EXPLOITATION OF POTENTIALS OF MICRO-ORGANISMS WITH VOLATILE FATTY ACID DEGRADING GENES IN BIOGAS PRODUCTION

BY

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CERTIFICATION

This is to certify that this work "Exploitation of potentials of Micro-organisms with volatile fatty acid degrading genes in Biogas production" was carried out by Kemka, Ugochi Nneka, Reg. number (ACE-FUELS/20/PhD/13000116) in partial fulfillment for the award of Ph.D. in Future Energies (Bio-energy option) in Africa Centre of Excellence in Future Energies and Electrochemical systems (ACE-FUELS) of the Federal University of Technology, Owerri.

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DEDICATION

I dedicate this work to field of Microbiology and its application in renewable energy as I contribute to the improvement of lives within and outside Africa.

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ABSTRACT

Anaerobic digestion of cow rumen waste and human fecal slurry was carried out to evaluate the cumulative bigas yield over a 60-day retention, to isolate and identify bacterial species with volatile fatty degrading genes with molecular characterization. Microbial analysis, biochemical tests, isolation, characterization and physicochemical analysis were carried out. The total aerobic and anaerobic bacterial counts were 3.5 x 10^7 cfu/ml and 1.6 x 10^7 cfu/ml respectively. Bacterial and fungal isolates were identified as *Paenibacillus lautus, Moellerella wisconsensis, Providencia alcalifaciens, Shimwellia blattae, Micrococcus yunnanensis, Bacillus barbaricus, Proteus vulgaris, Paenibacillus septentrionalis, Paenibacillus curdlanolyticus, Budvicia aquatica, Azotobacter beijerinekii and Acinetobacter iwoffii*. Fungal isolates were identified as *Aspergillus niger, Aspergillus flavus* and *Trichophyton rubrum.* Most of the organisms were facultative anaerobes. Temperature was maintained at mesophilic rate. pH and total viable count were recorded over the period. Biogas yield for cow rumen waste increased steadily from the 6th day (31.403ml) to the 42nd day (247.039ml) arriving its peak generation of 251.226ml at the 45th day. The biogas yield of the substrate began to decline steadily from the 48th day till the 60th day showing reduced volumes of 215.636ml to 167.016ml. Biogas yield for human fecal slurry substrate increased steadily from the 6th day (27.216ml) to the 45th day (221.916ml) arriving its peak generation of 226.103ml at the 48th day. A decline occurred steadily on the 51st day till the 60th day showing reduced volumes of 200.981ml to 125.613ml. Analysis of response surface methodology for cow rumen waste showed a mean optimal biogas production of 162.245ml can be achieved at a pH of 6.36, retention time of 10.5 days and microbial count of 1.03 x 10^9 cfu/ml. A mean optimal biogas production of 145.711ml can occur using human fecal slurry at a pH of 7.45, retention time of 10.5 days and microbial count of 5.9 x 10^8 cfu/ml. GC-MS analysis showed methane contents of 57.99% and 50.39% and carbon dioxide contents of 17.12% and 19.21% for human fecal slurry and cow rumen waste respectively. GC-FID results of VFA production at three intervals of 20 days each within the 60-day retention time showed the presence of acetic, isobutyric, valeric, isovaleric and caproic acids amongst others. The cumulative volumes of the VFAs reduced steadily for cow rumen waste in the ranking order of 745.797ppm > 372.539ppm > 86.366ppm while that of human fecal slurry ranked 509.405ppm > 133.627ppm > 71.318ppm. GC-FID results of LCFA production showed presence of saturated fatty acids such as palmitic, stearic acids and unsaturated acids such as linoleic, cervonic acids amongst others. A reduction in LCFA content was also observed. At the 20th and 60th day, LCFA fell from 108.216 to 60.499 ppm, and from 113.195 to 44.94ppm for cow rumen waste and human fecal slurry respectively. Polymerase chain reaction results identified 4 potential VFA degrading bacteria. The reductions in VFA concentrations demonstrated the influence of VFA degrading bacteria on the VFA and LCFA content within the digesters. More so, presence of high volumes of unsaturated acids in cow rumen waste led the lower levels of methane in cow rumen waste since presence of unsaturated acids are toxic to methane generation. It is therefore concluded that these microbial isolates possess ample capacity to degrade VFA and LCFA in these substrates and could be excellent inoculum for bioaugmentation purposes in enhancing biogas production.

Keywords: Biogas, Biomass, Long chain fatty acids, Methane, Microorganisms, Volatile fatty acid

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Energy is essential to economic development. It is necessary since energy is a fundamental input in both production and consumption processes. Energy is important for daily human operations like cooking and electricity production, among other things (Gürsan & Gooyert, 2021). Over reliance on fossil fuels has depleted these sources seeing that they are non-renewable. As such governments rest on a section of energy potentials while the other part remains unexplored, though more reliable (Rai, 2014). Interest in biogas as a substitute renewable energy source has increased due to the environmental worries about greenhouse gas emissions and climate change (Pasternak, 2021; Deepanraj, Sivasubramanian & Jayaraj, 2017). The most efficient and successful options appear to be biofuels like biogas, biodiesel, and bioethanol as well as renewable energy sources such as geothermal, wind, solar and hydropower (Choong, Chou & Norli, 2017; Hafida, Abdul-Rahmana, Shaha, Baharuddinb & Ariffc, 2017). Recently, there has been interest in appropriating organic waste to generate biogas via anaerobic digestion as a way to reduce this reliance. This might boost the portfolio of renewable energy sources in several nations while also increasing the consumption of renewable fuels. Additionally, it will improve the local economy and ecology, resulting in reduced greenhouse gas (GHG) emissions, less pollution of the air and water and job creation (Kuo & Dow, 2017). Since there is a plentiful supply of feedstocks, biogas is a feasible and typically sustainable energy source. With advantages like lower carbon emissions, more effective and practical organic waste management, and increased efficiency, the conversion of biomass wastes to biogas provides a sustainable route to a more circular economy (Mydeen, Venkateshwaran, Velan, Vigneshwari & Kirubakaran, 2016; Akbulut, Arslan, Arat, & Erbaş, 2021).

Biogas is generated by the deterioration of organic matter by microbial communities under anaerobic conditions, resulting in the generation of energy-rich biogas (Pasternak, 2021; Guo, Majeed, Xu, Zhang, Kakade, Khan, Hafeez, Mao, Liu & Li, 2019). It differs from natural gas being a renewable source of energy generated biologically through the process of anaerobic digestion rather than a fossil fuel produced by geological processes. Biogas is primarily composed of methane gas, carbon dioxide and trace amounts of hydrogen sulphide, nitrogen, hydrogen and carbon monoxide (Guenther-Lübbers, Bergmann & Theuvsen, 2016). Biogas has the capacity to be a significant player in the emerging renewable energy sector. Compressed biogas (CBG) and liquid biogas (LBG) for power generation and transportation fuel can be produced from upgraded biogas (biomethane) (Kabeyi & Olanrewaju, 2022). Any economy will certainly benefit from the commercial production of biomethane from biogas as these resources are plentiful, affordable, safe, clean, and ecologically benign in preventing emissions of greenhouse gas and the ozone layer's consequent thinning. The intent is to build global energy systems which are carbon-free and sustainable (Kuo & Dow, 2017). By effectively managing accumulated organic waste and converting it, biogas technology reduces greenhouse gas emissions and lowers carbon emissions while generating cleaner fuel for electricity, lighting, and cooking. Harnessing biogas is a cutting-edge renewable energy source with enormous present and promise. The sustainable development goals (SDGs) 6, 7, 11, and 13 of the UN, which focus on creating clean and renewable energy, creating sustainable cities and communities, and reducing climate change, would be aided by this green technology strategy. Both developed and developing countries must continue to utilize the potential of biogas generation and enhancement to prevent a worldwide energy calamity (Achinas, Achinas & Euverink, 2017; Kumar, Singh, Zularisam & Hai, 2018).

1.2 Statement of the problem

In a biodigester setup, methanogens are in charge of producing methane. Volume of biogas depletes due to the production of long-chain fatty acids, that slow down the development rate of methanogens while in the digester (Westerholm & Schnürer, 2019). When volatile fatty acids (VFA) build up, the environment becomes too acidic, which is harmful to methanogenic bacteria. Due to the slowed microbial activity, this will have a knock-on effect on the ideal methane output (Al-Sulaimi, Nayak, Alhimali, Sana & Al-Mamun, 2022). Therefore, it is advised to add a prospective methanogen that might break down these fatty acids and thereby increase the generation of methane. Screening bacterial species with functional genes for fatty acid breakdown is the first step in this process.

1.3 Objective of the study

The main objective of this study is to determine potential organisms with volatile fatty acid degrading genes to enhance biogas production in anaerobic digestion systems.

The following specific objectives of this study are:

I. Isolate and identify microorganisms from human fecal slurry and cow rumen wastes that target genes for volatile fatty acid degradation.
II. Determine the volatile fatty acid and long chain fatty acid degradation over a specified period.
III. Extract DNA from the isolates and carry out polymerase chain reaction (PCR) to amplify the acsl (acyl-CoA synthetase long chain) gene of interest.
IV. Determine biomethane quality using gas chromatography analysis.

1.4 Justification of the study

Optimizing the production of biogas in anaerobic digestion systems requires the effective breakdown of volatile fatty acids (VFA). In addition to impairing overall process stability, the increase of volatile fatty acids (VFAs) content lowers methane volumes and cumulative biogas output. This presents a serious obstacle to the economic feasibility and sustainability of producing biogas, a promising energy source. High ammonia concentrations experienced during anaerobic digestion are the cause of VFA formation (Tampio, Blasco, Vainio, Kaala & Resi, 2018; Al-Sulaimi, Nayak, Alhimali, Sana & Al-Mamun, 2022). Propionate-degrading methanogenic cultures were added to the biodigester in a study by Li, Wang, Xu, Sun, and Xing (2022a) to enhance the methane production of ammonia-rich substrates. This resulted in a methane yield that was 17–26% higher than the control. Methane output dropped to less than 18% when VFA levels were high. Anaerobic digestion (AD) efficacy, biogas output, and VFA degradation were all successfully improved by bioaugmentation using propionate-degrading methanogenic cells.

High volumes of VFA concentrations were seen in similar AD investigations using different substrates, which significantly slowed down the generation of methane (Wei, Li, Yu, Zou & Yuan, 2015; Park, Lee, Jo, Lee & Jun, 2018; Hou, Wang, Wei, Zhang, Liu, Zhang, Wang, Chen, Xu & Guo, 2021).

1.5 Scope of the study

This scope will cover the bacteriological analysis of microorganisms from fecal slurry and cow rumen waste that exhibit high activity of VFA. The isolates will be subjected to DNA extraction and polymerase chain reaction. The study will also cover the degradation of volatile fatty acid and long chain fatty acid over a 60-day retention time. The biogas production volume over the same period of time will be evaluated daily. The biomethane quality of the biogas generated using gas chromatography analysis will equally be determined.

CHAPTER TWO

LITERATURE REVIEW

2.1 The process of Anaerobic Digestion

Anaerobic digestion (AD) is a multi-step biological process that turns organic waste, including sewage, agricultural waste, municipal trash, animal manure, and industrial effluent, into valuable end products (Mata-Alvarez, Dosta, Romero Güiza, Fonoll, Peces & Astals (2014); Eboibi (2020). Anaerobic digestion uses a wide range of specific microbial consortia to interact and degrade organic materials without oxygen; at the same time, biogas a flexible renewable fuel, is recovered (Yang, Wu, Yao, He, Chen, Ma, Shu, Hou, Wang, & Li, 2019). Biogas is a mixture of methane (50–70%) and carbon dioxide (30–50%), and a minute number of other gases like carbon monoxide (CO), ammonia (NH_3), oxygen (O_2), hydrogen (H_2), nitrogen (N_2), hydrogen sulfide (H_2S), moisture, and digestate (Chasnyk, Sołowski & Shkarupa, 2015; Sun, Li, Yan, Liu, Yu & Yu, 2015). As a source of heat, power, and electricity, biogas offers substantial value (Ruggeri, Tommasi & Sanfilippo, 2015; Li, Fang, Liu, Xie, Lou & Xing, 2019). Thus, a variety of organic wastes, including as agro-industrial wastes, animal manure and municipal solid wastes can be treated using anaerobic digestion technology. These wastes can then be used as a substrate for the anaerobic digestion process (Abdeshahian, Lim, Ho, Hashim & Lee, 2016). Through the actions of microbes, organic resources are also split into simpler molecules in order to create digestate, or bio-fertilizer, and biogas. Microorganisms like *Aspergillus* sp., *Bacillus* sp., *Pseudomonas* sp., *Salmonella* sp., *Klebsiella* sp., and *Methanococcus* sp., which use a comparatively broad spectrum of substrates like hydrogen, methanol, and formate to produce methane, are usually present in the digestate that results from the partially degraded organic and inorganic matter (Ho, Jensen, & Batstone, 2014).

The efficient use of digestate in an integrated agricultural system helps to bridge the gap in nutrient requirement (Rouhollahi, Ebrahimi-Nik, Ebrahimi, Abbaspour-Fard, Zeynali & Bayati, 2020). By contrast, digestate contains microorganisms with a higher proportion of accessible macronutrients for plant growth and development than untreated organic waste, and it also reduces land pollution (Risberg, Cederlund, Pell, Arthurson & Schnürer, 2017). Particularly in Africa and the rest of the developing world, such a strategy would enhance agricultural production, waste reduction, soil fertility and rural populations' standard of living.

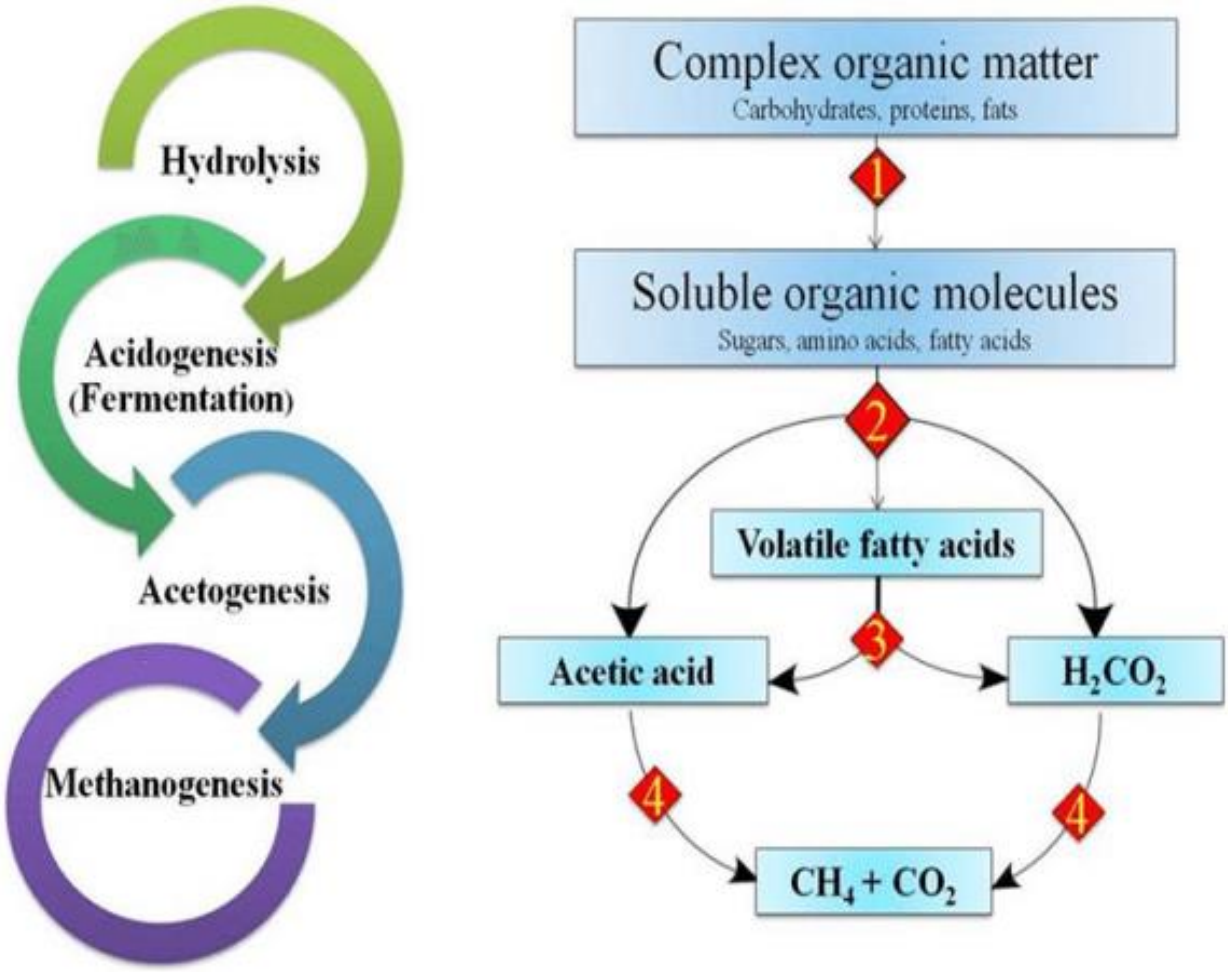

Figure 2.1: Diagram of the flow of anaerobic digestion (culled from Dussadee et al., 2017)

Hydrolytic bacteria and possibly fungi carry out the first step, hydrolysis, which is the conversion of macromolecules and components (proteins, lipids, and carbohydrates) found in organic materials into simple sugars, long chain fatty acids (LCFA), glycerol, and amino acids (Dussadee et al., 2017; Schnürer, 2016; Kazda, Langer & Bengelsdorf, 2014). Bacteria attach extracellular enzymes to their cell walls and secrete them into the bulk solution, which facilitate the hydrolytic reaction. Proteins are broken down into amino acids by extracellular enzymes called proteases (Schnürer, 2016). Hydrolytic bacteria release lipases, which catalyze the hydrolysis of lipids to produce glycerol and unsaturated/saturated LCFA (Westerholm & Schnürer, 2019). The soluble micro molecules undergo acidogenesis, which produces acetic acid, volatile fatty acids CO_2, and H_2. The microbial cell membranes of acetogenic bacteria carry LCFAs, which undergo beta-oxidation to produce acetate, carbon dioxide, and hydrogen (Ma, Zhao, Laurens, Jarvis, Nagle, Chen & Craig, 2015). The acidogenic and hydrolytic processes further produce intermediate products from the breakdown of the soluble monomers. They consist mainly of alcohols, formate, volatile fatty acids (such as acetate, propionate, butyrate, lactate, valerate, and caproate), carbon dioxide and hydrogen. Volatile fatty acids undergo acetogenesis, which produce additional acetic acid, CO_2, and H_2S. Numerous electron acceptors such as CO_2, sulfate, nitrate, and protons, are employed in this process; the latter is important for the production of biogas (Westerholm & Schnürer, 2019). Methanogens are the primary means by which the acidogenic products, acetate, H_2/formate, and certain methylated substances are eliminated (Westerholm & Schnürer, 2019). Using the CO_2 and H_2S created from the second and third steps, methane is synthesized in the presence of methanogenic archaea at the latter step of methanogenesis (Costa & Leigh, 2014).

2.2 Microorganisms engaged in anaerobic digestion

Anaerobic fungi and a variety of bacteria are equally active in biogas processes during the hydrolysis of polysaccharides (Kazda et al., 2014; Azman, Khadem, Van Lier, Zeeman & Plugge, 2015). *Acetivibrio, Halocella, Ruminoclostridium Butyrivibrio, Caldanaerobacter, Clostridium, Eubacterium, Caldicellulosiruptor* and *Ruminococcus, Bacteroides, Thermotoga, Fibrobacter, Spirochaetes, Fervidobacterium,* and *Paludibacter* are amongst the bacteria that break down starch and cellulose (Westerholm & Schnürer, 2019). It has also been noted that members of the phylum *Proteobacteria*, the candidate phylum *Cloacimonetes* (Westerholm & Schnürer, 2019), and Actinomyces possess genes for cellulose degradation (Wang, Zhang, Xi, Sun, Xia, Zhu, He, Li, Yang, Wang & Zhang, 2015; Liu, Wachemo, Tong, Shi, Zhang, Yuan & Li, 2018). *Neocallimastigomycota* are anaerobic fungi that have been proposed as viable options to better the production of biogas from lignocellulosic material; they are also found in ruminants (Cheng, Shi, Sun, Liang, Li, Li, Jin & Zhu, 2018). Numerous genera, including *Anaeromusa, Anaerosphaera, Aminobacterium, Thermanaerovibrio, Aminomonas, Clostridium, Gelria, Peptoniphilus, Proteiniborus,* and *Sporanaerobacter*, have been shown to degrade amino acids and proteins in anaerobic digesters (Hahnke, Langer, Koeck & Klocke, 2016; Hahnke, Langer & Klocke, 2018). However, it has also been suggested that in anaerobic digesters *Cloacimonetes Proteiniphilum, Fusobacteria*, and *Fermentimonas* have an active metabolism based on amino acids (Stolze, Bremges, Maus, Pühler, Sczyrba & Schlüter, 2017). Thus far, it has been proposed that the lipolytic bacteria found in anaerobic digesters are members of the *Trichococcus, Caldilineaceae,* and Bacteroidaceae families (Petropoulos, Dolfing, Yu, Wade, Bowen, Davenport & Curtis, 2018). *Methanobacteriales, Methanomicrobiales, Methanosarcinales, Methanonococcales,* and *Methanomassiliicoccales* are the orders of methanogens that are frequently found in biodigesters

(Schnürer, 2016; Enzmann, Mayer, Rother & Holtmann, 2018). The lineup of anaerobic microorganisms involved in various degradation pathways is constantly being updated due to continuous advancements in cultivation studies and molecular techniques (De Vrieze, Pinto, Sloan & Ijaz, 2018; Hassa, Maus, Off, Puehler, Scherer, Klocke & Sculter, 2018; Maus, Rumming, Bergmann, Heeg, Pohl, Nettmann, Jaenicke, Blom, Puhler, Schluter, Sczyrba & Klocke, 2018). The generation of biogas in an anaerobic set up can reduce over time with nutrient loss from the substrate or volatile fatty acids accumulation which invoke an extremely acidic environment within the digester. Methanogens get uncomfortable and hence unproductive. Hence the need for optimization approaches.

2.3 Factors affecting methanogenic activity

In anaerobic digestion, microbial diversity depends on feedstock type, seed inoculum, temperature, granulation, aeration, mixing speed, pre-treatment type, digester design, hydraulic retention time (HRT), trace elements, solids retention time (SRT), and organic loading rate (OLR). These factors are important for the generation of biomethane; deficits or surpluses in either of these factors can lead to imbalances in the process.

2.3.1. Substrate type

The inclusion of major macronutrients like lipids, carbohydrates, and proteins have notable effect on the theoretical production of biogas. Aside from this categories, lignocellulosic wastes, of which cellulose and hemicelluloses are the main nutrients have been extensively investigated for co-digestion processes. These nutrients vary widely in their biodegradability. Lipids, proteins and carbohydrates wastes have caused a major issue for the anaerobic digesting process. The fermentation of wastes causes notable rise in ammonia content. When reactors are fed wastes high in protein, this situation becomes much more problematic (Yeshanew, Frunzo, Pirozzi, Lens &

Esposito, 2016). Methanogenic activity has been shown to be inhibited by high ammonia concentrations in animal manure (Jiang, McAdam, Zhang, Heaven, Banks & Longhurst, 2019). For methane-forming bacteria to develop, certain nutrients are required. Methanogenic activity and optimal growth are stimulated by trace metals that include nickel, iron, cobalt, and molybdenum. In order to generate a nutrient-rich media, some metals have been proposed as extra ingredients, such as boron, manganese, aluminum, molybdenum, and selenium. The performance of the AD system can be enhanced by adding metal ion solutions to anaerobic digesters (Rabii, Aldin, Dahman & Elbeshbishy, 2019). A significant increase in ammonia concentration occurs due to fermentation of wastes. This problem becomes particularly serious when reactors are fed with wastes rich in protein (Yeshanew, Frunzo, Pirozzi, Lens & Esposito, 2016). Some nutrient elements are important for the growth of methanogens. Metals with molybdenum, nickel, iron and cobalt are essential for optimal growth and methane production. Trace metals stimulate methanogenic activity. Some metals, including molybdenum, manganese, aluminum, selenium and boron, have been noted to achieve a nutrient-rich medium when used as additional components. To improve the performance of the AD system, addition of metal ion solutions can aid (Rabii, Aldin, Dahman & Elbeshbishy, 2019).

2.3.2 Temperature

Temperature is a key operational factor that accelerates the methanogenic activity. Thermophilic or mesophilic treatment can be applied in anaerobic digestion. According to Grubel and Suschka (2018), thermophilic treatment works best at temperatures between 49 and 57^{o}C to 70^{o}C, while mesophilic treatment works best at temperatures between 30 and 35^{o}C (Kougias & Angelidaki, 2018). In lieu of mesophilic digestion, thermophilic digestion offers several benefits: faster fermentation, a higher biogas yield (shorter retention time) because of increased biochemical reactions, efficient reduction of organic matter and volatile suspended solids (VSS), improved

rheological properties of the digested sludge, the capacity to handle higher organic loads because of faster reaction rates, and a high pathogen inactivation efficiency (Kasinath, Fudala-Ksiazek, Szopinska, Bylinski, Artichowicz, Remiszewska-Skwarek, & Luczkiewicz, 2021). The thermophilic treatment has certain disadvantages as well, such as the need for additional energy to maintain the temperature, the requirement that the feeding biomass enter at a high temperature, and the difficulty of temperature changes for the creation of biogas. The many benefits of maintaining the right temperature throughout AD demonstrate that the mesophilic digester's performance offers improved parameter stability and financial advantages, such as steady biogas generation. The thermophilic process's increased temperature causes organics to decompose more quickly, which shortens the retention period and increases methane generation. The anaerobic process is quite sensitive to temperature; slight temperature changes will not have significant impact on conversion to acetic acid, but they will have an effect on the conversion of acetic acid (acetate) to methane (Matheri, Belaid, Seodigeng & Ngila, 2016). In accordance with Grimberg, Hilderbrandt, Kinnunen and Rogers (2015), anaerobic microbial systems are significantly impacted by ambient temperature, which has an impact on substrate solubility, ionization equilibria, and metabolic rate. Higher temperatures enrich hydrogen-producing and spore-forming bacteria and influence the activity of hydrogenotrophic methanogens in the anaerobic process (Grimberg et al., 2015). The ideal temperature range for mesophilic digestion in anaerobic digestion is thought to be between 35 and 37 ^{o}C. 55 ^{o}C is thought to be optimal for thermophilic digestion (Meegoda, Li, Patel & Wang, 2018). Provenzano, Daniela Malerba, Buscaroli, Zannoni and Senesi (2013), however, assessed the digestion of municipal solid wastes and sewage sludge and found that the thermophilic system performed better. The apparent discrepancy in the data could be attributed to the nitrogen content and substrate differences. Increases in temperature have a bigger negative impact on the process

outcome because the higher nitrogen content of manures causes higher ammonia levels during digestion (Provenzano et al., 2013).

2.3.3 pH

A solution's alkalinity or acidity is indicated by its pH value. Since the ideal pH range for each group of bacteria varies, it has an impact on biogas generation. The ideal pH range for methanogenic bacteria is 6.5 to 7.5. Their sensitivity to pH is very high (Matheri et al., 2016). Fermentative bacteria are less sensitive to pH due to their broad ideal pH range of 4.0 to 8.5. Acids like butyric, propionic, and acetic acids are very likely to be produced at low pH levels (pH 4.0, mainly). The primary production of ammonia occurs at pH values greater than 8.0. According to Matheri et al. (2016), the presence of volatile fatty acids tends to lower pH and could decrease the activity of methanogenic bacteria, thereby limiting the production of biogas. Methanogens thrive in environments that are neutral to slightly alkaline. Acidic circumstances cause them to deteriorate. The ideal pH range for the system once the anaerobic digestion process stabilizes is between 7.0 and 8.5, with values close to 7 for maximum activity (Sun et al., 2022). After the digester was run for two weeks in an acidic environment (pH 5.4), it took almost 40 days to recover the biogas output, according to Sun, Liu, Yanagawa, Ha, Goel, Terashima, and Yasui (2020). The digester's feeding was briefly decreased until the volume of organic acids returned to normal in a bid to address the acidic failure.

2.3.4 Volatile fatty acid (VFA)

Volatile fatty acids frequently build up, in anaerobic digestion systems, causing a pH drop, unstable operation, and subpar biogas output. VFA concentration and anaerobic digester performance are correlated so much so that VFAs lower the anaerobic digesters' total pH, which impacts methanogenic bacterial activity and results in unstable digester performance (Park et al., 2018). Thus, the ratio of acidogenic to methanogenic processes is crucial for the anaerobic digestion of

substrates (Das & Mondal, 2016). Fatty acids produced by acidogenic bacteria during anaerobic digestion, are broken down by syntrophic bacteria into carbon dioxide, hydrogen, and acetate. Currently, 23 distinct genera have been revealed to be capable of functioning as syntrophic bacteria, with the majority of these genera belonging to *Firmicutes* (Schuchmann & Müller, 2016). VFA accumulation impact on the methanogenesis pathway has been studied. Acetic acid concentration inhibits the production of biogas from kitchen garbage, according to a recent study. Al-Sulaimi et al. (2022) performed a study to assess the production of VFA from sludge and its effects on the efficiency of biogas generation and biodegradation. To evaluate the buildup of VFAs and methane gas yield, three various types of sewage sludge, primary, secondary, and mixed sludge were utilized as substrates (Al-Sulaimi et al., 2022). Methane generation began at each batch on day 11, when the overall VFA accumulation reached its peak, and it persisted at a considerable rate until day 32. When the system reached its maximum yield of VFAs, it was found that no methane was generated. Maximum total VFA accumulation from PS and MS was 824.68 ± 0.5 mg/L and 236.67 ± 0.5 mg/L, respectively, according to the system. Acetic acid, the primary intermediate by-product of methane synthesis, was found to be the most prevalent VFA present (Al-Sulaimi et al., 2022). Since methanogenic bacteria are susceptible to propionic acid concentrations exceeding 1000–2000 mg/L, another study suggested considering propionic acid as a deleterious volatile fatty acid in anaerobic digestion (Lee, Lee, Bae, Kang, Kim, Rhee, Park, Cho, Chung & Seo, 2015). The accumulation of VFA was taken as a warning sign and signal of a potential system imbalance, according to both studies. LCFAs can be degraded by 14 species in two families of syntrophs, *Syntrophomonadaceae* and *Syntrophaceae* (Duarte, Silva, Salvador, Cavaleiro, Stams, Alves & Pereira, 2018). According to thermodynamics, LCFA fermentation is non-spontaneous and endothermic. Thus, β-oxidation and syntrophy with hydrogenotrophic or acetoclastic methanogens enable the breakdown of LCFAs to

acetate and hydrogen (Chen et al., 2014; Treu, Campanaro, Kougias, Sartori, Bassani & Angelidaki, 2018; Ziels, Nobu & Sousa, 2019). According to certain studies, hydrogenotrophic methanogens are more prevalent during inhibition brought on by high VFA and ammonia content and at thermophilic temperatures (Pap, Györkei, Boboescu, Nagy, Bíró, Kondorosi & Maróti, 2015; de Jonge, Moset, Møller & Nielsen, 2017). VFAs at 5.8 - 6.9 g L^{-1} were found to be totally inhibitory to methanogens in a study that examined the impact of VFA accumulation brought on by a high organic load in a batch reactor processing kitchen waste (Xu, Zhao, Miao, Huang, Gao & Ruan, 2014). On the contrary, at 8.7 g L^{-1} of propionate, VFAs were shown to be non-inhibitory to methanogens in a full-scale reactor that processed food waste and cow manure (Franke-Whittle, Walter, Ebner & Insam, 2014). At high LCFA concentrations, the abundance of syntrophic acetogens decreases, indicating that syntrophic bacteria are especially vulnerable to LCFA (Ma et al., 2015). According to Ma et al. (2015), mass transfer and substrate access are restricted by inhibition brought on by LCFA attachment to cell surfaces. According to certain research (Kougias, Treu, Campanaro, Zhu, & Angelidaki, 2016; Ziels, Karlsson, Beck, Ejlertsson, Yerta, Bjorn, Stensel & Svensson, 2016), LCFA inhibition may be reversible. For instance, a study that used sodium oleate to mimic high LCFAs in the digestion of cattle manure found that the disturbance was reversible (Kougias et al., 2016). Additionally, inoculation with sludge that has previously been acclimated to high LCFAs reduced inhibition indicating that resilience can result from microbial community adaptation. The study conducted by Fernandez-Gonzalez, Huber and Vallino (2016) revealed that resilience was a crucial component in sustaining a syntrophic population subjected to disruptions in a full-scale reactor. The syntrophs recovered after experiencing stress. According to a further study, a rise in the relative abundance of syntrophic β-oxidizing bacteria, particularly *Syntrophomonas,* may be the cause of the reversibility of LCFA inhibition (Ziels et al., 2016).

Furthermore, according to the same study, adding 100–1570 mg of oleic acid g VS^{-1} had no effect on the number of methanogens, with 70% of them being hydrogenotrophic (*Methanomicrobiales*) and 30% being acetoclastic (*Methanosaeta*).

2.3.5 Carbon/Nitrogen (C/N) ratio

The relationship between the amounts of carbon and nitrogen in organic substrate is represented by the Carbon Nitrogen **(**C/N) ratio. As stated by Zahan, Georgiou, Muster and Othman (2018), the ideal C/N ratio for anaerobic digestion is between 15 and 30. Since a high C/N ratio shows that methanogens are using nitrogen quickly and producing less gas, it causes a shortage in the AD system (Kangle, Kore, Kore & Kulkani, 2012). Conversely, a decreased C/N ratio causes ammonia to build up and the digestate's pH to rise to levels that are harmful to methanogens. It is possible to co-digest substrates with high and low C/N ratios in order to keep the digester substrate's C/N level at optimal levels (Zahan et al., 2018). In an effort to better the C/N ratio and destruction of volatile solids, which would lead to a larger biogas yield, researchers have tried co-digesting wastes with cattle manure (Das & Mondal, 2016). To determine the ideal C/N ratio for biogas production, Qiang, Wang, Ding and Zhang (2020) investigated corn stalks and swine manure co-digestion in the ratio of VS manure / VS corn-stalks at 1:1, 1:2, 1:3, and 1:4 (Qiang et al., 2020). The optimum C/N ratio to generate biogas was attained when the proportion of VS manure to VS maize stalks was 1:3. According to Hagos, Zong, Li, Liu and Lu (2017), the ideal C/N ratio for the efficient metabolic activities of microbial groups was between 20 and 30. This was adequate to preserve system stability and satisfy the expected energy and nutrient needs for cell growth.

2.3.6 Retention time

Retention time comprises the solid retention time (SRT) and hydraulic retention time (HRT). HRT is the average amount of time it takes for slurry to move from the digester's entrance to its exit. SRT

is defined as the total amount of time bacteria spend in the digester; it is an extra retention time (Auma, 2020). One crucial element in the digesting process is the amount of time volatile solids remain in an anaerobic digester. A substrate will degrade more the longer it is maintained under optimal reactor conditions. However, as retention time increases, the reactor rate will drop. For microorganisms to convert organic substrates into the intended products, they require appropriate retention time (Khoo, Chia, Chew & Show, 2021). The effluent quality is improved by long HRT; nevertheless, its reaction rate diminishes with longer residence time (Kawan, Suja', Pramanik, Yusof, Rahman & Abu, 2022). Low biogas yields can arise from an accumulation of VFAs that suppress bacterial activity when HRT falls below the optimal level (Christou, Vasileiadis, Karpouzas, Angelidaki & Kotsopoulos, 2021). The primary source of the process disruption seen at brief HRTs is the increase of acetic acid. A longer retention time has the drawback of increasing output rate per reactor volume unit while decreasing total degradation. However, if the retention period is too short, the digester's bacteria are "washed out" before they can proliferate, bringing the fermentation process to a near complete halt (Megallaa, 2019).

2.3.7 Organic loading rate (OLR)

The mass of organic matter divided by the digester volume over time is the organic loading rate (OLR), which is the organic material flowing into the digester over time (Kangle et al., 2012). More still, the quantity of volatile solids (VS) that must be continuously fed into the digester each day is known as the organic loading rate. Although the biogas output somewhat increases as the OLR rises, overloading causes the volatile solids to degrade and the biogas yield to fall over the ideal organic loading rate. The process temperature and retention duration determine the highest OLR that can be attained. The effect of OLRs on biogas was investigated at a pilot scale in ambient conditions by Kesharwani & Bajpai (2021). Co-digesting food waste with cow dung produced the maximum

biogas yield when compared to mono-digesting cow dung without disturbing inhibitory chemicals; while the pattern was the contrary when cow dung and grass silage were co-digested, the laboratory investigation found that methane production from mono-digesting cow dung was higher at lower OLRs (Hajizadeh, 2021; Kesharwani, & Bajpai, 2021).

2.3.8 Toxicity

Among the substances that prevent bacteria from thriving in a digester are mineral ions, particularly those of trace elements. The growth of microorganisms is stimulated by small amounts of minerals (calcium, sodium, potassium, sulfur, magnesium, and ammonium), but larger concentrations have an inhibitory and hazardous impact. Triclosan, an antibacterial drug used in healthcare, was found to suppress the methanogenic activity in AD technology in a 2019 study by Reyes-Contreras, Leiva, and Vidal. The findings demonstrated a correlation between a decrease in methane production and an increase in triclosan concentrations (Reyes-Contreras et al., 2019). In extremely small amounts, heavy metals including zinc, nickel, cobalt, copper, lead, and chromium are necessary for bacterial growth; but, in larger amounts, they have a harmful bacterial effect. The microorganisms are also inhibited by organic solvents and antibiotics. Only by draining the contents, ceasing feeding, or diluting the contents to bring the concentration of inhibitory chemicals down below the hazardous level can digesters be recovered (Appels, Baeyens, Degrève & Dewil, 2008).

2.3.9 Co-digestion of biomass

Increased volumes of methane can be produced by co-digesting biomass waste than by manure alone, but this method presents a problem because organic material must completely break down during the hydrolysis stage. Stabilizing circumstances or other parameters in the digestive process, such as the C/N ratio and pH, is reason why co-digestion is significant. Co-digestion can reduce HRT and increase methane generation, according to studies. Manure, which has a high concentration

of organisms capable of hydrolyzing lignocellulose material, should be considered as an alternative substrate (Awasthi, Sar, Gowd, Rajendran, Kumar, Sarsaiya, Li, Sindhu, Binod, Zhang, Pandey & Taherzadeh, 2023). Rodriguez, C., El-Hassan, Z., and Olabi (2017) investigated using co-substrates to influence the generation of methane when waste paper (WP) and microalgal biomass (MA) were co-digested. Their study, which was carried out in batch mode, aimed to examine the impacts of the feedstock to inoculum (F/I) ratio and the feedstock mixing ratio (WP/MA). At the F/I and WP/MA ratios of 0.2 and 50:50, respectively, they obtained the highest methane output of 608 mL CH4/g VS. The production of methane that was achieved at this feedstock mixing ratio was greater than that of the feedstock's mono-digestion. At the same F/I ratio of 0.4 and co-digestion ratio of 50:50, the methane output increased by a maximum of 49.58%. According to Rodriguez et al. (2017), their investigation confirmed the synergistic impact for all F/I ratios of 0.2, 0.3, and 0.4 as well as the feedstock mixing ratio of 50:50.

2.4.0 Pre-treatment techniques

Pretreatment, also known as the conditioning process, is used to improve dewatering, speed up and improve digestion, and improve the digestate's quality. By eliminating the barriers and enabling the microbial population to readily access and utilize the substrate's organic contents, the pretreatment process promotes microbial digestion (Patinvoh, Osadolor, Chandolias, Sarvari Horv´ath & Taherzadeh, 2017). Thermal, mechanical, and biochemical pre-treatments are all possible (Deepanraj et al., 2017; Zhen, Lu, Kato, Zhao & Li, 2017). The grade of cellulose polymerization and its crystallinity are the two main physical and compositional characteristics of lignocellulosic biomass that affect its degradability. It is beneficial to administer treatment before anaerobic digestion in order to avoid challenges.

Any pretreatment technique aims to increase the accessibility of substrates to microorganisms by disrupting the composite structure of lignocellulosic components (Harris, 2017). Prior to the AD process, a pretreatment step should increase the digestibility of the processed material, reduce environmental waste, and increase total biogas. The goal of pretreatment methods is to increase waste's digestibility. The bacteria release exoenzymes during this period to reduce triglycerides, diglycerides, and LCFA and increase the amount of VFA (Sunny & Sankar, 2019). Many strains of microalgae, for instance, have shown effectiveness in enhancing their methane output throughout the digestion process by diverse pretreatment processes. The firm cell walls of most microalgae species, which are made of slowly biodegradable material, slow down digestion (Uggetti, Passos, Solé, Garfí & Ferrer, 2017).

2.4.1 Other Microbial relationships

Since methanogens have a limited range of substrates, they rely on anaerobic cellulose decomposers and mixed acid fermenters to create varieties of short-chain fatty acids from glucose and other fermentable carbohydrates. These are further fermented producing H_2 and CO_2 as well as other byproducts by bacteria working in syntrophy with methanogens. This process is known as syntrophism, and it occurs when an organism's ability to grow depends largely on the production of one or more metabolic products or nutrients by another organism growing within the environment.

Jihen, Baligh, Amel, Said, Moktar, Maher and Hassib (2015) examined the structure and dynamics of the microbial community in the anaerobic co-digestion of dairy wastewater and cattle dung. Their investigation yielded a maximum VS reduction of 88.6% and biogas production of 0.87 L/g VS removal, which translates to a C/N ratio of 24.7 at 20 days of HRT. Numerous uncultured *Bacteroidetes, Firmicutes*, and *Synergistetes* bacteria were found in the bacterial profile study. Additionally identified were the strains of *Syntrophomonas* linked to H_2-using bacteria, which

include *Methanospirillum* sp., *Methanosphaera* sp., and *Methanobacterium formicicum*. In anaerobic digestion reactors, these syntrophic relationships are necessary for maintaining low hydrogen partial pressure. In 2015, Ács, Bagi, Rákhely, Minárovics, Nagy, and Kovács investigated the anaerobic digestion performance after being inoculated with *Enterobacter cloacae* cells. Using continuously stirred reactors in a fed-batch format, these investigators were able to achieve a 20% increase in biogas production after operating the inoculated reactor for six weeks (Ács et al., 2015). Additionally, Kovács and coworkers investigated the efficacy of thermophilic reactors utilizing *Caldicellulosiruptor saccharolyticus* and mesophilic digestion systems using the same organism (*E. cloacae*) as the inoculant. They stated that because the inoculated microflora could not compete with the microbial consortium, it was washed out of the systems. As a result, the improvements that were made were only limited (Kovács, Ács, Kovács, Wirth, Rákhely, Strang, Herbel & Bagi, 2013).

2.5 Biogas from waste for sustainable energy

Biogas from waste is a renewable and sustainable energy source and is readily generated by trapping the gas which oozes when organic materials such as sewage sludge, animal or plant manure, agricultural residues, food remains are degraded by microbes in a process of anaerobic digestion. This process turns wastes into usable sources of biofuel hence contributing to sustainability by managing waste and cutting down dependence on fossil fuels (Alengebawy, Ran, Osman, Jin, Samer & Ai, 2024). Several sustainability benefits are created from the biodegradation of organic wastes. These include:

- Reduction of waste disposal needs: By putting organic waste materials to use, biogas generation helps minimize waste from landfills and dumpsites and curtails greenhouse gas emissions that occur due to indiscriminate and prolonged dumping of waste.

- Renewable energy source: Since organic materials can be replenished continuously, biogas is considered as a sustainable source of energy which can be utilized in place of non-renewables.
- Providing a circular economy: With further processing of the anaerobic digestion process, the leftover materials such digestate could be transformed to green biofertilizers for farmers, increase in agricultural yield thereby promoting sustainability.

Some applications of biogas consist of electricity generation, heat production, directly for heating in homes and businesses, vehicle fuel, as a transportation fuel for cars and trucks (Alengebawy et al., 2024).

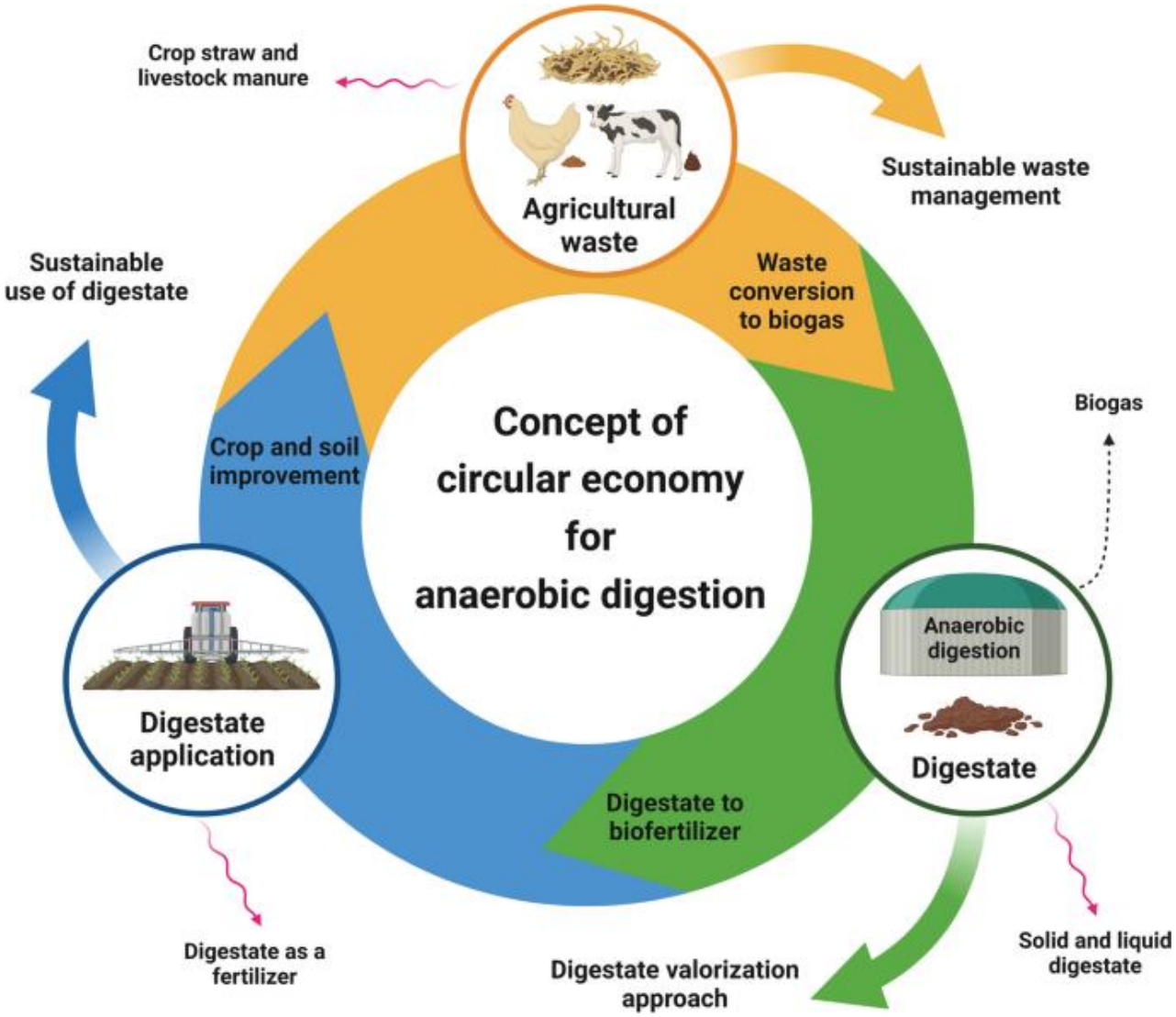

Figure 2.2: Integrated biogas generation and digestate use in a circular economy (culled from Alengebawy et al., 2024)

2.6 Evaluation of approaches to enhancing biogas production rates

Upgrading and testing specific techniques to optimize methane yields is crucial to develop the amount and quality of biogas required. The following are some ways that biogas production might be enhanced.

a. Variation in operational parameters

The activity of the biogas plant and the production of biogas can be improved by monitoring and modifying a number of variables, including pH, temperature, OLR, and agitation. Any major changes to these can have adverse effects on biogas production. To successfully operate the plant effectively and produce the required quantity of biogas, these limitations need be adjusted within the intended range. The ideal pH range for plants is 6.8 - 7.2, and they are healthier when grown in the thermophilic temperature range of 45 - 65 ^{o}C than when grown in the mesophilic temperature range of 15 - 45 ^{o}C (Sunny & Joseph, 2018).

b. Recycling of slurry and slurry filtrate

The utilization of digested slurry into the reactor has revealed enhancement in the gas production in small volumes, as the microbes which had been swept away are restored back, thus providing supplementary microbial inhabitants. The utilization of the processed slurry with filtrate has been tried out to preserve water and to increase biogas production. Reutilizing of digested slurry with new fresh substrate will help in decreasing the problem of starving biogas plants as well as in dealing with low gas production (Montgomery & Bochmann, 2014).

c. Pretreatment

The key compositional and physical features of lignocellulosic biomass, that shake their degradability, are the grade of cellulose polymerization and its crystallinity. In order to overcome

these complications, it is healthier to apply treatment preceding to anaerobic digestion. The aim of any pretreatment method is to disorder the composite structure of lignocellulosic constituents thereby boosting the accessibility of substrates to microbes (Harris, 2017). A pretreatment procedure should attract an enhancement in the digestibility of the treated material, lessen ecological trash and surge over-all biogas prior in the AD process. Pretreatment techniques aim at improving the digestibility of waste. During this time, exoenzymes are liberated by the bacteria to slash triglycerides, diglycerides, and LCFA, and raise the concentration of VFA present (Sunny & Sankar, 2019).

d. Use of Additives

The use of various chemical and biological additives under various operating conditions to promote microbial action and boost biogas production has proven to be very beneficial. The kind of substrate will probably determine whether an additive is appropriate. Crushed leaves of several leguminous plants, such as *Gulmohar, Leucaena leucocephala, Acacia auriculiformis, Dalbergia sissoo,* and *Eucalyptus tereticonius*, are examples of biological additives that have shown potential in increase biogas generation by 18–40% (Isaac & George, 2024). It has been shown that chemical additives such as iron salts ($FeSO_4$, $FeCl_2$) increase the rate of gas production (Liu, Wei & Leng, 2021). Since Ni-dependent metalloenzymes are involved in the formation of biogas, biogas was increased by up to 54% when exposed to nickel ions (2.5 and 5 ppm). Accumulative amounts of silica gel increased process stability, indicating that volatile acid was absorbed more quickly when an adsorbent was present. Methane generation was enhanced and foaming was prevented by employing calcium and magnesium salts as energy supplements (Pandey & Soupir, 2012). Research on the enzymatic treatment of lignocellulosic material for enhanced biogas production was conducted by Speda, Johansson, Odnell, and Karlsson (2017). Cellulolytic enzymes were employed, overproduced, and

subsequently extracted from a methanogenic microbial community so as to investigate the impact of endogenous enzymes. Through the method of an in situ anaerobic digester, a solution with a very high endogenous microbial cellulolytic activity was generated and its impact on biogas production from lignocellulose was studied. The pace and yield of biomethane generation were greatly enhanced by the addition of enzymes. Enzymes that were stable and active in the anaerobic digesting environment were discovered in the induced enzyme solution which was extracted from a microbial methanogenic colony. The pace and yield of biogas generation were greatly impacted by the enzymatic activity, which was equivalent to the outcomes of other pretreatment techniques. Accordingly, it was asserted that using these enzymes could make it possible to treat lignocellulosic material with an efficient low-energy in situ anaerobic digester, thereby increasing the generation of biomethane (Speda et al., 2017).

e. Use of Zeolites

Undesired compounds and other gases are usually contained in biogas; they are unwanted pollutants. To get rid of the trace contaminants and undesired components from the biogas, thus expanding its range of applications, the gas needs to be cleaned/scrubbed. The cleaning of the biogas entails elimination of impurities and acidic gases. Biogas needs to be cleaned to fulfill the requirements of gas appliances (gas engines, boilers, fuel cells, vehicles, etc.), increase the heating value of the gas, and standardize the gas. Zeolites are crystalline, nanoporous aluminosilicates composed of (TO_4) and tetrahedral (T = Si, Al) (Ameh, 2019). The zeolite rock sizes range from 3Å to 12Å which provides good selectivity for molecular transport into the zeolite crystals. Furthermore, the existence of active metal-phase and Brønsted acid sites in zeolite micro structures determine the activity of zeolite. The composition of Si/Al ratio also determines the acidity level and adsorption process (Ameh, 2019). In a study by Tira, Padang, Salman, Wirawan, Wijana and Anggara (2018), raw

biogas was purified using zeolite rocks, they observed that activating zeolite increased the upgrading properties of the rocks. Because of the presence of $Na^{2+,}$ Ca^{+}, and Mg^{2+} cations in their crystalline structure, zeolites were found to have advantageous properties for microbe adhesion and the ability to stimulate ion exchange during AD (Montalvo, Huiliñir, Borja, Sánchez & Herrmann, 2020). These properties are useful in improving AD of wastewaters with high concentrations of nitrogen, such as poultry manure, as it prevents process inhibition. The usage of biogas is affected by the presence of CO_2 and other trace gases (Bharathiraja, Sudharsana, Jayamuthunagai, Praveenkumar, Chozhavendhan & Iyyappan, 2018). Raw biogas contains trace levels of hydrogen sulfide (H_2S) and carbon dioxide (CO_2), which reduce its calorific value, induce corrosion, and make it difficult to compress biogas into the cylinder. To prevent corrosion from interaction with the residual SO_2, the moisture content of raw biogas must be completely eliminated. However, the residual CO_2 in biogas reacts with water vapor to produce carbonic acid, which corrodes metal surfaces (Vuppaladadiyam, Jena, Hakeem, Patel, Veluswamy, Thulasiraman, Surapaneni & Shah, 2024). Natural zeolites have been proven to boost microbial population density and offer more opportunities for microbial growth, adhesion, and co-metabolism when used as support medium in digesters that treat wastewater (Montalvo et al., 2020). According to a study, adding natural zeolite at concentrations ranging from 2 to 4 g/L enhanced the generation of CH_4, with the inhibition rising at concentrations more than 6 g/L (Achi et al., 2020). During the dark fermentation process, zeolites were used to improve energy recovery from cassava-ethanol waste water in the form of hydrogen (H_2) (Achi, Hassaneim & Lansing, 2020; Ramprakash, Lindblad, Eaton-Rye & Incharoensakdi, 2022).

f. Effect of nanoparticles

Nanotechnology is considered one of the very useful advances in science over the last years. However, the involvement of nanomaterials in bioenergy production is very deficient. Nanomaterials such as metal oxides, zero valent metals, carbon-based nanomaterials, nano-composites and nano-ash are vital candidates to enhance biogas production and can be used with biodegradation of wastes such as municipal solid waste, cattle manure, wastewater sludge, lake sediments and sanitary landfills (Rahman, Melville, Huq & Khoda, 2016; Zaidi, Feng, Malik, Khan, Shi, Bhutta & Shah, 2019a; Wu, Peng, Li, Yang, Peng, Liu & Wang, 2021). Jadhav, Muhammad, Bhuyar, Krishnan, Razak, Zularisam and Nasrullah (2021) studied the impact of metallic nanoparticles (NPs) on microbial direct interspecies electron transfer for biogas production enhancement. The use of metallic NPs was found to be cost-effective, efficient and sustainable for biogas production. Metallic NPs were highlighted as the most known NPs for their potential to decrease lag time and improve the biogas production and stability (Hassanein, Kumar & Lansing 2021). NPs interact with the cell membrane of sludge, leading to structural changes in the cells that give rise to bacteria permeable membranes. In this way, a greater number of bacteria find their way to the sludge hence increasing overall biogas production (Faisal, Yusuf Hafeez, Zafar, Majeed, Leng, Zhao, Saif, Malik & Li, 2019). Su, Zhen, Zhang, Zhao, Niu & Chai (2015) investigated the influence of 0.05, 0.10 and 0.20wt% nano-scaled zero-valent iron (NZVI) (60-120 nm) on waste activated sludge (WAS) anaerobic digestion for 20 days at a temperature of 32±1℃. The results showed that 0.05wt % and 0.10wt% NZVI increased the methane production by 9.8% and 4.6% respectively. 0.20wt% NZVI decreased methane production by 8.8%. The researchers suggest that NZVI stimulates methanogenic populations and sulfate reducers and accelerate sludge stabilization in AD which gives rise to increased biogas and methane production.

2.7 Bioengineering approaches to enhancing biogas generation

The ability to genetically manipulate methanogens has allowed them to serve as models for archaeal transcription, translation, physiology, and biochemistry. This development has been made possible by the availability of genetic tools such as DNA delivery systems, shuttle vectors, gene deletion and insertion systems, positive and counter selections, transposon insertion and regulated gene expression (Leigh, Albers, Atomi & Allers, 2011; Lyu & Whitman, 2019). Proteome, RNA- seq and microarray- based analyses have been used in studies of *Methanococcus maripaludis* to reveal the genome- wide patterns of expression, regulatory systems, the 50 and 30 boundaries of transcripts, and the 50- untranslated regions (50 UTR). These studies identified key features of the promoters and terminators, which helped decipher the functions of a conserved RNA- binding protein, and helped to understand the responses of *M. maripaludis* to specific nutrient limitations such as H_2, phosphate, specific amino acids, and formate. These studies provide a solid basis for enhancing the activity of *Methanococcus* sp. via metabolic engineering. More importantly, methanogens have been studied to contain the mcrA gene. This gene allows the expression of the enzyme methyl-coenzyme M reductase, the main agent in converting by-products of anaerobic digestion into methane. Xavier, Souza, de Aquino Santos and Diniz (2020) carried out molecular studies of microbial environmental samples to allow for greater functional and structural understanding of the microbiome. They obtained mcrA and 16S rRNA sequences genes from NCBI. Sequence alignment was carried out using BioEdit v.7.2.6. Then, the sequences of each gene were grouped relating to the country of collection and its environment. 59 sequences were a match for the mcrA gene; the largest sequence number of 14 belonged to the mesophilic sludge group. The gene equaled matched samples from thermal water, rice, beer, marine and terrestrial sediments, oil, biogas reactor samples (Xavier et al., 2020). The sequences showed similarities to those from the databank

and hence possessed inherent capacity to enhance methane production in a bioreactor set up (Xavier et al., 2020).

2.8 Anaerobic digestion and inhibitions

Since a variety of inhibitors are seen in significant amounts in biomass materials, they are the main cause of anaerobic digester interruption or failure (Hou, Ji & Zang, 2018). The mechanism and controlling elements of inhibition have been the subject of extensive study. Ammonia, heavy metals, light metal ions, organics and sulfide are among the inhibitors frequently found in anaerobic digesters (Pilarska, Pilarski, Waliszewska, Zborowska, Witaszek, Waliszewska, Kolasiński & Szwarc-Rzepka, 2019). The physiology, dietary needs, growth kinetics, and environmental sensitivity of the acid-forming and methane-forming microbes in anaerobic digestion are very different (Meegoda et al., 2018). The primary cause of reactor instability is inability to maintain equilibrium between acid-forming and methane-forming microbes. High concentration of inhibitory chemicals in sludges are also frequently identified as primary cause of anaerobic reactor upset and failure (Zeb et al., 2017).

2.8.1 Ammonia

The biological breakdown of nitrogenous materials, primarily in the form of urea and proteins, produces ammonia. Methanogens are the least tolerant and most likely to stop growing as a result of ammonia inhibition among the four categories of anaerobic microbes (Jiang et al., 2019). According to a study, methanogenic populations in granular sludge experienced an activity loss of 56.5% as ammonia concentrations increased between 4051 and 5734 mg NH_3-N L1, but acidogenic populations were hardly impacted (Wang, Sun, Li, Yin, Liu, Zhang, Li & Zheng, 2014). Air stripping and chemical precipitation are two physical-chemical techniques used to extract ammonia from the substrate. These methods have been demonstrated to be technically possible at high ammonia

concentrations and in complex wastewater matrix (Palakodeti, Azman, Rossi, Dewil & Appels, 2021). More so, it has been shown that immobilizing microorganisms using various inert materials (clay, activated carbon, and zeolite) lessen biogas process inhibition and increase process stability (Sari, 2019). Ammonia inhibition can also be reduced by adsorbents which can eliminate inhibitors or by the addition of ionic exchangers. As an ionic exchanger for ammonia, natural zeolite and glauconite exhibit great selectivity for ammonium ions (Yenigun & Demirel, 2013).

2.8.2 Sulfide

Sulfate is a common component of many industrial wastewaters. In anaerobic reactors, sulfate reducing bacteria (SRB) convert sulfate to sulfide (Zhang, Zhang, Yang, Zhang, Tang, Su, P. & Lin, 2022). Incomplete oxidizers and complete oxidizers are two main categories of SRB which carry out sulfate reduction; they break down lactate and other chemicals into acetate and CO_2 and which turn acetate entirely into CO_2 and HCO_3 respectively. Sulfate decrease results in two levels of inhibition. Primary inhibition occurs as a result of SRB's competition for common inorganic and organic substrates, which inhibit methane formation. Sulfide's toxicity to several bacterial species causes secondary inhibition (Yuan & Zhu, 2016). To encourage the elimination of dissolved sulfate, several types of procedures can be used. Diluting the wastewater stream is one way to avoid sulfide toxicity, although this strategy is generally seen as undesirable because of the increase in overall amount of wastewater which needs to be treated. Employing a sulfide removal phase to the entire process is another method of lowering sulfide concentration in an anaerobic system (Yuan & Zhu, 2016). Techniques for removing sulfides also include chemical processes (such as oxidation, precipitation, coagulation), biological conversions (for instance from partial oxidation to elemental sulfur), and physio-chemical methods (stripping) (Onodera, Takemura, Aoki & Syutsubo, 2023).

2.8.3 Ions of light metals (Mg, Ca, Na, K, and Al)

Anaerobic digester effluent contains ions of light metals such as calcium, sodium, potassium, and magnesium. They can be added as chemicals to modify pH or released as organic matter (such biomass) breaks down (Wikandari, Sanjaya, Millati, Karimi & Taherzadeh, 2019). Like all other nutrients, they impact on microbial growth rate because they are necessary for development and synthesis of microbes. Microbial growth is stimulated by moderate quantities of light metals ions, slowed by excessive levels, and severely inhibited or poisoned by even greater concentrations (Elbeshbishy, Dhar, Nakhla & Lee, 2017). According to the experimental data, the short chain fatty acids output dropped from 212.2 to 138.4 mg COD/g volatile suspended solids as the poly aluminum chloride addition increased from 0 to 40 mg Al per gram of total suspended solids. Investigating the mechanism proved that poly aluminum chloride helped sludge floc aggregation and resulted in more extracellular polymeric materials that were both loosely and strongly bound remaining in sludge cells (Chen, Wu, Wang, Li, Wang, Liu, Peng, Yang, Li, Zeng & Chen, 2018). Calcium plays an important role in the growth of microbial aggregates; some methanogenic strains require calcium to grow. However, excessive levels of calcium cause phosphate and carbonate to precipitate, which causes reactors, biomass and pipes to scale, activity of specific methanogenic to decrease, buffer capacity to be lost, and vital nutritional elements for anaerobic degradation to be stripped away (Jing, Li, He, Shankar, Saxena, Tiwari, Maturi, Solanki, Singh, Eissa, Ding, Xie & Awasthi, 2024). The anaerobic bacterium, *Methanosarcina thermophila* TM1 and a *Methanosarcinae*-dominated UASB reactor were shown to require an optimal magnesium content of 720 mg/L to enhance its stability (Romero-Güiza, Mata-Alvarez, Chimenos & Astals, 2016).

2.8.4 Organics

Anaerobic processes can be negatively impacted by a variety of chemical substances. In anaerobic digesters, organic compounds that are hardly soluble in water or that get adsorbed to the surfaces of sludge solids can build up to large concentrations. Bacterial membranes stretch and leak when apolar contaminants build up in them, upsetting ion gradients and ultimately leading to cell lysis (Czatzkowska, Harnisz, Korzeniewska & Koniuszewska, 2020). Alkyl benzenes, nitrobenzene, phenol and alkyl phenols, halogenated phenols, alkanes, amines, nitriles, halogenated aliphatic, alcohols, halogenated alcohols, carboxylic acids, aldehydes, ethers, ketones, acrylates, amides, pyridine and its derivatives, and other organic compounds have all been shown to be harmful to anaerobic processes (Ghattas, Fischer, Wick & Ternes, 2017). Additionally, it has been noted that certain LCFAs, surfactants, and detergents have a negative effect on anaerobic digestion (Badmus, Amusa, Oyehan & Saleh, 2021; Elsamadony, Mostafa, Fujii, Tawfik & Pant, 2021). Certain factors that can affect the toxicity of organic compounds include exposure time, biomass concentration, toxicant concentration, feeding pattern, acclimation, temperature, cell age, and toxicant concentration (Chen, Ortiz, Steel & Stuckey, 2014). Biodegradation of some toxicants can halt inhibition when in low concentrations, while higher concentrations of toxicants typically result in significant inhibition of anaerobic processes. In the presence of toxic shocks, reactors with higher biomass concentrations show greater process stability (Thengane, Kung, Gomez-Barea & Ghoniem, 2022). Microbial acclimation is another crucial factor in evaluating the inhibitory impacts of organic chemicals, just like it is for other inhibitory substances. According to Czatzkowska et al. (2020), there are four interconnected ways that adaptation can take place: genetic engineering, induction of specific enzymes for the degradation, enrichment of organisms that can break down toxic compounds, and exhaustion of preferential substrates before transforming to a xenobiotic substrate.

2.9 Inhibition of metabolic pathways in anaerobic digestion

2.9.1 Hydrolysis

Using extracellular enzymes such as cellulases, proteases, and lipases, hydrolytic fermentative bacteria carry out the initial stage of anaerobic digestion by breaking down complex polymers into oligomers and monomers (Amha, Anwar, Brower, Jacobsen, Stadler, Webster & Smith, 2018). Many AD feedstocks contain complex insoluble polymers that can cause hydrolysis to be rate-limiting (Kamperidou & Terzopoulou, 2021). According to Azman et al. (2015), the most prevalent taxa of hydrolytic bacteria in AD are usually *Firmicutes* and *Bacteroidetes,* albeit the relative abundance of these taxa is frequently determined by the kind of reactor and inoculum. After hydrolysis, oligomers and monomers are broken down by fermentative bacteria into intermediates such alcohols (acidogenesis) and volatile fatty acids (VFAs). Elevated hydrogen partial pressure, humic acids, VFAs, and LCFAs inhibit hydrolytic bacteria (Varjani, Sivashanmugam, Tyagi & Gunasekaran, 2022; Amha et al., 2018; Azman et al., 2015). Activity loss, reversible hydrolase decrease (for example, when inhibitors attach to substrate-enzyme complexes or enzyme active sites), or irreversible effects brought on by modifications in the chemical structure of the enzyme are the three ways by which inhibition happens (Azman et al., 2015). The latter can be challenging to mediate and necessitates the inhibitor's elimination from the system. According to Li, Zhang, Zhang, Tang, Wang, and Jin (2017a), cellulose hydrolysis was 75% inhibited at VFA concentrations of 2 g L^{-1}. Similarly, when VFAs above 1.8 g L^{-1}, cellulose hydrolysis was inhibited, according to another study (Amha et al., 2018). Additionally, it was shown that high hydrogen partial pressure inhibited hydrolytic bacteria, lowering wheat straw breakdown without causing an accumulation of acidogenic bacteria metabolites (Varjani et al., 2022). In a different study, high quantities of humic acid caused a 40% reduction in hydrolysis, which was caused by a drop in the relative abundance of bacterial

communities that are hydrolytic or fermentative, such as *Clostridiales*, *Bacteroidales*, and *Anaerolineales* (Amha et al., 2018). There have also been reports of LCFA-induced suppression of acidogenic bacteria at a concentration range of 2.1–7.9 kg COD m^{-3} and hydrolytic bacteria at 2.6–9.4 kg CO m^3 (Ma et al., 2015).

2.9.2 Syntrophy and methanogenesis

In AD, fatty acids produced by acidogenic bacteria are broken down by syntrophic bacteria into carbon dioxide, hydrogen, and acetate. As of right now, 23 distinct genera have been revealed to be capable of functioning as syntrophic bacteria, with the majority of these genera belonging to the Firmicutes (Schuchmann & Müller, 2016). LCFAs can be broken down by 14 species in two families of syntrophs, *Syntrophomonadaceae* and *Syntrophaceae* (Duarte, Silva, Salvador, Cavaleiro, Stams, Alves & Pereira, 2018). Thermodynamically, LCFA fermentation is both endothermic and nonspontaneous (Chen et al., 2014). As a result, β-oxidation and syntrophy with hydrogenotrophic or acetoclastic methanogens facilitate LCFA degradation to acetate and hydrogen (Chen et al., 2014; Treu, Campanaro, Kougias, Sartori, Bassani & Angelidaki, 2018; Ziels, Nobu & Sousa, 2019). According to some studies, hydrogenotrophic methanogens are dominant at thermophilic temperatures (Pap, Györkei, Boboescu, Nagy, Bíró, Kondorosi & Maróti, 2015) and during inhibition caused by high VFA and ammonia (de Jonge, Moset, Møller & Nielsen, 2017). A study that examined the effect of VFA accumulation due to high organic load in a batch reactor treating kitchen waste found that VFAs at 5.8 - 6.9 g L-1 had been completely inhibitory to methanogens (Xu, Zhao, Miao, Huang, Gao & Ruan, 2014). In contrast, high VFAs were shown to be non-inhibitory to methanogens in a full-scale reactor treating cow manure and food waste, even when propionate concentrations reached 8.7 g L-1 (Franke-Whittle, Walter, Ebner & Insam, 2014). Syntrophic bacteria have been found to be particularly sensitive to LCFA, with syntrophic acetogens

decreasing in abundance at high LCFA concentrations (Ma et al., 2015). In accordance with Ma et al. (2015), mass transfer and substrate access are restricted by inhibition brought on by LCFA attachment to cell surfaces. According to certain research (Kougias, Treu, Campanaro, Zhu, & Angelidaki, 2016; Ziels, Karlsson, Beck, Ejlertsson, Yerta, Bjorn, Stensel & Svensson, 2016), LCFA inhibition may be reversible. For instance, a study that used sodium oleate to mimic high LCFAs in the digestion of cattle manure found that the disturbance was reversible (Kougias et al., 2016). Additionally, inoculation with sludge that has previously been acclimated to high LCFAs reduced inhibition (Kougias et al., 2016), indicating that resilience can result from microbial community adaptation. The study conducted by Fernandez-Gonzalez, Huber, and Vallino (2016) revealed that resilience was a crucial component in sustaining a syntrophic population subjected to disruptions in a full-scale reactor. The syntrophs recovered after experiencing stress. According to another study, a rise in the relative abundance of syntrophic β-oxidizing bacteria, particularly Syntrophomonas, may be the cause of the reversibility of LCFA inhibition (Ziels et al., 2016). Furthermore, the same study found that adding 100–1570 mg of oleic acid g VS-1 had no effect on the quantity or composition of methanogens, with 70% of them being hydrogenotrophic (*Methanomicrobiales*) and 30% being acetoclastic (*Methanosaeta*). According to a related study, hydrolytic bacteria were more impacted by an increase in LCFA than methanogens (Ma et al., 2015). Observations showing differing inhibition according to LCFA type and methanogen taxonomy further complicate LCFA inhibition. Long-term exposure to microbial communities suggests that the action of inhibitors could significantly affect the microbes. This shows that presence of inhibitors is caused by variations in the inoculum or from earlier inhibitor exposure. (Silva, Lima, Sartoratto, de Sousa, Torres, de Souza, de Paula, de Oliveira & da Silva, 2018). Comparing inhibition in AD is particularly difficult since variations in the structure of the microbial community exist.

Microbial community structure during AD inhibition has only been studied using DNA-based techniques in most research thus far (Li, He, Ma, Wang & Peng, 2015; Ma et al., 2015; Ziels et al., 2016; de Jonge et al., 2017; Treu et al., 2018).

2.9.3 Inhibition mitigation strategies

Inhibition can lower energy recovery. Numerous inhibitory mechanisms in AD have been modified by new molecular techniques, which have also assessed the impacts of common inhibitors on the function and structure of microbial communities. To lessen the inhibitory effects of microbiota in AD, a variety of mitigating techniques have been investigated. One tactic used to improve AD system function and reduce inhibitor sensitivity is bioaugmentation, which involves adding important enriched cultures. According to De Vrieze and Verstraete (2016), there are conflicting reports about the efficacy of bioaugmentation. In comparison to a reactor that was not bioaugmented, a study that used bioaugmentation by adding a methanogenic propionate degrading community (0.3 g dry cell weight L^{-1} d^{-1}) under high ammonia stress conditions (3.0 g N L^{-1}) found that after 45 days, the methane recovery rate increased by 21% and the degradation of propionic acid increased by 51% (Li et al., 2022a). The enrichment of *Methanosaetaceae,* the most prevalent methanogenic population in the bioaugmentation culture (> 90% relative abundance), was partially responsible for improved performance. Furthermore, by regularly injecting a doubled dosage (0.6 g dry cell weight L^{-1} d^{-1}) of the bioaugmentation culture, the non-bioaugmented reactor recovered after near failure (nearly nil methane production after 75 days). According to another study, methane production increased by 31.3% when *Methanoculleus bourgensis* MS2 was bioaugmented in a CSTR with a higher ammonia concentration of 5g NH_3 L^{-1} as opposed to a reactor that was not bioaugmented (Fotidis, Wang, Fiedel, Luo, Karakashev & Angelidaki, 2014). After 39 days following bioaugmentation, the relative abundance of *Methanoculleus* increased fivefold, indicating that the

bioaugmented culture was operationally active in the CSTR. A previous study by the same authors found that bioaugmentation of a UASB reactor exposed to ammonia stress with *Clostridium ultunense* spp. nov., *Methanoculleus* spp. strain MAB1, and an ammonia-tolerant SAOB co-culture did not prevent system failure (Fotidis, Karakashev & Angelidaki, 2013). The slow growth of methanogens in the co-culture, according to the authors' hypothesis, limited the trials' success. In-situ acclimation technique resulted in tolerance to ammonia concentrations of up to 4.2 g L^{-1}, and the relative abundance of *Firmicutes* and hydrogenotrophic methanogens increased in response to elevated ammonia in the digestion of a protein-rich substrate in a CSTR (Gao, Zhao, Chen, Yu & Ruan, 2015). Another study compared bioaugmentation and acclimation as a strategy to reduce inhibition of LCFA and found that while bioaugmentation of a co-culture of *Syntrophomonas zehnderi* and *Methanobacterium formicum* had no significant effect, long-term acclimation of >100 days resulted in a reduced lag phase in biogas production by applying an increasing load of oleate (Silva, Cavaleiro, Pereira, Stams, Alves & Sousa, 2014). A reasonably simple method to dilute substrate supply that may contain inhibitors is to co-digest different substrates. For instance, compared to mono-digestion reactors, a study applied slaughterhouse waste feed and found that dilution of inhibitory chemicals with co-digestion enhanced methane output (Astals, Batstone, Mata-Alvarez & Jensen, 2014; Pages-Diaz, Pereda-Reyes, Taherzadeh, Sarvari-Horvath & Lundin, 2014). Other approaches for mitigating inhibition include alkaline pre-treatment (Koyoma, Watanabe, Kurosawa, Ishikawa, Ban & Toda, 2017), enzyme addition (Meng, Luan, Yuan, Chen & Li, 2017), and heat pretreatment (Ennouri, Miladi, Diaz, Güelfo, Solera, Hamdi & Bouallagui, 2016). Using two of lipases enhanced methane production by 81-158% in animal fat, 27-54% in vegetable oil, and 37-41% in floatable grease waste digestions, according to a study that assessed the addition of three lipases to hydrolyze food waste rich in crude lipid (Meng et al., 2017).

In order to recover from VFAs inhibition (pH 6.0) of an anaerobic digestion system, photosynthetic bacteria (PSB) were employed. The methane content recovered from 33.3 - 60.5% and from 32.1 - 59.3%, respectively, after adding PSB for 12 days with and without light conditions. The pH rose to 7.1 and 6.8, and the system alkalinity quickly increased to 2238 and 1921 mg/L, respectively. The sCOD dropped from 5600 to 995 mg/L and from 5575 to 2025 mg/L, respectively. Additionally, the amounts of formic acid, acetic acid, propionic acid, and total VFA were significantly reduced. According to microbiological investigation, PSB bioaugmentation could preserve the system's microbial diversity (Zhao, Huang, Hua, Huang, Droste, Chen, Wang, Yang & Yang, 2020). PSB bioaugmentation may be an efficient way to reduce the accumulation of acids and promote the synthesis of methane. With or without bioaugmentation, light has also been noted to hasten recovery (Zhao et al., 2020). Thus, the generation of green energy depends on the functional activity of a number of bacteria and archaea, so it is critical to set up and operate AD systems in a way that supports microbial populations.

2.10 Response surface methodology (RSM)

The modelling and optimization of bioprocesses help to better the understanding of process inputs for achieving optimal yields while also improving output and production rates. Response surface methodology plays a crucial role in experimental design and has been effectively used in many areas requiring optimization and development of new processes. It is a useful statistical technique for analyzing several parameters and different optimization conditions (Ishola et al., 2017; Eterigho, Musa, Ejejigbe, & Okokpujie, 2019). It serves as an effective tool for managing and encouraging the complex influential factors of anaerobic digestion (Falowo, Oloko-Oba, & Betiku, 2019). To determine the interactive effects of variables, the RSM design is primarily employed for

experimental designs, allowing for the analysis and modelling of data to optimize the response (Tetteh & Rathilal, 2019).

CHAPTER THREE

MATERIALS AND METHODS

3.0 Materials and methods

3.1 Chemicals and equipment

All the chemicals involved in this research were of analytical grade. These included growth media such as Nutrient Agar, Brain Heart Infusion agar (BHIA) (BDH Chemicals Ltd., Poole England), distilled water, MacConkey Agar, Sabouraud dextrose agar (Merck, Germany), 99% acetone, Gram staining reagents. Equipment and materials used include Mercury in glass thermometer (0-100^{o}C), pH meter (Search Tech, model PHS 3C, US), Microscope, weighing balance Z051599, GC-MS Agilent Model 1100 Technologies, USA, GC-FID Agilent Model 6890 Technologies USA, 5ml bacterial broth culture, LB medium, sterile water, PCR thermal cycler, dNTPs, Taq polymerase, Agarose powder, gel loading dye, primers, 100bp DNA ladder, ethidium bromide, absolute Ethanol, TBE buffer (1x), UV- transilluminator, electrophoresis gel tank, microcentrifuge rotor, microcentrifuge tubes, micropipettes of varying ranges and tips, vortex machine (model-IKA vortex 3), Eppendorf tubes, marker pen, sterile hand gloves, face masks, gas burner, autoclave, incubator. Other equipment used include test-tubes, beakers, conical flasks, measuring cylinders, funnel, plastic hose pipe, graduated (transparent) bucket and 25-litre sterile cans amongst others. Bioreactors (prototype) of 54-liter capacity constructed at the Federal University of Technology Owerri Nigeria, was used.

3.2 Fabrication of digester and digester set up

Four steel digesters with working volume of 54 liters were set up. They consisted of a gas collection system connected to the digester vessel via a gas outlet pipe allowing for collection and measurement of biogas produced. A steel stirrer was connected at the top for the proper stirring of the content

inside in the digester to ensure uniform decomposition and prevention of scum formation. A digital thermometer was also set up at the biogas plant for monitoring of temperature of the biogas plant.

3.3 Collection and processing of samples

Fresh human fecal slurry was obtained from the septic tank at residential areas around Obinze, Owerri and fresh cow rumen waste was obtained from the Cattle market at Obinze, Owerri, Nigeria using aseptic plastic cans which were washed before use with soap and clean water and surface sterilized with 70% ethanol and kept at -20^{o}C temperature in the lab refrigerator until use. Both substrates were weighed out and thoroughly mixed before charging into different biogas reactors. Appropriate quantities of water were used to loosen the sludge; there were four set ups: human fecal slurry only (set ups 1 and 2) and cow rumen waste only (set ups 1 and 2). Quantification of biogas by water displacement method was conducted as described by Sarker and Moller (2013). A transparent plastic pipe was connected toa nozzle on the biodigester. The pipe was filled with water leaving about 10% of the volume empty and then inverted. The initial water level was measured and recorded. As biogas is produced, the water in the pipe is displaced. The volumes displaced were measured and recorded daily at 12noon. Biogas production rate was calculated by multiplying biogas volume by the number of measurements taken per day divided by time interval. Upon biogas production, samples were collected for microbiological analysis, gram staining and subsequent biochemical tests according to the methods described in Cheesbrough (2006).

3.4 Proximate Analysis

Physiochemical parameters for both samples were determined in order to check their suitability for bigas production. Such parameter includes organic carbon contents, total solids, ash, nitrogen,

moisture content, protein content, fat content, total volatile solids and organic carbon contents. The proximate description of the substrates was determined by the method outlined by AOAC (2010).

3.5 Measurements of operational parameters

3.5.1 Total viable count and pH of digester contents

Total viable count and pH of the digester contents during decomposition were assessed at the Microbiology Laboratory, Federal University of Technology, Owerri. The pH was assessed using a pH meter. The electrode was inserted in buffer solutions of 4.00 and 6.87 and calibrated. The electrode was properly rinsed after each insertion into the buffer solutions. After calibration with the buffer solutions, the electrode was inserted inside the samples and the readings recorded. Total viable count was enumerated using standard plate count technique as outlined by Cheeseborough (2006). The samples for testing were taken with sterile sample bottles through a pipe outlet under the digester after appropriate stirring and then taken to the laboratory.

3.5.2 Media preparation and determination of the microbial count of samples

A 10-fold serial dilution was done using the samples with peptone water broth media. Thereafter, 0.1ml aliquot of appropriate dilutions were plated out onto Nutrient agar, MacConkey Agar, Brain heart infusion agar (BHIA) plates and Sabouraud Dextrose agar media plates (for fungal isolation) and spread evenly on the agar surface using a sterile L shaped glass rod. Petri plates were inverted, labelled and incubated at 35-37^{o}C for 24-48hrs for Nutrient agar and MacConkey media and at 37^{o}C for 72hrs for fungal growth. BHIA Petri plates were incubated inside an anaerobic jar for an incubation period of 5-7 days in the presence of Gas pack anaerobe paper sachet containing carbon (Becton, Dickinson and Company, Sparks, USA) (Cross, Campbell, Balachandran, Campbell, Cooper, Griffen, Heaton, Joshi, Klingeman, Leys & Yang, 2019).

Visible colonies were sub-cultured into stoppered tubes with Nutrient agar slant. Colonies were characterized and identified using biochemical tests (Cheesbrough, 2006).

3.5.3 Isolation and identification of bacterial isolates

Exactly 1 ml of the test sample was pipetted into 10 ml of peptone in a sterile test tube. This served as the stock. A total of 9ml of peptone water was distributed into 12 sterile test tubes, which were labelled with dilution factors ranging from 10^{-1} to 10^{-12}. From the stock solution, 1 ml was extracted and serially diluted across the 12 tubes containing 9ml each. an aliquot of 0.1ml from dilution factors 10^{-5}, 10^{-7}, 10^{-9} and 10^{-11} was spread plated onto all agar media plates using the spread plate technique. The Nutrient and MacConkey agar plates were incubated invertedly at 35 ± 2^{o}C for 24-48hours, while the Sabouraud dextrose plates were incubated at 30- 35^{o}C for 48-96hours. Brain heart infusion plates were incubated under anaerobic conditions using an anaerobic jar with a gas pack for 5-7 days. After incubation, visible colonies were observed on various plates; they were counted and recorded. Bacterial colonies were isolated and sub-cultured onto fresh sterile medium of Nutrient agar and coded. Stock cultures slants were prepared from the sub-cultured pure colonies and stored at 4^{o}C in a refrigerator. Sub-cultured isolates were subjected to Gram staining and microscopic test and the following biochemical/physicochemical tests: catalase, oxidase, motility, citrate utilization, indole production, methyl red-vogues Proskauer (MR-VP), hydrogen sulphide test, sugar fermentation tests using glucose, sucrose, lactose, mannitol, mannose, maltose and xylose. The tests were done as described by Peekate (2022). Results got were keyed into the search dialogue of the online bio-database software, advanced bacterial identification software (ABIS) so as to reveal the possible identity of the isolates. Stock cultures of identified isolates were also subjected to bio-molecular characterization.

3.5.4 Identification of fungal isolates

A minute segment of the mycelial growth was tactically taken from the surface of the fungus using a sterile teasing needle and placed on a drop of Lactophenol cotton blue on a grease free slide. The mycelial growth was further teased apart and covered with a cover slip. It was then observed under x10 Objective and then with x40 Objective. Hyphae and arrangement of structures were observed and noted.

3.5.5 Gram staining and microscopic examination of bacteria

Gram staining technique was performed on bacterial isolates. This test classifies bacteria as Gram positive or Gram negative, and demonstrates their cellular morphologies and shapes. A thin smear of bacterial culture was prepared by smearing it into 1-2 drops of distilled water. The smear was placed to air dry and heat fixed by passing the slide over a burner flame about 2-3 times. The slide was placed on a staining rack and covered with crystal violet stain for one minute. The stain was washed with clean distilled water and covered with Gram's iodine and allow to stand for one minute. The stain was washed with clean distilled water and decolorized rapidly with 95% ethyl alcohol for about 30 seconds. The stain is washed with quickly with clean distilled water. The stain is then covered with safranin for 30 seconds for one minute. The stain is then washed with clean distilled water and placed to air dry. The stain was observed under a microscope using oil immersion objective. The Gram-positive isolates stained purple while the gram-negative stained pink. Morphological characteristics such as rod in chain or in singles and cocci in clusters, chains or singles were noted.

3.6 Biochemical characterization of bacterial isolates

3.6.1 Catalase Test

This test identifies catalase producing organisms that can break down hydrogen peroxide (H_2O_2) to water and oxygen. Two drops of 5% hydrogen peroxide were placed on a grease free slide. By the aid of the edge of a clean slide, the test isolate was put in a drop of hydrogen peroxide. Effervescence or gas bubbling indicated that the organisms was positive for catalase.

3.6.2 Coagulase Test

This test identifies organisms that produce coagulase. Coagulase is capable of clotting blood plasma within 10 secs of its contact with the organism by converting fibrinogen into fibrin. A few drops of physiological saline were put on a grease free slide. The test isolate was emulsified on the saline. Then a little quantity of plasma was added to it and mixed gently. Clumping or agglutination within 10seconds indicated a positive reaction.

3.6.3 Oxidase Test

This test identifies bacteria that can produce oxidase enzyme which is used to show the electron transporter, cytochrome c in organisms. A piece of filter paper was placed on a clean Petri dish and impregnated with 2-3 drops of oxidase reagent. Using a sterile wire loop, the test organism was smeared on a filter paper and observed for the appearance of a dark blue to maroon color within 10seconds to indicate a positive reaction.

3.6.4 Sulphur Indole Motility Test

Sulphur Indole Motility (SIM) medium is differential bacterial growth medium for detecting three different characteristics of a bacterium: sulfur reduction and sulfide production, indole production and motility of a bacterium. The test isolate was aseptically stabbed once to a depth of about ¼ inch in the middle of a test tube containing the semi-solid SIM medium, by making a straight-line stab. The set up was incubated for 24- 48h at 37^{o}C and examined for fuzzy growth along the line of stab

and diffusion into the medium away from the line of stabbing. The tube was examined for any location of black color indicating sulfur reduction. 3-4 drops of 0.5 ml Kovac's reagent were placed on the agar and observed for a red ring appearance.

3.6.5 Sugar Fermentation Test

This test was used to evaluate the production of acid and gas or either by the test isolate when metabolizing different carbohydrate sources as glucose, sucrose, lactose, mannitol, mannose and xylose. The test isolates were inoculated into a broth containing the test sugar with an inverted Durham tube and incubated for 24h at 37^{o}C. The presence of a color change from red to bright yellow indicated acid production from fermentation of the sugar dropping pH to 6.9 or less. The production of gas bubbles is determined with the Durham tube. Gas produced during fermentation of the sugar is trapped in the Durham tube and appears as a bubble.

3.6.6 Methyl Red- Voges Proskauer Test

The test isolate was inoculated into 2 ml sterile glucose phosphate peptone water in duplicate tubes (A and B) and incubated for 48h at 37^{o}C. Thereafter, 4 drops of methyl red reagent were added into the tube A and 1ml of 40% KOH and 3ml of 5% alpha-naphthol into tube B using a Pasteur pipette. Color formation of red and pink indicated positive reactions respectively.

3.6.7 Citrate Utilization Test

This test is based on the capacity of the test isolate to use citrate as its only source of carbon. Slants containing citrate agar medium were prepared and a loop filled with the test isolate were streak-inoculated onto the slant surface and then stabbed at the butt. The set up was incubated for 24-48h at 35 -37^{o}C. A color change from green to bright blue and growth of the test isolates indicated a positive reaction.

3.6.8 Urease Test

Urea agar medium was carried out by suspending 2.4g of agar in 90ml distilled water. It was boiled to dissolve completely. The solution was autoclaved at 121℃ at 15psi for 15mins. 10ml of filter-sterilized urea base was added to the cooled agar solution and swirled thoroughly. It was distributed into 5ml sterile test tubes and left to solidify. The slants were inoculated with the test bacteria. The set up was incubated for 24-48h at 35 -37^{o}C. A pink coloration indicated a positive result.

3.7 Molecular characterization

3.7.1 DNA Extraction protocol

DNA extraction was carried out using PowerSoil DNA isolation kit method (Inqaba Biotech, South Africa) following manufacturers instruction. Exactly 100 µl of test isolate was put in a microcentrifuge tube. Then, 500 µl of lysis buffer was added, vortexed and incubation was done at 56^{o}C for 10min. Centrifugation of the tube and its content was done again at 10,000 rpm for 1 min. After spinning, 200 µl of absolute ethanol was put to the tube. The mixture was then transferred into the spin column and centrifuged at 10,000 rpm for 30 sec. The flow-through was discarded and tube was blotted on a tissue paper. Thereafter, 500 µl of wash buffer 2 was added to the spin column and centrifuged again at 12,000 to 14,000 rpm for 3 min. This was done to take away all traces of ethanol. The spin column was put in another microcentrifuge and 50 µl of nuclease-free water was added till the center of the column. This set up was incubated at 30℃ for 1 to 2 min, then centrifuged for 1 min at 10, 000 rpm to elute the DNA. The DNA was stored at -20^{o}C for polymerase chain reaction (PCR) analysis and other applications (Gupta, 2019). The extracted DNA was sequenced using Sanger sequencing technique. PCR was conducted to amplify the DNA (Albertsen, Karst, Ziegler, Kirkegaard & Nielsen, 2015; Gupta, 2019).

3.7.2 Amplification of DNA using Polymerase Chain Reaction

The amplification of the DNA extracts was carried out using polymerase chain reaction. The following specific primers were designed using Primer3plus application, to detect genes involved in degradation of fatty acids. The functional gene, *acsl* enzyme (acyl-CoA synthetase long chain) was picked from the conversed domain of *Bacillus licheniformis* strain SRCM 100115. The primers GC content and melting temperature were duly noted.

The specific primers were as follows: 5' GTGCTCTACCTCCATCCAAA '3 (primer 1), 5' GTGCTCTACCTCCATCCAAAA '3 (primer 2) and 5' GGTGCTCTACCTCCATCCAA '3 (primer 3). A mix of the template DNA obtained from each isolate, primers, dNTPs and polymerase was prepared in a PCR tube. The tube was placed in a thermal cycler and settings was inputted for 30 cycles. Initial denaturation temperature to separate DNA strands were held at 105^{o}C for 1 min. Denaturation temperature was then set to 95^{o}C for 30 secs; annealing temperature was set at 55^{o}C for 1 minute, this was to enable the primers bind to target sequences. Elongation temperatures were set at 72^{o}C for 1 min to enable polymerase synthesize new DNA strands and final elongation temperatures at 72^{o}C for 10 mins. The amplicons were analyzed via gel electrophoresis; bands were read using a UV- transilluminator.

3.7.3 Agarose gel electrophoresis protocol

Agarose gel was prepared by weighing out 1g of agarose powder. This was dissolved with 100ml of TBE 1x buffer and heated in a microwave for 3-5mins. The gel was allowed to cool to about 55℃ after which 10µl of ethidium bromide was added and gently mixed by swirling. The gel was cast into an electrophoresis tank with comb in place to obtain a gel thickness of about 4.5mm. This was allowed to stand for 20mins to solidify. Thereafter, the comb was gently removed and the tray placed in the electrophoresis tank with TBE buffer prior to loading of the amplicons. 20µl of each amplicon was mixed with 2µl of loading dye and carefully loaded into the 13 wells (lanes 1-13) created by the

comb alongside with the marker in a different well to serve as ladder to extrapolate the molecular weight. The electrodes were connected to the power pack ensuring that the negative terminal is positioned at the well where the samples were loaded. The gel was run at 65V for 45mins until the loading dye had migrated almost to the end. The power supply was turned off and the electrodes disconnected. The gel was removed from the gel tray and observed under UV-transilluminator. The gel field was then labelled appropriately.

3.8 Determination and quantification of biogas composition using GC- MS

Biogas composition and biomethane quality were estimated using gas chromatography mass spectrometry (GC- MS) according to Westerholm & Schnürer (2019). These were determined using a gas chromatograph (Agilent Model 1100 Technologies USA) equipped with a flame ionization detector and a 100m × 0.25mm × 0.20µm capillary column (SP^{TM}-2560). Biogas sample was collected from source, filtered and then dried to remove impurities and moisture. Sample was injected into the gas chromatograph and components were separated using GC column. The temperatures of the injector and detector were both set at 250^{o}C. The oven temperature was initially at 60^{o}C for 1 min, followed by a ramp up of 15^{o}C for 1 min to 165^{o}C for 1 min and held at a final temperature of 225^{o}C for 20 mins. Biogas components were detected and identified using MS. Helium was the carrier gas with a 0.8ml/min flow rate. Retention times and mass spectra were compared to standards and peaks were identified using GC-MS software.

3.8.1 Determination and quantification of VFA and LCFA using GC-FID

The volatile fatty acids and long chain fatty acids were observed and estimated via gas chromatography flame ionization method according to Westerholm and Schnürer, (2019). These were determined using a gas chromatograph (Agilent Model 6890 Technologies USA) equipped with a flame ionization detector and a 100m × 0.25mm × 0.20µm capillary column (SP^{TM}-2560).

Biogas samples were collected from source and filtered to remove particulates. Sample was acidified to convert VFAs to free acids. Methanol was used as organic solvent. Sample was injected into GC-FID and VFA and LCFA components were separated using GC column. The temperatures of the injector and detector were both set at 250^{o}C. Helium was the carrier gas with a 0.8ml/min flow rate. The oven temperature was initially at 60^{o}C for 1 min, followed by a ramp up of 15^{o}C for 1 min to 165^{o}C for 1 min and held at a final temperature of 225^{o}C for 20 mins. VFA and LCFA components were detected and quantified using FID and calibration curves. Retention times were compared to standards and peaks were identified using GC-FID software.

3.9 Statistical Analysis

The data were subjected to one- way analysis of variance (one-way ANOVA) using Origin Pro 2022 statistical software. Standard deviation and mean values were separated using Bonferrni test, Tukey test, Sidak test and Fisher test methods at P = 0.05 respectively. Response surface methodology (RSM) using Design Expert version 13 statistical software was applied to model this biological process.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.0 Results and discussion

4.1 Results

4.1.1 Proximate analysis of the substrates

The results of the proximate analysis carried on both substrates are shown below in Table 4.1.

Table 4.1: Proximate analysis of the substrates

Parameters	Cow rumen waste (%)	Human fecal slurry (%)
Moisture content	98.67	99.39
Ash content	1.09	0.034
Fat content	0.113	0.041
Protein content	0.475	0.363
Nitrogen content	0.076	0.058
Organic Carbon content	0.391	0.094
Organic Matter content	0.673	0.162
Total Solids	1.33	0.61
Total Volatile Solids	0.24	0.576

4.1.2 Isolation, identification and characterization of bacterial and fungal isolates from cow rumen waste (CRW) and human fecal slurry (HFS)

The morphological and biochemical characterization test results of the bacterial isolates are represented in Table 4.2 while the macroscopic and microscopic characterization of fungal isolates are represented in Table 4.3. A total of 13 bacteria were isolated from both substrates: 8 isolates from CRW and 5 from HFS. The bacterial isolates were identified as *Paenibacillus lautus, Moellerella wisconsensis, Providencia alcalifaciens, Shimwellia blattae, Micrococcus yunnanensis, Bacillus barbaricus, Proteus vulgaris, Paenibacillus septentrionalis, Paenibacillus curdlanolyticus, Budvicia aquatica, Azotobacter beijerinekii* and *Acinetobacter iwoffii*. The fungal isolates were identified as *Aspergillus niger, Aspergillus flavus* and *Trichophyton rubrum.*

Table 4.2: Bacterial isolates and their biochemical test results

ISOLATE	MORPHOLOGY	GRAM RXN	CATALASE	COAGULASE	CITRATE UTILIZTION	OXIDASE	METHYL RED	VOGES PROSKAUER	UREASE	HYDROGEN SULPHIDE	INDOLE PRODUCTION	MOTILITY	GLU.COSE	SUC.ROSE	LACTOSE	MANNITOL	MALTOSE	XYLOSE	PROBABLE ORGANISM
1	Creamy colored shinny colonies with entire edges	- rods	+	+	-	-	+	-	+	+	+	+	**A**	**A**	**A**	**A**	**A**	**A**	*Paenibacillus lautus*
2	Large creamy colonies with entire edges	- rods	-	-	+	-	+	-	+	-	-	-	**A**	**A**	**A**	-	**A**	**A**	*Moellerella wisconsensis*
3	Creamy colony	- rods	+	-	-	-	+	-	+	-	+	+	**A**	-	-	-	-	-	*Providencia alcalifaciens*
4	Creamy flat colony with crenated edges	- rods	+	-	+	-	+	-	+	-	-	+	**A**	**A**	-	-	-	-	*Shimwellia blattae*
5	Creamy colony with raised center with irregular edges	+ cocci	+	-	-	-	-	-	-	-	-	-	-	-	-	-	**A**	-	*Micrococcus yunnanensis*
6	Creamy slightly raised colony	+ rods	+	-	-	-	-	-	+	-	-	-	**A**	**A**	-	-	**A**	-	*Bacillus barbaricus*
7	Creamy flat colony with lobate margin	- rods	+	-	-	-	+	-	+	-	-	-	**A**	**A**	-	-	**A**	-	*Proteus vulgaris*
8	Shinny creamy small raised colony	+ rods	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	*Paenibacillus septentrionalis*
9	Shinny small colony	+ rods	+	-	-	-	+	+	+	-	-	+	**A**	**A**	**A**	-	**A**	-	*Paenibacillus curdlanolyticus*

10	Large colony with crenated edges	- rods	+	-	-	-	-	-	+	-	-	-	**A**	**A**	**A**	**A**	**A**	-	*Budvicia aquatica*
11	Tiny colony with entire edges	+ rods	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	*Paenibacillus septentrionalis*
12	Creamy tiny colony with crenated edges	- rods	+	-	-	+	+	-	+	-	-	-	**A**	**A**	-	-	-	-	*Azotobacter beijerinekii*
13	Reddish flat colony with entire edges	- rods	-	-	-	+	-	-	+	+	+	+	-	-	-	-	-	-	*Acinetobacter iwoffii*

Table 4.3: Macroscopic and microscopic characterization of fungal isolates

Isolate	Macroscopic characteristics	Microscopic characteristics	Probable organism
1	Black cottony growth with white edges and milky on reverse	Round conidia head with septate hyphae	*Aspergillus niger*
2	Green cottony growth with white radial edges and light yellow on reverse	Septate hyphae with conidia head	*Aspergillus flavus*
3	White creamy color with powdery texture and yellow brown on reverse	Slender microconidia	*Trichophyton rubrum*

These *Enterobacteriaceae – Shimwellia blattae, Paenibacillus* spp., *Moellerella wisconsensis*, *Budvicia aquatica*, *Bacillus* spp., *Azotobacter beijerinekii* are key microbial indicators in the anaerobic digestion process of waste products. In the study carried out by Pietruszka, Maslanko and Ciecholewska-Jusko (2023) *Paenibacillus* spp., *Moellerella wisconsensis*, *Budvicia aquatica* were observed at the initial hydrolytic stages of anaerobic digestion. Microbial counts began to reduce as the process moved towards its final stages of methanogenesis. These results suggests that *Enterobacteriaceae* are abundant in both substrates and play a crucial role when it comes to microbial activity in the anaerobic digestion process of these substrates. In the research conducted by Abuhena, Azim, Barman, Khan, Kabir, Rasul, Huang, Akter and Hug (2022), *Bacillus subtilis* was chosen as a bioaugmentation tool to treat anaerobic digestion residue because of its rapid growth on low-cost substrates, strong protein secretion ability, waste degradability, prolonged survival and favorable downstream processing (Abuhena et al., 2022). More still, *Bacillus* spp. is relatively stable in fermentation processes due to their stability as spore-formers and their ability to produce a variety of hydrolytic enzymes (Prenafeta-Boldu, Fernandez, Vinas, Lizardo, Brufau, Owusu-Asiedu, Walsh & Awati, 2017). *Moellerella wisconsensis* is an anaerobic bacterium thriving well in anaerobic conditions. *Moellerella wisconsensis* plays a vital role in degrading organic matter such as human fecal slurry (Zhang, Xing & Li, 2018). More still, *Moellerella wisconsensis* is an acid-tolerant microbe which makes it able to outcompete other microbial lives leading to its proliferation in the digester (Zhang et al., 2018). *Moellerella wisconsensis*, which indicated a negative catalase result, is an anaerobic bacterium that thrives well in anaerobic conditions. It plays a vital role in degrading organic matter such as human fecal slurry (Zhang et al., 2018).

According to Hadidi, Bahlaouan, Asbai, Benjelloun, El Antri and Boutaleb (2022), *Aspergillus niger* is a thermotolerant and osmotolerant fungus potentially useful in fermentation of various substrates producing citric and gluconic acids and enzymes. In their study, *Aspergillus niger* was attributed to have successfully increased biogas production using agro-industrial waste. Microbial counts began to reduce as the process moved towards its final stages of methanogenesis; hence the reduction of bacterial count. This reduction in microbial count can be due to continued decline of available nutrients in the substrates which could lead to competition among bacterial populations and ultimately decrease their number (Yu, Hu, Deng, Li, Xiong, Ye, Han & Li, 2015; Wang, Oehmen, Freitas, Carvalho & Reis, 2017). The indole test conducted showed that some of these bacteria were able to hydrolyze the tryptophan present in methane. Positive results of the MR test showed the presence and activity of bacteria in the acid phase during biogas production. Positive citrate utilization tests suggest the presence of citrate producers, which break down citrate into oxaloacetate and acetate, which is further converted into pyruvate and carbon dioxide, producing ammonia with sodium citrate during biogas production.

4.2 Biogas yield over a 60-day retention time

The biogas yield for cow rumen waste substrate increased steadily from the 5th day (27.216ml) to the 35th day (198.887ml) arriving its peak generation of 267.974ml at the 42nd day. After the 33rd and the 39th day, results show a progressive increase in biogas production, this continued to the 45th and the 48th day for cow rumen waste and human fecal slurry respectively. This indicates an exponential stage in the growth of the microbes (Tambuwal & Okoh, 2018; Soyingbe, Olayinka, Bamgbose & Adetunjim, 2019; Eboibi, 2020; Adjama, Derkyi, Uba, Akologo & Opuko, 2022). The biogas yield using cow rumen waste began to decline steadily on the 47th day till the 60th day showing reduced volumes from 217.636ml to 167.016ml.

On the other hand, biogas yield for human fecal slurry increased steadily from the 5th day (27.21ml) to the 35th day (198.887ml) arriving its peak generation of 230.291ml at the 49th day; while that of human fecal slurry began to decline steadily on the 50th day till the 60th day showing reduced volumes from 221.916ml to 125.613ml. The results of the biogas yield over the 60-day retention time are shown in figure 4.1 below.

4.2.1 Influence of pH and total viable count (TVC) on biogas yield

The pH of both cow rumen waste and human fecal substrates were measured in an interval of 3 days over a 60-day retention time. pH values declined from 8.1 to 4.61 for cow rumen waste indicating a change of slightly alkaline environment to a moderately acidic over the period; though pH rose to 5.19 on the 39th day and dropped to 4.92 on the 30th day all through to 4.61. Conversely, pH values of human fecal slurry from dropped from 8.1 to 6.27 on the 39th day and rose to 6.94 on the 51st day only to drop to 6.80 on the 60th day. The total viable count for cow rumen waste experienced a sharp drop from 1.64x10^{9} cfu/ml on the 3rd day to 4.2x10^{8} cfu/ml on the 60th day. In the case of human fecal slurry, total viable counts equally recorded a drop from 9x10^{8} cfu/ml on the 3rd day to 2.8x10^{8}cfu/ml on the 60th day. This fall in pH levels created microbial stress leading to decreased numbers of their total viable count. It can be inferred that extreme acidic ranges are detrimental to bacterial populations leading to their reduction in the digester over time. Oxygen exposure during the process can kill obligate anaerobes leading to a drop in microbial count (Auma, 2020). Similarly, Costa, Gusmara, Gardoni, Zaninelli, Tambone, Sala and Guarino (2017) reported that in an anaerobic digestion process, there was a significant reduction in bacterial population. This reduction in microbial count can be due to continued decline of available nutrients in the substrates and overwhelming acidity within the digester which could lead to competition among bacterial populations and ultimately decrease their number (Yu et al., 2015; Wang et al., 2017).

However, certain methanogens thrive in slightly acidic to neutral environments, typically pH values between 6.5-7.5; within this range, lower pH values can stimulate biogas production (Lackner et al., 2020; Mao et al. 2015). Also, lower pH values can suppress the growth of competing microorganisms, allowing acidophilic methanogens to strive, dominate and produce more biogas (Chen, Jiang, Xiao Shen, Zheng & Zhou, 2017; Bahira, Baki & Bello, 2018; Ali, Hua, Huang, Droste, Zhou, Zhao & Chen, 2019). Dwindling pH values in both samples and the reduction in microbial count can be linked to the accumulation of acids such as acetic and butyric acids during the breakdown of organic matter, which can lower the pH (Franke-Whittle et al., 2014; Chen et al., 2017; Huang, Dang, Wang & Feng, 2018). Figures 4.2a and 4.2b below represent these outcomes.

4.3 Gas chromatography mass spectrometry (GC-MS) for components of the biogas from substrates

GC-MS analysis was carried out on both samples to identify and quantify the components of the biogas produced from the substrates over the 60-day retention time. Methane, carbon-dioxide, carbon-monoxide, hydrogen sulphide, ammonia and sulphur oxide contents were identified in both samples after 60 days. The ranking order for the biogas components recorded for cow rumen waste was 50.39% (CH_4) > 19.21% (CO_2) > 6.52% (H_2S) > 5.33% (NH_4) > 3.84% (CO); that of human fecal slurry was 57.99% (CH_4) > 17.21% (CO_2) > 5.35% (CO) > 1.80% (H_2S) > 1.14% (SO_2) as seen in figure 4.3. The findings of this research show higher methane values of 57.99% for human fecal slurry compared to cow rumen waste of 50.39%. Dhugana, Adhikari, Shrestha and Shrestha (2019) recorded 53.11% high methane volume using human fecal slurry in their study. Kilucha, Cheng, Minza, Nasiruddin, Vempini, Li, Wang, Doroth and Li (2022), Adjama et al. (2022) and Soyingbe et al. (2019) all supported high levels of methane production in human fecal slurry when used in combinations with other substrates.

Ammonia was not observed in human fecal slurry unlike in some literature; reason being that ammonia were lost through dilution (Kupper, Hani, Neftel, Kincaid, Bühler, Amon & VanderZaag, 2020). This is supported by the water content of the slurry (99.3%) recorded from its proximate analysis as seen in Table 4.1.

4.4 Gas chromatography with flame ionization detection (GC-FID) for VFA production

Samples were subjected to GC-FID analysis to identify and quantify the reduction of volatile fatty acids (VFAs) volumes produced over a 60-day retention time. The GC-FID analysis was measured and recorded in three intervals of 20 days each. At 20^{th} day, cow rumen waste recorded the following VFAs - isobutyric (233.36) > valeric (183.96) > isovaleric (165.14) > hexanoic (72.09) > capronic (45.17) > butyric (30.58) > phosphonic acid (15.50). At the 40^{th} day, VFAs ranked as follows capronic (138.86) > isovaleric (73.19) > phosphonic (64.03) > butyric (61.80) > valeric (15.16) > isobutyric (12.78) > acetic (5.32) > hexanoic (1.39). at the 60^{th} day, VFAs ranked as follows hexanoic (19.81) > valeric (18.85) > capronic (16.51) > phosphonic (14.53) > acetic (8.52) > isobutyric acid (3.15) > isovaleric (3.06) > butyric (1.88). The cumulative volumes of VFAs generated from cow rumen waste within the three intervals of retention time reduced in the order of 745.797 > 372.539 > 86.366 (ppm). Figures 4.6 represents a progressive reduction of VFAs in the three intervals. For human fecal slurry, the following VFAs were observed at the 20^{th} day - isovaleric (177.58) > valeric (174.30) > phosphonic (55.76) > hexanoic (45.02) > capronic (26.47) > butyric (21.05) > isobutyric (9.23). VFAs at the 40^{th} day ranked as butyric (51.87) > acetic (24.07) > capronic (19.84) > phosphonic (15.58) > isobutyric (12.68) > valeric (5.18) > isovaleric (2.33) > hexanoic (2.08). At the 60th day, the ranking order of VFAs observed was butyric (27.05) > capronic (14.41) > valeric (8.24) > isovaleric (7.77) > phosphonic (6.08) > isobutyric (5.91) > acetic (5.61) > hexanoic (3.22). The cumulative volumes of VFAs generated using human fecal slurry

within the three intervals of retention time, reduced in the order of 509.405 > 133.627 > 71.318 (ppm). Figures 4.4a and 4.4b show a progressive reduction of VFAs in cow rumen waste and human fecal slurry respectively over a three-range interval of 20 days each. The chromatograph of figure 4.5a further showed isobutyric, isovaleric and valeric acids as the dominant VFAs in cow rumen waste at the onset with no acetic acid content. However, as the retention time progressed, acetic acid was generated with reduction of the previous dominant VFAs as seen in figure 4.5b and 4.5c. The chromatograph of figure 4.6a recorded isovaleric and valeric acids as the dominant VFAs in human fecal slurry at the onset with no acetic acid trace. Acetic acid was generated with reduction of the previous dominant VFAs as seen in figure 4.6b and 4.6c. The reduction of initial VFA volumes of 745.797ppm and 509.404ppm, for cow rumen waste and human fecal slurry respectively to 86.366ppm and 71.318ppm support the findings of Pham and Chang (2020) where VFA consuming bacterial strains were subjected to VFA degradation of butyric, isovaleric, valeric, isocaproic, acetic, propionic, isobutyric and caproic acids. They observed a 60% reduction of VFA volumes after 15 days of retention time (Pham & Chang, 2020). In the study of Al-Sulaimi et al. (2022) initial VFA accumulation in primary and secondary sewage sludge was 824.68mg/L and 236.67mg/L respectively with a methane production of 66.75% and 52.29% respectively. In their study, the dominant VFA was acetic acid hence the high methane yield since acetic acid is the main intermediate by product of methane production (Al-Sulaimi et al., 2022).

4.4.1 Gas chromatography with flame ionization detection (GC-FID) for LCFA content

The GC-FID analysis was carried out at day 20 and day 60. From the results of the chromatographs of long chain fatty acids (LCFA), it was observed that both substrates experienced a reduction in LCFA content over the 60-day retention time. In figures 4.7a, 4.7b, 4.8a and 4.8b cow rumen waste and human fecal slurry generated high volumes of LCFAs comprising C -18:1, C – 18:2, C – 20:4, C – 22: 4 (oleic, linoleic, arachidonic and docosatetraenoic acids) amongst others. Six saturated LCFA and nine unsaturated LCFA accounting for 62.52% of the cumulative fatty acid were recorded in cow rumen waste while human fecal slurry had six saturated LCFA and five unsaturated LCFA accounting for 55.38% of cumulative fatty acid content. These findings support the study of Erdirencelebi (2023) who recorded high presence of unsaturated and saturated LCFA in sewage sludge which contributed to inhibition in biogas production. High levels of long chain fatty acids and volatile fatty acids are the primary source of concern in the instability of anaerobic digestion process causing inhibitions and decline of bacterial activity (Cai, Zheng & Wang, 2021; Elsamadony et al, 2021). Reports have it that presence of LCFAs inhibit several reactions during anaerobic degradation process (Salama, Jeon, Kurade, Patil, Usman, Li & Lim, 2020; Usman, Salama, Arif, Jeon & Li, 2020). Inhibitory effects of unsaturated acids on substrates in anaerobic digestion processes are more deleterious compared to that of saturated LCFAs (Dasa, Westman, Millati, Cahyanto, Taherzadeh & Niklasson, 2016). Unsaturated LCFAs can inhibit methanogenesis leading to process imbalance and reduced biogas production (Zheng, Wu, Wang, Wei, Jia, Zhang, Shi, Zhang & Li, 2024). Elevated levels of unsaturated LCFAs can cause toxicity leading to reduced microbial activity (Szabo-Corbacho, Sharma, Miguez, de la Sovera, Brdjanovic, Etchebehere, Garcia & van Lier, 2024). More so, the inhibitory effects of LCFA on biogas production can be attributed to its ability to adsorb onto the microbial cell or cell membrane causing damage to the

microbes' transport and protective functions (Alibardi & Cossu, 2016, Dasa et al., 2016). Unsaturated fatty acids possess more double bonds and can be more problematic to microbes than same length saturated fatty acids with single bonds. The inhibition effect of LCFAs is positively related to the number of double bonds in LCFAs (Ma et al., 2015). The proximate analysis done using the samples, as seen in Table 4.1, show a higher fat percentage of 0.114% in cow rumen waste as compared to human fecal slurry of 0.041%. This result supports the findings of Yang, Xu, Zheng, Chen, Zhao and Li (2016) who mentioned that samples with high levels of unsaturated LCFAs may have high lipid content which can adversely affect anaerobic digestion. Figure 4.9 shows the reduction of LCFA from the 20^{th} day and the 60^{th} day. Upon further digestion at the 60^{th} day, the fatty acid volume fell from 108.216ppm to 60.499ppm in cow rumen waste while that of human fecal slurry reduced from 113.195ppm to 44.94ppm.

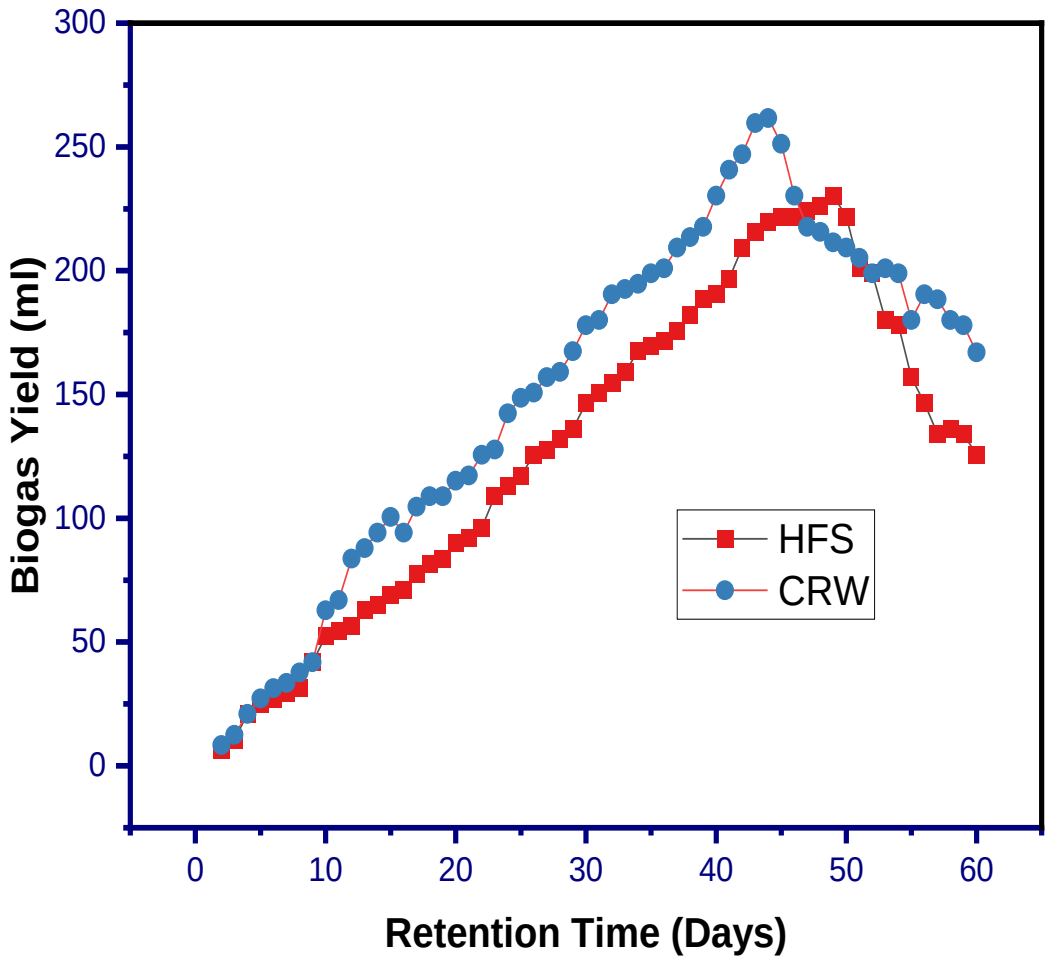

Figure 4.1: Comparison of biogas volumes generated in both substrates of cow rumen waste and human fecal slurry over a 60-day retention time.

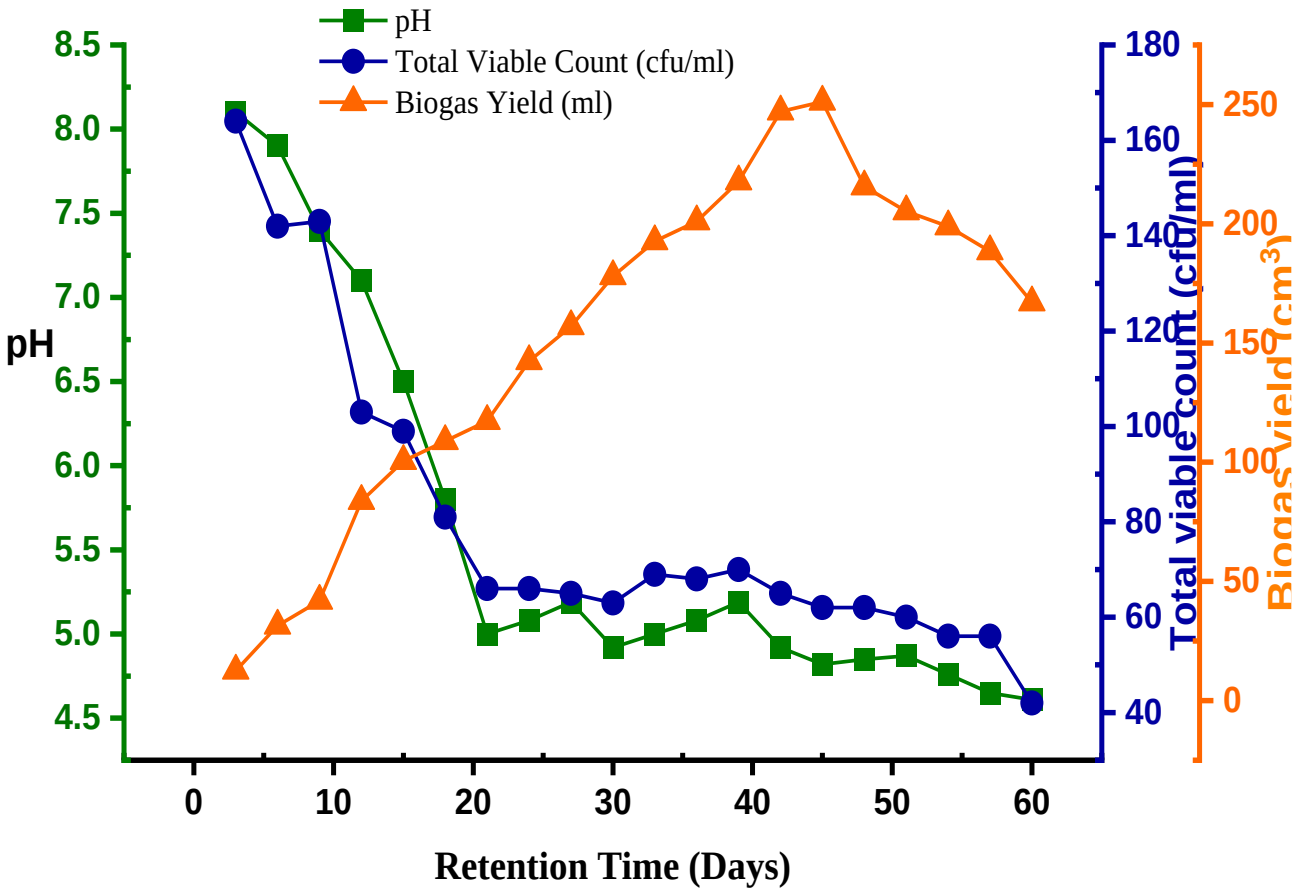

Figure 4.2a: Relationship between pH, total viable count (TVC), biogas yield and retention time in cow rumen waste substrate

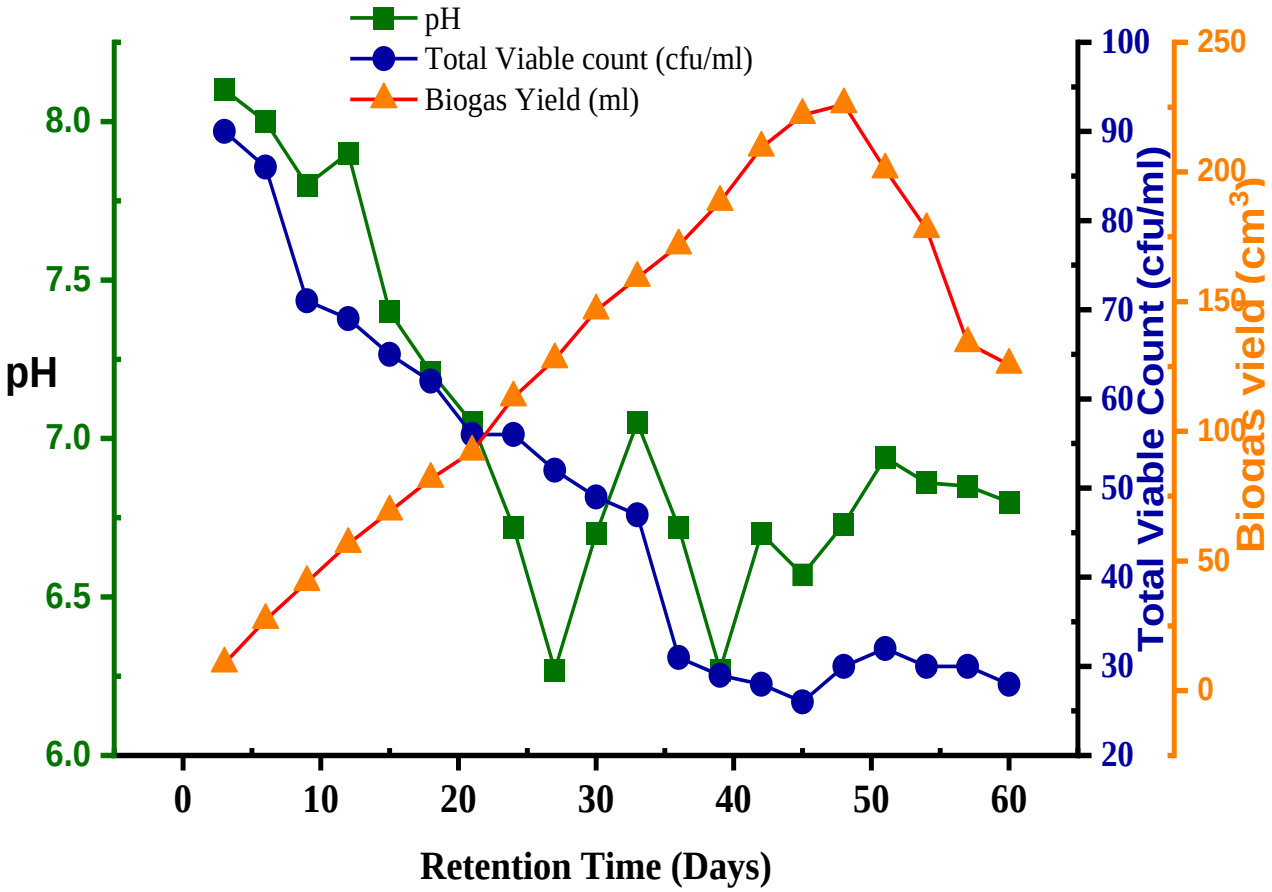

Figure 4.2b: Relationship between pH, total viable count (TVC), biogas yield and retention time in human fecal slurry substrate

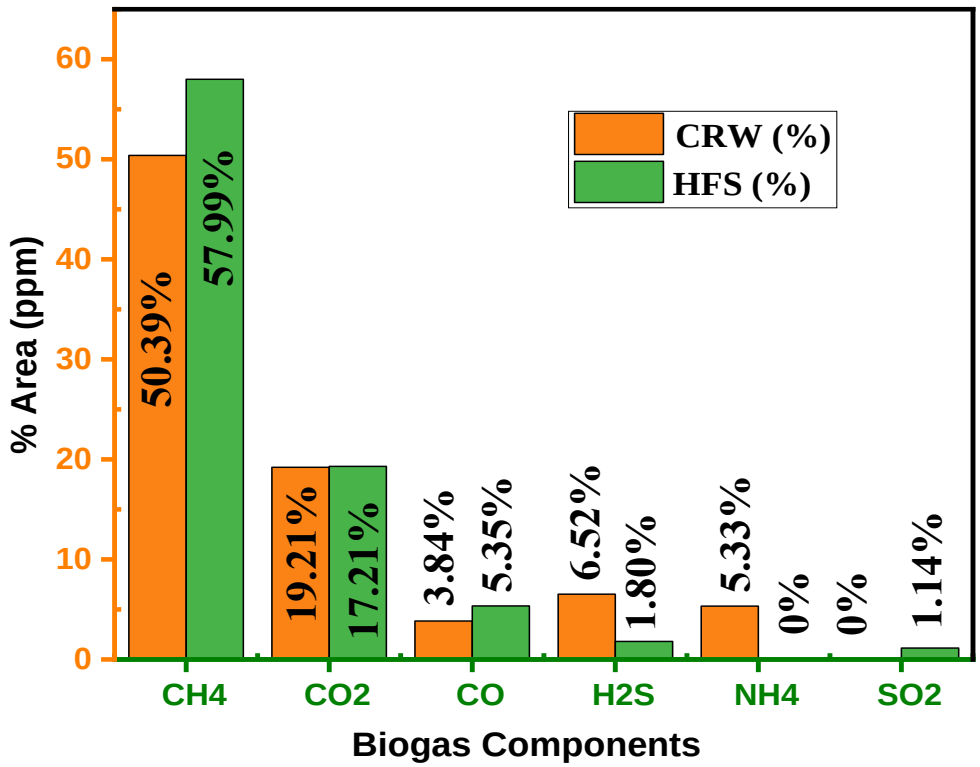

Figure 4.3: Comparison of GC-MS results in % area on biogas composition generated in both substrates.

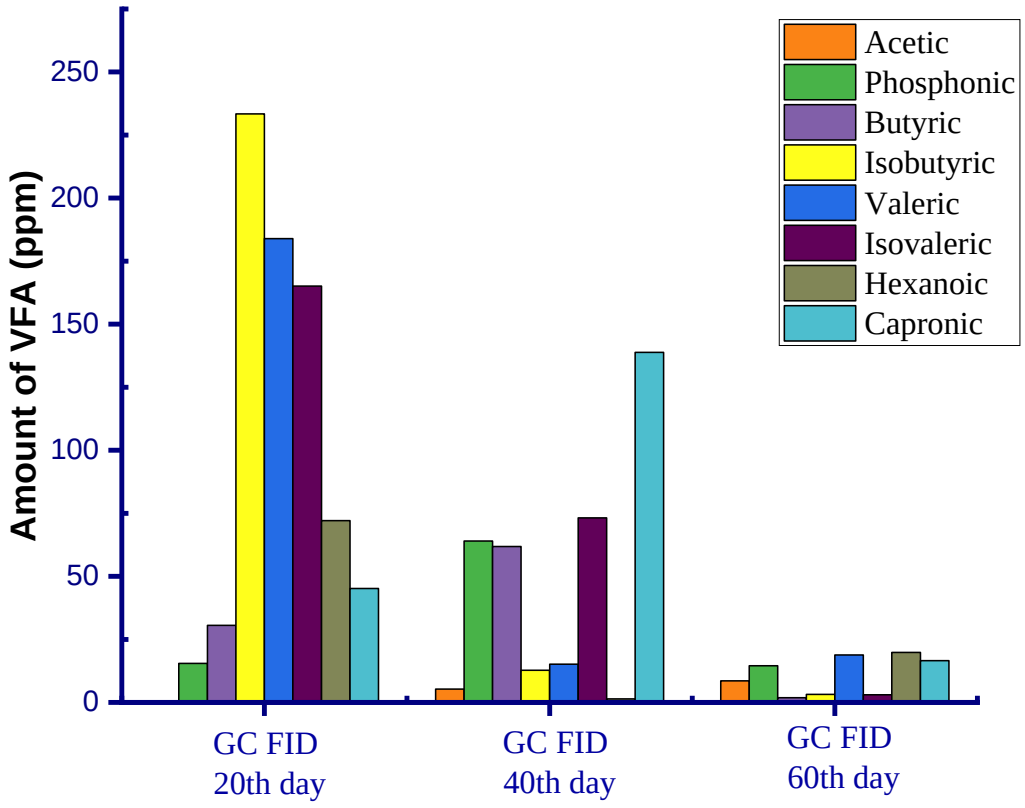

Figure 4.4a: Volatile fatty acid reduction in cow rumen waste substrate over three intervals of 20 days each.

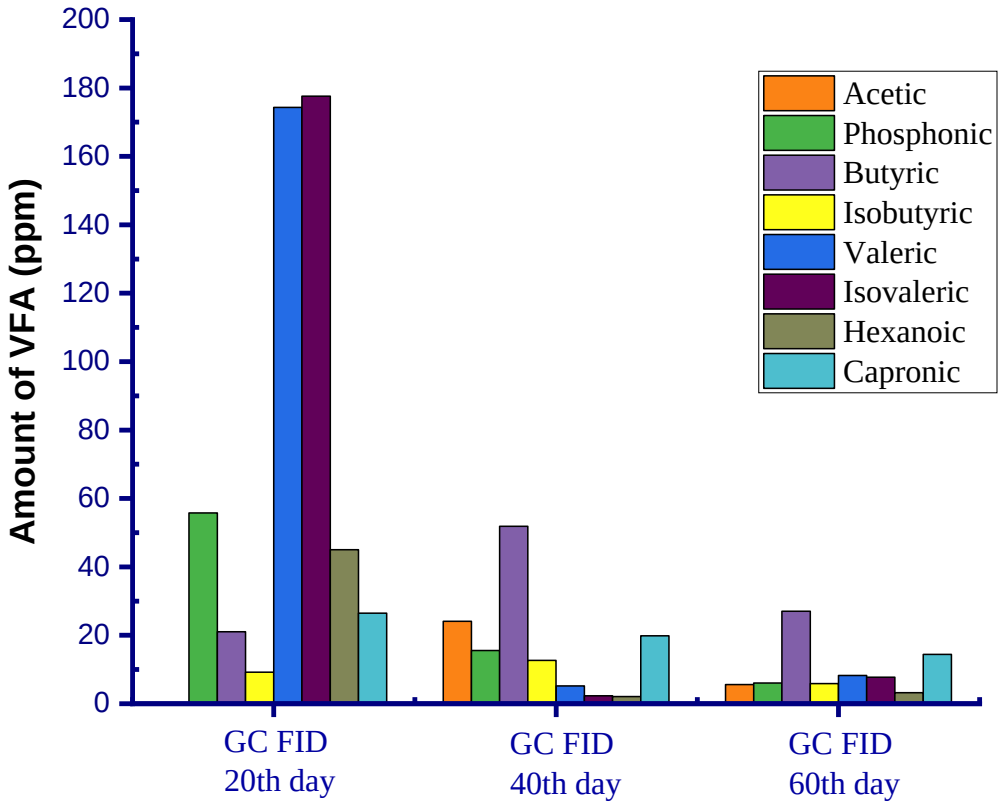

Figure 4.4b: Volatile fatty acid reduction in human fecal slurry substrate over three intervals of 20 days each.

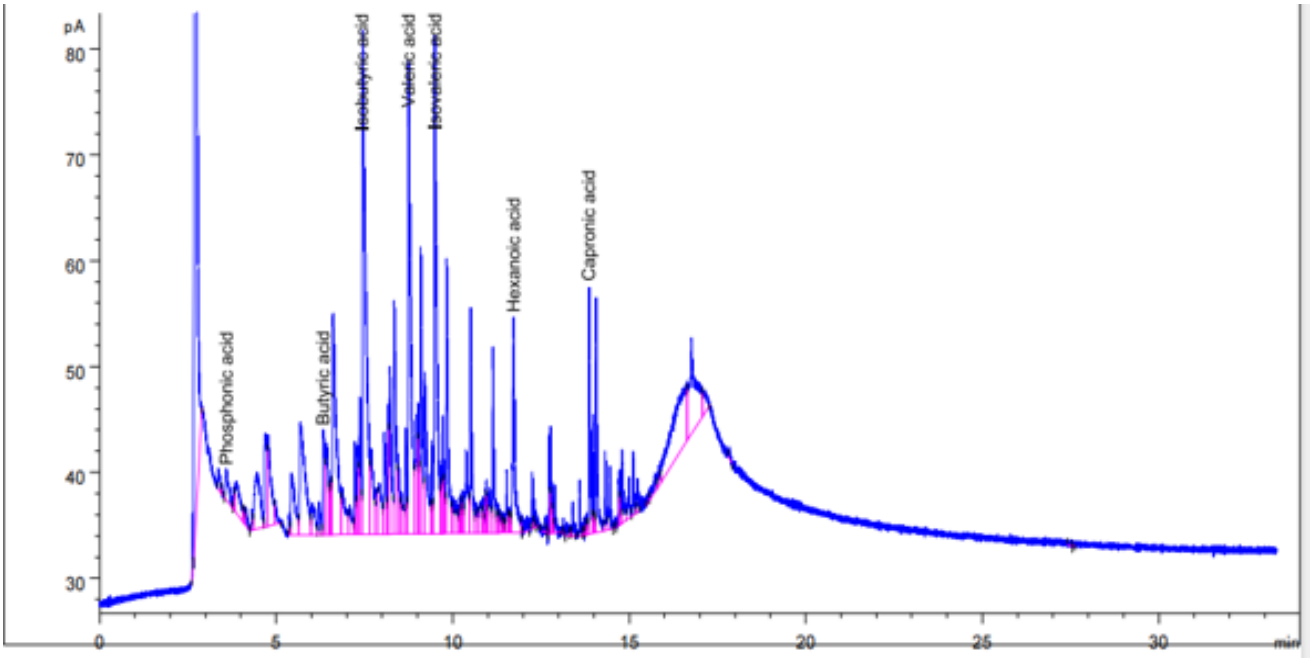

Figure 4.5a: GC-FID chromatogram of volatile fatty acids in cow rumen waste at the 20th day

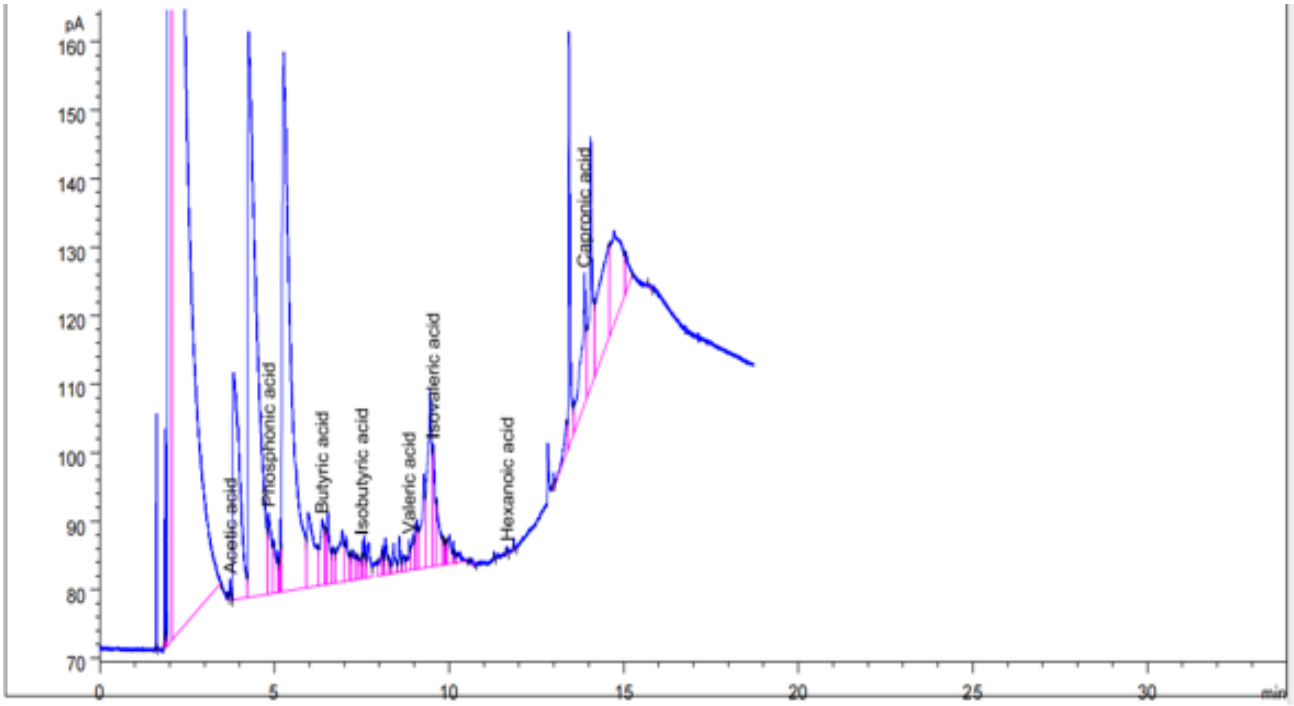

Figure 4.5b: GC-FID chromatogram of volatile fatty acids in cow rumen waste on the 40th day

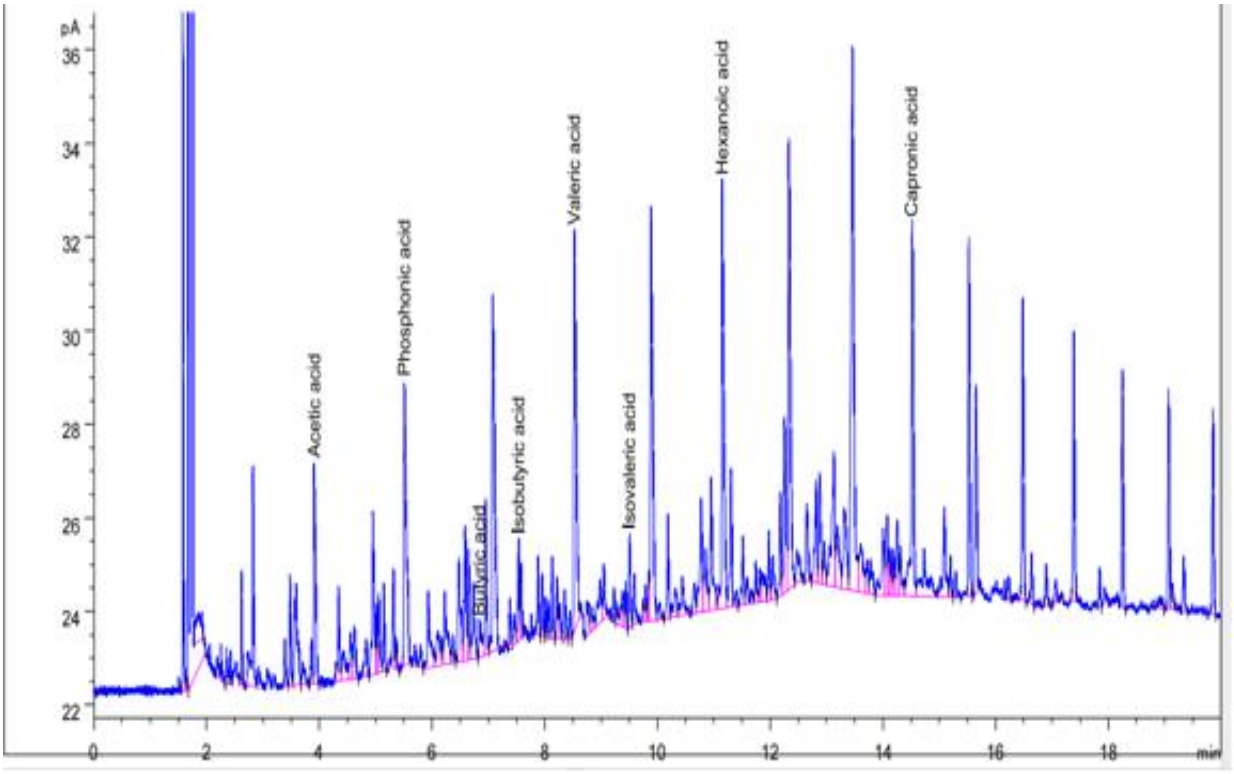

Figure 4.5c: GC-FID chromatogram of volatile fatty acids in cow rumen waste on the 60th day

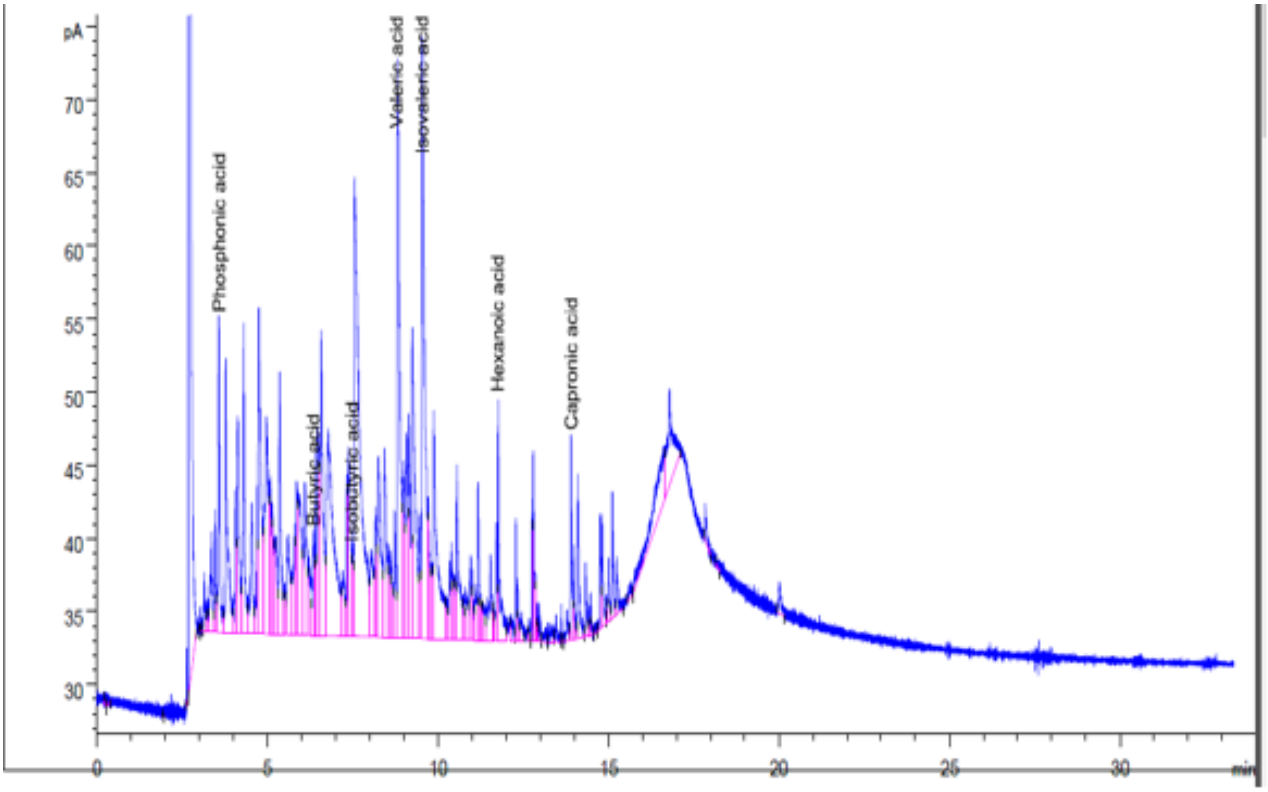

Figure 4.6a: GC-FID chromatogram of volatile fatty acids in human fecal slurry on the 20th day

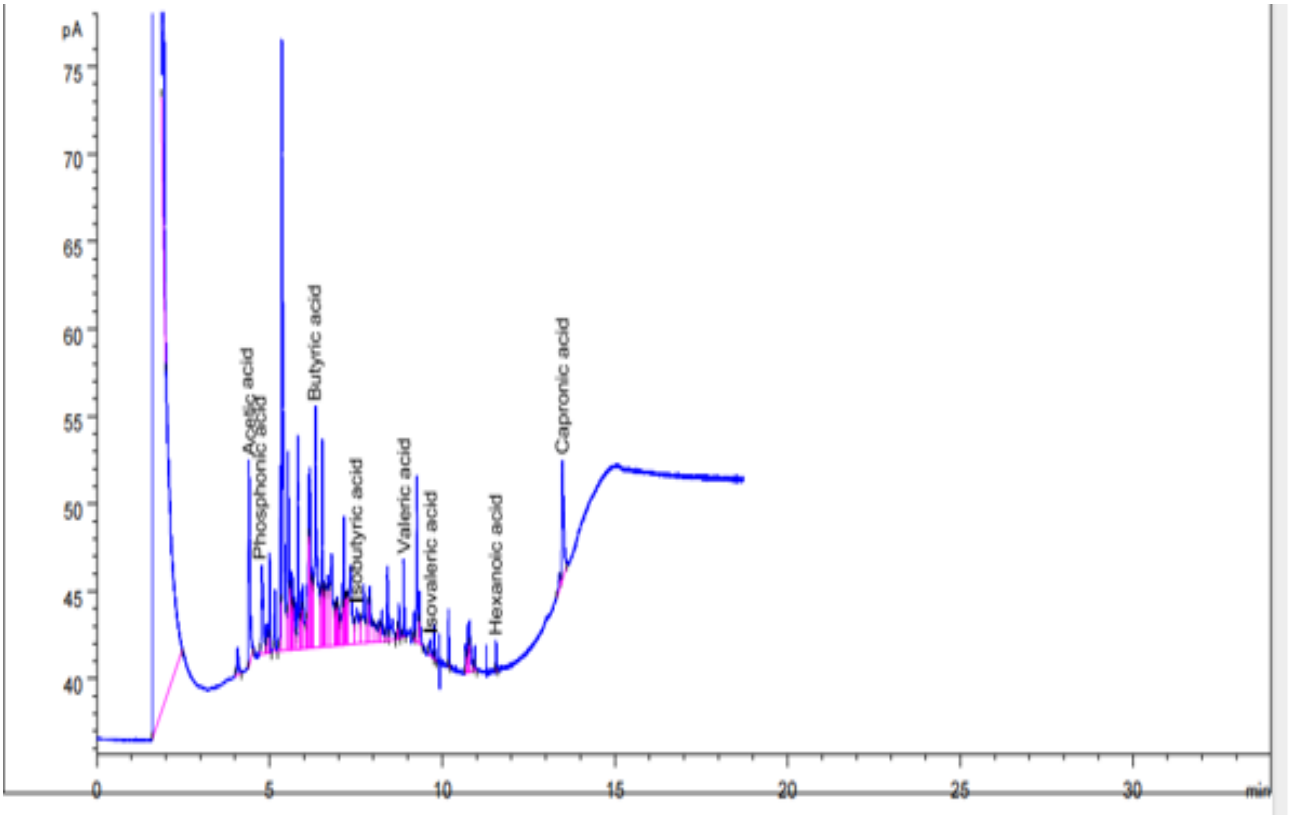

Figure 4.6b: GC-FID chromatogram of volatile fatty acids in human fecal slurry on the 40th day

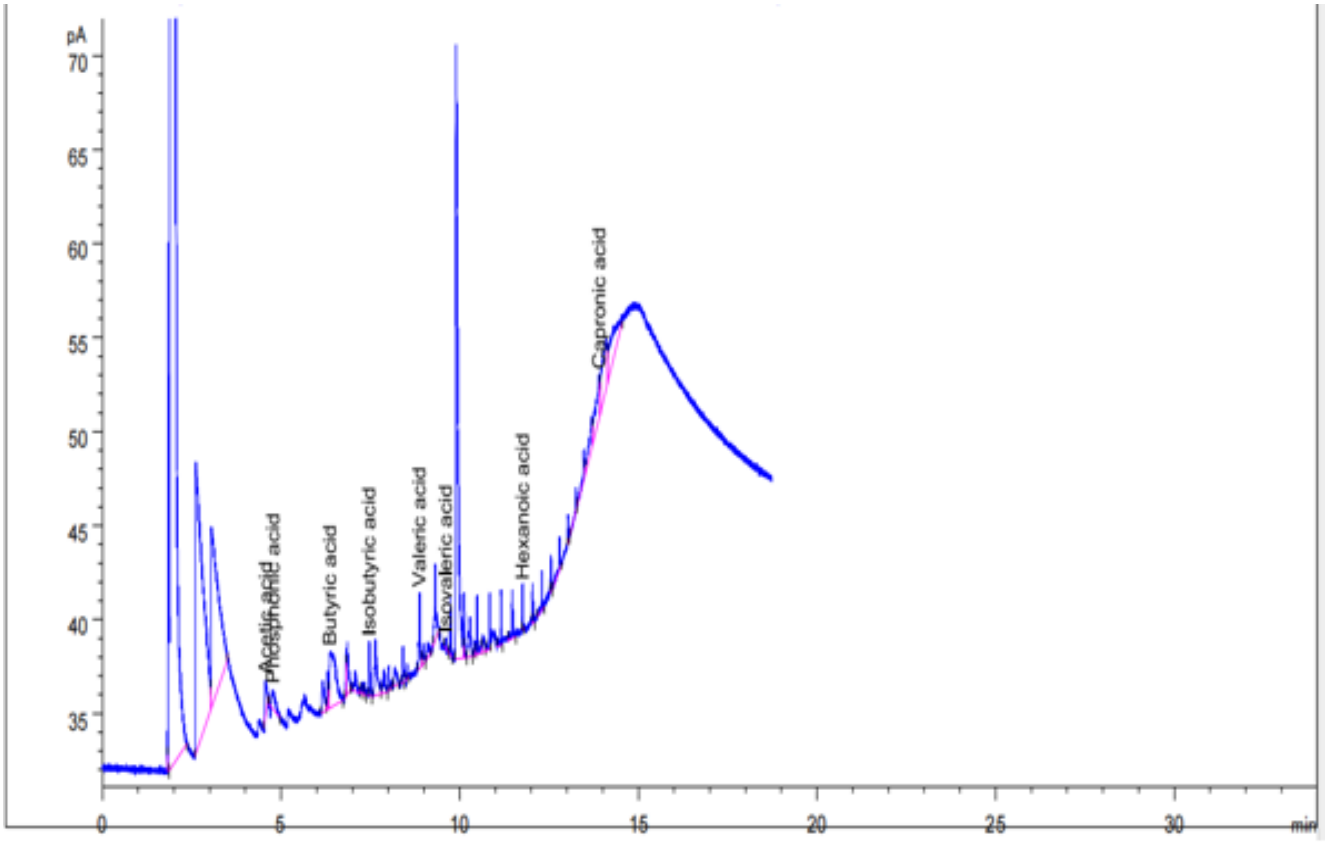

Figure 4.6c: GC-FID chromatogram of volatile fatty acids in human fecal slurry on the 60th day

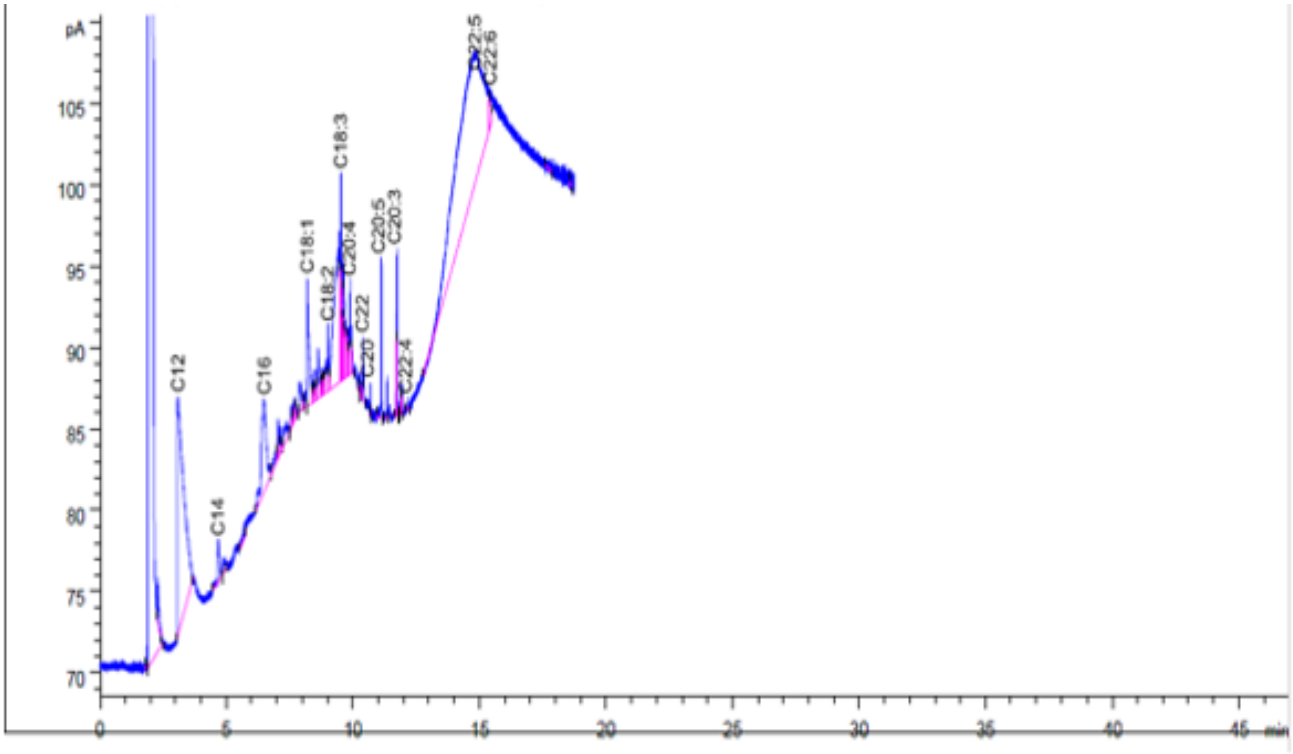

Figure 4.7a: GC-FID chromatogram of long chain fatty acids in cow rumen waste on the 20th day

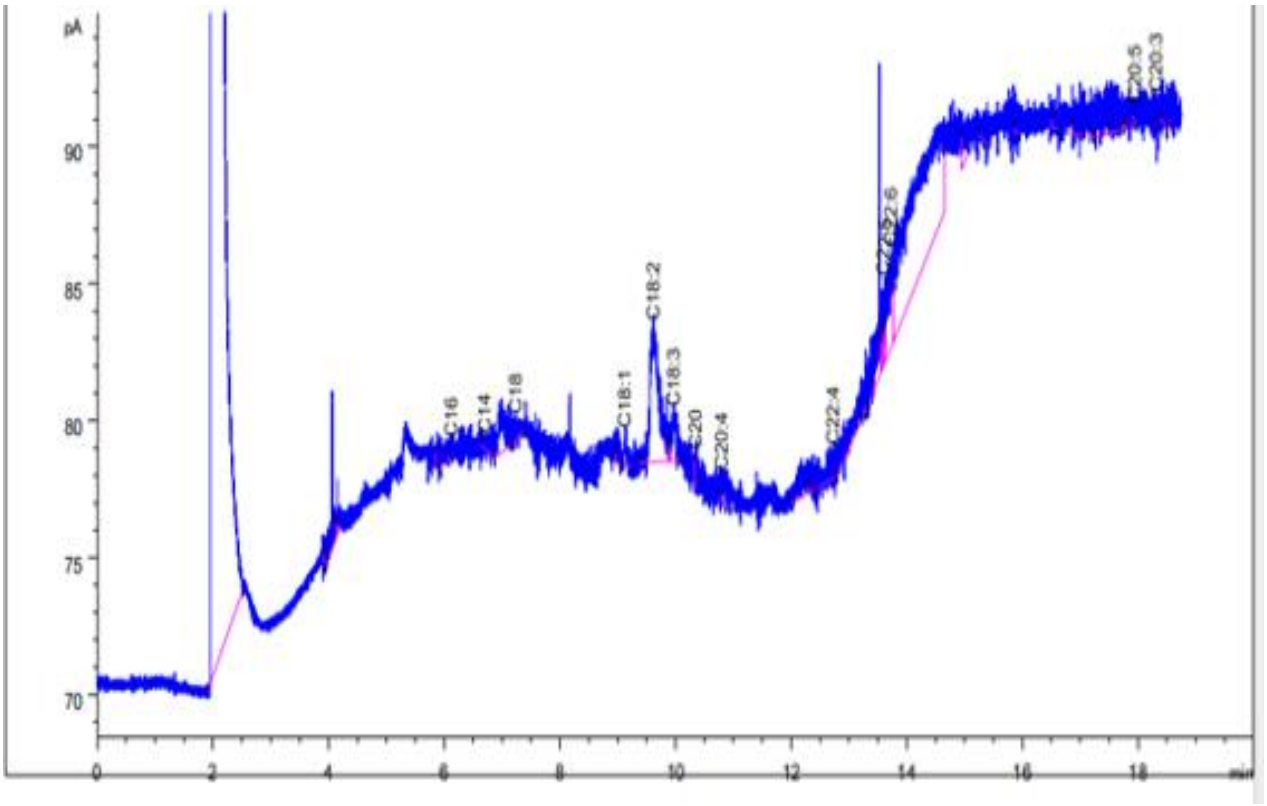

Figure 4.7b: GC-FID chromatogram of long chain fatty acids in cow rumen waste on the 60th day

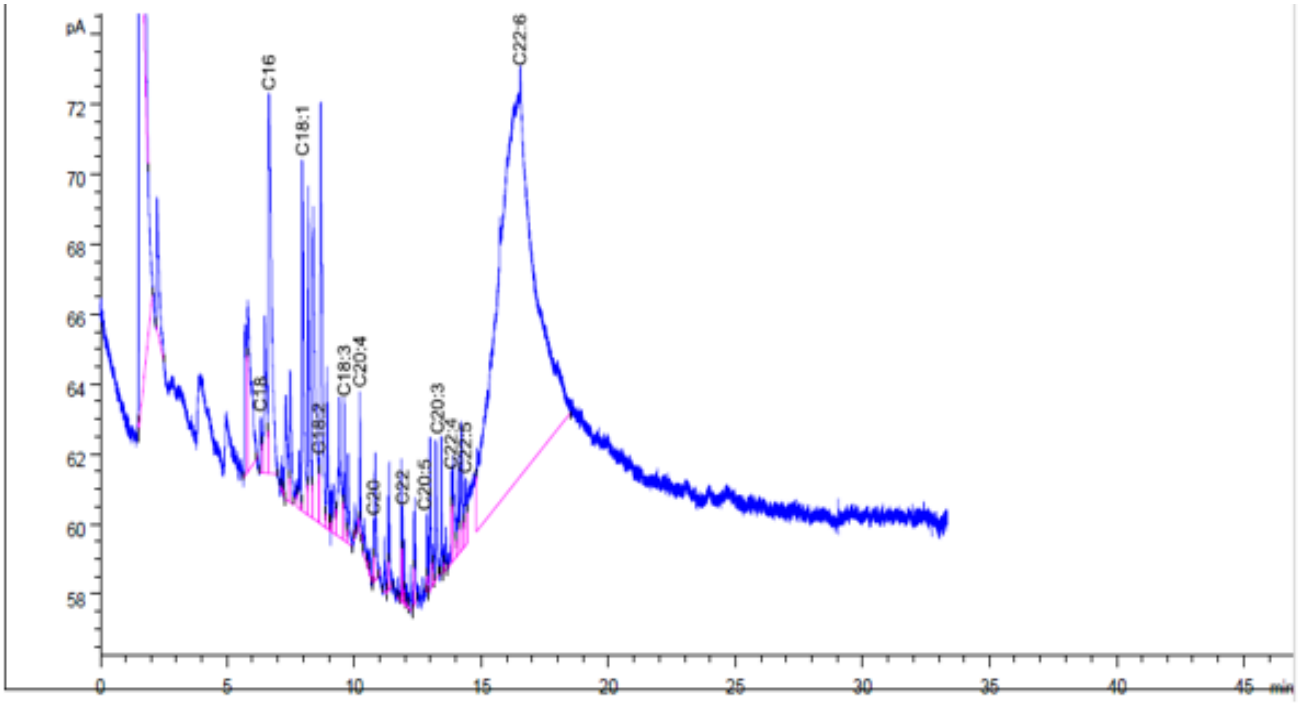

Figure 4.8a: GC-FID chromatogram of long chain fatty acids in human fecal slurry on the 20th day

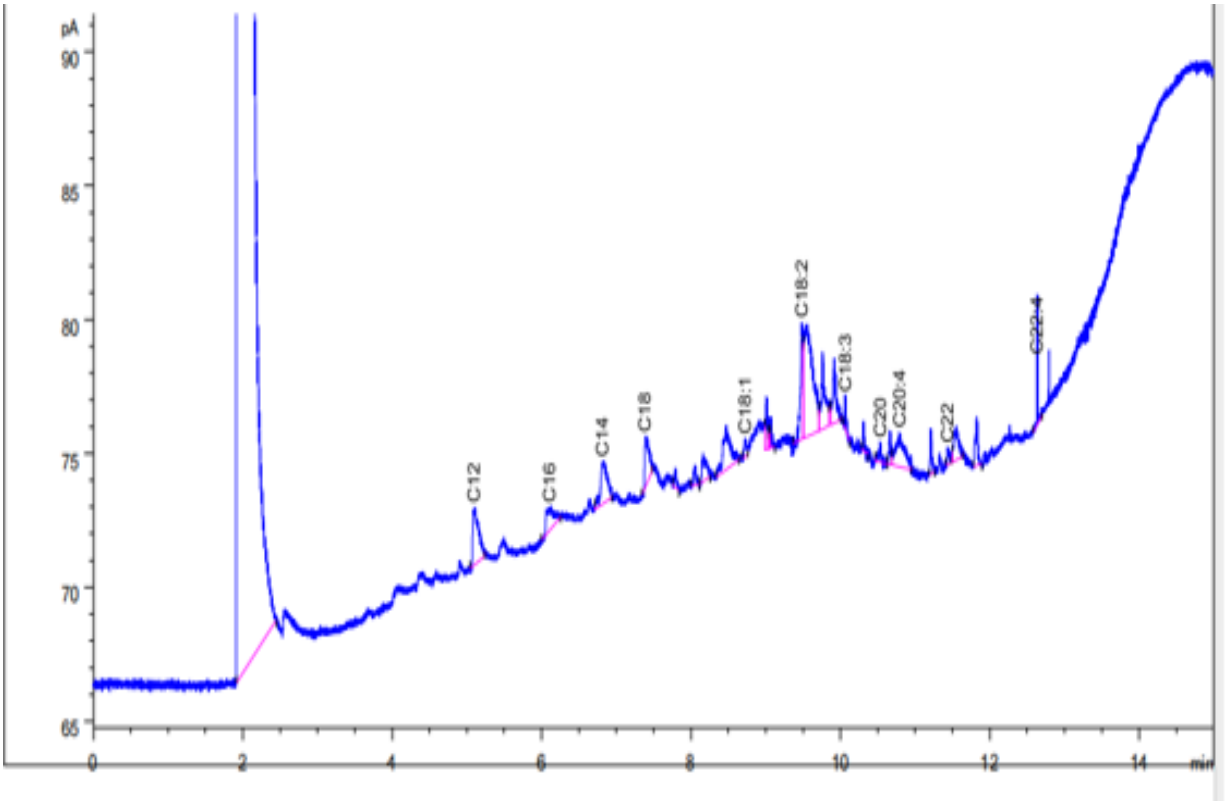

Figure 4.8b: GC-FID chromatogram of long chain fatty acids in human fecal slurry on the 60th day

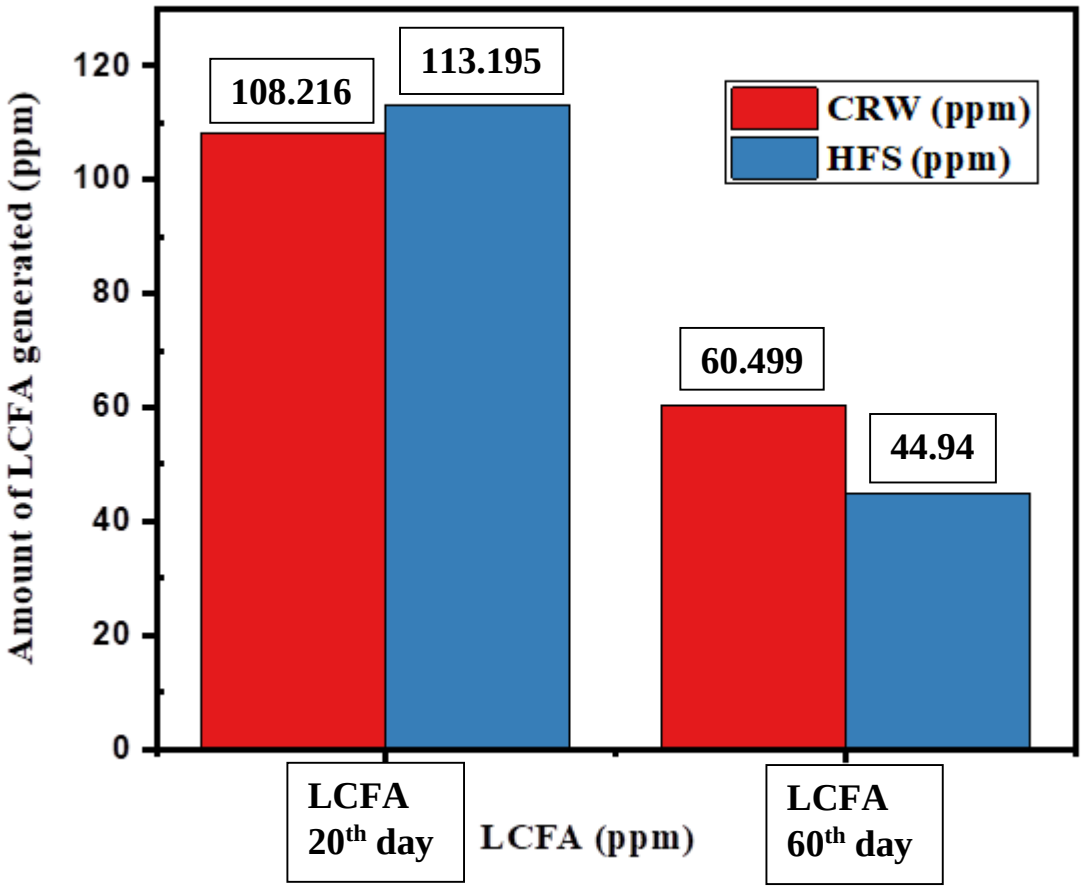

Figure 4.9: Cumulative long chain fatty acid reduction in both substrates over a 60-day retention time

4.5 – Agarose electrophoresis of bacterial isolates

Plates 4.1 and 4.2 showed agarose electrophoresis of a few particular bacterial isolates. Different bands were detected under UV-transilluminator with the molecular ladder (100bp) represented in lane M. A clear ladder pattern with distinct bands at 3000, 1000, 800 and 100 bp were shown in lanes 2, 9, 10 and 11 was detected using forward and reverse primers for detection of acsl genes. Isolates 1, 3, 4, 5, 6,7,8,12 and 13 showed no bands which shows that they are possibly not typable by the primers; the sequence of the primer used was not able to anneal to any complimentary sequence of the DNA fragment.

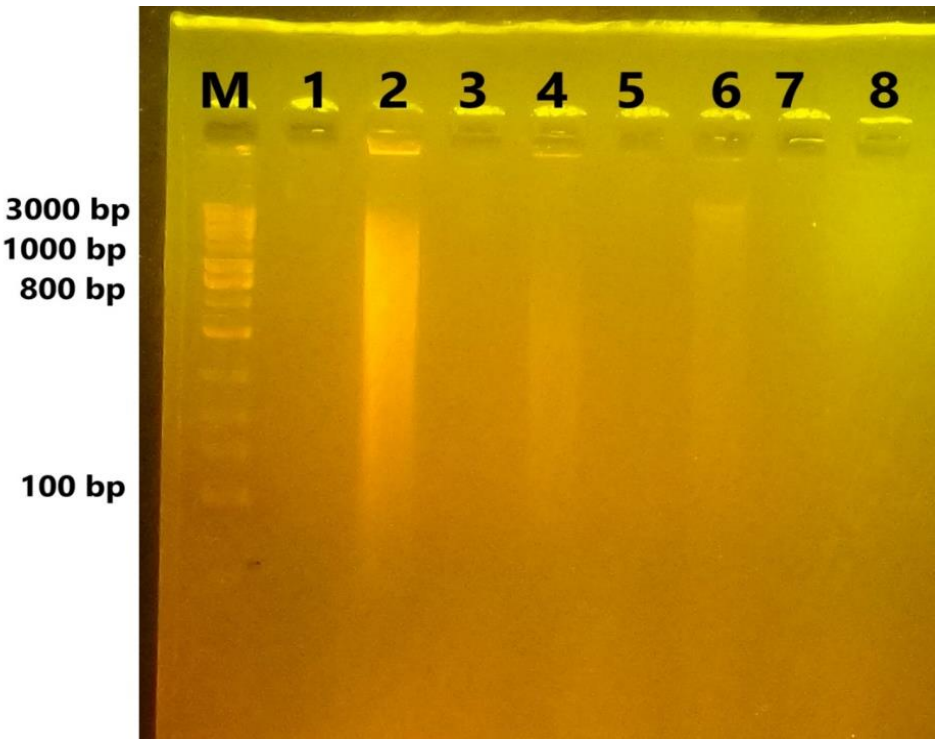

Plate 4.1: Gel profile of *Moellerella wisconsensis* on lane 2 showing amplification of acsl-encoding gene shown using primer 1 (5' GTGCTCTACCTCCATCCAAA 3'). Lane M is the 100bp DNA ladder.

Key:
M – marker
Lane 1 – *Paenibacillus lautus*
Lane 2 – *Moellerella wisconsensis*
Lane 3 – *Providencia alcalifaciens*
Lane 4 – *Shimwellia blattae*
Lane 5 – *Micrococcus yunnanensis*
Lane 6 – *Bacillus barbaricus*
Lane 7 – *Proteus vulgaricus*
Lane 8 – *Paenibacillus septentrionalis*

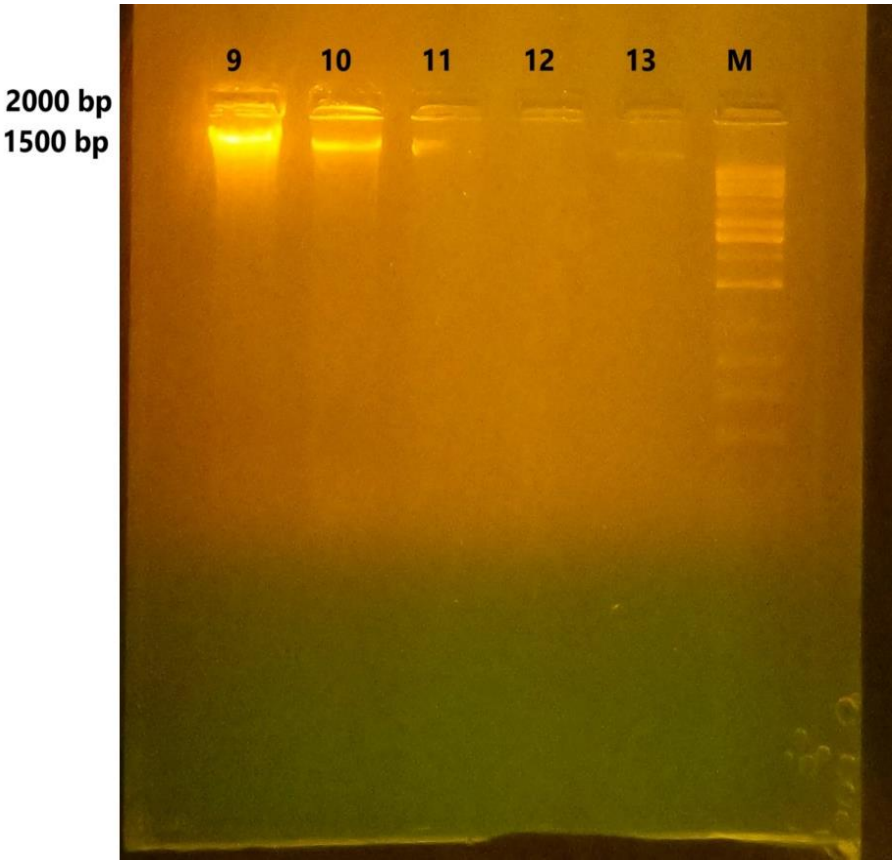

Plate 4.2: Gel profile of *Paenibacillus curdlanolyticus*, *Budvicia aquatica* and *Paenibacillus septentrionalis* on lane 9, 10 and 11 showing amplification of acsl-encoding gene shown using primer 1 (5' GTGCTCTACCTCCATCCAAA 3'). Lane M is the 100bp DNA ladder.

Key:
M – marker
Lane 9 – *Paenibacillus curdlanolyticu*
Lane 10 – *Budvicia aquatica*
Lane 11 – *Paenibacillus septentrionalis*
Lane 12 – *Azotobacter beijerinekii*
Lane 13 - *Acinetobacter iwoffii*

4.6 Statistical Analysis

The data were subjected to one- way analysis of variance (one-way ANOVA) using Origin Pro 2022 statistical software. Standard deviation and mean values were separated via Bonferrni test, Tukey test, Sidak test and Fisher test methods at P = 0.05 respectively (see Appendix). According Bonferrni and Tukey tests, the mean biogas yield values share the same letter which means that there is no significant difference. A *p*-value of 0.07301 was obtained. The null hypothesis shows that all levels are equal and the alternative hypothesis observes that one or more levels are different. At the 0.05 level, the means are not significantly different.

4.7 Optimization of process parameters using response surface methodology

Response surface methodology and a matrix of central composite design (CCD) using Design Expert version 13 statistical software was used to study the effects of three variables (retention time, pH and total viable count) of the both substrates. The optimization results showed that the process parameters can interact thereby improving the biogas production significantly. By applying ANOVA on the experimental data, a quadratic model and a 2FI model was generated to represent the biogas yield from the two substrates (see appendix). The cow rumen waste and the human fecal slurry substrates had a standard deviation of 64.99, mean of 152.91, coefficient of variance of 42.50% and standard deviation of 63.447, mean of 129.067 and coefficient of variance of 49.16% respectively. The adequate precision values were 4.493 and 4.705 for cow rumen waste and human fecal slurry respectively. Precision values of 4 and above imply that they are desirable and indicate an adequate model for optimization. Studies conducted by Eterigho et al. (2019) on biogas production form co-digestion of cow rumen and municipal waste recorded values of 13.843 and 14.136.

From the figures 5.0a and 5.0b, the confirmation location of optimal production using cow rumen waste is 10.5 days (retention time), 6.355 (pH) and 1.03 $x10^9$ cfu/ml (total viable count) while that of human fecal slurry is 10.5 days (retention time), 7.45 (pH) and 5.9 $x10^8$ cfu/ml (total viable count). Below are the equations in terms of actual factors which can be applied in order to ascertain predictions about response for given levels of either of the factors. The final model equations, in terms of actual factors, for both events, are given below:

Biogas yield = 209.93924 + 3.88227 (retention time) – 14.61494 (pH) – 0.047716 (total viable count) ……*Equation* 1 (cow rumen waste)

Biogas yield = -125.58540 + 9.17990 (retention time) + 47.37088 (pH) + 2.13147 (total viable count) – 2.79704 (retention time x pH) + 0.149285 (retention time x total viable count) – 0.441563 ……*Equation* 2 (human fecal slurry).

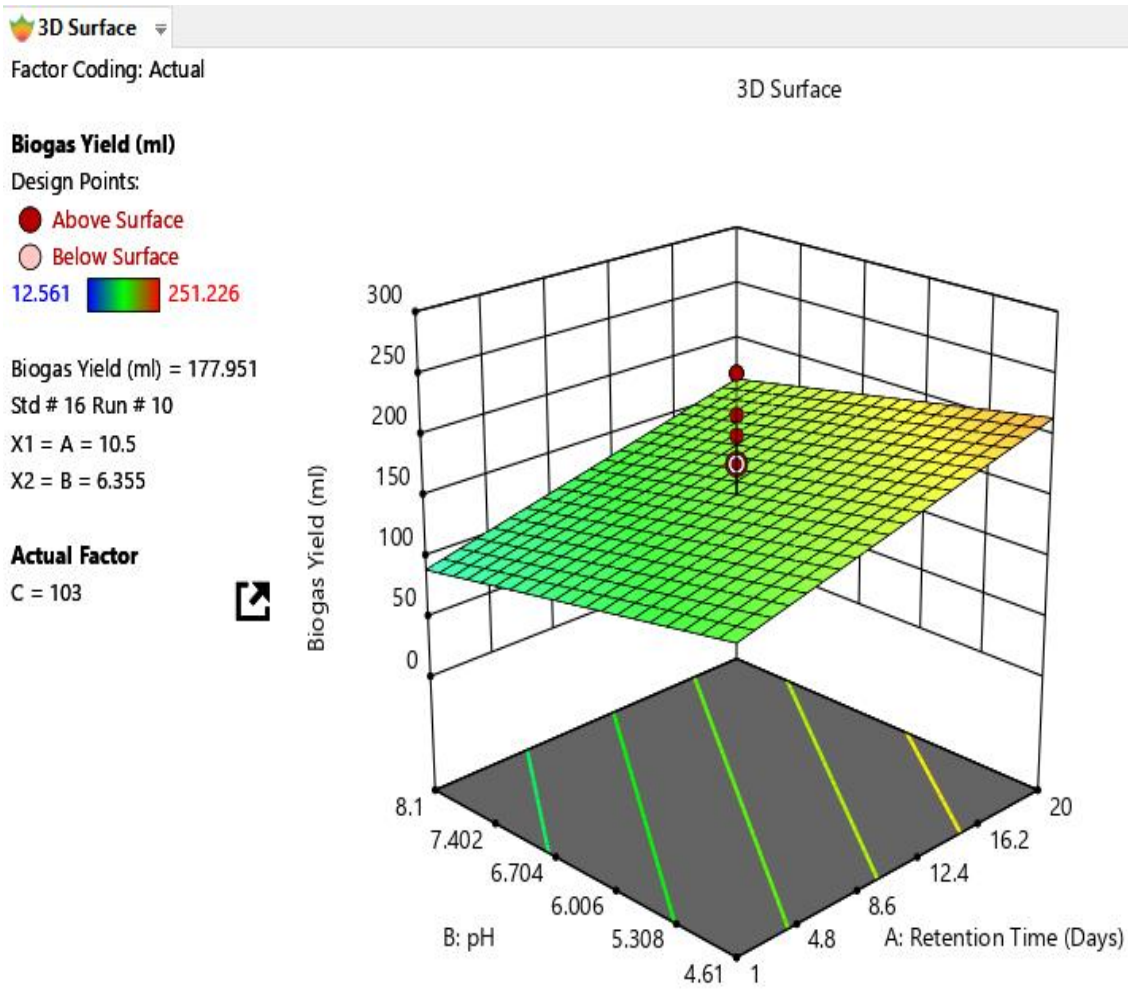

Figure 5.0a: 3D response surface plot showing the interactive effects of pH, retention time and biogas yield for cow rumen waste substrate

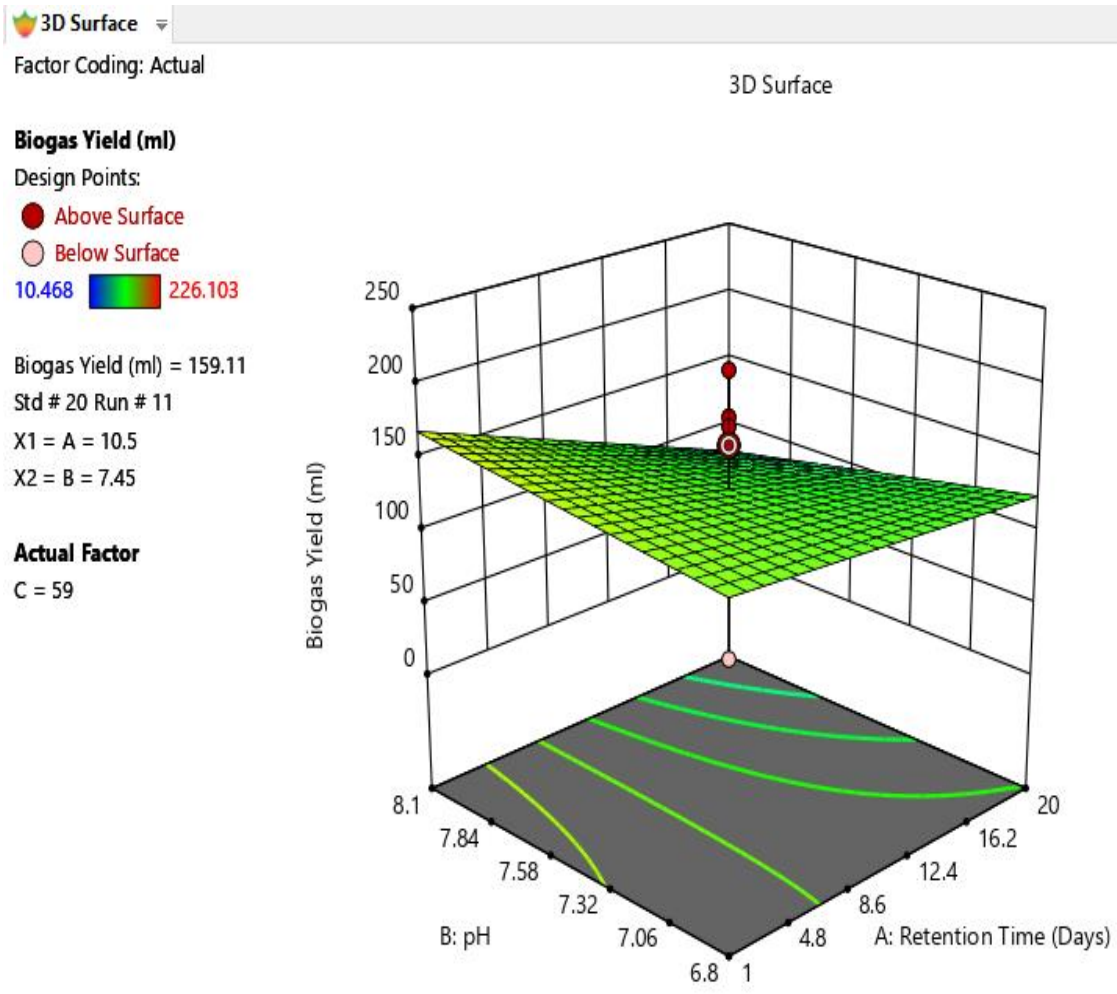

Figure 5.0b: 3D response surface plot showing the interactive effects of pH, retention time and biogas yield for human fecal slurry substrate

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSION

The results of this research on the exploitation of bacteria with volatile degrading genes has shown that specific bacterial isolates in cow rumen waste and human fecal slurry can degrade VFAs as the pH level rises and can continue with biogas production in anaerobic digestion processes when inoculated under appropriate conditions. *Moellerella wisconsensis, Paenibacillus curdlanolyticus, Budvicia aquatica* and *Paenibacillus septentrionalis* succeeded in the degradation of volatile fatty acids and long chain fatty acids in anaerobic digestion due to the availability of specific VFA degrading genes. This study successfully isolated and characterized these that organism possessed the target genes. Moreso, the degradation of volatile fatty acids and long chain fatty acids was also monitored using gas chromatography. The study demonstrated the efficacy of producing biogas from using cow rumen waste and human fecal slurry and highlighted the quality of biomethane generated over a 60-day retention. Meanwhile, cow rumen waste and human fecal slurry were considered potential feedstocks because they are readily available in large quantities, contain high percentage of organic matter and their use provides environmentally friendly solution for managing human and slaughter wastes, reducing the environmental and health impacts associated with inadequate sanitation. The findings of this study greatly advance our knowledge of the genetic basis of the breakdown of volatile fatty acids during anaerobic digestion and how it affects the generation of biogas. The discovery of these genes that break down VFA has significant ramifications for the creation of a novel bioaugmentation inoculum and a simple strategy for tracking the effectiveness of an anaerobic system setup. By investigating the functional roles of these VFA degraders and

their possible industrial or residential applications, future research can expand on these recent findings. All things considered, this research emphasizes how important biogas generation is as a long-term option for waste management and energy independence. When employing these organic substrates, the RSM determined the ideal microbe count for effective digestion and maximum biogas production, controlled pH and retention time, and identified fundamental performance indicators.

5.2 RECOMMENDATIONS

Further studies can be carried out on these VFA degrading bacteria. The strains of the isolates with VFA degrading genes can be subjected to sequencing to identify the exact species, purify and use them to test their efficiency using agro-wastes materials such as corn cobs, maize/rice stalks, other lignocellulosic waste materials from farms. Bacterial isolates possessing volatile fatty acid degrading genes should be sequenced and genetically engineered and used as bioaugmentation tools for use by biogas plants in industrial scale. Biogas systems can equally be designed with specific parameters can equally enhance the production of biogas. Seeing that cow rumen waste and human fecal slurry disposal can pose nuisance and offensive odor in the environment, this study has recorded that these substrates are a good source of biogas for domestic activities when effectively upgraded. The digestate can be used in agricultural activities as a biofertilizer since it is a rich source of nitrogen and phosphorus. The biogas produced from the anaerobic digestion of these substrates can be upgraded to increase its quality as a green fuel source, prevent corrosion of metal pipes, reduce health hazard and produce biomethane for domestic activities and power generation especially for low-income earners. Several indigenous zeolites are used for upgrade; however, a more sustainable approach would be the use of an algae scrubbing system comprising algal sp. such as *Chorella* sp., *Scenedesmus* sp. and *Neochloris* sp. since algae consume large quantities of CO_2

and H_2S and needs CO_2 for its growth and development. This helps support a sustainable circular economy. According to this research, both substrates generated high volumes of valeric, isobutyric, isovaleric, caproic and acetic acids at the initial stages of hydrolysis and acidogenesis, making them interesting biomass options for production of green chemicals. These waste-derived VFAs such as acetic and butyric acids can be used supplements in animal feeding. Phosphonic acid is used as a corrosion inhibitor in metal working fluids for iron and steel since they show great chemisorption on metals, creating a protective layer on the metal surface. Jobs, businesses and training opportunities in the area of biogas production and waste management are highly recommended.

5.3 CONTRIBUTIONS TO KNOWLEDGE

1. This research has identified *Moellerella wisconsensis, Paenibacillus curdlanolyticus, Budvicia aquatica* and *Paenibacillus septentrionalis* to possess acsl-encoding gene for VFA degradation using primer 5' GTGCTCTACCTCCATCCAAA 3'. These organisms can hence be part of consortia utilized for bio inoculation purposes for enhancing biogas production.

2. The study highlighted the potential of cow rumen waste and human fecal slurry as feedstocks for renewable energy production.

3. This study provided insights into the degradation of VFA and LCFA and its effect on biogas production over a 60- day retention time.

4. The study used response surface methodology to determine the ideal microbe count, pH and retention time for effective digestion and maximum biogas production using cow rumen waste and human fecal slurry.

5. The study used response surface methodology to provide model equations in terms of actual factors that can be applied in order to make predictions about biogas yield when using cow rumen waste and human fecal slurry.

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APPENDICES

Appendix 1: Data conversion using ideal gas equation (combined gas equation)

$\frac{P1V1}{T1} \quad \frac{P2V2}{T2}$ (Mesophilic condition)

T1 = 30℃ = 303K

V1 = Gas storage volume

P1 = Pressure of gas

$\frac{P2V2}{T2}$ = Standard temperature and pressure

T2 = 273K

Pressure (P) = 101325 Pa

P2 = 1032.875cm H_2O

V2 = Volume of gas at STP = $\frac{P1V1}{T1} \quad \frac{P2V2}{T2}$

$V2 = \frac{P1V1T2}{T1P2}$

$V2 = \frac{P1 \times 24 \times 273}{303 \times 1032.875}$

V2 = 0.02093550754 L (20.9355075 ml)

Appendix 2: Conversion table for mean values of volume of biogas generated over a 60-day using cow rumen waste substrate

Retention Time (Day)	Pressure (mmH_2O)	V_{STP} (ml)	Retention Time (Day)	Pressure (mmH_2O)	V_{STP} (ml)	Retention Time (Day)	Pressure (mmH_2O)	V_{STP} (ml)	Retention Time (Day)	Pressure (mmH_2O)	V_{STP} (ml)
1	-	-	16	4.5	94.210	31	8.6	180.045	46	11.0	230.290
2	0.4	8.374	17	5.0	104.678	32	9.1	190.513	47	10.4	217.729
3	0.6	12.561	18	5.2	108.865	33	9.2	192.607	48	10.3	215.636
4	1.0	20.936	19	5.2	108.865	34	9.3	194.700	49	10.1	211.448
5	1.3	27.216	20	5.5	115.145	35	9.5	198.887	50	10.0	209.355
6	1.5	31.403	21	5.6	117.239	36	9.6	200.981	51	9.8	205.168
7	1.6	33.497	22	6.0	125.613	37	10.0	209.355	52	9.5	198.887
8	1.8	37.684	23	6.1	127.707	38	10.2	213.542	53	9.6	200.981
9	2.0	41.871	24	6.8	142.362	39	10.4	217.729	54	9.5	198.887
10	3.0	62.810	25	7.1	148.642	40	11.0	230.290	55	8.6	180.045
11	3.2	66.994	26	7.2	150.736	41	11.5	240.758	56	9.1	190.513
12	4.0	83.742	27	7.5	157.016	42	11.8	247.039	57	9.0	188.420
13	4.2	87.929	28	7.6	159.110	43	12.4	259.600	58	8.6	180.045
14	4.5	94.210	29	8.0	167.484	44	12.5	261.694	59	8.5	177.952
15	4.8	100.490	30	8.5	177.951	45	12.0	251.226	60	8.0	167.016

Appendix 3: Conversion table for mean values of volume of biogas generated over a 60-day using human fecal slurry substrate

Retention Time (Day)	Pressure (mmH_2O)	V_{STP} (ml)	Retention Time (Day)	Pressure (mmH_2O)	V_{STP} (ml)	Retention Time (Day)	Pressure (mmH_2O)	V_{STP} (ml)	Retention Time (Day)	Pressure (mmH_2O)	V_{STP} (ml)
1	-	-	16	3.4	71.181	31	7.2	150.736	46	10.6	221.916
2	0.3	6.281	17	3.7	77.461	32	7.4	154.923	47	10.7	224.009
3	0.5	10.468	18	3.9	81.648	33	7.6	159.110	48	10.8	226.103
4	1.0	20.936	19	4.0	83.742	34	8.0	167.484	49	11.0	230.291
5	1.2	25.123	20	4.3	90.023	35	8.1	169.578	50	10.6	221.916
6	1.3	27.216	21	4.4	92.116	36	8.2	171.671	51	9.6	200.981
7	1.4	29.310	22	4.6	96.303	37	8.4	175.858	52	9.5	198.887
8	1.5	31.403	23	5.2	108.865	38	8.7	182.139	53	8.6	180.045
9	2.0	41.871	24	5.4	113.052	39	9.0	188.420	54	8.5	177.952
10	2.5	52.339	25	5.6	117.239	40	9.1	190.513	55	7.5	157.016
11	2.6	54.432	26	6.0	125.613	41	9.4	196.794	56	7.0	146.549
12	2.7	56.526	27	6.1	127.707	42	10.0	209.355	57	6.4	133.987
13	3.0	62.807	28	6.3	131.894	43	10.3	215.635	58	6.5	136.081
14	3.1	64.900	29	6.5	136.081	44	10.5	219.823	59	6.4	133.987
15	3.3	69.087	30	7.0	146.549	45	10.6	221.916	60	6.0	125.613

Appendix 4: Amount of volatile fatty acids generated in three intervals of 20 days each

	GC-FID on 20th day	**GC-FID on 40th day**	**GC-FID on 60th day**	**GC-FID on 20th day**	**GC-FID on 40th day**	**GC-FID on 60th day**
Substrate/ VFA (ppm)	CRW	CRW	CRW	HFS	HFS	HFS
Acetic	-	5.32	8.52	-	24.07	5.61
Phosphonic	15.50	64.03	14.53	55.76	15.58	6.08
Butyric	30.58	61.80	1.88	21.05	51.87	27.05
Isobutyric	233.36	12.78	3.15	9.23	12.68	5.91
Valeric	183.96	15.16	18.85	174.30	5.18	8.24
Isovaleric	165.14	73.19	3.06	177.58	2.33	7.77
Hexanoic	72.09	1.39	19.81	45.02	2.08	3.22
Capronic	45.17	138.86	16.51	26.47	19.84	14.41

Appendix 5: Volumes of long chain fatty acids from cow rumen waste and human fecal slurry at the 20th and 60th day

CRW	LCFA on the 20th day	LCFA on the 60th day	HFS	LCFA on the 20th day	LCFA on the 60th day
C12	8.297	-	C12	-	2.37
C14	5.995	1.578	C14	-	1.80
C16	27.323	7.295	C16	36.127	1.25
C18	-	1.398	C18	2.697	5.67
C18:1	14.894	1.293	**C18:1**	21.080	3.11
C18:2	4.668	17.067	**C18:2**	11.006	7.41
C18:3	10.161	3.663	**C18:3**	15.100	4.82
C20	1.317	5.416	C20	1.132	5.03
C20:3	5.843	4.265	**C20:3**	3.216	-
C20:4	6.370	2.328	**C20:4**	3.148	4.56
C20:5	6.711	3.950	**C20:5**	1.250	-
C22	3.048	-	C22	2.296	3.93
C22:4	3.150	4.078	**C22:4**	2.899	4.99
C22:5	8.177	1.178	**C22:5**	4.813	-
C22:6	5.097	6.990	**C22:6**	8.521	-

KEY: Boldened LCFA codes are unsaturated LCFAs

Appendix 6: GC-MS result in % Area on biogas composition generated in both substrates after a 60-day retention time

Substrate	**Cow rumen waste**	**Human fecal slurry**
Methane	50. 39	57.99
Carbon-dioxide	19.21	17.21
Carbon-monoxide	3.84	5.35
Hydrogen Sulphide	6.52	1.80
Ammonia	5.33	-
Sulphur Oxide	-	1.14

Appendix 7: Values of pH and total viable count (TVC) of both substrates after a 60-day retention time

	Cow rumen waste (CRW)					Human fecal slurry (HFS)	
Retention time (Days)	pH Value	Biogas yield (ml)	Total viable count (cfu/ml) $x10^7$	Retention time (Days)	Biogas yield (ml)	pH Value	Total viable count (cfu/ml) $x10^7$
3	8.1	12.561	164	3	10.468	8.1	90
6	7.9	31.403	142	6	27.216	8	86
9	7.4	41.871	143	9	41.871	7.8	71
12	7.1	83.742	103	12	56.526	7.9	69
15	6.5	100.490	99	15	69.087	7.4	65
18	5.8	108.865	81	18	81.648	7.21	62
21	5	117.239	66	21	92.116	7.05	56
24	5.08	142.362	66	24	113.052	6.72	56
27	5.19	157.016	65	27	127.707	6.27	52
30	4.92	177.951	63	30	146.549	6.7	49
33	5	192.607	69	33	159.110	7.05	47
36	5.08	200.981	68	36	171.671	6.72	31
39	5.19	217.729	70	39	188.420	6.27	29
42	4.92	247.039	65	42	209.355	6.7	28
45	4.82	251.226	62	45	221.916	6.57	26
48	4.85	215.636	62	48	226.103	6.73	30
51	4.87	205.168	60	51	200.981	6.94	32
54	4.76	198.887	56	54	177.952	6.86	30
57	4.65	188.420	56	57	133.987	6.85	30
60	4.61	167.016	42	60	125.613	6.80	28

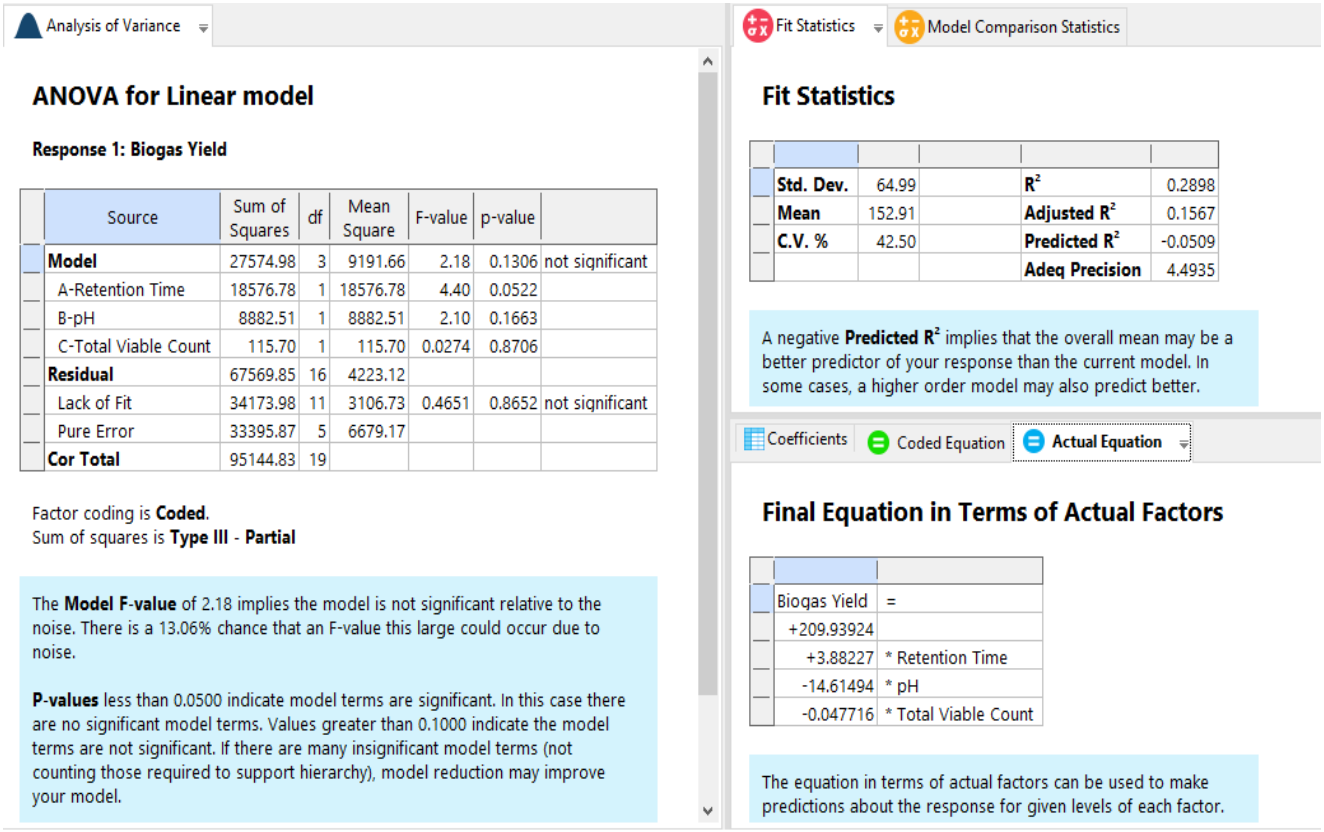
Appendix 8: CCD Matrix for three variables with actual biogas production for cow rumen waste

Run	Factor 1 A:Retention Time Days	Factor 2 B:pH	Factor 3 C:Total Viable Count cfu/ml	Response 1 Biogas Yield ml
1	20	4.61	164	12.561
2	26.477	6.355	103	31.403
3	20	8.1	164	41.871
4	10.5	3.42027	103	83.742
5	10.5	6.355	103	100.49
6	10.5	6.355	103	108.865
7	1	4.61	42	117.239
8	10.5	6.355	103	142.362
9	10.5	6.355	103	157.016
10	1	8.1	42	177.951
11	1	4.61	164	192.607
12	10.5	6.355	0.410637	200.981
13	10.5	9.28973	103	217.729
14	10.5	6.355	205.589	247.039
15	20	4.61	42	251.226
16	10.5	6.355	103	215.636
17	10.5	6.355	103	205.168
18	20	8.1	42	198.887
19	1	8.1	164	188.42
20	-5.47703	6.355	103	167.016

Appendix 9: CCD Matrix for three variables with actual biogas production for human fecal slurry

Run	Factor 1 A:Retention Time Days	Factor 2 B:pH	Factor 3 C:Total Viable Count cfu/ml	Response 1 Biogas Yield ml
1	31.5	7.45	111.136	10.468
2	31.5	7.45	59	27.216
3	60	8.1	28	41.871
4	31.5	7.45	59	56.526
5	31.5	6.35683	59	69.087
6	3	8.1	28	81.648
7	31.5	7.45	6.86442	92.116
8	79.4311	7.45	59	113.052
9	3	8.1	90	127.707
10	3	6.8	28	146.549
11	31.5	7.45	59	159.11
12	60	6.8	28	171.671
13	31.5	7.45	59	188.42
14	31.5	7.45	59	209.355
15	31.5	8.54317	59	221.916
16	60	8.1	90	226.103
17	-16.4311	7.45	59	200.981
18	3	6.8	90	177.952
19	60	6.8	90	133.987
20	31.5	7.45	59	125.613

Appendix 10: ANOVA on variables from cow rumen waste substrate



Analysis of Variance

ANOVA for Linear model

Response 1: Biogas Yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	27574.98	3	9191.66	2.18	0.1306	not significant
A-Retention Time	18576.78	1	18576.78	4.40	0.0522	
B-pH	8882.51	1	8882.51	2.10	0.1663	
C-Total Viable Count	115.70	1	115.70	0.0274	0.8706	
Residual	67569.85	16	4223.12			
Lack of Fit	34173.98	11	3106.73	0.4651	0.8652	not significant
Pure Error	33395.87	5	6679.17			
Cor Total	95144.83	19				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 2.18 implies the model is not significant relative to the noise. There is a 13.06% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case there are no significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Fit Statistics | Model Comparison Statistics

Fit Statistics

Std. Dev.	64.99	**R^2**	0.2898
Mean	152.91	**Adjusted R^2**	0.1567
C.V. %	42.50	**Predicted R^2**	-0.0509
		Adeq Precision	4.4935

A negative **Predicted R^2** implies that the overall mean may be a better predictor of your response than the current model. In some cases, a higher order model may also predict better.

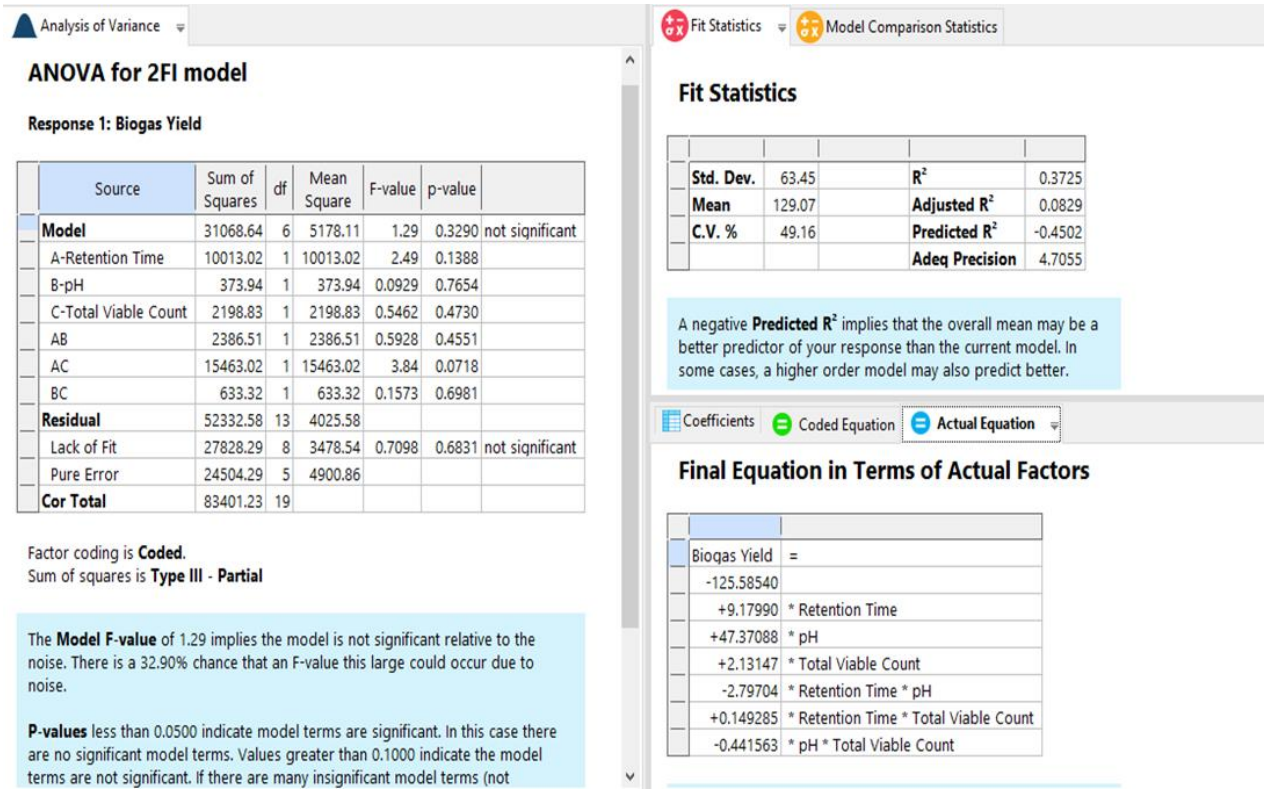
Coefficients | Coded Equation | **Actual Equation**

Final Equation in Terms of Actual Factors

Biogas Yield	=
+209.93924	
+3.88227	* Retention Time
-14.61494	* pH
-0.047716	* Total Viable Count

The equation in terms of actual factors can be used to make predictions about the response for given levels of each factor.

Appendix 11: ANOVA on variables from human fecal slurry substrate



Analysis of Variance

ANOVA for 2FI model

Response 1: Biogas Yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	31068.64	6	5178.11	1.29	0.3290	not significant
A-Retention Time	10013.02	1	10013.02	2.49	0.1388	
B-pH	373.94	1	373.94	0.0929	0.7654	
C-Total Viable Count	2198.83	1	2198.83	0.5462	0.4730	
AB	2386.51	1	2386.51	0.5928	0.4551	
AC	15463.02	1	15463.02	3.84	0.0718	
BC	633.32	1	633.32	0.1573	0.6981	
Residual	52332.58	13	4025.58			
Lack of Fit	27828.29	8	3478.54	0.7098	0.6831	not significant
Pure Error	24504.29	5	4900.86			
Cor Total	83401.23	19				

Factor coding is **Coded**.
Sum of squares is **Type III - Partial**

The **Model F-value** of 1.29 implies the model is not significant relative to the noise. There is a 32.90% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case there are no significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not

Fit Statistics | Model Comparison Statistics

Fit Statistics

Std. Dev.	63.45		**R^2**	0.3725
Mean	129.07		**Adjusted R^2**	0.0829
C.V. %	49.16		**Predicted R^2**	-0.4502
			Adeq Precision	4.7055

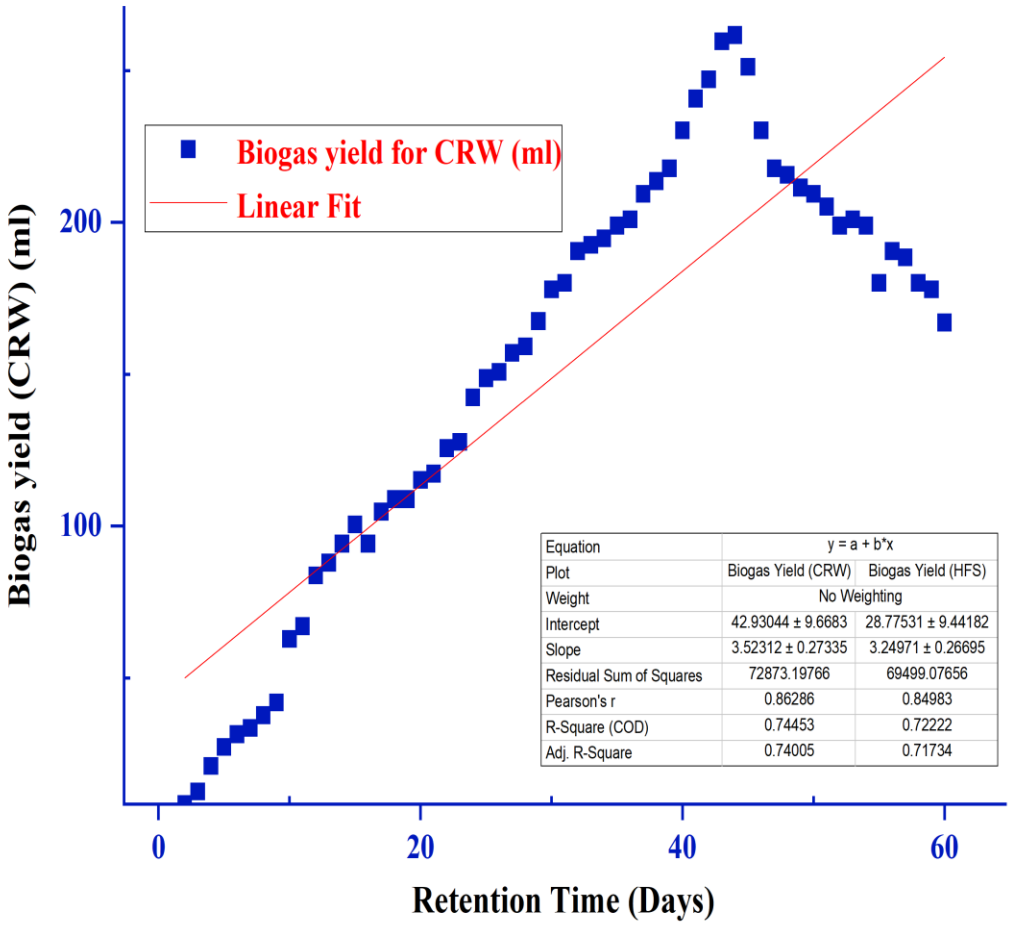
A negative **Predicted R^2** implies that the overall mean may be a better predictor of your response than the current model. In some cases, a higher order model may also predict better.

Coefficients | Coded Equation | Actual Equation

Final Equation in Terms of Actual Factors

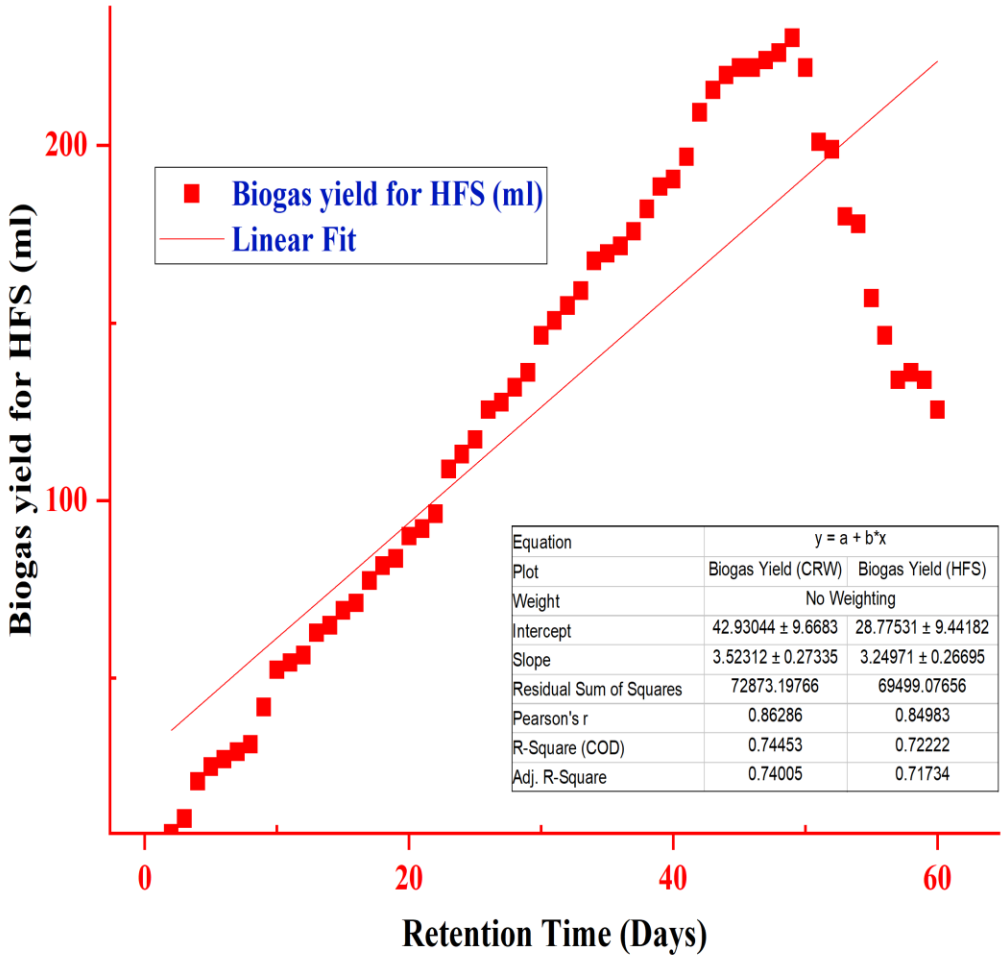
Biogas Yield	=
-125.58540	
+9.17990	* Retention Time
+47.37088	* pH
+2.13147	* Total Viable Count
-2.79704	* Retention Time * pH
+0.149285	* Retention Time * Total Viable Count
-0.441563	* pH * Total Viable Count

Appendix 12: Linear fit for biogas yield for cow rumen waste



Equation	y = a + b*x	
Plot	Biogas Yield (CRW)	Biogas Yield (HFS)
Weight	No Weighting	
Intercept	42.93044 ± 9.6683	28.77531 ± 9.44182
Slope	3.52312 ± 0.27335	3.24971 ± 0.26695
Residual Sum of Squares	72873.19766	69499.07656
Pearson's r	0.86286	0.84983
R-Square (COD)	0.74453	0.72222
Adj. R-Square	0.74005	0.71734

Appendix 13: Linear fit for biogas yield for human fecal slurry



Equation	y = a + b*x	
Plot	Biogas Yield (CRW)	Biogas Yield (HFS)
Weight	No Weighting	
Intercept	42.93044 ± 9.6683	28.77531 ± 9.44182
Slope	3.52312 ± 0.27335	3.24971 ± 0.26695
Residual Sum of Squares	72873.19766	69499.07656
Pearson's r	0.86286	0.84983
R-Square (COD)	0.74453	0.72222
Adj. R-Square	0.74005	0.71734

Appendix 14: One way ANOVA report

ANOVAOneWay (6/14/2024 20:28:00)

Notes

Description	Perform One-Way ANOVA
User Name	NKEMKA
Operation Time	6/14/2024 20:28:00
Report Status	New Analysis Report
Data Filter	No

Input Data

	Data	Range
Cow Rumen Waste BGY	[Book1]Sheet1!B"Biogas Yield"	[1*:60*]
Human fecal slurry BGY	[Book1]Sheet1!C"Biogas Yield"	[1*:60*]

There exist missing values in the input data.

Descriptive Statistics

	N Analysis	N Missing	Mean	Standard Deviation	SE of Mean
Biogas Yield	59	1	152.14708	70.12902	9.13002
	59	1	129.51629	65.6783	8.55059

ANOVA

Overall ANOVA

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	1	15108.51218	15108.51218	3.27317	0.07301
Error	116	535439.7452	4615.85987		
Total	117	550548.25738			

Null Hypothesis: The means of all levels are equal.
Alternative Hypothesis: The means of one or more levels are different.
At the 0.05 level, the population means are not significantly different.

Fit Statistics

R-Square	Coeff Var	Root MSE	Data Mean
0.02744	0.48242	67.94012	140.83169

Means Comparisons

Bonferroni Test

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
Human fecal slurry BGY Cow Rumen Waste BGY	-22.6308	12.50879	-1.80919	0.07301	0.05	0	-47.40603	2.14444

Tukey Test

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Human fecal slurry BGY Cow Rumen Waste BGY	-22.6308	12.50879	2.55858	0.07301	0.05	0	-47.40604	2.14445

Sidak Test

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
Human fecal slurry BGY Cow Rumen Waste BGY	-22.6308	12.50879	-1.80919	0.07301	0.05	0	-47.40603	2.14444

Fisher Test

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
Human fecal slurry BGY Cow Rumen Waste BGY	-22.6308	12.50879	-1.80919	0.07301	0.05	0	-47.40603	2.14444

Grouping Letters Table

Bonferroni Test

	Mean	Groups
Cow Rumen Waste BGY	152.14708	A
Human fecal slurry BGY	129.51629	A

Means that do not share a letter are significantly different.

Tukey Test

	Mean	Groups
Cow Rumen Waste BGY	152.14708	A
Human fecal slurry BGY	129.51629	A

Means that do not share a letter are significantly different.

Sidak Test

	Mean	Groups
Cow Rumen Waste BGY	152.14708	A
Human fecal slurry BGY	129.51629	A

Means that do not share a letter are significantly different.

Fisher Test

	Mean	Groups
Cow Rumen Waste BGY	152.14708	A
Human fecal slurry BGY	129.51629	A

Means that do not share a letter are significantly different.

Sig equals 1 indicates that the difference of the means is significant at the 0.05 level.
Sig equals 0 indicates that the difference of the means is not significant at the 0.05 level.

Homogeneity of Variance Test

Levene's Test(Absolute Deviations)

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	1	457.87007	457.87007	0.36157	0.54881
Error	116	146894.21887	1266.32947		

At the 0.05 level, the population variances are not significantly different.

Appendix 15: Procedures for media preparation

Peptone Water

3.75g of peptone water media was measured and dissolved in 250mls of water and swirled properly in order to dissolve well. 9mls was pipetted into 12 sterile test tubes to perform a serial dilution of the samples.

Nutrient Agar

28g of Nutrient Agar was measured and suspended in 1000mls of distilled water in a conical flask and heated properly on a Bunsen burner to dissolve. The agar media was sterilized using autoclaving at 121^{o}C at 15psi for 15mins. After autoclaving the agar was allowed to cool and then poured into sterile petri dishes and allowed to solidify.

MacConkey Agar

56g of MacConkey Agar was measured and suspended in 1000mls of distilled water in a conical flask and heated properly on a Bunsen burner to dissolve. The agar media was sterilized using autoclaving at 121^{o}C at 15psi for 15mins. After autoclaving the agar was allowed to cool and then poured into sterile petri dishes and allowed to solidify.

Sabouraud Dextrose Agar

15.5g of Sabouraud Dextrose Agar was measured and suspended in 250mls of distilled water in a conical flask and heated properly on a Bunsen burner to dissolve. The agar media was sterilized using autoclaving at 121^{o}C at 15psi for 15mins. After autoclaving the agar was allowed to cool and 2mls of antibiotics was added to inhibit bacterial growth on the plates; then it was poured into sterile petri dishes and allowed to solidify.

Brain heart infusion agar (BHIA) agar

56g of Brain heart infusion Agar was measured and suspended in 500mls of distilled water in a conical flask and heated properly on a Bunsen burner to dissolve. The agar media was sterilized using autoclaving at 121^{o}C at 15psi for 15mins. After autoclaving the agar was allowed to cool and then poured into sterile petri dishes and allowed to solidify.