ANAEROBIC DIGESTION OF SPINELESS CACTI (*Opuntia ficus-indica* (L.) Mill*)* BIOMASS IN TANZANIA: THE EFFECTS OF AEROBIC PRE-TREATMENT

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A Thesis submitted to Pan African University Institute for Basic Science, Technology and Innovation in partial fulfilment of the requirements for the degree of Master of Science in Molecular Biology and Biotechnology of the Pan African University

2018

DECLARATION

I, the undersigned, declare that this is my original work and has not been submitted to any other college, institution or university for academic credit.

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DEDICATION

To my family, my daughter Sofia and everyone who have supported me throughout the process. Your love, support and encouragement have immeasurably guided me throughtout the process.

ACKNOWLEDGEMENT

I would like to thank God for providing me the patience and endurance in the writing of this thesis. I convey my deepest appreciation to Pan African University Institute of Science, Technology and Innovation, particularly the Department of Molecular Biology and Biotechnology. I am thankful to the Coordinator of the Molecular Biology Department Prof. Naomi Maina for her help and assistance throughout the process. My deepest appreciations to my supervisors Prof. Anthony Mshandete and Dr. Samuel Imathiu for giving me constructive advice during the period of research and thesis writing without which completion of this work would not have been possible. I would also like to extend my gratitude to Mr. Kelvin Shitindi who has been a great help during my bench work. I am thankful to my colleagues, especially Mr. R. Chamedjeu for their continuous support during the whole research period.

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ABBREVIATIONS AND ACRONYMS

AD	Anaerobic Digestion
ASAL	Arid and Semi-arid Land
BLAST	Basic local alignment search tool
BMP	Biochemical methane potential
BOD	Biological oxygen demand
CAM	Crassulacean acid metabolism
C/N	Carbon/Nitrogen
COD	Chemical Oxygen Demand
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
GHG	Greenhouse gas
ITS	Internal transcribed spacer
MEGA	Molecular Evolutionary Genetics Analysis
MJ	Megajoule
PCR	Polymerase chain reaction
RuBisCO	Ribulose-1, 5-bisphosphate Carboxylase Oxygenase
STP	Standard Temperature and Pressure
TAE	Tris-acetate-EDTA
TS	Total Solid
V	Volume
VFA	Volatile Fatty Acids
VS	Volatile Solid

ABSTRACT

One of the best options for African countries to meet rural energy needs is to grow on a massive scale in waste lands, care-free crassulacean acid metabolism plants, which can enable bioenergy production without disrupting food supplies and hence sustainable energy supply for the future. *Opuntia ficus-indica* (L.) Mill is an ideal plant for arid regimes but has barely been studied as a potential bioenergy source. This study analysed *Opuntia*'s physicochemical parameters which are important for anaerobic digestion and then investigated the effect of aerobic pretreatment on methane yield of *Opuntia ficus indica* biomass. This effect was investigated in batch bioreactors by varying time from 0 to 72 h. Reducing sugar content and dissolved oxygen levels after pretreatment and methane forming potential through anaerobic digestion was analysed. Reducing sugar content in bioreactors increased with increase in pretreatment time from 12.22 ±0.69 g/l to 59.08 ± 5.35g/l in the control and 72 h pretreated batches respectively. Methane yields after pretreatment were observed to range from 0.286 m^3 CH_4/kg VS to 0.702 m^3 CH_4/kg VS at 9 and 72 h of pre-treatment respectively. A 9 h pre-treatment of feedstock prior to anaerobic digestion yielded 123% higher methane when compared to the control. The findings that there was an increase in reducing sugar production and methane yield at 9 h of aerobic pre-treatment suggests that there was increased hydrolysis with pretreatment and subsequently improved methane yield. Hence short pre-treatment period could be an option to increasing solubilisation of *Opuntia ficus indica* cladodes and promoting methane productivity. Pre-aeration of *Opuntia ficus indica,* therefore, was shown to be an effective method for enhancing its digestibility and improved methane yield during anaerobic digestion.

CHAPTER ONE

1.0 INTRODUCTION

1.1 General Background Information

One requirement for sustainable development in any country is the availability of adequate energy services for satisfying basic needs, improving social welfare, and achieving economic development (Rogner *et al*., 2004). Presently, about 80% of total global energy share is contributed by fossil fuels, whereby the transportation sector consumes the main part of energy (REN21, 2014). These fuels main component is hydrocarbon which include things such as petroleum, coal or natural gas derived from organic matter accumulated over time. Some of the issues with these sources of fuels is the fact that they are non-renewable and therefore can be exhausted, in fact by 2100 the global fossil fuel reserve is projected to be depleted (Saxena *et al*., 2009). Another issue with these fuel sources is that they contribute to the generation of greenhouse gases leading to global warming, and consequently climate change and its negative environmental impacts.

The search for renewable energy source such as biofuels, which are favored alternative to fossil fuels, safer and easily available is currently a necessity (Moshi *et al.*, 2015). Biofuel covers a wide range of fuels which in one way or another are derived from biomass. This includes bioethanol, biobutanol, biodsiesel, biogas, and biohydrogen (Nigam and Singh, 2011). Among these bioethanol, biogas and biodiesel are the ones which are mostly produced on commercial scale.

Biogas typically refers to a gas produced by the biological breakdown of organic matter in the absence of oxygen (Chynoweth *et al.*, 2001). Its composition is mainly methane (CH_4) and carbon dioxide (CO_2) and may have small amounts of hydrogen sulphide (H_2S), moisture and siloxanes (Chynoweth *et al*., 2001). Methane, hydrogen and carbon monoxide (CO) can be combusted or oxidised with oxygen to release energy. This energy release allows biogas to be used as a fuel for various purposes such as heating, for example cooking (Moshi *et al.*, 2015). Like natural gas biogas can be compressed and used to power motor vehicles Bio methane can also be made from it through cleaning and upgrading it to natural gas standards (Plugge, 2017). The nutrient-rich digestate that is left by biogas or anaerobic digestion process can be used as fertiliser (Eriksson *et al*., 2005).

When it comes to their classification biofuels can be classified as the first, second, third, and fourth generation (Davis, 2012). Food crops rich in sugar or starch or vegetable oil are the ones producing first generation biofuel (Davis, 2012). The major drawback of the first generation biofuel crops is that they are important food crops and their use for fuel can have adverse impacts on food supply. These crops are also intensive in the use of one or more inputs such as land and water which has other environmental implications (Ziolkowska and Simon, 2011). All these create a challenge in using biofuels mainly due to the cost of feedstock, which takes more than 50% of the total cost of biogas production (Moshi *et al*., 2014).

Up to now, corn and sugarcane are the main feedstock used in the biofuel production (Moshi *et al.,* 2015). As these main feedstock are also food crops, there is speculation that

using such crops for fuel can drive up the price of food. Therefore, there is a need to find alternative sources in order to reduce competition for these raw materials which are also used as human food and animal feed. The solution to this is identification and characterization of cheap and readily available feedstocks in the environment which are not used as food.

The current status of global warming makes crassulacean acid metabolism (CAM) plants that can withstand and resist drought more attractive as feedstock for anaerobic digestion (Jigar *et al*., 2011; Calabr *et al*., 2017; Ramos-Suarez *et al*., 2014; Consoli *et al*., 2013). Hence the best option to meet rural energy needs and overcome energy shortage is to grow on a massive scale in waste lands these care-free growth plants (Yang *et al.*, 2015). Among these CAM plants is the fast-growing *Opuntia ficus-indica* which is known to have high water use efficiency. *Opuntia ficus indica* is the most widely distributed species of the cactus family and the most potential species in terms of its usage (Nobel and Bobich, 2002). These are desert plants that can survive where most other plants cannot hence are suitable plant resource for climate change adaptation (Tarisse, 2008).

Use of these plant have great potential to mitigate climate changes associated with droughts especially in developing countries. Great areas of these countries are arid and semi-arid (ASALs). According to the FAO (2012), Tanzania's ASALs cover more than 50% of the country, whereas that of its neighbouring country Kenya is about 80%. These represents areas where *Opuntia* plants can be easily grown and subsequently be used as feedstock for biofuel production.

In this study a Crussulacean Acid Metabolism (CAM) plant, *Opuntia ficus indica* also known as spineless cacti was characterized as feedstock and the effect of aerobic pretreatment was evaluated for biogas production. These plants, known for their high water use efficiency, have cladodes which are covered with thick epidermis preventing water loss. Their stomata close during the day but open at night to prevent water loss through transpiration. The fact that these are desert plants that can survive where nothing else can grow makes them a suitable plant resource for climate change adaptation (Tarisse, 2008). Using spineless *Opuntia* as an energy crop is offering serious perspectives to countries prone to drought and relying on imports for their energy consumption (Tarisse, 2008).

1.2 Statement of the problem

Availability of suitable energy source to sustain the needs of rural communities in African countries remain one of the greatest obstacles for development. The continual use of fossil fuels and its effect of greenhouse gases (GHGs) on the environment necessitate more efforts in the production of alternative fuels from bio resources. Global energy demand have led to the increase in the use of fossil fuels making up to approximately 88% of the energy produced presently, this in turn drastically increases the amount of GHG emission into the atmosphere (IEA 2015; UNEP 2014). Reliability of fossil fuels should be reduced for the security of energy supply and because most of natural energy resources including oil are non-renewable.

The prospects of an increasingly hotter and drier climate has led many researchers to re-evaluate heat and drought tolerant CAM species for use as feedstocks for bioenergy

production on semi-arid and arid lands. Of these group of CAM plants Agave species have been most studied and the potential of *Opuntia ficus indica,* which is one of the species under cacti group have been for many years overlooked.

Studies have been carried out to evaluate the potentials of *Opuntia* in anaerobic digestion for biogas production but limited studies have dealt with pretreatment of the plant cladodes prior to anaerobic digestion and the effect they could have on both methane production and yield. Currently, there are limited scientific reports on the biological pre-treatment of *Opuntia* plant with regard to biogas production and methane yield. There is scarce documentation on the enhancement of biogas production and methane yield using *Opuntia* as feedstock by aerobic pre-treatment. It is therefore important to assess the impact that aerobic pre-treatment have on the anaerobic digestion of *Opuntia.*

1.3 Justification and significance of the study

The need for clean energy and phasing out the fossil fuels which have high amount of GHG emissions in the atmosphere is continuously raising. This necessitates turning to 'greener alternatives' which will have sustainable clean energy production, the use of *Opuntia* as feedstock being one of them.

Opuntia ficus indica is one of the heat and drought-durable CAM species suitable for use as bioenergy feedstocks on semi-arid and arid lands (Consoli *et al*., 2013; Russell and Felker 1987). The plant is found in abundance in these parts and therefore the feedstock for biogas production is not limited. In addition, the plant may not be significantly affected by climate change and its cultivation requires low agronomic input (Nobel and Bobich, 2002). These plants are recognized as ideal crops for arid regimes because they are

extremely efficient at converting water into biomass (Cushman *et al*., 2015). The fact that spineless cacti is not used as food in most areas would reduce the competition of food vs fuel use and represent an inexpensive renewable energy source, which, through anaerobic digestion and biogas production, has a very good potential to contribute to sustainable energy supply.

The conversion of lignocellulosic biomass in methane production usually requires some form of pretreatment prior to anaerobic digestion to facilitate enzymatic hydrolysis (Hahn-Ha¨gerdal *et al.,* 2006). Biological pre-treatment reduces the problems caused by other forms of pre-treatment such as chemical, thermal and mechanical methods which have high financial or environmental cost, while increasing the hydrolysis of the feedstock during anaerobic digestion and increase the overall methane yield (Carlsson *et al.,* 2012). This study provides detailed information on the effect of aerobic pre-treatment on methane yield during anaerobic digestion of *Opuntia ficus indica*. In addition the study characterized *Opuntia ficus indica* as a feedstock, which included molecular characterization and compositional analysis.

Using these species as feedstocks would inform future biofuel production plans on waste or bare land that is currently not used for the production of C_3 and C_4 crops and provide the possibility of targeted cultivation, harvesting, and utilization strategies of the species as feedstock in biogas production. This in turn will provide or increase employment to the growing young generation who can take part and participate in the whole production process.

1.4 Research objectives

1.4.1 General objective

To perform anaerobic digestion of *Opuntia* biomass focussing on the effects of aerobic pre-treatment.

1.4.2 Specific objectives

i. To perform physicochemical analysis of *Opuntia* used as feedstock for anaerobic digestion.

ii. To perform molecular identification of *Opuntia* used as feedstock for anaerobic digestion.

iii. To determine the effects of aerobic pre-treatment of *Opuntia* on the extent of biogas production and methane yield in batch anaerobic bioreactors.

1.5 Research hypotheses

i. There is no difference in key physicochemical properties of *Opuntia* samples collected from two sites.

ii. Difference in physicochemical properties of *Opuntia* feedstock is not caused by differences at molecular level.

iii. There is no improvement in methane yield of anaerobic digestion of *Opuntia* with aerobic pre-treatment.

iv. There is no significant difference in methane yield between *Opuntia* biomass under different period of aerobic pre-treatment.

1.6 Scope of the study

The study focused on the *Opuntia* found in Tanzania as the domain of interest. The research included an analysis of the plant as feedstock with particular emphasis on physicochemical analysis and molecular identification. The study also conducted anaerobic digestion using the plant as the feedstock after aerobic pre-treatment.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Biogas as a biofuel

Biogas is a renewable energy source produced from anaerobic digestion of various organic materials (Plugge, 2017). The gas has various potential uses including fuel for cooking in combined heat and power gas engines or upgraded to natural gas quality bio methane (Claassen, 1999). The utilisation of biogas as a fuel helps to replace fossil fuels. Biogas major composition is methane and carbon dioxide with a small amount hydrogen and trace hydrogen sulphide (Madu and Sodeinde, 2001: Plugge, 2017) (Table 2.1).

Table 2.1: Percentage compositions of each biogas constituent

Constituents	% Composition
Methane (CH_4)	55 – 75
Carbon dioxide (CO_2)	30 – 45
Hydrogen sulphide (H_2S)	1 – 2
Nitrogen (N_2)	0 – 1
Hydrogen (H_2)	0 – 1
Carbon monoxide (CO)	Traces
Oxygen (O_2)	Traces

Source: (Madu and Sodeinde, 2001)

Recent evaluations indicate that biogas produced by anaerobic digestion (AD) provides significant advantages over other forms of bioenergy because AD is an energy-efficient and environmentally friendly technology (van Foreest, 2012). Production of this gas

reduces greenhouse gas (GHG) emissions by utilizing locally available sources (Dhingra *et al*., 2011).

Most of the developed countries including United States, China, and India are investing in alternative technologies for biogas production from cellulosic resources, and are likely future producers (Soetaert and Vandamme, 2009). Comparing with the annual global consumption the amount of biogas produced is limited. Hence the search for a good feedstock that is most appropriate for the biogas production is still on (Calabr *et al*., 2017). There is a wide range of materials that can be used as substrates for biogas production using AD technology. These include agricultural wastes, municipal waste, animal manure and slurry (Table 2. 2).

In Africa specifically Tanzania, as in many developing countries there is limited knowledge on biogas production from other substrates other than the traditionally used animal manure hence conform production in animal rearing areas (Nzila *et al.*, 2010). There are studies that have been done to explore other feedstocks potentials in Tanzania but more efforts are still needed to find reliable feedstock source (Muthangya *et al*., 2009; Nalinga and Legonda, 2016). Currently coal, biogas and other renewable energies account for 0.8% of energy consumption in Tanzania while 90% comes from fuel wood and charcoal (Nzila *et al.*, 2010; Mshandete, 2011). Thus, this calls for development of multi-feedstock for sustainable bioconversion of the vast amounts of organic wastes to renewable energy thus substituting (especially in the rural sector) the unsustainable conventional sources of energy (Nzila *et al*., 2010). The use of feedstock that is readily available with minimum expenses has a potential of increasing energy yield per unit

production cost, which can subsequently lead to availability of energy covering rural communities (Mshandete and Parawira, 2009).

Table 2.2: Comparison of biogas yield and electricity produced from different potential substrates for anaerobic digestion.

Type	Biogas yield per ton fresh matter (m^3)	Electricity produced per ton fresh matter [a](kW·h)
Cattle dung	55–68	122.5
Chicken litter/dung	126	257.3
Fat	826–1200	1687.4
Food waste (disinfected)	110	224.6
Fruit wastes	74	151.6
Horse manure	56	114.3
Maize silage	200/220	409.6
Municipal solid waste	101.5	207.2
Pig slurry	11–25	23.5
Sewage sludge	47	96.0

Source: (Stucki, 2011)

2.2 Anaerobic digestion of feedstocks for biogas production

Anaerobic digestion is the conversion of organic material directly to a gas, termed biogas, a mixture of mainly methane and carbon dioxide with small quantities of other gases such as hydrogen sulphide (McKendry, 2002b). This is a process that involves a series of steps in which in the absence of oxygen microorganisms break down biodegradable material and release energy (Bouallagui *et al*., 2010; Plugge, 2017). Gaseous oxygen is excluded

from the reactions by physical containment. The whole process of digestion begins with bacterial hydrolysis of the feedstock materials and produce insoluble organic polymers such as carbohydrates which are broken down and become available for bacteria metabolism (Plugge, 2017; Lettinga, 1995; Adney *et al.,* 1991). Sugars and amino acids are then converted into carbon dioxide, hydrogen, ammonia, and organic acids (Lettinga, 1995). Next bacteria in action are the acetogenic bacteria which convert these resulting organic acids into acetic acid, along with additional ammonia, hydrogen, and carbon dioxide (Bouallagui *et al.,* 2010). At the end the methanogens come into play and convert these products to methane and carbon dioxide (Gerardi, 2003). The process is widely used as a source of renewable energy producing biogas and the nutrient-rich digestate. The digestate produced can be used as fertilizer (Ziemińsk, 2012; Eriksson *et al*., 2005).

Anaerobic digestion (AD) can be carried out in three different temperature conditions. That which is carried out at a temperature range of 45–60 °C is referred to as 'thermophilic', whereas that carried out at a temperature range of 20–45 °C is known as 'mesophilic', which is the most used process (Ward *et al*., 2008; Gerardi, 2003). The AD of organic matter at low temperatures (<20°C) is known as 'psychrophilic' digestion (Ward *et al*, 2008). Most digesters are operated at mesophilic temperature due to greater stability of operation and lower energy requirements (Gavala *et al*., 2003; Knottier, 2003). Anaerobic digestion requires specific incubation time known as retention time (Schink, 2002). Retention time is the number of days the organic material stays in the digester. Under mesophilic conditions the process of degradation requires at least 10-30 days (Salminen and Rintala, 2002).

2.2.1 Biochemical and microbial fundamentals of anaerobic digestion

The process of AD consists of liquefaction and hydrolysis of insoluble compounds and gasification of intermediates (Plugge, 2017). This is accompanied by a partial or complete mineralization and humification of organic substance (Lyberatos and Skiadas, 1999). There are four classes of microorganisms involved in synergistic interaction which include hydrolytic, acidogenic, acetogenic and methanogenic bacteria in a multi-step process (Adney *et al.*, 1991) (Figure 2.1).

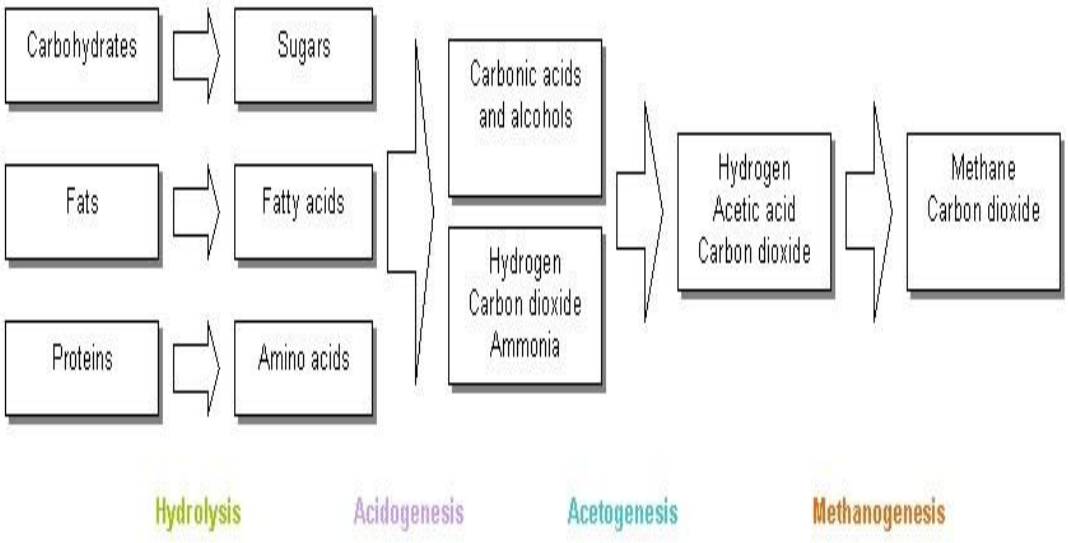

Figure 2.1: The key process stages of anaerobic digestion. Source: (Adney *et al*., 1991)

2.2.1.1 Hydrolysis

Most of the feedstock comprises of complex organic polymers, to allow access of these compounds by microorganisms the feedstock first has to be broken down into simple sugars, amino acids, and fatty acids (Gerardi 2003; Azman *et al.,* 2015). Through this process called hydrolysis monomers are made available to other microorganisms in the processes that follows (Parawira *et al.*, 2005; Plugge, 2017). This step is important since

fermentative microorganisms cannot absorb complex organic polymers directly into their cells (Sleat, R and Mah 2006).

This stage involves several steps, including enzyme production, diffusion, adsorption, reaction and enzyme deactivation (Batstone *et al*., 2002). Organic material size, shape, surface area, enzyme production and competitive adsorption of enzyme by inert substrates like lignin which reduce the efficiency of hydrolysis (Converse and Optekar, 1993; Song *et al*., 2005). This stage is the rate limiting step during anaerobic digestion process of highly particulate substrates like agro-industrial residues (Bjornsson *et al.*, 2001).

2.2.1.2 Acidogenesis

The process that results in further breakdown of the remaining components after hydrolysis by fermentative bacteria is what is called acidogenesis (Gerardi, 2003). Under this process volatile fat acids (VFAs) are produced, along with ammonia, carbon dioxide, and hydrogen sulphide, as well as other by products (Igoni *et al*., 2009). Sugars, long chain fatty acids and amino acids resulting from hydrolysis are used here as substrates by fermentative microorganisms to produce organic acids (Christy *et al*., 2014). The products of this stage depends on several factors such as substrate concentration, pH and dissolved hydrogen concentrations (Rodriguez *et al.*, 2005).

2.2.1.3 Acetogenesis

In anaerobic digestion the third stage is acetogenesis. Simple molecules created through the acidogenesis phase are further digested here by acetogens to produce largely acetic acid, as well as carbon dioxide and hydrogen (McInerney *et al*., 2008; Christy *et al*., 2014). Under this process the organic acids produced during acidogenesis are converted into

hydrogen and acetate by the acetogenic bacteria (Parawira *et al*., 2005). These conversions of volatile fatty acids are important as these acids, are mainly lethal to the methanogenic bacteria (Stams *et al.*, 2005; Gerardi, 2003). The products from this step consist mainly of acetate and hydrogen with more reduced products such as higher VFA, alcohols or lactate (Angelidaki *et al.*, 2002; Gerardi, 2003).

2.2.1.4 Methanogenesis

The final stage of anaerobic digestion is methanogenesis (Schink, 2002). Intermediate products of the acetogenesis are used by methanogens and converted to methane, carbon dioxide, and water which are the major components of the biogas emitted from the system (Christy *et al*., 2014; Franke-Whittle *et al.*, 2009). Key microbes in this stage are methanogenic archaea, a group of microorganisms, phylogenetically different from the main group of prokaryotic microorganisms (Wheeler and Rome, 2002). Bioreactor operating conditions such as pH, temperature, hydraulic loading rate, organic loading rate, and feed composition can affect their functioning (Murto, 2003). There is also homoacetogenic bacteria also involved in methane formation pathway through catalysing conversion between hydrogen and acetate. Depending on pH these organisms can either oxidize or synthesize acetate (Schink, 2002).

2.3 Batch bioreactor

Anaerobic digestion can take place in many different types of digesters. The majority of these digester types can be split into two groups based on the mode of feeding: batch-fed

and continuously-fed which differ primarily in the loading rate of the digester (Ziemińsk, 2012: Brown, 2006).

In a batch system, biomass is added to the reactor at the start of the process and then the bioreactor is sealed for the duration of the process (Brown, 2006). In this way batch processing needs inoculation with already processed material to start the anaerobic digestion. In a typical scenario, biogas production is formed with a normal distribution pattern over time (Igoni, 2009) this is used to determine when the process of digestion of the organic matter has completed. As the batch digestion is simple and requires less equipment and lower levels of design work, it is typically a cheaper form of digestion (Guendouz *et al*., 2010). Setting up of the bioreactor is an important step in the anaerobic digestion process as it is one among the factors that can affect the process performance (Plugge, 2017).

2.4 Crassulacean acid metabolism (CAM) plants

As much as there is a need to meet rural energy needs, bioenergy is widely seen as being in competition with food for land resources, as most of the feedstock used in its production are food crops (Calabr *et al.*, 2017; Michael *et al*., 2015). To overcome this the potential of plants that use the mode of photosynthesis known as crassulacean acid metabolism (CAM) to generate globally significant quantities of renewable electricity without displacing productive agriculture and perhaps even increasing food supply is now the focus of researchers (Calabr *et al*., 2017).

CAM plants require 10-fold less water per unit of dry biomass produced than do common C_3 and C_4 crops, and because of their succulence are endowed with substantial water-

storage capacities that helps to buffer intermittent water availability(Borland *et al.*, 2009; Michael *et al*., 2015). CAM pathways have low water requirements and are productive in semiarid regions because they assimilate carbon at night thereby decreasing the diffusive gradient of water out of leaves and improving water use efficiency (Nobel and Bobich, 2002; Consoli *et al*., 2013).

Although as a group these plants are understudied, there is nevertheless enough data available to evaluate the contribution they could possibly make to global electricity supply if used as feedstock for anaerobic digestion.

2.4.1 *Opuntia ficus indica*

Anaerobic digesters can be fed with various organic biomass such as purpose grown energy crops like maize but due to food insecurity the focus is moving mostly toward non-food crops including CAM plants (Mata-Alvarez *et al.*, 2000). *Opuntia ficus indica* (L.) Mill (Plate 2.1) is found under genus *Opuntia*, which belongs to the subfamily Opuntioideae, family cactaceae which is xerophytic family consisting of about 200 to 300 species (Stintzing and Carle, 2005: Gibson and Nobel, 1990).

Plate 2.1: *Opuntia ficus indica* used in this study

Opuntia ficus indica is one of the species found under CAM group of plants which is widely distributed in the arid and semi-arid regions throughout the world (Borland *et al*., 2009; Stintzing and Carle, 2005). *Opuntia ficus indica* is the most widely distributed species of the cactus family and at the same time the most economically important (Nobel and Bobich, 2002). The plant, which has succulent and thick stems called cladodes grows up to 3-5m in height (Borland *et al*., 2009). It normally produces flowers when they are 1-2 years old, and later on form fruits (Stintzing and Carle, 2005). Natural hybridization, associated with polyploidy and geographic isolation, has led to a great genotypic variability of *Opuntia*, displaying at the same time high levels of phenotypic plasticity (Wallace and Gibson, 2002).

Opuntia ficus indica is native to Mexico, but it is widely distributed and adapted to the arid and semi-arid regions of South and Central America, Africa and the Mediterranean area (Mohamed *et al.*, 1995). Due to the trend of Mediterranean area moving towards global desertification and decline of water resources, *Opuntia ficus-indica* has a great potential as feedstock in anaerobic digestion (Jigar *et al*., 2011; Ramos-Suarez *et al*., 2014; Yang *et al*., 2015; Calabr *et al*., 2017). Using spineless cacti as an energy crop is offering serious perspectives to countries prone to drought and relying on imports for their energy consumption (Tarisse, 2008).

There are studies which have been done to evaluate the potential of *Opuntia ficus indica* in anaerobic digestion for biogas production. During an experiment in a semi-continuous $1m^3$ mesophilic digester, the biogas potential of *Opuntia* with methane yield equal to around 500 mLCH4/gVS was reported (Obach and Lemus, 2006). Other studies have reported as low as 244 NmLCH4/gVS in the production of methane from *Opuntia* (Ortiz-Laurel *et al*., 2014).

To allow the conversion of lignocellulosic substrates to the highest possible degree through biological processes, it is necessary to increase the accessibility of cellulose by bacteria in the digester (Antonopoulou *et al*., 2015). To enhance the biodegradation of cellulose, several pretreatment techniques have been used which enhance hydrolysis, while avoiding degradation of other readily biodegradable compounds (Kumar *et al.*, 2009). This also avoids the formation of by-products that are inhibitory to subsequent processes (Calabr *et al*., 2017).

While using thermal, alkali and acid pretreatment it was found that methane yields ranged from 289 to 604 mL/gVS added where only the acidic pretreatment (Hydrochloric acid) was found to significantly increase methane generation (Calabr *et al*., 2017). While neither thermal nor alkaline pretreatment produced noticeably affect methane yield (an average reduction of 8% was recorded for NaOH pretreated substrate).

These studies suggests that there is availability of biodegradable material in *Opuntia* that can serve as a source of energy for microbes in anaerobic digestion ,since biogas production is a function of the feedstock's organic content and its biodegradability (Macias-Corral *et al*., 2008; Yeole and Ranande, 1992). However further studies are needed on pretreatment of *Opuntia ficus indica* so as to enhance its productivity. Special focus should be on biological pretreatments (such as aerobic pretreatment) which have an added advantage of being less expensive and environment friendly. Scientific literature on issues related to exploitation of pretreatment methods on anaerobic digestion of *Opuntia* is very scarce.

2.5 Pretreatment of lignocellulosic materials before anaerobic digestion

During the whole process of anaerobic digestion the step that can be termed as the rate-limiting step is the hydrolysis step (Raposo *et al.*, 2011). Hydrolysis is the step whereby the organic materials used in anaerobic digestion are produced (Gerardi, 2003). Hence it can be said that the whole process of biogas production depends on the crucial step of degradation, the hydrolysis step (Ghyoot and Verstrate, 1997). There have been reports on correlation between the biodegradability fraction and the lignocellulotic nature of the feedstock (Hartman and Ahring, 2005).

Lignocellulosic material represents significant barriers to hydrolysis hence its digestion is greatly enhanced by the pretreatment (Montgomery and Bochmann, 2014). Pretreatment prior to anaerobic digestion has been proven to be one of the simple and effective methods to improve biodegradability and biogas production of lignocellulosic materials (Muthangya *et al*., 2009; Mshandete *et al*., 2005; Calabr *et al*., 2017; Montgomery and Bochmann, 2014; Sun and Cheng, 2002). Alteration of physical structures as well as chemical characteristics of lignocellulosic materials could be done through a number of methods of pretreatment. Pretreatment increase biodegradability of lignocellulosic material by anaerobic microorganisms which in turn will increase efficiency of biogas production (Bougrier *et al*., 2008).

There are several ways in which lignocellulosic material can be pre-treated including thermal, chemical, mechanical and biological processes (Ariunbaatar *et al.*, 2014). All of these processes are said to be causing the disintegration of materials in the feedstock causing the release of the soluble organic matters and hence making them easily available to microorganisms involved in further processing downstream (Montgomery and Bochmann, 2014).

The usefulness of a pre-treatment in a specific system is determined by the mass and energy balance and the associated financial or environmental costs/values of inputs and outputs (Carlsson *et al.*, 2012). Pretreatment method should enhance hydrolysis, without degrading carbohydrates or forming by-products that are inhibitory to other processes downstream (Taherzadeh and Karimi, 2008).

2.5.1 Physical pretreatment

Physical pretreatment is done to reduce the particle size of the influent substrate and include techniques like mechanical, thermal, ultrasonic and electrochemical (Montgomery and Bochmann, 2014). A physical pretreatment process does not only increase the available surface area but also decrease the crystallinity and degrees of polymerization of cellulose (Carlsson *et al*., 2012). This pretreatment method increases biogas yield and reduce particle size which has an effect on the digester viscosity. Physical pretreatment reduce floating layers formation which cause problems in bioreactors as they can block outlets and interfere with gas release (Kamarad *et al.*, 2010). Nonetheless it has a downside which is increased energy demand high maintenance costs, as well as sensitivity to stones of mills used in mechanical pretreatment (Montgomery and Bochmann, 2014).

2.5.2 Chemical pre treatment

Chemical pretreatment methods include acid hydrolysis, alkaline hydrolysis and oxidant treatments (Montgomery and Bochmann, 2014). Acid treatment can significantly improve the reaction rate of the subsequent process of cellulose hydrolysis, while treatment with base increases the internal surface by swelling; decrease of polymerization degree and crystallinity; destruction of links between lignin and other polymers and the breakdown of lignin (Sanchez and Cardona, 2008). Some success with chemical pretreatment have been reported (Sun and Cheng, 2002; Montgomery and Bochmann, 2014). About 100% increase in biodegradability was observed when wheat straw was pretreated with NaOH (Montgomery and Bochmann, 2014).

Alkaline hydrolysis with NaOH has been successfully applied to treat lignocellulosic materials (Sun and Cheng, 2002). Most of these experiments were carried out using small-scale batch tests, but salt build up and increased pH can occur if alkali pretreatment is applied on continuous fermentation (Montgomery and Bochmann, 2014). High salt concentration and the resulting effect on the ammonium-ammonia balance inhibits methanisation (Chen *et al*., 2008). Generally, these pretreatment technologies are economically unattractive due to the high costs of chemicals that are used as well as the negative impact they can have on the environment (Montgomery and Bochmann, 2014; Chang *et al*., 1997).

2.5.3 Biological pretreatment

Biological pre-treatment methods have not been researched, studied and developed as extensively as physical and chemical methods for pretreatment of lignocellulosic feedstocks before anaerobic digestion (Muthangya *et al*., 2009; Mshandete *et al*., 2005). The general advantages of biological pretreatment over chemical or thermal pretreatment is that biological pretreatment can take place at low temperature without using chemicals and hence they do not require major capital investments (Mshandete *et al*., 2005; Montgomery and Bochmann, 2014).

Effect of the thermal, alkaline and acidic pretreatments on the composition and on the biochemical methane potential of the *Opuntia* cladodes have been evaluated (Calabr *et al.*, 2017).The authors found that only the acidic pretreatment (HCl) had significantly increase in methane generation, while neither thermal nor alkaline pretreatment produced noticeabe affect methane yields (an average reduction of 8% was recorded). These

pretreatment methods may have some draw backs since for example the use of hydrochloric acid can lead to a very low final pH, which could have a negative effect on the anaerobic digestion process (Calabr *et al*., 2017).

2.5.3.1 Aerobic pretreatment

Among many available pretreatment methods the one that is most favoured is the one that provides low financial or environmental values of inputs and outputs (Carlsson *et al.,* 2012). This is one of advantages posed by biological pretreatment methods, which takes into accounts both anaerobic and aerobic methods as well as the use of specific enzymes in enhancing the AD system (Ariunbaatar *et al.,* 2014). These methods aim at enhancing hydrolysis via the additional biological stage prior to a main digestion process (Carrere *et al.,* 2010).

Pretreatment using aerobic methods includes techniques like composting or micro-aeration before performing AD. These techniques cause higher production of hydrolytic enzymes which cause hydrolysis of substrates in the feedstock (Lim and Wang, 2013). There have been researches on aerobic pretreatment of feedstock prior to anaerobic digestion done by various researchers experimenting on feedstock other than *Opuntia.* Research have reported increase of about 20% in cumulative methane yield after 24 h of pre-aeration compared to the blank when working with sewage sludge (Ahn *et al.,* 2014). On the other hand sisal pulp waste had and an increase of 26% in methane yield after aerobic pre-treatment compared with control (Mshandete *et al.,* 2005). Similar results have also been reported whereby cumulative biogas production after 20 days of study was 465mLgVS-1 for pretreated waste mixture and 340mLgVS-1 for control (Subramani and

Ponkumar, 2012). All of these results show significant improvement in the methane composition and biogas production.

Evaluations of substrate solubilisation after pretreatment have been based on several measures such as total solids, volatile solids, or organic composition, e.g. contents of proteins, carbohydrates and lipids (Bougrier *et al*., 2008; Salsabil *et al*., 2010). Total solids, volatile solids and pH have been evaluated after pretreatment to evaluate the extent of solubilisation (Subramani and Ponkumar, 2012). Sugar content and enzymatic activities at the termination of pretreatment period have also been shown as useful parameter to determine the effect of pretreatment on substrate hydrolysability (Mshandete *et al*., 2005).

2.6 Description of feedstock physico-chemical composition

Suitability of any biomass as feedstock for processing in anaerobic digestion depends very much on its chemical characteristics (Dandikas *et al*., 2015). Feedstock variability can lead to operational uncertainty, which can affect energy recovery or ultimately result in system failure (Skaggs *et al*., 2018). The major fractions of lignocellulosic biomass are three major components of plant cell wall, namely cellulose (35-45% w/w), hemicellulose (25-45% w/w) and lignin (15-30% w/w) (Betts *et al*., 1992). Presence of adequate quantities of nitrogen, micronutrients, and water is also important in organic substrate that is to be anaerobically digested and generate methane rich biogas (Singh *et al*., 1999).

2.6.1 Cellulose

This is one of the major component of lignocellulose cell walls (Jorgensen *et al*., 2007). The anaerobic depolymerization of cellulose is conducted by hydrolytic bacteria, which produce cellulolytic enzymes in order to degrade the polymer into cellobiose and glucose units (Procházka *et al*., 2012). Studies have shown that cladode tissues of *Opuntia ficus-indica* have a lower crystalline cellulose content and a higher amorphous and paracrystalline (disordered) cellulose content (Cushman *et al*., 2015). This suggest its lignocellulosic biomass would be even more readily hydrolysable into fermentable sugars than would biomass from traditional herbaceous or woody feedstocks (Yang *et al*., 2015). Cellulose content of 8%, 11% and 21.6% have reported in *Opuntia ficus indica* (Calabr *et al*., 2017; Malainine *et al*., 2003).

2.6.2 Hemicellulose

In contrast to cellulose, hemicellulose is not entirely consisted of one monosaccharide. A variety of C6 sugars (e.g. D-glucose, D-mannose, Dgalactose, L-rhamnose), C5 sugars (e.g. D-xylose, D-arabinose) and sugar acids (D-glucuronic acid, 4-*O*-methyl-D-glucuronic acid) are the dominant polymers (Hendriks and Zeeman, 2009; Zheng *et al*., 2014). A variety of enzymes are needed to conduct hemicellulose breakdown due to increased heterogeneity (Azman *et al*., 2015). But its properties such as the short length, low molecular weight and amorphous shape make its units the easiest hydrolyzed components compared to cellulose and lignin (Fengel and Wegener, 1984).

2.6.3 Lignin

This is one of the abundant organic polymers in lignocellulosic biomass (Zheng *et al*., 2014). It has amorphous heteropolymer cross-links among its polysaccharides which creates an impermeable and resistant structure acting as the main barrier for biomass deconstruction (Jorgensen *et al*., 2007). Lignin is generally considered as the non-degradable organic matter in feedstocks though its oligomeric and polymeric components can be partially degraded under anaerobic conditions (Angelidaki and Sanders, 2004; Monlau *et al*., 2013). Cladode tissues of *Opuntia ficus-indica* contain relative low range of lignin content (0.01–16%), making the hydrolysis of this feedstock easier (Yang *et al.*, 2015).

2.6.4 Moisture content

Opuntia have very high moisture content, their fresh cladodes have been reportedly to have water content in the range of 88% to 95% (Stintzing and Carle, 2005). This high water content could help to reduce water inputs needed for downstream processing of *Opuntia* lignocellulosic biomass during mechanical, physiochemical, or enzymatic hydrolysis treatment steps (Parish and Felker, 1997).

2.6.5 Organic content

The key parameter that is used in determining the potential for a feedstock as a suitable digester substrate is its organic content, usually measured in terms of volatile solids (VS). This is basically the organic matter that get degraded into biogas. It’s important to note that not all organic matter have equal digestibility in the digesters (Burke, 2001).

Other components of cladodes on dry matter basis include crude protein content 5- 12 %, and carbohydrate content 2-6 % (Stuart, 2003). This plant shows both within site and inter-site variability in its physical properties. These properties vary from plants in one area to another and are strongly influenced by the environment (Parish and Felker, 1997). In this context, it is of great interest to study these properties before performing anaerobic digestion.

2.7 Molecular identification of *Opuntia*

Agricultural changes and modern agricultural practices have led to genetic diversity, degradation and gene pools loss among organisms (Labra *et al.,* 2003). Several *Opuntia* species have been described morphologically preceding molecular marker assessment (Gallegos–Vásquez *et al*., 2011). Most of researches have been oriented towards physicochemical and nutritional characterisation of the cladodes as fodder crop as well as a feedstock for anaerobic digestion in arid areas (Jigar *et al*., 2011; Calabr *et al*., 2017). Analysing to molecular level to for species variation and identification is limited. Due to the multiple uses and the ability of cacti to thrive in arid and semiarid environments, it has become increasingly important to describe and characterize these valuable resources (El Finti *et al*., 2013). In the recent years, molecular markers have been shown to be powerful in species identification (Charcosset, 2004).

Molecular studies on the genus *Opuntia* have been conducted in some studies using ISSR and AFLPs (Labra *et al*., 2003; Zoghlami *et al*., 2007; Bendhifi *et al*., 2013; Valadez-Moctezuma *et al*., 2014). A widely used method is based on the internal transcribed spacer

of nuclear ribosomal genes (nrITS) for phylogenetic analysis (Lyra *et al*., 2013). Ribosomal internal transcribed spacer (ITS) sequences and rRNA sequences have conserved lengths and a high degree of variability and are well suited for identification and classification studies (Lyra *et al*., 2013; Martínez *et al*., 2017). Physicochemical properties of a plant used as feedstock material for AD may differ based on genetic diversity. Since molecular characterisation of *Opuntia* in Tanzania has been scarcely studied, it's important to perform molecular identification of plants used in anaerobic digestion in this study alongside physicochemical analysis.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study site: the source of *Opuntia* samples

Opuntia used as the substrate for anaerobic digestion was obtained from two sites in Tanzania which were located in two different agroecological zones: Dar es salaam, Oysterbay (Site A) which is located at -6°49'24.56" S 39°16'10.24" E under Southern Guinea Savanna and the Ulongoni (Site B), which is located at -6°54'0.00" S 39°04'0.01" E under derived savanna (Figure 3.1).

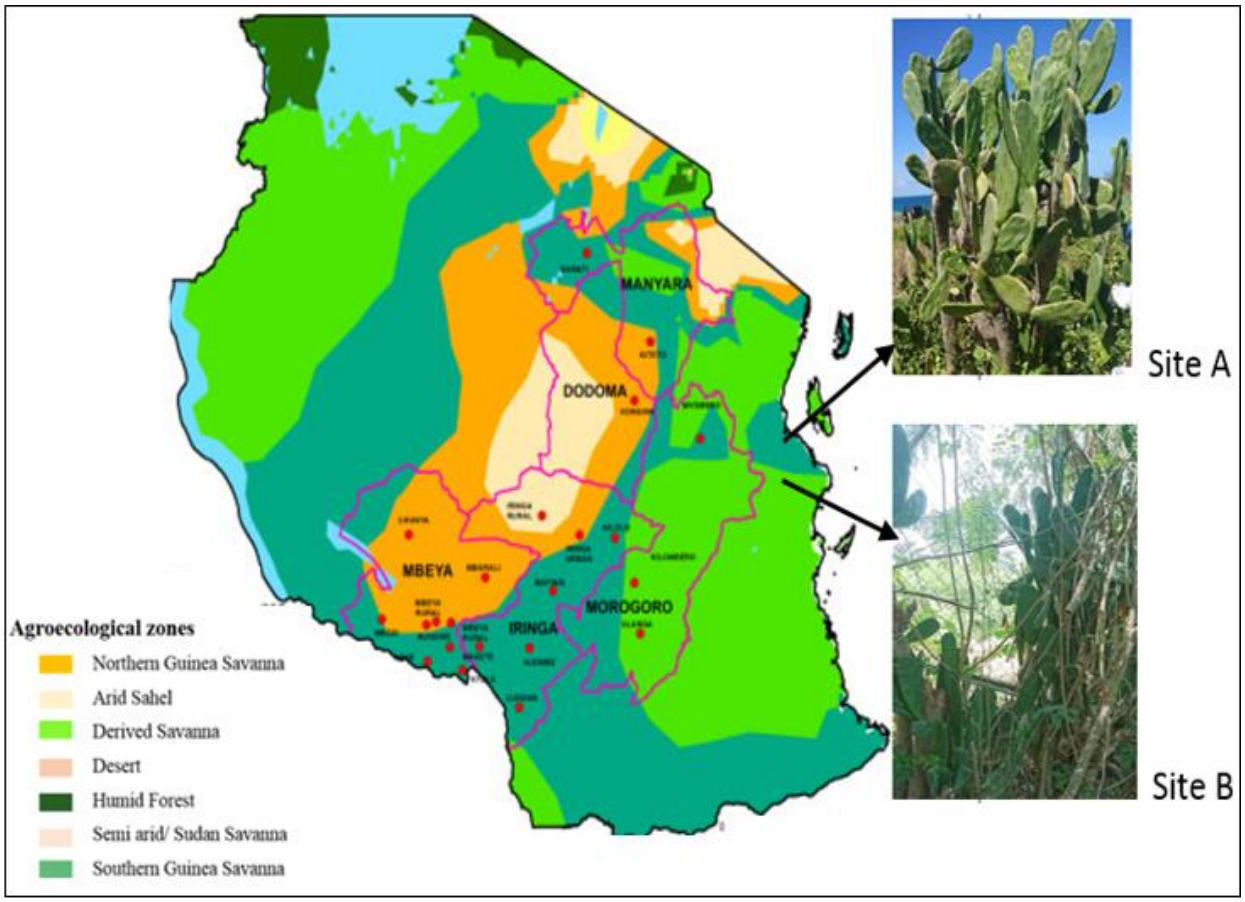

Figure 3.1: Locations of sample collection sites (source IITA Tanzania-GIS Unit)

3.2 Feedstock collection and preparation

A total of six *Opuntia* were sampled randomly from non-irrigated and non-cultivated land found in two sites, three samples per site were collected and numbered (sample one to three from site A and sample four to six from site B). Above the ground plants parts were collected and evaluated according to morphological characteristics list of descriptors by the International Union for the Protection of New Varieties of Plants (UPOV) including plant height, cladode length, cladode width, total weight, presence or absence of spines, firmness, and cladode colour. Plant samples were further identified and authenticated at the herbarium unit of the Department of Botany, University of Dar es salaam. The samples were immediately transferred to the laboratory and stored at 4°C for further analysis.

3.3 Physico-chemical characterization of substrate

3.3.1 Determination of total solids and volatile solids

Total solids (TS) and Volatile solids (VS) of the substrate were determined by the oven-drying and ignition methods, respectively according to standard methods (APHA, 2005). For TS determination clean empty porcelain crucibles were heated at 550 °C for about one h and cooled in a desiccator to room temperature. The empty crucibles was weighed and the fresh, 3g of sample added. They were then oven dried (Memmert UF 110 Schwabach Germany B413.1251) for 24 h at 105 °C after which the crucibles with the content were cooled in the desiccator and their weights recorded. For VS determination same samples were further dried in a furnance (Advantec KL-420, S/N FG32R) at 550 °C for two h. The samples were cooled in the desiccator before weighing and the following equations used in calculating VS and TS.

Equation 1: Total solids $TS(\%) = \left(\frac{B-A}{W}\right) \times 100$

Equation 2: Volatile solids $VS(\%) = \left(\frac{B-C}{W}\right) \times 100$

Where, A = Average weight of empty crucible (g).

B = Average weight of residue dried at 105°C + crucible weight (g)

C = average weight of residues/ ash after ignition at 550 °C + crucible weight (g)

W= Sample weight.

3.3.2 Determination of moisture content.

The moisture content was determined according to the methods of (AOAC, 2016). About 2 g of fresh sample was weighed and placed in a clean dry moisture dish and the weight of the sample and dish taken. These were placed in a moisture oven and the temperatures adjusted to 1050C. The samples were dried for 3 h and removed, cooled and weighed. The amount of moisture in the samples was calculated using the formula:

Equation 3: Moisture content $\%\mathrm{MC} = \left(\frac{\text{Weight before drying} - \text{weight after drying}}{\text{sample weight}}\right) * 100$

3.3.3 Total carbon determination

Total carbon determination was done by using the Walkley-Black potassium dichromate method as described by Schumacher (2002). Whereby 0.1g of dry samples was put in 250 cornical flasks and 10mls of 1N K2Cr2O7 was added and swirled. Then 15mls of H2SO4 was added in a fume hood swirled again three times. The flasks were allowed to stand for 30 min, then 150mls distilled water is added, followed by addition of 5 mls of Ortho-phosphoric acid. The contents were titrated with 0.5N ferrous ammonium sulphate

solution till the colour changes from blue to green was observed. Simultaneously, a blank was run without sample. Organic carbon was calculated using equation 1

Equation 4 $\%C = \big((B - C) \times (V \times 0.3 \times 1.33)\big) \div WB$

Where:

% C = Organic carbon

B = Blank reading (mls)

C = Sample reading (mls)

W = Weight of sample weighed (0.1g)

V = Volume of 1N $K_2Cr_2O_7$ (mls)

1.33 = a constant for the organic carbon on assumption that there is 77% recovery.

3.3.4 Determination of total nitrogen

Kjeldahl method (AOAC,2016) was used to determine the total nitrogen content which involved the sample digestion and volumetric determination whereby about 1 g of sample was weighed into a digestion flask together with a catalyst composed of 5 g of K_2SO_4 and 0.5 g of $CuSO_4$ and 15ml of concentrated H_2SO_4. The mixture was heated in a fume hood till the digest colour turned blue signifying the end of the digestion process. The digest was cooled, transferred to a 100 ml volumetric flask and topped up to the mark with distilled water. A blank digestion with the catalysts and acid was also made. Ten (10) ml of diluted digest was transferred into a distilling flask and washed with about 2 ml distilled water. 15 ml 0f 40% NaOH was added and this was also washed with about 2 ml distilled water. Distillation was done to a volume of about 60 ml distillate. The distillate was titrated using 0.02N-HCl to an orange colour of the mixed

indicator which signified the end point. Calculations were done using the following formula;

Equation 5: Total nitrogen $\mathrm{N}(\%) = (\mathrm{V1} - \mathrm{V2}) \times \mathrm{N} \times \mathrm{f} \times 0.014 \times (\frac{100}{\mathrm{V}}) \times (\frac{100}{\mathrm{S}})$

Where V_1 = Titer for the sample (ml);

V_2 = Titer for blank (ml)

N = Normality of standard HCl solution

F = Factor of standard HCl solution

V = Volume used for distillation

S = Weight of sample taken (g)

3.3.5 Total carbohydrates determination

Total carbohydrates was determined using a procedure described by Allen (1989). In this method, 1 g of dried sample was weighed into a conical flask, followed by 30ml of distilled water. The flask was then heated for two h after which filtration was carried out using Whatman paper No44 filter paper. Colour development was done using anthrone reagent. The absorbance was measured at 630 nm using a spectrophotometer (Jenway 6305 UK). Finally total carbohydrates was deduced using a standard curve using glucose as standards.

3.3.6 Determination of crude fiber

For the determination of crude fibre, AOAC (2016), Method 920.86-32.1.15 was used. A 2 g of the sample was weighed into a 500 ml conical flask (W) and 100 ml of boiling 0.25% H_2SO_4 added, after which boiling was done for 30 min under reflux condenser

(GF-6 SANSHIN INDUSTRIES LTD s/n 02926). Filtration was done under slight vacuum with Pyrex glass filter and the residue washed to completely remove the acid with boiling water. A 200 ml of boiling 0.25% NaOH was added to the washed residue and boiling done under reflux for another 30 min. Filtration was done using the same glass filter previously used with the acid. The residue was rinsed with boiling water followed by 0 . 1% HCL, and again washed with boiling water to rinse the acid from the residue. The residue was washed twice with alcohol and thrice with ether. It was then dried in an oven at 105°C in a porcelain dish to a constant weight (W_1). Incineration was done in a muffle furnace at 550°C for 3 hrs after which the dish was then cooled in a desiccator and the final weight (W_2) taken.

The crude fibre calculation was carried out as shown in the following equation;

Equation 6: Crude fiber $CF(\%) = (\frac{W_1 - W_2}{W}) \times 100$

Where:

W_1 = Weight of acid and alkali digested sample

W_2 = Weight of incinerated sample after acid and alkali digestion

W = Weight of sample

3.3.7 Determination of cellulose, hemicellulose and lignin

Direct method of cellulose, hemicelluloses and lignin was used (Moubasher *et al*., 1982). Two grams of ground samples were boiled in 8 ml ethanol for 15min and then washed thoroughly with distilled water. The samples were oven dried at 40ºC overnight and dry weight of the samples recorded as A. Samples were then treated with 24% KOH for 4hrs

at 25°C and residues washed thoroughly with distilled water. Samples were then dried at 80°C overnight and the dry weight taken as B fraction. Residues (B fractions) were further treated with 72% H_2SO_4 for 3hrs to hydrolyse the cellulose and the refluxed with 5% H_2SO_4 for 2hrs. H_2SO_4 was removed completely by washing with distilled water and then dried at 80°C in an oven for 24h and dry weight taken as fraction C.

Cellulose = B – C

Hemicellulose = A – B

Lignin = C

3.3.8 Determination of reducing sugar

Reducing sugar was determined using the Hagedorn-Jenson method based on quantitative oxidation by potassium ferricyanide and titration with sodium thiosulphate as described by Allen (1989). Where by 5ml of glucose standard, 5ml of water and 5ml of samples were poured into boiling tubes. All tubes were treated the same from this point. 5 mL of potassium ferricyanide was added to all the tubes and the tubes covered with glass bubble and left in boiling water for 15 minutes. Contents were then cooled to room temperature and 5 mL of Potassium iodide solution added and mixed by shaking. 3mL of 5% acetic acid was added, and then titration performed using 0.01M Sodium thiosulphate ($Na_2S_2O_3$) using the starch indicator until the blue colour disappeared. The reading were noted and values used to calculate sugar concentration of samples.

Equation 7: Reducing sugar

$$=((\mathrm{A}-\mathrm{C})\times \text{extract vol(ml)})\ /\ ((\mathrm{A}-\mathrm{B})\times \text{aliquot(ml)}\times \text{sample wt(g)}\times 2)$$

Where: A = Volume of thiosulphate in titration of water blank

B = Volume of thiosulphate in titration of glucose standard

C = Volume of thiosulphate in titration of sample

3.4 Molecular identification of the *Opuntia* samples used as feedstock for anaerobic digestion

This was done for species identification and confirmation, so as to identify which species of *Opuntia* was used in this study.

3.4.1 Sample preparation for DNA extraction

To reduce the mucilage content, cuticle and all the spongy like internal tissues were removed using a sterile blade and outer most layer of cladode was used for DNA extraction. Then about 20mg of samples were homogenized in porcelain mortar and pestle using liquid nitrogen. This was done for all the six samples.

3.4.2 DNA extraction

Genomic DNA was extracted using a Quick-DNA Plant/Seed Miniprep Kit (ZYMO RESEARCH CORP), following the manufacturer's protocol (www.zymoresearch.com). To allow maximum recovery of required DNA 120 to 150 mg of starting material was used.

3.4.3 DNA integrity analysis

The integrity of extracted gDNA from 6 samples of *Opuntia* was analysed by electrophoresis (45 minutes with 87 V cm−3 current) using 1.5 agarose gels prepared with TAE buffer (Tris Acetate-EDTA) which was melted on microwave and then cooled to 60ºC where the red fluorescence dye was added and the gel poured on the gel casting tray.

Samples for loading were prepared by mixing 8µl of the DNA sample with 2 µl loading dye. For marker a volume of 5 µl was used. The obtained bands were visualized under UV light with an UVIDOC HD5 (UVITEC Cambridge- UK).

3.4.4 Evaluation of gDNA concentration and quality

The quantity of DNA was estimated by spectrophotometric measurement of absorbance at 230, 260 and 280nm. This was done with the spectrophotometric analysis using a Nano Drop, PCR max Lambda (Bibby Scientific ltd-UK).

3.4.5 PCR amplification

Two molecular markers were used i.e. nDNA internal transcribed spacer (ITS, 600 bp and cpDNA RuBisCO gene (*rbc*L, 500 pb). A negative control which did not have the target gDNA was included to ensure no sample contamination took place. The primer pairs used were adapted from a work where the conditions were already optimized (Table 3.1). Primer blast search was done prior to primer development to confirm the specificity of primer sequences. For PCR reactions the final volume of the reaction mixture was 25 µL containing (1X One Taq MasterMix with standard buffers, 0.2 µM of forward primer, and 0.2 µM of reverse primer). PCRs were performed on a thermal cycler (ProFlex^{TM} Base PCR system (APPLIED BIOSYSTEM) which consisted of an initial denaturation step at 96 °C for 2 min, followed by 35 cycles at 94 °C for 1 min, annealing temperature was 57ºC for nDNA internal transcribed spacer and 56ºC for cpDNA RuBisCO gene (*rbc*L, 500 pb) for 1 min, 72 °C elongation temperature for 1 min and final extension at 72ºC for 10 min.

Table 3.1: Primers for the amplification and sequencing of the genomic DNA of *Opuntia* samples

Region	Name	Primer sequence(5'-3')	Reference
ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG	Martínez *et al*., 2017 ; Lyra *et al.,* 2013; White *et al*., 1990
	ITS4	TCCTCCGCTTATTGATATGC	Martínez *et al.,* 2017; Lyra *et al.,* 2013; White *et al*., 1990
*rbc*L	1f	ATGTCACCACAAACAGAAAC	Martínez *et al.*, 2017
	724r	CGCATGTACCTGCAGTAGC	Martínez *et al*., 2017

3.4.6 Gel electrophoresis of the PCR amplicons

After amplification, the products were subjected to electrophoresis using 1.5 agarose gels prepared with TAE buffer (Tris Acetate-EDTA), stained with Gel red and visualized under UV light with an UVIDOC HD5 (UVITEC Cambridge- UK) gel documentation system and the picture of the gels taken.

3.4.7 DNA sequencing and sequence analysis

The amplicons were sequenced (Inqaba biotech SA) and the obtained sequences cleaned using sequence cleaner application (bio-web: http://www.cellbiol.com/). BLAST search was done (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using default parameter so as to assess the identity of the sequences for all samples with reference to the accessions in GenBank. Best hit on the conducted searches based on E-value, similarity and identity was recorded. Sequences were aligned by multiple sequence alignment using Muscle (Robert, 2004) and further relatedness of samples was confirmed by evolution relationship analysis using Molecular Evolutionary Genetics Analysis, MEGA X (Kumar *et al*., 2018).

3.5 Determination of the effects of aerobic pre-treatment of *Opuntia* on the extent of methane production in batch anaerobic bioreactors.

A composite mixture of the six *Opuntia* samples was prepared and used for the anaerobic digestion process. The mixture was prepared by mixing 500g of each sample and then blended together.

3.5.1 Inoculum used in anaerobic bioreactors

The source of inoculum used to feed the system was cow rumen fluid obtained from Vingunguti abattoir, Ilala Municipal Dar es Salaam, Tanzania. A twenty litre plastic container with airtight lids was used to carry the inoculum at ambient temperature (31±1º C) to the laboratory. The fresh rumen fluid was filtered through a sieve of 2 mm pores (Endecott's Test Sieve Limited, BS 410, England) to separate solid content from the slurry. Prior to use, the inoculum was left to mature for sixteen days at 31±1º C to remove the easily degradable volatile solid present in inoculum (LoNiee-Liew, 2011), and later its TS and VS determined.

3.5.2 Batch bioreactor configuration

Anaerobic digesters were constructed in lab-scale experiments where biogas was produced out of the degradation of organic matter in 500 ml bioreactors (Plate 3.1) consisting of wide mouth Erlenmeyer conical flasks which was connected to gas-tight aluminium bag via a gas tight-plastic tubes for biogas collection. Gas sampling port was fitted in the bioreactor with n-butyl stoppers and sealed with aluminium caps as explained by Mshandete *et al.*, (2005). Each bioreactor had a sampling septum made of rubber stopper for taking biogas samples and a gastight bag for collecting the gas.

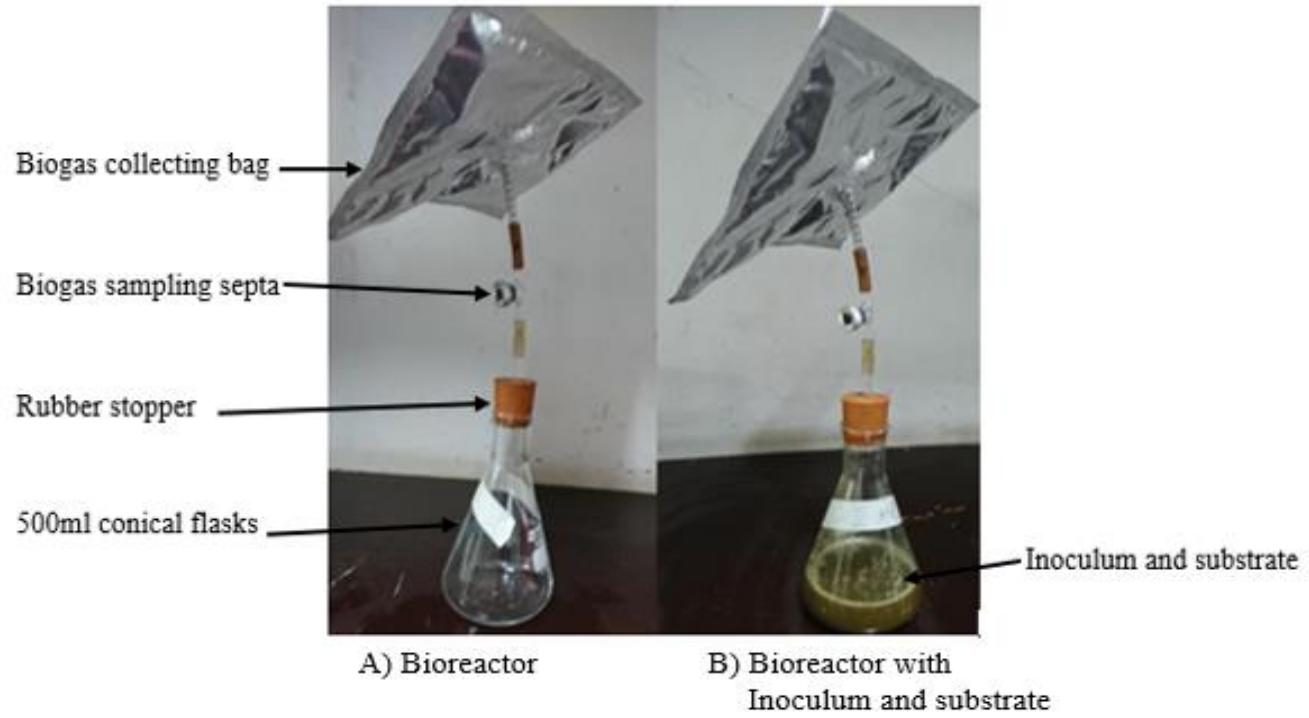

Plate 3.1: Bioreactor used in this study

3.5.3 Experimental set up of bioreactors

The experiment, which was carried out in a laboratory at a temperature of 31±1 °C was set up in twenty eight bioreactors organized into two sets. First series including four digesters which were charged with untreated *Opuntia* substrate for the conventional method of digestion and four served as controls (inoculum only) used to obtain the background biogas production from the inoculum, which was subtracted from that of the test. Second series consisted of five groups of bioreactors with aeration in five different time intervals consisting of four digesters each.

3.5.4 Substrate loading calculations

Substrate was manually chopped into pieces averaging 1 cm^3 with a sharp knife and then blended at maximum speed using kitchen blender (Philips HR2067/04 600W). Based on the total solids (TS) and volatile solids (VS) of both the inoculum and substrate the weight of substrate to be loaded was determined as follows:

TS of substrate (g/l) = A

TS of Inoculum (g/l) = B

VS of substrate (% of A) = C

VS of inoculum (% of B) = D

Then; (a) VS (g) of inoculum = D × B

(b) VS (g) of substrate = C × A

If the volume of inoculum used =V: Thus; VS (g) in V of inoculum = [D × B] × V

For loading ratio of 1:1 (Inoculum VS (g): substrate VS (g))

The VS (g) of the substrate = VS (g) of the Inoculum.

VS (g) of the substrate = [D × B] × V

Therefore; Equation 8: weight of substrate loaded $\frac{([D \times B]V)}{(C \times A)}$

By substituting the values in the formula the substrate needed to be loaded to the bioreactor was established. Which is in this case:

TS of cow inoculum = 2.7

VS (%TS) of inoculum = 26.7

TS of *Opuntia* feedstock = 11.95

VS (%TS) of *Opuntia* feedstock = 73.95

Therefore: Weight of the substrate loaded $= \frac{(0.027 * 0.267 * 250)}{(0.1195 * 0.7395)}$ = 20 g

Hence 20 g of the *Opuntia* feedstock were added to make 250ml of reactors volume.

3.5.5 Determination of effect of aeration pre-treatment on methane yield

To determine the effect of pre-treatment on the subsequent performance of batch anaerobic digestion of *Opuntia* biomass, experiments were carried out at five different times of aerobic exposure: 3, 9, 24, 48 and 72 h. For each of the bioreactor. The selected aeration periods for the experiment was in the interval to secure a period with sufficient length to accommodate proper statistical evaluation as aeration period should be long enough to build up measurable levels of reducing sugar increases in the aerobic periods. Ahn et al., (2014) presented seven different aeration time (0, 0.5, 1, 6, 24, 48 and 96h) for a mesophilic process (35°C):higher aeration time (96 h) resulted in no changes in hydrolysis rate and methane production whereas the lower aeration time (6 to 48 h) to increased biogas generation. 20g of the feedstock was added followed by inoculum to make up to 250 ml of working volume. The bioreactors were surface aerated by leaving the bioreactors open and shaking at 130 rpm using a shaking incubator at 31ºC (Orbital Incubator S150, Stuart Scientific, UK) for the aerobic period. The amount of reducing sugars produced after every pre-treatment period was evaluated. Relative amount of dissolved oxygen was determined with an oximeter (OXI 3205, Weilheim 2009, Germany).

Immediately after the aeration periods the content in each digester was flushed with nitrogen for 3 min to replace the oxygen and provide anaerobic conditions. Subsequently,

the bioreactors openings were closed with stoppers to ensure gas tightness. Bioreactors were kept at a temperature of 31±1°C. These bioreactors were then compared in terms of their biogas and methane content after every 72 h. Biogas and methane measurements was done until no significant amount of biogas was produced (30 days).

3.6 Analytical determinations

3.6.1 Determination of pH of bioreactors content.

The pH of the biomass and effluent were determined before and after anaerobic digestion using pH meter (Hanna Hi 2211).

3.6.2 Volume of biogas formed during anaerobic digestion

The volume of biogas formed during the experiment was measured using a graduated 100 mL glass syringe (SGE International Pty Ltd., Ringwood, Australia) according to Pham *et al*., (2013). A needle plugged at the tip of the graduated syringe was pierced through the air-tight n-butyl stopper in gas sampling septum and then the syringe plunger was pulled to draw the gas from the bag. The readings were done on the volume of the gas which corresponded to the graduated syringe and then the gas released. The process was repeated until the bag was empty.

3.6.3 Biogas compositional analysis

The methane content in the biogas produced from all experimental batch anaerobic bioreactors was estimated by the concentrated alkaline absorption method (Erguder *et al*., 2001). In this method only methane (CH_4) is selectively allowed to pass through the KOH solutions while other biogas components are dissolved in the concentrated alkaline

solution. From a biogas bag, 5 ml of biogas sample was withdrawn and then injected into a closed 10 ml serum bottles containing 8 ml concentrated KOH solution (20g/L). The bottles were shaken manually for 3 min facilitating absorption and allowed to settle for one more min. The pressure of the undissolved gas at the headspace pushed the syringe upwards. The amount of gas collected gives the approximate methane content in the process. Hence the percentage of methane in a whole biogas bag was established by taking the final volume divided by the initial volume taken from bioreactor multiplied by 100.

Equation 9: Methane content $\%CH_4 = \frac{undissolved\ volume}{5ml\ of\ biogas} * 100$

Methane yield was also calculated as methane yield in m3 per kilogram of volatile solid

Equation 10: Methane yield $CH_4\left(\frac{m3}{kgVS}\right) = (\frac{V}{M*TS*VS})$

Where: V = Total volume of methane

M = Weight of the biomass added (kg)

VS = Volatile solids of substrate.

TS = Total solids of substrate.

3.7 Data analysis methods

Data was expressed as mean ± standard error (SE) of the triplicate measurements. Differences between mean values were examined by one-way analyses of variance (ANOVA) and significance was set at P = 0.05. All statistical analyses were performed using Prism version 6.01 for Windows, Graph Pad Software, La Jolla California USA, www.graphpad.com.

CHAPTER FOUR

4.0 RESULTS

4.1 Morphological characterization of *Opuntia*

The cacti used in this study were found growing randomly dispersed intermittently. There were relatively short almost invisible spines on the cladodes. The sampled cacti morphological characteristics are shown in Table 4.1. The observed features together with the information from herbarium unit of the Department of Botany, University of Dar es salaam enabled the grouping of the collected samples into *Opuntia* genera.

Table 4.1: Morphological characteristics of *Opuntia* samples collected from two sites

Site	Sample	Cladode width(cm)	Cladode length(cm)	Height (m)	Weight (kg)	Spine	Peal colour	Firmness
	1	12.33±2.52	25.67±1.15	1.69	4.4	Absent	Yellowish green	Firm
	2	11.33±1.52	28±2.65	1.28	3.5	Absent	Yellowish green	Firm
A	3	11.67±1.15	27±200	1.21	5	Absent	Yellowish green	Firm
	4	13.3±0.58	23±1.73	1.50	3.2	Absent	Green	Firm
	5	14.83±1.53	24.3±2.08	1.94	7.5	Absent	Yellowish green	Firm
B	6	15.33±1.53	23.67±1.53	2.46	12	Absent	Green	Firm

All samples had greenish cladodes which were firm with no spines. Plant height and weight varied from two sites ($P<0.05$) whereby, plant height ranged from 1.21m to 2.46m and plant weight was 3.5 to 12 kg. There was significant difference in the cladode width and cladode height for the samples from two sites ($P<0.05$).

4.2 Physico-chemical properties of *Opuntia* cladodes

This was done to assess the suitability of plants found in these areas to be used for anaerobic digestion as compared to what has been reported from the literature. Physico-chemical characteristics of *Opuntia* which include the moisture content, total nitrogen,

crude fibre, hemicellulose, cellulose, total carbon, carbohydrate, total solid (TS) and volatile solid (VS), were determined to evaluate the use of samples for AD (Figure 4.1).

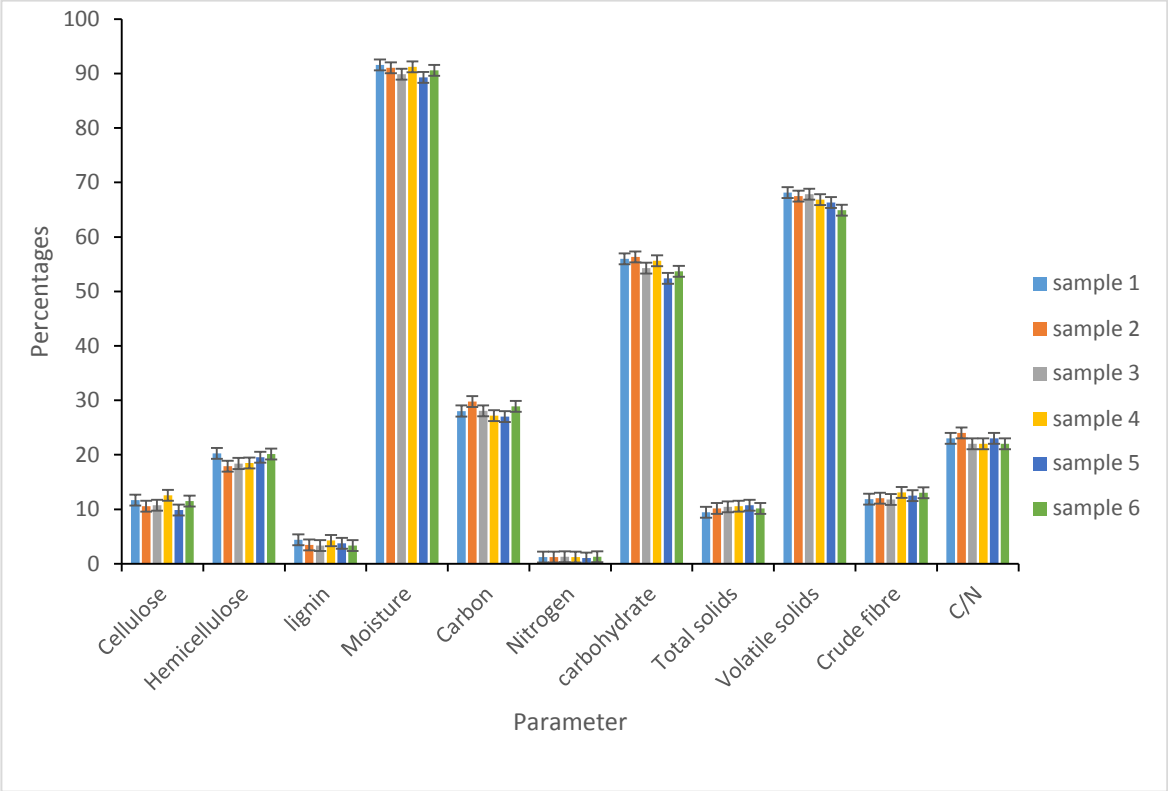

Figure 4.1: Physico-chemical composition of *Opuntia* samples obtained from the two sites in this study. C/N stands for carbon and nitrogen ratio.

With respect to physicochemical properties with outmost importance in anaerobic digestion there was no significant difference ($P>0.05$) on the samples from two sites. It can be seen that the plant samples were largely composed of water reflected by their high moisture content 89.29±0.58% to 91.58±0.33%. Cellulose, hemicellulose as well as the total carbohydrate content of the samples was also quite high: 9.85±0.03% to 12.56±0.25%, 17.89±0.18 to 20.26±0.25%, 53.68±0.20 to 55.96±0.91% respectively.

Low lignin contents was also obtained from all samples (3.32±0.062 to 4.4± 0.10%) and their difference between sites was not significant (P>0.05)

4.3 Molecular identification of the six *Opuntia* samples used in the study.

4.3.1 DNA extraction

Good quality genomic DNA free of contaminants was obtained from the *Opuntia* samples using plant extraction kit and confirmed with Agarose gel electrophoresis (Plate 4.1) and its concentration evaluated with spectrophotometry.

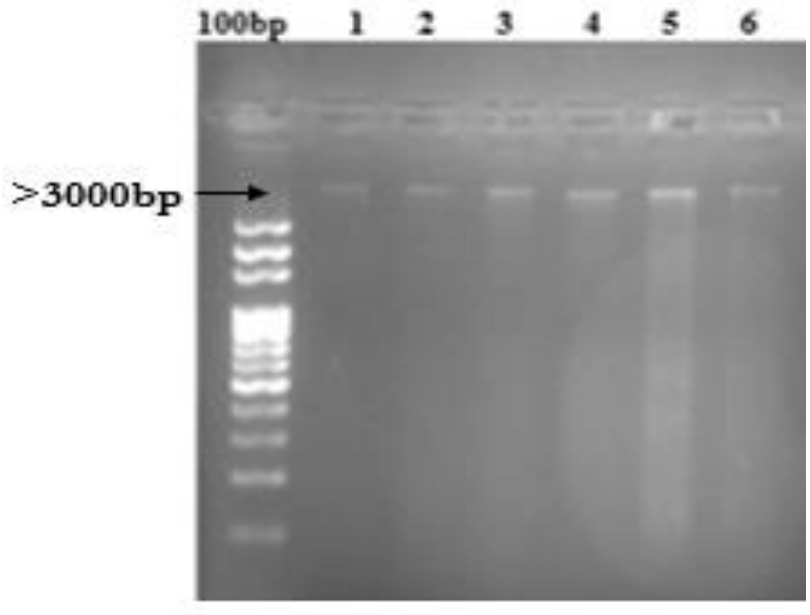

Plate 4.1: Gel electrophoresis picture of the genomic DNA from 6 *Opuntia* samples used in this study. Lane 1 to 6 stands for sample numbers one to six, DNA ladder of 100 bp Molecular Weight Marker was used.

4.3.2 Spectrophotometric analysis

Spectrophotometric analysis yielded a mean gDNA concentrations between 6.7–24.5 µg/ml for all of the samples used (Table 4.2). The absorbance readings from the spectrophotometric analysis was also obtained. From our results absorbance analysis for

the samples gave values within the range 1.5-2.5 for the 260/280 wave length and 0.4-1.2 for the 260/230 wavelength.

Table 4.2: Genomic DNA (gDNA) concentration and quality of six *Opuntia* samples from this study.

Sample	Concentration(µg/ml)	A_{260}/A_{280}	A_{260}/A_{230}
1	21.225	2.148	0.723
2	6.734	2.518	1.245
3	19.476	1.517	0.665
4	9.099	2.659	0.46
5	19.129	2.001	1.074
6	24.57	1.992	0.919

4.3.3 PCR amplification of the extracted DNA

Genomic DNA obtained from all samples was used as template in PCR and RuBisCO and ITS gene amplified. PCRs of RuBisCO and ITS regions were successful for all samples (Plate 4.2) expected size of about 600bp and 700bp was obtained.

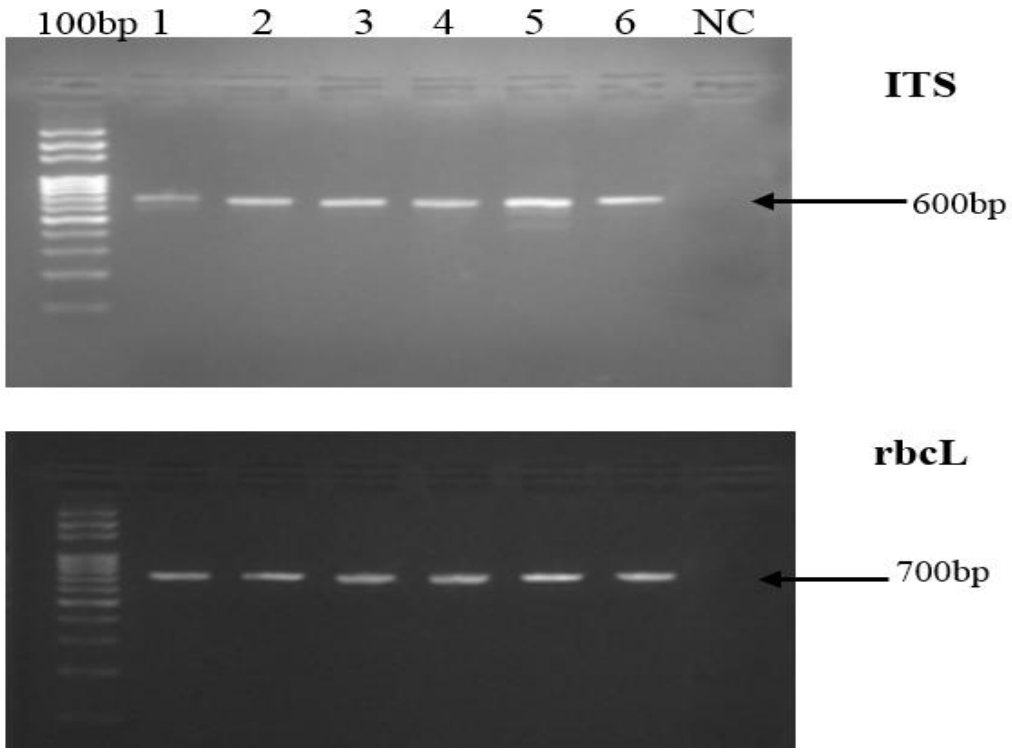

Plate 4.2: Gel electrophoresis picture of PCR amplicons of six *Opuntia* samples: ITS and rbcL. Lane 1 to 6 stands for sample numbers one to six and NC is negative control used. DNA ladder of 100 bp Molecular Weight Marker was use.

4.3.4 DNA sequencing and sequence analysis

4.3.4.1 Blast search

DNA sequencing for ITS and RuBisCO gene was successful, these sequences were then compared with those in Genbank. The corresponding accessions to our samples were recorded based on Query cover, E value and percentage Identity (Table 4.3) and all six samples of *Opuntia* were determined with reference to the accessions in GenBank. Sequences of *Opuntia ficus indica* were found as hit for all samples ITS whereas for RuBisCO gene sequences of *Opuntia maxima* (synonym used for *Opuntia ficus indica*) were found for the samples with the similarity values between 92% and 100%.

Table 4.3: Blast results using ITS and RuBisCO sequences of *Opuntia* samples used in this study

Sample	Identity	Query cover (%)	E value	Ident (%)	Accession
ITS					
1	*Opuntia ficus-indica*	99%	0.0	92%	EU930379.1
2	*Opuntia ficus-indica*	100%	0.0	94%	EU930379.1
3	*Opuntia ficus indica*	99%	0.0	96%	EU930379.1
4	*Opuntia ficus-indica*	98%	9e-59	96%	JF787101.1
5	*Opuntia ficus-indica*	99%	0.0	94%	EU930379.1
6	*Opuntia ficus-indica*	99%	0.0	99%	EU930379.1
RBCL					
1	*Opuntia maxima*	93%	0.0	99%	HM850212.1
2	*Opuntia maxima*	91%	0.0	99%	HM850212.1
3	*Opuntia maxima*	97%	0.0	99%	HM850212.1
4	*Opuntia maxima*	98%	0.0	99%	HM850212.1
5	*Opuntia maxima*	98%	0.0	99%	HM850212.1
6	*Opuntia maxima*	92%	0.0	100%	HM850212.1

4.3.4.2 Phylogenetic analysis

ITS sequences obtained ranged from 570 – 656 bp whereas for RuBisCO the length of about 740 bp was obtained. These sequences were further analyzed with MEGA X (Kumar et al., 2018) to check for the relatedness of the six samples with reference to accessions retrived from Genbank. Phylogenetic trees were rooted using KU382728.1 *Streptomyces spp* as outgroup and The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Figure 4.2 and Figure 4.3). From both phylogenetic trees, sequences from this study showed close relationship and were found in the same group.

For RuBisCO gene in the first clusters (bootstrap value =91%) consisted of eight accessions retired from GenBank and six samples studied in this work, whereby accessions KR737563.1, *Opuntia ficus-indica (*Kartzinel *et al*., 2015*)* and HM850212.1,

Opuntia maxima(Schaefer *et al.*, 2011) showed closely relatedness with the studied samples.

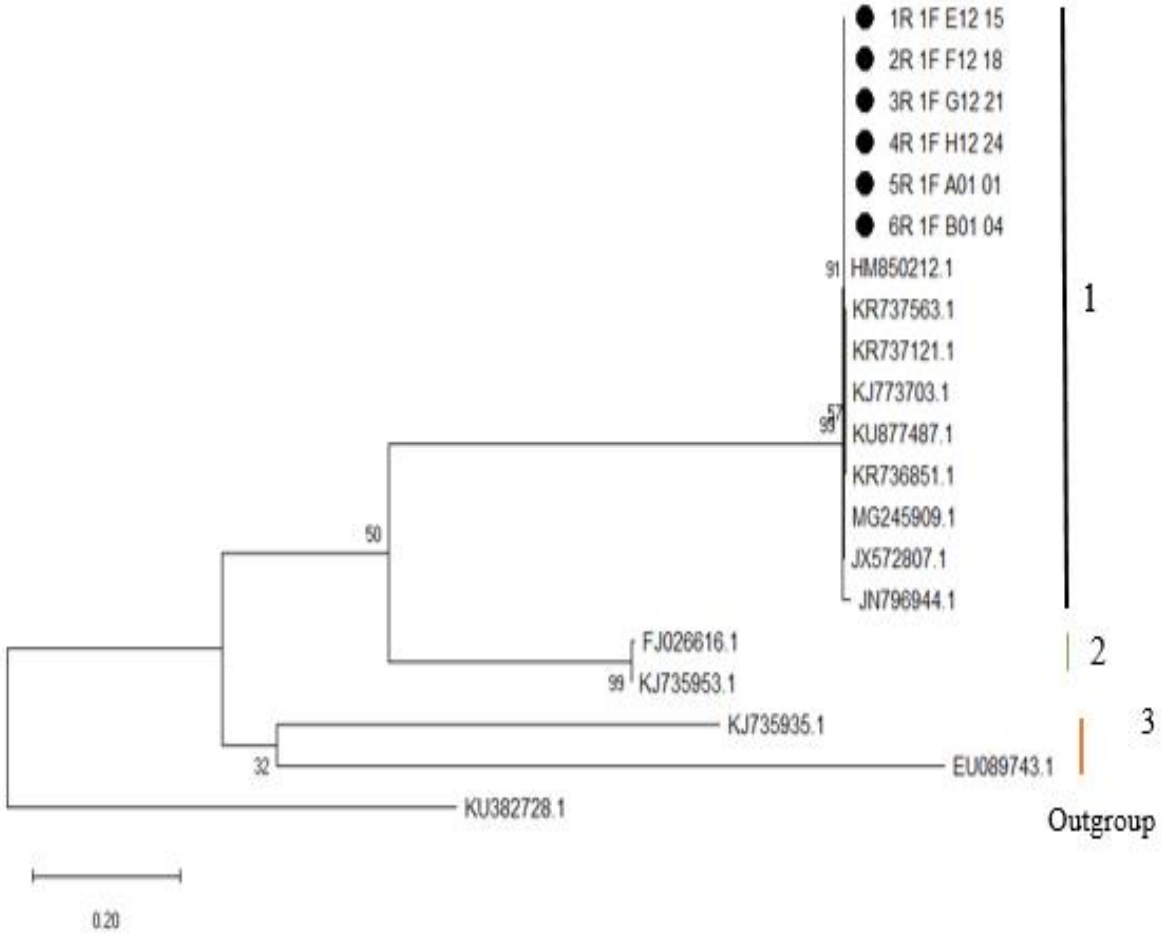

Figure 4.2: Maximum likelihood tree showing the evolutionary relatedness of RuBisCO sequences from samples used in this study.

For ITS gene, accessions JF786944.1 *Opuntia ficus-indica* (Majure *et al.*, 2012) was closely related to the sequences of samples from this study.

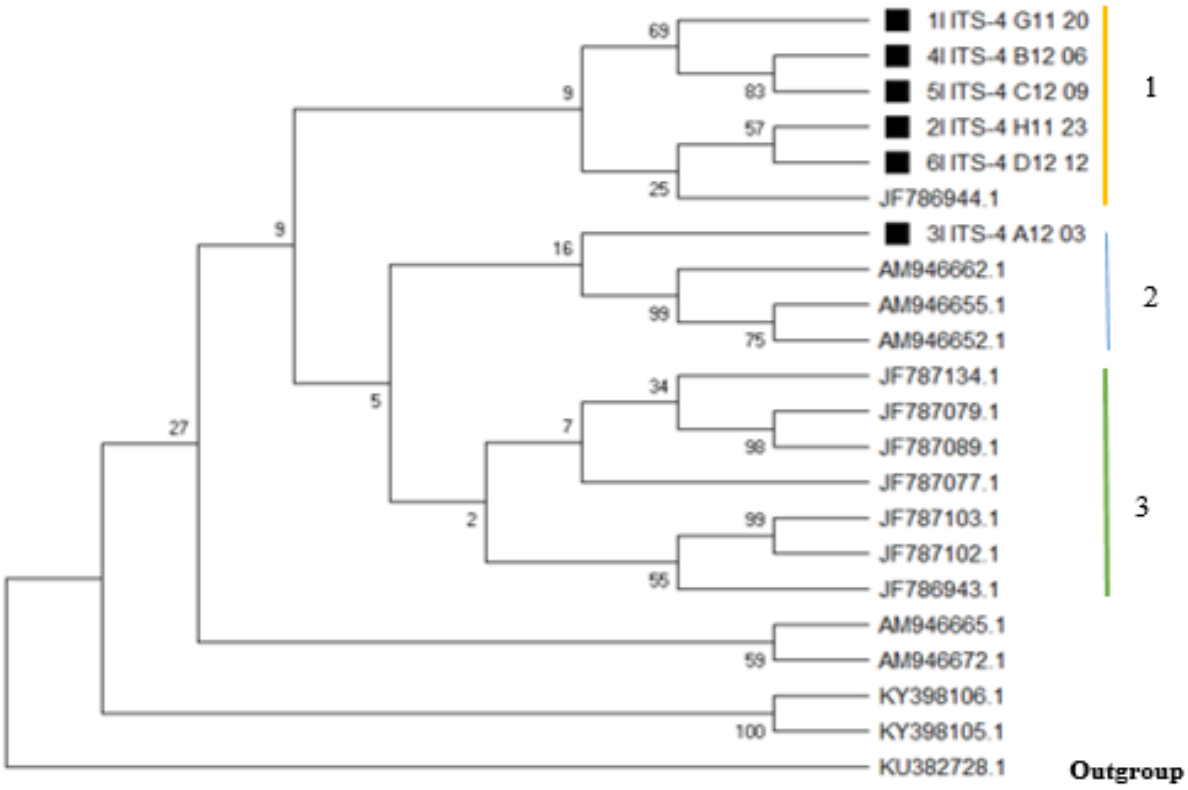

Figure 4.3: Maximum likelihood tree showing the evolutionary relatedness of ITS sequences from samples used in this study.

4.4 Anaerobic batch tests

4.4.1 Inoculum and substrate for anaerobic digestion

Since there was no significant difference in the major physicochemical composition of the six sample no significant difference in performance was suspected and hence the focus was on analyzing the effect of aerobic pretreatment of all *Opuntia* samples together treated as one substrate for anaerobic digestion. Total solids and volatile solids content of inoculum used anaerobic digestion and substrate were determined (Table 4.4)

Table 4.4: Composition of *Opuntia* composite substrate and cow rumen fluid inoculum used in the study

Parameter	*Opuntia* composite substrate	Inoculum
Total Solids (TS)%	11.95± 2.23	2.67 ±1.3
Volatile solids(VS)%	73.95± 6.33	26.6±0.1
pH	7.52± 0.02	7.7±0.3

Total solids of inoculum was 2.67 ±1.3 with its corresponding volatile solids of 26.6±0.1, these values together with the Total solids and volatile solids were used to calculate the loading weight using equation 6. And the total amount of weight to be loaded in 250ml working volume was found to be 20g.

4.4.2 *Opuntia* biomass hydrolysates composition after pre-treatment

4.4.2.1 Sugar content of hydrolysate.

The amount of reducing sugar in the bioreactors after pre-treatment was between 12.22 ±0.69g/l to 59.08±3.35g/l on the control and 72 h pretreatment, respectively. There was significant increase (P = 0.0033) in sugar content with increase in pretreatment time, reaching the highest amount on the bioreactor pretreated for 72 h (Figure 4.4). The rise in reducing sugar contents can be seen as early as the 3 h pretreatment time, though the difference between 9h and 24h is small compare to others (36.46±4.46g/l and 40.97±1.34g/l respectively).

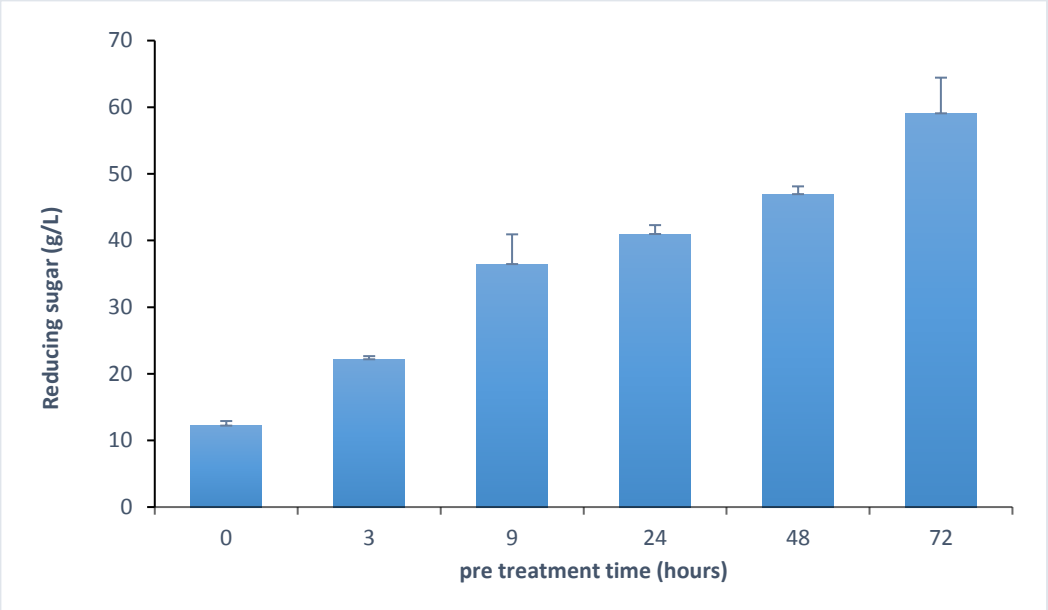

Figure 4.4: Sugar content of the hydrolysate after pretreatment of composite *Opuntia* substrate.

4.4.2.2 Dissolved oxygen contents of hydrolysate after pre-treatment period.

In this study dissolved oxygen measured ranged from 0.10 to 0.16 mg/l the highest and the lowest value found in 3h and 72 h pretreated batch respectively (Table 4.5), difference in actual values were very minimum. The amount of dissolved oxygen increased with increase in pretreatment time, there was no difference in dissolve oxygen levels for 24 h and 48 h of pretreatment

Table 4.5: Dissolved oxygen level in bioreactors after pretreatment

Pretreatment (h)	Dissolved oxygen(mg/l)
0	0.00
3	0.10 ±0.01
9	0.13± 0.009
24	0.14± 0.01
48	0.14±0.015
72	0.16± 0.01

4.4.3 Effect of pretreatment on total biogas and methane production

Total methane yield (as meter cubic of methane per kilogram of VS used) from the different pretreatment periods during the anaerobic digestion is presented in Figure 4.5. Significant differences in methane yield was observed (P = 0.0025). There was increase in the methane yield with increase pretreatment time up to 9th h of pretreatment and thereafter, a steady decrease up to the 72nd h where the value was 0.286 m3CH4/kgVS. Methane yield varied with feedstock pretreatment time ranging from 0.286±0.022 to 0.702±0.053 m^{3}CH4/kgVS, whereas that of control was 0.315±0.016 m^{3}CH4/kgVS.

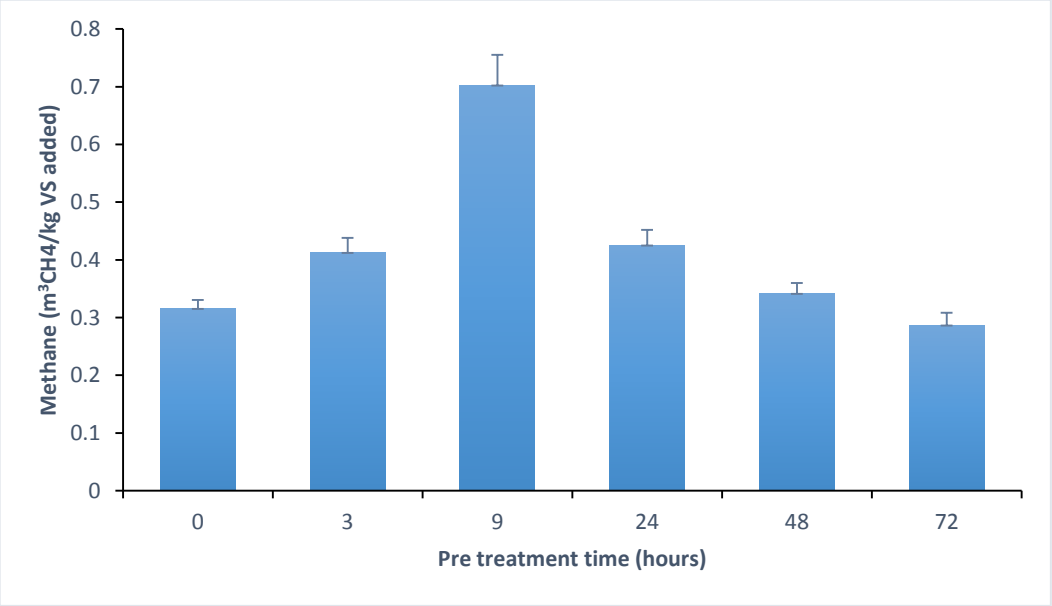

Figure 4.5: Total methane yield of *Opuntia* biomass from different pre-treatment period after 30 days of anaerobic digestion. (Values are means ± SE)

The highest methane yield was observed on the 9th h of the pre-treatment of *Opuntia* feedstock, the increase of which was about 123% (Table 4.6) compared to the blank. No

significant change in methane yield was observed after 24 h pretreatment. Further increase in pretreatment time to 72 h prior to anaerobic digestion, led to potential decrease in methane yield of 9%.

Table 4.6: Potential increase in methane yield from all treatments with reference to control

Pretreatment (h)	Methane (m^3/kg VS)	% increase from control	Energy value of CH4 (MJ kg-1 VS)
0	0.315±0.016	0	12.55
3	0.412±0.026	30.79	16.41
9	0.702±0.053	122.86	27.97
24	0.425±0.027	34.92	16.93
48	0.341±0.019	8.25	13.59
72	0.286±0.022	-9.21	11.39

*higher heat value of methane taken as 39.84 MJ m-3 at STP (Khanh, 2017).

4.4.4 Biogas production from pretreated *Opuntia* substrate during anaerobic digestion period.

4.4.4.1 Total biogas production

Significant differences were observed in the amount of biogas produced with the amount produced at the 9^{th} h being significantly higher (P = 0.0060) than in the rest of the pretreatment time. The total biogas production for 0 h, 3 h, 9 h, 24 h, 48 h and 72 h were 0.919, 1.068, 1.645, 1.029, 0.844 and 0.823 in m^3 respectively. The maximum total biogas production was observed with 9 h pretreatment period (1.645 m^3), followed by 3 h pretreatment period (1.068 m^3) and the least in 72 h pretreatment (0.823 m^3) (Figure 4.6). Bioreactors which were pretreated in 3, 9, and 24 h had higher total biogas production as

compared to the control. On the other hand, bioreactors pretreated for 48 and 72 h produced lower amount of total biogas 0.844±0.024m^3 and 0.823±0.027m^3 respectively compared to the control.

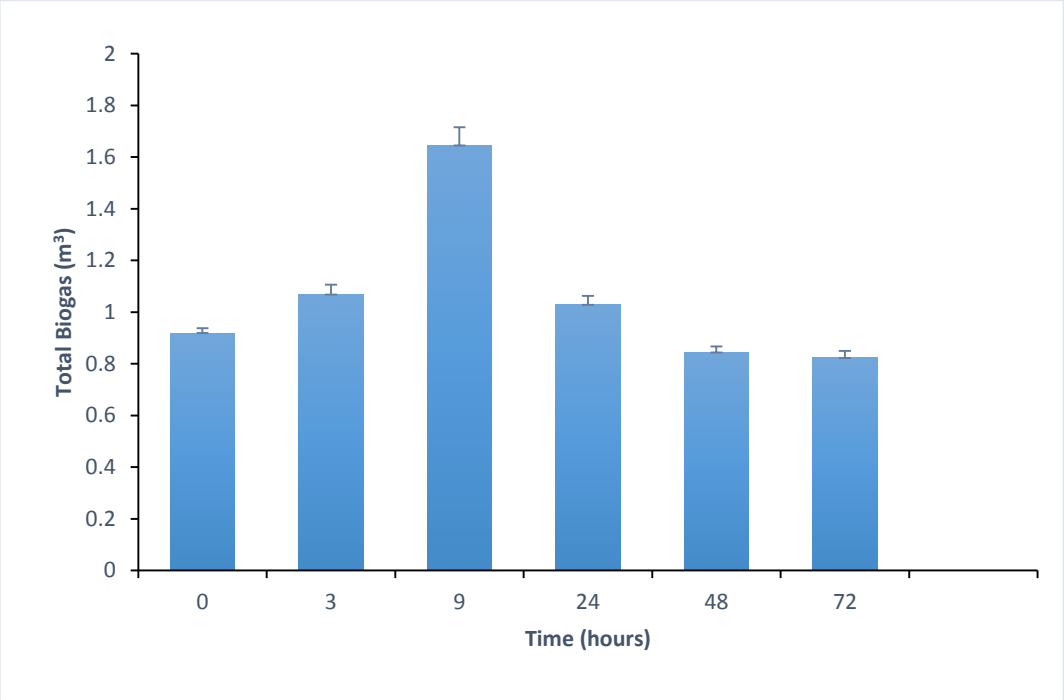

Figure 4.6: Total biogas yield of the *Opuntia* biomass from different pre-treatment time

Methane content of biogas varied according to pretreatment time, ranging from 69–77% (Table 4.7). Biogas with the highest methane content was found in the bioreactors pretreated for 9, 24, 48 and 72 h whereby for 3 h pretreatment period and control produced biogas with relatively low methane content 67 and 69 respectively.

Table 4.7: Total biogas production and highest methane content during anaerobic digestion of pre-treated O*puntia* biomass.

Pretreatment time (h)	Total biogas production (m^3)	Highest methane content of the biogas (%)
0	0.919±0.019	69
3	1.068±0.039	67
9	1.645±0.703	76
24	1.029±0.345	77
48	0.844±0.024	74
72	0.823±0.027	72

4.4.4.2 Daily biogas production

Gas production was seen from initial days of the experiment in all treatment. However, the amount of biogas measured varied among the treatments (Figure 4.7). The average daily biogas production observed from 0, 3, 9, 24, 48, 72 h treatments in this experiment were 0.031, 0.036, 0.055, 0.034, 0.028 and 0.027 m^3 respectively. On average, it was found that 9 h pretreatment produced the highest volume (0.055m^3) per day, followed by 3 h pretreatment (0.036 m^3) and the lowest was observed on 72 h pretreatment (0.027m^3). Daily production was different with different pretreatment time, whereby, in 3, 9 and 48 h pretreatment highest biogas production was seen in the initial seven days of incubation, and then gradually decreased to the lowest amount on the final day of incubation. On the other hand 24 and 72 h pretreatment started with the low biogas production which then increased from day 11^{th} to 14^{th} and later proceed to gradual decrease and lowest amount on the final day of incubation.

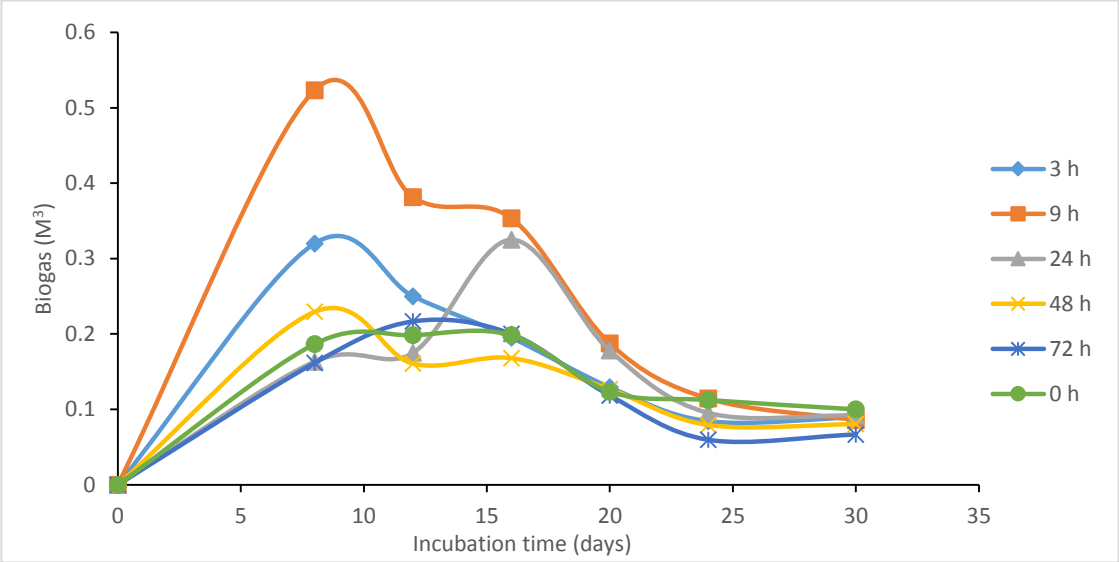

Figure 4.7: Daily biogas production from different treatments and control. Biogas production gradually decreased to the lowest amount on the 30 day of incubation.

4.4.5 pH changes of bioreactors

The pH values at the beginning of digestion were 7.31, 7.52, 7.60, 7.55, 7.21 and 7.72 for 0,3,9,24,48 and 72 h pre-treated bioreactors respectively (table). The final pH after 30 days of anaerobic incubation remained fairly the same for 3,9 and 48 h pre-treated bioreactors while and increase to 8.14, 8.16 and 8.23 was observed in 0, 24 and 72 h pre-treated bioreactors respectively.

Table 4.8: pH readings of digesters before and after anaerobic digestion

Pretreatment (h)	Initial pH	Final pH
0	7.31± 0.05	8.14±0.04
3	7.52± 0.08	7.65±0.05
9	7.60± 0.02	7.71±0.09
24	7.55± 0.01	8.16±0.08
48	7.21± 0.02	7.62±0.02
72	7.72± 0.04	8.23±0.08

4.4.6 *Opuntia* biomass energy value in relation with other biogas substrates

Taking into account the highest methane yield obtained on 9 h pretreated batches from this study (Table 4.8), expected energy value was compared with other feedstocks which have been reported to have potential of biogas production. The corresponding energy values of CH_4 from biochemical methane potential tests as MJ kg-1 VS was calculated with the higher heat value of CH_4 taken as 39.84 MJ m-3 at STP (Khanh, 2017).

Table 4.9: Methane yield and corresponding energy value from *Opuntia* in this study in relation to substrate reported from other studies.

Substrate	Reactor volume	Methane yield (m^3CH_4/kg VS added)	Energy value of CH4 (MJ kg-1 VS)	Reference
Opuntia ficus indica	250ml	0.702±0.053	27.97	This study
Opuntia spp	200ml	0.600	23.904	Calabr *et al* .,2017
Sisal waste	350ml	0.301	11.99	Muthangya *et al.*,2013
Maize grains	1000ml	0.72	28.68	Hutňan *et al*., 2010
Corn silage	600ml	0.872	34.74	Li *et al*., 2018

After the calculation of the energy value of CH_4 from BMP (MJ kg-1 VS), the potential energy per tonne production from each substrate was obtained from each substrate (Figure 4.8). Calculated energy contents are is higher in corn silage (obtained from 600ml bioreactors). Energy content from *Opuntia ficus indica* substrate used in this study is more or less the same with that obtained from maize grains using 1000ml bioreactor.

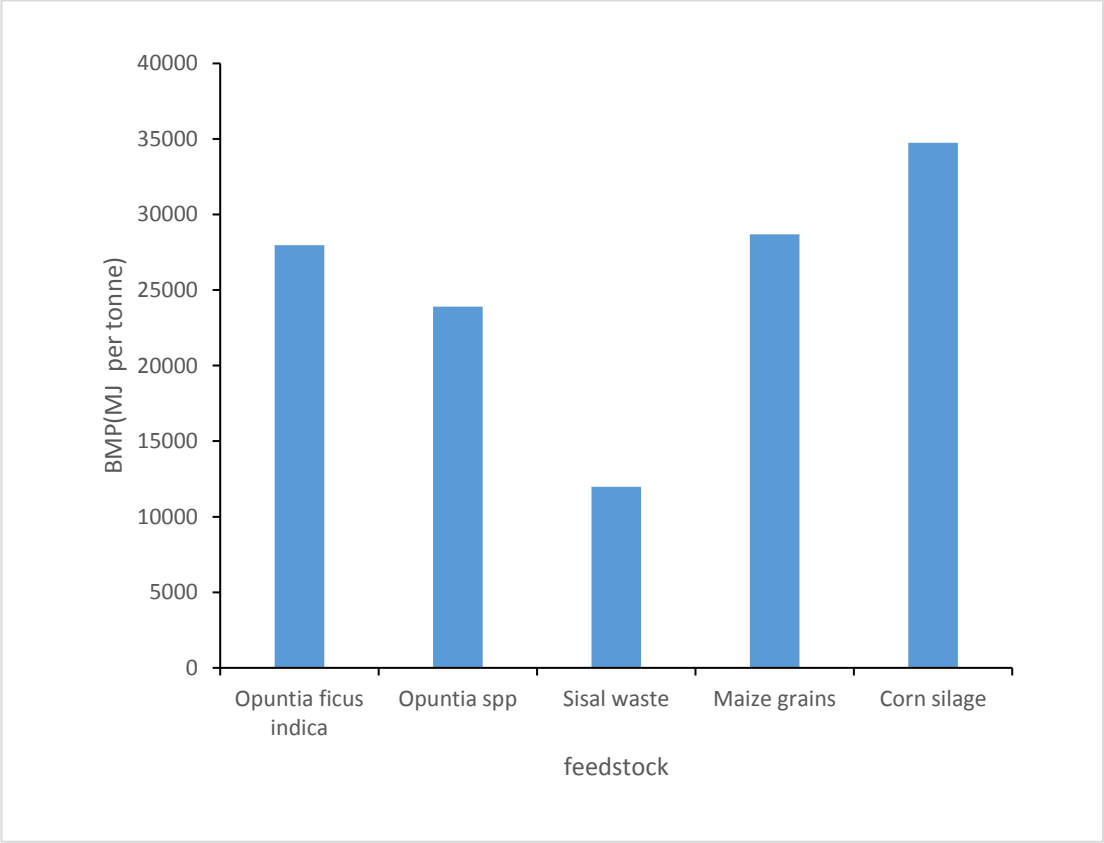

Figure 4.8: Calculated energy yields values per tonne of volatile solids based on experimental methane yield values for various substrates for anaerobic digestion relative to *Opuntia ficus indica* used in this study.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Morphological analysis of *Opuntia* samples

In this study plant height from all six samples ranged from 1.21 to 2.46 m similar and within the range of 1.10 to 3m of *Opuntia ficus indica* reported from ASALs of Kenya (Omweri *et al.,* 2016). Cladode characteristics have been shown to be useful in distinguishing species given that cladode size has been suggested to be species dependent (Peña-Valdivia *et al*., 2008). In this study cladode width was 11.33±1.52 to 15.33±1.53cm and cladode height was 23±1.73 to 28 ±2.65cm for the all samples. This is similar to the reported values of *Opuntia ficus indica*, 12 to 29 cm and 24 to 67cm for cladode width and height respectively (Omweri *et al.*, 2016). The main attributes used in this study are in agreement with those used by Gallegos-Vasquez *et al*., (2011) and Omweri *et al.* (2016) who utilized cladodes and other characters such as fruits to characterize cactus species. Greenish colour, firmness of cladode as well as the plant height of 1.21 to 2.46 m suggest that *Opuntia* samples analysed in this study were of age between one and two years (Holmes, 2016; Reis *et al*., 2016). It have been reported that physicochemical composition of *Opuntia* can vary depending on species of the plant and climate of where they are found (Stintzing *et al.,* 2005).

5.2 Physical chemical analysis of six *Opuntia* samples.

Based on the morphological differences initially noted it was necessary to perform physicochemical analysis and find out whether differences were reflected on their physicochemical properties. Organic content of a feedstock determines the theoretical

yield of biogas that can be produced from it (Dandikas *et al.,* 2015). The most important of these nutrients being carbon and nitrogen, and the C/N ratio of feedstock is a vital factor for the choice of feedstock (Zupancic and Ros, 2012; Friehe *et al*., 2010). Excess availability of nitrogen during the degradation leads to the formation of NH_3, which inhibits microbial growth at higher concentrations and its deficiency causes the biogas process to fail (Friehe *et al*., 2010). From physicochemical analysis of samples in this study, there were no significant differences ($P>0.05$) among the major organic components of the six *Opuntia* samples analyzed for their potential for use as substrate in anaerobic digestion. Overall *Opuntia* from this study had relatively high amounts of important organic matter for anaerobic digestion and hence signifying its suitability for anaerobic digestion.

5.2.1 Moisture content

Water make possible the movement and growth of bacteria facilitating the dissolution and transport of nutrient. In addition water reduces the limitation of mass transfer of non-homogenous or particulate substrate (Lu *et al.,* 2008). Mean moisture content of samples in this study were 90.84 and 90.37% for site A and site B respectively, with minimum and maximum ranges of 89.16 and 91.95% for site A, and 88.64 and 91.39% for site B respectively. Differences between sites was not significant ($P >0.05$). Values obtained in this study are comparable with other studies done on *Opuntia* plants. Moisture content of 86% from *Opuntia* in Ethiopia have been reported (Jigar *et al*., 2011). Moisture content in the range of 88.7 ± 1.2 to 92.6 ± 0.7% *Opuntia* species have also been reported in other studies (Filho *et al.,* 2016). This result shows that the moisture content of *Opuntia* samples

were relatively high, which can aid anaerobic digestion as it can increase the degree of digestion since microorganisms in the digestate can easily access liquid substrate for relevant reactions to take place (Sadaka and Engler, 2003).

5.2.2 Cellulose and lignin

The relative contents of cellulose and lignin are key factors to identify the suitability of lignocellulosic biomass for processing, particularly the biochemical conversion processes (McKendry 2002a). Mean cellulose content of samples in this study were 11.02 ± 0.61% and 11.32 ± 1.22% for site A and site B respectively. The differences between sites was not significant (P >0.05). These values fall in close range to those reported in literature such as 7.7% ± 0.41% and 11% (Calabr *et al.*, 2017). Higher values such as 21.6% have also been reported (Malainine *et al*., 2003). Among the major components of all plant materials cellulose cannot be left out, which forms about half to one third of plant tissues. Celluloses are resistant to hydrolysis by enzymes or acids because of their structure and the lignin barrier, hence the necessity of using pre-treatments at the initial stages of hydrolysis (Gujer and Zehnder, 1983). The mean lignin content found in this study, whose results were not significantly different from each other (P> 0.05) were 3.73 ± 0.52% for samples from site A and 3.79 ± 0.39% for samples from site B . These are relatively low values which add to the advantage of using this plant for anaerobic digestion in these areas. This is because low lignin content indicate minimum barrier to hydrolysis of these polysaccharides into fermentable sugars (Filho *et al*., 2016). Lignin value of *Opuntia* between 3.6 and 7.9% have been reported (Malainine *et al*., 2003; Kuloyo *et al*., 2014). Even though lignin content represents a potentially large energy source, current techniques

involving hydrolysis/enzymatic systems cannot convert the lignin into gas (McKendry, 2002a). The biodegradation of cellulose is greater than that of lignin and therefore, the overall conversion of biomass with a higher content of cellulose is greater than biomass with a higher content of lignin (Mahalaxmi *et al.,* 2014).

5.2.3 Hemicellulose

Mean hemicellulose content between the two sites was not significantly different (P> 0.05), with mean values of 18.85 ± 1.16% for samples from site A and 19.41 ± 0.74% for samples from site B. These values are similar to 17.3% hemicellulose content of *Opuntia* reported in Morocco (Malainine *et al*., 2003). Lower values (8.6% ± 0.43%) of these plants have been reported in Italy (Calabr *et al.,* 2017). The difference here could have been attributed to differences in climates where these plants were found as it is reported that lignin, hemicellulose and cellulose levels increase with growth temperature (Ford *et al*., 1979). Higher values seen from this study and those reported by Malainine *et al*. (2003) were obtained from plants in tropical and subtropical climates respectively with higher temperatures of more than 30ºC. On the other hand *Opuntia* located in the Mediterranean climate with temperatures ranging between 3 to 5 ºC in winter and 17 to 21 ºC in summer gave lower values (Calabr *et al.,* 2017). Hemicellulose protect cellulose, hence removal of hemicellulose by pre-treatment can increase the contact area of cellulose to enzymes and subsequently improve the hydrolysis rate (Taherzadeh and Karimi, 2008).

5.2.4 Carbohydrates

Mean carbohydrate content of samples in this study were 56.42±0.51% and 56.67±0.79 % for site A and site B respectively. Differences between sites was not significant (P

>0.05). The values obtained here are comparable 69% total carbohydrates that have been reported (Malainine *et al*., 2003). Substrates hydrolyzed in the first stage during anaerobic digestion consist of carbohydrates, lipids, and proteins (Lu *et al.*, 2008). In the anaerobic digestion process structural carbohydrates are split into their component sugars that can be utilized by microorganisms (Gerardi, 2003). Carbohydrates are converted to equal amounts of methane and carbon dioxide (Gujer and Zehnder, 1983). Hence the amount of carbohydrate content of feedstock is of important in evaluation of the viability of the biomass as a feedstock for anaerobic digestion. The values obtained from *Opuntia* sample in this study gives a good indicator that these plants can be used for anaerobic digestion processes.

5.2.5 Carbon and nitrogen

Methane yield and its production rates are highly influenced by the balance of carbon and nitrogen in the feedstock (Gerardi, 2003). From physicochemical analysis carbon content was between 27.05 ± 1.95% and 28.64 ± 0.1% in sample from both sites, values that were not statistically different (P> 0.05). The nitrogen content values ranged from 1.24 ± 0.07% and 1.20 ± 0.12%, and they did not differ significantly from each other (P>0.05). These values resulted into the ratio of carbon and nitrogen of *Opuntia* samples in the range of 22:1 to 25:1. This ratio does not fall far from the carbon to nitrogen ratio of organic biomasses recommended by most researchers to provide enough nutrients for microorganisms performing anaerobic digestion (Costa *et al*., 2012; Marchaim, 1992). Nevertheless the ratio obtained under this study is similar to 26.7:1 reported from *Opuntia* (Calabr *et al.*, 2017). Higher values of around 48 have been reported, though carbon was

not experimentally determined but rather derived from total solids that they obtained from the substrate (Jigar *et al*., 2011). It has been shown that various feedstocks used for anaerobic digestion can be optimized at a C/N ratio of approximately 6:1 to 30:1 (Costa *et al*., 2012; Marchaim, 1992). Nitrogen and carbon are among the key components needed by microorganisms in the development of their cell structures (Marchaim, 1992). It plays a key role in cell growth which is very crucial in methane production and provide buffering capacity through releasing ammonium cation contributed by nitrogenous compounds (Mshandete *et al*., 2005). Often the C: N ratio is used as an index of the suitability of organic feeds for methane production (Zhang *et al*., 2011).

5.2.6 Total solids (TS) and Volatile solids (VS)

Large fraction of *Opuntia* samples analysed here were biodegradable indicated which was indicated by TS values of 10.02 ± 0.49% to 10.50 ± 0.43%, and VS values of 67.83 ± 0.90% to 66.02 ± 1.54%. The TS values obtained from this study were similar to the values 10.9 to 14% reported elsewhere (Ramos-Suarez *et al*., 2014; Jigar *et al.*, 2011). Less productivity of the digestion of substrates having 7.6% have been reported (Costa *et al.*, 2012). These values indicated that large fraction of *Opuntia* is biodegradable and thus it can serve as an important feedstock for biogas production.

5.3 *Opuntia* identification by molecular markers

Molecular tools have been proven to be effective and efficient way of identification of various organisms. Based on the molecular marker, all six plant samples in this study were able to be identified as *Opuntia ficus indica*, which were later shown to be closely related as they clustered very closely together with bootstrap value of 91%. It is important to note

that closeness of the different species in natural environments has created a favourable environment for the gene flow between cultivars (Lyra *et al*., 2013). This necessitate the use of molecular identification as morphological characters alone are inconclusive and insufficient. Various other studies have been able to study and identify different *Opuntia* species using ITS gene and RuBisCO gene (Lam and Zechman, 2006; Lu and Wang, 2011; Martínez *et al*., 2017; Lyra *et al*., 2013). Phylogenetic analyses of RuBisCO sequences have been reported to be useful in clarifying a number of long standing issues related to the phylogeny and evolution of *Bryopsidales* plant (Lu and Wang, 2011).

It has been observed that ITS sequence variation levels are suitable for phylogenetic inference at the specific, generic or even family levels (Baldwin *et al*., 1995). This is attributable mostly to nucleotide polymorphisms and insertion–deletion polymorphisms (indels) are also common (Alvarez and Wendel, 2003). These sequences have been shown to have variation at the level that makes it suitable for evolutionary studies at the species or generic level (Baldwin *et al*., 1995; Maggini *et al*., 1998; Liston *et al*., 1996). Reportedly ITS sequences high rate of divergence which is an important source to study population differentiation (Yamaji *et al*., 2007). This goes hand in hand with its high copy numbers which allow for highly reproducible amplification and sequencing results. ITS have been shown to have high sequence divergence than other markers in the studying of *Opuntia* species phylogeny and were recommended in studying of *Opuntia* (Majure *et al*., 2012).

Molecular markers are important in order to both confirm the differences or similarity of organisms and provided the means for certification purposes of the organism in question.

These results enabled identification of plant sample used to species level which enabled confirmation with certainty that the substrate used for anaerobic digestion in this study was *Opuntia ficus indica*. The use of morphological characters makes evaluations difficult and the use of genetic markers have facilitated the classification within the genus *Opuntia* (Lyra *et al*., 2013).

5.4 Bioreactor hydrolysate composition after pretreatment

5.4.1 Changes in sugar contents of hydrolysate after pretreatment of *Opuntia* biomass

Pre-treatments are aimed at either directly releasing sugars or improving the enzymatic access to the structural carbohydrate polymers (Kumar *et al*., 2014). After pretreatment, variations in the sugar concentration was observed from different periods where by sugar levels increased with the increase in pretreatment time from 12g/l in the control to 59g/l in the 72 h pretreated batch . Increase in concentration of total sugar during the first 48 h of aeration have been reported, where an increase of 192% was obtained with respect of control value (Montalvo *et al.,* 2016). The rise in sugar content observed here can be accounted for by factors like increasing hydrolysis of polysaccharides to monosaccharides.

Increased in enzymatic activity of two hydrolytic enzymes related to the cellulase and protease have been observed when studying the effects of preaeration on the thermophilic anaerobic digestion of municipal solid wastes (Charles *et al*., 2009). Limited aeration can successfully be used to enhance hydrolysis during anaerobic digestion, about 50% increase in hydrolysis was obtained in the aerated reactors compared to unaerated control

(Johansen and Bakke, 2006). Nevertheless amount of sugar formed is also affected by the hydrolytic conditions and the microbial community present (Ahn *et al.,* 2014).

Similar observations have been reported using other forms of pretreatments. Sugar concentration in the liquid phase increased with increasing pretreatment temperature up to 200ºC, from 59.2 ±12.2mg/lin the batch that was not pretreated to 1224.5 ±67.3mg/l for the 180ºC pretreatment while performing thermal pretreatment (Bochmann *et al*., 2015). Highest amount of reducing sugar was obtained after pretreatment (about 80 g/l) while using corn pericarp under acid and temperature pretreatment (Granados-Arvizu *et al.*, 2017).

The low amount of reducing sugars obtained in this study during initial h of pretreatment can be due to the consumption of the sugars by the aerobic microorganisms, which are active due to the provided aerobic conditions. As the pretreatment is stopped the amount of sugar used is reduced and hydrolytic enzymes they produced are still active and continue to make soluble sugar available for the proper functioning of microorganism for anaerobic digestion (Mshandete *et al.,* 2005).

It should be well noted that even though methane producing microorganism can hydrolyse insoluble carbohydrates, lower methane yields can be observed when the lignocellulosic materials are utilized without any kind of pretreatment (Zheng *et al.,* 2014). Indeed, the pretreatment increased the solubility of polysaccharide present in the bioreactor as a result increasing sugar levels (Antonopoulou *et al*., 2015). On one hand, this facilitate the anaerobic digestion by increasing the accessibility of these sugars to microorganisms (Ahn *et al.,* 2014). On the other hand, if pretreatment is carried out by increasing period of time

above optimum, this could become problematic in systems as significant amounts of organic material will be aerobically degraded before anaerobic digestion period is started (Botheju and Bakke, 2011; Mshandete *et al*., 2005). Therefore, a compromise between increasing the solubility of substrate by aerobic pretreatment and prevention of overconsumption of soluble sugars due to consumption by microorganisms prior to anaerobic incubation needs to be found (Mshandete *et al*., 2005).

5.4.2 Dissolved oxygen changes in relation to hydrolysate properties

The concept behind aerobic pretreatment is to enhance growth of some aerobic organisms that produce cellulose, hemicellulose and/or lignin degrading enzymes (Montgomery and Bochmann 2014). In this study dissolved oxygen measured ranged from 0.10 to 0.16 mg/l the highest and the lowest value at 3 h and 72 h pretreatment. Dissolved oxygen values of 0 in control to 0.22mg/l after 96 h (with 0.16 after 48 h pretreatment) have been obtained after pre-treatment of sewage sludge (Ahn *et al.,* 2014). Though it has been reported that the dissolved oxygen (DO) values higher than 0.15 mg/l can start to inhibit methanogenic activities, a range of DO concentration levels required to inhibit 50% of methanogenic activity was 4.9 to 6.4 mg/L (Ahn *et al.,* 2014; Celis-Garcia *et al.,* 2004). The dissolved oxygen values obtained from this study were still in the range that is safe for methanogenic microorganisms. It is suggested that anaerobic digestion systems can have considerable oxygen tolerance due to several deterrence mechanisms (Botheju and Bakke, 2011). There studies which also support and suggest that some methanogenic species can have a certain extent of intrinsic tolerance to oxygen exposure (Kato *et al*., 1993; Kiener and Leisinger 1983; Conklin *et al.,* 2007). Methanogens might not have been killed by the introduction

of oxygen but rather the activity was inhibited for a short period (Zitomer, 1998). All these facts suggest that even though they work under anaerobic conditions, exposure to oxygen does not significantly affect the methanogen population in the bioreactor. Though methanogens cannot function in the presence of oxygen, they can survive longer time durations at oxygen exposure (Peter and Conrad, 1995). But still care must be taken as there exists an optimum oxygenation level for maximum methane yield of an anaerobic digestion system a point which vary depending on several factors such the reactor biomass concentration and feed composition (Botheju *et al.*, 2010b).

5.5 Anaerobic digestion of pretreated hydrolysate of *Opuntia ficus indica*

5.5.1 Changes in Methane yield with pre treatment

It is necessary to determine the methane potential of the feedstock in order to estimate the extent to which the specific feedstock can be degraded (Angelidaki *et al*., 2009; Nielfa *et al.*, 2015). In this study biochemical methane potential (BMP) test analysis confirmed the positive influence of pretreatment on the methane potential of *Opuntia ficus indica* biomass. Methane yields after pre-aeration were observed to range from 0.286±0.022 to 0.702±0.053 m^3CH4/kgVS, whereas for the untreated one was 0.315±0.016 m^3CH4/kgVS. Total methane yields were higher with 9 h pre-aerated batch as compared to the others and lower on 72 h. When compared to the batch without pretreatment, potential increase of methane yield in the 9 h bioreactor was about 123 %. This increase was significant ($P<0.05$) and is comparable to the reported 110% increase in the production of methane when using mixed sludge aerobic hydrolysis compared to the digestion process of non-aerated sludge (Montalvo *et al.*, 2016). It can be seen that pre-

aeration increased the methane yield till around 9th h of pretreatment and then levelled off to around 0.286 m^3CH4/kgVS, a decrease which is about 9% for the pretreatment time of 72 h.

Since limited research findings on aerobic pre-treatment of *Opuntia ficus indica* feedstock before anaerobic digestion are available, no direct comparison can be made. Nevertheless various researchers have reported increase in methane content in biogas with the incorporation of an aerobic process in anaerobic digestion (Miah *et al*., 2005; Dumas *et al*., 2010; Jang *et al*., 2014; Ahn *et al.,* 2014). The highest amount of methane yield in this study was on 9 h pretreated batch. Slightly different from the findings have been reported when working aerobic pretreatment of sewage sludge samples where the highest methane yield was obtained after 24 h of pretreatment (Ahn *et al*., 2014). The difference could have been attributed to differences in the type of feedstock used since sewage sludge contain a complex mix of protein, lipids and carbohydrate, as maximum methane yield of an anaerobic digestion vary depending on several factors include feed composition (Botheju *et al.,* 2010a). On the other hand the results are similar what have been reported when working with aerobic pretreatement of sisal pulp waste where highest methane yield was after 9 h of pretreatment (Mshandete *et al.,* 2005). Both of these studies showed no significant increase in methane potential with further increase in pretreatment time above the optimum obtained (9 h and 24 h respectively).

Increased methane yield can be attributed to improved biodegradability of aerobically pretreated *Opuntia* feedstock that came as a result of biological role played by microbes. It has been shown in some studies that short-term oxygen exposure of bioreactor does not

affect methanogenic activity and methanogens can survive longer than previously reported (Conklin *et al.,* 2007; Kato *et al*., 1993). Improvement of microbial growth have been explained in several other studies as a factor that might be causing the improvement in the yield during anaerobic digestion. The increase in methane yield as a result of increase of methanogenic activity can also be due to an improvement in the growth of facultative anaerobes, which can keep a low redox potential, providing the best conditions for the growth of strict anaerobes (Montalvo *et al.,* 2016). It has been reported that oxygen at low concentrations is necessary for synthesizing oleic acid and ergosterol which are essential membrane components for certain anaerobic bacteria and, hence, stimulate the growth under anaerobic conditions (Tango and Ghaly, 1999). Aerobic processes have also been reported to facilitate degradation of materials that do not degrade under anaerobic conditions, further improving stabilization (Carrere *et al*., 2010).

Methane yield was lowest at 72 h pretreatment (0.286 m^3CH4/kgVS) which corresponded to the potential decrease of 9 percent. This shows that further exposure to oxygen above optimum doesn't have an added advantage but rather negatively impact methane yield. The reduction of methane yield in bioreactors, which were pre aerated for longer periods have been reported by other researches. Reduction in methane yield of 26% and 37% following pre-treatment for 48 and 72 h, respectively has been reported (Mshandete *et al.*, 2005). Similarly 48 and 96 h of pre-aeration have been reported to cause reduction in methane yield which was linear with period of pre-aeration (Ahn *et al.*, 2014). Decreased in amounts of methane generation under increased aeration conditions have been reported as a linear reduction within the oxygenation range 0–10.1% (Botheju *et al.*, 2010b).

These results are in agreement with some authors findings who showed that long time exposure to oxygen does not significantly improve the methane yield (Xu *et al.,* 2014; Botheju and Bakke, 2011). Inhibition of the activities of methane forming bacteria and decrease the methane yield can occur under longer exposure to oxygen (Xu *et al.*, 2014). This decrease in methane yield observed here could have been as a result of several factors such as the substrate oxidation of readily available substrates by facultative acidogenic organisms and the partial inhibition of the activity of strictly anaerobic biomass (Botheju and Bakke, 2011). Under high "hydrolysability" conditions, there would be no positive response by oxygen addition on the methane yield and the overall effect would become negative (Botheju *et al.,* 2010b). Methane consumption by aerobic methanotrophs can also be a contributing factor to the decrease in methane yield observed (Fu *et al.*, 2015). Limited aeration can be used to enhance the production and minimize the formation of byproducts like glycerol, high aeration levels on the other hand are disadvantageous due to the high aerobic respiration rates leading to increased biomass and CO_2 generation (Franzen *et al*., 1996).

Based on these findings the best pretreatment time can be said to lie in the period between 9 and 24 h as these provide enough time for the microorganisms during pretreatment to thrive and produce their effect. It can be seen that pretreatment had an advantage in increasing the methane yield and more optimization of the pretreatment time is required to ensure maximum yield of methane from biomass while avoiding the inhibition of microbial activities involved in methane production. The advantage of an aerobic process is that it is considerably faster, but the disadvantage is that a lot of the organic matter that

could be degraded to methane is instead degraded to CO_2 if the pretreatment phase is too long (Montgomery and Bochmann, 2014).

5.5.2 Daily and total biogas production during anaerobic digestion period

The maximum total biogas production was observed with 9 h pretreatment period (1.645 m^3), followed by 3 h pretreatment period (1.068 m^3), and the least in 72 h pretreatment (0.823 m^3). Though the sugar content at 72 h pretreatment was higher (59.08± 3.35 compared to 36.46±1.46 for 9 h), it did not result in more biogas than the 9 h pretreatment period. This might be due to the less favorable situation caused by the dissolved oxygen in the bioreactor content of 72 h pretreatment bioreactor to microorganisms as compared to 9 h. As the pretreatment time increased from 9 h to 72 h, the dissolved oxygen levels increased suggesting that higher amount of dissolved oxygen provided less favorable condition for the microorganisms regardless of the level of hydrolysis performed in the bioreactor .This shows that aerobic pretreatment of *Opuntia ficus indica* is more productive with period not exceeding 24 h. Biogas production had relative high methane content, ranging from 69% to 77%.

After the first day of measurement, the production of biogas started to fluctuate and eventually reached the lowest level on the 30th day of the incubation. The fluctuation observed here might be due to several factors such as depletion of readily decomposable substrate after the first day (Ahn *et al*., 2014). It is also possible there was accumulation of toxic wastes due to increasing microbial population in the digester which as a result might have inhibited gas production (Gerardi, 2003). Soluble biodegradable organic substances are consumed throughout the anaerobic incubation period therefore at the end

of the experiment, biogas production declined due to their depletion. Optimal pH levels of digesters ranges between 6.8 and 7.6, in this study final pH values for control, 24 h and 72 h bioreactors were above 8.08. The pH value above this interval restrain microbial activities hence the observed decrease in biogas production (Gerald, 2003).

5.5.3 Changes in pH after anaerobic digestion

Initial pH for all digesters was around 7.5. This pH is suitable and within the range recommended for proper functioning of microorganisms in anaerobic digestion and ideal for anaerobic digestion and normal functioning anaerobic bioreactors (Ward *et al.*, 2008). Methanogens growth is greatly reduced below a pH of 6.4 due to toxic effects of the hydrogen ions, which are related to the accumulation of volatile fatty acids (Anderson and Yang, 1992). These values are also in agreement with a pH range of input mixture in the digester between 6.50 and 7.50, reportedly suitable for most methanogenic bacteria (Mahanta *et al*., 2004).

While the pH of 3, 9 and 48 h pretreatment bioreactors remained relative around the initial pH value (7.7, 7.62, and 7.63 respectively) there was noticeable increase in the pH of the batch without pretreatment and those of 24 h and 72 h pretreatment (8.1, 8.08 and 8.15 respectively) after the completion of anaerobic incubation period. The increase in pH value observed here can be attributable to various factors within the bioreactors. One among them being the production of alkali compounds, such as ammonium ions during the degradation of organic compounds in the digester (Gerardi, 2003). Another factor is that during fermentation carbonic gas is formed and when it combines with water forms carbonates in anaerobic environment which cause rise in pH (Gerardi, 2003). It is also

possible that the formation of methane results in an increase in pH (Jensen *et al.*, 2017). Though microorganisms can still be functioning, values of pH below 6 or above 8 are restrictive and somewhat toxic to methane-forming bacteria (Gerardi, 2003).

5.6 Energy value from *Opuntia* in this study in relation to other reported substrates.

One way to determine heating value of substrate is to first determine the methane potential of the substrate and then use the heating value of methane to calculate heating value of the substrate (Nordlander *et al.*, 2011; Fantozzi and Buratti, 2009). This method though does not reflect the complete energy content of the substrate, since a part of the substrate might be combustible even though it is not digestible, it still provide a way for comparison between biogas production plants using their methane potentials (Nordlander *et al*., 2011). To get the energy value of the substrates, methane yield values found in literature were used and converted to its corresponding energy value using highest reported energy value of methane (Khanh, 2017).

Corresponding energy values from highest methane produced using *Opuntia* in this study using batch bioreactors are comparable to the values in literature for several biomass (Muthangya *et al*., 2013; Li *et al*., 2018; Morris *et al*., 1977; Calabr *et al*., 2017). Though using higher bioreactor volume (1000ml) the energy value obtained from the use of maize grains as substrate is more or less comparable with the value obtained from *Opuntia* used in this study (Hutňan *et al.*,2010). This shows the potential posed by *Opuntia ficus indica* as a feedstock for anaerobic digestion especially when coupled with pretreatment.

A bit lower energy values were obtained using *Opuntia* as feedstock compared to what was obtained from this study (Calabr *et al*., 2017). The difference might be due to the

aerobic pre-treatment stage performed in this study which is lacking in Calabr *et al*., (2017). This further signifies the importance aerobic pretreatment in improvement of anaerobic digestion of *Opuntia ficus indica*.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

The composition of *Opuntia ficus indica* cladode samples was determined where it was found to have relatively high proportion of biodegradable substrates; cellulose, hemicellulose and carbohydrates which were found to range from 11.02 ± 0.61 to 11.32 ± 1.22%, 18.85 ± 1.16 to 19.41 ± 0.74%, and 56.42±0.51 to 56.67±0.79 % on dry weight basis respectively. This shows that *Opuntia* used in this study have large fraction which is biodegradable with great potential in biogas for energy generation. Moreover, lignin content of samples were 3.73 ± 0.52 to 3.79 ± 0.39% dry weight basis which is relatively low value indicate minimum barrier to hydrolysis of polysaccharides into fermentable sugars, providing an added advantage of using these *Opuntia* plant for anaerobic digestion. These results shows a great potential of *Opuntia* as feedstock which can be harnessed for low cost methane production.

Based on the molecular marker all six *Opuntia* samples in this study were identified as *Opuntia ficus indica* and they were closely related as they clustered very closely together in phylogenetic tree. Molecular markers are important in order to both confirm the differences or similarity in plants and provide means for certification purposes of the plant in question as these identification cannot rely only on morphological characters.

Aerobic pretreatment could be used as an effective low cost technology for preparation of organic materials prior to anaerobic digestion. It increases the digestibility of the biomass as well as efficiency of biogas production from it. The results of the study show that

aerobic pretreatment was effective in increasing digestibility of *Opuntia* feedstock which can be seen through sugar concentration changes as well as productivity where higher methane yields with an increase of 123% from the control when the pre-treatment was performed in 9 h period.

The results indicate that methane potentials per m^3CH4/kg volatile solids added decreased with increasing aerobic pretreatment periods above 9 h, the reasons of which could be inhibition of activities of methane forming bacteria (Xu *et al.*, 2014), substrate oxidation of readily available substrates by facultative acidogenic organisms and the partial inhibition of the activity of strictly anaerobic biomass (Botheju and Bakke, 2011) and methane consumption by aerobic methanotrophs (Fu *et al.*, 2015). Hence there is a need of optimization of pre-aeration time and levels in order to avoid the inhibition of methano-genic activity, which can lead to the potential decrease in methane yield.

6.2 Recommendations

Recommendations for further work include:

- Since limited records were found when identifying this plant; more studies on the available *Opuntia* resources should be done in Tanzania and East Africa as whole. No significant difference was found on the studied samples probably due to the few number of study sites, future studies can be conducted in an increased number of sampling sites covering all the seven major agro ecological zones of Tanzania.
- There was observed reduction in methane yield with longer exposure to aeration conditions. Optimization of the aerobic pretreatment time needed to result in significant cellulose digestibility at small scale, with minimal loss of useful sugars

can be further evaluated. This is so as to avoid reduction in methane yield as it was observed from 72h pretreatment from this study.

- There is need to evaluate the applicability of *Opuntia ficus indica* on an industrial scale for the production of biogas by levelling up these experiments to pilot scale.
- Soluble portions in the substrates in this study were determined as sugars only without evaluating which type at what concentration is present in the feedstock. Further analyses are needed to be done so as to determine the components of these soluble portions and their concentrations.
- In this study only cow rumen inoculum was used. It will be interesting to investigate this aerobic pretreatment effect using different types of inoculum and analysed the dominant microbial communities influenced by all inocula type which might probably influence the changes in solubility of the feedstock as well as its methane yield.
- Moreover investigations on this pretreatment effect while exploiting the synergistic effects of co-digestion of *Opuntia ficus indica* substrate with other types of substrates under appropriate mixing ratio C/N ratio and feedstock biodegradability will be of great value in its anaerobic digestion.
- Digestate composition after preatreatment should also be investigated, this is due to the fact that acceptability of the digestate residue after biogas production is important for the agricultural sector hence the compositional and applicability of digestate for agriculture purposes should not be affected by the pretreatment method.

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APPENDICES

Appendix 1: Physicochemical values for the six Opuntia samples from this study

sample	MC	TS	VS	C	N	Cellulose	Hemicellulose	lignin	CF	carbohydrate	C/N
A1	91.58 ±0.33	9.43± 0.11	68.15± 0.35	28.05 ±0.14	1.20± 0.04	11.72 ±0.28	20.26±0 .25	4.40± 0.10	11.88 ±0.16	55.96± 0.91	23.33 ±0.78
A2	91.06 ±0.18	10.18 ±0.05	67.49± 0.05	29.78 ±0.41	1.23± 0.05	10.59 ±0.17	17.89±0 .18	3.48± 0.07	12.05 ±0.31	56.33± 1.56	24.20 ±0.89
A3	89.89 ± 0.47	10.43 ±0.18	67.856 ±0.92	28.09 ±0.40	1.27± 0.02	10.74 ±0.12	18.41±0 .37	3.32± 0.06	11.83 ±0.55	54.29± 1.84	22.19 ±0.67
B1	91.24 ±0.13	10.56 ±0.05	66.85± 0.52	27.22 ±0.92	1.24± 0.07	12.56 ±0.25	18.51±0 .16	4.26± 0.04	13.10 ±0.28	55.62± 1.08	21.96 ±0.57
B2	89.29 ±0.58	10.76 ± 0.22	66.29± 1.15	25.01 ±0.53	1.08± 0.04	9.85± 0.03	19.59±0 .12	3.75± 0.05	12.51 ±0.19	52.40± 0.21	23.11 ±0.34
B3	90.56 ±0.18	10.18 ± 0.33	64.92± 0.76	28.91 ± 0.34	1.29± 0.04	11.53 ±0.22	20.13±0 .05	3.37± 0.041	13.03 ± 0.36	53.68± 0.22	22.47 ± 0.63

Appendix 2: Table for two sample t test results for cladode width and height

Unpaired t test	Cladode length	Cladode width
P value	0.0138	0.0161
P value summary	*	*
Significantly different? (P < 0.05)	Yes	Yes
One- or two-tailed P value?	Two-tailed	Two-tailed
t, df	t=4.187 df=4	"t=4.000 df=4"

Appendix 3: ANOVA results on the physicochemical properties

	Sample collection site		
	Southern guinea savanna	Derived savanna	P-Value
MC	90.84 ± 0.91	90.37 ± 1.01	0.309
TS	10.02 ± 0.49a	10.50 ± 0.43b	0.038 *
VS	67.83 ± 0.90a	66.02 ± 1.54b	0.044 *
C	28.64 ± 0.1	27.05 ± 1.95	0.051
N	1.24 ± 0.07	1.20 ± 0.12	0.521
Carbohydrate	56.42±0.51	56.67±0.79	0.137
Cellulose	11.02 ± 0.61	11.32 ± 1.22	0.522
Hemicellulose	18.85 ± 1.16	19.41 ± 0.74	0.242
Lignin	3.73 ± 0.52	3.79 ± 0.39	0.781
CF	11.92 ± 0.57a	12.88 ± 0.51b	0.01*

Appendix 4: Table for one sample t test results for sugar contents

One sample t test	
Theoretical mean	0.0
Actual mean	297.1
Discrepancy	-297.1
95% CI of discrepancy	151.7 to 442.5
t, df	t=5.253 df=5
P value (two tailed)	0.0033
Significant (alpha=0.05)?	Yes

Appendix 5: Table of ANOVA result for methane yield:

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.01917	5	0.003835	F (5.000, 25.00) = 5.049	P = 0.0025
Individual (between rows)	0.04350	5	0.008700	F (5, 25) = 11.46	P < 0.0001
Residual (random)	0.01899	25	0.0007594		
Total	0.08166	35			

Appendix 6: Table of ANOVA result for biogas production

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	77734	5	15547	F (5.000, 25.00) = 4.272	P = 0.0060
Individual (between rows)	187204	5	37441	F (5, 25) = 10.29	P < 0.0001
Residual (random)	90985	25	3639		
Total	355923	35			

Appendix 7: Table of pairwise comparison test

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
0 vs. 3	-0.01633	-0.05910 to 0.02643	No	ns
0 vs. 9	-0.06467	-0.1074 to -0.02190	Yes	**
0 vs. 24	-0.0185	-0.06126 to 0.02426	No	ns
0 vs. 48	-0.004496	-0.04726 to 0.03827	No	ns
0 vs. 72	0.004833	-0.03793 to 0.04760	No	ns