MOLECULAR CHARACTERIZATION OF FILAMENTOUS BACTERIA ISOLATED FROM FULL-SCALE ACTIVATED SLUDGE PROCESSES.

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Dissertation submitted in compliance with the requirements for the Master's Degree in Technology in the Department of Biotechnology, Durban University of Technology, Durban.

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Declaration

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ZINHLE MARRENGANE

I hereby declare that this dissertation represents my own work. It has not been submitted before for any diploma/degree or examination at any other University of Technology/University.

ZINHLE MARRENGANE **Date**

2007

Approval

I hereby approve the final submission of the following dissertation

PROF. FAIZAL BUX

Supervisor
Doctoral Degree in Technology: Biotechnology
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This _____ day of _____, 2007, at the Durban University of Technology.

DEDICATION

The work presented here is dedicated to Junior

With so much love and acknowledgement

for her valuable insight…

&

My loving family

My Mom, Sisters and Brother.

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PREFACE

Some of the work presented in this dissertation has been accepted for a poster presentation:

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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
EPS	Extracellular polymeric substances
FISH	Fluorescent *In Situ* Hybridization
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*
21 N	*Type* 021 N

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CHAPTER ONE- INTRODUCTION

1.1 WATER AND THE SOUTH AFRICAN WATER POLICY

Availability of clean water is a basic requirement for the establishment and maintenance of a healthy community. It is not only as a source of potable water but also provides valuable food supplements through supporting the growth of aquatic life and in agriculture (Horan, 1990). South Africa is famous for its sunshine and the climate varies from dessert to semi-dessert with an annual rainfall of 464 mm compared to world average of about 860 mm (MacKay *et al*., 2003). Water resources are scarce and extremely limited although the sea accounts for about 60% of the planet. Sea water has not been used as potable water because of its high salinity content. Desalinization of sea water is not cost-effective but because South Africa is a water stressed country, desalinization for potable consumption is soon to be implemented in semi-dessert region of the Cape (MacKay *et al*., 2003).

The South African national government is the public trustee of water resources stating that it is responsible for the protection, development, utilization and control of water. The core function of water policy is to ensure that all South Africans have equitable access to water and sanitation and that all water resources are managed in a sustainable manner (MacKay *et al*., 2003). The government provides 6 000 kiloliters free water to all households. However, industrial and domestic effluent needs to be treated before being discharged into receiving waters in order to prevent waterborne illnesses primarily

because an estimated 50% of South Africans are still faced with poor sanitation and rely on dams, boreholes and rivers for the water (MacKay *et al*., 2003).

1.2 WASTEWATER TREATMENT

Treatment of wastewater has become a necessity for healthy modern society in order to protect public health. Domestic and industrial effluents require extensive treatment to convert waste materials into stable oxidized end products that can be safely disposed off into inland waters whilst eliminating adverse ecological effects (Gray, 2002). Wastewater treatment involves three steps: primary, secondary and tertiary treatment. Primary treatment involves physical unit processes which range from balancing, screening, sedimentation, filtration and centrifugation amongst others. The most commonly used unit particularly for domestic effluent is screening where screen bars spaced 20-30 mm apart physically remove floating solids (Gray, 2002).

Secondary treatment involves the biological treatment processes. This treatment relies on microorganisms to degrade soluble organic matter. Biological treatment can be anaerobic as in anaerobic digestion and anaerobic stabilization ponds. Biological filtration and the activated sludge process both rely on aeration whether natural ventilation as in filtration or mechanical as practiced in the activated sludge process. The activated sludge process allows microorganisms to degrade about 95% organic polluting matter (Hartmann, 1999).

Biologically treated effluent undergoes further treatment by tertiary treatment where the remaining biological oxygen demand, suspended solids, bacteria and toxic compounds are removed. This can be achieved through chemical treatment by chlorination, precipitation, ion exchange, coagulation, oxidation reduction or neutralization although it can also be biological in constructed wetlands. All these measures are employed to ensure that final effluent meets effluent standards and safe to be discharged into receiving waters (Seviour and Blackall, 1999; Gerhardi and Frank, 1990). There are various processes employed worldwide for wastewater treatment including ozonation, trickling filters, membrane bioreactors and the activated sludge process. The activated sludge is the most common particularly in urban areas (Horan, 1990).

1.3 THE ACTIVATED SLUDGE PROCESS

The activated sludge (AS) process is a widely applied biotechnology application for the treatment of wastewater (Gerhardi and Frank, 1990). Treatment is achieved through the removal of soluble and insoluble organics from wastewater and to convert this material into a flocculant microbial suspension that settles well in a conventional gravity clarifier (Ramothokang *et al*., 2003). Microorganisms form associations of microbial aggregates which consist of wide variety of many species of bacteria, protozoa, metazoa, viruses, algae and fungi. The diversity of microbial community in the activated sludge plant depends on the influent wastewater, environmental parameters such as pH and temperature and prevalent operational conditions (Wilderer *et al*., 2002; Martins *et al*., 2004).

Treatment of wastes with the activated sludge process represents a component of the largest biotechnology industry in the world. The activated sludge is a suspended growth system comprising a mass of microorganisms constantly supplied with organic matter and oxygen (Horan, 1990). The removal of organic matter in the activated sludge process comprises of three mechanisms:

- Adsorption and also agglomeration onto microbial flocs
- Assimilation, where the substrate is converted to new microbial cell material.
- Mineralization which is the complete oxidation of the substrate (Gray, 2002).

The conventional AS plant consists of a reactor which can be a lagoon, ditch or a tank. The microbial biomass within the aeration tanks is referred to as the mixed liquor suspended solids which grows as a flocculant suspension. The final clarifier or sedimentation tank separates the microbial biomass from the treated effluent. The clarifier serves two functions: clarification and thickening. Clarification is the ability of the activated sludge flocs to produce a clear overflow that meets the discharge standards (Seviour and Blackall, 1999; Gray, 2002).

Return activated sludge is an important component of the AS process as it maintains the microbial population at a required concentration to facilitate continuation of the process by recycling the settled sludge in the sedimentation tank back to the reactor. This is done in order to achieve sufficient biomass to ensure efficient biological oxygen demand removal and thickening of the AS (Bitton, 1994).

A number of parameters are used to operate the AS plants. Biomass control, plant loading, sludge activity and most importantly sludge settleability are process parameters that affect the efficiency of the AS process (Seviour and Blackall, 1999). Most problems associated with the activated sludge process involve poor settleability (Bitton, 1994; Martins *et al*., 2004). This necessitates the need for a rapid assessment process to ensure good separation in the secondary settlement so that the final effluent has a low suspended solids concentration and that sufficient biomass is returned to the aeration tank (Horan, 1990; Bitton, 1999).

1.4 MOTIVATION FOR THIS STUDY

Activated sludge flocs are responsible for flocculation, settling and dewaterability. It is important to maintain the growth of floc-forming bacteria for efficient sludge settleability and compaction for good quality effluent. Filamentous bacteria on the other hand are believed to provide rigid support network or backbone upon which floc-forming bacteria adhere to form stable activated sludge flocs (Wilderer *et al*., 2002; Ramothokang *et al*., 2003).

Filamentous bacteria can also be detrimental to the process when they outgrow floc-forming bacteria. Morphologically filamentous bacteria are at an advantage as they have higher outward growth velocity and can extend freely to bulk liquid substrate. Proliferation of filamentous bacteria causes foaming and bulking (Martins *et al*., 2004).

Although chemical alleviation measures to circumvent bulking are present, they are symptomatic (Chang *et al*., 2004).

Eikelboom (1975) developed the first identification keys for the classification of filamentous bacteria that is primarily based on morphological characteristics and microscopic examination. Although very useful, this type of identification has its limitations. For instance some filamentous bacteria can change morphology in response to changes in the environment and although some of them can be morphologically similar they may vary considerably in their physiology and taxonomy (Martins *et al*., 2004).

A vast number of filamentous bacteria are still very poorly understood which could be due to the problems of cultivation due to their slow growing nature and maintenance of cultures (Rossetti *et al*., 2006). This limitation necessitates a molecular approach to resolve the taxonomy of filamentous bacteria as it is a culture-independent technique which is highly accurate.

This project was undertaken to verify the identity of pure cultures of filamentous bacteria isolated previously through the application of molecular techniques. The 16S rDNA are conserved regions in bacterial cells and they can be extracted and specific nucleic acid fragments amplified. Denaturation gradient gel electrophoresis enabled the separation of fragments of identical length but different size and served as an indication of purity (Muyzer *et al*., 1993). Single bands were excised and sequenced as was achieved in this

study. Whole cell hybridization or Fluorescent *in situ* hybridization was also done on all isolates using specific probes.

The accessibility to applying these novel molecular techniques to correctly identify and characterize microorganisms in the activated sludge has taken microbial community analysis to the next level. Current taxonomic status of filamentous bacteria that rely on conventional identification technique such as staining, morphological characteristics and cell inclusions has created much confusion. Novel molecular techniques will aid to rectify this and allow accurate classification of these important organisms.

1.5 AIM OF THE STUDY

- To determine the genotypic characteristics of selected filamentous bacteria using relevant molecular techniques.

1.6 OBJECTIVES

- To verify the purity of pure cultures of filamentous bacterial isolates.
- To apply fluorescent *in situ* hybridization for the identification of filamentous bacteria.
- To extract and amplify rDNA.
- To sequence amplified DNA.
- To compare the sequenced data using an established database.

CHAPTER TWO - LITERATURE REVIEW

2.1 ACTIVATED SLUDGE FLOC

The activated sludge (AS) wastewater consists of dispersed organic and inorganic substrates which are well mixed in the system to allow intimate contact time between microbes and substrates (Jenkins *et al*., 1993). Microorganisms grow as three-dimensional aggregated microbial communities called flocs (Seviour and Blackall, 1999). The size of the flocs varies between < 1μm to >1,000μm. The activated sludge flocs contain a wide range of prokaryotic and eukaryotic microorganisms (Horan, 1990; Bitton 1999).

Bioflocculation is the ability of microorganisms to adhere to one another (Jenkins *et al*., 1993); bacteria constitute the major component of AS flocs. Thousands of bacterial strains thrive in activated sludge but only a relatively small function can be detected by culture-based techniques (Seviour and Blackall., 1999; Hugenholtz *et al*., 2001). Culture based techniques have revealed major genera in the flocs to be *Zooglea, Pseudomonas, Flavobacterium, Alcaligenes, Achromobacter, Corynebacterium, Acinetobacter, Bacillus species* and filamentous bacteria (Seviour and Blackall, 1999). Previous investigations maintain that the active fraction of bacteria in activated sludge flocs represent only 1-3% of total bacteria (Horan, 1990; Seviour and Blackall, 1999). However, fluorescently labelled oligonucleotide probes show that a higher percentage of the microbial biomass is metabolically present (Amann, 1995; Amann and Ludwig, 2000). Denitrifying bacteria are a group of taxonomically diverse chemoheterotrophs that utilize nitrates in the

absence of oxygen in the anoxic zone in biological nutrient removal activated sludge. Photoautotrophic and photoheterotrophic bacteria are present in large numbers as purple non-sulphur bacteria in activated sludge systems and are known to be metabolically versatile and can denitrify (Seviour and Blackall, 1999).

Good settling sludge is formed of roughly spherical, compact, firm flocs with an average size of >100μm (Bitton, 1994). Neisser and Gram stained samples will often reveal the filamentous backbone of compact flocs that floc-forming bacteria adhere to (Jenkins *et al*., 1993). Filamentous organisms are characterized by their long thread-like shape. Filamentous fungi are sometimes visualized but filamentous bacteria are mostly present in activated sludge processes (Wanner, 2006). Normal flocs represent a balance between floc-forming bacteria and filamentous bacteria which results in strong flocs that can keep their integrity in the aeration basin and settle well in the sedimentation tank. This results in clear effluent because filaments are restricted largely to the floc interior and only limited extension to the bulk liquid (Bitton, 1994; Seviour and Blackall, 1999).

Well flocculating activated sludge processes, however, have been observed in the absence of filamentous bacteria. It was first thought that the production of intracellular storage product poly-β-hydroxybutyric acid was responsible for floc-formation. But extracellular slimes that are produced by *Zoogleal ramigera* and other activated sludge microorganisms play a role in the bacterial flocculation (Friedman *et al*., 1969, as cited by Bitton, 1994).

Lack of filamentous bacteria can result in dispersed growth where no true flocs are formed. The microbial cells freely suspended in bulk liquid. Thus resulting in turbid effluent. This condition results in poor settling sludge. This is due to lesser percentage of filamentous bacteria which signifies the importance of filamentous bacteria in flocs by improving clarifier efficiency (Gerhardi and Frank, 1990).

2.2 ACTIVATED SLUDGE SEPARATION PROBLEMS

2.2.1 Problems related to floc structure.

2.2.1.1 Poor floc microstructure

In some activated sludge samples, the compact core of the flocs that are created by bacterial biomass is absent and the flocs exhibit a loose open structure. When the compact core of the activated sludge is 20-80 μm, the cause of poor settling is referred to as poor floc microstructure (Wanner, 2006).

2.2.1.2 Dispersed growth

The activated sludge process sometimes acquires problems that are attributed to microbial biomass quality where microorganisms are dispersed freely in the bulk liquid as individual cells (Gray, 2002). This condition is detrimental to the process because the separation in the secondary settling tank is very poor. Pin-point flocs with a diameter of

10-20 μm produce a final effluent that is turbid because they are too slow to be removed efficiently by gravity sedimentation (Seviour and Blackall, 1999). Dispersed growth is attributed to very high organic loading since bacteria do not need to produce a glycocalyx under such conditions of high food to microorganism ratio (Gerhardi and Frank, 1990; Wanner, 2006).

2.2.1.3 Unsettleable microflocs

Unsettleable microflocs are similar to dispersed growth in that both these conditions produce a final effluent that is not very clear, although unsettleable microflocs are larger than dispersed growth. These microflocs result from the disintergaration of firm and sound flocs. This disintergration of flocs is attributed to:

- Insufficient glycocalyx production or its consumption by bacteria inside the flocs.
- Total absence of organic microorganisms.
- Disintergration of flocs by shearing effects due to high speed mechanical aerators (Gerhardi and Frank, 1990; Horan, 1990; Wanner, 2006).

2.2.1.4 Viscous bulking

Extracellular polymeric substances (EPS) production is the characteristic of most floc-forming bacteria. Zoogloeal bacteria produce large amount of EPS that are essential for the formation of firm flocs. However, under stressful conditions an overproduction of EPS occurs. These excessive amounts of slimy, jelly-like consistency are hydrophilic

which causes the activated sludge to be highly water retentive. Such a glutinous sludge imparts low settling and poor compaction velocities. A viscous sludge causes further mechanical problems as it foams when aerated (Wanner, 2002 as cited by Wanner, 2006).

2.2.2 Foaming

Foaming is detrimental to the activated sludge process as it poses a major solid separation problem (Jenkins *et al*., 1993; Blackall, 1994). It can be visualized as a thick viscous stable foam or scum. Foam can be more than 1 m deep and overflowing onto walkways creates unhygienic and unsafe conditions (Gray, 2002). Biological foaming is mostly reported in biological nutrient removal plants with aerobic, anoxic and anaerobic stages but also occurs in conventional activated sludge systems (Gray, 2002).

Foaming has been attributed to filamentous bacteria (Jenkins *et al*., 1993; Bitton, 1994). Stabilization of biological foams is caused by the following features of foam-foaming filaments:

- Production of extracellular materials such as lipids, lipopeptides, proteins and carbohydrates act as biosurfactants.
- The cell walls of foam-foaming bacteria are strongly hydrophobic (Wanner, 2006).

Microscopic examination proposed by Eikelboom (1975) has been widely used for the isolation and identification. These rely on Gram stain, Neisser stain and sulphur test.

Initially Nocardioform-actinomycetes were primarily reported as foam formers (Bitton, 1994). But recent developments in molecular biology have implicated a number of filaments including *Microthrix parvicella* as a dominant filament in most foaming plants (Kämpfer and Wagner, 2002). *Gordonia amarae* has also been implicated in most foaming plants as the next most important cause of foaming (Davenport *et al*., 2000). Eikelboom *Type* 0675, *Type* 0041, *Nostocoida limicola* and *Haliscomenobacter hydrossis* were also were regularly detected in foams in most surveys by Seviour and Blackall, (1999). Dominant filaments will vary from region to region depending on type of influent as they strive on different biochemical and physiological needs, environmental conditions and operating parameters (Martins *et al*., 2004).

Microthrix parvicella can use long-chain fatty acids as carbon and energy sources and can grow under aerobic and anaerobic conditions. All these properties enable *M. parvicella* to outcompete other bacteria present in activated sludge (Rossetti *et al*., 2006). A chemical method like the addition of polyaluminium chloride is another way of controlling foaming but it tends to be undesirable due to unreasonable financial implications (Chang *et al*., 2004). Recent developments in molecular biology do not only allow identification of *M. parvicella* but also the quantification of this organism (Kaetzke *et al*., 2005). The primer set for the quantification of 16S RNA gene of *M. parvicella* was designed. Using real-time PCR, *M. parvicella* was correctly estimated (Kaetzke *et al*., 2005). This has great application to combat foaming. Findings reveal that *M. parvicella* 16S rRNA gene copies of above 3% of total bacterial 16S rRNA gene copies could cause foaming (Kaetzke *et al*., 2005). Further developments in this field can enable quick yet

reliable measure of concentration of problematic filaments before they are detrimental to the process.

2.2.3 Filamentous Bulking

The ideal floc is the one where filamentous bacteria and floc forming bacteria grow in balance. Poor sludge settling, as expressed by sludge volume index (SVI), is observed when the length of extended filaments exceeds 10^7 μm/mg (Seviour and Blackall, 1999). The proliferation of filamentous bacteria in the aeration tank results in the suspension of cell biomass in the sedimentation tank. Both these conditions are detrimental to the success of activated sludge operation.

Sludge volume index (SVI) is defined as the measurement of settleability of the sludge after allowing the mixed liquor to stand for thirty minutes in a 1 L measuring cylinder. This is a classical measure of sludge quality by determining sludge settleability. Sludge with an SVI of less than 100 is considered sludge with good settleability. The growth of filamentous bacteria has been related to SVI. Decreased levels of SVI show that there is a possible proliferation of filamentous bacteria and hence bulking (Seviour and Blackall, 1999; Gerhardi and Frank, 1990).

In nitrification-denitrification biological enhanced phosphorus removal activated sludge systems, competition between filamentous bacteria and floc forming bacteria exists. Incomplete denitrification favours the proliferation of filamentous bacteria. Inhibition of

substrate utilization under aerobic conditions occurs which allows the proliferation of filamentous bacteria (Casey *et al*., 1999).

Initially *Sphaerotilus natans* was believed to be the main course of filamentous bulking by holding apart flocs in activated sludge system (Seviour and Blackall, 1999). Recent developments reveal that a number of different filamentous bacteria cause bulking (Sekiguchi *et al*., 2001). Pure cultures reveal that *Microthrix parvicella*, which is a gram-positive unbranced, unsheated filament, is abundant in most bulking AS processes. Its proliferation is difficult to prevent because pure culture studies on physiology have revealed that *M. parvicella* can utilize long chain fatty acids as carbon and energy source and grows under aerobic and anaerobic conditions. This enables *M. parvicella* to outcompete other bacteria present in activated sludge when there is an abundance of fatty acids in the influent (Kaetzke *et al*., 2005; Dionisi *et al*., 2006). Other filamentous bacteria in bulking sludges include *Thiothrix sp., N. limicola I. II and III; Type* 021 N and *Flexibacter sp*. among others (Kanagawa *et al*., 2000).

Thiothrix sp. is a filamentous bacterium that can grow mixotrophically, heterotrophically and litotrophically and is often present in activated sludge plants that are characterized by a high concentration of sulphides and thiosulphates. *Thiothrix* is an unsheathed filament and accumulates sulphur granules when incubated in the presence of reduced sulphur compounds. They also produce gliding gonidia from the filament apex (Rossetti *et al*., 2006).

2.2.4 Treatment Measures for Bulking

Chang *et al.*, (2004) evaluated the effect of chlorination on bulking control. Various chlorine levels were conducted. Increased levels of chlorination had adverse effects on sludge settling properties and supernatant water quality. Increased doses of chlorine gradually reduced the SVI owing to deterioration of larger flocs into smaller ones because chlorine is not specific to filamentous bacteria. The effect of chlorination is believed to be a function of penetration through the cell wall (Seviour and Blakall, 1999). *Microthrix parvicella* is particularly difficult to control through chlorination which is due to its hydrophobic cell wall that prevents the biocide agent from penetrating into the cytoplasm (Chang *et al*., 2004). Higher concentrations of chlorine are highly detrimental to floc-formers and results in pin-point flocs. This clearly shows that bulking cannot be controlled by chemical means particularly because effluent quality is also important (Chang *et al*., 2004).

One of the most frequent causes of filamentous bulking is the low concentration of dissolved oxygen in the aeration tank (Bitton, 1994). The amount of dissolved oxygen inside the floc is much lower than in the bulk liquid thus the growth of floc formers is lower than that of filamentous bacteria that protrude into bulk liquid. The filaments believed to be responsible for low dissolved oxygen bulking are *S. natans*. *H. hydrossis* and *Type* 1701 and their proliferation can be combated by increasing dissolved oxygen level in the plant (Dionisi *et al*., 2006).

The use of aerobic or anoxic selectors have been proven to be effective at controlling bulking sludge attributed to excessive growth of *Type 021N*, *Thiothrix sp*. and *S. natans*. Another method that has been applied to combat bulking by *M. parvicella* is by the addition of polyaluminium chloride although undesirable as a precautionary measure because it is not economical (Martins *et al*., 2006).

Domestic wastewater is normally rich in nitrogen (N) and phosphorus (P) whereas industrial influent can be low in both N and P (Martins *et al*., 2004). These nutrients are essential for biomass synthesis. Bulking has been proven to occur due to low nutrient concentration. A study by Wagner *et al*., (1982) and Richard *et al*., (1985) as cited by Rossetti *et al*., (2006) revealed that filament growth is enhanced with respect to floc-formers when N and P concentrations are low. The latter study further revealed that *Type* 021N has a high N uptake rate when grown under N deficient conditions. This type of bulking can simply be combated by the addition of the lacking nutrient (Rossetti *et al*., 2006). *N. limicola II* was found to proliferate due to P deficiency and this is controlled by increasing sludge age so that the rate of nutrient utilization by biomass is decreased elucidating P limitation (Rossetti *et al*., 2006).

Floc- formers are favored when substrate concentration is high yet filamentous bacteria are favored when substrate concentration is low (Martins *et al*., 2004). Floc-forming microorganisms face lower substrate concentrations than filamentous bacteria due to diffusion limitations inside the floc. In AS processes characterized by low food to microorganism (F/M) ratio in the aeration tank, filaments like *H. hydrossis*, *Norcadia sp*,

Thiothrix sp, *Types* 0041, 0675, 0803, 0914 and 1851 are believed to outgrow floc-formers (Seviour and Blackall, 1999; Rossetti *et al*., 2006). The most useful control measures include creating a substrate concentration gradient inside the aeration tank or continuous plug flow reactors, sequencing batch reactors and completely mixed reactors with a contact zone placed ahead of the main reactor (Martins *et al*., 2006; Rossetti *et al*., 2006).

2.3 TAXONOMY OF FILAMENTOUS BACTERIA

Classification of filamentous bacteria in activated sludge process has been a major limitation with regards to accurate taxonomic groupings of the organisms.

2.3.1 Lack of Availability of Pure Cultures

Reliable identification requires the availability of pure cultures yet not all filamentous are culturable. The lack of appropriate growth medium to support filamentous growth has magnified this problem. Some media has been able to successfully support growth of specific filament types but fail in supporting other filament types (Horan, 1990; Seviour and Blackall, 1999). Yet some culturable filamentous bacteria are unable to grow axenically (Seviour and Blackall, 1999). This provides great limitation to their nutritional requirements and bacterial classification. Chemically defined medium by Slijkuis (1983) for the cultivation of *M. parvicella* has never led to successful isolation and growth of filaments with morphological features of *M. parvicella* (Seviour and Blackall, 1999).

2.3.2 Characterization of filaments

Filaments that have not been grown in pure culture are almost exclusively restricted to microscopic examination. Cell dimension and shape, absence or presence of septa; sheath; cellular inclusions (poly-ß hydroxybutyrate, polyphosphate and sulphur), motility, trichome, branching and Neisser and gram staining results are characteristics commonly used for microscopic characterization of filamentous bacteria. Even with filaments that are culturable, characterization is still limited to morphological and physiological properties (Seviour and Blackall, 1999).

Eikelboom (1975) made one of the first attempts but influential attempt by dividing the first 29 different morphological types he recognized into several groups based on morphology and microscopy. These groupings are:

1. **Sheath- forming, Gram negative filaments**: *S. natans*, *Type* 1701, 1702, 0321.
2. **Sheath- forming, gram positive filaments**: *Type* 0041, 0675, 1851.
3. **Sheathless, curled, multicelled filaments**: *Thiothrix sp., N. limicola*, Cyanobacteria, *Type* 021 N.
4. **Slender coiled filaments**: *M. parvicella*, *Types* 0581, 0912.
5. **Straight, short, multicelled filaments**: *Types* 0803, 1091, 0092, 0961.
6. **Gliding, motile filaments**: *Beggiatoa sp*., *Types* 0941, 1111, 1501.
7. **Others**: *Nocardia sp*., *Types* 1863 0411 (Seviour and Blackall., 1999).

This classification is incomplete and it cannot differentiate between phylogenetically related bacteria. However, filaments like *G. amarae,* amongst a few, have been characterized chemically, biochemically, physiologically and at a genetic level (Kämpfer *et al*., 1996).

2.3.3 Identification and nomenclature of filaments

Generally identification requires that a well-established, comprehensive source of information be available against which comparisons can be made. Such databases do not exist for filamentous bacteria especially Eikelboom types. There are no references or type strains held in culture collection or described in reference manuals. Although there are a few exceptions that include *S. natans*, *Beggiatoa spp*. *H. hydrossis*, *T. nivea*, *G. amarae*, *S.pinifomis* and various *Rhodococcus sp* (Seviour and Blackall, 1999).

2.4 MOLECULAR CHARACTERIZATION OF ORGANISMS IN WASTEWATER TREATMENT.

The application of molecular techniques is a tool that can greatly increase our understanding of the phylogeny of filamentous bacteria. Morphological similarities are unreliable indicators of true relatedness of filamentous bacteria because each type may represent a group of quite different bacteria all having the same or similar appearance (Seviour *et al*., 1997; Martins *et al*., 2004).

2.4.1 Fluorescent *in situ* Hybridization (*FISH*).

Over the past years many rRNA targeted nucleotide probes for *in situ* detection of bacteria, particularly filamentous bacteria, present in activated sludge processes have been designed (Rossetti *et al*., 2006). One use of *FISH* or whole cell probing in an activated sludge has been to show that culture-dependant methods were inadequate for describing the microbial diversity in mixed liquor (Hahn *et al*., 1992). This technique for monitoring microbial population is not limited by problems inherent in pure culture techniques but is influenced by the abundance and accessibility of the intracellular rRNA target molecules (Roller *et al*., 1994). Probes are designed to be complementary from domain to species (Seviour and Blackall, 1999).

Fifteen isolates morphologically classified as Eikelboom *Type* 021N were isolated from bulking activated sludge. These filamentous bacteria were highly similar by morphological, staining and physiological characteristics making differentiation impossible thus referred to as one genetic entity. However, two of the Eikelboom Type isolates differed from the other isolates genotypically (Kanagawa *et al*., 2000). *FISH* revealed that these isolates can be divided into three distinct evolutionary groups (Kanagawa *et al*., 2000). *Thiothrix sp*. form a monophyletic group within the gamma-subclass of Proteobacteria in which the three Eikelboom *Types* 021N belong to. It is therefore important to note that the morphotype known as Eikelboom *Type* 021N is now named *Thiothrix eikelboomii*. The phylogeny of *Nostocoida limicola* had been unresolved. 16S rRNA genes of *N. limicola* I, II and III were sequenced and the results

showed that *Nostocoida limicola* I, II and III are three phylogenetically different bacteria. A phylogenetic tree was done which placed *N. limicola* I in the low G+ C gram positive bacteria, *N limicola* II as high G+ C gram positive bacteria and *N. limicola* III as members of *Planctomycetes* (Seviour *et al*., 2002).

Eikelboom *Type* 1863 is routinely identified on the basis of its distinctive morphology where filaments are made up of chains of gram-negative coccobacilli. A study by Wagner *et al*., (1994b) revealed that filaments which were identified microscopically as *Type* 1863, hybridized *in situ* with oligonucleotide probes designed specifically for 16S rRNA gene of *Acinetobacter sp*. Further studies have unequivocally shown that their isolate of *Type* 1863, from an Italian plant has phenotypic characteristics and 16S rDNA of *Acinetobacter johnsonii* (Rossetti *et al*., 1997 as cited by Levantesi *et al*., 2006). Seviour *et al*., (1997) isolated *Type* 1863 filaments that were not members of *Acinetobacter* sp. in order to explore the taxonomic diversity of this morphotype. Five bacteria identified microscopically as *Type* 1863 were isolated through micromanipulation. The results presented clearly revealed that this morphotype does not describe one bacteria but a collection of several phylogenetically unrelated organisms all sharing very similar morphological characteristics in activated sludge. These three genera are believed to be *Chryseobacterium*, *Acinetobacter* and *Moraxella (*Seviour *et al*., 1997). All these findings clearly illustrate the risks associated with the reliance on microscopic appearance to identify filamentous bacteria and how molecular techniques can revolutionize the phylogeny and nomenclature of filamentous bacteria.

2.4.2 Bacterial16S rRNA

Protein synthesis occurs at the ribosomes which are made up of proteins and rRNA. In prokaryotic ribosomes, 5S rRNA, 16S rRNA and 23S rRNA molecules are found (Prescott *et al*., 1999). Gene of 16S rRNA represent cell viability and particular genes in the cells which carry the genetic information that codes for the different synthesis of different rRNA molecules (Olsen and Woese, 1993). 16S rRNA is a gene that is normally sequenced. It is about 1500 bases of nucleotides and has been sequenced for a number of bacteria (Toranzos, 1997). Once the 16S rRNA of a bacterium is known, it can be compared with all other 16S rRNA sequences of other bacteria. This allows for differentiation at a taxonomic level and a specific probe can be constructed using an oligonucleotide synthesizer (Seviour and Blackall, 1999; Prescott *et al*., 1999). Thus providing information on evolutionary history of the organism and differentiation at a taxonomic level (Olsen and Woese, 1993).

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2.4.3 Polymerase Chain Reaction (PCR)

The study of genetic organizations requires that sufficient quantities of DNA be generated (Prescott *et al*., 1999). Once DNA has been extracted it is important to amplify DNA by generating copies of DNA. PCR is the method widely used for in-vitro amplification of extracted DNA. This is an enzyme-based process and it requires a DNA polymerase enzyme called Taq polymerase. There are three stages of PCR: template denaturation, primer annealing and extension (Toranzos, 1997).

Real-time PCR offers the opportunity to monitor the amount of specific bacteria present in activated sludge process in order to monitor bulking and foaming before it occurs (Kaetzke *et al*., 2005). A primer set for the amplification of *M. parvicella* has been designed and it is employed to quantify the 16S rRNA of this filament in real time PCR assays. Results showed that foaming occurs when percentage 16S rRNA of *M. parvicella* rises above 3% of the total activated sludge bacterial community (Kaetzke *et al*., 2005). Advances into real-time PCR provide a highly sensitive method that can quantify 16S rRNA gene copies irrespective of physiological state of the filament, on-site.

2.4.4 Denaturation Gradient Gel Electrophoresis (DGGE).

The separation principle of DGGE involves the separation of DNA fragments of identical length on the basis of differences in denaturant sensitivity in an acrylamide gel matrix, resulting from differences in the primary sequence (Stephen *et al*., 1998). The melting behavior is detected as a reduction in the mobility of the DNA fragment as it passes through an acrylamide gel containing denaturing agents: urea and formamide. DGGE can detect differences in melting behavior of fragments that differ even by one base pair (Gray, 2002).

The DGGE method offers the following advantages over the other methods that can detect changes in single base pairs:

- The fragment sizes can be size 100 – 1000 base pairs.
- Labeling with radioactivity is unnecessary.

- It can detect differences in the melting behavior of fragments that differ by even one base pair (Myers *et al*., 1989)

DGGE provides a complementary tool for the analysis of complex microbial communities. PCR products generated by from various samples can be compared directly, based on their mobility without the need for cloning. Differences between communities in dune soil samples were successfully demonstrated by DGGE through differences in mobility (Stephen *et al*., 1998).

2.4.5 rDNA sequencing

This is probably the most important technique available to the molecular biologist. DGGE separates DNA into single base pairs which can be excised and sequenced. The precise order of nucleotides in a piece of DNA can be determined by DNA sequencing. The Sanger Coulson Dideoxy chain- termination method and Maxam Gilbert chemical degradation methods are the two valuable methods employed for determining the exact order of nucleotide arrangement in a DNA strand (Prescott *et al*., 1999). The chain termination method requires single stranded DNA. The Sanger Coulson method requires double stranded DNA but it does not require a primer because the basis of this method is not synthesizing a new strand but cleaving an existing DNA molecule using chemical reagents that act specifically at a particular nucleotide (Prescott *et al*., 1999). DNA sequencing allows for the phylogenetic characterization of the filamentous bacteria which aids in a number of molecular techniques like designing probes (Snaidr *et al*., 2002).

CHAPTER THREE: CONVENTIONAL METHODS FOR THE IDENTIFICATION OF FILAMENTOUS BACTERIA.

3.1 INTRODUCTION

Isolating filamentous organisms is tedious and time consuming. In order to elucidate bulking and foaming in wastewater treatment plants, it is imperative that pure cultures of problematic filamentous bacteria be isolated and studied (Ramothokang *et al*., 2003). A number of filamentous microorganisms have been successfully isolated. van Veen (1973) isolated some of filamentous bacteria that cause bulking and foaming in activated sludge systems on, D-medium, I-medium, and SCY-medium. The cultures were grown on broths and agar plates and incubated them for 3-8 weeks between 17°C and 20ºC. Thirty eight filamentous bacterial strains were isolated from activated sludge processes that had bulking and foaming. *Sphaerotilus natans* dominated as one of the problematic bacteria.

Seviour *et al*., (1999) isolated filaments by micromanipulation, followed by plating on a specific growth medium. Richard *et al*., (1981) isolated the predominant types of problematic filamentous bacteria found in bulking activated sludge samples. Samples were obtained from bulking activated sludge processes taken from 89 wastewater treatment plants around USA. CGYA agar plates were inoculated using the spread plate method. Plates were then incubated for 3 weeks at 28°C. Colonies that grew on the plates

were examined on 100X phase contrast microscope for cellular morphological characteristics. These microorganisms were identified using the key of Eikelboom (1975). More than 200 filamentous bacteria were isolated using this method. From the results 33% of *Type* 1701 and 29% of *Nocardia sp.* dominated. This supported the hypothesis that these two microorganisms are the most common cause of bulking in activated sludge treatment plants. However, other different filamentous bacteria have been reported to cause bulking and are not in large numbers and common as the above mentioned organisms (Richard *et al.,* 1981). Kämpfer (1997) suggested that direct plating on different media was not successful because the majority of filamentous bacteria are overgrown by more rapid growers, or their full nutritional requirements were not provided by the chosen medium. Van Veen (1973) and Williams and Unz (1985) as cited by Kämpfer (1997) developed a method for isolation of filaments based on specific dilution procedures and suggested some additional pretreatment procedures to reduce the surface tension between the filaments and the floc-formers, therefore facilitating the separation of floc-formers from the filaments. These procedures make it easier to isolate filamentous organisms. The pretreatment procedures that were applied were sonication, where samples were sonicated at 30W for 10 seconds and washed three times by centrifugation at 1900Xg for 2-5 minutes. The sample was then viewed under microscope.

The other method that was applied was the micromanipulation technique, which is a selective method for isolation of filamentous bacteria. The cultures were inoculated by spread plate method on TGY agar, CGYA and the media with moderate nutrient content

like R2A agar. The incubation period was 6 weeks at 20°C. The isolation was successful as *Microthrix parvicella, Nocardioform* organism *(Gordonia amarae, Skermenia piniformis), Sphaerotilus-Leptothrix, Type* 1701, *Type* 0803, *Leucothrix-Thiothrix-Beggiatoa, Type* 021N, *Type*1863 and other organisms were isolated. The aim of this chapter was therefore, to presumptively identify filamentous bacteria on the basis of morphological and cellular characteristics in accordance with key and protocol by Jenkins *et al*., (1993).

3.2 EXPERIMENTAL PROCEDURE

Cultures were isolated previously by other researchers in the department as part of culture collection (Ramothokang, 2004). Filamentous bacteria were isolated from five treatment plants around Durban, South Africa. The spread plate technique was applied for initial inoculation of media R2A (Appendix 1), CGYA (Appendix 2), TGY agar (Appendix 3). The incubation temperature that was used was 20ºC for at least 10 days. Thirty eight cultures were isolated based on microscopic examination and morphology from a previous study by Ramothokang (2004). The protocol that was followed for the isolation was as follows:

3.2.1 Preliminary Isolation

3.2.1.1 Indirect inoculation (pretreatment procedures)

Two methods were used for pretreatment of samples, Nonidet pretreatment and Sonication.

(a) Nonidet Pretreatment

Nonidet is a non-ionic detergent that alleviates cell clumping without damaging the cells. Pure Nonidet was used (Ramothokang *et al.*, 2003). Nine parts of mixed liquor sample were mixed with 1 part of undiluted Nonidet detergent. The sample was then mixed for 1 minute with a vortex mixer to ensure complete homogenization (Stahl and Amann, 1991). The sample was then centrifuged at 1500 rpm for 10 minutes so as to separate floc formers from filaments. Serial dilutions were made (10^{-1}-10^{-5}) and 0.1mL from the centrifuged sample was used to inoculate the R2A (Appendix 1), CGYA (Appendix 2), TGYA (Appendix 3) and SCY (Appendix 4) plates using the spread plate method. The plates were incubated for at least 3 weeks at room temperature.

(b) Sonication

Sonication is the other method that can be applied as pretreatment method to disperse or separate floc formers from filaments (Kämpfer, 1997). The mixed liquor sample was sonicated at 20W for 10s. A volume of 0.1mL of sonicated sample was used for the inoculation of the plates same as above (for Nonidet method) (Munch and Pollard, 1997).

3.2.1.2 Direct Inoculation

The untreated mixed liquor samples were serially diluted and homogenized with a vortex mixer for 5 to10 seconds. Again 0.1 mL of the sample was used for the inoculation of plates and only the spread plate method was employed as above (Mulder and Deinema, 1981; Kämpfer 1997). The plates were incubated at room temperature for at least 3 weeks.

3.2.2 Selective Isolation

After incubation, individual colonies were isolated onto respective media. These individual isolated colonies were continuously sub-cultured in order to obtain pure cultures. Stains (Jenkins *et al*., 1993) that are routinely practiced to characterize filamentous bacteria were employed to determine whether or not isolates possessed filamentous characteristics and the cultures that maintained filamentous morphology were maintained in pure culture for further study.

3.2.3 Characterization

The cultures were examined for their gram reactions and cell morphology by performing Gram stain (Appendix 5). Neisser stain (Appendix 6) was employed to observe the presence or absence of polyphosphate granules in filamentous bacteria and other bacteria (Bitton, 1994).

The PHB stain (Appendix 7) which detects polyhydroxybutyrate granules in filamentous bacteria was performed. Sulfur oxidation test (Appendix 8) for presence of sulphur granules and crystal sheath stain (Appendix 9) was performed for the presence of a sheath on filamentous bacteria (Jenkins *et al.,* 1993).

3.2.4 Identification

The presumptive identification was as per Jenkins *et al*., (1993). Colonial morphology served as a useful additional tool in identification of filaments since some filaments displayed similar characteristics (Ramothokang, 2004).

3.2.5 Culture maintenance

Isolates were maintained on CGYA (Ziegler *et al.,* 1990) slopes and plates and stored at 4°C. These were re-cultured onto fresh media every four weeks. The Gram stain was done throughout sub-culturing in order to ensure purity of cultures.

3. 3 RESULTS

Table 3.1: Conventional characterization of filamentous bacteria through staining

Filament number	Gram Stain	Neisser stain (granules)	PHB stain	Crystal Sheath Stain	Comments
1	-	-	+	-	Filaments with barred shaped cells
2	+	-	-	-	Bacillus in chains
3	-	-(-)	+	+	Oval shaped cells
4	+	-	+	-	Round ended rods
5	+	-	+	-	Small rectangular shaped cells
6	+	-	-	+	Short filaments with discoid
7	+	-	-	-	Filaments had 'shifted' to single cells
8	+	-	+	-	Long rectangular cells
9	+/-	-	+	-	Rectangular cells
10	+	-(+)	+	-	Filaments with discoid shape
11	+/-	-	+	-	Small filaments with true branching
12	+	-	-	+	Discoid spherical cells
13	+/-	-(+)	+	-	Small rectangular cells
14	+	-	+	-	Rectangular cells

Table 3.1 continued: Conventional characterization of filamentous bacteria through staining

Filament number	Gram Stain	Neisser stain (granules)	PHB stain	Crystal Sheath Stain	Comments
15	+	-	-	+	Oval shaped cells
16	+	-	+	+	Long rectangular cells
17	-	-	+	+	Rod shaped cells
18	-	-	-	+	Rectangular cells
19	+	-	-	-	Long rectangular cells
20	+	-(+)	+	-	Rod shaped cells
21	+	-	+	-	Rectangular cells
22	+	+(+)	+	+	Very small rectangular cells
23	+	-	-	-	Long rectangular shaped cells
24	+	-	-	-	Round ended rods
25	+/-	-(+)	+	+	Very big spherical cells with short branches
26	+	-(+)	+	+	Short filaments with curled ended rods
27	+	-	+	-	Round ended rods
28	+	+(-)	-	-	Filaments with round ended rods
29	+	-	-	-	Rod shaped cells
30	+	+(+)	-	+	Filaments with rectangular cells

Table 3.1 continued: Conventional characterization of filamentous bacteria through staining

Filament number	Gram Stain	Neisser stain (granules)	PHB stain	Crystal Sheath Stain	Comments
32	+	+	-	-	Very small rectangular cells
33	+	-	+	-	Smoothly curved rods in chains
34	+	-	+	-	Smoothly curved rods in chains
35	+	-	+	-	Smoothly curved rods in chains
36	+	-	-	+	Straight and smoothly curved rods in chains
37	+	-	-	+	Straight rods in chains
38	+	-	+	-	Straight rods in chains

Keys: + positive, - negative. +/- variable. Neisser –(+) negative with positive granules. +(-) positive with negative result for intracellular accumulation of PolyPgranules. +(+) Neisser positive also accumulates PolyP intracellularly as granules.

The above tables represent microscopic examination using light microscopy at 1000X magnification. Isolates that store polyphosphate granules are indicated in brackets. The shape and arrangement of isolates is explained in Comments.

3.3.2 Gram stain images of the isolates

The isolated cultures were obtained from biological nutrient removal plants. It is generally accepted that filamentous morphotypes found in BNR systems are mostly gram positive (Martins *et al*., 2004). This was the case as 24 of the 38 isolates were gram positive. (Refer to Table 3.1).

Selection of these specific photo-micrographs is random. It is not selective, just representatives for the other cultures.

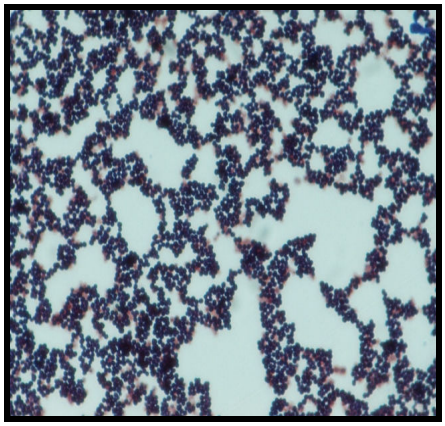

Figure 3.1: Photo-micrograph of gram positive Culture 6

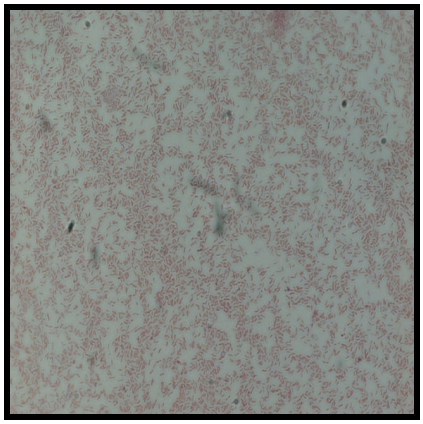

Figure 3.2: Photo-micrograph of gram negative Culture 9

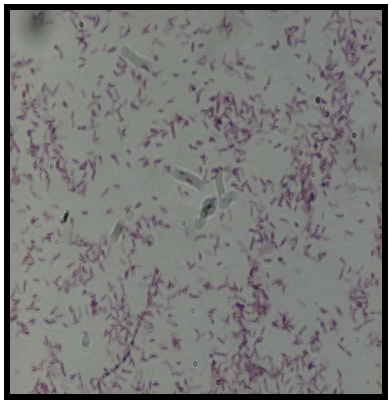

Figure 3.3: Photo-micrograph of gram positive Culture 20

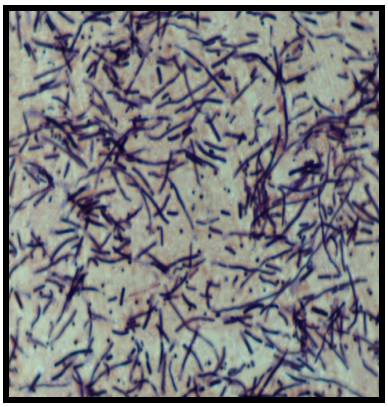

Figure 3.4: Photo-micrograph of gram positive Culture 24

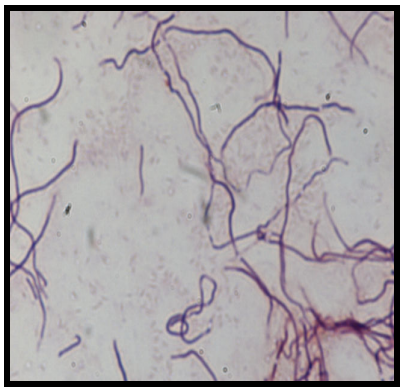

Figure 3.5: Photo-micrograph of gram positive Culture 30

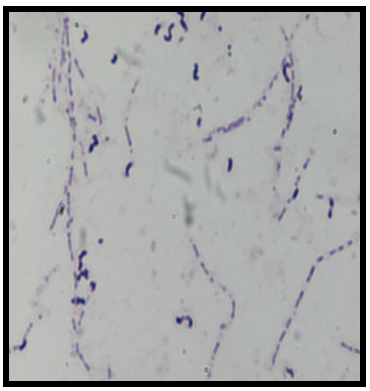

Figure 3.6: Photo-micrograph of gram positive Culture35

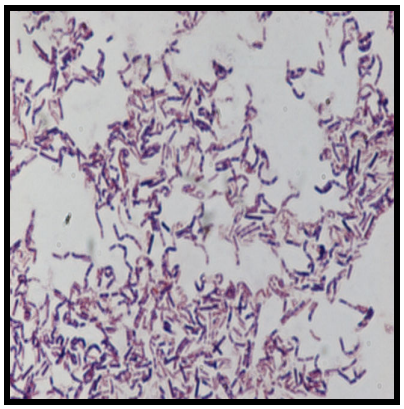

Figure 3.7: Photo-micrograph gram positive Culture 36

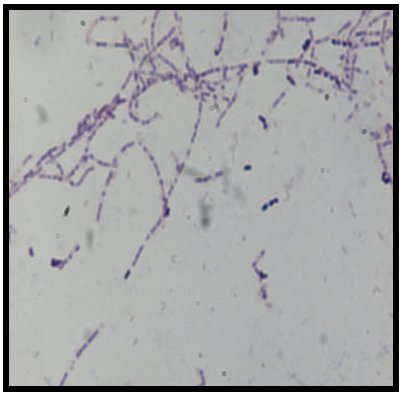

Figure 3.8: Photo-micrograph of gram positive Culture 38

3.4 DISCUSSION

Bacteria constitute the main components of the activated sludge and most often bacteria in the activated sludge are quite variable in cell morphology (Rossetti *et al*., 2006). Various distinct shapes were observed (Table 3.1) that varied from rods in chains to cocci in clusters. It is not unusual though to observe irregular shaped cells in activated sludge (Jenkins *et al*., 1993). Culture 25 (Table 3.1) exhibited larger than normal cocci with branching throughout the study. Curved rods and rectangular cells or tetrads are also observed in activated sludge processes (Jenkins *et al*., 1993; Seviour and Blackall, 1999; Rossetti *et al*., 2006). Rectangular cells were isolated in this study with variations in length. Cultures 5, 13, 22 and 32 had very short rectangular cells (Table 3.1). Culture 30 exhibited filamentous arrangement of rectangular cells as illustrated in Table 3.1 and Figure 3.5. Cultures 8, 16 and 23 had quite long rectangular cells as illustrated in Table 3.1.

Dominant and most frequent filaments were identified according to staining and morphological characteristics and were presumed to be *Microthrix parvicella*, *Types* 0092, 0675, 0041, 0914 and 1851 (Blackbeard *et al*., 1988). Bux and Kasan, (1994) found the most frequently occurring filaments to be *M. parvicella*, *Type* 0041 and *Norcadia*.

A number of isolates (Cultures 1, 2, 3, 4, 13, 16, 19 and 27) that were isolated during this study were left unclassified because their characteristics failed to match with known

filaments as characterized by Jenkins *et al*., (1993) for presumptive identification. However, it is generally accepted that many filamentous bacteria have not been taxonomically classified due to fastidious growth kinetics of filaments (Martins *et al*., 2004; Rossetti *et al*., 2006).

Identification keys that were developed by Eikelboom (1975) were developed to identify filamentous bacteria based on phenotypic characteristics. Most of these isolates in Table 3.1 were proven to be gram positive than those that were mentioned by Jenkins *et al., (*1993). This could be due to the fact that samples were collected from biological nutrient removal (BNR) plants supporting the findings by Eikelboom *et al*., (1998) that morphotype filamentous bacteria in BNR plants are mostly gram positive.

A filament presumed to belong to *Nocardia sp*. (Culture26) was isolated with very short filaments that are bent, the cell shape sometimes vary, because there are different types of filaments related to NALOs (*Nocardia amarae like organisms*) (Ziegler *et al* ., 1990; Martins *et al*., 2004). Other isolates that were presumptively identified are culture 12, culture 23 that were presumed to be *Type* 1863 or *Acinetobacter sp*.

Microscopic examination was done monthly on all isolates to verify gram reactions. It was noted that certain cultures had gram variable results. Culture 6 (Figure 3.1) were cocci in chains and its morphology and gram reaction was consistent throughout the study. Culture 9 (Figure 3.2) often had gram variable results. This was visualized as weakly gram positive or gram negative reactions. Realizing the importance of cell

morphology is vital because it is the only way to understand how it will affect the ecology (Martins *et al*., 2004). Maintaining stock cultures of these filamentous isolates proved to be a tedious task as their nutritional and growth parameters are not yet well understood, only limited information about their physiology and biochemistry is available (Ziegler *et al*., 1990) and it is also difficult to keep the cultures free from contamination as they are easily outgrown by contaminants (Ramothokang, 2004).

Initially when all the isolation was done, the isolates displayed filamentous morphology. However, morphological 'shifts' were observed from filamentous bacterial state to single cells. Initially culture 36 (Figure 3.7) was in filamentous form but later shifted to single cell rods that were slightly curved and grew in pairs and in chains. It is unclear though whether it was the continuous sub-culturing or the preservation that caused permanent morphological shift because nutritional conditions were constant throughout.

Variations in morphology were also observed on *Microthrix parvicella* (Foot *et al*., 1992 as cited by Seviour *et al*., 1994). Recently morphological shifts were observed on isolates that were positively identified through *FISH* to be filamentous bacteria. *Gordonia amarae* underwent variations from filamentous form to coccoidal and rod shaped cells (Ramothokang *et al*., 2006). Culture 20 (Figure 3.3) underwent variations from filamentous form to rod shaped cells and was later confirmed to be *Gordonia amarae*. Seviour and Blackall, (1999) also mentioned that certain morphotypes like *S. natans*, *Types* 1701, 0092 and 0961 can change morphology in response to changes in environmental conditions.

Cultures 1, 7, 9 (Figure 3.2), 10 and 20 (Figure 3.3) were all isolated in filamentous form and through continuous sub-culturing, the filamentous form 'shifted' to single cells permanently. Although these isolates could not be presumptively identified, they could not be excluded from the study as not all organisms in the activated sludge have been identified (Seviour and Blackall, 1999). Bradford *et al*., (1996) isolated and analyzed the 16S rRNA of gram negative bacteria that were isolated from activated sludge through micromanipulation. They were identified *in situ* according microscopic and phenotypic characteristics (Eikelboom, 1975; Jenkins, 1993). *Type* 0803, *Type* 0092, *Type* 0041 and *Herpetosiphon* sp. were micromanipulated and cultivated in different media. The growth was confirmed to be as a result of growth of specific identified filaments. It is widely accepted that variations may occur in filament morphology when filaments are grown in axenically (Seviour and Blackall, 1999; Davenport *et al*., 2000). But through methods employed in this study for isolation, it was found to be difficult to determine whether the colonies on the agar arose from the original filament observed in the sample. Hence micromanipulation is a method where one can be confident that the isolated bacteria originated from the specific filament in the sample.

Figure 3.4 represents gram positive, round ended rods. This culture exhibited two distinct shapes depending on the incubation period. It was rod- shaped if incubated for 4 days and if allowed to grow for 7 days it would be in filamentous form. Such variations in morphology could be indicative of the stage of growth in the isolates cycle. Therefore reliance on morphological traits can be misleading as isolates may look quite similar but varying considerably in their taxonomy and physiology.

CHAPTER FOUR- APPLICATION OF FLUORESCENT *in situ* HYBRIDIZATION (FISH) FOR THE IDENTIFICATION OF FILAMENTOUS BACTERIA

4.1 INTRODUCTION

Detection of microorganisms by using FISH with rRNA-targeted oligonucleotide probes is a very useful molecular tool for rapid, reliable and cultivation-independent monitoring of phylogenetically defined populations in environmental samples (Amann, 1995). The method has been used in activated sludge for the detection of microbial community structure, detection and in some cases enumeration of specific groups of microorganisms of special interest (Wagner *et al*., 1993, Wagner *et al*., 1994a). FISH has enabled progressions in taxonomic diversity among morphologically indistinguishable filamentous bacteria (Levantesi *et al*., 2006).

Probes can be designed to target narrow to broad phylogenetic groups. The FISH protocol involves application of oligonucleotide probes to permeabilized whole microbial cells. The probes enter the cells and specifically hybridize to their complementary target sequence. If no target sequence is present in cells ribosomes, probes are unable to hybridize and unbound probe is removed in subsequent wash step. Hence only specifically targeted cells retain the probes under appropriate stringency conditions (Hugenholtz *et al*., 2001). The technique basically consists of three main steps: fixation and dehydration of the sample, hybridization with the specific probes and successive

washing, and finally, microscopic examination through epifluorescent microscopy (Amann, 1995).

Gram positive filamentous bacteria are believed to have hydrophobic cell walls that are likely to be found in bulking and foaming BNR processes (Carr *et al*., 2005). The usefulness of FISH is greatly reduced due to lack of permeabilization. A study conducted by Carr *et al*., (2005) assessed combinations of mild acid hydrolysis and enzyme treatment for the permeabilization of pure cultures and foam samples. Lysozyme, mutolysin, lipase or proteinases K were the most effective for the permeabilization of cells to the probe (Carr *et al*., 2005).

The aim of this chapter is therefore to verify the identification of filamentous isolates using rRNA-targeted oligonucleotide probes that are domain and species-specific.

4.2 EXPERIMENTAL PROCEDURE

4.2.1 Oligonucleotide probes

The following oligonucleotide probes were synthesized according to specification and were labeled with fluorescein at the 5' end (Inqaba Biotech Pty Ltd., South Africa). Probes that were used are given in Table 4.2.1 and 4.2.3 and stringencies for each probe are given in Table 4.2.2. and Table 4.2.4 respectively. Probe selection was based on filamentous bacteria normally found in South African activated sludge processes (Blackbeard *et al*., 1988; Bux and Kasan, 1994) and presumptive identification of the isolates based on the Eikelboom-Type filament identification system.

All isolates were subjected to the EUB probe mix.

Table 4.2.1 EUB probes, target specificity and sequences.

TARGET SPECIFICITY	PROBE NAME	SEQUENCE (5'-3')
Most Bacteria	EUB 338	GCT GCC TCC CGT AGG AGT
Planctomycetales	EUB 338 II	GCA GCC ACC CGT AGG TGT
Verrucomicrobiales	EUB 338 III	GCT GCC ACC CGT AGG TGT

Table 4.2.2 Oligonucleotide probe stringencies

PROBE NAME	FORMAMIDE %	REFERENCES
EUB 338	20	Amann *et al*., 1990
EUB 338 II	20	Daims *et al*., 1999
EUB 338 III	20	Daims *et al*., 1999

Table 4.2.3. Species-specific Oligonucleotide probes, target specificity and sequences.

TARGET SPECIFICITY	PROBE NAME	SEQUENCE (5'-3')
Microthrix parvicella	Mpa645 MP223	CCG GAC TCT AGT CAG AGC GCC GCG AGA CCC TCC TAG
Nostocoida limicola I	NLIMI 91	CGC CAC TAT CTT CTC AGT
Nostocoida limicola II	NLIMII 175 NLIMII 192	GGC TCC GTC TCG TAT CCG AGA CTT TCC AGA CAG GAG
Nostocoida limicola III	NLIMIII 301 NLIMIII 729 NLIMIII 830	CCC AGT GTG CCG GGC CAC AGC ATC CAG AAC CTC GCT CCA TCG GCG AGC CCC CTA
Type 021N	21N G1B G2M G3M	TCC CTC TCC CAA ATT CTA TGT GTT CGA GTT CCT TGC GCA CCA CCG ACC CCT TAG CTC AGG GAT TCC TGC CAT
Sphaerotilus natans	SNA	CAT CCC CCT CTA CCG TAC
Gordonia amarae	GA	ATG A(CT)G TCC CCT CTG A
Thiothrix fructosivarans	TFR	CTC CTC TCC CAC ACT CTA
Type 1851	CHL 1851	AAT TCC ACG AAC CTC TGC CA
Type 1863/*Acinetobacter species*	ACA	ATC CTC TCC CAT ACT CTA
Haliscomenobacter hydrossis	HHY	GCC TAC CTC AAC CTG ATT
Thiothrix nivea	TNI	CTC CTC TCC CAC ATT CTA
Gordonia	Gor596	TGC AGA ATT TCA CAG ACG C
T. eikelboomii, T.nivea, T eii, T. fructosivarans, T. defulvii, Eikelboom Type 021N group I, II, III.	G123T Competitor	CCT TCC GAT CTC TAT GCA CCT TCC GAT CTC TAC GCA

Table 4.2.4. Oligonucleotide probe stringencies

PROBE NAME	FORMAMIDE %	REFERENCE
Mpa645	20	Erhart *et al*., 1997
MP223	20	Erhart *et al*., 1997
NLIMI 91	20	Liu and Seviour, 2001
NLIMII 175	40	Liu and Seviour, 2001
NLIMII 192	20	Liu and Seviour, 2001
NLIMIII 301	20	Liu and Seviour, 2001
NLIMIII 729	20	Liu and Seviour, 2001
NLIMIII 830	20	Liu and Seviour, 2001
21N	35	Wagner *et al*., 1994a
G1B	30	Kanagawa *et al*., 2000
G2M	35	Kanagawa *et al*., 2000
G3M	30	Kanagawa *et al*., 2000
SNA	45	Wagner *et al*., 1994a
GA	45	Kampfer *et al*., 1996
TFR	40	Kim *et al*., 2002
CHL 1851	35	Beer *et al*., 2002
ACA	35	Wagner *et al*., 1994b
HHY	20	Wagner *et al*., 1994a
TNI	45	Kanagawa *et al*., 2000
Gor596	20	De los Reyes *et al*., 1994
G123T Competitor	40	Kanagawa *et al*., 2000

4.2.2 *In situ* Hybridization

Fluorescent *in situ* hybridization was conducted as per Amann, 1995. However, the protocol was modified as follows (Prof. Valter Tandoi, CNR- Italy. Personal communication):

4.2.2.1 Pre-treatment of slides

Teflon coated slides were cleaned and pre-treated by detergent and Poly-L-lysine prior to use. The 0.1% gelatin and 0.01% chromium sulphate were substituted with 10% solution of poly-L- Lysine (Sigma Diagnostics, USA). Gelatin coated slides cannot be stored for long periods as the gelatin slowly begins to detach from the slides.

4.2.2.2 Sample preparation

The thirty eight bacterial isolates were fixed in ethanol and 4% paraformaldehyde depending on the gram reaction of the isolate. Gram negative bacteria were suspended in 4% paraformaldehyde whereas gram positive bacteria were fixed in absolute ethanol.

4.2.2.3 Whole cell Hybridization

Hybridization was carried out in a 50 ml polypropylene centrifuge tube, which served as a chamber for hybridization. The tube was covered with foil to prevent light from interfering with the fluorescence of the probe and paper towel was dampened with the hybridization buffer to create a moist environment in the chamber. It also eliminates non-specific binding of the probes to the cell by preventing evaporation of the buffer (Amann, 1995). All the probes were diluted accordingly to create a working concentration of 50 ng/µl. Hybridization was performed according to the protocol stated by Amann (1995) (Appendix 12).

Whole cell hybridization was followed by the staining of cells with a DNA-intercalating dye 4', 6-diamidino-2-phenylindole (DAPI) (Appendix 13). The staining procedure was modified from the methods of Hicks *et al*., (1992). The slides were viewed with the Zeiss (Germany) Axiolab microscope fitted for epifluorescence and Zeiss filter sets (02 for DAPI and 09 for fluorescein). Image analysis was carried out using the Zeiss KS 300 Imaging system.

4.3 RESULTS

DAPI is a DNA intercalating dye hence it binds to the DNA. Therefore all cells present can be visualized. It has been mostly applicable quantification of cells (Snaidr *et al*., 1997). Because DAPI binds to DNA, which is present in inactive or dead cells, it is no measure of cellular activity (Wilderer *et al*., 2002). Whereas oligonucleotide probes target the rRNA, implying that only intact, active cells that are homologous to the probe will hybridize (Amann, 1995).

The micrographs (Figures 4.1 and 4.2) represent Culture 3 that was ethanol fixed as a gram positive bacterium. The intensity of the fluorescence using DAPI therefore depends on the proper binding to the cells. The cells were intact and active and the morphology is clearly visualized (Figure 4.2).

The intensity of fluorescence with a probe depends on the direct correlation with the growth rate and cellular rRNA content. During hybridization procedure certain variations are expected in morphology. In Figure 4.3 and 4.4 (Culture 16), the arrangement is clearly visualized of rods in chains and this arrangement was evident throughout the study.

Figure 4.5 and 4.6 are chromatographs for culture 20. The mid-log phase of this organism was reached after seven hours of growth. It was not a particularly slow growing organism as the average mid-log phase for most organisms ranged between six and eight hours. But

the intensity of fluorescence was weak. This was attributed to inefficiency of the fixation to permeabilize the cell. Enzymatic treatment for the permeabilization of gram positive bacteria is vital although it should be mentioned that the morphology can be compromised.

Figure 4.7 (DAPI chromatograph for culture 30) and Figure 4.13 (DAPI chromatograph for culture 38) has strong fluorescence but figure 4.8 and Figure 4.14 have a weak fluorescence. This could be attributed to insufficient cellular rRNA content or lack of permeabilization. Figure 4.9 and Figure 4.11 have very strong DAPI fluorescence and the morphology of the isolates remained rods in chains through out the study. The intensity if the EUB mix fluorescence is quite intense representing a high level of cellular activity.

Throughout the application of species-specific probes for hybridization, EUB probe mix hybridization served as positive control. Gram positive isolates that were not treated for permeabilization served as a negative control for unspecific binding that can often be mistaken for positive hybridization (Results not shown). The sample was also not introduced into the well to observe autofluorescence as a negative control.

4.3.1 Whole cell probing using DAPI and EUB mix oligonucleotide probes

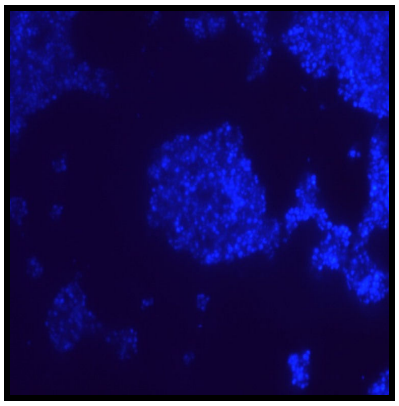

Figure 4.1: Photo-micrograph of DAPI for Culture 3

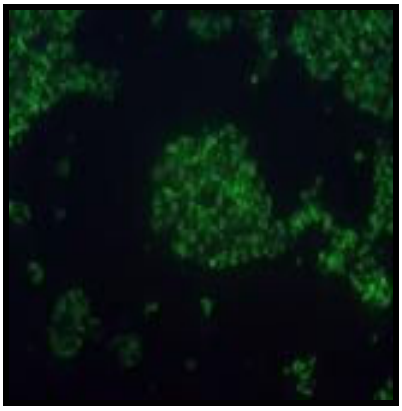

Figure 4.2: Photo-micrograph of EUB probe mix for Culture 3

Figure 4.3: Photo-micrograph of DAPI for Culture 16

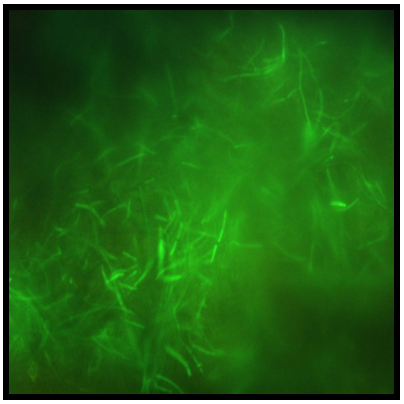

Figure 4.4: Photo-micrograph of EUB probe mix for Culture 16

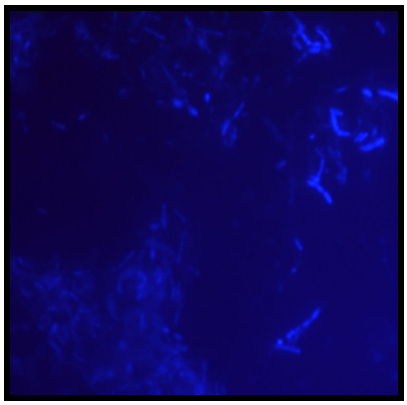

Figure 4.5: Photo-micrograph of DAPI for Culture 20

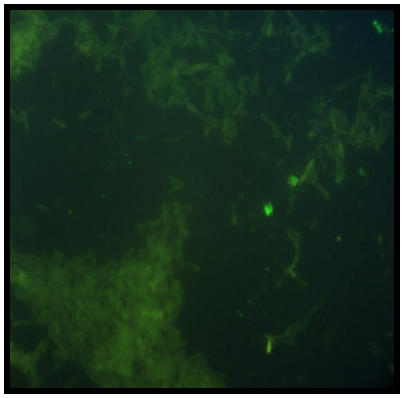

Figure 4.6: Photo-micrograph of EUB probe mix for Culture 20

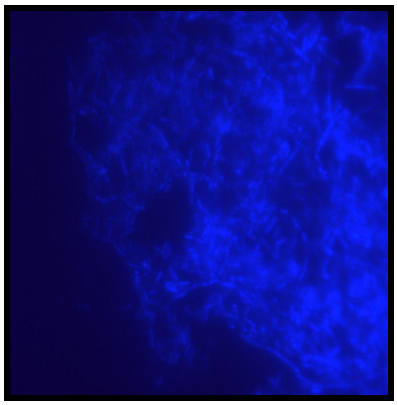

Figure 4.7: Photo-micrograph of DAPI for Culture 30

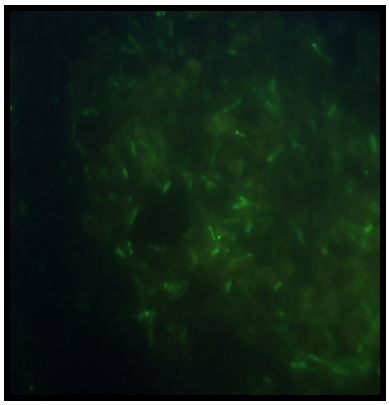

Figure 4.8: Photo-micrograph of EUB probe mix for Culture 30

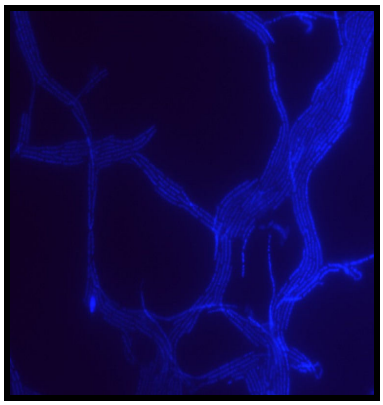

Figure 4.9: Photo-micrograph of DAPI for Culture 34

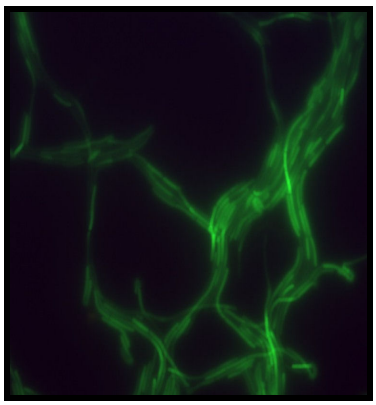

Figure 4.10: Photo-micrograph of EUB probe mix for Culture 34

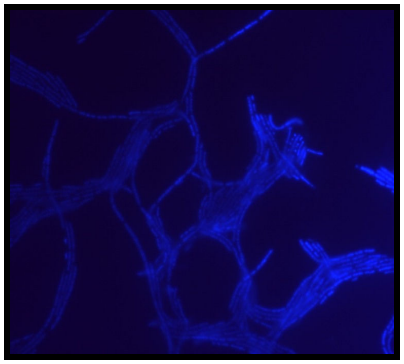

Figure 4.11: Photo-micrograph of DAPI for Culture 35

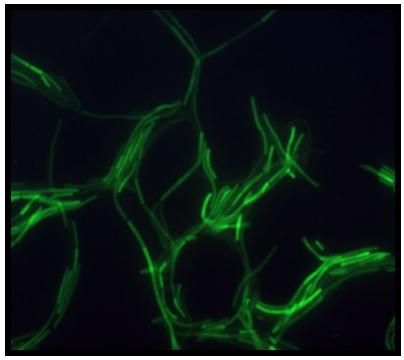

Figure 4.12: Photo-micrograph of EUB probe mix for Culture 35

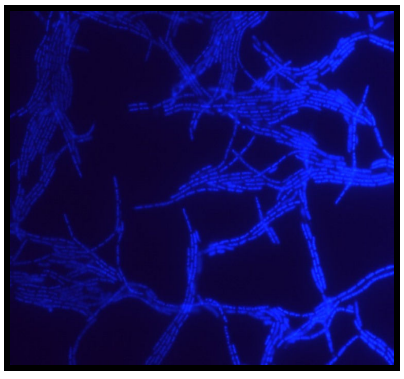

Figure 4.13: Photo-micrograph of DAPI for Culture 38

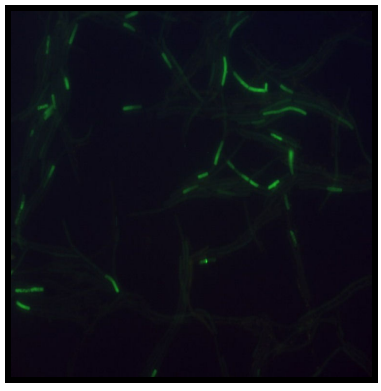

Figure 4.14: Photo-micrograph of EUB probe mix for Culture 38

4.3.2 *In situ* hybridization using species-specific probes.

Table 4.3.2 *FISH* with species-specific probes

Culture no.	SNA	TFR	TNI	HHY	21 N	ACA	GA	Myc657	NLIMI 91	Mpa 645, Mpa 222
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-		-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-		-	-	-	-
16	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-		-
18	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	+	-	-	-
21	-	-	-	-	-	-	-	-	-	-
22	-								-	-
23	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-	-
32	-	-	-	-	-	-	-	-	-	-
33	-	-	-	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-	-	-
35	-	-	-	-	-	-	-	-	-	-
36	-	-	-	-	-	-	-	-	-	-
37	-	-	-	-	-	-	-	-	-	-
38	-	-	-	-	-	-	-	-	-	-

Keys:
- Negative hybridization, +Positive hybridization

Table 4.3.2 clearly illustrates that none of the isolates had positive hybridizations when targeted with the above species-specific probes except for Culture 20. It showed positive hybridization to GA probe.

4.4 DISCUSSION

Probe sequences for filamentous bacteria that were used for this study were obtained from ProbeBase, an online public database and the oligonucleotide probes (Table 4.2.1 and 4.2.3) used in this study target the 16S rRNA. A positive and negative control was done throughout the experimentation to elucidate false positive and negative results.

During the past years, many rRNA targeted oligonucleotide probes have been designed for *in situ* detection of bacteria present in biological treatment systems. This technique consists basically of three steps, fixation and dehydration of the sample, hybridization with probes and successive washing and finally sample examination by epifluorescent microscopy (Amann, 1995; Rossetti *et al*., 2006).

rRNA targeted oligonucleotide probes are ideally suited to investigate the diversity of a microbial community and phylogenetic characterization. Many *FISH* studies have been done with domain and group specific oligonucleotide probes by targeting more conserved sites of the rRNA (Amann, 1995; Liu and Seviour, 2001). Cultures were initially subjected to EUB probe mix as listed in Table 4.1. These was done to confirm staining results and ensure that the isolates belonged to the bacterial kingdom as it is known that

cell size variations between some filamentous bacteria and filamentous fungi may be quite marginal (Prescott *et al*., 1999). All cultures showed positive homogeneity with the EUB probe mix.

Fixation of cells assures rRNA content preservation of cellular integrity and increases cell permeability (De Los Reyes *et al*., 1997). Although it should be mentioned that fixation normally compromises cell morphology due to cross-linking of proteins in the cell wall by the fixatives particularly paraformaldehyde (De los Reyes *et al*., 1997). Poor hybridization signals were obtained due to the lack of accessibility of rRNA within the cells particularly for mycolic-acid containing Actinomycete. This was circumvented by treatment with enzymes in order to permeabilize the cells (Carr *et al*., 2005). Culture 20 was identified as *Gordonia amarae* (Figure 4.16), although during the course of the study this bacterium maintained single cell morphology. Davenport *et al*., (2000) stated that certain changes in the environment, can induce coccoidal and rod shaped cells. This single cell morphology of *Gordonia amarae* has also been observed during foaming (Davenport *et al*., 2000).

Figure 4.3 has a weak signal with DAPI which is a DNA intercalating dye due to inaccessibility of the cells. But a strong hybridization signal was observed with the EUB probes (Figure 4.3). This is due to the fact that cultures were fixed only when they were at the mid-logarithm phase of growth. This was done in order to achieve strong fluorescence intensity as the probes bind to cellular rRNA (Amann, 1995; Wilderer *et al*., 2002).

The activated sludge floc consists of numerous constituents of bacteria such as extracellular polymeric substances (EPS). EPS consists of polysaccharides, proteins, lipids, nucleic acids and humic substances (Nielsen *et al*., 2004). Due to the EPS matrix around the cells, it was observed that the probes become embedded in the EPS and can not be washed off during the subsequent washing step. This EPS matrix yields false positive results that necessitate enzymatic treatment prior to hybridization. The probes are entrapped in the EPS matrix and cannot be washed off during subsequent washing step. Figure 4.8 shows positive hybridization with EUB probes but yielded false positive results with species-specific probes when it revealed false positive homology with more than two species-specific probes. This was eliminated through enzymatic treatment.

Fluorescent *in situ* hybridization can also be applied to study morphology and arrangement of cells. Figure 4.9 and Figure 4.10 show a strong hybridization signal and the morphology was not compromised. This was also evident for culture 4.11 and 4.12 as rods in chains were clearly visible. Figure 4.13 has very strong fluorescence intensity whereas Figure 4.14 has cells that did not fluoresce at all. Throughout staining this organisms had spore like cells that did not take up stains this could be due to the hydrophobic nature of the cell wall (Wagner *et al*., 1994a).

There are many filamentous bacteria that have not been phylogenetically classified. The specificity of the probe may be compromised because there are many unknown filaments that may be phylogenetically related to the probe target group because the 16S rRNA may not be enough to guarantee sufficient species identity (Rossetti *et al*., 2006).

Pure cultures of bacterial isolates presumed to be filamentous bacteria were subjected to species-specific oligonucleotide probes (Table 4.3.3.1). All samples were found to be non homologous to all probes. Upon isolation Culture 12 was presumed to be *Type* 1863/*Acinetobacter sp*. (Ramothokang *et al*., 2004). But *FISH* showed no positive hybridization to this probe. Culture 14 was presumed to be *Nostocoida limicola* but no positive hybridization was visualized. Martins *et al*., (2004) stated that variations in the conserved region of the 16S rRNA may exist considerably between different geographical areas. The probes were synthesized using the sequences obtained from probeBase, which is an international database. Mismatches may exist such that positive hybridizations are not possible.

CHAPTER FIVE- DETERMINING THE NUCLEOTIDE SEQUENCES OF ISOLATES

5.1 INTRODUCTION

Agarose and polyacrylamide gel electrophoresis is a rapid method used to identify, quantity and purify nucleic acids. DNA molecules are negatively charged due to dissociation of the phosphate backbone (Prescott *et al*., 1999). During electrophoresis they migrate towards the positively charged electrode. Small DNA fragments migrate more rapidly in the gel matrix compared to large ones. Hence DNA molecules are separated based on their size. Agarose is a non-toxic polysaccharide extracted from seaweed. It is easy to use and is relatively inexpensive. A wide range of DNA fragments (10-50 000 bp) can be separated on agarose gels of various concentrations (Fermentas, 2006; Gray, 2002).

DGGE is one of several DNA/RNA fingerprinting methods used to investigate microbial communities and has become more popular in molecular microbial ecology as an alternative to more laborious analysis of 16S rRNA gene libraries. It is based on the amplification of specific nucleic acid fragments by PCR and analysis by gel electrophoresis (Wilderer *et al*., 2002).

DGGE allows for the separation of 16SrDNA that is unique for each organism. DGGE has been widely applied in activated sludge research in order to characterize the

complexity of a microbial community. Even fluctuations in microbial populations can be monitored through DGGE but for the purposes of this study it was done to determine the purity of the culture followed by sequencing of the culture in order to determine its taxonomic status (Martins *et al*., 2004). In DGGE, single-stranded DNA molecules can be separated on polyacrylamide gels in varying concentrations of polyacrylamide gels. In contrast to conventional electrophoresis that allows the separation of DNA fragments of identical length but different sequence (Wilderer *et al*., 2002). Thus, microorganisms differing in their 16S rDNA genes will produce unique gel bands. Selected bands from the polyacrylamide gel are then sequenced where the exact nucleotide order is determined.

Modern sequencing methods are based on the chain–termination method but employ dNTP that are labeled with a fluorescent chemical marker. Each dNTP is labeled with a different colored marker. The resultant fragments are detected as the amplified PCR fragments pass through a stationery laser. The sequence of the strand is recorded as a chromatogram where each peak corresponds to a single nucleotide. The chromatogram is recorded through a computer and converted into a sequence data, which can be compared with sequences in the database (Howe and Ward, 1991).

This chapter serves to determine the nucleotide sequences of isolates in order to phylogenetically classify isolates.

5.2 EXPERIMENTAL PROCEDURE

5.2.1 Determining mid-log phase of cultures.

The age of the culture is important for successful extraction of rDNA. In general, late log growth phase should not be used for preparing DNA as nucleases tend to accumulate in older cultures (Moore *et al*., 2004).

Working cultures were prepared by inoculating stock cultures on CGYA agar plates (Appendix 2) for 48hours. Sufficient inoculum was then introduced to a starter broth of 2ml nutrient broth substituted with glucose (5%). Starter culture was left for about 48 hours or until sufficient culture is observed.

The starter culture was then introduced to 90 ml of nutrient broth containing 5% glucose. This was placed on a rotary shaker at 110 rpm at room temperature which ranged from 20º C to 25º C depending on weather conditions. The growth was monitored hourly by determining the absorbance which was read at 620 nm. The mid-logarithm phase was determined by calculating the average of the absorbance readings during the logarithm phase.

The mid log-phase for the isolates varied from 5.5 hours to about 7.5 hours for the slow growers. Growth was halted at the mid-log phase and sufficient biomass was obtained for DNA extraction.

5.2.2 Protocol for the extraction of DNA

DNA was extracted using QIAamp stool Mini kit (Qiagen, Southern Cross Biotechnology Pty Ltd) as per protocol as explained in Appendix 15.

5.2.3 Agarose gel electrophoresis

Subsequent to DNA extraction, the samples were run on Agarose gels to ensure the presence of DNA and purity verification. This procedure was also done after amplification of DNA fragments through PCR (Section 5.2.4). A 2.5% agarose gel was used. The gel was run at 80V for 60 minutes. The gel was viewed using a Hoefer® MacroVue UV-20 Transilluminator (Pharmacia-Biotech AB, South Africa).

5.2.4 Polymerase Chain Reaction

The DNA that was extracted from the isolates was amplified through PCR. The method employed was a method published by Giovanni (1991) that was modified by Naidoo (2005) for optimum results. Taguchi method (Cobb and Clarkson, 1994) has also been optimized by Naidoo (2005) for filamentous bacteria. Both these methods were used for the amplification of extracted 16S DNA.

Primers used for this study were universal primers that are complementary to the region conserved among the bacterial domain. A 40 base pair GC clamp was attached to the 5' end of the forward (f) primers. The reverse (r) primer that was used is 1492r. The following primers were used for all isolates:

- Primer 27f : 5' GAG TTT GAT CCGGC TCA G 3' (Blackall, 1994).
- Primer 341f : 5' ATT ACC GCG GCT GCT GG 3' (Sigler and Turco, 2002).
- Primer 1492r: 5' TAC GGC TAC CTT GTT ACG ACT T 3' (Blackall., 1994).
- 40 bp GC-clamp: CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCG GGG GCC GGG GGG C (Muyzer *et al*., 1993).

Table 5.1: PCR reaction mixture according to Giovanni (1991, modified for filamentous bacteria by Naidoo, 2005).

SOLUTION + CONCENTRATION	**AMOUNT µL**
10 X PCR Buffer without Mg^{+}	10
1.5 mM $MgCl_2$	6
10 ng/ml Genomic DNA	10
2mM dNTP (mix of all 4 dNTPs)	10
25 pmol Stock solution of each probe	2
Sterile distilled water	59
Taq polymerase 1 unit/ µl	1

PCR was carried out in an automated PCR Sprint Temperature Cycling System as explained in Table 5.2.

PCR Cycling parameters (Giovanni, 1991)

Stage 1	Step 1	94^0C	4 minutes	x 1 cycle
Stage 2	Step 1	94^0C	1 minute	x 35 cycles
	Step 2	53^0C	1 minute	
	Step 3	72^0C	2 minutes	
Stage 3	Step 1	72^0C	4 minutes	x 1 cycle

Table 5.2: PCR reaction mixture according to Cobb and Clarkson (1994), modified by Naidoo, 2005).

SOLUTION + CONCENTRATION	**AMOUNT µL**
10 X PCR Buffer without Mg^+	10
1.5 mM $MgCl_2$	6
10 ng/ml Genomic DNA	20
2mM dNTP (mix of all 4 dNTPs)	15
25 pmol Stock solution of each probe	2
Sterile distilled water	44
Taq polymerase 1 unit/ µl*	1

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

PCR Cycling parameters (Blackall, 1994)

Stage 1	Step 1	98^0C	1 minute	x 1 cycle
Stage 2	Step 1	48^0C	45 seconds	x 35 cycle
	Step 2	72^0C	2 minutes	
	Step 3	93^0C	1 minute	
Stage 3	Step1	48^0C	10 minutes	x 1 cycle
	Step 2	72^0C	10 minutes	x 1 cycle
	Hold	4^0C		

5.2.5 Denaturing Gradient Gel Electrophoresis (DGGE)

The amplified 16S rRNA or DNA was separated by DGGE using the BIO-RAD Gene™ Denaturing Gel Electrophoresis System and Power Pac 300. Method was performed in accordance with BIO-RAD D Gene instruction manual and applications guide (catalog numbers 170-9000 through 170-9070). Parallel gels were used of 7.5%. The gels contained a linearly increasing concentration gradient of formamide and urea where a 100% denaturant is defined as a 40% formamide + 7M urea. The optimum concentration of the denaturing solutions was determined experimentally by varying concentration of urea and formamide respectively. A 30% low density denaturing solution and a 60%

high denaturing solution was used. Gels were delivered between two glass plates with 1.5mm spacer using the Bio-Rad Model 475 Gradient Delivery System (Bio-Rad, South Africa), Immediately prior to pouring the gels, ammonium persulphate (200 mg/l) and TEMED, which are responsible for polymerizing the gel, were added to the high and low density solutions. The solutions that were used are available in Appendix 17. Once the gels had polymerized, the gel wells were washed with 1X TAE buffer. The sample and dye mix was then loaded and a molecular marker of 72- 1353 base pair molecular weight marker (IX, version 3, 2000, 0.25 µg/µl, Roche Products Pty Ltd., South Africa) was used. All the chemicals used were of electrophoretic grade (Bio-Rad, South Afirca). The gels were electrophoresed at 200 V for 1 hour at a constant temperature of 60º C.

Staining of Gel

After electrophoresis the gels were removed from the electrophoresis tank and core. The gel was then stained 250 ml 1X TAE buffer containing 10 µl of Ethidium Bromide for 1-2 minutes. The gels were then rinsed in a container containing 250 ml 1 X TAE for destaining. The gels were viewed using G-Box BioImaging System and analyzed using GeneSnap (Syngene, Europe)

5.2.6 Nucleotide Sequencing

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 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 to the nucleotide sequences in the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) 2.2.10. Algorithm (Altschul *et al*., 1997).

5.3 RESULTS

Of the original 38 cultures, 30 were subjected to sequencing due to high quality DNA yields from these organisms. All cultures were sequenced using the primers 27f and 1492r.

5.3.1 Introduction to BLAST

Query sequence is the sequence from an unknown isolate that has revealed alignments or homology to a subject sequence that an unknown bacterium has shown homology to. The degree of homology varies hence the level of significance. Phylogenetic analysis based nucleotide sequences can reveal only genus classification when an unknown isolate has no close relatives at species level when sequence similarity is less than 96.1 %.

A query sequence, which is a set nucleotide sequences of the unknown bacterium, can have homology at various intervals of the subject sequences. Such positions that have significant alignments are listed as illustrated below.

Culture 3

Subject sequence: *Bacillaceae bacterium*

1. Query sequence, Positions 30 - 89

AGC TTG CTC TCA AGA AGT TAG CGG CGG ACG GGT GAG TAA CAC GTG GGT AAC CTG CCC ATA

Subject sequence, Positions 73 -132

AGC TTG CTC TCA AGA AGT TAG CGG CGG ACG GGT GAG TAA CAC GTG GGT AAC CTG CCC ATA

2. Query sequence, Positions 150 – 209

TCG AAA TTG AAA GGC GGC TTC GGC TGT CAC TTA TGGA TGG ACC CGC GTC GCA TTA GCT AG

Subject sequence, Positions 193 -252

TCG AAA TTG AAA GGC GGC TTC GGC TGT CAC TTA TGGA TGG ACC CGC GTC GCA TTA GCT AG

3. Query sequence, Positions 210 – 268

TTG GTG AGG TAA CGG CTC ACC AA-G CAA CGA TGC GTA GCC GAC CTG AGA GGG TGA TCG GC

Subject sequence, Positions 253 – 312

TTG GTG AGG TAA CGG CTC ACC AA**G** GCA ACG ATG CGT AGC CGA CCT GAG AGG GTG ATC GGC

4. Query sequence, Positions 269 - 327

CAC ACT GGG ACT GAG ACA CGG CCC AGA CTC CTA CGG GAG GCA GCA GTA –GG AAT CTT CCG

Subject sequence, Positions 313 - 372

CAC ACT GGG ACT GAG ACA CGG CCC AGA CTC CTA CGG GAG GCA GCA GTA **G**GG AAT CTT CCG

5. Query sequence, Positions 328 - 384

CAA T-G ACG AAA GTC TGA CGG AGC AAC GCC GCG TGA GTG ATG AA-GCT TTC –GG TCG TAA

Subject sequence, Positions 373 - 432

CAA T**G**G ACG AAA GTC TGA CGG AGC AAC GCC GCG TGA GTG ATG AA**G** GCT TTC **G**GG TCG TAA

Culture 6

Subject sequence: *Micrococcus luteus*

1. Query sequence, Positions 59 - 118

GGG TTA GGC CAC CGG CTT CGG GTG TTA CCG ACT TTC GTG ACT TGA CGG GCG GTG TGT ACA

Subject sequence, Positions 1415 - 1356

GGG TTA GGC CAC CGG CTT CGG GTG TTA CCG ACT TTC GTG ACT TGA CGG GCG GTG TGT ACA

2. Query sequence, Positions 119 - 177

AG- CCC GGG AAC GTA TTC ACC GCA GCG TTG CTG ATC TGC GAT TAC

TAG CGA CTC CGA CTT

Subject sequence, Positions 1355 - 1296

AG**G** CCC GGG AAC GTA TTC ACC GCA GCG TTG CTG ATC TGC GAT TAC

TAG CGA CTC CGA CTT

3. Query sequence, Positions 178 - 237

CGT GGG GTC GAG TTG CAG ACC CCA ATC CGA ACT GAG ACC GGC TTT

TTG GGA TTA GCT CCA

Subject sequence, Positions 1295 - 1236

CAT GGG GTC GAG TTG CAG ACC CCA ATC CGA ACT GAG ACC GGC TTT

TTG GGA TTA GCT CCA

4. Query sequence, Positions 238 - 297

CCT CAC AGT ATC GCA ACC CAT TGT ACC GGC CAT TGT AGC ATG CGT

GAA GCC CAA GAC ATA

Subject sequence, Positions 1235 - 1176

CCT CAC AGT ATC GCA ACC CAT TGT ACC GGC CAT TGT AGC ATG CGT

GAA GCC CAA GAC ATA

5. Query sequence, Positions 298 - 357

GGG GGG CAT GAT GAT TTG ACG TCG TCC TCA CCT TCC TCC GAG TTG

ACC CCG GCA CGT CTC

Subject sequence, Positions 1175 - 1118

G**A**G GGG CAT GAT GAT TTG ACG TCG TCC TCA CCT TCC TCC GAG TTG

ACC CCG GCA CGT CTC

Culture 12

Subject Sequence: *Aeromonas hydrophila*

1. Query sequence, Positions 32 -91

AAA GTA GCT TGC TAC TTT TGC CGG CGA GCG GCG GAC GGG TGA GTA

ATG CCT GGG AAA TTG

Subject sequence, Positions 16 -75

AAA GTA GCT TGC TAC TTT TGC CGG CGA GCG GCG GAC GGG TGA GTA

ATG CCT GGG AAA TTG

2. Query sequence, Positions 92 -151

CCC AGT CGA GGG GGA TAA CAG TTG GAA ACG ACT GCT AAT ACC GCA

TAC GCC CTA CGG GGG

Subject sequence, Positions 76 – 135

CCC AGT CGA GGG GGA TAA CAG TTG GAA ACG ACT GCT AAT ACC GCA

TAC GCC CTA CGG GGG

3. Query sequence, Positions 152 - 211

AAA GCA GGG GAC CTT CGG GCC TTG CGC GAT TGG ATA TGC CCA GGT GGG ATT AGC TAG TTG

Subject sequence, Positions 136 - 195

AAA GCA GGG GAC CTT CGG GCC TTG CGC GAT TGG ATA TGC CCA GGT GGG ATT AGC TAG TTG

4. Query sequence, Positions 212 – 271

GTG AGG TAA TGG CTC ACC AAG GCG ACG ATC CCT AGC TGG TCT GAG AGG ATG ATC AGC CAC

Subject sequence, Positions 196 -255

GTG AGG TAA TGG CTC ACC AAG GCG ACG ATC CCT AGC TGG TCT GAG AGG ATG ATC AGC CAC

5. Query sequence, Positions 272 – 331

ACT GGA ACT GAG ACA CGG TCC AGA CTC CTA CGG GAG GCA GCA GTG GGG AAT ATT GCA CAA

Subject sequence, Positions 256 - 315

ACT GGA ACT GAG ACA CGG TCC AGA CTC CTA CGG GAG GCA GCA GTG GGG AAT ATT GCA CAA

Culture 13

Subject sequence: *Bosea minatitlanensis*

1. Query Sequence, Positions 51 - 110

CTC CTT GCG GTT AGC GCG ACG CCT TCG GGT AAA CCC AAC TCC CAT

GGT GTG ACG GGC GGT

Subject Sequence, Positions 1398 - 1339

CTC CTT GCG GTT AGC GCG ACG CCT TCG GGT AAA CCC AAC TCC CAT

GGT GTG ACG GGC GGT

2. Query sequence, Positions 111 -168

GTG TAC AAG CCC –G GAA CGT ATT CAC CGT GGC ATG CTG ATC CAC GAT

TAC TAG CGA TTC

Subject sequence, Positions 1338 - 1279

GTG TAC AAG **G**CC **CG**G GAA CGT ATT CAC CGT GGC ATG CTG ATC CAC

GAT TAC TAG CGA TTC

3. Query sequence, Positions 169 - 225

CAC CTT CAT GCA CTC GAG TTG CAG AGT GCA ATC TGA ACT GAG ACG –

CT TTT –G GAT TAG

Subject sequence, Positions 1278 - 1219

CAC CTT CAT GCA CTC GAG TTG CAG AGT GCA ATC TGA ACT GAG ACG

GCT TTT **TG**G GAT TAG

4. Query sequence, Positions 226 - 280

CTC GAG –TC GCC CTT TCG CTG CA- --T GTC ACC GCC AT-GTA GCA CGT GTG TAG CCC AGC

Subject sequence, Positions 1218 - 1159

CTC GAG GTC GCC CTT TCG CTG C**C**C **AT**T GTC ACC GCC AT**T** GTA GCA CGT GTG TAG CCC AGC

5. Query sequence, Positions 281 - 333

CTG TAA G-- -CA TGA G-A CTT GAC GTC ATC CCC AC- TTC CTC GCG –CT TAT CAC –GG CAG

Subject sequence, Positions 1158 - 1099

CTG TAA G**GG C**CA TGA G**G**A CTT GAC GTC ATC CCC AC**C** TTC CTC GCG **G**CT TAT CAC **C**GG CAG

Culture 15

Subject sequence: *Pseudomonas aeroginosa*

1. Query sequence, Positions 41 – 100

GGA GCT TGC TCC TGA GAT ACA GCG GCG GGA GGG TCG AGT AAT GCC TGG GAA TCT GGC CTG

Subject sequence, Positions 51 - 107

GGA GCT TGC TCC TGA GAT **T**CA GCG GCG G**AC** GGG TCG AGT AAT GCC **A**GG GAA TCT GGC CTG

2. Query sequence, Positions 101 - 160

GTA GGT GGG GGA TAA CGT CCG GAA ACG GGC GCT AAT GAC CGC ATA

CGT CCT GAG GGG AGA

Subject sequence, Positions 108 - 164

GTA GGT GGG GGA TAA CGT CCG GAA ACG GGC GCT AAT GAC CGC ATA

CGT CCT GAG GGG AGA

3. Query sequence, Positions 161 - 220

AAG TGG GGG GAT CTT CGG ACC TCA CGC TAT CAG GAT GAG CAC TAG

GTC GGG ATT AGC ATA

Subject sequence, Positions 165 - 220

AAG TGG GGG GAT CTT CGG ACC TCA CGC TAT CAG GAT GAG CAC TAG

GTC GGG ATT AGC ATA

4. Query sequence, Positions 221 – 280

GTA TGG TGG GGT AAA GGC GCT ACC AGA GGC GAC GAT CCA TAA CTG

GTC TGA GAG GAT GAT

Subject sequence, Positions 221 – 277

GTA TGG TGG GGT AAA GGC GCT ACC AGA GGC GAC GA**G** CCA TAA CTG

GTC TGA GAG GAT GAT

5. Query sequence, Positions 281 -340

CAG TCA CAC TGG AAC TGA GAC ACG GTC CAG ACT CCT ACG GGA GGC

AGC AGT GGG GAA TAT

Subject sequence, Positions 278 - 337

CAG TCA CAC TGG AAC TGA GAC ACG GTC CAG ACT CCT ACG GGA GGC

AGC AGT GGG GAA TAT

Culture 19

1. Subject sequence: *Erwinia chrysanthemi*

Query sequence, Positions 218 - 238

GAA ACG CTG ACG CTG CTC TTT

Subject sequence, Positions 5438 - 5458

GAA ACG CTG ACG CTG CTC TTT

2. Subject sequence: *Agrobacterium tumefaciens*

1. Query sequence, Positions 188 -207

GCT GCC TCA CTG AGC TGT TG

Subject sequence, Positions 3583 – 3564

GCT GCC TCA CTG AGC TGT TG

2. Query sequence, Positions 188 - 207

GCT GCC TCA CTG AGC TGT TG

Subject sequence, Positions 4211 -4230

GCT GCC TCA CTG AGC TGT TG

Culture 20

Subject sequence: *Gordonia amarae*

1. Query sequence, Positions 56 - 114

ACT CGA GTG GCG AAC GGG TGA GTA ACA CGT GGG TGA TCT GCC CCA AAC TCT –GG ATA AGC

Subject sequence, Positions 71 - 130

ACT CGA GTG GCG AAC GGG TGA GTA ACA CGT GGG TGA TCT GCC CC**T** **G**AC T**T**T **G**GG ATA AGC

2. Query sequence, Positions 115 - 172

CTG GGA AAC TGG G-C TAA TAC TGG ATA TGA CCT TCT GCT TCA TGG TGG TTG G-G GAA AGC

Subject sequence, Positions 131 - 190

CTG GGA AAC TGG G**T**C TAA TAC **C**GG ATA TGA CCT **G**CT **C**CT **G**CA TGG **G**GG T**G**G G**T**G GAA AGC

3. Query sequence, Positions 173 -230

TTT TGC GGT TTG GGA TGG GCC CGC GGC CTA TCA GCT TGT –GG GGG GTA ATG GCC TAC TC

Subject sequence, Positions 191 - 249

TTT TGC GG**C** **A**TG GGA TGG GCC CGC GGC CTA TCA GCT TGT **TG**G TGG GGT AAT GGC CTA CTC

4. Query sequence, Positions 231 – 287

ATA GGC GAC GAC GGG TAG CCG ACC TGA GA- -GT GAT CGG CCA CAC T-G GAC TGA GAC ACG

Subject sequence, Positions 250 – 308

ATA GGC GAC GAC GGG TAG CCG ACC TGA GA**G** **G**GT GAT CGG CCA CAC T**G**G GAC TGA GAC ACG

5. Query sequence, Positions 288 -343

CCC CAG ACT CCT ACG GGA GGC AGC CGT –G GAA TTT TGC –CA ATG GGC GCA AGC CTG A-G

Subject sequence, Positions 309 -368

GCC CAG ACT CCT ACG GGA GGC AGC **A**GT **GG**G GAA T**A**T TGC **A**CA ATG GGC GCA AGC CTG A-G

Culture 26

Subject sequence: *Enterobacter spp*

1. Query sequence, Positions 25 - 84

TTA AGC TAC CTA CTT CTT TTT GCA ACC CAC TCC CAG TGG TGT GAC GGG CGG TGT GTA CA

Subject sequence, Positions 1385 - 1328

TTA AGC TAC CTA CTT CTT TTT GCA ACC CAC TCC CAG TGG TGT GAC GGG CGG TGT GTA CA

2. Query sequence, Positions 85 - 143

G-C CCG GGA ACG TAT TCA CCG TAG CAT TCT GAT CTA CGA TTA CTA GCG ATT CCG ACT TCA

Subject sequence, Positions 1341 – 1282

G**G**C CCG GGA ACG TAT TCA CCG TAG CAT TCT GAT CTA CGA TTA CTA GCG ATT CCG ACT TCA

3. Query sequence, Positions 144 - 203

TGG AGT CGA GTT GCA GAC TCC AAT CCG GAC TAC GAC GCA CTT TAT GAG GTC CGC TTG CTC

Subject sequence, Positions 1281 - 1222

TGG AGT CGA GTT GCA GAC TCC AAT CCG GAC TAC GAC GCA CTT TAT GAG GTC CGC TTG CTC

4. Query sequence, Positions 204 - 261

TCG CGA G—CGC TTC TCT TTG TAT GCG CCA TTG TAG CAC GTG TGT AGC CCT ACT CGT AAG

Subject sequence, Positions 1221 – 1162

TCG CGA G**GT** CGC TTC TCT TTG TAT GCG CCA TTG TAG CAC GTG TGT AGC CCT ACT CGT AAG

5. Query sequence, Positions 262 – 321

GGC CAT GAT GAC TTG ACG TCA TCC CCA CCT TCC TCC AGT TTA TCA CTG GCA GTC TCC TTT

Subject sequence, Positions 1161 - 1102

GGC CAT GAT GAC TTG ACG TCA TCC CCA CCT TCC TCC AGT TTA TCA CTG GCA GTC TCC TTT

Culture 36

Subject sequence: *Bacillus cereus*

1. Query sequence, Positions 25 - 82

GGC GGA CGG GTG AGT AAC ACG TGG GTA ACC TGC CCA TAA GAC TGG – AT AAC TCC GG- AAA

Subject sequence, Positions 15 - 74

GGC GGA CGG GTG AGT AAC ACG TGG GTA ACC TGC CCA TAA GAC TGG **G**AT AAC TCC GG**G** AAA

2. Query sequence, Positions 83 - 141

CCG GG- CTA ATA CCG GAT AAC ATT TTG AAC CGC ATG GTT CGA AAT

TGA AAG GCG GCT TCG

Subject sequence, Positions 75 - 134

CCG GG**G** CTA ATA CCG GAT AAC ATT TTG AAC CGC ATG GTT CGA AAT

TGA AAG GCG GCT TCG

3. Query sequence, Positions 142 - 201

GCT GTC ACT TAT GGA TGG ACC CGC GTC GCA TTA GCT AGT TGG TGA

GGT AAC GGC TCA CCA

Subject sequence, Positions 135 – 194

GCT GTC ACT TAT GGA TGG ACC CGC GTC GCA TTA GCT AGT TGG TGA

GGT AAC GGC TCA CCA

4. Query sequence, Positions 202 - 261

AGG CAA CGA TGC GTA GCC GAC CTG AGA GGG TGA TCG GCC ACA CTG

GGA CTG AGA CAC GGC

Subject sequence, Positions 195 - 254

AGG CAA CGA TGC GTA GCC GAC CTG AGA GGG TGA TCG GCC ACA CTG

GGA CTG AGA CAC GGC

5. Query sequence, Positions 262 - 314

CCA GAC TCC TAC GGG AGG CAG CAG TA- GGA ATC TTC CGC AAT GGA

CGA AAG TCT GAC GGA

Subject sequence, Positions 255 -314

CCA GAC TCC TAC GGG AGG CAG CAG TA**G** GGA ATC TTC CGC AAT GGA

CGA AAG TCT GAC GGA

Organism 38

Subject sequence: *Bacillus cereus*

1. Query sequence, Positions 68 - 127

AAG TTA GCG GCG GAC GGG TGA GTA ACA CGT GGG TAA CCT GCC CAT

AAG ACT GGG ATA ACT

Subject sequence, Positions 1458 – 1399

AAG TTA GCG GCG GAC GGG TGA GTA ACA CGT GGG TAA CCT GCC CAT

AAG ACT GGG ATA ACT

2. Query sequence, Positions 128 - 187

CCG GGA AAC CGG GGC TAA TAC CGG ATA ATA TTT TGA ACT GCA TGG

TTC GAA ATT GAA AGG

Subject sequence, Positions 1398 - 1339

CCG GGA AAC CGG GGC TAA TAC CGG ATA ATA TTT TGA ACT GCA TGG

TTC GAA ATT GAA AGG

3. Query sequence, Positions 188 - 247

CGG CTT CGG CTG TCA CTT ATG GAT GGA CCC GCG TCG CAT TAG CTA GTT GGT GAG GTA ACG

Subject sequence, Positions 1338 - 1279

CGG CTT CGG CTG TCA CTT ATG GAT GGA CCC GCG TCG CAT TAG CTA GTT GGT GAG GTA ACG

4. Query sequence, Positions 248 - 307

GCT CAC CAA GGC AAC GAT GCG TAG CCG ACC TGA GAG GGT GAT CGG CCA CAC TGG GAC TGA

Subject sequence, Positions 1278 - 1219

GCT CAC CAA GGC AAC GAT GCG TAG CCG ACC TGA GAG GGT GAT CGG CCA CAC TGG GAC TGA

5. Query sequence, Positions 308 - 367

GAC ACG GCC CAG ACT CCT ACG GGA GGC AGC AGT AGG GAA TCT TCC GCA ATG GAC GAA AGT

Subject sequence, Positions 1218 - 1159

GAC ACG GCC CAG ACT CCT ACG GGA GGC AGC AGT AGG GAA TCT TCC GCA ATG GAC GAA AGT

Positive control

Subject sequence: *Escherichia coli*

A culture of Escherichia *coli* was also sent for sequencing to serve as a control.

1. Query sequence, Positions 25 - 83

CAG CTT GCT GCT TTG CTG ACG AGT GGC GGA CGG GTG AGT AAT GTC

TGG –AA ACT GCC TGA

Subject sequence, Positions 3860052 – 3859993

CAG CTT GCT GCT TTG CTG ACG AGT GGC GGA CGG GTG AGT AAT GTC

TGG **G**AA ACT GCC TGA

2. Query sequence, Positions 84 - 143

TGG AGG GGG ATA ACT ACT GGA AAC GGT AGC TAA TAC CGC ATA ACG

TCG CAA GAC CAA AGA

Subject sequence, Positions 3859992 – 3859933

TGG AGG GGG ATA ACT ACT GGA AAC GGT AGC TAA TAC CGC ATA ACG

TCG CAA GAC CAA AGA

3. Query sequence, Positions 144 - 203

GGG GGA CCT TCG GGC CTC TTG CCA TCG GAT GTG CCC AGA TGG GAT

TAG CTA GTA GGT GGG

Subject sequence, Positions 3859932 - 3859873

GGG GGA CCT TCG GGC CTC TTG CCA TCG GAT GTG CCC AGA TGG GAT TAG CTA GTA GGT GGG

4. Query sequence, Positions 204 - 263

GTA AAG GCT CAC CTA GGC GAC GAT CCC TAG CTG GTC TGA GAG GAT GAC CAG CCA CAC TGG

Subject sequence, Positions 3859872 - 3859813

GTA AAG GCT CAC CTA GGC GAC GAT CCC TAG CTG GTC TGA GAG GAT GAC CAG CCA CAC TGG

5. Query sequence, Positions 264 -323

AAC TGA GAC ACG GTC CAG ACT CCT ACG GGA GGC AGC AGT GGG GAA TAT TGC ACA ATG GGC

Subject sequence, Positions 3859812 - 3859753

AAC TGA GAC ACG GTC CAG ACT CCT ACG GGA GGC AGC AGT GGG GAA TAT TGC ACA ATG GGC

5.3.2. Comprehensive Overview of conventional identification and sequencing results.

Table 5.3: Comparison of Presumptive Identification and Sequencing

Culture no.	PRESUMPTIVE IDENTIFICATION/MORPHOLOGY	SEQUENCING
3	Oval shaped cells	*Bacillus anthracis* strain JH16
4	Round ended rods	*Bacillus pumilus*
6	Short filaments with discoid	*Micrococcus luteus*
10	Filaments with discoid shape	*Bacillus clausii* KSM-K16
12	*Type* 1863/ACA	*Aeromonas hydrophila*
13	Small rectangular cells	*Bosea minatitlanensis*
14	*Nostocoida limicola*	Unknown bacterium
15	Oval shaped cells in clusters	*Pseudomonas aeroginosa*
16	Slightly coiled rods in chains	*Bacillus thuringiensis*
19	Long rectangular cells	*Alcaligenes sp*.
19(2)	Long rectangular cells	*Agrobacterium tumefaciens* C58
20	Rod shaped cells	*Gordonia amarae*
21	Rectangular cells	*Klebsiella oxytoca* strain
24	*Type* 1863/ACA	*Bacillus cereus* strain
25	*Type* 1863/ACA	*Bacillus cereus* strain
26	Short filaments with curled ended rods	*Enterobacter sp*
27	Round ended rods	*Bacillus cereus*
28	Filaments with round ended rods	*Bacillus cereus*
30	Filaments with rectangular cells	*Bacillus cereus*
31	Rectangular cells	*Bacillaceae bacterium*
32	Smoothly curved rods in chains	Failed sequencing reaction
33	Smoothly curved rods in chains	*Bacillaceae bacterium*
34	Smoothly curved rods in chains	*Bacillaceae bacterium*
35	Smoothly curved rods in chains	*Bacillus sp*.
36	*Type* 1863/ACA. Straight and smoothly curved rods in chains	*Bacillus cereus*
37	Straight rods in chains	*Bacillus cereus*
38	Straight rods in chains	*Bacillus sp*.

5.3.3 Whole cell probing using species-specific probes.

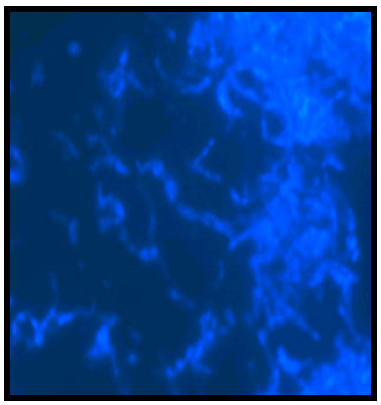

Figure 5.1: Photo-micrograph of DAPI for Culture 20

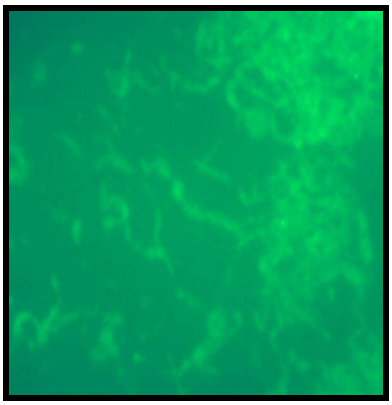

Figure 5.2: Photo-micrograph of GA for Culture 20

5.4 DISCUSSION

Conventional cultivation methods do not guarantee that isolated organisms will belong to the same genus or even species. A large number of morphologically different filamentous bacteria have been reported to be present in the activated sludge (Amann and Ludwig, 2000; Wilderer *et al*., 2002). Apart from these common filamentous bacteria, filamentous *Bacillus* species have been isolated from bulking sludge (Storm and Jenkins, 1984; Trick *et al*., 1984 as cited by Ajithkumar *et al*., 2001).

Phylogenetic analysis of ten in twenty six sequenced cultures belonged to the genus *Bacillus*. 16S rDNA sequencing revealed that culture 38 was a member of the genus Bacillus with no close relatives at the species level. This culture maintained a filamentous form when cultivated on solid medium and exhibited some unusual morphological traits. Spore stains were negative but gram reactions that were done at regular intervals on this culture revealed spore like cells that were present mostly during preservation at 4ºC. *Bacillus strain* NAF 001 at all stages of growth demonstrated a filamentous form as illustrated by Ajithkumar *et al*., (2001).

Bacillus is frequently found in activated sludge systems. Culture dependant methods of cultivation can be misleading because colonies that were isolated had to be slow-growing as it is believed that filamentous bacteria are slow growing but so are most *Bacillus sp* (Seviour and Blackall, 1999; Ajithkumar *et al*., 2001).

Culture 4 had considerable alignments with the nucleotide sequence of the bacterium *Bacillus pumilus*. *Bacillus pumilus* is a naturally occurring bacterium found in soil and water environments that is widely used in agriculture as an active ingredient as a fungicide in crops. *Bacillus pumilus* is a gram-positive rod shaped bacterium (USA, EPA, 2004).

Bacillus anthracis is a gram positive spore forming, rod shaped bacterium. It also belongs to the genus *Bacillus* and is a natural soil dwelling bacterium. Culture 3 (Refer to Table 5.3) was identified to be *B. anthracis*. *Bacillus anthracis* has as least 89 known strains with varying levels of virulence (Prescott *et al*., 1999).

Culture 10 showed close relation of >96% to *Bacillus clausii*, a gram positive alkaliphilic rod (Senesi *et al*., 2001). *Bacillus clausii* is a natural inhabitant of the soil and has received much attention in the pharmaceutical field. Various strains of *B. clausii* have been phylogenetically classified and marketed for application in oral bacteriotherapy.

The genus *Bosea* is phylogenetically placed in the alpha Proteobacteria subclass (Ouattara *et al*., 2003). It was initially described with one species *Bosea thiooxidans* from agricultural soils of free-living microorganisms (Outtara *et al*., 2003). *B. thiooxidans* was capable of oxidising reduced inorganic sulphur compounds. *Bosea minatitlanensis* (Culture 13) is a gram-negative bacterium that is strictly aerobic but was isolated from the anaerobic digester sludge. It has 97.3% similarity to 16S rRNA sequence of *Bosea*

thiooxidans. It is a motile, non-sporulating straight to curved rods with one polar flagellum (Outtara *et al*., 2003). Culture 13 had a significant similarity to *Bosea minatitlanensis* but motility was not observed with this isolate. It is unclear though whether it was motile or not when it was initially isolated.

Other traditional aerobic bacteria that were found genotypically identified include *Pseudomonas aeruginosa*, *Alcaligenes sp A72*, *Aeromonas hydrophila* and *Micrococcus luteus* (Table 5.3) that are all believed to be main bacterial components of the activated sludge (Pike, 1972 as cited Rossetti *et al*., 2006) (Refer to Table 5.3). They are all part of the floc-forming population. *Pseudomonas aeruginosa* and *Aeromonas hydrophila* (Culture 15 and 13 respectively) that was isolated from Biological Nutrient Removal (BNR) plant belong to the gamma subclass of Proteobacteria. It should also be mentioned that Culture 6 revealed significant similarity of (>98%) to *Micrococcus luteus* had a filamentous form throughout the study.

According to literature, plating of mixed liquor into nutrient rich media such as CGYA can lead to an overestimation of *Pseudomonas*, *Bacillus* and *Aeromonas* sp. (Wagner *et al*., 1994b; Kämpfer *et al*., 1996). This can be attributed to the non-fastidious nature of these organisms to culture media. *Pseudomonas sp*. has been found to have denitrifying and polyphosphate accumulating capabilities (Wagner *et al*., 1994b; Pike 1972 –as cited by Rossetti *et al*., 2006). A study by Brodisch and Joyner (1983) found *Pseudomonas sp*. and *Aeromonas sp*. to be dominant constituting 50% of total aerobic microbial population. Although this was not particularly the case in this study, but the isolation of

these species can be attributed to the fact that samples were taken in the aerobic reactor where these organisms are present in considerable numbers.

Comamonas testosteroni is a gram negative, aerobic rod that was previously referred to as *Pseudomonas testosteroni*. This bacterium has been isolated from activated sludge (Tabrez and Hiraishi, 2001). *C. testosteroni* is a denitrifying bacterium that is capable of degrading PHB (Tabrez and Hiraishi, 2001). A slight probability exists that some organisms that are believed to be in single cells can sometimes exist as filaments (Martins *et al*., 2004). This can be attributed to substrate limiting conditions that may persist in the activated sludge causing non-filamentous forms to exist in filamentous forms under stressful conditions when substrate limiting conditions persist (Wilderer *et al*., 2002; Ajithkumar *et al*., 2001).

Actinomycetes are a very large group of bacteria that grow as elongated cells and show some degree of true branching (Prescott *et al*., 1999). But it should be mentioned that branching may occur at some certain point growth cycle of certain *Bacillus* sp. (Ajithkumar *et al*., 2001). This further illustrates the fact that microscopic examination for presumptive identification is inadequate due to complexity that exists in the activated sludge system. *Actinomycetes* are also characterized by slow growth when grown on readily biodegradable sugars (Gerhardi and Frank, 1990). However, slow growth is characteristic of most filamentous bacteria isolated from activated sludge (Gerhardi and Frank, 1990).

The bacterial morphotype "*Nostocoida limicola*" is a coiled filament consisting of coccal or discoid cells as described by van Veen (1973) and further work subsequently split *Nostocoida limicola* into three morphotypes based on cell size (Eikelboom and van Buijsen, 1983 as cited by Jenkins *et al*., 1993). It has been concluded through 16S rRNA sequencing of pure cultures that these are phylogenetically quite different bacteria that share very similar morphology (Liu and Seviour, 2001; Seviour *et al*., 2002). The three morphotypes were shown to belong to *Actinobacteria* but recently they have been shown to belong to *Chloroflexi* and the alpha *Proteobacteria* as the members of the *Alisphaera* (Seviour *et al*., 2006). These findings by Seviour *et al*., (2002) and Seviour *et al*., (2006) demonstrate the discrepancy that exists when classification is based on phenetic classification. It raises concerns on whether bacteria that are indistinguishable microscopically should be classified as the same species.

Seviour *et al*., (1997) isolated *Type* 1863 filaments that were not members of *Acinetobacter* sp in order to explore the taxonomic diversity of this morphotype. Five bacteria identified microscopically as *Type* 1863 were isolated through micromanipulation. The findings presented clearly revealed that this morphotype does not describe one bacterial species but a collection of several phylogenetically unrelated organisms all sharing very similar morphological characteristics in activated sludge. These three genera are believed to be *Chryseobacterium*, *Acinetobacter* and *Moraxella* (Seviour *et al*., 1997). All these findings clearly illustrate the risks associated with the reliance on microscopic appearance to identify filamentous bacteria and how molecular techniques can revolutionize the phylogeny and nomenclature of filamentous bacteria.

DNA sequencing of Culture 20 (Refer to Table 5.3) revealed significant homology to *Gordonia amarae*. Sequence similarity was >98 %. 16S rDNA sequencing results were confirmed using a probe specific for *G. amarae*. Positive hybridization was observed as illustrated in Figure 5.1 and 5.2. *G. amarae* is an aerobic, gram-positive bacterium (Gerhardi and Frank, 1990; Kämpfer and Wagner, 2002). It is an *Actinomycete* and is mostly found in foaming sludges (Jenkins *et al*., 1993; Kämpfer and Wagner, 2002).

CHAPTER SIX

6.1 GENERAL DISCUSSION

The identification of filamentous bacteria by conventional taxonomy is based on several characteristics such as morphology, Gram reaction, presence or absence of cell inclusions and biochemical fingerprinting (Jenkins et al., 1993; Rossetti *et al*., 2006). The method of identification first proposed by Eikelboom for filamentous bacteria empirically sorted these bacteria. Morphological features such as cell size, shape, width and response to microscopic staining characteristics formed a dichotomous identification key that is still applicable today as a method of presumptive identification (Bitton, 1999; Wilderer *et al*., 2002).

However, it is generally accepted that morphology is strongly dependant on specific environmental conditions. Variations in temperature, pressure, pH, salinity and the concentration of nutrients have been shown in previous studies to initiate an intricate series of cellular events including changes in cellular morphology (Alonso *et al*., 2002 as cited by Ramothokang *et al*., 2006). For instance, many *S. natans* morphotypes can change morphology in response to environmental conditions (Ziegler *et al*., 1990) Seviour and Blackall, 1999).

The diversity of microbial species in an activated sludge plant is the result of the composition of influent wastewater (Seviour and Blackall, 1999). On the other hand, microscopic identification, for presumptive identification requires a well trained person otherwise wrong judgment can be made (Martins *et al*., 2004).

S. natans exists in filamentous state as bacillus in chains and *N. limicola* as filamentous cocci in chains. *Type* 1863 has been observed as coccobacilli in pairs and sometimes in chains and sometimes as single cells (Seviour *et al*., 1997; Kämpfer and Wagner, 2002). But the work presented here clearly illustrates that due to environmental conditions in the activated sludge process normal bacteria can resume filamentous form. Such discrepancies of conventional methods of isolation are insufficient as a method of pre – identification because all cultures that were isolated had exhibited cellular morphology that is typical of filamentous bacteria. Above all, when cultures were maintained on CGYA agar plates, depending on the stage of growth, different shapes were observed. After 2 days of cultivation at 25ºC, the culture was in single cells and after 72 hours it would be in filamentous form. This clearly illustrates that chances of false identification exist. Therefore, the use of morphological criteria for the classification of prokaryotes particularly filamentous bacteria is an unreliable method for the measure of relatedness because organisms that may look the same may not be genetically related (Ramothokang *et al*., 2006).

FISH using species-specific oligonucleotide probes was performed after DNA sequencing for culture 20 with homology to *Gordonia amarae* (Figures 5.1 and 5.2). This

served as a confirmation of DNA sequencing results. It should also be mentioned that due to geographic variations, some filamentous bacteria that are morphologically similar to known filaments may not hybridize to available specific probe as mutations may have occurred (Wilderer *et al*., 2002). *FISH* using a mixture of EUB mix probes was important in order to confirm the kingdom of the isolates (Amann *et al*., 1990; Daims *et al*., 1999).

Isolates that were isolated for this study were presumed to be filamentous bacteria. The screening method relied firmly on phenotypic characterization as per Eikelboom (1975). It has been documented in literature that true relatedness of bacteria can not be measured through morphological characterization as some bacteria may look morphologically alike but vary considerably in their physiology and taxonomy (Martins *et al*., 2004, Ramothokang *et al*., 2003). *Nostocoida limicola* can be divided into phylogenetically different bacteria that belong to low and high molecular percentage G+C gram positive bacteria (Liu and Seviour, 2001; Seviour *et al*., 2002), *Planctomycetes* and alpha subclass of *Proteobacteria* (Snaidr *et al*., 2002).

Such findings in literature (Lui and Seviour, 2001; Seviour *et al*., 2002; Snaidr *et al*., 2002) clearly illustrate that microscopic characterization is not just misleading but should form the basis of presumptive identification. Normal floc- forming bacteria like *Micrococcus* and *Bacillus* have the potential to accumulate polyphosphate in BNR systems (Atkinson, 1999). There have also been reports of *Bacillus* sp. that can accumulate PHB during sporulation (Ajithkumar *et al*., 2001). Therefore since bacteria

that are believed to be non-filamentous in nature, can also exhibit cellular and morphological traits of filamentous bacteria, it is therefore imperative that unknown isolates be sequenced. Sequencing can then be supported by morphological, cellular and physiological characteristics to aid in the phylogenetic characterization of unknown bacteria.

6.2 CONCLUSION AND RECOMMENDATIONS

6.2.1 CONCLUSION

The phenotypic classification of filamentous bacteria had been well established and in practice for some time. Therefore it had been common practice for researchers in the field to refer to Eikelboom classification keys when identifying filamentous bacteria in wastewater treatment processes. The latter was based primarily on morphological and staining characteristics. However, with the advent of novel molecular techniques and the application of phylogenetic approach, the systematics of filamentous identification and contribution in wastewater treatment has become highly fluid.

Therefore the current research attempted to bridge the gap and apply a phylogenetic approach to verify isolates of that had previously been characterized by the conventional methods. Although the using *FISH* and sequencing did not support previous identification, it could be regarded as making a valuable contribution to exposing the limitation of conventional identification method with respect to accurate identification of filamentous bacteria at a genetic level. The findings certainly question the taxonomic status of filamentous bacteria as per Eikelboom keys and has set the foundation for further research in the field, that could shift the paradigm of accurate filament identification and phylogenetic status in the Bergeys manual of Systematics Bacteriology.

In summary the findings of the current research showed:

- Using Eikelboom keys the current research verified the identification of the filaments from the culture collection.
- The probes that were used were synthesized and specific for the filaments that were identified as per Eikelboom keys. Unexpectedly, the designated species-specific probes that were used according to established *FISH* protocol did not support and verify any of the Eikelboom identified isolates.
- Ultimately, the sequencing results revealed that the majority of the isolates were non-filamentous Eubacteria belonging to Firmicutes, Actinobacteria, alpha, beta and gamma subclasses of Proteobacteria.
- Therefore these findings has set the foundation for further investigating and re-evaluating Eikelboom classification keys in the context of accurate phylogenetic classification.
- The findings also showed that morphological 'shifts' amongst filamentous bacteria need to be accorded and recognized when identifying filamentous bacteria.

6.2.2 RECOMMENDATIONS

As shown in the current research, using only conventional identification keys based on morphological characteristics has its limitations. Therefore the accurate classification of filamentous bacteria necessitated a holistic approach using both conventional (phenotypic) and molecular (phylogenetic) identification methods. However, pure culture studies are still important as physiological properties provide guidelines to the conditions that allow the proliferation of filamentous bacteria *in situ*.

It should be realized that Eikelboom's classification of filamentous bacteria did not align to taxonomic classification as per the Second edition of Bergeys Manual. It was rather based on morphological characteristics which was typical of the First edition of Bergeys Manual.

It is recommended not to completely disregard Eikelboom's method of presumptive identification. On confirmation of presumptive identification, micromanipulation should be the norm with respect to selective isolation of desired filaments from the mixed liquor The subsequent proposed order of analysis should be the filamentous bacterium then be grown axenically followed by DNA extraction and sequencing.

Probes can then be designed and synthesized targeting South African isolates in order to prevent non-hybridizations due to mismatches in conserved regions caused possibly by geographical differences.

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APPENDIX 1

R2A agar

Composition per litre

Agar	15.0g
Yeast Extract	0.5g
Acid hydrolysate of casein	0.5g
Glucose	0.5g
Soluble starch	0.5g
K_2 HPO $_4$	0.3g
Sodium pyruvate	0.3g
Pancreatic digest of animal tissue	0.25g
Peptic digest of animal tissue	0.25g
$MgSO_4$, anhydrous	0.024g

Preparation of medium

Add components to distilled /de-ionized water and bring volume to 1.0L .mix thoroughly. Gentle heat with mixing and bring to boiling Distribute into tubes or flasks. Autoclave for 15 minutes at psi pressure –121^{0}C. Do not over heat. Pour into sterile petri dishes (Atlas, 1993).

APPENDIX 2

Casitone Glycerol Yeast Autolysate Agar (CGYA)

Composition per litre

Bacto casitone (Difco)	5.0g
Yeast Aoutolysate	10.g
Glycerol	10.0ml
Agar	16.0g

Preparation of medium

Add components to distilled/de-ionized water and bring volume to 1.0L. Mix thoroughly. Gently heat with mixing and bring to boiling. Distribute into tubes or flasks. Autoclave for 15 minutes at 15psi pressure –121^0 C. Do not over heat. Pour into sterile petri dishes (Bridson, 1995).

APPENDIX 3

TYGA

Composition per liter:

Agar	15.0g
Pancreatic digest of casein	5.0g
Yeast Extract	3.0g
Glucose	1.0g
K_2HPO_4	1.0g

PH 7.0 ± 0.1 at 25°C

Preparation of medium

Add components to distilled/de-ionized water and bring volume to 1.0L. Mix thoroughly. Gently heat with mixing and bring to boiling. Distribute into tubes or flasks. Autoclave for 15 minutes at 15 psi pressure –121°C. Do not overheat. Pour into sterile petri dishes (Atlas, 1993).

APPENDIX 4

SCY medium

Components per litre	
Agar	10.0g
Sucrose	1.0g
Pancreatic digest of casein	0.92g
Yeast Extrast	0.25g
NaCl	0.05g
Papaic digest of soybean meal	0.03g
K_2HPO_4	0.025g
Thiamine	0.4g
Cyanocobalamin	0.01g

PH 7.3 ± 0.2 at 25°C

Preparation of medium:

Add components (except for the vitamins) to distilled/de-ionized water and bring volume to 1.0L. Mix thoroughly and autoclave at psi pressure –121°C for 15 minutes. Filter-sterilize the vitamins separately and then add aseptically to the cooled autoclaved agar. Pour into petri dishes and allow to set (Atlas, 1993).

APPENDIX 5

GRAM STAIN (modified Hucker method)

Reagents

Solution I

A. Crystal violet 2g
Ethanol, 95% 20 ml

B. Ammonium oxalate
Distilled water 80ml

The above (A and B) are prepared separately and then combined

Solution II:

Iodine	1g
Potassium Iodide	2g
Distilled water	300ml

Solution III:

Safranin 0(2.5% in 95% ethanol)	10ml
Distilled water	100ml

Method

Prepare a thin sample smears on microscope slides and allow to air dry. These slides are then stained 1 minute with solution 1 and then rinse with water for 1 second. Stain with solution 2 for 1 minute and then rinse well with water. Decolorize preparation for 25 second with 95% ethanol. Then stain slide for 1 minute with solution 3, rinse well and blot dry (Jenkins *et al*., 1993).

APPENDIX 6

NEISSER STAIN

Reagents

Solution 1:

A: Methylene Blue	0.1g	B: Crystal violet (10%w/v in	
Ethanol, 95%	5 ml	95% ethanol)	3.3 ml
Glacial acetic acid	5ml	Ethanol 95%	6.7 ml
Distilled water	100 ml	Distilled water	100 ml

The above (A and B are prepared separately and then stored). Two parts by volume of A are mixed with 1 part by volume of B. fresh stock to be prepared monthly.

Solution 2:

Bismark Brown (1%w/v aqueous)	33.3 ml
Distilled water	66.7 ml

Method:

Prepare thin sample smears on microscope slides and allow to air dry. Then stain slides for 30 seconds with solution 1 and then rinse with water for 1 second. Stain this preparation with solution 2 for 1 minute, rinse well with water and then blot dry.

APPENDIX 7

POLYHYDROXYBUTYRATE (PHB) STAIN

Reagents

Solution 1:

Sudan Black B (IV)	0.3% w/v in 60 % ethanol

Solution 2:

Safranin 0	0.5%w/v aqueous

Method:

Prepare thin sample smears on microscope slides and allow to air dry. Then satin slides for 10 minutes with solution 1 and the rinse well with water for 1 second. Stain this preparation with solution 2 for 10 seconds, rinse well with water and then blot dry (Jenkins *et al.,* 1993).

APPENDIX 8

SULPHUR STORAGE TEST

Solution 1:

Na2S solution

200 mg of Na_2S. 7 H_2O per 100 ml

Method:

Mix equal volume of activated sludge together with Solution 1. Shake and keep the sludge in suspension. Examine microscopically.

APPENDIX 9

CRYSTAL VIOLET SHEATH STAIN

Reagent

Crystal violet 0.1%w/v aqueous solution

Method:

Mix a wet preparation of the sample with 1drop crystal violet solution on a microscope slide and then cover with a cover slip.

Examine all of the above stains under oil immersion for filament characteristics

(Jenkins *et al.*, 1986).

APPENDIX 10

PRE-TREATMENT OF SLIDES

*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 hr.
2. Rinse with water and air dry.
3. Place clean slides in a 1: 10 diluted solution of Poly-P-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 11

CELL FIXATION

(Amann, 1995 modified by De Los Reyes, 1997)

Solutions

- 1 x Phosphate Buffered saline (PBS) pH 7.2

  10 ml 10X PBS

  90 ml sterile deionized water

- 3 x Phosphate Buffered Saline (PBS) pH7.2

  30 ml 10X PBS

  70 ml sterile deionized water

- 4% Paraformaldehyde

- 98% Ethanol

- 99,99% Ethanol

Preparation of paraformaldehyde Fixative

Preparation of 4% paraformaldehyde:

- heat 33 ml of deionized water to 60-65^{o}C
- add 2g paraformaldehyde while stirring
- add 2M NaOH drop-wise until paraformaldehyde is dissolved completely
- add 16.5ml 3 x PBS buffer
- adjust the pH to 7.2 - 7.4 at 20^{o}C
- filter through 0.45µm filter
- use within 24hrs, store in ice until use

Fixation:

- harvest cells by centrifugation(3500rpm, 4min) and discard the supernatant.
- wash the pellet with 1 x PBS, centrifuge again and resuspend pellet in an appropriate volume of fresh 1 x PBS
- add 3 volumes of fresh paraformaldehyde
- incubate for 3hrs or overnight at 4^{o}C
- spin down the cells (5mins)
- discard supernatant
- wash the pellet with 1 x PBS, centrifuge again
- resuspend the pellet in fresh 1 x PBS
- add 1 volume of ice-cold $EtOH_{abs}$
- store at -20^{o}C

Ethanol Fixative for gram positive bacteria

Paraformaldehyde fixative is most suitable for gram negative cells, gram positive cells are fixed ethanol only.

1. Harvest cells by centrifugation.
2. Wash the pellet in 1 X PBS, and centrifuge.
3. Resuspend the pellet in an appropriate volume of fresh 1 X PBS.
4. Add one volume of ice-cold $EtOH_{abs}$.
5. Store at -20^0C.

APPENDIX 12

IMMOBILISATION OF CELLS (Amann, 1995)

1. Prepare specimen on a Teflon coated slide: spot fixed cells (2-15 µl, 10 µl more ideal).
2. Spread the sample evenly in the well and dry for about 10 mins at 46^0C.
3. Dehydrate the cells by successive passages through increasing alcohol series 50, 80 and 99.99% ethanol washes for 3 minutes each.
4. Allow to air dry. The slides can be stored at room temperature.

APPENDIX 13

WHOLE CELL HYBRIDISATION (Amann, 1995)

Materials

- 50 ml Polypropylene screw top tube
- Whatman 3MM paper
- Hybridisation buffer pH 7.2

  5 M NaCl 360 µl

  1 M Tris HCl 40 µl

Add formamide and ultra pure water depending on applied stringency

Preparation of hybridisation buffer for in situ *hybridisation at 46^{o}C*

Pipette into a 2mL Eppendorf reaction tube:

- ✓ 5 M NaCl 360µl
- ✓ 1 M Tris HCl pH 8.0 40µl
- ✓ add formamide and MQ (ultra pure water), depending on the applied stringency:

%formamide(v/v)	Formamide[µl]	MQ [µl]
0	0	1.600
5	100	1.500
10	200	1.400
15	300	1.300
20	400	1.200
25	500	1.100
30	600	1.000
35	700	0.900
40	800	0.800
45	900	0.700
50	1000	0.600
60	1100	0.500
65	1200	0.400
70	1300	0.300

- ✓ 10% (w/v) SDS 4µl

(Add last; add onto lid of the tube and don't mix too much as the surfactant (SDS) will form bubbles.

- Thaw the oligonucleotide probes
- Drop 9µl of hybridization buffer onto the wells (*cover the well without touching the surface of the wells; change tips each time)*
- Add 1µl of each probe (working solution, concentration 50ng/µl for FLOUS labeled probes) without scratching the Teflon-coated surface (Mix the 1 µl probe with 9 µl of the hybridization buffer. Foe EUB, add 1 µl of each probe to 7 µl of the hybridization buffer.
- Prepare a hybridization tube (50mL sterile falcon tube) by folding a piece of tissue or Whatmann 3MM paper, put it into the tube and pour the rest of the hybridization buffer onto the tissue.
- Immediately transfer the slide into the hybridization tube (chamber) and incubate in the hybridization oven (46^{o}C) for 3 hours.

  - ✓ *Protect the slide in chamber with foil from hybridization oven*
  - ✓ *Transfer into washing buffer very quickly*

- Prepare the washing buffer and preheat this buffer at 48^{o}C in a water bath.

Preparation of washing buffer for in situ *hybridisation at 48°C*

Mix in a 50mL Falcon tube:

- ✓ 1mL of 1M Tris/HCl pH 8.0
- ✓ 5M NaCl and 0.5M EDTA pH 8.0 according to the following table:

%Formamide in hybridization buffer	**[NaCl] in mol/L**	**NaCl [µl]**
0	0.900	9.000
5	0.636	6.300
10	0.450	4.500
15	0.318	3.180
20	0.225	2.150
25	0.159	1.490
30	0.112	1.020
35	0.080	0.700
40	0.056	0.460
45	0.040	0.300
50	0.028	0.180
55	0.020	0.100
60	0.008	0.400
70	0.000	No NaCl, only 350µl EDTA

- ✓ for formamide concentrations of 20% and higher in the hybridization buffer add 500µL *(not critical though)* of 0.5M EDTA (for stabilization of probe)
- ✓ 50µL of 10% (w/v) SDS
- ✓ preheat the washing buffer at 48°C prior to use

- Rinse the hybridization buffer with the washing buffer from the slide and incubate the slide in the washing buffer for 10mins in a 48^{o}C preheated water bath
- Remove the washing buffer with distilled water without detaching the cells and dry the slide quickly with compressed air
- Embed the slide with embedding liquid *and* put a cover slip onto the slide.

APPENDIX 14

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

1. Spread 10 µl of DAPI (0.25 µg/ml) on each well and allow to stain for 5 minutes.
2. Wash the slides with 1 X PBS and allow to air dry.
3. Mount the slide with Vectashield Mounting Media (Vector Laboratories, Burlingame) and cover with a cover slide.
4. View immediately.

APPENDIX 15

DNA EXTRACTION PROTOCOL

DNA was extracted using QIAamp stool Mini kit (Southern Cross Biotechnology, Pty Ltd) as per following protocol:

1. A volume of 500 µl of bacterial culture was pippetted into a microcentrifuge tube and placed the tube on ice.
2. A volume of 1.4 ml Buffer ASL was added. Vortexed continuously for 1 min or until the culture is completely homogenized.
3. The suspension was then heated at 70^0 C (gram negative bacteria) or 90^0 C (gram positive) for 5 minutes.
4. Vortexed for 15 seconds and the sample was centrifuged at 10.3 X 10^3 rpm for 1 minute.
5. A volume of 1.2 ml of the supernatant was pipetted into a new 2 ml microcentrifuge tube and the pellet discarded.
6. One inhibitEX tablet was added 1 to each sample and vortexed immediately and continuously for 1 minute or until the tablet is completely suspended. The suspension was incubated for 1 minute at room temperature to allow inhibitors to allow inhibitors to adsorb to the InhibitEX matrix.
7. The sample was centrifuged at 10.3 X 10^3 rpm for 3 minutes to pellet the inhibitors bound to InhibitEX.
8. The entire supernatant was pipetted into a new microcentrifuge tube and the pellet discarded. Centrifuge sample at 10.3 X 10^3 rpm for 3 minutes.
9. Fifteen microlitres Proteinase K was pipetted into a new microcentrifuge tube.

10. A volume of 200 µl Supernatant from step 8 was introduced into the new microcentrifuge tube containing Proteinase K.
11. A volume of 200 µl Buffer AL was added and vortexed for 15 seconds.
12. The mixture was then incubated at 70^0 C for 10 minutes.
13. A volume of 200 µl Ethanol (96-100%) was added to the lysate, and mixed by vortexing.
14. The complete lysate from step 13 was carefully introduced to the QIAamp spin column without moistening the rim. The cap was closed and centrifuged at 10.3 X *g* rpm for 1 minute. The QIAamp spin column was placed in a new collection tube and the tube containing the filtrate was discarded.
15. The QIAamp spin column was carefully opened and 500 µl Buffer AW 1 was added. The sample was centrifuged at 10.3 X *g* rpm for 1 minute. The QIAamp spin column was placed in a new collection tube and the collection tube containing the filtrate discarded.
16. The QIAamp spin column was carefully opened and 500 µl Buffer AW 2 added. The sample was centrifuged at 10.3 X *g* rpm for 3 minutes. The collection tube containing the filtrate discarded.
17. The QIAamp spin column was transferred into a new, labeled microcentrifuge tube and 200 µl Buffer AE added directly onto the QIAamp membrane. Incubated for 1 minute at room temperature, then centrifuged at 10.3 X *g* rpm for 1 minute to elute the DNA.
18. The eluted DNA was then stored at -20 C.

APPENDIX 16

AGAROSE GEL ELECTROPHORESIS

Solutions

- 50x Tris Acetate Buffer (TAE) (1L)

  242g Tris Base

  57.1 ml Glacial Acetic Acid

  37.2g EDTA

  Add sterile distilled water to 1l.

- Gel loading buffer

  0.25% Bromophenol Blue

  0.25% Xylene Cyanol

  30% Glycerol

- Electrophoresis buffer

  275 ml 1x TAE

Agarose gel

1. 0.8g Agarose powder.
2. 50ml 1xTAE buffer.
3. Microwave for 60 seconds or until solution becomes clear.
4. Cool and add 1µl Ethidium bromide.
5. Mix gently.
6. Pour into a 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 electrophoresis submarine.
9. Add the electrophoresis buffer. Ensure the gel is completely covered with buffer.

Sample Loading

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

- Set the voltage at 80 V and time (45 min) and allow the gel to run.

APPENDIX 17

DENATURING GRADIENT GEL ELECTROPHORESIS

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

Solutions

- 40% Bis-Acrylamide

  38g Acrylamide

  2g Bis- Acrylamide

  Add distilled water to 100ml.

  Filter though Whatman Filter No.1 and store at $4^{o}C$.

- 30% Low density Denaturing Solution (DNS)

  18.8 ml 40% Bis-Acrylamide

  2.0 ml 50x TAE buffer

  12.0 ml Formamide

  12.6g Urea

  Distilled water to 100ml

- 60% High Density Denaturing Solution (DNS)

  18.8 ml 40% Bis-Acrylamide

  2.0 ml 50x TAE buffer

  24.0 ml* Formamide

  25.2g* Urea

Distilled water to 100ml

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

- 10% Ammonium Persulphate

  0.1 g Ammonium Persulphate

  Add distilled water to make up 1.0ml. Solution should be fresh for every use.

- 50x Tris Acetate Buffer (TAE) (1l)

  242 g Tris Base

  57.1 ml Acetic Acid Glacial

  37.2 g EDTA

  Distilled water to 1l

- D Gene Dye Solution

  0.05g Bromophenol Blue

  0.05g Xylene Cynol

  1x TAE to 10.0 ml

  Store at room temperature

- TEMED (Bio-Rad, South Africa)

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

Sample Loading

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

Electrophoresis Buffer

140ml 50 x TAE buffer
6860 ml distilled water

Pre-heat the electrophoresis buffer to 65^{o}C prior to the addition of gel core to the electrophoresis tank. Add 350ml 1x Tae buffer chamber once the core is added and the samples are loaded.