PCR DETECTION, DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) FINGERPRINTING AND IDENTIFICATION OF THE MICROBIAL CONSORTIUM IN DIFFERENT TYPES OF UASB GRANULES

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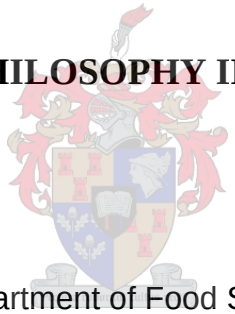
DOCTOR OF PHILOSOPHY IN FOOD SCIENCE



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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that it has not previously, in its entirety or in part, been submitted at any other university for a degree.

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ABSTRACT

High-rate anaerobic bioreactors are used for the treatment of various wastewaters, of which the upflow anaerobic sludge blanket (UASB) bioreactor has the widest application, especially in the food and beverage industries. In an UASB bioreactor sludge develops in a particular granular or flocculent form and the success of the anaerobic process relies on the formation of active and settable granules. These granules are formed by self-aggregation of bacteria that can be divided into different trophic groups that are responsible for the metabolic breakdown of organic substrates.

The successful performance of a bioreactor is influenced by the composition of the substrate which subsequently may have an impact on the microbial consortium present in the UASB granules. In order to determine if a change in the structure of the non-methanogenic microbial community takes place, UASB brewery granules were subjected to the sudden addition of different carbon sources at different concentrations. A shift in the microbial community did occur when the granules were subjected to lactate medium (5 $g.l^{-1}$). No changes in the microbial community were observed when the granules were stressed with glucose medium as carbon source, regardless of an increase in the glucose concentration.

In order to better understand the effect that different wastewaters may have on the microbial consortium present in different UASB granules, the polymerase chain reaction (PCR) based denaturing gradient gel electrophoresis (DGGE) technique and sequence analysis were used to fingerprint and identify the *Bacteria* and *Archaea* present in either, winery, brewery, distillery or peach-lye canning UASB granules. Each granule type showed distinct PCR-based DGGE fingerprints with unique bands, while other bands were found to be present in all the granules regardless of the wastewater being treated. *Bacillus*, *Pseudomonas*, *Bacteroides*, *Enterococcus*, *Alcaligenes*, *Clostridium*, *Shewanella*, *Microbacterium*, *Leuconostoc*, *Sulfurospirillum*, *Acidaminococcus*, *Vibrio*, *Aeromonas*, *Nitrospira*, *Synergistes*, *Rhodococcus*, *Rhodocyclus*, *Syntrophobacter* and uncultured bacteria were identified, representing different acidogenic, acetogenic and homoacetogenic *Bacteria*.

Different methanogenic bacteria such as *Methanosaeta*, *Methanosarcina*, *Methanobacterium* and uncultured bacteria belonging to the group *Archaea* were also fingerprinted and identified from different UASB granules. In both these studies a DGGE marker was constructed that may be used to assist in the identification of bacteria. The DGGE marker can also be used to monitor the presence of bacteria over a time period during anaerobic digestion. Bioaugmentation or the enrichment of granules results in tailor-made granules that may be used for the treatment of specific wastewaters.

One of the most important contributions to the maintenance and enhancement of UASB granule formation is the inclusion of suitable microbes in the granule structure. *Enterobacter sakazakii* was isolated from raw winery wastewater and was found to produce sufficient amounts of desired fatty acids. This bacteria was, therefore, incorporated into batch cultured granular sludge. In order to identify and monitor the presence of the incorporated *E. sakazakii* in the tailor-made granules, 16S rRNA gene sequence primers and PCR conditions were developed.

The use of molecular techniques such as PCR-based DGGE and sequence analysis proved to be successful methods to fingerprint and identify the microbial consortium present in the different UASB granules.

UITTREKSEL

Hoë-tempo anaerobiese bioreaktors word gebruik vir die behandeling van verskillende tipes afvalwater, waarvan die “upflow anaerobic sludge blanket” (UASB) bioreactor die wydste toepassing het, veral in die voedsel en drank industrie. In ‘n UASB bioreaktor ontwikkel slyk in ‘n bepaalde granulêre of flokkulente vorm. Die sukses van die anaerobiese proses hang af van die vorming van aktiewe en afgesakte granules. Hierdie granules word gevorm deur self-aggregasie van bakterieë wat in verskillende trofiese groepe verdeel is. Elke trofiese groep is verantwoordelik vir die metaboliese afbraak van organiese substrate.

Die suksesvolle werkverrigting van ‘n bioreaktor word beïnvloed deur die samestelling van die substrate, wat dan die mikrobiese konsortium wat teenwoordig is in die UASB granules kan beïnvloed. Om te bepaal of ‘n verandering in die struktuur van die nie-metanogeniese mikrobiese gemeenskap plaasvind, was UASB brouery granules blootgestel aan die skielike byvoeging van verskillende koolstofbronne teen verskillende konsentrasies. ‘n Verandering in die mikrobiese gemeenskap het wel plaasgevind nadat die granules blootgestel is aan laktaat medium (5 $g.l^{-1}$). Geen verandering in die mikrobiese gemeenskap was waargeneem nadat die granules onder spanning geplaas is nie, deur gebruik te maak van glukose medium as koolstof bron, ongeag verhoogde glukose konsentrasies.

Om ‘n beter begrip te kry oor hoe verskillende afvalwaters ‘n invloed kan hê op die mikrobiese konsortium wat teenwoordig is in verskillende UASB granules is die polimerase kettingreaksie (PKR) gebaseerde denaturerende gradiënt jelelektroforese (DGGE) analise en DNS volgorde bepalings gebruik vir die vingerafdrukking en identifisering van die *Bakterieë* en *Archaea* wat teenwoordig is in wyn, brouery, stokery en perske-loog inmaak UASB granules. Elke granule tipe het spesifieke PKR-gebaseerde DGGE vingerafdrukke met unieke bande gewys, terwyl sommige bande teenwoordig was in al die granules ongeag die afvalwater wat behandel is. *Bacillus*, *Pseudomonas*, *Bacteroides*, *Enterococcus*, *Alcaligenes*, *Clostridium*, *Shewanella*, *Microbacterium*, *Leuconostoc*, *Sulfurospirillum*, *Acidaminococcus*, *Vibrio*, *Aeromonas*, *Nitrospira*, *Synergistes*, *Rhodococcus*,

Rhodocyclus, *Syntrophobacter* en onkultiveerbare bakterieë was geïdentifiseer wat verskillende asidogene, asetogene en homoasetogene *Bakterieë* verteenwoordig.

Verskillende metanogeen bakterieë soos *Methanosaeta*, *Methanosarcina*, *Methanobacterium* en onkultiveerbare bakterieë wat aan die groep *Archaea* behoort was geïdentifiseer en vingerafdrukke daarvan bepaal van die verskillende UASB granules. In beide die studies was ‘n DGGE merker saamgestel wat moontlik ‘n bydrae kan lewer tydens die identifikasie van bakterieë. Die DGGE merker kan ook gebruik word om die teenwoordigheid van bakterieë oor ‘n sekere tydperk te monitor gedurende anaerobiese vertering. Verryking van granules kan lei tot “tailor-made” granules vir die behandeling van spesifieke afvalwater.

Een van die belangrikste bydraes vir die handhawing en verbetering van UASB granule vorming is die insluiting van sekere spesifieke mikrobes in die granule struktuur. *Enterobacter sakazakii* is geïsoleer vanuit rou wyn-afvalwater en daar is gevind dat hierdie bakterieë genoegsame hoeveelhede vetsure produseer. As gevolg hiervan is *E. sakazakii* geïnkorporeer in lot gekultiveerde granulêre slyk. Om die teenwoordigheid van die geïnkorporeerde *E. sakazakii* te identifiseer en te monitor in die “tailor-made” granules, is daar 16S rRNS geen volgorde peilers en PKR kondisies ontwikkel.

Hierdie studie bewys dat die gebruik van molekulêre tegnieke soos die PKR gebaseerde DGGE metode en volgorde bepalings suksesvol aangewend kan word vir die vingerafdruk en identifikasie van die mikrobiese konsortium teenwoordig in verskillende granules.

CONTENTS

Language and style used in this dissertation are in accordance with the requirements of the International Journal of Food Science and Technology. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

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CHAPTER 1

INTRODUCTION

The development of technologies for processing of food and food related products has lead to an increase in the production of wastewater (Kroyer, 1995). These types of wastewaters may result in the pollution of soil and surface waters, therefore, South African regulations have been designed to impose restrictions on the nature, amount and methods of their disposal (Coetzee *et al*., 2004). If food manufacturers do not comply with these regulations they may face high disposal charges and, in order for the food industries to minimise these disposal charges, it is essential to implement wastewater treatment processes.

Anaerobic digestion is a biological process, which has successfully been used for the treatment of a variety of industrial wastewaters (Lettinga, 2004). Anaerobic digestion has several advantages when compared to conventional aerobic processes that treat high-strength industrial wastewater. Anaerobic systems may have higher loading rates, from 5 - 20 kg $COD.m^{-3}.d^{-1}$, whereas the normal loads of aerobic systems are around 0.5 - 3 kg $COD.m^{-3}.d^{-1}$. This implies a substantial reduction of the reactor volume and the available space (“foot print”) required and, therefore, lower installation costs (Lema & Omil, 2001). Anaerobic digestion also ultimately results in the production of biogas which may serve as a fuel to offset the growing demand and cost for energy (Sawayama *et al*., 2000). Interest in anaerobic wastewater treatment has increased over the last few decades mostly as a result of the successful development of high-rate reactors of which the upflow anaerobic sludge bed (UASB) bioreactor has received the most commercial interest and widest application (Kolukirik *et al*., 2004).

The successful operation of an UASB bioreactor depends on the formation and activity of highly flocculated and compact sludge granules. These granules are formed by self-aggregation of anaerobic bacteria (Batstone *et al*., 2004). One of the most important parameters that have been shown to contribute to the maintenance and enhancement of UASB granule formation is the inclusion of suitable microorganisms in the granule structure.

The microorganisms present in UASB granules can be divided into different trophic groups, each with complex nutritional requirements and specialised ecological roles in the bioreactor. The microbial groups are responsible for the metabolic breakdown of organic material and involve several degradation phases including hydrolysis, acidogenesis, acetogenesis and methanogenesis (Batstone *et al*., 2002). Variations in the composition of the different trophic groups as a result of changes in one or more environmental operating conditions, such as pH, substrate composition and temperature of an UASB bioreactor, may impact the entire microbial community structure and metabolic stability that will subsequently affect the bioreactor performance (Casserly & Erijman, 2003).

Acidogens play a role in the production of volatile fatty acids (VFA), which may have an influence on the pH of the reactor. If the microbial consortium in a granule are dominated by the acidogens, too much VFA may be produced due to a high chemical oxygen demand (COD) influent or to an organic overload, resulting in a too low pH. This subsequently inhibits the methanogenic step and may lead to process inhibition and reactor failure. The methanogens on the other hand play an important part during the granulation of the UASB granules and also have a drastic shortening time on the start-up of a bioreactor (Zhang & Fang, 2004). If certain methanogens are not present, granulation may be unsuccessful which will lead to reactor failure. It is, therefore, important that the right microbial population is present in the granules to optimise the metabolic activity which will result in a successful UASB treatment.

Different types of wastewaters, when treated with the UASB design, may impact the microbial consortium structure of the granules. Wastewaters may be comprised of different constituents that are used as substrates by the microorganisms belonging to the different trophic groups present in the UASB granules. Each type of wastewater treated may result in different dominant granule species which may have a major metabolic impact on granules that have to be used as seed sludge for the start-up of new bioreactors. Each type of wastewater will, therefore, favour the growth of specific microorganisms that may have an impact on the success of the granulation process and subsequently the successful operation of the UASB bioreactor.

When an UASB bioreactor is operated under stressed environmental conditions, such as the sudden addition of different substrates at different

concentrations, this may also have an effect on the composition of the microorganisms present in the granules. It is thus essential to gain more insight into what effect these stressed environments have on the microbial community in the granules. It is, therefore, important to identify and detect the various microorganisms in the UASB granules.

Since the UASB treatment of different wastewaters may result in the growth of only certain microorganisms it may, therefore, be essential to incorporate microorganisms into the granules to ensure a rapid and successful granulation and subsequent UASB operation. For example, the addition of selected natural bacterial strains, which are known to produce sufficient amounts of desired fatty acids in specific wastewaters, could lead to an enhancement of bioreactor efficiency (Britz *et al*., 2004). This can in addition then lead to the development of granules tailored to a specific wastewater. Tailor-made granules can, therefore, play an important role in optimising the treatment of certain wastewaters and to ensure the successful working of the UASB bioreactor. Thus it is important in order to detect and fingerprint the different microorganisms present in UASB granules to use reliable and reproducible techniques.

In a previous study done by Ronquest & Britz (1999) it was attempted to optimise granules to treat winery effluent which contained no carbohydrates. This was done by isolating various organisms that could metabolise raw winery wastewater and produce VFAs. Of all the organisms isolated, *Enterobacter sakazakii* was found to produce the highest VFA concentration and was, therefore, incorporated into batch cultured granular sludge using the method of Britz *et al*. (2002). However, in their study Ronquest & Britz (1999) were not able to show that the added *E. sakazakii* was present in the final granules.

Traditional microbiological techniques have in the past been used to determine the bacterial populations present in anaerobic digesters but the success of these techniques have always been limited as many organisms are not readily cultured on selective media (Briones & Raskin, 2003). Molecular techniques, such as the polymerase chain reaction (PCR) based denaturing gradient gel electrophoresis (DGGE), have shown to be a promising method to study complex microbial communities present in natural environments (Zhang & Fang, 2000; Gonzalez *et al*., 2003).

The primary objective of this study was to typify both the microbial non-methanogenic and methanogenic populations present in different types of UASB granules by using a non-cultured molecular approach. The secondary objectives of this study were to gain insight into the microbial populations present in UASB granules that had been used to treat different types of wastewaters; to evaluate the influence of changes in substrates on the microbial structures present in UASB granules; and to detect incorporated microbes in tailor-made granules.

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CHAPTER 2

LITERATURE REVIEW

A. Background

The volume of water used world-wide has tripled over the last half-century (Brown, 2001) and according to the United Nations, water use in the 20th century grew at twice the rate of the world population (Van der Merwe, 2003). The supply of freshwater will become a major natural resource management issue in the 21st century (Jacobson, 2003).

South Africa is a semi-arid country in which the average rainfall of 450 mm per year is well below the world average of about 860 mm per year. As a result, South Africa's water resources are in global terms scarce and limited. Currently, South Africa is categorised as a water stressed country with an annual fresh water availability of less that 1 700 m^3 per capita. It is forecasted that in 2025 the country will be among the countries in the world that will experience a physical water scarcity scenario with an annual freshwater availability of less that 1 000 m^3 per capita (Otieno & Ochieng, 2004). Sustainable water development and management is a critical component of development for all societies (Otieno & Ochieng, 2004). The cost of supplying water to domestic and industrial users will rise dramatically over the coming decades (Coles, 2003) and as industrial operations expand around the world, they are increasingly faced with stricter legislation on water usage and disposal of wastes (Tebo, 2001).

Current South African legislation such as the National Environmental Management Act 107 of 1998 (Anon, 1998a) and Environmental Impact Assessment Regulations (Anon, 2004) insists on the need for every South African company to have a structured approach or strategy in place to reduce the environmental impacts of its activities, services and products. The development of an effective "Environmental Management System" such as ISO 14001 has, therefore, been identified as one of the numerous tools to assist in meeting the legal requirements (Walsdorff *et al*., 2004) The Department of Water Affairs and Forestry has developed a National Resource Strategy as set out in Section 5 of the National Water Act 36 of 1998 (Anon, 1998b) which

propose strategies to achieve equity, sustainability and efficiency in the use of South Africa's water resources (Maharaj & Pietersen, 2004).

The nature of a country's economy plus its specific climate, dictates the population it can support. People themselves need very little water to survive, with 25 litres per person per day being the minimum for basic domestic requirements (Bezuidenhout, 2004). The most water is used by agriculture, mining, industries and power generation. To prevent water shortage in the next century it is, therefore, important to maximise the use of available water (Ketrick, 2003).

This has led to development and improvement of methods for the treatment and use of wastes (Perez *et al*., 2000; Britz *et al*., 2004). These wastes can be either treated using physical-chemical or biological processes. Physical-chemical processes are mostly used for the treatment of inorganic wastes or wastes with non-biodegradable organic substances. Biological treatment is used when the pollutants can be removed by microorganisms under aerobic or anaerobic conditions. Anaerobic digestion is increasingly recognised as the most important method for environmental protection and resource preservation (Seghezzo *et al*., 1998). At present, anaerobic treatment has been implemented successfully in different industries around the world, including countries in Europe, South and South-East Asia and Latin America (Ince & Ince, 2000; Lettinga, 2004).

B. Anaerobic digestion

Anaerobic digestion is a biological process (Lettinga, 2004) in which the organic matter is broken down to form mostly biomass and biogas. The volume of material to be treated is reduced by the removal of much of the volatile components (Stein & Malone, 1980). Ultimately anaerobic digestion results in the production of biogas consisting mainly of methane (CH_4) (50-70%) and carbon dioxide (CO_2) (25-45%) and small volumes of hydrogen, nitrogen and hydrogen sulphide (Price, 1985). The methane as by-product can serve as fuel to offset the growing demand for energy (Sawayama *et al*., 2000). However, no single parameter can be used as a process control measure as the degradation of organic matter to CH_4 and CO_2 is brought about by a heterogeneous microbial population (Ince *et al*., 2004).

It is thus essential that suitable microbial communities with specialised ecological roles be established and maintained to ensure and sustain the digestion process (Dabert *et al*., 2002; Briones & Raskin, 2003; Ince *et al*., 2004). The microbial community may include many diverse genera (Cowley & Wase, 1981; McCarty, 2001) which greatly influence the digestion efficiency, bioreactor pH, alkalinity, volatile fatty acid content, solids and volatile solids content, chemical oxygen demand (COD) of the effluent and the rate and composition of biogas produced (Ince *et al*., 2004).

Anaerobic digestion can be carried out at different temperatures, including psychrophilic (4° - 15°C), mesophilic (20° - 40°C) and thermophilic (45° - 70°C) ranges (Batstone *et al*., 2002). Anaerobes are most active at the optimal mesophilic (35°C) and thermophilic (55°C) temperatures (Yadvika *et al*., 2004), where higher loading rates may be applied and a decrease in the amount of pathogens occurs (Bitton, 1994). In municipal wastewater treatment plants, anaerobic digestion is carried out at temperatures between 25°C and 40°C, with an optimum temperature at approximately 35°C. The temperature inside a bioreactor also has a major influence on the biogas production (Azbar *et al*., 2001).

The gas production rate in anaerobic bioreactors is highly dependent on the organic loading rate (OLR) which is the amount of biodegradable material or substrate per unit volume that will be introduced to the bioreactor (Hickey *et al*., 1991). Bioreactors have an optimum feed rate and maximum gas production rate, however an increase in the quantity of the substrate will not necessarily result in proportionally increased gas production (Yadvika *et al*., 2004).

The hydraulic retention time (HRT) is the average time that the input sludge spends in the bioreactor (Yadvika *et al*., 2004). The HRT depends on the wastewater characteristics and environmental conditions and must be sufficient to allow digestion of material by the anaerobic bacteria.

The optimal pH for a bioreactor should be kept within a desired range of 6.8 - 7.2 (Yadvika *et al*., 2004). pH values below 6.5 or above 7.5 may be harmful to the bacteria, especially to the methanogens. The amount of CO_2 and volatile fatty acids (VFA) produced during the anaerobic process also affects the pH of the bioreactor. For fermentation to proceed normally VFA concentrations, in particular the acetic acid, should be below 2 000 $mg.l^{-1}$ (Yadvika *et al*., 2004). The addition of $NaHCO_3$ to the waste is very effective in stabilising the pH of the bioreactor (Lin & Yang, 1991).

The availability of micronutrients such as Fe, Ni, Cu, Zn, Mo, Co, Se, Si, F, Mg, Na and W are important for the bacteria in a bioreactor in order to achieve efficient degradation of organic wastes and ensure the successful operation of a bioreactor (Nel *et al*., 1985). The presence of exchangeable form of metals plays an important role in the physiological functions of microorganisms in an UASB bioreactor. Ilangovan & Noyola (1993) found that during the anaerobic digestion of molasses stillage the sequence of metals in exchangeable form were K>Na>Fe>Ca>Mg>Ni>Cu=Zn.

Anaerobic digestion has several advantages compared to the conventional aerobic processes that are used to treat high-strength industrial wastewater (Lettinga, 1995). Anaerobic systems have higher loading rates, from 5 - 20 kg $COD.m^{-3}.d^{-1}$, whereas the usual loads of aerobic systems are around 0.5 – 3.0 kg $COD.m^{-3}.d^{-1}$. This implies a substantial reduction of the bioreactor volume and the available space required and, therefore, lower installation costs (Lema & Omil, 2001). Another advantage of anaerobic digestion is that the amount of solids produced is far less than when using aerobic treatments and these could be stabilised for land application and are widely used as soil conditioners (McCarty, 2001). The low nutrient requirements of anaerobic digestion and the production of methane gas are also significant advantages over aerobic digestion (Azbar *et al*., 2001).

Anaerobic processes have also been reported to remove inorganic pollutants such as nitrates and perchlorates, and most chlorinated hazardous compounds, including pesticides and chlorinated solvents. Polychlorinated biphenyls can also be converted to less harmful compounds during anaerobic digestion (Verstraete *et al*., 1996; McCarty, 2001). Several groups of xenobiotics, including halogenated organics can be eliminated from wastes using reductive dehalogenation, an energy yielding process that only occurs under anaerobic conditions (Stergar *et al*., 2003). Highly chlorinated organic compounds from the pulp and paper industry were successfully dehalogenated (Parker *et al*., 1993), while wastewaters with high levels of formaldehyde were effectively detoxified through its conversion to methanol that can then be transformed into methane (Omil *et al*., 1999). In addition, phenol derivatives from herbicides, pesticides and coal conversion could be successfully removed (Fang *et al*., 1996) from wastewater at 37°C, and more recently 98% phenol removal at 26°C using anaerobic digestion (Fang *et al*., 2004).

C. Operational principles of the UASB design

Since the 1970's, 1 215 full-scale industrial high-rate anaerobic bioreactors were operated around the world (Franklin, 2001; Casserly & Erijman, 2003). Of the existing full-scale plants, 72% are based on the upflow anaerobic sludge bed (UASB) design, emphasizing that the anaerobic granular sludge bed design has been the most successful for scale-up and implementation. The removal efficiencies, which largely depend on the wastewater type, are generally in the excess of 85 - 90% (Franklin, 2001), hence, high-rate anaerobic systems are widely used in municipal and industrial wastewater treatment (Tay & Zhang, 2000; Liu *et al*., 2003). The UASB bioreactor was first put into commercial use in the Netherlands for the treatment of industrial wastewater generated by food factories processing sugar beet, corn starch and potato starch (Bitton, 1994). These systems are mostly used by breweries, the beverage industry, distilleries, fermentation and food industry and the pulp and paper industries (Franklin, 2001).

The term 'high-rate' is widely used to refer to anaerobic treatment systems where the HRT is "uncoupled" from the solids retention time (SRT) (Stergar *et al*., 2003). High-rate bioreactors have a high retention of the sludge under high loading conditions, and adequate contact between the incoming wastewater and the retained sludge is important (Lettinga *et al*., 1987). All the modern high-rate UASB processes are based on bacterial sludge immobilisation or the formation of highly settleable sludge aggregates, gas separation, internal sludge settling and bacterial attachment to high density particulate carrier materials (Lettinga *et al*., 1980).

The UASB design can typically be divided into four compartments, as illustrated in Fig. 1: the granular sludge bed; the fluidised zone or sludge blanket; the gas-liquid separator; and the settling compartment (Lin & Yang, 1991; Schmidt & Ahring, 1996). The granular sludge bed is located at the bottom of the bioreactor. Wastewater is pumped in at the bottom of the bioreactor and passes through the granular sludge bed where the first part of the organic compounds is biologically degraded and biogas is produced. Just above the granular sludge bed a fluidised zone or sludge blanket develops (Schmidt & Ahring, 1996). The sludge blanket is a suspension of sludge

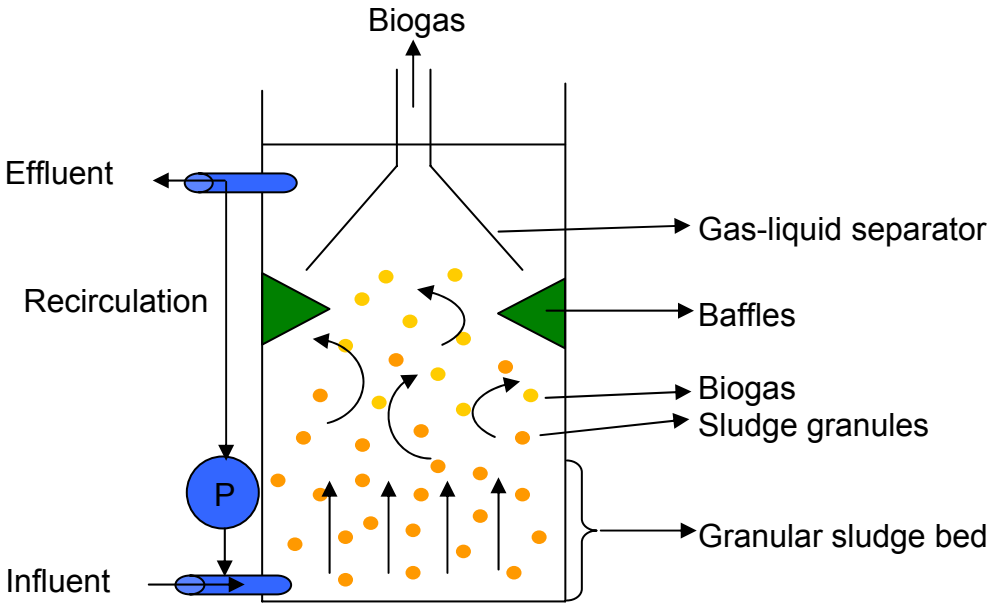

Figure 1. The upflow anaerobic sludge bed (UASB) bioreactor. P = recirculation pump.

particles mixed with the bio-gases produced during the degradation process. In this zone further biological degradation takes place. The biogas is separated from the liquid in the gas-liquid separator, which acts as a physical barrier (Morgan-Sagastume *et al*., 1997). The biogas is then collected outside the bioreactor (Lin & Yang, 1991).

The success of the UASB bioreactor design relies on the establishment of a dense granular sludge bed. This is formed by the accumulation of incoming suspended solids and bacterial cells (Seghezzo *et al*., 1998) that are retained as granules (Trnovec, & Britz, 1998). These granules vary in size from 0.1 to 5 mm depending upon the wastewater treated and the operational conditions applied. The granules vary in shape but usually have a spherical form (Trnovec & Britz, 1998). Granules with good settling abilities settle back to the granular sludge bed, while flocculated and dispersed bacteria are washed out of the bioreactor with the effluent (Lin & Yang, 1991).

Insufficient internal mixing in UASB bioreactors leads to dead space and the resulting reduction in the treatment efficiency (Seghezzo *et al*., 1998). In order to improve the granular sludge-wastewater contact and the use of the entire bioreactor volume efficiently, a better influent distribution is required. Therefore, different feed inlet devices, more feed inlet points per square meter or higher superficial velocities have been proposed. The use of effluent recirculation, combined with taller bioreactors (or height:diameter ratio) results in the expanded granular sludge bed (EGSB) bioreactor (Fig. 2) (Van der Last & Lettinga, 1992; Dinsdale *et al*., 2000). In these bioreactors upflow velocities of 8 - 30 $m.h^{-1}$ (Van Lier *et al*., 2001) causes the granular sludge bed to expand, eliminating dead zones and resulting in better sludge-wastewater contact (Seghezzo *et al*., 1998). Due to the high liquid and gas upflow velocities in the expanded bed bioreactors, high OLRs of up to 20 - 40 kg $COD.m^{-3}.d^{-1}$ are achieved (Van Lier *et al*., 2001). Soluble pollutants can be efficiently treated in EGSB bioreactors, but suspended solids cannot substantially be removed from the wastewater stream due to the high upflow velocities (Seghezzo *et al*., 1998).

UASB and EGSB bioreactors have extensively been applied for the treatment of wastewater from meat packing factories, canneries, wineries, breweries, slaughterhouses and paper industries (Sigge *et al*., 2002; Tagawa *et al*., 2002). These wastewaters are classified as non-toxic with 85 - 90% COD removal efficiencies for bioreactors operating at 20°C or higher at an OLR of around 5 – 15 kg $COD.m^{-3}.d^{-1}$ and HRT of 6 to 12 h.

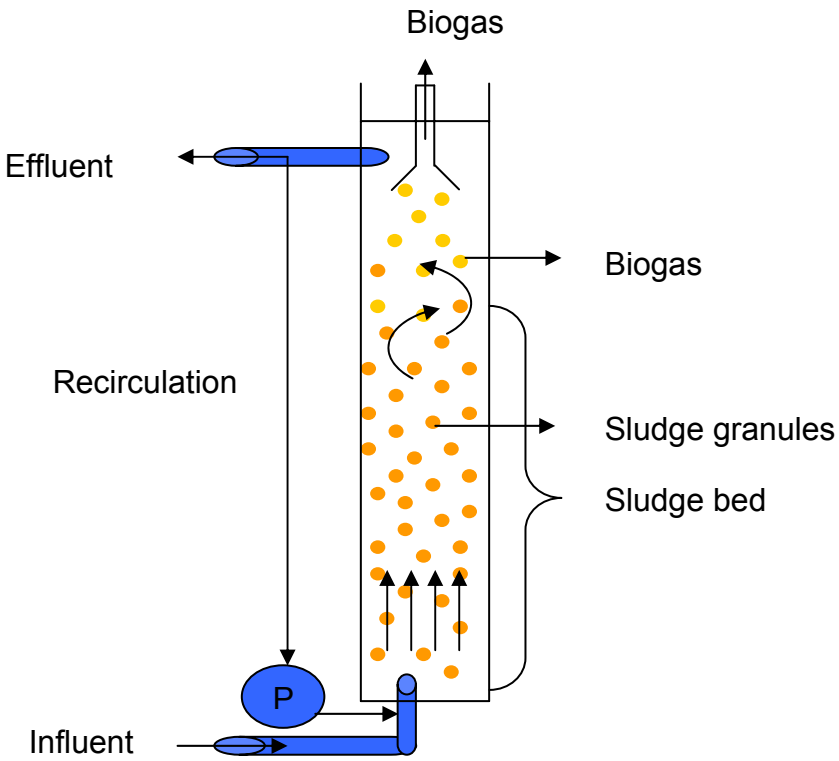

Figure 2. The expanded granular sludge bed (EGSB) bioreactor. P = recirculation pump.

Kim *et al*. (2003) obtained over 70% COD removal efficiency for the wastewater from a paper factory using a UASB bioreactor. During start-up the HRT was 23 h, but was reduced to 6 h with the OLR at 18 kg $COD.m^{-3}.d^{-1}$. As a result, effluent COD levels were below 1 500 $mg.l^{-1}$. The efficiency of this treatment of a wastewater from a sunflower oil factory in Turkey was also investigated in a pilot-scale mesophilic UASB bioreactor by determination of removal of total lipids (TL) and fatty acids (FA). The removal efficiencies of TL and FA were above 70% at OLRs between 1.6 and 7.8 kg $COD.m^{-3}d^{-1}$ and at an optimum HRT of between 2.0 and 2.8 d (Saatci *et al*., 2003). Treatment of seasonal fruit cannery wastewaters by UASB bioreactors resulted in COD reductions of up to 93% at OLR of 10.95 kg $COD.m^{-3}.d^{-1}$ and a HRT of $<$12 h was achieved (Trnovec & Britz, 1998). Similarly, COD removal of 93% at an OLR of 11.05 kg $COD.m^{-3}.d^{-1}$ and a HRT of 14 h were achieved when treating winery wastewater (Ronquest & Britz, 1999). Studies on the treatment of dairy wastewater using UASB bioreactors were done by Ramasamy *et al.* (2004). Their bioreactors were operated at OLRs from 2.4 kg $COD.m^{-3}d^{-1}$ to 10.8 kg $COD.m^{-3}d^{-1}$ and HRTs of 3 and 12 h. At the 3 h HRT, the maximum COD reduction was 96.3%.

Nunes & Martinez (1999) investigated the performance of an EGSB bioreactor for treating slaughterhouse wastewater under 35°C. The average COD removal obtained was 67% for OLRs of up to 15 kg $COD.m^{-3}.d^{-1}$ and HRT of 5 h. Of the fats, 85% were removed and no accumulation of the fats was observed. Pereira *et al*. (2002) also successfully treated oleic acid-based synthetic effluent with an EGSB bioreactor and an OLR of 8 kg $COD.m^{-3}.d^{-1}$ was obtained with a COD removal efficiency of 70%.

D. Granulation of anaerobic sludge

One of the biggest concerns of UASB and EGSB high-rate bioreactors is the stability of the granular conglomerates during continuous operation, where loss of biomass might occur due to granule disintegration, wash-out of hollow granules, occurrence of fluffy granules and scaling by inorganic precipitates (Van Lier *et al*., 2001). This dependency on the maintenance and growth of the sludge granules initiated various studies on the mechanism of granulation (Alphenaar *et al*., 1994; Thaveesri *et al*., 1995; Britz *et al*., 2002; Hulshoff Pol *et al*., 2004).

A highly settleable and active granular sludge (Lin & Yang, 1991) or an active biomass (Quarmby & Forster, 1995) is essential for UASB wastewater treatment (Lin & Yang, 1991; Schmidt & Ahring, 1996). The granulation of the sludge plays an important role to ensure successful UASB operation (Lin & Yang, 1991). For the process to operate at high OLRs and HRTs, the sludge should be in a highly flocculated, granular form (Alphenaar *et al*., 1994). The main advantage of the UASB process is that no support material is required for the retention of the activated anaerobic sludge. In the high-rate processes, the sludge retention time should be longer than the HRT to prevent wash-out. In the UASB process this can be achieved by the formation of settable aggregates of microorganisms, known as granules (Fukuzaki *et al*., 1991). Various theories and models on anaerobic granulation have been proposed and include physico-chemical models, structural models and the proton translocation-dehydration theory (Liu *et al*., 2003; Hulshoff Pol *et al*., 2004).

Physico-chemical models

Microbial adhesion or self-immobilisation is the starting point of the anaerobic granulation process, and can be defined in terms of the energy involved in the interaction of bacterium-to-bacterium or bacterium-to-solid surface. The interaction between the bacteria includes repulsive electrostatic force, Van De Waals force and repulsive hydration interaction. Based on these thermodynamic forces, certain physico-chemical models for anaerobic granulation have been developed which include the inert nuclei model, selection pressure model, multi-valence positive ion-bonding model and the extracellular polymers (ECP) bonding model (Liu *et al*., 2003).

Inert nuclei model - The inert nuclei model for anaerobic granulation was initially proposed by Lettinga *et al*. (1980) (Fig. 3). They suggested that in the presence of inert micro-particles in an UASB bioreactor, anaerobic bacteria could attach to the particle surfaces to form the initial biofilms, namely embryonic granules. Mature granules could further develop through the growth of these attached bacteria under given operation conditions. Yu *et al*. (1999) proposed that the inert materials to be used should have a high specific surface area, a spherical shape, good hydrophobicity and the specific gravity of the inert materials should be similar to the gravity of the anaerobic sludge. The inert nuclei model suggests that the presence of nuclei or micro-size bio-carriers for bacterial attachment is a first step towards anaerobic granulation.

Selection pressure model – In this model it was suggested that the basis of anaerobic granulation was the continuous selection of sludge particles that occurred in the bioreactors. Light and dispersed sludge would be washed out, while heavier components remain in the system (Visser *et al*., 1991; Hulshoff Pol *et al*., 2004). The selection pressure model suggests that microbial aggregation in UASB bioreactors may be an effective protection strategy against high selection pressures. Selection pressure may be created by the upflow flow pattern. In another study it was found that the absence of granulation was observed when the hydraulic selection pressure was weak (O'Flaherty *et al*., 1997). Noyola & Mereno (1994) reported that a rapid production of granules could be achieved through physical aggregation due to hydraulic stresses applied to the anaerobic flocculent sludge. High selection pressure in terms of upflow velocity seems to be in favour of fast formation and production of anaerobic granules.

Multi-valence positive ion-bonding model – This model is based on a simple electrostatic interaction between negatively charged bacteria and positive ions (Fig. 4). The positive ions added to sludge would partially neutralise negative charges on bacterial surfaces by adsorption thereby the electrical repulsion between bacteria would be decreased in a significant way. The electrostatic repulsion between negatively charged bacteria is reduced by introducing multi-valence positive ions, such as calcium, ferric, aluminium or magnesium ions into seed sludge. Positive ions could initiate cell-to-cell interaction, which is a decisive step to increase granulation (Liu *et al*., 2003). Reduced electrostatic repulsion between bacteria then promotes anaerobic granulation (Schmidt & Ahring, 1993; Yu *et al*., 2001b). Addition of Ca^{2+} at concentrations of 80 – 200 $mg.l^{-1}$, Mg^{2+} at 12 – 120 $mg.l^{-1}$ or Al^{3+} at 300 $mg.l^{-1}$ was found to increase the rate of anaerobic granulation in UASB bioreactors (Schmidt & Ahring, 1993; Yu *et al*., 2001a). In addition, the multi-valence positive ion may promote sludge granulation by bonding ECPs (Schmidt & Ahring, 1996).

Extracellular polymers model – It has been reported that the formation of ECP may mediate cohesion and adhesion of cells, and play a crucial role in maintaining structural integrity of the microbial matrix (Forster, 1991; Cammarota & Sant'Anna Jr., 1998) (Fig. 5). These polymers are of bacterial origin and contain

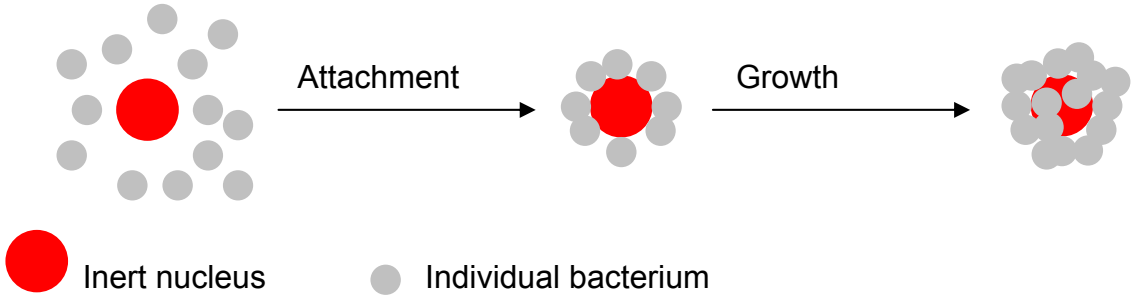

Figure 3. Schematic representation of the inert nuclei model.

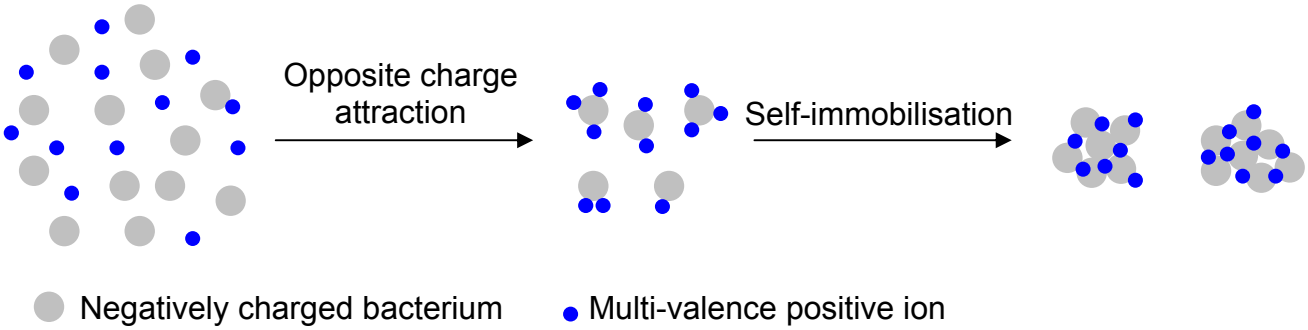

Figure 4. Schematic representation of the multi-valence positive ion-bonding model.

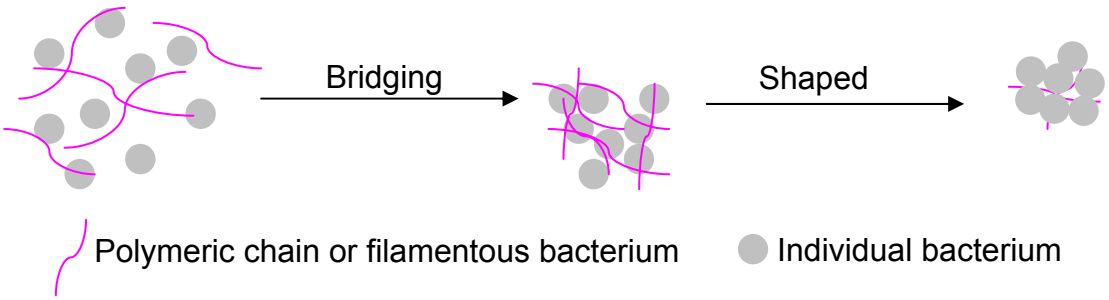

Figure 5. Schematic representation of the polymer or filament bonding model.

polysaccharides found outside the outer membrane of Gram-negative cells and the peptidoglycan in Gram-positive cells (Shen *et al*., 1993). Extracellular polymers are high molecular weight compounds produced by lysis, excretion or biosynthesis and may be present in a rigid or flexible form (Quarmby & Forster, 1995). Extracellular polymers promote the formation of bacterial aggregates and mediate cell to cell adhesion by forming a bridge between different microbial surfaces, thereby producing a three-dimensional floc-matrix (Shen *et al*., 1993). Chen & Lun (1993) observed that with increases in the organic loading rate, *Methanosarcina* grew in a significant way and secreted much more ECP to form larger clumps onto which *Methanosaeta* tended to attach (Hulshoff Pol *et al*., 2004).

It appears that each physico-chemical model only accounts for the contribution of one or two factors to the initial granulation process in UASB bioreactors. Since these factors exert their influences under specific environmental conditions and in specific steps during the whole granulation process, the physico-chemical models just provide some simplified description for anaerobic granulation. Anaerobic granulation is a complex process and other than physico-chemical forces, biological and microbiological factors are probably also involved.

Structural models

A series of structural models for anaerobic granulation have been developed to interpret the granulation phenomena. These include the Cape Town model, the spaghetti model, syntrophic microcolony model and the multi-layer model (Wiegant & de Man, 1986; Sam-Soon *et al*., 1988; Hulshoff Pol *et al*., 2004).

Cape Town model - The Cape Town model suggested that the ECP is produced by *Methanobacterium* strain AZ (Sam-Soon *et al*., 1988). This organism utilises hydrogen as its sole energy source and can produce all its amino acids, with the exception of cysteine. When *Methanobacterium* strain AZ is in an environment of high hydrogen partial pressure, excess substrates, cell growth and amino acid production will be stimulated. However, as *Methanobacterium* strain AZ cannot produce the essential amino acid cysteine, cell synthesis will be limited by the rate of cysteine supply (Hulshoff Pol *et al*., 2004). The overproduced amino acids which *Methanobacterium* strain AZ secretes as ECP, binds the *Methanobacterium* strain AZ and other bacteria together to form granules (Hulshoff Pol *et al*., 2004). In the Cape Town model, therefore, the

overproduction of ECP is considered a key step for initiating anaerobic granulation (Sam-Soon *et al*., 1988).

Spaghetti model – This is based on the microstructure of UASB granules as observed by scanning electron microscopy (Wiegant & de Man, 1986). This model suggets that granulation can be divided into two phases. The first step is considered the crucial part of the granule formation where filamentous *Methanosaeta* attach on precursors to form very small aggregates. The granules in this phase have a filamentous appearance, like a ball of spaghetti formed by the very long *Methanosaeta* filaments, of which part is loose and part in bundles. With time, rod-type granules are formed from these filamentous granules at high biomass retention time, due to the increase in the density of the bacterial growth (Wiegant & de Man, 1986).

Syntrophic microcolony model - Different microbial species are involved in the anaerobic digestion process. These species have to live in a close synergistic relationship and this may lead to the formation of granules. The close packing of bacteria in granules facilitates the exchange of metabolites and products such as hydrogen and other intermediates so that they are efficiently transferred between the bacterial groups. In the UASB granules, different groups of bacteria carry out sequential metabolic processes. In order to maintain a high metabolic efficiency the granule-associated cells must be present in an organised structure (Shapiro, 1998). It was demonstrated in the syntrophic microcolony model that the driving force for sludge granulation should be a result of the needs of bacterial survival and optimal combination of different biochemical functions of multiple species under the culture conditions (Liu *et al*., 2003).

Multi-layer model - Based on microscopic observations, a multi-layer model for anaerobic granulation was initially proposed by MacLeod *et al*. (1990) and Guiot *et al*. (1992) (Fig. 6). According to this model the granules can be described as near-spherical biofilms consisting of three concentric layers, each possessing different bacterial trophic groups (MacLeod *et al*., 1990). The granule core or inner layer mainly consists of methanogens such as the rod-shaped bacteria from the genus *Methanosaeta* that may act as the nucleation centre necessary for the initiation of granule development. Hydrogen-producing and hydrogen-utilising bacteria are dominant species in the middle layer, which also consists of a large number of syntrophic cocci (El-Mamouni *et al*., 1997). Finally, the outermost layer contains a variety of species

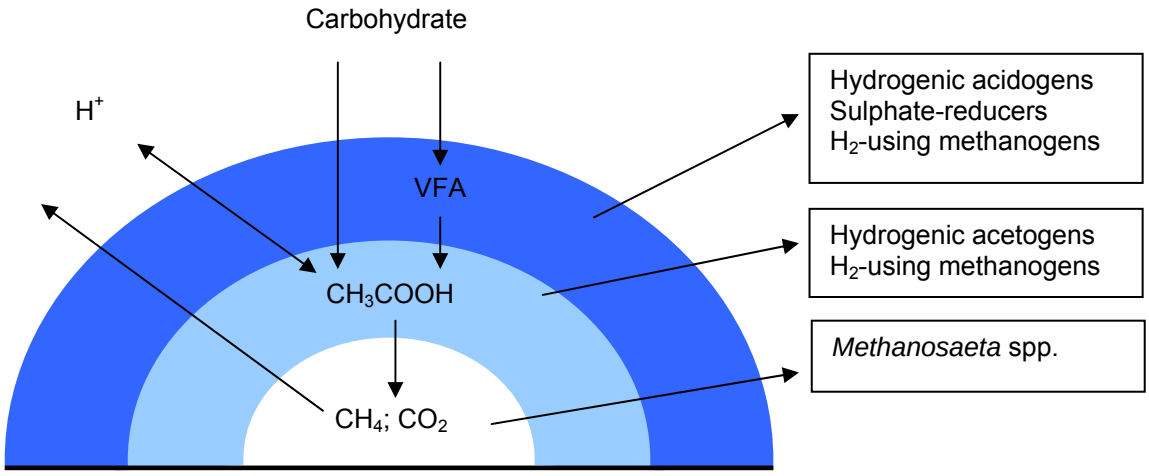

Figure 6. Schematic representation of the multi-layer model.

including rods and cocci that represent mostly acidogenic bacteria and filamentous bacteria such as *Methanosaeta* and *Methanosarcina* spp. (El-Mamouni *et al*., 1997).

Depending on the original inoculum, the composition of the substrate and the process conditions, different types of granules may develop (Hulshoff Pol *et al*., 1983). Different types of granules can also be formed from the same substrate (Thaveesri *et al*., 1995) and these include black granules mainly composed of methanogens, white granules and multi-layered grey granules (Verstraete *et al*., 1996). Rocheleau *et al*. (1999) found UASB granules which consisted of large dark non-staining centers, in which neither archaeal nor bacteria could be found. A possible explanation of the formation of these dark centers might be as a result of the accumulation of metabolically inactive, decaying biomass and inorganic materials (Sekiguchi *et al*., 1999).

Proton translocation-dehydration theory

The proton translocation-dehydration theory for anaerobic granulation was proposed to be based on the proton translocating activity on bacterial membrane surfaces (Tay *et al*., 2000). The overall granulation process starts from dehydration of bacterial surfaces, and followed by embryonic granule formation, granule maturation and post-maturation.

During the hydrolysis and degradation of complex organic compounds, the electron transport on the respiration chain of bacterial surfaces is activated. This couples with the activation of proton pumps on the membranes of these bacteria. Proton translocation can establish a proton gradient across the bacterial cell surface and cause surface protonation (Teo *et al*., 2000). The energized bacterial surfaces may result in the breaking of the tightly bonded water between negatively charged groups. This may result in partial neutralisation of the negative charges on bacterial surfaces and, therefore, induce the dehydration of the bacterial surfaces (Van Loodsrecht *et al*., 1987). By the action of external hydrodynamic shear forces, these relatively neutral and hydrophobic acidogens, acetogens and methanogens may adhere to each other due to the weakened hydration repulsion. These initial bacterial aggregates are embryonic granules (Liu *et al*., 2003). Continuous growth of the bacteria in the embryonic granule occurs. Some dispersed bacteria in the medium may also adhere to the embryonic granule and be integrated into the bacterial consortia. In embryonic granules the distribution of each group of bacteria depends on the orientation of intermediate

metabolites transference, which is the most efficient way for anaerobes to transfer their intermediates. This will result in the formation of well-organised bacterial consortium as mature granules (Liu *et al*., 2003; Hulshoff Pol *et al*., 2004). In the post-maturation stage the proton translocation activity keeps the bacterial surfaces at a relatively hydrophobic state and is the main factor in maintaining the structure of the mature granules (Hulshoff Pol *et al*., 2004).

There is general consensus that the initial stage of granulation is bacterial adhesion, which is a physico-chemical process. However, it is important to consider that the granulation process in total consists of more than one ongoing process that include the physico-chemical as well as the different structural theories. All these different methods contribute to a successful granulation process.

E. Microbial consortium

The efficient anaerobic degradation of complex organic matter to biogas is a result of the combined and coordinated metabolic activity of a specific bacterial consortium. This microbial consortium has complex nutritional requirements and specialised ecological roles in a bioreactor (Zeikus, 1980; Iannotti *et al*., 1987). During the consecutive stages of anaerobic digestion the metabolic products of one microbial group are assimilated by another microbial group. These consecutive stages consist of hydrolysis (acidogenesis), acetogenesis, homoacetogenesis and methanogenesis (Fig. 7) (Gerardi, 2003). During hydrolysis complex organic substrates are broken down to simple organic substrates. This may be followed by acidogenesis which is the process during which these simple organic substrates are metabolised to form acetate, formate, methanol, methylamine, propionate and butyrate (Batstone *et al*., 2002). During acetogenesis the organic acids and alcohols are converted to acetate, hydrogen and CO_2 (Van Andel & Breure, 1984; Sam-Soon *et al*., 1990), followed by homoacetogenesis during which hydrogen and CO_2 is used to form acetate (Zeikus, 1982; Forday & Greenfield, 1983). Methanogenesis completes the anaerobic digestion cycle, during which CO_2 and hydrogen are converted to CH_4 and water, and acetate is converted to CH_4 and CO_2 (Van Andel & Breure, 1984).

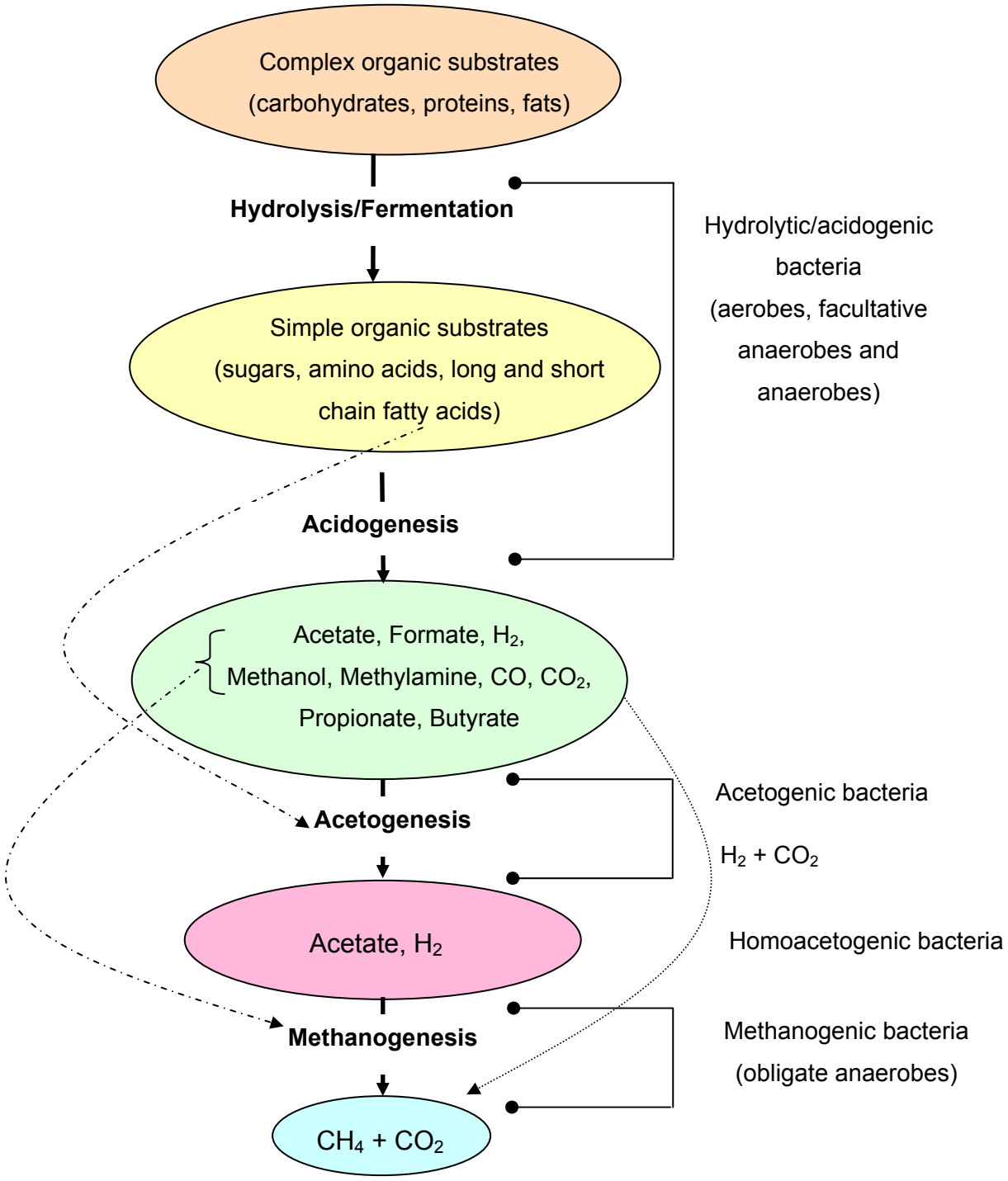

Figure 7. The basic stages of the anaerobic digestion process. These stages include the solubilisation of complex organic substrates or hydrolysis, the production of simple acids and the formation of biogas.

The different bacterial populations involved in the different stages of anaerobic digestion are comprised of the acidogenic, acetogenic, homoacetogenic and methanogenic bacteria. The groups can be distinctly recognised on the basis of the substrates that they ferment and metabolic end-products formed (Zeikus, 1982). Sulphate-reducing bacteria may also be present in anaerobic bioreactors along with acetate and methane utilising bacteria. If sulphates are present in the bioreactor, sulphate-reducing bacteria will reproduce by using hydrogen and acetate as substrates (Gerardi, 2003) and thus may be seen as competition for the methanogens.

The major trophic groups must be considered in unison when studying their respective metabolisms (Gorris *et al*., 1989). Variations in the composition of one trophic level due to changes in one or more operating conditions, such as pH, substrate composition, temperature and solids retention time will influence the entire microbial community structure, thus directly affecting the bioreactor performance. Complete insight into the microbial ecology of the anaerobic processes is, therefore, essential to make effective and reliable control of the bioreactor performance possible (Casserly & Erijman, 2003). Thus it is important to determine the different microbial populations present in UASB granules, especially if the granules have been used to treat different wastewaters.

Acidogenic population

The acidogenic population is by far the largest of the trophic groups, and consists about 90% of the total bioreactor community (Zeikus, 1980). In 1969 Toerien & Hattingh isolated acidogenic bacteria from sewage bioreactors and found that most of these bacteria are strict anaerobes.

Acidogenic bacteria play a role in acidogenises, which is the first stage in anaerobic digestion (Van Andel & Breure, 1984). In the anaerobic bioreactor complex insoluble compounds are hydrolysed (Cowley & Wase, 1981). The term hydrolysis is used to describe the degradation of macromolecular substrates, such as carbohydrates, proteins and lipids to their soluble monomer sugars, amino acids and fatty acids (Batstone *et al*., 2002). The hydrolysis process is catalysed by enzymes produced by the acidogens (Price, 1985; Batstone *et al*., 2002). The International Water Association (IWA) Task Group for Mathematical Modelling of Anaerobic Digestion (Model 1) (Batstone *et al*., 2002) suggested two models for the hydrolysis by the enzymatic activity of acidogens. In the first model the organisms secrete enzymes into the bulk liquid in the bioreactor where the enzymes

adsorb onto particles or macromolecular substrates such as carbohydrates, proteins and lipids or react with a soluble substrate. In the second proposed model the organisms attach to particles, produce enzymes in the vicinity of the particles and benefit from the soluble products released by the enzymatic reaction. Although the Task Group proposed both models, it was thought that in normal anaerobic mixed culture systems the dominant mechanism to be found will be model two as shown by Vavilin *et al*. (1996) and Sanders *et al*. (2000). The organisms growing on the particle surface, rather than the enzyme produced, are regarded as the effective catalyst for hydrolysis (Batstone *et al*., 2002). After hydrolysis of the complex substrates, acidogenesis takes place.

Acidogenises is the process during which more “simple” organic material is broken down to form CO_2, hydrogen, acids and alcohols (Van Andel & Breure, 1984). Amino acids and polysaccharides are broken down to sugar monomers, the oils and long chain fatty acids are catabolised to polyols and short-chain carboxylic acids or volatile fatty acids (including acetic, propionic and butyric acids) (Van Andel & Breure, 1984), (Table 1). Smaller quantities of formic, valeric, iso-valeric and caproic acids are also produced. Ammonia, sulphide, iso-butyrate, iso-valerate, n-valerate, 2-methylbutyrate and certain aromatic compounds may also be produced from the amino acids. Many of the acidogenic bacteria produce hydrogen for the disposal of the excess electrons generated during the energy yielding oxidation of organic materials. This is normally in the presence of hydrogen consuming bacteria, the methanogenic and homoacetogenic bacteria. The excessive activity of the acidogenic population can result in bioreactor failure (Forday & Greenfield, 1983), and considerable attention had been focused on the relationships between VFA concentrations and the anaerobic bioreactor performance (Cobb & Hill, 1991). Hill *et al*. (1987) reported that organic acids are the immediate precursors of CH_4 and that organic acids may cause microbial stress and even process failure if present in high concentrations. The inhibition of hydrogen consuming methanogens results in an increase in the hydrogen partial pressure in the bioreactor, which in turn inhibits the degradation of long chain fatty acids (Kasper & Wuhrmann, 1978). This would result in an accumulation of VFA and a decrease in the pH, ultimately resulting in the complete failure of the bioreactor (Cobb & Hill, 1991). Thus, is it important to determine the structure of the acidogenic populations which may play a huge role in the overall success of the UASB bioreactor.

Table 1. Fermentation reactions associated with the anaerobic conversion process in a successful operating UASB bioreactor (Van Andel & Breure, 1984; Schulz & Conrad, 1996; Batstone *et al*., 2002; Gerardi, 2003).

Reaction	**ΔG (kJ.mol^{-1})**
Acidogenesis:	
$C_6H_{12}O_6 + 2H_2O \rightarrow 2$ ethanol $+ 2HCO_3^- + 2H^+$	-225.4
$C_6H_{12}O_6 + 2H_2O \rightarrow$ butyrate$^-$ $+ 2HCO_3^- + 3H^+ + 2H_2$	-254.4
$C_6H_{12}O_6 \rightarrow 2$ lactate$^-$ $+ 2H^+$	-198.1
$C_6H_{12}O_6 \rightarrow 3$ acetate$^-$ $+ 3H^+$	-310.6
$C_6H_{12}O_6 + HCO_3^- \rightarrow$ succinate^{2-} + acetate$^-$ + formate$^-$ $+ 3H^+ + H_2O$	-144.0
3 lactate$^-$ $\rightarrow 2$ propionate$^-$ + acetate$^-$ $+ HCO_3^- + H^+$	-164.8
2 lactate$^-$ $+ 2H_2O \rightarrow$ butyrate$^-$ $+ 2HCO_3^- + H^+ + 2H_2$	-56.2
Acetogenesis:	
ethanol $+ 2HCO_3^- \rightarrow$ acetate$^-$ + 2 formate$^-$ $+ H_2O + H^+$	+7.0
ethanol $+ H_2O \rightarrow$ acetate $+ 2H_2 + H^+$	+9.6
lactate$^-$ $+2H_2O \rightarrow$ acetate$^-$ $+ 2H^+ + H^+$	-3.9
butyrate$^-$ $+ 2H_2O \rightarrow 2$ acetate$^-$ $+ 2H_2 + H^+$	+48.1
benzoate$^-$ $+ 6H_2 \rightarrow 3$ acetate$^-$ $+ 3H_2 + CO_2 + 2H^+$	+53.0
succinate^{2-} $+ 4H_2O \rightarrow$ acetate$^-$ $+ 2HCO_3^- + 3H_2 + H^+$	+56.1
propionate$^-$ $+ 3H_2O \rightarrow$ acetate$^-$ $+ HCO_3^- + 3H_2 + H^+$	+76.1
Homoactogenesis:	
$4H_2 + 2HCO_3^- + H^+ \rightarrow$ acetate $+ 4H_2O$	-104.5
Methanogenesis:	
acetate$^-$ $+ H_2O \rightarrow$ methane $+ HCO_3^-$	-31.0
$4H_2 + HCO_3^- + H^+ \rightarrow$ methane $+ 3H_2O$	-135.6
$4HCO_2^- + H^+ + H_2O \rightarrow$ methane $+ 3HCO_3^-$	-130.4
4 methanol $\rightarrow 3$ methane $+ HCO_3^- + H^+ + H_2O$	-312.8

The acidogens have a generation time of about two hours and an average doubling time of acidogens under normal operating conditions during anaerobic digestion, of 30 minutes (Mosey & Fernandes, 1989; Sam-Soon *et al*., 1990). It is well known that the pH of the substrate can influence the metabolic activity of the acidogens. Most of the studies on the effect of the pH on acidogenesis were conducted for the degradation of simple substrates, such as glucose, sucrose and lactose. Zoetemeyer *et al*. (1982) found that acidogenesis of glucose at pH 5.7 – 6.0 produced stable intermediates favoured by the bacteria in the down-stream methanogenic bioreactor. Similarly, the optimum pH for the acidification of sucrose and lactose were reported to be pH 6.5 (Joubert & Britz, 1986) and pH 6.0 – 6.5 (Kisaalita *et al*., 1987). During the acidogenesis of dairy wastewater the production of propionate and ethanol were favoured at pH 4.0 – 4.5, whereas the production of acetate and butyrate were favoured at pH 6.0 – 6.4 (Sam-Soon *et al*., 1990; Strydom *et al*., 1997; Yu & Fang, 2001). Thus, once again it is important to determine which microorganisms are present in the bioreactor to ensure optimum conditions for the successful degradation of the wastewater being treated.

Various acidogenic bacteria have been isolated from anaerobic bioreactors (Iannotti *et al*., 1987) and may include members of the following genera: *Bacillus*, *Pseudomonas*, *Klebsiella*, *Peptococcus*, *Escherichia*, *Acinetobacter*, *Bacteriodes* and *Propionibacterium* (Zeikus, 1980; Iannotti *et al*., 1987; Britz *et al*., 1988; Noeth *et al*., 1988; Riedel & Britz, 1993; Riffat *et al*., 1999; Lapara *et al*., 2000). *Peptostreptococcus* and *Eubacterium* are bacterial strains that were isolated from a bioreactor that treated swine manure (Iannotti *et al*., 1987). *Erwinia*, *Sarcina*, *Zynomonas*, *Enterobacter* and *Serratia* species are known to produce ethanol during the anaerobic digestion of glucose (Iannotti *et al*., 1987; Gerardi, 2003). Strict anaerobes of the genera *Clostridium* and *Butyrivibrio* ferment a variety of sugars to produce butyrate (Gerardi, 2003). The major genera of lactate forming and utilising bacteria include *Bifidobacterium*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Sporolactobacillus*, *Streptococcus* and *Propionibacterium* (Iannotti *et al*., 1987; Britz *et al*., 1988; Gerardi, 2003).

Acetogenic population

The obligate hydrogen-producing acetogenic bacteria are responsible for the second stage of the anaerobic digestion (Pipyn & Verstraete, 1981). The acetogenic bacteria are the slowest growing of the trophic groups, with a generation time of 2.3 days for butyrate

oxidisers (Gujer & Zehnder, 1983) and 4.6 to 5.8 days for propionate oxidisers (Boone & Bryant, 1980). The acetogenic bacteria obtain their energy from the oxidation of organic acids and alcohols to acetate, hydrogen and CO_2 (Table 1) (Van Andel & Breure, 1984), the substrates used by the methanogenic bacteria. Conversion of the short-chain fatty acids, butyric and propionic acid to acetic acid and hydrogen can only take place at a low pH, but at different partial pressures of hydrogen less than 10^{-5} atmospheres (Van Andel & Breure, 1984; Sam-Soon *et al*., 1990).

Inhibition of the acetogenic population especially due to hydrogen ion accumulation will result in an environment unfavourable for the methanogens (Forday & Greenfield, 1983) as the unionised forms of the short-chain fatty acids are particularly toxic to the methanogenic bacteria. Acetogens include members of the genera *Syntrophobacter*, such as *S. wolinii*, *Syntrophomonas*, such as *S. wolfei*, *Syntrophus*, such as *S. buswellii*, *Natroniella acetigena* and *Syntrophococcus* (Iannotti *et al*., 1987; Pitryuk & Pusheva, 2001; Karnholz *et al*., 2002; Parshina *et al*., 2003; Sirianuntapiboon *et al*., 2004).

Homoacetogenic population

The importance of the homoacetogenic bacteria in the anaerobic digestion process is not fully understood (Ryan *et al*., 2004) and relatively little is known about the functional significance of the homoacetogenic metabolism in anaerobic digestion (Zeikus, 1982). The main characteristic of homoacetogenic bacteria is their ability to utilise CO_2 and hydrogen to acetate as an end-product (Table 1) (Batstone *et al*., 2002). Although homoacetogens are found in lower numbers than the methanogens, they compete with the methanogens for a small amount of hydrogen (Batstone *et al*., 2002). As a consequence of the consumption of hydrogen by the homoacetogens, limited hydrogen availability can inhibit the hydrogenotrophic methanogenic bacteria (Forday & Greenfield, 1983) which maintain a low partial pressure in an anaerobic bioreactor that is required by the acetogenic bacteria.

The homoacetogenic bacteria also catabolise carbohydrates, aromatic compounds, alcohols and fatty acids and the main products that are formed by homoacetogens from methanol and CO_2 are acetate and butanol (Iannotti *et al*., 1987). Acetate is an important intermediate of the anaerobic degradation process and may be the main substrate for methanogenic bacteria (Weber *et al*., 1984).

Taxonomically, the homoacetogens are an extremely heterogenous group, including both Gram-positive and Gram-negative bacteria, classified in genera that also include non-

homoacetogenic bacteria (Drake *et al*., 2002; Ryan *et al*., 2004). Homoacetogens are found in a wide variety of anaerobic environments, and include psychrophilic, mesophilic and thermophilic species. The genera *Clostridium*, including *C. thermoaceticum*, *C. aceticum*, *C. formicoaceticum* and *C. thermoautotrophicum*; *Butyribacterium*, with species *B. methylotrophicum*; *Peptostreptococcus*; *Acetobacterium*, with species *A. woodii*; *A*. *bakii*; *A*. *paludosum*; *A*. *fimertarium*; *A*. *tundrae*; and *Sporomusa* are the most studied homoacetogens (Zeikus, 1982; Kotsyurbenko *et al*., 2001; Boga *et al*., 2003; Ryan *et al*., 2004). Homoacetogenic bacteria also include members of the genera *Eubacterium*, with species *E. limosum*; *Acetoanaerobium*, with species *A. noterae*; and *Acetogenium* species (Iannotti *et al*., 1987); *Sporobacter termitidis* (Grech-Mora *et al*., 1997); and *Holophaga foetida* that degrade methoxylated aromatic compounds (Liesack *et al*., 1994).

Methanogenic population

Methane forming bacteria are some of the most ancient bacteria and are grouped in the domain *Archaea*, a term which was derived from the word "*arachae*" meaning "ancient". *Archaea* comprise all known methane producing bacteria, extremely halophilic bacteria, thermoacidophilic bacteria, and thermophilic bacteria (Gerardi, 2003). Methanogens have several unique characteristics that are not found in the *Bacteria* and are morphologically a diverse group of bacteria (Zehnder *et al*., 1980; Zeikus, 1982; Gerardi, 2003). Methanogens are classified into five orders within the domain *Archaea* and include the *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales* and the *Methanopyrales* (Yu *et al*., 2004). Methanogens can be divided into three principal groups, and these different methanogens play an important role since they control the overall rate and the stability of anaerobic digestion (Sam-Soon *et al*., 1990; Gerardi, 2003).

A successful operating bioreactor requires the presence of not only a large amount of methane forming bacteria, but also a diversity of methane forming bacteria (Gerardi, 2003). The terminal metabolic reactions in anaerobic bioreactors are carried out by methanogenic bacteria (Albagnac, 1990) which also occur naturally in the digestive tract of animals, particularly the rumen of herbivores, in marshes, in brackish water, as well as in deep sea volcanic vents (Stafford *et al*., 1980; Gerardi, 2003). Methanogens comprise approximately 5% of the total population in a mixed bioreactor (Iannotti *et al*., 1987), they are particularly sensitive to pH values above 7.5 and below pH 6.0 (Moosbrugger *et al*., 1993) and require a lower oxidation-reduction potential for growth (-300 mV) than most

anaerobic bacteria (Forday & Greenfield, 1983). Methanogens have long generation times and thus long retention times are required in an anaerobic bioreactor to ensure the development of a large methanogenic population to facilitate the degradation of organic compounds (Zeikus, 1982; Albagnac, 1990; Lettinga, 1995; Gerardi, 2003).

The first methanogenic group comprises of the hydrogenotrophic methanogens and includes species belonging to the order *Methanobacteriales*, *Methanomicrobiales* and *Methanococcales* (Garrity & Holt, 2001; Casserly & Erijman, 2003). Various hydrogen utilising methanogens have been detected or isolated from different types of anaerobic bioreactors, and includes the genera *Methanobacterium*, *Methanospirillum*, *Methanococcus* and *Methanosarcina* spp. (Zeikus, 1980; MacLeod *et al*., 1990; Chan *et al*., 2001; Liu *et al*., 2002; Huang *et al*., 2003; Kolukirik *et al*., 2004; Sawayama *et al*., 2004; Zhang & Fang, 2004). In anaerobic bioreactors hydrogenotrophic methanogens use hydrogen to convert CO_2 to CH_4 (Table 1) (Sam-Soon *et al*., 1990). Although the hydrogenotrophs can operate over a wide hydrogen partial pressure range, by converting CO_2 to CH_4, these organisms help to maintain the low partial hydrogen pressure in the anaerobic bioreactor required for the survival of the acetogenic bacteria (Gerardi, 2003). Less than 30% of the CH_4 in an anaerobic bioreactor is produced by the hydrogen consuming methanogens, although it is a more effective process of energy capture by the methane forming bacteria (Weber *et al*., 1984). The reason for this is the limited supply of hydrogen in an anaerobic bioreactor because of the homoacetogens that compete with methanogens for the small amount of hydrogen (Iannotti *et al*., 1987). Delbès *et al*. (2001) analysed the *Archaeal* microbial populations present in an anaerobic bioreactor that had an accumulation of acetate. It was found that species closely related to *Methanobacterium formicicum* and belonging to the family *Methanobacteriales* were the dominant and most active methanogens in the bioreactor during the acetate accumulation period. Dominance of hydrogenotrophic methanogens suggests that hydrogen-dependent methanogenesis was the major route of CH_4 production, whereas acetate dependent methanogenesis was negligible. Such pathways have been observed in laboratory-scale anaerobic bioreactors treating various effluents (Hansen *et al*., 1999; Schnürer *et al*., 1999) and two bioreactors fed with glucose (Raskin *et al*., 1996; Fernandez *et al*., 1999). Thus, it is important to identify the different methanogenic populations present in the UASB granules to ensure successful anaerobic degradation of the wastewater being treated and obtaining a successful UASB process.

The second methanogenic group responsible for aceticlastic methanogenesis are the obligate aceticlastic methanogens. Only two genera of aceticlastic methanogens have been described and include *Methanosaeta* (previously known as *Methanothrix*; Huser *et al*., 1982) and the *Methanosarcina*, that utilise acetate to produce CH_4 and CO_2 (Table 1) (Zheng & Raskin, 2000). Smith & Mah (1966) found that 70% of CH_4 generated in an anaerobic bioreactor is derived from acetate. *Methanosaeta* spp., such as *M. concilii* and *M. thermophila* can only utilise acetate as substrate, whereas *Methanosarcina* spp., such as *M. barkeri*, *M. mazeii* and *M. thermophila* can utilise acetate, as well as methanol, methylamines, hydrogen and CO_2 (Schmidt & Ahring, 1999; Garrity & Holt, 2001). *Methanosaeta* spp. have a lower growth rate at high acetate concentrations than *Methanosarcina* spp., but their affinity for acetate is five to ten times higher (Jetten *et al*., 1992). *Methanosaeta* spp will, therefore, dominate when acetate concentrations are low and *Methanosarcina* spp. will dominate when acetate concentrations are high (Gujer & Zehnder, 1983). Conklin *et al*. (2004) showed that due to the low acetate concentrations in most anaerobic bioreactors, the slower growing, *Methanosaeta* spp. dominated in a stable continuously stirred tank bioreactor, although both *Methanosaeta* and *Methanosarcina* spp. have been identified in granules from UASB bioreactors under stable conditions treating various wastewaters (MacLeod *et al*., 1990; Fang *et al*., 1994; Fang *et al*., 1995; Schmidt & Ahring, 1999; Fang, 2000; Ince & Ince, 2000; Chan *et al*., 2001; McHugh *et al*., 2003; Sakamoto *et al*., 2004).

In a bioreactor, the sulphate-reducing bacteria also compete for hydrogen and acetate as substrates to reduce sulphate to hydrogen sulphide (Gerardi, 2003). The need for hydrogen results in competition between the sulphate-reducing and methane producing bacteria (Fukui *et al*., 2000). When sulphate and methane producing bacteria compete for hydrogen and acetate, sulphate-reducing bacteria obtain hydrogen and acetate more easily than methane forming bacteria under low acetate levels, which in turn may lead to bioreactor operational problems since sulphide can cause toxicity when present at high concentrations (Gerardi, 2003).

The third group of methane forming methanogenic species are known as the methylotrophic methanogens. The methylotrophic methanogens, such as *Methanosarcina barkeri* (Nishio *et al*., 1992), produce CH_4 directly from methyl groups ($-CH_3$) and utilise substrates such as methanol (CH_3OH) and methylamines [$(CH_3)_3$-N] (Gerardi, 2003). Methanol is an important organic pollutant present in several wastewaters (Minami *et al*.,

1991) but can be utilised as substrate by methanogens, as well as the acetogens (Jarrel & Kalmokoff, 1988; Florencio *et al*., 1995). In anaerobic bioreactors the concentration of the available substrate is one of the most important factors that influence the competition between methanogens and acetogens (Florencio *et al*., 1994).

In the literature it has been shown that a diversity of methanogens may be present in granules from pilot and full-scale bioreactors that had been exposed to the treatment of different wastewaters (Fang *et al*., 1995; Leclerc *et al*., 2001; Casserly & Erijman, 2003; Zhang & Fang, 2004). Each effluent treated should result in different dominant methanogen species in the granules. Thus, it is important to determine which methanogens are present in the granules and what the impact of stress conditions will be on these methanogens. The different methanogens present in UASB granules play a major metabolic role during granulation and thus the ability to be able to monitor the methanogens and understand their activities is essential to effectively control the start-up and operation of anaerobic bioreactors (Yu *et al*., 2004).

F. Molecular techniques

In order to obtain a better understanding of granulation and UASB bioreactor performance is it essential to know the composition of the microbial consortium present in UASB granules (Batstone *et al*., 2004). Traditional microbiological techniques have been used in the past to determine population identity in anaerobic digesters (Liu *et al*., 2002), but these techniques are limited and time consuming as many organisms, especially the anaerobes, are not readily cultured on selective media (Briones & Raskin, 2003). Molecular techniques are, therefore, used more and more to study the complex microbial populations that are present in natural environments (Gonzalez *et al*., 2003). Among available fingerprinting techniques, denaturing gradient gel electrophoresis (DGGE) is used. It is based on the electrophoresis of polymerase chain reaction (PCR)-amplified 16S rRNA gene fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants (Muyzer *et al*., 1993). By comparing community fingerprints obtained from different samples, the amplified DNA fragments of the dominant DGGE bands can be subjected to cloning and sequencing for identification of the different microorganisms (Gonzalez *et al*., 2003). Fluorescent *in situ* hybridisation (FISH) has also been used for the quantitative analysis and spatial distributions of microorganisms (Briones & Raskin, 2003).

This technique is based on the design of labelled oligonucleotides which specifically target 16S rRNA molecules of a single microorganism or a group of them. Hybridisation is performed on fixed whole cells with fluorescently labelled oligonucleotides and the targeted cells are observed by epifluorescence microscopy (Dabert *et al*., 2002).

PCR-based DGGE and FISH analyses have been applied to anaerobic sludge (Sekiguchi *et al*., 2001) and UASB bioreactors (Chan *et al*., 2001; Casserly & Erijman, 2003) to detect microbial population dynamics during the anaerobic process. Roest *et al*. (2005) found that the *Bacteria* and *Archaea* populations present in a full-scale UASB bioreactor treating paper mill wastewater were relatively stable over a period of three years, with *Methanosaeta* as the most abundant genus. DGGE fingerprints for *Bacteria* and *Archaea* were also obtained by Liu *et al*. (2002) during the start-up of an acidogenic anaerobic bioreactor. Among the methanogens monitored, the class *Methanomicrobiales* was the most abundant followed by *Methanobacteriales* and *Methanococcales*. Significant microbial population changes took place during the first 13 days for both bioreactors, but a longer period of up to 71 days was required to establish a microbial community with a stable metabolic activity. Etchebehere *et al*. (2003) also studied the changes that occurred in the bacterial community during UASB granule formation using the FISH technique.

The PCR-based DGGE method can be used to monitor the changes that may take place to the microbial consortium present in UASB granules that have been used to treat different wastewaters. The impact of stress conditions on the bioreactor can also be assessed by monitoring the stability or changes that may take place in the microbial populations present in the granules. Therefore, this may be a useful method to predict UASB bioreactor performance.

G. Conclusions

The development of technologies for the processing of food and food related products has led to an increase in the production of organic wastes (Perez *et al*., 2000). Anaerobic digestion has been used since the early 1970's and is an environmentally-friendly treatment which may help to ensure a more sustainable environment (Lettinga, 2004). Several types of wastewaters have been treated and many full-scale high-rate anaerobic bioreactors are operated successfully world-wide of which the UASB bioreactor has received the most commercial interest (Van Lier *et al*., 2001; Kolukirik *et al*., 2004).

The overall performance of an anaerobic bioreactor is determined by the composition of the substrate (Ince & Ince, 2000). Together with this, variation in the composition of the different trophic groups as a result of changes in one or more environmental operating conditions, impacts the entire microbial community structure and metabolic stability which will subsequently affect the bioreactor performance (Casserly & Erijman, 2003). In order to obtain a better understanding of the granulation process and improve UASB bioreactor performance and process stability, it is essential to recognize the diversity of the microbial consortium in UASB granules.

Traditional microbiological techniques have in the past been used to determine population characteristics in anaerobic digesters (Oude Elferink *et al*., 1998; Liu *et al*., 2002), but these techniques are limited as many organisms are not readily cultured on selective media (Briones & Raskin, 2003). Various molecular techniques have been applied to study the complex microbial communities present in natural environments (Gonzalez *et al*., 2003), UASB bioreactors (Casserly & Erijman, 2003) and anaerobic sludge (Sekiguchi *et al*., 2001). PCR-based DGGE of 16S rRNA gene fragments has become a popular method among microbial ecologists to study the diversity of natural microbial populations (Kisand & Wikner, 2003) and has also been applied to study the microbial community in granules in anaerobic bioreactors (Onuki *et al*., 2000).

The PCR-based DGGE technique may be applied successfully to determine the changes that may occur in the composition of the microbial populations during stressed conditions in the UASB bioreactor. It may also be a helpful method to determine the fingerprints of the various microbial populations present in UASB granules that have been used to treat different types of wastewaters. The different microbial populations present in the UASB granules play different roles during the granulation of UASB granules which is a key factor in the successful operation of the UASB bioreactor. The monitoring of the microbial consortium in UASB granules is, therefore, an important and useful method to assist anaerobic digestion and enable the successful operation of the UASB bioreactor.

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CHAPTER 3

PCR-BASED DGGE EVALUATION OF CHANGES IN THE NON-METHANOGENIC POPULATION OF STRESSED UPFLOW ANAEROBIC SLUDGE BLANKET GRANULES

Abstract

The performance of upflow anaerobic sludge bed (UASB) bioreactors is influenced by the composition of the substrate and the microbial species present in the granules. The aim of this study was to determine if a change in the structure of the non-methanogenic microbial community takes place when UASB brewery granules are subjected to the sudden addition of different carbon sources at different concentrations. A shift in the microbial community did occur when the granules were subjected to lactate medium (LM). The granules that were stressed with glucose medium (GM) did not show changes in the microbial consortium regardless of the increase in the glucose concentrations. The polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) method was successfully applied to show changes in the structure of the microbes present in UASB granules that were cultivated under different environmental conditions.

Introduction

The development of technologies for the processing of food and food related products has lead to an increase in the production of organic wastes (Kroyer, 1995). These wastes may be treated using either physical-chemical and/or biological processes (Perez *et al*., 2000). Interest in anaerobic wastewater treatment has increased considerably over the last decades as a result of the successful development of high-rate bioreactors in the early 1970's of which the UASB bioreactor has received the most commercial interest (Van Lier *et al*., 2001; Kolukirik *et al*., 2004).

The successful operation of an UASB bioreactor depends on the formation of highly flocculated compact sludge granules. These granules are formed by self-

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aggregation of anaerobic bacteria (Schmidt & Ahring, 1999; Liu *et al*., 2003; Batstone *et al*., 2004; Hulshoff Pol *et al*., 2004). These bacteria can be divided into different trophic groups that are responsible for the metabolic breakdown of organic chemicals that usually involves several degradation phases such as hydrolysis, acid-forming stage and methanogenesis (Batstone *et al*., 2002; Liu *et al*., 2002; Gerardi, 2003). In the first phase proteins, carbohydrates and lipids are hydrolised to amino acids, simple sugars and intermediate short-chain fatty acids such as acetic, butyric and propionic acid (Gerardi, 2003). The acid-forming degradation of the compounds produced through hydrolysis results in the production of carbon dioxide, hydrogen, alcohols, acetate, lactate, butyrate, propionate and formate (Gerardi, 2003). During methanogenesis methane is formed mostly from acetate, carbon dioxide and hydrogen. Anaerobic digesters are prone to failure when a rapid increase in the volatile fatty acids takes place, resulting in a drastic decrease in the pH (Liu *et al*., 2002). This inhibits the methanogenic step and leads to process failure (Liu *et al*., 2002). The overall performance of an anaerobic bioreactor is, therefore, determined by the composition of the substrate (Ince & Ince, 2000). Together with this, variations in the composition of the different trophic groups as a result of changes in one or more environmental operating conditions, impacts the entire microbial community structure and metabolic stability that will affect the bioreactor performance (Casserly & Erijman, 2003). Regardless of the substrate composition, the organisms that will eventually predominate will be those that can survive and compete under the changing environmental conditions. In order to obtain a better understanding of granulation and UASB bioreactor performance it is essential to have more insight into the effect of the sudden addition of different carbon sources at different concentrations on the microbial community in these granules.

Traditional microbiological techniques have in the past been used to determine population characteristics in anaerobic digesters (Oude Elferink *et al*., 1998; Liu *et al*., 2002), but these techniques are limited as many organisms are not readily cultured on selective media (Briones & Raskin, 2003). Molecular techniques may, therefore, be used to study the complex microbial communities present in natural environments (Onuki *et al*., 2000; Zhang & Fang, 2000; Gonzalez *et al*., 2003), UASB bioreactors (Röling *et al*., 2000; Domingues *et al*., 2001; Frignon *et al*., 2002; Casserly & Erijman, 2003) and anaerobic sludge

(Delbès *et al*., 2001). The aim of this study was to determine population shifts in non-methanogenic UASB granule microbial community that had been subjected to the sudden addition of different carbon source concentrations using PCR-based DGGE fingerprinting.

Materials and methods

DNA extraction

DNA was extracted using the modified method of Van Elsas *et al*. (1997), from UASB bioreactor granules that were homogenised with 0.6 g sterile glass beads (0.2 – 0.3 mm diameter) (Sigma) using a mortar and pestle. The granules were further homogenised after adding 800 µl of 120 mM phosphate buffer (1 part 120 mM NaH_2PO_4 (Saarchem) and 9 parts 120 mM Na_2HPO_4 (Merck), pH 8) and 100 µl 20% (m/v) sodium dodecyl sulphate (SDS) (BDH). The resulting suspension was vortexed for 2 min and incubated at 60ºC for 20 min and repeated. The mixture was centrifuged and extracted with 600 µl phenol (Saarchem), followed by 600 µl of a phenol:chloroform:isoamylalcohol (25:24:1) solution until the interphase was clean. The DNA was precipitated with 0.1 volume 3 M NaAc (pH 5.5) and 0.6 volume isopropanol (Merck). The DNA was pelleted by centrifugation, washed with 70% cold ethanol, air-dried and redisolved in 100 µl TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0).

PCR-based DGGE analysis

PCR reactions were performed on the extracted DNA using the primers F341 (5' CC TAC GGG AGG CAG CAG 3') with GC-clamp (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G 3') and R534 (5' ATT ACC GCG GCT GCT GG 3') as described by Muyzer *et al*. (1993). These primers amplify the V3 variable region within the 16S rRNA gene (corresponding to positions 341 to 534 of the 16S gene in *Escherichia coli*) from all *Bacteria*.

PCR reactions were performed in 25 µl reaction volumes, containing 0.75 µl of Expand *Taq* DNA polymerase (5 $U.\mu l^{-1}$) (Roche Diagnostics), 2.5 µl of 10 x PCR reaction buffer supplied with the enzyme, 1 µl of each of the primers (10 µM), 1 µl dNTPs (10 mM) (Promega), 1 µl BSA (10 $mg.ml^{-1}$) (Promega) and 1 µl of the extracted DNA.

The PCR amplification conditions were as follows: initial denaturation was performed at 94ºC for 3 min; followed by denaturation at 94ºC for 30 sec; primer annealing at 54ºC for 1 min; and chain elongation at 72ºC for 1 min. These three steps were repeated for 35 cycles. Final chain elongation was performed at 72ºC for 5 min and the PCR reactions were cooled to 4ºC (Muyzer *et al*., 1993). The amplified products were separated on 1% (m/v) agarose gels containing ethidium bromide, using 0.5 x TBE electrophoresis buffer and visualised under UV light.

The PCR fragments were separated using DGGE, performed with the BioRad DCode^{TM} Universal Mutation Detection System (BioRad Laboratories, USA). The PCR products were applied to 8% (m/v) polyacrylamide gels in 0.5 x TAE buffer, with a gradient of between 45 and 70%. Gradients were created by polyacrylamide containing 0 to 100% denaturant (7 M urea and 40% (v/v) formamide). Electrophoresis was performed at 130 V for 5 h at a constant temperature of 60°C. The DNA was stained with ethidium bromide and visualised under UV light.

Growth medium

The UASB granules were cultivated in lactate growth medium (LM) (Riedel & Britz, 1993) (pH 7) which consisted of ($g.l^{-1}$) 20 lactate, 5 yeast extract, 2 peptone, 10 KH_2PO_4 and ($ml.l^{-1}$) 1 Tween 80 and 10 of a trace element solution (Nel *et al*., 1985). A separate addition of glucose (GM) in place of the lactate was used at specific times to stress the granules. Glucose was specifically used to replace the lactate as it known to be a favourable substrate for the growth of the acidogens and other non-methanogenic bacteria present in the granules (Forday & Greenfield, 1983; Fernandez *et al*., 2000).

Batch cultivation and granules stress

Three studies were performed where UASB granules were batch cultivated for 14, 30 and 40 days, respectively. A linear shake-table (Department of Chemical Engineering, Stellenbosch University) at 35°C at 140 rpm was used to batch cultivate the granules. Four containers labelled A, B, C and D were used during the batch cultivation studies. Each 500 ml container contained 400 ml LM and 20 g of UASB granules obtained from an industrial size UASB treating brewery effluent (Amanzimtoti, Kwazulu-Natal). A 100 ml of the liquid volume of

each container was replaced daily with 100 ml fresh sterile LM. In the different studies the granules were stressed with 100 ml GM (pH 7). In Studies 1 and 2, the granules were stressed for four and nine days, respectively. In both these studies 5 $g.l^{-1}$ GM was used to stress the granules. In Study 3, the granules were stressed for only one day using 5, 10 and 15 $g.l^{-1}$ GM, respectively.

The 500 ml containers used in Study 1 were incubated for 14 days and labelled A1-14 and A2-14. In Study 2, the containers were incubated for 30 days and labelled B1-30 and B2-30. In Study 3, where the containers were incubated for 40 days, they were labelled C1-40, C2-40, C3-40 and C4-40. Containers labelled A1-14, B1-30 and C1-40 served as the controls for the different studies and only received LM.

Experimental procedures

In the first study of 14 days the granules in container A2-14 were cultivated in LM from days 1 to 5 and then stressed with 5 $g.l^{-1}$ GM from day 6 to 9 and then cultivated in LM from day 10 to 14. DNA was extracted from the granule samples in each container on days 0, 3, 7, 9, 12 and 14 just before the growth media was replaced.

In the second study (B2-30) the incubation time was extended to 30 days and the granules cultivated in LM from day 1 to 10 then stressed with 5 $g.l^{-1}$ GM from day 11 to 20 and then cultivated in LM for the remainder of the study. DNA was extracted from the granules on days 0, 5, 10, 15, 20, 25 and 30 specifically just before the growth media was replaced.

In the third study over 40 days, containers C2-40, C3-40 and C4-40 received LM from day 1 to 9. On day 10, the granules were stressed with GM containing glucose at different concentrations {C2-40 (5 $g.l^{-1}$), C3-40 (10 $g.l^{-1}$) and C4-40 (15 $g.l^{-1}$)}. During the remainder of the cultivation period (day 11 to 40), the granules received 5 $g.l^{-1}$ GM. In Study 3 the pH of the container contents was measured before replacing the growth medium. DNA was extracted from the granules on days 0, 5, 10, 15, 20, 25, 30, 35 and 40.

Reproducibility of molecular techniques

DNA isolations from the UASB granules were done in duplicate for each container for all three studies. Direct PCR amplifications were done on all the sample DNA, and DGGE profiles were obtained.

Results and discussion

PCR amplification

DNA was successfully isolated from the UASB granules before and during batch cultivation. PCR reactions performed on the extracted DNA resulted in amplification products of 200 base pairs (bp) and these were successfully separated using DGGE.

Batch cultivation and granules stress

Study 1 (14 days) - In this study UASB granules were cultivated in LM and stressed with 5 $g.l^{-1}$ glucose. The DGGE profile of the extracted DNA on day 0, was taken as representive of the microbial population present in the granules before growth medium were added (Fig. 1a). This profile shows six detectable bands on day 0. The DNA profiles obtained for the granules cultivated in LM (day 3 and day 7) show more bands than the profile of day 0. These additional DGGE bands may represent fermentative bacteria dominating under these environmental changes. A similar amount of new DGGE bands were obtained by Ren *et al*. (2004) who did a study to show the effect of organic loading rate on the start-up phase of bioreactors and the changes that occur in the microbial community.

The DGGE profiles of the stressed granules in A2-14 are similar to the profiles of the granules in A1-14 (control). The data for A2-14 showed no new bands in the microbial fingerprints, during or after the granules had been stressed with GM (Fig. 1b). The lack of change of the microbial structure in the UASB granules could have been a result of a too short stress period, or a too low glucose concentration used to induce stress. It was, therefore, expected that a longer cultivation and stress period would show a change in the structure of the microbial DGGE fingerprints.

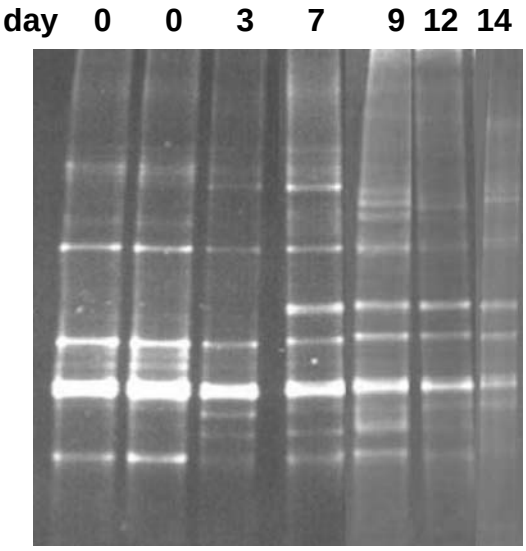

Figure 1a. DGGE fingerprints of UASB granules (A1-14) (control) cultivated in LM from day 1 to 14.

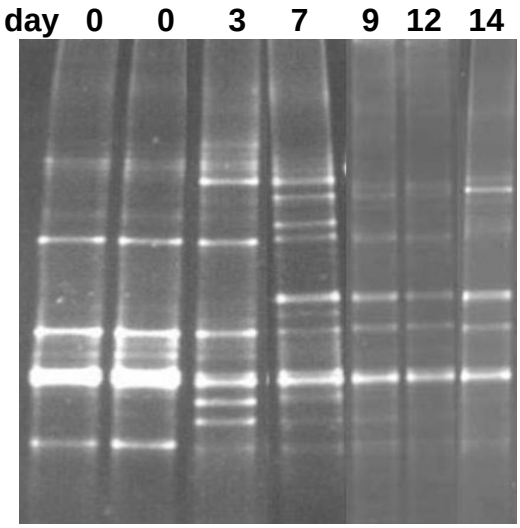

Figure 1b. DGGE fingerprints of UASB granules (A2-14) cultivated in LM from day 1 to 5 and day 10 to 14 and stressed with 5 $g.l^{-1}$ GM from day 6 to 9.

Study 2 (30 days) - The granules in this study received LM and were also stressed with 5 $g.l^{-1}$ GM but for six days longer than in Study 1. As in Study 1 the only differences in DGGE fingerprints were observed between day 0 and day 5 to 10 where the granules had been cultivated in LM. Three new DGGE bands were detected in the fingerprints of day 5 and day 10 that did not appear in the fingerprint of day 0. This suggests that the microbial structure may have changed because of the changes in the environment. The DGGE fingerprints from day 5 to 30 were identical in all respects, even though the granules were stressed for 9 days with 5 $g.l^{-1}$ GM. A third study was, therefore, conducted for a longer cultivation period, as well as higher glucose concentrations expecting to show a change in the structure of the microbes present in the granules.

Study 3 (40 days) - The DGGE fingerprints of C1-40 (control), C2-40 (stressed with 5 $g.l^{-1}$ glucose), C3-40 (stressed with 10 $g.l^{-1}$ glucose) and C4-40 (stressed with 15 $g.l^{-1}$ glucose) showed that certain DGGE bands were present in the fingerprints throughout the study, regardless of the content of the cultivation medium. Sakamoto *et al*. (2004), while evaluating the microbial community in UASB bioreactors, also found that the microbial community was complex and that the majority of the population was similar and persisted during the study.

In the fingerprints obtained for Study 3 the DGGE bands were labelled P, S and T to simplify the comparisons of the different bands (Figs 2, 3, 4 and 5). In all four the containers bands P1 to P4 were dominant (present in all the fingerprints). This suggests that these microorganisms that were not influenced by the changing cultivation conditions may, therefore, be important in the optimal functioning of the UASB granules. Bands S1 to S3 were present in several of the profiles, however, the occurrence of these bands were less common than the bands P1 to P4. These bands (S1 to S3) may represent microbial species that may have been be more susceptible to a changing environment, and it can be speculated that these microbes are of less importance in the functioning of the granules. Bands occurring infrequently in the fingerprints were the bands T1 to T7. These bands represented microbes that were sensitive to changing environments, possibly requiring a longer period to adjust to environmental changes.

The data in Fig. 2 shows the different DGGE fingerprints obtained from the DNA isolated from the granules in container C-40 that served as the control

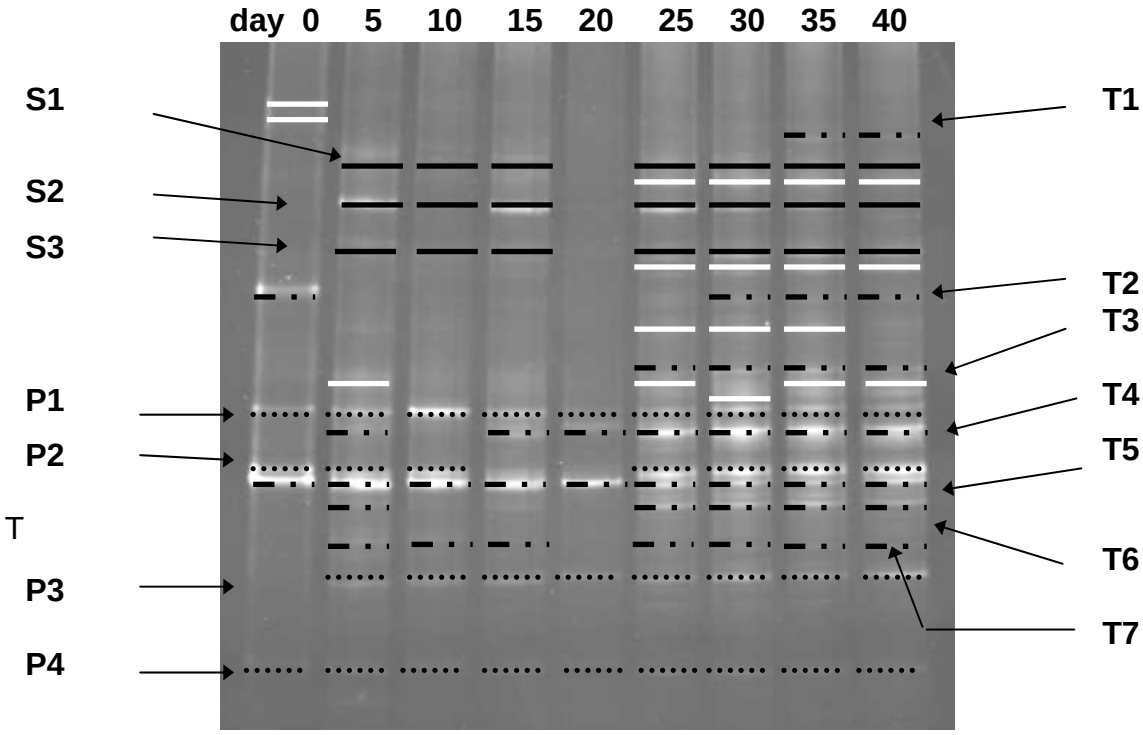

Figure 2. DGGE fingerprints of the UASB granules from C1-40 (control), cultivated in LM for 40 days. P1, P2, P3 & P4: dominant bands; S1, S2 & S3: less consistent bands; T1, T2, T3, T4, T5, T6 & T7: variable bands.

during the 40 day batch enrichment study. Bands P1 to P4 were dominant in all the fingerprints with the exception of P2 that was not observed in the fingerprints of days 15 and 20 and P3 that was not observed in day 0. The fingerprint of day 5 showed the additional bands S1, S2, S3, T5, T6 and T7 when compared to day 0, which indicates that a change in the microbial structure had or might have occurred after 5 days of batch cultivation. The number of bands in the fingerprints from days 10, 15 and 20 were less than the bands that were observed for the cultivation period of day 25 to 40. A reason for this could be the extended time the granules were subjected to LM that allowed lower cell numbers to increase to detectable cell numbers. Band T1 were observed from day 35 to 40, T2 from day 30 to 40, and T3 from day 25 to 30. It is clear that the extended cultivation period of the UASB granules to 40 days in LM caused the changes in the DGGE fingerprints, suggesting a change in the structure of the microbial population. This change may have occurred because of the prolonged adaptation time of the microbial species in LM that caused the microbial species to dominate.

The data in Fig. 3 represents the DGGE fingerprints of the microbes that were present in the granules that received LM from day 1 to 9 and 5 $g.l^{-1}$ GM from day 10 to 40. The fingerprints from days 5, 10, 15 and 20 are similar, with the exception of three bands. The DGGE band T1 can be seen in the fingerprint from day 25 and was present up to day 40, suggesting that the environmental conditions during that period were as such that it resulted in the increase in numbers of these organisms. When the fingerprint of C2-40 is compared to the fingerprint of C1-40 it is evident that no shift occurred in the microbial community regardless of the stressed conditions that the microbes in the granules were subjected to. Bands P1 to P4 were present in all the fingerprints. Bands S1, S2 and S3 were present from day 5 to 35 and day 10 to 40, respectively and band T1 appeared from day 25 to 40. In C2-40, T2 was observed from day 5 to 40, T4 from day 15 to 40 and T5 from day 5 to 40. Bands T3, T6 and T7 were present in the fingerprints of the granules in C1-40, but were not detected in the fingerprints of the granules in C2-40.

In the fingerprints of the granules in C3-40, bands were observed in the fingerprint of day 5 that do not appear in the fingerprint of day 0 (Fig. 4). These results are similar to the results obtained for the granules in C1-40 and C2-40. The DGGE profile of day 10 differed from the profile of day 5.

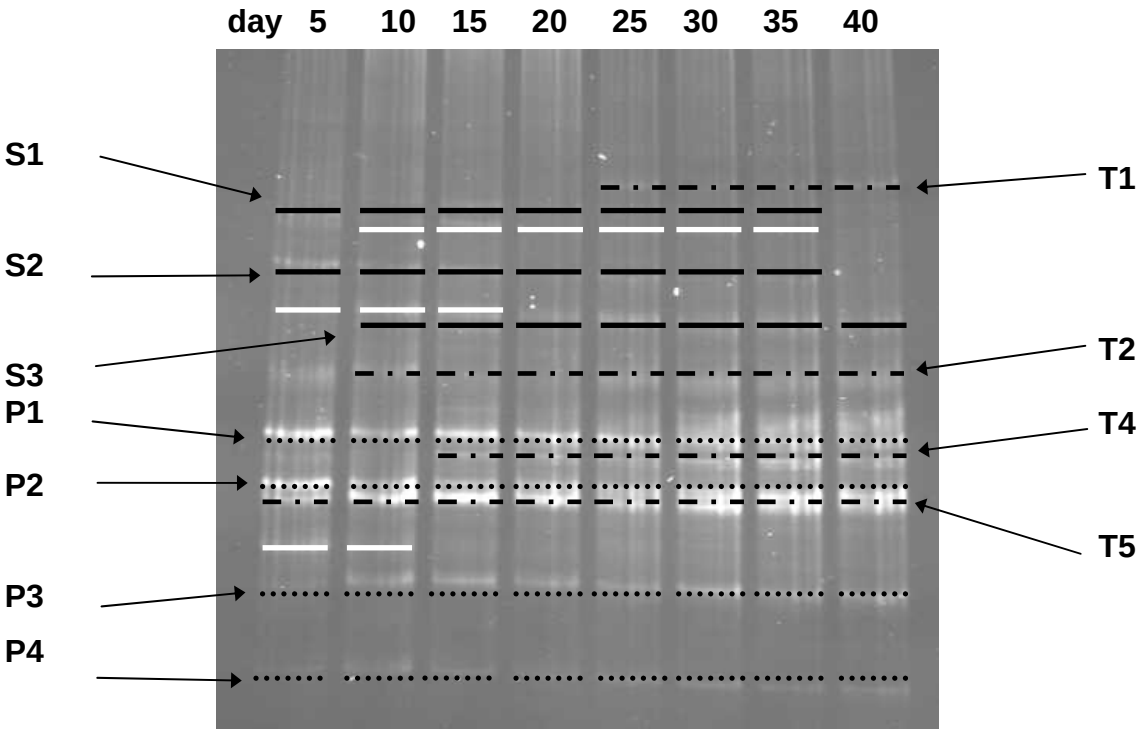

Figure 3. DGGE fingerprints of the UASB granules from C2-40 cultivated in LM: day 1 to 9; stressed with 5 $g.l^{-1}$ GM: day 10; cultivated in 5 $g.l^{-1}$ GM: day 11 to 40. P1, P2, P3 & P4: dominant bands; S1, S2 & S3: less consistent bands; T1, T2, T4 & T5: variable bands.

This was not expected, because the granules were treated with the same growth medium (LM). In the fingerprints from day 15 to 40 it was observed that the number of bands gradually decreased suggesting a decrease in the number of species in the UASB microbial community with only the more acclimatised microorganisms prevailing. P1 to P4 were present from day 5 to 40. Bands S1 and S3 were present in the fingerprints from day 5 to 20 and S2 from day 30 to 40. T1 was only observed on day 40, and T2 in the fingerprints of day 5 to 20.

In the data obtained for the granules in C4-40, dominant bands P1 to P4, as well as the less consistent bands S1 to S3 were observed from day 5 to 40. The fingerprints of the granules from day 5 to 10, with the exception of two additional bands observed in the DGGE profile of day 5, were identical (Fig. 5). The bands from day 10 and day 15 were identical, suggesting that the addition of the 15 $g.l^{-1}$ GM to the granules did not have an immediate impact on the diversity of the microbial structure. The fingerprints from day 20 to 25 were identical. Band T2 was only observed in the fingerprints from day 5 to 15 which suggest that the environment became unfavourable for the growth of this organism. The environment became favourable for the growth of the species represented by band T4 which can be seen in the profiles from day 20 to 40. The profiles of day 30 to 40 showed a new band, T3. The environmental conditions in C4-40 most likely became favourable for the growth of this new organism that is represented by band T3.

The pH profile of each container throughout the 40 day study is shown in Fig. 6. A decrease in the pH in all the containers occurred for the first two days. This decrease from pH 6.9 to pH 6.45 can be ascribed to the fermentation of carbohydrates, lipids and proteins by the acidogens to form volatile fatty acids and amino acids (Gerardi, 2003; Wangnai *et al*., 2004), as well as the homoacetogens that can catabolise 1-C compounds and complex carbohydrates to acetate. In contrast, this decrease was followed by an increase in the pH (day 2 to 10) of the medium for all four containers. This might be a result of acetogenic bacteria catabolising the fatty acids to acetate and hydrogen (Gerardi, 2003). The pH of the LM in which the granules were cultivated in C1-40, stabilised at pH 7.0 for the remainder of the experiment.

The granules in C2-40, C3-40 and C4-40 that were stressed with GM containing 5, 10 and 15 $g.l^{-1}$ glucose respectively, on day 10, showed a different

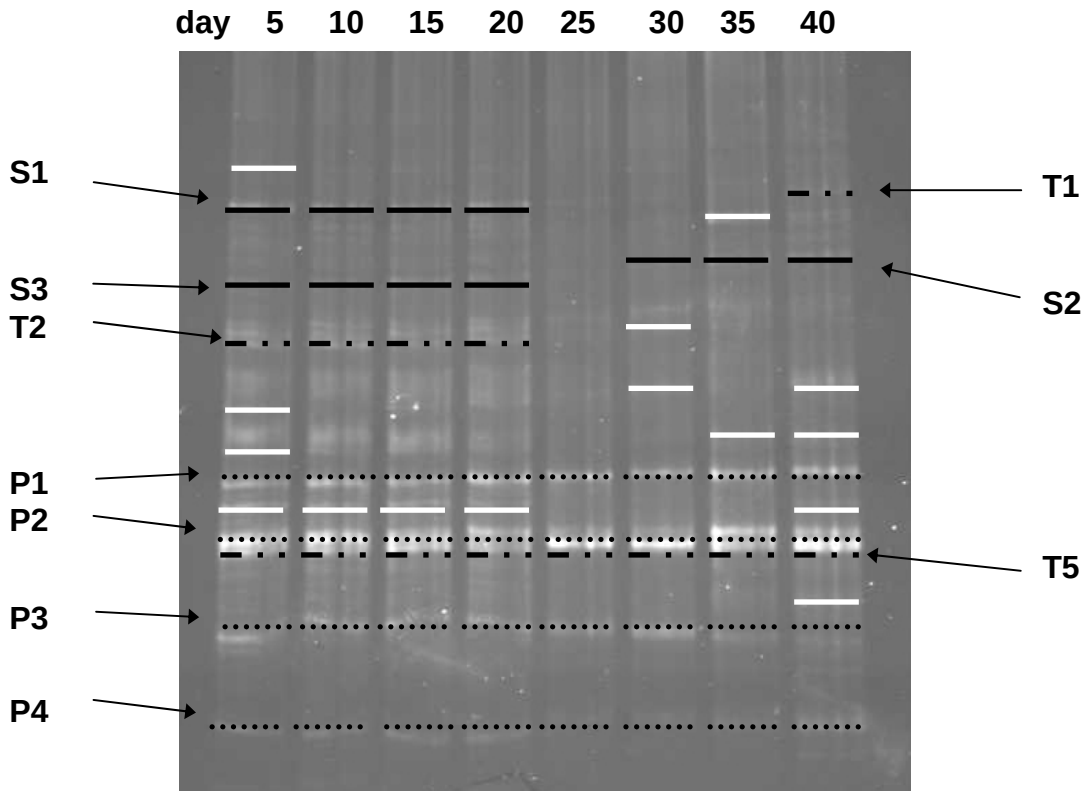

Figure 4. DGGE fingerprints of the UASB granules from C3-40 cultivated in LM: days 1 to 9; stressed with 10 $g.l^{-1}$ GM: day 10; cultivated in 5 $g.l^{-1}$ GM: day 11 to 40. P1, P2, P3 & P4: dominant bands; S1, S2 & S3: less consistent bands; T1, T2 & T5: variable bands.

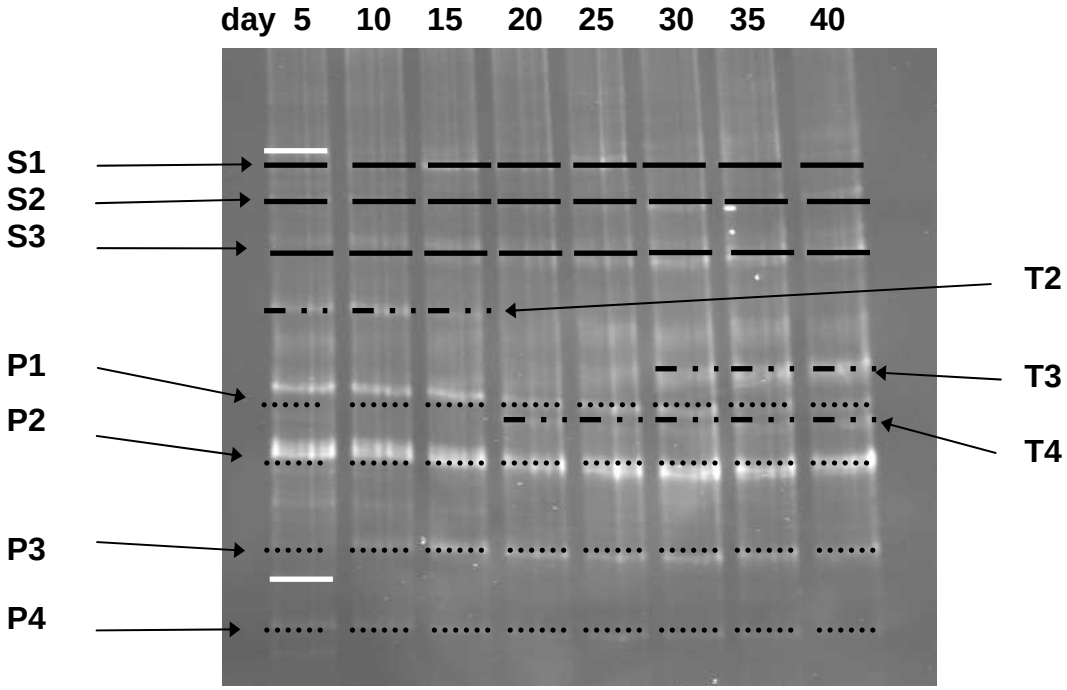

Figure 5. DGGE fingerprints of the UASB granules from C4-40 cultivated in LM: day 1 to 9; stressed with 15 $g.l^{-1}$ GM: day 10; cultivated in 5 $g.l^{-1}$ GM: day 11 to 40. P1, P2, P3 & P4: dominant bands; S1, S2 & S3: less consistent microbes; T2, T3 & T4: variable bands.

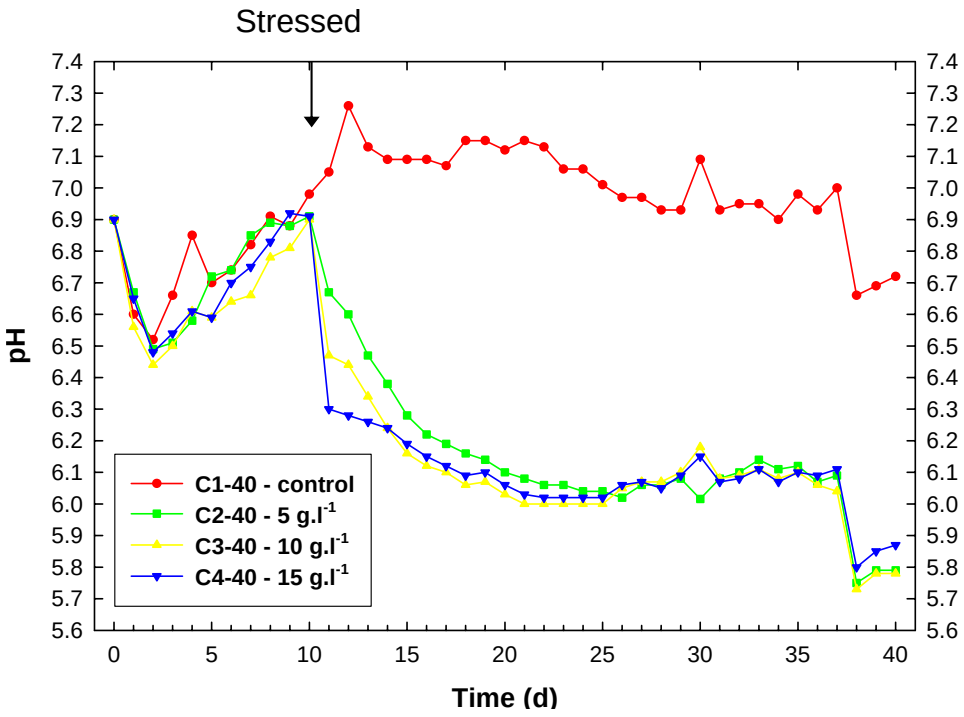

Figure 6. pH profiles of the medium in C1-40, C2-40, C3-40 and C4-40 during the 40 day cultivation period. C1-40 served as the control. On day 10 the granules in containers C2-40, C3-40 and C4-40 were stressed with 5, 10 and 15 $g.l^{-1}$ glucose, respectively.

decrease in pH, with the largest decrease in the profile of C4-40. This showed that the higher glucose concentration used resulted in the production of more organic acids by the acidogenic bacteria present in the granules. The pH for C1-40, C2-40 and C3-40 stabilised at ~pH 6.1 over the next 30 days. It is known that digester stability is influenced by pH and a lower pH of 6.1 could have a negative influence on the performance of a bioreactor. Anaerobic digestion is generally applied under pH conditions that can vary from pH 6.5 – 8 (Van Lier *et al*., 2001). It is also known that acceptable enzymatic activity of the acid forming bacteria occurs above a pH of 5.0, but acceptable enzymatic activity of the methane forming bacteria do not occur below pH 6.2. Methane forming bacteria metabolise organic acids such as acetate and formate that result in the increase of the pH and help to stabilise the performance of the bioreactor.

Conclusions

The successful operation of a UASB bioreactor depends on granules that have a stable, well settled, mixed microbial community. Various environmental factors such as different carbon sources used in a bioreactor can influence the metabolic stability of the microbes present in the granules and can cause changes in the microbial community structure.

In Study 1 done over 14 days and Study 2 over 30 days UASB brewery granules were cultivated in LM and stressed with 5 $g.l^{-1}$ GM. The DGGE fingerprints obtained showed changes in the profiles from day 0 to day 7. For these studies no other changes in the DGGE fingerprints occurred even when the granules were stressed with the glucose. This may be because the stress period was not long enough to have an observable effect on the growth of the different species present in the granules. In the third experiment the cultivation period was lengthened to 40 days, and the granules stressed only on day 10, using different glucose concentrations. The fingerprints of the granules in C1-40 (control) showed that a shift in the microbial community did occur when subjected to batch cultivation. The granules in C2-40, C3-40 and C4-40 that were stressed with glucose did not result in a similar microbial shift as observed in C1-40. It is clear from the different DGGE profiles obtained that changes in the microbial community occurred in the granules in the first 20 days after cultivation in LM, but that a

longer period of 35 to 40 days were required to establish a stable microbial community.

It is clear that using molecular techniques, identification of the mixed microbial communities in UASB granules is a daunting task. For improvement of process stability in UASB bioreactors, it is important to determine the diversity of the microbial community in a system. Only by acquiring a better understanding of the functional diversity and interactions between microbes and components can we hope to achieve the necessary understanding required to predict UASB performance under certain conditions.

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CHAPTER 4

FINGERPRINTING AND IDENTIFICATION OF *BACTERIA* PRESENT IN FOUR DIFFERENT TYPES OF UASB GRANULES

Abstract

The effective operation of the anaerobic digestion process in an upflow anaerobic sludge blanket (UASB) bioreactor is dependent on the microbial composition of the UASB granules. The granules contain a consortium of bacteria, with a specific metabolic function for each group, contributing to the overall efficiency and stability of the bioreactor. The aim of this study was to fingerprint and identify the *Bacteria* present in four different types of South African UASB granules treating winery, brewery, distillery and peach-lye canning wastewaters. This was done by combining conventional microbiological platings with PCR-based denaturing gradient gel electrophoresis (DGGE) and DNA sequence analysis. Each granule type showed distinct PCR-based DGGE fingerprints with unique bands, while other bands were found to be present in all the granules regardless of the wastewater being treated. A wide variety of bacteria were identified from the four granule types. Thirty five percent of the identified bacteria represented the unculturable bacteria and 65% the culturable bacteria which included members of the following genera: *Bacillus*, *Pseudomonas*, *Bacteroides*, *Enterococcus*, *Alcaligenes*, *Clostridium*, *Shewanella*, *Microbacterium*, *Leuconostoc*, S*ulfurospirillum*, *Acidaminococcus*, *Vibrio*, *Aeromonas*, *Nitrospira*, *Synergistes*, *Rhodococcus*, *Rhodocyclus* and *Syntrophobacter*. A DGGE marker was successfully constructed, representing members of the *Bacterial* consortium in UASB granules.

Introduction

The upflow anaerobic sludge blanket (UASB) process has been widely used for the biological treatment of food and beverage processing wastewaters (Wolmarans & De Villiers, 2002; Batstone *et al*., 2004). These types of

wastewaters normally have a high organic content and contain little or no toxic solids, providing the ideal conditions for bioreactor operation (Trnovec & Britz, 1998). The efficiency and stability of UASB bioreactors are dependent on the microbial composition (Roest *et al*., 2005). The microorganisms present in the seeding sludge form dense aggregates or granules through a granulation process characteristic to these bioreactors. The formed granules consist of different trophic groups necessary for anaerobic digestion (Sekiguchi *et al*., 1998). These are the acidogenic, acetogenic, homoacetogenic and methanogenic bacteria. These microbial groups are responsible for executing the consecutive stages of the anaerobic digestion process, where the metabolic products of one microbial group are assimilated by the next microbial group (Gerardi, 2003).

Acidogens are responsible for the degradation of organic material to form carbon dioxide, hydrogen, acids and alcohols (Wangnai *et al*., 2004). The acetogenic bacteria convert the fatty acids to acetic acid and hydrogen (Van Andel & Breure, 1984). To prevent hydrogen accumulation, the homoacetogenic bacteria utilise carbon dioxide and hydrogen to form acetate as an end-product. The methanogens convert the acetate to methane and carbon dioxide (Batstone *et al*., 2002). Variations in the composition of one trophic group as a result of changes in substrate composition, reactor temperature, retention time and even pH may influence the entire microbial community structure (Casserly & Erijman, 2003). It is, therefore, important which microbes of each granule population are present in the granules so as to optimise the metabolic activity which will result in the successful operation of an UASB bioreactor.

Different types of wastewaters when treated also have an influence on the microbial consortium present in the granules (Chapter 5 of this dissertation - Keyser *et al*., 2005). Each type of wastewater favours the growth of specific bacteria that may have an impact on the success of the granulation process and subsequently the successful operation of the UASB bioreactor. Each type of bacteria present in the granules performs a specific metabolic role in the granules and contributes to the successful performance of the granules and ultimately the UASB bioreactor. Several of these bacteria that form part of the mixed microbial community in the granules, have not been cultured before, and therefore, could not be identified. Thus, is it essential to identify the various microorganisms in a bioreactor, since they play a role in the successful operation of the bioreactor.

The use of conventional culture-dependent methods to isolate microbes from the UASB environment is limited due to the inability of many microbes to grow on synthetic media. Culture-independent methods, therefore, have over the last few years been proven to be of value for the identification of bacteria in complex samples (Roest *et al*., 2005). The aim of this study was firstly to use PCR-based DGGE and DNA sequence analysis to fingerprint and identify the different culturable and unculturable *Bacteria* present in four different types of UASB granules that had been used to treat either winery, brewery, distillery or peach-lye canning wastewaters. A further aim was to construct a DGGE marker representing some of the *Bacteria* present in the four different UASB granule types so as to facilitate future rapid identification of UASB microbes.

Materials and methods

UASB granules

Three of the different types of UASB granules were obtained in 20 kg batches from three industrial-scale UASB bioreactors. The granule batches that had been used in the treatment of winery and distillery wastewaters were obtained from Distell, Stellenbosch, Western Cape, RSA. The brewery granules were obtained from the SAB brewery plant in Amanzimtoti, Kwazulu-Natal, RSA. The fourth granule type was originally from a full-scale brewery UASB bioreactor and then used as inoculum for a 60 L lab-scale UASB bioreactor treating peach-lye canning wastewater at the Department of Food Science, Stellenbosch University. This 60 L UASB bioreactor was operated for 15 months on this peach-lye canning effluent before granules were taken for analysis in this study.

Isolation of the microbial population

Granules from each of the four different types of UASB granules were homogenised with a sterile pestle in 9 ml of sterile saline solution (0.85% (m/v) NaCl) and a dilution series ($10^{-1} \rightarrow 10^{-8}$) was prepared. Pour-plates were made for each of the dilutions using nutrient agar (NA) (Biolab) and deMan, Rogosa and Sharpe agar (MRS) (Biolab). The plates were incubated aerobically and anaerobically using the Anaerocult A system (Merck) at 35°C for 2 days. Although MRS is selective for lactic acid bacteria, it facilitates the growth of many bacteria

as it is a “rich” growth medium (Van der Merwe & Britz, 1994). Bacterial colonies were randomly selected and streaked until pure isolates were obtained. These were Gram-stained and microscopically examined to confirm isolate purity.

DNA extraction

DNA was extracted from the four different types of UASB bioreactor granules using the method of Van Elsas *et al*. (1997) as modified by Keyser *et al*. (2005). All the DNA extractions were done in duplicate.

PCR-based DGGE analysis

PCR reactions were performed on the extracted DNA from the UASB granules and from the pure isolates. PCR reactions were performed using the primers F341 (5’ CC TAC GGG AGG CAG CAG 3’) with GC-clamp (5’ CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G 3’) and R534 (5’ ATT ACC GCG GCT GCT GG 3’) as described by Muyzer *et al*. (1993). The primers amplify approximately 200 base pairs (bp) of the V3 variable region within the 16S ribosomal RNA (rRNA) gene (corresponding to positions 341 to 534 of the 16S rRNA gene of *Escherichia coli*). PCR reactions and conditions are as described by Keyser *et al*. (2005), using 1 μl 99% (v/v) dimethyl sulphoxide (DMSO) (Merck) instead of bovine serum albumin (BSA).

In order to obtain DNA sequence data of the bacteria present, a 1.5 kilobase pair (kb) part of the 5’ end of the 16S rRNA gene was amplified using the primers F8 (5’ – CAC GGA TCC AGA CTT TGA TYM TGG CTC AG – 3’) and R1512 (5’ – GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT – 3’) (Felske *et al*., 1997). PCR reactions were performed in 50 μl reaction volumes, containing 1 μl *Taq* DNA Polymerase (5 U/μl) (Promega), 5 μl 10 x PCR reaction buffer, 2 μl of each of the primers (10 μM), 2 μl dNTPs (10 mM) (Promega), 2 μl 99% (v/v) DMSO (Merck) and 2 μl of the extracted DNA. The PCR amplification conditions were as follows: initial denaturation was at 92ºC for 3 min; followed by denaturation at 92ºC for 30 sec; primer annealing at 54ºC for 30 sec; and chain elongation at 68ºC for 1 min. These three steps were repeated for 35 cycles. Final chain elongation was performed at 72ºC for 7 min (Felske *et al*., 1997). All the amplified PCR products were separated on 1% (m/v) agarose gels and

visualised under UV light (Vilber Lourmat). A nested PCR reaction amplifying a 200 bp part of the 5' end of the 16S rRNA gene was performed on the amplified 1.5 kb PCR fragments, using the primers F341 and R534 (Muyzer *et al*., 1993). This was done to confirm that each 200 bp band in the DGGE fingerprints was represented by a 1.5 kb PCR product.

The 200 bp PCR fragments were separated using DGGE, performed with the BioRad DCode^{TM} Universal Mutation Detection System (BioRad Laboratories, USA). The PCR products were applied to 8% (m/v) polyacrylamide gels with a gradient of between 45 and 70% as described by Keyser *et al*. (2005).

Cloning and DNA sequencing

The amplified 1.5 kb PCR fragments were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics) according to the manufacturer's instructions. Only the 1.5 kb PCR fragments amplified from the granule DNA were cloned into the pGemT-Easy Vector System II (Promega) since the PCR product contained a mixture of fragments of different sequence. Transformed cells were screened for the correct sized insert using the primers T7 and SP6. Amplification reactions were performed in a total reaction volume of 50 µl containing 1 µl *Taq* DNA Polymerase (5 U/µl) (Roche Diagnostics), 5 µl 10 x PCR buffer, 2 µl of each of the primers (10 µM) and 2 µl dNTPs (10 mM) (Promega). The PCR amplification conditions consisted of an initial denaturation at 92ºC for 3 min, followed by 35 cycles of denaturation at 92ºC for 30 sec, annealing at 54ºC for 30 sec, elongation at 68ºC for 1 min, and a final 7 min elongation at 72ºC. The amplification products were purified using the High Pure Purification Kit of Roche Diagnostics according to the manufacturer's instructions. These were sequenced using the ABI PRISM 377 DNA Sequencer (Perkin-Elmer) at the DNA Sequencing facility at Stellenbosch University. The sequences obtained were compared to 16S rRNA gene sequences available in the National Center for Biotechnology Information (NCBI) database using the BLASTn search option (Altschul *et al*., 1990).

Phylogenetic analysis

The 16S rRNA gene sequences were manually aligned by inserting gaps and the phylogenetic analyses were conducted using MEGA 2.1 (Kumar *et al*., 2001). Phylogenetic distances were calculated based on the Kimura 2-parameter (Kimura, 1980) substitution model. Phylogenetic trees were generated from the distance matrixes using a neighbour-joining tree building algorithm (Saitou & Nei, 1987) and the statistical support for the branching nodes was obtained by bootstrap (1 000 replicates) (Felsenstein, 1985).

DGGE marker

Nineteen DGGE bands were selected from the profiles obtained for the different granules. These bands represented cloned PCR fragments, as well as isolates from the granules. The selected DGGE bands were excised from the gels, purified and re-amplified using the primers F341 and R534 (Muyzer *et al*., 1993). The PCR products were separated using DGGE by loading 2 µl of each reaction mixture in the same well.

Results and discussion

PCR-based DGGE fingerprinting of the different UASB granules

The 200 bp PCR amplification products were successfully separated using DGGE to produce unique fingerprints for each of the granule types evaluated. PCR-based DGGE fingerprints of duplicate DNA extractions of each granule type were found as shown in Fig. 1, to be reproducible. Distinct fingerprints, containing unique bands, were observed for the four different UASB granules (Fig. 1).

Certain bands were in the same position on the gel and present in all the granules, suggesting that a section of the microbial composition, under the conditions employed in this study, were identical and constant, irrespective of the wastewater treated (Fig. 1). This suggests that the organisms represented by those bands are constant, irrespective of the wastewater being treated.

Certain bands in the fingerprints from the peach-lye canning granules were similar to bands found in the fingerprints of the brewery granules. Other bands present in the fingerprints of the peach-lye canning granules were not observed in

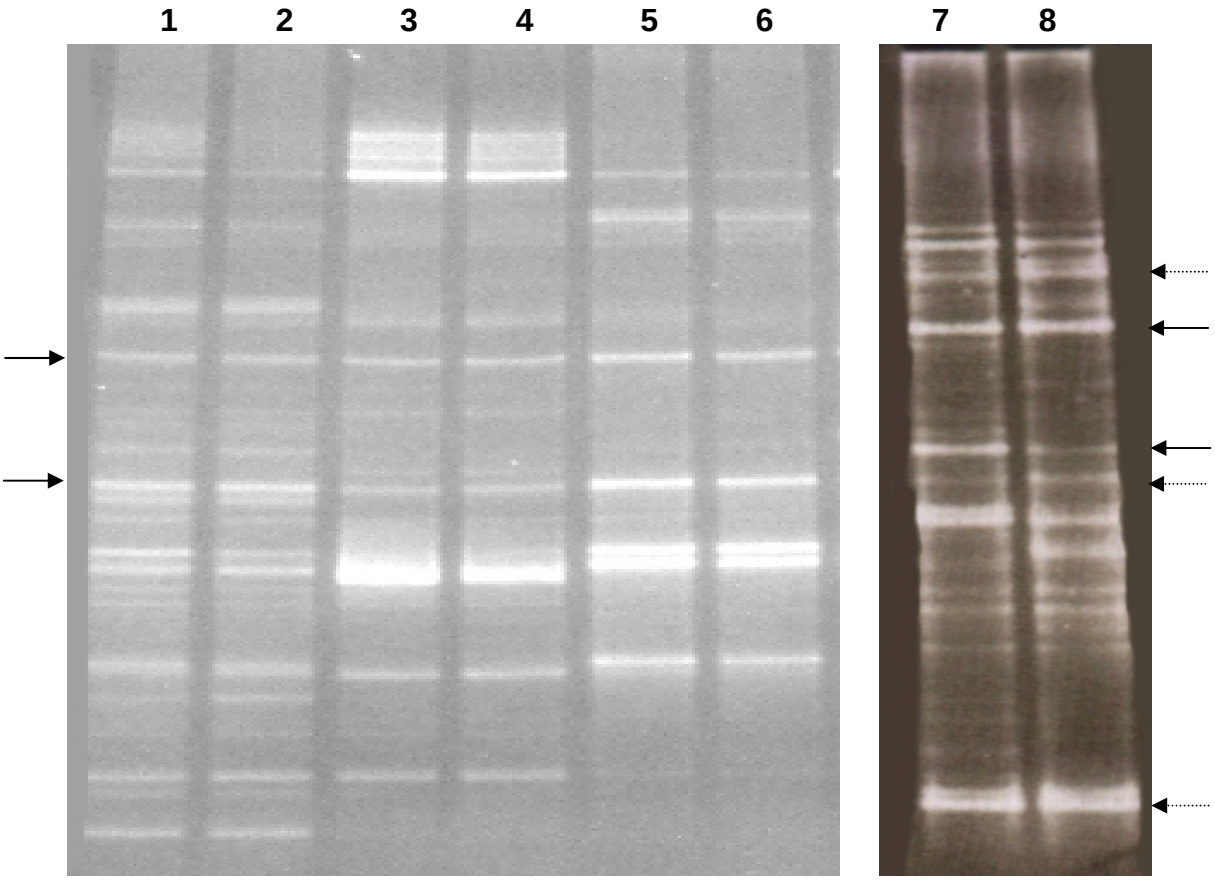

Figure 1. PCR-based DGGE fingerprints (in duplicate) of the different UASB granules treating different wastewaters. Lanes 1 and 2: winery wastewater granules; Lanes 3 and 4: distillery wastewater granules; Lanes 5 and 6: brewery wastewater granules; and Lanes 7 and 8: peach-lye canning wastewater granules. The bands common to all the fingerprints are indicated by the solid arrows. The bands present in the fingerprints of the peach-lye canning granules that were not observed in the brewery granules are indicated by the dashed arrows.

the fingerprints of the brewery granules (Fig. 1). These similarities and differences are clear indications of changes in the microbial consortium when granules are subjected to a change in wastewater composition. This confirms that the composition of the wastewater treated had had an impact on the microbial species present in the granules.

Species identification and phylogeny

The 1.5 kb PCR fragments amplified from the total DNA isolated from each granule type were cloned. The successfully transformed cells and the cells from the isolates from the selective media plated from the four different granules were subjected to PCR amplification (Muyzer *et al*., 1993). The amplified 200 bp PCR products were separated using DGGE. Single DGGE bands were obtained at different positions on the gel, confirming the purity of the isolates and transformants from the different UASB granules (data presented in the Appendix, Figs. A1 to A4).

Sixty nine different bacteria were identified from the winery, brewery, distillery and peach-lye canning granules. Of the 69 bacteria, 24 were representative of uncultured bacteria, constituting 35% of the identified bacteria. The remaining 65% were culturable bacteria. In Table 1, the number given to each unique DGGE band, the number of bases of each fragment sequenced and its GenBank accession number, as well as the closest relative and the percentage sequence similarity, are summarised.

Certain bacteria were specific to a certain granule type while other bacteria were found to be present in more than one granule. *Clostridium* was only identified in the distillery granules, while *Enterococcus*, *Leuconostoc*, *Aeromonas*, *Vibrio*, and species related to *Rhodocyclus*, *Nitrospira*, *Rhodococcus* and *Syntrophobacter* were present only in the brewery granules. *Microbacterium* species were found only in the winery granules. *Sulfurospirillum* and species related to *Acidaminococcus* were found only in the peach-lye canning effluent granules. The most numerous bacteria present in all four granule types were species of *Bacillus* and *Pseudomonas*. A total of 20 *Bacillus* and 12 *Pseudomonas* were identified in the various granules used in this study.

<u>*Bacillus:*</u> Twenty members of the genus *Bacillus* were found to be present in four different UASB granule types (Table 1). Seven of these

Table 1. Identification of *Bacteria* present in different UASB granules.

DGGE Band[a]	Sequence Length (bp)	GenBank Accession Number (DGGE Band)	% Sequence Similarity	Closest Relative	GenBank Accession Number (Closest Relative)
Bacillus					
W1	510	DQ238239	99.6	*Bacillus pycnus* sp. NRS-1695	AF169535
W2	510	DQ238238	99.8	*Bacillus megaterium* KL-197	AY030338
W3	506	DQ238244	98.2	*Bacillus* sp. TKSP21	AF411341
W6	511	DQ238240	100	*Bacillus cereus* G9667	AY138273
W9	511	DQ238242	100	*Bacillus cereus* J-1	AY305275
W10	510	DQ238243	99.2	Unidentified bacterium clone W4B-B03	AY345491
W20	511	DQ238241	100	*Bacillus subtilis* ATCC21331	AB018487
B6	511	DQ238237	100	*Bacillus cereus* J-1	AY305275
B7	510	DQ239796	100	*Bacillus fusiformis* DSM2898T	AJ310083
B12	510	DQ238236	96.5	*Bacillus sphaericus* PLC-5	AY161044
D1	511	DQ238249	100	*Bacillus cereus* B412	AJ577281
D2	511	DQ238248	100	*Bacillus subtilis* KL-077	AY030331
D3	491	DQ238247	99.8	*Bacillus* sp. 19497	AJ315065
D5	511	DQ238246	100	*Bacillus subtilis* C15	AF274248
D16	511	DQ238251	100	*Bacillus cereus* RIVM BC00068	AJ577283
D17	511	DQ238250	100	*Bacillus cereus* RIVM BC00068	AJ577283
PL2	511	DQ238255	100	*Bacillus cereus* ATCC535221	AF290551
PL3	511	DQ238254	100	*Bacillus* sp. A24	AF397399
PL4	508	DQ238253	99.6	*Bacillus pumilus*	AF393657
PL6	511	DQ238256	100	*Bacillus* sp. TKSP21	AB017591
Pseudomonas					
B4	682	DQ238235	99.9	*Brevundimonas bullata*	AB023428
D4	739	DQ238233	98.1	Sulphide-oxidizing bacterium N9-1	AF393509
D6	731	DQ238232	97.8	*Pseudomonas* sp. AMSN	AF438148
D10	729	DQ238270	94.7	*Burkholderia pyrrocinia* strain R13058	AJ440714
D13	730	DQ238271	98.5	*Pseudomonas* sp. NZ112	AY014826
PL1	736	DQ238231	99.2	*Pseudomonas fluorescens*	AF094726

[a]W = UASB granules treating winery wastewater, B = UASB granules treating brewery wastewater, D = UASB granules treating distillery wastewater, PL = UASB granules treating peach-lye canning wastewater.

Table 1. (cont.)

DGGE Band[a]	Sequence Length (bp)	GenBank Accession Number (DGGE Band)	% Sequence Similarity	Closest Relative	GenBank Accession Number (Closest Relative)
Pseudomonas					
PL5	732	DQ238230	99.2	*Pseudomonas fluorescens* bv. C	AF228367
PL8	736	DQ238234	96.0	*Pseudomonas* sp. 7-1	AF521651
PL11	734	DQ238263	99.6	Uncultured Gamma *Proteobacterium*	AB015570
PL14	729	DQ238262	99.6	*Pseudomonas veronii*	AF064460
PL17	733	DQ238260	99.3	*Pseudomonas* sp. NZ024	AY014806
PL19	736	DQ238264	97.8	Uncultured bacterium KM94	AY216460
PL20	734	DQ238259	99.6	*Pseudomonas putida*	D85999
PL22	732	DQ238261	98.4	*Pseudomonas viridiflava*	AF364097
Bacteroides					
W19	886	DQ238265	99.9	Uncultured bacterium clone IIB-29	AJ488088
D9	886	DQ238269	99.3	Uncultured bacterium clone IIIB-28	AJ488099
D15	886	DQ238268	99.9	Uncultured bacterium clone IIB-29	AJ488088
PL16	885	DQ238258	81.9	Uncultured *Bacteroidetes* clone ML635J-40	AF507859
Enterococcus					
B1	738	DQ238227	96.8	Uncultured bacterium clone P-1938-s962-3	AF371532
B11	737	DQ238229	98.1	*Enterococcus* sp. ALE-1	AY017051
B13	740	DQ238228	98.1	*Enterococcus durans*	Y18359
Alcaligenes					
W7	841	DQ238224	99.8	*Achromobacter spanius*	AY170848
B2	855	DQ238226	93.6	Uncultured bacterium clone ZZ14AC10	AY214198
B3	848	DQ238225	99.8	*Alcaligenes faecalis*	AF155147
Clostridium					
D7	894	DQ191233	98.0	*Clostridium butyricum* strain VPI3266	AJ458420
D8	873	DQ191234	97.2	*Clostridium bifermentans*	AF320283
Shewanella					
W8	626	DQ191239	99.5	*Shewanella putrefaciens*	U91551
B8	628	DQ191238	91.2	*Shewanella putrefaciens*	U91553

[a]W = UASB granules treating winery wastewater, B = UASB granules treating brewery wastewater, D = UASB granules treating distillery wastewater, PL = UASB granules treating peach-lye canning wastewater.

Table 1. (cont.)

DGGE Band[a]	Sequence Length (bp)	GenBank Accession Number (DGGE Band)	% Sequence Similarity	Closest Relative	GenBank Accession Number (Closest Relative)
Microbacterium					
W4	746	DQ191236	99.5	*Microbacterium* sp. PRLIST4	Y15325
W5	742	DQ191237	100	*Microbacterium oxydans*	Y17227
Leuconostoc					
B14	659	DQ191235	85.1	Uncultured *Leuconostoc* sp. clone LabS38	AF335916
Sulfurospirillum					
PL12	577	DQ191240	98.9	*Sulfurospirillum arsenophilum*	U85964
PL13	569	DQ191241	98.1	*Sulfurospirillum halorespirans*	AF218076
Acidaminococcus					
PL9	640	DQ191232	91.8	Unidentified eubacterium clone vadinHB04	U81750
PL21	639	DQ191231	94.8	Uncultured bacterium clone ER1_17	AY231317
Vibrio					
B9	852	DQ191248	99.9	*Vibrio parahaemolyticus*	AY245192
Aeromonas					
B10	848	DQ191247	96.2	*Aeromonas salmonicida*	X74681
Syntrophobacter					
B21	794	DQ191246	99.0	Uncultured bacterium clone R1p32	AF482435
Rhodocyclus					
B18	647	DQ191242	95.5	Uncultured bacterium clone HP1B54	AF502232
Rhodococcus					
B16	722	DQ191245	86.1	Uncultured bacterium clone BA149	AF323777
Nitrospira					
B15	661	DQ191243	88.2	Uncultured bacterium DCE29	AF349765
B19	661	DQ191244	99.8	Uncultured bacterium clone SR_FBR_L1	AY340834
Synergistes					
W11	663	DQ238267	98.2	Uncultured bacterium TA19	AF229792
W17	658	DQ238266	85.6	Uncultured bacterium clone SHA-104	AJ306760
D11	675	DQ238272	89.5	Uncultured bacterium clone TTA_B6	AY297966

[a]W = UASB granules treating winery wastewater, B = UASB granules treating brewery wastewater, D = UASB granules treating distillery wastewater, PL = UASB granules treating peach-lye canning wastewater.

Table 1. (cont.)

DGGE Band[a]	Sequence Length (bp)	GenBank Accession Number (DGGE Band)	% Sequence Similarity	Closest Relative	GenBank Accession Number (Closest Relative)
Synergistes					
D12	668	DQ238273	99.7	Uncultured bacterium mle1-42	AF280863
Uncultured					
W18	1069	DQ238245	92.3	Uncultured bacterium clone BSA2B-20	AB175392
B20	972	DQ238252	63.1	Uncultured bacterium clone W31	AY770971
PL15	857	DQ238257	66.8	Uncultured bacterium	AB195900

[a]W = UASB granules treating winery wastewater, B = UASB granules treating brewery wastewater, D = UASB granules treating distillery wastewater, PL = UASB granules treating peach-lye canning wastewater.

were present in the winery granules, six in the distillery granules, four in the peach-lye canning granules and three in the brewery granules. A comparative analysis of the sequences retrieved from GenBank was used to construct a phylogenetic tree. The data set contained 56 sequences. The phylogenetic tree (Fig. 2) was composed of six major clusters, and the outgroup. Band W3 was closely related to *B. licheniformis*, and served as the outgroup. The sequence of the following bands W6, W9, W10, B6, D1, D16, D17, PL2 and PL3 formed Cluster I, supported by a 99% bootstrap value. Band W10 showed a 99.2% sequence similarity to an unidentified bacterium (Accession number AY345491) (Table 1) previously isolated from a sediment sample and all the other bands were found to be closely related to *B. cereus*.

In Cluster II (99% bootstrap support), band PL4 was found to be closely related to *B. pumilus*, supported by a 99.6% sequence similarity.

In Cluster III, with a bootstrap support of 99%, band D3 was found to be closely related to a *Bacillus* sp. (Accession number AJ315065) (99.8% sequence similarity). Band W2 was also grouped in this cluster and was found to be closely related to *B. megaterium* (99.8% sequence similarity).

In Cluster IV, with a bootstrap support of 99%, band W1 was found to be closely related to *B. pycnus*. In Cluster V, band B7 was identified as *B. fusiformis* supported by a 100% sequence similarity and B12 was found to be closely related to *B. sphaericus*. The bands W20, D2, D5 and PL6 in Cluster VI (96% bootstrap support) were all closely related to *B. subtilis*.

It is generally accepted that most of the bacteria that can survive and be metabolically active in bioreactors are anaerobes, but the aerobic and facultative anaerobic bacteria still form a significant and constant part of the total bioreactor population. Species of *Bacillus* are metabolically active during the anaerobic digestion process and can degrade different types of organic compounds such as proteins, cellulose, starch or lipids (Price, 1985; Gerardi, 2003). Li *et al*. (2003) found that members of the genus *Bacillus* were prevalent species present in the biofilm of a batch reactor and that the genus was associated with phosphorus removal. The presence of *Bacillus* spp. in a bioreactor may also play a role in the formation of immobilised microbial populations or facilitate clumping because of their adhesion ability (Petruccioli *et al*., 2000; Gerardi, 2003). Noeth *et al*. (1988)

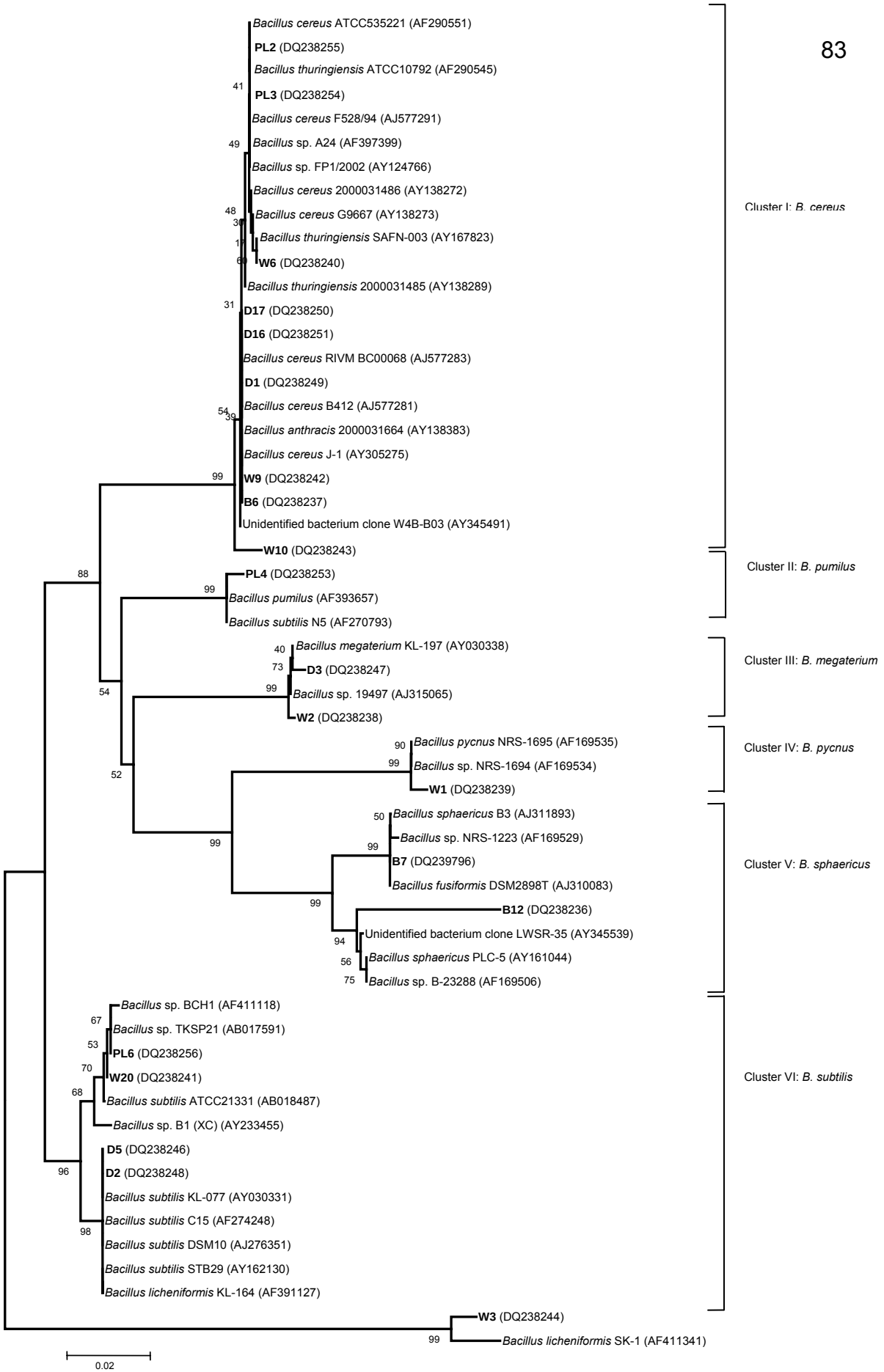

Figure 2. Neighbour-joining tree of partial 16S rRNA gene sequences of species of *Bacillus*. The numbers above the branches refer to the bootstrap values. Scale bar: substitution per site. (Un = Uncultured).

isolated *B. pumilus*, *B. subtilis*, *B. megaterium* and *B. sphaericus* from an anaerobic, fixed-bed bioreactor. They ascribed the growth of the *Bacilli* to sufficient oxygen in the bioreactor that was probably introduced by the substrate. *Bacillus coagulans* and *B. sphaericus* were also identified from a lab-scale UASB bioreactor (Thierry *et al*., 2004).

Pseudomonas: Fourteen members of the genus *Pseudomonas* were found as part of the population of the four different UASB granules (Table 1). Nine *Pseudomonas* species were present in the peach-lye canning granules, four in the distillery granules and one was isolated from the brewery granules. Together a comparative analysis of the 49 sequences retrieved from GenBank was used to construct a phylogenetic tree (Fig. 3). In the phylogenetic tree the band B4 (Cluster III) from brewery granules, was used as the outgroup and was identified as *Brevundimonas bullata* (99.9% sequence similarity).

In Cluster I, band PL11 was found to be closely related to an uncultured Gamma *Proteobacterium* (Li *et al*., 1999), while PL19 was found to be closely related to an uncultured bacterium previously isolated from wetland sediments (Accession number AY216460). Band PL5 was closely related to *P. fluorescens*, a potential phenol degrading bacteria (Heinaru *et al*., 2000) and band PL17 showed a sequence similarity of 99.3% to a *Pseudomonas* sp. (Accession number AY014806). PL14 was closely related to *P. veronii*, PL22 to *P. viridiflava* and band PL20 was closely related to *P. putida*. The distillery band D13 was closely related to a *Pseudomonas* sp. (Accession number AY014826), while D4 was found to be closely related to a sulphide–oxidising bacterium, previously isolated from an environmental sample (Accession number AF393509). Also part of Cluster I, D6 and PL8 were found to be closely related to these *Pseudomonas* spp. (Accession numbers AF438148 and AF521651, respectively) and PL1 was closely related to *P. fluorescens*.

In Cluster II (100% bootstrap support), band D10 was found to be closely related to *Burkholderia pyrrocina*.

Several members of the *Pseudomonas* are known for their ability to degrade aromatic compounds and to produce exo-polysaccharides. This ability might explain why *Pseudomonas* might play an important role in granulation (Petruccioli *et al*., 2000). Li *et al*. (2003) showed that *Pseudomonas* spp. play a role in the release and uptake of phosphorus in acidogenic bioreactors.

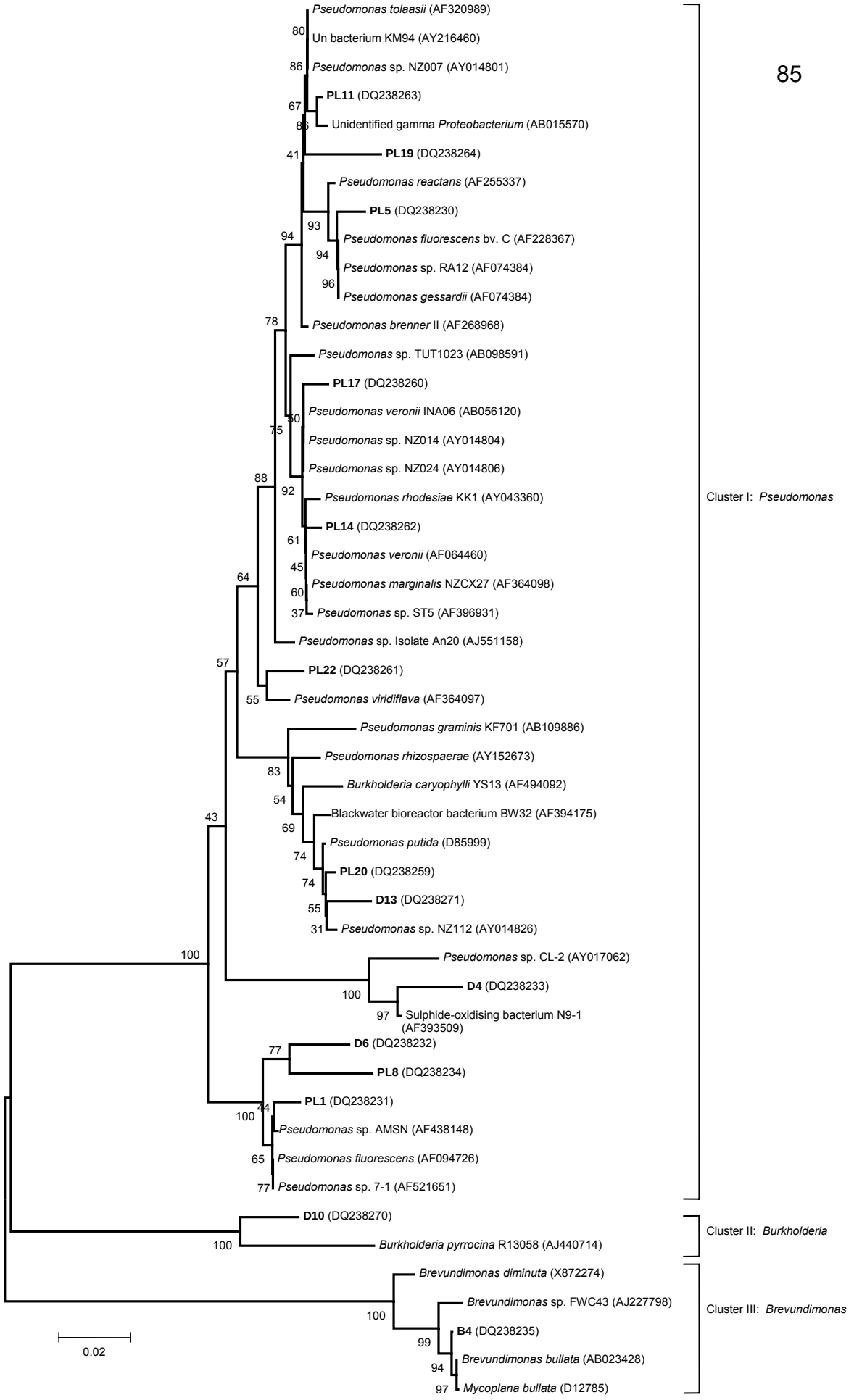

Figure 3. Neighbour-joining tree of partial 16S rRNA gene sequences of species of *Pseudomonas*. The numbers above the branches refer to the bootstrap values. Scale bar: substitution per site. (Un = Uncultured).

Phosphorus is an important macronutrient that is required by bacteria for growth in biological treatment processes (Gerardi, 2003). *Pseudomonas stutzeri*, *P. aeruginosa* and *P. putida* were isolated from UASB bioreactors in the past (De Haast & Britz, 1986; Pereira *et al*., 2002; Muthumbi *et al*., 2001; Thierry *et al*., 2004). *Pseudomonas* were identified from the brewery, distillery and the peach-lye canning granules, but since most of these bacteria were identified from the peach-lye caning granules, is it apparent that the *Pseudomonas* found the environmental conditions in these peach-lye canning granules favourable for growth. A possible explanation for their presence may be that these identified *Pseudomonas* may be halotolerant (Mioni *et al*., 2003; Lo Nostro *et al*., 2005) and also could have withstand the alkaline environment (pH 8.5) caused by the lye in the wastewater (Sigge *et al*., 2001). Although Pseudomonas are not classified as alkalophiles it seemed that these identified *Pseudomonas* were able to metabolise and grow in more alkaline environments.

Bacteroides: Four *Bacteroides* species were identified from the UASB granules analysed (Table 1) and two were present in the distillery granules, one in the winery and one in the peach-lye canning granules. Comparative analysis of the sequences retrieved from GenBank was used to construct a phylogenetic tree (Fig. 4) and the data set contained 11 sequences.

Band D9 formed a cluster, supported by a bootstrap value of 100%, with an uncultured bacterium (Accession number AJ488099) to which it showed the highest sequence similarity (99.8%). The DGGE band PL16 and an uncultured *Bacteroidetes* bacterium (Accession number AF507859) formed a cluster with a bootstrap support of 69%. The third cluster, with a 100% bootstrap support, included the bands W19 and D15. These two bands were found to be closely related to an uncultured bacterium previously isolated from environmental samples (Accession number AJ488088).

Gram-negative *Bacteroides* spp. are acidogenic, anaerobic bacteria (Krieg & Holt, 1984). They can metabolise carbohydrates and peptone to form acetate, lactate, formate or propionate (Krieg & Holt, 1984). Members of this genus have been isolated from sewage (Krieg & Holt, 1984), anaerobic bioreactors (Joubert & Britz, 1987; Lapara *et al*., 2000; McHugh & O'Flaherty, 2004) and activated sludge (Liu *et al*., 2005) and include the species *B. fragilis*, *B. distasonis*, *B. uniformis*, *B. splanchnicus* and *B. forsythus*.

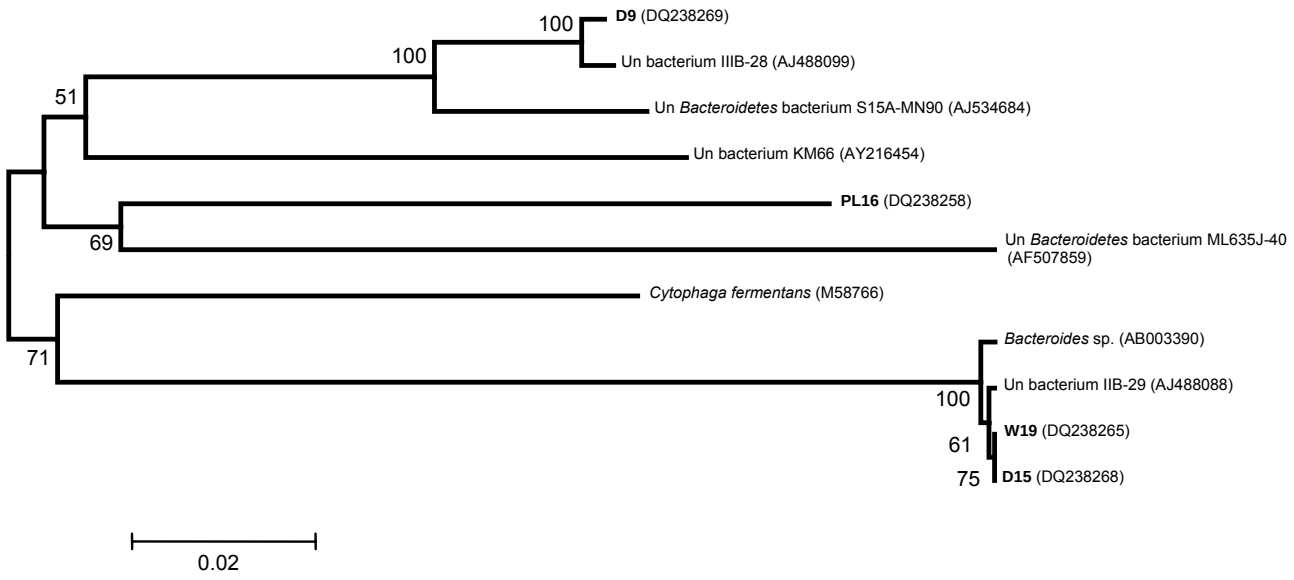

Figure 4. Neighbour-joining tree of partial 16S rRNA gene sequences of members of the *Bacteroides*. The numbers above the branches refer to the bootstrap values. Scale bar: substitution per site. (Un = Uncultured).

Enterococcus: Three *Enterococcus* species were isolated from the brewery granules (Table 1). Band B1 showed a 96.8% sequence similarity to an uncultured bacterium closely related to the genus *Enterococcus* (Leser *et al*., 2002), while bands B11 and B13 represent species that are closely related to *Enterococcus* sp. (98.1% sequence similarity) (Chee-Sanford *et al*., 2001) and *E. durans* (98.1% sequence similarity) (Collins *et al*., 1984), respectively.

Enterococcus are Gram-positive and facultative anaerobic that ferment carbohydrates, to mainly form lactic acid. *Enterococcus durans*, *E. avium* and *E. faecium* were isolated from raw bakers' yeast effluent (Van der Merwe & Britz, 1994). Chan *et al*. (2001) studied the microbial community of granular sludge treating brewery wastewater, but did not identify any *Enterococcus* species. In this study the brewery wastewater favoured the growth of *Enterococcus*.

Alcaligenes: Two species of this genus were identified from the brewery granules and one from the winery granules (Table 1). Band B2 showed a 93.6% sequence similarity to an uncultured bacterium (Accession number AY214198), related to the genus *Alcaligenes*. Band B3 showed a 99.8% sequence similarity to *A. faecalis*. *Alcaligenes* spp. can utilise a variety of organic acids and amino acids as carbon sources and certain strains are also capable of anaerobic respiration in the presence of nitrate or nitrite, although *A. faecalis* can only reduce nitrite (Krieg & Holt, 1984). *Alcaligenes faecalis* has also previously been isolated from bioreactors (De Haast & Britz, 1986) and the presence of this bacteria was ascribed to the sewage sludge used as inoculum at the start-up of the bioreactor.

Winery band W7 was found to be closely related to *Achromobacter spanius*, supported by a 99.8% sequence similarity. Coenye *et al*. (2003) found that *Achromobacter spanius* resembled *Alcaligenes faecalis* phenotypically, but protein and fatty acid analyses showed it to be distinct to *Achromobacter*. *Achromobacter* spp. can reduce nitrate and metabolise gluconate, acetate, propionate, butyrate, iso-butyrate and succinate during acetogenesis (Coenye *et al*., 2003). It may be in this study that the winery granules favoured the growth of *Achromobacter*, and the brewery granules favoured the growth of *Alcaligenes*.

Clostridium: Two *Clostridium* species were isolated and only from the distillery granules (Table 1). Band D7 was found to be closely related to *C. butyricum* and band D8 to *C. bifermentans*. Clostridia produce organic acids and alcohols from carbohydrates or peptones (Sneath *et al*., 1986) and are regarded

as acidogenic or homoacetogenic bacteria in anaerobic bioreactors. *Clostridium bifermentans* was previously isolated from oil mill wastewater (Chamkha *et al*., 2001) and *C. butyricum* was a prevalent hydrolytic bacterium in an anaerobic bioreactor treating cheese whey (Chartrain & Zeikus, 1986). Distillery granules favoured the growth of *Clostridium* species that can possibly be ascribed to a favourable redox potential for the development and growth of Clostridia. *C. butyricum* probably produced organic acids and *C. bifermentans* probably produced hydrogen during the anaerobic process.

Shewanella: In this study two *Shewanella putrefaciens* strains were isolated, band W8 from the winery granules and band B8 from the brewery granules (Table 1). Band W8 showed a 99.5% and band B8 a 91.2% sequence similarity to the genus *Shewanella*. All *Shewanella* species are Gram-negative and can reduce nitrate to nitrite. *Shewanella putrefaciens* can reduce trimethylamine *N*-oxide and sulphur and can produce hydrogen sulphate from thiosulphate (Venkateswaran *et al*., 1999).

Microbacterium: Two *Microbacterium* species were isolated and only from the winery granules (Table 1). Band W4 was found to be closely related to a *Microbacterium* sp. (Accession number Y15325) supported by a 99.5% sequence similarity and band W5 was identified as *M. oxydans* (100% sequence similarity). *Microbacterium oxydans* can produce acid from glucose, fructose, galactose, mannose, sucrose, maltose, mannitol, glycerol, salicin and dextrin (Schumann *et al*., 1999). Therefore, the bands W4 and W5 formed part of the acidogenic population in the winery granules.

Leuconostoc: One *Leuconostoc* sp. was isolated from the brewery granules (Table 1). Band B14 was found to be related to an uncultured *Leuconostoc* sp. (Accession number AF335916), supported by a sequence similarity of 85.1%. This *Leuconostoc* sp. showed a 99.8% sequence similarity to *Leuc. mesenteroides*, and it was thus concluded that B14 was related to *Leuc. mesenteroides*.

Leuconostoc species are Gram-positive, facultative anaerobes and growth is dependent on the presence of amino acids and fermentable carbohydrates, such as glucose (Sneath *et al*., 1986). Chartrain & Zeikus (1986) found that *Leuc. mesenteroides* formed part of the hydrolytic bacteria in an anaerobic bioreactor, making band B14 a member of the acidogenic bacteria in the brewery granules.

Sulfurospirillum: Two *Sulfurospirillum* species were cloned and only from the peach-lye canning granules (Table 1). Band PL12 showed a 98.9% sequence similarity to *S. arsenophilum* (Stolz *et al*., 1999) and band PL13 a 98.1% sequence similarity to *S. halorespirans*. Lactate, pyruvate and fumarate can be used by *S. arsenophilum* and *S. halorespirans* as electron donors but when acetate is used as the carbon source, hydrogen and formate serve as the electron donors (Luijten *et al*., 2003).

Acidaminococcus: Two uncultured species, related to the genus *Acidaminococcus* were identified from the peach-lye canning granules (Table 1). Band PL9 showed a 91.8% sequence similarity to an unidentified eubacterium (Accession number U81750) which was found to be related to *Acidaminococcus fermentans*, previously found in anaerobic bioreactors (Godon *et al*., 1997) and paper mill wastewater (Roest *et al*., 2005). Band PL21 showed a 94.8% sequence similarity to an uncultured bacterium (Accession number AY231317) previously isolated from an anaerobic bioreactor, which showed a 92% sequence similarity to *A. fermentans*.

Vibrio: Band B9 showed a 99.9% sequence similarity to *Vibrio parahaemolyticus* (Table 1). *Vibrio parahaemolyticus* has a fermentative metabolism (Krieg & Holt, 1984), so it may be possible that band B9 may have been part of the acidogenic bacteria in the brewery granules.

Aeromonas: Band B10 showed a 96.2% sequence similarity to *Aeromonas salmonicida* (Table 1). *Aeromonas* have been isolated from winery wastewater (Petruccioli *et al*., 2000) and raw bakers' yeast wastewater (Van der Merwe & Britz, 1994). Species of *Aeromonas* were found to be partly responsible for phosphorus uptake and release in bioreactors (You *et al*., 2002; Li *et al*., 2003).

Syntrophobacter, Rhodocyclus, Rhodococcus, Nitrospira: Brewery bands B21, B18, B16, B15 and B19 were all identified as uncultured bacteria. Brewery band B21 showed a 99% sequence similarity to an uncultured bacterium (Accession number AF482435) (Table 1), previously found in granular sludge and to uncultured bacterium SJA-172 (89.8% sequence similarity) (Accession number AJ009502). Roest *et al*. (2005) also identified uncultured bacterium SJA-172 in an UASB bioreactor and suggested that this bacterium might be involved in the oxidation of propionate. Propionate oxidation is an energetically unfavourable reaction and microorganisms involved in the degradation of propionate play a

crucial role in the anaerobic degradation process in methanogenic bioreactors. Uncultured bacterium SJA-172 was found to be closely related to *Syntrophobacter wolinii*. It is, therefore, possible that band B21 and the uncultured bacterium R1p32 may be related to *Syntrophobacter*.

Band B18 showed a 95.5% sequence similarity to an uncultured bacterium (Accession number AF502232) previously present in activated sludge with high phosphorus content. This uncultured bacterium was found to be closely related to a *Rhodocyclus* sp., which was associated with phosphorus removal in sludges (McMahon *et al*., 2002). This supports the finding that band B18 may be related to *Rhodocyclus*. The growth rate of *Rhodocyclus* species can be increased in the presence of complex organic nutrients or even yeast extract (Staley *et al*., 1989). Since band B18 was found in UASB granules treating brewery wastewater, it is possible that these bacteria could have used the yeast cells for their metabolism.

Band B16 showed an 86.1% sequence similarity to uncultured bacterium clone BA149 previously found in environmental samples (Accession number AF323777). Band B16 and bacterium clone BA149 showed sequence similarity of 74.9% and 74.1%, respectively to *R. rhodochrous*. *Rhodococcus* spp. can utilise various organic compounds as sole carbon sources. *Rhodococcus rhodochrous* can produce acid from dextrine, ethanol, fructose, glucose and sucrose (Sneath *et al*., 1986) and may have played an important role during acidogenesis in the brewery granules. Hawari *et al*. (2000) found that *Rhodococcus* produced formaldehyde or methanol as end-products during anaerobic biodegradation of anaerobic sludge.

Bands B15 showed an 88.2% sequence similarity to uncultured bacterium DCE29 (Gu *et al*., 2004) and B19 showed a 99.8% sequence similarity to uncultured bacterium SR_FBR_L1 (Kakosen *et al*., 2004) (Table 1). Gu *et al*. (2004) reported that the uncultured bacterium DCE29 used hydrogen as an electron donor and could be affiliated with the genus *Nitrospira*. Uncultured bacterium SR_FBR_L1 was isolated as part of a lactate-utilising sulphate-reducing fluidised-bed bioreactor bacterial community (Kakosen *et al*., 2004).

<u>*Synergistes:*</u> Four uncultured bacteria were identified from the winery and distillery granules and found to be related to the genus *Synergistes*. These are acidogenic bacteria associated with anaerobic bioreactors and soil (Godon *et al*., 2005) (Table 1). Band W11 showed a 98.2% sequence similarity to the uncultured

bacterium TA19, previously identified in an UASB bioreactor (Wu *et al*., 2001) and found to be closely related to *Synergistes jonesii*. Band D12 showed a 99.7% sequence similarity to the uncultured bacterium mle1-42 which was found to be present in bioreactors (Lapara *et al*., 2000; Pereira *et al*., 2002). Uncultured bacterium mle1-42 formed part of a cluster containing *S. jonesii* (Lapara *et al*., 2000). The band W17 showed a high sequence similarity of 94.4% to DGGE band D11, which also showed an 89.5% sequence similarity to the uncultured bacterium clone TTA_B6. This bacterium was found to be the second "most dominant" bacteria in an anaerobic reactor (Chen *et al*., 2004). This organism was closely related to an environmental clone MUG10 (Sekiguchi *et al*., 1998) which formed a cluster with *S. jonesii*.

Other uncultured bacteria: Band W18 showed a 92.3% sequence similarity to the uncultured bacterium clone BSA2B-20 (Accession number AB175392). Band B20 and PL15 showed a sequence similarity of 63.1% and 66.8% respectively to an uncultured bacterium clone W31 (Accession number AY770971) and an uncultured bacterium (Accession number AB195900). The sequence similarities of 63.1% and 66.8% are not high and the identification of these two bacteria are uncertain.

DGGE marker

The developed DGGE marker was constructed using selected DGGE bands from the profiles obtained from the winery, brewery, distillery and peach-lye canning effluent granules (Fig. 5). This marker represents the *Bacteria* that were present in the granules used in this study but can be of great value for the identification of members of the microbial consortium in other UASB bioreactors.

Since it is an extremely time consuming process to identify microorganisms, especially the not readily cultured microorganisms, the use of the DGGE marker can be of great assistance to provide a quick method to verify the presence of these microorganisms where each bacteria have a specific role to play during anaerobic digestion. Knowledge of the composition of the microbial consortium can be of great value during the start-up of a new bioreactor, and the marker can be used as a reference to monitor the various microorganisms during the

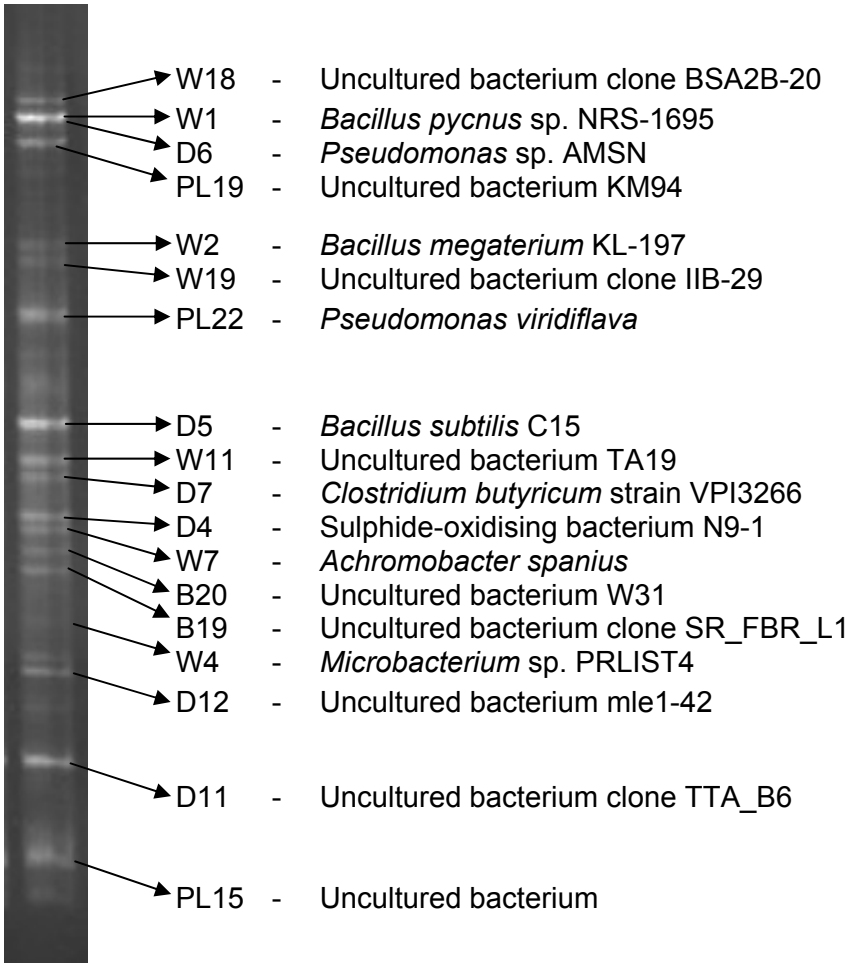

Figure 5. DGGE marker constructed from the different *Bacteria* present in the UASB granules that were used to treat winery, brewery, distillery and peach-lye canning wastewaters.

adaptation period of microorganisms in a new bioreactor or a bioreactor treating a new type of wastewater.

Conclusions

During the last decade advances in molecular biology have provided better insight into the structure of complex microbial communities. In this study PCR-based DGGE proved useful to fingerprint the various UASB granules. It is evident from the results obtained that the use of culture-independent molecular techniques is essential since 35% of the total identified bacteria were unculturable bacteria.

The different bacteria that were isolated and identified from the different granules emphasize the fact that the composition of each type of wastewater has a major impact on the microbial species present in the granules. Fingerprinting and identification of the complex microbial *Bacterial* community in UASB granules may lead to a better understanding of the influence that the treatment of various wastewaters may have on the structure of the different populations present in the UASB granules. A better understanding of the diversity of the *Bacteria* in different UASB granules can improve the anaerobic process stability and bioreactor performance. The metabolic activity of the different groups of bacteria play a major role during anaerobic digestion, and if the *Bacteria* are identified it is possible that tailor-made granules may be used to enhance bioreactor process stability. Tailor-made granules may also be used to reduce the start-up period. The survival of these incorporated microorganisms used to enhance bioreactor efficiency may be monitored by using the DGGE marker that was constructed in this study.

A major advantage of this DGGE marker is that it could be complemented by additional DGGE bands found in UASB bioreactors. The DGGE marker can also be used to assist in the monitoring of selected species during bioaugmentation or enrichment of granules for the treatment of specific wastewaters. The DGGE marker has to be used in combination with sequence analysis when analysing new granule batches. It is possible that some overlapping of the microorganisms can take place in the DGGE profiles under certain conditions.

The data obtained in this study should be of value in future identification of the microbial community present during anaerobic digestion studies, as well as process optimisation of UASB bioreactors.

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APPENDIX

TO CHAPTER 4

To simplify the discussion of the results, the data illustrated in Figs. A1 – A4 have been included in this Appendix.

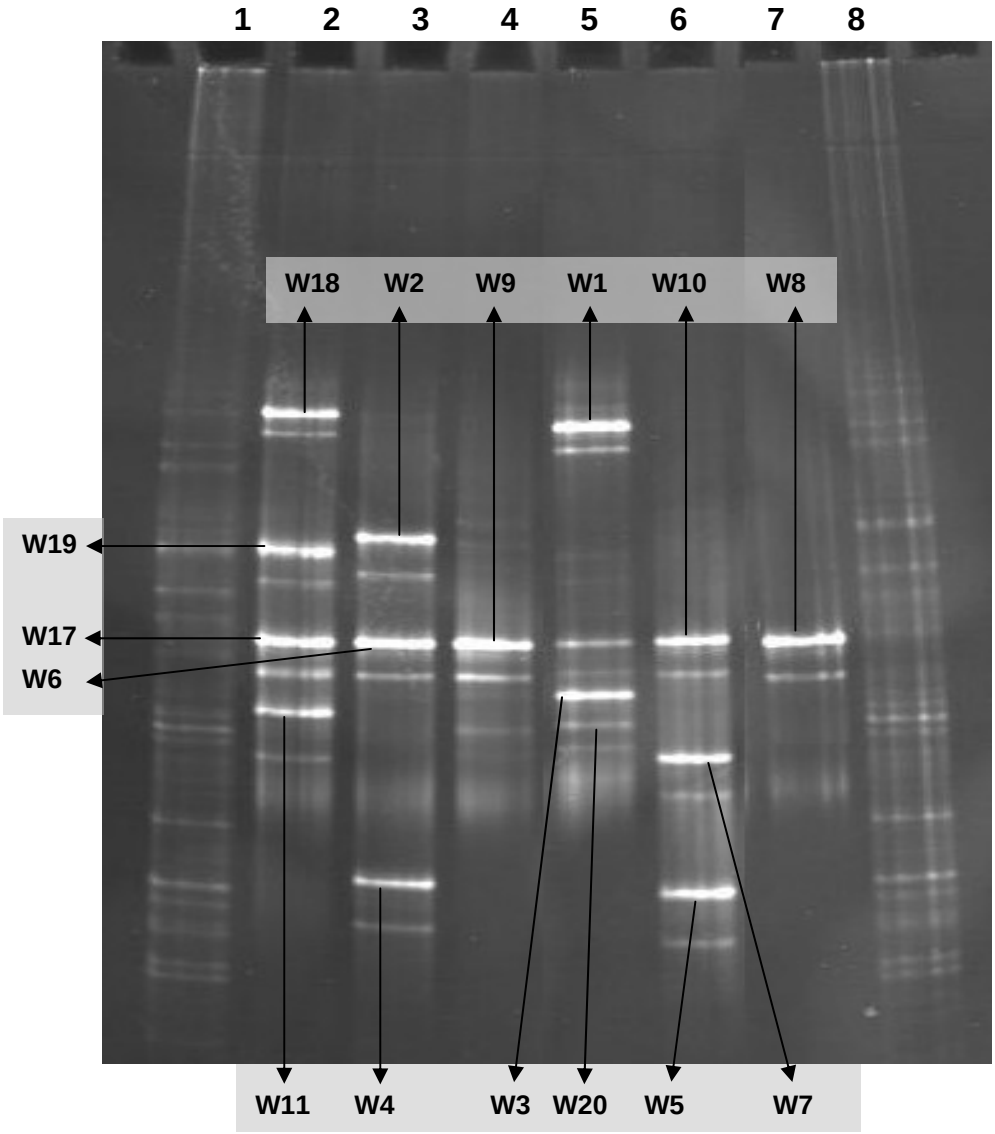

Figure A1. Single 200bp DGGE bands from the PCR products of the cloned inserts and isolates obtained of the DNA extracted from the winery granules. Lanes 1 & 8: fingerprints of the winery granules; Lane 2: bands W11, 17, 18 & 19; Lane 3: W2, 4 & 6; Lane 4: W9; Lane 5: W1, 3 & 20; Lane 6: W5, 7 & 10; and Lane 7: W8.

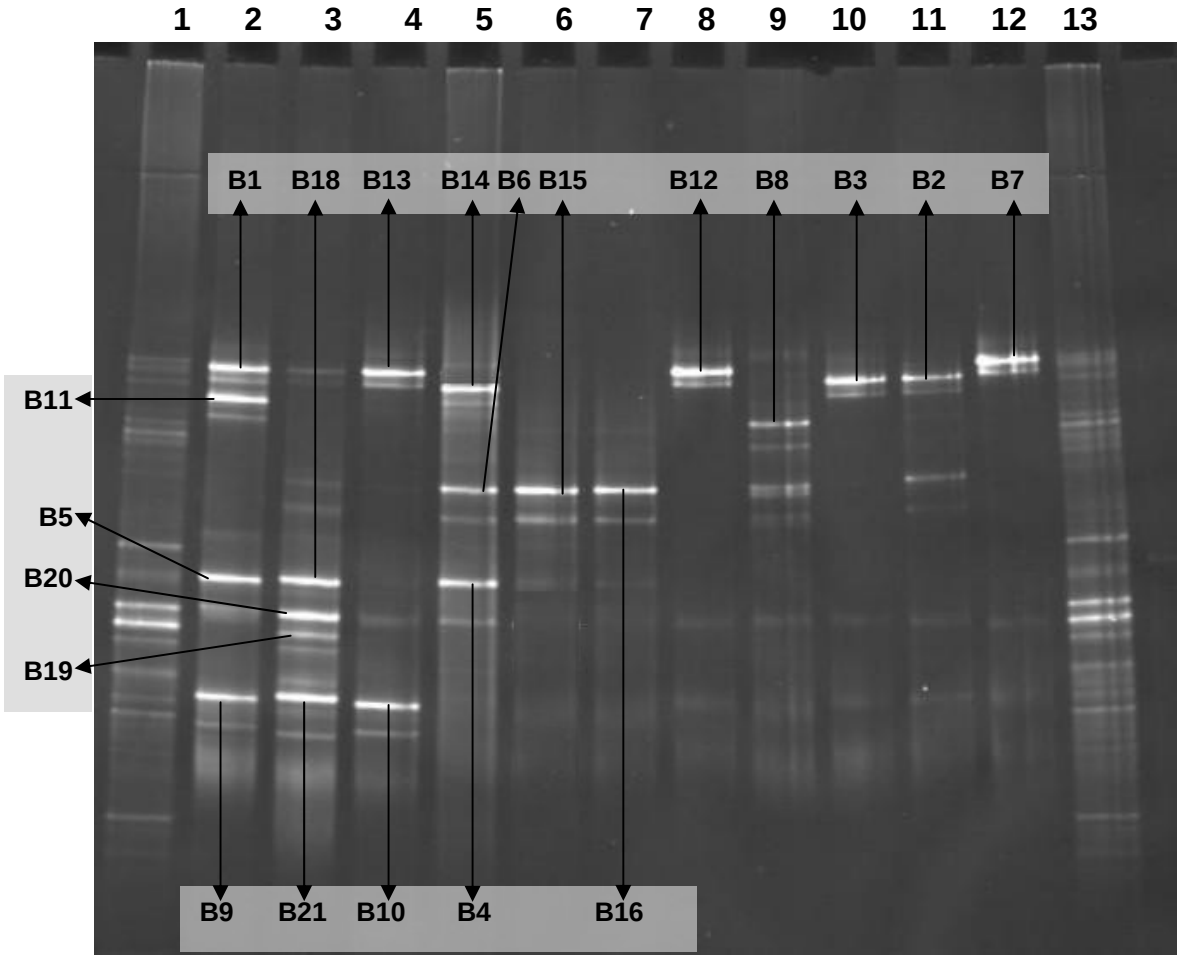

Figure A2. Single 200bp DGGE bands from the PCR products of the cloned inserts and isolates obtained of the DNA extracted from the brewery granules. Lanes 1 & 13: fingerprints of the brewery granules; Lane 2: bands B1, 5, 9 & 11; Lane 3: B18, 19, 20 & 21; Lane 4: B10 & 13; Lane 5: B4, 6 & 14; Lane 6: B15; Lane 7: B16; Lane 8: B12; Lane 9: B8; Lane 10: B3; Lane 11: B2; and Lane 12: B7.

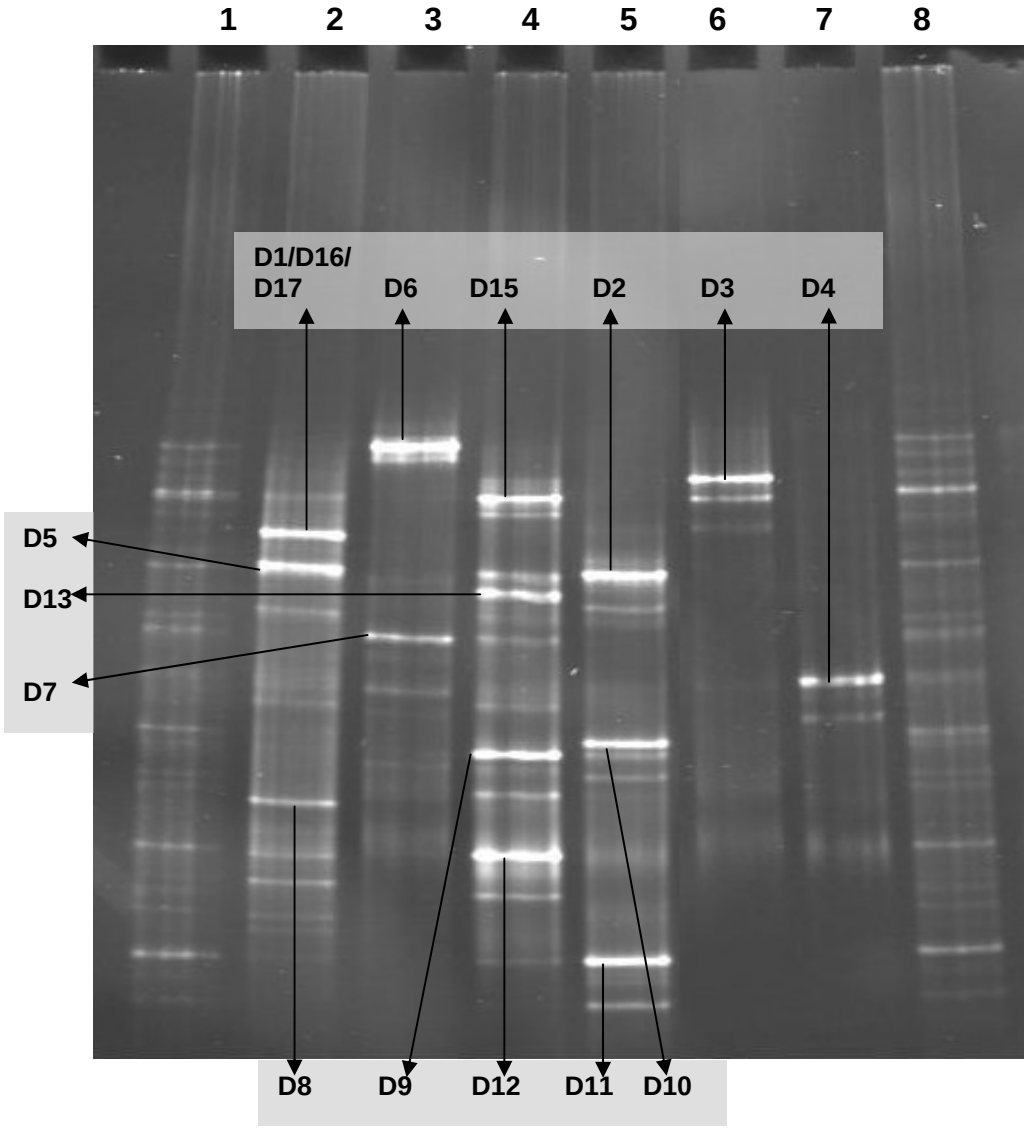

Figure A3. Single 200bp DGGE bands from the PCR products of the cloned inserts and isolates obtained of the DNA extracted from the distillery granules. Lanes 1 & 8: fingerprints of the distillery granules; Lane 2: bands D1, 5, 8, 16 & 17; Lane 3: D6 & 7; Lane 4: D9, 12, 13 & 15; Lane 5: D2, 10 & 11; Lane 6: D3; and Lane 7: D4.

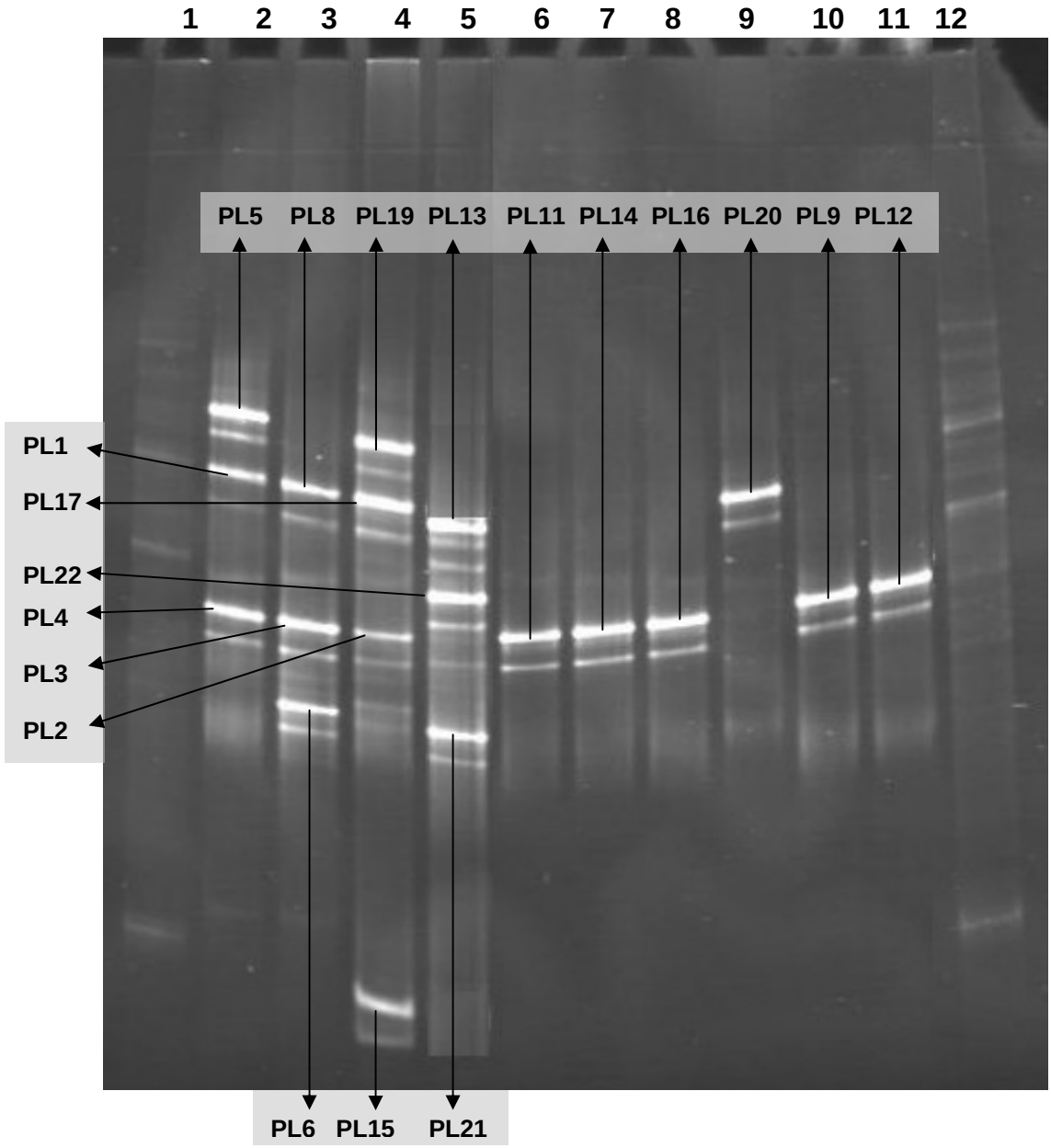

Figure A4. Single 200bp DGGE bands from the PCR products of the cloned inserts and isolates obtained of the DNA extracted from the peach-lye canning granules. Lanes 1 & 12: fingerprints of the peach-lye canning granules; Lane 2: bands PL1, 4 & 5; Lane 3: PL3, 6 & 8; Lane 4: PL2, 15, 17 & 19; Lane 5: PL13, 21 & 22; Lane 6: PL11; Lane 7: PL14; Lane 8: PL16; Lane 9: PL20; Lane 10: PL9; and Lane 11: PL12

CHAPTER 5

PCR-BASED DGGE FINGERPRINTING AND IDENTIFICATION OF METHANOGENS PRESENT IN THREE DIFFERENT TYPES OF UASB GRANULES

Abstract

Methane is produced by various methanogenic bacteria present in upflow anaerobic sludge blanket (UASB) bioreactors. Methane can be used to predict and improve UASB bioreactor efficiency. The methanogen population in the granules can be influenced by the composition of the substrate. The aim of this study was to fingerprint and identify the methanogens present in three different types of UASB granules that had been used to treat winery, brewery and peach-lye canning effluents. This was done using PCR-based denaturing gradient gel electrophoresis (DGGE) and DNA sequence analysis. The DGGE fingerprints obtained from the methanogen reference cultures of *Methanosaeta concilii*, *Methanosaeta thermophila*, *Methanosarcina barkeri*, *Methanosarcina mazeii* and *Methanobacterium formicicum* were compared to the DGGE profiles of the *Archaea* in the different granules. The positions of the DGGE bands that did not correspond well to the bands of the known species, were sequenced and compared to sequences available on GenBank using the Blastn search option. The aligned DNA sequences were used to construct a phylogenetic tree. Based on the data obtained a DGGE marker was constructed, which was used to provide a method to identify the *Archaeal* members of the microbial consortium in UASB granules.

Introduction

High-rate anaerobic bioreactors are used for the treatment of various wastewaters (Tay & Zhang, 2000). Many full-scale high-rate anaerobic bioreactors are used world-wide, of which the upflow anaerobic sludge bed (UASB) bioreactor has the widest application (Kolukirik *et al*., 2004).

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In an UASB bioreactor sludge develops in a particular granular or flocculent form and the success of the anaerobic process relies on the formation of active and settleable granules (Hulshoff Pol *et al*., 2004). The active granules then form a blanket through which the effluent flows and then diffuses into the sludge granules where it is degraded by the bacteria (Gerardi, 2003). Bacterial metabolic activity can be divided into two major distinct phases in the anaerobic digestion, namely acidogenesis (during which acid forming bacteria reduce complex organic matter to organic acids) and methanogenesis (during which specific methanogens may convert the acetate into methane and carbon dioxide) (Casserly & Erijman, 2003). As methane production is an end-product of UASB bioreactors it can be used to predict bioreactor efficiency. Thus, it is important to be able to define the methanogenic species present in these UASB bioreactor configurations (Smith *et al*., 2004; Jupraputtasri *et al*., 2005). Approximately 70% of the methane formed during the UASB process is produced by members of the acetoclastic *Methanosarcina* and *Methanosaeta* species (Gerardi, 2003; Conklin *et al*., 2004).

The diversity of the methanogenic granule population depends mainly on the composition of the substrate (Lévesque & Guiot, 2004), changes in temperature, pH stability and indicators as well as the solids retention time (Casserly & Erijman, 2003). In the literature it has been shown that a diversity of methanogenic species may be present in granules from pilot and full-scale bioreactors that had been exposed to the treatment of different wastewaters (Fang *et al*., 1995; Leclerc *et al*., 2001; Casserly & Erijman, 2003; Zhang & Fang, 2004). These different methanogens present in UASB granules play a major metabolic role during granulation and will have a significant influence on the start-up of a bioreactor. Each effluent treated will result in different dominant methanogen species in the granules which can have a major impact on granules that have to be used as seed sludge for the start-up of new bioreactors.

The use of traditional microbiological techniques in determining population structures and characteristics are limited as it has been shown that many organisms are not readily cultured on selective media (Briones & Raskin, 2003). The aim of this study was to identify the methanogens present in three different types of UASB granules that had been used to treat industrial winery, brewery and peach-lye canning effluents, using PCR-based DGGE. A DGGE marker will also

be constructed that can be used to assist in the identification of the *Archaea* species present in UASB granules.

Materials and methods

UASB granules

The different UASB granules used in this study were obtained in 20 kg batches from three industrial scale UASB bioreactors situated in different climatic areas of South Africa and treating different effluents. The granules obtained from Stellenbosch and Ceres in the Western Cape were from plants treating winery and peach-lye canning effluent, respectively. The third UASB granule batch was obtained from the Amanzimtoti Kwazulu-Natal brewery.

Methanogen reference cultures

The methanogen reference cultures used in the study were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). The five cultures included *Methanosaeta concilii* (DSMZ 3671), *Methanosaeta thermophila* (DSMZ 4774), *Methanosarcina barkeri* (DSMZ 800), *Methanosarcina mazeii* (DSMZ 2053) and *Methanobacterium formicicum* (DSMZ 1535).

DNA extraction

DNA was extracted using a modified method of Van Elsas *et al*. (1997) from UASB bioreactor granules treating winery, brewery and peach-lye canning effluent wastewaters. The granules were homogenised with 0.6 g sterile glass beads (0.2 – 0.3 mm diameter) (Sigma) using a mortar and pestle. The granules were further homogenised by vortexing, in 800 µl of 120 mM phosphate buffer (1 part 120 mM NaH_2PO_4 (Saarchem) and 9 parts 120 mM Na_2HPO_4 (Merck)) at a pH of 8 and 100 µl 20% (m/v) sodium dodecyl sulphate (SDS) (BDH). The resulting suspension was vortexed for 2 min and incubated at 60ºC for 20 min and the step repeated. The mixture was centrifuged and extracted with 600 µl phenol (Saarchem), followed by 600 µl of a phenol:chloroform:isoamylalcohol (25:24:1) mixture until the interphase was clean. The DNA was precipitated with 0.1 volume 3 M NaAc (pH 5.5) and 0.6 volume isopropanol (Merck). The DNA was centrifuged, washed with 70% cold ethanol, air-dried and redisolved in 100 µl TE

buffer (10 mM Tris, 1 mM EDTA; pH 8.0). DNA extractions from the different UASB granules were done in duplicate.

PCR amplification

PCR reactions were performed on DNA extracted from the three different granule types, as well as from the five methanogen reference cultures. The aim was to obtain the DGGE profiles for the unidentified *Archaeal* species in the different UASB granules, therefore, universal DGGE *Archaeal* primers were used in the amplification reactions. Initially the universal primers ARC622F and ARC915R were used in a PCR amplification reaction to produce a 293 base pair (bp) fragment (Chan *et al*., 2001). The DGGE profiles obtained from these PCR reactions resulted in only one or two bands for each profile. Therefore, the primers PRA46f and PREA1100f (Øvreås *et al*., 1997), based on the *Escherichia coli* 16S rRNA gene sequence, were used in a PCR amplification reaction to produce a 1 072 base pair (bp) fragment. This PCR product was then used as a template for the PCR amplification of a 179 bp fragment using the primers PARCH340f and PARCH519r (Øvreås *et al*., 1997) as recently used by Høj *et al*. (2005) and Stadnitskaia *et al*. (2005) for the amplification of *Archaea* in environmental samples. The GC clamp, 5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG–3' described by Chan *et al*. (2001) was included on the 5' end of the forward primer PARCH340f to enable the separation of the fragments using DGGE. PCR reactions for both primer sets were performed in 25 µl reaction volumes containing 0.35 µl of *Taq* DNA Polymerase (5 U/µl) (Promega), 2.5 µl of 10 x PCR reaction buffer (Roche Diagnostics), 1 µl of each of the primers (10 µM), 1 µl dNTPs (10 mM) (Promega), 1 µl of 99% (v/v) dimethyl sulphoxide (DMSO) (Merck) and 1 µl of the extracted DNA.

The modified PCR amplification conditions of Øvreås *et al*. (1997) for both the primer sets were as follows: initial denaturation was performed at 92ºC for 2 min; followed by denaturation at 92ºC for 1 min; primer annealing at 55ºC for 30 sec; and chain elongation at 72ºC for 1 min. These three steps were repeated for 30 cycles. Final chain elongation was performed at 72ºC for 6 min and the PCR reactions were cooled to 4ºC. The amplified products were separated on 1% (m/v) agarose gels containing ethidium bromide, using 0.5 x TBE electrophoresis buffer

and visualised under UV light. DNA was isolated in duplicate for each granule type and followed by PCR reactions of each isolation to test the reproducibility of the technique.

DGGE

The 179 bp PCR fragments were separated using DGGE, performed with the BioRad DCode™ Universal Mutation Detection System (BioRad Laboratories, USA). The PCR products were applied to 8% (m/v) polyacrylamide gels in 0.5 x TAE buffer, with a gradient of between 45 and 70%. Gradients were created by polyacrylamide containing 0 to 100% denaturant (7 M urea and 40% (v/v) formamide). Electrophoresis was performed at 130 V for 5 h at a constant temperature of 60°C. The DNA was stained with ethidium bromide and visualised under UV light.

DNA sequencing

The dominant DGGE bands from the different profiles were punched from the DGGE gels with sterile pipette tips and used as a template in a re-amplification using the primers PARCH340f and PARCH519r. The resultant PCR products were purified using the SigmaSpin™ Post-Reaction Clean-Up Columns (Sigma-Aldrich) according to the manufacturer's instructions and sequenced using the ABI PRISM 377 DNA Sequencer (PerkinElmer) at the DNA Sequencing Facility at Stellenbosch University. The sequences obtained were compared to 16S rRNA gene sequences in the National Center for Biotechnology Information (NCBI) database using the BLASTn search option (Altschul *et al*., 1990).

Phylogenetic analysis

The 16S rRNA gene sequences obtained were manually aligned by inserting gaps. Phylogenetic analyses were conducted using MEGA 2.1 (Kumar *et al*., 2001). Phylogenetic distances between the cultures were calculated based on a Kimura 2-parameter (Kimura, 1980) substitution model. Phylogenetic trees were generated from the distance matrixes using a neighbour-joining tree building algorithm (Saitou & Nei, 1987). Statistical support for the branching nodes was obtained by bootstrap (1000 replicates) (Felsenstein, 1985).

DGGE marker

A DGGE marker was constructed based on the profiles obtained of the methanogens present in the three different types of granules used in this study. The five dominant unidentified bands obtained from the different fingerprints were re-amplified using the DGGE primers PARCH340f and PARCH519r. A direct PCR amplification was also done on the five methanogen reference cultures. These ten PCR products were then separated using DGGE by loading 7 µl of each PCR product together in one well. This served as the DGGE marker.

Results and discussion

PCR-based DGGE analysis

Duplicate PCR reactions performed on the duplicate extracted DNA from the different granules and the five methanogenic reference cultures gave repeatable DGGE banding patterns. Based on the banding position, the five methanogen reference cultures (*Methanosaeta concilii*, *Methanosaeta thermophila*, *Methanosarcina barkeri*, *Methanosarcina mazeii* and *Methanobacterium formicicum*) were separated using PCR-based DGGE and the positions of the bands from the five different reference cultures, were determined (Fig. 1).

The methanogen consortium from the different granules that had been used to treat winery, brewery and peach-lye canning effluents showed distinct *Archaeal* population fingerprints (Fig.1). The individual fingerprints suggest that the composition of the different types of effluent probably had an influence on the methanogens detected in the granules. In this study, *Methanosaeta concilii* was found to be detected in the fingerprints of the winery and brewery granules, while *Methanosaeta thermophila* was detected in the fingerprint of the brewery granules (Fig.1). Several researchers have reported the presence of members of the *Methanosaetaceae* in anaerobic bioreactors (Chan *et al*., 2001; Batstone *et al.*, 2004; Hulshoff Pol *et al*., 2004). It is also generally known that the presence of *Methanosaeta* species lead to an improved granulation process and this results in a more stable bioreactor performance (Hulshoff Pol *et al*., 2004). Fang *et al*. (1995) found that the interior of brewery granules they examined was composed mainly of *Methanosaeta* (formerly known as *Methanothrix (*Huser *et al*., 1982)).

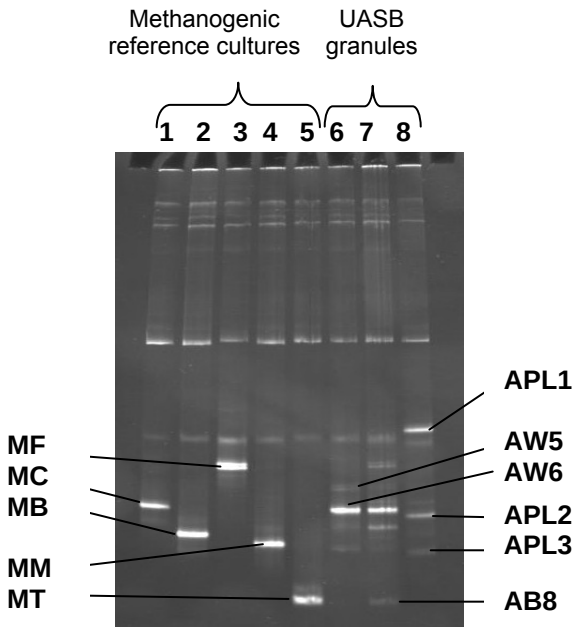

Figure1. PCR-based DGGE fingerprints of the methanogenic reference cultures and the methanogenic *Archaea* present in the three different UASB granule types. Lane 1: *Methanosaeta concilii* (MC); Lane 2: *Methanosarcina barkeri* (MB); Lane 3: *Methanobacterium formicicum* (MF); Lane 4: *Methanosarcina mazeii* (MM); Lane 5: *Methanosaeta thermophila* (MT); Lane 6: winery granules, band AW5, band AW6; Lane 7: brewery granules, band AB8; Lane 8: peach-lye canning granules, bands APL1, APL2 and APL3.

This is one of the main species responsible for the conversion of acetate to methane. Batstone *et al*. (2004) also analysed two sets of brewery granules using fluorescence *in situ* hybridisation (FISH), and only observed low numbers of members of the *Methanosaeta* species and no *Methanosarcina*. The brewery wastewater treated by the granules used in this study may have had a sufficient amount of substrate available, which can possibly be correlated with the presence of acetate, which is the only substrate that supports the growth of *Methanosaetaceae* (Garrity & Holt, 2001).

Methanosarcina mazeii was only detected in the fingerprint of the winery granules (Fig. 1). Although the *Methanosarcinaceae* can utilise a variety of substrates (Rocheleau *et al*., 1999), *Methanosarcina barkeri* was not detected in any of the studied UASB granules.

Methanobacterium formicicum was only detected in the fingerprint of the brewery granules (Fig.1). Gonzalez-Gil *et al*. (2001) found *Methanobacterium*-like organisms in expanded granular sludge bed granules that treated brewery wastewater. These organisms usually grow by utilising hydrogen, although some species can also oxidise formate, secondary alcohols and carbon monoxide (Garrity & Holt, 2001). *Methanobacterium* strains may have difficulty to compete with the faster-growing non-methanogens such as acetogenic bacteria that also utilise hydrogen as substrate (Gerardi, 2003). If the *Methanobacterium* cannot survive in the bioreactor due to a lack of hydrogen, their absence can have a negative influence on granulation. *Methanobacterium* is responsible for the production of extracellular polymers that play a role in binding other bacteria together to form granules (Hulshoff Pol *et al*., 2004).

Not one of the five methanogen reference cultures was observed in the peach-lye canning effluent granules examined in this study. In contrast, Batstone *et al*. (2004) detected *Methanosarcina* and *Methanosaeta* species present in cannery granules taken from an UASB bioreactor that treated fruit and vegetable product wastewaters. The absence of these methanogens species in the fingerprint of the peach-lye canning granules in this study may be due to undetectable low numbers of these species or unfavourable environmental growth conditions.

The profiles obtained in Fig. 1 show that the bands APL1 and APL2 were unique for the fingerprints of the granules treating peach-lye canning effluent and

band AB8 was only observed in the fingerprint of the brewery granules. In contrast bands APL3 and AW5 were detected in more than one granule type, suggesting that a portion of the *Archaeal* microbial composition is similar, irrespective of the effluent being treated. Band AW5 was detected in the winery fingerprint, as well as brewery fingerprint. Band AW6, which correlated with the band of the reference culture *Methanosaeta concilii*, was also detected in the fingerprints obtained for the winery and the brewery granules. Band APL3 was detected in both the fingerprints of the granules that had been obtained from UASB bioreactors treating winery and peach-lye canning effluents.

The excised DGGE bands (APL1, APL2, APL3, AW5 and AB8 (Fig. 1)) were successfully sequenced. The total number of sequenced nucleotides obtained ranged between 124 bp and 132 bp. Since phylogenetic assignments based on partial sequences of 130 bp may give less reliable results than sequences of larger data sets new PCR primers should be developed that amplify a larger part of the 16S rRNA gene. The sequences obtained in this study were compared to those available on GenBank and the sequence similarities are presented in Table 1. Although band AW6 correlated with the reference culture *Methanosaeta concilii*, it was also excised and sequenced to confirm the identification of the band as *Methanosaeta concilii*.

The DGGE bands were identified as: an uncultured Banisveld landfill archaeon (APL1) (109 bp of 124 bp identical); uncultured archaeon SYA-13 (APL2) (104 bp of 123 bp identical); uncultured euryarchaeote ArcSval_5 (APL3) (110 bp of 125 bp identical); uncultured archaeon clone SSADM_AG7 (AW5) (107 bp of 132 bp identical); *Methanosaeta concilii* (AW6) (117 bp of 124 bp identical); and an uncultured euryarchaeote ArcSval_7 (AB8) (95 bp of 129 bp identical). The data in Table 2 shows the presence of the different species found in the different UASB granule types. The sequence data obtained for the excised DGGE bands in this study are available in EMBL/GenBank/DDBJ databases under the accession numbers AY904330 to AY904334.

Table 1. Percentage (%) similarity based on the sequence alignments of the partial 16S rRNA gene sequences of the unidentified bands from the different UASB granules to their closest bacterial relatives present in the NCBI nucleotide sequence database.

DGGE band	Phylogenetic affiliation	GenBank accession number	% Sequence similarity
APL1	Uncultured Banisveld landfill archaeon	AY013585	88.0%
APL2	Uncultured archaeon SYA-13	AF126840	85.0%
APL3	Uncultured euryarchaeote ArcSval_5	AJ749954	88.0%
AW5	Uncultured archaeon clone SSADM_AG7	AY161260	81.0%
AB8	Uncultured euryarchaeote ArcSval_7	AJ749956	74.0%

Table 2. Methanogenic *Archaea* present in the three different UASB granules types. Results were obtained by DGGE fingerprinting and sequence analysis.

UASB granule type	**Methanogenic *Archaea* present**
Brewery	*Methanosaeta concilii*
	Preliminary *Methanosaeta thermophila*
	Preliminary *Methanobacterium formicicum*
	Uncultured euryarchaeote ArcSval_7 (AB8)
	Uncultured archaeon clone SSADM_AG7 (AW5)
Winery	Preliminary *Methanosarcina mazeii*
	Methanosaeta concilii
	Uncultured euryarchaeote ArcSval_5 (APL3)
	Uncultured archaeon clone SSADM_AG7 (AW5)
Peach-lye canning	Uncultured Banisveld landfill archaeon (APL1)
	Uncultured archaeon SYA-13 (APL2)
	Uncultured euryarchaeote ArcSval_5 (APL3)

Phylogenetic analysis

Comparative analyses of the sequences retrieved from GenBank revealed that all the species could be grouped into the class of methanogenic *Archaea* (Fig. 2). The sequence data set contained 28 sequences, including *Methanobacterium formicicum* that was used as the outgroup. The DGGE band APL2 showed 85% sequence similarity to uncultured archaeon SYA-13 that had previously been found in sediment samples taken from a lake in South Korea (NCBI database). Band APL3 showed 88% sequence similarity to the uncultured euryarchaeote ArcSval_5. This uncultured strain had recently been recovered from two arctic wetlands (NCBI database) and the organisms clustered together with other representatives of the *Methanomicrobiaceae* in the neighbour-joining tree with a 76% support value. This methanogenic family includes strictly anaerobic species that obtain energy by carbon dioxide reduction to methane, with hydrogen, formate or at times secondary alcohols as electron donors (Garrity & Holt, 2001). They are found in a wide range of environments including marine sediments and anaerobic sewage bioreactors (Nishihara *et al*., 1995; Garrity & Holt, 2001).

Band APL1 showed an 88% sequence similarity to the uncultured Banisveld landfill archaeon. The latter was previously recovered from samples taken from a landfill leachate-polluted aquifer (Röling *et al*., 2001). These, together with the *Methanocorpusculum* species, formed a cluster in the phylogenetic tree that was supported by a 100% bootstrap value. Species within this genus are strictly anaerobic and produce energy by the reduction of carbon dioxide to methane with hydrogen, formate and secondary alcohols as electron donors. Acetate and methylamines are not catabolised by these species (Garrity & Holt, 2001). *Methanocorpusculaceae* have been reported to be present in anaerobic bioreactors or anoxic lake sediments (Garrity & Holt, 2001).

In the phylogenetic tree, band AW5 and band AB8 formed a cluster, but a poor bootstrap support (52%) yielded a low confidence in this grouping. Band AW5 showed an 80% sequence similarity to *Methanosaeta concilii*. A sequence similarity of 81% was obtained between sequences from AW5 and that of the uncultured archaeon clone SSADM_AG7 that had been isolated from a psychrophilic expanded granular sludge bed (EGSB) bioreactor (Collins *et al*., 2003). In the study by Collins *et al*. (2003), strain SSADM_AG7 was found to be closely related to a *Methanosaeta concilii* strain and it is, therefore, possible that

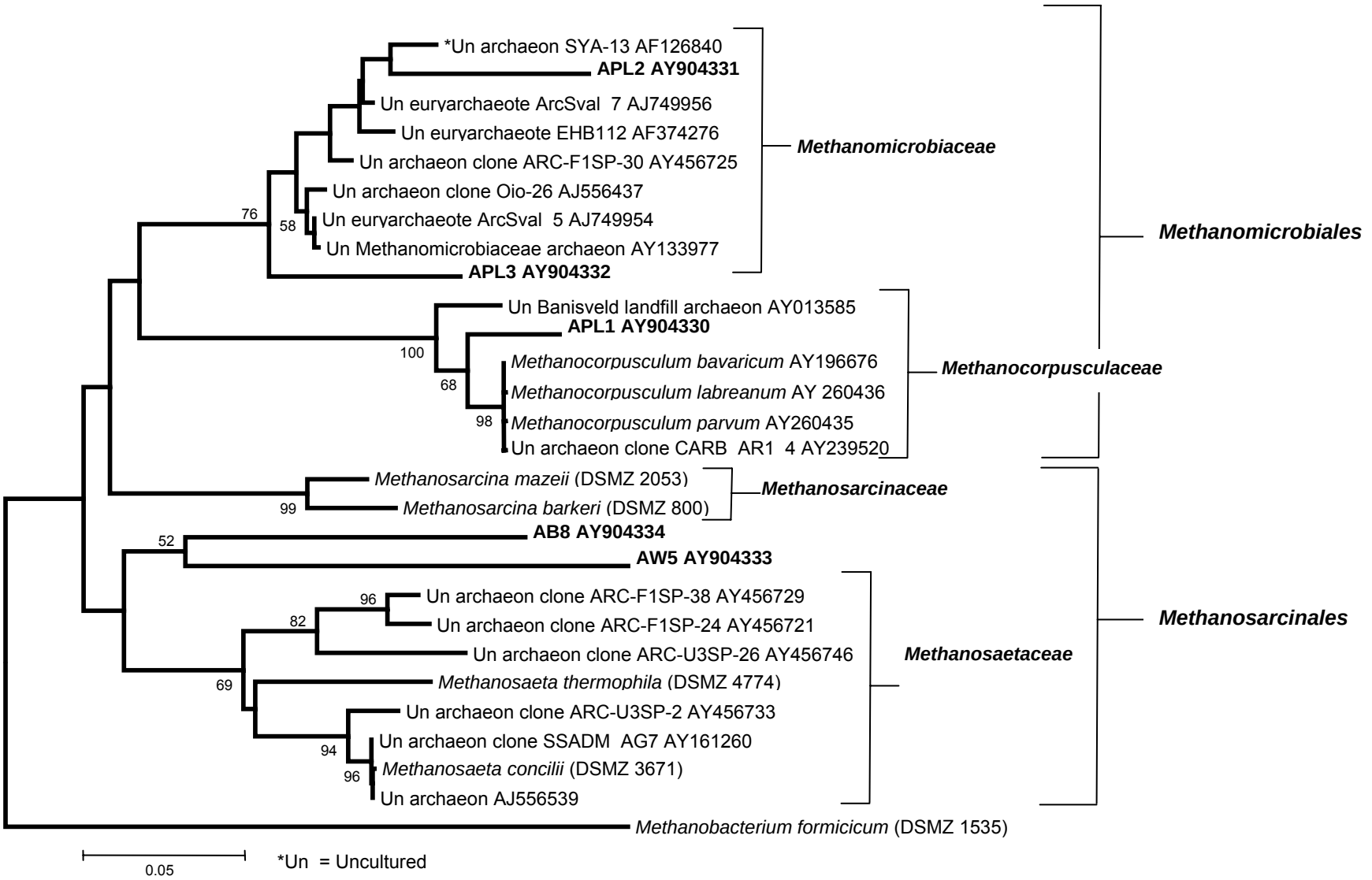

Figure 2. Neighbour-joining tree of partial 16S rRNA gene sequences of *Archaea*. The numbers on the branches refer to the bootstrap values. Scale bar: substitution per site.

the sequence of band AW5 is related to *Methanosaeta concilii*. This species formed part of a cluster that included other members of the *Methanosaetaceae* (69% bootstrap support) (Fig. 2).

Species within this family use acetate as their sole energy source, which is metabolised into methane and carbon dioxide. These organisms can be found in anaerobic sediments and anaerobic sewage sludge bioreactors (Garrity & Holt, 2001). Band AB8 showed a 74% sequence similarity to the uncultured euryarchaeote ArcSval_7 that had been previously found in environmental samples taken from an arctic wetland (NCBI database). The high sequence similarity of band AB8 indicated that it may be a member of the family *Methanomicrobiaceae*.

DGGE marker

The DGGE marker developed in this study was constructed so as to represent the dominant *Archaea* that may be present in UASB granules treating winery, brewery and peach-lye canning wastewaters (Fig. 3). This marker was constructed to provide a quick method to identify these members of the methanogenic population in different UASB granules. Identification of the methanogenic *Archaea* in UASB granules should lead to a better understanding of the population shift, especially during the start-up of a bioreactor. A major advantage of this DGGE marker is that it can be complemented by additional DGGE bands found in other UASB bioreactors. The DGGE marker can also be used to assist in the monitoring of selected species during bioaugmentation or enrichment of granules, which can lead to improved bioreactor performance. Even though Rittmann & Whiteman (1994) showed improved process performance with full-scale bioaugmentation experiments, these studies have often been hampered by a lack of ecological data about the activity and the fate of the inoculated organisms (Dabert *et al*., 2002). The DGGE marker, therefore, holds great potential for the molecular monitoring of individual microorganisms during bioaugmentation or population shifts that may occur in anaerobic bioreactors.

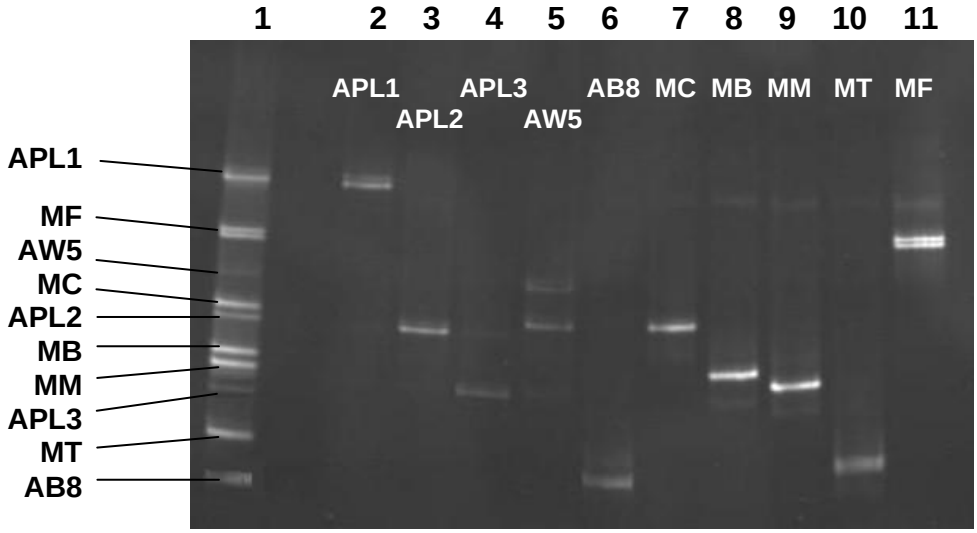

Figure 3. DGGE marker constructed from the *Archaea* present in different UASB granules. Lane 1: Developed DGGE marker; Lane 2: APL1 (Uncultured Banisveld landfill archaeon sp.); Lane 3: APL2 (Uncultured archaeon SYA-13 sp.); Lane 4: APL3 (Uncultured euryarchaeote ArcSval_5 sp.); Lane 5: AW5 (Uncultured archaeon clone SSADM_AG7 sp.); Lane 6: AB8 (Uncultured euryarchaeote ArcSval_7 sp.); Lane 7: *Methanosaeta concilii*; Lane 8: *Methanosarcina barkeri*; Lane 9: *Methanosarcina mazeii*; Lane 10: *Methanosaeta thermophila*; Lane 11: *Methanobacterium formicicum*.

Conclusions

The survey of *Archaea* in the different UASB granule types that had been used to treat brewery, winery and peach-lye canning effluents showed that the composition of the different wastewaters have a strong influence on the diversity of the *Archaea* present in the UASB granules. It is evident from the species identified in the different UASB granules that the presence of unidentified and uncultured species in the UASB granules underlines the need for further research on classical isolation and characterisation studies.

A better understanding of the diversity of the methanogenic *Archaea* in UASB granules can improve the anaerobic process stability. The methanogens are responsible for the terminal metabolic reactions in a bioreactor and are considered to be the key organisms in the anaerobic process. The ability to monitor methanogens and understand their ecology is essential to effectively control the start-up and operation of anaerobic bioreactors.

Molecular methods, such as the PCR-based DGGE technique and sequence analysis have been successfully applied to monitor and identify methanogens in bioreactors. The PCR-based DGGE marker constructed in this study can be successfully used to determine if changes have occurred in the *Archaeal* populations present in a bioreactor. This marker could also be used to observe if enriched methanogenic populations in UASB granules were maintained, to enhance bioreactor efficiency.

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CHAPTER 6

TREATMENT OF WINERY EFFLUENT WITH UPFLOW ANAEROBIC SLUDGE BLANKET (UASB) - GRANULAR SLUDGES ENRICHED WITH *ENTEROBACTER SAKAZAKII*

Abstract

Three upflow anaerobic sludge beds (UASBs) were evaluated for the treatment of winery wastewater: the first was seeded with granular sludge enriched with *Enterobacter sakazakii* and reached a 90% COD removal within 17 d at HRT of 24 h; the second was seeded with brewery granules and achieved 85% COD removal within 50 d, the third was seeded just with sludge and showed the typical problems encountered with conventional sludge seeding and had to be re-seeded continuously. A PCR based technique was developed for the rapid detection of *E. sakazakii* in the granular sludge.

Introduction

Wineries produce large amounts of acidic wastewater with an organic peak over the harvesting season which makes them potential candidates for effluent reuse. The upflow anaerobic sludge bed (UASB) process has successfully been used to treat a variety of effluents (Seghezzo *et al*., 1998), but this process is often limited by the biodegradability of complex organic substrates. An efficient treatment process requires a specific microbial community, but the microbial structure is dependent on the composition of the effluent fed, as well as the presence of competitive organisms in the effluent.

One of the most important parameters that contribute to the maintenance and enhancement of UASB granule formation is the inclusion of suitable microbes in the granule structure. The addition of selected natural bacterial strains, which are known to produce sufficient amounts of desired fatty acids in specific wastewaters, could enhance the production of volatile fatty acids (VFAs) and

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subsequent bioreactor efficiency. This could then lead to the development of granules tailored to a specific wastewater.

The aim of this study was to isolate organisms that could metabolise raw winery wastewater and produce VFAs and then incorporate the best strain into batch cultured granular sludge. The success of the tailor-made granular sludge was then determined by comparing the COD removal efficiency of three similar mesophilic laboratory-scale UASBs inoculated with either raw sludge, granules from a full-scale brewery UASB, or the tailor-made granular sludge containing the selected best VFA producing strain. The presence of the incorporated microbe in the tailor-made granules was monitored using PCR technology.

Materials and methods

Winery effluent characteristics

Winery wastes consist of pomace, lees, stillage bottle washings, cooling waters and saltwaters from ion-exchange processes. Wine waste is a high strength organic waste that is low in nitrogen and phosphorous with sufficient trace elements for bacterial growth (Tofflemire, 1972). Other compounds include alcohol, hexose sugars (glucose and fructose), organic acids (acetic, lactic, citric, malic, succinic and tartaric), soluble proteins, peptides and tartrates (Vaughn & Marsh, 1956; Moosbrugger *et al*., 1993).

Isolation and characterisation

Microbes were enumerated in raw winery effluent and the dominant members isolated using a winery effluent media (WE media), (filtered raw winery effluent), with the pH poised at 7.0. Before sterilisation, 12 g agar/L was added. A pour-plate dilution series was performed in duplicate in sterile WE-medium and the plates were incubated at 35 ºC for 24 h. Colony forming units from the highest dilution, yielding a count of over 30 but less than 300, were counted. Colonies were then selected and streaked out on the WE-medium (pH 7.0) until pure colonies were obtained.

The morphology of the cultures was determined by bright field microscopy of Gram-stained preparations and the following tests performed: catalase; oxidase; endospore formation and growth on MacConkey agar. The isolates were

characterised using combinations of the API 20 E, 20 NE and 50 CHB systems (BioMérieux). The identification of each culture was confirmed using standard identification systems (Krieg & Holt, 1984; Sneath *et al*., 1986).

For volatile fatty acid (VFA) determination the isolates were cultivated in WE-medium in duplicate sets and one was kept stationary at 35°C, while the other was incubated in a waterbath at 35°C and shaken at 150 r.p.m. Every two hours the pH was determined and samples for VFA analysis were made. The VFAs were determined on a gas chromatograph, equipped with a flame ionisation detector and a 30 m fused silica capillary column with a free fatty acid phase (FFAP) (Quadrex Co., New Haven). The column temperature was initially held at 105°C for 2 min, then increased, at a rate of 8°C per minute, to 190°C. Detector and inlet temperatures were set at 300°C and 130°C respectively and nitrogen gas was used as carrier gas at a flow rate of 6.1 $ml.min^{-1}$.

Batch granular sludge production

Batch granular sludge production was done according to the method of Britz *et al*. (2002). A linear-shake waterbath was used to cultivate biomass in a batch system at 35 °C and $150r.min^{-1}$. The batch system consisted of units containing 400 ml of each specific sterile growth medium inoculated with 50 ml sludge from the anaerobic tank of a local sewage works. After allowing the sludge to settle, 100 ml of the units upper volume was removed daily and replaced with one of the following: Lactate medium (Riedel & Britz, 1993); Sucrose medium (Quarmby & Forster, 1995); and glucose medium (Lens *et al*., 1993). A trace element solution (Nel *et al*., 1985) was added to each of the media used. To prevent the too rapid acidification of the units, 10g KH_2PO_4/L was added to each medium. The pH of all the medium was poised at 7.0 using 1M NaOH and the media steam sterilised at 121 °C for 15 min.

The *Enterobacter sakazakii* strain, identified as the best VFA producer from raw winery effluent, was added to the granule biosolid combination. The presence of the strain in the granular sludge was confirmed using a PCR detection technique developed for *E. sakazakii*. The PCR technique was developed and tested on pure control cultures and on the tailor-made UASB granules.

Control strains

Bacterial strains previously isolated from UASB bioreactors were used to test the specificity of the PCR detection method and were obtained from the University of Stellenbosch Food Science Culture Collection, Stellenbosch, South Africa. These strains included *E. sakazakii* (1039), *E. agglomerans* (826), *E. aerogenus* (63), *E. cloacae* (68) and a *Klebsiella* strain (513) and were cultivated on violet red bile agar (VRBA) (Biolab) and incubated at 35°C for 2 - 3 d. *E. agglomerans* was cultivated on MRS agar (Biolab) as it did not grow well on VRBA. The MRS plates were incubated at 30°C for 2 - 3 d. The species identification of the *E. sakazakii* (1039) strain used in this study was confirmed using the API 20 E system (API System S.A.). The isolated *E. sakazakii* that were cultivated on VRBA formed white colonies and not yellow colonies. According to the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) yellow colony forming *E. sakazakii* belongs to risk group 2. Organisms belonging to risk group 2 are subjected to distribution restrictions in several countries, therefore, users of *E. sakazakii* should take note of possible distribution hazards and obey the govermental laws of these countries.

Polymerase chain reaction

The PCR technique was developed and tested on the pure control cultures and the tailor-made UASB granules. The PCR primers Esak2 (5' CCC GCA TCT CTG CAG GAT TCT C 3') and Esak3 (5' CTA ATA CCG CAT AAC GTC TAC G 3') were developed for the specific amplification of *E. sakazakii* based on the DNA sequence of the 16S ribosomal RNA gene of *E. sakazakii* obtained from GenBank (http://www.ncbi.nlm.nih.gov/) (Accession number AB004746). Esak2 and Esak3 bind to position 169 bp and 1001 bp of the 16S rRNA gene of *E. sakazakii*, respectively.

The PCR reactions were performed, without the extraction of DNA, directly on the bacterial cells of all the isolates studied. The PCR reaction mixture (50 µl total volume) contained 1U *Taq* DNA polymerase (Bioline Technologies), the buffer supplied with the enzyme, 2 mM $MgCl_2$, 1 mM dNTPs (Promega) and 0.5 µM of each primer. The PCR reactions were performed on a Mastercycler (Eppendorf, Germany).

Initial denaturation was at 95 °C for 2 min. The following three steps were repeated for 35 cycles: denaturation at 95 °C for 35 s, primer annealing at 61 °C for 1 min and chain elongation at 72 °C for 1 min. The final chain elongation was performed at 72 °C for 10 min. All the PCR products were electrophoresed on a 1% (m/v) agarose gel containing ethidium bromide using 0.5 x tris-borate (TBE) electrophoresis buffer and the separated PCR fragments were visualised under UV light.

A dilution series ($10^2 \rightarrow 10^9$) of the *E. sakazakii* culture was prepared in physiological salt solution (0.85% NaCl). The McFarland Standard 3 (BioMérieux) was used to determine the initial cell concentration. A PCR reaction was performed on each of the dilutions using 1 μl of the cell suspension to determine the minimum cell concentration of *E. sakazakii* that can be detected using the newly developed PCR protocol.

UASB bioreactors

Three 2.3 l bioreactors (Trnovec & Britz, 1998) were used. The design combined an UASB system with an opengas/solids separator at the top of the bioreactor. The gas exited via the top, while substrate was introduced at the bottom of the bioreactor. The overflow of the bioreactor drained through a U-shaped tube to prevent any atmospheric oxygen from entering the system. The upflow velocity in the reactors was set at 2 $m.h^{-1}$ and operated at 35 °C.

The Control bioreactor was seeded with 1 000 g raw anaerobic sludge from a local sewage works and then fed with winery waste water supplemented with 5g sodium lactate/L, 100mg K_2HPO_4/L, 100mg urea/L and 1 ml trace elements for 5 d during start-up. The pH was adjusted to 6.0 and the HRT was set at 2.2 d. The average winery substrate COD throughout the experiment was 2 595 mg/L.

The Normal start-up bioreactor was seeded with 700 g of anaerobic granules from a full-scale UASB bioreactor treating brewery effluent. The bioreactor was allowed to stabilise for 24 h. One the bacteria had acclimatised, the reactor was fed winery waste water supplemented with 5g sodium lactate/L, 100mg K_2HPO_4/L, 100mg urea/L and 1 ml trace elements for 5 d during start-up. The pH was adjusted to 6.0 and the HRT was set at 2.2 d. The average substrate COD was 2 595 mg/L.

The Accelerated Bioreactor was seeded with 700 g anaerobic batch produced granular sludge (Britz *et al*. 2002) and the bioreactor set at a hydraulic retention time (HRT) of 24 h. Standard monitoring methods were used (APHA, 1992).

Results and discussion

Strain selection

The average viable bacterial counts from the WE-plates varied from 1.7 x 10^3 to 1.0 x 10^8 cfu.ml^{-1}. Only six strains used the winery effluent. They were identified as *Enterobacter sakazakii*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus licheniformis*, *Brevibacillus laterosporus* and *Staphylococcus* species. *Brevibacillus laterosporus* strain was the fastest grower (T_d = 45 min); *B. megaterium* (T_d = 60 min); *E. sakazakii* (T_d = 90 min) were average growers and the two *B*. *licheniformis* strains were the slowest (T_d = 135 – 165 min) on WE-medium. The *Staphylococcus* isolate was excluded from further studies, as it grew poorly on the raw winery effluent.

VFA production

Granules consist of complex bacterial communities with specialised ecological roles where the metabolic products, like VFAs, of one species are assimilated by the next species (Iannotti *et al*., 1982). VFA production was used as the most representative indicator of biodegradation as it reflects a major part of the metabolite production by the acidogenic population as part of the microbial community (Moosbrugger *et al*., 1993). Gas chromatography was used to analyse the concentrations of VFAs produced. This gave an indication of the effectiveness of degrading in the granules, but cannot be used to quantify the products, considering that as the products are produced it is utilised by other organisms in granules. The results obtained showed that of the six strains, *E. sakazakii* proved to be the most effective degrader of WE-medium and produced the most VFAs. It was found that with agitation at 150 rpm of the WE-medium, this strain produced 455 mg total VFAs/L, while in stationary WE-medium the strain produced 477 mg VFAs/L. This strain was thus included during the batch production of granular

sludge. Although it is proved that *E. sakazakii* contributes to the degradation of winery waste water, is it difficult to quantify this contribution.

UASB efficiency

Control bioreactor - The Control bioreactor, seeded only with sewage sludge, was used to represent a conventional UASB bioreactor that has no selected seeding specific for the treatment of winery wastewater. The pH in this bioreactor varied widely between 5.5 and 7.5 throughout the study clearly indicating an unstable state. The COD removal, even after 90 d, never reached 70%. This clearly showed a long and poor start-up period as the bioreactor continually needed re-seeding due to biomass wash-out.

Normal start-up bioreactor - In the case of the Normal start-up bioreactor, seeded with granules from a full-scale brewery UASB, the addition of the already formed granules prevented washout and facilitated start-up. From the start the COD removal steadily increased and by day 50, a stable COD removal of between 80 – 86% was reached. However, even after a 100 d of operation, this UASB still did not reach a COD removal efficiency of above 90%. The COD removal never improved above 86% with an HRT of 30 h and an OLR of 5.1 kg $COD.m^{3}.d^{-1}$. Biogas production was low. The data from this study suggested that the granules used as seeding inoculum were not selective enough to treat a substrate such as winery effluent. This was probably due to the fact that brewery granules are accustomed to brewery effluent (Moosbrugger *et al*., 1993). Therefore, some type of conditioning step for the granules to the winery wastewater needs to be implemented in order to improve the start-up time and bioreactor efficiency.

Accelerated Bioreactor - The Accelerated Bioreactor that had been seeded with 700 g anaerobic granular sludge produced in the anaerobic batch reactor (Britz *et al*., 2002) and containing the selected *E. sakazakii* strain took about 20 d to reach optimum operational conditions in terms of COD removal (90%), bioreactor pH (average 7.3), biogas production (2.3 $l.d^{-1}$) and an OLR of 6.3 kg $COD.m^{3}.d^{-1}$. This stable state was further maintained for 100 d. This bioreactor was successfully used in further studies where the HRT was shortened from 24 to 14 h with an increase in OLR to 10.12 kg $COD.m^{-3}.d^{-1}$.

PCR detection

The species position of the *E. sakazakii* strain (1039) used in this study was confirmed using the API 20 E system and the results obtained showed a 99.9% correlation with the *E. sakazakii* strain in the API 20 E system databank. The PCR amplification using the primers Esak2 and Esak3 resulted in a 900 bp amplification product in only the *E. sakazakii* strain, with no amplification products observed for the *E. agglomerans*, *E. aerogenus*, *E. cloacae* and *Klebsiella* strains (Fig. 1). The PCR results also confirmed that the Esak2 and Esak3 primers are specific for the amplification of the *E. sakazakii* strain from cells of a pure culture. The number of *E. sakazakii* cells present in the PCR reaction mixture must be equal to or exceed 2 000 cells.ml^{-1} in the presence of the sludge, without extraction of DNA for the reaction to give repeatable positive and reliable amplification results.

Detection of E. sakazakii in tailor-made UASB bioreactor granules

PCR amplifications were also done directly on the supernatant and the sediment of the granular sludge obtained during the batch production of UASB granules. *Enterobacter sakazakii* was detected in the supernatant and the sediment of the inoculated samples that were taken on day 20 by the amplification of a 900 bp fragment (Fig. 2). The 900 bp PCR fragment was not amplified in the supernatant or the sediment of the control. These results clearly show that the developed PCR technique can be used for the rapid detection of *E. sakazakii* in tailor-made UASB granules or granular sludge. As control DNA was isolated according to the method of Van Elsas *et al*. (1997) from granules taken from the normal start-up bioreactor. A PCR reaction was performed on the extracted DNA using primers Esak2 and Esak3. No amplification products were obtained as in the case of the UASB granules enriched with *E. sakazakii*.

Conclusions

Granule seeding plays an important role in reducing bioreactor start-up time. When using a granule inoculum, a microbial conditioning step was necessary to help acclimatise the granules to the carbohydrate deficient winery effluent. This reduced the start-up time to only 17 d with a COD removal of >90%. UASB is an

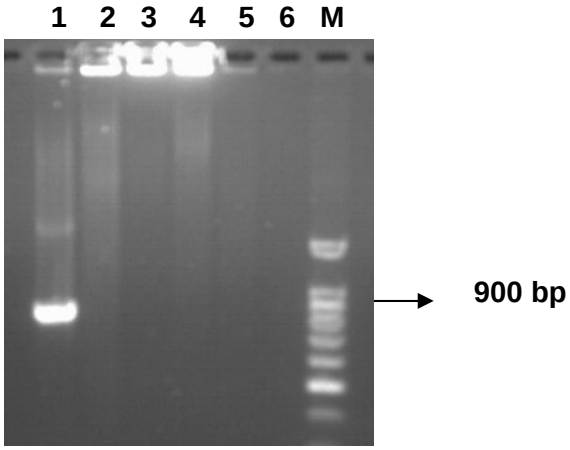

Figure 1. A 1% (m/v) agarose gel showing the PCR amplification of a 900 bp fragment of the 16S rRNA gene of *E. sakazakii* using the primers Esak2 and Esak3. Lane 1: *E. sakazakii*; Lane 2: *E. cloacae*; Lane 3: *Klebsiella* sp.; Lane 4: *E. aerogenus*; Lane 5: *E. agglomerans*; Lane 6: PCR negative control; Lane M: 100 bp ladder (Promega).

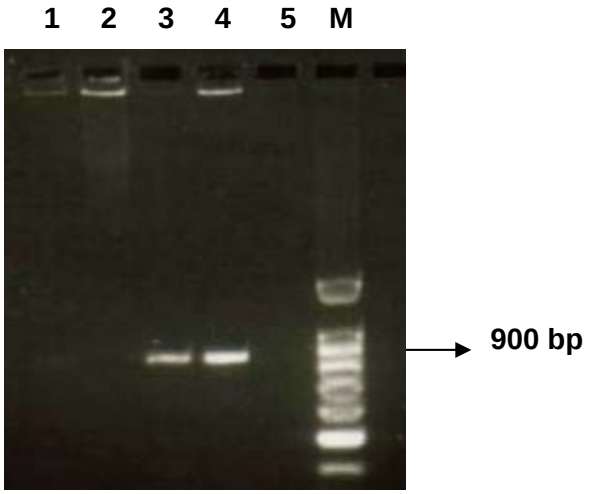

Figure 2. A 1% (m/v) agarose gel showing the PCR amplification of a 900 bp fragment of the 16S rRNA gene of *E. sakazakii* that had been inoculated into tailor-made UASB granules. Lane 1: control (supernatant); Lane 2: control (sediment); Lane 3: tailor-made UASB granules (supernatant); Lane 4: tailor-made UASB granules (sediment); Lane 5: PCR negative control; Lane M: 100 bp ladder (Promega).

effective treatment option, which does not involve the production and subsequent removal of excess sludge, which greatly contributes to the total operating costs.

PCR amplification can be successfully used to detect *E. sakazakii* in tailor-made UASB granules using the primer set Esak2 and Esak3. *E. sakazakii* strain could be incorporated into granules during the batch production of tailor-made UASB granules that may be used for the treatment of winery effluent, without the effluent inhibiting the PCR detection technique.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Anaerobic digestion, and in particular the upflow anaerobic sludge blanket (UASB) process, has been widely used for the biological treatment of food and beverage processing wastewaters. The operational efficiency and performance of UASB bioreactors rely on the formation of granules with good settling properties.

The composition of the wastewater treated by the bioreactor has an influence on the microbial consortium present in the UASB granules, which will influence granulation and ultimately affect the bioreactor performance. In order to determine if a change in the structure of the non-methanogenic microbial community takes place, UASB brewery granules were subjected to the sudden addition of different carbon sources at different concentrations. A shift in the microbial community did occur when the granules were subjected to lactate medium (5 $g.l^{-1}$). No changes in the microbial community were observed when the granules were stressed with glucose medium as a carbon source, regardless of an increase in the glucose concentration. It was clear from the different DGGE profiles obtained that changes in the microbial community occurred in the granules in the first 20 days after cultivation in lactate medium, but that a longer period of 35 to 40 days was required to establish a stable microbial community. This is of great importance for the start-up of a new bioreactor treating different wastewater.

The influence that different wastewaters have on the microbial consortium present in the UASB granules were analysed by fingerprinting and identifying the different *Bacteria* present in winery, brewery, distillery or peach-lye canning granules. Each granule type showed distinct PCR-based DGGE fingerprints with unique bands, while other bands were found to be present in all the granules regardless of the wastewater being treated. *Clostridium* was only identified in the distillery granules, while *Enterococcus*, *Leuconostoc*, *Aeromonas*, *Vibrio*, and uncultured species related to *Rhodocyclus*, *Nitrospira*, *Rhodococcus* and *Syntrophobacter* were present only in the brewery granules. *Microbacterium* species were found only in the winery granules. *Sulfurospirillum* and species related to *Acidaminococcus* were found only in the peach-lye canning effluent

granules. Various *Bacilli* were identified in all the granules and their presence in granules may play a role in the facilitation of clumping because of their adhesion ability. Most of the *Pseudomonas* were identified from the peach-lye canning granules which may play a role in the successful operation of bioreactors treating more alkaline wastewaters, since it seems as if these bacteria can survive and grow in granules treating alkaline wastewaters. It is evident from the study that the composition of each type of wastewater had a major impact on the diversity of the *Bacteria* present in the different UASB granules and that each bacteria present may play a specific metabolic role during the degradation process of that specific wastewater.

The different *Archaea* present in the different UASB granules were also fingerprinted and identified. *Methanosaetaceae*, which are known to lead to an improved granulation process, were found to be present in the winery and brewery granules. *Methanobacterium*, which plays a role in binding other bacteria together to form granules, were also detected. Not one of the five methanogen reference cultures was observed in the peach-lye canning effluent granules examined in this study. The absence of these methanogens in the fingerprint of the peach-lye canning granules may be due to undetectable low numbers of these species or unfavourable environmental growth conditions.

The various uncultured bacteria that were identified may also play a role during granulation, and further studies to determine their metabolic activities are necessary.

The different bacteria that were isolated and identified from the different granules emphasize the fact that the composition of each type of wastewater has a major impact on the diversity of the *Archaea* and *Bacteria* present in the different UASB granules. It is important to know which bacteria are present in the granules, especially when the granules are used as seed sludge for the start-up of a new bioreactor. If the species and their metabolic activities are known, it can have an influence on the choice of the granules to be used as seed sludge for the treatment of certain wastewaters.

The DGGE markers were developed to represent the dominant *Archaea* and *Bacteria* present in the South African UASB granules used in this study that treated winery, brewery and peach-lye canning wastewaters. These markers were constructed to provide a quick method to identify these members of the microbial

consortium present in the different UASB granules. Identification of the different microorganisms in UASB granules should lead to a better understanding of the population shift, especially during the start-up of a bioreactor. A major advantage of this DGGE marker is that it can be complemented by additional DGGE bands found in other UASB bioreactors. The DGGE marker can also be used to assist in the monitoring of selected species during bioaugmentation or enrichment of granules, which can lead to improved bioreactor performance. Bioaugmentation studies have often been hampered by a lack of ecological data about the activity and the fate of the inoculated organisms. The DGGE marker, therefore, holds great potential for the molecular monitoring of individual microorganisms during bioaugmentation or population shifts that may occur in anaerobic bioreactors.

It has in the past been argued that bacterial species may be selected and incorporated into UASB tailor-made granules which will improve the specificity of the granules for the treatment of a certain wastewater. To prove this *Enterobacter sakazakii*, identified as a bacterium that could metabolise winery wastewater and produce volatile fatty acids, was inoculated into winery granules. The presence of the incorporated *E. sakazakii* was successfully monitored in the tailor-made granule by using a newly developed PCR assay. Tailor-made granules or the incorporation of certain specific bacteria into UASB granules should in the future play a very important role during enhancement and stabilization of an UASB bioreactor, especially in reducing bioreactor start-up time.

Recommendations

Further studies are necessary to determine the metabolic activities and substrate requirements of the uncultured *Bacteria* and *Archaea* present in the different UASB granules. The bioreactor efficiency can be enhanced by enrichment of the granules with these bacteria. Tailor-made granules for the treatment of specific wastewaters can enhance bioreactor efficiency and reduce start-up time of the bioreactor and also reduce the adaptation time of the microbial consortium during the treatment of new substrates.

This study showed that the uncultured microorganisms make out an intrinsic part of the different UASB granules microbial community. Further studies should be conducted to try and cultivate these microorganisms and determine their

microbiological characteristics, metabolic activities and growth factors in order to understand the role they play during anaerobic digestion.