MOLECULAR ANALYSES OF PURE CULTURES OF FILAMENTOUS BACTERIA ISOLATED FROM ACTIVATED SLUDGE

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I hereby declare that this dissertation represents my own work, and that it has not been submitted before for any diploma/degree or examination at any other Technikon/University.

DASHIKA NAIDOO

I hereby approve the final submission of the following dissertation

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PROF. FAIZAL BUX
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This day ———— day of ———— 2005, at Durban Institute of Technology, Durban.

DEDICATION

The work presented here is dedicated with

deepest love and appreciation

to my parents…

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to the following people and institutions:

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A special acknowledgement to the late Lester Rasch, for his friendship, support and invaluable words of encouragement…and… for showing me that all things are possible with perseverance and that, the greatest glory is not in never falling, but in rising stronger when you fall…

'Thank You'

PREFACE

Aspects of the work covered in this dissertation have and will be published and presented elsewhere:

Conferences:

D. Naidoo, T.R. Ramothokang and F. Bux. *Title:* Ribosomal RNA targeting oligonucleotide probing and biochemical characterization of filamentous bacteria in pure culture. Poster presentation at: 4th IWA Activated Sludge Population Dynamics Specialist Conference – Microbial Population Dynamics in Biological Wastewater Treatment Systems, 17 – 20 July 2005 (accepted).

Publications:

D.Naidoo, T.R. Ramothokang and F. Bux. *Title*: Identification and morphological characterization of pure cultures of filamentous bacteria isolated from activated sludge. *In preparation for submission toApplied Microbiology and Biotechnology.*

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ABSTRACT

The activated sludge process is the mostl used biological treatment process. Engineers and microbiologists are constantly seeking ways to improve process efficiency, which can be attributed to the increasing demand for fresh water supplies and proper environmental management. Since the inception of the activated sludge process, bulking and foaming have been major problems affecting its efficiency. Filamentous bacteria have been identified as the primary cause of bulking and foaming. Numerous attempts have been made to resolve this problem. Some of these attempts were effective as interim measures but failed as long term control strategies. The identification of filamentous bacteria and the study of their physiology have been hampered by the unreliability of conventional microbiological techniques. This is largely due to their morphological variations and inconsistent characteristics within different environments. To fully understand their role in promoting bulking and foaming, filamentous bacteria need to be characterized on a molecular level.

The aim of this study was, therefore, to identify filamentous bacteria in pure culture with the purpose of validating these findings to the physiological traits of the pure cultures when they were isolated. Fourteen different filamentous cultures were used for this study. The cultures were identified using specific oligonucleotide probes via fluorescent *in situ* hybridisation and nucleotide sequencing. Prior to sequencing, an agarose gel and a denaturing gradient gel Electrophoresis profile were determined for each isolate. The various techniques were optimised specifically for the filamentous isolates. The isolates were identified as *Gordonia amarae, Haliscomenobacter hydrossis, Acinetobacter* sp./Type 1863, Type 021N, *Thiothrix nivea, Sphaerotilus natans* and Nocardioform organisms.

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LIST OF ABBREVIATIONS

ACA	*Acinetobacter* species
BNR	Biological Nutrient Removal
DAPI	4', 6-diamidino-2-phenylindole
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
dsDNA	Double Stranded DNA
DLP	Nocardioform filamentous bacteria
FISH	Fluorescent In Situ Hybridisation
F:M	Food to Microorganism Ratio
GA	*Gordonia amarae*
HHY	*Haliscomenobacter hydrossis*
NALO	*Nocardia amarae*-like organisms
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SNA	*Sphaerotilus natans*
SVI	Sludge Volume Index
ssDNA	Single Stranded DNA
TFR	*Thiothrix fructosivarans*
TNI	*Thiothrix nivea*
21N	Type 021N

CHAPTER ONE

INTRODUCTION

1.1'Water' A Precious Natural Resource

Earth is often referred to as the water planet. This is due to the fact that about three fifths of the planet is covered by water (Sanders *et al*., 1996). While this may appear to be an abundance, not all the water is available for use. The oceans account for about 97.2% of the earth's water. Due to the high salinity, this water is not very useful for daily applications. Approximately 2.1% is tied up in glaciers and it is the remaining 0.6% that is only available to us as either fresh water in rivers and lakes or as groundwater (Sanders *et al*., 1996).

Water is a critical natural resource in the world since without it life will cease to exist. Water resource shortages are a major problem that is plaguing the world. Water demand and availability is affected by a number of factors namely geographies, population demographics, climatic conditions, socio-economic development, technological developments, environmental conservation and water pricing.

The geographical location of South Africa has labeled it a water-stressed country. South Africa experiences an annual rainfall of about 500 millimeters, which is unevenly distributed across the country. In addition, a large portion of the water received is lost through evaporation (UK Trade and Investment, 2002). Since 1994 a number of political, social and economic changes have occurred. These changes have prompted people to flock too more urbanized areas, in search of better lifestyles. The modernization and maintenance of society has increased the demand for water. A number of water augmentation projects have been implemented to help facilitate water

entrapment between catchments. Consequently, the industrialization of society and unsustainable practices have also been the source of pollution of many of our water resources.

Our natural water resources are disappearing at an unprecedented rate. Based on current trends, it is estimated that by the year 2030 most of our water resources would be exhausted or totally destroyed (UK Trade and Investment, 2002). It must be noted that all water resources contain a variety of natural contaminants, which stem from erosion, leaching and weathering processes (Tebbutt, 1998). Most water bodies have the ability to self-purify whereby they are capable of assimilating a certain amount of these contaminants without any detrimental effects (Tebbutt, 1998). This is, however, dependent on environmental conditions, pollutant load and water retention times (Sanders and Yevjevich, 1996). If these natural contaminants are coupled with artificial pollutants, which are a result of human activities, the pollution load becomes too much for the water body to handle. The most significant form of artificial pollution is the return flows of wastewater, sewage and industrial effluents into our aquatic environment.

A common problem experienced around the world is eutrophication of water bodies. Eutrophication may be defined as the nutrient enrichment of a water body (Sala and Mujeriego, 2001). Eutrophic waters usually have high concentrations of nitrogen and phosphorus, which can be attributed to the discharge of poor quality effluents and wastewater and, agricultural practices (Forsberg, 1998). Eutrophication usually results in the excessive proliferation of algal blooms. These algal blooms exhaust both the nutrients and the dissolved oxygen content in water. This leads to death of some aquatic life and may possibly result in the collapse of the entire aquatic ecosystem concerned (Sala and Mujeriego, 2001). Extensive research and development have been invested into remediating eutrophically affected waters by reducing the nutrient load discharged (Tebbutt, 1998).

South Africa has been recognized as an imperative market for water and wastewater treatment and management. This is largely due to the high level of importance the government has placed in improving water services and sanitation, which is evident in the National Water Act (1998). The National Water Act aims at actively promoting the values enshrined in the Bill of Rights by providing equitable use of water and wastewater management services while still protecting the environment. The advent of investment into this sector has also been a motivating factor.

1.2. Treatment Of Wastewater

Wastewater management has become a necessity for the maintenance of a modern society. Due to ongoing urbanization and industrialization, there has been an increase in the use of products that have a high toxicity and low biodegradability. Huge amounts of wastewater are generated and thus require the application of physical, chemical or biological treatments or a combination of the three (Hartmann, 1999). Wastewater treatment processes can be classified into three groups: primary, secondary and tertiary (Sanders *et al*., 1996). Primary treatment is essentially composed of physical processes such as the screening, sedimentation and filtration to remove large, insoluble solids that may clog and damage the system. Primary treatment removes about 55% of the total suspended solids. From here the wastewater usually flows slowly to the primary settling basins where more suspended particles are removed. Some particles float to the surface and are removed by skimmers while, others settle at the bottom of the basin and are removed by dredges (Sanders *et al*., 1996; Tebbutt, 1998; Cheremisinoff, 2002).

Secondary treatment is primarily based on biological processes. This method employs biochemical reactions to further oxidize and remove dissolved particles, which are usually organic in nature (Sanders *et al*., 1996; Tebbutt, 1998). There are two types of biological

processes: suspended and attached growth processes. Attached growth processes are those where the microbial population is allowed to grow on a surface. This method is employed in trickling filters. In suspended growth processes, the microbial population is continuously mixed in suspension in the wastewater (Cheremisinoff, 2002). A well-known suspended growth process is the activated sludge process.

Tertiary treatment is usually a chemically based process. However a biological process in the form of constructed wetlands is also common (Sanders *et al*., 1996). The choice of the chemical process depends on the chemical characteristics of the wastewater and the financial feasibility of running the process (Cheremisinoff, 2002). The biological process is Tertiary treatment entails either, nitrification-denitrification, coagulation, precipitation, phosphorus removal and desalination, or a combination of these (Sanders *et al*., 1996; Cheremisinoff, 2002).

The type and extent of treatment depends on the nature of the wastewater and effluent standards. Thus in most cases it is necessary to use a combination of the above methods to achieve an acceptable quality of effluent for discharge.

1.3. The Activated Sludge Process

In the early 1900's the trend was to treat wastewater by aeration (Kayser, 1999). There was an escalating need for more sophisticated methods to treat wastewater in larger cities. In 1914 two engineers, Ardern and Lockett explored the principle of treatment with trickling filters and by aeration, and used this as a basis to design a new process, the 'Activated Sludge Process.' (Hartmann, 1999). They used the aeration/agitation method to mix wastewater with a microbial biomass. After a certain period of time they stopped agitation and allowed the biomass to settle

out. The remaining supernatant was removed and replaced with more wastewater. This process was continuously repeated and thus facilitated the accumulation of microbial biomass. Biomass refers to the aggregation of microorganisms. In an activated sludge process, the microorganisms grow as three-dimensional aggregated microbial communities called 'flocs' (Seviour *et al*., 1999b). The result was a fully nitrified effluent (Tebutt, 1998; Kayser, 1999). This process is capable of treating larger volumes of wastewater and producing higher quality effluent.

The activated sludge process has been adopted worldwide for the treatment of domestic and industrial effluents. A conventional activated sludge plant consists of four key phases (Fig 1.1.) (Kayser, 1999):

1. An aeration tank ~ where the biomass is kept in suspension by continuous agitation to ensure that the dissolved oxygen is maintained at an optimum level and to keep the biomass in continuous contact with the influent (Muyima *et al*., 1997).
2. The final clarifier ~ the flocs are carried over with the influent into the clarifier where the biomass is allowed to settle out. It is therefore important that these flocs have good settling properties.
3. The collection of return sludge to re-supply the aeration tank.
4. The removal of excess sludge.

A portion of the settled sludge is drawn off as waste while the remaining sludge is recycled to the aeration basin. This helps maintain a proper food to microorganism ratio (F : M) (Eckenfelder *et al.,* 1985; as cited by Muyima *et al*., 1997). The sludge that is wasted determines the average amount of time the bacteria will spend in the system. This maybe defined as 'sludge age' and is determined by the following equation:

$$\text{Sludge age (d)} = \frac{\text{Total solids in reactor (kg)}}{\text{Total solids wasted (kg/d)}}$$

(Muyima *et al.*, 1997)

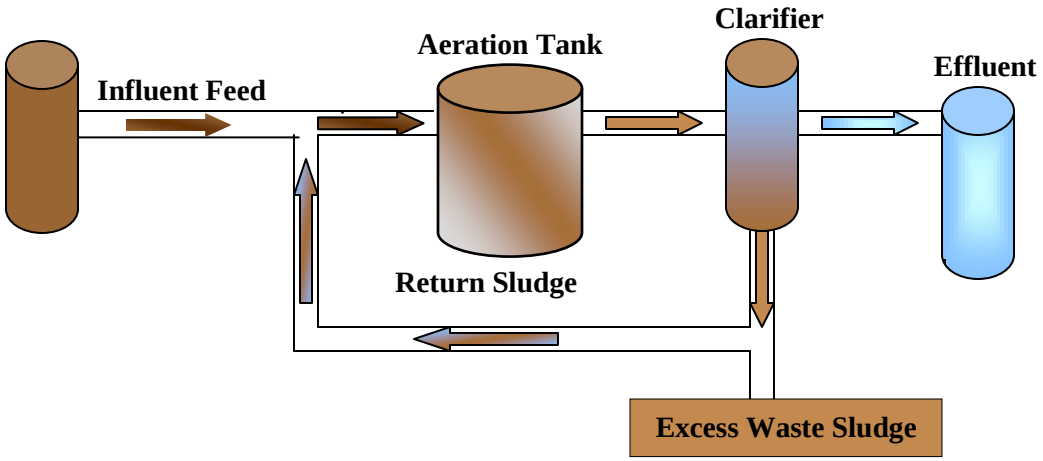

Figure.1.1. The basic configuration of a typical Activated Sludge Process (Kayser, 1999)

A major advantage of the activated sludge process is its flexibility with regard to process configurations. The configurations of the process can be modified according to the influent type and effluent standards. Most activated sludge processes have been configured to facilitate Biological Nutrient Removal (BNR). BNR refers to the removal of carbon, nitrogen through nitrification-denitrification and phosphorus (Muyima *et al*., 1997). Nitrification-denitrification is probably one of the most important processes that occur in activated sludge systems. Most nitrogen removing systems are single sludge processes; they employ a single amount of biomass (Seviour *et al*., 1999b). The configuration of the conventional activated sludge nitrification-denitirification system was changed by introducing an anoxic reactor in front of the aerobic reactor, to increase nitrification in the aerobic reactor. In addition two sludge recycles were introduced. Such a system is referred to as a double sludge system associated with the Modified Ludzack-Ettinger Process (Muyima *et al*., 1997).

1.4. Motivation For This Study

The success of any activated sludge wastewater process depends on the metabolic activity and physical characteristics of the microbial population in suspension and hence, the ability of the microbial biomass to settle out of the effluent stream (De Los Reyes *et al*., 1997). A significant number of activated sludge plants, irrespective of their configurations, are plagued by poor separation of solids. Bulking and foaming are direct consequences of poor solid separation, which is caused by the excessive proliferation of filamentous bacteria (Blackall, 1994; De Los Reyes *et al*., 1997). Bulking and foaming have drawn considerable interest to both the engineer and microbiologist. Both are major process problems as they result in poor effluent quality, loss of active biomass, increased costs and, pose a number of environmental and health hazards.

Engineers and microbiologists are constantly seeking ways to improve system design and performance by controlling or preventing bulking and foaming**.** The first step to control bulking and foaming is to identify the cause of the problem and to acquire as much information on it as possible; in this study it is "filamentous bacteria." This information will lead to the development of specific remedies, appropriate for the filament(s) involved. Interim control techniques are often used to promptly stop a bulking and foaming problem. However, the preeminent approach is to explore the long-term control methods with an overall aim to eradicate bulking and foaming.

The isolation and characterization of filamentous bacteria in activated sludge has attracted much attention, but has proven to be an arduous task. Eikelboom was the first to provide the foundation for filamentous related work with the development of the Eikelboom-Type filament identification system using microscopic analysis (Jenkins *et al*., 1993). Microscopic examination is useful for determining the physical nature of the activated sludge floc and the types of filaments that may be present.

A number of discrepancies exist in the inability of these techniques to accurately quantify and identify filamentous bacteria. Filaments that occur within the floc may go undetected**.** Identification based on phenotypic characteristics such as morphology and staining characteristics, have a major drawback. The morphology and staining characteristics of the same organism may vary significantly, depending on environmental conditions (Woese, 1987; Oerther *et al*., 2001). Furthermore a single species may exhibit polymorphisms, resulting in different species appearing alike. Many filamentous bacteria found in activated sludge are morphologically similar (Stainsby *et al*., 2002).

To overcome these problems, a molecular approach has been adopted. The rRNA approach has been a major break-through in culture-independent technique analysis. The sequence variation in rRNA has been exploited for inferring phylogenetic relationships amongst very similar species and for designing oligonucleotide probes for identifying different isolates and uncultured microbes (Woese, 1987; Muyzer *et al*., 1993). Probes allow for the identification of organisms *in situ* without the need to culture them which is particularly appropriate for filamentous bacteria since they are difficult to isolate and culture (Maszenan *et al*., 2000). The use of molecular techniques for filament identification is a relatively new idea. 16S rRNA-targeted oligonucleotide probes have been developed for some of the common filamentous bacteria. Filamentous bacteria that have morphologically been identified as Type O21N had bound the probe specific for *Thiothrix nivea* and not Type O21N (Nielsen *et al*., 1998). Without molecular identification, such cases would result in incorrect identification. With the development of sequencing techniques, new filaments are constantly being discovered.

This project was undertaken in an effort to identify and hence aid in the acquisition of information on the growth of filamentous bacteria in pure culture with an overall aim to apply this knowledge in activated sludge systems. Hence the aim of this study was to optimize molecular

techniques to identify and monitor filamentous bacteria in pure culture; which can be adapted for application in activated sludge plants.

Two types of rRNA-based techniques were used for analyses:

1. Fluorescent *In Situ* Hybridisation (FISH)
2. Denaturing Gradient Gel Electrophoresis (DGGE) of Polymerase Chain Reaction (PCR) amplified 16S rRNA genes followed by nucleotide sequence analysis.

1.5. Objectives Of This Study

This project was conducted in two parts using different molecular techniques. *Part A* involved the optimization of FISH to identify filamentous bacteria and evaluate the use of FISH for filament identification in pure culture.

Part B of this project involved the optimization of PCR and DGGE for filamentous bacteria and , nucleotide sequencing to verify the identification of the isolates.

PART A

1. To optimize conditions for FISH.
2. To identify filamentous bacteria in pure culture using FISH.
3. To evaluate the use of FISH for filament identification.

PART B

1. To extract DNA from pure cultures of filamentous bacteria using the phenol extraction method.
2. To amplify the DNA fragments coding for the 16S rRNA genes, using PCR with universal primers.
3. To profile and determine the purity of the isolates by dissociating the amplified fragments of the isolates into discrete bands via DGGE.
4. To excise and purify the prominent bands of DNA for nucleotide sequencing, to verify identification of the isolates.

CHAPTER TWO

LITERATURE REVIEW

2.1. The Activated Sludge Floc

Since the inception of the activated sludge process, the separation of solids has been a major problem affecting biological wastewater treatment. Solids separation is a process where activated sludge solids separate by flocculation and gravitational sedimentation from treated wastewater in the clarifier (Wanner, 1994). The objective of solid separation is to produce a secondary effluent that is low in suspended solids and a thickened activated sludge which settles at the bottom of the clarifier and can be recycled back into the system as return activated sludge (RAS) (Droste, 1997). Most solids separation problems can be attributed to the nature of the activated sludge floc.

Activated sludge flocs have two components: a biological component consisting of microorganisms, and a non-biological component consisting of organic and inorganic particulates (Jenkins *et al*., 1993). It has been proposed that there are two structural levels in the floc, the microstructure and the macrostructure. The microstructure is composed of microorganisms, mainly floc forming bacteria (Jenkins *et al*., 1993). Most of the cell biomass in the system is organised into these flocs through bioflocculation, which is the ability of microbes to adhere to one another (Seviour *et al*., 1999b). Although the process of bioflocculation is not well understood, it is assumed that it occurs through the interaction of extra-cellular microbial polymers (Seviour *et al*., 1999b). Sezgin *et al*. (1978) (as cited by Jenkins *et al*., 1993) found that filamentous bacteria provide the macro-structure of the activated sludge floc. Filamentous bacteria were found to provide a network of support to which the floc forming bacteria adhere (Jenkins *et al*., 1993). Filamentous bacteria preserve the integrity of the floc structure.

Floc morphology is critically important since it directly determines vital qualities of the activated sludge. These include settling characteristics of the sludge, thickening and dewatering of the sludge and, the clarity of the final effluent (Wanner, 1994; Seviour, 1999a). Filamentous bacteria also affect the sludge volume index (SVI). SVI is the reciprocal sludge concentration in the sludge phase after thirty minutes settling. A low SVI is indicative of good flocculation and settling characteristics (Wanner, 1994). Jenkins *et al*. (1993) described an ideal floc as being large with a strong macrostructure. All the organisms within the floc live in a balanced ecosystem. The presence of 'ideal flocs' is an indication of low SVI, between 80 to 120 mL/g. This produces a clear supernatant (Jenkins *et al*., 1993). A high SVI, greater than 150mL/g, is often associated with bulking and foaming (Jenkins *et al*., 1993).

2.2. Problems Associated With The Separation Of Solids

The efficient separation of solids from treated wastewater is a critical factor in determining the success of an activated sludge process. There are several problems encountered with the separation of solids after biological treatment. The most common problems are,

1. **Dispersed growth:** No floc formation. The microorganisms are dispersed within the system. The effluent is turbid as a result of unsettled solids.
2. **Slime Bulking/Non-Filamentous Bulking:** The microorganisms produce large amounts of exopolysaccharides, which largely contributes to the slimy consistency of the sludge. Often causes an overflow of sludge from the clarifier.
3. **Pinpoint flocs:** No macrostructure or weak microstructure. The effluent produced is turbid and has a very low SVI, less than 70 mg/L.

4. **Filamentous Bulking:** Filamentous bacteria extend out of the floc into the surrounding solution. There is poor compaction, resulting in unsettled sludge. The SVI is very high, usually greater than 150mg/L.
5. **Blanket Rising:** The production of nitrogen during denitrification, in the clarifier, causes the flocs to float to the surface of the clarifier.
6. **Foaming:** Foaming brings the biomass/flocs to the surface. It often results in the carry-over of solids from the clarifier. It is caused by the presence of non-biodegradable surfactants reacting with filamentous bacteria, mostly *Nocardia spp*.

(Jenkins *et al*., 1993; Seviour, 1999b)

2.3. Bulking And Foaming

Of all the problems associated with the separation of solids, bulking and foaming are considered to be the most prevalent ones. Problems arise when the normal microbial community balances within the system, more specifically within the floc, are disturbed and filamentous bacteria proliferate at a more rapid rate than floc forming bacteria (Blackall, 1999). Bulking and foaming pose a number of potential health and environmental hazards. In addition, they cost municipalities and private companies millions. Factors such as low Food:Microorganism (F:M) ratios, low dissolved oxygen (DO), low nutrient concentrations and the presence of high concentrations of sulphides, favour the growth of filamentous bacteria (Kämpfer and Wagner, 2002). Bulking and foaming in South Africa is largely attributed to low F:M ratios (Casey *et al*., 1995).

2.3.1. Bulking

Eikelboom and Van Büijsen (1983) (as cited by Jenkins *et al*., 1993) defined a bulking sludge as one, which settles slowly and compacts poorly because of excessive growth of filamentous

bacteria. Some filamentous bacteria grow out of the floc into the surrounding medium forming either bridges with neighbouring flocs or, diffuse or open flocs (Figure 2.1.) (Jenkins *et al*., 1993; Blackall, 1999). Previous research has indicated that the same morphological types of filaments are rife in most plants, which accounts for 90% of all bulking incidences (Blackall, 1999).

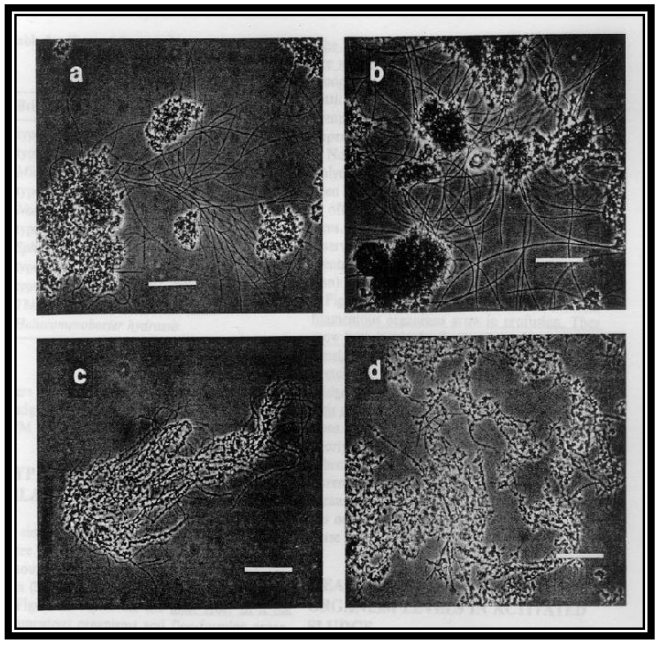

Figure. 2.1. Activated sludge flocs

a) and b) inter-floc bridging

c) and d) open flocs. Bar = 100μm (Jenkins *et al*., 1993)

Although bulking may provide a clear effluent it still causes significant operational problems, such as;

1. loss of solids may produce an increase in the substrate-loading rate per unit mass of sludge (Blackall, 1999).
2. loss of nitrification and reduced oxidation of organic matter, which leads to poor effluent quality (Wanner, 1994).
3. poor compaction and settling may lead to over-flowing of the tanks (Blackall, 1999).

Hence the control of bulking has attracted immense attention. There are two approaches, which can be adopted for bulking control. These are either specific or non-specific. 'Non-specific' methods involve dosing the system with a toxicant such as chlorine or hydrogen peroxide. This usually eliminates most of the filamentous bacteria in the system. Optimal dosing concentrations need to be determined. If the toxicant is incorrectly applied it may eliminate all viable biomass thus inhibiting treatment. 'Specific' methods require identification of the root of the problem, which are the causes for the growth of the filamentous bacteria. Non-specific control methods are generally used as a last resort or when it proves to be economically feasible or, until a specific method can be developed and applied (Lakay *et al*., 1988).

2.3.2. Foaming

Foaming is the development of a thick, dense floating scum/foam (Soddell, 1999). Foaming occurs through a flotation process that is facilitated by filamentous bacteria (Soddell, 1999). The production of gas bubbles by aeration or metabolic activities assists in the process. The hydrophobic nature of the cell walls of the microorganisms enables the floc particles to attach to air bubbles with the aid of biosurfactants or synthetic surfactants, which maybe present in the wastewater. The surfactants also stabilise the hydrophobic floc-air bubble aggregates. The hydrophobic nature of the aggregates keeps them at the surface. At the surface they accumulate thus forming thick, dense foams or scum's (Jenkins *et al*., 1993; Bitton, 1994; Soddell, 1999). There are three basic types of foams encountered in activated sludge processes:

1. White foams that occur as a result of the presence of slow or unbiodegradable detergents.
2. Foaming or scum due to rising sludge that occurs as a result of denitrification in the clarifier.
3. Biological, brown foams due to the excessive growth of actinomycetes (Bitton, 1994).

It is the biological foam that is the most problematic as it is highly viscous and very stable.
It is difficult to specifically identify which filaments cause foaming as some filaments also cause bulking. However, biological foaming due to actinomycetes has been ascribed to the mycolic acid-producing genus *Nocardia*. *M. parvicella* has also been implicated in foaming (Kämpfer and Wagner, 2002).

Foam production in activated sludge plants causes a number of problems:

1. Excess foam passes into the sludge effluent resulting in increased biochemical oxygen demand (BOD) and solids in the effluent.
2. Foam may carry opportunistic pathogens such as *Nocardia aesteroides*.
3. There may be over-flowing of sludge from the tanks resulting in hazardous working conditions and potential environmental damage (Bitton, 1994).

Foaming has been found to occur in plants that vary considerably in influent composition, temperature, aeration, sludge age and solid content. However, foaming is frequently associated with processes that employ retention times greater than 9 days and process temperatures that are higher than 18°C as well as high concentrations of grease and oil in the influent (Pipes *et al*., 1978; as cited by Bitton, 1994). The lack of information on the causes of foaming and filamentous bacteria has lead to the use of non-specific methods to control foaming. The commonly used methods are the regulation of operational parameters such as aeration and the reduction of sludge age (Wanner, 1994). The use of toxicants such as chlorine was extensively used but is now only employed in severe cases. Physical methods such as skimming and water spraying have also been applied, but with limited success (Soddell, 1999).

2.4. Activated Sludge Microbiology

Activated sludge comprises a complex biocoenosis of microorganisms, which include bacteria, protozoa, fungi and algae. Bacteria make up approximately 95% of the total microbial population in activated sludge (Bitton, 1994). Within the domain, bacteria, there are many groups that may be found in activated sludge for example floc formers, filamentous bacteria and denitrifiers. Collectively bacteria make up such a large percentage because they are the most versatile of all organisms in terms of their nutrient requirements and, catabolic and metabolic activities.

Floc forming and filamentous bacteria are the crucial bacteria involved in the activated sludge process. Under optimum conditions floc forming bacteria predominate resulting in good settling characteristics of the sludge. A change in process conditions can trigger an increase in the growth of filamentous bacteria, which can lead to bulking and foaming. Although the distribution of filamentous bacteria varies from plant to plant, the presence of certain filament types is an indication of conditions prevalent within the system. Certain operational conditions may stimulate the growth of specific filaments (Table 2.1.).

Table 2.1. Dominant filament types that are indicative of activated sludge operational conditions (Bitton, 1994)

FILAMENTOUS BACTERIA	CONDITIONS
M. parvicella, *Nocardia spp.*, *Haliscomenobacter hydrossis*, 0041, 0675, 0092, 0581, 0961, 0803	Low F:M
1701, *S phaerotilus natans*, 021N, *Thiothrix spp.*	Low DO
Thiothrix spp., *Beggiatoa spp.*, 021N	Presence of Sulphides
Thiothrix spp., 021N	Nutrient Deficiency

2.4.1. Bulking Microbiology

Sphaerotilus natans was initially thought to be the sole cause of bulking (Blackall, 1999). However recent findings indicate about thirty different types of filaments responsible for bulking. Due to different operating parameters and influent loading, a variety of filaments were found to be predominant in different plants. *Microthrix parvicella*, which is a Gram-negative unbranched filament, was found to be dominant in a number of plants across Europe, Australia and South Africa (Casey *et al*., 1995; Blackall, 1999). Type 021N is a common filament found in the United States, Japan and Germany. Type 021N has been reported in some plants in South Africa but is generally quite rare in this region (Casey *et al*., 1995; Blackall, 1999).

Table 2.2. Filamentous bacteria causing bulking in activated sludge systems (Blackall, 1999)

Sphaerotilus natans	Type 1851
Flexibacter spp.	Type 1702
Thiothrix spp.	Type 1852
Beggiatoa spp.	Type 0803
Nocardia spp.	Type 0092
Nostocoida limicola I, II, III	Type 0961
Haliscomenobacter hydrossis	Type 0581
Cyanobacteria	Type 1863
Type 0041	Type 0411
Type 0675	Type 0914
Type 021N	Type 1701

2.4.2. Foaming Microbiology

Nocardia spp. has been reported to be the major causes of foaming or scum formation (Blackall, 1994). *Nocardia spp*. are Gram-positive branched filaments, which belong to the group, mycolic-acid containing actinomycetes (Stainsby *et al*., 2002). The most prevalent species found in foam is *Nocardia spp*., *Nostocoida limicola* and Type 0041, all of which have the ability to produce biosurfactants (Blackall, 1999). *M. parvicella* is also a major cause of foaming. High incidence of *M. parvicella* induced foaming has been reported in South Africa and France (Davenport *et al*., 2000). Filamentous bacteria, found in bulking sludge, have also been implicated in foam formation. A number of filaments resembling the mycolic-acid producing actinomycetes have been observed and termed '*Nocardia amarae*-like organisms' (NALO) and 'Pine tree-like organisms' (PTLO) (Stainsby *et al*., 2002). However, recent studies have distinguished 'Actinomycetes' from other *Nocardioforms* by the appearance of branched rods rather than the distinctive branching of NALO and PTLO (Soddell, 1999).

Table 2.3. Filamentous bacteria causing foaming in activated sludge systems (Soddell, 1999)

Nocardia spp.	Type 1851*
PTLO	Type 1701*
NALO	Type 0581*
'Actinomycetes'	Type 0803*
Type 0041	Type 021N*
Type 0675	*H. hydrossis**
Type 0092	*Sphaerotilus natans**
Type 0413*	*Nostocoida limicola* I, II, III*
Type 0914*	Type 0581*

*These filaments have been identified in some foams but their incidence is low.

2.5. The Taxonomic Status of Filamentous Bacteria

The conventional approach for identifying filamentous bacteria was largely based on morphological characterisation. Eikelboom and co-workers characterised twenty-six types of filamentous bacteria into seven groups based on morphology, cell inclusions, motility and staining reactions (Kämpfer and Wagner, 2002).

Table 2.4. Eikelboom grouping of filamentous bacteria in activated sludge (Kämpfer and Wagner, 2002)

GROUP 1: SHEATH FORMING, GRAM-NEGATIVE BACTERIA
Sphaerotilus natans
Haliscomenobacter hydrossis
Type 1701
Type 1702
Type 0321
GROUP 2: SHEATH FORMING, GRAM-POSITIVE BACTERIA
Type 0041
Type 0675
Type 1851
GROUP 3: SHEATHLESS, CURLED, MULTICELLULAR BACTERIA
Type 021N
Nostocoida limicola
Cyanophyceae

GROUP 4: SLENDER, COILED BACTERIA
Microthrix parvicella
Type 0581
Type 0192
GROUP 5: STRAIGHT, MULTICELLULAR, GRAM-NEGATIVE BACTERIA
Type 0803
Type 1091
Type 0092
Type 0961
GROUP 6: MOTILE BY GLIDING
Type 0914
Type 1111
Type 1501
Beggiatoa sp.
GROUP 7: ADDITIONAL/UNCLASSIFIED TYPES
Type 1863
Type 0411
Nocardia sp.
Fungi

The identification and classification of filamentous bacteria based on the above criteria have proven to be inadequate with their inability to distinguish between phylogenetically unrelated

bacteria. *Thiothrix spp.* containing sulphur granules cannot be morphologically differentiated from *Leucothrix spp.* (Kämpfer and Wagner, 2002). In addition some filamentous bacteria are gram-variable while some have displayed non-filamentous growth forms (Seviour and Blackall, 1999; Kämpfer and Wagner, 2002). It has been hypothesized that some filamentous bacteria undergo 'morphological shifts' to unicellular rods or cocci in response to changes in their environment (Wagner *et al*., 1994a). There have also been incidences of contradiction with regards to individual filament descriptions. The reference manuals of Eikelboom and Van Büijsen (1983) and that of Jenkins *et* al. (1986, 1993) sometimes provide different morphological descriptions for the same filament.

Majority of the filamentous bacteria are very difficult to isolate and culture under laboratory conditions. The lack of pure cultures has hampered the construction of detailed physiological, biochemical profiles, and reference strains for each filament type. Hence, there are no established databases for comparative studies. Some filaments have been successfully isolated, however, not much information could be obtained from their culturing because they are characterized by relatively slow growth rates on solid medium and rarely grow in broth cultures (Seviour and Blackall, 1999). A number of filamentous bacteria have not even been classified by Bergey's Manual of Systematic Bacteriology (Seviour and Blackall, 1999). In accordance with the international code of nomenclature, a substantial percentage of filaments could not be named and are therefore referred to as numerical types, for example Type 1863.

The development of molecular techniques has lead to the re-classification of some filaments based on their phylogenetic characteristics, for example *Skermania piniformis* has been renamed *Nocardia pinensis* (Wagner and Cloete, 2002). According to the international code of nomenclature's bacterial naming standards, '*M. parvicella'* is not a true name based on taxonomy. Molecular techniques have allowed for the resolution of the taxonomic status of

M. parvicella (Rosetti *et al*., 1997a). It was suggested that *M. parvicella* was an actinomycete. With the use of 16S rDNA sequencing it was confirmed that it was indeed an actinomycete (Soddell, 1999).

2.6. Molecular Analyses

Nucleic acid based techniques have been applied to circumvent the problems encountered when using culture-dependent techniques. Advances in molecular biology and phylogenetics have provided a more effective approach for the examination of species diversity and spatial distribution of bacteria in their respective environments. Molecular analyses allows for the analysis or detection of bacteria without the need for cultivation. The primary objective of molecular analyses is the comparative analyses of genotypes or genetic characteristics.

2.6.1. Bacterial Ribosomal RNA's

Molecular analyses is based, inter alia, on the nucleotide sequence comparison of ribosomal RNA's (rRNA) (Olsen and Woese, 1993). The rRNA's are frequently targeted because:

1. They are key elements involved in protein synthesis.
2. Their genes are highly conserved across all phylogenetic domains. Some regions within the rRNA are more conserved than other regions and thus allow for differentiation at any taxonomic level.
3. The conserved nature of the genes provides a convenient target for cloning specific genes and for primer-directed sequencing as well as for providing information on the evolutionary history of the organism.
4. The amount of rRNA present in a cell is directly proportional to the metabolic activity and hence the viability of the cell. (Olsen *et al*., 1986; Schram and Amann, 1999).

Bacterial ribosomes contain a 50S subunit and a 30S subunit. Each subunit consists of one or two rRNA molecules: 50S ⇨ 23S rRNA + 5S rRNA

30S ⇨ 16S rRNA (Prescott *et al*., 1999)

The 5S rRNA contains ± 120 nucleotides and is considered too small to infer phylogenetic information. The 23S rRNA contains ± 3000 nucleotides. Although it contains a lot of information, it is too large and cumbersome to work with. The 16S rRNA is the most commonly targeted genes because it contains about 1600 nucleotides, which is a convenient size to work with, and contains sufficient information for phylogenetic inference (Wanner, 1997).

The application of rRNA based nucleic acids techniques for the analysis of environmental samples ranges from simple bacterial identification to full-scale population dynamics. These techniques encompass the extraction of DNA or RNA from a sample followed by amplification via PCR. The resultant gene fragments are then subjected to gel electrophoresis for the formation of a sample profile (Ferris *et al*., 1996; Schramm and Amann, 1999). The 16S rRNA genes may also be targeted by oligonucleotide probes for hybridization (Amann, 1995).

2.6.2. Polymerase Chain Reaction (PCR)

The study of genetic organisation and function requires that sufficient quantities of DNA be available for analyses. Microorganisms were used to produce multiple copies of genes of interest, which was a laborious task. Between 1983 and 1985 a new cell-free technique called polymerase chain reaction was developed by Kary Mullis (Prescott *et* al., 1999). PCR is the in-vitro amplification of a specific locus. DNA is amplified by repeated cycles of heating and cooling and therefore large amounts of DNA can be obtained from minute quantities of DNA or when the DNA is of poor quality (Erlich, 1989). PCR can also be used for the rapid screening or sequencing of fragments directly form bacterial colonies or phage plaques. PCR involves the

combination of DNA template with two oligonucleotide primers, four deoxynucleotide triphosphates (dNTP's), magnesium (added in the form $MgCl_2$) and DNA polymerase in a reaction buffer. PCR is an enzyme-mediated process that is catalysed by the thermo-stable DNA polymerase, usually Taq DNA polymerase (Steffan and Atlas, 1991). The entire PCR process takes place within a thin-walled PCR tube to allow for the transfer of heat in a thermal cycler, which can reproducibly maintain PCR temperatures.

PCR comprises three main stages: template denaturation, primer annealing and extension:

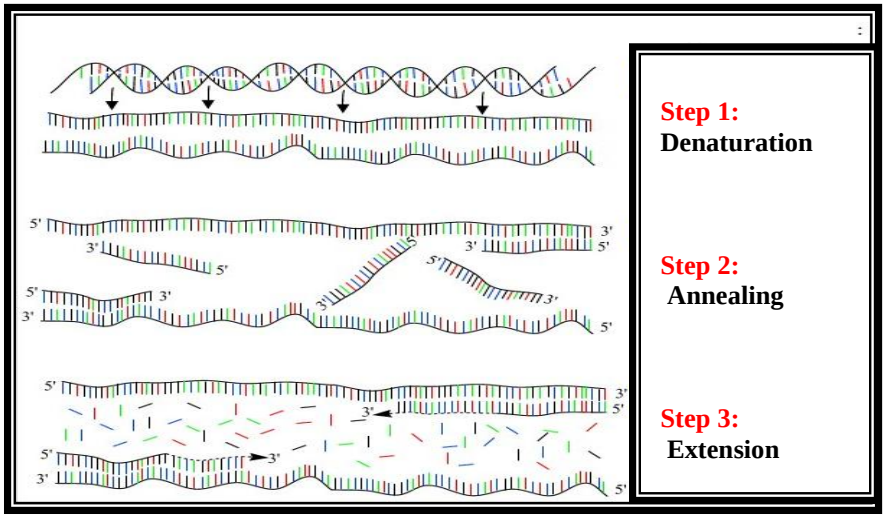

Figure 2.2. Polymerase Chain Reaction (Vierstraete, 1999)

Template Denaturation

The double stranded DNA (dsDNA) containing the sequence to be amplified is heat denatured to separate the complementary strands to produce a single stranded DNA (ssDNA) molecule. It is critical that the template DNA is completely denatured to ensure proper annealing of the primers to the template. If the DNA is partially denatured it will increase the chances of it reverting to the double stranded form thereby preventing primer annealing and extension or, it may lead to primer-primer binding. Many researchers use a 2 to 5 minute denaturation step before the actual

PCR cycling starts. Denaturation at 90°C to 98°C for 20 to 45 seconds is usually sufficient, but this step must be adapted for the thermal cycler, the tubes and the template DNA being used. Higher temperatures or longer denaturation times may be more appropriate for GC-rich samples. However, very high temperatures or increased exposure to elevated temperatures may decrease the activity of the Taq DNA polymerase (Erlich, 1989; Saiki, 1989a; Innis and Gelfand, 1990).

Primer Annealing

During this stage, the temperature is decreased. The primers move around in the solution by Brownian motion. The oligonucleotide primer anneals to the complementary sequence of the DNA strand by strong hydrogen bonding to provide an initiation site for the elongation of a new DNA strand. Primer-template annealing is highly temperature dependent. The annealing temperature is dependent upon the base composition, the length and concentration of the primers and hence, the melting temperatures of the primers. An annealing temperature that is about 5°C below the melting temperature of the primers generally yields the best results. The temperature is usually about ±54°C. For optimum amplification, it is advantageous to use two primers that have very similar melting temperatures. However, if the temperature is too high no annealing will occur but if the temperature is too low, non-specific annealing will occur (Innis and Gelfand, 1990; Vierstraete, 1999).

Extension

This is the final step of the PCR cycle. The primers are extended across the target sequence in the presence of free dNTP's. The primer adds dNTP's from the 5' to the 3' end, reading the template from the 3' to the 5' end. The temperature is adjusted for optimum polymerase activity, usually about ±72°C. This results in the formation of a complementary copy of the DNA template in the region specified by the annealed primer. The final PCR product is a double stranded DNA

molecule. Each strand produced acts as a template for the next cycle. DNA is amplified at an exponential rate (Figure 2..3.) (Erlich 1989; Steffan and Atlas, 1991; Vierstraete, 1999). In addition to the optimization of the above cycling parameters the total number of cycles has to also be established (Table 2.5). The number of cycles is dependent on the initial concentration of the template. The execution of too many cycles may exhaust the supply of primers, dNTP's and *Taq* and, may result in the accumulation of non-specific products while very few cycles will result in a low product yield. Most PCR applications use between 25 to 35 cycles (Innis and Gelfand, 1990).

Table 2.5. Guidelines for determining the number of PCR cycles (Innis and Gelfand, 1990)

NUMBER OF TARGET MOLECULES	NUMBER OF CYCLES
3×10^5	25 to 30
1.5×10^4	30 to 35
1×10^3	35 to 40
50	40 to 45

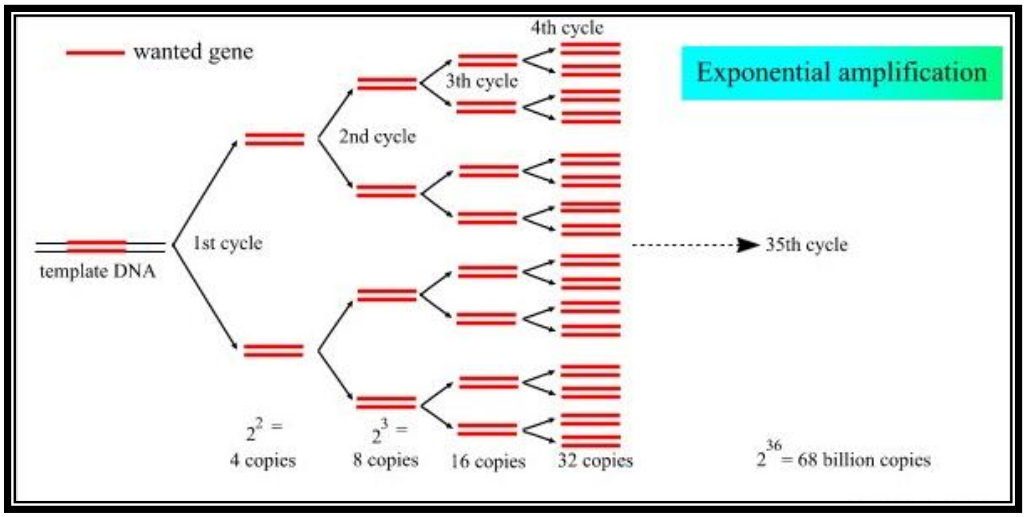

Figure 2.3. Exponential amplification of the template DNA (Vierstraete, 1999)

2.6.2.1. Critical Factors For A Successful PCR

PCR is a relatively complicated procedure. There are a several parameters which are interdependent that need to be considered and optimsed accordingly to increase specificity, fidelity and yield of the desired product.

Template

The template used in PCR should be of the highest purity possible. Sometimes the template may contain substances that may inhibit polymerase activity. When working with a DNA template there should be no trace of RNA in the sample as the RNA could chelate Magnesium (Mg^{2+}) and inactivate the polymerase and, thus reduce the yield of the desired product (Giovannoni, 1991). Most PCR's use DNA as a template rather than RNA because DNA is more stable and easier to work with. The concentration of template used also influences the reaction. For bacterial genomic DNA, a concentration between 0.1 – 1 μ*g* is sufficient and for plasmid or phage DNA a concentration of 0.01 – 1 ng (Fermentas Life Sciences, 2003).

Oligonucleotide Primers

Primers are short synthetic segments of nucleotides (Giovannoni, 1991). They are usually about 15 to 30 bases long (Fermentas Life Sciences, 2003). In PCR the primer is complementary to that portion of the DNA that is to be amplified. Primers can either be specific or universal. Universal primers are complementary to sequences that are common amongst a particular set of DNA molecules. For example the universal primer 1492R: 5' CCTACGGGAGGCAGCAG 3', is complementary to the region conserved among the members of the domain, bacteria (Muyzer *et al*., 1993). Primers are designed such that they are not complementary to each other at their 3' ends. This prevents the formation of primer-dimers. Primer-dimers are duplex PCR products that are composed mainly of primers and lack the template. Primer-dimers are easily amplified and

may become the primary PCR product (Gelfand and White, 1990). For optimum PCR, the primers should have between 50 to 60% G/C content. Primer concentrations have to optimised in accordance with other reaction components. Optimal concentrations are usually between 0.1 and 0.5 μM. Very high concentrations of the primer may result in the formation of non-specific products and primer-dimers. However, for some reactions a concentration of up to 1μM may improve the overall yield. Low concentrations of the primer will result in the exhaustion of the primers before the reaction is complete and would thus result in very low yields of the desired product (Innis and Gelfand, 1990).

DNA Polymerase

The first PCR experiments used the Klenow fragment (PolI-Kf)[23] of *E.coli* DNA Polymerase I (Gelfand, 1989). These experiments attested to be laborious as fresh enzyme had to added after each denaturation step because the enzyme is irreversibly denatured at 94°C (Giovannoni, 1991). PCR has now gained widespread application largely due to the substitution of PolI-Kf[23] with a DNA polymerase form the thermophilic eubacterium, *Thermus aquaticus* (*Taq*) (Giovannoni, 1991). *Thermus aquaticus* is a thermophile capable of growth at temperatures up to 75°C. *Taq* DNA Polymerase is highly stable and can withstand repeated exposures at high temperatures (94 to 75°C) and has a high extension rate, more than 60 nucleotides per second at 70°C (Gelfand and White, 1990). The optimum concentration of *Taq* is dependent on the template and primers that are used. Concentrations usually vary between 0.5 and 5 units/100 μl reaction (Innis and Gelfand, 1990). High concentrations of *Taq* may cause the formation of non-specific products (Fermentas Life Sciences, 2003).

With the advancement of technology, the demand for PCR with higher yields and fidelity increased. Today there are a number of variations of the standard, single *Taq* Polymerase

available. For example, Tth DNA Polymerase for Reverse Transcriptase - PCR (RT-PCR), Pwo for high fidelity PCR and the blend of two polymerases which has been optimized for Expand High Fidelity PCR (Burke, 1996).

Magnesium Concentration

The magnesium concentration is a critical component in PCR. The free Mg^{2+} ions form complexes with dNTP's, together with the primers and the DNA template to produce a substrate for polymerase activity. The amount of dNTP's in solution will determine the amount of free Mg^{2+} ions (Fermentas Life Sciences, 2003). Incorrect concentrations affect the product specificity and enzyme activity. High concentrations may increase denaturation and annealing temperatures, and the chance of primer-dimers forming (Saiki, 1989b; Innis and Gelfand, 1990). Concentrations vary between 1 and 5mM. The $MgCl_2$ has to be adjusted in relation to the dNTP concentration (Innis and Gelfand, 1990). For most PCR a concentration of 1.5mM is optimal with a dNTP concentration of 200µM (Saiki, 1989b).

dNTP Concentration

Deoxynuleotide triphosphates consist of dATP, dCTP, dGTP and dTTP. They are usually present at the optimal concentrations of 200µM (Innis and Gelfand, 1990). dNTP's can be added to the reaction mixture individually or as a mixture. Whichever method is chosen each dNTP should be present in the reaction mixture at equivalent concentrations. Very high concentrations and imbalanced dNTP mixtures will result in Taq Polymerase infidelity (Saiki, 1989b). Low concentrations reduce the likelihood of mispriming and the extension of misincorpoarted nucleotides (Innis *et al*., 1988; as cited by Innis and Gelfand, 1990).

2.6.3. Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE offers a convenient method for analysing PCR amplified products to infer sequence diversity amongst bacteria. In agarose and acrylamide gel electrophoresis DNA fragments are separated according size. In DGGE, the separation of fragments is based on their melting properties (Myers *et al*., 1989). DGGE offers the following advantages over other methods:

1. It has a high detection rate and sensitivity.
2. There is no need for radioactive labeling for detection of fragments.
3. It can detect fragments that range from 100 to 1000 base pairs and maybe even longer fragments.
4. It can detect differences in the melting behaviour of fragments that differ by even one base pair. (Myers *et al*., 1989; Bio-Rad, 1994; Spanevello, 1997).

2.6.3.1.Principle of DGGE

DGGE relies on the electrophoretic mobility of the denatured molecules, which is caused by differences in the rate at which the respective DNA molecules melt. The dsDNA molecules separate or melt when heat and a chemical denaturant, that is Urea and Formamide, is applied (Sheffield *et al*., 1990). DNA molecules melt in segments called 'melting domains'. The specific temperature at which a dsDNA dissociates is influenced by:

1. The strength of the hydrogen bonds between complementary base pairs. The melting domain in which an AT pair is substituted by a GC pair will melt at a higher temperature.
2. The force of attraction between adjacent bases on the same strand. (Myers *et* al., 1987).

Hence, the point at which the dsDNA begins to denature is dependent on the nucleotide sequence in that particular melting domain.

In a DGGE system, DNA fragments are electrophoresed through a polyacrylamide gel at a temperature between 50 and 60°C. The polyacrylamide gel contains a linear gradient of increasing denaturant concentrations, which range from 0% to 40% for Formamide and 0M to 7M for Urea (Bio-Rad, 1994). The dsDNA migrates through the gel until it reaches the concentration where it begins to partially melt. At this point the rate of mobility of the fragment slows down. As the denaturing conditions increase, the partially melted DNA will completely dissociate into single strands (Figure.2.4.) (Myers *et al*., 1989; Bio-Rad, 1994). The rate of mobility of the partially melted fragments is dependent on their physical shape; dsDNA fragments will migrate faster than the ssDNA fragments (Spanevello, 1997). The conformational change from dsDNA to ssDNA occurs in a step-like manner (Myers *et al*., 1989). The nucleotide sequence of the melting domain will determine the position of the DNA fragment in the gel. Different nucleotide sequences will result in different positions in the gel.

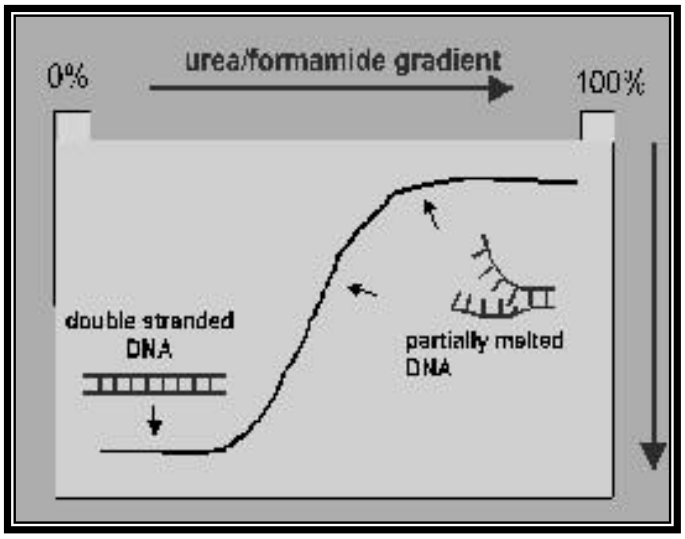

Figure 2.4. DNA melting properties during DGGE (Bio-Rad, 1994).

Strand dissociation only occurs in the region of low melting domains. High melting domains prevent complete strand separation. Some fragments are characterised by more than one melting

domain. Nucleotide base changes that do not occur in the first melting domain may go undetected due to a loss of fragment migration upon complete strand dissociation (Myers *et al.*, 1989). High melting domains are artificially created by incorporating a GC-rich sequence to one end of the molecule to overcome this problem (Myers *et al.*, 1989). The GC-rich sequence which is called a GC-clamp, is about 40 base pairs and is usually attached to the 5' end of forward PCR primer (Ferris *et al*., 1996). The GC-clamp imparts melting stability and allows for the formation and visualization of more discreet bands.

2.6.3.2. Variations Of DGGE

There are three types of denaturing gradient gels:

1. Perpendicular gradient gels: the gradient of denaturants is perpendicular to the direction of electrophoresis (Sheffield *et* al., 1990). It employs a broad range of denaturants such as a low density denaturing solution of 20% and a high density denaturing solution of 70% (Bio-Rad, 1994). Perpendicular gels are used to determine optimum denaturing conditions and the number of melting domains within the fragment
2. Parallel gradient gels: the gradient of denaturants is parallel to the electric current and employs a narrower range of denaturants to allow for better separation of fragments (Myers *et al*., 1987).
3. Constant gradient gel: the concentration of the denaturants is kept constant throughout the gel. The optimum denaturing conditions for the perpendicular DGGE is employed for this gel (Bio-Rad, 1994).

2.6.4. DNA Sequencing

DNA sequencing is the process of determining the precise order of nucleotides (A, T, G, C) on a DNA fragment (Howe and Ward, 1991). The comparative sequence analysis for identification and phylogenetic charcterisation of microorganisms has been a major advancement in molecular biology. It has provided the foundation for the development of a number of molecular techniques. DNA sequencing relies on the successful isolation and amplification of a DNA fragment. Two different techniques were developed, the dideoxy chain-terminating method by F. Sanger and A. R. Coulson and, and the chemical degradation method by A. Maxam and W. Gilbert (Dale, 1998).

Chemical Degradation Method

This method uses chemical reagents to cleave double stranded DNA. The DNA fragments are usually labelled with a radioactive phosphorus group at the 5' end of each strand. The addition of a chemical reagent causes the dsDNA to dissociate. The single strands are separated by gel electrophoresis. One strand is selected, purified and divided into four portions. Each portion is subjected to a chemical cleavage reagent. These reagents are nucleotide specific. The reagent renders the strand susceptible to cleavage at the particular nucleotide when the chemical piperidine is added (Lodish *et al*., 2000). The resultant fragments are separated electrophoretically and visualized. Four gel lanes each corresponding to one of A, G, C and T will contain a series of bands with only one at each level. The sequence is read from the bottom of the gel, from the fragment that has migrated the furthest, progressing up the gel to the fragment that has migrated the shortest distance (Prescott *et al*., 1999; Untereiner and Vancanneyt, 1997).

There are a number of variations of this method with regards to the type of chemicals that are used. However, this method is not frequently employed as the chemicals utilised are extremely toxic and may cause irreversible damage.

Dideoxy Chain-terminating Method

This method employs dideoxynucleotide triphosphates (ddNTP's). These ddNTP's are labeled with a compound (α ^{32}P) and differ from the normal dNTP's in that they lack the hydroxyl group (-OH) on the carbon atom at the 3' end. When ddNTP's are added to an extending DNA strand, chain elongation stops because there are no 3' –OH groups available for the next nucleotide to attach to (Prescott *et* al., 1999). Single stranded DNA is used for this method. DNA polymerase, a primer, the four dNTP's (dATP, dGTP, dCTP, dTTP) and one ddNTP are added to the single stranded template. Chain elongation proceeds normally due to the presence of the dNTP's but terminates when the ddNTP is incorporated instead of a dNTP. For example. if ddATP is incorporated into the mixture, then termination will occur at positions that are opposite thymidines (T).

Termination does not always occur at the first T because dATP may have been incorporated instead of ddATP. During the reaction the ratio of ddNTP to dNTP is such that the single stranded DNA is allowed to undergo elongation before a ddNTP is added. This results in fragments of varying length. Four synthesis reactions have to be carried out, each with a different chain-terminating inhibitor (Untereiner and Vancanneyt, 1997; Dale, 1998; Prescott *et* al., 1999). The resultant fragments are electrophoresed and visualized as per chain-terminating method.

Modern sequencing procedures are based on the chain-termination method but employ ddNTP's that are labelled with a fluorescent chemical marker. Each ddNTP is labelled with a different coloured marker. The resultant fragments are detected as the amplified PCR fragments pass through a stationary laser. The sequence of the strand is recorded as a chromatogram where each peak corresponds to a single nucleotide. The chromatogram is recorded by a computer and converted into sequence data, which can be compared with sequences in the various databases (Howe and Ward, 1991).

2.6.5. Fluorescent *In Situ* Hybridisation (FISH)

Fluorescent in situ hybridisation of whole cells using 16S rRNA-targeted oligonucleotide probes provides an effective tool for phylogenetic identification, enumeration and for analyzing the spatial distribution of organisms in the environment without the need for cultivation (Hugenholtz *et al*., 2001). Oligonucleotide probes are short, synthetic sequences of nucleic acid that are designed to be complementary to a conserved region of RNA in a particular organism or group of organisms (Coskuner, 2002). The probes can be designed to target different phylogenetic groups ranging from family to species level (Keller and Manak., 1989). The probes are frequently labelled with fluorochromes for example, fluorescein at the 5' end. This allows for the easy detection of hybridized cells. Oligonucleotide probes have a number of advantages:

1. They are very stable; they are not easily degraded by rNases.
2. They are easily available to specification.
3. They permit the incorporation of fluorochromes. Fluorochrome labelled probes do not interfere with the hybridisation process as compared with cDNA and RNA probes which often hinder the hybridisation process.

2.6.5.1. Principle of FISH

FISH entails the application of an oligonucleotie probe to whole cells. The cells are usually pre-treated with aldehydes (formalin, paraformaldehyde, glutaraldehyde) or alcohols (ethanol, methanol) to make them more permeable for penetration by the probes (Amann, 1995). The hybridisation step occurs under an elevated temperature in the presence of the hybridisation buffer. The probes penetrate the cells. Once inside the cells, they hybridise to their complementary target sequence via hydrogen bonding. If the complementary sequence is absent, the probes are unable to hybridise and are removed during the subsequent washing step (Amann, 1995).

The specificity of the probe binding to the target is not only dependent on the sequence of the probe but also the hybridization and washing stringencies. The concentration of formamide in the hybridization buffer and sodium chloride in the wash buffer are inter-dependent and, have to carefully be optimized for each probe (Amann *et al*., 1995). Cells that have undergone successful hybridisation can be visualised under epifluorescence microscopy using the appropriate filter set. The white light of the microscope is filtered which allows only the relevant wavelengths to pass through. This results in excitation of the fluorochromes, which is evident from the brightly coloured signals against a dark background. With the use of more sophisticated filter sets it is possible to visualise multiple flurochromes. Hence, it is possible to apply multiple probes of varying specificity, containing different flurochromes, on the sample (Hugenholtz *et al*., 2001).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Samples

Previous research undertaken at the Centre for Water and Wastewater Technology involved the isolation of filamentous bacteria from activated sludge samples. The samples were obtained from various activated sludge wastewater treatment plants, in particular those plants experiencing bulking and foaming problems, in Durban and surrounding areas (Kwa-Zulu Natal, South Africa).

The samples were screened using different culture medium and various staining procedures. Fourteen filamentous bacteria were isolated and presumptively identified using the Eikelboom-Type filament identification system, based on morphology and staining characteristics. The isolates were maintained on nutrient agar and casitone-gylcerol-yeast extract agar (CGYA), slopes and plates. They were also inoculated onto various other media, which have been reported to be successful for the growth of specific filamentous bacteria (Ramothokang, 2004).

These fourteen isolates provided the basis for this project. The fourteen isolates are given in Table 3.1. This project was conducted in two parts. In the first part of the project the fourteen isolates were subjected to FISH. For the second part of the project, the isolates were subjected to DGGE profiling of their PCR amplified fragments coding for the 16S rRNA genes, which was followed by nucleotide sequence analysis.

Table 3.1. Filamentous Bacterial Isolates

HCBTY
NGCD
NGCD01
SWNCGO1
SWNCGO2
HCBCG01
UCSRO1
OSIOO1a
OSIOO1b
OSIOO4b
DLNT01
TTBCGO1
TTBCG02
TTBCGO3

The above isolates were named according to the coding system derived during previous research at the Centre for Water and Wastewater Technology (Ramothokang, 2004). The isolates were given codes based on the activated sludge plant they were isolated from, the pre-treatment method of the isolate prior to culturing and the medium they were cultured on. For example, the isolate TTBCGO2, *TT* refers to Amanzimtoti (Toti) wastewater works, *B* refers to the bead-beating method of pre-treatment, *CG* refers to CGYA medium and *O2* is the numbering of the culture because three cultures were isolated from that plant.

PART A

3.2. Oligonucleotide Probes

The following oligonucleotide probes were synthesised according to specification and were labelled with fluorescein at the 5' end (MWG-Biotech, Germany; subsidiary company of Roche Products (PTY) LTD, South Africa). Probes used are given in Table 3.2 and the formamide stringencies for each probe are given in Table 3.3. Probe selection was based on the filamentous bacteria that are commonly found in activated sludge and presumptive identification of the isolates based on the Eikelboom -Type Filament identification system.

Table 3.2. Oligonucleotide probes and target specificity

PROBE NAME	TARGET SPECIFICITY
SNA	*Sphaerotilus natans*
TNI	*Thiothrix nivea*
GA	*Gordonia amarae*
HHY	*Haliscomenobacter hydrossis*
21N	Type 021N
ACA	*Acinetobacter* species
TFR	*Thiothrix fructosivarans*
DLP	Nocardioform filamentous bacteria

Table 3.3. Oligonucleotide probe sequences and stringencies

PROBE	SEQUENCE (5'- 3')	FORMAMIDE%	REFERENCE
SNA	CATCCCCCTCTACCGTAC	45	Wagner *et al.*, 1994a
TNI	CTCCTCTCCCACATTCTA	45	Kanagawa *et al.*, 2000
GA	ATGA(CT~Y)GTCCCCTCTGA	45	Kampfer *et al*., 1996
HHY	GCCTACCTCAACCTGATT	20	Wagner *et al.*, 1994a
21N	TCCCTCTCCCAAATTCTA	35	Wagner *et al.*, 1994a
ACA	ATCCTCTCCCATACTCTA	35	Wagner *et al.*, 1994b
TFR	CTCCTCTCCACACTCTA	40	Kim *et al.*, 2002
DLP	CCACCATGCGGCAGGAGCTCA	40	Schüppler *et al*., 1998.

3.3. *In Situ* Hybridisation

Pre-treatment of slides

The slides were cleaned and pre-treated using a modified version of the procedure stated by Amann (1995) (APPENDIX 1). The 0.1% gelatin and 0.01% chromium sulphate were substituted with a 10% solution of poly-L-lysine (Sigma Diagnostics, USA). Slides that are coated with gelatin and chromium sulphate are usually used for samples that contain a relatively low concentration of biomass. Gelatin coated slides cannot be stored for long periods as the gelatin slowly begins to detach from the surface of slide.

Sample Preparation

The fourteen isolates were fixed in 4% paraformaldehyde (APPENDIX 2). Approximately 0.2g of each of isolate was suspended in 4% paraformaldehyde fixative and allowed to fix for 1 minute only.

Aliquot samples of the fixed isolates were sonicated for 10 seconds at 30 Watts. Sonication was used as a method of pre-treatment for filamentous bacteria prior to hybridization. The sonicated and unsonicated samples of the isolates were immobilized onto the Poly-L-Lysine heavy Teflon coated slides (APPENDIX 3). Both sonicated and un-sonicated samples were used to determine whether sonication as a pre-treatment would increase permeability and hence provide better FISH results.

Whole Cell Hybridisation and DAPI Staining

Hybridisation was carried out in a 50 ml polypropylene centrifuge tube, which served as a chamber for whole cell hybridisation. The tube used for hybridization has to be properly sealed to prevent evaporative loss of the hybridisation buffer, which might result in non-specific binding of the probes to the cells. (Amann, 1995). The concentration of formamide in the hybridization buffers and sodium chloride in the wash buffers was optimised accordingly for each probe. If a high stringency is required formamide (up to 59%) can be added to the hybridisation solution (Amann, 1995). All the probes were diluted accordingly with the appropriate hybridization buffer. All eight probes were applied to each of the fourteen isolates and to the activated sludge samples. Hybridisation was performed according to the methods stated by Amann (1995) (APPENDIX 4).

Once whole cell hybridization was complete, the cells were stained with a DNA-intercalating dye called called 4',6-diamidino-2-phenylindole (DAPI) (APPENDIX 4). This method of staining was modified from the methods of Hicks *et al*., (1992). The slides were then viewed using the Zeiss (Germany) Axiolab microscope fitted for epifluorescence and Zeiss filter sets (Filter set numbers: 02 for DAPI and 09 for fluorescein). Image analysis was carried out using the Zeiss KS 300 imaging system.

PART B

3.4. Extraction of DNA

During this study the DNA extraction methods of Kuhn *et al*. (2002) and, Mayer and Palmer (1996) were combined and modified to produce a single method , that would yield a high concentration of pure DNA.

1. Approximately 0.2g of cells from each isolate was added to 500*ul* of sterile de-ionised water and centrifuge (3500 rpm, 5 minutes).
2. The cell pellet was resuspend in 75*ul* of Tris-EDTA buffer (10mM Tris-HCl, 1mM EDTA, pH8.0) and 25*ul* of 10% sodium dodecyl-sulphate (SDS).
3. The tubes were mixed and incubated for 2 hours at 48°C.
4. 500*ul* of lysis buffer (10% SDS, 0.1M NaCl, 0.5M Tris-HCl) was added after incubation.
5. The cells were then lysed using the freeze-thaw method, which involves immersing the tubes in a dry ice-ethanol slurry for 2 minutes followed by incubation at 48°C for 5 minutes. This cycle was repeated 5 times.
6. 500*ul* of Tris-saturated phenol was then added, mixed by inversion and centrifuge.
7. The upper layer was then removed to a clean tube. This is followed by consecutive treatments using phenol-chloroform (25 : 24 v/v), chloroform and 100% Isopropyl-alcohol. With each treatment, the upper layer was immediately transferred to a clean tube and centrifuged (3500 rpm, 5 minutes). Following the addition of Isopropanol the tubes were stored at -20°C for 1 hour.
8. The DNA was pelleted by centrifugation (3500 rpm, 5 minutes) and resuspended in 30*ul* of sterile de-ionised water and stored at -20°C.

3.5. Agarose Gel Electrophoresis

Subsequent to DNA extraction, the samples were run on agarose gels to ensure the presence of DNA and to check the purity of the extracted samples. Some PCR amplified fragments (section 3.6. PCR) were also subjected to agarose gel electrophoresis. A 2.5% agarose gel was used (APPENDIX 5). Agarose gel electrophoresis was accomplished using an HG/02 Mini Gel System comprising a single place horizontal submarine gel (OMEG Scientific, South Africa) and Power Pac 300 (Bio-Rad, South Africa). The gel was run at 80 volts for 60 minutes. Following electrophoresis, the gel was viewed using a Hoefer® MacroVue UV-20 transilluminator (Pharmacia- Biotech AB, South Africa).

3.6. Polymerase Chain Reaction (PCR)

DNA that was successfully extracted from the isolates was amplified via PCR. PCR had to be optimized for each of the filamentous isolates. The PCR reaction conditions, published by Giovannoni (1991), were used as a basis to determine the optimum amplification conditions of the 16S rDNA of some of the isolates whilst conditions for the optimum amplification of the remaining isolates were determined and optimsed using Taguchi methods (Cobb and Clarkson, 1994).

Three primers were used in this study. The primers are all universal primers that are complementary to the region conserved among the members of the domain, bacteria. A 40 base pair GC-clamp was attached to the 5' end of the forward primers. The primers used for each isolate is summarized in Table 3.4. (f – denotes forward primer, r- denotes reverse primer).

- ✧ Primer 27f : 5' GAGTTTGATCC GGCTCAG 3' (Blackall, 1994)
- ✧ Primer 341f : 5' ATTACCGCGGCTGCTGG 3' (Sigler and Turco, 2002)

- ✧ Primer 1492r: 5’ TAC GGC TAC CTT GTT ACG ACT T 3’ (Blackall, 1994)
- ✧ 40bp GC-clamp: 5’ CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCC GGG GGG C (Muyzer *et al*, 1993).

PCR was accomplished using a 10 × PCR buffer without Mg^{2+}, $MgCl^{2+}$, dNTP mix and proof reading Taq Polymerase (Fermentas, USA – distributed by Inqaba Biotech Pty. Ltd., South Africa); oligonucleotide primers (27f and 1492r - Roche Diagnostics, Germany; 341f and 1492r – Fermentas, USA – distributed by Inqaba Biotech Pty. Ltd., South Africa). PCR was carried out in an automated PCR Sprint Temperature Cycling System (Hybaid, United Kingdom).

Table 3.4. Primers used in PCR

ISOLATE	PRIMERS
HCBTY	341f + GC-clamp & 1492r
NGCD	341f + GC-clamp & 1492r
NGCD01	341f + GC-clamp & 1492r
SWNCGO1	27f + GC-clamp & 1492r
SWNCGO2	27f + GC-clamp & 1492r
HCBCG01	27f + GC-clamp & 1492r
UCSRO1	27f + GC-clamp & 1492r
OSIOO1a	27f + GC-clamp & 1492r
OSIOO1b	341f + GC-clamp & 1492r
OSIOO4b	27f + GC-clamp & 1492r
DLNT01	27f + GC-clamp & 1492r
TTBCGO1	27f + GC-clamp & 1492r
TTBCG02	27f + GC-clamp & 1492r
TTBCGO3	341f + GC-clamp & 1492r

PCR Reaction conditions for Batch 1: filamentous bacterial isolates

Table 3.5. PCR reaction mixture for Batch 1 isolates (Giovannoni, 1991; modified)

FILAMENTOUS ISOLATES	SOLUTION + CONCENTRATION	AMOUNT µl
HCBTY TTBCGO3 OSIOO1a OSIOO1b OSIOO4b DLNTO1	10× PCR Buffer without Mg^{+}	10
	1.5mM $MgCl_2$	6
	10ng/ml Genomic DNA	10
	2mM dNTP (mix of all 4 dNTP's)	10
	25pmol Stock Solution of each probe	2
	Sterile distilled water	59
	Taq Polymerase 1unit/*ul* *****	1

PCR Cycling Parameters (Giovannoni, 1991)

Stage 1 *****	Step 1	94°C	4 minutes	× 1cycle
Stage 2	Step 1	94°C	1 minute	× 35 cycles
	Step 2	53°C	1 minute	
	Step 3	72°C	2 minutes	
Stage 3	Step 1	72°C	4 minutes	× 1 cycle
	Hold	4°C		

***** The 1µl of Taq Polymerase was added after stage 1, which was the initial denaturation step.

PCR Reaction conditions for Batch 2: filamentous bacterial isolates

Table 3.6. PCR reaction mixture for Batch 2 isolates (as determined using Taguchi Methods; Cobb and Clarkson, 1994)

FILAMENTOUS ISOLATES	SOLUTION + CONCENTRATION	AMOUNT µl
NGCD NGCD01 SWNCGO1 SWNCG02 HCBCG01 UCSRO1 TTBCGO1 TTBCG02	10× PCR Buffer without Mg^{+}	10
	1.5mM $MgCl_2$	6
	10ng/ml Genomic DNA	20
	2mM dNTP	15
	25pmol Stock Solution of each probe	2
	Sterile distilled water	44
	Taq Polymerase 1unit/*ul* *****	1

PCR Cycling Parameters (Blackall, 1994)

Stage 1 *****	Step 1	98°C	1 minutes	× 1 cycle
Stage 2	Step 1	48°C	45 seconds	× 35 cycles
	Step 2	72°C	2 minute	
	Step 3	93°C	1 minutes	
Stage 3	Step 1	48°C	10 minutes	× 1 cycle
	Step 2	72°C	10 minutes	× 1 cycle
	Hold	4°C		

***** The 1µl of Taq Polymerase was added after stage 1, which was the initial denaturation step.

3.7. Denaturing Gradient Gel Electrophoresis (DGGE)

PCR amplified DNA fragments coding for 16S rRNA genes were separated by DGGE using the *BIO-RAD* D Gene™ Denaturing Gel Electrophoresis System (Bio-Rad, South Africa) and Power Pac 300 (Bio-Rad, South Africa). DGGE was done in accordance with the *BIO-RAD* D Gene instruction manual and applications guide (catalog numbers 170-9000 through 170-9070). 7.5%, 16 ×16-cm parallel gels were used. The gels contained a linearly increasing concentration gradient of formamide and urea where a 100% denaturant is defined as 40% formamide + 7M urea. The optimum concentration of the denaturing solutions was determined experimentally by varying the concentrations of urea and formamide, respectively. Good separation and resolution of DNA into clear bands on the gel demonstrated optimum concentrations. A 30% low density denaturing solution and a 60% high density denaturing solution were used.

The gels were poured between two glass plates with a 1.5mm spacer using the Bio-Rad Model 475 Gradient Delivery System (Bio-Rad, South Africa). Immediately prior to pouring the gels, ammonium persulphate (200mg/ml) and TEMED were added to the high and low density solutions. These two chemicals had to be added last because these chemicals are responsible for the polymerisation of the gel. The solutions used for DGGE is detailed in APPENDIX 6. Once the gel had polymersied, the wells were washed with 1× TAE buffer. The samples/dye mix were then loaded (APPENDIX 6). A 72-1353 base pair DNA molecular weight marker (IX, version 3, 2000, 0.25µg/µl; Roche Products Pty Ltd., South Africa) was used. All the chemicals used were of electrophoretic grade (Bio-Rad, South Africa). The gels were electrophoresed at 200V for 1 hour at a constant temperature of 60°C.

Staining of Gel

After electrophoresis, the gel was removed from the electrophoresis tank and core. The gel was then stained with 250 mL 1× TAE buffer containing 10 *ul* ethidium bromide (10 mg/mL) for 1-2 minutes. The gel was then transferred to another container containing 250 mL 1× TAE buffer where it was de-stained for 10 minutes. The gel was then viewed using a Hoefer® MacroVue UV-20 transilluminator (Pharmacia- Biotech AB, South Africa).

3.8. Excision and Purification of Bands

After DGGE, selected bands were excised from the gel using a sterile scalpel. The excised fragments were placed in a 1mL Eppendorf® tubes, each containing 40*ul* sterile distilled water. The excised bands were incubated at -20°C for 15 minutes. This was followed by an overnight incubation at 37°C. The samples were then centrifuged (5000 rpm for 7 minutes) and the supernatant was removed to a clean tube. The supernatant contained the DNA. The samples were re-amplified using the respective PCR reaction conditions as summarized in Table 3.5. and Table 3.6., and the accompanying cycling parameters. A sample of each PCR product (4µl) was subjected to agarose gel electrophoresis to ensure purity and the presence of sufficient DNA for sequencing reactions.

3.9. Nucleotide Sequencing

After a DGGE profile was established and purity was confirmed the bacterial genomic DNA of the filamentous isolates was sequenced. The samples were sent to a commercial sequencing facility (Inqaba Biotech Pty Ltd., South Africa). Nucleotide sequencing was done using the Applied Biosystems, ABI Prism® BigDye version 3.1. Dye™ Terminator Cycle Sequencing Ready Reaction kits. A Spectrumedix SCE 2410 automated DNA sequencer equipped with 24 capillaries (SpectruMedix LLC, Pennsylvania, USA) was employed to separate the fragments and to read the sequences.

The complied sequences were sent to us in an scf. file format. The sequences were then analysed using the programs, Chromas version 2.3 and Chromas Pro version 1.2 (Technelysium Pty Ltd.). The derived sequences were compared with the nucleotide sequences in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) 2.2.10. Algorithm (Altschul *et al*., 1997).

CHAPTER FOUR

RESULTS

PART A

4.1. Fluorescent *In Situ* Hybridization

Table 4.1. FISH results of the filamentous bacterial isolates

ISOLATES	PROBE HOMOLOGY
HCBTY	-
NGCD	HHY
NGCD01	Type 021 N
SWNCGO1	HHY
SWNCGO2	ACA
HCBCG01	GA
UCSRO1	DLP
OSIOO1a	Type 021 N
OSIOO1b	-
OSIOO4b	TNI bound to cells only
DLNTO1	SNA & HHY
TTBCGO3	-
TTBCGO2	-
TTBCGO1	GA

The following figures (Figure 4.1 to Figure 4.35) are randomly selected fields that depict the respective filamentous bacterial isolates that have been subjected to FISH and stained with DAPI respectively.

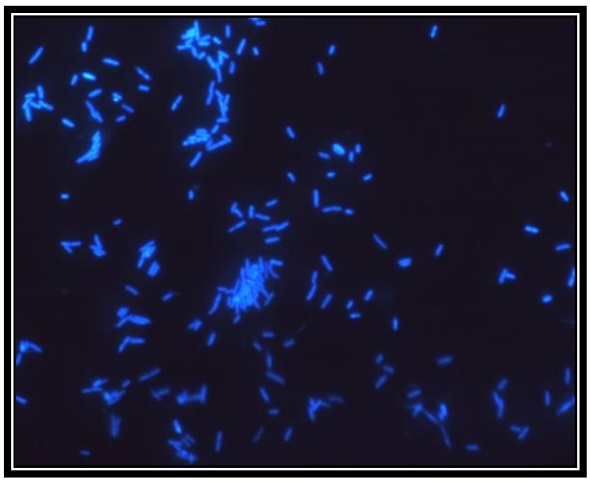

Figure 4.1. Micrograph of the isolate, HCBTY after DAPI staining.

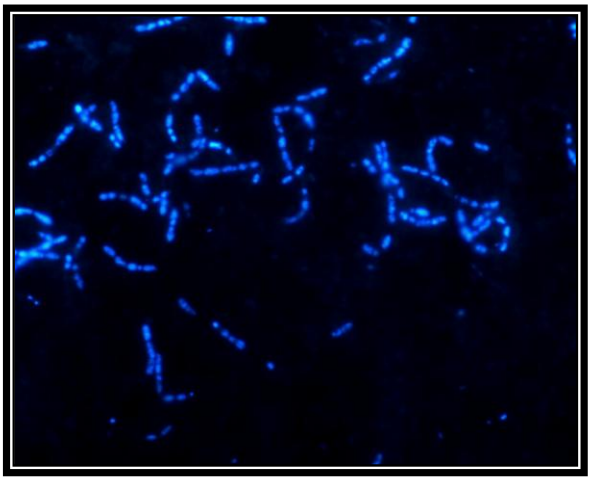

Figure 4.2. Micrograph of the isolate, TTBCGO3 after DAPI staining.

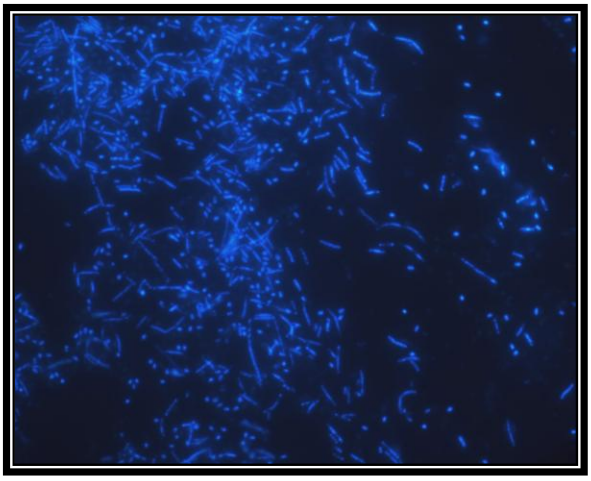

Figure 4.3. Micrograph of the isolate, TTBCGO2 after DAPI staining.

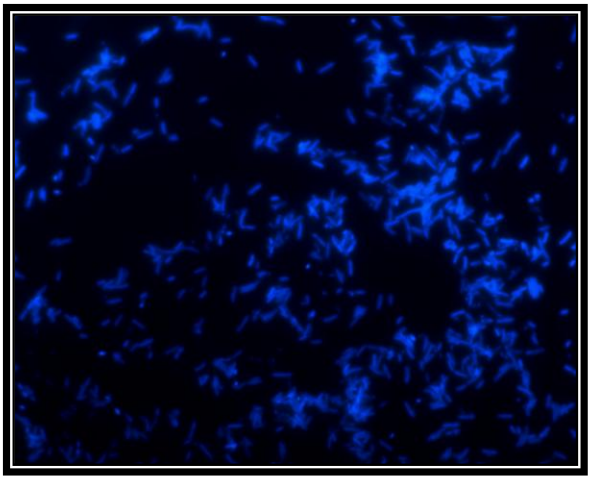

Figure 4.4. Micrograph of the isolate, OSIOO1b after DAPI staining.

Cultures HCBTY, TTBCGO2, TTBCGO3 and OSIOO1b all showed no homology with any of the oligonucleotide probes that were applied (Figures 4.1, 4.2, 4.3 and 4.4). DAPI staining allowed for the visualization of the morphology and distribution within the culture.

Cultures HCBTY and OSIOO1b had similar morphological characteristics (Figures 4.1 and 4.4). The cultures contained thin, long rods that were scattered while some cells were present in clumps. TTBCGO2 (Figure 4.3) contained thin, slightly longer rods but there was also an abundance of single cells. The single cells were present in pairs or chains. After an initial observation of the sample under DAPI it was hypothesized that TTBCGO2 was a mixed culture. However, careful inspection of the culture plates and the gram reaction indicated that it was a pure culture. When working with filaments, the possibility of a morphological shift cannot be excluded. The culture TTBCGO3 (Figure 4.2) displayed very different morphological characteristics from the other three cultures. Medium length filaments that appeared to be segmented were observed. These filaments were sparsely distributed as compared with the other cultures.

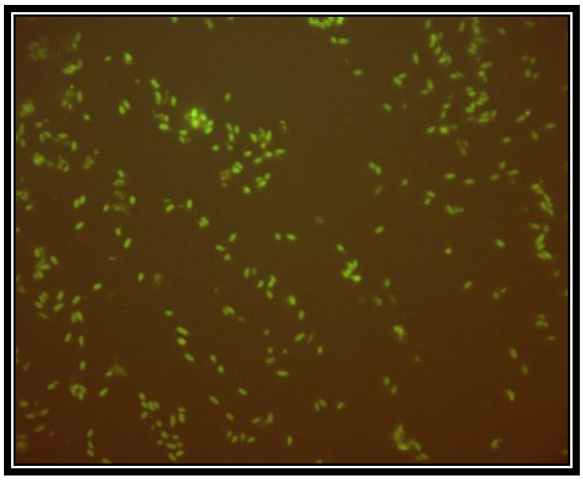

Figure 4.5. Micrograph of the isolate, NGCD after hybridization with fluorescein (green) labelled probe HHY.

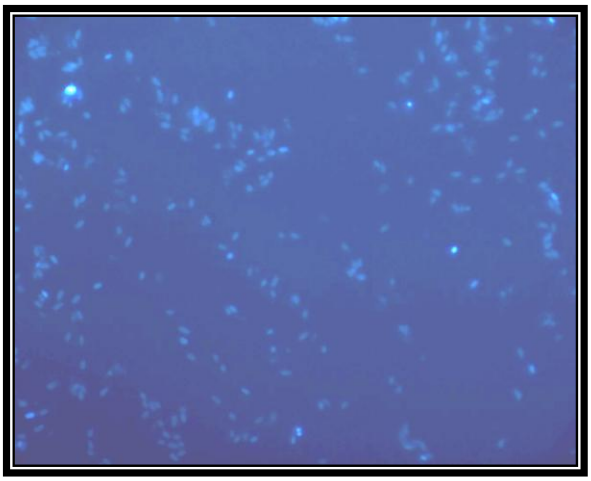

Figure 4.6. Micrograph of the isolate, NGCD after DAPI staining.

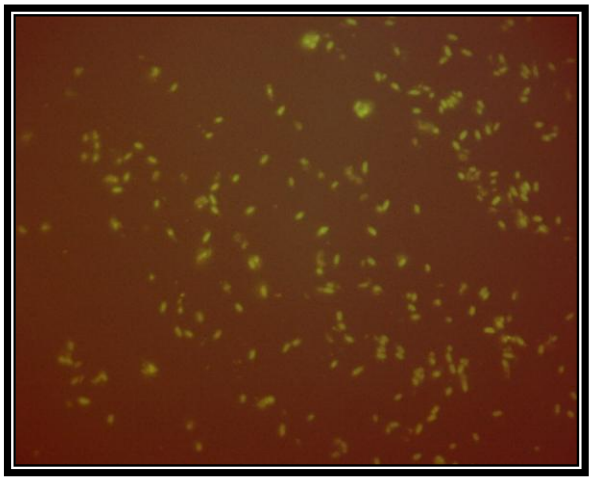

Figure 4.7. Micrograph of the isolate, SWNCGO1 after hybridization with fluorescein (green) labelled probe HHY.

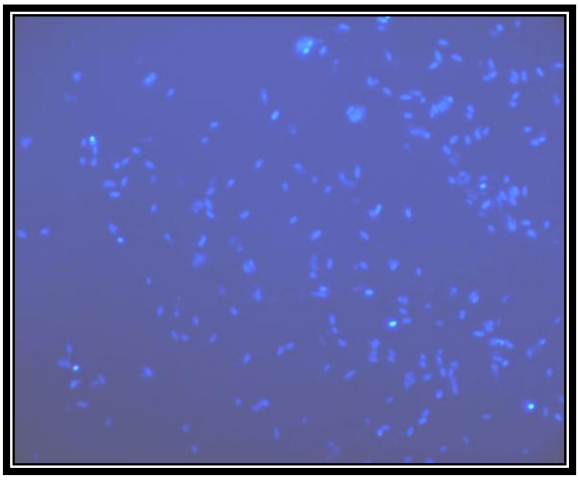

Figure 4.8. Micrograph of the isolate, SWNCGO1 after DAPI staining.

Isolates NGCD and SWNCGO1 showed positive hybridization signals only for the HHY probe (Figures 4.5 and 4.7). Both the cultures displayed similar morphological characteristics. The morphologies of these pure cultures were not characteristic with *H. hydrossiss* or other filamentous bacteria. Single, oval shaped and rectangular shaped cells were observed.

Although relatively good signals were obtained during FISH, the corresponding DAPI stains were very faint (Figures 4.6 and 4.8). The faint DAPI stains indicate that the dye did not bind properly to the cells. The cultures were stored at 4°C, which rendered them inactive or dormant. DAPI binds only to DNA. If the cells are inactive, they have a lower concentration of DNA present and hence, less DNA available for the dye to bind with (Porter and Feig, 1980). The cells were not metabolically active and hence, there was insufficient DNA for the dye to form a complex with. Good signals were received during FISH because the probes targeted the RNA, which is more stable and conserved than DNA.

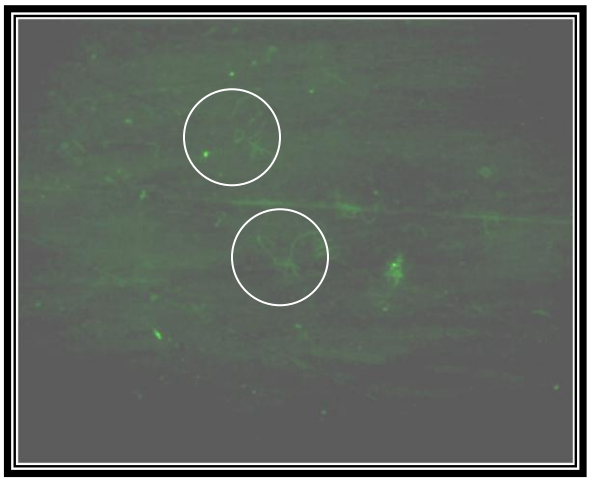

Figure 4.9. Micrograph of the isolate NGCD01 after hybridization with fluorescein (green) labelled probe O21N.

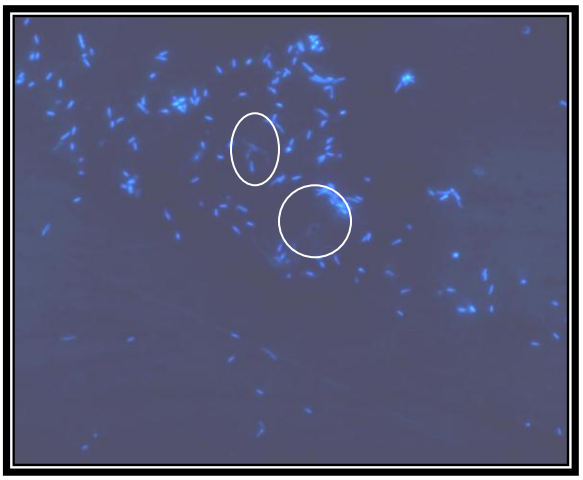

Figure 4.10. Micrograph of the isolate NGCDO1 after DAPI staining.

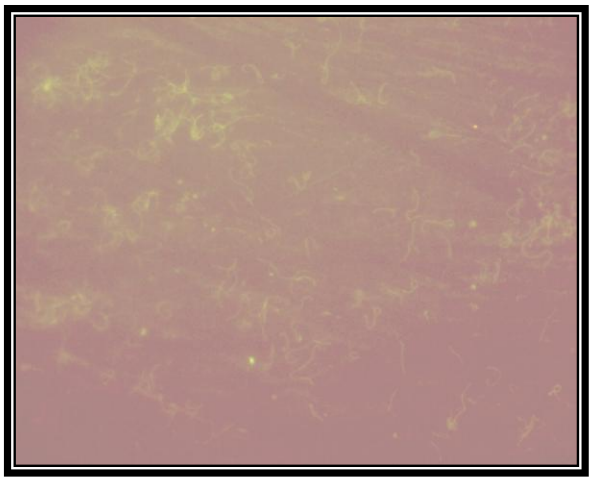

Figure 4.11. Micrograph of the isolate OSIOO1a after hybridization with fluorescein (green) labelled probe O21N.

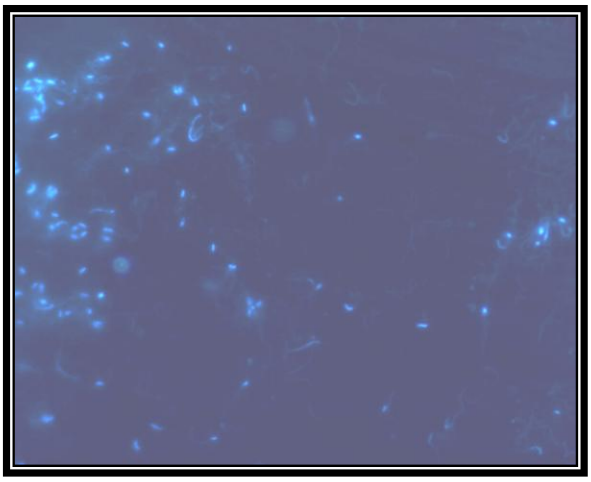

Figure 4.12. Micrograph of the isolate OSIOO1a after DAPI staining.

NGCDO1 and OSIOO1a showed positive hybridization signals for the 21N probe only. Figures 4.9 and 4.11(cells indicated by the white circles) show the probe had hybridized to long fibre-like cells. The corresponding DAPI images (Figures 4.10 and 4.12) show the presence of other cells in conjunction with the fibre-like cells. NGCDO1 and OSIOO1a appear to be mixed cultures. It is possible that the single cells maybe a contaminant. However, the possibility of a contaminant was not apparent from physical examinations of the agar slants and plates. Gram stains of these cultures did not provide any conclusive evidence of a contaminant (results not shown). The fibre-like cells appeared very faint, almost unnoticeable under DAPI, indicating that they maybe metabolically inactive because there was less DNA available for the DAPI to bind. They were more apparent under fluorescence because the probes had targeted the rRNA and not DNA. These two cultures displayed different morphological characteristics from those described by Jenkins *et al* (1993) for Type O21N.

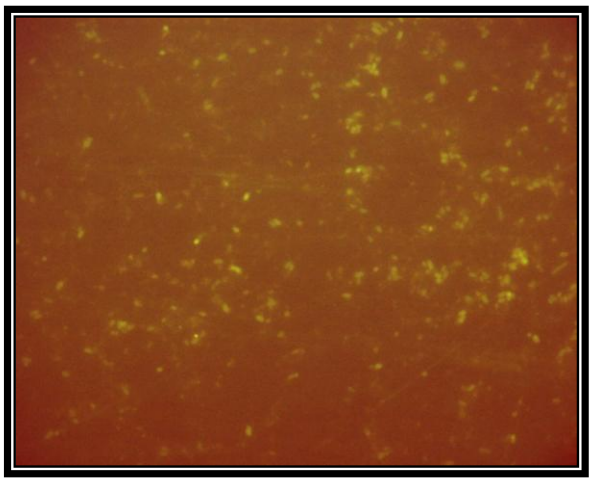

Figure 4.13. Micrograph of the isolate HCBCGO1 after hybridization with fluorescein (green) labelled probe GA.

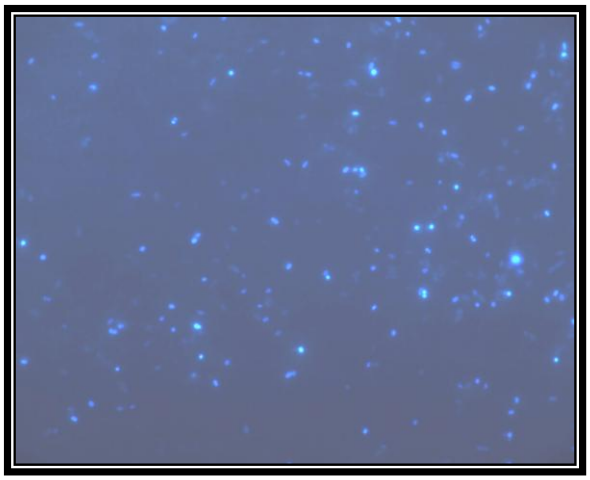

Figure 4.14. Micrograph of the isolate HCBCGO1 after DAPI staining.

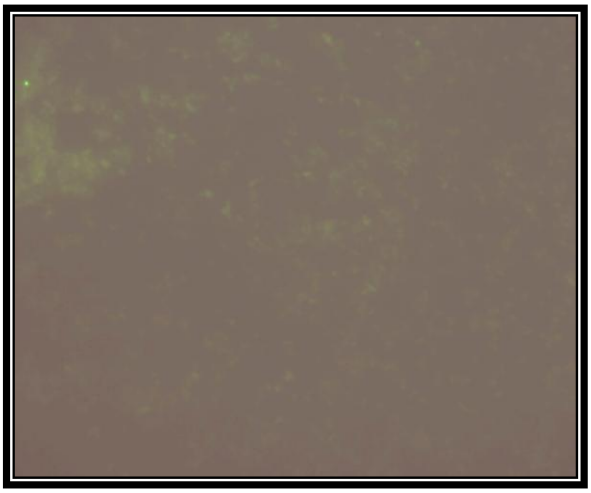

Figure 4.15. Micrograph of the isolate TTBCGO1 after hybridization with fluorescein (green) labelled probe GA.

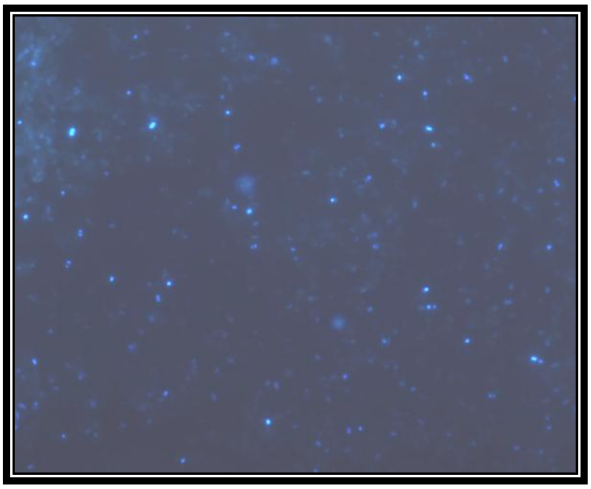

Figure 4.16. Micrograph of the isolate TTBCGO1 after DAPI staining.

TTBCGO1 and HCBCGO1 showed homology with only the GA probe (Figure 4.13 and 4.15). No filamentous morphology was observed with both the cultures. Instead single, variable shaped cells were observed. The differentiation of individual cell morphology, of both the cultures, proved to be a difficult task as the cultures were very dense and the some cells were clumped together. Sonication was used to pre-treat the sample, but this method proved in adequate, as it had destroyed the cells. The morphological characteristics, as shown in figures 4.13 and 4.15, are similar to those described by Jenkins *et al* (1993) for *Nocardia amarae* species. The comparative analysis of Figures 4.13 and 4.15 with Figures 4.13 and 4.16 show that more cells were visualized under fluorescence than DAPI. This can once again be attributed to the cells having a lower concentration of DNA because they are inactive.

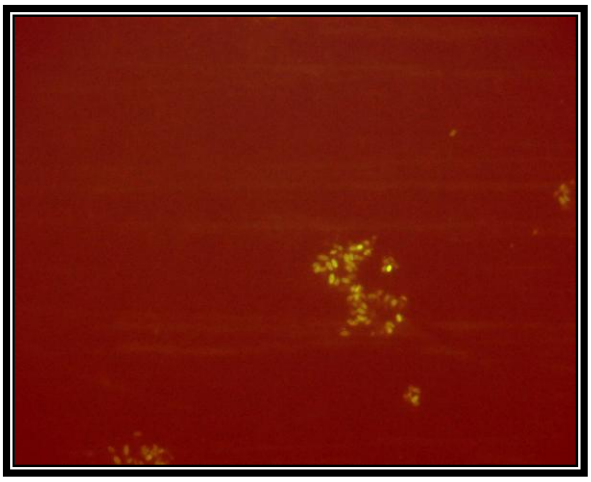

Figure 4.17. Micrograph of the isolate OSIOO4b after hybridization with fluorescein (green) labelled probe TNI.

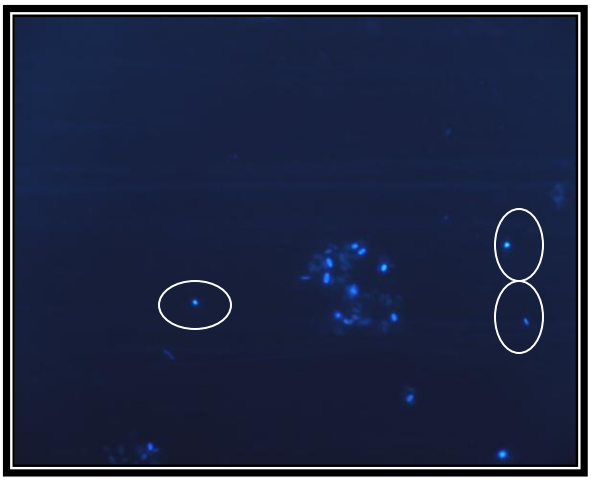

Figure 4.18. Micrograph of the isolate OSIOO4b after DAPI staining.

OSIOO4b displayed homology with only the TNI probe (Figure 4.17). The TNI probe had bound to oval shaped cells. The cells that displayed a positive hybridization signal were clustered. DAPI staining had revealed the presence of other single cells, as indicated by the white circles in figure 4.18. These single cells had shown no homology with TNI or any other probe. However, physical examination of the culture plates and slants and Gram reactions displayed no evidence of contamination. The morphology as observed in the above figures is not atypical of the normal morphological appearance of *Thiothrix nivea*.

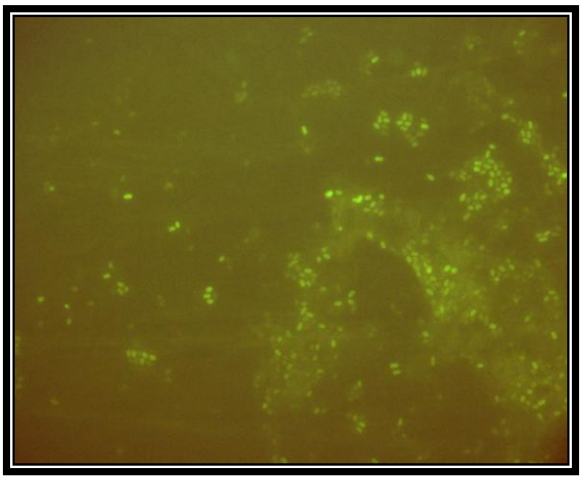

Figure 4.19. Micrograph of the isolate DLNTO1 after hybridization with fluorescein (green) labelled probe SNA.

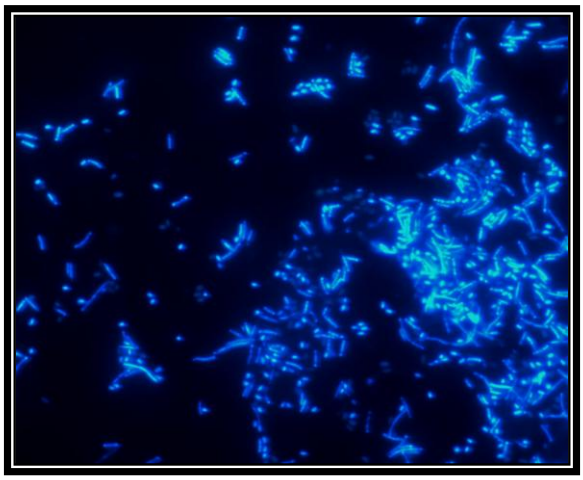

Figure 4.20. Micrograph of the isolate DLNTO1 after DAPI staining.

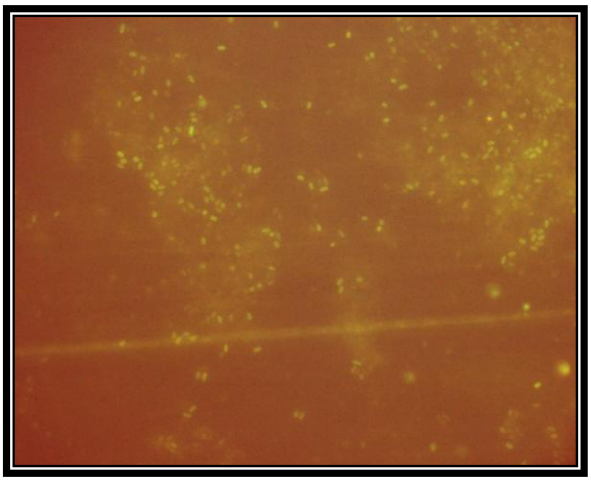

Figure 4.21. Micrograph of the isolate DLNTO1 after hybridization with fluorescein (green) labelled probe HHY.

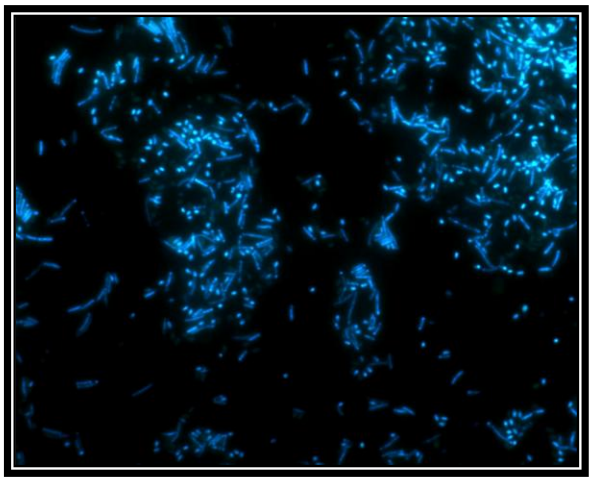

Figure 4.22. Micrograph of the isolate DLNTO1 after DAPI staining.

The isolate DLNTO1 had appeared to be a mixed culture. The culture had shown positive hybridization signals for the SNA probe and the HHY probe (Figures 4.19 and 4.21). DAPI staining, as shown in figures 4.20 and 4.22, showed the presence of single, almost rectangular shaped cells and thin, long rod shaped cells.

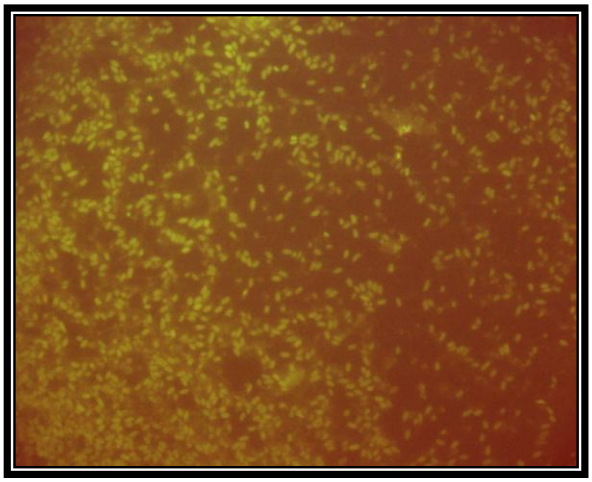

Figure 4.23. Micrograph of the isolate SWNCGO2 after hybridization with fluorescein (green) labelled probe ACA.

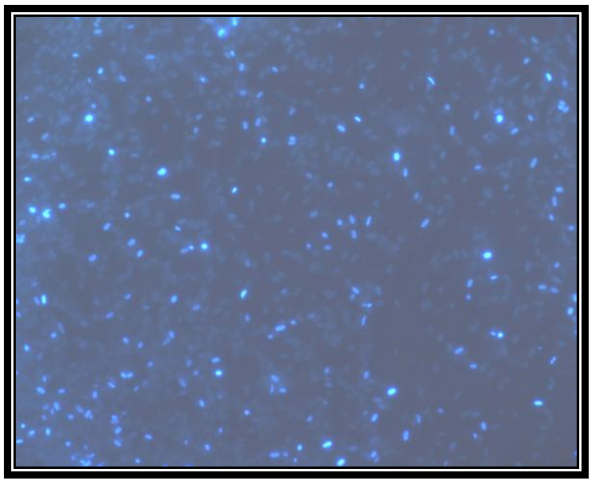

Figure 4.24. Micrograph of the isolate SWNCGO2 after DAPI staining.

The isolate SWNCGO2 had shown a good fluorescent signal for hybridization with only the ACA probe (Figure 4.23). The culture had exhibited round-ended to oval shaped cells. The morphology of the hybridized cells is analogous with that of *Acinetobacter* cells. Under DAPI a number of the cells had appeared very faint (Figure 4.24), this again indicates that a very low concentration of DNA was present in the cells possibly due to an inactive metabolism.

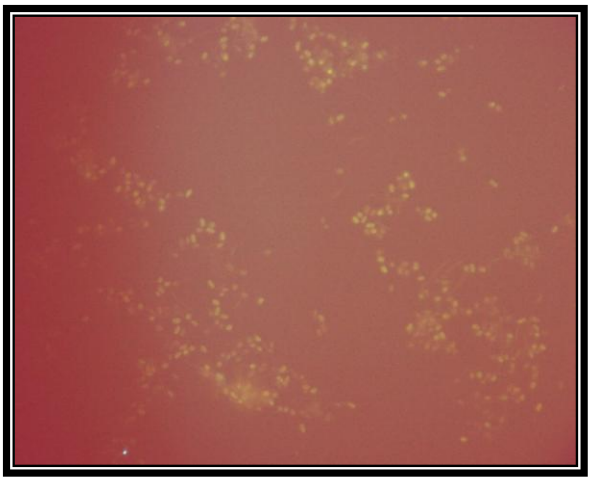

Figure 4.25. Micrograph of the isolate UCSRO1 after hybridization with fluorescein (green) labelled probe DLP.

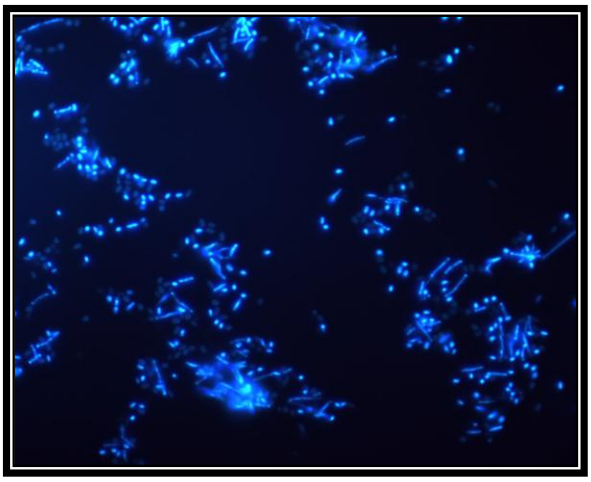

Figure 4.26. Micrograph of the isolate UCSRO1 after DAPI staining.

FISH of UCSRO1 with the DLP probe showed positive signals under fluorescence (Figure 4.25). UCSRO1 displayed variable morphology. Thin, long rod shaped cells and some single, oval shaped cells were observed. Both the cells had taken up the probe. The rods shaped cells displayed a relatively weak hybridization signals as compared with the single cells. *Nocardioform* species are generally characterized by variable morphology (Jenkins *et al*, 1993).

During the subsequent application of the different probes (as per chapter three) and DAPI staining of UCSRO1, it was noticed that the morphology of the culture was changing. The isolate, UCSRO1, was then subjected to FISH and DAPI staining at seven day time intervals. The time intervals were calculated from the first application of DLP (as shown in figure 4.25) over a three week period, which was considered sufficient time to observe any changes in the culture.

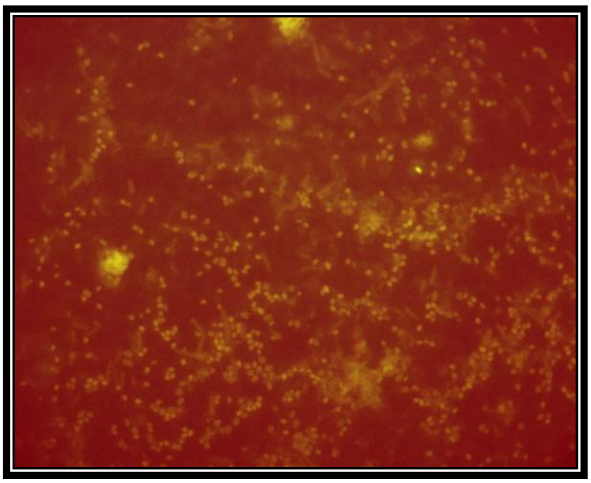

Figure 4.27. Micrograph of the isolate UCSRO1 after hybridization with fluorescein (green) labelled probe DLP, after 7 days following initial hybridization with DLP.

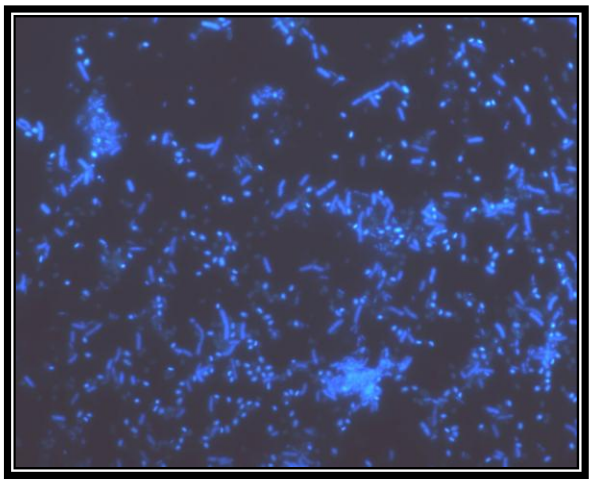

Figure 4.28. Micrograph of the isolate UCSRO1 after DAPI staining, after 7 days following initial staining.

Application of the probe DLP to UCSRO1 following seven days after initial FISH, the culture showed strong signals under fluorescence (Figure 4.27). UCSRO1 once gain displayed variable morphology. Very few rod shaped cells were present. There was abundance of single cells. The culture appeared more dense than initially observed.

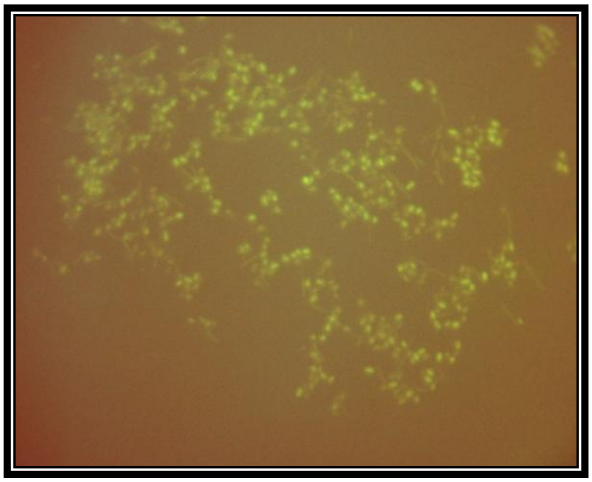

Figure 4.29. Micrograph of the isolate UCSRO1 after hybridization with fluorescein (green) labelled probe DLP, after 14 days following initial hybridization with DLP.

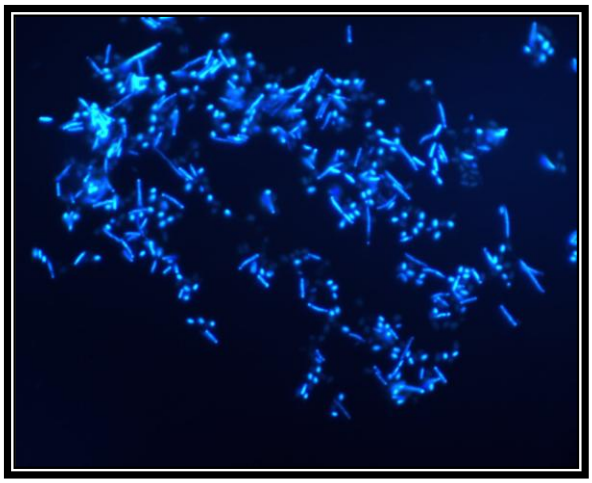

Figure 4.30. Micrograph of the isolate UCSRO1 after DAPI staining, after 14 days following initial staining.

Application of the probe DLP to UCSRO1 following fourteen days after initial application of the probe, the culture showed good signals under fluorescence (Figure 4.29). Variable morphology was observed. The culture was not as dense as compared to that after seven days (Figures 4.27 and 4.28). There were more rod shaped cells present after 14 days (Figures 4.29 and 4.30) than after 7 days (Figures 4.27 and 4.28). The culture was very similar, with regards to morphology and cell distribution, when compared with the initial FISH with DLP (Figures 4.25 and 4.26).

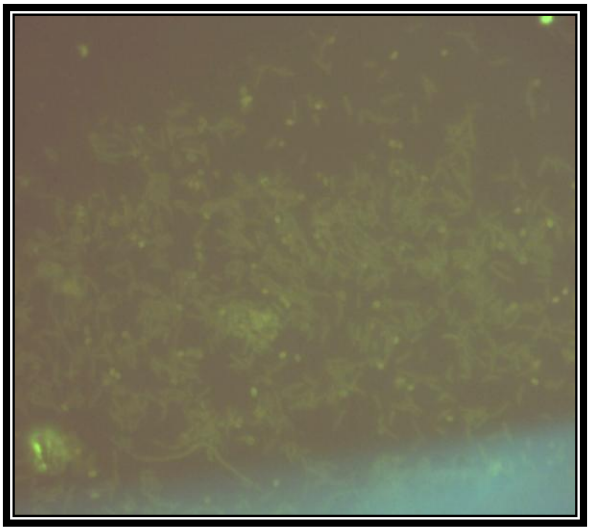

Figure 4.31. Micrograph of the isolate UCSRO1 after hybridization with fluorescein (green) labelled probe DLP, after 21 days following initial hybridization with DLP.

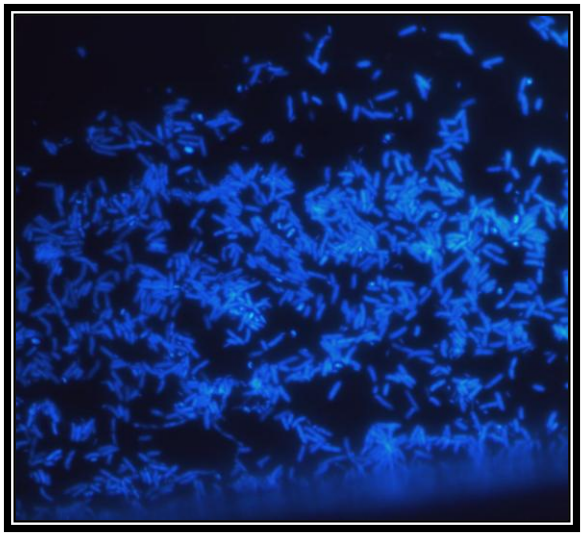

Figure 4.32. Micrograph of the isolate UCSRO1 after DAPI staining, after 21 days following initial staining.

Application of the probe DLP to UCSRO1 after twenty one days following initial FISH analysis with the same probe, the culture showed positive signals under fluorescence (Figure 4.31). Variable morphology was observed. There was now an abundance of rod shaped cells of variable length. Very few single cells were observed during this analysis. The culture was more dense as compared to that after fourteen days.

PART B

4.2. Gel Electrophoresis

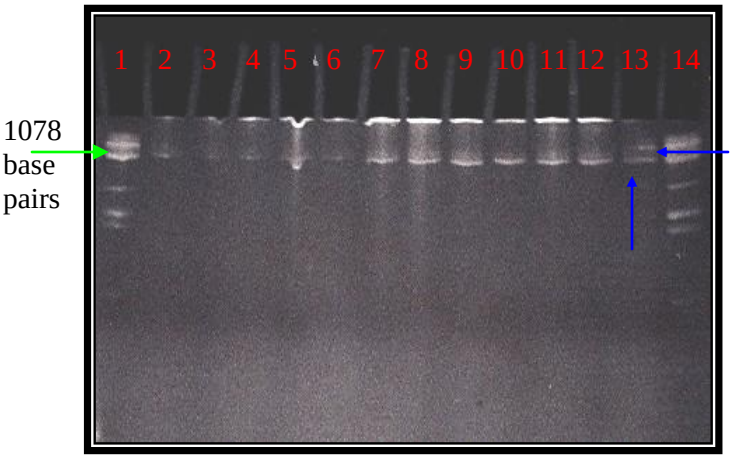

Table 4.2. DGGE samples and lane numbers

1	DNA Marker
2	HCBTY
3	TTBCGO3
4	HCBCGO1
5	UCSRO1
6	SWNCG01
7	SWNCG02
8	NGCD
9	NGCDO1
10	OSIOO4b
11	OSIOO1b
12	OSIOO1a
13	DLNTO1
14	DNA Marker (1353-310 bp)

Figure 4.33. DGGE profile of the PCR amplified 16S rRNA gene segments from DNA isolated from the filamentous bacterial isolates.

Twelve of the fourteen isolates are shown in the above DGGE profile. There were not enough samples wells available to accommodate all fourteen samples. The remaining two isolates, TTBCGO1 and TTBCG02 were electrophoresed on another denaturing gradient gel under the same conditions. Electrophoresis of TTBCGO1 and TTBCGO2 revealed the presence of DNA smears for these two samples. The gel was run properly as was evident from the good separation of the DNA molecular weight marker. The smears were initially believed to be the result of a high concentration of DNA in the wells. The samples were then diluted and electrophoresed. The smears were once again observed (results not shown).

The smears on the gel were possibly the result of proteolysis of cellular proteins, which are activated by either calcium or magnesium nucleases. The nucleases cause multiple nicks and breaks within the DNA, resulting in smears when subjected to gel electrophoresis. Another

possible cause of smears is that the linear DNA could be forming looped structures. Thus during PCR the primers are not able to recognize the looped structure resulting in poor quality PCR products or no PCR products (Myers *et al*., 1998). To circumvent these problems, DNA was re-extracted from TTBCGO1 and TTBCGO2. The samples were re-amplified using the reaction conditions stated in Table 3.6. A 1% solution of dimethyl sulfoxide (DMSO) was added to the PCR reaction mixture. DMSO reduces the occurrence of secondary structures thus maintaining the linear shape of the DNA (PCR Additives, 2004). The resultant PCR products were electrophoresed (Figure 4.34).

With reference to figure 4.33., the white bands on the gel indicate the presence of DNA. Lanes 2 through to 6 had very faint bands present. This was indicative of low concentrations of DNA. Lanes 7 through to 12 had more prominent bands, which was indicative of high concentrations of DNA. Lanes 2 through to 12 all displayed similar banding patterns. They exhibited the presence of two bands. The first band shows DNA that was still present in the well. During the optimization of DGGE, the gels were run at different time periods. Even after electrophoresis at 200 volts for 4 hours, the DNA had not migrated out of the well. However, electrophoresis of the same samples with agarose gel showed only one band for each sample. All DNA had migrated out of the respective wells. It was therefore decided to regard the DNA present in the denaturing gradient gel wells as negligible. Lane 13 displayed a different banding pattern. This sample displayed two distinct bands (indicated by the two blue arrows).

Based on the comparative analysis of the samples with the DNA molecular weight marker, it was estimated that the second band in lanes 2 through to 12 (indicated by the green arrow) were approximately 1078 base pairs. The first band in lane 13 was approximately 1353 base pairs and the second band was approximately 1078 base pairs, which was the same as the other isolates. Based on the above profile, the DNA extracted from all the isolates were about the same size with

the exception of the first band from the isolate DLNTO1. Bacteria that display the same banding pattern in DGGE profile are presumed to belong to the same group or species (Muyzer *et al*., 1993). Based on the above results it can deduced that the isolates all belong to the same group of organisms, in this case filamentous bacteria.

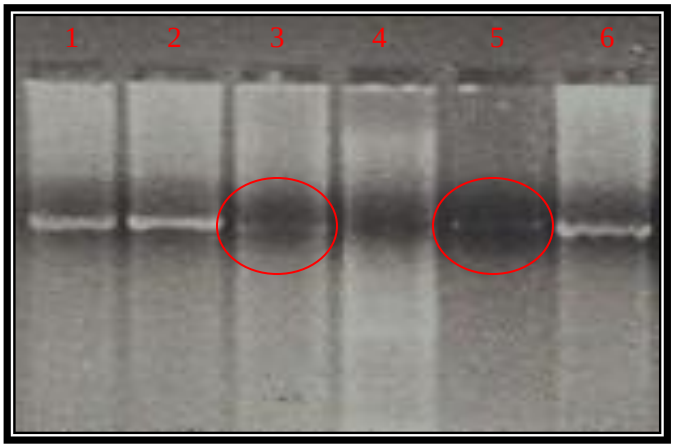

Table 4.3. Agarose samples and lane numbers

1	HCBTY
2	TTBCG01
3	TTBCG02
4	TTBCGO3
5	HCBCGO1
6	SWNCG01

Figure 4.34. Agarose gel electrophoresis profile of the PCR amplified 16S rRNA gene segments from DNA isolated from the filamentous bacterial isolates.

Samples HCBTY, TTBCGO3, HCBCGO1 and SWNCGO1 displayed very faint bands with DGGE. DNA was re-extracted and the samples were re-amplified using the previously stated PCR protocols for each sample respectively (Pages 46 and 47). The amplified products are shown in the above figure. More prominent bands were evident with samples HCBTY and SWNCGO1 as compared with the DGGE profile. There was no DNA present in lane 4 (TTBCGO3). Lanes 3 and 5 had very faint bands, which are indicated, by the red circles. It can be seen that the samples had bands with the same alignment. DNA was re-extracted from samples TTBCGO2, TTBCGO3 and HCBCGO1 and re-amplified because there was insufficient DNA for sequencing. The samples were then subjected to agarose gel electrophoresis (Figure 4.35).

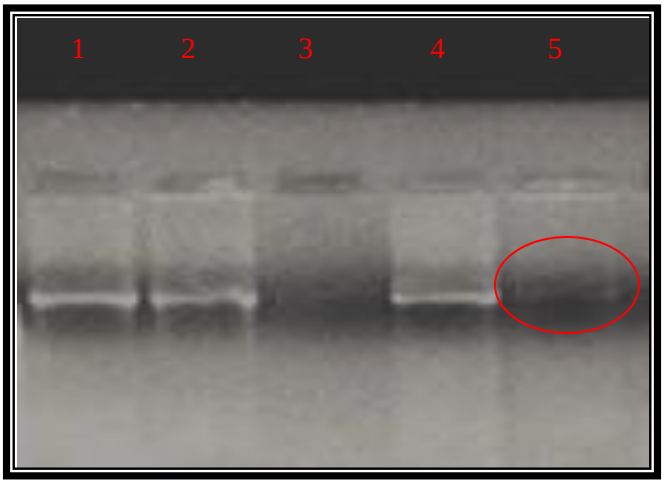

Table 4.4. Agarose samples and lane numbers

1	TTBCG02
2	TTBCGO3
3	DLNTO1- first band
4	DLNTO1- second band
5	HCBCGO1

Figure 4.35. Agarose gel electrophoresis profile of the PCR amplified 16S rRNA gene segments from DNA isolated from the filamentous bacterial isolates.

More prominent, high intensity bands were visualized in lanes 1 and 2 as compared with the previous gels. Lanes 3 and 4 contained the excised samples of culture DLNTO1 from lane 13 of the denaturing gradient gel (Figure 4.33). Only the second band that was excised displayed a prominent band after subsequent purification and amplification (Lane 4). Lane 5 had a very faint band. The above samples displayed the same banding patterns.

4.3. Nucleotide Sequencing

DNA from all fourteen isolates were sent for sequencing. But, good yields of high quality genomic DNA was only obtained from five of the fourteen isolates. Thus only five of the isolates were successfully sequenced. From the BLAST analysis, only the closest matching sequences were selected.

HCBTY

The sample was sequenced using primers 341f and 1492r. The resultant sequences showed significant alignments with the bacterium *Comamonas testosteroni.*

HCBTY – Query Sequence

Comamonas testosteroni – Subject Sequence

1. Query Sequence, Positions 133 – 192

GAT TAG CTC CCC CTG GGG GGT GGG CAA CCC TTT GTA CCA GCC ATT GAA TGA TGT GTG TAC

Subject Sequence, Positions 913 – 854

GAT TAG CTC CCC CTC GCG GGT TGG CAA CCC TTT GTA CCA GCC ATT GTA TGA CGT GTG TAG

2. Query Sequence, Positions 193 – 252

CCC AAC AAT ATA AGG GCC ATG AGG ACT TGA CTT CAT CCC CAC CTT CCT CGG GTT TGT CAC

Subject Sequence, Positions 853 – 795

CCC CAC CTA TAA GGG CCA TGA GGA CTT GAC GTC ATC CCC ACC TTC CTC CGG TTT GTC AC

3. Query Sequence, Positions 253 – 312

GGG CAG TCC CAT AAA GAG GGC CCA ACT GAA TGT AGC AAC TAA GGG CAG GGG TTG CGC TCC

Subject Sequence, Positions 794 – 737

CGG CAG TCC CAT TAG GTG CTC AAC TGA ATG TAG CAA CTA ATG GCA AGG GTT GCG CTC

4. Query Sequence, Positions 313 – 372

GTT GCG GGA CTT AAC CCA ACA TTC TCA CGA CAC GAG CTG ACG ACA GCC ATG CAG CAC CGG

Subject Sequence, Positions 736 – 678

GTT GCG GGA CTT AAC CCA ACA TCT CAC GAC ACG AGC TGA CGA CAG CCA TGC AGC ACC TG

5. Query Sequence, Positions 373 – 432

GGT GCG GGT TCT CTT TCG AGC ACC AAA CCA TTT TGT GGA AAG TTC CTG CCA TGT CAA AGG

Subject Sequence, Positions 677 – 619

TGT GCA GGT TCT CTT TCG AGC ACC AAA CCA TCT CTG GTA AGT TCC TGC CAT GTC AAA GG

6. Query Sequence, Positions 433 – 457

GGG GAA AGG TTT TTC GCG TTG CAT C

Subject Sequence, Positions 618 – 594

TGG GTA AGG TTT TTC GCG TTG CAT C

7. Query Sequence, Positions 516 – 575

AAC CTT GGG GCC GTA CTC CCC GAG GGG GGC AAT TAC ACG CGT AAG TTT CGT TAC TGA GTC

Subject Sequence, Positions 536 – 478

AAC CTT GCG GCC GTA CTC CCC AGG CGG TCA ACT TCA CGC GTT AGC TTC GTT ACT GAG TC

8. Query Sequence, Positions 576 – 607

AGT TAA CAC CCA ACA ACC AGT TGA CAT CGT TT

Subject Sequence, Positions 477 – 446

AGT TAA GAC CCA ACA ACC AGT TGA CAT CGT TT

TTBCGO2

The sample was sequenced using the primers 27f and 1492r. The resultant sequences showed alignments with *Bacillus* spp.

TTBCGO2 – Query Sequence

Bacillus spp. – Subject Sequence

1. Query Sequence, Positions 445 – 463

TAA AAG AAT TAA AGA AAA A

Subject Sequence, Positions 3020715 – 3020733

TAA AAG AAT TAA AGA AAA A

TTBCGO3

The sample was sequenced using primers 341f and 1492r. The resultant sequences showed alignments with the bacterium Nostoc spp.

TTBCGO3 – Query Sequence

Nostoc spp. – Subject Sequence

1. Query Sequence, Positions 402 – 419

TAA AGC CCA GTA AAA TTT

Subject Sequence, Positions 4386634 – 4386651

TAA AGC CCA GTA AAA TTT

UCSRO1

The sample was sequenced using primers 27f and 1492r. The resultant sequences showed alignments with *Nocardia farcinica*.

UCSRO1– Query Sequence

Nocardia farcinica – Subject Sequence

1. Query Sequence, Positions 791 – 809

TCG TGT TCG CGC TGT TGT T

Subject Sequence, Positions 37391 – 37373

TCG TGT TCG CGC TGT TGT T

OSIOO1b

The sample was sequenced using primers 341f and 1492r. The resultant sequences showed alignments with an uncultured bacterium.

OSIOO1b – Query Sequence

Uncultured bacterium – Subject Sequence

1. Query Sequence, Positions 424 – 441

ATT GGC TTG TGG CCA GTG

Subject Sequence, Positions 770 – 787

ATT GGC TTG TGG CCA GTG

CHAPTER FIVE

DISCUSSION

Presently there is an inadequate supply of phylogenetic information available on filamentous bacteria. This is largely attributed to the fact that filamentous bacteria are probably one of the most difficult organisms to isolate, culture and study. The research that is being done and the data that is slowly becoming available is placing some doubt on the classification and characterization of filamentous bacteria based on phenotypic characteristics.

Most filamentous bacteria are morphologically different from other identified bacteria. Therefore, most of them have been classified by type numbers e.g. Type 0411. The filamentous sulphur bacteria of *Thiothrix* species, *Leucothrix mucor* and Type 1863 are morphologically similar which makes physical microscopic differentiation difficult (Nielsen, 1984; Brock 1992; as cited by Kämpfer and Wagner, 2002). Some filamentous bacteria have been found to be Gram variable (Wagner and Amann, 1997). It has also been suggested that some filamentous characteristics used for identification, such as the sheath forming ability of *Leptothrix discophora*, may be plasmid encoded (Emerson and Ghiorse, 1992; as cited by Wagner and Amann, 1997). Plasmids can be easily lost from the cell, which would result in the loss of a vital identification tool.

The genetic approach for analysis of filamentous bacteria has immense practical value to resolve conflicting reports. The combination of fluorescent *in situ* probing and direct retrieval of nucleotide sequences allows for identification and proper phylogenetic characterization of filamentous bacteria without the need for cultivation.

FISH provides a means for the rapid identification of organisms. This method has the potential for on-site application to identify problematic filamentous bacteria within the plant. Oligonucleotide probes have the ability to target organisms at different phylogenetic levels. Samples of the fourteen isolates were first subjected to FISH without any pre-treatment. Poor hybridization signals were obtained which can be attributed to the reduced accessibility of the probe to the rRNA within the cell. Some filamentous bacteria are characterized by cell envelopes that contain straight-chained saturated and unsaturated fatty acids and storage polymers such as gylcogen that either inhibit or make infiltration of the probe difficult. The cell walls of actinomycetes contain in addition to storage polymers, tuberculostearic acid and 3-hydroxyl 2-alkyl fatty acids, which is commonly known as mycolic acid (De Los Reyes *et al*., 1997). The combination of these acids with storage polymers makes penetration of the cell wall complicated.

To overcome this problem, the isolates were pre-treated in 4% paraformaldehyde(PFA) for 1 minute. The pre-treatment of cells with PFA or any other fixative results in the cross-linking of proteins in the cell wall, which increases cell permeability and maintains cell integrity (De Los Reyes *et al*., 1997). Filamentous bacteria are very sensitive to their environments e.g. changes to temperature, pH and the addition of chemicals. Previous research conducted by De Los Reyes *et al.* (1997) found that pre-treatment of filamentous bacteria with PFA for the normal fixation period of 2 hours compromised the cell morphology. This was possibly due to the excessive cross-linking of proteins in the cell wall by the PFA molecules.

Sonication as a method of pre-treatment is normally used to break up clusters of cells to make them more accessible to the probe and to allow for better visualization of cells after hybridization. Sonication of the filamentous bacterial isolates prior to FISH proved unsuccessful. Sonication had disrupted the cells. Individual cells could be distinguished. FISH and DAPI staining of the

sonicated samples had resulted in lots of background staining and auto-fluorescence (results not shown).

All the probes listed in Table 3.2. were applied to all fourteen isolates to ensure specificity of the probes and identities of the isolates. The probe TFR, specific for *T. fructosivarans* showed no homology with any of the isolates. The species *T. fructosivarans* is not very common in South Africa. Nine of the isolates were identified using FISH. The FISH results were compared with the morphological and staining results for the isolates obtained by Ramothokang (2004), as a means of verifying their identities as determined by FISH.

Isolates NGCD and SWNCGO1were identified as *H. hydrossis* using the species specific probe HHY (Figures 4.5 and 4.7). *H. hydrossis* is typically Neisser negative and Gram negative (Seviour *et al.*, 1999a). Both these cultures were Neisser negative and Gram positive (Ramothokang, 2004). There is no evidence to suggest that *H. hydrossis* is Gram variable. These isolates also exhibited different morphological characteristics to those described by Jenkins *et al.* (1993).

The cultures NGCDO1 and OSIOO1a were identified as Type O21N (Figures 4.9 and 4.11). Type O21N is generally Neisser negative but sometimes contains Neisser positive granules and, Gram negative cells that sometimes stain Gram positive. Type O21N has displayed variable morphology (Williams *et al.*, 1987; as cited by Seviour *et al.*, 1999a). According to Ramothokang (2004) the cultures NGCDO1 and OSIOO1a were Gram positive and were Neisser negative and positive respectively. The cultures also presented variable morphological characteristics.

The two isolates, HCBCGO1 and TTBCGO1, were identified as *Gordonia amarae* (Figures 4.13 and 4.15). These cultures displayed characteristics similar to the species *Nocardia amarae*.

Nocardia amarae was renamed *Gordona amarae* (Wagner and Cloete, 2002). However, based on correct etymology, the genus *Gordona* was renamed *Gordonia* (De Los Reyes *et al*., 1998). Gordonia species form part of the mycolic-acid containing actinomycetes group (Davenport *et al*., 2000). This group of organisms has exhibited filamentous, coccoidal and rod shaped forms in mixed liquor and foaming samples (Davenport *et al*., 2000). Both the cultures identified as *Gordonia amarae* had single coccoidal and rod shaped cells. The comparative analyses of staining reactions and FISH results coincided with that of *Gordonia amarae.*

The culture OSIOO4b was identified as *T. nivea*. *Thiothrix* species belong to the filamentous sulphur bacterial group (Jenkins *et al*., 1993). The TNI probe had bound to coccoidal cells (Figure 4.17). A previous study of a plant in Høgeland, Germany, found the TNI probe had bound to coccoidal cells. It was hypothesized that the single cells represented gonidia, which is a non-filamentous form of *Thiothrix* (Nielsen *et* al., 1998). According to the criteria routinely used for morphological identification of *Thiothrix* sp., the cells are either square or longer than they are wide and are sometimes present as rosettes or gonidia (Pernelle *et al*., 1998). *Thiothrix nivea* cells are Gram variable when significant amounts of intracellular sulphur granules are present and, they often contain Neisser positive granules (Seviour *et al*., 1999a). OSIOO4b was identified as being positive for Gram and Neisser stains (Ramothokang, 2004). These phenotypic characteristics provide verification that the culture, OSIOO4b, is *T. nivea.*

The culture SWNCGO2 showed positive hybridization for the ACA probe only (Figure 4.23). The morphology of the hybridized cells was oval shaped. The morphology of this culture is analogous with that of *Acinetobacter* cells which are characterized by oval shaped cells commonly referred to as cocco-bacilli (Blackall *et al*., 1998). *Acinetobacter* cells have been implicated in enhanced biological phosphorus removal since they accumulate phosphate during

aerobic growth on acetate. *Acinetobacter* cells are Gram negative and they are Neisser positive for polyphosphate (Blackall *et al.*, 1998).

The probe ACA has also been reported to show positive hybridization signals for the filament Type 1863. Type 1863 has demonstrated variable morphology ranging from cocco-bacilli to rods in pure culture (Seviour *et al.*, 1999a). The cells are generally Gram negative but some cells within the filament have stained positive (Rosetti *et al.*, 1997b). Type 1863 cells are Neisser negative (Seviour *et al.*, 1999a). Research conducted by Ramothokang (2004) showed that culture SWNCGO2 was Gram positive and Neisser negative. It has been suggested that *Acinetobacter* and Type 1863 share a very close relationship (Wagner *et al*, 1994b). An Eikelboom Type 1863 strain RT2 was isolated, identified and phylogenetically placed in the gamma sub-class of the proteobacteria *Acinetobacter* johsonii, based on its 16S rDNA sequence (Rossetti *et* al., 1997a). Based on the above results and comparison with literature it can be deduced that SWNCGO2 is Type 1863. Previously published research has also identified an Australian Type 1863 as *Acinetobacter* using the Biolog System (Seviour and Blackall, 1999).

The culture DLNTO1 had showed positive hybridization signals for the SNA and HHY probes. Both the probes had hybridized to the single cells in the culture. DGGE profiling of the culture had given two distinct bands with different molecular weights. Based on these results it was concluded that this was a mixed culture. The cells that bound the HHY probe had similar morphology as the cells that bound the same probe in cultures SWNCGO2 and NGCD (Figures 4.5 and 4.7). Both *S. natans* and *H. hydrossis* are negative for Gram reactions and Neisser stains. This concurs with the results obtained by Ramothokang (2004) for DLNTO1.

Hybridization of UCSRO1 at seven-day intervals for twenty-one days had showed that a morphological shift had taken place. During the first hybridization with the DLP probe, small

cocci cells and a few rod-shaped cells were noticed (Figure 4.25). Hybridization after seven days revealed numerous oval shaped cells (Figure 4.27). Fewer rod shaped cells were detected. After fourteen days fewer single cells and more rod shaped cells of variable length were observed (Figure 4.29). The rods showed very weak hybridization signals on days one, seven and fourteen. The cells may have been physiologically inactive and as a result would have a low polysomal content, which would account for the weak hybridization signals. On day twenty-one there was an abundance of rod shaped cells, of variable length (Figure 4.31). Only a few cocci cells were observed. UCSRO1 had indeed undergone a morphological shift from a filamentous form (when it was first isolated) to cocci cells, then to oval cells and finally to rod shaped cells.

Based on FISH results, UCSRO1 was identified as a Nocardioform bacterium. Nocardioform bacteria are Gram positive and Neisser negative with Neisser positive granules (Seviour *et al.*, 1999a). According to Ramothokang (2004) UCSRO1 was Gram positive and Neisser negative. Nucleotide sequence analysis of UCSRO1 confirmed this identification as it showed a close alignment with the bacterium *Nocardia farcinica*. *Nocardia farcinica* is a Gram positive coccal/rod shaped pathogenic organism that has been implicated in foaming of activated sludge (Stratton *et al.*, 1996). *N. farcinica* is a member of the mycolic acid-producing bacterial group (Davenport, 2000). Unidentified Gram positive cocci was reported to be dominant in some foams in Australia (Soddell, 1999). Soddell and Seviour (1990) had speculated that the cocci cells might be some stage in the life cycle of some Nocardioforms (as cited by Soddell, 1999).

Of the fourteen isolates, only five isolates were successfully sequenced. Of the five isolates, UCSRO1 was the only isolate where nucleotide sequencing verified its identification as determined by FISH. The remaining four isolates showed no homology with any of the applied probes. Many of the isolates had failed sequencing reaction because they had very low concentrations of DNA. Various extraction methods and PCR protocols were employed to

maximize the concentration of DNA. Some sequencing reactions had terminated early because of short fragments. While other reactions gave sequences with lots of variable bases.

The culture HCBTY showed considerable alignments with the nucleotide sequence of the bacterium *Comamonas testosteroni* (Pages 72 to 74). *Comamonas testosteroni* is a Gram negative, aerobic rod (Medical Web, 2004). It was previously known as *Pseudomonas testosteroni* and is distinguished from other *Comamonas* species by its ability to assimilate testosterone (Medical Web, 2004). *C. testosteroni* has been isolated from activated sludge and characterized as a denitrifying bacterium that is capable of degrading poly –3-hydroxybutyrate (Tabrez and Hiraishi, 2001).

The culture, HCBTY, was isolated in a filamentous form, however DAPI staining had indicated the presence of rods and not filaments (Figure 4.1). The Gram reaction of the culture had identified it as Gram negative rods. From the results obtained during physical examination of the agar plates and slants, Gram staining of the cultures and the DGGE and Agarose profiles (Figures 4.33 and 4.34), it was concluded that there was no contamination. The probability that *C. testosteroni* had undergone a morphological shift from a filamentous form in mixed liquor to rod shaped cells in pure culture is probable but can not be substantiated as there is no evidence to date, to suggest that it is capable of growing in a filamentous form.

The nucleotide sequence of the culture TTBCGO3 had the closest match to the nucleotide sequence of *Nostoc* sp (Page 75). *Nostoc* sp. is also known as *Anabaena* sp. *Nostoc* sp. have been found in activated sludge. *Nostoc* sp. has been identified as filamentous nitrogen-fixing cyanobacterium (Kaneko *et al*., 2001). Segmented, filamentous-like growth was observed under DAPI (Figure 4.3).

The nucleotide sequence of the culture TTBCGO2 showed alignment with the nucleotide sequence of *Bacillus* sp (Page 75). *Bacillus* is frequently found in activated sludge. They are typically Gram negative rods (Prescott *et al*., 1999). There have been reports on the occurrence of filamentous *Bacillus* sp. in wastewater treatment plants and in bulking sludge (Farquhar and Boyle, 1971; Storm and Jenkins, 1984; Trick *et al*., 1984; as cited by Ajithkumar *et al*., 2001). This would account for the isolation of the culture which was in a in a filamentous form. After subsequent sub-culturing and growth in pure culture the filamentous form was no longer evident. Under DAPI staining rods and some cocci cells were observed. These cells stained positive for the Gram reaction (Ramothokang, 2004).

A filamentous *Bacillus* sp. strain NAF001 was isolated from a domestic wastewater treatment plant (Ajithkumar *et al.*, 2001). This strain had Gram negative cells but occasionally some parts of the cells stained positive. During the study two types of cells were observed. During the dormant phase of growth the filamentous NAF001produced endospores. The endopsores germinated to form short filaments that subsequently formed longer filaments. The other form of growth that was observed was round and cylindrical cells. These were not spores and were termed Spore Like Resting Cells (SLRCs) (Ajithkumar *et al*., 2001). Filamentous spore-forming iron bacteria were also identified in bulking sludge (Emtiazi *et al*., 1989). These strains could not be assigned to any recognized taxa and therefore remain novel.

The culture TTBCGO2 was stored at 4°C, which implies the culture was in a dormant phase. The cocci-shaped cells of TTBCGO2 could therefore be endospores or spore like resting cells. The rods could possibly be some phase in the life cycle of the *Bacillus* where the endospores or the spore like resting cells had germinated or grew by budding to form rods. With TTBCGO2 a non-filamentous growth form was observed in pure culture but with NAF001 at no stage was a non-filamentous growth form observed (Ajithkumar *et al*., 2001).

OSIOO1b could not be assigned a valid name because the culture showed alignment with an uncultured bacterium. According to Ramothokang (2004) the culture was positive for Gram and Neissser stains. The culture was presumptively identified as *M. parvicella* based on morphology and staining characteristics. The morphology of the culture as viewed under DAPI was different from those described by Jenkins *et al.* (1993) for *M. parvicella.*

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

During this study none of the cultures displayed the same morphology as when they were first isolated. During subsequent sub-culturing and storage, the cultures had undergone a 'morphological shift' from a filamentous form to single cocci cells or rods and in some cases a combination of both. With culture UCSRO1 a change in morphology was observed over a 21-day period. The cultures had undergone a 'morphological shift' possibly in response to the change in environment. Many bacteria form spores during adverse conditions and when favourable conditions prevail they revert to their normal morphological form or to vegetative cells. When they were isolated onto solid media there was a change in the supply of nutrients and pH, amongst other factors. All these factors could contribute to a stressful environment, which could trigger a morphological change, as a survival tactic. The theory of 'morphological shifts' has been speculated but there is no detailed published literature to support these findings.

The use of contemporary molecular techniques for the analysis of filamentous bacteria is constantly opening new doors to microbiology and taxonomic classification. The combination of FISH with gel electrophoresis and nucleotide sequencing provided a powerful means for the identification of the filamentous cultures. As with all techniques used to study bacteria, these methods are not without limitations. Although, DGGE has a major advantage of being able to separate DNA fragments that differ by as little as one base pair and allows for rapid comparisons of different bacterial communities; it has a major limitation in that some sequence types that are known to be quite different but are from a related family may migrate to the same position in the gradient. Sequences with multiple base pair differences are also not easily resolved as they sometimes show identical migration in DGGE gels (Jackson *et al.*, 2000). The combination of gel

electrophoresis and nucleotide sequencing eliminates the possibility of false profiles due to co-migrations of different species as well the occurrence of identical species.

FISH conversely provided a faster and easier tool for the identification of the filamentous cultures. The successful application of FISH on filamentous bacteria is dependent on the specificity of the probe and the pre-treatment method of the sample e.g. pre-treatment with PFA for 4 minutes at 4°C.

Using FISH, ten of the fourteen isolates were identified. The identity of the culture UCSRO1was verified by nucleotide sequencing. The identities of the other nine isolates could not be verified by sequencing because they had all produced failed sequencing reactions. The isolates HCBTY, OSIOO1b, TTBCGO3 and TTBCGO2 could not be identified by FISH due to the limited number of olignucleotide probes that are available nationally as well as internationally. Currently, probes are only available for the common or frequently encountered bacteria/ filamentous bacteria.

To substantiate the findings of this study further, more pure culture work needs to be done. 'Morphological shifts' needs to be examined in more detail to provide more definitive answers to the following questions:

1. Why do 'morphological shifts' occur?
2. What triggers the change in morphology?
3. Do these changes occur in pure culture only or in activated sludge as well?
4. Is there any link between the changes in filamentous bacterial morphology and process performance?

In light of the findings of this study it is imperative that the extent of diversity and the taxonomy of filamentous bacteria be reviewed. The result of pure culture studies forms the basis for

applications in activated sludge plants. Thus it is vital that conditions prevalent in the system are mimicked as closely as possible during pure culture studies.

Further studies are required to expand the 16S rDNA database of filamentous bacteria with the goal to generate oligonucleotide probes for all filamentous bacteria. The use of automated molecular techniques such as microarray/DNA chips, confocal laser scanning microscopy and flow cytometry in combination with other molecular techniques, biochemical assays and micro-autoradiography to monitor substrate uptake provides a more promising approach for determining and monitoring the factors affecting the growth of filamentous bacteria. The comparative analysis of these results with plant conditions and wastewater characteristics will provide a better understanding of these bacteria and hence, bulking and foaming. This would ultimately result in the development of more effective and long-term control strategies.

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APPENDIX 1

PRE-TREATMENT OF SLIDES

(Amann, 1995; modified)

*Heavy Teflon coated slides with eight wells (Merck, Germany) were used for experimental work.

1. Clean the slide surface by soaking in warm detergent solution for 1 hour.
2. Rinse with distilled water and air dry.
3. Place clean slides in a 1 : 10 diluted solution of poly-L-lysine (Sigma Diagnostics, USA).
4. Allow the slides to soak for 5 minutes at room temperature.
5. Remove and allow the slides to air dry.

APPENDIX 2

CELL FIXATION WITH 4% PARAFORMALDEHYDE

(Amann, 1995, modified De Los Reyes *et al.*, 1997)

Solutions

- 1 × Phosphate Buffered Saline (PBS) pH 7.2

  130mM sodium chloride (NaCl)

  10mM sodium phosphate buffer

- 3 × Phosphate Buffered Saline (PBS) pH 7.2

  390mM NaCl

  30mM sodium phosphate buffer

- 4% paraformaldehyde (Merck, Germany)
- 98% ethanol (Merck, Germany)

Preparation of Paraformaldehyde Fixative

1. Heat 65mL of double distilled water to 60°C.
2. Add 4g of paraformaldehyde.
3. Add one drop of 2M NaOH solution and stir until the solution is clear.
4. Remove from the heat source and add 33mL of 3 × PBS.
5. Adjust the pH to 7.2 with HCl.
6. Filter the solution through a 0.2µm filter.
7. Quickly cool down to 4°C and store in the refrigerator.

Note: This solution should not be stored for long periods of time and ideally should be prepared fresh for every fixation.

Fixation of Cells

1. Add three volumes of paraformaldehyde fixative to one volume of sample.
2. Allow to fix for 1 minute at 4°C.
3. Pellet cells by centrifugation (3500 rpm, 4 min) and remove the supernatant.
4. Wash cells in 1 × PBS, centrifuge and then resuspend in 1 × PBS to 50% of the original sample volume.
5. Add 50%, of the original sample volume, of ice-cold 98% ethanol and gently invert to mix.
6. The cells maybe stored at -20°C for several months or spotted onto slides.

APPENDIX 3

IMMOBILISATION OF CELLS

(Amann, 1995)

1. Add10µl of the sample to the well. Spread the sample evenly in the well.
2. Allow the smears to air dry.
3. Dehydrate the cells by successive passages through 50, 80 and 98% Ethanol washes for 3 minutes each.
4. Allow to air dry. The slides can be stored at room temperature.

APPENDIX 4

WHOLE CELL HYBRIDISATION

(Amann, 1995)

Materials

- 50mL Polypropylene screw top tube
- Whatman 3MM paper
- Hybridisation buffer pH 7.2 (100 mL)

  0.01g sodium dodecyl-sulphate

  0.24g Tris

  0.186g ethylenediaminetetra-acetic Acid (EDTA)

  5.26g sodium chloride (NaCl)

  × ml formamide

  Sterile distilled water to 100mL

20% Buffer ~ 20mL formamide

35% Buffer ~ 35mL formamide

40% Buffer ~ 40mL formamide

45% Buffer ~ 45mL formamide

- Wash Buffer pH 7.2 (1000 mL)

  0.1g sSodium dodecyl-sulphate

  1.86g EDTA

  2.42g Tris

  X NaCl * depending on Formamide concentration used in the hybridization buffers.

Where 0% formamide = 0.9M NaCl

= 52.6g NaCl

Hybridisation Procedure

1. Soak a strip of the Whatman 3MM paper in the appropriate hybridization buffer and place in the polypropylene tube.
2. Allow the chamber to equilibrate for 15 minutes at 46°C.
3. For each spot to be hybridized mix 1µl of probe with 9µl of hybridization buffer.
4. Spread the 10µl of the probe/buffer mix on each spot of fixed cells.
5. Carefully place the slide in the pre-warmed moisture chamber and allow it to hybridise for 2 hours at 46°C.
6. After 2 hours, remove the slide from the chamber and rinse off the probe/buffer mix with the pre-warmed hybridization wash buffer. The wash buffer should be pre-warmed to the 48°C.
7. Place the slide in a screw cap polypropylene tube containing the wash buffer and incubate for 20 minutes at 48°C.
8. Remove the slide and briefly rinse with double distilled water. Remove excess water and allow to air dry briefly.

DAPI Staining (Hicks *et al*., 1992; modified)

1. Spread 10µl of DAPI (0.25µg/ml) on each spot and all to stain for 5 minutes.
2. Wash the slides with 1 × PBS and allow to air dry.
3. Mount the slide with Kallestad™ Mounting Media (Bio-Rad, South Africa) and cover with a cover slip.

APPENDIX 5

AGAROSE GEL ELCTROPHORESIS

Solutions

- 50 × Tris Acetate Buffer (TAE) (1L)

  242g Tris Base

  57.1mL Glacial Acetic Acid

  37.2g EDTA

  Distilled water to 1L

- Gel loading buffer

  0.25% bromophenol blue

  0.25% xylene cyanol

  30% Gylcerol

- Electrophoresis Buffer

  275mL 1 × TAE

Agarose Gel

1. 0.8g Agarose powder
2. 50 mL 1 × TAE buffer
3. Microwave for 60 seconds or until the solution becomes clear.
4. Cool and add 1*ul* ethidium bromide
5. Gently mix
6. Pour into the gel tray and carefully set the well comb in place.
7. Allow the gel to set for 15-20 minutes.
8. Remove the well comb and place the gel in the electrophoresis submarine.

9. Add the electrophoresis buffer. Ensure that the gel is completely covered with buffer.

Sample Loading

1. 2 *ul* of Gel loading buffer + 4*ul* of sample.
2. Carefully load the loading buffer/sample in the wells.

- Set the voltage and time and allow to gel to run.

APPENDIX 6

DENATURING GRADIENT GEL ELCTROPHORESIS

BIO-RAD D Gene instruction manual and applications guide (catalog numbers 170-9000 through 170-9070)

Solutions

- 40% Bis-Acrylamide

  38g acrylamide

  2 g bis-acrylamide

  Add distilled water to 100 mL.

  Filter through Whatman Filter No. 1 and store at 4°C

- 30% Low Density Denaturing Solution (DNS)

  18.8 mL 40% bis-acrylamide

  2.0 mL 50 × TAE Buffer

  12.0 mL* formamide

  12.6 g* urea

  Distilled water to 100 mL

- 60% High Density Denaturing Solution (DNS)

  18.8 mL 40% bis-acrylamide

  2.0 mL 50 × TAE Buffer

  24.0 mL* formamide

  25.2 g* urea

  Distilled water to 100 mL

The concentration of urea and formamide are varied according to the required concentration of the denaturing solutions, which is given in the manual.

- 10% Ammonium Persulphate

  0.1 g ammonium persulphate

  Distilled water to 1.0 mL. Solution should be fresh for every use.

- 50 × Tris Acetate Buffer (TAE) (1L)

  242g Tris Base

  57.1mL glacial acetic acid

  37.2g EDTA

  Distilled water to 1L

- D Gene Dye Solution

  0.05 g bromophenol blue

  0.05 g xylene cyanol

  1 × TAE to 10.0 mL

  Store at room temperature

- TEMED (Bio-Rad, South Africa)

Denaturing Gel → High Density DNS (60%)	**+**	**Low Density DNS (30%)**
DNS	15 mL	15 mL
Ammonium Persulphate	150 *ul*	150 *ul*
TEMED	15 *ul*	15 *ul*
Dye	300 *ul*	No dye added

Sample Loading

1. 2µl of *D* Gene Dye solution + 5µl of sample
2. Wash the wells with 1 × TAE buffer before loading the samples.
3. Carefully load the D Gene Dye solution/samples into the wells.

Electrophoresis Buffer

140 mL 50 × TAE buffer

6860 mL distilled water

Pre-heat the electrophoresis buffer to 65°C prior to addition of the gel core to the electrophoresis tank. Add 350 mL 1 ×TAE buffer to the upper buffer chamber once the core is added and the samples are loaded