using PCR have been considered for elucidating population diversity. An investigation into selective pressure generated by different growth conditions identified *T. thiooxidans* and *L. ferrooxidans* as predominant species in low ferrous iron copper concentrates by selective amplification of the spacer region between the 16S and 23S genes. Identification of the bacteria was achieved by electrophoretic separation of

the amplified spacer regions, which on the basis of their unique and different sizes made the discrimination of species possible (Pizarro *et al.*, 1996).

Another approach to the detection of specific bacteria in environmental samples is by the construction of species-specific primers. What complicates the construction of species-specific primers for chemolithoautotrophic bacteria is the genetic diversity of certain species. For instance, *T. ferrooxidans* is genomically very diverse and comprises 5 DNA homology groups, while *T. thiooxidans* comprises 2 DNA homology groups (Harrison, 1982). This factor must be considered and primers designed that are specific to a phylotype group. Such considerations allowed for the possible detection of six groups of chemolithotrophs responsible for the biooxidation of chalcopyrite ore at very low target DNA concentrations (De Wulf-Durand *et al.*, 1997). This PCR based method was not designed to detect as yet unidentified bacteria in the biomining environment.

A novel method, however, attempts to overcome the problem associated with PCR quantification of uncultured microorganisms by using PCR-generated templates for the *in vitro* transcription of 16S rRNA. These transcribed templates serve as standards in quantitative probing of uncultured microorganisms (Polz and Cavanaugh, 1997). Although, this technique does not require pure cultures of the microbe, it does require the 16S rRNA gene of the unidentified bacterium to be cloned. Therefore, this technique is also indirectly subject to problems associated with PCR selection. This method may be quantitative for particular species in a population, but may fail to account for all members of a community.

Thus, the use of the PCR for the isolation of unidentified bacteria and enumeration of these bacteria in an environmental sample has not completely overcome problems experienced with other techniques. The PCR also suffers from the inability to distinguish between viable and non-viable cells and is prone to limitations. Such limitations include the inhibition of various commercially available *Taq* DNA polymerases by environmental components found in biological samples (Abu Al-Soud and Rådström, 1998).

In order to determine the predominant species present in the biooxidation pilot plant (UCT) the protocol established by Rawlings (1995) was used. This protocol involves the restriction enzyme profiling of PCR amplified 16S rDNA for the rapid identification of chemolithotrophic bacteria in bioreactors. In this protocol bacterial 16S rRNA gene primers amplify the 16S rDNA from total DNA extracted from the bioreactor biomass. The amplified 16S rDNA is then systematically digested with a range of restriction enzymes that results in 16S rDNA fragment profiles in agarose gels that can be correlated to known profiles of representative bacteria. In this way the identification of the apparently predominant bacteria can be made. As each restriction enzyme generates a unique 16S rDNA fragment profile for each known bacterium, an unrecognized profile will immediately signal the possibility of a previously unidentified bacterium. However, this PCR technique is almost certainly not quantitative due to problems associated with multi-template PCR selection. Although the amount of PCR product cannot reliably be used to estimate the number of bacteria in a sample, analysis of the PCR product using restriction enzymes reflects the bacterial diversity in a sample (Rawlings, 1995).

In conclusion, Pizarro *et al.* (1996) argued that it is important to identify and characterize bacteria involved in biomining since some properties in a few popular laboratory strains are extrapolated for the entire microbial consortium and considered for process design.

The aim of the presented work in this chapter is two-fold. Firstly, an attempt was made at identifying bacteria in the biooxidation pilot plant (UCT) using the restriction enzyme based PCR product profiling technique of Rawlings (1995). Secondly, to determine the level of rhodanese activity of the newly identified *T. caldus* strain since it was possibly the major rhodanese producer in the biooxidation pilot plant (UCT).

3.2) Materials and Methods

3.2.1) Preparation of chromosomal DNA: The biomass was harvested by centrifugation. The cells were washed in acid water pH 1.8, and resuspended in 560µl TE (0.01M Tris, 0.001M EDTA)-0.15M NaCl pH 7.6 buffer. After heating the cells to 70°C for 15 minutes (Pizarro *et al.*, 1996), 15µl of a 20mg.ml^{-1} solution of Proteinase K and 30µl of 10% SDS was added to the sample. The sample was incubated at 42°C for 30 minutes until cell lysis was complete. The DNA was precipitated using 10% (v/v) 3M KAc pH 5.2 and absolute ethanol. It was washed with 70% ethanol, air-dried and resuspended in TE buffer pH 7.6.

3.2.2) DNA manipulations: The standard methods of Sambrook *et al.* (1989) and Ausubel *et al.* (1993) were used for restriction enzyme digests, agarose gel electrophoresis, purification of DNA fragments from agarose gels by electroelution, and ligations. All restriction enzymes and their buffers were acquired from Boehringer Mannheim. The restriction enzymes and other enzymes used for DNA manipulations were used according to manufacturer's specifications. The bacterium *E. coli* JM109 was made competent using the standard rubidium chloride method of Sambrook *et al.* (1989).

3.2.3) Polymerase chain reaction protocol: The PCR was carried out in 50µl total volume using the primers and a modified protocol of Rawlings (1995). Total 16S rDNA was amplified from the sample in a Biometra® Personal Cycler using 1µl genomic DNA, 0.25µM of each primer, 2.5mM $MgCl_2$, 0.25mM dNTPs and 1 unit Redhot polymerase (Advanced Biotechnologies). After initial denaturation for 60s at 94°C, 25 cycles of amplification were carried out as follows: 30s at 94°C, 30s at 52°C, 90s at 72°C. A final elongation step of 120s at 72°C followed by a cooling step for 60s at 25°C ended the reaction. The 16S rDNA primers, designed from bacterial 16S rRNA gene conserved regions (bold), are given below. Primer fDD2 contains the restriction enzyme sites *Bam*HI and *Sal*I, and primer rPP2 contains the sites *Hin*dIII and *Xba*I (Italics) upstream of the 16S rRNA conserved sequence to facilitate cloning (Rawlings, 1995).

primer fDD2 5' CC *GGATCCGTCGAC***AGAGTTTGATCITGGCTCAG** 3' 34-mer
*Bam*HI *Sal*I

primer rPP2 5' CC *AAGCTTCTAGA***CGGITACCTTGTTACGACTT** 3' 33-mer
*Hin*dIII *Xba*I

PCR restriction enzyme analysis for the identification of species, as described by Rawlings (1995), was used to determine the dominant species in the bioreactors. Restriction enzyme maps of the strains considered are shown diagrammatically (Fig 3.3) and simulated 16S rDNA restriction enzyme analyses, to determine fragment numbers and sizes, is presented in Table 3.1. Simulated restriction enzyme digests and restriction enzyme maps were constructed using the DNAMAN version 3.0 (Lynnon BioSoft) software.

3.2.4) Isolation of *T. caldus* MNG: The bacterium *T. caldus* MNG was isolated in our laboratory (Thiobacillus Research Unit) by Dr Deane. The liquor fraction from the biooxidation pilot plant (UCT) was plated onto solid FeSo medium (Johnson, 1995) and the plates incubated at 37°C until colony formation. Liquid culture was in 10mM tetrathionate media pH 2.5 (Appendix A[i]).

3.2.5) Cloning of *T. caldus* MNG 16S rRNA gene: The 1.5kb 16S rDNA fragment amplified from the *T. caldus* MNG isolate was cloned into the vector pBluescriptSK at the *Sal*I and *Hin*dIII restriction enzyme sites. Recombinant plasmids were generated by subcloning to facilitate sequencing of the 16S rDNA insert. All recombinant plasmids were transformed into *E. coli* JM109 and grown on Luria-Bertani medium containing 100μg.ml^{-1} ampicillin and 2μg.ml^{-1} X-gal (Appendix A) for selection of transformants. Recombinant plasmids were purified using the High Pure Plasmid Isolation Kit (Boehringer Mannheim). Strains and vectors used in this study are described in Appendix B.

3.2.6) Nucleotide sequencing of *T. caldus* MNG 16S rRNA gene: The 16S rDNA was sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977), using the thermosequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia

Biotech UK Ltd.). The Cy5 end-labeled primers used for sequencing were obtained from the Synthetic DNA Laboratory, Department of Biochemistry (University of Cape Town). Sequencing reactions were run on an ALFexpress automated DNA sequencer (Pharmacia Biotech, Uppsala, Sweden). As sequencing was used for identification purposes, and not for direct comparison to other *T. caldus* 16S rDNA sequences, the 16S rDNA was cloned and sequenced to completion only once in both directions. The Genetics Computer Group Inc. Winconsin Package version 9.1 (GCG, Madison Wis.) sequence analysis software was used for sequence assembly and analysis. The 16S rRNA gene sequence was submitted to the Ribosomal Database Project (RDP) for comparison with other bacterial 16S rDNA sequences. The result returned (Fig 3.7) was assembled using the Aladdin Ghostscript version 5.1 graphical interface software. For sequence comparison, the GeneDoc version 2.3 multiple sequence alignment software was used to produce Appendix D[ii].

3.2.7) Substrate range of *T. caldus* MNG rhodanese: The substrate range of *T. caldus* MNG rhodanese was determined using assays (section 2.2.3) in which the thiosulfate substrate was substituted with the sulfur substrate in question. Sulfur substrates investigated include: SO_3^{2-}, SO_4^{2-}, $S_2O_5^{2-}$, and $S_4O_6^{2-}$. All sulfur substrates for this investigation were made as stocks of 100mM $S_nO_x^{2-}$ + 20% NaCl, except $S_4O_6^{2-}$ which was made to 1mM.

3.2.8) *T. caldus* MNG growth kinetics and rhodanese activity during batch growth: The *T. caldus* MNG batch culture growth rate was determined using a 3 litre jacketed Z61103CT04 Applikon® autoclavable bioreactor (Fig 3.1). The bioreactor had a working volume of 2 litres and three baffles orientated 120° to one another. The head plate contained a number of ports which allowed for media injection, and continuous pH (Mettler Toledo Combination pH Electrode) and temperature monitoring. A Grant Y6 constant temperature bath was used to maintain the temperature in the bioreactor at 37°C by circulating water through the bioreactor jacket. Temperature and pH were monitored by an Applikon® ADI 1030 Bio Controller. A pitched (45°) six-bladed turbine impeller rotating at 400rpm was used for mixing and gas dispersion. The impeller was located

2cm from the base of the bioreactor and driven by an Applikon® P100 motor and an Applikon® ADI 1032 stand alone speed controller. A Peak Scientific OAG2000DA Oilless Air Generator supplied the inlet gas, and the flow rate of 315ml.min^{-1} was controlled by a Brooks Model 5850S Mass Flow Controller and a Brooks Model 0154 Microprocessor Control and Read Out Unit. The air entered the bioreactor through an air-inlet pipe located under the impeller. The off-gas was dried using a reflux condenser containing an ethylene:glycol (75%:25%) coolant chilled to 6°C by a Grant LTD6G low temperature bath. Prior to entering the gas analyzers the off-gas was passed through a cloth filter and a Hartmann & Braun CGEK Sample Gas Conditioner fitted with a CGKA 1 Automatic Condensate Outlet. Off-gas CO_2 concentration from the bioreactor was determined using a Hartmann & Braun Uras 4 NDIR (non-dispersive infrared) Industrial Photometer. The off-gas O_2 concentration was determined using a Hartmann & Braun Magnos 6 G Oxygen Analyzer.

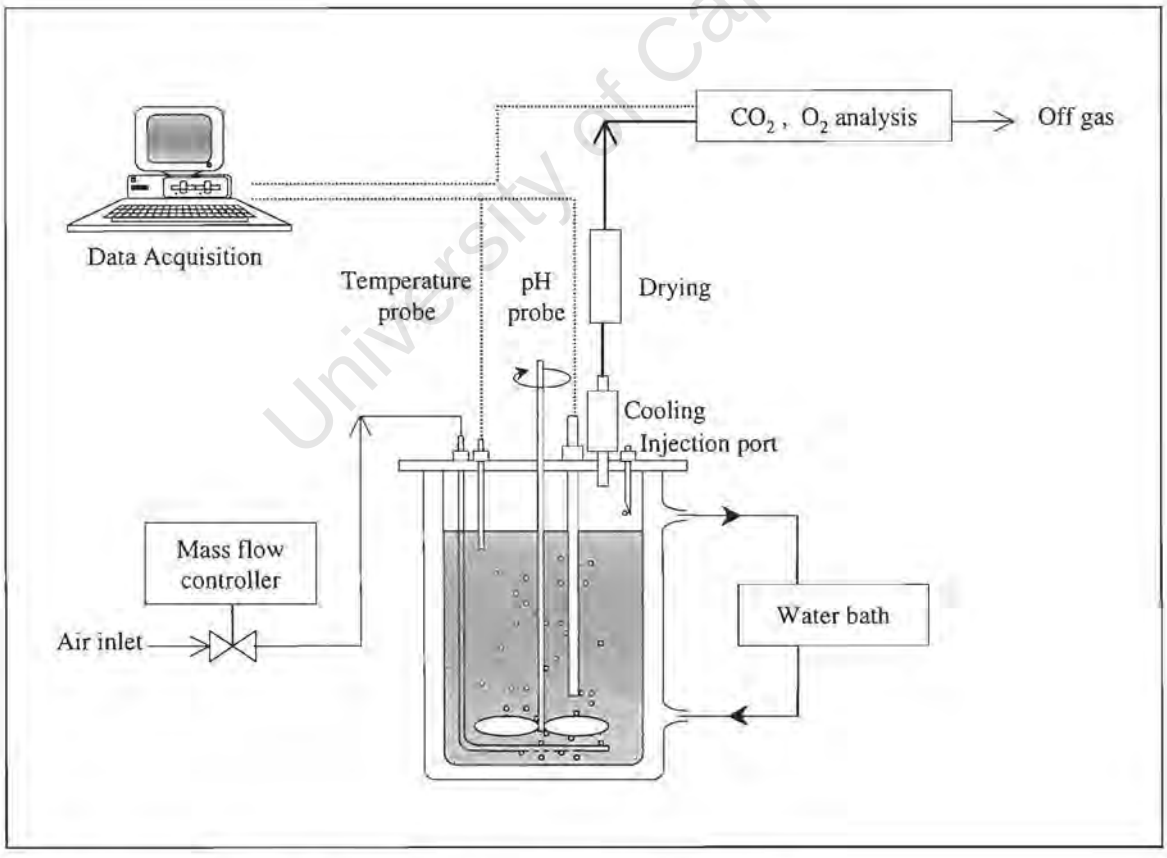


Fig 3.1: Schematic of 3 litre jacketed Applikon® autoclavable bioreactor
[Adapted with permission from Breed *et al.* (1998)]

Calculations for CO_2 and O_2 utilization rates are found in Appendix E. The CO_2 and O_2 off-gas concentrations and the inlet air were alternately logged onto computer. The sampling period for the bioreactor was set at six minutes. The switching and data logging hardware and software used was both designed, built, and written by the Department of Chemical Engineering (University of Cape Town).

To ensure sterility, the bioreactor was filled with the make-up volume of double distilled water (Milli-Q^{TM}), sealed, the pH probe attached, and autoclaved. Once the bioreactor had cooled the 10mM tetrathionate media (Appendix A[i]) was injected through the injection port. All media entering the bioreactor was passed through a 0.22µm membrane filter (Micron Separations Inc.). After the bioreactor had reached the growth temperature of 37°C and the required gas flow rate had been achieved the pH of the bioreactor was adjusted to pH 2.5 by injection of conc. H_2SO_4 (no further pH adjustment was made during an experiment). *T. caldus* MNG (15ml) was inoculated into the media through the injection port, and data logging initiated.

Due to the low biomass, the bioreactor was stopped at various bacterial growth stages, as determined by mmolC.l^{-1} analysis, and the entire biomass harvested (section 2.2.2) for a rhodanese assay. Rhodanese assays of *T. caldus* MNG during batch growth were as described in section 2.2.3.

3.3) Results

To determine whether shifting species dominance in the biooxidation pilot plant (UCT) caused by the oxidation state of the concentrate could have influenced the deviation between biooxidation pilot plant (UCT) rhodanese assays, a ten day batch culture was initiated in an attempt to observe shifting species dominance during prolonged concentrate biooxidation.

3.3.1) Detection of a *T. caldus*-like bacterium in the biooxidation pilot plant (UCT)

Using the substrate described in section 2.2.1, and an inoculum from previous biooxidation experiments, a ten day batch culture was initiated in the biooxidation pilot plant (UCT). A batch culture of only ten days was possible as an extended period resulted in bioreactor foaming. Samples were withdrawn from the bioreactor every 48 hours, the bacteria harvested from the liquor fraction, and restriction enzyme analysis performed on the PCR amplified total 16S rDNA obtained. It was not possible to amplify directly from the concentrate due to the inability to remove the bacteria from the ore particles, and/or interfering non-biological compounds. The method of García and Jerez (1995), who demonstrated release of ore-bound *T. ferrooxidans* using the non-ionic detergent Triton-X100, was unsuccessful. As the bacteria from the biooxidation pilot plant (UCT) were harvested only from the liquor fraction for rhodanese assays, it was the microbial composition of the liquor fraction that was of most interest.

In the Rawlings (1995) protocol the restriction enzymes *Eco*RI, *Stu*I, and *Eco*RV were used to generate unique fragment profiles to identify *T. ferrooxidans*, *T. thiooxidans*, and *L. ferrooxidans* from a mixed culture. Initially, it was thought that over the ten day batch culture the predominant bacterium was *T. thiooxidans* (Fig 3.2) as comparison of the culture samples with the control 16S rDNA profiles gave *Eco*RI and *Stu*I profiles identical to that of *T. thiooxidans*. However, the emerging importance of *T. caldus* and the suggestion that this bacterium has been underestimated in biooxidation bioreactors (P.

Norris per. comm.) prompted further investigation to confirm that the bacterium thought to be *T. thiooxidans* was indeed *T. thiooxidans*.

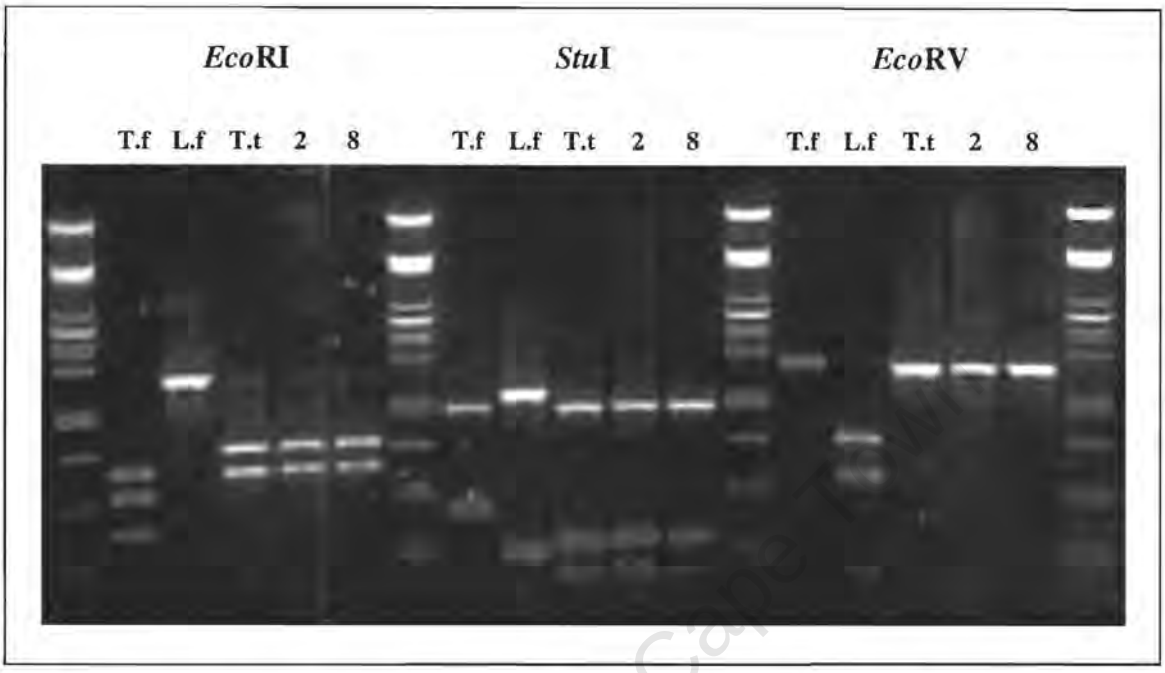


Fig 3.2: Restriction enzyme digestion profiles of PCR amplified 16S rDNA digested with *Eco*RI, *Stu*I and *Eco*RV from samples harvested at 48 and 192 hours (samples **2** and **8**) from the bioreactor. The restriction enzyme profiles of the controls *T. ferrooxidans* ATCC 33020 (**T.f**), *L. ferrooxidans* DSM 2705 (**L.f**) and *T. thiooxidans* ATCC 19377 (**T.t**) are present in all restriction enzyme groupings. *Pst*I–digested Lambda marker is included in each group.

There is currently only one *T. caldus* 16S rRNA gene sequence lodged in the NCBI database; that of *T. caldus* DSM 8584 (Hallberg and Lindström, 1994). Analysis of this 16S rRNA gene sequence against the *T. ferrooxidans*, *T. thiooxidans*, and *L. ferrooxidans* sequences revealed two possible restriction enzymes that would generate unique fragment profiles for *T. caldus*. The restriction enzymes are *Bam*HI and *Nar*I. Although *Nar*I is inherently a poor DNA digesting enzyme it was included as a second diagnostic restriction enzyme for *T. caldus*. It has subsequently been established that the 16S rDNA of a strain classified as *T. thiooxidans* (Accession no.: X72851) physiological group II, and which contains a *Bam*HI and a *Nar*I site, is in fact a strain of *T. caldus*. The *Bam*HI and *Nar*I sites are absent from the *T. thiooxidans* DSM 612 sequence (Accession no.: M79401) and the 16S rRNA genes of *T. thiooxidans* ATCC 19377, DSM 504, and TI (Rawlings, 1995). Thus, with this range of restriction enzymes unique 16S rDNA

profiles could be generated for *T. ferrooxidans*, *T. thiooxidans*, *L. ferrooxidans*, and *T. caldus* (Fig 3.3 and Table 3.1).

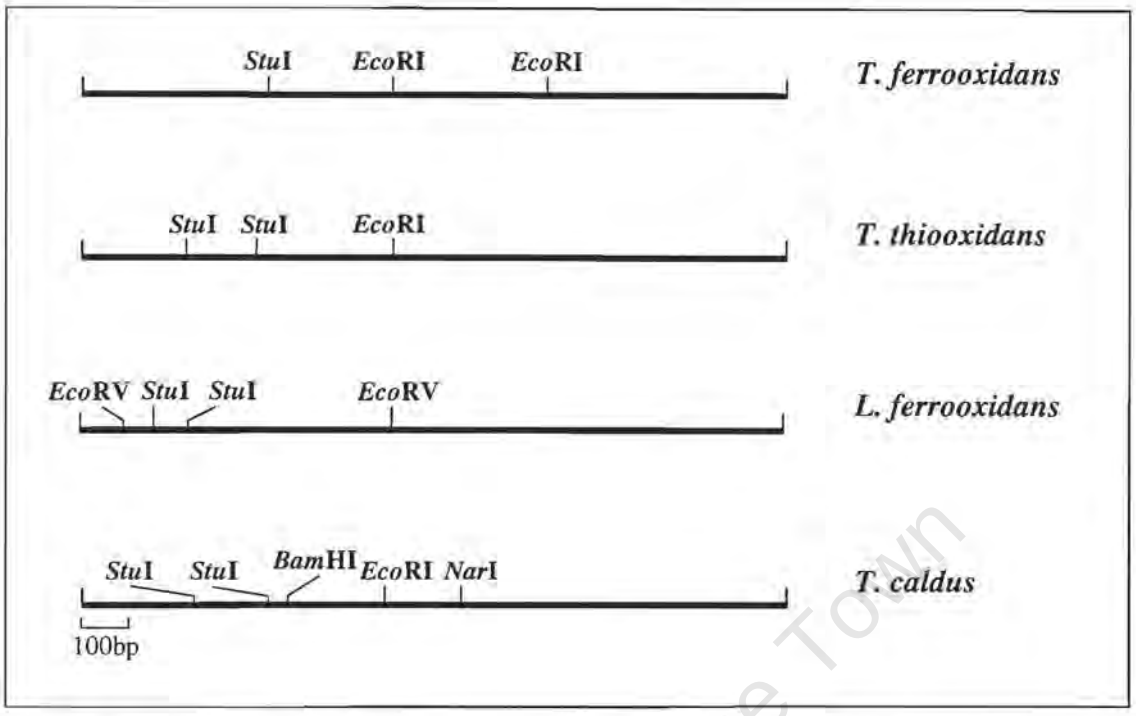


Fig 3.3: Restriction enzymes used to create unique 16S rDNA profiles for the strains considered. Restriction enzyme maps were generated using published sequences in the NCBI database.

Table 3.1: 16S rDNA fragment sizes (bp) as determined for each strain by computer simulation using available NCBI database sequences. The number of fragments (**Frag No.**) generated and the size of these simulated fragments (**Size**) in base pairs following restriction enzyme digestion of a particular strain's PCR amplified 16S rDNA is presented.

	T. ferrooxidans		*T. thiooxidans*		*L. ferrooxidans*		*T. caldus*	
Enzyme	Frag. No.	Size (bp)	Frag. No.	Size (bp)	Frag. No.	Size (bp)	Frag. No.	Size (bp)
*Eco*RI	3	329 497 644	2	634 838	0		2	641 819
*Stu*I	2	387 1083	3	151 226 1095	3	65 164 1250	3	151 233 1076
*Eco*RV	0		0		3	96 572 811	0	
*Bam*HI	0		0		0		2	425 1035
*Nar*I	0		0		0		2	656 804

PCR amplified total 16S rDNA products obtained from cells harvested 48 and 192 hours from the bioreactor were digested with this range of restriction enzymes to tentatively determine whether the initial *T. thiooxidans* identification was in fact *T. caldus*. According to the computer simulated profiles (Table 3.1) and the fragment profiles obtained for these two samples, it appeared that the amplified 16S rDNA was that of a *T. caldus*-like bacterium (Fig 3.4).

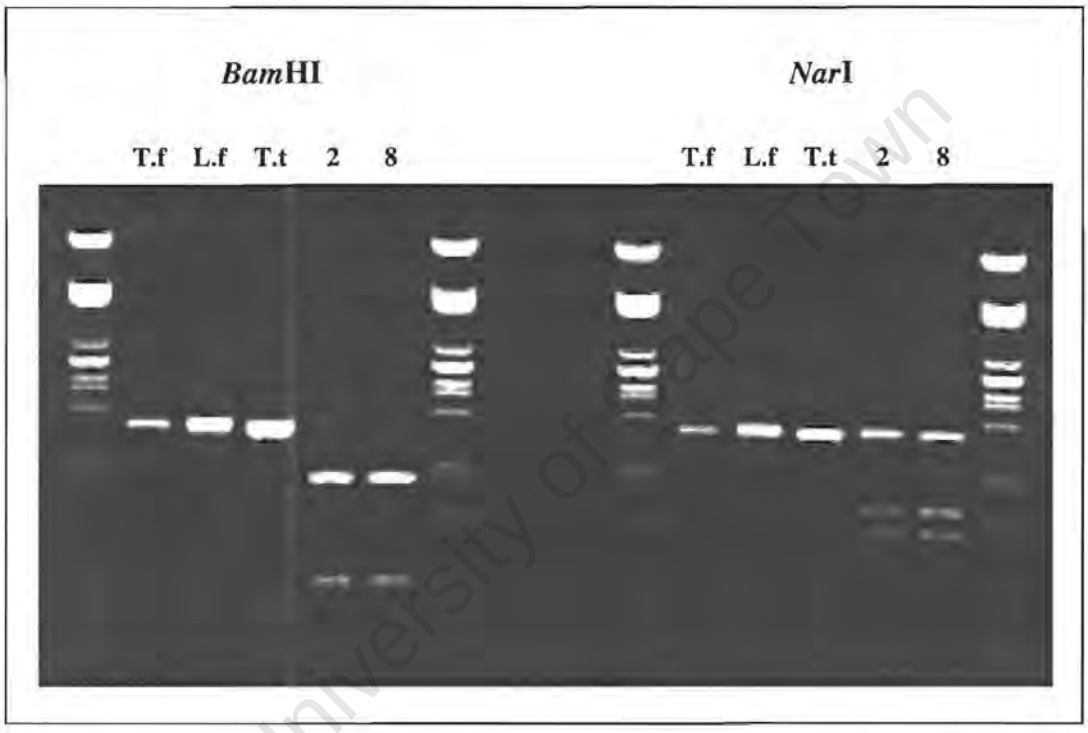


Fig 3.4: Restriction enzyme digestion profiles of the 16S rDNA amplified from samples harvested at 48 and 192 hours (samples **2** and **8**) generated by digestion with *Bam*HI and *Nar*I. The restriction enzyme profiles of the controls *T. ferrooxidans* ATCC 33020 (**T.f**), *T. thiooxidans* ATCC 19377 (**T.t**) and *L. ferrooxidans* DSM 2705 (**L.f**) are present in both restriction enzyme groupings. *Pst*I-digested Lambda marker is included in each group.

The same five PCR amplified 16S rDNA samples from the ten day batch culture were digested with the five diagnostic restriction enzymes. The digestion profiles generated from total 16S rDNA PCR amplified from samples harvested every 48 hours indicated the apparent predominance of a *T. caldus*-like bacterium throughout the batch culture period. In Fig 3.5 only the digestion profiles obtained after digestion with *Stu*I and *Nar*I are given to demonstrate the similarity between a *Stu*I profile of *T. thiooxidans* and *T.*

caldus, and that digestion of the same PCR product with *Nar*I (or *Bam*HI, data not shown) can be used to differentiate between these two species.

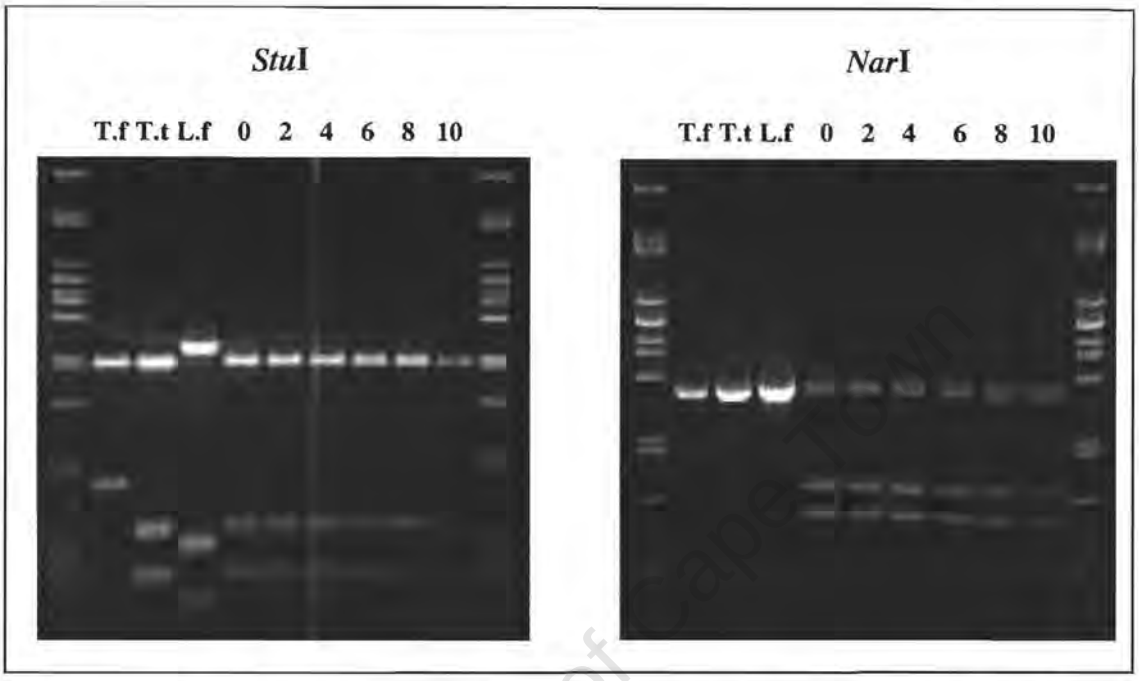


Fig 3.5: *Stu*I and *Nar*I restriction enzyme digestion profiles of PCR amplified total 16S rDNA from the biooxidation pilot plant (UCT). Samples were prepared every 48 hours over a ten day batch culture (lanes **0-10**). The 16S rDNA profiles of *T. ferrooxidans* ATCC 33020 (**T.f**), *T. thiooxidans* ATCC 19377 (**T.t**), and *L. ferrooxidans* DSM 2705 (**L.f**) are included for identification purposes. *Pst*I-digested Lambda marker is included in each group.

It was unusual not to have detected the iron-oxidizer *L. ferrooxidans* in the biooxidation pilot plant (UCT) (Fig 3.5) as during the establishment of the PCR protocol this bacterium was commonly identified in the leach liquor (see Appendix D[i]). Possible reasons why *L. ferrooxidans* was not detected during the ten day batch culture are discussed in section 3.4.

3.3.2) Identification of a *T. caldus* strain from the biooxidation pilot plant (UCT) by 16S rRNA gene sequencing and analysis

The predominant bacterium in the biooxidation pilot plant (UCT) leach liquor was isolated by Dr Deane (Fig 3.6). The 16S rRNA gene was amplified from this isolate,

cloned, and sequenced. The isolate showed a 99.5% nucleotide similarity (Table 3.2) to *T. caldus* DSM 8584 and seven changes in the nucleotide sequence of this bacterium's 16S rRNA gene were identified (Appendix D[ii]).

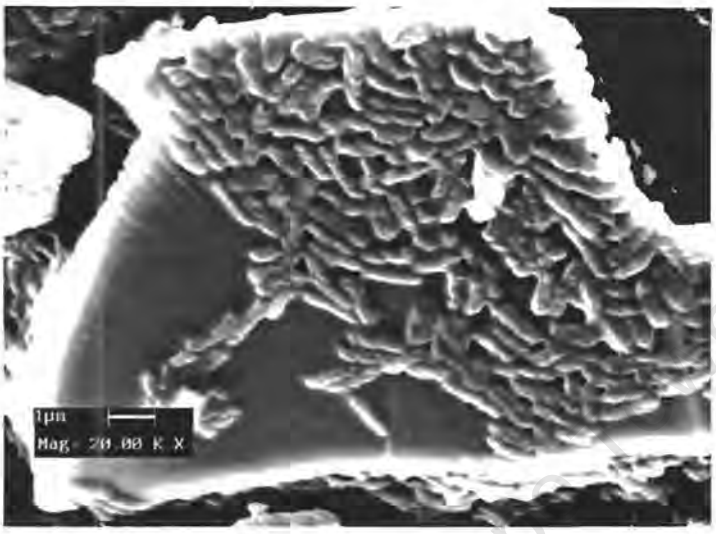


Fig 3.6: SEM of *T. caldus* MNG.

Table 3.2: FASTA of the *T. caldus* MNG 16S rDNA sequence to determine percentage (%) identity to 16S rDNA sequences lodged in the NCBI database.

NCBI sequence description	% Identity	Accession Number
T. caldus DSM 8584	99.5	Z29975
Thiobacillus strain C-SH12 (now recognised as *T. caldus*)	99.7	X72851
T. ferrooxidans DSM 9465	95.4	Y11595
T. thiooxidans ATCC 19377	95.3	Y11596
T. ferrooxidans N-Fe4 (Partial)	95.4	X75267
T. ferrooxidans N-Fe3 (Partial)	95.5	X75268
T. ferrooxidans N-B3 (Partial)	95.1	X75269
T. ferrooxidans N-Fe2 (Partial)	95.2	X75266
T. thiooxidans DSM 612	93.4	M79401

The phylogenetic relationship of the new isolate, designated *T. caldus* MNG, to other bacteria based on 16S rRNA gene sequences is shown in Fig 3.7.

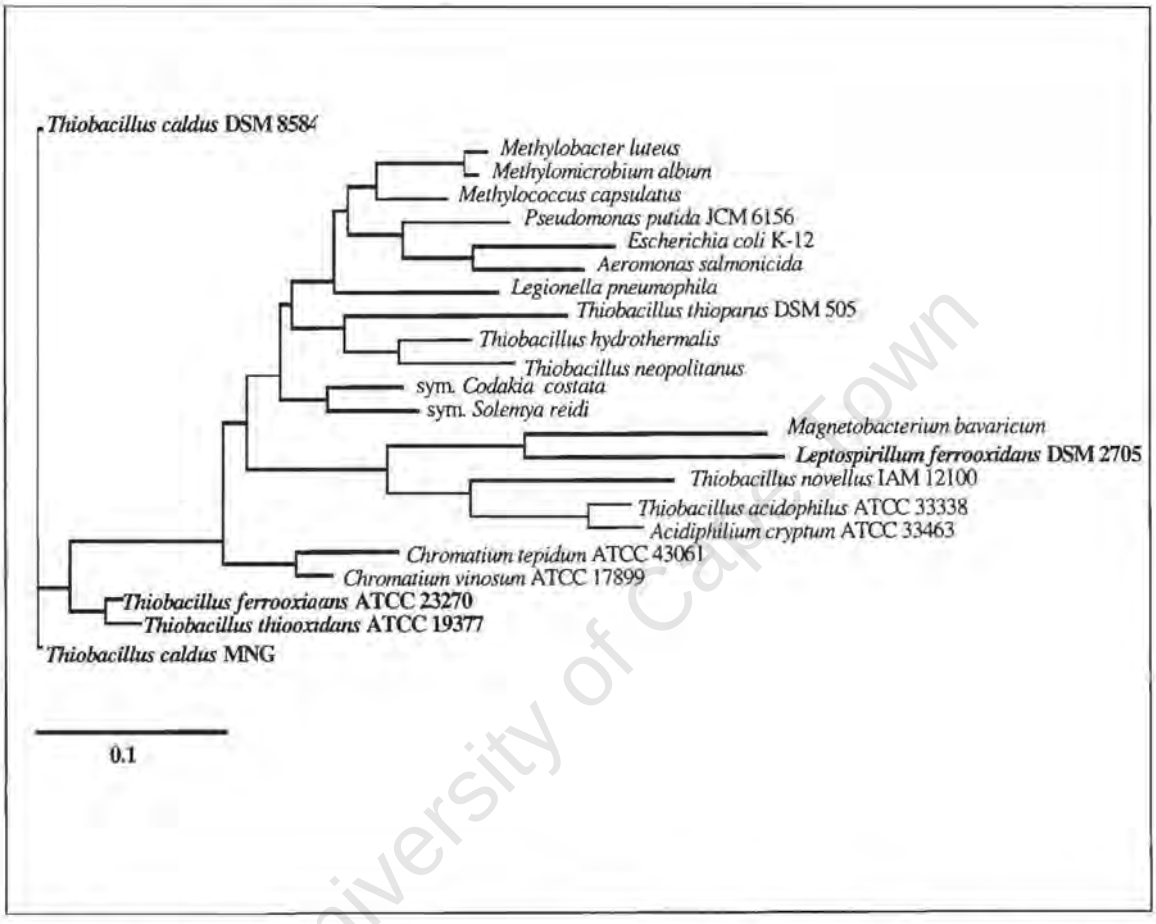


Fig 3.7: Phylogenetic relationship of *T. caldus* MNG to the thiobacilli and related species. The scale of 0.1 represents a 10% difference in the nucleotide sequence.

3.3.3) *T. caldus* MNG rhodanese activity and rhodanese substrate range

Having isolated a *T. caldus* strain from the biooxidation pilot plant (UCT) and noted its predominance it was of interest to determine whether this bacterium demonstrated rhodanese activity (Fig 3.8).

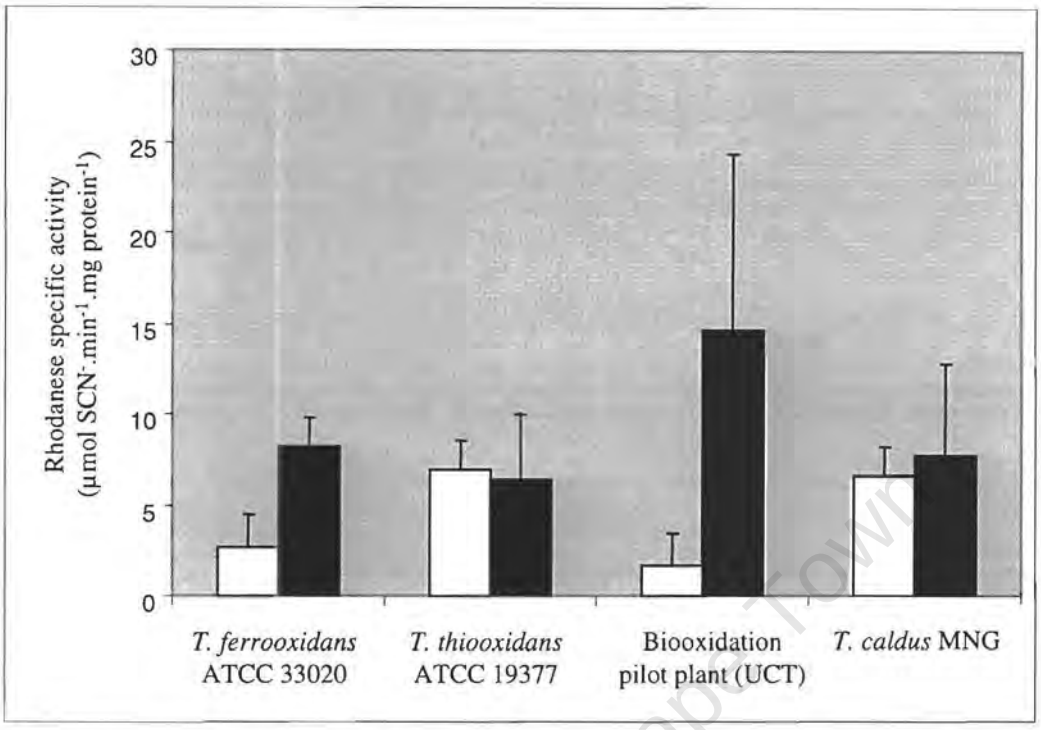


Fig 3.8: Rhodanese specific activity of *T. caldus* MNG and comparison to pure culture rhodanese activities determined previously. Error bars represent the mean standard deviation between (□) whole cell and (■) crude enzyme extract assays.

As *T. caldus* MNG demonstrated the ability to convert cyanide to thiocyanate, and because of its predominance throughout batch culture in the biooxidation pilot plant (UCT), the rhodanese substrate range of this bacterium was investigated (Table 3.3).

Table 3.3: *T. caldus* MNG rhodanese activity as determined using a range of sulfur substrates.

Sulfur substrate	µmol $SCN^-.min^{-1}$. mg $protein^{-1}$	
	Whole cell assay	Crude enzyme extract
SO_3^{2-}	0	0
SO_4^{2-}	0	0
$S_2O_3^{2-}$	6.66 ±1.58	7.73 ±5.09
$S_2O_5^{2-}$	0	0
$S_4O_6^{2-}$	0	0

It was found that tetrathionate ($S_4O_6^{2-}$) reacted spontaneously with cyanide in the assay resulting in a molar concentration of thiocyanate outside the range of the assay. Therefore, the concentration of tetrathionate in the assay reaction mixture was lowered so that rhodanese activity might be detected above the spontaneous chemical reaction. Even when the assay concentration of tetrathionate was as low as 50μM, as opposed to the normal 5mM assay sulfur substrate concentration, no rhodanese activity could be detected above the spontaneous formation of thiocyanate (Table 3.3). The cyanolytic degradation of tetrathionate to form thiocyanate and sulfate is well characterized (Roy and Trudinger, 1970).

3.3.4) Effect of bacterial growth phase on rhodanese activity

The rhodanese specific activity of *T. caldus* MNG (Table 3.3) had the same large deviation between assays as observed for the sulfur-oxidizers in chapter two. Therefore, in an attempt to address whether the physiological state of the cell affected rhodanese activity *T. caldus* MNG was cultured in a 3 litre Applikon® bioreactor. A 3 litre bioreactor was required for growth phase experiments for two reasons. Firstly, the low biomass yield of the chemolithoautotrophic bacterium required that the entire bioreactor be harvested to determine rhodanese activities during early growth phases. Secondly, as optical density readings on such low biomass was unreliable (absorbance did not correlate with assays of total protein, data not shown), off-gas analysis was required to assist in estimating the growth phase of the culture.

The initial *T. caldus* MNG rhodanese activity result (Table 3.3) had been determined using shaking flask experiments. Due to available laboratory equipment limitations and the volume of culture media required for sufficient rhodanese assay biomass, the growth of *T. caldus* MNG in shaking flask experiments had been restricted to a temperature of 37°C. Consequently, it was decided that although *T. caldus* grows at an optimum growth temperature of 45°C (Hallberg and Lindström, 1994) the bioreactor experiments would be undertaken at 37°C to minimize growth condition changes between shaking flask and bioreactor experiments. It was hoped that a correlation between shaking flask and

bioreactor rhodanese activity might assist in explaining the large standard deviation between assays.

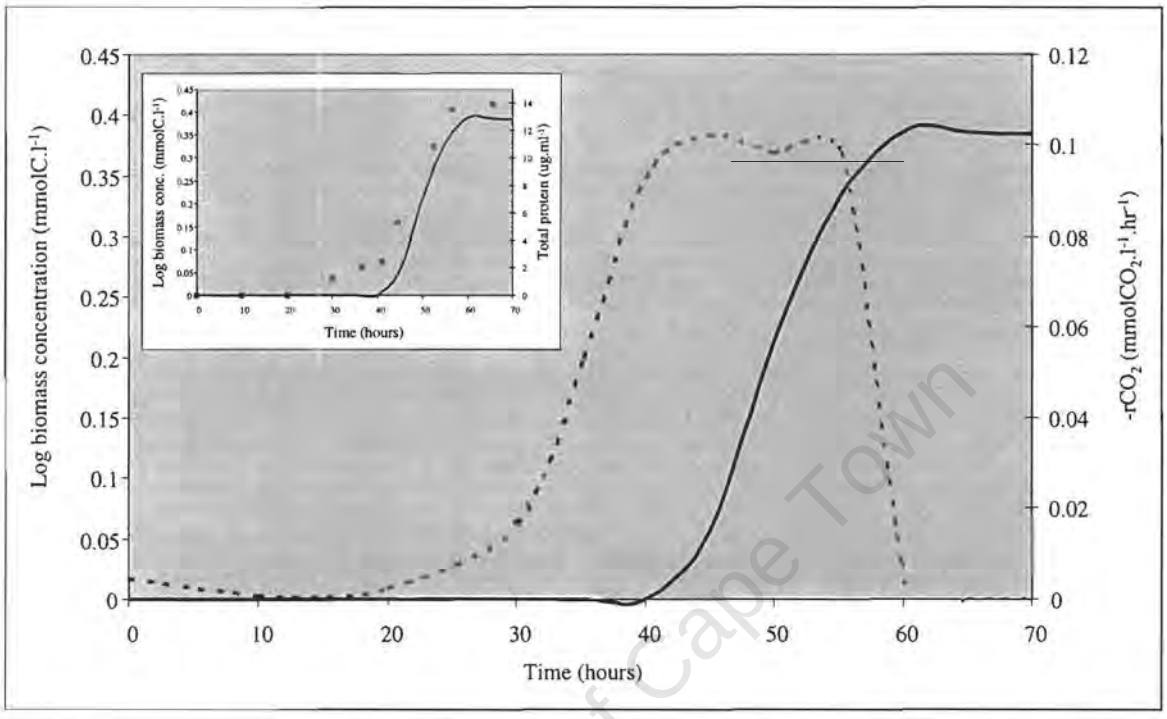


Fig 3.9: Biomass concentration (mmolC.l^{-1}; solid line) derived from off-gas analysis and CO_2 utilization rate (-rCO_2 mmolCO_2.l^{-1}.hr^{-1}; dotted line) during batch growth of *T. caldus* MNG. Insert graph shows the biomass concentration (mmolC.l^{-1}; solid line) and protein concentration (µg.ml^{-1}; points) versus time.

From the growth kinetic data (Fig 3.9) the generation time of *T. caldus* MNG could be determined for the particular growth conditions (described in section 3.2.8). A generation time of approximately 4 hours at 37°C, with no pH adjustment, was estimated which correlated well with the reported approximate 3.5 hour generation time at 37°C and pH 2.0 for *T. caldus* DSM 8584 reported by Hallberg and Lindström (1994). This value, along with off-gas analysis, assisted in estimating which growth phase *T. caldus* MNG had entered so that the biomass from different growth phases could be harvested for rhodanese assays.

It was noted from Fig 3.9 that CO_2 was limiting during bioreactor growth, and had this not been the case it is possible that the generation time could have been shortened. As

generation time is a reflection of the growth rate constant in exponential growth, a low growth rate will result in an extended generation time. CO_2 limitation is argued on the observation that the maximum carbon dioxide utilization rate was reached during early exponential growth (Fig 3.9). The exponential growth phase is the most metabolically active phase of the culture. Therefore, considering that the carbon dioxide utilization rate was unchanged during exponential growth, rather than increasing (as would be expected if CO_2 was available in excess), it appears that the *T. caldus* MNG growth rate was constrained by CO_2 limitation. As inlet gas entered the bioreactor at a constant rate of 315ml.min^{-1} throughout batch growth, the maximum growth rate for *T. caldus* MNG would be relative to the amount of available CO_2. Data from the oxygen consumption rate (see Appendix D[iii]) showed that the oxygen consumption rate increased for a further 12 hours after the carbon dioxide consumption rate had stabilized (presumably due to an increase in cell mass). This indicated that O_2 did not have as profound an effect on the growth rate as CO_2 limitation. As the culture entered stationary phase (Fig 3.9) the rate of carbon dioxide fixation stabilized which is reflected in the steady decrease in the carbon dioxide utilization rate to basal levels by 60 hours.

Having determined the growth kinetics under the particular growth conditions described above, batch cultures of *T. caldus* MNG were terminated during various growth phases and the cells harvested for rhodanese assays. Basal levels of rhodanese activity were detected prior to exponential growth (Fig 3.10), but it was only after 45 hours of culture growth in the bioreactor, when the culture had entered the exponential growth phase, that rhodanese activity began to increase. The greatest fluctuation in rhodanese activity was found when the culture was in early stationary phase (Fig 3.10). The variation in whole cell rhodanese activities during early stationary phase was not as pronounced as for crude enzyme extracts (see section 3.4). Prolonged growth in stationary phase resulted in both whole cell and crude enzyme extract rhodanese activities returning to basal levels (Fig 3.10).

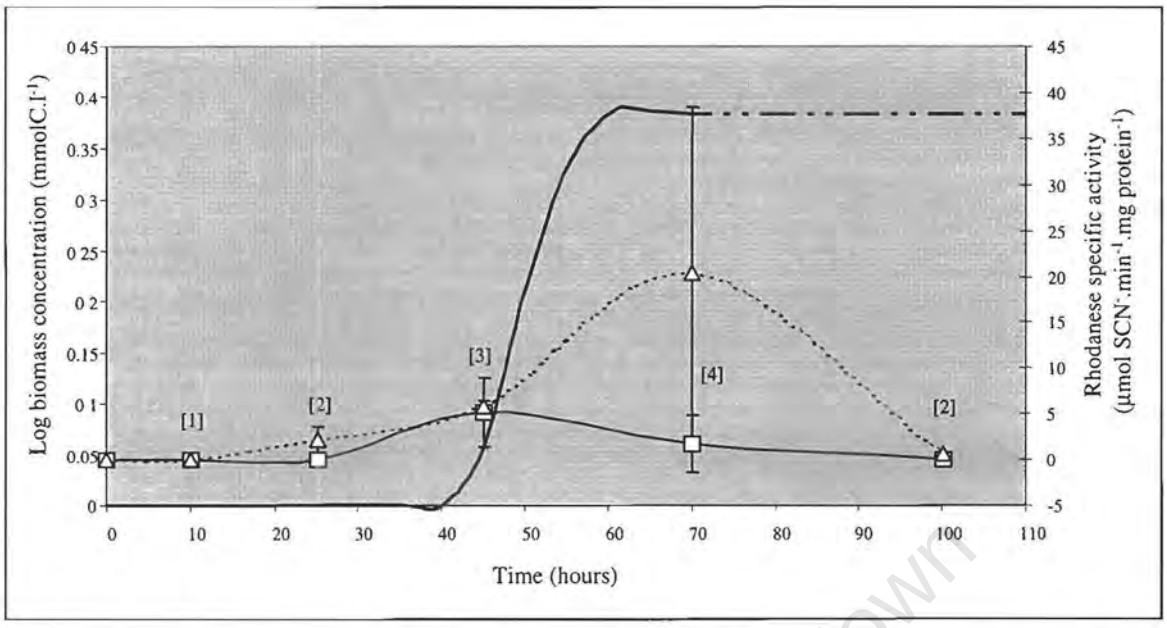


Fig 3.10: Composite diagram demonstrating the specific activity of rhodanese produced during different growth phases of *T. caldus* MNG in batch culture. The growth data are represented by biomass concentration (mmolC.l^{-1}; solid line), with both whole cell (□) and crude enzyme extract (Δ) rhodanese specific activities expressed in µmol SCN^-.min^{-1}.mg $protein^{-1}$. Dashed line for biomass concentration is an extrapolation of growth data allowing for the assays performed in late stationary phase to be represented. Numbers in square brackets represent the number of rhodanese assays at each point shown. Error bars represent the mean standard deviation between rhodanese assays.

3.4) Discussion

Using the restriction enzyme based 16S rDNA profiling technique described by Rawlings (1995) a *T. caldus* strain was identified as the predominant species in the leach liquor of a biooxidation pilot plant treating a pyrite-arsenopyrite concentrate. The identification was based on the finding that the restriction enzyme sites *Nar*I and *Bam*HI are unique sites for *T. caldus* (Fig 3.3 and Table 3.1). The complete digestion of the PCR amplified 16S rDNA product by *Bam*HI (Fig 3.4) showed that the PCR products obtained over the ten day biooxidation pilot plant (UCT) batch culture were not a heterologous mixture of templates and were composed entirely of *T. caldus* 16S rDNA. The restriction enzyme *Nar*I is recognised as a poor DNA digesting enzyme which would account for the undigested 1.5kb fragment in lanes 2 and 8 of Fig 3.4. Therefore, *Nar*I can only be used in a confirmatory capacity and not for determining the heterogeneity of a PCR 16S rDNA product. It was surprising that during the ten day biooxidation pilot plant (UCT) batch culture that *L. ferrooxidans* was not detected. Previous investigations had revealed that this iron-oxidizer was present in the leachate (see Appendix D[i]). Possibly unequal lysis of species during chromosomal DNA extraction and/or PCR selection factors resulting from a high template ratio could have resulted in *L. ferrooxidans* not being detected. These are recognised problems associated with this identification technique (Rawlings, 1995).

The sequence of the 16S rRNA gene of the *T. caldus* isolate that was consistently present during the ten day batch culture in the biooxidation pilot plant (UCT) was determined to establish that this bacterium was indeed a *T. caldus* strain. Sequence analysis revealed seven nucleotide changes (Appendix D[ii]) when compared to the published sequence of *T. caldus* DSM 8584. The *T. caldus* isolate showed a 99.5% nucleotide sequence identity to *T. caldus* DSM 8584 (Table 3.2). However, higher nucleotide identity to a sequence lodged in the databases as "*T. thiooxidans* gene for 16S ribosomal RNA" (Accession no. X72851) was found. Analysis of this *T. thiooxidans* physiological type II sequence (Goebel and Stackebrandt, 1994) has led to the proposal that it is the 16S rRNA gene sequence of a *T. caldus* strain. The *T. thiooxidans* physiological type II 16S rRNA gene

sequence has a 99.7% nucleotide sequence similarity to the *T. caldus* isolate (Table 3.2). A FASTA analysis of this *T. thiooxidans* physiological type II sequence against *T. caldus* DSM 8584 confirmed a 99.7% nucleotide similarity supporting the previously inaccurate designation of this sequence. The 16S rRNA gene for *T. thiooxidans* ATCC 19377 and *T. thiooxidans* DSM 612 showed sequence similarities of only 95.3% and 93.4%, respectively, to the *T. caldus* isolate (Table 3.2). Simulated computer restriction enzyme mapping of the 16S rRNA gene of this *T. thiooxidans* physiological type II strain revealed a *Bam*HI and *Nar*I site at the same base pair location (taking published sequence length into consideration) as found for *T. caldus* DSM 8584 (Fig 3.3). These restriction enzyme sites are not found in the 16S rRNA gene sequence of *T. thiooxidans* ATCC 19377, DSM 612, DSM 504 or TI. Having an explanation for the high sequence similarity to *T. thiooxidans* physiological type II and *T. caldus* DSM 8584, the biooxidation pilot plant (UCT) isolate was designated *T. caldus* MNG. Phylogenetic analysis of *T. caldus* MNG showed that after *T. caldus* DSM 8584 it was more closely related to *T. ferrooxidans* ATCC 23270 than *T. thiooxidans* ATCC 19377 (Fig 3.7).

Having confirmed the identification of this species found in the leach liquor, the pure culture of *T. caldus* MNG was assayed for rhodanese activity. No rhodanese activity for a *T. caldus* strain has been reported but considering the ubiquity of rhodanese in the thiobacilli (section 1.10) it was not surprising that *T. caldus* MNG demonstrated *in vitro* rhodanese activity (Fig 3.8). The substrate range of the *T. caldus* MNG rhodanese was investigated (Table 3.3) and it was found that within the sulfur substrates tested, this rhodanese behaved exclusively as a thiosulfate: cyanide sulfurtransferase. The specific activities determined for whole cell and crude enzyme extract *T. caldus* MNG rhodanese assays were found to be comparable with that of *T. thiooxidans* ATCC 19377 (Table 2.1 and Fig 3.8). However, as was experienced with previous rhodanese assays (section 2.3), the *T. caldus* MNG rhodanese assays also demonstrated fluctuations between assays, although the standard deviations within replicates of the same sample were much lower.

As *T. caldus* MNG appeared to be the dominant sulfur-oxidizer in the biooxidation bioreactor, and could be cultured in a shorter time period than the other pure cultures

investigated, it was chosen for growth related rhodanese assays. It was hoped that growth related rhodanese assays would indicate whether the physiological state of the cell affected rhodanese activity. Rhodanese specific activities for both whole cell and crude enzyme extracts of *T. caldus* MNG were first detected in late lag phase, but this may be attributed to the low biomass yield (Fig 3.10). The accumulation of reactive sulfur species during stationary phase growth may have progressively influenced the quantitative sensitivity of the rhodanese assay. Spontaneous chemical formation of thiocyanate catalyzed by these reactive sulfur species could have affected the whole cell assays (see section 2.4), as after exponential growth these activity levels began to decrease. This spontaneous chemical formation of thiocyanate may also have contributed to the very high standard deviation between crude enzyme extract assays observed in early stationary phase. Boiled sample control levels of spontaneous thiocyanate formation for stationary phase *E. coli* BMH 71-18 rhodanese assays (data not shown) were in the order of 1.5-fold and 19.4-fold lower for crude enzyme extract and whole cell assays, respectively, than for stationary phase (70 hour, Fig 3.10) *T. caldus* MNG rhodanese assays. This suggests that accumulated reactive sulfur species affected *T. caldus* MNG rhodanese assays as the culture aged. Both whole cell and crude enzyme extract rhodanese activity decreased to basal levels as the culture aged during prolonged growth in stationary phase (Fig 3.10). Thus, if the shaking flask experiments to determine rhodanese activity for the various pure cultures used in this study were not harvested at a similar stage of the growth cycle, this may have contributed to the large variation observed in rhodanese activity for the pure cultures. In other words, indiscriminate timing for the harvesting of pure cultures for rhodanese assays contributed to the high standard deviation between assays, as rhodanese activity was strongly influenced by the physiological state of the culture.

A further observation from the *T. caldus* MNG bioreactor rhodanese assays was the high mean specific activity of the crude enzyme extract at 70 hours. Although a large standard deviation between assays demonstrated fluctuations in rhodanese activity during stationary phase growth (Fig 3.10), a mean crude enzyme extract specific activity of 20.31 (±18.23) μmol $SCN^-.min^{-1}.mg\ protein^{-1}$ was calculated. This mean value was 5

units higher than that determined for the biooxidation pilot plant (UCT) mixed culture (Table 2.1). Considering the assay fluctuations observed for the biooxidation pilot plant (UCT) culture (Fig 2.2), and the optimal growth conditions in both the Applikon® bioreactor and the biooxidation pilot plant bioreactor (which was dominated by *T. caldus* MNG), the levels of rhodanese activity are of the same order. This suggests that rhodanese activity in the biooxidation pilot plant (UCT) is primarily due to *T. caldus*. Shake flask experiments to determine the rhodanese activity of *T. caldus* MNG (Table 3.3) did not demonstrate activities of this magnitude.

Chapter Four

Genetic studies for the isolation of a rhodanese gene from biomining bacteria

Contents

4.1) Introduction

As thiosulfate: cyanide sulfurtransferase activity had been demonstrated for the sulfur-oxidizers *T. ferrooxidans* ATCC 33020, *T. thiooxidans* ATCC 19377 and *T. caldus* MNG an attempt was made to identify and clone the rhodanese gene(s) from one of these bacteria. It was hoped that should a rhodanese gene be cloned, sequence analysis would assist in the characterization of the first rhodanese gene isolated from a *Thiobacillus*.

An approach considered for the isolation of a rhodanese gene was the polymerase chain reaction (PCR) amplification of the gene using primers designed from the available sequences in the National Center for Biotechnology Information (NCBI) database. However, there were two difficulties with this approach. Firstly, besides *T. ferrooxidans* (Rawlings *et al.*, 1991), the codon usage preference is not well characterized for thiobacilli. Secondly, the available rhodanese sequences in the NCBI database have low sequence similarity, and therefore it would be difficult to design primers that were likely to be specific. The *A. vinelandii rhdA* gene isolated by Colnaghi *et al.* (1996) had been acquired from these authors and the potential to identify the rhodanese gene by Southern hybridization using the *rhdA* gene as a probe was considered to be a better option. Once a restriction fragment which gave a positive hybridization signal to the probe had been identified, it was envisaged that a partial gene library would be constructed using fragments of a similar size, and the gene isolated.

4.2) Materials and Methods

4.2.1) Preparation of chromosomal DNA for Southern hybridization: Chromosomal DNA from the pure and biooxidation pilot plant (UCT) cultures were prepared using the method described in section 3.2.1, with the slight modification of two to three additional washes of the chromosomal DNA with ammonium acetate. The ammonium acetate precipitation protocol for the removal of bound extraneous protein involved the addition of 50% (v/v) 7.5M ammonium acetate pH 7.5 to chromosomal DNA resuspended in TE buffer pH 7.6. The solution was incubated for 60 minutes, and then centrifuged for 15 minutes at 14K. The supernatant was transferred to a new Eppendorf tube and 2.5 volumes absolute ethanol added. The solution was incubated for two hours before being centrifuged to pellet the chromosomal DNA. The chromosomal DNA pellet was then washed with 70% ethanol, air-dried and resuspended in TE buffer pH 7.6. The ammonium acetate precipitation protocol was carried out at room temperature.

4.2.2) DNA manipulations: The standard methods of Sambrook *et al.* (1989) and Ausubel *et al.* (1993) were used for restriction enzyme digests, agarose gel electrophoresis, purification of DNA fragments from agarose gels by electroelution, and ligations. All restriction enzymes and their buffers were acquired from Boehringer Mannheim. The restriction enzymes and other enzymes used for DNA manipulations were used according to manufacturer's specifications. The bacterium *E. coli* JM109 was made competent using the standard rubidium chloride method of Sambrook *et al.* (1989).

4.2.3) Southern hybridization probe preparation: The pRC9189 recombinant plasmid containing the *A. vinelandii rhdA* gene (Fig 4.1) was acquired from Colnaghi *et al.* (1996) and used to construct a probe for Southern hybridization studies.

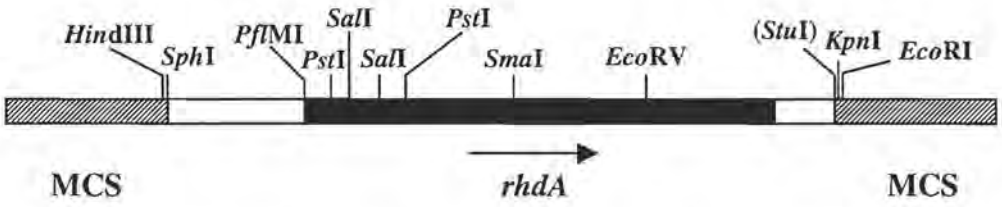


Fig 4.1: Restriction enzyme map of the 1.3kb insert containing the *A. vinelandii rhdA* gene cloned into the *Sph*I and *Sma*I sites of the cloning vector pTZ18. Bracketed *Stu*I is the *Stu*I-*Sma*I blunt-end cloning site. The 813bp *rhdA* open reading frame is represented by the black rectangle and the flanking regions are represented by unshaded rectangles. The multiple cloning site (**MCS**) of the pTZ18 cloning vector is represented by the hatched rectangles.

The alkaline lysis cell disruption method (Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981) and plasmid purification by CsCl-EtBr gradient centrifugation (Ausubel *et al.*, 1993) was used for the large scale preparation of pRC9189 plasmid DNA from *E. coli*. For Southern hybridization against chromosomal digests the *rhdA* probe was prepared by digesting the plasmid pRC9189 with the restriction enzymes *Sph*I and *Eco*RI, followed by the electroelution of the fragment from a 0.8% agarose gel. The fragment was labeled according to the digoxigenin-11-dUTP (DIG) random primed DNA labeling method using the DIG DNA labeling kit (Boehringer Mannheim). When screening the partial gene library the *rhdA* probe was digested with *Sal*I and *Kpn*I to remove as much of the *A. vinelandii rhdA* gene flanking regions and vector sequence as possible, so as to increase hybridization specificity (see section 4.3). This *SalI-Kpn*I *rhdA* probe was labeled using a scaled-up DIG random primed DNA labeling protocol (Boehringer Mannheim).

4.2.4) DNA:DNA hybridization (Southern hybridization): Restriction enzyme digested chromosomal and recombinant plasmid DNA fragments were separated by electrophoresis in a 0.8% agarose gel at 25V overnight, together with *Pst*I-digested Lambda DNA as a marker. After electrophoresis, the DNA was depurinated by washing the gel in two volumes 0.25M HCl for 15 minutes, rinsing with water, and washing the gel in two volumes of 0.4N NaOH for 15 minutes at room temperature to denature the DNA. The DNA was then capillary blotted overnight onto Hybond^{TM}-N^{+} nucleic acid transfer membrane (Amersham) using 0.4N NaOH. The membrane was prehybridized with DIG Easy Hyb (Boehringer Mannheim) solution for one hour at 35°C. Following

prehybridization, the denatured probe was added to the DIG Easy Hyb solution and hybridization was carried out at 35°C overnight. The membrane was then washed twice for ten minutes with 2X SSC (300mM NaCl, 30mM sodium citrate pH 7.0) containing 0.1% SDS at room temperature. A second set of higher stringency washes using 0.1X SSC (15mM NaCl, 1.5mM sodium citrate pH 7.0) containing 0.1% SDS were carried out at 65°C for ten minutes. After these washes the probe was detected using the DIG luminescent detection kit and protocol of Boehringer Mannheim. The chemiluminescent substrate used was CSPD® (Boehringer Mannheim) and the signal was detected by autoradiography using 3M X-ray film (Trimax).

4.2.5) Partial gene library of *T. caldus* MNG: Chromosomal DNA of *T. caldus* MNG was digested with *Bam*HI and *Pst*I. DNA fragments in the size range of 1.8-2.3kb, which corresponded to the approximate hybridization signal size, were gel excised and electroeluted from a 0.8% agarose gel. The DNA fragments were cloned into the vector pUCBM20 and the recombinant plasmids transformed into *E. coli* JM109. Strains and vectors used in this study are described in Appendix B. Transformants were selected on Luria-Bertani medium (Appendix A) containing 100µg.ml^{-1} ampicillin and 2µg.ml^{-1} X-gal. A total of 500 positive transformants were replica-plated in groups of 30 per plate.

To screen the partial gene library, each group of 30 transformants was pooled and recombinant plasmids purified from the pool by the alkaline lysis and CsCl-EtBr gradient centrifugation methods (Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981; Ausubel *et al.*, 1993). *Bam*HI-*Pst*I digests of the recombinant plasmids from each pool were separated by electrophoresis in a 0.8% agarose gel and the pools screened by Southern hybridization using the *SalI-Kpn*I *rhdA* probe. Lack of a hybridization signal from a pool resulted in the elimination of that pool from further rounds of screening.

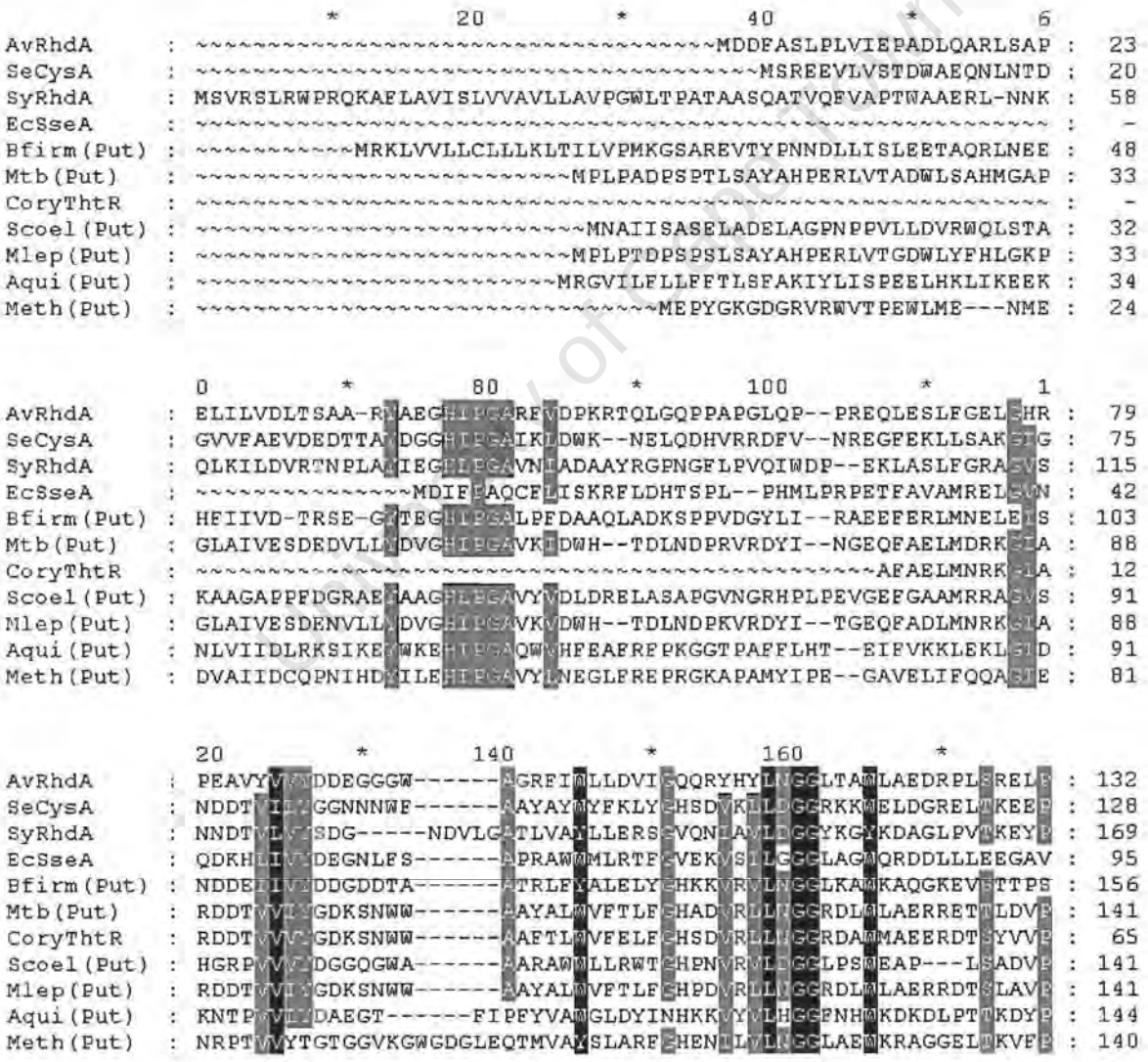
4.2.6) Nucleotide sequencing of the recombinant plasmid pUC350-34: The recombinant plasmid pUC350-34 was sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using the thermosequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech UK Ltd.). The Cy5 end-labeled primers

used for sequencing where obtained from the Synthetic DNA Laboratory, Department of Biochemistry (University of Cape Town). Sequencing reactions were run on an ALFexpress automated DNA sequencer (Pharmacia Biotech, Uppsala, Sweden). The Genetics Computer Group Inc. Winconsin Package version 9.1 (GCG, Madison Wis.) and the GeneDoc version 2.3 multiple sequence alignment software were used for sequence construction and analysis. Simulated restriction enzyme digests and restriction enzyme maps were constructed using the DNAMAN version 3.0 (Lynnon BioSoft) software.

4.3) Results

4.3.1) PCR primer design for the amplification of a *Thiobacillus* rhodanese gene

Using the protein sequences available in the NCBI database, confirmed prokaryotic rhodanese and rhodanese-like amino acid sequences, including putative thiosulfate: cyanide sulfurtransferase sequences from various genome sequencing projects, were aligned using PILEUP (Fig 4.2).



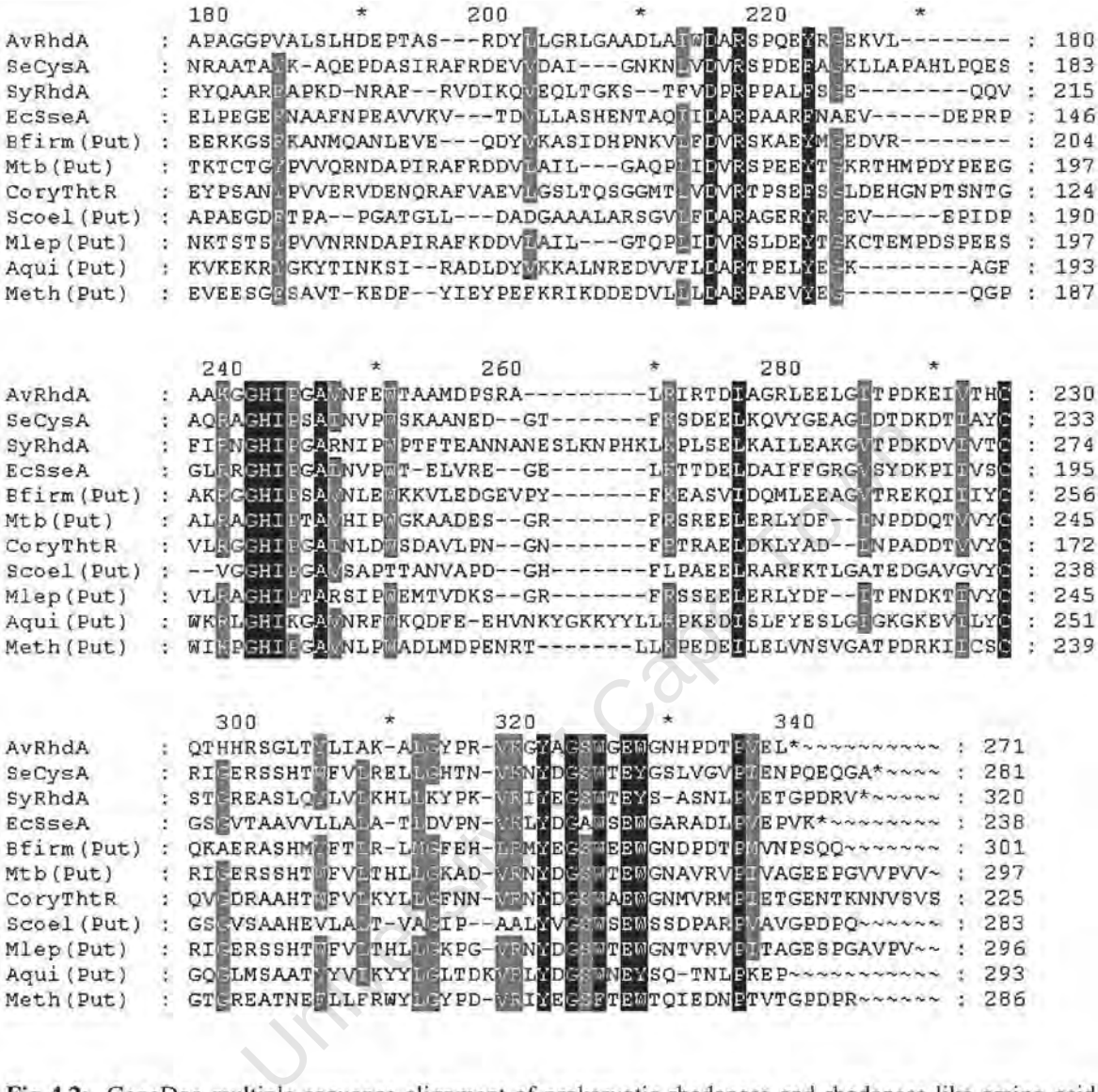


Fig 4.2: GeneDoc multiple sequence alignment of prokaryotic rhodanese and rhodanese-like amino acid sequences. Residues that are conserved are shaded in black, while residues that show a greater than 80% conservation are shaded in grey. Sequences with the suffix (**Put**) are putative thiosulfate: cyanide sulfurtransferase polypeptides identified in genome sequencing projects. Abbreviations used are: *A. vinelandii* rhodanese (**AvRhdA**); *S.* erythraea CysA (**SeCysA**); *Synechococcus* PCC 7942 RhdA (**SyRhdA**); *E. coli* SseA (**EcSseA**); *Bacillus firmus* (**Bfirm**), *Mycobacterium tuberculosis* (**Mtb**), *Corynebacterium glutamicum* ThtR (**CoryThtR**), *Streptomyces coelicolor* (**Scoel**), *Mycobacterium leprae* (**Mlep**), *Aquifex aeolicus* (**Aqui**), *Methanobacterium thermoautotrophicum* (**Meth**).

Attempts were made to design PCR primers to the partially conserved sequences. The conserved sequences considered were at approximately amino acid residues 158-166, 240-251, and 319-328. However, no primers of sufficient length and specificity could be

designed due to the lack of adequate conserved sequence length. As a result a Southern hybridization approach was adopted.

4.3.2) Southern hybridization using the *A. vinelandii rhdA* probe

Initially the *A. vinelandii rhdA* gene and its flanking regions were used to probe total chromosomal digests of the pure cultures and biooxidation (UCT) mixed culture (Fig 4.3). As *A. vinelandii* is taxonomically distantly related to the bacterial species considered in this study the hybridization temperature was lowered to 35°C according to the DIG Easy Hyb (Boehringer Mannheim) protocol for heterologous probes. The hybridization temperature of 35°C and a stringency wash in 0.1X SSC containing 0.1% SDS at 65°C were the upper limits for a hybridization signal to be detected.

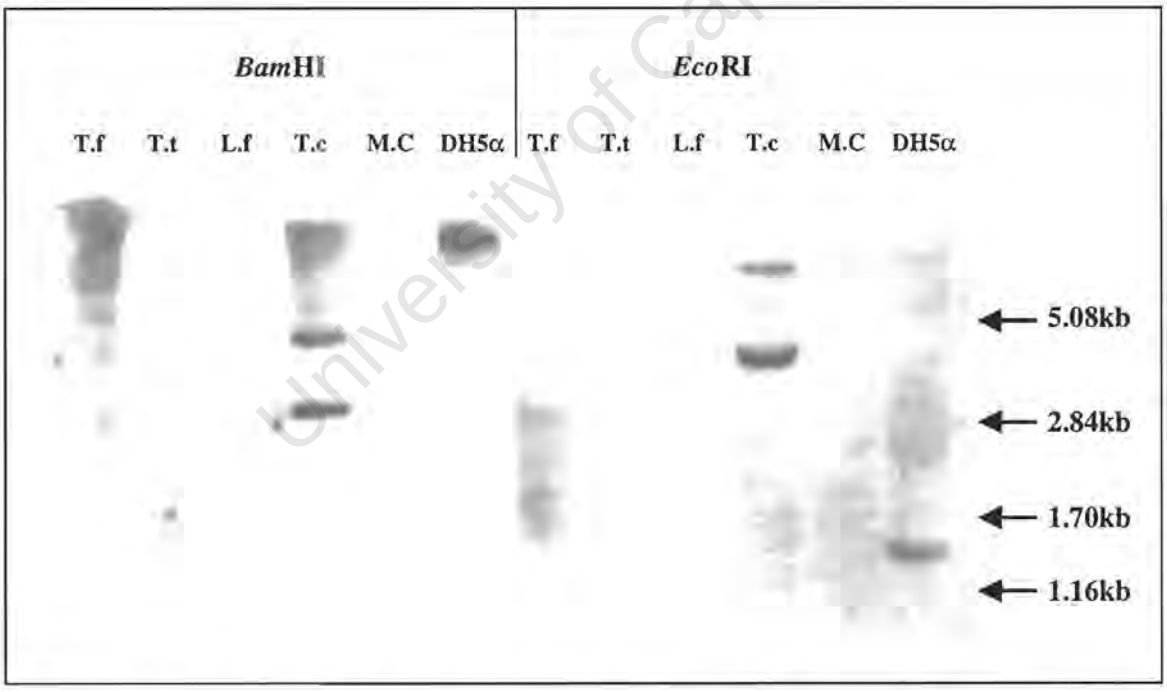


Fig 4.3: Southern hybridization using the *Sph*I-*Eco*RI digested *A. vinelandii rhdA* probe. Total chromosomal DNA of the pure cultures and biooxidation pilot plant (UCT) were digested with either *Bam*HI or *Eco*RI. An *E. coli* DH5α (**DH5α**) total chromosomal DNA control was included in each group. Southern hybridization was attempted against total chromosomal DNA of: *T. ferrooxidans* ATCC 33020 (**T.f**), *T. thiooxidans* ATCC 19377 (**T.t**), *L. ferrooxidans* DSM 2705 (**L.f**), *T. caldus* MNG (**T.c**), and the biooxidation pilot plant (UCT) mixed culture (**M.C**). A *Pst*I-digested Lambda marker was used to identify fragment sizes.

A hybridization signal was detected for the *E. coli* DH5α control (Fig 4.3) which probably indicates hybridization to the *E. coli sseA* gene. Less clear signals were obtained for *T. ferrooxidans* and the biooxidation pilot plant (UCT) mixed culture. However, a clear hybridization signal in both *Bam*HI and *Eco*RI digested *T. caldus* MNG total chromosomal DNA was detected, and it was decided to create a *T. caldus* MNG partial gene library in an attempt to clone the DNA which gave the clear signal.

4.3.3) Partial gene library of *T. caldus* MNG

In order to facilitate cloning, total chromosomal DNA of *T. caldus* MNG was digested with a range of restriction enzymes that would generate 5'- and 3'-overhangs. Combinations of these restriction enzymes were used for double-digests of the total chromosomal DNA because it would be much easier to clone a DNA fragment with different sticky ends than fragments having similar sticky ends. The rationale for this approach was that after Southern hybridization and probing with the *Sph*I-*Eco*RI *rhdA* probe, double-digested total chromosomal DNA that showed a hybridization signal size shift to a smaller size, when compared to total chromosomal DNA digested with a single restriction enzyme, would allow for that particular combination of restriction enzymes to be considered for cloning purposes. Such a hybridization signal size shift was found when total chromosomal DNA was digested with *Bam*HI and *Pst*I (data not shown).

As the positive signal for *Bam*HI-*Pst*I digested DNA was approximately 2kb, a partial gene library of 1.8-2.3kb size fragments was created in *E. coli* JM109 using the cloning vector pUCBM20. A total of 500 positive transformants were screened by Southern hybridization. However, under the hybridization conditions described in section 4.3.2, the *Sph*I-*Eco*RI *rhdA* probe also hybridized to digested pUCBM20 vector control DNA (data not shown). As an increase in hybridization temperature and wash stringency resulted in the loss of hybridization signals from the insert DNA, the only means of increasing hybridization specificity was to remove the flanking regions of *rhdA* gene present on the *Sph*I-*Eco*RI *rhdA* probe. Therefore, a *Sal*I-*Kpn*I *rhdA* probe was

constructed and used to screen the recombinant plasmids. Although the *Stu*I site in the recombinant pRC9189 plasmid might appear to be a more logical choice for the removal of the downstream flanking region (Fig 4.1), this restriction enzyme site was lost due to the *Stu*I-*Sma*I blunt-end cloning to generate the pRC9189 plasmid (Colnaghi *et al.*, 1996). As the full nucleotide sequence of the *A. vinelandii rhdA* insert in pRC9189 (Fig 4.1) is known, a simulated restriction enzyme map was created in order to determine possible restriction enzymes that would remove the flanking regions of the *A. vinelandii rhdA* gene; thereby increasing hybridization specificity. The downstream flanking region posed the greatest problem as no suitable restriction enzyme site could be found that was not also present in the *rhdA* open reading frame. Because no suitable alternative restriction enzyme sites were found it was decided to use the *Kpn*I site present in pTZ18. This meant that ten nucleotides of the pTZ18 multiple cloning site (the cloning vector from which the recombinant plasmid pRC9189 was constructed [Colnaghi *et al.*, 1996]) remained attached to the downstream flanking region. Thus, this flanking region of approximately 127 nucleotides could not be removed. However, the larger *rhdA* upstream flanking region was removed by digestion with *Sal*I. Although there is a *Sal*I restriction enzyme site 138bp into the *A. vinelandii rhdA* open reading frame (Fig 4.1) this restriction enzyme was used as little amino acid similarity to other prokaryotic rhodanese and rhodanese-like enzymes had been demonstrated within the region to be deleted (Fig 4.2). This *Sal*I-*Kpn*I *rhdA* probe hybridized at exactly the same positions as the previous probe when used on the blot shown in Fig 4.3 (data not shown). Unfortunately, this probe continued to hybridize under the same conditions used for the *Sph*I-*Eco*RI *rhdA* probe to the digested pUCBM20 vector control (data not shown), and further increases in wasn stringency resulted in the loss of the insert DNA hybridization signals.

In spite of this background hybridization to vector DNA, using the *Sal*I-*Kpn*I *rhdA* probe, one recombinant plasmid, designated pUC350-34, was isolated which appeared to give a stronger hybridization signal than the vector control. This recombinant plasmid contained a 1.9kb insert which was sequenced for 990bp from the insert 5-'end and 978bp from the 3'-end. However, sequence homology comparisons using the BLAST

program yielded non-rhodanese-like sequences. A BLAST of the 5'-end indicated a 48% amino acid identity to 209 amino acids of the *E. coli* penicillin-binding protein 1A sequence. A BLAST of the 3'-end indicated a 39% amino acid identity to 104 amino acids of the *Neisseria meningitidis* penicillin-binding protein 1 sequence.

4.4) Discussion

The attempt to isolate a rhodanese gene from one of the sulfur-oxidizers that possessed rhodanese activity was unsuccessful. Initially, enzyme purification was attempted, but proved to be difficult. The major difficulty was the low protein concentrations of soluble enzyme extracts (as a consequence of poor biomass yields), even after $(NH_4)_2SO_4$ precipitation of soluble extracts and combining the concentrates. Given the low concentration of enzyme, the buffer ionic strength at which the *T. caldus* MNG enzyme suspension would bind efficiently to either DE-52 or CE-52 Whatman ion-exchange resins, or to the stronger anion-exchange Sepharose-Q Fast Flow resin, could not be determined. If a rhodanese enzyme could have been purified then N-terminal sequencing would have allowed for the design of degenerate PCR primers that would have facilitated the isolation of a rhodanese gene.

No sufficiently specific PCR primers could be designed on the basis of published rhodanese and rhodanese-like sequence data. A multiple sequence alignment of both eukaryotic and prokaryotic rhodanese and rhodanese-like enzymes (data not shown) showed no conserved amino acid regions of sufficient length for this purpose. Greater amino acid sequence similarity was found when only prokaryotic rhodanese and rhodanese-like amino acid sequences were aligned (Fig 4.2), but even in this case no conserved amino acid region large enough to be used for primer design was available. This point was made by Colnaghi *et al.* (1996) who also noted that the *E. coli* SseA protein, which demonstrated marginal rhodanese activity, was more closely related to vertebrate rhodaneses than other prokaryotic rhodanese sequences. Even so, Nagahara *et al.* (1995) showed that a partial sequence of rat mercaptopyruvate sulfurtransferase had higher amino acid similarity (87.5%) to human rhodanese than it does to other vertebrate rhodaneses (64-68%). This suggests that the human rhodanese is in fact a mercaptopyruvate sulfurtransferase. Thus, low rhodanese sequence similarity is found even within the vertebrate rhodanese group.

Therefore, the only other approach for the isolation of a rhodanese gene was the screening of a partial gene library using a rhodanese gene probe. Although a clear hybridization signal was detected when the *A. vinelandii rhdA* gene was used to probe digested total chromosomal DNA of the bacteria studied in this investigation (Fig 4.3), this signal was not specific as the probe also hybridized to a digested pUCBM20 cloning vector control, and to digested *E. coli* DH5α. However, as the hybridization signal detected in digested *T. caldus* MNG total chromosomal DNA was stronger than the signal for the vector control, a partial gene library was created using *Bam*HI-*Pst*I digested *T. caldus* MNG chromosomal DNA. Screening of recombinant plasmids by gene complementation of a mutant was not possible as the only prokaryotic rhodanese mutant available was *A. vinelandii* (Colnaghi *et al.*, 1996). This *A. vinelandii* mutant had no discernible phenotype distinct from wild-type *A. vinelandii* and the mutant strain demonstrated residual rhodanese activity (Colnaghi *et al.*, 1996) which suggested that *A. vinelandii* contained more than one enzyme with rhodanese activity (as has been found in other bacteria; see section 1.10). Therefore, screening of recombinant plasmids by complementation was deemed to be impractical. Screening of recombinant plasmids of the partial gene library by Southern hybridization with the *A. vinelandii rhdA* probe was the alternate approach.

Taxonomic distance, and therefore lack of sufficient nucleotide sequence similarity, probably prevented the Southern hybridization method of screening from being successful. BLAST scores of the isolated pUC350-34 plasmid insert DNA showed a 48% (5'-end) and 39% (3'-end) amino acid identity to an *E. coli* and *N. meningitidis* penicillin-binding protein, respectively. Although there was no obvious explanation for this, the rather convincing similarity to a penicillin-binding protein appeared to be a meaningful result, but was of no significance in the context of the probe used. To verify that the pUC350-34 insert DNA originated from *T. caldus* MNG, the pUC350-34 insert DNA was gel excised, labeled, and used to probe *Bam*HI-*Pst*I digested *T. caldus* MNG chromosomal DNA. A *T. caldus* MNG chromosomal signal of equal size to the pUC350-34 insert DNA (data not shown) confirmed that although a rhodanese-like sequence had not been identified, the insert DNA of pUC350-34 originated from *T. caldus* MNG.

Chapter Five

General discussion

Refractory gold-bearing metal-sulfide ores and concentrates require a pretreatment process to allow for efficient gold recovery by cyanidation. The establishment of biooxidation has presented mining operations with an economically viable alternative to costly metallurgical process, such as roasting and pressure-leaching, for the pretreatment of refractory gold-bearing ores and concentrates. As with all technologies, process optimization is required to maintain a competitive edge for the continued use of such technology. Although, economically and technologically successful, when compared to pyro and hydrometallurgical processes, one of the greatest drawbacks of the biooxidation process is the excessive wastage of cyanide during cyanidation.

A large number of factors appear to contribute to cyanide wastage. It is well documented that the formation of metallo-cyanides and the presence of reactive sulfur species contributes to cyanide wastage. As gold is locked up in a matrix of metallic sulfides, the sulfide content of the ore or concentrate is a major consideration when treating ores or concentrates with cyanide for the dissolution of gold. It is argued that the microbial population in the bioreactors contributes indirectly to the wastage of cyanide through the metabolic production of a range of reactive sulfur species that may readily complex with cyanide forming thiocyanate. Thiocyanate formation is presently recognised as accounting for the largest proportion of cyanide consumption during cyanidation (Hackl and Jones, 1997). Further investigation of the microbial contribution to cyanide wastage was deemed necessary to evaluate the potential for enzymatic depletion of cyanide. Enzymatic depletion of cyanide would implicate the biooxidation microbial consortium in contributing directly to cyanide wastage. A more complete understanding of the chemical and enzymatic depletion of cyanide during cyanidation of biooxidation treated ores and concentrates could result in process optimization.

To investigate the direct microbial contribution to cyanide wastage, the seemingly ubiquitous enzyme, rhodanese, was examined for its potential to deplete cyanide. This enzyme, which is defined by its *in vitro* activity as a thiosulfate: cyanide sulfurtransferase, has been found in many prokaryote species. The defining irreversible reaction that this enzyme is able to catalyze is the transfer of the sulfane-sulfur of thiosulfate to the thiophilic acceptor cyanide, by means of a double-displacement mechanism, forming thiocyanate. Although the various rhodanese enzymes reported have displayed such diverse catalytic ability that the physiological role of this enzyme is still debated, it was due to the catalytic ability of rhodanese to form thiocyanate that the rhodanese activity of the relevant biomining bacteria was investigated. As an enzyme family, the rhodaneses are probably best defined as general sulfurtransferases able to form a binary Michaelis-Menten complex by electrophilic attack on a sulfane-sulfur anion. The enzyme-substrate complex then transfers the bound sulfenyl sulfide to a thiophilic acceptor following discharge of sulfide.

Enzymatic depletion of cyanide

The assay protocol of Singleton and Smith (1988) for the detection of thiocyanate was adopted for this study. The sulfur-oxidizers *T. ferrooxidans* ATCC 33020, *T. thiooxidans* ATCC 19377, and *T. caldus* MNG demonstrated rhodanese activity, but it was difficult to quantify and, therefore, correlate rhodanese activities between pure cultures and a biooxidation pilot plant. However, the obligate iron-oxidizing chemolithoautotroph *L. ferrooxidans* DSM 2705 demonstrated no rhodanese activity. Although the assay was reliable within replicates of the same sample, activity fluctuations between assays made quantifying the rhodanese activity of the sulfur-oxidizers difficult. It appears from work based on the effect of growth phase on *T. caldus* MNG rhodanese activity that the large standard deviation between assays could be attributed to the physiological state of the cell. The accumulation of reactive sulfur species during growth is thought to be responsible for influencing the catalytic ability of rhodanese and, therefore, decreasing the quantitative nature of the assay. As the culture aged, background levels of spontaneously formed thiocyanate in the assays increased. This highlights the

importance of growth phase, and the accumulation of reactive sulfur species during growth, on rhodanese activity.

Possibly the major contributor to enzymatic depletion of cyanide is *T. caldus*. This sulfur-oxidizer is becoming increasingly important to biooxidation studies owing to its newly recognised dominance in the biomining microbial consortium. Throughout a ten day pyrite-arsenopyrite batch culture this bacterium was consistently present, and together with the high levels of rhodanese activity detected during pure culture bioreactor studies, this suggests that *T. caldus* is potentially the major contributor to rhodanese-mediated wastage of cyanide during cyanidation. Shifting species dominance in the Biox® bioreactor cascade (Dew *et al.*, 1997) towards a higher percentage of sulfur-oxidizers in the last bioreactor, would determine whether species such as *T. caldus* were present in proportionately higher numbers during cyanidation than species unable to catalyze the formation of thiocyanate. Optimizing conditions so that the microbial population in the slurry entering the cyanidation plant is predominantly composed of species lacking rhodanese activity, such as *L. ferrooxidans*, would assist in decreasing cyanide wastage.

Chemical depletion of cyanide

An alternative would be to ensure the complete oxidation of sulfur species so that available reduced sulfur sources, including cellular sulfur reserves, would be exhausted. This would serve to decrease the concentration of reactive sulfur species introduced to the cyanidation plant. However, this would require the complete mineralization of the ore, and, therefore, very long residence times. Possibly, the most influential sulfur species contributing to cyanide wastage is tetrathionate. Due to its instability in acid solutions, thiosulfate is barely detectable in leaching solutions (Schippers *et al.*, 1996), and is rapidly oxidized either chemically or enzymatically to tetrathionate. Enzymatic oxidation of thiosulfate to tetrathionate has been demonstrated in *T. ferrooxidans*, *T. thiooxidans*, and *T. caldus* (Pronk *et al.*,1990; Hallberg *et al.*, 1996). The reported stability of tetrathionate in acidic biooxidation conditions, as opposed to thiosulfate,

suggests that this reactive sulfur species, formed by both chemical and enzymatic oxidation reactions, is introduced to the cyanidation plant in proportionately higher concentration than thiosulfate, following biooxidation. Tetrathionate decomposition in the alkaline conditions (≈pH 10) of the cyanidation plant results in a range of reactive sulfur species that readily complex to cyanide forming thiocyanate. Alternatively, tetrathionate will react rapidly with excess sulfide to form thiosulfate and elemental sulfur (equation 4.1), or react spontaneously with cyanide (equation 4.2) forming thiocyanate (Roy and Trudinger, 1970).

$$S_4O_6^{2-} + S^{2-} \rightarrow 2S_2O_3^{2-} + S^0 \qquad (4.1)$$

$$S_4O_6^{2-} + CN^- + OH^- \rightarrow S_2O_3^{2-} + HSO_4^- + SCN^- \qquad (4.2)$$

Cyanolysis of thiosulfate and elemental sulfur would then form thiocyanate (equations 2.1 and 2.2). Thiosulfate may, however, decompose to sulfide and sulfite, or sulfate, as it is also unstable at high alkaline pH values (Roy and Trudinger, 1970).

Relative contribution of enzymatic and chemical depletion of cyanide

It is, therefore, likely that the major contributor to chemical thiocyanate formation in the cyanidation plant is tetrathionate. Chemical consumption of cyanide could account for proportionally greater depletion of the cyanide than rhodanese. Although the rhodanese substrate (thiosulfate) is formed in the cyanidation plant during the decomposition of tetrathionate, the activity of rhodanese at cyanidation pH (≈pH 10) is questionable. Rhodanese activity was detected in the predominant and relevant sulfur-oxidizers of the biooxidation process, but due to the instability of thiosulfate at alkaline pH values, rhodanese activity could not be assayed above pH 8.0. During biooxidation the cytoplasmic pH of the chemolithoautotrophic bacteria is maintained at pH 6.5 (Cox *et al.*, 1979). It would be expected that at pH 6.5 rhodanese is active, and cellular thiosulfate would not be as susceptible to decomposition at that pH. Rhodanese would, therefore, be

active in the biooxidation environment, provided it remained in the cell. However, should cellular integrity be affected at cyanidation pH, it is thought that rhodanese would probably be inactive. If rhodanese does contribute to the formation of thiocyanate in the cyanidation plant, the cyanide and thiosulfate will probably have to be transported into the cell for the reaction to take place in the cytoplasm. Rhodanese thiocyanate formation is unlikely to contribute as much to cyanide depletion as the spontaneous chemical formation of thiocyanate

Alternative gold dissolution processes

If the production of tetrathionate in the Biox® bioreactors cannot be avoided, it may be necessary to consider processes other than cyanidation for the dissolution of gold. Lixiviants, such as thiourea and chloride, offer the potential for dissolution of gold in acidic conditions (Nicol *et al.*, 1987). Although, the dissolution of gold by chlorination is approximately two-fold higher than cyanidation (Nicol *et al.*, 1987), both chloride and thiourea, like cyanide, are susceptible to oxidation resulting in unfavourable biooxidation end-products. Thiocyanate and thiosulfate may also be used as lixiviants. The dissolution of gold is faster in thiocyanate than in thiosulfate (Nicol *et al.*, 1987). However, ammonium thiosulfate dissolution of gold from heap biooxidation processes was found to be economical by the Newmont Gold Company (Brierley, 1997). The applicability of gold dissolution lixiviants for a particular ore or concentrate must be based on the cost of the lixiviant, efficiency of gold dissolution, and consumption (both chemical and microbial) of the lixiviant. Therefore, if it is not possible to reduce the consumption of cyanide following biooxidation, alternative gold dissolution processes may be required to improve the economics of the process.

Appendix A

All media, solutions and buffers were sterilized by autoclaving at 121°C for 20 minutes. Heat labile substances were filter sterilized using 0.22µm membrane filters (Micron separations Inc.)

A) Chemolithoautotroph liquid culture media

10X Mineral salt solution

$(NH_4)_2SO_4$	30.0g.l^{-1}
KCl	1.0g.l^{-1}
K_2HPO_4	5.0g.l^{-1}
$MgSO_4.7H_2O$	5.0g.l^{-1}
$Ca(NO_3)_2.4H_2O$	0.14g.l^{-1}
Na_2SO_4	14.5g.l^{-1}

pH to 2.5 with H_2SO_4 and autoclave.

1000X Trace elements solution

$ZnSO_4.7H_2O$	10.0g.l^{-1}
$CuSO_4.5H_2O$	1.0g.l^{-1}
$MnSO_4.4H_2O$	1.0g.l^{-1}
$CoCl_2.6H_2O$	0.5g.l^{-1}
$Cr_2(SO_4)_3.15H_2O$	0.5g.l^{-1}
$Na_2B_4O_7.10H_2O$	0.5g.l^{-1}
$NaMoO_4.2H_2O$	0.5g.l^{-1}

Acidify with 530µl.l^{-1} H_2SO_4 and autoclave.

i.) Tetrathionate medium

The mineral salt and trace element solutions were added aseptically to the required concentration of filter sterilized tetrathionate. The pH was then adjusted to the required pH with H_2SO_4.

Appendix A (cont.)

ii.) 9K iron medium

$FeSO_4.7H_2O$	$38g.l^{-1}$

Adjust pH of iron solution to pH 1.2 and autoclave.

The mineral salt and trace element solutions were added aseptically to the iron solution. The medium pH was then adjusted to pH 1.6 with H_2SO_4.

B) Luria-Bertani media

Bactotryptone	$10g.l^{-1}$
Yeast extract	$5g.l^{-1}$
NaCl	$5g.l^{-1}$

Solid media contained 1.5% (w/v) agar

C) Media additives

Media additive stocks were made as follows:

Ampicillin (Amp)	$100mg.ml^{-1}$

X-gal (5-bromo-4-chloro-3-indolyl-β-galactoside)

X-gal (2% w/v)	0.2g
Dimethylformamide	10ml

The solution was stored in aliquots at –70°C

D) SET buffer

Sucrose	50g
0.5M EDTA pH 8.0	0.8ml
1.0M Tris pH 8.0	10ml

Make to 200ml with distilled water and autoclave

Appendix B

Strains	Genotype	Reference/Origin
Thiobacillus ferrooxidans ATCC 33020	Wild type	Rockville, Md.
Thiobacillus thiooxidans ATCC 19377 (Type strain)	Wild type	Rockville, Md.
Leptospirillum ferrooxidans DSM 2705 (Type strain)	Wild type	Braunschweig, FRG
Thiobacillus caldus MNG	Wild type	This work
Escherichia coli JM109	*endA*1 *recA*1 *gyrA*96 *thi* *hsdR*17 (r_k^-, m_k^+) *relA*1 *supE*44 Δ(*lac-proAB*) [F' *trad*36 *proAB* *lacI*^{q}ZΔM15]	Promega Corp. USA
Escherichia coli DH5α	ϕ80d*lacZ*ΔM15 *endA*1 *recA*1 *gyrA*96 *thi*-1 *hsdR*17 (r_k^-, m_k^+) *relA*1 *supE*44 *deoR* Δ(*lacZYA-argF*) U169	Promega Corp. USA
Escherichia coli BMH 71-18	*thi supE* Δ(*lac-proAB*) [*mutS*::Tn10] [F' *proAB* *lacI*^{q}ZΔM15]	Promega Corp. USA

Plasmids	Description	Reference/Origin
pBluescriptSK	Amp^R, cloning vector	Stratagene, USA
pUCBM20	Amp^R, cloning vector	Stratagene, USA
pRC9189	Derivative of pTZ18 containing *rhdA* gene of *A. vinelandii*	Colnaghi *et al.* (1996)

Appendix C

The table below illustrates the range of spontaneous chemical formation of thiocyanate (SCN^-) formed during assays. Representative data is used to show the upper and lower limits of the spontaneous thiocyanate contribution to the rhodanese assays. For each assay the spontaneous chemical formation of thiocyanate (Denatured control) was subtracted from the biological sample (Non-denatured biological sample) to yield a result that reflected only the enzymatic formation of thiocyanate. Data is expressed as the molar formation of thiocyanate after a 30 minute reaction period at 30°C.

mM SCN^-		% spontaneous chemical SCN^- formation in the Non-denatured sample
Denatured control	Non-denatured biological sample	
0.01	0.04	25
0.07	0.26	27
0.01	0.03	33
0.13	0.36	36
0.10	0.26	38
0.28	0.61	46
0.25	0.53	47
0.02	0.04	50
0.31	0.60	52
0.16	0.30	53
0.90	1.67	54
0.43	0.74	58
0.20	0.31	64

Appendix D

i.) Detection of *L. ferrooxidans* and *T. caldus* in the biooxidation pilot plant (UCT)

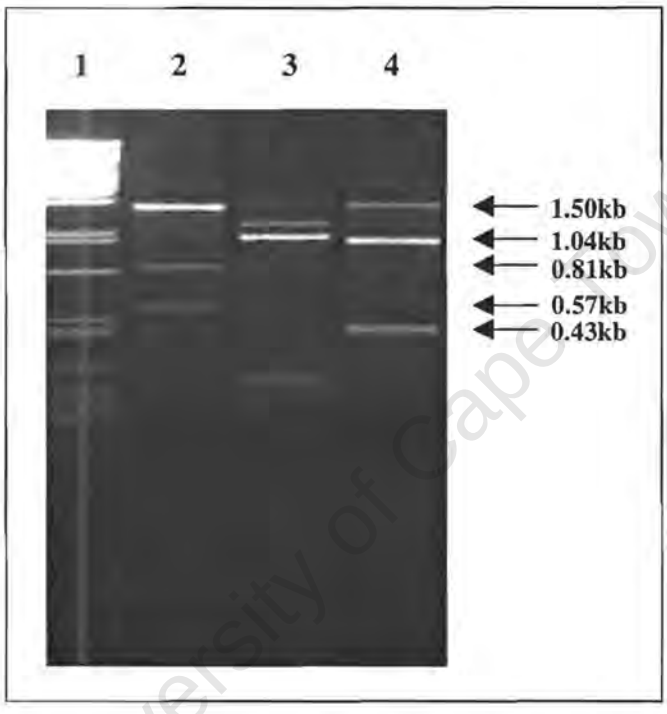
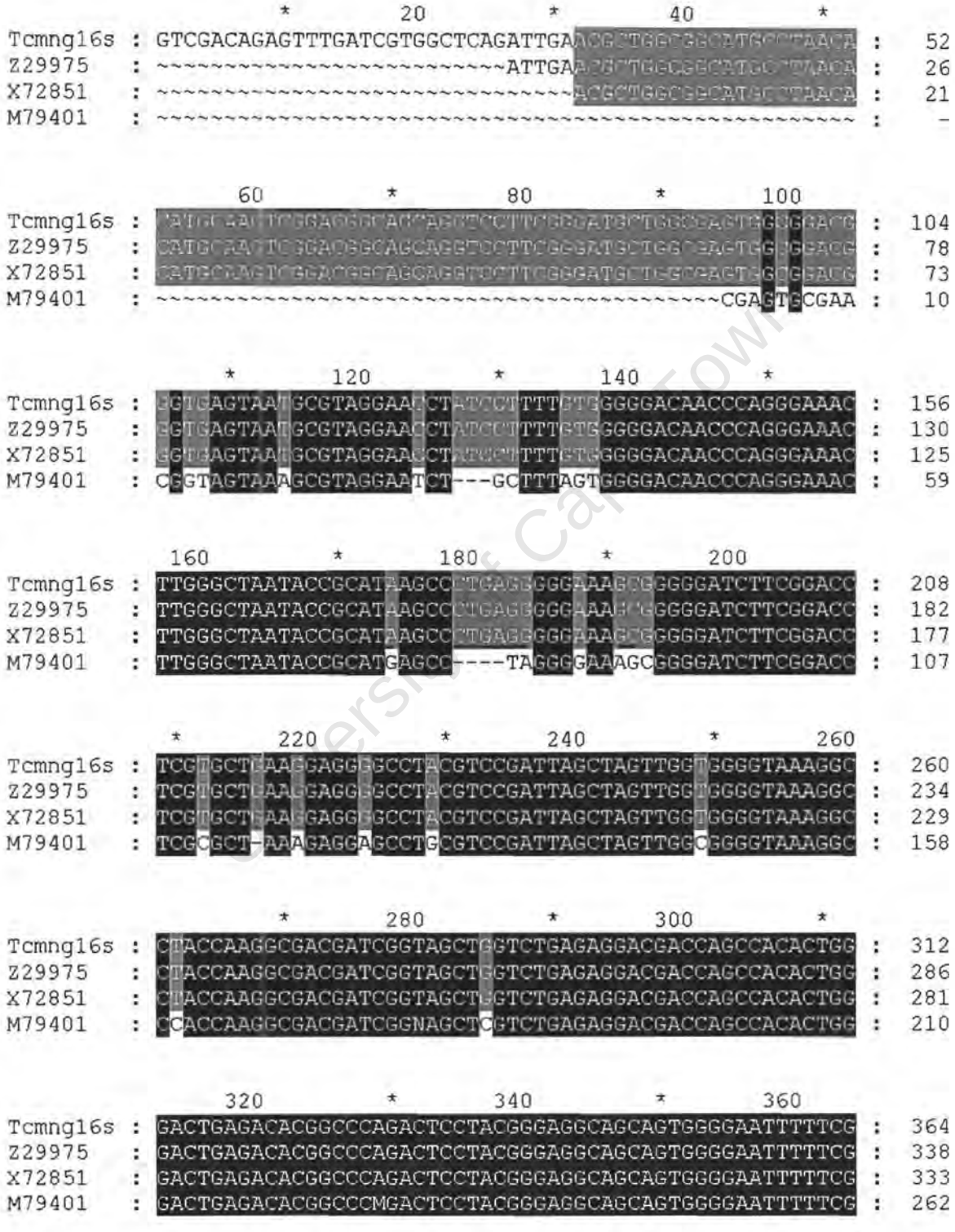
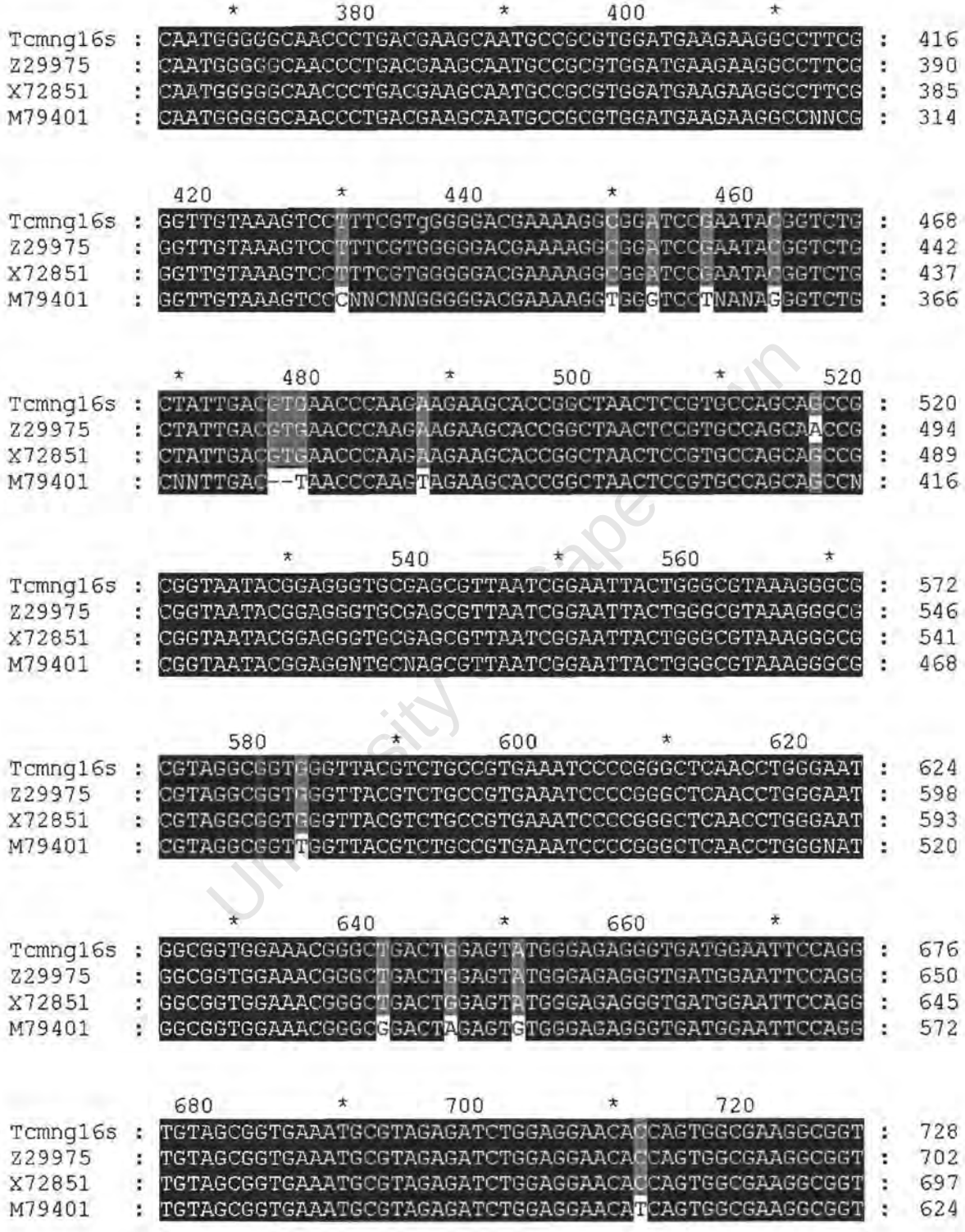


Fig A: Detection of *L. ferrooxidans* and *T. caldus* in the biooxidation pilot plant using the restriction enzyme based PCR amplified 16S rDNA profiling technique. Lane 1 is *Pst*I-digested Lambda marker. Lanes 2-4 are the same PCR product digested with *Eco*RV (**2**), *Stu*I (**3**), and *Bam*HI (**4**). Using Table 3.1 the typical profiles of *L. ferrooxidans* and *T. caldus* can be distinguished. For example, in Lane 2 *L. ferrooxidans* 16S rDNA digested with *Eco*RV is identified by the 0.57kb and 0.81kb bands (0.1kb band is to small to see in the gel). However, *T. caldus* is not digested by *Eco*RV resulting in the approximate 1.5kb undigested band in the same lane. Similarly, the 0.43kb and 1.04kb bands characteristic of *T. caldus* can be seen in the *Bam*HI digested samples (lane 4). The approximate 1.5kb undigested band in lane 4 is that of *L. ferrooxidans*.

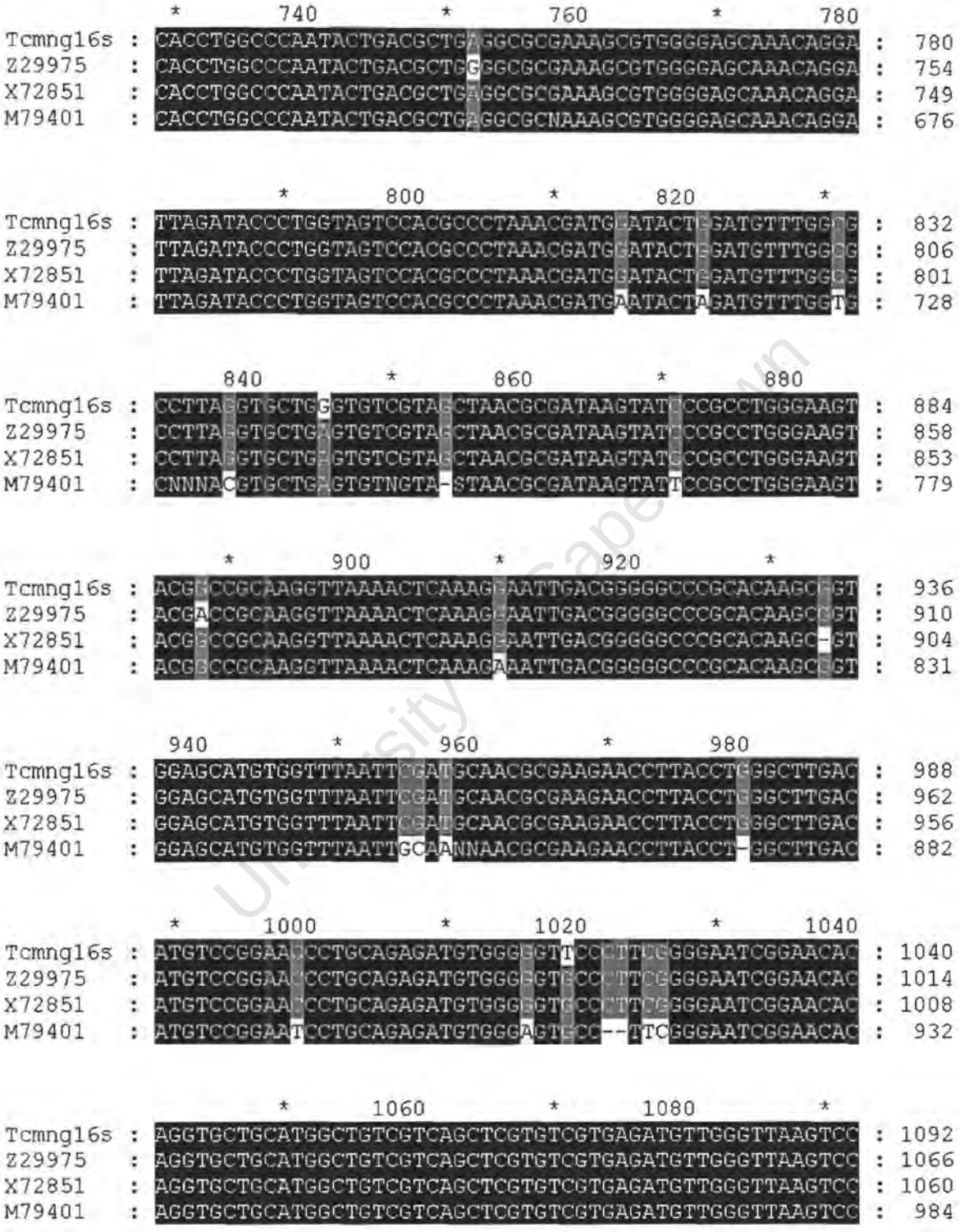
ii) Multiple sequence alignment of *T. caldus* MNG 16S rRNA gene



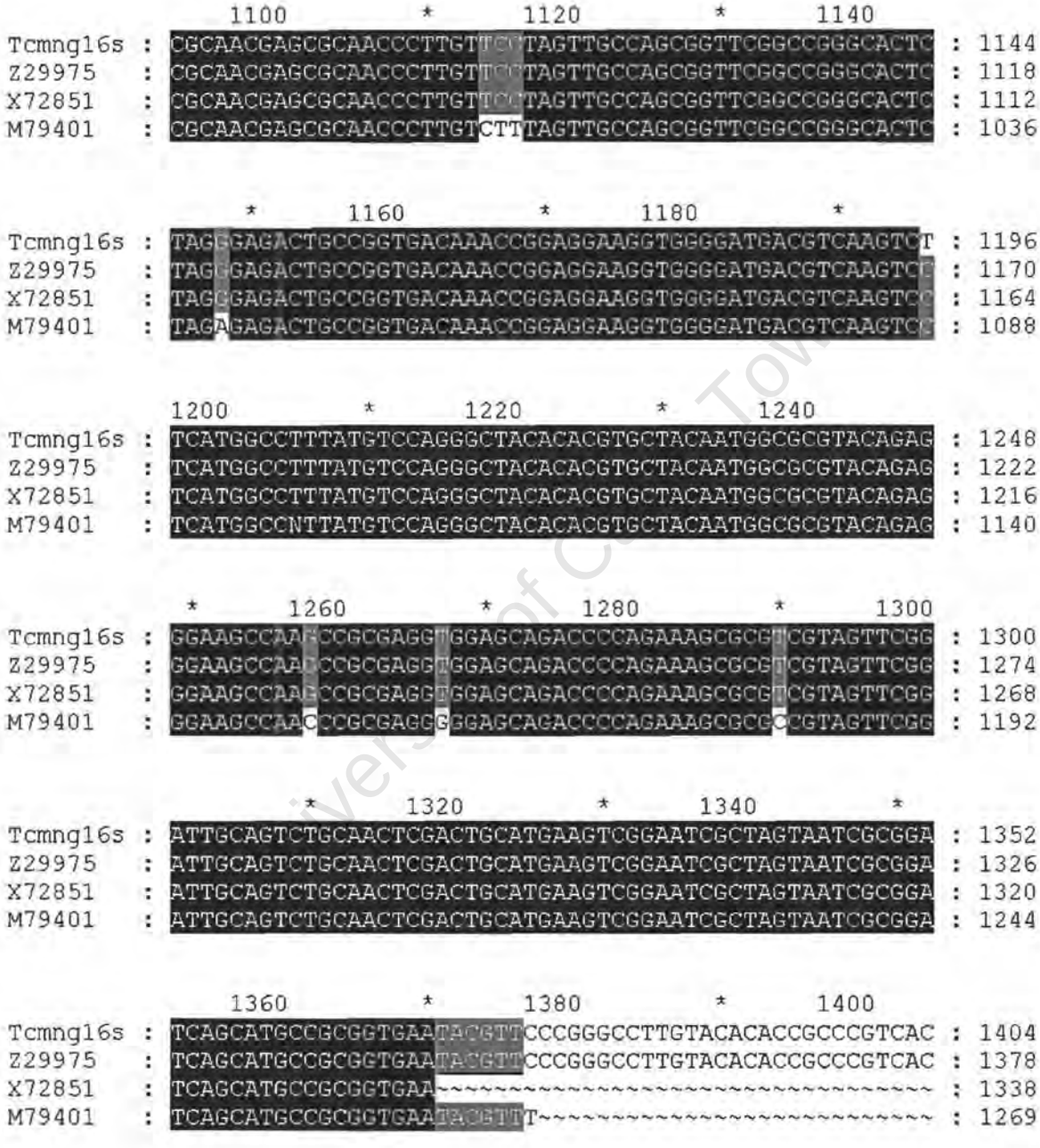
Appendix Dii (cont.)



Appendix Dii (cont.)



Appendix Dii (cont.)



Appendix Dii (cont.)

```
                *      1420          *      1440          *
Tcmng16s : ACCATGGGAGTGGATGGTACCAGAAGCCGTTAGCCTAACCTTCGGGGGGGCG : 1456
Z29975   : ACCATGGGAGTGGATGGTACCAGAAGCCGTTAGCCTAACCTTCGGGGGGGCG : 1430
X72851   : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ :    -
M79401   : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ :    -

           1460          *      1480          *      1500
Tcmng16s : ACGACCACGGTATGGTTCATGACTGGGGTGAAGTCGTAACAAGGTACCCGTC : 1508
Z29975   : ACGACCACGGTATAGTTCATGACTGGGGTG~~~~~~~~~~~~~~~~~~~~~ : 1460
X72851   : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ :    -
M79401   : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ :    -

            *
Tcmng16s : TAGAAGCTT : 1517
Z29975   : ~~~~~~~~~ :    -
X72851   : ~~~~~~~~~ :    -
M79401   : ~~~~~~~~~ :    -
```

Fig B: GeneDoc PILEUP of *T. caldus* MNG (**tcmng16S**) 16S rDNA sequence against *T. caldus* DSM 8584 (**Z29975**), *Thiobacillus* strain C-SH12 (**X72851**), and *T. thiooxidans* DSM 612 (**M79401**). Nucleotides shaded in black are conserved throughout sequences, while those in grey are found in three of the sequences.

iii.) Off-gas analysis of *T. caldus* MNG bioreactor batch growth

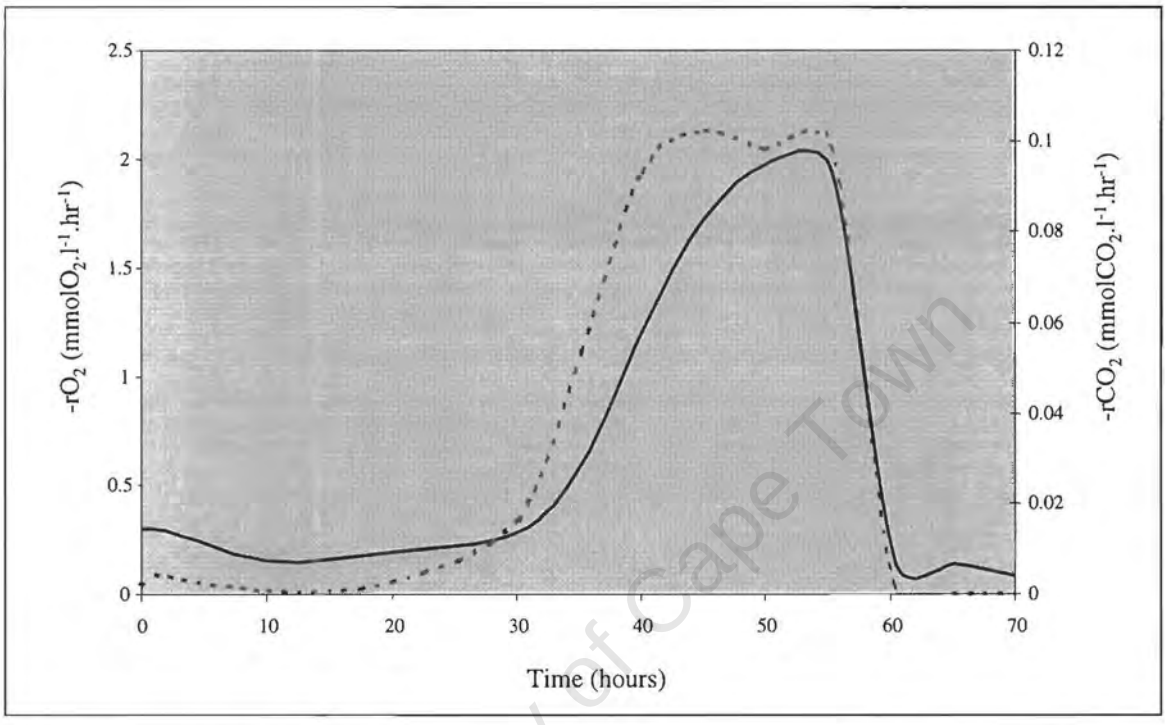


Fig C: Off-gas analysis of *T. caldus* MNG bioreactor during a 70-hour batch growth period. Off-gas analysis for the oxygen utilization rate ($-rO_2$; solid line) and the carbon dioxide utilization rate ($-rCO_2$; dotted line) is expressed as $mmol.l^{-1}.hr^{-1}$.

Appendix E

Equations used for the calculation of oxygen and carbon dioxide utilization rates, and biomass concentration in the Applikon® 3 litre bioreactor.

i) Oxygen utilization rate [$-rO_2$, $molO_2.l^{-1}.hr^{-1}$]

$$-r_{O_2} = \frac{1}{V_{liq}}\left(\phi_{gas,in}[O_2]_{ref} - \phi_{gas,out}[O_2]_{out}\right)$$

where:

r_{O_2}	oxygen production rate	$mol.l^{-1}.h^{-1}$
V_{liq}	liquid volume	l
$\phi_{gas,in}$	volumetric flow rate of the inlet gas	$l.h^{-1}$
$[O_2]_{ref}$	concentration of oxygen in the inlet gas	$mol.l^{-1}$
$\phi_{gas,out}$	volumetric flow rate of the off-gas	$l.h^{-1}$
$[O_2]_{out}$	concentration of oxygen in the off-gas	$mol.l^{-1}$

ii) Carbon dioxide utilization rate [$-rCO_2$, $molCO_2.l^{-1}.hr^{-1}$]

$$-r_{CO_2} = \frac{1}{V_{liq}}\left(\phi_{gas,in}[CO_2]_{ref} - \phi_{gas,out}[CO_2]_{out}\right)$$

where:

r_{CO_2}	carbon dioxide production rate	$mol.l^{-1}.h^{-1}$
$[CO_2]_{ref}$	concentration of carbon dioxide in the inlet gas	$mol.l^{-1}$
$[CO_2]_{out}$	concentration of carbon dioxide in the off-gas	$mol.l^{-1}$

and:

$$\phi_{gas,out} = \phi_{gas,in}\left(\frac{1-[O_2]_{ref}-[CO_2]_{ref}}{1-[O_2]_{out}-[CO_2]_{out}}\right)$$

Appendix E (cont.)

iii) Biomass concentration [c_X, molC.l^{-1}]

$$c_x(t) = c_x(0) - \sum_{t_{i=1}}^{t_{i+1}} \frac{r_{CO_2}(t_i) + r_{CO_2}(t_{i+1})}{2}\left(t_{i+1} - t_i\right)$$

where:

$c_X(t)$	bacterial concentration at time t	molC.l^{-1}
$c_X(0)$	bacterial concentration at time = 0 (inoculum)	molC.l^{-1}
$r_{CO_2}(t_i)$	carbon dioxide production rate at time = t_i	molC.l^{-1}.h^{-1}
$r_{CO_2}(t_{i+1})$	carbon dioxide production rate at time = t_{i+1}	molC.l^{-1}.h^{-1}
$t_{i+1} - t_i$	time between two successive carbon dioxide analyses	h

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