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Characterising biogas production from waste residues: understanding the role of microbial metabolism

By

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July 2017

University of the Free State

Bloemfontein

Republic of South Africa

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Submitted in fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

In the

Department of Microbial, Biochemical and Food Biotechnology

Faculty of Natural and Agricultural Sciences

University of the Free State

Bloemfontein

Republic of South Africa

July 2017

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Dr. M. Erasmus

Mr. T. Höppner

I dedicate this dissertation to my parents (Lawrence and Lillian Molaoa) and brother (S'thembile Molaoa), thank you for your support.

“Keep on, then, seeking first the Kingdom and his righteousness, and all these other things will be added to you.”

Matthew 6:33

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Declaration

I hereby declare that this thesis is submitted by me for the Magister Scientiae degree at the University of the Free State. This work is solely my own and hasn't been previously submitted by me at any other University or Faculty, and the other sources of information used have been acknowledged. I further grant copyright of this thesis in favour of the University of the Free State.

RR Molaoa

July 2017

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List of symbols and abbreviations

ºC	Degrees Celsius
%	Percentage
&	And
<	Less than
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
µL	Microlitre
®	Registered Trademark
A	Absorbance
AD	Anaerobic digestion
ATP	Adenosine triphosphate
ATCC	American Type Culture Collection
APS	Ammonium persulfate
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide Triphosphates
DGGE	Denaturing Gradient Gel Electrophoresis
dH_2O	Distilled water
DM	Dry Matter
DSMZ	German Collection of Microorganisms and Cell Cultures
Da	Dalton
DAPI	4’,6-diamidino-2-phenylindole
EDTA	Ethylene diaminetetraacetic acid
et al.	et alii/ and others
EtBr	Ethidium Bromide
FM	Fresh Matter
i.e.	That is

g	gram
g/kg	gram per kilogram
g/100 ml	Gram per 100 milliliter
g/L	Gram per liter
gDNA	Genomic DNA
GC	Gas Chromatography
h	Hour
HPLC	High Performance Liquid Chromatography
HRT	Hydraulic Retention Time
IPTG	Isopropyl β-D-1-Thiogalactopyranoside
l	Litre
M	Molar
mM	millimolar
ml	millilitre
µg/ml	microgram per millilitre
µl	microlitre
µm	micrometre
µM	micromolar
ND	NanoDrop
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NCBI	National Center for Biotechnology Information
NTA	Nitrilotriacetic Acid
PCR	Polymerase Chain Reaction
pH	Measure of the Acidity or Basicity of a Solution
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
s	Second
TAE	Tris-Acetic Acid-EDTA
TEMED	N,N,N’,N’-tetramethylethylenediamine
TCD	Thermal Conductivity Detector
TS	Total Solids
TYG	Tryptone, Yeast extract, Glucose
vDM	Volatile Dry Matter
vTS	Volatile Total Solids

UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
x	Times
x g	Acceleration due to Gravity
X-Gal	5-Bromo-4-Chloro-3-Indolyl-beta-D-Galactopyranosidehosphate

CHAPTER 1

1 Introduction

1.1 Biogas production

The current global energy supply is highly dependent on fossil fuels; these include crude oil, lignite, coal and natural gas (Ackah *et al.*, 2016; Sun *et al.*, 2013; Weiland, 2010). These are remains of dead plants and animals, which have been exposed to heat and pressure in the earth's crust over hundreds of millions of years (Seadi *et al.*, 2008). For this reason, fossil fuels are non-renewable resources which reserves are being depleted much faster than new ones are being formed (Höök & Tang, 2013; Owens *et al.*, 2010).

Over the years fossil fuelled energy has been used extensively across the world, however; concentrations of greenhouse gases (GHGs) in the atmosphere are rising rapidly, with carbon dioxide (CO_2) emissions being the most important contributor of greenhouse gases (Weiland, 2010). In order to minimize global warming and climate change impacts, GHG emissions must be reduced to less than half of global emission levels of 1990 (IPCC, 2000; Nazaries *et al.*, 2013). Another important global challenge is the security of energy supply, because most of the known conventional oil and gas reserves are concentrated in unstable regions such as mine areas. As a result there is a need for renewable and sustainable energy sources. In this regard, biogas from waste residues and energy crops will play a vital role in the future (Luo & Angelidaki, 2012; Weiland, 2010).

Simply put, biogas refers to a mixture of gases that is produced when microorganisms degrade organic material in an oxygen free environment (Hilkiah-Igoni *et al.*, 2008; Mursec, 2009). Biogas is composed mainly of methane (CH_4), carbon dioxide (CO_2) and low concentrations of other gases such as nitrogen (N_2), hydrogen (H_2), oxygen (O_2) and hydrogen sulphide (H_2S) (Bassey *et al.*, 2013; Luo & Angelidaki, 2012; Rasi, 2009). In 2011, Offor found that biogas is composed of methane (40 - 70 vol. %), carbon dioxide (30 - 60 vol. %) and other gases (1 - 5 vol. %) including hydrogen (0-1 vol. %) and hydrogen sulphide (0 - 3 vol. %) (Offor, 2011). In 2009, Mursec also reported the same percentage of methane and carbon dioxide while the other gases 0 - 3 vol. % (Mursec, 2009).

Biogas can occur naturally in the environment or at controlled conditions in specialized biogas plants; this is known as anaerobic digestion (AD). Swamps, marshes, landfills, river beds and rumen of herbivore animals are some of the areas where biogas is formed naturally (Bassey *et al.*, 2013). The same microbial activities are achieved in both natural and controlled conditions (Vindis & Mursec, 2009).

Biogas is a versatile renewable and environmentally friendly source of energy, which can be a viable alternative to fossil fuels (Abdel-Hadi, 2008). This form of energy production has several advantages, such as the use of local resources, the reduction of greenhouse gas emissions and it has been evaluated as a very effective form of bio-energy production. As a result, the production of biogas and the construction of biogas plants has increased in European, American, Asian and African countries over the past years (Ali Shah *et al.*, 2014). Moreover, the renewable energy sector has also gained more economic growth (Ackah *et al.*, 2016).

Anaerobic digestion of energy crops, residues, and wastes is of increasing interest in order to reduce greenhouse gas emissions and it is considered a feasible option to manage and treat organic waste (Weiland, 2010). Anaerobic digestion not only treats the organic waste but also produces clean energy (biogas). The digestion residues (digestate) obtained from the process can be used as soil conditioner or a nutrient rich organic fertilizer depending on its final quality (Dioha *et al.*, 2013; El-Mashad & Zhang, 2010).

Any organic material can serve as feedstock for biogas production, these include household waste, restaurant waste, municipal solid waste, industrial organic waste, garden waste, agricultural waste (manure and crop residue), energy crops like maize, sorghum and bran, cellulose rich biomass, algae and seaweed by-products of ethanol and bio-diesel production (Amon *et al.*, 2007; Beevi *et al.*, 2013; Hilkiah-Igoni *et al.*, 2008; Seadi *et al.*, 2008; Yong *et al.*, 2015; Zhang *et al.*, 2014).

2 An overview of anaerobic digestion process

During anaerobic digestion, different groups of microorganisms work synergistically to degrade organic matter. The biodegradation (**Figure 1.1**) mainly includes four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Wirth *et al.*, 2012; Yokoyama & Matsumura, 2008; Zeb *et al.*, 2013).

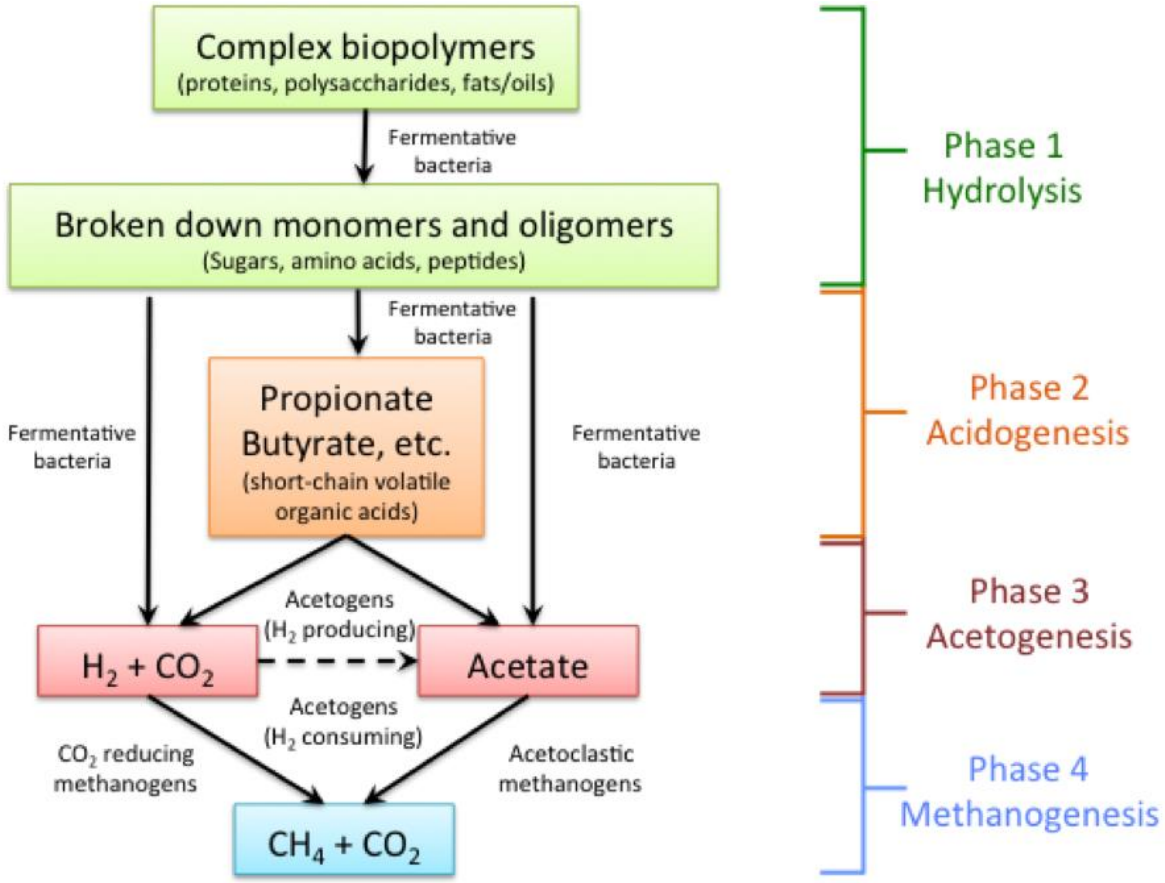

Figure 1.1: Anaerobic digestion of organic matter consists of four main phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Each stage involves a group of different microorganisms which work synergistically with each other, forming a microbial consortia. (Taken from https://www.e-education.psu.edu/egee439/node/727).

2.1 Hydrolysis

Hydrolysis is the first step of the anaerobic digestion process and it is regarded as the rate-limiting step during the anaerobic digestion of complex organic matter; this is because in order for the whole biogas production process to become successful, hydrolytic enzymes should be primarily adsorbed on the surface of solid substrates

(Sun *et al.*, 2013; Weiland, 2010; Zhang *et al.*, 2014), again during this stage bacterial action proceeds slower than the other following digestion steps. During hydrolysis, complex organic matter are hydrolysed into simple compounds by hydrolytic microorganisms. Proteins present in the substrate are converted into amino acids, fats into long chain fatty acids and carbohydrates into simple sugars (Liu & Whitman, 2008; VDI 4630, 2006). An approximate chemical formula for the mixture of organic waste is $C_6H_{10}O_4$ (Ostrem, 2004). **Equation 1.1** shows an example of a hydrolysis reaction where organic waste is broken down into a simple sugar, in this case, glucose.

Equation 1.1: $C_6H_{10}O_4 + 2H_2O \rightarrow C_6H_{12}O_6 + 2H_2$ (Merlin *et al.*, 2014)

In anaerobic digesters, proteins serve as a source of carbon, nitrogen and energy for bacterial growth. Proteins are hydrolyzed by proteolytic enzymes to peptides, amino acids, ammonia, and carbon dioxide. It has been shown that a specialized group of proteolytic clostridia such as *Clostridium perfringens, C. bifermentans, C. histolyticum, and C. sporogenes* are responsible for protein degradation in digesters (Bagge, 2009; Zverlov *et al.*, 2010). Proteins are generally hydrolyzed to amino acids by proteases. Microorganisms that are responsible for this reaction include species of the genera *Bacteroides, Butyrivibrio, Fusobacterium and Streptococcus* (Ali Shah *et al.*, 2014; Alvarado *et al.*, 2014).

Hydrolysis of lipids is catalyzed by enzymes called esterases, this reaction results in the production of saturated and unsaturated long chain fatty acids and glycerol. Glycerol is easily assimilated and metabolized by the bacteria, while the long chain fatty acids undergo an intracellular beta-oxidation mediated by a variety of enzymes, resulting in short chain fatty acids such as acetic acid, propionic acid and hydrogen. Anaerobic microorganisms that are capable of decomposing lipids include *Anaerovibrio lipolytica and Syntrophomonas wolfei* (Li *et al.*, 2017a; Wirth *et al.*, 2012).

Starch from food waste is readily biodegradable, however it requires multiple enzymes to complete its hydrolysis. Three main types of enzymes that act synergistically are: alpha-amylases, beta-amylases, gluco-amylases. A number of microorganisms found in anaerobic digesters capable of degrading starch are *Streptococcus bovis*,

Bacteriodes amylophilus, *Selenomonas ruminatium*, *Bacteriodes ruminocola* and serveral *Lactobacillus* species (Li *et al.*, 2017b; Niu *et al.*, 2016).

Hydrolysis of hardly decomposable polymers, that is cellulose and hemicellulose, is considered to be a stage which limits the rate of wastes digestion. During solid wastes digestion, only 50% of organic compounds undergo biodegradation. **Figure 1.2** shows a structure of cellulose, which is a linear homopolymer of glucose consisting of β-1,4 bonds, hemicellulose is a heteropolymer of pentoses (xylose and arabinose), hexoses (glucose, galactose and mannose) and sugar acids (acetic acid), which are hydrolysed by specialised bacteria (Zverlov *et al.*, 2010). The predominant bacteria found to degrade the hemicellulose in the rumen are *Bacterioides ruminicola*, *Butyrivibrio fibrisolvens*, *Ruminococcus flavenfaciens* and *Ruminococcus albus* (Alvarado *et al.*, 2014; Li *et al.*, 2017b; Niu *et al.*, 2016; Sun *et al.*, 2013).

In plant tissue both cellulose and hemicellulose are tightly packed in lignin and are therefore difficult for bacteria to access and digest. This is why only approximately 40% of the cellulose and hemicellulose in cow manure is decomposed in the biogas process. Normally the decomposition of organic matter to methane and carbon dioxide is not absolute and is frequently only about 30-60% for animal manure and other substrates that have a high concentration of complex molecules (Ali Shah *et al.*, 2014; Chen, 2014; Jørgensen, 2009).

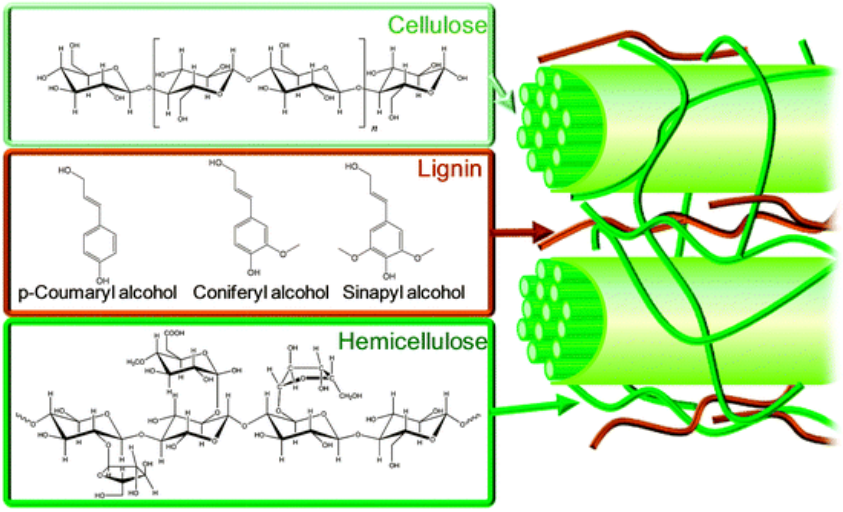

Figure 1.2: Cellulose and hemicellulose are long-chain polysaccharides that can be broken down by specific enzymes present in certain bacteria, but not in animals. Lignin has a compact structure and is practically biologically inert. (Taken from Alonso *et al.,* 2012).

2.2 Acidogenesis

In the Acidogenesis process, Acidogens consisting of facultative microorganisms and obligate anaerobes convert the products of hydrolysis into simple organic compounds, mostly short chain (volatile) fatty acids such as butyric acid ($CH_3CH_2CH_2COOH$), propionic acid (CH_3CH_2COOH), acetic acid (CH_3COOH), valeric acid ($CH_3(CH_2)_3COOH$), formic acid ($HCOOH$), lactic acid ($C_3H_6O_3$), ethanol (C_2H_5OH) methanol (CH_3OH), hydrogen (H_2) and carbon dioxide (CO_2) (Angelidaki *et al.*, 2009; Jørgensen, 2009; Raposo *et al.*, 2006; Weiland, 2010). From these products, the hydrogen, carbon dioxide and acetic acid will skip the third stage, acetogenesis, and be utilized directly by the methanogenic bacteria in the final stage (Ostrem, 2004). The transition of the substrate from organic material to organic acids in the acid forming stages causes the pH of the system to drop. This is beneficial for acidogenic and acetogenic bacteria that prefer a slightly acidic environment, with a pH of 4.5 - 5.5. However, if the acidogenesis process is unbalanced, this can cause acidification (Abdel-Hadi, 2008).

It is important to maintain constant pH in the start-up of the digestion process, this is because fresh waste undergoes hydrolysis and acidogenesis before any methane can be formed, which will lower the pH. In order to keep the value of pH on the equilibrium, a buffer such as calcium carbonate or lime has to be added into the system. Although it has been proven that the optimal range of pH for obtaining maximal biogas yield in anaerobic digestion is 6.5 - 7.5, the range is relatively wide in the plants and the optimal value of pH varies with substrate and digestion technique (Liu & Whitman, 2008). The pH value is a function of volatile fatty acid (VFA) concentration, bicarbonate concentration, and alkalinity of the system as well as the fraction of CO_2 in digester gas. In order to fix constant pH value it is crucial to adjust the relationship between the VFA and bicarbonate concentrations (De Mes *et al.*, 2003; Raposo *et al.*, 2006; VDI 4630, 2006).

Facultative anaerobic microorganisms can grow under acid conditions. In order to produce acetic acid, they need oxygen and a carbon source. For this, they use the oxygen dissolved in solution or bounded-oxygen. Hereby, the acid-producing bacteria create anaerobic conditions essential for methane producing microorganisms. Specific concentrations of products formed during acidogenesis differ with the type of

microorganisms as well as with culture conditions such as temperature and pH. Typical reactions in the acid-forming stages are shown below. **Equation 1.2, 1.3** and **1.4** represent three typical acidogenesis reactions where glucose is converted to ethanol, propionate and acetic acid, respectively (Ali Shah *et al.*, 2014; Dioha *et al.*, 2013; Liu & Whitman, 2008; Seadi *et al.*, 2008).

Equation 1.2: $C_6H_{12}O_6 \leftrightarrow 2CH_3CH_2OH + 2CO_2$ (Merlin *et al.*, 2014)

Equation 1.3: $C_6H_{12}O_6 + 2H_2 \leftrightarrow 2CH_3CH_2COOH + 2H_2O$ (Merlin *et al.*, 2014)

Equation 1.4: $C_6H_{12}O_6 \rightarrow 3CH_3COOH$ (Merlin *et al.*, 2014)

2.3 Acetogenesis

Acetogenesis is the third stage of anaerobic digestion whereby the products of acidogenesis i.e. the propionic acid, butyric acid and alcohols are transformed by acetogenic bacteria into hydrogen, carbon dioxide and acetic acid (Seadi *et al.*, 2008; Weiland, 2010). In anaerobic reactions, hydrogen plays an important intermediary role. H_2 gas formation occurs when long chain fatty acids are oxidized into propionate or acetate. However, this oxidation is inhibited by H_2 in the solution under standard conditions. Thus, reactions will only occur if the hydrogen partial pressure is low enough to thermodynamically allow the conversion of all the acids (Luo & Angelidaki, 2012; Pan *et al.*, 2008).

Such lowering of the partial pressure is carried out by hydrogen consuming microorganisms (Kern *et al.*, 2016). **Equation 1.5** shows the conversion of propionate into acetate, this is only achievable at low hydrogen pressure. Thus, hydrogen concentration which is measured by partial pressure, is an indicator of the digester's health. **Equation 1.6** and **1.7** show glucose and ethanol respectively, these are also converted to acetate during acetogenesis (Mata-Alvarez, 2003; Merlin *et al.*, 2014; Ostrem, 2004; Zeb *et al.*, 2013; Zeikus, 1977).

Equation 1.5: $CH_3CH_2COO^- + 3H_2O \leftrightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2$ (Merlin *et al.*, 2014)

Equation 1.6: $C_6H_{12}O_6 + 2H_2O \leftrightarrow 2CH_3COOH + 2CO_2 + 4H_2$ (Merlin *et al.*, 2014)

Equation 1.7: $CH_3CH_2OH + 2H_2O \leftrightarrow CH_3COO^- + 2H_2 + H^+$ (Merlin *et al.*, 2014)

2.4 Methanogenesis

Methanogenesis is the last step in the degradation of biomass. In this step, under strictly anaerobic conditions methanogens convert the carbon dioxide, hydrogen and acetic acid to methane. Methanogenesis can be divided into three major pathways: the hydrogenotrophic (CO_2 reduction) pathway, acetotrophic pathway and methylotrophic pathway. The hydrogenotrophic pathway is the most common metabolic pathway where CO_2 and H_2 are converted to methane (**Equation 1.8**). Besides H_2, most of the hydrogenotrophs can also use formate as the major electron donor. In the acetotrophic pathway (**Equation 1.9**), acetate is directly converted to methane. The carboxyl- group of the acetate is oxidized to CO_2 whereby the methyl group is reduced to methane. The microorganisms involved in the methanogenesis stage are called methanogens and belong to the domain of the Archaea (Ferry, 2010; Lessner, 2009).

Montero and co-workers found that the consumption of butyric acid, the main precursor of methane, is related to hydrogenotrophic methanogens during start-up phase and to acetotrophic methanogens during stabilization phase. It was then concluded that methanogenic population dynamics depend on the concentration of VFA, specifically butyric acid. Thus if concentration of VFA is high, hydrogenotrophic methanogens will prevail (Ma *et al.*, 2015; Montero *et al.*, 2010; Sousa *et al.*, 2013).

Methanogens are obligate anaerobes and very sensitive to environmental changes. In contrast to acidogenic and acetogenic bacteria, methanogens are sensitive to changes in pH and temperature, thus they prefer a neutral to slightly alkaline environment (Merlin *et al.*, 2014). If the pH is allowed to fall below 6, methanogens cannot survive. A better indicator is therefore methane production. The change in pH can be both an indicator and the cause of process imbalance (Liu *et al.*, 2003; Triantafyllou *et al.*, 2014).

Equation 1.8: $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$ (Merlin *et al.*, 2014)

Equation 1.9: $CH_3COOH \rightarrow CH_4 + CO_2$ (Merlin *et al.*, 2014)

3 Methanogenic diversity in biogas reactors

The production of biogas is highly dependent on the methanogenic community in the reactor, as a result the study of its diversity (**Figure 1.3**) is of growing interest. Nettmann and colleagues performed a study on ten agricultural biogas plants operated at a mesophilic temperature. The study revealed that the order Methanomicrobiales was predominate in nine of ten analysed reactors. The most abundant genus was the *Methanoculleus* (Nettmann *et al.*, 2008). In a study by Kröber and co-workers, it was found that most methanogens in a biogas plant operated on maize silage and liquid manure belong to the genus *Methanoculleus* (Kröber *et al.*, 2009).

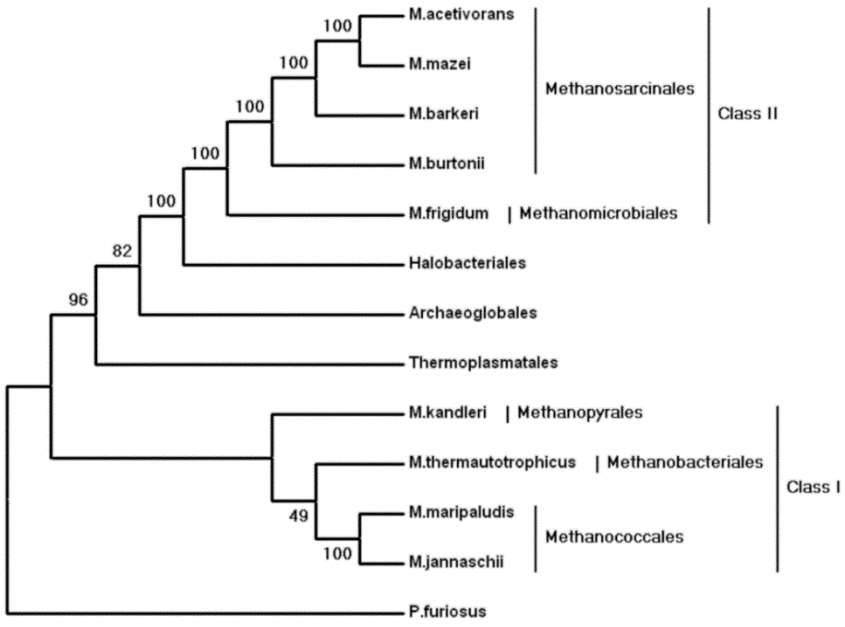

Figure 1.3: Phylogeny of methanogens inferred from fusion analyses of ribosomal protein (Taken from Luo *et al.,* 2009).

Other studies also revealed the Methanomicrobiales as the most abundant order, however in these studies the dominant genus was the *Methanocorpusculum* (Borrel *et al.*, 2011; Luton *et al.*, 2002; Wirth *et al.*, 2012). Most species in the order Methanomicrobiales grow by CO_2 reduction with H_2 as electron donor (Nettmann *et*

al., 2008), therefore this can imply that the CO_2 reduction (hydrogenotrophic) pathway was the main metabolic pathway in these reactors.

However, other orders are also present in agricultural biogas plants. According to Nettmann and colleagues, members of the order Methanobacteriales were found in seven of ten biogas reactors. Moreover members of the genus *Methanosaeta* were present in seven of ten reactors (Nettmann *et al.*, 2008). The presence of this genus introduces the acetotrophic pathway for the generation of methane. Nevertheless, Nettmann also alluded that the majority of methanogens in agricultural biogas reactors are expected to use the CO_2 reduction pathway for growth. This is in contrast to other literature sources in which the acetotrophic pathway was described as the dominant metabolic pathway (Bagi *et al.*, 2007; Vergara-Fernández *et al.*, 2008).

4 Microbiology and biochemistry of the methanogens

The taxonomy of methanogens has been extensively revised in the light of new information based on comparative studies of 16S rRNA oligonucleotide sequences, membrane lipid composition and antigenic fingerprinting data (Garcia, 1990; Kern *et al.*, 2016; Manyi-Loh *et al.*, 2013; Qiu *et al.*, 2014). These organisms are no longer considered prokaryotic as they share some similarities with the eukaryotic cells (**Figure 1.4**) (Triantafyllou *et al.*, 2014).

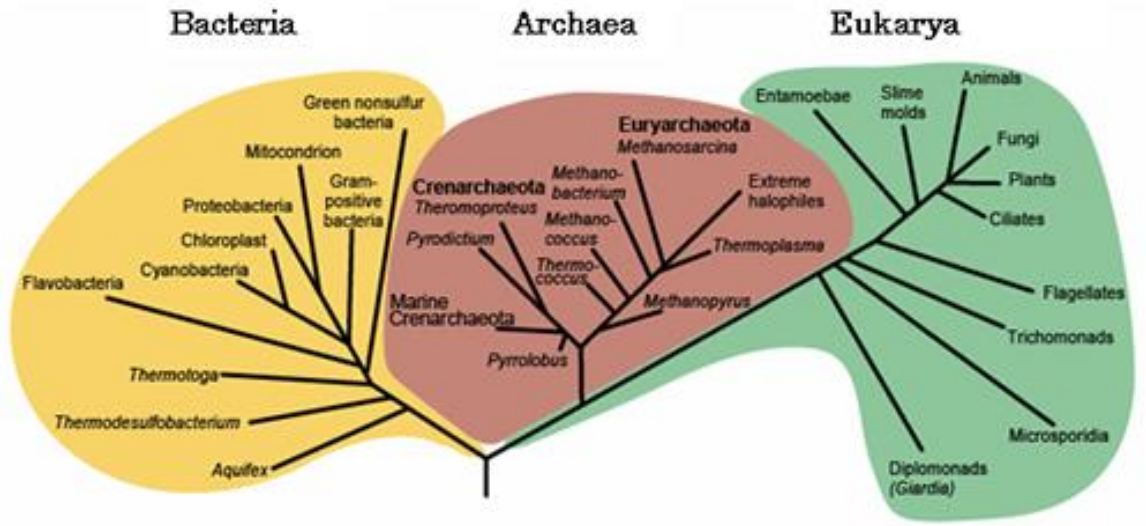

Figure 1.4: The phylogenetic tree of life as defined by comparative rRNA gene sequencing. The tree consists of three domains of organisms: Bacteria (prokaryotes), Archaea (Euryarchaeota and Crenarchaeota kingdom) and Eukarya (eukaryotes). (Taken from *Madigan et al.,* 2008, Brock Biology of microorganisms, 12th Edition).

Methanogens are anaerobic unicellular organisms (Garcia, 1990). These organisms belong to the domain Archaea in the Euryarchaeota kingdom (Shlimon *et al.*, 2004). They can be divided into five orders (**Figure 1.5**) *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales* and *Methanopyrales* (Franke-Whittle *et al.*, 2014; Nunoura *et al.*, 2008; Ziemiński & Frąc, 2012). A sixth order, the Methanocellales, was recently included (Pandey *et al.*, 2015). According to Borrel and co-workers, currently cultivated methanogenic archaea consist of six orders and 31 genera all belonging to the Euryarchaeota phylum based on comparative 16S rRNA gene sequence analysis (Borrel *et al.*, 2011; Jabłoński *et al.*, 2015).

The phenotypic characteristics of methanogens often do not provide a sufficient means of distinguishing among taxa (Garcia, 1990). They have unique properties such as living in strictly anaerobic conditions, they are difficult to cultivate, require specific conditions and their cell wall lacks peptidoglycan therefore making them susceptible to some antibiotics (i.e., chloramphenicol and bacitracin) and resistant to many common others like penicillin and aminoglycosides (Triantafyllou *et al.*, 2014). Methanogens have specialized growth requirements which includes several metals for use in a variety of metalloenzymes. Nickel is of particular importance as it is required for methyl–coenzyme M reductase, the enzyme catalysing the terminal step in all methanogenic pathways (Ferry, 2009).

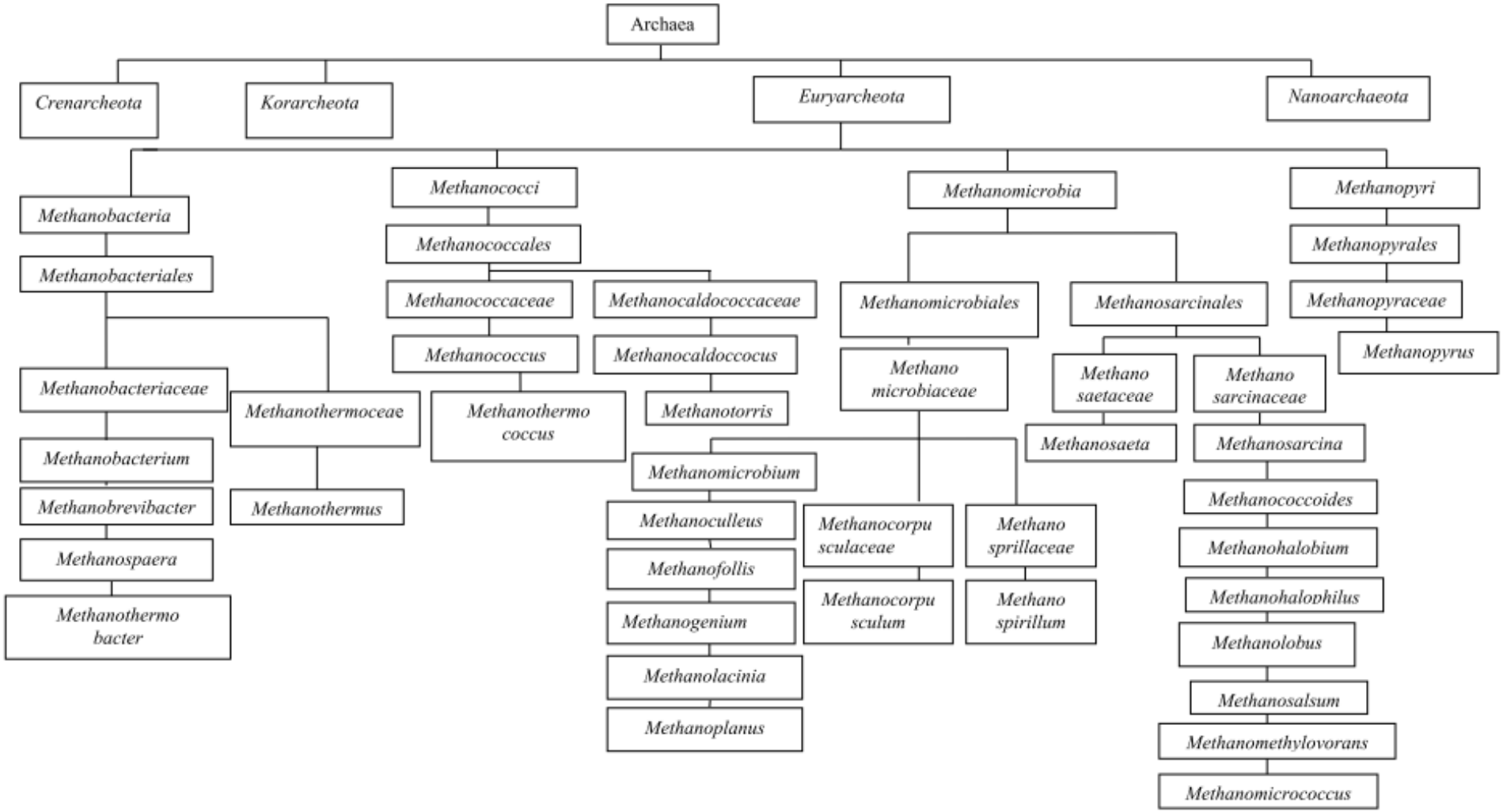

Figure 1.5: Phylogenetic hierarchy of methanogens. (Taken from Demirel & Scherer, 2008; Ziemiński & Frąc, 2012).

The methanogens are extremely diverse and range from psychrophilic species to thermophilic species, acidophilic to alkaliphilic species and from halophilic species to non-halophilic species (Van de Vossenberg *et al.*, 1998). An example of an acidophilic and thermophilic methanogen is the *Methanotorris igneus* with optimum growth conditions at a pH 5.7 and 88°C. By contrast, the *Methanobacterium subterraneum* has its optimal growth conditions at a pH between 7.8 - 8.8 and temperatures between 20 - 40°C (Mori & Harayama, 2011; Wright *et al.*, 2004). However, most methanogens are mesophiles and non-halophilic and grow at a neutral to slightly alkaline pH (Sunarto, 2015; Ventosa *et al.*, 1998). The most unifying characteristic of methanogens is that they all produce methane (Ravichandran *et al.*, 2015).

Methanogenesis is the last step of anaerobic digestion, it is a type of anaerobic respiration and requires six remarkable coenzymes namely; ferredoxin, methanofuran, methanopterin, coenzyme F_{420}, coenzyme M and coenzyme B. Methanogenesis also requires a number of specific membrane bound enzyme complexes coupled to the generation of a proton gradient driving ATP synthesis. Although the methanogens are very diverse, they can only utilize a restricted number of substrates (Ferry, 2011; Pandey *et al.*, 2015).

The three primary methanogenic substrates are CO_2, acetate and methyl group containing compounds such as methanol, methylated amines and methylated sulphides. For this reason, three distinct pathways for methane production exist; hydrogenotrophic, acetotrophic and methylotrophic pathway (Ali Shah *et al.*, 2014; Liu & Whitman, 2008). Even though the intermediates and enzymatic reactions of the three pathways are specific, they share common features in the final steps of CH_4 production. Most organic substances, for instance, carbohydrates and long-chain fatty acids and alcohols, are not substrates for methanogenesis. Instead, these compounds must first be processed by hydrolytic bacteria to produce the substrates actually used by the methanogens (Ferry, 2009; Liu & Whitman, 2008; Zverlov *et al.*, 2010).

4.1 Hydrogenotrophic (CO_2 reduction) pathway

Methanogens using CO_2 as a substrate follow the hydrogenotrophic pathway. Hydrogenotrophic methanogens reduce CO_2 to methane and H_2 serves as the primary electron donor. Hydrogenotrophic methanogens can also use formate as the major electron donor. In this case, four molecules of formate are oxidized to CO_2 by formate dehydrogenase (Fdh) before one molecule of CO_2 is reduced to methane. In hydrogenotrophic methanogenesis, CO_2 is reduced successively to methane through the formyl, methylene and methyl levels (**Figure 1.6 A**). The one carbon (C-1) moiety is carried by special coenzymes, methanofuran (MFR), tetrahydromethanopterin (H_4MPT), and coenzyme M (CoM) (Kern *et al.*, 2016; Liu & Whitman, 2008).

Initially, CO_2 binds to MFR and is reduced to the formyl level, at this step ferredoxin (Fd) is the direct electron donor. The formyl group is then transferred to H_4MPT, forming formyl-H_4MPT. The formyl group is then dehydrated to methenyl group, which is subsequently reduced to methylene-H_4MPT and then to methyl-H_4MPT. Reduced F_{420} ($F_{420}H_2$) acts as the electron donor in these two reduction steps. The methyl group is then transferred to CoM, forming methyl-CoM (Ferry, 2011). The last reduction step reduces methyl-CoM to methane by methyl coenzyme M reductase (*mcrA*), which is the key enzyme in methanogenesis. Coenzyme B (CoB) serves as the electron donor in this reduction, and the oxidized CoB forms a heterodisulphide with CoM (CoM-S-S-CoB). Lastly, the heterodisulphide is reduced to regenerate thiols (Liu & Whitman, 2008).

Thermodynamically, two reactions - the methyl transfer from H_4MPT to CoM and the reduction of the heterodisulphide are exergonic and are involved in energy conservation. The methyl transfer reaction is catalysed by methyl-H_4MPT:HS-CoM methyltransferase (Mtr), which is a membrane-bound complex. The reduction of the heterodisulphide is catalysed by heterodisulphide reductase (Hdr), which is a membrane-bound complex in *Methanosarcina* and coupled to $F_{420}H_2$ dehydrogenase (Fpo) when H_2 is present. The reduction of CO_2 to formyl-MFR is endergonic and driven by ion gradient via the membrane-bound energy conserving hydrogenase (Ech) (Ferry, 2011).

Some hydrogenotrophic methanogens can also use secondary alcohols, such as 2-propanol, 2-butanol, and cyclopentanol, as electron donors. A small number can use ethanol. The secondary alcohols are oxidized to ketones via coenzyme F_{420}-dependent secondary alcohol dehydrogenases (Adf). Ethanol is oxidized to acetate via a nicotinamide adenine dinucleotide phosphate (NADP)–dependent alcohol dehydrogenase. Although the growth on alcohols is poor compared to that on H_2, it is an important exception to the generalization that methanogens cannot directly metabolize most organic compounds. Even in this case, where the substrate is obviously assimilated, the oxidation is incomplete, and methane is derived from CO_2 reduction (Shlimon *et al.*, 2004; Wirth *et al.*, 2012).

Furthermore, CO can be used as a reductant for methanogenesis from CO_2. *Methanothermobacter thermoautotrophicus* and *Methanosarcina barkeri* have shown to utilize CO. In this instance; four molecules of CO are oxidized to CO_2 using CO dehydrogenase (CODH) before one molecule of CO_2 is reduced to methane. H_2 is an intermediate in this reaction and serves as the direct electron donor for the reduction of CO_2 (Lessner, 2009).

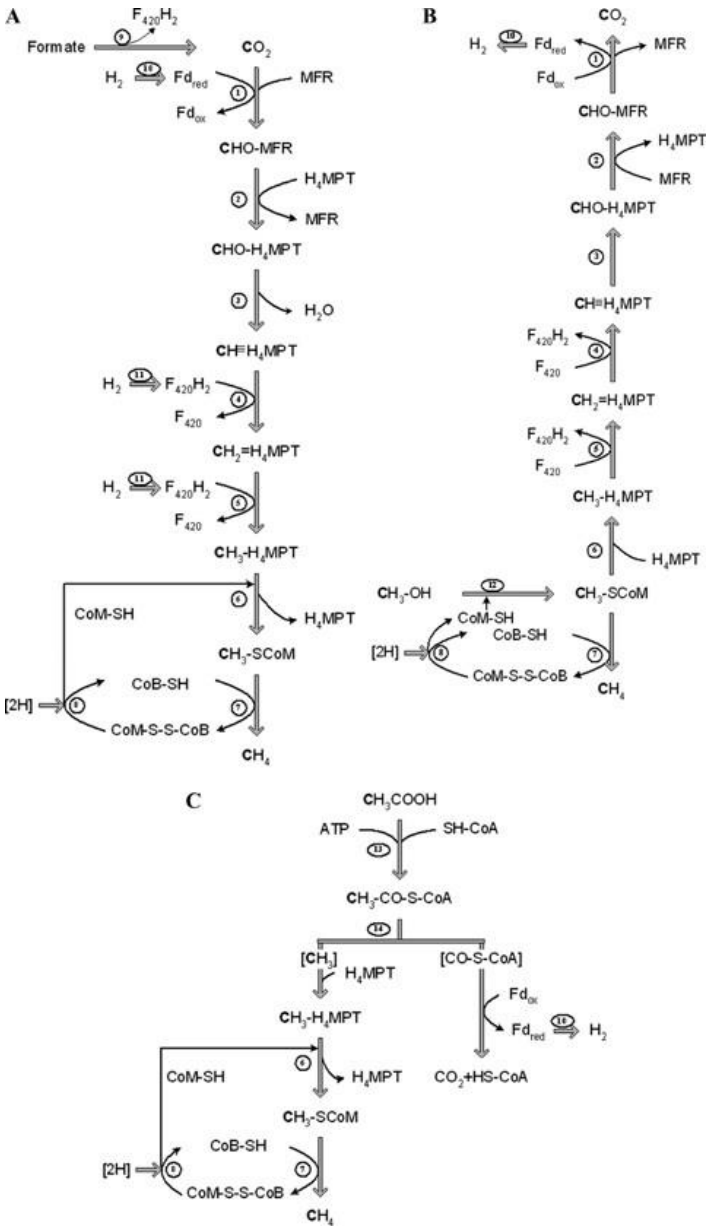

Figure 1.6: Pathways of methanogenesis. (**A**) Methanogenesis from H_2/CO_2 or formate. (**B**) Methanogenesis from methanol. (**C**) Methanogenesis from acetate. Abbreviations: Fdred= reduced form of ferredoxin; Fdox= oxidized form of ferredoxin; $F_{420}H_2$= reduced form coenzyme F_{420}; MFR - methanofuran; H4MPT = tetrahydromethanopterin; CoM-SH = coenzyme M; CoB-SH = coenzyme B; CoM-S-S-CoB = heterodisulphide of CoM and CoB; CoA-SH = coenzyme A. Enzymes: 1. formyl-MFR dehydrogenase (Fmd); 2. formyl-MFR:H4MPT formyl-transferase (Ftr); 3. methenyl-H4MPT cyclohydrolase (Mch); 4. methylene-H4MPT dehydrogenase (Hmd); 5. methylene-H4MPT reductase (Mer); 6. methyl-H4MPT:HS-CoM methyltransferase (Mtr); 7. methyl-CoM reductase (Mcr); 8. heterodisulphide reductase (Hdr); 9. formate dehydrogenase (Fdh); 10. energy-conserving hydrogenase (Ech); 11. F420-reducing hydrogenases; 12. methyltransferase; 13. acetate kinase (AK)-phosphotransacetylase (PTA) system in Methanosarcina; AMP-forming acetyl-CoA synthetase in Methanosaeta; 14. CO dehydrogenase/acetyl-CoA synthase (CODH/ACS). (Taken from Liu & Whitman, 2008).

4.2 Acetotrophic pathway

The second type of substrate is acetate. Acetate is a major intermediate in the anaerobic food chain, and as much as two-thirds of the biologically generated methane is derived from acetate. Only two genera are known to use acetate for methanogenesis: *Methanosarcina* and *Methanosaeta*. They carry out an acetotrophic reaction that splits acetate, oxidizing the carboxyl-group to CO_2 and reducing the methyl group to CH_4 (**Figure 1.6 C**). Acetate kinase and phosphotransacetylase function together to activate acetate to acetyl–CoA prior to cleavage by the CO dehydrogenase/acetyl–CoA synthase complex (Bialek *et al.*, 2011; Ma *et al.*, 2015).

The five-subunit CO dehydrogenase/acetyl–CoA synthase complex in the *methanosarcina* functions to cleave the C–C and C–S bonds in the acetyl moiety of acetyl–CoA, oxidize the carbonyl group to CO_2 (CO dehydrogenase activity), and transfer the methyl group to tetrahydrosarcinapterin (H_4SPT), an analogue of H_4MPT. The enzyme complex can also catalyse the synthesis of acetyl–CoA (Ravichandran *et al.*, 2015).

4.3 Methylotrophic pathway

Methyl-group containing compounds can also be used as substrate. These include methanol, methylated amines (monomethylamine, dimethylamine, trimethylamine, and tetramethylammonium), and methylated sulphides (methanethiol and dimethylsulphide). Methanogens that are able to use methylated compounds, or methylotrophic methanogens, are limited to the order Methanosarcinales, except for *Methanosphaera* species, which belong to the order Methanobacteriales. During methanogenesis, the methyl-groups from methylated compounds are transferred to a cognate corrinoid protein and then to CoM (**Figure 1.6 B**) (Ferry, 2011; Liu & Whitman, 2008).

Methyl-CoM subsequently enters the methanogenesis pathway and is reduced to methane. The activation and transfer of the methyl group requires substrate-specific methyltransferases. Interestingly, all known methylamine methyltransferases contain a UAG (amber codon)-encoded L-pyrrolysine designated the twenty-second

genetically encoded amino acid. This implies a connection between the abilities of amber codon translation and methylamine utilization. In most methylotrophic methanogens, the electrons required for the reduction of the methyl groups to methane are obtained from the oxidation of additional methyl-groups to CO_2, which proceeds stepwise as the reverse of hydrogenotrophic methanogenesis (Borrel *et al.*, 2011; Liu & Whitman, 2008).

In methylotrophic methanogenesis, three methyl groups are reduced to methane for every molecule of CO_2 formed. This process is termed a disproportionation, since the oxidation of a portion of the substrate is used to reduce the remainder. Different from this mode of growth, the methylotrophic growth of *Methanomicrococcus blatticola* and *Methanosphaera* species is H_2-dependent. They are obligate methylotrophic and hydrogenotrophic methanogens that are specialized to reduce methyl groups with H_2. The metabolism of *Methanosphaera* is restricted to methanol, while *M. blatticola* can use both methanol and methylamine (Franke-Whittle *et al.*, 2014; Nunoura *et al.*, 2008; Wright *et al.*, 2004).

All three methanogenic pathways have different enzymatic reactions and intermediates; however they share similar features in the final steps of CH_4 production (**Figure 1.6**). The hydrogenotrophic and acetotrophic pathways each result in the yield of a carrier bound methyl intermediate. In hydrogenotrophic pathway the carrier protein is methyl-tetrahydromethanopterin (CH_3-H_4MPT) and methyl-tetrahydrosarcinapterin (CH_3-H_4SPT), a derivative of methanopterin, is the carrier protein in the acetotrophic pathway (Ferry, 2011).

In this final step, the reduction of methyl-coenzyme M (CoM-S-CH_3) with a coenzyme B (CoB-SH), a secondary thiol coenzyme, to form CH_4 and the heterosulphide CoM-S-S- CoB is catalysed by methyl-coenzyme M reductase (MCR) making MCR the key enzyme in methanogenesis (Garcia, 1990). All known methanogenic Archaea express MCR.

Currently the presence of MCR is considered a diagnostic indicator of methanogenesis. The genomes of all methanogenic archaea encode at least one copy of the *mcrA* operon. Composed of two alpha (*mcrA*), beta (*mcrB*) and gamma (*mcrG*) subunits (**Figure 1.7**), it also contains a unique porphinoid nickel (Ni)-containing active site called coenzyme F_{430} with a molecular mass of 905 Da. Functional constraints on

its catalytic activity have resulted in a high degree of MCR amino acid sequence conservation, even between phylogenetically distant methanogenic lineages. This conserved primary structure has been used to develop degenerate PCR primers for recovering naturally occurring *mcrA* fragments from a variety of environments (Dhillon *et al.*, 2005; Hallam *et al.*, 2003; Ziemiński & Frąc, 2012). MCR has a molecular mass of 300 kDa. This enzyme is used as a functional gene marker for methanogenic Archaea (Baxter *et al.*, 2005; Luton *et al.*, 2002).

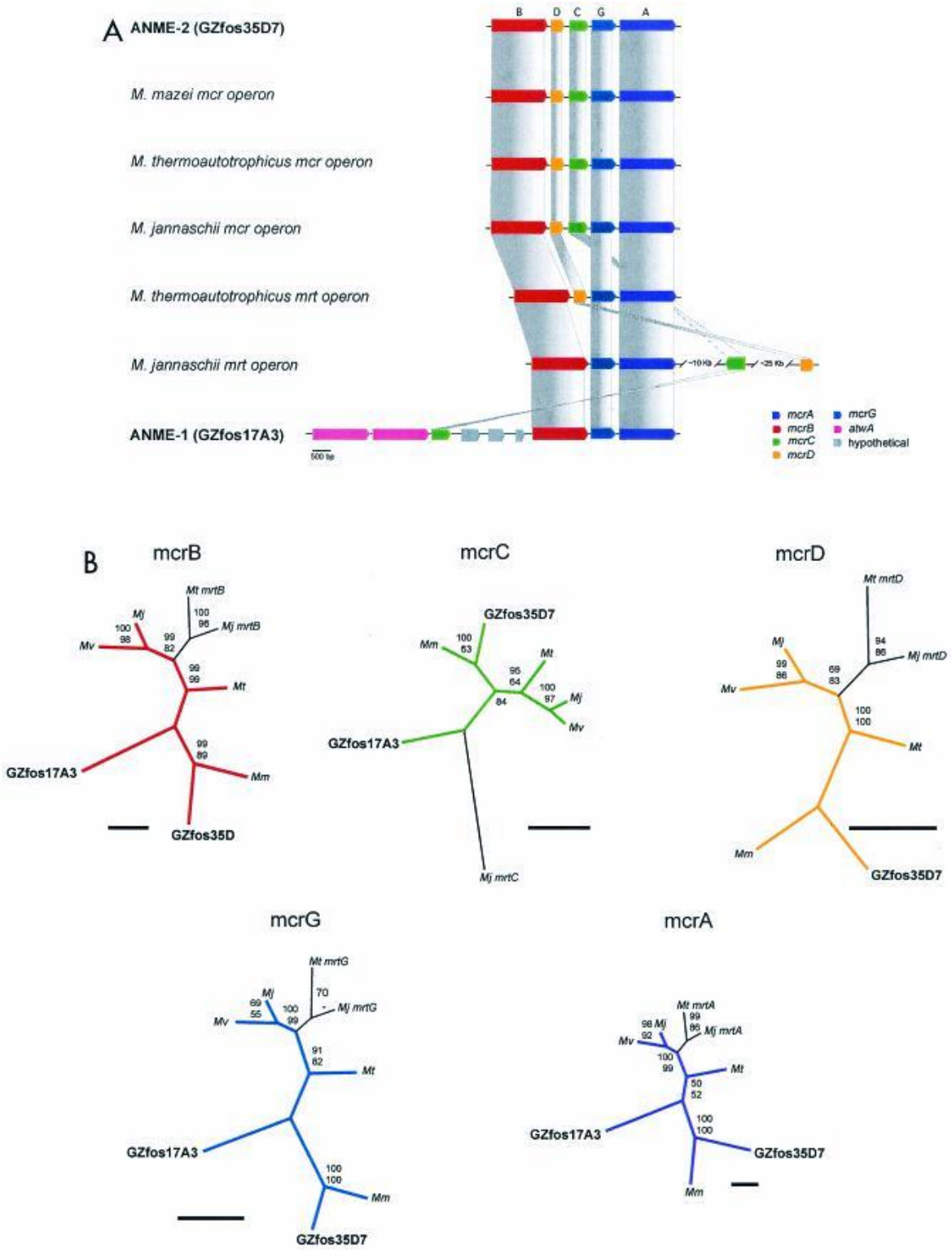

Figure 1.7: (**A**) Schematic depiction of *mcrA* methanogenic lineages. The *mcrA* operon consists of *mcrBDCGA*. (**B**) Gene trees for *mcrA* Abbreviations for methanogenic species harbouring these genes are *Mj, Methanocaldococcus jannaschii; Mv, Methanococcus vanni; Mt, Methanothermobacter thermautotrophicus*; and *Mm, Methanosarcina mazei*. (Taken from Hallam, 2003).

5 Factors affecting performance and biogas yield

5.1 Parameters and process optimisation

Anaerobic digestion of organic waste is prone to many inhibition problems which can be difficult to control. Therefore in order for the biogas process to be effective and productive, there are a number of parameters that have to be optimised. These include temperature, nutrients available, retention time, pH level, VFA's, antibiotics, heavy metals and metals (Drosg, 2013; Seadi *et al.*, 2008; VDI 4630, 2006; Weiland, 2010).

5.1.1 Incubation temperature

Temperature is a critical parameter for anaerobic digestion since it influences both system heat requirements and methane production. Anaerobic digestion can occur between 3°C and 70°C (Yokoyama & Matsumura, 2008). Differentiation is generally made between three temperature ranges; psychrophilic (15 - 25°C), mesophilic (35 - 40°C), and thermophilic (50 - 60°C) conditions. Mesophilic anaerobic digestion is the most common system which has a more stable operation. Mesophilic temperatures are also typically recommended for methane production from animal manure (Schievano *et al.*, 2012; Vindis & Mursec, 2009).

The rate of biogas production increases with temperature. If the temperature of an anaerobic digester is below 15°C, gas production will be so low that the biogas plant is no longer economically feasible. Thermophilic temperature ranges have sparked more interest in industry due to the fact that higher temperatures reduce pathogens, thermophilic temperatures also provide more rapid reaction rates than mesophilic temperatures. Thermophilic anaerobic digestion in general are more efficient in biogas production but associated with more energy requirements for heating therefore higher maintenance cost, increased sensitivity to thermal shock and higher odour formation resulting from a higher VFA's concentration (Lukehurst *et al.*, 2010; Luo & Angelidaki, 2012; Zeb *et al.*, 2013).

Temperature changes can affect optimum biogas production, however brief fluctuations not exceeding ± 1-2°C/h may be regarded not harmful to the process. The temperature fluctuations between day and night are no great problem for plants built

underground, since the temperature of the earth below a depth of one meter is practically constant (Igoni *et al.*, 2008; Krátký *et al.*, 2012).

5.1.2 Antibiotics and heavy metals

Antibiotics (Bacitracin, Flavomycin, Lasalocid, Monensin, Spiramycin) and detergents used in livestock husbandry are obvious inhibitors of the biogas production process, because by definition, antibiotics and detergents are both toxic to and are used to kill microorganisms (Shlimon *et al.*, 2004; Triantafyllou *et al.*, 2014). Both substances are used in livestock production to treat sick animals and to keep animal houses and milking parlours clean and can therefore also be found in the slurry, but only at concentrations; in fact so low that they do not have a negative impact on the biogas plant (Bialek *et al.*, 2011; Lukehurst *et al.*, 2010; VDI 4630, 2006; Zeb *et al.*, 2013).

Other substances such as heavy metals (e.g. cadmium (Cd), lead (Pb), chromium (Cr) and Mercury (Hg)) can also inhibit the process at high concentrations (Jørgensen, 2009). However, as previously mentioned, some metals such as nickel (Ni), cobalt (Co), copper (Cu), iron (Fe), zinc (Zn), molybdenum (Mo) are essential for the process at low concentrations, in the same way that vitamins are for human beings (Yadvika *et al.*, 2004).

5.1.3 Available nutrients

Incoming substrate does not only allow high methane production, but should also provide the necessary nutrients. In order to guarantee a sufficient supply of nutrients for specific microbial groups in biogas production, the substrate C: N: P: S ratio should be about 600:15:5:1 (Weiland, 2010). In addition, in order for microorganisms to thrive in a digester they need more than just a supply of carbon and energy. They also require certain trace elements. Therefore in addition to carbon; nitrogen, phosphorus and sulphur the generation of biogas iron, nickel, manganese, cobalt, selenium, molybdenum and tungsten are also required. In the case of methanogens, the presence of nickel and cobalt which are necessary for the cofactor F_{430} and the corrinoid factor III respectively are particularly important. In order to avoid the addition of artificial micronutrients, energy crops and manure must be used. Thereby the micronutrients are provided by the manure. However, the addition of artificial

micronutrients can still enhance the biogas production process (Bialek *et al.*, 2011; Garcia, 1990; Lessner, 2009; Weiland, 2010).

5.1.4 pH value

The pH value gives an approximate indication on the state of the digestion process (Drosg, 2013). The use of pH as a process indicator is normally based on the fact that a pH drop corresponds to VFA accumulation. Some anaerobic systems apply pH monitoring and control where acid or base are added to ensure suitable pH for microbial growth. Reduction in pH can be controlled by the addition of lime, sodium hydroxide or sodium bicarbonate (Hem, 1985). Chen and colleagues (2010) reported that in a biogas production reactor, alkalinity of about 2,500 mg $CaCO_3$/L and pH above 7 was maintained by adding 0.2 g NaOH/g VS (Volatile Solids) (Chen *et al.*, 2010). The results of this study indicated that it was necessary to use chemicals, such as sodium hydroxide (NaOH), to control the pH of the single-stage anaerobic digester treating the food waste.

In a reactor with low buffering capacity and no pH control, VFA accumulation can decrease pH quickly, therefore pH is an effective process indicator. The optimum environment for methanogens is slightly alkaline conditions. Once the anaerobic digestion process has stabilized, the pH will normally take on a value of between 6.5 and 8, and the preferred level is 7.2. Due to the buffer capacity in biogas plants, which is dependent on dissolved carbon dioxide-bicarbonate (CO_2 - HCO_3^-) and ammonia-ammonium (NH_3 - NH_4^+), a detectable pH change takes place only after process instability has started. Therefore; the measurement of the pH value is not suitable as early indicator of process imbalance, but gives important information for process monitoring. If the pH value drops below 6.2, the medium will have a toxic effect on the methanogenic species (Lee *et al.*, 2015; Raposo *et al.*, 2006; Zhang *et al.*, 2014).

As the digestion proceeds and reaches methanogenesis, protein degradation increases the ammonia concentration through release of amino groups. The produced ammonia acts as a buffer and during this time, pH can reach 8 or above. After the methanogenesis process has stabilized, the pH becomes stable between 7.2 and 8.2 (Dioha *et al.*, 2013; Igoni *et al.*, 2008; Merlin *et al.*, 2014; Wolfe, 2011; Yokoyama & Matsumura, 2008). Thus, in anaerobic digesters, ammonia is also responsible for

buffering, and stabilizes pH when present up to 1000 mg/L concentration (Fricke *et al.*, 2007).

5.1.5 Retention time

Retention time is defined as the time during which the feedstock remains in the biogas digester. The retention time can only be accurately defined in batch-type reactors. For continuous systems, the mean retention time is approximated by dividing the digester volume by the daily influent rate. Depending on the vessel geometry and the rate of mixing, the effective retention time may vary widely for the individual substrate constituents. Selection of a suitable retention time thus depends not only on the process temperature, but also on the type of substrate used (Athanasoulia *et al.*, 2012; Dhanya *et al.*, 2009; Lee *et al.*, 2015; Seadi *et al.*, 2008; Thy *et al.*, 2003).

5.1.6 Volatile fatty acids (VFA)

In a reactor, methanogens are susceptible to high concentrations of acids and as a result acidic conditions can inhibit their growth. VFA's are intermediate compounds of the biogas production process and their high concentration can have a negative effect on the microorganisms. The accumulation of VFA's in a reactor reflects a kinetic uncoupling between acid producers and consumers. This indicates organic overload; ideally, biomass should be added to the reactor in relation to the growth rate of the methanogens, also organic acids have to be used up by microorganisms at the rate at which they are produced (Ali Shah *et al.*, 2014; De Mes *et al.*, 2003; Weiland, 2010).

If more biomass is added than the bacteria are able to degrade, the process will become acidic. The biomass also has to be fed to the reactor at an even rate and volume, preferably as a continuous feed. If the substrate has to be changed, this must be done gradually, so that bacteria can adapt to the new conditions (Jørgensen, 2009). The main intermediates produced during anaerobic digestion of organic material are acetic, propionic, butyric and valeric acids (Weiland, 2010).

The presence of different volatile fatty acids is also affected by pH of reactor medium. Yu and colleagues (2008) reported that there was no effect of pH on propionic acid production, whereas with increase in pH, the yield of acetic acid slightly increased while that of butyric acid increased with decrease in pH (Yu *et al.*, 2008). However, if

the ammonia concentration in the medium is very high or substrate contains high concentrations of proteins, accumulation of VFA will not lead to acidification due to buffer capacity (high level of bicarbonate) provided by ammonia (Drosg, 2013; Hagens *et al.*, 2015; Pai *et al.*, 2001).

In a low buffered system, pH, partial alkalinity and VFA measurements are useful for process monitoring whereas in highly buffered system only VFA is reliable for indicating process imbalance (Franke-Whittle *et al.*, 2014). VFA is commonly measured by gas chromatography (GC) with flame ionization detection (FID), for individual VFA, or titration to give total VFA, which is cheaper and widely used in commercial biogas plants. Several titration methods for determination of total VFA have been proposed, e.g. a simple titration (Anderson & Yang, 1992), a 5-point titration (Moosbrugger *et al.*, 1993), and an 8-point titration (Lahav *et al.*, 2002).

6 Benefits of biogas production

6.1 Renewable energy source

Compared to fossil fuels, biogas is renewable, as it is produced on biomass. Biogas production will not only improve the energy balance of a country but also make an important contribution to the preservation of the natural resources and to environmental protection (Börjesson & Mattiasson, 2008; Zerihun, 2015).

6.2 Reduces greenhouse gas emissions and mitigation of global warming

An increase of the current CO_2 concentration in the atmosphere causes global warming as carbon dioxide is a greenhouse gas (GHG). The combustion of biogas also releases CO_2, however the carbon in biogas is utilized as a form of energy, and for this reason biogas technology has gained a lot of attention. Biogas production reduces emissions of methane (CH_4) and nitrous oxide (N_2O) from storage and utilisation of untreated animal manure as fertiliser. If biogas becomes a more favoured and widely used form of energy production and upgraded as fuel for transport, a reduction of emissions of CO_2, CH_4 and N_2O will occur thus contributing to mitigating

global warming (Ackah *et al.*, 2016; Börjesson & Mattiasson, 2008; Höök & Tang, 2013).

6.3 Reduced dependency on imported fossil fuels

Fossil fuels are limited resources and they are concentrated in limited geographical areas of our planet. As a result, many countries which lack these fossil fuel reserves become permanently dependent on import of energy from other countries. A lot of European countries are strongly dependent on fossil energy imports from regions rich in fossil fuel sources such as Russia and the Middle East. Thus, developing and implementing renewable energy systems such as biogas plants, based on national and regional biomass resources, will increase security of national energy supply and diminish dependency on imported fuels (Bagher *et al.*, 2015; Lantz *et al.*, 2007; Owens *et al.*, 2010).

6.4 Contribution to African Union (AU) and European Union (EU) energy and environmental targets

Climate change presents significant threats to the achievement of the Millennium Development Goals (MDGs) especially those related to promoting environmental sustainability. Fighting global warming is one of the main priorities of the United Nations (UN) and environmental policies. The European targets of renewable energy production, reduction of GHG emission, and sustainable waste management are based on the commitment of the EU member states to implement appropriate measures to reach the targeted goals. The production and utilisation of biogas has the potential to comply with all three targets (Amigun *et al.*, 2008; IPCC, 2000; Lantz *et al.*, 2007; Pegels, 2010; Raven & Gregersen, 2007).

6.5 Waste reduction

Biogas production has the ability to convert waste material into a valuable resource, by using it as substrate for anaerobic digestion. Many countries such as South Africa have an overproduction of organic wastes from industry, agriculture and households. Biogas production is an excellent way to dispose this organic waste. This is also a

good way to reducing the volume of waste and of costs for waste disposal (El-Mashad & Zhang, 2010; Krátký *et al.*, 2012; Krich *et al.*, 2005; Pegels, 2010).

6.6 Job creation

The collection and transport of feedstock, manufacturing of technical equipment, construction, operation and maintenance of biogas plants all require workforce. This means that the development of a national biogas sector contributes to the establishment of new enterprises, some with significant economic potential, increases the income in rural areas and creates new jobs (Rasi, 2009; Seadi *et al.*, 2008; Vindis *et al.*, 2009).

6.7 Flexible and efficient end use of biogas

Biogas is a flexible energy carrier, suitable for many different applications. One of the simplest applications of biogas is the direct use for cooking and lighting, but in many countries including Germany biogas is used heating and power generation or it is upgraded and fed into natural gas grids, used as vehicle fuel or in fuel cells (Luo & Angelidaki, 2012; Weiland, 2010).

6.8 Low water inputs

Compared to other biofuels, biogas has some advantages. The biogas production process needs the lowest amount of process water. This is an important aspect related to the current and expected future water shortages in many countries (Seadi *et al.*, 2008).

6.9 Digestate is an excellent fertiliser

A biogas plant is not only a supplier of energy. The digestate that comes out of biogas systems is rich in nutrients such as nitrogen, phosphorus, potassium and micronutrients [Boron (B), Copper (Cu), Chlorine (Cl), Selenium (Se) and Zinc (Zn)]. It can be applied directly on soil in order to help plants grow. Thereby the digestate replaces chemical fertilizers. Compared to raw animal manure, digestate has improved fertiliser efficiency due to higher homogeneity and nutrient availability, better C/N ratio

and significantly reduced odours. This saves money and is better for the environment, therefore biogas production can be perfectly integrated into conventional and organic farming (Bagge, 2009; Drosg, 2013; De Mes *et al.*, 2003).

6.10 Reduced odours and pathogens

Storage and application of liquid manure, animal dung and many organic wastes are sources of persistent unpleasant odours and pathogens. Anaerobic digestion reduces these odours by up to 80% and improves hygienic conditions through reduction of pathogens. In order to be suitable for use as fertiliser, digestate is submitted to a controlled sanitation process (Fleming *et al.*, 2006; Seadi *et al.*, 2008).

Depending on the type of feedstock involved, sanitation can be provided by the anaerobic digestion process itself, through a minimum guaranteed retention time of the substrate inside the digester, at thermophilic temperature, or it can be done in a separate process step, by pasteurisation or by pressure sterilisation. In all cases, the aim of sanitation is to inactivate pathogens, weed seeds and other biological hazards and to prevent disease transmission through digestate application. Digestate is almost odourless and the remaining ammonia odours disappear shortly after application as fertiliser (Bond & Templeton, 2011; Lukehurst *et al.*, 2010; Merlin *et al.*, 2014; Seadi *et al.*, 2008).

7 Conclusions

Anaerobic digestion from organic waste has become very popular due to the benefits offered by biogas production. Biogas is primarily composed of methane (CH_4) and carbon dioxide (CO_2). The quality of biogas generated by the organic waste material does not remain constant but varies with the period of digestion. However, the methane concentration also depends on substrate composition, temperature, pH and pressure. Temperature has a great influence on the methanogenic community in biogas reactors; therefore maintaining constant temperature is of high priority in any biogas digester. The main intermediates produced during anaerobic digestion of organic material are acetic, propionic, butyric, and valeric acid.

Many studies have highlighted the use of the *mcrA* gene as the target for the detection of methanogens in a wide range of environments, this is because all known methanogens express the enzyme methyl coenzyme M reductase (MCR), which catalyses the reduction of a methyl group bound to coenzyme-M, with the concomitant release of methane. This enzyme complex is unique to methanogens thus, making it a suitable tool for their specific detection.

All methanogenic pathways have in common the demethylation of methyl coenzyme M to methane and reduction of CoM-S-S-CoB to the sulfhydryl forms of the cofactors catalysed by heterodisulphide reductase. The heterodisulphide reductase accepts electrons from a membrane-bound electron transport chain coupled to proton extrusion providing a chemical gradient that drives ATP synthesis by a membrane-bound ATPase. Thus, the different methanogenic pathways (hydrogenotrophic, acetotrophic and methylotrophic) are distinguished by the method of acquiring the methyl group for conversion to methane.

The production and utilisation of biogas provides environmental and socio-economic benefits for the society as a whole, as well as for the involved farmers. Utilisation of the internal value chain of biogas production enhances local economic capabilities, safeguards jobs in rural areas and increases regional purchasing power. It improves living standards and contributes to economic and social development.

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CHAPTER 2

2 Introduction to study

2.1 Introduction

In recent years, the issue of global warming and climate change have received strong public attention and have become a major environmental concern globally. One of the main environmental problems facing society today is the increased greenhouse gas emissions (Chaudhary *et al.*, 2011; Krich *et al.*, 2005; Schittenhelm, 2008). Coal, natural gas and crude oil are fossil fuels which are major contributors to CO_2 emissions in the atmosphere (Dioha *et al.*, 2013; Vindis *et al.*, 2009; Weiland, 2010).

In addition, population growth and consequently increasing waste generated from food industries, agriculture and households has also become a concern. There is also an increasing demand for a sustainable and environmentally friendly source of energy; one option within the renewable energy spectrum is biogas.

Biogas is the gaseous product of the anaerobic digestion of organic material. It consists primarily of methane and carbon dioxide; it can also contain other gases such as nitrogen, ammonia, hydrogen sulphide, water vapour and other gases depending on the substrate (VDI 4630, 2006). Biogas production is an efficient way for waste management, it also offers considerable environmental and economic benefits. As a source of renewable energy, biogas production can improve the quality of the environment and it can also provide new job opportunities to local communities (Krátký *et al.*, 2012; Krich *et al.*, 2005).

Biogas can be used as an energy source for heating and generating electricity. The main advantage of biogas production is the ability to use different types of feedstock for anaerobic digestion. Livestock waste such as animal manure, slurries and waste water are good substrates for biogas production. Energy crops and agricultural waste from abattoirs, wineries and vegetable-processing plants have also been used to make biogas. Food waste from restaurants and hotels as well as the organic fraction of municipal solid wastes also serves as good substrate for biogas production.

It is international practice to characterize unusual substrate mixtures or organic waste to determine its feasibility as biogas substrate and pay special attention to the

microbiological processes as this remains a hurdle for implementation and ultimately the roll out of this technology. The biogas yield is highly dependent on the substrates fed to the digester. Organic dry matter content, the rate of digestion as well as the retention time have an influence on the final gas production and gas quality. The higher the methane content the higher the actual energetic value of the gas (Dhanya *et al.*, 2009; Owczuk *et al.*, 2013; VDI 4630, 2006; Vindis *et al.*, 2009).

Substrate characterization is necessary especially if the substrate contains interferents or if it is of a consistency which makes it unsuitable for the digestion process. This results in characterization features such as consistency and homogeneity and also the substrate's interferent or pollutant content (VDI 4630, 2006). Another important characterization feature is the chemical composition of the substrate. These include, dry matter content and volatile total solids (organic total solids) and from this, conclusions can be made regarding not only fermentability but also the gas yield to be expected from the substrate (VDI 4630, 2006). The N, P, K and Mg parameters are used for characterizing the substrate with regard to its fermentability and in assessing and classifying the fermentation product as fertilizers in accordance with the Fertilizer Ordinance (VDI 4630, 2006).

In biogas production the digester is the core part of every plant where the anaerobic digestion takes place. The digester can also be subjected to conditions necessary to enhance biogas production such as heating and stirring. The biogas production process can be divided into four major steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Abdel-Hadi, 2008; Oyewole, 2010; Zhang *et al.*, 2014).

Basic information is available to estimate energy yields for many feedstocks and combinations of feedstocks without full testing. However, in order to obtain an accurate estimate of the expected gas yield, laboratory tests need to be conducted. Biogas is produced from the organic (volatile) fraction of the feedstock, therefore in order to understand how this whole process comes about, it is important to have a full feedstock characterisation (VDI 4630, 2006).

Currently South Africa does not have an existing substrate database which can be used to estimate the biogas potential for electricity generation from the agro-waste industry. Therefore to gain insight on the phenomena occurring in the anaerobic digestion processes, to increase the process performance and methane production; it

is important to monitor the microbial diversity especially the methanogens in the digester (Dhillon *et al.,* 2005). To this effect, many other methods have been developed in order to increase the efficiency of the anaerobic digestion process and optimize the operating conditions of the anaerobic system.

2.2 Main Objectives

- The objectives of this study include the benchmarking of a spectrum of analytical techniques and evaluating various feedstocks for their biogas potential.

- This study also focused on characterizing the microbial diversity contributing to biogas production and to study the microbial population dominance during the biogas process.

- Lastly, the biogas production process was optimized using a selected substrate (Spent Mushroom Substrate).

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CHAPTER 3

3 Microbial diversity and the enrichment of cultures contributing to the biogas process

3.1 Introduction

Microbial diversity in biogas digesters is as vast as that of ruminating animals that in essence also harness the biogas process steps to digest and assimilate their food. However, the microbial community underlying the anaerobic digestion process is not well understood, especially with regards to microbial community diversity and dynamics in response to variations in feedstocks as well as operation conditions. Microorganisms are the driving force for the whole conversion process and thus specific microorganisms and their metabolic activities during anaerobic digestion depend on the chemical composition of the feedstock, environmental factors and digester operating conditions (Merlin *et al.*, 2014; Nunoura *et al.*, 2008).

The conversion of complex organic compounds to CH_4 is possible due to the cooperation of four different groups of microorganisms, hydrolytic bacteria, acid producing microorganisms, fermenting bacteria and methanogenic archaea. These microorganisms are ubiquitous in several environments and play various roles during the process of anaerobic digestion (Ali Shah *et al.*, 2014; Montero *et al.*, 2010; Zverlov *et al.*, 2010).

Several studies have established the widespread and fundamental role of bacteria in the anaerobic degradation processes in nature (Bialek *et al.*, 2011). During hydrolysis, various hydrolytic microorganisms degrade complex organic polymers to monomers such as amino acids and sugars. For example, microorganisms responsible for cellulose degradation use either free extracellular or cell-anchored enzyme complexes including cellulosomes, the latter more commonly found in anaerobic environments (Chojnacka *et al.*, 2015; Phan & Sabaratnam, 2012; Wirth *et al.*, 2012).

Medie and colleagues (2012) conducted a study on 1500 completed bacterial genomes, the study revealed that approximately 38% of the sequenced bacterial genomes encoded at least one cellulase gene, with a small fraction containing more than three cellulases. The cellulase gene product enables effective degradation of

cellulose (Medie *et al.*, 2012). The genes necessary for degradation of cellulose have been found in bacteria belonging to several different phyla: *Actinobacteria, Fimicutes, Bacteroidetes, Thermotogae, Choloroflexi and Proteobacteria* (Alvarado *et al.*, 2014; Sun *et al.*, 2015; Weiss *et al.*, 2008).

Bacterial communities specifically in biogas processes have been studied using various methods. These include cultivation, molecular methods including construction of clone libraries and sequencing by targeting 16S rRNA genes and functional genes involved specifically in each step of the biogas process (Manyi-Loh *et al.*, 2013; Sun *et al.*, 2015). In addition, the development and application of next-generation sequencing technologies has enabled time and cost-efficient studies of the microbial communities in various biogas processes (Wirth *et al.*, 2012).

The majority of cultivated hydrolytic bacteria isolated from different anaerobic environments mainly belong to the order *Clostridiales* (Bagge, 2009; Kröber *et al.*, 2009). *Clostridiales* play an important role specifically in the hydrolysis step, based on results obtained using a metagenomic approach (Weiss *et al.*, 2008). Bacteria from this order have also been shown to dominate in various anaerobic digestion processes operating with various lignocellulosic materials, such as wheat straw, cow manure, chicken manure, maize straw and hay (Chen, 2014; Ismail & Talib, 2014; Luostarinen *et al.*, 2011). Bacteria which belong to the order *Bacteroidales* have been suggested to be involved in the degradation of lignocellulose materials, such as straw and hay, in biogas processes (Kröber *et al.*, 2009; Sun *et al.*, 2015).

Different stages of anaerobic digestion contain considerably varied species of symbiotic microorganisms. This includes bacteria from the genera *Propionibacterium, Butyrivibrio, Acetivibrio, Eubacterium, Bifidobacterium and Lactobacillus* which are facultative anaerobes (Alvarado *et al.*, 2014; Merlin *et al.*, 2014; Sunarto, 2015; Weiland, 2010), they use the oxygen introduced into the system to create favourable conditions for the growth of obligate anaerobes of the following genera: *Pseudomonas, Bacillus, Clostridium, Micrococcus and Flavobacterium* (Ali Shah *et al.*, 2014; Ziemiński & Frąc, 2012). Fermenting bacteria such as syntrophic and acetogenic bacteria play an important role during biogas production. Interspecies hydrogen transfer is a symbiotic relationship between acidogenic, acetogenic and methanogenic

microorganisms. During acetogenesis, acetate-producing bacteria including those of the genera of *Syntrophomonas and Syntrophobacter* convert the acid phase products into acetate and hydrogen which may be used by methanogenic archaea.

As earlier mentioned, all microorganisms based on analysis and comparison of conservative phylogenetic features, through analysis of 16S and 18S rRNA genes, were classified to three main taxonomic units of the living world. The phylogenetic domains are distinguished as Archaea, Bacteria and Eukarya (Mori & Harayama, 2011; Nunoura *et al.*, 2008; Ziemiński & Frąc, 2012).

Methanogens have been studied more closely in order to elucidate their systematic relationship with prokaryotes. According to analysis of sequence 16S rRNA genes, methanogens were classified to domain Archaea. Archaea are involved in the production of methane and they also show synergistic relationships with other populations of microorganisms. Among the microorganisms within domain Archaea, four groups are distinguished, the most visible being Crenarchaeota and Euryarchaeota (Ali Shah *et al.*, 2014; Ferry, 2009; Luo *et al.*, 2009; Wright *et al.*, 2004; Zeikus, 1977).

3.2 The aims of the chapter

- In order to gain a better understanding of the microbial diversity in a biogas digester, each step was mimicked through enrichment in specific media to study the diversity using various techniques.

- The methanogenic populations were identified using 16S rRNA gene sequencing and specifically the *mcrA* gene was targeted, which encodes the alpha subunit of the methyl-coenzyme M reductase (MCR), catalyzing the terminal enzyme complex in methanogenesis.

3.3 Materials and Methods

3.3.1 Seeding material for reactors

A cow manure sample used as seeding material in the initial part of this study was obtained from a local farm. This manure sample was used for all enrichments and initial feedstock biogas potential analyses in order to asses if it contains an adequate supply of microbial population, nutrients and buffering capacity.

3.3.2 Microbial enrichments

The media used for the primary and secondary enrichments of possible anaerobic and/or aerobic microorganisms involved in each process of the biogas steps can be seen in **Section 3.3.3.1 – 3.3.3.6** All chemicals used were purchased from Sigma-Aldrich® (Pty) Ltd and Merck (Pty) Ltd., unless otherwise stated.

3.3.3 Enrichment procedure

Serum vials were prepared by soaking in 15% nitric acid overnight, then rinsing with distilled water three times, the distilled water (dH_2O) used was 15 $M\Omega.cm^{-1}$ Barnstead™ TII water (Thermo Scientific) that was filtered and UV treated. The vials were then allowed to dry overnight in an oven at 105°C. Approximately 50 ml of each liquid media described in **Section 3.3.3.1 – 3.3.3.6** was dispensed in prepared serum vials with a capacity of 120 ml and inoculated with manure (5 g). One vial was not inoculated with any manure and this served as the negative control. The vials were sealed with butyl rubber stoppers and aluminium crimp caps. The headspace in each sealed vial was subsequently purged with nitrogen (N_2). All enrichment procedures were carried out in the anaerobic chamber (Coy Laboratories, Grass Lake, M.I., U.S.) with a 10% CO_2, 10% H_2, 80% N_2 headspace (Air Liquid, Alrode, S.A.). Inoculated vials as well as the negative controls were incubated at 37°C. After three weeks of incubation, 10% (v/v) inoculum was re-inoculated into fresh media and incubated for another three weeks at 37°C. This process was repeated until the enrichment media was sediment free. The samples were subjected to microbial and molecular analysis to identify the enriched consortium.

3.3.3.1 TYG media

TYG media was used for aerobic enrichments, specifically the cultivation of hydrolytic bacteria. The media was prepared according to ATCC 741 (American Type Culture Collection). All components **Table 3.1** were prepared in serum vials however the media was not degassed or purged with nitrogen.

Table 3.1: Components of TYG media (ATCC 741).

Component	Concentration (g/L)
Tryptone	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
pH	7.4

3.3.3.2 Thioglycollate media

Thioglycollate media was prepared according to MERCK 108190. Thioglycollate is a reducing agent and as a result this medium supports the cultivation of anaerobic bacteria. The medium was prepared as described in **Table 3.2**. The medium was purged with O_2-free N_2 to create anaerobic conditions. Following purging, the media was autoclaved (20 min at 121°C) and cooled to room temperature before being transferred into the anaerobic chamber.

Table 3.2: Components of thioglycollate media (MERCK 108190).

Component	Concentration (g/L)
Yeast Extract	5.0 g
Pancreatic Digest of Casein	15.0 g
Glucose (anhydrous)	5.0 g
L-Cystine	0.5 g
Sodium Chloride	2.5 g
Sodium Thioglycollate	0.5 g
Resazurin	0.001 g
pH	6

3.3.3.3 Basal media

The media was prepared according to the specifications of DSMZ 1001 (https://www.dsmz.de). All components (**Table 3.3**) were prepared in separate Schott bottles and dispensed into serum vials (50 ml). The media was degassed by flushing with N_2 to create anaerobic conditions. Following degassing, the media was autoclaved (20 min at 121°C) and cooled to room temperature before being transferred into the anaerobic chamber. Autoclaving is not expected to cause any significant change to the properties of the various enrichment media used. The vitamin and mineral solutions were prepared separately by filter sterilization (0.22 µm, GVS Filter Technology) (Wolfe, 2011). The vitamin and mineral solutions were filter sterilized into sealed pre-evacuated serum vials and specific volumes were transferred into the media as specified on **Table 3.3**. Enrichments were carried out in the anaerobic chamber to ensure an O_2 free environment.

Table 3.3: Components of the basal medium (DSMZ 1001).

Component	Concentration (g/L)	Component	Concentration (g/L)
1. Basal medium		**5. Mineral Solution (10.00 ml)**	
$NaHCO_3$	2.5 g	Nitrilotriacetic acid (NTA)	1.5 g
NH_4Cl	0.25 g	$MgSO_4$	3.0 g
$NaH_2PO_4.H_2O$	0.6 g	$MnSO_4.H_2O$	0.5 g
KCl	0.1 g	NaCl	0.1 g
Distilled water	980.00 ml	$FeSO_4.7H2O$	0.1 g
2. 1. 0 M Acetate Solution (1 ml)		$CoCl_2.6H_2O$	0.1 g
$C_2H_3NaO_2$	13.6 g	$CaCl_2$	0.1 g
Distilled water	100 ml	$ZnSO_4.7H_2O$	0.1 g
3. 500 mM Fe (III) NTA (1 ml)		$CuSO_4.5H_2O$	0.01 g
$NaHCO_3$	8.2 g	$AlK\ (SO_4)_2.12H_2O$	0.01 g
Na_3Nitrilotriacetic acid (NTA)	12.8 g	H_3BO_3	0.01 g
$FeCl_3.6H2O$	13.5 g	$Na_2MoO_4.2H_2O$	0.01 g
Distilled water	1.0 L	Distilled water	1,0 L
4. Vitamin Solution (10.00 ml)		**6. pH**	6.8 – 7.0
Biotin	2.0 mg		
Folic acid	2.0 mg		
Pyridoxine hydrochloride	10.0 mg		
Thiamine	5.0 mg		
Riboflavin	5.0 mg		
Nicotinic acid	5.0 mg		
Calcium D-(+)-pantothenate	5.0 mg		
Vitamin B_{12}	0.1 mg		
p-Aminobenzoic acid	5.0 mg		
Thioctic acid	5.0 mg		
Distilled water	1.0 L		

3.3.3.4 Acidogenic media

A medium designed to support the growth of acidogenic bacteria was prepared according to Leedle & Greening (1988). All components (**Table 3.4**) were prepared in separate serum vials. Following purging, the vials were autoclaved and transferred into the anaerobic chamber. Inside the anaerobic chamber, the mineral and vitamin solutions were added by filter sterilization. The Acidogenic media had a pH of 6 and 1.4 g/L NaCl, therefore methanogenic microorganisms are not expected to thrive.

Table 3.4: Components of acidogenic media (Leedle & Greening, 1988).

Component	Concentration (g/L)	Component	Concentration (g/L)
1. Media for Acidogens		**5. Vitamin Solution (10.0 ml)**	
K_2HPO_4	0.94 g	p-aminobenzoic acid	0.01 g
KH_2PO_4	0.28 g	Nicotinic acid	0.01 g
NaCl	1.4 g	Calcium Pantothenate	0.01 g
KCl	1.6 g	Pyridoxine	0.01 g
$MgSO_4.7H_2O$	0.2 g	Riboflavin	0.01 g
$CaCl_2.\ 2H_2O$	0.1 g	Thiamine	0.01 g
NH_4Cl	1.0 g	Biotin	0.05
2. Cysteine.HCl (10.00 ml)		Folic acid	0.005 g
3. 10 % NaHCO3	1 ml	a-lipoic acid	0.05
4. Mineral Solution (10.0 ml)		Vitamin B_{12}	0.005 g
Nitrilotriacetic acid (NTA)	1.5 g	**6. Yeast extract**	20.0 g
$Fe(NH_4)_2(SO_4)_2$	0.8 g	**7. Resazurin (1.0 ml)**	
$NaSeO_3$	0.2 g	**8. pH**	6
$CoCl_2.6H_2O$	0.1 g		
$MnSO_4.H_2O$	0.1 g		
$Na_2MoO_4.2H_2O$	0.1 g		
$NaWO_4.2H_2O$	0.1 g		
$ZnSO_4.7H_2O$	0.1 g		
$NiCl_2.6H_2O$	0.1 g		
H_3BO_3	0.01 g		
$CuSO_4.5H_2O$	0.01 g		

3.3.3.5 ATCC Media for methanogens

The medium was designed for the cultivation of methanogens and was prepared according to ATCC 1340. All components (**Table 3.5**) were prepared in separate schott bottles and dispensed into serum vials. The media was purged with N_2 to create anaerobic conditions. Following purging, the media was autoclaved (20 min at 121°C) and cooled to room temperature before being transferred into the anaerobic chamber (Wolfe, 2011) along with the vitamin and mineral solutions. The vitamin and mineral solutions were filter sterilized (0.22 µm, GVS Filter Technology) before used.

Table 3.5: Components of the ATCC media for methanogens (ATCC 1340).

Component	Concentration (g/L)	Component	Concentration (g/L)
1. Mineral Solution #1 (15 ml)		**10. Vitamin Solution (4.0 ml)**	
K_2HPO_4	6.0 g	Biotin	2.0 mg
Distilled water	1.0 L	Folic acid	2.0 mg
2. Mineral Solution #2 (15 ml)		Pyridoxine hydrochloride	10.0 mg
KH_2PO_4	6.0 g	Thiamine	5.0 mg
$(NH4)_2SO_4$	6.0 g	Riboflavin	5.0 mg
NaCl	12.0 g	Nicotinic acid	5.0 mg
$MgSO_4.7H_2O$	2.6 g	Calcium D-(+) pantothenate	5.0 mg
$CaCl_2.2H_2O$	0.16 g	Vitamin B_{12}	0.1 mg
Distilled water	1.0 L	p-Aminobenzoic acid	5.0 mg
3. 20% Yeast Extract – Trypticase Solution (4 ml)		Thioctic acid	5.0 mg
Yeast extract	20.0 g	Distilled water	1.0 L
Trypticase Peptone	20.0 g	**11. Mineral Solution (4.0 ml)**	
Distilled water	100.0 ml	Nitrilotriacetic acid (NTA)	1.5 g
4. 20% Sodium formate (6.0 ml)		$MgSO_4$	3.0 g
5. 25% Sodium acetate (4.0 ml)		$MnSO_4.H_2O$	0.5 g
6. 0.2% $FeSO_4.7H_2O$ (0.4 ml)		NaCl	0.1 g
7. 0.1% Resazurin (0.4 ml)		$FeSO_4.7H_2O$	0.1 g
8. $NaHCO_3$ (2.4 g)		$CoCl_2.6H_2O$	0.1 g
9. Cysteine-Sulphide Reducing Agent (16.0 ml)		$CaCl_2$	0.1 g
N NaOH (4 g/100 ml)	30.0 ml	$ZnSO_4.7H_2O$	0.1 g
L-Cysteine.HCl	5.0 g	$CuSO_4.5H_2O$	0.01 g
12.5% Na_2S stock solution	40.0 ml	$AlK(SO_4)_2.12H_2O$	0.01 g
Distilled water	330.0 ml	H_3BO_3	0.01 g
		$Na_2MoO_4.2H_2O$	0.01 g
		Distilled water	1,0 L
		12. pH	6.5

3.3.3.6 Anoxic media for methanogens

Anoxic media was designed for the cultivation of methanogens. The media was prepared as described by Balch & Wolfe (1976). The procedure followed was the same as **Section 3.3.3.5**. **Table 3.6** shows all components of the media.

Table 3.6: Components of the anoxic media for methanogens (Balch & Wolfe, 1976).

Component	Concentration (g/L)	Component	Concentration (g/L)
1. Anoxic medium for methanogens		**5. Vitamin Solution (10.0 ml)**	
NH_4Cl	1.0 g	p-aminobenzoic acid	0.01 g
NaCl	0.6 g	Nicotinic acid	0.01 g
$NaHCO_3$	5.0 g	Calcium pantothenate	0.01 g
KH_2PO_4	0.3 g	Pyridoxine	0.01 g
K_2HPO_4	0.3 g	Riboflavin	0.01 g
$MgCl_2.6H_2O$	0.16 g	Thiamine	0.01 g
$CaCl_2.2H_2O$	0.009 g	Biotin	0.05
2. Resazurin 0.1% solution	1 ml	Folic acid	0.05
3. Cysteine HCl	4.0 g	a-lipoic acid	0.05
4. Trace Mineral Solution (10.0 ml)		Vitamin B_{12}	0.005 g
Nitrilotriacetic acid (NTA)	1.5 g	**6. pH**	7
$Fe(NH_4)_2(SO_4)_2$	0.8 g		
$NaSeO_3$	0.2 g		
$CoCl_2.6H_2O$	0.1 g		
$MnSO_4.H_2O$	0.1 g		
$Na_2MoO_4.2H_2O$	0.1 g		
$NaWO_4.2H_2O$	0.1 g		
$ZnSO_4.7H_2O$	0.1 g		
$NiCl_2.6H_2O$	0.1 g		
H_3BO_3	0.01 g		
$CuSO_4.5H_2O$	0.01 g		

3.3.4 Molecular characterization

3.3.4.1 Genomic DNA extraction

The NucleoSpin® Soil Kit - Macherey-Nagel was used to extract bacterial genomic DNA (gDNA) according to the manufacturer's instructions. Genomic DNA was extracted from the original seeding sample and enrichments. DNA was eluted in Nano-pure water (milliQ water). The Nano-pure water used was 18 MΩ.cm^{-1} Barnstead ™ Nano-pure™ water (Thermo Scientific) that was filtered and UV treated.

The concentrations and purity of the gDNA were determined on the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific). Genomic DNA extracts were visualized on a 0.8% (w/v) agarose gel stained with 3 µl ethidium bromide (3 µl of a 10 mg/mL of stock solution). Genomic DNA (5 µl) was added to 2 µl loading dye (Fermentas) before loading onto the gel. The GeneRuler™ DNA ladder (Fermentas) was added as reference, the gel was resolved at 90 volts for 1 hour and visualized using the ChemiDoc XRS (Bio-Rad Laboratories) gel documentation system. Genomic DNA samples were stored at -20°C until further use.

3.3.4.2 16S rRNA gene amplification

A conserved region of 16S ribosomal RNA (16S rRNA) gene fragment present in all bacteria was amplified using the Polymerase Chain Reaction (PCR). The primers used are listed in **Table 3.7** and the reaction mixture consisted of components shown in **Table 3.8**.

Table 3.7: Primers used for 16S rRNA gene fragment amplification.

Primer	Sequence	Reference
341F-GC Clamp	5'-CCT ACG GGA GGC AGC A -3'	(Muyzer *et al.*, 1993)
908R	5'- CCG TCA ATT CMT TTG AGT TT -3'	(Muyzer *et al.*, 1993)
GC clamp *	5'-CGCGCGCCGCGCCCCGCGCCCGTCCCG CCGCCCCCGCCCG-3'	(Muyzer *et al.*, 1993)

*GC clamp attached to the 5'end of the primer 341F.
F= Forward primer, R = Reverse primer and M=A/C.

Table 3.8: Composition of 16S rRNA gene fragment PCR.

Reaction components	Volume (µl)
Buffer (10X)	5
dNTPs (10 mM)	1
Primer 341F-GC (10 µM)	1
Primer 908R (10 µM)	1
BSA (10 µg/µl)	1
Taq DNA Polymerase (2 U/µl)	0.25
Template	Final concentration of (25 ng/µl – 50 ng/µl)
MilliQ water	Up to 50 µl

Amplifications were carried out in a thermal cycler PXE 0.2 (Thermo Electron Corporation). An initial denaturation at 95ºC for 5 min was followed by 30 cycles of 95ºC for 30 s, 52ºC for 30 s and 70ºC for 2 min and final extension at 72ºC for 10 min.

The PCR products were visualised on 1% (w/v) agarose gel stained with ethidium bromide. MassRuler™ or GeneRuler™ DNA ladder (Fermentas) was used to infer the size of the amplicons. The gel was resolved at 90 volts for 1 hour and visualized under UV illumination using the ChemiDoc XRS (Bio-Rad Laboratories) gel documentation system. Amplicons were then subjected to denaturing gradient gel electrophoresis (DGGE).

3.3.4.3 *mcrA* gene amplification

The alpha-subunit of MCR (*mcrA*) was proposed as a 'functional' marker gene for the identification of methanogens, alternatively by the 16S rRNA gene analysis (Luo & Angelidaki, 2012; Nettmann *et al.*, 2008). To determine the optimal annealing temperature, gradient PCR was performed for the primer set ME1 and ME2 in the temperature range 48ºC and 58ºC. The primers used are listed in **Table 3.9**. The reaction mixture and PCR program were performed according to **Table 3.10**.

Table 3.9: Primers used for *mcrA* gene amplification.

Primer	Sequence	Reference
ME1 (Forward)	5'--GCMATGCARATHGGWATGTC--3'	(Luton *et al.*, 2002)
ME2 (Reverse)	5'-TCATKGCRTAGTTDGGRTAGT-3'	(Luton *et al.*, 2002)

Table 3.10: Reaction components for *mcrA* PCR.

Reaction components		**PCR program**			
Reagent	**Volume (µL)**	**Step**	**T [°C]**	**Time**	**Cycles**
Buffer (10X)	5	Initial denaturation	94	3 min	
dNTPs (10 mM)	1	Denaturation	94	40 s	35x
Primer ME1 (10 µM)	1	Annealing	48 – 58	45 s	
Primer ME2 (10 µM)	1	Extension	72	2 min	
BSA (10 µg/µl)	1	Final extension	72	10 min	
Taq DNA Polymerase (2 U/µl)	0.25	Hold	4	∞	
Template	Final concentration of (25 ng/µl – 50 ng/µl)				
MilliQ water	Up to 50 µl				

3.3.4.4 Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is a method used for microbial community characterizations, it is based on the melting behaviour of DNA fragments in a special polyacrylamide gel (Kern *et al.*, 2016). The gel contains a linear gradient of denaturant, for example urea or formamide, which causes the denaturation of the DNA fragment according to GC content and nucleotide sequence; thereby generating band patterns that directly reflect the genetic biodiversity of the sample.

The number of bands corresponds to the number of dominant species (Ali Shah *et al.*, 2014). The most important application of DGGE is monitoring dynamic changes in microbial communities, especially when many samples have to be analysed (Ali Shah *et al.*, 2014).

Acrylamide/Bis-acrylamide denaturing solutions (stock solutions, 0 and 80% w/v) were prepared as indicated in **Table 3.11**. Working solutions (40 and 60% w/v) were prepared as shown in **Table 3.12**. The stacking gel was prepared using 5 ml (0% denaturing solution), 12.5% (w/v) APS (100 µl) and TEMED (10 µl) were added to catalyse the polymerization of acrylamide and bis-acrylamide. The gel was stored in the dark and allowed to polymerize for 5 hours.

Table 3.11: Urea-formamide composition (stock solution).

Reagent	0% denaturing solution	80% denaturing solution
40% acrylamide/bis	10 ml	10 ml
TAE (50X)	1 ml	1 ml
Formamide (deionised)	-	16 ml
Urea	-	16,8 g
MilliQ water	Up to 50 µl	Up to 50 µl

Table 3.12: Urea-formamide composition (working solution).

Component	40% denaturing solution	60% denaturing solution
0% denaturing solution	6 ml	3 ml
80% denaturing solution	6 ml	9 ml
Ammonium Persulfate Solution (APS)	84 µl	84 µl
Tetramethylethylenediamine (TEMED)	8.4 µl	8.4 µl

PCR products (20 µl) from **Section 3.3.4.2** were mixed with loading dye (7 µl) and loaded on the gel. Electrophoresis was performed in 1xTAE buffer at 100 Volts and a temperature of 60°C for 17 hours. The gel was stained for 20 minutes with 50 µl ethidium bromide (10 mg/ml) in 500 ml distilled water. The gel was washed 3 times with distilled water and visualized under UV illumination using ChemiDoc XRS (Bio-Rad Laboratories) gel documentation system.

DGGE bands were excised with a sterile blade, eluted in 50 µl Milli-Q water and incubated at 55°C overnight. The bands eluted in water were re-amplified. The re-amplification was performed using the primer combination 341 F and 908 R with the same PCR conditions used in the amplification of 16S rRNA gene in **Section 3.3.4.2**.

3.3.4.5 Exo/SAP clean up

The *mcrA* amplified products in **Section 3.3.4.3** and re-amplified DGGE products in **Section 3.3.4.4** were purified using the ExoSAP-IT® protocol. The ExoSAP-IT® mixture is composed of the hydrolytic enzymes Exonuclease I and Shrimp Alkaline Phosphates. The complex enzyme digests remaining excess forward and reverse primers and dNTPs from the post-PCR mixture. ExoSAP-IT® is routinely used for DNA sequencing and single nucleotide polymorphism analysis. Exonuclease I degrades single stranded DNA in the 3' to 5' direction producing 5' mononucleotides and a terminal 5'-dinucleotide, as long as the 3' terminus is not phosphorylated. Shrimp Alkaline Phosphatase dephosphorylates the 5' ends of dNTPs rendering them inactive in downstream applications (Fendt *et al.*, 2009; Wolff *et al.*, 2012).

The Exonuclease I (0.5 µl) and FastAP™ Thermosensitive Alkaline Phosphatase or Shrimp Alkaline Phosphates (2 µl) were added to the PCR product (10 µl) as recommended by the manufacturer. The mixture was incubated in a thermal cycler PXE 0.2 (Thermo Electron Corporation) at 37ºC for 15 min followed by heat inactivation of the enzymes at 85ºC for 15 min. The enzyme-treated amplified product was stored at -20ºC until next use.

3.3.4.6 Sequencing of 16S rRNA gene fragments

Sequencing PCR was performed by using the ABI BigDye Terminator v3.1 Ready Reaction Sequencing Kit (Applied Biosystems). Sequencing PCR was performed for products of **Section 3.3.4.5** (therefore 16S rRNA gene and *mcrA* fragments). The PCR reaction contained the components listed in **Table 3.13.** Each reaction contained either a forward or reverse primer, ME1 and ME2 respectively. The PCR was carried out in a thermal cycler PXE 0.2 (Thermo Electron Corporation), the PCR conditions entailed initial denaturation at 96°C for 1 min, 25 cycles of denaturation at 96°C for 10 s, followed by annealing at 50°C for 5 s and primer extension at 60°C for 4 min.

Table 3.13: Sequencing PCR reaction components.

Reaction component	Reaction size (µl)
Premix	1
Sequencing primer (3.2 pmol. μl^{-1})	1
Dilution buffer	2
Template	Final concentration (5 – 20 ng)
MilliQ water	Up to 10 µl

The EDTA/Ethanol precipitation protocol was used for sequencing clean up. The sequencing reaction volume was adjusted to 20 µl and transferred to a 1.5 mL Eppendorf tube containing 5 µl 125 mM EDTA (pH 8.0) and 60 µl absolute ethanol. The reaction was precipitated at room temperature for 15 min, centrifuged at 4°C (20 000 *x g*, 10 min), then the supernatant was completely aspirated. A volume of 60 µl ethanol (70%) was added to the tubes followed by centrifugation at 4°C (20 000 *x g*, 5 min). The supernatant was aspirated and the tubes were dried in the Speed-Vac (Eppendorf) for 5 min. Samples were stored at 4°C until they were sequenced.

3.3.4.7 Next Generation Sequencing (NGS)

Genomic DNA (**Section 3.3.4.1**) from the enrichments and the original seeding sample were sent for processing and sequencing at the Centre for Proteomic and Genomic Research (CPGR, Cape Town, South Africa) and Inqaba biotec-Africa's Genomics Company (Inqaba, Pretoria, South Africa). Briefly, the sequencing library was prepared by amplifying a ~460 bp region located in the hypervariable V3/4 region of the 16S rRNA gene using region of interest-specific primers (Klindworth *et al.*, 2013) with Illumina adapter overhang nucleotide sequences.

The 16S V3/4 amplicons were purified using the Agencourt AMPure XP bead clean up kit (Beckman Coulter Genomics, Danvers, MA, USA), followed by a second amplification to attach dual indices and Illumina sequencing adapters using the Nextera XT Index kit (Illumina, San Diego, CA, USA). Final purification using the AMPure XP bead clean up kit (Beckman Coulter Genomics) was done, followed by library quantification, normalization, pooling and denaturing before being subjected to 2 x 301 cycle sequencing on the Illumina MiSeq using the MiSeq v3 reagent kit (Illumina).

The obtained 16S rRNA gene sequence data was analysed using QIIME as described by Caporaso and colleagues (2010) with alterations. Briefly, before running the QIIME pipeline, the quality of the sequencing was assessed and quality control performed using PrinSeq-lite v0.20.4 (Schmieder & Edwards, 2011). All data sets were pre-processed and trimmed to obtain an average quality score of ≥20 using a 5 nt window with a 3 nt step. All sequences shorter than 200 bp were filtered out and paired end reads merged using PEAR 0.9.6 (Caporaso *et al.*, 2011).

The demultiplex and quality filtering script in QIIME was run without any additional inputs to obtain a FASTA output file that could be analysed in the QIIME pipeline. Chimeric sequences were identified, using usearch 6.1.544 (Edgar, 2010) as the chimera detection method against the RDP “Gold” database (Edgar, 2010), and filtered out of the quality trimmed reads by using identify_chimeric_seqs.py and filter_fasta.py commands, respectively, in QIIME. Operational Taxonomic Units (OTU) picking was carried out and taxonomy assigned to representative OTUs using the pick_open_refernece_otus.py script, at 97% sequence identity against the SILVA 119 database (Quast *et al.*, 2013). A stepwise workflow for the annotation of the data is shown in **Figure 3.1**.

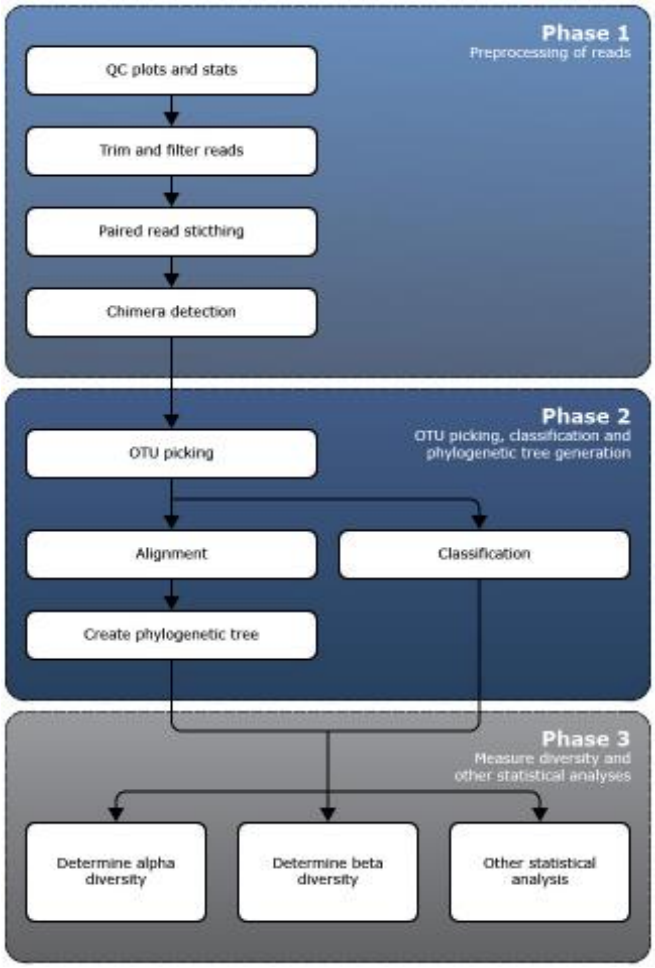


Figure 3.1: Stepwise workflow for analysis of using QIIME (http://h3abionet.org/).

3.3.5 DAPI staining

4´6-diamidino-2-phenylindole (DAPI) is a blue fluorescent dye and useful for nuclear counter-stain for multicolour fluorescent techniques. DAPI preferentially attaches to the double strand DNA specially the AT clusters of the minor grove (Chazotte, 2011). When stained with DAPI, the DNA appears as blue-white fluorescence under ultraviolet (UV) illumination, and the positions of cell nuclei and organelle nucleoids can therefore be determined (Suzuki *et al.*, 1997).

Briefly samples were fixed by adding 108 µl formaldehyde (37% v/v) to 1 ml sample from the enriched media, these were incubated in the dark at 4°C for 30 min. The fixed samples were filtered through a 0.20 µm pore size membrane filter. A sterile blade was used to excise a piece of the membrane filter and stained with 10 µl DAPI solution (10 µg/ml). The membrane filter containing the DAPI solution was then incubated in

the dark for 2 min at room temperature. The filter was rinsed with water and placed on a microscope slide containing 10 µl citifluor. The stained product was visualised with an epifluorescence microscope under 1 000X magnification.

DAPI epifluorescence microscopy allows the direct observation and total enumeration of viable and non-viable microorganisms in the enrichment media (Manyi-Loh *et al.*, 2013).

3.4 Results

3.4.1 Microbial enrichments

Enrichment media was prepared and inoculated as described in **Section 3.3.3**. Culture conditions were designed to select either hydrolytic bacteria, acidogenic bacteria, acetogenic bacteria or methanogenic archaea as per specified medium. Enrichment cultures were incubated at 37°C in order to create favourable conditions for the desired microorganisms. **Table 3.14** shows biomass accumulation over time for the media enrichments. The number of plus signs (+) indicate the increase in biomass, one + sign shows low turbidity of the media, two or three + signs show medium turbidity and more than four + signs show maximum turbidity if the media.

Table 3.14: Increase in biomass over time.

	Growth over time					
	Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**
TYG Media	++	++	+++	++++	+++++	+++++++++
Thioglycolate media	++	++	+++	++++	+++++	+++++++++
Basal media	++	++	+++	++++	+++++	+++++++++
Acidogenic media	++	++	+++	++++	+++++	+++++++++
Anoxic media	+	+	++	++	+++	++++
ATCC media for methanogens	+	+	++	++	+++	++++

[+]turbidity of the media

3.4.2 Molecular characterization

3.4.2.1 Genomic DNA extraction

Genomic DNA was extracted from the enriched media after 6 weeks of incubation using the NucleoSpin® Soil Kit Macherey-Nagel as described in **Section 3.3.4.1**. Genomic DNA was also extracted directly from the original seeding material. The DNA concentration (**Table 3.15**) was determined with NanoDrop Spectrophotometer ND-1000 (Thermo Scientific). **Figure 3.2** shows successful extraction of gDNA, however the visualization of the DNA showed faint bands and shearing indicating low concentration and low integrity of the DNA. Lanes 5 and 7 are both methanogenic media enrichments that visually showed very low cell biomass.

Table 3.15: DNA concentration of enrichment cultures.

Enrichment media	Concentration (ng/µl)
TYG Media	49.01
Thioglycolate media	48.66
Basal media	36.03
Acidogenic media	48.00
Anoxic media	35.33
ATCC media for methanogens	38.65

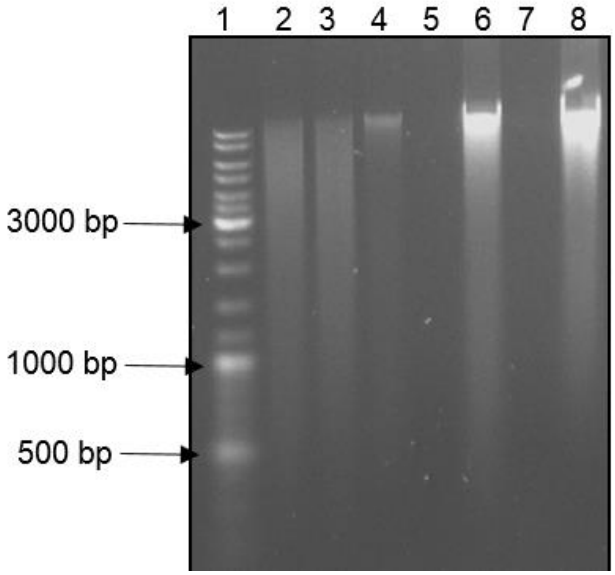


Figure 3.2: Genomic DNA extracted from the enrichments and the original seeding material samples. Lane 1: GeneRuler^{TM} DNA ladder, Lane 2: Basal medium, Lane 3: Acidogenic medium, Lane 4: Thioglycolate medium, Lane 5: ATCC medium for methanogens, Lane 6: original seeding material, Lane 7: Anoxic media for methanogens and Lane 8: TYG medium.

3.4.2.2 DGGE specific 16S rRNA PCR amplification

The 16S rRNA gene was amplified as described (**Section 3.3.4.2**) using universal primers 341F-GC and 908R as outlined previously. A DNA fragment of approximately 600 bp (**Figure 3.3**) was obtained for all samples as expected for bacterial DNA extractions. The amplicons were then subjected to DGGE.

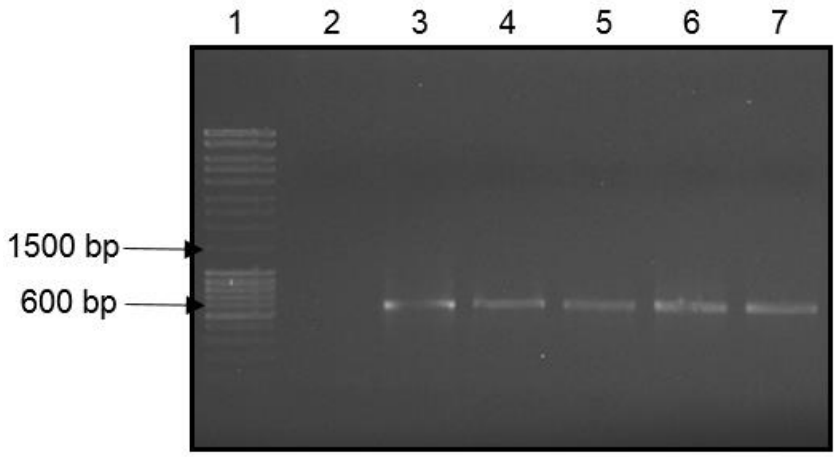


Figure 3.3: 16S rRNA gene amplification. Lane 1: MassRuler^{TM} DNA ladder, Lane 2: Negative control, Lane 3: Original seeding material, Lane 4: Basal medium, Lane 5: Acidogenic medium, Lane 6: Thioglycolate medium and Lane 7: TYG medium.

3.4.2.3 Amplification of *mcrA* gene and sequence analysis

All known methanogens express the enzyme methyl coenzyme M reductase (MCR), which is assumed to be a common enzyme in methanogens (Ozutsumi *et al.*, 2012). Successful amplification through gradient PCR confirmed the presence of the *mcrA* gene (**Figure 3.4**). An 800 bp product was obtained, these results are similar to Paul *et al.,* (2012). This was confirmed by repeating the PCR again with the optimized conditions (**Figure 3.5**) and products were subjected to Exo/SAP clean up.

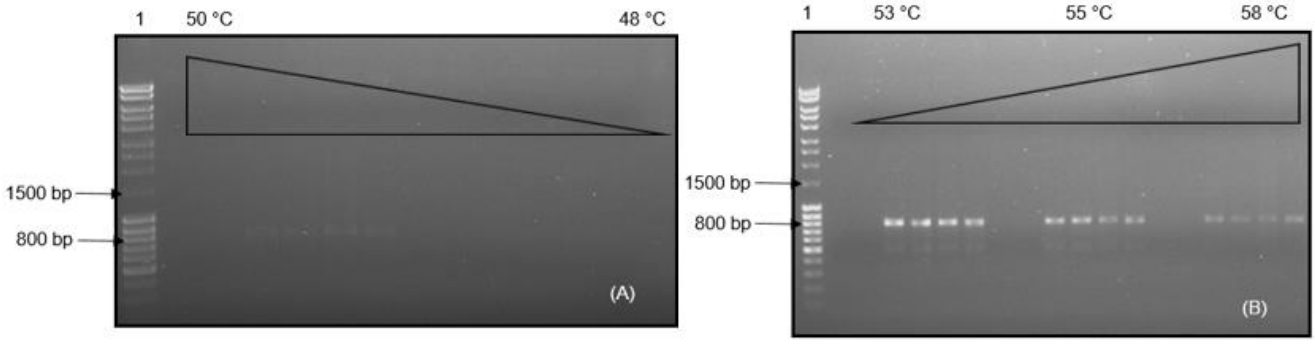


Figure 3.4: Gradient PCR amplification of the *mcrA* gene fragments from the enrichments. Lane 1: MassRuler^{TM} DNA ladder. (A) Decreasing temperature gradient, (B) Increasing temperature gradient

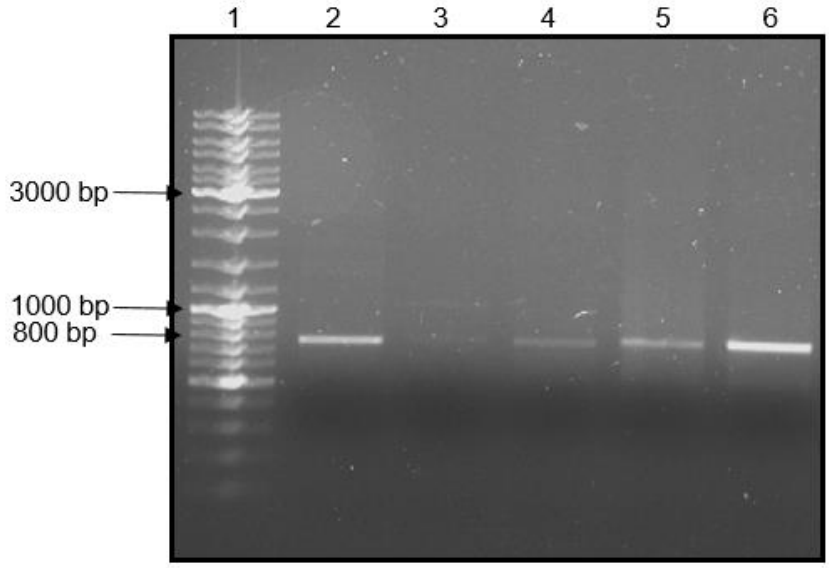


Figure 3.5: *mcrA* amplification. Lane 1: GeneRuler^{TM} DNA ladder, Lane 2: ATTC medium for methanogens, Lane 3: Original seeding material, Lane 4: Basal medium, Lane 5: Thioglycolate medium and Lane 6: Anoxic medium for methanogens.

The *mcrA* gene was detected in enrichments that selected for anaerobic growth conditions. Although the media is not selective for methanogens the original seeding sample itself according to the PCR results already contain species that have the gene. Therefore it is not inconceivable that the anaerobic media will still have methanogenic species that survive.

The *mcrA* gene sequences obtained in this study were compared with similar sequences of the reference organisms by a Basic Alignment Search Tool (BLAST) search (Nunoura *et al.*, 2008). Sequence data were aligned with the Multiple Alignment using Fast Fourier Transform (MAFFT) version 7 alignment tool. Phylogenetic tree was inferred with Molecular Evolutionary Genetics Analysis (MEGA) software version 6 (Katoh & Standley, 2013; Sun *et al.*, 2013) using the neighbour-joining method (Mori & Harayama, 2011), and a bootstrap analysis of 1 000 replicates. The K2-G model was shown appropriate to use the data (Crandall, 2001). The results of the *mcrA* gene phylogenetic analysis are shown in **Figure 3.6**. All the nucleotide sequences derived were classified into *Methanobacteriales* and *Methanosarcinales*. These findings are the same as described in literature (Ali Shah *et al.*, 2014; Bialek *et al.*, 2011; Borrel *et al.*, 2011; Dhillon *et al.*, 2005; Jabłoński *et al.*, 2015; Luo & Angelidaki, 2012; Nettmann *et al.*, 2008; Ziemiński & Frąc, 2012).

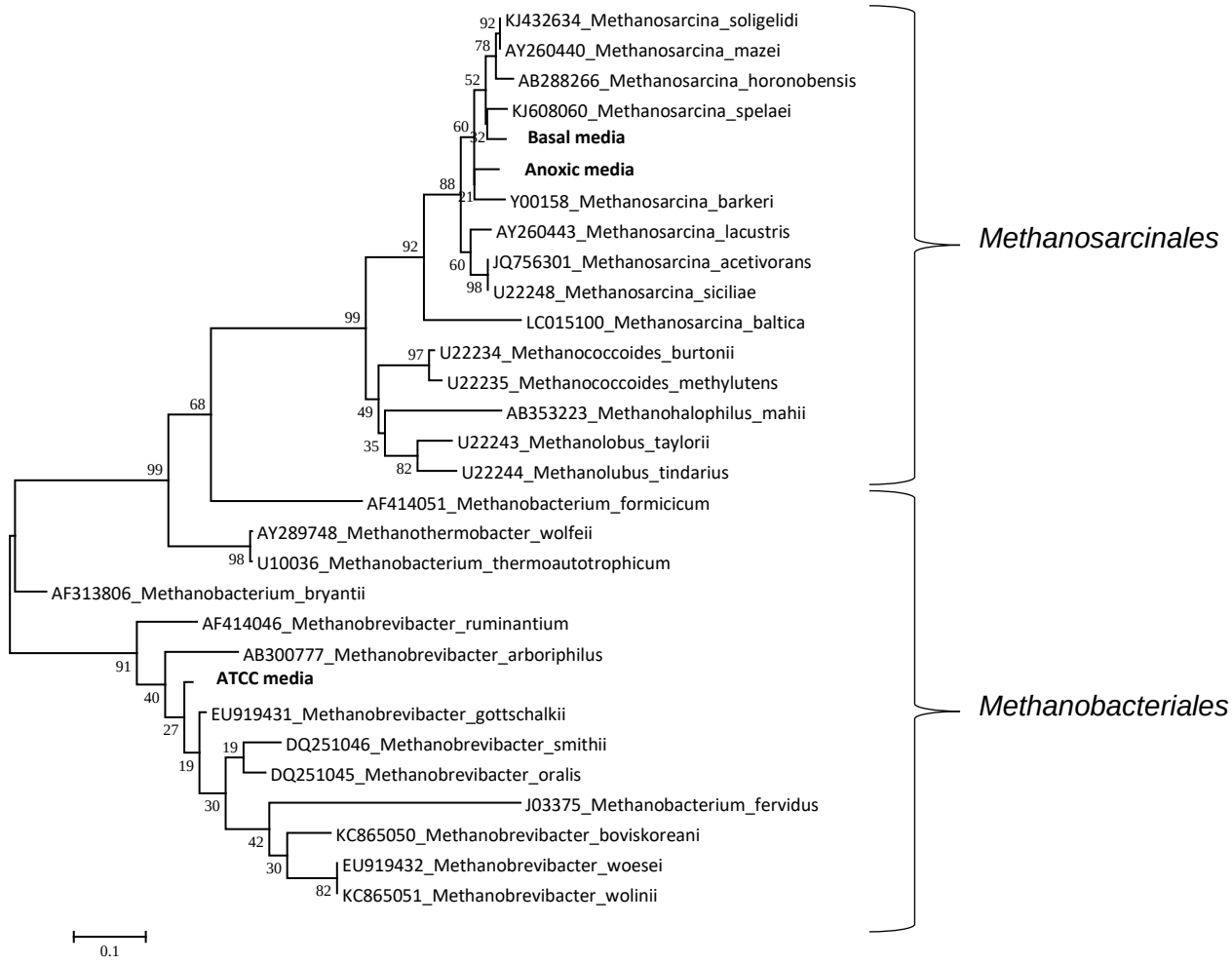


Figure 3.6: Phylogenetic tree showing the relationship between nucleotide sequences from the *mcrA* gene sequences of methanogens in the enriched media. The tree was constructed using neighbour-joining analysis. The scale bar = 0.1 substitutions per nucleotide position. Samples obtained from the enrichment media appear in bold letters. The accession number is given for each published sequence. *Methanobacterium bryantii* was used as the out group to root the tree.

3.4.2.4 DGGE Diversity

The microbial diversity in the samples was determined by DGGE as explained in **Section 3.3.4.4**. The results showed a clear difference in the bacterial populations in all enrichments and the original seeding sample. Dominant bands indicated in **Figure 3.7** were excised, DNA eluted and subjected to PCR re-amplification. The bands were sequenced and identified by comparing the gene sequences with DNA sequences in the National Centre for Biotechnology Information (NCBI) database using the BLAST algorithm.

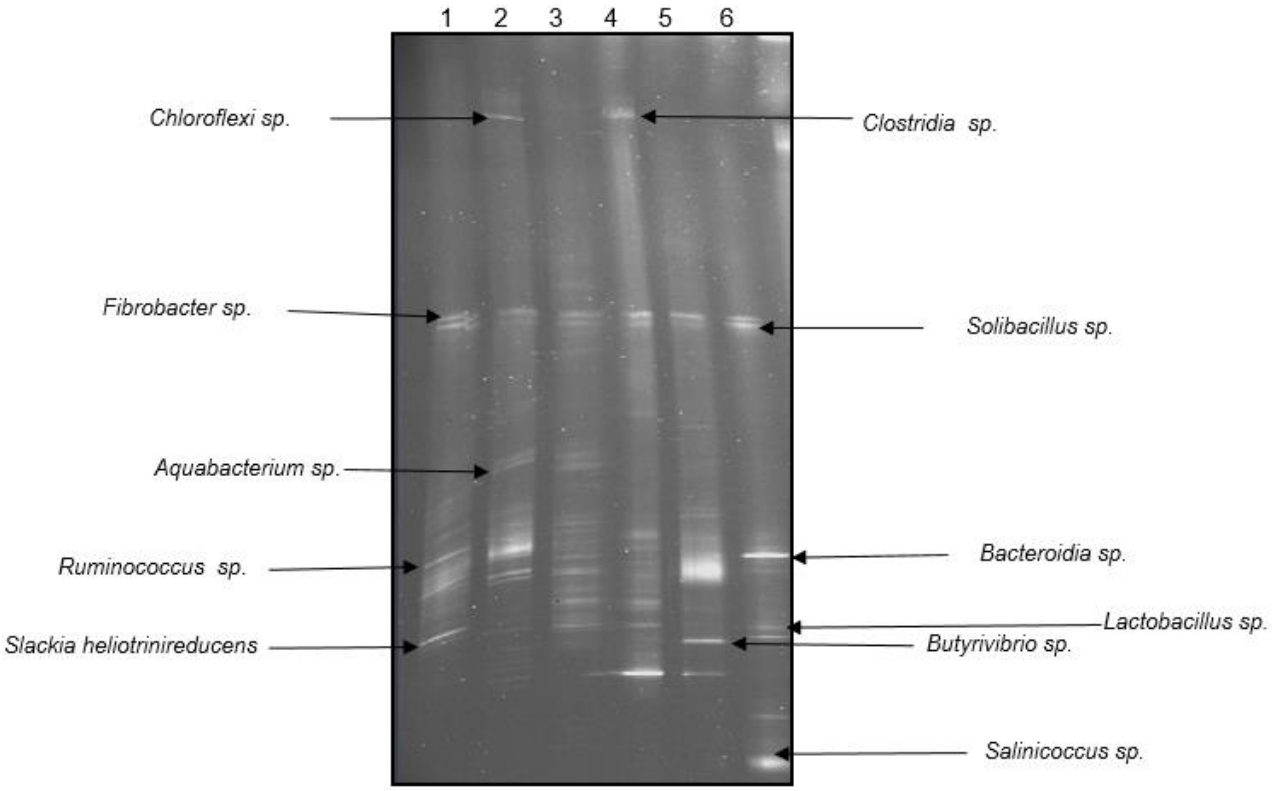


Figure 3.7: DGGE diversity profile. Lane 1: Original seeding material, Lane 2: Basal medium, Lane 3: TYG medium. Lane 4: TYG repeated, Lane 5: Thioglycolate medium, Lane 6: Acidogenic medium.

The DGGE profile from enriched media shows specific profile variation from the original seeding material. Profiles of bacterial communities showed several well-separated bands representing different phylogenetic groups of dominant bacteria in the samples. Microorganisms within *Firmicutes* are widely distributed throughout various anaerobic habitats, both natural and enriched, and have the ability to degrade a wide range of complex organic macromolecules, including both proteins and carbohydrates (Kröber *et al.*, 2009; Sun *et al.*, 2013; Weiss *et al.*, 2008).

Most bacteria which are capable of the efficient hydrolysis of plant biomass rich in lignocellulose belong in the classes of the *Clostridia* and *Bacteroidia* (Wirth *et al.*, 2012). As expected *Clostridia* and *Bacteroidia* species were identified (Sun *et al.*, 2015; Wirth *et al.*, 2012). Members of the *Bacteriodia* are common in nature at sites where degradable organic material is to be found, such as plants and other forms of biomass. *Bacteroides capillosus* is an intestinal bacterium that ferments lactate and produces H_2, and also displays cellulolytic activity (Wirth *et al.*, 2012).

Clostridium species can hydrolyze cellulose efficiently by means of its extracellular cellulases. Also, *Clostridium* species have been previously found in rumen and has been described to be responsible for the oxidation of acetate. Members of the *Actinobacteria* class are commonly found in soils and natural water (Chojnacka *et al.*, 2015). Some of them effectively break down complex organic material such as cellulose, and thereby play an important role in the carbon cycle. Furthermore, members of this group are known to produce lignin-degrading enzymes (Wirth *et al.*, 2012).

A species of *Actinobacteria* was identified: *Slackia heliotrinireducens*, this organism is a Gram-positive anaerobic bacterium which can reduce nitrate to ammonia if there are electron donors (H_2 or formate) in the system. This organism has also been reported to produce acetic acid and lactic acid, and contains a hydrogenase (Kuczynski *et al.*, 2012; Manyi-Loh *et al.*, 2013).

A number of strictly anaerobic bacteria known as fermenting bacteria which include *Lactobacillus* and *Butyrivibrio* species were found, these organisms are associated with the production of short chain fatty acids such as formate, acetate, lactate, butyrate, as well as H_2 production. Other dominant genera including *Aquabacterium, Salinicoccus and Solibacillus* were also identified. *Fibrobacter, Ruminococcus* and uncultured bacteria have also been suggested to play important roles in cellulose hydrolysis in the rumen (Sun *et al.*, 2015).

3.4.2.5 Next generation sequencing data analysis

The 16S rRNA gene NGS sequencing identified *Firmicutes, Bacteroidetes, Synergistetes and Proteobacteria* as the most dominant phyla within the bacterial community for the enrichments and seeding material (**Figure 3.8**). As explained previously the *Firmicutes and Bacteroidetes* have been suggested to be important in hydrolytic and acidogenic digesters fed with dried hay and straw. These two phyla have also been shown to be important during batch digestion of wheat straw and swine manure (Sun *et al.*, 2013; Wirth *et al.*, 2012).

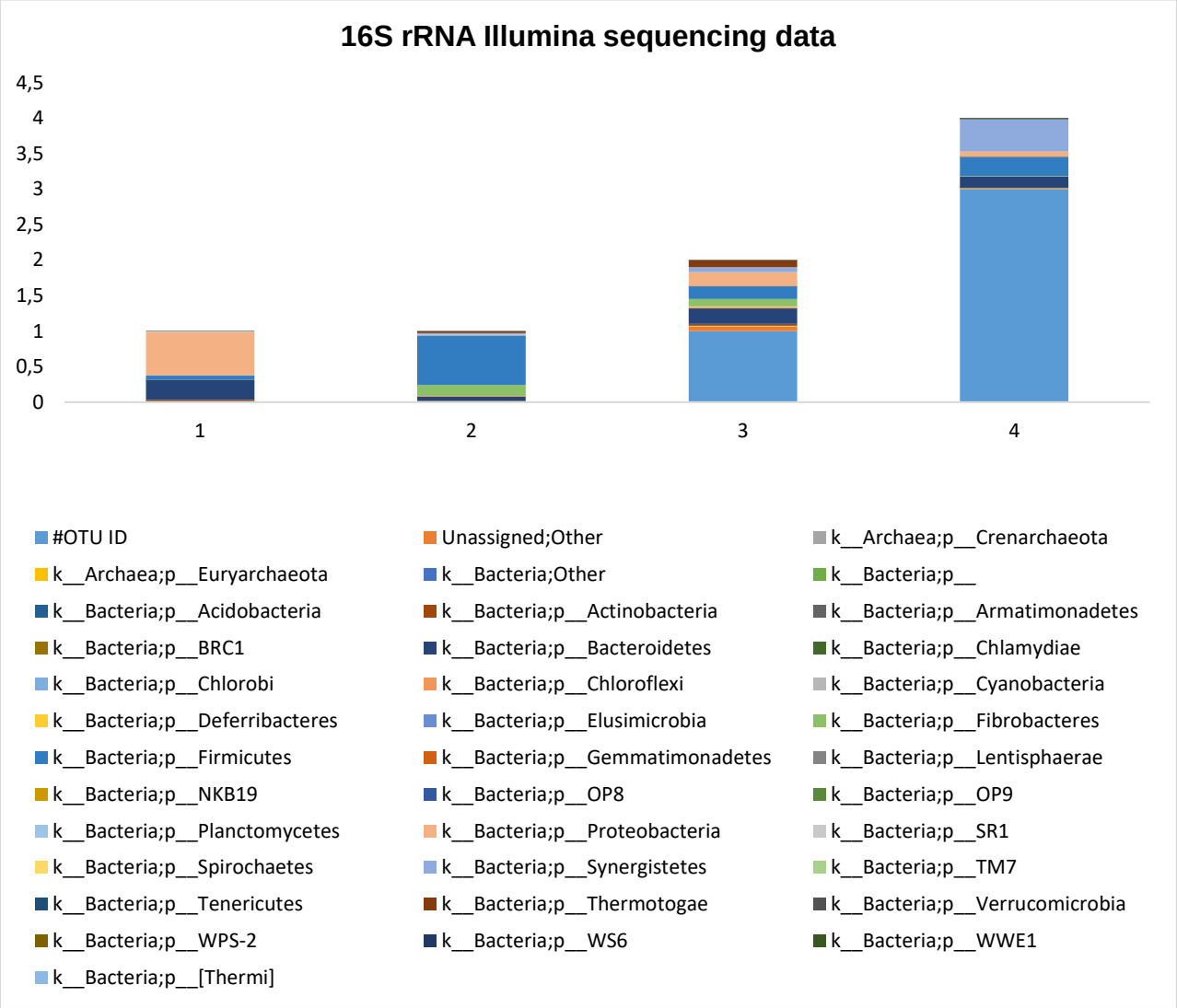


Figure 3.8:16S rRNA gene Illumina sequencing data classification by phyla. Lane 1: Seeding material, Lane 2: Thioglycollate medium, Lane 3: Acidogenic medium, Lane 4: Acetogenic medium.

In addition, sequences belonging to *Actinobacteria* and *Spirochaetes* were detected in the original seeding material. *Chloroflexi, Fibrobacteres* and *Chlamydiae* were

dominant in the Thioglycollate media. All these organisms have been described to be common in biogas production reactors digesting sewage sludge (Ali Shah *et al.*, 2014; Pukall *et al.*, 2009). *Synergistetes* was dominant in acidogenic media and basal media targeted at enriching acetogenic bacteria. *Synergistetes* have been found in a wide range of anoxic ecosystems, such as anaerobic wastewater treatment systems, soil, and gastrointestinal tracts. *Synergistetes* specifically ferment amino acids (Qiu *et al.*, 2014), this is true because this phylum was found in media enriching fermenting bacteria. The phylum *Thermotogae* was found in Thioglycollate media and acidogenic media, this phylum has been previously found in biogas digesters (Klindworth *et al.*, 2013; Weiss *et al.*, 2008).

The seeding material was dominated by *Proteobacteria* of the genus *Acinetobacter, Acrobacter, Sphingobium, Comamonas and Dechloromonas. Bacteroidetes* of the genus *Rhodococcus, Paludibacter and Flavobacterium* were also found in large quantities. The phylum *Firmicutes* was also present but in small quantities in seeding material, the *Firmicutes* of the genus *Clostridium* and *Coprococcus* were however selected in the Thioglycollate media. In the Acidogenic media, the phyla *Synergistetes, Bacteroidetes and Actinobacteria* were also found in the Basal media enriched to select the growth of acetogenic bacteria (**Figure 3.9**). These organisms have been previously associated with biogas digesters (Aly *et al.,* 2012; Chojnacka *et al.,* 2015; Kröber *et al.,* 2009; Ma *et al.,* 2015; Weiss *et al.,* 2008). **Figure 3.10 and Figure 3.11** shows reference colours to **Figure 3.9**.

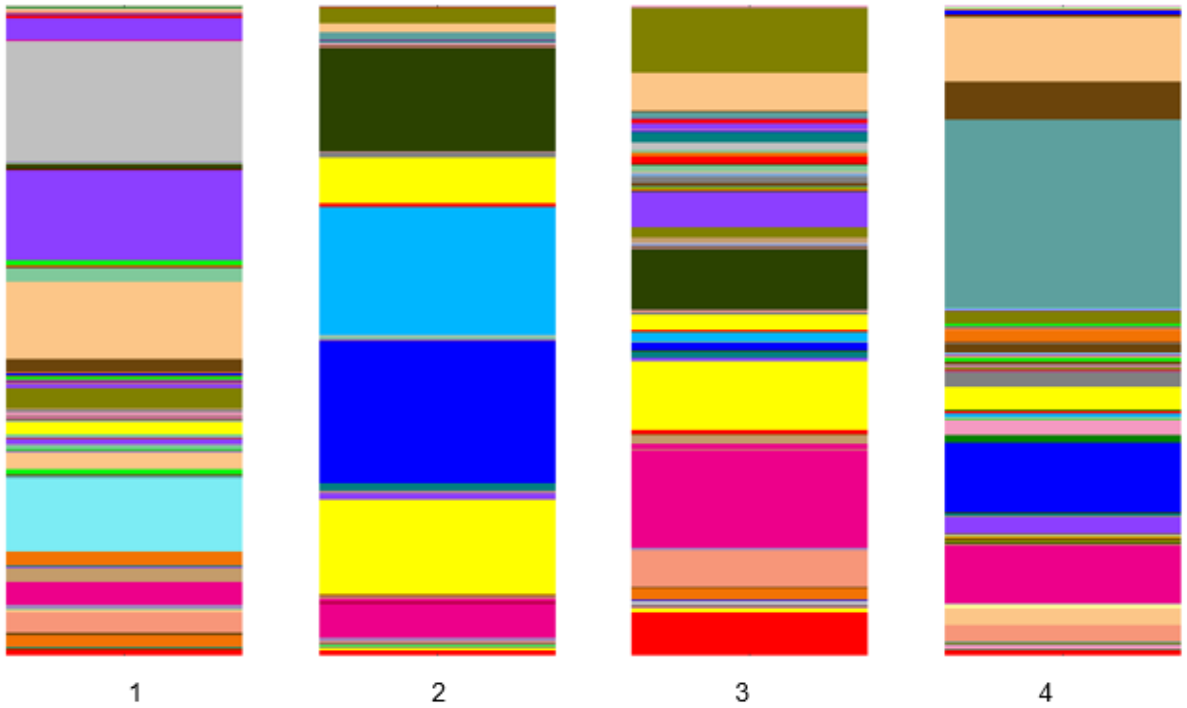
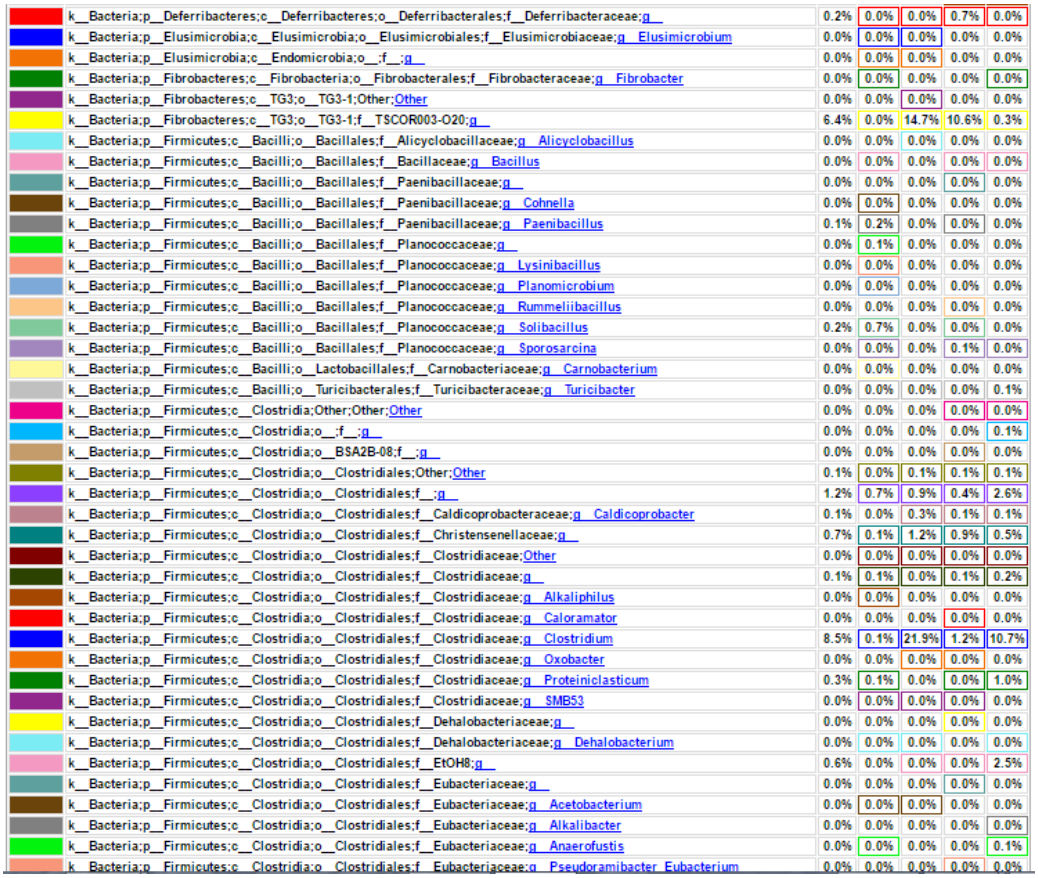


Figure 3.9: 16S rRNA gene Illumina sequencing data classification by genus. Lane 1: Seeding material, Lane 2: Thioglycollate medium, Lane 3: Acidogenic medium, Lane 4: Acetogenic medium.

Legend	Taxonomy	Total %	1 %	2 %	3 %	4 %
	Unassigned;Other;Other;Other;Other;Other	2.2%	1.0%	0.6%	6.5%	0.8%
	k__Archaea;p__Crenarchaeota;c__MCG;o__pGrfC26;f__;g__	0.1%	0.0%	0.1%	0.1%	0.0%
	k__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanobacteriaceae;g__Methanobacterium	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanobacteriaceae;g__Methanobrevibacter	0.0%	0.1%	0.0%	0.0%	0.0%
	k__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanobacteriaceae;g__Methanosphaera	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanomicrobiales;f__Methanomicrobiaceae;g__Methanoculleus	0.2%	0.0%	0.3%	0.4%	0.1%
	k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanomicrobiales;f__Methanomicrobiaceae;g__Methanofollis	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanosarcinales;Other;Other	0.3%	0.0%	0.0%	0.4%	0.7%
	k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanosarcinales;f__Methanosaetaceae;g__Methanosaeta	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanosarcinales;f__Methanosarcinaceae;g__Methanosarcina	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__YC-E6;f__;g__	0.2%	0.2%	0.3%	0.1%	0.0%
	k__Archaea;p__Euryarchaeota;c__Thermoplasmata;o__E2;f__[Methanomassiliicoccaceae];g__Methanomassiliicoccus	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Archaea;p__Euryarchaeota;c__Thermoplasmata;o__E2;f__[Methanomassiliicoccaceae];g__vadinCA11	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;Other;Other;Other;Other;Other	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__;c__;o__;f__;g__	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Acidobacteria;c__Acidobacteriia;o__Acidobacteriales;f__Koribacteraceae;g__Candidatus Koribacter	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Acidobacteria;c__BPC102;o__MVS-40;f__;g__	0.0%	0.0%	0.0%	0.1%	0.1%
	k__Bacteria;p__Acidobacteria;c__Solibacteres;o__Solibacterales;Other;Other	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Acidobacteria;c__Solibacteres;o__Solibacterales;f__AKIW659;g__	0.2%	0.0%	0.2%	0.6%	0.0%
	k__Bacteria;p__Acidobacteria;c__Solibacteres;o__Solibacterales;f__Solibacteraceae;g__Candidatus Solibacter	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales;f__;g__	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__;g__	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Dietziaceae;g__Dietzia	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Leucobacter	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Microbacterium	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Salinibacterium	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Arthrobacter	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micromonosporaceae;g__	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Nocardiaceae;Other	0.0%	0.0%	0.0%	0.2%	0.0%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Nocardiaceae;g__Rhodococcus	0.9%	1.6%	0.0%	1.8%	0.1%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Thermomonosporaceae;g__Actinocorallia	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Thermomonosporaceae;g__Actinomadura	0.0%	0.0%	0.0%	0.1%	0.0%
	k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;Other	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__	0.0%	0.0%	0.0%	0.0%	0.1%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__BF311	0.1%	0.2%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides	0.4%	0.5%	0.5%	0.4%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Marinilabiaceae;g__	0.2%	0.0%	0.0%	0.0%	0.6%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;Other	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__	8.3%	3.5%	5.3%	15.2%	9.1%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Dysgonomonas	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Paludibacter	0.6%	2.1%	0.0%	0.0%	0.1%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Parabacteroides	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella	0.1%	0.2%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Blvii28	0.1%	0.2%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__S24-7;g__	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__SB-1;g__	0.1%	0.0%	0.0%	0.0%	0.2%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Odoribacteraceae];g__Butyricimonas	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__[Paraprevotellaceae];g__YRC22	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__p-2534-18B5;g__	0.5%	2.1%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Adhaeribacter	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Flavobacterium	2.9%	11.5%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];g__	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];g__Chryseobacterium	0.1%	0.3%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];g__Cloacibacterium	0.0%	0.2%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__Pedobacter	0.2%	0.8%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__Sphingobacterium	0.0%	0.1%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__VC2_1_Bac22;o__;f__;g__	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;g__	0.6%	2.6%	0.0%	0.0%	0.0%
	k__Bacteria;p__Chlamydiae;c__Chlamydiia;o__Chlamydiales;f__Parachlamydiaceae;g__Candidatus Protochlamydia	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Chlorobi;c__OPB56;o__;f__;g__	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__;f__;g__	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolinaceae;g__	0.0%	0.0%	0.0%	0.1%	0.0%
	k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolinaceae;g__Anaerolinea	0.3%	0.0%	0.6%	0.8%	0.0%
	k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolinaceae;g__C1_B004	0.0%	0.0%	0.0%	0.0%	0.0%
	k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolinaceae;g__SHD-231	0.5%	0.0%	0.4%	1.4%	0.1%
	k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolinaceae;g__T78	0.1%	0.0%	0.0%	0.0%	0.6%
	k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolinaceae;g__WCHB1-05	0.0%	0.0%	0.0%	0.0%	0.0%

Figure 3.10: Diversity assessment of seeding material and enrichment culture from 16S rRNA gene Illumina sequencing data.



k__Bacteria;p__Deferribacteres;c__Deferribacteres;o__Deferribacterales;f__Deferribacteraceae;g__	0.2%	0.0%	0.0%	0.7%	0.0%
k__Bacteria;p__Elusimicrobia;c__Elusimicrobia;o__Elusimicrobiales;f__Elusimicrobiaceae;g__Elusimicrobium	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Elusimicrobia;c__Endomicrobia;o__;f__;g__	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Fibrobacteres;c__Fibrobacteria;o__Fibrobacterales;f__Fibrobacteraceae;g__Fibrobacter	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Fibrobacteres;c__TG3;o__TG3-1;Other;Other	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Fibrobacteres;c__TG3;o__TG3-1;f__TSCOR003-O20;g__	6.4%	0.0%	14.7%	10.6%	0.3%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Alicyclobacillaceae;g__Alicyclobacillus	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Paenibacillaceae;g__	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Paenibacillaceae;g__Cohnella	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Paenibacillaceae;g__Paenibacillus	0.1%	0.2%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae;g__	0.0%	0.1%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae;g__Lysinibacillus	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae;g__Planomicrobium	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae;g__Rummeliibacillus	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae;g__Solibacillus	0.2%	0.7%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae;g__Sporosarcina	0.0%	0.0%	0.0%	0.1%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Carnobacteriaceae;g__Carnobacterium	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Turicibacterales;f__Turicibacteraceae;g__Turicibacter	0.0%	0.0%	0.0%	0.0%	0.1%
k__Bacteria;p__Firmicutes;c__Clostridia;Other;Other;Other	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__;f__;g__	0.0%	0.0%	0.0%	0.0%	0.1%
k__Bacteria;p__Firmicutes;c__Clostridia;o__BSA2B-08;f__;g__	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;Other;Other	0.1%	0.0%	0.1%	0.1%	0.1%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__;g__	1.2%	0.7%	0.9%	0.4%	2.6%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Caldicoprobacteraceae;g__Caldicoprobacter	0.1%	0.0%	0.3%	0.1%	0.1%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Christensenellaceae;g__	0.7%	0.1%	1.2%	0.9%	0.5%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;Other	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__	0.1%	0.1%	0.0%	0.1%	0.2%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Alkaliphilus	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Caloramator	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Clostridium	8.5%	0.1%	21.9%	1.2%	10.7%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Oxobacter	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Proteiniclasticum	0.3%	0.1%	0.0%	0.0%	1.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__SMB53	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Dehalobacteriaceae;g__	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Dehalobacteriaceae;g__Dehalobacterium	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__EtOH8;g__	0.6%	0.0%	0.0%	0.0%	2.5%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae;g__	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae;g__Acetobacterium	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae;g__Alkalibacter	0.0%	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae;g__Anaerofustis	0.0%	0.0%	0.0%	0.0%	0.1%
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae;g__Pseudoramibacter_Eubacterium	0.0%	0.0%	0.0%	0.0%	0.0%

Figure 3.11: Diversity assessment of seeding material and enrichment culture from 16S rRNA gene Illumina sequencing data (continued).

Methanogenic archaea community was also detected in the Thioglycollate media (**Figure 3.9**). *Methanosaeta*, a strict acetotrophic methanogen genus prevailed in both of the communities of the seeding material and Thioglycollate media. *Methanosphaera* which belong to the order *Methanobacteriales* were also found to be dominating the manure sample, *Methanobacterium*, a hydrogenotrophic genus was detected in Thioglycollate media.

Methanosarcina was dominant in Thioglycollate media, the dominance of *Methanosarcina* is in line with previous studies of manure-based digesters (Karakashev *et al.*, 2005; Sun *et al.*, 2015). *Methanomassiliicoccus* were found in the Thioglycollate media, this organism represents the seventh recently described order of methanogens, the *Methanomassiliicoccales* (Chojnacka *et al.*, 2015; Pandey *et al.*, 2015). Initially isolated from human faeces, these Archaea have been shown to be widely distributed in the environment. They use methylated compounds (mono-, di-,

tri-methylamine and dimethylsulphide) as substrates for methanogenesis and the methyl group is reduced by hydrogen (Chojnacka *et al.*, 2015).

3.4.3 DAPI staining

Increased amount of bacterial cells (stained blue) was seen in TYG media (**A**) incubated aerobically **Figure 3.12**, this is because TYG media contains the necessary nutrients to support the growth of bacteria. A substantial amount of bacterial cells was also observed in Basal medium (**B**) and Thioglycollate medium (**C).** More growth was also seen in media supporting the cultivation of acidogenic bacteria (**D**). Fewer cells were seen for Anoxic media (**F**) and ATCC media for methanogens (**E**), this was due to low cell biomass and also this might be an artefact of the fixation treatment but might also reflect specific characteristics of archaeal cell walls or cytoplasm (Long *et al.*, 1999).

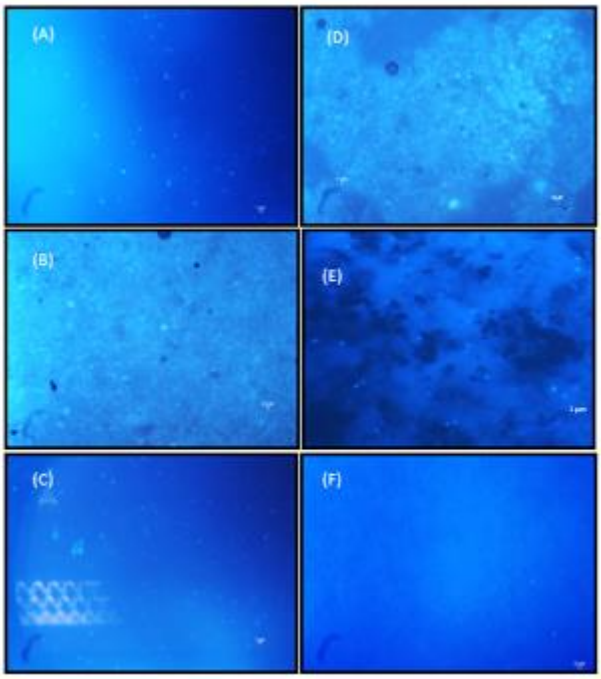


Figure 3.12: DAPI staining of various enrichments. (A) TYG medium, (B) Basal medium, (C) Thioglycollate medium and (D) Acidogenic medium, (E) ATCC media for methanogens, (F) Anoxic medium for methanogens. Scale bars were set at 1 µm.

3.5 Conclusions

Various enrichment were utilized to select for hydrolytic, acidogenic, acetogenic and methanogenic microorganisms under aerobic and anaerobic conditions. The presence of microorganisms was determined microscopically using DAPI staining and confirmed by molecular characterization.

All enrichment media was effective in enriching the desired microbial population. Anoxic media and ATCC media for methanogens were also effective in enumerating methanogenic archaea, this is because over some time a fair amount of growth was observed; however the growth rate or doubling time of methanogens was very slow, this could be due to a number of factors such as temperature and the substrate used, the substrate utilized can either be H_2 + CO_2, acetate or methyl-group containing compounds (Jabłoński *et al.*, 2015). The *mcrA* gene phylogenetic analysis revealed organisms which belong to the order *Methanobacteriales* and *Methanosarcinales*.

The DGGE analysis showed specific profile variations for enrichments and original seeding material. The analysis identified bacteria of the class *Clostridia* and *Bacteroidia*, these are associated with hydrolysis of the feedstock due to their hydrolytic enzyme activity. Bacteria from the genera *Aquabacterium, Butyrivibrio* and *Actinobacteria* were also identified; these are acidogenic and facultative anaerobic. They convert the products of hydrolysis and amino acids into carbon dioxide, hydrogen, ammonia, hydrogen sulphide, volatile fatty acids and organic acids like propionic, butyric acids and alcohols.

Hydrolytic bacteria found during DGGE also correlate with those found using Illumina sequencing, showing that the enrichment media used was specific and ideal for enriching the desired group of bacteria, thus further screening product accumulation of hydrolytic products such as sugars, amino acids and fatty acids would also confirm successful enrichment of hydrolytic bacteria. Furthermore, acid forming bacteria such as *Actinobacteria* found in DGGE and Illumina sequencing gave an indication of the presence of volatile fatty acids, subsequently these volatile fatty acids would be metabolised by various acetogenic and methanogenic microorganisms and finally CH_4 will be produced.

The 16S rRNA gene Illumina sequencing data clearly indicated a diverse bacterial community containing various bacteria, predominantly of the phylum *Firmicutes, Bacteroidetes, Synergistetes and Proteobacteria* which play a role in hydrolytic, acidogenic and acetogenic bacteria. The major bacterial genera found in the enrichments and original seeding material have previously been shown to be present in biogas digesters, these include members of *Rhodococcus, Butyricimonas, Fibrobacter, Solibacillus, Acetobacterium, Flavobacterium, Sphingobium, Dechloromonas and Salinibacterium*.

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CHAPTER 4

4 Characterization and analysis of different substrates for biogas production potential

4.1 Introduction

One of the major problems facing the world today, is the growing energy demand and reduced supply of substrates for fuel (Mursec, 2009; Oyewole, 2010). This has led to extensive research into the use of renewable energy sources, and consequently; the widespread use of new technology for energy production (Vindis, 2010). Today waste disposal alternatives include incineration, landfill, application to agricultural land and anaerobic digestion. Anaerobic digestion continues to be one of the most widely used processes for waste disposal due to waste stabilization ability (Ismail & Talib, 2014). Biogas production which is achieved by anaerobic digestion offers many environmental benefits and the production of biogas makes the process profitable. Among the different forms of renewable sources available, biogas is the most promising (Ismail & Talib, 2014).

The biogas production process is facilitated by different groups of microorganisms, which work synergistically to break down organic material (Ali Shah *et al.*, 2014; Igoni *et al.*, 2008; Zeb *et al.*, 2013). The organic material can be any raw material such as agricultural waste, manure, municipal waste, plant material, sewage and food waste (Offor, 2011). This organic material has high nutritional and calorific value necessary for the microorganisms to thrive (Yadvika *et al.*, 2004).

Biogas production follows the 4 stages of anaerobic digestion (**Figure 4.1**). Biomass is composed of macromolecules such as carbohydrates, proteins and fats. In order to start the digestion process, these long chain organic polymers should be broken down into their constituent monomers, this is necessary for the bacteria in the anaerobic digester to access the energy portion of the organic material. Monomers such as sugars, amino acids and fatty acids will thus be readily available for the microorganisms to metabolise (Dioha *et al.*, 2013; Zeb *et al.*, 2013). The process of breaking these large molecules into their smaller constituents is called hydrolysis. Therefore, hydrolysis of these long chain organic polymers is an important step in

anaerobic digestion. It is the first stage of anaerobic digestion and is carried out by hydrolytic bacteria, with the use of hydrolytic enzymes (Boe, 2006).

The process of acidogenesis further breaks down the remaining components by acidogenic bacteria. During this stage, volatile fatty acids (VFA) which are short-chained volatile organic acids such as acetic acid, propionic acid, butyric acid and valeric acid or branched isomers of them (iso- butyric acid), carbon dioxide, ammonia, and hydrogen, as well as other by- products are formed (Dioha *et al.*, 2013; Drosg, 2013).

The third stage of anaerobic digestion is acetogenesis. In acetogenesis, simple molecules formed during the acidogenesis stage are further catabolized into compounds that can be directly utilized by methanogens. During this stage acetogens produce acetate, carbon dioxide and hydrogen (Ali Shah *et al.*, 2014).

The terminal stage of anaerobic digestion is methanogenesis. Methanogens utilize the intermediate products of the preceding stages, such as acetate and hydrogen and convert them into methane, carbon dioxide, and water. It is these components that make up most of the biogas emitted from the system. Methanogenesis is sensitive to both high and low pH and occurs between pH 6.5 and pH 8 (Dioha *et al.*, 2013; Zeb *et al.*, 2013).

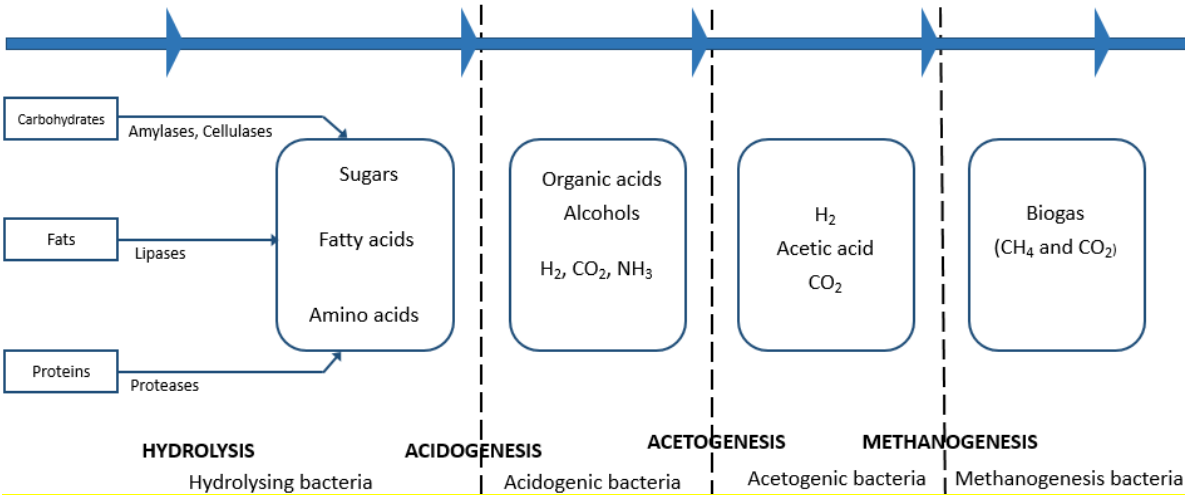


Figure 4.1: Key stages of biogas production (Adapted from Zeb *et al.,* 2013).

The composition of biogas varies depending on the chemical composition of the organic material used, as well as upon stages of the digestion process. The quality of biogas generated by organic waste materials does not remain constant but varies with the stage of digestion (Ismail & Talib, 2014). Nutrient composition and biodegradability are key factors for methane yield from energy crops and animal manures. Crude protein, fat, crude fibre, cellulose, hemi-cellulose, starch and sugar influence methane formation (Mursec *et al.*, 2006; Vindis *et al.*, 2007).

Following a general characterization and identification of the feedstock, the basic fermentability of the organic material can be estimated. Feedstock characterization is important as it gives information about handling the material and about special factors which need to be taken into consideration in the planning of a biogas installation in which feedstock of the same kind are to be used. Typically, feedstock is characterized by physical and chemical properties. Physical properties include consistency, homogeneity and particle size of the feedstock. Chemical properties include Total Solids, Volatile Solids, pH and Volatile Fatty Acids. Chemical analysis of an organic material allows a far-reaching assessment to be made of its potential fermentability.

Typically biogas contains 50 – 70% methane (CH_4), approximately 1% hydrogen (H_2), 25 – 50% carbon dioxide (CO_2), 0 – 2% oxygen (O_2) and 1 – 2% water (H_2O).The quality of biogas with regards to energy utilization is based on the percentage of the methane concentration, however this can be improved by reducing non-combustible matters such as carbon dioxide and hydrogen sulphide (H_2S) (Mursec, 2009).

Until recently, anaerobic digestion was a single substrate, single purpose treatment. For example, manure was digested to produce energy. Today, the limits and the possibilities of AD are better known and co-digestion has therefore become a standard technology (Rudolf & Arthur, 2001). Co-digestion is the simultaneous digestion of a homogenous mixture of two or more substrates. Several studies have been reported about the co-digestion of agricultural wastes and manure for biogas production (El-Mashad & Zhang, 2010; Ismail & Talib, 2014; Shehu *et al.*, 2012; Yong *et al.*, 2015; Zhang *et al.*, 2014).

Typically, so-called energy crops are chosen according to their net energy yield per hectare and in most cases a mixture of renewable primary products such as grass- or maize silage and manure is used, this co-digestion can optimize the production of

biogas (Ismail & Talib, 2014; Rudolf & Arthur, 2001). The advantages of energy crops such as maize and rye-whole crop silage are well-known harvesting practices and an easy storage by ensiling. The process of ensiling can be regarded as a pre-treatment of the substrate by which the pH value is considerably lowered to inhibit the growth of detrimental microorganisms. Another important fact concerning ensiling is the compacting of the silage and the sealing by means of plastic wraps to avoid aerobic degradation before usage.

In the study by Mursec and co-workers (2009), various agricultural substrates were analysed for processing into biogas. In the case of sunflower, the methane concentration reached about 62.85%; it means that out of 451 Nl/kg VS the methane quantity was 283 Nl/kgVS which was useful for utilization of the fuel, this substrate gave the most gas production and methane concentration, and this is particularly true due to the lipid content in sunflower. Two universally useful crops, maize and sorghum which are predominant animal feed were also tested. Maize and sorghum were also found to be suitable for the biogas production because they contain abundant proteins and carbohydrates, and these have a high energy potential. The amount of the biogas produced from sorghum was 350 Nl/kg VS, the methane concentration was 50 - 60%, therefore; about 180 - 205 Nl/kg VS energy potential (Mursec *et al.,* 2009).

Other alternative crops such as Jerusalem artichoke and amaranth were tested, which also have a high protein supply but cannot compete with predominant crops such as maize, produced considerably less gas and methane. The methane concentration in the amaranth sample was not excessively low, since it amounted to about 50% on an average.

Biogas production offers many advantages including the production of energy from waste at relatively low cost. A reduction of landfills and incineration of waste material contributing to carbon emissions. Airborne pathogens are also reduced while air quality is improved by odour control and/or reduction. Greenhouse gas emissions are reduced.

4.2 The aims of the chapter

- Standardizing analytical tests for biogas potential analysis according to VDI 4630 specifications.
- Evaluate various feedstocks for biogas potential test to start a local database.

4.3 Materials and Methods

4.3.1 Substrates/feedstocks tested

Different substrates were received from local farms and factories (**Table 4.1**). The sample was made homogeneous by cutting, shredding, chopping or grinding; this is an important step for both analytical aspects as well as a form of pre-treatment that will aid in the fermentation process (Mursec, 2009).

Table 4.1: List of substrates tested for biogas potential.

Name	Origin
Bran	Itau Milling
Hominy chop	Itau Milling
Dry paper pulp	Ibert Biogas
Liquid paper pulp	Ibert Biogas
Cow manure	Local farm
Swine manure	Local farm
Molasses	Ibert Biogas

4.3.2 Characterisation of substrate

4.3.2.1 Dry Matter (DM)

Clean porcelain crucibles were dried at 100°C in an oven overnight, cooled to room temperature in a desiccator and weighed accurately. Approximately 30 g of sample was weighed and placed into the dried and already weighed porcelain crucible. The sample was dried overnight at 105°C in an oven, cooled to room temperature in a desiccator and weighed again (Kreuger *et al.,* 2011). Dry matter was determined using the **Equation 4.1**.

$$Dry\ matter\ (g) = Mass\ (g)\ after\ drying - Mass\ (g)\ before\ drying \quad (\mathbf{4.1})$$

4.3.2.2 Volatile Dry Matter (vDM)

The dried sample that was used to determine the DM content was retained in the porcelain crucible in order to determine vDM. The sample of known DM weight was incinerated in the porcelain crucible for 1 hour at 550°C in a muffle furnace. After incineration, the porcelain crucible and ash were cooled to room temperature in a desiccator and weighed accurately. Volatile dry matter (vDM) also known as Organic Matter (OM) (Laskri *et al.,* 2015) was calculated using **Equation 4.2**.

$$Volatile\ dry\ matter\ (g) = mass\ (g)\ after\ incineration - mass(g)\ before\ incineration \quad (\mathbf{4.2})$$

4.3.2.3 Total Solids (TS)

Total Solids refers to the amount of solids present in the sample after the water present in it has been evaporated. It is the weight of the dry matter of a sample and is reported as a percent of the total weight of the sample. Total solids were determined following **Equation 4.3**. Fresh Matter refers to the mass of a material or the substrate in its original state with its natural water content (Kreuger *et al.,* 2011)

$$Total\ Soldis\ \% = \frac{Dry\ Matter}{Fresh\ Matter}\ \text{x}\ 100 \quad (\mathbf{4.3})$$

4.3.2.4 Volatile Total Solids (vTS)

The dried residue from Total Solid analysis was weighed and combusted for 1 hour at 550°C in a furnace (Peces *et al.,* 2014). After cooling the crucible residue was weighed, giving Volatile Total Solids. Volatile Total Solids were determined using **Equation 4.4**. Volatile Total Solids are reported as a percent of the total weight of the sample.

$$Volatile\ Total\ Solids\ (\%) = \frac{Volatile\ Dry\ Matter}{Dry\ Matter}\text{x}\ 100 \quad (\mathbf{4.4})$$

4.3.3 Nutrient composition analysis

Samples were also sent to the Department of Animal, Wildlife and Grassland Sciences for analysis (University of the Free State, South Africa). AOAC (2000) Official Methods of Analysis were used to determine fat (hexane extractives), acid detergent fibre (ADF), neutral detergent fibre (NDF), gross energy (GE) and crude protein (CP). Metrohm 744 pH meter was used to determine the pH.

4.3.4 Seeding material

The seeding material used for this experiment was a digestate received from Jan Kempdorp Meat to Market Abattoir biogas plant. The seeding material was stored at room temperature until used. The seeding material had TS and vTS content of 2% and 98% respectively. The pH of the seeding material was 7.41 determined using Metro-Hm 744 pH meter.

4.3.5 Experimental conditions for biogas production potential test

Biogas production potential of the various substrates was determined in a laboratory scale digester. In order to prevent inhibition in the fermentation batch, the European Standard VDI 4630 recommends that the substrate should not be overlarge in proportion to the seeding material (VDI 4630, 2006) and therefore samples are incubated on the basis of Organic Total Solids (oTS also known as vTS) which contributes to biogas. As a result, a ratio of 0.5 oTS substrate to seeding is recommended, shown in **Equation 4.5.** The VDI 4630 standard was used for the incubation of all tests, this was achieved by incubating specific ratios of substrate to seeding material, shown on **Table 4.2**.

$$\frac{oTS\ substrate}{oTS\ seeding\ material} \leq 0.5 \qquad (4.5)$$

Table 4.2: Recommended ratio for execution of test.

Sample name	Substrate (FM)	Seeding material (FM)	Water
Bran	216,45 g	1648,35 g	-
Hominy chop	300,12 g	1648,35 g	-
Dry paper pulp	54.17 g	1648,35 g	-
Liquid paper pulp	289,6 g	1648,35 g	-
Swine manure	1696,83 g	-	-
Cow manure	1696,83 g	-	300 ml
Molasses	555,55	1648,35 g	-

* FM = Fresh Matter (substrate in its original state with its natural water content).

The ratio of substrate to seeding material was 1:2 (15 g vDM substrate to 30 g vDM seeding material) according to the acceptable results of batch experiments by VDI 4630. The particle size of the organic material was reduced to <10 mm and was mixed with the seeding material. There was no addition of water during mixing. The results were presented according to standard conditions of temperature (273 K) and pressure (1013 mbar).

4.3.6 Digester design and construction

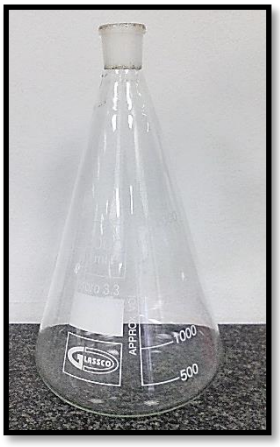
A laboratory bench scale digester set-up (**Figure 4.2 D**) was designed according to the European Standard VDI 4630. The eudiometer set-up consisted of a digester (2 L Erlenmeyer flask) with a NS 29/32 ground glass joint (**Figure 4.2 A**) (Mursec & Vindis, 2006). The gas pipe (eudiometer) was 400 ml volume graduated from the top down and contained the confining liquid (**Figure 4.2 C**). The levelling vessel (1 L Erlenmeyer flask) served as the reservoir tank (**Figure 4.2 B**). Distilled water was used as the confining liquid, 2 ml of methyl orange solution (0,1 g of methyl orange sodium salt dissolved in 100 ml of distilled water) were added to the water and the solution turned orange. The confining liquid is then stored at room temperature (Mursec, 2009).

The full experimental set-up is shown in **Figure 4.3 A**. The eudiometers were fixed to a metal stand with bosshead clamps, so that they do not overturn and can be easily removed and fixed for experimental purposes (Muršec & Vindiš, 2009; Vindis *et al.*,

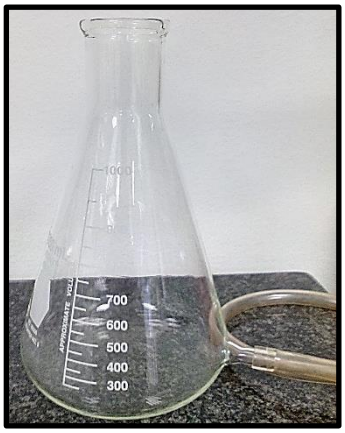
2008). On the upper edge of the eudiometer there is a tap cock for gas sampling and for adjustments of the zero mark (Mursec, 2009; Vindis, 2010).

Through the bottom of the eudiometer is a connecting tube which enables entry of the biogas from the digester into the eudiometer. The connecting tube was fixed in position by glass sticks located on four sides. The digesters were incubated under mesophilic conditions at a constant temperature of 38°C, which was maintained throughout the water bath (**Figure 4.3 B**); the water bath was covered in order to avoid heat loss and temperature fluctuations. On the lower edge of the eudiometer was a septum which links the eudiometer with the levelling bulb using a gas tight tubing. The biogas produced in the digester supplants the confining liquid in the eudiometer into the levelling vessel (Mursec & Vindis, 2006). The volume of the gas produced was read on the eudiometer.

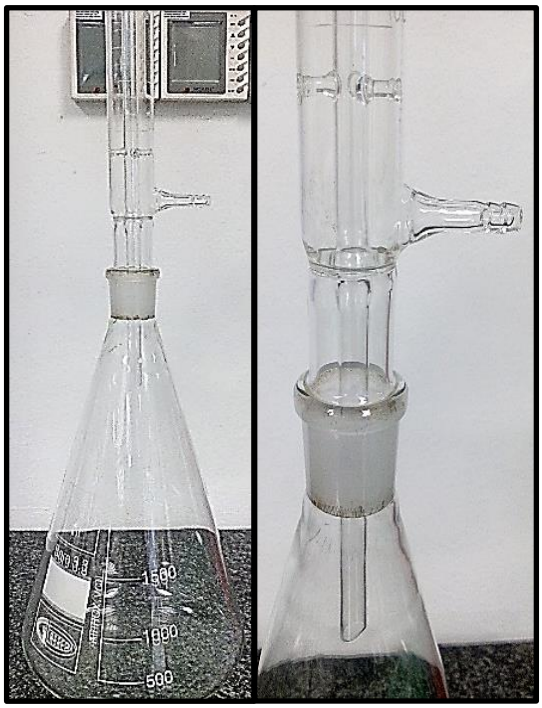
A 2 L Digester



B 1 L Levelling flask



D Digester fitted with eudiometer



C 400 ml Eudiometer

Figure 4.2: Components and equipment making up the biogas digester set-up.

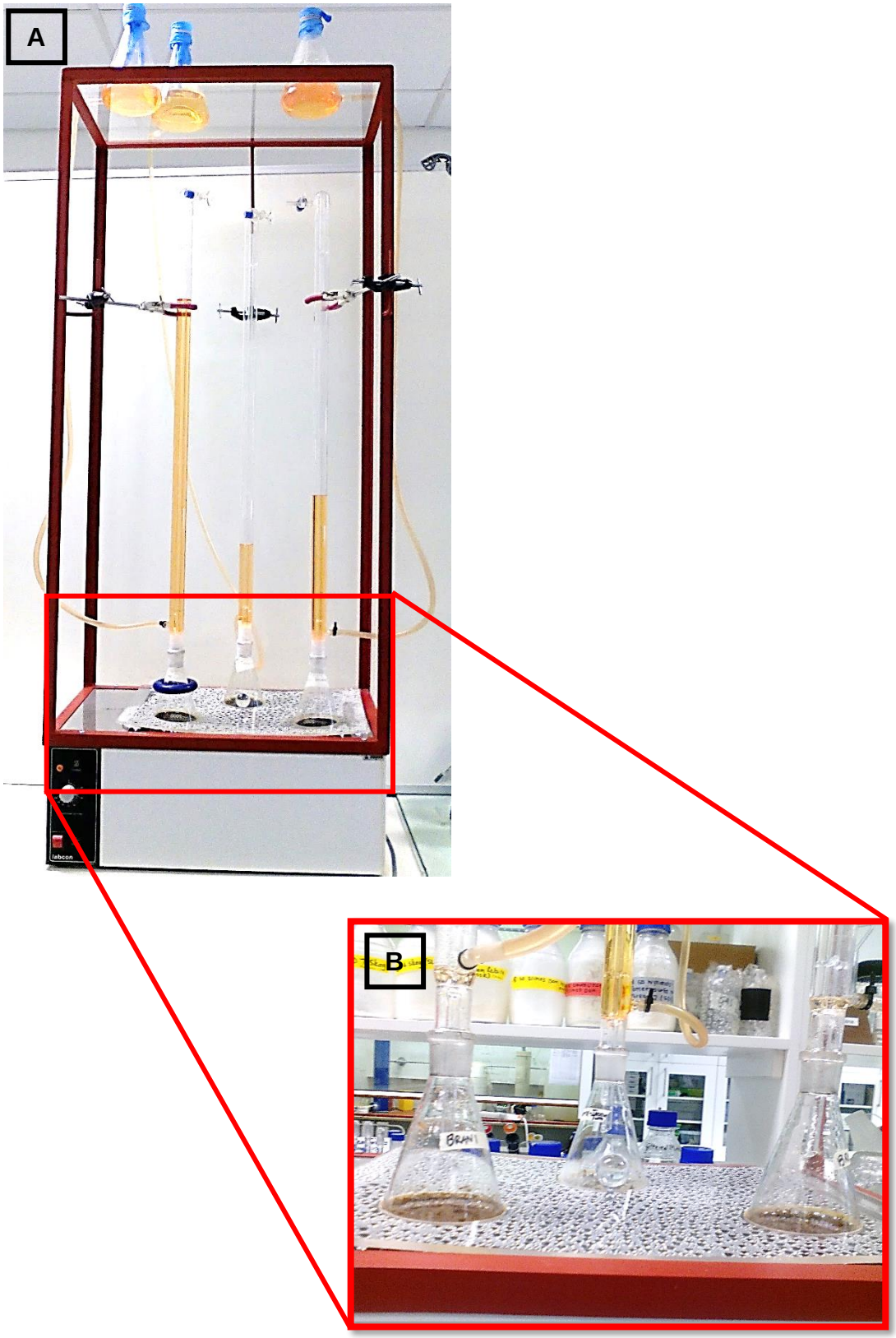


Figure 4.3: Laboratory scale digester set up for monitoring biogas production from substrate and seeding.

Biogas production was monitored daily and the biogas produced was collected from the eudiometer into a Tedlar bag. Biogas quality (CH_4, CO_2, and O_2) was analysed by gas chromatography.

4.3.7 Gas quantification and analysis

Gas samples were quantified by gas chromatography (GC). The analysis was carried out on a Shimadzu 2010 GC fitted with a Thermal Conductivity Detector (TCD) detector. The analytical column was a Restek ShinCarbon 80/100, 2 m x 0.53 mm, micro-packed. Injection was performed through a sampling valve with a 250 µL loop. Initial column temperature was 40°C raised at 8°C to 128°C. Argon was used as carrier gas at 133 kPa column head pressure. TCD temperature was 255°C.

4.4 Results

4.4.1 Substrate characterisation

All feedstocks from **Table 4.1** were analysed using methods described in **Section 4.3.2** to **Section 4.3.3** and the results are shown in **Table 4.3** and **Table 4.4**. Characterization of the substrate by Total Solids (TS) and Volatile Total Solids (vTS) is important owing to the influence on biogas production. The vTS represent the organic fraction of the substrate which is the source from which biogas is produced and is therefore very important. TS represent both the organic and inorganic fraction of the substrate like minerals, dust, glass and stones among others, varying according to the season and location in which the sample was harvested.

Literature has suggested that biogas production depends extensively on the composition of the substrates, the tables below (**Table 4.3** and **Table 4.4)** leads us to believe that a remarkable difference in the quantity of biogas would be produced by different substrates over the course of the experiments.

Following a general characterization and classification into groups the possibilities of a basic fermentability of the feedstock as well as the theoretical methane yield can be estimated. Feedstock characterization by elemental composition shown in **Table 4.3** could be used to estimate the biogas potential of a particular substrate. Buswell and

Mueller (1952) developed a model based on the chemical composition of the degraded waste to predict methane production and this is expressed as:

$$C_aH_bO_c + \left(a - \frac{b}{4} - \frac{c}{2}\right)H_2O \rightarrow \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4}\right)CH_4 + \left(\frac{a}{2} - \frac{b}{8} + \frac{c}{4}\right)CO_2$$

Where $C_aH_bO_c$ = organic matter and a, b and c are stoichiometric coefficients

In order for the Buswell's formula to function, the chemical and/or elemental compositions of the substrate need to be known. The elemental-analytical investigations into the titrimetric proportions of carbon (C), Hydrogen (H), Oxygen (O), Nitrogen (N), Sulphur (S), and phosphorus (P) in the organic part of a substrate are used in order to set up the empirical formula above. From this the stoichiometric relationships, the maximum biogas yield and composition (Buswell's formula) and the oxidation oxygen theoretically required (CSB) can be determined. Knowledge of the substrate's chemical and elemental fractions allows the determination of theoretical methane potential via three ways: (i) from protein, fat and carbohydrate distributions (ii) use of the maximal theoretical methane yield constant and (iii) substrate's elemental composition.

Anchinas and colleagues (2016) did a theoretical study of anaerobic digestion in order to predict the biogas amount of agricultural waste. The biochemical model by Buswell and Mueller (1952) was used. This model can predict biogas output assuming that a reaction goes to completion. From this study, it was found that the results using the Buswell model are unlikely to match the true results of a biogas digester because in practice no reaction goes to full completion and there is no 100% breakdown of cellulosic materials. The model predicts ideal settings that are not found in the real world. For this reason, the model was adjusted with the limiting factor in order to regulate the ideal conditions to more realistic ones (Achinas *et al.*, 2016).

Substrate characterisation

Table 4.3: Substrate analysis by Total Solids and Volatile Total Solids and chemical composition characterisation.

Sample name	Total Solids (TS) %	Volatile Total Solids (vTS) %	Phosphorus (P) g/kg DM	Potassium (K) g/kg DM	Sulphur (S) %	Nitrogen (N) %	Carbon (C) %	Hydrogen (H) %
Bran	7	99	12,84	3,16	0.52	1.15	53.68	0.24
Hominy chop	7	71,4	5.58	1,64	0.59	0.79	11.26	0.51
Dry paper pulp	39	71	0,77	0,10	0.103	1.05	17.98	1.80
Liquid paper pulp	7	74	2,18	0,35	0.22	2.25	31.90	2.18
Swine manure	1	73	23,78	3,59	0.15	3.41	33.56	2.63
Cow manure	34	78	5,26	1,84	0.102	5.20	49.19	2.84
Molasses	3	90	-	-	0.45	12.23	50.22	2.72

Substrate Characterisation

Table 4.4: Substrate characterisation by nutrient composition.

Sample name	Dry Matter (g/kg FM)	Organic Matter (g/kg DM)	Crude Protein (g/kg DM)	Gross Energy (g/kg DM)	Neutral Detergent Fibre (g/kg DM)	Acid Detergent Fibre (g/kg DM)	Fat (g/kg DM)	pH
Bran	900,63	955,77	154,86	18,09	374,41	113,80	28,49	7,04
Hominy chop	906,23	978,43	84,93	18,04	219,76	61,35	38,49	7,00
Dry paper pulp	393,80	755,49	16,63	12,04	637,03	578,75	12,73	7,4
Liquid paper pulp	72,89	770,20	21,71	11,66	637,8	583,87	14,67	5,3
Swine manure	7,70	-	206,97	17,97	-	-	36,06	6,58
Cow manure	180,96	780,13	54,11	15,7	548,10	322,31	23,82	7,31
Molasses	4,06	-	128,22	7.13	-	-	20,47	6,92

Total Solids (TS) and Volatile Total Solids (vTS) are commonly used to estimate the fermentability of the feedstock (**Table 4.3**). Total solids are used to determine the loading rate of the anaerobic digester and give clues as to when maintenance is needed. Typically, total solids amount to less than 10% of the total volume. The total volatile solids content can give an estimate on the amount of substrate that can potentially be turned into methane (VDI 4630, 2006). It only gives an estimate because volatile solids are made up of different organic compounds that have varying degradability speeds (Nelson, 2010). In order to elucidate the significance of TS and vTS various studies have been conducted.

The study by El-Mashad and co-workers (2004) did not list the final results of TS or vTS, but did indicate that TS was within the average range at 5%. The ratio of the TS/vTS was just slightly over one. For this trial, the hydraulic retention time (HRT) was 20 days and thermophilic bacteria were used (El-Mashad *et al.*, 2004).

Møllera and colleagues (2004) found that the average cow manure had approximately a third of the TS of a sow, and about half the TS of a pig. The cow also had the least methane production than the other animals. Volatile total solids for the three species were quite similar, not varying much more than 100 g/L from an average. The ratios of TS/vTS for all three trials were quite a bit lower than 1.0. The lowest ratio corresponded to the lowest methane production, but the highest ratio only gave the second highest methane production (Møllera *et al.*, 2004).

Hill and Bolte (2000) studied the effects of HRT on the production of methane. Their data is hard to draw conclusions from, as the different HRTs affect the production of methane. However, looking at the ratios of the TS/vTS the lowest ratio corresponds to the lowest production of methane (Hill & Bolte, 2000). As in the study by Molera *et al.,* (2004), the highest ratio corresponded to the second highest methane production.

Griffin and colleagues (1998) looked at start-up of an anaerobic digester for municipal solid wastes (MSW). The test used a mix of organic fraction of MSW, primary sludge, and waste activated sludge. The pH was controlled during this study with the addition of acid or base. They considered two types of digesters varied by temperature: mesophilic (with a constant temperature of 37˚C) and thermophilic (with a constant temperature of 55˚C). The methane production was very similar between the two

digesters; the highest ratio of TS/vTS corresponded to the highest production of methane (Griffin *et al.*, 1998).

For liquid samples like wastewater, vTS or TS are often not good parameters to try and follow because the volatile substances present (acetic acid, ethanol) cannot be determined. In these cases, a chemical oxygen demand (COD) measurement is applied. Chemical oxygen demand measurements are rarely applied for solid feedstocks as the analysis is more complex than the VS measurement and the reproducibility is quite poor for a solid or inhomogeneous feedstock (VDI 4630).

Characterization by nutritional composition is shown on **Table 4.4**. Protein varied from as low as 16,63 g/kg for dry paper pulp to as much as 206,97 g/kg for swine manure, this shows that swine manure contains 20% more protein than dry paper pulp; as a result swine manure would be a much favourable substrate than dry paper pulp. Methanogenesis is fastest in systems with excess of proteins followed by the reactors with excess of cellulose and carbohydrates respectively (Drosg, 2013; Kovács *et al.*, 2013; VDI 4630, 2006).

Fat content also gives a good indication of the theoretical biogas yield and CH_4 content. Fat content varied from as low as 12,73 g/kg for dry paper pulp to as much as 36,06 g/kg for swine manure, this is 3x (times) more fat than dry paper pulp. The highest methane yields have systems with excess of lipids but with the longest retention time. However, there are also inhibitory effects observed in the assays with excess of lipids and excess of proteins due to the VFA accumulation and ammonium nitrogen respectively. The lowest rates of the hydrolysis are the assays with an excess of lipids and cellulose, indicating that when these components are in excess, a slower hydrolysis is induced (Neves, 2007; VDI 4630, 2006). Similarly, the organic matter of the bran substrate is 20% more than dry paper pulp, indicating that bran contains more organic material in comparison to dry paper pulp, thus suggesting that bran is likely to yield more biogas than dry paper pulp.

4.4.2 Biogas production potential for various substrates

Substrates were mixed according to **Section 4.3.5.** The gas generated was measured daily using the water displacement method by reading the volume of water displaced

from the eudiometer into the levelling vessel that is equal to the volume of gas generated. The test proceeded for 30 days and biogas production was recorded at three days intervals. The biogas produced was expressed in norm litre per kg of volatile dry mass (Nl/kg vDM); the volume of the biogas production is based on norm conditions: 273 K and 1013 mbar (Vindis *et al.*, 2009).

4.4.2.1 Bran

Bran substrate was mixed with seeding material in the ratio 216,45 g bran and 1648,35 g seeding, this is in relation with the 0,5 oTS recommended by the European Standard VDI 4630. The data presented on **Table 4.5** shows the cumulative biogas yields. The gas production proceeded as shown in **Figure 4.4**. The final biogas production amounted to 885 Nl/kg vDM. Methane concentration was 75.55% CH_4, this means that out of 885 Nl/kg vDM the methane quantity was 669 Nl/kg vDM which was useful for utilization of the fuel. The remaining gas composition consisted of 18.60% CO_2, 3% N_2, 2.35% H_2 and 0.5% O_2.

Table 4.5: Biogas yield for bran substrate.

Weeks	Days	Gas yield			
		ml	**Nl/kg FM**	**Nl/kg DM**	**Nl/kg vDM**
Week 1	3	236	5,487	78,4	79,2
	6	475	11,0	157,8	159,4
Week 2	9	880	20,4	292,3	295,2
	12	1050	24,4	348,8	352,3
Week 3	15	1436	33,3	477,0	481,8
	18	1682	39,1	558,7	564,3
Week 4	21	2015	46,9	669,3	676,0
	24	2330	54,2	773,9	781,7
Week 5	27	2555	59,4	848,6	857,2
	30	2640	61,4	876,9	885,7

4.4.2.2 Hominy chop

Hominy chop substrate was mixed with seeding material. The mixture consisted of 300,15 g hominy chop and 1648,35 g seeding material. The hominy chop was already

ground and no further pretreatment was done. The experiment proceeded for 30 days. Biogas production was recorded every three days as depicted in **Table 4.6**. The final biogas yield shown in **Figure 4.4** was 727 Nl/kg vDM with 68.17% CH_4 content. Therefore 496 Nl/kg vDM methane quantity was useful as fuel. The remaining gas composition consisted of 12.35% CO_2, 11.89% N_2, 6% H_2 and 1.59% O_2.

Table 4.6: Biogas yield for hominy chop substrate.

Weeks	Days	Gas yield			
		ml	**Nl/kg FM**	**Nl/kg DM**	**Nl/kg vDM**
Week 1	3	125	3,3	47,0	65,8
	6	335	8,8	126,0	176,4
Week 2	9	442	11,6	166,2	232,8
	12	525	13,8	197,4	276,5
Week 3	15	600	15,8	225,6	316,0
	18	655	17,2	246,3	344,9
Week 4	21	750	19,7	282,0	395,0
	24	825	21,7	310,2	434,5
Week 5	27	1170	30,7	439,9	616,1
	30	1382	36,4	519,6	727,8

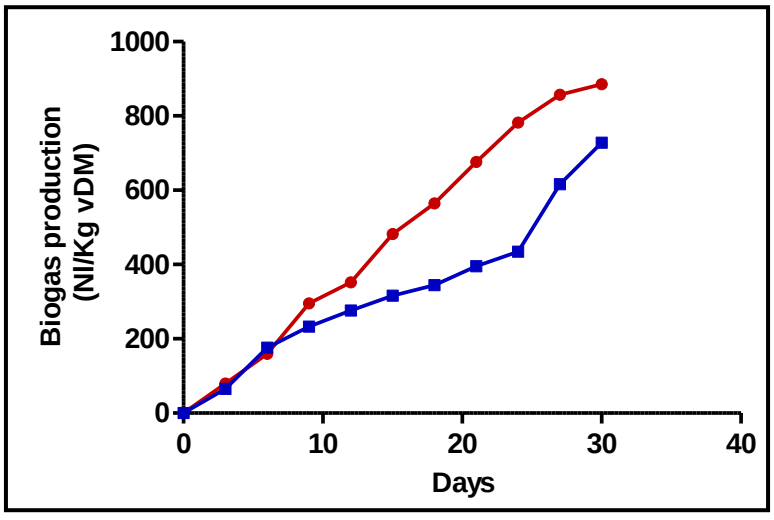


Figure 4.4: Biogas production for bran and hominy chop during 5-week digestion. Bran (—); Hominy chop (—).

Bran and hominy chop are universally useful agricultural products which are predominatly used in the production of refined grains, such as cereal, rice and wheat. These crops are also used as animal feed. Bran and hominy chop are also suitable for biogas production because they contain much protein and carbohydrate which have a high energy potential. Bran substrate produced more biogas than hominy chop, this could be expected because during substrate characterization, bran showed to have more vTS and crude protein than hominy chop.

4.4.2.3 Dry paper pulp

Dry paper pulp was mixed with seeding material, the mixture consisted of 54,17 g dry paper pulp and 1648,35 g seeding material. The dry paper pulp was shredded as a form of pretreatment. The experiment proceeded for 27 days. Biogas production was recorded every three days as depicted in **Table 4.7**. The final biogas production shown in **Figure 4.5** was 148,35 Nl/kg vDM with 53.4% CH_4 content. This means that out of 148,35 Nl/kg vDM the methane quantity was 77 Nl/kg vDM which was useful for utilization of the fuel. The gas composition also constituted of 6.11% CO_2, 40.23% N_2, 0.2% H_2 and 0.06% O_2. The high N_2 fraction comes from hydrogen producing acetogens, the hydrogen is further utilised by hydrogenotrophic methanogens to produce CH_4 (Angelidaki *et al.,* 2009; Luo & Angelidaki, 2012).

Table 4.7: Biogas yield for dry paper pulp.

Weeks	Days	Gas yield			
		ml	**Nl/kg FM**	**Nl/kg DM**	**Nl/kg vDM**
Week 1	3	170	3,1	14,6	20,5
	6	282	5,1	24,2	34,0
Week 2	9	470	8,5	40,2	56,7
	12	670	12,2	57,4	80,8
Week 3	15	880	16,0	75,4	106,2
	18	1000	18,2	85,7	120,6
Week 4	21	1190	21,6	101,9	143,5
	24	1230	22,4	105,4	148,3
Week 5	27	1230	22,4	105,4	148,3

4.4.2.4 Liquid paper pulp

Liquid paper pulp was mixed with seeding material and incubated at 38°C. The mixture consisted of 289,6 g dry paper pulp and 1648,35 g seeding material. The substrate was a homogeneous pulp and no further pretreatment was needed. The experiment proceeded for 27 days. Biogas production was recorded every three days as depicted in **Table 4.8**. The final biogas production shown in **Figure 4.5** was 722,1 Nl/kg vDM with 47.73% CH_4 content. This means that out of 722,1 Nl/kg vDM the methane quantity was 345 Nl/kg vDM which was useful for utilization of the fuel. After day 21 of the test the biogas production lagged behind and remained constant until the end of the experiment. This phenomenon may be caused by trouble in the digester due to presence of major concentration of carbon dioxide and oxygen. The gas composition also constituted of 50.82% CO_2, 0.15% N_2, 1.25% H_2 and 0.05% O_2.

Table 4.8: Biogas yield for liquid paper pulp.

Weeks	Days	Gas yield			
		ml	**Nl/kg FM**	**Nl/kg DM**	**Nl/kg vDM**
Week 1	3	150	0,5	74,7	101,2
	6	350	1,2	173,7	235,5
Week 2	9	485	1,7	239,8	325,0
	12	560	1,9	277,1	375,6
Week 3	15	620	2,1	307,2	416,5
	18	750	2,6	373,3	506,0
Week 4	21	1075	3,7	532,6	722,1
	24	1075	3,7	532,6	722,1
Week 5	27	1075	3,7	532,6	722,1

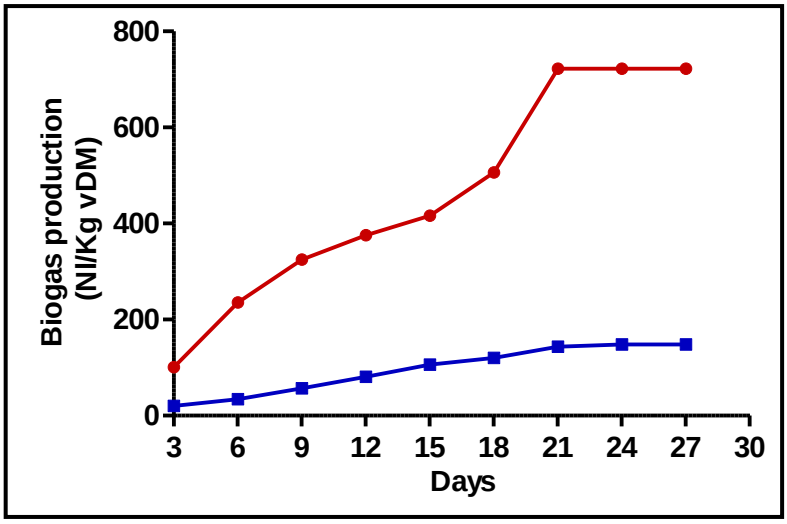


Figure 4.5*:* Biogas production for dry and liquid paper during 5 week digestion. Liquid paper pulp ( ); dry paper pulp ( ).

During substrate characterization, liquid paper pulp showed to have more organic dry matter, crude protein and vTS than dry paper pulp, thus it is expected that liquid paper pulp would yield more biogas than dry paper pulp.

4.4.2.5 Cow manure

Cow manure was incubated without seeding at 37°C. Cow manure (1696,83 g) was loaded into a batch anaerobic digester, 300 ml water was added to the manure in order to achieve a homogeneous, slurry consistency. The cow manure was not pre-treated. The experiment proceeded for 60 days. The experiment was given a longer retention time because as agreeable with literature, manure samples take longer to digest due to the complex composition like lignin (Amon *et al.*, 2007).

Biogas production was recorded every six days as depicted in **Table 4.9**. The final biogas production shown in **Figure 4.6** amounted to 350 Nl/kg vDM with 57% CH_4 concentration. This means that out of 350 Nl/kg vDM the methane quantity was 200 Nl/kg vDM which was useful for utilization as fuel. Most of the biodegradable carbon in cow feed is already digested in the rumen, thus cow manure has a lower potential to produce biogas than swine or poultry manure. CH_4 concentration in the biogas is lower (Weiland, 2010). The gas remaining gas composition was 40% CO_2, 2% N_2, 0.92% H_2 and 0.08% O_2.

Table 4.9: Biogas yield for cow manure.

Weeks	Days	Gas yield			
		ml	**Nl/kg FM**	**Nl/kg DM**	**Nl/kg vDM**
Week 2	6	400	6,7	19,6	25,1
	12	1000	16,7	49,0	62,9
Week 4	18	1520	25,3	74,5	95,5
	24	2830	47,2	138,8	177,9
Week 6	30	3000	50,0	147,1	188,6
	36	3600	60,0	176,5	226,3
Week 8	42	4205	70,1	206,2	264,3
	48	5025	83,7	246,4	315,9
Week 10	54	5200	86,8	255,0	326,9
	60	5580	93,0	273,6	350,7

In agreement with the above results, Amon and co-workers (2007) found the lignin and cellulose content of cow feeds to influence biogas and methane production from dairy cattle manure. A model was developed that estimates biogas and methane yield from carbohydrate, fat and protein content of cow manure. Lignin content in cow manure, which is determined by lignin content in the animal diet, was a key influence on biogas production (Amon *et al.*, 2007).

Feed lignin content correlates with the vegetation period and a variation can be observed in course of the year. Amon and colleagues (2001) measured methane production at a commercial biogas plant for 1 year. The biogas plant digested dairy cow and swine farmyard manure. Specific methane production was not constant throughout the year. When the dairy cow diet changed from winter feed to summer feed, specific methane production increased. Winter feed consisted mainly of hay. In spring and summer fresh clover grass was fed (Amon *et al.*, 2001).

4.4.2.6 Swine manure

Swine manure was incubated without seeding at 37°C. A weight of 1696,83 g was loaded into a batch anaerobic digester. The manure was a homogeneous liquid and no water was added. The manure was not pre-treated. The experiment proceeded for

60 days. Similar to cow manure the experiment was given a longer retention time. Biogas production was recorded every six days as shown in **Table 4.10**. The final biogas production depicted in **Figure 4.6** amounted to 400 Nl/kg vDM with 60% CH_4 concentration. This means that out of 400 Nl/kg vDM the methane quantity was 240 Nl/kg vDM which was useful for utilization as fuel. The remaining gas composition consisted of 35.2% CO_2, 3.8% N_2, 0.5% H_2 and 0.5% O_2.

Table 4.10: Biogas yield for swine manure.

Weeks	Days	Gas yield			
		ml	**Nl/kg FM**	**Nl/kg DM**	**Nl/kg vDM**
Week 2	6	360	0,2	17,5	23,9
	12	925	0,4	44,9	61,5
Week 4	18	1620	0,8	78,6	107,6
	24	2300	1,1	111,6	152,8
Week 6	30	2870	1,4	139,2	190,7
	36	3400	1,6	164,9	225,9
Week 8	42	4100	1,9	198,9	272,4
	48	5452	2,6	264,4	362,2
Week 10	54	6000	2,9	291,0	398,6
	60	6020	2,9	292,0	400,0

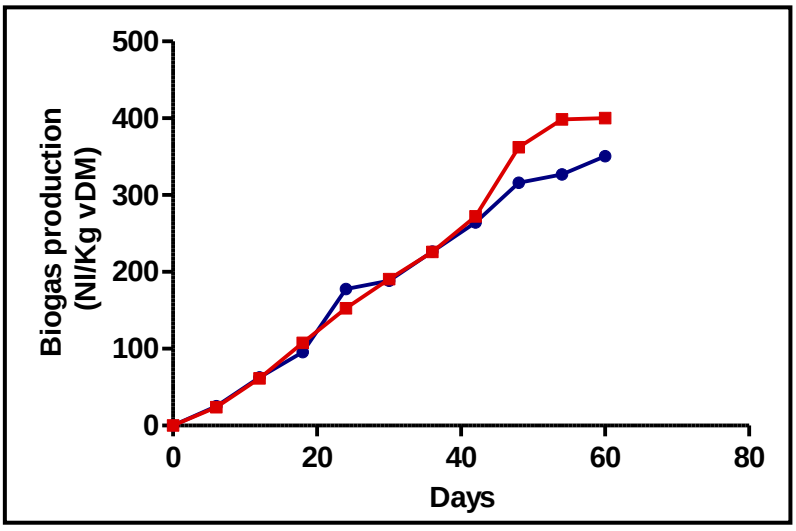


Figure 4.6: Biogas production for cow and swine during 10 week digestion. Swine manure ( ); cow manure ( ).

Swine manure yielded the most biogas and increased CH_4 than cow manure, this is because swine manure showed to have more crude protein and fat content than cow manure.

4.4.2.7 Molasses

Molasses was mixed with seeding material and incubated at 38°C. The mixture consisted of 555,55 g dry paper pulp and 1648,35 g seeding material. The substrate was homogeneous and no further pretreatment was necessary. The experiment proceeded for 30 days. Biogas production was recorded every three days as shown in **Table 4.11**. The final biogas production depicted in **Figure 4.7** was 233,3 g Nl/kg vDM with 72,75% CH_4 content. This means that out of 233,3 Nl/kg vDM the methane quantity was 170 Nl/kg vDM which was useful for utilization of the fuel. The remainng gas composition consisted of 23.58% CO_2, 3.3% N_2, 0.3% H_2 and 0.07% O_2.

It could be anticipated that molasses would yield the most biogas as it contains more carbohydrates (sugars) compared to other substrates. It is important to note that even though molasses did not yield maximum biogas; the CH_4 content was second highest at 72.75% after bran which yielded 75.55% CH_4.

Table 4.11: Biogas yield for molasses.

Weeks	Days	Gas yield			
		ml	**Nl/kg FM**	**Nl/kg DM**	**Nl/kg vDM**
Week 1	3	400	0,7	24,0	26,7
	6	928	1,7	55,7	61,9
Week 2	9	1200	2,2	72,0	80,0
	12	1500	2,7	90,0	100,0
Week 3	15	2000	3,6	120,0	133,3
	18	2333	4,2	140,0	155,5
Week 4	21	2500	4,5	150,0	166,7
	24	3000	5,4	180,0	200,0
Week 5	27	3200	5,8	192,0	213,3
	30	3500	6,3	210,0	233,3

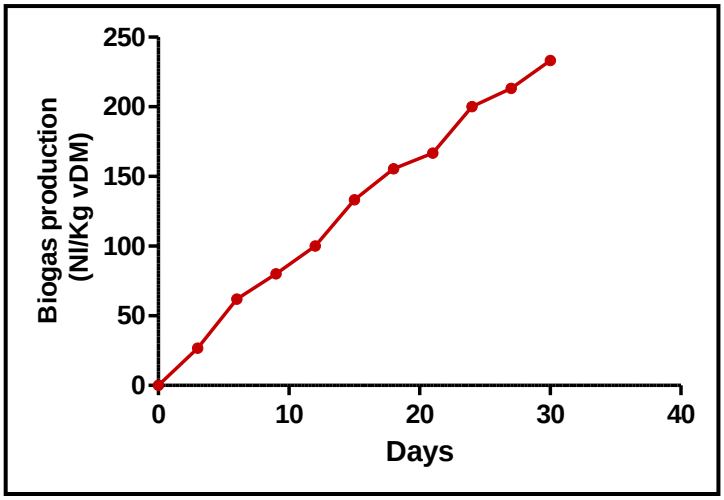


Figure 4.7: Biogas production for molasses during 5 week digestion.

Many experiments using carbohydrate-rich wastes including food wastes, molasses and sugarcane wastewater have been documented (Lee *et al.*, 2015; Singh & Bajpai, 2013). Singh and co-workers (2013) conducted a batch study at mesophilic conditions (35 ± 1°C) for 90 days, the results showed the maximum methane yield from molasses as 286 L CH_4 /kgVS which was approximately 63% of the calculated theoretical yield. The observed results were similar to the biogas yield and methane concentration obtained in the current experiment.

4.4.3 Seeding material (Blank gas production)

Table 4.12 shows the seeding material which was incubated separately as a reference test. The final biogas yield is depicted in **Figure 4.8** as 15,7 Nl/kg vDM with 51.2% CH_4 content. The gas also composed of 46.84% CO_2, 1.41% N_2, 0.42% H_2 and 0.13% O_2. This implies that only 15,7 Nl/kg vDM biogas from seeding material contributed to the final biogas yield when the various substrates were inoculated with this seeding material. This validates that adding digestible material enhances biogas production and consequently CH_4 concentration.

Table 4.12: Biogas yield for reference seeding material.

Weeks	Days	Gas yield			
		ml	**Nl/kg FM**	**Nl/kg DM**	**Nl/kg vDM**
Week 1	3	70	0,04	2,1	2,3
	6	150	0,1	4,5	5,0
Week 2	9	160	0,1	4,9	5,3
	12	160	0,1	4,9	5,3
Week 3	15	200	0,1	6,1	6,7
	18	240	0,2	7,3	8,0
Week 4	21	300	0,2	9,1	10,0
	24	350	0,2	10,6	11,7
Week 5	27	400	0,3	12,1	13,3
	30	470	0,3	14,3	15,7

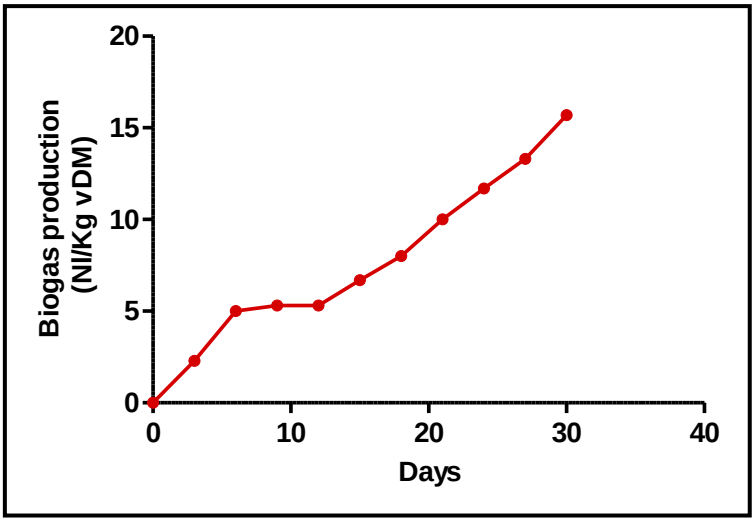


Figure 4.8: Biogas production for seeding material during 5 week digestion.

4.5 Conclusions

Agriculture biomass serves as good substrate for biogas production. It is important to note that feedstock characterization in terms of elemental and nutritional composition is necessary as this can give an estimate of gas potential for the particular substrate.

The findings of this study show than bran, liquid paper pulp and swine manure are feasible for gas production compared to their counterparts, this is due to the high organic content and vTS of the feedstock. Cow manure also has the potential to be a good substrate for biogas production, however the lignin content in the manure causes the process to yield low biogas.

The biogas potential test for molasses yielded approximately 72% CH_4. Although this is still relatively high, the overall biogas yield was however lower than expected for molasses, especially given than it is a sugar by-product, only 233,3 Nl/kg vDM biogas was produced. This low volume of biogas can be attributed to possible interferences either with the substrate and the fermantation process; the system could have been experiencing VFA over-load. The seeding material incubated as reference only produced 15,7 Nl/kg vDM biogas, therefore this confirms that co-digestion of digestible substrates is important for optimum biogas production and consequently more CH_4.

It is also evident that the retention time of the digester is important as more biogas yield can be expected if the digester is kept running for longer. Gas composition also varies depending on the retention time and as well as the chemical composition done during feedstock characterization.

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CHAPTER 5

5 The application of batch tests to assess the biogas production potential of spent mushroom substrate and upscaling to continuous-flow bioreactor system to optimize methane content

5.1 Introduction

The term “spent mushroom substrate” (SMS) is used to describe a by-product of mushroom production. Spent mushroom substrate is the left-over material after the harvesting of mushrooms. SMS is commonly made from varying amounts of agricultural residues such as sawdust, wheat straw, hay, poultry manure, corncobs and other substances.

Spent mushroom substrate is a good feedstock for producing biogas because there are many nutrients in SMS that provide the basis for the long term proliferation of methane producing bacteria. Furthermore, SMS has less lignin due to the digestion process by extracellular lignocellulosic enzymes during mushroom production (Phan & Sabaratnam, 2012). Extracellular lignocellulose degrading enzymes that have been found to be produced by mushrooms include xylanases, cellulases and cellobiases (Kerem *et al.*, 1992; Park *et al.*, 2012; Wood *et al.*, 1985).

Furthermore, it was found that the hemicellulose content of wheat straw decreased, from 26.3% to 7.4% of dry mass, 120 days after inoculation with mycelium of *Pleurotus ostreatus* while cellulose content decreased from 45.3% to 34.1%. The lower lignin content but high nitrogen and ash content make SMS more easily digested by hydrolysing microorganisms in order to yield more reducing sugars. Indeed, the resulting polysaccharides act as a suitable substrate for hydrolysis, since the production of SMS itself has served as a form of pre-treatment (Hayes & Hayes, 2009).

Lin and co-workers (2015) investigated the anaerobic digestion of SMS from Shiitake cultivation for enhanced biogas production. Enzymatic digestibility and cumulative methane yields (133–160 L kg−1 VS during 62 days) were achieved (Lin *et al.,* 2015).

5.2 The aims of the chapter

In order to optimize the conditions of biogas production using spent mushroom substrate (SMS), it is important to understand and describe the microbial diversity and growth dynamics inside the bioreactor.

This study was initiated with the following objectives:

- To assess the feasibility of using spent mushroom substrate as feedstock for biogas production using a bench scale digester.
- Up scaling the bioreactor system using 2-stage process and understanding how the microbial diversity changed.

5.3 Materials and Methods

5.3.1 Substrate characterization

5.3.1.1 Substrate and new Inoculum used as seeding material

Spent Mushroom Substrate (**Figure 5.1**) was received from Food Biotechnology (Department of Microbial, Biochemical and Food Biotechnology, UFS, South Africa). The seeding inoculum was collected from the Jan Kempdorp Meat to Market Abattoir biogas plant (Northern Cape, South Africa).

Figure 5.1: Spent Mushroom Substrate (SMS) used as feedstock.

5.3.1.2 Analytical methods

Dry matter (DM), Volatile dry matter (vDM), Total Solids (TS) and Volatile Total Solids (vTS) were determined according to standard methods on VDI 4630 as described in **Section 4.3.2**.

The SMS and inoculum were sent for nutrient composition analysis at the Department of Animal, Wildlife and Grassland Sciences (University of the Free State, South Africa). The analysis were performed using Official Methods of Analysis (AOAC 2000) to determine dry matter (DM), organic matter (OM), crude protein (CP), gross energy (GE), acid-detergent fibre (ADF), neutral detergent fibre (NDF), fat content and mineral content including potassium (K) and phosphorus (P). SMS and Inoculum were also sent for chemical composition analysis using LECO CHN628 elemental analyzer at the Department of Microbial, Biochemical and Food Biotechnology.

5.3.2 Bench-scale digester set up

A 2 L bench scale digester (**Section 4.3.6**) was used for the incubation of spent mushroom substrate. Digesters were set-up as triplicates and terminated at day 7, 14 and 28 respectively; this was done in order to assess how much gas was produced and assess the changes of the spent mushroom substrate over 28 days. This was also important to get a better understanding of the microbial population shift over the period of incubation. The digesters were incubated according to the organic loading ratio of 0.5 Organic Total Solids (OTS) substrate to inoculum as recommended by VDI 4630, therefore 11.5 g spent mushroom substrate was inoculated with 1 115 g inoculum.

5.3.3 Two-stage bioreactor set up

The system was optimised by upscaling to a 27 L two-stage anaerobic bioreactor (**Figure 5.2**), herein different parameters were monitored and controlled. Components of the two-stage reactor system included a 20 L vessel where the anaerobic digestion took place, the vessel was fitted with pH and DO probes. The vessel was also equipped with baffles and impellors for stirring. The reactor gas outlet pipe was fitted to a 400 ml eudiometer for gas quantification and sampling. The effluent sample from the 20 L vessel was pumped into a second bioreactor (7 L) and inoculated with 1 L basal media (**Section 3.3.3.3**). Thus Bioreactor 1 served as the hydrolysis vessel and the second bioreactor (7 L) was regarded as the acetogenesis and methanogenesis vessel as basal media enhances the growth of acetogenic bacteria thus enhancing the system for acetogenesis and methanogenesis stages. The second bioreactor was also fitted with a 400 ml eudiometer for gas quantification and sampling. Incubation ratios were done as recommended by VDI 4630, therefore 100 g spent mushroom substrate was inoculated with 9 920 g (approximately 10 L) seeding material.

Temperature was controlled in the bioreactors. Parameters such as pH, stirring and dissolved oxygen (DO) were monitored over the incubation period in the 20 L and 7 L bioreactors.

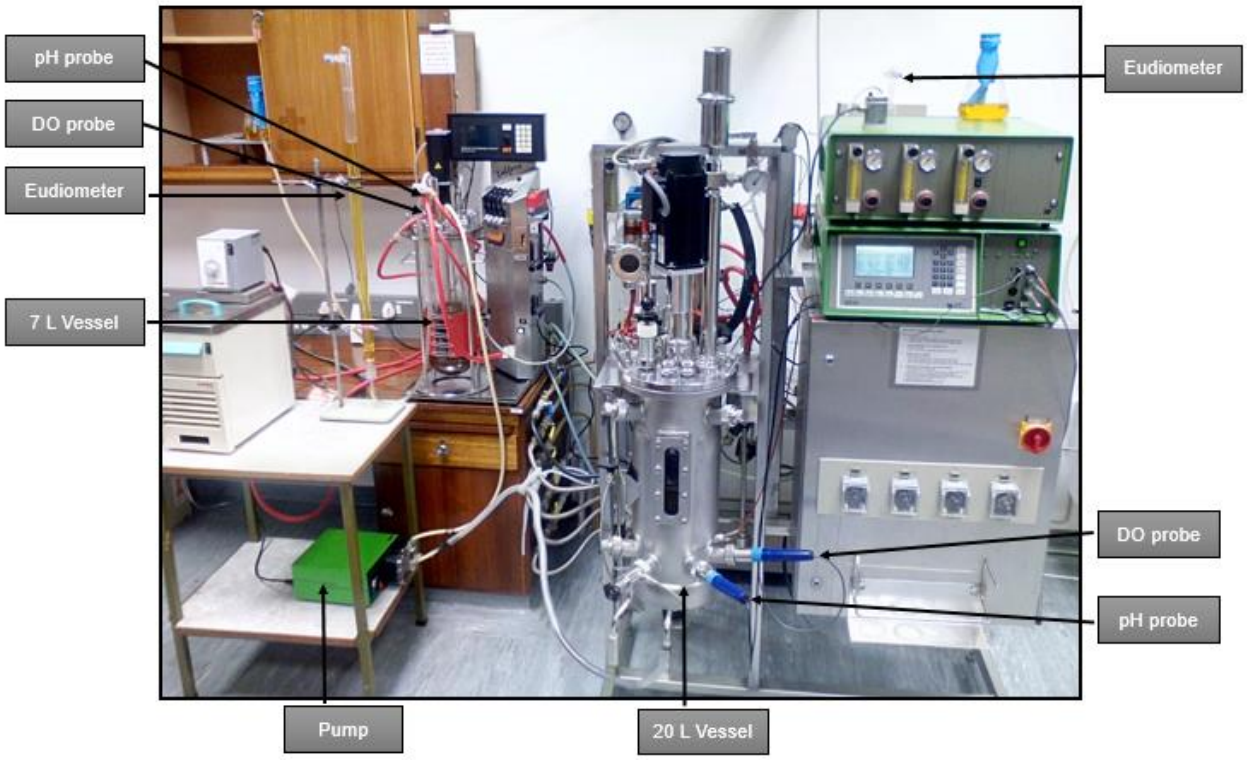

Figure 5.2: Two-stage bioreactor comprising of a 20 L vessel fitted with a pH and DO probe and eudiometer for gas quantification, connected to a 7 L vessel also fitted with a pH and DO probe and eudiometer.

5.3.4 Process monitoring

The monitoring of anaerobic digesters is necessary to ensure optimised operations. Given that anaerobic digestion is a complex process involving several groups of microorganisms which are sensitive to many operating factors as discussed in **Chapter 3**, it is important to be able to detect process imbalances in the early stages so that action can be taken to optimise. As with other biological processes, anaerobic digestion can be monitored by measuring parameters discussed in **Section 5.3.4.1 – 5.3.4.6.**

5.3.4.1 pH

Upon digester termination, pH was measured on the digestate or effluent samples. Changes in pH can be used as an indicator because the microorganisms can function only in a specific pH range as discussed in **Chapter 3,** also in the biogas process;

specific sections like acidogenesis function optimally at certain pH and thus if each step is optimised then kinetically optimum biogas can be produced; conversely the pH of the feed can also improve or impair biogas production. The pH of the digestate was measured using a calibrated MetroHM 744 pH meter.

5.3.4.2 Alkalinity

Alkalinity or buffering capacity is a better alternative than pH for indicating volatile fatty acids (VFA) accumulation, since the increased VFA will directly consume alkalinity before any pH changes. Alkalinity was measured using MetroHM 665 Dosimat, the titration was performed using 37% hydrochloric acid (HCl) to an endpoint of pH 4.5. **Equation 5.1** was used to calculate the buffering capacity of the digesters (Drosg, 2013; Yoon *et al.,* 2014).

$$Total\ alkalinity\ (CaCO_3 mg\ per\ L) = \frac{\alpha(Volume\ HCl\ displayed\ on\ on\ Dosimat)*0.1\ (Normality\ of\ acid)*50\ 000}{100ml\ (Sample\ volume)} \quad \textbf{(5.1)}$$

5.3.4.3 Long chain fatty acids (LCFAs)

The digestate samples (40 ml) were centrifuged at 3 000 *x g* for 10 min and then at 15 000 *x g* for 20 min at 4°C. The supernatants were filtered through 0.22 µm membrane filter (GVS Filter Technology) in order to remove any particulates that can affect the column and thereby tampering with the results. Fatty acids were analysed by gas chromatography after derivatization with trimethylsulfonium hydroxide. The instrument used was a Shimadzu 2010 chromatograph fitted with a SGE BPX70 analytical column with dimensions, length: 30 m, inner diameter: 0.25 mm and film thickness of 0.25 µm. Flame ionization detection was employed with detector temperature at 260°C and an injection port temperature at 250°C. Hydrogen carrier gas flow rate was 60 cm/s. Column temperature programme was as follows: initial temperature 100°C held for 1 min then raised at 5°C/min to 256°C. Instrument control and data collection and analysis was carried out with Shimadzu GC Solution software.

5.3.4.4 Volatile fatty acids (VFAs)

The supernatants were further separated by solvent extraction (Chloroform: Methanol 2:1) and analysed for volatile fatty acids (VFAs). The analysis was carried out on a Thermo Finnagan Survyeor HPLC consisting of a pump, autosampler, photodiode array detector and a refractive index detector connected in series. The analytical column was a Biorad Aminex HPX 87H of 300 mm by 7.6 mm which was held at 65°C. Sulfuric acid, 5mM, was used as mobile phase at a flow rate of 0.6 ml/min. Acids were detected at 202 nm wavelength. Instrument control and data recording and processing was carried out with Thermo Xcalibur software.

5.3.4.5 Protein quantification

The digestate samples (supernatants in the preparation step of **Section 5.3.4.3**) were used to analyse proteins extracted from the spent mushroom substrate. A standard curve was constructed and protein quantification was performed according to the manufacturer's instructions using Pierce BCA Protein Assay Kit (Thermo Scientific). Spectronic® Genesys™ 5 was used to measure the absorbance at 562 nm.

5.3.4.6 Carbohydrate quantification

Total carbohydrate concentration was determined by AOAC Method 988.12 (44.1.30). The phenol–sulfuric acid assay is an easy method for measuring all classes of sugars (i.e. sugar derivatives, oligosaccharides, polysaccharides). The procedure detects soluble sugars as well as oligomeric sugars because the high sulfuric acid concentration can hydrolyse oligomeric sugars into monomers. A standard curve was constructed using D-xylose.

The supernatants in **Section 5.3.4.3** were used to quantify total carbohydrates. Briefly, 1 ml of sample was pipetted into a test tube, followed by the addition of 5 ml of 96% H_2SO_4 (v/v) and 1 ml phenol, all tubes were shaken vigorously and then incubated in a water bath for 20 min at 30°C to develop orange-yellow colour, after cooling at room temperature, absorbance was measured at 480 nm. When treated with phenol and concentrated sulfuric acid, the reducing groups give an orange yellow colour which absorbs light in the ultraviolet visible range.

5.3.5 Molecular characterization

5.3.5.1 Genomic DNA extraction

Genomic DNA was extracted from the digestate collected from the three digesters terminated at day 7, 14 and 28 as well as from the two upscaling bioreactors (20 L and 7 L) using NucleoSpin® Soil Kit - Macherey-Nagel according to the manufacturer's specifications. The concentration and quality of the extracted gDNA was assessed using the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific) and visualized on a 0.8 % (w/v) agarose gel as described in **Section 3.3.4.1**.

5.3.5.2 16S rRNA gene amplification

The amplification of 16S rRNA gene fragments for bacteria and archaea were performed on the gDNA extracts (**Section 5.3.5.1**) using primer sets shown in **Table 5.1**. A final reaction volume of 50 µl was performed (**Table 5.2**). Amplifications were carried out in a thermal cycler PXE 0.2 (Thermo Electron Corporation) according to PCR conditions described in **Section 3.3.4.2**. The PCR products obtained were visualized on 1% (w/v) agarose gel and then subjected to DGGE.

Table 5.1: Primers used for 16S rRNA gene fragment amplification.

Primer	Sequence	Reference
341F-GC Clamp	5'-CCT ACG GGA GGC AGC A -3'	(Muyzer *et al.*, 1993)
908R	5'- CCG TCA ATT CMT TTG AGT TT -3'	(Muyzer *et al.*, 1993)
GC clamp [a]	5'-CGCGCGCCGCGCCCCGCGCCCGTCCCG CCGCCCCCGCCCG-3'	(Muyzer *et al.*, 1993)
344F-GC Clamp	5'-ACGGGGCGCAGCAGGCGCGA-3'	(Raskin *et al.*, 1994)
934R	5'-GTGCTCCCCCGCCAATTCCT-3'	(Raskin *et al.*, 1994)

Table 5.2: Composition of 16S rRNA gene fragment PCR.

Reaction components	Volume (µl)
Buffer (10X)	5
dNTPs (10 mM)	1
Forward Primer F-GC (10 µM)	1
Reverse Primer (10 µM)	1
BSA (10 µg/µl)	1
Taq DNA Polymerase (2 U/µl)	0.25
Template	Final concentration of (25 ng/µl – 50 ng/µl)
MilliQ water	Up to 50 µl

5.3.5.3 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was performed as described in **Section 3.3.4.4**. The gel was stained for 20 minutes with 50 µl ethidium bromide (10 mg/ml) in 500 ml distilled water and washed 3 times with distilled water then visualized under UV illumination using ChemiDoc XRS (Bio-Rad Laboratories) gel documentation system. Microbial population shift was assessed and dominant bands from the DGGE profile were excised. Bands were eluted in 50 µl milliQ water and incubated 55°C overnight. The eluted DGGE bands for 16S bacteria were re-amplified using primer set 341F and 908R for bacteria and 344F and 934R archaea, with the exception that the forward primer did not contain the GC-clamp. The re-amplifications were carried out according to PCR conditions described in **Section 3.3.4.4**. The re-amplified PCR products were evaluated on a 0.8% (w/v) agarose gel, the PCR product was then purified using Exo/SAP clean up method.

5.3.5.4 Exo/SAP clean up

Exonuclease I (0.5 µl) and FastAP^{TM} Thermosensitive Alkaline Phosphatase (2 µl) were added to the PCR product (10 µl) as recommended by the manufacturer. The mixture was incubated in a thermal cycler PXE 0.2 (Thermo Electron Corporation) at 37ºC for 15 min followed by heat inactivation of the enzymes at 85ºC for 15 min. The enzyme-treated amplified product was stored at -20ºC until next use.

5.3.6 Cloning 16S rRNA gene into pGEM®-T Easy vector

5.3.6.1 Ligation

The purified PCR products (**Section 5.3.5.4**) were ligated into the pGEM®-T Easy vector system (**Figure 5.3**) according to the manufacturer's instructions (Promega Corporation, USA) but the reaction was modified by the addition of Adenosine triphosphate (ATP), this was done in order to maximise the efficiency of the T4 DNA ligase in catalysing the synthesis of phosphodiester bonds between the 3'hydroxyl of one nucleotide and 5'phosphate of another. The ligation reaction was prepared as described in **Table 5.3** and incubated at 16°C for 2 h, 20°C for 15 min and 4°C for 16 h.

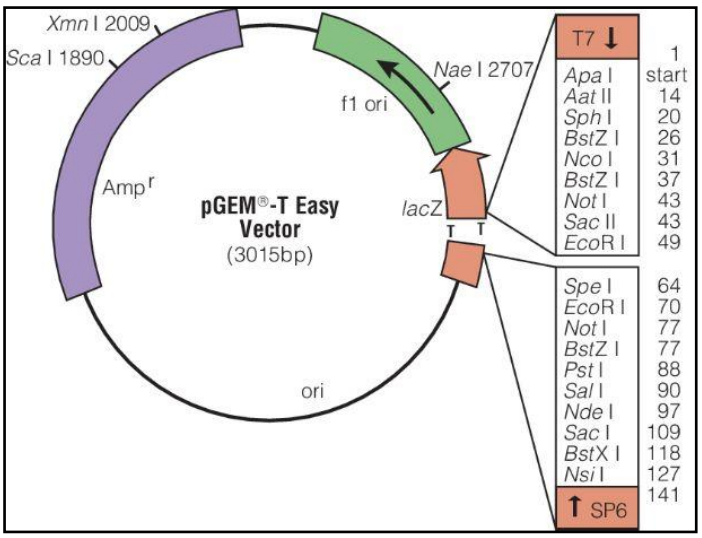

Figure 5.3: pGEM®-T Easy Vector System (Promega).

Table 5.3: Ligation mixture composition for the pGEM®-T Easy vector system.

Reaction component	Volume (µl)
2X Rapid ligation Buffer, T4 DNA ligase	5
pGEM®-T Easy Vector (50 ng)	1
PCR product	2
T4 DNA Ligase (3 Weiss units/µl)	1
ATP (10 mM)	0.5
Milli-Q water	0.5
Total	10

5.3.6.2 Transformation

Escherichia coli (*E. coli*) Top 10 competent cells (50 µl), were thawed on ice and 5 µl of the ligation product was added. This mixture was incubated on ice for 20 min, heat shocked for 40 s at 42ºC followed by immediate cooling on ice for 2 min. Thereafter 950 µl of SOC medium [LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) supplemented with 50 µl 2 M magnesium and 100 µl 1 M glucose] was added. The transformation reaction was incubated at 37ºC for 1 h while shaking at 175 rpm.

The transformation reaction (100 µl) was plated onto AIX plates (LB medium, 10 g/l tryptone, 5 g/l yeast extract, 10 g/ NaCl, 5 g/l agar) supplemented with 30 mg/ml ampicillin, 24 mg/ml IPTG (isopropylthio-β-D-galactoside), 20 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). The plates were incubated at 37˚C 16 h. Positive clones (white colonies) were selected and inoculated into 5 ml LB media, supplemented with 10 mg/ml ampicillin and incubated at 37˚C for 16 h.

5.3.6.3 Plasmid DNA extractions and Restriction digestion

Plasmid DNA was isolated using the Plasmid DNA Extraction Kit (Biospin) according to the manufacturer's specifications. Plasmid DNA concentrations were determined on the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific). The purified plasmid DNA was subjected to restriction enzyme digestion to confirm the presence of the ligated insert. *Eco*RI was used to confirm the insertion of the 16S rRNA gene into the pGEM®-T Easy Vector system. The final reaction volume of the restriction digest was 10 µl, the digestion mixture consisted of *Eco*RI enzyme (0.2 µl), *Eco*RI buffer (1 µl), plasmid DNA (6.8 µl) and MilliQ water (2 µl). The restriction digests were incubated at 37ºC for 3 hours and the products were evaluated on a 1% (w/v) agarose gel.

5.3.6.4 Sequencing of 16S rRNA gene fragments

Sequencing PCR was performed by using the ABI BigDye Terminator v3.1 Ready Reaction Sequencing Kit (Applied Biosystems). Sequencing PCR was performed on

products of **Section 5.3.6.3** (therefore 16S rRNA genes from bacteria and archaea). The PCR reaction contained the components listed in **Table 3.13** (**Section 3.3.4.6**). Each reaction contained either a forward or reverse primer; the only exception was that the forward primer did not contain the GC-clamp. The PCR was carried out in a thermal cycler PXE 0.2 (Thermo Electron Corporation), the PCR conditions entailed initial denaturation at 96°C for 1 min, 25 cycles of denaturation at 96°C for 10 s, followed by annealing at 50°C for 5 s and primer extension at 60°C for 4 min.

The EDTA/Ethanol precipitation protocol was used for sequencing clean up. The sequencing reaction volume was adjusted to 20 µl and transferred to a 1.5 ml Eppendorf tube containing 5 µl 125 mM EDTA (pH 8.0) and 60 µl absolute ethanol. The reaction was precipitated at room temperature for 15 min, centrifuged at 4°C (20 000 *x g*, 10 min), then the supernatant was completely aspirated. A volume of 60 µl ethanol (70%) was added to the tubes followed by centrifugation at 4°C (20 000 *x g*, 5 min). The supernatant was aspirated and the tubes were dried in the Speed-Vac (Eppendorf) for 5 min. Samples were stored at 4°C until they were sequenced.

5.4 Results

Characterization was done according to standard methods described in **Section 4.3.2** and **Section 4.3.3**.

5.4.1 Substrate characterization

Table 5.4: Substrate characterization by nutrient composition.

Sample	Dry matter (g/kg)	Organic matter (g/kg)	Crude protein (g/kg)	Gross energy (g/kg)	Neutral detergent fibre (g/kg)	Acid detergent fibre (g/kg)	Fat (g/kg)
SMS	965.17	955.87	134.28	16.12	663.88	308.47	7.34

Table 5.4 shows that SMS contains 95,57% organic matter which is combustible into energy, this is the same amount of organic matter found in the bran substrate at 95. 57% discussed in **Chapter 4.** Crude protein for SMS at 13.4% is also comparable to crude protein for bran substrate at 15.5%. This also confirms the composition of SMS, as it is made up of agriculture products such as wheat straw, bran and hay (Lin *et al.,* 2014). Therefore; bran, SMS and hominy chop contain almost the same amount of organic matter proving that co-digestion of manure with agricultural residue can increase the biogas yield. According to Amon and colleagues (2007), methane production is a function of protein, fat, cellulose (CEL), and hemicellulose (HEM) concentration of the whole-plant biomass (Amon *et al.*, 2007).

As shown in **Table 5.4** the organic fraction of the SMS is mostly made of crude protein which is 134.28 g/kg (13.4%), therefore this means that the majority of the biogas from this substrate will be formed from protein. In addition to protein, biogas generation could also be due to other OM in SMS. However, the concentration of protein in relation to fat is too high; this could mean that SMS is a protein-rich substrate. Kovács and co-workers (2013) warn that protein-rich biomass substrates should be avoided due to inevitable process inhibition (Kovács *et al.*, 2013), however; this problem can be overcome when microorganisms, capable of degrading proteins, are available. Decomposition of proteins to amino acids is a critical step, and one of the most important preconditions in the utilization of the protein-rich substrate is therefore the level of protease activity in the system, thus it is important that there is enough protein degraders in the system. The fat content in SMS (**Table 5.4**) is relatively low compared to crude protein.

Another parameter to look at is the Acid detergent fibre (ADF) which is a measure of cellulose and lignin content. Neutral detergent fibre (NDF) is a measure of total insoluble fibre and includes cellulose, lignin and hemicellulose. It is a better indicator of total fibre than ADF. It is well known and documented that ADF and NDF are indigestible and do not contribute to biogas production (Chen, 2014; Phan & Sabaratnam, 2012; Schittenhelm, 2008).

Table 5.5: Chemical composition characterisation.

Sample	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Sulphur (%)	Phosphorus (%)	Potassium (%)
SMS	20	6.01	0.85	0.51	0.31	4.58

Table 5.6: Substrate analysis by Total Solids and Volatile Total Solids.

Sample	Total solids (%)	Volatile total solids (%)
SMS	36	91

Characterization by chemical composition **Table 5.5** is especially important for the fertiliser value of the feedstock and can also be used to calculate the expected biogas yield using Buswell's empirical formula. Analysis by Volatile Total Solids (vTS) (**Table 5.6**) shows that spent mushroom substrate has the ability to produce maximum biogas because the analysis shows that more than 90% of the feed is combustible.

Compared to dry paper pulp in **Chapter 4**, SMS contains nitrogen (N), Phosphorus (P) and Potassium (K) in moderation (**Table 5.5**) and could serve as a good organic fertiliser; these findings also indicate that agricultural waste can be a better fertilizer than industrial waste, this is because industrial waste is known to contain impurities such as lead (Pb) and antibiotics (Lukehurst *et al.*, 2010).

SMS is also a better substrate for biogas production than paper pulp, this is shown in **Table 5.6**, SMS contains less Total Solids and more Volatile Total Solids contrary to paper pulp (**Chapter 4**).

5.4.2 Reactor start up

5.4.2.1 Bench scale digester

Spent mushroom substrate was incubated according to the organic loading ratio of 0.5 substrate to inoculum (0.5 OTS) as recommended by VDI 4630, therefore 11.5 g spent

mushroom substrate was inoculated with 1 115 g inoculum. Three digesters were incubated with the same substrate to inoculum ratio. The digesters were kept at 37°C, no stirring or agitation was applied on the digesters; gas production was monitored and captured as needed. Biogas was collected in a Tedlar bag (Sigma-Aldrich) for analysis using gas chromatography (Shimadzu 2010 GC fitted with a TCD detector) described in **Section 4.3.7**. **Table 5.7** shows gas production and methane production for the respective digesters. At day 7 gas production was seen, however no CH_4 was detected. Gas production increased at day 14 to day 28 and CH_4 content also increased significantly from 45.81% to 67.60%. Following the Nl/kg vDM gas production trend as seen on the table, gas production could still continue longer than 28 days and therefore more CH_4 concentration could be expected.

Table 5.7: Gas production volumes and methane percentage over 28 days.

	Gas yield			Methane [CH_4]
	***Nl/kg FM**	***Nl/kg DM**	***Nl/kg vDM**	**[%]**
Day 7	4,5	12,1	13,3	0 %
	5,7	15,7	17,3	
Day 14	4,5	12,1	13,3	45,81 %
	14,8	41,1	45,1	
	20,9	58,0	63,7	
	30,4	84,5	92,9	
	34,8	96,6	106,2	
Day 28	6,5	18,1	19,9	67,60 %
	16,9	47,1	51,8	
	23,5	65,2	71,7	
	30,8	85,8	94,2	
	36,5	101,5	111,5	
	46,5	129,2	142,0	
	54,4	151,0	165,9	
	60,9	169,1	185,8	
	63,1	175,1	192,4	

*Nl/kg FM refers to the fresh mass of the substrate, Nl/kg DM refers to the dry mass of the substrate and Nl/kg vDM refers to the volatile dry mass which contributes to biogas.

As shown in **Figure 5.4** the digesters were terminated at day 7, 14 and 28 respectively, while readings were taken at 3 day interval. At day 7, small amounts of approximately 17,3 Nl/kg vDM biogas was produced, this could be due to hydrolysis still taking place and therefore no significant gas production can be expected; also the gas composition during day 7 contained other gases such as CO_2 and N_2 but no CH_4 was present. This is also the period at which microorganisms acclimatize, especially protein degrading microorganisms as the spent mushroom substrate shown to have high protein content, therefore the enzyme proteases is key in this step.

Biogas production increased over 14 and 28 days at 106,2 Nl/kg vDM and 192,4 Nl/kg vDM respectively, this shows the importance of longer retention time, therefore more biogas yield can be expected when the retention is increased especially considering that only 17,3 Nl/kg vDM biogas was produced at day 7; this is also true looking at the methane percentage.

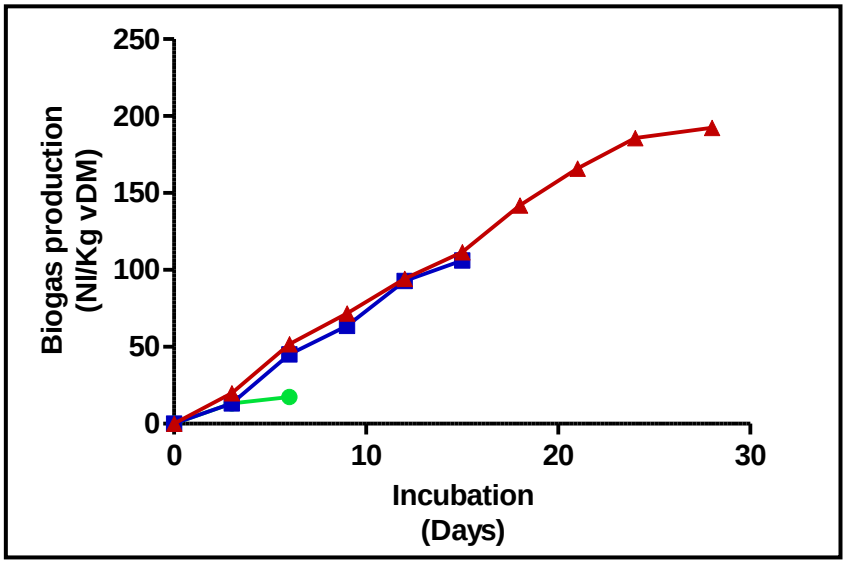

Figure 5.4: Biogas production from spent mushroom substrate. Day 7 (—); day 14 (—) and day 28 (—). At day 7 gas production was 17,3 Nl/kg vDM, at day 14, 106,2 Nl/kg vDM and day 28, 192,4 Nl/kg vDM gas was produced.

Lin and co-workers (2014) investigated the co-digestion of spent mushroom substrate with yard trimmings and wheat straw for biogas production. SMS was found to be highly degradable, which resulted in inhibition of the digestion process further downstream due to volatile fatty acid accumulation and a decrease in pH. This issue was addressed by the co-digestion of SMS with either yard trimmings or wheat straw. The anaerobic digestion of SMS/yard trimmings achieved a cumulative methane yield of 194 Nl/kg VS, which was 16 and 2 times higher than that from SMS and yard trimmings, respectively. The digestion of SMS/wheat straw obtained a cumulative methane yield of 269 Nl/kg VS, which was 23 times as high as that from SMS and comparable to that from wheat straw (Lin *et al.,* 2014).

Nguyen and Fricke (2012) also investigated the co-digestion of SMS with swine manure. The experiments were operated for 90 days, the average produced biogas was 4.1 L per day with 60% methane (Nguyen & Fricke, 2012).Thus the anaerobic digestion of SMS alone is possible however optimum results are achieved when the SMS is co-digested.

5.4.2.2 Two-stage bioreactor

Upon upscaling to the 20 L bioreactor, the same incubation recommendations by VDI 4630 were followed. Therefore 100 g spent mushroom substrate was inoculated with 9 920 g/kg (approximately 10 L) seeding material. The 20 L bioreactor was kept at 37°C and stirred at 100 rpm for mixing of substrate and inoculum. During incubation pH, DO and gas production were monitored. Biogas was collected in a Tedlar bag (Sigma-Aldrich) for analysis using gas chromatography (Shimadzu 2010 GC fitted with a TCD detector) described in **Section 4.3.7**. The substrate and seeding were allowed 14 days to acclimatize before being pumped into the 7 L bioreactor.

After 14 days the contents of the 20 L were pumped into a 7 L bioreactor (containing 1 L basal media, prepared according to **Section 3.3.3.3**). The 7 L bioreactor served as the methanogenesis vessel. The reactor was stirred at 70 rpm; pH, DO and gas production were also monitored. **Table 5.8** shows cumulative gas production over the 30 days of incubation for both bioreactor 1 (20 L) and bioreactor 2 (7 L). It can also be noted that CH_4 stabilized at 39,54% between day 10 and day 15, this could be due to the system switching to the acetogenesis phase and therefore products such as acetic acid being formed and metabolized. **Table 5.8** week 1 to week 2 shows Bioreactor 1, week 3 to week 5 shows Bioreactor 2.

Table 5.8: Biogas production over 30 days.

		Gas yield			Methane [CH_4]
	Days	**Nl/kg FM**	**Nl/kg DM**	**Nl/kg vDM**	**[%]**
Week 1	1	8,00	22,2	24,4	19,43
	2	16,00	44,4	48,8	19,66
	3	24,00	66,7	73,3	19,71
	4	32,00	88,9	97,7	23,3
	5	40,00	111,1	122,1	26,00
	6	48,00	133,3	146,5	29,20
	7	56,00	155,6	170,9	30,79
Week 2	8	64,00	177,8	195,4	34,72
	9	72,00	200,0	219,8	35,77
	10	80,00	222,2	244,2	39,54
	11	88,00	244,4	268,6	39,54
	12	96,00	266,7	293,0	39,54
	13	104,00	288,9	317,5	39,54
	14	112,00	311,1	341,9	39,54
Week 3	15	120,00	333,3	366,3	39,54
	16	128,00	355,6	390,7	51,49
	17	136,00	377,8	415,1	53,52
	18	144,00	400,0	439,6	55,4
	19	152,00	422,2	464,0	55,4
	20	160,00	444,4	488,4	57,9
	21	168,00	466,7	512,8	59,77
Week 4	22	176,00	488,9	537,2	60,46
	23	184,00	511,1	561,7	60,46
	24	192,00	533,3	586,1	60,46
	25	200,00	555,6	610,5	62,31
	26	208,00	577,8	634,9	63,04
	27	216,00	600,0	659,3	67,90
	28	224,00	622,2	683,8	72,81
Week 5	29	232,00	644,4	708,2	75,14
	30	240,00	666,7	732,6	76,84

As seen in **Table 5.8** and **Figure 5.5** gas production and CH_4 concentration increased significantly over time. Biogas production in Bioreactor 1 was only reported until 2 weeks or until the effluent was pumped into Bioreactor 2, this does not suggest that the biogas process was complete in Bioreactor 1, the CH_4 quality in Bioreactor 1 continued to be analysed, as each bioreactor was equipped with a eudiometer. As can be seen in **Figure 5.5** the CH_4 concentration continued to increase in Bioreactor 1 up to a maximum of 44% [CH_4] until the last day of incubation, this shows that the system was efficient and more optimized.

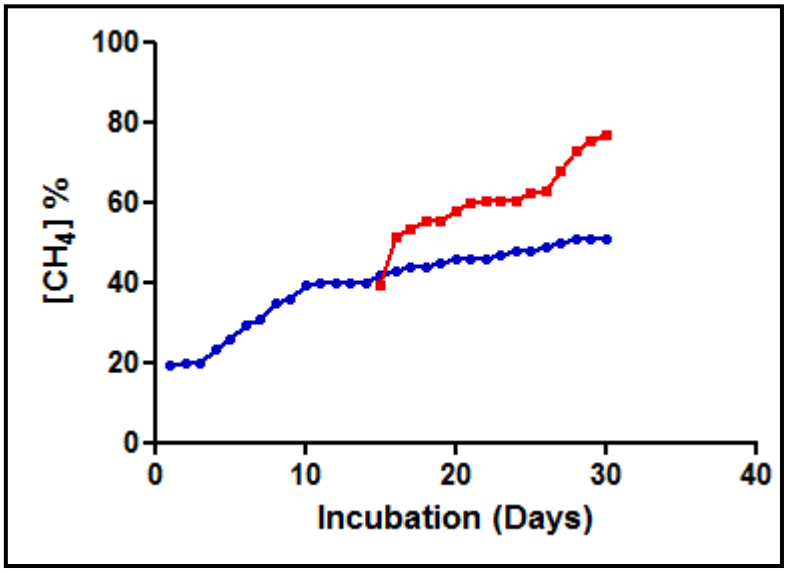

Figure 5.5: Methane concentration of spent mushroom substrate. The 20 L bioreactor (—) and 7 L bioreactor (—)

5.4.3 Factors affecting anaerobic digestion

5.4.3.1 pH

As part of process monitoring, pH was measured and used to better understand how it could affect the microbial diversity in a bioreactor. As discussed in **Chapter 3**, all organisms have an optimum pH for growth. Bacterial cells can survive in different pH conditions by keeping their internal pH constant (usually between pH 5 and pH 8). However, at pH levels other than their optimum, it becomes difficult for cells to grow, as they must expend energy to maintain that internal pH. At pH levels too acidic or alkaline it can become too difficult for cells to maintain the proper pH and thus cells will die.

Figure 5.6 depicts pH determined during incubation of SMS in a two-stage bioreactor. The pH in the digester is a function of retention time. The pH in the 20 L bioreactor decreased from 7.35 to 6.7. This could be due to the production of carbon dioxide and organic acids during acidogenesis step. At day 25, the pH increased again showing favourable conditions for methanogens (Manimegalai *et al.*, 2014). In the 7 L bioreactor, the pH remained above 7, this pH favours the growth of acetogenic and

methanogenic microorganisms. Methanogenic bacteria are very sensitive to pH and are inactive at pH values below 6.5 (Bialek *et al.*, 2011; Manyi-Loh *et al.*, 2013). They do not favour sudden increases in pH, even if they are within the range of their operation. However they can also regulate the pH if the environment becomes too acidic so that their environment may be suitable for them by using fatty acids as their food source. However, a pH below 5.5 causes the bacteria activity to decrease drastically (Sousa *et al.*, 2013).

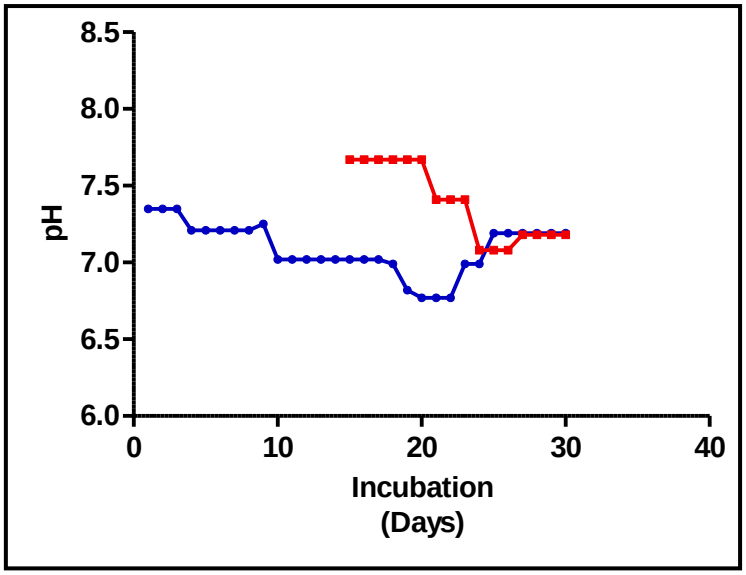

Figure 5.6: Two-stage bioreactor pH determination. The 20 L bioreactor (—) and 7 L bioreactor (—)

5.4.3.2 Alkalinity

The alkalinity or buffering capacity was performed as described in **Section 5.3.4.2**. It is important that the system has a high bicarbonate alkalinity in order for the pH to remain high enough for methanogens to survive. Alkalinity is expressed in terms of an equivalent amount of calcium carbonate. **Table 5.9** shows the buffering capacity of the three digesters and the two-stage bioreactors. The 7 L bioreactor had the highest buffering capacity of 9 881,4 $CaCO_3$ mg per L, this high buffering capacity ensures that the pH is stabilized and also it is expected that the 7 L should produce increased methane due to the methanogens present in the system (Lee *et al.*, 2015; Nazaries *et al.*, 2013; Weiland, 2010).

Table 5.9: The buffering capacity of the digesters and bioreactor.

Sample	pH	Alkalinity
Day 7	7,54	4 449,4 $CaCO_3$ mg per L
Day 14	7,53	4 518,75 $CaCO_3$ mg per L
Day 28	7,55	6 244,4 $CaCO_3$ mg per L
Bioreactor 1 (20 L)	See Figure 5.4	8 586,9 $CaCO_3$ mg per L
Bioreactor 2 (7 L)	See Figure 5.4	9 881,4 $CaCO_3$ mg per L

5.4.3.3 Dissolved Oxygen (DO)

Dissolved oxygen (DO) was monitored over the incubation period. **Figure 5.7** shows the DO measured in Bioreactor 1 and Bioreactor 2. From day 0 to day 13 no oxygen was present in the system, this is seen by the negative percentage of DO, however oxygen was detected at day 14 when the effluent was pumped from Bioreactor 1 to Bioreactor 2. This could be due to the air that was present in the tubing used for pumping and the air that was already present in Bioreactor 2 upon transferring the effluent between the two bioreactors.

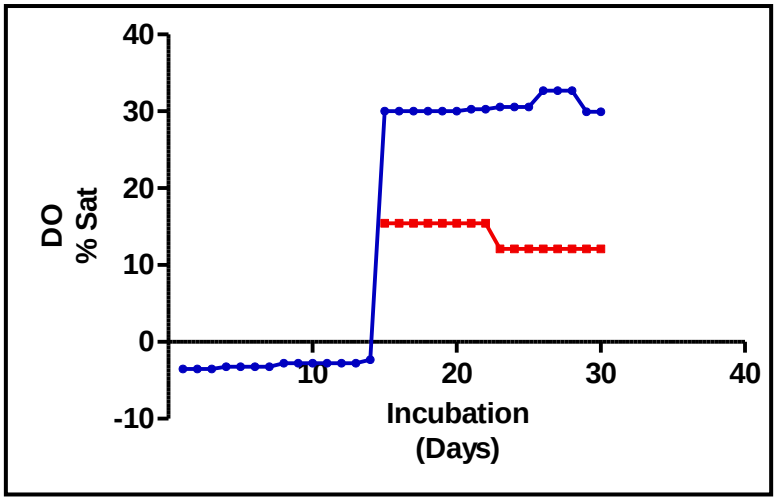

Figure 5.7: Two-stage bioreactor DO monitoring. The 20 L bioreactor (—) and 7 L bioreactor (—)

The acidogenic bacteria belonging to facultative anaerobes use the intrusion oxygen to create favourable conditions for the development of obligate anaerobes of the genera *Pseudomonas*, *Bacillus*, *Clostridium*, *Micrococcus* and *Flavobacterium* (Ziemiński & Frąc, 2012).

5.4.3.4 Volatile fatty acids (VFAs)

Organic acids or volatile fatty acids (VFAs) were determined by HPLC as described in **Section 5.3.4.4**. The accumulation of VFAs reflects a syntrophic relationship between acidogenic and acetogenic bacteria. Acetic acid and propionic acid were detected in the two-stage bioreactors (Bioreactor 1 & Bioreactor 2), however the specific concentrations were not quantified (**Figure 5.8**). Acetic acid is a major precursor for methanogenesis; methane is produced from substrates such as acetic acid, despite the fact that only few bacteria are capable of producing methane from acetic acid, a vast majority of CH_4 arising in the methane digestion process results from acetic acid conversions by heterotrophic methane bacteria (Demirel & Scherer, 2008). Propionic acid is subsequently converted to acetic acid during acetogenesis (Yadvika *et al.*, 2004). In the digester samples of day 7, 14 and 28 no organic acids were detected and therefore not quantified.

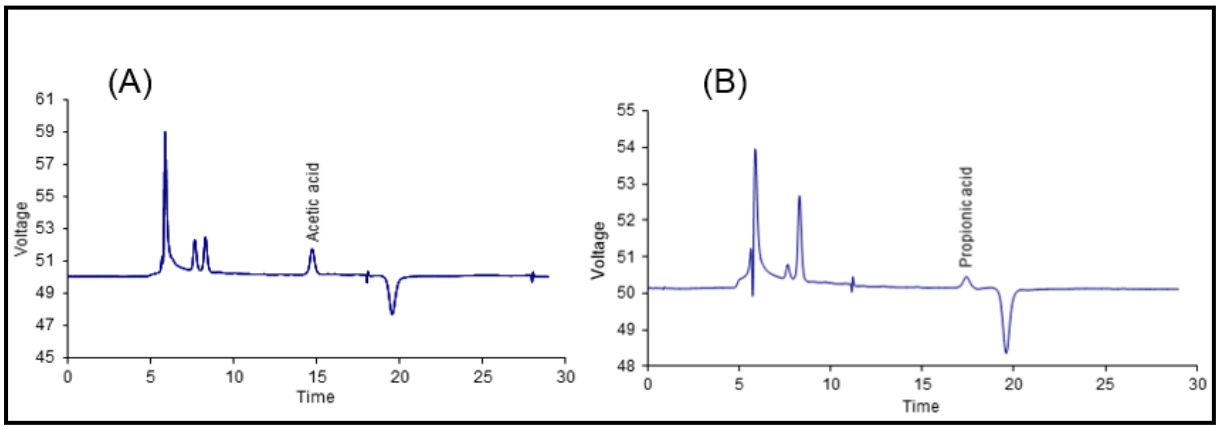

Figure 5.8: Organic acids detection in the two-stage bioreactor system. (A) Acetic acid detection, (B) Propionic acid.

Acetic acid and propionic acid have been previously detected in many biogas digesters (Athanasoulia *et al.*, 2012; Kreuger *et al.*, 2011; Owczuk *et al.*, 2013; Weiss *et al.*,

2008; Yadvika *et al.*, 2004) and these are important as they show the efficiency of the acidogenic bacteria and acetogenic bacteria and also CH_4 is produced from these intermediates. However the concentration of total VFA must be kept at 1000 mg/l for a more stable biogas production process (Ali Shah *et al.*, 2014; Zhang *et al.*, 2014).

5.4.3.5 Long chain fatty acids (LCFAs)

Long chain fatty acids (LCFAs) are typically produced from lipids. Long chain fatty acids were determined as described in **Section 5.3.4.3**. LCFA are amphiphilic compounds produced via the rapid hydrolysis of lipids by extracellular lipases. The anaerobic degradation of LCFA proceeds through the β-oxidation pathway, in which a repetitive cleavage of 2-carbon fragments occurs with concomitant release of acetyl-CoA (Kougias *et al.*, 2016).

No LCFAs were detected in the two-stage bioreactor and also in the day 7, 14 and 28 digesters; this is not surprising because previous studies indicated inhibitory problems caused by LCFAs. Inhibition occurs when major products of β-oxidation accumulate and thermodynamically-limiting levels that prevent LCFA (and propionate) to be further oxidized (Nielsen & Angelidaki, 2008) and inhibition can be easily designated by a distinct lag phase during which the methane productivity is decreased (Rasit *et al.*, 2015). The negative effect of the LCFA accumulation on the anaerobic microbial consortium in biogas reactors is well known (Kougias *et al.*, 2016).

It has been previously documented that LCFA could inhibit the activity of hydrolytic, acidogenic and acetogenic bacteria as well as methanogenic archaea (Angelidaki & Ahring, 1992; Lalman & Bagley, 2002; Pereira *et al.*, 2005). However, the archaeal community was found to be more tolerant to increased LCFA concentration levels compared to the bacterial community. Moreover, it was shown that the hydrogenotrophic methanogens are more resilient to the toxic effects from LCFA compared to the acetotrophic methanogens (Angelidaki & Ahring, 1992; Lalman & Bagley, 2002; Małgorzata Worwag *et al.*, 2011). The inhibition of methanogenesis was initially proposed to be permanent and was attributed to the lipophilic properties of

LCFA that permits them to be absorbed into the surface of microbial cell causing membrane lysis (Kougias *et al.*, 2016).

5.4.3.6 Protein quantification

Protein concentrations were determined using the BCA Protein Assay Kit as described in **Section 5.3.4.5**. **Figure 5.9 (A)** shows how the protein concentration changed over 28 days. At day 7, approximately 135 µg.ml^{-1} peptide bonds were detected in the sample, 141 µg.ml^{-1} was detected at day 14 and 214 µg.ml^{-1} was detected at day 28. It is worthy to note that protein concentration was increasing during incubation of the bench-scale digesters, the same phenomenon was seen in the work done by Kovács et al., (2013). In the two-stage bioreactor [**Figure 5.9 (B)**] protein concentration was decreasing, this was anticipated due to protein degradation, free amino acids were formed and thus a small amount of peptide bonds were present in the sample.

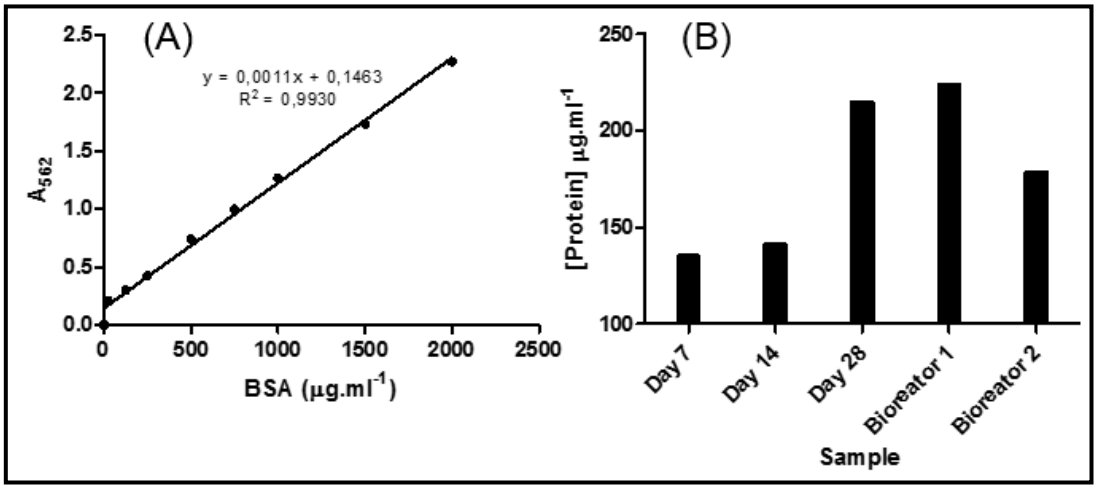

Figure 5.9: Protein quantification. (A) Standard curve, (B) Protein concentration in respective digesters.

5.4.3.7 Quantification of sugars

The carbohydrate phenol-sulfuric acid assay, discussed in **Section 5.3.4.6** was used to quantify sugars. As shown in **Figure 5.10**, a standard curve was prepared using standard D-xylose solution ranging 100 to 2000 µg/ml. The relative absorbance obtained from the samples was compared to the standard curve. An increased sugar

concentration was observed in the samples: day 7, 14, 28 and bioreactor 1 (20 L), this shows that the hydrolysis step was effective as a result of complex sugar polymers being broken down into monomers. However in bioreactor 2 (7 L), lower sugar concentrations were detected than in bioreactor 1, this is because the sugars were metabolised in the methanogenic step.

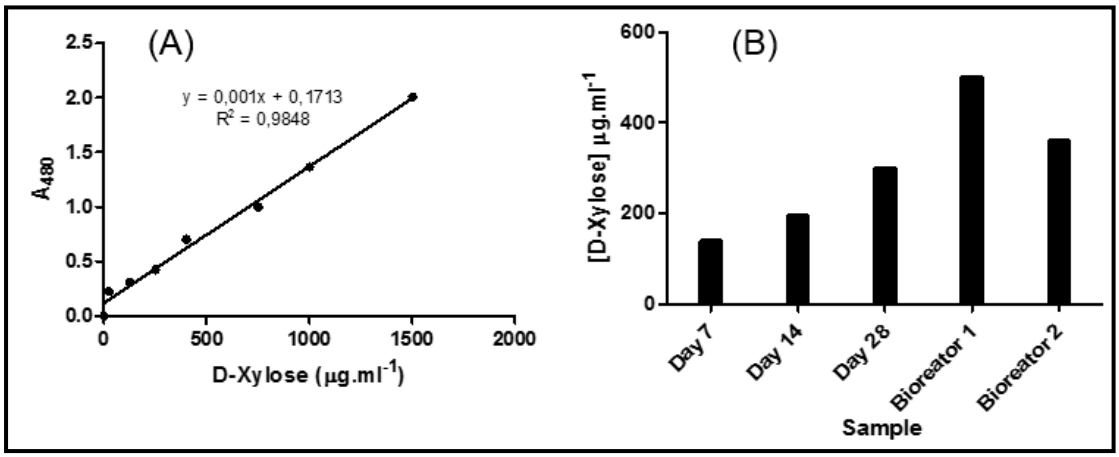

Figure 5.10: (A) Carbohydrate phenol – sulphuric acid Standard curve (B) Quantification of sugars in effluent samples.

5.4.4 Molecular characterisation

5.4.4.1 Genomic DNA extraction and 16S rRNA gene amplification

Genomic DNA was successfully extracted from the digestate or effluent samples obtained from the termination of the small digester at Day 7, Day 14 and Day 28. Genomic DNA was also successfully extracted from the digestate obtained from the upscaling bioreactors. The gDNA was evaluated on a 0.8 % (w/v) agarose gel as described in **Section 5.3.5.1**. The visualization of the DNA showed low integrity with shearing as shown in **Figure 5.11** indicating low DNA concentrations.

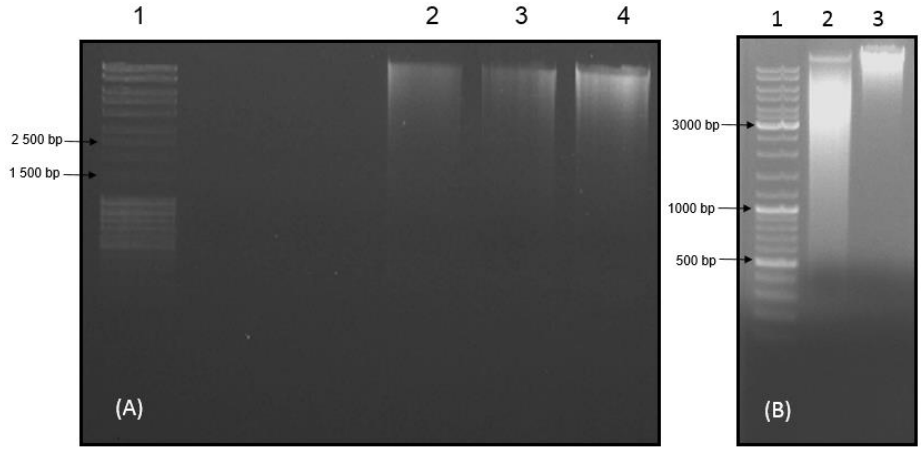

Figure 5.11: Genomic DNA extracted from the digestate samples. (A) Bench scale digester. Lane 1, MassRulerTM DNA ladder (Fermentas), Lane 2: day 7 effluent; Lane 3: day 14 effluent; Lane 4: day 28 effluent. (B) Upscale bioreactor. Lane 1: GeneRuler^{TM} DNA ladder (Fermentas); Lane 2: effluent from 20 L bioreactor; Lane 3: effluent from 7 L bioreactor.

The amplification of the 16S rRNA genes were performed as described in **Section 5.3.5.2**. The PCR products obtained were evaluated on 1% (w/v) agarose gel as shown in **Figure 5.12.** Amplicons of approximately 600 bp were obtained as expected for bacterial and archaeal 16S rRNA gene amplifications.

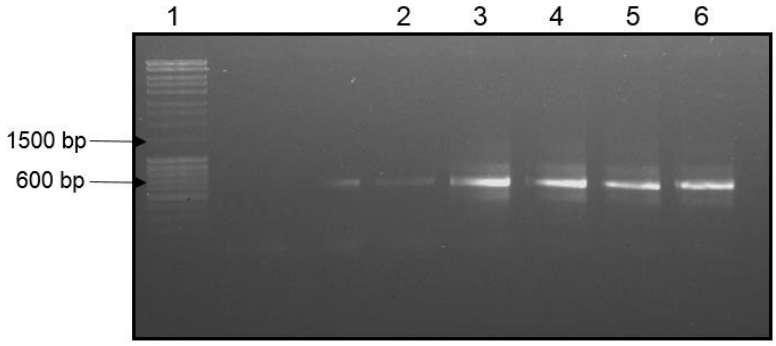

Figure 5.12: Amplification of partial bacterial 16S rRNA gene and archaeal fragments. Lane 1, MassRulerTM DNA ladder (Fermentas), Lane 2: day 7 effluent; Lane 3: day 14 digester effluent; Lane 4: day 28 digester effluent; Lane 5: the 20 L bioreactor effluent; Lane 6: the 7 L bioreactor effluent.

5.4.4.2 DGGE

PCR products were loaded onto a DGGE gel as described in **Section 5.3.5.3**. DGGE was performed in order to assess the diversity of microorganisms and understand the population shift over day 7, 17, 28 and in the upscale bioreactors (**Figure 5.13 A &**

B). The DGGE was performed in 1xTAE buffer at 100 Volts and a temperature of 60°C for 17 hours, the gel was visualized under UV illumination using ChemiDoc XRS (Bio-Rad Laboratories) gel documentation system.

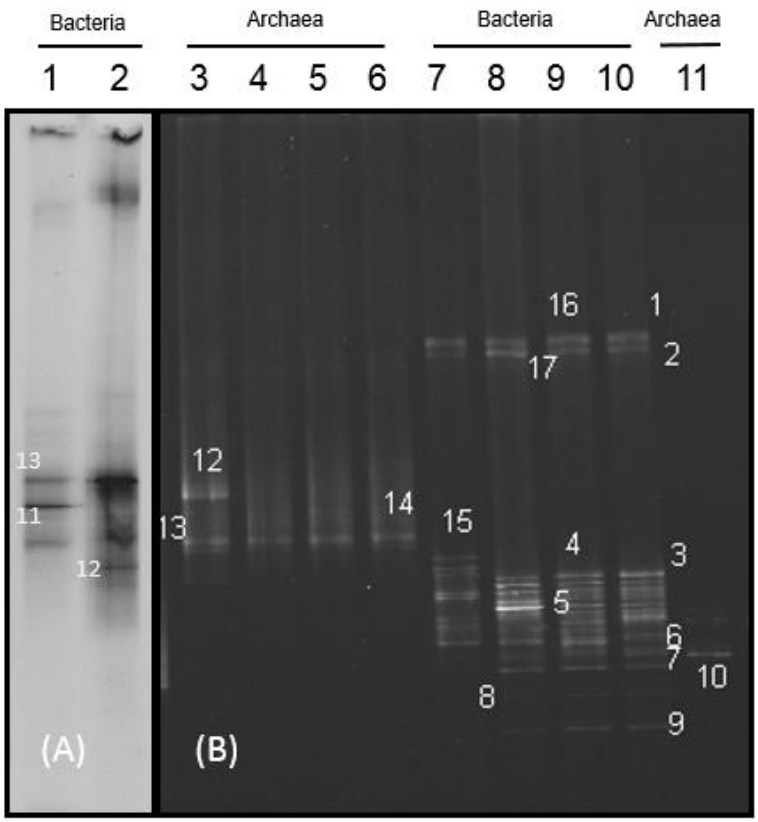

Figure 5.13: DGGE profile. (A) Lane 1: Bioreactor 1 (20 L); Lane 2: Bioreactor 2 (7 L); (B) Lane 3: Bioreactor 1 (20 L); Lane 4: Bioreactor 2 (7 L); Lane 5: day 7; Lane 6: day 14; Lane 7: Inoculum; Lane 8: day 7; Lane 9: day 14; Lane 10: day 28; Lane 11: day 28.

The bands from the DGGE profile were excised, eluted in 50 µl MilliQ water and subjected to PCR re-amplification. The re-amplified products were purified using the Exo/SAP method as described in **Section 5.3.5.4**. Purified PCR products were then ligated into pGEM®-T Easy and transformed into competent Top 10 *E. coli* cells as described in **Sections 5.3.6.1** and **Section 5.3.6.2** respectively. Positive clones were selected from the LB-AIX media plates (IPTG, X-Gal and ampicillin) and grown for 16 h in 5 ml LB media supplemented with 10 mg/mL ampicillin. Plasmid extractions were carried out on cultures grown on LB media as described in **Section 5.3.6.3**.

Restriction digest was performed as described in **Section 5.3.6.3** on products recovered from the plasmid extractions. A 1% (w/v) agarose gel was run to visualize the digested products shown in **Figure 5.14**. The backbone of the vector (~3000 bp) and insert of varying sizes but adding up to 600 bp were released with restriction enzymes *Eco*RI. The confirmed clones were then used in the subsequent sequencing reaction for identification purposes as described in **Section 5.3.6.4**.

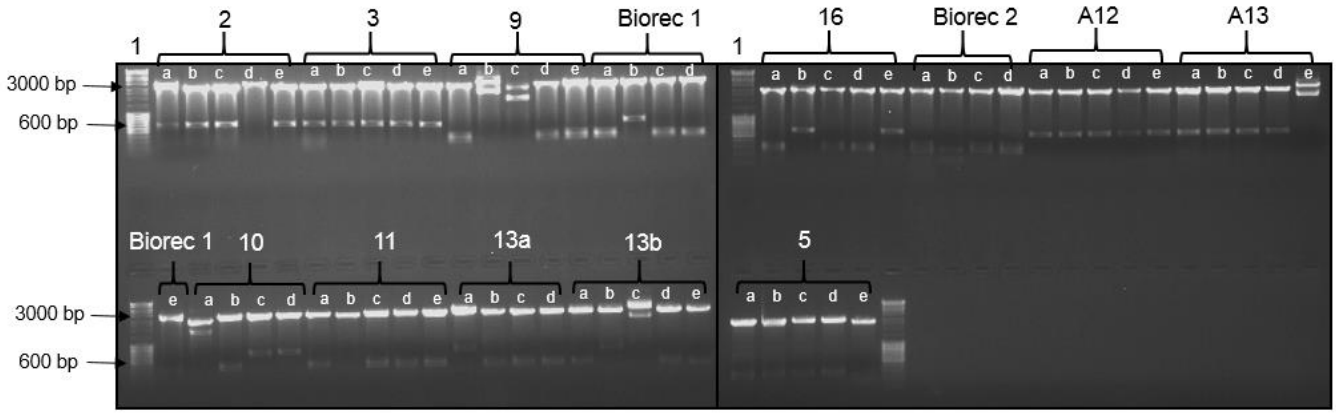

Figure 5.14: Restriction digest analysis. Numerical labelling corresponds to the number identified on the DGGE gel. The alphabets represent the number of colonies picked per AIX plate.

5.4.4.3 DNA sequencing results

Total community DNA was prepared from digestate samples obtained from Bioreactor 1 and Bioreactor 2, as well as from digesters (day 7, 14, 28 and the inoculum digester). To analyse the community structure of the samples, 16S rRNA clone libraries were constructed covering bacterial as well as archaeal 16S rRNA gene fragments. Purified total community DNA was used as template in the PCR reactions to generate 16S rRNA gene amplicons by means of a universal primer pair (341F-GC and 908R) and the Archaea-specific primers (344F-GC and 344R). Bacterial and archaeal clone libraries were constructed using the vector pGEM-T Easy.

Clones were sequenced and analysed using BLASTn NCBI database (2017). Results shown in **Table 5.10** showed to have a wide diversity of bacteria and archaea. The 16S rRNA Archaeal clones labelled A12E and A13A were identified as *Methanosarcina barkeri* and *Methanoregula boonei*. It has been reported that stirred tank reactor conditions affect the conglomeration and structure of *Methanosarcina* and

Methanoregula and thereby decreased the acetotrophic activity (Kampmann *et al.*, 2012).

Table 5.10: 16S rRNA clone library sequences from the biogas digesters and corresponding NCBI nucleotide sequence database entries by means of BLASTn.

DGGE band no:	Organism	Maximum Identity	Accession number
2C	*Pedobacter alpinus*	82%	NR_144597.1
3D	*Proteiniphilum acetatigenes*	93%	NR_043154.1
5A	*Cellulosibacter alkalithermophilus*	96%	NR_116826.1
9E	*Sphaerochaeta globosa*	99%	CP002541.1
10B	*Sphaerochaeta associata*	89%	JN944166.1
10C	*Hydrogenispora ethanolica*	90%	NR_125455.1
11C	*Comamonas denitrificans*	99%	NR_025080.1
13AA	*Pseudomonas japonica*	99%	KT825519.1
13AD	*Comamonas denitrificans*	98%	NR_025080.1
13BB	*Hydrogenispora ethanolica*	90%	NR_125455.1
16B	*Thermincola carboxydiphila*	92%	NR_043010.1
16C	*Pedobacter alpinus*	82%	NR_144597.1
A12E	*Methanosarcina barkeri*	99%	CP009528.1
A13A	*Methanoregula boonei*	99%	NR_074180.1
D11A	*Sphaerochaeta globosa*	89%	CP002541.1
D11B	*Pedobacter alpinus*	82%	NR_144597.1
D12B	*Sphaerochaeta globosa*	88%	CP002541.1
D12E	*Sphaerochaeta globosa*	89%	CP002541.1

The predominance of hydrogenotrophic methanogens such as *Methanoregula boonei* suggested that there was an increase in hydrogen concentration in Bioreactor 1 and Bioreactor 2; it is possible that the type of feedstock as well as the presence and activity of syntrophic bacteria resulted in additional hydrogen and promoted hydrogenotrophic methanogenic activity.

Methanosarcina species have been reported to grow better under high loading rates, with high acetate turnover. *Methanosarcina* has been defined as “the robust methanogen” because it’s proven ability to tolerate the four most common causes of stress in digesters, which are; temperature variations, organic loading rates, concentration of ammonium, and other salts (Alvarado *et al.,* 2014). Moreover, *Methanosarcina* has also been observed in high acidic environments, including natural wetlands (Alvarado *et al.,* 2014), which suggest that this type of acetotrophic, acid-resistant methanogen might represent a good choice as inoculum for waste treatment at higher organic loads and to overcome adverse acidic pH conditions. This is also true considering that the pH in Bioreactor 2 dropped to as low as pH 6.7 and *Methanosarcina* was able to thrive. Also in agreement to **Chapter 3** (ATCC media) methanogens were enriched at pH 6.5.

Moreover the bacterial clones found in the batch digesters (day 14 & day 28) and Bioreactor 2 included *Sphaerochaeta globose*, *Comamonas denitrificans*, *Pedobacter alpinus* and *Hydrogenispora ethanolica*. These acetogens have been previously found in biogas digesters (Gumaelius *et al.,* 2001; Liu *et al.,* 2017; Ringo *et al.,* 2008; Troshina *et al.,* 2015; Yong & Jiang, 2016). These acetogens were able to thrive due to the optimum pH in the digesters (pH 7 – 7.5) also intermediates such as acetate were present in the digesters and were consequently metabolized by these acetogenic bacteria.

The maximum identity shown in **Table 5.10** is low, this could be attributed to the fact that most of the species found during this research have not been identified in large numbers previously or that they have been identified as unculturable.

5.5 Conclusions

Feedstock characterization by nutritional composition as well as by volatile total solids (vTS) could already give an estimate of what could be expected when SMS is used as substrate for biogas production; protein and fat content of the SMS could also give the calorific value of the substrate and thereby an estimate of the energy value to be expected. The vTS determination showed that 90% of the SMS will be converted into biogas, this was confirmed by gas composition analysis showing 76.84% methane in Bioreactor 2 (7L) and 67.60% in the day 28 digester. This finding also indicates that the biogas production system becomes more efficient when the reactor volume and retention time are increased.

Process monitoring is an important factor, this helps to monitor and ensure that the biogas production process is efficient and not experiencing inhibition. The pH and alkalinity of the biogas system can give an indication of the anaerobic digestion stage of the system, for example if the pH is low this indicates that acidogenesis is taking place, this can be seen by VFA accumulation and if the pH increases, this indicates acetogenesis and the start of methanogenesis; it was for this reason that microbial diversity profiles were performed to give clear insight on the performance of the system. The carbohydrate and protein assay can also indicate the efficiency of the process. This was seen particularly in Bioreactor 1 and Bioreactor 2 when the concentration of proteins was decreased, indicating less detection of peptide bonds as they were being broken down and assimilated by bacteria and archaea as amino acids. The comparison between 2 L and 2 stage bioreactor shows that when using the 2-stage reactor there is better control of the respective steps of biogas production and consequently higher gas yields are achieved.

Methanogens such as *Methanosarcina* and *Methanoregula* species were found to be more dominant in Bioreactor 2, this was expected as Bioreactor 2 was supplemented with basal media, and therefore additional nutrients were available for acetotrophic bacteria and methanogenic archaea to thrive. Acetogenic bacteria identified included *Sphaerochaeta globose*, *Proteiniphilum acetatigenes*, *Pedobacter alpinus* and

Hydrogenispora ethanolica. Acidogenic bacteria identified were *Comamonas denitrificans* and *Thermincola carboxydiphila*. The hydrolytic bacteria that was identified in the bioreactors is *Cellulosibacter alkalithermophilus*.

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CHAPTER 6

6 Summary

Biogas production follows the normal anaerobic digestion process, this process can be divided into four major steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. The components of biogas are primarily methane and carbon dioxide, but it may contain small amounts of hydrogen, nitrogen, hydrogen sulphide and moisture; the composition of biogas also depends upon feed material. Biogas is a low-cost energy source derived from renewable resources; this is because it can be produced from any organic waste, including household food waste, animal waste and agricultural residue. These are wastes that will continue to be produced as long as humans live on earth and keep livestock. Cow dung, for example, is known to contain the necessary microorganisms, such as acid and methane formers, for biogas production. The biogas can be harnessed (and made environmentally friendly) by converting it to a fuel.

Biogas digester systems provide a residue organic waste, after the anaerobic digestion (AD), this effluent is called digestate and has superior nutrient qualities over normal organic fertilizer. Biogas digesters also function as waste disposal systems, and can therefore prevent potential sources of environmental pollution and the spread of pathogens and disease-causing bacteria.

The first step of AD is hydrolysis, during which the complex organic matter (polymers) are degraded into smaller units (mono and oligomers). During hydrolysis, polymers like carbohydrates, fats and proteins are converted into glucose, lipids and amino acids. Hydrolytic microorganisms release hydrolytic enzymes, converting biopolymers into simpler and soluble compounds. A variety of microorganisms are involved in hydrolysis, these bacteria are mostly strict or facultative anaerobes such as *Bacteriocides*, *Clostridia* and *Streptococc*i. The products produced from hydrolysis are further metabolized by the microorganisms involved in the subsequent step.

During acidogenesis, the products of hydrolysis are converted by acidogenic (fermentative) bacteria into methanogenic substrates. Simple sugars, amino acids and fatty acids are converted into volatile fatty acids (VFA) such as acetic, propionic and butyric acid. In acetogenesis, VFA and alcohols are oxidised into methanogenic

substrates like acetate, hydrogen and carbon dioxide. VFA, with carbon chains longer than two units and alcohols, with carbon chains longer than one unit, are oxidized into acetate and hydrogen. The production of hydrogen increases the hydrogen partial pressure. This can be regarded as a waste product of acetogenesis and inhibits the metabolism of the acetogenic bacteria. During methanogenesis, hydrogen is converted into methane. Acetogenesis and methanogenesis usually run parallel, as a result of the symbiosis of the two groups of microorganisms. Examples of acetogenic bacteria are *Acetobacterium woodii* and *Clostridium aceticum*.

The production of CH_4 and CO_2 from intermediate products is carried out by methanogenic archaea. Methanogenesis is a critical step in the entire anaerobic digestion process, as it is most prone to imbalance. Methanogenesis is severely influenced by operation conditions like temperature, pH, composition of feedstock and feeding rate. Digester overloading, temperature changes or large entry of oxygen can result in termination of methane production. Other factors which highly influence the biogas production are the process conditions at which the digestion process is carried out, these include the retention time, nutrients available and intermediates which are generated during the digestion process

A close interaction of all the involved microorganisms is of absolute importance for the biogas production. This is especially true for a well-balanced partial pressure of hydrogen. A too high concentration of hydrogen can hinder the metabolism of the acetogenic bacteria. Therefore, it is important that the hydrogen is constantly being used up by the methanogens in order to avoid a breakdown of the whole process.

In this study, specific enrichment media was used in order to select for microorganisms participating in all four stages of the biogas production process, these are hydrolytic, acidogenic, acetogenic and methanogenic microorganisms. Proliferation of the enriched microorganisms was confirmed microscopically as well as amplification of 16S rRNA genes and *mcrA* genes. Basal media specific for acetogenic bacteria was used as inoculum for the upscaling and optimization using Spent Mushroom Substrate (SMS).

Various feedstock including bran, hominy chop, paper pulp, molasses, cow and swine manure were characterized by nutritional and chemical composition, and the feedstock was also tested for biogas potential in 2 L digesters. Spent Mushroom Substrate

(SMS) was used for the upscaling and optimization of CH_4 in a 20 L bioreactor while monitoring pH and Dissolved Oxygen (DO).

Biogas production was monitored and quantified by GC. The process was also monitored by studying product formation. Total carbohydrate assay was used to look at the production of sugars, BCA assay was used to quantify protein degradation while LCFA and VFA were monitored by HPLC.

Out of all feedstock tested bran yielded the most biogas of 885,7 Nl/kg vDM, this would be expected as following nutritional composition bran contained 955,77 g/kg organic matter, thus over 95% of organic material. Bran also contained 99% vTS, this means that 99% energy value contained in bran. The results obtained during this research also show that when using the 2-stage bioreactor, there is better control of the respective steps of biogas production and consequently higher gas yields are achieved than using a 2 L digester.

The digestates were screened for bacteria and archaea using specific primers and the analysis was done using Sanger sequencing and Next Generation Illumina Sequencing. The NGS data confirmed the presence of *Firmicutes* and *Actinobacteria* which participate in hydrolysis and acetogenesis respectively. Methanogenic species such as *Methanosaeta, Methanobacterium* and *Methanosarcina* were also detected.

Key words: biogas, feedstock, digester, CH_4, 16S rRNA gene, *mcrA*