THE STRUCTURE AND MICROBIOLOGY OF FLOATING SULPHIDE OXIDISING BIOFILMS

THESIS

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ABSTRACT

Floating sulphur biofilms are observed as surface layers in numerous aquatic sulphide-rich environments, and apparently play an important role in the cycling of sulphur in its various oxidation states. In addition to the conversion of sulphide to sulphur and/or sulphate species, it has been suspected that subsequent reduction back to sulphide may occur within the floating sulphur biofilm in organic-rich environments. The use of sulphur biofilms for the harvesting of elemental sulphur from wastewater treatment systems has also been suggested. There is, however, little documented information in the literature on the structure of floating sulphur biofilms, or the microbial species responsible for their occurrence. In this study, floating sulphur biofilms were generated in a continuous flow baffle reactor and their structure was examined using scanning electron microscopy. It was found that they occur as layered structures with morphologically distinct bacterial forms present in different layers of the biofilm. The biofilm structure was also found to be dynamic, with structural changes observed as feed conditions were altered. An enriched culture derived from the biofilm demonstrated rates of sulphide oxidation comparable to values reported in the literature for liquid culture systems. The microbiology of the biofilm was studied using traditional plate culture techniques and analysis of rRNA genes. Identification of plate culture isolates as representatives of the biofilm community proved to be limited, leading to a PCR-based cloning approach. The majority of the organisms present in the sulphur biofilm were classified as species in the genus *Pseudomonas*, and a number of other bacterial species whose sulphide oxidising capacity has been noted previously. Surprisingly, only 2% of the clone library consisted of *Thiobacillus* spp., and no sulphate reducing bacteria were identified in the biofilm at all. These results indicate that in organic sulphide-rich environments facultative chemolithoheterotrophic bacterial forms predominate in floating sulphur biofilms, and that the complete biological cycling of sulphur may not occur in these systems.

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ABBREVIATIONS

A	adenine
AMD	acid mine drainage
Amp	ampicillin
bp	base pair(s)
C	cytosine
°C	degrees Celsius
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
ds	double stranded
EDTA	ethylenediaminetetraactetic acid
EPS	extracellular polymeric substance
EtBr	ethidium bromide
FAME	fatty acid methyl ester
FISH	fluorescent *in situ* hybridisation
G	guanine
G+C	glutamine:cytosine ratio
IPTG	isopropyl-β-D-galactosidase
Kb	kilobase pair(s)
L	litre
LA	Luria agar
LB	Luria broth
M	molar
mg	milligram
min	minutes
mL	millilitres
mM	millimolar
PCR	polymerase chain reaction
rDNA	ribosomal deoxyribonucleic acid

RDP	ribosomal database project
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SCLM	scanning confocal laser microscopy
SEM	scanning electron microscopy
SRB	sulphate reducing bacteria
T	thymine
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
µg	microgram
µL	microlitre
µM	micromolar
µm	micrometer
X-Gal	5-bromo-4-chloro-3-indolyl-β-galactosidase

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 SULPHUR IN THE ENVIRONMENT

The distribution of sulphur in the environment has been greatly affected by human urbanisation and industrialisation over the last 100 years. The major source of sulphur pollution is the burning of fossil fuels, which releases sulphur dioxide into the atmosphere, and eventually reaching water-bodies and the soil (Campbell, 1983). Many industries, including tanneries, petrochemical plants and paper mills also release hydrogen sulphide and sulphate into waste water streams (Kuenen and Robertson, 1992). In addition, gold and coal mining operations expose large surface areas of minerals to pyrite-oxidising bacterial activity, which results in the production of sulphate, heavy metal and acid pollution. The resultant acidification of ground waters and surrounding soils is known as acid mine drainage (AMD), or acid rock drainage (Pulles *et al.*, 1995). The current practice in South African mines is either not to treat sulphate containing wastewaters, or to use evaporation ponds, which serves to concentrate rather than eliminate the problem (Pulles *et al.*, 1995).

While sulphate can cause taste and alimentary problems in high concentrations in drinking water, it is not cyto-toxic (Visser *et al.*, 1997). It does, however, contribute to salinity and its presence in the waters of arid countries such as South Africa is therefore a problem (Pulles *et al.*, 1995). Furthermore, sulphate is biologically reduced to sulphide, which is a highly toxic, corrosive chemical, and in high concentrations can cause overloading of the biological sulphur cycle.

Industrial effluents containing sulphide are problematic when released into freshwater systems due to its cyto-toxicity, rotten-egg odour, high oxygen demand and corrosivity (Janssen *et al.*, 1997). At low doses hydrogen sulphide causes injury to the central nervous system of mammals, and death at higher doses (Chung and Huang, 1997). Table 1.1 documents the effects of hydrogen sulphide on human health at various concentrations:

Table 1.1: Effects of sulphide concentration on human health (Guidotti, 1996)

Concentration (ppm)	Effects
0.01-0.3	Odour threshold
1-5	Moderate odour, nausea, headache
10	8 hour occupational exposure limit in Alberta
15	15 minute occupational exposure limit in Alberta
20	Evacuation limit in Alberta, very strong odour
20-50	Eye and lung irritation, digestive upset, loss of appetite
100	Eye and lung irritation, odour disappears
150-200	Severe eye and lung paralysis, sense of smell paralysed
250-500	Pulmonary oedema
500	Severe eye and lung damage, unconsciousness and death in 4-8 hours, amnesia
1000	Breathing stops in 1-2 breaths, immediate collapse

The high oxygen demand of reduced sulphur products such as hydrogen sulphide causes anaerobiosis of river systems, leading to death of the ecosystem (Eckenfelder, 1996). Sulphides may also not be released into sewer pipes as a result of concrete pipe corrosion caused by sulphuric acid, which is formed when hydrogen sulphide reacts with oxygen or nitrate (Dart and

Stretton, 1980), as illustrated in reaction 1 below:

$$2S^{-} + 7O_2 + 2H_2O \rightarrow 2SO_4^{2-} + 2H_2SO_4 \quad \text{..... (1)}$$

This reaction also results in acid rain formation when H_2S is released into the atmosphere. Industries involved in sulphide release into the environment are petroleum refining, wastewater treatment, food processing, pulp and paper manufacturing and coal gasification (Chung and Huang, 1997). South African coal gasification produces 100 000 tonnes of hydrogen sulphide per day (Steudel, pers. comm). Although industrial output of sulphur compounds is relatively low compared to biogenic outputs (de Zwart and Kuenen, 1992), it is important to remove the pollutants produced industrially before they enter delicately balanced eco-systems.

1.2 THE SULPHUR CYCLE

An understanding of the biological sulphur cycle is important in the development of strategies for excess sulphur removal from the environment. Sulphur is of major importance in living systems as it is one of the twelve major bio-elements, and is a constituent of cysteine, methionine, thiamine, pyrophosphate, co-enzyme A, biotin and α-lipoic acid (Gottschalk, 1986). The sulphur cycle involves the cycling of sulphate, organic sulphur, sulphide, and elemental sulphur, as depicted in Figure 1.1. The reductive side of the cycle involves assimilatory and dissimilatory sulphate reduction, where sulphate and sulphur are used as electron acceptors by anaerobic bacteria (Widdel, 1988). The reduced sulphur compounds, including sulphide, produced on the reductive side of the cycle by volcanic emissions, and by microbial activity, are then used in the

oxidative side of the cycle as electron donors for anaerobic and phototrophic bacteria, or to provide energy to the colourless sulphur bacteria (Robertson and Kuenen, 1991).

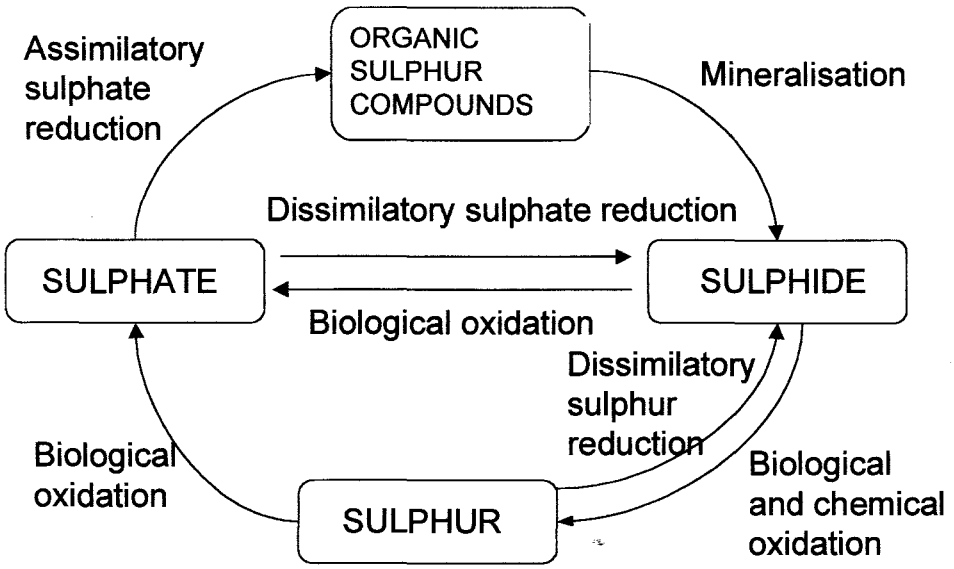


Figure 1.1: The biological sulphur cycle (Janssen *et al.*; 1999)

1.2.1 Sulphate reduction

Dissimilatory sulphate reduction is carried out by the group of bacteria known as sulphate reducing bacteria (SRB), which are found in soil, water and in the ocean. The most important genera within this group are *Desulfovibrio*, *Clostridium* and *Desulfotomaculum* (Campbell, 1983). These heterotrophic anaerobic organisms use sulphate as a terminal electron acceptor, converting it to hydrogen sulphide, as illustrated in the following equation:

$$2CH_2O + 2SO_4^{2-} \rightarrow 2HCO_3^- + H_2S \quad \text{..... (2)}$$

The ability of SRBs to remove sulphate has been exploited in many biotechnological applications,

particularly in the treatment of AMD (Maree and Hill, 1989; Du Preez *et al.*, 1992). Shell Research Ltd. and Paques Co. developed a biological sulphate removal reactor, used at a zinc refinery in the Netherlands, where the sulphide generated by the SRBs is used in heavy metal precipitation (Barnes *et al.*, 1991)

1.2.2 Sulphide oxidation

The hydrogen sulphide produced during dissimilatory sulphate reduction may react with metals in a chemical reaction, forming black metal sulphide precipitates; or alternately the sulphide is converted to sulphur and sulphate by bacterial oxidation. The phylogenetic relationship among sulphur oxidizing bacteria was studied by Lane *et al.* (1992) using 16s rRNA analyses. The genera included in the study were *Thiomicrospira, Thiobacillus, Thiothrix, Acidiphilum, Leptospirillum, Thiovulum, Chromatium* and *Chlorobium.* The authors found that the bacterial species were spread across the archaebacteria and eubacteria, illustrating that the traditional physiological groupings based on metabolism create inconsistencies, with overlapping groupings for many unrelated bacterial species. The three groups of bacteria involved in sulphide oxidation are:

(i) anaerobic phototrophic bacteria;

(ii) colourless sulphur bacteria;

(iii) Certain heterotrophic bacterial groups which have sulphide oxidising capabilities, although they are not as well documented as the colourless sulphide oxidising bacteria.

1.2.2.1 Photosynthetic sulphur bacteria

Photosynthetic sulphide oxidising bacteria, including green and purple sulphur bacteria, use

sulphide as an electron donor for photosynthesis, with CO_2 as a carbon source in a reaction powered by light, as illustrated in reaction 3:

$$CO_2 + H_2S \rightarrow CH_2O + H_2O + 2S \quad \text{..... (3)}$$

Sulphide is converted to sulphur, which may be located intracellularly (e.g. *Chromatium* sp.) or as extracellular sulphur globules (e.g. *Chlorobium* sp.). Alternately, sulphide may be fully oxidised to sulphate. The photosynthetic sulphide oxidisers are of importance in nature only in anaerobic shallow waters, although they are one of the few means to oxidise reduced sulphur compounds under anaerobic conditions (Campbell, 1983). These organisms grow abundantly in stratified lakes with a chemocline not deeper than 15m, which allows light penetration (Widdel, 1988). The genera commonly found in these environments are *Chromatium, Chlorobium, Rhodobacter* and *Thiospirillum.*

The sulphide oxidising potential of photosynthetic sulphur bacteria such as *Chlorobium limicola* have been used in sulphide oxidising bioreactors (Cork *et al.*, 1986; Kim *et al.*, 1990). This efficient photosynthetic sulphide oxidiser transforms 90% of inlet hydrogen sulphide into sulphur (Kim *et al.*, 1990). Photosynthetic sulphur bacteria are not, however, the first choice in biotechnological sulphide removal processes because light is a prerequisite for photosynthesis, increasing energy costs and making reactor design difficult, and also because sulphide oxidation is strictly coupled to growth. Photosynthetic sulphide oxidising bacteria use carbon dioxide as a terminal electron acceptor with 1-2g of sulphur being produced per 1g of cells (Kuenen and Robertson, 1992).

1.2.2.2 Colourless sulphur bacteria

The most widely studied sulphide oxidisers are the colourless sulphur bacteria, a very diverse group which includes both archaebacteria and eubacteria, and which exists due to convergent rather than divergent evolution (Robertson and Kuenen, 1991). Colourless sulphur bacteria were given their name because they do not have photo-pigments, although in dense cultures, the colonies can appear pink or brown because of the presence of large amounts of cytochrome (Robertson and Kuenen, 1991). The genera in the colourless sulphur bacteria group include *Thiobacillus*, *Thiomicrospira*, *Thiospaera*, *Sulfolobus*, *Acidianus*, *Thermothrix*, *Thiovulum*, *Beggiatoa*, *Thiothrix*, *Thioploca*, *Thiodendron*, *Thiobacterium*, *Macromonas*, *Achromatium* and *Thiospira* (Widdel, 1988; Robertson and Kuenen, 1991; Janssen, 1995; Gardner, 1998). The members of these genera have differing pH and thermal requirements for growth, and some are capable of denitrification while others are not.

The thiobacilli are the most well documented group of colourless sulphide oxidising bacteria. They mainly obtain energy from the chemolithotrophic oxidation of inorganic sulphur compounds, which is used to support autotrophic growth using CO_2 (Kelly, 1985). *Thiobacilli* are gram negative, rod-shaped eubacteria, with very high diversity amongst members of the genus. The six obligate chemolithotrophic species have a variation in G+C content from 51-68%, have pH optima from pH 2 to7 and grow at optimal temperatures ranging from 20-30°C (Kelly, 1985). The wide variation in the genus has made it a challenging task to elucidate the enzymatic pathway involved in sulphur metabolism (Kelly, 1985).

Colourless sulphur bacteria may be aerobic or anaerobic, the latter using other inorganic

compounds rather than sulphide as electron donors, such as hydrogen and ferrous iron. *T. denitrificans* has been characterised as an anaerobe, although Sublette (1987) found that aerobic cultures can reduce the sulphide content of gas to a very low level. Under anaerobic conditions, nitrate is used by *T. denitrificans* as a terminal electron acceptor, while producing nitrogen, as described by the following reaction:

$$5H_2S + 8KNO_3 \rightarrow 4K_2SO_4 + H_2SO_4 + 4N_2 + 4H_2O \quad \text{..... (4)}$$

The colourless sulphur bacteria are present wherever reduced sulphur compounds, usually sulphides or sulphur, are found. These environments include hydrothermal vents and hot springs, wastewater treatment plants and soil, where the sulphate produced by sulphide oxidising bacteria is important for soil fertility (Dart and Stretton, 1980; Robertson and Kuenen, 1991; Weller *et al.*, 1991; Voordouw *et al.*, 1996; Taylor *et al.*, 1999). Basu *et al.* (1995) confirmed that a symbiotic relationship existed between sulphate reducing bacteria and the microaerophilic sulphide oxidising *Beggiatoa* sp. in a micro-aerophilic sulphate reducing bioreactor with organic matter removal, using metabolic tests and microscopy. The healthy population of *Beggiatoa,* observed in sludge granules using phase contrast microscopy, converted sulphide produced by the SRBs to sulphate, or to intracellular sulphur, while the SRB population was involved in anaerobic sulphate reduction using organic acids as a carbon and energy source (Basu *et al.*, 1995). Williams and Unz (1995) also found filamentous sulphide oxidising bacteria (*Thiothrix* and *Beggiatoa*) in activated sludge.

Hydrogen sulphide is a commonly used growth substrate because of its abundant formation by the

sulphate reducing bacteria. The problem with hydrogen sulphide as a substrate for bacterial growth is the ready reaction of hydrogen sulphide with oxygen, which explains why the bacteria are found growing at the interface between anoxic, sulphide containing waters and oxygen rich waters (Robertson and Kuenen, 1991; Tonolla *et al.*, 1999). The gradients between the two interfaces can range from micrometers to metres in thickness. Besides competition with oxygen, the sulphide oxidising bacteria also have to compete for sulphide with sulphide-metal precipitation (Stefess *et al.*, 1996).

Colourless sulphur bacteria are able to use reduced sulphur compounds, including organic and inorganic sulphides and also sulphur, as an energy source. The products of sulphide oxidation are elemental sulphur and sulphate. Colourless sulphur bacteria can compete with the chemical oxidation of sulphide due to their high affinity for sulphide, but the amount of sulphur formed is species and strain dependent (Janssen *et al.*, 1997). For example, *Thiobacillus* sp. Strain W5 has been found to stoichiometrically convert sulphide to elemental sulphur under optimal conditions, whereas *T. neapolitanus* is only able to convert 50% of the sulphide supplied into sulphur (Visser *et al.*, 1997). *T. denitrificans* converts sulphide to sulphate only, with no formation of elemental sulphur (Sublette, 1992). Sulphide oxidation is not linked to growth in *Thiobacilli*-like bacteria, because oxygen or nitrate is the terminal electron acceptor, rather than carbon dioxide, as is the case with photosynthetic sulphide oxidising bacteria. This allows colourless sulphur bacterial cells to produce at least 20g of sulphur per 1g cells (Kuenen and Robertson, 1992).

The sulphide oxidising capacity of an organism is dependent on its maximum electron transporting

capacity (Visser *et al.*, 1997). The two reactions occurring in an aerobic sulphide removal system are described in equations 5 and 6 (Janssen *et al.*, 1998):

$$2HS^- + O_2 \rightarrow 2S^0 + 2OH^- \quad \Delta G^0 = -169.35 \text{ kJ.mol}^{-1} \quad \text{..... (5)}$$

$$2HS^- + 4O_2 \rightarrow 2SO_4^{2-} + 2H^+ \quad \Delta G^0 = -732.58 \text{ kJ.mol}^{-1} \quad \text{..... (6)}$$

Under oxygen limiting conditions (dissolved oxygen (DO) values below 0.1 mg/L), sulphur is the major end-product of sulphide oxidation, while sulphate is formed under sulphide limiting conditions. Sulphur formation is also favoured under high sulphide loading rates because it is formed faster than sulphate, allowing the bacteria to remove harmful sulphide faster, although sulphate formation is preferred under optimal conditions because the cells benefit from the higher Gibbs free energy release (Gommers *et al.*, 1988; Boogerd *et al.*, 1991; Robertson and Kuenen, 1991; Visser *et al.*, 1997; Janssen *et al.*, 1998). Because detection of oxygen by DO sensors is not sensitive below 0.1 mg/L, the redox potential can be used to control conditions favourable for sulphur formation. Redox potential is a measure of a solutions' tendency to accept or donate electrons, although in a sulphide-oxidising system, the redox potential is mainly determined by the sulphide concentration, with oxygen having much less of an effect (Janssen *et al.*, 1998).

1.2.2.3. Heterotrophic sulphide oxidisers

Examples of heterotrophic organisms that are capable of oxidising sulphide are: *Pseudomonas, Streptomyces, Arthrobacter, Bacillus, Flavobacterium, Micromonas, Micrococcus, Alcaligenes, Brevibacterium, Achromobacter, Mycobacterium, Aquaspirillum, Xanthobacter, Paracoccus* and

Escherichia coli (Friedrich and Mitrenga, 1981; Gommers and Kuenen, 1988; Chung *et al.*, 1996a; Chung *et al.*, 1996b; Chung *et al.*, 1997; Gallardo *et al.*, 1997). These heterotrophs, which gain metabolically useful energy from oxidation of reduced sulphur compounds, are classified as chemolithoheterotrophs (Gommers and Kuenen, 1988). The heterotrophic *Xanthomonas* sp. strain DY44 studied by Cho *et al.* (1992) is able to oxidise sulphide to elemental sulphur. The rate of conversion is not as high as that by *Thiobacillus* species, although the *Xanthomonas* sp. could be considered for use in sulphide oxidation reactors in cases where organic material is readily available, for example sewage water. *Pseudomonas putida* CH11 was used by Chung *et al.* (1996a) for sulphide removal in a biofilter, with results of above 95% sulphide removal, which is higher than the autotrophic *Thiobacillus* strain tested.

1.3 BIOLOGICAL SULPHIDE CYCLING IN BIOTECHNOLOGY

1.3.1 Chemical sulphide oxidation

Traditional techniques for removing sulphide from industrial waster streams are chemical and physical methods, including chemical oxidation, aeration, ion-exchange resins and absorption with aqueous or organic solvents. Chemical oxidation commonly used in sulphide removal include chlorination, ozonation, potassium permanganate treatment and hydrogen peroxide treatment (Buisman *et al.*, 1989). These methods are not desirable, however, because they are expensive, have high energy requirements, and usually only transfer the waste to another stream, thus involving further disposal costs (Buisman *et al.*, 1989).

The products from chemical oxidation include elemental sulphur, thiosulphate and sulphate (Sublette, 1992). Chemical oxidation reactions are usually exothermic and may cause explosions (Sublette, 1992). Chemical precipitation is also used to remove sulphide from waste streams, but this method results in an iron sulphide sludge which must be disposed of (Buisman *et al.*, 1989). Most removal techniques at present are chemical removal processes, such as the Claus process, where sulphide is converted into liquid sulphur (Guoqiang *et al.*, 1994).

1.3.2 Biological sulphide oxidation

Because of the chemical and energy costs of physico-chemical methods for sulphide removal from waste streams, microbiological removal of sulphides has been investigated. Biological processes can be carried out at atmospheric pressure and at temperatures close to room temperature, thereby reducing energy costs. Biological sulphide removal methods have a higher removal efficiency and also give rise to less secondary pollution than physico-chemical methods (Buisman *et al.*, 1989). Sulphur, rather than sulphate, formation is preferred in the removal of sulphide from industrial waste streams for environmental reasons because it is insoluble and can be removed from the water stream, thus reducing the total amount of sulphur from the wastewater. Sulphate formation also uses four times more oxygen, resulting in higher energy consumption for aeration (Janssen *et al.*, 1998). Another advantage of sulphur formation is that hydroxide ions are formed together with the sulphur, which can be used to absorb sulphide gas from waste gas streams. Sulphate-rich effluent also cannot be discharged into freshwater bodies as, already noted, it contributes to salinity.

Sulphide removal as elemental sulphur by sulphide oxidising bacteria is therefore the preferable route, as the sulphur cycle is broken (Kuenen and Robertson, 1992). The production of biological sulphur rather than sulphate as an end product of sulphide oxidation is also advantageous because the sulphur, which is a potentially valuable commodity, can be recovered as a source of income for the industry involved. An example of a possible use for biologically produced sulphur is in soil-bioleaching processes where biological sulphur is oxidised much faster by *Thiobacilli* than inorganic flowers of sulphur (Tichý,1994). Biological sulphur can also be purified by melting at high temperatures and used in the production of sulphuric acid (Janssen *et al.*, 1999).

Steudel (1988) suggests that elemental sulphur produced by bacteria should be called "hydrophilic sulphur" in order to emphasize the difference between bacterial sulphur and other types of elemental sulphur. The sulphur produced by bacteria is hydrophilic and differs from elemental sulphur which is hydrophobic and has a solubility in water of only $5\mu g.kg^{-1}$ (Hazeu *et al.*, 1988). Biological sulphur is pale yellow or white and forms spherical globules able to dissolve in organic solvents (Janssen *et al.*,1998). Biological sulphur has been classified as liquid-like according to X-ray diffraction, although it does convert to crystalline S_8 when allowed to stand or when dried (Janssen *et al.*, 1998).

The Shell-Paques process operated at the Budelco zinc refinery in the Netherlands is an example of the use of sulphide oxidising bacteria's natural role in the sulphur cycle in a biotechnological application (Scheerem *et al.*, 1993). In the process, illustrated in Figure 1.2, hydrogen sulphide from waste streams is collected in a scrubber, which is then fed to sulphide oxidising bacteria able to convert the sulphide into elemental sulphur. The sulphur is collected by a titled plate settler, resulting in a 99.5% removal of H_2S from the gas stream (Janssen *et al.*, 1999).

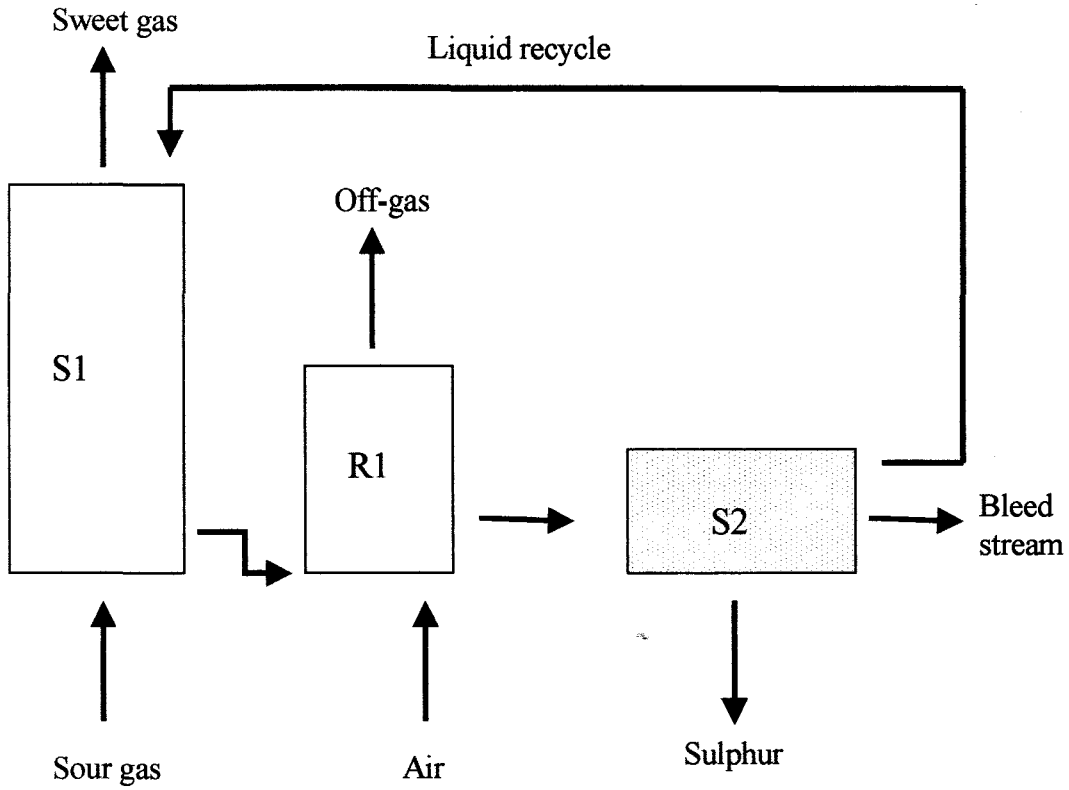


Figure 1.2: The Shell-Paques process for high pressure natural gas desulphurisation: S1=High pressure scrubber, R1=Bioreactor; S2=Tilted plate settler for sulphur removal

Guoqiang *et al.* (1994) developed a desulphurisation process using *Thiobacillus ferrooxidans*, with an iron sulphate feed being converted into sulphur. The bacteria catalysed the conversion of ferrous sulphate to ferric sulphate, which oxidised hydrogen sulphide to elemental sulphur while reducing the ferric sulphate to ferrous sulphate. This reduced solution was then recycled. The following two reactions describe this process:

$$2FeSO_4 + \tfrac{1}{2}\, O_2 + H_2SO_4 \rightarrow Fe_2(SO_4)_3 + H_2O \quad \text{- catalysed by bacteria at pH 2} \quad \ldots\ldots (7)$$

$$H_2S + Fe(SO_4)_3 \rightarrow 2FeSO_4 + H_2SO_4 + S^o \quad \ldots\ldots (8)$$

Sublette (1992) investigated the anaerobic oxidation of a sulphide sludge by *Thiobacillus*

denitrificans, a chemoautotrophic, facultative anaerobe. This bacterium reduces sulphide to sulphate under anaerobic conditions with nitrate as the terminal electron acceptor. The growth of *Thiobacillus denitrificans, T. thioparus, T. versutus, T. neapolitanus* and *T. thiooxidans* on hydrogen sulphide was compared, and it was found that none of these species were better sulphide oxidisers than *T. denitrificans* (Cadenhead and Sublette, 1990).

1.4 BACTERIAL BIOFILMS

Bacterial biofilms are defined as a non-uniform group of cells immobilised in an extracellular polymeric matrix (Nivens *et al.,* 1995). Biofilms consist of solids with a liquid phase in the interstitial spaces between the solids and bacterial colonies. They are mostly spatially heterogenous systems with complex groupings of cells ranged along physical and nutrient gradients (Zhang and Bishop, 1994). Microbial communities within biofilms may have very high metabolic rates due to dense microbial populations and the high availability of nutrients which occur at times (Kühl and Jørgensen, 1992).

The formation of a biofilm is advantageous to cells because being fixed in a flowing system increases the availability of nutrients and creates micro-niches within the biofilm which allows the growth of organisms which would otherwise not be able to grow in the surrounding environment. An example would be the creation of anaerobic micro-niches in an aerobic environment (Nivens *et al.,* 1995). Biofilms also allow metabolic co-operation between different species within the biofilm (Costerton, 1995), although there is great competition for nutrients and a susceptibility to predators (Nivens *et al.,* 1995).

1.5 METHODS USED IN THE STUDY OF BIOFILMS

Due to their fragility, complex organisation and small size, biofilms are difficult to study. There are nevertheless several tools available to biofilm researchers, including microscopy, micro-profiling, traditional isolation and culture techniques, and molecular techniques.

1.5.1 Microscopy

Biofilms are difficult to study using light microscopy, although the use of differential stains gives useful information about the chemical composition and the structure of the biofilm. Scanning electron microscopy (SEM) is a powerful tool for observing the internal structure of a biofilm, because this form of microscopy has a large depth of field and very high resolution. However, preparation techniques may leave artifacts (Stewart *et al.*, 1995). The form of microscopy which gives the most detailed information to date is scanning confocal laser microscopy (SCLM). SCLM allows a detailed, non-destructive examination of biofilms, giving qualitative information on the structure of fully hydrated biofilms (Massol-Dayà *et al.*, 1995). This microscope uses confocal apertures to create a thin plane of focus, with all other light blocked out, resulting in very clear images (Chalmers *et al.*, 1997). Fluorescent oligonucleotide probes can be used to measure gene expression and cellular conditions in three dimensions within the living biofilm (Nivens *et al.*, 1995). SCLM combined with fluorescent probes allows a detailed investigation into the three-dimensional structure of a biofilm because it allows the simultaneous sequestering of images using a range of wavelengths and emission filters (Lawrence *et al.*, 1998). SCLM is, however, limited to thin biofilms because laser light can only penetrate less than 200μm, therefore most biofilms

studied to date using this technique have been young, thin ones (Bishop, 1997).

1.5.2 Micro-profiling

The use of micro-electrodes was introduced into microbial ecology by Revsbech (1989), who constructed O_2 micro-electrodes for profiling chemical gradients within biofilms. Since then micro-electrodes have been developed which can measure pH, N_2O, NH_4^+, NO_3^-, S^{2-}, H_2S, NO_2, CH_4, and CO_2 (de Beer and Schramm, 1999). The ideal micro-electrode would have a small tip (size of bacterium), be stable, sensitive, measure only one chemical species, and be reproducible and accurate (Nivens *et al.*, 1995). In reality micro-electrodes measure interferences, the signal drifts, they are fragile, must be calibrated and are difficult to manufacture. Another disadvantage is that their insertion into a biofilm can cause structural damage (Nivens *et al.*, 1995). Sulphide, pH and oxygen microelectrodes have been used to study the internal sulphur cycle in a 3-5mm thick wastewater biofilm from a trickle filter, and to measure the gradients within green sulphur bacteria biofilms and aerobic biofilms from wastewater treatment plants (Kühl and Jørgensen, 1992; Pringault *et al.*, 1998; Yu and Bishop, 1998). Schramm *et al.* (1997) used oxygen and nitrate microprobes to determine the metabolic activity of a nitrifying biofilm.

1.5.3 Molecular techniques

The traditional method of studying microbial populations within biofilms begins with the isolation of pure cultures of bacterial strains from the environment of interest. Strain isolation has, however, been replaced by molecular techniques because isolation may introduce serious biases

into a study. Salhani and Uelker-Deffur (1998) have noted that less than 10% of microbes isolated from environmental samples can be cultured . When the organisms can be grown in pure culture, phenotypes may be altered because gene expression is strongly influenced by environmental constraints and the growth mode, and the cultivated organisms, which may also adapt genetically to the culture conditions, would then not be representative of the naturally occurring microbes (Lee *et al.*, 1999). Procedures currently used in microbial ecology are mostly molecular techniques which do not require the isolation of pure cultures from the environment, including deoxy/ribonucleic acid (DNA/RNA) extractions, polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), cloning, denaturing gradient gel electrophoresis (DGGE) and nucleic acid probing (Kane *et al.*, 1992; Rawlings, 1995; Kalmbach *et al.*, 1997; Muyzer and Ramsig, 1998; Ramsig, 1998; Salhani and Uelker-Deffur, 1998; Eichner *et al.*, 1999; Heuer *et al.*, 1999; Schönhuber *et al.*, 1999)

1.5.4 Fluorescent *in situ* hybridisation

A very powerful technique for studying the structure of bacterial populations within a biofilm is fluorescent *in situ* hybridisation (FISH), where labelled (^{32}P, fluorescent dye, or enzyme linked antibodies) oligonucleotide probes are used in conjunction with fluorescence microscopy, usually SCLM. This method has been used to identify unculturable organisms and to characterise bacteria. Lee *et al.* (1999) combined FISH performed with rRNA targeted probes and micro-autoradiography for simultaneous *in situ* identification and determination of substrate uptake patterns in complex microbial communities. FISH does not disrupt the biofilm, or remove cells from their growth conditions, compared to traditional methods where disruption of the biofilm is

required in order to cultivate the microorganisms.

Problems with FISH include uneven cell penetration, low signal intensity, high levels of background autofluorescence from phototrophs, and unspecific staining of inorganic particles giving a signal stronger than the probe signal. Another problem in the use of FISH is that quantification is difficult. With irregularly shaped cells or clusters of cells, there can be insufficient permeability of cells and 10000 cells/mL are needed to detect one cell because of microscopy limitations (Ramsig,1998).

The microbial populations of sludge granules have been studied using FISH to visualise uncultured organisms and methanogens by making rDNA probes of dominant species from clone libraries (Amann *et al.*, 1996; Rocheleau *et al.*, 1999; Sekiguchi *et al.*, 1999). Okabe *et al.* (1999) studied micro-aerophilic wastewater biofilm consisting of ammonia-oxidising, nitrate reducing and sulphate reducing bacteria, while Neef *et al.* (1996) examined the community in a denitrifying sand filter using whole cell hybridization

1.6 BIOFILM STRUCTURE

An attached biofilm reaches steady state when the rate of biofilm growth is equal to the detachment by sloughing off of the biofilm particles (Jahn and Neilsen, 1998). Biofilms are highly hydrated structures composed mostly of interstitial space and extracellular materials, with only 2-17% of the biofilm consisting of biomass. The extracellular matrix consists of polysaccharides (~65%), proteins, nucleic acids and lipids (Lazarova and Manem, 1995). In a heterotrophic

biofilm studied by Zhang and Bishop (1994), the ratio of viable cells to total biomass was highest in the top layers (72-91%) and decreased down into the biofilm (31-39%), with the biofilm also becoming less porous from the top to the bottom. Most biofilms are attached, although aggregates are a special type of biofilm, where the attachment is to the biofilm itself rather than to a substrate, and the biofilm floats freely in liquid medium (Nivens *et al.*, 1995).

The structure of the biofilm is influenced by substrate loading on the biofilm surface, shear forces and the types of organisms in the biofilm (van Loosdrecht *et al.*, 1995). A higher shear rate leads to a more dense biofilm because the protruding pieces are "shaved" off (van Loosdrecht *et al.*, 1997). Increasing shear rates also result in increased eddy diffusion, thus bringing more substrate into contact with the biofilm. This is a contributing factor to the less porous biofilm structure at a higher shear rate, along with mechanical removal of biofilm (Wimpenny and Colasanti, 1997). Active cells deep within the biofilm help to keep the biofilm attached, making it more resistant to sloughing off. The biofilm population is uneven, with non-uniform distribution of biomass. Species composition is important in determining biofilm structure (Murga *et al.*, 1995).

1.6.1 Biofilm growth

There are two main models of biofilm growth:

1. The "continuum model", which states that biofilms consist of living and dead biomass, cell debris and extra-cellular polysaccharide, with nutrients diffusing through a liquid boundary layer at the biofilms' surface. The diffusion is influenced by density of the biofilm, age, porosity, thickness and the microbes present within the biofilm. Growth of the biofilm begins with

attachment of bacteria to a surface by the polysaccharide-rich glycocalyx, which also acts as an ion-exchange matrix trapping nutrients and collecting digestive enzymes released by the bacteria, thus increasing metabolic activity (Morton and Surman, 1994). The cells then undergo horizontal and vertical growth, leading to the convergence of the separate colonies, and continued upwards growth (Bishop, 1997). The biofilm is assumed to be continuous and homogeneous. New research has, however, indicated that biofilms are much more heterogenous than previously thought, leading to gradients of cell types and physical and chemical characteristics such as porosity, microbial density and species, presence of water channels and availability of nutrients and gases (De Beer *et al.*, 1994; Massol-Deyà *et al.*, 1995; Murga *et al.*, 1995; Okabe *et al.*, 1996; Bishop, 1997). The bacterial species present change with depth into the biofilm. At the surface there is an actively dividing population and towards base of the biofilm, cells begin to die because the substrate is limiting (Bishop,1997);

2. The "continuum model" has now been replaced by the cluster model since scanning confocal laser microscopy (SCLM) has given more detail on the internal structure of biofilms, and has shown that biofilms do not grow as a continuum. The findings suggest the biofilms grow as separate colonies between actively maintained pores or channels which facilitate transport of nutrients and gases (Bishop, 1997). The maintenance of these channels is a microbial strategy to increase the biological surface area relative to volume and may facilitate the transport of substrates, nutrients and gases right into the biofilm (Massol-Deyà *et al.*, 1995). The channels also allow end-product toxins to be removed from the cells (Silyn-Roberts and Lewis, 1997). It is possible that the channels seen under SCLM are filled with a substance transparent to laser light, such as extracellular polysaccharide material (Bishop,1997).

Channels into the biofilm are actively maintained for long periods of time, which conflicts with the theory that diffusion is uniform within the biofilm (Massol-Deyà *et al.*, 1995). The development of micro-colonies is controlled so as to prevent the closure of the water channels (Costerton,1995). This organised pattern of growth suggests that cell-to-cell communication may take place within the biofilm community (Massol-Deyà *et al.* 1995). Some biofilms consist of mushroom shaped colonies which indicates a level of growth control through cell-to-cell communication (Costerton, 1995). Massol-Deyà *et al.* (1995) found that after 63 days, the biofilm became lobed, which became more pronounced with time, revealing maintenance of the channels separating microbial communities by colony growth.

Biofilm structure represents an optimal arrangement for influx of nutrients and efflux of by-products. Aerobic biofilms consist of microbial clusters and interstitial voids which transport oxygen and liquid to the microbial cells (de Beer *et al.*, 1994). Interstitial voids supply 50% of the O_2 consumed by microbes in a sulphate reducing biofilm grown on domestic wastewater (Okabe *et al.*, 1996). Specific activity within the biofilm decreases rapidly with biofilm thickness because the number of active bacteria in the biofilm remains constant with age, but inactive bacteria accumulate (Moreau *et al.*, 1994).

1.6.2 Mass transport

The traditional description of mass transport within a biofilm assumes that there is a film of liquid (diffusive boundary layer) flowing adjacent to the boundary which provides the same mass transfer

resistance as in the flowing liquid, therefore mass transport occurs only as diffusion (Rasmussen and Lewandowski, 1998). Nuclear magnetic resonance (NMR) studies which showed that water moves within the biofilm through channels, have helped to prove that convection is one of the important factors in mass transport within the biofilm, although it is very difficult to quantify because of complicated hydrodynamics (Stoodley *et al.*, 1997). Rasmussen and Lewandowski (1998) found that convective mass transport is active near the biofilm/liquid interfaces, and is affected by the spacial distribution of microbes and other non-viable material (Zhang and Bishop, 1994).

1.7 MIXED POPULATION BIOFILMS

Most biofilms found in the environment, including floating sulphur biofilms, are mixed population biofilms. Biofilms consisting of mixed populations have been found to be thicker and more stable than single species biofilms. This may be due to one of the species having a stabilising function, for example, by the production of a polysaccharide (James *et al.*, 1995). Microbial communities often have a greater capacity to survive than individual species of microbes. James *et al.* (1995) has described the different types of interaction within mixed population communities:

- Neutralism - populations are not affected by each other which can be because of spacial separation but can also occur between closely situated populations (e.g. activated sludge);
- Competition - two or more populations compete for nutrients, which can lead to dominance of one species, but the other still remains present;
- Commensalism - the presence of one population is advantageous to another population. This is common in biofilms, for example, when aerobic organisms utilise O_2, thus creating

an anaerobic micro-environment for obligate anaerobes;

- Protocooperation - both species benefit from the presence of each other, e.g. phototrophic and heterotrophic interactions. Protocooperation is important for biofilm structure, where production of attachment particles, such as sulphur crystals, by one species allows the attachment of another species.

Biofilms can support a mixed population of microbes, allowing the development of micro-niches which enables the growth of important slow-growing organisms, such as autotrophic sulphide oxidising bacteria. It is possible that within a multispecies biofilm individual species are present as single species micro-colonies adjacent to each other (Bishop, 1997). This is evident in waste-wasters, where the presence of organic substrates results in constant competition between autotrophs and heterotrophs for space in the biofilm (Rittmann and Manem, 1992). In a multispecies biofilm the different species present may compete for the same substrate or utilise different substrates, but they will always compete for space in the biofilm. In a competitive situation, the species with the higher growth rate will grow closer to the liquid film than a slower growing organism (Rittmann and Manem, 1992).

1.7.1 Sulphate reducing biofilms

Sulphate reducing biofilms, in which sulphide oxidation also takes place, are an example of mixed-population biofilms. These biofilms occur in domestic waste-water treatment plants where organic material is readily available, and where sulphate is present. Sulphate reduction will take place wherever sulphate is present, even if the water is aerated, resulting in the production of hydrogen

sulphide (Okabe *et al.*, 1999)

The sulphur cycle in aerobic and anaerobic wastewater biofilms is very complex and presently not well described, although sulphate reducing biofilms have been documented by a number of researchers (Kühl and Jørgensen, 1992; De Beer *et al.*, 1994; Goebel and Stackebrandt, 1994; Kolmert *et al.*, 1997; Okabe *et al.*, 1998; Yu and Bishop, 1998; Okabe *et al.*, 1999). Okabe *et al.* (1998) used FISH probes and kinetic data to study an attached sulphate reducing biofilm grown on domestic wastewater, and found that the surface was covered with a grey coloured sulphur oxidising *Beggiatoa* spp. mat with the highest concentration of SRBs in the middle of the porous biofilm. SRBs were most prominent 500-600μm from the unattached surface. Sulphide was produced 700-950μm from the surface, and depleted 450 - 200μm from the surface, with a specific sulphide oxidation rate of 12.2 H_2S μmol. cm^{-3}. h^{-1} (Okabe *et al.*, 1999).

1.7.1.1 Sulphide oxidation in sulphate reducing biofilms

Data on sulphide oxidation within biofilms can be obtained from experiments carried out on sulphate reducing biofilms, which have been extensively studied. The sulphide produced in sulphate reducing biofilms grown in wastewater treatment plants does not diffuse out of the biofilm as it is completely oxidised in the sulphide oxidising zone. The bacterial oxidation of sulphide in sulphate reducing biofilms occurs in an anaerobic zone just below the oxygen respiration zone and just above the sulphate reduction zone (Kühl and Jørgensen, 1992; Santegoeds *et al.*,1998; Okabe *et al.*, 1999).

Kühl and Jørgensen (1992) measured the sulphide turnover rate in the biofilm, and found that it

was extremely fast (7 seconds), compared to the chemical oxidation rate of sulphide in water at room temperature (half life of 30 minutes to several hours), which confirms that aerobic microbial oxidation of the sulphide must be responsible for the very high sulphide turnover rates (Santegoeds *et al.*,1998; Okabe *et al.*, 1999).

1.8 ATTACHMENT OF SULPHIDE OXIDISING BACTERIA

The attachment of sulphide oxidising bacteria to surfaces is an important consideration in the design of sulphide-oxidising bio-reactors. Nemati and Webb (1997) reported the immobilisation of *Thiobacillus ferrooxidans* onto polyurethane biomass support particles, while Ongcharit *et al.* (1989 and 1990) immobilised *Thiobacillus denitrificans* by mixing a culture with washed sludge, resulting in this autotroph being immobilised in macroscopic flocs by co-culture with floc-forming heterotrophs. Photosynthetic sulphide oxidising bacteria have also been immobilised in sulphide oxidising bioreactors. *Chlorobium limicola* has been immobilised to the inside of a transparent vinyl plastic tubing, which allowed light through to the bacterial cells, while *Chlorobium thiosulfatophilum* was immobilised in strontium alginate beads (Kim *et al.*, 1990; Henshaw *et al.*, 1999). Chung *et al.* (1996b) immobilised *Thiobacillus thioparus* and *Pseudomonas putida* in calcium alginate beads, where the heterotrophic *Pseudomonas* was shown to be a more successful sulphide oxidiser.

1.9 FLOATING SULPHUR BIOFILMS

Sulphate reducing bio-reactors developed at Rhodes University to treat mine drainage wastewaters

using complex carbon sources have been described by Boshoff *et al.*; 1996; Rose *et al.*, 1996; Van Hille and Duncan, 1996; Molipane, 1998; and Rose *et al.*, 1998. Floating sulphur biofilms, visible as a white biofilm layer, have been observed on top of these sulphate digesting bioreactors and on the surface of sulphide generating facultative ponds and tannery waste stabilisation ponds (P. Rose, pers. comm.). A white film adhering to the glass walls of a sulphide rich reactor at the gas-liquid interface was also reported by Gadre (1989). These biofilms are suspected to consist of a sulphide oxidising bacterial community involved in the conversion of the high concentrations of sulphide to elemental sulphur and their use as a means of final removal of sulphate in wastewater treatment has been suggested.

Studies on sulphide oxidising biofilms relate mainly to attached biofilms, with few reported studies in the literature on floating biofilms. In natural environments, such as aquatic sediments, bogs, marshes and lakes, hydrogen sulphide rarely reaches the atmosphere because mats of sulphide oxidising organisms grow at the sulphide-oxygen interface, and convert the sulphide to sulphate or elemental sulphur. The importance of this process is reflected in the fact that many natural sulphur deposits on earth originate from microbiological action.

Sulphur biofilms which float on the surface of sulphide-rich waters could also play a role in nutrient cycling within the biological sulphur cycle. Rapid cycling of sulphur through both its oxidised and reduced forms within attached biofilms grown on domestic wastewaters has been documented by numerous authors (Kühl and Jørgensen, 1992; De Beer *et al.*, 1994; Goebel and Stackebrandt, 1994; Kolmert *et al.*, 1997; Okabe *et al.*, 1998; Yu and Bishop, 1998; Okabe *et al.*, 1999). This cycling could play an important role in the overall reduction of the organic load in

these systems. Little information is available relating to the microbial forms occuring in floating sulphur biofilms.

1.10 RESEARCH OBJECTIVES

Given the poor availability of information about floating sulphur biofilms the current study was undertaken to investigate their structure and microbiology. It is unknown whether the biofilms are composed of a complex assortment of micro-organisms which grow in separate parts in the biofilm, or whether they are simple, amorphous single species systems. It may be expected that the structure of the biofilm would determine its function, with a multi-species system involved in both sulphur and nutrient cycling. Single species biofilms would be expected to perform only limited chemical reactions without complete sulphur or nutrient cycling taking place. The types of micro-organisms present in sulphide oxidising biofilms have not been documented, and it is unknown whether the bacterial species occurring are autotrophic, mixotrophic or heterotrophic sulphide oxidisers.

Sulphur biofilms may be examined by studying the biofilm structure and the microbial populations found within them, thus enabling a determination of the microbes' roles within the biofilm. The environmental conditions which affect the biofilms' structure and composition also need to be known. After the physical, chemical and biological properties of the floating sulphur biofilm have been determined, sulphur biofilm formation could be optimised for use in industrial waste water treatment processes to oxidise sulphide and facilitate the final removal of sulphate salinity as sulphur.

The following research objectives were identified for this study of the structure and microbiology of floating sulphur biofilms:

1. To develop and optimise a laboratory-scale bio-reactor for the cultivation of floating sulphur biofilms to enable the experimental evaluation of these systems;
2. To undertake an examination of the structure of sulphur biofilms and to determine structure-function relationships occurring within the biofilms;
3. To determine the sulphide oxidising capacity of the microorganisms occurring in the biofilm;
4. To identify the bacterial species present in sulphur biofilms.

CHAPTER 2

DEVELOPMENT AND PERFORMANCE OF AN EXPERIMENTAL FLOATING SULPHUR BIOFILM

2.1 INTRODUCTION

While there have been some observations of sulphur biofilms in hydrothermal vents, hot springs, on the surface of sulphidogenic ponds, in domestic wastewater plants and in the Rhodes Biosure Process pilot study (Weller *et al.*, 1991; Taylor *et al.*, 1999; P.Rose and C. Corbett, pers. comm), very little is known about their development and performance. The study of floating sulphur biofilm development required a laboratory system which allowed the maintenance of a continuous flow of nutrient medium to the biofilm, without disrupting or causing washout of the biofilm. To date, there have been no reports of such an experimental system for these biofilms in the literature.

This chapter describes the development of a baffled reactor model system which satisfied the growth requirements of a sulphur biofilm layer as well as a sulphate reducing bacterial (SRB) sludge bed. The SRB sludge produced the constantly high levels of sulphide necessary for the formation of a sulphur biofilm. The baffled reactor design allowed a comparative study of the biofilm where sulphide and COD gradients occurred across the reactor baffles, while operating conditions and the inoculum

remained identical through the reactor. This study of floating sulphur biofilms was undertaken in order to describe their growth and performance.

2.2 METHODS AND MATERIALS

2.2.1 Development of an experimental biofilm model system

A schematic diagram of the laboratory-scale baffled reactor used to grow a model sulphur biofilm is shown in Figure 2.1. The reactor was fed at a flow rate of 2.16 L/day (90 mL/hr), resulting in a hydraulic retention time of approximately 4 days. The bottom each of the four valleys was filled with a SRB sludge obtained from the Grahamstown Disposal Works. The baffles were designed to force the lactate feed (Appendix A) to circulate throughout the reactor, thereby ensuring that the feed reached the SRB sludge bed at the bottom of the valleys, and thus preventing a flow which only passed over the surface (Figure 2.1).

The SRB growing in the reactor produced sulphide from the sulphate in the feed, resulting in a continuous high concentration of sulphide, which was utilised by the sulphide oxidising bacteria on the surface of the reactor. The sulphide oxidising bacteria formed a sulphur biofilm on the surface of the reactor, which was used in this study. After 45 days of reactor operation, the lactate supplied in the feed was increased by 50%. The sampling positions are indicated as V1-V5 in Figure 2.1, where five samples of the biofilm were taken, between the four baffles. These five sampling points were

given the names "valley 1" to "valley 5", although they do not correspond directly to the actual valleys of the reactor.

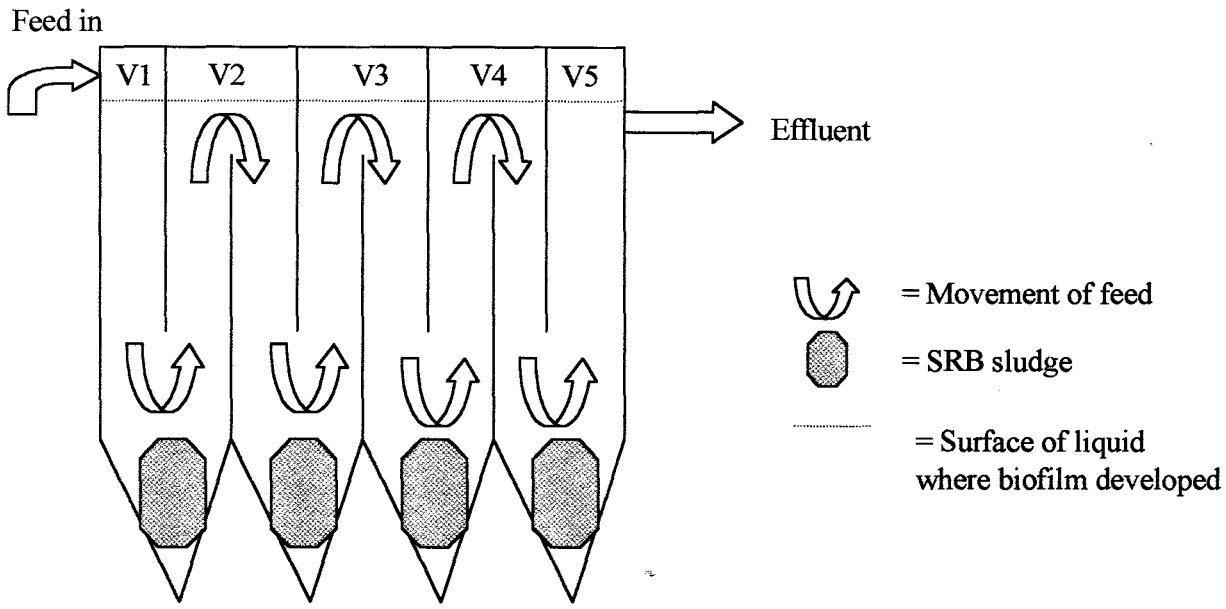


Figure 2.1: Schematic diagram of the baffled reactor used to cultivate the sulphur biofilm. The dimensions of the reactor were 140 cm x 180 cm x 170 cm, with a volume of 8.5L

Brittleness, coverage and colour were subjective observations made of the biofilm at 4, 6 and 8 weeks after start-up of the reactor. Brittleness was measured on a scale of 1-5 according to how easily the biofilm broke after disruption with the tip of a Pasteur pipette. Coverage indicated the percentage of the biofilm covering the surface of each valley, and colour was described as the biofilm changed from transparent to white to yellow.

2.2.2 Sulphide, sulphate and sulphur determination

Sulphide in the reactor was monitored each day using the Methylene blue method (Trüper and

Schlegel, 1964):

A 100 µL sample was added to 100 µL of 15% zinc acetate and 4800 µL of dH_2O. 500 µL ferric chloride (16 g/L in 6M HCl) and 500 µL amine-sulphuric acid stock solution (4 g/L N-N dimethyl-p-pheylenediamine dihydrochloride in 6M HCl) was then added to the solution and left to stand for 1 hour before reading the absorbance at 670 nm (See Appendix F for standard curve).

The sulphate concentration was determined using the turbidometric barium chloride method (APHA, 1976):

A 200 µL sample was added to 4800 µL dH2O, 1 mL buffer solution A (30 g magnesium chloride, 5 g sodium acetate, 1 g potassium nitrate, 20 mL acetic acid, made up to 1L with dH_2O) and 500 µL of a 40 g/L barium chloride solution. The solution was vortexed for 60 seconds, before reading the absorbance at 420 nm (See Appendix F for standard curve).

Sulphur was determined using an HPLC method, modified from Möckel (1984):

A 5 mL sample was centrifuged in a Beckman JA-21 rotor at 18 000 rpm (37 000 g) for 20 minutes before extracting the pellet overnight at 4°C in 1 mL acetone. The following day the suspension was vortexed and microfuged for 5 minutes at 13 000 rpm. 20 µL of the supernatant was injected into the sample chamber of the HPLC (Beckman Instruments, USA) fitted with a reverse-phase Nucleosil 5 µm C_{18} column (Machery-Nagel, Germany). The mobile phase consisted of 97% methanol in dddH_2O with a flow rate of 1 mL per minute. Peaks were detected using a Beckman 168 diode array detector at 270 nm and analysed using Beckman System Gold software version 6.0 (See Appendix F for standard curve).

2.2.3 Chemical oxygen demand (COD)

The Spectroquant COD kit (Merck) was used in the determination of COD concentration, according to the manufacturer's instructions.

2.2.4 Inoculum for stirred fed-batch reactor studies

Sulphide oxidising bacteria were isolated from the biofilm by growing a biofilm inoculum on a minimal salt medium with 166 mg/L sulphide, in the HS^- form at pH 8, as the sole electron donor and energy source, described in Appendix A. This was done in a 1L stirred reactor on a fed batch basis; every day a 20 mL sample was removed from the reactor and 20 mL fresh medium and sulphide was added, to give a final sulphide concentration of 166 mg/L. When anaerobic conditions were required, the reactor was flushed each day with N_2 gas for 15 minutes, after which all ports in the reactor were closed with rubber stoppers.

2.2.5 Flask studies to determine sulphide toxicity

10 mL inoculum (as described above) was added to each of seven 100 mL Erlenmeyer flasks containing 75 mL minimal salts medium (Appendix A). The flasks were operated on a fed-batch system, by adding 5 mL diluted sulphide stock solution (12 g/L sulphide in 0.2 M NaOH) to give

a specific final sulphide concentration for each flask, ranging from 65 to 265 mg/L. 5 mL samples were removed from the flasks each day for sulphur, sulphide and sulphate analysis. The pH was maintained at pH 7.8, and the flasks were incubated at 25°C on an orbital shaker set at 100 rpm. The sulphide removal ability of the inoculum in the flasks was monitored by measuring the sulphide remaining in the flasks each day.

After 5 days, the sulphide concentration of the growth media was increased by 66% in each flask, for example, the flask with a starting concentration of 65 mg/L was increased to 110 mg/L. The experiment was run for 9 days and each sulphide concentration was done in triplicate.

2.2.6 Flask studies to determine the optimal sulphide oxidation temperature

75 mL autoclaved autotrophic medium (Appendix A) with a sulphide concentration of 166 mg/L, and 10 mL inoculum (described in 2.2.4) was added to a 100 mL Erlenmeyer flask, and placed in a shaking incubator (100 rpm) set at a determined temperature (10, 15, 20, 25, 30 and 37°C), for 9 hours. The sulphide removal rate, determined by measuring sulphide remaining in solution each hour for a nine hour period, was used to measure the activity of the inoculum at different temperatures. Controls, used to calculate chemical sulphide oxidation, contained 75 mL autoclaved media and 10 mL autoclaved ddH_2O. All experiments were repeated in triplicate. Biological sulphide removal was calculated by subtracting chemical sulphide oxidation from the total sulphide oxidation, thereby removing the effect of chemical sulphide oxidation, which can be considerable, especially at higher temperatures.

2.2.7 Biomass determination

Bacterial cells were counted using a Neubauer chamber (Marienfeld, Germany). The cells were observed at 100X magnification with a Olympus CH2 (Japan) light microscope, using phase contrast to visualise the unstained cells.

2.3 RESULTS AND DISCUSSION

2.3.1 Monitoring the surface layer of the reactor supporting sulphur biofilm growth

Biofilm growth was studied using the laboratory-scale baffled reactor. The SRB present in the sludge bed in the valleys of the reactor produced sulphide as a by-product of sulphate reduction, as described by equation 1 below. The sulphide was utilised by the sulphide oxidising bacteria on the surface of the reactor.

$$2(CH_2O) + SO_4^{2-} \rightarrow 2HCO_3^- + H_2S \quad \text{..... (1)}$$

It is assumed that the redox potential would drop dramatically at the liquid-air interface, from the aerobic head-space to the liquid of the reactor, which is high in sulphide and anaerobic due to the metabolism of organic compounds by SRB. For this reason, sulphide oxidising bacteria would form a sulphur biofilm on the surface of the liquid, at the sulphide-oxygen interface, because the cells require both oxygen and sulphide. Grobicki and Stuckey (1991) have reported the effective

separation of microorganisms within each zone of a baffled reactor, which proved useful in the study of the sulphur biofilms as these are retained by the baffles in each compartment and cannot float off the surface of the reactor.

2.3.1.1 Sulphide concentrations in the baffled reactor

Figure 2.2a illustrates the sulphide concentration in the five valleys of the baffled reactor over a 50 day period, where the sulphide concentration increased from valley 1 to valley 5. The results in Table 2.1 illustrate that from valleys 1 to 5 the biofilm became more brittle and the colour changed from transparent or white to yellow as more sulphur was produced. This may be related to the increase in sulphide concentration across the valleys, which was available for conversion to elemental sulphur by sulphide oxidising bacteria in the biofilm.

The sulphide produced by the SRB sludge moved along the length of the reactor with the feed, resulting in higher sulphide concentrations in valleys 4 and 5 than in valleys 1 and 2. The sulphide concentration in valleys 4 and 5 could also have been higher than valley 1 and 2 as a result of a more metabolically active SRB sludge, although it is more likely that the metabolic activity of all the sludge beds were similar, and the sulphide produced in the earlier valleys was moved along to

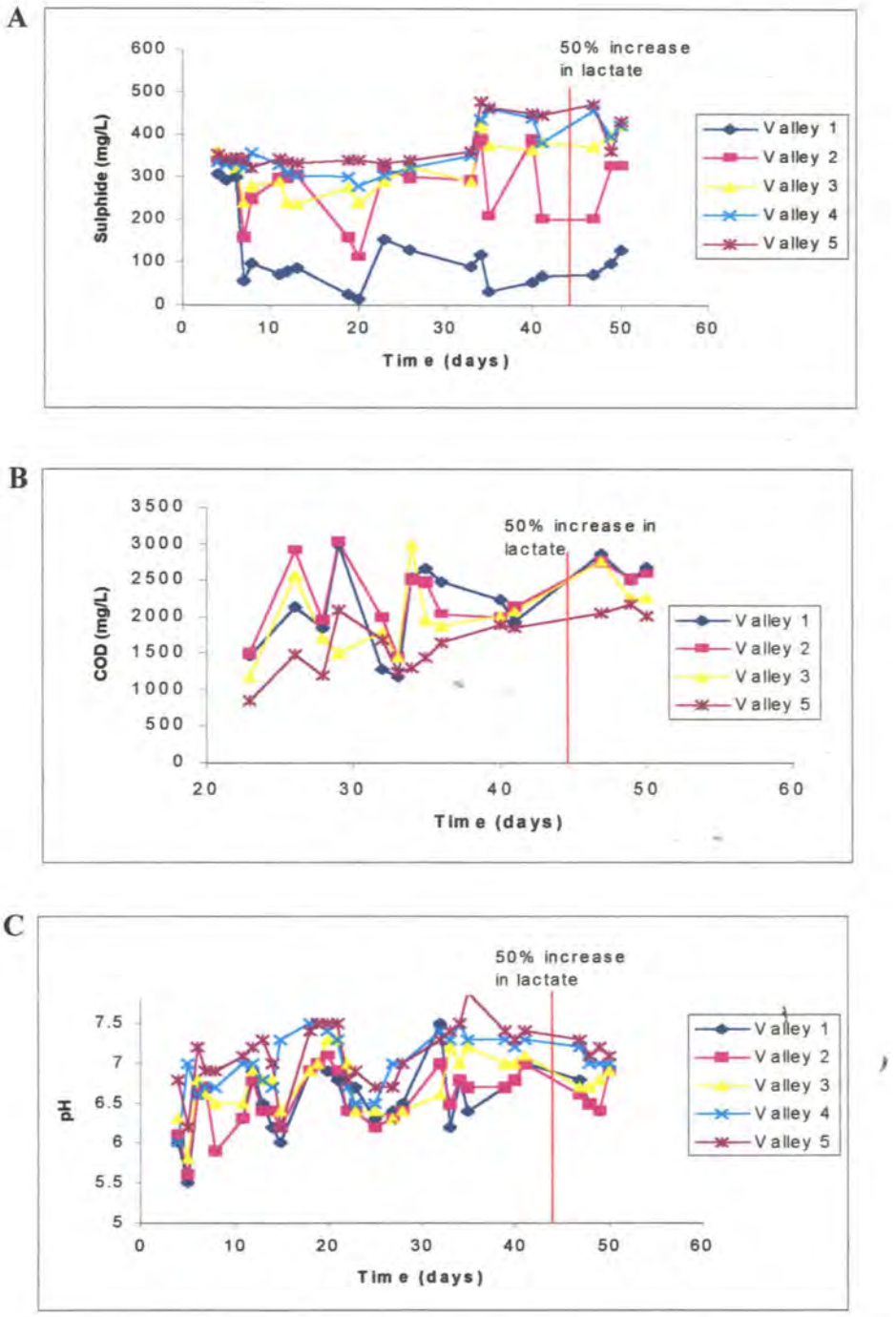


Figure 2.2: Measurements of the feed directly below the sulphur biofilm in the baffled reactor of: A-Sulphide; B-COD and C-pH

the end of the reactor with the flow of the feed. This sulphide gradient achieved was useful in comparative biofilm studies as the effects of the five different sulphide and COD concentrations could be determined on the same biofilm population. Biofilms grown on the higher sulphide concentrations in valleys 4 and 5 were more brittle and yellow in colour than the other biofilms, with full coverage of the biofilm occurring (Table 2.1). Although the biofilm sank easily when the biofilm became more brittle, the high sulphide concentrations resulted in rapid re-formation of the biofilm on the reactor surface.

The increase in lactate from day 45 caused an increase in sulphide concentration in valleys 1, 2 and 3, although in valleys 4 and 5 the sulphide concentrations decreased initially before increasing. The higher sulphide concentrations resulted in an increase in the biofilm coverage, while the brittleness decreased, probably due to a matrix production, as observed in SEM photographs in chapter 3 (Table 2.1). The sulphide concentrations measured in the baffled reactor, particularly in valleys 4 and 5 (350mg/L - 450mg/L) were higher than reported feed values in the literature for laboratory-scaled reactors, which range from 20 mg/L to 240 mg/L (Janssen *et al.*, 1997; Chung *et al.*, 1996b; Lee and Sublette, 1993; Buisman *et al.*, 1991a). This suggests that the sulphide oxidising bacteria in the biofilm may be adapted to high sulphide concentrations and can withstand levels which are toxic to most organisms.

The high sulphide concentrations present in the reactor after the increase in lactate resulted in the rapid formation of a more pronounced sulphur biofilm (Table 2.1). This may have be due to sulphide oxidising bacteria being forced to convert sulphide to sulphur rather than sulphate.

Sulphate formation is preferred under optimal conditions because of the higher Gibbs free energy available to the cells: -772.43 kJ/mol for sulphate formation compared to -129.50 kJ/mol for sulphur formation (Janssen *et al.*, 1998). The sulphur forming reaction is, however, favoured under oxygen limiting and high sulphide conditions because sulphur is formed faster than sulphate, allowing the bacteria to remove harmful sulphide, and the lower Gibbs energy is favoured when the electron transport chain is already overloaded. Sulphur is an intermediate in the sulphate formation reaction, and under more favourable conditions where oxygen is available and sulphide loading values are not as high, the reaction will be completed with sulphur being converted to sulphate by the bacteria (Gommers *et al.*, 1988; Boogerd *et al.*, 1991; Visser *et al.*, 1997). The increase in biofilm coverage after the increase in lactate could also be a result of an increased matrix production, which reduced sinking of the biofilm.

2.3.1.2 COD

Figure 2.2b illustrates the COD concentration of the system, beginning on day 23 after the reactor start-up. The COD values were erratic. This could be due to the system still stabilising, as Okabe *et al.* (1996) found that their reactor reached steady state after 40 days. The COD concentration, which was a measure of the amount of organic material present, was higher where the feed entered the system compared to the effluent because lactate was consumed by the SRB population as an energy and carbon source in order to reduce sulphate to sulphide.

The 50% increase in the feed lactate on day 45 resulted in a less brittle biofilm (Table 2.1). This could have been as a result of a stabilising matrix produced by the biofilm, as noted in the SEM

study described in chapter 3. The formation of the matrix was dependent on organic material from the feed, and has been found to play a very important role in maintaining the biofilm structure by previous authors (Costerton, 1995; Bishop, 1997). The increase in lactate also resulted in a greater sulphide production by the SRB sludge, as described by Reaction1, indicating that the SRB were not receiving an optimal lactate concentration before the increase.

Although the sulphide concentration was higher after the increase in lactate, which usually led to biofilm brittleness, the increased organic content of the feed reduced biofilm brittleness as a result of the matrix constructed by the cells making up the biofilm (Table 2.1). The increased organic concentration also led to a greater coverage by the biofilm, as a result of better growth and also because the stabilised biofilm did not sink to the bottom of the reactor as easily as the more brittle biofilms did. Okabe *et al.* (1996) found that in a nitrifying biofilm, low carbon made the biofilm fragile, and a higher organic load caused more intensive stratification of bacteria within the biofilm accompanied by the biofilm becoming less brittle.

2.3.1.3 pH

The pH of the medium in the baffled reactor directly beneath the biofilm was monitored over a 50 day period (Figure 2.2c). The pH ranged from pH 6-7.5, with the highest pH values measured in valleys 4 and 5, corresponding to the trend found with sulphide concentrations. The pH values are within the optimal sulphide oxidation pH range of pH 6.5 - 8.5 (Buisman *et al.*, 1989; Janssen *et al.*, 1997). Sulphate reduction is an alkalinity generating reaction (Reaction1) which probably accounts for the increasing pH levels accompanying the increase in sulphide levels discussed in

2.3.1.1.

Table 2.1: Feed details and subjective biofilm measurements over a two month period. The eight week measurement was taken four days after the lactate added to the feed was increased by 50%

Valley	Time after start up	Brittleness (Scale of 0-5)	Colour	Surface area	[COD] (mg/L)	[Sulphide] (mg/L)
1	4 weeks	0	Transparent	50%	2142	301.395
	6 weeks	0	Transparent	50%	2244	53.92
	8 weeks	0	White	100%	2682	129.26
2	4 weeks	1	White	100%	2916	336.279
	6 weeks	0	Transparent	50%	1998	287.66
	8 weeks	0	White	100%	2590	325.55
3	4 weeks	1	White	100%	2568	324.729
	6 weeks	3	Yellow-white	75%	2008	364.26
	8 weeks	2	Yellow	100%	2260	421.71
4	4 weeks	3	Yellow-white	100%	2180	322.248
	6 weeks	5	Yellow	100%	1860	439.31
	8 weeks	3	Yellow	100%	430.83	431.7
5	4 weeks	4	Yellow-white	100%	1486	339.225
	6 weeks	5	Yellow	100%	1884	449.93
	8 weeks	3	Yellow	100%	2014	430.83

2.3.2 Fed batch reactor studies on the kinetics of the biofilm microbial population

After monitoring the laboratory scale biofilm, studies were carried out on a mixed culture of sulphide oxidising bacteria enriched from the biofilm in order to confirm that the biofilm is involved in sulphide oxidation, by measuring sulphide removal along with the production of

sulphur and sulphate. The inoculum was grown in a 1L stirred fed-batch reactor under aerobic and anaerobic conditions as described in 2.2.4, while the redox potential, sulphide, sulphate and sulphur were measured. The formation of sulphate by sulphide oxidising bacteria is described by the reaction:

$$2HS^- + 4O_2 \rightarrow 2SO_4^{2-} + 2H^+ \quad \text{..... (2)}$$

Under aerobic conditions, the sulphide concentration in the reactor before the addition of new feed each day (effluent sulphide) was constantly close to zero over the nine day period, indicating that the sulphide in the feed was oxidised almost completely to sulphur and sulphate (Figure 2.3a). The effluent sulphate concentration was comparable to the sulphide concentration fed to the reactor, indicating most of the sulphide was converted to sulphate under aerobic conditions, as described by reaction 2, although the sulphur concentration also increased over the 10 day measurement period (Figure 2.3b).

Under anaerobic conditions, however, not all the sulphide in the feed was utilised by the inoculum (Figure 2.4a), indicating that sulphide oxidation occurs more rapidly under aerobic than anaerobic conditions. The sulphur concentrations, relative to the sulphate produced, were higher under anaerobic conditions, compared to the aerobic conditions (Figures 2.3b and 2.4b), which can be explained by Reaction 3, as four times less oxygen is used in the sulphur forming reaction than in the formation of sulphate (Reaction 2). Sulphate production is favoured by cells under optimal conditions because of the higher Gibbs free energy release, however in oxygen limited conditions,

sulphur formation is favoured.

$$2HS^- + O_2 \rightarrow S^\circ + 2OH^- \quad \text{.....(3)}$$

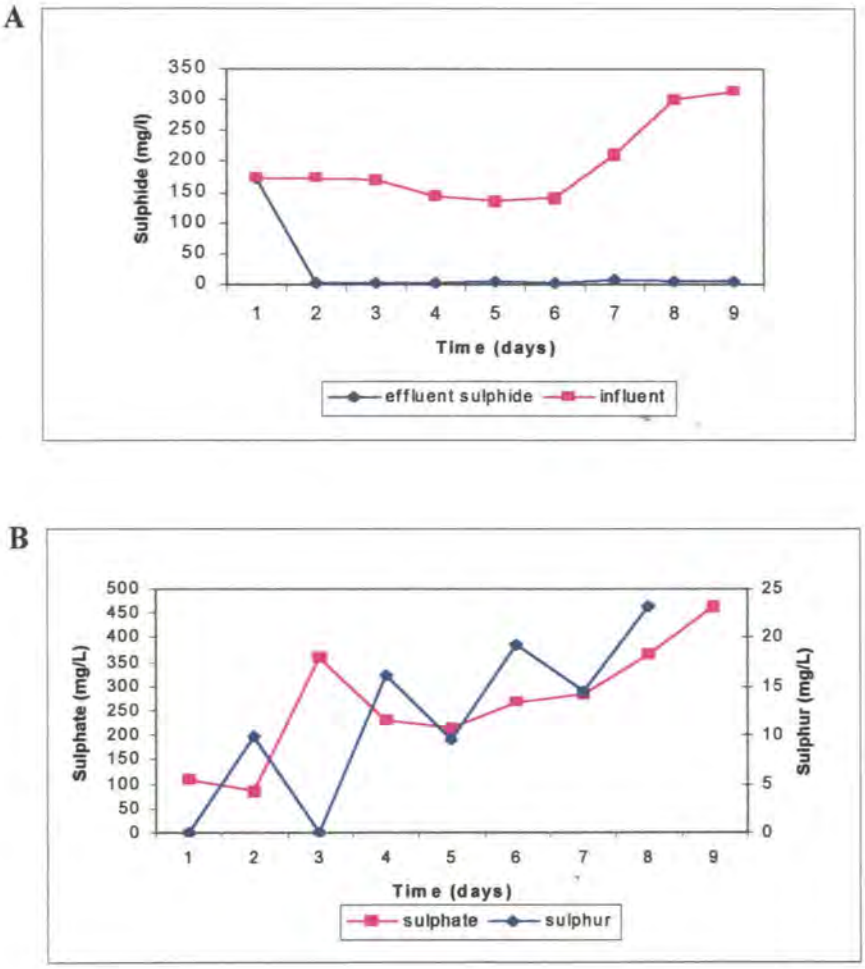


Figure 2.3: Concentrations of sulphur species in the fed-batch reactor under aerobic conditions: A=sulphide feed and effluent; B=sulphate and sulphur concentrations

Sulphur production under anaerobic conditions was correlated to a decrease in the redox potential of the medium (Figure 2.5), illustrating the increase in sulphur formation under a low redox

potential. Because detection of oxygen by DO (dissolved oxygen) sensors is not sensitive below 0.1 mg/L, redox potential can be used instead to control conditions favourable for sulphur formation (Janssen *et al.*, 1998). Redox potential is a measure of the solutions tendency to accept or donate electrons, although in a sulphide-oxidising system, the redox potential is determined mainly by the sulphide concentration, rather than oxygen (Janssen *et al.*, 1998). Sulphur production is therefore dependent on the presence of a low redox potential, which occurs under low oxygen and high sulphide concentrations, because sulphide is a powerful reducing agent.

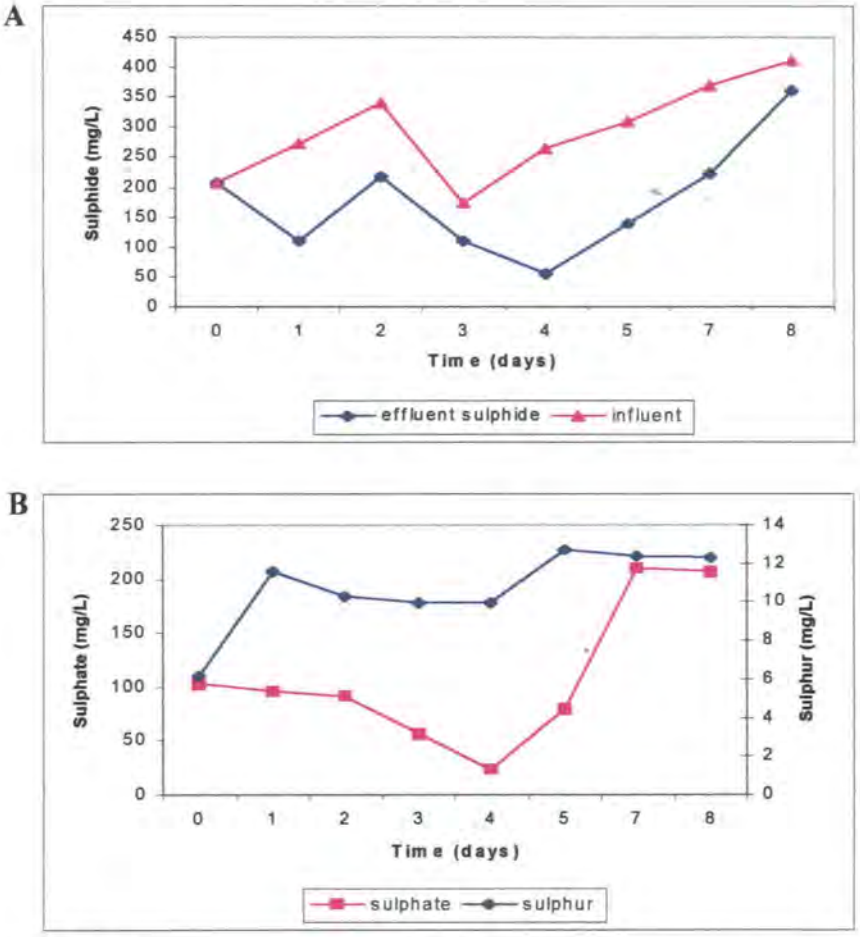


Figure 2.4: Concentrations of sulphur species in the fed-batch reactor under anaerobic conditions: A=sulphide feed and effluent; B=sulphate and sulphur concentrations

The sulphur concentrations measured under both anaerobic and aerobic conditions are not indicative of actual amounts of sulphur produced. Other researchers have also noted that measurements of sulphur are problematic because of its tendency to adhere to surfaces, especially to glass reactor walls (Janssen *et al.*, 1997).

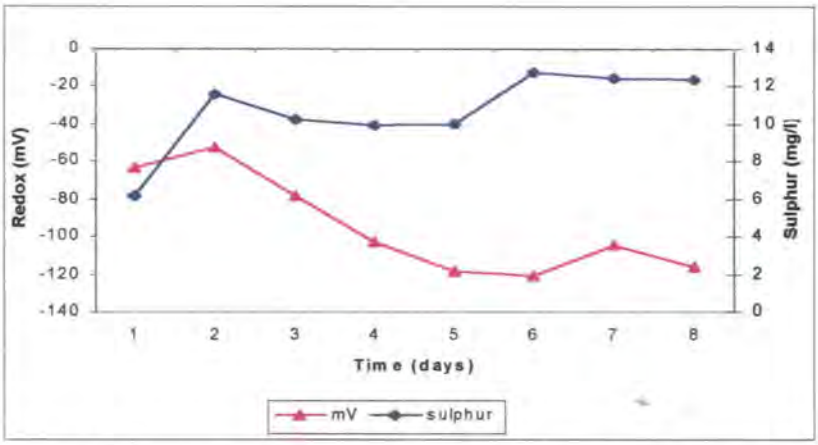


Figure 2.5: Relationship between redox potential and sulphur production

2.3.3 Flask studies to determine the optimal sulphide concentration and temperature for the biofilm inoculum

2.3.3.1 Sulphide toxicity

The aim of this set of experiments was first to determine the toxic concentrations of sulphide for sulphide oxidising bacteria present in the biofilm, and second, to determine the effect of the initial sulphide concentration on the subsequent tolerance of the bacterial population to an increase in the sulphide concentration of the growth media. The inoculum was grown in a series of sulphide concentrations in shake flasks operated on a fed-batch system, with the sulphide removal rate measured after six hours every day over a nine day period. After five days, the sulphide

concentration in each flask was increased by 66%. The increase in sulphide concentrations is given in Table 2.2

Table 2.2: The increase in sulphide concentration in each flask after day 5

Initial sulphide concentration (mg/L)	**New concentration after a 66% increase in sulphide after day 5 (mg/L)**
65	110
100	165
130	215
165	275
200	330
230	380
265	440

The cells grown at initial sulphide concentrations below 230mg/L showed good sulphide removal performance, with 92-97% of the sulphide removed in six hours on day 4 (Figure 2.6). The flasks with sulphide concentrations of 230 and 264mg/L showed sulphide removal figures below 50% on day 4. In addition to poor performance, this result could be influenced by the bacterial strains adapted to very high sulphide concentrations not having had sufficient time to establish themselves as the dominant strains.

This hypothesis was tested by increasing the sulphide concentration by 66% on day 5. After the

increase in sulphide concentrations in all the flasks on day 5, the sulphide removal decreased dramatically in the flasks with lower initial sulphide concentrations (Figure 2.6). Sulphide removal dropped significantly from day 4 to day 6 in flasks with sulphide concentrations of 65 mg/L (from 94.5% to 19.83%) , 100 mg/L (from 93.6% to 15.6%), 130 mg/L (from 92.36% to 16.91%) and 165 mg/L (95.51% to 21.48%). The flasks with initial sulphide concentrations of 200 and, in particular, 230 and 265 mg/L, did not exhibit a great decrease in sulphide removal efficiency (97.87% to 25.7%; 57.3% to 40.6% and 4.69 to 27.65% respectively). By the end of the nine day experimental period, the sulphide removal efficiencies of these three flasks had improved and stabilised, whereas the flasks with the four lowest sulphide concentrations did not recover from the increased sulphide concentrations.

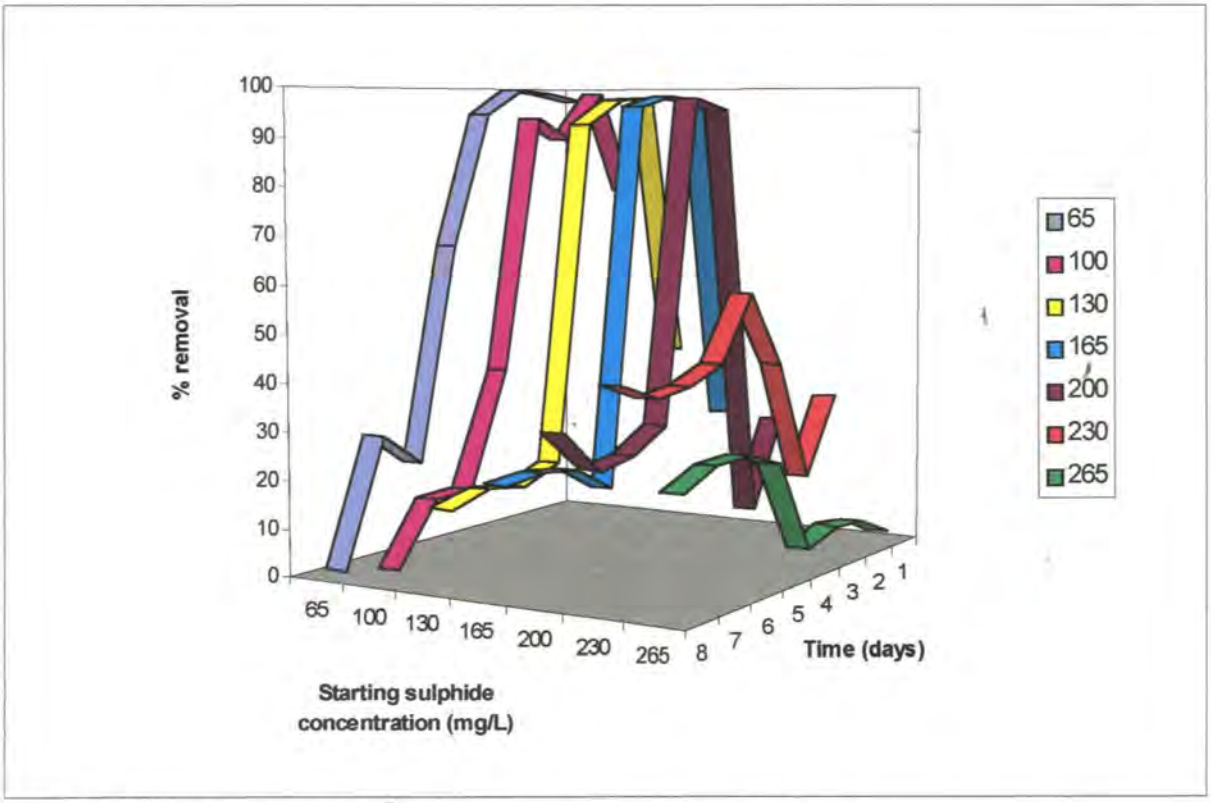


Figure 2.6: Effect of starting sulphide concentration on sulphide oxidation and sulphide toxicity

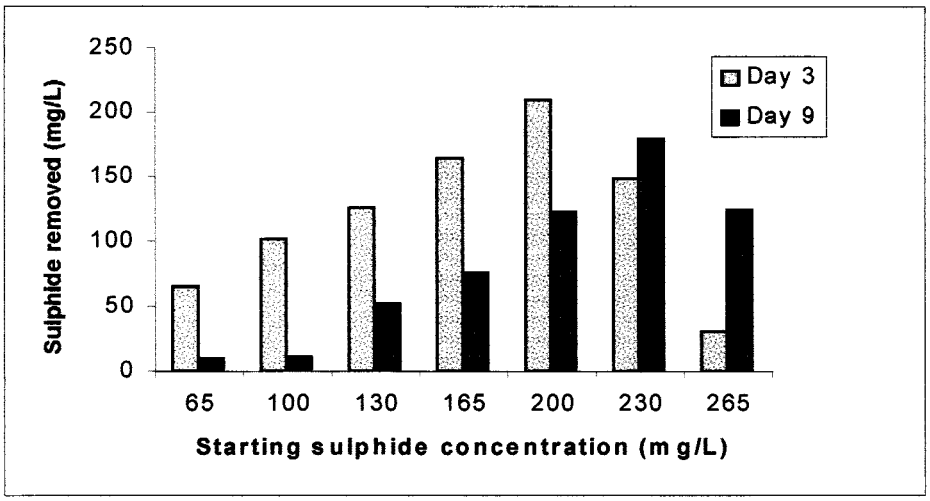


Figure 2.7: Concentration of sulphide oxidised by the cultures in each flask. Samples presented in this graph were taken on day 3, and on day 9, comparing the original sulphide concentrations and the 66% increase in sulphide after day 5

The concentration of sulphide oxidised by the cultures in each flask, illustrated in Figure 2.7, gives an indication of the tolerance of the cultures grown in higher sulphide concentrations. At the end of the nine day period, the cultures in flasks "230" and "265", which were growing on 380 mg/L and 440 mg/L sulphide respectively after day 5, were oxidising over 100 mg/L sulphide in six hours (Figure 2.7). The cultures in flasks "65" and "100" did not, however, survive in sulphide concentrations of 110 mg/L and 160 mg/L. This suggests that the cultures which are tolerant to high concentrations are not very efficient sulphide removers, while the cultures which remove lower concentrations of sulphide very efficiently are not tolerant to high sulphide concentrations.

These sulphide toxicity flask experiments also suggest that the sulphide concentration upon which sulphide oxidising strains are grown is important in determining the final sulphide toxicity levels of that culture. The strains grown on lower concentrations of sulphide were much less able to

function with an increase in sulphide, even though the final sulphide concentration was lower than the initial concentrations present in other flasks. With starting concentrations of 200, 230 and 265mg/L, the cultures were able to cope with a 66% increase in sulphide concentration, which may suggest an adaptation of the culture to higher sulphide concentrations. These results also suggest that different sulphide concentrations select for different bacterial cultures, which are capable of living at higher or lower sulphide concentrations, or which have faster oxidation rates. Spread plates (minimal salts agar) of the inoculum present in each flask after the nine day incubation revealed the presence of two different coloured colonies. Orange colonies were dominant at the lower sulphide concentrations, while white colonies were dominant at higher sulphide concentrations. This suggests the selection of different strains from the culture depending on the sulphide concentration present. However, further studies would be necessary in order to identify and characterise the dominant species at each sulphide concentration.

The optimal sulphide concentration for growth of bacterial species which originated from the sulphur biofilm appears to be between 100-250mg/L. Between these concentrations the cell numbers remained constant while sulphide removal continued (not shown), because the sulphide oxidised was not strictly coupled to growth, as is the case with phototrophic sulphide oxidising bacteria (Kuenen and Robertson, 1992). Colourless sulphur bacteria are able to produce 20g of sulphur for each gram of cells produced (Kuenen and Robertson, 1992). At higher concentrations (265 mg/L and above), sulphide probably became toxic to the cells, as reflected by the incomplete sulphide removal.

As the sulphide concentrations increased, an increase in sulphur production was also observed. The growth media became milky at the three highest sulphide concentrations with white sulphur deposits occurring on the sides of the glass flasks. At lower sulphide concentrations the medium was clear with no noticeable sulphur deposition on the flasks. Milkiness of the solution has been used by other researchers (Gommers and Kuenen, 1988; Buisman *et al.*, 1989) as an indication of the presence of biological sulphur in solution. Sulphur concentrations of 3.31 mg/L were measured by HPLC on day 8 in the flask with the lowest initial sulphide concentration (65 mg/L), while the flask with the highest initial sulphide concentration (265 mg/L) had a final sulphur concentration of 14.55 mg/L on day 8 (Figure 2.8). Cells grown on the highest final sulphide concentration therefore produced over 4 times the amount of sulphur than cells grown on the lowest sulphide concentration. It must be noted that the samples taken on day 8 were grown on the increased sulphide concentration; therefore the 65 mg/L flask was receiving 110 mg/L sulphide, while the 265 mg/L flask was receiving 440 mg/L sulphide.

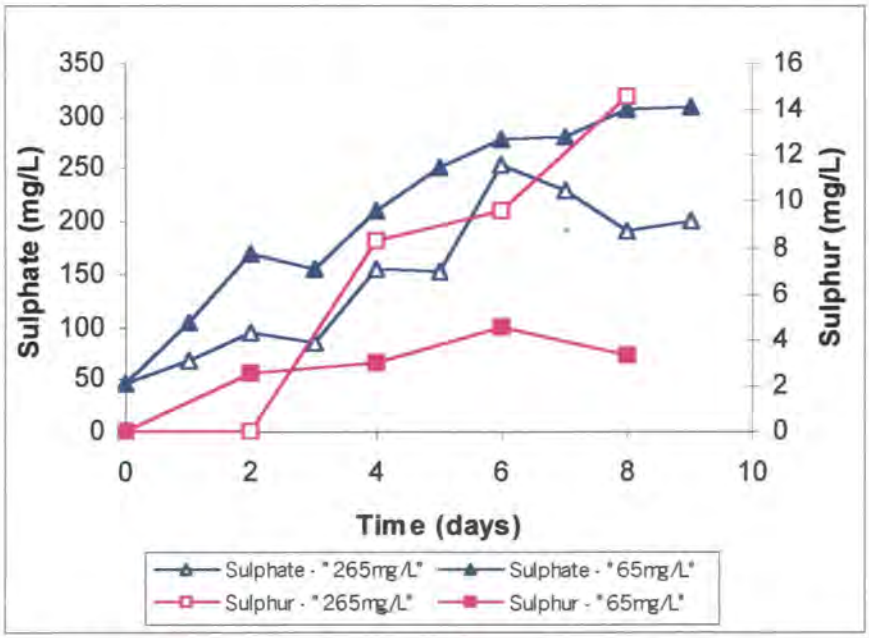


Figure 2.8: Sulphate and sulphur concentrations at the lowest and highest sulphide concentrations (65mg/L and 265 mg/L)

More sulphur was produced at higher sulphide concentrations because the cells were forced to stop the sulphide oxidation reaction at sulphur, instead of a complete conversion of the sulphide to sulphate. This is a result of the lowered redox potential accompanying an increase in sulphide concentration, which favours reaction 2, described in section 2.3.2. The production of sulphur allows the cells to remove toxic sulphide while collecting sulphur, which is converted to sulphate for energy at a later stage, when less sulphide is present, as documented in the literature (Gommers *et al.*, 1988; Boogerd *et al.*, 1991; Robertson and Kuenen, 1991; Visser *et al.*, 1997; Janssen *et al.*, 1998).

Although these results do give an indication of the sulphide oxidation capacity of the biofilm, further studies using the whole biofilm would have to be carried out in order to determine the actual sulphide oxidising capacity of the biofilm. The isolated population used in this study does not represent the entire biofilm population, and the culture would not have the same metabolic activity in aerated flask cultures as in the floating biofilm.

2.3.3.2 Optimal temperature for biological sulphide oxidation

The optimal temperature for sulphide oxidation by a mixed culture enriched from the biofilm was determined in this set of experiments, where sulphide oxidation at 10°C, 15°C, 20°C, 25°C, 30°C and 37°C was measured (Figure 2.9 a-f). Chemical sulphide oxidation, as well as biological sulphide oxidation, was determined in order to calculate a rate of biological sulphide which completely eliminated the effect of chemical oxidation.

Biological sulphide oxidation rates without the effect of chemical oxidation were calculated by subtracting the rate of chemical sulphide oxidation from the total sulphide oxidation. At each of the temperatures tested, the biological sulphide removal rate was greater than the chemical sulphide removal rate, as reflected by the control which contained no inoculum (Figure 2.10). Increasing the temperature resulted in an increased rate of both biological and chemical oxidation (Figure 2.10).

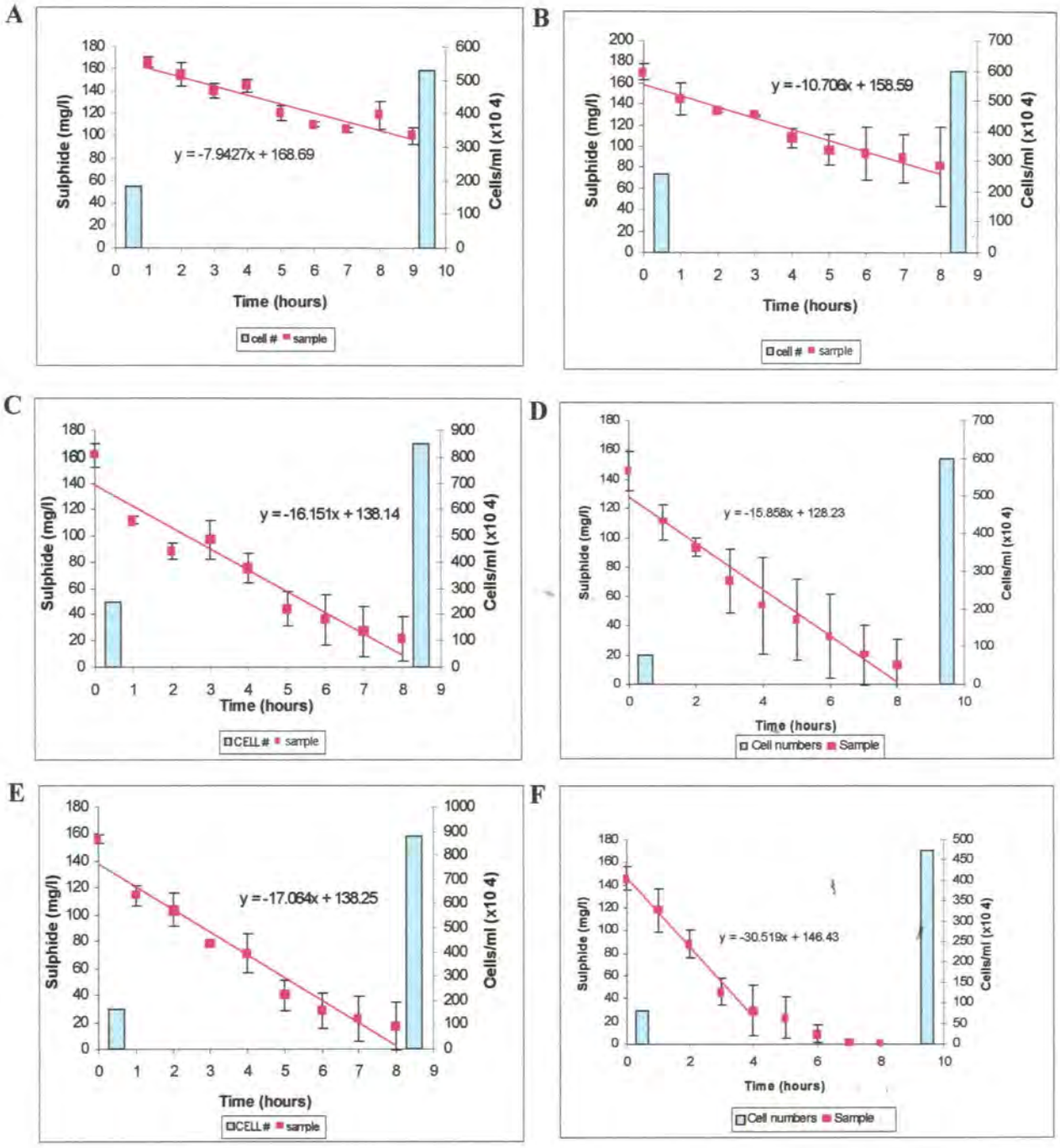


Figure 2.9: Biological sulphide removal rates at various temperatures: A=10°C; B=15°C; C=20°C; D=25°C; E=30°C; F=37°C. The biological sulphide oxidation rate was calculated by subtracting the chemical oxidation rate from the total oxidation rate.

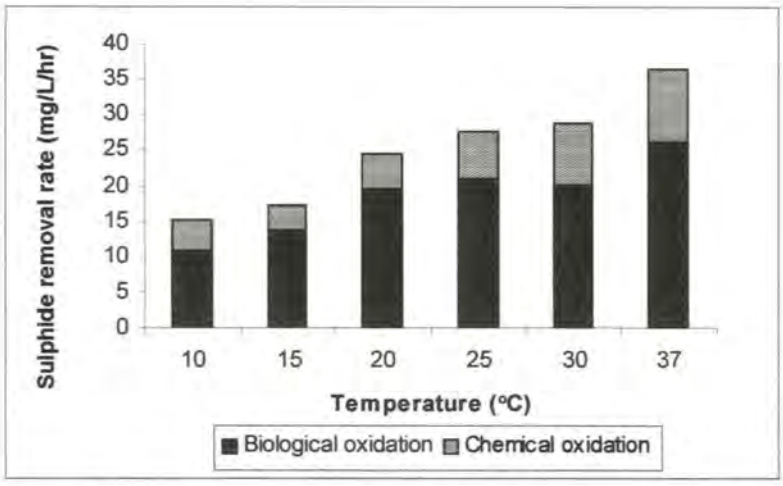


Figure 2.10: Biological and chemical oxidation rates as parts of the total sulphide oxidation rate at varying temperatures

At each temperature tested cell growth increased over the 9 hour measurement period, indicating utilisation of the energy released by sulphide oxidation by the cells for growth (Figure 2.11). The increase in cell numbers did not, however, appear to be related to temperature, and was influenced to a greater extent by the starting number of cells (Figure 2.11). The higher rate of sulphide oxidation at higher temperatures was therefore not correlated to cell growth, but rather to increased sulphur production, as described by Kuenen and Robertson (1992).

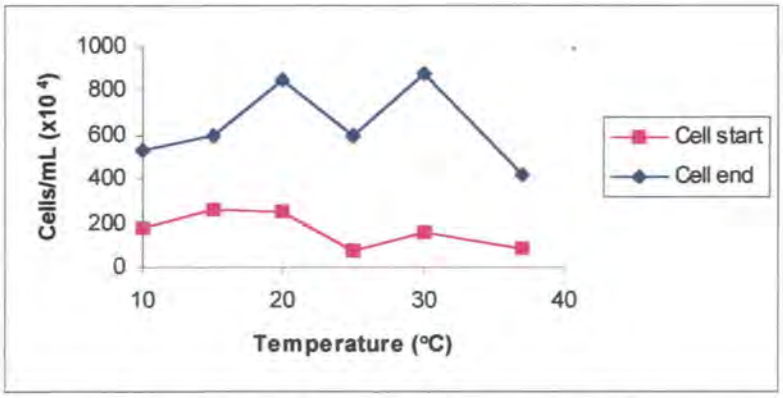


Figure 2.11: Numbers of cells at each temperature at T = 0 and T = 9 hrs

This set of experiments indicates that the optimal temperature for sulphide oxidation by bacteria originating from the biofilm is 37°C (Figure 2.10). Biological sulphide oxidation proceeded at a higher rate than chemical oxidation, even though the calculation of the biological oxidation rate used in this study was conservative. Previous researchers have found that biological sulphide oxidation occurs within seconds, whereas chemical oxidation occurs over minutes to hours (Kühl and Jørgensen,1992; Okabe *et al.*, 1998).

The regression fit ($R^2 = 0.86$) of the line plotting the sulphide oxidation slopes at various temperatures follows a fairly linear trend. The trend in Figure 2.12 suggests a relationship between biological sulphide oxidation and temperature, and has the potential be used to determine biofilm sulphide oxidation rates at any temperature, however, this prediction would only be valid if the microbial population was the same as the one used in this study.

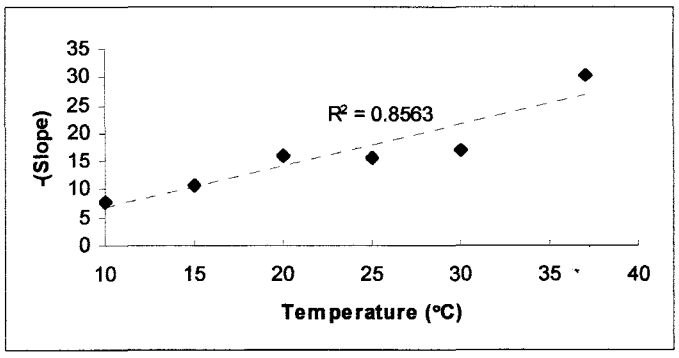


Figure 2.12: The slope of biological sulphide oxidation trend lines at varying temperatures

The point at 37°C does not, however, fit into the trend formed by the slopes of the lines for 10° to 30°C. This could imply the presence of a different dominant species at the increased temperature, which is capable of higher oxidation rates than the consortium present below 30°C. These

preliminary results could be expanded by further studies in order to determine a more precise trend, and determine the species present at each temperature.

The temperature chosen to do further experimental work on sulphide oxidising bacteria was 25°C because the bacterial sulphide removal efficiency at this temperature was very similar to the efficiency at 20°C and 30°C (Figure 2.10), and 20-30°C is the temperature range likely to occur in laboratory conditions and in the field for further work. The temperature optimum of naturally occurring sulphide oxidising bacteria varies from 4°C to 95°C (Robertson and Kuenen, 1991). Thermophilic sulphide oxidising bacteria are found in terrestrial warm springs and hydrothermal vents in the ocean, with extreme thermophiles growing in vents which reach a temperature of 100°C and moderate thermophiles growing in water with a temperature of 45-55°C (Robertson and Kuenen, 1991). Janssen *et al.* (1997) and Buisman *et al.* (1989) used room temperature (22-25°C) to achieve maximum sulphur production rates, and found that oxidation occurred at temperatures between 20°C and 35°C. The sulphide oxidising *Pseudomonas putida* strain CH11 used by Chung *et al.* (1996c) for sulphide removal achieved optimal removal from 20-37°C.

2.4 CONCLUSION

An anaerobic baffled reactor with a SRB sludge bed was used in this study to cultivate a sulphur biofilm. The reactor provided a continuous flow of nutrients to the biofilm without disruption of the biofilm, while the SRB sludge supplied a constant high concentration of sulphide. The baffled reactor also produced a gradient of sulphide and COD across the reactor, separated by the baffles,

where different biofilm types were observed in the different valleys, which contained varying amounts of sulphide and COD.

Controlling the redox potential of the growth media of sulphide oxidising bacteria had a great effect on the sulphur producing capacity of the culture. A low redox potential, created by a low oxygen and high sulphide concentrations, resulted in the formation of more sulphur than sulphate, confirming the link between redox potential and sulphur production. The optimal sulphide concentration for the inoculum isolated from the biofilm and grown in shake flasks was 100-250 mg/L, above which concentration sulphide became toxic to the cells. Biological sulphide oxidation was also shown to be more rapid than chemical oxidation, as reported in the literature, and the optimum temperature for biological sulphide oxidation by the biofilm inoculum was 37°C, although sulphide oxidation also occurred at a rapid rate between 20 and 30°C.

The development of this experimental system in which a gradient of increased sulphide concentration and decreased COD concentration was achieved, resulted in the formation of five potentially different biofilms to study. The conditions in all of the valleys, with the exception of valley 2, remained fairly constant, creating an ideal system for the comparative study of sulphur biofilms. Having determined aspects of biofilm performance, it became necessary to examine the structure and microbiology of the sulphur biofilm.

CHAPTER 3

MORPHOLOGY OF A FLOATING SULPHUR BIOFILM

3.1 INTRODUCTION

While the structure of biofilms has been difficult to study due to their fragile nature, recent technological advances have been applied in this area. The structure, physiology and microbiology of sulphate reducing biofilms has been studied extensively using light and electron microscopy, micro-electrodes, nucleic acid probes and fluorescent in-situ hybridisation (FISH) techniques (Kühl and Jørgensen, 1992; De Beer *et al.*, 1994; Goebel and Stackebrandt, 1994; Kolmert *et al.*, 1997; Okabe *et al.*, 1998; Yu and Bishop, 1998; Okabe *et al.*, 1999). Anaerobic sulphate reducing bacteria have been reported to be found in the bottom layers of sulphate reducing biofilms, near the site of attachment, with sulphide oxidising bacteria occurring near the middle of the biofilm, at the oxygen-sulphide interface (Okabe *et al.*, 1998).

Since the structure and function of sulphur-producing sulphide-oxidising biofilms, and in particular, floating biofilms, are sparsely reported in the literature, this study undertook an investigation of their structure. Understanding the structure of a biofilm is important in understanding aspects of its functioning. All models of biofilm growth are based on biofilms attached to substratums, therefore

it was worthwhile determining the structure of a free floating biofilm, using microscopy to determine the internal structure (Marshall, 1992; Costerton *et al.*, 1994).

Scanning electron microscopy (SEM) was used in this study to observe sulphur biofilm morphology and structure. SEM has a large depth of field which allows a considerable amount of the sample to be in focus at a time and a high resolving power, making this type of microscopy useful in structural studies. The fixation and dehydration steps in sample preparation can, however, alter the morphology of the biofilm, and lead to shrinkage and loss of polysaccharide matrices (Stewart *et al.*, 1995). While techniques needed to be developed for this application, SEM provided good morphological and structural detail on the biofilm components.

In this chapter, a protocol for the observation of sulphur biofilms using SEM was developed. The morphology and effect of feed conditions on the biofilm was studied.

3.2 METHODS AND MATERIALS

3.2.1 Biofilm sampling

The laboratory-scaled baffled reactor described in Chapter 2 was used to grow a sulphur biofilm, which formed within days. Once the biofilm had developed on the surface of the reactor, samples were taken four, six and eight weeks after start-up of the reactor, from three of the five valleys in the

baffled reactor. The last biofilm sample (eight weeks) was taken four days after the 50% increase of lactate in the feed.

3.2.2 Scanning electron microscopy

Since no protocols for SEM preparation of floating sulphur biofilms could be found in the literature, a process of method development was undertaken in this study. The first sample preparation procedure evaluated for observing the biofilm in cross section involved the use of an agarose (3.5% agarose, Techcomp, molecular biology grade in ddH_2O solution) coated microscope slide. The slide was slipped under the biofilm and lifted, allowing the biofilm to rest on the agarose coating. A layer of molten agarose (3.5%) was then poured over the biofilm and allowed to set. This agarose "sandwich" with the biofilm in the middle was sliced manually into sections approximately 1-2 mm thick with a sterile blade, and prepared for SEM, as described in Appendix B. The sample was placed on a stub, and gold coated in this position. Alternately, a 1.75% sodium alginate solution was used instead of agarose. The whole alginate "sandwich" was then submersed in a cold calcium chloride solution (2%) to allow the alginate to harden. The alginate preparation was used because it was found to be more rigid than agarose, and was therefore more amenable to sectioning. The preparation was not, however, firm enough for sectioning with a LKM UM III microtome (LKB AB Bromma, Sweden), so a freezing microtome (Leitz 1320, Germany) was used.

The third method attempted was to lift a 1cm^2 section of the biofilm onto a 0.22 micron supported

nylon membrane (Micron Separations Inc), with another membrane placed on top of the biofilm, again creating a "biofilm sandwich". The two membranes were stapled together, and the sample fixed in 2.5% gluteraldehyde in 0.1M phosphate buffer (pH 7.0) overnight, before being processed through the alcohol dehydration series described in Appendix B. After critical point drying, cross-sections were cut through the membranes using sharp scissors and placed between two copper "bookends". The copper structures were anchored to a sample stub using colloidal graphite, with the cross section of the biofilm facing upwards (Figure 3.1). The sample was sputter coated with gold in this position.

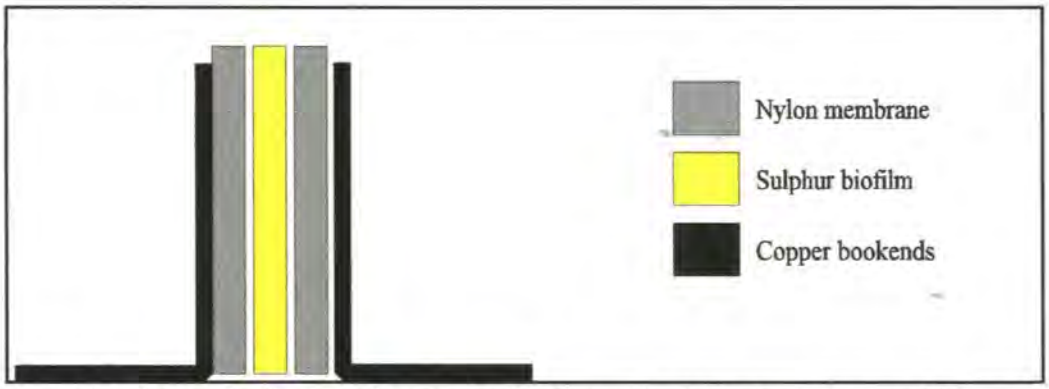


Figure 3.1: Schematic diagram of "copper bookend" sample positioning system used for viewing biofilm samples by SEM

3.3 RESULTS AND DISCUSSION

3.3.1 Optimisation of SEM sample preparation

The agarose coating method (Figure 3.2a) was useful for the observation of individual cellular morphologies within the biofilm. The biofilm structure was not, however, well preserved, as the molten agarose destroyed the biofilm structure, making the top and bottom surfaces of the biofilm difficult to observe. The alginate sections could not be observed using the electron microscope as the alginate casing around the biofilm disintegrated once frozen on the freezing microtome, and this method was subsequently discarded. The nylon membranes proved to be the most effective support for the biofilm during sectioning. The two membranes prevented the biofilm from disintegrating during the fixation and alcohol dehydration procedures. Copper "bookends" held the biofilm in such a way to enable a full view of the cross-sections (Figure 3.2b). Colloidal graphite was then used to anchor the edges of the copper bookends to prevent charging of the sample under the electron beam, which was noted before the graphite was added (Figure 3.2c).

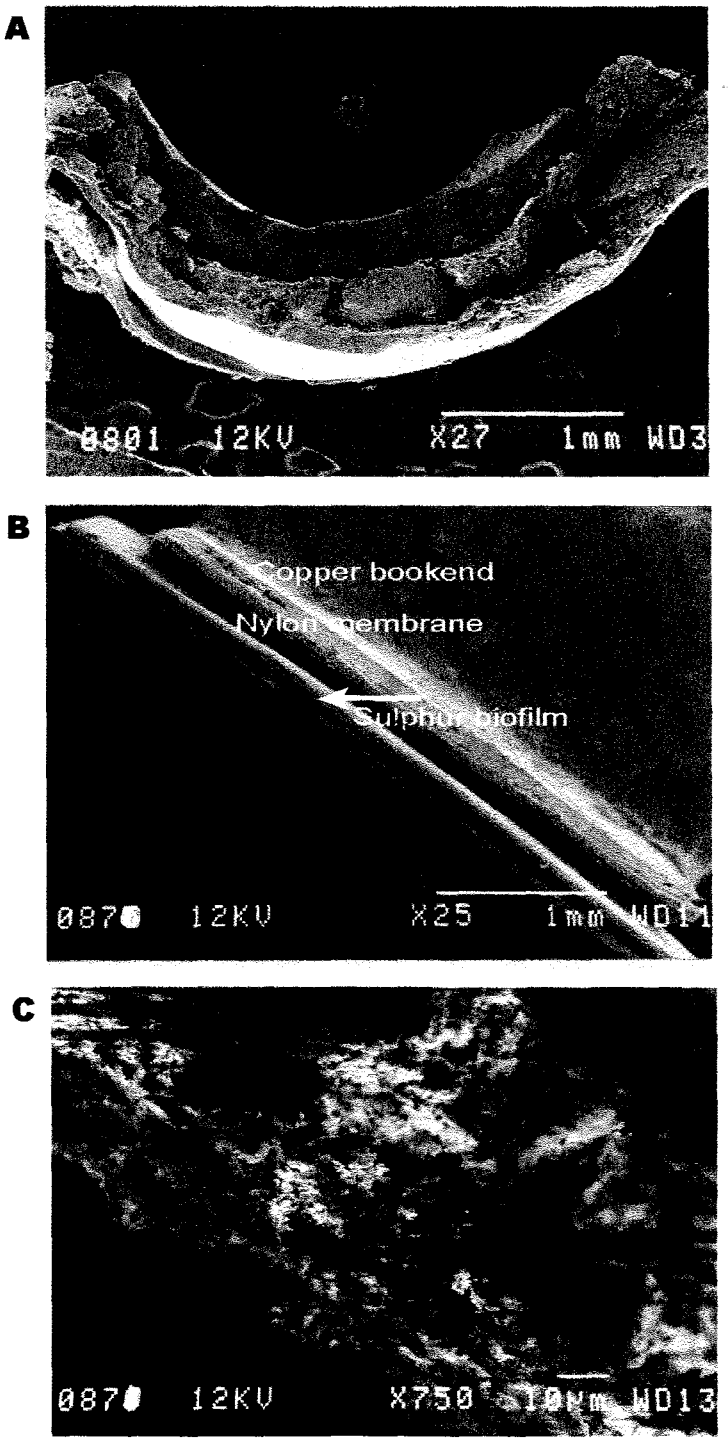


Figure 3.2: Optimisation of SEM preparation. Micrograph A: The agarose casing surrounding the sulphur biofilm. Micrograph B: Structure of copper "bookends" supporting the biofilm. Micrograph C: Effect of charging on photograph quality

3.3.2 Description of SEM observation of biofilm structure at three time periods

3.3.2.1 Four weeks after start-up:

The upper surface of the biofilm in valley 1 was composed of 10 -70μm wide square crystals with concave centres and 1μm long bacilli, which were covered in an extracellular matrix. Bacterial cells on the lower surface were 3μm long bacilli with few cocci and spirillum amongst 10μm circular crystals (Figure 3.5). The upper surface of the biofilm in valley 3 was a mixture of spirillum (2μm), cocci (1μm) and bacilli (1.5μm). Voids and the matrix were visible throughout the biofilm structure. The lower surface of the biofilm in valley 3 consisted mostly of spirillum (2μm) in an extracellular matrix with tightly packed aggregates of cells visible, with orthorhombic crystals in places (Figures 3.6a and b). The cross section of valley 5's biofilm measured 650μm in diameter with voids present throughout cross-section (Figure 3.3). Bacterial cells on the upper surface were 3μm long bacilli with fewer 0.75μm cocci present (Figure 3.4a). A matrix was visible between the cells and covered the surface completely in some areas. 1-3μm rhomboid crystals were visible on the biofilm surface. The middle section of the biofilm in valley 5 was composed of very long (>20μm) spirillum, with clusters of 1-2μm cocci, bacilli and spirillum also seen attached to the longer filaments (Figures 3.4b and c). On the lower surface smaller bacteria, mostly 2μm long bacilli, were predominant, with the sporadic appearance of cocci in the matrix.

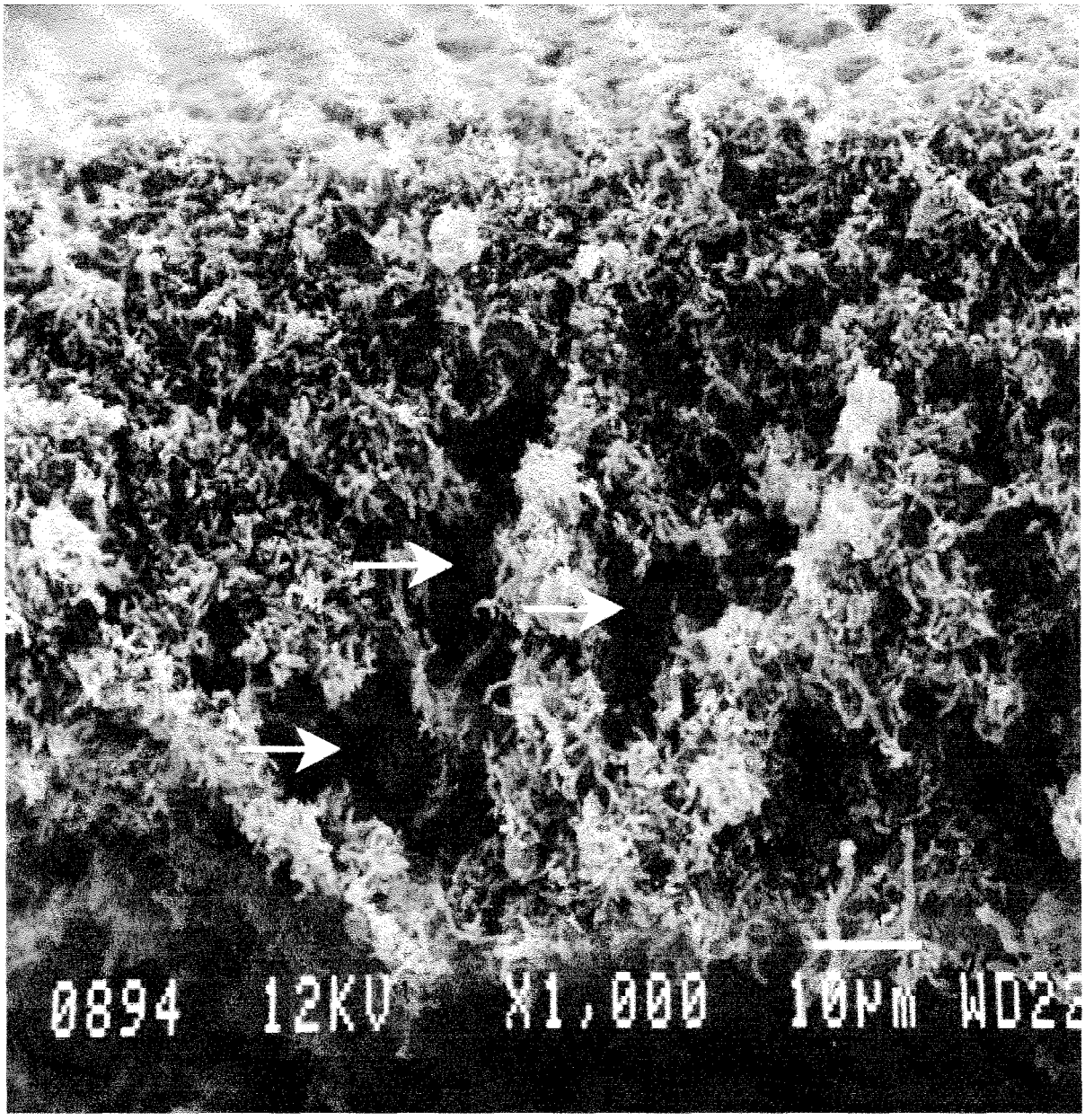


Figure 3.3: SEM cross section of the sulphur biofilm in valley 5 with arrows indicating the voids. The surface of the biofilm exposed to the air is at the top of the picture, moving down to the surface in contact with the liquid

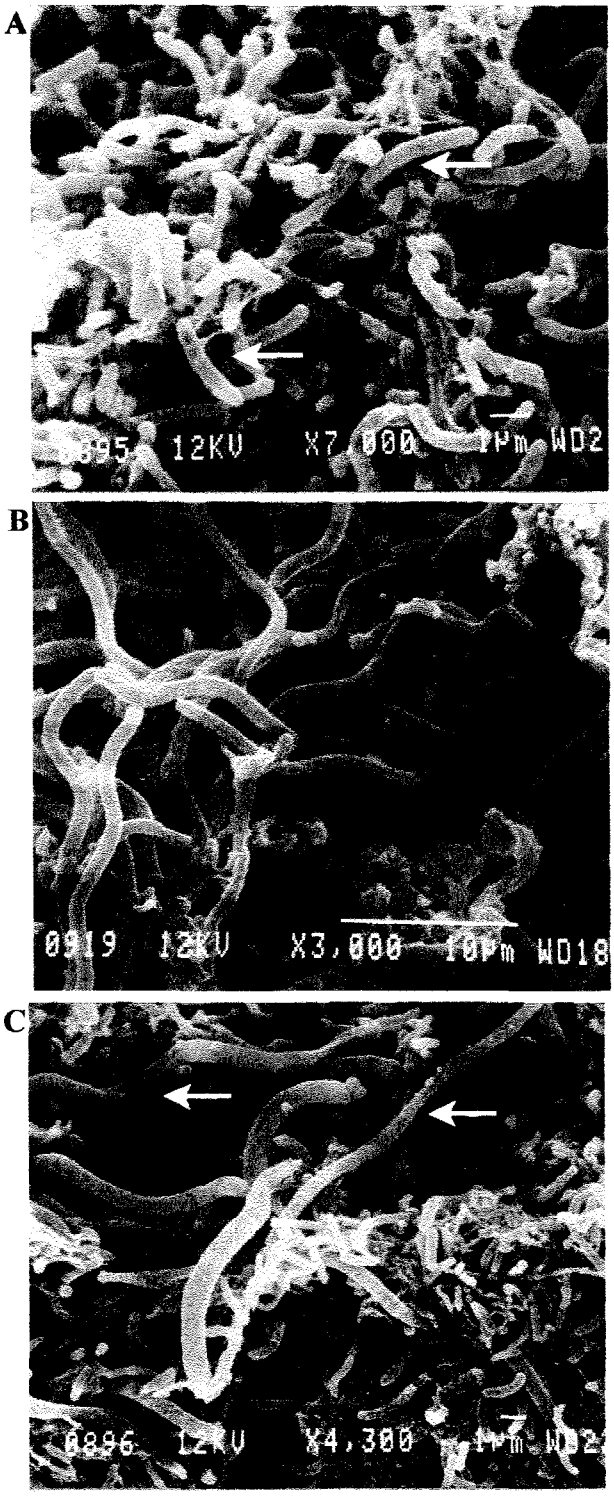


Figure 3.4: SEM of the biofilm from valley 5. A = Top layer of biofilm, arrows indicate 3μm bacilli; B =filamentous bacteria in the middle of the biofilm; C = Lower surface of biofilm, arrows indicate 10 μm filamentous bacteria

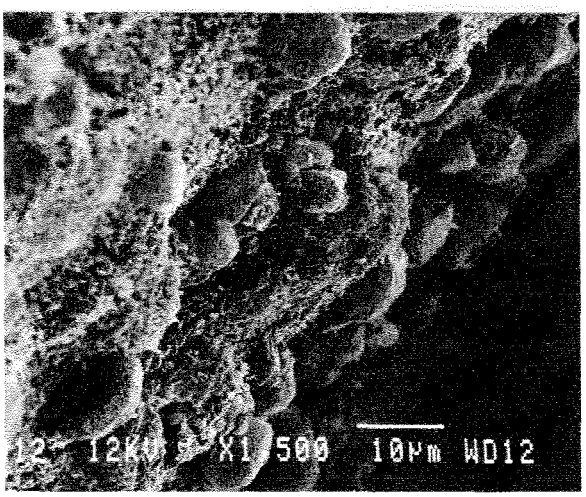


Figure 3.5: SEM of the lower surface of a floating sulphur biofilm showing attachment of crystals

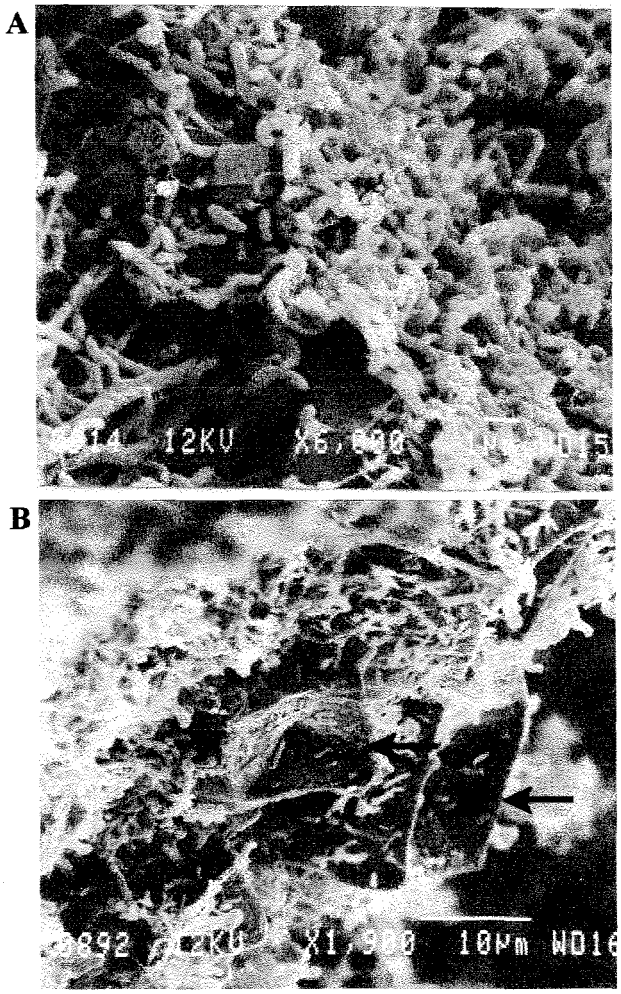


Figure 3.6: SEM of biofilm from valley 3. A = lower surface of biofilm; B = lower surface, with arrows indicating orthorhombic crystals

3.3.2.2 Eight weeks after start-up, 4 days after the 50% increase in lactate:

A continuous extracellular matrix layer was present on valley 1, entrapping 0.5 -1μm bacilli (Figure 3.7a). Distinct cellular aggregates were visible on the upper as well as the lower surface, where groups of cells and matrix (2-3μm wide) were joined by a web-like matrix structure. The upper surface of valley 3 was composed of a thick EPS web, trapping clumps of cocci and bacilli <1μm long (Figure 3.7b). Large rhomboid crystals (25μm), interspersed between 1μm long cocci and bacilli in a matrix, protruded from the lower surface of the biofilm. The upper surface of valley 5 consisted of 3μm long bacilli covered by the matrix. The middle appeared to have a more open structure with porous crystalline structures (1-2μm) distributed between 3μm long bacilli.

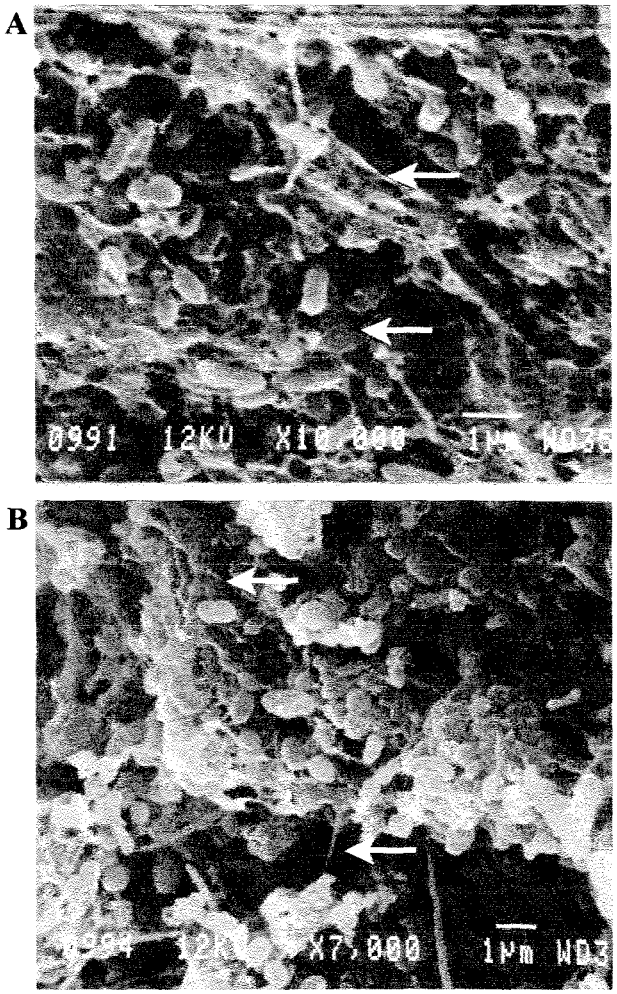


Figure 3.7: SEM of the biofilm after an increase in lactate, with arrows illustrating matrix development. A = Valley 1; B = Valley 3

3.3.2.3 Six weeks after start-up:

A very thick matrix covering was present on the biofilm in valleys 1, 2 and 3. No bacterial cells were visible in the biofilms from valley 1 and 3, although rod shapes of bacteria were discernable in valley 2. At a magnification of 2500X, only sulphur crystals, and no bacteria, were visible in the biofilm from valley 5 (Figure 3.8). The crystals were assumed to be sulphur because a comparison to previously collected biological sulphur shows very similar crystal shapes (Figure 3.9). At 7000X magnification, 1 μm bacilli were visible. A marked decrease in bacterial numbers was noted, compared to the observations two weeks prior to this sample. Valley 5's biofilm had a crystalline surface, with very few bacilli present (1-2μm). Bacilli (1-3μm) were present in the middle of the biofilm, held together by an EPS matrix with fewer sulphur crystals present. The lower surface was composed of an EPS, crystal and bacterial matrix.

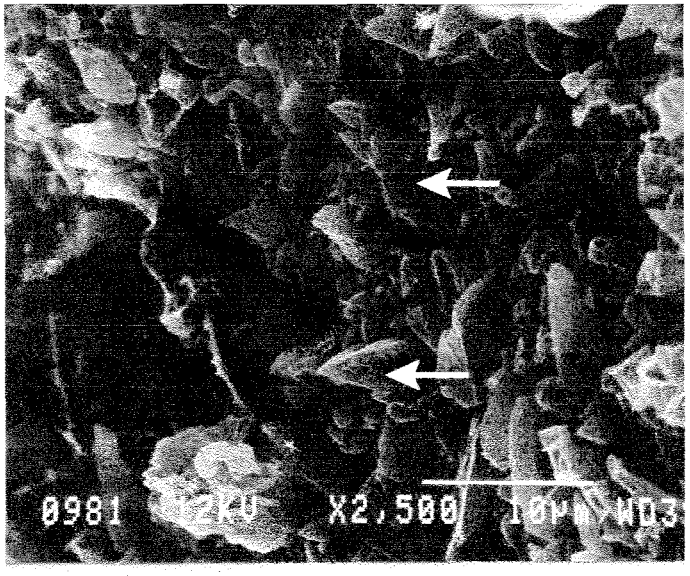


Figure 3.8: Biological sulphur crystals on the upper surface of a biofilm from valley 5; taken 6 weeks after start-up. Arrows indicate sulphur crystals

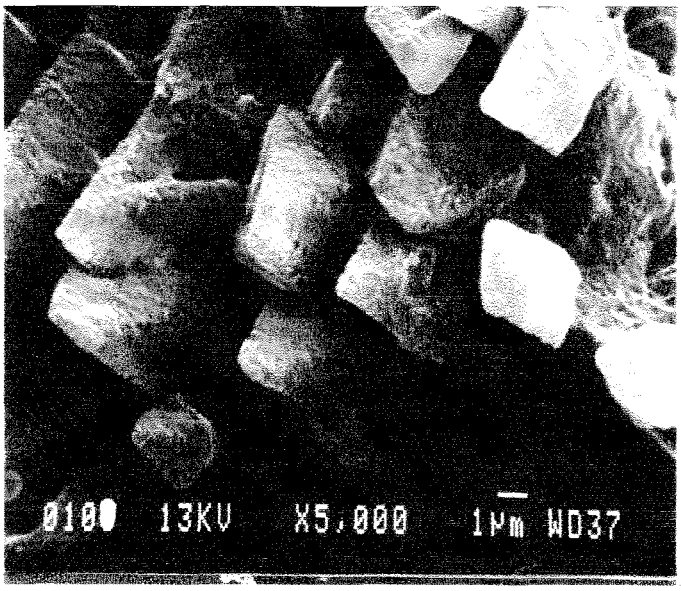


Figure 3.9: Magnification of biological sulphur crystals

3.3.4 Effect of changes in sulphide and COD concentration, and aging on biofilm morphology

Distinct bacterial morphological differentiation was observed through the biofilm cross section (Figure 3.3a), four weeks after start-up. Bacilli and cocci were the dominant bacterial morphologies, with long, filamentous bacteria and spirilla observed in the middle and lower sections of the biofilm (Figures 3.4a-c; 3.5; 3.6a and b). At lower sulphide concentrations present in valley 1 and 3, sulphide was converted to sulphur as well as sulphate, creating a less brittle biofilm, although orthorhombic crystals, which may be sulphur, were present (Figure 3.6b). The biofilm cross section also provided evidence of voids, which facilitate transport of nutrients and gases (Bishop, 1997). Okabe *et al.* (1996) found that up to 50% of the O_2 consumed by microbes was supplied by the interstitial voids. These channels are actively maintained by the species present within a biofilm for long periods of time, which further supports the idea that they are used for delivery of nutrients to the cells, rather

than passive diffusion (Costerton, 1995).

The sulphide gradient from valley 1 to 5 was not very pronounced after four weeks, resulting in less morphological differentiation across the reactor than in the sample taken after six weeks (Table 2.1). The higher sulphide concentrations in valley 5 six weeks after start-up resulted in a change in the dominant bacterial morphologies present. Bacterial cells were mostly rod-shaped, with very few cocci visible, suggesting bacillus forms were the dominant sulphide oxidising and sulphide tolerant types present. Biofilms grown in higher sulphide concentrations (valleys 4 and 5 after six weeks) also showed a greater concentration of sulphur crystals present, apparently contributing to increased biofilm brittleness (Figure 3.8). The appearance of sulphur crystals observed using SEM, and the increase in biofilm brittleness, was accompanied by the biofilm changing colour from white to yellow as a result of biological sulphur accumulation (Table 2.1). This is probably due to the increase in sulphide concentration, because it would lower the redox potential, thereby favouring biological sulphur production. The metabolism of the increased COD, after eight weeks, by the SRB would result in increased oxygen consumption, thereby decreasing the oxygen concentration and also lowering the redox potential, leading to an increase in sulphur production.

Aging of the biofilm from 4 to 6 weeks resulted in the presence of fewer long spirillum-shaped bacterial cells, and smaller cocci and bacilli became more visible, suggesting that the species involved in sulphur biofilm functioning were cocci and rod shaped cells. As the biofilm aged, it also became more brittle, and sank down from the surface, thereby reducing the amount of coverage (Table 2.1). The sinking of the biofilm is probably a result of sulphur and biomass accumulation. The large

sulphur crystals observed in Figure 3.8 would disrupt the biofilm structure and the surface tension of the liquid feed would not be able to support the increasing weight.

When the amount of lactate in the feed increased, however, the biofilm coverage increased along with a decrease in brittleness. The increase in lactate resulted in a greater production of the matrix surrounding the biofilm (Figures 3.7a and 3.7b), which was still visible with SEM despite the preparative techniques which have been found to dramatically reduce the amount of matrix visible in a biofilm (Stewart *et al.*, 1995). This matrix appeared to have a stabilising effect on the biofilm and prevented the otherwise brittle biofilm from breaking apart and sinking. After the increase in lactate, the only apparent bacterial shapes were small (<1 μm) rods which, linked to increased sulphur production, implicated rod forms as the principal sulphide oxidising forms present in the biofilm. The composition of the matrix was difficult to determine because staining for polysaccharides using light microscopy was not possible due to the opaque nature of sulphur-rich biofilm. Other authors have found that biofilm matrixes are composed of exopolysaccharide glycocalyx polymeric substances (Costerton *et al.*, 1987; Marshall, 1992), therefore it is likely that the composition of the matrix in this sulphur biofilm is similar, although no chemical tests were performed on the biofilm to confirm this.

3.4 CONCLUSION

This SEM study indicates that the floating sulphur biofilms generated in this study were layered, heterogenous structures with morphologically differentiated bacterial populations apparent through

their cross section. The microbial community was observed to change with varying levels of sulphide and COD, modifying the internal biofilm structure. This was apparent as the biofilm became more brittle with increased sulphur crystal formation at higher sulphide concentrations, and less brittle when the COD concentration was increased as a result of pronounced matrix formation. As both sulphide and COD increased, morphological differentiation was observed to decrease, with rod shaped bacterial cells dominating. Once the dominant rod-shaped morphologies were distinguished by SEM, the next step was the identification of these bacterial species to gain more information on their role in the biofilm.

CHAPTER 4

ISOLATION AND CHARACTERISATION OF BACTERIAL STRAINS IN A FLOATING SULPHUR BIOFILM

4.1 INTRODUCTION

An understanding of the biology of floating sulphur biofilms depends in part on a characterisation of the microbial community members occurring in these systems. Identifying microbial types occurring in the biofilm is the first step in determining their individual performance, and what their combined role in the biofilm would be. A broad range of procedures are available for studying the components of mixed microbial communities. These include traditional isolation techniques, where microorganisms are grown and separated on solid media to obtain pure cultures of the species present in the system. Pure cultures obtained from complex environmental samples may be useful as a control for other procedures, for example with nucleic acid probes (usually rDNA) that are used to confirm the presence of certain species in an environment. The probes' stringency needs to be tested against a reference organism by hybridisations, making a pure culture of an organisms from the environment a great advantage (Schönhuber *et al.*, 1999). Isolates from the microbial community are also helpful as controls in molecular techniques, where the presence of the organisms in the total community can be cross-checked.

The pure cultures isolated from environmental samples can be identified using fatty acid methyl ester (FAME) analysis, or rRNA gene analysis. FAME analysis, which classifies organisms based on their fatty acid profiles, assumes that the phospholipids present in bacterial cell membranes vary between taxonomic groups, resulting in unique "signatures" (Haack *et al.*, 1994). A disadvantage of this method is that lipid profiles vary within individual taxa with changes in environmental conditions, and there is a lack of information on the fatty acid profiles of various taxa (Haack *et al.*, 1994).

Analysis of rRNA sequences is favoured by microbial ecologists because rRNA is found in all organisms and has both conserved and variable regions, allowing the construction of general and specific probes (Muyzer and Ramsig, 1995). Using this approach, single cellular organisms may be divided into Archaea (Archaebacteria), Bacteria (Eubacteria) and Eucaryote (Eucarya). Sulphate and sulphur reducing bacteria are grouped mostly in the delta subclass of proteobacteria within the Bacterial kingdom (Busse *et al.*, 1996). The rRNA gene is preferred over other genes as genetic markers because there are large public rRNA databases with known sequences, and rRNA genes are highly conserved and can therefore be used to examine phylogenetic relationships between species (Britschgi and Giovannoni, 1991). In order to identify a species, the rRNA gene is sequenced, and the sequence is compared to known sequences in the public databases to match the unknown sequence to the closest sequence available.

PCR amplification of rRNA genes is one of the RNA-based species detection and identification methods. Once the sequence of an organism has been determined, primers specific to the conserved region of that species can be designed. A positive PCR reaction using those specific primers would

therefore indicate the presence of that particular organism.

Restriction enzyme digests are another method used in species identification, where specific banding patterns of DNA after digestion with a restriction endonuclease can be used to differentiate between strains, species and genera (Busse *et al.*, 1996). Gardner (1998) used this protocol to identify the dominant thiobacilli species in a biomining reactor. This rapid identification technique does, however, require a prior knowledge of the restriction sites of the target organisms in order to recognise the banding pattern on a specific DNA fragment.

Traditional plate isolation techniques were used in this study as part of a structured approach to identify the microbial components of a floating sulphur biofilm grown in the baffled reactor. The procedures for identification of bacterial species present in the biofilm included strain isolation on solid media, followed by their morphological and metabolic characterisation. Identification of these strains was attempted using Bergeys manual, after which the molecular techniques of ribosomal DNA extraction and PCR amplification of DNA fragments corresponding to parts of the 16S rRNA gene. The sequences of these PCR fragments were then determined and compared with other sequences in the Ribosomal Database.

4.2 METHODS AND MATERIALS

4.2.1 Strain isolation

Strains were isolated from a floating sulphur biofilm grown on a lab scale reactor, described in Chapter 2. Approximately 2cm^2 of the biofilm was vortexed in 1 mL phosphate buffer (61.5 mL 1M K_2HPO_4 and 38.5 mL 1M KH_2PO_4, pH 7.0), after which serial dilutions were made in phosphate buffer, with 100 µL plated onto minimal salts agar (Appendix A) and incubated at 25°C. Minimal salts agar with the addition of thiosulphate as an energy source was used in order to select for mainly autotrophic sulphide oxidising bacteria. Individual colonies were picked and restreaked onto minimal salts agar at least three times to obtain pure cultures. Isolated colonies were classified according to colony shape and colour, with six morphologically distinct colonies being chosen as representative species in the biofilm. These isolates were plated out on three types of growth media (nutrient agar without thiosulphate addition, nutrient agar with the addition of 12.4 mg/L thiosulphate and a minimal salts medium with the addition of 12.4 mg/L thiosulphate) in order to determine their growth requirements and to determine whether the isolates were heterotrophs, facultative heterotrophs or autotrophs. Gram stains were made by air drying a smear of the cells on a glass slide, followed by the protocol set out in Appendix B.

4.2.2 Polysaccharide determination

The amount of polysaccharide produced by each isolate was determined under autotrophic and

heterotrophic conditions. The cells were grown in 75 mL minimal salts medium or alternately in nutrient broth in a 100 mL Erlenmeyer flask for 24 hours shaking at 90 rpm at 37°C, in order to achieve optimal growth conditions, as determined in Chapter 2, and then left to stand for 48 hours at 37°C to stress the cells. To measure the capsular polysaccharide, 5 mL of the cells were centrifuged at 5 000 rpm (Beckman JA-21 rotor) for 15 minutes, then resuspended in 5mL ddH_2O. This suspension was heated to 50°C for 30 minutes before centrifuging at 5 000 rpm for 15 minutes. The supernatant was then used for further analysis.

The phenol-sulphuric acid method was used to determine the glucose-equivalent production by the cells (Dubois *et al.*, 1956). A 1 mL sample was added to an equal volume of 5% unbuffered phenol and mixed by vortexing before adding 5 mL concentrated sulphuric acid. The solution was cooled to room temperature in a water bath, before reading the absorbance (495nm). The equation used to determine polysaccharide concentration, calculated from the standard curve (Appendix F) was:

Glucose equivalent = $\text{Absorbance}_{(495nm)} \times 123$

4.2.3 DNA Extraction procedures

4.2.3.1 Chromosomal DNA extraction:

The six isolates were each grown in 5 mL nutrient broth with 12.4 mg/L (50 mM) added sodium thiosulphate and incubated for 24 hours at 37°C. DNA was extracted using the DNeasy Tissue Kit (Quigen). Bacterial cells (not more than 2×10^9 cells) were pelleted, then resuspended in 180 µL enzymatic lysis buffer (20 mM Tris-Cl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, 20mg/mL

lysozyme) and incubated at 37°C for 30 minutes. The manufacturer's instructions for bacterial DNA extraction were then followed. DNA concentrations between 50 and 110 µg/mL were routinely extracted from the isolates, determined as in Appendix C.

4.2.3.2 Plasmid DNA extraction:

Two methods of plasmid extraction were used in this study. For routine screening of clones, the Easyprep method (Berghammer and Auer, 1993) was used. For cloning and sequencing, a High Pure Plasmid Extraction kit (Roche) was used. In both methods, colonies were picked and grown in 5 mL Luria broth (LB) + Ampicillin (Appendix A) in a test tube overnight. The Easyprep method is described in Appendix C, and the High Pure Plasmid Extraction kit was used according to the manufacturer's instructions.

4.2.4 PCR reactions and restriction fragment length polymorphism (RFLP) analysis

Primers GM5F (5'-CCT ACG GGA GGC AGC AG-3') and P2 (5'-ATT ACC GCG GCT GCT GG), which correspond to positions 341 and 534 on the 16s rRNA gene of *E. coli* were used to amplify a 193 bp DNA fragment from the 16s rRNA gene. The PCR reactions contained 250 ng chromosomal DNA, 50 pmol of each primer and 1U *Taq* DNA polymerase (Roche) in a final volume of 100 µL. A touchdown program (Table 4.1) was used for a total of 28 cycles with a final annealing temperature of 58°C. In the case of amplifying the 190 bp insert from plasmid DNA, approximately 50 ng of plasmid DNA was used, and the program was run for 25 cycles. The presence of the 190 bp fragment was detected by electrophoresing 5 µL of the PCR reaction on a 1% agarose gel (Appendix C)

Table 4.1: Touchdown PCR program used for primers P2 and GM5F

Temperature	Time	Number of cycles
98°C	5 min	1 - Hot start
80°C	2 min	1- Add *Taq* polymerase
94°C 66°C 72°C	30 sec 45 sec 3 min	4 cycles
94°C 64°C 72°C	30 sec 45 sec 3 min	4 cycles
94°C 62°C 72°C	30 sec 45 sec 3 min	4 cycles
94°C 60°C 72°C	30 sec 45 sec 3 min	4 cycles
94°C 58°C 72°C	30 sec 45 sec 3 min	12 cycles
72°C	5 min	1 - Final extension

The 190 bp fragments amplified from the six isolates were analysed using RFLP with the restriction enzymes *Sau* 3A and *Msp* I, which recognise the sequences GATC and CCGG respectively. *Msp* I and *Sau* 3A both have 4 bp recognition sites, which means that in the 1.5 Kb rRNA gene, there

would be a restriction site on average once every 256 bp. One μg amplified DNA was digested with 2U enzyme, in a final volume of 20 μL. The reaction was incubated at 37°C for 3 hours, before electrophoresis on 2% MetaPhor agarose (Bioproducts). The molecular weight marker run with the restriction digests was a digests of 1 μg pUC 18 with 2U *Msp* I, which yielded 10 fragments ranging in size from 501 bp to 34 bp.

4.2.5 Cloning of the 193 bp fragment into pGEM-T Easy vector

The amplified 190 bp fragments were cloned into the pGEM-T Easy Vector System (Promega) (Figure 4.1). 150 ng PCR fragment was inserted into 50 ng of the vector in a ligation reaction with one Weiss unit of T4 DNA ligase in a final volume of 5 μL, which was incubated at 4°C overnight. The ligation mix was then electroporated into electrocompetent DH5α *E. coli* cells (Appendix C) and plated out onto Luria agar containing ampicillin, 5-bromo-4-chloro-3-indolyl-β-galactosidase (X-gal) and isopropyl-β-D-thiogalactosidase (IPTG) (Appendix A).

The pGEM-T Easy vector carries the β-lactamase gene, which confers ampicillin resistance, allowing any DH5α *E. coli* cells with the plasmid to grow on the Luria agar plates containing ampicillin. The plasmid also has a multiple cloning region within the α-peptide coding region of β-galactosidase. When the fragment is inserted into the coding region, the α-peptide is inactivated, allowing colour screening of recombinant plasmids. In the presence of X-Gal and IPTG, the α-peptide of the β-galactosidase peptide produces a blue product, resulting in blue colonies where there is no insert in the plasmid. If the fragment has been inserted into the multiple cloning region, resulting in a

recombinant plasmid, the α-peptide is inactivated, producing white colonies.

4.2.6 Sequencing of rRNA fragments

Sequencing reactions were performed using half reactions from the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer), as described in Appendix C. The DNA fragments were sequenced in one direction using the -21 M13 forward primer. The 16S rDNA sequences were submitted to the Ribosomal Database Project (RDP) for comparison with other 16S rDNA sequences. Sequence alignments were performed using ClustalW version 1.5 software.

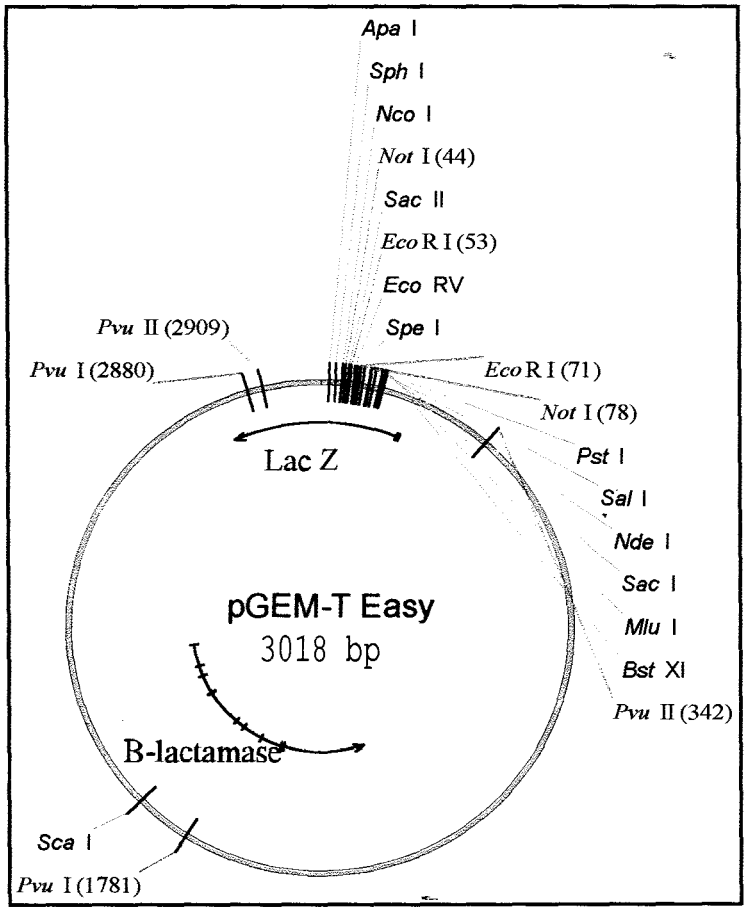


Figure 4.1: Restriction map of pGEM-T Easy Vector

4.3 RESULTS AND DISCUSSION

4.3.1 Isolation and culture of strains from the sulphur biofilm

Single colonies were selected based on colony shape and colour, cell shape and size and gram stain reactions. Six morphologically distinct colonies were chosen, designated as A, B_1, B_2, C, D and E (Figures 4.2a-f). The colonies were re-streaked onto minimal salts agar plates regularly to select for autotrophic sulphide oxidising bacteria and to ensure that pure cultures were retained. All of the isolates grew at similar rates on nutrient agar with or without thiosulphate, and on minimal salts agar plates. Growth on minimal salts agar was, however, slow for all the isolates, with colonies only appearing one week after inoculation, compared to a day when grown on nutrient agar. Growth of these organisms on the minimal salts agar could have been supported by trace organic compounds known to be found in the Bacteriological Agar (Saarchem) used to make up the plates. The minimal salts agar plates were therefore not strictly autotrophic, and could have supported the slow growth of heterotrophic organisms for a period of time.

Table 4.2: Comparison of the six strains isolated from a sulphur biofilm

	A	B_1	B_2	C	D	E
Colour and shape of colony (figures 1a-f)	White, layered colony shape	Yellow, round colonies	Light coloured, round colonies	Cream colonies with frilly edges	Light purple and cream colonies with irregular edges	Cream, round colonies
Cell shape (figures 2a-f)	Rod	Filamentous rods	Cocci	Rods	Cocci and rods	Cocci
Cell size	2 µm	10 µm	1 µm	1 µm	1-2 µm	1 µm
Gram stain	Positive	Positive	Negative	Negative	Positive	Negative

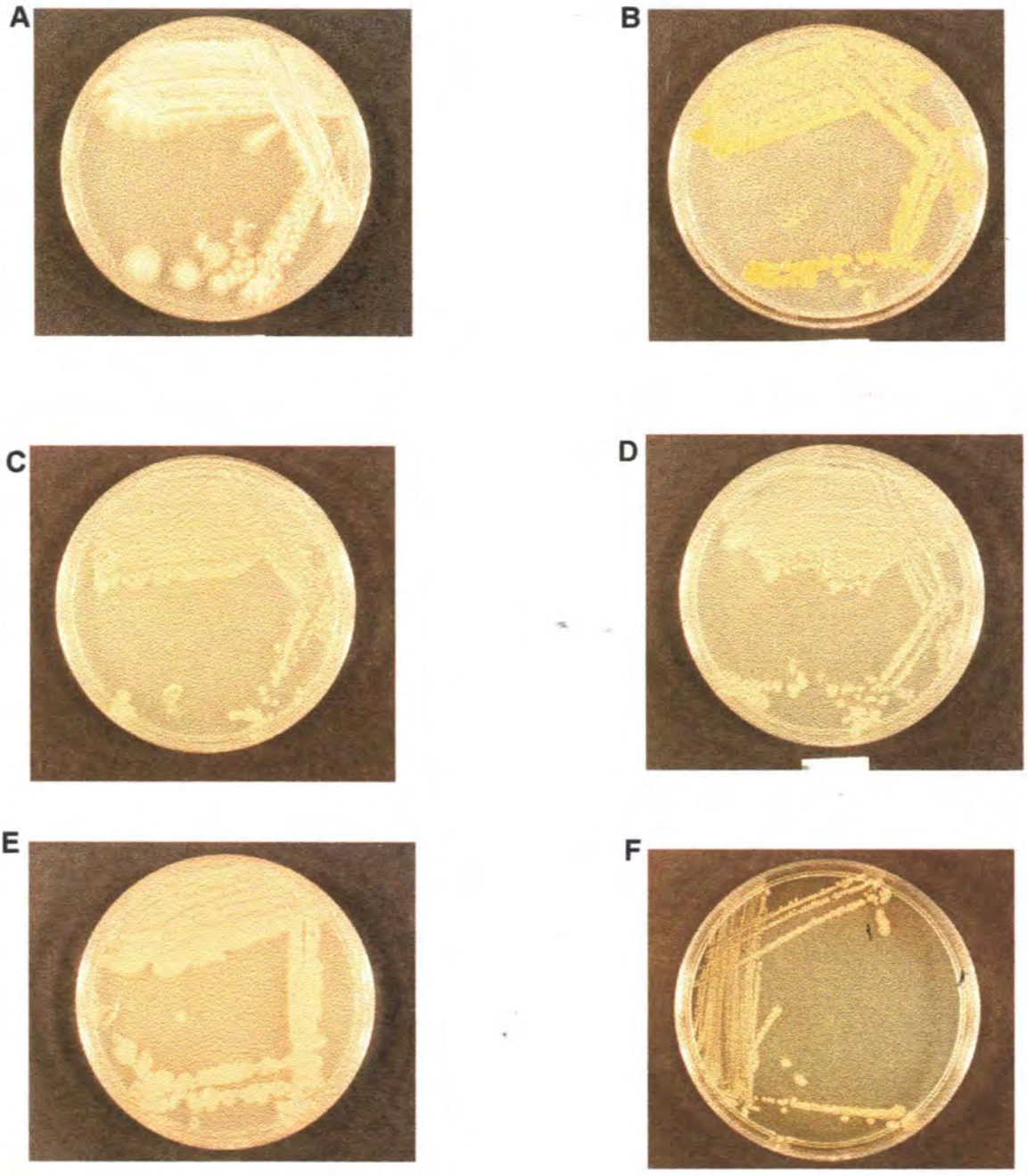


Figure 4.2: Colonies of isolates grown on NA plates with the addition of 50 mM thiosulphate. A=Isolate A; B=Isolate B_1; C=Isolate B_2; D=Isolate C; E=Isolate D; F=Isolate E

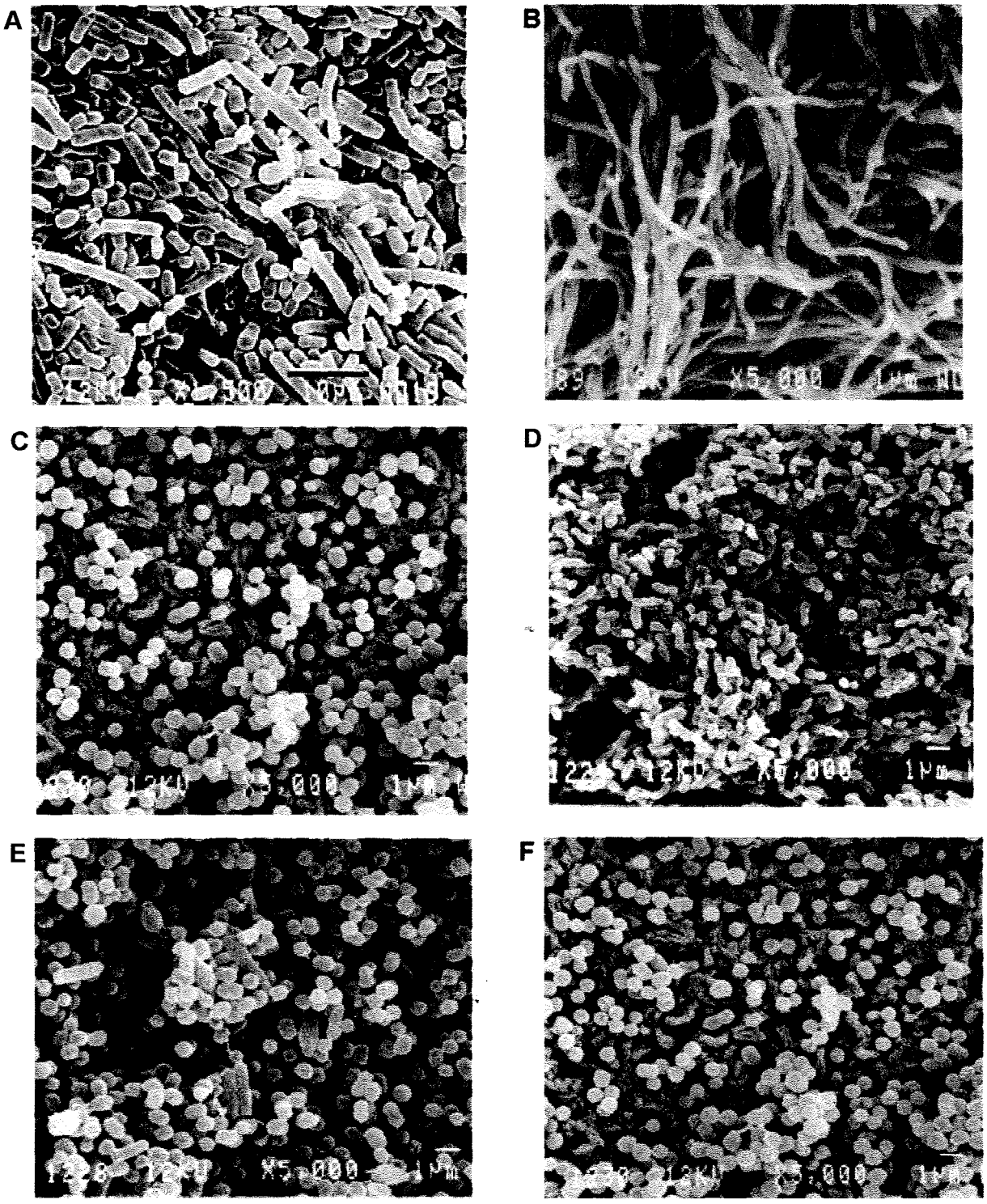


Figure 4.3: Scanning electron micrographs of the six isolates, illustrating cellular morphology. A=Isolate A; B=Isolate B_1; C=Isolate B_2; D=Isolate C; E=Isolate D; F=Isolate E. Scale bar for B-F = 1μm. Scale bar for A = 10 μm

The SEM micrographs reveal that isolates A and D are composed of two morphologically distinct cell types (Figures 4.3a and 4.3e). Isolate A contains cylindrical rods over 1 μm long and shorter coccobacilli, while Isolate D consists of 1 μm cocci and 2 μm rods. Although the colonies of A and D were restreaked numerous times in order to establish pure colonies, the mixed cultures persisted. This is visible in the colonies of Isolate D, where the colonies consist of distinct purple and yellow areas (Figure 4.4). The species in Isolates A and D could be co-dependant, and therefore are unable to survive alone. The cells of Isolates B_2 and E are very similar in shape (Figures 4.3c and 4.3f), and the colony shapes are similar, however the colour of E's colonies is darker than B_2.

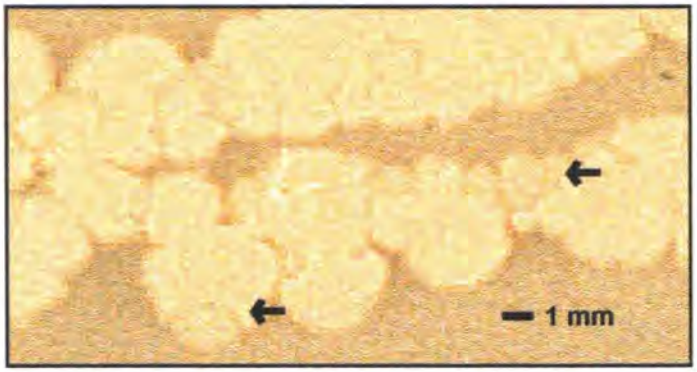


Figure 4.4: Magnification of Isolate D colonies, where arrows indicate the purple and yellow areas. Scale bar = 1mm

4.3.2 Polysaccharide production by the six isolates

Polysaccharide production by the six isolates was studied in order to determine their possible role within the biofilm. Biofilm structures are supported by some form of matrix, as observed in Chapter 3, which is most often a polysaccharide matrix (Costerton *et al.*, 1987; Marshall, 1992). The type and quantity of the EPS present in the biofilm affects the physico-chemical properties of the biofilm,

making an understanding of the bacteria involved in this function important (Jahn and Neilsen,1998). Because a polysaccharide matrix plays a central role in maintaining the biofilm, it is possible that non-polysaccharide producers would be selected against, making polysaccharide production a differential criterion for active biofilm species. The isolates were tested for polysaccharide production to test this hypothesis, and to compare the amount of polysaccharide produced by each isolate. Although SEM revealed a thick matrix surrounding the cells in a sulphur biofilm (Figures 3.5a and 3.5b), electron microscopy cannot be used to study polysaccharides as the sample preparation protocol involving an alcohol dehydration series tends to destroy the EPS matrix. For this reason, polysaccharide production by each of the strains was measured using the phenol-sulphuric acid method to determine whether any of the isolates were involved in the important role of polysaccharide matrix production within the biofilm.

Capsular polysaccharide production by the isolates was greater when grown on complete medium than on minimal salts medium (Figure 4.5), which is to be expected when more organic material is present. Isolate D had the lowest polysaccharide production under both growth conditions, with isolate A producing the next lowest amount of polysaccharide. When grown on complete medium, isolate B_1 produced the most polysaccharide, while on minimal salts medium, isolate E produced the most. Under the complete medium conditions, simulating the heterotrophic conditions present in the biofilm environment, Isolate B_1 produced the most polysaccharide, and could be involved in the important role of producing a polysaccharide matrix, which holds the otherwise brittle biofilm together. Isolates A and D are probably not important in this role, as they produce the least polysaccharide, while B_2, C and E may be involved to some extent. Although the isolates produce

varying amounts of polysaccharide, they do all probably produce enough to account for biofilm adhesion purposes. Biofilm formation has been found to be responsible for switching on polysaccharide production genes, therefore the study of polysaccharide production in liquid culture is inherently problematic, although growing the cells in a biofilm would have been much more difficult.

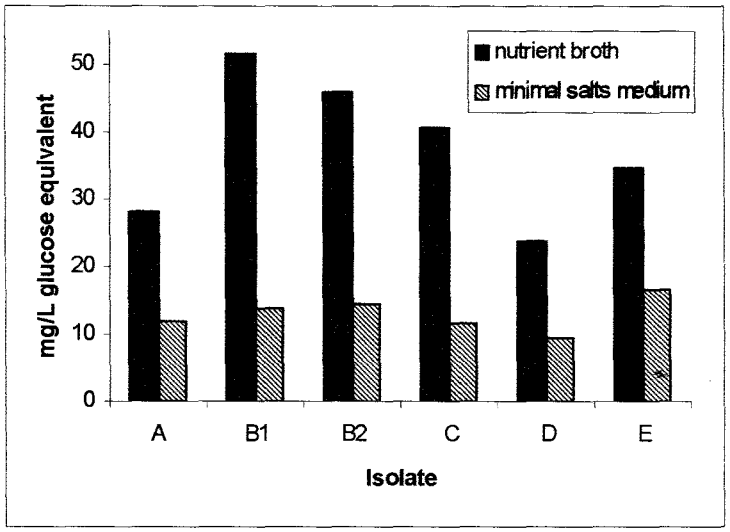


Figure 4.5: Capsular polysaccharide production by isolates from the biofilm under heterotrophic and autotrophic conditions

Identification of the isolates using the conventional techniques of morphological and metabolic tests and gram stains indicated the presence of six distinct strains present in the biofilm. Identification of the isolates using Bergey's Manual of Determinative Bacteriology on this information alone was difficult as all six of the isolates could grow on minimal salts and complete media, and the presence of thiosulphate did not appear to affect cell growth.

4.3.3 Genetic analysis and strain identification

A 190 bp DNA fragment corresponding to a portion of the 16S rRNA gene from the different isolates

The presence of more than one species in at least one of the isolates was problematic for identification of the isolates by DNA sequencing. In order to ensure single species, the amplified 190 bp fragments were inserted into the pGEM-T Easy vector. After ligation of the 190 bp fragment into the vector, and transformation of the vector into electrocompetent *E. coli* DH5α cells, 10 white colonies (which contained recombinant plasmids) were picked from each isolate. The recombinant plasmids were screened for the 190 bp insert by extracting the plasmid DNA and digesting it with *Eco* RI, which has recognition sites on either side of the p-GEM-T Easy vectors'cloning cassette (Figure 4.1), and therefore releases the inserted fragment from the plasmid.

Between 2-4 recombinants with the correct inserts were chosen for PCR amplification from each isolate, depending on whether or not the isolates appeared to comprise more than one species from SEM work and restriction fragment analysis. The inserted fragments were amplified using primers P2 and GM5F, in order to confirm the correct insert and also to amplify sufficient DNA for restriction enzyme analysis of the fragments with *Sau* 3A. The restriction analysis confirmed that the target DNA fragment had the same restriction sites as the isolates from which they were amplified. One or two recombinants from each isolate were chosen for sequencing, depending on whether or not the isolate was suspected to consist of more than one species.

DNA fragments isolated from High Pure plasmid extractions of 9 recombinant plasmids were sequenced using the Big Dye kit, and the sequences were submitted to the Ribosomal Database Project in order to identify the sequences. The results of this study are reported in Table 4.4.

Table 4.4: Sequencing results of the 9 recombinants

Recombinant	Best match species	Similarity Index
A4	*Bacillus sphaericus*	0.96
A6	Environmental clone	0.96
$B_1$1	*Flavobacterium breve*	0.89
$B_1$2	*Flavobacterium breve*	0.87
$B_2$6	*Pseudomonas putida*	0.98
C5	*Alcaligenes faecalis*	0.97
D1	*Bacillus subtilus*	0.87
D3	*Bacillus sphaericus*	0.84
E4	*Acinetobacter sp.*	0.88

Because all six isolates were able to grow on the minimal salts medium, the sequencing results obtained were surprising, as none of the species were classic autotrophic sulphide oxidisers. It was suspected, however, that the majority of the strains would be heterotrophic as all six of the isolates could grow better on the complete medium. Also, the minimal salts agar plates were not entirely autotrophic, and could probably support the slow growth of heterotrophic organisms. Some heterotrophic organisms whose sulphide oxidising capabilities have been studied include: *Pseudomonas, Xanthomonas, Streptomyces, Arthrobacter, Bacillus, Flavobacterium, Micromonas, Alcaligenes, Brevibacterium, Achromobacter, Mycobacterium, Aquaspirillum, Xanthobacter, Paracoccus* and *Escherichia coli* (Friedrich and Mitrenga, 1981; Gommers and Kuenen, 1988; Cho *et al.*, 1992; Chung *et al.*, 1996a; Chung *et al.*, 1996b; Chung and Huang, 1997; Gallardo *et al.*, 1997).

Chung *et al.* (1996a) used a heterotrophic *Pseudomonas putida* CH11 immobilised in a biofilter for sulphide removal and found that it achieved a sulphide removal efficiency of 97%, which was better than the autotrophic *Thiobacillus* sp CH11. Mizoguchi *et al.* (1976) found that two *Thiobacillus* species (*T. rubellus* nov. sp. and *T. delicatus* nov.sp.) which were thought to be autotrophs were able to oxidise thiosulphate when organic material was added to the medium.

16S rDNA fragments from both isolates A and D were identified as belonging to the genus *Bacillus*. *Bacillus sphaericus*, found in isolates A and D, is a gram positive endospore forming anaerobe with elliptical or cylindrical shaped cells (Krieg and Holt, 1989), which is in agreement with the observations of isolate A, and one of the strains in isolate D, made in this study (Table 4.2; Figures 4.3a and e). The second recombinant of isolate D was identified as *Bacillus subtilus*, which is a gram positive rod shaped bacterium, accounting for the bacilli observed in the SEM of isolate D (Figure 4.3e). The two morphologically separate species observed in isolate D using SEM gave the impression that the isolate consisted of two different species, which was confirmed by sequencing a portion of the clones rDNA. Both species were *Bacillus*, which accounts for the restriction analysis not indicating the presence of more than one species.

Isolate B_1 was identified as *Flavobacterium breve*, which is a gram positive, rod-shaped, non-motile bacterium with a yellow pigmentation, commonly isolated from sewage (Krieg and Holt, 1989). The cell shape of B_1 observed using SEM (Figure 4.4b) was a long, filamentous rod shape, which is possible in this species, according to Krieg and Holt (1989) and the colonies were yellow (Figure 4.3b). An alignment of the sequences of $B_1$1 and $B_1$2, using ClustalW multiple alignment program, revealed that the two clones were identical species. One of the species in isolate B_1 was not, however,

detected in the clones, as the restriction enzyme analysis clearly indicated the presence of more than one species.

Pseudomonas putida is a gram negative chemoorganotrophic organism, isolated from soil and water, with cells not longer than 4 μm (Palleroni, 1975). This supports the findings of the study on isolate B_2, which was identified as *Pseudomonas putida*. Isolate C was identified as *Alcaligenes faecalis*, a chemoorganotrophic obligate aerobe with coccal-rod shaped cells, and most strains have colonies with thin spreading irregular edges (Krieg and Holt, 1989). The colonies of isolate C also had irregular edges and the same shaped cells as described for *Alcaligenes faecalis*.

The genus *Acinetobacter*, of which isolate E4 is a member, is characterised by coccobacilli cells which become spherical in stationary growth phase, as in Figure 4.3d. They are chemoorganotrophic, gram negative aerobes, as confirmed by the studies on isolate E (Table 4.2). Identification of the clones from their sequences using the Ribosomal Database Project (RDP) was substantiated using Bergeys Manual of Systematic Bacteriology (Krieg and Holt, 1989), indicating correct identifications.

4.4 CONCLUSIONS

Traditional plate culture techniques were used to isolate six morphologically distinct strains from the biofilm. These isolates were characterised morphologically and metabolically, and then identified by sequencing a 190 bp portion of the rRNA gene. The isolates were all identified as heterotrophic organisms, many of which have been reported as having sulphide oxidising capabilities. This preliminary study on biofilm organisms could be useful in future studies, e.g. FISH, to corroborate

cellular morphology and species identification.

Traditional isolation techniques are, however, problematic in microbial ecology because the exact conditions of the biofilm are not duplicated, leading to different recovery patterns on solid media. Also, pure culture performance may be different compared to mixed cultures growing in proximity with each other. Kalmbach *et al.* (1997) have estimated that less than 10% of microscopically visible bacteria are culturable on standard media, which leads to a huge discrepancy between direct counts and plate counts. These factors have made traditional plate isolation techniques less widely used in microbial ecology studies (Salhani and Uelker-Deffur, 1998).

Because of the known limitations of the traditional isolation techniques used in this study, it was necessary to identify components of the biofilm microbial community using molecular techniques. The next stage in this project would be the construction of a clone library of 500 bp 16S rDNA fragments amplified directly from the biofilm DNA, and to sequence those fragments. This would present a more reliable picture of the microbes present in the biofilm and could be used to confirm the results obtained using traditional isolation techniques.

CHAPTER 5

PHYLOGENETIC DIVERSITY OF A FLOATING SULPHUR BIOFILM DETERMINED BY ANALYSIS OF 16S rRNA GENES EXTRACTED DIRECTLY FROM THE BIOFILM

5.1 INTRODUCTION

Because most organisms from environmental samples are not culturable, microbial ecologists have replaced traditional cultivation techniques with molecular techniques which do not require the organisms to be isolated and cultured in the laboratory. This is especially true in the study of biofilms where spacial heterogeneity and aggregation also hinder adequate separation of microbes in the biofilm (Amann *et al.*, 1992). Analysis of rRNA genes, as described in Chapter 4, has become the favoured technique in microbial ecological studies because it is the most fundamental and straightforward method for the classification and evolutionary study of organisms (Olsen *et al.*, 1986).

However, problems with this method may be anticipated when used on complex environmental samples, such as the sulphur biofilm. One of the problems with the application of rRNA gene analysis is that bias may be introduced, by incomplete lysis of community members before nucleic acid extraction, preferential amplification of certain sequences by PCR, and poor detection of less common

organisms (Weller *et al.*, 1991; Amann *et al.*, 1995). Another problem encountered in complex environmental samples where diversity is high is that the large majority of retrieved sequences do not match the approximately 9700 sequences stored on public databases (Amann *et al.*, 1996; Maidak *et al.*, 1999). There is insufficient information on rRNA sequences for the microbial world because most organisms have not yet been isolated and/or sequenced (Mau and Timmis, 1998). Despite these limitations, the analysis of rRNA genes is the most accurate method available for microbial population identification in the study of microbial ecology.

A powerful tehnique used for species identification within complex communities is flourescent *in situ* hybridisation (FISH). Single stranded DNA or RNA probes, which are derived from the sequence of the target organism, have been used in FISH with great success in microbial ecology (Poulsen *et al.*, 1993; Muyzer and Ramsig, 1995; Amann *et al.*, 1996; Lee *et al.*, 1999; Okabe *et al.*, 1999; Rocheleau *et al.*,1999; Sekiguchi *et al.*,1999). In this technique labelled oligonucleotide probes bind to a target nucleic acid, which can be used for identification purposes and to determine the spacial distribution of the target organism. rDNA is the favoured target molecule for *in situ* hybridisation because it is not affected by environmental or physiological conditions, whereas ribosome content is dependent on growth rate and stage. The amount of rRNA in dormant cells is also usually too low to be detected (Amann *et al.*, 1992), although this can be used to determine the metabolic state of a specific organism. Nucleic acid probes are detected when the labelled probes anneal to form a double stranded helix with the single stranded nucleic acid target molecule. Probes can be labelled in a variety of ways, including fluorescence, colorimetry, bioluminescence and radiolabelling (Ealkinham, 1994). In the environment, nucleic acid probes are used to identify bacteria from mixed

cultures. This is necessary because plate counts often reflect less than 1% of cell counts determined using direct counting methods, which may be due to viable cells being in a non-culturable state and because many cells cannot be grown since the right media/conditions are not known (Schönhuber *et al.*, 1999). Tagged oligonucleotide probes can be used to simultaneously measure single-cell identity and growth rate within mutlispecies biofilms, as there is a correlation between cellular rRNA and growth rate. This technique has been used by Poulsen *et al.* (1993) on a sulphate reducing biofilm.

The design of phylogenetically nested oligonucleotide probes does not necessarily require cultivation or isolation of the target organism, and can therefore can be used to monitor population dynamics directly from environmental samples (Schönhuber *et al.*, 1999). Dot blot hybridisations to membrane bound nucleic acids from reference organisms are used to test the stringency of these probes (Schönhuber *et al.*, 1999)

Problems with probing when used in conjunction with FISH include uneven cell penetration, low signal intensity, high levels of background autofluorescence from phototrophs, and non-specific staining of inorganic particles can give a signal stronger than the probe signal. Other problems with using FISH are that quantification is difficult with irregularly shaped cells or clusters of cells, there can be insufficient permeability of cells and cell densities of 10000 cells/mL are needed to detect one cell because of microscopic limitations (Ramsig,1998). Scanning confocal laser microscopy can, however, be used to enhance a specific fluorescent signal. It is necessary to use a negative non-hybridising control and well known reference strains in order to evaluate staining efficiency. Digital imaging and "blind" experiments help to eliminate subjective bias by the evaluator of the signal.

Denaturing gradient gel electrophoresis (DGGE) is becoming a frequently used technique in molecular ecology to compare the structures of complex microbial communities (Heuer *et al.*, 1999), and is a powerful tool when used in conjunction with DNA sequencing. DGGE is a culture-independent approach based on the separation of 16S rDNA fragments amplified directly from the whole community DNA extracts on a polyacrylamide gel containing a linearly increasing gradient of denaturants. The fragments are separated according to their melting point, which is dependent on their G+C content (Fantroussi *et al.*, 1999). DGGE allows the separation of fragments of the same size, but with different sequences. The electrophoretic pattern can also be hybridised with taxon-specific oligonucleotide probes (Muyzer and Ramsig, 1995).

Under optimised conditions, one point mutation in a 1000bp fragment can be detected by DGGE (Eichner *et al.*, 1999). The rRNA sequences from environmental samples can also be compared using sequence similarities detected by DGGE to elucidate phylogenetic information, as it is assumed that fragments denaturing in the same position have a high probability of being the same, or closely related species. The distribution of SRBs in a water column have been studied using this method (Muyzer and Ramsig, 1995).

In simple communities, e.g. hot springs, each DGGE band can be assigned to cultured organisms, or retrieved ribosomal sequences, but in more complex environments, e.g. sludges, DGGE becomes limited because the banding patterns become too complicated (Eichner *et al.*, 1999). The number, position and relative abundance of the bands allow comparison of number and relative abundance of dominant rDNA types between communities. However, the number and intensity of bands do not

equal the number and abundance of species within the community because of an inherent bias of PCR amplification from complex template mixtures where one organism can produce more than one band because of multiple, heterogenous rRNA operons (Eichner *et al.*, 1999). In a complex mixture of species, less abundant sequences are not amplified sufficiently to be visualised as bands, therefore bands only reflect the most dominant species (Eichner *et al.*, 1999). One band may also represent several species with the same rDNA sequences. Because of the possibility that one band might contain more than one sequence, especially when analysing complex communities, DGGE should be used in conjunction with cloning (Felske *et al.*, 1998), and only limited sequence information is obtained because fragments longer than 500bp are not separated properly (Muyzer and Ramsig, 1995).

The molecular techniques described above are useful not only for species identification, but also for the information that can be inferred from the species identification. The metabolic role of an organism could be deduced from previous studies on a particular organism, and the sequence can be used to design specific probes for an organism. Numerous authors (for example Tsien *et al.*, 1990; Jurtshuk *et al.*, 1992; Wagner *et al.*, 1993; Wagner *et al.*, 1994; Raskin *et al.*, 1995; Mau and Timmis, 1998; Sekiguchi *et al.*, 1998) have reported the use of probes designed from nucleotide sequences for rapid species identification and FISH to study the population structure and dynamics within biofilms.

In this study, the genomic DNA from a sulphur biofilm was extracted and amplified to yield a 586 bp product with a GC-clamp attached to the 5' end, corresponding to a portion of the 16S rRNA gene. These fragments were used to construct a clone library consisting of 100 clones, which were analysed

using DGGE, and assigned into groups, from which representatives were selected and sequenced. The resultant sequences were used to determine taxonomic identities of the bacterial types associated with the floating sulphur biofilm and to begin to determine their phylogenetic relationships.

5.2 METHODS AND MATERIALS

5.2.1 Genomic DNA extraction from the sulphur biofilm

Bacterial genomic DNA was obtained from the biofilm, grown on the lab-scale baffled reactor (described in Chapter 2), as follows: 0.25 - 0.5 g of the biofilm from valleys 2 and 4 was colleted using sterile techniques and resuspended in 500 µL of TE buffer (10mM Tris-HCL, 1mM EDTA, pH8). After the addition of 0.3 mg lysozyme, the samples were incubated at 37°C for 30 minutes and mixed every 10 minutes by vortexing for three seconds. The tubes were then boiled for 1 minute, followed by four freeze-thaw cycles performed at -196°C, in liquid nitrogen, and at 80°C. The homogenate was mixed with 10 mL chilled extraction buffer (0.01M Tris-HCL pH 8.0, 5 mM EDTA pH 8.0 and 0.5% SDS). Proteinase K (50µg/mL), was added and the suspension was incubated for 12 hrs at 37°C, after which 1% (v/v) CTAB and 1M NaCl final concentration were added and the suspension was incubated at 55°C for 1hr. An initial phenol-chloroform-isoamyl alcohol (1:24:1 v/v) extraction was performed, followed by chloroform-isoamyl alcohol (24:1 v/v) extractions to remove proteins until a clear interphase was obtained. This was followed by a final chloroform extraction (without isoamyl-alcohol). The DNA was precipitated by adding 2.25 volumes of ethanol (96%) and incubated at -20°C for 12 hrs. The precipitate was collected by centrifugation (Beckman JA 20

rotor) at 10 000 rpm for 30 min at 4°C, washed with 70% ethanol, dried and resuspended in 500 µL TE buffer (10mM Tris-HCl, 1 mM EDTA, pH8). A RNase digestion (40 µg/mL DNase free Rnase, Roche) was performed at 37°C for 90 minutes to remove RNA. DNA concentrations (calculated as in Appendix C) between 200-300 ng/µL were routinely extracted from the biofilm.

5.2.2 PCR amplification of the 16S rRNA genes

Primers GM5F (5'-CCT ACG GGA GGC AGC AG-3') and 907R (5'-**CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC** GCC GTC AAT TCC TTT RAG TTT-3'), which correspond to positions 341 to 358, and 927 to 997 on the 16S rRNA gene of *E. coli* respectively, were used to amplify a 586 bp fragment from chromosomal rDNA isolated from the floating sulphur biofilm. The GC-clamp (indicated in bold, on primer 907R) is necessary for use with DGGE to prevent complete strand separation during electrophoresis through the denaturing gel. The PCR reactions contained 250 ng chromosomal DNA, 50 pmol of each primer and 1U *Taq* DNA polymerase (Roche) in a final volume of 100 µL. A touchdown program was run for a total of 28 cycles with a final annealing temperature of 60°C (Table 5.1). In the case of amplifying the 586 bp insert from plasmid DNA, approximately 50 ng of plasmid DNA was used, and the program was run for 25 cycles. The presence of the amplified fragment was detected by electrophoresing 5 µL of the PCR reaction on a 1% agarose gel, as described in Appendix D.

Table 5.1: Touchdown PCR program used for primers GM5F and 907R

Temperature	Time	Number of cycles
98°C	5 min	1 - Hot start
80°C	2 min	1- Add *Taq* polymerase
94°C	30 sec	
68°C	45 sec	
72°C	3 min	4 cycles
94°C	30 sec	
66°C	45 sec	
72°C	3 min	4 cycles
94°C	30 sec	
64°C	45 sec	
72°C	3 min	4 cycles
94°C	30 sec	
62°C	45 sec	
72°C	3 min	4 cycles
94°C	30 sec	
60°C	45 sec	
72°C	3 min	12 cycles
72°C	5 min	1 - Final extension

5.2.3 Cloning of the amplified fragments

Recombinant plasmids were generated as described in Chapter 4. Plasmids containing the 586 bp

inserts were classified into 2 groups: (i) GI, where the fragments did not contain an *Eco* R1 site and (ii) GII-E, with inserts containing an *Eco* R1 site, as determined by *Eco* RI restriction digests performed on all the recombinants. The inserts from each group were then classified according to their separation on DGGE gels, as described below, in order to eliminate identical recombinants from each group.

5.2.4 Denaturing Gradient Gel Electrophoresis analysis of the 586 bp fragments

DGGE analysis was performed using a Bio-Rad Protean II system, as previously described (Muyzer *et al.,* 1993 and 1995). The 586 bp DNA fragments were loaded directly onto 6% (w/v) polyacrylamide gels in 0.5 X TAE (0.4 M Tris-acetate, 0.01 M EDTA, pH 8.3). Gradients were formed with 6% acrylamide solutions (acrylamide-bisacrylamide 37:1 w/w) that contained variations of between 10-80% denaturant (Appendix D). The optimised concentration of denaturant used for fragments in GI and GII-E was a 35% - 70% gradient. Appendix D contains details on reagents and the gel pouring procedure. The samples from GI (non *Eco* RI site containing fragments) were loaded directly onto the acrylamide gel after the plasmid extraction was digested with *Eco* RI to separate the fragment from the plasmid. 10 µL of GI restriction digests, containing approximately 500 ng DNA, were loaded onto the gel with 5 µL tracking dye. The fragments which contained internal *Eco* RI sites (GII-E), were amplified directly from the purified plasmid using primers GM5F and 907R before loading 7 µL of the PCR product (approximately 500 ng DNA) with 3µL tracking dye onto the gel. Electrophoresis was performed at a constant temperature of 60 °C and at a constant voltage of 100V for 17 hours. After electrophoresis, the gels were stained in 1X Tris-acetate-EDTA (TAE) buffer

with ethidium bromide (0.5 mg/l) for 15 min. Fluorescence of the dye bound to DNA was excited by UV irradiation from a UV transilluminator, and was then photographed with a digital image gel documentation system (Kodac ds Electrophoresis documentation and analysis system 120 camera) and analysed using Kodac digital science 1D version 2.0.3 software.

5.2.5 Nucleotide sequencing and analysis of the amplified 586 bp fragment from the 16S rRNA gene

Sequencing reactions were performed using half reactions from the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer), as described in Chapter 4 and Appendix C, and analysed on the ABI 377 automated DNA sequencer (PE Biosystems) with assistance from Carel van Heerden, Stellenbosch University. Alternately, the Thermosequenase fluorescent labelled primer cycle sequencing kit (Amersham Pharmacia Biotech) was used, and analysed on an ALFexpress automated DNA sequencer (Pharmacia Biotech) with assistance from Mrs Di James, from the Microbiology Department of UCT. The DNA fragments were sequenced in one direction. The 16S rDNA sequences with the two primer sequences removed were submitted to the Ribosomal Database Project (RDP) for comparison with other 16S rDNA sequences (Maidak *et al.*, 1999). Sequence alignments were performed using ClustalW version 1.5 software.

5.3 RESULTS AND DISCUSSION

5.3.1 Analysis and classification of the 16S rRNA gene fragments

Screening of the 100 recombinant plasmids obtained from shotgun cloning of the amplified 586 bp rDNA fragments originating from biofilm bacteria was carried out using restriction enzyme analysis and DGGE, as outlined in Figure 5.1. The inserts were digested with *Eco* RI restriction enzyme, and it was found that 48 of the fragments contained an internal *Eco* RI recognition site, while the remaining 52 fragments did not. This information was used to classify the inserts into two groups, namely GI (without the *Eco* RI site, and GII-E, which contained the *Eco* RI site). After duplicate inserts were eliminated using DGGE analysis, 14 sub-groups remained in GI and 11 sub-groups remained in GII-E.

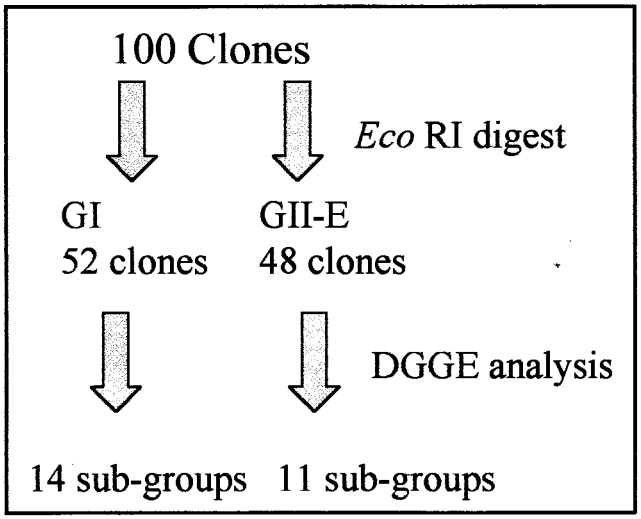
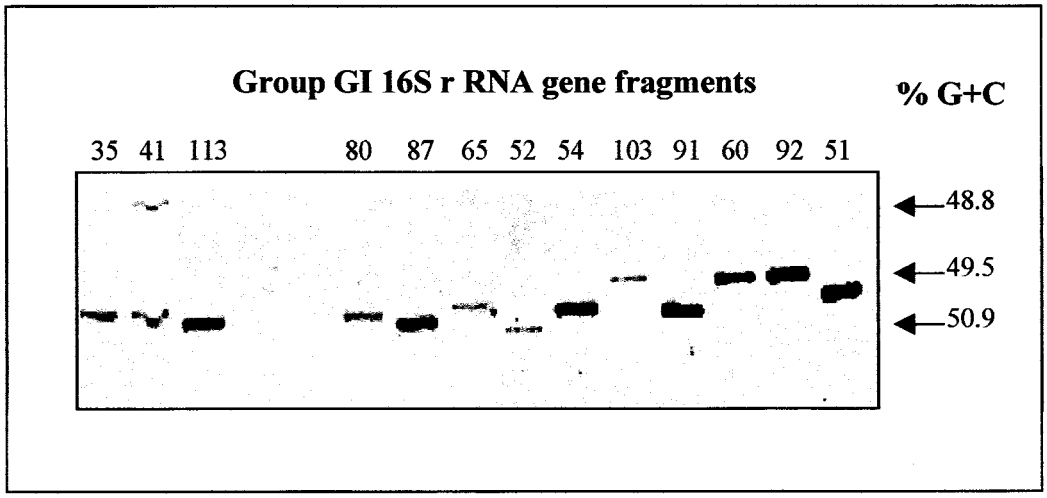


Figure 5.1: Flow chart of clone library organisation

A



B

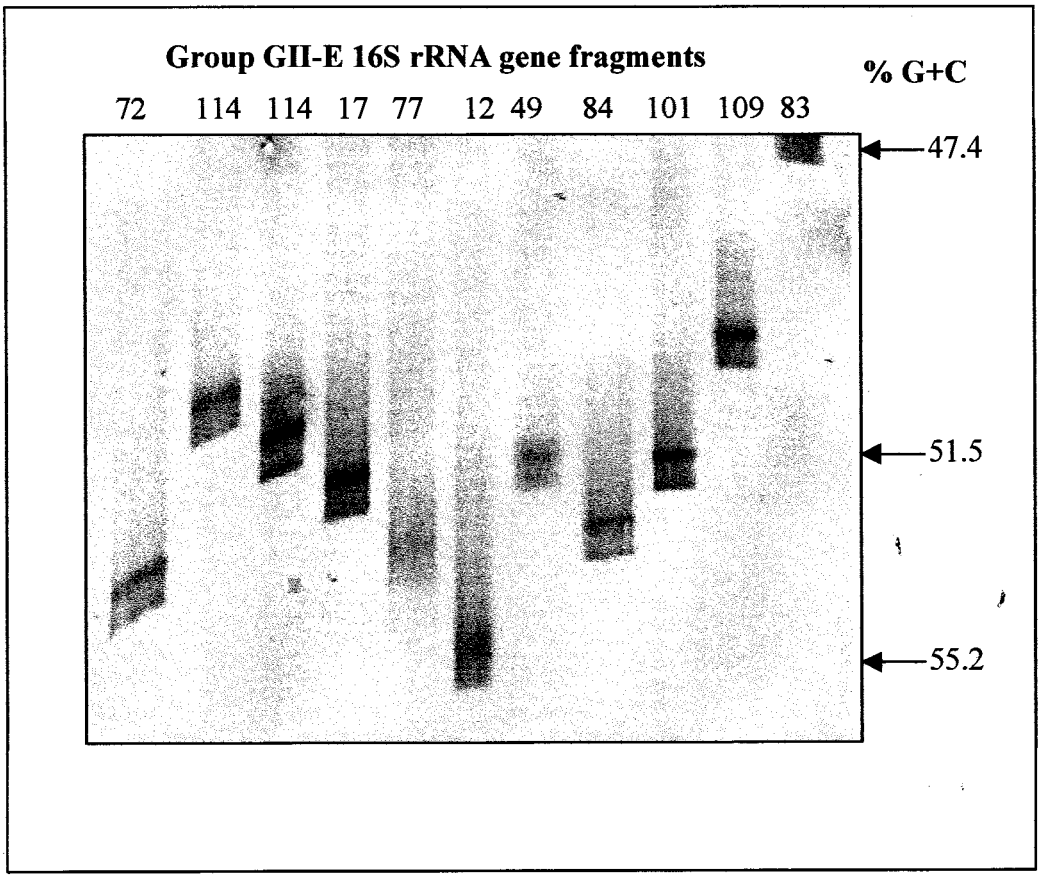


Figure 5.2: Negative image of ethidium bromide stained 6% acrylamide gels with a denaturant gradient of 35-70%. Panel A displays 13 of the 16 sub-groups in GI, and Panel B displays the 11 sub-groups in GII-E. The sub-groups are named by the numbers at the top of the gels, with the G+C content of a low, medium and high content band on each gel indicated by the arrows

Figure 2a and b illustrates DGGE gels of GI and GII-E, on which 586 bp DNA fragments have separated, giving information on their G+C contents. In GI, sub-groups 60, 92 and 103 would be expected to have a lower G+C content than sub-groups 52, 113 and 87. In GII-E, sub-group 83 would be expected to have a much lower G+C content than sub-group 12, which was corroborated by analysing the fragments sequence. A G+C content analysis of the fragments (Table 5.3) does, however, reveal that the DGGE system is not entirely reliable. For example, the G+C content of fragment 52 is 49.1%, although the fragment migrated further down the denaturing gel than fragments 80, 87, 65 and 54 surrounding it, which all had higher G+C contents than fragment 52. Fragments 35, 80 and 52 on the gel illustrating GI (Figure 5.2a), and fragment 72 on the gel for GII-E (Figure 5.2b), had incongruous banding positions and G+C contents. This could, however, be a result of unknown bases in the sequence giving an inaccurate reading of the G+C content of the sequence. The remaining fragments in both groups did, however, migrate to positions in the denaturing gel which corresponded with their G+C contents.

Fragment number 41 appeared to consist of more than one species, as two bands were seen in the sample lane. After this was noted, the clone was re-streaked, and the process was repeated ensuring a single colony was used. When the clone representing sub-group 41 was run on a DGGE gel again, only one band was observed, confirming the presence of a single species fragment. There appears to be more variation between the sub-groups in GII-E than in GI, based on the greater degree of band separation in GII-E, and according to the range in G+C contents (Figures 5.2a and b, Table 5.3).

5.3.2 DNA sequence determination

The 27 clones whose DGGE band positions were different were sequenced after DGGE analysis (Table 5.2a and b). The 586 bp fragments sequenced were short enough to be separated efficiently by DGGE, but were long enough to give reliable phylogenetic information on the species (Muyzer *et al.*, 1995; Sekiguchi *et al.*, 1998). According to Amann *et al.* (1996) there are two main categories of diversity: (i) similarities below 0.8 and (ii) similarities between 0.93-0.99. Sequences which fall into the second category are considered closely related. Of the 27 sequences, only 2 had a similarity index above 0.9, suggesting that most of the species in the biofilm are unclassified organisms. In this study, the minimum similarity index used was 0.3, below which the organisms (15% of the total library) were labelled as previously unclassified. The groupings of the organisms were therefore not true identifications, but were given the names of the organisms which had the closest sequence matches, designated by the name of the organism in inverted commas.

In order to confirm the assumption that clones which had the same band position after DGGE, a randomly selected duplicate (113) from sub-group 54 was chosen and sequenced along with the other sub-groups. The sequences of clones 113 and 54 both matched *"Pseudomonas monteilii"*, although the similarity matches differed by 0.033, suggesting that the DGGE analysis is accurate for species identification, although different strains of a species may not be distinguished.

Table 5.2a: Identification of GI sub-groups

Sub-group representative	Other clones in the sub-group	Species identification according to the RDP	Similarity index (0-1)	G+C content (%)
7	28, 27, 19, 10, 2, 21	Unclassified		46.2
29		Unclassified		47.4
41	38	*Xanthomonas campestris*	0.46	48.8
52		Unclassified		49.1
118		*Bacteriodes fragilis*	0.317	49.1
103	53	*Bacteriodes forsythus*	0.51	49.2
92	98, 82, 88	Environmental clone - *Xanthomonas* group	0.67	49.5
60		*Bacteriodes merdae*	0.552	49.6
54	100, 102, 105, 108, 54, 117, 111, 45, 87, 89, (113)	*Pseudomonas monteilii*	0.767	50.9
91	57, 97, 96, 90, 79, 40	*Pseudomonas flavescens*	0.519	51.4
65	71, 76, 63, 50, 58	*Pseudomonas putida*	0.585	51.6
51		*Pseudomonas monteilii*	0.957	52.3
32	42, 34, 43, 47	Environmental clone - unclassified	0.518	52.5
113 (54)		*Pseudomonas monteilii*	0.873	53.8
80	35	*Pseudomonas amygdali*	0.629	55.4

Table 5.2b: Identification of GII-E sub-groups

Sub-group representative	**Other clones in the sub-group**	**Species identification according to the RDP**	**Similarity index (0-1)**	**G+C content (%)**
83		Environmental clone - *Bacteriodes* group	0.764	47.4
109	95, 110, 99, 104	Environmental clone - *Azoarcus* group	0.616	50.7
114		Unclassified		51.4
101		*Xanthomonas theicola*	0.512	51.5
72	18, 37, 20, 23, 81, 74, 78, 26, 11, 4, 14, 48	*Pseudomonas caricapapayae*	0.921	52.2
116		Unclassified		52.6
49		*Thiobacillus hydrothermalis* str.	0.594	52.6
84	86, 112, 106, 94, 70, 69, 66	Environmental clone - *Xanthomonas* group	0.67	52.9
77	115, 15, 61	Unclassified		53.1
68		*Thiobacillus hydrothermalis*	0.567	55.1
12	33, 8, 1, 3, 55, 59, 62, 25, 56, 17, (36)	*Leucobacter komagatae*	0.822	55.2
36 (12)		*Leucobacter komagatae*	0.822	55.2

The primers used in PCR to amplify the 16S rRNA genes were prokaryote specific. All of the 20 different sub-groups in the clone library which could be classified were *Eubacteria*, with no *Archaea*

detected. 62% of the fragments in the clone library were classified into Proteobacteria, which is the largest and most highly evolved group of bacteria (Table 5.3). Wagner *et al.* (1993) also found that Proteobacteria accounted for about 60% of microbial cells found in activated sludge communities. The largest group of clones (40% of the total) fell into the *"Pseudomonas"* group of Proteobacteria, whose sulphide oxidising capacity has been described in the literature (Chung *et al.* 1996a; Chung *et al.* 1996b; Chung *et al.* 1996c; Gallardo *et al.*, 1997).The high number of *"Pseudomonas"* species related to 16S rRNA fragments from the sulphur biofilm suggests that *"Pseudomonas"* species could be involved in sulphide oxidation. The next largest groups were *"Xanthomonas"* and *"Arthrobacter"*, which comprised 15% and 13% respectively of the clone library. The genera found in the biofilm which are known to oxidise sulphide, including *"Thiobacillus"*, *"Pseudomonas"*, *"Xanthomonas"* and *"Arthrobacter"* (Gommers and Kuenen, 1988; Cho *et al.*, 1992;), made up 83% of the 85 clones which could be classified. This result indicates that the majority of the bacterial species present in the sulphur biofilm are probably involved in sulphide oxidation.

It was surprising that so few thiobacilli were present in the clone library (2%), and no known sulphate reducing bacteria were detected. Sulphate reducing bacteria were expected to exist in the sulphur biofilm, as an anaerobic, organic and sulphate-rich-niche would be created within the biofilm for SRB growth.

The only genus the clone library and isolated species from Chapter 4 had in common was *Pseudomonas putida.*

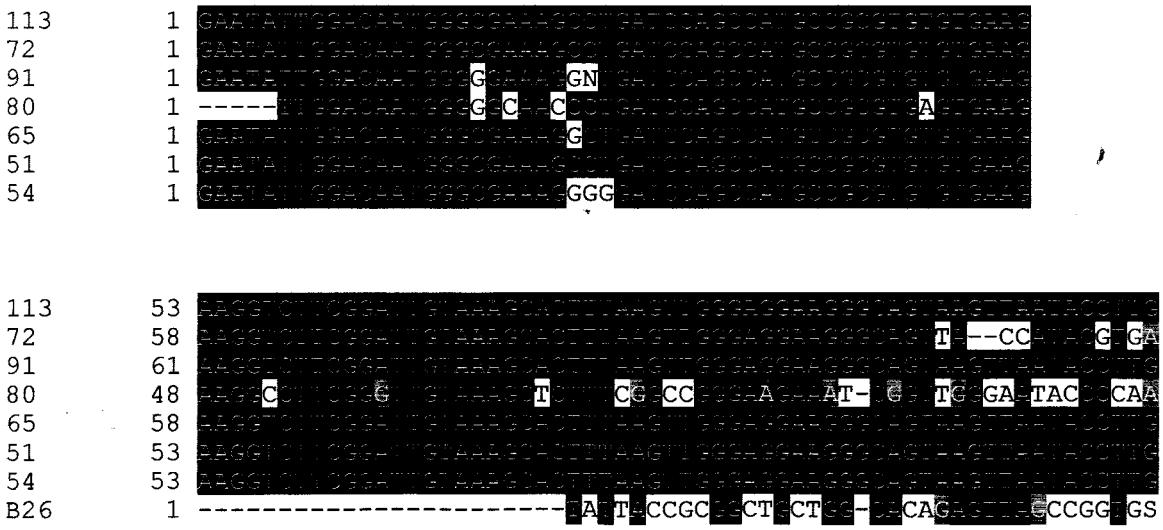
Table 5.3: Phylogenetic distribution of the 16S rRNA clones from the sulphur biofilm

Phylogenetic group	**No. of sub-groups**	**No. of clones**	**% Total clones**
PROTEOBACTERIA			
Beta subdivision -			
Azoarcus group	1	5	5
Gamma subdivision -			
Pseudomonas group	6	40	40
Xanthomonas group	4	15	15
Thiobacillus group	2	2	2
FLEXIBACTER-CYTOPHAGA-BACTERIODES			
Bacteriodes group	4	5	5
GRAM POSITIVE HIGH G+C			
Arthrobacter subdivision	2	13	13
UNCLASSIFIED	7	15	15

5.3.3 Sequence alignments of phylogenetic groups

An alignment was performed on all sub-groups classified as "*Pseudomonas*" (72 from GII-E; 65, 54, 113, 51, 80, 91 from GI, and isolate B_2 described in Chapter 4). Evolutionary relationships deduced from nucleotide sequences assume that a particular nucleotide sequence was derived by a series of mutations from an ancestral nucleotide (Olsen *et al.*, 1986). Many mutations are insertions or

deletions, thereby changing the length of the molecule, which means that alignments are regional and alignment gaps often have to be introduced. The ClustalW alignment of all the *"Pseudomonas"* groups identified in this study and strain B_2 from Chapter 4 (Figure 5.3), shows that the isolated strain B_2 was not as similar to the other *"Pseudomonas"* species found in the biofilm which were identified by the culture-independent technique of PCR and cloning. A possible explanation for this could be that isolate B_2 was present in the biofilm in very low numbers, and so was not detected in the clone library. The *"Pseudomonas"* species identified in the clone library could have been co-dependent strains, or unculturable. The alignment of the duplicates 54 and 113 confirms the slight variation in nucleotide sequence, suggesting that the fragments are from different strains of the same species. The GGG sequence at position 24-26 of the nucleotide sequence of clone 54 could be used as a site for primer design for use in further FISH studies. There are many positions in the sequence of isolate B_2 where primers could be designed for future hybridisation studies, for example, position 29-33.



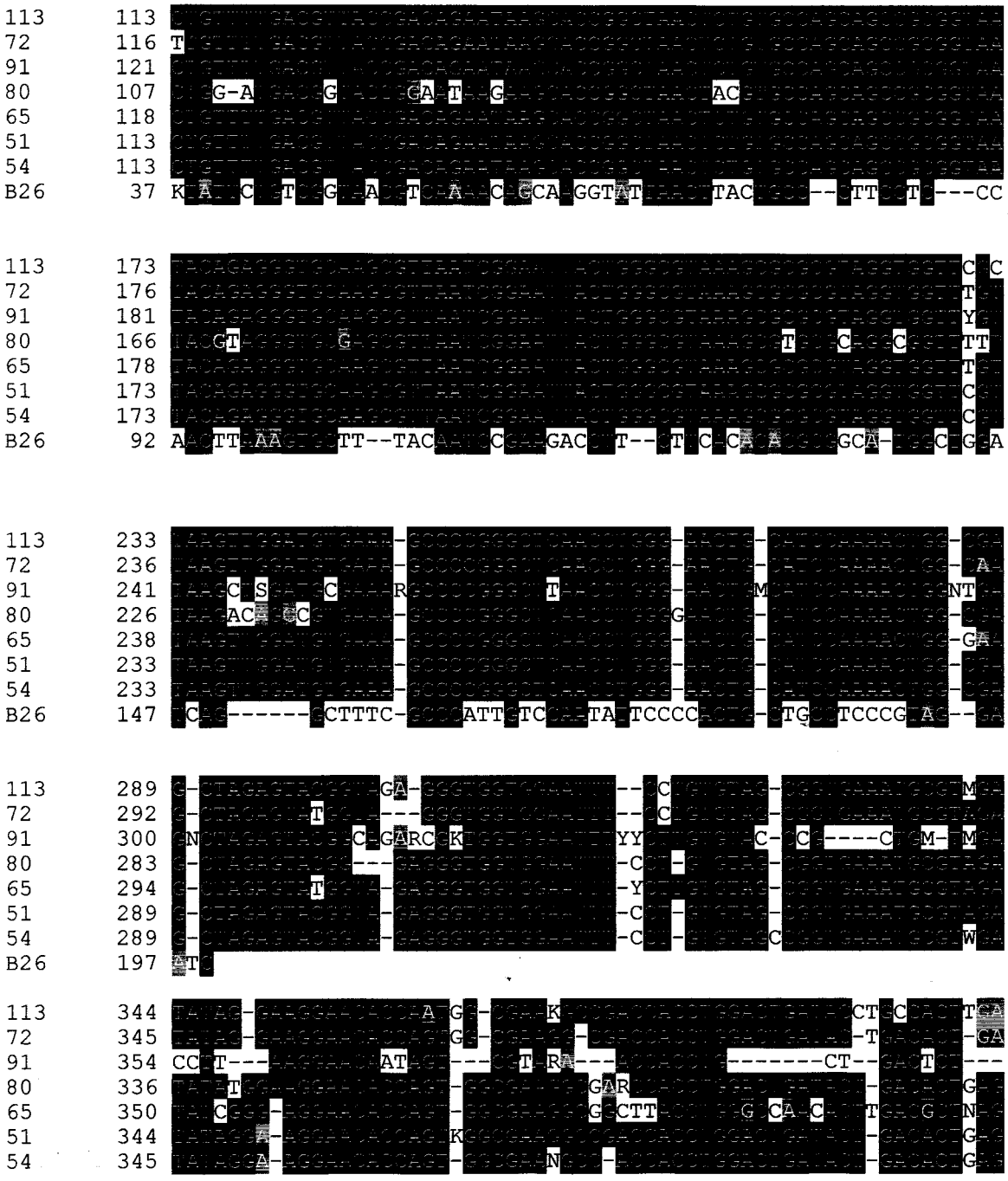


Figure 5.3: ClustalW alignment of the Pseudomonas groups' 16S rRNA sequences from the biofilm against isolate B_2 described in Chapter 4. Nucleotides shaded in black are conserved throughout the sequences.

An alignment of the three clones placed in the *Pseudomonas monteilii* group; 54, 113 and 51 confirmed the similarity of the organisms placed in this group (Figure 5.4). The differences suggest that the all three clones contain sequences of slightly different strains of *"P. monteilii"*.

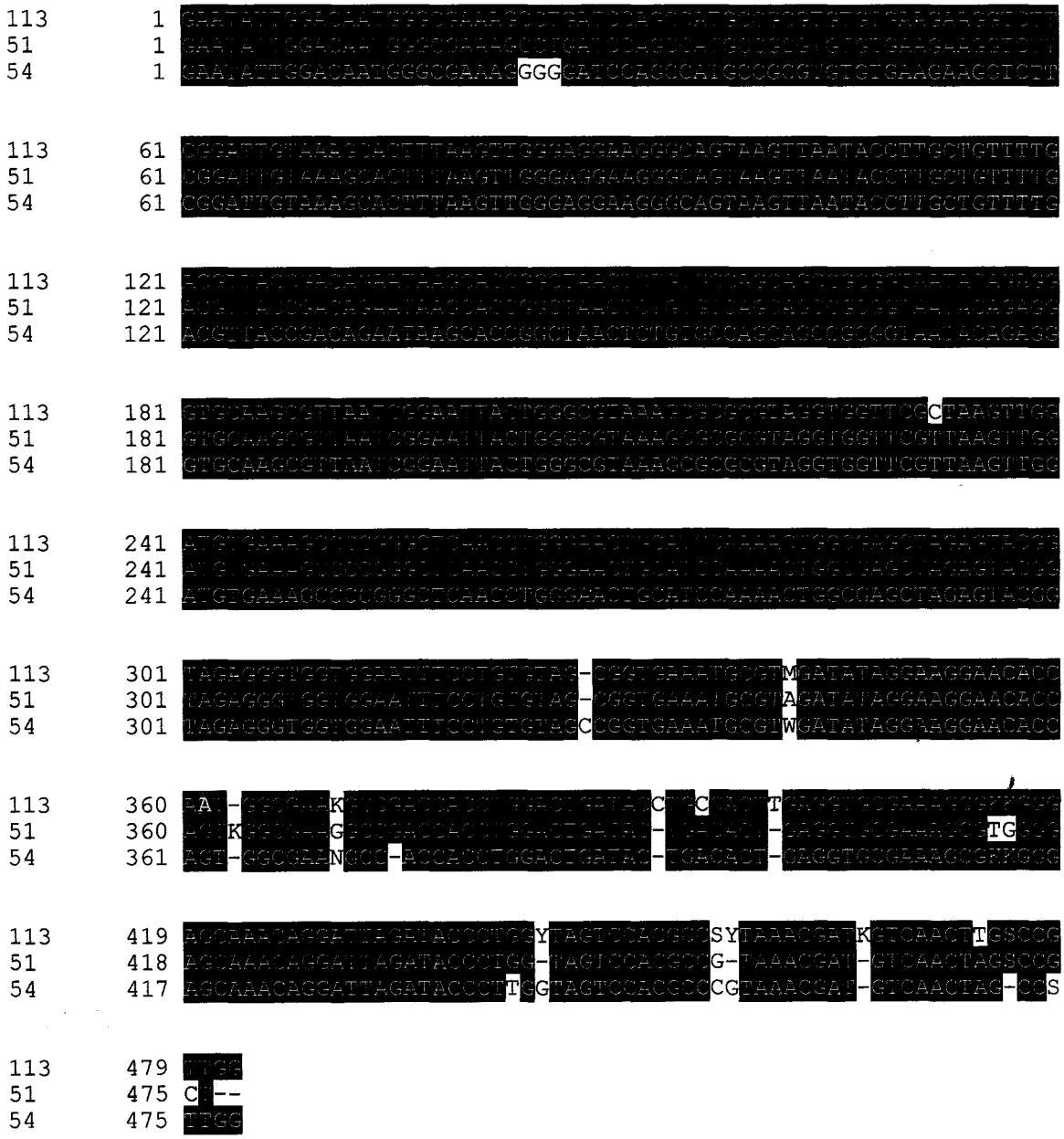


Figure 5.4: ClustalW alignment of the sequences of *Pseudomonas monteilii* clones 54, 113, 51

The similarity between the two *"Thiobacillus hydrothermalis"* fragments was confirmed by aligning the two sequences (Figure 5.5). Differences between the two sequences highlighted by the alignment suggests that the clones contain sequences of two different strains of the *"Thiobacillus hydrothermalis"* species.

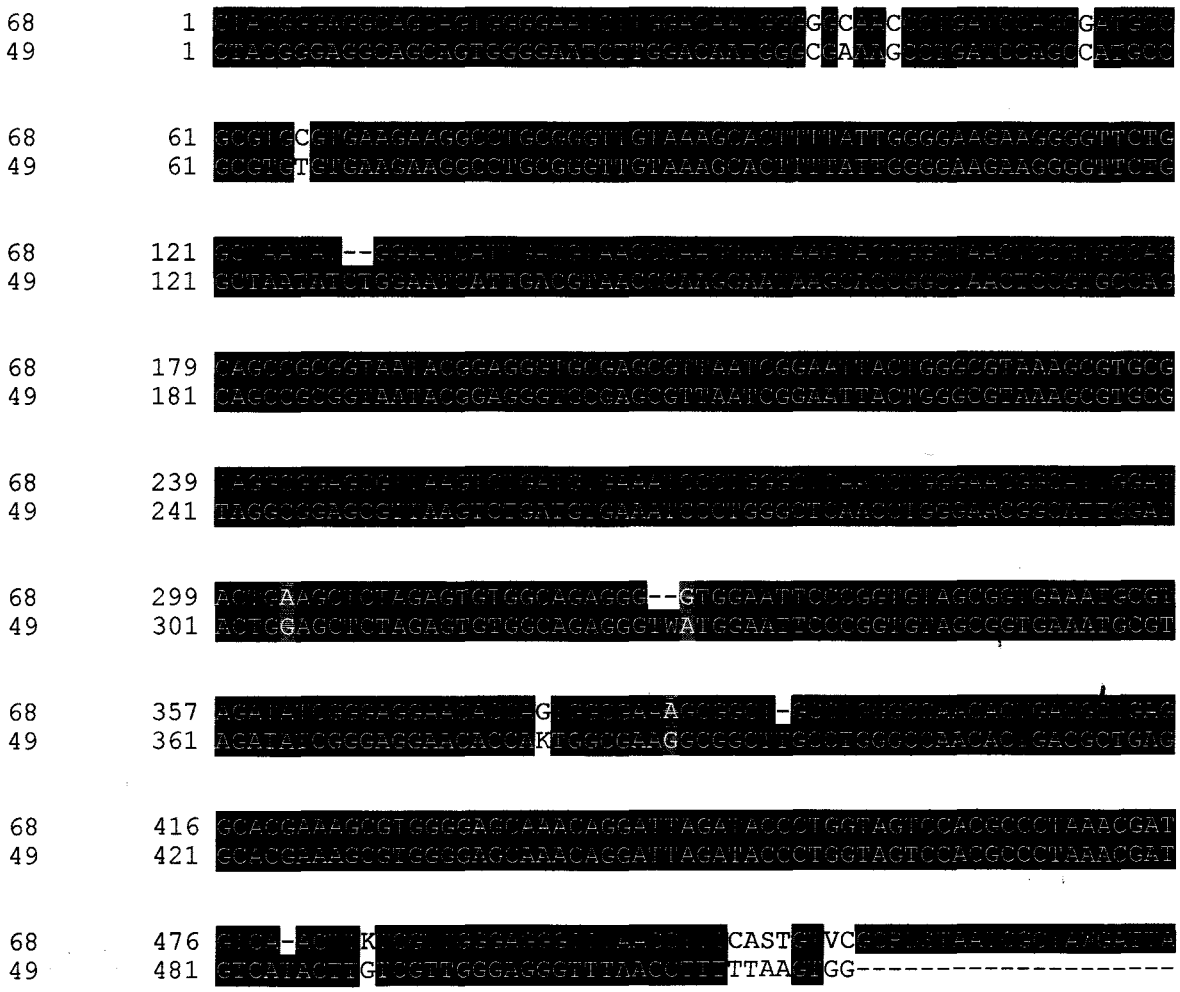


Figure 5.5: ClustalW alignment of the sequences of *Thiobacillus hydrothermalis* clones 68 and 49

An alignment of the four clones placed in the *"Xanthomonas"* group (Figure 5.6) shows that clone 84 has the most sequence variation compared to clones 101, 41 and 92. Although clones 84 and 92 were identified as environmental clones in a *"Xanthomonas"* group after submission to the RDP, and both clones had a similarity index of 0.67, the sequences of the clones was shown to vary significantly (Table 5.3a and b). The alignment therefore indicates that the clones are different, although their identification appeared to be identical. This confirms the initial observation made that the two clones were different with respect to the presence of an *Eco*RI site in the DNA fragment.

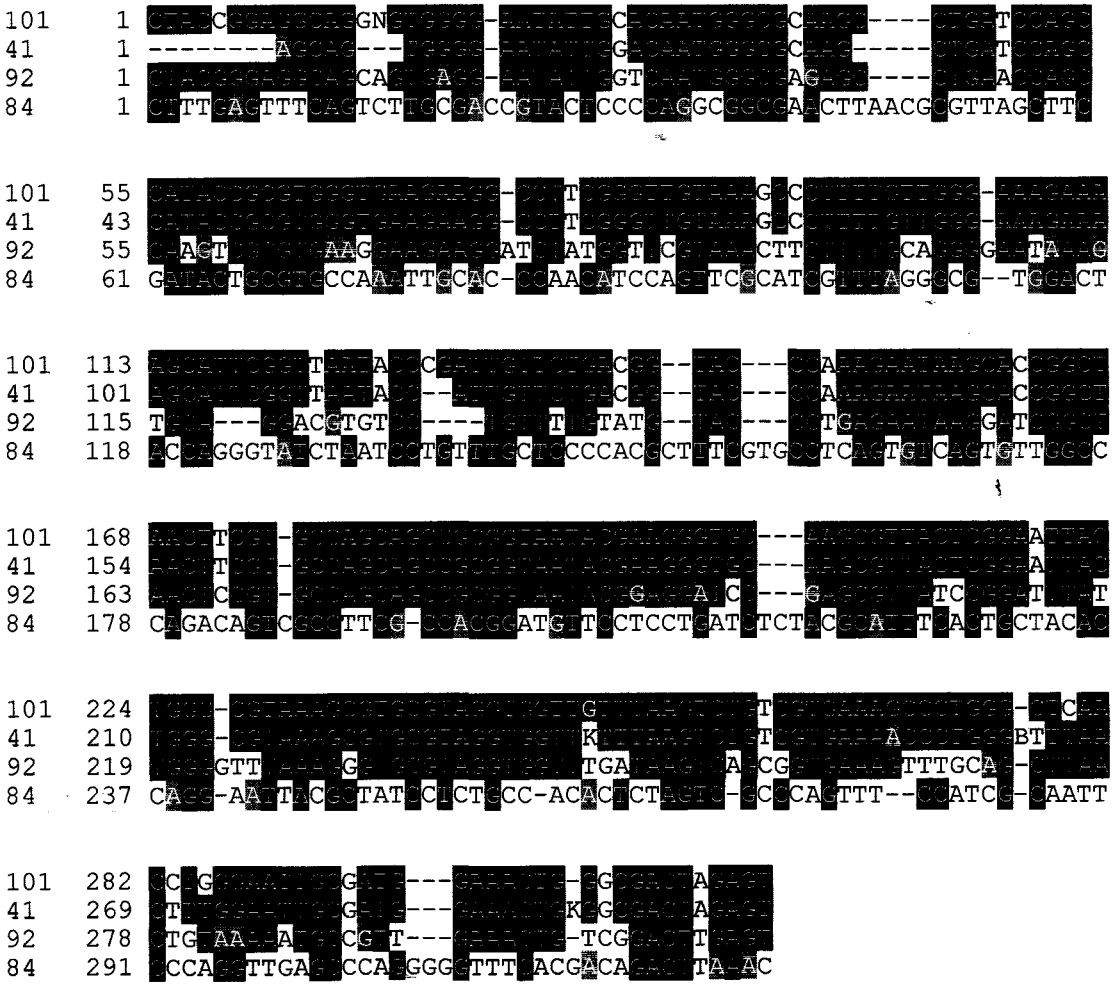
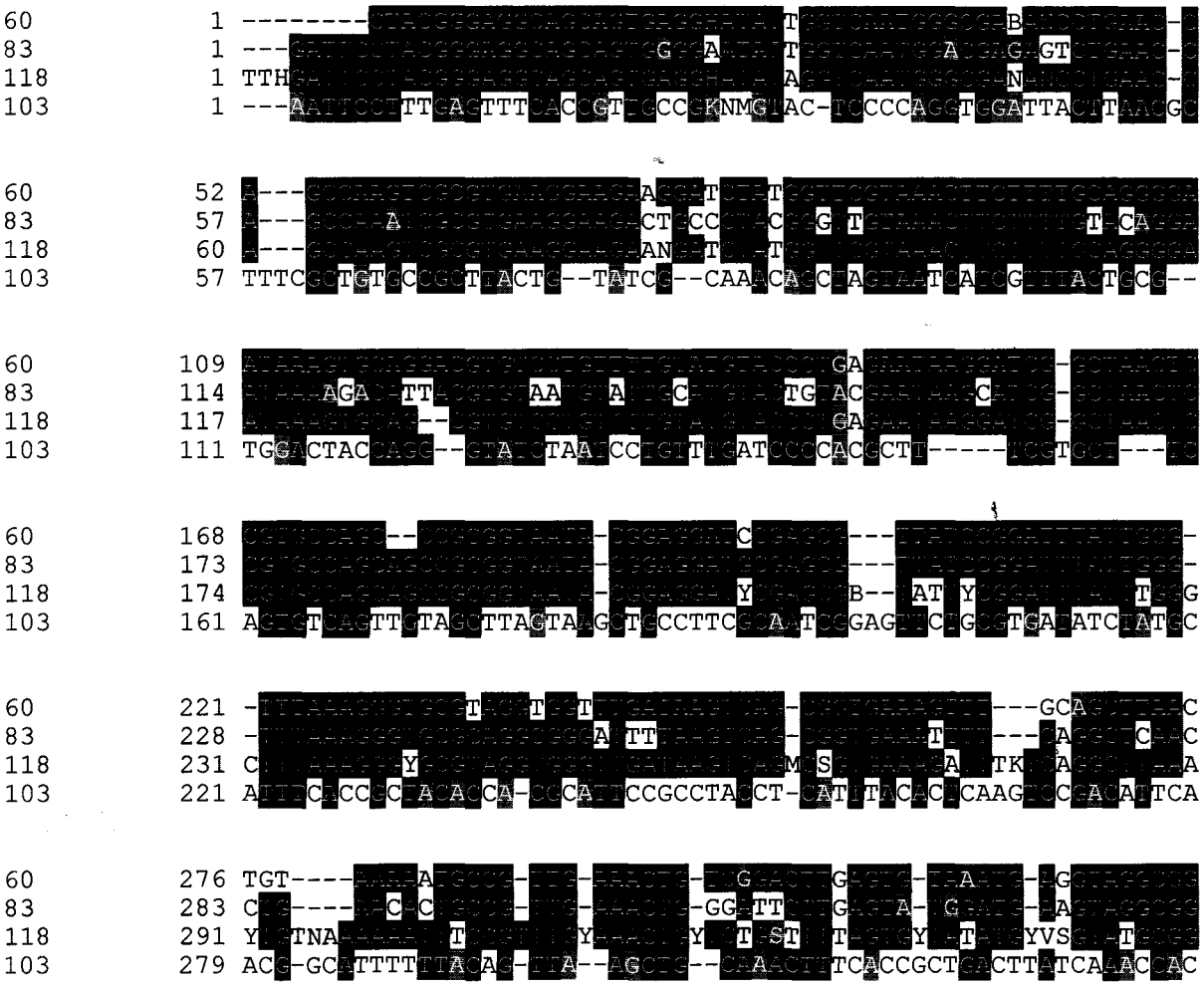


Figure 5.6: ClustalW alignment of the sequences of *Xanthomonas* clones 101, 41, 92, 84

The *"Bacteriodes"* group exhibits the most diversity between members of the group. None of the clones placed in this group had similarity indexes above 0.76, indicating that the species are only distantly related to *"Bacteriodes"*, although clones 60 and 118 are similar to each other. Primers could be designed for each of the clones, in order to distinguish between them in the biofilm. Alternatley, a primer for the whole group could be designed in order to determine where the clones in this group are situated in the biofilm.



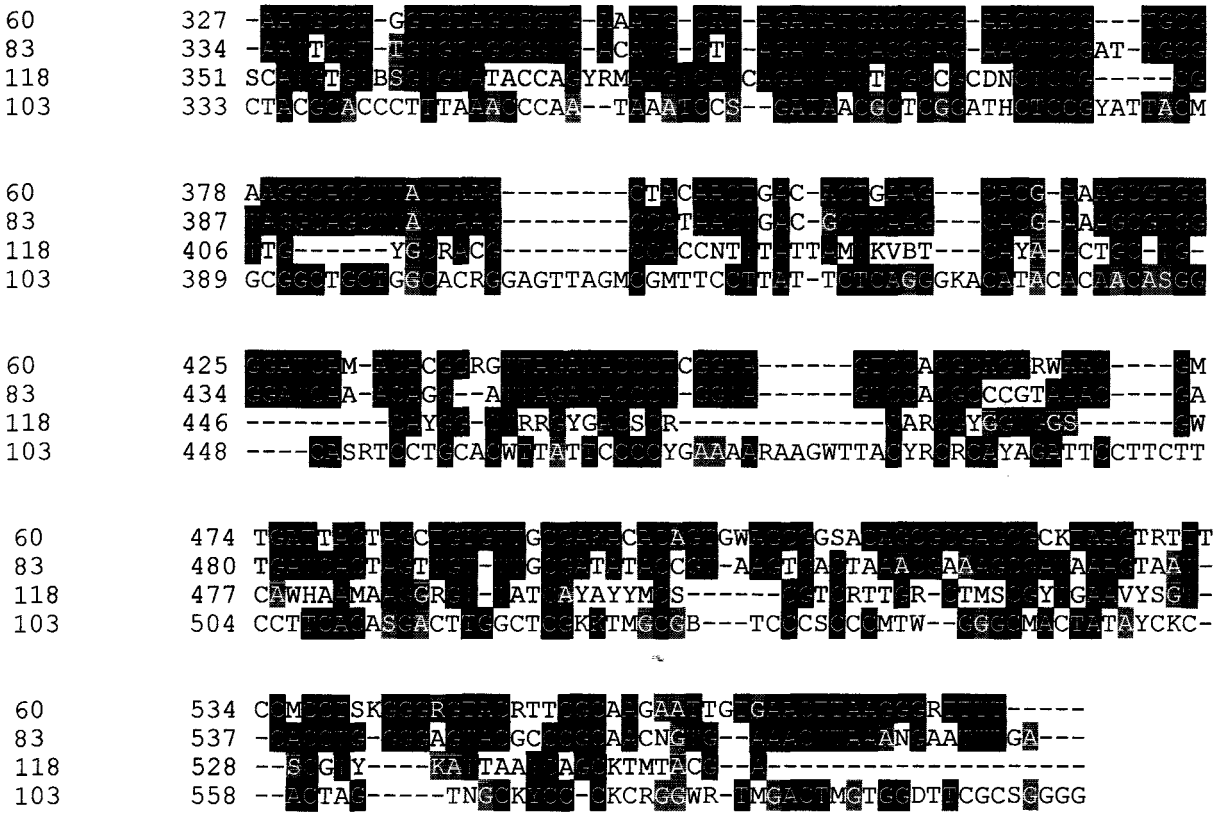


Figure 5.7: ClustalW alignment of the sequences of *Bacteriodes* clones 60, 83, 118, 103

An alignment of representative species from each of the three major groups within the clone library: *"Pseudomonas" "Xanthomonas"* and the Arthrobacter subdivision. The portion of the "*Pseudomonas"* fragment (54) from bases 120 to138 could be a possible position for a probe design as it is dissimilar to the other two sequences at that region. A probe for the "*Xanthomonas*" group could be designed at position 494 to 515, which corresponds to a unique region on the rRNA gene compared to the other two species.

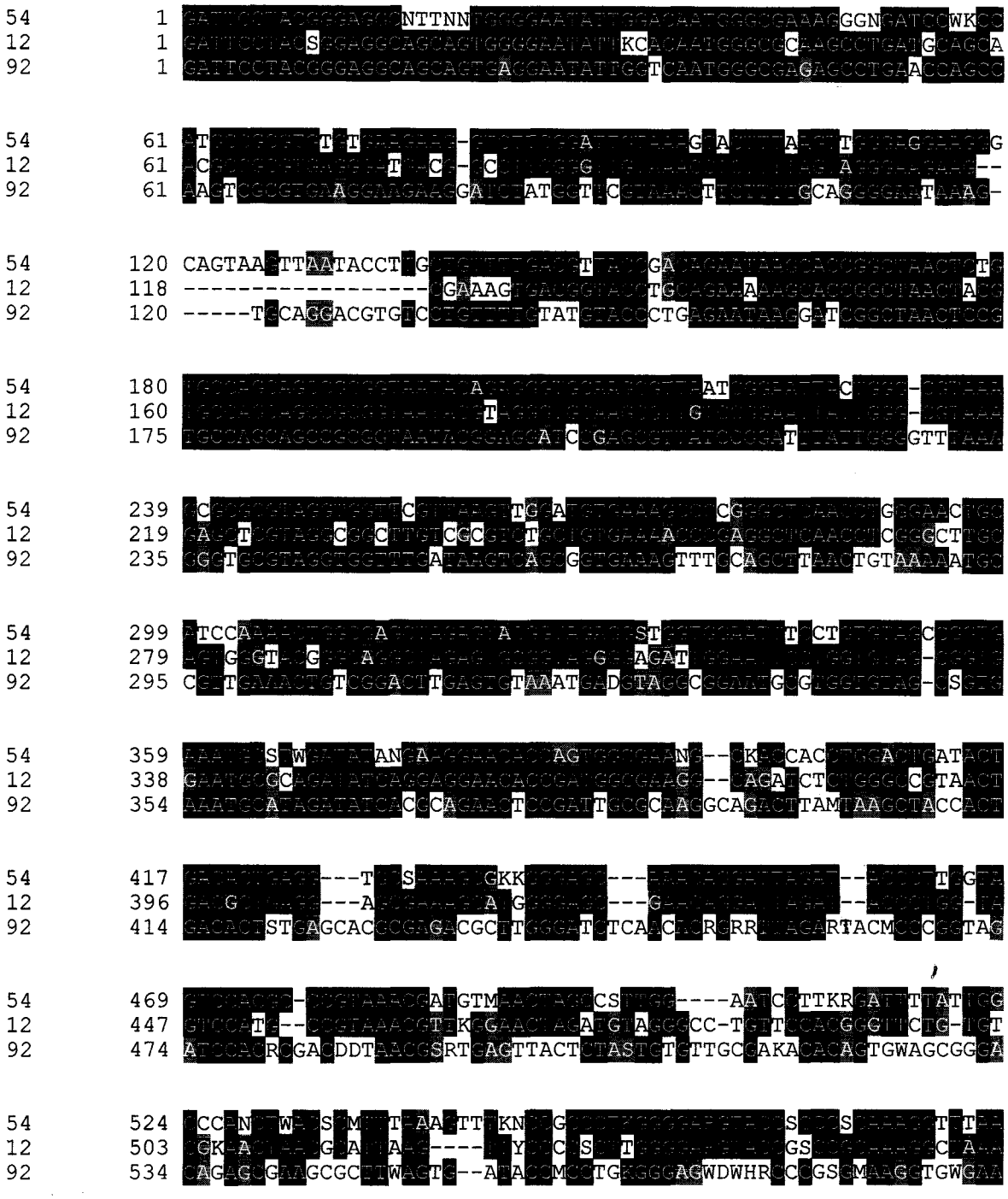


Figure 5.8: ClustalW alignment of representative fragments from each of the three major groups within the clone library: *"Pseudomonas"* (54), *"Xanthomonas"* (92) and the Arthrobacter subdivision (12)

5.4 CONCLUSION

The floating sulphur biofilm studied in this project was composed of a heterotrophic sulphide oxidising microbial community. The dominant genus in the biofilm was *"Pseudomonas"*, which has been documented in the literature as a genus consisting of species which are able to oxidise sulphide. The other two important groups were "*Xanthomonas*" and "*Arthrobacter*". Very few thiobacilli were present, and no sulphate reducing bacteria were found in the clone library. This result was interesting as the initial theory of the floating sulphur biofilms microbial community being composed of classic autotrophic sulphide oxidisers and sulphate reducing bacteria in a nutrient cycling process was questioned. It appears that the biofilms are heterotrophic sulphide oxidising systems which participate in the sulphur cycle by depositing elemental sulphur. The elemental sulphur could be removed when pieces of the sulphur-rich biofilm sink to bottom of the reactor or pond, or alternately, when the biofilm on the surface of sulphidogenic ponds is blown onto the banks of the pond, resulting in elemental sulphur deposits in nature.

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSION

6.1 DISCUSSION

Floating sulphur biofilms have not been well documented in the literature, despite their substantial role in the natural sulphur cycle and their potential use in biotechnological processes. The presence of the biofilms has been noted on the surface of sulphide-rich waters, for example, sulphate reducing bioreactors and sewage and tannery waste stabilisation ponds. These sulphur biofilms are responsible for the oxidation of sulphide to sulphur and/or sulphate, thereby largely preventing the escape of toxic hydrogen sulphide gas into the atmosphere. Sulphide oxidising bacteria present in the biofilms also remove large amounts of sulphur from the sulphur cycle by converting thiosulphate and sulphide to sulphur, resulting over time in geological sulphur deposits. This function of sulphide oxidising biofilms could be harnessed in biological sulphide removal bioreactors, to convert sulphide in waste streams to elemental sulphur, which may thus be easily recovered by physical separation techniques.

In this study a baffled sulphate reducing bioreactor was used for the cultivation of sulphur biofilms and was fed with a synthetic lactate and sulphate feed. Sulphate reducing bacteria present in the sludge bed of the reactors' valleys reduced sulphate to sulphide, which was converted in turn by the sulphide oxidising bacteria in the biofilm to elemental sulphur. Floating sulphur biofilm formation

resulted from the sulphur crystals and matrix thus produced. The biofilm developed on the surface of the reactor as sulphide oxidising bacteria require a carefully poised redox balance between oxygen and sulphide concentrations. The cells must therefore grow in close proximity to the sulphide:oxygen interface. The presence of a too high oxygen concentration results in high rates of chemical sulphide oxidation to sulphate, thereby reducing the amount of sulphide available to the sulphide oxidising bacteria. A redox balance poised too low, and in the absence of oxygen, will result in no elemental sulphur formation at all.

The sulphur biofilm was examined by scanning electron microscopy using a technique developed for this study whereby the biofilm was sandwiched between two nylon membranes and supported by copper structures. The examination by SEM revealed the complex differentiated biofilm structure, where morphologically distinct cells were found to occur in different layers of the biofilm. Bacilli and cocci were the dominant bacterial morphologies, with long, filamentous bacteria and spirilla found in the middle and lower sections of the biofilm. The structure of the biofilm varied with the amount of sulphide and organic material supplied to the reactor. A higher sulphide concentration resulted in a more brittle biofilm with the deposition of visible sulphur crystals throughout the biofilm. Increasing the organic content made the biofilm less brittle with an increase in the amount of matrix surrounding the cells, and with an apparent reduction in the amount of visible sulphur crystals present.

In order to test the assumption that a floating sulphur biofilm would consist of sulphide oxidising bacteria, a biofilm inoculum was enriched in a minimal salts medium containing sulphide. The culture

was shown to oxidise sulphide in a fed-batch reactor, with conversion to sulphate and elemental sulphur. The optimal temperature for sulphide oxidation by the biofilm inoculum was found to be 37°C, although temperatures between 20-37°C result in closely comparable sulphide oxidation rates. The ideal sulphide concentration for bacterial growth and sulphide oxidation lay between 100-250 mg/L, which is similar to or greater than maximum sulphide concentrations reported in the literature (Buisman *et al.*, 1991; Lee and Sublette, 1993; Chung *et al.*, 1996b; Janssen *et al.*, 1997). It was therefore demonstrated that the bacterial species enriched from the biofilm had sulphide oxidising capabilities.

Having described the performance and morphology of the floating sulphur biofilm, the next stage of this project was to attempt to identify the sulphide oxidising bacterial species present in the biofilm. To achieve this, strains were isolated from the biofilm using traditional plate culture techniques. Six strains, chosen on the basis of colony morphology, were characterised morphologically and metabolically in an attempt to identify them. These preliminary studies revealed that all the isolates were able to grow on a minimal salts medium with the addition of thiosulphate and all isolates grew well on a complete medium containing organic compounds. Thereafter amplified 193 bp portions of the rRNA gene from each isolate were sequenced for identification purposes. None of the six strains isolated from the biofilm were found to be classic autotrophic sulphide oxidising bacteria, which was an unexpected result because a minimal salts medium had been used to isolate the strains. It had, however, been suspected that heterotrophic organisms would be amongst the isolated strains as they were able to grow on nutrient agar. The strains identified were *Pseudomonas putida/monteilii,*

Bacillus, Flavobacterium, Alcaligenes and *Acinetobacter* sp. All these heterotrophic species have been documented by previous authors to possess sulphide oxidising capabilities (Friedrich and Mitrenga, 1981; Gommers and Kuenen, 1988; Chung *et al.*, 1996a; Chung *et al.*, 1996b; Chung and Huang, 1997; Gallardo *et al.*, 1997). Heterotrophic organisms which gain energy from the oxidation of inorganic compounds are known as chemolithoheterotrophs, and are selected for in environments such as domestic waste water treatment facilities, which is where the inoculum for this study was obtained.

Although the species enriched from the biofilm for flask studies and the strains isolated on solid medium were sulphide oxidising bacteria, it was suspected that in a complex environmental system more than one metabolic type of organism would be present. For example, with the production of sulphate and sulphur by sulphide oxidising bacteria, a niche would expected to be created for sulphate reducing bacteria, where they would in turn recycle the oxidised sulphate back to sulphide. This is an example of protoco-operation, where two species benefit each other. In order to investigate the microbial population of sulphur biofilms without the biases imposed by traditional cultivation techniques, rRNA genes isolated directly from the biofilm were analysed. This approach involved the construction of a clone library of 586 bp fragments amplified directly from the biofilm, followed by restriction digests and DGGE analysis, where identical fragments were grouped together. After the analysis, 26 different groups were identified. A representative clone from each group was then sequenced. Identification of the microbial community members using the rDNA approach gave results similar to the isolation of individual strains. The clone library was constituted of many

unidentified species, whose sequences had not been added to the Ribosomal Database Project. 40% of the clones were found to belong to the "*Pseudomonas*" group, which corresponded to one of the strains isolated on solid media. The sequence alignment of all the fragments in the "*Pseudomonas*" group against the isolated strain indicated that the solid medium isolate differed from the clones described in this group. Only two fragments fell into the "*Thiobacillus*" group, and none of the other clones identified belonged to the autotrophic colourless sulphur bacteria group. Surprisingly, no sulphate reducing bacterial species were detected in the clone library, suggesting that nutrient cycling of biologically produced sulphur and sulphate back to sulphide by SRBs does not play a major role in floating sulphur biofilms. The majority (83%) of the rDNA fragments which could be identified did, however, belong to genera which have been reported in the literature as having the ability to oxidise sulphide. This result indicates that the microbial community of the floating sulphur biofilm studied consisted of heterotrophic sulphide oxidising bacteria, which oxidise most of the sulphide to sulphur and not sulphate.

6.2 CONCLUSIONS

The following objectives were achieved in this study:

1. The development of a reasonably reliable laboratory-scaled reactor for cultivation of a stable sulphur which could be studied comparatively;
2. A determination of the sulphide oxidising capacity of a sulphur biofilm and the optimal growth conditions of the biofilm inoculum;
3. The development of a protocol for observation of fragile sulphur biofilms using SEM;
4. Identification of the bacterial species which are members of the sulphur biofilm microbial community using molecular techniques.

This study was a preliminary investigation into the structure and microbiology of floating sulphur biofilms and opened many possibilities for future studies into these complex structures. The combination of FISH and micro-electrodes would be ideal for a study of this nature. Differences between the sequences of the dominant biofilm genera determined in this study could be used to design nucleic acid probes for use in FISH, in conjunction with SCLM. The probes could confirm the presence of the microbes identified in this study in biofilms grown in the laboratory and, more importantly, in biofilms found in nature on sulphide rich waters. This would be useful in comparative biofilm studies and in determining the spacial distribution of various genera within the biofilm. Micro-profiles of O_2, H_2S and the redox potential measured using micro-electrodes would illuminate the chemical system existing within and around the biofilm. This would confirm the theories of sulphur

production in relation to sulphide concentration and redox potential postulated in this study, or enable the creation of new hypotheses on sulphur biofilm function.

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APPENDIX A: Growth media

1. MINIMAL SALTS MEDIUM

Table 6.1: Minimal salts medium with thiosulphate or sulphide (per L), modified from Atlas (1993)

	Reagent	Quantity	Final volume
Solution A	NH_4Cl	4 g	800 mL
Autoclaved	$MgCl_2$	1 g	
	$NaHCO_3$	3 g	
	Trace element solution	10 mL	
Solution B	KH_2PO_4	2 g	100 mL
Aotoclaved			
Solution C	Na_2SO_4	50 mM	100 mL
Autoclaved		(12.4 g)	

Adjust the of solution A to pH to 8.0 with 1M KOH

Combine solutions once cooled to below 60°C

When sulphide is required as the electron donor rather than thiosulphate, a 50mM (166 mg/L) sulphide solution was prepared in 0.1M NaOH and filter sterilised.

2. TRACE ELEMENT SOLUTION

Modified from Atlas (1993)

50 g Na_2-EDTA

22 g $ZnSO_4.7H_2O$

5.54 g $CaCl_2.2H_2O$

5.06 g $MnCl_2.4H_2O$

5 g $FeSO_4.7H_2O$

1.1 g $(NH_4)_6Mo_7O_{24}.4H_2O$

1.16 g $CoCl_2.6H_2O$

1.1 g $CuSO_4.5H_2O$

Adjust to pH6 with 1M KOH and make up to 1L with ddH_2O

3. COMPLETE MEDIUM

Nutrient agar/broth (Difco) made up to 900 mL with ddH_2O and autoclaved separately

12.4 g thiosulphate in 100 mL ddH_2O, autoclaved separately

The two solutions are cooled to 60°C before combining.

4. LACTATE SRB MEDIUM

Modified from: Atlas (1993)

3.5 g 70% sodium lactate solution

2 g $MgSO_4.7H_2O$

1 g NH_4Cl

1 g Na_2SO_4

1 g yeast extract

0.5 g K_2HPO_4

0.1 g $CaCl_2.2H_2O$

Combine components and make up to 1L with ddH_2O. The medium was not autoclaved as the reactor was not operated under sterile conditions.

5. LURIA BROTH

Luria broth with ampicillin (per L):

10 g Tryptone

5 g Yeast extract

5 g NaCl

Adjust to pH 7.0 with NaOH

Autoclave and cool to 50°C

Add ampicillin to a final concentration of 100 mg/L

Luria agar (LA) with ampicillin

Add 15 g agar to 1L LB

Autoclave and cool to 50°C before adding ampicillin to a final concentration of 100 mg/L

Luria agar (LA) with ampicillin, IPTG and X-Gal

Make LA with ampicillin plates as described above. When agar is set, spread 10 µL of 100 mg/mL X-Gal (100 mg 5-bromo-4-chloro-3-indolyl-β-D-galactosidase dissolved in 1 mL N,N-dimethylformamide, stored at -20°C) and 12 µL of 200 mg/mL IPTG solution (200 mg IPTG dissolved in 1 mL ddH_2O) over the plates half an hour before the transformed cells are spread onto the plates.

APPENDIX B

1. SAMPLE PREPARATION FOR SCANNING ELECTRON MICROSCOPY

The sample preparation protocol was modified from Cross (1999). The process started with sample fixation in a 2.5% gluteraldehyde solution in 0.1 M phosphate buffer (pH7.0) overnight. The next day the samples were washed twice in 0.1 M phosphate buffer for ten minutes. The samples were then sent through an alcohol dehydration series; 30%, 50%, 70%, 80%, 90% and 100% ethanol for 10 minutes each, followed by two 100% ethanol washes for 10 minutes. Following the alcohol dehydration, samples were critical point dried from liquid CO_2 in a Polaron E3000 Critical Drying Apparatus, and sputter coated in gold with a Polaron E 5100 Sputter Coating Unit, after which the samples were ready for observation in the JEOL JSM 840 Scanning Electron Microscope.

2. GRAM STAIN (Voet and Voet, 1990)

Table 6.2: Protocol followed for Gram stains

Chemical	Time
Grams crystal violet (Saarchem)	30 sec
dH_2O	rinse for 5 sec
Grams iodine (Saarchem)	45 sec
95% ethanol	approximately 4 drops
dH_2O	rinse for 5 sec
Safrinin (Saarchem)	30 sec
dH_2O	rinse for 10 sec

APPENDIX C: Recombinant DNA techniques

1. SPECTROPHOTOMETRIC QUANTIFICATION OF NUCLEIC ACIDS (Maniatis, 1989)

DNA quantification was performed at the wavelengths of 260 nm and 280 nm. The reading at 260nm allows calculation of the concentration of nucleic acid in the sample, where an OD of 1 corresponds to approximately 50u g/mL for double-stranded DNA. The ratio between the readings at 260nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid, with pure preparations of DNA giving OD_{260}/OD_{280} values of 1.8. Accurate quantification of the amount of nucleic acid is only possible if there is no protein or phenol contamination of the sample.

2. RESTRICTION DIGESTS:

The reaction consisted of 1µg DNA, 2U enzyme, 2 µL 10X buffer for the enzyme and ddH_2O to bring the volume to 20 µL. The mixture was incubated at 37°C for three hours.

3. PCR AMPLIFICATION OF A FRAGMENT FROM THE 16S RRNA GENE

DNA extracted from the biofilm was used as target DNA in the polymerase chain reaction to amplify the 16S rRNA gene. Two sets of primers were used for cloning and DGGE analysis. GM5F and P2 (Muyzer *et al,* 1993) were used to amplify a fragment of 193 bp, which corresponds to position 341-534 on the 16S rDNA of *E. coli*. GM5F and 907R (Muyzer *et al* 1995) were used to amplify a 586-bp rDNA fragment, corresponding to positions 341-927 on the 16S rDNA of *E. coli*, with a 40bp GC-clamp attached to the 5'-end of the 907R primer. A combination of GM5F and P2 was thus useful for cloning since it eliminated the incorporation of a GC-clamp into the sequence. Sequences and exact

positions of primers are given in Appendix F

PCR amplifications were performed as follows: 100-250 ng of purified genomic DNA, 50 pmol of each of the appropriate primers, 20nmol of each deoxyribonucleoside triphosphate, and 10 ul of 10X PCR buffer + $MgCl_2$ were added to a 0.2 mL tube, where the volume was adjusted to 50 ul using $dddH_2O$. The tubes were then transferred to the Hybaid PCR Sprint Thermal Cycler.

A touchdown program was used in order to increase specificity and prevent the amplification of non-target sequences during the amplification process. Samples were first incubated for 5 min at 98 °C for the hot start where the template is denatured completely. The cycle then cooled to 80 °C, at which point 1 U of *Taq* DNA polymerase (Roche) was added. The temperature was then lowered to 8 °C above the expected annealing temperature (63 °C for P2/GM5F and 68 °C for GM5F/907R) and decreased by 2 °C every third cycle until the desired annealing temperature of 55 (P2/GM5F) or 60 °C(GM5F/907R), where 16 additional cycles were carried out. Denaturing was performed at 94 °C for 30 seconds, primer extension at 72 °C for 3 min and a final extension was carried out at 72 °C for 5 min. The total number of cycles was 30.

4. CLONING

Cloning was done into the p GEM-T Easy Vector System (Promega), see Figure 4.2 for map
The 193bp fragment was cloned into the vector using the manufacturers protocol, although half the recommended quantities of reagent were used. 150 ng of target DNA was ligated into 50 ng plasmid. The ligations were incubated at 4°C overnight to ensure maximum number of transformants.

The controls included: * Positive control, using the control DNA insert given with the kit. More than 60% of the cells should be white when competent cells with 1 x 10^8 cfu/μ g DNA are transformed.

* Back ground control, with no insert to ensure the plasmid does not re-circularise

* Transformation efficiency, where an insert was added with 2 pg pUC18 vector and no pGEM-T vector. The transformation efficiency should be at least 1 x 10^8 cfu/μ g DNA.

* Negative control, where water is added to the *E. coli* cells with no ligation reaction, and no cells should grow.

The recombinant plasmids were electroporated into electrocompetent *E. coli* DH5α cells using a Stratagene Electroporator 1000 set at 2000V. The ligation reaction was first diluted 1 in 5 with $dddH_2O$, in order to dilute the buffer salts, and then heated to 68°C for 1 min to inactivate the ligase. The mixture was then vortexed briefly and cooled to room temperature. 3 μL of the ligation mix was added to a 40 μL aliquot of electrocompetent *E. coli* DH5α cells, which was then transferred to a cold electroporation cuvette and electroporated at 2000V. After electroporation, 200 μL cold LB medium was pipetted into the cuvette and mixed with the cells, before returning then to the original eppendorf and incubating at 37°C for 30 min. 100 μL of the cells in broth were plated onto a LB agar plate with 10 μL of 100 mg/mL 5-bromo-4-chloro-3-indolyl-β-galactosidase (X- Gal) and 12 μL of 200 mg/mL isopropyl-β-D-galactosidase (IPTG) and incubated overnight at 37°C.

5. PREPARATION OF ELCTROCOMPENENT DH5α *E. COLI* CELLS

A 100 µL aliquot of a 5 mL overnight culture of DH5α *E. coli* cells in Luria broth was plated out onto Luria agar. The next morning, the lawn of cells was scraped off the agar and added to the Luria broth. Four 100 mL Erlenmyer flasks containing Luria broth were inoculated with 1.5, 1.0, 0.7 and 0.3 mL of the pre-inoculum and incubated for 3 hours at 37°C on an orbital shaker set at 100 rpm. Once the absorbance (600nm) of the flask inoculated with 1.5 mL of pre-inoculum had reached 0.6-0.8, the flasks were cooled for 5-10 minutes in an ice bath and were then processed separately until the final step. After centrifugation at 5 000 rpm (Beckman centrifuge with JA 14 rotor) for 10 minutes, the supernatant was discarded and the pellet was resuspended in 50 mL RF1 (100 mM KCl, 50 mM $MnCl_2$, 30 mM CH_3COOH; 10 mM $CaCl_2$, 15% m/v glycerol pH 5.8), followed by a 20 minute incubation on ice. The cells were pelleted by a 10 minute centrifugation at 5 000 rpm (Beckman centrifuge with JA 14 rotor). The supernatant was discarded and each of the four pellets was resuspended in 4 mL RF2 (10 mM MOPS, 10 mM KCl, 75 mM $CaCl_2$, 15% m/v glycerol pH 6.8) and the flask content were pooled. Aliquots of 40 µL were pipetted into sterile Eppendorf tubes and stored at -70°C for further use.

6. EASYPREP PLASMID EXTRACTION (Berghammer and Auer, 1993)

1.5 mL overnight culture was spun for 1 minute in a microfuge at 13 000 rpm, and repeated, before resuspending the pellet in 75 µL lysis buffer (10mM Tris-HCl pH8, 1mM EDTA, 15% (w/v) sucrose, 2 mg/mL lysozyme, 0,2 mg/mL pancreatic RNase, 0.1 mg/mL BSA. Stored at -20°C) . The cells were incubated at 37°C for 10-15 min before boiling for 90 seconds then chilling on ice for 60 seconds. The

mix was then microfuged at 13 000 rpm for 15 min, after which the supernatant was transferred to a clean eppendorf and stored at -20°C for further work.

7. SEQUENCING REACTIONS

Sequencing was performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Approximately 200 ng DNA was used per reaction, and half shots were used (Table 6.2)

Table 6.3: Sequencing reactions, as described in the Perkin-Elmer ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit manual (1998)

Reagent	Quantity
Terminator Ready Reaction mix (dye terminators, dUTP*, dCTP, dATP, dITP**, AmpliTaq DNA polymerase, r*Tth* pyrophoshatase, magnesium chloride, buffer)	8 µL
DNA template	100 - 200 ng
-21 M13 Primer (forward)	1.6 pmol
$dddH_2O$	x
Total volume	10 µL

* dITP is used in place of dGTP to minimise band compressions

** dUTP is used in place of dTTP as it results in a better T patterns because dUTP improves incorporation of T terminators.

The reagents were vortexed then spun briefly before the PCR sequencing reaction was started (Table 6.4)

Table 6.4 Sequencing reaction using a Hybaid PCR Sprint Thermal Cycler

	Temperature	Time
Denaturing	96°C	10 seconds
Annealing	50°C	5 seconds
Extension	60°C	4 minutes
Repeat this sequence for 25 cycles, then store at 4°C before purification (Table 8)		

Table 6.5: Precipitation protocol

Reagent	Quantity	Time
3 M Sodium acetate (pH 4.6)	2 µL	
96% Rectified ethanol	50 µL	
PCR product	10 µL	Vortex and keep at room temperature for 1 hour, before spinning down at 13 000rpm for 20 minutes and discarding the supernatant
70% Ethanol	200 µL	Spin for 5 min and discard supernatant

The resultant pellet was air dried overnight before sending off for sequencing.

APPENDIX D: GEL ELECTROPHORESIS

1. AGAROSE GEL ELECTROPHORESIS

Agarose gels were made by dissolving the appropriate amount of agarose in 1X TBE buffer (12.1 g Tris, 0.37 g EDTA and 5.14 g Boric acid made up to 1L and adjusted to pH 8.4 with 1 M HCl) for 0.8 - 1.5 % gels, depending on the fragment size loaded onto the gel. Genomic DNA was run on 0.8% gels, whereas 1.5% gels were used with fragment sizes of 190 bp and 550 bp. The agarose gels were electrophoresed in TBE buffer at a voltage range between 80 - 120 V for approximately 1 hour. In the case of restriction digests, 2% MetaPhor agarose was used as it gives better resolution of smaller fragments. Samples were loaded into the wells with 10% tracking dye. 0.5 µg/mL ethidium bromide added to the gels to allow visualisation of DNA when it was placed on a UV transilluminator, which caused any DNA bound to ethidium bromide to fluoresce.

Tracking Dye III (Maniatis *et al*, 1993)

0.25% bromophenol blue

0.25% xylene cyanol FF

30% glycerol in ddH_2O

Store at 4°C

Markers

The markers were made using Lambda DNA cut either with *Eco*RI and *Hin*d III (resulting in a fragment

size range from 0.56 to 24.76 Kb), or with *Pst* (resulting in a fragment size range from 0.3 to 20 Kb). 25 μg λ DNA (Roche) was digested with either 40 U *Pst* or a mixture of 20 U each of *Eco* RI and *Hind* III in a final volume of 50 μL, using the buffers specified by the maufacturers. The mixture was incubated for 3 hours at 37°C, after which the reaction was stopped by adding 5 μL 0.5 M EDTA and 5 μL tracking dye.

2. DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

Reagents for DGGE analysis (Adapted from Myers *et al* 1987)

- 10x TAE electrophoresis buffer

  [0.4 M Tris acetate, 0.01 M EDTA, pH 8-8.3). For 1 litre: 48.45 g Tris base, 3.72 g Na_2EDTA and water to 1 litre. Adjust pH to 8-8.3 with glacial acetic acid (11.5 mL).

- Acrylamide stock solution:

  40% acrylamide (37.5:1 acrylamide: bisacrylamide). For 250 mL, dissolve 100 g electrophoresis-grade acrylamide and 2.7 g bisacrylamide in water to 250 mL.

- Denaturant stock solutions:

  0% denaturant: 6 or 8% acrylamide (depending on fragment size) in TAE buffer

  100% denaturant: 6 or 8% acrylamide, 7M urea, and 40% (v/v) formamide in TAE buffer

  - To deionize formamide: Gently mix 250 mL formamide with approximately 12.5 g Dowex mixed-bed Resin AG 501X8 for 1 hour at room temperature. Filter to remove resin.

- Ammonium persulfate stock (20%)

  For 50 mL: 20 g ammonium persulphate to 50 mL with water.

- TEMED (*N,N,N',N'*-tetramethylethylenediamine)

Preparation of polyacrylamide gels and Polymerization (Adapted from Myers *et al* 1987)

The Bio-Rad Protean II system was assembled as described by the manufacturers, with ethanol and acetone cleaned glass plates. Two solutions of equal volume (12 mL each, which was the volume that filled the void between the glass plates) were prepared from the stock solutions of denaturants and acrylamide to give the desired denaturant concentration range. The chilled solutions were degassed under vacuum for 10 min, before adding 100 μL 20% ammonium persulphate and 10 μL TEMED to each denaturant and gel solution. The solution of higher denaturant concentration was poured into the chamber of the gradient maker which was the first to exit the gradient maker and enter the plate cavity. The solution of lower denaturant concentration was poured into the remaining chamber of the gradient maker. A syringe needle was placed on the end of the tubing and inserted between the glass plates. The needle was kept approximately 10mm above the level of the gel as it was being pumped between the plates by a peristaltic pump. The solution with the highest denaturant concentration was stirred during pouring by a magnetic stirrer, allowing effective mixing of the two denaturant solutions in the chamber. The entire gel was poured in less than 10 minutes, to ensure that polymerization did not occur in the tubing or in the chambers.

After completion of polymerization, the gel was prepared for electrophoresis by removing the comb and heating it to 60°C. The buffer (1X TAE) level was adjusted to be slightly above the level of the wells in the gel. The buffer was pumped by a peristaltic pump, allowing the buffer to circulate from the lower buffer chamber (anode) to the upper buffer chamber (cathode), thereby avoiding increases in buffer pH during electrophoresis.

The wells were flushed with buffer using a syringe in order to remove non-polymerized acrylamide before pre-running the gel for approximately 15-20 min at 120V. The wells were then flushed again before loading the samples, and running the gel at the determined voltage.

Table 6.6: Components of 6% acrylamide DGGE gels in 1X TAE buffer used for separating amplified DNA fragments from the sulphur biofilm

% Gel	Quantity of reagent	
	Formamide	Urea
0	4%	0.7 M
20	8%	1.4 M
30	12%	2.1 M
35	14%	2.45 M
60	24%	4.2 M
70	28%	4.9 M
80	32%	5.6 M
100	40%	7 M

APPENDIX E: DNA SEQUENCES

1. PRIMER SEQUENCES

P2: 5'-ATT ACC GCG GCT GCT GG-3' (position 341: annealing temp =55°C (Muyzer *et al*, 1997)

GM5F: 5'-CCT ACG GGA GGC AGC AG-3' (position: 341-357; annealing temp = 60°C) (Muyzer *et al*, 1995)

907R: 5'-**CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G**CC GTC AAT TCC TTT RAG TTT-3' position: 907-927; annealing temp = 60°C (Muyzer *et al*, 1995) CG clamp in indicated in bold font

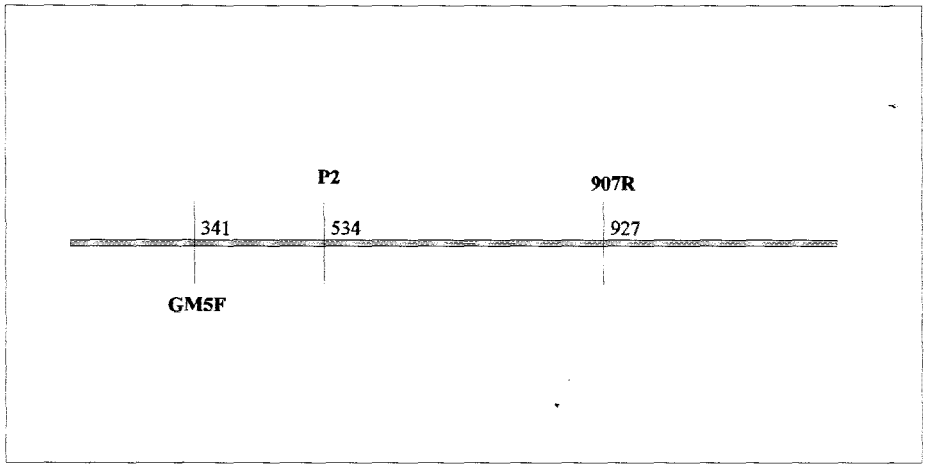

Figure 6.1: Relative positions of primer sets on the 10s rRNA gene from the 5' to the 3' end

The primer used in sequencing reactions was the forward primer - **21 M13:** 5' -TGT AAA ACG ACG GCC AGT -3'.

APPENDIX F: Standard curves

1. SULPHIDE DETERMINATION

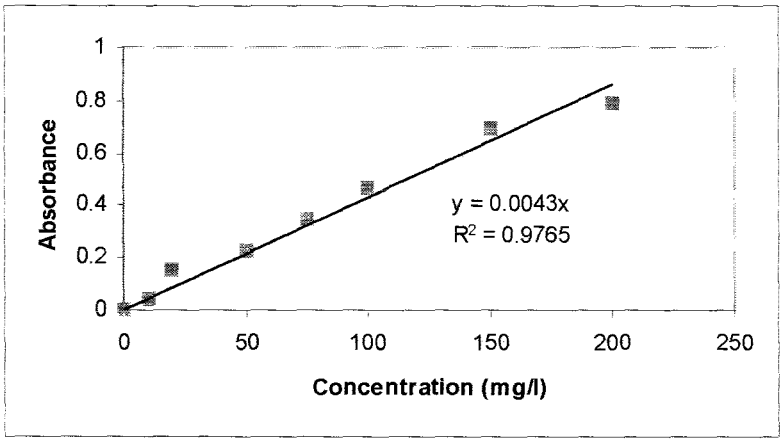

Figure 6.2: Standard curve for sulphide determination, with a dilution factor of 50

2. SULPHATE DETERMINATION

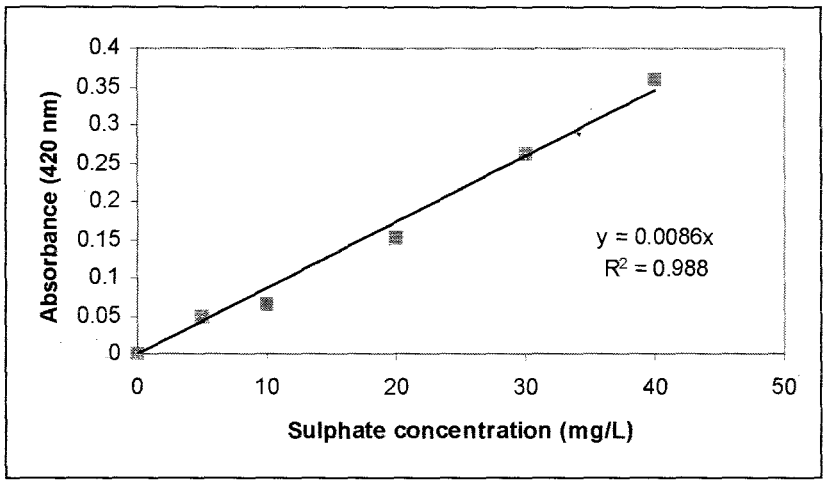

Figure 6.3: Standard curve for sulphate determination

3. SULPHUR DETERMINATION

The sulphur standard curve was constructed using standard sulphur flower solutions extracted in acetone overnight and processed as in the protocol described in chapter 3

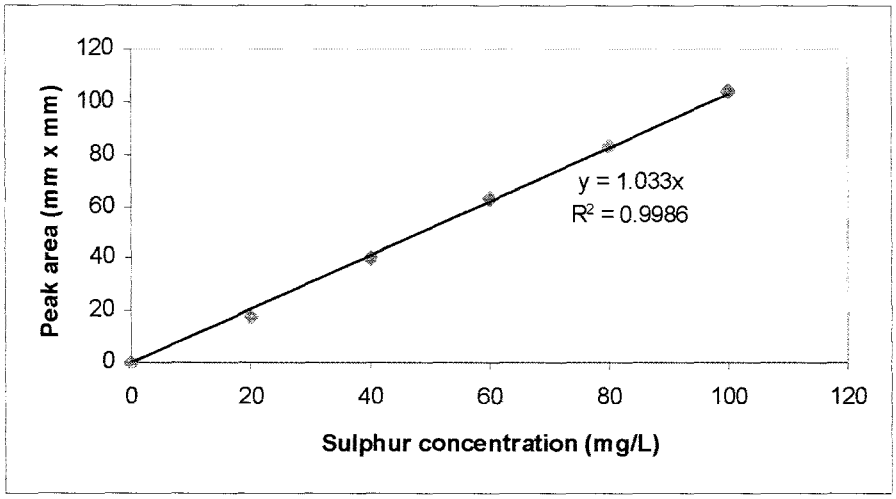

Figure 6.4: Standard curve for sulphur determination

4. POLYSACCHARIDE DETERMINATION

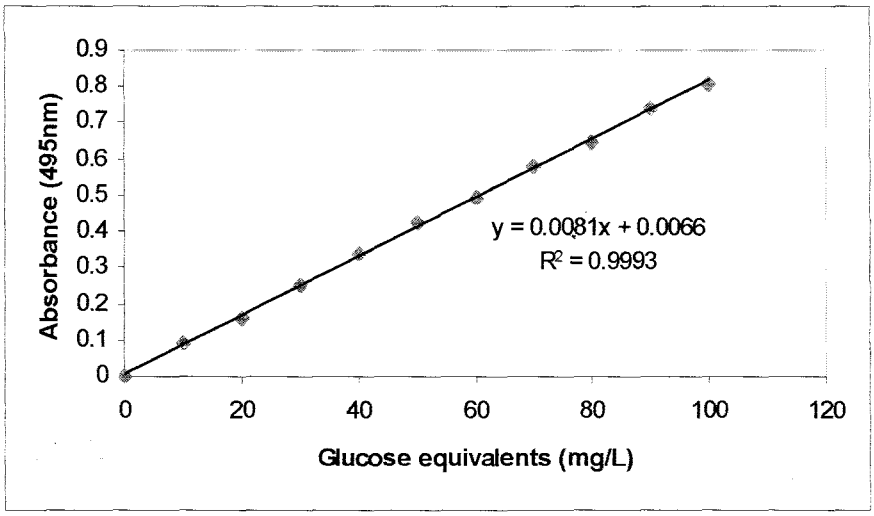

Figure 6.5: Standard curve created for polysaccharide assay