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Microbial community structure and dynamics within sulphate-removing bioreactors

by

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in

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University of Pretoria
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DECLARATION

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

Signature – G.N. van Blerk: ________________________

Date: ____________________

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SUMMARY

Mining activities, particularly coal mining, lead to the excavation of large volumes of pyrite rich soils. When exposed to air (oxygen) and water these pyrite complexes are oxidised to form highly acidic and corrosive wastewaters collectively termed acid mine drainage (AMD). Containing elevated levels of sulphates (SO_4^{2-}) and toxic dissolved heavy metals, AMD seeping from mining sites, active or abandoned, poses a major environmental risk to aquatic bio-systems – not only in South Africa but globally.

Chemical neutralization of AMD is expensive and often challenging. Biological sulphate reduction provides a promising and cheaper alternative to the treatment of sulphate rich wastewaters. Little, however, is known about the microbial communities involved in biological treatment systems and the effect of external factors thereon. Studying microorganisms in their natural

environment is extremely difficult. The limitations of culture-based methods only provide a limited insight into the bacterial diversity of natural habitats and the microbial communities present. With the progressive advances in molecular biology, non culture-based tools such as DGGE, FISH and more recently t-RFLP allow easier and much more accurate studies of microbial communities within their natural as well as man-made environments.

This study specifically investigated the use of t-RFLP to study microbial communities and dynamics within sulphate removing bioreactors. The set up and optimization of a t-RFLP system to specifically study microbial communities from sulphate removing bioreactors were investigated and the applicability of t-RFLP demonstrated.

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LIST OF ABBREVIATIONS

^{o}C	–	degrees Celsius
^{o}C/min	–	degrees Celsius per minute
%	–	percentage
µg	–	microgram
µl	–	microliter
µM	–	micromolar
16S	–	16S ribosomal RNA
AFLP	–	amplified fragment length polymorphism
AMD	–	acid mine drainage
APS	–	ammonium persulphate
bp	–	base pairs
CB	–	cellulolytic bacteria
COD	–	chemical oxygen demand
CSIR	–	Council for Scientific and Industrial Research
C-TAB/NaCl	–	hexadecyltrimethylammonium bromide-NaCl
Cy5	–	sulphoindocyanine dye
ddH_2O	–	distilled and deionised water
DGGE	–	denaturing gradient gel electrophoresis
DMSO	–	dimethylsulfoxide
DNA	–	deoxyribonucleic acid
dNTP	–	deoxynucleic-5′-triphosphate
EDTA	–	ethylenediamine-tetra-acetic acid
EtBr	–	ethidium bromide
FISH	–	fluorescent *in situ* hybridization
g	–	gram
g/ml	–	gram per milliliter
GC/FID	–	gas chromatograph equipped with a flame ionization detector
gDNA	–	genomic deoxyribonucleic acid

H_2O	–	water
L	–	liter
m	–	meter
M	–	molar
mA	–	milli ampere
mg	–	milligram
mg/L	–	milligram per liter
mg/ml	–	milligram per milliliter
$MgCl_2$	–	magnesium chloride
$MgSO_4 \bullet 7H_2O$	–	magnesium sulphate
min	–	minute
ml	–	milliliter
ml/min	–	milliliter per minute
mM	–	millimolar
N_2	–	nitrogen gas
NaCl	–	sodium chloride
ng	–	nanogram
ng/µl	–	nanogram per microliter
nm	–	nanometers
P:C:I	–	phenol chloroform iso-amyl alcohol
PAGE	–	polyacrylamide gel electrophoresis
PAT	–	phylogenetic assignment program
PCR	–	polymerase chain reaction
pH	–	potential of hydrogen
rDNA	–	ribosomal deoxyribonucleic acid
RDP	–	Ribosomal Database Project
RNA	–	ribonucleic acid
rRNA	–	ribosomal ribonucleic acid
SDS	–	sodium dodecyl sulphate
sp	–	species
SRB	–	sulphate reducing bacteria
SSU	–	small sub-unit

TAP-t-RFLP	–	t-RFLP analysis program
Taq	–	*Thermus aquaticus*
TBE	–	tris boric acid ethylenediamine-tetra-acetic acid
TE	–	tris ethylenediamine-tetra-acetic acid
TEMED	–	tetramethylethylenediamine
t-RF	–	terminal restriction fragment
t-RFLP	–	terminal restriction fragment length polymorphism
Tris	–	tris(hydroxymethyl)aminomethane
UV	–	ultraviolet
V	–	volt
VFA	–	volatile fatty acids
W	–	watt

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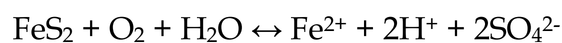
CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

Complex geo-chemical and microbiological processes associated with many mining activities, especially coal mining activities, result in the formation of highly acidic and sulphate-rich wastewaters commonly known as acid mine drainage (AMD). The accelerated oxidation of complex iron sulphides such as pyrite (FeS_2) leads to the formation of sulphuric acid and the dissolution of heavy metals (Santos *et al.* 2004). Formation of AMD can be summarized by the following equation:



$$FeS_2 + O_2 + H_2O \leftrightarrow Fe^{2+} + 2H^+ + 2SO_4^{2-}$$

These toxic effluents from active, but especially abandoned mining sites pose a major environmental risk leading the contamination and pollution of natural surface waters (Johnson and Hallberg, 2005). The extremely low pH (often pH<4) and high concentrations of sulphates and dissolved heavy metals contributes to the destructive properties of AMD to aquatic environments.

Effective treatment of AMD is dictated by many factors including the chemical properties of the AMD. Currently, strategies to treat AMD include chemical neutralization processes using calcium carbonate (lime), soda ash, calcium peroxide etc. These chemical treatments are, however, very expensive and often lead to the formation of heavy metal laid sludge that requires additional treatment and disposal measurements. A promising alternative to chemical treatment processes is biological sulphate reduction (Johnson *et al.* 2005; Luptakova *et al.* 2004; Kalin *et al.* 2003). During biological sulphate reduction, the unique capabilities of special sulphate reducing bacteria (SRB) are utilized to convert sulphates (SO_4^{2-}) into solid hydrogen sulphide (H_2S)

resulting in an increase in pH and the sub sequential precipitation of heavy metals in a solid form.

A disadvantage of biological sulphate reduction systems is that it often requires specific carbon and energy sources (e.g. ethanol), which could be expensive. Alternative carbon and energy sources for biological sulphate removal have been investigated during previous studies (Chang *et al.* 2000; Genschow *et al.* 1996; Boshoff *et al.* 2004).

The microbiology of biological sulphate reduction proves to be complex. Although studies have been conducted on the microbial communities within wetland systems treating AMD (Johnson *et al.* 1995; Hallberg *et al.* 2005), little is known about the structure and dynamics of SRB communities within sulphate removing bioreactors. The effects of alternative carbon and energy sources on the dominant bacterial members of SRB communities are for instance not well known.

Conventional culturing techniques only provided a very limited view on sulphate reducing communities within natural environments or environments such as sulphate removing bioreactors. Culture-independent DNA based techniques such as denaturing gradient gel electrophoresis (DGGE) and fluorescent *in situ* hybridisation (FISH) on the other hand, now provide much more accurate insights into the true compositions and dynamics of SRB communities present in their natural surroundings (Geets *et al.* 2006; Dar *et al.* 2007). Terminal restriction fragment length polymorphism (t-RFLP) combines 16S ribosomal gene nucleotide sequence information and the unique restriction sites of specific restriction endonucleases to identify bacterial members and construct profiles of communities (Marsh *et al.* 1999). By comparing terminal restriction fragment (t-RF) profiles from different

communities or from the same community over periods of time, changes and stabilities within the community structure can be observed and studied.

Community studies using a tool such as t-RFLP could provide valuable information in the biological treatment of AMD, as the structure and stability of the SRB community within a sulphate removing bioreactor plays such an important role in the behaviour and performance of the bioreactor. (Marsh *et al*. 2000; Kent *et al*. 2003; Nakano *et al*. 2006)

The specific aims of each experiment conducted during this study will be stated in each separate chapter.

CHAPTER 2

Literature review

CHAPTER 2

Literature review

1. Introduction

Since the industrial revolution, many human activities have not only polluted our skies and soils but also our streams, rivers, lakes and oceans. Enormous volumes of liquid waste are produced daily by everyday human activities. When left untreated, domestic, agricultural and especially industrial and mining wastewaters end up in natural fresh water bodies altering their oxygenation, turbidity, temperature, salinity and pH. Ironically, humans need these same water bodies as sources of drinking water, for recreational purposes and to drive agricultural, industrial and mining operations.

A number of industrial processes, such as tanneries, flue-gas scrubbing, galvanic processes, fermentation and the detoxification of metal-contaminated soils, lead to the formation of wastewaters with high levels of sulphate (Genschow *et al*. 1996; Pol *et al*. 1998). In mineral rich countries, such as South Africa, the generation of sulphate rich wastewaters by mining activities is of particular and growing concern. Being also a country that faces water shortages, preserving our natural fresh water systems is critical and pressure to do so is increasing (Akcil *et al*. 2006).

2. Acid mine drainage:

2.1. Formation of acid mine drainage

The mining industry, especially the mining of coal and metals, are the most significant source of sulphate-rich effluents. Water draining from mining sites, notwithstanding whether the sites is operational or abandoned, often contains elevated sulphate levels (100 to 5 000 mg/dm^3), has a very low pH (pH 4 or less) and is enriched with dissolved heavy metals such as Hg, Pb, Fe

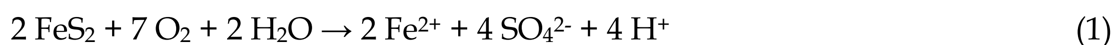
and Cu (Burgess *et al*. 2002). These wastewaters, often referred to as acid mine drainage (AMD) or acid rock drainage (ARD), are highly polluting and toxic to most life. The typical chemical composition of AMD from a coalmine is given in Table 2.1.

Table 2.1. Typical chemical composition of AMD from a coalmine (Richards *et al*. 1993)

Constituent:	*Concentration:*
pH	3.0 – 5.5
Mg^{2+} mg/L	80
Ca^{2+} mg/L	200
Al_{Total} mg/L	50
Fe_{Total} mg/L	50 – 300
Mn^{2+} mg/L	20 – 300
SO_4^{2-} mg/L	20 – 2000

Large volumes of soil are excavated during the mining of coal or metal ores such as gold. Unfortunately, metal ores and coal are commonly found in iron sulfides predominately in the form of pyrite (Ward, 2002). When exposed to water and air, these mine tailings undergo a series of complex geo-chemical and microbiological reactions leading to the formation of acid mine drainage. Although the process of pyrite oxidation and AMD formation is complex, it can be summarized in three basic steps.

Firstly, pyrite is oxidized chemically by oxygen as well as biologically by bacteria (e.g. *Acidithiobacillus ferrooxidans*) attached to the pyrite (Blodau, 2006). The oxidation of pyrite can be summarized by the following equation:



$$2\ FeS_2 + 7\ O_2 + 2\ H_2O \rightarrow 2\ Fe^{2+} + 4\ SO_4^{2-} + 4\ H^+ \qquad (1)$$

Secondly, ferrous iron (Fe^{2+}) is oxidized into ferric iron (Fe^{3+}) as summarized by Equation (2):

$$4\,Fe^{2+} + O_2 + 4\,H^+ \rightarrow 4\,Fe^{3+} + 2\,H_2O \qquad (2)$$

Although at first very slow, the oxidation of pyrite is accelerated by a drop in pH, the formation of elemental sulphur, ferric iron and further bacterial interactions (e.g. *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*) which could drastically increase the reaction rate. (Pronk and Johnson 1992; Johnson *et al*. 1993; Evangelou *et al*. 1995). Therefore, the rate of pyrite oxidation is highly dependant on the amount of ferric iron available.

Lastly, ferric iron is hydrolysed to form solid ferric hydroxide ($Fe(OH)_3$) and in the process releases further acidity. This step is pH dependant and when at a pH lower than 3.5 the ferric iron would stay in solution. At lower pH values, ferric iron will precipitate as ferric hydroxide, giving the acid mine drainage a typical yellow colour (Blodau, 2006). Equation (3) summarizes this reaction:

$$4\,Fe^{3+} + 12\,H2O \rightarrow 4\,Fe(OH)_3 + 12\,H^+ \qquad (3)$$

If left untreated, AMD running into streams, rivers, dams and lakes will alter the water bodies' quality and can potentially destroy fragile ecosystems. The effective treatment thereof is thus high priority for many mining companies. Figure 2.1 shows an example of AMD from an abandoned mining site.

Figure 2.1: Acid mine drainage from an abandoned mine, South Africa (Akcil *et al*. 2006).

2.2. Treatment of acid mine drainage

2.2.1 Chemical treatment strategies

Chemical treatments are still the most widely accepted strategy for the treatment of industrial wastewaters (Johnson and Hallberg, 2005; Akcil *et al*. 2006). The traditional approach for the treatment of acid mine drainage is by so-called chemical neutralization. (Herrera *et al*. 2007). The chemical treatment of acid mine drainage strongly relies on the principles of neutralization. The addition of basic chemicals to acidic mine effluents results in an increase in the pH of the wastewater. This rise in pH cause toxic heavy metals in solution to precipitate as sludge (Utgikar *et al*. 2000; Maree *et al*. 1994). The toxic sludge can be separated from the water component by a variety of methods including settlement. Treated water is then either recycled for further use within the mine itself or is discharged into surrounding rivers or dams. The sludge, which contains high levels of precipitated heavy metals, requires further treatment or could simply be pumped into storage dams or landfill sites (Green *et al*. 2006).

Although commonly used and fairly effective, the chemical treatment of acid mine drainage could be expensive and has a number of drawbacks. Neutralization leaves wastewaters with low concentrations of heavy metals and therefore suitable for re-use or safe discharge, but the large volumes of sludge created by the process still requires further attention (Utgikar *et al*. 2000; Li *et al*. 2007). The treatment of sludge is very expensive and in many cases the operational costs of AMD treatment plants are overshadowed by the management and treatment costs associated with the sludge. Because sludge is of little or no value, it is usually stored in dams or landfill sites. This pose a particular problem to many mining companies as this strategy requires large areas of land, which is often not available. The approval of new landfill sites is also becoming more difficult in many countries due to strict legal and environmental requirements that have to be met.

To overcome the challenges associated with chemical neutralization, many studies have been performed to improve current technologies and techniques used (Maree *et al*. 1994; Li *et al*. 2007). Some studies suggest the use of other basic substances for the neutralization process such as fly ash or even wastewater sludge (Theodoratos *et al*. 2000; Gabr *et al*. 2000). The sludge created after neutralization with traditional bases such as $CaCO_3$ is often not stable and other studies propose the use of specific chemicals for neutralization of AMD that will result in a more stable sludge or by-products of higher value (Herrera *et al*. 2007).

2.2.2. Biological treatment strategies

Effective management and treatment of acid mine drainage specifically demands a range of active and passive remediation technologies to minimize the impact thereof on the surrounding environments (Gitari *et al*. 2007). Because of the high costs involved in the chemical treatment of acid mine drainage as well as the many associated problems therewith, alternative

methods such as biological treatment have been investigated. In biological treatment the combined metabolic efforts of different microorganisms, especially sulphate reducing bacteria (SRB), are utilised. Currently, research on the biological treatment of acid mine drainage is largely focussed on passive systems (e.g. wetland systems) or active systems (e.g. bioreactor systems) (Chang *et al*. 2000; Burgess *et al*. 2002; Johnson and Hallberg, 2005; Akcil *et al*. 2006; Kaksonen *et al*. 2006).

During the early eighties it was observed that acidic mine water running through natural wetland systems greatly improved in quality (Hallberg *et al*. 2005). Microbial sulphate reduction was identified as one of the reasons and the following years saw many research projects focussing on the biological processes active in wetlands and the engineering of wetland systems specifically for AMD treatment. Natural wetland systems have always served as biological filters for environmental wastewaters (Gray *et al*. 2000). Combining the metabolic efforts of both plants and microorganisms, contaminants are removed restoring the wastewater to its original quality. While slowly flowing through a wetland system, nutrients from the wastewater are metabolised by plants while bacteria oxidises and degrades chemical contaminants (Tichy *et al*. 1998). Sedimentation also allows for the adsorption of heavy metals and dissolved particles in the system. Artificially constructed wetlands have shown to remediate acidic mine waters more efficiently. Due to better controlled and manipulated conditions, load rates and removal efficiencies were in general greater in constructed wetlands than in natural wetlands (Stoltz *et al*. 2006; Whitehead *et al*. 2005).

Although a much more cost effective alternative to traditional chemical treatments of acid mine drainage, wetland systems still pose major obstacles. Firstly, the treatment process is much slower typically resulting in the treatment of much smaller volumes of wastewater. Secondly, wetland

systems require large areas for operation – often not available to mining companies. In many cases the topography of many mining sites would also not allow for wetlands to be constructed. Attempting to overcome these problems, many research projects aimed at combining conventional chemical treatments with wetland technology (Maree *et al*. 1994; Evangelou *et al*. 1995). Here, raw wastewaters would typically undergo some chemical conditioning before entering the wetland allowing for higher loads and faster remediation rates.

A probably more costly, but also more effective biological approach to AMD treatment involves the use of bioreactors. The basic function of bioreactors would not only be to provide containment for the biological processes to occur, but also to provide optimal environmental conditions for the desired cellular metabolism within the reactor (Lidén, 2002). Bioreactors have been used in wastewater treatment for over 100 years and many different reactor designs were implemented ranging from trickling filter systems, rotating designs and completely suspended reactors, all operating at both aerobic and anaerobic conditions (Langwaldt *et al*. 2000). However, since the 1970's the use of anaerobic processes for the treatment of wastewaters increased rapidly. The successful use of anaerobic technology for treatment is dependent on the bioreactor design and rate. Bacteria attached to a support substrate show higher activity than cells in suspension (Langwaldt *et al*. 2000). This observation led to reactors with a fixed substrate design such as fluidized bed reactors which has been used with success in wastewater treatment (Massol-Deya *et al*. 1997). In contrast to Continually Stirred Tank Reactors (CSTR), high rate reactor designs allow for a high reaction rate in terms of kg COD/m^3 per day as well as biomass retention (Barber *et al*. 1999). A breakthrough came with the development of the up-flow anaerobic sludge blanket (UASB) reactor (Oude *et al*. 1998; Saravanan *et al*. 2006). Using small granules (1-4mm) to immobilize anaerobic bacteria, this reactor design does

not require any support material such as clay or Raschig rings (Nicolella *et al*. 2000). Intuitive reactor design, well-controlled environments and the exploitation of selected bacterial traits can result in very effective treatment systems. However, very few changes in bioreactor design have been made the past 40 years (Lidén, 2002).

Currently, some of the main challenges in sulphate removal bioreactor studies include finding effective and stable microbial consortiums, cheap and readily available energy sources to fuel and operate the treatment process and reactor or plant designs allowing for high load rates, fast treatment times and in some cases the recovery of valuable heavy metals (McHugh *et al*. 2003; Foresti *et al*. 2006).

Expensive carbon sources such as ethanol is currently used in full-scale plants. Studies have shown very promising results using cheap alternative energy sources such as plant material or waste products such as wastewater sludge or municipal wastes in the treatment of AMD (Peppas *et al*. 2000; Burgess *et al*. 2002; Boshof *et al*. 2004; Johnson and Hallberg, 2005;). As in wetlands, some studies also suggested combining chemical and biological efforts as an effective treatment strategy.

3. Studying microbial communities within their natural environments:

The optimal operation and control of biological treatment systems require a good understanding of the macro- and microbiological actions and interactions within the reactor. For many years research and design of biological treatment systems had a "black box" approach where the desired results were achieved only by changing the external parameters. Only recently have scientists focussed their research on the microbiology of biological treatment systems. However, insights into the microbiological

workings of biological treatment systems were, for many years, restricted to the limitations of the methods used. Fortunately, as we entered the era of molecular biology, much more reliable and accurate tools were developed for microbial ecology studies.

3.1. Culture-based techniques

For decades the only way to study microorganisms was to use conventional techniques such as microscopy, special staining techniques or to isolate and culture them in a pure form. A pure culture of a specific microorganism would then allow for other characteristics to be determined such as physiological and biochemical properties.

Being one of the few ways to study bacteria, scientists quickly discovered that many bacteria share the same metabolic trademarks, making it difficult to differentiate one from the other. This observation lead to the development of more specialised culturing techniques and artificial growth media allowing selective isolation of specific bacteria. However, as the knowledge of the microbial world expanded, the immense diversity thereof became more apparent. Realising the limitations, some studies suggest that current culturing techniques and media only allow for the cultivation of 1% of all bacteria occurring in nature (Torsvik *et al*. 1998; Rölleke *et al*. 1999).

Using culturing methods, studies of microorganisms were also limited to the controlled environments of the laboratory. Investigating the effects of external environmental factors on specific microorganisms or microbial communities are very difficult if not impossible using culturing methods. Just determining the composition of a microbial community using culture-based methods would be a laborious and complex task and taking into account that culturing is limited to certain microbes, probably inaccurate. By entering a viable but

non-culturable state, many bacterial members within a community could also be overlooked (Kirk *et al*. 2004; Keep *et al*. 2006).

Understanding the composition and dynamics of the microbial community within sulphate-removing bioreactors treating acid mine drainage and the effects external parameters might have thereon could be a crucial step in obtaining optical treatment conditions as well as the maintenance thereof. It is important to get an idea of all the microbes involved and using culturing techniques to study the community dynamics would not be ideal as it would be a laborious process and the results would be inaccurate. Studying microorganisms and microbial communities using a molecular approach would be a much more powerful alternative (MacGregor, 1999; Fontana *et al*. 2005).

3.2. Molecular-based techniques

Targeting the genetic traits of bacteria rather than their physiological capabilities will result in a more accurate analysis of the community composition and dynamics. By not relying on the culturing of specific bacteria, molecular techniques often resolve the issue of detecting viable but non-culturable cells. Genomic DNA can be extracted directly from samples taken from the environment or bioreactor. Several molecular techniques have proven to be useful tools for studying microbes and microbial communities from a variety of environments (Dent *et al*. 2004; Marschner *et al*. 2003; Hiraishi *et al*. 2000). Commonly used, these included techniques such as DGGE, FISH and more recently t-RFLP.

Degenerate Gradient Gel Electrophoresis (DGGE):

By combining the denaturing properties of DNA molecules with gel electrophoresis, DGGE is a useful tool to obtain microbial community profiles. In studies used, a selected gene (usually the 16S rRNA gene) is amplified by

means of PCR (Fasoli *et al*. 2003; Farnleitner *et al*. 2004). After purification, the amplicons are subjected to gel electrophoresis using a poly-acrylamide gel with denaturing agents. This denaturing gradient of the gel is gradual and usually consist of urea. When prepared, the poly-acrylamide gel would have a urea concentration ranging from low at the sample loading point to high at the opposing end of the gel. Amplicons are differentiated and separated from one another based on their GC content (Muyzer *et al*. 1993). Fragments with a lower GC content will denature faster causing them to migrate slower through the poly-acrylamide gel matrix, resulting in a separation of the identical sized fragments.

Different bacterial communities are studied by comparing their DGGE band profiles generated. Additional bands present in one profile and not the other are considered to be different bacterial members present in the community. The same principle is used when investigating community dynamics where profiles generated over specific time periods would be compared. Differences in the DGGE profiles would then be interpreted as a change in the community structure (Nikolcheva *et al*. 2005).

Unfortunately, DGGE has many pitfalls still leaving a number of questions regarding microbial community structures unanswered. The identification of specific bacterial members of a community using DGGE is a strenuous process. Represented by a single band in the DGGE profile, the identity of the bacterial member could only be determined by sequence analysis. This process require that the fragment has to be cut from the poly-acrylamide gel, further purified and sequenced after which the sequence data can be compared to a database for identification.

Separation of the PCR amplicons generated by supposedly different bacteria is also not very accurate using a denaturing gradient. Although different in

origin and sequence, the denaturing of two or more fragments could be very similar resulting in the co-migration of the different fragments. Appearing then as a single band pattern on the gel, the true diversity of the microbial community studied could actually be misrepresented.

Fluorescent in situ Hybridization (FISH):

The advancements in oligonucleotide probe and fluorescent dye technologies led to the development of the fluorescent in situ hybridization (FISH) technique in the late 1980's (Rogers *et al*. 2007). During a typical FISH analysis of a microbial community, fluorescently labelled probes can be designed to target bacterial ribosomes or ribosomal RNA (rRNA) and bind to it (Lenaerts *et al*. 2007; Rogers *et al*. 2007). After hybridization of the probes to their targets, the dyed cells can then be detected and enumerated using fluorescent microscopy or flow cytometry. Probes can be designed in such way as to target whole bacterial domains or be more precise and only detect specific species within a bacterial community. The use of 16S rDNA FISH to analyse microbial community structure and dynamics have been demonstrated (Amann *et al*. 1995; Morter *et al*. 2000; Daims *et al*. 2005).

Several obstacles and difficulties in analysing microbial communities using FISH have been identified, one of the main problems being associated with background fluorescence. In their studies of soil microbial communities using FISH, the detection of fluorescent cells was mainly hampered by auto fluorescent soil particles (Hahn *et al*. 1992; Zarda *et al*. 1997). These problems could be overcome by extracting the bacterial cells from the soil and studies where FISH was used to analyse soil communities have been performed successfully (Stein *et al*. 2001). Bacteria from environmental samples may also be slow growing and hence, would contain less ribosomes. Consequently this could lead to low detection signals making it difficult to distinguish between target cells and debris using FISH. Accordingly, there

has been some effort to improve the fluorescent signal-to-noise ratio (MacDonald *et al*. 2000). One such improvement included the use of molecular beacons instead of linear oligonucleotide probes. Still, the use of FISH to analyse microbial communities within their natural environment is limited to members with high ribosome or rRNA content and would thus result in a skewed representation of the true diversity of the community studied.

Terminal Restriction Fragment Length Polymorphism (t-RFLP):

Another emerging tool for microbial ecology the last decade was terminal restriction fragment length polymorphism (t-RFLP). This technique is largely based on a combination of technologies including comparative genomics, PCR and nucleic acid electrophoresis, especially high resolution electrophoresis as obtained by automated DNA sequence analysers (Marsh, 1999). Comparative sequence analysis of 16S rRNA greatly contributed to our understanding of microbial evolution, and diversity. One of the first techniques reported to study microbial communities and diversity combined automated sequencing technology, PCR and 16S rRNA sequence information (Avaniss-Aghajani *et al*. 1994).

In t-RFLP, universal or near-universal eubacterial primers are used to amplify conserved regions of the 16S rDNA gene from the pool of genomic DNA extracted from the community studied. By fluorescently labelling one of the primers (usually the forward primer) the resulting amplicons are terminally labelled. Following purification, the homologues are subjected to a series of individual restriction enzyme digestions. Sequence data reveals small differences in the 16S rDNA sequence between different bacterial species and the unique sequence-specific restriction sites of the restriction enzymes are used to discriminate between different bacterial members. This species discrimination is based on the differently sized restriction fragments that will

result due to the small sequence differences. Since the amplicons were terminally labelled, resulting restriction fragments will also be terminally labelled, hence the term terminal restriction fragments (t-RF's). Complete separation of closely sized t-RF's (±1 bp) is crucial in obtaining accurate and representative results. To achieve this, high-resolution gel electrophoresis is used. Many earlier studies relied on automated gel-based DNA sequencing systems to perform this task (Ayala-del-Rio *et al*. 2004; Rademaker *et al*. 2006) but in later studies capillary-based sequencing systems became the method of choice (Dollhopf *et al*. 2001; Smalla *et al*. 2007). The subsequent gel images or output data is analysed, t-RF size data compiled and then compared to existing 16S ribosomal databases for possible bacterial species identification. Popular 16S databases such as the Ribosomal Database Project (RDP) also developed analysis tools specific for t-RFLP data analyses (Maidak *et al*. 2001).

Using t-RFLP to study microbial communities from environmental samples appears to have several advantages opposed to DGGE and FISH. Firstly, the high resolving power of DNA sequencing systems allows for the separation of closely related restriction fragments. This is hardly achieved during DGGE analyses. Secondly, the gel image or peak data obtained after electrophoresis is available in digital format and allows for easy export to alternative computer software for further analysis. Like DGGE, t-RFLP profiles can also be analysed using powerful software to obtain the most accurate results. Unlike DGGE, however, t-RFLP results can be compared to online databases to retrieve bacterial group or species identities (Kent *et al*. 2003; Nakano *et al*. 2006). The results are thus immediate and do not require further processing such as excision from PAGE gels, band purification and sequence analysis to obtain species identifications. Contradictory to FISH, t-RFLP analyses are not reliable on ribosome or rRNA content meaning that bacterial cells in low numbers or metabolic states can still be detected. Results are thus more representative and accurate and since the fluorescent labelling is restricted to

only the 16S amplicons and t-RF's, background fluorescence has less influence than during a FISH analysis. Lastly, apart from being less expensive and unlike DGGE or FISH, t-RFLP has the potential for full automation allowing a high throughput of samples.

Terminal restriction fragment length polymorphism has already been used successfully in several studies of microbial communities. These include communities from soil samples, river sediments, plants, dairy samples and even samples from the human intestines and oral cavities (Sakamoto *et al.* 2003, 2004; Graff *et al.* 2005; Sakata et al. 2005; Park *et al.* 2006; Rademaker *et al.* 2005, 2006; Schwarz *et al.* 2007). Studies on wastewater treating bioreactors and the microbial communities within them have also been performed using t-RFLP (Fedi *et al*. 2005; Collins *et al*. 2006). The use of t-RFLP in sulphate removing bioreactors using t-RFLP is however limited.

CHAPTER 3

SET UP AND OPTIMIZATION OF T-RFLP FOR MICROBIAL COMMUNITY ANALYSIS OF SULPHATE REMOVING BIOREACTORS

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3.1 INTRODUCTION:

Studying microorganisms and microbial communities within their natural environment can be an extremely difficult task. Numerous factors have to be taken in consideration to obtain an accurate picture of the microbial diversity within a habitat. In years, many studies have been conducted to study microorganisms within their natural habitats e.g. sediments, soil, plants and animals, wetlands and even extreme environments such as deep ocean thermal vents and hot water springs (Bart *et al.* 2000; Elshahed *et al.* 2003; Webster *et al.* 2003; Dent *et al.* 2004; Lloyd *et al.* 2004) Because microorganisms, whether individually or as a community, play such important roles in biochemical cycling it is important to get an idea of the diversity in the habitat to try and understand its specific characteristics. Thus, by studying microorganisms and microbial communities within their natural habitat, we might gain a better understanding of the complex biological and chemical processes involved - even if the habitat being studied is a man-made bioreactor.

Like any natural habitat, the bioreactors used in this study provided a unique surrounding for the microorganisms present. As, for example, climate changes or fluxes in nutrients would alter the microbial community of a natural habitat, so would external factors or changes to the bioreactors, or their content, influence and alter the microbial community within. An understanding of the changes in the microbial community due to the changing external factors might therefore provide some answers to questions related to the reactor's functioning.

Various methods have been developed to study microbial communities within their natural habitat, most notably techniques such as DGGE and FISH (Hill *et al.* 2000; Webster *et al.* 2003; Lyautey *et al.* 2005). These techniques, however, may only provide a limited insight into the full diversity of the microbial community and its individual members. In most cases further methods such as cloning and sequencing are required to finally identify most of the members present within the community. Apart from being labor intensive, these methods are also fairly expensive to conduct.

Currently, ever growing ribosomal sequence databases provide researchers with valuable information regarding different bacterial genera. By comparing 16S rDNA sequences the unique genetic properties of different bacteria can be identified and exploited using the unique features of restriction enzymes for instance. Hence, the development of a technique such as terminal restriction fragment length polymorphism (t-RFLP). By combining the ability to label and detect fragments of DNA, the unique cutting properties of restriction enzymes and the unique 16S nucleotide sequence differences between different bacteria, t-RFLP provides a powerful tool capable of separating bacterial communities into its individual members (Marsh, 1999). Like DGGE, t-RFLP results in fingerprint profiles of microbial communities consisting of labeled terminal restriction fragments (t-RF's). These community profiles can easily be compared to other community profiles or be monitored over specified time periods to observe any changes within the community.

As an additional feature, t-RFLP allows the researcher to further identify dominant bacterial members within communities by comparing t-RF size data to existing databases. Terminal Restriction Fragment Length Polymorphism has been used to study microbial communities from numerous environments including soil (Graff *et al.* 2005), the rhizospheres of plants (Park *et al.* 2006),

lake sediments (Schwarz *et al.* 2007), even cheeses and yoghurts (Rademaker *et al.* 2005, 2006) and the oral cavities of humans (Sakamoto *et al.* 2003, 2004). Few studies on sulphate removing bioreactors and the microbial communities involved therein have been conducted. Because of the applicability of t-RFLP to so many different environments, it appears as a very useful tool for studying the unique and in many cases changing community within sulphate-removing bioreactors. By using t-RFLP, community profiles generated can easily be compared over time periods of bioreactor operation to study the stability of the community, changes that might have occurred and even identify the dominant members within the bioreactor. Combining this data with the chemical parameters measured in the bioreactor, a much clearer understanding of controlled sulphate removal can be gained. The aim of this experiment was to set up and optimize t-RFLP in the laboratory for specific use in studying microbial communities and dynamics within sulphate removing bioreactors.

3.2 MATERIALS AND METHODS:

3.2.1 Bioreactor sample collection and storage

All samples were collected at the division Natural Resources and the Environment, CSIR, Pretoria, where personnel from the CSIR operated the sulphate removing bioreactors. Samples from bioreactors investigated in this study were collected in sterile Schott bottles (100 ml). Sample volumes ranged from 50 ml to 100 ml. Samples were transported on ice to the University of Pretoria, where all t-RFLP analyses were performed. All samples were stored at 4°C before analysis.

3.2.2 Genomic DNA extraction

Genomic DNA (gDNA) was extracted from samples obtained from each bioreactor studied. A modified C-TAB/NaCl method (*personal communication Estie Eloff, CSIR*) was initially used to extract gDNA from the bioreactor samples, but further modifications were made to optimize gDNA extractions and to increase the quality and yield of extracted DNA.

Firstly, 1 to 3 ml of reactor sample was centrifuged at 6440 x g for 5 minutes to pellet bacterial cells. Because the reactors samples contained a lot of grass cuttings and/or biomass, an additional sonication step was later added before collecting bacterial cells by means of centrifugation. Sonication is known to break clumps of cells and it was anticipated that sonication would loosen and break clumps of bacteria attached to biomass particles or grass cuttings.

Samples collected in 100ml sterile glass bottles, were placed on ice for approximately 10 minutes. A sonication probe was used to sonicate each sample separately for a period of 30 seconds. One to three ml of sonicated reactor samples was then subjected to centrifugation to collect loosened bacterial cells. The resulting cell pellet was re-suspended in 565 μl TE buffer. Lysozyme at a concentration of 50mg/ml was added and the suspension incubated at 37°C for a minimum period of 30 minutes. After incubation, 30 μl of a 10% SDS solution was added to the suspension as well as Proteinase K at a concentration of 20mg/ml. The suspension was incubated at 45°C for a minimum of 1 hour.

After incubation, 100 μl of a 5M NaCl solution was added. After mixing thoroughly, 80 μl of C-TAB/NaCl solution, which was pre-heated to 65°C, was added and the suspension mixed well again by vortexing for 10 seconds. This was followed by incubation at 65°C for a minimum of 25 minutes.

A phenol/chloroform/iso-amyl alcohol (P:C:I) extraction followed. The initial protocol stated that an equal volume of P: C:I should be used but, due to a lack in volume within the 1.5 ml microfuge tube, only 700 µl of P:C:I was added to the suspension. Suspensions were mixed thoroughly by means of vortexing after which centrifugation at 6440 x g for 5 minutes followed to separate the different phases formed. The clear, top phase was transferred to a fresh microfuge tube. The P:C:I extraction was repeated.

Genomic DNA was precipitated from the aqueous top phase adding isopropanol at 0.6 of the total volume. Isopropanol was later substituted with 96% ethanol, as this appeared to deliver better results. The initial extraction protocol suggested gentle mixing by means of inverting until a stringy, white DNA precipitate formed. Centrifugation at 6440 x g for 20 minutes then followed. The supernatant was carefully removed from the resulting DNA pellet to which 200 µl 70% ethanol was added. Centrifugation followed once again for 5 minutes at 6440 x g after which the supernatant was removed and the pellet air-dried completely. The initial extraction protocol suggested that the DNA pellet being re-suspended in TE buffer. Sterile de-ionized water was used instead. The microfuge tubes were also incubated overnight with agitation at 37°C to facilitate the re-suspension of the DNA pellet.

3.2.2 16S ribosomal DNA gene amplification

The t-RFLP technique is based on small 16S nucleotide sequence differences between different taxonomic groups (Marsh, 1999). Therefore, the 16S ribosomal gene needed to be amplified before being subjected to restriction enzyme digestions and further analysis. Amplification of the 16S gene was performed using the polymerase chain reaction (PCR). Various primers have been used and suggested for 16S gene amplification in previous studies (Muyzer *et al.* 1993; Heuer *et al.* 1997; Gomes *et al.* 2001). However, web-based analysis tools and *in silico* enzyme restrictions suggested using forward

primer 341F and reverse primer 16R1522pH in combination would deliver the most hits to 16S rDNA sequences in the database as well as a high diversity of different bacteria. The online analysis tool allows the user to perform an *in silico* primer (probe) match to all 16S sequences present in the database as well as to perform a restriction enzyme digestion. Table 3.1 shows the nucleotide sequences for each primer used in this study.

Table 3.1: Nucleotide sequences for forward primer 341F and reverse primer 16R1522pH

Primer name:	*Sequence:*
341F	5' CCT ACG GGA GGC AGC AG 3'
16R1522pH	5' ACG CCG ACC TAG TGG AGG A 3'

Polymerase chain reactions were performed using SuperTherm Taq (J.M.R Holdings, U.S.A). The Taq polymerase was supplied with a 10x reaction buffer and separate $MgCl_2$ allowing adjustments to the final concentration of $MgCl_2$ in each reaction. Initial PCR reactions were performed using a final concentration of 1.5 mM $MgCl_2$, 0.5 µM of each primer and 0.2 mM of each dNTP. The forward primer 341F was labeled with sulphoindocyanine dye Cy5 resulting in a terminally (5′) fluorescent-labeled PCR amplicon.

The first PCR results obtained indicated that several adjustments would have to be made to obtain the desired 16S gene amplification. The first adjustments were made to the $MgCl_2$ final concentration. Several PCR reactions were performed with varying final concentrations of $MgCl_2$ ranging from 0.5 mM to 3.0 mM.

To facilitate 16S gene amplification, the addition of a co-solvent, such as DMSO, have also been investigated. Two and an half micro liters of a 10% DMSO solution was added to PCR reactions.

As an excess of primer might cause non-specific binding (Sambrook and Russell, 2001), the amounts of primer used were reduced from 0.5 µM to 0.2 µM final concentration. PCR reactions typically had a total volume of 50 µl and contained reaction buffer (10x) and dNTP's at a final concentration of 0.2 mM.

At first undiluted template DNA was used but later results indicated that diluted genomic DNA delivered much better 16S gene amplification. Genomic DNA was diluted ten times using sterile de-ionized water. Of these dilutions 5 µl was used in each PCR reaction.

Some adjustments were also made to the PCR reaction conditions. A hot-start PCR was performed at 94°C for 3 minutes followed by 30 cycles of denaturing at 94°C for 30 seconds, primer annealing at 58°C for 30 seconds and extension at 72°C for 30 seconds. A final extension step was done at 72°C for 4 minutes. PCR products were stored at 4°C.

3.2.4 16S ribosomal DNA purification

Successful amplification of 16S ribosomal DNA gene fragments was followed by a purification step to remove all excess primers and PCR reagents. At first, the purification was performed using a QIAquick gel purification kit (Qiagen). The manufacturers' instructions were carefully followed. Briefly, the total volume of the PCR reaction was loaded onto a 1% agarose gel stained with ethidium bromide (10mg/ml). Amplified 16S gene fragments were then carefully excised from the agarose gel and melted in a supplied buffer. This was followed by the addition of further supplied buffers, purification through a supplied column and elution of purified 16S rDNA. Amplified 16S gene fragments were also purified directly from the PCR reactions using the QIAquick PCR purification kit (Qiagen). In this procedure

only a tenth of the total PCR reaction volume was loaded onto an ethidium bromide stained agarose gel (1%) to confirm amplification of the 16S gene. The remainder of the PCR reaction was then subjected to purification following the manufactures' instructions.

After both purification methods, one tenth of the final elution volume was visualized on an ethidium bromide stained agarose gel (1%) to confirm the 16S rDNA product and to estimate the DNA concentration.

3.2.5 Restriction enzyme digestions

Different taxonomic groups represented by different 16S gene fragments, can be distinguished from one another by performing a restriction enzyme digestion. Because restriction enzymes recognize specific restriction sites with a specific nucleotide sequence, unique differences between different bacteria would result in restriction fragments differing in size. A fluorescently labeled forward primer was used to amplify the 16S gene, resulting in PCR products fluorescently labeled at the 5′ end. After restriction enzyme digestion, only the terminally labeled, or terminal restriction fragments (t-RF's) will be detected and sized. The size data of each individual t-RF and corresponding restriction enzyme is then used to assign possible bacterial members to each t-RF in the whole t-RFLP profile generated.

The choice of restriction enzymes used in this specific study was based on both the literature and using web-based analysis tools provided by the RDP website. Literature frequently suggested the use of restriction enzymes *RsaI*, *MspI* and *HhaI* (Hiraishi *et al.* 2000; Fedi *et al.* 2004; Rademaker *et al.* 2005, 2006). After further consultation of the literature and by performing further *in silico* restriction enzyme digestions, an additional two restriction enzymes

were also used to generate t-RF profiles. Thus, 5 different restriction enzymes were used in this study – *RsaI*, *MspI*, *HhaI*, *HaeIII* and *Sau96I*.

Digestion reactions typically contained restriction buffer (10X), 1 unit of restriction enzyme and purified 16S rDNA. Adjustments to the amount of 16S rDNA used were made according to results obtained after PAGE analysis. Sterile deionised water was used to make to reaction volume up to 25 µl in total.

Digestions were initially performed for a period of three hours but later results indicated no significant differences if digestion was performed for one hour only. No heat inactivation was performed and digestion reactions was merely cooled down and held on ice until it was loaded onto the PAGE gel for electrophoresis.

3.2.6 Polyacrylamide gel electrophoresis (PAGE)

The exact size of each t-RF generated by restriction enzyme digestion is very important for data analysis and high-resolution gel electrophoresis is used to accurately separate the differently sized t-RF's. For this study, polyacrylamide gel electrophoresis (PAGE) was chosen. Polyacrylamide gel electrophoresis was performed using the IR^2 Global Edition DNA Analyzer (LI COR, Lincoln, U.S.A). This apparatus is equipped with a laser capable of constantly scanning the PAGE gel and detecting fluorescently labeled fragments as they migrate through the acrylamide gel. All electrophoretic settings and conditions were automatically controlled and all data generated processed by computer software to form a complete gel image.

An existing protocol used for AFLP analysis of restriction fragments was used (Brady *et al*. 2007). An 8% polyacrylamide gel was prepared using 20 ml gel

solution (Long Range Gel), 150 µl APS (0.1g/ml) and 15 µl TEMED. The gel solution was poured between two special glass plates, cleaned thoroughly with a 1% SDS solution, and allowed to set for 1 hour. The solidified gel, together with the glass plates, was mounted in a vertical position in the LI COR sequencer. The buffer tanks were filled with 0.8 X TBE buffer, obtained by diluting 160 ml 10 X TBE buffer (108 g Tris-base, 55 g ortho-boric acid, 7.44 g EDTA, 1 L ddH_2O, pH 8.0) with 1840 ml ddH_2O. The gel was subjected to a 30-minute pre-run with the following settings. Set voltage: 1500 V; set current: 35 mA; set power: 35 W; set temperature 45°C. Following the pre-run, 0.8 – 1.5 µl of the digested 16S rDNA was loaded in triplicate onto the gel together with an internal size standard (700bp standard). Prior to loading the digested 16S rDNA was denatured at 90°C for 3 minutes after which it were kept on ice until loaded. Electrophoresis was performed at the mentioned settings for a period of 4 hours.

3.2.7 PAGE gel image analysis

Gel images generated by PAGE were processed and analyzed using Bionumerics® computer software supplied by Applied Maths, Belgium. Although the Bionumerics® computer software is specifically designed for the analysis of AFLP data and gel images, the software provides many features applicable to t-RFLP data analysis. This software, for instance, allows the user to perform various functions such as lane selections, background subtractions and band selections. Most importantly the software provide tools to compensate for skew gel images (normalization), set standard size markers and determine the sizes of individual t-RF bands. These features were of particular importance for t-RFLP data analysis. Gel images can also be adjusted to increase and decrease band intensities and compare different lanes. Image and t-RF band data can also be exported to other software applications such as Microsoft Word and Excel.

After downloading the PAGE gel image from the LI COR local server, the image was inverted and opened with the Bionumerics® software. Each sample lane was then carefully selected after which a background subtraction was performed to reduce background noise. The internal size standard was used as a reference and individual bands formed by the internal size standard were then used to normalize the gel image. By aligning corresponding bands from the size standard loaded in lanes across the gel, the software automatically adjusts each lane so that the image is pulled straight. This step is particularly important as t-RF profiles from different samples loaded in different lanes need to be compared for similarities and differences.

Normalization of the gel image was followed by identification of individual t-RF bands. By adjusting the intensity settings, t-RF bands were made as clearly visible as possible. Although the Bionumerics® software provides a standard cut-off value function for band identification, this feature was not used, as the software did not recognize many lighter bands as true t-RF bands. The identification of t-RF bands was thus done manually and was mainly based on the visualization of the specific t-RF band. The known size of each band from the internal size standard was put in as an external reference. Bionumerics® then calculates the sizes of unknown t-RF bands by using the % of migration of each band from the sample and the given size of each band from the internal size standard.

After selecting and sizing the t-RF bands for each sample loaded, band size data was exported to Excel for further analysis. Gel images and individual lanes were selected and compared using the features provided by Bionumerics®. Processed comparisons were exported to Word for further annotations and processing.

3.2.8 Further data analysis and t-RF fragment assignment

Terminal restriction fragment length profiles were analyzed for similarities and differences between different reactor samples or between samples from the same reactor taken on different time periods during a specific experiment. This was done by comparing the t-RF profiles generated using Bionumerics® and identifying similar bands or different bands between samples. By using specialized online analysis tools, possible bacterial members represented by individual t-RF bands were also assigned.

The Ribosomal Database Project II (RDP II), release 8.0, at the Michigan State University in East Lansing, Michigan, contains more than 10 700 small subunit (SSU) rRNA sequences in an aligned format and more than 21 000 sequences in unaligned format (Maidak *et al.*, 2001). The RDP II also provides data, programs and online analysis services that relate to the bacterial ribosome. The TAP-t-RFLP online analysis tool was used to perform *in silico* digestions of the data set with the selected restriction endonucleases *RsaI*, *MspI*, *HhaI*, *HaeIII* and *Sau96I*. *In silico* digestion results were used to predict t-RF sizes for specific bacterial members and to compose a database for further use. The t-RF size data exported from the Bionumerics software was then compared to the database constructed by the TAP-t-RFLP program to assign possible bacterial members to each t-RF profile generated. This task, however, soon proved to be extremely complex and laborious.

Thus, another on-line analysis tool, the Phylogenetic Assignment Tool (Wisconsin University), was used to assign possible bacterial species to specific t-RF bands and profiles. This online program was specifically designed for t-RFLP data analysis and allows the user to import t-RF band sizes as tab delimited Excel files. The software then uses restriction data from all five enzymes to compute all possible combinations and represented

bacterial species for each set of values. All data generated by the analysis tool was then exported to Excel where further modifications were made.

Lists of possible bacterial members represented by t-RF profiles were edited based on the biology of the bacteria and only the most probable bacterial members were selected to represent the communities within each reactor. Bacterial members with physiological characteristics that did not correspond to conditions within the bioreactors were excluded from the analysis. Special attention was also paid to t-RF fragments that remained stable throughout an experiment as well as to t-RF fragments that appeared or disappeared from profiles during the specific experimental time period.

3.3 RESULTS AND DISCUSSION:

3.3.1 Genomic DNA extraction

The genomic DNA extractions proved to be fairly successful with only a few adjustments made to the initial C-TAB/NaCl method. The addition of the sonication step resulted in a higher yield of gDNA as demonstrated by Figure 3.1. Although a little less phenol-choloroform-iso-amyl alcohol was used than the suggested volume, very little effect could be observed as high gDNA yields were still obtained. An additional P:C:I extraction step did seem to improve the gDNA extractions however. The quality of gDNA extracted seemed to have improved and fewer problems occurred during the 16S PCR.

When precipitating the gDNA with isopropanol no stringy, white precipitate could be observed as the protocol claimed. Even when the isopropanol was substituted by 96% ethanol, no DNA precipitate could be observed. Only after the 20 minute centrifugation step was a precipitate (presumably that of

gDNA) visible. The use of ethanol rather than isopropanol seemed to deliver higher yields of gDNA.

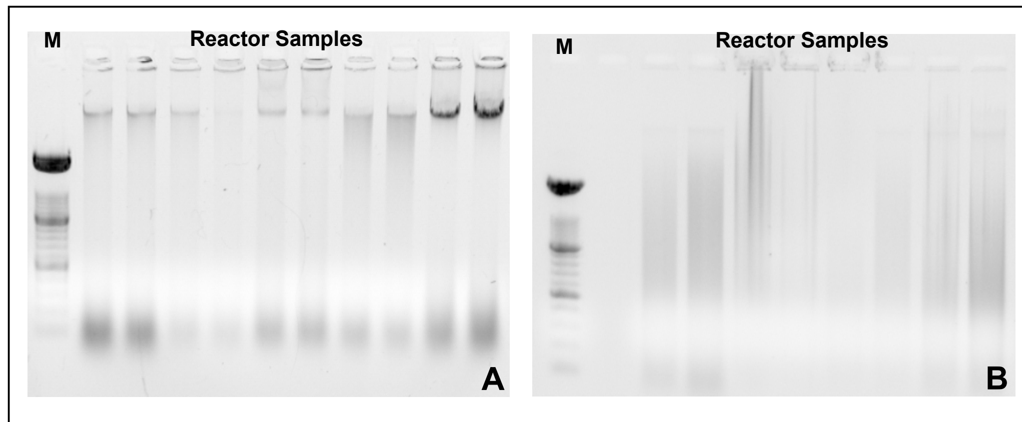

Figure 3.1: Comparison between gDNA extracted with (A) and without (B) sonication.

To achieve the most representative results, the extraction of genomic DNA from the microbial community should be as thorough as possible. Poor gDNA extractions with low yields might lead to an under representation of the true microbial diversity within the community when subjected to t-RFLP analysis. Though it is very difficult to determine whether gDNA was actually extracted from every bacterial representative within a community, the modified C-TAB/NaCl method used in this study delivered exceptionally high yields of gDNA. It is thus assumed that the higher the yields of gDNA extracted from the community, the better the representation of diversity. Although high in yield, the quality and purity of the extracted gDNA should also be considered as this could greatly influence subsequent 16S PCR reactions.

3.3.2 16S ribosomal DNA gene amplification

Various primer pairs exist for the specific amplification of the 16S gene. Using the RDP II database and accompanying online analysis tools, forward primer 341-F and reverse primer 16R1522pH delivered the best results. This primer pair matched a high number of 16S sequences from different bacteria in the

database and in combination with the selected restriction enzymes delivered the highest degree of diverse t-RF profiles.

Successful amplification of the 16S gene target proved to be much more difficult however. Several adjustments had to be made to the PCR reaction setup to finally obtain the desired result. At first, very little or no 16S amplification could be observed as shown in Figure 3.2. The addition of DMSO also seemed to have very little effect on the success of the PCR reaction and it seemed to have rather favored non-specific priming. When amplification was achieved, a lot of non-specific amplification could also be observed (Figure 3.3).

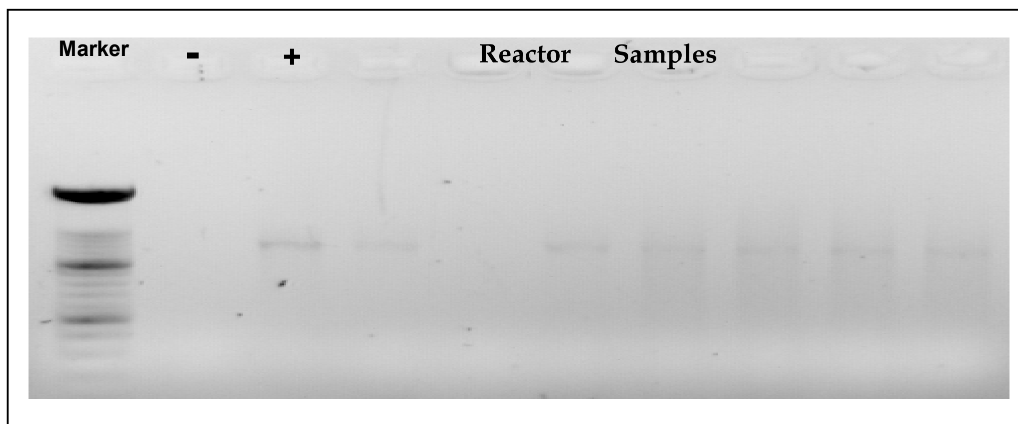

Figure 3.2: Poor PCR results with little or no 16S rDNA amplification observed.

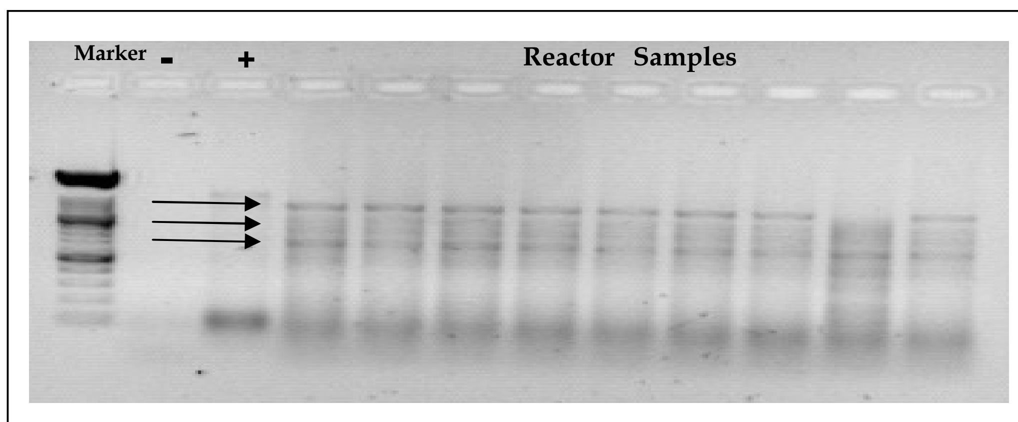

Figure 3.3: Non-specific binding (indicated by arrows) observed during 16S rDNA PCR reactions.

When primer concentrations were lowered to a final concentration of only 0.1 µM, amplification showed some improvement. The only real improvement however was observed when the gDNA used as template for each reaction was diluted 1:10. Figure 3.4 shows the difference in 16S amplification using diluted and undiluted gDNA.

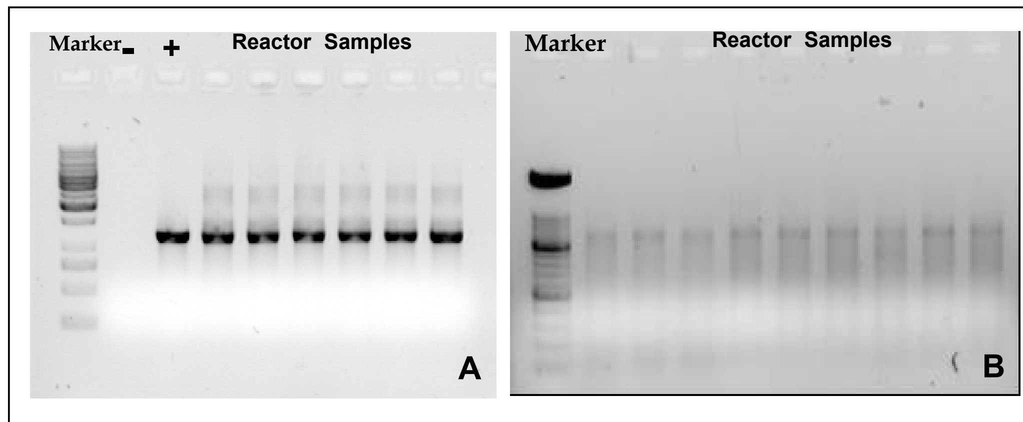

Figure 3.4: Differences in 16S PCR results using diluted (A) and undiluted (B) gDNA as template.

Changes in the primer annealing temperature showed little effect on the results and it was found that an annealing temperature of 58°C delivered the best results. This temperature is 4°C lower than the Tm of reverse primer 16R1522pH, the highest of the primer pair and thus corresponded well to theory regarding annealing temperatures and annealing (Sambrook and Russell 2001).

In addition to extra P:C:I extraction procedures, the dilution of gDNA used as template during PCR reactions also greatly improved results. Not only are the high concentrations of gDNA, which can be inhibitory to the PCR reaction (Sambrook and Russell 2001), reduced but so the concentrations of inhibitory chemicals. The most important disadvantage to take into consideration, however, is that the dilutions of gDNA might also lead to under representative results. The gDNA of bacterial members less dominant within

the community could be diluted so much that it is no longer detectable during the 16S PCR amplification, thus leading to a misrepresentation of these bacteria. The 16S PCR reaction is therefore biased towards gDNA present at high concentrations. Issues of biases associated with profiling microbial communities using t-RFLP have been investigated in some previous studies (Frey *et al.* 2006). Because PCR is such a sensitive reaction though, it was assumed for the purposes of this study that even highly diluted copies of gDNA from less dominant bacterial members would still be detected. The true effects of using diluted gDNA as template deserves further investigation to determine the impact it has on the t-RFLP results.

3.3.3 16S ribosomal DNA purification

Purification of the PCR reaction itself delivered much better results and higher yields of purified 16S rDNA than purification from excised gel fragments. A high concentration of 16S rDNA was required for subsequent restriction enzyme digestions and PAGE analysis. The QIAquick PCR purification kit was preferred as the means of purification. The concentration of purified DNA was usually estimated at 200 - 500 ng/µl.

After successful amplification, it is important to retain most of the 16S rDNA for further enzyme digestions and PAGE. This would help to ensure a more representative result which will include the less dominant bacterial members within the community as well. Although many methods for nucleic acid purification exist, the use of a commercially available purification kit delivered the best results. The QIAquick PCR purification kit also delivered the highest yield of purified 16S rDNA.

3.3.4 Restriction enzyme digestions

The success of the digestion reactions was evaluated by the PAGE results obtained. A variety of differently sized t-RF bands suggested a successful and complete restriction enzyme digestion of the 16S rDNA. Initial results often delivered t-RF bands with a too low intensity. Increasing the purified 16S rDNA concentrations form 300 ng to approximately 1 μg per digestion reaction greatly improved t-RF band intensities on the PAGE gel as is demonstrated by Figure 3.5. Digestion of the amplified 16S rDNA fragments by restriction enzymes is an important step during t-RFLP analysis. Complete digestion of 16S fragments is crucial to result in a truly representative t-RF profile of the community. To achieve this, the ratio between restriction enzyme and the amount of DNA to be digested should be optimized. One unit of the enzymes used in this study was defined as the amount of enzyme required to completely digest 1 μg of DNA within 1 hour at the specified temperature. Thus, by digesting approximately 1 μg of purified 16S rDNA with one unit of restriction enzyme, delivered the best results. The effects of these adjustments to the t-RFLP protocol were well reflected by the results obtained by PAGE.

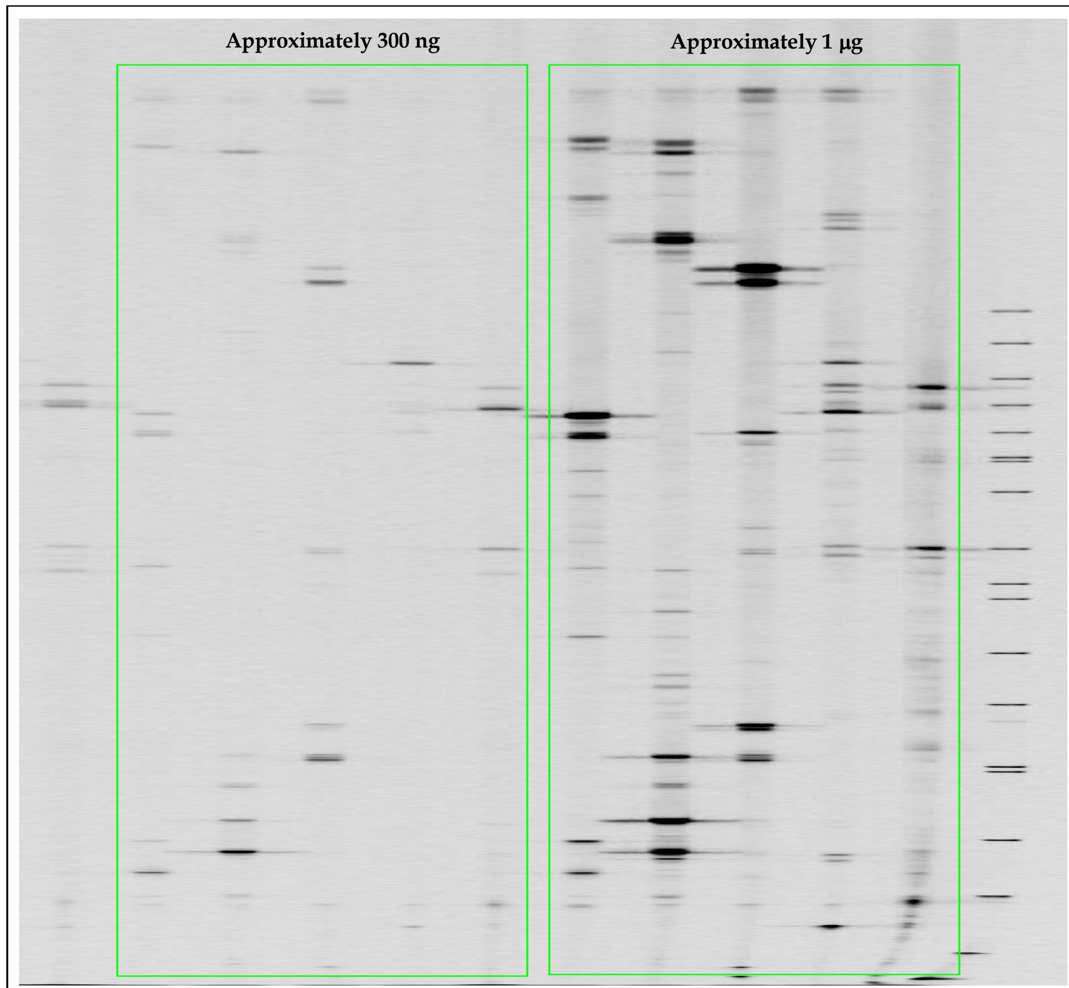

Figure 3.5: Increase in t-RF band intensity by using approx. 1 µg purified 16S rDNA per digestion compared to 300ng.

3.3.5 Polyacrylamide gel electrophoresis (PAGE)

Results obtained using the existing AFLP protocol were satisfactory. Good separation of t-RF bands was observed using the initial machine settings and t-RF bands appeared sharp and bright.

3.3.6 PAGE gel image analysis, further data analysis and t-RF fragment assignment

The Bionumerics® computer software used to analyze t-RFLP gel images in this study proved to be a very useful tool. Many of the features provided by

Bionumerics proved to be crucial during the analysis procedure. Skew PAGE gel images were easily corrected to allow for proper comparisons between t-RF profiles from different bioreactor samples. Sizing of the t-RF bands also proved to be accurate as control samples delivered the expected t-RF band sizes. Figure 3.6 shows such a control sample and the expected band pattern.

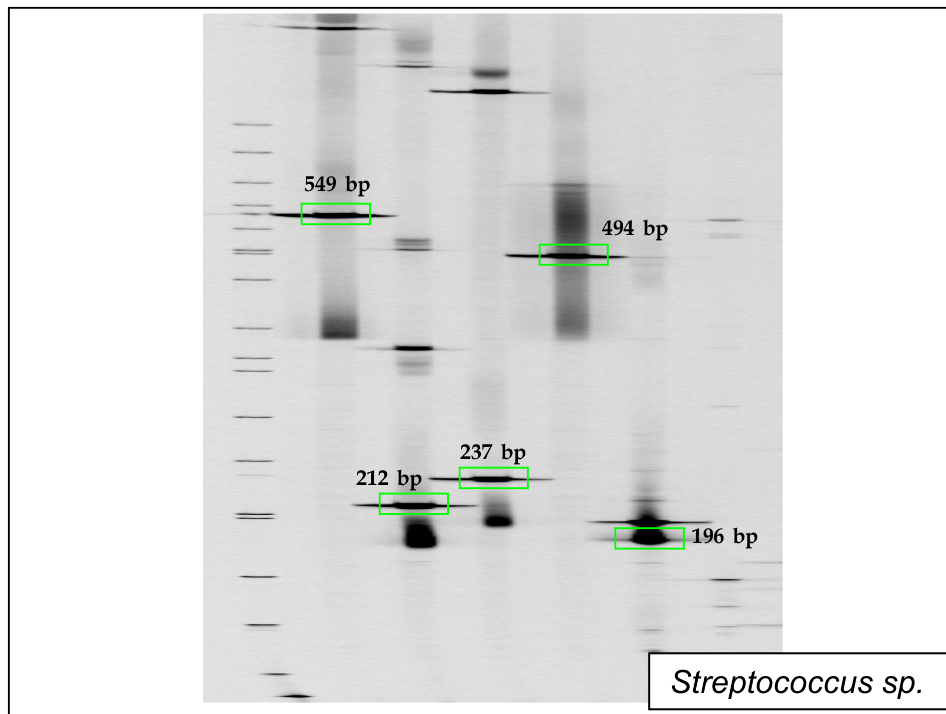

Figure 3.6: t-RF band pattern for a Streptococcus control.

The use of the additional two restriction enzymes clearly improved the analysis of t-RFLP results obtained. Engebretson *et al.* (2003) showed in their study that *HaeIII* and *Sau96I* generated a highly diverse set of t-RF's when tested against a mixed bacterial population. The additional t-RF band size data generated by the additional restriction enzymes eased the assignment of possible bacterial members to corresponding t-RF patterns.

The Phylogenetic Assignment Tool (PAT-tool) provided by the Wisconsin University appeared to be a far much more useful tool than the TAP-t-RFLP analysis tool provided by the Michigan State University. The TAP-t-RFLP tool

delivered impractical large amounts of data that needed processing. Possible combinations of restriction to identify bacterial members had to be performed manually. This proved an extremely difficult, if not impossible, task as very long lists of possible bacterial members for each t-RF band size were obtained and the data provided could also not be easily edited.

The PAT analysis tool, on the other hand, proved much more useful when processing the t-RFLP data generated. The most useful feature of this tool was the t-RF band size input utility. The software delivered more accurate and compact results, which were easier to handle as it automatically combined all size data provided by the user. Data could also easily be exported to other Microsoft application software such as Notepad and Excel.

Even if good, clear t-RF profiles are obtained, the most complex part of the t-RFLP analysis is the processing and interpretation of these results. The amount of data delivered by t-RFLP is in many cases overwhelming. Because the 16S databases contain thousands of sequences, t-RFLP data can consist of hundreds of differently sized t-RF fragments in various combinations representing possible bacterial members. In this particular experiment, online tools such as the PAT tool helped to resolve this problem. The computer software automatically combines and sorts all data, which would be an extremely laborious task to perform manually.

Like a natural habitat, very little is known about the bacterial members present within the reactor, even less about the members within the feed to the reactor (e.g. grass cuttings). It is thus difficult to predict how a t-RFLP profile of such an unknown community should look like and which bacterial members to expect.

Another problem is that often background fluorescence may complicate correct t-RF band identifications.

This makes the decision of which bands to include in the analysis and which to discard difficult. Because the t-RF band data generated from each restriction is combined, excluding some bands from the analysis could lead to a loss in diversity and representation of possible bacterial members.

It is known that the same bacterial species can contain more than one copy of the 16S rDNA gene in its genome. Different copies of the 16S gene are also not completely similar in nucleotide sequence, which has profound effects on the t-RF profile. Sequence differences between different 16S gene copies would result in more than the expected single t-RF band pattern for a particular restriction enzyme as is clearly demonstrated in Figure 3.6. Even when a pure culture of *Streptococcus* sp. was used in the analysis and a single t-RF fragment was expected for each restriction enzyme used, multiple 16S copy numbers resulted in more than one t-RF fragment to be observed. Band patterns created as a result of multiple 16S copy numbers could lead to the misrepresentation of bacterial members, which actually do not exist within the microbial community.

3.4 CONCLUSIONS:

There are many important factors to consider when setting up a t-RFLP analysis system – all of which can greatly influence the outcome of results. Every step during the process, from sampling the environment studied to the final analysis of results, should be considered crucial.

Chemical impurities originating from the bioreactor itself proved to be a great obstacle during this study. Therefore, when studying sulphate removing bioreactors or any other environments with high levels of impurities:

- Care should be taken when extracting genomic DNA from reactor samples. High yields of fairly pure gDNA are important to obtain good 16S rDNA amplification during PCR and well-represented t-RFLP results of the microbial community studied.

- Selecting a PCR primer pair which matches a high number of 16S sequences from many different bacteria in the database used is important as it will aid in profiling the microbial community studied.

- Diluting gDNA to be used as template during 16S PCR seemed to improve amplification during this particular experiment but the effects on reflecting the true community diversity should be considered as dilution of the gDNA could lead to the exclusion of bacterial members in low numbers within the community.

- Purification of the amplified 16S rDNA should allow a high recovery rate and the use of a specially designed kit for this step proved helpful during this experiment. Complete digestion of purified 16S rDNA is important in generating a well-represented t-RF profile of the community studied.

- Separation of different t-RF fragments should be thorough and during this experiment PAGE proved effective in achieving this. Gel images generated by PAGE would also allow for easy comparison between different samples and for investigating the dynamics within a specific community studied.

- Gel image analyses of t-RFLP profiles are complex but computer software designed for these purposes such as the Bionumerics® software proved to be very helpful.

- Analysis of t-RFLP data can be very difficult but web-based software currently available and specifically designed for t-RFLP data analysis proved to be of great help.

Despite many challenges setting up and optimizing, t-RFLP still provide a powerful alternative to conventional culturing methods.

CHAPTER 4

THE EFFECTS OF DAILY ADDITION OF GRASS CUTTINGS ON VOLATILE FATTY ACID PRODUCTION, SULPHATE REMOVAL AND THE MICORBIAL COMMUNITIES INVOLVED

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4.1. INTRODUCTION:

Sulphate reducing bacteria (SRB) can utilize a range of different organic compounds as a source of carbon and energy. Volatile fatty acids such as acetate, propionate and butyrate are the main carbon sources for SRB while other short-chain fatty acids (lactate, pyruvate, malate) and fermentative end products such as methanol or ethanol can also function as a suitable energy source. (Zagury *et al.* 2006). Polysaccharides such as cellulose and hemicellulose are not known to be degraded by any known SRB. Sulphate reducers thus have to rely on hydrolytic fermentative organisms to degrade the polysaccharides to fatty acids or alcohols, which would then support sulphidogenesis (Johnson and Hallberg, 2005).

Studies have been done on the use of biowaste substances as an energy source for sulphate reduction by SRB. Organic substrates such as oak leaves, poultry manure, sheep and cow manure, hay and municipal compost have proven to all support SRB metabolism (Waybrant *et al.* 1998; Cocos *et al.* 2002; Amos *et al.* 2003). The biodegradability of the organic substrate plays a major role and might drastically influence the production of fatty acids and alcohols for SRB use. In a recent study done by Gilbert *et al.* (2004) the authors concluded that lower lignin content, results in higher degradability of the substrate.

Most of the previous studies on the use of biowaste products only focused on the substrate composition and utilization and the effects thereof on the sulphate removal from a metabolic viewpoint. Very few studies identified the

microorganisms involved in the fermentation and sulphate removal processes as well as the dynamics of the microbial community present in these reactors. The aim of this study was to investigate what effect the addition of grass cuttings on a daily basis to the bioreactor would have on the microbial community structure and dynamics.

4.2. MATERIALS AND METHODS:

4.2.1. Bioreactor specifications

Two anaerobic bioreactors (R10 and R100) were operated simultaneously, in batch mode, for a period of 24 days. Each bioreactor received 15 ml compost bacteria (CB), 300 ml sulphate reducing bacteria (SRB), 5 ml hydroponic nutrients and 2000 mg/L $MgSO_4 \cdot 7H_2O$. The CB used was obtained from the University of Stellenbosch and consisted of a consortium of cellulolytic bacteria isolated from compost by selective culturing methods. The SRB bacteria used was isolated from sulphate rich wastewaters originating from a coal mine in Witbank, South Africa. The volume of each bioreactor was 2 litres. Additional sulphate solution at the above-mentioned concentration was added whenever the sulphate level in any of the two bioreactors dropped below 500 mg/L during operation. Bioreactor R100 received 100 g of fresh grass cuttings once off while bioreactor R10 received 10 g of fresh grass cuttings on a daily basis.

4.2.2. Determination of pH, COD, VFA and sulphate removal levels

The pH, chemical oxygen demand (COD) and sulphate levels were determined by the CSIR for each bioreactor. All procedures were carried out on filtered samples and according to procedures as described by Standard

Methods (APHA, *1985*). Samples for COD testing were pre-treated to eliminate the sulphide contribution to the COD concentrations.

Volatile fatty acid (VFA) levels were determined using a gas chromatograph (Hewlett Packard. HP 5890 Series II) equipped with a flame ionisation detector (GC/FID), while the data analyses were done using the Chem. Station software package, supplied by Hewlett Packard. The column used was a HP-FFAP, 15 m x 0.530 nm, 1 micron. An outline of the GC/FID programme used is depicted in Table 4.1. The N_2 flow rate was set at 1 ml/min. The specific volatile fatty acids measured during this experiment were acetate, butyrate and propionate.

Table 4.1: The GC/FID specifications for the detection of volatile fatty acids

Parameter:	***Setting:***
Initial oven temperature (°C)	30
Initial time (min)	2
Temperature programme (°C)	80
Rate (°C/min)	25
Final temperature (°C)	200
Final time (min)	1
FID temperature (°C)	240

4.2.3. Molecular analysis of microbial communities

Terminal restriction fragment length polymorphism (t-RFLP) was used to determine the microbial community composition and investigate the dynamics of the community. Samples were collected from both bioreactors R10 and R100 on day 1 and day 24 of operation and were not filtered. A sample of the initial cellulolytic compost bacteria added to the reactor was also collected for t-RFLP analysis. All samples were collected in sterile containers and were kept at a storage temperature of 4°C.

4.2.4. Total genomic DNA extraction

The total genomic DNA was extracted from the samples obtained for bioreactors R10 and R100. Genomic DNA was extracted from each sample by means of an adapted CTAB method (*personal communication, Mrs. E. Eloff, CSIR*). One and a half milliliters of biomass was centrifuged at 6440 x g to obtain a pellet. The biomass pellet was re-suspended in 565 µl of TE buffer (pH 8.0) and 5 µl lysozyme (50 mg/ml) was added. The suspension was incubated at 37°C for 30 minutes. Thirty µl 10% SDS and 5 µl Proteinase K (20 mg/ml) were added; the suspension was mixed and incubated at 45°C for 1 hour. After incubation 100 µl 5M NaCl was added. The suspension was mixed thoroughly after which 80 µl CTAB/NaCl solution was added. The suspension was mixed and incubated at 65°C for 25 minutes. After incubation an extraction was performed using an equal volume chloroform/isoamyl alcohol. The suspension was centrifuged at 6440 x g for 5 minutes to separate the different phases after which the top aqueous phase was transferred to a new Eppendorf tube. Precipitation of the genomic DNA was achieved by adding isopropanol at 0.6 of the volume to the aqueous phase followed by centrifugation for 20 minutes at 6440 x g.

The DNA pellet obtained was washed by adding 200 µl 70% EtOH and centrifugation at 6440 x g for 5 minutes. The supernatant was removed, the DNA pellet air-dried and re-suspended in 50 µl ddH_2O.

The concentration of the extracted DNA was estimated by loading 5 µl suspended DNA on a 1% agarose gel. One micro liter molecular weight DNA marker (250 µg/ml, Roche Diagnostics) was also loaded onto the agarose gel. Electrophoresis was performed at 100 V and 100 mA for 30 minutes to separated the DNA fragments. The DNA concentration (ng/µl) was estimated

by comparing the brightness of the molecular weight DNA marker bands to the DNA band obtained form the extraction.

4.2.5. 16S ribosomal gene amplification

Amplification of the 16S rDNA (rRNA) gene was performed using the polymerase chain reaction (PCR). Specifically designed eubacterial universal primers were used and Table 4.2 shows the forward and reverse primer sequences.

Table 4.2: Nucleotide sequences for forward primer 341F and reverse primer 16R1522pH

Primer name:	***Sequence:***
341F	5' CCT ACG GGA GGC AGC AG 3'
16R1522pH	5' ACG CCG ACC TAG TGG AGG A 3'

PCR reactions contained 5 µl reaction buffer (10x), dNTP's (5 mM each), $MgCl_2$ (25 mM), both forward and reverse primers (50 µM) and *Taq* DNA polymerase (1 unit). Two µl extracted genomic DNA from the bioreactors was used as template for the amplification and the final reaction volume was 50 µl. Amplification was performed in a 2700 GeneAmp thermocycler (Applied Biosciences).

A hot-start PCR was performed at 94°C for 3 minutes followed by 30 cycles of denaturing at 94°C for 30 seconds, primer annealing at 58°C for 30 seconds and extension at 72°C for 30 seconds. A final extension step was done at 72°C for 4 minutes. PCR products were stored at 4°C.

4.2.6. Amplified 16S rDNA fragment purification

The amplified 16S rDNA fragments were purified from the PCR reagents using the QIAquick PCR Purification Kit (Qiagen). The manufacturer's

instructions were followed during the purification process. A final volume of 50 µl purified 16S rDNA was retained.

4.2.7. Restriction endonuclease digestions

Five different restriction endonucleases were selected to digest the purified 16S rDNA. The 5 different restriction endonucleases were *RsaI*, *MspI*, *HhaI*, *HaeIII* and *Sau96I*. All five restriction endonucleases are four base-pair cutters. Restriction endonuclease reactions contained 1x reaction buffer, 200-300 ng purified 16S rDNA and 2-3 units of the specific restriction endonuclease. The total reaction volume was 20 µl and the digestions were performed at 37°C for a period of 2 hours. Digestion reactions were not terminated and were only placed and hold on ice until loading on the LI COR DNA sequencing system.

4.2.8. Polyacrylamide gel electrophoresis (PAGE)

After digestion of the 16S rDNA by the selected restriction endonucleases, the differently sized terminal restriction fragments (t-RF's) were separated using PAGE and the IR^2 Global Edition DNA Analyzer (LI COR, Lincoln, U.S.A). An 8% polyacrylamide gel was prepared using 20 ml gel solution (Long Range Gel), 150 µl APS (0.1g/ml) and 15 µl TEMED. The gel solution was poured between two special glass plates, cleaned thoroughly with a 1% SDS solution, and allowed to set for 1 hour. The solidified gel, together with the glass plates, was mounted in a vertical position in the LI COR sequencer. The buffer tanks were filled with 0.8 X TBE buffer, obtained by diluting 160 ml 10 X TBE buffer (108 g Tris-base, 55 g ortho-boric acid, 7.44 g EDTA, 1 L ddH_2O, pH 8.0) with 1840 ml ddH_2O. The gel was subjected to a 30-minute pre-run with the following settings. Set voltage: 1500 V; set current: 35 mA; set power: 35 W; set temperature 45°C. Following the pre-run, 0.8 – 1.5 µl of the digested 16S rDNA was loaded in triplicate onto the gel together with an internal size

standard (700bp standard). Prior to loading the digested 16S rDNA was denatured at 90°C for 3 minutes after which it were kept on ice until loaded. Electrophoresis was performed at the mentioned settings for a period of 4 hours.

4.2.9. PAGE gel image analysis

Bionumerics® computer software was used to analyze the generated PAGE image. The identification of t-RF bands was done manually and was mainly based on the visualization of the specific t-RF band. Terminal restriction fragment lengths were determined in the following way: The internal size standard was used as a reference. The known size of each band from the internal size standard was put in as an external reference. Bionumerics® then calculates the sizes of unknown t-RF bands by using the % of migration of each band from the sample and the given size of each band from the internal size standard.

4.2.10. Further data analysis and t-RF fragment assignment

The Ribosomal Database Project II (RDP II), release 8.0, at the Michigan State University in East Lansing, Michigan, contains more than 10 700 small subunit (SSU) rRNA sequences in an aligned form and more than 21 000 sequences in unaligned form (Maidak *et al.*, 2007).

The RDP II also provides data, programs and online analysis services that relate to the ribosome. The probe match analysis tool analyses the occurrence of a specific probes sequence in the data set. This online analysis tool was used to analyse the forward and reverse primer sequences for priming efficiency to the known database. The TAP-t-RFLP online analysis tool was used to perform *in silico* digestions of the data set with the selected restriction

endonucleases. *In silico* digestion results were used to predict t-RF sizes for specific bacterial members and to compose a database for further use.

Another on-line analysis tool, the Phylogenetic Assignment Tool (Wisconsin University), was used to assign bacterial species to specific t-RF bands. T-RF band length values were fed to the program in the form of tab delimited Excel files. The software then uses restriction data from all five enzymes to compute all possible combinations and represented bacterial species for each set of values.

4.3. RESULTS

4.3.1. Chemical oxygen demand (COD)

The COD measurements for bioreactors R10 and R100 differed over the 24-day period of operation. This was expected though, since COD measurement reflects the organic load and, as stated, bioreactor R100 received 100 grams of grass cuttings once at the start of the experiment while bioreactor R10 was fed 10 grams of grass cuttings on a daily basis. Although the COD measurement on day one was similar for both bioreactors, it quickly increased to approximately 8000 mg/L within bioreactor R100 as more of the COD became soluble. The COD levels in bioreactor R100 stabilized within 2 days and for the remainder of the experimental period ranged between approximately 8000-9000 mg/L. The COD levels within bioreactor R10, on the other hand, were initially lower than in bioreactor R100. On day 14 of the experimental period, the mass of grass cuttings in bioreactor R10 totaled approximately 100 g and the COD levels were very similar to that of bioreactor R100. The COD levels in bioreactor R10 further increased and by day 24 of operation reach levels of 16 000 mg/L. Figure 4.1 shows a graphical representation of COD levels measured in R10 and R100.

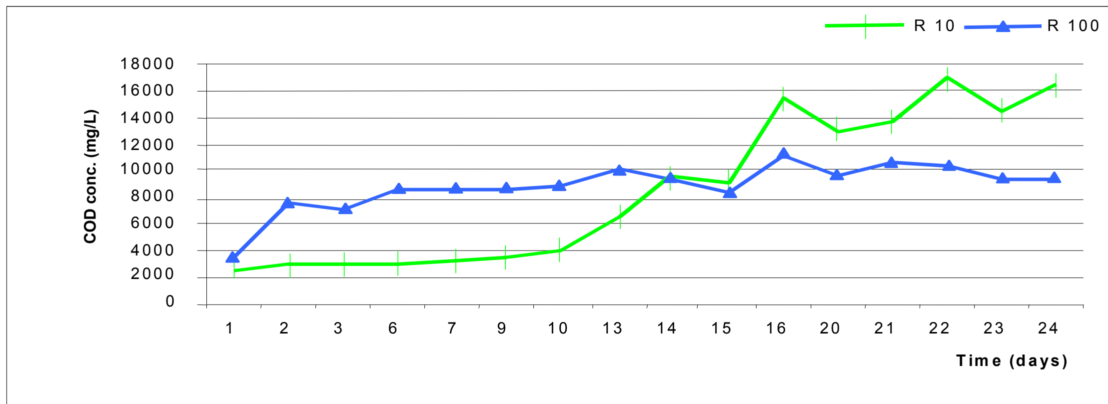

Figure 4.1: COD levels measured for bioreactors R10 and R100.

4.3.2. Volatile fatty acids (VFA)

Results showed significantly different concentrations of acetate in bioreactors R10 and R100. Figures 4.2 and 4.3 shows the acetate concentrations measured for bioreactor R10 and R100 respectively.

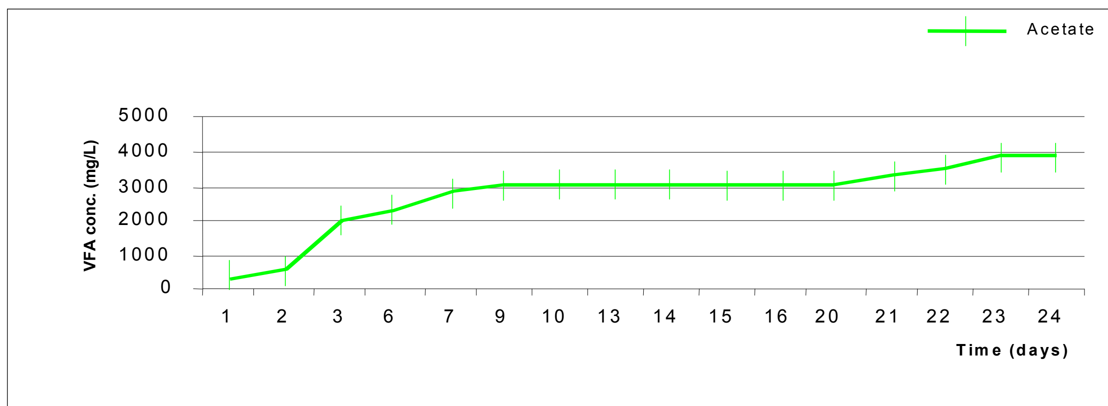

Figure 4.2: Acetate concentration measured in bioreactor R10.

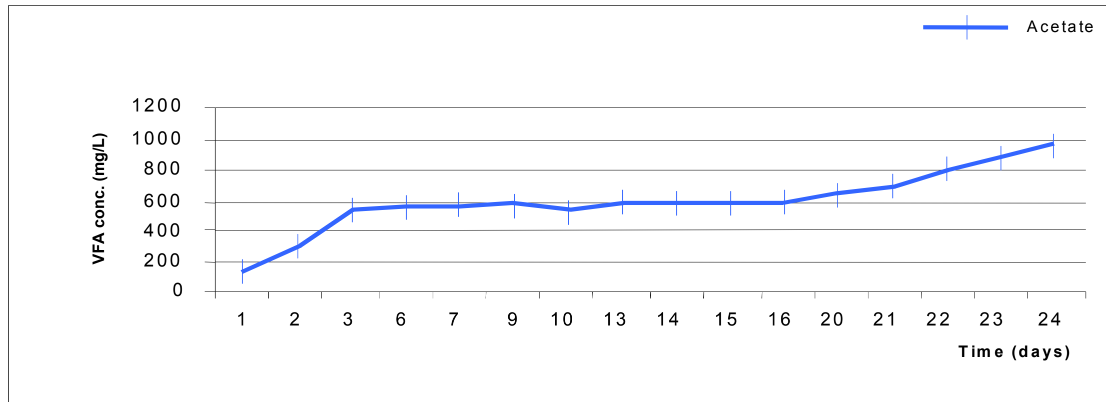

Figure 4.3: Acetate concentration measured in bioreactor R100.

It is clear that the acetate production in bioreactor R10 was much higher than in bioreactor R100 (Figures 4.2 and 4.3). On day 3 of the experimental period, bioreactor R10 already achieved an acetate concentration exceeding 1 000 mg/L. Bioreactor R100 could only achieve an acetate concentration of approximately 1 000 mg/L by day 24 of the experimental period. Bioreactor R10, on the other hand, had an acetate concentration of approximately 4 000 mg/L by day 24.

Unlike acetate, the concentrations of propionate and butyrate produced in bioreactors R10 and R100 were very similar. This can be seen from Figures 4.4 and 4.5.

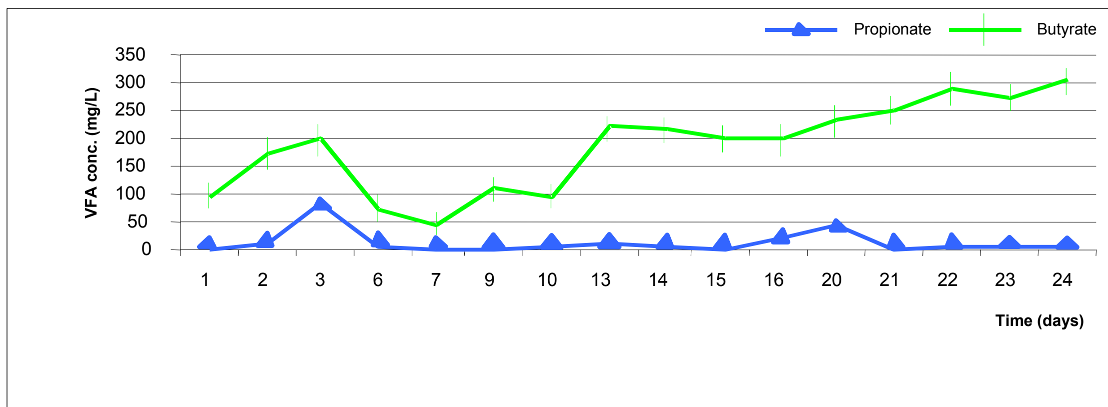

Figure 4.4: Propionate and butyrate concentrations measured in bioreactor R10.

Figure 4.4 indicates that within bioreactor R10 propionate concentrations remained largely below 50 mg/L throughout the experiment while butyrate concentrations increased up to approximately 300 mg/L by day 24.

The same scenario could be seen in bioreactor R100 as Figure 4.5 shows. By day 24, a butyrate concentration of approximately 300 mg/L was measured.

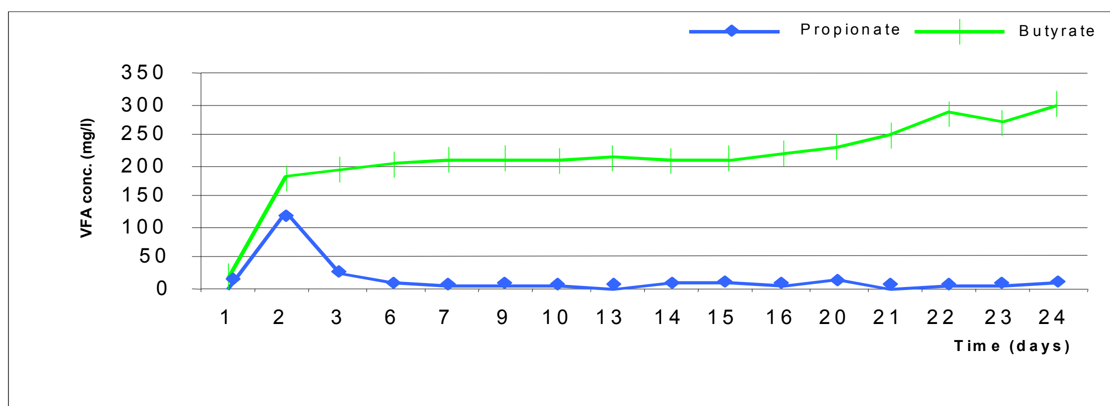

Figure 4.5: Measured propionate and butyrate concentrations within bioreactor R100.

In bioreactor R100 results shows that propionate concentrations largely remained below 50 mg/L. Although butyrate concentrations showed an increase, a sudden decrease was observed between days 3 and 10 of the experimental period.

4.3.3. Sulphate removal

Figure 4.6 shows the results for sulphate removal from both bioreactors R10 and R100. Initially the removal of sulphate in both bioreactors was very similar. Sulphate was reduced from 2 000 mg/L to less than 50 mg/L within a period of 3 days.

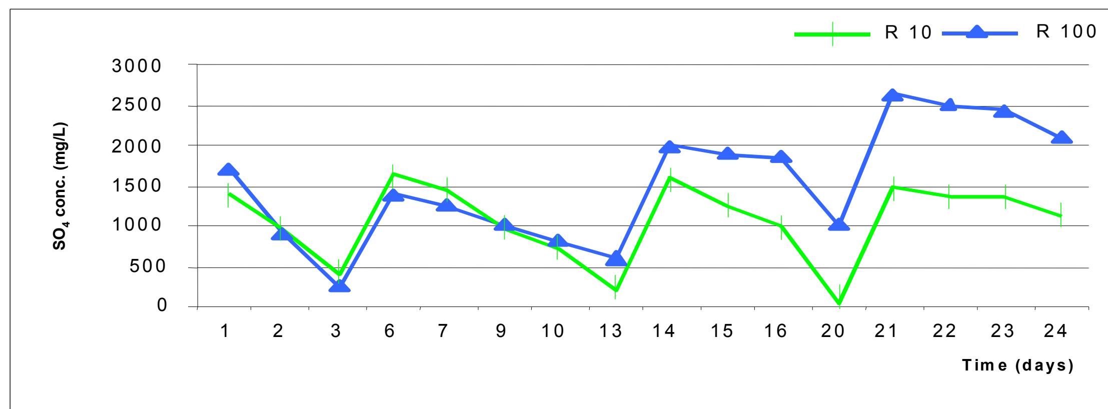

Figure 4.6: Sulphate removal from bioreactors R10 and R100.

Although sulphate reduction continued in both bioreactors, Figure 4.6 also showed that the rate of reduction decreased in both bioreactors. From day 13 sulphate reduction in bioreactor R100 suddenly became less than for bioreactor R10.

4.3.4. Microbial community composition and dynamics

Terminal restriction fragment length polymorphism results indicated significant differences, not only between the communities of bioreactors R10 and R100, but also between the start and end communities within each bioreactor. Results also showed differences and similarities between the starter community and the bioreactor communities. Figure 4.7 shows the t-RF pattern obtained for the cellulolytic starter community.

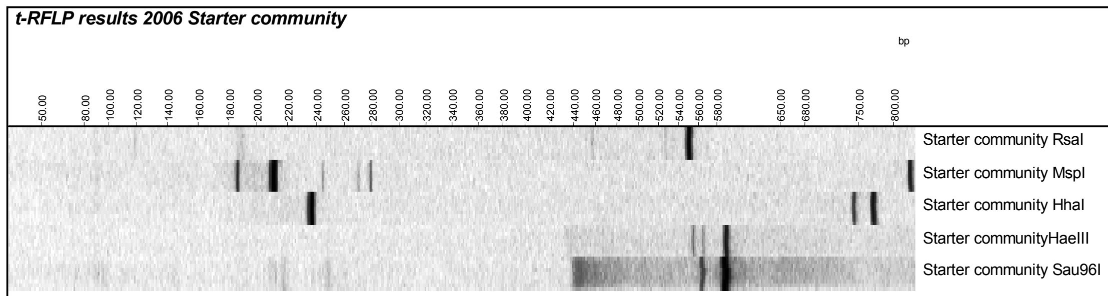

Figure 4.7: t-RF patterns obtained for cellulolytic starter community using five different restriction enzymes.

Analysis of t-RF size combinations for each individual restriction endonuclease used revealed a list of possible bacterial members comprising the starter community. Table 4.3 shows a list of possible bacterial members comprising the cellulolytic starter community.

Table 4.3: Possible bacterial members comprising the cellulolytic starter community.

Cellulolytic starter community:
Bacillus sp.
Brevibacillus sp.
Brevibacterium sp.
Clostridium sp.
Eubacterium sp.
Lactobacillus sp.
Planococcus sp.
Sporolactobacillus sp.
Sporosarcina sp.

Some of these possible bacterial members within the cellulolytic starter community used as initial inoculum remained stable within bioreactors R10 and R100 throughout the experimental period and were still detected on day 24 of bioreactor operation. Others were only detected on day 1 of operation within bioreactors R10 and R100, while some of the original members within the cellulolytic community were not detected in the one or both of the bioreactors at all.

Figure 4.8 shows a PAGE gel image of the differences in t-RF patterns obtained for the cellulolytic starter community and the bioreactor R10

community on day 1 and day 24 of operation while Figure 4.9 shows similarities in t-RF patterns for the cellulolytic community used as inoculum and the bioreactor communities.

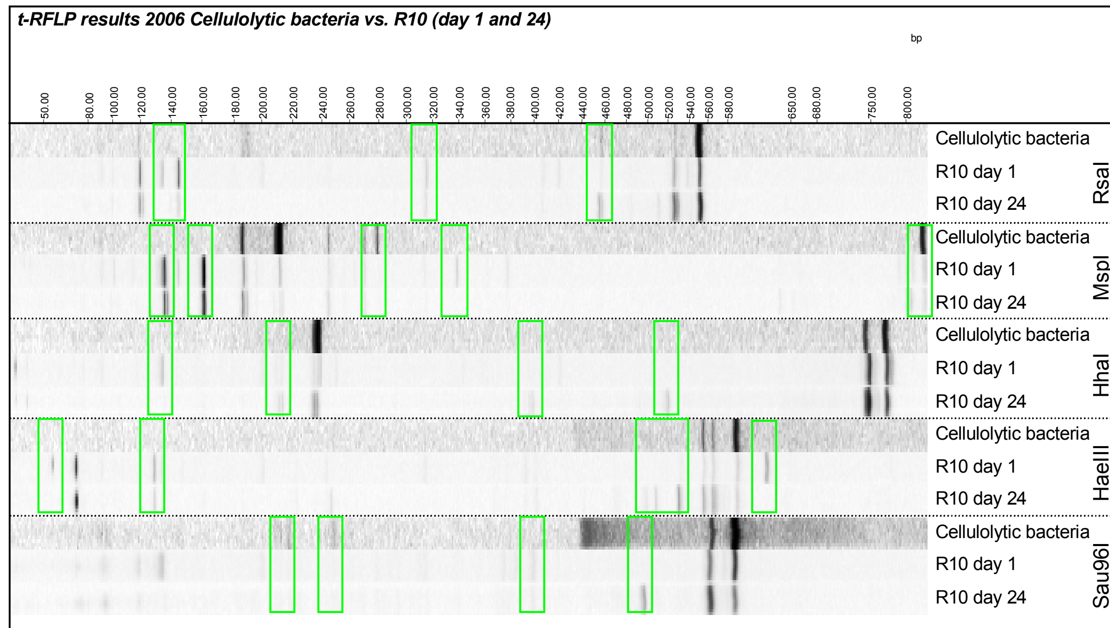

Figure 4.8: Differences in t-RF patterns for the cellulolytic starter community and the bioreactor communities.

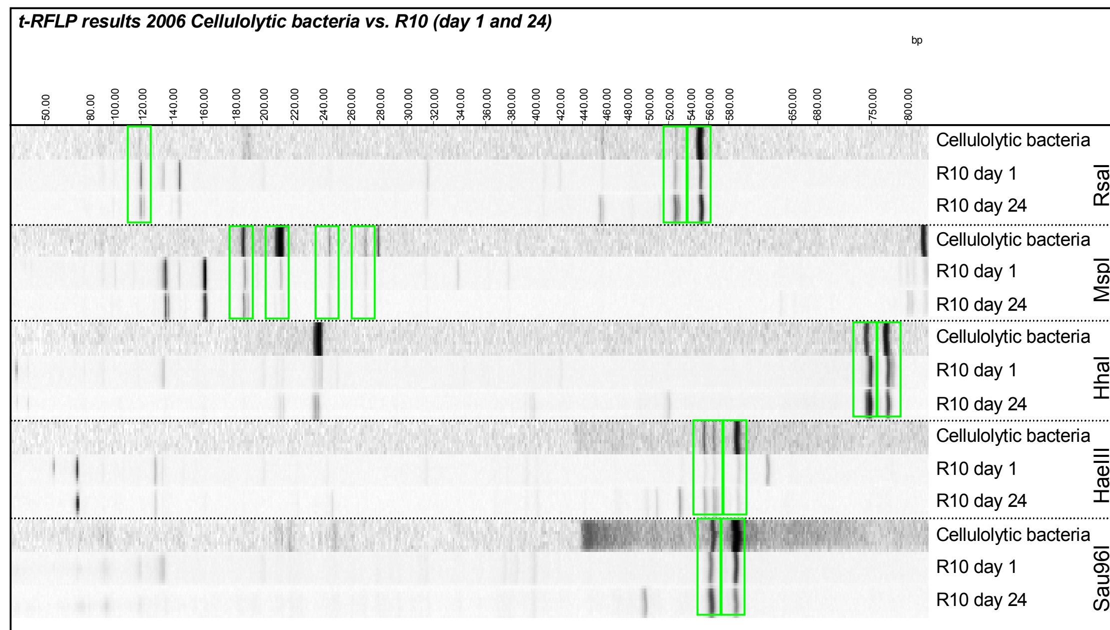

Figure 4.9: Similarities in the t-RF patterns for the cellulolytic starter community and the bioreactor communities.

Figure 4.10 shows a diagrammatical representation of the microbial community dynamics within bioreactor R10 based on the possible data for day 1 and day 24. Some of the bacterial members from the cellulolytic starter community remained stable throughout the experimental time period and were still detected in samples taken after 24 days of operation. This might be attributed to the continuous addition of fresh grass cuttings to the reactor. Green shaded blocks in the flowchart indicate these members. Members indicated by light blue shaded blocks originated from the cellulolytic starter community, but were only detected during day 24 of bioreactor operation and not during day 1. The possible *Eubacterium sp.* originating from the starter community was not detected within the bioreactor R10 at all. The *Nitrococcus sp.* was detected within the original bioreactor community but seemed to have disappeared and was not detected on day 24 of operation. Bacterial members indicated by light purple shaded blocks appeared stable within the bioreactor communities from day 1 to day 24 and did not originate from the cellulolytic starter community while possible members indicated by the pink shaded blocks only appeared on day 24 of operation.

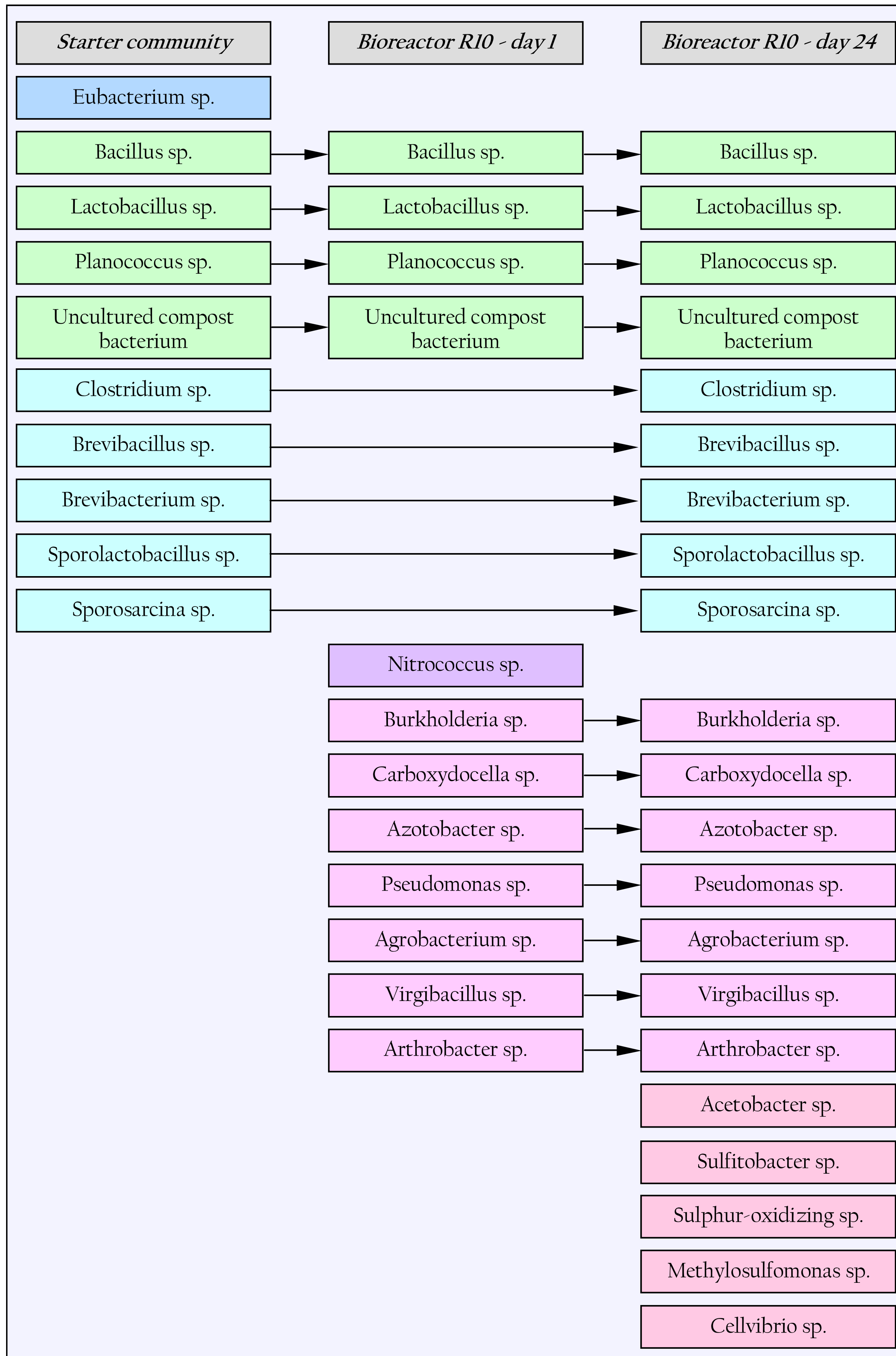

Figure 4.10: Microbial community dynamics within bioreactor R10.

Poly-acrylamide gel electrophoresis images indicating differences and similarities within the t-RF patterns for bioreactor R100 on day 1 and day 24 are shown by Figures 4.11 and 4.12 respectively.

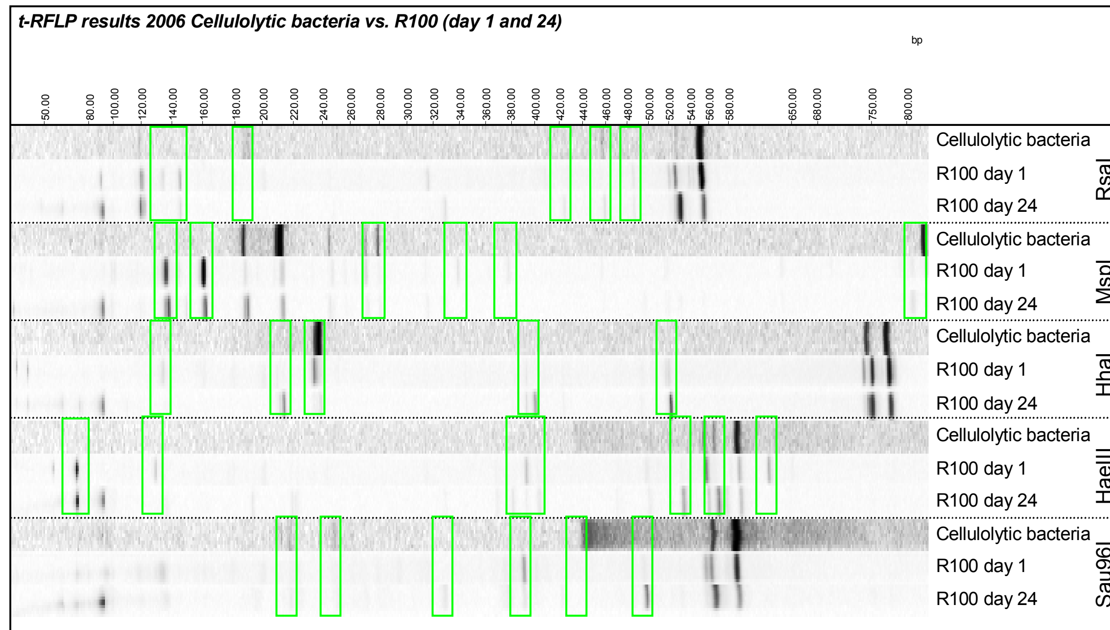

Figure 4.11: Differences in the t-RF patterns for the cellulolytic starter community and the bioreactor communities.

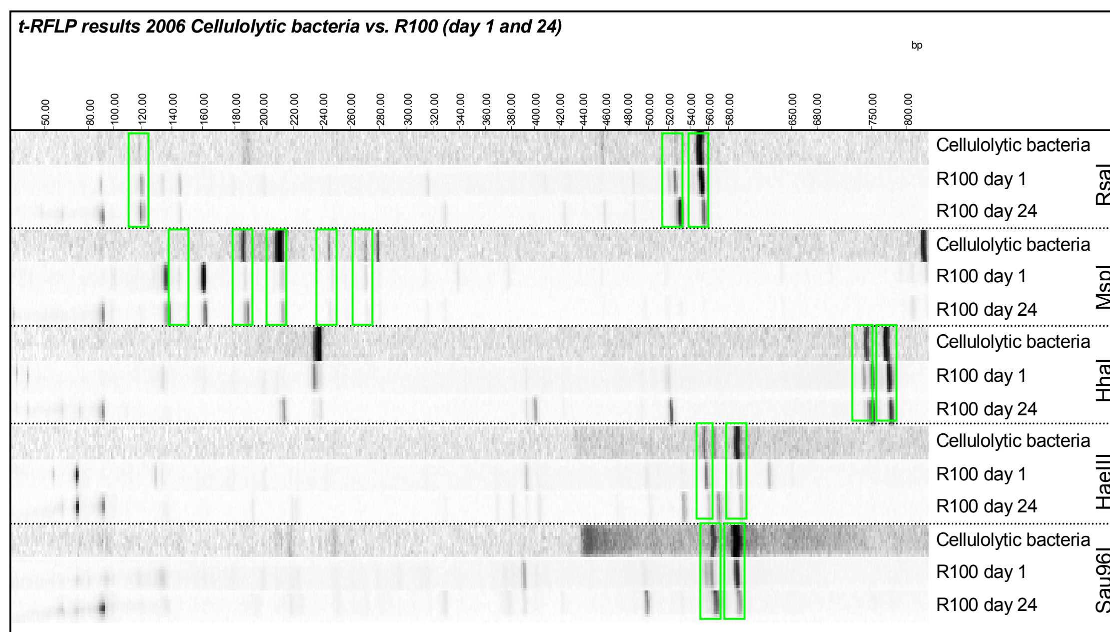

Figure 4.12: Similarities in the t-RF patterns for the cellulolytic starter community and the bioreactor communities.

The microbial community dynamics within bioreactor R100 are represented in Figure 4.13. Once again green shaded blocks indicate possible stable bacterial members originating from the cellulolytic starter community. The *Brevibacterium* and *Clostridium spp*. originated from the cellulolytic starter community, but were not detected within bioreactor R100. The *Planococcus* and *Eubacterium* spp., indicated by the yellow shaded blocks, were only detected during day 1 of operation while the *Brevibacillus sp*. was only detected on day 24 of operation. The *Pseudomonas sp*. originating from the bioreactor community remained stable while the *Caryophanon* and *Cellvibrio spp*. was not detected any more on day 24. The *Sulfitobacter* and *Desulfotomaculum spp*. appeared on day 24.

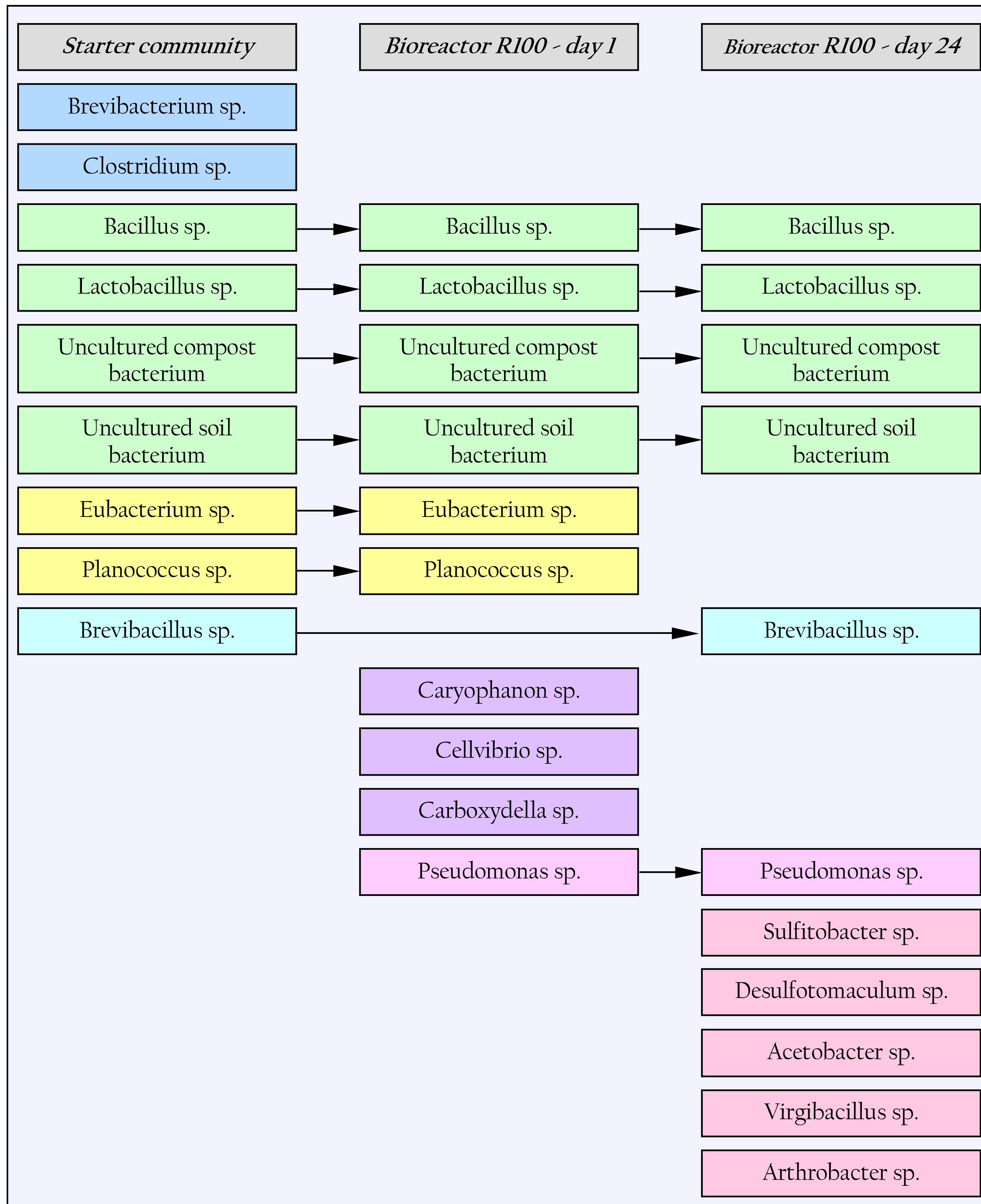

Figure 4.13: Microbial community dynamics within bioreactor R100.

4.4. DISCUSSION

As expected, bioreactors R10 and R100 behaved chemically quite differently. While COD levels remained fairly constant within reactor R100 for most of the time, an increase in COD levels over the 24 day period was observed within reactor R10. These observations were due to the constant adding of grass cuttings to reactor R10. Chemical oxygen demand is an indication or measurement of organic load and the increasing COD levels within reactor R10 thus correlates well to the increase of soluble organics due to the daily addition of grass cuttings. Both reactors displayed a short lag phase before COD levels gradually increased. This could be due to the fact that only soluble COD was measured. Volatile fatty acid production in both reactors differed. Although propionate and butyrate levels were similar in both reactors throughout the time period, acetate levels within reactor R10 were approximately four times higher by day 24 than within reactor R100. This might indicate the continued bacterial degradation of the grass cuttings while t-RFLP analysis also indicated the presence of acetate producing bacteria as dominant members of the community within bioreactor R10. Sulphate removal in reactor R10 remained fairly stable while the removal seemed to have decreased halfway through the experimental period within reactor R100. A possible explanation for this observation might be a build-up of toxic compounds within the reactor or a depletion in the nutrients required for sulphate reduction. On day 20, only about half of the added sulphate was removed within bioreactor R100.

These chemical differences and similarities observed between the two reactors show a possible correlation with the microbiological similarities and differences observed within each reactor. Terminal restriction fragment profiles can be seen as a fingerprint of each microbial community. With each t-RF band representing a possible bacterial member of the community, many

bands thus reflect high bacterial diversity. This can be easily demonstrated by comparing the t-RF profile obtained for the starter community to the t-RF profile obtained for the bioreactor communities. The bioreactor communities comprised of a variety of microorganisms occurring naturally on the grass cuttings as well as accompanying soil, thus a t-RF profile with many bands. The starter community, on the other hand, comprised of fewer members selected by specific culturing procedures and resulted in a t-RF profile with fewer bands.

By comparing the t-RF profiles of the bioreactors another difference was revealed. Although the profiles for the two reactors looked very similar, differences did occur. Analysis of the t-RF size combinations for each restriction endonuclease also revealed a higher diversity within reactor R10 after the 24 day time period than in reactor R100. Most probably the daily addition of fresh grass cuttings to reactor R10 attributed to this higher diversity. The extent of grass degradation within bioreactor R10 might also have remained similar throughout the experimental period resulting in the required nutrients for sulphate reduction to continue at a steady rate. The once off volume of grass cuttings to bioreactor R100 was probably detrimental to the development and stabilization of the microbial community resulting in a limited ability of the community to degrade refractory parts of the grass cuttings. By adding grass cuttings daily to the reactor one of or a combination of the following could have contributed to the higher diversity:

→ the numbers of certain bacteria occurring on the grass cuttings could have increased substantially over the 24 day time period so that it could be detected within reactor R10 but not within reactor R100.

→ the addition of fresh grass cuttings may have helped to maintain the necessary nutrient levels within reactor R10, thus supporting a larger

variety of microorganisms than in reactor R100 where a depletion of certain nutrients available carbon sources may have lead to the disappearance of certain members in the community.

→ the higher acetate levels in reactor R10 might thus have supported the bacterial members not detected within reactor R100.

By comparing the t-RF profiles, the survival or establishment of the starter community could be monitored within each reactor. The starter community seemed to have established and survived better within reactor R10 than in reactor R100. Most of the possible bacterial members from the starter community could be detected within reactor R10 by day 24 of operation. Only the *Eubacterium sp*. could not be detected in reactor R10. Although the *Eubacterium sp*. was detected within reactor R100 on day 1, it also did not survive and could not be detected anymore on day 24. The *Brevibacterium* and *Clostridium spp*. did not survive within reactor R100, while these members established well within reactor R10 and were still detected within the reactor by day 24. The *Bacillus*, *Lactobacillus* and uncultured compost species seemed to have survived quite well in both reactors R10 and R100 and could be detected by day 24 of operation in both reactors.

These results thus suggest that bacterial starter communities can survive well and out compete natural bacteria entering the bioreactor to establish as a community. The survival of the starter community might be linked though to the availability of nutrients as these results also suggested. In bioreactor R100 where grass cuttings was not added on a daily basis the survival of the starter community was poor compared to the survival in bioreactor R10 which received grass cuttings (nutrients) on a regular basis. Starter communities could play an important role in bioreactor set up and in achieving the desired community characteristics and reactor functioning.

When comparing the original reactor communities, it appeared as if the community within reactor R10 remained more stable than the community within reactor R100. Similar start and end conditions within bioreactor R10 might explain this observation. Only the possible *Pseudomonas sp*. remained detectable within both reactors R10 and R100 on day 24. Within reactor R100, the possible *Caryophanon*, *Cellvibrio* and *Carboxydella spp*. were lost after 24 days of operation. These members were still present within reactor R10 however. The only possible member lost from the R10 original community seemed to have been the *Nitrococcus sp*. In both reactors possible members also appeared after the 24 day period that were not detected within the original communities. Some of these possible members included *Acetobacter* and *Sulfitobacter spp*. In reactor R10 a possible sulphur-oxidizing species and a *Methylosulfomonas sp*. appeared on day 24 and within reactor R100 a *Desulfotomaculum sp*. The appearance of these possible members might be attributed to the changing conditions within both reactors.

The effects of adding grass cuttings on a daily basis could be demonstrated by analysis of the t-RFLP data. The natural bacteria occurring on the grass seemed to have played a prominent role within the reactor R10 community after 24 days of operation while this was not so much the case within reactor R100. As mentioned, the daily addition of fresh grass cuttings to reactor R10 could have stabilized crucial nutrient levels, created an semi anoxic environment and increased bacterial numbers to such an extend that the additional members identified within this reactor could form a functional component of the community.

It is, however, very important to realize that the results and conclusions drawn from this experiment should serve as guidelines for future research. The identification of dominant bacterial members within each community was merely based on 16S sequence and restriction site data. Similar 16S

ribosomal sequences and restriction sites are shared between a number of different genera and species. By taking the specific environmental factors within each reactor in consideration, only the most probable members were identified. Certain species actually present within the reactors might thus have been overlooked.

The t-RFLP data and results do provide insights into the different community dynamics however. Comparing the t-RF profiles generated could monitor the success of the starter community used in this study and clearly demonstrated the stability and loss of bacterial members within each reactor.

CHAPTER 5

SURVIVAL OF BACTERIAL STARTER COMMUNITIES IN FERMENTATION REACTORS AND THE EFFECT OF NATURAL COMMUNITIES ON SUCCESSION

CHAPTER 5

SURVIVAL OF BACTERIAL STARTER COMMUNITIES IN FERMENTATION REACTORS AND THE EFFECT OF NATURAL COMMUNITIES ON SUCCESSION

5.1. INTRODUCTION:

Sulphate reducing bacteria can utilize the end products of cellulose fermentation, such as acetate and propionate, as sources of energy (Zagury *et al*. 2006). Biological sulphate reduction could thus be driven by a strategy of co-metabolism where cellulolytic bacteria break down cellulose (e.g. from grass cuttings) into suitable metabolites for SRB. Containing and controlling this process of co-metabolism in a reactor set up, could provide a useful alternative to chemical methods in the treatment of sulphate rich wastewaters such as AMD. The microbial breakdown of cellulose and chitin into substrates for dissimilatory sulphate reduction have already been demonstrated (Boyer *et al*. 1986; Beguin *et al*. 1993; Mohanty *et al*. 2000; Johnson and Hallberg 2005), however, the majority of these studies have focused on the efficiency and kinetics of biological sulphate reduction using cellulose rich materials without considering the microbial communities involved. Understanding the structure and dynamics of microbial communities is important for the successful implementation of treatment strategies since particular microbial communities can be responsible for the success or failure of the process (Ayala-del-Rio *et al*. 2004). Studying the succession of natural occurring bacterial communities or communities originating from starter cultures used in a controlled environment, such as a fermentation reactor, is necessary in understanding and predicting the functionality of the reactor. Often, reactors are operated for extended periods of time assuming that the communities involved have remained stable. A previous study on a methanogenic reactor has, however, revealed that microbial communities in a functionally stable reactor could even be very dynamic (Fernandez *et al*. 1999). Few studies have

been performed on the survival and succession of starter communities in cellulose fermenting reactors. The aim of this study was to investigate the dynamics of starter cultures used in the set up of different fermentation reactors using t-RFLP. The effects on microbial succession due to the addition of SRB to the reactor or due to the natural bacterial population occurring on the grass cuttings have been investigated specifically.

5.2. MATERIALS AND METHODS:

5.2.1. Sample collection and bioreactor specifications

Samples were collected from four different fermentation bioreactors used for volatile fatty acid production. Bioreactors SRB and No-SRB differed only in one aspect, being the fact that the starter culture used in bioreactor SRB also contained sulphate-reducing bacteria. The starter culture used in bioreactors R1 and R2 were the same but bioreactor R1 received 70 grams of autoclaved grass instead of the 70 grams non-autoclaved grass received by bioreactor R2. The same compost and sulphate reducing bacteria were used as starter culture as were described in Chapter 4, Section 4.2.1. The specifications for each bioreactor are summarised in Table 5.1. All samples were collected in sterile containers and were kept at a storage temperature of 4°C until analysis.

5.2.2. Determination of pH, COD, VFA and sulphate removal levels

The pH, COD, VFA and sulphate removal levels were determined for bioreactors R1 and R2 only, as the main objective of the SRB and No-SRB bioreactors was only to analyze the microbial community and dynamics within the bioreactors using t-RFLP. The same analytical procedures were followed as described in Chapter 4, section 4.2.2.

Table 5.1: Specifications of the different bioreactors sampled from.

	Bioreactors:			
Specifications:	**SRB**	**No-SRB**	**R1**	**R2**
Carbon source:	Grass cuttings	Grass cuttings	70 g autoclaved grass	70 g non-autoclaved grass
Starter culture:	Compost bacteria and SRB	Compost bacteria	50 ml compost bacteria	50 ml compost bacteria
Bioreactor volume:	10 L reactor	10 L reactor	2 L sterile reactor	2 L sterile reactor
Feed water:	–	–	2 L sterile feed water	2 L sterile feed water
Additional nutrients:	–	–	10 ml sterile nutrients	10 ml sterile nutrients

5.2.3. Molecular analysis of microbial communities

Molecular analysis of the microbial communities in each bioreactor was performed using Terminal Restriction Fragment Length Polymorphism (t-RFLP). The same procedures were followed as described in Chapter 4, sections 4.2.3 to 4.2.10.

5.3. RESULTS:

SRB vs. No-SRB bioreactor – effect of adding SRB on starter culture

5.3.1. Microbial community composition and dynamics

Figures 5.1 shows results of the t-RFLP analysis of the starter cultures used to spike bioreactors SRB and No-SRB. Analysis of the t-RF fragment size data also revealed the possible composition of the starter community used as shown in Tables 5.2.

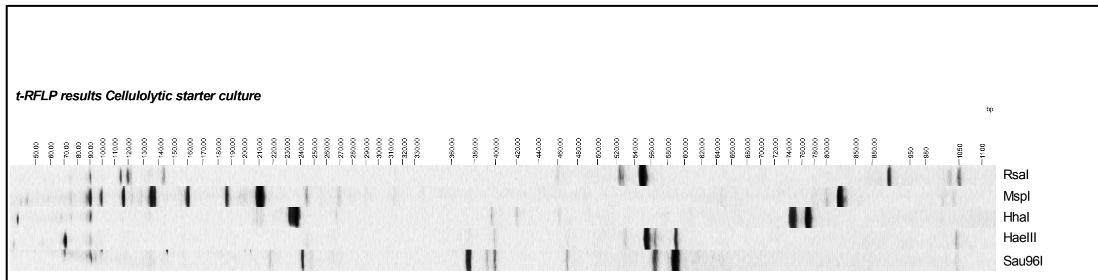

Figure 5.1: t-RFLP patterns obtained for the starter culture used in bioreactors SRB and Non-SRB.

Table 5.2: Possible bacterial members comprising the cellulolytic starter community used in fermentation reactors SRB and Non-SRB.

Cellulolytic starter community:
Acetobacter sp.
Agrobacterium sp.
Bacillus sp.
Carboxydocella sp.
Clostridium sp.
Gluconacetobacter sp.
Lactobacillus sp.
Nitrococcus sp.
Phyllobacterium sp.
Planococcus sp.
Pseudomonas sp.
Rhizobium sp.

Some of the bacterial members within the cellulolytic starter communities remained stable within each of the fermentation bioreactors and could still be detected after 15 days of operation. Other members within the starter communities could not be detected in the bioreactors at all after the experimental period.

Figure 5.2 shows a PAGE gel image of the t-RF patterns obtained for the starter community compared to the communities in both bioreactors SRB and Non-SRB on day 1 of operation. The t-RF pattern obtained for the starter community compared to the communities in bioreactors SRB and Non-SRB on day 15 of operation is shown in Figure 5.3. The differences in the t-RF patterns are indicated by rectangles.

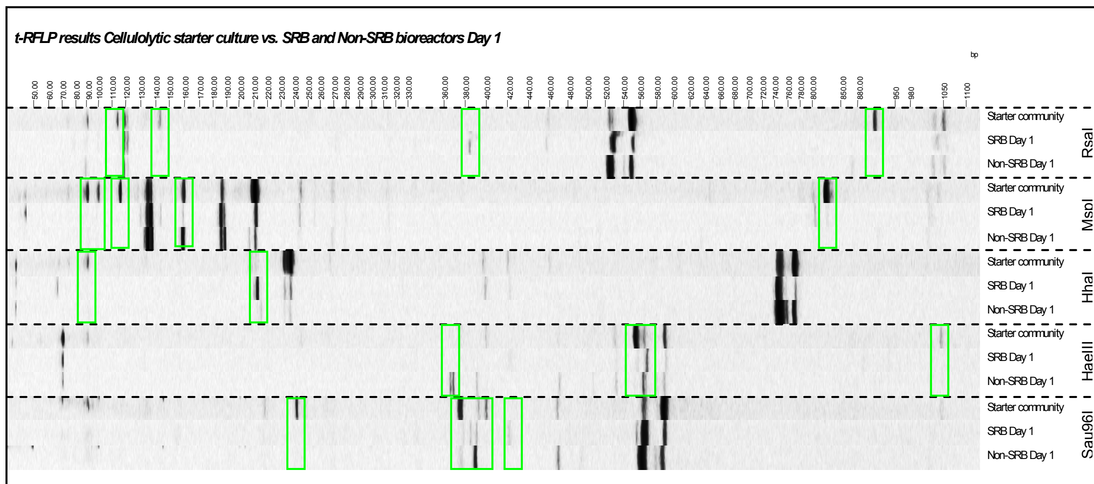

Figure 5.2: Differences in t-RF patterns for the cellulolytic starter community and the bioreactor communities.

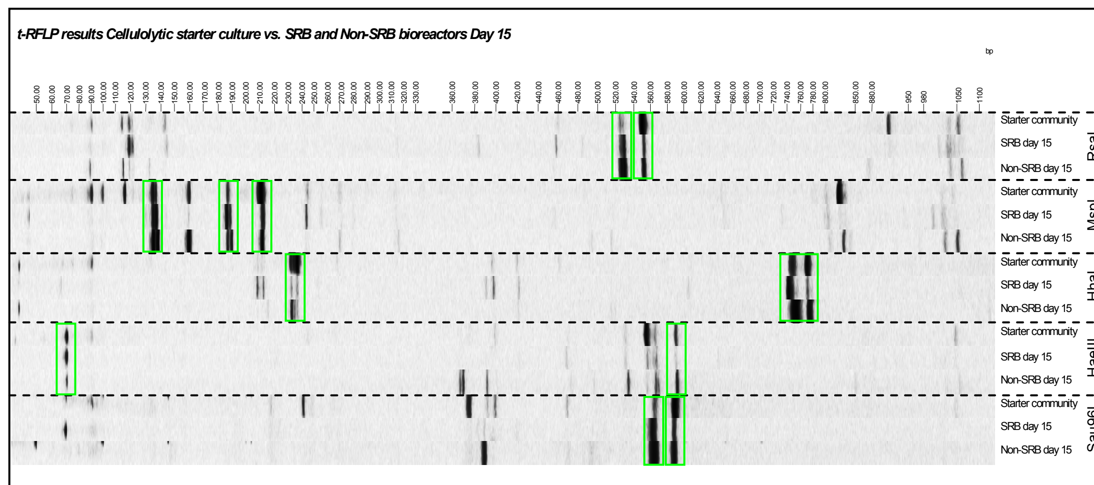

Figure 5.3: Similarities in the t-RF patterns for the cellulolytic starter community and the bioreactor communities.

Figure 5.4 shows a diagrammatical representation of the microbial community dynamics within bioreactors SRB and Non-SRB over the 15-day period of operation. Some of the bacterial members from the starter community remained stable throughout the experimental time period and were still detected in samples taken after 15 days of operation in both bioreactors. Green shaded blocks in the flowchart indicate these members. Members indicated by light blue shaded blocks originated from the starter community, but were only detected in bioreactor Non-SRB and not in

bioreactor SRB during day 15 of bioreactor operation. Some of the possible bacterial members in the starter community could not be detected at all in both bioreactors SRB and Non-SRB after the 15 day time period. The dark blue blocks indicate these possible bacterial members.

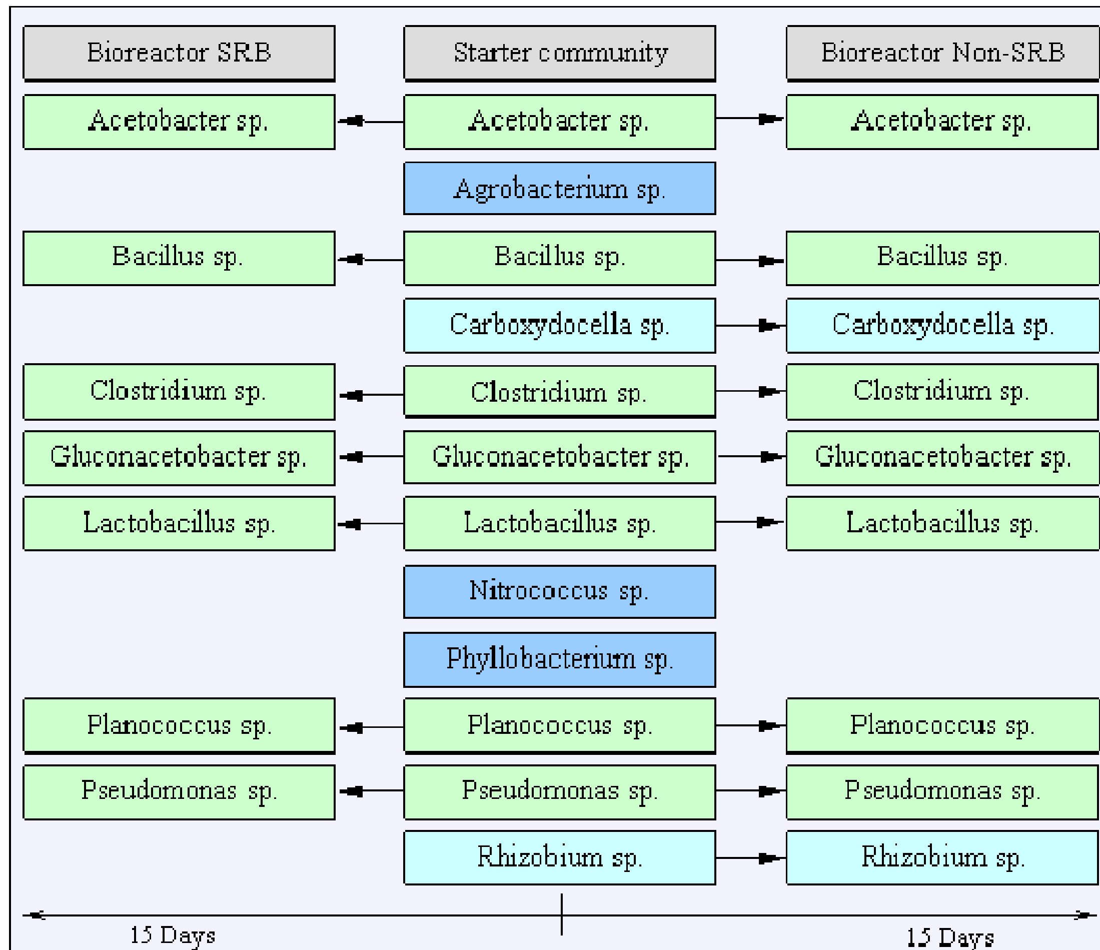

Figure 5.4: Community dynamics of possible bacterial members within bioreactor SRB and Non-SRB after 15 days of operation.

Bioreactor R1 vs. bioreactor R2 – effect of using autoclaved grass cuttings on starter culture

5.3.2. Chemical oxygen demand (COD)

The COD measured for bioreactors R1 and R2 is shown in Figure 5.5. The start COD concentration measured for bioreactor R1 was ca. 2000 mg/L, which was maintained at that level till day 13. After that the COD increased slightly to ca. 2800 mg/L. The measured COD concentrations in bioreactor R2 increased from ca. 3000 mg/L to ca. 4000 mg/L over the 17 day time period.

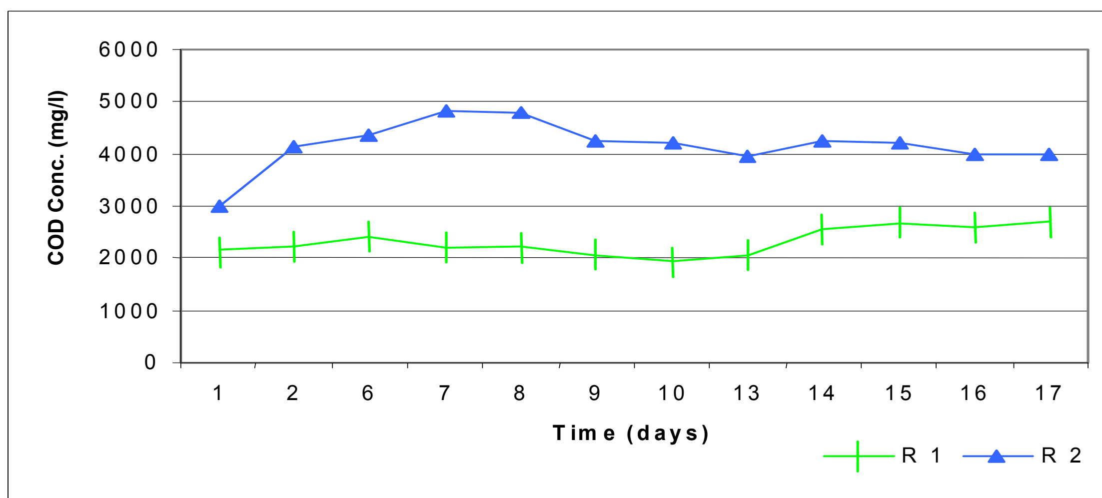

Figure 5.5: COD levels measured for bioreactors R1 and R2.

5.3.4. Volatile fatty acids (VFA)

Acetic Acid

Initially no acetic acid was observed in bioreactor R1. The acetic acid concentration only increased after day 7 of operation while a sharp increase in acetate concentration was measured in bioreactor R2 after day 2. The highest acetate concentration in bioreactor R1 was observed on day 15 (400 mg/L). The highest acetate concentration measured in bioreactor R2 was ca. 500

mg/L on day 6 but levels then dropped to ca. 380 mg/L on day 17 of operation. Figure 5.6 shows the acetate levels for both bioreactors measured.

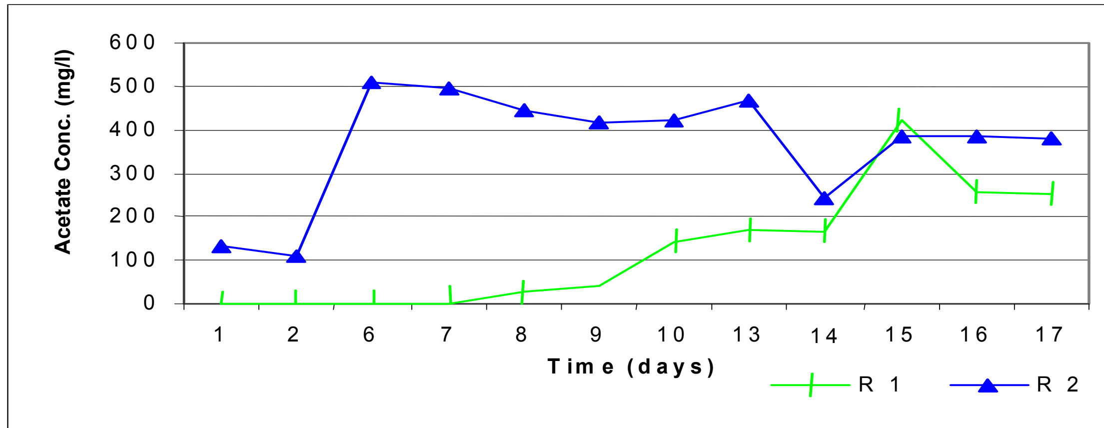

Figure 5.6: Acetate concentration measured in bioreactors R1 and R2.

Propionic Acid

The propionic acid pattern in bioreactors R1 and R2 were similar to the acetate pattern in the two reactors (Figure 5.7). Again, propionate was only measured in bioreactor R1 after 7 days of operation while a sharp increase in propionate levels were detected in bioreactor R2 after 2 days. Over the 17 day operation period, the level of propionic acid in bioreactor R2 dropped from ca. 100 mg/L to ca. 50 mg/L though – roughly the same as in bioreactor R1 after 17 days of operation. The concentration of propionate measured was lower than the acetate concentrations measured in both bioreactors

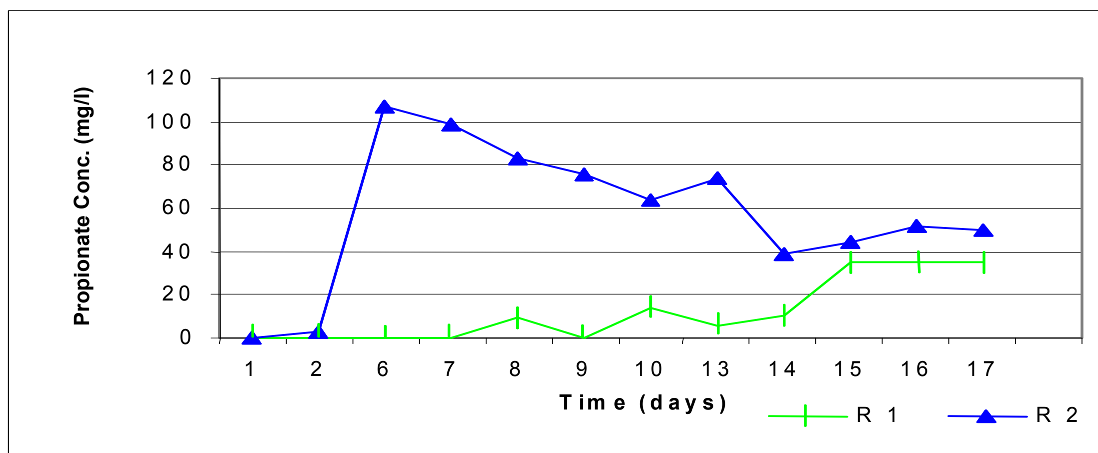

Figure 5.7: Propionate concentration measured in bioreactors R1 and R2.

Butyric Acid

Characteristically, the compost bacteria used in this experiment were butyrate producers. Although the butyric acid concentrations measured were low, the production thereof in both bioreactors R1 and R2 was faster than the production of the other acids. The butyric acid concentration measured in bioreactor R1 remained more stable than that in bioreactor R2 where a drop in butyrate levels was observed after 10 days of operation. Figure 5.8 shows the butyric acid levels for both bioreactors.

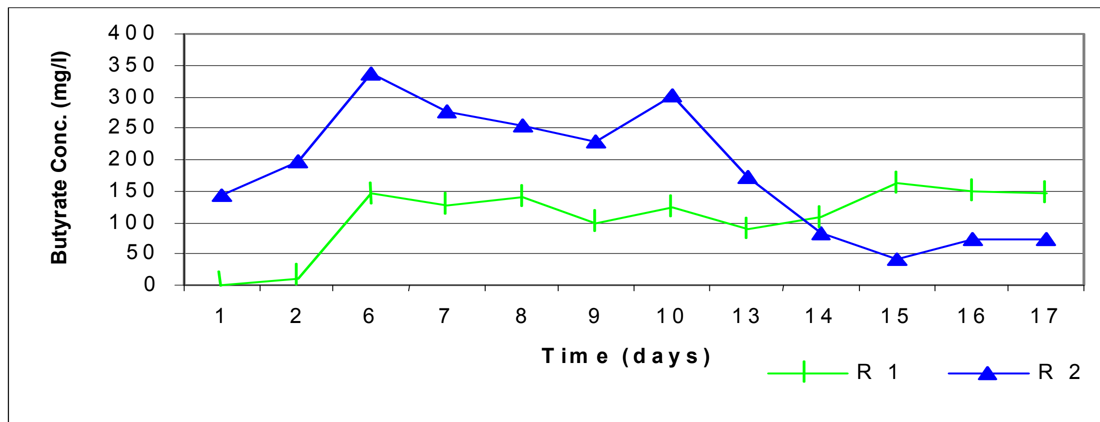

Figure 5.8: Butyrate concentrations measured in bioreactor R1 and R2.

5.3.5. Microbial community composition and dynamics

Figures 5.9 shows results of the t-RFLP analysis of the starter cultures used in bioreactors R1 and R2.

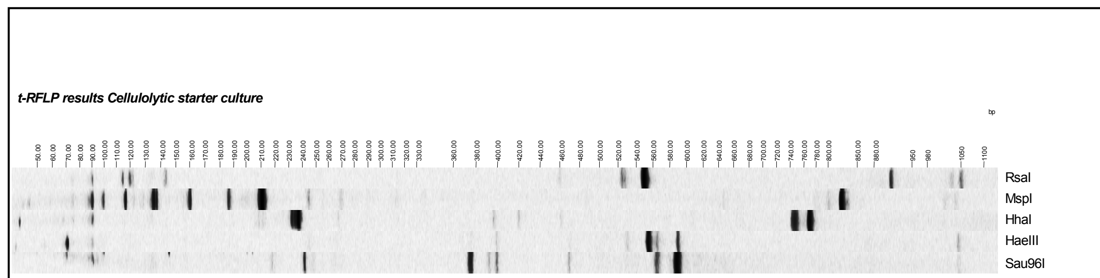

Figure 5.9: t-RFLP patterns obtained for the starter culture used in bioreactors R1 and R2.

Analysis of the t-RF fragment size data also revealed the possible composition of the starter community used as shown in Tables 5.2.

Table 5.3: Possible bacterial members comprising the cellulolytic starter community used in fermentation reactors R1 and R2.

Cellulolytic starter community:
Agrobacterium sp.
Bacillus sp.
Brevibacillus sp.
Carboxydocella sp.
Clostridium sp.
Eubacterium sp.
Lactobacillus sp.
Pseudomonas sp.
Sporolactobacillus sp.

Poly-acrylamide gel electrophoresis images showing a comparison of the t-RF patterns for bioreactors R1 and R2 after 17 days of operation are shown by Figures 5.10 and 5.11 respectively.

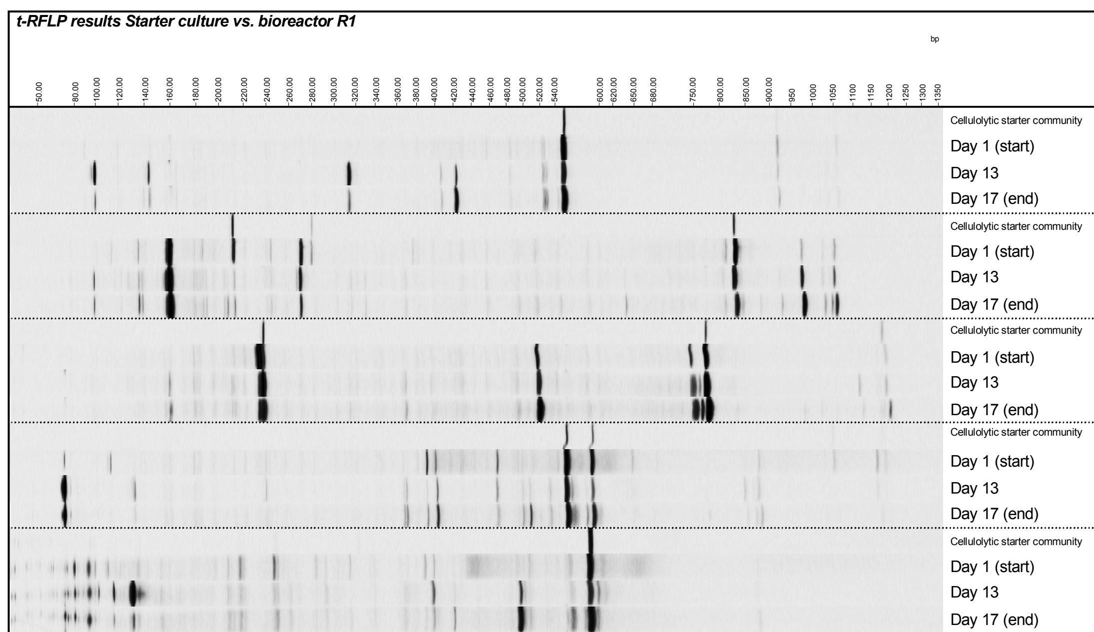

Figure 5.10: t-RF patterns obtained for the starter community and the bioreactor R1 community.

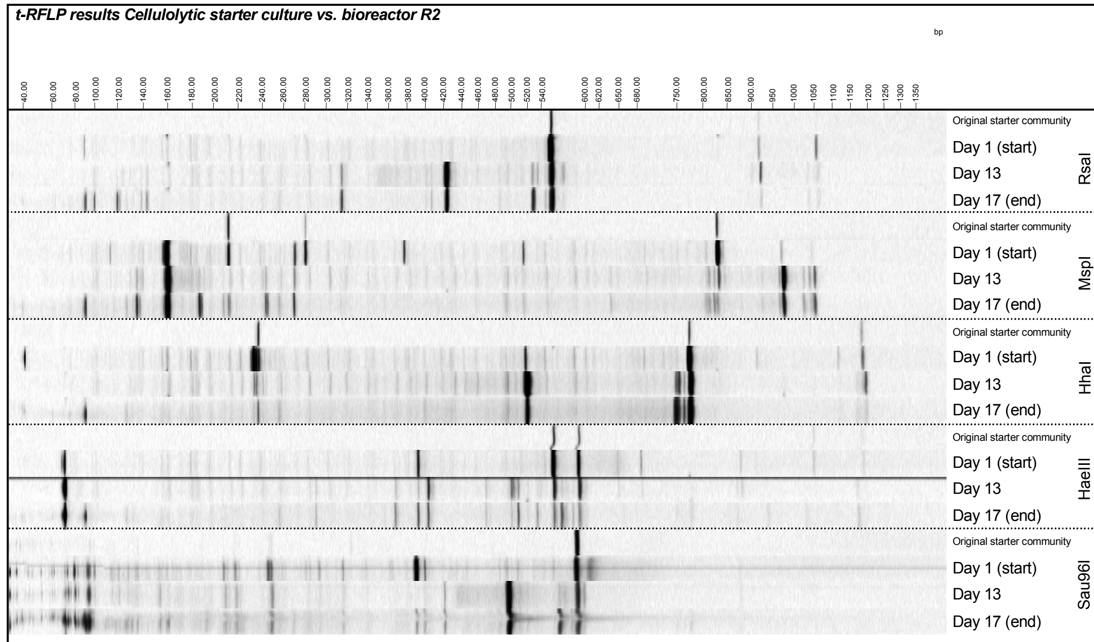

Figure 5.11: t-RF patterns obtained for the starter community and the bioreactor R2 community.

The microbial community dynamics within bioreactors R1 and R2 are represented in Figure 5.12. Once again green shaded blocks indicate possible stable bacterial members originating from the starter community, still detected in both bioreactors R1 and R2 after 17 days of operation. Possible bacterial members indicated by the purple shaded blocks could only be detected in bioreactor R1 on day 17 while the possible *Agrobacterium* and *Brevibacillus* spp., indicated by the light blue shaded blocks, were only detected in bioreactor R2 during day 17 of operation.

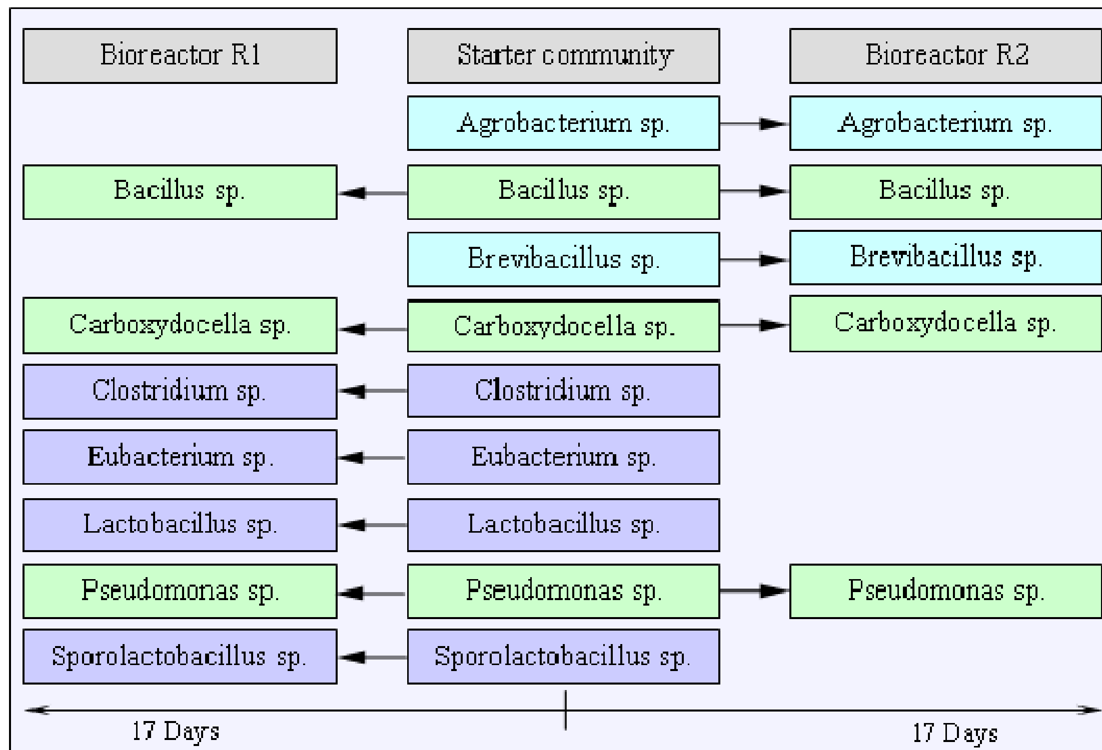

Figure 5.12: Microbial community dynamics within bioreactors R1 (autoclaved grass cuttings) and R2 (non-autoclaved grass cuttings).

5.5. DISCUSSION

Analyses of the samples from bioreactors SRB and Non-SRB by means of t-RFLP strongly suggests that the starter culture used in the set up of these reactors survived and established well in the both reactors. Terminal restriction fragment length polymorphism results showed that most of the bacterial members of the starter community could still be detected in samples from both reactors after 15 days of operation. The addition of additional sulphate reducing bacteria to bioreactor SRB did not seem to have any direct affects on the survival of the starter community within the reactor. Interestingly, the natural micro flora on the grass cuttings used, as carbon source in the bioreactors also seemed to have little affect on the success of the starter cultures' establishment and survival. This was clearly not the case in bioreactors R1 and R2. Here, the natural populations occurring on the grass

cuttings affected the survival of the starter community. The higher bacterial diversity and numbers of the starter culture used in bioreactors SRB and Non-SRB might be an explanation for this finding as t-RFLP results have showed.

Results for bioreactors R1 and R2 suggest that the starter culture used in the set up of these reactors had difficulty surviving and stabilizing in reactor R2. Terminal restriction fragment length polymorphism results showed that most of the bacterial members from the starter community could not be detected after 17 days of operation in bioreactor R2 while of all the members identified using t-RFLP, only the possible *Agrobacterium* and *Brevibacillus* strains could not be detected in bioreactor R1. These two members from the starter community could, however, be detected in bioreactor R2.

In this specific experiment the presence of the natural microbial community on the grass cutting used as carbon source did seem to have an effect on the survival and establishment of the starter community within the reactor. Although autoclaving the grass cuttings used in bioreactor R1 did not kill off all microbes present, the likely reduction of bacterial counts and specific population members did seem to favor the survival of the starter community within the reactor.

These findings are also supported by the chemical analyses for bioreactors R1 and R2. The COD measurements for bioreactor R2 were clearly higher than for bioreactor R1. These higher COD levels for bioreactor R2 can be attributed to the higher bacterial numbers and diversity on the non-autoclaved grass cuttings. Interestingly, though, only a slight increase in the COD level within bioreactor R1 was observed. The reason for this observation is not clear.

The levels of each of the volatile fatty acids measured within the two bioreactors also support the suggestion that the starter community survived

better in bioreactor R1. For each VFA measured, a sudden sharp increase in the measurements was observed for bioreactor R2 with steady decrease in levels thereafter. The sharp increase in VFA levels might be explained by the presence of the possible members from the starter community during the earlier stages of operation. As these bacterial members from the starter community did not survive within the bioreactor the production of VFA's decreased. As mentioned, the compost bacteria used as starter culture were typical butyrate producers. As t-RFLP results indicate, these butyrate producers probably did not survive within bioreactor R2, thus causing a drop in butyric acid levels.

Contrary to these results, low levels of VFA was initially measured within bioreactor R1 with a steady increase over the 17-day period. Again, these findings support the t-RFLP results suggesting that the starter community did survive better within bioreactor R1. As the starter community established within the reactor over time the production of VFA's increased. A possible explanation for the lag in VFA production within bioreactor R1 might be due to low bacterial numbers and diversity caused by autoclaving the grass cuttings. Natural bacteria present on the grass cuttings could also have attributed to VFA production causing the sharp increases as was observed in bioreactor R2 where non-autoclaved grass cuttings was used.

These experiments clearly demonstrate how t-RFLP can be used to monitor the survival and stabilization of starter cultures used in the set up of bioreactor systems.

CHAPTER 6

CONCLUSIONS

CHAPTER 6

CONCLUSIONS

For many years, cultured-based techniques only provided us with a limited view and understanding of microbial communities and their behavior within natural or synthetic environments. In situations where the microbial community present is primarily responsible for the processes taking place within that environment, a good understanding of the microbiology of the system is crucial. This especially applies to biological reactors, which require strict operational control. External factors affecting the microbial community involved could cause alterations in the biological processes and alter the desired outcome and results of the operating system.

In this study, reactors removing sulphate from wastewater was specifically investigated. To drive the biological sulphate reduction within these reactors, sulphate removing microbial communities involved were dependant on the metabolic byproducts produced by cellulolytic microbial communities degrading grass cuttings added to the reactors – mainly volatile fatty acids such as propionate, acetate and butyrate. An understanding of community stability, dynamics and functionality within each reactor was thus extremely important.

Nowadays, DNA based techniques provides a promising alternative for studying microbial communities. Researchers commonly use techniques such as denaturing gradient gel electrophoresis (DGGE), fluorescent *in situ* hybridization (FISH) and even cloning strategies to study microbial communities. Apart from being laborious and expensive, these techniques lack the ability to rapidly investigate community dynamics and to identify possible dominant bacterial members comprising the community. This study demonstrated the usefulness of terminal restriction fragment length

polymorphism as a tool for studying microbial communities within fermentation and sulphate removing bioreactors.

During this study is was noted that:

- Terminal restriction fragment length polymorphism could be optimized for analyses of samples from harsh environments such as sulphate removing bioreactors.

- Terminal restriction fragment length polymorphism allowed for the analysis of microbial community composition as well as dynamics within fermentation and sulphate removing bioreactors over operational time periods.

- T-RFLP allowed for the identification, to some extent, of dominant bacterial members comprising a microbial community - the drawback being that many bacteria share the same restriction sites thus making accurate identifications complex.

In terms of the operation of sulphate removing bioreactors:

- T-RFLP results suggested that the daily addition of grass cuttings to the reactors helped to better stabilize the microbial community and VFA production within the reactor.

- The natural microbial populations present on grass cuttings used as carbon source in the bioreactors could affect the survival and stabilization of starter cultures within the reactors.

As with any other molecular technique, t-RFLP has certain very important aspects and shortcomings that require the necessary attention and further investigation:

- 16S rDNA PCR reactions might be biased towards the dominating microbial members thus not presenting the true composition of the community studied.

- Accurate t-RF profiles and subsequent identification of bacterial members comprising the community is dependant on complete restriction digestions. Poorly digested 16S rDNA fragments due to reaction failure for instance, could lead to drastic alteration and inaccurate results.

- Identification of bacterial members is based on t-RF length data but because many bacteria share restriction sites, the process is complex. Combining t-RFLP analysis with e.g. cloning strategies could greatly improve microbial community analyses and deliver more accurate identifications of dominant bacterial members.

To fully understand the potential and limitations of t-RFLP, it is very important to take these into consideration when studying microbial communities.

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