COMPARISON OF ANAEROBIC DIGESTION APPROACHES USING SELECTED FIBROUS AND NON-FIBROUS ORGANIC WASTE

by

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I, Noluthando Nonwabiso Dube, declare that the contents of this thesis represent my own unaided work and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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ABSTRACT

While biogas production through anaerobic digestion (AD) is becoming more auspicious as a sustainable approach for energy production and waste management, finding a suitable feedstock and the right anaerobic digestion approach that results in maximum biogas production is still a challenge. Generally, any feedstock that contains carbon and hydrogen can be used in anaerobic digestion. However, some feedstock such as fibrous materials (lignocellulose materials) poses a challenge due to their complex structure that offers recalcitrance during hydrolysis, even though they have the potential to yield higher biogas yields. On the other hand, non-fibrous materials that have been explored such as abattoir waste also pose a challenge because they are oftentimes high in nutrient content which introduces an imbalance in the C:N ratio and thereby hinders the process. Several anaerobic digestion approaches have been suggested to deal with these hurdles, such as the pre-treatment of feedstock prior to anaerobic digestion and the co-digestion of fibrous and non-fibrous feedstock.

The aim of this study was to compare different anaerobic digestion approaches using Napier grass as the fibrous feedstock and abattoir waste as the non-fibrous feedstock. To achieve this aim, biomethane potential tests were carried out to compare the biogas yields between non-pre-treated Napier grass and thermally pre-treated Napier grass (TPN) that was treated with heat in an autoclave at 151℃ for 15 minutes. Biomethane potential tests were further used to compare the biogas yield when thermally pre-treated Napier grass was co-digested with abattoir waste at ratios of 1:1, 1:2 and 2:1 AW:TPN. Central composite design was used to find the optimum conditions for maximum biogas yields using a three-factor level design (operation temperature, co-digestion ratio, inoculum substrate ratio) which gave rise to 20 experimental runs and was conducted using BMP for 30 days. The obtained experimental results were then fitted into the model and a second order polymonial equation was obtained. The conditions that resulted in maximum biogas yield from the optimisation were further tested for their feasibility when the process was scaled up in a 5L single-stage batch reactor that was allowed to take place for 30 days. Furthermore, the bacterial community present in the 5L single-stage batch reactor was studied. Inoculum samples were collected on day 1 and day 30 of the experiment and sent to Inqaba Biotech laboratory for 16s ribosomal ribonucleic acid (rRNA) analysis to compare the different bacterial communities present.

At 38℃ mono-digested thermally pre-treated Napier grass yielded the highest biogas yield of 70.3 Nml/g•VS_{added} while mono- digested raw Napier grass accumulated the least biogas of 46 Nml/g•VS_{added}. Moreover, pre-treated Napier grass accumulated a total of 72% of methane while raw Napier grass only accumulated 61% of methane. The co-digestion ratio that proved to be the most effective was 50:50 which resulted in a total of 117 Nml/g•VS_{added} biogas yield. The optimum range was determined to be 35℃ with a 50:50 co-digestion ratio and an F/M of 5 with predicted biogas yields of 188 NmL/g•VS_{added}. This optimum range was still feasible even after the conditions were scaled up in a 5L single-stage reactor.

Finally, microbial analysis showed that the phyla present in the process confirmed consistency on both day 1 and day 30 even though there were discrepancies in read count, with day 1 having a higher read count than day 30. This study highlighted some of the possible options that can result in optimum biogas yield when using Napier grass and abattoir waste; moreover, it highlighted the most effective options which led to the creation of templates that could be used in future studies when researching biogas using Napier grass and abattoir waste as substrate. Even though these optimums may be applicable when using other substrate other than Napier grass and abattoir waste, they may not necessarily be applicable to all potential biogas substrate.

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- And finally, myself, for pushing through it all and believing that it was possible, even when it did not seem possible. You have done well for yourself. Keep working hard and believing.

DEDICATION

I would like to dedicate this work to two amazing women in my life who have moulded me and made me the woman that I am today:

- *To my mother*: Mother K, thank you so much for being the epitome of hard work. You have embodied hard work throughout your life. You have taught me the importance of hard work; but most importantly, you have shown me hard work at its optimum through all the sacrifices you have made and continue making for your children, even today. This achievement is the fruit of your hard work and sacrifices. All that I am or ever hope to be I owe to you. I love you so much.
- *To my grandmother:* Gogo, thank you for all your prayers that are carrying me even now, though you are no longer with us. I was only a little girl when I heard you asking God that He give your grandchildren strength to do well in school. This work is your answered prayer. May your beautiful soul continue resting in peace.

Mkhonza Mhlungwane

RESEARCH OUTPUTS

The following research outputs represent the contributions of the author to the scientific knowledge and development during her master's candidacy (2020-2022)

1. Dube, N.N., Okudoh, V.I., Van Rensburg, E. (2021). Comparison of anaerobic digestion approaches using selected fibrous and non-fibrous organic waste in *Proceedings of the 2021 CPUT Postgraduate conference*, November 30, 2021, Cape Town – South Africa (Oral presentation)
2. Dube, N.N. Okudoh, V.I. Mewa Ngongang, M. Van Rensburg, E. (2022). Comparing Different Anaerobic Digestion Approaches Using Fibrous Napier Grass And Non-Fibrous Abattoir Waste As Feedstocks. Article submitted for publication: *Biomass and Bioenergy.* (Submitted)

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CLARIFICATION OF BASIC TERMS AND CONCEPTS

Acidogens	Acidogens are bacterial that metabolise and convert simple and soluble compounds into CO_2, H_2 VFA's and simple alcohols
Acetogens	Acetogens bacteria metabolise and convert acidogenic bacteria synthesis by-products into acetate/acetic acid and H_2
Anaerobic digestion	A process where a consortium of microorganisms, under anaerobic conditions, break down organic waste (i.e., substrate) to its most reduced carbon state and produce gaseous waste biogas
Anaerobic co-digestion	When two or more substrates are mixed and simultaneously digested
Biomethane potential test	Specially designed experimental tests for the methane potential of a substrate under a set of reproducible conditions
Fibrous material	Fibrous materials are rich in lignocellulose material such as lignin, cellulose and hemicellulose; these materials cannot be easily digested by microorganisms during AD
Inoculum	An active source of microorganisms that can be used in anaerobic digestion process
Non-fibrous material	Materials that contain little or no fibrous properties and are thus easily degraded during the hydrolysis stage

GLOSSARY

Abbreviation	Full description
AcoD	Anaerobic co-digestion
AD	Anaerobic digestion
AW	Abattoir waste
BMP	Biomethane potential
F/M	Food-to-microorganisms ratio
GC	Gas chromatography
GHG	Greenhouse gases
HPLC	High-performance liquid chromatography
HRT	Hydraulic retention time
Mono-AD	Mono-Anaerobic digestion
OLR	Organic loading rate
RNG	Raw Napier grass
TPN	Thermally pre-treated Napier grass
VFA	Volatile fatty acid
VS	Volatile solids

CHAPTER 1

1 INTRODUCTION

1.1 Background

Since ancient times, the primary source of energy has been through the burning of fossil fuel; in present times, this has changed little as fossil fuels are still widely used to produce energy in the form of electricity. Electricity is the lifeblood of modern living, as it is needed for a plethora of human activities such as lighting, cooking and many other industrial operations. However, in recent years, a fallout in electricity supply has been observed. Several factors contribute to this fallout: the fact that fossil fuel is a non-renewable source, the increase in population and the steady economic growth (Braun *et al.*, 2010). Moreover, the burning of fossil fuels is not environmentally friendly as it introduces greenhouse gases (GHG) into the atmosphere engendering environmental issues such as global warming (Mukumba *et al.*, 2016). As a result of the above mentioned, the International Partnership for Energy Efficiency Cooperation (IPEEC) is encouraging all countries across the world to seek alternatives that are cost-effective and sustainable forms of energy production (IPEEC, 2018). Amongst other alternative energy sources such as hydropower and solar energy, biogas produced by the anaerobic digestion (AD) of organic carbon sources using microorganisms is considered a possible source of energy (Mukumba *et al.*, 2016).

Even though the production of biogas through AD is regarded as an alternative energy source, that is not the only desirable trait it possesses. During AD, organic material, referred to as feedstock, is broken down by a consortium of microorganisms to produce biogas. This feedstock is usually organic waste generated from the agricultural industry, food industry, pharmaceutical industry and sewage works (Khalid *et al.*, 2011; Amano *et al.*, 2017; Xu *et al.*, 2019). If not handled correctly, such waste can have a negative effect on the environment, such as environmental pollution (Matheri *et al.*, 2019). Thus, AD provides a channel for this waste to be treated correctly while producing a valuable commodity –energy in the form of biogas.

1.2 Problem statement

Even though the production of biogas through AD offers a promising solution to the current energy crisis and environmental issues, there are several challenges in this process. One challenges, for example, pertains to the type of feedstock used. During AD, a consortium of microorganisms breaks down the feedstock in a series of four-stage events (Hydrolysis, acidogenesis, acetogenesis and methanogenesis) to produce biogas. Generally, all kinds of feedstock with protein, cellulose, carbohydrates, fats and hemicelluloses can be used as substrates in the biogas production process (Akuzuo *et al.*, 2016). However, most of the commonly available feedstocks, referred to as fibrous material, are agricultural waste and energy crops containing compounds like cellulose, hemicellulose and lignin with complex structures which aggravate the digestion process (Sawasdee & Pisutpaisal, 2014a; Zhang *et al.*, 2007). The complex structure of these feedstocks renders the feedstock recalcitrance to enzymatic hydrolysis by the microorganisms involved in anaerobic digestion. Moreover, hydrolysis is the most rate-limiting stage among the four stages in anaerobic digestion, and as a result, no or little biogas is produced (Triolo *et al.*, 2012). Another type of feedstock is non-fibrous material, which includes abattoir waste, winery waste and other food waste. While these types of feedstocks can be easily digested and degraded by the microorganisms, in most cases when these types of feedstocks are used, problems such as the excess accumulation of volatile fatty acids (VFA), carbon-to-nitrogen (C:N) ratio imbalance, and the introduction of toxic inhibitors in the system result in little biogas production or even process failure in some cases (Belaid *et al.*, 2018). Even though both the fibrous and non-fibrous materials have the potential to produce an adequate amount of biogas in spite of the challenges they present, they increase the difficultly of implementation in the biogas industry (Maragkaki *et al.*, 2018).

To combat these feedstock challenges, several anaerobic digestion approaches have been proposed: the pre-treatment of feedstock, co-digestion of feedstocks and different operation temperatures. Thus, this study seeks to compare different anaerobic digestion (AD) approaches using Napier grass as fibrous material and abattoir waste as non-fibrous material.

1.3 Justification

Recent renewable energy studies have shown much interest in the use of Napier grass as a feedstock for producing bioenergy and bio-bases products; this is due to its desirable traits. For instance, Napier grass has a high yield (up to 40 tons/10,000 m^2/year) and requires low attention

for plantation. Napier grass (*Pennisetum purpureum*) is a perennial C-4 grass species native to Africa, growing between 2 and 3.5 m tall (Sawasdee & Pisutpaisal, 2014a). The ability to grow Napier grass with little required input and its ability to yield a high amount when cultivated renders it a suitable feedstock in biogas. Moreover, Napier grass is a good carbon source due to the presence of an adequate amount of lignocellulose (Sawasdee & Pisutpaisal, 2014a; Sittijunda, 2015).

Abattoirs produce large quantities of liquid and solid organic waste during their operations. This waste poses significant environmental pollution if not treated correctly before discarding in running water. Even though in South Africa the use of abattoir waste as a feedstock in AD is not guided by any clear legislation, there is, however, legislation guiding the disposal and environmental protection acts of abattoir waste, such as the National Environmental Management: Air Quality Act (Act No. 39, 2008); Meat Safety Act (Act No. 40, 2000); and the National Environmental Management: Waste Act (Act No. 59, 2008). Such legislation is used interchangeably to regulate the disposal of abattoir waste, but as a result of inconsistency of application, some unacceptable disposable activities have been observed with detrimental effects on the environment (Munganga *et al.*, 2014). Considering this, the use of abattoir waste in AD can be regarded as a treatment strategy of waste produced in abattoirs, protecting the environment and synergy producing biogas. The diversity of nutrients present in abattoirs waste, such as nitrogen and carbon, make it ideal to be used in AD because it allows a wider range of microorganisms to grow in the rich nutrient content present. Moreover, studies have shown that abattoir waste has the potential to produce an adequate amount of methane when a suitable AD approach is used (Rabah *et al.*, 2010; Ibrahim, 2014).

1.4 Hypothesis, aims and objectives

1.4.1 Hypothesis

The following hypotheses for this study were formulated:

Hypothesis 1: Thermal pre-treatment of Napier grass will improve the rate of anaerobic digestion and concomitantly improve the biogas yield.

Hypothesis 2: Co-digestion of thermally pre-treated Napier grass and abattoir waste will balance the nutrient ratio and thereby improve biogas yield.

1.4.2 Aims and objectives

This research aims to compare and uncover the appropriate AD approach that has the potential to be used in the AD of fibrous material (Napier grass) and non-fibrous material (abattoir waste).

The research work will be conducted under the following objectives:

Objective 1: Compare biogas yield between thermally pre-treated and non-thermally pre-treated Napier grass;

Objective 2: Compare biogas yield between different AcoD ratios of abattoir waste and Napier grass;

Objective 3: Optimise the production of biogas using response surface methodology (RSM);

Objective 4: Evaluate the feasibility of optimised conditions when the volumes are scaled up to a 5L single-stage digester; and

Objective 5: Investigate the microbial population dynamics during co-digestion of abattoir waste and Napier grass.

1.5 Significance of research

Previous studies have explored various anaerobic digestion approaches singularly. For instance, Ali *et al.* (2019) investigated different pH levels that yield maximum biogas; while Dussadee *et al.* (2017) and Palatsi *et al.* (2011) investigated mono-AD of Napier grass and abattoir, respectively. Begum *et al.* (2020) evaluated the different AcoD ratios which resulted in maximum biogas yields; and Córdoba *et al.* (2018) studied different inoculum substrate ratios that yield maximum biogas. Even so, there is a gap in the literature that fails to answer if combining all the optimum factors improves biogas yields and if so, what are the optimum ranges these factors can be combined at to yield maximum biogas? Thus, this study seeks to find the best AD approach when two or more factors are combined. This study will pave the way for future studies of anaerobic digestion of fibrous and non-fibrous materials possessing properties like that of Napier grass and abattoir waste. Moreover, it will offer recommendations of the optimum ranges that can be followed when co-digesting fibrous and non-fibrous materials like Napier grass and abattoir waste.

1.6 Project delineation

The downstream processing of the biogas as well as an economic feasibility study will not be carried out. Moreover, biochemical pathways followed by microorganisms will not be evaluated.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Definition of feedstock

Feedstock in the context of anaerobic digestion can be defined as any substrate that can be digested by anaerobic microorganisms, mostly bacteria, to produce methane. The desired attribute in any feedstock is that it should contain a substantial amount of organic matter that can be degraded by the microorganisms to produce methane (Steffen *et al.*, 1998). Research has uncovered that different kinds of feedstocks vary considerably in composition, homogeneity, fluid dynamics and biodegradability. The biodegradability of a feedstock can range from easily degradable organic material to intricate high-solid material (Klimiuk *et al.*, 2010; Zhang *et al.*, 2007). For this research, the feedstocks will be classified into two main groups: fibrous material and non-fibrous material.

2.1.1 Fibrous material and its properties

Fibrous materials such as grass and wood are omnipresent and form part of an essential resource for humankind in terms of material and fuel. Fibrous materials are essentially comprised of lignocellulose, making them ideal for AD because they provide enough carbon for the microorganisms to degrade and thus produce higher biogas yields (Esposito *et al.*, 2012b; Filer *et al.*, 2019). Even though this is the case, studies by Sawatdeenarunat *et al.* (2015) have confirmed that fibrous materials are hard and complex to degrade due to the presence of lignocellulose.

Sawatdeenarunat *et al.* (2015) describe lignocellulose as a material composed of carbohydrate polymers called cellulose and hemicelluloses and an aromatic polymer, lignin. These carbohydrate polymers, containing different sugar monomers, are tightly bound to lignin. Figure 2-1 shows a picture of how Sawatdeenarunat *et al.* (2015) describe the arrangement of cellulose, hemicellulose and lignin. The arrangement of these components creates a highly hydrolysis recalcitrant and resistant biomass structure. Consequently, the hydrolysis of lignocellulose often becomes the rate-limiting step during traditional AD (Esposito *et al.*, 2012b). Even though studies by Mönch-Tegeder *et al.* (2014) suggest that the consortium of microbes works synergistically to

deconstruct recalcitrant biomass structures (like lignocellulose) into their respective fundamental components, this is a slow and energy-consuming process, rendering it ineffective in AD processes, especially on large scales (Ziganshin *et al.*, 2013).

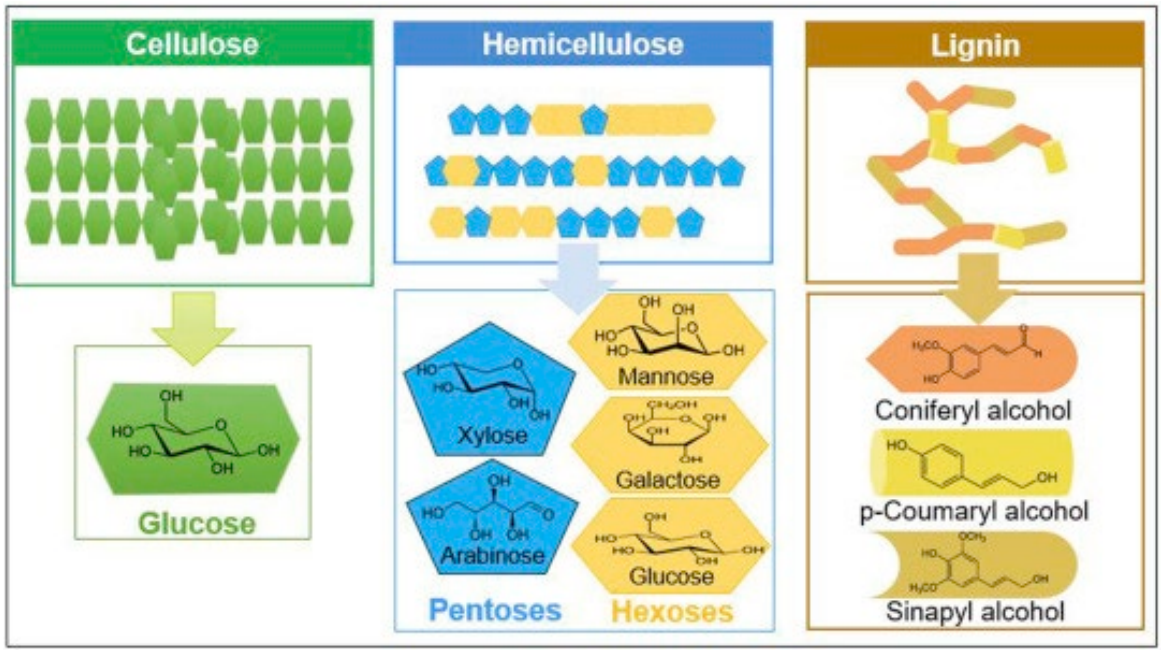


Figure 2-1: Schematic drawing showing the arrangement of cellulose, hemicellulose and lignin in fibrous material (Klimiuk *et al.*, 2010)

Lignocelluloses have been classified into three main groups: the virgin biomass (which includes all naturally occurring terrestrial plants such as grass, bushes and trees); the low value by-product from various industrial sectors such as agriculture (corn stover, sugarcane bagasse and straw); and lastly, forestry (sawmill and paper mill discards) which are classified under waste biomass (Braun *et al.*, 2010). Energy crops are crops with a high yield of lignocellulosic biomass produced to serve as a raw material for the production of second-generation biofuel, with examples such as switchgrass and Napier grass (Braun *et al.*, 2010; Sittijunda, 2015).

2.1.1.1 Napier grass

Napier grass (*Pennisetum purpureum*), shown in Figure 2-2, is a species commonly grown and used for energy crops, rich in carbohydrate and proteins content, and easy to cultivate, making it favourable for biogas production (Sawasdee & Pisutpaisal, 2014). Napier grass is one of the auspicious feedstocks in biogas production. One of the desired characteristics of Napier grass is

that it can be cultivated under a wide spectrum of conditions. As it endures a wide variety of land types, growing seasons and weather, it does not require many inputs for growth (Braun *et al.*, 2010; Sittijunda, 2015; Domingo & Giné Bordonaba, 2011). The AD of Napier grass in a continuously stirred tank reactor is able to produce 238.17 L/KgVS$_{added}$ methane with 56% volatile solids (Sittijunda, 2015). Despite these desirable characteristics of Napier grass, as it is composed of hemicellulose, cellulose and lignin, it is a lignocellulose plant and thus not easily biodegradable (Sawasdee & Pisutpaisal, 2014).

Figure 2-2: Picture of Napier grass before harvesting

The approximate and ultimate chemical composition of Napier grass post-harvest is shown in Table 2-1. The by-products from the hydrolysis of the cellulose and hemicellulose found in Napier grass and other lignocelluloses are xylose, arabinose, mannose, galactose and glucose (Prapinagsorn *et al.*, 2017). These sugars serve as carbon sources in the production of energy. Napier grass has a higher carbon content but a low nitrogen content, resulting in an imbalanced C:N ratio, which is one of the challenges associated with fibrous materials (Sawasdee & Pisutpaisal, 2014).

Table 2-1: Proximate and ultimate composition of Napier grass (Dussadee *et al.*, 2017)

Property	Biomass
pH	4.85
Moisture	77.74 (wt.%)
Ash	3.18 (wt.%)
Carbon (C)	44.19 (wt.%)
Hydrogen (H)	6.00 (wt.%)
Nitrogen (N)	2.00 (wt.%)
Oxygen (O)	43.80 (wt.%)
Sulphur (S)	0.06 (wt.%)

The lignocellulosic structure of the cell wall of Napier grass and other fibrous materials is a defence mechanism by which the plants protect themselves against degradation by microorganisms and enzymes. This then offers plants like Napier grass its recalcitrance. The molecular structure of the cell walls is the main reason behind the recalcitrance (van Beilen & Poirier, 2008). The microfibrils are surrounded by a variety of polymers, such as lignin, hemicellulose and pectin. These polysaccharides are linked to each other through covalent and non-covalent bonds, forming a 3-D structure (Prapinagsorn *et al.*, 2017; Pokój *et al.*, 2018). These celluloses containing microfibrils are joined to the matrix polymers, and these diverse polymers in the gel matrix are linked to each other. This connection between multiple polymers in the cell wall has a significant impact on biomass recalcitrance (Pokój *et al.*, 2018).

2.1.2 Non-fibrous organic waste and its properties

Non-fibrous organic waste is a polysemy phrase; however, in the context of this research, non-fibrous organic waste refers to organic waste that does not contain any lignocellulose properties. This type of waste is typically easy to digest and contains a suitable amount of carbon and other essential nutrients to be used by the microorganisms (Triolo *et al.*, 2012). This waste usually originates from municipal sewage and industrial processes (Ibrahim, 2014). Thus, some studies suggest that due to its origin, non-fibrous organic waste contains excess amounts of other

nutrients causing an imbalance in the nutrients in the AD system. This acts as a hurdle in the AD system resulting in little or no biogas production (Zhu *et al.*, 2009; Esposito *et al.*, 2012a; Ware & Power, 2016).

Nonetheless, data presented in Table 2-2 shows that non-fibrous organic waste contain excess nutrients, and thus, it can still be used in AD and produce adequate biogas. However, in most cases, such processes wherein fibrous organic waste is used require additional attention such as the continuous adjustment of pH, the addition of other nutrients and pre-treatment before the AD process (Haroldsen *et al.*, 2012).

Table 2-2: Biogas yields from different non-fibrous material

Feedstock	Methane yield (l/ kg VS)	References
Municipal solid waste	360	Municipal solid waste 360 Vogt *et al.* (2002)
Abattoir wastewater	850	Fruit and vegetable waste, and abattoir wastewater 850 Forster-Carneiro *et al.* (2007)
Food waste	396	Food waste 396 Zhang *et al.* (2011)
Winery waste	348	Swine manure and winery wastewater 348 Riano *et al.* (2011)
Household waste	350	Household waste 350 Ferrer *et al.* (2011)

Due to the challenges that are encountered when using non-fibrous organic waste, this type of waste has had limited anaerobic digestion, and as a result, it has accumulated over the past years and now poses environmental issues (Roberts *et al.*, 2009 & Haroldsen *et al.*, 2012). Public health concerns and environmental awareness has brought to light that organic waste is now an ecological dilemma. A study conducted in 23 developing countries shows that on average a single person produces 0.77 kg of organic waste per day (Khumalo *et al.*, 2020). To date, worldwide municipalities generate about two billion tons of organic waste per year, a number predicted to increase to three billion tons by 2025. Amongst other contributors to the organic waste concerns, waste from animal processing industries such as abattoirs is a major contributor to this organic waste generation (Bouallagui *et al.*, 2009).

2.1.2.1 Abattoir waste

Abattoir waste can be classified into two main groups – liquid abattoir waste and solid abattoir waste – as shown in Figure 2-3. Liquid abattoir waste can include urine, bile, blood and chemical detergents used in slaughterhouses, while solid abattoir waste includes small bones, carcasses, fat, dead foetuses, hair and some small flesh from the slaughtering process (Matheri *et al.*, 2019). This type of waste can be used in anaerobic digestion as a substrate because it contains high levels of organic matter that can be anaerobically digested to produce biogas. Rabah *et al.* (2010) have determined that 225m^3 of biogas can be produced from digesting 200 g of fresh rumen content of cattle.

Figure 2-3: Abattoir waste: (a) liquid abattoir waste; (b) solid abattoir waste

Abattoir waste is suspected to be a serious health risk if consumed by humans or animals. South Africa produces an estimated 113,750 tons of abattoir waste each day (Commission of the European Communities, 1990). Meat and meat products are not sterile, and bacteria can be found on and within them. The high nutrient and water content of meat render it particularly susceptible to bacterial growth and spoilage. Thus, nations have ventured into finding ways to deal with abattoir waste (Roberts *et al.*, 2009). Ware and Power (2016) believe that anaerobic digestion of this waste could be a brilliant solution in combating this environmental issue because in synergy this process produces a valuable commodity: biogas. However, Sittijunda (2015) argues that even though abattoir waste can be used in anaerobic digestion, it brings other operational challenges, such as the accumulation of VFA and the imbalance in nutrients, as discussed in sections to follow. According to Sittijunda (2015), unless these hurdles are monitored and rectified, the process becomes unfeasible, resulting in little biogas production or sometimes even no biogas production at all. Table 2-3 shows the major components of abattoir waste.

Table 2-3: Major abattoir waste composition (Zafar, 2015)

Property	Composition
pH	6.8-7.0
Chemical Oxygen Demand (COD)	5200-11400 mg/l
Total Solids (TS)	570-1690 mg/l
Total Kjeldal Nitrogen (TKN)	19-74 mg/l
Protein	3250-7860 mg/l

2.2 Anaerobic digestion process

According to Ibrahim (2014), anaerobic digestion follows a series of events where a consortium of bacteria convert the organic compounds to a mixture of gases. The gas is composed primarily of methane (CH_4) and carbon dioxide (CO_2). This series of events is a four-stage process, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 2-4).

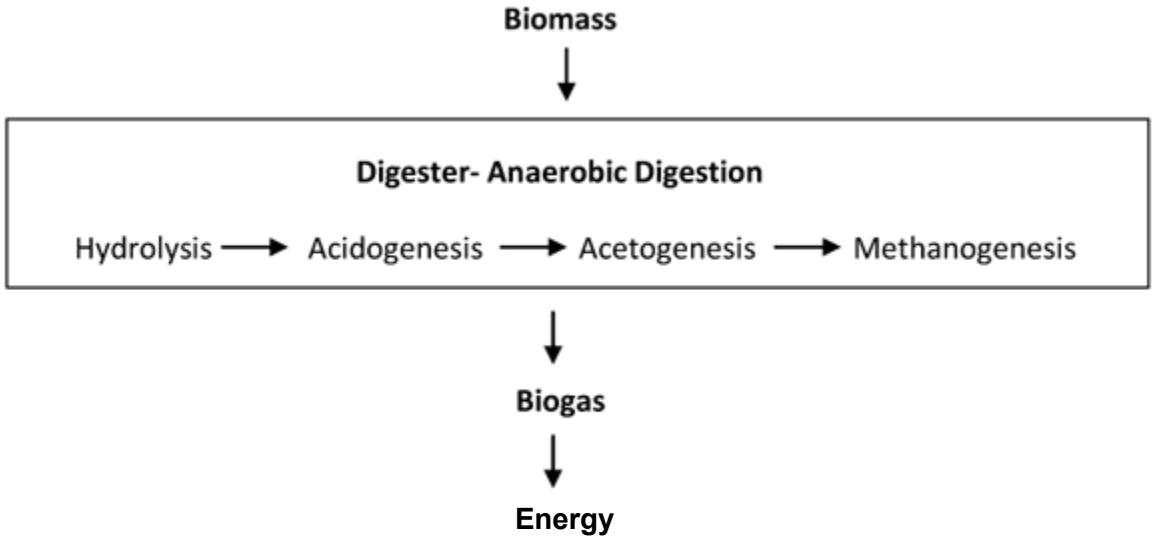


Figure 2-4: Flow diagram illustrating the anaerobic digestion process

2.2.1 Stages in anaerobic digestion

The chemical degradation pathways that take place in AD are clarified in Figure 2-5.

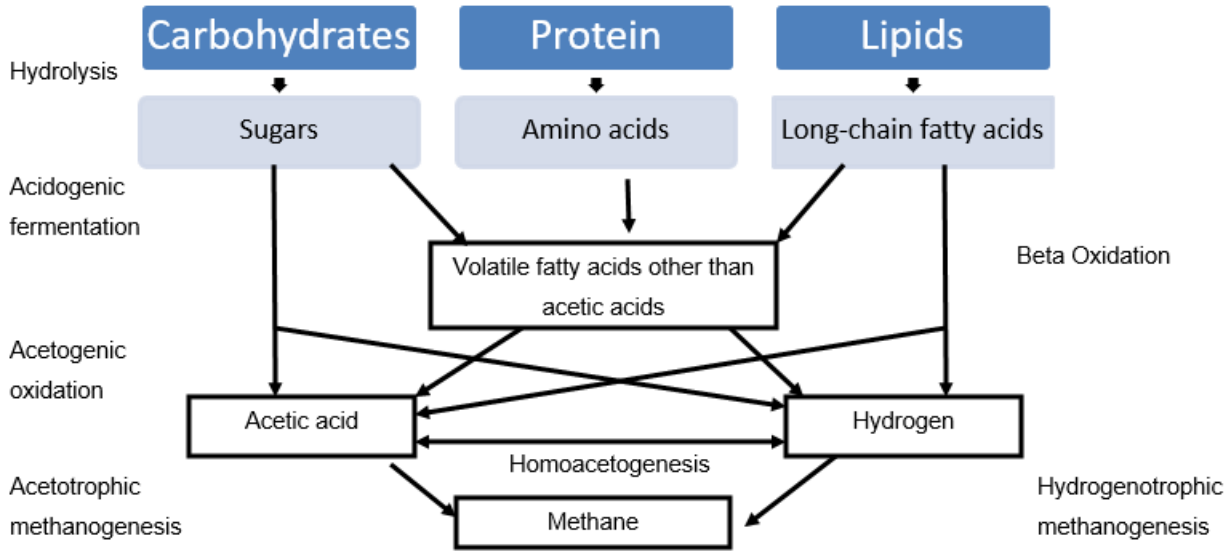


Figure 2-5: Degradation pathways in anaerobic digestion

I. *Hydrolysis*

Belaid *et al.* (2018) explain that during the hydrolysis stage, fibrous and/or non-fibrous organic compounds are broken down by hydrolysing enzymes into fatty acids, amino acids and sugars, as is illustrated in Equation 1:

$$Biomass + H_2O \rightarrow Monomers + H_2 \quad \textbf{[1]}$$

Extracellular hydrolytic enzymes from bacterial cells cleave the covalent bonds in the polymers of the biomass, resulting in simple monomers (Roopnarain & Adeleke, 2017; Zhang *et al.*, 2016; Belaid *et al.*, 2018). Fibrous biomass may require weeks for hydrolysis to occur, due to the complexity of their structure (discussed previously) thereby rendering it recalcitrant to hydrolysis (Sawasdee & Pisutpaisal, 2014b). This hurdle is rarely observed in non-fibrous organic biomass (Boadzo *et al.*, 2011).

II. *Acidogenesis*

In acidogenesis, the products produced from the hydrolysis stage are then further metabolised by fermentative bacteria resulting in short-chain organic acids consisting of two to six carbon atoms. Hydrogen (H_2), alcohols, ammonia and CO_2 are the end products of acidogenesis, as shown in Equations 2 and 3 (Velásquez Piñas *et al.*, 2018).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CH_2OH + 2CO_2 + 4H_2 \quad \text{(-116.3 kj/mol)} \quad \textbf{[2]}$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad \text{(-36.5 kj/mol)} \quad \textbf{[3]}$$

III. *Acetogenesis*

Some products that result from acidogenesis may go straight to the methanogenesis phase and be further used by methanogenic microorganisms. However, certain compounds such as fatty acids with more than two carbons, alcohols with more than one carbon, as well as aromatic and branched-chain fatty acids, are further degraded by acetogenic bacteria in the acetogenesis phase into acetic acid, CO_2 and H_2 (Equations 4-6) (Velásquez Piñas *et al.*, 2018).

$$CH_4CH_2OH + 2H_2 \rightarrow CH_3COO^- + 2H_2 + H^+ \quad (\Delta G = +19 kj / mol) \quad \textbf{[4]}$$

$$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + H^+ + HCO_{3^-} + 3H_2 \ (\Delta G = -104.9 kj / mol) \quad \textbf{[5]}$$

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \quad (\Delta G = -112 kj /mol) \quad \textbf{[6]}$$

IV. *Methanogenesis*

The final stage is the methanogenesis stage, where mainly the acetic acid produced in the acetogenesis stage is used by the methanogenesis bacteria to produce CH_4 and CO_2 (Palatsi *et al.*, 2011). However, this is not the only pathway in methanogenesis. Antonelli *et al.* (2016) have explained other possible ways in which methane can be produced: hydrogenotrophic methanogenesis, methylotrophic methanogenesis, and acetotrophic methanogenesis. Hydrogenotrophic methanogenesis uses hydrogen and carbon dioxide to produce only methane, and methylotrophic methanogenesis uses methanol to produce methane and water, while acetotrophic methanogenesis cleaves acetate to produce methane. Approximately 70% of the produced methane is from acetolactic methanogenesis and 30% is from hydrogenotrophic methanogenesis (Equations 7 and 8) (Antonelli *et al.*, 2016; Filer *et al.*, 2019)

$CH_3COOH \rightarrow CH_4 + CO_2$ (ΔG = -112.55kj / mol) **[7]**

$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$ (ΔG = -135.6kj / mol) **[8]**

Even though this process is not linear, process literature has successfully managed to explain it in these four-stage processes.

2.2.2 Types of anaerobic digestion systems

An AD system's design should allow for the growth and activity of microorganisms under specified conditions. Moreover, it should accommodate the biogas accumulation by trapping the produced biogas and preventing other external gasses from being introduced inside the system (Zamanzadeh *et al.*, 2017). Previous AD studies have managed to collate the various types of digestion systems that are available (Ngumah *et al.*, 2013; McCabe *et al.*, 2014; Dussadee *et al.*, 2017).

2.2.2.1 Batch system

According to Dussadee *et al.* (2017), a *batch system* is any system that is operated in a closed system where nothing is added or removed from the system until the duration of the process is completed. A batch system in anaerobic digestion is no different, where the feedstock is digested in a close digester for a long time, usually from a week to several months (Weinrich *et al.*, 2018). During this time, nothing new should be added or removed from the digester. This type of system is advantageous because it is easy to operate and has proven to have a high removal efficacy of contamination (Liebetrau *et al.*, 2016). However, the problem associated with a batch digester is that the biogas production rate is irregular. It is noted as high at the start of the process and very low near its end (Holliger *et al.*, 2016), a phenomenon due to the growth pattern that microorganisms follow in a batch system, as shown in Figure 2-6. Narayan and Sahana (2009) explain that when microorganisms are introduced in a batch system, they firstly remain in a lag phase. During this phase, the microorganisms are still adapting to the new environment. Once they have adapted, they grow exponentially, multiplying in number and digesting the feedstock, until they reach the stationary phase. During this phase, the rate of death is equivalent to the rate of new cells. This occurs until the substrate is almost depleted and then they enter the death phase where cell division slows down and more cells die. This system is appropriate and popular in biogas production.

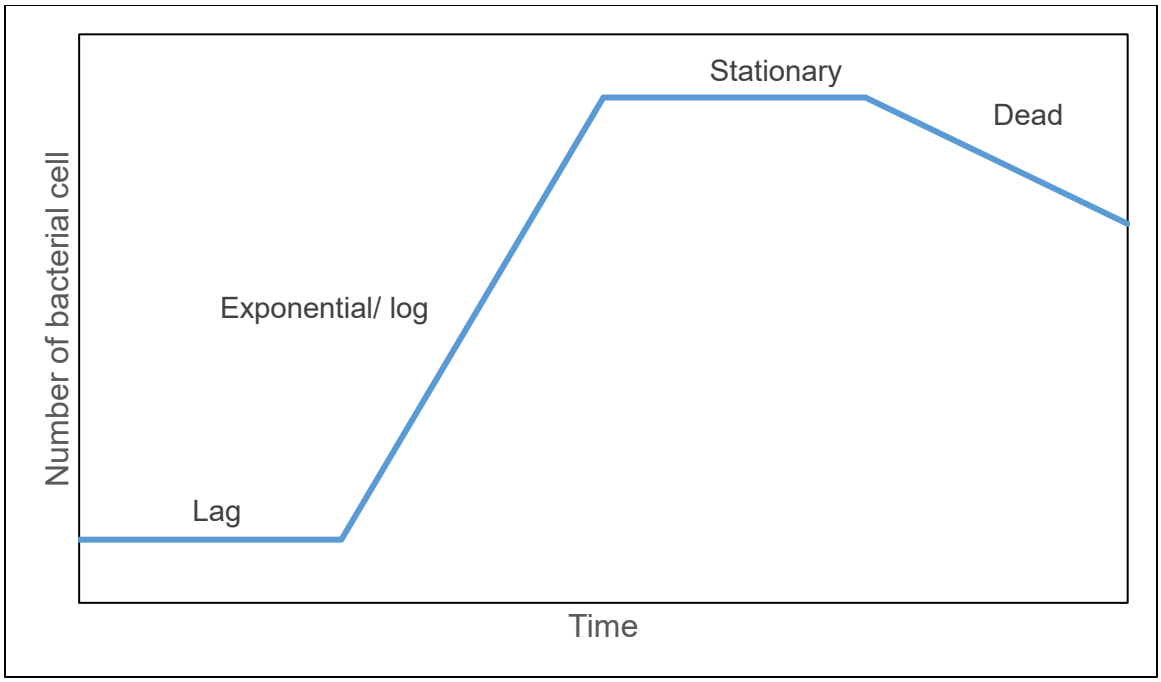


Figure 2-6: Microbial growth curve in a batch system

2.2.2.2 Continuous system

Chaudhari, Suryawanshi and Kothari (2011) define a *continuous system* as one where the substrate is continuously added with a continuous removal of waste or product from the system. A continuous system in biogas production follows the same principle: the fresh feedstock is added and the digested mixed liquor is regularly removed. The biogas production rate will be constant with little variation, provided that the conditions inside the digester remain the same and the digester volume is kept constant. Continuous systems have a disadvantage, though, because they have a limited amount of feedstock that can be digested. According to Zhu *et al.* (2009), the concentration of the feedstock should not exceed 100 kg dry matter per m^3 of effluent, as at a higher concentration the feedstock cannot be pumped. Another disadvantage of this system is that the digested liquor that is removed from the digester bears some bacteria with it, which then lowers the bacterial population inside the digester (Cai *et al.*, 2016). The methanogenic bacteria have a low specific growth rate and to multiply and increase in number, they require approximately two to five days (Henniges *et al.*, 2014). As a result, the active biomass remains restricted in a continuous system, limiting the maximum possible biogas production rate (Zhu *et al.*, 2009).

I. Single stage digestor

In a single digester configuration, all four reaction steps take place inside a single digester (see Figure 2-7). It is, however, important that the pH inside the tank be constantly monitored as it can be lowered by the presence of acidogenic bacteria inside the tank (Ware & Power, 2016).

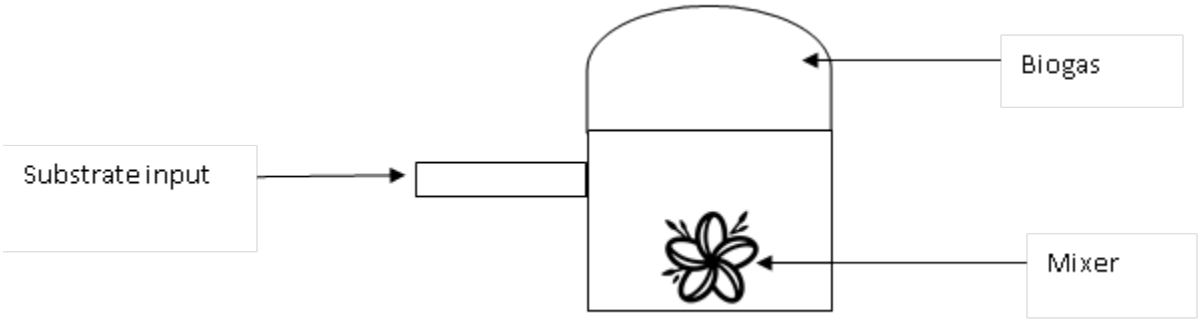


Figure 2-7: A schematic drawing representing a single-stage digester

II. Two-stage digestor

In a two-stage configuration digester, the digestion process is separated into two digesters. Hydrolysis and acidogenesis take place in the first digester and acetogenesis and methanogenesis takes place in the second digester (Figure 2-8) (Begum *et al.*, 2020). The two reactors are connected in series: digester (a) cascading into digester (b). Rajendran and Balasubramanian (2011) contend that separating the reactors into different stages assists in optimising conditions favourable to the growth of each group of organisms in each reactor.

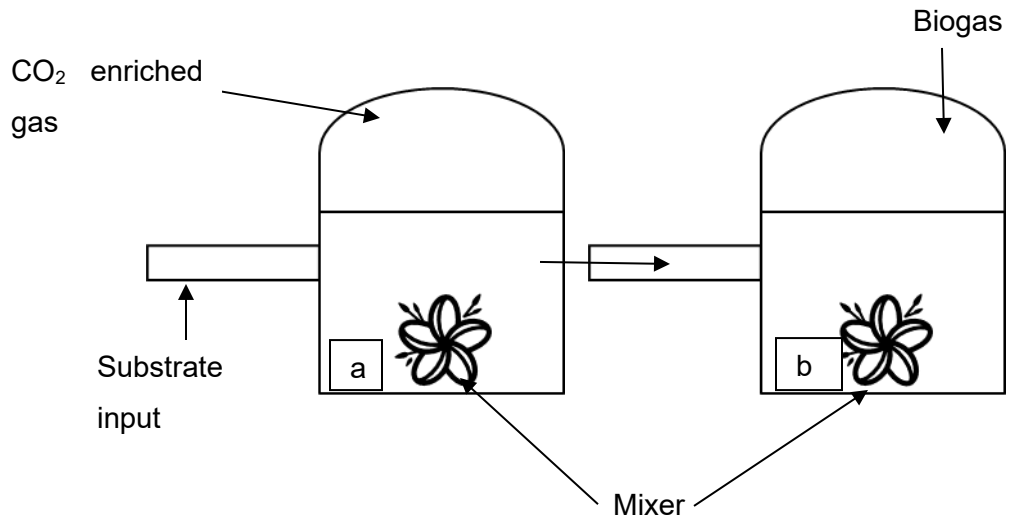


Figure 2-8: Schematic drawing of a two-stage digester: (a) acidification reactor; (b) methane reactor

2.3 Microbiology and enzymes involved in anaerobic digestion

A variety of microorganisms contribute to the AD process, including members of archaea and eubacteria (Cai *et al.*, 2016). Each stage involves different types of microorganisms. Bacterial such as *Bifidobacterium, Clostridium*, *Desulfovibrio*, *Corynebacterium*, *Peptococcus*, *Actinomyces* and *Escherichia coli* use their enzymes for the degradation of the substrate (Chaudhari *et al.*, 2011). Endoenzymes and exoenzymes are the two types of enzymes involved in the catabolic reaction of substrates (Ziganshin *et al.*, 2013). While all bacteria produce endoenzymes, a distinctive number of bacteria can produce both endoenzymes and exoenzymes. However, no bacterium has the genetic coding to produce all the exoenzymes required to degrade an organic substrate (Cai *et al.*, 2016).

2.4 Important parameters to consider in AD

In any AD digestion process, the primary aim is to digest the substrate in synergy producing maximum biogas. Several factors and parameters need to be considered when designing an AD process to ensure that adequate digestion of the substrate is achieved and maximum biogas produced. These factors are outlined in Figure 2-9. If no special attention is given to these factors and parameters, the likely result is little biogas production or in the worst case, complete process failure (Bernat *et al.*, 2017).

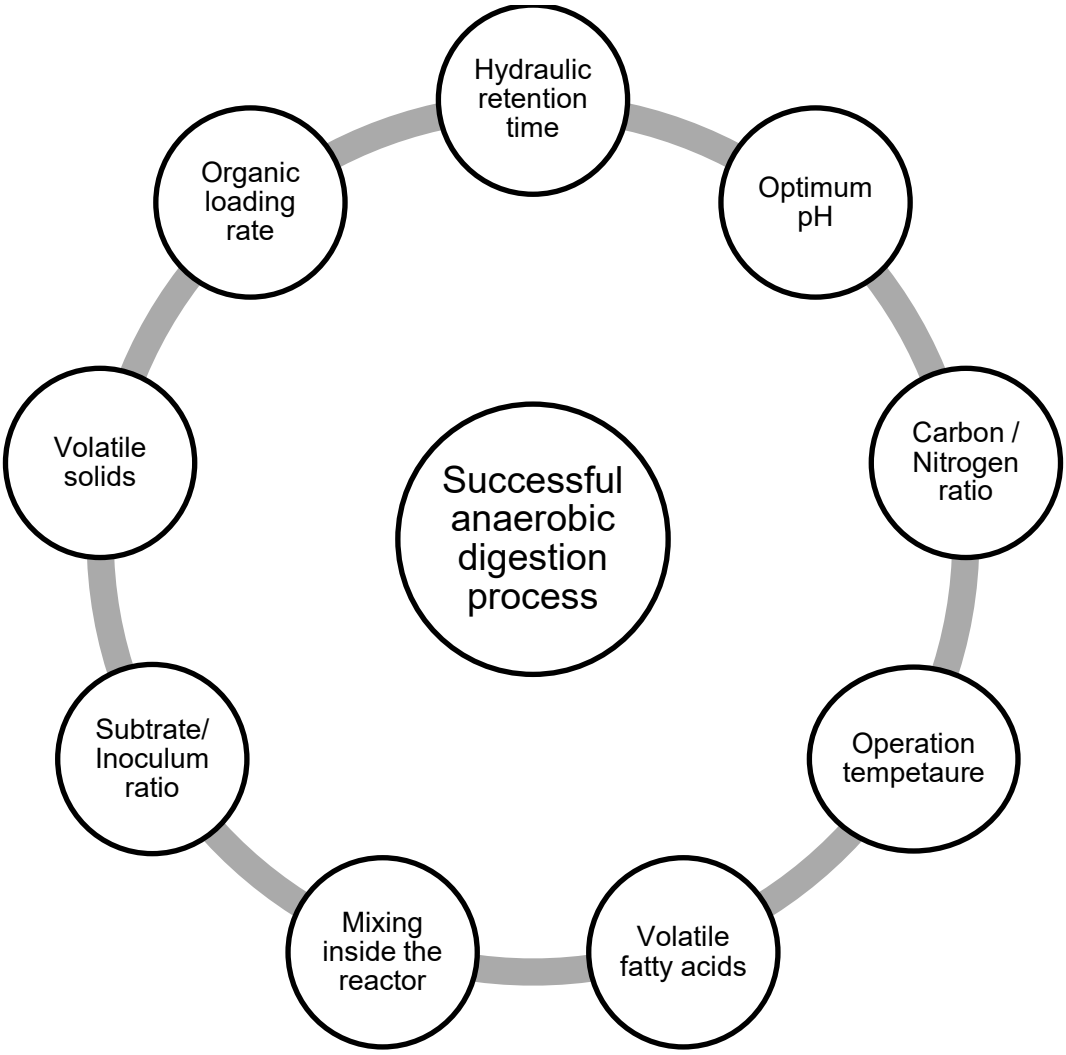


Figure 2-9: Important parameters when designing an anaerobic digestion process

2.4.1 Operation temperature

As the anaerobes responsible for the digestion of the substrates are temperature-dependent, the anaerobic digestion process is similarly temperature-dependent. It is therefore of utmost importance to consider the temperature when designing the process to achieve stability and feasibility (Sanchez *et al.*, 2019). The rate of digestion and biogas production is directly proportional to the operating temperature. However, it is worth noting that there is a maximum temperature under which the process can operate; exceeding this maximum temperature results in process destabilisation (Chen *et al.*, 2008; Sanchez *et al.*, 2019). The anaerobic digestion process can take place in three different temperature ranges: mesophilic (30℃-45℃), thermophilic (44℃-57℃) and psychrophilic (<20℃) (Bouallagui *et al.*, 2009). Conversely, after

intensive research, Sawasdee and Pisutpaisal (2014) and Klimiuk *et al.* (2010) insist that operation temperature should be either mesophilic, or at most, in thermophilic temperature ranges. However, the mesophilic temperature range is highly recommended as this temperature range offers good stability and thus optimal biogas production. The substrate composition and the number of days in which the process will run (hydraulic retention time) need to be taken into consideration when deciding on the operating temperature of the process (Sanchez *et al.*, 2019). Table 2-4 highlights the different temperature ranges with their average hydraulic retention time. Operating at the mesophilic range is economically feasible because it does not require much energy input and it operates for fewer days as supposed to thermophilic which operates for fewer days but still requires a high energy input during that time (Schmidt *et al.*, 2019). Chae *et al.* (2008) elucidate that elevated temperature accelerates the digestion, thereby reducing the hydraulic retention time; however, Bouallagui *et al.* (2009) suggest that elevated temperature results in the accumulation of ammonia, causing process inhibition. This inhibition is common when substrates rich in protein are employed (Bouallagui *et al.*, 2009).

Table 2-4: Different temperature range with average hydraulic retention time

Thermal stage	Process temperature	Hydraulic retention time (days)
Psychrophilic	< 20	70 - 80
Mesophilic	30 -45	14 - 40
Thermophilic	44 - 57	14 - 20

2.4.2 Hydraulic retention time

Hydraulic retention time (HRT) indicates the time required for bacterial growth and to degrade the substrate completely inside a digester (Friehe *et al.*, 2012). Its numeric value is defined as:

$$\theta = V / Q \quad \textbf{[9]}$$

Where ɵ, hydraulic retention time in days;

V, volume of reactor (m^3); and

Q, influent flow rate (m^3/d).

HRT has a remarkable impact on biogas production. Methanogens require longer retention time as compared to hydrolysis and acidogenesis bacteria; therefore, the HRT must be long enough to ensure that the methanogens are retained longer and thus produce biogas (Chen, Cheng & Creamer, 2008). In previous studies by Rajendran and Balasubramanian (2011) when the HRT was under two days, it failed due to the growth limitation of methanogens. Various studies have verified optimum HRT at about 14 days or more (Palatsi *et al.*, 2011; Rajendran & Balasubramanian, 2011; Rodriguez *et al.*, 2017). Even though this is the case, other parameters influence the length of the HRT such as the substrate composition and operation temperature, as already discussed. For example, Belaid *et al.'s* (2018) digested fruit waste and mesophilic temperature of 37℃ had an HRT of 35 days and accumulated a total of 350 Nml of biogas. When Matheri *et al.* (2019) digested abattoir waste at 37℃, they reported a 14 days HRT with a total of 722.1 Nml of just biomethane. Even though both these studies operated at the same temperature due to the variation in the substrate used, the HRT was different. Matheri *et al.* (2019) suggest that complex substrates such as fibrous materials have longer HRT because this time allows the hydrolysing bacteria to hydrolyse the substrate, while non-fibrous material tends to have a lower HRT.

2.4.3 Optimum pH

The anaerobic digestion processes take place under well-defined values of pH. During hydrolysis, the acidogenic bacteria require a pH ranging between 5.5-7.0 while in the final stage, the methanogenic bacteria require a pH value ranging between 6.5-8 (Liebetrau *et al.*, 2016). A low pH value (below 5) can be a major limitation in a single-stage anaerobic digestion process. The lower pH value is usually due to acidification brought about by the accumulation of organic acids such as volatile fatty acids (VFA). A lower pH counteracts and inhibits the activity of methanogenic bacteria; this results in lower or no biogas production (Esposito *et al.*, 2012b). The concentration of ammonia also tends to increase as the digestion process continues; this can cause an increase in the pH value. If the pH value exceeds the optimum pH levels, above 8.5, the digestion process might be stopped because the activity of all the anaerobes will be inhibited due to this high pH level. This phenomenon is generally observed when digesting substrate with a high nitrogen content such as abattoir waste, due to the presence of protein from the meats (Ware & Power, 2016). A single-stage reactor must meet the requirements of the population of microorganisms that coexist (hydrolytic and methanogenic bacteria). To achieve this, typically the addition of NaOH or HCl is required, depending on the initial pH and the desired pH level. Another factor that

can be controlled to ensure the stability of the pH is the carbon-to-nitrogen ratio (C:N) (Zhang *et al.*, 2007).

2.4.4 Carbon-to-nitrogen ratio

The C:N ratio, representing the correlation between the 'mass of carbon' to the 'mass of nitrogen' present in feed materials, has been shown to influence the biogas yield. Besides being used for methane production, carbon is also essential for microbial energy production, while nitrogen is essential to promote microbial growth. Therefore, neither nitrogen nor carbon should be insufficient or in excess. The ultimate C:N ratio for AD ranges between 20:1 and 30:1 (Zhang *et al.*, 2016; Holliger *et al.*, 2016). According to Alexopoulou *et al.* (2015) who evaluated the influence of the C:N ratio of biomass samples, the C:N ratios around 30:1 are preferential for biogas production. However, research by Hills (1979) has concluded that the optimum C:N ratio is 25:1 for cattle manure feedstock. Similar results were obtained by Khalid *et al.* (2011), who evaluated the effect of biogas yield for co-digestion of swine manure and wheat straw for different C:N ratios, and determined that the best biogas yield occurs for C:N ratios of 27.2:1, for mixtures of 40.3% swine manure and 59.7% wheat straw. The evaluations carried out by different researchers verify that the optimum C:N ratio may change depending on the temperature, being that the models developed for mixtures of swine manure and rice straw have optimum methane yield for C:N ratios of 26:1 for temperatures of 35°C and 30:1 for temperatures of 55°C. A substrate that has a higher carbon content such as Napier grass presents a higher C:N ratio synchronous substrate, with higher protein content such as abattoir waste having a lower C:N ratio due to the excess presence of nitrogen.

If the C:N ratio is high, more carbon than nitrogen present, the nitrogen will be consumed rapidly by methanogenic bacteria; this leads to lower biogas production (Krutov *et al.*, 2014). Other studies suggest that a high C:N ratio results in the accumulation of organic acids which also hinders the activity of the methanogens because of the decrease in pH level. A high C:N ratio is common when fibrous materials such as grass are mono-digested (Maragkaki *et al.*, 2018). At a lower C:N ratio, more nitrogen than carbon present, nitrogen will accumulate in the form of ammonia (Chaudhari *et al.*, 2011). The ammonia raises the pH levels in the digester, as explained in section 2.4.3, and if the pH level becomes higher than 8.5 the conditions become unfavourable for the methanogenic bacteria and will thus reduce the biogas production. This phenomenon is usually observed when digesting waste that contains high protein content such as abattoir waste (Escalante *et al.*, 2018). To maintain the C:N ratio, it is important to choose a substrate that has

a balanced C:N ratio or to mix substrate of high C:N ratio with a substrate of low C:N ratio (Krutov *et al.*, 2014).

2.4.5 Volatile fatty acids

Volatile fatty acids (VFAs) are intermediate compounds produced during acidogenesis in the AD process. They are usually composed of carbon chains comprised of six-carbons. Some examples of VFAs produced are acetate, butyrate and propanoate. They are used to monitor biogas production in anaerobic digestion, an indication of the state and activity of microorganisms inside a digester (Chen *et al.*, 2008; Bernat *et al.*, 2017; Mhlanga, 2018). If the VFA concentrations are high, exceeding 13000 mg/L, they result in the decrease of the pH level, which then directly affect the methanogenic population, as explained in the previous section with a discussion of pH and C:N ratios (Sawasdee & Pisutpaisal, 2014b). Propionic acids and butyric acids are methanogenic bacteria inhibitors obstructing biogas production. In contrast, acetic acid promotes methanogenesis resulting in biogas production. It is worth noting that a decrease in pH levels is not always due to the accumulation of VFAs. Thus, it is important to measure VFAs to identify if the decrease of the pH level is brought about by the accumulation of VFAs or not, in a case where low pH levels are experienced. The commonly used methods to measure VFAs are gas chromatography (GC), high-performance liquid chromatography (HPLC) and titration (Mönch-Tegeder *et al.*, 2014).

2.4.6 Mixing inside the reactor

Although mixing is not compulsory, the main purpose of mixing is to achieve homogeneity inside the digester. Mixing allows the system to maintain uniformity in the substrate's concentration and temperature within the digester (Maragkaki *et al.*, 2018). To achieve homogeneity, a mixing device can be used. Propellers and paddles are the commonly used mixing devices. However, when working in small digesters, for example biomethane potential tests, it is advisable that the flask content be shaken once or twice a day for about one to two minutes (Manyi-Loh *et al.*, 2015).

2.4.7 Food to microorganism ratio

Food-to-microorganism (F/M) ratio refers to the amount of substrate being digested to the amount of inoculum added in the process, typically reported on the basis of volatile solids. The F/M ratio will determine the yield and rates of biogas production (Escalante *et al.*, 2018). Literature suggests that a feasible F/M ratio is between two and six. The provision of adequate inoculum ensures that

the AD processes is proficient. Adding too little inoculum can result in process failure because as this means there are not enough microorganisms to digest the substrate; moreover, the substrate can become too toxic to the microorganisms present as it is present in an excess amount (Velásquez Piñas *et al.*, 2018). On the other hand, adding too much inoculum will mean that the system is overloaded with microorganisms and will compete for food and space; this will result in unsuccessful microbial growth and thus a failure in the AD process (Liebetrau *et al.*, 2016).

2.4.8 Volatile solids and organic loading rate

Volatile solids are part of the organic material solids that can be biodegraded by microorganisms while the other part of the solids is non-biodegradable and fixed. The rate of biogas production is influenced by the concentration of volatile solids loaded into the digester (Liebetrau *et al.,* 2016).

The quantity in kilograms of volatile solids that can be loaded into a digester at a working volume m^3 per unit of time is represented by the organic loading rate. The organic loading rate is expressed as kg VS/m^3d. The usual values of organic loading rates are between 0.2 and 2 kg VS/m^3d. This will be determined by the type of feedstock fed into the digester (Zhang *et al.*, 2007).

2.5 Improving biogas yields

Optimisation through more appropriate monitoring and regulation of the biogas process is one way to improve the efficiency of the process. Another way to improve the process is by making the biodegradable compounds easily accessible to the microorganisms by applying pre-treatment techniques (Lindmark *et al.*, 2012). A third way: co-digestion of the substrate can be applied to limit the inhibition from the substrate, balancing the nutrients and also enhancing biogas production (Bernat *et al.*, 2017).

2.5.1 Anaerobic co-digestion

Anaerobic co-digestion (AcoD) refers to the mixing of two or more substrates in the AD process. This approach is well-established, proven to be effective to overcome the challenges that rise from mono-AD of a substrate such as the imbalance in nutrient content and reducing toxic compound accumulation (Lindmark *et al.*, 2012; Esposito *et al.*, 2012a; Esposito *et al.*, 2012b). AcoD allows a diversity of microorganisms to be present in a system, thereby improving the rate of the process and the methane content produced. Different authors have reported different results concerning different co-digested substrates; however, all the results prove that AcoD does enhance biogas yields. In some cases, not only was biogas improved but the quality of the biogas

was improved, meaning a higher methane content was also improved (Bouallagui *et al.*, 2009; Belaid *et al.*, 2018; Matheri *et al.*, 2019). Jordan and Kell (2019) suggest that when deciding on the type of substrate to mix, it is important to use substrates with different compositions e.g., fibrous and non-fibrous materials. Many authors prefer to use fibrous material such as grass, straws and waste from the paper industry and non-fibrous material or material with lesser lignocellulose such as manure and/or abattoir waste (Matheri *et al.*, 2019; Khumalo *et al.*, 2020; Thaemngoen *et al.*, 2020). Abattoir waste is preferred because it has high nutrient content which is necessary for microbial growth.

2.5.2 Substrate pre-treatment

Pre-treatment means that before anaerobic digestion, the substrate is subjected to a step that will help improve AD (Rodriguez *et al.*, 2017). An ideal pre-treatment method should improve the degradability of the fibrous material by improving the accessibility of the enzymes to the cellulose and hemicellulose; in so doing, it should, however, avoid the degradation or loss of carbohydrates and avoid the production of potential inhibitors. Moreover, because AD is considered a greener, cleaner and more cost-effective technology, the pre-treatment method should have a low or zero impact on the environment and should be cost-effective and energy-efficient (Rodriguez *et al.*, 2017; Patinvoh *et al.*, 2017; Jordan & Kell, 2019). The pre-treatment of a substrate can be divided into chemical, physical, thermal and biological methods.

2.5.2.1 Chemical pre-treatment

Chemical pre-treatment includes the introduction of chemicals to the substrate before the first AD stage, hydrolysis. The chemicals used can be grouped into two groups, namely acidic pre-treatment and alkaline pre-treatment. *Alkaline pre-treatment* is more effective in lignin removal. The effectiveness of alkaline pre-treatment depends on the content of lignin present in the biomass (Rodriguez *et al.*, 2017). Alkaline pre-treatment cleaves the lignin-carbohydrate linkages and produces the saponification, increasing the porosity and internal surface area of biomass, and decreasing the degree of polymerisation and crystallinity of feedstock. In addition, the alkaline residual remaining in the biomass can help prevent a drop in pH during acidogenesis (Khalid *et al.*, 2011). The most frequently used alkalis are ammonium, calcium, potassium hydroxides and sodium (Patinvoh *et al.*, 2017).

Acid pre-treatment, more effective in hemicellulose solubilisation, can be conducted by either concentrated acid or diluted acid. The most frequently used acid is sulphuric acid even though

acetic acid, hydrochloric acid and nitric acid have shown to be effective (Sawasdee & Pisutpaisal, 2014a; Rodriguez *et al.*, 2017). Chemical pre-treatment is considered less attractive because it is economically infeasible. Moreover, the chemicals used in most cases harm the environment; for example, concentrated acid is highly corrosive and very toxic (Palatsi *et al.*, 2011).

2.5.2.2 Biological pre-treatment

Biological pre-treatment refers to introducing the substrate to biological substances such as bacterial, fungi and enzymes, before the AD process. White and soft rots fungi are effective in lignin and cellulose degradation while brown rots attack cellulose (Maile *et al.*, 2015). Anaerobic microbial pre-treatment is another biological pre-treatment method where the first two stages of the AD process occur separately from the final methane production step. The separation of the stages eliminates inhibitory effects on methanogens such as the accumulation of VFAs, thereby improving biogas production (Velásquez Piñas *et al.*, 2018). Although enzymes are already produced by the consortium of microorganisms present in the digester, the addition of one enzyme or a mixture of enzymes can aid in substrate degradation and as a result, improve biogas production. Enzymes at a pre-treatment method can be used solely or combined with other pre-treatment methods. However, although the use of enzymes is deemed environmentally friendly, the process is time-consuming and requires more space: it requires 10-14 days, and at a higher residence time, a larger reactor volume is required (Moody *et al.*, 2009).

2.5.2.3 Physical pre-treatment of fibrous material

Unlike chemical and biological pre-treatment, *physical pre-treatment* does not include the use of external compounds during the pre-treatment stage but rather focuses on reducing the substrate particle size via mechanical commination, or the surface area of the substrate increases without size reduction (Patinvoh *et al.*, 2017). Physical pre-treatment can be classified into mechanical, ultrasonic and thermal pre-treatment.

I. *Mechanical pre-treatment*

   Mechanical pre-treatment, mainly disrupting weak physical bonds, can be achieved by chopping and grinding substrate to reduce particle size, increasing the substrate's surface area and enabling substrate-bacteria contact (Jordan & Kell, 2019).

II. *Ultrasonic pre-treatment*

In ultrasonic pre-treatment, also referred to as microwave pre-treatment, waves disrupt the substrate's cells to promote cavitation inside the cell, thereby enhancing the contact between the substrate and anaerobic bacteria (Thaemngoen *et al.*, 2020). Microwave irradiation hydrolysis lipids to oleic acid, palmitic acid and stearic acid. Proteins are hydrolysed into saturated and unsaturated acids, ammonia and carbon dioxide; while carbohydrates are hydrolysed into polysaccharides with lower molecular weight (Patinvoh *et al.*, 2017). In a study by Rodriguez *et al.* (2017), Napier grass pre-treated by microwave resulted in a decrease in methane yields from 189.7 mL/gVS of untreated Napier grass to 163.6 mL/gVS of three-minute wave pre-treated. These results suggest microwave pre-treatment has no significant effect on the volume of methane production. However, from this study, the methane kinetics showed a significant increase. In addition, the time needed to produce 80% methane was reduced by 4.5 days.

III. *Thermal pre-treatment*

In thermal pre-treatment, heat breaks down the hydrogen bonds in a crystalline complex of cellulose and lignocellulose. This results in the substrate swelling, thereby increasing the surface area. Thermal pre-treatment is commonly used and most effective in the treatment of lignin and hemicellulose (Patinvoh *et al.*, 2017; Rodriguez *et al.*, 2017). Jacketed reactors, pressure cookers or autoclaves are normally used to carry out thermal pre-treatment, though this may vary from lab to lab depending on the type of equipment available. When Rodriguez *et al.* (2017) thermally pre-treated Napier grass, results revealed an increase in methane yield from 189.7 mL/gVS of raw grass to 198.3 mL/gVS of 30-minute water vapour pre-treatment.

Not only is heat applied as a pre-treatment, but it also eliminates pathogens that might be present in a feedstock; this is important when using feedstock that contains a great deal of nutrients and thus becomes favourable for the growth of other undesired microorganisms and pathogens, i.e., sewage waste and abattoir waste (Palatsi *et al.*, 2011). Thermal pre-treatment is also favourable because it can be used with chemical and/or biological pre-treatment without counteracting with the other pre-treatment but rather improving the biogas yields generally. The equipment needed for thermal pre-treatments is also easily accessible, enhancing its favourability. Thermal pre-treatment

does not usually result in the production of other undesired substances (Patinvoh *et al.*, 2017).

2.6 Biomethane potential tests

To investigate the ultimate potential of an organic feedstock for producing methane in anaerobic digestion, biomethane potential (BMP) tests are employed. Biomethane potential tests are used universally to determine the methane potential and biodegradability of organic feedstocks. In these tests, the substrate is combined with the inoculum in small digesters with volumes ranging between 120 mL-2000 mL depending on the substrate and the inoculum volume (Esposito *et al.*, 2012b). These tests, operated in a batch system, are allowed to run for 15-55 days, depending on the operating temperature and the feedstock used. The central issue with BMP tests is the lack of standardised procedures. Many national and international procedures have been proposed; in all of these procedures different serum bottles are used, different inoculum from different sources is used, the S/I ratios vary and the biogas measuring device is different (Esposito *et al.*, 2012b; Velásquez Piñas *et al.*, 2018). In addition to the lack of standardised procedures, there is an absence of instructions for new operators to start BMP. Most BMP guidelines provide a generalised methodology to accommodate most feedstocks, but it would be useful to provide methodologies that cover a wide range of substrates.

2.7 Conclusions

While biogas offers a promising prospect as an alternative energy source, the issue of process efficiency constrains implementation in most parts of the world. The issue of efficiency can be addressed by consolidating both the engineering aspect and the microbiology aspect of the process. There is a gap in the literature that neglects to address both these aspects concurrently. Moreover, the feedstock used in the process has been identified as the major decider in whether the anaerobic process will be a success. It is important, therefore, to study the different feedstocks that can be used in the process and explore different approaches that can be implemented in the process to ensure maximum biogas production. Although different feedstocks have different limitations, literature has proven that these limitations can be combated with the right approach such as pre-treatment and co-digestion of the feedstocks. Studying these approaches will fill this gap in the present knowledge of the anaerobic digestion of fibrous and non-fibrous organic waste.

CHAPTER 3

3 MATERIAL AND METHODS

This chapter unpacks the materials used and the methods followed in this study. It presents a detailed description of the sample collection and preparation, BMP set-up, bioreactor set-up and all the experimental protocols followed. The analytical methods used for data analysis and the justification of the use of material and techniques are also presented in the chapter.

The steps followed in determining substrates and co-substrates suitable for enhanced biogas production are outlined in chronological order as follows:

1. Substrate characterisation (proximate and ultimate analysis) was carried out under reproducible conditions.
2. The ultimate results, Carbon, Hydrogen, Nitrogen and Sulphur (CHNS) were used to determine the theoretical methane potential (TMP) of the substrates and co-substrates using the Buswell and Boyle equation.
3. Biomethane potential assays based on mono- and co-digestion of the substrate were conducted in triplicate under reproducible conditions (Chapter 4 and 5).
4. Optimisation of experiments using DesignExpect for experimental design was carried out using BMP under reproducible conditions (Chapter 6).
5. Up-scaling using a two-stage 5L bioreactor to determine the feasibility of optimised condition at up-scale was conducted under reproducible conditions (Chapter 7).
6. Microbial analysis at the start and end of the up-scaling stage was sent for microbial analysis to the Inqaba Biotech Laboratory.

3.1 Reagent solution description

The preparation of all the reagents used in this study is described below.

I. Sodium hydroxide stock solution

To prepare various dilutions, a 10M sodium hydroxide (NaOH) stock solution was prepared from lab-grade pellets (NaOH, CAS-NO. 1310-73-2, B & M Scientific) and distilled water and was then flushed with nitrogen gas.

II. Hydrochloric acid stock solution

A hydrochloric (HCL) acid lab-grade concentrated solution (HCl: 36.46, 32% CP-grade, Batch-No. 15477W0, B & M Scientific) was used to prepare 5 M hydrochloric acid. The 5 M HCL stock solution was further diluted to 1 M HCL which was used to prepare an acidified-saline solution as well as a pH-control/adjustment solution.

III. Acidified-saline solution

An acidified-saline solution was used to decrease the solubility, and diffusion of gasses was used as discharge fluid in the gasometers (Strömberg *et al.*, 2014). The acidified solution was prepared with normal table salt. The salt was dissolved in warm water (30 ±5℃) with constant stirring until completely dissolved. The pH was then adjusted to 2 using the HCL solution.

IV. Scrubbing saline solution

Before measuring the gas produced in BMP tests, it is important to upgrade the quality of the gas by removing the CO_2 present in the produced gas. This is achieved by passing the produced biogas in a scrubbing saline solution, where the CO_2 dissolves in the solution and the biomethane and other trace gasses remain in a gaseous form. The saline solution was prepared by dissolving three pellets of NaOH in 1L of distilled water; this was then mixed until the pellets were completely dissolved, and three drops of phenol indicator were added. The phenol indicator was used to indicate the presence of OH^- available to react with CO_2 and thus dissolve it. A pink colour indicated the presence of OH^- while a faded pink indicated that the OH^- has been depleted (Khumalo *et al.*, 2020)

3.2 Substrate collection, preparation and storage

3.2.1 Abattoir waste

An abattoir waste (AW) sample containing meat waste, cow blood and manure was collected from Deon Groenland Meat Traders Grabouw (Cape Town, Western Cape, SA) in sterile plastic containers which were kept in a cooler box at 0 ℃ for an hour during transit to the lab (Waste-2-Energy CPUT, Bellville, Western Cape, SA). The meat waste, cow blood and manure were all mixed using a food processing blender (Kenwood Multi-Pro, FDM780BA, 1000W, SA) to obtain a slurry. Several authors suggested that to rid the slurry of pathogens that might be present in the abattoir waste, it should be sterilised (Valta *et al.,* 2015; Escudero *et al.,* 2014; Salminen & Rintala,

2002). This was achieved by autoclaving the slurry in Schott's bottles in a vacuum autoclave (Moss Eagleguard EG6018 Retort) set at 151℃ for 15 minutes. The autoclaved waste was then stored in a refrigerator at 4℃ until required for use.

3.2.2 Napier grass

The Napier grass was collected from Somerboch wine farm (Stellenbosch, Western Cape, SA). Napier grass weed was cut into small pieces of about 4 cm, and then put in a food processor to grind into a smaller particle size of about 0.5 cm in diameter. The Napier grass was divided into two portions: one portion was transferred into four 500 mL Schotts bottles which were then thermally pre-treated in an autoclave at 151℃ for 15 minutes; while the other portion was transferred into plastic bags. Both thermally pre-treated (TPN) and untreated/raw Napier grass (RNG) were stored in a refrigerator at 4℃ until required for further use.

3.3 Inoculum collection, preparation and storage

3.3.1.1 Inoculum collection

The inoculum was collected from global energy, Malmesbury Farm, Cape Town, 7299, in a sterile 20 L container and kept at room temperature during the 1-hour transit to the lab. Upon arrival, the inoculum was prepared further following three stages. These stages were all carried out in a laboratory-induced anaerobic environment.

3.3.1.2 Inoculum preparation and storage

I. Stage one: mixing

The inoculum was mixed with Zebra dung obtained from Vredenburg Game Reserve in Stellenbosch. The Zebra dung had been kept in the lab and used in previous studies. This mixture was prepared by mixing approximately 200 g of Zebra dung with 2L of inoculum in a food processing blender for 10 minutes. This blended mixture was then transferred into a 3L degassed plastic bottle. It was then flashed with N_2 and sealed and stored at room temperature for 10 days.

II. Stage two: inoculum acclimatisation

The produced inoculum seed (3 L) was transferred to a 5 L plastic container. Then, 5 g of thermally pre-treated grass, 5 g of raw grass and 10 g of abattoir were added to acclimatise the inoculum

to the substrates. This inoculum and substrate mixture was kept in a water bath at 37℃ for 20 days.

III. Stage three: screening and characterisation of inoculum

After stage two, the inoculum was sieved to remove large particles (>2mm) before proximate and ultimate analyses. After characterisation, the inoculum was preserved in a 2 L plastic container and refrigerated at -4±1℃ for later use in all the experiments.

3.4 Analytical methods

3.4.1 Assumptions applied and scientifically standardised methods

The standard temperature and pressure (STP) conditions were applied according to the International Union and Pure and Applied Chemistry (IUPAC) by assuming perfect mixing and ideal gas behaviour inside the digester:

- Standard pressure (Ps) = 101.325 KPa (1 atm)
- Standard temperature (Ts) = 0℃ (273.15 K)
- Standard gas volume (Vs) = 22.4 m^3

To simplify the complex nature of anaerobic digestion, the following assumptions were drawn:

- adequate microbiological conditions inside the digester, meaning complete digestion;
- adequate mixing resulting in homogeneity inside the digester;
- feedstock composed of C, H, O, N and S; and
- biogas produced consisting only of CH_4, CO_2, NH_3; and H_2S; and H_2.

3.5 Feedstock and inoculum characterisation

3.5.1 Proximate analysis

The TS and VS analysis were performed using standard methods described by Sluiter *et al.* (2008). To determine TS, a known weight/volume of sample was placed in crucibles and dried in a laboratory convective oven at 105 ±5℃ to a constant weight. The samples were then cooled in a desiccator before weighing them to determine the TS. These samples were further incinerated in a muffle furnace at 575 ±25℃ for four hours, after which the samples were allowed to cool before weighing them for VS determination.

3.5.2 Ultimate analysis

The elemental composition of C, H, N and S analysis was done at Central Analytical Facilities (CAF) in Stellenbosch by combustion using CHNS element analyser with oxygen determined as the difference. The metal concentration was analysed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). These results were used to estimate the carbon-to-nitrogen (C/N) ratio.

3.6 Experiment set-up and procedure

3.6.1 BMP set-up and procedure

This study followed the standard BMP protocol as described by Owen *et al.* (1979) and later modified by other scientists (Angelidaki *et al.*, 2009; Hansen *et al.*, 2004; Strömberg *et al.*, 2014) with minor changes to fit the current study (see Figure 3-1) for directly measuring methane yield. All BMP assays were carried out at 38 ±5℃ under anaerobic conditions which were achieved by flushing the headspace of the digester for five minutes with N_2 gas (Nitrogen Baseline 5.0, UN No. 1066, Afrox gas, Epping, SA), before adding the sample and inoculation and before sealing and incubation. The BMP assay was set up as shown in Figure 3-1 in a three-processing unit.

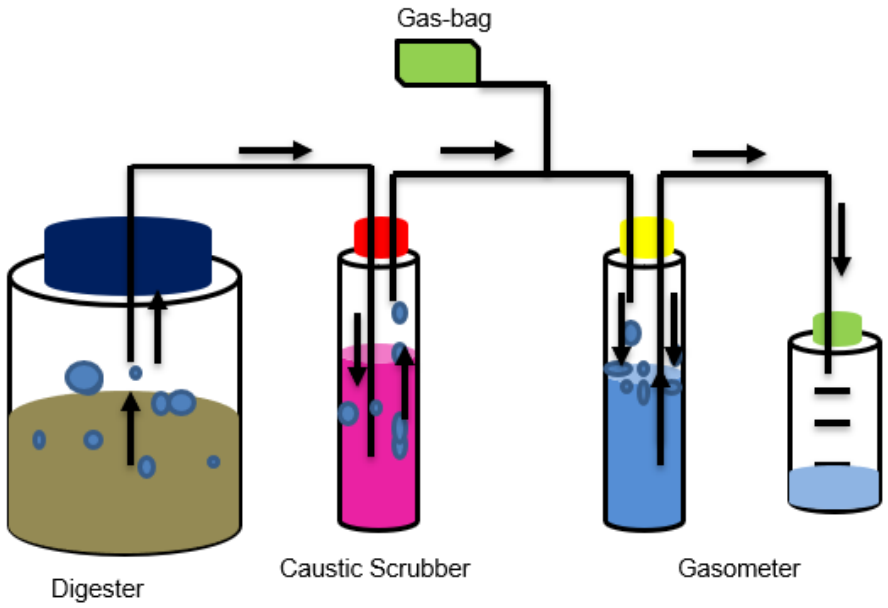


Figure 3-1: Schematic representation of the BMP assay experimental set-up

I. Anaerobic digestion section

Temperature-controlled water baths were filled with tap water and fitted with 500 mL digesters (Duran Schott bottle); a gas production line was fitted with a plastic clamp. To monitor the temperature inside the water baths, circulating immersion thermostats (FMH electronics) were fitted inside the water bath.

II. Gas scrubbing section

A scrubbing solution (described in section 3.1) was filled into 500 mL scrubbing bottles, which were directedly connected by a tube to the digester.

III. Gas measuring unit section

Gasometers made from usable 2 L plastic bottles were filled with acidified-saline solution (described in section 3.1) and connected to the scrubbing solution. A 100 mL Tedlar gas bag (Sigma-Aldrich) was attached to each gasometer and used as gas storage for the long term.

3.7 Biomethane potential tests to compare biogas yields at the different substrates

Thermally pre-treated, raw Napier grass and abattoir waste were used in this study. A detailed description of substrate collection, preparation and preservation is described in section 3.2.1. A working volume of 450 mL was maintained. The pH was adjusted before incubation to a neutral pH with the addition of HCL or NaOH, depending on the initial measured pH level. These tests were allowed to run for 30 days at 37℃ with daily shaking of the digesters to ensure adequate mixing of content. A control test was used which contained only inoculum.

3.7.1 Comparison of biogas yields between thermally pre-treated and raw grass

All tests were undertaken in triplicate and were set up as described in Table 3-1. The substrates used, raw and thermally pre-treated Napier grass, were measured on VS basis.

Table 3-1: Biomethane potential inoculation for mono-digestion of raw and thermally pre-treated Napier grass

Digester number	Substrate description	Organic load (g V^{-1})	F/M (g VS_{sub} g^{-1} VS_{inoc})
1-3	Raw Napier grass	20	2
4-6	Thermally pre-treated Napier grass	20	2

3.7.2 Co-digestion of thermally pre-treated Napier grass and abattoir waste

All tests were conducted in triplicate. The TPN and AW were co-digested at different co-digestion ratios (see Table 3-2). The ratio was on a VS basis.

Table 3-2: Biomethane potential inoculation for co-digestion of thermally pre-treated Napier grass and abattoir waste

Digester number	Co-digestion ratio (TPN:AW)	Organic load g/VS	F/M ratio
1-3	1:0	20	2
4-6	0:1	20	2
7-9	1:1	20	2
10-12	1:2	20	2
13-14	2:1	20	2

3.7.3 Biogas optimisation set-up and procedure

Biogas yields were optimised using central composition. Table 3-3 shows the chosen parameters for optimisation which were temperature (A), co-digestion ratios (B) and F/M ratio (C).

Table 3-3: Selected optimisation factors and levels

Factors	Symbol	Factor level		
		-1	0	1
Temperature (℃)	A	35	36.5	38
Co-digestion ratio	B	30:70	50:50	70:30
F/M ratio	C	2	3.5	5

3.8 Upscaling experiment with 5 L batch digester

The optimised conditions were upscaled in duplicate in a 5 L single-stage batch reactor (GlassChem Pty) shown in Figure 3-2. The pH levels and the temperature were monitored and controlled by an integrated pH probe and heating mantle, respectively. The digesters were operated at a 20 g VS^{-1} organic load with a co-digestion ratio of 50:5 (thermally pre-treated Napier grass:abattoir waste), an F/M of 5 and an operating temperature of 35℃. Before the start of the experiment, the pH was adjusted to 7 with 1M NaOH or HCL, depending on the initial pH level. Pure N_2 (99.9%) was passed through the digester for 2-3 minutes and sealed immediately to create an anaerobic condition. The digestor was continuously stirred at 200 rpm to achieve homogeneity. The digestion was conducted for 30 days, and the biogas production was recorded daily using the water displacement method (described in section 3.2). A Geotech 5000 Biogas Analyser was used to analyse the quality of the biogas produced.

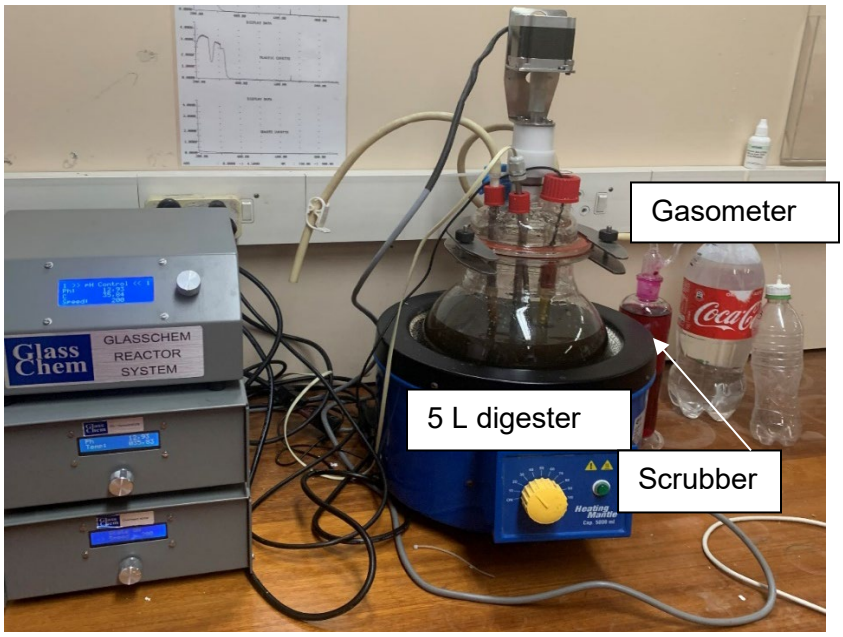


Figure 3-2: 5L single-stage batch digestor set-up for upscaling

CHAPTER 4

4 COMPARISON OF BIOGAS YIELDS BETWEEN THERMALLY PRE-TREATED AND RAW NAPIER GRASS

4.1 Abstract

Biogas can be produced from any organic substance; however, fibrous material poses a challenge to the AD process due to the complex arrangement of cellulose, hemicellulose and lignin. To combat this challenge, the substrate must be subjected to pre-treatment before the AD. Pre-treating the substrate with heat, referred to as thermal pre-treatment, is one of the favoured and most adopted techniques in AD. Biomethane potential tests were used to compare the biogas yields between thermally pre-treated and raw Napier grass. The pre-treated Napier grass was pre-treated using an autoclave for 15 minutes at 151℃ prior to digestion, while the raw Napier grass was not pre-treated. These two substrates were then digested at a 20 g VS/L organic load with an F/M of 2 for 30 days. The thermally pre-treated Napier grass had a VS content of 88.24% while the raw Napier grass had 90.67%. The C:N ratio for the thermally pre-treated and raw Napier grass was 23.40 and 20.57, respectively. Both these substrates managed to yield biogas. Thermally pre-treated Napier grass accumulated a total of 70.3 Nml/g•VS_{added} while raw Napier grass accumulated the least biogas of 46 Nml/g•VS_{added}. In addition, pre-treated Napier grass accumulated a total of 72% of methane while raw Napier grass only accumulated 61% of methane. The BMP tests indicated that thermal pre-treatment of Napier grass improved biogas yields. And in addition to the improved biogas yield, the quality of the biogas was also improved over that of raw Napier grass. This study revealed the significant effect of pre-treating a substrate before the AD.

4.2 Introduction

To overcome the challenge of recalcitrance in fibrous material, several techniques have been proposed which involve the treatment of the substrate before digestion. These techniques have been grouped into physical, chemical and biological pre-treatments. Biological and chemical pre-

treatments involve the use of biological entities or chemical substances, respectively, to treat the substrate before the digestion stage (Patinvoh *et al.*, 2017). Physical pre-treatment includes the use of mechanical, thermal and ultrasonic operations to reduce the particle size of the substrate and thus increase the surface area, making it easily accessible by the microorganisms for hydrolysis (Antonelli *et al.*, 2016).

With the world moving to a cleaner and greener environment, the desired pre-treatment technique should not harm the environment. Chemical pre-treatment is less desirable because the chemicals tend to harm the environment (Rodriguez *et al.*, 2017; Phuttaro *et al.*, 2019). Even though biological pre-treatment might be perceived as environmentally-friendly, the process is slow, reducing its desirability. In recent years, the use of physical pre-treatment has been acknowledged as the best option as this pre-treatment technique has been proven effective. Mechanical and thermal pre-treatment are the ones commonly used due to their easy accessibility (Rodriguez *et al.*, 2017).

However, there is a gap in the literature that does not investigate whether the pre-treatment of a substrate influences the carbon composition and thus diminishes the biogas yield. Therefore, this study compares the physicochemical properties of thermally pre-treated Napier grass and raw Napier grass. Likewise, the biogas yields and quality of the two substrates are compared to each other.

4.3 Aim and objectives

This chapter aimed to compare biogas yields between thermally pre-treated and raw Napier grass.

The aim was achieved by following these objectives:

- Thermal pre-treatment of Napier grass at 151℃ for 15 minutes.
- Assessing the physicochemical properties of both TPN and RNG.
- Anaerobically digesting the substrate to compare the biogas yields as well as the amount of methane produced.

4.4 Materials and methods

Napier grass was collected and pre-treated (a detailed description of substrate collection, preparation and storage is in section 3.2). The substrate was then digested using BMP tests for 30 days, with daily measuring of the produced biogas (a detailed description is in section 3.7.1).

4.5 Results and discussions

4.5.1 Substrate ultimate and approximate analysis

The physical and chemical characterisation of the substrate is presented in Table 4.1. The AW, RNP and TPN showed major differences in their properties. This can be a verifiable indication in co-digestion because it means that when one substrate lacks one property when mixed with a second substrate, the second substrate can make up for the lacking property. Bouallagui *et al.* (2009) suggest that when two or more substrates are co-digested, it is important that they have different properties so that they can offer compatibility and result in an efficient AcoD. Matheri *et al.* (2019) define *total solids percentage* as the organic and inorganic material present in the feedstock, while the *volatile solids* represent only the organic matter in the feedstock. Thus, a higher VS content is expected to produce a high methane yield due to the presence of organic matter for the microorganism to use. Both the RNG and TPN have a VS content of 90.67% and 88.24%, respectively. In contrast, AW only had 20.49% VS content, which is relatively low when compared to that RNG and TPN. The TPN, however, had a lower VS% as compared to the RNG, suggesting that during the heat pre-treatment stage some organic contents were lost through volatilisation.

The RNG and TPN both have a C:N ratio of 20.57 and 23.40, respectively, a ratio within the suggested ratio of 20-30. This indicates that these substrates will result in an efficient AD process. However, that alone is not conclusive that a higher biogas yield will be obtained from the digestion of these substrates because these substrates cannot be easily digested by the microorganisms due to their complex structural arrangement. In contrast to the high C:N ratio of Napier grass, AW had a low C:N ratio of 4.5 which falls below the recommended ratio. This lower C:N ratio was expected because AW has a higher nitrogen concentration due to the presence of small meat pieces in abattoir waste introducing ammonia in the AW. With a lower C:N ratio, abattoir waste will need to be co-digested to achieve a stable AD process.

Table 4-1: Substrate and inoculum physicochemical characteristics

	AW	RNG	TPN	Inoculum
TS (%) wet basis	22.409	24.2581	15.1111	0.821
VS (%) wet basis	20.485	90.676	88.242	0.798
VS/TS wet basis	0.9141	3.7379	5.8395	0.9719
Ca (mg/kg)	2829	5871	5805	n.d
Cu (mg/kg)	20.62± 0.003	n.d	n.d	n.d
Fe (mg/kg)	1752	103	117	n.d
K (mg/kg)	2944	17794	17638	n.d
Na (mg/kg)	5946	146	197	n.d
Zn (mg/kg)	36.02± 6.63	n.d	n.d	n.d
P (mg/kg)	2304	2322	2283	n.d
C (%)	50.59	45.06	44.7	n.d
N (%)	11.05	2.19	1.91	n.d
H (%)	8.304	6.217	6.989	n.d
S (%)	0.596	BDL	BDL	n.d
C:N ratio	4.5	20.57	23.403	n.d

n.d. = not determined; TS = total solids; VS = volatile solids

The Napier grass and AW both vitally contain micronutrients which are essential for bacterial growth, indicating that the addition of nutrients will not be required when these substrates are used. Even though some elements were present in relatively lower concentrations in the Napier grass, co-digesting it with abattoir waste, which is rich in nutrients, can compensate for the lacking nutrients in Napier grass.

4.5.2 Biomethane potential tests

The biogas yield between TPN and RNG over 31 days is represented in Figure 4.1.

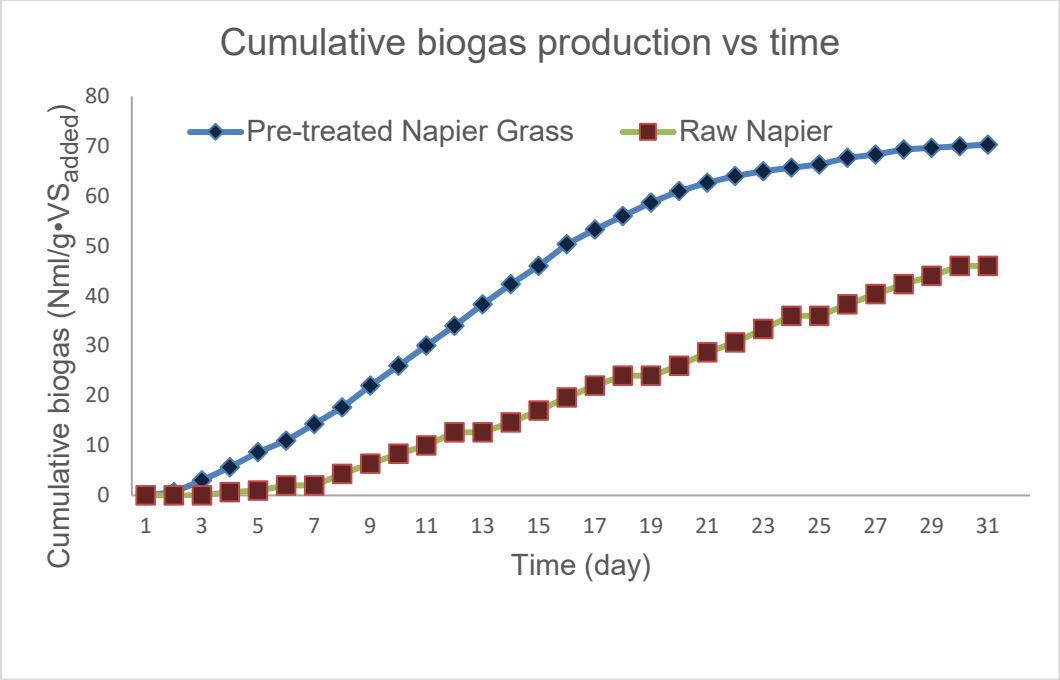


Figure 4-1: Cumulative biogas production of pre-treated and non-pre-treated Napier grass

Biogas production for RNG took five days before there was biogas production, with the maximum biogas produced at the end of 31 days being 46 Nml/g•VS_{added}. Comparing this to the TPN which started producing biogas after day 3 and had maximum biogas of 70.3 Nml/g•VS_{added} shows that while thermal pre-treatment might have the potential to enhance biogas production, this is not conclusive as yet. Dussadee *et al.* (2017) recorded 164.04 and 150.69 Nml/g•VS_{added} from RNG and TPN, respectively. These yields, compared to those obtained in this study, were higher. However, the Napier grass used in the study of Dussadee *et al.* (2017) was first pulverated into particles of 1.0 mm. The pulverisation acted as a pre-treatment stage by increasing the surface area; therefore, improved biogas yields can be expected.

The Napier grass that was used in this study was not pulverated but instead was shredded in a food blender to obtain a slurry. The study by Dussadee *et al.* (2017) showed that thermal pre-treatment was not effective because the thermally pre-treated Napier grass yielded less biogas than the raw Napier grass. This was expected in this case, however, because the decrease in VS content from 81.67% to 68.35% after pre-treatment indicated a loss in organic material. Even though there was still a loss in organic material in this study (see Table 4-1) from 90.68% to 88.25% after pre-treatment, this was not as drastic as in the studies of Dussadee *et al.* (2017).

Because in this study there was still a relatively high organic content which was also broken down by heat for easy access for the microorganisms, higher biogas yields were obtained from thermally pre-treated Napier grass. These results contradict the results of Dussadee *et al.* (2017). They do, however, match with other results where thermal pre-treatment improved the biogas yields using different fibrous substrates (Rodriguez *et al.*, 2017; Maragkaki *et al.*, 2018).

The time taken before biogas was produced by the RNG shows that the microorganisms had difficulty degrading the substrate due to its complex structure. The literature explains that fibrous materials are difficult to hydrolyse and as a result, when such substrates are used in AD, the process might be hindered or will result in little biogas production. Consequently, in this case, little biogas was produced (Ismail & Talib, 2016). Lindmark *et al.* (2012) suggest that fibrous substrate must be pre-treated before AD to eliminate the recalcitrance that is brought about by the structural arrangement of fibrous material. Thus, in this study, TPN produced biogas after day 2, and in addition, produced the highest biogas as compared to RNG. This supports the suggestion of pre-treatment of a substrate before AD.

Figure 4-2 shows the cumulative production of methane of the RNG and TPN. Of the TPN which had the highest biogas production, 72% of that gas was methane. The most desirable gas in biogas is methane, and to date, researchers are exploring ways to enhance the methane yield through different substrates and engineering the AD process so that maximum methane levels are reached (Patinvoh *et al.*, 2017). In this case, TPN has demonstrated the potential to produce higher methane yields as compared to RNG which only produced 61% of methane of the total accumulated gas. However, even though RNG produced the least biogas when compared to TPN grass, the amount of methane produced is still within the range of that which other researchers obtained. This is a clear indication that Napier grass has the potential to be used in AD for biogas production because it can produce good quality biogas, rich in methane.

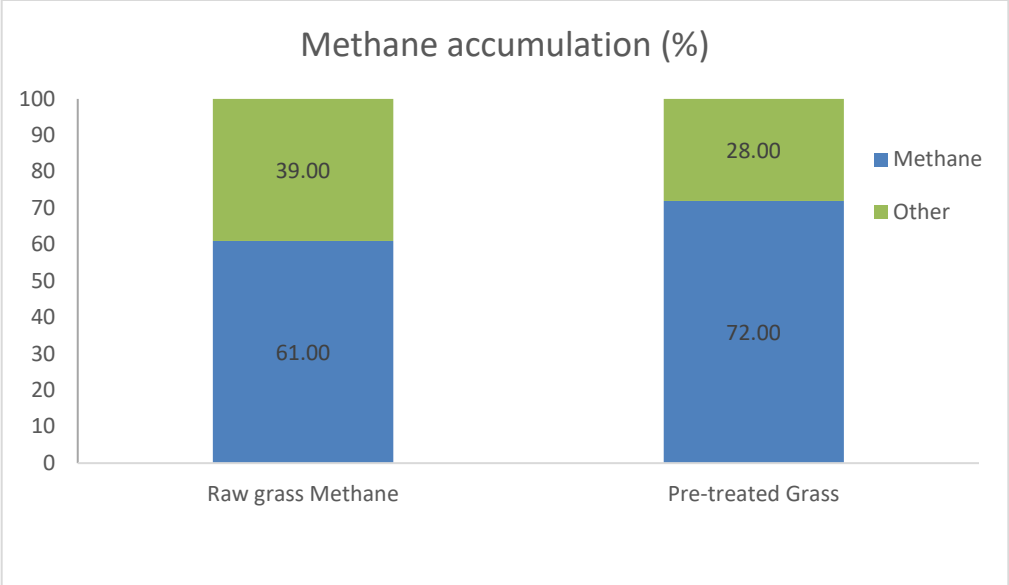


Figure 4-2: Cumulative methane percentage of biogas produced by RNG and TPN after 31 days

4.6 Conclusions and recommendations

It is evident from this objective that not only does pre-treated Napier grass have the potential to produce a high amount of biogas, it also has the potential to produce good quality biogas rich in methane. Physical pre-treatment through the milling of Napier grass has proven to be more effective because when comparing the biogas yields of this current study to those of studies by Dussadee *et al.* (2017) in which the Napier grass was milled, milled Napier grass produced higher biogas yields. Future studies can investigate the use of other physical pre-treatment stages, and if possible, combine these to observe the effect this might have on biogas yield.

CHAPTER 5

5 COMPARISON OF BIOGAS YIELDS BETWEEN DIFFERENT CO-DIGESTION RATIOS OF THERMALLY PRE-TREATED NAPIER GRASS AND ABATTOIR WASTE

5.1 Abstract

AcoD of substrates is often used to overcome the challenge of C:N ratio imbalance in the digester and to enhance biogas production by allowing different substrates to be digested in parallel, thereby allowing a diversity of microorganisms to be active. This study aimed to compare different co-digestion ratios of thermally pre-treated Napier grass (Thermally pre-treated under the same conditions as mentioned in Chapter 3) and abattoir waste. Using a locally designed single-stage batch mesophilic digester, BMP tests were undertaken on TPN co-digested with AW at ratios TPN:AW (100:0, 0:100, 70:30, 50:50 and 30:70). Results showed that the mono-AD of the AW (0:100) produced 23.33 Nml/g•VS_{added} of biogas while that of TP produced 70.33 Nml/g•VS_{added} of biogas after 30 days' retention time at 37^{o}C. When the two substrates were co-digested, the biogas yields were improved by 80% to 117 Nml/g•VS_{added} for the mono-AD of AW a 50:50 ratio while Napier grass increased by 40% at the same conditions. In conclusion, the highest biogas yields in this study were achieved during AcoD of TPN and AW using a substrate ratio of 50:50 at mesophilic conditions for 30 days' retention time. However, the quality of the biogas produced was compromised (a lower methane content) through inhibition when higher concentrations of AW were present in the substrate mixture. This study confirms the importance of deciding on the right co-digestion ratio when employing substrates rich in nitrogen and carbon during anaerobic digestion.

5.2 Introduction

The production of biogas through the AD of organic waste is a promising alternative energy source. Most parts of the world have successfully adapted this technology, while the remaining

parts are still facing challenges with this technology (Nzila *et al.*, 2012). Again, the anaerobic digestion of organic waste is associated with several challenges which have hindered its establishment in certain parts of the world. Among these challenges is imbalance in the C:N ratio (Nzila *et al.*, 2012; Matheri *et al.*, 2017).

The C:N ratio is a ratio intended to gauge the amount of carbon present relative to the amount of nitrogen present in a substrate: the recommended C:N ratio is between 20:30. While both carbon and nitrogen are important in AD, when one becomes more excessive than the other, the process results in little or no biogas production (Matheri *et al.*, 2017; Maragkaki *et al.*, 2018; Jordan & Kell, 2019). This is because when the C:N ratio is above the recommended range, indicating excess carbon, the carbon will result in the accumulation of VFAs, causing a drop in the pH levels. Similarly, if the C:N ratio is below the recommended range, indicating excess nitrogen, the pH levels will increase beyond the desirable pH levels, hindering the activity of the methanogens. In such a case, methane production will be inhibited, resulting in the production of gases such as H_2S (Chen *et al.*, 2008; Matheri *et al.*, 2017).

The AcoD of substrates has been proven to be effective to overcome the issue of C:N ratio. AcoD is when two or more substrates are digested together in one system to produce biogas. Even though AcoD is employed to combat the issues of imbalance in the C:N ratio, another desirable trait is the ability to allow a wider diversity of microorganisms to be present in a system. When more than one substrate is used in a system, that system will have a wider diversity of microorganisms due to the presence of different nutrients in the system (Antonelli *et al.*, 2016).

In this chapter, fibrous and non-fibrous materials were co-digested at different co-digestion ratios to compare the biogas yields. The fibrous material, Napier grass, was thermally pre-treated under the conditions mentioned in Chapter 3 and the non-fibrous material was abattoir waste.

5.3 Aims and objectives

This study aimed to compare biogas production between different co-digestion ratios of fibrous and non-fibrous materials. This aim was achieved by following these objectives:

- Mixing the substrates at different co-digestion ratios in terms of the VS%.
- Using BMP to digest the co-substrate and measure the biogas yields.

5.4 Materials and methods

A detailed description of how the BMPs were set up and the experimental procedure is outlined in section 3.7.3.

5.5 Results and discussion

The biogas yields from the mono and co-digestion of substrates is shown in Figure 5-1. The mono-AD of AW produced very small quantities of biogas of 23.33 Nml/g•VS_{added}. These results were expected because AW had a C:N ratio of 4.5 which is lower than the recommended range of 20-30. Velásquez Piñas *et al.* (2018) explain that a lower C:N ratio indicates an excess amount of nitrogen present in the form of ammonia which causes a rise in pH to 8.5 or higher; this then becomes a toxic environment for the methanogenic bacteria resulting in little or no biogas production. This phenomenon was observed when AW was mono-AD. At the beginning of the test, the pH was adjusted to a pH level of 7, a slight increase in biogas production was observed up until day 12 when the biogas production seized. It can be assumed that the pH adjustment at the start offered a desired pH environment for the microorganisms to produce biogas; moreover, there was still carbon present for the microorganisms to use. However, after day 12, the carbon depleted while the nitrogen remained in excess amounts, causing an increase in the pH levels, consistently inhibiting biogas production.

The mono-AD (100:0) of TPN has proven effective in biogas production with no major inhibition observed, even though hydrolysis recalcitrance remains an issue resulting in little biogas yields, even though high biogas is expected based on the presence of carbon content. Nonetheless, there was still biogas produced.

Abattoir waste, however, is rich in other nutrients that are present in small quantities in Napier grass or absent completely, rendering it suitable as a co-digester with other substrates such as Napier grass.

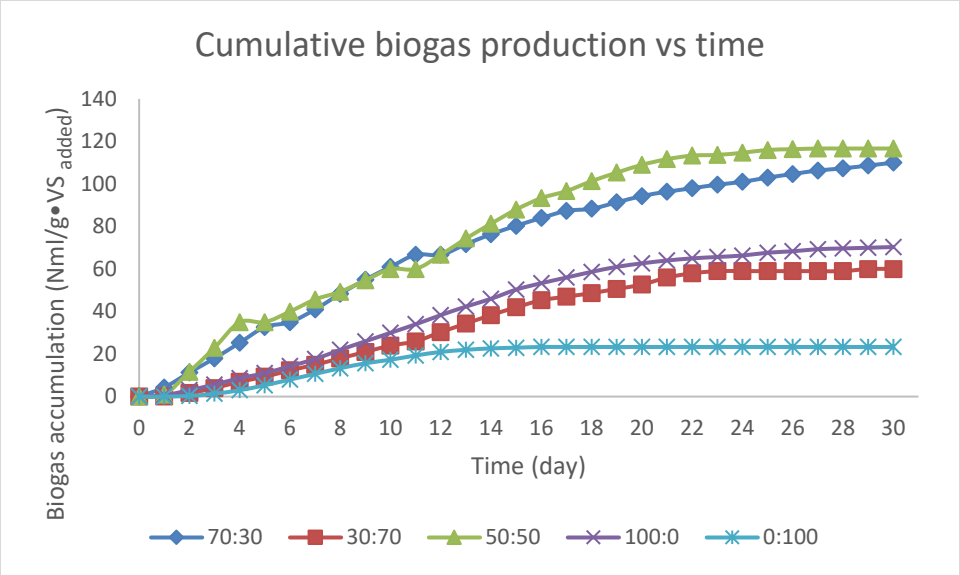


Figure 5-1: Cumulative biogas production of the mono- and co-digestion of thermally pre-treated Napier grass and abattoir waste

The AcoD of AW and TPN have proven effective in biogas production because when the two substrates were co-digested there was improvement in biogas production. The 30:70 co-digestion ratio yielded the lowest biogas of 60 Nml/g•VS_{added}, which can be expected because this AcoD ratio was dominated by AW at 70%. AW contains a lower C:N ratio as already specified, so if it is present at a higher percentage than TPN, there is ammonia accumulation inhibiting biogas production. The presence of TPN at only 30% allowed biogas production to occur from day 2 up until day 22, whereafter biogas production was inhibited. This suggests that the carbon was depleted at this stage and ammonia was accumulating in an excess amount, inhibiting the activity of the methanogen.

At a 70:30 (AW:TPN) AcoD ratio, a significant improvement in biogas yield is observed, with a total of 117 NmL/g•VS_{added}. This observation is due to the higher TPN content (70%). Moreover, adding the AW at a little concentration of 30% allows a larger diversity of microorganisms to be present because of the higher nutrient content of AW (Velásquez Piñas *et al.*, 2018). However, the issue of the C:N ratio is still present, even in this instance, because the C:N ratio of 70:30 and AcoD ratio estimated at 10:11 which is still lower than the recommended ranges. However, for the duration of this test, no inhibition was observed. A possible explanation for these observations is perhaps that the tests were stopped before the carbon content was depleted beyond levels that

result in ammonia accumulation. Looking at the carbon content of the 70:30 co-digestion ratio, it is evident that even though the C:N ratio is below recommended levels, there is still a substantial amount of carbon which allowed the biogas to be produced. Perhaps if the test was allowed to run more than 31 days, the carbon would eventually be depleted and ammonia would accumulate, increasing pH levels and inhibiting biogas production.

The 50:50 co-digestion ratio had the highest biogas yield as compared to all the other mono-AD and AcoD ratios that were studied. Even after day 26, biogas production was inhibited. This inhibition might be a result of the imbalanced C:N ratio because even at the 50:50 co-digestion ratio, the C:N was still lower than the recommended range: it was estimated to have a C:N ratio of 15. However, because this ratio is quite close to the recommended range, this could account for why this AcoD ratio was still a success. When compared to the 70:30 AcoD ratio, the 50:50 AcoD ratio has lower carbon content, which explains why biogas production inhibition was observed after day 26, before the test was stopped. The carbon was depleted which resulted in ammonia accumulation. Despite the imbalance in the C:N ratio, the 50:50 AcoD ratio had the maximum biogas yields of 117 Nml/g•VS_{added}.

Zamanzadeh *et al.* (2017) emphasise that even though the amount of biogas produced is important, the quality of that biogas is of paramount importance. Biogas contains other gasses such as H_2S, CO_2, NH_3, H_2 and N_2, and the quality of biogas can be influenced by the type of feedstock used (Klimiuk *et al.*, 2010). In this study, to improve the quality of the biogas, the CO_2 was scrubbed out using the scrubbing solution, with the other gasses included, as mentioned here, are mainly H_2S, NH_3, H_2 and N_2. The accumulated methane percentage is represented in Figure 5.2.

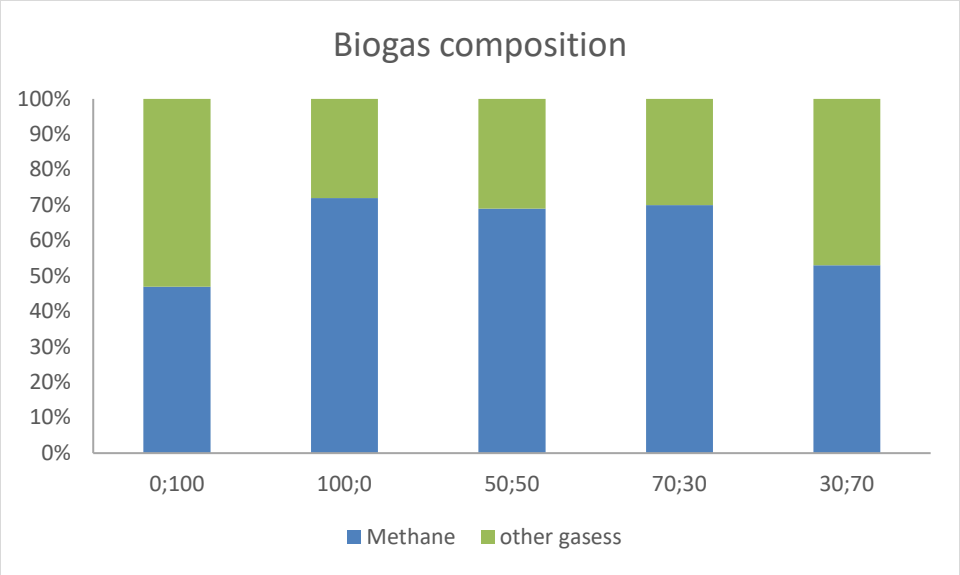


Figure 5-2: Biogas composition of the mono- and co-digestion of substrates

When compared with co-digestion ratios, the 0:100 mono-AD had the lowest methane production of 47%. This implies that only 12.35 CH4 Nml/g•VS_{added} was produced. This can be expected because the total biogas produced from this mono-AD ratio was not satisfactory. As already elucidated, a possible reason for this low biogas yield could be the low C:N ratio which inhibits methanogen activity. If the methanogen activities are inhibited, other microorganisms with different biochemical pathways, such as sulphur reducing bacteria, will be able to establish and thrive, which in this case can explain why there were more other gases (at 53%) than the desired methane. Sulphides are one of the gases that are shown to be common in the anaerobic digestion of abattoir waste; their presence indicating the presence of sulphur reducing bacteria. Sulphur reducing bacteria competes with the methanogenic bacteria and thereby inhibits the activity of methanogenic bacteria (Chen *et al.*, 2008). Moreover, AW contains small meat pieces, introducing amino acids, which when broken down further can result in the accumulation of ammonia gas.

The mono-AD of TPN (100:0) had a 72% methane accumulation, which was the highest methane percentage accumulation when compared to the other AcoD ratios, even though the mono-AD did not yield the highest biogas quantities. This suggests that a higher biogas yield does not equate to a higher methane content.

With the presence of TPN, the quality of the biogas improved. The AcoD ratios with higher TPN percentage resulted in relatively higher methane content, even though when TPN was mono-

digested, it yielded a higher methane content than when co-digested at 50:50, 70:30 and 30:70. This suggests that even though co-digestion with the abattoir waste improves biogas yield, it has a potential negative influence on the quality of biogas produced.

5.6 Conclusions and recommendations

The AcoD of TPN with AW showed evidence of the ability to increase biogas yields; however, the presence of the AW had an undesirable effect on this. The TPN had a C:N ratio that fell within recommended ranges, while the AW had a lower C:N ratio: when these two were co-digested the AW lowered the C:N ratio, and thus brought challenges with the process. But it is undeniable that the presence of AW has some beneficial properties, such as introducing other nutrients in the system and allowing for the presence of a wider diversity of microorganisms.

CHAPTER 6

6 OPTIMISATION OF BIOGAS YIELD

6.1 Abstract

Previous studies in the field of AD have identified the optimum conditions that can lead to the success of AD and the maximum biogas productions. Amongst these conditions are optimum temperature, F/M ratio and AcoD ratio. However, little is known about combining all these factors at different suggested optimum conditions. Moreover, the relationship between these factors is also not well understood. This chapter seeks to determine the optimum temperature, F/M ratio and AcoD ratio that could result in optimum biogas yield when all these factors are considered. CCD was used to find the optimum conditions that would lead to maximum biogas yields using a three-factor level design. DesignExpect version 10.0.0.1 was used to design an experiment with 20 runs using CCD. These experiments were conducted using BMP for 30 days. The obtained results were then fitted into the model and a second order polynomial equation was obtained. The model that was designed was significant. The optimum range was determined to be 35℃ with a 50:50 co-digestion ratio and an F/M of 5 with predicted biogas yields of 188 NmL/g•$VS_{added.}$ The optimised ranges resulted in optimum biogas production. The quadratic equation could be used in future studies when designing AD experiments, as it can act as a prescription when deciding on the operation conditions to yield maximum biogas.

6.2 Introduction

In a quest to find alternative energy sources, scholars have investigated a way to enhance biogas production since it is acknowledged as an alternative energy source. Scientists have investigated all the important parameters in AD such as the AcoD of substrates and the pre-treatment of substrates and have tried to find optimum F/M ratios for yielding maximum biogas.

Using all the suggested optimum ranges that have been investigated in a quest to enhance biogas yields, this chapter uses CCD to optimise three factors to improve biogas yields.

6.3 Aims and objectives

This study aimed to determine the optimum conditions that would result in the highest possible biogas production. The following objectives were followed to achieve this aim:

- use of DesignExpect to design experiment al runs using three factors: temperature, F/M ratio and co-digestion ratio; and
- use of BMP to set up the experiments to determine the biogas yields.

6.4 Materials and methods

A detailed description of the materials used and methods followed is described in section 3.7.3.

6.5 Results and discussion

The experimental values obtained were plotted into a response surface analysis to evaluate the relationship between the chosen factors – temperature (A), co-digestion ratio (B) and F/M ratio (C) – as represented in Table 6-1.

Table 6-1: Coded and actual values of temperature, co-digestion ratio and F/M ratio with their predicted and experimental results

	Coded values			Actual values			Biogas yield NmL• kg VS_{added}	
Run order	A	B	C	A	B	C	Actual Value	Predicted Value
1	0	0	0	36.5	50	3.5	150.00	148.53
2	-1	-1	1	35	30	5	181.00	171.68
3	1	1	-1	38	70	2	78.00	82.59
4	-∞	0	0	33.9773	50	3.5	157.00	171.20
5	0	0	0	36.5	50	3.5	150.00	148.53
6	0	0	0	36.5	50	3.5	150.00	148.53
7	-∞	0	0	39.0227	50	3.5	137.00	115.44
8	0	0	0	36.5	50	3.5	150.00	148.53
9	0	-∞	0	36.5	16.3641	3.5	79.00	70.14
10	0	0	-∞	36.5	50	6.02269	188.00	170.94
11	-1	-1	1	35	30	5	169.00	171.68

12	1	-1	1	38	30	5	101.00	129.97
13	1	1	-1	38	70	2	83.00	82.59
14	0	0	0	36.5	50	3.5	141.00	148.53
15	-∞	0	0	33.9773	50	3.5	165.00	171.20
16	0	0	0	36.5	50	3.5	154.00	148.53
17	0	0	0	36.5	50	3.5	154.00	148.53
18	1	-1	-1	38	30	2	69.00	66.15
19	0	0	0	36.5	50	3.5	154.00	148.53
20	1	1	1	38	70	5	92.00	96.04

Actual results obtained from the experiments were then fitted into a second-order polynomial equation using multiple regression analysis. Equation 10, the resultant of mathematic regression models for biogas production, was determined in terms of coded factors:

$$Biogas = 148.53 - 16.57A - 0.963B + 27.01C - 3.41AB - 7.69AC - 12.59BC - 1.84A^2 - 28.29B^2 - 8.13C^2 \quad [\mathbf{10}]$$

The statistical significance and adequacy of the quadratic model were ascertained from the data obtained by analysis of variance (ANOVA), as illustrated in Table 6-2.

Table 6-2: Variance analysis of the quadratic model for biogas production

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	39883.83	9	4431.54	22.40	< 0.0001	Significant
A	4718.63	1	4718.63	23.85	< 0.0001	
B	11.43	1	11.43	0.0578	0.8125	
C	9712.31	1	9712.31	49.09	< 0.0001	
AB	83.05	1	83.05	0.4198	0.5244	
AC	381.57	1	381.57	1.93	0.1802	

BC	1134.68	1	1134.68	5.74	0.0265
A^2	82.55	1	82.55	0.4173	0.5256
B^2	13189.73	1	13189.73	66.67	< 0.0001
C^2	1090.81	1	1090.81	5.51	0.0293
Residual	3956.87	20	197.84		
Lack of Fit	3614.62	4	903.66	42.25	< 0.0001
Pure Error	342.25	16	21.39		
Cor Total	43840.70	29			

The model F-value of 22.40 is evidence that the model is significant. There is only a 0.01 chance that an F-value this large could occur due to noise. A P-value less than 0.0500 indicates model terms are significant. In this case, A, C, BC, B^2 and C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms, model reduction may improve the model. The lack of fit F-value of 42.25 confirms that the lack of fit is significant. There is only a 0.01% chance that a lack of fit F-value this large could occur due to noise.

Table 6-3: Fit summary for biogas production

Std. Dev.	14.07	R^2	0.9097
Mean	128.90	Adjusted R^2	0.8691
C.V. %	10.91	Predicted R^2	0.3724
PRESS 27513.23		Adeq Precision	12.9958

In the results obtained, the coefficient of determination, adjusted R^2 was found to be 0.8691 which implies that 86.91% of the observed variation in the biogas yield response could be explained by the model. A statistical measure of fit, R^2 ranging from 85% to 100%, indicates that the stock performance moves relatively in line with the index. The predicted R^2 of 0.3724 is not as close to the adjusted R^2 of 0.8691 as required. The difference between the two should be less than 0.2. This may indicate a large block effect. In such a case, model reduction or response transformation should be considered. All empirical models should be tested by undertaking confirmation runs. A ratio greater than 4 is desirable. As the ratio of 12.996 indicates an adequate signal, this model can be used to navigate the design space. The coefficient of variation (CV) expresses the ratio of the standard deviation to the mean. A higher percentage of CV indicates a greater level of dispersion around the mean. Thus, a relatively low percentage (10.91%) is an indication of a more precise estimate. Figure 6-1 shows the proximity between the actual biogas volume values and the predicted ones: the biogas yields were closer to the predicted values.

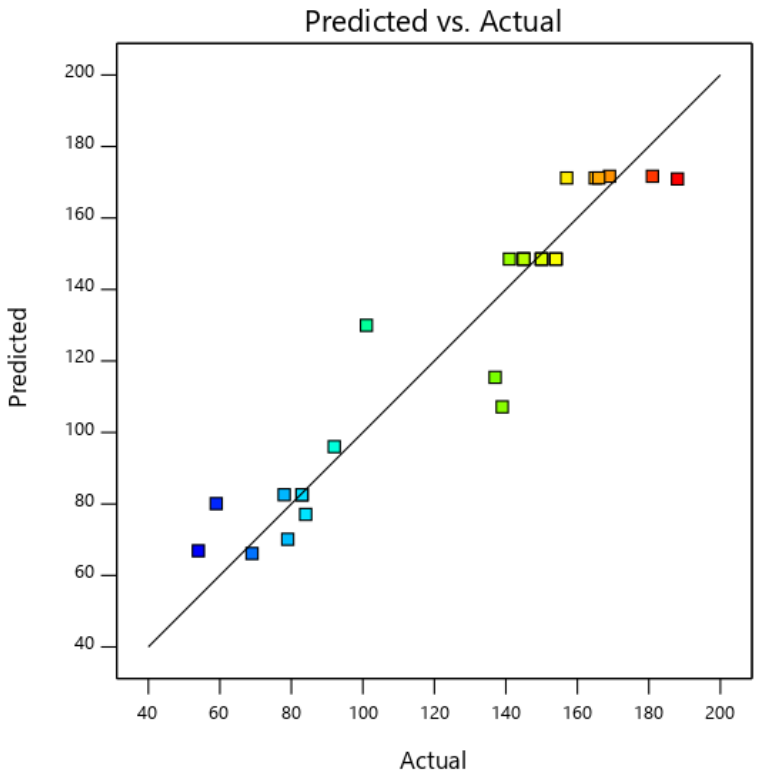


Figure 6-1: Proximity between actual biogas yields and predicted biogas yields

6.5.1 Response surface analysis and interactions among factors

6.5.1.1 Interaction between temperature and substrate co-digestion ratio

The interaction between temperature and substrate co-digestion ratio (illustrated in Figure 6-2) was demonstrated using the 3D response surface and the corresponding contour plot by maintaining the F/M ratio at a central level plot at 3.5 and contrasting the temperature and the substrate AcoD ratio inside the expected experimental range. While the effect of the temperature was statistically significant on the digester's performance, the effect of the AcoD ratio was insignificant. Moreover, when both these factors were combined, the statistical difference was insignificant. Several studies suggest that the operating temperature during AD affects biogas production even though there are contradictions where other scholars suggest that the temperature has a linear effect on biogas production (Chae *et al.*, 2008). Schmidt *et al.* (2019) suggest that although there is rapid degradation at a higher temperature, this results in reduced biogas yields due to the increased inhibition of free ammonia (NH_3) which increases with temperature.

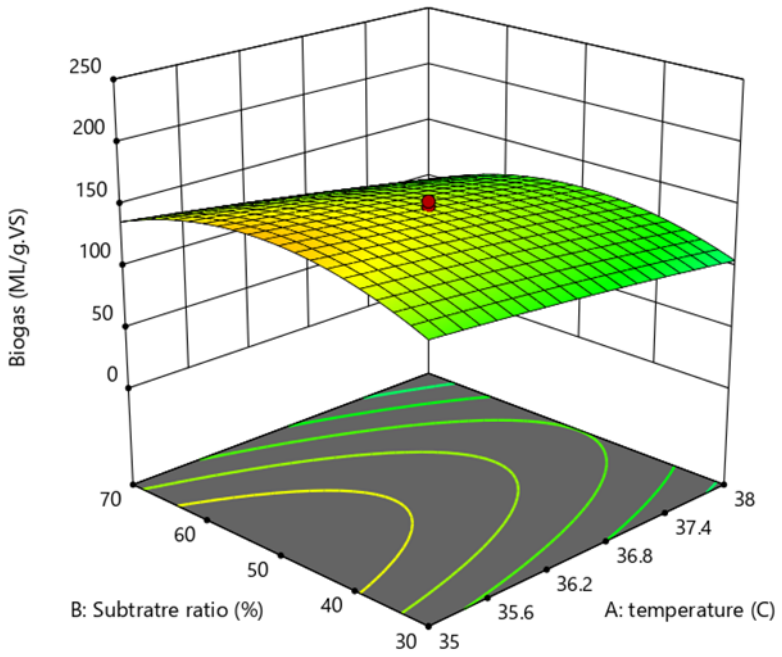


Figure 6-2: Three-dimensional response surface and contour lines of the effect of substrate co-digestion ratio and temperature on biogas yield

Based on the results presented in Figure 6-2, the increase in temperature from 35℃ to 38℃ resulted in the decrease in biogas production from 163.16 mL/g • VS_{added} to100 mL/g • VS_{added} which could verify Schmidt *et al.'s* (2019) suggestions as true. However, the AcoD of substrates is intended to prevent issues such as the accumulation of ammonia by balancing the C:N ratio. Thus, it can be expected that even at higher temperatures, higher biogas yield can still be obtained because the AcoD of the substrate allows a balanced C:N ratio; however, this was not observed in this current study. The maximum biogas yield of 163.16 mL/g • VS_{added} was obtained at 35℃ and 53:47 AcoD ratio, 35℃ was the lowest operation temperature, with 53:47 falling within the mid-point of the AcoD ratio. Maximum biogas yields were achieved at a lower temperature, a good indication for economical purposes because higher temperatures require more energy input, even though literature suggests that higher temperatures increase the rate of reaction.

6.5.1.2 Relationship between F/M ratio and temperature

The interactive effect between the F/M ratio and temperature is shown in Figure 6-3. The darker region (in red) identifies the maximum amount of biogas predicted, 188.826 NmL/vs_{added} at 35℃ with an AcoD ratio of 50:50. According to ANOVA, the F/M ratio and the temperature are both exclusively significant, both having P-values less than 0.0001. However, when both these factors are taken into consideration, they are non-significant because their P-value is greater than 0.1000. As previously mentioned, a higher temperature accelerates the digestion process; however, it is associated with the accumulation of ammonia, which then hinders the process. This can be used as evidence of the low biogas yield at higher temperatures. Shahbaz *et al.* (2019) contend that it is important to maintain a balanced F/M ratio to prevent substrate limitation or organic load overload. Substrate limitation, brought about when there are more microorganisms than substrate, results in a very slow AD process, while an overload in organic load results in total inhibition of microbial activity or at least a longer lag phase for acclimatisation of the microorganisms to substrate.

Maximum biogas yields were obtained at an F/M ratio of 5 which means that there was more substrate (food) than microorganism. This means that the microorganisms will have more food and remain active and multiply, which will subsequently result in better digestion and higher biogas yield. A lower F/M ratio of 2 signifies lesser food (substrate) but more microorganisms,

and in such a case, the food will eventually be depleted, and the microorganism will have nothing to digest: little biogas will be produced. Even though literature recommends an F/M ratio of 2-5 many scholars have worked on an F/M ratio of 2 and have achieved higher yield (Córdoba *et al.*, 2018). In this study though, maximum biogas yields were achieved at an F/M ratio of 5 with a low

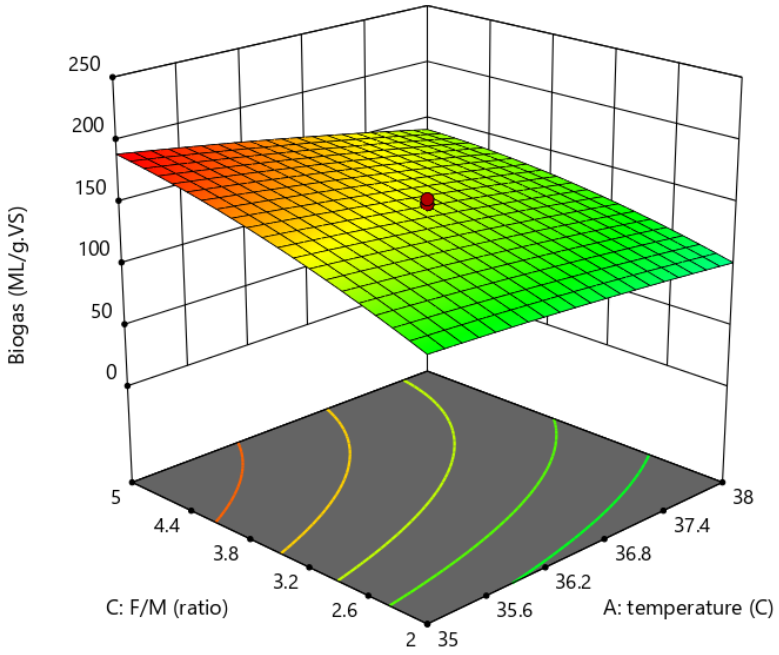


Figure 6-3: Three-dimensional response surface and contour lines of temperature and F/M ratio on biogas production

temperature.

6.5.1.3 Interaction between F/M ratio and substrates ratio

The interaction between the F/M ratio and substrate ratio is presented in Figure 6-4. The substrate co-digestion ratio has a significant effect on the digester with a P-value of 0.0261 which is less than 0.0500. This interaction is the only significant interaction. The benefit of co-digestion is to maintain an optimal pH for the bacteria by providing a better C:N ratio in the substrate and diluting potential toxic compounds as well as allowing a wider diversity of microorganisms to be present. A higher F/M ratio means that there are fewer microorganisms to start with and more food (substrate) present; however, the substrate must have nutrients that are present at desirable concentrations, and thus AcoD issues happen. Even more, because of the abundant nutrients,

even though the starting microorganism is small, a wider diversity of microorganisms will emerge and allow for efficient degradation. In this case, the maximum biogas yield of 169 NmL/gVS $_{added}$ was achieved with a 50:50 AcoD ratio and an F/M ratio of 5, which is the optimum F/M ratio. At a lower AcoD ratio of 30:70, where there is more AW than TPN, and a lower F/M ratio of 2, there was a lower biogas yield. This can be expected because a lower F/M ratio means that more microorganisms are added at the start and because of the abattoir waste, and as the pH is higher, the activity of the microorganisms is inhibited.

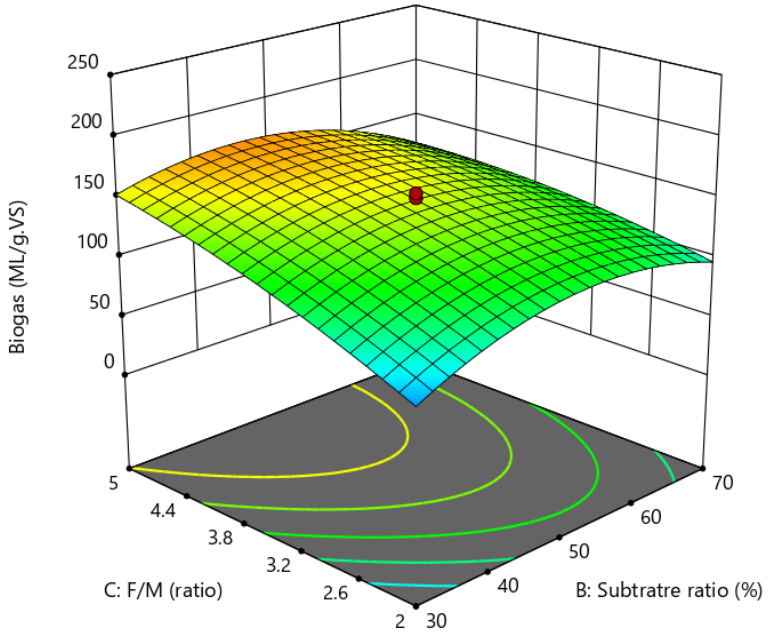


Figure 6-4: Three-dimensional response surface and contour lines of co-digestion substrate ratio and F/M ratio effect on biogas production

6.6 Conclusions and recommendation

Maximum biogas yields were achieved at lower thermal temperatures. This can be supported by the fact that even though higher temperatures have been shown to accelerate the digestion process in other studies, this causes an increase in pH in the presence of nitrogen-rich substrate. Even though AcoD of substrate averts this, in this instance, AcoD had no effect because maximum biogas yields were obtained at lower temperatures. The most effective AcoD ratio was 50:50 AW:

TPN while maximum yields of 188 NmL/kVS$_{added}$ were achieved at an F/M ratio of 5, an AcoD ratio of 50:50 and a mesophilic temperature of 35℃.

Perhaps future studies should study the effect of different inoculums instead of just one inoculum. Another parameter that could be studied concerns the retention time since scholars suggest that a higher temperature encourages a fast digestion process.

CHAPTER 7

7 UP-SCALING IN A 5L SINGLE-STAGE BATCH DIGESTER

7.1 Abstract

BMP tests are traditionally employed as a tool to evaluate the potential of a particulate substrate or substrates to produce biogas under set conditions. BMP tests alone do not generate sufficient evidence to conclude that a particular substrate and set conditions can be used commercially to produce biogas; thus, one measure to test the feasibility of a substrate to be digested under set conditions would be to up-scale it, thereby allowing the process to take place at a bigger scale. This chapter intends to test the feasibility of the optimised conditions determined when the volume was scaled up. The optimised conditions from Chapter 6, with the following conditions – AcoD ratio of AW:TPN at 50:50; F/M ratio of 5; and 35℃ – was scaled-up in a 5 L single-stage batch digester with an operation volume of 4.5 L for 30 days. The pH was monitored and adjusted daily with the addition of HCL or NaOH, depending on the initial pH level. The total biogas accumulation was 1830 NmL/g•VS_{added} with 1334.05 NmL/g•VS_{added} as methane. The up-scaling of the optimised conditions resulted in the success of the process even though the yields were lower than the predicted ones. Nonetheless, biogas was still produced in satisfactory quantities. This study determined that even when volumes are scaled up, the success of AD can still be achieved.

7.2 Introduction

Most biological processes work at a laboratory scale but as soon as the volumes are scaled up, challenges are usually encountered. This is because bigger volumes mean more of the substrate and if the substrate has toxic compounds that would not be detrimental at a smaller scale, they would be detrimental at a larger scale. These observations are true even in AD. To solve the energy crisis biogas would need to be produced at a larger scale, and thus it is important to evaluate the feasibility of producing biogas at larger scale to meet the energy demands. In this chapter, the optimised conditions are up-scaled to observe the effect of bigger volumes.

7.3 Aims and objectives

The aim was to evaluate the feasibility of optimised conditions on a larger scale. The aim was achieved through the following objective:

- use a 5 L single-stage batch digester to simulate the optimum conditions and allow the system to operate for 30 days with continuous adjustment monitoring of the pH level.

7.4 Materials and methods

A detailed description of the steps followed is found in section 3.8.

7.5 Results and discussion

The optimised conditions – 50:50 AcoD ratio, F/M ratios at 5, and an operating temperature of 35℃ with a predicted output of 188 NmL/gVS_{added} – were further simulated in triplicate in the BMP test and the actual output, the biogas yield, was 172 NmL/$gVS_{added.}$ Such small discrepancies are expected when working with microorganisms because it is not feasible to always have the same microorganisms that possess the same qualities in every test. A little variation in the microorganisms can be expected and as a result, a variation in the process as well.

The optimised condition was further up-scaled, with results presented in Table 7-1.

Table 7-1: Daily data of optimum conditions for up-scaling system

Day	Biogas in Nml.VS $_{added}$	Methane yield	Temperature ℃	pH
1	0	0	35	7,01
2	0	0	35	7,01
3	0	0	35,1	7
4	62	43,4	35,1	7,1
5	62	43,4	35	7
6	70	49	35	6,98
7	75	52,5	34,9	6,98
8	75	52,5	34,9	6,89
9	74	51,8	35	6,88
10	70	49	35	6,88

11	75	52,5	35	6,89
12	76	53,2	35	6,98
13	80	56	34,9	6,97
14	81	56,7	35,2	6,98
15	79	59,25	35,2	6,97
16	78	58,5	35	7
17	80	60	35	7
18	80	60	35	6,98
19	75	56,25	34,9	6,99
20	74	55,5	34,9	6,97
21	74	55,5	34,9	7
22	60	45	35	7
23	59	44,25	35	7,02
24	58	43,5	34,9	6,98
25	58	43,5	35,3	6,99
26	50	37,5	35	6,99
27	50	37,5	35	6,98
28	51	38,76	35,1	6,98
29	52	39,52	35	6,97
30	52	39,52	35	6,97
Total	1830	1334,05		

While biological processes have a trend of working efficiently on small scales, as soon as the volumes are increased, their pattern is one of minimal success, or in some instances, complete failure. Esteban-Gutiérrez *et al.* (2018) explain that one possible reason for this is that certain substances which would not be toxic in small volumes can be toxic when the volumes are increased due to the excess of such substances when the volumes are increased. It has already been mentioned that AB contains nitrogen in the form of ammonia, and when ammonia accumulates, this increases pH levels and hinders the process. According to the results in Table 12, the maximum biogas obtained was 1830 Nml/kg.VS_{added} ;however, based on mathematical estimation, the total biogas yield was supposed to be 169 200 NmL/kg.$VS_{added.}$. The obtained

value was lower than the estimated value, which agrees the findings of Esteban-Gutiérrez *et al.* (2018).

Napier grass, due to the presence of lignocellulose, is associated with VFA accumulation which when present in higher concentrations can bring process failure. A larger volume allows more lignocellulose to be present and thus the issue of VFA accumulation can be expected. The pH levels, in this case, suggest a factor that constantly caused the pH levels to drop below pH 7. It can be suggested that VFAs were the leading cause of the pH drop; however, the presence of ammonia prevented the drop of the pH level to an acidic level. This suggests that the co-digestion of the substrate is significant because it solves several substrate-associated issues.

7.6 Conclusions and recommendations

Up-scaling of the optimised factors was feasible because biogas was produced under such conditions; however, the produced biogas was not the estimated biogas yield. The AcoD of the fibrous and non-fibrous substrate also aided in maintaining the pH during the test, preventing a drop or increase in pH levels.

Instead of up-scaling only the optimised condition, it would be beneficial to also up-scale the condition that yielded the lowest and the middle biogas volumes to compare their feasibility in up-scaled volumes.

CHAPTER 8

8 COMPARISON OF BACTERIAL COMMUNITY STRUCTURE PRESENT IN AD

8.1 Abstract

While most of the world is finding AD attractive, and more research is underway to optimise the process, little is known concerning the microbial community that is integral in the process. This chapter aimed to compare the bacterial community at the start of the process (day 1) and the end of the process (day 30). An inoculum sample, collected on day 1 and day 30 of the experiment, was sent to Inqaba Biotech laboratory for 16s rRNA analysis to analyse and compare the different bacterial communities present. The bacterial phylum that was present showed consistency, though there was a shift in the read count, showing a decrease in the bacterial community as the process progressed. The dominating species present on day 1 and day 30 were unknown. Bacteria that are commonly known for their ability to degrade macromolecules were present, though in minority quantities. In addition, anaerobic bacteria were also observed as present, an indication that the process was anaerobic. This study has highlighted that the bacterial community present at the start of the process is most likely to remain constant throughout the process though a shift in the read count can be observed. In addition, this study has opened a door for future research, as unknown species could be carefully studied and classified. The bacterial community that is commonly known for their ability to degrade macromolecules could also be studied in an attempt to render them the most dominating species in the process. Better knowledge and understanding of the bacterial community involved in AD could ease the optimisation of the AD in future ventures.

8.2 Introduction

Researchers in the AD field have investigated various parameters that can be optimised in an AD process such as temperature and AcoD ratios. Even though a few researchers have managed to explore the microorganisms that are involved in the process in the quest to optimise the AD process, there are as of yet no consistent results in terms of present microorganisms. These discrepancies are due to the different sources of inoculum that are employed, the different

substrates that are used, and the different operating temperatures. This study thus investigates and compares the different bacteria that were involved when conditions were scaled-up to a 5 L single-stage reactor.

8.3 Aims and objectives

This study aimed to investigate and compare the different bacteria that were involved when TPN was co-digested with AW in a 5 L single-stage bioreactor from day 1 and day 2 inoculum. The aim was achieved through the following objective:

Inoculum samples were collected on days 1 and 30 of the experiment and sent to Inqaba Biotech Laboratory for metagenomic analysis.

8.4 Materials and methods

A detailed description of the steps followed is found in section 3.8.

8.5 Results and discussion

The comparison of the top phylum classification between day 1 and day 30 in a 5 L single-stage reactor is outlined in Table 8-1. While phylum classification in both samples (day 1 and day 30) was relatively similar, a variation was observed in the read count and thus the percentage composition.

During AD, different microorganisms play different roles in different stages of the process, so each stage of the process is expected to have different microorganisms. It is important to note that the four stages in AD are non-linear, but rather they occur in parallel. The results in phyla comparison between day 1 and 30 show that even though the process was at different stages, some of the phyla were consistent in both days reckoning that the stages occur parallel.

The *Bacteroidetes*, the most dominant phylum on both day 1 and day 30, are gram-negative rod-shaped bacteria that can be both anaerobic and aerobic. These bacteria are highly abundant in intestines, playing a significant role in metabolic conversions such as the degradation of complex sugar polymers and protein. This explains why *Bacteroidetes* were dominant in this AD process because the root of AD is the degradation of substances, and thus bacteria that are heavily involved in degradation are expected to be present in abundance to ensure the success of the AD process. The presence and dominance of *Bacteroidetes* on both day 1 and day 30 suggests

that degradation of the substrate not only takes place at the start of the process, but continues through the process in parallel with other processes. In addition, *Bacteroidetes* were the dominating phylum on day 30, moving from 28.81% on day 1 to 41.62% on day 30.

The *Firmicutes* were also present in abundance, but only at the start of the process (day 1); after day 30, these decreased in read count, although they were still the second dominating phylum percentage on day 30.

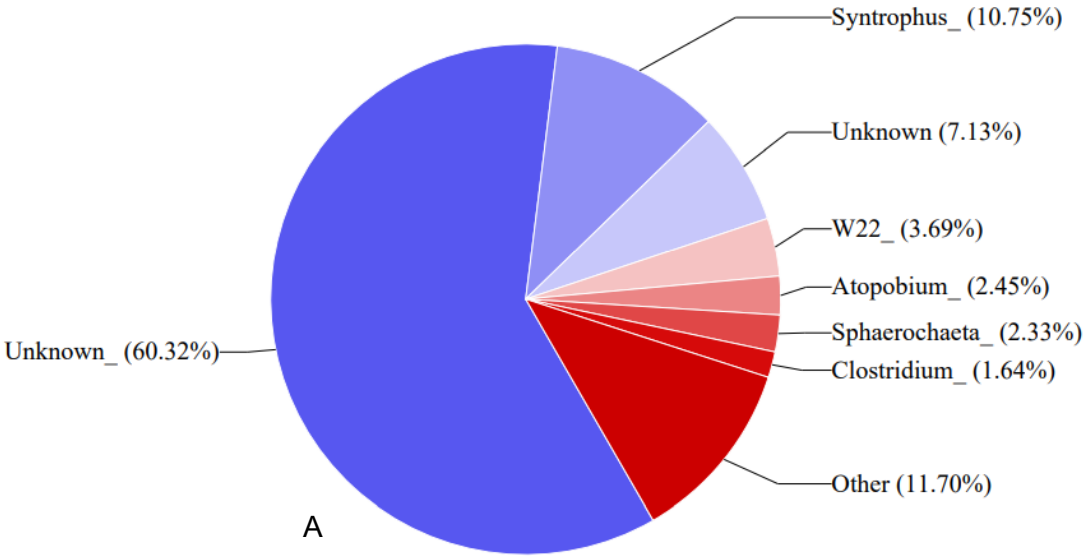
The bacterial population on the two different days showed a decline in the read count, where day 1 had more bacterial read count as compared to day 30. This may suggest that as the substrate decreases, so does the bacterial community because on day 1 the substrate was present in an adequate amount, but as the process progressed the substrate was converted to biogas and a lesser substrate was available for degradation, causing a decrease bacterial read count.

Table 8-1: Comparison of top phylum classification for day 1 and day 30

Phyla Classification	Read Count		%	
	Day 1	Day 30	Day 1	Day 30
Bacteroidetes	2179.0	2069.0	28.81	41.62
Firmicutes	2023.0	1792	26.75	36.05
Proteobacteria	1749.0	573	23.13	11.53
Actinobacteria	302.0	14	3.99	0.28
WWE1	287.0	215	3.79	4.33
Verrucomicrobia	249.0	6.0	3.29	0.12
Spirochaetes	247.0	53.0	3.27	1.07
Synergistetes	181.0	41.0	2.39	0.82
Unknown	65.0	43.0	0.86	0.87
Chloroflexi	54.0	52.0	0.71	1.05
Lentisphaerae	49.0	2.0	0.65	0.04
Thermotogae	46.0	6.0	0.61	0.12
OP9	34.0	4.0	0.45	0.08
Tenericutes	29.0	45.0	0.38	0.91
Planctomycetes	21.0	19.0	0.28	0.38
OP11	13.0	0.0	0.17	0.0

WS1	8.0	1.0	0.11	0.02
OD1	8.0	0.0	0.11	0.0
WPS	6.0	0.0	0.08	0.0
Cyanobacteria	4.0	12.0	0.05	0.24
LD1	2.0	0.0	0.03	0.0
Armatimonadetes	2.0	15.0	0.03	0.30
WS6	2.0	0.0	0.03	0.0
TM7	1.0	0.0	0.01	0.0
SR1	1.0	0.0	0.01	0.0
NKB19	1.0	1.0	0.01	0.02
Fibrobacteres	0	7.0	0	0.14
Acidobacteria	0	1.0	0	0.02

The top species classification on day 1 and day 30 is outlined in Figure 8-1, comparing the different species that were present on day 1 and day 30.



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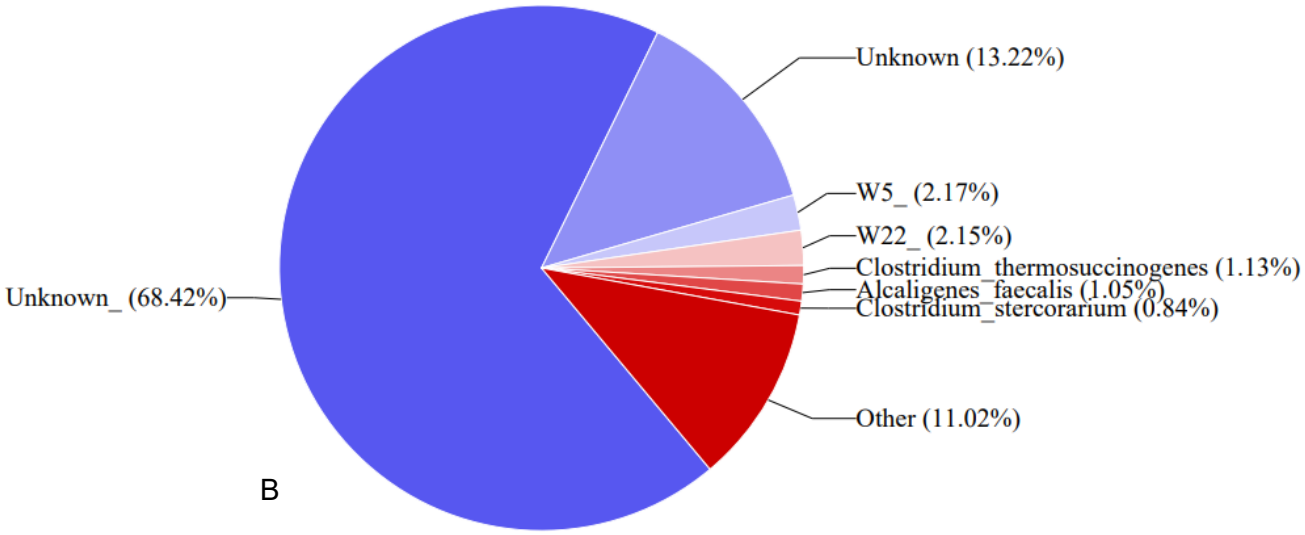

Figure 8-1: Top species classification chart for microbial population on day 1 (A) and day 30 (B)

While some parts of the world are adopting AD and substantial research has been done to optimise the process, there is a dearth of published research on the microbial species that are involved in AD. From the microbial analysis from days 1 and 30 of this current study, 67.45% and 81.64%, respectively, were classified as unknown species. This comprises most of the species that were present in the process.

A huge variation is observed in the species present from days 1 and 30. Day 1 had 10.75% *Syntrophus* species, a species that comes from the Phylum *Proteobacteria*, which are obligate anaerobic bacteria. The presence of this species suggests that the conditions other than at which the process was operating were anaerobic.

W22, accounting for 3.69% and 2.15% on day 1 and day 30, respectively, and based on 16S rRNA, is closely related to species of the genus *Chryseobacterium* with 87.5% to 90.2% similarity within the family *Flavobacteriaceae*. The *Flavobacteriaceae* is commonly known for its ability to degrade various biomacromolecules such as protein and polysaccharides. The presence of this species suggests that degradation was occurring, likely driven by this species; however, ideally,

to allow efficient degradation, this species should be present in relatively large amounts as they are integral to the degradation of the substrate.

8.6 Conclusions and recommendations

The phyla that were present in the process showed consistency on both day 1 and day 30 even though there was a discrepancy in the read count, suggesting that the bacterial count is affected by the stage of the process. For instance, on day 1 more bacterial counts were recorded, but as the process progressed and the substrate lessened, lower bacterial counts were recorded. Species that are known for their ability to degrade large macromolecules were present, suggesting that degradation did take place. Most of the species that were present in this process were unknown species. This could open a new door into research wherein these unknown species could be scrutinised and classified, perhaps even engineered to produce optimum biogas.

CHAPTER 9

9 CONCLUSIONS AND RECOMMENDATIONS

9.1 Introduction

This chapter highlights important findings from each set objective, drawing conclusions based on those findings. In addition, recommendations for future studies are proposed.

9.1.1 Comparison of biogas yield between thermally pre-treated and non-thermally pre-treated Napier grass

While the thermal pre-treatment of Napier grass reduces the VS%, this approach has demonstrated the ability to improve the efficiency of AD and thereby improve biogas production. It appears that the thermal pre-treatment reduced the VS content because the RNG had a VS% of 90.676 and after thermal pre-treatment, the VS% was 88.242% suggesting a loss in VS during heat pre-treatment. However, TPN still produced the highest biogas yield of 70.3 Nml/g•VS_{added} while RNG only produced 46 Nml/g•VS_{added}. The thermal pre-treatment approach not only improves the biogas yield but also improves the total methane production from 61% in RNG to 72% in TPN. The Napier grass used was not subjected to physical pre-treatment such as pulverisation. Thus, when the results obtained are compared to literature of studies with pulverised Napier grass, findings shows that pulverisation coupled with thermal pre-treatment also improves biogas yield.

9.1.2 Compare biogas yield between different co-digestion ratios of abattoir waste and Napier grass.

The co-digestion of TPN and AW succeeded in improving the biogas yield even though in some ratios the C:N ratio was not balanced. However, it was not far from the recommended ratio ranges of 20-30. Not only did the AcoD ratio of 50:50 yield the highest biogas, it also resulted in the highest methane production. The 70:30 TPN:AW produced the second-highest biogas yield. In contrast, the 30:70 AcoD produces the least biogas and the least methane production. While AW proved to have the potential to co-digest fibrous material, an undesirable trend was observed: when the AW is dominating, it causes inhibits biogas production as well as methane yield. In

cases where the C:N ratio is imbalanced, inhibition does not occur at the start of the process but rather after a couple of days. It can be assumed that this is after the one substrate is depleted and the other one is present in excess.

9.1.3 Optimisation of biogas production using response surface methodology (RSM)

The design model obtained is competed enough to be used within the design space. Moreover, the actual biogas yield values were very close to the predicted biogas yield values. Maximum biogas yields were achieved at the lowest thermal temperature of 35℃, an F/M ratio of 5 and AcoD ratio of 50:50. But these factors are not the only factors with significant roles in AD. Other factors such as the retention time and pH warrant evaluation. Thus, instead of a three-factor level design, a four or five-factor level design could be evaluated.

9.1.4 Up-scaling in a 5L single-stage batch digester

Even though the obtained biogas yields were not the same as the predicted biogas yield, up-scaling of the optimised factors was feasible because biogas was still produced. AcoD of substrates allowed the pH level to remain constant. Future studies could compare the biogas yields from at least the top three conditions that resulted in maximum biogas yield from the optimised conditions.

9.1.5 Microbial population dynamics during co-digestion of abattoir waste and Napier grass

While there might be variation in the bacterial community present at the different stages of the process, the most dominant species, are always present in dominancy in all the stages. The substrate concentration might influence the bacterial count because it was observed that at the beginning of the process, while the substrate was still present in a relatively high concentration, the bacterial count was high; as the process progressed, the bacterial count decreased. Lastly, a consortium of anaerobic bacteria was present suggesting that the process was indeed anaerobic, even though aerobic bacteria were still present, suggesting that the bacterial community present during AD is not exclusively anaerobic bacterial. Even facultative aerobic bacterial can be present. As bacterial species are not the only microbial community present during AD, though they appear to be the most dominant, future studies could investigate other microbial communities present during AD. In addition, it would be necessary to study and understand the complex biochemical

pathways that microorganisms undergo. Perhaps future studies could attempt to engineer the microorganisms (the enzymes involved) so that instead of having a consortium of microorganisms, these might in turn offer competition to each other, using lesser microorganisms engineered with all the enzymes. Future studies could also evaluate the current findings on other fibrous and non-fibrous material other than Napier grass and abattoir waste.

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APPENDICES

APPENDIX A: Raw data from BMP tests

Table A1: Average biogas and methane yield for Abattoir waste

Day	Average biogas yield per day (ml)	Average methane yield weekly (ml)
1	0	0
2	0	
3	0.33	
4	1	
5	1.67	
6	2.33	2.67
7	2.67	
8	2.67	
9	2.67	
10	2.33	
11	1.67	
12	2	5.67
13	1.67	
14	0.33	
15	0.33	
16	0	
17	0	
18	0	0.67
19	0	
20	0	
21	0	
22	0	
23	0	
24	0	
25	0	0
26	0	
27	0	
28	0	
29	0	
30	0	
31	0	0

Table A2: Average biogas and methane yield for raw Napier grass

Day	Average biogas yield per day (ml)	Average methane yield weekly (ml)
1	0	0
2	0	
3	0	
4	0.67	
5	0.33	
6	1	
7	0	1
8	2.33	
9	2	
10	2	
11	1.67	
12	2.67	
13	0	10
14	2	
15	2.33	
16	2.67	
17	2.33	
18	2	
19	0	13.67
20	2	
21	2.67	
22	2	
23	2.67	
24	2.67	
25	0	
26	2.33	18
27	2	
28	2	
29	1.67	
30	2	
31	0	21

Table A3: Average biogas and methane yield for thermally pre-treated Napier grass

Day	Average biogas yield per day (ml)	Average methane yield weekly (ml)
1	0	0
2	0.66	
3	2.33	
4	2.66	
5	3	
6	2.3	
7	3.3	
8	3.3	2
9	4.3	
10	4	
11	4	
12	4	
13	4.33	
14	4.	
15	3.66	11
16	4.33	
17	3	
18	2.66	
19	2.66	
20	2.33	
21	1.66	25
22	1.33	
23	1	
24	0.66	
25	0.66	
26	1.33	
27	0.66	
28	1	
29	0.33	
30	0.33	
31	0.33	11

Table A4: Average biogas and methane yield for AcoD 1:1 ratio AW:TPN

Day	Average biogas yield per day (ml)	Average methane yield weekly (ml)
1	0	0
2	1	
3	11	
4	11	
5	12	
6	0	
7	5	26.67
8	6	
9	4	
10	5	
11	5	
12	6	
13	4	
14	5	18.33
15	5	
16	0	
17	7	
18	8	
19	7	
20	7	19.33
21	3	
22	5	
23	4	
24	4	
25	3	
26	2	
27	0	
28	1	
29	0	
30	0	
31	0	10.33

Table A5: Average biogas and methane yield for AcoD 1:2 ratio AW:TPN

Day	Average biogas yield per day (ml)	Average methane yield weekly (ml)
1	0	0
2	0	
3	2	
4	2	
5	3	
6	2	
7	3	5
8	3	
9	3	
10	3	
11	3	
12	2	
13	4	16
14	4	
15	4	
16	4	
17	3	
18	2	
19	2	
20	2	28
21	2	
22	1	
23	1	
24	0	
25	0	
26	0	34
27	0	
28	0	
29	0	
30	0	
31	0	34

Table A6: Average biogas and methane yield for AcoD 2:1 ratio AW:TPN

Day	Average biogas yield per day (ml)	Average methane yield weekly (ml)
1	0	0
2	4	
3	7	
4	7	
5	7	
6	7	
7	7	25
8	6	
9	7	
10	7	
11	6	
12	6	
13	0	27
14	5	
15	5	
16	4	
17	4	
18	3	
19	2	12
20	3	
21	2	
22	2	
23	2	
24	2	
25	2	9
26	2	
27	2	
28	2	
29	1	
30	1	
31	1	3

APPENDIX B: Raw data for biogas optimisation

Table B1: DesignExpect showing actual value vs. predicted value

coded values			real values					
A	B	C	A	B	C	Run Order	Actual Value	Predicted Value
0	0	0	36.5	50	3.5	1	150.00	148.53
-1	-1	1	35	30	5	2	181.00	171.68
1	1	-1	38	70	2	3	78.00	82.59
-∞	0	0	33.9773	50	3.5	4	157.00	171.20
0	0	0	36.5	50	3.5	5	150.00	148.53
0	0	0	36.5	50	3.5	6	150.00	148.53
-∞	0	0	39.0227	50	3.5	7	137.00	115.44
0	0	0	36.5	50	3.5	8	150.00	148.53
0	-∞	0	36.5	16.3641	3.5	9	79.00	70.14
0	0	-∞	36.5	50	6.02269	10	188.00	170.94
-1	-1	1	35	30	5	11	169.00	171.68
1	-1	1	38	30	5	12	101.00	129.97
1	1	-1	38	70	2	13	83.00	82.59
0	0	0	36.5	50	3.5	14	141.00	148.53
-∞	0	0	33.9773	50	3.5	15	165.00	171.20
0	0	0	36.5	50	3.5	16	154.00	148.53
0	0	0	36.5	50	3.5	17	154.00	148.53
1	-1	-1	38	30	2	18	69.00	66.15
0	0	0	36.5	50	3.5	19	154.00	148.53
1	1	1	38	70	5	20	92.00	96.04
0	0	0	36.5	50	3.5	21	145.00	148.53
-1	1	-1	35	70	2	22	139.00	107.18
1	1	-1	38	70	2	23	83.00	82.59
-1	-1	-1	35	30	2	24	84.00	77.10
0	-∞	0	36.5	83.6359	3.5	25	54.00	66.90

0	0	-∞	36.5	50	0.977311	26	59.00	80.10
-∞	0	0	33.9773	50	3.5	27	166.00	171.20
0	0	0	36.5	50	3.5	28	145.00	148.53
0	0	0	36.5	50	3.5	29	145.00	148.53
0	0	0	36.5	50	3.5	30	145.00	148.53

APPENDIX C: Bacterial analysis data

Table C1: Phylum classification on day 1

Phyla Classification	Read count	%
Bacteroidetes	2179.0	28.81
Firmicutes	2023.0	26.75
Proteobacteria	1749.0	23.13
Actinobacteria	302.0	3.99
WWE1	287.0	3.79
Verrucomicrobia	249.0	3.29
Spirochaetes	247.0	3.27
Synergistetes	181.0	2.39
Unknown	65.0	0.86
Chloroflexi	54.0	0.71
Lentisphaerae	49.0	0.65
Thermotogae	46.0	0.61
OP9	34.0	0.45
Tenericutes	29.0	0.38
Planctomycetes	21.0	0.28
OP11	13.0	0.17
WS1	8.0	0.11
OD1	8.0	0.11
WPS	6.0	0.08
Cyanobacteria	4.0	0.05
OD1	8.0	0.11
WPS	6.0	0.08
LD1	2.0	0.03

Armatimonadetes	2.0	0.03
WS6	2.0	0.03
TM7	1.0	0.01
SR1	1.0	0.01
NKB19	1.0	0.01

Table C2: Phylum classification on day 30

Phyla Classification	Read count	%
Bacteroidetes	2069.0	41.62
Firmicutes	1792.0	36.05
Proteobacteria	573.0	11.53
WWE1	215.0	4.33
Spirochaetes	53.0	1.07
Chloroflexi	52.0	1.05
Tenericutes	45.0	0.91
Unknown	43.0	0.87
Synergistetes	41.0	0.82
Planctomycetes	19.0	0.38
Armatimonadetes	15.0	0.30
Actinobacteria	14.0	0.28
Cyanobacteria	12.0	0.24
Fibrobacteres	7.0	0.14
Thermotogae	6.0	0.12
Verrucomicrobia	6.0	0.12
OP9	4.0	0.08
Lentisphaerae	2.0	0.4
NKB19	1.0	0.02
Acidobacteria	1.0	0.02
WS1	1.0	0.02

Table C3: Class classification on day 1

Class	Read count	%
Bacteroidia 2165.0 28.63	2165.0	28.63
Clostridia 1959.0 25.90	1959.0	25.90
Deltaproteobacteria	931.0	12.31
	567.0	7.50
Betaproteobacteria	358.0	4.73
Coriobacteriia	269.0	3.56
Spirochaetes	246.0	3.25
Epsilonproteobacteria	201.0	2.66
Gammaproteobacteria	195.0	2.58
Synergistia	181.0	2.39
Unknown	94.0	1.24
Alphaproteobacteria	56.0	0.74
Thermotogae	46.0	0.61
Anaerolineae	46.0	0.61
Erysipelotrichi	44.0	0.58
Verruco	41	0.54
Actinobacteria	32.0	0.42
RF3	24.0	0.32
OPB46	24.0	0.32
Planctomycetia	19.0	0.25
OP11	11.0	0.15
Dehalococcoidetes	8.0	0.11
JS1	8.0	0.11
OPB54	7.0	0.09
Flavobacteriia	5.0	0.07
Mollicutes	4.0	0.05
4C0d	4.0	0.05
Bacilli	3.0	0.04
WCHB1	2.0	0.03
SJA	2.0	0.03
SC72	2.0	0.03
Phycisphaerae 2.0 0.03	2.0	0.03
MVP	1.0	0.01

Verrucomicrobiae	1.0	0.01
AHT28	1.0	0.01
Sphingobacteriia	1.0	0.01
Acidimicrobiia	1.0	0.01
VC2_1_Bac22	1.0	0.0

Table C4: Class classification on day 30

Class	Read count	%
Bacteroidia 2057.0 41.38	2057.0	41.38
Clostridia	1702.0	34.24
Betaproteobacteria	507.0	10.20
	222.0	4.4
Unknown	91.0	1.83
Spirochaetes	53.0	1.07
Anaerolineae	51.0	1.03
RF3	42.0	0.84
Synergistia 41.0 0.82	41.0	0.82
OPB54	33.0	0.66
Bacilli	29.0	0.58
Epsilonproteobacteria	23.0	0.46
Gammaproteobacteria	19.0	0.38
Phycisphaerae	17.0	0.34
Deltaproteobacteria	15.0	0.30
SJA	12.0	0.24
4C0d	11.0	0.22
Actinobacteria	8.0	0.16
Alphaproteobacteria	8.0	0.16
TG3	7.0	0.14
Coriobacteriia	6.0	0.12
Thermotogae	6.0	0.12
Verruco	2.0	0.04
TSBW08	1.0	0.02
Solibacteres	1.0	0.02
OPB46	1.0	0.02
Dehalococcoidetes	1.0	0.02

Mollicutes	1.0	0.02
JS1	1.0	0.02
Cytophagia	1.0	0.02
Verrucomicrobiae	1.0	0.02
Planctomycetia	1.0	0.02