

**PHYSIOLOGICAL AND BIOCHEMICAL EVALUATION
OF PURE CULTURES OF PROBLEMATIC
FILAMENTOUS BACTERIA ISOLATED FROM
ACTIVATED SLUDGE**

TSHIRELETSO R RAMOTHOKANG

January 2004

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TSHIRELETSO R RAMOTHOKANG

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

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TSHIRELETSO R RAMOTHOKANG

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

TSHIRELETSO R RAMOTHOKANG

I hereby approve the final submission of the following dissertation.

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This 2 day of FEB., 2004, at Durban Institute of Technology,
Durban.

DEDICATION

*The work presented here is dedicated to my Mother, Ntloheleng Ramothokang and my
Brother, Tlholohelo Lesetla.....*

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Greatest and sincere thanks and acknowledgements I send to God, for his unfailing love, from whom my strength I draw and my courage I get. Thank you Lord for choosing me to deliver this work!

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PREFACE

Of the work presented in this dissertation various aspects have and will be published and presented elsewhere:

Publications

Ramothokang, TR, Drysdale, GD and Bux, F. (2003). Isolation and Cultivation of Filamentous Bacteria Implicated in Activated Sludge Bulking. *Wat. SA*, Vol. 29, No. 4, pp 405-410.

Ramothokang, TR, Drysdale, GD and Bux, F. *Title*: Evaluation of the Metabolism of Selected Filamentous Bacteria Associated with Activated Sludge Bulking. *In preparation for Water SA*.

Conferences: Oral Presentations

Ramothokang, TR, Drysdale, GD and Bux, F. (2002). Isolation of Problematic Filamentous Bacteria Implicated in Bulking and Foaming During Wastewater Treatment. South African Society for Microbiology. KZN Regional Symposium, Durban Institute of Technology, Durban.

Ramothokang, TR, Drysdale, GD and Bux, F. *Title: Biochemical And Physiological Fingerprinting Of Pure Cultures Of Filamentous Bacteria Isolated From Wastewater Treatment Plants In Durban, South Africa. Submitted to International Water Association (November, 2003).*

EXECUTIVE SUMMARY

Since the development of the activated sludge process, bulking and foaming have been a major problem affecting treatment efficiency. Filamentous bacteria have long been known to be the primary cause of bulking and foaming problems in activated sludge wastewater treatment systems. Attempts to cure filamentous bulking and foaming have thus far not shown great success in effective long-term control measures due to a lack of understanding of these organisms. Chemical methods such as chlorination and the use of hydrogen peroxide are still used to cure bulking but are only effective as interim measures. This could be due to the main factors stimulating filamentous bacterial growth not being changed by these methods for curing bulking and also, a lack of in-depth understanding of filamentous bacteria by scientists. It is therefore important to gain a proper understanding of these bacteria on the basis of their physiological, biochemical and growth characteristics. For all this to be successfully attained, filamentous bacteria need to be studied in pure culture so as to facilitate a better understanding of bulking and foaming and the control thereof during wastewater treatment.

The aim of this study was therefore, to isolate and cultivate problematic filamentous bacteria and determine the physiological, biochemical and morphological traits of these organisms in pure culture, with the purpose of being able to integrate these findings to *in situ* analysis.

Using four different isolation techniques, a total of 14 isolates from 7 different wastewater systems were obtained and evaluated for a range of physical, chemical, redox and substrate conditions. Results of the study indicate that filamentous survival and proliferation in BNR systems is largely due to varied phosphate uptake capacities and widespread ability to denitrify both nitrate and nitrite. Lipid hydrolysis is also a major component of filamentous bacterial metabolism with hydrolysis of other large compounds, as revealed by Biolog, such as starch, dextrin, proteins/peptides, Tween 40, Tween 80 and nucleosides indicating an affinity for larger slowly biodegradable substrates. They also thrive on a variety of amino acids and sugars.

The results obtained in this study revealed that filamentous bacteria are more diverse and complex in their biochemistry and physiology hence the difficulty in achieving long-term optimal control of filamentous bulking in activated sludge. It was concluded that filamentous bacteria have the ability to survive during times of starvation where growth factors are limiting and, this may be attributed to their ability to store storage compounds such as PHB, glycogen and polyP. The filaments' ability to store storage compounds and denitrify, suggests that they may in fact play significant roles in denitrification and EBPR. It is also concluded from this study that the filamentous bacteria under study are sensitive to aromatic compounds and that they have an affinity for slowly biodegradable polymers such as lipids, nucleosides, proteins/ peptides, dextrin and starch. Also concluded is that, the use of Biolog for biochemical profiling/ fingerprinting of filamentous bacteria is useful, however, due to the possibility that some organisms may in fact, not grow and/ or may give negative results on some and/ or all substrates, other

strategies for biochemical profiling be established and used in this regard. Identification and evaluation of filamentous bacteria based on morphological traits is limiting and requires development and optimization of *in situ* techniques, such as DNA/RNA based probes and microautoradiography.

Bulking and BNR are elaborate and still not fully understood. The filaments' ability to take up phosphates and denitrify means that an advanced understanding of the roles they play in BNR systems and AA- bulking (Anoxic- Aerobic) is still required. Physiological and biochemical fingerprinting of pure cultures of filamentous bacteria is an important basis to understanding these organisms, and establishing potential bulking and foaming criteria for *in situ* evaluation and verification. It is from a study such as this that the main goal of curing bulking and gaining an enhanced understanding of BNR may be achieved.

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CHAPTER 1

INTRODUCTION

1.1 THE ACTIVATED SLUDGE PROCESS

The activated sludge process has found immense application as an effective means of biological wastewater treatment in the developed world, treating both domestic and industrial wastewaters (Gray, 1990). Due to public concern about environmental protection, and its (the public's) increasing exposure to pollution and subsequent consequences, an extensive re-evaluation of existing wastewater management concepts has come about (Orhan and Artan, 1994). A need for the consideration of new substrate and biomass components also arose so as to gain a deeper understanding of measures such as BOD, COD, VSS, nutrients, priority pollutants, inert organics, etc., and also the development of advanced processes for their removal (Orhan and Artan, 1994). It is also necessary to acquire a much better understanding of complex biochemical processes such as nitrification-denitrification and biological phosphorous removal as they affect the performance of more thorough wastewater treatment systems (Orhan and Artan, 1994).

The objective of the activated sludge process is to remove soluble and insoluble organics from the wastewater and to convert this material into a flocculent microbial suspension that settles well in a conventional gravity clarifier. As a general rule, the nature of the

wastewater will dictate the preferred process modifications, primarily for the purpose of maintaining mixed liquor settling quality (Eckenfelder and Musterman, 1995). Basically, activated sludge comprises of a microbiological enrichment culture consisting of a mixed, and largely uncontrolled, consortium of micro- and macro-organisms that remove wastewater inorganics and organics and transform them into environmentally acceptable forms (Richard, 1989). The major concern is therefore the development and maintenance of suitable microbial populations or cultures (Schroeder, 1997). Microorganisms also provide the functional basis for sludge settling, biological nitrification-denitrification and biological phosphorus removal.

1.2 ACTIVATED SLUDGE ORGANISMS

The activated sludge process has a large population of both micro- and macroorganisms, and, these include protozoa, metazoa and bacteria. The protozoa (e.g. Stalked ciliates, *Vorticella* spp., *Epistylis* spp. and *Opercularia* spp.) and metazoa (e.g. Rotifers) are vital because, amongst other things, they feed on pathogenic bacteria and other protozoa, and also serve as indicator organisms in the process; giving an indication of the state of health of the activated sludge treatment process. These organisms now provide a food source for holozoic protozoa and a food chain has been initiated (Horan, 1990).

As these protozoa and metazoa (higher activated sludge organisms) feed on the bacteria, they keep the bacterial population in a steady and healthy state. Depending on which of the higher organisms are present in abundance in the system, one could use them to determine the state of health of the activated sludge system. If there are any perturbations to the system, such as a sudden increase or decrease in the influent BOD concentration, or sudden change in sludge age caused by excessive solids wasting, the food chain will adjust itself to the new conditions (Horan, 1990). Because they are large in size, protozoa may be identified easily by inspection under a suitable light microscope (Horan, 1990). Bacteria are, however, the most versatile of all the organisms associated with wastewater treatment in terms of the conditions under which they can thrive and the substrates they can metabolize.

It is with no doubt that it is the bacteria that are the most important group of organisms in biological treatment systems (Gray, 1989). Amongst bacteria found in the activated sludge process, there are filamentous bacteria as well. These bacteria are of interest due to the role they play in wastewater treatment and their implication in activated sludge bulking and foaming. With unconstrained food and oxygen, extremely high rates of microbial growth and respiration can be achieved, resulting in the utilization of the organic matter present either as oxidized end product (CO_2 , NO_3 , SO_4 and PO_4) or the biosynthesis of new microorganisms (Gray, 1990).

Some studies on the physiology of filamentous bacteria have been published (e.g. van Veen *et al.*, 1973, Slikhuis, 1983 and Lemmer and Kroppenstedt, 1984 as cited by Kämpfer *et al.*, 1995) but, because the isolation of filamentous bacteria as a necessary requirement for the investigation of nutritional and growth parameters is still difficult, only limited information is available (Ziegler *et al.*, 1990) and, systematic approaches to the nutritional requirements of different filamentous organisms are therefore scarce (Kämpfer *et al.*, 1995).

1.3 AIMS AND OBJECTIVES

The aim of this research was therefore, to assess different techniques for efficient isolation of monocultures of filamentous bacteria from activated sludge and, to obtain, maintain and study the filamentous bacterial monocultures on the basis of their physiology and biochemistry. The objectives of the study were: *i)* to screen different plants in the Durban and surrounding areas for filament identification and sample selection, *ii)* to assess the use of various procedures for activated sludge floc break-up during sample pretreatment, i.e. Nonidet, cellulase, sonication and bead beating. *iii)* to investigate optimal conditions for culture maintenance and storage and *iv)* to investigate possible variations in individual filamentous bacterial physiology- by growing them at varying temperature and pH conditions- and biochemistry by growing them on different substrates for the purpose of biochemical profiling/ fingerprinting of filamentous isolates.

CHAPTER 2

REVIEW OF RELATED LITERATURE

2.1 FILAMENTOUS BACTERIA

Bacteria are of main or primary interest because a large variety of them are pathogenic and also because they can be used to remove organic material and some unwanted minerals from wastewaters (Schroeder, 1997). It is important to maintain the growth of floc-forming bacteria on wastewater organics that will settle under gravity in the final clarifier so as to obtain or sustain a clarified supernatant (final effluent) and a thickened return sludge. Not all bacteria that develop in the activated sludge process are floc-formers. Many different types of filamentous bacteria have been acknowledged as well. These filamentous bacteria play important roles in wastewater treatment (Richard, 1989).

The importance of filamentous bacteria is basically twofold:

- They have the potential to interfere with separation and compaction of the activated sludge leading to bulking and foaming problems
- They also affect the sludge volume index (SVI) (Gerardi, 1990)

The sludge volume tells something about the flocculation and settling characteristics of the activated sludge (Henze *et al*, 1997). Filamentous bacteria will therefore affect settling because, they make provision for the rigid support network or backbone upon which floc-

forming bacteria can adhere and grow into suitable activated sludge flocs (Gerardi, 1990). While these filamentous bacteria may be considered detrimental to wastewater treatment when they occur in excessive quantities, leading to activated sludge bulking and foaming, they are just as important in the development of an activated sludge floc with proper settling and clarification properties.

Due to the filamentous macrostructure allowing for branching and irregular shape of the floc, the floc is permitted to filter out particulate matter in the wastewater. The result is inter-floc bridging which is the actual action that greatly improves clarifier efficiency and provides for a clear supernatant. Less adequate quantities of filamentous microorganisms result in the formation of pin-point flocs (Gerardi, 1990). Pin-point flocs are due to macrostructure failure i.e. no filamentous backbone or when there is very few filamentous organisms present (Jenkins *et al*, 1986). These pin-point flocs are sheared (broken off under pressure) in the turbulent environment of the aeration basin, resulting in a turbid effluent (Gerardi, 1990).

The growth of filamentous microorganisms or total extended filament length (TEFL), has been found to relate to SVI. SVI is the reciprocal sludge concentration in the sludge phase after 0.5 hour's settling in a glass cylinder. The lower the SVI is, the better the flocculation and settling characteristics (Gerardi, 1990). The relationship between SVI and filamentous microorganisms can be of use as a general indicator of the filamentous condition of the activated sludge. Because the SVI depends on the individual treatment

plant, as well as other operating criteria, the critical SVI value at which the plant's operation is affected depends or may vary from plant to plant (Gerardi, 1990).

Filamentous microorganisms are also an indication of the conditions prevailing in the activated sludge system on the microbiological level.

The indications given by the filamentous bacteria could be of:

- *Low D.O. (Dissolved oxygen):*

This condition can cause the growth of several filamentous organism types in activated sludge. *S. natans* and type 1701 are certainly associated with low DO bulking (Jenkins *et al*, 1986).

- *Low F/M (food-to-microorganism) ratio:*

The specific cause(s) for the growth of the filamentous organisms that appear in continuously- fed, completely mixed, low F/M activated sludge systems are poorly understood (Jenkins *et al*, 1986).

- *Presence of septic waste:*

The presence in abundance of filamentous bacteria such as type 021N and *Thiothrix* sp. is normally an indication of septic waste (Jenkins *et al*, 1986).

- *Nutrient deficiency:*

This usually means deficiency of nitrogen and/or phosphorus. Some workers have reported deficiencies of 'trace' nutrients such as iron (Wood and Tchanoglous, 1974 and Carter and McKinney, 1973 as cited by Jenkins *et al*, 1986)

- *Low pH in the system:*

When the pH is low, fungal proliferation takes place (Jenkins *et al*, 1986).

2.2 BULKING AND FOAMING

2.2.1 Bulking

Since the introduction of continuous-flow reactors, sludge bulking has been one of the major problems affecting biological waste treatment (Slykes, 1989 as cited by Bitton, 1999). As previously described, poor settlement of activated sludge flocs adversely affects the operation of sewage purification plants; a voluminous sludge is hard to control. The primary reason for the poor settling of properties (bulking) of activated sludge is the presence of filamentous microorganisms, either free-floating in the mixed liquor or protruding from the flocs (Pipes, 1967 as cited by Eikelboom, 1975). A study conducted by Seviour *et al.* (1994) confirmed that bulking and foaming are common and occur in plants with a wide range of operational characteristics. It has been pointed out by other such authors as Williams and Unz (1989, as cited by Kämpfer *et al.*, 1995) that understanding the complexity of factors responsible for filamentous bulking in activated sludge begins with a comprehensive knowledge of the growth requirements of the filamentous bacteria.

A bulking sludge may be defined as one that settles and compacts slowly due to filamentous organisms dominating the floc-formers within the mixed liquor. They go far beyond the boundaries of the floc in to the bulk liquid of the mixed liquor, which results in an interference with the compaction of the floc, which in return produces sludge with a poor settling rate (Richard, 1989). Attempts to control bulking have been rather confused due to the failure to identify the causative organisms (Eikelboom, 1975). The first authors dealing with bulking attributed the poor settling properties of the sludge to the presence of the sheathed bacterium *Sphaerotilus natans* (Arden and Lockett, 1923, Buswell and Long, 1923, Martin, 1927, Kolkwitz, 1928, Morgan and Beck, 1928 and Agersborg and Hatfield, 1929 as cited by Eikelboom, 1975). Since then, various types of filamentous organisms, either dominating or growing in combination with *Sphaerotilus natans*, have incidentally been recorded (Eikelboom, 1975). Two toxicants, chlorine and hydrogen peroxide, have been used to selectively kill filamentous organisms and therefore eliminate symptoms of activated sludge bulking (Jenkins *et al*, 1986). Chlorination is however, a last resort in the control of bulking. It should only be contemplated when effluent from the bulking plant is likely to cause environmental damage to the receiving water; if used correctly it provides a rapid alleviation of bulking problems, but, if used incorrectly, it may completely inhibit all treatment (Horan, 1990).

2.2.2 Foaming

Besides sludge bulking, biological wastewater treatment performance is also disturbed by scum production, which, cannot be destroyed by water trickling as in the case of detergent foams. The phenomenon of scumming usually coincides with abundant actinomycete growth. Their long branched hyphae link together the sludge flocs enclosing gas bubbles, leading to buoyancy (Lemmer and Kroppenstedt, 1984). Actinomycetes were isolated from 14 sewage treatment plants suffering from scum production in the aeration tank in southern Germany, Berlin and Switzerland by Lemmer and Kroppenstedt (1984). Some of the strains isolated by Lemmer and Kroppenstedt (1984) belonged to the *Rhodococcus rhodocrous* group, some to the *Gordona aurantiaca* group and 1 strain was identified as *Nocardia amarae*. The formation of foams or scums on the surface of activated sludge aeration basins and secondary clarifiers has been ascribed to a variety of causes. A white, frothy foam is observed during the start-up of activated sludge plants, possibly due to the presence of undergraded surface-active organic matter in the aeration basin. A viscous, stable and often chocolate-colored foam or scum has been noted widely on activated sludge aeration basins and secondary clarifiers (Anon, 1969, Wells and Garrett, 1971, Lechevalier and Lechevalier, 1975, Pipes, 1978 and Dhaliwal, 1979 as cited by Jenkins *et al*, 1986). The occurrence of this foam has been associated with the presence of large numbers of bacteria of the genus *Nocardia* (Jenkins *et al*, 1986).

2.3 BIOLOGICAL NUTRIENT REMOVAL (BNR)

The biological nitrogen and phosphorus removal system has been intensely studied and applied for the treatment of municipal wastewater (Daigger and Polson, 1991, Wanner *et al.*, 1992, Cooper *et al.*, 1994 and Bortone *et al.*, 1996 as cited by Chuang and Ouyang, 2000). Nutrient removal reactions in activated sludge processes require appropriate chemical, biological and biochemical conditions which are facilitated by aerobic, anoxic and anaerobic conditions (Demoulin *et al.*, 1997). These conditions allow for microorganisms to respectively exhibit their various characteristic tendencies such as storage of polyhydroxyalkanoate (PHA) or polyphosphate (polyP) cleavage, denitrification and nitrification or phosphate uptake. For this reason, wastewater treatment systems comprise a more complex community of microorganisms (Bond *et al.*, 1995 and Wanner, 1997 as cited by Chuang and Ouyang, 2000).

BNR processes are currently being designed and implemented worldwide using established mathematical models and related software. These BNR processes refer to nitrification/ denitrification/ biological excess phosphorus removal processes (Gujer and Kappler, 1992 as cited by Drysdale *et al.*, 1999). While the activated sludge process was originally designed as a single aerobic reactor for the removal of organic matter from wastewater (Lu and Leslie Grady (Jr), 1988; Gray, 1990; Ekama *et al.*, 1992 and Wentzel *et al.*, 1992 as cited by Drysdale *et al.*, 1999), it has, thus far, been improved to multi-reactor processes consisting of anaerobic, anoxic and aerobic zones with inter-reactor recycles thus allowing the process to gradually include nitrification, denitrification and

phosphorus removal (Wentzel *et al.*, 1992 as cited by Drysdale *et al.*, 1999). Very precise information is provided by these models on the basis of process design and performance and, can result in the development of effective BNR processes. However activated sludge models go amiss in that they do not take into account the biomass structure present in the process (Henze, 1992 and Kristensen *et al.*, 1992 as cited by Drysdale *et al.*, 1999). Effluent quality in the nutrient removal system is directly affected by the complexity of the microorganism population in wastewater treatment which, has been rendered important in the operation and control of a biological nitrogen and phosphorus removal process (Jenkins and Tandoi, 1991 and Liu *et al.*, 1997 as cited by Chuang and Ouyang, 2000).

2.3.1 Denitrification

When untreated, discharged $\text{NH}_4\text{-N}$ may aggravate algal growth, deplete dissolved oxygen (DO) and have toxic effects on aquatic life (Bae *et al.*, 1997). $\text{NH}_4\text{-N}$ may be removed chemically/ physically or biologically. However, chemical/ physical processes have disadvantages such as odour, air pollution, high chemical cost, and excess sludge production (Ehrig, 1989, Bae *et al.*, 1995 and Hwang, 1995 as cited by Bae *et al.*, 1997). A sequence of aerobic and anoxic processes provides for conventional biological removal of nitrogen in activated sludge processes. Simultaneous denitrification results in carbon dioxide (CO_2) production, and the remaining available DO provides nitrifying conditions

(Münch *et al.*, 1996, Isaacs *et al.*, 1994 and Isaacs and Henze, 1995 as cited by Fuerhacker *et al.*, 2000).

The exposure of humans to nitrates is a subject of great concern. Health agencies have issued reports that, domestic water that is safe to use, should contain no more than 10 mgN/L (equivalent to 45 mg/L as NO₃) (Fraser *et al.*, 1980 and Fan *et al.*, 1981 as cited by Terblanche, 1991). Nitrates, together with nitrites, are known to hinder phosphorus removal during activated sludge treatment (Gruenebaum and Dorgeloh, 1992 and Kuba *et al.*, 1996 as cited by Drysdale *et al.*, 2001). Also, denitrification by heterotrophic bacteria in activated sludge treatment is of particular interest in that nitrates and nitrites are eutrophic (Gray, 1990 as cited by Drysdale *et al.*, 1999).

Nitrogen removal by biological means has been applied for domestic wastewater since the research by Wuhrmann (1964 as cited by Bae *et al.*, 1997) and, is now applied for wastewater with high ammonia concentrations (Glas *et al.*, 1993, Clifford and Liu, 1993, Imai *et al.*, 1993, Robinson *et al.*, 1995, Abeling and Seyfried, 1992 and Mahne *et al.*, 1996 as cited by Bae *et al.*, 1997). Two stages make feasible the biological removal of nitrogen from wastewaters, i.e. **a) nitrification**: the bacterial oxidation ammonia to nitrite and then nitrate and **b) denitrification**: the microbiological reduction of the nitrite/ nitrate to gaseous nitrogen compounds (Hippen *et al.*, 1997 and Cole, 1994 as cited by Barber and Stuckey, 2000). Denitrification may therefore be defined as a process whereby nitrogen oxyanions- nitrates, nitrites, nitrous oxide, nitrogen dioxide and nitric oxide- are

reduced to gaseous nitrogen (Tiedjie, 1988 as cited by Barber and Stuckey, 2000) with simultaneous chemical oxygen removal (COD) removal (Ketchum, 1988; Wanner and Grau, 1988; Cappuccino and Sherman, 1992 as cited by Drysdale *et al.*, 2001). The term “denitrification” was first introduced by Gayon and Dupetit (1886 as cited by Robertson and Kuenen, 1984) for a phenomenon, which had been known for some years, by then. Denitrification is achieved in the anoxic zone/s of the BNR processes whereby some heterotrophic bacteria are simulated into utilizing nitrates and nitrites as final electron acceptors for cellular respiration in place of oxygen (Ketchum, 1988 and Cappuccino and Sherman, 1992 as cited by Drysdale *et al.*, 1999). Assimilatory nitrogen removal is defined as the reduction of nitrate to ammonium (NH_4^+), whereby the ammonium is used for synthesis of cellular material. Dissimilatory nitrogen reduction on the other hand, is defined as the reduction of nitrate to nitrite, or nitrite to one of the more reduced gaseous nitrogen oxide compounds, nitric oxide (NO), nitrous oxide (N_2O) or nitrogen (Casey *et al.*, 1999).

The classic definition of denitrification assumes that the denitrification under the consumption of carbon is carried out by heterotrophic microorganisms (Hippen *et al.*, 1997). Hesitation exists however, in the wastewater industry pertaining the bacteria involved in denitrification as well as the extent to which these bacteria contribute to nitrate and nitrite attenuation under anoxic conditions. The general idea is normally that *Pseudomonas* spp., as well being involved in EBPR (Osborn *et al.*, 1989; Kavanaugh and Randall, 1994 and Jørgensen and Pauli, 1995 as cited by Drysdale *et al.*, 1999) are the

ubiquitous microorganisms through which denitrification is achieved (Janda *et al.*, 1988; gray, 1990 and Lazarova, 1992 as cited by Drysdale *et al.*, 1999). According to Otlanabo (1993 as cited by Drysdale *et al.*, 1999) however, an assortment of other species of bacteria are accountable for denitrification in soil and they include *Vibrio*, *Bacillus*, *Flavobacterium* etc. It consequently seems unlikely that only *Pseudomonas* spp. are accountable for denitrification taking place in such an exceedingly miscellaneous microbial consortia as that of activated sludge (Drysdale *et al.*, 1999). The coexistence and/ or competitive behaviour of the wide variety of microorganisms contained in a nitrogen and phosphorus (N and P) removal system complicate the reaction mechanism in the system (Cech and Hartman, 1993, Mino *et al.*, 1994, Chuang *et al.*, 1996 and Kim and Pagilla, 1997 as cited by Chuang and Ouyang, 2000).

2.3.2 Phosphorus removal

The notion of modern activated sludge processes for industrial and human wastewater has extended from the simple degradation of organic matter to removal of nutrients such as nitrogen and phosphate (Nielsen *et al.*, 1999). There are two ways in which biological phosphorus removal (P- removal) can be attained in wastewater, i.e. stoichiometric coupling to microbial growth or enhanced storage in the biomass as polyphosphate (PolyP). The latter is the key device in the enhanced biological P- removal (EBPR) process (Mino *et al.*, 1998). Enhanced biological phosphorus removal (EBPR) is an activated sludge process modification that allows a high degree of phosphate removal from wastewater (Jenkins, <http://www.ce.berkeley.edu/~jenkins/>). This results in low

effluent phosphorus levels, which is highly favourable when the wastewater treatment plant is discharging effluent into sensitive systems, like inland rivers and lakes (Bond, <http://www.uea.ac.uk/env/studentships/bondp1.htm>).

In EBPR phosphate accumulating organisms (PAOs) are selected for and can dominate the microbial community. In the anaerobic zone, PAOs may, amongst other functions remove readily biodegradable organic carbon from the mixed liquor and store these intracellularly as poly-hydroxybutyrate (PHB) and, degrade intracellular stores of polyphosphate (polyP) and glycogen. In the aerobic zone, the stored PHB is utilized and polyP is removed from the mixed liquor, a result to which is phosphorus removal (Bond, <http://www.uea.ac.uk/env/studentships/bondp1.htm>). Jenkins (<http://www.ce.berkeley.edu/~jenkins/>) states that research conducted in their laboratory has determined the limiting activated sludge growth rate for EBPR to be a range of temperatures. From a study conducted by Erdal (2002), it was shown that EBPR systems perform better at colder temperatures (5°C as compared to 20°C) and the reason for this behaviour was determined to be related to reduced competition for substrate in the non- aerobic zones which resulted in an increased population of PAOs, leading to greater EBPR efficiency. Their work (Bond *et al.*, 1999) suggested that pH is an important factor in determining whether PAOs or GAOs are favoured in EBPR systems.

One difficulty in the optimization of the EBPR process is the probable participation of microbial competitors for substrate uptake under anaerobic conditions. Under these conditions, some bacteria can store carbon at the expense of other, previously stored compounds, such as glycogen (Cech and Hartman, 1993, Liu *et al.*, 1996 and Satoh *et al.*, 1992 as cited by Nielsen *et al.*, 1999). These bacteria can compete with the phosphate accumulating organisms (PAOs) for anaerobic uptake of the carbon source and, under certain conditions, can cause the failure of EBPR (Liu *et al.*, 1997 as cited by Nielsen *et al.*, 1999). Glycogen has been reported to be an essential requirement for the EBPR mechanism (Erdal, 2002). Glycogen accumulating organisms (GAOs) can also confiscate carbon substrate in the anaerobic zone without contributing to phosphate removal and have been reported to compete with PAOs for substrate in the anaerobic zone and is therefore suggested that GAOs in EBPR cause decreased phosphorus removal performance (Bond, <http://www.uea.ac.uk/env/studentships/bondp1.htm>).

Biological P- removal has become one of the most important processes but, little is known about the microbial groups participating in the reactions (Nielsen *et al.*, 1999). While the EBPR process provides a more lucrative solution than the chemical precipitation processes previously employed, the process operation has not yet been fully optimized and therefore often fails. The inability to isolate the responsible microorganisms and to verify the biochemical metabolism for the observed EBPR activity tends to be yet another one of the difficulties in process optimization (Nielsen *et al.*, 1999). Investigations to understand the metabolism and physiology of either PAO or GAO are restricted to mixed culture studies

as representative isolates of pure cultures thereof are not available (Bond, <http://www.uea.ac.uk/env/studentships/bondp1.htm>).

Fuhs and Chen (1975 as cited by Mino *et al.*, 1998) were the first to identify *Acinetobacter* spp. as the bacterium responsible for EBPR. Many researchers accordingly reported its predominance in EBPR processes based on culture dependent identification methods such as the API system (Buchan, 1983; Lotter, 1985 and Wentzel *et al.*, 1988 as cited by Mino *et al.*, 1998) and reported its ability to accumulate polyphosphates during aerobic growth on acetate (Brodisch, 1985, Fuh and Chen, 1975, Lötter, 1985 and Wentzel *et al.*, 1988 as cited by Nielsen *et al.*, 1999). *Acinetobacter* spp. have, nevertheless, failed to display the key biochemical transformation observed in EBPR sludge (Jenkins and Tandoi, 1991 and Van Loosdrecht *et al.*, 1997 as cited by Nielsen *et al.*, 1999). However, only those bacteria that are culturable on the artificial media used under the defined circumstances can be isolated and identified by these methods. It is therefore expected that only a small fraction of bacteria in activated sludge can grow under such circumstances and hence can be detected (Wagner *et al.*, 1993 and Kämpfer *et al.*, 1996 as cited by Nielsen *et al.*, 1999). But, as in other natural systems, this comes as no surprise as this preconceived notion is caused by the lack of culturability of the majority of the microorganisms in the activated sludge (Wagner *et al.*, 1993 as cited by Nielsen *et al.*, 1999). Wentzel *et al.* (1986) concluded that *Acinetobacter* spp. are able to compete productively with other organisms for substrate in entirely aerobic sludge systems, whereby they metabolize glucose by means of the Entner- Doudoroff pathway. They

(Wentzel *et al.*, 1986) established that with the introduction of an anaerobic reactor into the system, the anaerobic reactor selects a multitude of facultative organisms capable of fermenting sugars to lower fatty acids (LFA) through the glycolytic (Embden- Meyerhof) pathway. According to Wentzel *et al.* (1986) *Acinetobacter* spp. do not possess the glycolytic pathway and consequently are not capable of producing energy for survival in the anaerobic zone by the e.g. glucose fermentation. Accordingly, the *Acinetobacter* spp. would be at a distinct drawback in the anaerobic/ aerobic system due to this deficiency of the glycolytic pathway and would be outcompeted by the facultative organisms in such a system (Wentzel *et al.* 1986). With the implementation of molecular techniques, it was revealed that *Acinetobacter* spp. represent only a small fraction of the total population in the activated sludge system (Wagner *et al.*, 1994 and Wallner *et al.*, 1995 as cited by Nielsen *et al.*, 1999). A much higher microbial diversity was identified by the molecular criteria (Bond *et al.*, 1995, Liu *et al.*, 1997 and Schuppler *et al.*, 1995 as cited by Nielsen *et al.*, 1999), which showed that bacteria from the subclasses of the *Proteobacteria* accounted for up to 80% of the total sludge population (Wagner *et al.*, 1994 and Wallner *et al.*, 1995 as cited by Nielsen *et al.*, 1999).

In addition, Mino *et al.* (1998) stated that it has been demonstrated that *Acinetobacter* is NOT primarily accountable for EBPR. Some of the experimental evidences reported that point to this include:

- (1) A fluorescent antibody staining technique for *Acinetobacter* spp. revealed (Cloete and Steyn, 1987 as cited by Mino *et al.*, 1998) that the number of *Acinetobacter* spp. in the EBPR process studied was less than 10% of total bacteria, and could not account for the EBPR observed.
- (2) The governing respiratory quinones in PAO- enriched sludges were quinone-8 (Q-8) and menaquinone-8 (MK-8 (H₄)), whereas *Acinetobacter* spp. has Q-9 (Hiraishi *et al.*, 1989; Hiraishi and Morishita, 1990 and I Made *et al.*, 1998 as cited by Mino *et al.*, 1998). Quinones are one of electron carriers in the respiratory chain. The type of quinone is species specific, and therefore, by analyzing the quinone profile of a microbial community the overriding populations can be identified.
- (3) Auling *et al.* (1991 as cited by Mino *et al.*, 1998) used a polyamine, diaminopropane (DAP), as a biomarker for *Acinetobacter* spp. and showed that EBPR plants eliminating phosphate very proficiently had nearly no DAP in the polyamine pattern.
- (4) Application of a 16s-rRNA targeted oligonucleotide probe specific for *Acinetobacter* spp. showed *Acinetobacter* spp. was less than 10% of total bacteria and not dominant in the EBPR processes studied (Wagner *et al.*, 1994; Bond *et al.*, 1995; Kampfer *et al.*, 1996 and I Made *et al.*, 1998 as cited by Mino *et al.*, 1998).

The above-mentioned evidences against *Acinetobacter* were all obtained through non-culture-dependent methods (Mino *et al.*, 1998). Meticulous research has furthermore established that no pure cultures of *Acinetobacter* have shown the representative characteristics of EBPR sludges with high P- removal capability (Jenkins and Tandoi, 1991 and Van Loosdrecht *et al.*, 1997 as cited by Mino *et al.*, 1998). Hence, *Acinetobacter* spp. need no longer be considered the principle organisms accountable for the EBPR process (Mino *et al.*, 1998). It is also possible that PAOs and GAOs are the same organism, in which case variable phosphorus removal could result from an alteration in the phosphate- accumulating capabilities of that particular organism (Bond *et al.*, 1999). Brodisch and Joyner (1983 as cited by Wentzel *et al.*, 1986) have shown that a number of genera are associated with biological P- removal. For instance, Stante *et al.* (1996 as cited by Mino *et al.*, 1998) isolated a PHB storing strain from sequencing batch reactor (SBR) designed for EBPR and identified it as *Lampropedia* spp. It is Neisser staining positive, indicating polyP storage, and has a ability to take up acetate and store it as PHA under anaerobic conditions. Functionally this isolate resembles PAOs, but morphologically it has a very exclusive sheet- like cell arrangement which is not common in EBPR processes (Mino *et al.*, 1998). A better understanding therefore, of the diversity of microorganisms prevalent in EBPR processes is a key element for optimizing these processes (Nielsen *et al.*, 1999).

Current literature proves beyond reasonable doubt that a lot of research has been done and a lot of data documented on the denitrification process and EBPR, while the organisms responsible have been partly studied. However, very little, if anything at all, is known about filamentous bacteria and the role they play in denitrification and EBPR in BNR processes. The possibility that filamentous bacteria may be involved in such processes has not adequately been explored and studied. It is therefore very important to acquire a detailed understanding of filamentous bacteria and the precise role/s they play in denitrification and EBPR in BNR systems. Findings from such studies may indeed be the key element required to fully understand BNR and activated sludge bulking.

2.4 ISOLATION, CULTIVATION AND ASSESSMENT OF FILAMENTOUS BACTERIA

Isolation of filamentous organisms is a pre-requisite for the investigation of these organisms and is still difficult (Kämpfer, 1997). As much as isolation is a pre-requisite, it is however, not common practice as it is very laborious but, it is a technology that has shown promise as an essential tool in studying filamentous bacteria on the basis of morphology, nutritional requirements and physiology. Such knowledge is imperative in order to eventually fully understand activated sludge bulking and foaming and BNR. The great variety of nutrients present in industrial and domestic sewage water creates excellent conditions for the development of many unicellular and filamentous bacteria (van Veen, 1973). Because the majority of filamentous bacteria is overgrown by more rapid growers, samples are pretreated and diluted before plating on solid media. Narrow and short filaments or filaments scarce in the activated sludge may be concentrated by centrifugation.

Another method for selective isolation of filamentous bacteria is micromanipulation with special microtools under a microscope (Kämpfer, 1997). Although the latter method is one of the latest and commonly used methods for isolation of filamentous bacteria, it requires highly specialized equipment and is therefore expensive. It also tends to be labour intensive and difficult (Seungbum, 2001). Micromanipulation requires practice to master and the success rate for isolation is very low (<1%) (Lindrea *et al.*, 1999).

It is important that the flocs be broken up before isolation so as to facilitate easier separation of filamentous bacteria from floc-forming bacteria. Nonidet is a non-ionic detergent that alleviates cell clumping without any obvious damage to the cells when used at correct concentrations i.e. 0.1% (Stahl and Amann, 1991). Cellulase is an enzyme complex capable of decomposing cellulosic polysaccharides into smaller fragments (<http://www.nutritionfocus.com>, accessed on 06/04/2001). The majority of studied microbial cellulases has an optimum pH that generally lies between 4 and 5 but is effective between pH 3 and 7 (<http://www.serva.de/products/knowledge/061097.shtml>, accessed on 06/04/2001). Nonidet and cellulase reduce surface tension in the microbial flocs and therefore ease separation of filaments from floc-formers. Sonication of activated sludge samples is a convenient method for dispersing the flocs. It is also the least disruptive to the bacterial cell integrity (Pike, 1975 and Jorand *et al*, 1995 as cited by Munch and Pollard, 1997). After breaking up of the flocs, it is important to separate filamentous bacteria from floc-formers. This is so as to avoid or minimize contamination by floc-formers, so that when the filaments are being grown on different media, there are no interferences caused by floc-forming bacteria. One method of separating filaments from floc-formers is by centrifugation. Centrifugation separates particles on the basis of their size. The supernatant containing free filaments after centrifugation can be used as the inoculum (Kämpfer, 1997).

2.5 MORPHOLOGY, PHYSIOLOGY AND BIOCHEMISTRY OF FILAMENTOUS BACTERIA

Procedures for the identification of filamentous bacteria rely on morphological characteristics and their response to a number of simple staining techniques (Eikelboom and van Buysen, 1981; Jenkins *et al.*, 1984 as cited by Seviour *et al.*, 1994). The use of morphological criteria for prokaryotes is, however, unreliable as indicators of relatedness (Woese, 1987 as cited by Seviour *et al.*, 1994), and organisms that look the same, even to a trained eye, may not necessarily be so. It has become clear that organisms like the nocardioform bacteria, which look very similar in mixed liquor or foam samples under the microscope, differ widely in their taxonomy, physiology and biochemistry in pure culture (Soddell *et al.*, 1992 as cited by Seviour *et al.*, 1994). Similarly, some foam producing organisms like *Nocardia pinensis* (Soddell and Seviour, 1994 as cited by Seviour *et al.*, 1994) may look quite different in foams, and yet very similar in pure culture. Variations in morphology have now also been reported in several other filaments including *Microthrix parvicella* (Foot *et al.*, 1992 as cited by Seviour *et al.*, 1994), Types 0961 and 0092 (Buali and Horan, 1989 as cited by Seviour *et al.*, 1994) and Type 021N (Ziegler *et al.*, 1990 as cited by Seviour *et al.*, 1994). A chance of incorrect identifications exists, with possibilities that the filaments presently recognized are much more diverse in their properties, and may in fact represent groups of quite different organisms all looking similar (Williams and Unz, 1985 as cited by Seviour *et al.*, 1994).

Models for controlling filamentous growth (e.g. Jenkins, 1992 as cited by Seviour *et al.*, 1994) may have limited value, as they fail to take account of possible variations in organism ecology, physiology and biochemistry. Only some single strains of various filamentous bacteria have been isolated and physiologically characterized (Richard *et al.*, 1981, Aufderheide, 1982, Salcher *et al.*, 1982, Trick, 1982, Williams *et al.*, 1984 and Williams and Unz, 1985a as cited by Ziegler *et al.*, 1990), whereas other authors did so with the *Leucothrix-Thiothrix* type 021N-group (Poffé *et al.*, 1979, Richard *et al.*, 1985, Williams and Unz, 1985b,c and Williams *et al.*, 1987 as cited by Ziegler *et al.*, 1990), *Haliscomenobacter hydrossis* (van Veen *et al.*, 1971, 1973 as cited by Ziegler *et al.*, 1990), *Microthrix parvicella* (Slijkhuis and Deinema, 1982, Slijkhuis, 1983 and Slijkhuis *et al.*, 1984 as cited by Ziegler *et al.*, 1990), *Actinomyces* (Lemmer and Kroppenstedt, 1984 and Lemmer, 1986 as cited by Ziegler *et al.*, 1990) and, type 0041 (Brand *et al.*, 1987 as cited by Ziegler *et al.*, 1990). Ziegler *et al.* (1990) isolated and characterized type 021N, *Sphaerotilus natans*, type 1701, *Haliscomenobacter hydrossis*, nocardioforms and unidentified pigmented colonies.

The majority of microorganisms in their natural habitat cannot be cultured and remain unidentified (Haldeman and Amy, 1993 and Wagner *et al.*, 1993 as cited by Van Heerden *et al.*, 2000). This has led to a lack of knowledge on microbial community composition and function. Molecular techniques have recently been used in microbial ecology studies in an attempt to overcome the limitations of culture techniques (Wagner *et al.*, 1993, Amann *et al.*, 1995 and Muyima *et al.*, 1997 as cited by Van Heerden *et al.*, 2000). The

use of gene probes for the *in situ* identification of filamentous bacteria in activated sludge has been found successful. Oligonucleotides (short strands of nucleic acids, usually 15-30 nucleotides in length), complementary to 16S rRNA and 23S rRNA sequence regions with an intermediate degree of conservation and characteristic for phylogenetic entities like genera, families and subclasses, have been used successfully for rapid identification of bacteria. The technique is called fluorescent *in situ* hybridization (FISH) or whole cell probing (Lindrea *et al.*, 1999).

Such identification techniques as FISH are however, expensive and require highly specialized equipment and, are therefore not viable to use in small laboratories with limited funds. It is therefore vital to optimize microbial techniques for isolation of the majority of organisms from their natural sources. Isolation of filaments will allow comprehensive studies on the biochemistry, physiology and morphology of these bacteria, as there is currently a serious lack of data on these subjects hence a lack of controlling bulking and understanding BNR in depth.

2.6 BIOLOG FOR FINGERPRINTING OF ORGANISMS

One or more species exist in any given system, each performing a particular task. The more species, the more functions related to their metabolism. It can therefore be said that a specific microbial community has a specific metabolic capacity. The objective is therefore to relate this microbial capacity of the community to utilize certain selected substrates with the hypothesis being that the more substrates utilized the higher the diversity due to the collective action of individual species (Van Heerden, 2000).

One system that affords the opportunity to generate metabolic patterns for a specific community, is the Biolog system (Garland and Mills, 1991, Haack *et al.*, 1994, Winding, 1994, Zak *et al.*, 1994, Bossio and Scow, 1995, Ellis *et al.*, 1995, Wünsche and Babel, 1995, Guckert *et al.*, 1996, Insam *et al.*, 1996, Smalla *et al.*, 1996, Garland, 1997, Glimm *et al.*, 1997, Heuer and Smalla, 1997, Kersters *et al.*, 1997 and Engelen *et al.*, 1998 as cited by Van Heerden, 2000). The Biolog system relies on the potential utilization of 95 substrates in a microwell plate by the test organisms (Biolog, Inc., Hayward, CA. as cited by Van Heerden, 2000). The Biolog method can reflect the activities of a broad range of bacteria (Zak *et al.*, 1994 as cited by Van Heerden, 2000). Oxidation of the substrates is monitored by the colourimetrically determinable conversion of the redox dye tetrazolium violet into a vividly purple formazan (Biolog, Inc., Hayward, USA. as cited by van Heerden, 2000). Metabolism of a substrate results in the formation of NADH (reduced nicotinamide adenine dinucleotide) and through an electron transport chain, in the irreversible reduction of the redox dye to the purple formazan. The Biolog system has

already been used for characterization of naturally occurring bacteria and for classification of bacterial communities of different environments (Fredrickson *et al.*, 1991, Garland and Mills, 1991, Verniere *et al.*, 1993, Winding, 1994 and Zak *et al.*, 1994 as cited by Van Heerden *et al.*, 2000). This system allows the testing of carbon sources utilization's of bacteria not producing pH change and it works equally well with fermenting and non-fermenting bacteria. However, oligotrophic bacteria may produce false positive or negative results (Bochner, 1989 as cited by van Heerden *et al.*, 2000). An imperative benefit of the Biolog method is its ease of use and thus viability for use in large-scale field studies (van Heerden *et al.*, 2000).

CHAPTER 3

MATERIALS AND METHODS

3.1 SAMPLING

Different plants in the Durban and surrounding areas were screened microscopically for constituent filamentous bacteria. Based on the results, plants with different populations of filamentous bacteria were chosen for sampling and experimentation. Sterile schott bottles were used to obtain 1L Grab samples of mixed liquor from each of the three chosen plants. Under no circumstances were samples chemically preserved or frozen as these would alter the characteristics of filamentous organisms (Jenkins *et al.*, 1986). The mixed liquor samples were stored half-full in the schott bottles so as to allow for aerobic conditions (Eikelboom, 2001). Filamentous bacteria were identified using staining procedures, i.e. Gram Stain, Neissser Stain, PHB (Polyhydroxybutyrate) Stain and Crystal violet sheath Stain (Appendices A-D) and cell morphology (Jenkins *et al.*, 1986).

3.2 SAMPLE PRETREATMENT (FLOC BREAK-UP)

Nonidet (now known as Igepal), cellulase, bead- beating and sonication were used to break up the flocs so as to facilitate easier separation of filamentous bacteria from floc-formers.

Method 1

Nonidet (Igepal CA-630, Sigma- Germany) is a non-ionic detergent that alleviates cell clumping without any obvious damage to the cells when used at correct concentrations i.e. 0.1%. For the purposes of this project, various concentrations (0.1, 1.0, 10 and 100%) of the nonidet were assessed. 9 Parts of mixed liquor sample were mixed with 1 part of Nonidet (Igepal CA-630, Sigma- Germany) (Stahl and Amann, 1991). The samples were then mixed for a minute using a vortex mixer so as to assure complete homogenization. Samples were then centrifuged at 1500 rpm for 10mins to minimize the amount of floc-formers from the samples.

Method 2

Cellulase is an enzyme complex capable of decomposing cellulosic polysaccharides into smaller fragments (<http://www.nutritionfocus.com>, accessed on 06/04/2001). The enzyme cellulase was mixed with the mixed liquor sample(s) using the procedure detailed below: The enzyme was dissolved in distilled water at a concentration of 1mg/L. The mixed liquor sample was centrifuged at 3500 rpm for 5 minutes and 200mg of the pellet sludge

was measured into a clean dry test tube. To this sludge, 4.0ml of a 0.05M acetic acid solution and 1.0ml of the enzyme dilution were added and mixed, followed by incubation at 37°C for 2hrs (<http://www.worthington-biochem.com/manual/C/CEL/html>, accessed in March 2001). The samples were then centrifuged at 1500rpm for 10mins to minimize the amount of floc-formers from the samples whereby floc-formers remained in the bulk liquid and filamentous bacteria were mostly concentrated in the pellet. This result facilitated easier separation of filamentous bacteria from floc-forming bacteria by permitting decanting of a considerable amount of the floc-formers that remained in the bulk liquid subsequent to centrifugation.

Method 3

Samples containing filaments were diluted 1:5 in sterile mineral-salt-vitamin solution (MSV) (Appendix M), sonicated at 30W for 10 seconds and washed three times by centrifugation at 4470rpm for 2-5mins. The final pellet was resuspended in fresh MSV and centrifuged at 1180rpm for 2-5mins. The supernatant containing free filaments was used as the inoculum (Kämpfer, 1997).

Method 4

The mixed liquor samples were whirl-mixed in the presence a few small glass beads in a tube. The whirl-mixed sample was then microscopically checked for filamentous bacteria in the supernatant and the mixing was repeated where necessary. An aliquot was directly plated onto the isolation media (Ziegler *et al.*, 1990).

3.3 SEPARATION OF FLOC-FORMERS FROM FILAMENTOUS BACTERIA

After breaking of the flocs (sample pretreatment), the samples were centrifuged (except for the whirl-mixed samples) so as to separate the filaments from the floc-formers. These samples were centrifuged at 4470rpm for 2-5 minutes. The supernatant was discarded and the samples were suspended in sterile MSV and further centrifuged for 2-5 minutes at 1180rpm. The supernatant containing free filaments was therefore be used as the inoculum (Kämpfer, 1997).

3.4 INOCULATION AND INCUBATION

Method 1

With an inoculation loop dipped into the activated sludge sample, separate streaks were made on the various isolation media (Ziegler *et al.*, 1990). This was done on samples that had not been pretreated. Samples were therefore directly inoculated without anything having been done to them.

Method 2

Serial dilutions (10^{-1} - 10^{-5} in MSV) of the filamentous bacterial samples obtained after their separation from floc-formers were made and each was plated individually on the different media that were chosen. Media used for isolation and cultivation are listed in table 3.1. These media were chosen because they have been reported successful in supporting the

growth of a wide range of filamentous bacteria. The technique that was employed for the inoculation of agar plates was the spread plate technique (Mulder and Deinema, 1981; Kämpfer, 1997) whereby a 0.1ml aliquot of the dilute sample was spread with a sterile glass “hockey stick” on sterile agar media. All plates were incubated aerobically at 20-25°C for three to six weeks where after discrete colonies were subcultured onto fresh media and screened microscopically for filamentous morphology (Kämpfer, 1997).

3.5 IDENTIFICATION OF ISOLATES

Microscopic verification of isolates was performed as per methods for the identification of filamentous bacteria (Jenkins *et al*, 1986) using staining techniques (Appendices A- D).

Cell morphology of the isolates was also observed as an aid in identification. The possibility of morphological shifts i.e. a shift from filamentous form to single- celled form or any other morphological shift was determined using the Gram stain. This was done mainly on isolates that executed positive results for the Biolog substrates (Section 3.7).

Table 3.1: Table of media

| Media | Filaments that have been reported to grow on these media |
|---|---|
| 1 R2A agar (Appendix E) | <i>M. parvicella</i> , <i>S. natans</i> , type 1701, 0803, 1863, 0092, 0411 and <i>Leptothrix</i> spp. (Kämpfer, 1997) |
| 2 CGYA (Casitone glycerol yeast agar) (Appendix F) | <i>S. natans</i> (Kämpfer, 1997) |
| 3 SCY Medium (Appendix G) | <i>Leptothrix</i> spp (Kämpfer, 1997) and <i>H. hydrossis</i> (Mulder and Deinema, 1981) |
| 4 I Medium (Appendix H) | <i>Thiothrix</i> spp., <i>Beggiatoa</i> spp. and type 021N (Kämpfer, 1997) and also <i>H. hydrossis</i> (Mulder and Deinema, 1981) |
| 5 TYGA (Tryptone yeast glucose agar) (Appendix I) | <i>Nocardia</i> spp. (Kämpfer, 1997) |
| 6 Nocardia Medium (Appendix J) | <i>Nocardia</i> spp. (Atlas, 1993) |
| 7 D-medium (Appendix K) | van Veen (1973) |
| 8 C-medium (Appendix L) | Eikelboom (1975) |
| 9 Actinomycete Isolation Agar (commercially available, Difco) | Difco (USA) |

Antibiotic sensitivity

Isolates were also tested for antibiotic sensitivity using ampicillin, furazalidone, gentamycin and penicillin G on Antibiotic Medium No. 1 (Appendix O).

Aromatic compounds as carbon source

Growth of isolates on aromatic compounds as sole carbon source was also determined. The aromatic compounds tested were Phenol, o-cresol and 2,5-dihydroxybenzoic acid which were supplemented on modified I-Medium (Appendix P) that contained CO_3 with phenol, o-cresol and 2,5-dihydroxybenzoic acid (separately) as carbon sources, and for the fact that there was CO_3 in the medium, two controls were employed. The first control was the same medium without any aromatic compound (medium plus CO_3); the second control was medium without CO_3 . The I-medium was modified by adding 0.05g/L NaCl and 2.0g/L aromatic compound (either phenol, o-cresol or 2,5-dihydroxybenzoic acid) that had been filter-sterilized and added to the cooled agar after autoclaving. This medium excluded glucose.

Hydrolyses of polymers

In addition, isolates were tested for the ability to hydrolyse macromolecules and, these included Nutrient gelatin on Nutrient Gelatin dip (Appendix Q), Starch on Starch agar (Appendix R), Casein on Skim Milk agar (Appendix S) and various lipids on Spirit Blue agar (Appendix T). The lipids used for the purposes of this study include Olive oil, Sunflower oil, Peanut oil, Grapeseed oil, Avocado oil and a mixture of Sesame and Soya oil.

Phosphatase

The ability for isolates to cleave phosphate i.e. presence of the enzyme phosphatase using Phenolphthalein Phosphate agar (Appendix U) was determined. To detect phosphatase, 1% phenolphthalein diphosphate was included in the agar. The organisms were streaked onto the plates and incubated. With this test the principle is that, if the organism produces phosphatase, the phosphate in the medium will be cleaved from the phenolphthalein thus liberating free phenolphthalein, which is an indicator in the free state. Following incubation the plate is exposed to ammonia, which is alkaline, which turns the free phenolphthalein bright, pink immediately.

Urease, pH and temperature

The presence of the enzyme urease on Christiansen's urea agar slope (Appendix V) was also tested. Nutrient pH broths (Appendix W) were prepared at different pHs i.e. pH 5.5, 6.5, 7.0 and 8.0 using either acid or base to alter the pH. For the determination of temperature tolerance, isolates were grown on nutrient agar and incubated at 12°C, 20°C and 30°C respectively.

Denitrification

A colorimetric test was employed to determine the ability of isolates to denitrify with nitrate (NO_3) and nitrite (NO_2) respectively (Appendix X).

Glycogen

All isolates were stained and tested for the storage product glycogen using the Periodic Schiff's Method (Appendix Y).

Biolog

In addition, 95 different substrates presented by Biolog MicroPlates for Gram positive and Gram Negative bacteria were used respectively as a means of biochemical profiling/fingerprinting of the isolates under study (Appendices Z and AA). These included:

- ❖ *Amino sugars* - N- Acetyl- D- Glucosamine and N- Acetyl- D- Mannosamine.
- ❖ *Glucosides* - Amygdalin, Arbutin, α - Methyl- D- Glucoside, β - Methyl- D- Glucoside and Salicin.
- ❖ *Sugar alcohols* - D- Arabitol, D- Mannitol and D- Sorbitol.
- ❖ *Sugar acids* - D- Galacturonic acid and D- Gluconic acid.
- ❖ *Glycosides* - α - Methyl- D- Galactoside, β - Methyl- D- Galactoside and α - Methyl- D- Mannoside.
- ❖ *Acid amides* - Lactamide and Succinamic acid.
- ❖ *Dicarboxylic acids* - Succinic acid and Succinic Acid Mono- methyl Ester.

- ❖ *Peptides* - L- Alanyl- Glycine, Glycyl- L- Glutamic acid and L- Pyroglutamic acid.
- ❖ *Aminoalkanes* - Putrescine.
- ❖ *Organic glycols* - 2,3- Butanediol.
- ❖ *Alcohols* - Glycerol.
- ❖ *Phosphate esters* - D- Fructose- 6- Phosphate, α - D- Glucose- 1- Phosphate, D- Glucose- 6- Phosphate and D- L- α - Glycerol Phosphate
- ❖ *Sugars* - L- Arabinose, D- Cellobiose, D- Fructose, L- Fucose, D- Galactose, Gentiobiose, α - D- Glucose, m- Inositol, α - D- Lactose, Lactulose, Maltose, Maltotriose, D- Mannose, D- Melezitose, D- Melibiose, Palatinose, D- Psicose, D- Raffinose, L- Rhamnose, D- Ribose, Stachyose, Sucrose, D- Tagatose, D- Trehalose, Turanose, Xylitol, D- Xylose and 3- Methyl- Glucose.

- ❖ *Organic acids* - Acetic acid, α - Hydroxybutyric acid, β - Hydroxybutyric acid, γ - Hydroxybutyric acid, p- Hydroxy- Phenylacetic acid, α - Ketoglutaric acid, α - Ketovaleric acid, L- Lactic acid, D- Malic acid, L- Malic acid, Propionic acid, Pyruvic acid, D- Lactic Acid Methyl Ester, Pyruvic Acid Methyl Ester and N- Acetyl- L- Glutamic Acid.
- ❖ *Nucleosides* - Adenosine, 2'- Deoxy Adenosine, Inosine, Thymidine, Uridine, Adenosine- 5'- Monophosphate, Thymidine- 5'- Monophosphate, and Uridine- 5'- Monophosphate.
- ❖ *Polymers* - β - Cyclodextrin, Dextrin, Glycogen, Inulin, Mannan, Tween 40 and Tween 80
- ❖ *Amino acids* - L- Alaninamide, D- Alanine, L- Alanine, Asparagine, L- Glutamic acid, L- Pyroglutamic Acid and L- Serine

Each Biolog MicroPlate comes with a water control. Cultures were suspended in the Biolog inoculating fluid and homogenized. 150µl of the homogenate were aseptically inoculated into each well of the MicroPlates depending on the organism's Gram reaction. Only one isolate was Gram negative (DLNT01) so, only it was inoculated into the GN MicroPlate, the rest of the isolates were inoculated into the GP MicroPlates respectively. This was followed by incubation at 20-25°C under moist conditions. Plates were incubated for a maximum of 10 days. The MicroPlates also have a water control (Appendices Z and AA).

CHAPTER 4

RESULTS

Fourteen isolates from seven different systems were studied. Of all the fourteen isolates, four were isolated from a laboratory-scale treatment system (MLE system or Modified Ludzack-Ettinger system). These isolates are OSI001a, OSI001b, OSI004a and OSI004b. This MLE system was treating edible oil effluent and was in fact bulking at the time of sampling for isolation of filamentous bacteria. The remaining ten isolates were obtained from full-scale wastewater treatment plants in Durban and surrounding areas except for the isolate coded UCSR01, which was obtained from the UCT process.

Table 4.1: Representation of isolate source, pre-treatment procedures and isolation media

| Filament code | Sampling plant | Pre-treatment procedure | Medium of isolation | Type of waste treated by plant |
|----------------------|-----------------------|------------------------------------|----------------------------|---|
| TTBCG01 | Amanzimtoti | Bead- beating | CGYA | Domestic waste |
| TTBCG02 | Amanzimtoti | Bead- beating | CGYA | Domestic waste |
| TTBCG03 | Amanzimtoti | Bead- beating | CGYA | Domestic waste |
| SWNCG01 | Southern Works | Nonidet | CGYA | Domestic waste |
| SWNCG02 | Southern Works | Nonidet | CGYA | Domestic waste |
| NGCD | New Germany | Cellulase | D-Medium | Industrial (Activated sludge process) |
| NGCD01 | New Germany | Cellulase | D- Medium | Industrial (Activated sludge process) |
| UCSR01 | UCT | Sonication | R2A | |
| DLNT01 | Darvil | Nonidet | TYGA | Industrial and Domestic waste |
| HCBCG01 | Hillcrest | Bead- beating | CGYA | Mainly domestic (Extended aeration) |
| OSI001a | MLE process | Sonication | I- Medium | Edible oil effluent |
| OSI001b | MLE process | Sonication | I- Medium | Edible oil effluent |
| OSI004a | MLE process | Sonication | I- Medium | Edible oil effluent |
| OSI004b | MLE process | Sonication | I- Medium | Edible oil effluent |

4.1 TEMPERATURE

With the exception of 4 isolates (OSI001a, OSI001b, OSI004a and OSI004b), good growth was observed with the other 10 isolates at 12°C and excellent growth at 20°C with most isolates (8 of the 10) (Table 4.3). The other two, i.e. SWNCG01 and SWNCG02, grew excellently at 30°C as compared to 20°C. OSI001a, OSI001b, OSI004a and OSI004b did not grow at all at 12°C and showed moderate growth at 20 and 30°C with the exception of OSI001a and OSI004a, which grew better at 30°C.

4.2 pH

All isolates were grown on broths of pHs 5.5, 6.5, 7.0 and 8.0 (Appendix U). Two isolates (TTBCG01 and TTBCG03) showed excellent growth at all pHs. At pH 5.5 TTBCG02, SWNCG01, SWNCG02, UCSR01 and HCBCG01 showed good growth. NGCD and NGCD01 showed moderate growth whereas DLNT01 showed little growth. OSI001a, OSI001b, OSI004a and OSI004b showed no growth. At pH 6.5 an increase in growth was noted with most of the isolates except for TTBCG02 and HCBCG01 whose growth was the same as that at pH 5.5. Isolate HCBCG01 actually exhibited the same amount of growth at pHs 7.0 and 8.0 as well. Excellent growth at pH 6.5 was given by all the other isolates except OSI001a, OSI001b, OSI004a and OSI004b, which, however, demonstrated an increase in growth to little growth as compared to no growth at pH 5.5. Excellent growth was achieved by most isolates at pH 7.0. OSI001a, OSI001b, OSI004a and

OSI004b showed moderate growth. At pH 8.0, most organisms gave excellent growth still. Overall, isolates seemed to grow best at neutral to alkaline pHs although some grew equally well at all the different pHs.

4.3 AROMATIC COMPOUNDS AS CARBON SOURCE

The control with CO₃ showed good growth with most isolates, moderate growth with two isolates and little growth with one isolate. The control without CO₃ exhibited moderate growth with most isolates, little growth with three isolates and good growth with one isolate (UCSR01). All three aromatic compounds were inhibitory to the growth of all isolates as opposed to the controls.

4.4 PHOSPHORUS UPTAKE

As seen on table 4.2, most of the isolates are positive for PHB and glycogen storage, and although only a few of the isolates demonstrated a positive result for polyP accumulation (Neisser stain), it may be deduced that these filamentous bacterial isolates have an involvement in phosphorus removal (Appendices B, C and N).

4.5 DENITRIFICATION

The results obtained from this study, as seen on table 4.3, show that all filamentous isolates under observation are denitrifiers. Seven of the isolates demonstrated sequential denitrification, three isolates showed true denitrification and four are nitrate respirers.

4.6 EFFECT OF NaCl

As seen on the table, all isolates grew very well on the control with the exception of OSI001a, OSI001b, OSI004a and OSI004b, which showed little to moderate growth. At 5% NaCl the few organisms that grew demonstrated little to moderate growth and the rest did not grow at all. At 10% NaCl only TTBCG03, SWNCG02 and HCBCG01 showed little growth whereas the rest of the isolates did not grow. At 15 and 20%, only HCBCG01 showed slight growth.

4.7 LIPID HYDROLYSIS

A qualitative test to determine the ability of the isolates to hydrolyze lipids was conducted. As seen on table 4.3, all isolates were able to hydrolyze all tested lipids. They all hydrolyzed the lipids to glycerol and fatty acids, and most of them actually hydrolyzed the lipids so much that the fatty acids no longer attracted the dye used in the medium as an

indicator. These results were obtained within 48 hours of growing the isolates on the lipid medium.

4.8 ANTIBIOTIC SENSITIVITY

Apart from the antibiotic medium with the test antibiotic, a control was also prepared to assess the ability of organisms to grow on this medium. With the exception of HCBCG01 and OSI004b, which did not grow on the control plates, all isolates demonstrated excellent growth on the control plates. Results for antibiotic sensitivity for HCBCG01 and OSI004b are therefore not yet known. However, OSI001a, OSI001b and OSI004a were completely sensitive to all four antibiotics as the plates were completely clear after incubation with the antibiotic discs. Most isolates were most sensitive to furazalidone and most resistant to penicillin G. Isolates TTBCG03 and NGCD were resistant to ampicillin, furazalidone and penicillin G. DLNT01 was more sensitive to penicillin G than all the other isolates (second to OSI001a, OSI001b and OSI004a).

4.9 UREASE AND PHOSPHATASE

For the urease test, a positive result was given by isolates TTBCG01, TTBCG02 and TTBCG03. Isolates DLNT01, HCBCG01, OSI001a, OSI001b, OSI004a and OSI004b demonstrated a negative result for the presence of this enzyme. A weak positive result was given by isolates SWNCG01, SWNCG02, NGCD, NGCD01 and UCSR01. This may

mean that the organisms required more time to break down the urea completely than did those that gave a positive result.

Only isolates UCSR01, OSI 001a, OSI001b, OSI004a and OSI004b were negative for the production of phosphatase.

4.10 STARCH HYDROLYSIS, CASEIN HYDROLYSIS AND GELATIN LIQUEFACTION

Only isolate HCBCG01 gave a negative reaction for hydrolysis of starch/ presence of the enzyme amylase. Isolates TTBCG01, TTBCG02, TTBCG03 and HCBCG01 gave a negative result for hydrolysis of casein / presence of the enzyme caseinase. Only TTBCG03, SWNCG02, NGCD, NGCD01 and UCSR01 were positive for gelatin liquefaction/ presence of the enzyme gelatinase.

4.11 BIOLOG AS A MEANS OF BIOCHEMICAL PROFILING/ FINGERPRINTING

Table 4.4: Various groups of substrates as carbon source for filamentous isolates

| Group | Substrates | TTBCG01 | TTBCG02 | TTBCG03 | SWNCG01 | SWNCG02 | NGCD | NGCD01 | UCSR01 | HCBCG01 | OSI001a | OSI001b | OSIO04a | OSIO04b |
|--------------------|-------------------------------------|---------|---------|---------|---------|---------|------|--------|--------|---------|---------|---------|---------|---------|
| Amino sugars | N- Acetyl- D- Glucosamine | - | - | - | - | + | - | + | + | - | - | + | - | - |
| | N- Acetyl- D- Mannosamine | - | - | - | - | + | - | + | + | - | - | + | - | - |
| Glucosides | Amygdalin | - | - | - | - | + | - | - | - | - | - | + | - | - |
| | Arbutin | - | - | - | - | + | - | - | - | - | - | + | - | + |
| | α - Methyl- D- Glucoside | - | - | - | - | + | - | - | - | - | - | + | - | + |
| | β - Methyl- D- Glucoside | - | - | - | - | + | - | - | + | - | - | + | - | + |
| | Salicin | - | - | - | - | + | - | + | + | - | - | + | - | + |
| Sugar alcohols | D- Arabitol | - | - | - | - | + | + | - | + | - | - | + | - | + |
| | D- Mannitol | - | - | - | - | + | + | - | + | - | - | + | - | + |
| | D- Sorbitol | - | - | - | - | + | + | + | + | - | - | + | - | + |
| Sugar acids | D- Galacturonic acid | - | - | - | - | + | + | - | + | - | - | + | - | - |
| | D- Gluconic acid | - | - | - | + | + | + | - | + | - | - | + | - | - |
| Glycosides | α - Methyl- D- Galactoside | - | - | - | - | + | - | - | + | - | - | + | - | + |
| | β - Methyl- D- Galactoside | - | - | - | - | + | - | + | + | - | - | + | - | + |
| | α - Methyl- D- Mannoside | - | - | - | - | + | - | - | + | - | - | + | - | + |
| Acid amides | Lactamide | - | - | - | - | + | - | + | - | - | - | + | - | + |
| | Succinamic acid | - | - | - | + | + | + | + | + | - | - | + | - | + |
| Dicarboxylic acids | Succinic acid | - | - | - | + | + | + | + | + | - | - | + | - | + |
| | Succinic Acid Mono- methyl Ester | - | - | - | - | + | + | + | + | - | - | + | - | + |
| Peptides | L- Alanyl- Glycine | - | - | - | + | + | + | + | + | - | - | + | - | + |
| | Glycyl- L- Glutamic acid | - | - | - | - | + | - | + | - | - | - | + | - | + |
| | L- Pyroglutamic acid | - | - | - | - | + | + | + | + | - | - | + | - | + |
| Aminoalkanes | Putrescine | - | - | - | - | + | + | + | + | - | - | + | - | + |
| Organic glycols | 2,3- Butanediol | - | - | - | - | + | - | + | - | - | - | + | - | + |
| Alcohols | Glycerol | - | - | - | + | + | + | + | + | - | - | + | - | + |
| Phosphate esters | D- Fructose- 6- Phosphate | - | - | - | - | + | - | + | + | - | - | + | - | + |
| | α - D- Glucose- 1- Phosphate | - | - | - | - | + | - | + | + | - | - | + | - | + |
| | D- Glucose- 6- Phosphate | - | - | - | - | + | - | + | + | - | - | + | - | + |
| | D- L- α - Glycerol Phosphate | - | - | - | - | + | + | + | + | - | - | + | - | + |

Table 4.5: Sugars and sugar derivatives as carbon source for filamentous isolates

| Substrates: | TTBCG01 | TTBCG02 | TTBCG03 | SWNCG01 | SWNCG02 | NGCD | NGCD01 | UCSR01 | HCBCG01 | OS1001a | OS1001b | OS1004a | OS1004b |
|------------------------------|---------|---------|---------|---------|---------|------|--------|--------|---------|---------|---------|---------|---------|
| Sugars and sugar derivatives | | | | | | | | | | | | | |
| L- Arabinose | - | - | - | - | + | + | - | + | - | - | + | - | - |
| D- Cellobiose | - | - | - | - | + | - | - | + | - | - | + | - | + |
| D- Fructose | - | - | - | - | + | + | + | + | - | - | + | - | + |
| L- Fucose | - | - | - | - | + | - | + | + | - | - | + | - | - |
| D- Galactose | - | - | - | - | + | + | - | + | - | - | + | - | - |
| Gentiobiose | - | - | - | - | + | - | + | - | - | - | + | - | - |
| α - D- Glucose | - | - | - | + | + | + | - | + | - | - | + | - | + |
| m- Inositol | - | - | - | - | + | + | + | + | - | - | + | - | - |
| α - D- Lactose | - | - | - | - | + | - | - | - | - | - | + | - | + |
| Lactulose | - | - | - | - | + | - | - | - | - | - | + | - | + |
| Maltose | - | - | - | + | + | + | + | + | - | - | + | - | + |
| Maltotriose | - | - | - | + | + | + | + | + | - | - | + | - | + |
| D- Mannose | - | - | - | + | + | + | + | + | - | - | + | - | + |
| D- Melezitose | - | + | - | - | + | - | + | + | - | - | + | - | + |
| D- Melibiose | - | - | - | - | + | - | - | + | - | - | + | - | + |
| Palatinose | - | - | - | + | + | - | + | + | - | - | + | - | + |
| D- Psicose | - | - | - | - | + | - | + | + | - | - | + | - | + |
| D- Raffinose | - | - | - | - | + | - | + | + | - | - | + | - | + |
| L- Rhamnose | - | - | - | - | + | - | + | + | - | - | + | - | + |
| D- Ribose | - | - | - | - | - | + | - | + | - | - | + | - | - |
| Stachyose | - | - | - | - | + | - | + | + | - | - | + | - | + |
| Sucrose | - | - | - | - | + | - | + | + | - | - | + | - | + |
| D- Tagatose | - | - | - | - | + | - | - | + | - | - | + | - | + |
| D- Trehalose | - | - | - | + | + | + | + | + | - | - | + | - | + |
| Turanose | - | - | - | + | + | - | + | + | - | - | + | - | + |
| Xylitol | - | - | - | + | + | - | + | - | - | - | + | - | + |
| D- Xylose | - | - | - | - | - | + | - | + | - | - | + | - | + |
| 3- Methyl- Glucose | - | - | - | - | + | - | + | + | - | - | + | - | + |

Table 4.6: Organic acids as carbon source for filamentous isolates

| Substrates: | TTBCG01 | TTBCG02 | TTBCG03 | SWNCG01 | SWNCG02 | NGCD | NGCD01 | UCSR01 | HCBCG01 | OSI001a | OSI001b | OSIO04a | OSI004b |
|--------------------------------|---------|---------|---------|---------|---------|------|--------|--------|---------|---------|---------|---------|---------|
| Organic acids | | | | | | | | | | | | | |
| Acetic acid | - | - | - | + | + | + | + | + | - | - | + | - | + |
| α - Hydroxybutyric acid | - | - | - | + | + | + | + | + | - | - | + | - | + |
| β - Hydroxybutyric acid | - | - | - | + | + | + | + | + | - | - | + | - | + |
| γ - Hydroxybutyric acid | - | - | - | + | + | - | + | - | - | - | + | - | + |
| p- Hydroxy- Phenylacetic acid | - | - | - | - | + | + | - | + | - | - | + | - | + |
| α - Ketoglutaric acid | - | - | - | + | + | + | + | + | - | - | + | - | + |
| α - Ketovaleric acid | - | - | - | + | + | + | + | + | - | - | + | - | + |
| L- Lactic acid | - | - | - | - | + | + | + | + | - | - | + | - | + |
| D- Malic acid | - | - | - | - | + | + | + | + | - | - | + | - | + |
| L- Malic acid | - | - | - | - | + | + | + | + | - | - | + | - | + |
| Propionic acid | - | - | - | + | + | + | + | + | - | - | + | - | + |
| Pyruvic acid | - | - | - | + | + | + | + | + | - | - | + | - | + |
| D- Lactic Acid Methyl Ester | - | - | - | - | + | - | + | - | - | - | + | - | + |
| Pyruvic Acid Methyl Ester | - | - | - | - | + | + | + | + | - | - | + | - | + |
| N- Acetyl- L- Glutamic Acid | - | - | - | + | + | - | + | + | - | - | + | - | + |

Table 4.7: Nucleosides as carbon source for filamentous isolates

| Substrates: | TTBCG01 | TTBCG02 | TTBCG03 | SWNCG01 | SWNCG02 | NGCD | NGCD01 | UCSR01 | HCBCG01 | OSI001a | OSI001b | OSIO04a | OSI004b |
|------------------------------|---------|---------|---------|---------|---------|------|--------|--------|---------|---------|---------|---------|---------|
| Nucleosides | | | | | | | | | | | | | |
| Adenosine | - | - | - | + | + | + | + | + | - | - | + | - | + |
| 2'- Deoxy Adenosine | - | - | - | + | + | + | + | + | - | - | + | - | + |
| Inosine | - | - | - | + | + | + | + | + | - | - | + | - | + |
| Thymidine | - | - | - | - | + | - | + | + | - | - | + | - | + |
| Uridine | - | - | - | - | + | + | + | + | - | - | + | - | + |
| Adenosine- 5'- Monophosphate | - | - | - | + | + | + | + | + | - | - | + | - | + |
| Thymidine- 5'- Monophosphate | - | - | - | + | + | - | + | + | - | - | + | - | + |
| Uridine- 5'- Monophosphate | - | - | - | + | + | + | + | + | - | - | + | - | + |

Table 4.8: Polymers as carbon source for filamentous isolates

| Substrates: | TTBCG01 | TTBCG02 | TTBCG03 | SWNCG01 | SWNCG02 | NGCD | NGCD01 | UCSR01 | HCBCG01 | OS1001a | OS1001b | OS1004a | OS1004b |
|-------------------------|---------|---------|---------|---------|---------|------|--------|--------|---------|---------|---------|---------|---------|
| Polymers | | | | | | | | | | | | | |
| α - Cyclodextrin | - | - | - | - | + | - | - | - | - | - | + | - | - |
| β - Cyclodextrin | - | - | - | - | + | - | - | - | - | - | + | - | - |
| Dextrin | - | - | - | + | + | - | + | + | - | - | + | - | - |
| Glycogen | - | - | - | - | + | - | + | - | - | - | + | - | - |
| Inulin | - | - | - | - | + | - | - | - | - | - | + | - | - |
| Mannan | - | - | - | - | + | - | - | - | - | - | + | - | - |
| Tween 40 | + | - | - | + | + | + | - | + | - | - | + | - | + |
| Tween 80 | - | - | - | + | + | + | + | + | - | - | + | - | + |

Table 4.9: Amino acids as carbon source for filamentous isolates

| Substrates: | TTBCG01 | TTBCG02 | TTBCG03 | SWNCG01 | SWNCG02 | NGCD | NGCD01 | UCSR01 | HCBCG01 | OS1001a | OS1001b | OS1004a | OS1004b |
|----------------------|---------|---------|---------|---------|---------|------|--------|--------|---------|---------|---------|---------|---------|
| Amino acids | | | | | | | | | | | | | |
| L- Alaninamide | - | - | - | + | + | + | + | + | - | - | + | - | + |
| D- Alanine | - | - | - | + | + | + | + | + | - | - | + | - | + |
| L- Alanine | - | - | - | + | + | + | + | + | - | - | + | - | + |
| Asparagine | - | - | - | - | + | + | + | + | - | - | + | - | + |
| L- Glutamic acid | - | - | - | - | + | + | + | + | - | - | + | - | + |
| L- Pyroglutamic Acid | - | - | - | - | + | + | + | + | - | - | + | - | + |
| L- Serine | - | - | - | + | + | + | + | + | - | - | + | - | + |

4.12 MORPHOLOGICAL SHIFTS/ VARIATIONS

Microscopic evaluation of isolates that gave positive results for substrate utilization on Biolog showed no shift in morphology of the isolates. Isolates were still in filamentous form after substrate utilization.

CHAPTER 5

DISCUSSION

5.1 EFFECT OF TEMPERATURE AND pH

Temperature and pH are very important parameters in the growth of all living organisms. For biochemical reactions to occur efficiently, enzymes are required that specifically perform functions that are unique to all individual enzymes. For these enzymes to perform at their optimum, temperature and pH need be exactly right, as enzymes are sensitive to environmental changes. Enzymes are found in all living systems and are responsible for catabolism (breakdown of substrates) and anabolism (building up of substrates). The rate at which all biochemical reactions will take place depends largely on, amongst others, the temperature and pH of the reaction medium. In general, it may be said that successful metabolism is dependent on temperature and pH. Bond *et al.* (1999) suggested that pH is an important factor in determining whether PAOs or GAOs are favoured in EBPR systems.

In this study, the effect of temperature and pH on filamentous bacterial growth was investigated. With the exception of 4 isolates (OSI001a, OSI001b, OSI004a and OSI004b), good growth was observed at 12°C and excellent growth at 20°C and 30°C (Table 4.3). OSI001a, OSI001b, OSI004a and OSI004b, however demonstrated optimal

growth at higher temperature as compared to all the other isolates. This may be due to the nature of the treatment system that these four isolates were obtained in i.e. a laboratory scale anoxic-aerobic system treating high organic strength industrial waste. These findings however, lead to doubt that these four isolates are in fact *M. parvicella* strains, as this filamentous bacterium is known to proliferate in cold conditions. Isolates obtained from the full-scale municipal systems showed temperature preference typical of most known filaments. In general, it may be deduced that these isolates exhibit good growth over a wide range of temperature. It is noted however, that most of these isolates grew best at 20°C. This characteristic of growing well over a wide range of temperature may present these isolates with a growth advantage over other or some microorganisms.

Similarly, with the exception of the 4 laboratory-scale isolates (OSI001a, OSI001b, OSI004a and OSI004b), the filamentous isolates demonstrated a wide pH tolerance (table 4.3). A broad pH tolerance is important for these types of organisms to survive, and even proliferate in treatment systems. The narrow range of pH tolerance observed in isolates OSI001a, OSI001b, OSI004a and OSI004b again may be explained by the unique conditions being experienced in the parent system. There may be more ways than one of interpreting these findings, i.e. lower pHs may be considered inhibitory to the growth of some of these isolates as they gave better growth at higher pHs or, given that most of the isolates showed good growth (and some with excellent growth) at these low pHs, it may as well be said that they grow well at a wide range of pHs in which case it may be deduced that this characteristic poses a growth advantage in favour of these filamentous organisms.

5.2 AROMATIC COMPOUNDS AS CARBON SOURCE

A carbon source is essential for microorganisms for survival. In this study, various aromatic compounds were tested as a way of profiling the bacterial isolates and partially attempting to understand their complex metabolic behaviours. Isolates were grown on I medium (excluding carbon substrate addition) with phenol, o-cresol and 2,5-dihydroxybenzoic acid, respectively, serving as carbon sources. Two controls were employed, the first being the test medium without any aromatic compound addition, and the second medium excluding aromatic as well as carbonate (CO_3) addition. Phenol, O-cresol and 2,5-dihydroxybenzoic acid proved to be inhibitory to the growth of all the isolates (Table 4.3). However, it was noted in these experiments that almost all the isolates had an affinity for CO_3 . CO_3 (lime) is routinely used in wastewater and is known to improve sludge settleability and flocculation of suspended solids. It is noteworthy to point out that the growth of isolates on a control without CO_3 may suggest that they have the ability to survive without a carbon source. This may be attributed to the fact that most of these organisms have storage products such as glycogen, PHB and poly-P (Table 4.2). The observed little to no growth on the broth control may be attributed to lack of dissolved oxygen (DO) in the broths but this deduction may not be considered conclusive. More studies are still required. Further research is required, however, to determine if CO_3 can be utilized as a carbon substrate or simply provides ideal alkalinity for filamentous bacterial growth. Furthermore, filamentous growth observed on the control media without CO_3 addition may be attributable to storage polymers such as glycogen, PHB and polyP (Table

4.2), which enhance organisms survival rates under substrate limiting conditions (Ballicora *et al.*, 2003). It was also stated by Belanger and Hatfull (1999) that bacterial glycogen is generally considered a storage compound as it accumulates in stationary phase and under growth limiting properties and is therefore presumed to serve as a reservoir of carbon and energy and a strategy to cope with transient starvation conditions in the environment (Ballicora *et al.*, 2003). In addition, it has been reported that some organisms e.g. some actinomycetes, scavenge from nonnutrient substances such as purified agar (Labeda and Shearer, 1990). The possibility that this scenario may also apply with these organisms is therefore worth a comprehensive study.

5.3 PHOSPHORUS UPTAKE

The microorganisms of the activated sludge wastewater treatment process experience rapidly changing conditions and availability of nutrients (feast/ starvation regime with respect to carbon source). The production of storage polymers such as glycogen and poly- β -hydroxyalkanoates (PHAs) has been shown to be a means by which sludge active biomass respond to feast/ starvation regimes (Dawes and Senior, 1973 as cited by Carta *et al.*, 2001). As previously discussed in section 2.3.2, phosphate-accumulating organisms (PAOs) are selected for in EBPR and can dominate the microbial community. They store intracellular organic substrates (in the anaerobic zone) such as poly-hydroxybutyrate (PHB) and, degrade intracellular stores of polyphosphate (polyP) and glycogen, which brings about the release of phosphate from the mixed liquor. In the aerobic zone, the

stored PHB is utilized and polyP is removed from the mixed liquor, a result to which is phosphorus removal (Bond, <http://www.uea.ac.uk/env/studentships/bondp1.htm>).

In essence, the ability of activated sludge microorganisms to store polymers such as PHB, PHA and glycogen has a great effect on the performance of EBPR processes. In fact, it is known that the removal of phosphorus from wastewaters depends largely on the ability of the microbes to actually store these compounds and perform such functions under the designated activated sludge environments (i.e. aerobic/ anaerobic zone/s).

PAOs have an advantage in BNR systems, in that they are able to sequester carbon substrates and store these (i.e. PHB, glycogen) for low nutrient conditions. PolyP acts as an energy source facilitating storage of such compounds. Therefore, filaments containing these storage compounds would have a selective advantage over other organisms, particularly under conditions of anaerobic-aerobic sequencing and low substrate conditions (low food to microorganism ratio). Most of the isolates were positive for PHB and glycogen storage (table 4.2) therefore, giving indication of added survival and proliferation abilities of filamentous bacteria, particularly in BNR systems. However, only isolates OSI001a, OSI001b, OSI004a and OSI004b were positive for polyP storage as well as being negative for the production of phosphatase. This would infer added advantage for these organisms therefore, increasing risk of bulking. Beccari *et al.* (1998), while investigating storage response in a bulking sludge; found that bulking sludge presented a high storage capacity whereby PHB was stored up to about the dry weight of the biomass,

therefore storage capacity would remain mainly unsaturated under usual organic load. According to their findings, Beccari *et al.* (1998) state that both high storage capacity and resistance to starvation can provide further competitive advantage for filaments in a large range of operating conditions, thus it is unlikely that kinetic control of such a bulking could be easily achieved by increasing or decreasing organic load or frequency of feed cycles. It is quite evident that filamentous bacteria may therefore be capable of EBPR but this has not been conclusively proven. There is therefore, a need for further research. Beccari *et al.* (1998) also point out that, too little is still known about the importance of storage under steady state conditions, with particular reference to its influence on population dynamics.

5.4 DENITRIFICATION

Microbial denitrification is the process that achieves removal of nitrates and nitrites from wastewater under anoxic conditions. When DO becomes limiting, facultative heterotrophs switch from oxygen to nitrate/nitrite as terminal electron acceptors and respire anoxically (Drysdale *et al.*, 2001). It has however, been proven that denitrification is more complex than a simple step- wise reduction of nitrate, via nitrite, to gaseous nitrogen by individual bacteria exposed to anoxic conditions. A novel colorimetric test designed by Drysdale *et al.*, (2001) showed that denitrifying organisms can be sub-divided into four functional groups based on their ability to reduce nitrate and/or nitrite. Those organisms with the ability to reduce both nitrate and nitrite have a selective advantage over nitrate and

exclusive nitrite reducers in that more electron acceptor is available for growth and survival. All of the isolates, except OSI001a, OSI001b, OSI004a and OSI004b (nitrate respirers), demonstrated both nitrate and nitrite reduction using the Drysdale *et al.*, (2001) method (Table 4.3). These results may explain why anoxic-aerobic sequencing in BNR systems can induce bulking (Ekama *et al.*, 1999). From the results obtained, it is evident that the isolates under study are capable of denitrification. They belong to three different groups of denitrifiers i.e. sequential denitrifiers, true denitrifiers and nitrate respirers. The sequential denitrifiers reduce nitrate to nitrite and nitrite is also further reduced but when grown in a nitrite medium they do not reduce it at higher concentrations. The true denitrifiers are able to reduce both nitrates as well as nitrites. The nitrate respirers on the other hand are only capable of reducing nitrates to nitrites with no further reduction of the nitrites produced (Drysdale *et al.*, 2001). It is noteworthy to point out that contrary to the notion that filaments are not known to denitrify past NO_2 (Nielsen, 2003), the results obtained in this study disprove this. The novel test employed in this study (Drysdale *et al.*, 2001) has been proven to demonstrate nitrite reduction capacity in numerous environmental bacterial strains not previously known to do so. Findings emerging from this work also call for a detailed investigation geared into determining the exact role played by filamentous bacteria in BNR.

5.5 EFFECT OF NaCl AND ANTIBIOTICS

Relatively low NaCl tolerance was generally observed amongst the isolates, with only TTBCG03, SWNCG02 and HCBCG01 proving a bit more resistant. It may therefore be said that the isolate HCBCG01 has a higher threshold for NaCl and that NaCl is inhibitory to the growth of these organisms. OSI001a, OSI001b and OSI004a appeared to be completely sensitive to all four antibiotics tested. Most isolates seemed to be most sensitive to furazalidone and most resistant to penicillin G. Isolates TTBCG03 and NGCD appeared to be resistant to ampicillin, furazalidone and penicillin G, while DLNT01 seemed to be very sensitive to penicillin G. It is not clear as to the extent of antibiotics occurring in activated sludge systems, and further research is required to understand any possible *in situ* effects of antibiotics mediated microbial competition.

Previous studies have shown that waste effluents from hospitals contain higher levels of antibiotic- resistant enteric bacteria than waste effluents derived from other sources (Fontaine *et al.*, 1976, Grabow and Prozesky, 1973, Linton *et al.*, 1974 and Walter and Vennes, 1985 as cited by Guardabassi *et al.*, 1998). Extensive investigations have not been conducted concerning the effects on the indigenous bacterial microflora of sewage caused by the release into sewers of effluents from hospitals or other potential sources of antibiotic- containing effluent such as pharmaceutical plants (Guardabassi *et al.*, 1998).

5.6 LIPID HYDROLYSIS

The edible oil industry has a very high specific water intake compared to other industries in South Africa and as much as 40% of this water is released as effluent into the municipal sewer systems (Steffen, Roberts and Kirsten, 1989 as cited by Reddy *et al.*, 2003). Treatment of edible oil effluent preceding discharge into the sewers is imperative as such effluents contain a mixture of high concentrations of pollutants such as fats, oils, sodium, phosphorus, sulphates and many others (Hrudey, 1981 and Horan, 1990 as cited by Reddy *et al.*, 2003). Efficient treatment of edible oil effluent is usually achieved via a combination of physical, chemical and biological treatment processes (Dalzel, 1994 as cited by Reddy *et al.*, 2003) but, biological treatment of edible oil is seen as a cost-effective and efficient alternative (Mkhize *et al.*, 2000 as cited by Reddy *et al.*, 2003).

The composition of the substrate in the influent wastewater is accepted as one of the parameters that determine the proliferation of filamentous types in activated sludge process (Eikelboom, 2000 as cited by Bux, 2003). Filaments such as *M. parvicella* have been reported to be specialized lipid consumers and consume long chain fatty acids (LCFA) (Nielsen *et al.*, 2002 as cited by Bux, 2003). Recent studies also showed that *M. parvicella* preferred long chain fatty acids especially oleic acid (major LCFA in sunflower oil) as compared to simpler substrates like glucose and acetate (Andreasen and Nielsen, 1997 as cited by Bux, 2003). Due to its hydrophobic nature, *M. parvicella* is also known to play a role in foaming.

Given this information, it is quite evident that some filamentous bacteria do survive conditions where the wastewater contains a high amount of fats, oils and grease (FOG) and in fact, may prefer those as their substrate of choice to other substrates that may be available in the system. The isolates under study for this research have proven to have a high affinity for all the different edible oils tested (table 4.1). Essentially, while lipids are often hydrolyzed to glycerol and fatty acids, most of the isolates hydrolyzed the lipids completely so that not even fatty acids remained to react with the indicator dye used in the test media. It was also noted that isolates OSI001a, OSI001b, OSI004a and OSI004b were isolated from a bulking laboratory-scale system treating high organic strength edible oil effluent. It is therefore apparent that the affinity of filamentous bacteria for larger slowly degradable compounds, particularly lipids, is a widespread characteristic of numerous (if not all) of these organisms.

In a study conducted by Reddy *et al.* (2003), while evaluating activated sludge treatment in remediation of edible oil effluent, their MLE (Modified- Ludzack Ettinger) process (treating the edible oil) experienced washout of large amounts of biomass due to filamentous bulking. This resulted in high effluent TSS (Total Suspended Solids) and temporary loss in COD removal efficiency at the peak of the bulking period. No changes were made to the operating parameters of the MLE unit, the wastewater composition was hypothesized as the key factor responsible for inducing bulking. This filamentous bulking was noted to coincide with new edible oil effluent batch (soybean oil in addition to sunflower seed oil). In fact, at the time of filamentous bulking during treatment of edible

oil, Reddy *et al.* (2003) microscopically confirmed extensive inter-floc bridging with *Sphaerotilus* sp. being largely predominant and *Haliscomenobacter hydrossis*, *Sphaerotilus natans*, *Nostocoida limicola* and Type 1851 present in low numbers. It is apparent therefore, that *M. parvicella* is not the only filament that may be expected to thrive in bulking situations where the FOG (lipids) is high.

The fact that the filaments under scrutiny are able to breakdown complex substrates such as oils, presents them with an advantage of survival even in what could be referred to as complex environments. This finding may also be useful to industries producing edible oil where their effluent needs to be treated before discharge into municipal sewers. This information may also be useful to plant operators of wastewater treatment plants receiving and treating such industrial wastes, especially during periods of operation upset.

5.7 UREASE AND PHOSPHATASE

Urease is an enzyme that breaks down the carbon-nitrogen bond of amides (urea) to form carbon dioxide, ammonia and water (<http://medic.med.uth.tmc.edu/path/urease.htm>). Urea comprises a large component of urine and animal/human waste, which naturally enters most municipal systems in high concentrations. Therefore, it is understandable that most of the isolates tested positive for urease, as they were isolated from full-scale municipal systems. However, isolates DLNT01, HCBCG01, OSI001a, OSI001b, OSI004a and OSI004b demonstrated negative results for the presence of this enzyme. The absence of

this enzyme in isolates OSI001a, OSI001b, OSI004a and OSI004b is again most likely due to the nature of the industrial effluent being treated, with no domestic waste present. Phosphatase facilitates the cleavage of phosphate by the possessor, which implies that it's presence in an organism means that that organism may in fact store or utilize phosphate as a substrate at any given point in its growth. The presence of these enzymes in the organisms therefore suggests that they have more substrates they can utilize in wastewater treatment systems, which may be considered a growth advantage.

5.8 STARCH HYDROLYSIS, MILK HYDROLYSIS AND GELATIN LIQUEFACTION

Hydrolysis of starch leads to production of readily degradable sugars. The enzyme amylase is responsible for starch hydrolysis. A lot of wastewaters are expected to contain starch as one of their components especially those treating domestic waste. Isolates were therefore tested for their ability to hydrolyze starch and only one out of fourteen isolates gave a negative reaction (table 4.3). It is possible that filamentous bacteria have an affinity for larger and more slowly biodegradable compounds, such as starches and lipids. This would infer a strong selective advantage over other floc-forming organisms in systems with high concentrations of such compounds, thus leading to potential bulking and foaming problems.

The ability to hydrolyze milk protein was seen in all but four of the isolates. This again substantiates the general ability of filamentous bacteria to hydrolyze larger slowly biodegradable compounds. Systems treating dairy waste may be partial to bulking from such isolates.

Gelatin is a protein derived from collagen. The exoenzyme gelatinase first hydrolyzes gelatin into polypeptides, and then further break down the polypeptides into smaller amino acid molecules. These can then be easily transported into the bacterial cells (<http://www2.austin.cc.tx.us/microbugz/38nutgel.html>). While gelatin hydrolysis was not widespread, specific populations may be present in selected systems, such as those treating abattoir wastes.

5.9 BIOLOG AS A MEANS OF BIOCHEMICAL PROFILING/ FINGERPRINTING

Five of the isolates were negative for all the substrates presented by the Biolog MicroPlates. These were DLNT01, TTBCG03, OSI004a, OSI001a and HCBCG01. Results for DLNT01 are not shown as it was negative for all substrates presented by the Biolog GN MicroPlate and, was the only Gram negative isolate. The remaining 9 isolates showed positive results for some of the substrates provided by the Biolog MicroPlates. The fact that some isolates did not show any metabolism with any of the substrates may be because filamentous bacteria may have a complex metabolism and are slow-growers; also, Biolog is not optimized for filamentous bacteria. However, this system may be used, not

necessarily for identifying but for metabolic profiling of filamentous bacteria. The fact that it has so many organic substrates as carbon sources, which may be tested all at once, makes the system very convenient to use for profiling/ fingerprinting purposes. Van Heerden *et al.* (2000) also stated that, any one organism will not necessarily utilize all the available substrates in a system, nor does the utilization of some of the substrates suggest that this is the complete set of substrates that a particular organism can use. Also, substrates might not match the activity of a particular organism (van Heerden *et al.*, 2000). Toxic effects of the redox dyes may in fact inhibit the growth of the organism (Ullrich *et al.*, 1996 as cited by van Heerden *et al.*, 2000).

From the results obtained for the Biolog in this study, it is self-evident that filamentous bacteria can and do metabolize a wide range of substrates including slowly biodegradable polymers such as starch, dextrin, peptides and lipids (tables 4.4- 4.9) which, makes understanding the metabolic patterns of these bacteria even more complex than ever thought possible. Oligopeptides and polypeptides are formed by polymerization of amino acids. All proteins are polypeptides (Mathews and Van Holde, 1996). The results show that some of the isolates are positive for the utilization of peptides. This result suggests that filaments have the ability to strive on proteins, breaking them down to amino acids, which they can utilize further for other cellular functions. This coincides with the results obtained for amino acid utilization whereby isolates show an affinity for amino acids. These results put forward the idea that, in conditions where they need to strive on polymers, filaments would be able to break them down into monomers that they can use

for survival and cellular functions. This fact alone means that they have the ability to survive distressing conditions with limited resources which gives them a growth advantage over other organisms. They also show positive results for growth on a variety of sugars, organic acids and especially nucleosides. Coupling nucleosides, which are the monomers, forms the polymers Deoxyribonucleic acid and Ribonucleic acid (Mathews and Van Holde, 1996). Knowing the substrates filaments utilize, is one vital stepping stone into characterizing and understanding filamentous bacteria and, eventually, the processes that take place in the activated sludge process. Also, this is important in that it brings about insight to researchers on issues relating to the nutritional requirements of filamentous bacteria which in turn, may lead the way to finally understanding exactly what is it that enhances and/or inhibits the growth of filaments in activated sludge processes.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

- (1) Based on the results obtained during this study, it is evident that filamentous bacteria are more diverse and complex in their biochemistry and physiology hence the difficulty in achieving long- term optimal control of filamentous bulking in activated sludge.
- (2) Filamentous bacteria have the ability to survive during times of starvation where growth factors are limiting and, this may be attributed to their ability to store storage compounds such as PHB, glycogen and polyP. Such a study as this may in fact aid in an in-depth understanding of these bacteria therefore lead to a better understanding of bulking and foaming, and the control thereof.
- (3) The filaments' ability to store storage compounds and denitrify, suggests that they may in fact play significant roles in denitrification and EBPR, and these areas still need a more detailed study to establish the exact role played by filamentous bacteria in these processes.

- (4) It is also concluded from this study that the filamentous bacteria under study are sensitive to aromatic compounds and that they have an affinity for slowly biodegradable polymers such as lipids, nucleosides, proteins/ peptides, dextrin and starch.
- (5) It is concluded that, the use of Biolog for biochemical profiling/ fingerprinting of filamentous bacteria is useful, however, due to the possibility that some organisms may in fact, not grow and/ or may give negative results on some and/ or all substrates, other strategies for biochemical profiling be established and used in this regard.

6.2 RECOMMENDATIONS

- (1) Molecular techniques need to be employed as a means of identifying these filamentous bacteria using molecular probes and fluorescent *in situ* hybridization (FISH).
- (2) Pure culture results need to be verified *in situ* in both laboratory- and full-scale processes treating real sewerage. This may be achieved by means of micro-autoradiography (MAR) with the use of radioactively- labeled substrates.
- (3) In addition, it is recommended that focus be directed towards studying the complex macromolecules that filamentous bacteria utilize as substrates such as lipids, starch and proteins as they demonstrate an affinity for such substrates, which, earns them a growth advantage over other bacteria.
- (4) Batch tests are recommended for BNR purposes i.e. denitrification and phosphorus removal, so as to understand in depth all the roles played by filamentous bacteria in activated sludge processes/ wastewater treatment, especially AA- bulking (anoxic- aerobic).
- (5) It is also recommended that, in a case where Biolog is used for biochemical profiling/ fingerprinting of filamentous bacteria and a negative result is executed

by an organism, a different approach be used in testing utility of that substrate by that organism as a negative result with the Biolog may not necessarily mean that the organism under study does not utilize that particular substrate.

- (6) Also, numerous other substrates may be used in addition to the Biolog as it (Biolog) does not necessarily mean that the substrates presented by Biolog are the sole substrates any given organism can metabolize at any given time.

REFERENCES

Atlas, RM. (1993). *Handbook of Microbiological Media*. USA, CRC Press.

Bae, J, Kim, S and Chang, H. (1997). Treatment of Landfill Leachates: Ammonia Removal via Nitrification and Denitrification and further COD Reduction via Fenton's Treatment Followed by Activated Sludge. *Wat. Sci. Tech.*, Vol. 36, No. 12, pp 341-348.

Ballicora, MA, Iglesias, AA and Preiss, J. (2003). ADP-Glucose Prophosphorylase, A Regulatory Enzyme for Bacterial Glycogen Synthesis. *Microbiol. Mol. Biol. Rev.*, Vol 67, No. 2, pp 213-225.

Barber, WP and Stuckey, DC. (2000). Nitrogen Removal in a Modified Anaerobic Baffled Reactor (ABR): 1, Denitrification. *Wat. Res.*, Vol. 34, No. 9, pp 2413-2422.

Beccari, M, Majone, M, Massanisso, P and Ramadori, R. (1998). A Bulking Sludge with High Storage Response Selected under Intermittent Feeding. *Wat. Res.*, Vol. 32, No. 11, pp 3403-3413.

Belanger, AE and Hatfull, GF. (1999). Exponential-Phase Glycogen Recycling is Essential for Growth of *Mycobacterium smegmatis*. *Journ. of Bact.*, Vol 181, No. 21, pp 6670- 6678.

Biolab Catalogue- Version 2. (1997). Merck, Version 11.

Biolog Inc. (2001). Instructions for use. USA.

Bitton, G. (1999). *Wastewater Microbiology* (2nd edition). Canada, Wiley-Liss.

Bond, P. (<http://www.uea.ac.uk/env/studentships/bondp1.htm>, accessed November 2003).

What's upsetting the Activated Sludge Biological P Removal? Determining Factors in the Competition Between Bacterial Polyphosphate and Glycogen Accumulation.

Bond, PL, Erhart, R, Wagner, M, Keller, J and Blackall, L L. (1999). Identification of Some of the Major Groups of Bacteria in Efficient and Nonefficient Biological Phosphorus Removal Activated Sludge System. *Appl. and Enviro. Micro.*, Vol. 65, No. 9, pp 4077- 4084.

Bux, F. (2003). *Development and Microbial Community Analysis of a Biological Treatment Process for Edible oil Effluent*. PhD thesis, Department of Biotechnology, Durban Institute of Technology.

Bridson, EY. (1995). *The Oxoid Manual* (7th edition). England, Unipath Limited.

Carta, F, Beun, JJ, van Loosdrecht, MCM and Heijnen, JJ. (2001). Simultaneous Storage and degradation of PHB and Glycogen in Activated Sludge Cultures. *Wat. Res.*, Vol. 35, No. 11, pp 2693-2701.

Casey, TG, Wentzel, MC and Ekama, GA. (1999). Filamentous Organism Bulking in Nutrient Removal Activated Sludge Systems, Paper 9: Review of Biochemistry of Heterotrophic Respiratory Metabolism. *Wat. SA*, Vol. 25, No. 4, pp 409-424.

Chuang, S and Ouyang, C. (2000). The Biomass Fractions of Heterotrophs and Phosphate –Accumulating Organisms in a Nitrogen and Phosphorus Removal System. *Wat. Res.*, Vol. 34, No. 8, pp 2283-2290.

Demoulin, G, Goronszy, MC, Wutscher, K and Forsthuber, E. (1997). Co-current Nitrification/Denitrification and Biological P-Removal in Cyclic Activated Sludge Plants by Redox Controlled Cycle Operation. *Wat. Sci. Tech.*, Vol. 34, No. 1, pp 215-224.

Department of Microbiology- Durban Institute of Technology. (2003). South Africa. (Personal communication).

Drysdale, GD, Kasan, HC and Bux, F. (1999). Denitrification by Heterotrophic Bacteria during Activated Sludge Treatment. *Wat. SA*, Vol. 25, No. 3, pp 357-362.

Drysdale, GD, Kasan, HC and Bux, F. (2001). Assessment of Denitrification by the Ordinary Heterotrophic Organisms in an NDBEPR Activated Sludge System. *Wat. Sci. Tech.*, Vol. 43, No. 1, pp 147-154.

Eckenfelder, WW and Musterman, JL. (1995). *Activated Sludge Treatment of Industrial Wastewater*. England, Technomic Publishing Company, Inc.

Eikelboom, DH. (1975). Filamentous Bacteria Observed in Activated Sludge. *Wat. Res.*, Vol 9, pp 365-388.

Eikelboom DH. (2001). TNO- MEP, Netherlands. (Personal communication).

Ekama, GA, Wentzel, MC, Lakay, MT, Pilson, RA, Mellin, HKO and Casey, TG. (1999). *Causes and control of low F/M filament bulking in nutrient removal activated sludge systems*. WRC Report No. 542/1/99, South Africa, Water Research Commission.

Erdal UG. (2002). *The Effects of Temperature on System Performance and Bacterial Community Structure in a Biological Phosphorus Removal System*. PhD thesis, Department of Civil and Environmental Engineering, Virginia Polytechnic Institute and State University.

Fuerhacker, M, Bauer, H, Ellinger, R, Sree, U, Schmid, H, Zibuschka, F and Puxbaum, H. (2000). Approach for a Novel Control Strategy for Simultaneous Nitrification/Denitrification in Activated Sludge Reactors. *Wat. Res.*, Vol. 34, No. 9, pp 2499-2506.

Gerardi, MH. Horsfall, FL III, Barron, TR, Baumgardner, MT, Boyt, L *et al.* (1990). *Wastewater Biology: The Microlife*. USA, The Water Pollution Control Federation.

Gray, N F. (1989). *Biology of Wastewater Treatment*. USA, Oxford Science Publications

Gray, NF. (1990). *Activated Sludge: Theory and Practice*, USA, Oxford University Press.

Guardabassi, L, Petersen, A, Olsen, JE and Dalsgaard, A. (1998). Antibiotic Resistance in *Acinetobacter* spp. Isolated from Sewers Receiving Waste Effluent from a Hospital and a Pharmaceutical Plant. *Appl. Environ. Microbiol.*, Vol. 64, No. 9, pp 3499-3502.

Henze, M, Harremoes, P, la Cour, J and Arvin, E. (1997). *Wastewater Treatment: Biological and Chemical Processes* (2nd edition). USA, Springer- Verlag.

Hippen, A, Rosenwinkel, K, Baumgarten, G and Seyfried, CF. (1997). Aerobic Deammonification: A new Experience in the treatment of Wastewaters. *Wat. Sci. Tech*, Vol. 35, No. 10, pp 111-120.

Horan, N J. (1990). *Biological Wastewater Treatment Systems: Theory and Operation*. England, John Wiley and Sons Ltd.

<http://www.nutritionfocus.com> (Accessed on 06/04/2001).

<http://www.serva.de/products/knowledge/061097.shtml> (Accessed on 06/04/2001).

<http://medic.med.uth.tmc.edu/path/urease.htm> (Accessed November 2003).

<http://128.175.57.161/Harding/401.rtf>. (Accessed November 2003).

<http://www.hoslink.com.histo/6.HTM>. (Accessed May 2003). The Periodic Acid Schiff's Method.

<http://www2.austin.cc.tx.us/microbugz/38nutgel.html> (Accessed November 2003).

<http://www.worthington-biochem.com/manual/C/CEL/html> (Accessed in March 2001)

Jenkins, D, Richard, M G and Daigger, G T. (1986). *Manual on the Causes and Control of Activated Sludge Bulking and Foaming*. South Africa, Water Research Commission

Jenkins D. (<http://www.ce.berkeley.edu/~jenkins/>, Accessed November 2003).

Kämpfer, P, Weltin, D, Hoffmesiter, D and Dott, W. (1995). Growth Requirements of Filamentous Bacteria Isolated from Bulking and Scumming Sludge. *Wat. Res.*, Vol 29, pp 1585-1588.

Kämpfer, P. (1997). Detection and Cultivation of Filamentous Bacteria from Activated Sludge. *FEMS Microbio. Ecol.*, Vol 23, pp 169-181.

Labeda, DP and Shearer, MC. (1990). 'Isolation of Actinomycetes for Biotechnological Applications', *In* Labeda, DP (ed), *Isolation of Biotechnological Organisms from Nature*. USA, McGraw-Hill.

Lemmer, H and Kroppenstedt, M. (1984). Chemotaxonomy and Physiology of Some Actinomycetes Isolated from Scumming Activated Sludge. *System. Appl. Microbiol.*, Vol 5, pp 124-135.

Lindrea, KC, Seviour, EM, Seviour, RJ, Blackall, LL and Soddell, JA. (1999). 'Practical Methods for the Examination and Characterization of Activated Sludge', *In* Seviour, RJ and Blackall, LL (eds), *The Microbiology of Activated Sludge*. USA, Kluwer Academic Publishers, pp 283-293.

Mathews, CK and van Holde, KE. (1996). *Biochemistry* (2nd edition). USA, The Benjamin/Cummings Publishing Company, Inc.

Mino, T, Van Loosdrecht, MCM and Heijnen, JJ. (1998). Microbiology and Biochemistry of the Enhanced Biological Phosphorus Removal Process. *Wat. Res.*, Vol 32, No. 11, pp 3193-3207.

Mulder, EG and Deinema, MH. (1981). 'The Sheathed Bacteria', In Starr, P. M. *et al.* (eds), *The Prokaryotes: A Hand book on Habitats, Isolation, and Identification of Bacteria*. USA, Springer-Verlag, pp 425-440.

Munch, EV and Pollard, PC. (1997). Measuring Bacterial Biomass-COD in Wastewater Containing Particulate Matter. *Wat. Res.*, Vol 31, No 10, pp 2551-2556.

Nielsen, AT, Liu, W, Filipe, C, Grady, L (Jnr), Molin, S and Stahl, DA. (1999). Identification of a Novel Group of Bacteria in Sludge from Deteriorated Biological Phosphorus Removal Reactor. *Appl. Enviro. Microbiol.*, Vol 65, No 3, pp 1251-1258.

Nielsen, AT. (2003). Aarlborg University. (Personal Communication).

Orhan, D and Artan, N. (1994). *Modelling of Activated Sludge Systems*. England, Technomic Publishing Company, Inc.

Reddy, K, Drysdale, GD and Bux, F. (2003). Evaluation of Activated Sludge Treatment and Settleability in Remediation of Edible Oil Effluent. *Wat. SA*, Vol 29, No. 3, pp 245-250.

Richard, M. (1989). *Activated Sludge Microbiology*. USA, The Water Pollution Control Federation.

Robertson, LA and Kuenen, JG. (1984). Aerobic Denitrification- Old Wine in New Bottles?. *Ant. Van Leeuw.*, Vol. 50, pp 525-544.

Schroeder, ED. (1997). *Water and Wastewater Treatment*. USA, Mc-Graw-Hill, Inc.

Seunghum, K. (2001). CABI Bioscience, United Kingdom. (Personal communication).

Seviour, EM, Williams, C, DEGrey, B, Soddell, JA, Seviour, RJ and Lindrea, KC. (1994). Studies on Filamentous Bacteria from Australian Activated Sludge Plants. *Wat. Res.*, Vol 28, No. 11, pp 2335-2342.

Stahl, DA and Amann, R. (1991). 'Development and Application of Nucleic Acid Probes', In Stackenbrandt, E and Goodfellow, M (eds), *Nucleic Acid Techniques in Bacterial Systematics*. England, John Wiley and Sons Ltd.

Terblanche, APS. (1991). Health Harzards of Nitrate in Drinking Water. *Wat. SA*, Vol. 17, No. 1, pp 77-82.

Van Heerden, J, Ehlers, MM, Korf, C and Cloete, TE. (2000). *Biolog for the Determination of Microbial Species Diversity and Evenness in Activated Sludge Systems*, South Africa, Water Research Commission.

Van Veen, WL. (1973). Bacteriology of Activated Sludge, in Particular the Filamentous Bacteria. *Ant. Van Leeuw.*, Vol 39, pp 189-205.

Wentzel, MC, Lötter, LH, Loewenthal, RE and Marais, GvR. (1986). Metabolic Behaviour of *Acinetobacter* spp. in Enhanced Biological Phosphorus Removal- A Biochemical Model. *Wat. SA*, Vol 12, No. 4, pp 209-224.

Ziegler, M, Lange, M and Dott, W. (1990). Isolation and Morphological and Cytological Characterization of Filamentous Bacteria from Activated Sludge. *Wat. Res.*, Vol 24, No. 12, pp 1437-1451.

APPENDICES

Appendix A: Gram stain (Modified Hucker method)

Reagents

Solution 1:

A. Crystal violet 2g B. Ammonium oxalate 0.8g

Ethanol, 95% 20ml Distilled water 80ml

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

Solution 2:

Iodine 1g

Potassium iodide 2g

Distilled water 300ml

Solution 3:

Safranin O (2.5% in 95% ethanol) 10ml

Distilled water 100ml

Method:

Prepare 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 seconds with 95% ethanol. Then stain slide for 1 minute with solution 3, rinse well and blot dry.

Appendix B: Neisser stain**Reagents****Solution 1:**

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

The above (A and B) are separately prepared 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) 33.3ml

Distilled water 66.7ml

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 C: Polyhydroxybutyrate (PHB) stain**Reagents****Solution 1:**

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

Solution 2:

Sarfanin O 0.5% w/v aqueous

Method:

Prepare thin sample smears on microscope slides and allow to air dry. Then stain slides for 10 minutes with solution 1 and then rinse well with water for 1 second. Stain this preparation with solution 2 for 10 seconds, rinse well with water and then blot dry.

Appendix D: Crystal violet sheath stain**Reagent**

Crystal violet 0.1% w/v aqueous solution

Method:

Mix a wet preparation of the sample with 1 drop 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 E: R2A agar**Composition per liter:**

| | |
|---------------------------------|--------|
| 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 casein | 0.25g |
| Peptic digest of animal tissue | 0.25g |
| MgSO ₄ , anhydrous | 0.024g |

pH 7.2 ± 0.2 at 25°C

Preparation of medium: Add components to distilled/deionized 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 F: CGYA (Casitone Glycerol Yeast Autolysate Agar)**Composition per liter**

| | |
|------------------------|--------|
| Bacto casitone (Difco) | 5.0g |
| Yeast autolysate | 1.0g |
| Glycerol | 10.0mL |
| Agar | 16.0g |

Preparation of Medium: Add components to distilled/deionized 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 (Bridson, 1995).

Appendix G: SCY Medium**Components per liter:**

| | |
|---------------------------------|--------|
| Agar | 10.0g |
| Sucrose | 1.0g |
| Pancreatic digest of casein | 0.92g |
| Yeast Extract | 0.25g |
| NaCl | 0.05g |
| Papaic digest of soybean meal | 0.03g |
| K ₂ HPO ₄ | 0.025g |
| Thiamine | 0.4mg |
| Cyanocobalamin | 0.01mg |

pH 7.3 \pm 0.2 at 25°C

Preparation of Medium: Add components (except for the vitamins) to distilled/deionized water and bring volume to 1.0L. Mix thoroughly and autoclave at psi pressure- 121°C for 15 mins. 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 H: I Medium

Composition in grams per liter:

| | |
|---|--------------------|
| Agar (Oxoid) | 10 |
| Glucose | 0.15 |
| (NH ₄) ₂ SO ₄ | 0.5 |
| Ca(NO ₃) ₂ | 0.01 |
| K ₂ HPO ₄ | 0.05 |
| MgSO ₄ ·7H ₂ O | 0.05 |
| KCl | 0.05 |
| CaCO ₃ | 0.1 |
| Vitamin B12 | 10 ⁻⁵ |
| Thiamine | 4x10 ⁻⁴ |

(van Veen, 1973 as cited by Mulder and Deinema, 1981)

Preparation of Medium: Add components (except for the vitamins) to distilled/deionized water and bring volume to 1.0L. Mix thoroughly and autoclave at psi pressure- 121°C for 15 mins. Filter sterilize the vitamins separately and then add aseptically to the cooled autoclaved agar . Pour into petri dishes and allow to set (Atlas, 1993; van Veen, 1973 as cited by Mulder and Deinema, 1981).

Appendix I: TYG Agar**Composition per liter:**

| | |
|---------------------------------|-------|
| Agar | 15.0g |
| Pancreatic digest of casein | 5.0g |
| Yeast extract | 3.0g |
| Glucose | 1.0g |
| K ₂ HPO ₄ | 1.0g |

pH 7.0 ± 0.1 at 25°C

Preparation of medium: Add components to distilled/deionized 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 J: *Nocardia* Medium**Composition per liter:**

| | |
|--------------|-------|
| Agar | 20.0g |
| Peptone | 10.0g |
| Beef extract | 5.0g |
| NaCl | 2.5g |

Preparation of medium: Add components to distilled/deionized 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 K: D-medium**Composition** per liter:

| | |
|--|-------|
| Trypticase Soy Broth without dextrose (B.B.L.) | 0.25g |
| Casitone (Difco) | 0.25g |
| Yeast extract (Difco) | 0.25g |
| Nutrient broth (Oxoid) | 0.25g |

Preparation of medium: Add components to glass distilled water and bring volume to 1.0L. Mix thoroughly (van Veen, 1973). Gently heat with mixing and bring to boiling. Autoclave for 15 minutes at 15 psi pressure- 121°C. Do not overheat. Pour into sterile tubes.

Appendix L: C-medium**Composition per liter:**

| | |
|--|------|
| Glucose | 0.5g |
| Lactose | 0.5g |
| Casamino acids (Difco) | 0.6g |
| Peptone (Difco) | 0.3g |
| Yeast extract (Difco) | 0.3 |
| Vitamin stock solution (MSV; Appendix M) | 10ml |

Preparation of medium: Add components (except for the vitamin stock solution) to distilled water and bring volume to 1.0L. Mix thoroughly (Eikelboom, 1975). Gently heat with mixing and bring to boiling. Autoclave for 15 minutes at 121°C. Sterilize the vitamin stock solution through a Jena G5 glass filter and add aseptically to the cooled media. Pour into sterile tubes.

Appendix M: Mineral-Salt-Vitamin (MSV) solution**Composition per liter:**

| | |
|-----------------------------|----------------------|
| Ca pantothenate | 10^{-4} g |
| Niacin | 10^{-4} g |
| Biotin | 5×10^{-6} g |
| Cyanocobalamin | 5×10^{-6} g |
| Folic acid | 5×10^{-6} g |
| Pyridoxine | 10^{-4} g |
| <i>p</i> -aminobenzoic acid | 10^{-4} g |
| Coccarboxylase | 10^{-4} g |
| Inositol | 10^{-4} g |
| Thiamine | 10^{-4} g |
| Riboflavin | 10^{-4} g |

Preparation of solution: Add components to distilled water and bring volume to 1.0L.

Mix thoroughly. Sterilize the vitamin stock solution through a Jena G5 glass filter and add aseptically to the cooled media (Eikelboom, 1975).

Appendix N: NaCl medium

Make up different concentrations (5, 10, 15, and 20%) of NaCl medium by preparing commercially available nutrient agar as per manufacturer's instructions. Make it up to the desired salt concentration by adding the required amount of salt (NaCl) respectively. Bring medium to boil. Autoclave for 15 minutes at 15 psi pressure and 121°C. pour cooled medium into sterile petri dishes.

Appendix O: Antibiotic medium No. 1 (Bridson, 1995)

Preparation of Medium: Prepare commercially available Antibiotic Medium No.1 (Oxoid- CM327) as per instructions by manufacturer. Pour into sterile petri dishes and allow to set. Inoculate by making lawns on the surface of the agar plate with the test organism and then place the antibiotic disc/s (commercially available- Oxoid). Incubate.

Appendix P: Modified I-Medium (C- source medium)**Composition** in grams per liter:

| | |
|---|--------------------|
| Agar (Oxoid) | 10 |
| C- source | 0.2 |
| (NH ₄) ₂ SO ₄ | 0.5 |
| Ca(NO ₃) ₂ | 0.01 |
| K ₂ HPO ₄ | 0.05 |
| MgSO ₄ ·7H ₂ O | 0.05 |
| KCl | 0.05 |
| CaCO ₃ | 0.1 |
| Vitamin B12 | 10 ⁻⁵ |
| Thiamine | 4x10 ⁻⁴ |
| NaCl | 0.05 |

Preparation of Medium: Add components (except for the vitamins and the c-source/s) to distilled water and bring volume to 1000ml. Mix thoroughly and autoclave at 15psi pressure- 121°C for 15 minutes. Filter sterilize the vitamins and the c-source/s separately and then add aseptically to the cooled autoclaved agar. Pour into petri dishes and allow to set. Inoculate plates.

Appendix Q: Nutrient Gelatin (Bridson, 1995)

Composition in grams per liter:

| | |
|---------------------|-------|
| 'Lab- Lemco powder' | 3.0 |
| Peptone | 5.0 |
| Gelatin | 120.0 |

pH 6.0 ± 0.2

Preparation of Medium: Add components to 1000ml distilled water and bring to boil. Dispense 10ml aliquots into tubes and autoclave at 15psi pressure- 121°C for 15 minutes. Leave to set in a refrigerator. Inoculate by making a stab inoculation with the test organism and incubate. Refrigeration follows incubation and a positive result is given by a dip that remains liquid after refrigeration.

Appendix R: Starch agar (<http://128.175.57.161/Harding/401.rtf> accessed 6/11/2003)

Preparation of medium:

10g/L soluble starch in 1000ml of Nutrient Agar prepared as per manufacturers instructions. Autoclave for 15 minutes at 15 psi pressure and 121°C. Allow to cool and pour into sterile petri dishes.

Appendix S: Skim milk agar (<http://128.175.57.161/Harding/401.rtf> accessed 6/11/2003)**Preparation of medium:**

Mix 10g skim milk powder with 100ml distilled water and autoclave. Add 20ml of this suspension to 100ml sterilized nutrient agar prepared as per manufacturer's instructions.

The nutrient agar should be cooled to 50 °C prior to addition of the skim milk solution.

Appendix T: Spirit Blue agar (<http://128.175.57.161/Harding/401.rtf> accessed 6/11/2003)**Composition per litre**

| | |
|-----------------|--------|
| Tryptone | 10g |
| Yeast extract | 5g |
| Spirit blue dye | 0.15g |
| Agar | 20g |
| Distilled water | 1000ml |

Preparation of medium: Bring medium to boil. Autoclave for 15 minutes at 15 psi pressure and 121°C. Allow cooling to 50 °C and add aseptically, 30ml of lipid homogenate. Pour lipid medium into sterile petri dishes.

Lipid homogenate:

| | |
|----------------------|-------|
| Warm distilled water | 400ml |
| Tween 80 | 1ml |
| Test oil | 100ml |

Homogenise and autoclave for 10 minutes at 15 psi pressure and 121°C.

Appendix U: Phenolphthalein Phosphate Agar (Dept. of Microbiology- DIT, 2003)**Preparation of medium:**

0.12g of Phenolphthalein diphosphate in 20ml distilled water is prepared to make up a 0.6% solution. This 20ml of the 0.6% solution is added (filter-sterilized) to 1000ml sterile nutrient agar prepared as per manufacturer's instructions. Allow to cool and then pour into sterile petri dishes.

Method and principle:

Streak test organisms onto agar plate and incubate. If the organism produces phosphatase, the phosphate will be cleaved from the phenolphthalein during incubation, thus liberating the free phenolphthalein, which is an indicator in the free state. After incubation the plate is exposed to ammonia, which is alkaline, which will turn the free phenolphthalein bright pink immediately.

Appendix V: Christiansen's urea agar slopes (Dept. of Microbiology- DIT, 2003)**Preparation of medium:**

A 40% urea solution is made up by adding 20g urea crystals to 50ml distilled water. This 40% solution is then added (filter- sterilized) to 950ml of urea agar base prepared as per manufacturer's instructions. Dispense 15ml of this mixture into sterile universal bottles and make slopes.

Method and principle:

Streak test organisms onto agar slope and incubate. If the organism produces urease, the slope will turn deep pink. The medium contains a pH indicator phenol red. The ammonia released on urea breakdown creates an alkaline environment, which causes the phenol red to turn a deep pink. No deep pink colour is a negative reaction.

Appendix W: pH broth**Preparation of medium:**

Prepare commercially available nutrient broth as per instructions by manufacturer. Bring to boil. Adjust pH by adding a few drops of acid or base with a pH probe in the medium until desired pH is reached. Dispense 10ml aliquots of the broth into tubes and autoclave for 15 minutes at 15 psi pressure and 121°C. Allow to cool and inoculate.

Appendix X: Nitrate and Nitrite medium (Drysdale *et al.*, 2001)

Composition per litre

| | |
|--|--|
| Bacto casitone | 5.0g |
| Yeast autolysate | 1.0g |
| Glycerol | 10.0mL |
| Agar | 1.0g |
| Nitrate (NO ₃)/ Nitrite (NO ₂) | 0.2, 0.5 and 1.0g respectively, as Potassium salts |

Preparation of Medium: Add components to distilled water and bring volume to 1000ml. Mix thoroughly. Gently heat with mixing and bring to boiling. Distribute 10ml aliquots into tubes. Autoclave for 15 minutes at 15 psi pressure- 121°C. Cool and inoculate.

Method and principle:

Inoculate the test organism into nitrate medium. After incubation add 5 drops of solution A followed by 5 drops of solution B to cultures. If a red colour develops then the nitrate has only been reduced to nitrite. If no red colour develops then add zinc powder to culture. If a red colour develops then no nitrate was reduced at all. However, if no red colour develops then nitrate was reduced to nitrite, which in turn was further, reduced to either ammonia, nitrogen gas or a less oxidized form of nitrogen.

Preparation of reagents:

Solution A

8g Sulphanilic acid

1litre Acetic acid, 5N (1 part glacial acetic acid to 2.5 parts distilled water)

Solution B

5g Alpha-naphthylamine

1litre Acetic acid, 5N (1 part glacial acetic acid to 2.5 parts distilled water)

Appendix Y: Glycogen stain (The Periodic Acid Schiff's Method)

(<http://www.hoslink.com.histo/6.HTM>).

Reagents

1% Periodic acid solution:

2ml Commercially available Periodic acid solution

98ml distilled water

Add the acid to water. Mix the solution. Filter, bottle and label solution. Important: solution should be double- filtered before use.

Schiff's reagent:

This solution is commercially available and should be double- filtered before use.

Method:

Make sample smears of the sample on a clean, glass, microscope slide. Heat- fix the smears. Treat smears with 1% Periodic Acid Solution for 10 minutes. Wash treated slides in running tap water for 3- 5 minutes. Treat smears with Schiff's reagent for 10 minutes. Wash with running tap water for 5- 10 minutes. Allow slides to dry and view under oil for glycogen storage by test organisms.

Appendix Z: Biolog microplate for Gram Negative organisms (Biolog, Inc., 2001)

Gram Negative Identification Test Panel

GN MicroPlate

| | | | | | | | | | | | |
|---------------------------------------|------------------------------|-------------------------------|--------------------------------|------------------------------------|------------------------------|------------------------------------|----------------------------------|----------------------------|--------------------------------------|-------------------------------------|---|
| A1 Water | A2 ex-Cyclodextrin | A3 Dextrin | A4 Glycogen | A5 Tween 40 | A6 Tween 80 | A7 N-Acetyl-D- Galactosamine | A8 N-Acetyl-D- Glucosamine | A9 Adonitol | A10 L-Arabinose | A11 D-Arabinol | A12 D-Cellobiose |
| B1 L-Erythritol | B2 D-Fructose | B3 L-Fucose | B4 D-Galactose | B5 Gentiobiose | B6 ex-D-Glucose | B7 ...Inositol | B8 ex-D-Lactose | B9 Lactulose | B10 Maltose | B11 D-Mannitol | B12 D-Mannose |
| C1 D-Melibiose | C2 D-Meth- DoGlucoside | C3 D-psicose | C4 D-Raffinose | C5 L-Rhamnose | C6 D-Sorbitol | C7 Sucrose | C8 D-Trehalose | C9 Turannose | C10 Xylitol | C11 Pyruvic Acid Meth Ester | C12 Succinic Acid Mono-Meth- Ester |
| D1 Acetic Acid | D2 Chl. Acetic Acid | D3 Citric Acid | D4 Formic Acid | D5 DoGalactonic Acid Lactone | D6 DoGalacturonic Acid | D7 DoGluconic Acid | D8 DoGlucosaminic Acid | D9 DoGlucuronic Acid | D10 ex- Hydroxybutyric Acid | D11 p- Hydroxybutyric Acid | D12 y- Hydroxybutyric Acid |
| E1 p-Hydroxy Phenacetic Acid | E2 Iaconic Acid | E3 ex-Keto Butyric Acid | E4 ex-Keto Glutaric Acid | E5 ex-Keto Valeric Acid | E6 D,L-Lactic Acid | E7 Malonic Acid | E8 Propionic Acid | E9 Quinic Acid | E10 D-Saccharic Acid | E11 Sebacic Acid | E12 Succinic Acid |
| F1 Bromosuccinic Acid | F2 Succinamic Acid | F3 Glucuronamide | F4 L-Alaninamide | F5 D-Alanine | F6 L-Alanine | F7 L-Alan. glycine | F8 L-Asparagine | F9 L-Aspartic Acid | F10 L-Glutamic Acid | F11 Gly.L. Asparic Acid | F12 Gly.L. Glutamic Acid |
| G1 L-Histidine | G2 Hydroxy-L- Proline | G3 L-Leucine | G4 L-Ornithine | G5 L- Phenalanine | G6 L-Proline | G7 L-Pyroglutamic Acid | G8 D-Serine | G9 L-Serine | G10 L-Threonine | G11 D,L-Carnitine | G12 y-Amino Butyric Acid |
| H1 Urocanic Acid | H2 Inosine | H3 Uridine | H4 Thymidine | H5 Phenyth. amine | H6 Putrescine | H7 2-Aminoethanol | H8 2,3-Butanediol | H9 Glycerol | H10 D,L-Glycerol Phosphate | H11 ex-D-Glucose. 1-Phosphate | H12 D-Glucose. 6-Phosphate |

Appendix AA: Biolog microplate for Gram Positive organisms (Biolog, Inc., 2001)

BiOLOG

Gram Positive Identification Test Panel

GP MicroPlate

| | | | | | | | | | | | |
|------------------------------|-----------------------------------|------------------------|--------------------------|-----------------------|-------------------------------------|---|------------------------------------|------------------------------------|---|-------------------------------------|--|
| A1 Water | A2 ex.cyclodextrin | A3 p.cyclodextrin | A4 Dextrin | A5 Glycogen | A6 Inulin | A7 Mannan | A8 Tween 40 | A9 Tween 80 | A10 N-Acetyl-D- Glucosamine | A11 N-Acetyl-p.D- Mannosamine | A12 Amygdalin |
| B1 L-Arabinose | B2 D-Arabinol | B3 Arbutin | B4 D.cellobiose | B5 D.Fructose | B6 L.Fucose | B7 D-Galactose | B8 D-Galacturonic Acid | B9 Gentiobiose | B10 D-Gluconic Acid | B11 ex-D-Glucose | B12 ...Inositol |
| C1 ex-D-Lactose | C2 Lactulose | C3 Maltose | C4 Maltotriose | C5 D.Mannitol | C6 D.Mannose | C7 D-Melibiose | C8 D-Melibiose | C9 ex.Meth.D- Galactoside | C10 p-Meth.D- Galactoside | C11 3-Meth Glucose | C12 ex-Meth.D- Glucoside |
| D1 p-Meth.D. Glucoside | D2 ex.Meth.D. Mannoside | D3 Palatinose | D4 D.Psicose | D5 D.Raffinose | D6 L.Rhamnose | D7 D-Ribose | D8 Salicin | D9 Sedoheptulose | D10 D.Sorbitol | D11 Stachyose | D12 Sucrose |
| E1 D-Tartrate | E2 D-Trehalose | E3 Turranose | E4 Xylitol | E5 D-Xylose | E6 Acetic Acid | E7 ex- Hydroxybutyric Acid | E8 p- Hydroxybutyric Acid | E9 y- Hydroxybutyric Acid | E10 p-Hydroxy. Phenylacetic Acid | E11 ex- Ketoglutaric Acid | E12 ex. Ketovaleric Acid |
| F1 Lactamide | F2 D.Lactic Acid Meth Ester | F3 L.Lactic Acid | F4 D.Malic Acid | F5 L.Malic Acid | F6 Pyruvic Acid Meth Ester | F7 Succinic Acid Mono.methyl Ester | F8 Propionic Acid | F9 Pyruvic Acid | F10 Succinamic Acid | F11 Succinic Acid | F12 N-Acetyl-L- Glutamic Acid |
| G1 L-Alaninamide | G2 D-Alanine | G3 L-Alanine | G4 L-Alan- Glycine | G5 L-Asparagine | G6 L-Glutamic Acid | G7 G1-L. Glutamic Acid | G8 L-Pyrogutamic Acid | G9 L-Serine | G10 Putrescine | G11 2,3-Butanediol | G12 Glycerol |
| H1 Adenosine | H2 2'-deoxy Adenosine | H3 Inosine | H4 Thymidine | H5 Uridine | H6 Adenosine.S' Monophosphate | H7 Thymidine.s' Monophosphate | H8 Uridine.S' Monophosphate | H9 D-Fructose-6- Phosphate | H10 ex-D-Glucose- 1-Phosphate | H11 D-Glucose- 6-Phosphate | H12 D-L...oGlycerol Phosphate |

Appendix AB: Nutrient Agar (Biolab, 1997 and Bridson, 1995)**Composition in grams per liter (Biolab, 1997):**

| | |
|-----------------|------|
| Meat extract | 1.0 |
| Peptone | 5.0 |
| Yeast extract | 2.0 |
| Sodium chloride | 8.0 |
| Agar | 15.0 |

pH 7.1 ± 0.1

Preparation of medium: Add components to 1000ml of distilled water. Bring to boil to dissolve completely and autoclave at 15psi pressure- 121°C for 15 minutes.

Composition in grams per liter (Bridson, 1995):

| | |
|---------------------|------|
| 'Lab- Lemco powder' | 1.0 |
| Peptone | 5.0 |
| Yeast extract | 2.0 |
| Sodium chloride | 5.0 |
| Agar | 15.0 |

pH 7.4 ± 0.2

Preparation of medium: Add components to 1000ml of distilled water. Bring to boil to dissolve completely and autoclave at 15psi pressure- 121°C for 15 minutes.