Cape Peninsula University of Technology

DEVELOPMENT AND BENCH-SCALE OPTIMISATION OF A REACTOR SYSTEM FOR RUMEN-BASED ANAEROBIC DIGESTION

By

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DECLARATION

I, **Gustav Ludwig Meyer**, 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.

2021/11/11

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ABSTRACT

Biomass is becoming an important feedstock to be used in anaerobic digesters throughout the world due to its abundance and fast growth. Most common anaerobic digesters do not have the hydrolytic organisms to digest fibrous biomass, which leads to a high retention time required for biogas production from these feedstocks.

This study aimed to evaluate the use of a rumen-based anaerobic digester to decrease the retention time required for the anaerobic digestion of lignocellulosic biomass. The digester was designed and constructed to run similarly to the rumen simulation technique (RUSITEC) which was used in other studies to study the rumen dynamics in animals. The designed digester was operated for 15 days to determine the digestion characteristics of the feedstock and the amount of biogas produced. Rumen fluid was used as an inoculum, with barley straw being used as the feedstock. The biogas production was high in the designed semi-continuous rumen reactor at 12.69 mL/gVS/day, with a methane content of 41.5% with over 50% of the feedstock undigested. The solid feedstock was removed after digestion and the protein content was measured in the digested grass to determine if it would be suitable as a feedstock for animals. The protein in the solid digestate increased from 4.75% of total solids to 7.5% in the early stages of the AD process but later decreased to 1.5% of total solids due to a lack of nitrogen in the feedstock for microbial growth.

Batch digesters were used to test the effect of different organic loadings of barley straw on the biogas production in mesophilic digesters using rumen fluid as inoculum. The biogas produced from the different organic loadings, indicated that the highest biogas production of 269 mL/gVS was obtained at an organic loading of 16.24 gVS/L, which is extremely high loading rate for anaerobic digesters. The lowest biogas production was obtained from an organic loading of 2.04 gVS/L and 24.41 gVS/L, which amounted to 185 mL/gVS and 205 mL/gVS added, of biogas respectively. The organic loading of 24.41 gVS/L led to an increase in the total VFA and drop in pH below 6, which had detrimental effects on the amount of biogas produced having 25% less than the organic loading of 16.24 gVS/L. The organic loading of 16.24gVS/L was used to determine the microbial capability of rumen fluid to degrade different type of lignocellulose biomass. The biogas production was measured for three different grass feedstocks namely: Napier grass, barley straw and kikuyu grass. The biogas potential from different lignocellulosic biomasses did not differ significantly between one another and the biogas production were 275 mL/gVS for barley straw, 282 for napier grass, and 289 mL/gVS for kikuyu grass.

The experimental data obtained by digesting different biomass feedstocks were fitted to various kinetic models (modified Gompertz, two-fraction first order, Monod type, and first order) efficiently simulated the biogas production from different organic loadings and ISRs with coefficient of determination values above 91%, with the modified Gompertz model having the

best coefficient of determination value above 98%. The kinetic modelling revealed a good fit from all models with a coefficient of determination (R^2) above 95%.

The use of rumen fluid for the mono-digestion of lignocellulosic biomass has proved to be an effective tool to decrease the retention time required for biogas production and increase the rate of biogas produced. The decrease in retention time can potentially lead to smaller reactor systems to be built or increase the organic loading for more biogas production.

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AD	Anaerobic digestion
BMP	Biochemical methane potential
BOD	Biological oxygen demand
BPP	Biogas production potential
CP	Crude protein
C:N	Carbon-to-nitrogen ratio
CH_4	Methane
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
DM	dry matter
FA	Free ammonia
FOS/TAC	Ratio of volatile organic acids to alkaline buffer capacity
GHG	Greenhouse gas
H_2	Dissolved hydrogen gas
HRT	Hydraulic retention time
ISR	Inoculum-to-substrate ratio
NH_4	Ammonium
NDF	Neutral detergent fibre
NFC	Non-fibre carbohydrate
OL	Organic load
OLR	Organic loading rate
OM	Organic matter
ORP	Oxidation reduction potential
PFR	Plug flow reactor
RDP	Rumen degradable protein
RUDAD	Rumen derived anaerobic digestion
RUP	Rumen undegradable protein
RUSITEC	Rumen simulation technique
TAN	Total ammonia nitrogen
TKN	Total Kjeldahl nitrogen

TS	Total solids
TSS	Total suspended solids
UASB	Up-flow anaerobic sludge blanket
VFAs	Volatile fatty acids
VS	Volatile solids
WW	Wet weight

CHAPTER 1

INTRODUCTION

The demand for energy is continually increasing due to a rise in the global population. Most of the energy produced in African countries is derived from fossil fuels which has a negative impact on the environment and leads to global warming. The use of fossil fuels in countries like South Africa accounts for 78.4% of the total energy produced (DOE, 2015). The rest of the energy is renewable and nuclear energy, amounting to 19.3% and 2.3%, respectively. Biogas produced from biomass as a source of renewable energy only accounts to 4.2% of the 19.3% (DOE, 2015). There is a definite need to increase the energy being generated from the renewable energy sector to decrease the use of fossil fuels and diminish the impact of global warming on the earth. Global warming is an increasing problem as can be seen by rising temperatures caused by the emission of greenhouse gasses (GHG) from fossil fuels. The year 2019, recorded the second highest average surface temperature since the 1870s (Lindsey & Dahlman, 2020).

One of the most widely recognised greenhouse gasses is carbon dioxide (CO_2), but another greenhouse gas which poses similarly devastating potential is methane gas (CH_4). Methane, short-lived in the atmosphere, has 86 times more global warming effect compared to CO_2 over a period of 20 years (EESI, 2021). Methane gas is emitted from various locations such as landfills, cattle farms and compost heaps through microorganisms that make use of organic matter for growth and metabolism. Most of the methane (60%) is caused by anthropogenic activities that influence the carbon cycle on earth – such as the piling up of organic wastes and the lack of effective waste management strategies (Dean *et al.,* 2018).

Fibrous agricultural waste is considered an important feedstock for biogas production as it is readily available throughout the world and its potential for energy production in developing countries is under-utilised. Farms make use of agricultural waste by burning or making compost heaps, resulting in more methane and carbon dioxide being released in the atmosphere. However, farms can make use of lignocellulose biomass generated through farming activities in anaerobic digestion as a renewable resource to produce methane, which is combusted to produce electricity. There has been little research focussing on the use of stover and other fast growing, fibrous grasses for the production of biogas due to the slow degradation of the grasses in common anaerobic digesters (Wyman & Yang, 2009; Chiumenti *et al.,* 2018). This is a common problem from fibrous lignocellulose biomass as it consists of hemicellulose, cellulose and lignin polymers which form a crystallin structure recalcitrant to microbial degradation (Zou *et al.,* 2018; Sayara & Sánchez, 2019). However, some grasses like barley straw have high carbon content and have reported a methane yield of 280 mL/gVS added and a methane percentage of 64% (Dubrovskis *et al.,* 2013).

Like anaerobic digesters, cows are known to produce methane gas from grasses through the diverse microbial ecosystem found in the rumen of the cow. However, cows digest their food in a couple of days as opposed to 30-60 days which is seen for the digestion of various lignocellulose biomasses in anaerobic digesters (Bayané & Guiot, 2010; Kaur *et al.,* 2016; Nguyen *et al.,* 2019; Putri *et al.,* 2019; Vargas *et al.,* 2020). Therefore, this thesis focusses on the digestion of lignocellulose biomass through anaerobic digestion by making use of rumen fluid as an inoculum for a fast biogas production.

1.1 Background

Anaerobic digestion is a progressive technology to utilise waste generated from industries and farm activities to produce biogas. Biogas is considered a renewable source of energy for electricity production and can be used to alleviate organic waste from piling up in landfills (Comparetti *et al.,* 2013). The use of anaerobic digesters for the production of electricity have grown significantly in countries like Germany, China, USA and throughout Europe as there is a growing awareness of the impact of global warming in society (Auer *et al.,* 2016). Africa, however, is still lagging in its switch to more renewable fuels as it is more expensive than fossil fuels such as coal for the production of electricity (Owen, 2006).

Anaerobic digestion adheres to complex chemical reactions where microorganisms exchange nutrients such as volatile fatty acids and produce various by-products such as a digestate and methane gas for energy production (Gerardi, 2003). The production of biogas depends on various factors such as pH, temperature, the type of inoculum used and the feedstock used as these can have a significant impact on the efficiency, stability and amount of biogas produced (Al Seadi *et al.,* 2008). African countries have a high amount of farming activities which has the potential to add to the renewable energy sector by making use of agricultural biomass to generate biogas for electricity through anaerobic digestion (Rösch *et al.,* 2009).

1.2 Problem statement

An increasing world population means more waste is generated and the demand for energy in a country like South Africa is escalating. More waste is accumulating in landfills, which leads to more greenhouse gasses such as methane being emitted and the acceleration of global warming. South Africa makes use of fossil fuels (78.4%) for the generation of its energy (DOE, 2015). The use of fossil fuels leads to the emission of CO_2 which worsens global warming and unbalances the carbon cycle (Dean *et al.,* 2018). Most waste generated in the world consists of organic fractions (46%) which is degradable through microorganisms (Thompson, 2012). South Africa will soon be running out of landfill space as the country generates roughly 54.2 million tons of general waste per year (IWMSA, 2019). According to the Institute of Waste

Management of Southern Africa (IWMSA), South Africa is in a deep crisis mode: no additional landfills have been dug in the past 24 years and only 10% of the general waste is recycled (IWMSA, 2019). This is concerning from any perspective. This situation requires immediate action to expand the recycling of waste and minimise the waste piling up in landfills. Thankfully, South Africa forms part of the Paris Climate Agreement which aims to keep global temperatures from rising more than 1.5^{o}C and to reduce the emission of greenhouse gasses.

1.3 Justification

South Africa lags far behind the rest of the world in switching to renewable fuels for the generation of electricity. With solar energy becoming less expensive, more businesses are making use of the technology for electricity production. However, if there is no sunlight in the area, there will be no electricity produced. Anaerobic digestion, therefore, is an intelligent alternative in cases where there is no sunlight to generate electricity, as anaerobic digestion can generate methane gas when supplied with a waste-feedstock. Most solar farms and wind farms cover large swatches of land due to the nature of energy production, but anaerobic digesters do not occupy large pieces of land and can be built even where land is limited.

Anaerobic digestion has other benefits as well: it removes organic waste from piling up in landfills and provides a digestate which can be used in agricultural settings (Nanda *et al.,* 2018). Fibrous grasses such as barley straw, Napier grass and kikuyu grass are fast growing, high yielding and commonly available crops, making them attractive feedstock for anaerobic digestion in African countries. Even though these feedstocks are readily available, the crystalline structure of the grasses makes it difficult to break down in anaerobic digestion and achieve high methane yields (Sayara & Sánchez, 2019). So, the solution to this problem is to make use of rumen fluid as an inoculum in the anaerobic digester as it contains specific microorganisms that produce various exolytic enzymes to digest the fibrous grasses in cows in a matter of days (Saleem *et al*., 2019). The quick retention time of the feedstock in the digester means smaller volumes of digesters to be built; and higher organic loadings added into the digesters to effectively reduce the capital cost of the anaerobic digestion plant. The addition of higher organic waste which is degradable will ultimately lead to an increased biogas yield produced (Gijzen *et al.,* 1988). The by-products produced from anaerobic digesters also have the potential to be used as a fertilizer, beneficial for farmers, and it is an additional source of income or savings to the anaerobic digester.

1.4 Hypothesis, aims and objectives

1.4.1 Hypotheses

In this study, the following hypotheses were formulated:

i) The use of rumen fluid will increase the biogas produced and lower the retention time required for anaerobic digestion of lignocellulose biomass.
ii) The increase of substrate loadings in the reactors, lowering inoculum to substrate ratios (ISR), will increase the VFA produced, leading to improved biogas production.
iii) The biogas production potential from various lignocellulose biomass samples will differ.
iv) The by-product digestate from the anaerobic digestion will be good fertilizer and protein source from its nitrogen, phosphorus and potassium content.

1.4.2 Aims

The main aim of this study is to establish to what extent the natural process in ruminants such as cows can be exploited in a bioreactor system for the production of biogas and fertiliser from lignocellulose biomass.

1.4.3 Objectives

- Design and build a rumen based anaerobic digester to digest lignocellulose biomass.
- Test the biogas production and volatile fatty acids (VFA) from various organic loadings (gVS/L) and inoculum-to-substrate-ratios (ISRs), using rumen fluid as inoculum.
- Test the biogas production of three different lignocellulose feedstocks – Napier grass, barley straw and kikuyu grass – from rumen fluid and test the nitrogen, phosphorus and potassium content of the effluent from different lignocellulose biomass
- Finally, compare the protein content in the digestate after the mono-digestion from different grass samples from the digestion of rumen fluid.

1.5 Significance of study

The use of anaerobic digestion to produce biogas from biomass will provide an alternative renewable energy source and enhance the production of electricity. This research will evaluate the reduction of retention time in anaerobic digestion for biomass. To the best of our knowledge, there have only been a few studies assessing the mono-digestion of biomasses by making use of rumen fluid as inoculum for biogas production. Therefore, this study will give more information surrounding the use of rumen fluid in a commercial digester with grass as a feedstock to increase biogas production. This study will also contribute to the effective management of agricultural biomass to reduce the emissions of greenhouse gasses and reduce their impact on global warming.

1.6 Delineation of study

- This research will not assess the specific microbial populations in the anaerobic digestion process, but rather the product and digestate of the system.

- This study will only look at lab-scale digestion of the lignocellulose biomass and will reserve pilot-scale digestion for future studies.
- This research will not assess the overall safety of the by-product as a fertiliser, but rather the NPK composition of the effluent to determine if it is comparable with other fertilizers.

CHAPTER 2

LITERATURE REVIEW

2.1 Anaerobic digestion process

Anaerobic digestion provides several positive attributes such as lowering the toxicity and virulence of organic and hazardous waste; lowering the amount of waste sent to landfills; providing a source of energy; and the potential production of a fertilizer (Zheng *et al.,* 2017; Iocoli *et al.,* 2019). The process in an anaerobic digester can be subdivided into four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Anukam *et al.,* 2019). Overall, the four stages involve the growth of complex microbial consortiums to ensure the degradation of various feedstocks to produce biogas and digestate **(Figure 2.1)**. During these stages, the microbes form a synergistic relationship with one another to acquire the necessary nutrients and compounds for their growth and metabolism.

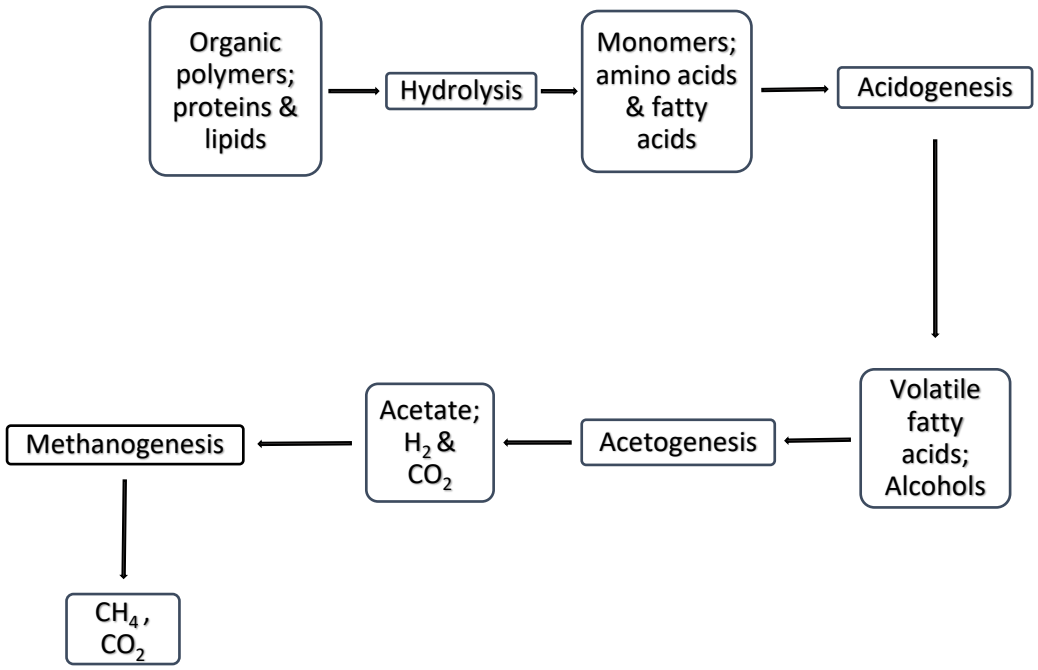


Figure 2.1: Overview of the different stages and products in the process of anaerobic digestion

2.1.1 Hydrolysis

Hydrolysis is the first step in anaerobic digestion and involves the degradation of polymers such as polysaccharides, proteins, and lipids to monomers such as sugars, amino-acids, and long chain fatty acids.

Hydrolysis is considered the rate-limiting step in the anaerobic digestion process as it requires a great deal of time and energy to break down the various polymers to its monomers (Shrestha *et al.,* 2017). Due to the lack of oxygen in an anaerobic digester, hydrolysis is performed by facultative anaerobes and strict anaerobes secreting extracellular enzymes to break down polymers (Adekunle & Okolie, 2015). Hydrolysis, mainly achieved through various exo- and endo-enzymes to break down these polymers, needs the presence of a liquid substance for the successful working of the enzymes (Gerardi, 2003). However, not all bacteria produce the exoenzymes required to break down these complex polymers (Gerardi, 2003). A diverse range of microorganisms, in fact, are needed to break down the complex polymers to their respective monomers. Most enzymes are extremely sensitive to environmental conditions. A slight change in the environmental conditions such as temperature, pH and micronutrients can lead to the degradation or enhancement of the enzymes and can restrict or increase the rate of hydrolysis (Al Seadi *et al.,* 2008).

The hydrolysis of lignocellulose is generally a time-consuming process due to the structural components of the polymers found in biomass and lignin's resistance to microbial degradation (Nizami, Korres & Murphy, 2009). There are, however, various methods that can be applied in the hydrolysis process to enhance the degradation of biomass. Separating the first-phase (acid production) and second-phase (methane production) of the anaerobic digestion in two different tanks has been shown to increase the production of methane and lead to a lower hydraulic retention time for digestion (Nizami *et al.,* 2009).

It has also been suggested that the use of rumen fluid will promote hydrolysis of biomass due to the digestion strategies used by ruminant animals (Nizami *et al.,* 2009). This will lead to the addition of various enzymes and microorganisms capable of degrading lignocellulose biomass more effectively to achieve a higher biogas production rate. The common equation suggested for the reaction of hydrolysis is highlighted in **Equation (2.1)** (Anukam *et al.,* 2019):

$$(C_6H_{10}O_5)\ n + n\ H_2O \rightarrow n\ C_6H_{12}O_6 + n\ H_2 \qquad (2.1)$$

Most polymers consist of complex bonds of glucose, arabinose, fructose and various other sugars. Glucose is the building block of most polymers and the most common substrate in downstream reactions as it can be used during glycolysis for the formation of ethanol and acetate. The products are then used during acidogenesis and acetogenesis to produce methane gas (**Figure 2.1**).

2.1.2 Acidogenesis

Acidogenesis, the second stage in an anaerobic digester, utilises the products from hydrolysis to produce short chain organic acids such as acetic acid, propionic acid and butyric acid and various alcohols (Adekunle & Okolie, 2015). This stage is also referred to the *acidification stage*, as the pH usually drops during this stage with the production of various volatile fatty acids (VFAs) (Jin *et al.,* 2018).

. As the polymers are degraded in hydrolysis, various acid-forming microorganisms metabolise the monomer sugars to produce acetate and propionic acid. The high rate of acidification from the microorganisms in anaerobic digesters is not preferred for biogas production as it can lead to digestion failure from a low pH which will kill the methanogens. A study testing the influence of different pH values (5, 7, 9, 11) on hydrolysis and acidification in anaerobic digestion has shown that a pH of 7 has led to 86% of the total organic carbon (TOC) and 82% of chemical oxygen demand (COD) being solubilized (Zhang *et al.,* 2005). The study further indicated that at a pH of 7, most of the protein is degraded into ammonia nitrogen (NH_3^+- N), which serves as an additional buffer for the anaerobic digester. The summary of equations associated with this stage is shown in **Equation (2.2)** to **(2.4)** (Anukam *et al.,* 2019):

$$C_6H_{12}O_6 \leftrightarrow 2\ CH_3CH_2OH + 2\ CO_2 \quad (2.2)$$

$$C_6H_{12}O_6 + 2\ H_2 \leftrightarrow 2\ CH_3CH_2COOH + 2\ H_2O \quad (2.3)$$

$$C_6H_{12}O_6 \rightarrow 3\ CH_3COOH \quad (2.4)$$

These products form the substrate for acetogenic bacteria to further metabolise the products to acetate.

2.1.3 Acetogenesis

The second and third stages are closely related to one another, leading to the production of various acids. However, during *acetogenesis*, the primary products are acetate and hydrogen gas (H_2) (Adekunle & Okolie, 2015). The product that receives the most interest during this stage is acetate because acetate accounts for 70% of methane production during methanogenesis (Jin *et al.,* 2018). The bacteria during this stage are obligate anaerobes and facultative anaerobes which can utilise their products in no oxygen or minimal oxygen (Gerardi, 2003). This stage is also known as the *dehydrogenation* stage as the metabolism of the various acetogenic bacteria are inhibited through high amounts of H_2 produced. The hydrogen gas is metabolised by various methanogenic bacteria to produce methane. Therefore, the symbioses of the methanogenic bacteria and acetogenic bacteria are crucial for the successful digestion of the feedstock for biogas production. The equations of acetogenesis are summarised in **Equations (2.5)** to **(2.7)** (Anukam *et al.,* 2019):

$$CH_3CH_2COO^- + 3\ H_2O \leftrightarrow CH_3COO^- + H^+HCO_3^- + 3\ H_2 \quad (2.5)$$

$$C_6H_{12}O_6 + 2\ H_2O \leftrightarrow 2\ CH_3COOH + 2\ CO_2 + 4\ H_2 \quad (2.6)$$

$$CH_3CH_2OH + 2\ H_2O \leftrightarrow CH_3COO^- + 3\ H_2 + H^+ \quad (2.7)$$

2.1.4 Methanogenesis

Methanogenesis is the most important step during anaerobic digestion for the formation of methane gas. Methanogenic archaea will metabolise the various organic acids and various gasses to produce methane and carbon dioxide. The various methanogenic archaea are sensitive to changes in the environment and require certain criteria to be met for optimal metabolism and methane production (Liu & Whitman, 2008). The optimal production of methane is preferred for commercial anaerobic digesters to increase the potential of biogas that can be used to produce electricity. The production of methane is entirely dependent on the growth and metabolism of the microbial consortium in the reactor. Methanogenesis is the rate limiting step-together with hydrolysis due to the slow growth rate of archaea. Methanogens, able to tolerate high salt concentrations, are known to naturally grow on decaying material and the rumen of ruminants (Gerardi, 2003). Methanogenic bacteria, known to grow in a microbial consortium, are obligate anaerobes; therefore, a hint of oxygen can kill the methanogens present. Methanogenesis is shown in **Equations (2.8)** to **(2.10)** (Anukam *et al.,* 2019):

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (2.8)$$

$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O \quad (2.9)$$

$$2\ CH_3CH_2OH + CO_2 \rightarrow CH_4 + 2\ CH_3COOH \quad (2.10)$$

According to Gerardi, (2003), there are three main classes of methane-forming archaea: a) hydrogenotrophic methanogens; b) acetotrophic; and c) methylothropic methanogens. These classifications are in accordance with the substrate the various methanogens use. The conversion of acetate to methane and carbon dioxide in **Equation (2.8)** is performed by acetotrophic methanogens (Gerardi, 2003) whereas hydrogenotrophic methanogens will follow **Equation (2.9)** to reduce H_2 and CO_2 to form methane. **Equation (2.10)** results in methane production through the decarboxylation of ethanol to form methane and acetate. Under optimal conditions, the generation time of methanogens may be several days to a few weeks (Gerardi, 2003). It is important to consider the growth rate of these methanogens as a low hydraulic retention time will result in the complete washout of the methanogens present in the reactor and lead to low methane production. Again, methanogens are extremely sensitive to changes in the environment and need a set environment for gas production.

2.2 Classification of anaerobic digesters

Anaerobic digesters are classified according to size, function, type of feedstock used, energy availability and cost of the digesters (Al Seadi *et al.,* 2008). The size of the digesters can be divided into household digesters (small to medium); farm-scale digesters (medium to large); or centralised digesters (large). Digesters can also be classified according to the functioning of the digester such as a batch or continuous culture, wet or dry processing and plug-flow or continuous stirrer. Every anaerobic digester is classified through its manner of acquiring its feedstock, functioning as a batch or continuous digester, for example. Different digesters have various advantageous and disadvantageous for biogas production from any given feedstock.

2.2.1 Batch digester

Anaerobic batch cultures are typically grown in batch reactors with no oxygen in the tank with feedstock added to the digester for a set time before being discarded. The batch reactor is the most commonly used digester in chemical industries to study the effect of various parameters during the production phase (Dimian, Bildea & Kiss, 2014). The design of a batch-based anaerobic digester is illustrated in **Figure 2.2**.

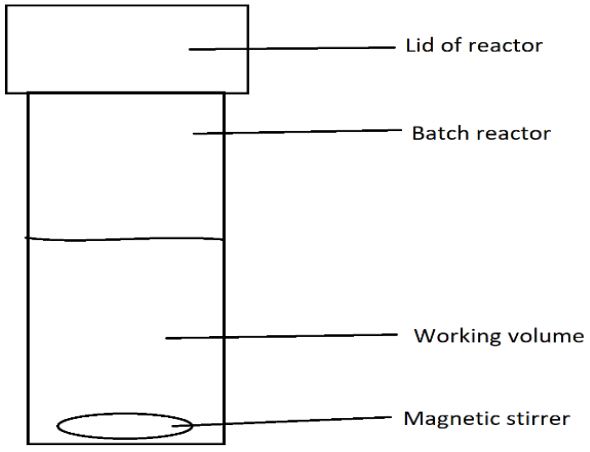


Figure 2.2: Design of a batch reactor

The digester can function as wet-based digester where the total solids (TS) content is between 10% - 25%, or a dry-based digester where the TS content is 30%-40% (Karagiannidis & Perkoulidis, 2009). The total solids depend on the mixture of water and dry matter added that will be able to degrade in the digester. A batch-based digester requires an initial feedstock added at the start and mixed with water in an anaerobic tank. The tank must be gas tight without any oxygen to create an anaerobic environment. Industries usually purge the tanks with a gas mixture of CO_2 and N_2 gas to remove the oxygen present in the reactor.

The absence of oxygen will lead to the growth of facultative anaerobes and obligate anaerobic microorganisms to degrade the feedstock in anaerobic digesters to produce biogas. Batch-cultures can simply be defined according to the limited amount of feed they receive at the start of the digestion process. Therefore, the microbes must compete with one another for growth and to acquire the various nutrients. The feedstock can either be mixed in the reactor or left to ferment on its own with no mixing action required. However, mixing the contents will result in an even distribution of the feedstock in the tank and optimal surface contact for microbial growth to increase the rate and amount of biogas produced. The period in which the batch cultures are in contact with the feedstock is defined as the *hydraulic retention time* (HRT). A low HRT can lead to a low production of biogas and an excessively high HRT can minimise the economic feasibility of a anaerobic digester as it leads to increased cost for the running of the reactor (Ezekoye *et al.,* 2011). Batch cultures, with various advantages and disadvantages, are summarised in **Table 2.1.** Batch-based digesters are usually the go-to method for biochemical methane potential tests and biogas potential tests in laboratories as this requires the least amount of effort and can give an indication of the biogas potential from different feedstocks for anaerobic digestion (Angelidaki *et al.,* 2009; Holliger *et al.,* 2016).

Table 2.1: Advantages and disadvantages of batch digesters

Advantages	Disadvantages
Reduced risk of contamination (Holliger et al., 2016)	Frequent sterilisation after use (Angelidaki *et al.,* 2009)
Low capital investment required (Al Seadi *et al.,* 2008)	Lower productivity (Holliger et al., 2016)
Higher raw material conversion (Angelidaki *et al.,* 2009	Long HRT

2.2.2 Continuous culture

Continuous culture digesters are the preferred industrial digesters with the continuous addition of new feedstock in the reactor. The design of a continuous digester is typically more expensive as it requires extra maintenance costs and additional equipment for the continuous flow of feedstock. After initial microbial growth is initiated in the continuous reactor, a continuous supply of new feedstock is added to maintain the microbial growth. The volume of fluid in the reactor is maintained with an overflow to remove the degraded feedstock. This continuous flow of feedstock in the reactor leads to the formation of a continuous culture of microorganisms.

These microorganisms are not limited with available nutrients and can therefore optimally utilise all the nutrients in the reactor leading to a continuous microbial consortium (Gerardi, 2003). However, an abundance of nutrients can lead to the growth of unfavourable microbes that compete with the desired microbes and result in low performance or failure of the digester.

Therefore, much research has focussed on finding the optimal organic loading rate (OLR) in various continuous anaerobic digesters for different feedstocks (Koch *et al.,* 2009; Zhou *et al.,* 2017; Liu *et al.,* 2018; Martí-Herrero *et al.,* 2019). This is because the chemical reactions in anaerobic digester do not have the same rate and the feedstock may be limited in its degradability. Therefore, the OLR is important in continuous culture digesters to allow enough time for growth of the microorganisms and prevent the cultures from washing out. The various advantages and disadvantages of continuous cultures are summarised in **Table 2.2.**

Table 2.2: Advantages and disadvantages of continuous digesters

Advantages	**Disadvantages**
High concentration of nutrients (Koch *et al.,* 2009)	Expensive to construct (Al Seadi *et al.,* 2008)
High rate of biodegradability (Gijzen et al., 1988)	Complex to control
High biogas production (Comparetti *et al.,* 2013)	Risk of contamination (Al Seadi *et al.,* 2008)
Shorter HRT compared to batch reactors (Liu *et al.,* 2018)	Removal of undigested feedstock (Liu *et al.,* 2018)
Increase productivity (Ward *et al.,* 2008)	

2.2.3 Continuous flow stirred tank reactor

The continuous flow stirred tank reactor (CFSTR) has the same concentration of feedstock throughout the tank. The continuous addition of new feedstock results in a short HRT of the feedstock in an anaerobic digester. Continuous mixing leads to a homogenous environment of feedstock in the tank.

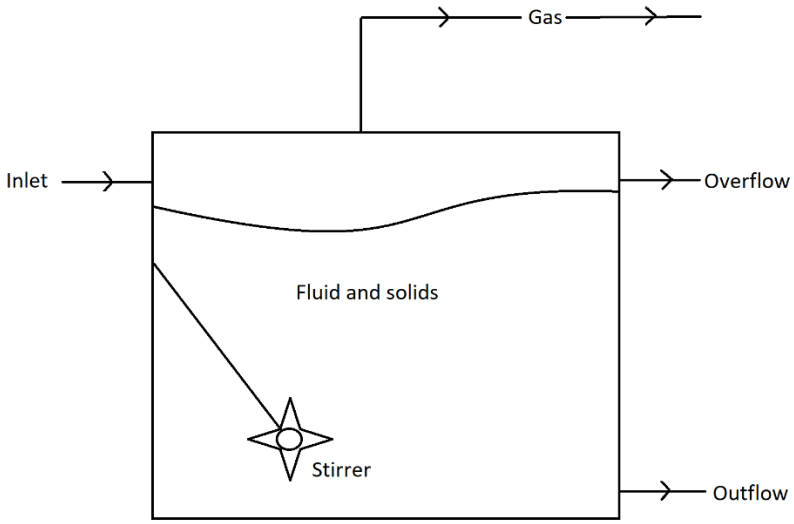


Figure 2.3: Schematic view of continuous stirred tank reactor

The homogenous environment will lead to optimal feedstock utilisation and growth of the different microorganisms. While this continuous flow of new reagents increases the productivity for various chemical reactions in a number of industries (Ward *et al.,* 2008), the continuous flow in anaerobic digestion can be time consuming as the environment in the tank needs requires adjustment for favourable growth of microorganisms during the different phases of anaerobic digestion. This can result in longer hydraulic retention times (HRT) and low organic loading rates (OLR) in the digester due to the waiting period for the microorganisms to adapt. However, methods have been developed to optimise the growth of microorganisms and enhance the process stability. One such method is a multi-stage anaerobic digester (Ward *et al.,* 2008). The stages of anaerobic digestion are separated from one another to create favourable environments for growth of the different microorganisms, as shown in **Figure 2.4** (Ward *et al.*, 2008; Van *et al.,* 2020).

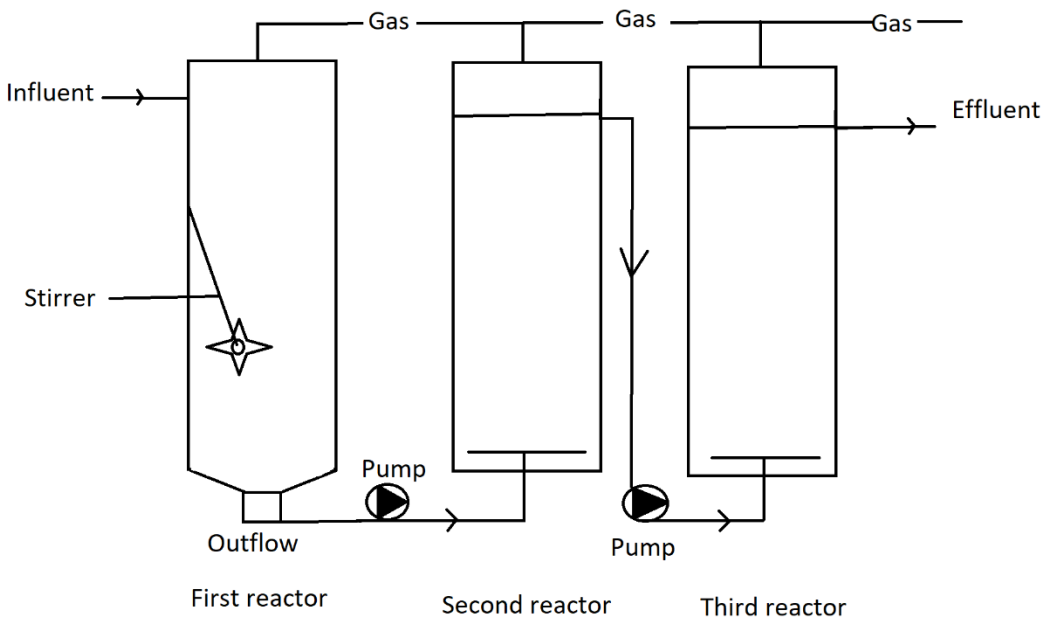


Figure 2.4: Schematic view of a multi-stage anaerobic digestion process

Multi-stage digesters have numerous advantages: higher methane production, for example, and better methane quality compared to single-stage digesters (Nizami & Murphy, 2011). Two disadvantages of multi-stage digesters over single-stage digesters, though, is that they are more expensive and require precise control measures to ensure a biogas is continually produced (Ward *et al.,* 2008). It might also be a disadvantage to separate the different stages to produce biogas, as all the microorganisms will develop in their own tank and therefore, the synergistic relationship will be difficult to maintain in a multiple-stage reactor.

2.2.4 Plug flow reactors

Plug flow reactors (PFR) differ from a CFSTR as the fluid in the tank is not homologous. The plug flow reactor (PFR), otherwise known as the plug bed reactor (PBR), is a long, slim, narrow tank which allows the feedstock to flow through the reactor in a plug-like manner (Ward *et al.,* 2008). The PFR functions in a horizontal position with a series of mixers creating a diverse environment for the growth of different microorganisms**. Figure 2.5** illustrates the functioning of a plug flow digester (Logan & Visvanathan, 2019). As new feedstock is being added from one side of the reactor, it pushes the old feedstock through the outflow in a plug-like manner.

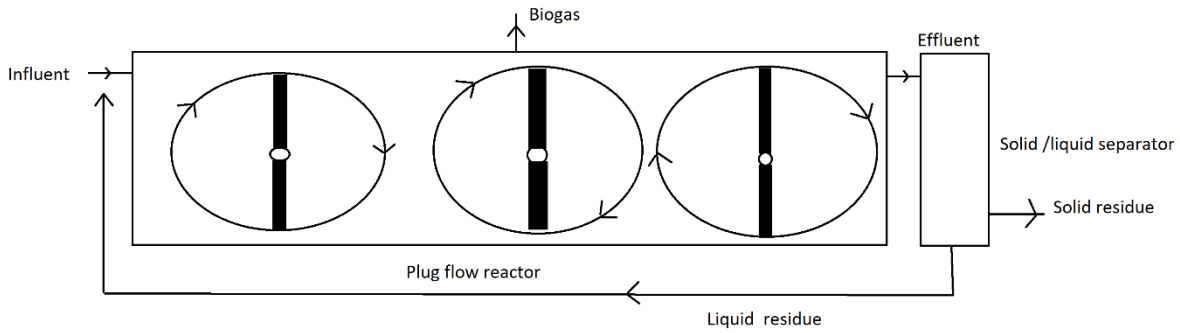


Figure 2.5: Example of the functioning of a plug flow digester

The first documented use of this digester in South Africa was in 1957 (Ghosh & Bhattacherjee, 2013). The feedstock fed from the one side creates different environments within the plug flow digester and enables a batch-like environment for the variety of microorganisms to adjust to the feedstock. The addition of new feedstock in the digester generates an environment in which acidogenic and hydrolytic bacteria can grow, as there is an abundance of newly-fed feedstock to metabolise. As the feedstock is broken down to its monomers and respective acids, it gives rise to methanogenic bacteria further in the plug flow digester to metabolise the acids and optimise methane production. The plug flow digester is an optimal low-cost digester with little to no interference necessary during the process. However, it can become clogged with sand and other solids and will therefore require occasional cleaning periods to limit the amount of solids from building up in the digester (Ghosh & Bhattacherjee, 2013).

2.3 Rumen-based anaerobic digestion

Ruminants are mammals able to acquire their food from a variety of different lignocellulose products such as grass and leaves. However, mammals do not contain the cellulase enzymes which break down lignocellulose biomass (de Ondarza, 2001). This has led to the symbiotic relationship between the microorganisms in the gut and the ruminants to enhance the digestion of lignocellulosic biomass and maximise the nutrients obtained from their food. This method of food digestion, called *rumination,* occurs in the part of the stomach called the rumen (Ørskov, 1986). Rumination leads to the production of VFA which is taken up by rumen papillae (finger-like structures on the rumen wall) and used as a source of energy in the cow (de Ondarza, 2001). Interestingly, the biomass of the microbial cells is not absorbed in the rumen but is later absorbed in the intestines as a source of protein for the cow. It has been found that the amino acid composition in the rumen is like the amino acid composition of milk and meat of the cow (de Ondarza, 2001). This suggests that the rumen microorganisms are a high-quality protein source.

Rumen-based anaerobic digestion of lignocellulosic biomass has been studied extensively with little success due to a lack of understanding of the process in ruminants. Al Mamun *et al.* (2018), for example, have analysed the biogas production of rumen content obtained from cows, chicken and goats. Their study determined that the rumen of chickens produced the highest cumulative yield of biogas compared to the other rumens. However, the feedstock used in this study was just rumen fluid and solid rumen contents inserted into a batch digester which may therefore already be degraded. The contents in the chicken rumen may have been high in nutrient composition which could have accounted for the high biogas yield, although this is not reported in the study. They have found that the addition of rumen fluid in a anaerobic digester increased the amount and rate of biogas produced (Zhang *et al.,* 2017; Zou *et al.,* 2018). A study on the digestion of cereals in a two-staged anaerobic digester, termed the *rumen derived anaerobic digestion* (RUDAD), has shown that rumen fluid can digest roughly 42%-57% of all cereals in just 60 hours of incubation (Kivaisi *et al.,* 1992). Another study comparing the biogas produced from rumen fluid and anaerobic sludge found that rumen fluid had a lower biogas production potential compared to anaerobic sludge (Nguyen *et al.,* 2019). As these results are contradictory, more clarity on the use of rumen fluid as an inoculum during anaerobic digestion is necessary.

2.3.1 Lignocellulosic biomass as feedstock in an anaerobic digestion

Grass is one of the most used lignocellulose products in anaerobic digesters (Nizami & Murphy, 2010). Perennial grasses are favourable feedstocks for the use of anaerobic digesters due to their high growth rate, high yield and availability throughout the year. Switchgrass (*Panicum virgatum*) is commonly grown grass in warm climates and used for biofuel production in North-America (Fedenko *et al.,* 2013). Switchgrasses utilise the C4 photosynthetic pathway and are known to grow in warm, dry conditions reaching heights of up to three meters (Karp & Shield, 2008).

Other perennial grasses used to produce biogas are Napier grass and kikuyu grass, scientifically known as *Pennisetum purpureum* and *Pennisetum clandestinum,* respectively. Napiergrass, grown across the African grasslands, is used as an erosion inhibitor, windbreaker and feed for livestock due to its high nutrition values (Dussadee *et al.,* 2016). A study by Dussadee *et al.* (2016) has analysed the biogas production from Napiergrass in a leachate circulating digester and found the biogas production to be 20.62 L/kg fresh grass, which is relatively low compared to other biogas potentials of grasses. The density in which these grasses grow enables high biomass yields per hectare. Perennial grasses are also preferrable for biogas production due to their lower lignin concentrations compared to other woody crops (Fedenko *et al.,* 2013). It has been reported that the biogas from agricultural residue and grass has the highest content of methane, reaching up to 84% (Vintila *et al.,* 2012).

Pennisetum clandestinum, better known as kikuyu grass, is one of the most widely available grass feedstocks. However, little research has concerned the use of kikuyu grass in anaerobic digestion for biogas production. Kikuyu is considered high in nutrient content, which is why it is considered one of the best grasses to grow for dairy and sheep pastures. There have been some studies pertaining to the digestion of kikuyu grass in rumen fluid and faeces (Posada, Noguera & Segura, 2012), finding that the gas produced from kikuyu grass ranges between 40-45 mL/gTS over 96 hours of incubation. However, the study did not determine the biogas potential from kikuyu when incubating it for longer periods in an anaerobic digester, until there is no more biogas being produced.

2.3.2 Continuous culture system for rumen based anaerobic digestion

I) Rumen simulation technique (RUSITEC)

Originally designed and constructed by Czerkawski and Breckenridge in (1977), the *rumen simulation technique* (RUSITEC) has been used as an *in vitro* technique to study rumen-based microbes and their interactions (Martínez *et al.,* 2010; Lengowski *et al.,* 2016; Duarte *et al.,* 2017; Saleem *et al.,* 2019; Vargas *et al.,* 2020). The whole RUSITEC system consists of four to eight reaction vessels, where a single reactor vessel consists of three different parts (**Figure 2.6**). The first part is the main reaction vessel with a working volume of 1 ℓ. The main reaction vessel has a removable lid; an inlet pipe at the bottom of the reactor for the inlet of artificial saliva; an overflow pipe at the top of the reactor connected to an overflow vessel of at least 1 ℓ; and a sample valve at the top (**Figure 2.6**). A stainless-steel shaft through the middle of the lid of the main reactor is connected to the second part of the reactor, which consists of a cylindrical PET bottle with small holes through which the rumen fluid can move. The PET bottle, with a removable lid and fitting snuggly into the main reaction vessel, is connected to the steel shaft to create up and down movements of the bottle. Inside of the PET bottle is the third part of the reactor, nylon bags (pore size 100 µm) containing the lignocellulose feedstock. The overflow vessel has an outlet pipe which is connected to a gas bag to collect the gas produced.

For the start-up of the RUSITEC system, the four to eight reaction vessels are filled with rumen fluid and placed in a thermostatically controlled water bath at 39ºC to maintain mesophilic conditions. The lignocellulose biomass is added into a permeable nylon bag and the nylon bag is then added to the PET bottle. Another permeable nylon bag, filled with rumen solids, is also added to the PET bottle and the lid of the PET bottle is closed. Then the PET bottle is connected to the stainless-steel shaft and lowered into the digester. The main reaction vessel is closed and flushed with 50% CO_2 and 50% N_2-gas to create an anaerobic environment. After flushing the reactors, the gas bag is connected to the gas pipe from the overflow vessel. The RUSITEC system is then turned on and the PET bottle moves up and down intermittent intervals through a motor pump. The peristaltic pump will also pump fresh artificial saliva in the system (McDougall, 1948). After 24 hours, the RUSITEC system is turned off. The gas bag is

loosened, and the lid of the main reaction vessel is opened to remove the one nylon bag with solid rumen content. A new permeable nylon bag filled with feedstock is added to the PET bottle and the main reaction vessel is closed and flushed with CO_2-N_2 gas. After the flushing of the main reaction vessel, the gas bag is connected to the pipe from the overflow vessel and the reaction continues. Every subsequent day, the nylon bag which has been in the digester for 48 hours is removed and replaced by a new nylon bag containing new feedstock for the duration of the experiment.

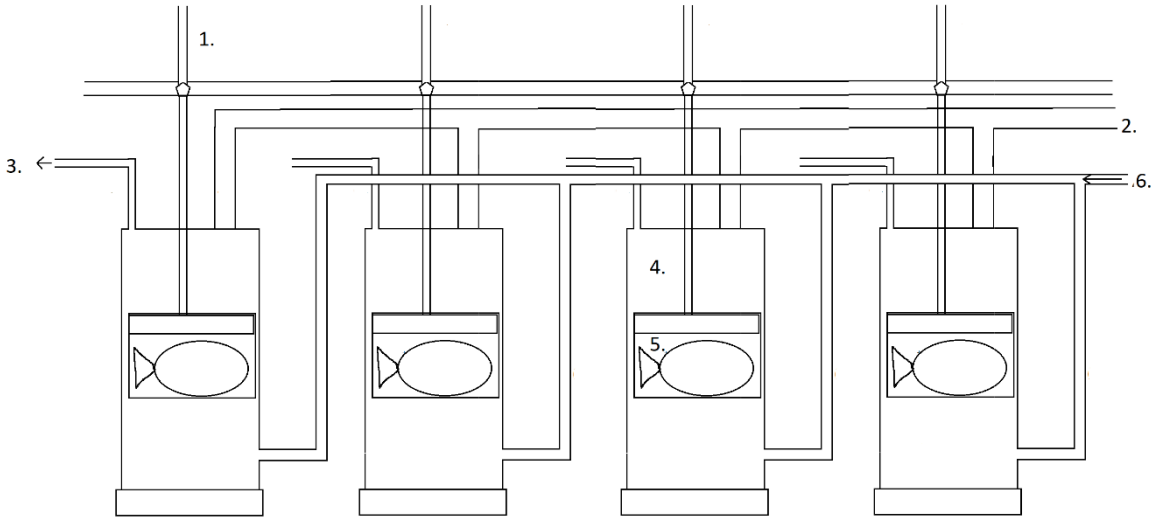


Figure 2.6: RUSITEC system with four reactors: 1) stainless steel shaft 2) biogas outflow 3) overflow pipe of one vessel 4) main reactor vessel 5) stainless steel mesh vessel with nylon bag 6) artificial saliva inflow pipe

The lignocellulose product in the bag is degraded by the various microorganisms present in an anaerobic digester to mimic the digestion of lignocellulose products in living animals. The digestion of the feedstock in a RUSITEC system can be divided into three stages – solid state, semi-solid and fluid state – which serve as a dilution of the feedstock in the reactor. The solid state digestion is optimal for the growth of cellulolytic bacteria to effectively degrade lignocellulose products (Bayané & Guiot, 2010). The semi-solid state is found close to the nylon bags and in the second vessel where some pieces of feedstock floating in the reactor will allow various microorganisms to degrade the feedstock. The fluid state digestion is in the rumen fluid throughout the whole reactor vessel where various microorganisms will exchange nutrients and minerals to degrade the material. The artificial saliva supplements the microorganisms with various nutrients and enzyme functioning. Artificial saliva is a mixture of various chemicals to mimic the saliva found in ruminants. It has been reported that the concentration of Na^+/K^+ in artificial saliva has an impact of protozoa populations. The study

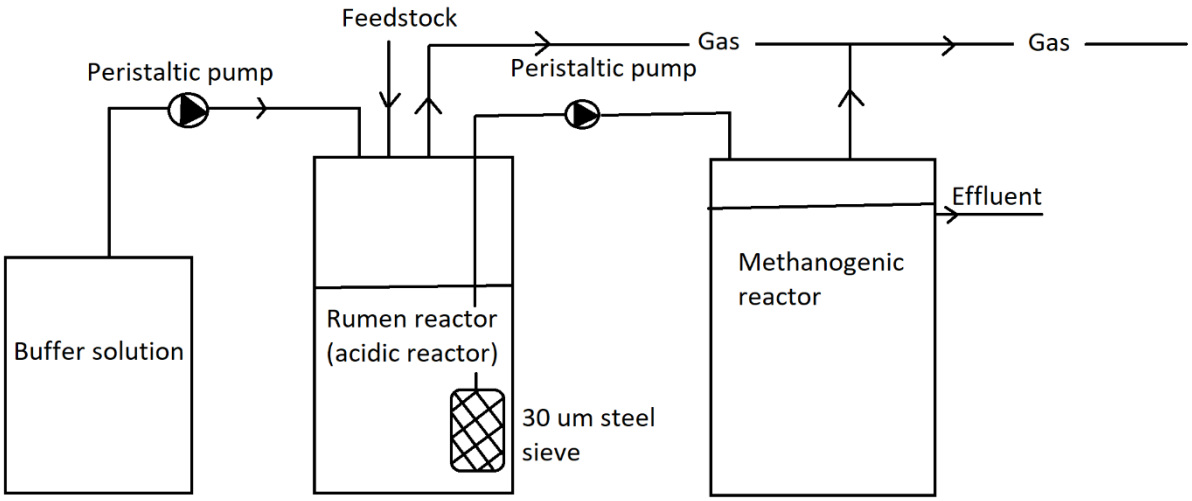
also found that HCO_3^- and HPO_4^- concentrations have an impact on the pH of the anaerobic digester (Broudiscou *et al.,* 1999).

Even though the RUSITEC system have been used over the years, most studies were conducted to compare the microbial populations to that of living animals and the production of methane in ruminants (Czerkawski & Breckenridge, 1977; Martínez *et al.*, 2010; Vargas *et al.*, 2020). Only a few studies have focussed on the protein content in the effluent of the RUSITEC system (Martínez *et al.,* 2010; Lengowski *et al.,* 2016). The effluent in the RUSITEC system might contain similar microbial populations of that found in the rumen of living animals and may thus be of high-quality protein used as a potential protein source in animal feed. Therefore, future studies should focus on the composition of the effluent in the RUSITEC system and its possible use as a fertiliser.

II) Rumen derived anaerobic digestion (RUDAD)

Several years later an *in vitro* continuous fermentation reactor, called rumen-derived anaerobic digestion (RUDAD), was designed and developed by Gijzen *et al.* (1987). This reactor is designed to replicate conditions found in the rumen to enhance the degradation of lignocellulosic biomass. The RUDAD system consist of two reaction vessels (**Figure 2.7**). The first part of the system consists of a 3 ℓ vessel which has a working volume of 1,5 ℓ and is kept at 39^{o}C to maintain mesophilic conditions (Gijzen *et al.,* 1987). The first reaction vessel has an overflow tube for the removal of effluent; an inlet tube for adding artificial saliva; a gas-pipe for the gas to escape; and a tube connected to a filter for the removal of effluent into the second reactor. The filter unit is cylindrical and consists of stainless-steel wire gauze (pore size of 0.30 mm) and a glass tube with a Perspex disc on top of the cylinder, with the whole unit wrapped in a single layer of nylon gauze (pore size of 30 µm) (Gijzen *et al.,* 1987). The filter unit is inserted to the reactor (**Figure 2.7**). The second part of the system consists of a common anaerobic digester such as the up-flow anaerobic sludge blanket (UASB) with a total volume of 2.5 ℓ, as described by Gijzen *et al.* (1987). Reactor one (acidogenesis) and reactor two (methanogenesis) are connected with the tube connected to the filter. The flow of the effluent from reactor one to reactor two is controlled with a peristaltic pump.

At the start of the RUDAD system, the parts are assembled, and the reactors are filled with strained rumen fluid and artificial saliva. The feedstock is then added to the acidogenic reactor with the use of filter paper cellulose after being reduced to a particle size of 5-10 mm. Every day new feedstock is added, and the contents mixed at intermitted intervals with a rotary shaker. The volume of 1.5 ℓ is maintained with an automated peristaltic pump to automatically remove the homogenous fermenter contents to the overflow vessel. The first stage of the system consists of a mixture of rumen bacteria and ciliates to implement hydrolysis and acidogenesis of lignocellulose biomass. This is followed by the second stage which is the production of methane gas from the volatile fatty acids.



As this two-staged digester has been shown to have high loading rates, it can thus be applied in small digester volumes, together with a UASB, to enhance the biogas production (Gijzen *et al.,* 1987). A few RUDAD process systems have been applied to industrial uses (Deublein & Steunhauser, 2008). Different optimisations have been undertaken in the RUDAD system, such as the OLR, HRT and dilution rate of artificial saliva inserted (Gijzen *et al.,* 1987; Gijzen *et al.,* 1988). The RUDAD system gives an indication of the possibility of using a multi-staged digestion with rumen-based microorganisms which could be used to degrade lignocellulose biomass and enhance the production of biogas. The RUDAD system is more advantageous than the RUSITEC system due to the possible higher organic loading rates and the method of loading compared to the RUSITEC system. However, the RUDAD system is more expensive than the RUSITEC system due to the phase separation.

2.3.3 Production of protein in digestate of rumen based anaerobic digestion

The protein in rumen can be categorised as rumen degradable protein (RDP) and rumen undegradable protein (RUP). RDP is described as the proteins from the feedstock that are degraded in the rumen of the cow and used as a nitrogen source for the growth of the microorganisms (Tedeschi *et al.,* 2015). In contrast, RUP is described as the proteins from the feedstock that move through the rumen of the cow and form part of the metabolizable protein (MP) in the intestines of the cow (Tedeschi *et al.,* 2015). The metabolizable protein, a combination of the RUP of the feedstock and the single cell protein, ultimately serves as a quality protein in the cow to produce milk (de Ondarza, 2001). In common anaerobic digestion, the proteins are degraded by the microorganisms in the digester and cause an accumulation

of ammonia that can result in system failure due to a low C:N ratio (Al Seadi *et al.,* 2008). Because of the short HRT of the feedstock in the RUSITEC and RUDAD systems, there is a continuous production of microorganisms as the microbes are continually fed. According to Czerkawski and Breckenridge (1977), the more feed introduced in the RUSITEC system, the higher the protein content in the effluent. This can be accounted for the microbial populations increasing in the system. Other studies have shown that the microbial populations differ for different feedstocks used in an *in vitro* system (Martínez *et al.,* 2010; Lengowski *et al.,* 2016). This is an interesting observation as it may indicate that different feedstocks may have a higher production of protein due to the presence of more microorganisms. Lengowski *et al.* (2016) have also affirmed that crude protein from corn silage and grass silage solid digestate in a RUSITEC system increased from 8.1% DM to 67.4% DM and 17.1% DM to 66.5% DM, respectively. The increase in crude protein has significant implications as the solid digestate can potentially be mixed with animal feed to increase the RUP in feed. The increase of RUP will lead to higher metabolizable protein for the cow and better milk production.

2.4 Factors affecting the anaerobic digestion of lignocellulose biomass

There are a range of different factors summarised in **Table 2.3** that impact the digestion and overall biogas production of lignocellulose biomass during anaerobic digestion. Some of these parameters will be discussed in the following section.

Table 2.3: Factors affecting anaerobic digestion of lignocellulose biomass

Factor	References
pH	(Cioabla *et al.,* 2012; Nizami *et al.,* 2009; Zhang *et al.,* 2017)
Temperature	(Duarte *et al.,* 2017; Banks *et al.,* 2008; Madigou *et al.,* 2019)
VFA concentrations	(Shi *et al.,* 2017; Madigou *et al.,* 2019; Nguyen *et al.,* 2019)
Dissolved hydrogen	(Boe *et al.,* 2010)
C:N ratios	(Shi *et al.,* 2017; Sayara & Sánchez, 2019)
Hydraulic retention time (HRT)	(Liu *et al.,* 2018; Ezekoye, Ezekoye & Offor, 2011)
Organic loading rate (OLR)	(Liu *et al.,* 2018; Zhou *et al.,* 2017)
Total solids and volatile solids	(Yi *et al.,* 2014; Dioha *et al.,* 2013; Koch *et al.,* 2009)
Lignin content	(Chiumenti *et al.,* 2018; Lizasoain *et al.,* 2017; Lehtomäki *et al.,* 2008)

2.4.1 pH

The optimal pH for the first two steps during anaerobic digestion varies greatly, but has been reported to be between pH 4 and 6.5 (Nizami *et al.,* 2009). However, one study determined that the optimal pH for the functioning of the enzymes during hydrolysis of lignocellulosic biomass is around pH 6, but this may vary based on the inoculum source used for treating the feedstock (Dewar *et al.,* 1963). Even though the pH differs between the different phases of the anaerobic digestion process, it serves as an indicator of the efficiency of the system and the presence of inhibitory products.

The effect of pH between 5.5-7.5 on the degradation of cellulose and production of hydrogen has been studied by Zhang *et al.* (2017). The study collected rumen residue from a buffalo cow and four different pH treatments were implemented on the rumen in a batch reactor. It was found that a pH of 6.5 had the highest amount of cellulose degradation and hydrogen production. A pH below 6.5 is due to the higher concentration of acetate and butyrate which can inhibit the functioning of an anaerobic digester in high concentrations. A similar study assessing the effect of different pH, conducted in batch reactors using grass as a feedstock and cow dung as an inoculum, revealed that optimal biogas production was carried out at a pH of 6.5 (Sibiya *et al.,* 2014). Another study found that an adjustment of the pH to 6 in a two-stage leach bed reactor led to a lower rate of hydrolysis, acidification and methanogenesis (Lehtomäki *et al.,* 2008).

2.4.2 Temperature

The effect of temperature on an anaerobic digester has also been studied extensively. An anaerobic digester can be run in thermophilic or mesophilic or psychrophilic reactors. The thermophilic bacteria in a thermophilic digester function at a temperature of 50-60^{o}C compared to mesophilic bacteria which are optimal in a digester run at 32-42^{o}C. Psychrophilic digesters

function at a temperature lower than 20°C (Martí-Herrero *et al.,* 2019). One study, analysing the effect of temperature on the digestion of food waste in pilot-scale anaerobic digesters (Banks *et al.,* 2008), shows that the mesophilic reactor is much more stable compared to the thermophilic reactor. Though the thermophilic reactor had a faster hydrolysation time, this is risky due to the fast rate of volatile fatty acid production, leading to a lower pH and overall process instability. Another study investigating the effect of modifying the temperature in an anaerobic digester from 35°C to 55°C and then restoring it to 35°C (Madigou *et al.,* 2019), found that the increase in temperature from 35°C to 55°C increased the biogas produced for a couple of days, but afterward had detrimental effect where it lowered the biogas production. However, while a steady rate was achieved at each temperature, the increase in temperature led to an increase in acetate produced. This is expected in thermophilic reactors as they increase the hydrolysis of the feedstocks which will result in an increase in VFAs, as evident in the study.

2.4.3 C:N ratio

The carbon-to-nitrogen (C:N) ratio of the feedstock has an effect on the ammonia concentrations which can inhibit the performance of the digester (Sayara & Sánchez, 2019). The sole purpose of the C:N ratio is to serve as an indicator of the availability of the feedstock for growth for the various microorganisms. Most lignocellulose products have a high C:N ratio and may result in a low gas yield due to too low nitrogen for microorganisms to grow (Deublein & Steunhauser, 2008). However, if the ratio is too low it could lead to high ammonia production due to too much nitrogen available. Although it is difficult to ascertain the optimal C:N ratio as it differs for different processes and feedstocks, it has been suggested that the optimal range of C:N ratio for successful anaerobic digestion of grass should be between 20-30 (Dussadee, Unpaprom & Ramaraj, 2016; Bhandari, 2017; Sayara & Sánchez, 2019). Lignocellulosic biomasses have a high C:N ratio due to the high carbon and low nitrogen content in the biomass. Consequently, the co-digestion of lignocellulosic biomass with feedstocks high in nitrogen can engender a more stable anaerobic digester process.

2.4.4 Hydraulic retention time and organic loading rate

Two main factors contributing to the functionality of the digester are the hydraulic retention time (HRT) and organic loading rate (OLR) (Liu *et al.,* 2018). The HRT is the number of days the given feedstock is in the digester, resulting in the formation of methane. However, the HRT differs between different feedstocks and different digesters based on its degradability. The HRT is correlated to the volume of the tank and the amount of feedstock fed to the reactor (Al Seadi *et al.,* 2008). The following **Equation 2.11** represents the HRT of a reactor (Al Seadi *et al.,* 2008):

$$HRT = V_R / V \qquad (2.11)$$

Where HRT is the hydraulic retention time; V_R is the volume of the digester in (m^3); and V is the volume feedstock fed per time unit (m^3/d). A low HRT can lead to a low production of biogas and too high HRT can minimise the economic feasibility of a digester (Ezekoye, Ezekoye & Offor, 2011).

Equation (2.11) suggests that an increase in the feedstock fed per time unit will decrease the HRT. Together with the HRT, the OLR is one of the most important parameters to increase the biogas yield and justify the use of the digester and feedstock. However, as few studies have investigated the optimisation of the OLR and HRT of different rumen-based systems, more are required. **Equation (2.12)** below indicates how to calculate the OLR of a digester:

$$OLR = m * c / V_R \quad (2.12)$$

Where OLR is the organic load in (kg/d/m³); m is the mass of substrate fed per time unit in (kg/d); c is the concentration of organic matter in (%); and V_R is the digester volume in (m³).

2.4.5 Total solids and volatile solids content

The digester can function as a wet-based digester (total solids [TS] content is 10%-25%) or a dry-based digester, where the TS content is 30%-40%) (Karagiannidis & Perkoulidis, 2009). The TS in an anaerobic digester refers to the dry matter, whereas the volatile solids refer to the total amount of organic compounds in the feedstock that can be degraded. A study analysing the effect of an increase of total solid content from 5% to 20% in an mesophilic anaerobic digester found that an increase led to higher volatile solid reduction and methane yields (Yi *et al.,* 2014). However, the higher methane yields are due to higher organic loading rate leading to a higher TS content which increases the feedstock availability and microbial growth. A study analysing the effect of the harvest period of different grasses has shown that an increase of 10% VS content in summer grass compared to spring grass did not have a significant increase in the biogas production (Chiumenti *et al.,* 2018). The study also indicated that the lignin content in the grass is an attributing factor to the rate and amount of biogas produced.

2.5 Inhibiting substances on anaerobic digestion

Different substances are known to inhibit anaerobic digestion and must be monitored to provide adequate and optimal biogas production. Various microorganisms in an anaerobic digester require optimal concentrations of nutrients for their growth and metabolism. A deviation from the optimal range of these elements may indicate low performance and lead to digester failure.

2.5.1 Ammonia concentration

The ammonia in the digester comes primarily from the broken-down proteins and urea (Al Seadi *et al.,* 2008). The ammonium ion (NH_4^+) and free ammonia/ NH_3 (FA) are the two main

ammonia compounds found in liquid form (Chen *et al.,* 2008). Free ammonia is known for its ability for process inhibition. It has been suggested that due to the inhibitory effect of free ammonia, the concentration should be maintained below 80 mg/L (Al Seadi *et al.,* 2008). The temperature and pH of the reactor have a significant effect on the free ammonia in the digester and therefore, lowering the temperature or pH can lead to lower formation of ammonia, but it might also inhibit the growth of microbes in a thermophilic digester (Al Seadi *et al.,* 2008; Shi *et al.,* 2017). It has also been indicated that the propionate and valerate are VFA that are influenced by free ammonia concentration in the digester (Shi *et al.,* 2017).

2.5.2 Oxygen concentration

In any anaerobic digestion process the amount of oxygen in the system will limit the growth of anaerobic organisms. Facultative anaerobic microbes are able to grow in low concentrations of oxygen, but methanogens are strictly anaerobic organisms and need an optimal oxygen reduction potential (ORP) of between -200 and -400 millivolts (mV) (Gerardi, 2003).

2.5.3 Sulphate concentration

The sulphate concentration is reduced by sulphate reducing bacteria (SRB) during anaerobic digestion to sulphide and termed assimilatory sulphate reduction (Gerardi, 2003). SRB, also known to be direct competitors to methanogens for the organic and inorganic substrates in an anaerobic digester, will lead to less methane production (Harada *et al.,* 1994). Even though sulphate is non-inhibitory to the anaerobic digestion process, its reduction leads to the formation of dissolved hydrogen sulphide (H_2S) by the SRB (Gerardi, 2003). This reduction reaction has a toxic effect on other microorganisms in the anaerobic digester and can limit the rate and amount of biogas produced (Colleran *et al.,* 1998).

2.6 Pre-treatments to enhance biogas production from lignocellulosic biomass

There are different ways to enhance the biogas production of lignocellulosic biomass. Lignocellulosic biomass contains lignin which is resistant to degradation in the anaerobic digestion process. Therefore, different pre-treatments could be used to improve the degradability of lignocellulosic biomass. The pre-treatments of biomass can be divided into three categories – biological, mechanical or chemical pre-treatments (Wyman & Yang, 2009) – implemented to increase the target surface for enzyme degradation and accelerate the digestion process (Mood *et al.,* 2013; Rodriguez *et al.,* 2017).

2.6.1 Biological pre-treatment

The biological pre-treatment of lignocellulosic biomass involves treating the biomass with bacterial and fungal communities to enhance the excretion of enzymes and improve the digestibility of the biomass. It has been reported that the pre-acidification step, otherwise known as anaerobic microbial pre-treatment, which separates the first stage from the second

stage in anaerobic digestion led to an increase the biogas produced (Ghanimeh *et al.,* 2019). This strategy enables optimal growth for microorganisms in the reactors. Other studies have likewise evaluated the pre-treatment of three different microbial consortia and their effect on biogas production of Napier grass (Wen *et al.,* 2015). After developing their own microbial consortiums, WSD-5, XDC-2 and MC1, from different environments, the Napier grass was pre-treated in the microbial consortium for 21 days. The pre-treatments of microbial consortiums had a higher methane yield compared to the control sample. Another biological pre-treatment involves the addition of different enzymes to improve the degradation of the substrate. Enzymes require an optimal pH, temperature and incubation time to achieve an effective result (Parawira, 2012). There are many different enzymes produced by different microorganisms which can be used to treat lignocellulose biomass in an anaerobic digester. Among the different enzymes used in anaerobic digestion, the most common enzymes are cellulase, beta-glucosidase and xylanase, acknowledged to improve the methane production from biomass (Parawira, 2012). Only a few enzymes have been tested on Napier grass (Wen *et al.,* 2015) and *Miscanthus giganteus* (Michalska *et al.,* 2015) with great efficiency to increase the amount of methane produced.

2.6.2 Physical pre-treatment

The physical pre-treatment involves physically treating the feedstock before adding it to the reactor to improve the digestibility of the lignocellulosic biomass. Some physical pre-treatments involve mechanical and ultrasound pre-treatments (Rodriguez *et al.,* 2017). Mechanical pre-treatment involves the reduction of the particle size of the feedstock, enhancing its availability for microorganisms to metabolise. Reducing the particle size of forage has an impact on the rate of anaerobic digestion and increases the biogas production in a RUSITEC system (Duarte *et al.*, 2017). It has,, however been reported that the particle size varies greatly for the anaerobic digestion (Raposo *et al.,* 2012). Thus, it is difficult for long fibrous grasses to be milled as this results in clogging and the cost to run the mill escalates beyond that of the biogas produced. A recent study, evaluating the use of ultrasound alkaline as a pre-treatment to improve the delignification of the different substrates (Subhedar *et al.,* 2018), found that the alkaline pre-treatment had a 38%-41% delignification, whereas the ultrasound alkaline pre-treatment had a 70%-80% delignification on the substrates with a lignin content ranging between 29-32%. Only one study thus far has tested the use of ultrasound-acid pre-treatment on grass to improve H_2 production, finding that the combined effect of ultra-sound and acid pre-treatment increases H_2 production to the control by 318% (Subhedar *et al.,* 2018). However, the use of only ultrasound pre-treatment showcased a small increase compared to the control, whereas the diluted acid pre-treatment had a significant effect on H_2 production. Therefore, combining the two treatments can significantly affect the production of biofuels.

2.6.3 Chemical pre-treatment

Treating the feedstock with different alkalis or acids can open the lignocellulose contents for enzymatic degradation. Alkali pre-treatment has been identified as more effective to open the lignin content whereas acidic pre-treatment is known to increase hemicellulose solubilisation (Michalska *et al.,* 2015; Rodriguez *et al.,* 2017). However, the use of chemical pre-treatments is not economically viable on an industry scale, as highly concentrated acids are expensive. The use of diluted alkali or acids may be of more importance and more economically viable in the chemical pre-treatment of feedstocks. Therefore, mainly using biological and physical pre-treatments as opposed to chemical pre-treatments has received more attention in recent years. It has been suggested that diluted acids are more economically viable and can therefore be used in combination with physical and biological pre-treatments to increase the biogas production (Rodriguez *et al.,* 2017).

CHAPTER 3

MATERIALS AND METHODS

This chapter presents a detailed description of the methods followed in this study. The feedstock samples, inoculum collection, digester design, digester set-up, buffer medium, gas measuring system, experimental design and data analysis are explained in this chapter. The experiments are divided into four phases: i) digester design and functioning; ii) biogas production potential and VFA production from different inoculum to substrate ratios using rumen fluid as inoculum; iii) evaluation of the biogas production from three different lignocellulose feedstocks; and iv) testing of the protein and NPK content in the digestate from three different lignocellulose feedstocks.

3.1 Design and functioning of rumen digester

The adapted designed RUSITEC system consisted of two high density polyvinyl chloride (HDPVC) reactors (**Figure 3.1**). The two reactors are interlinked with two gas-tight HDPVC pipes at the top and bottom where rumen fluid can freely flow through without clogging the pipes. Reactor 1 has a screw cap lid where the nylon bags (100µm) containing the feedstock and rumen solids can be added (**Figure 3.1**). The lid of reactor 2 has three ports, each sealed with a PVC gland to ensure it is gas-tight and to maintain anaerobic conditions (**Figure 3.1**). The functioning of the reactor is similar to the functioning of other RUSITEC reactor systems (Czerkawski & Breckenridge, 1977; Martínez *et al.,* 2009, 2010; Duarte *et al.,* 2017). Both reactors are sealed by means of a screw cap and the pipes are connected to the lid of reactor 2 where the gas and effluent will be able to move through the overflow pipe into the overflow vessel and then the gas bag (**Figure 3.2**). The entire assembled RUSITEC system is placed in a thermostatically controlled water bath at 40ºC to function under mesophilic conditions (**Figure 3.2**). On the first day of incubation, barley straw (20 g) and rumen solids (40 g) were added to separate nylon bags with a size of 8 cm x 15 cm (pore size of 100 µm) and the two nylon bags were inserted in reactor 1 (**Figure 3.1**).

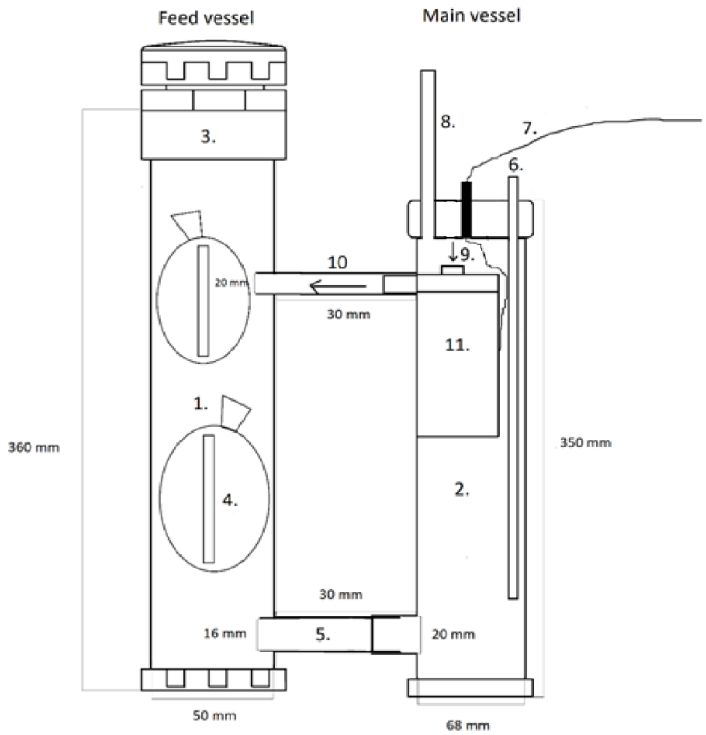


Figure 3.1: Design of rumen-anaerobic digester 1) Reactor one 2) Reactor two 3) Screw cap lid of reactor one 4) Nylon bag with rigid tube 5) Bottom flow through pipe 6) Artificial saliva input port 7) Electrical cord for magnetically coupled pump 8) Overflow pipe 9 & 10) Flow direction 11) Magnetically coupled pump (submersible)

The reactor was filled with 1.5 L strained rumen fluid. The artificial saliva solution (**Table3.1**) with adjusted pH of 8.2 (van Soest, 1970) was infused with a peristaltic pump (Watson Marlow sci 323) at 1500 mL/day where the inlet tube extended to the bottom of reactor 2 to mix the contents (**Figure 3.1**). Each day the vessel was opened, and the nylon bags removed and replaced with a new nylon bag filled with barley straw (20 g). The original nylon bag with rumen solids was rejected. After every second day, the nylon bag that was incubated for two days was removed and gently squeezed and washed with 40mL artificial saliva solution. After the wash, the fluid is added back into the reactor. The overflow pipe is connected to an overflow vessel, which is placed in an ice bath at 3-4^{o}C for the storage of effluent (**Figure 3.2**). The pressure from the peristaltic pump to add artificial saliva in reactor 2 will cause the effluent to flow through the overflow pipe to the overflow vessel at the same rate that the peristaltic pump adds artificial saliva in the system. The overflow vessel has a gas outlet pipe connected to a Tedlar bag (**Figure 3.2**). For the testing of the reactor system the reactor was run for 15 days for biogas production (seven days adaptation and eight days testing), similar to other RUSITEC systems (Duarte *et al.,* 2017).

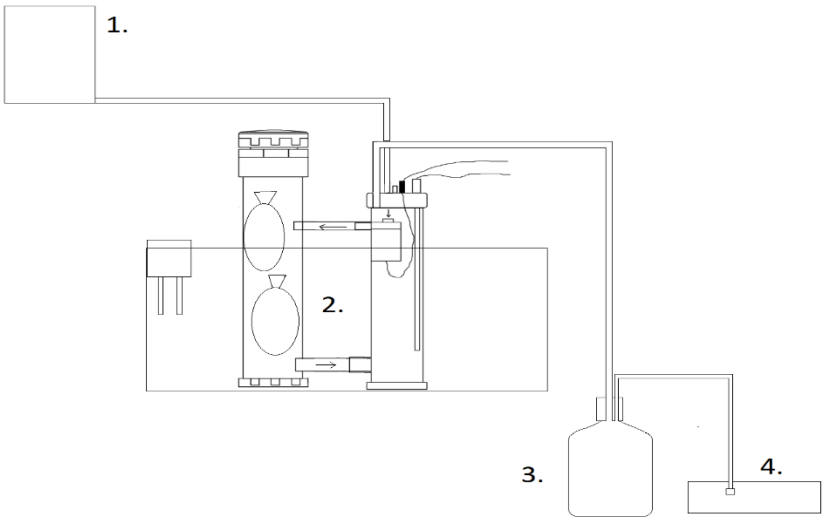


Figure 3.2: Layout of system 1) Peristaltic pump for input of artificial saliva 2) Rumen digesters in a thermostatically controlled water bath (37°C) 3) Overflow vessel in ice bath (2.5L) 4) 5 L Tedlar bag

3.2 Sampling of lignocellulosic feedstocks

Different lignocellulose feedstocks – Napier grass, kikuyu grass and barley straw – were sampled from farms outside Stellenbosch and Malmesbury, South Africa, respectively. The feedstocks were cut with scissors and transported in air-tight plastic containers to the laboratory where they were stored at -4°C. The feedstocks were milled through a hammer mill with a mesh size of 4 mm to ensure the feedstocks had similar particle sizes. The particle size is chosen from other studies that had the highest biogas production from various grass samples (Tavakoli *et al.,* 2009; Raposo *et al.,* 2012) as the cost to sieve these long grasses through a smaller sieve size would increase the energy cost for the pre-treatment significantly (Tavakoli *et al.,* 2009).

3.3 Sampling of inoculum

Fresh rumen fluid was collected prior to each *in vitro* run from two ruminal cannulated lactating Holstein cows located on the Welgevallen Experimental Farm at University of Stellenbosch, South Africa. All rumen collections were in accordance with the rumen extraction protocol of the University of Stellenbosch, with the help of a research assistant. A thermos flask was prewarmed with boiling water before being transported to the farm. The boiling water was removed and rumen fluid collected from the rumen of the cows and filtered through two layers of cheesecloth to remove all solid materials before being transported to the laboratory in prewarmed thermos flask (2L). The flask was filled to keep the contents free of oxygen. The rumen fluid was gassed with CO_2 before use to maintain an anaerobic environment and the pH and temperature were measured.

3.3 Incubation medium and solutions

All solutions were prepared as described by Goering and Van Soest (1970) and Van Soest, Robertson, and Lewis (1991). The composition of the buffer, macro-mineral, micro-mineral and reducing solution can be seen in **Table 3.1**. Resazurin solution was made by adding 0.1g Resazurin in 100mL distilled water to create a 0.1% solution. The solution was stored at 4ºC in a glass container.

Table 3.1: Chemical composition of the solutions (Goering & Van Soest, 1970)

Reagent	Quantity
1 L Buffer solution:	
Distilled water (dH_2O)	1000 mL
Ammonium bicarbonate (NH_4HCO_3)	4 g
Sodium bicarbonate ($NaHCO_3$)	35 g
1 L Macro-mineral solution:	
Distilled water (dH_2O)	1000 mL
Di-sodium hydrogen orthophosphate (Na_2HPO_4) (anhydrous)	5.7 g
Potassium dihydrogen orthophosphate (KH_2PO_4) (anhydrous)	6.2 g
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.6 g
100 mL Micro-mineral solution:	
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	13.2 g
Manganese chloride tetrahydrate ($MnCl_2.4H_2O$)	10 g
Cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$)	1 g
Ferric chloride hexahydrate ($FeCl_3.6H_2O$)	8 g
3.7 L Incubation medium (30 samples):	
Distilled water (dH_2O)	600 mL
Tryptose	3 g
Micromineral solution	150 µL
Buffer solution	300 mL
Micromineral solution	300 mL
Resazurin	1.5 mL
160 mL Reducing solution (30 samples):	
Flask A:	
Distilled water (dH_2O)	30 mL
Cysteine hydrochloride ($C_3H_7NO_2$ HCl)	0.375 g
Potassium hydroxide (KOH) pellets	15
Flask B:	
Distilled water (dH_2O)	30 mL
Sodium sulphide nonahydrate (NaS)	0.375 g

3.4 Equipment set-up

3.4.1 Biogas production potential set-up

Glass vials (with a total volume of 120 mL) were used as anaerobic digesters for the various feedstocks. The vials were incubated in a controlled incubator which maintained a temperature of 38.8°C. Each vial had a magnetic stirrer (0.2 mm) added which stirred the contents for two hours in three-hour intervals at 250 rpm. The exact volume of each vial was predetermined as it was required for the calculation of the total gas produced. Prior to the experimental set-up, each vial was filled with milled feedstock and 40 mL of the reduced incubation medium was added into each (**Table 3.1**). This was followed by the addition of10 mL of the inoculum to each vial, making up a total working volume of 50 mL.

3.4.2 Gas measurement system

The reading pressure technique (RPT) was used to determine the gas produced (Mauricio *et al.,* 1999). The pressure in each vial was measured with a surgical needle and precision digital pressure gauge model (CPG1500) daily. To prevent the pressure from becoming too high in the head space of the vial (≥ 9 psi), the pressure was released on regular intervals with a surgical needle.

3.4.3 Gas quality measurement

The gas quality was measured at the end of the experimental run. The gas, sampled from the pressured vessels with a surgical needle and 50mL syringe, was analysed with a portable Biogas Analyzer 5000 (Geo. Tech, UK). The methane content was adjusted with **Equation 3.1** according to German standard methods (Kafle & Kim, 2012):

$$CH_{4\,Corr.} = \frac{CH_4.100}{CO_2 + CH_4} \quad (3.1)$$

Where: $CH_{4\,Corr.}$ = corrected methane content in the gas (%)

CO_2 = Measured carbon dioxide content from Biogas Analyzer 5000 (%)

CH_4 = Measured methane content from Biogas Analyzer 5000 (%)

3.4.4 Conversion of pressure to gas volume

The linear equation (**Equation 3.2)** was developed for the setup in the Department of Animal Sciences, University of Stellenbosch, and was used to convert the pressure in the glass vial to the gas volume at standard temperature and pressure (STP):

$$Y = \frac{[(1000\,((0.0977\,X)\,C)]}{VS} \quad (3.2)$$

Where: Y = Gas volume (mL/g VS)

X = Gas pressure (psi)

C = Vial head space (mL)

VS = Volatile solids (mg)

3.4.5 Kinetic coefficients, modelling and theoretical biogas potential

The cumulative biogas in the biogas potential tests were determined by subtracting the biogas produced from the control to that of the different samples, as demonstrated in **Equation 3.3**.

$$B = Bt - Bi \quad (3.3)$$

Where: B = Total real volume of biogas

Bt = Biogas from feedstock

Bi = Biogas from inoculum

The biogas production from the experimental data was fitted to various non-linear models to determine the rate and other kinetic parameters of the different reactions and the best fit. The models used in this study (summarised in **Table 3.2**) are the first-order model, Monod type model and modified Gompertz model.

Table 3.2: Models used to describe gas production and kinetics

Model name	Equation	Reference
First order (FO)	$B(t) = B_0\,(1 - \exp^{-kt})$	(Strömberg, Nistor & Liu, 2015)
Monod	$B(t) = B_0 \frac{(k.t)}{(1+k.t)}$	(Junker, Coors & Schüürmann, 2016)
Modified Gompertz (GM)	$G(t) = Gmax\,.\,e^{-e^{(\frac{Rm.e}{Gmax}.(\lambda - t)+1)}}$	(Zwietering *et al.,* 1990)
Two-fraction first order (TFFO)	$B(t) = B_0\left(1 - \alpha.\exp^{-k_F.t} - (1-\alpha).\exp^{-k_S.t}\right)$	(Ponsá, Gea & Sánchez, 2011)

Where: B(t) = cumulative biogas volume at time t

B_0= maximum biogas produced (mL g^{-1} VS)

μ_m = maximum rate of biogas production

λ = lag time in days

t = time (day)

exp = natural exponent (2.7183)

k, k_S, k_f, and α are fitted constants

3.5 Experimental set-up

3.5.1 Effect of ISR and organic load on biogas production

Various concentrations of barley straw were used to determine the optimal substrate to inoculum ratio for biogas production when using rumen fluid as inoculum and barley straw as a feedstock. The values were obtained from a previous experiment done by Gjizen et al., 1987 while using a continuous reactor system. The experiment was conducted under mesophilic conditions (38-39^{o}C) to replicate the conditions in the rumen of a cow. Each treatment was run in triplicate in 120 mL nominal volume reactors and a 50 mL working volume as described in **Section 3.4.1** and the gas was measured as described in **Section 3.4.2**. **Table 3.3** shows the experimental layout to test the different inoculum to substrate ratios. The digestion was terminated when the gas production plateaued in all the reactors. At the end of the run, samples were taken from each vial to determine the volatile fatty acids (VFA) and the total solids digested. The quality of the gas produced was calculated and corrected (as described in **Section 3.4.3**). The control was inoculated with the buffer medium and inoculum with no substrate added.

Table 3.3: Different inoculum to substrate ratios experimental design

Samples	Inoculum (mL)	Substrate added (gWW)	Organic load (gVS/L)	Inoculum-to-substrate-ratio (VS)
1-3	10	0	n/a	n/a
4-6	10	0.125	2.03	1.36
7-9	10	0.250	4.07	0.68
10-12	10	0.500	8.14	0.34
13-15	10	0.750	12.21	0.23
16-18	10	1.000	16.24	0.17
19-21	10	1.500	24.41	0.11

3.5.2 Biogas production from different lignocellulose feedstocks

Napier grass, kikuyu grass and barley straw were incubated in 120 mL nominal volume and 50 mL working volume in triplicate (as described in **Section 3.4.1**) and the gas was measured (as described in **Section 3.4.2**) to determine their biogas production. The experimental set-up is summarised in **Table 3.4**. The substrates had the same number of volatile solids added to achieve the same inoculum to substrate ratio based on the volatile solids added. The quality of the gas volume produced was measured (as described in **Section 3.4.3**). The control was inoculated with the buffer medium and inoculum with no substrate added.

Table 3.4: Experimental layout for biogas production tests of different lignocellulosic biomass

Samples	ISR	Inoculum (mL)	gWW* substrate	gVS substrate
Napier grass (1-4)	0.17	10	1.14	0.81
Kikuyu grass (5-9)	0.17	10	1.45	0.81
Barley straw (10-13)	0.17	10	1.00	0.81
Control (14-16)	0	10	0	0

*gram wet weight

3.5.3 Total nitrogen, phosphorus, and potassium in digestate of different feedstocks

Napier grass, kikuyu grass and barley straw were incubated in triplicate (as described in **Section 3.4.1**) and the gas was measured (as described in **Section 3.4.2**) to determine their biogas production potential. The gas volume produced was calculated (as described in **Section 3.4.3**). The experimental layout that was followed is shown in **Table 3.4**. After the digestion of the lignocellulosic biomass, the digestate was analysed to determine the NPK content in the digestate and its protein. The total protein in the sample is calculated by multiplying the total ammonium nitrogen by a factor of 6.25 (Lengowski *et al.,* 2016).

3.6 Input and output analysis

The total solids (TS), total suspended solids (TSS) and ash were determined for both the inoculum and feedstocks according to standard procedures (APHA, 2005). The ethanol and water extractives, lignin, cellulose and hemicellulose were determined for the different feedstocks according to the NREL laboratory analytical procedure (Sluiter *et al.,* 2008). The carbon, nitrogen, hydrogen, sulphur and oxygen of the different feedstocks were determined by central analytical facilities (CAF), Stellenbosch University, using an Elementar Vario EL Cube Analyzer. The pH was measured with a portable digital pH Pen Meter Tester (accuracy: +/- 0.1). The VFA of the inoculum and digestate were analysed with a high performance liquid chromatography (HPLC) using standard procedures (APHA, 2005). The total suspended solids (TSS) in the digestate were subtracted from the total solids (TS) added to the reactors to calculate the percentage digested total solids. The digestate of the reactors were sent to Elsenburg, Stellenbosch, South Africa, for analysis of the total Kjeldahl ammonium nitrogen, which can be converted to crude protein (CP) as N x 6.25, phosphorus and potassium content.

3.7 Data and statistical analysis

All samples were run in triplicate. The values are reported as the average of the triplicates, except if stated differently. The goodness of fit (GoF) from the experimental data and the models was evaluated by examining the residual sum of squares (RSS) and coefficient of determination (R^2). The Solver option in Microsoft Excel was used to evaluate the best fit

between the different models, with the R^2 indicating the efficiency of the fit. The results were statistically evaluated with the use of STATISTICA Software Version 13 through the Tukey-test to determine if the means of the samples are statistically different from one another. The results were statistically significant if the p-values were below 0.05.

CHAPTER 4

DESIGN OF RUMEN BASED ANAEROBIC DIGESTER FOR THE DIGESTION OF LIGNOCELLULOSE BIOMASS

Abstract

Lignocellulosic biomass, one of the most widely available resources in the world, exhibits much potential for biogas production using anaerobic digestion. The common designs of anaerobic digesters using energy crops are not suitable due to the recalcitrance of the feedstock, which requires high retention times for biogas production. This study aimed to design and build a rumen-based anaerobic digester working similarly to the RUSITEC digester. The rumen-based digester was operated for 15 days to determine the digestion characteristics of the feedstock and the biogas production of the digester. Rumen fluid was used as an inoculum, with barley straw used as the feedstock. The buffer solution (pH 8.2) was added at a rate of 1500 mL/day for the duration of the experiment to create a hydraulic retention time of one day. For the start-up of the reactor, 40 g rumen solids and 20 g wet weight barley straw was placed into nylon bags with a pore size of 100 µm and added into the reactor, resulting in two nylon bags continuously present in the reactor system. After one day of incubation, the nylon bag containing the rumen solids was removed and replaced with a new nylon bag with 20 g barley straw. Every subsequent day, the nylon bag with barley straw that was incubated for two days was removed and replaced with a new nylon bag with 20 g barley straw, resulting in a solid retention time of two days to determine the biogas production in the reactor. The biogas quantity and quality produced was measured with the water displacement method and biogas analyser 5000, respectively. The solid residue and liquid effluent were collected and analysed for the total nitrogen to determine the protein content. The average biogas produced from the digester was 12.63 mL/gVS/day, with an average of 43.95% of the total solids digested after two days solid retention time. The VFA in the reactor ranged between 0.85 g/Land 1.73 g/L throughout the 15 days. The protein content in the solid digestate increased from 4.75% of total solids to 7.5% of total solids on day ten but decreased to 1.5% on day 15 due to the low nitrogen content of the feedstock. The semi-continuous designed reactor had low cumulative biogas production of just over 180 mL, due to the incomplete digestion of the feedstock. Therefore, it would be of more value to digest the barley straw in a batch mode with rumen fluid to determine the biogas production of the feedstock while using rumen fluid as inoculum.

Keywords: barley straw, design, RUSITEC, rumen fluid, biogas

4.1 Introduction

There is an ever-increasing need for alternative energy in the world to reduce the detrimental impact of fossil fuels on global warming and reduce organic wastes from piling up in landfills. A great alternative source for energy production is the use of agricultural biomass in anaerobic digestion to produce biogas (Galván-Arzola *et al.,* 2019). The use of agricultural biomass in anaerobic digestion has the potential to serve as an alternative feedstock due to its fast growth and its high availability throughout the world (Dussadee *et al.,* 2016).

The design of the digester is important when digesting different feedstocks and to improve the anaerobic process and increase biogas yield (Ramatsa *et al.,* 2014). A wide range of digesters to lower cost and maximise the efficiency of the digestion process have been designed. There are certain factors to consider when designing a digester such as the hydraulic retention time, solid retention time, mixing and feedstock used (Ramatsa *et al.,* 2014). Lignocellulose feedstock is high in solids and can result in clogging and improper mixing in a digester (Bayané & Guiot, 2011). This has led to some rumen based anaerobic digester designs.

Rumen based anaerobic digestion has received some interest due to the high number of hydrolytic microorganisms found in the rumen of cows which can digest lignocellulose biomass in a shorter time than common anaerobic digesters (Gijzen *et al.,* 1988). The design of the rumen-based anaerobic digestion is based on how the rumen functions in an ruminant.

Ruminants are mammals that acquire food from a variety of different lignocellulose products such as grasses and leaves. However, these mammals do not contain the cellulase enzymes necessary to break down lignocellulose biomass (de Ondarza, 2001). There is a symbiotic relationship between the microorganisms in the gut and the ruminants to enhance the digestion of lignocellulose biomass and maximise the nutrients obtained from food. This method of food digestion is called rumination as it occurs in the part of the stomach called the rumen (Ørskov, 1986).

The rumen simulation technique (RUSITEC) and rumen derived anaerobic digestion (RUDAD) are the most famous methods for studying the working of the rumen (Czerkawski & Breckenridge, 1977; Gijzen *et al.,* 1988b). The RUSITEC system has been designed successfully on a small scale and used primarily to study microbial populations and ruminant feeding strategies. However, the use of the rumen-based anaerobic digester has not been tested for biogas production. Therefore, the functioning of the RUSITEC system can be used to test if the biogas production will increase due to the design. No study has yet been identified that has focused on building a single-stage digester similar to the functioning of the RUSITEC system for the production of biogas. Therefore, this present study intended to design and build a rumen-based anaerobic digester for the digestion of lignocellulose biomass using rumen fluid for biogas production.

4.2 Materials and methods

4.2.1 Feedstock and inoculum

The feedstock and inoculum used in this study are described in **Section 3.1** and **Section 3.2**, with barley straw being the sole feedstock used to test the reactor.

4.2.2 Incubation medium and solutions

The incubation medium and artificial saliva solution used in this study are described in **Section 3.3.** The total amount of artificial saliva solution made was 20L.

4.2.3 Rumen digester design and functioning

The design and functioning of the rumen digester are explained in **Section 3.1**.

4.2.4 Input and output analysis

The characteristics of the feedstock and inoculum were determined according to **Section 3.6**. The pH was measured daily with a portable digital pH Pen Meter Tester (accuracy: +/- 0.1). After the first day the rumen solids were removed and discarded. The nylon bags filled with barley straw were removed every two days from the digester and dried at 60^{o}C for two days to determine the total solids and prevent structural changes in the lignocellulose biomass. The solid residue samples were pooled together in three phases of the run: days 2-5, days 6-11 and days 12-15. The biomass samples were then analysed according to NREL analysis to determine the composition of the remaining residue in each sample. The liquid effluent was also pooled into three phases – days 1-5, days 6-11 and days 12-15 – and the volatile fatty acids were determined according to **Section 3.6**. The quantity of the biogas produced was according to the water displacement method (Mamun *et al.,* 2018) and quality of the biogas produced was determined according to **Section 3.4.3**. The solid residue was sent to central analytical facilities (CAF), Stellenbosch University, to determine the nitrogen content using an Elementar Vario EL Cube Analyzer. The liquid effluent was collected and frozen at -20^{o}C before being sent to Elsenburg, Stellenbosch, for the determination of the total nitrogen content of the effluent according to the Kjeldahl method. The protein content was then calculated as described in **Section 3.5.3**.

4.3 Results and discussion

4.3.1 Feedstock and inoculum characteristics and digestion

The characteristics of the inoculum and feedstock are summarised in **Table 4.1**. The inoculum had a total solids (TS) content of 4.01%, of which 68.65% were volatile solids (VS), and a pH value of 7.4 which makes it a suitable inoculum in anaerobic digestion. The barley straw feedstock had a high TS content of 89.72%, of which 97.18% was VS. The high VS indicates that most of the total solids are digestible in anaerobic digestion. However, a deeper look into the structure of barley straw reveals that the cellulose, hemicellulose, and lignin content were 33.78%, 29.74% and 20.23% of TS, respectively. The total extractives of barley straw were 17.61% of the TS. The extractives, hemicellulose and cellulose percentages are similar to that reported by other studies characterising barley straw (Serna-Díaz *et al.,* 2020). The digested barley straw was pooled in three different stages: days 2-5, days 6-10 and days 11-15. Samples of the digestate were taken and analysed to determine the loss in cellulose, hemicellulose, and lignin content. After 2-5 days, the cellulose and hemicellulose both decreased by 10% (**Table 4.1**). This decrease is expected due to the cellulose and hemicellulose being degraded by the rumen fluid microorganisms to its various monomers and the sugars used for the growth of the microbes. After 6-15 days, the cellulose and hemicellulose had a similar decrease of 10% and 9%, respectively (**Table 4.1**). The decrease in cellulose and hemicellulose reflects the wide range of different hydrolytic microbes in the rumen to digest the lignocellulose biomass (Nguyen *et al.,* 2019). The lignin content of the barley straw increased as the barley straw was digested (**Table 4.1**). The increase in the lignin content in the feedstock is due to its indigestibility: the microorganisms would rather digest the easier available resources such as cellulose and hemicellulose. Another study digesting different grasses found similar trends where the cellulose and hemicellulose decreased by 7% and 5% in the digestate, respectively (Chiumenti *et al.,* 2018). The lignin content in the digestate also increased slightly by 1%. The higher lignin content indicates that it is undegradable in anaerobic digesters which leads to a higher solid content in the reactor.

The total solids digested in the first few days in the digester (days 1-5) was 37.74% and the total solids digested increased to 48.40% as the digester continued to day 15 (**Table 4.1**). This increase in total solids digested is due to more microorganisms adapting to the conditions of the reactor system and establishing a colony in the reactor surface area which led to increased digestion of the feedstock. Another study of digesting lignocellulose in rumen fluid found a TS degradation of 30%-41% over a period of 48 hours (Rambau *et al.,* 2016). And another study of digesting ryegrass found a TS degradation of 60%-70% in rumen fluid (Duarte *et al.,* 2017).

Table 4.1: Characterisation of inoculum and feedstock on different days in the reactor

Feedstock	TS (%)	VS (%TS)	TS digested (%)	pH	Extractives (% TS)	Cellulose (% TS)	Hemicellulose (% TS)	Lignin (% TS)	Total VFA (g/L)
Inoculum	4.01±0.05	68.65±0.78	nd	7.4±0.1	nd	nd	nd	nd	4.04±0.02
Barley straw	89.72±0.04	97.12±0.41	nd	nd	17.61±2.27	33.78±0.52	29.74±3.52	20.23±1.49	nd
Day 2-5	nd	nd	37.74±10.40	6.88±0.16	10.73±0.64	23.6±1.88	18.98±1.93	27.84±0.08	1.75±0.39
Day 6-10	nd	nd	45.71±4.48	6.96±0.09	12.12±2.47	22.93±0.49	20.33±0.30	31.025±0.36	0.85±0.28
Day 11-15	nd	nd	48.40±5.79	6.78±0.11	9.01±2.30	23.44±1.85	20.26±1.24	31.385±1.28	1.73±0.43

**nd – not determined*

The volatile fatty acids (VFAs) decreased during the experiment to day 10 which is an indication of the already established methanogenic archaea found in the rumen of the cow that digested the VFA to produce carbon dioxide and methane gas. The significant increase in VFA from 0.85 g/L on day 10 to 1.73 g/L on day 15 may be caused by the short retention time of the biomass feedstock and a higher population of hydrolytic microorganisms in the reactor (**Table 4.1**). Another reason for the increase in VFAs may be due to more total solids digested and not enough methanogenic bacteria to convert the volatile fatty acids into methane gas, due to the longer reproduction time of the methanogenic bacteria (Gerardi, 2003). The overall pH of the reactor during the experiment was stable between 6.78 and 6.96.

4.3.2 Biogas and methane production from rumen digester

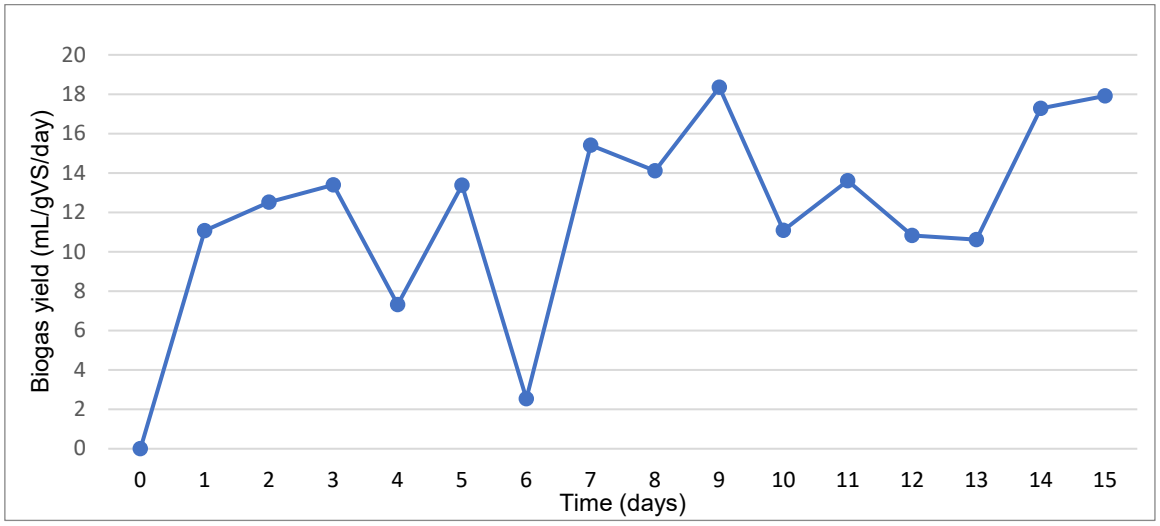


Figure 4.1: Daily biogas production in the designed reactor

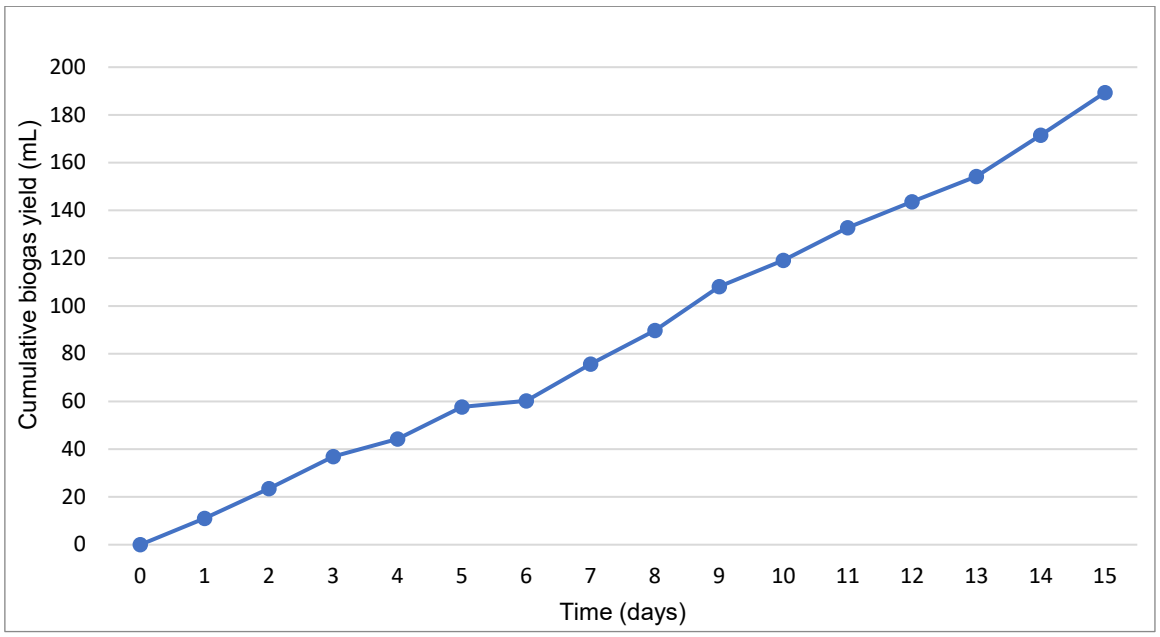


Figure 4.2: Cumulative biogas yield over time of barley straw in the designed rumen digester

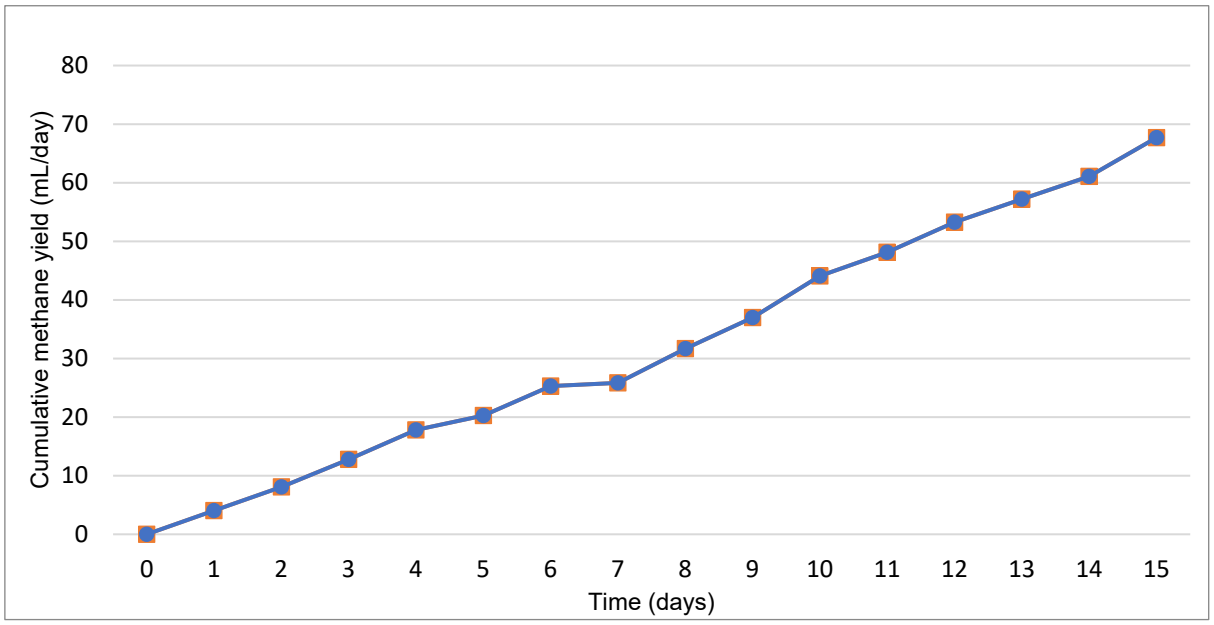


Figure 4.3: Cumulative methane yield over time of barley straw in the designed rumen digester

The biogas produced over the different days in the rumen digester can be seen in **Figure 4.1**. The cumulative biogas and methane produced from the designed digester followed a linear trend for the duration of the experiment (**Figure 4.2 & Figure 4.3**), respectively. The biogas produced daily was 12.63 mL/gVS/day. The biogas production was low compared to other studies digesting barley straw as a substrate which produced 130 mL/gVS, at an HRT of 12 hours (Kivaisi *et al.,* 1992). A study digesting various straw in batch digesters found that the biogas produced ranged between 25 mL/gVS and 60 mL/gVS (Nguyen *et al.,* 2019). The sudden drop of biogas production on day 4 and day 6 is due to a malfunction in the peristaltic pump which was unable to add artificial saliva to the reactor, resulting in a lower the pH of the

reactor. The average biogas produced each day was 12.63 mL/gVS/day. The average methane percentage of the biogas produced each day was 41.5%. The methane percentage of other rumen reactors ranged between 30% and 42% when digesting cereals and other lignocellulosic waste (Gijzen *et al.,* 1987, 1988b; Kivaisi *et al.,* 1992). Less than 50% of the added substrate was digested during the running of the reactor, resulting in a lower biogas production than expected. However, the remaining solid residue was analysed to determine the crude protein which can be used for animal feed.

4.3.3 Nitrogen content and protein production of solid residue

The nitrogen content in the digested solid residue increased from the initial feedstock from 0.76% to between 1.28% and 1.20% on days 1-10 (**Table 4.2**). The crude protein in the digestate was calculated by multiplying the total ammonium-nitrogen content obtained from the Kjeldahl method with a factor of 6.25 (Lengowski *et al.,* 2016). The increase in nitrogen content in the solid residue between days 2-10 is perhaps due to added buffer and quick replication of microorganisms which are being removed with the solid residue after being digested. The nitrogen content in the solid residue decreased between days 11-15 to 0.24%, a potential indication of the washout of the microorganisms or other nitrogen sources in the liquid being depleted as can be seen in **Table 4.2**.

Table 4.2: Nitrogen and protein of solid residue

Barley straw samples	Nitrogen of solids residue (% of TS)	Protein content (% of TS)	Liquid nitrogen content (%)
Undigested	0.76	4.75	0.78
Days 2-5	1.28	8	0.08
Days 6-10	1.20	7.5	0.02
Days 11-15	0.24	1.5	0.03

The total Kjeldahl nitrogen content in the liquid effluent decreased from the initial day 1 to day 15 (**Table 4.2**). This decrease of the nitrogen in the effluent is because it is readily available for microorganism growth and therefore the microorganisms will utilise the nitrogen in the liquid before making use of the solid nitrogen from the feedstock, resulting in the lower nitrogen content. The carbon-to-nitrogen ratio (C:N) in this digester was at 60. This is very high compared to the suggested C:N ratio of 30 for stable digestion of a feedstock (Al Seadi *et al.,* 2008). It is possible that the nitrogen content in the rumen digester is too low for optimal biogas production. The continuous addition of the buffer solution containing a nitrogen content can be substituted with the co-digestion of a low carbon-to-nitrogen ratio content feedstock to lower the C:N ratio.

4.4 Conclusion and recommendations

The anaerobic digestion of barley straw was tested with the use of a newly designed rumen-based anaerobic digester. The digester was able to digest on average 42.7% of the barley straw in a solid retention time of two days and hydraulic retention time of one day. The low retention time resulted in stable biogas production of 12.63 mL/gVS/day. Furthermore, the nitrogen content of the solid residue after digestion increased slightly which may serve as a product for animal feed. Even though the running of the designed reactor may not be industrially profitable for biogas production, the reactor was able to prove that rumen fluid is able to digest a major portion of barley straw in a short hydraulic and solid retention time. However, for biogas production, the use of a two-stage system may be more profitable with rumen fluid used for the acidogenesis reactor for the quick hydrolysis and a separate methanogenic reactor used for biogas production. The biogas production potential of barley straw could not accurately be determined through the designed digester due to the low retention time and undigested solid residue. Therefore, batch digesters may give valuable information about the biogas production potential of barley straw and other grasses when digested with rumen fluid.

CHAPTER 5

EFFECT OF DIFFERENT INOCULUM TO SUBSTRATE RATIOS ON THE ANAEROBIC DIGESTION OF LIGNOCELLULOSE BIOMASS USING RUMEN FLUID AS INOCULUM

Abstract

Lignocellulosic biomass, one of the most widely available resources in the world, holds great potential for biogas production during anaerobic digestion. However, lignocellulosic biomass is high in fibre which makes it difficult to break down during anaerobic digestion leading to an increase in the retention time and input costs for pre-treatment. The use of rumen fluid poses great potential to minimise the retention time for biogas production from lignocellulosic biomass, as it is one of the most diverse microbial ecosystems on earth, resulting in the fast digestion of lignocellulosic biomass. This study investigated the biogas production potential from the mono-digestion of barley straw with rumen fluid at different ISR's (0.11-1.36) and organic loadings (2.03 gVS/L-24.41 gVS/L). This was to determine the most effective ISR for biogas production when using rumen fluid as inoculum. The biogas production potential was measured at mesophilic conditions (38^{o}C) to replicate the conditions found in a cow. The highest biogas production potential was obtained from an ISRs of 0.17, resulting in 269 mL/gVS biogas produced. The lowest ISR (0.11) led to an increase in the total VFAs from the lignocellulosic biomass and had detrimental effects on the biogas production potential, lowering it by 25%. A too high ISR led to low rate of biogas production in the anaerobic digesters and low substrate available for the microbes to digest which resulted in a decrease in the efficiency of biogas production potential. This study has shown that the fast mono-digestion of lignocellulose biomass is possible with a degradation percentage of between 54% and 76% when using rumen fluid as an inoculum. However, further research should be conducted to determine if rumen fluid can digest a wide range of fibrous lignocellulosic biomasses effectively.

Keywords: lignocellulose; rumen fluid; biogas; inoculum-to-substrate ratio, organic loading; barley straw

5.1 Introduction

Anaerobic digestion is a growing technology wherein companies can lower their carbon emission footprint throughout the world and minimise organic waste generation. The use of anaerobic digesters for the production of electricity has grown significantly in countries like Germany and throughout Europe as they are cognisant of the impact of global warming and the benefits of anaerobic digestion for the environment to achieve a zero-waste policy (Auer *et al.,* 2016).

There are a wide range of feedstocks used in anaerobic digestion to generate biogas. The use of lignocellulosic waste as feedstock in anaerobic digestion has received substantial attention due to its overall availability and fast growth (Kaparaju *et al.,* 2009; Dussadee *et al.,* 2016; Chiumenti *et al.,* 2018). Barley straw and wheat straw have similar compositions and are regarded as the most widely grown crops throughout the world (Lucas, 2012). After harvesting the barley, stover is left behind as a by-product from harvesting. The wasteful stover (straw) is then used as animal feed or worked in the ground to add organic matter in the soil. However, in addition to this, the stover from this widely grown crop has great potential to be used in anaerobic digestion which will increase its overall usage.

Lignocellulose biomass has a structure that consists of three different polymers: hemicellulose, cellulose, and lignin. Lignin is known to limit its degradability during anaerobic digestion and decrease the rate and amount of biogas produced (Wyman & Yang, 2009). This has led to the use of different pre-treatment techniques to increase the available cellulose and hemicellulose to be degraded from lignocellulose to achieve more biogas (Rodriguez *et al.,* 2017). The function of the different pre-treatments is to enable the microorganisms to reach the nutrients more freely thereby ensuring a higher degradation efficiency of the feedstock. However, these pre-treatments are expensive and require a great deal of time and effort when fibrous biomass is used. Therefore, turning to nature, rumen-based anaerobic digestion has been suggested to increase the digestibility and rate of hydrolysis of fibrous feedstocks, leading to an increase in the rate and amount of biogas produced (Yue, Li & Yu, 2013).

Ruminants are animals like cows and sheep known to feed off lignocellulosic biomass and which have been perfecting the art of digesting lignocellulosic biomass for centuries (O'Kiely *et al.,* 2015). However, ruminants do not produce their own cellulase enzyme and are thus unable to break down lignocellulose biomass. Therefore, ruminants live with various microorganisms in their stomach (rumen) to digest the biomass (de Ondarza, 2001). Ruminants are known to digest their food in a couple of days rather than months, which is completely opposite to anaerobic digesters which digest various grasses for 40 to 60 days (Dussadee, Unpaprom & Ramaraj, 2016; Kaur, Phutela & Goyal, 2016). The cellulolytic microorganisms in the rumen produce a number of enzymes and lead to the quick digestion of lignocellulose biomass and the production of volatile fatty acids (VFAs), which are then used

as the intermediate products for biogas production (Bayané & Guiot, 2010). There is a scarcity of research exploring the efficiency of rumen fluid for the mono-digestion of lignocellulose biomass for biogas production. Different organic loadings may have various effects on the biogas production and overall fermentation kinetics when digesting biomass. Therefore, this study aimed to evaluate the mono-digestion of lignocellulose biomass to find the most effective inoculum-to-substrate ratio and organic loading for biogas production by using rumen fluid as an inoculum.

5.2 Materials and methods

5.2.1 Feedstock and inoculum

The feedstock used in this study is outlined in **Section 3.1** and **Section 3.2**. Only barley straw was used in this study as it is one of the most widely grown crops in the world and has similar properties to wheat straw (Lucas, 2012). Barley straw is also considered a wasteful product, readily available throughout the world.

5.2.2 Incubation medium and solutions

The incubation medium and solutions used in this study are explained in detail in **Section 3.3**.

5.2.3 Experimental set-up

The experimental set-up of this study is outlined in **Section 3.5.1**. The biogas production potential set-up, explained in **Section 3.4.1**, is to test the biogas production potential on different inoculum-to-substrate ratios and organic loadings.

5.2.4 Gas measuring system and gas quality and conversion

The gas measuring system and gas conversion used are described in **Section 3.4.2** to **Section 3.4.4**.

5.2.5 Input and output analysis

All input and output analyses were undertaken as described in **Section 3.6**.

5.2.6 Modelling and statistical analysis

The various kinetics of the data were analysed as outlined in **Section 3.4.4** and **Section 3.7.**

5.3 Results and discussion

5.3.1 Feedstock and inoculum characterisation

The characteristics of the inoculum and feedstock are summarised in **Table 5.1**. Barley straw has a high total solids (TS) and volatile solids (VS) content, comprising a total of 86.83% and 81.37% of the wet weight (WW), respectively. Similar results has been reported in other studies involving barley straw showing a TS and VS of 94.4% and 89.66% dry weight, respectively (Serna-Díaz *et al.,* 2020). The cellulose, hemicellulose and lignin of barley straw in the present

study was 33.78%, 29.74% and 20.7% of TS, respectively. The high cellulose and hemicellulose content indicate the available sugars in the feedstock and give an indication of what can potentially be used in the barley straw for the generation of biogas. Barley straw has 16.4% extractables in water and only 1.96% extractables in ethanol. The ethanol extractives are quite low in barley straw due to the low amount of chlorophyll and waxes in straw. Similar results have been published in another stsudy which determined total extractives of 14% (Serna-Díaz *et al.,* 2020). Based on the elemental analysis of barley straw, the empirical formula can be extrapolated as follows: $C_{60}H_7O_{59}N$. The carbon-to-nitrogen ratio is 60:1 for barley straw, which is lower than other studies having a C:N ratio of 80:1 and 145:1 in barley straw (Christensen, 1988; Serna-Diaz *et al.,* 2020). This may be due to the nature of barley straw in various areas having a different composition from different growing areas (Contreras-López *et al.,* 2008).

Table 5.1: Characterisation of feedstock and inoculum

Characteristic	Inoculum (rumen fluid)	Barley straw
Total solids (%)	2.27 ± 0.22	86.83 ± 0.13
Volatile solids (%)	1.47 ± 0.16	81.37 ± 0.13
Ash (% of TS)	39.22 ± 2.97	7.26 ± 0.23
Water extractives (% of TS)	n/a	16.24 ± 1.11
Ethanol extractives (% of TS)	n/a	1.96 ± 1.29
Total Extractives (% TS)	n/a	17.61 ± 1.85
Hemicellulose (% of TS)	n/a	29.74 ± 2.88
Cellulose (% of TS)	n/a	33.78 ± 0.42
Lignin (% of TS)	n/a	20.70 ± 0.6
Carbon (% of TS)	n/a	45.89
Nitrogen (% of TS)	n/a	0.76
Kjeldahl-NH_4^+ (% WW)	0.148	n/a
Oxygen (% of TS)	n/a	47.81
Hydrogen (% of TS)	n/a	5.54
Sulphur (% of TS)	n/a	BDL
pH	6.22 ± 0.07	n/a

**BDL- below detection limit*

5.3.2 Biogas production from different inoculum-to-substrate ratios and organic loadings

To test the effect of different inoculum-to-substrate ratios on biogas production, barley straw was digested at different feedstock loadings in batch anaerobic digesters. The results from the biogas potential tests are indicated in **Figure 5.1.**

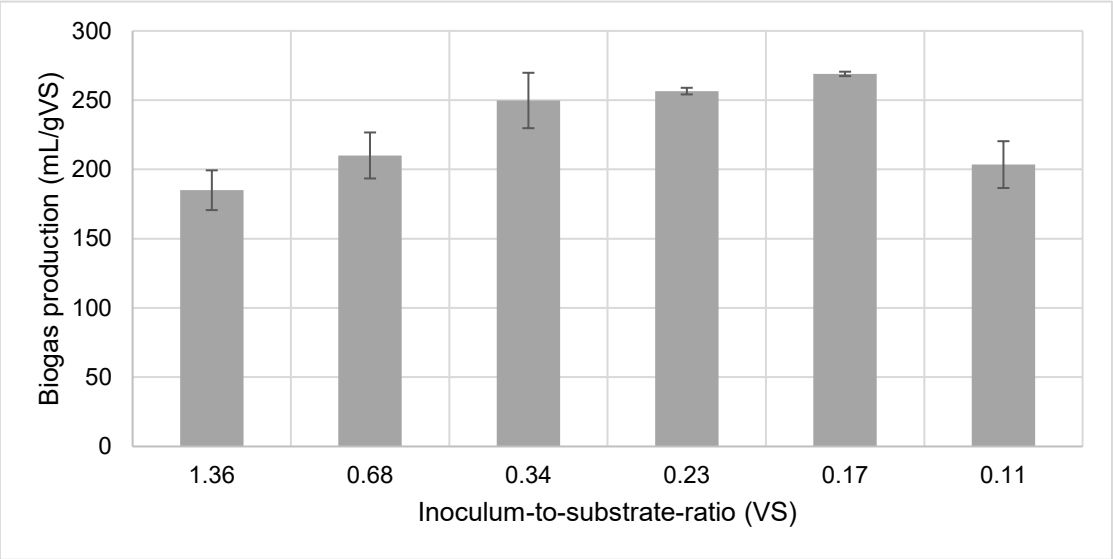


Figure 5.1: Biogas production from different inoculum-to-substrate-ratios (ISRs) after 8 days based on the VS added. The biogas production values are expressed in mL/gVS of feedstock added. The error bars indicate the standard deviation (SD), n=3

There was a difference in the means of the cumulative biogas production potential from the different inoculum-to-substrate ratios (**Figure 5.1**). The biogas potential with an ISR of 1.36 and 0.68 yielded a total of 185 mL/gVS and 210 mL/gVS, respectively, whereas the ISR of 0.34, 0.23, 0.17 and 0.11 yielded 250 mL/gVS, 257 mL/gVS, 269 mL/gVS and 203 mL/gVS biogas, respectively (**Figure 5.1**). The biogas production potential is similar to what is reported in other studies from digesting biomass with rumen fluid, having a biogas production of barley straw between 220 mL/gVS and 178 mL/gVS (Kivaisi *et al.,* 1992; Dubrovskis *et al.,* 2013).

The biogas production increased with a decrease in the ISR of 1.36 to 0.17. The ISR of 0.17 had the highest biogas production. A further decrease of the ISR to 0.11 had detrimental effects on the biogas production. The biogas production from the ISRs of 0.11, 0.68 and 1.36 are not statistically different. There is a significant difference in the biogas production of 0.1 and 0.11, and 0.17 and 1.36, respectively ($p<0.05$) (**Appendix A, Table 1A**). This shows that the ISR between 0.17 and 0.68 is likely ideal for biogas production when using rumen fluid due to the high biogas production and pH stability during gas production. The methane content in all the reactors was 22% based on the gas measurement from the Biogas Analyzer 5000. This is substantially lower to what has been reported in biogas plants, requiring a methane percentage

of at least 50%. Other studies on the digestion of lignocellulose biomass with rumen fluid have reported similar methane percentages ranging between 28% and 30% (Zwart *et al.,* 1988; Kivaisi *et al.,* 1992). Even though rumen fluid produced biogas in a short retention time, it has been suggested that the use of anaerobic sludge is better for the quality of biogas production because of the excess of methanogenic archaea present (Nguyen *et al.,* 2019).

The ISR of 0.17 in this study is relatively low compared to other studies suggesting an inoculum-to-substrate-ratio close to 1 and 2 when digesting lignocellulosic biomass (Raposo *et al.,* 2012; Holliger *et al.,* 2016). The low ISR indicates the efficiency of the inoculum, as a small amount of inoculum is needed for the digestion of lignocellulose biomass. The low ISR has also been reported in a study testing the biogas production of cow manure with rumen fluid as an inoculum. Researchers found that a biogas production of 191 mL/gVS is achieved when an ISR of 0.05 is used (Seno & Nyoman, 2010).

5.3.3 Biogas production from different organic loadings

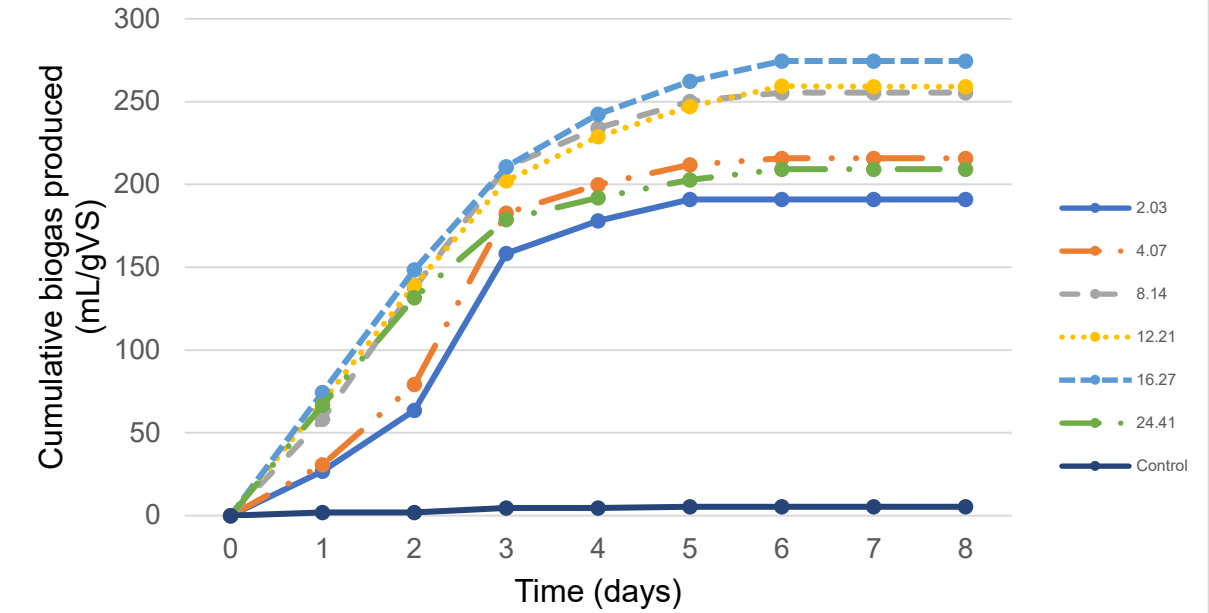


Figure 5.2: Cumulative biogas produced from different organic loadings (gVS/L). Gas volumes are expressed in mL/gram volatile solids (VS) added. n=3

The organic loading and inoculum to substrate ratio is directly proportional to one another. **Figure 5.2** presents the biogas production curves when looking at the organic loadings (gVS/L) in the anaerobic digesters. It is expected that the biogas production will increase as the organic load increases in the reactor (Gijzen *et al.,* 1988). This is because there is more substrate available for the microorganisms to digest and this will lower the competition that will lead to more biogases produced. However, there will also be a greater mass transfer limitation as a result of less feedstock available. **Figure 5.2** shows that with the increase in the organic

loading, the biogas production increased as expected but only up to 16.24 gVS/L. The control, with no feedstock added, did not produce a significant amount of biogas. The organic loading of 2.03 gVS/L to 16.24 gVS/L led to an increase in the biogas production, but a further increase of organic loading from 16.24 gVS/L to 24.41 gVS/L lowered the biogas production by 25% compared to the organic loading of 16.24 gVS/L (**Figure 5.1**). The biogas production of 185 mL/gVS and 269 based on the volatile solids added is similar to what is reported in other studies of digesting barley straw having a biogas production of 210 mL/gVS (Gijzen *et al.,* 1987; Kivaisi *et al.,* 1992).

The results suggest that the organic loading of 8.14 gVS/L and 16.24 gVS/L had a good biogas production when making use of rumen fluid as inoculum. However, another study found an organic loading between 2 gVS/L and 6 gVS/L to be the most effective for the production of biogas from the mono-digestion of wheat straw (Rajput & Sheikh, 2019). This low organic load may be due to the inoculum used. The biogas production in other studies was tested over a 20 to 40-day period, whereas the biogas production in this study lasted only eight days before it plateaued. Other studies had a higher biogas production of 297 mL/gVS and 556 mL/gVS with the use of different inoculums (Rajput & Sheikh, 2019). Rumen fluid led to an increase in the hydrolysis and acidification phase during anaerobic digestion which is also supported in other studies (Pertiwiningrum *et al.,* 2017; Zou *et al.,* 2017).

5.3.4 Kinetic coefficients of different organic loadings and inoculum-to-substrate ratios

The kinetics of the various reactions fitted to different models from the different organic loadings (gVS/L) are shown in **Table 5.2.** The non-linear regression curves fitted with the experimental results with the first order, Monod type, modified Gompertz, and two-fraction first order models from different organic loadings. The model with the least error for all organic loadings between predicted biogas and measured biogas and best fit the experimental data was the modified Gompertz (GM) model, revealing error values between -0.4% and 1.34%. This was followed by the two-fraction first order model (1.54% and 6.35%), first order (5.66% and 21.27%) and Monod type (35.98% and 80.52%) which had the largest margin of error. The lag phase between the different organic loadings decreased with a concomitant increase in organic loading (**Table 5.2**). The lower lag phase during start-up indicated that there is more than enough substrate available for the microorganisms to digest, resulting in less competition. The lag phase at an organic load of 2.03 gVS/L was 1.129 days and decreased to 0.197 days at an organic loading of 24.41 gVS/L (**Table 5.2**). This suggests that the competition at low organic loadings led to a higher lag phase as the microorganisms do not survive in the reactor. It can also be noted that the rate constant (k) increased as the organic loading increased. The rate constant at an organic load of 8.14 gVS/L, 12.21 gVS/L and 16.24 gVS/L is similar, ranging between 0.310-0.313 for the first order model and 0.367 and 0.376 for the Monod type model.

This confirms an adequate number of microorganisms in the specific organic loadings to digest the biomass, further supporting that an organic loading between 8.14 gVS/L and 16.24 gVS/L is sufficient for biogas production when using rumen fluid.

The maximum biogas production rate per day (μ_m) was the highest between an organic loading of 4.07 gVS/L and 8.14 gVS/L (91.295 mL/gVS/day and 90.433 mL/gVS/day). The lowest maximum biogas production rate per day (μ_m) was achieved for the organic load of 24.41 gVS/L which was 75.74 mL/gVS/day, but this may be due to inhibiting factors that influenced the total amount of biogas produced. The maximum rate of biogas production in this study is 23 times higher compared to a study digesting cow manure with rumen fluid (Budiyono *et al.,* 2014; Seno & Nyooman, 2010). All models overestimated the biogas production except for the modified Gompertz model at an organic load of 24.41 gVS/L.

The coefficient of determination (R^2) at an organic loading of 2.03 gVS/L and 4.07 gVS/L was the lowest for the first order model at organic loading of 0.908 and 0.915, respectively. The highest coefficient of determination for all organic loadings was the modified Gompertz model (0.988 and 0.999), followed by the two-fraction first order model (0.965 and 0.999). These results suggest that the modified Gompertz model is a sufficient tool to estimate biogas production during anaerobic digestion from lignocellulose biomass, having an error between predicted and measured biogas of only -0.4 % and 1.34%.

Table 5.2: Kinetics from different models and coefficient of determination between different models

Organic loading (gVS/L)	Model	Specific Biogas Production			Error (%)	μ_m (mL/gVS/day)	λ(Day)	k_s	k_f	R^2
		k (day-1)	Predicted	Measured						
2.03	FO	0.281	225	186	21.27	nd	nd	nd	nd	0.919
	MT	0.199	335	186	80.52	nd	nd	nd	nd	0.908
	GM	nd	188	186	1.34	84.950	1.129	nd	nd	0.988
	TFFO	nd	197	186	6.35	nd	nd	35.561	0.556	0.965
4.07	FO	0.295	250	210	19.02	nd	nd	nd	nd	0.927
	MT	0.214	368	210	74.66	nd	nd	nd	nd	0.915
	GM	nd	213	210	1.00	91.295	0.990	nd	nd	0.989
	TFFO	nd	221	210	5.21	nd	nd	35.561	0.581	0.972
8.14	FO	0.376	277	250	10.71	nd	nd	nd	nd	0.973
	MT	0.313	378	250	51.07	nd	nd	nd	nd	0.960
	GM	nd	252	250	0.65	90.433	0.430	nd	nd	0.999
	TFFO	nd	258	250	2.99	nd	nd	35.561	0.625	0.996
12.21	FO	0.367	279	254	10.18	nd	nd	nd	nd	0.988
	MT	0.310	380	254	49.90	nd	nd	nd	nd	0.979
	GM	nd	255	254	0.61	79.494	0.234	nd	nd	0.998
	TFFO	nd	264	254	3.98	nd	nd	35.561	0.526	0.998
16.24	FO	0.367	296	269	10.14	nd	nd	nd	nd	0.990
	MT	0.310	403	269	49.71	nd	nd	nd	nd	0.981
	GM	nd	271	269	0.72	82.675	0.203	nd	nd	0.998
	TFFO	nd	281	269	4.28	nd	nd	35.561	0.512	0.998
24.41	FO	0.469	215	204	5.66	nd	nd	nd	nd	0.989
	MT	0.445	277	204	35.98	nd	nd	nd	nd	0.976
	GM	nd	203	204	-0.40	75.740	0.197	nd	nd	0.998
	TFFO	nd	207	204	1.54	nd	nd	35.561	0.674	0.999

FO – First order; MT – Monod Type; Gm – modified Gompertz; TFFO – two-fraction first order; k- first order kinetic constant; μ_m – maximum biogas production rate; λ- lag phase; k_s and k_f are reaction rate constants in TFFO model and nd -no data.

5.3.5 Production of volatile fatty acids and total solids digested from different inoculum-to-substrate ratios and organic loadings

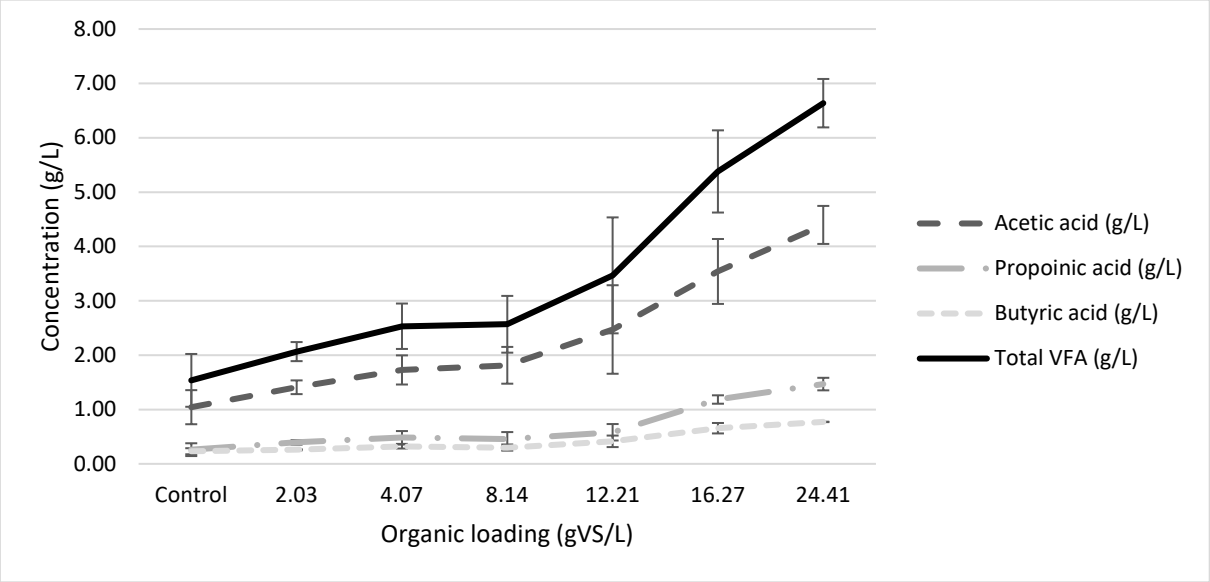


Figure 5.3: Total volatile fatty acids produced from different organic loads in the reactors (gVS/L). The error bars indicate the standard deviation of the samples, n=3

Table 5.3: Specific production of biogas, volatile fatty acids (VFAs), pH, percentage degraded total solids from different inoculum-to-substrate ratios (ISR) and organic loadings (OL). The standard deviation (SD) is also indicated

OL (gVS/L)	ISR (VS)	Digested total solids (%)	Total VFA in digestate (g/L)	VFA/gVS added	pH after digestion	Biogas production mL/gVS
2.03	1.36	74	2.07 ± 0.1	1.02	6.83 ± 0.03	185 ± 14
4.07	0.68	65	2.53 ± 0.24	0.62	6.83 ± 0.03	210 ± 17
8.14	0.34	69	2.57 ± 0.30	0.32	6.7	250 ± 20
12.21	0.23	59	3.47 ± 0.62	0.28	6.56 ± 0.03	257 ± 2
16.24	0.17	60	5.38 ± 0.62	0.33	6.3	269 ± 2
24.41	0.11	54	6.64 ± 0.44	0.27	5.97 ± 0.03	226 ± 17

The total volatile fatty acids (VFAs) in the digestate provides evidence of the efficiency of hydrolysis and acidification of the microorganisms in the system. It also serves as an indicator of the methanogenic activity in the reactor, as this is the intermediate step for methane production (Nguyen *et al.,* 2019). For biogas production, a low VFA concentration is desirable, as a too high concentration will lead to acidification in the reactor and system failure. An organic loading of 2.03 gVS/L, 4.07 gVS/L and 8.14 gVS/L yielded a total of 2.07 g/L, 2.53 g/L and 2.57 g/L VFAs, respectively (**Figure 5.3**). The organic loading of 12.21 gVS/L, 16.24 gVS/L and 24.41 gVS/L yielded a total of 3.47 g/L, 5.38 g/L and 6.64 g/L VFAs, respectively (**Figure 5.3**). Acetic acid and propionic acid are the highest producing volatile fatty acids in the digesters, making up a total of 68% and 19% of the VFAs, respectively. The VFAs produced

in the digestate correlates well with the drop in the pH of the digestate (**Table 5.2**). The total VFAs increased as the organic load increased from 2.03 gVS/L to 24.41 gVS/L (r=0.97). Similar results have been reported in other studies involving rumen fluid, with a rapid increase in the VFAs during anaerobic digestion of biomass (Gijzen *et al.,* 1988; Kivaisi *et al.,* 1992; Nguyen *et al.,* 2019; Rajput & Sheikh, 2019). However, it seems high organic loadings of barley straw will not lead to acidification in the reactor (Dubrovskis *et al.,* 2013). This is contrary to the results reported in this study, as the higher organic loadings of barley straw led to acidification of the reactor (**Figure 5.3**). The cellulolytic microorganisms in rumen fluid produce a high amount of VFAs from the biomass in a short amount of time and led to acidification in the reactor.

The highest gVFA/gVS ratio was obtained for an organic load of 2.03 gVS/L which yielded a ratio of 1.02. This is followed by an organic loading of 4.07 gVS/L which yielded a ratio of 0.62. An organic loading between 8.14 gVS/L and 24.41 gVS/L yielded between 0.27 gVFA/gVS and 0.33 gVFA/gVS. The gVFA/gVS in this study is similar to an anaerobic digestion of citrus waste which ranged between 0.161 and 0.28 gVFA/gVS (Eryildiz *et al.,* 2020). Rumen fluid is a better inoculum for the fast production of VFAs from lignocellulosic biomass. This is also confirmed by Nguyen *et al.* (2019), where the VFA produced is four times higher from rumen fluid compared to anaerobic sludge.

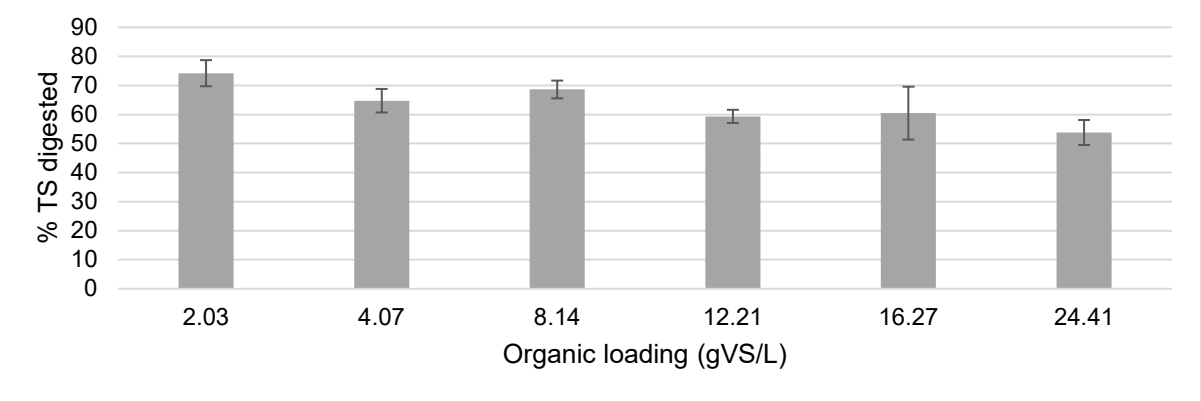


Figure 5.4: The percentage of total solids that has been digested from different organic loadings (gVS/L). The error bars indicate the standard error of replicates. n=3

The percentage of total solids degraded from the lignocellulose biomass ranged between 54% and 74% irrespective of the organic load and ISR. The organic load of 2.03 appears to have the highest percentage of degraded total solids: 74%. The affirms that the microorganisms had limited substrate and are trying to digest the undegradable parts of the biomass, resulting in a higher percentage of total solids being digested. The organic load of 4.07 gVS/L, 8.14 gVS/L, 12.21 gVS/L, 16.24 gVS/L and 24.41 gVS/L had a degradability of total solids of 65%, 69%, 59%, 60% and 54%, respectively (**Figure 5.4**). In this study, the percentage of total solids

degraded is similar to other studies testing the degradation of lignocellulose biomass in rumen fluid, with values ranging 55%-60% (Czerkawski & Breckenridge, 1977; Gijzen *et al.,* 1988a; Kivaisi *et al.,* 1992). The low degradation of biomass at a high organic load (24.41gVS/L) may be attributed to the low pH value and high VFAs in the digester inhibiting the further degradation of the biomass. This is reflected through another study showing how a low pH inhibits further degradation of cellulose when using rumen fluid (Zhang *et al.,* 2017). There is no correlation with the percentage of degraded total solids and the biogas production at different organic loadings or ISRs (r^2= -0.3).

5.4 Conclusion and recommendations

The biogas production of different ISR and organic loadings differed substantially when using rumen fluid as an inoculum. The ISR is inversely proportional to the organic loading (gVS/L) in the reactor. The organic loading between 8.14 and 16.24 had no significant difference in the biogas production; however, 16.24 gVS/L (ISR of 0.17) had the highest biogas production of all the organic loads, producing 269 mL biogas/gVS for the mono-digestion of barley straw. It is suggested that an ISR between 0.34 and 0.17 be used when digesting lignocellulose biomass. The increase of the organic loading to 24.41 gVS/L (ISR of 0.11) had detrimental effects on the biogas production, leading to an increase in VFA in the reactor and a drop in the pH. The results indicated that the use of rumen fluid decreased the retention time significantly for the digestion of lignocellulose biomass and led to a significant increase in the maximum biogas production rate per day as compared to other studies. The methane content in the rumen fluid is 22% when digesting lignocellulose. Further research should evaluate if rumen fluid is effective to degrade various lignocellulose biomasses for biogas production.

CHAPTER 6

BIOGAS PRODUCTION AND COMPOSITION OF DIGESTATE FROM DIFFERENT LIGNOCELLULOSE FEEDSTOCKS BY USING RUMEN FLUID AS INOCULUM

Abstract

Anaerobic digestion of various feedstocks differs in the amount of biogas produced and the rate of biogas production. This has an impact on the amount of energy that can be generated from various feedstocks during anaerobic digestion. The efficiency of a feedstock in anaerobic digestion can be determined through its biogas production and is dependent on the feedstock used. This study aimed to test the biogas potential, degradability and digestate composition for the mono-digestion of three different lignocellulosic feedstocks – Napier grass, barley straw and kikuyu grass – by using rumen fluid as an inoculum. The study was conducted at mesophilic conditions (38°C) to replicate the conditions found in a cow. The biogas production of the experimental data was simulated through to various non-linear regression models, Monod type, first order, modified Gompertz, and Two-Fraction First Order model, with the least square non-linear regression analysis for the different feedstocks. The results in this study indicated that while a slightly higher biogas potential was reached for kikuyu grass (289 ± 3.83 mL/gVS), followed by Napier grass (285 ± 2.56 mL/gVS) and barley straw (279 ± 10.3 mL/gVS), there were no significant differences between the biogas produced from the different feedstocks. The composition of the digestate from different grasses did not differ significantly. The ammonium nitrogen in the digestate of Napier grass was higher at 0.115 ± 0.005 mg/L than the other two lignocellulose biomasses ranging between 0.086 and 0.09 mg/L. Rumen fluid proved to reduce the digestion time of all lignocellulosic biomasses to eight days, in which the biogas production plateaued irrespective of the biomass feedstock used. Further research should focus on scaling-up the system to determine if rumen fluid can be used effectively on a larger scale for the mono-digestion of lignocellulosic biomass.

Keywords: lignocellulose; biomass; anaerobic digestion; rumen fluid; digestate

6.1 Introduction

Anaerobic digestion is a highly advantageous technology which utilises wasteful products for the production of digestate and biogas. Biogas is considered a renewable energy resource consisting of carbon dioxide and methane gas (Comparetti *et al.,* 2013). The feedstocks used in anaerobic digestion come from different waste streams, like fruit and vegetable waste, municipal waste, food waste and agricultural waste (Al Seadi *et al.,* 2008). The use of agricultural waste such as straw and fast growing fibrous grass can be a beneficial feedstock for energy production due to its high carbon content and overall availability throughout the year (Clauser *et al.,* 2021). The use of lignocellulose biomass in anaerobic digestion has grown over recent years due to its availability and the high methane potentials it can generate (Stopp *et al.,* 2011; Wall *et al.,* 2016; Kholif *et al.,* 2017). However, the application of lignocellulose biomass in anaerobic digestion is limited due to the resistance of the structure of lignocellulose biomass to hydrolysis (Eryildiz, Lukitawesa & Taherzadeh, 2020). The structure of lignocellulose leads to higher retention times required for biogas production and reduces the feasibility of anaerobic digestion from these agricultural wastes.

The use of rumen fluid from a cow has been shown to be highly beneficial, leading to higher biogas production rates and increasing the digestion of lignocellulosic biomass in an anaerobic digester (Okeh *et al.,* 2014; Pertiwiningrum *et al.,* 2017; Zou *et al.,* 2018; Nguyen *et al.,* 2019). Rumen fluid, posing significant advantages for the mono-digestion of grasses, can be used with great effect to produce biogas. Cows have been known to digest their food in just over two days, and are known for the high production of volatile fatty acids (VFAs) from the biomass they digest as an energy source (Yue, Li & Yu, 2013). This production of VFA is achieved through the rich cellulolytic microbial ecology found in the rumen fluid of a cow (Bayané & Guiot, 2010). The volatile fatty acids (VFAs) are used by methanogens for the production of methane gas and carbon dioxide. Feedstocks used for the production of biogas are dependent on availability and degradability and competition in other sectors. For example, crops such as maize and sugarcane are not good crops for biogas production because the biogas will be in direct competition with food demand. Therefore, other fibrous grasses which are not used for food production may present an alternative source of biogas production.

Barley straw, a by-product from farming activities, is typically used as bedding and a food source for farm animals. Despite being considered a waste product; barley straw can be implemented in anaerobic digestion to produce high value by-products such as fertilizer and electricity. Other fast growing fibrous grasses can also be used as an alternative method for biogas production. Studies on Napier grass (*Pennisetum purpureum*) have shown that this crop can be used in anaerobic digestion due to its fast growth, high yield and overall availability (Sawasdee & Pisutpaisal, 2014; Deshmukh *et al.,* 2016; Dussadee *et al.,* 2016). Another possible feedstock for use is kikuyu grass (*Pennisetum clandestinum*) because it is one of the

highest yielding perennial grasses under unfavourable conditions, yielding 17tDM/ha (Neal *et al.,* 2009). These feedstocks do not compete directly with the food crops and are readily available. Therefore, they can be of value in the biogas production industry due to their availability. However, to evaluate if the feedstocks can be used in anaerobic digestion, it is important to measure the biogas production from these grasses in a batch reactor.

There are a few different methods used in research to determine the biogas production and biochemical methane potential of different feedstocks (Angelidaki *et al.,* 2009; Holliger *et al.,* 2016). The batch fermentation of feedstocks are used to determine the biogas production and biodegradability of a feedstock in anaerobic digestion for larger scale digesters (Strömberg *et al.,* 2015). Anaerobic digesters are not only considered for the biogas they produce but also other high value by-products. The use of rumen fluid to digest lignocellulosic biomass will also lead to other highly desirable products such as an organic fertilizer with a high nitrogen, phosphorus, and potassium content. With a need for sustainable farming in developing countries, the digestate will be an important source to add value to the anaerobic digestion process and provide an alternative income.

This study therefore aimed to determine the biogas production from three different lignocellulosic biomasses using the rumen fluid as an inoculum. The use of these grasses can help anaerobic digestion gain traction and increase its use in various settings. This study went further to analyse the ammonium nitrogen, phosphorus, and potassium of the digestate after the mono-digestion of different lignocellulose feedstocks in anaerobic digestion.

6.2 Materials and methods

6.2.1 Feedstock and inoculum

The feedstocks used in this study – barley straw, kikuyu grass and Napier grass (elephant grass) – were milled as outlined in **Section 3.1** to obtain a uniform sample size. The inoculum used in this study was obtained from two-cannulated-Holstein cows as outlined in **Section 3.2**. The inoculum was transported directly to the laboratory and maintained at 38^{o}C under anaerobic conditions to preserve the microorganisms.

6.2.2 Incubation medium and solutions

The incubation medium used in this study is explained in detail in **Section 3.3**.

6.2.3 Experimental set-up

The experimental set-up in this study is outlined in **Section 3.5.2**. The biogas production experimental set-up, explained in **Section 3.4.1**, tested the biogas production of different lignocellulosic biomasses.

6.2.4 Gas measuring system, gas quality and gas conversion

The gas measuring system, gas quality and gas conversion used in this study are described in **Section 3.4.2** to **Section 3.4.4**.

6.2.5 Input and output analysis

All analysis was conducted as described in **Section 3.6**. The total solids (TS), total suspended solids (TSS) and ash were determined for both the inoculum and feedstocks according to standard procedures (APHA, 2005). The ethanol and water extractives, lignin, cellulose and hemicellulose were determined for the different feedstocks according to the NREL laboratory analytical procedure (Sluiter *et al.,* 2008). The carbon, nitrogen, hydrogen, sulphur and oxygen of the different feedstocks were determined by central analytical facilities (CAF), Stellenbosch University, using an Elementar Vario EL Cube Analyzer. The pH was measured with a portable digital pH Pen Meter Tester (accuracy: +/- 0.1).

The total suspended solids (TSS) in the digestate were subtracted from the total solids (TS) added to the reactors to calculate the percentage digested total solids. The digestate of the reactors were sent to Elsenburg, Stellenbosch, South Africa, for analysis of the total Kjeldahl ammonium nitrogen, phosphorus and potassium content. The crude protein is calculated by multiplying the total Kjeldahl ammonium nitrogen with 6.25.

6.3 Results and discussion

6.3.1 Feedstock composition

The composition of the feedstocks and inoculum used in this study is summarised in **Table 6.1**. The different feedstocks vary in their composition according to the NREL analysis. Barley straw, Napier grass and kikuyu grass samples presented a total solids (TS) content of 86.83%, 79.21% and 61.62%, respectively; VS resulted in 81.37% of wet weight (WW), 71.39% of WW, and 56.24% of WW, respectively. This is due to the moisture found in green grasses compared to straw which is not necessarily rich in moisture content. The grass samples differed in their total extractives with Napier and kikuyu showing similar percentages of total extractives with 26.02% and 25.50% of TS. Barley straw had the lowest percentage of total extractives with only 17.61% extractives of the TS. This is due to the chlorophyll found in green grasses such as Napier and kikuyu grass, whereas barley straw will have little chlorophyll, resulting in lower extractives and waxes in the grass. The lignin content was reported to be the lowest in barley straw with 20.70% of TS, followed by kikuyu, 24.71% of TS, and Napier gras, 28.53% of TS. The hemicellulose and cellulose of barley straw were 33.78% and 29.74% of TS, respectively. Napier grass had a hemicellulose and cellulose content of 13.94% and 25.60% of TS, respectively, whereas kikuyu revealed hemicellulose and cellulose concentrations of 18.74% and 25.71% of TS, respectively. The TS in the inoculum was 2.45% and the VS 1.47% of WW

after being strained through two layers of cheesecloth. Barley straw, Napier grass and kikuyu has an empirical formula of $C_{60}H_7O_{59}N_1$, $C_{14}H_2O_{17}N_1$, and $C_{19}H_3O_{23}N_1$, respectively. The C:N ratio for the different feedstocks could then be calculated as 60, 14 and 19 for barley straw, Napier grass and kikuyu grass, respectively. Studies have reported quite similar values for the carbon-to-nitrogen ratios from these grasses; however, barley straw has been reported to have a higher C:N ratio of 80:1 (Christensen, 1985; Dussadee, Unpaprom & Ramaraj, 2016). This might be due to natural variability in the grasses and different seasons of harvesting.

Table 6.1: Feedstock and inoculum characteristics

Characteristic	Inoculum (rumen fluid)	Barley straw	Napier grass	Kikuyu grass
Moisture (%)	97.53	13.56 ± 0.49	20.79 ± 0.49	38.38 ± 0.93
Total solids (%)	2.27 ± 0.22	86.83 ± 0.13	79.21 ± 0.49	61.62 ±0.93
Volatile solids (%)	1.47 ± 0.16	81.37 ± 0.13	71.39 ± 0.44	56.24 ± 0.80
Ash (% of TS*)	39.22 ± 2.97	7.26 ± 0.23	12.46 ± 0.07	14.16 ± 0.21
Water extractives (% of TS)	n/a	16.24 ± 1.11	20.93 ± 0.04	20.62 ± 0.09
Ethanol extractives (% of TS)	n/a	1.96 ± 1.29	4.56 ± 0.02	5.39 ± 0.02
Total Extractives (% of TS)	n/a	17.61 ± 1.85	26.02 ± 0.08	25.50 ± 0.05
Hemicellulose (% of TS)	n/a	29.74 ± 2.88	13.94 ± 0.04	18.74 ± 0.1
Cellulose (% of TS)	n/a	33.78 ± 0.42	25.60 ± 0.16	25.71 ± 0.23
Lignin (% of TS)	n/a	20.70 ± 0.6	28.53 ± 0.08	24.71 ± 0.03
Carbon (% of TS)	n/a	45.89	41.33	41.15
Nitrogen (% of TS)	n/a	0.76	2.87	2.12
Kjeldahl-NH_4^+ (% WW)	0.148	n/a	n/a	n/a
Oxygen (% of TS)	n/a	47.81	49.33	49.42
Hydrogen (% of TS)	n/a	5.54	6.47	7.31
Sulphur (% of TS)	n/a	BDL	BDL	BDL
pH	6.22 ± 0.07	n/a	n/a	n/a

Error bars indicate the standard deviation of the samples (n=3);

**TS- total solids*

6.3.2 Biogas production from different lignocellulosic biomasses

The biogas production of Napier grass, barley straw and kikuyu grass were tested with the use of cow rumen fluid as inoculum. The grasses represent long fibrous grasses which are not known to be quickly digested during anaerobic digestion; however, these grasses hold great potential for biogas production due to their vast availability.

Figure 6.1 indicates the biogas production from the different biomass samples after eight days of incubation. After eight days of incubation, the biogas production plateaued, and the grasses did not produce any more biogas, so the experiment was terminated (**Figure 6.1**). It was

expected that barley straw would have the highest biogas production due to the high amount of VS in the feedstock (**Table 6.1**). However, the three different grasses had only minor differences in biogas production (**Figure 6.1**). Kikuyu grass had a slightly higher biogas production in terms of the volatile solids added, followed by Napier grass and then barley straw. Napier grass had a biogas production of 282 mL/gVS added. Kikuyu and barley straw had a biogas production potential of 289 ml/gVS and 275 mL/gVS, respectively. A Tukey-test determined that the differences between the means are not statistically significant (**Appendix A, Table 2A**). Even though the hemicellulose and cellulose content in the grasses differed significantly, this was not reflected in the biogas production.

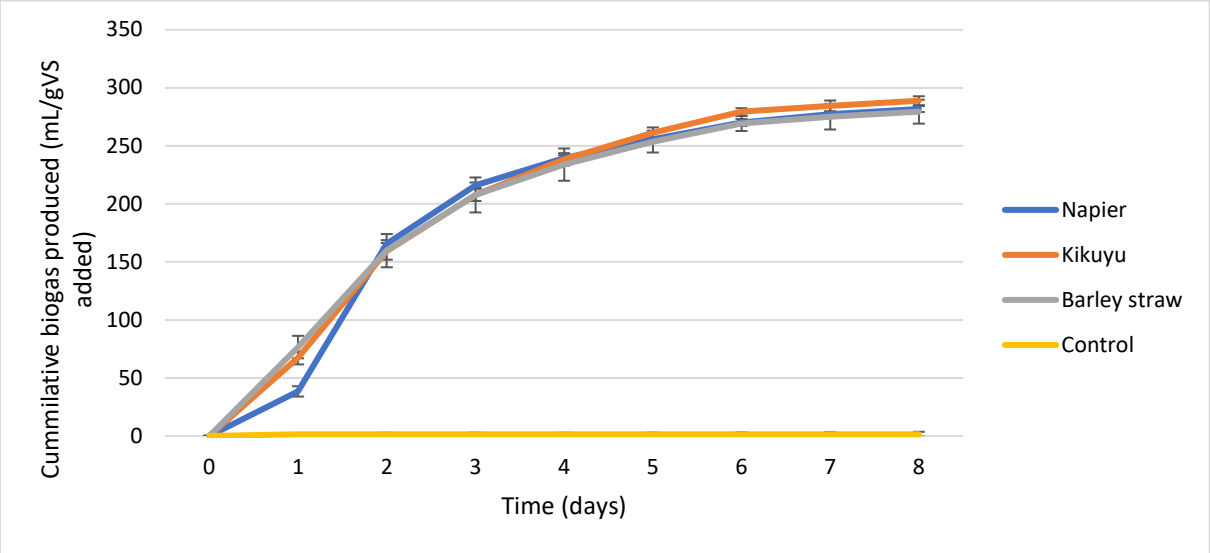


Figure 6.1: Cumulative biogas production from different lignocellulose biomass samples: blue line represents Napier grass; red line represents kikuyu grass; grey line represents barley straw. The error bars indicate the standard deviation of samples. n=4

The gas composition of the different feedstocks revealed only a slight difference in the methane percentage between the different biomasses. Barley straw had the highest methane percentage of 22.79%, followed by kikuyu grass at 22.49% and Napier grass at 21.14%. Other studies digesting grasses through rumen fluid have reported higher methane percentages ranging between 28% and 32% (Zwart *et al.,* 1988; Kivaisi *et al.,* 1992). Studies of digesting Napier grass through anaerobic digesters revealed a biogas production ranging between 92.4 L/kg VS-190.25 L/kg VS (Dussadee *et al.,* 2016; Kaur *et al.,* 2016). A study on the anaerobic digestion of other green grasses revealed a higher biogas production, reaching a biogas yield of over 600 L/kg VS (Chiumenti *et al.,* 2018). This is significantly higher than what has been reported in this study which may be due to the inoculum used and different feedstock composition. However, the biogas production of barley straw has been shown to be similar to what is reported in other studies (Gijzen *et al.,* 1988a; Kivaisi *et al.,* 1992). A study on the

digestion of kikuyu grass (*Pennisetum clandestinum*) revealed that the use of two different inoculums, rumen and cow dung, had a biogas potential of 45 mL/gTS added after three days of incubation (Posada, Noguera & Segura, 2012). The biogas production for kikuyu grass was likewise in this study after three days close to 200 mL/gVS.

Figure 6.2 indicates the biogas production for the different lignocellulosic biomass samples in different units. The biogas production potential of Napier grass, kikuyu grass and barley straw in terms of the wet weight added are 201 mL/gWW, 162 mL/gWW, 226 mL/gWW, respectively. These values are similar to figures reported in other studies of digesting different grasses which yielded a biogas production ranging between 164-186.1 NL/kg wet weight (Chiumenti *et al.,* 2018).

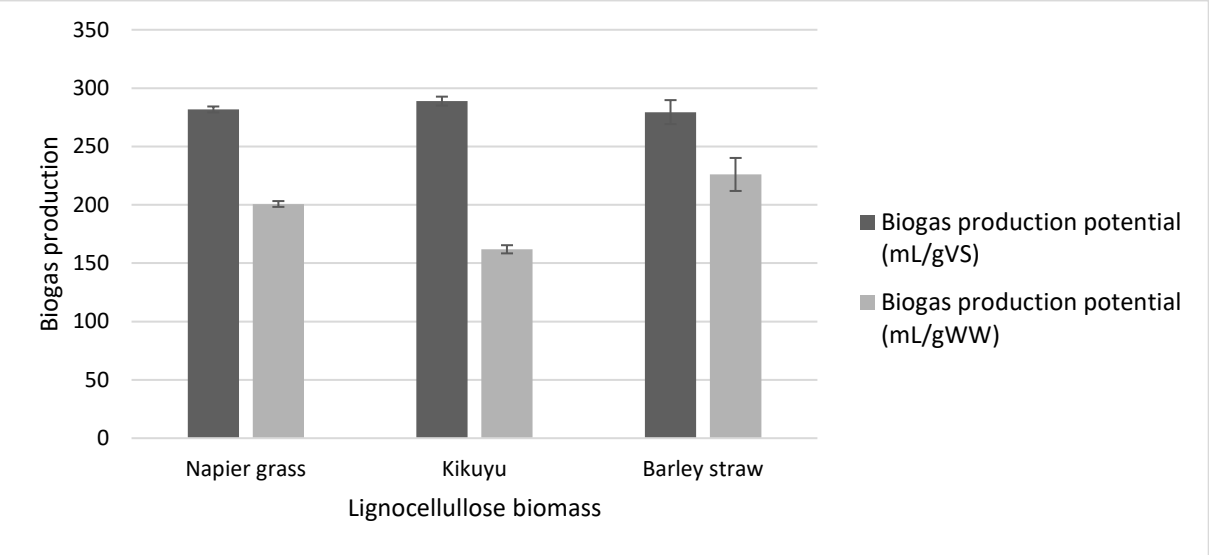


Figure 6.2: Biogas production of different lignocellulose biomass samples. The biogas produced is expressed in mL/gVS for the black bar and in mL/gWW for the grey bar. n=3

Barley straw has the highest biogas production in terms of the wet weight added, followed by Napier grass and kikuyu grass. This might be due to the high VS-content of barley straw compared to the other grasses. Therefore, less wet-weight of barley straw is needed for the same amount of biogas production, which renders it more viable for biogas production compared to the other fibrous feedstocks. This also leads to lower total solids in the reactor and will provide favourable conditions for microorganisms to digest the biomass.

The amount of biogas produced from the number of volatile solids added, indicated that rumen fluid is able to digest a wide range of grasses effectively. However, the composition of the different biomass samples shows no clear relationship between the biogas produced and the lignin content, which has been suggested by other studies as a limiting factor for gas production in anaerobic digestion (Shrestha *et al.,* 2017). Barley straw had the highest hemicellulose and cellulose content, but there is no evidence of a difference in the biogas production of the other grasses with lower cellulose and hemicellulose contents (**Table 6.1**). This may be an indication

that the sugars that are able to be degraded from the microorganisms in the rumen fluid are quickly degraded and the rest of the sugars are bound to lignin or woven in the structure of the biomass, strengthening its resistance to attacks from various microorganisms.

6.3.3 Degradability and reaction kinetics from different lignocellulosic biomass

The total solids and volatile solids digested from the different lignocellulose biomasses differ from one another, as can be seen in **Figure 6.3**. Kikuyu grass had the highest number of total solids digested (75%), followed closely by Napier grass (74%) and barley straw (68%) (**Figure 6.3**). The percentage TS digested of kikuyu grass and Napier grass was not significantly different to one another but both Napier grass and kikuyu grass were significantly different to barley straw ($p<0.05$).

The degradation values between 66% and 75% are similar to other studies digesting Napier grass and barley straw (Gijzen *et al.,* 1987; Kivaisi *et al.,* 1992; Sawasdee & Pisutpaisal, 2014). The degradation of TS correlates well with its biogas produced from the volatile solids added **(Figures 6.2 & 6.3**). This is possibly due to the structure of barley straw which lessens the available of the sugars and resistance to enzymatic attacks from the microorganisms compared to fresh green grasses. Both green grasses, kikuyu, and Napier grass, have a similar degradability percentage between 74%-75%. Another reason for higher degradability from the fresh green biomasses may be due to the moisture content in the grasses which may improve its digestibility. The kinetics of the different biomasses may also give an indication of the efficiency of rumen fluid as an inoculum.

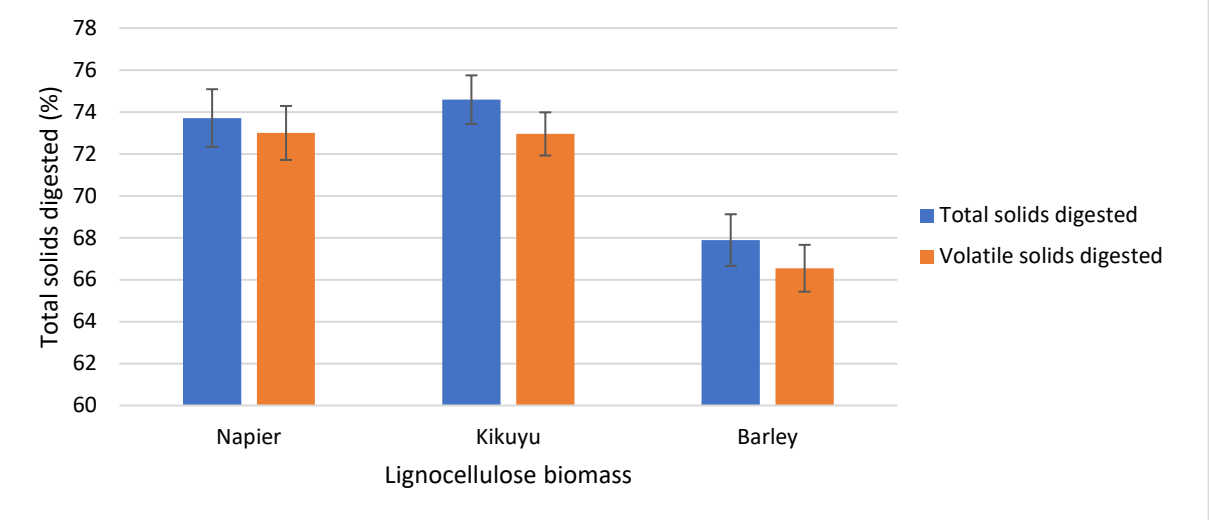


Figure 6.3: The percentage total solids and volatile solids digested from the different lignocellulose biomasses: blue bar indicates the total solids digested; orange bar indicates the volatile solids digested. The error bars indicate the standard deviation between the samples. n=4

The kinetics of the digestion from the different biomass samples are summarised in **Table 6.2**. The measured data for all the biomass samples had a high goodness of fit (GoF) in all models

(R^2) ranging between 0.954 and 0.999. The predicted biogas potential compared to the measured biogas potential was higher for all models, except for the modified Gompertz model and the two-fraction first order model. The modified Gompertz model had the least error between the measured values and predicted values (-3.81 to -1.92). The highest maximum rate of biogas production was observed for Napier grass (μ_m = 107.269 mL/gVS/day). This was followed by kikuyu grass and barley straw, which had similar maximum biogas production rates. However, the maximum rate of biogas potential for Napier grass may be due to the longer lag time for biogas production as seen in the modified Gompertz model (0.580 days) compared to the other two grasses. Studies exploring the maximum rate of biogas production from lignocellulosic biomasses have reported values that range between 0.613 mL/gVS/day and 0.899 mL/gVS/day (Ghatak & Mahanta, 2014). This is quite a low rate compared to the results reported in this study, serving as an indication into how rumen fluid can increase the overall rate of biogas production in anaerobic digestion.

The coefficient of determination fit best for the two-fraction first order model which had a coefficient of determination value between 0.997 and 0.999. This was followed by the modified Gompertz model and first order model. The Monod model also had a good coefficient of determination ranging between 0.954-0.986 for all biomasses. The reaction rate of biogas production per day (k) ranged between 0.26 and 0.33 for all biomass samples for the first order and Monod type models. Overall, the rate did not differ between the different lignocellulosic biomasses, highlighting the potential use of rumen fluid for a wide range of lignocellulosic biomasses in anaerobic digestion.

Table 6.2: Kinetics characteristics from different models

Biomass	Model	Specific biogas production			Error (%)	μm (mL/gVS/day)	λ(Day)	ks	kf	R^2
		k (day^{-1})	predicted	measured						
Napier grass	FO	0.339	310	282	-10.21	nd	nd	nd	nd	0.993
	MT	0.269	435	282	54.41	nd	nd	nd	nd	0.955
	GM	nd	271	282	-3.81	107.269	0.580	nd	nd	0.991
	TFFO	nd	278	282	-1.20	nd	nd	35.56	0.672	0.998
Kikuyu grass	FO	0.334	318	289	9.99	nd	nd	nd	nd	0.991
	MT	0.273	440	289	52.35	nd	nd	nd	nd	0.986
	GM	nd	285	289	-1.40	81.988	0.186	nd	nd	0.994
	TFFO	nd	298	289	3.02	nd	nd	35.56	0.472	0.999
Barley straw	FO	0.334	318	279	13.71	nd	nd	nd	nd	0.992
	MT	0.320	404	279	44.42	nd	nd	nd	nd	0.990
	GM	nd	274	279	-1.92	81.235	0.108	nd	nd	0.994

	TFFO	nd	286	279	2.42	nd	nd	35.56	0.481	0.9997

FO – First order; MT – Monod Type; Gm – modified Gompertz; TFFO – two-fraction first order; k- first order kinetic constant; μ_m – maximum biogas production rate; λ- lag phase; k_s and k_f are reaction rate constants in TFFO model and nd – no data

6.3.4 Chemical composition of digestate after digestion and protein produced in digestate

The total Kjeldahl ammonium nitrogen, total phosphorus and total potassium were measured to see if the digestate can potentially be used as a fertilizer for agricultural purposes. The digestate of kikuyu grass, barley straw and Napier grass were compared to one another to test if the composition of the digestate differs from different feedstocks used in an anaerobic digester. The results of the potassium, Kjeldahl ammonium nitrogen and phosphorus are presented in **Table 6.3**.

Table 6.3: Characteristics of digestate from different lignocellulose feedstocks

Digestate	pH	Total suspended solids (TSS) in digestate (g/L)	Kjeldahl ammonium nitrogen NH_4^+ (%)	Total phosphorus (%)	Total potassium (%)
Inoculum	6.22 ± 0.07	nd*	0.15	0.05	0.19 ± 0.01
Kikuyu grass	6.38 ± 0.05	4.53 ± 0.21	0.09 ± 0.01	0.065 ± 0.01	0.21 ± 0.01
Napier grass	6.60 ± 0.08	4.75 ± 0.25	0.115 ± 0.01	0.065 ± 0.01	0.20 ±0.01
Barley straw	6.25 ± 0.06	5.55 ± 0.21	0.086	0.07	0.18

*- *Nd- not determined*

The digestate of kikuyu grass had a final pH of 6.38 with a total suspended solids content of 4.53 g/L. The total ammonium nitrogen (NH_4^+) content of kikuyu digestate was 0.09%; total phosphorus content was 0.065%; and a total potassium content was 0.21% (**Table 6.3**). Napier grass digestate had a total suspended solids (TSS) content of 4.75 g/L and a pH of 6.60. The total ammonium nitrogen, phosphorus, and potassium content in the digestate of Napier grass was 0.115%, 0.065% and 0.20%, respectively. The digestate of barley straw had a pH of 6.25 and total suspended solids of 5.55 g/L (**Table 6.3**). The total ammonium nitrogen in the digestate of barley straw was 0.086%, phosphorus was 0.07% and potassium was 0.18%.

The potassium and phosphorus concentrations did not differ between the different digestates (**Table 6.3**). Napier grass had the highest percentage of ammonium nitrogen in the digestate compared to the other biomasses. Other studies have reported values of ammonium nitrogen in the ranges of 2.92 g/L – 2.53 g/L in the digestate when digesting different grass samples in anaerobic digestion (Chiumenti *et al.,* 2018; Lehtomäki *et al.,* 2008). However, when investigating *in vitro* digestion of lignocellulose biomass using rumen fluid, similar ammonium

nitrogen values have been reported in literature compared to this present study (Saleem *et al.,* 2019). The low nitrogen in the digestate might be due to the high carbon-to-nitrogen ratio found in grasses. The demand for nitrogen in rumen digestion is an important factor for consideration when using rumen fluid as an inoculum source. This is due to the fast digestion and fast growth of these bacteria, the nitrogen in the reactor may be limiting during longer periods of anaerobic digestion for microbial growth and metabolism.

The crude protein in the digestate can be calculated by multiplying the total ammonium-nitrogen content obtained from the Kjeldahl method with a factor of 6.25 (Lengowski *et al.,* 2016). Based on the abovementioned calculation, the crude protein in the digestate for Napier grass, kikuyu grass and barley straw are 720 mg/L, 560 mg/L and 540 mg/L, respectively. The crude protein in the digestate from Napier grass was the highest compared to the digestate of the other biomasses, likely due to this feedstock having the lowest carbon-to-nitrogen ratio. Napier grass had a carbon-to-nitrogen ratio of 14, whereas the carbon-to-nitrogen ratio for barley straw was the highest, an indication why the ammonium nitrogen and crude protein in the digestate are the lowest for barley straw.

The results suggest that when examining the digestate of a batch culture, microorganisms will utilise all the available nutrients for their own growth and metabolise the feedstock until there is a lack of nutrients or other inhibiting factors limiting the metabolism of the microorganisms.

6.4 Conclusions and recommendations

The biogas potential from the anaerobic digestion of three different lignocellulose biomass samples are presented in this study. This study determined no statistically significant differences when digesting various lignocellulose biomasses with the use of rumen fluid as an inoculum. Kikuyu grass had the highest reported biogas production (289 mL/gVS) and barley straw had the lowest biogas production (275 mL/gVS). The methane percentage of the different biomasses ranged between 21.14% and 22.79%. The composition of the biomass did not have an effect on overall rate of biogas produced and the biogas potential when using rumen fluid as an inoculum. This study has determined that the use of rumen fluid has a significantly shorter retention time for the digestion of lignocellulosic biomass compared to other studies. While the potassium and phosphorus of the digestate from the different feedstocks did not differ, the ammonium nitrogen and crude protein differed between the feedstocks. The digestate of Napier grass had the highest crude protein content compared to the other feedstocks. Rumen fluid is a highly effective inoculum source for the digestion of a range of different lignocellulose biomasses. Future research should scale-up the mono-digestion of lignocellulosic biomasses to see if this would result in a feasible use of rumen fluid in a large-scale digester.

Chapter 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

7.1.1 Designed rumen based anaerobic digester

The designed digester had an average biogas production of 12.63mL/gVS/day with a solid degradation percentage between 37.74% and 48.40% with rumen fluid. The protein content of the solid residue increased after 10 days of incubation, but then decreased after 15 days which is an indication of a nitrogen demand for the mono-digestion of barley straw. The biogas production was not able to be determined with the use of the digester as it did not digest the feedstock completely; therefore, it will be of more value to determine the biogas production of the feedstock through batch digesters by using rumen fluid as an inoculum. The functioning of the designed digester is not suitable for biogas production as it is opened daily and the biogas is able to escape from the reactor during the running of the digester, causing additional stress on the bacterial population.

7.1.2 Effect of different ISR and organic loadings on biogas production

The biogas production differed at different ISRs and organic loadings. There were no statistically significant differences in biogas production between organic loadings of 8.14 gVS/L (ISR of 0.34), 12.21 gVS/L (ISR of 0.23) and 16.28 gVS/L (ISR of 0.17). However, the organic loading of 16.28 gVS/L (ISR of 0.17) had the highest biogas production of 269 mL/gVS for the mono-digestion of lignocellulosic biomass. The increase of the organic load to 24.41 gVS/L (ISR of 0.11) had detrimental effects on the biogas production by decreasing the pH and increasing the concentration of volatile fatty acids (VFAs) in the reactor leading to 25% loss in biogas production. The increase in the organic loadings and decrease in ISR led to a higher concentration of volatile fatty acids (VFAs) in the digestate. For effective biogas production from the mono-digestion of lignocellulose, it is advised to have an organic loading between 8.14 gVS/L-16.28 gVS/L and an ISR between and 0.17-0.34. The kinetic modelling showed that all models had a coefficient of a determination value above 91%, with the modified Gompertz model having the best fit and least error between the measured and predicted values.

7.1.3 Biogas potential from different lignocellulosic biomasses

There were no significant differences in the biogas production from the different feedstocks. However, the biogas production for kikuyu grass (289 mL/gVS) was higher than both Napier grass (285 mL/gVS) and barley straw (279 mL/gVS).

The reaction kinetics, similar for all feedstocks, had a high coefficient of determination for all models with a value above 95%. The two-fraction first order and modified Gompertz model had the least error between measured and predicted values.

7.1.4 Digestate composition between different lignocellulose biomasses

The digestate after anaerobic digestion of the lignocellulosic biomass samples did not differ significantly. Napier grass had the highest ammonium concentration (115 mg/L) compared to barley straw and kikuyu grass. All the biomass samples had similar potassium and phosphorus concentrations. The crude protein differed in the digestate with Napier grass having the highest crude protein compared to other feedstocks.

7.2 Recommendations

The recommendations for future studies are as follows:

- Lignocellulose biomass has a high carbon-to-nitrogen ratio and may require an additional source of nitrogen to lower the ratio to the optimal.
- The co-digestion of lignocellulose biomass with other waste streams should be considered to minimise the input cost for additional nutrients.
- The use of a two-staged system to separate the hydrolysis and methanogenesis phases may provide a solution to minimise the acidification experienced when adding a too high organic load in the reactor.
- To understand which bacterial strain assists in the production of the hydrolytic enzymes to enable the fast degradation of lignocellulose biomass.
- Future studies should test the effect of adding the digestate as a fertilizer to plants, to evaluate the growth of the plants.
- A cost analysis should determine if it will be economically feasible to grow long, fibrous grasses for biogas production.
- A test should be conducted to use the solid digestate in animal feed to evaluate the quality of the meat being produced.

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APPENDIX A

Table 1A – Tukey's test to test the significant differences (p<0.05) between the means of the biogas production potential of ISRs

Samples	Significant difference (p<0.05)	P-value
ISR 1.36 vs ISR 0.68	no	0.8255
ISR 1.36 vs ISR 0.34	yes	0.0432
ISR 1.36 vs ISR 0.23	yes	0.0222
ISR 1.36 vs ISR 0.17	yes	0.0065
ISR 1.36 vs ISR 0.11	no	0.9518
ISR 0.68 vs ISR 0.34	no	0.3710
ISR 0.68 vs ISR 0.23	no	0.2194
ISR 0.68 vs ISR 0.17	no	0.0718
ISR 0.68 vs ISR 0.11	no	0.9998
ISR 0.34 vs ISR 0.23	no	0.9997
ISR 0.34 vs ISR 0.17	no	0.9338
ISR 0.34 vs ISR 0.11	no	0.2225
ISR 0.23 vs ISR 0.17	no	0.9920
ISR 0.23 vs ISR 0.11	no	0.1234
ISR 0.17 vs ISR 0.11	yes	0.0380

Table 2A – Tukey's test to test the significant differences (p<0.05) between the means biogas production potential of different biomass feedstocks

Samples	Significantly different (p<0.05)	P-value
Napier vs Kikuyu	no	0.3595
Napier vs Barley	no	0.8274
Kikuyu vs Barley	no	0.1021