Molecular and Physiological Characterization of Thiosulphate-Oxidizing Microbial Associations Prior to Use in Hydrogen Sulphide Biofiltration

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September 2000

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Chapter 2

Materials and Methods

Abstract

Interacting microbial associations capable of utilizing thiosulphate as an energy source were enriched/isolated from activated sludge, landfill site final covering soil and soil from an acid mine water drainage site. The isolates were designated Lf-1, Ws-2 and Am-3, respectively. Although hydrogen sulphide was the target molecule for gas biofiltration, thiosulphate, which is a key oxidized intermediate, was used in this study due to the difficulty of working with a toxic gas. Together with thiosulphate oxidation, the microbial associations were assessed for their abilities to oxidize dissolved sulphide to elemental sulphur. Physiological analyses (temperature, pH and substrate concentration optimization) were made with closed and open cultures while morphological characterization and species compositional changes were monitored by light and scanning electron microscopy (SEM). To investigate further functional and structural responses to physiological changes, denaturing-gradient gel electrophoresis (DGGE) separation of PCR-amplified 16S DNA gene fragments and Biolog GN microtitre plates were used. The associations were found to be active metabolically between 0 and 35^{0}C, 15 and 50^{0}C, and 15 and 45^{0}C, with optimum temperatures of 25, 40 and 35^{0}C for Lf-1, Ws-2 and Am-3, respectively. The optimum pH range for microbial association Lf-1 was between 3 and 4. The maximum specific growth rates of associations Lf-1, Ws-2 and Am-3 were 0.08, 0.06 and 0.03 h^{-1}, respectively. Components of all three Gram negative rod-dominated associations were motile and displayed anaerobiosis. During open culture cultivation the species complement of Lf-1, as determined by morphological analysis, changed. The same association oxidized sulphide (40 ppm) to sulphur although Ws-2 and Am-3 did not have this capacity.

Biolog GN plates detected pH-effected species compositional changes in Lf-1 and these were confirmed by DGGE. The same technique showed that enrichment had occurred in the Biolog GN wells. Species composition changes also resulted in response to different pH values (2 to 9), temperatures (5 to 40^{0}C) and dilution rates (0.003 to 0.09 h^{-1}), but activity changes were not always accompanied by population profile changes.

Acknowledgements

Professor E. Senior for his expert advice and editing and Dr R. Daneel for his enthusiasm and encouragement.

My family for their encouragement and support.

Staff at the Centre for Electron Microscopy, University of Natal (Pietermarizburg) for all their help and enthusiasm.

The technical, support staff and fellow students of the School of Applied Environmental Sciences, University of Natal (Pietermarizburg).

The Department of Molecular Cell Physiology, Vrije Universiteit Amsterdam.

The School of Molecular and Cellular Biosciences, University of Natal.

The National Research Foundation and the University of Natal for their financial support.

Declaration

I declare that this dissertation is my own unaided work. It is being submitted for examination for the Degree of Master of Science (School of Applied Environmental Sciences, University of Natal). It has not been submitted before, for any degree or examination, at any other university.

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September 2000

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Chapter 1

Literature Review

1.1 Introduction

Bioremediation is a pollution treatment technology which uses biological systems to catalyze the destruction or transformation of various chemicals to less harmful forms (Atlas, Cohen, Hershberger, Hu, Sherman, Wilson and Wu, 1999). Extensive descriptions of actual cases exist where the uses, limitations and scientific and engineering underpinnings of bioremediation are reviewed (Pomeroy, 1982; Ottengraf, 1987; Saker and Herson, 1994; Deshusses, Hamer and Dunn, 1995b; Swanson and Loehr, 1997; van Lith, Leson and Michelsen, 1997). The current approach to bioremediation of contaminated sites relies primarily on the use of indigenous strains and the modification of environmental variables to enhance microbial growth (Greer, Robinson and Shelton, 1992). The harnessing of microbiological activities and their implementations in advanced engineering applications form the basis for the increasing use of biotreatment systems for the biodegradation of even the most difficult pollutants.

Since 1980, there has been a rapid advance in the technology of odour removal, with biofiltration described as the 'best available control technology' (BACT) (Singhal, Singla, Walia and Jain, 1999). Gas biofiltration is an innovative technology in which polluted air passes through a reactor which contains microbial films on support material (Webster, Devinny, Torres and Basrai, 1997). Biofiltration is most effective with dilute labile waste gases. Over the past two decades, biofilters have developed from systems which abated odours to technically sophisticated and controlled units (Swanson and Loehr, 1997).

Most industrial and agriculture processes, transport functions and energy-production systems generate gaseous emissions which are often pollutants. The focus of this research programme was sulphide-containing waste streams which are generated by a number of industries and processes such as petrochemical plants, tanneries, viscose rayon manufacture, coal gasification for electricity production, landfills and anaerobic treatment of sulphate-containing wastewaters (Rinzema and Lettinga, 1988; Brimblecombe, 1989; Kuenen and Robertson, 1992). In addition to the obvious odour nuisance, problems such as health impacts, crop damage, the

greenhouse effect and others must be considered. Public concern about air pollution is growing rapidly even in so-called developing countries. In many countries environmental protection legislation has been introduced. This is especially true for Europe where biofilters were developed for odour abatement and in the United States of America (USA) where, in 1990, the Clean Air Act amendments were promulgated (Swanson and Loehr, 1997).

Biofiltration has been used successfully to control a number of air contaminants such as noxious odours, volatile organic compounds (VOCs) and hazardous air pollutants (HAPs) originating from a wide range of industrial and public sector sources (http://trgbiofilter.com 21/2/00). Atmospheric emissions of volatile sulphur compounds, such as hydrogen sulphide (H_2S), methanethiol, dimethyl sulphide and dimethyl disulphide cause offensive odour problems (Zhang, Kuniyoshi, Hirai and Shoda, 1991) in concentrations above their odour detection thresholds of ppb (Smet, Lens and van Langehove, 1998). The solubilization of discharged sulphur dioxide (SO_2) to sulphuric acid results in acid (pH 2 to 3) rain which decreases the viability and species diversity of poorly-buffered receiving waters (Lens and Pol, 1999). Sulphuric acid can be formed also from H_2S by a microbial process which is responsible for the corrosion of many materials (Hao, Chen, Huang and Buglass, 1996).

Significant contributors to odour are sulphur compounds and, among these, H_2S is often the most abundant (Oh, Kim and Lee, 1998). There is, therefore, a great need to develop processes for H_2S removal because of its toxicity (Reiffenstein, Hulbert and Roth, 1992) and highly odorous and corrosive properties (Chung, Huang and Tseng, 1996a). Hydrogen sulphide becomes progressively more dangerous as its concentration exceeds the human toxic limit (70 ppm) and, finally, becomes lethal at 600 ppm (Janssen, de Hoop and Buisman, 1997). However, compared to atmospheric pollution by carbon, nitrogen and phosphorus, pollution by sulphur compounds has received far less attention despite the fact that the biological sulphur cycle offers a wide range of unexploited opportunities for pollution control (Lens and Pol, 1999). Furthermore, many bacteria are capable of H_2S oxidation and, hence, serve as potential candidates for gas desulphurization technology (Gadre, 1989).

Since the mid-1980s, biofiltration technology for the treatment of off-gases containing odours, or low concentrations of volatile organic compounds (VOC), and hazardous air pollutants has been accepted in Europe as an often cost-effective and

benign alternative to traditional air pollution control techniques (APC) (van Lith *et al.*, 1997). The, so-called, other APC technologies refer to physical and chemical treatments such as activated carbon, combustion, incineration and acid-alkali processes which, together with producing greenhouse gases and depleting the stratospheric ozone, are simply uneconomical (van Groenestijn and Hesselink, 1993). In contrast, biofiltration processes have low capital and operating costs, high removal efficiencies and are reliable and, through the use of natural cycles, secondary pollution is minimized (Bohn, 1992). The spent biofilter medium, if not contaminated with pollutants such as heavy metals, can be disposed of as a non-hazardous waste or can be used as a fill for land reclamation (Torres, 1997). Today, low-cost biological deodorization processes are the obvious choice for Africa.

With increasing emphasis on air pollution due to public awareness and subsequent political intervention, biofiltration as a treatment may become a necessity. Fortunately, biofiltration is a safe, environmentally-friendly "green" technology. Microbial activities within biofilters must, however, be better understood as must their interactions with attendant physical and chemical parameters. Most biofilter designs are based conservatively on the blanket "rule of thumb" criteria (Yang and Allen, 1994). As a result, the desired control efficiencies are frequently not achieved due to a lack of understanding of the processes involved and the improper designs and maintenance of the systems. The biodegradative capacities of associations are thought to be greater both in quantitative and qualitative terms, than monospecies populations (Bull and Slater, 1982). For H_2S oxidation, it is important to screen effective associations, and investigate and optimize the physiological conditions. Molecular techniques such as denaturing-gradient gel electrophoresis (DGGE) can be used to estimate rapidly the global diversity of each association in complex environments (Santegoeds, Darmgaard, Hesselink, Zopfi, Lens, Muyzer and de Beer, 1999) and, therefore, facilitate population studies at the genetic level.

1.2 History of Gas Biofiltration

The biological treatment of malodorous air started in the 1920s and the first patent was registered a few years later (Degorce-Dumas, Kowal and Le Cloirec, 1997). Suggestions to treat odorous off-gases by biological methods can be found in literature as early as 1923 where Bach discussed the basic concept of H_2S emissions

control from sewage treatment plants (Leson and Winer, 1991). Reports on treatment applications dating back to the 1950s were published in the USA and Europe (van Lith *et al.*, 1997). In 1959, a soil bed installation was commissioned at a municipal sewage treatment plant in Nuremberg, West Germany for the control of odours from an incoming sewer main (Leson and Winer, 1991).

In the USA, Carlson and Leiser (1966) reported the first research on biofiltration (Langenhove, Wuyts and Schamp, 1986). The work resulted in the successful installation of several soil filters at a wastewater treatment plant near Seattle and demonstrated that biodegradation rather than sorption accounted for odour removal. During the next two decades, scientists in the USA demonstrated the usefulness of soil beds in several full-scale applications. Much of the fundamental knowledge of the technology is attributed to Hinrich Bohn who investigated, for more than 15 years, the theory and potential applications of soil beds (Leson and Winer, 1991).

In the 1970s, more advanced, open biofilters were developed in Europe (van Lith *et al.*, 1997) while the basic processes determining filter efficiency were understood qualitatively since the 1960s. However, the approaches to designing biofilter systems were usually empirical with no practical applications (Leson and Winer, 1991). In the 1980s, practical experience and the increasing desire of regulatory agencies to quantify biofilter performance stimulated the development of a new generation of biofilters (van Lith *et al.*, 1997). This development was motivated, primarily, by USA federal regulations, which require the control of emissions of VOCs and air toxins from new and existing sources, together with a well-funded development programme run by the West German Federal Environmental Agency, Umweltbundesamt (UBA), and the establishment/alliance of several engineering firms which addressed and resolved some of the initial technical problems.

Today, biofiltration is an accepted and mature technique for air pollution control and carries much industrial confidence. Increasingly stringent regulation requirements in the Netherlands with regards to off-gas treatment are demonstrated by the exponentially-increased capacity of biofilters installed by two major Dutch biofiltration companies which resulted in the treatment of 330 000 m^3 h^{-1} in 1990 (Fig. 1.1) (van Groenestijn and Hesselink, 1993). Developing countries are inevitably associated with growing industrialization, urbanization and motorization. The history of biofiltration makes it the ideal tool to reduce the pollution associated with these factors as it has economic viability and a proven track record.

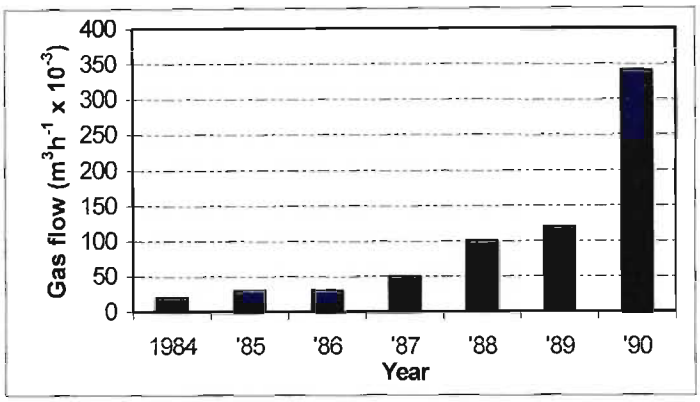


Fig. 1.1: Total gas flow treated per year with biofilters by two major Dutch companies (van Groenestijn and Hesselink, 1993).

1.3 Theory and Basic Design of Gas Biofilters

Biofiltration technology involves the passage of a polluted air stream through a packed bed which contains microorganisms immobilized within biofilms attached to the bed packing materials which are, traditionally, mixtures based on compost, peat or soil (Corsi and Seed, 1995). Improved removal efficiencies have been achieved with activated carbons and calcium alginate beads as carrier materials due to low pressure drops and high microbial contents (Chung, Huang and Li, 1997). The filter beds are, typically, 1 metre deep. A conceptual design of an open biofilter is shown in Fig. 1.2. Raw gas enters a blower system which is used to control the flow rate to facilitate optimum biodegradation. The gas may need to be heated or cooled to the optimal range for microbial activity. Such temperature adjustment is incorporated often into the humidification step. If not, and gas heating is required, temperature regulation precedes humidification to prevent lowering the relative humidity of the gas. Humidification ensures that the gas is fully saturated as it enters the biofilter to prevent water stripping from the biofilter medium. Following humidification, the gas enters an air distribution network which is designed to feed uniformly the gas to the biofilter medium. The gas distribution system often serves a second purpose of leachate collection which may drain from the biofilter medium.

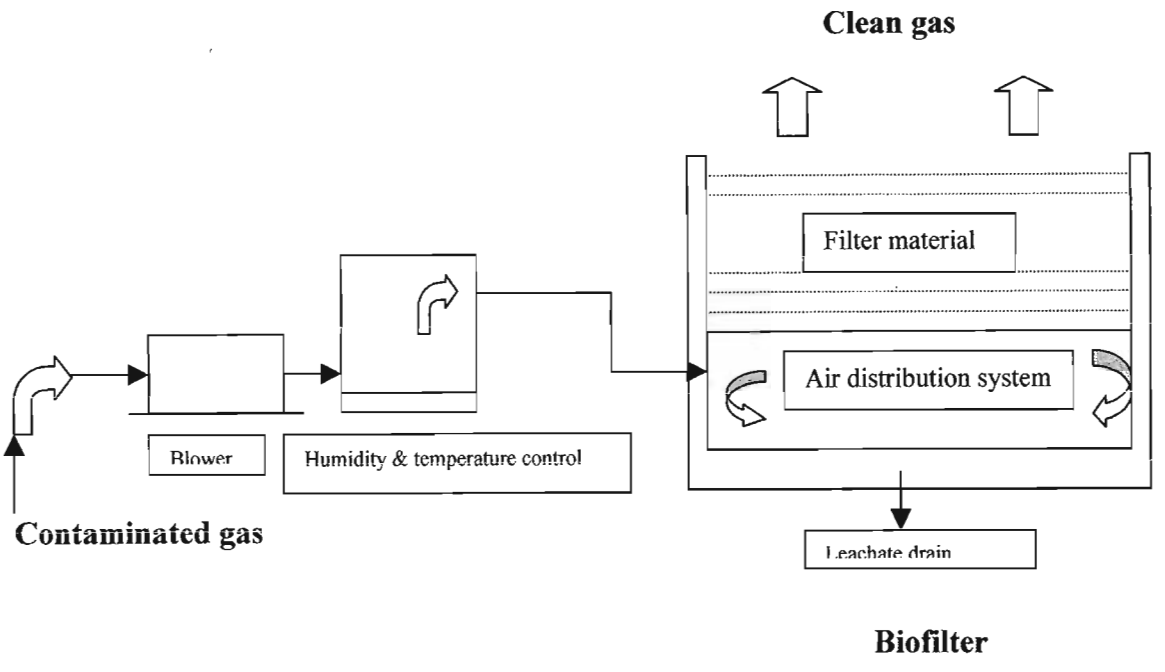


Fig. 1.2: Schematic diagram of an open single-bed biofilter system (adapted from Leson and Winer, 1991).

After distribution to the bed, the gas enters the pores of the biologically-active medium which supports the microbial population. The biofiltration process can be divided into three steps (Leson and Winer, 1991). First, a pollutant in the gas phase crosses the interface between gas flowing in the pore space and the aqueous biofilm surrounding the solid medium. Then, the pollutant diffuses through the biofilm to an association of acclimated microorganisms. The "uptake" of a contaminant (substrate) by the microorganisms creates a concentration gradient within the biofilm which promotes molecular diffusion of substrate molecules from the gas-biofilm interface toward the biofilm-solid interface (Corsi and Seed, 1995). Finally, the microorganisms obtain energy from oxidation of the chemical as a primary substrate (Swanson and Loehr, 1997). Simultaneously, there is diffusion and uptake of nutrients, such as nitrogen and phosphorus in available forms, and oxygen within the biofilm. Continuous utilization of the chemical, electron acceptor(s) and nutrients maintains concentration gradients which drive diffusive transport in the biofilm (Swanson and Loehr, 1997).

1.4 Factors Affecting Elimination Rates

Biofiltration, like all biotechnologies, is a combination of two unit processes, namely mass transfer and biodegradation. Therefore, treatment success is not dependent solely on the biodegradability of the compound since gas/liquid interfacial mass transfer rates and the partition coefficients of the substrate and oxygen are also important. Transfer rates and partition coefficients are used to model biofilter operations and select appropriate filter materials for specific applications (Hodge and Devinny, 1997). The transfer rate describes the rate at which compounds leave the air phase of the biofilter and is determined by the properties of both the contaminant compound and the packing material (Hodge and Devinny, 1997). The absorption rate depends on Henry's coefficient. Poorly-soluble compounds with Henry's coefficients > 10 are assumed unsuitable for treatment in gas biofilters because of low interfacial mass transfer (van Groenestijn and Hesselink, 1993). Hydrogen sulphide has a solubility of 1.496 g l^{-1} (Lide, 1998) and a Henry's coefficient of 1.02 x 10^{-1} mol l^{-1} atm^{-1} at 298 K (http://www.ecs.umass.edu/cee/reckhow/ courses/370/ 37015/ sld004.htm 1/18/2000). These factors affect the, so-called, empty bed contact time (EBCT) which is a simplified, relative measure of the chemical residence time in a biofilter (Swanson and Loehr, 1997). Sufficient EBCT is necessary to allow transport and degradation of the pollutant to occur and is, thus, a critical design and operation parameter. Mass transfer dictates the retention time or EBCT necessary for effective H_2S removal. However, a dramatic increase in the EBCT can result in cracking and drying of the filter bed, particularly in the corners and along the walls of the vessel, and thus decreased efficiency (Bohn, 1976). Chung, Huang and Tseng (1996b) reported that biofilters effected high removal efficiencies (> 97%) when the retention time was in the range of 40 to 140 seconds. When the retention time was lowered to 14 seconds, the removal efficiency decreased by 25%. Related studies have indicated that microorganisms can metabolize H_2S within 1 to 2 seconds (Chung *et al.*, 1996b). This suggests that any lowering of H_2S removal efficiency at shorter residence times was not necessarily due to insufficient contact time between the H_2S molecules and the biomass but could have been due to the slow step of H_2S diffusion from the gas phase into the liquid phase (mass transfer) where the microorganisms existed (Yang and Allen, 1994). Chung *et al.* (1996b) presented a similar hypothesis.

From a biological point of view, the biodegradation rate of a compound in a biofilter depends on the type(s) of microorganism involved, the amount of biomass present, the micro-environment of the microorganisms, the cellular and metabolic regulatory conditions and the type of filter material. These factors are discussed in detail in the following sections together with other parameters which affect biofilter design and H_2S removal efficiency.

1.4.1 Transient Behaviour and Biomass Development

Acclimation time can be defined as the time it takes for a biofilter to reach a pseudo-steady state or maximum removal efficiency after start-up (Corsi and Seed, 1995). The acclimation time depends on a number of factors including the components of a gas stream, the properties of specific contaminants and the characteristics of the bed material. Very few publications have reported details of the start-up periods of biofilters (Deshusses, 1997). This situation exists probably because start-up is not considered to influence future performance. However, most biofilters are limited by reaction rate rather than by mass transfer. Hence, optimization of the active biomass density, through a better understanding of the initial colonization of the support material by the growing the biomass, may alter such views. The presence of the pollutant in the waste gas gives rise to changes in the existing microbial populations on the carrier material. Microbiological associations which metabolize efficiently the target pollutant(s) normally grow spontaneously (Smet, Chasaya, van Langenhove and Verstraete, 1996). This occurs since the microorganisms adapt enzymes and degradative pathways to metabolize the substrate present in the off-gas. The acclimation time is often measured as the time necessary to reach $\geq 95\%$ of some maximum removal capacity. Acclimation becomes an issue in two instances: during the initial start-up; and during restart after shutdown. It is important that both these acclimation periods are minimized.

Cho, Hirai and Shoda (1992) recorded an enhanced removal efficiency of malodorous gases in a pilot-scale biofilter inoculated with *Thiobacillus thioparus* compared with an uninoculated biofilter. For the former no adaptive period was needed. This contrasts with uninoculated biofilters where acclimation periods of 2 weeks to 6 months are often required (Cho *et al.*, 1992).

The actual biomass is a critical factor since too little militates against degradation of the target compound(s) while excess may clog the filter and cause a high pressure drop across the filter bed. Different methods have been investigated to control the biomass. High ionic strength and nitrogen limitation have been shown to prevent clogging (Torres, 1997) as too has intermittent reversal of the inlet air flow (Ottengraf and Diks, 1992).

1.4.2 Microorganisms

In many cases, gas biofiltration relies on the intrinsic degradative capacity of the indigenous microorganisms in the organic support medium. In other cases, such as with soil which is often used as a carrier material, the rates of metabolism of the indigenous microbial community are increased through environmental engineering (biostimulation). Finally, the microbial community may be altered through seeding with specialized cultures (bioaugmentation). To date, most commercial applications of biofiltration have depended on indigenous groups of microorganisms including bacteria, actinomycetes and fungi (Wainwright, 1978). Many of the responsible bacteria possess unique metabolic and eco-physiological features as clearly illustrated by the discovery of *Thiomargarita namibiensis*, the largest prokaryotic organism discovered so far with a size of up to 0.75 mm (Schulz, Brinkhoff, Ferdelman, Hernandez, Teske and Jorgenson, 1999). Compost-based filter material, typically, shows significantly higher population densities of microorganisms compared with soil and peat. Inorganic support medium must be inoculated (seeded) with a monoculture or microbial association from a suitable source material, e.g. sewage sludge, or with an inoculum from an adapted filter. Growth and metabolic activity of microorganisms in a filter depend primarily on: the presence of dissolved oxygen in the biofilm; the absence of compounds which are toxic to the microorganisms; the availability of nutrients; the presence of sufficient moisture; and suitable ranges for temperature and pH. Therefore, it is imperative that these parameters are investigated thoroughly to maximize biofiltration.

The types of microorganisms (i.e. heterotrophs or autotrophs) dominating a particular biofilter depend largely on the presence of an organic or inorganic constituent in the off-gas. The predominant microorganisms in biofilters treating off-gas with organic constituents are heterotrophs (Kosky and Neff, 1988; Leson and

Winer, 1991). Such organisms can be divided into two groups: those which obtain energy from the oxidation process (chemoheterotrophs); and those which, at first, do not benefit measurably from it. The former includes *Pseudomonas* spp, *Streptomyces* spp, *Bacillus brevis, Micrococcus* spp, *Xanthomonas* spp and *Arthrobacter* spp (Chung *et al.*, 1996a). It was reported by Chung *et al.* (1996a) that *Pseudomonas putida* has a high potential to remove H_2S in the range of 5 to 100 ppm. It, therefore, represents an effective means to remove both high and low concentrations of H_2S. For the latter, it may be beneficial still to oxidize sulphur compounds because of their toxicities. In the *Beggiatoa* species which lack catalase, the oxidation of sulphide may protect the cells from reactive harmful oxygen compounds such as hydrogen peroxide (Kuenen and Beudeker, 1982).

For off-gases with inorganic constituents, the microorganisms are chemoautrotrophs and use CO_2 as the carbon source. In such cases, mesophiles and thermophiles predominate (Swanson and Loehr, 1997). Autotrophs include microorganisms with widely different types of physiology and morphology and consist of specialist obligate chemolithotrophs and facultative chemolithotrophs. The obligate chemolithotrophs belong to the genera *Thiobacillus* and *Thiomicrospira.* They are able to generate energy from the oxidation of reduced inorganic sulphur compounds such as sulphide, thiosulphate and elemental sulphur. Organic carbon is synthesized by these bacteria from CO_2 via the Calvin cycle, and exogenous carbon compounds may contribute, at most, 20 to 30% of the total cell carbon (Kuenen and Beudeker, 1982). Autotrophic *Thiobacillus* species and *Hyphomicrobium* 155 have been seeded into different packing materials of biofilters to enhance H_2S removal (Torres, 1997). Facultative chemolithotrophs are not only able to grow autotrophically, with reduced inorganic sulphur compounds as energy sources, but are also capable of heterotrophic growth. Such bacteria belong to the genera *Thiobacillus, Sulfolobus, Thermothrix* and *Paracoccus*. Several *Thiobacillus* species are able to utilize simultaneously mixtures of inorganic and organic compounds (mixotrophic growth) (Kuenen and Beudeker, 1982). A study by Cadenhead and Sublette (1989) to determine whether other *Thiobacilli* spp offered any advantages compared with *T. denitrificans* in the aerobic oxidation of H_2S did not reveal any. However, a mixed association of these organisms may offer a wider range of applications and advantages compared with a monoculture. Strains of *Thiobacillus, Thiothrix, Beggiatoa* and *Hyphomicrobium* can oxidize H_2S to elemental sulphur and store it intracellularly.

The elemental sulphur may be oxidized further to sulphate when the concentration of hydrogen sulphide is low (Chung *et al.*, 1996b) although the resulting acidity will have adverse effects on microbial activity (Nelson, 1990).

In addition, some photosynthetic bacteria have demonstrated the ability to oxidize reduced inorganic sulphur compounds (Das, Mishra, Tindall, Rainey and Stackebrandt, 1996). *Chlorobium thiosulfatophilum*, for example, has shown an ability to degrade H_2S (Cadenhead and Sublette, 1989) while photoautotrophic bacteria have been shown to effect 100 % conversion of S^{2-} to S^0 (Henshaw, Bewtra and Biswas, 1998). The photoautotrophic bacteria, including *Chlorobium, Chromatium, Ectothiorhodospira* and *Rhodobacter* species have been used also (Chung *et al.*, 1996b) to convert H_2S to elemental sulphur under anaerobic conditions which supplies the reducing power for carbon dioxide assimilation during photosynthesis (Das *et al.*, 1996). However, there are significant problems in using phototrophic bacteria in a large-scale operation due to their slow growth rates and the need for a strong light source (Gadre, 1989; Oh, Kim and Lee, 1998). Various fungal species, namely *Alternaria tenuis*, *Aureobasidium pullulans, Cephalosporium* spp, *Epicoccum nigrum* and *Penicillium* spp have also demonstrated H_2S-oxidizing ability (Wainwright, 1978).

Many bacteria capable of H_2S oxidation serve as potential candidates for microbial desulphurization technology although the ideal microorganism must possess several other characteristics for the technology to be viable economically. For example, the microorganism should be a strict autotroph to simplify the nutritional requirements (Gadre, 1989). The ideal microorganism should be: a facultative anaerobe capable of H_2S oxidation under either aerobic or anaerobic conditions; small in size, with simple morphology and the absence of a capsid for suspension maintenance and transportation; a neutrophile, to minimize bioreactor corrosion; and characterized by a high specific activity for H_2S oxidation (Sublette and Sylvester, 1987a). Use of chemostats to study the growth characteristics of such microorganisms is common practice (Gommers and Kuenen, 1988).

1.4.3 Micro-Environmental Conditions

Temperature, pH and moisture content are important micro-environmental factors for the optimal functioning of a biofilter.

Temperature

Operation of cellular biotechniques should be preferably between 20 and 40^{0}C (Leson and Winer, 1991). The effects of temperature on H_2S removal efficiency were reported by Yang and Allen (1994). In the range of 25 to 50^{0}C, high H_2S removal efficiencies were observed although these decreased by approximately 80% when the temperature was reduced from 25 to 7.5^{0}C. Reduced performance of biofilters at low temperatures may limit their application in cold climates. It has been reported that some filter bed temperatures may be 10 to 20^{0}C higher than the ambient temperature due to chemical and microbial activity in the absorption/catabolism of the various gaseous components (Rands, Cooper, Woo, Fletcher and Rolfe, 1981). Chung *et al.* (1996a) reported high H_2S removal efficiencies with little variation in the temperature range of 20 to 37^{0}C. A decrease in H_2S removal efficiency at higher temperatures was lower than that observed at lower temperatures (Yang and Allen, 1994). While degradation rates, typically, increase with temperature, this potential gain in efficiency can, depending on the chemical, be counter balanced by a decrease in the water solubility of the target pollutant (Leson and Winer, 1991). Hydrogen sulphide water solubility decreases as the temperature increases (Britton, 1994). Yang and Allen (1994) reported H_2S removal efficiencies of 40% at 100^{0}C compared with 97.4 % removal at 50^{0}C. The removal of H_2S at elevated temperatures is due probably to both increased chemical oxidation and biological oxidation. Another important consideration is that the operation of a filter at high temperatures may repress microbial enzyme activity and accelerate cell death. It has been reported that population size reductions were lower at 42 than at 50^{0}C while enzyme activity was also affected at temperatures $\geq$ 42^{0}C (Chung *et al.*, 1996a).

pH

Since different groups of microorganisms proliferate at different pH values, changes in the pH of the filter material will affect strongly their activity. Sulphur-oxidizing bacteria can survive in environments with a wide pH range (1 to 8) depending on the species present. Optimum pH values for the oxidation of sulphur compounds fall within the range for growth of 2 to 5 (Meulenberg, Pronk, Hazeu, Bos and Kuenen, 1992).

It was reported by Yang and Allen (1994) that maximum H_2S removal occurred at a compost pH value of 3.2. It is probable, therefore, that the dominant active species

in the compost were acidophiles with an optimum pH value of 3. At higher pH values, chemical reaction between H_2S and the compost material or reaction products can enhance significantly its removal and biological oxidation can be promoted. As a result of this dual action, higher removal efficiencies for H_2S can be expected. The partitioning of H_2S between the liquid and gas phases depends mainly on the pH, the initial dissolved H_2S concentration and the temperature. Hydrogen sulphide solubility in the liquid phase decreases as the pH decreases. At pH 7, H_2S represents 50% of the dissolved sulphides in the liquid phase (Britton, 1994). If the biotechnology is used to oxidize H_2S by the action of acidophilic thiobacilli only, lower pH values can be tolerated. A pH range of 2.5 to 3.5 was measured in biofilters which removed successfully H_2S (van Groenestijn and Hesselink, 1993). Microorganisms, such as *Thiobacillus thiooxidans*, predominate at very low pH values and can biodegrade contaminants, such as H_2S, under such conditions (Torres, 1997). Furthermore, the results of Chung *et al.* (1997) indicated that particular *Thiobacillus* species possess better pH adaptability in a mixotrophic than in an autotrophic environment. In contrast, Wada, Shoda, Kubota, Kobayashi, Katayama-Fujimura and Kuraishi (1986) researched a peat biofilter fed with H_2S and found that once the pH fell below 3, the number of facultatively autotrophic bacteria decreased markedly and a breakthrough of H_2S at the outlet resulted. Other observations, that a neutral pH value improved biofilter efficiency, were made by Degorce-Dumas *et al.* (1997) and Oh *et al.* (1998). Furthermore, the study by Degorce-Dumas *et al.* (1997) on the evolution of thiobacilli in a biofilter showed that non-acidifying autotrophs were able to multiply efficiently until acidification of the packing occurred which then resulted in a decrease in the population. As a result, acidifying autotrophs multiplied and became dominant. By buffering the packing material at neutral pH the length of the high-efficiency period (>95% removal) doubled. Under these conditions, the non-acidifying thiobacilli were favoured which suggested a correlation between the biofilter removal efficiency and the presence of non-acidifying bacteria. The reason why acidophilic autotrophs are unable to oxidize sulphide effectively when the pH falls is unclear. Some possibilities include oligo-nutrient depletion caused by leaching, sulphur deposition and a higher rate of sulphur oxidation than sulphide oxidation (Degorce-Dumas *et al.*, 1997). Another consideration is that at low pH values fungi may overgrow the bacteria and affect the biofilter performance either negatively or positively (Cox, Houtman, Doddema and Harder, 1993).

It has been found that the biodegradation of H_2S and ammonia promotes acidic conditions within the filter bed via oxidative reactions (Carlson and Leiser, 1966). Excessively acidic conditions (< 3) have been shown in some cases to be detrimental to H_2S removal efficiencies (Cho, Zhang, Hirai and Shoda, 1991; Yang and Allen, 1994; Chung *et al.*, 1996b; Oh *et al.*, 1998). The City of Los Angeles experimented in the 1950s with a bed of beach sand. After a time, the sand became acidic with sulphuric acid and the removal efficiency declined (Pomeroy, 1982). Attempts to reduce acidification have involved routine filter washing with aqueous sodium bicarbonate solution followed by deionized water to remove excess sulphur (Torres, 1997). According to van Groenestijn and Hesselink (1993), biofilter acidification can be coped with by buffering with calcium carbonate combined with incidental sprinkling of H_2O and draining. Dolomitic limestone was used by Lang and Jager (1982) at, approximately, 1% (m/m) of the total medium mass to control the pH in a biofilter.

Moisture Content

Biofilter medium moisture content was identified as the single most important parameter in biofilter operation (Swanson and Loehr, 1997; van Lith *et al.*, 1997). An off-gas entering the filter medium at less than 100% relative humidity evaporates moisture from the filter adjacent to the entry port until it reaches full saturation (van Lith *et al.*, 1997) and, therefore, quickly dries out the bed. Furthermore, as in other combustion processes, the enzymatic bio-oxidation of organic off-gas constituents in a biofilter is an exothermic process. The net resulting increase in off-gas enthalpy causes an increase in off-gas temperature and, consequently, evaporation of filter moisture to maintain complete off-gas saturation. Rands *et al.* (1981) observed that without adequate watering, drying and channeling of a compost support medium occurred and resulted in deceased H_2S removal efficiency. Moisture is essential for the survival and metabolism of the resident microorganisms and contributes to the buffering capacity (Leson and Winer, 1991). A sub-optimum moisture content can result in: high backpressure and low gas retention time; oxygen transfer problems due to a reduced air/water interface; creation of anaerobic zones which promote odour formation; and production of high-strength, low-pH leachate which requires disposal. A moisture content between 40 and 60% by mass is deemed optimal (Leson and Winer, 1991). Langenhove *et al.* (1986) found that the optimum moisture content for

wood bark in the removal of H_2S was 65% (m/m) while Yang and Allen (1994) demonstrated removal efficiencies of 99.9% for compost when the water content was increased from 32 to 62%. However, when the water contents were reduced below 30%, the H_2S removal efficiencies decreased proportionally.

Characteristically, moisture contents have been maintained in the following three ways: influent gas humidification to minimize its drying potential; direct water addition to the surface of the biofilter medium with a spray-like irrigation system; and a combination of humidification and periodic water addition (Swanson and Loehr, 1997). Water consumption by a biofilter is generally low and is approximately 19 to 76 *l* of water per 165 000 m^3 gas h^{-1} (Leson and Winer, 1991).

1.4.4 Inhibition by High Concentrations of Substrate and Product

Although many industrial waste gas streams contain multiple substrates, most laboratory studies have used "synthetic" gases of one or a few constituents. Attempts have been made to model the interactive kinetics of simultaneous contaminant removal in biofilters although there has been a paucity of published data related to the effects of multiple substrates on biofilter performance (Corsi and Seed, 1995). The maximum substrate concentration in a bioreactor depends on the concentration in the gas phase and Henry's coefficient (van Groenestijn and Hesselink, 1993). The effects of various H_2S concentrations in the range 5 to 2 650 ppm in the inlet gas on its removal efficiency were investigated by Yang and Allen (1994). Removal efficiencies >99% were recorded for all the concentrations tested in the selected range. The efficiencies did not change provided that the H_2S total loading was less than the maximum elimination capacity, i.e. high concentrations of H_2S required low gas flow rates. Elimination capacity is defined as the maximum amount of compound degraded per unit volume of bed material per unit time (Corsi and Seed, 1995). The maximum H_2S elimination capacity is a function of the type of filter material used, its properties and the operating conditions. For example, the maximum elimination capacity of an aged, acidic compost biofilter with a sulphate content of 70.4 $mgSg^{-1}$ was found to be 11.5 $gSm^{-3}h^{-1}$ compared to 130 $gSm^{-3}h^{-1}$ for a young, neutral compost biofilter (Yang and Allen, 1994). Various studies have been made to determine the effects of sulphide concentrations on growth in batch cultures (Sublette and Slyvester, 1987b; Chung,

Huang and Tseng, 1997). The common conclusion of these studies was that high sulphide concentrations inhibit enzymatic activity and, thus, decrease the sulphide oxidation rate. Hence, the consumption of H_2S gas is limited by microbial activity (reaction control) rather than diffusivity of the target compound (Chung *et al.*, 1997).

Catabolic repression may occur due to the accumulation of an end product which has a detrimental effect on the microorganisms present. A study by Yang and Allen (1994) demonstrated that a sulphate content of 25 $mgSg^{-1}$ was a critical concentration for the microbial populations. Pilot plant studies confirmed that a sulphate content of $25mgg^{-1}$ was essential for the microbial environment (Singhal *et al.*, 1999) since higher concentrations were toxic. This observation is extremely important in H_2S biofiltration as sulphate may accumulate if no action is taken. Yang and Allen (1994) reported that H_2S removal efficiencies were reduced from 99.9 to about 35% when the sulphate concentration was increased from 45 to 200 $mgSg^{-1}$.

1.4.5 Filter Material

The filter bed provides a nutrient source (organic media only) and site of attachment for microorganisms (Corsi and Seed, 1995). For maximum compound removal, the bed material should have the following characteristics: it must provide optimum environmental conditions (1.4.3) for the resident microbial population to achieve and maintain high degradation rates; the particle size distribution and pore structure should provide large reactive surfaces and low pressure drops; it should contain a diverse microbial population (Corsi and Seed, 1995); and compaction should be minimized to reduce maintenance and filter replacement (Leson and Winer, 1991).

A wide range of biofilter packing materials has been studied (Carlson and Leiser, 1966; Langenhove *et al.*, 1986; Lee and Shoda, 1989). Soil was the first packing material used in the biodegradation of H_2S. In a study of soil beds for odour removal made by Carlson and Leiser (1966), moist loam soils effected excellent removals while clay beds performed poorly. Smith (1973) demonstrated the capability of soil to sorb H_2S, carbon monoxide, ethene and ethyne. Sulphur-containing gases were oxidized quite rapidly compared to the other gases. Although high removal efficiencies were achieved with soil, only a low volumetric load was possible due to the low air permeability of the soil (Chung *et al.*, 1996a).

Compost, like soil, is inexpensive, has an indigenous microbial population and contains adequate microbial nutrients. In addition, it has good adsorptive characteristics and its structure is conducive to good airflow (Torres, 1997). Although compost was shown to perform well over short periods, its aging curtailed its long-term use (Langenhove, Bendinger, Oberthur and Schamp 1992). Ottengraf (1986) recommended the addition of polystryrene spheres (3 to 5 mm diameter) to prolong the life of a compost bed. Composts from various sources have been used to demonstrate their unique capabilities as biofilters for H_2S removals (Yang and Allen, 1994). Meat waste treatment plant emissions were investigated by Rands *et al.* (1981) who recorded H_2S removals > 97.4% in bench-scale studies. In a full-scale study at Palmar Santa Elena Palm Oil Mill in Colombia, a PVC liner was used to cover a lagoon and capture 1 250 m^3 of biogas per day. The H_2S concentration of 400 ppm was removed efficiently by gas biofiltration (Conil, 1999). Langenhove *et al.* (1986) investigated the potential of compost compared with wood bark and fibrous peat and found that the wood bark had the best air permeability characteristics. With a wood bark filter bed height of 0.9 m and a surface load of 65 m^3m^{-2} h^{-1}, 10 ppm H_2S were eliminated from odorous air. Although fibrous peat packing has been demonstrated to be preferable to soil, compost and activated carbon (Chung, Huang and Tseng, 1996c), the filter size needs to be large because of high air permeability (Hodge and Devinny, 1994).

New and improved biofilter media are being introduced. One such medium is LCP-11X which is a blend of marine algae, derived from calcified seaweed, peat fibre, sand and other essential compounds. The calcareous material has a high porosity, high surface area and a rough surface which make it an ideal medium for odour removal. Hydrogen sulphide (10 to 150 ppm) removal efficiencies of 99%, over a period of 150 days, have been achieved with this medium (http:indigo.ie/~hibernia/index.html, 24/12/1999).

1.4.6 Back Pressure

The main problem with large biofilters is the provision of a homogeneous air flow through the filter bed to prevent uneven drying and, thus, differential permeability. If this occurs then the same volume of air flows through a reduced area of filter, the

backpressure increases and the removal efficiency decreases. Since this problem is often inherent in the physico-chemical characteristics of the filter material, it can be resolved by either conditioning the existing materials or searching for new materials with better air permeability characteristics.

In preliminary testing by Langenhove *et al.* (1986), household composts showed back pressures which were ten times higher than those of other materials. Yang and Allen (1994) made a similar observation. A comparison between wood bark and fibrous peat showed that the latter had a backpressure of 385 Pa for every metre height of material while the bark had a backpressure of only 70 Pa m^{-1}. This means that wood bark should be the choice both in terms of cost and efficiency. A note of warning is that backpressure increases with moisture content and a water content < 60% by mass was shown (Langenhove *et al.*, 1986) to result in a severe loss of efficiency. Therefore, it was suggested that the relative humidity of gas entering the biofilter should be controlled (Langenhove *et al.*, 1986).

1.4.7 Residence Time

To ensure maximum H_2S elimination capacity, the gas should stay in the bed for ≥ 25 seconds. With residence times of 10 and 7 seconds, efficiency decreases of about 80% were recorded by Singhal *et al.* (1999) and Yang and Allen (1994). In contrast, Sublette and Slyvester (1987a) reported that H_2S can be metabolized by a monoculture of *T. denitrificans* within 1 or 2 seconds. From these studies it may be concluded that a decrease in H_2S removal efficiency with a lower residence time is not due necessarily to insufficient reaction time between the molecule and the biomass but may be due to rate-limiting H_2S diffusion from the gas phase into the liquid phase.

1.4.8 Filter Construction and Size

The type, construction and installation of a biofilter (e.g. open single-bed, enclosed multiple bed, multi-stage system and modular system) for a given application depend, primarily, on the availability of space relative to the required filter

volume (Leson and Winer, 1991). Other criteria include differences in the capital cost and maintenance requirements.

Single-layer, open systems consist of a single, open bed, approximately 1 m deep, composed of composts or porous soils and vented to the open air (Figs. 1.3 and 1.4).

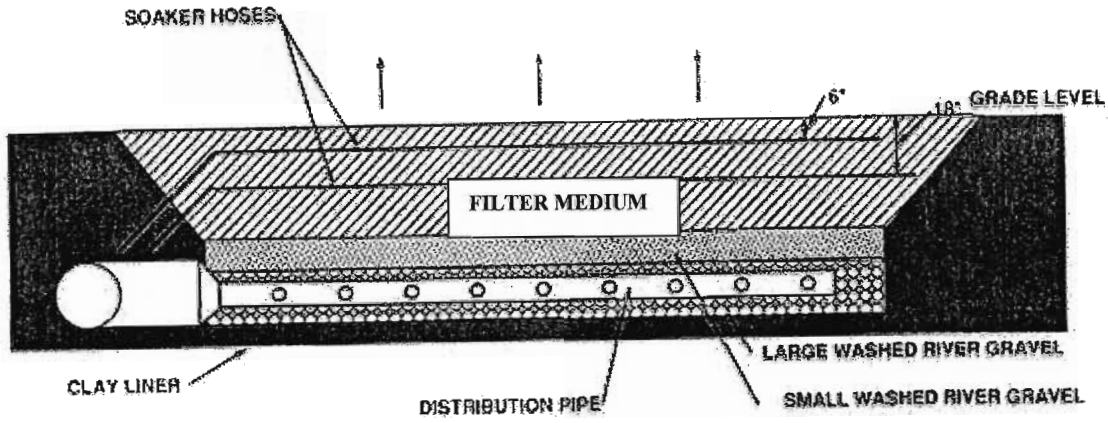


Fig. 1.3: Design of a single-layer open biofilter. (From http://www.odor.net/Information/Biofilter/biofilter.html. 27/12/1999).

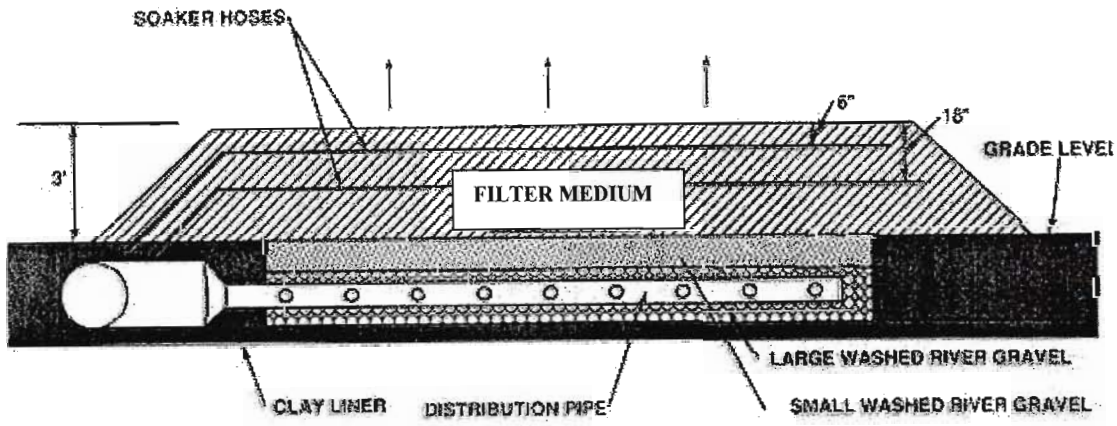


Fig. 1.4: Design of a single-layer open biofilter (above ground level). (From http://www.odor.net/Information/Biofilter/biofilter.html. 27/12/1999).

These biofilters are open and rainfall is the sole source of water (Swanson and Loehr, 1997). The effectiveness of a single open-bed, full-scale biofilter for the removal of odours for high waste gas flows was described by Swanson and Loehr (1997). The biofilter was sited on the roof of an abattoir and consisted of bark mulch mixed with timber waste to a depth of 1 m. The biofilter treated a waste gas flow of 80 000 m^3h^{-1} with a surface loading of $40m^3m^{-2}h^{-1}$. A 99% removal efficiency was achieved despite the corrosive nature of the waste gas. This system was expected to run for 4 years

before medium replacement was required. Effective biofiltration was demonstrated also by Rands *et al.* (1981) with a compost open-bed filter which removed ≤ 820 ppm H_2S, with a removal efficiency of 99%, over a 4-month trial period at the Moerewa Meat Waste Treatment Plant. Application of such a system to a landfill site seems a possibility.

Enclosed single-layer systems exist where gas humidification, overhead sprinkling for moisture, and nutrient and pH controls are incorporated.

Multi-layer, closed systems consist of multiple, separately-supported layers of biofilter medium to create beds without compaction (Fig. 1.5). Moisture control of each layer can be maintained independently. Multi-stage systems consist of multiple single-layer, closed biofilters in series. These are the least common of the gas biofilters but they allow the most flexible configurations. The separate reactors facilitate optimal temperature, moisture and microbial population control specific to the different contaminants in the off-gas. For landfill gas the microbial populations include methane-and hydrogen sulphide oxidizers.

Modular systems are commercially available units which are similar to enclosed, multiple–layer systems with the exception that each layer is contained in a separate removable 'tray'. The stacked tray system permits the cycling or removal of different layers of medium. Humidification and heating of the off-gas is possible as well as moisture control.

Biofilters are designed usually for volumetric loads < $200 m^3 m^{-2} h^{-1}$. This means at least 50 m^2 of filter area are needed for every 10 000 $m^3 h^{-1}$ of air (Langehove *et al.*, 1986). The La Zoreda landfill at Astrurias in Spain produces gas at a rate of 3 500 $m^3 h^{-1}$. The site has a surface area of 70 hectares (Martin, Maranon and Sastre, 1997). Theoretically, a biofilter with a surface area of 17.5 m^2 is needed to treat the gas generated by such a site. Practical experience with the treatment of H_2S from a landfill was reported by van Groenestijn and Hesselink (1993) where a flow rate of 300 $m^3 h^{-1}$, a volumetric load of 17 $m^3 m^{-2} h^{-1}$ and an elimination efficiency of >99% were recorded.

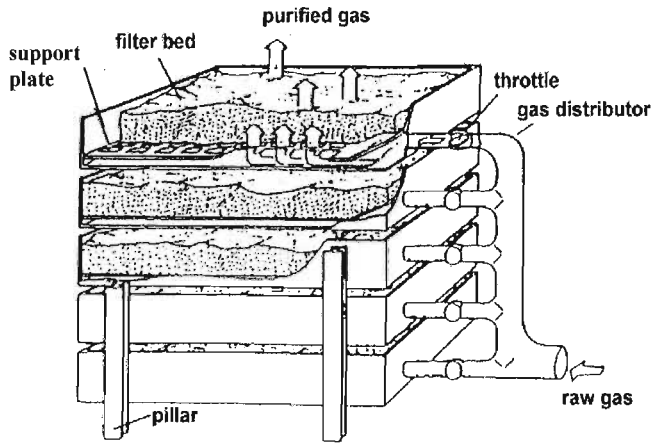


Fig. 1.5: Multi-layer closed system. (From http://chemengineer.miningco.com/ education/ chemengineer/library/weekly/ aa031698.htm 24/12/1999).

The first floating gas biofilter was installed at a farm lagoon operated by Anglian Water at Balsham, London in April 1999. This application demonstrates the flexibility of the biofilter system. The installed design encompasses a floating double-chambered mattress. One chamber contains a floating medium which supports the second chamber which contains the filter material (Plate 1.1) (http://www.rhenipal. com/productserv/Biofilter/casestudybiofilter.htm. 24/12/99).

Plate 1.1: Floating biofilter used to reduce gaseous emissions (including H_2S). (From http://www.rhenipal.com/productserv/Biofilter/ casestudybiofilter. htm. 24/12/99).

1.5 Oxidative Processes of Sulphur Compounds

Winogradsky's observation some seventy years ago was correct when he envisioned an ecological niche in which a microorganism derived all its energy from the oxidation of sulphur compounds and all its carbon from CO_2 (Kuenen, 1975). The ability to oxidize reduced sulphur compounds is not just limited to autotrophic bacteria. The work of Guittonneau revealed that the oxidation of reduced sulphur compounds in nature can be effected also by heterotrophic microorganisms (Vishniac and Santer, 1957). It is likely that in most soils the oxidation of sulphur compounds mediated by members of the genus *Thiobacillus* is insignificant compared to that of heterotrophic organisms (Vishniac and Santer, 1957). The name of Martinus Beijerinck and Cornelis van Neil will be associated forever with the pioneering studies of the oxidation of sulphur (Kelly, Shergill, Lu and Wood, 1997).

The oxidation of inorganic sulphur compounds is carried out by a spectrum of sulphur-oxidizing organisms which include: obligately chemolithotrophic microorganisms; facultative chemolithotrophic microorganisms; mixotrophs; heterotrophs which do not gain energy from the oxidation of sulphur compounds but benefit in other ways from this reaction; and heterotrophs which do not benefit from the oxidation of sulphur compounds. The spectrum is completed by a hypothetical group of heterotrophic organisms which may have a symbiotic relationship with thiobacilli and related bacteria (Kuenen, 1975). Such heterotrophs may stimulate the growth of colourless sulphur bacteria and thereby contribute to the oxidation of sulphur compounds.

The oxidation of H_2S by chemosynthetic, photosynthetic and various heterotrophic microorganisms gives rise to a number of reduced sulphur compounds:

SH^-	$\longrightarrow$	S	$\longrightarrow$	$S_2O_3^{2-}$	$\longrightarrow$	$S_4O_6^{2-}$	$\longrightarrow$	SO_4^{2-} (1.1)
sulphide		elemental sulphur		thiosulphate		tetrathionate		sulphate
(-2)		(0)		(-2 to + 6)		(–3 to + 1.7)		(+6)

It is, essentially, the above oxidation process which is employed for the degradation of H_2S. A sulphur pathway, proposed for the thiobacilli by Suzuki (1974), is given below and demonstrates that thiosulphate and elemental sulphur may be utilized as energy sources by oxidation to sulphate (Fig. 1.6).

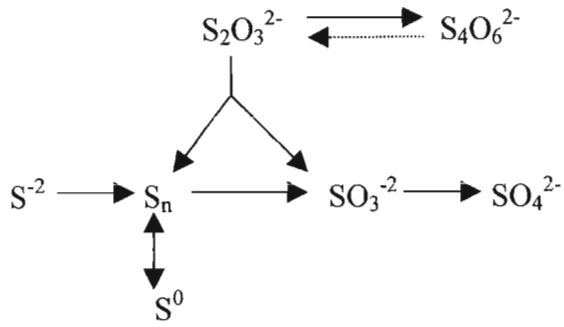


Fig. 1.6: Sulphur pathway in *Thiobacillus* (adapted from Suzuki, 1974).

Recent literature on sulphur oxidation by various neutrophilic and acidophilic thiobacilli demonstrates clearly that there is no uniformity in the pathways employed and major differences exist (Kelly, 1985). Also, the products of H_2S oxidation are dependent on the strain of *Thiobacillus* spp employed (Sublette and Sylvester, 1987c; Cho, Hirai and Shoda, 1991).

All of the unicellular, chemolithotrophic, sulphur-oxidizing bacteria appear to be able to oxidize sulphite to sulphate with sulphite:cytochrome oxidoreductases (Nixon and Norris, 1992) and so provide energy for growth. The intermediate compounds formed, and the relation of the oxidative steps to energy-storing and energy-utilizing reactions are still largely unknown. The oxidation reactions by aerobic species under standard conditions are as follows:

$$H_2S + 2O_2 = SO^{2-}{}_4 + 2H^+ \quad \Delta\dot{G} = -188.7 \text{ Kcal mol}^{-1} \qquad (1.2)$$

$$S + H_2O + 1.5\ O_2 = SO^{2-}{}_4 + 2H^+ \quad \Delta\dot{G} = -140.6 \text{ Kcal mol}^{-1} \qquad (1.3)$$

$$S_2O_3^{2-} + H_2O + 2O_2 = 2\ SO^{2-}{}_4 + 2H^+ \quad \Delta\dot{G} = -97.7 \text{ Kcal mol}^{-1} \qquad (1.4)$$

(Oh *et al.*, 1998). Most thiobacilli are capable of the enzymatic conversion of thiosulphate to tetrathionate (Pronk, Meulenberg, Hazeu, Bos and Kuenen, 1990) and this could be the first step in the conversion of thiosulphate to sulphate:

$$2S_2O_3^{2-} + 0.5\ O_2 + 2H^+ = S_4O_6^{2-} + H_2O \qquad (1.5)$$

(Nixon and Norris, 1992). Aerobic bacteria of the genus *Thiobacillus* perform a bio-catalyzed redox reaction in which H_2S is oxidized to elemental sulphur under oxygen-limited conditions (oxygen concentration < 0.1 mgl^{-1}) and/or high sulphide loads according to the following equation:

$$H_2S + 0.5\ O_2 = S^0 + H_2O \quad \Delta\dot{G} = -169.4 \text{ Kcal mol}^{-1} \qquad (1.6)$$

(Janssen, Marcelis and Buisman, 1999). The elemental sulphur particles formed are hydrophobic in nature and, therefore, can be separated from the aqueous stream by

gravity sedimentation (Janssen, De Keizer and Lettinga, 1994; Janssen, Ma, Lens and Letttinga, 1997). The sulphur cake subsequently produced is suitable as a soil fertilizer (Tichy, Janssen, Grotehuis, Lettinga and Rulkens, 1994). Plugging of the biofilter due to sulphur particle aggregation is a major disadvantage (Janssen *et al.*, 1997). Where the final product is sulphate, acidification of the biofilter occurs while a final product of sulphur leads to a neutral pH environment. The production of sulphate is an advantage in processes such as ore refinement by bioleaching which operate at low pH values. Furthermore, it allows refinery alkaline process streams, such as spent caustics, to be neutralized (Janssen *et al.*, 1999). Langenhove *et al.* (1986) noted that most of the sulphate runs out of the biofilter into the percolate.

Together with biological sulphide oxidation, chemical oxidation of sulphide must be considered which results in the formation of thiosulphate as follows:

$$2\ HS^- + 2\ O_2 = H_2O + S_2O_3^{2-}\ \Delta G = -387.35\ Kcal\ mol^{-1} \qquad (1.7)$$

(Janssen, Sleyster, Van der Kaa, Jochemsen, Bontsema and Lettinga, 1995). In earlier publications by the same group, it was reported that the biological oxidation of sulphide with oxygen occurs at a significantly faster rate than the abiotic non-catalyzed oxidation of sulphide with oxygen (Buisman, Ijspeert, Hof, Janssen, ten Hagen and Lettinga, 1991). The latter slow reaction rate may reflect the high activation energy needed to break the oxygen-oxygen bond as well as the low solubility and diffusion of oxygen in water (Dell'Orco, Chadik, Britton and Neumann, 1998). Metals such as nickel, cobalt and manganese catalyze sulphide oxidation but iron, lead and copper are the most active catalysts (Chen and Morris, 1972). By such catalysis, the length of time needed to oxidize sulphide can be reduced from several days to a few minutes (Dell'Orco *et al.*, 1998).

After nearly a century of studies on bacterial sulphur oxidation, questions remain unresolved particularly since the complexity and diversity of the processes have become progressively more apparent (Kelly *et al.*, 1997).

1.6 Hydrogen Sulphide Toxicity

Hydrogen sulphide is an extremely toxic gas (Cadena and Peters, 1988) and has the potential to cause injury to the central nervous system at low-dose exposures (Chung, Huang and Tseng, 1997). Because the human threshold exposure limit is 10 ppm for 7 to 8 h periods, excess H_2S must be removed for reasons of health and safety

(Chung, Huang and Tseng, 1996c). Hydrogen sulphide is a colourless gas which is heavier than air and is characterized even at low concentrations by a nauseating smell of rotten eggs. The gas is soluble in both organic and aqueous solutions. Due to its lipid solubility, it readily penetrates biological membranes (Reiffenstein *et al.*, 1992). Exposure to H_2S has a tendency to dull the olfactory nerve and thus the sense of smell which can be lost after 2 to 15 minutes of exposure to low concentrations and 60 seconds at higher concentrations (http://www.instanet.com/~pfc/files/h2s.htm 24/12/1999). Hydrogen sulphide poisons by accumulation in the blood stream in the same way as carbon monoxide. Hydrogen sulphide is, however, between 5 and 6 times more toxic than carbon monoxide and as toxic as hydrogen cyanide (http://www.instanet.com/~pfc/files/h2s.htm 24/12/1999). The molecule paralyzes the nerve centres in the brain which control breathing. As a result, the lungs are unable to function and the individual is asphyxiated. Hydrogen sulphide poisoning has been referred to as one of the major occupational health hazards (Roth, Skrajny and Reiffenstein, 1995). Although it has been almost 300 years since the first description of hydrogen sulphide toxicity was recorded, few reviews have been made and only one research conference has been held (Reiffenstein *et al.*, 1992).

Although exposure to H_2S at high concentrations can be lethal details of low-dose exposure effects are lacking. Similarly, the risks from repeated exposures have not been well defined (Roth *et al.*, 1995). The central nervous system (CNS) appears to be the major target and there is a greater potential for injury to the developing or immature CNS. The general opinion is that sulphide inhibits oxidative enzymes, particularly enzymes involved with oxidative phosphorylation, in a manner similar to cyanide (Reiffenstein *et al.*, 1992).

Considerable volumes of H_2S are produced by industrial activities such as petrochemical processing, wastewater and refuse treatment, paper and pulp manufacture and fuel production. Therefore, there is a great need to develop processes for H_2S removal because of its toxicology to humans as well as animals.

1.7 Economic Considerations

Biological deodorization has become big business. The market research firm Frost and Sullivan Inc. (London) predicted that Europe's market for biological waste gas cleaning processes would reach almost £54 millions in 1997 (Fouhy, 1992). The

reasons are simple. Biological deodorization is straightforward and has low capital and operating costs. For low contaminant concentrations, biofilters cost approximately 80 to 90% less than thermal oxidation treatments (Fouhy, 1992). In a market-driven world this translates to demand.

An economic comparison of available control options should always be made on a case-by-case basis and this requires some initial knowledge of the physical and chemical characteristics of the off-gas. As an example, the filter size and the capital cost of a biofilter are determined mainly by the pollutant load in the off-gas whereas for incineration systems the off-gas flow rate is the major design parameter (Leson and Winer, 1991). Prior to gas biofiltration, a sophisticated conditioning of the raw gas may be required which results in higher capital costs. For example, if large amounts of dust need to be removed, or if a lot of cooling is necessary for microbial activity, then conventional scrubbing or thermal oxidation may be more viable economically.

For a biotechnology approach, the costs reported vary greatly and are difficult to compare due to differences in a particular country's legislation and energy costs, etc. Information on capital and operating costs of various biofilter systems installed in Germany and the Netherlands has been reported (Leson and Winer, 1991). The operating costs for a biofilter include charges for electricity, replacement of spent medium, consumption of water, monitoring, maintenance and repairs (van Lith *et al.*, 1997). The data suggest total operating and maintenance costs of approximately US$ 0.60 to 1.50 per 2 832 m^3 of off-gas depending on the size of the biofilter. Comparable total costs of US$ 0.25 to 1.25 per 1 000 m^3 of waste gas were reported by Ottengraf and Diks (1992). Capital costs for open single-bed filters installed in Germany were estimated in 1991 to be US$ 25 to 95 per 9.3 x 10^{-2} m^2 of filter area (Leson and Winer, 1991). As the installation of an open-bed filter is labour intensive, the low labour costs of South Africa would mean much lower costs compared with Germany.

1.8 Experimental Theory

1.8.1 Growth Kinetics

The capacity to grow, and ultimately to multiply, is one of the most fundamental characteristics of living cells (Tempest and Neijssel, 1984). A population of

microorganisms in a suitable chemical and physical environment should utilize the available nutrients for energy production and biosynthetic purposes. The result of such metabolic activity will be an increase in the amount of cellular material and an increase in the size of the population. Microbial growth kinetics are important in simulation models for the predication of bioreactor system behaviour (Deshusses, Hamer and Dunn, 1995a). However, the methods to determine microbial growth kinetics were developed originally for monocultures (Panikov, 1995) while bioreactors often make use of microbial associations (Andreoni, Origgi, Colombo, Calcaterra and Colombi, 1997). The presence of such associations, complicates significantly the determination of microbial growth kinetics. However, the use of mixed cultures in gas desulphurization simplifies the process by removing the requirements for aseptic precautions (Sublette and Sylvester, 1987b).

The effect of microbial numbers and certain environmental parameters on the rate of growth of a population may be described by basic growth equations. The growth rate (k) can be calculated as follows:

$$\log_{10}N = kt/2.303 + \log_{10}N_0 \qquad (1.8)$$

(Note: variables defined in Appendix A) (Ingraham, Maaloe and Neidhardt, 1983) from a graphical plot. The graphical plot is the simplest way to express experimental results since it is easier to fit a straight line than an exponential curve to the data. The specific growth rate (μ) can be calculated subsequently from the following equation:

$$\mu = k/0.693 \qquad (1.9)$$

(Ingraham *et al.*, 1983). There is an essential difference between μ and k: μ only refers to the growth at a certain moment, and can be different before and after, whereas k is an average value for a population over time.

Under defined and constant growth conditions, the numbers (or biomass) of a particular monoculture or association will double in a given period of time. This characteristic time period is known as the culture doubling time (t_d) and is represented by the following equation:

$$t_d = 0.693/k \qquad (1.10)$$

(Ingraham *et al.*, 1983).

The growth of bacteria is a consequence of a complex and highly regulated set of chemical reactions. Hence, the effect of temperature on the rate of growth might be expected to reflect its effect on the velocity of chemical reactions. The Swedish

chemist Arrhenius discovered this relationship and the Arrhenius plot came into being. The equation reflecting this plot is given as:

$$\ell n\ v = (-\Delta E^*/R)\ (1/T) + \text{constant} \qquad (1.11)$$

(Ingraham *et al.*, 1983). The general form of the Arrhenius plot of growth rate is typical for all bacteria studied although the absolute values of the defining parameters vary widely.

Since the fundamental work of Novick and Szilard (1950) and Herbert, Elsworth and Telling (1956) on the theory of continuous culture, the advantages and potentials of this type of cultivation have been recognized widely (Atlas *et al.*, 1999). As a research tool, continuous culture provides well-defined cultivation conditions for genetic, biochemical and physiological characterization of cells (Dykhuizen and Hartl, 1983; Nyns, 1989; Zeng, Biebel and Deckwer, 1990; Weusthuis, Pronk, van den Broek and van Dijken, 1994) as well as representing a highly efficient method for the selection and consolidation of high-producing microorganism which underpin the majority of biotechnological processes (Novak, 1987). Furthermore, it allows independent variation of growth parameters thus enabling reliable kinetic studies of cell growth and metabolism for process optimization (Zeng and Deckwer, 1992). Interaction and competition between different species within an association can be studied more easily in continuous culture than in batch culture (Gottschal, 1992). Microbiologists recognize the chemostat as the most appropriate system for studying such interactions (Veldkamp and Jannasch, 1972). Prolonging a culture of microorganisms by the continuous addition of fresh medium and removal of product has been discussed for more than half a century (Pirt, 1975) and yet the major applications of continuous culture are still found in fundamental studies and process optimizations (Gottschal, 1992). Continuous culture study has been used also for bioreactor characterization, control and scale-up (Weusthuis *et al.*, 1994).

Knowledge of the basic principles of a chemostat is necessary since continuous cultivation of microorganisms is usually based on it (Enfors, 1991). The ideal chemostat contains a perfectly-mixed suspension of biomass into which fresh medium is introduced continuously at a constant flow rate F, while the volume V of the culture is kept constant by the continuous removal of the culture at the same rate as fresh medium enters the culture vessel (Atlas *et al.*, 1999). Cell division within the chemostat can be varied by a factor of 10 by appropriate adjustment of the rate of inflow but an equilibrium will be reached eventually in which the number of new cells

exactly balances the number of cells removed in the overflow (Dykhuizen and Hartl, 1983). Chemostats provide an environment in which cell division is continuous but the population size is held constant and the two parameters can be manipulated independently. Cells cultured in a chemostat are all in the same physiological condition which is maintained throughout growth. This makes chemostat cultures ideal for (comparative) physiological studies of microorganism as well as for studies of interactions between association members. An important aspect of a chemostat is that it represents an evolving system or, at least, a potentially evolving one (Dykhuizen and Hartl, 1983).

The most important parameter of the chemostat is the dilution rate D:

$$D = F/V \tag{1.12}$$

(Ingraham *et al.*, 1983; Britton, 1994; and Atlas *et al.*, 1999). A closely related parameter is the residence time T_r:

$$T_r = 1/D = V/F \tag{1.13}$$

where the reciprocal value of the dilution rate equals the time required for one volume change (Weusthuis *et al.*, 1994). Surprisingly, fresh medium can be added to the vessel over a wide range of flow rates while the culture within the vessel maintains a constant density. Thus, the rate of growth of the culture is just sufficient to balance the rate of loss of cells through the overflow. Once a steady state is achieved, i.e. the rate of production of cells through growth = the rate of loss of cells through the overflow, the growth rate of the culture (μ) = the dilution rate (D) (Hong and Lee, 1987). Thus, by maintaining a constant volume and changing the influent flow rate, the specific growth rate of a culture over a range up to the maximum specific growth rate (μ_{max}) can be manipulated (Atlas *et al.*, 1999). If the medium is added at a rate that requires growth of the culture at a higher rate than it is able to attain under conditions of unrestricted growth in the same medium, the culture density decreases and, eventually, all the cells wash out. The critical value for the dilution rate, or D_c, occurs when the culture just washes out ($D_c > \mu_{max}$). The critical dilution is dependent on temperature, pH and the chemical composition of the medium as well as the concentration of the growth-limiting substrate. Washout enables calculation of the maximum specific growth rate (μ_{max}) as follows:

$$\mu_{max} = \frac{(\log_e x_t - \log_e x_0)}{t_t - t_o} + D \tag{1.14}$$

The relationship between μ_{max} and D_c is given in Equation 1.15:

$$D_c = \mu_{max} \frac{S_r}{K_s + S_r} \quad (1.15)$$

Under certain conditions, the assumptions made in the derivation of the above equations may be fulfilled only approximately. The deviations may originate from interactions of the cells with the equipment and reactor hydrodynamics such as wall growth and imperfect mixing (Atlas *et al.*, 1999). Wall growth, caused by the adhesion of cells to glass and metal surfaces, can increase the steady-state biomass concentration and enable a cultivation to be carried out at dilution rates > μ_{max} (Topiwala and Hamer, 1971). Simple chemostat theory assumes perfect mixing of the medium and, quantitatively, this means that the time required for mixing should be lower than the time for mass transfer and bioreaction (Zeng and Deckwer, 1996). In large-scale bioreactors, however, imperfect mixing is encountered inevitably which, as with wall growth, results in an operational dilution rate > μ_{max}. Often biological effects are responsible for these, so-called, unexplained deviations. Among others these include growth inhibition by products and/or substrate (Yang and Humphrey, 1975), variations of growth yield due to metabolic overflow (Zeng and Deckwer, 1995) and maintenance requirements (Pirt, 1965).

Bacteria in natural habitats are faced with fluctuating nutrient availabilities with temporary excesses followed by periods of deficiency. Under such conditions, a successful life strategy depends not only on the fast and effective uptake and conversion of nutrients but also on an appropriate starvation survival strategy (Tappe, Laverman, Bohland, Braster, Rittershaus, Groeneweg and van Verseveld, 1999). This strategy should include a maintenance energy demand that is as low as possible while remaining ready for a fast response to nutrient increases. In the 1920s, Buchanan and Fulmer noted that bacteria with low energy capitals were not subcultured effectively, even if the transfer interval was short, and suggested that they needed some energy to "maintain" the cells (Russel and Cook, 1995). Maintenance energy is the energy which a bacterium expends on functions such as the constant hydrolysis and resynthesis of macromolecules which are not directly growth related (Russel and Cook, 1995). With the advent of continuous culture techniques, estimation of maintenance energy has become a more straightforward exercise (Tempest and Neijssel, 1984).

In designing a continuous culture for long-term operation, the possibility of spontaneous mutations in response to specific selection pressures must be taken into

consideration. Sooner or later cells with a competitive advantage will displace the original strain. Thus, if the physiology of a population is to be studied, the operation of a culture should be time limited otherwise metabolic responses of cells to changes in environmental conditions may be indistinguishable from genetic changes due to mutation (Atlas *et al.*, 1999).

Some cultures form aggregates or exhibit strong wall growth after prolonged cultivation under metabolic stresses (e.g. low or high pH) (Atlas *et al.*, 1999). Care should, therefore, be taken when interpreting data obtained under these conditions.

Finally, many continuous-flow culture enrichments result in the isolation of highly-stable microbial communities which contain more than one species capable of surviving and growing on the energy and/ or carbon source (Bushell and Slater, 1981). That is, there is more than one primary population. In addition, there are often a number of additional populations, secondary microorganisms, which are unable to utilize the primary substrate but are nevertheless stable members of the community. Senior, Bull and Slater (1976) reported the isolation of such a community (Bushell and Slater, 1981).

1.8.2 Phenotypic Characterization of Microbial Communities

The classification and characterization of animal and plant communities have helped define the factors which regulate both the structures and functions of such communities (Garland and Mills, 1991). Microbial ecologists have been less successful in identifying and classifying microbial communities (especially bacteria) where highly structured communities, composed of microorganisms of distinct morphology or nutritional strategy or both have been reported (Margulis, Chase and Guerrero, 1986). In most cases, a culture-based taxonomic approach is inappropriate since, currently, only very small proportions of environmental bacteria are amenable to cultivation. Nonetheless, it is important to delineate the constituents of microbial communities since changes in functionality may be caused by either shifts in community structure or physiological change (Choi and Dobbs, 1999).

Biolog GN microtitre plates (Biolog Inc., Hayward, California) were developed originally for classification of bacterial isolates based on their ability to oxidize 95 different carbon sources (Victorio, Gilbride, Allen and Liss, 1996). The GN substrates are divided into 11 categories: polymers (substrates 1-5); carbohydrates (substrates 6-

33); esters (substrates 35 & 35); carboxylic acids (substrates 39-59); brominated chemical (substrate 60); amides (substrates 61-63); amino acids (substrates 64-83); aromatic chemicals (substrates 84-87); amines (substrates 88-90); alcohols (substrates 91-92); and phosphorylated chemicals (substrates 93-95) (Haack, Garchow, Klug and Forney, 1995). Although Biolog Inc. produce 'ecology' (ECO) plates which were designed specifically to characterize environmental communities, GN plates have been adapted and used to characterize the functional potential of microbial communities (Smalla, Wachtendorf, Heuer, Liu and Forney, 1998) and to quantify differences among specific environmental samples (Konopka, Oliver and Turco, 1998). In a comparative study by Choi and Dobbs (1999), GN and ECO plates demonstrated an equal capacity to discriminate microbial diversity in a community. In such cases the inoculum is a mixture of microorganisms rather than a cell suspension of a monoculture. The metabolism of carbon sources in the microtitre plates, indicated by the reduction of tetrazolium dye, affords a detailed appraisal of the catabolic activities of complex bacterial communities (Engelen, Meinken, von Wintzingerode, Heuer, Malkomes and Backhaus, 1998). The test yields a characteristic pattern of purple wells which constitutes a "metabolic fingerprint" of the capacities of the inoculated association. Quantitative measurement of colour is superior to visual observations in providing a more accurate set of data for analysis (Konopka *et al.*, 1998). Bacterial growth occurs in the wells during the course of the assay and, hence, the pattern of substrate use may reflect only the functional characteristics of the microorganisms that are able to grow under the assay conditions used (Smalla *et al.*, 1998) rather than the numerically-dominant bacterial species (Konopka *et al.*, 1998). Similar observations were made by Haack *et al.* (1995) and Verschuere, Fievez, Van Loren and Verstraete (1997) who found evidence that only a limited range of microorganisms from a community influence the carbon utilization pattern. Another fundamental problem is that substrate utilization patterns are insensitive to changes in population structure which result from metabolic redundancy in communities (Konopka *et al.*, 1998). Thus, large changes in species genetic diversity may express very small changes in "functional diversity" as assayed by carbon substrate utilization patterns. Furthermore, when testing whole communities, the adaptation and turnover velocity of the fastest substrate utilizers will be more decisive for detecting a "functional potential" within a given incubation time (Becker and Stottmeister, 1998). The data should, therefore, be interpreted cautiously as conditions of the assay do not

necessarily reflect those found *in situ*. The data obtained with Biolog plates are amendable to multivariate statistical analyses to quantify differences among samples (Konopka *et al.*, 1998; Choi and Dobbs, 1999).

1.8.3 Genotypic Profiling of Microbial Communities

To obtain a better understanding of microbial diversity in associations, techniques which complement traditional microbiological procedures are needed. Only an estimated 20% of naturally-occurring bacteria has been isolated and characterized so far (Muyzer, De Waal and Uitterlinden, 1993) possibly because selective enrichment cultures fail to mimic the conditions which particular microorganisms require for proliferation in their natural habitats (Fantroussi, Verschuere, Verstraete and Top, 1999). To overcome the drawbacks of these culture-dependent methods, interest is focused currently on the use of molecular biological techniques where the development of increasingly advanced techniques has provided new, very powerful, tools for microbial diversity research. Instead of phenotypic characterization these methods allow characterization on a genetic basis (phylogenetic). The application of molecular biological techniques, such as DGGE, to detect and identify microorganisms is now used more and more frequently to explore the sequence diversity of complex natural microbial populations and to classify the species complements (Muyzer and Ramsing, 1995; Kowalchuk, Stephen, De Boer, Prosser, Embley and Woldendorf, 1997). This has many advantages compared with characterization based on phenotypic features (Woese, 1987), particularly since the approach does not necessitate isolation and cultivation of the microorganisms to be classified.

Separation techniques on which DGGE is based were first described by Fischer and Lerman (1983). Deoxyribonucleic acid (DNA) fragments of the same length, but with different sequences, can be separated. The separation is based on the decreased electrophoretic mobility of a partially-melted double-stranded DNA molecule in a polyacrylamide gel which contains a linear gradient of DNA denaturants (a mixture of urea and formamide) or a linear temperature gradient (Muyzer and Smalla, 1998). The melting of DNA fragments proceeds in discrete, so-called, melting domains (base pairs with identical melting temperatures). Once a domain with the lowest melting temperature reaches its melting temperature (T_m) at a particular position in the

denaturing-gradient gel, a transition from a helical to a partially- melted molecule occurs and migration of the molecule practically ceases (Muyzer and Smalla, 1998). Sequence variations within such domains result in different melting temperatures and, hence, molecules with different sequences stop migrating at different positions in the gel.

Denaturing-gradient gel electrophoresis enables the study of community complexity. A fragment from the bacterial genome is exploited as an indicator of diversity as it meets specific criteria necessary for community analysis: it is present in all species of the population; it varies between species; it contains sufficient information for good classification; and it is accessible by a universal method for all species of the population (Woese, 1987).

The fragment that meets the above criteria is the gene 16S rDNA which codes for the 16S ribosomal RNA (Woese, 1987). Together with 21 proteins, the 16S rRNA makes up the small sub-unit of the prokaryote ribosome. The primary structure can be considered as a string of domains which vary in their level of conservation while the secondary structure is very conserved. The very conserved regions permit universal access with general primers. The intermediately conserved regions are conserved only in specific phylogenetic groups and thus can be useful for detecting these groups. The highly variable domains show specific variations and can be used for the identification of different species. In the last decade, analysis of the 16S rRNA molecule or its corresponding gene (16S rDNA) has been by far the most widely used approach (Amann, Ludwig and Schleifer, 1995).

Genomic DNA is extracted from natural samples and segments of the 16S rDNA are amplified by the polymerase chain reaction (PCR). This results in a mixture of products from the different bacteria present in the sample. The individual PCR products are, subsequently, separated by DGGE. This results in a pattern of bands for which the number of bands corresponds to the number of predominant members in the microbial community. By subsequent hybridization analysis, with group-specific oligonucleotide probes, particular constituents of the population may be identified. This procedure allows for the first time direct identification of the presence and relative abundance of different species and, thus, to profile microbial populations in both a qualitative and a semi-quantitative way (Muyzer *et al.*, 1993).

Denaturing-gradient gel electrophoresis analysis of PCR-amplified 16S rRNA has been used to study the activities of members of a sulphate-reducing bacterial

association (Teske, Wawer, Muyzer and Ramsing, 1996). A misconception is that if a member of the association is dominant numerically it will be the most active member of that association. With DGGE analysis of rRNA rather than rDNA, the difference between numerical dominance and the most active species can be distinguished (Muyzer and Smalla, 1998). This was confirmed by Felske, Engelen, Nubel and Backhaus (1996).

Microbial studies often require sampling at different time points over a protracted period to identify community changes. By use of DGGE, many samples taken at different time intervals can be analyzed simultaneously. This makes the technique a powerful tool for monitoring community response to environmental changes.

Ferris, Nold, Revsbech and Ward (1997) used DGGE to study population structure and monitor physiological changes within a hot spring mat community following the removal of the top 3.0 mm. They concluded that disturbances resulting from anthropogenic influences affected the composition, structure and function of the community. Of the 16S rDNA- based methods used to study complex microbial populations, DGGE has received the most attention and has been applied successfully to several natural habitats (Wawer and Muyzer, 1995; Teske, Wawer, Muyzer and Ramsing, 1996; Brinkhoff and Muyzer, 1997; Kowalchuk *et al.*, 1997).

Molecular techniques, as with every method, are not free from errors and biases. Biases may be introduced by sample handling and through the extraction of nucleic acids from bacterial cells present in the sample. Problems are encountered with respect to a reliable and reproducible lysis of all bacterial cells as well as with the extraction of intact nucleic acid and the removal of substances, such as humic acids and bacterial exopolysaccharides, which may inhibit both DNA digestion with restriction enzymes and PCR amplification (Muyzer and Smalla, 1998). The polymerase chain reaction is another potential source of errors and biases in molecular studies of environmental samples.

Despite its limitations, DGGE is a means to obtain useful information from very complex communities and, as such, was chosen as an important tool to study the complexities and dynamics of thiosulphate-oxidizing communities prior to their use in gas biofiltration to attenuate the H_2S component of landfill gas and industrial off-gases.

1.9 Objectives

The objectives of this research programme were to enrich and isolate H_2S-oxidizing microbial associations for use in biofilters for landfill/industrial gas treatment and to make a detailed study of the physical and chemical factors which affect ultimately the biofiltration process. Physiological, phenotypic and genetic characterizations of the associations were central to the programme.

Chapter 2

Materials and Methods

2.1 Inoculum Source Materials

The source materials for the enrichment/isolation programme were: activated sludge, sampled from the wastewater return channel of an aerobic wastewater treatment reactor (Darvill, Pietermarizburg, Republic of South Africa), which was separated from the wastewater by centrifugation at 5 000 rpm x g for 10 minutes at 25^0C; a landfill site covering soil (Darvill); and soil from an acid mine water drainage site (Pretoria, Republic of South Africa). The inocula were designated Lf-1, Ws-2 and Am-3, respectively and these designations were retained throughout the study.

2.2 Growth Media

Four different media were used:

Autotrophic enrichment/isolation medium (gl^{-1} distilled water):

$Na_2S_2O_3$. $5H_2O$, 10.0; KH_2PO_4, 1.0; NH_4Cl, 1.0; and $MgCl_2$. $7H_2O$, 0.5 (pH 6.8 ±0.2 at 25^0C)(Atlas and Parks, 1993).

Heterotrophic enrichment/isolation medium (gl^{-1} distilled water):

$Na_2S_2O_3$. $5H_2O$, 10.0; K_2HPO_4, 4.0; KH_2PO_4, 4.0; $CaCl_2$, 0.1; $MgSO_4$. $7H_2O$, 0.1; $(NH_4)_2SO_4$, 0.1; $FeCl_3.6H_2O$, 0.02; $MnSO_4.4H_2O$, 0.02; and $NaHCO_3$, 0.5 (pH 6.6 ±0.2 at 25^0C) (Atlas and Parks, 1993). The $FeCl_3.6H_2O$ and $CaCl_2$ were autoclaved (121^0C, 15 min) separately, allowed to cool, then mixed aseptically with the bulk solution.

Solid medium (gl^{-1} distilled water):

agar, 15.0; $Na_2S_2O_3$. $5H_2O$, 10.0; NH_4Cl, 1.0; yeast extract; 1.0; K_2HPO_4, 4.0; $MgCl_2$, 0.5; KH_2PO_4, 0.4; chlorophenol red, 0.08; and $FeCl_3$, 0.02 (Atlas and Parks, 1993).

Sulphide thiosulphate maintenance medium (gl^{-1} distilled water):

$Na_2S_2O_3$. $5H_2O$, 5.0; KH_2PO_4, 1.0; NH_4Cl, 1.0; $MgCl_2$. $7H_2O$, 0.5; with increased concentrations (10, 15, 20, 25, 30 and 40 ppm of S^{2-}) of sodium sulphide ($Na_2S.9H_20$, Saarchem) (pH 6.8 ±0.2 at 25^0C).

The pH values of all the media were adjusted with 1N NaOH or 1 N HCl prior to autoclaving at 121^0C (205 kPa) for 15 minutes.

2.3 Analytical Methods

2.3.1 Optical Density

Culture growth was monitored by optical density (OD_{540}) (Baloo and Ramkrishna, 1991) in 1 cm light path cuvettes with a Milton Roy Spectronic 301 spectrophotometer. Distilled water was used as the blank.

2.3.2 Biomass Determination

Culture biomass was determined directly by dry weight measurement. Prior to use, 25 mm glass fibre pre-filters (Osmonics Inc.) and 0.2μm filters (Micron Separations Inc.) were washed with distilled water, dried at 105^0C, weighed and stored in a desiccator (Ongcharit, Sublette and Shah, 1991). Replicate aliquots (5 m*l*) of culture were then filtered under reduced pressure through the filters in series which were, subsquently, washed twice with sterile distilled water. The filters were dried at 105^0C for 2 hours and cooled in a desiccator before re-weighing. The biomass was then determined by difference.

2.3.3 Elemental Sulphur, Thiosulphate and Sulphate

Extraction of elemental sulphur from an aqueous culture suspension (Lf-1) and qualitative analysis by high-performance liquid chromatography (Thermo Separation Products: P-2000 Pump and Ultra-Violet 6000 LP Detector) were made as described by Henshaw, Bewtra and Biswas (1997) with the exception that the C_{18} column had the dimensions of length 3.9 cm and internal diameter of 150 mm and the injection volume was 20 μ*l*. The retention time of the sample was then compared with that of standard (99.99 %) elemental sulphur (Aldrich Chemical Company, Wisconsin). For thiosulphate and sulphate, culture supernatant samples were filter sterilized (Cameo 0.22μm sterile filters, Micron Separations Inc.) and stored at -20^0C prior to quantatitive analysis by ion chromatography (Controlled Method) by Umgeni Water (Pietermarizburg, Republic of South Africa).

2.3.4 pH

Batch culture pH values were monitored with pH test strips (Macherey-Nagel pH-Fix 1-14) while the continuous cultures were monitored directly with a pH probe (Mettler Toledo Proceed Analytical Inc.).

2.4 Batch Culture Enrichment

A series of enrichments with the respective media was made to isolate H_2S-oxidizing associations. For the autotrophic medium, the inocula (10% v/v) were activated sludge and landfill site covering soil while for the heterotrophic medium the inoculum was acid mine water drainage site soil. All the enrichments were made in 500 m*l* conical flasks which were closed with non-absorbent cotton wool bungs, shaken (150 rpm) and incubated at 30^0C. Prior to subculturing, the sediment in each flask was allowed settle to allow future monitoring of growth by optical density. All subcultures (10% v/v into fresh medium) were made at the mid-exponential point. For each enrichment/isolation programme five successive subcultures were made.

2.5 Culture Maintenance

After batch cultivation the isolated microbial associations were stored at 4^0C. Culture maintenance was practised by subculturing into fresh medium every two weeks. In addition, the isolated associations were freeze dried (Gamma 1 freeze-dryer, Martin Christ).

2.6 Batch Cultures

All batch cultures were made in triplicate as follows. A 10% (v/v) inoculum was transferred from each stock culture to a 100 m*l* conical flask which contained 50 m*l* of the respective medium. The cultures were incubated at 30^0C in a gyratory water bath shaker (150 rpm) (IncoShake-Labotec).

2.6.1 Specific Growth Rate Determination

Batch cultures were monitored by optical density (2.3.1), sulphate formation (2.3.3) and pH change (2.3.4). The optical densities recorded during the exponential phase of growth were plotted against time (h) and the growth rate (k), specific growth rate (μ) and doubling time (t_d) calculated according to Equations 1.8, 1.9 and 1.10, respectively.

2.6.2 Substrate Concentration

Batch cultures with different concentrations of thiosulphate (0, 0.1, 1.0, 5.0 and 10.0 gl^{-1}) were made to determine the effect of substrate concentration on growth. Growth was monitored by optical density and pH change.

2.6.3 Glucose-Supplemented Cultures

Batch cultures supplemented with glucose (1 000 ppm) were made and growth monitored by optical density, sulphate production and pH change.

2.6.4 Sulphide Inhibition

Threshold toxicity concentrations of sulphide were determined with inoculated sulphide thiosulphate maintenance medium (2.2). The pH was maintained at 6 (±0.2) and the temperature at 30 ^{0}C, in a bioreactor (Bioflo III, New Brunswick Scientific Co. Inc.) equipped with pH and temperature control. Each culture was monitored at regular intervals for growth (OD_{540}).

2.6.5 Temperature Optimization

Batch cultures were made at various temperatures and growth monitored by optical density (OD_{540}). The growth rate (k) was calculated for each temperature and plotted against temperature (^{0}C) (Arrhenius plot)(Ingraham *et al.*, 1983).

2.6.6 pH Optimization

Batch cultures were initiated and monitored as above with the exception that the respective medium was combined with 50 m*l* of buffer. The buffers (0.1 M sodium phosphate, 0.1 M acetate) were prepared as described by Chambers and Rickwood (1993) to final pH values of 6, 5 and 4. To test for possible catabolism of buffer (acetate) carbon components, microbial associations Lf-1 and Ws-2 were inoculated individually into autotrophic medium (minus the thiosulphate component) plus buffer and the cultures were monitored for growth (O.D.). In addition, the two buffers used were tested for toxicity to the cultures. The optical densities of the six cultures were monitored to mid-exponential at which point 50 m*l* of the respective buffer were added and growth monitoring continued.

2.6.7 Thermal Death Point

For each association, a 500 m*l* conical flask with 300 m*l* of culture in mid-exponential phase, maintained in a shaker water bath, was heated progressively from 35 to 100^{0}C in increments of 5^{0}C. A sample was taken from each flask 10 min after each temperature had been reached and inoculated (10% v/v) into a 100 m*l* flask which contained 50 m*l* of the same medium. The flasks were monitored for growth (OD_{540}).

2.6.8 Establishing a Relationship Between the Base Consumed and Sulphate Produced

The Bioflo III bioreactor was used to batch cultivate (30^{0}C, pH 6) Lf-1 to explore the relationship between the base consumed and sulphate produced during exponential growth. The initiation of exponential growth was determined by optical density (2.3.1). Over a period of 24 h, six readings were taken of the volume of 1N NaOH used to maintain the culture pH at 6. At each reading a sample was taken for sulphate concentration determination (2.3.3).

2.7 Continuous Cultures

2.7.1 Bioreactors

Continuous culture of association Lf-1 in the presence of thiosulphate as an energy source was investigated with a Bioflo III 5 *l* bench-scale bioreactor with a working volume of 4.8 *l*. The bioreactor was equipped with pH (Type 465 pH sensor, Mettler Toledo Process Analytical Inc.) and temperature controls together with a dissolved oxygen probe (Ingold) (Plate 2.1). Filtered air, supplied via a Regent 9500 air pump, was introduced via a ring sparger at a rate of 1.8 to 1.9 *l* min^{-1} (0.45 v/v/min) and the agitation rate was maintained at 150 rpm to ensure aerobiosis. Gas exited the bioreactor through a water-cooled stainless steel condenser which returned the condensed water vapour to the culture. A bacterial filter was located on the exit side of the condenser. An in-house positive displacement pump, fitted with a graduated pipette to monitor the flow rate, controlled the addition of influent medium from a 10 *l* Schott bottle (Duran). A line break was also present to prevent any back growth (Atlas *et al.*, 1999). The pH was maintained by means of two auto-titrators by additions of 2N HCl and 2N NaOH.

A simplified, water-jacketed 200 m*l* glass chemostat (working volume 155 m*l*) was used for association Ws-2 and a water-jacketed quickfit glass 1 *l* vessel (working volume 765 m*l*) for Am-3 (Fig. 2.1 and Plate 2.2). For both, agitation was achieved with a magnetic stirrer (Amroh) and the bioreactors were maintained at 30^0C by means of a thermo-circulator (Haake D1) connected to the water jackets. The medium was introduced by means of a peristaltic pump (Watson-Marlow) through 2.79mm (Am-3) and and 0.8mm (Ws-2) i.d. silicone tubing (Watson-Marlow). The aeration rates were, typically, 700 to 800 m*l* min^{-1} and were maintained with a Regent 9500 air pump through a diffuser.

All three reactors were maintained at 30^0C ($\pm$ 0.5^0C) unless otherwise stated. Prior to inoculation (10% v/v) each reactor was autoclaved (121^0C, 205 kPa) for 50 min (Baloo and Ramkrishna, 1991). Initially, each culture was maintained under batch conditions until the mid-exponential point was reached when continuous flow operation was initiated (Atlas *et al.*, 1999). The bioreactors were operated at various discrete dilution rates and monitored by optical density and sulphate concentration

determinations. Three culture volume displacements were made before each sampling (Brannan and Caldwell, 1983; Sublette and Sylvester, 1987c).

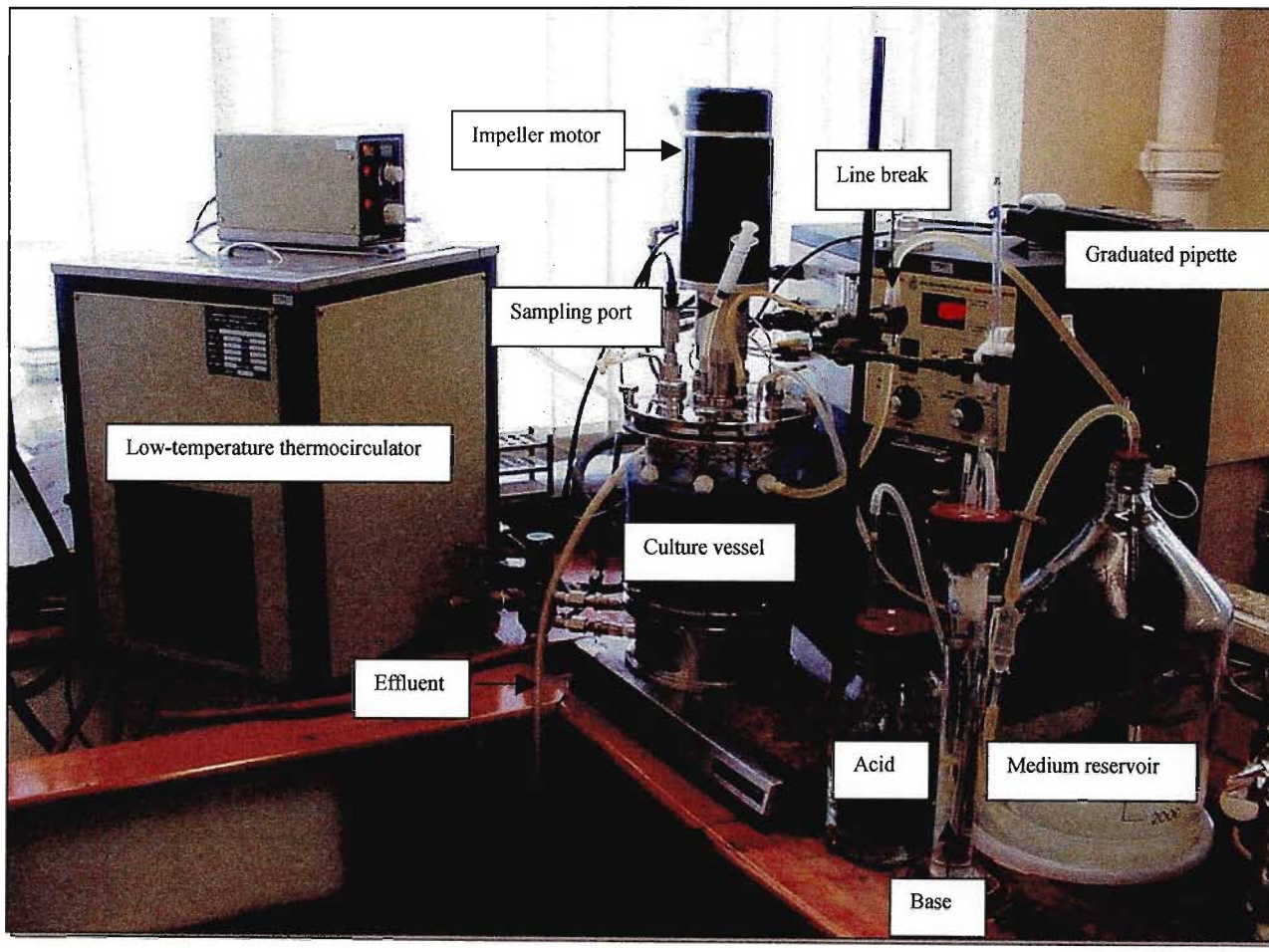


Plate 2.1: Bioflo III bioreactor (New Brunswick).

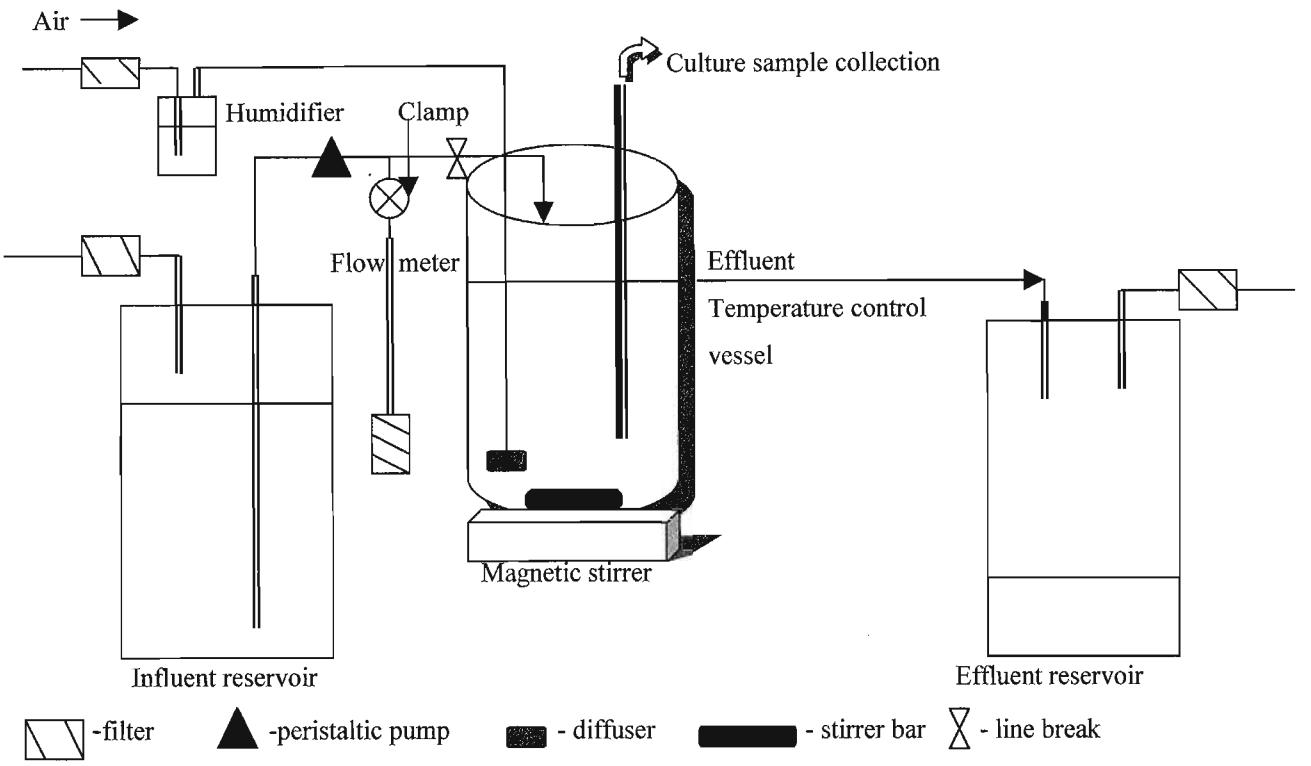


Fig. 2.1: Schematic diagram of all-glass continuous culture apparatus.

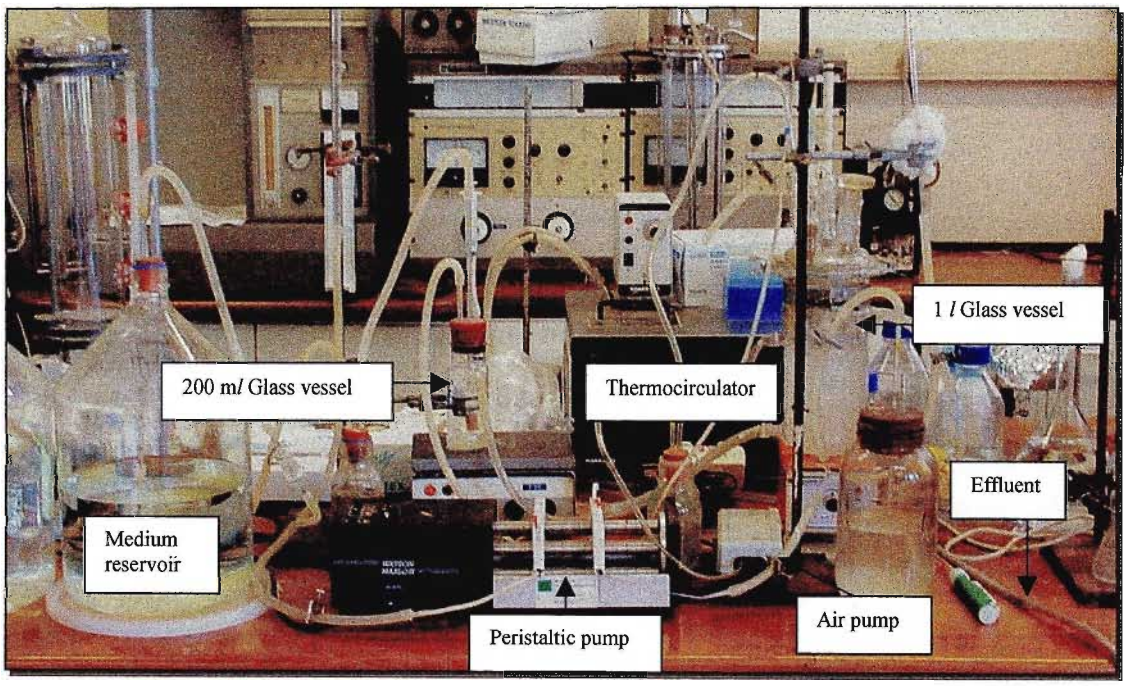


Plate 2.2: All-glass continuous culture apparatus.

2.7.2 Measurement of Influent Medium Flow Rate

The influent medium flow was measured by means of a 10 m*l* pipette attached to the influent tube by a "Y" piece. The medium reservoir was isolated with a gate clamp and the mean value of three readings for the time taken to pump 10 m*l* from the pipette into the bioreactor was recorded.

2.7.3 Steady States

Steady states, as defined by Atlas *et al.* (1999), were judged by optical density (OD_{540}) and biomass dry weight (g l^{-1}).

2.7.4 Determination of Maximum Specific Growth Rate (μ_{max}) by Washout Kinetics

In continuous culture, the maximum specific growth rate was calculated from the rate of cell washout at $D > \mu_{max}$ (Tros, Bosma, Schraa and Zehnder, 1996) once a steady state was reached according to the standard method for washout kinetics. Samples were taken at 1-h intervals over a period of 12 hours and assayed for biomass dry weight and optical density.

2.7.5 Physiological Factors Effecting Sulphate Formation

Base consumption was used as an indirect measure of the impacts of pH and temperature on product formation (sulphate). Once a steady state was achieved, the volume of base consumed (1N NaOH) was measured over a 24-h period for different pH values (range 3 to 9) and temperatures (5 to 40^0C). To negate the influence of the influent medium pH, it was adjusted to the appropriate experimental value.

2.7.6 Maintenance Energy

Growth yields (Y) of association Lf-1 were calculated at various dilution rates (D) as grams dry weight per mole thiosulphate substrate oxidized in steady-state conditions. These were calculated from the steady-state biomass dry weight and the concentration

of thiosulphate used. Values of the maximum or true growth yield, Y_{max} (grams (dry weight) per mole), and the maintenance coefficient, m (in moles per gram (dry weight) per hour) were obtained from the regression parameters of the relationship:

$$1/Y = m/\mu + 1/ Y_{max} \quad (2.1)$$

with Y (mass ratio of cells produced to substrate utilized) the apparent growth yield at different dilution rates (μ= D) in the bioreactor (Tros *et al.*, 1996).

2.8 Characterization of Microbial Associations

2.8.1 Gram Stain

Gram stains (Hucker's modification) of unknown species and controls were examined with an Olympus (type) light microscope fitted with an oil immersion lens and the images were captured with a microscope digital camera system (Olympus DP10). Analysis 3.0 computer software (Soft Imaging System) was used to manipulate the images.

2.8.2 Motility

Standard hanging-drop tests were carried out to determine motility in the associations.

2.8.3 Anaerobiosis

The ability of the associations to grow under anaerobic conditions was investigated with a gaspak anaerobic apparatus (Das *et al.*, 1996). Replicate inoculated plates of solid medium (2.1.2) were maintained (30^0C, 3 days) under anaerobic conditions (CO_2), produced with a gas-generating kit and catalyst (Oxoid), and confirmed with anaerobic test strips (Merck). The controls were maintained under aerobic conditions.

2.8.4 Electron Microscopy

Scanning Electron Microscopy (SEM)

Continuous culture samples (2 m*l*) were taken at different dilution rates from each of the three bioreactors and filtered (0.2μm) under reduced pressure. Each filter was placed in 3% (v/v) glutaraldehyde in cacodylate buffer (pH 6.96) for 30 minutes, washed twice in cacodylate buffer (pH 7.2) for 30 minutes and then subjected to critical point drying (CPD) as follows:

10 minutes in 30% (v/v) ethanol;
10 minutes in 50% (v/v) ethanol;
10 minutes in 70% (v/v) ethanol;
10 minutes in 80% (v/v) ethanol;
10 minutes in 90% (v/v) ethanol; and
3x10 minutes in 100% (v/v) ethanol (adapted from Hayat, 1981; Dawes, 1979).

The filters were then placed in CPD baskets under 100% ethanol and critically dried in a Hitachi HCP-2 CPD dryer at 30^0C and 80 kgfcm^{-2} for 1 h. The samples were placed in a desiccator overnight prior to mounting on stubs. The stubs were sputter-coated (Polaron E5100 coating unit) and viewed with a Hitachi S-570 SEM. Isolated colonies from solid medium were subjected to the same fixation/dehydration procedure with the exception that the glutaraldehyde step was extended to 12 hours.

Environmental Scanning Electron Microscopy (ESEM)

A sample of the mucilangeous material found in the effluent of Am-3 which resulted over a prolonged period of cultivation at different dilution rates (0.01 to 0.06 h^{-1}) was examined with a Phillips XL ESEM at an accelerating voltage of 20.0 kV and pressure of 5.1 Torr.

Electron Beam X-Ray Micro-analysis (EDX)

Culture precipitates from the effluent reservoir were filtered (Whatman no. 1) under reduced pressure and then washed twice with sterile distilled water to remove any residual chemical contaminants. The solids/filters were then placed on carbon-

coated stubs and carbon coated (Edwards E 306A) to reduce interference, i.e. charging (Chandler, 1977). Subsequently, the preparations were analyzed with a SEM (Hitachi S-570) and the spectrum collected with an EDX Link exl ∏ system (Hitachi).

Transmission Electron Microscopy (TEM)

The presence of flagella was visualized by transmission electron microscopy (TEM) after samples of the continuous culture were subjected to different negative staining techniques. Prior to staining, a drop of cell suspension was placed on a 100-mesh formvar-coated grid (Wirsam Scientific) and excess liquid removed with filter paper. Staining was by one of three methods. Two methods simply differed in the use of 2% (v/v) phosphotungstic acid (PTA) (Das *et al.*, 1996) and 2% (v/v) uranyl acetate. The former stain was applied for 20 seconds and the latter for 1 minute (Hayat and Miller, 1990). The third method, referred to as shadowing, involved coating the dried specimen with 0.5 mm gold palladium wire (Hayat and Miller, 1990). The preparations were then viewed by TEM (Jed 100CX) at an accelerating voltage of 80 kV (Willison and Rowe, 1980).

2.8.5 Biolog GN Microplates

Inoculation and Monitoring of Biolog Plates

Culture samples were ultra-centrifuged (Beckman J2-HS centrifuge) for 10 min at 15 000 rpm x g (20^0C). The pellets were then bulked and re-suspended in 15 m*l* of sterile (121^0C, 15 min) 0.85% (m/v) sodium chloride solution (Becker and Stottmeister, 1998; Engelen *et al.*, 1998). Prior to inoculation (0.15 m*l* per well) of Biolog GN microplates, the suspension was adjusted to a specific OD_{630} of 0.5 nm (Haack *et al.*, 1995). The microplates were incubated in the dark at 30^0C in the presence of a beaker of distilled H_2O to prevent the wells from drying out. Optical density readings (630 nm) were taken after 6, 24, 48 and 72 h (Konopka *et al.*, 1998; Smalla *et al.*, 1998) with a Bio-kinetics EL312 reader (Bio-Tek Instruments).

Biolog Data Processing

Optical density values were processed by average well colour development (AWCD) ($\Sigma_{(i = 1-95)}$ (R_i - C)/95), where C is the colour of the control well and R_i is

the colour in the i-th of the 95 wells) (Garland and Mills, 1991; Konopka *et al.*, 1998; Choi and Dobbs, 1999). Wells with no colour development in all observations (OD ≤ 0) were eliminated (Engelen *et al.*, 1998). The substrate utilization pattern was then compared with samples of equal AWCD (Konopka *et al.*, 1998). Comparisons of microbial samples on the basis of their patterns of sole carbon source utilization were assessed for their statistical significance by a multivariate t-test (principal component (PC) test) (Engelen *et al.*, 1998). Principal component analysis of the data, using a correlation matrix, was made with Genstat five statistical software (Numerical Algorithms Group Ltd). Prior to analysis, the values of each replicate were subtracted from their respective control values. The analysis was used to reduce the dimension of data (95 variables) to the number of PC scores which accounted for > 75% of the variance (Glimm *et al.*, 1997). Each PC score extracts a percentage of the variance from the original data with the most being extracted by the first PC score (Victorio *et al.*, 1996). A plot of the first two PC scores was made to visualize differences in the samples. A statistical test for community differences was based on principal component scores of 1 to 6. Classical two-way anovas and a manova were applied to determine if a statistically significant difference existed between the PC scores relative to the samples.

2.9 Genotypic Profiling of Sulphur-Oxidizing Associations

2.9.1 Storage and Extraction of Deoxyribonucleic Acid

Continuous culture samples were centrifuged (11 500 rpm x g)(Eppendorf Geratebau) in eppendorf tubes repeatedly until pellets were present. The supernatants were discarded and the pellets resuspended in 500μl of TE buffer (Appendix B) prior to storage at -20^0C.

The isolation of total genomic DNA from the samples was made with a Nucleospin C & T kit (Macherey-Nagel) with one modification to the protocol. The nucleospin column and elution buffer were incubated for 5 minutes rather than 1 minute as per the instructions.

2.9.2 Detection and Quantification of Isolated DNA

Detection

A 0.8% (m/v) agarose gel was prepared by combining 0.2 g of electrophoresis grade agarose (Whitehead Scientific Ltd.) with 24.5 m*l* of distilled water and 0.5 m*l* of 50x TAE buffer (Appendix C). The mixture was heated in a microwave (Tedelex) for 40 seconds until all the agarose had dissolved. After cooling to ± 50^0C, 0.5 µ*l* of ethidium bromide stock solution (Appendix C) were added. The solution was mixed carefully to ensure that no air bubbles were present and poured into a gel chamber (Hoefer Scientific Instruments). The comb was placed at one end of the chamber and the gel was left for 30 minutes at ambient temperature to solidify. Once solidified, the gel was placed in an electrophoresis chamber (Hoefer Scientific Instruments) which contained 200 m*l* of running buffer (Appendix C).

Loading buffer (Appendix C) (3 µ*l*) was micropipetted onto a clean surface (strip of tinfoil or parafilm), mixed with 10 µ*l* of extracted DNA and loaded into the gel wells. A marker (10 kB marker, Boehringer Mannheim) and a positive control were loaded into Lanes 1 and 2, respectively. The gel was run at 90 volts (BioRad Power Pac 300) for 1 h. The bands were visualized with an UV-transilluminator (Chromo-Vue TM-36, San Gabriel, USA) and the image stored (Imagestore 5000 software, Ultra Violet Products) on disc. These DNA preparations were used as templates in the PCR.

Quantification

The extracted DNA was quantified with a Gene Quant II (Pharmacia Biotech). The DNA was blanked against 2.995 µ*l* of Milli-Q water before 5µ*l* of sample were added. Samples which contained ≥ 50 ngμl^{-1} of purified genomic DNA were amplified (Reed, Holmes, Weyers and Jones, 1998).

2.9.3 16s rDNA Amplification by the Polymerase Chain Reaction

Step 1: *$MgCl_2$ Optimization*

The PCR for the DGGE marker and associations was optimized by adjusting the $MgCl_2$ concentration (Table 2.1).

Step 2: *Polymerase Chain Reaction Amplification*

Deoxyribonucleic acid isolated at discrete pH values, temperatures and dilution rates for association Lf-1 were amplified. For associations Ws-2 and Am-3, DNA isolated at 30^0C only was amplified.

The GC clamp was incorporated into the forward primer by the addition of a 40-nucleotide GC-rich sequence (GC clamp) at its 5' end (Muyzer *et al.*, 1993). The variable V3 region of 16S rDNA (Saiki, Scharf, Faloona, Mullis, Horn, Erlich and Arnheim, 1985), which corresponds to positions 341 and 534 in *E.coli* (Muyzer *et al.*, 1993), was amplified enzymatically in the PCR with primers to conserved regions of the 16S rRNA genes (Medlin, Elwood, Stickel and Sogin, 1988).

Table 2.1: Reagents used for PCR amplification of 16S rDNA.

Reagent per reaction tube	Volume (μl)
10x reaction buffer without $MgCl_2$ (Boehringer Mannheim)	2.5
DNTPs (Promega, 10 mM)	0.5
Target DNA	1
Expand (Expand^{TM} High Fidelity PCR system)	0.5
Primer (forward) (0.01 M)	1
Primer (reverse) (0.01 M)	1
Bovine serum albumin (Boehringer Mannheim)(10 mgml^{-1})	1
Sub total	**7.5**
$MgCl_2$	variable (see below)
Milli-Q water	variable (see below)
Total	**25**

mM $MgCl_2$	Vol $MgCl_2$ stock (25 mM)(μl)	Vol Milli-Q water (μl)
1.0	2	15.5
1.5	3	14.5
2.0	4	13.5
2.5	5	12.5
3.0	6	11.5

Properties of forward primer: 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3'; $T_m > 75^0$C; and length 57-mer.
Properties of reverse primer: 5'-ATT ACC GCG GCT GCT GG-3'; $T_m = 57.6^0$C; and length 17-mer.
The primers were synthesized and supplied by Roche Diagnostics, Randburg.

Polymerase chain reaction amplification was made in an automated thermal cycler (Perkin-Elmer GeneAmp PCR System 2400, Norwalk, USA) with the following programme adapted from Konopka, Bercot and Nakatsu (1999):

Time Delay File	Initial denaturation	94^0C	5 min
Step Cycle File	Denaturation	94^0C	30 sec
	Annealing	55^0C	30 sec
	Elongation	72^0C	30 sec
	30 CYCLES		
Time Delay File	Final elongation	72^0C	7 min
Soak File	Cooling		4^0C

The cycle repeated itself 30 times and the products were stored at -20^0C. Two control tubes were included in each PCR run: a positive control, which contained DNA from a reference culture of *E.coli*; and a negative control with sterile (121^0C, 15 min) distilled water.

Step 3: *Visualization of Polymerase Chain Reaction Products*

A 1.5% (m/v) gel was prepared by mixing 24.5 m*l* of distilled water, 0.5 m*l* of 50x TAE (Appendix C) and 0.375 g of agarose gel (Whitehead Scientific Ltd.). The gel preparation and running process were the same as those described in 2.9.2. with the exception that a different molecular marker (DNA molecular weight marker X, Boehringer Mannheim) and run time (1.5 h at 70 volts) were used.

2.9.4 Parallel Denaturing-Gradient Gel Electrophoresis

The following four steps were applied to the running of all the denaturing-gradient gels unless stated otherwise.

Step 1: *Initial Set Up*

The electrophoresis tank was filled with 7 *l* of fresh running buffer (Appendix D). The temperature control module was placed on top of the tank and the pump and the heater were switched on. The temperature controller was set to 60^0C with a ramp rate of 200^0C h^{-1}.

Step 2: *Gel Sandwich Assembly*

The glass plates were cleaned with soap and water, rinsed with ethanol and finally with acetone and assembled. The sandwich assembly was placed in the alignment slot of the casting stand and an alignment card was inserted to keep the spacer bars parallel to each other. The clamp was then tightened until it was finger-tight. The handles to lock the assembly in the casting stand were tightened.

Step 3: *DGGE Gel Casting*

To prepare gradients of 45 and 65%, the desired volumes of the 0% and 100% gel solutions (Appendix D) (9.375 and 5.625 m*l*, respectively to prepare a 45% gradient, and 5.966 and 11.080 m*l*, respectively to prepare a 65% gradient) were pipetted into beakers. A stacking gel of 5 m*l* of 0% denaturant and 50 μ*l* of gel dye constituted the top 2 cm of each gel. Ammonium persulphate (APS)(100 μ*l*) and N, N, N', N'-tetramethylethylenediamine (TEMED-Sigma, electrophoresis grade)(10 μ*l*) were added to the beakers. The solutions were then delivered to the gel sandwich with a gradient delivery system. Following comb insertion, the gels were left to polymerize for 2 h before comb removal and well washing with running buffer (Appendix D).

Step 4: *Running DGGE Gels*

Each gel was released from the casting stand and, with the short plate facing the core, inserted into the core apparatus. The control module was turned off and removed. Running buffer (1 *l*) was removed from the chamber and 300 m*l* of it were added to the upper chamber of the core. The core, together with the attached gel assemblies, was placed in the buffer chamber. The control module was then placed on top of the tank and the unit switched on. The system was allowed to reach the initial temperature of 60^0C (approximately 2 h) and pre-electrophoresed for 15 min at 70 volts. Gelsavers tips (Whitehead Scientific Ltd) were used to load 3μ*l* of loading buffer and 15 μ*l* of sample into the wells of the 8% (m/v) polyacrylamide gel. The system was run for 16 h at 70 volts (H.W. van Verseveld, personal communication). After electrophoresis, the gel was incubated (room temperature) in the dark for 30 min in Milli-Q water which contained ethidium bromide (10 μ*l* l^{-1}). The image was then captured with UV-transillumination and Imagestore 5000 software (Ultra Violet Products) and stored on disc.

2.9.5 Denaturing-Gradient Gel Electrophoresis Optimization

Time Optimization

Amplified marker (50 μl) was loaded into adjacent lanes of a parallel denaturing-gradient gel with 1 h intervals between loadings for a total of 10 h. After electrophoresis (total run time 16 h), the gel was viewed as above (2.9.4) to determine the optimum run time to achieve maximum band resolution and separation.

Voltage Optimization

Two polyacrylamide gels were prepared and loaded with identical PCR products. One was run at 200 volts for 4 h (Wawer and Muyzer, 1995; Straub and Buchholz-Cleven, 1998) and one at 70 volts for 16 h (H.W. van Verseveld, personal communication). Subsequently, the gels were viewed to determine the better voltage for resolution and separation.

2.9.6 Denaturing-Gradient Gel Electrophoresis Data Analysis

The phylotype profiles of the samples were compared visually and pairwise similarities were computed with the Sorenson's index (Konopka *et al.*, 1999).

2.9.7 Characterization of Biolog GN Communities by DGGE

The cultures of randomly chosen Biolog GN wells (α-D-glucose, D-galactose, D-fructose and sucrose) together with a control of the inoculum were subjected to DNA isolation and amplification prior to DGGE.

Chapter 3

Results and Discussion (Part 1) Batch and Continuous Cultivation

3.1 Enrichment/Isolation and Batch Cultivation of Microbial Associations

"The number of mixed cultures in commercial use which are well studied and understood, and deliberately constituted, is extremely small" (David Harrison, 1978). In certain aspects, some 20 years later, these words still hold true (Weimer, 1991).

Emphasis was placed on microbial association Lf-1 due to equipment and financial constraints. Thus, unless stated otherwise, all experimentation was carried out with association Lf-1.

3.1.1 Enrichment

The aim of the primary enrichments was to select H_2S-oxidizing associations through the imposition of specific selection pressures. Theoretically, the enrichment/isolation conditions should mirror those existing in nature pertinent to that particular niche. Since the exact duplication of a specific niche is impossible, all enrichments will, therefore, be imperfect (Hungate, 1961) and lead to the cultivation of, so-called, laboratory artifacts. Another consideration when using a batch enrichment is that the substrate is present in excess. Therefore, competition, and hence microbial selection, is based solely on maximum specific growth rate. Thus, microorganisms with low maximum specific growth rates are excluded (Jannasch, 1967). The isolated microorganisms, therefore, have high growth efficiencies at high substrate concentrations.

The batch culture enrichments (2.4) resulted in the isolation of microbial associations capable of oxidizing reduced sulphur compounds. Thiosulphate, a common substrate in sulphide-oxidizing microbial association isolation, was used as the energy source (Kuenen and Robertson, 1992). This molecule is readily soluble, non-toxic at high concentrations and stable at neutral pH values (Visser, Stefess, Robertson and Kuenen, 1997).

3.1.2 Batch Cultivation

It is well known that with elevated sulphur compound concentrations, many chemical interactions occur amongst the sulphur compounds (Roy and Trudinger, 1970). Thus, before culturing the isolated associations the possible contribution of

chemical oxidation in substrate conversation rates was investigated. Sulphate concentration analyses were made of an uninoculated (control) and inoculated (Lf-1) autotrophic medium (2.2) during incubation for 96 h (2.6). The control indicated that chemical oxidation of thiosulphate to sulphate contributed, on average, 8.49 % of the total substrate converted (Table 3.1).

Table 3.1: Chemical and chemical plus microbiological oxidation of thiosulphate to sulphate during 96 h incubation at 30^0C.

Replicate	$[SO_4^{2-}]$ ppm	
	Chemical oxidation	Chemical plus microbiological oxidation
1	642	7 137
2	595	7 267
3	614	7 389

Autotrophic Growth

The maximum specific growth rate (μ_{max}) was determined only from exponential growth data points.

(a) Microbial Association Lf-1

The results of batch cultivation of microbial association Lf-1 in autotrophic medium (2.2) are shown in Fig. 3.1. It is probable that growth occurred chemolithotrophically by utilizing energy from the oxidation of thiosulphate (3.1) and obtaining carbon from CO_2 by the energy-consuming Calvin cycle

$$S_2O_3^{2-} + H_2O + 2O_2 = 2SO_4^{2-} + 2H^+ \qquad (3.1)$$

(Lu and Kelly, 1988; Oh *et al.*, 1998). If the product formation route is well defined, as is the case for sulphate production, a simple stoichiometric equation (3.1) can be used to estimate the theoretically possible maximum production from the substrate (Atkinson and Mavituna, 1991). In this study, conversion of thiosulphate to sulphate was stoichiometric with every mole of thiosulphate producing two moles of sulphate. Therefore, based on the mass of thiosulphate supplied in the autotrophic medium, the theoretical maximum production of sulphate was 7 685 ppm. This corresponded closely with the maximum sulphate concentration (ppm) obtained (Fig. 3.2). Exponential growth was accompanied by a marked pH reduction which correlated closely with an increase in sulphate formation (Fig. 3.2). Similar observations were

reported by Cho *et al.* (1991) and Chung *et al.* (1996a). The decrease in the pH, from 6.5 to 1 suggested that the accumulation of sulphate overcame the buffer capacity of the medium. The culture absorbance reached a maximum of 0.328 on the third day and then decreased. Near the same time, the pH reached one which suggested that either the association was sensitive to the low pH or the substrate was exhausted. The growth rate (k) of Lf-1, which was obtained from the slope (k = slope x 2.303) of the straight line in Fig. 3.3 was calculated as 0.05 h^{-1} (Equation 1.8). The specific growth rate (0.08 h^{-1}) and doubling time (13.25 h) were calculated from Equations 1.9 and 1.10, respectively.

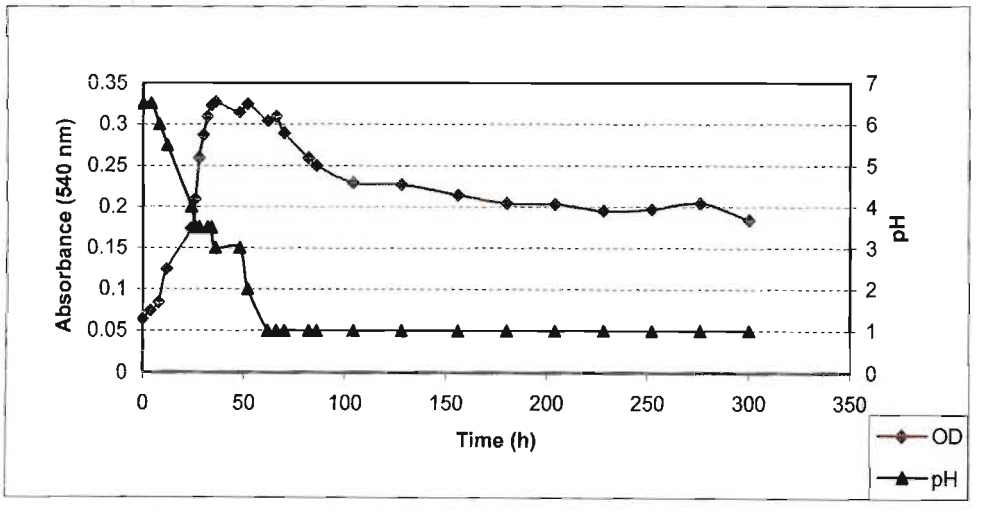


Fig. 3.1: Changes in pH (▲) and A_{540} (◆) during batch cultivation (30^0C, 150 rpm) of microbial association Lf-1 in the presence of autotrophic growth medium.

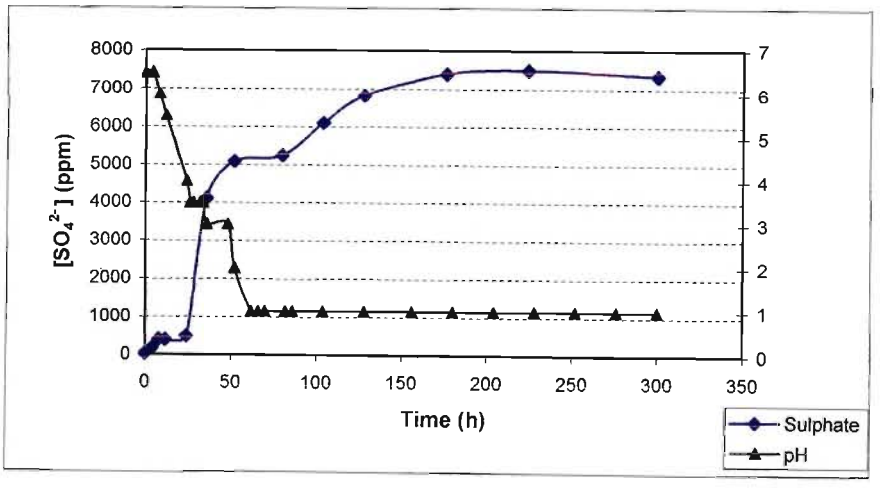


Fig. 3.2: Changes in sulphate concentration (◆) and pH (▲) during batch cultivation of microbial association Lf-1 in the presence of autotrophic growth medium.

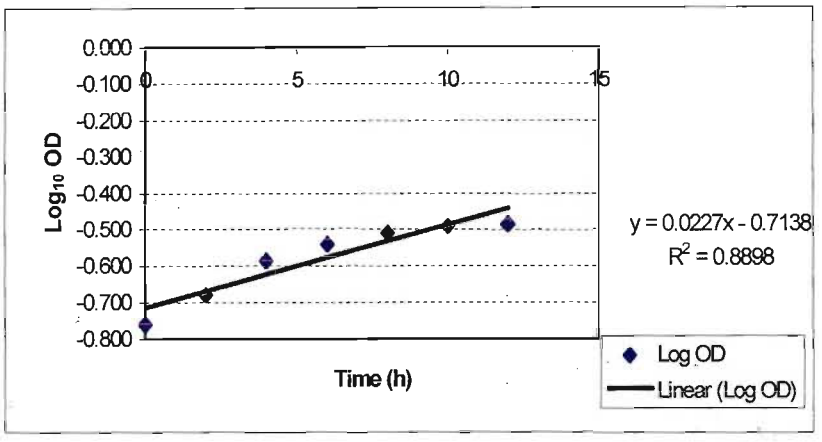


Fig. 3.3: Linear representation of exponential growth of microbial association Lf-1.

To determine whether pH had a negative effect on growth and sulphate production, a series of batch cultures (30^0C, 150 rpm) at pH 7 and 3 were made in the Bioflo III bioreactor (2.7.1) and the results compared to those above. At neutral pH (Fig. 3.4) decreases in the growth rate (0.04 h^{-1}) and specific growth rate (0.06 h^{-1}) and an increase in the doubling time (17 h) were recorded. At pH 3 (Fig. 3.5), the values (k = 0.06 h^{-1}, μ = 0.08 h^{-1} and t_d = 12 h) were comparable to the results obtained in the absence of pH control. This indicated that the fast-growing components of the association were probably acidophilic. It was interesting to note that the rates of sulphate formation under pH-controlled conditions were comparable to those where no pH control was imposed. The ability of the association to maintain a high conversion rate of thiosulphate to sulphate over a broad pH range would be an advantage in the degradation of H_2S in a biofilter where pH fluctuations occur frequently (Wada *et al.*, 1986). Furthermore, the ability of the association to oxidize thiosulphate at a pH value as low as three is of fundamental interest as it indicates that the periplasmic enzymes involved are extremely acid tolerant.

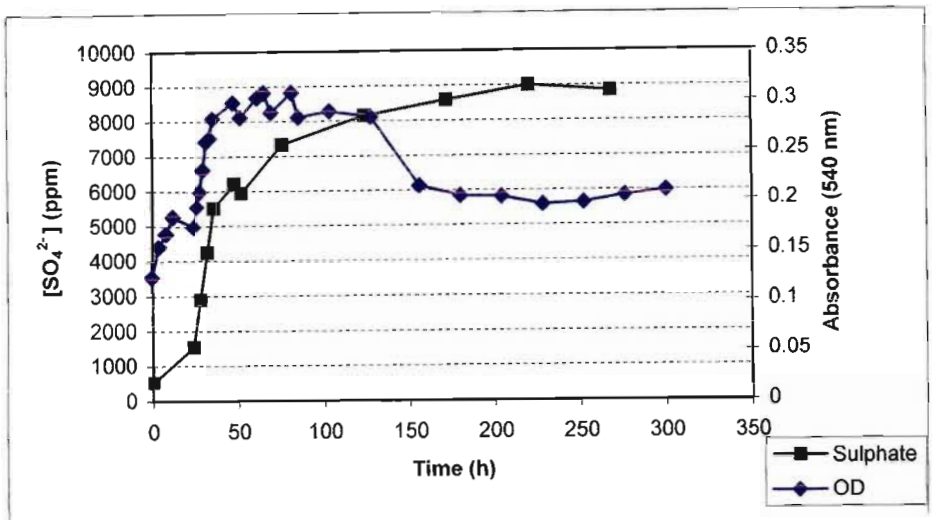


Fig. 3.4: Changes in A_{540} (◆) and sulphate concentration (■) during batch cultivation (30^0C, pH 7) of microbial association Lf-1 in the presence of autotrophic growth medium.

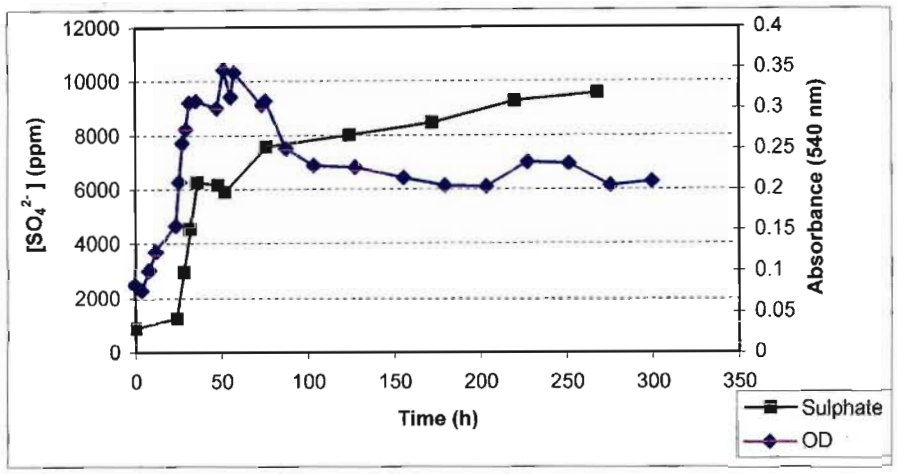


Fig. 3.5: Changes in A_{540} (◆) and sulphate concentration (■) during batch cultivation (30^0C, pH 3) of microbial association Lf-1 in the presence of autotrophic growth medium.

(b) Microbial Association Ws-2

Like Lf-1, batch cultivation (2.6) of Ws-2 resulted in a decreased pH (Fig. 3.6) concomitant with SO_4^{2-} accumulation (Fig 3.7). Thiosulphate was the energy source and CO_2 the sole carbon source for growth and, therefore, the association was characterized as chemolithoautotrophic. The growth rate (k) of Ws-2, obtained from the slope (k = slope x 2.303) of the straight line in Fig. 3.8, was calculated as 0.04 h^{-1}

while the specific growth rate and doubling time were calculated to be 0.06 h^{-1} and 17.33 h, respectively.

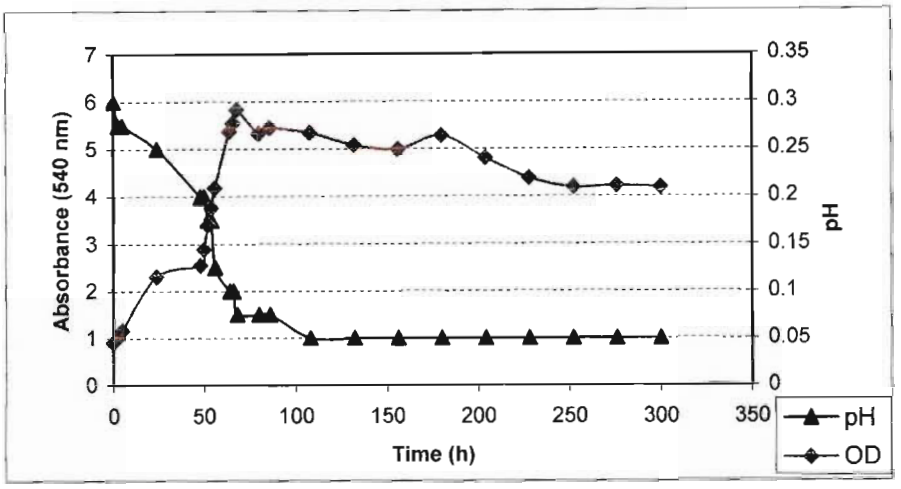


Fig. 3.6: Changes in pH (▲) and A_{540} (◆) during batch cultivation (30^0C, 150 rpm) of microbial association Ws-2 in the presence of autotrophic growth medium.

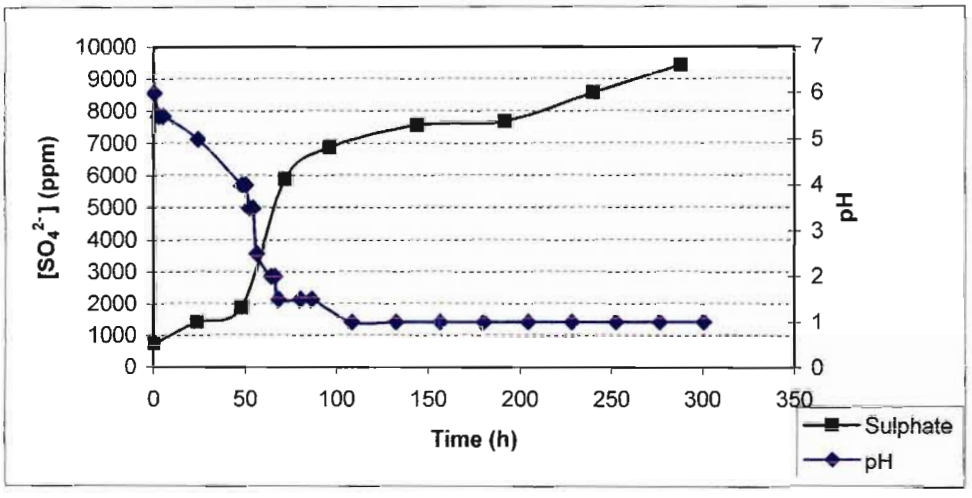


Fig. 3.7: Changes in sulphate concentration (◆) and pH (▲) during batch cultivation of microbial association Ws-2 in the presence of autotrophic growth medium.

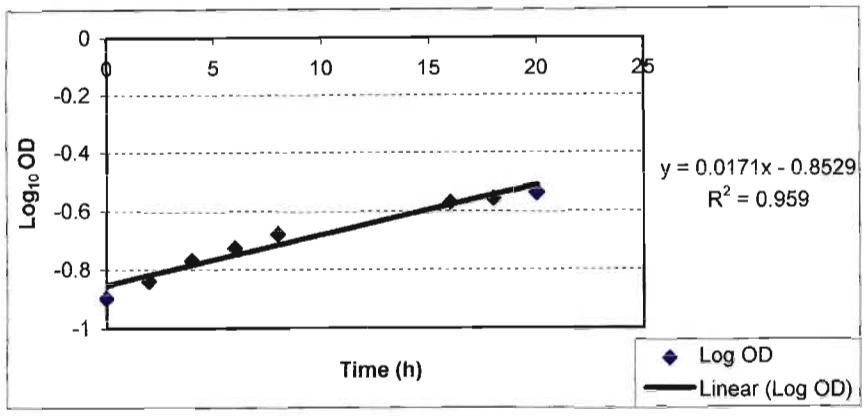


Fig. 3.8: Linear representation of exponential growth of microbial association Ws-2.

(c) Microbial Association Am-3

As with the other two associations a similar growth pattern was recorded and was accompanied by a reduced pH and sulphate accumulation (Figs. 3.9 and 3.10). The optical density plateau maintained between 25 and 100 h coincided with a continuous decline in the pH. Therefore, the subsequent OD increase possibly represented enrichment and growth of acidophilic microorganisms i.e. a population shift.

When the same association was cultivated in the heterotrophic medium (2.2), growth also resulted and, therefore, it was classified as a facultative heterotroph. The growth rate (k) for Am-3, obtained from the slope (k = slope x 2.303) of the straight line in Fig. 3.11, was calculated as 0.02 h^{-1} while the specific growth rate and doubling time were calculated to be 0.03 h^{-1} and 36.28 h, respectively.

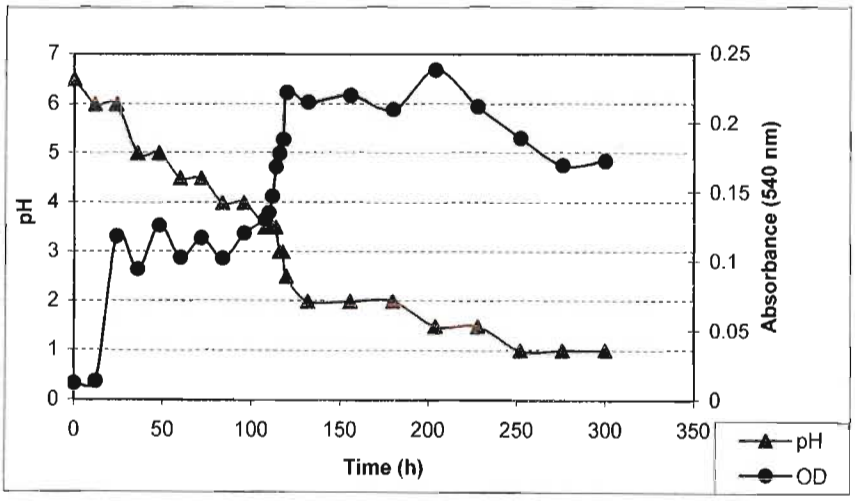


Fig. 3.9: Changes in pH (◆)and A_{540} (●) during batch cultivation (30^0C, 150 rpm) of microbial association Am-3 in the presence of autotrophic growth medium.

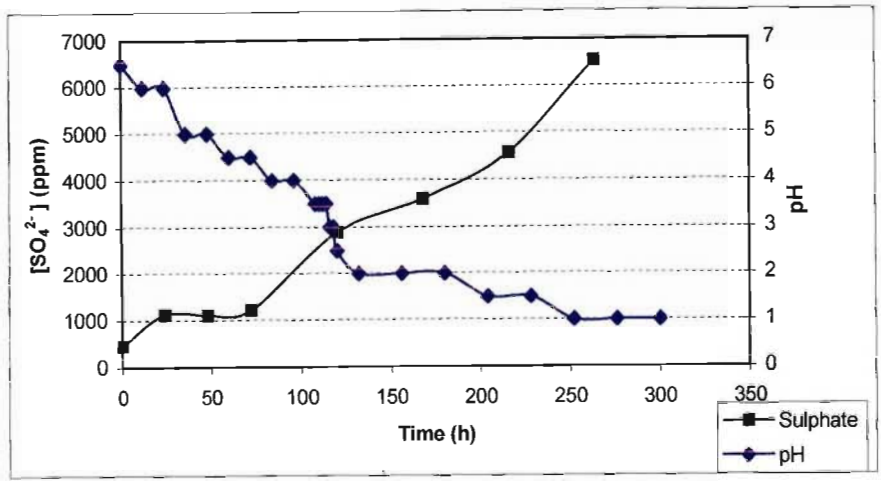


Fig. 3.10: Changes in sulphate concentration (■) and pH (◆) during batch cultivation of microbial association Am-3 in the presence of autotrophic growth medium.

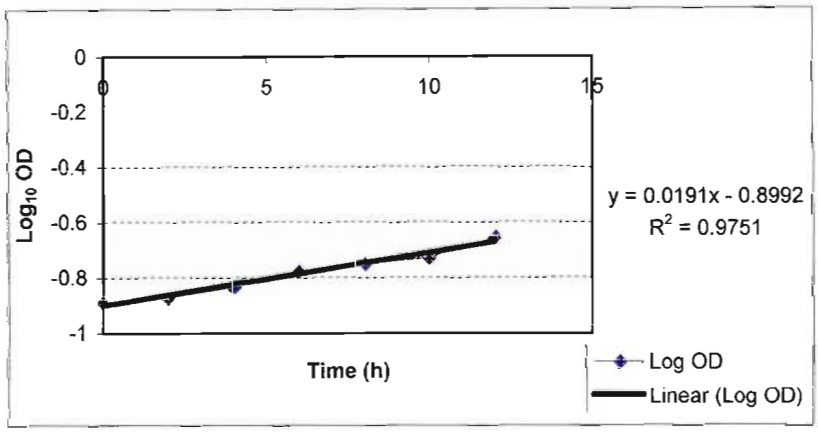


Fig. 3.11: Linear representation of exponential growth of microbial association Am-3.

Heterotrophic Growth

The ability of facultatively chemolithotrophic bacteria to grow autotrophically on reduced sulphur compounds as well as heterotrophically on organic compounds is well known (Rittenberg, 1969). To obtain more insight of such metabolic versatility, a wide variety of organic compounds were screened as possible sole carbon sources for heterotrophic growth using Biolog microtitre plates. Samples of stock cultures were subjected to the procedure described in 2.8.5. The sole exception was that one reading only was taken after 48 h of incubation. The criterion used to judge catabolism of a particular carbon source was the corrected OD value (i.e. substrate OD - control well OD). Thus, a positive OD was indicative of microbial catabolism. The effect of the

addition of glucose (1 000 ppm)(2.6.3) to the autotrophic medium (2.2) on product formation was investigated for microbial association Lf-1.

(a) Microbial Association Lf-1

With the exceptions of α-cyclodextrin, lactulose, D-meliobiose, D-raffinose, acetic acid, α-ketovaleric acid, glucuronamide, glycyl-L-aspartic acid, thymidine, 2-amino ethanol and 2, 3-butanediol association Lf-1 catabolized the remaining 84 Biolog GN substrates. This indicated that components of microbial association Lf-1 were facultative chemolithotrophic. Characteristically, facultative chemolithotrophs are members of the genera *Thiobacillus*, *Sulfolobus*, *Thermothrix* and *Paracoccus* (Kuenen and Beudeker, 1982).

When glucose (1 000 ppm) was supplied to the autotrophic medium (2.2), the association grew heterotrophically with accelerated growth (OD_{540}, SO_4^{2-})(Fig. 3.12). The growth rate (k), μ and t_d were calculated as 0.2 h^{-1}, 0.29 h^{-1} and 3.47 h, respectively. This acceleration in growth by the addition of an organic molecule has been reported previously (Rittenberg, 1969; Mason and Kelly, 1988). The sulphate concentration (ppm) at 80 h was approximately 10-fold lower, with no substantial decrease in pH (Fig. 3.13) compared with the equivalent autotrophic culture. Interestingly, the pH of the medium increased between 36 and 48 h (Fig. 3.13), a phenomenon also reported by Das *et al.* (1996) for *Bosea thiooxidans* cultured on sodium succinate-supplemented medium. The results suggested that thiosulphate-oxidizing activity was repressed in the presence of excess organic carbon. A review by Matin (1978) of batch studies of facultative chemolithotrophs gave many examples of the repression of both autotrophic carbon assimilation and energy generation during oxidation of sulphur compounds in the presence of organic substrates. Similar observations were made by Pronk *et al.* (1990) where three- to eight-fold lower thiosulphate oxidation rates were observed in the presence of an organic carbon source (glucose, fructose or L-malate). The degree of repression was reported by Gottschal and Kuenen (1980) to be dependent on the organic carbon:thiosulphate ratio in the growth medium. It has been postulated by Chung *et al.* (1997) that under mixotrophic conditions the cells obtain energy from both reduced sulphur compounds and organic molecules (e.g. glucose oxidation). This would explain the low sulphate concentration which resulted during mixotrophic growth compared to the autotrophic control. In the autotrophic culture, the predominant oxidized product was sulphate.

The inorganic substrate (thiosulphate) was the only energy source available. Therefore, for the association to survive, complete oxidation was necessary as indicated by the high conversion to the end product, sulphate. This inferred that active oxidation was necessary to obtain energy. The 10-fold reduction in thiosulphate oxidation in the presence of glucose could have severe repercussions in the treatment of an off-gas which contains an organic constituent. Therefore, the maintenance of an autotrophic environment within the biofilter may be essential.

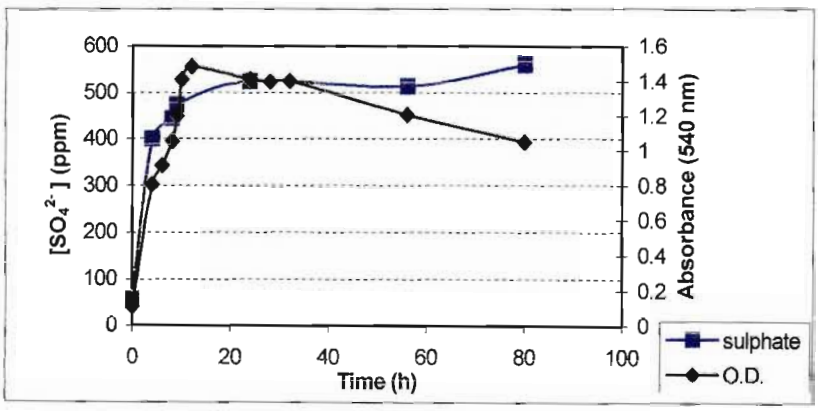


Fig. 3.12: Changes in A_{540} (◆) and sulphate concentration (■) during batch cultivation of microbial association Lf-1 in the presence of autotrophic growth medium supplemented with 1 000 ppm glucose.

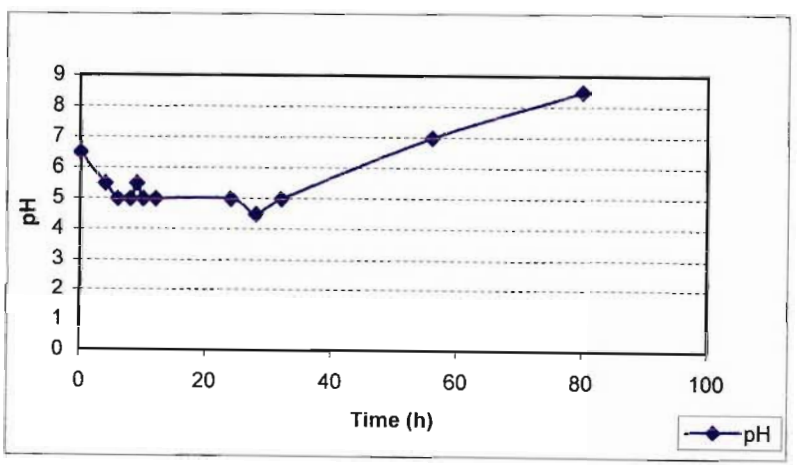


Fig. 3.13: Changes in pH (◆) during batch cultivation of microbial association Lf-1 in the presence of autotrophic growth medium supplemented with 1 000 ppm glucose.

It has been reported that facultative autotrophs which utilize a simple sugar such as glucose as the sole source of energy can lose permanently their ability to grow autotrophically (Tabita and Lundgren 1971; Shafia, Brinson, Heinzman and Brady,

1972). Since this 'transition' from autotrophy to heterotrophy has raised many arguments and counter-claims (Tabita and Lundgren 1971), it was decided to investigate this phenomenon. Samples from the glucose-supplemented batch cultures were used to inoculate autotrophic medium (2.2). Growth was monitored by OD and pH for 300 h. Comparable growth curves to the original autotrophic batch cultures (Fig. 3.1) were recorded although the lag phases were extended to 30 h.

(b) Microbial Association Ws-2

With the exceptions of α-cyclodextrin, glycogen, α-D-lactose, methyl pyruvate, meliobiose, D-raffinose, α-hydroxybutyric acid, α-ketobutyric acid, α-ketovaleric acid and butanediol, association Ws-2 catabolized the remaining 85 Biolog GN substrates.

(c) Microbial Association Am-3

With the exceptions of α-D-lactose, acetic acid, formic acid, α-ketobutyric acid, α-ketovaleric acid, glycyl-L-aspartic acid and 2, 3-butanediol, association Am-3 catabolized the remaining 88 Biolog GN substrates.

Toxicity of organic acids, such as α-ketobutyric acid and α-ketovaleric acid, to acidophilic bacteria is a well-known phenomenon (Pronk *et al.*, 1990). Such effects have been explained by acid accumulation in the cytoplasm which effects a decrease in the internal pH (Alexander, Leach and Ingledew, 1987). Interestingly, pyruvate which is salt of an organic acid only inhibited microbial association Ws-2. Pyruvate has been reported to inhibit growth and respiration of the obligate autotrophs *T. ferrooxidans* and *T. thiooxidans* (Rao and Berger, 1970).

Sulphide Inhibition

For practical reasons, in the past most researchers have used dissolved elemental sulphur, thiosulphate or tetrathionate as a substitute for sulphide due to its poor chemical stability and its toxicity to microorganisms (Janssen *et al.*, 1995). Threshold sulphide toxicity determinations (2.6.4) were made in sulphide thiosulphate maintenance medium (2.2). The effect of increasing sulphide concentrations on the growth of microbial association Lf-1 are shown in Fig. 3.14 (a-f). The higher the sulphide concentration, the greater was the apparent inhibition recorded while total

inhibition resulted at concentrations > 40 ppm. These results indicated that if sulphide concentrations > 40 ppm were present in an off-gas then inhibition of growth of microbial association Lf-1 would probably occur. In the absence of oxidation, sulphide would accumulate in the biofilter thus exacerbating its toxicity. According to Buisman *et al.* (1991) sulphide inhibited thiobacilli in the range of 5 to 30 ppm while Janssen *et al.* (1995) reported that concentrations < 5 ppm were inhibitory. Visser *et al.* (1997) also reported that soluble sulphide is toxic to thiobacilli and quoted a toxicity threshold of 10 to 64 ppm. For the microbial association used in the present study inhibition by sulphide occurred when the concentration reached 40 ppm which indicated that the association had similar susceptibility as monocultures.

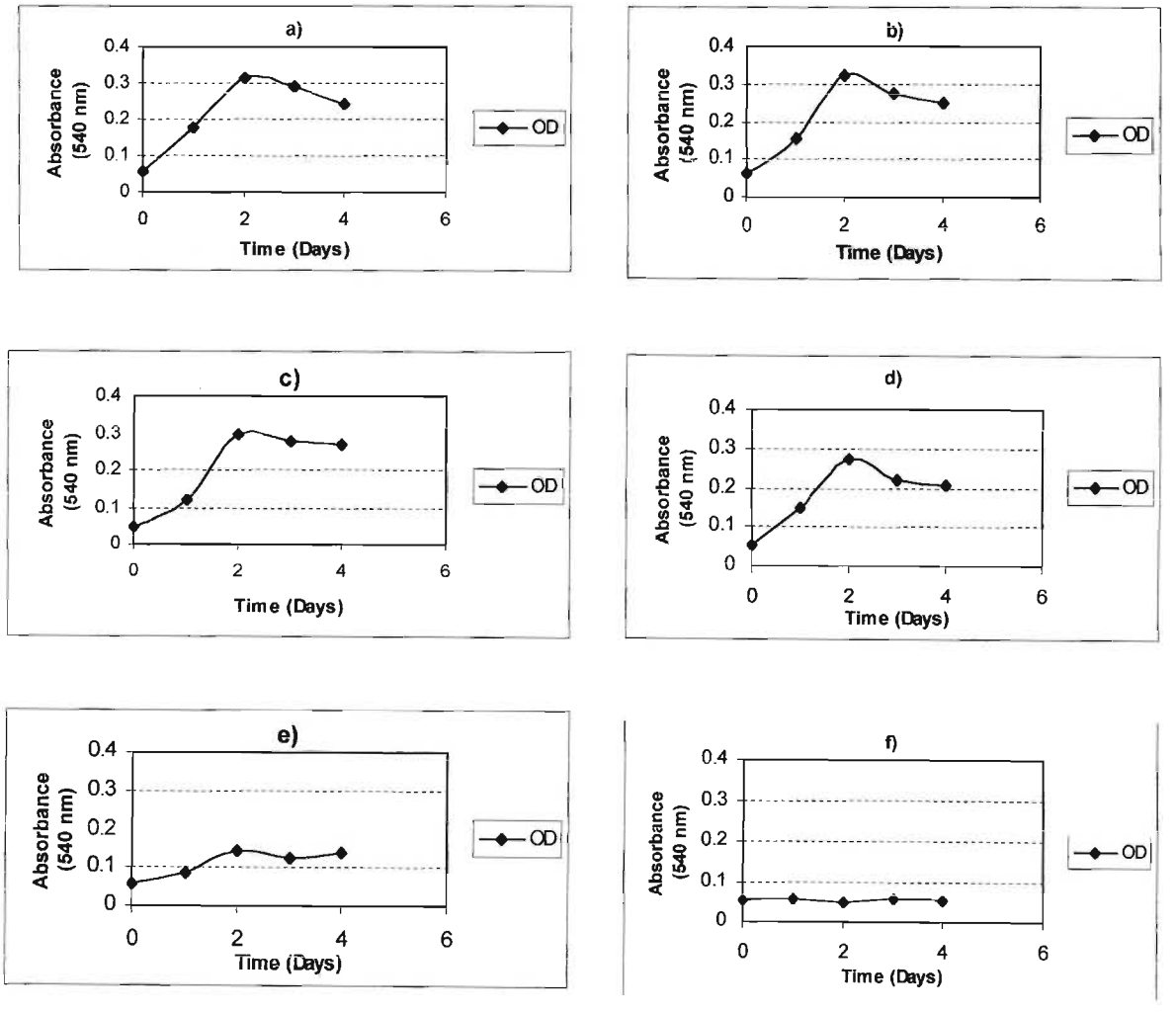


Fig. 3.14: Changes in optical density during batch cultivation of microbial association Lf-1 in the presence of autotrophic medium supplemented with 0 (a), 10 (b), 20 (c), 30 (d), 35 (e) and 40 (f) ppm sulphide.

Substrate Concentration

The effect of substrate concentration on the growth of microbial association Lf-1 was determined (2.6.2) and population size increases were observed with increased concentrations of thiosulphate (≤ 5 000 ppm) (Fig. 3.15). The culture pH appeared to be linked directly to the biomass produced (Fig. 3.16). The ability of association Lf-1 to catabolize different concentrations of thiosulphate has important implications in gas biofiltration since the influent gas is often variable in concentration. Comparable results with respect to OD and pH were obtained with microbial associations Ws-2 and Am-3.

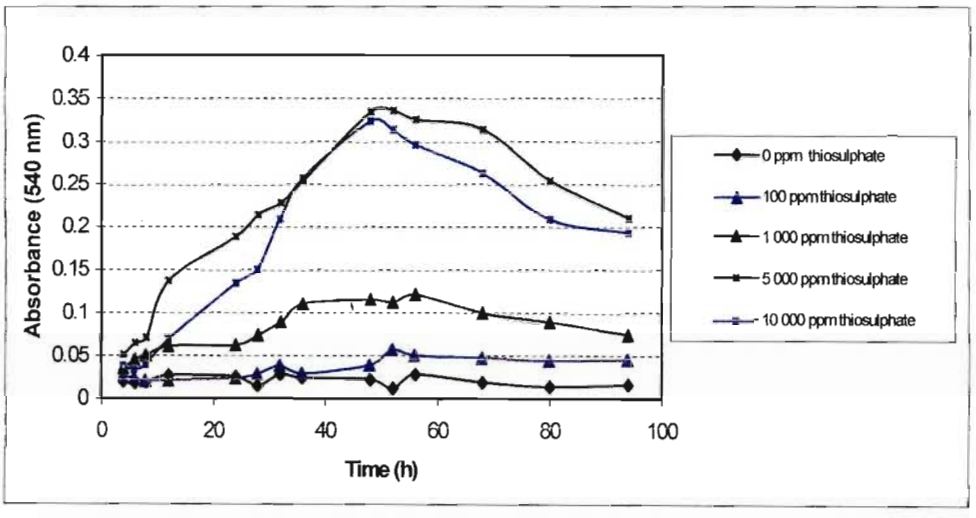


Fig. 3.15: Changes in optical density during batch cultivation (30^0C, 150 rpm) of microbial association Lf-1 in the presence of 0(◆), 100 (▲), 1 000 (▲), 5 000 (■) and 10 000 (■) ppm thiosulphate.

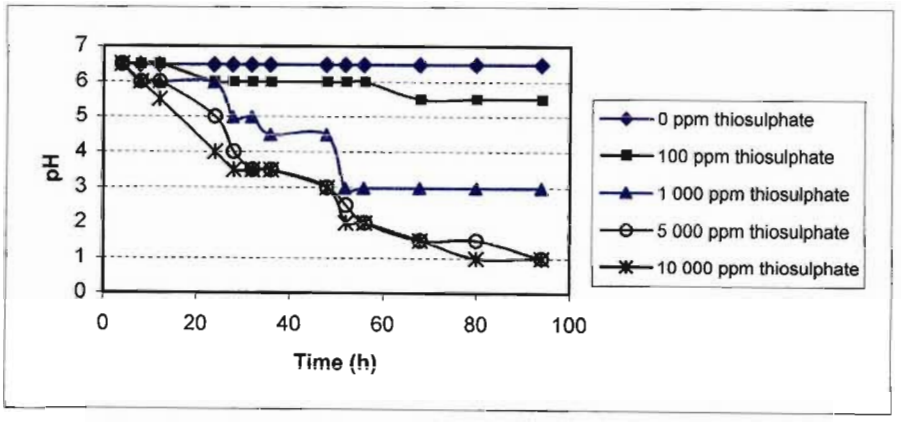


Fig 3.16: Changes in pH during batch cultivation (30^0C, 150 rpm) of microbial association Lf-1 in the presence of 0 (◆), 100 (■), 1 000 (▲), 5 000 (o) and 10 000 (*) ppm thiosulphate.

Temperature

A temperature profile predicts the effects of changes on the growth rate. This is particularly important with an inhibitory substrate (H_2S) where a decrease in growth rate could result in accumulation to toxic concentrations. The temperature profiles and thermal death points of the three microbial associations were determined (2.6.5 and 2.6.6).

(a) Microbial Association Lf-1

A temperature profile for the association subjected to autotrophic cultivation is shown in Fig. 3.17. The three key temperatures were an optimum of 25^{0}C, a minimum of -5^{0}C and a maximum between 35 and 40^{0}C. The growth rate decreased rapidly once the optimum temperature was exceeded. Numerous studies have established that the maximum temperature for growth is fixed by the stability of the primary structure of proteins (Ingraham *et al.*, 1983). Figure 3.17 shows that association Lf-1 was more susceptible to higher than lower temperatures. The ability of the association to grow at low temperatures could be advantageous in a biofilter subjected to extreme cold. A similar temperature profile was reported by Cho *et al.* (1991) for *Thiobacillus* sp. HA43 which was isolated from an H_2S-acclimitated peat biofilter. The thermal death point (2.6.6) of the association was found to be 65^{0}C.

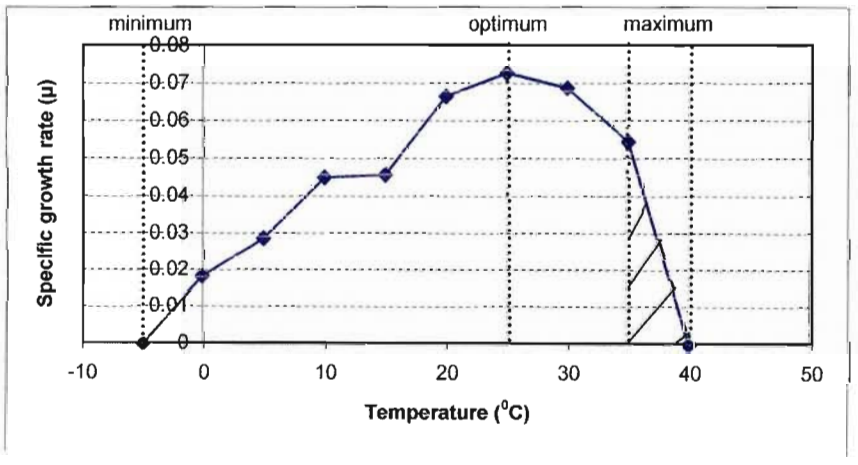


Fig. 3.17: Changes in specific growth rate of microbial association Lf-1 in response to different temperatures.

(b) Microbial Association Ws-2

A temperature profile for the association is shown in Fig. 3.18. The three key temperatures were an optimum of 40^0C, a minimum of 10^0C and a maximum between 50 and 55^0C. The thermal death point (2.6.6) of the association was found to be 80^0C.

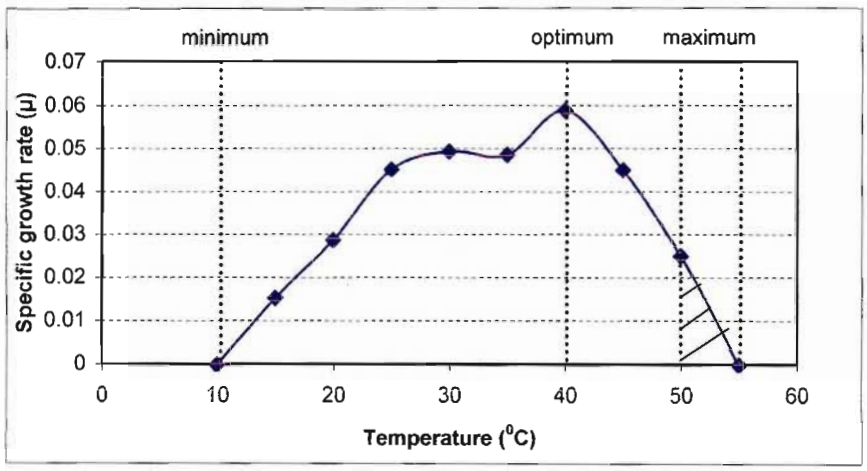


Fig. 3.18: Changes in specific growth rate of microbial association Ws-2 in response to different temperatures.

(c) Microbial Association Am-3

A temperature profile for the association is shown in Fig. 3.19. The three key temperatures were an optimum of 35^0C, a minimum of 10^0C and maximum between 45 and 50^0C. The thermal death point was found to be 75^0C.

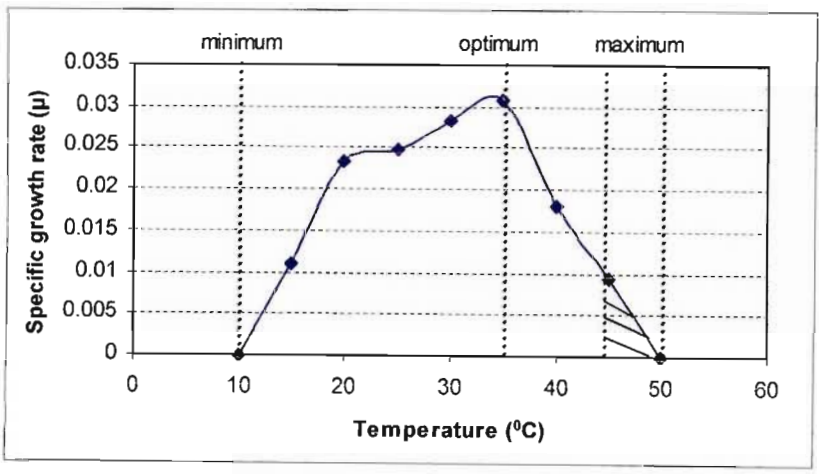


Fig. 3.19: Changes in specific growth rate of microbial association Am-3 in response to different temperatures.

pH

It was found that sulphate production overwhelmed the buffering capacity of the autotrophic and heterotrophic media as well as various buffers (sodium phosphate and acetate) (2.6.6). Also, with acetate buffer-supplemented autotrophic medium, catabolism of the carbon component resulted. As a consequence, a pH profile with respect to the maximum specific growth rate was determined for microbial association Lf-1 in pH-controlled continuous culture (3.2.5a).

Establishing a Relationship Between Sulphate Production and NaOH Consumption

Replicated batch experiments (2.6.8) demonstrated a direct relationship between OH^- equivalents consumed and sulphate produced as a result of the biological oxidation of thiosulphate. Sublette and Sylvester (1987c) and Cadenhead and Sublette (1989) reported similar observations. The volumes of base consumed and the sulphate produced during the same time intervals were used to construct a standard curve (Fig. 3.20). This curve was derived as an indirect means (base consumed) to measure sulphate formation as a result of thiosulphate oxidation in continuous culture (3.2). By monitoring the consumption of base which was required to neutralize the acid produced an inexpensive method of monitoring sulphate production resulted. The equivalent cost of a single sulphate concentration determination by HPLC was ± R33.

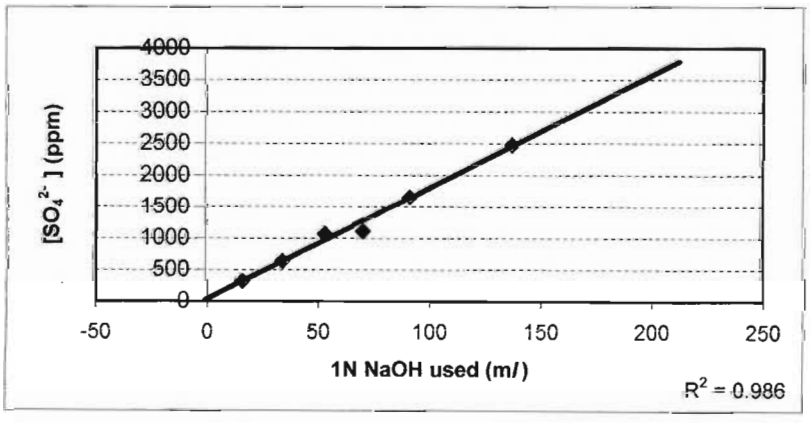


Fig. 3.20: Standard curve of sulphate production versus NaOH consumption for microbial association Lf-1 during batch cultivation at 30^0C and pH 6.

3. 2 Continuous Cultivation of Microbial Associations

"The physiological state of a population is dependent on the route which led to the present state, the history of individual cells and the history of the culture as a whole. Such information can be obtained by studying the dynamic responses of continuous culture to short-term perturbations or to step-wise changes" (Novak, 1987).

3.2.1 Characterization of Microbial Associations

Based on the descriptions in Bergey's manual (Hart, Krieg, Sneath, Staley and Williams, 1994), the component species of microbial associations Lf-1 and Ws-2 were classified as members of the genus *Thiobacillus.*. This characterization was based on morphology, Gram character, motility, temperature and pH optimum.

Association Lf-1

The association consisted of Gram-negative and Gram-positive, non-sporing, rod-shaped cells, of which the Gram-negative species were the more dominant numerically (Plate 3.1). This dominance was expected since it is the major factor which links the genera of the "colourless sulphur bacteria" (Hart *et al.*, 1994). The presence of the Gram-positive bacteria may have been supported by the putative products of the chemolithotrophs (Hart *et al.*, 1994). The dimensions of the predominant single cells varied from 0.4 to 0.6μm in width and 1.3 to 1.6μm in length (Plate 3.2 **A**). The hanging-drop test (2.8.2) showed that some members of the association were motile and this was confirmed by TEM (2.8.4) visualization of single polar flagella (Plate 3.2 **B**). Non-flagellated cells were also present. Components of microbial association Lf-1 isolated under continuous cultivation grew under anaerobic conditions (2.8.3).

With time (8 months continuous culture, $D = 0.02\ h^{-1}$), morphological changes were apparent and the distribution of short (≈ 0.5 to 1.5) and long rods (≈ 0.63 x 7.6μm) plus cocci (Plate 3.2 **C**) was replaced by rods only.

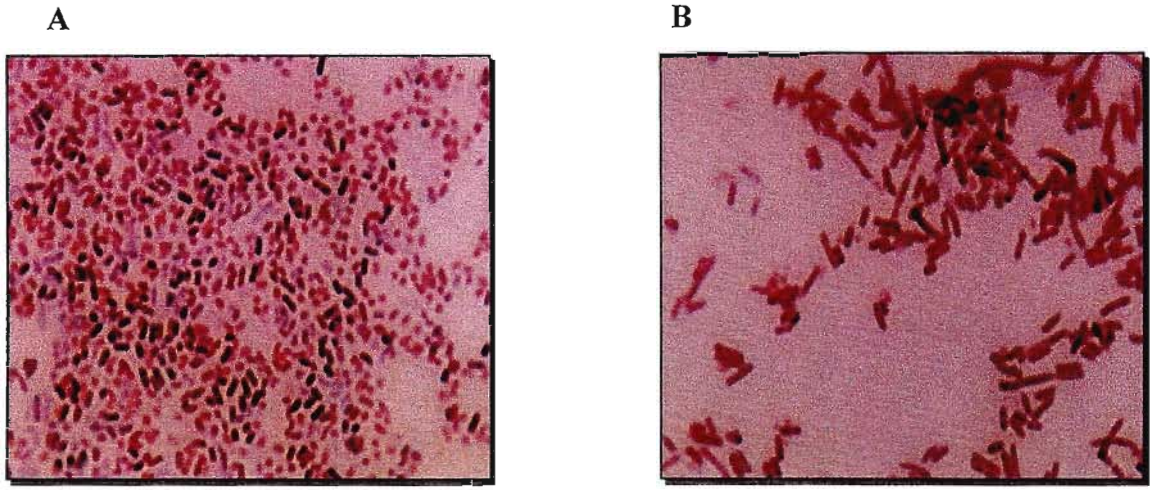
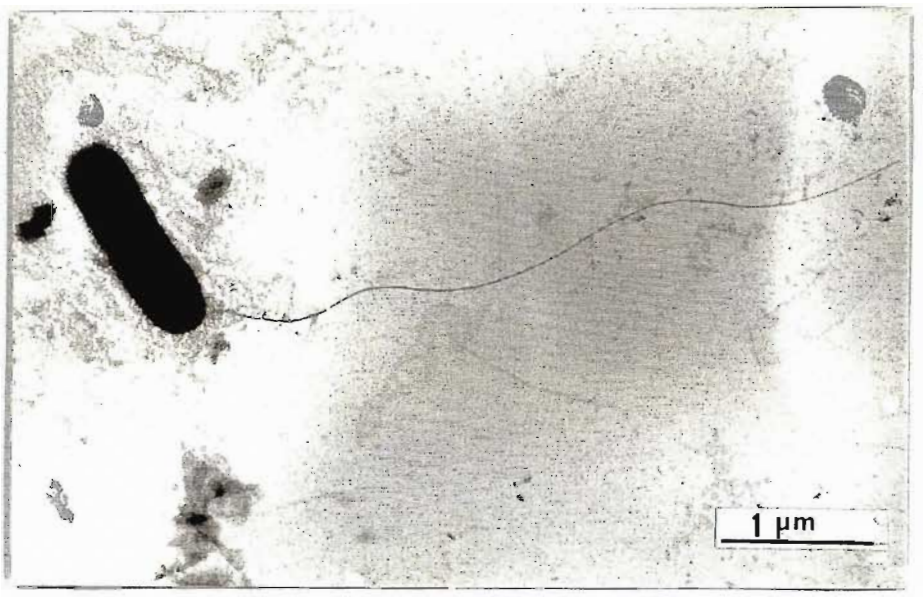


Plate 3.1: Gram-stained microbial association Lf-1 (**A**) and *Escherichia coli* control (**B**) viewed under oil immersion at 1 000 x magnification.

Plate 3.2: Transmission electron micrograph of microbial association Lf-1 stained negatively showing non-flagellated, rod-shaped cells with rounded ends (**A**).

B



C

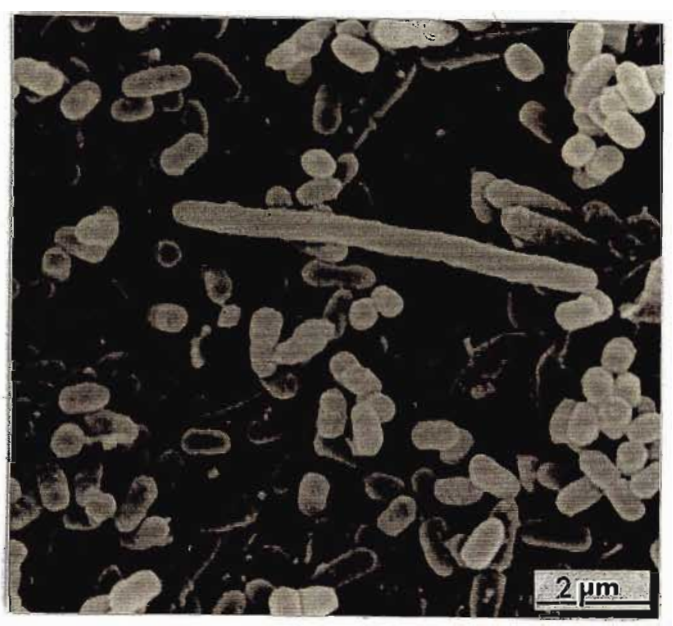


Plate 3.2: Transmission electron micrograph of microbial association Lf-1 stained negatively showing a single, polar flagellated, rod-shaped cell (**B**). Scanning electron micrograph of the initial isolated microbial association showing long and short rods plus cocci (**C**).

Association Ws-2

Association Ws-2 consisted of Gram-negative (Plate 3.3 **A**), non-sporing, motile (single apical polar flagellum)(Plate 3.4 **A**) and non-flagellated (Plate 3.4 **B**) rods. The cell dimensions varied from 0.3 to 0.7μm in width and 0.6 to 2.8μm in length. The association was capable of growth under anaerobic and aerobic conditions. An interesting phenomenon was the aggregation of cells (Plate 3.4 **C**) which resulted at pH values < 2 (30^0C, 0.015 h^{-1}). This may be attributed to a protective mechanism against extreme conditions (pH < 2) (Atlas *et al.*,1999).

A **B**

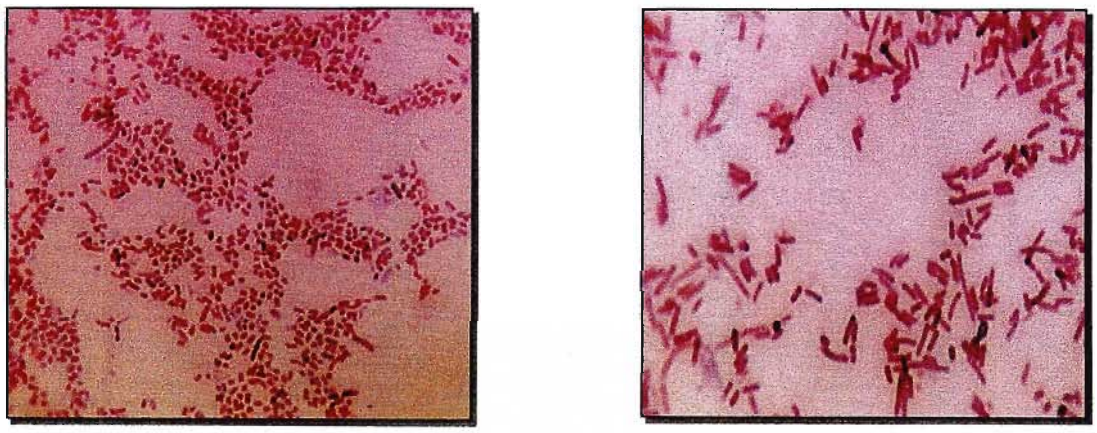


Plate 3.3 : Gram-stained microbial association Ws-2 (**A**) and *Escherichia coli* control (**B**) viewed under oil immersion at 1 000 x magnification.

A

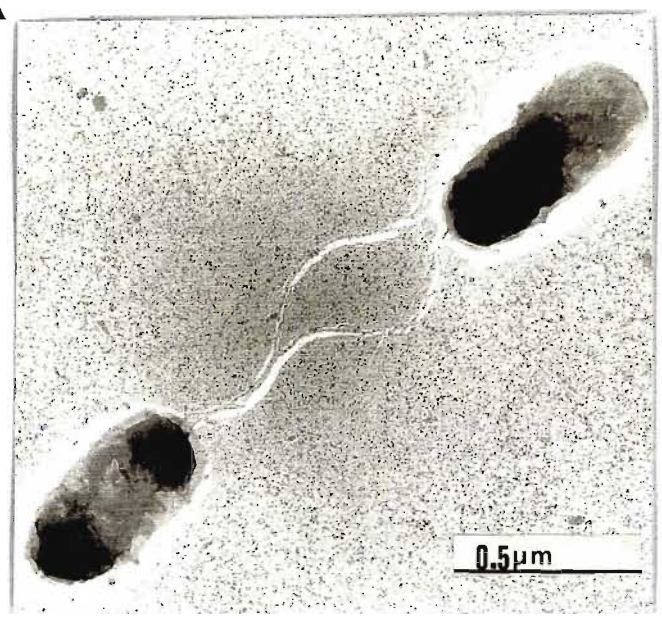


Plate 3.4: Transmission electron micrograph of negatively-stained cells of microbial association Ws-2 subjected to continuous cultivation (30^0C, 0.015 h^{-1}, pH 1.7) showing single apical polar flagellated rods (**A**).

B

C

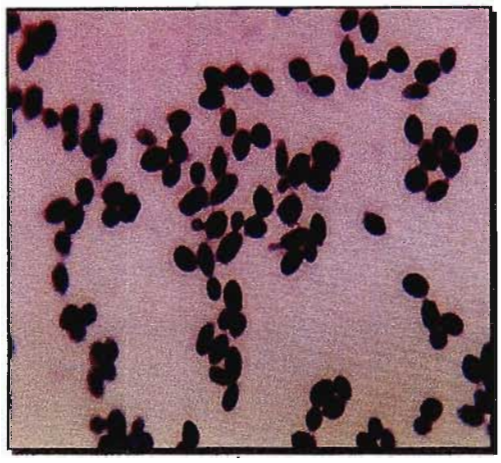
Plate 3.4: Transmission electron micrograph of negatively-stained cells of microbial association Ws-2 subjected to continuous cultivation (30^0C, 0.015 h^{-1}, pH 1.7) showing nonflagellated rods (**B**). Scanning electron micrograph (**C**) of aggregated cells of the same association subjected to continuous cultivation (30^0C, 0.015 h^{-1}, pH 1.5).

Association Am-3

The association was dominated, initially, by a yeast (Plate 3.5 **A**) which, subsequently, was displaced by another fungal species plus Gram-negative bacterial rods. The elimination of the yeast could, possibly, be accounted for by a low maximum specific growth rate. The fungal component (Plate 3.5 **B**) was dominant at dilution rates < 0.02 h^{-1} and when cultured (30^0C, 48 h) on solid medium (2.2) was identified tentatively as *Trichoderma harzianum* (M.D. Lang, personal communication) based on reproductive ornamentation and hyphal branching. Fruiting bodies (Plate 3.6 **A**) and spores (Plate 3.6 **B**) of the fungal species were examined by SEM (2.8.4). Fungal species are capable of oxidizing both elemental sulphur and thiosulphate to sulphate (Wainwright, 1978). The most commonly reported sulphur-oxidizing fungi are *Alternaria tenuis*, *Aureobasidium pullulans*, *Cephalosporium* sp., *Epicoccum nigrum* and a range of *Penicillium* species (Wainwright, 1978). When sampled at a dilution rate of 0.048 h^{-1}, following continuous cultivation at 30^0C and pH values > 3, and, subsequently, inoculated onto agar-set medium, the primary fungal species was interspersed with Gram-negative bacterial colonies following three days of incubation at 30^0C. The composition of the association was comparable to other reports in the literature (Tuovinen, Kelley and Groudev, 1991).

Of interest was the development of a whitish/yellowish mucilaginous oily substance in the effluent reservoir. The only previously cited reference to such a phenomenum was a whitish mucilaginous mass (bare'gene) found in the waters of Bare'ges, France (Hedoin, Coute, Kaiser and Laugier, 1996). It was reported that a thiobacterium-like microorganism, which used reduced sulphur compounds as a source of energy, produced this substance (Hedoin *et al.*, 1996). Filamentous species of *Thiothrix* were associated often with the production of the major components of bare'gene. The mucilaginous mass, which was viewed with environmental scanning electron microscopy, contained chains of rod-shaped cells resting on an unknown substance (Plate 3.7). Montaner, studied the mucilaginous substance of Bare'ges (France) and also reported the presence of rod-shaped bacteria (Hedoin *et al.*, 1996). Electron beam X-ray microanalysis (2.8.4) of the mucilaginous mass showed that it contained elevated concentrations of sulphur atoms (Plate 3.8). The development of such a mucilaginous mass within a biofilter would result in increased backpressure which would impact negatively on the performance. Fortunately, the literature suggests that such masses only develop in liquid medium (Heroin *et al.*, 1996).

A



B

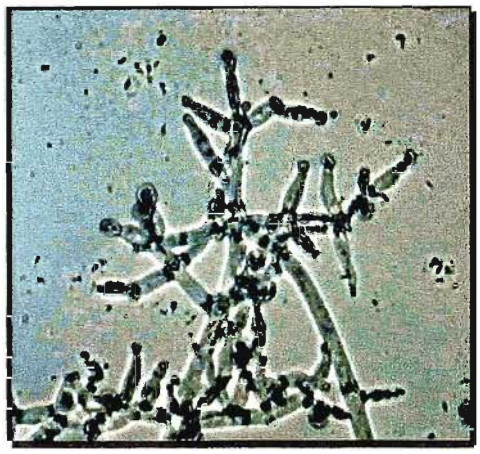


Plate 3.5: Gram-stained microbial association Am-3 viewed under oil immersion at 1 000 x magnification (**A**) and a wet mount of the same association after cultivation on solid medium (2.2)(**B**).

A

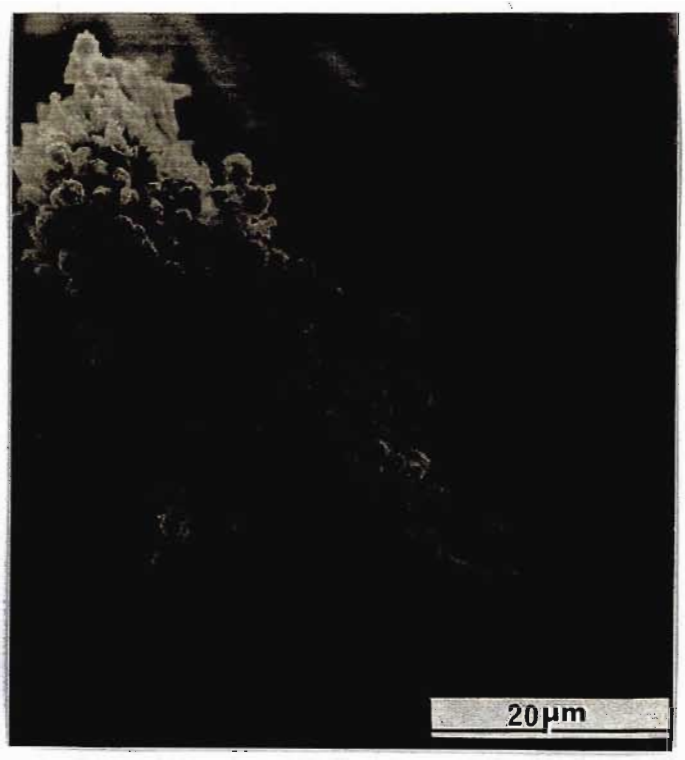


Plate 3.6: Scanning electron micrograph of fruiting bodies (**A**) of a fungal species, cultivated (30^0C, 48 h) on solid medium (2.2), isolated from the continuously-cultured (30^0C, < 0.02 h^{-1}) microbial association Am-3.

B

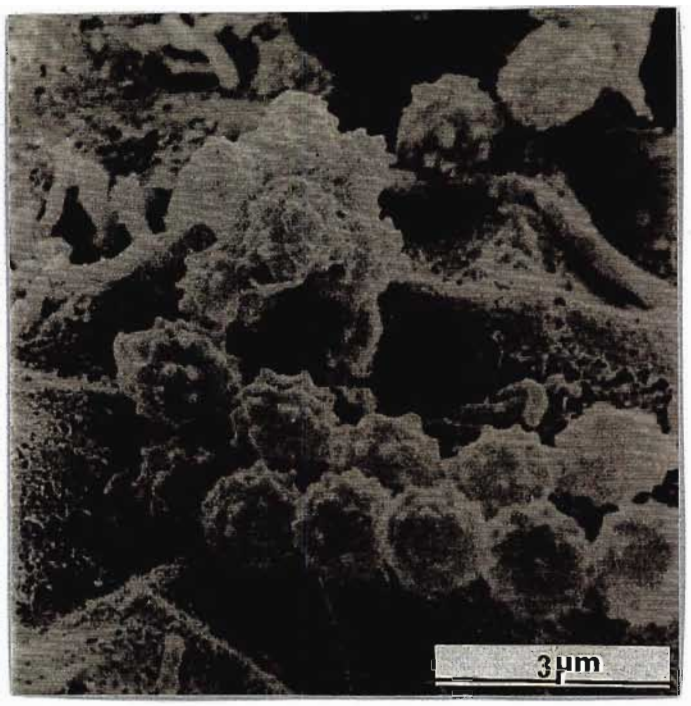


Plate 3.6: Scanning electron micrograph of spores (**B**) of a fungal species, cultivated (30^0C, 48 h) on solid medium (2.2), isolated from the continuously-cultured (30^0C, < 0.02 h^{-1}) microbial association Am-3.

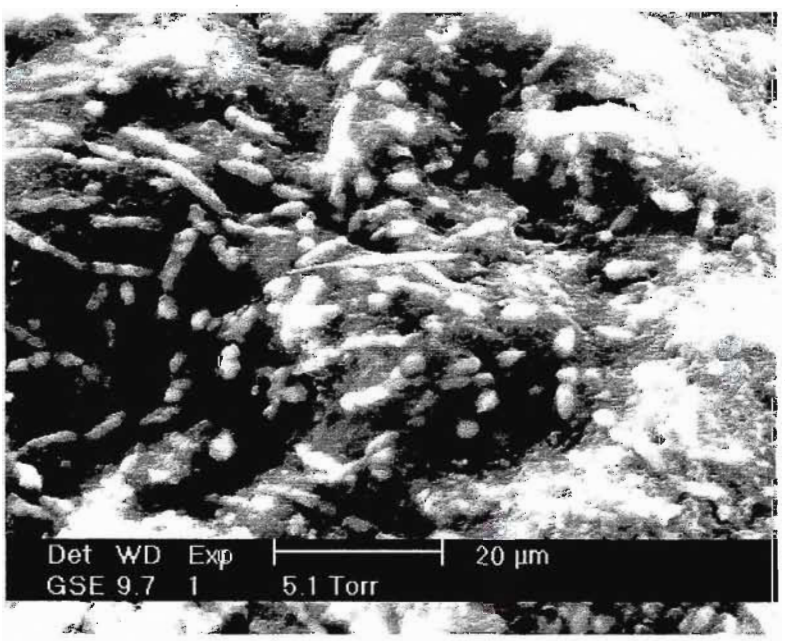


Plate 3.7: Environmental scanning electron micrograph of mucilaginous material which developed in the effluent reservoir of continuously-cultured microbial association Am-3.

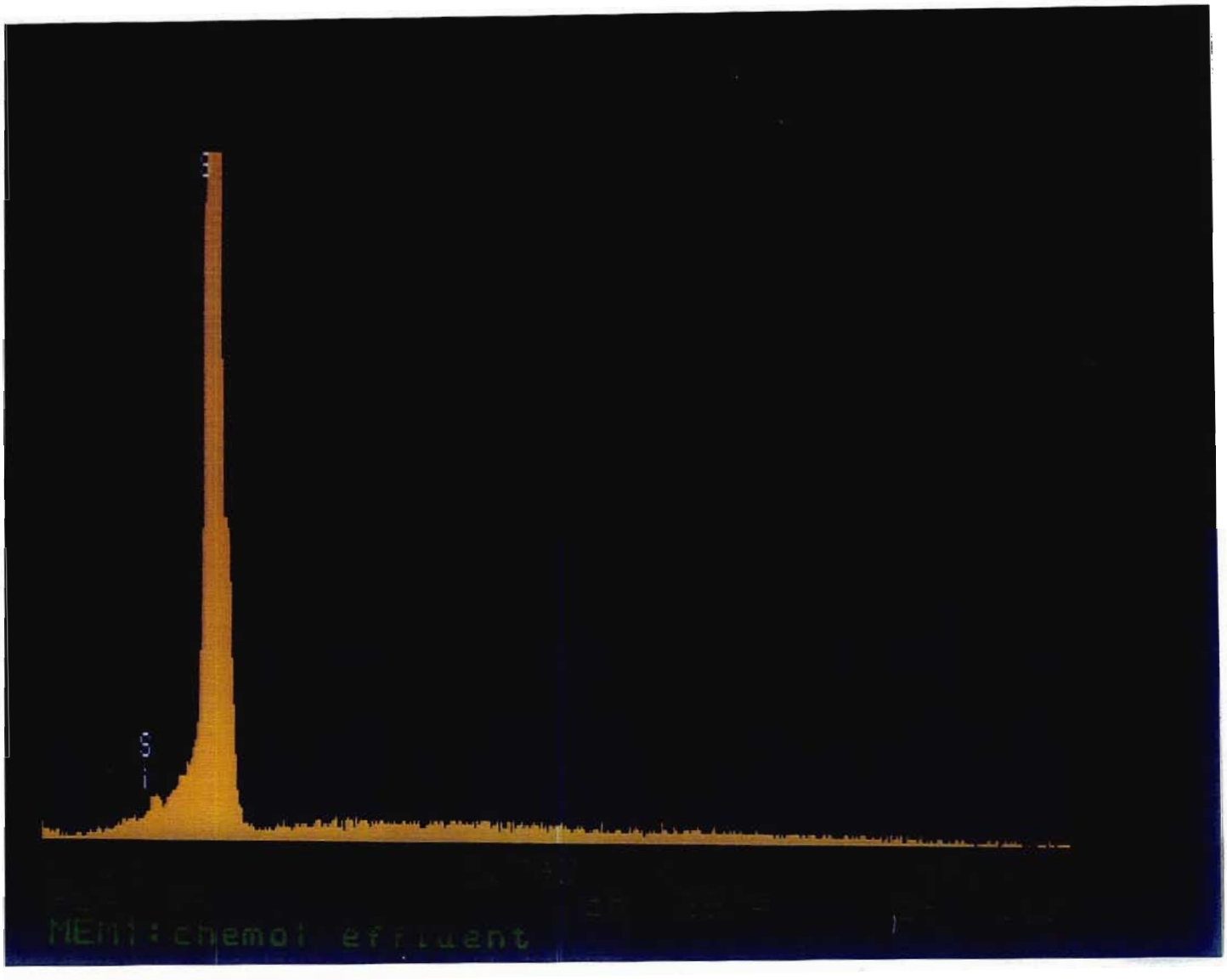


Plate 3.8: Electron beam X-ray microanalysis (2.8.4) of the mucilaginous mass which developed in the effluent reservoir of continuously-cultured microbial association Am-3.

3.2.2 The Effect of Step-Wise Increases in Dilution Rate

Association Lf-1

Association Lf-1 was cultivated (25^0C and pH 6) in the autotrophic thiosulphate medium (2.2) at an initial dilution rate of 0.01 h^{-1}. After steady-stable state conditions were observed (2.7.3), the dilution rate was increased incrementally until washout occurred at 0.12 h^{-1}. The influent thiosulphate concentration was maintained at 10 $g\ell^{-1}$ throughout. An atypical curve (Fig. 3.21) was observed at washout as described by Pirt (1975). Such deviations may originate from the interactions of the cells with the equipment and/or the reactor hydrodynamics (Atlas *et al.*, 1999). It was possible in this case that the atypical curve was due to the accretion of biomass at the liquid/gas interface (Plate 3.9) and biomass growth on the baffles and probes (Plate 3.10 **A** and **B**). This resulted in heterogeneous conditions within the bioreactor. Any adhering cells represented potential for continuous/continual inoculation of the medium thus facilitating growth in the bioreactor at dilution rates > μ_{max} (Topiwala and Hamer, 1971).

Dominance of different members of the association at different dilution rates was to be expected due to the changed selection pressures (Novick and Szilard, 1950; Chiu, Fan, Kao and Erickson, 1972). This was confirmed by genetic analysis (4.3.2).

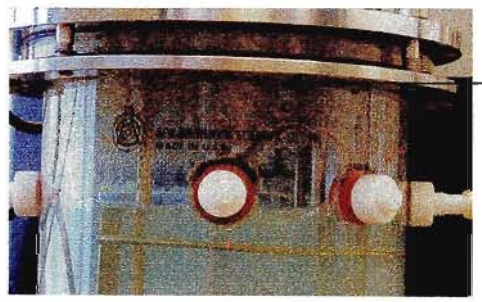
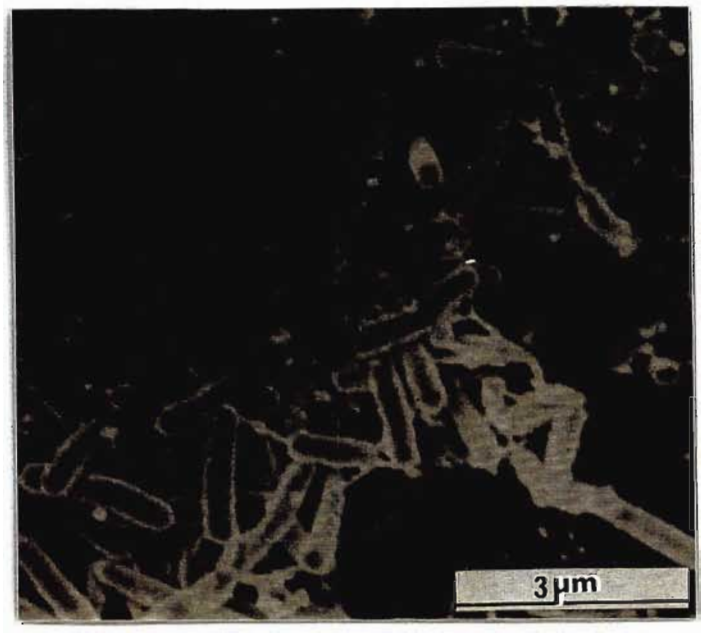


Plate 3.9: Digital photograph of biomass accretion above the liquid/gas interface of the Bioflo III reactor.

A



B

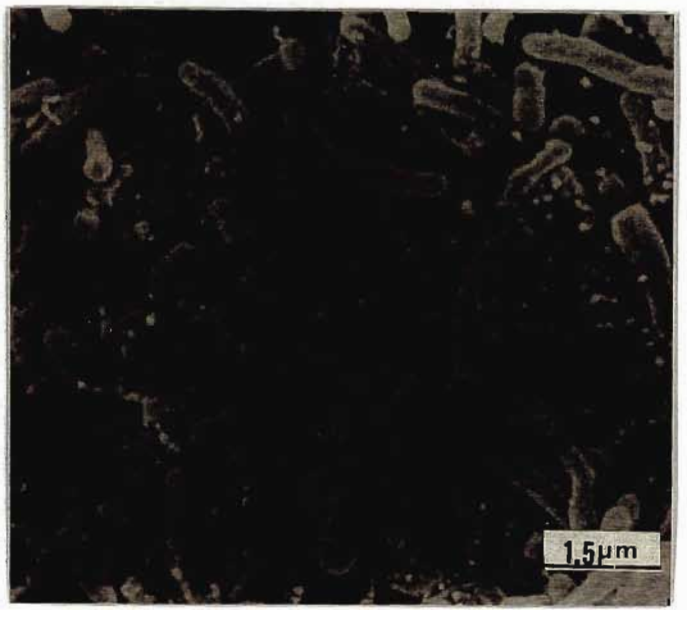


Plate 3.10: Scanning electron micrographs of microbial association Lf-1 biofilm which developed on the baffles of the Bioflo III reactor during 2 (**A**) and 8 (**B**) months continuous cultivation (25^0C, pH 6, autotrophic growth) with incremental dilution rate increases from 0.01 to 0.12 h^{-1}.

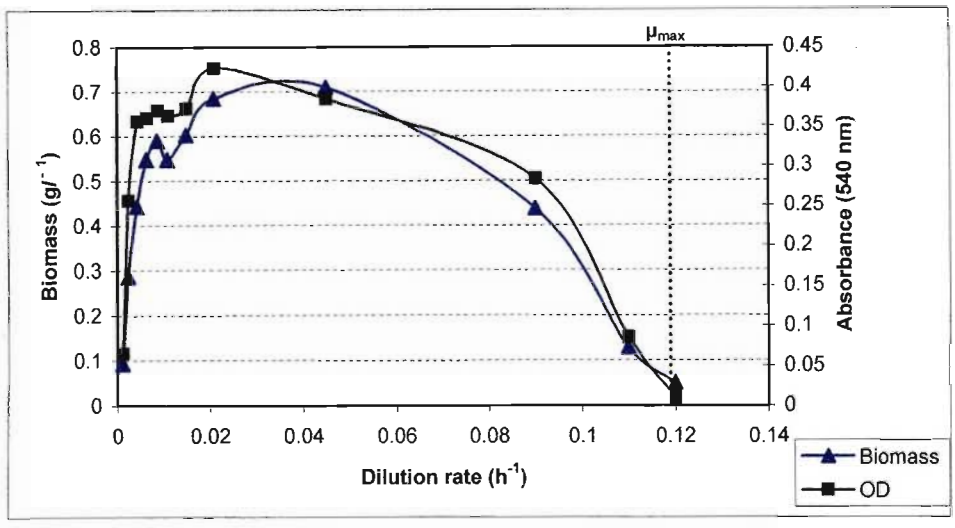


Fig. 3.21: Stable-state optical densities (■) and biomass concentrations (▲) of microbial association Lf-1 subjected to continuous cultivation (pH 6, 25^{0}C) on autotrophic growth medium.

Association Ws-2

Association Ws-2 was also subjected to regular step-wise increases in dilution rate in the range of 0.01 to 0.03 h^{-1}. Figures 3.22 and 3.23 show the changes in steady-state absorbance and biomass, and pH, respectively. A dilution rate > 0.01 h^{-1} was marked initially by a decline in pH to ±1.8. Thereafter, as the dilution rate exceeded 0.02 h^{-1}, the pH increased progressively to 5 at washout. The pH change of a culture is a good indication of cell growth and metabolic activity (Atlas, 1999). In general, the absorbance and biomass both increased with increased dilution rates although slight declines in both parameters were apparent when the pH fell below 1.5. Thereafter, continued upward trends were observed with increased dilution rate. The second increases could have be in response to pH changes and indicative of selective enrichment of species which could tolerate the low culture pH values.

The absence of marked pH changes at dilution rates < 0.02 h^{-1} suggested more rapid stabilization than at higher dilution rates where significant changes in the pH values were observed. Marked rises in culture pH preceded the maximum specific growth rate.

Washout of the culture, as determined by culture absorbance (2.3.1) and biomass dry weight (2.3.2), occurred at a dilution rate > 0.03 h^{-1} (Fig. 3.22). After washout, the dilution rate was decreased to 0.02 h^{-1} and a concomitant pH reduction to

1.5 resulted. Increases in biomass and culture absorbance were apparent also. This suggested the presence of wall growth as described by Pirt (1975). Wall growth represents an extreme case of segregation within the bioreactor and tends to be a description of both attachment to and growth on surfaces by microorganisms (Hamer, 1975). As the chemostat had a working volume of 155 m*l* only, and a high surface area, the wall growth probably represented a considerable fraction of the total population. With culture volumes < 500 m*l*, sampling can reduce this and so affect the dilution rate (Atlas *et al.*, 1999).

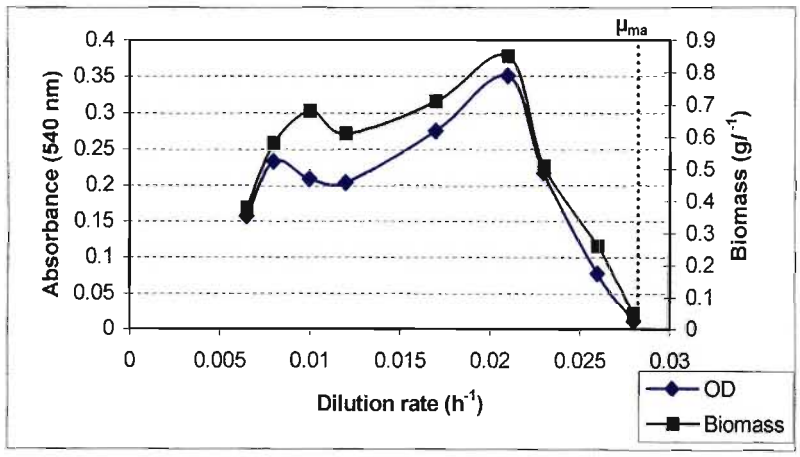


Fig. 3.22: Stable-state optical densities (◆) and biomass concentrations (■) of microbial association Ws-2 subjected to continuous cultivation (30^0C) on autotrophic growth medium.

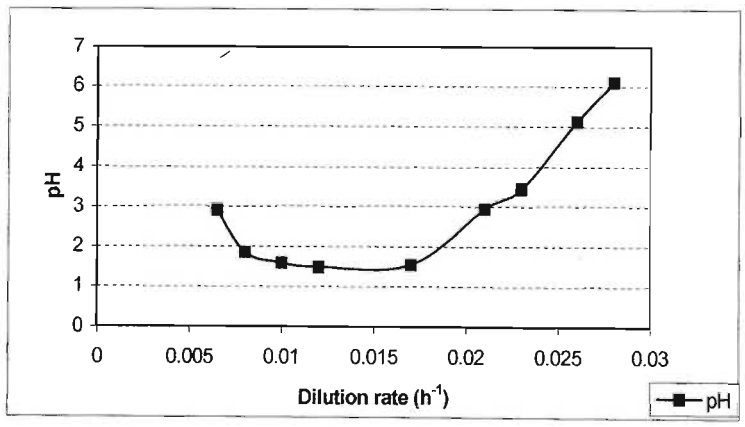


Fig. 3.23: Stable-state pH values of microbial association Ws-2 subjected to continuous cultivation (30^0C) on autotrophic growth medium.

Association Am-3

Association Am-3 was dominated by a fungal component to such an extent that genetic analysis revealed it to be the sole component at dilution rates < 0.02 h^{-1} (4.3.3). In most situations, fungal growth in continuous culture follows the same kinetics as bacteria (Prosser, 1982). Differences which may arise are due to growth form. For example, as a result of the filamentous growth, fungal wall growth presents much greater problems due to increased segregration and imperfect mixing compared with bacterial cultures. In addition, mycelium growth effects medium viscosity (Prosser, 1982). This has negative implications for a biofilter where excessive mycelium growth would cause plugging of the support material.

The association was grown in a open culture (30^0C) and subjected to incremental increases in dilution rate from 0.015 to 0.06 h^{-1}. Statistically, the biomass dry weight and culture absorbance (Fig. 3.24) correlated closely. Substantial wall growth (Plate 3.11 **A** and **B**) was visible and was probably the determining factor in the atypical behaviour observed at washout. At dilution rates < 0.02 h^{-1}, the fungal component was dispersed homogeneously throughout the medium whereas dilution rates close to washout (± 0.06 h^{-1}) were marked by an absence of the fungal component in suspension and attachment to the chemostat walls. The development of wall growth was more pronounced at pH values < 3. As discussed above the development of heavy wall growth probably invalidated the determination of μ_{max} by washout kinetics.

It was interesting to note that biomass dry weight and culture absorbance values were maximum at a dilution rate of 0.02 h^{-1}. Thereafter, steady declines were apparent until washout occurred (Fig. 3.24). This suggested that the dominant fungal component was capable of growth at the lower dilution rates only and that pH changes with increased D (Fig. 3.25), probably effected population changes within the microbial association. This was corroborated by the finding that at low dilution rates (< 0.02 h^{-1}) culture samples inoculated onto solid medium (2.2) were exclusively fungal while with higher dilution rates (> 0.02 h^{-1}) the fungal growth was interspersed with Gram-negative bacterial rods. The appearance of Gram-negative colonies possibly reflected changes in the selection determinants. One possiblity is that at the pH values < 3 the fungal component outgrew the bacteria (Cox *et al.*, 1993).

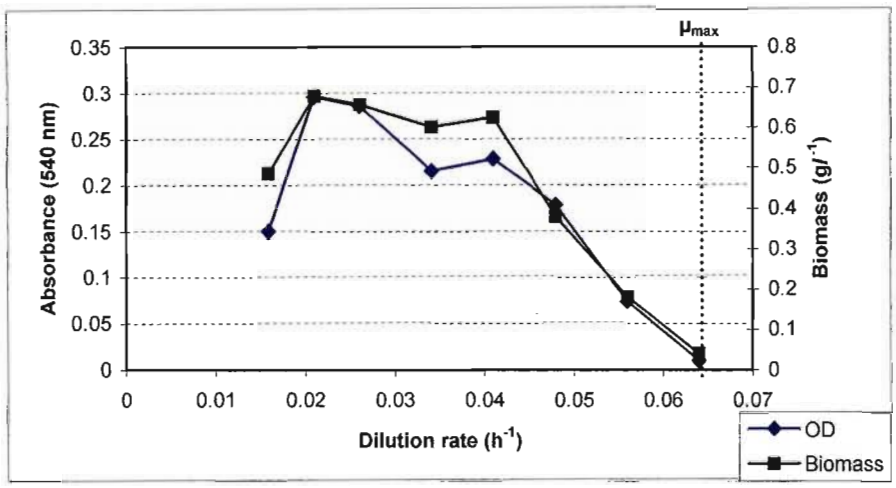


Fig. 3.24: Stable-state optical densities (◆) and biomass concentrations (■) of microbial association Am-3 subjected to continuous cultivation (30^0C) on autotrophic growth medium.

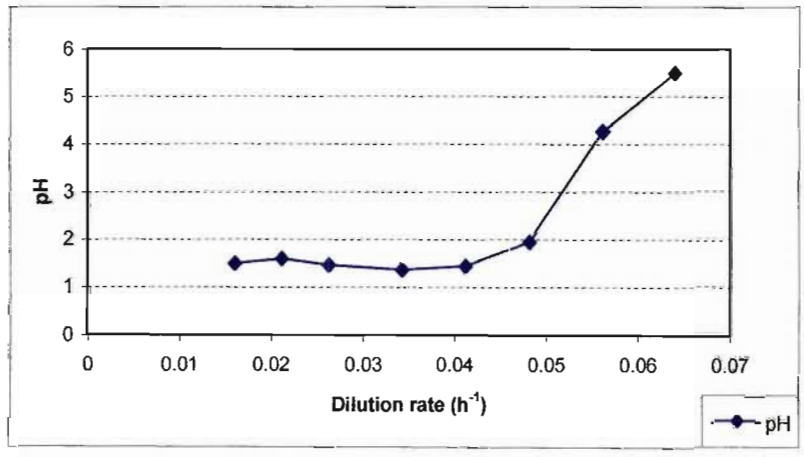


Fig. 3.25: Stable-state pH values of microbial association Am-3 subjected to continuous cultivation (30^0C) on autotrophic growth medium.

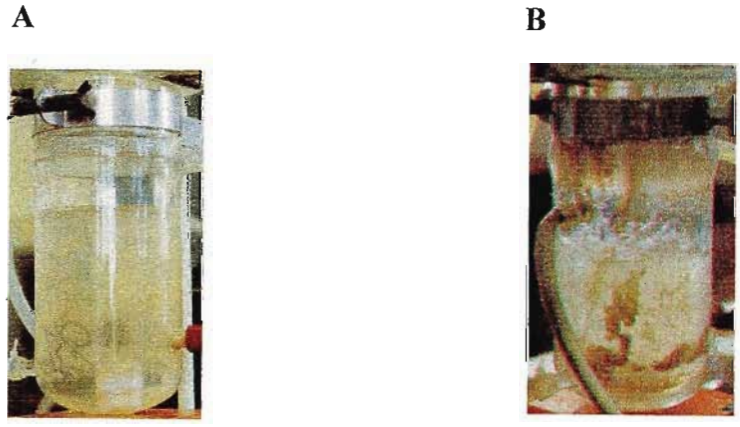


Plate 3.11: Wall growth generated by microbial association Am-3 during 2 (**A**) and 6 (**B**) months continuous cultivation (30^0C, autotrophic growth medium) with incremental dilution rate increases from 0.02 to 0.06 h^{-1}.

3.2.3 Product Formation in Chemostat Culture

Association Lf-1

If a product is strictly growth dependent, it follows that its concentration will vary with D in the same way as biomass does (Pirt, 1975). This relationship was demonstrated clearly (Fig 3.26) at constant temperature (25^0C) and pH (6) since the sulphate concentration (2.3.3) correlated closely the biomass dry weight (2.3.2). Theoretically, an increase in product formation is achievable with an increase in biomass. This can be effected by increasing the concentration of the growth-limiting substrate or by using some form of biomass feedback mechanism. In a biofilter, an increase in substrate concentration (H_2S) would be impractical due to its inhibitory effects (Buisman *et al.*, 1991). Furthermore, Yang and Allen (1994) reported that sulphate accumulation in a biofilter bed can impede its biological function. Therefore, a mechanism to prevent such an accumulation is extremely important.

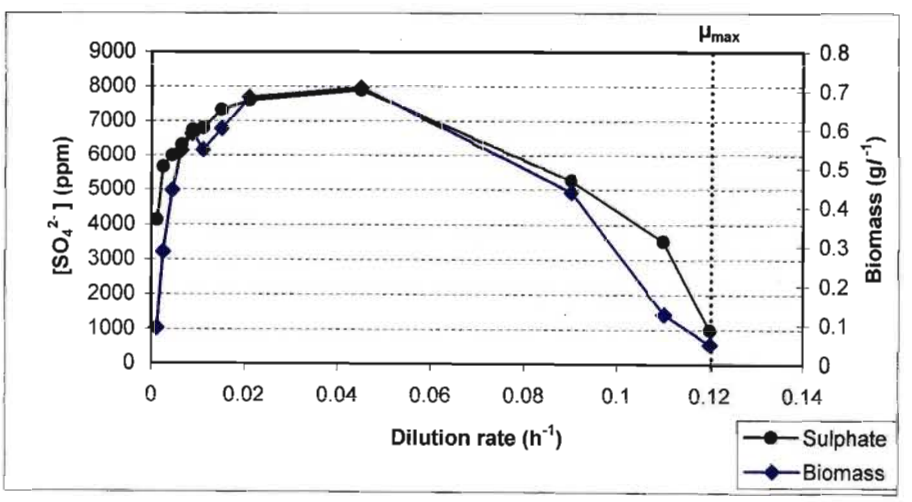


Fig. 3.26: Stable-state sulphate (●) and biomass concentrations (◆) of microbial association Lf-1 subjected to continuous cultivation (25^0C, pH 6) on autotrophic growth medium.

3.2.4 The Concept of a 'Steady State'

Steady state is defined as: "the condition of a continuous culture in which changes in the process parameters and the physiological state of cells are no longer detectable" (Atlas *et al.*, 1999). The criteria to determine a steady state are, however, not

extensive and largely arbitrary (Pirt, 1975). Cultivation (25^0C, pH 6) of microbial association Lf-1 at constant dilution rates of 0.004 h^{-1} (initial D, 0.002 h^{-1}) and 0.013 h^{-1} (initial D, 0.009 h^{-1}) was carried out to determine if a "true" steady state could be reached and if the magnitude of the pertubation affected the time needed to reach such a steady state. Marked changes in the biomass and the optical density were recorded in the period directly after the dilution rate increase (Figs. 3.27 and 3.28). With a constant dilution rate of 0.04 h^{-1}, changes in the two parameters were apparent for 20 days after a rate increase of 0.002 h^{-1}. Subsquently, the oscillations decreased and became damped. Since 28 days were required to effect three culture volume changes, it appeared that a steady state was reached earlier (Pirt, 1975; Brannan and Caldwell, 1983; Sublette and Sylvester, 1987c). Following the second dilution rate change to 0.013 h^{-1} a steady state was not achieved even after six culture volume changes (Fig. 3.28). Although, tacitly it has been accepted generally that five culture volume changes are excessive for the achievement of a steady state, many examples of longer periods are recorded (Barfold, Pamment and Hall, 1982). The inability of the culture to reach a steady state was attributed partly to the magnitude of change in the dilution rate which has been shown to influence the length of time needed to achieve a stable state (Herbert *et al.,* 1956) by inducing an extended lag period (Mateles, Ryu and Yasuda, 1965). The lag period is often indicative of the time required for the members of the association to alter their nucleic acid and/or enzyme content and other structures to the new steady-state levels (Pirt, 1975). The stability of the association, in terms of the number of component species, in relation to different dilution rates is discussed in 4.3.2.

It is not unusual for microbial populations to oscillate frequently in natural environments and to an extent this can be simulated in a chemostat (Meers, 1971). The oscillations are caused by factors such as predatory interactions between the component species which, in a constant environment, occur with regular periodicity. Oscillations may also be caused by changes in the chemostat environment, e.g. the dilution rate which may alter the dominance of particular members. The assumption made in theoretical analyses of mixed systems, that cultures reach a stable equilibrium or "true" steady state is, therefore, explicitly flawed (Cassel, Sulzer and Lamb, 1966). For such a steady state to be reached, the environment must remain unaltered with time and the components of the association must remain inert to one another. Although it is possible to maintain a stable environment, interactions between the

component species will always be present. A "true" steady state can, therefore, be considered unrealistic.

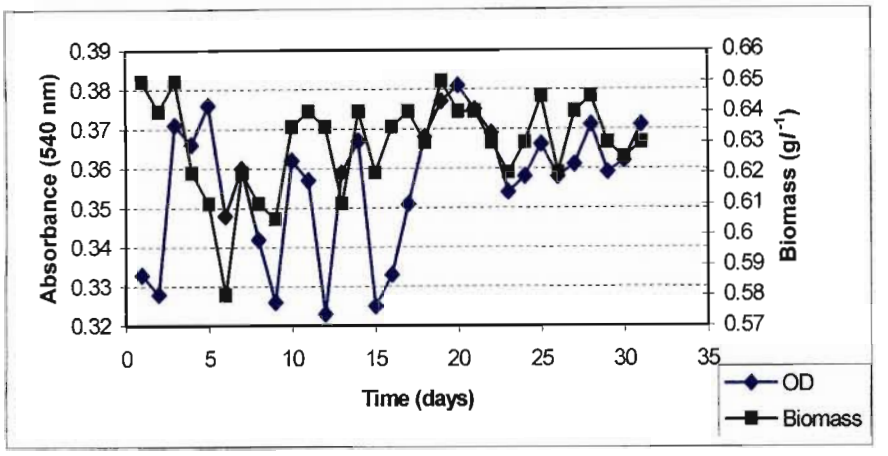


Fig. 3.27: Changes in optical density (◆) and biomass (■) of microbial association Lf-1 subjected to continuous cultivation (25^{0}C, pH 6), following a dilution rate increase from 0.002 to 0.004 h^{-1}, on autotrophic growth medium.

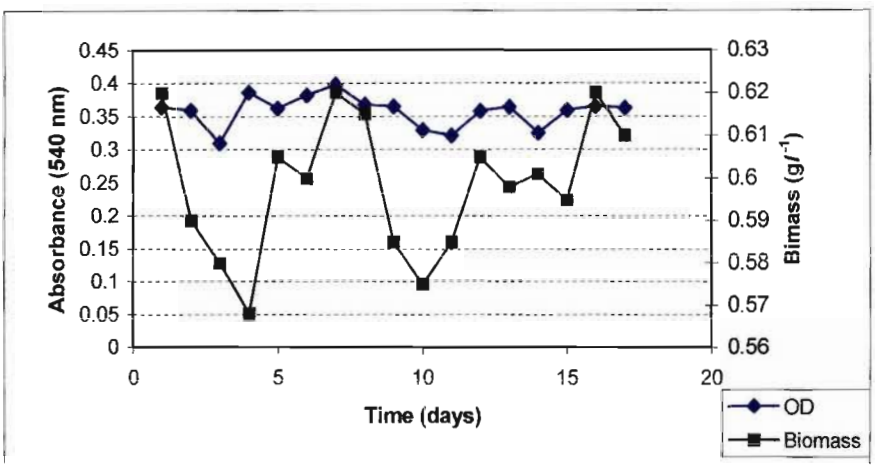


Fig 3.28: Changes in optical density (◆) and biomass (■) of microbial association Lf-1 subjected to continuous cultivation (25^{0}C, pH 6), following a dilution rate increase from 0.009 to 0.013 h^{-1}, on autotrophic growth medium.

For associations Ws-2 and Am-3 steady states were not achieved even after 5 culture volume changes, probably due to the small working volumes of the culture vessels and the presence of substantial wall growth particularly with Am-3.

3.2.5 Determination of Maximum Specific Growth Rate by Washout Kinetics

Washout kinetics (2.7.4) were used to determine the effects of temperature and pH on the maximum specific growth rate. Prior to the commencement of washout, the respective associations were cultured at dilution rates < D_c until steady states (2.7.3) in the presence of the different parameters were attained. At these points product formation (sulphate) was determined (2.3.3). Maximum specific growth rate determinations, in reponse to temperature changes, were made for Ws-2 and Am-3. Washout is reliant on a dilution rate which is higher than the critical dilution rate. Thus, as the association is washed out, its growth rates is equivalent to the maximum specific growth rate (Pirt, 1975).

pH

(a) Association Lf-1

Very small variations in pH can cause sub-optimum activity of enzyme systems thus exemplifying the importance of pH in the growth and metabolism of microorganisms (Pickett, 1982). The effects of pH change (1 to 9) on the maximum specific growth rate, as determined by washout kinetics (0.14 h^{-1}), and steady-state (0.012 h^{-1}) product formation of the microbial association Lf-1 were determined at constant temperature (25^{0}C). The optimum pH for growth rate and product formation occurred in the range of 3 to 4 (Fig. 3.29). Therefore, it is probable that the dominant species in microbial association Lf-1 were acidophiles with optimum pH values near 3 (Yang and Allen, 1994). Optimum pH values for the control of H_2S emissions from biofilters are generally between 3 and 8 (Yang and Allen, 1994, Chung *et al.*, 1996c). The specific growth rate and product formation were relatively high over an extended pH range of 1 to 7. This was in agreement with published literature which has reported sulphur-oxidizing bacterial activity over a pH range of 1 to 8, depending on the species (Yang and Allen, 1994). Significant decreases in pH values have been reported in H_2S biofilters. Thus, microbial association viability at low pH values is highly beneficial. However, if a biofilter is operated under acidic conditions, the production of leachate could corrode biofilter housing, ductwork and drainage and air distrubition works (Ergas, Schroeder, Chang and Morton, 1995).

Previous research has demonstrated that pH has a significant influence on the formation of end products (Pirt, 1975). This can be clearly seen in Fig. 3.29 where pH

values > 6 resulted in dramatic decreases in sulphate formation. The volumes of base consumed (2.7.4) at pH values from 3 to 9 over 24 h periods showed clearly that high consumptions resulted between pH values of 3 and 6 with an maximum recorded between 4 and 5 (Fig. 3.30). At pH values > 6 substantial decreases in base consumption occurred which correlated with decreased sulphate formation (Fig. 3.29). With pH values < 3 components of the influent medium precipitated therefore similar determinations were not made. In general, the association, with respect to sulphate production and maximum specific growth, was more sensitive to pH values > 6 than pH values < 4. The association was able to grow at a pH value as low as 1. A similar observation was made by Meulenberg *et al.* (1992) who reported that acidophilic thiobacilli can proliferate in environments with pH values as low as 1.5. When compared to reported values for monocultures of sulphur-oxidizing bacteria, the μ_{max} value of 0.08 h^{-1} of association Lf-1 (pH 4, 25^{0}C) was > that of *T. acidophilus* (0.05 h^{-1}) but considerably less than those of *Thermothrix thiopara* (0.55 h^{-1})(Mason *et al.*, 1987), *Thiobacillus denitrificans* (0.84 h^{-1}) and *Thiobacillus ferroxidans* (thiosulphate-limiting conditions)(0.94 h^{-1})(Brannan and Caldwell, 1983).

Isolation of the component species and the subsquent replication of the above experiment would determine whether association Lf-1 had a higher μ_{max} value than the individual components. There is evidence in the literature that microbial associations have higher maximum specific growth rates than monocultures of the primary species (Sheehan and Johnson, 1971; Tsuchiya, Tivedi and Schuler, 1974).

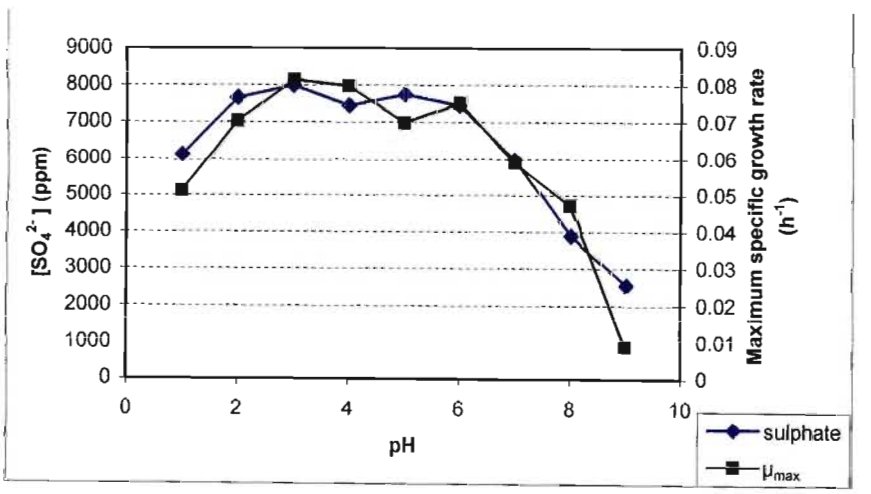


Fig. 3.29: Steady-state sulphate concentrations (◆) and maximum specific growth rates (■) of microbial association Lf-1 following open cultivation (0.012 h^{-1}, 25^{0}C) at discrete pH values between 1 and 9.

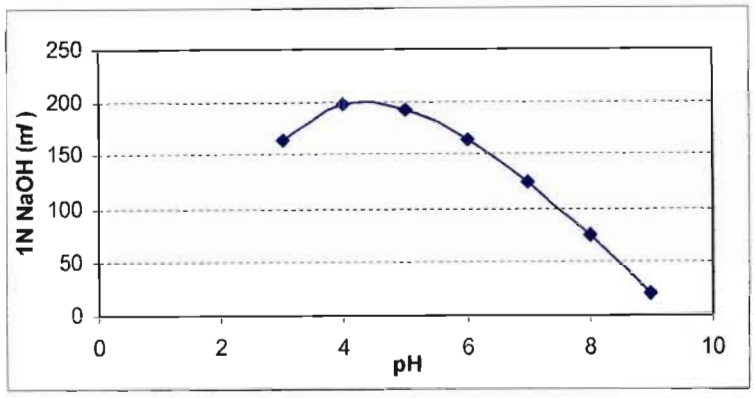


Fig. 3.30: 24-h NaOH consumption by microbial association Lf-1 at discrete pH values between 3 and 9 during continuous cultivation (25^0C, 0.012 h^{-1}) on autotrophic growth medium.

Temperature

Environmental temperature is a factor to which the biomass is inescapably subjected to since cell temperature must equilibrate with the temperature of the culture medium (Pirt, 1975). The physiology of continuous-grown cultures can be affected by temperatures in ways which are not predicted from observations made on the activities of batch-grown cultures (Hunter and Rose, 1972). It was, therefore, essential to investigate the effects of temperature on maximum specific growth rate and product formation before use of any microbial association in a biofilter.

(a) Association Lf-1

The effects of temperature on the maximum specific growth rate and sulphate formation of association Lf-1 are shown in Fig. 3.31. The highest μ_{max} recorded was at 25 ^{0}C while the rate approached zero at 40^0C. Such a decrease usually reflects either a disruption of metabolic regulation or cell death (Pirt, 1975). Increased temperature will eventually cause breakdown of protein structures and, thus, adversely affect the affinity of the cells for the substrate (Pirt, 1975). This was apparent (Fig. 3.31) when a sharp decline in sulphate concentration occurred when the temperature exceeded 30^0C. At temperatures < 30^0C, decreases in sulphate concentration correlated with decreases in the maximum specific growth rate. The results indicated that the optimum temperature (10^0C) for sulphate production did not coincide with the highest μ_{max} which was recorded at 10^0C. This phenomenon was

corroborated by the base consumption data since maximum consumption occurred at 10^0C (Fig. 3.32). At temperatures < 10^0C, decreases in sulphate concentration were recorded and these were attributed to sub-optimal enzyme activity. A similar scenario was described by Demain (1972) who reported that maximum product formation did not always coincide with the highest μ_{max} value.

Temperatures > 30^0C had greater effects on sulphate formation and μ_{max} values than temperatures < 20^0C. The negative affects of temperatures at values > 35^0C were disappointing as off-gas often has a temperature > 35^0C and, therefore, gas cooling prior to biofiltration would be necessary. Association Lf-1 recorded high μ_{max} and sulphate concentration values over the temperature range of 10 to 30^OC. This would be beneficial in off-gas biofiltration were temperature flunctuations are common, particularly for open biofilters where the temperatures can vary with the weather conditions.

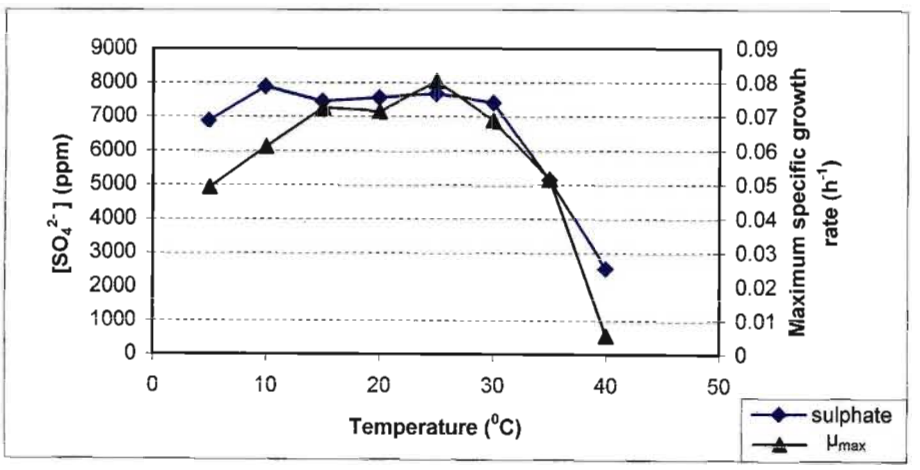


Fig. 3.31: Steady-state sulphate concentrations (◆) and maximum specific growth rates (▲) of microbial association Lf-1 following open cultivation (0.012 h^{-1}, pH 6) at discrete temperatures between 5 and 40^0C.

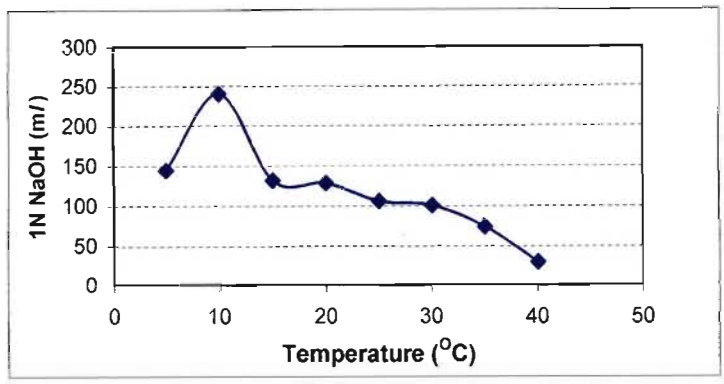


Fig. 3.32: 24-h NaOH consumption by microbial association Lf-1 at discrete temperatures between 5 and 40^{0}C during continuous cultivation (pH 6, 0.012 h^{-1}) on autotrophic growth medium.

(b) Associations Ws-2 and Am-3

Associations Ws-2 and Am-3 were subjected to incremental (5^{0}C) increases in temperature over the range of 10 to 60^{0}C (Fig. 3.33) and 5 to 50^{0}C (Fig. 3.34), respectively. Optimum μ_{max} occurred at 40 and 35^{0}C for Ws-2 and Am-3, respectively. The maximum specific growth rate approached zero at 10 and 60^{0}C for association Ws-2 and at 5 and 50^{0}C for Am-3. For both associations, near zero maximum specific growth rates coincided with increased pH values. Like association Lf-1, this was probably indicative of low product formation.

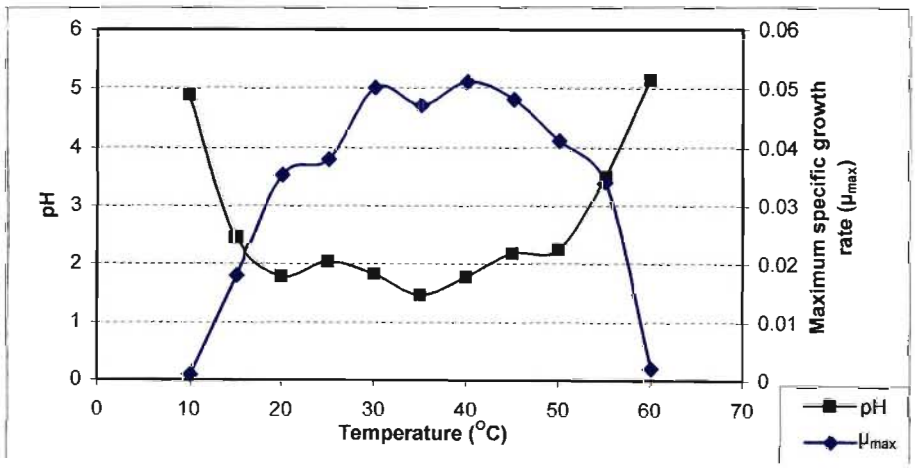


Fig. 3.33: Steady-state pH values (■) and maximum specific growth rates (◆) of microbial association Ws-2 following open cultivation (0.015 h^{-1}) at discrete temperatures between 10 and 60^{0}C.

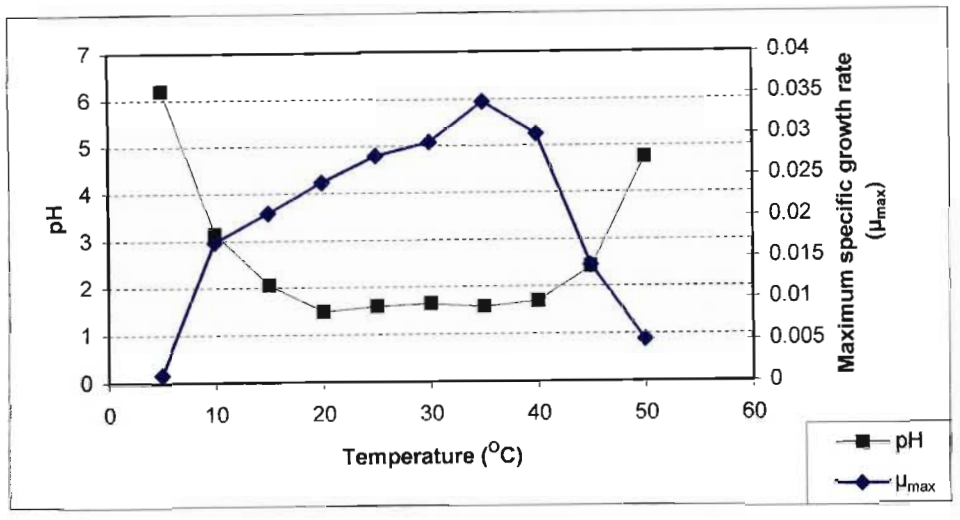


Fig. 3.34: Steady-state pH values (■) and maximum specific growth rates (◆) of microbial association Am-3 following open cultivation (0.012 h^{-1}) at discrete temperatures between 5 and 50^0C.

3.2.6 Maintenance Energy

In non-sporing bacteria such as those which constituted microbial association Lf-1, a successful life strategy should include satisfaction of a maintenance energy demand which is as low as possible while remaining ready for a fast response to nutrient upshifts (Tappe *et al.*, 1999). As populations in biofilters are faced with fluctuating nutrient availablities, with periods of substrate excess and deficiency, a low maintenance requirement would be beneficial.

The observation by Pirt (1975), that when the specific growth rate is varied by changing the dilution rate in a chemostat a straight-line relationship between 1/Y and 1/μ is generally observed, proved to be valid for association Lf-1 (Fig. 3.35). The biomass production rate increased with increased dilution rate (0.011 to 0.06 h^{-1}, 30^0C, pH 6, fixed substrate concentration) and the yield increased from 14.481 to 23.841 with a theoretical maximum (Y_{max}) of 27.248 (g dry wt $mole^{-1}$ thiosulphate). The Y_{max} value was higher than reported values for monocultures of sulphur-oxidizing chemolithoautotrophs. The highest recorded Y_{max} for a monoculture is 22.8 g dry wt $mole^{-1}$ thiosulphate for *Thx. thiopara* (Mason *et al.*, 1987). The observation by the same workers that for thiobacilli the yield increased with increased dilution rate was thus in agreement with the results of this study. Also the biomass yield

increase on thiosulphate was within the range generally observed for thiobacilli (5 to 13 g dry wt $mole^{-1}$) (Kuenen and Beudeker, 1982). Although the yields calculated here were not typical of aerobic sulphur oxidizers (7.5 ± 1.5 g dry wt $mole^{-1}$) (Kelly , 1982), higher yields have been reported for *T. dentrificans* and *Thx. thiopara* (Mason *et al.*, 1987). Due to the relatively low μ_{max} value of 0.08 h^{-1}, the range of dilution rates for determination of the maintenance energy were relatively low (0.011 to 0.06 h^{-1}). Extremely low dilution rates in chemostats (< 0.05 h^{-1}) give rise to inhomogeneities due to mixing problems and low steady-state biomass concentrations thereby making maintenance calculation problematic (Bulthuis, Frankena, Koningstein, van Versveld and Stouthhamer, 1988). Tomaschewski demonstrated that for *Nitrosomonas europaea* more substrate was needed for maintenance requirements at higher growth rates than at lower μ values (Tappe *et al.*, 1999). As the growth rate range of association Lf-1 was ≤ 0.08 h^{-1}, it was probable that the maintenance requirements were low. A maintenance energy value of 0.0004 (g dry wt $mole^{-1}$) (Fig. 3.35) was calculated for association Lf-1. This was in close agreement with values quoted for monocultures of *T. acidophilus* (0.0005 g dry wt $mole^{-1}$) and *T. thiooxidans* (0.0004 g dry wt $mole^{-1}$) grown on thiosulphate (Mason *et al.*, 1987).

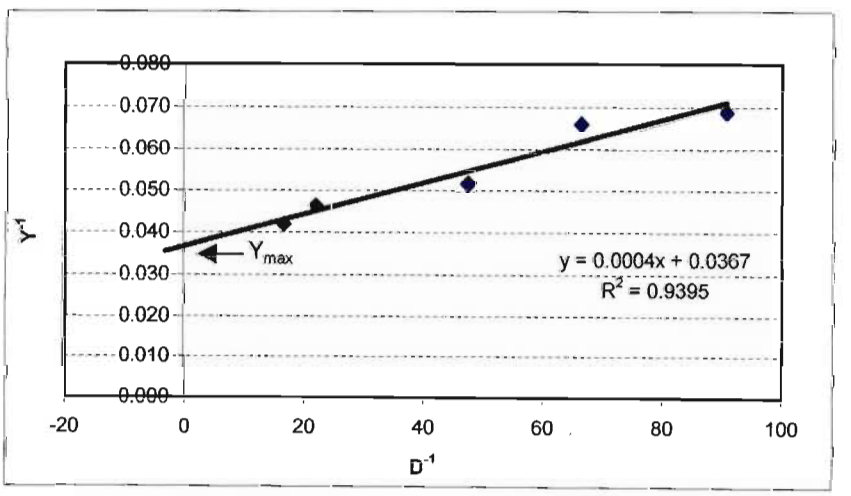


Fig. 3.35: Maximum growth yield and maintenance value for microbial association Lf-1 during continuous cultivation (D = 0.01 to 0.06 h^{-1}, 30^0C, pH6) on autotrophic growth medium.

3.2.7 Biolog Analysis at Discrete pH Values

Previous studies have shown that microbial communities produce habitat-specific and reproducible patterns of carbon source oxidation thus enabling their classification with respect to carbon source metabolism (Garland and Mills, 1991; Zak, Willig, Moorhead and Wildman, 1994). Questions do, however, remain of the effectiveness of the Biolog system in distinguishing changes in a single microbial association due to growth under different physiological conditions, such as temperature and pH. It was, therefore, decided to investigate this further with microbial association Lf-1 at the pH values of 3, 6 and 9. Steady states, as judged by optical density, were reached at a dilution rate of 0.045 h^{-1} and a temperature of 30^0C for each of the pH values. Samples were then taken and assayed by the method described in 2.8.5. Prior to the analysis, DGGE of each sample revealed distinct differences in their structures (4.5).

Plate replication for each sample pH value was necessary to determine the extent of variation due to measurement errors and other miscellaneous influences which could not be assessed (Glimm *et al.*, 1997). Since a plate has more wells than the number of replicate plates used, i.e. replications, the number of variables per observation was higher than the number of observations. Principal component analysis (PCA) was used to reduce the dimensionality of the data enabling the determination of relationships among and within samples on the basis of optical density utilizing far fewer variables. Principal component analysis is an ordination method which facilitates the projection of the original cluster of data points into new axes or principal components so that the intrinsic patterns become visually apparent (Pielou, 1984). To achieve a greater degree of accuracy, PCA was carried out by means of a correlation matrix enabling standardization of the data. Principal component scores (PC scores) were calculated with Genstat 5 statistical software (Numerical Algorithms Group Ltd). Plotting sample values in two dimensions on the basis of their scores for the first two PCs allows relationships among samples to be observed (Victorio *et al.*, 1996). Principal component analysis identified the reproducibility and uniqueness of the association at the three discrete pH values. Replicate Biolog plates inoculated with samples of similar optical density tended to cluster (Figs 3.36 to 3.38) demonstrating the reproducibility. Reproducibility of the substrate oxidation pattern has been reported previously by Haack *et al.* (1995).

Visual separation of the association after cultivation at the three discrete pH values was possible using just two PCs (Figs. 3.36 to 3.38) demonstrating uniqueness.

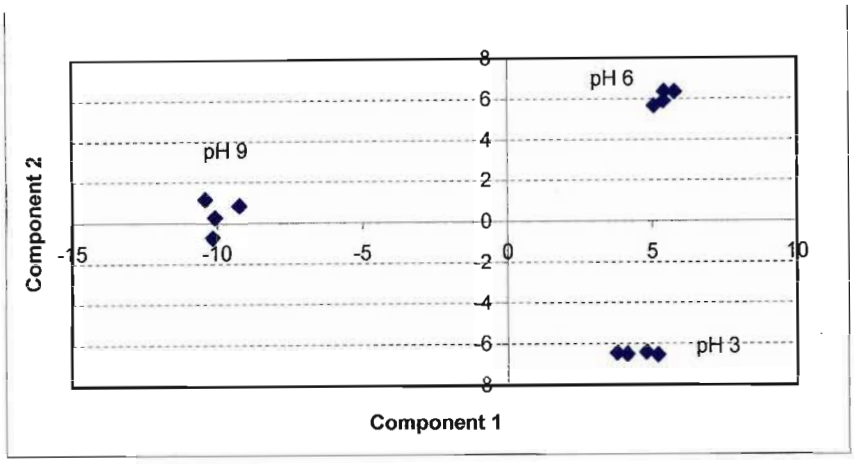


Fig. 3.36: Ordination produced from principal component analysis of PC scores 1 and 2 after 24 h incubation of microbial association Lf-1 previously cultured at pH values of 3, 6 and 9.

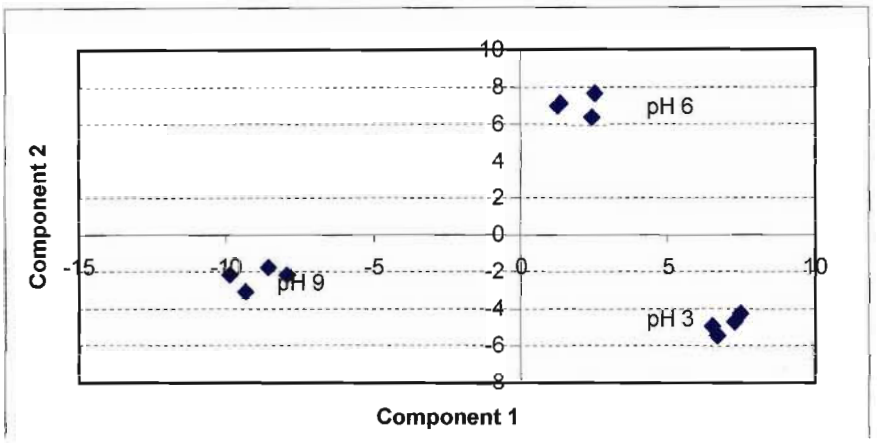


Fig. 3.37: Ordination produced from principal component analysis of PC scores 1 and 2 after 48 h incubation of microbial association Lf-1 previously cultured at pH values of 3, 6 and 9.

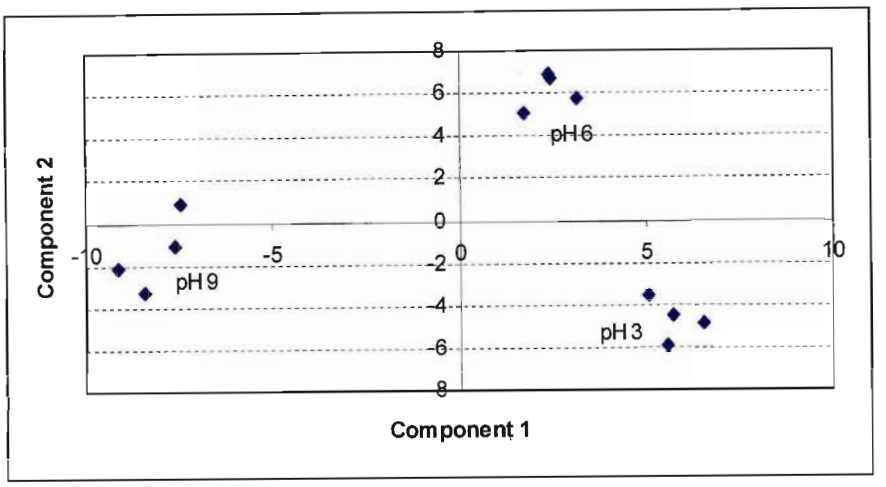


Fig. 3.38: Ordination produced from principal component analysis of PC scores 1 and 2 after 72 h incubation of microbial association Lf-1 previously cultured at pII values of 3, 6 and 9.

Generally, visual separation is not possible with two PCs as reported by Heuer and Smalla (1997). Differences between the fingerprints of the association after cultivation at the three discrete pH values were probably due to shifts in the microbial population as demonstrated by DGGE (4.3.2). After 24 h, the first correlation PC axis accounted for 57.35% and the second accounted for 30.53% of the total variation in the data for a cumulative total of 87.88%. Similar values were obtained after 48 and 72 h of incubation. The minimum number of PCs used should explain at least 75% of the total variation observed (Glimm *et al.*, 1997).

By examining all the possible correlations of the original variables in relation to the PC scores, the separation of samples in PC space can be related to differences in carbon source utilization (Victorio *et al.*, 1996). The carbon sources with high positive or negative correlations are the dominant differential factors in discerning structural population changes at the different pH values (Tables 3.2 to 3.4). These were substrates for which there were consistent differences in the amount of substrate oxidation between the association after cultivation at the three pH values. Variability in the first PC was due predominately to carbohydrates, carboxylic acids and amino acids. Poor correlation of a carbon source is not necessarily a reflection of poor utilization but rather that it was not useful in differentiating between samples (Garland and Mills, 1991).

It was noted that substrates which were significant in distinguishing the community changed over the course of the assay as reported by Haack *et al.* (1995). As with traditional bacterial growth curves, microbial growth in Biolog wells often

exhibit a lag phase, an exponential phase and a stationary phase (Haack *et al.*, 1995). Therefore, several substrates which were significant to the PC scores at the 24 h incubation point were not at the 72 h point (Table 3.2 and 3.4).

Table 3.2: Correlation between the most heavily loaded carbon source and the loading values (24 h) of the first two principal components. The absence of substrates corresponds to a loading value < 0.13 and > -0.13.

PC1		PC2	
Carbohydrates		***Polymers***	
N-acetyl-D-glucosamine	-0.134	Tween 80	0.147
B-methyl D-glucoside	-0.131	***Carbohydrates***	
D-trehalose	-0.133	Adonitol	-0.156
Carboxylic acids		L-arabinose	-0.177
Cis-aconitic acid	0.132	L-fructose	-0.162
Citric acid	0.133	M-inositol	-0.165
D-galactonic acid lactone	-0.132	D-psicose	-0.161
D-saccharic acid	0.135	D-sorbitol	-0.168
Succinic acid	0.130	Turanose	0.179
Amino acids		N-acetyl-D-galactosamine	0.138
L-glutamic acid	0.134	***Amines***	
L-proline	0.131	Phenyl ethylamine	0.145
D-serine	-0.131	Putrescine	0.155
Miscellaneous		2-Amino ethanol	0.143
D, L-α-glycerolphosphate	-0.132	***Amino acids***	
		D-alanine	0.144
		L-alanyl glycine	0.175
		Glycyl-L-glutamic acid	0.178
		L-ornithine	0.177
		L-phenylalanine	-0.138
		Miscellaneous	
		Bromo-succinic acid	0.181

Table 3.3: Correlation between the most heavily loaded carbon source and the loading values (48 h) of the first two principal components. The absence of substrates corresponds to a loading value < 0.2 and > -0.2.

PC 1		PC 2	
Polymers		***Carbohydrates***	
Glycogen	-0.238	N-acetyl-D-galactosamine	-0.204
Dextrin	-0.214	D-psicose	-0.272
Carbohydrates		Turanose	0.216
Cellobiose	-0.200	***Carboxylic acids***	
Gentiobiose	-0.209	D-galactonic acid lactone	-0.231
α-D-lactose	-0.266	D-glucosaminic acid	-0.272
D-meliobiose	-0.236	***Amines***	
B-methyl D-glucoside	-0.215	2-Amino ethanol	0.311
D-raffinose	-0.215	***Amino acids***	
Carboxylic acids		D-serine	-0.284
Formic acid	0.218		

Table 3.4: Correlation between the most heavily loaded carbon source and the loading values (72 h) of the first two principal components. The absence of substrates corresponds to a loading value < 0.2 and > -0.2.

PC 1		PC 2	
Carbohydrates		***Carbohydrates***	
Lactulose	-0.262	N-acetyl-D-glucosamine	-0.243
D-meliobiose	-0.283	D-psicose	-0.406
B-methyl D-glucoside	-0.246	D-raffinose	0.206
D-raffinose	-0.254	D-sorbitol	-0.205
Xylitol	0.205	***Carboxylic acids***	
Carboxylic acids		D-glucosaminic acid	-0.348
α-Hydroxybutyric acid	0.259	***Amines***	
α-Ketovaleric acid	-0.280	2-amino ethanol	0.200
Sebacic acid	0.217	***Amino acids***	
Amino acids		D-serine	-0.382
L-leucine	-0.243		

An analysis of variance was made with the PC scores as the response variable. This enabled examination of significant differences between the three inocula as a function of time. Two-way anovas were made on PC scores one to six which accounted for 95% of the observed variation. Principal component one scores for the three inocula were (statistically) significantly different at the 95% level of confidence ($F_{pr} < 0.05$) while for two to six the degree of significance was 99% ($F_{pr} < 0.001$). A manova was made to allow multiple analysis of all six PC scores for the three inocula. The Wilkes lambda was extremely small (0.00022) confirming that the pH values as a function of time did differ across the six PC scores.

Most Biolog microtitre plate studies have been used to resolve changes in heterotrophic microbial associations. Thus, the ability to discriminate changes in an autotrophic microbial association based on heterotrophic capabilities was an interesting phenomenon particularly since component species changes in response to discrete pH values were demonstrated (4.5). Although additional work is necessary to establish the effectiveness of phenotypic fingerprinting to monitor gas biofilters, incorporation of such a meaningful technique may be beneficial. As with DGGE, it may serve as a high-tech', diagnostic tool for troubleshooting biofilters performing substandard.

3.2.8 Sulphide

Few studies have appraised the biological oxidation of sulphide in chemostats probably due to its poor chemical stability and toxicity to microorganisms (Janssen *et al.*, 1995). In at least two aerobic (facultatively) autotrophic *Thiobacillus* spp., $S_2O_3^{2-}$ and S^{2-} were shown to be equivalent energy sources (Beudeker, Gottschal and Kuenen, 1982).

Sodium sulphide (10 ppm S^{2-}) was used to replace $S_2O_3^{2-}$ in the autotrophic growth medium (2.2) for the cultivation (0.045 h^{-1}, 30^{0}C and pH 6) of microbial association Lf-1. Timmer-Ten Hoor (1981) demonstrated previously the ability of sulphur-oxidizing bacteria to grow on sulphide (Na_2S) as an energy source in a chemostat. Even after a high $S_2O_3^{2-}$ (8 000 ppm)-oxidizing potential was pre-induced (cultures on $S_2O_3^{2-}$ for 6 volume changes), visible accumulations of sulphur (S^0) were apparent within 24 h. High-performance liquid chromatography (2.3.3) was used to confirm the presence of precipitated S^0 in the culture of Lf-1. Previous literature has indicated that S^{2-} reacts with S^0 to form polysulphides (S^{2-}_x) thereby interfering with the partitioning of S^0 into an organic phase (Henshaw *et al.*, 1997). Elemental sulphur must be partitioned into an organic phase for HPLC to be a successful quantitative method. The addition of 0.02 m*l* 1N HCl per 10 m*l* of culture was used to alleviate S^{2-} interference (Henshaw *et al.*, 1997). The HPLC retention times of the sample precipitate and S^0 standard were 5.844 and 5.881 min, respectively (Appendixes E 1 and 2). When equal volumes (5 m*l*) of sample and standard were mixed and assayed by HPLC, a single peak was obtained with a retention time of 5.807 min (Appendix E 3). This result proved that the precipitate in the culture was S^0. When the culture was underloaded (hydraulic) by reducing the flow rate (0.005 h^{-1}, 30^{0}C and pH 6), the S^0 particles were oxidized to SO_4^{2-}, as confirmed by an increase in base consumption and SO_4^{2-} concentrations (2.3.3). Gadre (1989) reported that during the oxidation of sulphide in a bioreactor, the pH of the medium decreased from 7.3 to 3.3. In contrast, in this study when the pH control of the bioreactor was switched off for two weeks the pH dropped from 6 to 5. This was of particular significance since at low pH values H_2S solubility is near negligible (Gadre, 1989).

Associations Ws-2 (0.01 h^{-1}) and Am-3 (0.03 h^{-1}) were unable to oxidize S^{2-} and washout resulted.

One of the major advantages of elemental sulphur formation is that it is non-corrosive and non-toxic and contains more sulphur per unit mass than any other form (Henshaw *et al.*, 1998). The precipitated sulphur can be recovered and re-used as a feedstock for chemical, fertilizer and materials manufacture (Henshaw *et al.*, 1998) or as a raw material for sulphuric acid production (Lens and Pol, 1999). The efficacies of autotrophic bacteria, such as those found in association Lf-1, has promoted the development of biotechnological methods.

Chapter 4

Results and Discussion (Part 2) Genotypic Profiling

4.1 Deoxyribonucleic Acid Isolation

Genomic DNA, subjected to various physiological conditions, was isolated from the open cultures and equal volumes were analyzed on an ethidium bromide-stained agarose gel. Prior to use, both the gel and running buffer were refrigerated at 4^0C to reduce distortion of the bands in the peripheral lanes. A typical gel of the total genomic DNA isolated from association Lf-1 is shown in Plate 4.1. The genomic DNA consolidated in the upper position of the gel while the plasmid DNA appeared as two distinct lower bands at the 1 and 2 kB DNA ladder marker positions. Similar results were obtained for associations Ws-2 and Am-3.

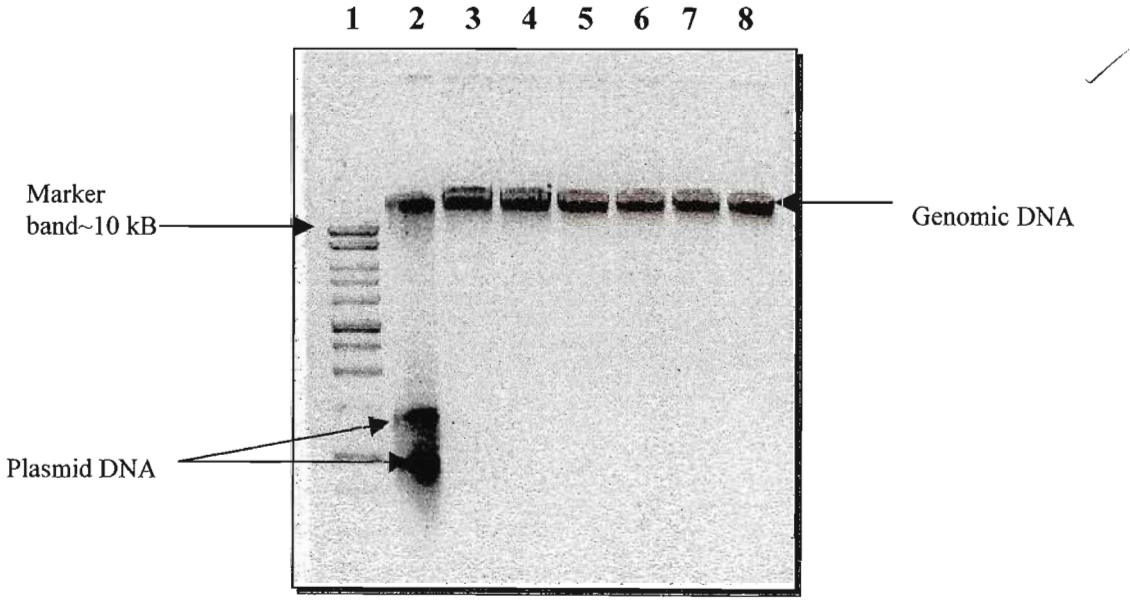


Plate 4.1:Visualization, by agarose gel electrophoresis, of total genomic DNA of chemostat-cultured Lf-1. Lane 1 contained the 1 kB ladder molecular weight marker. Lane 2 contained the positive control (*E. coli*) while Lanes 3 to 8 contained the total genomic DNA isolated from the microbial association after cultivation under different physiological conditions.

4.2 Amplification by Polymerase Chain Reaction of 16S rDNA

Polymerase fidelity, Mg^{2+} concentration, template concentration, primer annealing temperature and cycle number have all been shown to influence PCR

specificity (Arnheim and Erlich, 1992). Optimization of these parameters can be made by testing the range of each parameter:

Polymerase Fidelity

Expand™ high fidelity enzyme, a unique mixture of thermostable Taq DNA and two DNA polymerases (Barnes, 1994), was preferred to Taq DNA polymerase due to its inherent 3' to 5' exonuclease proof-reading activity which results in a 3-fold increased fidelity for DNA synthesis (Expand™ high fidelity PCR system package insert, Boehringer Mannheim).

Mg^{2+} Concentration

Plates 4.2 to 4.5 show ethidium bromide-stained agarose gels and demonstrate the $MgCl_2$ concentration optimization which was necessary to reduce the formation of spurious by-products and maximize yield (McPherson, Quirke and Taylor, 1991). The optimal $MgCl_2$ concentrations for the DGGE marker and microbial associations were 1.5 and 2 mM, respectively. Product size (193 bp) was in close agreement with the size calculated from the published sequence of Muyzer *et al.* (1993).

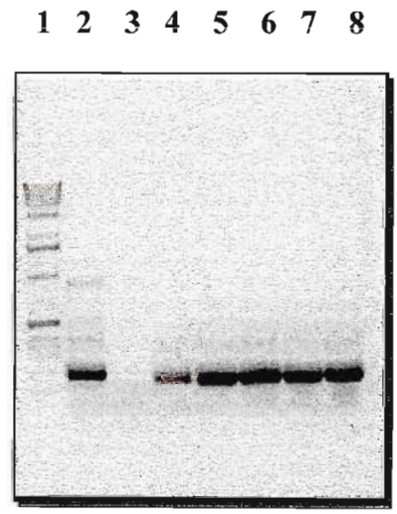


Plate 4.2: Analysis, by agarose gel electrophoresis, of the PCR optimization products of the DGGE marker obtained from Vrije Universiteit of Amsterdam. Lane 1, molecular marker. Lane 2, *E.coli* (+ ve control). Lane 3, negative control. Lanes 4 to 8, increased concentrations of $MgCl_2$ from 1 to 3mM in increments of 0.5.

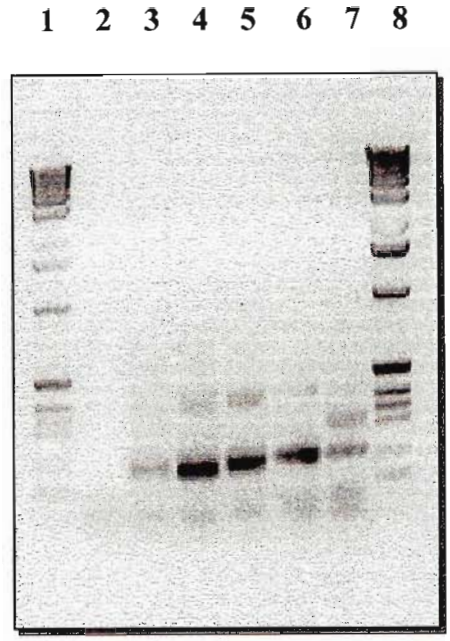


Plate 4.3: Analysis, by agarose gel electrophoresis, of the PCR optimization products of microbial association Lf-1. Lanes 1 & 8, molecular marker X. Lane 2, negative control. Lanes 3 to 7, increased concentrations of $MgCl_2$ from 1 to 3mM in increments of 0.5.

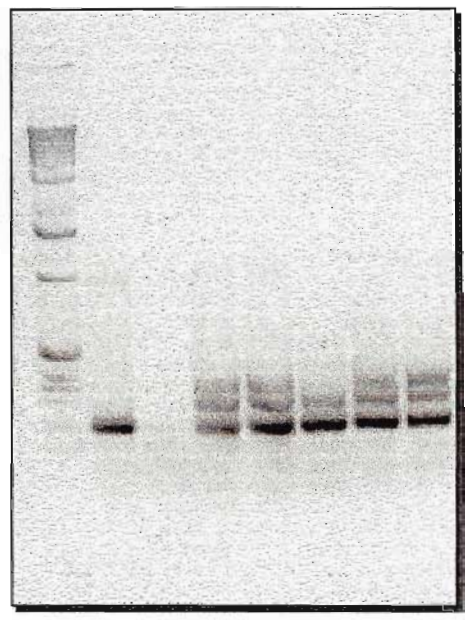


Plate 4.4: Analysis, by agarose gel electrophoresis, of the PCR optimization products of microbial association Ws-2. Lane 1, molecular marker x. Lane 2, *E.coli* (+ ve control). Lane 3, negative control. Lanes 4 to 8, increased concentrations of $MgCl_2$ from 1 to 3mM in increments of 0.5.

1 2 3 4 5 6 7 8

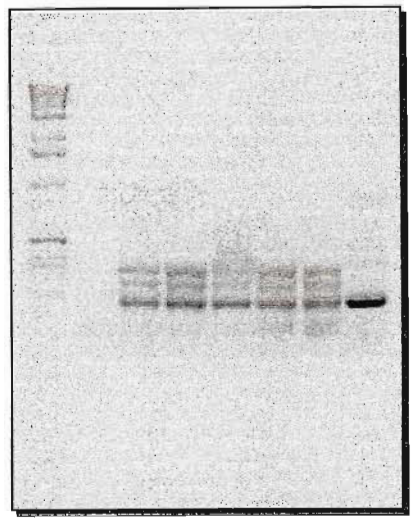


Plate 4.5: Analysis, by agarose gel electrophoresis, of the PCR optimization products of microbial association Am-3. Lane 1, molecular marker x. Lane 2, negative control. Lanes 3 to 7, increased concentrations of $MgCl_2$ from 1 to 3mM in increments of 0.5. Lane 8, *E.coli* (+ ve control).

Template Concentration

Ovreas, Forney, Daae and Torsvik (1997) reported that when the amount of template DNA used for PCR amplification was increased 100-fold, similar DGGE patterns were observed. It was, therefore, decided not to standardize the template concentration although only samples which contained $\geq$ 50 ng μl^{-1} of purified genomic DNA were amplified to ensure sufficient product formation (Reed *et al.*, 1998).

Primer Annealing Temperature and Cycle Number

Cycle number is important to maximize the efficiency of the reaction (McPherson *et al.*, 1991). An excessively high cycle number can result in aspecific products, the appearance of small deletion mutant products and, occasionally, the disappearance of specific products (Arhheim and Erlich, 1992). The cycle number and annealing temperature were obtained from the Department of Molecular Cell Physiology, Vrije Universiteit of Amsterdam.

Substances which inhibit enzyme activity are present in many materials and, thus, can limit the use of the PCR (Kreader, 1996). As a consequence of inhibition, extensive purification is often required to generate PCR-compatible material. However, because purification adds to the time and expense of sample preparation, and may effect template loss, a more efficient approach would be to minimize the

interference rather than attempt to remove all the offending substances. To this end, bovine serum albumin (BSA) was included in the reaction mixture as it has widespread use for minimizing such interference in the PCR (Hoss, Kohn, Paabo, Knauer and Schroeder, 1992; Romanowski, Lorenz and Wackernagel, 1993).

It is during the PCR amplification process that the 40-bp GC clamp is attached to the amplified genomic DNA isolated from the different associations to prevent complete separation in the denaturing gradient (Myers, Fisher, Lerman and Maniatis, 1985). Without the attachment of the GC-clamp, DGGE would detect only ± 50% of all possible single-base changes in DNA fragments ranging from 50 to ± 1 000 bp (Sheffield, Cox, Lerman and Myers, 1989). The primers used in this study were specific for all eubacteria although, if required, primers for a specific group of bacteria, such as sulphur-oxidizing bacteria, can be designed to determine specific genetic diversity.

'Primer-dimer' artifacts are frequent in most PCR reactions (McPherson *et al.*, 1991). In Plate 4.6 primer-dimer formation can be seen clearly at the leading edge of the migrated bands. To circumvent primer-dimer formation, primers should be designed so that there is no complementarity of their 3' ends. This precaution reduces the incidence of primer-dimer caused when one primer is extended by the polymerase with the other primer or itself as a template which results in a short, incorrect product (Newton and Graham, 1994).

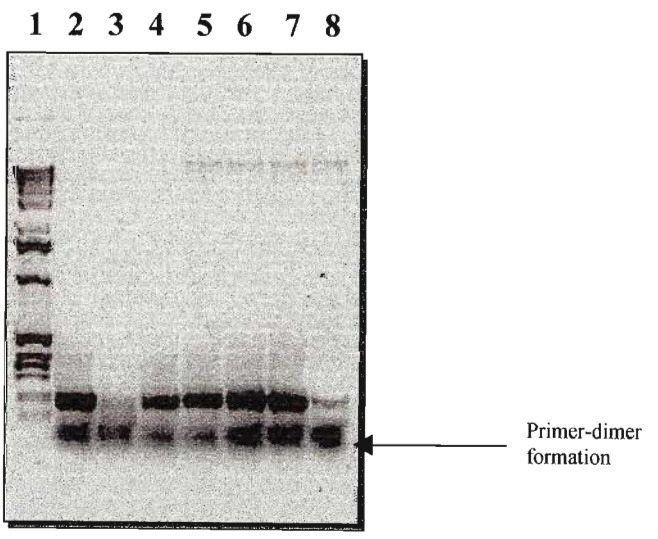


Plate 4.6: Analysis, by agarose gel electrophoresis, of the PCR optimization products of association Lf-1. Lane 1, molecular marker 10. Lane 2, *E.coli* (+ ve control). Lane 3, negative control. Lanes 4 to 8, Lf-1 products subjected to discrete physiological conditions.

4.3 Denaturing-Gradient Gel Electrophoresis

Denaturing-gradient gel electrophoresis was used to profile the genetic diversity of the sulphur-oxidizing microbial associations after open culture in the presence of different physiological conditions. It must be noted that, like most techniques, there are limitations and areas of possible errors in the protocol. Firstly, polymerase chain reaction amplification of DNA from diverse samples can lead to errors (Wintzingerode, Gobel, Stackebrandt, 1997). The numbers of bands may overestimate diversity because of: the formation of heteroduplex or chimeric molecules (Liesack, Weyland and Stackebrandt, 1991); the preferential amplification of target DNAs from some bacteria (Reysenbach, Giver, Wickham and Pace, 1992); and sequence heterogeneities in different copies of the 16S gene (Konopka *et al.*, 1999). Heteroduplexes are double-stranded fragments which are formed by association of fragments from two different organisms or from two different fragments from a single species (Newton and Graham, 1994). On a DGGE gel these heteroduplexes result in bands at a relative low denaturant concentration due to the fact that the double-stranded fragments have a number of mismatches which lead to a lower melting point (Newton and Graham, 1994). Conversely, diversity may be underestimated because all the sequences are not amplified equally or because the PCR products of phylogenetically-related strains may have similar or identical electrophoretic mobilities. Therefore, the sequences of the amplified region of the 16S rDNA genes are identical or nearly so and co-migrate in the gel (Smalla *et al.*, 1998). Another limitation is that only relatively small fragments, up to 500 bp, can be analyzed (Wawer and Muyzer, 1995).

Although not completely free of biases, the advantages of DGGE analysis of PCR-amplified gene fragments are many: the use of universal PCR primers in the amplification step, combined with the effective separation of different PCR products, enable DGGE to distinguish a wide range of microorganisms in a given ecosystem without the constraints of specific media or the laborious sequencing of clone libraries; and it provides an immediate display of the constituents of a population in both a qualitative and semi-quantitative way and the method is rapid (about 18h for the PCR and DGGE), simple (once the technique is established in the laboratory) (Wawer and Muyzer, 1995) and inexpensive relative to other genetic techniques. Sample-to-sample comparison is possible as multiple samples (up to 12 samples) can

be analyzed simultaneously on one gel thus allowing changes in population profiles after subjection to different physiological conditions to be analyzed. Furthermore, DGGE has been shown to produce reproducible patterns (Konopka *et al.*, 1999).

The different intensities of the DGGE bands which are derived from different bacterial species do not allow a quantitative estimate of the abundances of the different bacteria. This is due to a possible unknown PCR bias in the amplification of the different templates. (Teske *et al.*, 1996). In contrast, Suzuki and Giovannoni (1996) suggested that because environmental DNA samples contain highly diverse templates, it is unlikely that the amplification of any particular 16S rDNA will produce products at concentrations which are high enough to produce the reannealing inhibition effect and, therefore, introduce bias. Furthermore, Muyzer *et al.* (1993) stated that the relative intensity of each band and its position most likely represented the relative abundance of a particular species in the population. To resolve these different views a semi-quantitive interpretation for band intensity was made.

4.3.1 Denaturing-Gradient Gel Electrophoresis Optimization

Time Optimization

To determine the optimum electrophoresis time a, so-called, "time travel" experiment was made (Plate 4.7). The amplified marker was loaded onto the gel every hour for a total of 10 h (total run time 16 h). Note that in Lanes 10 and 11 complete separations of the marker did not occur while for Lanes 6 to 9 the bands were still in the process of migrating down the gel. For Lanes 2 to 5 no further migration of the bands occurred which suggested that this was the optimal run time for the marker (14 to 16 h), i.e. the bands of the marker had stopped abruptly due to PCR fragment branching caused by transition from helical to a partially melted state. The strands did not dissociate completely due to the GC clamp which had a melting point of about 95^0C. Without the GC clamp the fragments would have dissociated completely and, as single strands, continued to migrate down the gel. The sequence variants of the melting domains of each fragment determine the point at which migration stops and, hence, allows effective separation of the fragments (Muyzer *et al.*, 1993). The marker had seven bands which represented seven different general species. A far better option would have been to create a marker which consisted of the most common/influential sulphur-oxidizing bacteria. This would have facilitated direct comparisons.

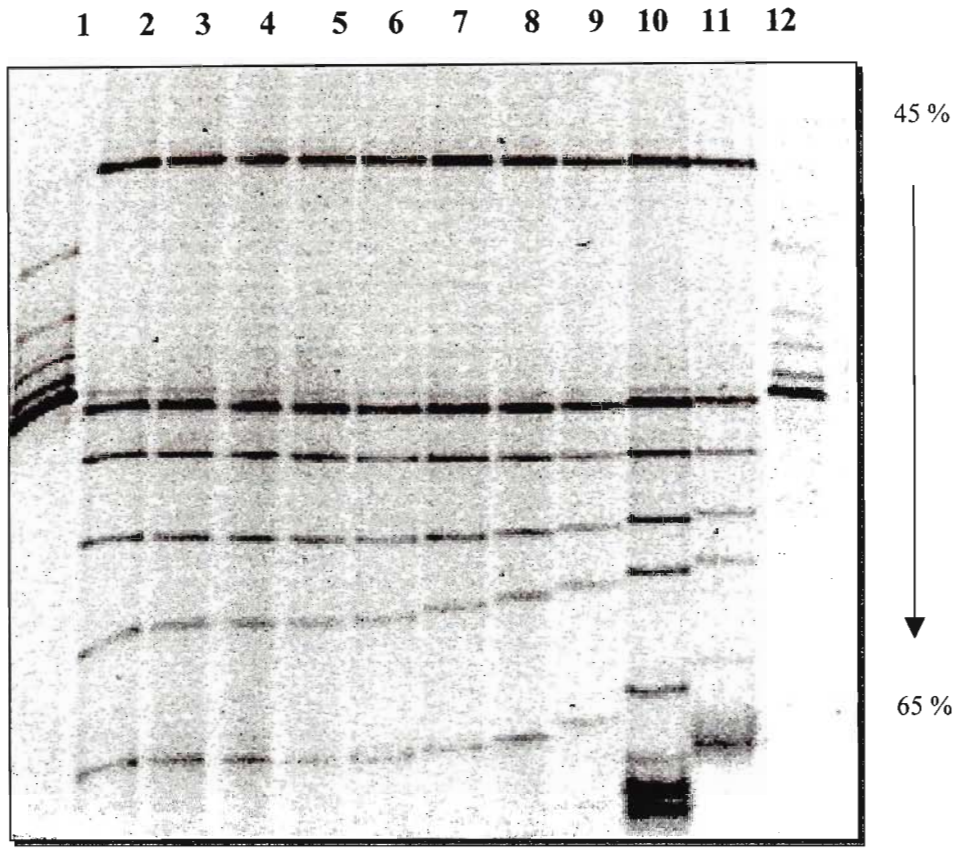
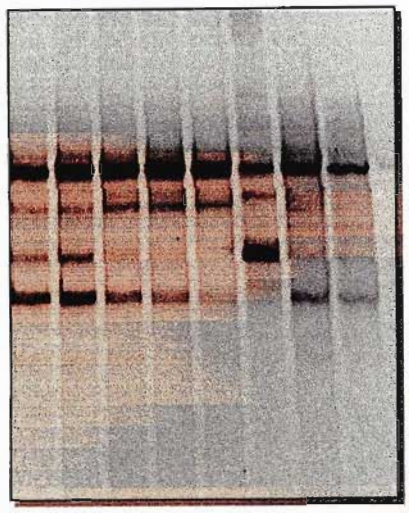


Plate 4.7: "Time travel" experiment. Image of an ethidium bromide-stained DGGE separation pattern of PCR-amplified DGGE marker. Lanes 1 and 12: *E.coli* (+ ve control).

Voltage Optimization

For distortion-free banding patterns, effective heat dissipation must be maintained. Heat is a function of power and power is the product of current and voltage. Therefore high voltages result in poor resolution (Plate 4.8 **A**) (Hoefer Electrophoresis Catalogue, 1990-1991). Distinct improvements in resolution and separation were observed when a DGGE gel was run with 70 volts for 16 h compared with one run with 200 volts for 4 h (Plates 4.8 **A** & **B**). Brinkhoff and Muyzer (1997) reported a similar observation.

A



B

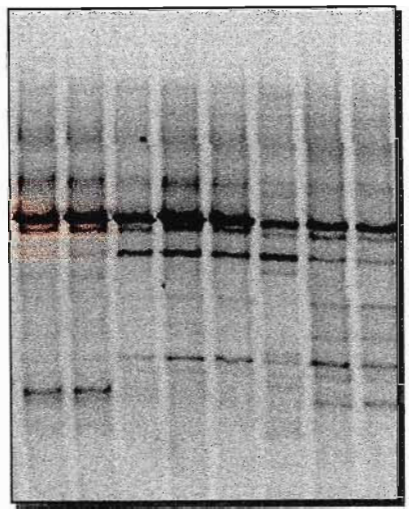


Plate 4.8: DGGE gels, each loaded with the same series of 16S rDNA PCR products. Gel **A** was run with 200 volts for 4 h while gel **B** was run with 70 volts for 16 h.

4.3.2 The Effects of Physiological Factors on Genetic Diversity

Visual comparisons were made of DGGE profiles of microbial association Lf-1 after it had been subjected to continuous cultivation with various pH values (2 to 9), temperatures (5 to 40^0C, increments of 5^0C) and at dilution rates (0.003, 0.004, 0.011, 0.015, 0.021, 0.045 and 0.09 h^{-1}). The Sorenson's index, $C_s = 2j/(a + b)$ which is a pairwise similarity coefficient, was used also to make a statistical comparisons between lanes; j is the number of sequences found common to both sites, a is the number of sequences at site A, and b is the number of sequences at site B (Konopka *et al.*, 1999). A C_s value of 1 indicates homogeneity while one of 0 indicates complete heterogeneity (Murray, Hollibaugh and Orrego, 1996). Deoxyribonucleic acid was isolated after incremental increases in pH, temperature and dilution rate and subjected to the procedures described in 2.9.1 to 2.9.6.

pH

Different bands of the DGGE profile correspond to different PCR-amplified 16S rDNA fragments of bacterial species or strains. The complex pattern of the DGGE fragments is, thus, derived from the bacterial populations and their nucleic acids. Very small differences in the sequences of amplified 16S rDNA fragments,

ranging from 1 to 10 nucleotides, are sufficient usually to separate similar PCR products (Uitterlinden and Vijg, 1989).

In Plate 4.9, Lane 2 represents the control bacterium which was assumed to be a monoculture of *E.coli*. However, the purity of the sample was questioned since 5 bands were present. Teske, Sigalevich, Cohen and Muyzer (1996) demonstrated the effectiveness of the DGGE system for testing the purity of bacterial isolates.

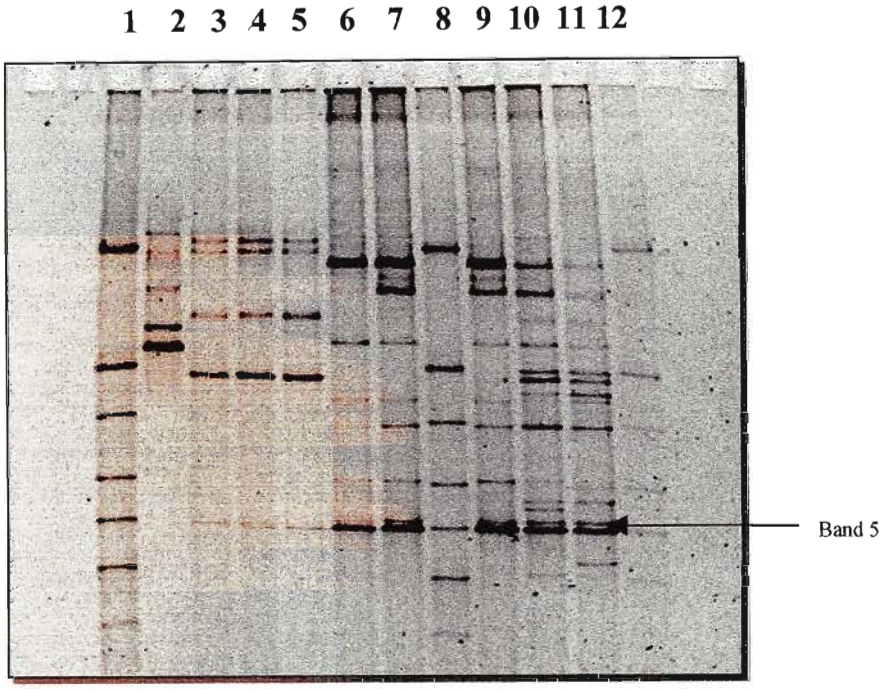


Plate 4.9: DGGE gel of bacterial 16S rDNA PCR products of microbial association Lf-1 after cultivation at different pH values. Lanes 1, 8 and 12, marker. Lane 2, *E.coli* (+ ve control). Lane 3, pH 2. Lane 4, pH 3. Lane 5, pH 4. Lane 6, pH 5. Lane 7, pH 6. Lane 9, pH 7. Lane 10, pH 8. Lane 11, pH 9.

The DGGE pattern (Plate 4.9) can be interpreted as specific bands which emerged and disappeared at different pH values. At the lower pH range (2 to 4), 5 distinct bands were present while the population profile remained homogeneous as confirmed by the C_s value of 1. After cultivation at pH 5, a rather dramatic change in the population profile occurred with an increase in band number and, thus, association diversity. A pair-wise comparison of the profiles for pH 4 and 5 revealed a C_s value of 0.32 which emphasised the change. This increase in species diversity continued through to pH 8 when the association was characterized by 16 distinct bands. For the pH values of 7 and 8, a C_s value of 0.55 indicated that the population profiles had unique phylotypes but also shared common members. There was, however, one

distinct band (5) which was common to all the pH values. Therefore, this was designated a primary species. Similar common bands were apparent for the more limited pH ranges of pH 2 to 4 and 5 to 9. The median C_s values were highest (and similar in magnitude) for comparisons made between the population profiles found near to pH 7. Lower C_s values were found when the profiles of the association, after subjection to acidic conditions, were compared with post-basic condition cultivation. These results suggested that the majority of the component species of microbial association Lf-1 favoured neutral to basic conditions. The gel shows clearly that after cultivation at different pH values, the population profile of the thiosulphate-oxidizing microbial association, changed rather dramatically.

Previous studies (Yang and Allen, 1994; Chung *et al.*, 1996 a; b; c; Degorce-Dumas *et al.*, 1997) have shown that the efficiency of a biofilter to remove H_2S is effected by physio-chemical parameters such as pH. Furthermore, a correlation between biofilter removal efficiency and the presence of non-acidifying bacteria was suggested by Degorce-Dumas *et al.* (1997). The reasons why acidophilic bacteria are unable to ensure effective oxidation of sulphide when the pH falls are not clear (Degorce-Dumas *et al.*, 1997). The observed structural changes in the profile of the association at a pH > 4 supported the assumption that non-acidifying thiosulphate-oxidizing bacteria act more effectively in the pH range of 4.5 to 7.5. Taking these factors into consideration, it is probable that association Lf-1 would be more effective in sulphide oxidation when the packing material of a biofilter was maintained at a pH value > 4.

Temperature

Physiologically, microorganism which exhibit the capacity to utilize reduced sulphur as an energy source are very diverse with a wide range of optimum temperatures for growth (Kelly, 1989). It was, therefore, expected that at various temperatures different subsets of the microbial association would be dominant. This was illustrated clearly in Plate 4.10 where the species composition of the microbial association Lf-1 changed as the temperature increased from 5 to 40^0C. A general trend of increased diversity was apparent with temperature increase. Two primary subsets of the association were apparent (Bands 1 and 2) which dominated over the temperature range. This dominance would be advantageous in a biofilter where temperature fluctuations were possible.

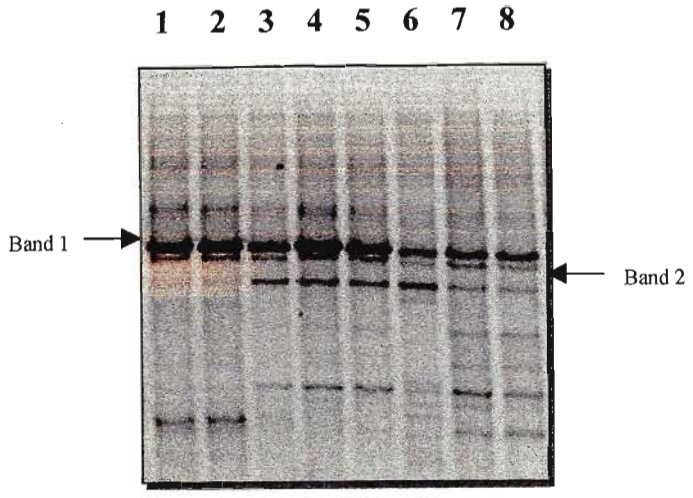


Plate 4.10: DGGE gel of bacterial 16S rDNA PCR products of microbial association Lf-1 following continuous cultivation at discrete temperatures. Lanes 1 to 8, temperatures 5 to 40^{0}C in increments of 5 degrees.

Dilution Rate

The isolation and profiling of association Lf-1 was carried out after continuous cultivation at discrete dilution rates to determine if any qualitative changes in species composition had resulted. The objectives were to determine if: any species was washed out of the bioreactor; any species was enriched at low dilution rates; and the faster and slower growing species could be discriminated.

As the dilution rate increased, the faster-growing species dominated as clearly shown in Plate 4.11. It is interesting to note that the greatest change in the genetic profile occurred at dilution rates < 0.011 h^{-1} ($C_s = 0.50$) through elimination of a number of species. Conversely, at dilution rates ≥ 0.011 h^{-1} the profiles achieved a degree of homogeneity ($C_s = 0.94$) due possibly to comparable maximum specific growth rates of the component species and the displacement of the slower-growing species. An association which contains species with different maximum specific growth rates may be an advantage in a biofilter where the hydraulic loading changes. Furthermore, the stabilization of the structural components of the association over a broad dilution rate range (0.011 to 0.09 h^{-1}) suggests that a biofilter would cope with different loading rates. Lane 12 shows the profile of Lf-1 after maintenance in the continuous culture effluent reservoir. The dramatic changes in the profile were attributed to high concentrations of sulphate, low pH, oxygen limitation and no residual substrate. However, an interesting feature was the increase in species diversity under conditions which one would have assumed to be unfavourable. An

unexplained phenomenon of this gel was the increase in band number for the marker possibly due to PCR bias (4.3).

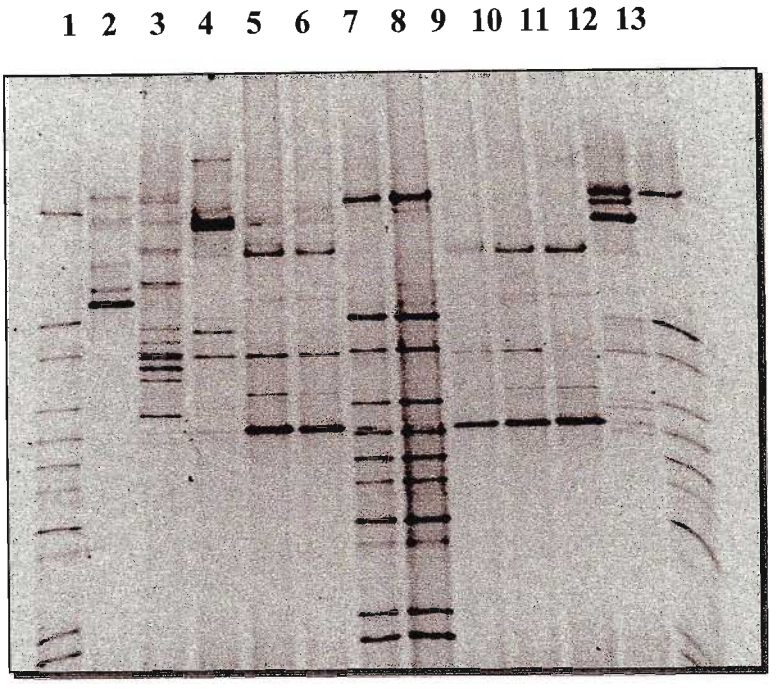


Plate 4.11: DGGE gel of bacterial 16S rDNA PCR products of microbial association Lf-1 at different dilution rates. Lane 1, marker. Lane 2, *E. coli*. Lane 3, 0.003 h^{-1}. Lane 4, 0.004 h^{-1}. Lane 5, 0.011 h^{-1}. Lane 6, 0.015 h^{-1}. Lanes 7 and 8, marker. Lane 9, 0.021 h^{-1}. Lane 10, 0.045 h^{-1}. Lane 11, 0.09 h^{-1}. Lane 12, effluent of Lf-1 at 0.09 h^{-1}. Lane 13, marker.

4.3.3 Comparison of Phylotypes

The genetic diversity of the 16S rDNA amplified from samples of the three microbial associations was examined. Monthly samples of bioreactor (0.02 h^{-1}, 30^{0}C) cultures were taken for 3 consective months. For this period the pH for Lf-1 was maintained at 6 (±0.2) while for Ws-2 and Am-3 the pH values were consistently in the range of 1.5 to 2. For Lf-1, 8 bands were obtained while for Ws-2 and Am-3 4 and 1 bands were visualized, respectively (Plate 4.12). The detection of 8 bands in Lf-1 indicated the presence of at least 8 different species. This result was contrary to the generally accepted rule that a single growth-limiting substrate permits only one species to become established in a continuous culture (Taylor and Williams, 1974; Yoon, Klinzing, Blanch, 1977). Dramatic changes in the population profiles of Lf-1 and Ws-2 were apparent between months 1 and 2 with C_s values of 0.5 and 0.25 recorded, respectively. These changes could be attributed possibly to an acclimation

period and/or a selective advantage of the species under these conditions. The highest ability to obtain energy from reduced sulphur compounds should confer an advantage to the specific microorganism during competition (Pirt, 1975). The surprising result with Am-3 may be attributed to the presence of a fungal species. Fast growing species, such as fungi, can displace competitively bacteria at low pH values (Cox *et al.,* 1993). A pair-wise comparison of the acclimated population profiles of Lf-1 and Ws-2 (month 2) gave a C_s value of 0 which indicates dissimiliar phylotypes. Differences in the initial inoculum source material, pH and medium were probably the main contributors to the phylotype differences.

Although DGGE visualizes both the dominant species of an association and species which constitute < 1% of the total population (Muyzer *et al.,* 1993), it may not visualize the composition of the whole community. Factors, such as the possibility of positional overlap of bands of two different species and, with very diverse samples, the mass of individual products being too small to be visualized in a gel (Konopka *et al,* 1999), account for imperfect phylotypic profiles. Thus, the profiles of Plate 4.12 should not be regarded as comprehensive.

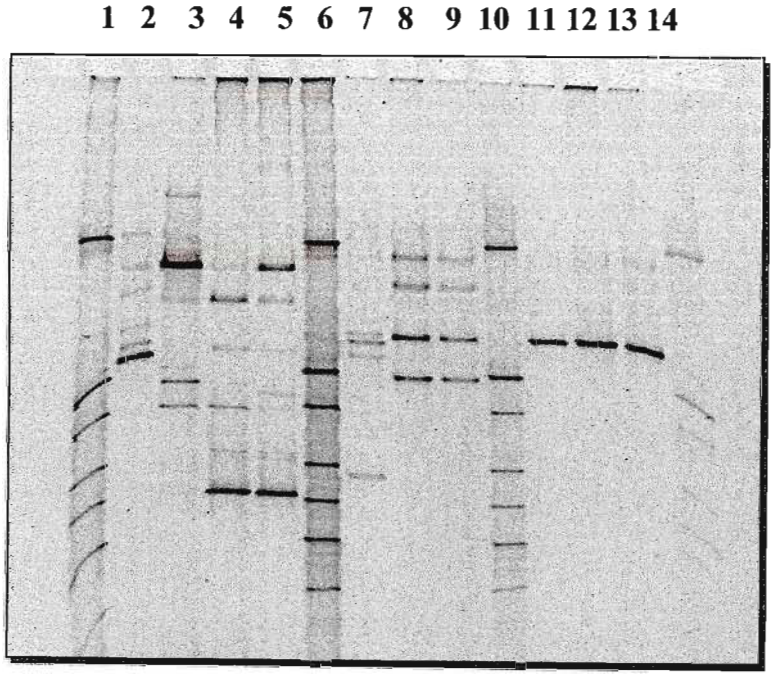


Plate 4.12: Negative image of ethidium bromide-stained DGGE gel showing separation patterns obtained for microbial associations Lf-1, Ws-2 and Am-3. Lanes 1, 6 and 14, DGGE marker. Lane 2, *E.coli* (+ ve control). Lanes 3 to 5, samples of Lf-1 taken at one-month intervals. Lanes 7 to 9, samples of Ws-2 taken at monthly intervals. Lanes 11 to 13, samples of Am-3 taken at monthly intervals.

4.3.4 Biolog GN Plate Analysis

When the effects of environmental perturbations on microbial associations are estimated by the patterns of sole carbon source utilization (Biolog), it is important to determine whether the population profile present in the community at the time of sampling is the same as that which accounts for the results of the assay (Smalla *et al.*, 1998). Although it is accepted generally that during the course of the assay substantial growth occurs, it is still assumed that the profile reflects the initial catabolic potential of the community. Any species composition change invalidates possible extrapolations (Smalla *et al.*, 1998).

In this study (2.9.7), DGGE comparison of the four different Biolog wells with the control (initial inoculum) clearly demonstrated a significant alteration in profile (mean C_s =0.59)(Plate 4.13). Although the population profiles of microbial association Lf-1 after batch cultivation in the presence of glucose and sucrose were identical (C_s =1), and comparable after cultivation with other substrates (D-fructose and D-galactose), there were differences in the numbers and mobilities of the bands in the profiles. Since changes were apparent compared with the control, it appeared that various subsets of the profiles had been selected/enriched.

From the results it can be concluded that substrate oxidation reflected changes in the community structure, thus interpretation of Biolog data must be made with caution.

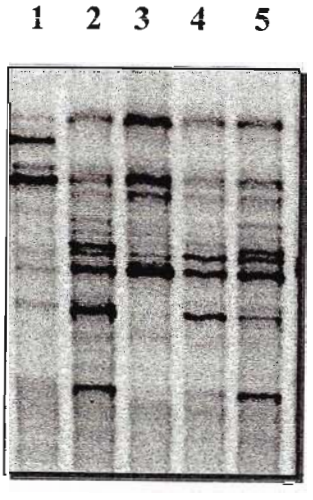


Plate 4.13: 16S rDNA DGGE profiles of microbial association Lf-1 after batch culture (30^0C, 72 h) in a Biolog plate. Lane 1, source inoculum. Lane 2, α-D-glucose. Lane 3, D-fructose. Lane 4, D-galactose. Lane 5, sucrose.

4.4 Future Research

Additional work is necessary to characterize the major bands observed in the DGGE analyses. Sequencing the fragments should facilitate the determination of the phylogenetic positions of the bacteria and, subsequently, enable an evaluation of the ecological roles of the different species. Recently, the sequencing of six *Thiomicrospira* species resulted in the creation of a phylogenetic framework to characterize *Thiomicrospira*-related molecular isolates from hydrothermal vent microbial communities (Muyzer, Teske, Wirsem and Jannasch, 1995). Sequencing is one of the major advantages of DGGE as the bands can be excised directly from the DGGE gel, re-amplified and sequenced directly without the need for cloning. Thus, an accumulating database of nucleotide sequences can be built from which species-specific oligonucleotide probes can be designed. This allows identification *in situ* of particular constituents of a population (Muyzer and Ramsing, 1995).

The creation of a species-specific probe can be used in a genus-specific PCR where amplification of the 16S rDNA of the bacterium of interest allows detection. The formation of product indicates the presence of such a bacterium. A polymerase chain reaction specific for *Thiomicrospira* sp, a sulphur-oxidizing bacterium, was used by Brinkhoff and Muyzer (1997) to detect this species in different habitats. This emphasizes the importance of sequencing, identifying and creating species-specific probes for the major bands present in the isolated associations. This is particularly important since many microorganisms are unculturable. This approach would rapidly and efficiently characterize microbial associations.

By using the above technique, attempts can be made to study enriched and isolated microorganisms during biofilter optimization by monitoring any changes at the genetic level. Furthermore, the results of this study demonstrated that this technique is a powerful tool to elucidate genetic diversity changes of uncharacterized microbial associations in response to various physiological conditions. Thus, together with providing fundamental insights, the use of DGGE might, in future, serve as a high-tech', diagnostic tool for troubleshooting biofilters exhibiting poor performance. The immediate extension of the study is to determine the impacts of profile changes on the efficacy of thiosulphate oxidation.

Chapter 5

General Discussion

Biofiltration is being applied increasingly in the purification of waste gases although the fundamental processes involved are still poorly understood. This has led to unsuccessful or suboptimum operation of large-scale biofilters (Deshusses, 1997). It was in this context that physiological and molecular techniques were used to gain insight of microbial associations capable of catabolizing thiosulphate.

Of the three potential hydrogen sulphide-oxidizing associations evaluated and characterized in this study, microbial association Lf-1 exhibited the greatest efficacy due to its ability to oxidize sulphide at concentrations < 40 ppm to elemental sulphur (3.2.8) and its maximum specific growth rate of 0.08 h^{-1} (3.2.5). Although the μ_{max} was higher than those of associations Ws-2 and Am-3 it would still present a limitation for gas biofiltration. Since concentrations > 40 ppm may be encountered with landfill/industrial off-gas, gas dilution may be required before biofiltration. There is, therefore, a need to develop and configure a biofiltration system which would minimize the limitations imposed by slow growth rate and sulphide inhibition.

The ability of microbial association Lf-1 to retain its catabolic potential at pH values of 3 to 6 and temperatures of 5 to 30^{0}C demonstrated its resilience to possible perturbations (3.2.5). Such characteristics are particularly important as pH decreases in H_2S-oxidizing biofilters are common (Degorce-Dumas *et al.*, 1997), especially after prolonged use or in response to temperature fluctuations. Since hydrogen sulphide solubility decreases as the pH decreases, and both biological and chemical oxidation are promoted at higher pH values, the maintenance of a near-neutral pH environment is important. For Lf-1, the pH range/value which facilitated the maximum specific growth rate and the highest sulphate production were 3 to 4 and 5, respectively. Thus, optimum biofilter operation must take cognizance of all these variables.

For the same association, a temperature optimum of 25^{0}C was recorded although maximum sulphate production (catabolic potential) occurred at 10^{0}C. In addition, the association survived temperatures up to 65^{0}C for an hour which commend it for use in landfill/industrial off-gas treatment where temperature fluctuations are apparent. Since hydrogen sulphide solubility decreases with increases

in temperature (Britton, 1994), the ability of Lf-1 to retain its catabolic potential at low temperatures may improve removal efficiencies. Contrary to the findings of Yang and Allen (1994) a dramatic reduction in the catabolic potential of the association was not recorded when the temperature was lowered from 25 to 7.5^{0}C. It is probable, therefore, that association Lf-1 could be successful in colder climates. As landfill and industrial off-gas temperatures often exceed 25^{0}C, influent gas cooling may be essential to maximize biofiltration efficacy.

Industrial/landfill production of hydrogen sulphide is often intermittent and of variable composition. Biofilter populations must, therefore, cope with variable loadings. Therefore, a successful H_2S-oxidizing association should have: a low maintenance energy requirement; respond quickly to increased loadings (Tappe *et al.*, 1999); and catabolize different concentrations of the target gas. The low maintenance energy value of 0.0004 g dry weight per mole (3.2.6) and the ability of association Lf-1 to catabolize different concentrations of thiosulphate (3.1.2) again commend it for possible application in gas biofiltration.

Although Lf-1 was categorized as a facultative chemolithotroph, growth in the presence of an organic carbon source (3.1.2) had a negative effect on the oxidation of thiosulphate to sulphate. Therefore, the maintenance of an autotrophic environment within the biofilter may be essential.

Denaturing-gradient gel electrophoresis was used to define the catabolic profile of Lf-1 and its changes in response to physiological variables. Following enrichment/isolation, species composition changes were apparent and a similar result was recorded for association Ws-2 (4.3.3). It thus appeared that biofilters may require an initial period of acclimation. As a result of different physiological (pH and temperature) and kinetic (dilution rate) conditions population changes in microbial association Lf-1 manifested. Although structural changes were effected between pH values of 4 and 5, they were not accompanied by marked functional potential or μ_{max} changes. Similar analyses following exposure to different temperatures showed that this variable rather than the attendant population change dictated the functional potential. Also, it became apparent that a general trend of increased species diversity, but not functional potential, resulted from pH and temperature increases. Thus, for biofiltration of H_2S, these physiological parameters are the dominant efficacy determinants.

Together with demonstrating DGGE as an efficient tool to study compositional changes in biofilters, Biolog GN plates proved effective for determining species composition changes (3.2.7). Since, most Biolog GN microtitre plate studies have been used to resolve changes in heterotrophic associations, the ability to discriminate changes in an autotrophic microbial association, based on heterotrophic capabilities, was an interesting phenomenon. Although successful for detecting compositional changes, a fundamental flaw in the Biolog assay was demonstrated by DGGE since analyses of randomly-selected Biolog wells identified substrate-mediated species diversity increases and, possibly, changes in dominance through selective enrichment (Smalla *et al.*, 1998).

Although several fundamental flaws are inherent with both DGGE and the Biolog assay, both are useful tools for microecophysiology research. Thus, together with traditional microbiological techniques, they should be used routinely to evaluate underperforming biofilters and probe the complex microbial dynamics and attendant functional potentials.

The results of this study, particularly the physiological characterizations, can be extrapolated to a model biofilter and so facilitate the development of a specific operation protocol for H_2S biofiltration.

References

Alexander, B., Leach, S. and Ingledew, W.J. (1987). The relationship between chemiosmotic parameters and sensitivity to anions and organic acids in the acidophile Thiobacillus ferrooxidans. *Journal of General Microbiology* 133, 1171-1179.

Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews* 59, 143-169.

Andreoni, V., Origgi, G., Colombo, M., Calcaterra, E. and Colombi, A. (1997). Characterization of a biofilter treating toluene contaminated air. *Biodegradation* 7, 397-404.

Arnheim, N. and Erlich, H. (1992). Polymerase chain reaction strategy. *Annual Review of Biochemistry* 61, 131-156.

Atlas, R.M., Cohen, G., Hershberger, C.L., Hu, W.-S., Sherman, D.H., Wilson, R.C. and Wu, J.H. (1999). Manual of Industrial Microbiology and Biotechnology. ASM Press, Washington, D.C.

Atlas, R.M. and Parks, L.C. (1993). Handbook of Microbiological Media. CRC Press, Boca Raton.

Baloo, S. and Ramkrishna, D. (1991). Metabolic regulation in bacterial continuous culture. *Biotechnology and Bioengineering* 38, 1337-1352.

Barfold, J.P., Pamment, N.B. and Hall, R.J. (1982). Lag phases and transients. In: Microbial Population Dynamics. Bazin, M.J. (editor). CRC Press, Boca Raton, pp 55-90.

Barnes, W.M. (1994). PCR amplification of up to 35-kb DNA with high fidelity and high yield from λ bacteriophage templates. *Proceedings of the National Academy of Siences USA* 91, 2216-2220.

Becker, P.M. and Stottmeister, U. (1998). General (Biolog GN) versus site-relevant (pollutant-dependent) sole-carbon-source utilization patterns as a means to approaching community functioning. *Canadian Journal of Microbiology* 44, 913-919.

Beudeker, R.F., Gottschal, J.C. and Kuenen, J.G. (1982). Reactivity versus flexibility in thiobacilli. *Antonie van Leeuwenhoek* 48, 39-51.

Bohn, H.L. (1976). Compost scrubbers of malodorous air streams. *Compost Science* 17, 5-6.

Bohn, H. (1992). Consider biofiltration for decontaminating gases. *Chemical Engineering Progress* 88, 34-40.

Brannan, D.K. and Caldwell, D.E. (1983). Growth kinetics and yield coefficients of the extreme thermophile *Thermothrix thiopara* in continuous culture. *Applied and Environmental Microbiology* 45, 169-173.

Brimblecombe, P. (1989). Evolution of the Global Biogeochemical Sulfur Cycle. Wiley-Liss Publishers, New York.

Brinkhoff, T. and Muyzer, G. (1997). Increased species diversity and extended habitat range of sulphur-oxidizing *Thiomicrospira spp. Applied and Environmental Microbiology* 63, 3789-3796.

Britton, G. (1994). Wastewater Microbiology. Wiley-Liss Publishers, New York.

Buisman, C.J.N., Ijspeert, P., Hof, A., Janssen, A.J.H., ten Hagen, R. and Lettinga, G. (1991). Kinetic parameters of a mixed culture oxidizing sulphide and sulphur with oxygen. *Biotechnology and Bioengineering* 38, 813-820.

Bull, A.T. and Slater, J.H. (1982). Microbial Interactions and Communties, Volume 1. Academic Press, London.

Bulthuis, B.A., Frankena, J., Koningstein, G.M., van Verseveld, H.W. and Stouthamer, A.H. (1988). Instability of protease production in a rel^+/rel^- pair of *Bacillus licheniformis* and associated morphological and physiological characteristics. *Antonie van Leeuwenhoek* 54, 95-111.

Bushell, M.E. and Slater, J.H. (1981). Mixed Culture Fermentations. Academic Press, London.

Cadena, F. and Peters, R.W. (1988). Evaluation of chemical oxidizers for hydrogen sulphide control. *Journal of Water Pollution Control Federation* 60, 1259-1263.

Cadenhead, P. and Sublette, K.L. (1989). Oxidation of hydrogen sulfide by *Thiobacilli. Biotechnology and Bioengineering* 35,1150-1154.

Carlson, K.Y. and Leiser, C.P. (1966). Soil beds for the control of sewage odours. *Journal of Water Pollution Control* 38, 829-834.

Cassel, E.A., Sulzer, F.T. and Lamb, J.C. (1966). Population dynamics and selection in continuous mixed cultures. *Journal of Water Pollution Control* 38, 1398-1409.

Chambers, J.A.A. and Rickwood, D. (1993). Biochemistry Labfax. Bios Scientific Publishers, Oxford.

Chandler, J.A. (1977). X-Ray Microanalysis in the Electron Microscope. North-Holland Publishers, Amsterdam.

Chen, K.Y. and Morris, J.C. (1972). Kinetics of oxidation of aqueous sulfide by O_2. *Environmental Science and Technology* 6, 529-529.

Chiu, S.Y., Fan, L.T., Kao, I.C. and Erickson, L.E. (1972). Kinetic behaviour of mixed populations of activated sludge. *Biotechnology and Bioengineering* 14, 179-199.

Cho, K.S., Hirai, M. and Shoda, M. (1991). Degradation characteristics of hydrogen sulphide, methanethiol, dimethyl sulphide and di-methyl disulphide by *Thiobacillus thioparus* DW44 isolated from peat biofilter. *Journal of Fermentation and Bioengineering* 71, 384-389.

Cho, K.S., Hirai, M. and Shoda, M. (1992). Enhanced removal efficiency of malodorous gasses in a pilot-scale peat biofilter inoculated with *Thiobacillus thioparus* DW44. *Journal of Fermentation and Bioengineering* 73, 46-50.

Cho, K.S., Zhang, L., Hirai, M. and Shoda, M. (1991). Removal characteristics of hydrogen sulphide and methanethiol by *Thiobacillus sp.* isolated from peat in biological deodorization. *Journal of Fermentation and Bioengineering* 71, 44-49.

Choi, K.H. and Dobbs, F.C. (1999). Comparison of two kinds of Biolog microplates (GN and ECO) in their ability to distinguish among aquatic microbial communities. *Journal of Microbiological Methods* 36, 203-213.

Chung, Y.C., Huang, C. and Li, C.F. (1997). Removal characteristics of H_2S by *Thiobacillus novellus* CH 3 biofilter in autotrophic and mixotrophic environments. *Journal of Enviromental Science and Health* 32, 1435-1450.

Chung, Y.C., Huang, C. and Tseng, C.P. (1996a). Operation optimization of *Thiobacillus thioparus* CH11 biofilter for hydrogen sulphide removal. *Journal of Biotechnology* 52, 31-38.

Chung, Y.C., Huang, C. and Tseng, C.P. (1996b). Microbial oxidation of hydrogen sulphide with biofilter. *Journal of Environmental Science and Health* 31, 1263-1278.

Chung, Y.C., Huang, C. and Tseng, C.P. (1996c). Biodegradation of hydrogen-sulphide by a laboratory-scale immobilized *Pseudomonas putida* CH11 biofilter. *Biotechnology Progress* 12, 773-778.

Chung, Y.C., Huang, C. and Tseng, C.P. (1997). Removal of hydrogen sulphide by immobilized *Thiobacillus* sp. strain CH11 in a biofilter. *Journal of Chemical Technology and Biotechnology* 69, 58-62.

Conil, P. (1999). Sulphurous compounds in tropical wastewater treatment systems: problems and solutions. *Water* 21, 58-60.

Corsi, R.L. and Seed, L. (1995). Biofiltration of BTEX: media, substrate, and loading effects. *Environmental Progress* 14, 151-157.

Cox, H.H.J., Houtman, J.H.M., Doddema, H.J. and Harder, W. (1993). Enrichment of fungi and degradation of styrene in biofilters. *Biotechnology Letters* 15, 737- 742.

Das, S.K., Mishra, A.K., Tindall, B.J., Rainey, F.A. and Stackebrandt, E. (1996). Oxidation of thiosulphate by a new bacterium, *Bosea thiooxidans* (strain B1-42) gen. nov., sp. nov.: analysis of phylogeny based on chemotaxonomy and 16S ribosomal DNA sequencing. *International Journal of Systematic Bacteriology* 46, 981-987.

Dawes, C.J. (1979). Biological Techniques for Transmission and Scanning Electron Microscopy. Ladd Research Industries Publishers, Vermont.

Degorce-Dumas, J.R., Kowal, S. and Le Cloirec, P. (1997). Microbiological oxidation of hydrogen sulphide in a biofilter. *Canadian Journal of Microbiology* 43, 264-271.

Dell'Orco, M.J., Chadik, P.A., Britton, G. and Neumann, R.P. (1998). Sulfide-oxidizing bacteria: their role during air stripping. *Journal of the American Water Works Association* 90, 107-115.

Demain, A.L. (1972). Cellular and environmental factors affecting the synthesis and excretion of metabolites. *Journal of Applied Chemistry and Biotechnology* 22, 345-362.

Deshusses, M.A. (1997). Transient behaviour of biofilters: start-up, carbon balances, and interactions between pollutants. *Journal of Environmental Engineering* 123, 563-568.

Deshusses, M.A., Hamer, G. and Dunn, I.J. (1995a). Behaviour of biofilters for waste air biotreatment. 1. Dynamic model development. *Environmental Science and Technology* 29, 1048-1058.

Deshusses, M.A., Hamer, G. and Dunn, I.J. (1995b). Behaviour of biofilters for waste air biotreatment. 2. Experimental evaluation of a dynamic model. *Environmental Science and Technology* 29, 1059-1068.

Dykhuizen, D.E. and Hartl, D. (1983). Selection in chemostats. *Microbiological Reviews* 47, 150-168.

Enfors, S.O. (1991). European Federation of Biotechnology. B Kidric Institution of Chemistry, Germany.

Engelen, B., Meinken, K., von Wintzingerode, F., Heuer, H., Malkomes, H.P. and Backhaus, H. (1998). Monitoring impact of a pesticide treatment on bacterial soil communities by metabolic and genetic fingerprinting in addition to conventional testing procedures. *Applied and Environmental Microbiology* 64, 2814-2821.

Ergas, S.J., Schroeder, E.D., Chang, D.P.Y. and Morton, R.L. (1995). Control of volatile organic compound emissions using a compost biofilter. *Water Environment Research* 67, 816-821.

Fantroussi, S.E., Verschuere, L., Verstraete, W. and Top, E.M. (1999). Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. *Applied and Environmental Microbiology* 65, 982-988.

Felske, A., Engelen, B., Nubel, U. and Backhaus, H. (1996). Direct ribosomal isolation from soil to extract bacterial rRNA for community analysis. *Applied Environmental Microbiology* 62, 4162-4167.

Ferris, M. J., Nold, S. C., Revsbech, N. P. and Ward, D. M. (1997). Population structure and physiological changes within a hot spring microbial mat community following disturbance. *Applied and Environmental Microbiology* 63, 1367-1374.

Fischer, S. and Lerman, L. (1983). DNA fragments differing by single base pair substitutions are separated in denaturing-gradient gels: correspondence with melting theory. *Proceedings of the National Academy of Sciences USA* 80, 1579-1583.

Fouhy, K. (1992). Cleaning waste gas, naturally. *Chemical Engineering* 99, 41-46.

Gadre, R.V. (1989). Removal of hydrogen sulphide from biogas by chemolithoautotrophic fixed-film bioreactor. *Biotechnology and Bioengineering* 34, 410-414.

Garland, J.L. and Mills, A.L. (1991). Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Applied and Environmental Microbiology* 57, 2351-2359.

Glimm, E., Heuer, H., Engelen, B., Smalla, K. and Backhaus, H. (1997). Statistical comparisons of community catabolic profiles. *Journal of Microbiological Methods* 30, 71-80.

Gommers, P.J.F. and Kuenen, J.G. (1988). *Thiobacillus* strain Q, a chemolithoheterotrophic sulphur bacterium. *Archives of Microbiology* 150, 117-125.

Gottschal, J.C. (1992). Continuous culture. In: Encyclopedia of Microbiology. Lederberg, J. (editor). Academic Press, New York, pp 559-572.

Gottschal, J.C. and Kuenen, J.G. (1980). Mixotrophic growth of *Thiobacillus* A2 on acetate and thiosulphate as growth limiting substrates in the chemostat. *Archives of Microbiology* 126, 33-42.

Greer, L.E., Robinson, J.A. and Shelton, D.R. (1992). Kinetic comparison of seven strains of 2,4-dichlorophenoxyacetic acid degrading bacteria. *Applied and Environmental Microbiology* 58, 1027-1030.

Haack, S.K., Garchow, H., Klug, M.J. and Forney, L.J. (1995). Analysis of factors affecting the accuracy, reproducibility and interpretation of microbial community carbon source utilization patterns. *Applied and Environmental Microbiology* 61, 1458-1468.

Hamer, G. (1975). Problems of wall growth in industrial fermentations. *Society for General Microbiology: Proceedings* 3, 69.

Hao, O.J., Chen, J.M., Huang, L. and Buglass, R.L. (1996). Sulphate-reducing bacteria. *Critical Reviews in Environmental Science and Technology* 26, 155-187.

Harrison, D.E.F. (1978). Mixed cultures in industrial fermentation processes. *Advances in Applied Microbiology* 24, 129-164.

Hart, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. and Williams, S.T. (1994). Bergey's Manual of Determinative Bacteriology. Williams and Wilkins Publishers, Baltimore.

Hayat, M.A. (1981). Fixation for Electron Microscopy. Academic Press, New York.

Hayat, M.A. and Miller, S.E. (1990). Negative Staining. McGraw-Hill Publishing Company, United States.

Hedoin, H., Coute, A., Kaiser, P. and Laugier, R. (1996). Nature and occurrence of sulfoxidising bacteria in Bare'gine developing in sulphurated thermal waters at Bare'ges (France). *Hydrobiologia* 323, 75-81.

Henshaw, P.F., Bewtra, J.K. and Biswas, N. (1997). Extraction of elemental sulphur from an aqueous suspension for analysis by high-performance liquid chromatography. *Analytical Chemistry* 69, 3119-3123.

Henshaw, P.F., Bewtra, J.K. and Biswas, N. (1998). Hydrogen sulphide conversion to elemental sulphur in a suspended-growth continuous stirred tank reactor using *Chlorobium limicola*. *Water Research* 32,1769-1778.

Herbert, D., Elsworth, R. and Telling, R.C. (1956). The continuous culture of bacteria: a theorectical and experimental study. *Journal of General Microbiology* 14, 601-622.

Heuer, H. and Smalla, K. (1997). Evalution of community-level catabolic profiling using Biolog GN microplates to study microbial community changes in potato phyllosphere. *Journal of Microbiological Methods* 30, 49-61.

Hodge, D.S. and Devinny, J.S. (1994). Biofilter treatment of ethanol vapours. *Environmental Progress* 13, 167-173.

Hodge, D.S. and Devinny, J.S. (1997). Determination of transfer rate constants and partition coefficients for air phase biofilters. *Journal of Environmental Engineering* 123, 577-585.

Hong, J. and Lee, C.K. (1987). Unsteady-state operation of continuous fermentor for enhancement of cell mass production. *Biotechnology and Bioengineering* 30, 187-195.

Hoss, M., Kohn, M., Paabo, S., Knauer, F. and Schroeder, W. (1992). Excrement analysis by PCR. *Science* 359, 199.

http://chemengineer.miningco.com/education/chemengineeer/library/weekly/aa031698.htm (24/12/1999).Biofilter equipment and design.

http://indigo.ie/~hibernia/index.html (24/12/1999). Hibernia Export Trading House Limited.

http://trgbiofilter.com (21/2/00).

http://www.ecs.umass.edu/cee/reckhow/courses/370/37015/sld004.htmm (18/1/2000). Henry's Law constants.

http://www.instanet.com/~pfc/files/h2s.htm (24/12/1999). Hydrogen sulphide.

http://www.rhenipal.com/productserv/Biofilter/casestudybiofilter.htm (24/12/1999). Case study biofilter.

Hungate, R.E. (1961). Ecology of bacteria. In: The Bacteria. Gunsalus, I.C. and Stanier, R.Y. (editors). Academic Press, New York, pp 95-118.

Hunter, K. and Rose, A.H. (1972). Influence of growth temperature on the composition and physiology of microorganisms. *Journal of Applied Chemistry and Biotechnology* 22, 527-540.

Ingraham, J.E., Maaloe, O. and Neidhardt, F.C. (1983). Growth of the bacterial cell. Sinauer Associates Inc., United States of America.

Jannasch, H.W. (1967). Enrichments of aquatic bacteria in continuous culture. *Archiv fur Mikrobiologie* 59, 165-173.

Janssen, A.J.H., de Hoop, K. and Buisman, C.J.N. (1997). The removal of H_2S from air at a petrochemical plant. In: Biological Waste Gas Cleaning. Prins, W.L. and van Ham, J. (editors). VDI Verlag Gmbh Publishers, The Netherlands, pp 359-364.

Janssen, A.J.H., De Keizer, A. and Lettinga, G. (1994). Colloidal properties of a microbiologically produced sulphur suspension in comparison to a LaMer sulphate solution. *Colloids Surfaces B: Biointerfaces* 3, 111-117.

Janssen, A.J.H., Ma, S.C., Lens, P. and Letttinga, G. (1997). Performance of a sulphide-oxidizing expanded-bed reactor supplied with dissolved oxygen. *Biotechnology and Bioengineering* 53, 32-40.

Janssen, A., Marcelis, C. and Buisman, C. (1999). Industrial applications of new sulfur biotechnology. *Water* 21, 55-57.

Janssen, A.J.H., Sleyster, R., Van der Kaa, C., Jochemsén, A., Bontsema, J. and Lettinga, G. (1995). Biological sulphide oxidation in a fed-batch reactor. *Biotechnology and Bioengineering* 47, 327-333.

Kelly, D.P. (1982). Biochemistry of the chemolithotrophic oxidation of inorganic sulphur. *Philosophical Transactions. Royal Society of London Series B* 298, 473-497.

Kelly, D.P. (1985). Physiology of the thiobacilli: elucidating the sulphur oxidation pathway. *Microbiological Science* 2, 105-109.

Kelly, D.P. (1989). Physiology and biochemistry of unicellular sulphur bacteria. In: Autotrophic Bacteria. Schlegel, H.G. and Bowen, B. (editors). Science Tech Publishers, USA, pp 193-217.

Kelly, D.P., Shergill, J.K., Lu, W. and Wood, A.P. (1997). Oxidative metabolism of inorganic sulfur compounds by bacteria. *Antonie van Leeuwenhoek* 71, 95-107.

Konopka, A., Bercot, T. and Nakatsu, C. (1999). Bacterioplankton community diversity in a series of thermally stratified lakes. *Microbial Ecology* 38, 126-135.

Konopka, A., Oliver, L. and Turco, R.F. (1998). The use of carbon substrate utilization patterns in environmental and ecological microbiology. *Microbial Ecology* 35, 103-115.

Kosky, K.F. and Neff, C.R. (1988). Innovative biological degradation system for hydrocarbon treatment. Proceedings, NWWA/API Petroleum Hydrocarbons and Organic Chemicals in Ground Water Conference, Houston, pp 119-132.

Kowalchuk, G. A., Stephen, J. R., De Boer, W., Prosser, J.I., Embley, T.M. and Woldendorf, J.W. (1997). Analysis of ammonia-oxidising bacteria of the ß subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. *Applied and Environmental Microbiology* 63, 1480-1497.

Kreader, C.A. (1996). Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Applied and Environmental Microbiology* 62, 1102-1106.

Kuenen, J.G. (1975). Colourless sulfur bacteria and their role in the sulfur cycle. *Plant and Soil* 43, 49-76.

Kuenen, J.G. and Beudeker, R.F. (1982). Microbiology of *thiobacilli* and other sulfur-oxidising autotrophs, mixotrophs and heterotrophs. *Philosophical Transactions. Royal Society of London Series B* 298, 473-497.

Kuenen, J.G. and Robertson, L.A. (1992). The use of natural bacterial populations for the treatment of sulfur containing wastewater. *Biodegradation* 3, 239-254.

Lang, M.E. and Jager, R.A. (1982). Odor control for municipal sludge composting. *Biocycle* 33, 76-85.

Langenhove, H.V., Bendinger, B., Oberthur, R. and Schamp, N. (1992). Organic sulfur compounds: persistent odorants in biological treatment of complex waste gases. In: Biotechniques for Air Pollution Abatement and Odour Control Policies. Dragt, A.J. and van Ham, J. (editors). Elsevier Science Publishers, Amsterdam, pp 177-182.

Langenhove, H.V., Wuyts, E. and Schamp, N. (1986). Elimination of hydrogen sulphide from odorous air by a wood bark biofilter. *Water Research* 20, 1471-1476.

Lee, S.K. and Shoda, M. (1989). Biological deodorization using activated carbon fabric as a carrier of microorganisms. *Journal of Fermentation and Bioengineering* 68, 437-442.

Lens, P. and Pol, L. H. (1999). Challenges for environmental bioprocesses using the sulfur cycle. *Water* 21, 50-53.

Leson, G. and Winer, A.M. (1991). Biofiltration: An innovative air pollution control technology for VOC emissions. *Journal of Air Waste Management Association* 41, 1045-1053.

Lide, D.R. (1998). Handbook of Chemistry and Physics 79th Edition. CRC Press, Boca Raton.

Liesack, W., Weyland, H. and Stackebrandt, E. (1991). Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed culture of strict barophilic bacteria. *Microbial Ecology* 21, 191-198.

Lu, W.-P. and Kelly, D.P. (1988). Kinetic and energetic aspects of inorganic sulphur compound oxidation by *Thiobacillus tepidarius*. *Journal of General Microbiology* 134, 865-876.

Margulis, L., Chase, D. and Guerrero, R. (1986). Microbial communities. *BioScience* 36, 160-170.

Martin, S., Maranon, E. and Sastre, H. (1997). Landfill gas extraction technology: study, simulation and manually controlled extraction. *Bioresource Technology* 62, 47-54.

Mason, J. and Kelly, D.P. (1988). Mixotrophic and autotrophic growth of *Thiobacillus acidophilus* on tetrathionate. Archives of Microbiology 149, 317-323.

Mason, J., Kelly, D.P. and Wood, A.P. (1987). Chemolithotrophic and autotrophic growth of *Thermothrix thiopara* and some thiobacilli on thiosulphate and polythionates, and a reassessment of the growth yields of *Thx. thiopara* in chemostat culture. *Journal of General Microbiology* 133, 1249-1256.

Mataleles, R.I., Ryue, D.Y. and Ysuda, T. (1965). Measurement of unsteady state growth rates of microorganisms. *Nature (London)* 208, 263-264.

Matin, A. (1978). Organic nutrition of chemolithotrophic bacteria. *Annual Review of Microbiology* 32, 433-469.

McPherson, M.J., Quirke, P. and Taylor, G.R. (1991). PCR, a Practical Approach. Oxford University Press, New York.

Medina, V.F., Webster, T. and Devinny, J.S. (1995). Treatment of gasoline residuals by granular activated carbon-based biological filtration. *Journal of Environmental Science and Health* 30, 407-422.

Medlin, L., Elwood, H.J., Stickel, S. and Sogin, M.L. (1988). The characterization of enzymatically amplified eukaryotic 16S-like rRNA coding regions. *Gene* 71, 491-499.

Meers, J.L. (1971). Effect of dilution rate on the outcome of chemostat mixed culture experiments. *Journal of General Microbiology* 67, 359-361.

Meulenberg, R., Pronk, J.T., Hazeu, W., Bos, P. and Kuenen, J.G. (1992). Oxidation of reduced sulphur compounds by intact cells of *Thiobacillus acidophilus. Archives of Microbiology* 157, 161-168.

Murray, A.E., Hollibaugh, J.T. and Orrego, C. (1996). Phylogenetic compositions of bacterioplankton California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Applied and Environmental Microbiology* 62, 2676-2680.

Muyzer, G., De Waal, E.C. and Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59, 695-700.

Muyzer, G. and Ramsing, N.B. (1995). Molecular methods to study the organization of microbial communities. *Water Science and Technology* 32, 1-9.

Muyzer, G. and Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek* 73, 127-141.

Muyzer, G., Teske, A., Wirsem, C.O. and Jannasch, H.W. (1995). Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Archives of Microbiology* 164, 165-172.

Myers, R.M., Fisher, S.G., Lerman, L.S. and Maniatis, T. (1985). Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Research* 13, 3131-3145.

Nelson, D.C. (1990). Physiology and Biochemistry of Filamentous Sulphur Bacteria. In: Autotrophic Bacteria. Schegael, H.G. and Bowien, B. (editors). Springer-Verlag, Berlin, pp 219-234.

Newton, C.R. and Graham, A. (1994). PCR. Bios Scientific Publishers Ltd., United Kingdom.

Nixon, A. and Norris, P.R. (1992). Autotrophic growth and inorganic sulphur compound oxidation by *Sulfolobus* sp. in chemostat culture. *Archives of Microbiology* 157, 155-160.

Novak, M. (1987). Continuous cultivation of microorganisms-past and future propects. In: Continuous Culture. Kyslik, P., Dawes, E.A., Krumphanzi, V. and Novak, M. (editors). Harcourt Brace Jovanovich Publishers, London, pp 1-6.

Novick, A. and Szilard, L. (1950). Description of the chemostat. *Science* 112, 715.

Nyns, E.J. (1989). Concluding remarks. In: Continuous Culture. Kyslik, P., Dawes, E.A., Krumphanzi, V. and Novak, M. (editors). Harcourt Brace Jovanovich Publishers, London, pp 261-265.

Oh, J.K., Kim, D. and Lee, I. (1998). Development of effective hydrogen sulphide removing equipment using *Thiobacillus sp.* IW. *Environmental Pollution* 99, 87-92.

Ongcharit, C., Sublette, K.L. and Shah, Y.T. (1991). Oxidation of hydrogen sulfide by flocculated *Thiobacillus denitrificans* in a continuous culture. *Biotechnology and Bioengineering* 37, 497-504.

Ottengraf, S.P.P. (1986). Biological elimination of volatile xenobiotic compounds in biofilters. *Bioprocess Engineering* 1, 61-69.

Ottengraf, S.P.P. (1987). Biological Systems for Waste Gas Elimination. Elsevier Science Publishers, Amsterdam.

Ottengraf, S.P.P. and Diks, R.M.M. (1992). Process Technology of Biotechniques. In: Biotechniques for Air Pollution Abatement and Odour Control Policies. Dragt, A.J. and van Ham, J. (editors). Elsevier Science Publishers, Amsterdam, pp 17-31.

Ovreas, L., Forney, L., Daae, F.L. and Torsvik, V. (1997). Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Applied and Environmental Microbiology* 63, 3367-3373.

Panikov, N.S. (1995). Microbial Growth Kinetics. Chapman Hall, London.

Pickett, A.M. (1982). Growth in a changing environment. In: Microbial Population Dynamics. Bazin, M.J. (editor). CRC Press, Boca Raton, pp 91-124.

Pielou, E.C. (1984). The Interpretation of Ecological Data. Wiley Publishers, New York.

Pirt, J.S. (1965). The maintenance energy of bacteria in growing cultures. *Proceedings Royal Society of London Series B. Biological Sciences* 163, 305-314.

Pirt, J.S. (1975). Priniciples of Microbe and Cell Cultivation. Blackwell Scientific Publications, Oxford, United Kingdom.

Pomeroy, R.D. (1982). Biological treatment of odourous air. *Journal of Water Pollution Control Federation* 54, 1541-1545.

Pronk, J.T., Meulenberg, R., Hazeu, W., Bos, P. and Kuenen J.G. (1990). Oxidation of reduced inorganic sulphur compounds by acidophilic thiobacilli. *FEMS Microbiological Reviews* 75, 293-306.

Prosser, J.I. (1982). Growth of fungi. In: Microbial Population Dynamics. Bazin, M.J. (editor). CRC Press, Boca Raton, pp 125-166.

Rands, M.B., Cooper, D.E., Woo, C.P., Fletcher, G.C. and Rolfe, K.A. (1981). Compost filters for H_2S removal from anaerobic digestion and rendering exhausts. *Journal of Water Pollution Control Federation* 53, 185-189.

Rao, G.S. and Berger, L.R. (1970). Basis of pyruvate inhibition in *Thiobacillus thiooxidans*. *Journal of Bacteriology* 102, 462-466.

Reed, R., Holmes, D., Weyers, J. and Jones, A. (1998). Practical Skills in Biomolecular Sciences. Addison Wesley Longman Publishers, Hong Kong.

Reiffenstein, R. J., Hulbert, W. C. and Roth, S. H. (1992). Toxicology of hydrogen sulfide. *Annual Review of Pharmacology and Toxicology* 32, 109-134.

Reysenbach, A.L., Giver, L.J., Wickham, G.S. and Pace, N.R. (1992). Differential amplification of rRNA genes by polymerase chain reaction. *Applied Environmental Microbiology* 58, 3417-3418.

Rinzema, A. and Lettinga, G. (1988). Anaerobic treatment of sulfate containing waste water. In: Biotreatment Systems. Wise, D.L. (editor). CRC Press, Boca Raton, pp 65-109.

Rittenberg, S.C. (1969). The roles of exogenous organic matter in the physiology of chemolithotrophic bacteria. *Advances in Microbial Physiology* 3, 159-196.

Romanowski, G., Lorenz, M.G. and Wackernagel, W. (1993). Use of polymerase chain reaction and electroporation of *Escherichia coli* to monitor the persistence of extracellular plasmid DNA introduced into natural soils. *Applied and Environmental Microbiology* 59, 3438-3446.

Roth, S.H., Skrajny, B. and Reiffenstein, R.J. (1995). Alteration of the morphology and neurochemistry of the developing mammalian nervous system by hydrogen sulphide. *Clinical and Experimental Pharmacology and Physiology* 22, 379-380.

Roy, A.B. and Trudinger, P.A. (1970). The Biochemistry of Inorganic Compounds of Sulphur. University Press, Cambridge.

Russel, J.B. and Cook, G.M. (1995). Energetics of bacterial growth: balance of anabolic and catabolic reactions. *Microbiological Reviews* 59, 48-62.

Saiki, R.K., Scharf, S.J., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230, 1350-1354.

Saker, K.H. and Herson, D.S. (1994). Bioremediation. McGraw-Hill, New York.

Santegoeds, C.M., Darmgaard, L.R., Hesselink, G., Zopfi, J., Lens, P., Muyzer, G. and de Beer, D. (1999). Distribution of sulphate reducing and methanogenic bacteria in UASB aggregates determined by microsensors and molecular techniques. *Applied Environmental Microbiology* 65, 4618-4629.

Schulz, H.N., Brinkhoff, T., Ferdelman, T.G., Hernandez, M., Teske, A. and Jorgenson, B.B. (1999). Dense populations of a giant sulphur bacterium in Namibian shelf sediments. *Science* 284, 493-495.

Senior, E., Bull, A.T. and Slater, J.H. (1976). Enzyme evolution in a microbial community growing on the herbicide Dalapon. *Nature* (London) 263, 476-479.

Shafia, F., Brinson, K.R., Heinzman, M.W. and Brady, J.M. (1972). Transition of chemolithotroph *Ferrobacillus ferrooxidans* to obligate organotrophy and metabolic capabilities of glucose grown cells. *Journal of Bacteriology* 111, 56-65.

Sheehan, B.T. and Johnson, M.J. (1971). Production of bacterial cells from methane. *Applied Microbiolgy* 21, 511-515.

Sheffield, V.C., Cox, D.R., Lerman, L.S. and Myers, R.M. (1989). Attachment of a 40-base-pair G + C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proceedings of the National Academy of Sciences USA* 86, 232-236.

Singhal, V., Singla, R., Walia, A.S. and Jain, S.C. (1999). Biofiltration: An innovative air pollution control technology for H_2S emissions. http://www.exicom.org/cew/sep96/biofil.htm.

Smalla, K., Wachtendorf, U., Heuer, H., Liu, W. and Forney, L. (1998). Analysis of BIOLOG GN substrate utilization patterns by microbial communities. *Applied and Environmental Microbiology* 64, 1220-1225.

Smet, E., Chasaya, G., van Langenhove, H. and Verstraete, W. (1996). The effect of inoculation and the type of carrier material used on the biofiltration of methyl sulphides. *Applied Microbiology and Biotechnology* 45, 293-298.

Smet, E., Lens, P. and van Langehove, H. (1998). Treatment of waste gases contaminated with odourous sulphur compounds. *Critical Reviews in Environmental Science and Technology* 28, 89-116.

Smith, K.A. (1973). Sorption of gaseous atmospheric pollutants by soils. *Soil Science* 116, 313-314.

Straub, K.L. and Buchholz-Cleven, B.E.E. (1998). Enumeration and detection of anaerobic ferrous iron-oxidizing, nitrate-reducing bacteria from diverse European sediments. Applied and Environmental Microbiology 64, 4846-4856.

Sublette, K.L. and Sylvester, N.D. (1987a). Oxidation of hydrogen sulphide by *Thiobacillus denitrificans*: desulfurization of natural gas. *Biotechnology and Bioengineering* 29, 249-257.

Sublette, K.L. and Sylvester, N.D. (1987b). Oxidation of hydrogen sulphide by mixed cultures of *Thiobacillus denitrificans* and heterotrophs. *Biotechnology and Bioengineering* 31, 759-761.

Sublette, K.L. and Sylvester, N.D. (1987c). Oxidation of hydrogen sulfide by continuous cultures of *Thiobacillus denitrificans*. *Biotechnology and Bioengineering* 31, 753-758.

Suzuki, I. (1974). Mechanisms of inorganic oxidation and energy coupling. *Annual Review of Microbiology* 28, 85-99.

Suzuki, M.T. and Giovannoni, S.J. (1996). Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Applied and Environmental Microbiology* 62, 625-630.

Swanson, W.J. and Loehr, R.C. (1997). Biofiltration: Fundamentals, design and operations principles, and application. *Journal of Environmental Engineering* 123, 538-546.

Tabita, R. and Lundgren, D.G. (1971). Utilization of glucose and the effect of organic compounds on the chemolithotroph *Thiobacillus ferrooxidans*. *Journal of Bacteriology* 108, 328-333.

Tappe, W., Laverman, A., Bohland, M., Braster, M., Rittershaus, S., Groeneweg, J. and van Verseveld, H.W. (1999). Maintenance energy demand and starvation recovery dynamics of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* cultivated in a retentostat with complete biomass retention. *Applied and Environmental Microbiology* 65, 2471-2477.

Taylor, P.A. and Williams, P.J. (1974). Theorectical studies on the coexsistence of competeing species under continuous flow conditions. *Canadian Journal of Microbiology* 21, 90-98.

Tempest, D.W. and Neijssel, O.M. (1984). The status of Y_{atp} and maintenance energy as biologically interpretable phenomena. *Annual Review of Microbiology* 38, 459-486.

Teske, A., Sigalevich, P., Cohen, Y. and Muyzer, G. (1996). Molecular identification of bacteria from a coculture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. *Applied and Environmental Microbiology* 62, 4210-4215.

Teske, A., Wawer, C., Muyzer, G. and Ramsing, N.B. (1996). Distribution of sulfate-reducing bacteria in a stratified Fjord as evaluted by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. *Applied and Environmental Microbiology* 62, 1405-1415.

Tichy, R., Janssen, A.J.H., Grotehuis, J.T.C., Lettinga, G. and Rulkens, W. (1994). Possibilities for using biologically-produced sulphur for cultivation of thiobacilli with respect to bioleaching processes. *Bioresources and Technology* 48, 221-227.

Timmer-Ten Hoor, A. (1981). Cell yield and bioenergetics of *Thiomicrospira denitrificans* compared with *Thiobacillus denitrificans*. *Antonie van Leenwenhoek* 47, 231-243.

Topiwala, H.H. and Hamer, G. (1971). Effect of wall growth in steady-state continuous cultures. *Biotechnology and Bioengineering* 13, 919-922.

Torres, E. M. (1997). Biofiltration: Controlling Air Emissions through Innovative Technology. Water Environment Research Foundation, United States of America.

Tros, M.E., Bosma, T.N., Schraa, G. and Zehnder, A.J.B. (1996). Measurement of minimum substrate concentration (S_{min}) in a recycling fermentor and its prediction from the kinetic parameters of *Pseudomonas sp.* strain B13 from batch and chemostat cultures. *Applied and Environmental Microbiology* 62, 3655-3661.

Tsuchiya, H.M., Tivedi, N.C. and Schuler, M.L. (1974). Microbial mutalism in ore leaching. *Biotechnology and Bioengineering* 10, 991-995.

Tuovinen, O.H., Kelley, B.C. and Groudev, S.N. (1991). Mixed cultures in biological leaching processes and mineral biotechnology. In: Mixed Cultures in Biotechnology. Zeikus, G. and Johnson, E.A. (editors). McGraw-Hill Inc., New York, pp 373-420.

Uitterlinden, A.G. and Vijg, J. (1989). Two-dimensional DNA typing. *Trends in Biotechnology* 7, 336-341.

van Groenestijn, J.W. and Hesselink, P.G.M. (1993). Biotechniques for air pollution control. *Biodegradation* 4, 283-301.

van Lith, C., Leson, G. and Michelsen, R. (1997). Evaluating design options for biofilters. *Journal of Air and Waste Management Association* 47, 37-48.

Veldkamp, H. and Jannasch, H.W. (1972). Mixed culture studies with the chemostat. *Journal of Applied Chemistry and Biotechnology* 22, 105-123.

Verschuere, L., Fievez, V., Van Loren, L. and Verstraete, W. (1997). The contribution of individual populations to the Biolog pattern of model microbial communities. *FEMS Microbiology-Ecology* 24, 353-362.

Victorio, L., Gilbride, K.A., Allen, D.G. and Liss, S.N. (1996). Phenotypic fingerprinting of microbial communities in wastewater treatment systems. *Water Research* 30, 1077-1086.

Vishniac, W. and Santer, M. (1957). The *Thiobacilli. Bacteriological Reviews* 21, 195-209.

Visser, J.M., Stefess, G.C., Robertson, L.A. and Kuenen, J.G. (1997). *Thiobacillus* sp. W5. The dominant autotroph oxidizing sulfide to sulfur in a reactor for aerobic treatment of sulfidic wastes. *Antonie van Leeuwenhoek* 72, 127-134.

Wada, A., Shoda, M., Kubota, H., Kobayashi, T., Katayama-Fujimura, Y. and Kuraishi, H. (1986). Characteristics of H_2S oxidizing bacteria inhibiting a peat biofilter. *Journal of Fermentation and Biotechnology* 64, 161-167.

Wainwright, M. (1978). Sulfur-oxidising microorganisms on vegetation and in soils exposed to atmospheric pollution. *Environmental Pollution* 17,167-174.

Wawer, C. and Muyzer, G. (1995). Genetic diversity of *Desulfovibrio* spp. in environmental samples analyzed by denaturing gradient gel electrophoresis of [NiFe] hydrogenase gene fragments. *Applied and Environmental Microbiology* 61, 2203-2210.

Webster, T.S., Devinny, J.S., Torres, E.M. and Basrai, S.S. (1997). Microbial ecosystems in compost and granular activated carbon biofilters. *Biotechnology and Bioengineering* 53, 296-303.

Weimer, P.J. (1991). Use of mixed cultures for the production of commercial chemicals. In: Mixed Cultures in Biotechnology. Zeikus, G. and Johnson, E.A. (editors). McGraw-Hill Inc. Publishers, New York, pp 205-228.

Weusthuis, R.A., Pronk, J.T., van den Broek, P.J. and van Dijken, J.P. (1994). Chemostat cultivation as a tool for studies on sugar transport in yeasts. *Microbiological Reviews* 58, 616-630.

Willison, J.H.M. and Rowe, A.J. (1980). Replica, Shadowing and Freeze-Etching Techniques. North-Holland Publishing Company, Amsterdam.

Wintzingerode, F.V., Gobel, U. B. and Stackebrandt, E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbial-Review* 21, 213-229.

Woese, C. R. (1987). Bacterial evolution. *Microbiological Reviews* 51, 221-271.

Yang, R.D. and Humphrey, A.E. (1975). Dynamic and steady states of phenol biodegradation in pure and mixed cultures. *Biotechnology and Bioengineering* 17, 1211-1235.

Yang, Y. and Allen, E.R. (1994). Biofiltration control of hydrogen sulfide 1. Design and operational parameters. *Journal of Air and Waste Management Association* 44, 863-868.

Yoon, H., Klinzing, G. and Blanch, H.W. (1977). Competition for mixed substrates by microbial populations. *Biotechnology and Bioengineering* 19, 1193-1211.

Zak, J.C., Willig, M.R., Moorhead, D.L. and Wildman, H.G. (1994). Functional diversity of microbial communities: a quantitative approach. *Soil Biology and Biochemistry* 26, 1101-1108.

Zeng, A.P., Biebl, H. and Deckwer, W.D. (1990). 2,3-Butanediol production by *Enterobacter aerogenes* in continuous culture and the role of oxygen supply. *Applied Microbiology and Biotechnology* 33, 463-468.

Zeng, A.P. and Deckwer, W.D. (1992). Utilization of the tricarboxylic acid cycle, a reactor design criterion for the microaerobic production of 2,3-butanediol. *Biotechnology and Bioengineering* 40, 1078-1084.

Zeng, A.P. and Deckwer, W.D. (1995). Mathematical modelling and analysis of glucose and glutamine utilization and regulation in cultures of continuous mammalian cells. *Biotechnology and Bioengineering* 47, 334-346.

Zeng, A.P. and Deckwer, W.D. (1996). Bioreaction techniques under low oxygen tension and oxygen limitation: from molecular level to pilot plant reactor. *Chemical Engineering Science* 51, 2305-2314.

Zhang, L., Kuniyoshi, I., Hirai, M. and Shoda, M. (1991). Oxidation of dimethyl sulfide by *Pseudomonas acidovorans* DMR-11 isolated from peat biofilter. *Biotechnology Letters* 13, 223-228.

Appendix A

Nomenclature

Symbol	Definition	Unit
A	Gas constant (8.3145)	$J\ mol^{-1}K^{-1}$
D	Dilution rate	h^{-1}
D_c	Critical dilution rate	h^{-1}
E	Exponential	
ΔE	Activation energy	J
F	Flow rate	ml
K_s	Saturation constant	$g.l^{-1}$
k	Growth rate	h^{-1}
m	Maintenance energy (g dry wt (mole of substrate oxidized)$^{-1}$)	
N_0	Culture optical density at time 0	nm
N	Culture optical density at time t	nm
S_r	Substrate [] in influent medium	$g.l^{-1}$
R	Gas constant (8.3145)	$J\ mol^{-1}K^{-1}$
t	Time	h
t_d	Culture doubling time	h
t_0	Time at 0	h
t_t	Time at t	h
T	Temperature	^0C
T_r	Residence time	h
μ	Specific growth rate	h^{-1}
μ_{max}	Maximum specific growth rate	h^{-1}
v	Velocity of the reaction	ms^{-1}
V	Volume of open culture	l
x	Culture optical density	nm
x_o	Culture optical density at time 0	nm
x_t	Culture optical density at time t	nm
Y	Yield (g dry wt (mole of substrate oxidized)$^{-1}$)	
Y_{max}	Maximum yield (g dry wt (mole of substrate oxidized)$^{-1}$)	

Appendix B

Deoxyribonucleic Acid Isolation

All solutions used for DNA isolation were sterilized by autoclaving at 121^0C for 15 minutes and stored at 4^0C unless stated otherwise.

B.1 1mM EDTA:

Ethylenediaminetetraacetic acid disodium salt (0.0186 g)(EDTA, Saarchem) was dissolved in 40 m*l* of distilled water, the pH adjusted to 8.0 with 1N NaOH and diluted to 50 m*l* with distilled water.

B.2 0.01M Tris-HCl:

Tris(hydroxymethyl) aminomethane (0.606 g)(Tris-HCl, Aldrich) was dissolved in 40 m*l* of distilled water, the pH adjusted to 8.0 with 1N HCl and diluted to 50 m*l* with distilled water.

B.3 TE Buffer:

Equal volumes of 0.01 M Tris-HCl and 1mM EDTA (see above) were mixed and stored at room temperature.

Appendix C

Reagents Used in Agarose Gel Electrophoresis

All solutions were stored at 4^0C unless stated otherwise.

C.1 Ethidium Bromide Stock Solution:

Ethidium bromide (0.005 g)(Merck) was dissolved in distilled water and diluted to 10 m*l*.

C.2 Running Buffer:

TAE buffer (50 x)(4.0 m*l*) was combined with 196.0 m*l* of distilled water.

C.3 0.1 M EDTA:

Ethylenediaminetetraacetic acid disodium salt (1.95 g) was dissolved in 40 m*l* of distilled water, the pH adjusted to 8.0 with 1N HCl and diluted to 50 m*l* with distilled water.

C.4 Sample Loading Buffer:

Ficoll 400 (2 g)(Merck), 1 g sodium dodecyl sulphate (Saarchem), 0.25 g bromophenol blue (Merck) and 0.25 g xylene cyanol (Merck) were dissolved in 0.1M EDTA and diluted to 10 m*l*.

C.5 50x TAE Buffer:

Tris (hydroxymethyl) aminomethane (12.1 g) (Tris-HCl, Aldrich) and 1.86 g $Na_2EDTA.2H_20$ (Saarchem) were dissolved in 30 m*l* of distilled water plus 2.855 m*l* of glacial acetic acid and then diluted to 50 m*l* with distilled water.

Appendix D

Denaturing-Gradient Gel Electrophoresis

D.1 40% Acrylamide/Bis (37.5:1):

N, N'-Methylene-bis-acrylamide (1.07 g)(Sigma) and 38.93 g of acrylamide (Merck-Schuchardt) were dissolved in sterile Milli-Q water and diluted to 100 m*l*. The resulting solution was stored in dark bottles at 4^0C.

D.2 0% Denaturing Solution for 8% Gel:

Acrylamide/bis (20 m*l* of 40% (m/v) solution) and 2 m*l* of 50x TAE were added to 78 m*l* of sterile Milli-Q water.

D.3 100% Denaturing Solution for 8% Gel:

Urea crystals (42 g)(Associated Chemical Enterprises cc.) were dissolved in 20 m*l* of 40% (m/v) acrylamide/bis solution, 2 m*l* of 50x TAE and 40 m*l* of formamide (Sigma) and diluted to 100 m*l* with sterile Milli-Q water.

Both the 0% and 100% denaturant solutions were degassed under vacuum for 15 minutes and stored at 4°C in dark bottles.

D.4 10% (m/v) Ammonium Persulphate:

Ammonium persulphate (1 g)(Sigma, ultra-pure grade) was dissolved and diluted to 10 m*l* with sterile distilled water and stored in 0.5 m*l* aliquots at -20^0C.

D.5 10M NaOH:

Sodium hydroxide (40 g)(Saarchem) was dissolved and diluted to100 m*l* with sterile distilled water.

D.6 0.5M EDTA:

Ethylenediaminetetraacetic acid disodium salt (18.61 g) was dissolved in 75 m*l* of distilled water. The pH was adjusted to 8.0 with 10M NaOH and diluted to 100 m*l* with distilled water. The solution was autoclaved at 121°C for 15 minutes and stored at room temperature.

D.7 1x TAE:

TAE buffer (20 m*l* of 50x) was diluted to 1*l* with distilled water.

D.8 Gel Dye:

Bromophenol blue (hexadecyl trimethylammonium bromide, Sigma) (0.05 g) was dissolved and diluted to 10 m*l* with 1xTAE.

D.9 6x Loading Buffer:

Bromophenol blue (0.05 g), sucrose (40 g) and sodium dodecyl sulphate (SDS) (0.5 g) were dissolved in 50 m*l* of distilled water and 20 m*l* of 0.5M EDTA and then diluted to100 m*l* with distilled water.

D.10 Ethidium Bromide:

Ethidium bromide (10 mg)(Merck) was dissolved and diluted to 1m*l* with distilled water.

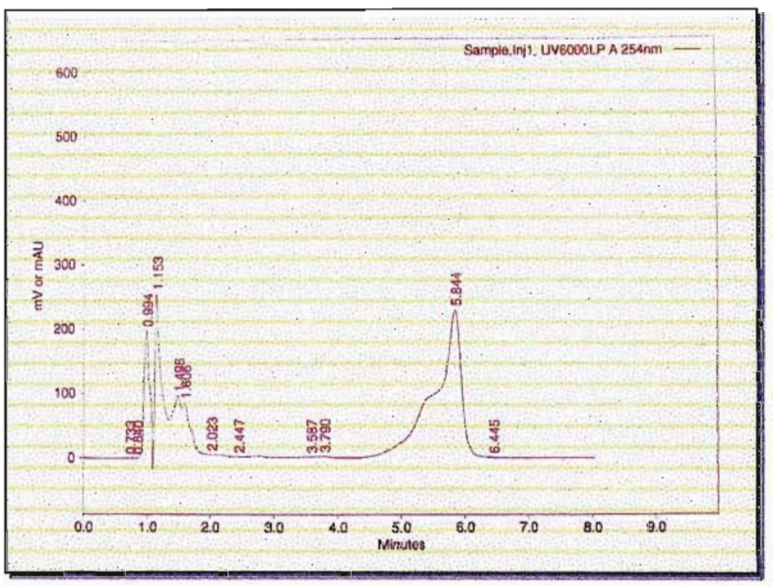
D.11 Running Buffer (Electrophoresis Tank):

TAE buffer (140 m*l* of 50x) was diluted with 6 860 m*l* of distilled water.

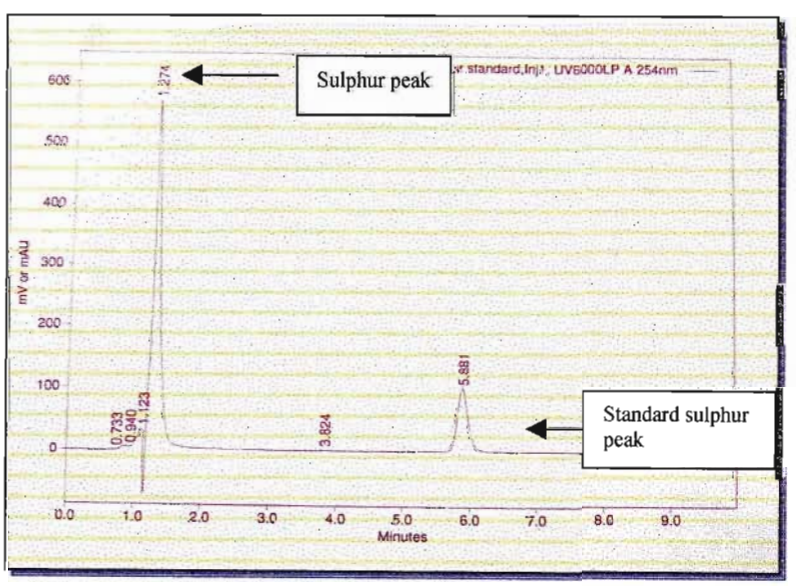
Appendix E

High-Performance Liquid Chromatograms

E.1 Chromatogram of sample precipitate:



E. 2 Chromatogram of sulphur standard:



E.3 Chromatogram of sample precipitate and standard sulphur: