PROFILING AND EVALUATION OF BACTERIAL CONSORTIA FOR EFFICIENT BIOGAS PRODUCTION IN SMALL SCALE BIODIGESTERS WITHIN UASIN-GISHU COUNTY

OSESO FLORENCE

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DECLARATION

Declaration by the Candidate

This thesis is my original work and has not been submitted for any academic award in any institution and shall not be reproduced in part or full or in any format without prior permission from the author and/or the University of Eldoret.

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Declaration by Supervisors

This thesis has been submitted with our approval as the university supervisors.

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DEDICATION

I dedicate this study effort entirely to my family and friends, who have provided me with moral support throughout this crucial academic step in my life. May the Almighty God bless you all.

ABSTRACT

Anaerobic digestion is a sequential biological activity that accepts the efficient capture of methane for energy production. The dependence on fossil and wood fuels as a primary energy source has led to multitudes of problems such as global warming, environmental degradation and human respiratory health complications. The aim of this research was to characterize the anaerobic bacterial present in small scale bio-digesters within Uasin Gishu County. The specific objective of the study is to profile and evaluate anaerobic bacterial consortia for their efficiency in biogas production in a small-scale bio-digesters within Uasin Gishu County. Six study sites namely Opande, Radar, Energy station, Langas, Nettos and Beta farm were selected within Uasin Gishu county, they varied in both volume and biogas production volume. The cow dung which had been fed into bio-digesters were collected aseptically in 250ml sterile flask and transported to the laboratory. Bacteria were isolated in Microbiology laboratory, University of Eldoret by spread plate method on methanogenic media and incubated under anaerobic conditions in a Gas-pak jar at a mesophilic temperature of 35^{o}C for seven days. Isolates were coded as per their study sites. Pure isolates were obtained using streak plate method and evaluation of physicochemical parameters was done *in situ*. Identification of isolates was done using cultural, morphological and biochemical characteristics. Laboratory scale set up of anaerobic digester for biogas production was done to evaluate the anaerobe efficiency in biogas production. Three, 500ml erlernymer flask were half filled with cow dung and inoculated with *Methanococcus* and *Methanosaeta* sp separately and in consortia of both. This was done in triplicate with different inoculum ratios at 10:500, 20:500 and 30:500ml respectively and a control experiment with cow dung only was allowed to run for 31 days. Gas generated was collected by volume displacement of water and measured at an interval of 0:10, 11:21 and 21:31 days in ml. The temperature and the pH were regulated and monitored regularly. Shannon Weiner diversity test and Chi-square test was used to analyze the morphological and diversity of the anaerobes in each study sites. Duncan Multiple Range Test was used to determine the physico-chemical parameters while the Analysis of variance and linear regression was used to analyze the quantity of biogas produced in the three set- ups. Study identified 7 different anaerobic bacteria genera as *Methanococcus, Sulfolobus, Methanosaeta, Methanospirillium, Methanosarcina, Methanomicrobium* and *Methanothrix.* The most predominant methanogenic bacterial genera, which occurred in at least 5 digesters were *Methanococcus and Methanosaeta* genera which also produced the highest volume of gas with maximum production being observed in 21-31 days at a ratio of 30:500. *Methanococcus and Methanosaeta* synergistic activity yield the highest gas production of 74.23 ml versus 22.50ml in control. The study on the 6 bio digesters showed that physico-chemical parameters play a paramount role in biogas production and should be maintained at an optimum range. Predominant methanogens *Methanococcus* and *Methanosaeta* genera when inoculated in the digesters increased the quantity of biogas produced. Research recommends that biogas technique is an effective way of providing solution to the increasing waste management and disposal problems apart from the liberation of fuel or energy from recyclable energy sources, promotes the use of organic fertilizer as compared to chemical based fertilizers Further research work should be done on methanogenic bacteria involved in the production of biogas, the anaerobes should be isolated, recognized, and characterized at the species and strain levels before being utilized to produce biogas.

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LIST OF ABBREVIATIONS

$(CH_3)_3$-N-Methylamines

AD - Anaerobic digestion

ANOVA-Analysis of variance

AOAC - Association of Analytical Chemist's

APHA - American Public Health Association

C: N - Carbon to Nitrogen ratio

CBB - Calvin Benson-Bassham

CH_{3-} Methyl group.

CH_3OH-Methanol

CH_4 – Methane

CHP-Combined heat power

CO_2 - Carbon (iv) oxide

COD - Chemical Oxygen Demand

C.V-Coefficient of variance

DMRT-Duncans Multiple Range Test

H_{2-}-Hydrogen

H_2S - Hydrogen Sulphide.

HRT - Hydraulic Retention Time

KNFAD - Kenya National Federation of Agricultural Producers

LP - Liquid Pressurized

LSD-Level of significance difference

MR - Methyl Red

Nacl- Sodium Chloride

OH^- - Hydroxide ion

OLR - Organic Loading Rate

SPSS-Statistical package for social sciences

SRT - Solids Retention Time

STP-Standard temperature and pressure

TS - Total Solids

TSI - Triple Sugar Iron

VP - Vogues Proskauer

VPA - Volatile fatty acids

VS - Volatile Solid

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I am especially grateful to Almighty God for strengthening my memory and giving me the strength to carry on, even when it looked like it was almost impossible. My supervisors Dr. Salinah Rono, Dr. Pixley Kipsumbai and Mr. Muturi deserves special recognition for providing me with the chance and resources to do this research, as well as their academic support, direction, and ideas, without which this project would not have been possible. I thank and acknowledge the entire University staff and students for a harmonious learning environment during the entire course. Thank you to everyone in the family for their financial and emotional assistance.

CHAPTER ONE

INTRODUCTION

1.1 Background information

The quest for renewable energy facilities to replace fossil fuels has gotten a lot of attention in recent years (Arutyunov & Lisichkin, 2017). Anaerobic digestion (AD) is a more appealing option in this regard. It enables the conversion of various organic waste materials and crop leftovers into a sustainable energy component of biogas as well as the generation of a nutrient-rich residue for agricultural use (Awasthi *et al*., 2020). Anaerobic digestion is a dual-function technology widely used in the treatment of various organic wastes and wastewaters (Awasthi *et al*., 2020; Ramos-Suárez *et al*., 2015).

Anaerobic digestion is a waste-to-energy method that is commonly used in the treatment of various organic effluents such as city-wide solid waste organic output, wastewater sludge, food waste, livestock waste (Adekunle & Okolie, 2015; Leung & Wang, 2016; Zamri *et al*., 2021). Biogas production has been going on for a period exceeding 30 years. However, its acceptability has been delayed by a lack of understanding of its fabrication, immediate benefits, and excessively high design costs (Arutyunov & Lisichkin, 2017; Omer, 2017; Zamri *et al*., 2021). Biogas is a combustible and sustainable energy source composed of methane, carbon dioxide, and some water vapor (Ghatak & Mahanta, 2016). It is created by the decomposition of organic waste, such as municipal garbage, agricultural by products, and animal manure (Adekunle & Okolie, 2015).

Anaerobic digestion is the most environmentally friendly technique of organic disposal due to its significant volume reduction and stability levels (Kadam & Panwar, 2017).

Depending on the qualities of these wastes, they can be converted into energy without the use of any extra fuels, and finally, anaerobic digestion. So far, naturally occurring fuels including coal, oil, and natural gas have given power to developing nations while simultaneously supporting the technologically advanced modern world (Barisa *et al*., 2020). However, fossil fuels are limited, and their ongoing usage has an influence on our ecosystem and the global climate owing to greenhouse gas emissions. Furthermore, oil and gas supplies are running low. To prepare for the transition to a more dependable energy source, appropriate conserving, augmenting, and exchanging technologies must be investigated (Shindell & Smith, 2019). In this sense, waste products have been shown to boost energy supply, aid in lowering growing dependency on fossil fuels, and ameliorate environmental and health concerns that have arisen as a result of the use of fossil fuels in many third-world and industrialized countries.

Anaerobic digestion of organic waste might have a substantial influence on renewable energy consumption (Barisa *et al*., 2020; Shinde ll & Smith, 2019; Zamri *et al*., 2021). The method has dismayed the interest of additional researchers in eliminating organic contaminants from solid waste and wastewater while also creating methane as an energy resource (Kumar & Samadder, 2020).

Anaerobic digestion comprises several succeeding and parallel steps carried out by majorly four groups of micro-organisms populations. They include hydrolyzing, acidogenic, acetogenic, and methanogenic archaea, which operates in syntrophic association with one another in order to yield methane gas (Huang et al, 2015).

Anaerobic digestion has recently attracted significant attention from governments in numerous countries, including the United Kingdom, Germany, and Denmark, due to the

re-use of waste as a resource and new technical advances that have decreased starting expenses on fossil fuels (Hermann *et al*., 2019; Shindell & Smith, 2019; Zamri *et al*., 2021). Sovacool *et al*., (2015). Biogas digesters in Kenya have used inputs from various sources, including slaughterhouses waste, trash from municipal landfills, and bagasse from sugar manufacturers, plants such as water hyacinth and animal as well as human excreta. Others include human waste, such as utilizing a public toilet block in Kibera, Kenya, where biogas reactors have been erected (Sharma & Biswas, 2016). However, issues have developed as a result of the poor quality of the installed units. Users' insufficient operational and maintenance capabilities has decreasing performance, resulting in the abandonment of biogas digesters (Wassie & Adaramola, 2020). In other circumstances, the demonstration effect has discouraged rather than encouraged the use of biogas.

The biggest issues confronting current biogas facilities in Kenya are inadequate design and construction, ineffective methane digestion, and low communal acceptability (Sharma & Biswas, 2016). Small-scale farmers sometimes do not have enough domestic animals to generate enough manure for the bio digester to generate enough gas for lighting and cooking. Even when households produce an adequate number of animals, many Sub-Saharan African tribes' semi-nomadic or free grazing practices make it impossible to collect excrement to feed digesters (Berhe *et al*., 2017). Several research have been undertaken to explore the anaerobic digestion start-up kinetics. Still, no profiling of anaerobic microbial community in small-scale biogas reactor has been carried out to the best knowledge. This study sought to profile anaerobic microorganisms responsible for biogas production. Physicochemical parameters were analyzed, and the

predominant microbes were evaluated for their efficiency in biogas production in a laboratory-scale set-up.

1.2 Statement of the problem

Global warming due to greenhouse gases and the high cost of fossil fuel is an area of concern that needs to be addressed. There is need to explore a renewable energy source that is self-reliant and environmentally friendly. This research was set to isolate and identify the anaerobic microbes involved in biogas digestion. Under anaerobic circumstances, a mixed microbial population produces methane from organic sources. However, information regarding the bacteria and their roles is scarce. The microbial community's synergism that leads to biogas generation is poorly understood. Kenya's population is quickly growing, creating large gap in energy demand and supply. In the absence of proper disposal techniques, livestock manure, cow dung, can pose environmental risks, includes pathogen contamination, airborne ammonia in the air, odour, and greenhouse gases emissions (Sharma *et al*., 2022; Sharma & Biswas, 2016). On the other hand, the expanding demand for fossil fuels, a key energy source, depletes them on a daily basis, necessitating a significant capital expenditure.

1.3 Justification

To provide the most efficient solutions to both energy crisis and environmental pollution challenges, biogas' creation using waste materials will deliver fuel's reliable production. This plan would minimize the usage of fossil-fuel-derived energy while also reducing environmental effect, such as global warming and pollution. It would also enhance cleanliness, reduce demand for wood and charcoal for cooking, and supply high-quality

organic fertilizer, all of which would help to alleviate food shortages, which are a serious problem in developing nations, and thus increase output. Reliable power is a determinant of economic development and prosperity because it ensures continuous company operations.

Deforestation is wreaking havoc on rural Kenya as people cut down trees for cooking. However, biogas created from animal and human waste is a feasible option. A thorough understanding of the microbiota participating in the anaerobic process of particular waste and by-products is a vital step in better understanding the overall process and the relationship of each microbial species. The situation outlined above justifies the use of alternative energy sources, specifically sun, water, wind, petroleum, and geothermal. Biogas offers the most potential as a low-cost domestic energy source since it is renewable, simple to create, simple to use, and economical. Several publications on biogas (methane) generation from cow dung are available today, almost no work has been done on assessing the likelihood of methanogenic bacteria composition for effective biogas production from cow dung.

1.4 Objectives

1.4.1 Broad objective

To characterize and evaluate effective bacterial consortia for efficient biogas production on small scale bio-digester within Uasin Gishu County.

1.4.2 Specific Objective

i. To characterize the anaerobic bacterial community involved in biogas production in six bio- digester within Uasin Gishu County.

ii. To determine the physico-chemical parameters favouring the growth of the anaerobic bacterial community involved in biogas production in bio digester within Uasin Gishu County.

iii. To evaluate effective bacterial consortia for biogas production under laboratory conditions.

1.5 Hypothesis

i. There is no significant difference in anaerobic bacterial community involved in biogas production.

ii. There is no significant difference in physico-chemical parameters for the anaerobic bacterial community involved in biogas production.

iii. There is significant difference in efficiency of bacterial consortia responsible for biogas production.

CHAPTER TWO

LITERATURE REVIEW

2.1 Biomass energy in Kenya

Anaerobic digestion is a biochemical process consisting of organic material degradation, organic fraction of municipal solid wastes, sewage sludge, cattle manure, and pig slurry into biogas (Ibrahim *et al*., 2016; A. Sharma *et al*., 2022; Zamri *et al*., 2021). This process occurs in an enclosed anaerobic environment, for example, in a landfill in storage tanks or anaerobic reactors. Organic waste, such as bovine dung and dead fish from fish farms, is abundant in Norway (Ahuja *et al*., 2020). Norwegian fish farms reported a shortage of 27.4 million dead salmon, while the overall yearly quantity of organic waste in Norway is 1.45 million tons. In the UK, Didcot Sewage Works was first to supply biogas to the national grid, helping up to 200 homes in Oxfordshire (Rasmussen, 2020). Ecotricity, a UK energy company, aimed to power 6000 houses with a digester fed by locally grown grass (Elliott, 2019).

Simple household and agricultural anaerobic digestion compositions provide the prospect of low-cost energy for lighting and cooking in third-world countries (Morgan *et al*., 2018). For biogas generation, anaerobic digesters may also be fed with particular cultivated plants, such as green forage feed. Most Asian nations have extensive, government-supported programs for modifying small biogas plants for use in residential cooking and lighting (Jeuland *et al*., 2021). Asia is home to technologies for small-scale energy and sanitation supplies. Since then, there has been a surge of interest in anaerobic digestion to help with waste management and energy generation, with municipal sewage disposal garnering the greatest attention (Huang *et al*., 2015; Zhen *et al*., 2017).

Currently, initiatives for anaerobic digestion in the poor world can receive financial support for biogas technology under the United Nations Clean Development Mechanism if they can demonstrate that they reduce carbon emissions (Edwards *et al*., 2015). In Africa, participation has been boosted by different international organizations' and foreign assistance agencies' promotional efforts, such as publications, meetings, and visits (Cornelissen, 2016). Due to low technical quality, the majority of the facilities have only been operational for a short time. Although animal manures are blended with energy crops in Denmark and Germany, they have been verified as a good source for biogas generation in Africa (Scarlat *et al*., 2018).

More over 75 % of Kenyans live in rural regions, with agriculture being their major source of income. Cash crops, fruits, vegetables, food crops, forages, cattle, and tree cultivation are the principal farming systems (Sovacool *et al*., 2015). A research done in the Embu District's coffee-based land-use system revealed that smallholder farmers rely extensively on trees for a variety of purposes, including fencing, construction, fuel, food/fruits, and aesthetics (Wanjira & Muriuki, 2020). The diversity of tree species and the quantity of trees per type were badly insufficient, forcing net farmers to import tree products, particularly fuelwood. Energy is very important in the life of smallholder farmers.

Replacement rates were predicted to be just 60 % within the same time period, indicating that accessible wood fuel stocks were fast depleting. The primary farm-based energy source is fuelwood (Whiting *et al*., 2017). Nonetheless, it is frequently in limited supply as a result of the increasingly problematic supply of inexpensive domestic energy in rural as well as in urban locations (Koirala *et al*., 2016). Fuelwood scarcity has an impact on

all mothers and children. They are forced to travel long distances, wasting time and energy in the process. Inhaling fumes and soot while cooking with lower-grade fuels, such as firewood, harms women's health. Furthermore, it has caused long-term environmental harm that is difficult to reverse through improved soils and forest loss, resulting in desertification (Wanjira & Muriuki, 2020).

Biomass meets over 75 percent of Kenya's entire energy consumption (Sovacool *et al*., 2015; Wanjira & Muriuki, 2020). A growing population is facing the effects of conventional fuel shortages in rural regions, where more than 80 % of the country's 25 million inhabitants live. Charcoal, Firewood and agricultural leftovers are the primary source of cooking fuels as well as and heating in rural and urban regions, with kerosene used for illumination. The industrial, domestic and the commercial sectors are the primary consumers of biomass. Rural families account for approximately 90 % and 25 % of overall fuelwood and charcoal usage in Kenya, respectively. It is also estimated that firewood provides 92 % of the energy needs of rural households (Wanjira & Muriuki, 2020).

In comparison, the industry utilizes almost 68 % of Kenya's total energy. Few rural families utilize electricity, owing to high supply costs as well as inaccessibility. The environmental effect of fuelwood consumption has significantly increased. Desertification threatens the basic base of agricultural output by increasing soil erosion, deforestation and crop-land loss (Amwata, 2020; Wanjira & Muriuki, 2020).

As fuel sources dwindle, cow dung and agricultural wastes are burned, depleting soil of important nutrients and organic matter (Barnard, 2019; Edwards *et al*., 2015). As a result, rural energy issues jeopardize the availability of food and other essential necessities. If

extensively implemented and used effectively, anaerobic digestion biogas generation technology can ease some of the concerns described above (Zuberi & Ali, 2015). It produces a clean combustible gas, for example, that may be used for lighting, cooking or to fuel an engine that runs a grain mill or agricultural pump-set, decreasing dependency on diesel, kerosene, petrol and other imported petroleum products (Barisa *et al*., 2020; Morgan Jr *et al*., 2018; Scarlat *et al*., 2018; Vijay *et al*., 2015). It will also reduce reliance on wood fuel, resulting in less environmental damage due to deforestation and soil erosion. The method is particularly useful for treating sanitary wastes, reducing serious public health issues if not initially properly disposed off (Berhe *et al*., 2017). Biogas technology, as opposed to the traditional direct burning method, recovers a more useable and practical sort of energy from cow dung and agricultural waste without compromising their fertiliser value (Barnard, 2019; Vijay *et al*., 2015; Wassie & Adaramola, 2020). As a result, it provides a low-cost fertilizer or soil conditioner.

Kenya has a population of above 54 million people, with rural regions accounting for 77.8 % of the population (Sarkodie and Ozturk, 2020). Kenya's National Federation of Agricultural Producers (KNFAP), has a residential biogas development effort as the sole implementing agency. The initiative aims to develop 8000 residential biogas plants with capacities ranging from $6m^3$ to $12m^3$ and favours high agricultural potential locations. Several demonstration plants are now being built and deployed. The efficiency of biogas digesters have been found to vary greatly on the substrate type and microorganism present and further by the physicochemical conditions the variation in this could provide the different gas production efficiency (Amwata, 2020; Scarlat *et al*., 2018; Sovacool *et al*., 2015). Sovacool *et al*., (2015) reported on five biogas case studies in Kenya that used

agricultural leaves, floriculture wastes, and vegetable production and canning waste. The Dutch government has indicated that it will fund two hundred million Kenyan Shillings to establish eight thousand biogas digesters around the country. The method was designed for farmers who practice zero-grazing (Diouf & Miezan, 2019).

2.2. Anaerobic Digester Technology

Making alternative energy carriers is gaining popularity across the world. Biogas is a potential participant because its production may combine the treatment of diverse organic wastes with an energy carrier for the most varied uses (Awasthi *et al*., 2020; Berhe *et al*., 2017; Diouf & Miezan, 2019; Vijay *et al*., 2015). Microbial CO_2 fixation and conversion to CH_4 in anaerobic digesters is becoming well acknowledged. An efficient way to turn waste into energy is anaerobic digestion commonly employed in the treatment of various organic wastes (Edwards *et al*., 2015; Ibrahim *et al*., 2016; Morgan Jr *et al*., 2018). It is the treatment process of organic waste and wastewater without the use of external electron acceptors such as oxygen, allowing for lower treatment costs as well as the production of energy in the form of 'biogas' methane from organic waste (Hagos *et al*., 2017). Over the last two decades, the technology has grown in popularity, and knowledge of the technology's microbiological components has grown dramatically. Significant progress has been achieved in understanding the diversity of yet-to-be cultivated microorganisms in anaerobic digesting systems. The cultivation of uncultured organisms is gaining popularity as a means of learning more about how these species work (Lillington *et al*., 2020). Microorganism’s breakdown organic material in the absence of oxygen during the anaerobic digestion process.

The process involves four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, and is carried out by communities of hydrolysing, acid-producing, acetate-producing bacteria and methane-producing Archaea (Akindolire *et al*., 2022). The profiles of eubacterial of anaerobic co-digesters and the anaerobic stage of sewage plants, like methanogens, are frequently unique in terms of bacteria. Microbial participation in CO_2 fixation has been described, as have six autotrophic CO_2 fixation biochemical processes. The Calvin–Benson–Bassham (CBB) cycle is critical in nature for autotrophic CO_2 fixation (Berg, 2011). The CBB cycle has been seen in Proteobacteria, certain Firmicutes, Actinobacteria, and Chloroflexi members, as well as plants, algae, and cyanobacteria (Agarwal *et al*., 2018).

The reductive acetyl-CoA route, which has been described in acetogenic prokaryotes, is another significant process for CO_2 fixation as well as autotrophic archaea belonging to the order Archaeoglobales. Acetogenic prokaryotes use this mechanism for energy conservation (Yang *et al*., 2021). Diverse microorganisms have been found in anaerobic digesters, including bacteria such as *Firmicutes, Proteobacteria, Chloroflexi* and *Actinobacteria* as well as archaea such as methanogens and acetogenes. They are intended to employ efficient CO_2 fixation and bioconversion methods. Understanding the destiny of CO_2 in the anaerobic environment is of tremendous scientific interest and importance since adverse bacteria dwells in these structures and microbial fixation and rotation of the sequestered CO_2 into CH_4 are very feasible (Lehman *et al*., 2015). The numerous and complicated anaerobic processes that result in methane generation are generally carried out by syntrophic interactions between methanogenic Archaea and

Bacteria. This syntrophic relationship offers substrates for methanogens and removes biochemical pathways from acid-forming bacteria (Mutungwazi *et al*., 2021).

2.3 Anaerobic digester design and operation

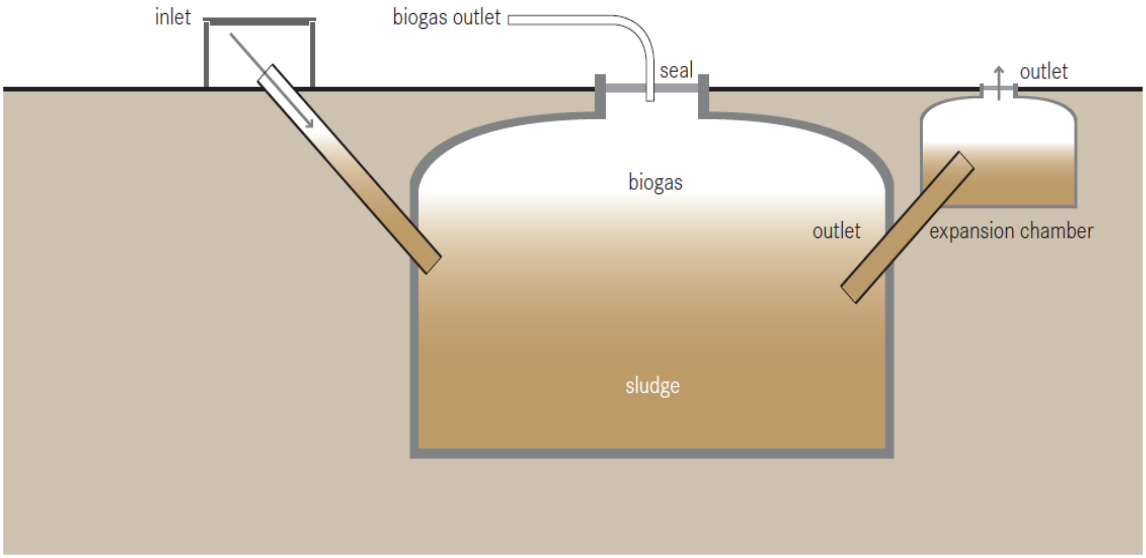

Figure 2.1: The layout of a typical small-scale bio-digester

German model digester (Ananthu, 2019).

Anaerobic construction and operation are critical factors in boosting process efficiency (Ananthu, 2019; Awasthi *et al*., 2020; Diouf & Miezan, 2019; Sovacool *et al*., 2015). Weiland (2010) various designs have been utilized effectively. They comprise or several reactor cores, closed batch or constant organic loading, and nutrient and biomass recycling (Fig 2.1). The balloon-type reactor was adapted for constant flow and batch operations (Aydin, 2017).

The batch reactor is the most basic, since the input material is fed into the reactor to begin digestion (Awasthi *et al*., 2020). The reactor is sealed, and biogas is sucked out continuously as it is created. Methanogenesis happens in stages. It starts with hydrolytic

bacteria, then moves on to acidogenic and acetogenic bacteria, and finally methanogenic archaea.

Another mutual anaerobic digester is an ending flow system with organic material continuously being added to the system (Li *et al*., 2021). All series of methanogenic pathways occur concurrently. In the early stages of an organization, there is minimal or no accumulation of volatile fatty acids. It implies that the acetogenic community's metabolic activity is the same as that of methanogenic archaea (Adekunle & Okolie, 2015; Ananthu, 2019; Sovacool *et al*., 2015; Yang *et al*., 2021).

2.4 Types of Bio-Digesters

A biogas digester is made up of one or several airtight chambers through which dung form animals such as cows animal is fed in batches or constantly (Pramanik *et al*., 2019). Digestion systems can be structured at various levels of complexity, such as single-stage or multi-stage, depending on whether they are fed continuously or in batches. The three steps of the anaerobic treatment occur in one reactor in the single-stage approach. Nevertheless, the fermentative bacteria growth rate is quicker than that of acetogenic and methanogenic bacteria. As a result of increasing organic rate of loading and unsuitable other process parameters, acids build, the pH decreases, and methanogenic bacteria growth is inhibited (Sun *et al*., 2017). All biological processes take place in a single, sealed reactor or holding tank. It lowers building costs but leads in less control over the system's reactions. Acidogenic bacteria lower the pH of the tank by producing acids. The biological processes of various species in a single-stage reactor might compete with one another.

An anaerobic bio digester is a one-stage reaction structure (Awasthi *et al*., 2020; Sun *et al*., 2017). These lagoons are pond-like earthen basins used for manure curing and long-term storage. The anaerobic processes are confined inside the pool's naturally anaerobic biomass. In multi-stage systems, two or more reactors partition the acetogenesis and methanogenesis phases in space. They make it possible to create conditions which are operational that reduce starting time and microbiota specialization in each of the reactor. The two-stage system generally includes two continuous flow reactors, one for the hydrolytic and fermentative communities and one for the methanogenic communities (Kadam & Panwar, 2017; Mutungwazi *et al*., 2021; Sun *et al*., 2017). The organic input is constantly fed into the acetogenic reactor, and the highly volatile fatty acid-containing product is transported to the methanogenic reactor. This technique allows for maximal organic matter division since the acidified material may be put into the methanogenic reactor in a widget fashion. For methanogenic activity, the volatile fatty acid concentration and pH are tuned. According to research, the two-stage method increases overall energy generation by 5-18 percent as compared to digestion utilizing a single continuous flow system (Joseph *et al*., 2019).

In biogas production units fed with various kinds of biomass, several microbial profiles have been observed. For example, the microbial diversity in a totally agitated digester fed with fodder beet silage as a mono-substrate has been demonstrated to be particularly rich in *Clostridiales*, *Deltaproteobacteria*, *Bacilli*, and *Bacteroidetes* (Ma *et al*., 2018). Other research indicates that bio waste sludge maturation affects the microbial composition in a thermophilic digester containing primarily Clostridia Firmicutes and Bacteroidetes dominate the microbial communities in laboratory-scale reactors fed casein, starch, and

cream (Joseph *et al*., 2019; Ma *et al*., 2018; Sun *et al*., 2017). Based on these findings, we may infer that the microbiological features of anaerobic digesters vary depending on the biogas reactor/biomass type. Firmicutes and Bacteroidetes are important in anaerobic digestion in eubacteria, and the most common group within Firmicutes is Clostridia (Ma *et al*., 2018).

2.5 Conditions and variables influencing anaerobic digestion

Anaerobic digestion (AD) is an organic process. To optimize microbial activity and hence anaerobic digestion efficiency, the digester's operational aspects must be regulated. Putrification of organic compounds by a microbial population happens in anaerobic (Sabliy *et al*., 2019). The many microorganisms in the microbial food chain progressively degrade the complex compounds required for a combination of CH_4 and CO_2. Environmental and internal influences synchronize the diverse microorganisms' systems, which include members of the Eubacteria and Archaea (Fuhrman *et al*., 2015). This microbial population composition is influenced by a variety of parameters, including substrate components, mixing, pH, temperature and the architecture of the anaerobic digester (Sabliy *et al*., 2019; Wassie & Adaramola, 2020).

The pH of the digester is determined by the concentration of volatile fatty acids generated, the system's bicarbonate alkalinity, and the amount of carbon dioxide produced. The optimal pH levels for acidogenesis and methanogenesis are distinct. The pH of the anaerobic digestion system is highly important. Methanogenic bacteria are particularly sensitive to acidic environments, and their development and methane synthesis are hampered in an acidic environment (Barisa *et al*., 2020; Gaby *et al*., 2017; Joseph *et al*., 2019; Morgan Jr *et al*., 2018). Methanogens like pH levels between 7 and

7.5. Hydrolysis and acidogenesis reduce the pH of the anaerobic digestion process before any methane can be created. The best pH range for getting maximum biogas generation in anaerobic digestion has been proved to be 6.5–7.5, the range is rather large in plants, and the optimal pH value changes with feedstock and digestion technique. On the other hand, an oversupply of methanogens might result in greater ammonia concentration, raising the pH above 8, which inhibits acidogenesis (Li *et al*., 2021; Sabliy *et al*., 2019; Sovacool *et al*., 2015).

A carbon-to-nitrogen ratio of 25–30:1 is ideal for biogas generation. A greater C:N ratio indicates that methanogens use nitrogen quickly and produce less gas (Matheri *et al*., 2018). Nitrogen is used by methanogens to meet their protein needs. Excess Nitrogen results in the creation of Ammonia at higher ratios. It elevates the pH level over 8.5, which inhibits microbial activity and, as a result, gas generation. Microorganisms use carbon 25–30 times quicker than nitrogen during anaerobic digestion, according to research. To meet this criterion, bacteria require a C to N ratio of 20–30:1, with the highest proportion of Carbon being quickly decomposable. Levels of also influence the optimal C: N ratio (Khayum *et al*., 2018; Matheri *et al*., 2018).

Temperature is by far the most important environmental element regulating microbe growth; each bacterium has a range of temperature in which it may grow and replicate (Awasthi *et al*., 2020; Khayum *et al*., 2018; Lillington *et al*., 2020; Sun *et al*., 2017). However, because enzymes are protein-like, crucial chemical processes in the multiple metabolic pathways known to be catalysed by enzymes are irreversibly destroyed above optimal temperatures. The anaerobic digester may operate at two different temperatures: mesophilic conditions and thermophilic conditions. Mesophilic digesters operate at

temperatures ranging from 25°C to 45°C, typically 350°C, whereas thermophilic digesters operate at temperatures ranging from 50°C to 65°C (Kim & Lee, 2016; Lillington *et al*., 2020). The optimal digestion temperature, might vary depending on feedstock content and digester type (Khayum *et al*., 2018; Li *et al*., 2021; Matheri *et al*., 2018; Pramanik *et al*., 2019). Nonetheless, it should be kept roughly constant in most anaerobic digestion processes in order to maintain the gas production rate. Thermophilic digesters perform better in terms of retention duration, loading rate, and gas output, but they require more heat and are more sensitive to operational and environmental factors, making the process more difficult than mesophilic digestion (Ghasimi *et al*., 2015; Leung & Wang, 2016).

The Organic Loading Rate (OLR) is a measure of the anaerobic digestive system's bio-conversion capability. When the biological system is overburdened, it might result in low biogas output (Ghasimi *et al*., 2015; Sun *et al*., 2017). The extra substrate at the beginning of the process causes the accumulation of undecomposed substances such as fatty acids. It lowers the pH and causes an imbalance throughout the breakdown chain. A sudden increase in organic loading rate causes system failure due to decreased chemical oxygen demand removal efficiency, methane generation, and pH. More specifically, an organic loading rate greater than the optimal capacity enhances the generation of intermediate products (fatty acids) by hydrolytic and acidogenic bacteria (Huang *et al*., 2015; Joseph *et al*., 2019).

As a result of the sluggish pace at which these fatty acids are used by methanogens, pH will fall, reducing methanogenic activity (Lehman *et al*., 2015; Sun *et al*., 2017). The retention time refers to how long the fermentable material is kept inside the digester. A

larger digester is required for a longer retention duration, and complete feed digestion is assigned. Retention period, also known as hydraulic retention time, is typically between 10 and 25 days. The process's particle retention time or Solids Retention Time (SRT) is sometimes mentioned. In most cases, Hydraulic Retention Time (HRT) and Solid Retention Time (SRT) are the same, with the exception of digesting tanks, where a portion of the residues are returned to the process, in which case (SRT) becomes longer than hydraulic retention time. In mesophilic and thermophilic digesters, waste retention times range from 15 to 30 days and 12 to 14 days, respectively. When the retention period is too short, the microorganisms cannot multiply at the pace at which the material is withdrawn from the process (Hagos *et al*., 2017; Joseph *et al*., 2019).

The extent to which incoming animal dung come into touch with a viable bacterial consortia as a result of mixing in the reactor is crucial in anaerobic digestion (Khayum *et al*., 2018; Lillington *et al*., 2020; Vijay *et al*., 2015). Several studies have established the economic benefits of mixing digester components during the anaerobic process, which include trying to prevent scum establishment inside the digester, ensuring homogeneous density of microorganisms and substrate throughout the mixture and reinforcing contact between them, trying to suppress stratification inside the digester, which allows for uniform heat distribution throughout the mixture, and ultimately assisting in the release of gas from the microbial community (Berhe *et al*., 2017; Pramanik *et al*., 2019; Sovacool *et al*., 2015). Mixing inside the digester enhances interaction between the microorganisms and the substrate, as well as the bacterial population's capacity to absorb nutrients; nevertheless, excessive mixing might displace the microorganisms, therefore gradual mixing is ideal (Awasthi *et al*., 2020; Matheri *et al*., 2018).

2.6 Stages of anaerobic degradation of organic wastes

The anaerobic microbiology organic of waste processing is a complex process involving several bacterial species (Figure 2.2). Hydrolytic, acid-forming, acetogenic, and CO_2 and CH_4 generating methanogenic bacteria are examples of these species (Hamad 2019). The phasing of methane digestion is a distinct feature. Each one is responsible for the breakdown of a distinct type of chemical.

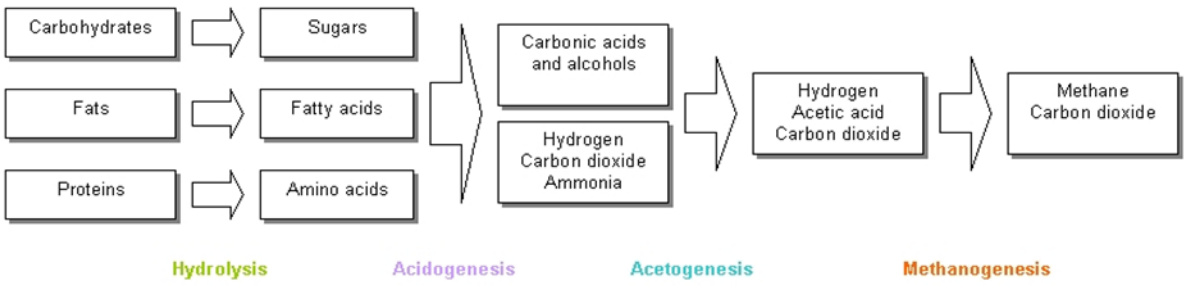

Figure 2.2: Description of the process of organic matter degradation during anaerobic digestion (Lu *et al*., 2018)

Hydrolysis is a chemical process that breaks down water to produce H+ cations and OH- anions. In the presence of an acidic catalyst, hydrolysis is frequently used to break down bigger polymers (Solovev *et al*., 2018). Big polymers like proteins, lipids, and carbohydrates are hydrolysed into smaller molecules like amino acids, fatty acids, and simple sugars. The presence of water causes chemical bonds to be broken. The digester feedstock can be made up of a variety of elements and materials (Adekunle & Okolie, 2015; Matheri *et al*., 2018; Morgan *et al*., 2018). As a result, there are several hydrolysis forms; carbohydrates, lipids, and proteins are first broken down into smaller molecules during anaerobic digestion. Polysaccharides and complex sugars are broken down into monosaccharides in the case of a carbohydrate. The hydrolysis of lactose into galactose

and glucose is one example (Leung & Wang, 2016; Li *et al*., 2021). Lactose, a polysaccharide, is hydrolysed to produce galactose and glucose, both monosaccharides. Triglycerides are generally broken down into three fatty acids and glycerol in lipids by adding three water molecules—triglyceride hydrolysis yields glycerol and three fatty acids In the case of proteins, peptide bonds are broken to separate amino acids. During protein hydrolysis, a peptide bond is broken to segregate amino acids (Khayum *et al*., 2018; Solovev *et al*., 2018).

Microorganisms involved in the hydrolysis stage have been researched in ruminants and, more recently, in the biogas process. In each slurry, these anaerobes were separated and identified. *Clostridium, Prevotella, Succinivibrio, Bacteroides* and *Ruminococcus* are the most commonly found taxa in bovine rumen (Chuang *et al*., 2020). *Fibrobacter*, formerly known *Ruminococcus* and *Bacteroides* as well as uncultured bacteria, have been implicated in cellulose breakdown in the rumen (Awasthi *et al*., 2020; Zhang *et al*., 2017). Only a few prior research on the biogas generation process have directly addressed the hydrolysis stage. While some hydrolysis products, such as hydrogen and acetate, may be used by methanogens later in the anaerobic digestion process, the majority of the molecules, which are still relatively bulk, must be further split down in the acidogenesis process to produce methane, according to a recent study (Huang *et al*., 2015; Solovev *et al*., 2018). Acidifying bacteria convert water-soluble chemical compounds, including hydrolysis products, to short-chain organic acids, alcohols, carbon dioxide, hydrogen and aldehydes, during the acidogenesis phase (Figure 2.3). Protein purification results in the production of amino acids and peptides, which anaerobic microbes can use as energy sources (Walsh, 2020; Wassie & Adaramola, 2020).

Acidogenic bacteria convert hydrolysis products to volatile fatty acids. Some hydrogen, carbon dioxide, and acetic acid are also created, allowing the acetogenesis stage to be skipped (Solovev *et al*., 2018). Acidogenesis is the process by which bacteria convert glucose into acetate and butyrate (volatile fatty acids). Bacteria produce acetate and butyrate during acidogenesis. Acidogenesis may be bidirectional due to the impact of diverse microorganism populations (Huang *et al*., 2015; Joseph *et al*., 2019; Sun *et al*., 2017). There are two forms of this process: hydrogenation and dehydrogenation. The primary transfiguration mechanism involves acetates, CO_2, and H_2, with minor contributions from other acidogenesis products (Aydin, 2017; Khayum *et al*., 2018; Scarlat *et al*., 2018). As a result of this transformation, methanogens may use the new products as substrates and energy sources directly. The bacterial reaction to a rise in hydrogen concentration in the solution is the accumulation of electrons by molecules such as ethanol, lactate, butyrate, propionate, and highly volatile fatty acids. Methanogenic bacteria may not immediately utilise the new output. It must be transformed by acetogenesis bacteria that produce hydrogen (Huang *et al*., 2015; Li *et al*., 2021). Among the acidogenesis products, ammonia and hydrogen sulphide give this phase an extremely disagreeable odour (Adekunle & Okolie, 2015; Walsh, 2020). In the acid phase, facultative anaerobes utilize the oxygen that was accidentally introduced into the process, creating ideal conditions for the obligatory anaerobes' formation of from the following genera: *Bacillus, Pseudomonas, Clostridium, Flavobacterium,* or *Micrococcus species* (Jikia *et al*., 2016).

Acetogenesis is the process by which acetate bacteria, such as those found in the genera *Syntrophomonas* and *Syntrophobacter*, transform acid phase products into acetates and

hydrogen for use by methanogenic bacteria (Pandey *et al*., 2020). Bacteria such as *Methanobacterium suboxydans* are in charge of converting *pentanoic* acid to propionic acid, whereas *Methanobacterium propionicum* is in charge of breaking down propionic acid to acetic acid (Lillington *et al*., 2020; Sabliy *et al*., 2019). Hydrogen is produced as a by-product of acetogenesis, which is harmful to the bacteria that carry out this activity. Acetogenesis according to Jikia *et al*. (2016) is a phase that improves the efficiency of biogas production since acetate reduction yields around 70 % of methane (Hagos *et al*., 2017; Kadam & Panwar, 2017). As a result, acetates are an important intermediate product of the methane digestion process. In the wastes degrading process, roughly 25 % of acetates are created during the acetogenesis phase, and 11 % of hydrogen is produced (Moestedt *et al*., 2019).

The process through which methanogenic bacteria make methane is known as methanogenesis. During methanogenesis, bacteria known as methane formers make methane in two ways: breakage of two acetic acid molecules to generate carbon dioxide and methane or reduction of carbon dioxide with hydrogen (Huang *et al*., 2015; Moestedt *et al*., 2019). Despite the fact that just a few bacteria can create methane from acetic acid, the vast majority of CH4 produced throughout the methane digestion process is due to acetic acid conversions by heterotrophic methane bacteria. Only 30 % of the methane produced in this process is the result of CO_2 reduction by autotrophic methane bacteria. H_2 is used up throughout the process, generating optimal circumstances for the development of acid bacteria that produce short-chain organic acids in the acidification phase and, as a result, minimal H_2 generation in the acetogenic phase. Because only a

portion of CO_2-rich gas is converted to methane, such conversions may result in CO_2-rich gas (Ghasimi *et al*., 2015; Khayum *et al*., 2018; W alsh, 2020).

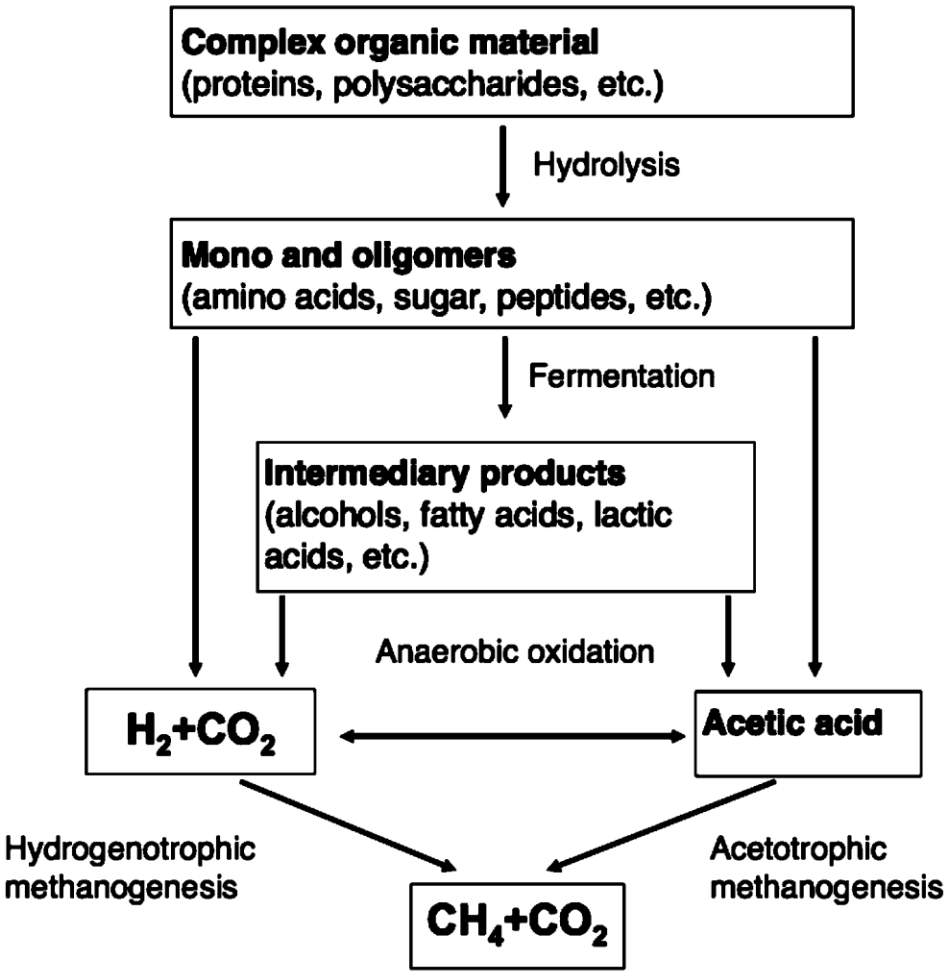

Figure 2.3: Anaerobic digestion process

Microorganisms decompose organic material through a series of biological processes in the absence of oxygen, resulting in the production of biogas. (Wang, 2017)

2.7 Output from the digestion process

The products from the digestion process are:

1. Biogas — a gas composed of 60 % methane (C_2H_4), 40 percent carbon dioxide (CO_2), 1 percent hydrogen sulphide, and trace amounts of other 'contaminant' gases (Hagos *et al*., 2017). This biogas is subsequently burnt to provide heat, electricity, or road fuel.

2. Digestate (slurry) - is a sterile, inert wet substance containing important plant nutrients and organic humus. This product can be split into "liquor" and "fiber" for land use or subsequent processing.

2.8 Biogas

The most valuable component of biogas is methane (CH_4), which generally accounts for 60 % of the total, with the remainder consisting of carbon dioxide (CO_2) and trace amounts of other gases (The amount of methane depends on the feedstock and the efficiency of the process, with a methane content range of 40 % to 70 % (Adekunle & Okolie, 2015; Berhe *et al*., 2017; Joseph *et al*., 2019). Biogas is concentrated and includes Hydrogen Sulphide (H_2S), which contributes to the pungent odour of rotten eggs and flatulence (Yang *et al*., 2021).

2.8.1 Feedstock for biogas production

Biogas is produced by the anaerobic conversion of organic molecules. All biodegradable components with a high lignin content (excluding wood) are suitable input materials for biogas operations. Manure and most plant biomass may be supplied to agricultural biogas plants, but food waste and sewage sludge are the most common material flows to urban biogas operations (Berhe *et al*., 2017; Khayum *et al*., 2018; Lillington *et al*., 2020). Biodegradable by-products from several sectors can be used in biogas facilities.

Depending on the amount of carbs, lipids, and proteins in the raw material, different amounts of biogas and methane will be produced (Aydin, 2017).

2.8.2 Biogas adoption in Kenya

Energy deficiency is a hindrance to economic and social growth in the majority of Kenyan homes, therefore finding an cheap and dependable residential energy source is vital (Sovacool *et al*., 2015). There is tremendous biogas potential in central and western Kenya, but water scarcity restricts opportunities in other areas. Despite the fact that agricultural waste has the ability to contribute to biogas production, cow dung was the most commonly used feedstock. In rural Kenya, the inflatable tubular digester was proven to be the most cost-effective kind for biogas generation (Momanyi & Benards, 2016; Sovacool *et al*., 2015; Wilkes & Dijk, 2017).

Wood fuel is the most widely used biomass fuel, resulting in a supply-demand mismatch. Overreliance on unsustainable wood fuel leads to unregulated tree and shrub cutting in rural areas, which is exacerbated by climate and rainfall vulnerability (Sovacool *et al*., 2015). Less than 5 % of Kenya population use liquid pressurized gas as their primary cooking fuel, 5 % use kerosene, 1 % use an improved biomass cook stove, and 90 % of rural communities use fire wood for cooking (Momanyi & Benards, 2016; Sovacool *et al*., 2015; Wilkes & Dijk, 2017). In the country, over 22,000 biogas plants have been built, with 20,000 depending on animal dung and the remaining on agricultural waste (Momanyi & Benards, 2016; Sovacool *et al*., 2015; Wilkes & Dijk, 2017). Approximately 90 % of these biogas plants are residential, with the remainder being institutional or on flower farms (Wilkes & Dijk, 2017).

2.8.3 Liquid displacement method to study biogas quantity

In this process of quantifying biogas yield, some significant parameters must be preserved to ensure that the data is accurate. The two most pertinent of these is picking a proper barrier concentration to prevent the loss of gases through decomposition and the standardization of volumetric measurements to standard temperature and pressure (Buraimoh *et al*., 2020; Dennis, 2015). The study has displayed that the ideal barrier solution for lowering gas solubility is an acidified, saturated NaCl solution. It is owing to the tendency of CO_2 to dissolve in water over time (Matheri *et al*., 2018; Wassie & Adaramola, 2020) studied different barrier concentration and how much CO_2 or CH_4 was lost through dissolution for 17 days. A 100 % concentrated NaCl solution was suitable for preventing the loss of CO_2 into the solution. Such a concentration can lead to amassing of salts on the equipment and thus not allowed. The reason for preventing the dissolution of gases into the barrier concentration is to ensure that gas-composition measurements are not negatively affected (Ananthu, 2019; Li *et al*., 2021).

2.8.4 Uses of biogas

Biogas replaces firewood as a cooking fuel, minimizing deforestation (Rupf *et al*., 2015). Biogas burns significantly cleaner than traditional wood stoves and helps to reduce eye and respiratory problems caused by smoke in unventilated dwellings the World Health Organization (WHO) estimates that indoor air pollution causes roughly 2 million additional deaths in developing nations (Mocumbi *et al*., 2019). Biogas may power an internal combustion gas engine in an Integrated Heat and Power unit, which produces both electricity and heat. The gas might potentially be expanded and utilised in gas distribution networks (Ananthu, 2019; Rafiee *et al*., 2021; Wilkes & Dijk, 2017).

2.9 Methanogens in the methane fermentation process

Methanogens are a distinct family of microorganisms classed as Archaea that differ from bacteria in several ways, the absence of essential cellular features, including the presence of membrane lipids, and the presence of specialized ribosomal RNA (Banach *et al*., 2019). Methanogens are obligate anaerobes that are rate-limiting organisms in anaerobic wastewater treatment. Furthermore, methanogens coexist or compete for substrate with sulfate-reducing bacteria in anaerobic treatment–laden effluent (Banach *et al*., 2019; Dennis, 2015; Gaby *et al*., 2017). Methanogens are anaerobic microorganisms that live in anaerobic environments such as marshlands, sewage sludge, swamps, wastewater deteriorated tanks, sandy lagoons, bottom deposits, tundras, rice fields, solid and ruminant stomachs (Millard & Regan, 2016). These bacteria are extremely sensitive to temperature and pH changes, and their growth is inhibited by high quantities of volatile fatty acids and other compounds in the environment such as hydrogen, ammonia, and sulfur hydrogen (Banach *et al*., 2019, 2019). Psychro-, meso-, and thermophilic bacteria were discovered among methanogenic microorganisms identified by morphological and biochemical examination (Joshi, 2020).

Mesophilic and thermophilic bacteria are highly active at temperatures ranging from 28°C to 42°C and 55°C to 72°C, respectively. So yet, no anaerobic psychrophilic bacteria has been discovered to be active at temperatures below 25°C (Ghasimi *et al*., 2015). Temperature is crucial for methanogenic bacteria (Matheri *et al*., 2018) due to the low temperature resistance of their enzyme complexes (Joshi, 2020). Methanogenic bacteria are frequently found in inert settings with pH values ranging from 6.8 to 7.2 this is not to argue that methanogenesis does not take place in acidic or alkaline environments.

Methanogens that degrade acetates, such as *Methanosarcina barkeri* and *Methanosarcina* species, were isolated in pH 5 environments. In contrast, methylotrophic and hydrogen-oxidizing methanogens have been identified in very alkaline environments. Methanogenic bacteria are classified as chemolithotrophs because they can obtain carbon from CO_2. (Zabranska & Pokorna, 2018).

Because of their capacity to convert organic resources to methane, methanogens are an important class of bacteria. As part of the waste treatment system, methanogenic bacteria are utilized in the anaerobic breakdown of wastewater. In aerobic wastewater treatment, sedimentation technologies are also employed for primary and secondary sludge (Matheri *et al*., 2018). The pharmaceutical sector is particularly interested in these bacteria because they may contain vitamin B12. Methanogenesis occurs in anaerobic digestion via two different routes: 1st, direct acetate cleavage to CH_4 and 2nd CO_2 and CO_2 reduction with hydrogen gas. Each route's contribution to methanogenesis is determined by the inoculum sludge, organic substrate, and process parameters. (Huang *et al*., 2015). There are three kinds of methane-forming bacteria. The three types of methanogens are hydrogen trophic, acetotrophic and methylotrophic methanogens. The term "trophic" (from trophe, "nutrition") refers to the bacteria food sources (Yang *et al*., 2021).

2.9.1 Hydrogenotrophic methanogens

Hydrogenotrophic methanogens use hydrogen to convert carbon dioxide to methane (Banach *et al*., 2019; Zabranska & Pokorna, 2018). By converting carbon dioxide to methane, these organisms help in maintaining a low partial hydrogen pressure in an anaerobic digester, which is necessary for acetogenic bacteria (Dennis, 2015; Rafiee *et al*., 2021; Walsh, 2020).

2.9.2 Acetotrophic methanogens

Acetotrophic methanogens convert acetate into methane and carbon dioxide. Hydrogen trophic methanogens may convert acetate-derived carbon dioxide to methane. Carbon monoxide is used by certain hydrogen trophic methanogens to generate methane (Huang *et al*., 2015; Zabranska & Pokorna, 2018). Acetotrophic methanogens reproduce at a slower rate than hydrogen trophic methanogens and are heavily controlled by hydrogen accumulation. As a consequence, keeping an anaerobic digester at a low partial hydrogen pressure benefits both acetate-forming bacteria and acetotrophic methanogens (Zhang *et al*., 2017). When the hydrogen partial pressure is high, the generation of acetate and methane is decreased (Zabranska & Pokorna, 2018).

2.9.3 Methylotrophic methanogens

Methanotrophic methanogens thrive on methylated (-CH3) substrates. These substrates include methanol (CH_3OH) and methylamines [$(CH_{3)3}$-N] (Huang *et al*., 2015; Zabranska & Pokorna, 2018). Methanogens in groups 1 and 2 create methane from CO_2 and H_2. Rather than CO_2, Group 3 methanogens produce methane directly from methyl groups (Li *et al*., 2021; Zabranska & Pokorna, 2018). More energy is gained by methane-forming bacteria through hydrogen-consuming methane formation than from acetate breakdown (Gaby *et al*., 2017; Mutungwazi *et al*., 2021). Although hydrogen methane synthesis is the most efficient energy capture process used by methane-forming bacteria, it only accounts for around 30 % while Acetate accounts for over 70 % of the methane produced in an anaerobic digester (Li *et al*., 2021). This is because hydrogen is in short supply in an anaerobic digester. *Methanosarcina* and *Methanothrix* are two acetotrophic methanogen species that create the majority of the methane produced by acetate.

The majority of bacteria that produce methane are mesophiles or thermophiles, with some flourishing at temperatures exceeding 100°C (Banach *et al*., 2019; Matheri *et al*., 2018)**.** Mesophiles like temperatures between 30°C and 35°C, whereas thermophiles prefer temperatures between 50°C and 60°C. Some methane-forming bacteria genera have thermophilic and mesophilic species (Joseph *et al*., 2019).

2.9.4 Methods of detecting methanogenic bacteria from bio digesters

Methanogenic bacteria have been identified and cultivated for centuries using the procedures described by Hungate, with a modification of the original approach given by (Banach *et al*., 2019, 2019; Dennis, 2015). These techniques offer the conditions required for the cultivation of the very O_2-sensitive methanogenic bacteria. In the absence of O_2., the approach comprises of preparing and inoculating medium (Gaby *et al*., 2017; Kim & Lee, 2016). A butyl rubber stopper isolates the medium from the aerobic environment. Agar was uniformly distributed across the inside surface of Roll tubes. Bacteria are embedded in the agar or streaked across the surface, equating the roll tube to a petri plate. The roll tube has two disadvantages: It is difficult to see and identify isolated colonies, especially at low dilutions where colonies are crowded; and it cannot be used for regular genetic operations such as replica plating (Ananthu, 2019; Sun *et al*., 2017).

Cow dung may also be cultured in medium to identify methanogens (Sharma *et al*., 2022) Standard aerobic approach is used to prepare the media, removing the need for time-consuming anaerobic preparation procedures. Using a fluorescent pigment unique to this metabolic type of bacteria, colonies of methanogenic bacteria are discovered on petri plates. They create F420, a low-molecular-weight molecule that fluoresces when stimulated by long-wave ultraviolet light (Li *et al*., 2021). Methanogenic organisms

develop quickly and can be detected earlier than using roll tube approaches. These organisms may be studied using standard genetic approaches such as replica plating (Ananthu, 2019; Leung & Wang, 2016).

2.9.5 Application of Anaerobic digestion

Biogas is a digester gas produced by an anaerobic bacterial consortium that decomposes organic materials. Its composition is determined by the type of raw material used in the digestion process as well as the manner used to carry it out. Rojas and colleagues (2010) Methane CH_4 (50–75) %, hydrogen sulfide H_2S (0-1) %, oxygen O_2 (0–2) %, carbon dioxide CO_2 (25–45) %, hydrogen H_2 (0-1) %, nitrogen N_2 (0–2) %, carbon monoxide CO (0–2) %, ammonia NH_3 (0-1) %, and water H_2O (2–7) %. The generated biogas may be employed in a variety of economic domains, mostly in technical processes and power engineering Using anaerobic digestion systems can assist to reduce greenhouse gas emissions (Buraimoh *et al*., 2020; Dennis, 2015; Rafiee *et al*., 2021).

The leftover solid waste is low in odour and rich in nutrients and serves two purposes: approximately half of the solid waste is reserved and mixed in with the incoming fresh feedstock as a source of inoculum, while the remaining half is further composted off-site and later can be applied as fertilizer or as soil supplement (Agarwal *et al*., 2018). Nutrient availability is greater in slurry than in untreated organic waste. The usage of slurry also enhances the soil's humus balance. This can also be used as fertiliser or soil supplements in agriculture and landscaping, allowing for nutrient cycling and enhanced soil structure due to the incorporation of organic matter. Replacement of fossil fuels, reduction of energy footprint for waste treatment plant, reduction or elimination of landfills methane emissions and replacement of chemical fertilizers produced industrially, reduction of

electrical grid transportation losses, reduction of vehicle movements, reduction of liquefied petroleum gas usage for cooking (Buraimoh *et al*., 2020; Momanyi & Benards, 2016).

2.9.6 Use of slurry

The slurry is high in plant nutrients including nitrogen, phosphate, and potassium. Nutrients are preserved, with more than 90 % of nutrients entering anaerobic digesters being conserved throughout digestion (Momanyi & Benards, 2016; Pramanik *et al*., 2019; Wilkes & Dijk, 2017; Zabranska & Pokorna, 2018). Fermented biogas slurry, on the other hand, enhances the physical, chemical, and biological qualities of the soil, resulting in higher qualitative and quantitative yields of food crops. Ponds as feed for algae, or ducks, water hyacinth, fish and mushroom culture are further uses for slurry.

CHAPTER THREE

METHODOLOGY

3.1 Study area

The research study was carried out in the Microbiology laboratory at the University of Eldoret. The University of Eldoret is situated in Uasin Gishu County, at a height of 2180 meters above sea level, between latitude 0^{o} 34' 35" North and longitude 35^{o}18' 13" East. The region receives between 900mm and 1600mm of rain from March to September, with two distinct peaks in May and August, and the average temperature is around 24 °C.

3.2 Sampling techniques

Six sampling sites within Uasin-Gishu County were selected these were Opande, Beta farm, Radar, Nettos, Langas and Ministry of Energy (Table 3.1). The digesters under study varied in both their sizes and biogas production volume. Thus, they were categorized into small, medium and big size. The samples of the cow dung were collected aseptically from the six identified digesters in a sterile 250ml flask in triplicates. This totalled into 18 samples. The bioreactor contents were mixed before each sampling. Physical parameters of biogas digester including the condition of biogas digester, temperature, and pH were recorded. In each sampling days, at least 250ml of the cow dung was collected in a sterile sampling bottle through a clean funnel and immediately closed with a sterile stopper and transported to the laboratory in a keep cool box containing ice packs, stored at 4°C-8°C and processed within 24hrs. Sampling was done three times a week during morning hours for three consecutive weeks. The bio-digesters from which the samples were collected had different characteristics.

Table 3.1: Characteristics of the six digesters under study

Digester	Location	Size	Type	Gas prodn	Uses
BIG (N)	Nettos	150M^3	Fixed dome	155m^2D	Cooking
(R)	Radar	150M^3	Fixed dome	150m^2D	Cooking
Medium (O)	Opande	135M^3	Floating gas holder	130m^2D	Cooking
(E)	Ministry of Energy.	135m^3	Floating gas holder	131m^2D	Cooking
SMALL (P)	Langas	120M^3	Plastic bag holder	121m^2D	Cooking
(B)	Beta farm	120M^3	Plastic bag gas holder	121m^2D	Cooking

3.3 Sample processing

The cow dung that was sampled from the bio-digesters and stored in a fridge at a temperature of 4^oC were removed to thaw and attain room temperature, 9ml of sterile distilled water was measured into clean test tubes, 1ml of the cow dung was added into the test tube and mixed. 1 ml of the mixture was drawn and added to the second tube making 10^1 they were serially diluted to 10^5 and then cultured anaerobically on sterile methanogenic media. The isolates were coded as E1m and E2m, for isolates from Energy. R1m, R2m, R3m, R4m and R5m for isolates from Radar. B1m, B2m, B3m and B4m for the isolates from Beta farm. O1m, O2m and O3m for the isolates from Opande. P1m and P2m for the isolates from Langas. N1m, N2m, N3m and N4m for the isolates from Nettos.

3.4 Isolation and identification studies

3.4.1 Spread plate method

The pre-sterilized methanogenic media (Appendix 1.0) was dispensed into respective plates. They were labelled and left to solidify after which 1 ml of the serially diluted sample of 10^5 was pipetted and dispensed into the solidified media. A sterile bend glass rod was used to spread the inoculum onto the media and let to stand for 5 minutes which allowed the media to absorb the inoculum as described in Bergey's manual 9^{th} (Whitman *et al*., 2015). The plates were labelled and packed into an anaerobic jar; this allows anaerobic condition (Plate 3.1). The jar was then placed into the incubator set at a mesophilic temperature of 35^{o}C to 55^{o}C for 3-4 days. The colonies were observed and photographed.

Plate 3.1: Anaerobic jar packed with plate ready for incubation

3.4.2. Isolation in axenic culture by streak plate method

After 3-4 days, the plates revealed mixed bacterial colonies. They were differentiated based on morphological characteristics and sub cultured into sterilized plates containing methanogenic media by streaking plate technique. A sterile inoculating loop was used to pick a pure colony differentiated by its colour from the mixed population and streaked on the solidified methanogenic media in the plate and labeled. It was then incubated anaerobically for 3-4 days.

3.5 Morphological studies

From the pure colonies morphological characteristics were noted using the key described in Bergeys Manual of determinative bacteriology 9th Edition (Whitman *et al*., 2015). The morphological characteristics included, colour, elevation, form, surface, margin, shape and gram reaction.

The pure isolated colonies were subjected to the Gram staining technique. This was done to differentiate between gram positive and negative bacteria cells. A sterile inoculating loop was used to pick a colony from a pure streak plate; a thin smear was made on a grease-free glass slide and allowed to dry by passing it on a heat source. A drop of crystal violet stain was added to the smear and allowed to stand for one minute. It was then washed off using slow running tap water. Lugols iodine was added for one minute and washed off. The smear was then decolourized by absolute alcohol briefly for 30 seconds after which safranin stain was added for one minute and washed off, the slide was dried and observed at ×100 oil-immersion-objective-lense. The positive bacteria cell was

identified by the bluish black colour while negative cells were noted because of the pink colouration.

3.6 Biochemical studies

The isolates were subjected to biochemical tests, which further assisted in their identifications as follows.

3.6.1 Catalase test

A sterile wire loop was used to pick a bacteria colony from a streak plate and transferred onto a grease-free glass slide. One drop of 15 % H_2O_2 was added, and the reaction was observed as described by Bergey's manual (Whitman *et al*., 2015). This was done to evaluate the microorganism's capacity to convert hydrogen peroxidases into oxygen and water, causing foaming owing to oxygen release. Positive test is indicated by presence of bubbles of oxygen while negative results show no bubble.

3.6.2. Motility test

Motility media (Appendix 1.0) was dispensed into test-tubes and allowed to solidify. An inoculating wire was used to pick pure colony and stabbed straight into the culture media, the tubes were later incubated at a temperature of 35^oC for 48hrs as described by Bergey's manual (Whitman *et al*., 2015). The positive test was depicted by cracks within the media while negative test develops no cracks.

3.6.3. Sugar fermentation test

Kliger iron agar (Appendix 1.0) was used to detect sugar fermentation. This was done to assess bacteria's capacity to consume three sugars, lactose, glucose, and sucrose, as well

as the creation of H_2S, which is the primary activity of anaerobic bacteria. This test comprises of various tests, including H_2S production, Glucose and Lactose test, and CO_2 production. In sulphur production test a positive reaction is shown by formation of a black colouration for H_2S on the butt region of a slant. Glucose presence detection, a positive test is depicted by a colour change from red to yellow while a negative test shows no colour change in the media. The CO_2 production test is exhibited by the media's cracks in the slant culture thus a positive reaction a negative reaction shows no cracks. The media was dispensed into a test tube in a slant position. A straight inoculating wire was used to stab halfway to the bottom of the tube with the pure isolated colonies from the streak plate; the tubes were covered with parafilm creating an anaerobic condition and incubated at 37^oC for 48 hrs.

3.6.4. Citrate utilization test

Simmon citrate agar (Appendix 1.0) was dispensed into respective tubes in a slanting position and let to solidify. A straight inoculating wire was used to pick the pure colony obtained from the streak plate and stabbed straight into the media inoculation. The tube's mouth was covered using sterile cotton wool. The tube was incubated at 35^oC for 48 hours. This was done to evaluate the capability of the bacterium that uses citrate as its only carbon source and breaks it down into oxaloacetate and acetate, which is then converted into pyruvate and carbon dioxide (Whitman *et al*., 2015). Positive results are indicated by colouration of the medium from green to blue while the negative result shows no colour change thus remains green.

3.6.5. Test for indole

Tryptophan broth (Appendix 1.0) was used in this test; the broth was dispensed into respective tubes, then the isolated colonies were emulsified into the broth and labeled accordingly. The tubes were later incubated at a temperature of 37^{o}C for 48 hrs. Half a mililitre (0.5ml) of Kovacs reagent was added into the broth culture and observed. This was done to locate bacteria that can deaminate and hydrolyze amino acids, producing pyruvic acid and ammonia as well as methane and CO_2, which is the main function of methanogens (Whitman *et al*., 2015). A positive test results in the formation of a cherry red hue in the top layer of the tube, whereas a negative test results in no red coloring.

3.6.6. Methyl red- Voges- Proskauer test (MR-VP)

The MR-VP broth (Appendix 1.0) was used with a 5 ml of broth being poured into each tube, and autoclaved then the tubes were divided into two pairs. The test organism was inoculated into each tube pair and labelled appropriately. The tubes were incubated at 37^{o}C for 48hrs. Five drops of methyl red indicator were put to one pair of tubes after 48 hours and assessed. To the other pair, ten drops of VP 1 reagent and 2-3 drops of VP 11 reagent were added. To finish the reaction, the tubes were gently shaken and plugged with cotton wool for 15-30 minutes. Using the techniques of, the bacterial isolates were identified by comparing their features to those of recognized species (Whitman *et al*., 2015). The methyl red test detects microorganisms that produce stable acid end products via glucose mixed acid fermentation. The Voges proskauer test is used to detect an organism's ability to produce acetoin, a neutral end product of glucose fermentation (Mallick, 2019). Positive test in MR is indicated by the tube maintaining the red colour while negative test is indicated by turning of the tube media to yellow.

3.7 Determination of physicochemical parameters

3.7.1. Measurement of pH

In each of the digester the substrate was mixed with distilled water in ratio of 2:1 and pH recorded. This was done in replicates to determine the mean pH value and the data obtained was recorded. It was determined for three consecutive days each morning in every digester. A pH meter was used for the analysis.

3.7.2. Determination of Temperature

The temperature was determined from the cow dung waste for all the digesters under study. Wet bulb thermometer was used. This was taken daily for three consecutive days and mean temperature recorded.

3.7.3. Moisture content assay

Moisture content was determined using the AOAC method. Fifteen-grams of slurry was weighed into the dried crucible dish and then heated at 98^{o}C-100^{o}C for two hours. After the time elapsed, the dish was removed and transferred to a desiccator weighed at room temperature and data was recorded. The moisture content was calculated using the formula;

Moisture content = B (mass of silica + sample A (mass of crucible)/wt. of sample.

Where;

A= Mass of the empty clean and dried crucible

B= Mass of silica + sample (g)

3.7.4. Volatile solids assay

The volatile solids were determined using APHA (1999) (Hobbs *et al*., 2018). Fifteen grams of the slurry were put in a crucible dish and burned for 30 minutes in a muffle furnace at 500°C-550°C. The crucible was removed from the muffle furnace, slightly cooled in air, and placed in a desiccator for a few minutes before being weighed and recorded.

Volatile solid was computed using formulae, shown below;

Volatile solid = B (mass of silica crucible +sample) – C (of silica crucible +sample after ignition) / B (mass of silica crucible +sample) –A (mass of the empty clean and oven-dried crucible)

Where;

A=mass of the empty clean and oven-dried crucible

B=mass of silica crucible +sample (g)

C=mass of silica crucible +sample after ignition.

3.7.5. Total solids assay

Total solid was determined by weighing 10gm of freshly collected sample and taken into the dish for 1 hour, the crucible was put in a hot air oven set at 105°C. It was cooled in a desiccator until it attained room temperature and weighed. It was analysed according to the methodology described by APHA, (1999) (Hobbs *et al*., 2018).

Total solid was computed using the formulae;

Total solids = C (Mass of silica crucible +sample after oven drying) –A (Mass of the empty clean and oven-dried crucible) / B (Mass of silica crucible + sample) – A (Mass of the empty clean and oven-dried crucible)

Where;

A - Mass of the empty clean and oven-dried crucible

B - Mass of silica crucible + sample

C - Mass of silica crucible + sample after oven drying.

3.8 To evaluate effective bacterial consortia for efficient biogas production

3.8.1 Lab-scale setups

The study estimated the amount of biogas produced, through synchronized and simulated lab-scale setups, using the cow dung waste (Plate 3.2). It was done in two parts. First, the cow dung was diluted with sterile distilled water in a 2:1 ratio and placed in a 500ml bottle for 30 days. The first setup acted as the control with no inoculum added. The second setup contained the same cow dung volume fed with different concentrations of isolated microbes at concentrations 10:500ml, 20:500ml, and 30:500ml of methanogens to cow dung waste volume. To maximize biogas production, incubation was carried out for 30 days. During the incubation stage, biogas was collected by displacing water downward at 10-day intervals for 30 days. In the course of the work, pH and temperature contents was monitored regularly.

Plate 3.2: A simulation of a laboratory-scale digester set up

3.8.2 Digester Design

Two 500ml Erlenmeyer flasks were utilized, each half filled with cow dung slurry, while plastic basins acted as water troughs. The flasks were immersed in a water bath to control and maintain the temperature between 35^{o}C and 40^{o}C. 1 M sodium hydroxide and a trace of phenolphthalein were used as pH indicators. The indicator became pinkish violet in dilute solutions with pH more than 8.2 and colorless in solutions with pH less than 8.2. To shut the flask's mouth, a rubber stopper was employed, and a polyvinyl chloride tube was inserted into the flask's arm. This acted as a repository for the biogas generated. The rubber tube's free end was then forced through water containing methyl red as an indicator in a halfway water filled trough into an inverted measuring cylinder filled with water to serve as the biogas collecting system. To catch the biogas, water was moved downhill. The displacement of water technique of biogas collection was a way by which gas could substitute water for the same quantity of water displaced, and it was used to calculate the amount of gas produced on a particular day.

3.8.3 Preparation of cow dung slurry

Fresh cow excrement was gathered from the Eldoret University farm. This was done in a big sterile plastic container and transferred to the laboratory for additional testing. All physico-chemical characteristics were measured in situ. A weighing balance was used to weigh 260 g of cow excrement, which was then combined with 520 ml of water in a plastic calibrated container in a 1:2 ratio. The pH of the slurry was measured with a pH meter that was continuously modified with alkaline buffer. A little quantity of 1 M sodium hydroxide was repeatedly added while the pH was measured until it reached a pH of roughly 7.03.

3.8.4 Feeding of digester

The prepared organic waste was put into the reactor bottle, which was connected to the digester through a hose. The loading technique used was discontinuous feeding. As a result, the digester was only filled once and stayed closed for the retention period. The slurry mixture was placed in the digester for anaerobic digestion. The daily gas productions were monitored for thirty-one (31) days.

3.8.5 Collection of biogas

The biogas was supplied by water displacement downwards. The displacement of water process of biogas collection was a method for gas to replace water with equivalent capacity, and it was used to compute the volume of gas generated each day. The reactor bottle's biogas was attached to an inverted 250 ml measurement cylinder. The amount of water displaced was used to compute the gas capacity.

3.8.6 Biogas Volume

The liquid displacement method was employed to quantify the biogas generated as specified by Boshagh & Rostami (2020). The displacement water in the inverted cylinder that had been filled with water to act as the biogas collecting system was used to measure biogas output on a volume basis at 10-day intervals.

3.9 Data analysis

Total Shannon diversity test was used to determine the abundance and diversity of the isolated methanogens in each bio digester under study.

Chi-square test was employed on characterization of the isolated methanogens to determine their p value on their morphological parameters e.g. elevation, margin, surface, shape and gram stain and to calculate their p value in each parameter.

The data from the physicochemical parameters was statistically analysed using Genstat discovery (10th edition 2008). Duncan Multiple range test (DMRT) and the means separated by Turkey's 95 % level of coefficient. Analysis of variance (ANOVA), Statistical Package for Social Sciences. SPSS, (2007) version 16.0.

The data obtained from the gas generated in three set ups was subjected to statistical analysis using Genstsat discovery (10^{th} edition, 2008) Duncan's Multiple Range Test (DMRT) the means will be separated by the Turkey's 95% level of coefficient.

Linear regression was used to plot the graph of biogas production in each treatment.

CHAPTER FOUR

RESULTS

4.1 Methanogenic anaerobic bacteria isolated from the bio-digesters under study

The seven genera of bacteria isolated from the different bio-digesters were as follows; *Methanococcus* sp. *Methanomicrobium* sp. *Methanosarcina* sp. *Methanosaeta* sp. *Sulfolobus* sp. *Methanothrix* sp. and *Methanospirrilium* sp. (Table 4.1).

The bio-digester located in Radar showed the highest bacterial population having 7 of the bacterial genera identified in this study. It was further noted that the highest proportion was with *Methanosaeta* sp. which was more prevalent while the least was *Methanospirillium* sp. and *Methanomicrobium* sp isolated from Radar and Energy bio-digester respectively.

Table 4.1: Bio-digester sites and methanogenic bacterial genera identified

Sites	Bacterial genera isolated	Total isolates
Radar	*Methanococcus, Methanosaeta, Methanosarcina, Methanothrix, Sulfolobus, Methanomicrobium and Methanospirrilium*	7
Energy	*Methanomicrobium and Methanothrix*	2
Opande	*Methanococcus, Methanosaeta and Methanomicrobium*	3
Beta farm	*Methanococcus, Methanosaeta, Methanosarcina and Sulfolobus*	4
Langas	*Methanococcus and Methanosaeta*	2
Nettos	*Methanococcus, Methanosaeta, Methanosarcina, Methanomicrobium, Methanothrix and Sulfolobus*	6

Total Shannon Weiner diversity of the bacteria was 1.76 H'. In terms of sites, Radar had the highest diversity (1.90 H') with seven genera recorded. (Figure 4.1.). The sites with

the lowest diversity were Energy (0.56 H') and Langas (0.69 H') with two genera recorded in each site. There was a significant difference in Shannon Weiner diversity between Radar and Opande (t=1.9062, p=0.0259), Radar and Energy (t=1.6021, p=0.0342), Radar and Langas (t=4.4233 p=0.0001), Radar and Beta farm (t=1.4245), p=0.0359), Energy and Beta farm (t=5.9782, p=0.0001), and Langas and Beta farm (t=1.9352, p=0.0001).

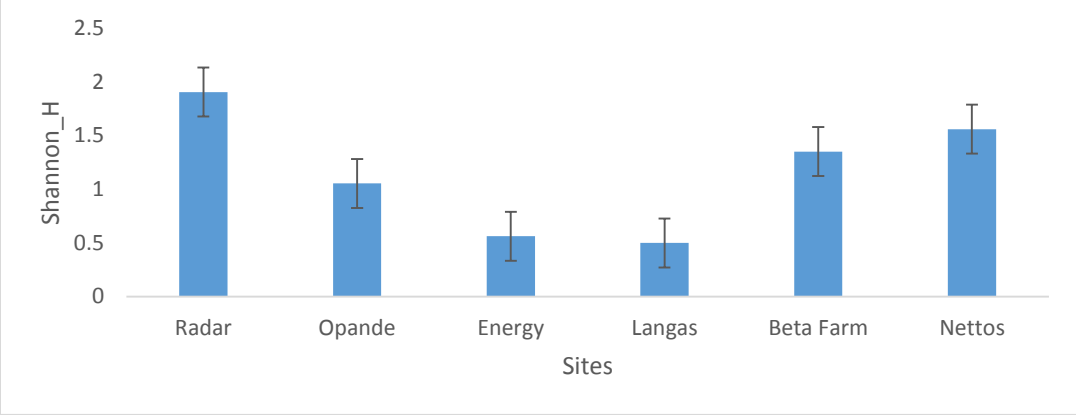

Figure 4.1: The diversity of bacterial community from bio-digesters

4.2 Characteristics of anaerobic bacterial community involved in biogas production in six bio-digester within Uasin Gishu County

4.2.1 Morphological characterization of bacterial isolates

There were seven morphological characteristics that were observed for the anaerobic microbial community involved in biogas production in six bio-digester within Uasin Gishu County. These were colour, form, margin, elevation, surface, shape and Gram stain. Five notable colours were identified in the morphological characterization of the bacterial isolates, these included cream, orange, pink, white and yellow. White colour was observed in most isolates 6 (30%), namely E1m, R1m, N4m, O1m, B3m, R5m,

followed by cream 5 (25%), and yellow 5 (25%), pink had 3 (15%) while orange was found in only 1 (5%) isolate (R4m) with a significant difference (χ^2 = 20.00, d.f = 4, p= 0.0005) as portrayed in (Table 4.2.) There were five forms that were noted in the morphological characterization of bacterial isolates. The punctiform was the dominate form in 9 (45%) of isolates characterizing *Methanosaeta* sp. *Methanococcus* sp. *Methanosarcina barkeri* and *Sulfolobus* sp. bacteria species while concentric rings had 1 (5%) isolate with *Methanosarcina barkeri.* Differing in significant difference (χ^2 = 32.0, d.f=3, p= 0.0005).

Table 4.2: Morphological characterization of bacterial isolates

Morphological characterization	Attribute	F	%f	Chi square (χ^2)
Colour	Cream	5	25.00	χ^2 = 20.0, df=4, p= 0.0005
	Orange	1	5.00	
	Pink	3	15.00	
	White	6	30.00	
	Yellow	5	25.00	
Form	Circular	5	25.00	χ^2 = 32.0 df=3 d.f, p= 0.0000
	Concentric rings	1	5.00	
	Irregular	5	25.00	
	Punctiform	9	45.00	
Margin	Entire	15	75.00	χ^2 = 78.5 df=2 p= 0.0000
	Lobate	2	10.00	
	Undulate	3	15.00	
Elevation	Convex	4	20.00	χ^2 = 70.0 df=3 p= 0.0000
	Flat	3	15.00	
	Pulvinate	1	5.00	
	Raised	12	60.00	
Surface	Dry / powdery	3	15.00	χ^2 = 110.0 df=3 p= 0.0000
	Rough	1	5.00	
	Smooth	2	10.00	
	Smooth / glitter	14	70.00	
Shape	Cocci	13	65.00	χ^2 = 54.5 df=2 p= 0.0000
	Lobed cocci	1	5.00	
	Rods	6	30.00	
Gram stain	Negative	3	15.00	χ^2 = 49.0 df=1 p= 0.0000
	Positive	17	85.00	

Three types of margins were noted in the study which included entire, lobate and undulate. Majority of the isolates (75%) were of entire margin characterizing, *Methanosaeta* sp., *Methanospirillium* sp. *Methanococcus* sp. *Sulfolobus* sp. *Methanothrix* sp. and *Methanosarcina barkeri.* Bacteria species while undulate types of margins characterized *Methanomicrobium* sp. with a significant difference (χ^2 = 78.5, d.f = 2, p= 0.0005). Raised elevation dominated many of the isolates 12 (60%) characterizing *Methanospirillium* sp., *Methanosaeta* sp. *Methanosarcina barkeri. Sulfolobus* sp. *Methanosaeta* sp. and *Methanomicrobium* sp. (χ^2 = 70.0, d.f=3, p= 0.0005). Majority of the isolates had smooth / glitter surface which characterizes *Methanospirillium* sp. *Sulfolobus* sp. *Methanosaeta* sp. *Methanomicrobium* sp. *Methanococcus* sp. and *Methanothrix* sp. in terms of shape, Cocci dominated majority of the isolates whose bacteria species were *Methanococcus* sp. *Methanosarcina barkeri and Sulfolobus* sp. likewise, most of the isolates were of gram positive.

4.2.2 Biochemical characterization of bacterial isolates

The biochemical test revealed that most isolates 70 % were catalase positive B1m, B2m, B4m, P3m, P4m, E2m, R1m, R2m, R3m, N3m, N4m, O1m, O2m and O3m with only 30 % being catalase negative E1m, P2m, P1m, N1m, N2m and B3m, N1m, N2m, N3m, N4m, P2m, P3m, E1m, R1m, R2m, R3m, O1m, O2m and O3m showed a negative reaction to catalase test (Table 4.3 and Plate 4.1a and b) respectively. Carbon dioxide production was also exhibited on 4 isolates R2m, N2m, N4m and R1m were positive to the test scoring 20 % while as the remaining 18 isolate 80 % B1m, B2m, B4m, P1m, P2m, P3m, P4m, E2m, N1m, N2m, N3m, N4m, E1m, E2m, R3m, O1m, O2m and O3m exhibited a negative reaction. (Table 4.3 and Plate 4.2 a). Ten isolate B2m, B3m, B4m,

P2m, P4m, E2m, R3m, O1m, O2m and O3m subjected to Methyl red test showed a positive test to the reaction exhibited by broth colour change from yellow to pink scoring 50 % the rest 10 isolates B1m, N1m, N2m, N3m, N4m, P1m, P3m, E1m, R1m and R2m showed a negative test to the reaction representing 50 % (Table 4.3, Plate 4.4 b).

Table 4.3: Biochemical characterization of bacterial isolates

Bacteria sp.	Sample	Catalase	Motility	H2S	Glucose	MR	Indole	Citrate	CO2
Methanococcus	P3m,	+	+	-	+	-	+	+	-
	N1m	-	+	-	+	-	-	+	-
Methanomicrobium	E2m,	+	-	+	+	+	-	+	-
	O1m	+	+	-	+	+	-	-	-
	O3m	+	+	-	+	+	-	-	-
Methanosaeta	N3m	+	+	-	+	-	-	+	-
	P1m,	-	+	+	+	-	-	+	-
	P2m,	-	+	-	+	+	-	+	-
	R2m,	+	+	-	+	-	-	+	+
	O2m,	+	+	-	+	+	+	-	-
	B1m,	+	+	-	+	-	-	+	-
	B2m,	+	+	-	+	+	-	-	-
Methanosarcina	B3m,	-	+	-	+	+	-	+	-
Methanospirilium	N2m	-	+	-	+	-	-	+	+
	N4m	+	+	-	+	-	-	+	+
Methanothrix	P4m	+	+	-	+	+	-	+	-
	R1m,	+	+	-	+	-	-	+	+
	E1m,	-	+	-	+	-	-	-	-
Sulfobolus	B4m,	+	+	-	+	+	-	+	-
	R3m,	+	+	-	+	+	-	-	-
Total positive (+ve)	% +ve	70	90	10	100	50	30	70	20
Total negative (–ve)	%-ve	30	10	90	0	50	70	30	80

Of the isolates 90 % were majorly motile except isolate E2m which showed negative motility. Sugar fermentation test done on the isolates exhibited different results which includes reaction to glucose showing 100 % synthesis in all the 20 isolates (Table 4.3). Hydrogen sulphide production was also exhibited on the test 10 % of the isolates P1m and E2m showed a positive reaction of black colouration of the butt (Table 4.3, Plate 4.2 b) while 90 % of the isolate R1m, R2m, R3m, R4m, B2m, B3m, B4m, B1m, P2m, P3m,

P4m, R2m, R3m, N3m, N4m, O1m, O2m and O3m showed a negative reaction to the test. Citrate test showed a positive reaction of 70 % of the isolates R1m, R2m, R4m, B3m, B4m, B1m, P2m, P3m, P4m, R2m, R3m, N3m, N4m, while negative had 30 %. R3m, E1m, B2m, O2m, O1m, and O3m. (Table 4.3, Plate 4.3 a and b) respectively. Indole test had 30% isolates positive O2m, while 70 % represented negative reaction to the test. R1m, R2m, R3m, R4m, B2m, B3m, B4m, B1m, P2m, P3m, P4m, R2m, R3m, N3m, N4m, O1m, O3m.P1m, N1m and N2m (Table 4.3, Plate 4.4a).

4.3 Physico-chemical parameters of the anaerobic bacterial community involved in biogas production in the different bio-digester within Uasin Gishu County

The highest pH was recorded for Beta farm (7.06±0.78) followed by Opande (7.20±2.00) and radar (7.20±0.92) while the lowest pH was recorded at Energy (6.86±1.22) with no signficant difference (p=0.0035) (Table 4.4.) The recorded temperature was highest in Radar (37.87±4.67) and Nettos (37.80±8.89) but lower in Energy (34.90±3.12) with a notable significant difference (p=0.0225). Mean significant difference was between Beta farm and Langas, Nettos and Opande. The mean volatile solids ranged from a lowest of 0.20±0.00 gms recorded at Beta farm to a maximum of 0.81±.00gms recorded at Energy which was found to differ significantly amongst the sites except in the case of Radar, Beta farm and Nettos (p=0.0213). The measured moisture content ranged from a lowest of 11.82±1.56gms recorded at Energy to a highest of 13.84±2.45 gms recorded at Radar with a significant variation noted among all the sites studied (p=0.0131). Total solids reported was in the range of 6.90±1.34 g in Opande and 9.46±1.32 g in Nettos which was significantly different among the sites (p=0.078)


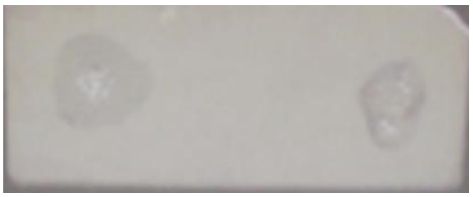

Plate 4.1: (a) Positive catalase (b) Negative catalase

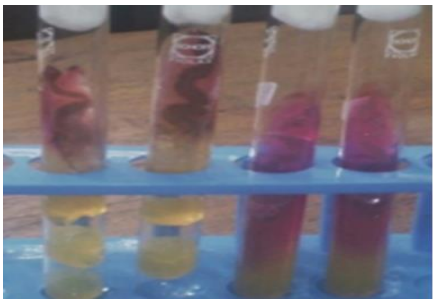
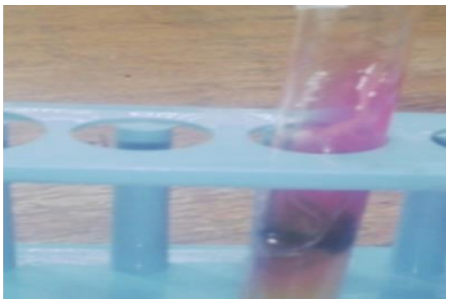

Plate 4.2: (a) Positive sugar fermentation with CO2 production (b) Positive H2S production


Plate 4.3: Citrate test (a) Positive (b) Negative

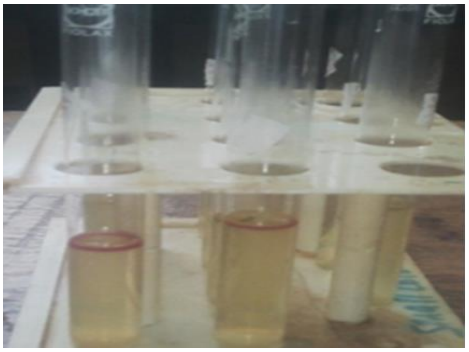
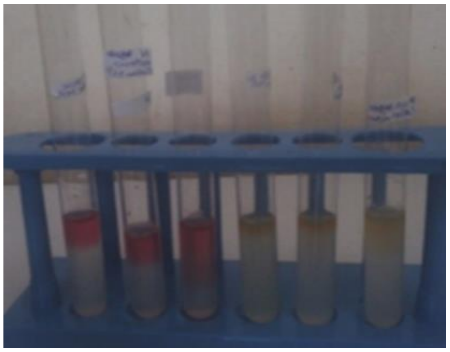

Plate 4.4: (a) Indole Positive test (b) Methyl red test Positive left Negative right

Table 4.4: Mean value of physico-chemical parameters

Site	pH	Temp (^{o}C)	VS (g)	MC (g)	TS (g)
Beta	7.06±0.78a	35.10±5.02ab	0.20±0.00a	12.72±1.89c	9.25±2.89c
Energy	6.86±1.22a	34.90±3.12a	0.81±.00d	11.82±1.56a	7.77±1.56ab
Langas	7.00±1.45a	36.23±6.67b	0.32±0.01b	13.19±1.25d	8.40±1.90c
Nettos	6.96±0.99a	37.80±8.89c	0.25±0.00a	13.46±2.75e	9.46±1.32c
Opande	7.20±2.00a	35.57±6.89ab	0.42±0.00c	12.47±2.56b	6.90±1.34a
Radar	7.20±0.92a	37.87±4.67c	0.22±0.00a	13.84±2.45f	8.64±1.62bc
p value	0.0035	0.0225	0.0213	0.0131	0.0078

Key: VS-Volatile solids, MC-Moisture content, TS-Total solids. Figures of means followed by the same letters are not significantly different at the 5% level of significance according to Duncan's Multiple Range Test (DMRT).

4.4 Effective bacterial consortia for biogas production under laboratory conditions

4.4.1 Amount of gas produced

From the findings, the highest amount of gas produced (74.23±12.85) was from *Methanosaeta* sp. and *Methanococcus* sp. at a treatment of 30:500 for a period of between 21 to 30 days. This was followed by *Methanosaeta* sp. producing 64.23±5.56 then *Methanococcus* sp. produced 50.00±3.56 while control produced 22.50±2.45 of gas in 30:500 (21-30) days with a significant difference (p=0.0002). In 20:500 (11-21) days, the highest amount of gas was produced in *Methanosaeta* sp. and *Methanococcus* sp. (54.00±3.75) followed by *Methanosaeta* sp. 51.40±4.81 while *Methanococcus* sp. produced 44.40±4.58 control produced the least (19.63±3.45) with a significant difference (p=0.0121). At 10:500 (0-10) days, *Methanosaeta* sp. and *Methanococcus* sp. produced the highest volume of gas (53.50±8.45) followed by *Methanosaeta* sp. (44.70±4.45) while *Methanococcus* sp. produced 37.83±7.85. The control had the least with a significant difference of 18.47±2.86.

Table 4.5: Amount of gas produced

Isolates /Ratio	10:500 (0-10) days	20:500 (11-21) days	30:500 (21-30) days
Methanosaeta sp.	44.70±4.45bc	51.40±4.81bc	64.23±5.56c
Methanococcus sp.	37.83±7.85b	44.40±4.58b	50.00±3.56b
Methanosaeta and *Methanococcus* sp.	53.50±8.45c	54.00±3.75c	74.23±12.85c
Control	18.47±2.86a	19.63±3.45a	22.50±2.45a
p value	0.0001	0.0121	0.0002

Means followed by different letters within a column are significantly different at p<0.05

4.4.2 Rate of gas production per 10-day interval

The rate of gas production was found to increase as the ratio of bacteria species that were added to the substrate increased (Figure 4.2). The *Methanosaeta* sp. was found to produce more biogas across the experiment set ups which in most cases was not significantly different from the biogas production by the consortia of *Methanococcu*s sp. and *Methanosaeta* sp. after 30 days. The gas produced by the substrate with no addition of the bacteria species was significantly very low in all the set-ups, in most cases less than 20ml after 20 days only surpassing 22-50 ml after 30 days. Further the gas production was found to increase as the days in the bio-digester fermentation increases.

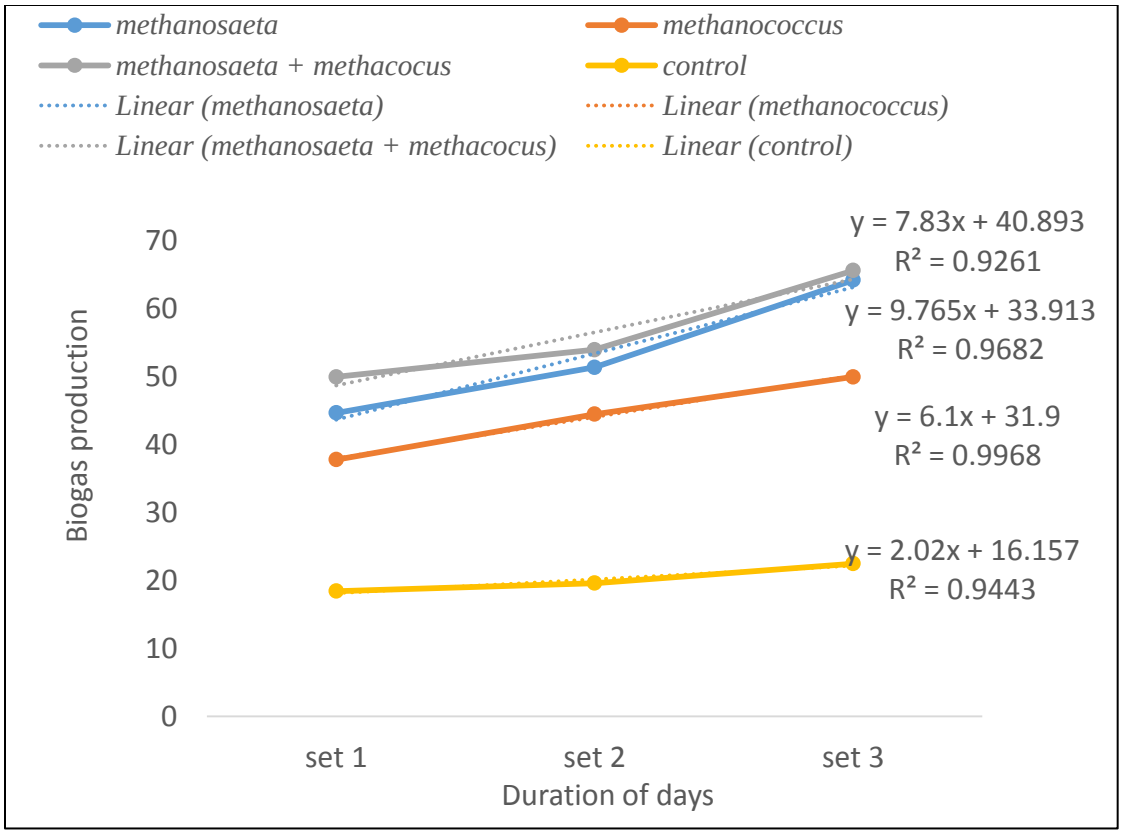

Figure 4.2: Rate of gas production per 10-day interval

CHAPTER FIVE

DISCUSSION

5.1 Characteristics of anaerobic microbial community involved in biogas production in six bio-digester within Uasin Gishu County

5.1.1 Morphological characterization of bacterial isolates

Results established different morphological characterization of bacterial isolates which included colour, form, margin, elevation, surface, shape and Gram stain. Results established that some bacteria were white others were cream, orange, pink, and yellow. Kern *et al*. (2016), indicated that there are some bacteria capable of producing pigment with different varieties of colours. *Methanosaeta* sp. has distinct characteristics such as cream colour, the form is puntiform and the margin is entire while surface is pulvinate. It is small cocci shaped and gram reaction is positive. The findings indicated that different bacteria produced different colours with example of *Methanosaeta* sp. producing cream colour. The findings are in line with those of Singh, (2020), that *Methanosaeta* sp. are cream in colour. Masaki *et al*., (2016) also added that *Sulfolobus* sp. are highly concentrated with yellow colour due to Sulphur element in them.

Bacteria can also be classified in terms of forms. The punctiform was the dominate form characterizing *Methanosaeta* sp. *Methanococcus* sp. *Methanosarcina barkeri* and *Sulfolobus* sp. bacteria species while concentric rings isolate characterized *Methanosarcina barkeri.* Three types of margins were noted in the study which included entire, lobate and undulate. The finding established that entire margin characterizes *Methanosaeta* sp. *Methanospirillium* sp. *Methanococcus* sp. *Sulfolobus* sp. *Methanothrix*

sp. and *Methanosarcina* sp. Bacteria species while undulate types of margins characterized *Methanomicrobium* sp. these findings are in line with those of (Norell *et al.,* 2003). Raised elevation dominated many of the isolates characterizing *Methanospirillium* sp. *Methanosaeta* sp. *Methanosarcina* sp. *Sulfolobus* sp. *Methanosaeta* sp. and *Methanomicrobium* sp. which is similar with the findings of Wang *et al*., (2018). Most of the isolates had smooth / glitter surface which characterizes *Methanospirillium* sp. *Sulfolobus* sp. *Methanosaeta* sp. *Methanomicrobium* sp. *Methanococcus* sp. and *Methanothrix* sp. in terms of shape, cocci dominated majority of the isolates whose bacteria species were *Methanococcus* sp. *Methanosarcina* sp. *and Sulfolobus* sp. likewise, majority of the isolates were of gram positive which concurs with the findings of (Kern *et al*., 2016) that methanogenic bacteria isolated on the sewage sludge appeared positive and cocci on shape when observed on a phase contrast microscope.

5.1.2 Biochemical characterization of bacterial isolates

The biochemical properties of microorganisms were investigated in order to determine the genus and species of an unknown bacteria. Microorganisms are extremely adaptable, with a wide range of metabolic capabilities (Brzeszcz & Kaszycki, 2018). These traits can be used to illustrate the tremendous metabolic diversity. *Methanosaeta* sp. and *Methanococcus* sp. were positive for indole, whereas the other isolates tested negative. These results were comparable to the study done by Khanthong *et al*., (2021) on test on methanogens ability to deaminate amino acids. He found that methanogens isolated on shrimp's base possessed the enzyme tryptophanase which hydrolyze tryptophan to pyruvate a major function of methanogens. Carbohydrate metabolism of isolates was

assessed by conducting tests involving Methyl red, Vogues Proskauer and Triple sugar iron test.

The test was positive for *Methanosaeta* sp. *Sulfolobus* sp. and *Methanomicrobium* sp. The results concur with the study done by Jaml & Ghalibi (2020) on methanogens isolated from cow dung which showed a positive result for *Methanosaeta* sp. and *Methanomicrobium* sp. and *sulfolobus* sp. when tested on carbohydrate metabolism. Citrate testing is used to assess a micro-organism's ability to utilize citrate as its primary carbon source.

The citrate negative test revealed that the isolates were unable to be acted upon by the enzyme citrase. *Methanothrix* sp. *methanosaeta* sp. and *Methanomicrobium* sp. showed a negative reaction to the test. *Methanosarcina* sp. *Methanospirrilium* sp. *Methanococcus* sp. and *Sulfolobus* sp. showed a positive test. Krzmarzick *et al*., (2018) on their study of diversity and niche of archaea in bioremediation found that *Methanosarcina* sp. *Methanospirrilium* sp. *Methanococcus* sp. and *Sulfolobus* sp tested positive on citrate reaction. The capacity of bacteria to swallow three sugars, glucose, lactose, and sucrose, as well as the formation of H_2S, which is the major function of anaerobic bacteria in biogas production, is evaluated using triple sugar iron. In the detection of glucose production all the identified isolate showed a positive reaction. While as they all showed a negative test reaction in lactose detection. Detection of H_2S *Methanosaeta* sp. *and Methanomicrobium* sp. showed a positive test reaction. Many organisms generate the enzyme catalase, which transforms hydrogen peroxidases into water and oxygen, creating foaming due to oxygen release. Except for *Methanosarcina* sp. *Methanothrix* sp. and *Methanosaeta* sp. all of the isolates showed a favorable response this concurs with the

study done by Semenova *et al.,* (2022) on their study on oil degrading methanogenic enrichment and reclassification found that *Methanosarcina* sp., *Methanothrix* sp. and *Methanosaeta* sp. were positive to catalase test.

5.1.3 Methanogenic bacteria from the sample bio-digesters

There were seven genera of bacteria isolated from the different bio-digester these were *Methanococcus* sp. *Methanomicrobium* sp. *Methanosarcina* sp. *Methanosaeta* sp. *Sulfolobus* sp. *Methanothrix* sp. and *Methanospirrilium* sp. these findings were similar to the work reported by Giongo *et al*., (2020) on their study on bacterial community in anaerobic digester of which they found that digester constitute diverse group of anaerobes including *Methanosaeta* sp. *sulfolobus* sp. and *Methanospiriilium* sp. The findings of having similar levels of abundance could have been contributed by the minimal distances between the sampling sites. Nierychlo *et al.,* (2020) on their study on anaerobic diversity found that distance between groups of taxa with comparable evolutionary histories shows the similarity between methanogenic anaerobes.

Total Shannon Weiner diversity of the bacteria was moderately high. In terms of sites, Radar had the highest diversity with seven genera recorded. The sites that had the lowest diversity were Energy and Langas. There was a significant difference in Shannon Weiner diversity between some sites. This could have been contributed by socio economic activities of an area whereby some farmers collect the cow dung together with the urine while others collect only the cow dung. The different feeds given to specific animals and the combination of different wastes from various domestic animals.

5.2 Physicochemical parameters of the anaerobic microbial community involved in biogas production in the different bio-digester within Uasin Gishu County

The present study looked at the moisture influence in biogas production. According to Seruga *et al*., (2020), water concentration is a crucial factor influencing solid waste anaerobic digestion. There are two fundamental reasons behind this first reason is that water enables bacteria to travel and grow, hence facilitating nutrient dissolution and transport and also water reduces the mass transfer limitation of non-homogeneous or particulate substrate. In overall, the moisture content of the slurry increased as the amount of volatile solid and total solid decreased. The kind of garbage determines the moisture content that must be maintained during the degrading process. Micro-organisms are categorized as per their optimal pH range (Ananthu, 2019; Buraimoh *et al*., 2020; Pramanik *et al*., 2019) and to maximize the CH_4 yield, pH typically varies from 6.85 to 7.2 with optimal values of 7.0 to 7.2.

The highest pH was recorded in Beta farm followed by Opande and Radar while the lowest was recorded in energy with no signficant difference. The difference in pH levels could have been attributed to different socio economic activities being carried in the areas.These activities include collection of the cowdung with the urine also mixed with other domesticated animal waste. The recorded temperature were highest in Radar and Nettos but lower in Energy with a notable significant difference ($p=0.0025$). Mean significant difference was between Beta farm and Langas,Nettos and Opande.

According to the research findings, the measured pH was largely within the permitted range for anaerobic digestion during the whole operation at mesophilic conditions. It denotes the mixed substrate's average buffering capacity. The pH levels are low during

the start of digestion; initially, the acid-forming bacteria will break down the organic matter and release volatile fatty acids. The methanogenic species are the most pH sensitive. An acidic pH might cause the sequence of biological events to halt during digestion, resulting in overall acidity of the digesting material; as a result, the pH will fall below neutral. This value enhances process stability and, as a result, the digester's proper operation. Bacterial species may live in a variety of environments.

The volatile solids and total solids ratio determine physical impediment caused by inorganic matter build up within the bioreactor. According to (Chae *et al*., 2011), the digester's volatile solids and total solids ratio is a good indicator of the buildup of undesired materials and the appropriateness of the mixing mechanism (Patel, 2017). As a result, these data indicate that the bioreactor is adequately mixed. Microbes may be responsible for the reduction in total solids and volatile solids. According to Hobbs *et al*., (2018), research, when methane emission increases, total solids and volatile solids decrease. Although there is still a propensity for further total solids and volatile solids decline with low or non-biogas generation, this is most likely due to the inherent scarcely biodegradable ingredients, and increased ammonia concentrations result in process inhibition. According to McVoitte (2018), the animal slurry employed in this study, such as cattle dung, contains lignocellulose rich components, rendering anaerobic digestion unsuitable.

The current study examined total solids for optimal gas generation. These findings were consistent with those Sun *et al*. (2017). The ideal solid concentration for biogas production was discovered to be between 7 % and 9 %. Another research Hagos *et al*., (2017), shows that there is no further increase in the volume of biogas generated at some

point when the % total solid increases. Furthermore, Dalkılıc & Ugurlu (2015) discovered that biogas production was unstable below a total solids level of 25 % (of manure) and more acidic than lower total solids concentrations in his study. Slurries with higher total solids concentrations were more acidic than those with lower total solids concentrations. The amount of methane produced is governed by the number of volatile compounds in the waste, the number of solids in the waste, and the degradability of the solids.

The current research found temperature variations in gas generation. Temperature has a significant impact on the anaerobic degradation process. Anaerobic digestion reactors are typically operated in the mesophilic (20°C to 42°C) and thermophilic (42°C to 75°C) temperature ranges. Temperature has little effect on the hydrolysis and acidogenesis processes. However, fewer specialized bacteria undertake the processes or stages of acetogenesis and methanogenesis; they are more temperature sensitive. The biogas unit's temperature varies from 32°C to 37°C. The temperature fluctuations inside the biogas unit were always larger than the ambient temperature, suggesting exothermic metabolism. The temperature within the digester has a big influence on the biogas generating process. Another research Kainthola *et al*., (2019), found that high ammonia concentrations are fatal to anaerobes, reducing digesting efficiency and upsetting the process.

5.3 Effective bacterial consortia for biogas production under laboratory conditions

From the findings, the highest amount of gas produced was from *Methanosaeta* sp. and *Methanococcus* sp. combined at a treatment of 30:500 for a period of between 21 to 30 days. It was observed that biogas production was slow at the start, and the fermentation process at a maximum of 20ml. This result corresponds to the study done by Fatima *et*

al., (2018) on biogas production on different wastes she found that the production of biogas was quite sluggish both at the start and the end of monitoring. This is because the pace at which methanogenic bacteria grow in a bio-digester under batch conditions is directly correlated with biogas production rate. In the ten days observation, in three proportions, 10:500ml, 20:500ml, and 30:500ml biogas production was less. This might be because most cows eat fibrous meals, which take longer for microbes to break down. This finding is similar with prior findings by Machado *et al*. (2021) indicates that the biogas-producing bacteria were in the lag phase of growth, during which cell changes take place. This outcome is similar to Zhou *et al*., (2021), whereas, in the range of 10 days of observation, biogas production increased substantially due to the exponential growth of the anaerobes and the seeded *Methanococcus* sp. and *Methanosaeta* sp. In this stage of the bacterial growth, the cells increase logarithmically and the cells divides at a maximum rate permitted by the composition of media and environmental conditions. This is also consistent with the findings of Fatima *et al.,* (2018), on her study on biogas production on various waste showed that the production of gas was postponed until the sixth day since it was thought that the micro-organisms were in their lag phase. Increase was observed in the second and third weeks, which was explained by the organisms' exponential phase, which is characterized by microbial growth and proliferation. The highest biogas production rate of 54 ml was attained on the 20^{th} day. This truth implies that the batch condition's biogas production rate is directly equal to methanogenic bacteria's specific growth.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

From the study, 7 different methanogens were identified which differed in both cultural, morphological and biochemical characteristics. These were *methanococcus, sulfolobus, Methanosaeta* sp., *Methanospirillium* sp., *Methanosarcina barkeri* sp., *Methanomicrobium* sp., *and Methanothrix* sp.

Study on the 6 bio digesters showed that physiochemical parameters play a paramount role in biogas production and should be maintained at an optimum range. The mean values for Temperature is 35.1^{o}C to 37.87^{o}C, pH is 6.86 to 7.2, volatile solids are 0.2 to 0.81, total solids are 8.4 to 9.25, and moisture content 11.82 to 13.84.

The study showed that predominant methanogens *Methanococcus* sp. and *Methanosaeta* sp.in consortium when inoculated into the bio-digesters increased the quantity of biogas produced followed by *Methanomicrobium* sp. and *Sulfolobus* sp.

6.2 Recommendations

The following recommendations were made following the findings of the study;

Anaerobic methanogens in bio-digesters operates best at a mesophilic temperature of 35.1^{o}C to 37.87^{o}C and at an alkaline pH of around 6.86 to 7.2. This must be kept constant and monitored regularly.

For efficient biogas production the consortium of *Methanococcus* sp. and *Methanosaeta* sp. proved to increase the quantity of biogas thus can be maintained as inoculum into the digesters.

Future study should be done on molecular characterization of the anaerobic genera to species level.

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APPENDICES

Appendix I: Media and reagents preparations

1.1 Methanogenic media

The composition of the methanogenic media for culturing *Methanogens archaea* was as follows, Ammonium Chloride 0.5g, Potassium di hydrogen phosphate 0.4g, Magnesium chloride 0.15g, Calcium chloride 0.05g, Sodium molybdate 0.01g, Biotin 0.01g, Resazurin 0.001g, cysteine 0.5g, Agar 14gms, pH 6.8 (15.62 g) dissolved to one-liter distilled water, sterilized by autoclaving at 121^0C for 15minute.

1.2 Motility media

The composition of the above media used to determine the motility of the anaerobes were as follows, Gelatin 10g, Beef extract 30g, NaCl 6g, Agar 4g (50gms) dissolved into 1lt of distilled water in a conical flask the pH was adjusted to 7.3 using a pH meter and autoclaved at 121^oC for 15min.

1.3 Kliger iron agar media

The above media is used to detects fermentation of sugars lactose, vs glucose with reduction of Sulphur and CO_2 production. Beef extract 3g, yeast extract 3g, peptone 15g, glucose 1g, lactose 10g, $Feso_4$ 0.2 g, $NathioS0_4$ 0.3g, Agar 12 g, phenol red 0.024g (44.52 g) was dissolved into 1ltr conical flask with distilled water and the pH adjusted to 7.2. autoclaved at 121^0C for 20min.

1.4 Simmons citrate agar

The above media is used to determine the utilization of citrate as a sole carbon source. Weigh Simmons citrate agar 24.28g and dissolve into 1ltr then autoclave at 121^0c for 20min.

1.5 MR-VP broth.

Used for methyl red and voges- proskauer test. peptone 7g, glucose 5g, K_2PO_4 5 g, the composition was dissolved in 1ltr distilled water and the pH adjusted to 6.9. it was autoclaved at 121^0C for 20min.

1.6 Tryptophan broth.

Tryptone 10 g is dissolved in distilled water and autoclaved at 121^0C for 20 min.

1.7 VP1 Reagent

Barritts reagent (5% alpha naphthol dissolved in absolute alcohol)

1.8 VP 11 Reagent

40 % KOH solution.

Appendix II: Similarity Report

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