ALKALINE AND BACTERIAL PRETREATMENT OF RICE STRAW AND WATER HYACINTH FOR BIOGAS PRODUCTION IN A BATCH BIOREACTOR

BY

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DEDICATION

To the glory of God Almighty, I dedicate this work to my late mum who could not see this piece come to accomplishment but played a major role at the unset of this work.

CERTIFICATION

This is to certify that this work "Alkaline and Bacteria pretreatment of Rice Straw and Water Hyacinth for biogas production in a batch bioreactor" was carried out by I Hans- Anukam, Uzunma Stephanie (20144940128) in partial fulfillment for the award of the degree of Ph.D. in Environmental Microbiology in the department of Microbiology of the Federal University of Technology Owerri.

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ABSTRACT

The Presence of lignin in lignocellulosic substrates greatly limits anaerobic digestion for production of biogas. The need for initial pretreatment to sufficiently remove it became inevitable. Alkaline and bacterial pretreatment methods were used to pretreat rice straw and water hyacinth samples for application in biogas production in a batch bioreactor. Optimum concentration of NaOH (%), mass of substrate (g) and duration (h) of the pretreatment were studied using Response surface methodology (Box Behnken design) Bacterial spp isolated from termite gut were identified by 16S rRNA gene sequencing and were used for biological pretreatment of the substrate. Each pretreated sample was co-digested with 1:1, 2:1 and 3:1 ratios of cow dung, pig waste and poultry droppings respectively, as amendments. Optimization studies on the biogas production process from the amendment that gave the highest yield in biogas was carried out. Proximate composition ,lignin, cellulose and hemicellulose content of the feedstock was determined by standard methods as well as microbial succession studies .Results obtained showed that 0.5M concentration of NaOH, 14g of sample and 39.5h exposure time were optimum of NaOH pretreatment of the samples which reduced lignin concentration from 17.4% to 8.3% and 17.4 % to 7.3% in bacteria pretreated samples while cellulose, reducing sugar and total sugar contents increased from 10.31% to 38.86%, 6.4%to 6.9% and 156.08% to 167.14% respectively, after chemical pretreatment of water hyacinth samples. Lignin and hemicellulose concentrations reduced from 18.01 % to 7.821 % and 11.01 % to 8.21%, while cellulose concentration increased from 10.31 % to 14.40 % after 30 days of bacterial pretreatment of water hyacinth sample. The 16S RNA gene analysis of bacterial consortium from termite gut indicated the presence of *Escherichia coli* and *Morgenela morganii* strain S4L2C (MH745964), with 100% and 98.6% similarity, respectively. There was a significant difference ($P \leq 0.05$) in biogas yield in all the Alkaline pretreated rice straw (APRS) and Bacteria pretreated rice straw (BPRS) amended with animal manure compared to APRS, BPRS and URS alone. For NaOH pretreated rice straw, best ratio of rice straw to cow dung was 2:1, which yielded 22.51dm^3 of biogas. For bacterial pretreated rice straw, 1:1 ratio of rice straw to cow dung was the best combination, which yielded 27.05dm^3 of biogas. For bacterial pretreated water hyacinth co-digested with cow dung, highest yield of biogas was recorded in 1:1 ratio, with an average of 12.03dm^3. Comparative analysis of biogas yield of APRS with BPRS with amendment at varying ratios gave 30.90% increase in biogas yield for APRS and BPRS with cow dung 2:1,45.45% for BPRS and APRS with cow dung 2:1 and 20.30% for APRS and BPRS with cow dung 3:1APRS/BPRS amended with poultry dropping at1:1,2:1and 3:1 recorded 25.01% ,42.01% and19.41% respectively while APRS/BPRS amended with pig waste at 1:1,2:1 and 3:1 recorded 1.43%, 3.05% and 16.37%.Result indicated that substrate concentration of 520g, Hydraulic Retention Time 22.57 days and cow dung content of 520 g were the optimum conditions with predicted biogas yield of 1.960 x10^4 mlfor bacterial pretreated rice straw. For NaOH pretreated rice straw, 216.7 g of substrate at HRT of 15.5 days and 520 g of cow dung as amendment were the optimum conditions, with predicted biogas yield of 1.517 x10^4 ml. Concentrations of components gases in biogas produced by rice straw/ cow dung 2:1 which produced highest volume of biogas in this study, were CO 1.149%, CO_2 13.556% and CH_4 64.960% .proximate characteristicsof digestate, such as nitrogen, phosphorus and potassium increased, while others including carbon, total solids and total volatile solid content decreased after anaerobic digestion. Finally, microbial load of slurry during digestion decreased during anaerobic digestion from 0, 14 and 28 days. These prove that rice straw and water hyacinth can be used for large-scale biogas production using Response surface methodology (BBD)from the use of bacterial isolate from termite gut which is significantly more effective in pretreatment than 6% NaOH.

Keywords: Lignocellulosic wastes, Animal manure, Pretreatments, Anaerobic digestion, Response Surface Model, Optimization and Biogas production

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Over the past century, the rapid rise in dependence on petroleum and its allied energy industries has contributed to rapid rise in the world economy. It has been under looked that the underlying energy resource in the form of fossil fuel can deplete, and therefore is a limited form of energy. It cannot be overemphasized also that combustion of these products contributes to emissions of greenhouse gases (e.g. fossil fuel-derived carbon dioxide (CO_2) emissions) which have become a global concern, since about 88% of global energy consumption is derived from fossil fuels (Achinas 2017). Therefore, developing and developed countries have made efforts towards overcoming the environmental challenges and overdependence on fossil fuels by increasing their proportion of renewable energy to between 20-40% by 2020 (Karmellos 2016). For instance, progress towards this target is measured every two years and was proposed on 30 November 2016 to reach at least 27% renewables in final energy consumption in the EU by 2030 (Scarlat 2018).

Among other renewable energy sources, biogas is seen as one of the most promising renewable energy resources that can replace part of the fossil fuel-based energy used today. It shows great potential and many advantages, including both climate and economic benefits (Meyer-Aurich 2016). Considering its accessibility to all, a biogas process can be implemented in small or large scale by the wealthy or poor, which is important when designing flexible and sustainable energy solutions in both industrialized and developing countries (Holm-Nielsen 2009) as opposed to fossil fuels. Substrates or raw materials usedin

the production of biogas can be sourced by rural dwellers which include but not limited to various types of waste products, such as manure, straw, municipal wastewater, food waste etc., and dedicated energy crops (Appels2011;Vasco-Correa 2018). This implies that if the technology is implemented, it can be a source of employment therefore reducing the poverty index of Nigeria which is currently on the increase (Ogujiuba, 2015). Among the substrates used in production of biogas, lignocellulosic materials, such as agricultural residues, are of great interest due to their high abundance and potential for biogas production (Azman 2015). Environmentally, adoption of biogas technology will effect controlled waste collection as opposed to dumping household waste in landfill or storing farm manure in open tanks. This can not only have a down surge on number of waste deposits, but also to decrease emissions of carbon dioxide and other greenhouse gases (Borjesson and Mattiasson, 2008).

Biogas production is a rich technology that employs complete diversification of organic waste into energy, economically and environmentally rich products. The Biogas produced, containing energy carrier methane, can be used for production of heat, electricity and vehicle fuel after upgrading (removal of carbon dioxide and trace gases) (Holm-Nielsen 2009). The residues left after biogas production are rich in mineral nutrients and can be used as fertilizer during crop production to replace fossil energy-requiring mineral fertilizers.Thus,enables recycling of nutrients between urban and rural areas (Holm-Nielsen 2009; Weiland, 2010; Möller and Müller, 2012; Vasco-Correa 2018).

The Bio-refineries of biogas technology are microorganisms which are vital in their consortium and their diversified metabolic capabilities for degrading the wastes or organic rich substrates into desired products. (De Francisci 2015). To obtain a stable biogas process, all these conversion steps and microorganisms must work in a synchronized manner (Vanwonterghem 2014). When plant-based materials (*e.g.* agricultural residues) are used for biogas production, the first step of microbiological process, hydrolysis, becomes rate-limiting.

This is the major limitation of use of cellulolytic materials since the crystalline structure of lignocelluloses obstructs degradation in the initial step, thus, slowing down hydrolysis of these insoluble compounds into lower polymers (Lynd 2002; Mulat and Horn, 2018;).

However, several efforts have been made to overcome the obstacle associated with the degradation of lignocellulosic and similar materials through adoption of several pretreatment methods that would expose the material and increase its accessibility to biological attack (Martínez-Gutiérrez, 2018). Similarly, engineering the microbial consortium is an alternative method which can be characterized by adding lignocelluloses degrading microorganisms to the batch digester. The quest for bacteria and fungi with lignocelluloses-degrading activities have been on-going in soil ecosystems (Lynd 2002; Tsavkelova and Netrusov, 2012; Ransom-Jones 2012; Do 2018)), with only a limited number having examined cellulose-degrading bacteria in biogas digesters (Yan 2012; Sun 2013; Bozan 2017;Jia 2018) Consequently, insufficient information is available on cellulose-degrading communities in biogas processes and on possibilities to enhance the degradation rate by 'microbial steering', *i.e*. by supporting the growth of highly efficient cellulose-degrading bacteria or communities. This study therefore seeks to employ mechanisms that would contribute to exposure of lignocelluloses to microbial attack through the adoption of several physicochemical and biological methods.

1.2 Statement of Problem

According to Adeleye and Okorondu (2015), energy and environmental sustainability are two most pressing needs in the world today. Available abundant energy in the form of fossil fuels are not sustainable, cheap, easily accessible and ecofriendly. There is therefore the need to explore other sources of energy with promising capabilities such as eco-friendliness, accessibility and cheaper cost. More researches have been directed towards the bioconversion of cellulolytic materials that are abundant in agro wastes (Braide 2016). Cellulose, trapped in

agricultural residues, has been accounted to be the most abundant organic compound on earth, estimated about 10^{11} tons of cellulose that are synthesized each year (Matsuoka 1996; Son 2002). This figure accounts for more than 50% of bound carbon on earth. According to US National Petroleum Council (2007) and Smeets *et al*. (2007), the estimated global annual production of biomass is 1×10^{11} tons, sequestering 2×10^{21} J of energy and when compared to annual petroleum production which amounts to 2×10^{20} J, the technically recoverable endowment of conventional crude oil is 2×10^{22} J (Smeets 2007). This means that in only one decade, Earth's plants can renew in the form of cellulose, hemicellulose, and lignin, all of the energy stored as conventional crude oil. Therefore, the challenge for scientists is to access these polymers and convert them into fuels and building blocks for civilization (US National Petroleum Council, 2007).

1.3 Aim and Objectives

The aim of this study is to evaluate the effect of alkaline and bacterial pretreatment of rice straw and water hyacinth on biogas production in a batch bioreactor system.

Specific objectives include the following;

i. To optimize effects of concentration of NaOH, mass of substrate and duration of pretreatment on chemical (NaOH) pretreatment of rice straw and water hyacinth, using Response Surface Model (Box Behnken design).
ii. To isolate and identify, using 16S RNA gene sequencing lignolytic bacteria inhabiting termites gut and scale up the inoculum for biological pretreatment of feed stock.
iii. To pretreat rice straw and water hyacinth substrates using inoculum raised from the isolated bacteria

iv. To determine the lignin, cellulose and hemicellulose contents of rice straw and water hyacinth substrates, before and after pretreatment by adopting standard methods.

v. To evaluate the effects of supplementation (co-digestion) of alkaline and bacterial pretreated feed stocks with different ratios of nitrogen sources on biogas production.

vi. To carry out optimization studies on biogas production from the most suitable substrate

vii. To study the variations inmicrobial load of slurries during anaerobic digestion.

viii. To determine the proximate compositions of slurry before and after anaerobic digestion.

1.4 Justification of Study

Several works have been done to isolate, characterize and identify isolates with cellulose - degrading systems, which is a component of this work. Similarly, the use of physicochemical pretreatment techniques wasalso adopted in this study. However, no available publication on biomethenation of biogas technologies adopted pretreatment of substrate with bacteria from termite gut for production of biogas and for improved yield.Also several combinations of different wastes from cellulosic plants and animal farms were used in the study as a waste management option.

1.5 Scope of study

This study will be restricted to the following boundaries:

1. Only rice straw and water hyacinth will be used as lignocellulosic biomass in this study. Cow dung, poultry dropping and pig wastewill serve as sources of nitrogen for co-digestion.

2. Only bacteria inhabiting guts of termites will be isolated and used to pretreat lignocellulosic biomass samples.
3. Box Behnken design (Minitab 17) will be used to design the study for optimization of effects of factors on pretreatment and biogas production.
4. Sodium hydroxide (NaOH) will be used as alkaline for chemical pretreatment of lignocellulose samples.

CHAPTER TWO

2.0LITERATURE REVIEW

2.1 INTRODUCTION TO BIOGAS PRODUCTION

The economy of every nation is dependent on its access to energy. People need access to energy supply to run various businesses, power machines, drive vehicles, cook food, light homes, etc. stated that Nigeria's heavy reliance on fossil fuel has made it vulnerable to fluctuations in price in the global oil market. This can be tagged as one of the reasons why the nation is still underdeveloped and its populace remains impoverished. Due to unstable prices of petrol, kerosene and cooking gas, many businesses have been shut down as they cannot keep up with the high operating cost as regards power; most homes now prefer cooking with firewood or charcoal which is not a healthy option due to the long term effects of inhaling smoke(Akinbomi *et al.,* 2014).

Nigeria is a large nation that should be doing better than other African countries in terms of power supply yet; many homes are off-grid and have to look for an alternative source of power. The nation produces enough waste every day that should be utilized in the production of biogas. Biogas technology has been available as far back as 1630 when Jan Baptista Van Helmont first tested the release of flammable gases from decaying organic matter (Khoiyangbam *et al.,* 2011). In 1776, Count Alessandro Volta experimented and found that the amount of inflammable gas produced had a direct correlation with the amount of decaying organic matter (Kothari *et al.,* 2010).

In 1808, Sir Humphrey Davy investigated that methane was available in the gases produced during the anaerobic digestion of cattle manure and collected 0.3 liter of methane from cattle manure (Khoiyangbam *et al.,* 2011). In 1875, Wouter Sluys, a Dutch farmer used methane

first for the purpose of illumination. In 1895, England recovered biogas by applying the sewage treatment and the same was used in street lamps (Fabien, 2003). During World War II, due to the shortage of crude oil, biogas was used as an alternate fuel to drive German trucks. After the war, biogas technology was not popular because of cheap crude oil availability. After the crude oil crisis in 1970s, research on biogas technology development picked up and again started taking part in the supply of energy for our daily usage (Khoiyangbam *et al.,* 2011).

Biogas can be produced from municipal solid waste, sewage, agricultural waste, and food waste. Lignocellulose materials such as agricultural plant residues which are widely available in Nigeria and other countries can also be used for the production of biogas. Biogas is a cheap, clean and renewable source of energy, which will also serve as a sustainable system of waste management.

2.1.1 BIOGAS PRODUCTION IN NIGERIA AND AFRICA

Nigeria, the largest country in Africa, is an oil exporting nation and rich in fossil fuels. Nigeria's economy revolves heavily around crude oil. This has in no small measure contributed to the economic growth and development of the nation. However, its negative effects on the health of the populace as well as the increasing concern on environmental impact of fossil fuel, coupled with the frequent fluctuations in price of fossil fuel products, has brought about increase in poverty of the populace. Most people who cannot afford fossil fuel products have resorted to use of firewood and charcoal for cooking their daily meals. The rising price of kerosene is making it less affordable daily and in many parts of Africa, firewood and charcoal is becoming scarce due to deforestation.

A lot of waste is being generated daily in Nigeria as a result of her large population. This includes municipal solid waste, sewage, agricultural waste, and food waste. Due to a lack of good waste management systems, these wastes are disposed in unhygienic ways that are even more harmful to health and environment. Wastes are littered on the streets and in gutters, burnt or dumped on a large expanse of land. However, these wastes can be utilized to generate clean and affordable energy for cooking, as vehicle fuel and lighting.Not much attention has been given to the development of biogas technology in Nigeria. Only few biogas pilot plants have been developed by different research centers (Sambo, 2005). Organizations and individuals are currently highlighting the need to promote production of biogas in Nigeria as a way of mitigating climate change. For farmers, biogas production help in solving the challenge of waste disposal as well as access to clean and affordable energy to power farm machineries.

In Abuja, the Federal Capital Territory of Nigeria, an agricultural firm, Ajima Farms, powered Rije village with 20 kilowatts of biogas from generators. This project called Ajima Farms Biogas Digester Off-Grid was inaugurated by the United States African Development Foundation (USADF). Furthermore, in Ondo state, the Federal College of Agriculture (FECA), Akure, Ondo State in collaboration with the West Africa Agricultural Productivity Program (WAAPP-Nigeria) empowered few farmers in Ibulesoro on generation of renewable electricity and cooking gas from cow dung (The Nation, 2018).Other biogas projects have been carried out. These include construction of biogas plants in Zaria Prison, Kaduna; Ojokoro in Lagos; Mayflower School, Ikene in Ogun State and at Usman Danfodio University, Sokoto with a digester capacity ranging between 10 and 20m^3. Many of these pilot projects are un-operational today and have failed to be commercialized. Its inability to penetrate the Nigerian energy market shows that biogas is not yet part of Nigeria's power supply options (Akinbomi *et al.,* 2014).

Sehgal (2018) pointed out that, access to electricity and clean cooking fuels is still a challenge in many African countries. This is so for both residential and commercial activities. Households in Sub-Saharan Africa (SSA) form the majority of over 2.7 billion people globally, that rely predominantly on traditional biomass as cooking fuel and only 38% of its population had access to electricity in 2014. Africa has a great opportunity to grant its populace access to energy owing to its vast biomass resources that can be used in the production of biogas. These resources are under-utilized. A review identified the critical barriers to commercial biogas production to include, high initial capital costs, weak environmental policies, poor institutional framework, poor infrastructure and a general lack of willpower to implement renewable energy policies and set challenging targets (Muyiwa *et al.*, 2018).

Some countries in the continent have made headway with small-scale or household biogas digesters but little effort has been made towards commercialization (Rupf *et al.*, 2016). Table 2.1 shows the number of household digesters in some African countries in 2007. Biogas technology in Africa has recorded very few successes as only few of the plants are operational (Akinbomi *et al.,* 2014).

Table 2.1: Number of household digesters in some African countries in 2007

Country	Number of household digester
Kenya	6749
Ethiopia	5011
Tanzania	4980
Uganda	3083
Burkina Faso	2013

Senegal	334
Cameroon	159
Benin	42

Surendra *et al.* (2014)

2.1.2 PROCESS PARAMETERS INVOLVED IN BIOGAS PRODUCTION

In order to achieve optimum efficiency in biogas production, the process should be properly overseen. This will allow for growth of various microorganisms as required for the entire process. There are many factors that affect the performance of an anaerobic digester. Process factors discussed in this study include some important monitoring parameters and some operating parameters that will ensure optimum performance. Monitoring parameters can serve as indicators of stability of the whole process, while operating parameters affect the growth of microorganisms within the anaerobic digester, which eventually affect overall performance and stability of the biogas process.

2.1.2.1 Parametersfor process monitoring

These parameters are necessary for finding reasons of process instability. They include quantity and composition of feedstock, biogas production and gas composition, fermentation temperature, TS (total solids)/DM (dry matter), pH value, and Ammonium nitrogen (NH_4-N). Consequently, there are parameters that give information on current stability of the process but may not often be the main reason. Such parameters include gas measurement (CH_4, CO_2, and H_2), volatile fatty acids (VFA), alkalinity ratio, and redox potential.

(a) pH: This is an expression ofintensity of the basic or acid condition of a liquid, a measure of the acidity of a solution. Commonly, methanogens in wastewater treatment systems are most active in the neutral pH range (7.0). The pH range suitable for most organisms is 6.0 – 9.0 (Adrien, 2008); beyond which, digestion can proceed but with less

efficiency. According to Chandra *et al.* (2012), this can result in unstable digester performance and sometimes even process failure. It is therefore important that the pH of digester be maintained between the required ranges as this parameter is very important in the production of methane gas. Reduction in pH can be controlled by addition of chemicals such as sodium carbonate, sodium bicarbonate, gaseous ammonia, ammonium hydroxide, lime, potassium and sodium hydroxide (Khanal, 2008).

(b) Volatile fatty acids (VFA): These are intermediates in methane formation pathway (Merlin *et al*, 2014). The levels of VFA within an anaerobic digester should be used as an indicator for monitoring the stability of digestion process. Acetic acid has been known as an important intermediate for overall anaerobic digestion process as it is directly related to the end products, methane and carbon dioxide (Wijekoon 2011). Studies have shown that higher concentration of acetic acid inhibited the degradation of propionic acid and inhibited the acetate-utilizing methanogenic bacteria .The accumulation of propionic acid might indicate the sign of disturbance in the process (Björnsson 2000 reported that accumulation of propionic acid is closely related to the concentration of hydrogen; therefore hydrogen concentration could be a possible parameter to monitor the accumulation of volatile fatty acid. Some studies have found that propionic acid should be treated as a toxic volatile fatty acid in anaerobic digester and the methanogenic bacteria have been shown to be vulnerable to propionic acid concentration greater than 1000 – 2000mg/L (Wijekoon 2011)

(c) Ammonium ($NH4^+$) It is a product of the anaerobic digestion of proteins and amino acids. Ammonium ion (NH^{4+}) exists in equilibrium with free ammonia (NH_3), and hydrogen ion (H^+), as seen in the equation below:

$NH^{4+} = NH_3 + H^+$

This equilibrium is a well-known inhibitor of the anaerobic digestion process (Chen *et al.*, 2008; Rajagopal *et al.*, 2013;Chen *et al.*, 2014). A review suggested that methanogens are

less tolerant to ammonia in the digestion process and the inhibition is believed to be caused by a proton imbalance and/or a potassium deficiency within the cell (Niu 2014). Methanogenesis is being overtaken by syntrophic acetate oxidation which is an alternative pathway during ammonia stress (Schnürer and Nordberg, 2008, Westerholm, 2012; Sun*et al.*, 2014.) In order for this to occur and to avoid instability of the process, a slow increase in ammonia concentration and a long acclimation period are suggested (Westerholm, 2012). The ammonium/ammonia equilibrium is affected by the pH and temperature, with a shift towards free ammonia with increasing temperature and pH. Ammonia and temperature have been suggested to be the main parameters surrounding the anaerobic digestion by bacterial community (De Vrieze 2015).

Hydrogen sulphide can either be produced from amino acids cysteine and methionine or by sulphate-reducing bacteria reducing sulphate, present in the process (Moestedt 2013). Hydrogen sulphide within the anaerobic digestion process can cause inhibition, either directly or indirectly by precipitate trace metals (Ramírez 2011). Trace metals are needed for enzymes involved in methanogenesis (Glass and Orphan, 2012) and limited availability may affect overall process performance (Demirel and Scherer, 2011). In addition, hydrogen sulphide is corrosive and causes bad odour; all of which makes hydrogen sulphide a highly undesirable compound present in digester and in the biogas. In practice, iron can be supplied to the process to reduce the level of hydrogen sulphide by precipitation (Nordell 2015).

(2.1.2.2) Parameters for Process Operation

(a) Hydraulic retention time (HRT) is also known as hydraulic residence time. It is a measure of average length of time that a soluble compound remains in a constructed bioreactor. In simple terms, it is the average time that the slurry remains in a biogas digester

(Yadvika *et al.*, 2004), hydraulic retention time is the volume of the aeration tank divided by the influent flow rate: as shown in the equation 2.1

$$HRT\ (d) = \frac{volume\ of\ aeration\ tank\ [m^3\ ]}{influent\ flow\ rate\ [^{m^3}/_{d}]} = \frac{V}{Q} \text{.....Equation 2.1}$$

Where HRT is hydraulic retention time (d) and usually expressed in hours (or sometimes days), the V is volume of aeration tank or reactor volume (m), and Q is influent flow rate (m/d).

For a continuously stirred tank reactor, the hydraulic retention time is the same as solid retention time. In general, the minimum HRT should be longer than the retention time requires a larger volume of reactor at a particular organic loading rate. A typical HRT is 15-30 days under mesophilic conditions and slightly shorter under thermophilic conditions (Braun 2010 and Mao 2015). HRT is a good operational parameter that is easy to control and also a macro-conceptual time for the organic material to stay in the reactor.

(b) Organic Loading Rate (OLR) is an important operating parameter which affects the biogas production in anaerobic digestion, especially when the digestion takes place in continuous flow mode (Abbasi *et al.*, 2012). It can be defined as the amount of organic material (volatile solids, VS) fed daily per liter of digester volume (g VS/L/day). When sewage sludge is used as the main substrate, the OLR typically ranges from 1.2 to 8.9 g VS/L/day (Mata-Alvarez *et al.*, 2014). During co-digestion of cattle manure with other organic wastes, such as municipal waste or crude glycerol, OLR up to 5.5-7.3 g VS/L/day have been reported (Mata-Alvarez *et al.*, 2014). For mono- or co-digestion of lignocellulose materials, reported OLR are typically lower, around 1.5-3.5 g VS/L/day (Ziganshin 2013; Lebuhn 2014;Lucas 2015).

An increase in OLR usually results in an increase in total methane yield. However, if a certain OLR value is exceeded, the process can be unstable and process failure may even occur (Mata-Alvarez *et al.*, 2014). According to Khoiyangbam (2011), overloading digester can easily affect digestion process as a result of acid accumulation. Optimum loading rate is in between 0.5 kg and 2 kg of total volatile solids per unit volume of the digester per day which can be chosen based on type of raw material, retention time and the process temperature.

In natural environments, activity of methanogens has been detected within a wide temperature range, from nearly 0 °C to over 100 °C. Constructed digesters are commonly run either at mesophilic temperature (~37 °C) or thermophilic temperature (~55 °C). In the thermophilic temperature range (45-55 °C), the reaction typically proceeds much faster than under mesophilic conditions (25-40 °C), in general allowing higher OLR compared with digesters operating at lower temperature. Furthermore, at higher temperatures pathogens are removed at higher efficiency, which leads to a more sanitary end product. On the other hand, mesophilic digestion requires lower energy input for heating and is commonly more stable and less affected by inhibition, *e.g*. ammonia inhibition (Chen 2008).

There are distinct merits and demerits of using various substrates for anaerobic digestion. For instance, digestion of animal manure or slaughterhouse waste may lead to ammonia inhibition due to the high nitrogen content. Plant residues, on the other hand, lack nitrogen and are also low in trace elements (Mata-Alvarez 2014;Sawatdeenarunat 2015). Therefore co-digestion has been proposed as a solution in this regard. Careful selection of the co-substrates and blending them in a ratio, synergizes the process, dilutes harmful compounds and optimizes methane production and digestate quality (Mata-Alvarez 2014).

In a study conducted by Li (2015), wheat straw was evaluated as a substrate in co-digestion with cattle manure, in different ratios. Wheat straw has a C/N ratio of almost 100, which is

far from the 15-30 suggested as optimal for anaerobic digestion (Chandra 2012). Co-digestion with cow manure gave a more optimal substrate mix in this regard but, interestingly, a stable process was observed even with a C/N ratio as high as 75.

2.2 ANAEROBIC DIGESTION

Anaerobic digestion is a biochemical process during which complex organic matter is decomposed in absence of oxygen, by various types of anaerobic microorganisms, anaerobic digestion of organic waste is of increasing interest as it offers an opportunity to deal with some of the problems regarding the reduction of the amount of organic waste, while diminishing the environmental impact and facilitating a sustainable development of the energy supply. Anaerobic digestion has been applied in agricultural sector at animal feeding operations and diaries to alleviate some of the impacts of manure and for energy production (Rapport *et al.,* 2008). Majority of these anaerobic digestion systems in operations are single stages (one stage), where all biological reactions occur within a single reactor or holding tank. Studies have shown that two-stage anaerobic digestion have great advantages over the single-stage digestion as a more rapid and more stable treatment can be achieved .

In practice however, it has been argued that two-stage digestion has not been able to validate its claimed advantages in the market, and the added benefits in increasing rate of hydrolysis and methanization have not been confirmed Owing to the fact that the solids can be introduced into the system at an acceptable concentration (this includes new installations and retrofits), anaerobic digestion systems are thus considered to be appropriate for all wastewater treatment systems. Most of the existing research on anaerobic digestion is aimed at retrofitting multi-stage systems into facilities where single-stage processes are already present. The concentration of the feed solids is the most important factor in determining whether a multi-stage anaerobic digestion process is achievable for a system. Given that a

multi-stage process could be sensitive to variation in feed solids, it might not be practicable if the characteristics of feed solids concentrations fluctuate extensively (US EPA, 2006).

There is an abundant availability of cellulose-based waste, which could be appropriate for biogas production such as lignocelluloses and waste textiles. These materials are carbohydrate-rich and could be used as a substrate for biogas production. However, they are very difficult to digest due to their reluctant nature, as their structure opposes microbial hydrolysis in biogas production. Today, the application of lignocellulosic materials in biogas production is limited and for waste textiles, it is nonexistent (Jeihanipour, 2011; Teghammar, 2013).

2.2.1 THE ANAEROBIC DIGESTION PROCESS AND ITS COMPLEXITIES

Anaerobic digestion as opined by Aslanzadeh (2014) can be considered to be a complex process as the digestion itself consists of a number of biochemical reactions which is based on a reduction process that takes place under anoxic conditions. Anaerobic digestion takes place in four stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The chemical reactions and the bacteria involved in all the four stages are given in Table 2.2 and the process flow chart is shown in Figure 2.1. However, the anaerobic digestion process can be divided into two stages as shown below:

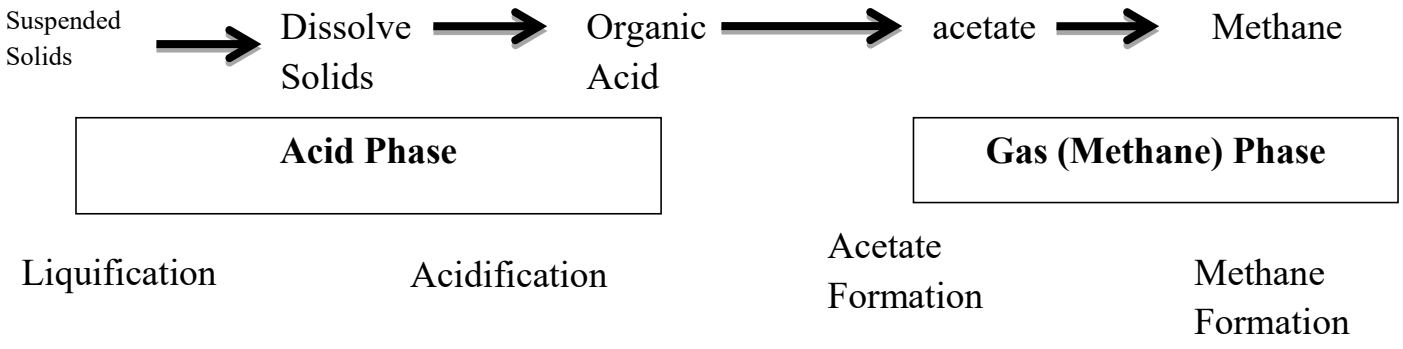


Figure 2.1: phase separation of the anaerobic digestion system.

Various microorganisms are involved in degradation process at each stage. They are distinct in terms of physiology, nutritional needs, growth kinetics, and sensitivity to environment. Demirel and Yenigun (2002) revealed that striking a delicate balance between these two groups - the acid forming and the methane forming microorganisms – is often difficult and can lead to reactor instability and consequently low methane yield.

Table 2.2: Chemical reaction and bacteria involved in the anaerobic digestion

Stage	**Type of conversion**	**Bacteria involved**
Stage-I Hydrolysis $(C_6H_{10}O_5)n+nH_2O$ $n(C_6H_{12}O_6)$	Proteins to soluble peptides and amino acids	*Clostridium, Proteus vulgaris, Vibrio, Bacillus, Peptococcus, Bacteriodes*
	Carbohydrates to soluble Sugars	*Clostridium, Acetovibrio celluliticus, Staphylococcus, Bacteriodes*
	Lipids to fatty acids or Alcohols	*Clostridium, Micrococcus, Staphylococcus*
Stage-II Acidogenesis	Amino acids to fatty	*Lactobacillus, Escherichia, Bacillus, Staphylococcus,*

$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2+CO_2$ $C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$ $C_6H12O_6 \rightarrow CH_3CH_2CH_2COOH + 2H_2+ 2CO_2$ $C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$ $C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH$	acids, acetate and NH_3 Sugars to intermediary fermentation products	*Pseudomonas, Sarcina, Desulfovibrio, Selenomonas, Streptococcus,Veollonella, Desulfobacter, Desulforomonas.* *Clostridium, Eubacterium limosum, Streptococcus*
Stage III Acetogenesis $CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2H_2$ $2CH_3CH_2OH + 2CO_2 \rightarrow CH_4 + 2CH_3COOH$ $CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + 3H_2 + CO_2$ $CH_3CH_2CH2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2$ $CH_3CHOHCOOH + H_2O \rightarrow CH_3COOH + CO_2 + 2H_2$	Higher fatty acids or alcohols to hydrogen and acetate Volatile fatty acids and alcohols to acetate or hydrogen	*Clostridium, Syntrophomonas Wolfeii* *Syntrophomonas wolfei, Syntrophomonas wolinii*
Stage IV Methanogenesis $CH_3COOH \rightarrow CH_4 + CO_2$ $CO_2 + 4\ H_2 \rightarrow CH_4 + 2H_2O$	Acetate to methane and Carbondioxide Hydrogen and Carbondioxide	*Methanosaeta, Methanosarcina* *Methanobacterium formicicum,*

to methane	*Methanobrevibacterium, Methanoplanus, Methanospirilum*

(Nayono, 2009; Abbasi *et al.,* 2012)

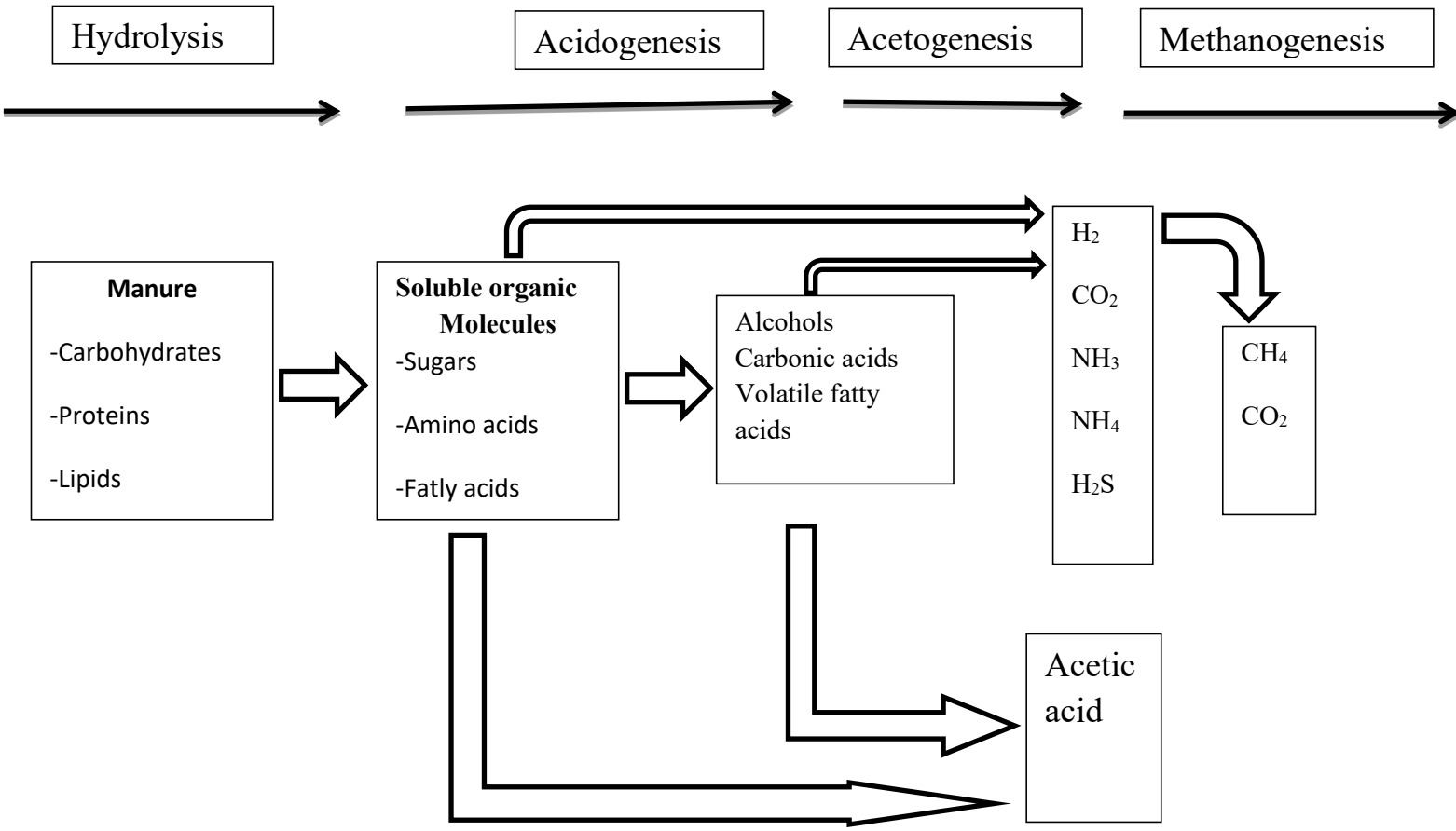


Figure 2.2: Flow chart for stages of anaerobic digestion process Girard *et al.*(2013)

2.2.1.1 Hydrolysis

This is the first stage in anaerobic digestion process. It involves conversion of complex organic polymers into simple soluble molecules. This step is carried out by strict anaerobes such as bacterides, clostridia and facultative bacteria such as streptococci etc (Merlin *et al.,* 2014). This first stage is considered as very important because large organic molecules are

rather too large to be directly absorbed and used by microorganisms as substrate/food source. In order to achieve biodegradation, some microorganisms need to secrete different types of enzymes called extracellular enzymes that will break down the large molecules into smaller pieces that microorganism can then take into the cell and use as sources of energy and nutrition.

According to Schnurer and Jarvis (2009), some microorganisms secrete several different enzymes, which allow them to break down different types of organic materials. Other microorganisms are specialized on just a type of organic material. For example, they secrete enzymes that break down either sugar or protein. Microorganisms that break down different sugars are called saccharolytic, while those that break down proteins are called proteolytic. There are different enzymes for sugars, proteins, fats etc. Table 2.3 below shows examples of some groups of extracellular enzymes. Each group contains several enzymes that are specialized in various substrates, such as different proteins. In the hydrolysis stage, the nature of the substrate determines the rate of decomposition.

Table 2.3:Some important groups of hydrolytic enzymes and their functions

Enzymes	**Substrates**	**Breakdown products**
Proteinase	Proteins	Amino acids
Cellulase	Cellulose	Cellobiose and glucose
Hemicellulase	Hemicellulose	Sugars, such as glucose, xylose, mannose and arabinose
Amylase	Starch	Glucose
Lipase	Fats	Fatty acids and glycerol
Pectinase	Pectin	Sugars, such as galactose,

	arabinose and polygalacticuronic acid

(Schnurer and Jarvis, 2009)

2.2.1.2 Acidogenesis

The products of hydrolysis are further degraded into short chain organic acids such as butyric acids, propionic acids, acetic acids, alcohols, hydrogen and carbon dioxide. The acetic acid formed in this stage is directly taken to last stage and the other products are taken to third stage for further degradation by acetogens (Deepanraj *et al.,* 2014). Hydrogen, one of the intermediate products of acidogenesis has the ability of influencing the type of final product of fermentation process. This final product is dependent on the concentration of hydrogen formed. For example, if the partial pressure of the hydrogen is too high, there would be decrease in amount of reduced compounds. In general, during this phase, simple sugars, fatty acids and amino acids are converted into organic acids and alcohols.

2.2.1.3 Acetogenesis

The products which cannot be directly converted to methane by methanogenic bacteria are converted into methanogenic substrates. Volatile fatty acids and alcohols (VFA) are oxidized into methanogenic substrates like acetate, hydrogen and carbon dioxide. With the help of acetogens, volatile fatty acids having more than two carbon atoms (from acidogenesis stage) are converted into acetic acids, hydrogen and carbon dioxide (Al Seadi *et al.,* 2008).

2.2.1.4 Metanogenesis

Methane and carbon dioxide are produced under strict anaerobic conditions through the action of methanogenic bacteria (methogens). Methanogenesis is a critical step in the entire anaerobic digestion process, as it is the slowest biochemical reaction of the process (Al Seadi *et al.,* 2008).

2.2.2 MICROBIOLOGY OF ANAEROBIC DIGESTION

There exists a complex microbial community within the anaerobic digester. This structure plays a very important role in the formation of biogas. Anaerobic breakdown of organic matter is done by various bacteria species and archeae. There is a coordinated interaction among these microbes and if any organism is inhibited, there may be a failure in the anaerobic digestion process. This anaerobic digestion takes place in four stages namely: hydrolysis, acidogenesis, acetogenesis and methanogenesis.

2.2.2.1 RESPONSE TO FREE MOLECULAR OXYGEN

Bacteria may be further classified into three groups. These groups are 1) strict aerobes, 2) facultative anaerobes, and 3) anaerobes, including the methane-forming bacteria.

(1) Strict aerobes are active and degrade substrate only in the presence of free molecular oxygen. This implies that strict aerobes will die in an anaerobic digester in which free molecular oxygen is absent.

(2) Facultative anaerobes are active in the presence or absence of free molecular oxygen. If present, free molecular oxygen is used for enzymatic activity and the degradation of wastes.

(3) Anaerobes become inactive when there are free molecular oxygen in the anaerobic digester. They are divided into two subgroups: oxygen-tolerant species and oxygen-intolerant species or strict anaerobes. Some anaerobes are strong acid producers, such as, *Streptococcus* spp., whereas other anaerobes, such as *Desulfomarculum* spp., reduce sulfate (SO_4^{2-})to hydrogen sulfide (H_2S). Although oxygen-tolerant anaerobes survive in the presence of free molecular oxygen, these organisms cannot perform normal cellular activities, including the degradation of substrate, in the presence of free molecular oxygen.

Table 2.4: Groups of bacteria according to their response to free molecular oxygen

Group	Example	Significance
Strict aerobes	Haliscomenobacter hydrossis	Degrades soluble organic compounds; contributes to filamentous sludge bulking
	Nitrobacter sp.	Oxidizes NO_2^- to NO_2^-
	Nitrosomonas sp.	Oxidizes NH_2^+ to NO_2^-
	Sphaerotilus natans	Degrades soluble organic compounds; contributes to filamentous sludge bulking
	Zoogloea remigera	Degrades soluble organic compounds; contributes to floc formation
Facultative anaerobes	Escherichia coli	Degrades soluble organic compounds; contributes to floc formation; contributes to denitrification or clumping
	Bacillus sp.	Degrades soluble organic compounds; contributes to denitrification or clumping
Anaerobes	Sesulfovibrio sp methanobacterium formicium	Reduces SO_2^{2-} to H_2S Produces CH_4

(Gerardi, 2003)

Table 2.5: Group of anaerobic bacteria

Group	Example	Significance
Oxygen tolerant	Desulfomarculum sp.	Reduces SO to H_2S
	Desulfomarculum sp	Reduces SO to H_2S
Oxygen intolerant	Methanobacterium formicium	Produces CH_4
	Methanobacterium propionicium	Produces CH_4

(Gerardi, 2003)

2.2.2.2 ENZYMATIC ABILITY TO DEGRADE SUBSTRATE

There are two types of enzymes that degrade organic matter in anaerobic digester. These are; endoenzymes and exoenzymes.

(a) Endoenzymes are produced in the cell and degrade soluble substrate within the cell while exoenzymes are also produced in the cell but are released through the "slime" coating the cell to the insoluble substrate attached to the slime. Once in contact with the substrate, the exoenzyme solubilizes particulate and colloidal substrates. Once solubilized, these substrates enter the cell and are degraded by endoenzymes (Gerardi, 2003).

With respect to the type of substrate to be degraded, bacteria can be divided into three groups:

(i) The acetate-forming (acetogenic) bacteria,

(ii) The sulfate-reducing bacteria, and

(iii) The methane-forming bacteria.

In the presence of sulphate, methanogens and acetogens multiply, often requiring hydrogen and acetate, which are the substrates utilized by methanogens (Gerardi, 2003). Owing to the fact that both groups need hydrogen, there is always a competition between the two bacterial groups for hydrogen. In such situation sulfate reducing bacteria reap hydrogen and acetate more effortlessly than methanogens (Gerardi, 2003). Hydrogen sulphide produced by sulfate reducing bacteria on degradation of sulphate exhibits inhibitory effects, at low levels, on methanogens and acetogens than on acidogens. Synergistic relationships exist between acetogens and methanogens for methane production. As a result of digestion, microorganisms metabolize fatty acids and alcohols during which syntrophic bacteria produce Adenosine Triphosphate (ATP) (Ziemiński and Frąc, 2012). Methanogens then utilize these compounds after being converted into acetate and hydrogen. *Syntrophomonas* genus produces acetate, hydrogen and CO_2 upon oxidation of organic acids, which are used by methanogens (Yamada,

2006). This syntrophic association of methanogens and acetogens play a role in oxidation of propionate, which is likewise a vital phase of methanogenesis process (Demirel and Scherer, 2008). Another kind of symbiosis is seen between methanogens and bacterial group, which is mostly sulfate reducing bacteria belonging to *a*sub division of *Proteobacteria*.

2.2.2.3 FACTORS INFLUENCING THE ANAEROBIC DIGESTION PROCESS

There are many factors that can influence anaerobic digestion process. They may be physical, chemical or biological. A change in temperature, pH and/or amount of substrate fed in to digester can disrupt the stability required for gas formation.

1. Temperature

This is a very important parameter in anaerobic digestion process. According to Nijaguna, (2011), different species of methogens function optimally in three different temperature ranges: 45–60°C thermophilic, 20–45°C mesophilic and below 20°C psychrophilic. The rate of biogas production increases with an increase in temperature. In biogas digestion process, only mesophilic and thermophilic temperature ranges are considered important because anaerobic digestion reaction essentially stops below 10°C. Influence of temperature on rate of anaerobic digestion process is shown in figure 2.4. Methanogens are very responsive to fluctuations in temperature. Hence, rapid change in operating temperature should be avoided.

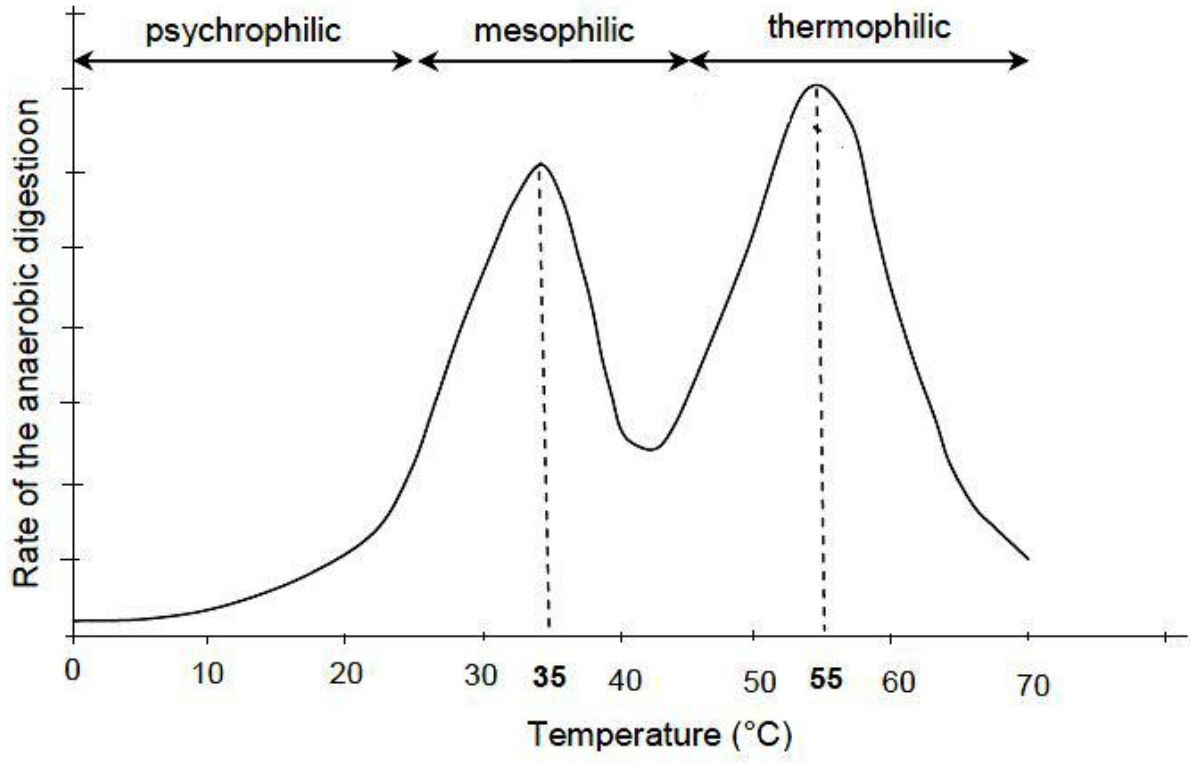


Figure 2.4: Influence of temperature on the rate of anaerobic digestion (Mata, 2002)

2. pH

This is a very important factor that should be considered in anaerobic digestion process. The performance and growth of certain organisms in digester is highly dependent of the pH of the digester. The pH of digester can be maintained at a desired range (7.0–8.5), by feeding the system at an optimal organic loading rate (OLR). According to Solmaz (2014), at the start of a biogas process, volatile acids are produced due to drop in the pH of digester below 6.0. The volatile acids formed can inhibit methanogens, because they are very sensitive to acid conditions. Reduction in pH can be controlled by addition of chemicals, such as sodium carbonate, sodium bicarbonate, gaseous ammonia, ammonium hydroxide, lime, potassium and sodium hydroxide (Nijaguna, 2012).

3. Volatile Fatty Acids

Volatile Fatty Acids, as important intermediates in anaerobic digestion process, exist in two forms. They can be dissociated or un-dissociated. The dissociated form dominates at a high pH level while the un-dissociated dominates at a lower pH. The levels of VFA within an anaerobic digester should be used as an indicator for monitoring the stability of digestion process.

4. C/N ratio and ammonia

C/N ratio is the relationship between the amount of carbon and ammonia that exist in a raw material. If C/N ratio is too high, microorganisms will quickly consume the nitrogen to meet their protein requirements and will no longer take care of available carbon content of the material, which will in return, reduce gas production. On the other hand, if C/N ratio is too low as a result of protein degradation and other nitrogenous materials, nitrogen will be released and built up in the form of ammonium ion (NH^{4+}) or ammonia (NH_3) in the system (Chandra *et al.,* 2012). A C/N ratio of 20 – 30 is considered to be appropriate for optimization of gas production in the anaerobic digestion process. The typical C/N ratios for different organic materials available are shown in Table 2.6.

Table 2.6: Typical C/N ratios of different materials

Materials	**N (%)**	C/N ratio
Cow, buffalo, sheep, pig and horse Manure	1.4 – 3.8	15 – 40
Poultry manure	6.3	5.2
Night soil	6	6 – 10
Fish scarps	6.5	5.1

Slaughter house waste	7 – 14	2 – 4
Sawdust	0.1 – 0.25	200 – 511
Grass clipping and hay	2 – 4	10 – 20
Bagasse, Wheat and rice straw	0.3 – 0.5	120 – 150
Corn stalks	0.8	60
Kitchen vegetable scraps	3.3	16

Nijaguna(2012) and Khoiyangbam *et al.* (2015)

5. Substrates

All biodegradable biomass materials are well suitable for anaerobic digestion. However, the type of feed material used directly affects the amount and composition of gas that will be produced. Substrates can be categorized into agricultural wastes and crop residues, animal wastes, aquatic waste, forest residues and municipal solid wastes. Table 2.7 shows a detailed list of feeds under each category.

Table 2.7: Feedstock for anaerobic digestion

Category	Types of waste
Agricultural wastes and crop residues	Crop Stubbles, straw, spoiled fodder, sugar cane trash and bagasse, weeds, tobacco waste, rice and coffee husks, fruit and vegetable processing wastes, oil cakes, etc.
Animal wastes	Cattle dung, goat and sheep manure, pig manure, elephant dune, fishery waste, slaughter house wastes.
Aquatic plants	Algae, sea weeds, water hyacinth, etc.
Forest residues	Dead trees, plants, twigs, barks, roots, leaves, etc.

(Khoiyangbam *et al.,* 2015)

6. Limiting factors in anaerobic digestion

Practical experience has shown that several factors can contribute to the failure of an anaerobic digestion process. These factors include: microbiological limitations which automatically affects the microbial community (*e.g.* ammonia inhibition, trace element insufficiency, etc.) or technical weaknesses of the equipment, such as insufficient mixing caused by the inappropriate particle size or rheological limitations (Braun *et al.,* 2010).

7. Organic loading rate (OLR)

Organic loading rate (OLR) is an important parameter which affects biogas production in anaerobic digestion, particularly when digestion takes place in continuous flow mode (Abbasi, 2012). It can be expressed as the amount of raw material (kg of volatile solids) fed to digester per unit volume per day. Due to accumulation of acids in digester, overloading the digester can easily affect digestion process. The Optimum loading rate is in between 0.5 kg and 2 kg of total volatile solids per unit volume of the digester per day, which can be chosen based on type of raw material, retention time and process temperature (Khoiyangbam *et al.,* 2011).

8. Retention time

Retention time is the period within which organic material stays inside the digester for biogas generation. Retention time varies with respect to the type of feedstock and the temperature used. Shorter retention times are usually preferred in order to reduce system cost. Retention time is usually expressed as: hydraulic retention time (HRT), which states the approximate time that the liquid sludge remains in the digester, and solid retention time (SRT), which is the time that the microorganisms/solids spend in the digester (Appels *et al.,* 2008). Solids retention time (SRT) and hydraulic retention time (HRT) are two significant retention times in anaerobic digestion process. Upon removal of sludge (mixture of biomass solids and water) from the digester, a portion of bacterial population is also removed. Since methanogenic microorganisms have a significantly longer generation time compared to hydrolytic and acid

forming microorganisms, shorter HRTs would cause a washout of slow growing biomass from the system, which would ultimately jeopardize the process stability and decrease conversion efficiency of the process (Chandra *et al.,* 2012). However, the risk for washout of microorganisms from the system can be prevented with phase separation.

2.2.2.4 PHASE SEPARATION

Anaerobic digestion can be divided into two phases, which are acid phase and gas phase. The Microorganisms involved in each stage differ with respect to physiology, nutritional needs, kinetic growth and sensitivity to the environment. Often times, it is difficult to maintain a balance between acid forming and methane forming microorganisms. Demirel and Yenigün (2002) revealed that such imbalance can lead to instability of the digester and results low methane yield. However, the separation of acid forming and methane forming microorganisms was first proposed with the aim of utilizing the difference in their growth kinetics. Techniques such as membrane separation, kinetic control, and pH control can be used in achieving phase separation (Cohen *et al.,* 1979).

The two-phase process holds a number of potential advantages. These include:

(i) Allowance for a decrease in the total reactor volume.

(ii) Appropriate control of the acidification, which improves the stability due to the abundance of heterogeneous bacterial population.

(iii) The process would tolerate organic and hydraulic overloading and fluctuations, as the first-phase will function as a metabolic buffer; and

(iv) Toxic materials and substances that can affect the more sensitive methanogenic microorganisms will be eliminated in the first phase.

2.3 BIOGAS FROM LIGNOCELLULOSIC MATERIALS

Biogas has been presented as a solution to global warming, as well as numerous impacts of fossil fuels to the environment. It can be produced using materials such as agricultural waste, manure, plant material, municipal waste, sewage, and food waste. Among these material sources, lignocellulosic biomass is an abundantly available carbon-rich and land-based feedstock, which can improve the independency on gas and oil (Pickett *et al*., 2008). Lignocelluloses are carbohydrate rich materials and can be found in abundance worldwide. Lignocelluloses have been estimated to account for approximately 50% of biomass in the world, and to have a yearly production of about 200 billion tons per year (Zang, 2008).

2.3.1 STRUCTURE OF LIGNOCELLULOSIC BIOMASS

Lignocellulosic biomass consists of three polymers: cellulose, hemicellulose and lignin, which correspond to approximately 90% of the total dry matter (Rubin, 2008). Apart from the three major components, some other compounds as ash, pectin and proteins are also presented in smaller amounts. Depending on the type of lignocellulosic biomass, these polymers are organized in complex, non-uniform and three-dimensional structures to different degrees and varying relative composition.

1. Cellulose

This is the major component of lignocellulose cell walls representing 17-50% of the total organic matter (Gnansounou and Dauriat, 2010; Mutschlechner *et al*., 2015). Its structure consists of extensive intramolecular and intermolecular hydrogen bonding networks, which tightly binds the glucose units. Since about half of the organic carbon in the biosphere is present in the form of cellulose, the conversion of cellulose into fuels and valuable chemicals has a paramount importance (Zhou *et al.,* 2011).

2. Hemicellulose

Unlike cellulose, hemicellulose has a random and amorphous structure, which is composed of several heteropolymers including xylan, galactomannan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan. The heteropolymers of hemicellulose are composed of different 5- and 6-carbon monosaccharide units; pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and acetylated sugars. Hemicelluloses are imbedded in plant cell walls to form a complex network of bonds that provide structural strength by linking cellulose fibres into microfibrils and cross-linking with lignin (Agbor *et al.,* 2011).

3. Lignin

Lignin is the most abundant non-polysaccharide organic matter and commonly the second most abundant organic polymer in lignocellulosic biomass (Jørgensen *et al*., 2007; Zheng *et al*., 2014). Lignin is a three dimensional polymer of phenylpropanoid units. It serves as the cellular glue which gives compressive strength to the plant tissue and the individual fibers, stiffness to the cell wall and resistance against insects and pathogens.Cellulose, hemicellulose and lignin are not uniformly distributed within the cell walls. The structure and quantity of these plant cell wall components vary according to species, tissues and maturity of plant cell wall (Barakat *et al.,* 2013). Generally, lignocellulosic biomass consists of 35–50% cellulose, 20–35% hemicellulose, and 10–25% lignin. Proteins, oils, and ash make up the remaining fraction. Table 2.8 summarizes particular types of lignocellulosic biomass and their chemical composition.

Table 2.8:Types of lignocellulosic biomass and their chemical composition

Lignocellulosic biomass		**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**
Hard wood	Poplar	50.8-53.3	26.2-28.7	15.5-16.3
	Oak	40.4	35.9	24.1
	Eucalyptus	54.1	18.4	21.5
Soft wood	Pine	42.0-50.0	24.0-27.0	20.0
	Douglas fir	44.0	11.0	27.0
	Spruce	45.5	22.9	27.9
Agricultural waste	Wheat hull	35.0-39.0	23.0-30.0	12.0-16.0
	barley hull	34.0	36.0	13.8-19.0
	Barley straw	36.0-43.0	24.0-33.0	6.3-9.8
	Rice straw	29.2-34.7	23.0-25.9	17.0-19.0
	Rice husk	28.7-35.6	12.0-29.3	15.4-20.0
	Oat straw	31.0-35.0	20.0-26.0	10.0-15.0

	Ray straw	36.2-47.0	19.0-24.5	9.9-24.0
	Corn cobs	33.7-41.2	31.9-36.0	6.1-15.9
	Corn stalks	35.0-39.6	16.8-35.0	7.0-18.4
	Sugarcane Bagasse	25.0-45.0	28.0-32.0	15.0-25.0
	Sorghum Straw	32.0-35.0	24.0-27.0	15.0-21.0
Grasses	Grasses	25.0-40.0	25.0-50.0	10.0-30.0
	Switch grass	35.0-40.0	25.0-30.0	15.0-20.0

Cherubini (2010)

2.3.2 PRE-TREATMENT METHODS

The complex structure of lignocelluloses, with the high crystallinity and lignin content, makes material difficult to digest naturally for microorganisms in anaerobic digester. This slow or incomplete digestion results in low biogas yield. In order to decrease the biomass recalcitrance, and thereby increase biogas yield, different pretreatment methods can be used (Hendriks and Zeeman, 2009).Pretreatment of lignocellulose materials is very important in the production of biogas. This is carried out in order to get rid of lignin and degrade hemicellulose, decrease the level of crystallinity in the cellulose, and enhance the porosity of lignocellulosic materials.

According to Kumar *et al.* (2009), pretreatment should meet some important requirements which include:

- Enhancement of sugar formation or facilitation of hydrolysis process after pretreatment
- Avoid degradation or loss of carbohydrates.
- Not cause formation of by products that could possibly give rise to inhibition in subsequent hydrolysis and fermentation processes.
- Be economical.

Pretreatments can have effects on physicochemical properties of a substrate, such as molecular size, cellulose crystallinity, surface accessibility, pore size distribution and particle sizes (Hendriks and Zeeman, 2009). Some pretreatments have an impact on chemical composition of the substrate, where lignin and/or hemicelluloses, to some extent, get soluble, while others have no effect on chemical composition of the substrate.

Identifying pretreatment methods which are suitable for lignocellulose based anaerobic digestion is essential for feasibility of a biogas plant. Pretreatments can be roughly categorized as physical, chemical and biological (Zheng 2014). Each of these methods serves a specific purpose. Hence, it is necessary to carefully identify and apply the most appropriate method based on operational characteristics (e.g. feedstock composition, temperature, reactor configuration). Table 2.9 lists a few treatment methods that are used to improve biomethanation process of lignocellulosic substrates. Combination pretreatment, such as physicochemical pretreatment by including two or more pretreatment techniques from the same or different categories is quite common, but combination pretreatment is not considered as an individual pretreatment category (Taherzadeh and Karimi, 2008).

Table 2.9:Applied methods to improve the biodegradability of lignocellulosic substrates

Methods	Substrate	Conditions	CH_4 Increase	Reference
Mechanical pretreatment				
Milling	Wheat straw	Size reduction from 5 to 0.2 cm	80%	(Menardo *et al*.,2012)
Grinding	Ley crop silage	Size reduction from 1-16 mm to 0.1- 2.0 mm	59%	(Lindmark *et al.*,2012)
Chemical pretreatment				
Alkali	Biofibers	6% CaO w/w, 15 °C, 10 days	66%	(Bruni et al., 2010)
Thermal alkali	Wheat Straw	4% NaOH (g/g TS), 37 °C, 5 days	112%	(Chandra *et al*.,2012)
Bioaugmentation				
Clostridium cellulolyticum	Wheat Straw	33% of the working volume	13%	(Peng *et al*., 2014)
Pseudobutyrivibrio xylanivorans Mz5T	Brewery spent grain	5% of the total Volume	18%	(Čater *et al*., 2015)

Micro-aeration				
Oxygen	Corn straw	12.5 mLO2/LR/day	17%	(Fu *et al*., 2016)
Oxygen	Sugarcane Bagasse	10 mLO2/gVS	17%	(Fu *et al*., 2015)

Zheng *et al*.(2014)

1. Physical pretreatment

The aim of physical treatment is to improve access to degradable organic matter by alternating biomass size (Kratky and Jirout, 2011). It is done to physically /mechanically reduce the particle size and crystallinity. This activity increases the accessible surface area and pore sizes of the biomass. Enhanced hydrolysis is achieved as crystallinity is reduced, and mass transfer characteristics are improved due to reduction of the particle size. Milling, grinding, irradiation, ultrasound and hydrothermal pretreatments are some of the methods that belong in this category (Taherzadeh and Karimi, 2008 and Brodeur *et al.,* 2011). However, a major drawback of physical pretreatment is that enormous amount of energy is consumed to carry out an efficient disintegration (Hidaka 2013; Rodriguez 2016).

2. Chemical pretreatment

Chemical pretreatment is based on ability of chemical compounds to disrupt the lignocellulosic polymers.Usage of acids or bases formed the foundation of the most widely studied chemical pretreatments. According to Zheng (2014), acid pretreatments solubilize hemicellulose units and break the bonds of lignin structure, but do not dissolve lignin and are typically applied in high temperature levels. Hence, inhibitors are generated as furfural and hydroxymethylfurfural (HMF).

Conversely, alkaline pretreatments can boost saponification, induce disruption of lignin-carbohydrate bonds, and form less severe inhibitors to methanogenesis (Hendriks and

Zeeman, 2009). Additionally, the efficacy can be enhanced if catalyst's usage is combined with application of thermal energy and more specifically, thermochemical methods are considered to be among the most appropriate for lignocellulose treatment (Biswas 2012).Apart from acid and base, wet oxidation, catalyzed steam-explosion and ionic liquids methods are included in this category. The chemical pretreated substrate had a greater absolute model yield than the bacterial pretreated. This is because chemical pretreatment with thermal treatment is important for increased hydrolysis, increased surface area, and easy establishment of acidogenic, acetogenic and methanogenic bacteria (Shoar 2019; Venturin *20*19; Novakovic 2020).

3. Physiochemical pretreatments

This pretreatment method combines chemical and physical processes in order to prevail over the recalcitrance of lignocellulosic biomass. The processes that belong to this group are: ammonia fiber explosion (AFEX), CO_2 explosion, SO_2 explosion, steam pretreatment/autohydrolysis, hydrothermolysis, and wet oxidation (Mohnen 2009).

4. Biological pretreatment

Biological pretreatments utilize the natural ability of microorganisms (*e.g.,* fungi) to degrade the lignin and hemicellulose, leaving the cellulose intact (Shi 2008; Kumar and Wyman, 2009; Sánchez, 2009). White-rot fungi which is the most studied microorganism is considered to be promising due to the substrate specificity of its ligninolytic enzymes (Sánchez, 2009). Lignin is then degraded through the action of lignin degrading enzymes secreted by the fungi. Although biological pretreatments involve mild conditions and are economically beneficial, the drawbacks connected to this pretreatment methods, such as low rates of hydrolysis and long pretreatment times, weigh against their advantages compared to other technologies (Sun and Cheng, 2002; Johnson and Elander, 2009).

2.3.3 CO-DIGESTION AND TECHNIQUES

Lignocellulosic materials are rich in carbon, have a poor buffering capacity and deficient in nutrients as stated by Mata-Alvarez (2014),. Mono-digestion of lignocellulosic materials often results in a slow process and low methane yield (Sawatdeenarunat 2015). However, this drawback can be overcome by using a co-substrate, such as animal manure to supplement lignocellulosic materials with macro- and micronutrients and buffering capacity (Mata-Alvarez *et al.*, 2014). Co-digestion has been investigated for wheat straw with dairy and chicken manure (Wang *et al.*, 2012), rice straw with kitchen waste and pig manure (Ye *et al.*, 2013) and oat straw with cattle manure (Lehtomäki *et al.*, 2007). These studies report higher methane yields (approximately 200-400 mL/g VS), compared with when using straw alone (~120-200 mL/g VS), as a consequence of higher energy content of the co-digestion materials and their complementary properties. In short, most recent attempts to optimize the breakdown of cellulolytic feedstock are predominantly engineered through co-digestion (Ai *et al.,* 2020; Almomani and Bhosale, 2020; Miryahyaei *et al.,* 2020; Zhong *et al.,* 2020).

Sun (2015), discovered that complementing manure with lignocellulosic materials do not always result in higher specific yield. However, Møller (2004) opined that high VS content of plant based materials can allow an increase in OLR without reducing the HRT, and thus increase volumetric biogas production. Lignocellulosic materials, such as agricultural residues are often low in trace elements such as iron, nickel, cobalt, molybdenum, selenium and tungsten, which are required for microbial enzyme activity (Demirel and Scherer, 2011). In a long-term mono-digestion of maize silage for biogas production, trace element deficiency was suggested as the cause of acidification observed after around 8 months of operation (Lebuhn 2008).Consequently, adequate knowledge on the composition of feedstock is critical in determining the most appropriate manure to co-digest lignocellulosic materials.

Cow dung and poultry droppings are good sources of natural inoculum for bio-digesters (Ona *et al.,* 2019; Aniekan 2020; Olatunji 2020) and have been strongly reviewed (Caruso 2019; Ghosh 2020; Kapoor 2020; Putatunda 2020).

2.3.4 BIOGAS DIGESTER

Biogas digester is a large tank inside which biogas is produced through breakdown of organic matter, in anaerobic condition. It is called a digester because organic matter are consumed and digested by microorganisms to produce biogas. Without the digester, there will be no production of biogas, hence, its importance. A typical biogas digester has a part that holds slurry which is a combination of water and organic matter. There is also a part that holds the gas that has been produced. There is a connecting system through which the digester is fed with organic matter. There are also pipes that connect the slurry part to gas holding part; moves the biogas to where it will be utilized; and for ejecting residues(Opurum 2014)

An increase in Organic Loading Rate (OLR) usually results in an increase in total methane yield, however, if a certain OLR value is exceeded, the process can be unstable and process failure may even occur (Astals *et al.,* 2014; Mata-Alvarez *et al.,* 2014; Dhar *et al.,* 2016; RomeroGu¨iza *et al.,* 2016). Also, HRT between 15 – 30 days, there was a continuous methane production. This corresponds with reports from Mao *et al.* (2015), Lochyn´ska and Frankowski (2018) and Braun *et al.,* (2010) who opined that a typical HRT is 15-30 days under mesophilic conditions and slightly shorter under thermophilic conditions. Also, a long HRT is required in anaerobic digestion of lignocellulosic wastes. However, a shorter HRT is desirable, in order to reduce the capital cost and increase the efficiency of the process (Shi *et al.,* 2017; Issah *et al.,* 2020). This shows that HRT and substrate concentration are important parameters for biogas production from both alkaline and biological pretreatment methods.

2.3.5 TYPES AND DESIGNS OF BIOGAS DIGESTERS

Different types of digesters have been designed and often times, they are classified as Liquid or solid state process; batch or continuous process; and single- or two-stage process.

1. Liquid/solid state process digester

Anaerobic digester can be categorized as liquid anaerobic digester (L-AD) process when it has a total solid (TS) content of less than 15%, and as solid anaerobic digester (S-AD) process when its TS content is 15% or higher (Li *et al.*, 2011, Brown *et al.*, 2012;Yang *et al.*, 2015).According to Sawatdeenarunat *et al.* (2015), it is best to use the L-AD process when digesting substrates with high moisture content, such as wastewater streams. Currently,semi-continuous stirred tank reactor(CSTR) is the most suitable digester for more diluted substrates. The substrates are typically pumped into the digester either continuously or semi-continuously, while residues are simultaneously removed. Solid materials can be fed into a CSTR directly at the top without pumping, but typically this reactor type is not optimal for handling lignocellulosic materials.

2. Single stage process

The semi- solid anaerobic digestion (SS-AD) is better for processing high solids content feedstock such as lignocellulosic materials. Compared with L-AD, SS-AD requires less process water and lower energy input for heating and mixing (Li *et al.*, 2011). Moreover, for the same solid loading rate, SS-AD requires less reactor volume and achieves a higher volumetric methane yield (Brown *et al.*, 2012).The up-flow anaerobic sludge blanket (UASB) reactor is a type of single-stage reactor, which is designed to process high-rate sewage wastewater streams (Chong *et al.*, 2012). The UASB reactor allows separation ofsolid retention time (SRT) from HRT, which minimizes washout of microbes (*e.g*. hydrolyzing group of bacteria and methanogens) due to short HRT.

Plug flow digester is another common type of SS-AD process, in which substrate is fed from a feeding port and moved as a plug through the reactor to the exit. This process requires heavy equipment that can handle dry, viscous material (Li *et al.*, 2011).

3. Two-stage process

Two digesters are used for degradation of substrates in two-stage process. The first digester is used for hydrolysis/ acidogenesis/acetogenesis and the second digester for methanogenesis (Kothari *et al.*, 2014). Due to difference in ideal pH ranges for hydrolysis (5.5-6.5) and methanogenesis (6.8-7.2), this type of anaerobic digestion process has been shown to achieve better hydrolysis of solid organic compounds (Weiland, 2010;Kothari *et al.*, 2014).

4. Batch digester

This is very suitable for digestion of lignocellulosic materials. The digester is filled once or several times and materials are allowed to be degraded before being taken out of the digester. A series (at least three) of garage-type batch digesters with percolation and without mechanical mixing can be applied for mono-fermentation of energy crops, for large-scale applications (Weiland, 2010). Heiermann *et al.* (2007) reported a specific methane yield of 0.34 L/g of VS in an experiment, using four boxes of digesters, running with a substrate mixture of maize silage, poultry manure and digested material from previous run. Laboratory-scale reactors of different designs can also be used to evaluate different substrates and substrate combinations.

Figure 2.5: Laboratory-scale semi-continuous stirred tank reactor (CSTR) (Sun, 2015)

2.4 MICROBIAL COMMUNITY DIVERSITY IN ANAEROBIC DIGESTION

2.4.1 METHODS FOR INVESTIGATING COMMUNITY STRUCTURE OF CELLULOSE-DEGRADING BACTERIA

As discussed previously, lignocellulosic materials are abundant in nature. However, their lack of certain nutrients required for biogas formation, as well as their complex biomass limits their efficiency for use as substrates in anaerobic digestion process. As a result of these, various studies have come up with solutions such as co-digestion and pretreatment, but further investigations need to be carried out to improve biodegradability of lignocellulosic materials while saving cost.

(A) Culture-dependent methods:

In studying the morphology, physiology and genetics of specific microorganisms, enrichment, isolation and cultivation of microorganisms in pure culture are very important steps to be taken, but this can be time consuming and laborious. In order to cultivate anaerobic microorganisms, special equipment and techniques are required to provide an anaerobic environment, and agar shake or role tube method is typically used for isolation.

In a study carried out by Sun (2015), for isolation of cellulose-degrading bacteria, cellulose or cellobiose was used as the sole carbon source during the whole isolation procedure.Isolation started with enrichment of bacterial consortia in a reduced mineral medium, with the purpose of enriching the bacteria that is able to metabolise cellulose/cellobiose. Serial dilution of the enrichment cultures was then performed in same mineral medium. For the highest dilution at which growth (visual) occurred, the agar shake method was applied for picking single colonies and cultivation in pure culture.

Sun (2015) further used industrial biogas plants as the inoculum source for isolation and from this, two cellulose-degrading bacteria were isolated. The 16S rDNA sequence revealed the two isolates to be closely related to *Clostridium straminisolvens* CSK1 (1368 bp, 98% identity) and *Clostridium clariflavum* DSM 19732 (1500 bp, 97% identity) respectively. Other cellulose-degrading bacteria have been isolated in a similar way, including *Clostridium cellulolyticum* ATCC 35319, *Clostridium cellulovorans* 743B*Clostridium papyrosolvens Clostridium populeti* and *Clostridium stercorarium.*

Figure 2.6: Agar shake with single colonies (Sun, 2015).

(B) Culture – independent methods:

Most of the microorganisms in anaerobic digestion process have not yet been cultivated, and using standard cultivation techniques, it has been estimated that 5% or less of microbial diversity in the biosphere can be cultured (Curtis *et al.*, 2002). Studies on microbial ecology and physiology of anaerobic digestion are most likely incomplete and biased because there are many factors that co-exist in this complex environment, and affect microbial activity which cannot be studied when using culture-based methods. When using isolated microorganisms, it is difficult to determine functions related to competition and interactions between microorganisms (Vanwonterghem *et al.*, 2014). However, based on available genomic data, a variety of molecular methods have been invented and developed for further investigations of microbial community structures existing within anaerobic digestion processes.

A type of culture-independent method – Clone library – was used in the investigation of DNA extracted from an environmental sample by cloning and subsequent sequencing (Chouari *et al.*, 2005). This technique has been used to target 16S rDNA of microbial

community in various anaerobic digesters, such as digesters processing beet silage (Krakat *et al.*, 2011), crops and cow manure (Wang *et al.*, 2009b), grass silage (Wang *et al.*, 2010), pig manure (Liu *et al.*, 2009) and organic solid waste (Sasaki *et al.*, 2011).

(Bi) Terminal restriction fragment length polymorphism (T-RFLP)

Iis a method based on polymerase chain reaction (PCR) technology where a selected target gene is amplified with PCR using the total DNA extracted from digester samples. However, different from clone library method, the primer in the PCR reaction is labeled with a fluorescent dye such as 6-carboxyfluorescein (FAM). In a second step, the amplicon generated from the PCR is digested with a selected restriction enzyme appropriate for the sequence of interest. The digestion products, which are called fluorescently labeled terminal restriction fragments (T-RFs) are separated and detected using capillary electrophoresis. The T-RFLP profile is visualized as relative abundance of each T-RF at a specific length. As different sequences possibly have different restriction sites, in the assay, each T-RF could represent a unique sequence, but occasionally the same T-RF can be represented by two different organisms. Differences in T-RFLP profile indicate the differences in structure between the communities. This method is usually combined with a clone library. Once the sequence for the environmental sample is available, the restriction site of each sequence can be analyzed *in silico* and thus the corresponding sequence for each T-RF can be decided. This technique has been used in combination with clone libraries in several previously mentioned studies (Wang *et al.*, 2009 and Wang *et al.*, 2010)

(Bii)Real-time quantitative PCR (qPCR)

It can be applied to study, detect and quantify a targeted DNA sequence such as 16S rDNA or the functional gene. For quantification, the real-time PCR technique is used, together with an intercalating dye such as SYBR green that fluoresces with double-stranded DNA. The fluorescence signal recorded increases as the double-stranded DNA increases after each

amplification cycle. A melting curve analysis is performed following the PCR program to inspect possible false positive signals, such as primer-dimer and amplification errors. By comparison against the standard curve, usually a solution of a known amount of target DNA cloned in the plasmid, absolute amount of gene of interest can be calculated (VanGuilder *et al.*, 2008). This method has also been used successfully to quantitatively analyse other bacterial populations in biogas processes (Westerholm *et al.*, 2011,Moestedt *et al.*, 2013;).

Following the development of Next-Generation Sequencing (NGS), such as 454-pyrosequencing, cost-effective massive parallel sequencing of environmental samples with comparatively high coverage of community can now be conducted (Schlüter *et al.*, 2008 and Zakrzewski *et al.*, 2012). The amplicon sequencing approach enables sequencing of PCR products without an extra clone step, thus eliminating the clone bias. Moreover, the multiplex technique allows the integration of barcode onto the primer, which enables processing of a considerable number of samples at the same time. Rapidly increasing numbers of studies are using amplicon sequencing targeting the 16S rDNA of samples from anaerobic digestion processes, including both laboratory-scale and industrial-scale biogas digesters. These include to date: laboratory-scale processes digesting straw and manure, industrial-scale digesters that operate with various substrates (Sundberg *et al.*, 2013;De Vrieze *et al.*, 2015) a full-scale plant processing energy crops (Lucas *et al.*, 2015), batch cultivation of wheat straw and swine manure (Li *et al.*, 2014) and laboratory-scale digesters processing straw and hay (Lebuhn *et al.*, 2014).

Metagenomic shotgun sequencing is another NGS approach, which directly sequences a library of sheared DNA fragments. Unlike the clone library or amplicon approach, the shotgun approach sequences random DNA fragments resulting from microbial genomes. Thus this method not only generates the sequences of phylogenetic genes, but also provides functional insights into the microbial community (Sharpton, 2014). A number of anaerobic

digestion processes have been investigated using this metagenomic approach and it has generated information leading to a specific understanding of the hydrolysis step in the anaerobic digestion process (Schlüter *et al.*, 2008,Hanreich *et al.*, 2013and Yan *et al.*, 2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 COLLECTION AND PRECESSING OF SAMPLES OFRICE STRAW (RS), WATER HYACINTH (WH), NITROGENOUS WASTES AND COW RUMEN LIQUOR

Rice straw was collected from a rice farm at Abakiliki in Ebonyi state, while Water hyacinth was collected from coastal areas of Ihiagwa River Owerri West L.G.A. of Imo State both in Nigeria. Both samples were transported to the laboratory for processing. The samples were sorted and washed then sundried after which they were milled to fine reduced sizes, using a crushing machine and subsequently stored in air tight polyethene bags.

Cow dung,pig waste and poultry droppings were used as amendmentsto co-digest the substrate in order to improve their biodegradability and nutritional composition. The cow dung was collected from an abattoir located around 34 Artillery Brigade, Obinze, in Owerri West Local Government Area of Imo State, Nigeria. Pig waste and poultry droppings were collected from piggery and poultry farms in Ihiagwa, respectively. All samples were sun-dried and ground into fine powder before storing in an air tight polyethylene bag.

Cow rumen liquor was used as inoculum in all the anaerobic digesters. Fresh cow rumen waste was collected as soon as the cow was slaughtered using 10 liter air tight buckets to preserve the anaerobes. The cow rumen sample was strained with white cloth filter and the filtrate (liquor)was used immediately to inoculate the processed substrates.

3.2DESIGN OF OPTIMIZATION OFALKALINE PRETREATMENT OF RICE STRAW AND WATER HYACINTH SAMPLES

Optimization of conditions for alkaline pretreatment of Rice straw and water hyacinth was carried out using Box Behnken Design; a type of Response Surface Model, in Minitab 17 version.The 3 factors, 3 level analysis of the parameters to determine the optimum conditions for the alkaline pretreatment of the substrate was adopted. This produced 15 runs with different values for each factor in each run, in other to determine the optimum condition which was used to pretreat the substrate for lignin degradation.

Table 3.1: Box Benken design for optimization of alkaline pretreatment of substrates

StdOrder	RunOrder	PtType	Blocks	NaOH %/ Concentration	Concentration of substrate (g)	Time (h)
1	1	2	1	2	10	48
2	2	2	1	6	10	48
3	3	2	1	2	14	48
4	4	2	1	6	14	48
5	5	2	1	2	12	24
6	6	2	1	6	12	24
7	7	2	1	2	12	72
8	8	2	1	6	12	72
9	9	2	1	4	10	24
10	10	2	1	4	14	24
11	11	2	1	4	10	72
12	12	2	1	4	14	72
13	13	0	1	4	12	48
14	14	0	1	4	12	48
15	15	0	1	4	12	48

Using the values for each factor as specified in Table 3.1 for each run, rice straw and water hyacinth samples were separately pretreated, and concentration of lignin obtained after pretreatment was used as the response.

3.3ALKALINEAND BIOLOGICAL PRETREATMENT OF RICE STRAW AND WATER HYACINTH SAMPLES

3.3.1 Alkaline Pretreatment of Rice Straw and Water Hyacinth Samples

Sodium hydroxide was used to pretreat each substrate at ambient temperature to improve their biodegradability, and their potential for anaerobic biogas production. Optimum conditions obtained from the optimization study were usedaccording to the method of Shetty (2016**)** with few modificationSix grams (6g) of NaOH dissolved in 100m/l of water was used to pretreat fourteen grams (14g) of the substrate for 40 hours after which the substrates were washed until the pH is brought to 7.0 and sun-dried. This process was repeated until the required mass of the substrate needed for anaerobic digestion was obtained.

Optimum values obtained from Response Optimizer were applied in the pretreatment process and the concentrations of lignin remaining in each case, after pretreatment was determined and recorded.

3.3.2 Bacterial Pretreatment of Substrate Rice Straw and Water Hyacinth

The method described by Girisha *et al.* (2017) was adopted with modifications. The bacterial isolates from the gut of termites were used to pre-treat Rice Straw and water hyacinth for 30 days. Nutrient broth was prepared and 500 ml of the freshly prepared nutrient broth was poured into a bowl containing 100 grams of sample. The contents of the bowl were properly mixed by gentle turning to form slurry. The contents of the bowl were left for biomass cellulose degradation. Samples were drawn from the bowl to determine lignin, hemicellulose and cellulose content on Day 0, Day 15 and Day 30 respectively.

3.4 DETERMATION OF LIGNIN, CELLULOSE, HEMICELLULOSE CONTENTS OF RICE STRAW AND WATER HYACINTH SUBSTRATES

Lignin, cellulose and hemicellulose contents of each substrate were measured before and during and after pretreatment at 3 intervals (i.e. before pretreatment, after 15days and after 30days of pretreatment). These were done according to methods described by Lin (2010).

Hemicellulose determination

Some 1g of sample (rice straw and water Hyacinth was weighed into a flask and 150ml of 0.5 MNaOH was added and boiled for 3hours with distilled water .the mixture was filtered after cooling before washing to neutrality, finally the residue is dried to constant weight at 105^0C.

Hemicellulose =weight before treatment- weight after treatment

Lignin determination

Some 0.3g of dried sample (rice straw and water Hyacinth) was weighed into a test tube and 3ml of 72%$H_2S\ O_4$ and kept at room temperature for 2hours and shaken at 30minutes interval for hydrolysis to take place, thereafter the mixture is autoclaved for 1 hour at 121^0C for second hydrolysis to occur and cooled and filtered. Residue is dried at 105^0Cto get the acid insoluble lignin thereafter the acid soluble lignin is determined by ashing the hydrolyzed sample at 575^0Cin a muffle furnace and measuring the absorbance of the acid hydrolyzed sample at 320nm.

Lignin content = Acid insoluble lignin + Acid soluble lignin

Then,

Cellulose content determination = Lignin content - Hemicellulose content.

3.5COLLECTION, ISOLATION AND IDENTIFICATION OF LIGNOLYTIC BACTERIA FROM TERMITE GUT

Red headed termites were collected from old and decaying woods with containers and taken to the laboratory for analysis.The termites were washedwith sterile distilled water dried on a filter paper and sterilized with 70% ethanol, thereafter their guts were accessed by removing singly, the heads and legs such that what is left is the gut which was then squashed with mortar and pestle. A 10 fold serial dilution was done, where 1 gram of the squashed termites gut wasput in 9mls of maximum recovery diluent (MRD) and left to stand for 30mins – 1hour. Then 0.1ml was collected from the maximum recovery diluent and dropped on an enriched medium(containing 2% w/v saw dust powder,0.05% w/v glucose,5% v/v stock salt solution,0.02 % v/v Hunter's trace element and 1.5%w/v agar)and incubated at ambient temperature for 15 days.

3.5.1 CULTURAL AND BIOCHEMICAL IDENTIFICATION

Bacterialisolates were characterized based on cultural (colonial), microscopic and biochemical methods with reference to standard manuals (Cheesbrough, 2000). The identities of the isolates were cross-matched with reference to standard manuals for the identification of bacteria (Buchanan and Gibbon, 2000).

3.5.2 MOLECULAR IDENTIFICATION OF ISOLATES

3.5.2.1. DNA Extraction (Boiling method)

Five milliliters of an overnight broth culture of bacterial isolate in Luria Bertani (LB) was spun at 14000rpm for 3 min. The cells were re-suspended in 500ul of normal saline and heated at 95 0 C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3

min at 14000rpm.The supernatant containing the DNA was transferred to a 1.5ml micro centrifuge tube and stored at -20 o C for other downstream reactions.

3.5.2.2. DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2 ul of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal; the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the “measure” button.

3.5.2.3 Amplification of 16S rRNA of Isolates

The 16s rRNA region of the rRNA gene of the isolates were amplified using the 27F: 5'- AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles.The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5uM and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extention, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light transilluminator.

3.5.2.4.Sequencing of samples

Sequencing was done using the Big Dye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10µl,

the components included 0.25µl BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10µM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing condition were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

3.5.2.5. Phylogenetic Analysis of Sequences

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using MAFFT. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969).

3.6. CO-DIGESTION OF SUBSTRATES WITH NITROGENOUS SOURCES (AMENDMENTS)

Cow dung, pig waste and poultry droppings which were sun-dried, weremixed in three ratios of 1:1, 2:1 and 3:1 with each of the primary substrates. The composites were loaded into the digesters after thorough mixing with water. Then 1.6 liters of freshly strained cow rumen waste which was used as source of inoculum was added to each composite and properly mixed. Each of these inoculated composites was charged into the biodigesterwhich wasdesigned and operated as described in 3.6.1 below.

3.6.1 BIOREACTOR DESIGN, SETUP AND OPERATION

Plastic containers of 10 L capacity each were used to construct the biodigesters. Two holes were bored on the cover of the plastic container, one for gas outlet and the other for thermometer. A three quarter inch gas hose was installed in one of the holes and held tight with an epoxy glue to ensure that there is no gas leakage. In the other hole, a thermometer was installed and also made air tight with epoxy glue. The other hose from the 10 liter container was connected to a calibrated water filled inverted 3liter mini bucket (container). The bioreactor was set up in triplicates. The gas collection was by water displacement method (Aragaw, 2013), pH and temperature changes during the course of anaerobic digestion was monitored with a digital hand held pH meter and the installed thermometer respectively.

Anaerobic digestion of the substrates was carried out under controlled and reproducible conditions using 10 liters capacity digester. Five hundred and twenty grams (520g) of each ratio of the mixed composites, as well as the control experiment, which was set up without amendment, was weighed separately with an electronic weighing balance and mixed thoroughly with 1.2liters of water for optimum gas production. This was loaded in the bioreactors to about ¾ of its volume (Ojolo, 2008). Fresh cow rumen waste was strained and 1.6liters of the inoculum was used to pitch the loaded substrate in the bio digester and made up to eight (8) liter mark.The digesters were made air tight to exclude oxygen. The hose from the digesters outlet was connected to the inverted water filled mini containers, and properly labeled. The digesters were periodically shaken to avoid stratification of the substrate and also ensure thorough mixing of the digester content, while maintaining intimate contact between the microorganisms and the substrates in order to enhance complete digestion of the substrates.

Anaerobic digestion of the substrates lasted for 35 days hydraulic retention time, during which daily ambient temperature remained around 28-32^{o}C. The daily volumes biogas yields from the bioreactors were monitored by water displacement method using an inverted calibrated water filled mini container as container by Aragaw(2013). Here volume of biogas yield is equivalent to the mean value of water displaced from the mini container after 24hours or before then if empty.

3.7 OPTIMIZATION OF CONDITIONSAFFECTING YIELD OF BIOGAS USING BOX BENHKEN DESIGN

Response Surface Model (Box Behnken design) was used to optimize the conditions that affectthe yield of biogas(concentration of substrate, concentration of nitrogen source and hydraulic retention time (HRT)) in a batch system.

3.7.1 DESIGN OF OPTIMIZATION OF STUDY

Using the three –factor mode of Box Behnken Design, 15 runs were obtained, each having definite combinations of the factors as shown in Table 3.2. Here, amendment which yielded highest quantity of biogas during co-digestion study was used.

Table 3.2: Design for optimization of parameters affecting biogas production

StdOrder	RunOrder	PtType	Blocks	Substrate concentration (g)	HRT	Amendment concentration (g)
4	1	2	1	520	30	325
10	2	2	1	325	30	130
3	3	2	1	130	30	325
11	4	2	1	325	15	520
15	5	0	1	325	22.5	325
9	6	2	1	325	15	130
14	7	0	1	325	22.5	325
13	8	0	1	325	22.5	325
6	9	2	1	520	22.5	130
2	10	2	1	520	15	325
5	11	2	1	130	22.5	130
7	12	2	1	130	22.5	520
12	13	2	1	325	30	520
1	14	2	1	130	15	325
8	15	2	1	520	22.5	520

After using the values prescribed in Table 3.2 to run biogas production in a batch system, recorded volume of biogas produced in each case was fed into Response Optimizer (Minitab 17) which predicted optimum values of each of the factors, as well as maximum volume of biogas that would be produced using the predicted optima. Furthermore, these optimum values for selected factors were used to run another set of biogas production, in triplicates, and results obtained were recorded.

3.8ANALYSIS OF COMPOSITION OF BIOGAS PRODUCED

Analysis of composition and percentages of each component ofbiogas produced in the study was carried out using Gas Chromatograph (GC).This was carried out in triplicates and their means computed. The GC used in this study was manufactured by Buck Scientific (M910, USA) equipped with FID detector and capillary column (Elite-5, 30m*0.25mm*0.25µm).

The workstation was Total chrom Navigator used for data processing. The temperature for column chamber, inlet chamber and detector were 150 ^{o}C, 200 ^{o}C and 250 ^{o}C, respectively. High purity nitrogen was used for carrier gas in this study, and the flow rate for nitrogen was 2.0 ml/min. The split ratio of gas sample in inlet chamber was 20:1, which is used to control the amount of biogas flew into column, and prevent the unconventional peak, such as flat peak, trailing peak. The flow rate was 450mL/min for air produced by automatic air source (BCHP, SPB-300, China) and 45mL/min for hydrogen produced by hydrogen generator. The temperature programme used is as follows;

Table 3.3: Temperature programme

Initial temp	Hold	Ramp	Final temp
50.00	5.000	10.000	180.00
180.00	2.000	5.000	220.00
220.00	0.000	5.000	310.00

3.9 PROXIMATE ANALYSIS OF SLURRY

Physicochemical analysis of the slurries was carried out before and after anaerobic digestion. The following parameters were determined;

3.9.1 Determination of Total Solid Content (TSC)

The total solid content of the slurry was determined by the procedure given by APHA (1995). Ten grams of freshly collected sample (Rice straw and Water hyacinth) was taken in a disc and placed inside a hot air oven at 105°C for one hour. Then it is taken out and cooled to room temperature in desiccators and weighed. The percent of total solids is computed using the formula;

$$\% \text{ Total solids} = \frac{C-A}{BA} X100 \quad \text{……..Equation 3.1}$$

Where, A= Mass of empty clean and oven dried silica crucible, g

B= Mass of silica crucible + sample, g

C= Mass of silica crucible + sample after oven drying, g

3.9.2 Determination of Total Volatile Solid Content

The volatile solid content of sample was determined following procedure furnished by APHA (1995). A known mass of residue obtained from the determination of total solids is placed in a silica crucible and ignited in a muffle furnace, partially cooled in air, kept in desiccators for few minutes and then weighed. Volatile solids(VS) is computed using the formula in equation 3.2

$$\% \text{ VS} = \frac{\text{B-C}}{\text{C-A}} \times 100\ \% \quad \text{.....Equation 3.2}$$

Where, A= Mass of empty clean and oven dried silica crucible, g

B= Mass of silica crucible + sample, g

C= Mass of silica crucible + sample after ignition, g

3.9.3 Determination of Total Moisture Content

The moisture content was determined using AOAC (1995) method, about 15g of sample was weighed in covered dish previously dried at 98-100°C, cooled in desiccator and weighed soon after reaching room temperature. Cover was loosened and heated at 98-100°C to constant weight. At the end of drying, the cover was immediately tightened on dish, transferred to desiccator and weighed soon after reaching room temperature.

$$\%\text{Moisture content} = \frac{\Delta SB - \Delta SA}{\text{Weight of sample}} \times 100 \quad \text{.....Equation 3.3}$$

ΔSB = weight of dish and sample before drying.

ΔSA = weight of dish and sample after drying.

3.9.4 Determination ofTotal Carbon Content

Sample powder (0.1g) was weighed into a 500ml conical flask and 10ml of 1N potassium dichromate solution and 20 ml sulphuric acid was added and mix by gentle rotation for 1 minute taking care to avoid throwing sample powder up onto the sides of the flask and left to stand for 30minutes thereafter diluted to 200 ml with distilled water.10ml phosphoric acid, 0.2 g ammonium fluoride and 10 drops diphenylamine indicator were also added and titrated with 0.5 N ferrous ammonium sulphate solutions until the color changes from dull green to a turbid blue. Add the titrating solution drop by drop until the end point is reached when the color shifts to a brilliant green and finally prepare and titrate a blank in the same manner.

$$\% \text{ Organic Matter content} = 10[1(S/B)] \times 0.67$$

Where,

S = sample titration

B = blank titration

3.9.5 Determination of Total Nitrogen Content

Nitrogen content was determined using Kjeldahl method, 1950. 0.5g of powdered sample was digested with concentrated H_2SO_4 in the presence of digestion mixture ($CuSO_4$ + K_2SO_4 + selenium powder in 20:10:1) till the digest gave clear green colour. The digested sample was further diluted carefully with distilled water to known volume. Then a known amount of aliquot was transferred to distillation unit (Micro Kjeldahl apparatus) and liberated ammonia will get trapped in boric acid containing mixed indicator. Later, it will be titrated against standard H_2SO_4 and the amount of ammonia liberated indicator.

3.9.6 Determination of Total Potassium Content

The potassium content of the samples was estimated using the standard procedure as suggested by flame photometer method which is explained below. For 10 ml of dried sample, 50 ml of 0.01N ammonium acetate was added. The sample got leached with an additional 50 ml of ammonium acetate and made up the volume to 100 ml. The standard of potassium is fed to the flame photometer and the readings were noted down using K-filter. The procedure was repeated thrice and the average values was reported.

3.9.7 Determination of Total Phosphorus Content

Plant and seed samples were digested with diacid mixture of $HNO_3$and $HClO_4$ in the ratio of 9:4 and the extract was made to a definite volume. Total phosphorous was determined by Vanado-Molybdate phosphoric acid yellow colour method at 730 nm (Yadav 2014)

3.9.8 pH Analysis

pH was measured using a hand held pH meter. The Electrodes were connected to the pH meter and calibrated using buffer solutions (pH 4 and pH 7) before taking pH reading.

3.10DETERMINATION OF MICROBIAL COMMUNITY IN SLURRY DURINGANAEROBIC DIGESTION

3.10.1 Preparation of Media and Diluents

Nutrient agar, Salmonella Shigella Agar(SSA), Eosin Methylene Blue Agar(EMBA), Mannitol Salt Agar(MSA) and Potato Dextrose Agar(PDA)used for the isolation, characterization and identification of the microorganisms in the digesting slurry were prepared according to manufacturer's specification. Nutrient agar was used in the isolation of

heterotrophic bacteria, PDA for heterotrophic fungi, MSA for *Staphylococcus* sp, EMBA for *Escherichia coli* and other enteric bacteria and SSA for the isolation of *Salmonella* and *Shigella* species. Physiological saline used as diluents was prepared by dissolving 9.8g of sodium chloride in 1000ml of distilled and dispensed in 90ml and 9ml portions. Both diluents and media were sterilized in an autoclave at 121^0C for 15mins.

3.10.2 Preparation of samples and inoculation

One milliliter (1 ml) samples were dispersed in 9 ml of sterile physiological saline. Ten-fold dilution method was prepared by transferring 1ml from each tube until the required dilution was obtained.Aliquot portion (0.1ml) of appropriate dilution was inoculated into the pre-sterilized and surface dried medium. Inocula were spread evenly to ensure uniform and countable colonies. Plates were incubated at ambient temperature for 48 hours for heterotrophic bacteria.

3.10.3 Determination of microbial population

Colony counts obtained on the media were expressed as colony forming units per gram (CFU/g) to obtain total population (Harrigan and McCance, 1987).

3.10.4 Characterization and identification of microbial isolates

Microbial isolates were characterized based on cultural (colonial), microscopic and biochemical methods with reference to standard manuals (Cheesbrough, 2000; Beishir, 1987). The identities of the isolates were cross-matched with reference to standard manuals for the identification of bacteria (Buchanan and Gibbon, 2000).

3.10.4.1Cultural characterization

(a) Gram staining test

The Gram staining technique was used for the bacterial isolates as described by Cheesbrough (2000). A smear of the isolate was made on grease free glass slide with a drop of water and allowed to dry. The smear was fixed by mild heating, flooded with crystal violet and allowed to stand for 30 seconds. The crystal violet was rinsed off with water; Lugol's iodine was added and allowed to stand for 30 seconds. This was washed off with water and acid alcohol, till discoloration. It was counter stained with Safranin for 10 seconds and rinsed with water. The wet slide was allowed to air dry. A drop of oil immersion was added on the slide and viewed using 100X objective lens of the microscope.

(b)Spore staining test

Spore stain was used to confirm the presence of spores when indicated in the Gram stain. Isolates were heat fixed on a slide and flooded with 5% malachite green. It was steamed for 3 minutes (without allowing it to boil), dried and cooled. It was then rinsed off and stained with Safranin for 30 seconds. This was rinsed, dried with filter paper and viewed under the microscope using oil immersion tens. The positive spores showed green while the vegetative cells were stained pink.

(c)Motility test

This test was used to determine the motility of bacteria isolated. The test was carried out on a semi-solid agar medium in which motile bacteria swarm and gave a diffuse spreading growth. The medium was dispensed into test tubes, sterilized and allow to set in an upright position. It was then inoculated using an inoculation needle by stabbing it into the medium in the test tube. This was incubated at 37°C for 24 hours. Diffuse growth from the straight line of inoculation was recorded as positive result (Cheesbrough, 2000).

3.10.4.2. Biochemical characterization of bacteria isolates

Microorganisms that were not identified by the colonial and microscopic characteristics were further subjected to few biochemical tests described by Cheesbrough (2000) and Beishir (1987).

(a) Catalase test

Catalase activity can be detected by adding the substrate H_2O_2 to an appropriately incubated (18-24 hours) tryptic soy agar slant culture. Organisms which produce the enzyme breakdown the hydrogen peroxide and the resulting O_2 production produces bubbles in the reagent drop, indicating a positive test. Organisms lacking the cytochrome system also lack the catalase enzyme and are unable to breakdown peroxide into O_2 and water and are catalase negative.

(b) Coagulase test

In the test, the sample is added to rabbit plasma and held at 37ºC for a specified period of time. Formation of clot within four hours is indicated as positive result and indicative of a virulent *Staphylococcus aureus* strain. The absence of coagulation after 24 hours of incubation is a negative result indicative of an avirulent strain.

(c) Oxidase test

Oxidase test is an important differential procedure that isperformed on all gram negative bacteria for their rapid identification. The test depends on the ability of certain bacteria to produce indophenol blue from the oxidation of dimethyl-p-phenylenediamine and ∞-naphthol. This method uses N, N-dimethyl-p-phenylenediamine oxalate in which all Staphlococci are oxidase negative. In the presence of the enzyme cytochrome oxidase (gram negative bacteria) the N, N-dimethhyl-p-phenylenediamine oxalate and ∞-naphthol react to indophenol blue. *Pseudomonas aeruginosa* is an oxidase positive organism.

(d)Sugar fermentation/oxidation

This test is used to differentiate between bacteria groups that oxidize carbohydrate such as members of Enterobacteriaceae. One milliliter (1ml) of 10% glucose, maltose, lactose, fructose, mannitol, and sucrose were separately under aseptic conditions transferred into duplicate tubes containing 9ml of sterile Hugh and Leifson's medium to obtain a final concentration of 1% of each of sugar. The tubes were stab-inoculated in duplicates while two uninoculated tubes serve as control. Vaseline was used to cover one set of the duplicate tubes, one control to discourage oxidative utilization of sugar. All tubes were incubated at 37^{o}c for 48h. After the incubation, they were observed for acid production in the culture. Yellow colouration indicates acid production in the open tubes only suggesting oxidative utilization of the sugar while acid production in the sealed tubes suggests a fermentative reaction.

(e)Hydrogen sulphide production (H_2S) test

The test isolates were aseptically inoculated into a tube containing triple sugar iron agar, started by stabbing the agar to the bottom and streaking the surface of the slant. The inoculated tube was incubated at 37^{o}c for 72h and was examined daily. Black precipitation and yellow colouration was checked for. Black precipitate indicates H_2S production and yellow colouration for sucrose, lactose and glucose fermentation.

(f) Urease test

Urease Agar slant in McCartney bottle was inoculated with the bacteria isolate at 30^{o}C for 4 hours and then overnight. A pink colour in the medium indicated a positive result.

(g) IMViC test

This test consists of four different test; they are Indole production, Methyl-Red test, Voges Proskaeur test and Citrate utilization test. This test is specifically designed to determine the physiological properties of microorganism. They are especially useful in the differentiation of Gram-negative intestinal bacilli, particularly *Escherichia coli* and the *Enterobacter-Klebsiella* group.

(i) Indole test

This test demonstrates the ability of certain bacteria to decompose the amino acid-Tryptophan to Indole. The bacteria isolates were inoculated into the medium and incubated at 37°C for 48 hours. At the end of incubation period, 3 drops of Kovac's reagents was added and then shaken. A red colour ring at the interface of the medium denotes a positive result.

(ii) Methyl red and Voges-Proskaeur test: They can be considered together since they are physiologically related. Opposite test is usually obtained from the MR and VP test, that is, MR+, VP-, or MR-, VP+.Methyl red test was performed to demonstrate the capacity of different organisms to produce acid from the fermentation of sugar (dextrose). Methyl-red positive organisms produce a red colouration when five drops of methyl-red indicator is added into 48h old MR-VP broth culture.The Voges-Proskaeur test demonstrates the ability of organisms to produce acetoin from glucose metabolism. Some organisms metabolise glucose to produce pyruvic acid which is further broken down to yield Butane-diol and acetyl-methyl carbinol as an intermediate product.Into one milliliter of the culture add one milliliter of six percent alcoholic solution of alpha-naphtol and one milliliter of 16% KOH and stand for 15-20 minutes. Development of red to pink color is a positive test.

(iii) Citrate utilization test

This is one of the several techniques used to assist in the identification of Enterobacteria.Principle of the test is based on the ability of an organism to use citrate as its only source of carbon. The test was carried out using Simmon's citrate agar. The slopes of the media were prepared in bijou bottles as recommended by the manufacturers. A sterile straight wire was used to the slope with a saline suspension of the test organisms before stabbing the butt. The bottles are incubated at 35^{o}C for 48 h. Bright blue colours in the medium means

positive test while no change in colour of medium indicates negative citrate test (Cheesbrough, 2000).

3.11 ANALYSIS OF DATA

MS excel, comparative statistics, Standard Error of Mean were applied in the analysis of generated data to establish the impact of the different parameters on biogas production, from the different substrate under test.

CHAPTER FOUR

4.0 RESULTSAND DISCUSSION

4.1 RESULTS

4.1.1OPTIMIZATION OF FACTORSAFFECTING ALKALINE (NaOH) PRETREATMENT OF RICE STRAW (RS) AND WATER HYACINTH (WH) SAMPLES USING BOX BEHNKEN DESIGN

From the results obtained using rice straw, main effects plot of interactions of the factors affecting the pretreatment process is as shown in Figure 4.1.

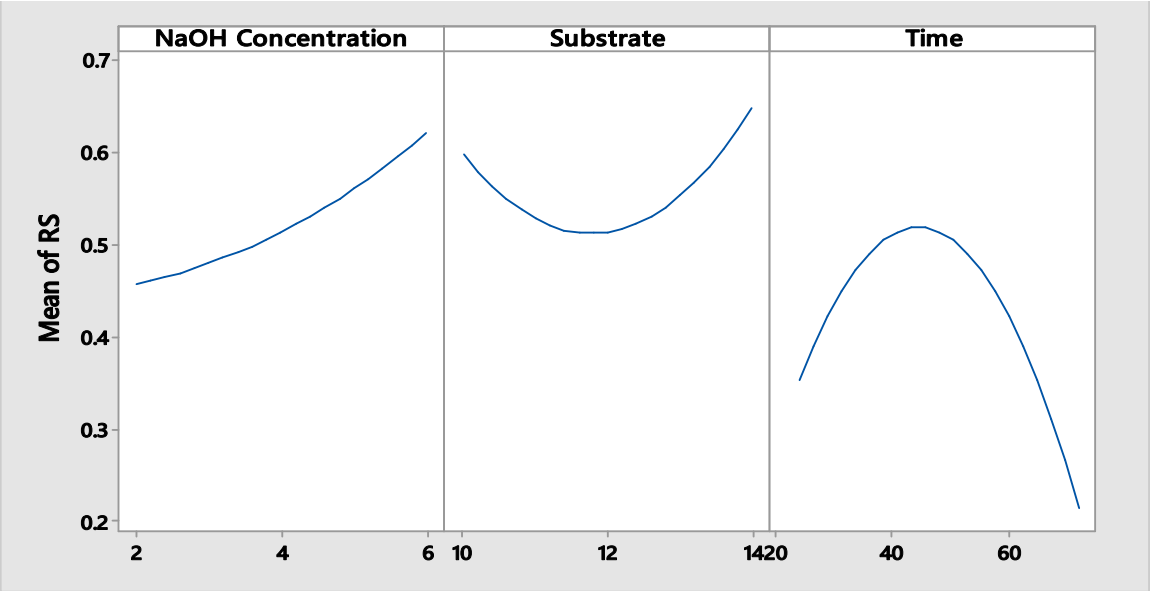


Fig.4.1: Main effect plot for the preliminary pretreatment of Rice Straw

Fig. 4.1 showed that increase in concentration of alkaline (NaOH) from 2% to 4% resulted in corresponding increase in degradation of the biomass. This also continued when the concentration increased to 6%. Initial increase in concentration of biomass from 10g to 12 g caused a reduction in pretreatment output. However, when the concentration of rice straw was increased to 12.2g, there was a sudden rise in rate of pretreatment, which continued up to

when concentration was14g. Time required for pretreatment increased proportionally with rate of pretreatment from 20h to 40h and then plateaued off. Further extension of time brought about a drastic fall in rate of reduction. This could imply that at constant biomass concentration using 6% concentration of NaOH and 14g of rice straw (RS), maximum of about 40h will suffice for pretreatment. The surface plots illustrating these interactions between the factors affecting lignocellulosic biomass pretreatment are as shown in Figure 4.2.

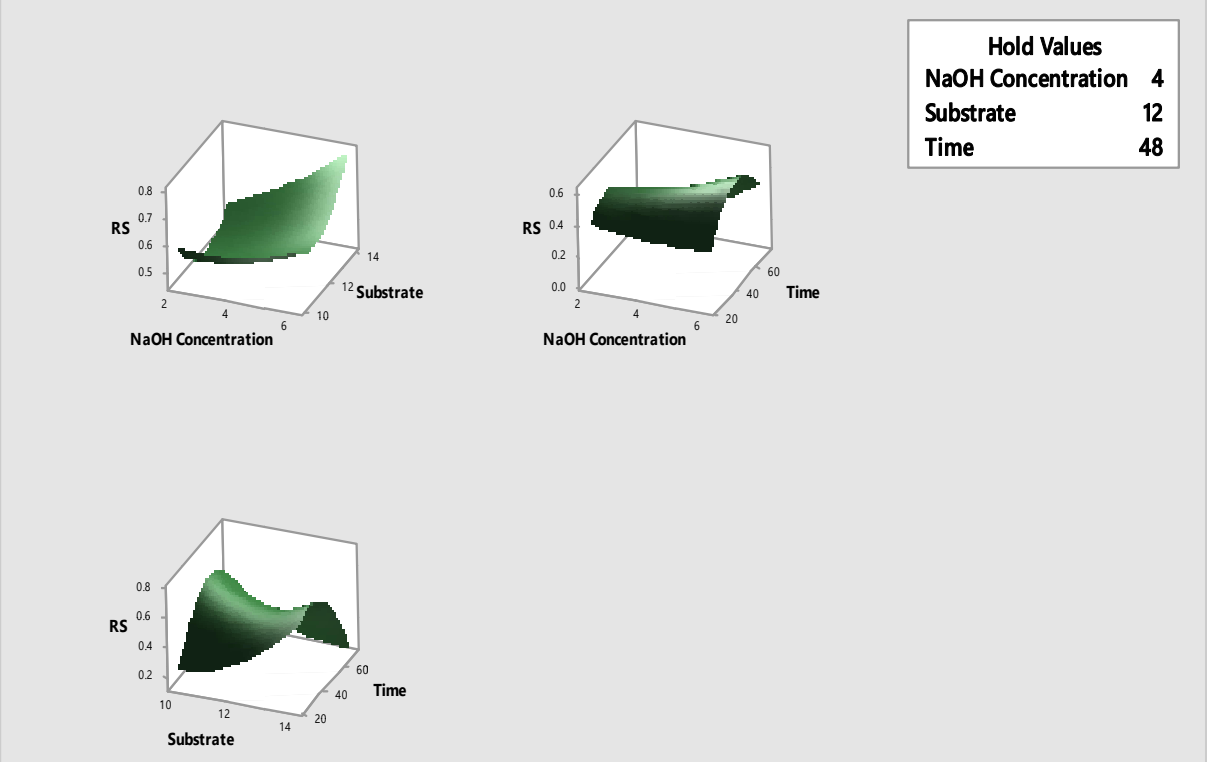


Fig.4.2: Surface plot for the preliminary pretreatment of Rice Straw

Using Response Optimizer (Minitab 17®) to analyze the concentrations of lignin remaining after pretreatment in each of the solutions from the 15 runs, it was observed that the optimum conditions were; 6% of NaOH, 14g of rice straw at 39.52 h of pretreatment. With these optimum conditions, the predicted minimum concentration of lignin that will remain after pretreatment is 0.83g, as shown in Figure 4.3.

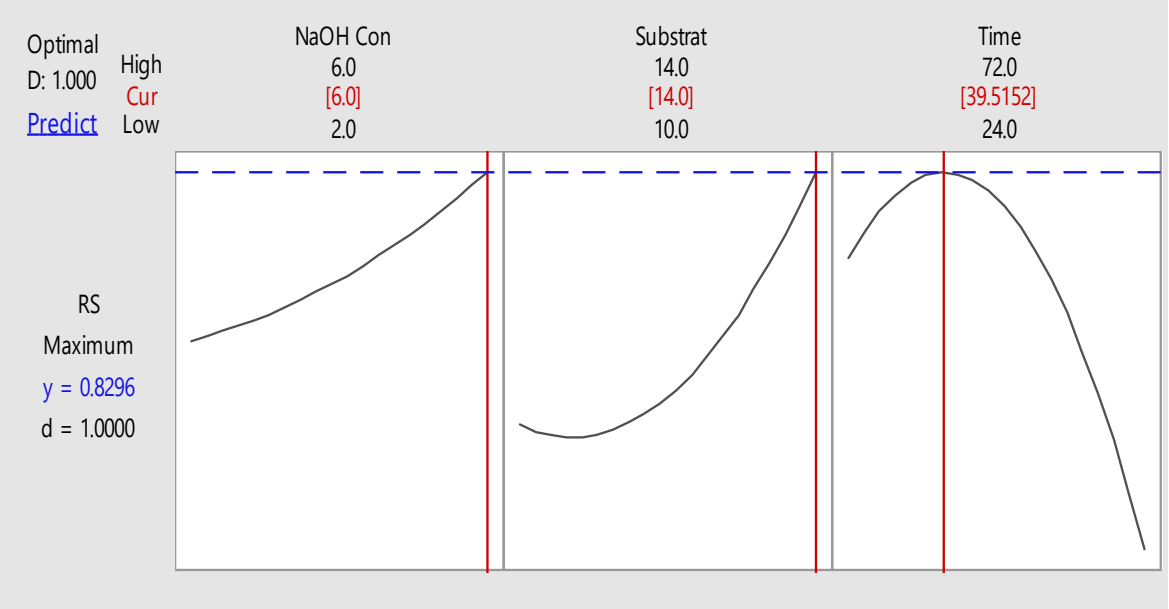


Figure 4.3: Optimization plot for preliminary alkaline pretreatment of Rice straw.

4.1.2 PRETREATMENT OF LIGNOCELLULOSIC BIOMASS

Rice straw and water hyacinth samples were both pretreated using alkaline (NaOH) and bacteria isolated from gut of termites.

4.1.2aAlkalinePretreatment of Substrates using Sodium Hydroxide

To enhance the yield of biogas from rice straw and water hyacinth biomass, 14g of each of the substrates was subjected to chemical pretreatment using 6% NaOH for 39.5 h,which are the optima for the factors, as determined earlierin 4.1. It was discovered that after chemical pretreatment with 6%NaOH,water hyacinth biomass was lost during washing to neutralize effect of NaOH, hence there was nothing to be charged into the biodigester. However, on analysis of the chemical compositions of the resulting slurry, the reducing sugarand total sugar concentrations were found to be 6.41 and 6.91 before pretreatment, but 156.08 and 167.14after pretreatment for 40hrespectively. These are illustrated in Table4.1.

Table 4.1: Sugar content of water hyacinth following chemical pretreatment.

Chemical	Reducing Sugar	Total Sugar
NaOH (charging)	6.41	156.08
After 40 hrs	6.91	167.14

Following alkaline (NaOH) pretreatment of rice straw, lignin content decreased to 8.3% of the total biomass.

4.1.2b Bacterial Pretreatment of Substrates

Lignin, hemicellulose and cellulose contents of rice straw were 17.439%, 10.278% and 57.709% respectively in the initial (un-pretreated) rice straw. After biological pretreatment for 14 days using bacteria consortium from termite gut, the lignin and cellulose content reduced to 11.259, and 33.552 respectively, while hemicellulose content increased slightly to 14.369. On the 30th day of pretreatment, lignin and hemicellulose content of RS reduced further to 7.299, 8.908 respectively, while the cellulose content increased to 38.860, as shown in Table 4.2.

Table 4.2: Chemical composition of rice straw following pretreatment with bacteria isolated from termite gut.

Anaerobic Digestion (days)	Lignin (%)	Hemicellulose (%)	Cellulose (%)
0	17.439	10.278	57.709
15	11.259	14.369	33.552
30	7.299	8.908	38.860

Table 4.3 depicts that lignin, hemicellulose and cellulose contents of water hyacinth (WH) were initially 18.01nm, 11.01nm and 10.31nm respectively for water hyacinth. Following pretreatment for 14 days, the lignin and cellulose content reduced to 11.22nm and 7.60nmrespectively, while the hemicellulose content increased slightly to 18.871nm. On the 30th day, lignin and hemicellulose contents of the WH substrate reduced further to 7.821nm and 8.21nm respectively, while the cellulose content increased to 14.40nm.

Table 4.3: Chemical composition of water Hyacinth following pretreatment with bacteria isolated from termite gut.

Anaerobic Digestion(days)	Lignin(%)	Hemicellulose (%)	Cellulose (%)
0	18.01	11.1	10.31
15	11.22	18.81	7.60
30	7.82	8.21	14.4

4.1.3 ISOLATION AND IDENTIFICATION OF MICROORGANISMS FROM TERMITES GUT USING 16S rRNA GENE SEQUENCE

The 16S rRNA of isolate F1 showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolate F1 within the *Morganella* sp. and revealed a closely relatedness to *Morganella morganii* strain S4L2C (MH745964) than other *Morganella* sp. as can be seen in Fig.4.4.Gel electrophoresis analysis of PCR products produced the bands shown in Figure 4.5.

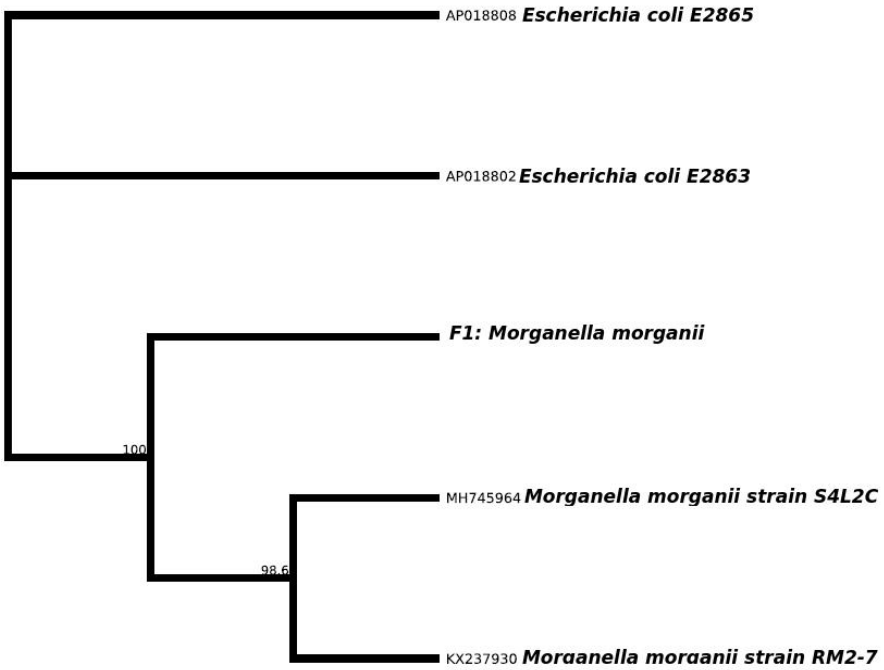


Fig 4.4: Phylogenetic tree showing the evolutionary distance between the bacterial isolate

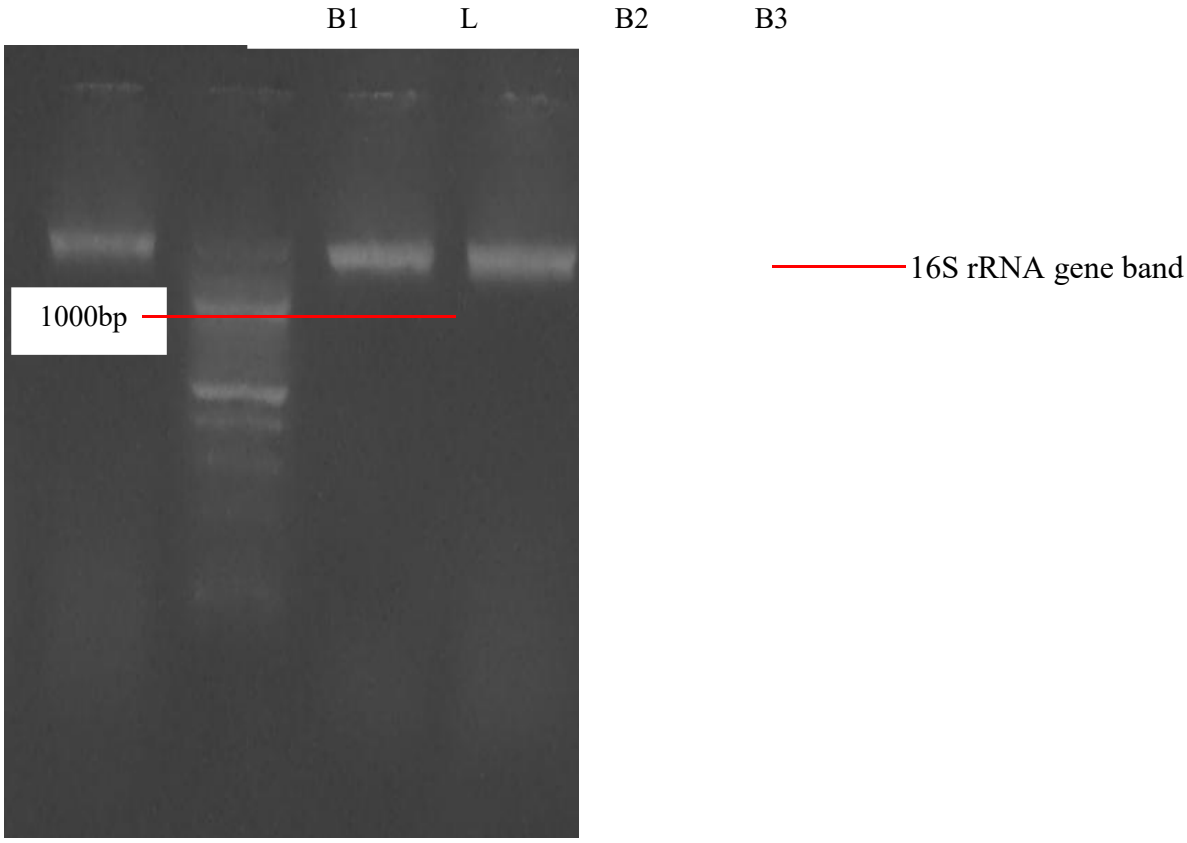


Figure 4.5: Bands obtained after gel electrophoresis of PCR products

4.1.4 AMENDMENT OF SUBSTRATES

4.1.4a AMENDMENT OF ALKALINE PRETREATED SUBSTRATES

A. Amendment of AlkalinePretreated Rice Straw with Cow Dung

Results of biogas produced from amendment of pretreated rice straw with cow dung are shown in figure 4.6. The pretreated substrate was amended with different ratios of cow dung and volume of gas produced was compared to those obtained from untreated substrate without amendment,treated substrate and cow dung alone which were used as controls. Results demonstrated that all amended substrates had higher yield of biogas than controls. The best combination being Rice straw/ cow dung in the ratio 2:1. Similarly, the treated substrate had a higher gas yield than untreated,but a lower yield when compared to the cow dung.

Considering individual combinations, rice straw/ cow dung in the ratio 1:1, did not produce any gas until day 5, when it produced 50 ml of gas. At day 10, it had produced 520ml of gas. The rate of production increased gradually until day 15 with an average volume of 2470 ml. There was a continuous increase until day 34 and 35, a constant volume of 18,870 ml of gas was produced. On the other hand, Rice straw/ cow dung in ratio 2:1 started producing on day 4, with a volume of 100ml of gas. It produced 1380 ml of gas on day 10. An average of 5580 ml of gas was produced on day 15. This combination showed continuous increases until 34th day when it produced 22,510 ml of gas; which was maintained to day 35.Rice straw/ cow dung 3:1 produced an average of 100 ml of gas on day 4. It produced 1,600 ml of gas on day 10 which was 13.75% greater than what rice straw/ cow dung 2:1 produced on day 10. On day 15, it had produced 5,050 ml of gas. By day 34 and 35, it had a constant volume of 17,440 ml of gas. Also, Cow dung alone started producing on day 6,80ml of gas, which is poor when compared to the performance of rice straw and cow dung combination in ratios

2:1 and 3:1. However, on day 10, it produced an average of 650 ml of gas, which is 20% more than what rice straw/ cow dung 1:1 produced same day. By day 15, it had produced an average of 2750 ml of gas. This is 10.18% more than what rice straw/cow dung 1:1 produced same day. On day 32, it produced 8,770 ml of gas and maintained it to day 35.

Treated rice straw produced 20 ml of gas on day 7, and an average of 170 ml on day 10. By day 15, it had increased to 1,090ml of gas. This increase continued gradually until day 34 where it produced 8,080 ml of gas which was maintained till day 35. Untreated rice straw produced 30 ml of gas on day 8 and 140ml on day 10. By day 15, it had produced 890 ml of gas. It produced 7,080 ml of gas from day 33 to day 35. The best combination in this batch was Rice straw/ cow dung 2:1 based on the data collected over 35 days and all combinations produced higher biogas volumes than controls.

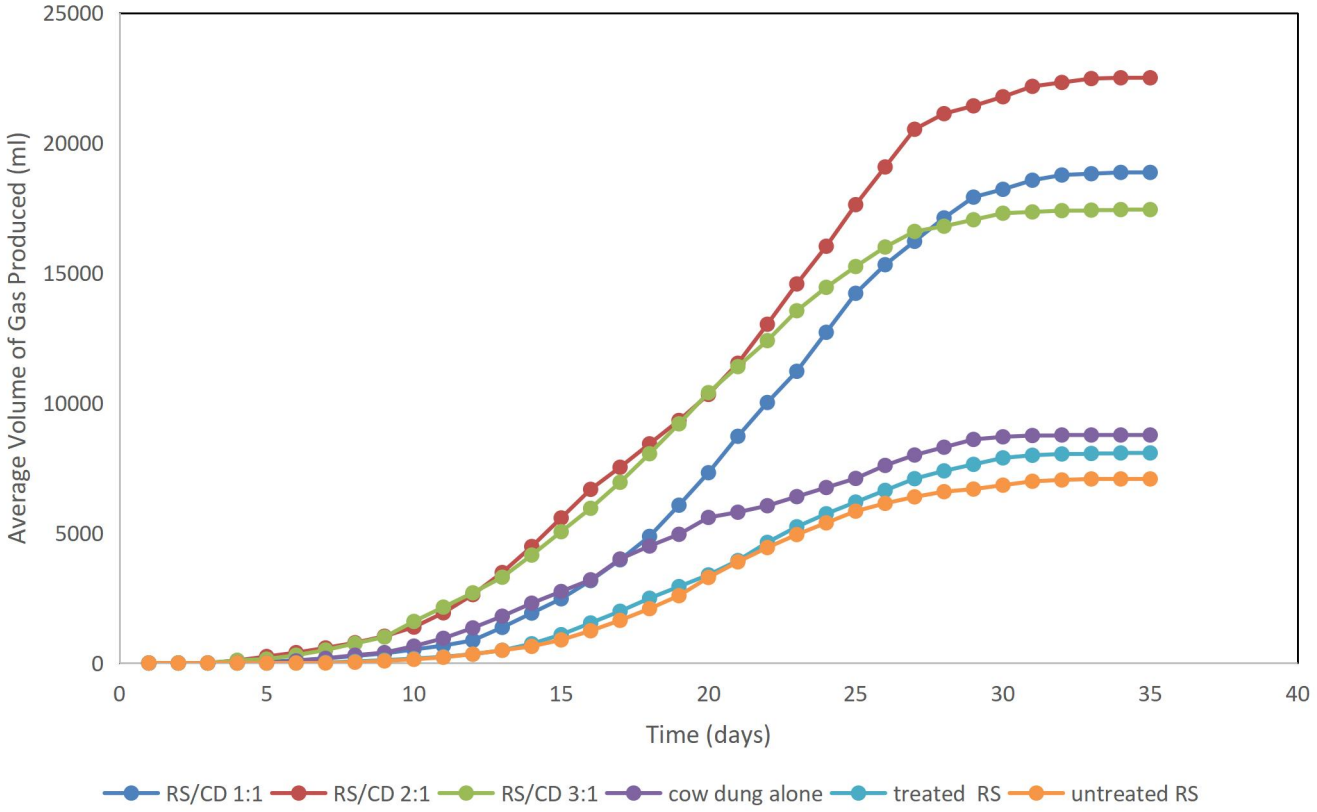


Figure4.6: Average volume of gas produced from pretreated rice straw amended with cow dung over time

B. Amendment of Alkaline Pretreated Rice Straw with Pig Waste

Results of gas produced from the amendment of alkalinepretreated rice straw with piggery waste are shown in figure 4.7. Rice straw/pig waste in ratio of 1:1 started producing on day 5 with an average volume of 150 ml and peaked to a cumulative average of 17,850 ml and 17,870 ml on day 34 and 35. Similarly, rice straw/ pig waste in ratio of 2:1 produced of 100 ml of gas on day 5 and 1,130 ml on day 10. It produced an average of 3,430 ml of gas on day 15 and 18,450 ml on day 35. Also, Rice straw/ pig waste in ratio of 3:1 started producing on day 1, an average of 50 ml of gas. By day 5, it had produced an average of 220 ml of gas and 5,010 ml on day 15. At the end of day 35, it had produced 12,220 ml of gas.

Pig waste started producing on day 6, an average of 50 ml of gas. On day 15, it produced 2,520 ml, and on day 35, it had produced an average of 8000 ml of gas. Treated rice straw started producing on day 7, an average of 20 ml of gas. By day 15, it had produced 1,090 ml and 8,080 ml on day 35. Finally, untreated rice straw started producing on day 8, an average of 30 ml of gas. By day 15, it had produced 890 ml and 7,080 ml on day 35. The best combination in this batch was Rice straw/ pig waste 3:1 based on the data collected over 35 days and all combinations produced higher biogas volumes than controls.

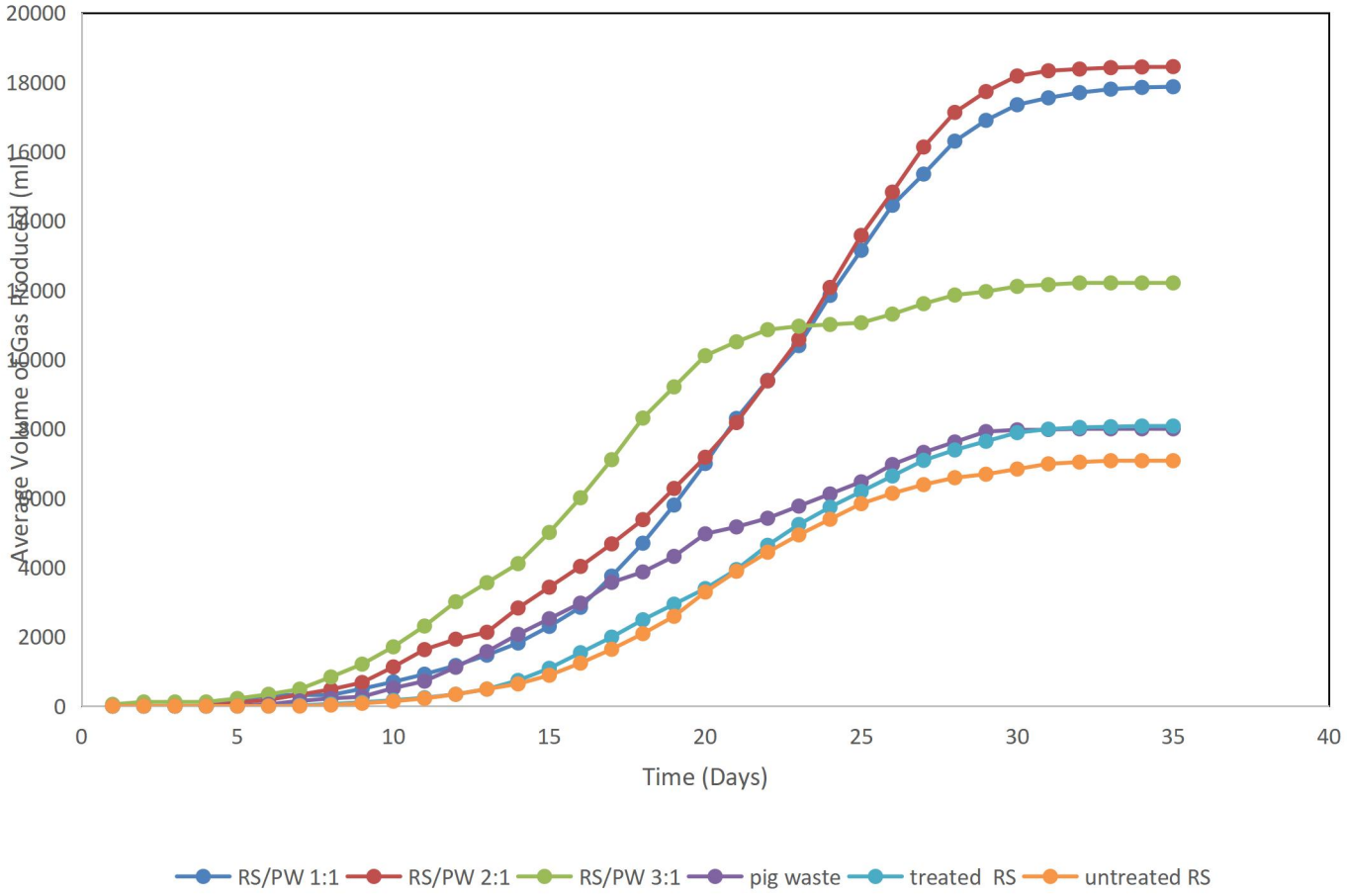


Figure4.7:Average volumes of gas produced from pretreated rice straw amended with pig waste over time

C. Amendment of AlkalinePretreated Rice Straw with Poultry Dropping

Results of gas produced from digestion of alkaline pretreated rice straw amended with Poultry dropping is shown in figure 4.8. Rice straw/poultry droppings in ratio of(1:1)started producing on day 5,with an average of 50 ml of gas. By day 15, it had produced an average of 1,920 ml of gas, and 14,660 ml of gas on day 35. On the other hand, Rice straw/poultry droppings in the ratio of 2:1 started producing on day 7,with 100 ml of gas and 5,000 ml of gas on day 15. By the end of day 35, it had produced 13,030 ml of gas.Rice straw/ poultry droppings in the ratio of (3:1) started producing on day 3,with an average of 50 ml of gas and 4,740 ml of gas on day 15. On day 35, it produced an average of 15,290 ml of gas. Similarly, Poultry droppings started producing on day 5, giving 50 ml of gas and 2,705 ml on day 15. On day 35, it had produced 8,145 ml of gas.

Pretreated rice straw without amendmentstarted producing on day 7, with 20 ml of gas and 1,090 ml of gas on day 15. On day 35, it produced an average of 8,080 ml of gas. Untreated rice straw started producing on day 8, an average of 30 ml of gas. On day 15, it had produced an average of 890 ml of gas and an average of 7,080 ml of gas on day 35.

The best combination was rice straw/ poultry droppings in the ratio of (3:1), closely followed by rice straw/poultry droppings in the ratio of (2:1) based on the data collected over 35 days All amended samples produced higher biogas volumes than control.

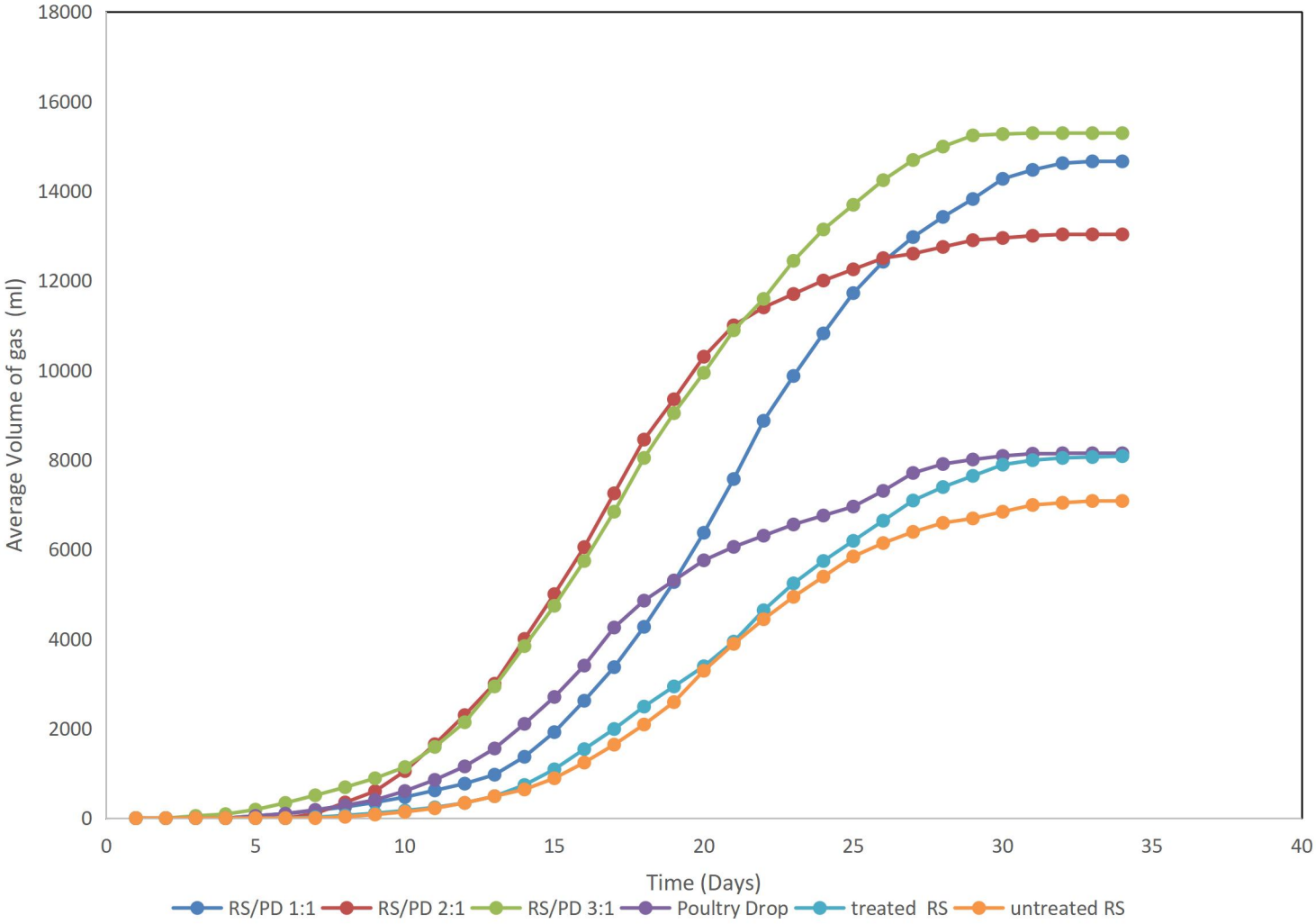


Figure 4.8: Average volume of gas produced from pretreated rice straw amended with poultry dropping over time

D. Anaerobic Digestion of Combinations of different Amendments (PW/CD)

Results of biogas produced from digestion of different ratios of amendment of poultry dropping and Cow dung, are shown in figure 4.9. Pig waste/ cow dung (1:1) combination produced 300 ml of gas on day 5. On day 15, it produced an average of 5,450 ml of gas and 16,600 ml of gas on day 35. Similarly, Pig waste/cow dung (2:1) produced an average of 100 ml on day 5 and 1730 ml of gas on day 15. By day 35, it had produced an average of 11,830 ml of gas.

Pig waste/cow dung(3:1) started producing on day 6, an average of 200 ml of gas. On day 15, it produced an average of 4,030 ml of gas and on day 35, an average of 15,980 ml of gas. Also, cow dung produced an average of 80 ml of gas on day 6 and 2,300 ml on day 15. By day 35, it had produced an average of 8,770 ml of gas. Finally, pig waste produced an average of 50 ml of gas on day 6 and 2,070 ml of gas on day 15. On day 35, it had produced an average of 8,000 ml of gas.

The best combination in this batch was Pig waste/ cow dung (1:1) based on the data collected over 35 days and all combinations produced higher biogas volumes than controls.

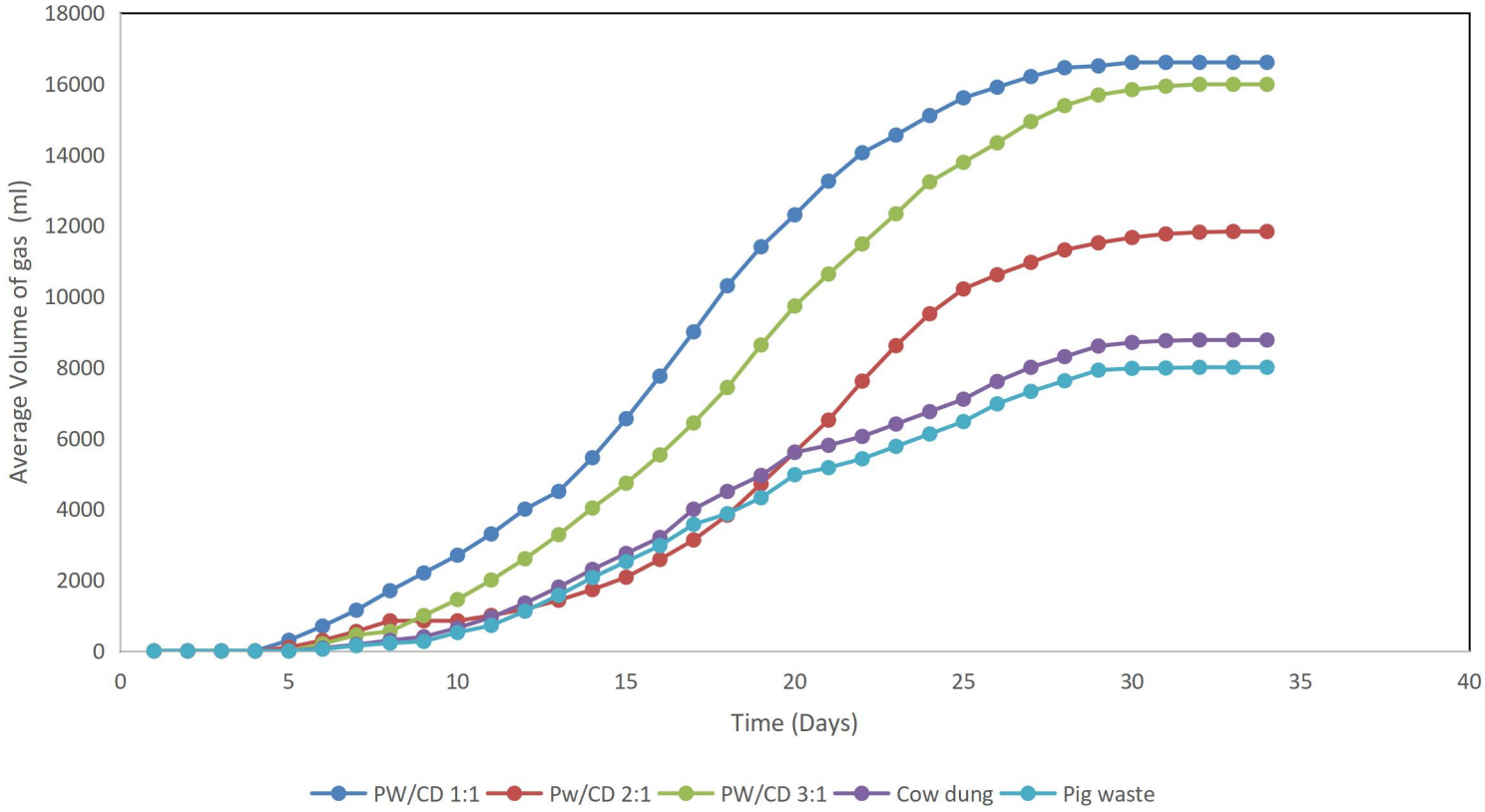


Figure4.9:Average volume of gas produced from co-digestion of pig waste and cow dung over time

E. Anaerobic digestion of combinations of different amendments (PW and PD)

Results of the gas produced from digestion of different ratios of amendments, of poultry dropping and piggery waste, are shown in Figure 4.10. Pig waste/poultry droppings (1:1) started producing on day 5, an average of 30 ml of gas and 5,180 ml of gas on day 15. By day 35, it had produced an average of 14,190 ml of gas.

Pig waste/poultry droppings (2:1) produced an average of 150 ml of gas on day 5, and 4,080 ml of gas on day 15. On day 35, it had produced 11,230 ml of gas. In addition, pig waste/poultry waste (3:1) started producing on day 4, yielding an average of 150 ml of gas. On day 15, it produced an average of 4,330 ml and on day 35, it had an average of 12,670 ml of gas.

Considering the controls, Pig waste started producing on day 6, with an average of 50 ml of gas. On day 15, it produced an average of 2,520 ml of gas, and 8,000 ml on day 35. Also, Poultry droppings produced an average of 50 ml on day 5 and an average of 2,705 ml of gas on day 15. By day 35, it had produced an average of 8,145 ml of gas.

The best combination in this batch was Pig waste/poultry droppings (1:1) based on the data collected over 35 days ratios of all amendment produced higher biogas volumes than controls. The different combinations that produced the highest yields were compared in figure 4.6. Rice straw amended with Cow dung in the ratio 2:1 ratio had the highest cumulative gas yield while Rice straw amended with poultry dung had the lowest cumulative yield of biogas.

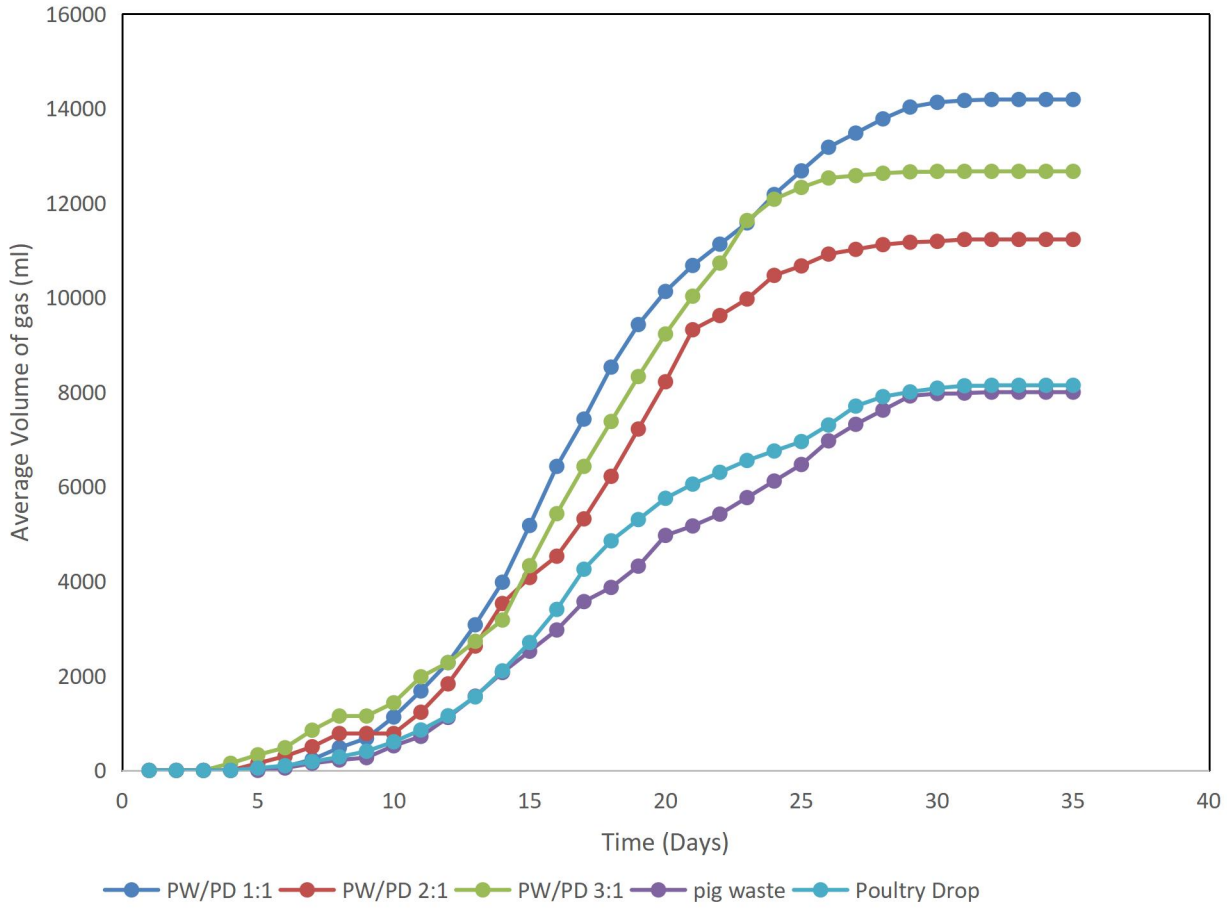


Figure 4.10: Average volume of gas produced from co-digestion of pig waste and poultry dropping over time

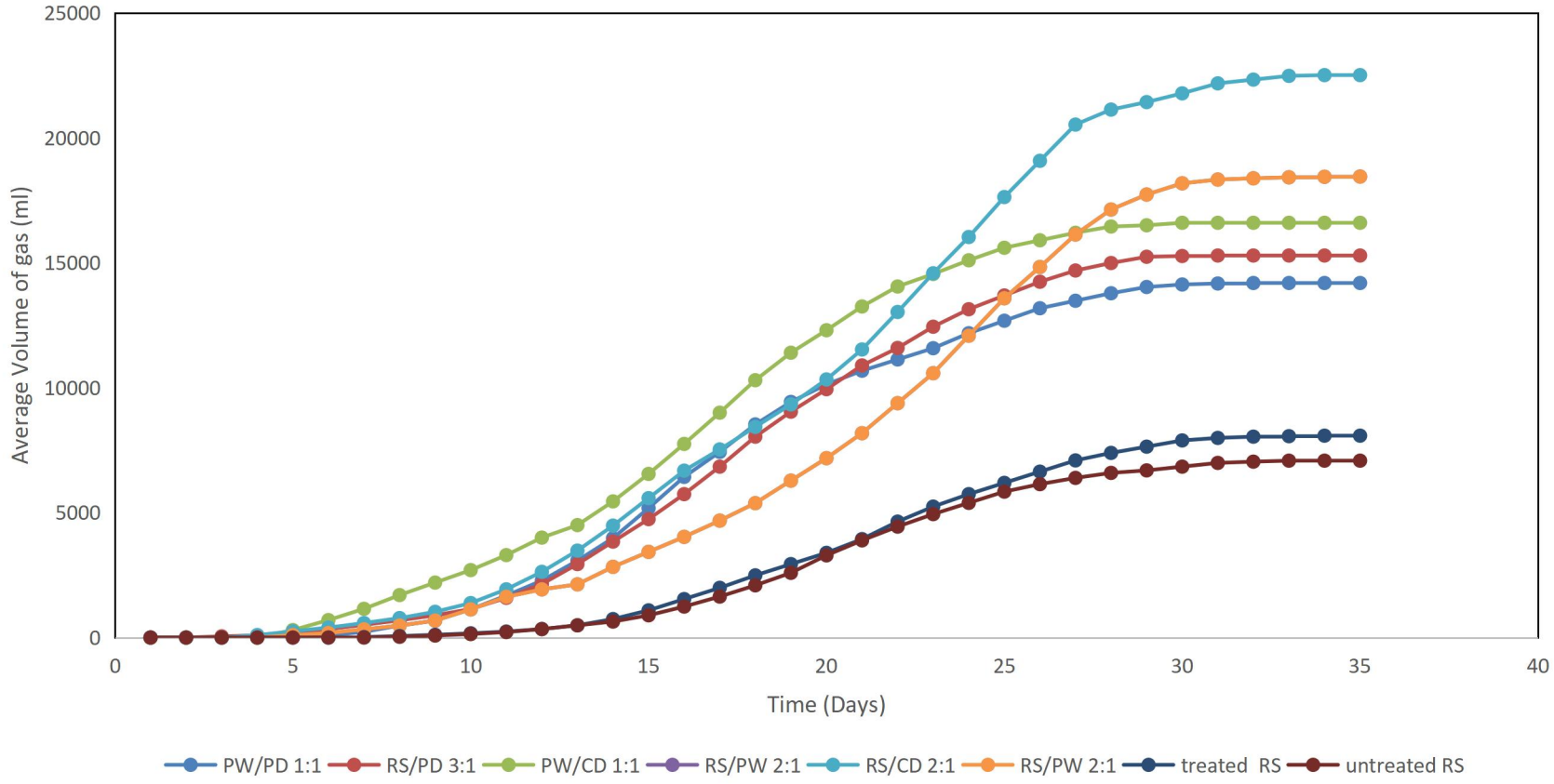


Figure 4.11: Summary of best co-digestions and control

Table 4.4 : Maximum Cumulative Biogas Yield (dm^3) from the Alkaline Pre-treated Rice Straw and the amendments.

Treatments	Biogas Yield(dm^3)
APRS	8.08
(URS)	7.08
APRS/CD 1:1	18.87
APRS/CD 2:1	22.51
APRS/CD 3:1	17.44
APRS/PD 1:1	14.66
APRS/PD 2:1	13.03
APRS/PD 3:1	15.29
APRS/PW 1:1	17.78
APRS/PW 2:1	18.45
APRS/PW 3:1	12.21

Legend: APRS = Alkaline Pretreated Rice Straw
URS = Untreated Rice Straw

From table 4.4 above it can be seen that alkaline pre-treatment enhanced biogas production by 12.38% though there was no statistical difference in biogas yield in APRS compared to the URS, there was a significant difference ($P \leq 0.05$) in biogas yield in all the APRS amended with animal manure compare to APRS and URS alone. The APRS co-digested with different animal manure, APRS/CD 2:1 showed the highest yield in biogas followed by APRS/CD 1:1, with a cumulative biogas yield of 22.51 and 18.87dm^3, respectively.

Table4.5: Maximum Cumulative Biogas Yield from the Biologically Pretreated Feedstock and Amendments.

Treatments	Biogas Yield (dm^3)
BPRS/CD 1:1	27.31
BPRS/CD 2:1	12.28
BPRS/CD 3:1	13.90
BPRS/PD 1:1	19.55
BPRS/PD 2:1	22.47
BPRS/PD 3:1	18.26
BPRS/PW 1:1	18.13
BPRS/PW 2:1	19.03
BPRS/PW 3:1	14.60
BPWH/CD 1:1	13.35
BPWH/CD 2:1	8.02
BPWH/CD 3:1	10.89
BPWH/PD 1:1	11.01
BPWH/PD 2:1	8.73
BPWH/PD 3:1	6.77
BPWH/PW 1:1	7.34
BPWH/PW 2:1	7.81
BPWH/PW 3:1	6.62

BPRS= Biologically Pretreated Rice Straw.
BPWH = Biologically Pretreated Water Hyacinth

From table 4.5 above it can be seen that bacteria pre-treatment enhanced biogas production by 45.45% though there was no statistical difference in biogas yield in BPRS compared to the URS, there was a significant difference ($P \leq 0.05$) in biogas yield in all the BPRS amended with animal manure compare to BPRS and URS alone. The BPRS co-digested with different animal manure, BPRS/CD 1:1 showed the highest yield in biogas followed by BPRS/PD 2:1, with a cumulative biogas yield of 27.31 and 22.47dm^3, respectively.

4.1.4aAMENDMENT OF BACTERA PRETREATED SUBSTRATES

A. Amendment of Bacteria Pretreated Rice Straw with Cow Dung

The Biogas yield of bacteria pretreated rice straw amended with cow dung is shown in figure 4.12. Rice straw/cow dung (1:1) produced an average of 1130 ml of gas on day 5; 8,950 ml on day 15; and on day 35, it produced 27,050 ml of gas. On the other hand, Rice straw/cow dung (2:1) produced an average of 50 ml on day 5; 4910 ml of gas on day 15; and an average of 12,280ml on day 35.Rice straw/ cow dung (3:1) started producing on day 6, with an average of 40 ml of gas; 1900 ml of gas on day 15; and an average of 13,600ml on the 35th day. Also, Cow dung started producing on day 6, giving an average of 80 ml of gas; with 2750 ml of gas on day 15; and 8770 ml of gas on day 35.

Bacterial treated rice straw with amended, started producing on day 7, yielding an average of 20 ml of gas; an average of 1090 ml of gas on day 15; and 8080 ml of gas on day 35.Untreatedrice straw started producinga day later than the treated sample, with an averaged 30 ml of gas on day 15; and a cumulative average of 890 ml of gas on day 35. The best combination in this batch was rice straw/cow dung (1:1) based on the data collected over 35 days and all amendment ratios produced higher biogas volumes than controls.

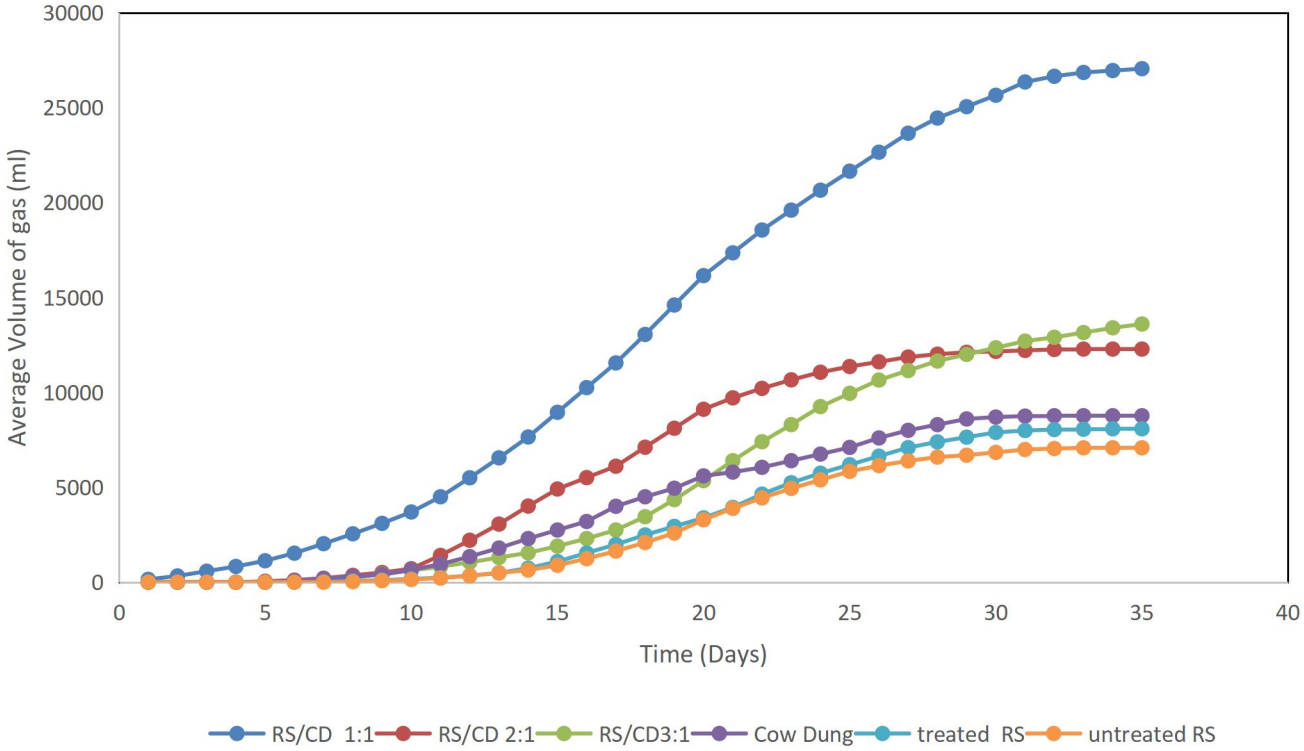


Figure 4.12: The biogas yield of bacterial pretreated rice straw amended with cow dung over time.

B. The biogas yield of bacterial pretreated rice straw amended with Poultry Droppings

The Biogas yield of bacterial pretreated rice straw amended with poultry droppings is shown in Figure 4.13. Rice straw/poultry droppings (1:1)combination produced an average of 170 ml of gas on day 5; an average of 4,000 ml of gas on day 15; and 19,050 ml of day 35.Similarly, Rice straw/poultry droppings (2:1) produced an average of 200 ml of gas on day 5; an average of 4400 ml of gas on day 15; and a cumulative average volume of 21,870 ml on the 35^{th} day.

Rice straw/poultry droppings (3:1) started producing on day 6, giving an average of 100 ml; an average of 2,780 ml on day 15; and an average of 17,810 ml on day 35. Also, Poultry droppings produced an average of 50 ml of gas on day 5; an average of 2,705ml on day 15; and an average of 8,145 ml on day 35.

Considering the controls, pretreated rice straw started producing on day 7, with an average of 20 ml of gas; 1090 ml on day 15; and 8,080 ml on day 35;Untreated rice straw started producing on day 8, with an average of 30ml of gas; an average of 890 ml on day 15; and 7,080 ml of gas on day 35.

The best combination in this batch was rice straw/poultry droppings (3:1) based on the data collected over 35 days all amendment combinations produced higher biogas volumes than controls.

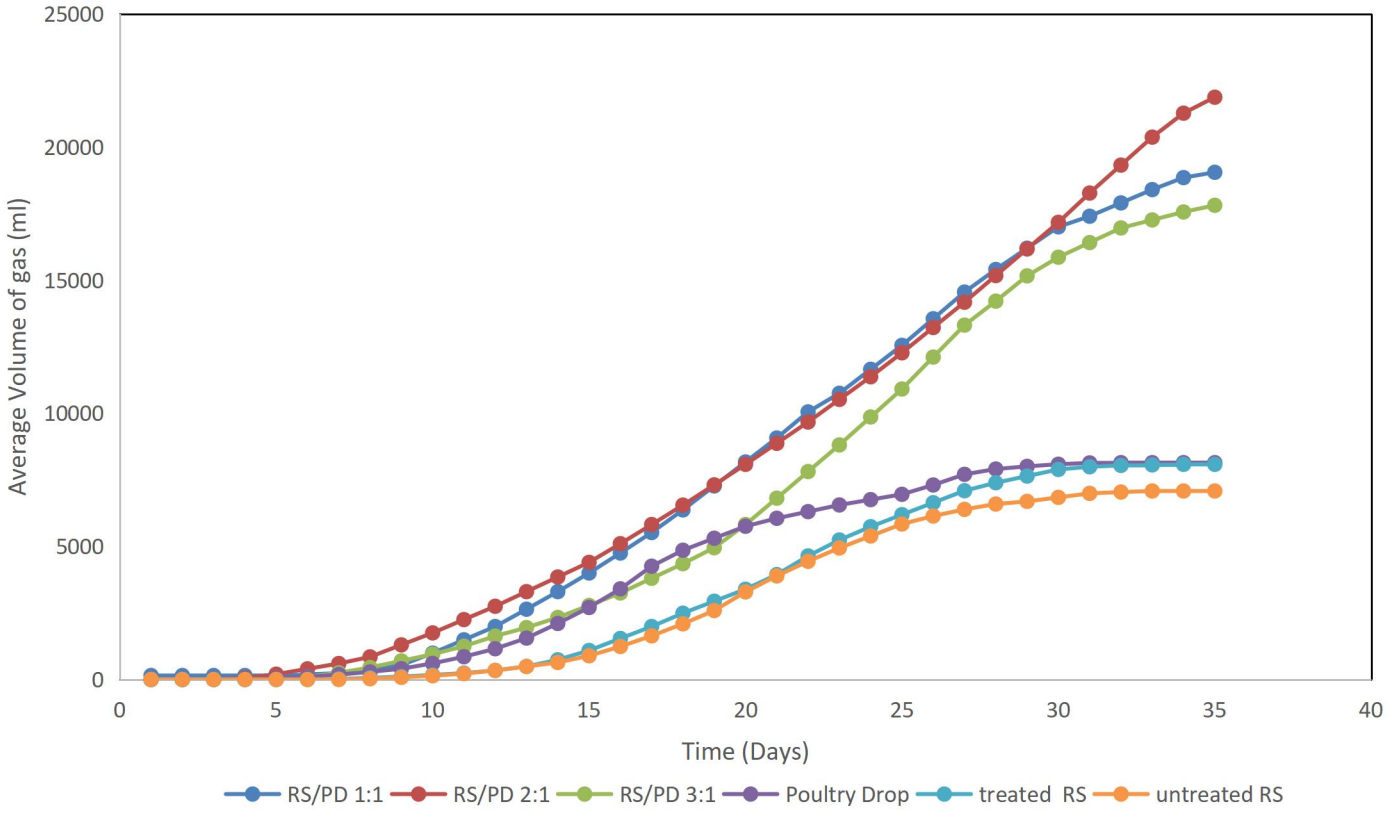


Figure 4.13: The biogas yield of bacterial pretreated rice straw amended with poultry droppings over time.

C.Biogas yield of bacterial pretreated rice straw amended with Pig Waste

Biogas yield of bacterial pretreated rice straw amended with Pig Waste is shown in Figure 4.14. The rice straw/pig waste in (1:1) combination produced an average of 700 ml of gas on day 5; an average of 5,530 ml of gas on day 15; and an average of 18,010 ml of gas on the 35th day. Also, Rice straw/pig waste (2:1) combination, produced an average of 100 ml of gas on day 5; 3,160 on day 15; and an average of 18,630 ml of gas on day 35. The rice straw/ pig waste (3:1) started gas production on day 7 with an average of 50 ml of gas; an average of 1,070 ml on day 15; and an average of 13,820 ml of gas on day 35.

On the other hand, the Pig waste started producing on day 6, with an average of 50 ml of gas; 2520 ml of gas on day 15; and 8,000 ml on day 35; Treated rice straw started producing on day 7, withan average of 20 ml of gas; 1,090 ml of gas on day 15; and 8,080 ml of gas on day 35. And Finally, untreated rice started producing on day 8,giving an average of 20 ml of gas; 8, 890 ml of gas on day 15; and 7,080 ml of gas on day 35.

The best combination in this batch was rice straw/ pig waste (2:1) based on the data collected over 35 days. All amendment combinations produced higher biogas volumes than controls.

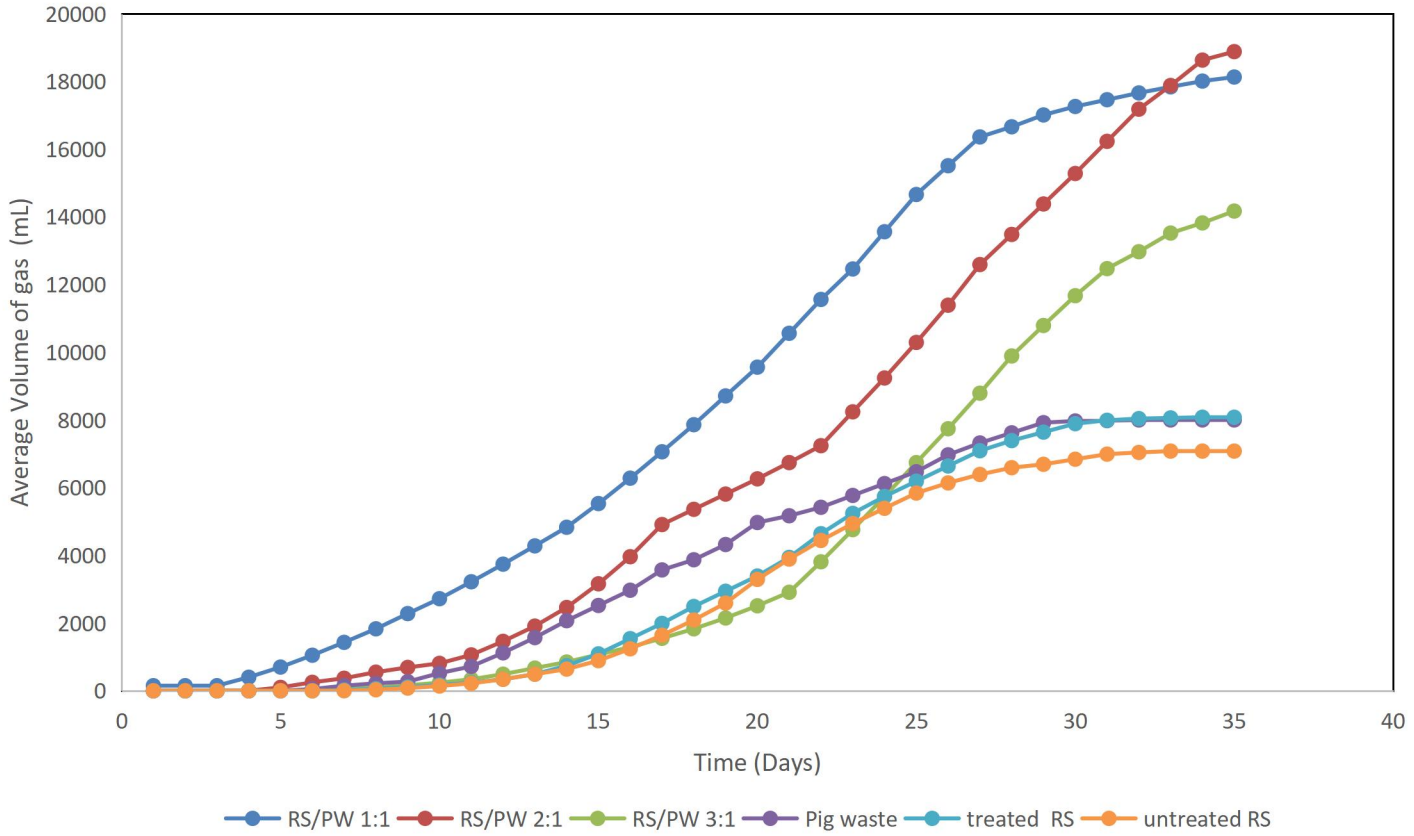


Figure 4.14: The biogas yield of bacterial pretreated rice straw amended with Pig Waste over time

D. Biogas Produced from Digestion of Water hyacinth and Cow Dung

Biogas produced from digestion of Water hyacinth and Cow Dung is shown in Figure 4.15. The Water hyacinth/cow dung (1:1) combination produced an average volume of 110 ml of gas on day 5; an average of 2350 ml of gas on day 15; and an average of 12,030 ml of gas on day 35. More so, Water hyacinth/ cow dung (2:1) started producing on day 6, an average of 20 ml of gas; an average of 910 ml of gas on day 15; and an average of 7,820 ml of gas on day 35. On the other hand, Water hyacinth/ cow dung 3:1 started producing on day 5,giving an average of 20 ml of gas; an average of 980 ml of gas on day 15; and an average of 9,990 ml of gas on day 35.

Controls which include Cow dung and water hyacinth started producing on day 6, an average of 80 ml and 50 ml of gas respectively; an average of 2750 ml and 1700 ml of gas respectively on day 15; and an average of 8,770 ml and 7370ml of gas respectively on day 35. The best combination in this batch was Water hyacinth/ cow dung (1:1) based on the data collected over 35 days and all amendment combination produced higher volumes of biogas than tested controls.

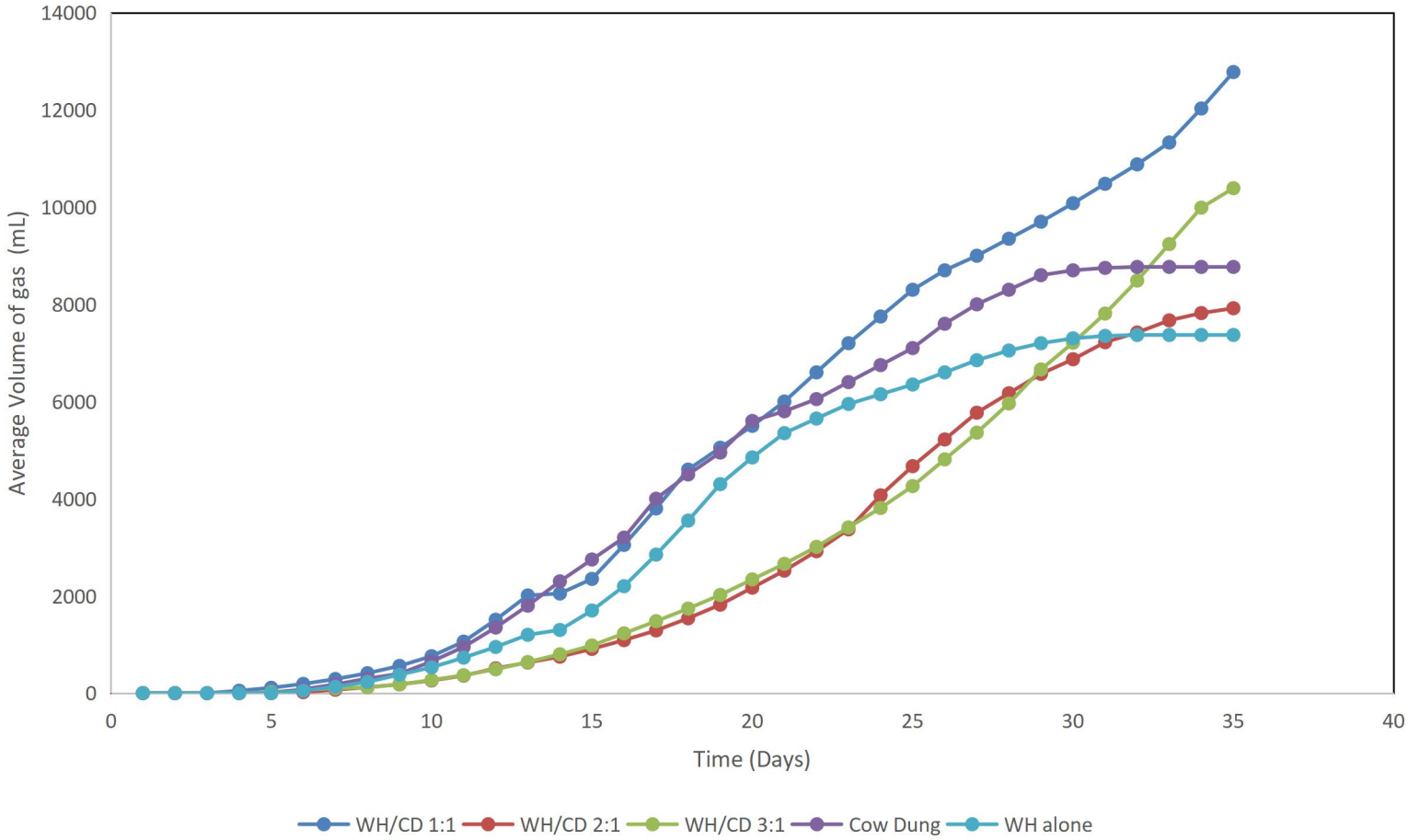


Figure 4.15: Volume of biogas produced from the digestion of water hyacinth and cow dung over time

E. Amendment of Water Hyacinth and Poultry Drop

Biogas produced from digestion of Water hyacinth and poultry droppings is shown in Figure 4.16.Water hyacinth/ poultry droppings in 1:1 combination, produced an average of 100 ml of gas on day 5; an average of 1780 ml of gas on day 15; and an average of 11,010 ml of gas on day 35. Also, Water hyacinth/ poultry Droppings in 2:1 produced an average of 20 ml of gas on day 5; an average of 1420 ml of gas on day 15; and an average of 8690 ml of gas on day 35. Similarly, Water hyacinth/ poultry droppings 3:1 produced an average of 60 ml of gas on day 5; an average of 1890 ml of gas on day 15; and an average of 6,760 ml of gas on day 35.

Considering the controls, Poultry droppings produced an average of 50 ml of gas on day 5; an average of 2705 ml of gas on day 15; and an average of 8145 ml of gas on day 35;Water hyacinth produced an average of 50 ml of gas on day 6; an average of 1,700 ml of gas on day 15; and an average of 7,370 ml of gas on day 35.

The best combination in this batch was water hyacinth/poultry droppings (1:1) based on the data collected over 35 days.

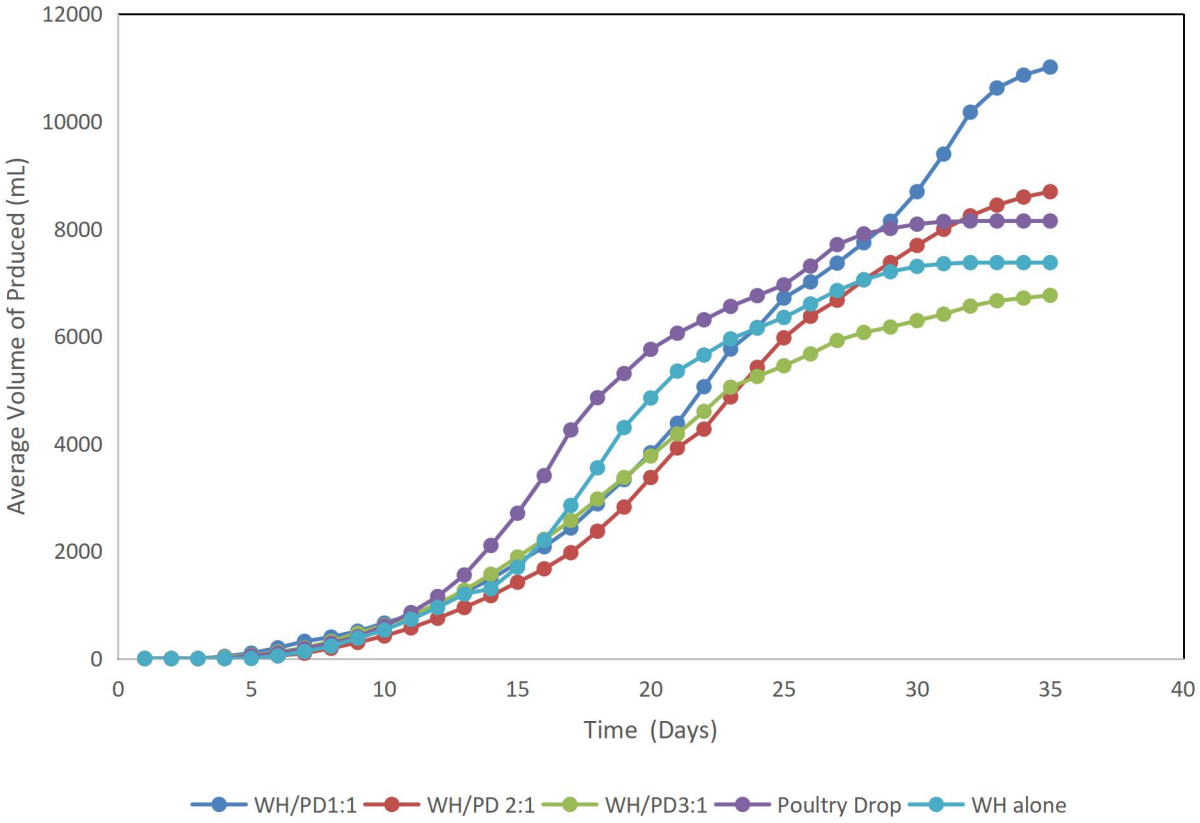


Figure 4.16: The volume of Biogas produced from the Digestion of Water hyacinth with poultry droppings over time

F. Amendment of Water Hyacinth and Pig Waste

Biogas produced from the digestion of Water hyacinth and Pig waste is shown in Figure 4.17. The Water hyacinth/ pig waste in ratio 1:1 produced an average of 40 ml of gas on day 5; an average of 1590 ml of gas on day 15; and an average of 7,340 ml of gas on day 35. On the other hand, Water hyacinth/ pig waste (2:1) produced an average of 20 ml of gas on day 5; an average of 1420 ml of gas on day 15; and an average of 7,770 ml of gas on day 35. Also, Water hyacinth/ pig waste (3:1) produced an average of 40 ml of gas on day 5; an average of 1820 ml of gas on day 15; and an average of 6,620 ml of gas on day 35.

For controls, which involved isolated use of Pig waste and Water hyacinth without amendment,produced an average of 50 ml each of gas on day 6; an average of 2,520 ml and 1700 ml of gas respectively on day 15; and an average of 8,000 ml and 7370 ml of gas respectively on day 35.

The best combination in this batch was pig waste, closely followed by Water hyacinth/ pig waste (2:1) based on the data collected over 35 days and none of the amendment's combination produced higher biogas volumes than tested controls.

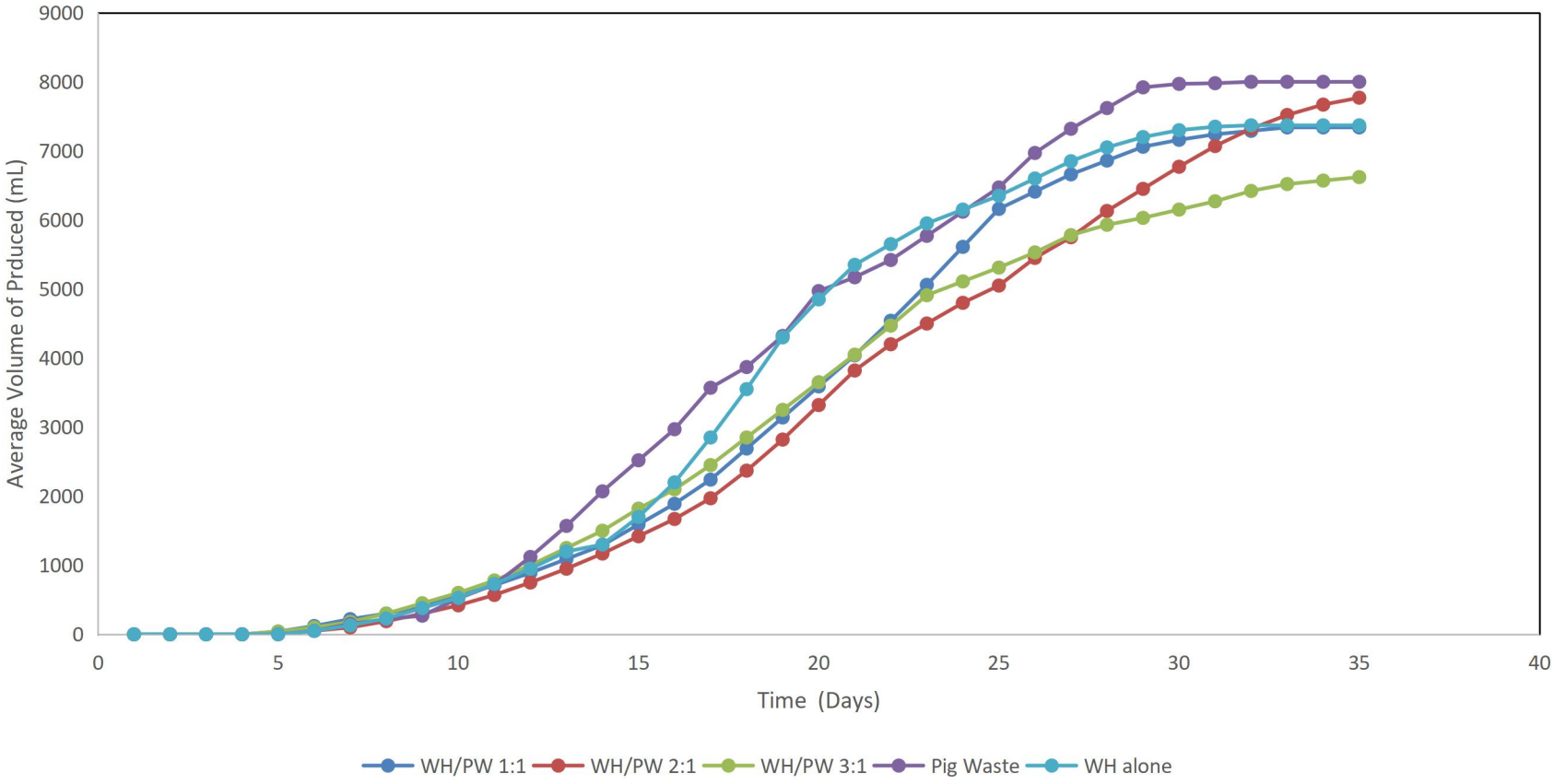


Figure 4.17:Volume of biogas produced from the digestion of water hyacinth and pig waste over time

G. Biogas yield of bacterial pretreated Water Hyacinth amended with Cow dung

Biogas produced from digestion of Water hyacinth and Cow Dung is shown in Figure 4.18Water hyacinth/cow dung (1:1) combination produced an average volume of 50 ml of gas on day 4; an average of 9460 ml of gas on day 15; and an average of,13560 ml of gas on day 38. More so, Water hyacinth/ cow dung (2:1) started producing on day 6, an average of 20 ml of gas; an average of 3720 ml of gas on day 15; and an average of 715560ml of gas on day 37. On the other hand, Water hyacinth/ cow dung 3:1 started producing on day 6,giving an average of 20 ml of gas; an average of 1140ml of gas on day 15; and an average of 13245 ml of gas on day 37.

Controls which include Cow dung started producing on day 6, an average of 80 ml of gasan average of 2750 ml on day 15; and an average of 8,770 ml on day 35.

The best combination in this batch was Water hyacinth/ cow dung (2:1) based on the data collected over 37 days and all amendment combination produced higher volumes of biogas than tested control.

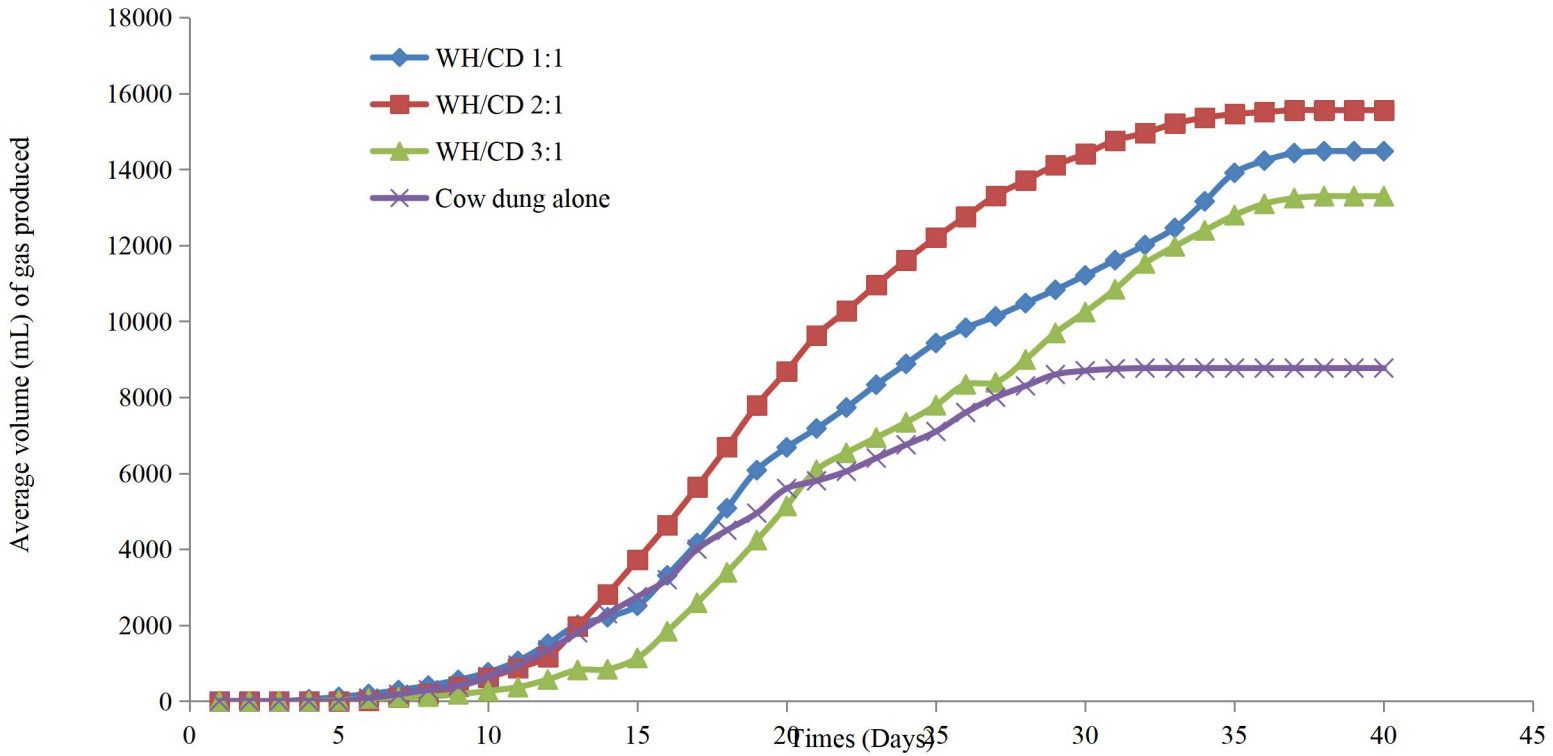


Figure 4.18: The volume of Biogas produced from the Digestion of Water hyacinth and cow dung over time

H. Biogas yield of bacterial pretreated Water Hyacinth amended with poultry dropping

Biogas produced from digestion of Water hyacinth and poultry droppings is shown in Figure 4.19.Water hyacinth/ poultry droppings in 1:1 combination produced an average of 100 ml of gas on day 4 an average of 2500 ml of gas on day 15; and an average of 12670 ml of gas on day 356. Also, Water hyacinth/ poultry droppings in 2:1 produced an average of 20 ml of gas on day 4; an average of 2500 ml of gas on day 15; and an average of 13830 ml of gas on day 36. Similarly, Water hyacinth/ poultry droppings 3:1 produced an average of 20 ml of gas on day 5; an average of 2790 ml of gas on day 15; and an average of 9010 ml of gas on day 35. Considering the controls, Poultry droppings produced an average of 50 ml of gas on day 5; an average of 2705 ml of gas on day 15; and an average of 8145 ml of gas on day 35.

The best combination in this batch was water hyacinth/poultry droppings (2:1) based on the data collected over 35 days

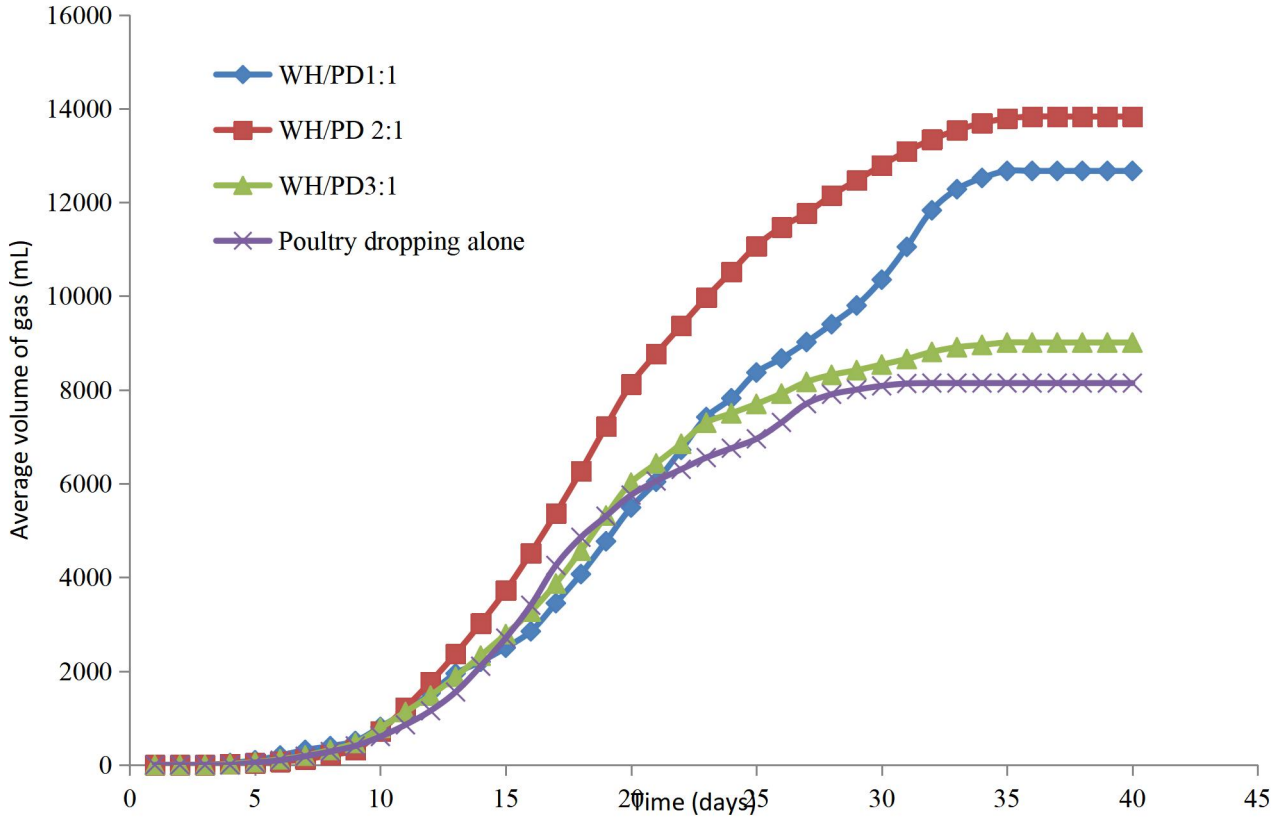


Figure 4.19: Volume of biogas produced from the digestion of water hyacinth and poultry dropping over time

I. Biogas yield of bacterial pretreated Water Hyacinth amended with pig waste

Biogas produced from the digestion of Water hyacinth and Pig waste is shown in Figure 4.20. The Water hyacinth/ pig waste in ratio 1:1 produced an average of 40 ml of gas on day 5; an average of 1990 ml of gas on day 15; and an average of 9860 ml of gas on day 33. On the other hand, Water hyacinth/ pig waste (2:1) produced an average of 20 ml of gas on day 5; an average of 1420 ml of gas on day 15; and an average of 7,770 ml of gas on day 35. Also, Water hyacinth/ pig waste (3:1) produced an average of 20 ml of gas on day 5; an average of 1820 ml of gas on day 15; and an average of 6,620 ml of gas on day 35.

For controls, which involved isolated use of Pig waste alone, produced an average of 50 ml each of gas on day 6; an average of 2,520 ml and 1700 ml of gas respectively on day 15 and 35 respectively.

The best combination in this batch was pig waste, closely followed by Water hyacinth/ pig waste (2:1) based on the data collected over 35 days and none of the amendments combinations produced higher biogas volumes than tested controls.

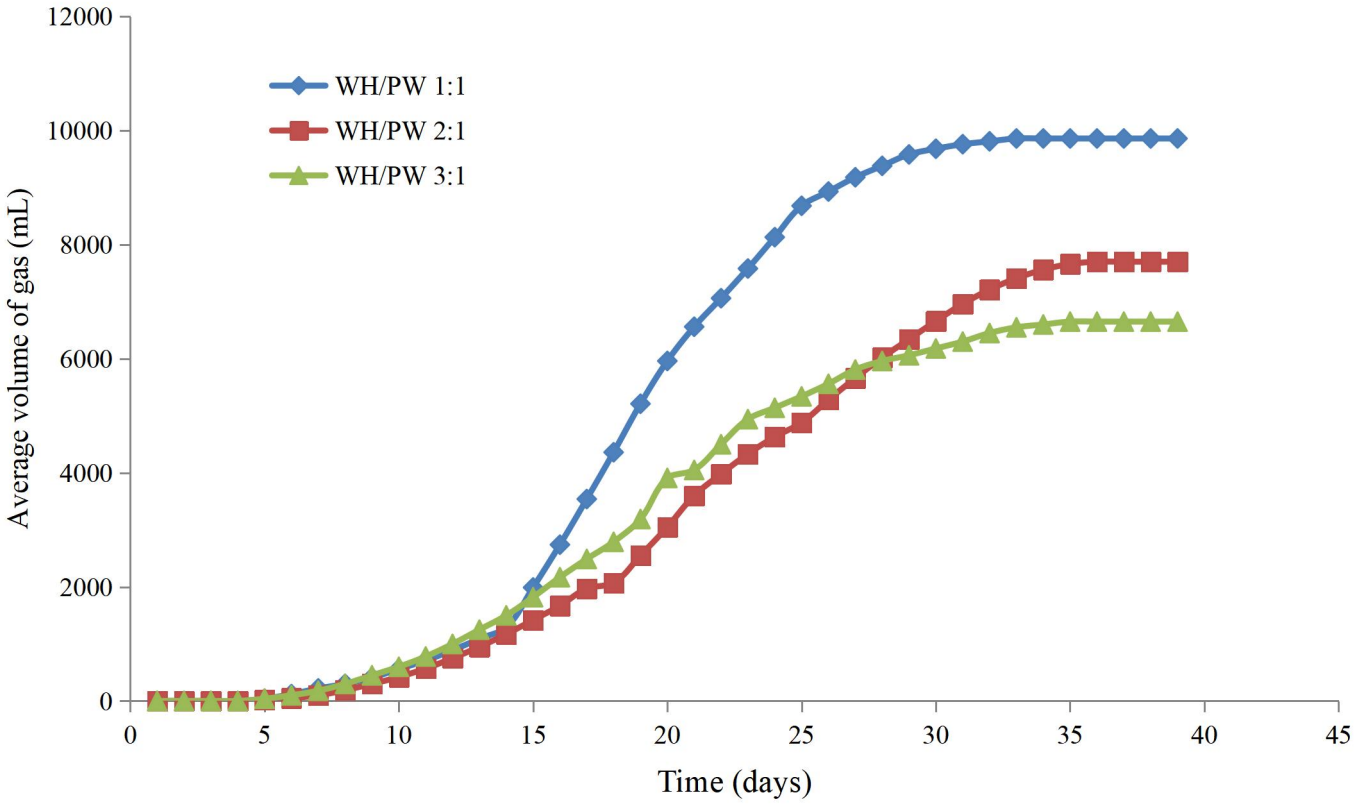


Figure 4.20: Volume of Biogas produced from the Digestion of Water hyacinth and poultry dropping over time

4.1.5OPTIMIZATION OF BIOGAS PRODUCTION FROM PRETREATED SUBSTRATES AMENDED WITH COW DUNG

4.1.5.1. OPTIMIZATION OF BIOGAS PRODUCTION FROM BACTERIA – PRETREATEDRICE STRAW (RS) AMENDED WITH COW DUNG

A.Main Effects and InteractionPlots ofFactors Affecting Methane (ppm) Production from Bacteria Pre-treated Rice Straw

To understand the effect of different parameters such as hydraulic retention time (HRT), substrate concentration and concentration of amendments on the production of methane gas, main effect plots were obtained from collected data using Minitab 17. The plots in Figure 4.21 show the single effects of tested factors.It indicated that the volume of methane produced increased from 5000ml to about 13500ml and began to plateau as the substrate concentration was increased from 100g to 500g. Similar results were obtained for the hydraulic retention time (HRT) over the 15 – 30 days showed that biogas production was initially low (about 10,000ml) at 15 days, but spiked to maximum of 11,500ml at 22 days before leveling off. It continued to gradually decrease from day 26 till the end of 30 days.Addition of cow dung as amendment resulted in a consistent increase in biogas production, which rose from below 5000ml at when 100g of cow dung was used to over 13000ml when 500g of it was used.

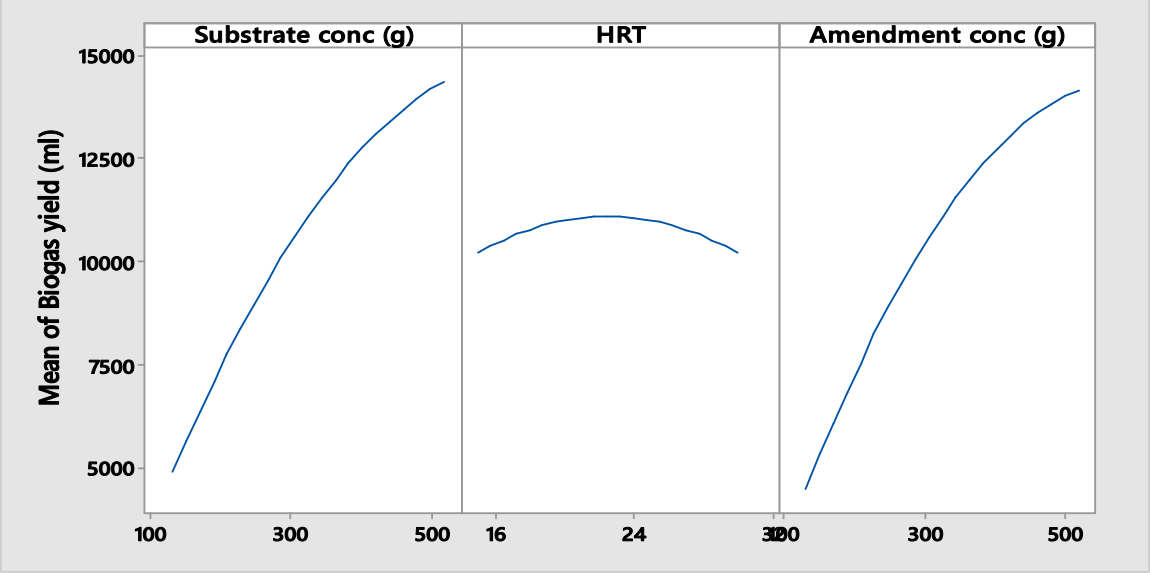


Figure 4.21: Main effects plot for the production of methane (ppm) following bacterial pre-treatment of the substrate

On the other hand, Figure 4.22 shows the interactions between substrate, HRT, and amendment which indicated that there are no considerable interactions between the substrate and HRT within the tested range hence the interactions were not destructive. This implies that measurement of production of gas is favourable at the tested HRT range of 15 – 30 days. Considering substrate and amendment, there were no interactions at different levels of amendment. These independent interactions between amendment concentrations of 130,325 and 520 and substrate concentration resulted in an increase in methane production. However, methane production reduced as a result of the interaction between amendments and HRT at concentration of 130,325 and 520.

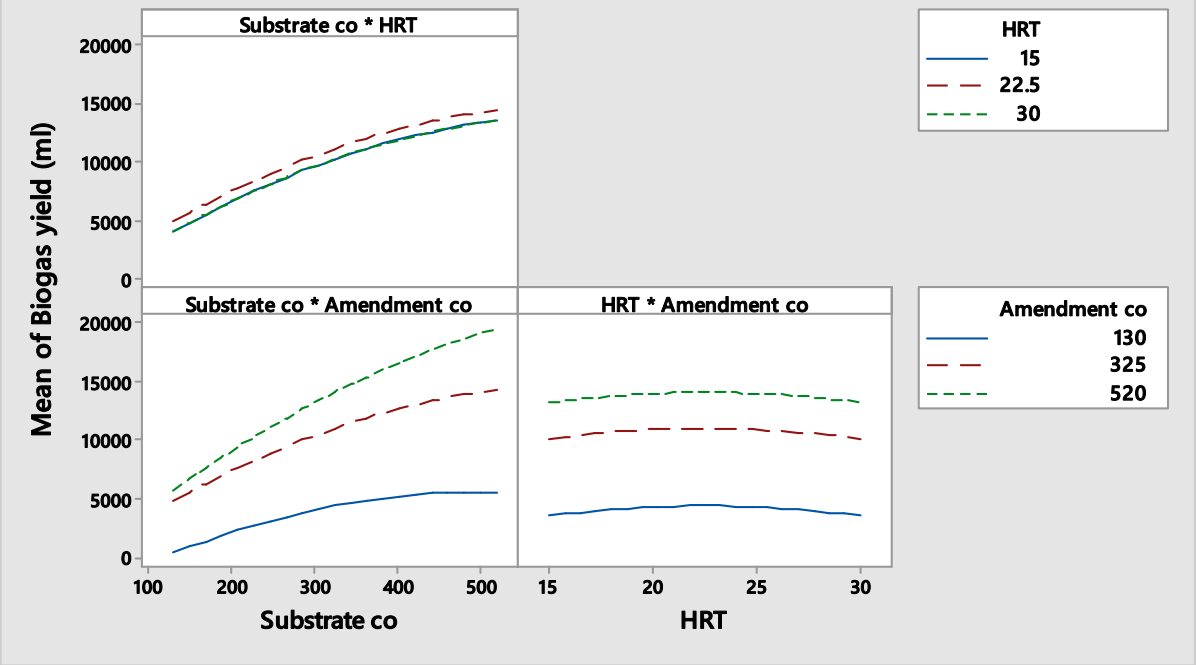


Figure 4.22: Interaction plot for Methane (ppm) production following bacterial pre-treatment of the substrate

Finally, for HRT and Amendment, interaction had it that at an amendment of 130,325 and 520, methane production had no visible interaction with other effects at other amendment. Consequently, there was no interaction between amendment which resulted to a decrease in methane production.

B. Response Surface Plots for the Production of Methane

The Response Surface Plots help in understanding the main effects and interaction plots by analysing each interaction separately. At constant holding values of substrate, the production of methane increased when amendment was 0 and continued till concentration of 520. This can be seen in Figure 4.24. Furthermore, Figure 4.23 shows that at constant holding values of HTR, methane production decreased as substrate concentration was increased. There was also an increase in methane production when amendment was at 130 and 520. Additionally,

Figure 4.23 explains that at constant holding values of amendment, the production of methane decreased with increase in substrate concentration and HRT.

Results of the response surface regression shows that none of the factors had significant effect on the production of methane at $P<0.05$ as shown in the appendix A. The regression had an r^2 value of 48.71% indicating that the results occurred by chance.The Regression Equation in Uncoded Units is given as;

Methane (ppm) = 18.3 + 0.0081 Substrate - 0.66 HRT - 0.0239 Amendment

+ 0.000010 Substrate*Substrate + 0.0106 HRT*HRT

+ 0.000070 Amendment*Amendment + 0.00094 Substrate*HRT

- 0.000083 Substrate*Amendment + 0.00044 HRT*Amendment

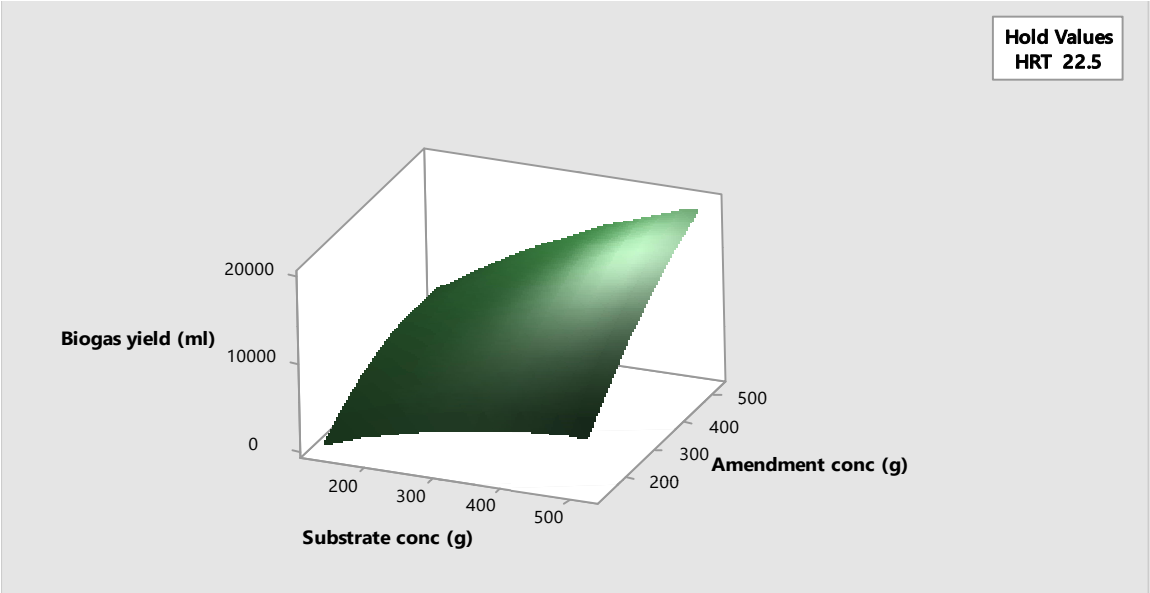


Figure 4.23: Surface Plot of methane (ppm) vs Amendment, HRT after bacterial pretreatment of the substrate

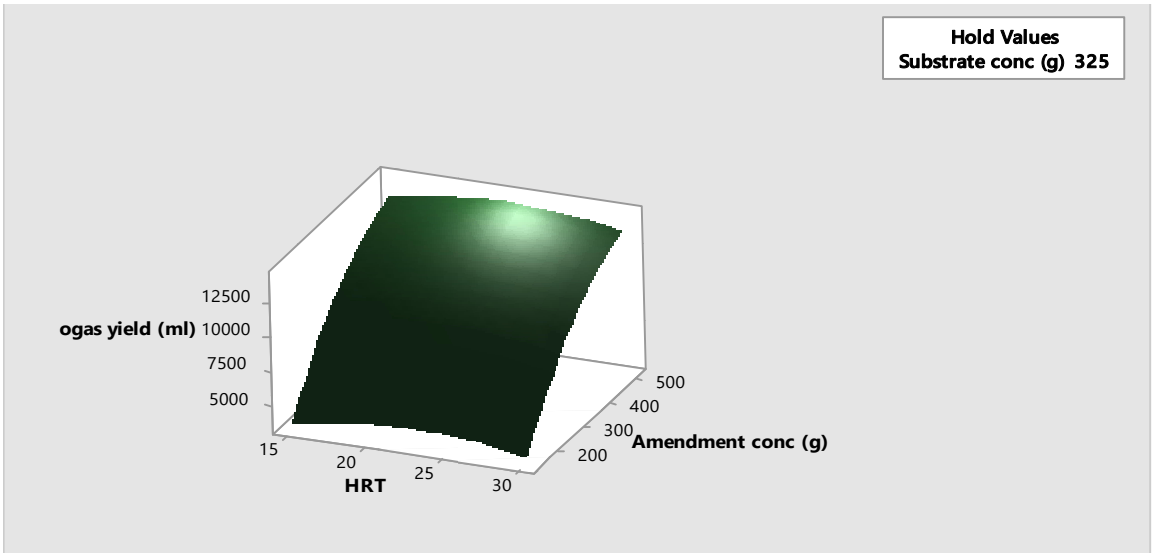


Figure 4.24: Surface Plot of Methane (ppm) vs Amendment, Substrate following bacterial pre-treatment of the substrate.

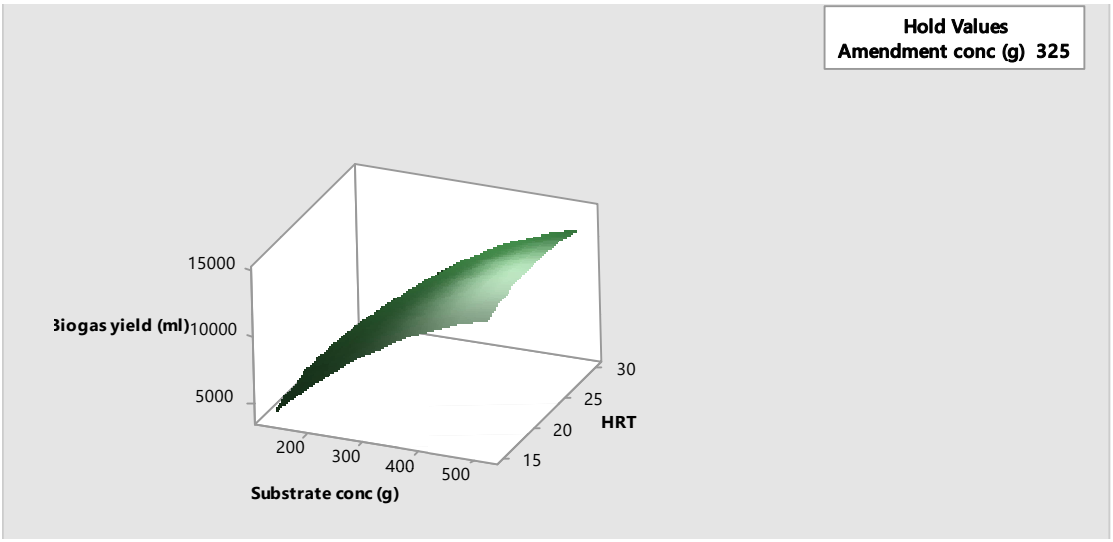


Figure 4.2.5:Surface plot of methane (ppm) vs HRT and substrate following bacterial pretreatment

C.Optimization of Methane Production

It was observed that it took between 3 – 6 days for production of biogas to start in various biodigesters used in this study. Figure 4.26 shows the optimization plots for methane production following bacteria pre-treatment of the substrate. The results show that the

optimum conditions for methane production are substrate concentration of 520g, HRT of 22.57 days and amendment of 520g. At these conditions, the predicted maximum yield that will be achieved will have a response of 1.960 x 10^4 ml.

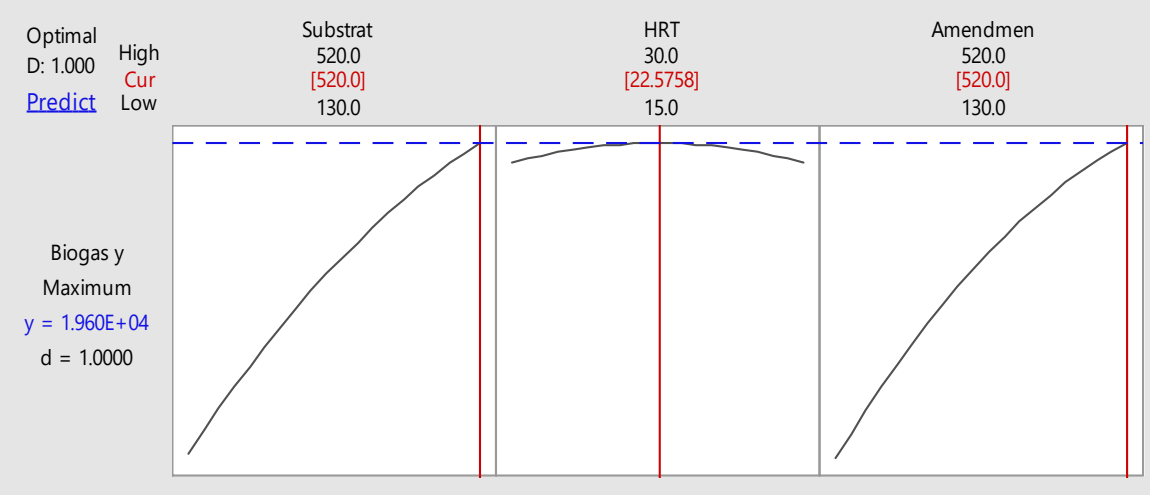


Figure4.26:Optimization plot for methane production after bacterial pretreatment and amendment with cow dung

4.1.5.2OPTIMIZATION OF FACTORS AFFECTING METHANE (PPM) PRODUCTION FROM ALKALINE - PRETREATED RICE STRAW

A. Main Effects Plot of Factors Affecting Methane (ppm) Production from Alkaline - Pretreated Rice Straw

As in the case of bacterial pretreated sample. Figure 4.26 showed that total methane production increased from 7200ml and peaked at 1130ml when substrate concentration increased from 340g. Furthermore, biogas production increased with HRT of 15 days to 27 days before plateauing. Addition of 100g of cow dung to the substrate produced a total of 9000ml of gas, which sharply increased to 13600ml when 500g of cow dung was used.

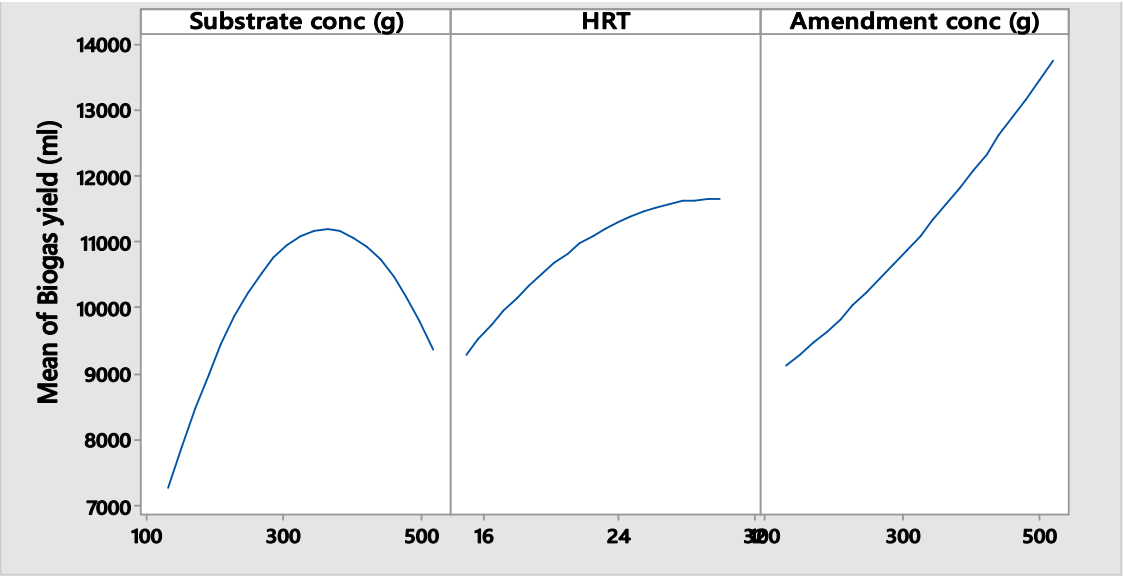


Figure 4.26: Main effect plot for methane (ppm) production following chemical pretreatment of the substrate

From Figure 4.27, when substrate is kept constant, that is, substrate * HRT, there is an interaction at HRT 30 days which leads to an increase in methane production. Also, in substrate * amendment, there is a similarity between amendment 0 – 520 which leads to increase in methane production. Consequently, in HRT * amendment, there is a visible similarity in the interaction of amendments 0 – 520 which led to a slight increase in methane production.

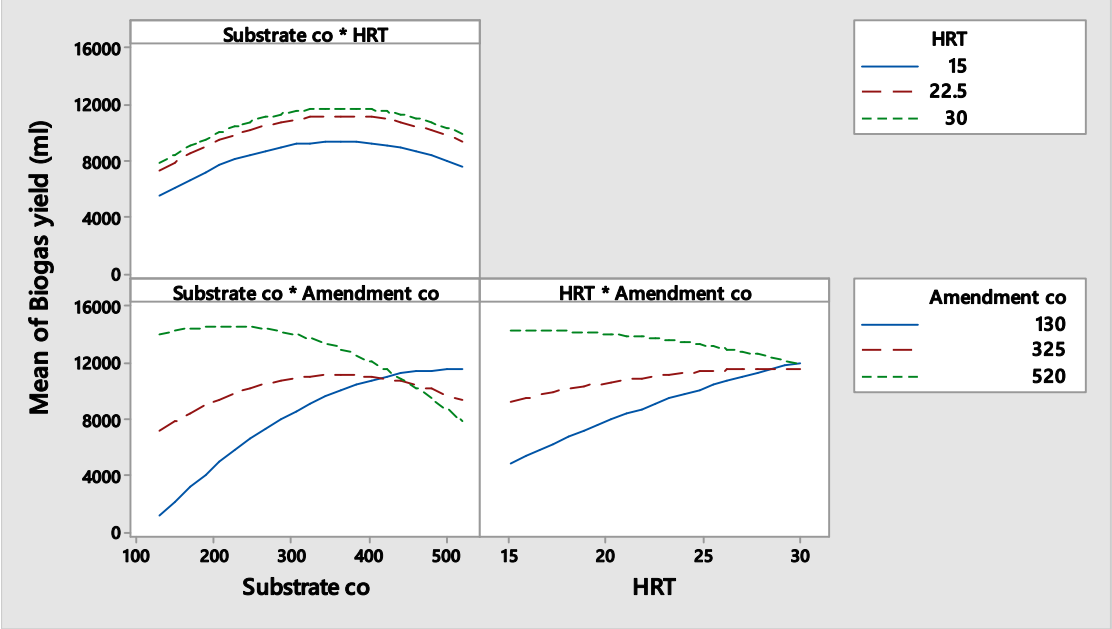


Figure 4.27.: Interaction plot for methane (ppm) production following chemical pretreatment of the substrate.

B. Response Surface Plots for Production of Methane

Results of the response surface regression shows that only substrate had significant effect on the production of methane (ppm) using chemical pretreatment at $P<0.05$ as shown in the appendix C. The regression had an r^2 value of 77.72% indicating that about 22.28% of the results occurred by chance.

The Regression Equation in Uncoded Units is given as

Methane (ppm)_Ch = 96.6 - 0.1417 Substrate - 7.58 HRT + 0.0209 Amendment

+ 0.000131 Substrate*Substrate + 0.150 HRT*HRT

- 0.000010 Amendment*Amendment + 0.00592 Substrate*HRT

- 0.000083 Substrate*Amendment + 0.00026 HRT*Amendment

From Figure 4.28, it can be deduced that at a constant holding value for substrate, methane production increased as HRT was increasing till HRT approached 30 days. On the other hand, an increase in amendment led to an increase in methane production.

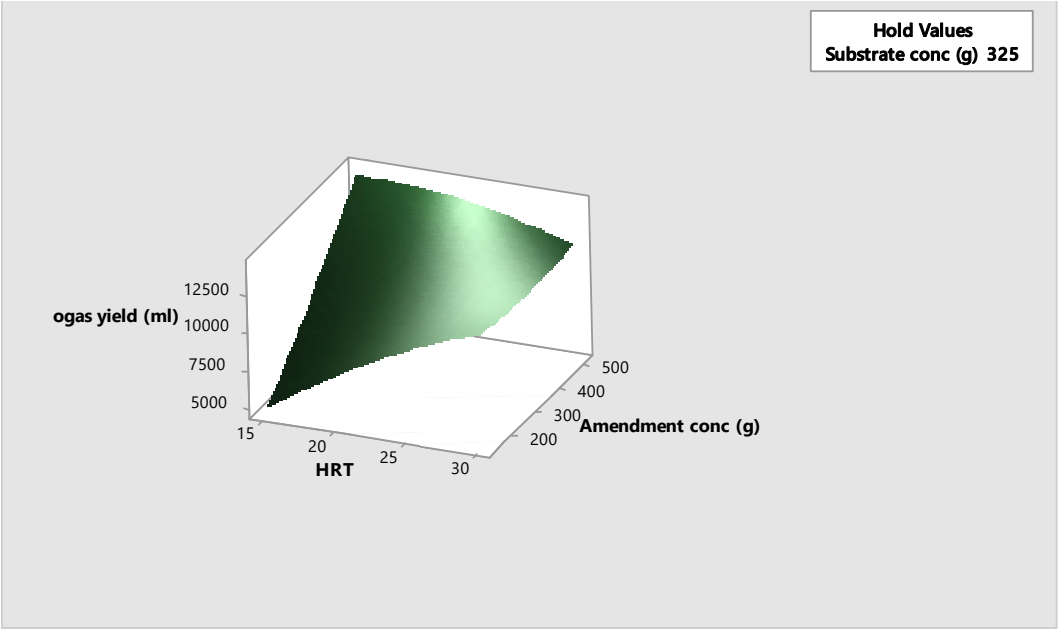


Figure 4.28: Surface Plot of Methane (ppm) Vs. Ammendment, HRT after chemical pretreatment of the substrate

It can be seen from Figure 4.29, for constant holding values for HRT that as the substrate concentration increased, methane production also increased. Furthermore, there was an increase in amendment which also led to a slight increase in methane production.

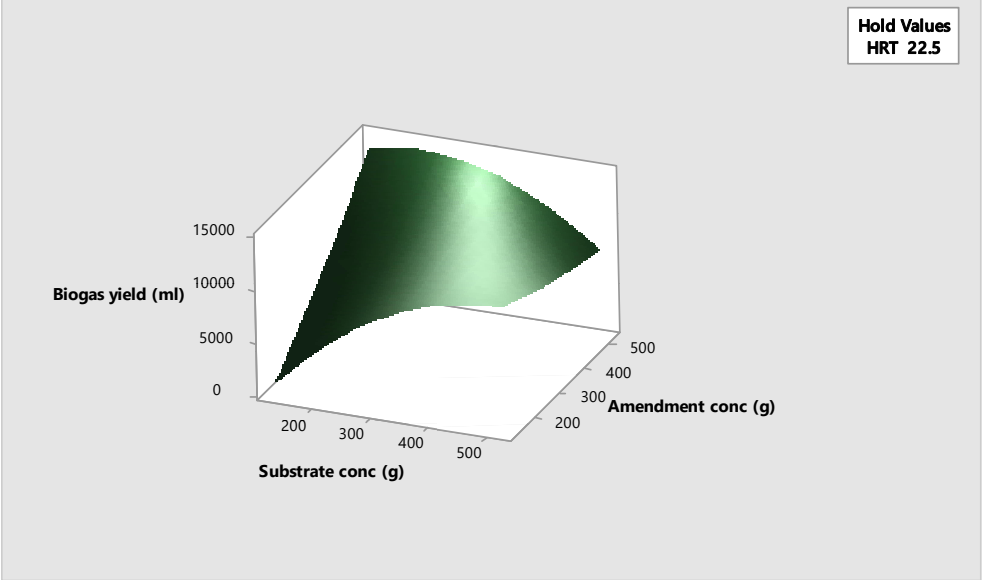


Figure 4.29: Surface Plot of Methane (ppm) Vs. Ammendment, Substrate after chemical pretreatment of the substrate

In Figure 4.30, at constant holding values of amendment, there was a slight increase in methane production as substrate concentration increased. On the other hand, methane production increased as HRT increased from 15 – 30 days.

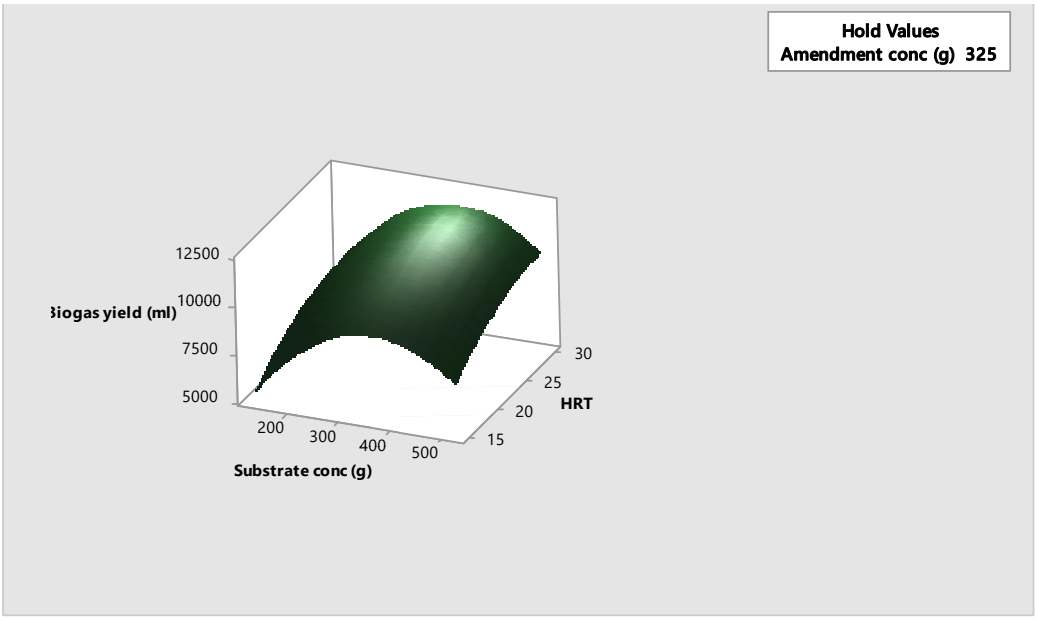


Figure 4.30: Surface Plot of methane (ppm) vs HRT, Substrate following chemical pretreatment of the substrate.

C.Optimization of the production of Methane (ppm)

Results of this study showed that production of biogas in biodigesters used started between 3 – 6 days. Figure 4.31 shows the optimization plots for the production of methane following chemical pre-treatment of the substrate. The results show that the optimum conditions for methane production are substrateconcentration of 216.7g, HRT of 15.5 days and 520g of cow dung as amendment. At these conditions the maximum yield that will be achieved will have a response of 1.517 x 10^4 ml.

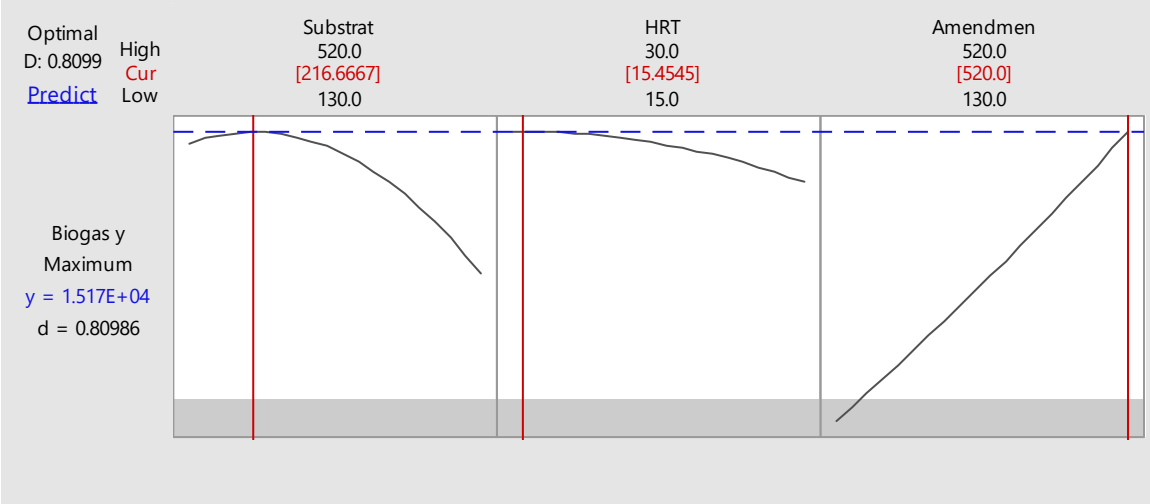


Figure 4.31: Optimization plot for methane production (ppm) after chemical pretreatment of the substrate

4.1.6 ANALYSIS OF COMPOSITION OF BIOGAS

Results obtained from Gas chromatography analysis of biogas sample collected from biodigester that gave highestyield in this study indicated the percentage composition as follows; 1.149 of CO, 13.556% of CO_2 and 64.960% of Methane as shown in table4.6.

Table 4.6: % composition of biogas

Component gas	%composition
CO	1.149
CO_2	13.556
CH_4	64.960

4.1.7PROXIMATE COMPOSITION OF SLURRY BEFORE AND AFTER ANAEROBIC DIGESTION

The physicochemical parameters of the samples before and after digestion were determined to ascertain if there was an increase or decrease in the parameters before and after digestion and whether the difference were significant. This was done for each digestion and the efficiency of digestion was obtained by comparing the nitrogen, phosphorus and potassium(NPK) ratio of the digestate (used slurry).

A. proximate Composition of Rice Straw and Cow Dung Before and After Digestion

Figure 4.36 shows that RS/CD (3:1) recorded the highest carbon content of 30.97 and 28.38; while RS had the least carbon content of 23.37 and 18.18 before and after digestion respectively. Similarly, results showed that Nitrogen content of 1.87 and 2.52; and Potassium concentration of 0.89 and 0.9 were recorded for RS/CD 2:1; while RS also recorded least Nitrogen values of 0.9 and 0.23; and least potassium values of 0.75 and 0.32 before and after digestion respectively. On the other hand, values (before digestion, after digestion) of phosphorus for the RS/CD 1:1 were (0.066 and 1.05) for RS values were (0.05 and 1.1) indicated that RS/CD 1:1 had the highest phosphorus content before digestion, contrary to TS values for RS/CD 1:1 were (98.2 and 93.67) and RS (89.22 and 82.15) showed that the RS/CD (1:1) had highest values while RS had least values before and after digestion.

Before digestion, RS/CD (1:1) had the highest volatile solids content (93.12) while RS had the least content (88.15); RS had the highest moisture content (16.52) while RS/CD (3:1) had the least content of (10.3); and RS/CD 3:1 had the highest NPK ratio of (34.67) while After digestion, RS/CD (1:1) had the highest volatile solids content of (83.31) while RS/CD 3:1 had the least (81.1); RS had the highest moisture content (18.89) while RS/CD 3:1 had the least content of (13.5) and RS/CD (2:1) had the highest NPK ratio of (2.62) while RS recorded the least value of (0.65).

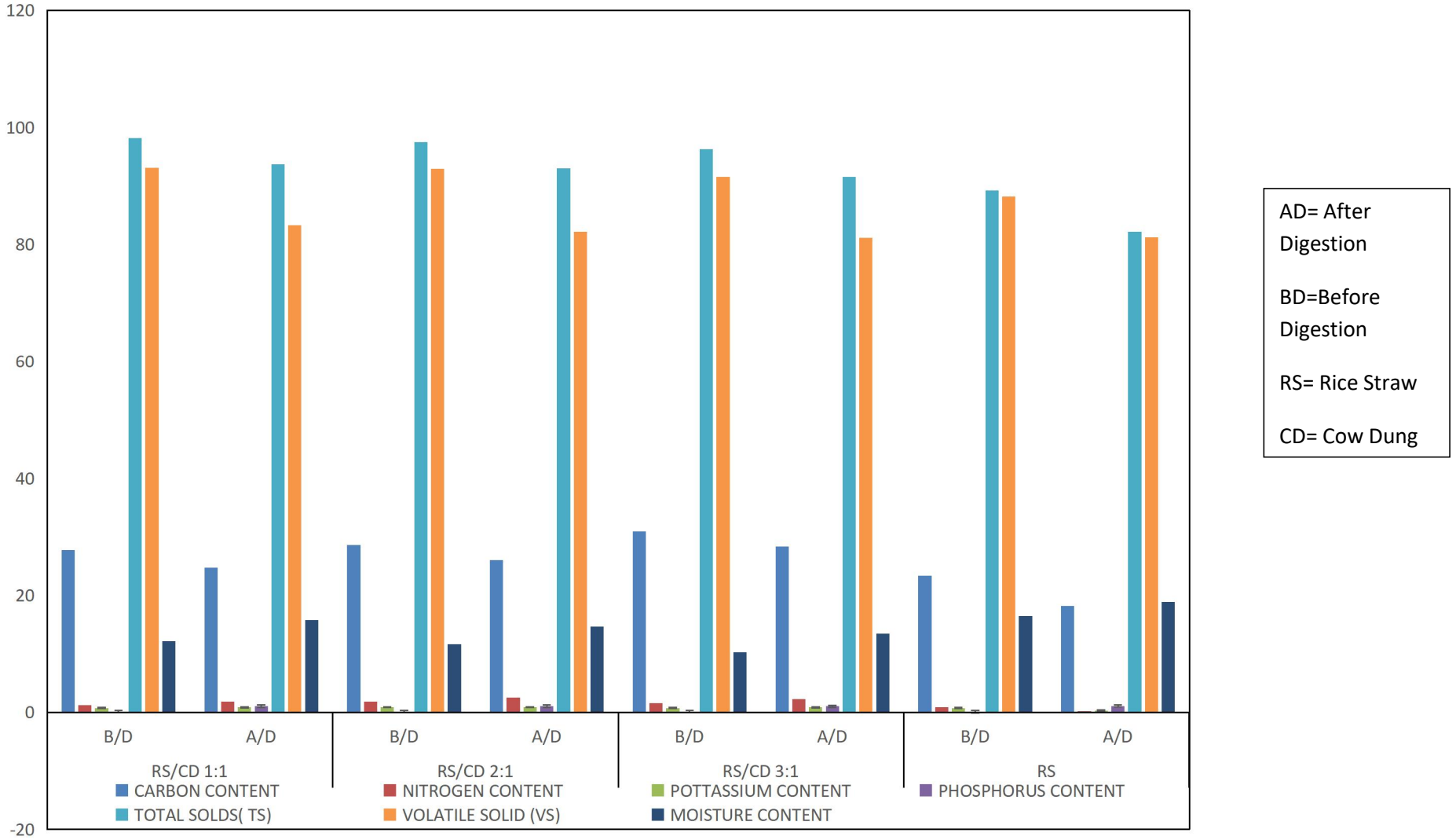


Figure 4.36: The proximate composition of the slurry before and after digestion for the pretreated rice straw amended cow dung.

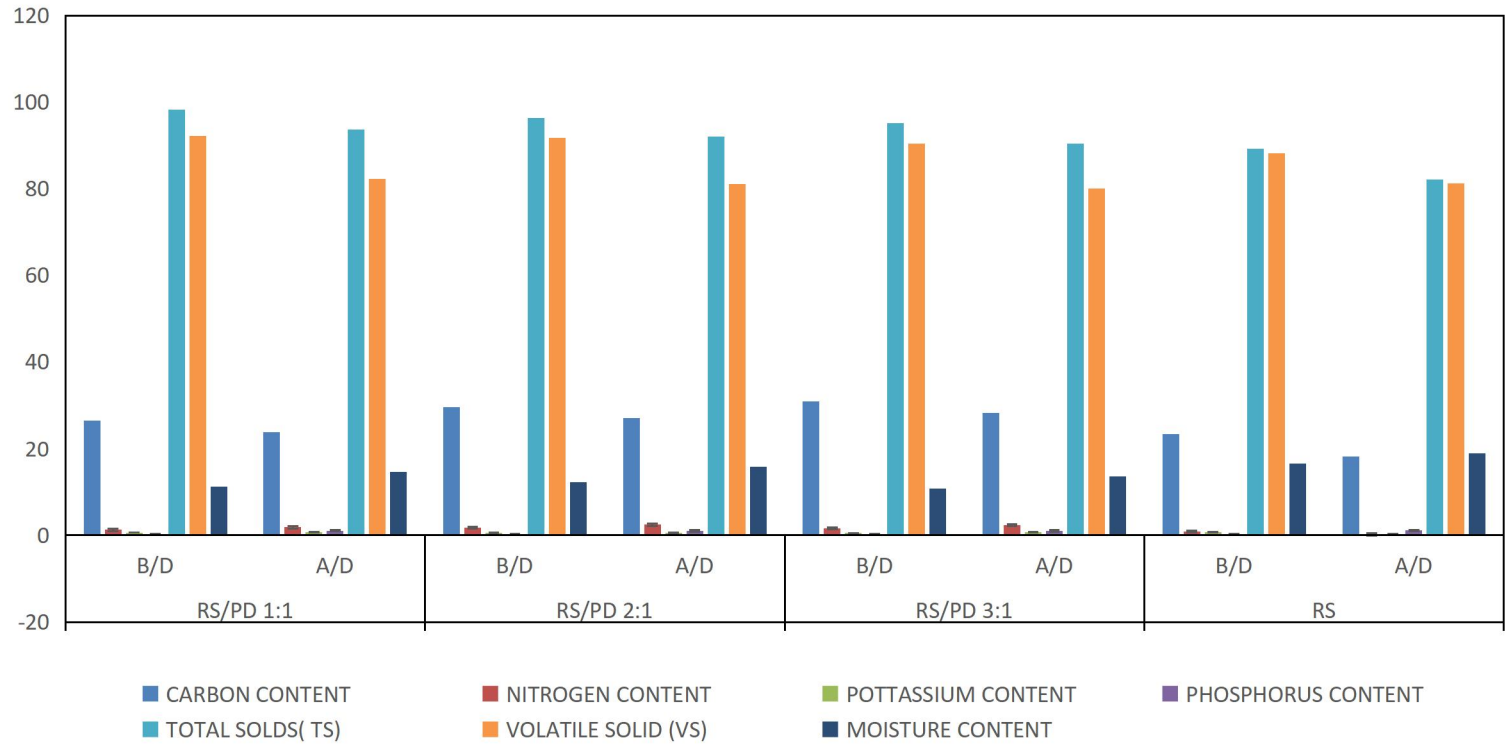
B.ProximateComposition of Mixture of Rice Straw and Poultry Droppings Before and After Digestion

Physicochemical composition of for rice straw and Poultry Droppings before and after digestion combination is shown in 4.37. it can be seen that before digestion, RS/PD (3:1) had the highest carbon content (30.98) while RS had the least (23.37). After digestion, RS/PD (2:1) had the highest carbon content (27.04) while RS had the least (18.18). Also, before digestion, RS/PD (2:1) had the highest nitrogen content (1.77) while RS had the least (0.9). After digestion, RS/PD 2:1 had the highest nitrogen content (2.44) while RS had the least (0.23).

Before digestion, RS had the highest potassium content (0.76) while RS/PD (3:1) had the least (0.51). After digestion, RS/CD (1:1) had the highest potassium content (0.77), while RS had the least (0.32). Also, RS/PD 1:1 had the highest phosphorus content (0.065), while RS/PD (3:1) and RS had the least values (0.05). After digestion, RS had the highest phosphorus content (1.1) while RS/PD 3:1 had the least (1.042).

RS/PD (1:1) had the highest Total Solids (98.2), while RS had the least (89.22). After digestion, RS/PD (1:1) had the highest Total Solids (93.66) while RS had the least (82.15). Similarly, RS/PD (1:1) had the highest volatile solid (92.11) while RS had the least (88.15). After digestion, RS/PD (1:1) had the highest volatile solid (82.21) while RS/PD (3:1) had the least (80).

Furthermore, before digestion, RS had the highest moisture content (16.52) while RS/PD (3:1) had the least (10.8). After digestion, RS had the highest moisture content (18.89) while RS/PD (3:1) had the least (13.6). Finally, it was deduced that before digestion, RS/PD (3:1) had the highest NPK ratio (64.71) while RS had the least (23.68). After digestion, RS/PD (2:1) had the highest NPKratio (3.82) while RS had the least (0.65).



AD= After Digestion

BD=Before Digestion

RS= Rice Straw

PD= Poultry Dropping

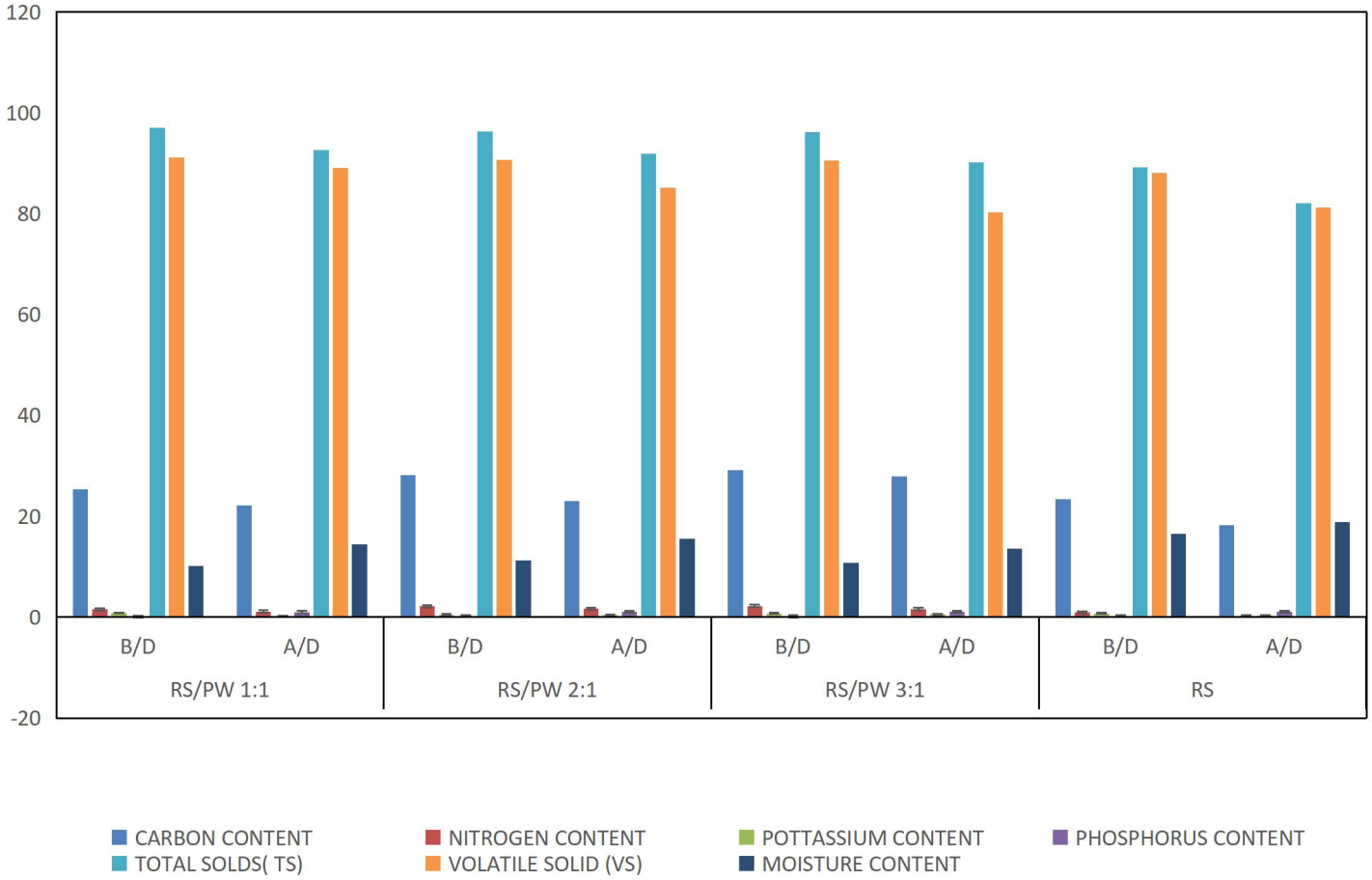
4.37proximate composition of the slurry before and after digestion for the pretreated rice straw amended poultry droppings

C. ProximateComposition of Rice Straw and Pig Waste Before and After Digestion

Physicochemical composition of the slurry before and after digestion for Rice Straw and Poultry Droppings combination is shown in Figure 4.38. Results show that RS/PW (3:1) had the highest carbon content (29.18), while RS had the least (23.37). After digestion, RS/PW (3:1) had the highest carbon content (27.98), while RS had the least (18.18). Similarly, before digestion, RS/PW 3:1 had the highest nitrogen content (2.23) while RS had the least (0.9). After digestion, RS/PW (2:1) had the highest nitrogen content (1.67), while RS had the least (0.23).

Consequently, before digestion, RS/PW (1:1) had the highest potassium content (0.85), while RS/PW (2:1) had the least (0.52). After digestion, RS/PW 3:1 had the highest potassium content (0.53) while RS/PW (1:1) had the least (0.25). Also, before digestion, RS/PW (2:1) had the highest potassium content (0.062) while RS/PW (1:1) had the least (0.023). After digestion, RS had the highest potassium content (1.1) while RS/PW 1:1 had the least (1.015). RS/PW (1:1) had the highest Total Solids (97.1) before digestion while RS had the least (89.22). After digestion, RS/PW (1:1) had the highest Total Solids (92.65) while RS had the least (82.15).

Before digestion, RS/PW 1:1 had the highest volatile solid (91.21) while RS had the least (88.15). After digestion, RS/PW (1:1) had the highest volatile solid (89.12) while RS/ PW (3:1) had the least (80.21). Also, RS had the highest moisture content (16.52) before digestion while RS/PW (1:1) had the least (10.21). After digestion, RS had the highest moisture content (18.89) while RS/PW (3:1) had the least (13.6). It was deduced that before digestion, RS/PW (1:1) had the highest NPK ratio (79.28) while RS had the least (0.65). After digestion, RS/PW 1:1 had the highest NPK ratio (4.34) while RS had the least (0.65).



AD= After Digestion

BD=Before Digestion

RS= Rice Straw

PW= Pig waste

Figure 4.38:proximatecomposition of the slurry before and after digestion for the pretreated rice straw amended pig waste

D. ProximateComposition of Mixture Water Hyacinth and Cow Dung Before and After Digestion

Physicochemical composition of the slurry before and after digestion for Water Hyacinth and Cow Dung combination is shown in figure 4.39. From the results, it was deduced that WH/CD(1:1) had the highest carbon content (18.6) while WH had the least (14.13). After digestion, WH/CD 1:1 had the highest carbon content (17.4) while WH had the least (13.31). Also, before digestion, WH/CD (2:1) had the highest nitrogen content (0.72) while WH/CD (3:1) had the least (0.05). After digestion, WH/CD (2:1) had the highest nitrogen content (0.5) while WH/CD (3:1) had the least (0.04).

Similarly, before digestion, WH/CD (1:1) had the highest potassium content (0.32,) while WH had the least (0.13). After digestion, WH/CD (1:1) had the highest potassium content (0.2) while WH had the least (0.11). The WH/CD (2:1) had the highest phosphorus content (0.05) before digestion while WH had the least (0.012). After digestion, WH/CD (1:1) had the highest phosphorus content (0.52) while WH/CD (3:1) had the least (0.16).

Before digestion, WH/CD (2:1) had the highest total solids (96.32) while WH had the least (85.12). After digestion, WH/CD (2:1) had the highest total solids (88.23) while WH had the least (80.1). Furthermore, WH/CD (2:1) had the highest volatile solid (90.23) before digestion, while WH had the least (83.21). After digestion, WH/CD (1:1) had the highest volatile solid (85.23) while WH had the least (79.25).WH had the highest moisture content (17.21) before digestion, while WH/CD (1:1) had the least (15.2). After digestion, WH had the highest moisture content (20.02) while WH/CD (1:1) had the least (17.3). However, it was deduced that before digestion, WH had the highest NPK ratio (76.92) while WH/CD (3:1) had (6.95). After digestion, WH/CD (2:1) had the highest NPK ratio (16.53) while WH/CD (1:1) had the least (0.91).

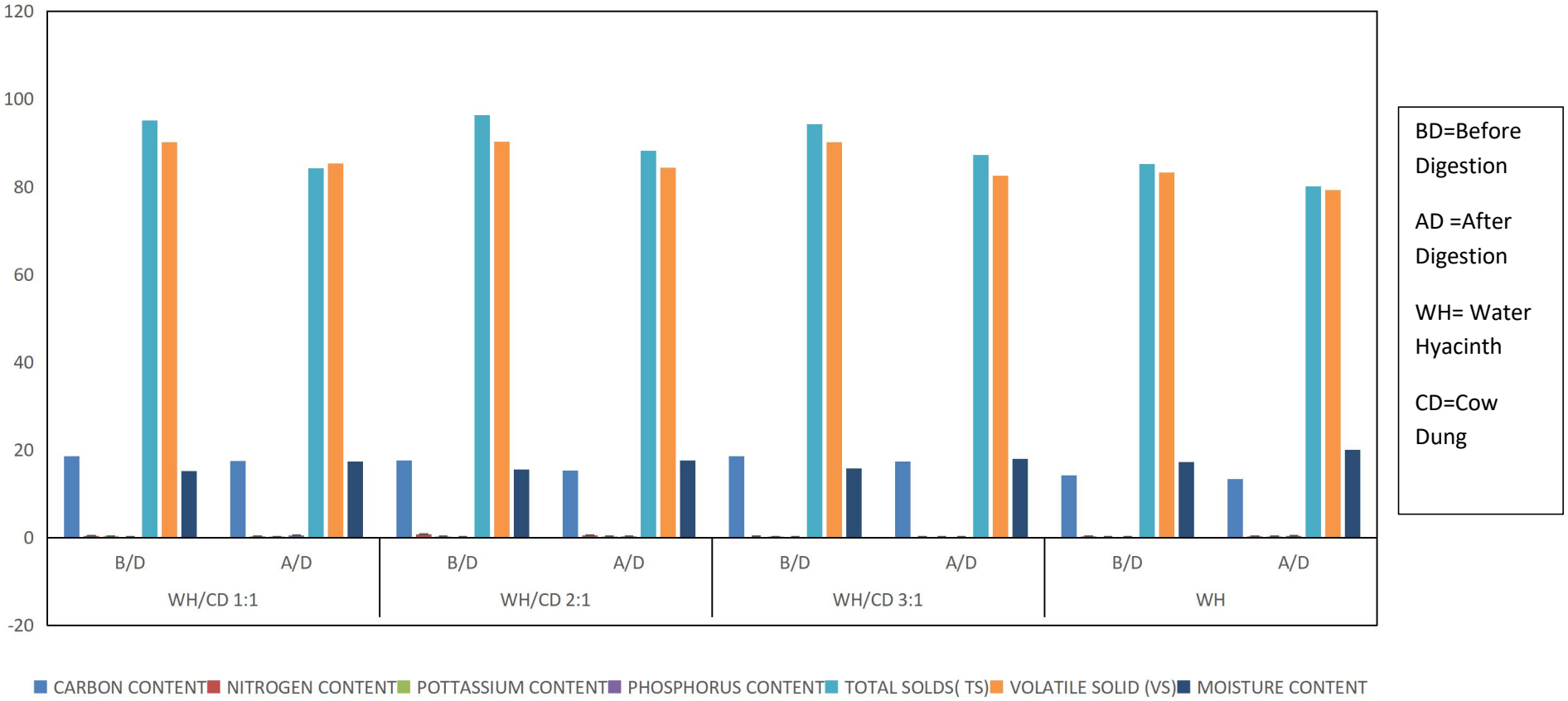


Figure 4.39: Proximate composition of the slurry before and after digestion for Water Hyacinth and Cow Dung combination

E. ProximateComposition of Mixture Water Hyacinth and Pig Waste Before and After Digestion

Physicochemical composition of the slurry before and after digestion for Water Hyacinth and Pig waste combination is shown in Figure 4.40. it can be seen that before digestion, WH/PW (1:1) had the highest carbon content (16.51) while WH had the least (14.13). After digestion, WH/PW (1:1) had the highest carbon content (15.41) while WH had the least (13.31). Before digestion, WH/PW (1:1) and(2:1) had the highest nitrogen contents (0.321) while WH had the least (0.13). After digestion, WH/PW (1:1) had the highest nitrogen content (0.111) while WH/PW (3:1) had the least (0.01). WH/PW (1:1) had the highest potassium content (0.321) before digestion, while WH had the least (0.13). After digestion, WH/PW (1:1) had the highest potassium content (0.4) while WH/PW (3:1) had the least (0.1). Also, before digestion, WH/PW (3:1) had the highest phosphorus content (0.025) while WH/PW (2:1) had the least (0.01). After digestion, WH/PW (1:1) had the highest phosphorus content (0.53) while WH/PW (3:1) had the least (0.01).

Before digestion, WH/PW (1:1) had the highest total solids (94.21) while WH had the least (85.12). After digestion, WH/PW (3:1) had the highest total solids (84.11) while WH had the least (80.1). Consequently, before digestion, WH/PW (1:1) had the highest volatile solid (91.12) while WH had the least (83.21). After digestion, WH/PW (1:1) had the highest total solids (86.62) while WH had the least (79.25).In addition, WH had the highest moisture content (17.21) before digestion, while WH/PW (1:1) had the least (15). After digestion, WH had the highest moisture content (20.02) while WH/PW (1:1) had the least (17.4). Finally, it was deduced that before digestion, WH had the highest NPK ratio (102.89) while WH/PW (3:1) had the least (40.13). After digestion, WH/PW (3:1) had the highest NPK ratio (10) while WH had the least (2.38).

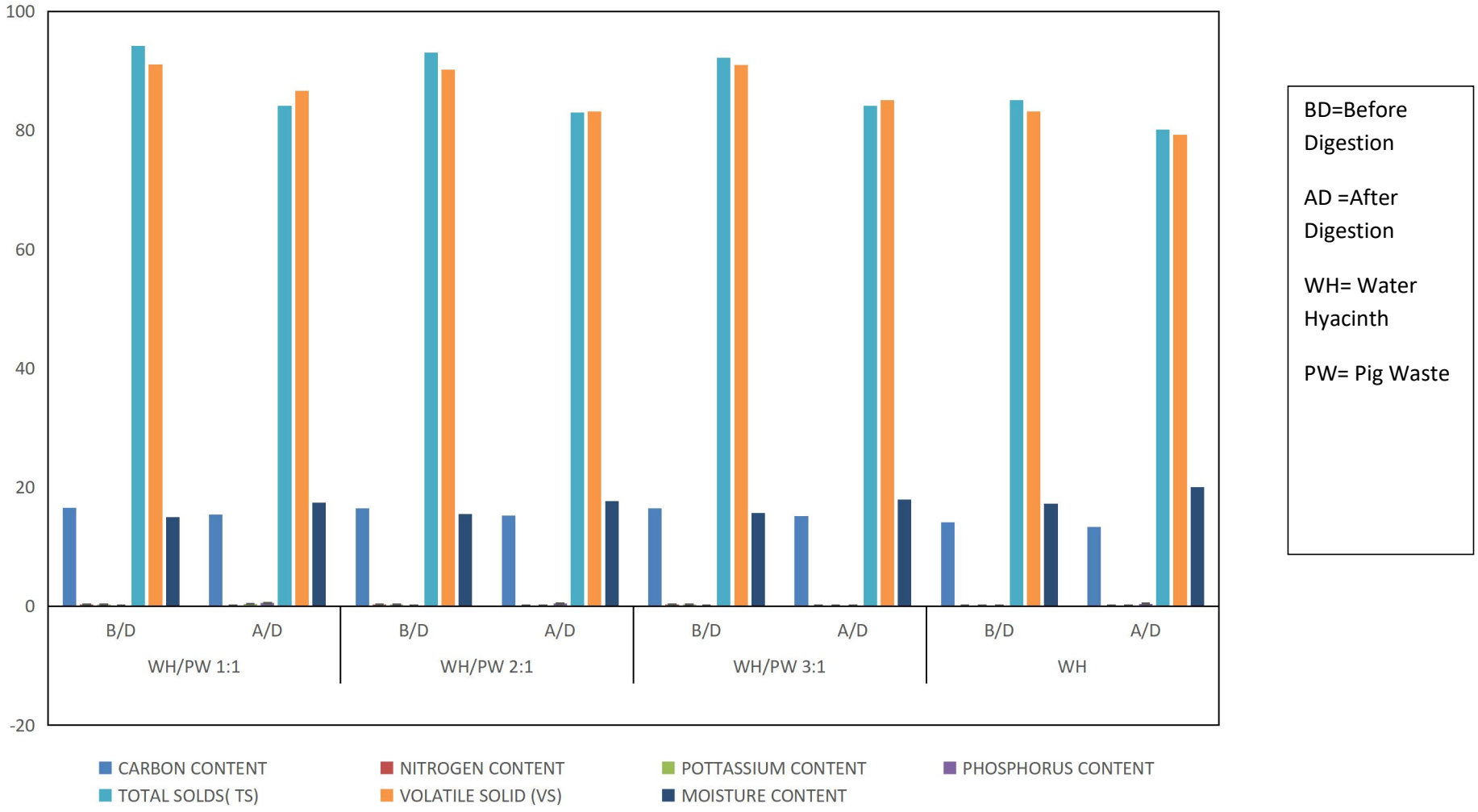


Figure 4.40: proximate composition of the slurry before and after digestion for Water Hyacinth and pig waste combination

F. ProximateComposition of Mixture of Water Hyacinth and Poultry Droppings

Physicochemical composition of the slurry before and after digestion for Water Hyacinth and poultry droppings combination is shown in figure 4.41. It can be seen that before digestion, WH/PD 3:1 had the highest carbon content (16.45) while WH had the least (14.13). After digestion, WH/PD (2:1) had the highest carbon content (15.85) while WH had the least (13.31). WH/PD 2:1 had the highest nitrogen content (0.32) before digestion, while WH had the least (0.12). After digestion, WH/PD (2:1) had the highest nitrogen content (0.21) while WH/PD (3:1) had the least (0.01). Before digestion, WH/PD (1:1) had the highest potassium content (0.36) while WH had the least (0.13). After digestion, WH/PD (1:1) had the highest potassium content (0.4) while WH/PD (3:1) had the least (0.1).

WH/PD (3:1) had the highest phosphorus content (0.026) before digestion,while WH/PD (2:1) had the least (0.01). After digestion, WH/PD (1:1) had the highest phosphorus content (0.43) while WH/PD (3:1) had the least (0.01). Before digestion, WH/PD (1:1) had the highest total solids (94.21) while WH had the least (85.12). After digestion, WH/PD (3:1) had the highest total solids (84.11) while WH had the least (80.1). Also, WH/PD 1:1 had the highest volatile solid (91.12) before digestion, while WH had the least (83.21). After digestion, WH/PD (1:1) had the highest total solids (85.62) while WH had the least (79.25).

Before digestion, WH had the highest moisture content (17.21) while WH/PD (1:1) had the least (15). After digestion, WH had the highest moisture content (20.02) while WH/PD (1:1) had the least (17.4). Finally, it was deduced that before digestion, WH had the highest NPK ratio (102.89) while WH/PD (3:1) had the least (28.07). After digestion, WH/PD (3:1) had the highest NPK ratio (10) while WH/PD (1:1) had the least (0.65).

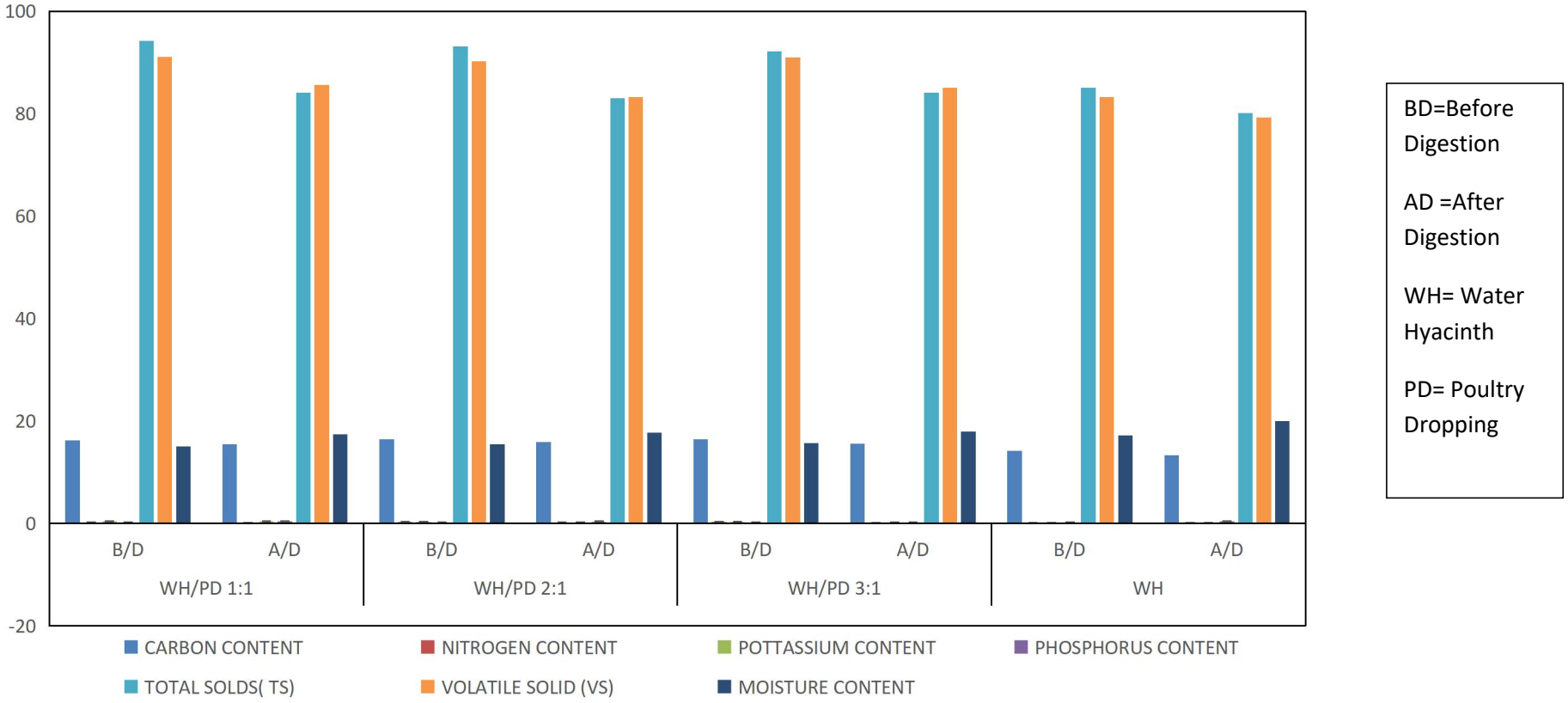


Figure 4.41: proximate composition of the slurry before and after digestion for Water Hyacinth and poultry droppings combination

G. NPK Ratio Plot for Different Treatments

NPK Ratio Plot for Different Treatments is shown in Figure 4.42. It shows that before digestion, RS/PW (1:1) had the highest NPK ratio (79.28) while RS/CD (1:1) had the least (24.72). After digestion, RS/PW (1:1) had the highest NPK ratio (4.33) while WH/PW (1:1) had the least (0.52).

Before digestion, WH/PW 2:1 and WH/PD (2:1) had the highest NPK ratios (102.89) while RS/CD (2:1) had the least (33.35). After digestion, WH/CD (2:1) had the highest NPK ratio (16.53) while WH/PW (2:1) had the least (2.12). Before digestion, RS/PW (3:1) had the highest NPK ratio (73.36) while WH/CD (3:1) had the least (6.95). After digestion, WH/PW and WH/PD (3:1) had the highest NPK ratio (10) while WH/CD 3:1 had the least (2.06).

Finally, in the control plot, before digestion, WH combinations had the highest NPK ratio (76.92) while RS combinations had the least (23.68). After digestion, WH combinations had the highest NPK ratio (2.38) while RS combinations had the least (0.65)

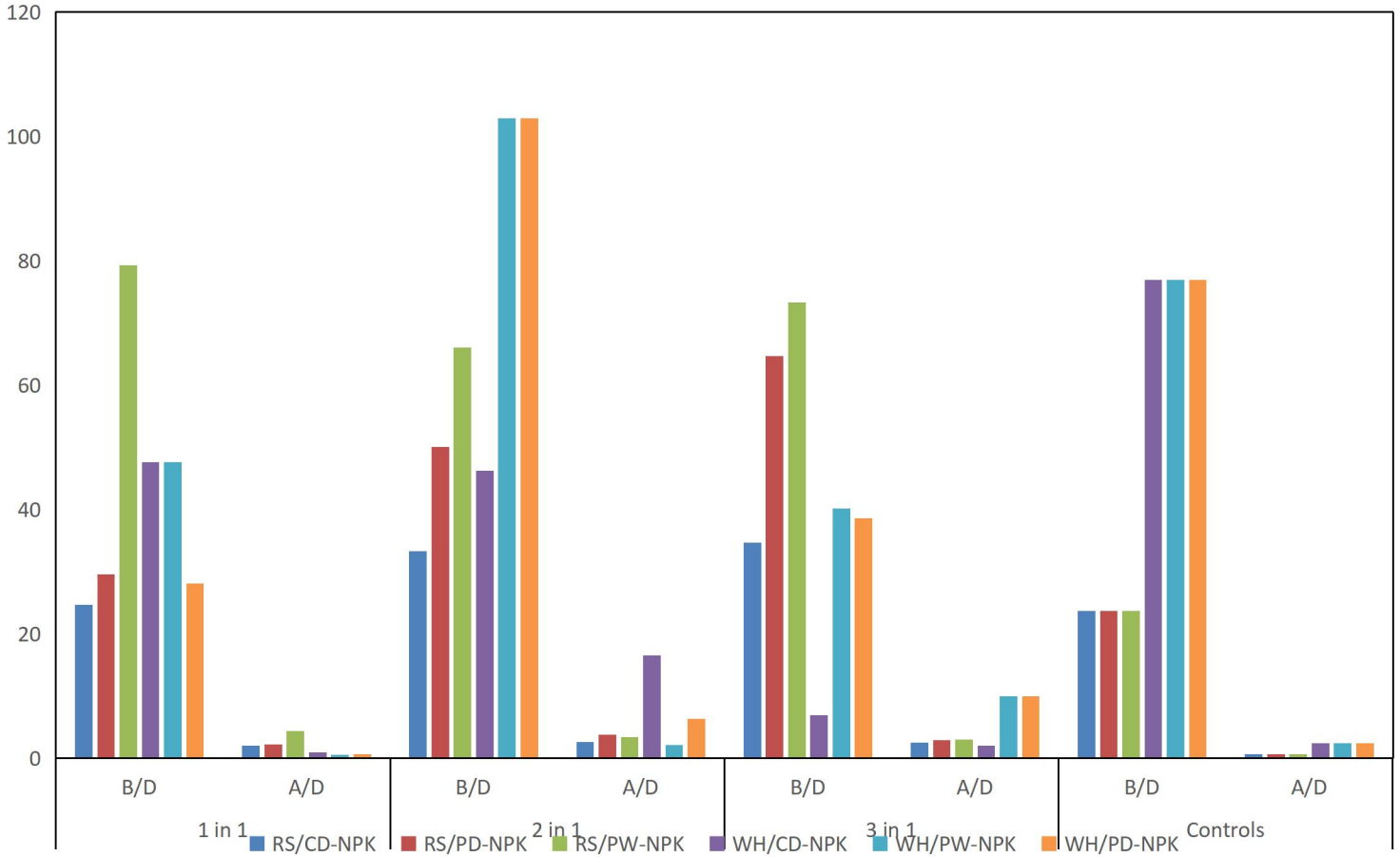


Figure 4.42:NPK ratios plot for the different treatments

4.1.8MICROBIAL LOAD AND ISOLATES OF SLURRY DURING ANAEROBIC DIGESTION

Tables 4.6, 4.7 and 4.8 below depict the microbial load and the microbial isolates of the different amendment at varying ratios during anaerobic fermentation at three intervals which is day 0(charging),14th day and 28th day which is significant in portraying the efficiency of the different amendments in not only producing biogas but also in the area of waste management.As can be seen from the table bioload of the microorganism decreased across the duration of fermentation.

In this study, it was observed that initial bacterial load of 3:1 ratio of rice straw/cow dung (RS/CD) was $2.11x10^{10}$ which is higher than those of other ratios. After day 14, it increased to $5.1x10^{10}$, before plunging to $1.08x10^{9}$ on day 28. For rice straw/poultry dropping (RS/PD), bacterial load was initially recorded as $2.11x10^{10}$, which also increased to $4.3x10^{10}$ on day 14 before plummeting to 8.1 x 10^{8} on day 28. Similarly, 3:1 rice straw/pig waste (RS/PW) gave the highest initial bacterial load of $2.17x10^{10}$. On day 14, it decreased to $6.0x10^{9}$ and further to $5.9x10^{8}$ on day 28. Meanwhile, bacterial load of untreated RS was $2.96x10^{10}$, $2.41x10^{11}$ and $3.8x10^{8}$ on days 0, 14 and 28 respectively.

Table4.6: Microbial load at three intervals of 0, 14th and 28th day in the different amendments

Treatments		0day		14th day		28th day	
		N/A	PDA	N/A	PDA	N/A	PDA
RS/CD	1:1	$7.2x10^{9}$	NIL	$5.1x10^{10}$	$1.28x10^{11}$	$9.8x10^{8}$	$3.2x10^{8}$
	2:1	$2.49x10^{10}$	$9.7x10^{9}$	$1.6x10^{11}$	$1.3x10^{10}$	$6.5x10^{8}$	$5.9x10^{8}$
	3:1	$2.11x10^{10}$	$1.28x10^{10}$	$5.1x10^{10}$	$6.1x10^{10}$	$1.08x10^{9}$	$4.8x10^{8}$
RS/PD	1:1	$2.11x10^{10}$	$2.72x10^{10}$	$4.3x10^{10}$	$2.5x10^{10}$	$8.1X10^{8}$	$4.1X10^{8}$
	2:1	$2.01x10^{10}$	$2.08x10^{10}$	$1.09x10^{11}$	$1.68x10^{11}$	$4.3X10^{8}$	$1.0X10^{7}$
	3:1	$1.49x10^{10}$	$6.1x10^{9}$	$3.1x10^{10}$	$8.0x10^{9}$	$4.5X10^{8}$	$4.0X10^{8}$
RS/PW	1:1	$1.02x10^{10}$	$2.0x10^{9}$	$2.89x10^{11}$	$7.2x10^{10}$	$1.2x10^{8}$	$5.5x10^{8}$
	2:1	$1.36x10^{10}$	$3.0x10^{8}$	$1.09x10^{11}$	$1.68x10^{11}$	$6.8x10^{8}$	$5.0x10^{8}$
	3:1	$2.17x10^{10}$	$2.72x10^{10}$	$6.0x10^{9}$	$1.0x10^{9}$	$5.9x10^{8}$	$1.2x10^{8}$
RS Treated		$1.59x10^{10}$	$1.0x10^{8}$	NIL	NIL	NIL	NIL
RS untreated		$2.96x10^{10}$	$2.17x10^{10}$	$2.41x10^{11}$	$4.7x10^{10}$	$3.8x10^{8}$	$3.0x10^{7}$

NA = Nutrient agar; PDA = potato dextrose agar; RS = Rice straw; PW = Pig waste; CD = Cow dung; PD = Poultry droppings; Nil, nogrowt

Table4.7: Microbial load at three intervals of 0, 14th and 28th day using specific media in the different treatments

Treatment	0 day		14th day		28th day	
	MSA	SSA	MSA	SSA	MSA	SSA
RS/ CD						
1:1	$2.4x10^5$	$1.69x10^7$	$1.2x10^5$	$2.61x10^6$	Nil	$2.5x10^5$
2:1	$1.6x10^{10}$	$1.45x10^7$	$1.4x10^{10}$	$7.2x10^5$	$1.0x10^4$	$3.0x10^5$
3:1	$3.0x10^4$	$5.5x10^6$	$2.51x10^4$	$4.2x10^5$	$1.41x10^3$	$1.9x10^5$
RS/PD						
1:1	$3.2x10^6$	$2.61x10^7$	$3.0x10^6$	$1.3x10^6$	$1.0X10^4$	$1.2X10^5$
2:1	$2.01X10^6$	$2.71x10^7$	$1.85x10^5$	$8.1x10^5$	NIL	$2.1X10^5$
3:1	$1.0X10^5$	$1.38x10^7$	NIL	$3.9x10^5$	NIL	$1.0X10^4$
RS/PW						
1:1	$4.3x10^5$	$3.5x10^6$	$3.5x10^5$	$9.6x10^5$	Nil	$3.2x10^5$
2:1	$3.0x10^4$	$8.9x10^5$	$1.0x10^4$	$8.1x10^5$	NIL	NIL
3:1	$4.4x10^5$	$9.6x10^5$	$2.8x10^5$	$4.6x10^5$	$2.4x10^5$	NIL
RS un-treated	$3.0x10^5$	$2.15x10^5$	$2.1x10^4$	$1.0x10^4$	NIL	NIL
RS treated	NIL	$3.0x10^5$	NIL	NIL	NIL	NIL

MSA = Minimal salt agar; SSA = Salmonella shigella agar; RS = Rice straw; PW = Pig waste; CD = Cow dung; PD = Poultry droppings

Dominant bacteria isolated from RS/CD samples were *Salmonella* sp*, Shigella sp, Enterococcus* sp*, Bacillus cereus, Enterobacter* sp*, Staphylococcus aureus* and *Micrococcus roseus*. On the other hand, fungal isolates were *Aspergillus* sp*, Penicillium* sp*, Mucor* sp, *Saccharomyces* sp and *Rhizopus* sp for RS/CD. In the case of RS/PD, bacterial isolates were *Salmonella* sp*, Shigella* sp, *Staphylococcus aureus, Enterococcus* sp*, Pseudomonas aeruginosa* and *Bacillus cereus*. Fungi isolated from the samples were *Mucor* sp*, Saccharomyces* sp*, Penicillium* sp and *Rhizopus* sp. Similarly, *Salmonella* sp*, Shigella* sp*, Enterococcus* sp, *Micrococcus roseus, Enterobacter* sp*, Staphylococcus aureus* and *Staphylococcus saprophyticus* were dominant bacteria isolates from RS/PW samples. Fungal isolates were *Mucor* sp*, Penicillium* sp, *Saccharomyces* sp and *Rhizopus* sp.

Table4.8:Microbial isolates from the different treatments at different ratios

Treatments	**Bacteria**	**Fungi**
RS/CD		
1:1	*Salmonella* sp*, Enterococcus* sp*, Micrococcus roseus, Staphylococcus aureus*	*Aspergillus* sp*, Penicillium* sp*, Saccharomyces* sp, *Rhizopus* sp
2:1	*Salmonella* sp*, Shigella sp, Enterococcus* sp*, Bacillus cereus, Enterobacter* sp*,*	*Mucor* sp *and Saccharomyces* sp.

	Staphylococcus aureus.	
3:1	*Enterobacter* sp, *Salmonella* sp, *Shigella* sp, *Staphylococcus aureus*.	*Mucor* sp *and Saccharomyces* sp.
RS/PD		
1:1	*Salmonella* sp, *Shigella* sp, *Staphylococcus aureus*	*Mucor* sp, *Saccharomyces* sp, *Penicillium* sp, *Rhizopus* sp
2:1	*Salmonella* sp, *Shigella* sp, *Enterococcus* sp, *Pseudomonas aeruginosa, Staphylococcus aureus*	*Saccharomyces* sp, *Rhizopus* sp
3:1	*Salmonella* sp, *Shigella* sp, *Enterococcus* sp, *Bacillus cereus, Staphylococcus aureus, Staphylococcus saprophyticus*	*Mucor* sp, *Saccharomyces* sp
RS/PW		
1:1	*Salmonella* sp, *Shigella* sp, *Enterococcus* sp, *Micrococcus roseus, Enterobacter* sp, *Staphylococcus aureus, Staphylococcus*	*Saccharomyces* sp

	saprophyticus	
2:1	*Salmonella* sp, *Shigella* sp*, Enterococcus* sp, *Bacillus cereus, Staphylococcus aureus*	*Saccharomyces* sp, *Rhizopus* sp
3:1		*Mucor* sp, *Saccharomyces* sp, *P.notatum*
RS TREATED	*Salmonella* sp*, Shigella* sp, *Enterococcus* sp,	*Mucor sp, Penicillium* sp, *Saccharomyces* sp*, Rhizopus* sp
RS UNTREARED	*Staphylococcus aureus, Enterococcus* sp*Shigella* sp	*Saccharomyces* sp

4.2 DISCUSSION

Studies have proven that when compared to many other available agricultural residues, rice straw has higher potential for conversion to biomethane (Pore *et al.,* 2015). Globally, it has become a choice source of lignocellulosic substrate for bioenergy production (Mustafa *et al.,* 2017). Similarly, water hyacinth has gained attention as a potential substrate for bioethanol

and biogas production, in view of its abundance in recent times (Ganguly *et al.,* 2012 and Rezania *et al.,* 2015).

Anaerobic digestion (AD) is a vital process of converting crop straws and manures into renewable energy, thus changing waste into useful resource (Forster-Carneiro *et al.,* 2008). Nevertheless some drawbacks against the performance of AD using crop residues, such as rice straw (RS) with high C/N ratio and presence of lignin, hemicellulose and cellulose have been identified. Lignin, hemicellulose and cellulose are responsible for their recalcitrance to degradation by microbes hence the need for the initial, rate-limiting step, hydrolysis (Sagarika and Venkateswara, 2020). These challenges can be minimized by first subjecting the biomass to some pretreatment methods and then co-digesting it with high nitrogen – containing substrates (manures) to balance the C/N ratio (Sagarika and Venkateswara, 2020).

Other factors affecting the performance of AD system include, feeding methods, quality of inoculums, C/N ratio, redox potential, organic loading rate (OLR), feedstock to inoculum ratio, source and characteristics of substrate, type of AD process, VFAs production, trace elements, hydraulic retention time (HRT), temperature, mixing patterns and pH (Terashima *et al.,* 2009, Luo *et al.,* 2018). However, their influences on AD process vary largely (Sagarika and Venkateswara, 2020).

4.2.1 PRETREATMENT OF LIGNOCELLULOSIC BIOMASS FOR BIOGAS PRODUCTION

The main reason for pretreatment of lignocellulosic biomass is to minimize the structural and compositional barriers to hydrolysis (Kaur and Phutela 2016). Steps involved include; hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Pretreatment methods break

complex lignocellulose chains to release cellulose, hemicellulose and lignin from its bond (Ma *et al.,* 2010). Pretreatment is necessary because ordinarily, this substrate could take between weeks to months to undergo degradation into simple sugars needed for biogas production . It has been well documented that several factors affect the yield of biogas in a bioreactor (Sarker *et al.,* 2019). In order to determine the optimal conditions required for the production of biogas, we decided to optimize several factors that are critical in stages of the production line. Biological pretreatment is much slower than the chemical pretreatment and is usually used alongside the inoculum in order to obtain a synergistic activity where one community of microorganisms degrade the waste and another community ferment the sugars to produce gas (Amin *et al*., 2017).

In this study, results obtained showed that increase in concentration of NaOH from 2% to 4% resulted in corresponding increase in degradation of rice straw biomass. Further increase degradation was recorded when concentration increased to 6%. Rate of pretreatment increased proportionally with increasing time, from 20h up to 40h when it peaked before plummeting. The reduction in rate of degradation implied that most of the biomass had been degraded. Generally, NaOH pretreatment aids biogas production, as has been attested to that methane production using 1.5M NaOH – pretreated rice straw was 3.17 times higher than that obtained from the untreated rice straw (Shu *et al.,* 2015). Results of this study are in line with those reported by Ben-lin *et al.*(2017) which indicated that a total biogas yield on 26th and 30th days for untreated and pretreated rice straw substrates using 2%, 4% and 6% NaOH were 3.40 and 3.38, 3.82 and 3.81, and 5.90 and 5.91 respectively. The 6% NaOH pretreated substrate yielded maximum total biogas.

Furthermore, results obtained from this study indicated that using 6% NaOH to pretreat 14g of rice straw sample for 39.5 h, as the optima, produced a reduction in lignin concentration from 17.4% to 8.3% of the total biomass. This is related to the findings of Shu *et al.* (2015) which reported that lignin, cellulose, hemicellulose and ash compositions of untreated rice straw were 10.64, 38.20, 30.85 and1.02. These reduced to 5.80, 81.12, 8.23 and 0.56 for NaOH – pretreated sample and 10.43, 37.29, 30.60 and 1.16 for biologically – pretreated rice straw. Thus, using 1.5M NaOH for pretreatment of rice straw samples improved their delignification by removing 45.5% of the lignin content (Shu *et al.,* 2015). Similarly, highest reduction of volatile solids (81.6 to 56.4 %), total solids (13.1 to 4.5 %), hemicellulose content (26.4 to 12.7 %) and cellulose content (22.8 % to 10.5 %) was recorded using 1 cm sized sample (Rozy *et al.,* 2017).

In the present study, it was observed that lignin, hemicellulose and cellulose contents of rice straw were 17.439nm, 10.278nm and 57.709nm respectively in the initial (un-pretreated) rice straw. After biological pretreatment using bacteria consortium from termite gut for 30 day, lignin and hemicellulose content of RS reduced to 7.299nm, 8.908nm respectively, while the cellulose content increased to 38.86. This is comparable to initial cellulose (%) content of rice straw sample reported as 38.3 ± 0.8(%), hemicellulose was 21.3 ± 0.5(%) while lignin was 12.5 ± 0.2(%). Following NaOH and cellulase pretreatments, there was reduction in contents of hemicellulose and lignin, compared to untreated rice straw substrates. The percentage content of cellulose showed a dramatic decrease, from 38.3 to 10.9%, after the cellulase pretreatment. Cumulative biogas production yields increased from 16–103, 25–122% for NaOH- and cellulase pretreated rice straw substrate, respectively (Ben-lin *et al.,* 2017).

For water hyacinth samples, loss of biomass due to washing could be reason some researchers either avoided the washing step to neutralize pH, or did not chemically pretreat it in their studies. Failure to wash after NaOH – pretreatment could lead to heavy metal toxicity due to the presence of Na^+. This finding lends credence to the report that there was decrease in volume of biogas produced due to toxic effect of specific ions present in substrate on microorganism. Concentrations of ions, such as Na^+ and Ca^+ in excess of 8,000 mg/L can inhibit growth of anaerobic bacteria (Rahmansyah *et al.,* 2017). In this study, analysis of chemical compositions of slurry remaining after washing of biomass pretreated with NaOH, revealed that reducing sugar and total sugar concentrations increased from 6.41 and 6.91 to 156.08 and 167.14 after 40h of pretreatment respectively. In a separate study by Okewale and Adesina (2019), hemicellulose (%) content of 22.5, 20.5 and 12.5, cellulose (%) content of 14.5, 8.5 and 20.8, lignin (%) content of 6.5, 8.2 and 11.5 and total fiber (%) content of 40.5, 38.3 and 19.5 were recorded for pig dropping, poultry dropping and water hyacinth respectively.

In some other previous studies, results obtained using samples of water hyacinth indicated that hemicellulose contentwas 27.0% while cellulose content was 34.2% (Mishima *et al.,* 2008). Also, reports by Cheng *et al.,* (2010) showed that cellulose content of 27.0% and hemicellulose contentof 20.3% were recorded. Furthermore, cellulose content obtained was 23.31%, while hemicellulose content was 22.11% in sample studied by (Xia *et al.,* 2013). The highest of sugar yield at 60 g sugar /g substrate was observed from 140^{o}C by using dry water hyacinth, whereas fresh hyacinth had yield at 36.7 g sugar /g substrate. Conversely, it has been reported that combination of alkaline pretreatment and five days of biological pretreatment

before thermophilic co-digestion gave rise to the highest amount of reducing sugars and methane production of 2.23 g/L and 1834.20 mL respectively (Shu *et al.,* 2015).

After 30 days of pretreatment using bacteria consortium from termite gut in this study, lignin and hemicellulose contents of WH substrate reduced to 7.821 and 8.21 respectively, while the cellulose content increased to 14.40. Water hyacinth biomass contains 23.31% cellulose and 22.11 % hemicellulose (Ao *et al.,* 2013). This is also corroborated by a separate report, which indicated that dry biomass of water hyacinth mostly has low lignin content (7–26%), but high cellulose content (18–31%) and hemicellulose content (18–43%), components that are easily hydrolyzed to produce fermentable reducing sugars for bioethanol/biogas production (Bergier et al., 2012).

Notably, OH- in NaOH is capable of weakening the hydrogen bonds of hemicellulose and cellulose, as well as ester and ether bonds lignin and polysaccharides, thereby separating and partially decomposing lignin, cellulose and hemicellulose (Xiao *et al.,* 2001). Resulting hemicellulose could be further broken down into a monomer structure, furfural and other volatiles products. Soluble hydroxyl lignin is also produced from insoluble lignin, hence making it easier for microbial biodegradation (Chen, 2014). The percentages of cellulose and hemicellulose decreased with increasing amounts of the biological reagents, and conversely, the percentage of lignin increased. The results showed that cellulase pretreatment was advantageous to the degradation of cellulose and hemicellulose (Ben-lin *et al.,* 2017).

4.2.2 THE 16S RNA IDENTIFICATION OF LIGNOLYTIC BACTERIA FROM TERMITE GUT

The 16S RNA analysis of bacterial consortium from termite gut indicated the present of *Escherichia coli, Morganella morganii* strain S4L2C (MH745964) with 100% and 98.6% similarity. Incidentally, these microorganisms have not been widely reported to have been isolated from termites gut. Reviewing results obtained by Azizi-Shotorkhoft *et al.,* (2016) showed that isolated bacteria strains from termite gut had 99, 97, and 97% similarity with *Ochrobactrum intermedium, Bacillus licheniformis,* and *Microbacterium paludicola,* respectively. They observed greatest and lowest ($P < 0.05$) acid detergent fibre content of wheat straw with *B. licheniformis* and *O. intermedium,* respectively. Again, unlike strains obtained in this study, Tsegaye *et al.* (2018) reported isolation of *Bacillus* sp. BMP01 and *Ochrobactrum oryzae* BMP03 strains from wood – eating termites which produced 69.96% and 53.74% maximum lignin removal from sample of rice straw.

Otani *et al.* (2014) have stated that 26 bacterial phyla dominated by Firmicutes, Bacteroidetes, Spirochaetes, Proteobacteria and Synergistetes made up gut communities of termite. They observed that gut communities of termites belonging to the same genus were more similar than distantly related species. Besides, three isolates belonging to the genus, Clostridium, one isolate from the groups, Lactobacillaceae, Mycobacteriaceae, or Coryneform and one from the genus, *Proteus*. Of all the 12 isolates obtained, six were cellulolytic (Peristiwati *et al.,* 2018).

Furthermore, reports given by Butera *et al.* (2016) revealed that the termite, *R. lucifugus* gut harbours members of five phyla in the domain Bacteria, which include; Bacteroidetes (1%), the candidatus TG1 phylum (12 %), Spirochaetes (14 %), Proteobacteria (24 %) and Firmicutes (49 % of clones). They observed that Firmicutes, in the genera *Bacillus* and *Paenibacillus* (67 %) formed largest proportion of cellulolytic bacteria of *R. lucifugus* that can be cultured. On the other hand, Ali *et al.* (2019) reported that when the 16s rRNA genes of

isolates of gut of the subterranean termite *Psammotermes hypostoma* Desneux were sequenced, *Lysinibacillus macrolides*, *Paenibacillus lactis*, *Lysinibacillus fusiformis,Bacillus cereus* and *Stenotrophomonas maltophilia.*

From the foregoing, it may be inferred that gut communities may be determined by species of termites studied. Marynowska *et al.* (2020) have clearly shown that prokaryotic communities were determined by carbohydrates metabolism, irrespective of feeding habit, and shared majority of their metabolic signatures.

4.2.3 SUPPLEMENTATION (CO-DIGESTION) OF PRETREATED SUBSTRATES WITH DIFFERENT SOURCES OF NITROGEN

Co-digestion is a technique which involves the mixing and treatment of many different feed stocks at a time to improve performance of anaerobic digestion (Kwietniewska and Tys, 2014). It helps to fill the gap created by the highs and lows in methane production to ensure stability in gas supply (Li *et al.,* 2015). Comparatively, co-digestion yields more total biomethane than obtainable from mono-digestions (Ye *et al.,* 2013). Mono-digestion of lignocellulosic materials often results in a slow process and low methane yield (Sawatfdeenarunat *et al.,* 2015; Surendra *et al.,* 2015; Sawatdeenarunat *et al.,* 2016). This is because C/N ratio is generally an essential factor used to characterize substrates (Atelge *et al.,* 2018), as it affects nutrient balance requirements for anaerobic bacteria, and maintenance of steady environment (Li *et al.,* 2018).

Low C/N ratio could cause the inhibition of ammonia nitrogen and accumulation of VFAs (Meng *et al.,* 2018), as obtainable in protein – rich substrates (Kwietniewska and Tys 2014).

Conversely, excessive C/N ratio results in accumulation of ammonia, thereby inhibiting bacteria growth, and may reduce concentration of nitrogen in the substrate leading to reduced utilization of carbon sources (Resch *et al.,* 2011). Essentially, recommendable C/N ratio range of 20–30 is considered suitable for AD (Kwietniewska and Tys, 2014). For hydrolysis, C/N ratio in the range of 16–45, as well as ratio in ranging from 20–30 for methanogenesis, is recommendable (Atelge *et al.,* 2018). Balance in C/N ratio is usually ensured by co-digestion of substrates with opposing nitrogen and carbon contents, such as co-digesting pig urine (Meng *et al.,* 2018), cow manure (Li *et al.,* 2015) and food waste (Zhang *et al.,* 2018) rice straw to improve system stability and enhance methane production.

This study was designed to determine the amendment and its ratio that is best for co-digestion with pretreated rice straw and water hyacinth. Using $NaOH^-$ pretreated rice straw, results obtained indicated that all substrates amended with cow dung showed higher yield of biogas than controls, with best combination being $NaOH^-$ pretreated rice straw/cow dung in the ratio 2:1. It produced an average of 5580 ml of gas on day 15, which continuously increased to 22,510 ml on 34^{th} day. This is in line with results reported by Omondi *et al.* (2019), which showed that at 24ºC, co-digesting rice straw with 30% ruminal slaughterhouse waste improved biogas production by 75%, from about 8.05 to 14.09L/Kg biomass and equally decreased the retention time for digestion of substrate by 36%. In related studies, Earnest and Singh (2013) have reported that co-digestion of vegetable wastes and fruit with cow dung in the ratio of 1:1 and 1:2 produced 230 and 245 ml of biogas respectively.

Again, co-digestion of cattle manure with organic kitchen wastes in the ratios of 1:1, 1:3, and 3:1 increased biogas production from 24.12 to 47.13% (Aragaw and Andargie, 2013). Using

pig waste, it was observed that amended substrates also performed better than control sample, with the best combination being rice straw/ pig waste in the ratio, 3:1. It produced an average of 220 ml of gas, and 5,010 ml on day 15 and on day 35, it produced an average of 12,220 ml of biogas. Using poultry droppings, the best combination was rice straw/ poultry droppings in the ratio of 3:1, closely followed by rice straw/poultry droppings in the ratio of (2:1). Again, all amended samples produced higher biogas volumes than control. This combination produced 4,740 ml of biogas on day 15 and on day 35, it increased to average of 15,290 ml of biogas.

For bacteria – pretreated rice straw, all substrates amended with cow dung in this study produced higher biogas than controls samples, with 1:1 ratio of rice straw/cow dung being the best combination. It produced an average of 1130 ml of biogas on day 5; 8,950 ml on day 15; and on day 35, it produced 27,050 ml of gas. This study also showed that using poultry droppings, all amended substrates were better than control, with the best combination being rice straw/poultry droppings in the ratio 3:1. It started producing biogas on day 6, giving an average of 100 ml; an average of 2,780 ml on day 15; and an average of 17,810 ml on day 35. Similarly, this study revealed that using pig waste as amendment, all bacteria – pretreated rice straw biomass produced more biogas than control sample, with the best combination of rice straw/ pig waste being 2:1. It produced an average of 100 ml of gas on day 5; 3,160 on day 15; and an average of 18,630 ml of gas on day 35. In a similar study by Natthanicha *et al.* (2017), results have shown that co-digestion was most suitable at ratio of pretreated plants to animal dung at 1:1 using samples of elephant dung, pig dung, and bat dung.

For bacteria – pretreated water hyacinth samples studied, it was recorded that samples amended with cow dung were better than control samples, with the best ratio for co-digestion of water hyacinth/cow dung being 1:1. It produced an average of 110 ml biogas on day 5; an average of 2350 ml on day 15; and an average of 12,030 ml on day 35. Co-digesting water hyacinth with 5-50% ruminal slaughterhouse waste significantly reduced the hydraulic retention time and increased biogas yield (Omondi *et al.,* 2019). Using poultry droppings, this study revealed that the best combination was water hyacinth/poultry droppings in the ratio 1:1, which all outperformed control treatments. It produced an average of 100 ml of biogas on day 5; an average of 1780 ml on day 15; and an average of 11,010 ml on day 35. When pig waste was used as amendment in this study, the best combination ratio was 2:1 of water hyacinth/pig waste and all amended substrates produced higher biogas than control. It yielded an average of 20 ml of biogas on day 5; an average of 1420 ml on day 15; and an average of 7,770 ml on day 35. In their study, Meng *et al.* (2018) have reported that co-digestion of substrates with pig urine yields highest biomethane at lower F/I ratios.

Co-digestion of water hyacinth with cow dungs reportedly produced methane concentration at 24% (Sugumaran *et al.,* 2014) while co-digestion of rice straws with pig dung produced 52.8–55.3% of methane (Dong *et al.,* 2015). From the foregoing, it is evident that different types of animal dungs have varying effects on biogas production, which is in support of results obtained by Natthanicha *et al.,* (2017). The variations recorded in effects of different amendments, as well as their ratios of combination with rice straw and water hyacinth sample pretreated chemically and biologically in this study, is thought to arise due to differences in efficiencies of both methods of pretreatment which resulting in differences in the compositions of each pretreated substrate. Earlier, report has shown that co-digestion of rice

straw with municipal solid wastes yielded best results at 1:2 ratio, which was better than results of rice straw alone; while co-digestion of rice straw gave highest cumulative methane yield of 65 ± 0.93% and lowest at rice straw/municipal solid wastes in a ratio of 1:1 (Negi *et al.,* 2018). As reported in previous studies, the findings of this study affirm that co-digestion of two or more different types of organic biomasses help to overcome deficiencies associated with use of single material (Surendra *et al.,* 2014), such as low C/N ratio found in water hyacinth. As observed, co-digesting water hyacinth and cow manure would correct the low C/N ratio for enhanced anaerobic digestion process, with optimum C/N ratio ranging from 25-30 (Wang *et al.,* 2014).

4.2.4 BIOGAS PRODUCTION AND OPTIMIZATION OF FACTORS AFFECTING IT

As observed in this study, biogas production using bacterial - pretreated rice straw started about 3 to 6 days after set up. This result confirms the report of Okewale and Adesina (2019) which stated that no biogas was produced in all the five digesters used during the first 4 days of fermentation. Such period of inactivity was regarded as the lag phase, during which the inoculums were growing, adapting to the environment and synthesizing enzymes necessary for their fermentation process. Here, the inoculum was also consuming methane precursors resulting from initial activity (Lalitha *et al.,* 1994).

Volume of methane produced increased from 5000ml to about 13500ml and began to plateau as the substrate concentration was increased from 100g to 500g. Similar results were obtained for the hydraulic retention time (HRT) over the 15 – 30 days showed that biogas production was initially low (about 10,000ml) at 15 days, but spiked to maximum of 11,500ml at 19 days before leveling off. It continued to gradually decrease from day 26 till the end of 30 days.

Addition of cow dung as amendment resulted in a consistent increase in biogas production, which rose from below 5000ml at when 100g of cow dung was used to over 13000ml when 500g of it was used. Similar results have stated that for cellulase – pretreated rice straw samples, biogas production was 3.52 and 3.62, 4.30 and 4.40 and 5.78 and 5.80 for 10 U/g, 20 U/g and 30 U/g on 26^{th} and 30^{th} days respectively (Ben-lin *et al.,* 2017). After optimization, substrate concentration of 520g, HRT of 22.57 days and amendment concentration of 520g, with predicted highest biogas production of 1.960×10^4 ml. On validating the predicted yield of biogas three experiments was performed at optimum conditions, the experiments yielded 1.60x104ml of biogas compared to the untreated Rice straw.

For alkaline pretreated rice straw, result of this study revealed that total methane production increased from 7200ml and peaked at 11300 when substrate concentration increased from 340g. Also biogas production increased with HRT of 15 days to 27 days before plateauing. On addition of 100g of cow dung to the substrate, a total of 9000ml of gas was produced, which sharply increased to 13600ml when 500g of cow dung was used. This is supported by the results reported by Ben-lin *et al.,* (2017) which indicated that NaOH – pretreated rice straw produced total biogas of 10.704, 12.053, 18.720, and 14.957 L for 2, 4, 6, and 8%, respectively, under an HRT of 30 days. However, above 99.0% of biogas production occurred in the first 26 days of HRT, with only about 1.0% of it produced in the first 4 days. It has also been reported that rice straw gave highest methane yield at 3% total solid (TS), which was around (141.4±3.70) mL CH_4/g volatile solids (VS) added (Darwin *et al.,* 2014).

In a comparative study, it was reported that highest methane production using silage maize straw was 67.83%, recorded on day 16, whereas the peak for rice straw was 63% on day 14 (Li *et al.,* 2017). Again it was reported that methane production using rice straw was highest

(0.154 L/CH_4) on day 32 and achieving 81.33% COD conversion ratio (Syazwani *et al.,* 2017). When compared with untreated rice straw substrate, NaOH and cellulose – pretreated rice straw gave total biogas yield of 3.38–5.91 and 3.62–6.45, at hydraulic retention time of 30 days respectively. However, highest volumes of biogas and methane were derived from 40 U/g total solids (TS) cellulase-pretreated rice straw (20.433 and 9.918 L respectively) (Ben-lin *et al.,* 2017).

Results of optimization of biogas production using NaOH – pretreated rice straw, amended with cow dung indicated that 216.7g of substrate at HRT of 15.5 days and 520g of cow dung as amendment were the optimum condition, with predicted maximum biogas yield of 1.517 x 10^4 ml however, on validating the predicted yield of biogas three experiments was performed at optimum conditions, the experiments yielded 20.05x 10^4ml of biogas compared to the untreated Rice straw.

. Studies have been previously undertaken to optimize certain parameters affecting biogas production. For instance, with the use of Central Composite Experimental Design for optimization of C/N ratio, Enzyme, and total solid, resulting 10 runs indicated that optimum factors were enzyme concentration of 6%, C/N ratio of 35, and TS of 1.59% with biogas production 202.51 mL/g TS was obtained (Syafrudin *et al.,* 2020).

Generally, it was observed in this study that after the reported optimum HRT of 22.5 and 15.5 days for bacteria – pretreated rice straw and NaOH – pretreated rice straw amended with cow dung respectively was attained, biogas production plateaued and gradually began decline from 26^{th} and 27^{th} days respectively. This observation has been attributed to possible exhaustion of nutrients in the biodigesters operated under batch system. Similar report revealed that biogas production reached peak on the 32^{nd} day as a result of growth of bacteria within the digester.

However, after 32nd day, the bacteria started to starve and competition for food gradually became intense. This decreased the population of microbes and resulting in significant decline in biogas production (Njogu *et al.,* 2015). This is in line with earlier reports that a sharp decrease in biogas production was recorded after 26 days hydraulic retention time, noting that biogas production stopped after 30 days of HRT for both untreated, blank, and pretreated substrates (Ben-lin *et al.,* 2017). Furthermore, Chandra *et al.* (2012), attributed it to consumption of volatile fatty acids by aerobic bacteria to produce methane, thereby changing the pH to alkalinity, which inhibits activities of microorganisms. On the other hand, it has been suggested that variation in population of fermentative bacteria and methanogenic bacteria with changing pH of substrates in the digester may explain the drop in production after 36th day, with production of only 26.5 ml on 38th day, after which no appreciable production of biogas was recorded (Iyagba *et al.,* 2009).

4.2.5 COMPOSITION OF BIOGAS PRODUCED

Concentration of methane is an important factor in this study because the quality of biogas is assessed from the methane content obtained (Rahmansyah *et al.,* 2017). Therefore, concentrations of component gases in biogas produced in this study are 1.149% of CO, 13.556 of CO_2 and 69.960% of methane. This is contrary to results obtained by Rathod *et al.,* (2018) which showed that biogas produced from different sources had carbon dioxide content ranging from 36% to 41%, methane content from 48% to 65%, and nitrogen from 1% to 17%, with oxygen content remaining below<1%.

Meanwhile, it has been reported that biogas content is much affected by pH and temperature (Bitton, 1999). Perhaps this could account for the difference between our results and those

reported by Syazwani *et al.,*(2017) which showed that the composition of biogas produced using co-digested rice straw leachate and domestic wastewater as amendment, with addition of urea was 76.02% nitrogen, 4.12% methane, 17.53% oxygen, 2.28% carbon dioxide, and 0.056% other gases on day 29 of anaerobic digestion. These changed to 2.07% carbon dioxide, 15.34% oxygen, 76.53% nitrogen, 6.01% methane, and 0.05% other gases on day 32. Finally, it became 76.34% nitrogen, 2.14% carbon dioxide, 16.43% oxygen, 5.05% methane, and 0.04% other gases on day 36 of anaerobic digestion.

Low CO_2 and an appreciable percentage of methane present in biogas produced in this study may imply that it can be used for cooking and heating purposes, even without thorough scrubbing, as suggested by Okewale and Adesina, (2019). It has been stated earlier that biogas containing more than 50% of methane in its total composition will support burning (Graaf and Fendler, 2010).

4.2.6 PROXIMATE COMPOSITION OF PRETREATED SLURRIES

Results obtained from this study indicated that anaerobic digestion of substrates increased the contents of various parameters of the substrates, such as nitrogen, phosphorus and potassium. Conversely, carbon content, total solid content and total volatile solid content decreased appreciably after anaerobic digestion. Above result is supported by results of earlier studies which revealed that there was decline in concentration of total solids, volatile solids. Total solids of control sample decreased from 15.5 % to 7.0 % in 40 days, while volatile solids reduced from 83.4% to 56.4%. Reduction in total solids from 13.1 to 4.5 %, and volatile solids from 81.6 to 56.4 % were highest when particle sizes of samples were smaller (Rozy *et al.,* 2017). Again, results of physicochemical analysis of swine manure before digestion were total

organic carbon (TOC) of 860 ± 121.29 mg/L,total Kjeldahl nitrogen of 566.75 ± 92.49 mg/L, pH of 7.29 ± 0.28, volatile solids (VS) of 78.19 ± 1.64% and total solids (TS) of 1.02 ± 0.08%. Following 31 days retention time, the pH was 6.92 ± 0.04, VS was 70.35 ± 0.56%, TKN was 734.06 ± 41.08 mg/L, TOC was 901 ± 21.67 mg/L and TS was 1.92 ± 0.02% (Darwin *et al.,* 2014).

Ben-lin *et al.,* (2017) have reported that initial C/N contents of cattle manure and rice straw samples they studied were 57.2 ± 0.4 and 42.5 ± 0.3, TS (%) were 87.4 ± 0.9 and 85.6 ± 2.1 whereas VS (%) were 56.8 ± 1.1 and 74.9 ± 1.4 respectively. In another study, it was revealed that optimal ratio of pig manure, kitchen waste, and rice straw was 1.6: 0.4:1, and C/N ratio was 21.7. Percentage of methane in resulting biogas was 45.9–70.0% while VS reduction rate was 55.8% (Ye *et al.,* 2013).

Gaseous nitrogen (N) is usually converted, during anaerobic digestion, to ammonia (NH_3), which is water-soluble form of nitrogen that can be utilized by plants as a nutrient (Ludwig, 1988). This gives nutrient ratio of about 1:0.5:1 for $N:P_2O_5:K_2O$. it has earlier been observed that fermented slurry containing lower C/N ratio has better fertilizing characteristics. Thus in comparison with fresh manure, increase in yield of 5% - 15% is obtainable (McCann *et al.,* 1996).

4.2.7 TREND OF MICROBIAL LOAD IN SLURRIES DURING DIGESTION

It has been revealed that inoculums used in anaerobic digestion system may significantly affect its overall performance (Darwin *et al.,* 2014). Also, inoculum plays significant roles in initiating anaerobic digestion process and production of biogas by balancing populations of

various bacteria, including *Syntrophobacter* which degrade propionate, as well as butyrate, and methanogens(Pandey *et al.,* 2011). The rich source of inoculum can be a key player in determining the duration of the lag phase (Gu *et al.,* 2014). A low inoculum source can result in slower and incomplete substrate digestion while a high inoculum size to substrate ratio will increase the hydraulic retention time (HRT) of the bioreactor. This implies that the feed to inoculum ratio plays an important role in determining increased biogas yield (Liu *et al.,* 2009). In addition, previous research has reported that the biogas yield from water hyacinth was independent of the particulate size but dependent on the amendment (nitrogen source) and inoculum volume (Moorhead and Nordstedt 1993).

Consequently, in this study, it was observed that initial bacterial load of 3:1 ratio of rice straw/cow dung (RS/CD) was $2.11x10^{10}$ which is higher than those of other ratios. After day 14, it increased to $5.1x10^{10}$, before plunging to $1.08x10^{9}$ on day 28. For rice straw/poultry dropping (RS/PD), bacterial load was initially recorded as $2.11x10^{10}$, which also increased to $4.3x10^{10}$ on day 14 before plummeting to 8.1×10^{8} on day 28. Similarly, 3:1 rice straw/pig waste (RS/PW) gave the highest initial bacterial load of $2.17x10^{10}$. On day 14, it decreased to $6.0x10^{9}$ and further to $5.9x10^{8}$ on day 28. Meanwhile, bacterial load of untreated RS was $2.96x10^{10}$, $2.41x10^{11}$ and $3.8x10^{8}$ on days 0, 14 and 28 respectively.

It was found in this study that dominant bacteria isolates from RS/CD samples were *Salmonella* sp*, Shigella* sp*, Enterococcus* sp*, Bacillus cereus, Enterobacter* sp*, Staphylococcus aureus* and *Micrococcus roseus.* On the other hand, fungal isolates were *Aspergillus* sp*, Penicillium* sp*, Mucor* sp, *Saccharomyces* sp and *Rhizopus* sp for RS/CD. In the case of RS/PD, bacterial isolates were *Salmonella* sp*, Shigella* sp, *Staphylococcus aureus, Enterococcus* sp*, Pseudomonas aeruginosa* and *Bacillus cereus.* Fungi isolated from the

samples were *Mucor* sp*, Saccharomyces* sp*, Penicillium* sp and *Rhizopus* sp. Similarly, *Salmonella* sp*, Shigella* sp*, Enterococcus* sp, *Micrococcus roseus, Enterobacter* sp*, Staphylococcus aureus* and *Staphylococcus saprophyticus* were dominant bacteria isolates from RS/PW samples. Fungal isolates were *Mucor sp, Penicillium* sp, *Saccharomyces* sp and *Rhizopus* sp.

Report shows that *Bacillus* sp., is responsible for the decomposition of simple organic compounds to inorganic matter. They can convert lignin into biological surface agents, enhance microbial decomposition of recalcitrant organic matter, and increase biogas production efficiency (Yuan, 2011; Yu *et al.,* 2016; Yuan *et al.,* 2016). As earlier presented, most members of phylum, Bacteroidetes are major groups of acidogenic bacteria which inhabit diverse anaerobic environments (Zhao *et al.,* 2013; Ren *et al.,* 2014). Although previous studies implicated Clostridium as capable of rapidly hydrolyzing substrates during anaerobic fermentation and degrade cellulose (Qiao *et al.,* 2013; Ren *et al.,* 2014), it was not isolated from any of the samples used in this study.

Furthermore, community genomic analyses of samples have implicated most members of the phylum, Firmicutes to be very versatile and contribute to degradation of several complex organic residues, including carbohydrates, lipids, and proteins (Ren *et al.,* 2014). In a related study, Li *et al.* (2017) had reported that most common phylogenetic groups present in samples of rice straw, silage corn straw, and tobacco straw, were *Methanoculleus,Methanobacterium, Halobacterium, Methanosarcina,* WCHA2-08_norank, *Methanosaeta,* and others. Again, *Methanosarcina* and *Methanosaeta* have been reported to be acetoclastic (Zhao *et al.,* 2013).

CHAPTER FIVE

5.0 CONCLUSION AND RECOMENDATION

5.1 CONCLUSION

Findings from this study indicated that biological pretreatment enhanced biogas production much better than chemical pretreatment.

Though reports are rare on biological pretreatment of lignocellulosic biomass using *Morgenela morganii* strain S4L2C (MH7459) to enhance biogas production in anaerobic digestion. The results of this research work have shown that*Morgenela morganii* strain S4L2C (MH745964) could be used in the pretreatment of lignocellulosic wastes for improved biogas yield even in large scale production process.

The model used to predict the optimum yield of biogas exhibited a favorable fit with the experimental value which led to increased biogas yield from the pretreated substrate hencesuitable for predicting biogas production process

5.3 RECOMMENDATION

In view of the successful deployment of Response Surface Model in this study, it is recommendable to apply it to other types of pretreatment techniques, such as ionic liquid, ultra-sonication and fungal pretreatment, to further harness their potentials. Also Further studies on this bacterial species are therefore required so as to harness its potentials in the biological pretreatment of lignocellulosic waste for improved biogas yield.

Development of bacterial consortium for anaerobic digestion of recalcitrant lignocellulosic wastes for biofuel production using *Morgenela morganii* strain S4L2C (MH745964) is recommended. This is because biological pretreatment is cheaper and eco-friendly compare to chemical pretreatment.

Furthermore, studies should be undertaken using digestates from anaerobic digestion as soil amendment to fertilize crops to better appreciate their effects on crop productivity

REFERENCES

Abbasi, T., Tauseef, S. M. and Abbasi, S. A. (2012).Anaerobic digestion for global warming control and energy generation-An overview. *Renew Sust Energ Rev*., **16**:228-3242

Adeyanju, A.A. (2008).Effect of seeding of wood-ash on biogas production using pig waste and cassava peels. *J. Eng. Appl. Sci.* **3**: 242 – 245.

Achinas, S., Achinas, V. and Euverink, G.J.W. (2017). *A technological overview of biogas production from biowaste*. *Engineering* **313**:299-307.

Adeleye S.A. and Okorondu S. I. (2015). Bioelectricity from students' hostel waste water using microbial fuel cell. *International Journal of biological and Chemical sciences.* **9**(2):1038-1049.

Adrien,N. G. (2008).Processing water,wastewater, residuals, and excreta for health and environmental protection. in *An Encyclopedic Dictionary* John Wiley and sons,Inc. USA. ISSN :9780470261934

Agbor, V. B., Cicek, N., Sparling, R., Berlin, A. and Levin, D. B. (2011). Biomass pretreatment: *Fundamentals towards application. Biotechnol. Adv.***29**: 675-685.

Agency, I.E. (2015). World energy outlook special report 2015: Energy and climate change. (Final report. OECD/IEA, Paris: International Energy Agency.

Ahring, B. K., Sandberg, M. and Angelidaki, I. (1995).Volatile fatty acids as indicators of process imbalance in anaerobic digesters. *Applied Microbiology and Biotechnology*, **43**(3): 559–565.

Akinbomi, J., Brandberg, T.,Sikiru, A. and Mohammad, J.T. (2014).Development and dissemination strategies for accelerating biogas production in Nigeria. *BioResources,* **9**(3): 5707 -5727.

Ali, H.R.K., Hemeda, N.F. and Abdelaliem, Y.F. (2019). Symbiotic cellulolytic bacteria from the gut of the subterranean termite *Psammotermes hypostoma* Desneux and their role in cellulose digestion. *AMB Expr,* **9**(111): 1-9.

Ao, X., Jun, C,, Wenlu, S., Cong, Y., Junhu, Z. and Kefa, C. (2013). Enhancing enzymatic saccharification of water hyacinth through microwave heating with dilute acid pretreatment for biomass energy utilization. *Energy,* **61**: 158-166.

Appels, L., Baeyens, J., Degrève, J. and Dewil, R. (2008). Principles and potential of the anaerobic digestion of waste activated sludge. *Progress in Energy and Combustion Science,* **34**:755-781.

Appels, L., Lauwers, J., Degrève, J., Helsen, L., Lievens, B., Willems, K., Van Impe, J. and Dewil, R. (2011). Anaerobic digestion in global bio-energy production: potential and research challenges. *Renewable and Sustainable Energy Reviews,* **15**(9):4295-4301.

Aragaw, T. and Andargiem M. (2013). Co-digestion of cattle manure with organic kitchen waste to increase biogas production using rumen fluid as inoculums. *Int J Phys Sci,* **8**: 443-450.

Aslanzadeh, S. (2011). Pretreatment for better biogas.*BioResources,* **6**(4): 5193-5205.

Aslanzadeh, S. (2014).Pretreatment of cellulosic waste and high rate biogas production. Doctoral Thesis on Resource Recovery, University of Borås, Borås, 1-50.

Atelge, M.R., Krisa, D., Kumar, G., Eskicioglu, C., Nguyen, D.D., Chang, S.W, Atabani, A.E., Al-Muhtaseb, A.H. and Unalan, S. (2018). Biogas production from organic waste: recent progress and perspectives. *Waste and Biomass Valorization,*

Azizi-Shotorkhoft, A., Mohammadabadia, T., Motamedib, H., Chajia, M. and Fazaeli, Q.H. (2016). Isolation and identification of termite gut symbiotic bacteria with lignocellulose-degrading potential, and their effects on the nutritive value for ruminants of some by- products. *Anim. Feed Sci. Tech.*, 0:0. DOI: http://dx.doi.org/10.1016/j.anifeedsci.2016.04.016.

Azman, S., Khadem, A.F., Lier, J.B., Zeeman, G. and Plugge, C.M. (2015). Presence and role of anaerobic hydrolytic microbes in conversion of lignocellulosic biomass for biogas production. *Crit Rev Environ Sci Technol,* **12**: 36-45.

Barakat, A., De Vries, H. and Rouau, X. (2013). Dry Fractionation process as an important step in current and future lignocellulose biorefineries: a review *Bioresour.Technol.*, **134**: 362-373.

Ben-lin, D., Xu-jing, G., Dong-hai, Y. and Ji-ming, X. (2018). Comparison of different pretreatments of rice straw substrate to improve biogas production. *Waste Biomass Valorization.* **9**(1) :1503-1512

Bergier, I., Salis, S.M., Miranda, C.H.B., Ortega, E., and Luengo, C.A. (2012). Biofuel production from water hyacinth in the Pantanal wetland. *Ecohydrol. Hydrobiol.,* 12: 77–84.

Biswas, R., Ahring, B.K. and Uellendahl, H. (2012). Improving biogas yields using an innovative concept for conversion of the fiber fraction of manure. *Water Science and Technology* 66:1751–1758.

Bitton, G. (1999). Wastewater microbiology. 2nd ed. Wiley Liss Inc. New York.

Björnsson, L., Murto, M. and Mattiasson, B. (2000). Evaluation of parameters for monitoring an anaerobic co-digestion process. *Applied Microbiology and Biotechnology*, **54**(6): 844–849.

Borjesson, P. and Mattiasson, B. (2008). Biogas as a resource-efficient vehicle fuel. *Trends Biotechnol,***26**(1):7-13.

Bozan, M., Akyol, C., Ince, O., Aydin, S. and Ince, B. (2017). Application of next-generation sequencing methods for microbial monitoring of anaerobic digestion of lignocellulosic biomass. *Appl Microbiol Biotechnol,* **101**(18), 6849-6864.

Braide W, Kanu I.A, Oranusi U.S and Adeleye S.A. (2016). Production of bioethanol from agricultural waste. *J. Fundam. Appl. Sci*. **8**(2): 372-386.

Braun, R., Drosg, B., Bochmann, G., Weiß, S. and Kirchmayr, R.(2010). Recent developments in bio-energy recovery through fermentation. In: B. D. R. Braun, G. Bochmann, S. Weiß, R. Kirchmayr (ed). Microbes at work: From wastes to resources: Springer. 35-58.

Brodeur, G., Yau, E., Badal, K., Collier, J., Ramachandran, K.B. and Ramakrishnan, S. (2011). Chemical and Physicochemical Pretreatment of Lignocellulosic Biomass: A Review. *Enzyme Research*. 1-17.

Butera, G., Ferraro, C., Alonzo, G., Colazza, S. and Quatrini, P. (2016). The gut microbiota of the wood-feeding termite *Reticulitermes lucifugus* (Isoptera; Rhinotermitidae). *Ann Microbiol.,***66**:253–260.

Chandra, R., Takeuchi, H. and Hasegawa, T. (2012). Methane production from lignocellulosic agricultural crop wastes: A review in context to second generation of biofuel production.*Renewable and Sustainable Energy Reviews*. **16**:1462-1476.

Chen, Y., Cheng, J.J. and Creamer, K.S. (2008). Inhibition of anaerobic digestion process: A review. *Bioresource Technology*, **99**:4044-4064.

Cheng, J., Xie, B., Zhou, J., Song, W., and Cen, K. (2010). Cogeneration of H_2 and CH_4 from water hyacinth by two-step anaerobic fermentation. *International Journal of Hydrogen Energy,* **35**: 3029-3035.

Chen, H. (2014). Chemical composition and structure of natural lignocellulose. In: Chen, H. (ed) Biotechnology of Lignocellulose, pp. 25–71. Springer, Dordrecht.

Cherubini, F. (2010). The biorefinery concept : Using biomass instead of oil producing energy and chemicals. *Energy Convers. Manage.*, **51**:1412-1421.

Claassen, P.A.M., Van Lier, J.B., Lopez Contreras, A.M., Van Niel, E.W.J., Sijtsma, L., Stams, A.J.M., De Vries, S.S. and Weusthuis, R.A. (1999). Utilisation of biomass for the supply of energy carriers. *Applied Microbiology and Biotechnology*. **52**(6):741-755.

Cohen, A.,Zoetemeyer, R.J., Van Deursen, A. and Van Andel, J.G. (1979). Anaerobic digestion of glucose with separated acid production and methane formation. *WaterResearch*. **13**:571-580.

Darwin, A.E., Cheng, J.J., Liu, Z.M., Gontupil, J., Kwon, O.S. (2014). Anaerobic co-digestion of rice straw and digested swine manure with different total solid concentration for methane production. *Int J Agric & Biol Eng,***7**(6): 79－90.

De Baere, L. (2000) Anaerobic digestion of solid waste: state of the art. *Water Science and Technology*,**41**: 283-290

De Francisci, D., Kougias, P.G., Treu, L., Campanaro, S. & Angelidaki, I. (2015). Microbial diversity and dynamicity of biogas reactors due to radical changes of feedstock composition. *Bioresource Technology,* **176**: 56-64.

De Vrieze, J., Saunders, A.M., He, Y., Fang, J., Nielsen, P.H.,Verstraete, W. and Boon, N.(2015). Ammonia and temperature determine potential clustering in the anaerobic digestion microbiome. *Water Research,* **75**(10): 312-323.

Deepanraj, B.,Sivasubramanian, V. and Jayaraj, S. (2014). Biogas generation through anaerobic digestion process - An overview. *Res. J. Chem. Environ,***18**(5): 333-545

Demirel,B. and Scherer, P. (2011). Trace element requirements of agricultural biogas digesters during biological conversion of renewable biomass to methane. *Biomass and Bioenergy,* **35**(3): 992-998.

Demirel, B.and Yenigün, O. (2002). Two-phase anaerobic digestion processes: A review. *Journal of Chemical Technology and Biotechnology*; **77**:743-755.

Demirel, B. and Scherer, P. (2008). The Roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: A review, *Reviews in Environmental Science and Biotechnology*, **7**(2):173-190.

Demirer, G.N. and Chen, S. (2005).Two-phase anaerobic digestion of unscreened dairy manure. *Process Biochem.*, **40**: 3542–3549.

Do, T.H., Le, N.G., Dao, T.K., Nguyen, T.M.P., Le, T.L., Luu, H.L., Nguyen, K.H.V., Nguyen, V.L., Le, L.A., Phung, T.N., van Straalen, N.M., Roelofs, D.and Truong, N.H. (2018). Metagenomic insights into lignocellulose-degrading genes through Illumina-

based de novo sequencing of the microbiome in Vietnamese native goats' rumen. *J Gen Appl Microbiol,* **64**(3): 108-116.

Dong, X., Xin, Y., Jian, W., Liu, X. and Ling, D. (2000). Bifidobacteriumthermacidophilum sp. nov., isolated from an anaerobic Digester, *International Journal of Systematic andEvolutionary Microbiology*, **50**(1): 119-125.

Dong, L., Shengchu, L., Li, M., Zhidong, L., Yuexiang, Y., Zhiying, Y. and Xiaofeng, L. (2015). Effects of feedstock ratio and organic loading rate on the anaerobic mesophilic co-digestion of rice straw and pig manure. *Bioresource Technology,***187**: 120–127.

Earnest, V.P. and Singh, L.P. (2013). Biomethanation of vegetable and fruit waste in co-digestion process. *Int. J. of Emerg. Technol. and Advanced Eng.,* 3(6): 493-495.

Fabien, M. (2003). An introduction to anaerobic digestion of organic wastes, Final report, Remade Scotland.

Retrieved from http//homepage2.nifty.com/biogas/cnt/refdoc/whrefdoc/d8feed.pdf on 2nd July, 2019.

Fernandes, I.A.P. (1986). Application of porous membranes for biomass retention in a two-phase anaerobic process. University of Newcastle upon Tyne. 50 – 70.

Florencio, L., Nozhevnikova, A., Van Langerak, A., Stams, A. J. M., Field, J. A. and Lettinga, G. (1993). Acidophilic degradation of methanol by a methanogenic enrichment culture. *FEMS Microbiology Letters*, **109**(1):1–6.

Fricke,K., Santen, H. and Wallmann, R. (2005). Comparison of selected aerobic and anaerobic procedures for MSW treatment, *WasteManage*., **25**:799-810.

Ganguly, A., Chatterjee, P.K., and Dey, A. (2012). Studies on ethanol production from water hyacinth: A review. *Renew. Sustain. Energy Rev.,* **16**: 966–972.

Gerardi, M.H. (2003).The microbiology of anaerobic digesters. Wiley, Hoboken. 89-92.

Ghosh,S. and Pohland, F.G. (1974).Kinetics of substrate assimilation and product formation in anaerobic digestion.*Journal of Water Pollution Control Federation*. 748-759.

Girisha,S.T.,Siddaramaiah,M.S. and Ramaiah, S.K. (2017) Efficacy of Cellulose degrading microbes in bioethanol production from Estuarine algea- Ulva lactuca *journal of Algal Biomass Utilization* **8**(4):110-117.

Girard,M., Palacios, J. H., Belzile, M., Godbout, S. and Pelletier, F. (2013). Biodegradation in animal manure management.*Biodegradation Engineering and Technology,***14**: 126-132.

Glass,J.B. and Orphan, V.J. (2012). Trace metal requirements for microbial enzymes involved in the production and consumption of methane and nitrous oxide. *Frontiers in Microbiology,* **3**: 61-75.

Gnansounou,E.and Dauriat, A. (2010). Technoeconomic analysis of lignocellulosic ethanol: A review. *Bioresource Technology,* 4980–4991.

Gourdon, R. and Vermande, P. (1987). Effects of propionic acid concentration on anaerobic digestion of pig manure. *Biomass*, **13**(1):1– 12.

Gerardi, H. (2003). The microbiology of anaerobic digesters. John Wiley & Sons, Inc., Hoboken, New Jersey. Pp. 11 – 16.

Graaf, D. and Fendler, R. (2010). Biogas production in Germany. Federal Environment Agency, p 29.

Gujer, W. and Zehnder, A.J.B. (1983). Conversion processes in anaerobic digestion. *Water Sci. Technol*., **15**:127–167.

Hendriks, A.T.W.M. and Zeeman, G.(2009).Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource technology***100**: 10–18.

Hidaka, T.,Arai, S., Okamoto, S. and Uchida, T. (2013).Anaerobic co-digestion of sewage sludge with shredded grass from public green spaces. *Bioresource Technology,***130**: 667–672.

Holm-Nielsen, J.B., Al Seadi, T. and Oleskowicz-Popiel, P. (2009). The future of anaerobic digestion and biogas utilization. *Bioresour Technol,* **100**: 234-241.

Iyagba, E.T., Mangibo, I.A. and Mohammad, Y.S. (2009). The study of cow dung as co-substrate with rice husk in biogas production. *Scientific Research and Essay,* **4**(9): 861-866.

Jagadish, H.P., Mal, A.R., Shankar, B.B., Mahesh, K.S. and Pradeep, K.B.P. (2014). Anaerobic co-digestion of water hyacinth and sheep waste. *Energy Procedia,***52**: 572 – 578.

Jeihanipour A. (2011).Waste textiles bioprocessing to ethanol and biogas.Doctoral Thesis, Chalmers University of Technology;Gothenburg.

Johnson, D.K. and Elander, R.T. (2009). Pretreatments for enhanced digestibility of feedstocks. In: M. E. Himmel (ed.)Biomass recalcitrance deconstructing the plant cell wall for bioenergy. Blackwell Publishing Ltd. USA. 436 - 453.

Jia, Y., Ng, S.K., Lu, H., Cai, M. and Lee, P.K.H. (2018). Genome-centric metatranscriptomes and ecological roles of the active microbial populations during cellulosic biomass anaerobic digestion. *Biotechnol Biofuels,* **11**(1): 117.

Jørgensen, H., Kristensen, J.B. and Felby, C. (2007). Enzymatic conversion of lignocellulose into fermentable sugars: Challenges and opportunities.*Biofuels,Bioproducts and biorefining* **1**(2): 119–134.

Ju, D. J., Jung, C. S., Park, J. J., Byun, I. G. and Park, T. J. (2008). Methane production and microbial community from vs concentration in anaerobically co-digesting of food waste and sewage sludge. *Proceedings of the Spring conference, Ulsan, Environmental EngineeringScience*, 443–450.

Kato, M.T, Field, J.A., Versteeg, P. and Lettinga, G. (1994). Feasibility of expanded granular sludge bed reactors for the anaerobic treatment of low strength soluble wastewaters. *Biotechnology and Bioengineering*, **44**(4): 469–479.

Karmellos, M., Kopidou, D. and Diakoulaki, D. (2016). A decomposition analysis of the driving factors of CO_2 (Carbon dioxide) emissions from the power sector in the European Union countries. *Energy,* **94**: 680-692.

Kaur, K. and Phutela, U.G. (2016). Enhancement of paddy straw digestibility and biogas production by sodium hydroxide-microwave pretreatment. *Renew Energy,* **92**:178–184.

Kengo, S., Masahiko, M., Shin-Ichi, H., Daisuke, S., Naoya, O. and Yasuo, I. (2010). Efficient degradation of rice straw in the reactors packed by carbon fiber textiles. *Appl. Microbiol. Biotechnol.,***87**: 1579-1586.

Khanal S.K. (2008).Anaerobic biotechnology for bioenergy production principles and applications, Wiley-Blackwell, Singapore. Pp. 234-237.

Khoiyangbam, R.S., Gupta, N. and Kumar, S. (2011). Biogas technology towards sustainable development, TERI , New Delhi.pp 200

Koo, T., Shin, S. G., Lee, J., Han, G., Kim, W., Cho, K. and Hwang, S. (2017). Identifying methanogen community structures and their correlations with performance parameters in four full-scale anaerobic sludge digesters, *Bioresource Technology*, **228**: 368-373.

Kothari, R., Tyagi, V. and Pathak, A. (2010). Waste-to-energy,away from renewable energy sources to sustainable development, *Renew SustEnerg Rev.,* **14**: 3164-3170.

Kratky, L. and Jirout, T. (2011). Biomass size reduction machines for enhancing biogas production. *Chemical Engineering & Technology.* **34**:391–399.

Kumar, P., Barrett, D.M., Delwiche, M.J. and Stroeve, P. (2009). Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial & Engineering Chemistry Research*; **48**:3713-3729.

Kumar, R. and Wyman, C.E. (2009). Effects of cellulase and xylanase enzymes on the deconstruction of solids from pretreatment of poplar by leading technologies. *Biotechnology Progress*; **25**:302-314.

Kurr, M., Huber, R., König, H., Jannasch, H.W., Fricke, H., Trincone, A., Kristjansson, J.K. and Stetter, K.O. (1991). *Methanopyrus kandleri*, Gen. and sp. nov. represents a novel group of hyperthermophilic methanogens, growing at 110°C. *Archives of Microbiology,* **156**(4):239-247.

Kwietniewska, E. and Tys, J. (2014) Process characteristics, inhibition factors and methane yields of anaerobic digestion process, with particular focus on microalgal biomass fermentation. *Renew Sust Energ Rev,***34**:491–500.

Lebuhn, M., Hanreich, A., Klocke, M., Schluter, A., Bauer, C. and Perez, C.M. (2014). Towards molecular biomarkers for biogas production from lignocellulose-rich substrates. *Anaerobe,* **29**:10-21.

Lebuhn, M., Liu, F., Heuwinkel, H. and Gronauer, A. (2008). Biogas production from mono-digestion of maize silage-long-term process stability and requirements. *Water Science and Technology,* **58**(8): 1645.

Li, D., Liu, S., Mi, L., Li, Z., Yuan, Y., Yan, Z. and Liu, X. (2015). Effects of feedstock ratio and organic loading rate on the anaerobic mesophilic co- digestion of rice straw and cow manure. *Bioresour Technol,***189**: 319–326.

Li, S. (2015). Biogas production from lignocellulosic materials. Doctoral Thesis. Swedish University of Agricultural Sciences, Uppsala. 26.

Li, X., Liu, Y.H., Zhang, X., Ge, C.M., Piao, R.Z., Wang, W.D., Cui, Z.J. and Zhao, H.Y. (2017). Evaluation of biogas production performance and dynamics of the microbial community in different straws. *J. Microbiol. Biotechnol.,* **27**(3), 524–534.

Li, Y., Li, L., Sun, Y. and Yuan, Z. (2018). Bioaugmentation strategy for enhancing anaerobic digestion of high C/N ratio feedstock with methanogenic enrichment culture. *Bioresour Technol,***261**:188–195.

Liu, C., Li, H., Zhang, Y., Si, D. and Chen, Q. (2016). Evolution of microbial community along with increasing solid concentration during high-solids anaerobic digestion of sewage sludge, *Bioresource Technology*, **216**: 87-94.

Lin, l., Yan, R., Liu,Y. and Jiang, W. (2010).In-depth investigation of enzymatic hydrolysis of biomass wastes based on three major components: Cellulose,hemicellulose and lignin.*Bioresource Technology,***101**(21): 8217-8223.

Lucas, R., Kuchenbuch, A., Fetzer, I., Harms, H. and Kleinsteuber, S. (2015). Long-term monitoring reveals stable and remarkably similar microbial communities in parallel

full-scale biogas reactors digesting energy crops. *FEMS Microbiology Ecology,***91**(3): 237-246.

Ludwig, S. (1988). Biogas plants: A publication of the Deutsches Zentrum für Entwicklungstechnologien - gATE. In: *Deutsche Gesellschaft für Technische Zusammenarbeit* (GTZ). 1-66. Retrieved on 3rd March, 2021 from https://www.susana.org/_resources/documents/default/2-1799-biogasplants.pdf

Lynd, L.R., Weimer, P.J., Van Zyl, W.H. and Pretorius, I.S. (2002a). Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews,* **66**(3): 506-577.

Ma, F., Yang, N., Xu, C., Yu, H., Wu, J. and Zhang, X. (2010). Combination of biological pretreatment with mild acid pretreatment for enzymatic hydrolysis and ethanol production from water hyacinth. *Bioresource Technology,* **101**: 9600-9604.

Martínez-Gutiérrez, E. (2018). Biogas production from different lignocellulosic biomass sources: advances and perspectives.*Biotech,* **8**(5): 34-47.

Matsuoka, M., T. Tsuchida, K.Matsushita, O. Adachi, and F. Yoshinage. 1996. A synthetic medium for bacterial cellulose production by *Acetobacter xylinum* Subsp. *sucrofermentans*. *Biosci. Biotech. Biochem.,***60**: 575-579.

Meyer-Aurich, A., Lochmann, Y., Klauss, H.and Prochnow, A. (2016). Comparative advantage of maize-and grass-silage based feedstock for biogas production with respect to greenhouse gas mitigation. *Sustainability,* **8**(7):617-625.

Mao, C., Feng, Y., Wang, X. and Ren, G. (2015). Review on research achievements of biogas from anaerobic digestion. *Renewable and Sustainable Energy Reviews,* **45**: 540-555.

Marynowska, M., Goux, X., Sillam-Dussès, D., Rouland-Lefèvre, C., Halder, R., Wilmes, P., Gawron, P., Roisin, Y., Delfosse, P. and Calusinska, M. (2020). Compositional and functional characterisation of biomass-degrading microbial communities in guts of plant fibre- and soil-feeding higher termites. *Microbiome,* **8**(96): 1-18.

Massey, M.L. and Pohland, F.G.(1978). Phase separation of anaerobic stabilization by kinetic controls. *Journal of Water Pollution Control Federation*. 2204-2222.

Mata A. J. (2002).Biomethanization of the organic fraction of municipal solid wastes, IWA Publishing, London pp 336**.**

Mata-Alvarez, J., Dosta, J., Romero-Güiza, M.S., Fonoll, X., Peces, M. and Astals, S. (2014). A critical review on anaerobic co-digestion achievements between 2010 and 2013. *Renewable and Sustainable Energy Reviews,* **36**(10):412-427.

Mawson, A. J.,Earle, R. L. and Larsen, V. F. (1991).Degradation of acetic and propionic acids in the methane fermentation. *Water Research*, **25**(12): 1549–1554.

McCann, J.A., Taylor, F., Mithe, D.E. and Willy, R. (1996). Nonindigenous aquatic and selected terrestrial species of florida-status, pathway, and time of introduction, present distribution, and significant ecological and economic effects. *Southeastern Biological Science Center, Gainesville,* 256 pp.

Meng, L., Xie, L., Kinh, C.T., Suenaga, T., Hori, T., Riya, S., Terada, A. and Hosomi, M. (2018). Influence of feedstock-to-inoculum ratio on performance and microbial community succession during solid-state thermophilic anaerobic co-digestion of pig urine and rice straw. *Bioresour Technol,***252**:127–133.

Merlin Christy, P., Gopinath, L.R. and Divya, D. (2014). A review on anaerobic decomposition and enhancement of biogas production through enzymes and microorganisms. *Renewable and Sustainable Energy Reviews*, **34**: 167-173.

Merlin, C.P., Gopinath, L.R. and Divya, D. (2014). A review on anaerobic decomposition and enhancement of biogas production through enzymes and microorganisms. *Renewable and Sustainable Energy Reviews,* **34**: 167-173.

Mishima, D., Kuniki, M., Sei, K., Soda, S., Ike, M. and Fujita, M. (2008). Ethanol production from candidate energy crops: Water hyacinth *(Eichhornia crassipes)* and water lettuce *(Pistia stratiotes L.). Bioresource Technology,***99**: 2495-2500.

Moestedt, J.,Nilsson, P. S. and Schnurer, A. (2013). The effect of substrate and operational parameters on the abundance of sulphate-reducing bacteria in industrial anaerobic biogas digesters. *Bioresource Technology,* **132**: 327-332.

Mohnen, D., Bar-Peled, M. and Somerville, C. (2009). Cell wall polysaccharide synthesis. In: M. E. Himmel (ed). Biomass recalcitrance: Deconstructing the plant cell wall for bioenergy. Blackwell Publishing Ltd. USA. 94-187.

Möller, K. and Müller, T. (2012). Effects of anaerobic digestion on digestate nutrient availability and crop growth: a review. *Engineering in Life Sciences,* **12**(3): 242-257.

Morrison, M., Pope, P.B., Denman, S.E.and McSweeney, C.S.(2009). Plant biomass degradation by gut microbiomes: more of the same or something new? *Curr Opin Biotechnol,* **20**(3):358-363.

Mulat, D.G. and Horn, S.J. (2018).Biogas production from lignin via anaerobic digestion. In: *Lignin Valorization* 391-412.

Mutschlechner, M., Illmer, P. and Wagner, A.O. (2015). Biological pre-treatment: Enhancing biogas production using the highly cellulolytic fungus *Trichoderma viride.Waste Management,* **43**: 98–107.

Mulat, D.G.and Horn, S.J. (2018). Biogas production from lignin via anaerobic digestion.*Lignin Valorization*, 391-412.

Muyiwa, S. A., Francis, K. and John, M. (2018). A review of commercial biogas systems and lessons for Africa. *Energies*, 11: 2984.

Natthanicha, S., Kingkan, K. and Suwimon, P. (2017). Biomethane recovery from fresh and dry water hyacinth anaerobic co-digestion with pig dung, elephant dung and bat dung with different alkaline pretreatments. *Energy Procedia,* **138**: 294–300.

Nayono, S.E. (2009). Anaerobic digestion of organic solid waste for energy production,*Bioresource Technology* **30**(10) 1828-1833**.**

Negi, S., Dhar, H., Hussain, A. and Kumar, S. (2018) Biomethanation potential for co-digestion of municipal solid waste and rice straw: a batch study. *Bioresour Technol,* **254**:139–144.

Nielsen, H. B. Uellendahl, H. and Ahring, B. K. (2007). Regulation and optimization of the biogas process: propionate as a key parameter. *Biomass and Bioenergy*, **31**(11-12):820–830.

Nijaguna, B. T. (2012). Biogas technology, New Age International (P) Limited Publishers, New Delhi. Pp. 23 – 50.

Niu, Q., Hojo, T., Qiao, W., Qiang, H. and Li, Y.-Y. (2014). Characterization of methanogenesis, acidogenesis and hydrolysis in thermophilic methane fermentation of chicken manure. *Chemical Engineering Journal,* **244**: 587-596.

Njogu, P., Kinyua, R., Muthoni, P. and Nemoto, Y. (2015). Biogas production using water hyacinth (*Eicchornia crassipes*) for electricity generation in Kenya. *Energy and Power Engineering*, **7**: 209-216.

Noike, T., Endo, G., Chang, J.E., Yaguchi, J.I. and Matsumoto, J.I. (1985). Characteristics of carbohydrate degradation and the rate-limiting step in anaerobic digestion. *Biotechnol Bioeng,* **27**(10):1482-1489.

Nordell, E., Nilsson, B., Nilsson Påledal, S., Karisalmi, K. and Moestedt, J. (2015). Co-digestion of manure and industrial waste – The effects of trace element addition. *Waste Management*.**47**:21-27

Ogujiuba, K. (2014). Poverty incidence and reduction strategies in Nigeria: Challenges of meeting 2015 MDG targets. *J Economics,***5**(2): 201-217.

Okewale, A.O and Adesina, O.A. (2019). Evaluation of biogas production from co-digestion of pig dung, water hyacinth and poultry droppings. *Waste Disposal & Sustainable Energy,***1**(4):271-277

Omondi, E.A., Njuru, P.G. and Ndiba, P.K. (2019). Anaerobic co-digestion of water hyacinth (*E*. crassipes) with ruminal slaughterhouse waste for biogas production. *International Journal of Renewable Energy Development,***8**(3): 253-259.

Otani, S., Mikaelyan, A., Nobre, A., Hansen, L.H., Kone, N.A., Søren, J.S., Aanen, D.K., Boomsma, J.J., Brune, A. and Poulsen, M. (2014). Identifying the core microbial community in the gut of fungus-growing termites. *Molecular Ecology,***23**: 4631–4644.

Pandey, P.K., Ndegwa, P.M., Soupir, M.L., Alldredge, J.R. and Pitts, M.J. (2011). Efficacies of inoculation the startup of anaerobic reactors treating dairy manure under stirred and unstirred conditions. *Biomass and Bioenergy,* **35**(7): 2705– 2720.

Peristiwati, D.J., Natamihardja, Y.S. and Herlini, H. (2018). Isolation and identification of cellulolytic bacteria from termites gut (*Cryptotermes sp.*). *IOP Conf. Series: Journal of Physics: Conf. Series,*1013: 1-7.

Pickett, J., Anderson, D., Bowles, D., Bridgwater, T., Jarvis, P., Mortimer, N., Poliakoff, M., Woods, J. (2008). Sustainable biofuels: prospects and challenges, The Royal Society. UK. London. Pp. 120.

Pohland, F.G. and Mancy, K.H. (1969). Use of pH and pE measurements during methane biosynthesis. *Biotechnology and Bioengineering*. **11**:683-699.

Pohland, F.G. and Ghosh, S. (1971). Developments in anaerobic stabilization of organic wastes—the two-phase concept. *Environmental Letters*, **1**: 255-266.

Qiao, J.T., Qiu, Y.L., Yuan, X.Z., Shi, X.S., Xu, X.H. and Guo, R. B. (2013). Molecular characterization of bacterial and archaeal communities in a full-scale anaerobic reactor treating corn straw. *Bioresour. Technol.,* **143**: 512-518.

Rahmansyah, M.S., Wilujeng, S.A., Warmadewanthi, R. and Pandebesie, E.S. (2017). Co-digestion of water hyacinth *(Eichhornia crassipes)* mixed with cow manureto enhance biogas production. *International Journal of ChemTech Research,***10**(9): 988-993.

Rajagopal, R., Massé, D.I. and Singh, G.A. (2013). Critical review on inhibition of anaerobic digestion process by excess ammonia. *Bioresour. Technol*., **143**: 632-641.

Ramírez, M., Fernández, M., Granada, C., Le Borgne, S., Gómez, J.M. and Cantero, D. (2011). Biofiltration of reduced sulphur compounds and community analysis of sulphur-oxidizing bacteria. *Bioresource Technology,* **102**(5): 4047-4053.

Ransom-Jones, E., Jones, D.L., McCarthy, A.J.and McDonald, J.E. (2012). The Fibrobacteres: An important phylum of cellulose-degrading bacteria. *Microbial Ecology,* **63**(2):267-281.

Rapport, J.,Zhang, R., Jenkins, B.M. and Williams, R.B. (2008). Current anaerobic digestion technologies used for treatment of municipal organic solid waste.California Environmental Protection Agency, Sacramento.pp 90

Rathod, V.P., Bhale, P.V., Mehta, R.S., Harmani, K., Bilimoria, S., Mahida, A. and Champaneri, H.(2018). Biogas production from water hyacinth in the batch type anaerobic digester. *Materials Today: Proceedings,* **5**: 23346–23350.

Ren, J., Yuan, X., Li, J., Ma, X., Zhao, Y. and Zhu, W. (2014). Performance and microbial community dynamics in a twophase anaerobic co-digestion system using cassava dregs and pig manure. *Bioresour. Technol.,* **155**: 342-351.

Resch, C., Wörl, A., Waltenberger, R., Braun, R. and Kirchmayr, R. (2011). Enhancement options for the utilisation of nitrogen rich animal byproducts in anaerobic digestion. *Bioresour Technol,* **102**:2503–2510.

Rezania, S., Ponraj, M., MdDin, M.F., Songip, A.R., Sairan, F.M., and Chelliapan, S. (2015). The diverse applications of water hyacinth with main focus on sustainable energy and production for new era: An overview. *Renew. Sustain. Energy Rev.,* **41**: 943–954.

Rodriguez, C., Alaswad, A., Benyounis, K.Y. and Olabi, A.G. (2016). Pretreatment techniques used in biogas production from grass.*Renewable andSustainable Energy Reviews*. 1–12.

Rozy, S., Rouf, A.D. and Urmila, G.P. (2017). Optimization of biogas production from water hyacinth (*Eichhornia crassipes*). *Journal of Applied and Natural Science,* **9**(4): 2062 - 2067.

Rubin, E.M. (2008).Genomics of cellulosic biofuels. *Nature,* **454**:841–845.

Rupf, G.V., Bahri, P.A., De Boer, K. and McHenry, M.P.(2016).Broadening the potential of biogas in Sub-Saharan Africa: An assessment of feasible technologies and feedstocks. *Renew. Sustain. Energy Rev*., **61**:556–571.

Sagarika, M. and Venkateswara, R.P. (2020). Review on anaerobic digestion of rice straw for biogas production. *Environmental Science and Pollution Research,* 0:0. DOI: https://doi.org/10.1007/s11356-020-08762-9.

Saha, B. C. (2005).Enzymes as biocatalysts for conversion of lignocellulosic biomass to fermentable sugars. In *Handbook of Industrial Biocatalysis*, ed. C. T. Hou, CRC Press. Pp. 45-71.

Sahlström, L. (2003). A review of survival of pathogenic bacteria in organic waste used in biogas plants. *Bioresource Technology,* **87**(2):161-166.

Sambo, A.S (2005). Renewable energy for rural development: The Nigerian perspective. *ISESCO Science and Technology Vision,* **1**:12-22.

Sánchez, C. (2009). Lignocellulosic residues: biodegradation and bioconversion by fungi. *Biotechnology Advances,* **27**:185-194.

Scarlat, N., Dallemand, J.F. and Fahl, F. (2018). Biogas: Developments and perspectives in Europe. *Renewable Energy,* **129**:457-472.

Smeets, E.M.W., Faaij, A.P.C, Lewandowski, I.M. and Turkenburg, G. (2007). A bottom-up assessment and review of global bio-energy potentials to 2050. *Prog Energ Combust,* **33**:56–106.

Sawatdeenarunat, C., Surendra, K.C., Takara, D., Oechsner, H. and Khanal, S.K. (2015). Anaerobic digestion of lignocellulosic biomass: Challenges and opportunities. *Bioresource Technology,* **178**(10): 178-186.

Schink, B. (1997). Energetics of syntrophic cooperation in methanogenic degradation, *Microbiology and Molecular Biology Reviews*, **61**(2):262-280.

Schnurer, A. and Jarvis, A. (2009) Microbiological Handbook for Biogas Plant. Swedish Waste Management, Swedish Gas Centre, Malmö. 1-74.

Schnürer, A. and Nordberg, A. (2008). Ammonia, a selective agent for methane production by syntrophic acetate oxidation at mesophilic temperature. *Water Science and Technology,* **57**(5): 735-740.

Sehgal, K. (2018).Current state and future prospects of global biogas industry. In Biogas, biofuel and biorefinery technologies, Springer: Cham, Switzerland. Pp.449–472.

Shetti D.J.Kshirsagar,p. Lanjekar,V.(2016) Alkali pretreatment at ambient temperature : Apromising method to enhance biomethanation of rice straw. *Bioresource Technology* ***226***:80-88

Shi, J., Chinn, M.S. and Sharma-Shivappa, R.R. (2008). Microbial pretreatment of cotton stalks by solid state cultivation of *Phanerochaete chrysosporium.Bioresource Technology*; **99**:6556-6564.

Shu, C.H., Jaiswal, R. and Shih, J.S. (2015). Improving biodegradation of rice straw using alkaline and *Aspergillus niger* pretreatment for methane production by anaerobic co-digestion. *J Bioprocess Biotech,* 5:1-7.

Stams, A.J., Oude Elferink, S.J. and Westermann, P. (2003). Metabolic interactions between methanogenic consortia and anaerobic respiring bacteria. *Advances in Biochemical Engineering/Biotechnology,* **81**: 31-56.

Sträuber, H.,Schröder, M. and Kleinsteuber, S. (2012).Metabolic and microbial community dynamics during the hydrolytic and acidogenic fermentation in a leach-bed process, *Energy, Sustainability and Society*, **2**(13): 246-263.

Son, Changjin, Seonyong Chung, Jieun Lee and Seongjun Kim. (2002) Isolation and cultivation characteristics of *Acetobacter xylinum* KJ-1 producing bacterial cellulose in shaking cultures.*Journal Microbioligical Biotechnol.,* **12**(5):722–728.

Sugumaran, P., Priya, E., Manoharan, D. and Seshadri, S. (2014). Biogas production from water hyacinth blended with cow dung. *Indian Journal of Energy,***3**(1): 134-139.

Sun, L., Muller, B., Westerholm, M. and Schnurer, A. (2014). Syntrophic acetate oxidation in industrial CSTR biogas digesters. *Journal of Biotechnology,* **171**: 39-44.

Sun,Y. and Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology*,**83**:1-11.

Sun, L.,Müller, B. and Schnürer,A. (2013). Biogas production from wheat straw: community structure of cellulose-degrading bacteria. *Energy, Sustainability and Society,* **3**(1): 1-11.

Surendra, K.C., Takara, D., Hashimoto, A.G. and Khanal, S. K. (2014).Biogas as a sustainable energy source for developing countries: Opportunities and challenges. *Renewable and Sustainable Energy Reviews,* **31**:846-859.

Syafrudin, W.D., Nugraha, H.H.A., Matin, F.M. and Budiyono, F. (2020). Optimization of biogas production from water hyacinth by liquid anaerobic digestion (L-AD) using response surface methodology. *IOP Conf. Series: Materials Science and Engineering,* **845**: 012046.

Syazwani, I., Sim, J.Z., Nurul, S.R., and Nik, N.N.D. (2018). Co-digestion of rice straw leachate and domestic waste water for biogas production with addition of urea as nitrogen source. *International Journal of Engineering and technology.* **10**(1): 76-81.

Taherzadeh,M.J. and Karimi, K. (2008). Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. *International Journal of Molecular Sciences*; **9**:1621-1651.

Tatton, M., Archer, D., Powell, G. and Parker, M. (1989). Methanogenesis from ethanol by defined mixed continuous Cultures, *Applied and Environmental Microbiology*, **55**(2):440-445.

Tehama A. (2013).Biogas production from lignocelluloses: pretreatment, substrate characterization, co digestion and economic evaluation. Sweden: Chalmers University of Technology; Doctoral thesis. pp 56

The Nation (2018). Farmers embrace biogas for electricity. Retrieved from thenationonlineng.net on July 1st, 2019.

Tsegaye, B., Balomajumder, C. and Roy, P. (2018). Biodelignification and hydrolysis of rice straw by novel bacteria isolated from wood feeding termite. *Biotech,* 8:447. DOI: https://doi.org/10.1007/s13205-018-1471-0.

Tsavkelova, E.A.and Netrusov, A.I. (2012). Biogas production from cellulose-containing substrates: A review. *Applied Biochemistry and Microbiology,* **48**(5):421-433.

Turovskiy, I.S. and Mathai, P.K. (2006). Wastewater sludge processing. John Wiley & Sons, Inc. USA. Pp.30-60.

US EPA Biosolids Technology Factsheet (2006). Multi stage anaerobic digestion. US Environmental Protection Agency,Washington DC, EPA 832-F-806-031.

US National Petroleum Council (2007). Facing the hard truths about energy (US National Petroleum Council, Washington, DC),10, 156–158.

Vanwonterghem,I., Jensen, P.D., Dennis, P.G., Hugenholtz, P., Rabaey, K. and Tyson, G.W. (2014). Deterministic processes guide long-term synchronised population dynamics in replicate anaerobic digesters. *ISME J,***8**: 201-213.

Vasco-Correa,J.,Khanal,S.,Manandhar,A.andShah,A.(2018).Anaerobic digestion for bioenergy production: Global status, environmental and techno-economic implications, and government policies. *Bioresource Technology,* **247**:1015-1026.

Vasco-Correa, J., Khanal, S., Manandhar, A. and Shah, A.(2018). Anaerobic digestion for bioenergy production: Global status, environmental and techno-economic implications, and government policies. *Bioresource Technology,***247**: 1015-1026.

Van Lier, J.B., Martin, J.L.S. and Lettinga, G. (1996).Effect of temperature on the anaerobic thermophilic conversion of volatile fatty acids by dispersed and granular sludge. *Water Research,***30**(1): 199-207.

Wang, X., Lu, X., Li, F. and Yang, G. (2014). Effects of temperature and carbon-nitrogen (c/n) ratio on the performance of anaerobic co-digestion of dairy manure, chicken manure and rice straw: focusing on ammonia inhibition. *PLOS ONE,* **9**(5): 1-7.

Westerholm, M.(2012).Biogas production through the syntrophic acetate-oxidising pathway-characterisation and detection of syntrophic acetate-oxidising bacteria.*Swedish University of Agricultural Sciences*.1**79**:124-135

Weiland, P. (2010). Biogas production: current state and perspectives. *Appl Microbiol Biotechnol,* **85**(4): 849-860.

Wijekoon, K.C, Visvanathan, C. and Abeynayaka, A. (2011).Effect of organic loading rate on VFA production.Organic matter removal and microbial activity of a two-stage thermophilic anaerobic membrane bioreactor. *Bioresource technology,* **102**(9): 5353 – 5360.

Xia, A., Cheng, J., Song, W., Yu, C., Zhou, J. and Cen, K. (2013). Enhancing enzymatic saccharification of water hyacinth through microwave heating with dilute acid pretreatment for biomass energy utilization. *Energy,***61**: 158-166.

Xiao, B., Sun, X.F. and Sun, R. (2001). Chemical, structural, and thermal characterizations of alkali-soluble lignins and hemicelluloses, and cellulose from maize stems, rye straw, and rice straw. *Polym. Degrad. Stabil.,***74**(2): 307–319.

Yan, L., Gao, Y., Wang, Y., Liu, Q., Sun, Z., Fu, B., Wen, X., Cui, Z. and Wang, W. (2012).Diversity of a mesophilic lignocellulolytic microbial consortium which is useful for enhancement of biogas production. *Bioresource Technology,* **111**: 49-54.

Yadvika, S., Sreekrishnan, T.R., Kohli, S. and Rana, V.(2004).Enhancement of biogas production from solid substrates using different techniques—A review. *Bioresour. Technol.***95**: 1–10.

Yamada,T.(2006).*Anaerolineathermolimosa* sp., *Levilineasaccharolytica*Gen. and *Leptolineatardivitalis*Gen.; Novel filamentous anaerobes, and description of the new Classes Anaerolineae classis nov. and Caldilineae classis nov.in the Bacterial phylum Chloroflexi, *International Journal of Systematic andEvolutionary Microbiology*, **56**:1331-1340.

Ye, J., Li, D., Sun, Y., Wang, G., Yuan, Z., Zhen, F. and Wang, Y. (2013). Improved biogas production from rice straw by co-digestion with kitchen waste and pig manure. *Waste Manag,***33**: 2653–2658.

Yu, J., Zhao, Y., Liu, B., Zhao, Y., Wu, J., Yuan, X. (2016). Accelerated acidification by inoculation with microbial consortia in a complex open environment. *Bioresour. Technol.,***216**: 294-301.

Yuan, X. (2011). Enhancing the anaerobic digestion of corn stalks using composite microbial pretreatment. *J. Microbiol. Biotechnol.,***21**: 746-752.

Yuan, X., Ma, L., Wen, B., Zhou, D., Kuang, M., Yang, W. and Cui, Z. (2016). Enhancing anaerobic digestion of cotton stalk by pretreatment with a microbial consortium (MC1). *Bioresour Technol.,***207**: 293-301.

Zeikus, J.G. and Winfrey, M.R. (1976).Temperature limitation of methanogenesis in aquatic sediments. *Applied and Environmental Microbiology,* **31**(1): 99-107.

Zhang, Y.H.P. (2008).Reviving the carbohydrate economy via multi-product lignocellulosebiorefineries. *Journal of Industrial Microbiology and Biotechnology,***35**:367-375.

Zheng, Y.,Zhao, J., Xu, F. and Li, Y. (2014). Pretreatmentof lignocellulosic biomass for enhanced biogas production. *Progress in Energy and Combustion Science* **42**: 35–53.

Zhang, H., Luo, L., Li, W., Wang, X., Sun, Y., Sun, Y. and Gong, W. (2018). Optimization of mixing ratio of ammoniated rice straw and food waste co-digestion and impact of trace element supplementation on biogas production. *J Mater Cycles Waste Manag,***20**:745–753.

Zhao, H., Li, J., Li, J., Yuan, X., Piao, R., Zhu, W. (2013). Organic loading rate shock impact on operation and microbial communities in different anaerobic fixed-bed reactors. *Bioresour. Technol.,***140**: 211-219.

Zhou, C. H., Xia, X., Lin, C. X., Tong D. S. and Beltramini, J.(2011). Catalytic conversion of lignolytic biomass to fine chemicals and fuels. *Chem. Soc. Rev.*, **40**:5588-5617.

Ziemiński, K. and Frąc, M.(2012). Methane fermentation process as anaerobic digestion of biomass: transformations, stages and microorganisms.*African Journal ofBiotechnology,***11**(18):4127-4139.

Ziganshin, A.M., Liebetrau, J.,Proter, J. and Kleinsteuber, S. (2013).Microbial community structure and dynamics during anaerobic digestion of various agricultural waste materials. *Applied Microbiology and Biotechnology,* **97**(11):5161-5174.

Zoetemeyer,R.J.(1982).*Acidogenesis of soluble carbohydrate-containing wastewater*university van Amsterdam. pp.132.

APPENDIX

Design and results of optimization of biogas production using chemically pretreated rice straw

StdOrder	RunOrder	PtType	Blocks	Substrate conc (g)	HRT	Amendment conc (g)	Biogas yield (ml)
4	1	2	1	520	30	325	12890
10	2	2	1	325	30	130	13190
3	3	2	1	130	30	325	2490
11	4	2	1	325	15	520	13190
15	5	0	1	325	22.5	325	11100
9	6	2	1	325	15	130	3710
14	7	0	1	325	22.5	325	11100
13	8	0	1	325	22.5	325	11100
6	9	2	1	520	22.5	130	7470
2	10	2	1	520	15	325	12890
5	11	2	1	130	22.5	130	5371
7	12	2	1	130	22.5	520	18150
12	13	2	1	325	30	520	13190
1	14	2	1	130	15	325	2490
8	15	2	1	520	22.5	520	3710

Design and results of optimization of biogas production using biologically pretreated rice straw

StdOrder	RunOrder	PtType	Blocks	Substrate conc (g)	HRT	Amendment conc (g)	Biogas yield (ml)
4	1	2	1	520	30	325	15050
10	2	2	1	325	30	130	3710
3	3	2	1	130	30	325	2490
11	4	2	1	325	15	520	13190
15	5	0	1	325	22.5	325	11100
9	6	2	1	325	15	130	3710
14	7	0	1	325	22.5	325	11100
13	8	0	1	325	22.5	325	11100
6	9	2	1	520	22.5	130	4010
2	10	2	1	520	15	325	15050
5	11	2	1	130	22.5	130	1900
7	12	2	1	130	22.5	520	7470
12	13	2	1	325	30	520	13190
1	14	2	1	130	15	325	2490
8	15	2	1	520	22.5	520	18150