Optimization of Biogas Production from Abattoir Waste by Bioaugmentation

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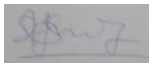
Sign:  Date 20/10/2022

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DEDICATION

I dedicate this work to my loving parents, the late Mr. Patrick M. Makokha and Mrs. Gladys N. Makokha, for their endless guidance and support, and to my siblings for their inspiring pieces of advice.

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ABSTRACT

Biogas production from abattoir waste using conventional technology is a very slow and inefficient process. The process can be improved by use of bioaugmentation and optimisation of operating parameters. The general objective of this study was to optimize biogas production from abattoir waste through bioaugmentation. The specific objectives were to: evaluate composition of abattoir waste, characterize physiochemical properties, analyze effects of using rumen inoculum and bioaugmentation utilizing *Bacillus subtilis* and *Escherichia coli* on biogas yield and composition and establish optimum temperature, percentage of rumen inoculum and hydraulic retention time for biogas production from mixed abattoir waste. Biogas was produced in 250ml flask reactors from mixed abattoir waste using 0, 20 and 50% rumen inoculum based on volume ratio at 35 ℃ for 30 days. The most effective percentage was applied in the second experiment that produced biogas at 37 ℃ in 25 days in 500ml flask reactors using *Bacillus subtilis*, *Escherichia coli*, *Bacillus subtilis* + *Escherichia coli* mixture for bioaugmentation and control experiment without any bioaugmentation. Box-Behnken design was used to optimize temperature, percent rumen inoculum and hydraulic retention time for biogas production from mixed abattoir waste in the last experiment. Analysis of the mean biogas volume, biogas potential, methane content and depletion of total solids and chemical oxygen demand were used to determine the most effective set up while response surface methodology was applied in determining optimum conditions for the Box-Behnken designed experiment. Rumen inoculum of 20 and 50% (v/v) achieve a significant increase in biogas potential and methane content over use of 0% rumen inoculum. Rumen inoculum of 20 and 50% achieve production potentials of 0.068 and 0.069 ml/mgTS with

methane content being 54.13 and 60.12% respectively compared to 0% rumen inoculum which attains a potential and methane content of 0.052 ml/mgTS and 48.91% respectively. Similarly, combining *Bacillus subtilis* and *Escherichia coli* significantly improves biogas yield and methane content compared to when each microbe is used separately during digestion of abattoir waste. The combined power of the microbes achieves a potential of 0.083 ml/mgTS and a methane content of 66.92%. Separately *E.coli* and *Bacillus subtilis* achieve a potential of 0.077 and 0.076 ml/mgTS and methane content of 62.71 and 62.24% respectively. Optimum levels of temperature, percent rumen inoculum and hydraulic retention are 37.93 ℃, 70.45% and 16.28 days respectively. This study recommends optimisation of biogas production from abattoir waste using a combination of *E.coli*, *Bacillus subtilis* and 70.45% rumen inoculum at temperature of 37.93℃.

Key words: Abattoir waste, Bioaugmentation, Biogas production, Optimisation

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ABBREVIATIONS

AD	Anaerobic digestion
AH	Alkaline hydrolysis
Ag_2SO_4	Silver sulfate
ANOVA	Analysis of variance
APHA	American public association
B. subtilis	*Bacillus subtilis*
BOD	Biological oxygen demand
BW	Blood waste
C3 and C4	Molecule with three and four carbons in the chain
C/N	Carbon/nitrogen ratio
C/N/P	Carbon/Nitrogen/Phosphorous Ratio
COD	Chemical oxygen demand
DO	Dissolved oxygen
DR-sol	Digestion reagent solution
EC	European commission
E. coli	*Escherichia coli*
FID	Flame ionization detector
G	Grams
H_2SO_4	Sulfuric acid
$HOOCC_6H_4COOK$	Potassium hydrogen phthalate

HRT	Hydraulic retention time
IMViC	Indole, methyl red, Voges-Proskauer, citrate utilization test
I/S	Inoculum/substrate ratio
IW	Intestinal waste
$K_2Cr_2O_9$	Potassium dichromate
KOH	Potassium hydroxide
$K_2S_2O_8$	Potassium Peroxidisulphate
KPH	Potassium hydrogen Phthalate
Ksh	Kenya shillings
Lbs	Pound unit mass
M	Molar solution
Mg	Milligram
mgL^{-1}	Milligram per litre
Ml/mgTS	Milliliter per milligram total solids
MgO_2/L	Milligram oxygen per litre
MgO_2/mg	Milligram oxygen per milligram
MW	Mixed waste
Nm	Nano Metre
^oC	Degree Celsius
PBS	Phosphate Buffered Saline fat
PMWR	Production Mixed Waste Ratio

pH	Hydrogen potential
PR	Percent Rumen fluid inoculum
RI	Rumen Inoculum
RO	Reverse Osmosis
RSM	Response Surface Method
RT	Retention Time
RW	Rumen Waste
SRT	Solids Retention Time
T	Temperature
TDS	Total Dissolved Solids
TN	Total Nitrogen
TOC	Total Organic Carbon
TP	Total Protein
TS	Total Solids
TSS	Total Suspended Solids
UN	United Nations
UV	Ultra violet
V/V	Volume per Volume
WW	Waste Water
W/W	Weight per Weight

CHAPTER 1. INTRODUCTION

1.0 Background information

Food security is a goal that is in line with achieving a better and more sustainable future for all (The UN general assembly, 2015). The supply of adequate meat products from abattoir facilities has a corresponding increase in the generated wastes. According to Adeyemi and Adeyemo, (2006), the major wastes generated at abattoirs are: blood from the slaughtering process, rumen solids, intestinal, wastes and wash water. The wastes not only contain pathogens that might cause disease outbreak but also cause both water and air pollution and therefore ought to be disposed sustainably. Biogas production through anaerobic digestion has the advantage of producing clean energy, organic fertilizer and reduction of both pathogens and odour hence a better option of disposing the waste. Abattoir waste has high biogas potential. However, the low carbon/nitrogen (C/N) ratio associated with the waste reduces this potential considerably (Klintenberg, Jamieson, Kinyaga & Odlare, 2014).

Bioaugmentation involves the addition of archaea and bacteria communities with stable metabolic pathways to a natural system such as fermentation to increase the speed and efficiency of the process (Ács et al., 2015; Bagi et al., 2007; Hubman and Plowman, 1997; Kovacs et al., 2013; Kovacs et al., 2015). This study is based on the application of bioaugmentation to improve biogas production from the protein rich abattoir waste.

The four steps which take place in anaerobic digestion (AD) process by natural consortia in order are hydrolysis, acidogenesis, acetogenesis and methanogenesis (Wang, 2014). Hydrolysis step is carried out by facultative anaerobes and the obligates which make

particulates soluble and break down large molecules (polymers) that include carbohydrates, fats and proteins into smaller molecules (monomers) allowing passage through bacterial cell wall (Ranalli, 2007). According to Adekunle and Okolie (2015), hydrolysis step becomes rate limiting if there is high concentration of large particulate organic matter (protein) in the system that may lead to the formation of toxic by-products. Abattoir wastes are characterized by high concentration of carbohydrates (mainly in the form of lactose), protein and lipids (Selormey, Barnes, Kemausnor and Darkwah, 2021). Protein digestion can be improved by addition of bacterial cultures with protein degrading abilities such as *Bacillus subtilis* whereas extremely large particles can be reduced physically by grinding (Mshandete, Bjornsson, Kivaisi, Rubindamayugi and Maltiasson, 2006; Kovacs et al., 2015). In acidogenesis sugars, amino acids and fatty acids produced in hydrolysis are converted to C_3 and C_4 volatile fatty acids (76%), H_2 (4%) and acetic acid (20%) where accumulation of fatty acids slightly consume alkalinity whereas ammonia gas (NH_3) may be produced from amino acids (Chen, Cheng and Creamer, 2008; Ranalli, 2007). The acetogenesis step converts volatile fatty acids from acidogenesis step to acetic acid (68%) and H_2 (32%) (Goethe University Frankfurt Release, 2016; Ranalli, 2007). High concentrations of ammonia, hydrogen sulphide and long chain fatty acids are major inhibitors of methanogenic activities and are known to be main cause for digester upsets. Ammonia and hydrogen sulphide are released from degradation of amino acids during acidogenesis and at elevated concentrations are toxic for different microorganisms in particular methanogens (Moestedt, Muller, Weslerholon and Schnurer, 2016). The biogas bacteria involved are known to be sensitive to increasing hydrogen (H_2) concentration and stop functioning at high concentrations (Bagi

et al., 2007; Chen, Cheng and Creamer, 2008; Ranalli, 2007). In methanogenesis step, obligate anaerobes (methanogens) which have the slowest growth rate form methane at pH range 6.7 to 7.4 and work well at optimum temperatures (35-40℃) (Ranalli, 2007). According to Chen, Cheng and Creamer (2008), an increase in concentration of hydrogen due to acetogenesis lowers the pH to acidic level which inhibits methane formation. Therefore, methanogenesis becomes rate limiting for easy biodegradable substance (C/N ratio between 25/1 to 30/1) (Ranalli, 2007; Adekunle and Okolie, 2015). A syntrophic relationship between acetogenesis and methanogenesis arise from the fact that methanogens remove H_2 from the system to produce methane (Schink, 1997). This causes the revival of acetogens that consume the volatile fatty acids from the system hence increasing the pH to neutral for continued biogas production (Brynt, Wolin, Wolin and Wolfe, 1967; Ranalli 2007). It is possible to control the pH of the system by artificial addition of acids or alkali solutions to maintain the robust pH range of Methanogens. Addition of tolerant hydrogen generating bacterial species is desirable to sustain acetogenesis (Ács et al., 2015; Bagi et al., 2007; Merlyn, Christy, Gopinath and Divya, 2014). *Escherichia coli* can be used in augmenting hydrogen generation leading to a stable anaerobic digestion (AD) process (Zang, Lv, Xing, 2011). This is required to achieve complete digestion corresponding to high biogas production with increased methane content for easy biodegradable substrates. several publications suggest that methanogenesis step follows a special route (Archaea 2018; Chapelle et al. 2002; Demirel, Scherer, Yenigun and Onay 2010; Ranalli 2007) which includes acetotrophic methanogens breaking down acetate to biogas, hydrogenotrophic methanogens Consuming carbon dioxide and hydrogen to release methane and water, then

methylotrophic methanogens Combining alcohols and hydrogen to release methane and water.

In decomposing organic wastes with ideal C/N ratio using natural consortia, hydrolysis and acidogenesis steps are robust (Kovacs et al., 2015). On the other hand, the acetogenesis step is sensitive to increasing hydrogen and thus is self-inhibiting to stop the four step fermentation process (Chen, Cheng and Creamer, 2008). The methanogenesis counterparts are sensitive to acidic pH caused by accumulation of fatty acids (Ranalli, 2007). Some methanogens use hydrogen, carbon dioxide or alcohols as substrate to form methane (Gerardi, 2003; Ranalli, 2007; Brynt, Wolin E., Wolin M. and Wolfe, 2014). The methane formation routes by methanogens suggest that a steady and fast hydrogen generation by acetogens in a balanced system is a solution to maximizing waste decomposition and therefore efficient biogas generation that contains higher methane content in a continuous AD process (Kovacs et al., 2013; Adekunle and Okolie, 2015). This imply that augmentation for hydrogen generation can provide a better system speed and efficiency for biogas production as sufficient hydrogen can now be produced from acetogenesis for use by hydrogenotrophic and methylotrophic bacteria to generate methane.

Rumen inoculum has microorganisms adapted to biogas production and may assist in raising the C/N ratio to acceptable level of 30/1. Complete digestion of the waste implied by Gerardi (2003) with a corresponding high biogas and methane yield can be achieved sustainably.

The research was motivated by failure of the current anaerobic digestion process to handle 100% of the daily generated abattoir waste, low conversion of the processed waste into biogas that has poor methane content on using rumen inoculum, protein degrading bacteria and hydrogen producing bacteria singly as well as the need to establish optimum levels of temperature, percentage rumen inoculum and hydraulic retention time that are absent in literature at this point in time.

The aim of this study was to demonstrate through experiments that rumen inoculum, *Bacillus subtilis* and *Escherichia coli* can be used in overcoming inhibitions and optimize the process of biogas production from abattoir waste singly and that the effect could be better when the three are applied together. The applicable optimum levels of temperature, percentage rumen inoculum and hydraulic retention time were determined using Box-Behnken experiment and response surface methodology.

1.1 Statement of the problem

Ideally, biogas production from the abundant energy-rich abattoir waste is expected to meet most of the renewable demands globally. Currently, utilization of abattoir waste is limited because it is slow, inefficient and often encounters inhibitions that can result to complete failure. A slow or inefficient process requires a large digester to handle small quantities of waste. A failed digester on the other hand has to be restarted implying time wastage. Moreover, there is a low gas output with poor methane content resulting from the high Carbon/Nitrogen (C/N) ratio. This is a problem because the anticipation by researchers that anaerobic digestion could be used appropriately to dispose of the entire abattoir waste from the world with the benefit of releasing renewable energy can no longer be relied upon. The process of anaerobic digestion just handles a very small

fraction leaving behind the bulk of it. More so, the small fraction of the waste handled in anaerobic digesters hardly undergo full degradation meaning the digestate is still a source of pollution once released to the environment. The biogas released from digesters contain low methane due to presence of unwanted impurities such as hydrogen sulphide and ammonia causing it to be of poor quality.

This research seeks to use bioaugmentation involving rumen inoculum, *Bacillus subtilis* and *Escherichia coli* to achieve optimisation of biogas production from the anaerobic digestion of abattoir waste. Levels of temperature, percentage of rumen inoculum and hydraulic retention time necessary for the optimisation are key and ought to be determined.

1.2 Justification of the study

This research is intended to benefit consumers of biogas as the sole form of renewable energy, abattoir operators, farmers and the general public. A faster and complete digestion makes anaerobic digestion a safe and reliable method for disposing abattoir waste. There will be reduction of odors and related pollution problems at the will be minimized at sites as the fully digested waste is now safe for application on farms as manure. More importantly, optimization ensures the use of small digesters in handling large throughputs in relatively shorter retention times implying that up to 100% of the waste generated can be processed. The biogas made available to consumers in the long run will be of high quality as desired.

1.3 Significance

Whereas *Bacillus subtilis* and *Escherichia coli* have been used singly in previous research work to optimize biogas production from protein rich abattoir waste, results of their use in coaugmentation have not yet been reported. Similarly, rumen inoculum has been shown to improve biogas production from abattoir waste though the optimum levels of temperature, percent rumen inoculum and hydraulic retention time for operation are yet to be determined. This study was motivated by the need to optimize biogas production from abattoir waste through bioaugmentation involving combined application of *Bacillus subtilis* and *Escherichia coli* as well as determine optimum levels of temperature, percentage of rumen inoculum and the hydraulic retention time necessary for an optimized process.

1.4 Research objectives

1.4.1 General Objective

To optimize biogas production from abattoir waste through bioaugmentation.

1.4.2 Specific objectives

The specific objectives were to: evaluate composition of abattoir waste, characterize physicochemical properties, analyze the effects of using rumen inoculum and bioaugmentation utilizing *Bacillus subtilis* and *Escherichia coli* on biogas yield and composition and finally to establish the optimum operating conditions of temperature, percentage rumen inoculum and hydraulic retention time for biogas production from abattoir waste.

1.5 Scope of the research

Batch system under mesophilic conditions was used for investigations. *Bacillus subtilis* and *Escherichia coli* bacterial species were applied for bioaugmentation. The choice of the bacterial species was based on ease of cultivation conditions and fast growth on inexpensive substrates, metabolic activity and the survival capability in the anaerobic digestion community. *Bacillus subtilis* was isolated from garden soils near plant roots and with decaying pieces of wood while *Escherichia coli* was from Moi University raw sewage. The key factors influencing this process include temperature, percentage of rumen inoculum and hydraulic retention time. The applicable levels of these factors can be achieved on a laboratory scale.

CHAPTER 2. LITERATURE REVIEW

Biogas production offers an opportunity to convert organic waste into renewable energy resource through which rising demand for energy can be met (IEA, 2020). The increasing demand for alternative energy sources makes biogas production from the rich organic abattoir waste necessary. Kenya has 63 abattoirs and a single facility is capable of producing up to 6000 kg of waste daily from which one tonne of animal waste can produce 100 cubic metres of biogas (Gachie 2020; Kabeyi and Olanrewaju, 2020). Less than 10 abattoirs in Kenya have installed biodigesters and an installed biodigester has been reported to process less than 7.5% of the waste generated daily at the site (Kabeyi and Olanrewaju, 2020). There is the need to improve the anaerobic digestion process so as to handle 100% of the daily abattoir waste. The main challenge is that anaerobic digestion of abattoir waste encounters inhibition leading to low yield of biogas and methane content. Process optimization is required to overcome inhibition and realize high biogas yield. Klintenberg et al. 2014 reported enhancement of biogas production from abattoir waste using rumen inoculum. The optimum value of rumen inoculum has not been mentioned in literature so far. Effectiveness of bioaugmentation utilizing *Bacillus subtilis* and *Escherichia coli* singly has been reported in literature (Bagi et al. 2007; Budiyono et al. 2009; Budiyono et al. 2014; Ács et al 2015; Kovacs et al. 2015). There is need to investigate effect of simultaneous use of the two bacterial species in combination with rumen inoculum in digestion of mixed abattoir waste. Once confirmed to produce superior outcome, the use of rumen inoculum and bioaugmentation involving protein synthesis and hydrogen generation could be applied in optimizing the process. Simultaneous augmentation for protein degradation and hydrogen generation is thought

of as a way for maintaining the delicate balance required by the acid forming and methane forming microorganisms leading to reactor stability. Rumen fluid has sufficient microbes that fasten hydrolysis and methanation. Use of rumen inoculum and right temperature is anticipated to fasten the rate of anaerobic digestion and thus its hydraulic retention time. Hydraulic retention time is proportional to size of digesters and hence any reduction in the hydraulic time corresponds to a reduction in size of the digester. It is worth noting that prolonged digestion at high temperatures is responsible for production of inhibition agents such as ammonia and hydrogen sulphide. It therefore means that reducing hydraulic retention time will assist in achieving a reduction in size of digester and attainment of desired process stability.

2.1 Assessment of abattoir effluent waste and use in anaerobic digestion

In Kenya cattle, sheep, goat, pigs and poultry are slaughtered to supply meat to the domestic and export market. Donkey meat and skin are processed at the Goldox abattoir for the Chinese market. The portion of each cow, sheep/goat, pig and chicken consumed by human beings is 52%, 60-62%, 68-72% and 78% respectively with the remainder being wastes. These wastes increase with the population and growth of the cities (Adeyemi and Adeyemo, 2007). The liquid waste streams generated in abattoirs cause significant impact towards pollution. They include blood, soft oval, rumen and wash water (Twumasi, Kwang, Boakye, Agyel-Frimpong, and Oppong-Twum, 2016). Abattoir operations produce characteristic high organic waste with relatively high levels of suspended solids, total solids, dissolved oxygen, biological oxygen demand (BOD), chemical oxygen demand (COD), proteins and fat (Adeyemi and Adeyemo, 2006; Omole and Longe, 2008; Budiyono, Johari and Sunarso, 2011). The high organic and moisture

content of abattoir effluent make the waste well suited for anaerobic digestion (Adeyemi and Adeyemo, 2006; Budiyono et al 2011). Budiyono et al (2011) gives chemical properties of abattoir wastes which suggest a highly concentrated waste water comprising of 45% soluble and 55% suspended organic composition. Further, blood being one of the major dissolved pollutants has a very high chemical oxygen demand (COD).Therefore, releasing abattoir wastewater to water bodies can increase BOD, COD, total solids, pH, temperature and turbidity. The amount of oxygen taken up by the microorganisms that decompose the organic matter in waste water is known as biological oxygen demand or biochemical oxygen demand while that required to completely oxidize chemical compounds present is referred to us the chemical oxygen demand (Ankur[a], 2008). It is used to measure the amount of organic water pollution. Biological oxygen demand is calculated using a sample of water containing a known amount of oxygen at 20 ℃. The oxygen content is measured again and BOD calculated using the modified Winkler method (Ankur[b], 2008). The higher the BOD, the more the pollution. The high concentration of suspended organic solids include pieces of fat, grease, hair, feathers, manure, grit and undigested food that will contribute to the slow biodegradable organic matter (Adesemoye, 2006).

Biogas production from abattoir waste has been enhanced using rumen inoculum and bioaugmentation utilizing *Bacillus subtilis* and *Escherichia coli* singly (Bagi et al. 2007; Budiyono et al. 2009; Budiyono et al. 2014; Klintenberg et al. 2014; Ács et al 2015; Kovacs et al. 2015). There is little information on bioaugmentation using mixed species of *Bacillus subtilis* and *Escherichia coli* and the optimum applicable percentage of rumen inoculum.

2.2 Microorganisms in abattoir waste

Abattoir waste water liquid is unpleasant to the surrounding communities and is highly contaminated with microbes (Adesemoye, 2006). Fransen, Elzen, Urlings, and Bijker (1996) reported that flocculated and aerobically activated sludge contain *enterobacteriaceae*, *enterococci*, *clostridium*, *salmonella*. *Yersinia enterolytica* serotypes O: 3 and O: 9 are found in sludge from most slaughterhouses. The prevalence of C*ampylobacter jejuni/coli* is prevalent in flocculated poultry sludge. Tomley and Shirley (2009) classified animal disease pathogens into bacteria, protozoa, fungi and viruses These microbes also affect humans especially people who have close contact with large numbers of animals, animal products, byproducts and waste products such as farmers, abattoir workers, shearers, knackery workers and veterinarians. Zoonotic diseases such as anthrax, bovine fever, tuberculosis and meningitis among others may be contracted from both ill and apparently healthy animals, animal products, by products and wastes. Fransen et al. (1996) suggests mandatory decontamination of sludge for further handling and use to prevent pathogen cycles from occurring in livestock as well as humans. Low temperature anaerobic treatment is efficient in reducing viable populations of indicator microorganisms (total *coliforms*, *Escherichia coli*) and selected pathogens (*Salmonella, Yersinia enterocolitica, cryptosporidium and Giardia*). Anaerobic digestion for biogas generation has the potential to reduce pathogen loadings to the environment (Avery, Yongabi, Tumwesige, Strachan and Goude, 2014). Anaerobic digestion of swine manure slurry at 20 ℃ for 20 days has been used to reduce indigenous populations of total *Coliforms* by 97.94-100%, *E.coli* by 99.67-100%, *Salmonella, cryptosporidium and Giardia* to undetectable levels (Cote, Masse and Quessy 2006). Poudel, Joshi, Raj

Dhakal, and Bahadur Karki (2010) demonstrated that anaerobic digestion can achieve a significant reduction of pathogenic microorganisms in sewage sludge mixture.

The rumen is a complex ecosystem composed of anaerobic bacteria, protozoa, fungi, methanogenic archaea and phages. These microbes interact closely to breakdown plant material (Gonzalez, Barraza, Viveros and Martinez, 2014; Huws, Creevey, Oyama, Mizrahi, Denman, Popova, Tamayo, …, Morgavi, 2018). Bacterial species involved in lignocellulose degradadtion that have been detected in the rumen include Ruminococcus florefaciens, Ruminococcus albus, Fibrobacter succinogenes and Prevollella ruminicola (Hu, Shi, Digkao, Wang, Li, Zhang, Luo, …, Lui, 2020). Abattoir wastewater microbes that take part in anaerobic digestion comprise of common fermentative bacteria that include *lactobacillus, Bacteroides, Clostridium, Escherichia coli, Leuconostoc and Klebsiella*; Acetogetogenic bacteria include *Acetobacterium, Clostridium and Desulfovibrio*; Methane producing organisms are classified under archaea (Jabari et al., 2016).

2.3 Abattoir waste disposal methods

The practiced disposal methods for abattoir wastes include burying, incineration, rendering, land spreading, dumping in landfills and draining in sewer lines. These methods of disposal are unsafe or require approved pretreatment and post treatment measures that attract heavy investment and high-tech expertise (Franke-White and Insam, 2013). Anaerobic digestion has emerged as a suitable method for disposal as it combines benefits for biogas production, decrease in odour, and elimination of pathogens and recovery of digestate for use as fertilizer (Ranalli, 2007).

2.4 Biogas systems

2.4.1 Psychrophilic or ambient temperature systems

The system produces less gas and operates in a temperature of12-24^{o}C (Ranalli, 2007). It is less efficient of the available biogas technologies and need a large system for digestion of large amounts of waste and improved gas production. Higher temperatures lead to high gas output from the system (Kashyap, Dadhich and Sharma, 2003).

2.4.2 Mesophilic temperature systems

Temperature range for mesophilic system is 30-42^{o}C (Ranalli, 2007). The system has the advantage of being stable and less sensitive to ammonia toxicity. Average gas is produced from these systems. Maximum gas production in these systems is observed at temperatures of 35-40^{o}C (Chae, Jang, Yim and Kim, 2008).

2.4.3 Thermophillic systems

Temperature of operation for thermophillic system is 43-60^{o}C (Ranalli, 2007). These systems have high energy recovery efficiencies and are faster. The disadvantage of these systems is that they exhibit sensitivity to ammonia toxicity and prevalent system instability. Maximum biogas production is observed at a temperature of 50-60℃ (Gebreeyessus and Jenicek, 2016). Fluctuations in temperature can result to either decrease in bacterial activity or death of bacteria subsequently leading to decrease in biogas production for the thermophillic system (Uzodinma, Ofoefule, Eze and Onwuka, 2007).

2.5 Factors affecting biogas production

The main factors influencing biogas yield and methane content in AD are Temperature, solid content, size of particles, pH range, retention time, mixing/agitation and C/N ratio (Igoni, Abowei, Ayotamuno and Chibuogwu, 2008; Jayaraj, Deepanraj and Velmurugan, 2014; Mshandete et al., 2006).

Several factors affect anaerobic digestion of wastes and they include feed stock characteristics, reactor design and operational conditions like temperature, pH, material size, dilution ratio, agitation factor and C/N ratio (Getahun et al., 2014; Doble, Nicolae and Matei, 2014). All these factors must be kept at their respective optimal levels for efficient biogas recovery (Ciobla, Lonel, Dumitrel, and Propescu, 2012). All operating parameters either as stand alone or together influence performance of anaerobic digestion systems. The levels of crucial factors ought to be carefully selected according to ranges that have meaningful impact on biogas production (Sarker, Lamb and Lien, 2019). Operating biogas digesters at pH 7.0 and temperature of 39℃ can give a hydraulic retention time of 30 days (Jayaraj et al., 2014). Solidification of substrate can stop gas production in AD systems (Onwuliri et al., 2013). Balmant and Ordonez (2013) stress the need to find an optimum hydraulic retention time for a given system of digestable wastes with all the other parameters fixed. Mohammed et al., (2013) showed that out of the many parameters affecting biogas production such as temperature, C/N ratio, hydraulic retention time, mixing ratio and pH, retention time is the only factor that is not involved in interaction.

2.5.1 Temperature

Temperature is a key factor in biogas production. Specific methane yield can be improved in mesophilic process by increasing temperature from 35 to 39 ℃ and any other increase in temperature negatively affects the production (Nielsen et al., 2017).Studies on thermophillic process suggest that methane production at 60 ℃is lower than that at 50 ℃ (El-Mashad et al., 2004). In mesophilic systems, maximum biogas is achieved at a temperature of 35-40℃ with peaks of biogas production at 36-37℃ and average at 31-32℃ (Uzodinma, Ofoefule, Eze and Onwuka, 2007). This can be explained by the fact that growth rate of bacteria can decrease by 50% for each 10℃ drop. There is a fall in biogas generated as temperature decrease towards 20℃ and even stops at 10℃. Further, increase in temperature from 40℃ lead to decrease in the rate of biogas generation in mesophilic systems (Ciobla et al., 2012). Temperature increase towards 40℃ with agitation in mesophilic systems can significantly increase biogas (Getahun et al., 2014).

2.5.2 Solid concentration

Solid concentration is one of the most important parameters in anaerobic digestion process that affect biogas yield. Biogas production has been found to be maximum at solid concentrations of between 20 and 25% (Paramaguru et al., 2017; Sathish, Chandrasekaran and Solom 2017; Deepanraj, SethilKumar and Ranjitha, 2019). Up to 10% total solids in liquid waste produce high biogas (Getahun et al., 2014; Paramaguru et al. 2017). The best digestibility is exhibited by wastes with a solid content of 7.4-9.2% (Budiyono, Widiasa, Johari and Sunarso, 2010).

2.5.3 Particle size

Size of particles has a profound effect on biogas production. At a given temperature, maximum biogas is produced from most finely grounded substrate (Sharma, S., Mishra, Sharma, M., and Saini 1988; Mshandete et al., 2006; Nalinga and Legonda 2016).

2.5.4 pH range

The range of pH in a digester affects performance on biogas production and degradation of substrate. Ranalli (2007) suggested an optimum pH range of 6.7 to 7.4. Biogas yield and substrate degradation efficiency have been found to be substantially higher for substrate of pH 7 compared to other values (Jayaraj et al., 2014). Optimum pH for biogas systems has been established to be in the range of 6.8-7.2 but can tolerate the range of pH 6.5 - 8.0 (Jayaraj, Deepanraj and Velmurugan, 2014). Digester pH of 7.2 or lower favors ammonia ion while digester pH greater than 7.2 favors formation of ammonia gas. Ammonia-nitrogen concentration beyond 1500-3000 mg/l is not only inhibitory but creates an additional problem of foam and scum generation (Suryawanshi, Chaudhari, Bhardwaj, and Yeole, 2013).

2.5.5 Hydraulic retention time

Hydraulic retention time usually varies from 10 to 30 days depending on temperature (Ezekoye, V., Ezekoye, B., and offor, 2011). Sing, Malik and Tauro (1985) suggested that total gas available at very long retention times is recovered in 20-25 days and a short retention time reduces the size and hence the costs of constructing digesters. It has been demonstrated that biogas production linearly increases with retention time (Ezekoye et al, 2011). Under good operation, more than 95% of biogas can be produced in less than a

month's time from mixed wastes (Getahun, Gebrehiwot, Ambelu, Van Gerven and Van der Bruggen, 2014).

2.5.6 Agitation

Agitation factor bears an influence on biogas production. Studies done by Rusin, Chamradova and Grycova (2017) revealed that there is an increase in anaerobic biogas and methane production by slow agitation. In the study, agitated batches produced 32.5% extra biogas and 28.5% higher methane yield than digesters without agitation. Biogas and methane production are increased by slow and continuous rotation and therefore agitation of the digester can be used effectively as an operating strategy to optimize biogas production (Keanoi, Hussaro and Teekasap, 2014). The period of daily mixing during AD process is beneficial in degassing the biogas that forms, preventing formation of dry and inactive layers, and may influence optimum retention time (Rusin, Chamradova and Grycova, 2017).

2.5.7 Effect of Carbon/Nitrogen ratio to anaerobic digestion of abattoir waste

Abattoir waste has low carbon/ nitrogen (C/N) ratio that is known to inhibit anaerobic digestion at high temperatures (Budiyono et al., 2011; Kovacs et al., 2015). High proteins in substrate are responsible for sulfide formation during anaerobic digestion (Fotidis et al., 2014). Increased concentrations of sulfides in the digester will result in formation of hydrogen sulfide in the biogas, a cause for inhibition of methanogens (Ranalli, 2007; Budiyono et al., 2011). In addition to sulfides, ammonia is formed during anaerobic digestion which may increase the pH of the digester (>8.0) which inhibits methane forming bacteria. Also, ammonia is a growth limiting factor for volatile fatty acid consuming and Acetotrophic bacteria (Budiyono et al., 2011). Methane potential

increases between C/N ratio 25:1 and 30:1 (Xiaojiao, Gashe, Yongzhang, Guangxin and Xinhui, 2012). Different interventions exist for maximizing AD to this low C/N ratio waste. Codigestion with high C/N ratio wastes optimizes feeding composition and improves the C/N ratio. Bioaugmentation is another promising method as it involves acclimatization or addition of species to adopt the digestion of low C/N ratio (Fotidis et al., 2014; Kovacs et al., 2015). Alkaline hydrolysis induces a fraction of protein breakdown and it can be used along with bioaugmentation to influence better protein breakdown (Biosafe engineering, 2019).

2.6 Pretreatment of wastes for efficient anaerobic digestion

Several procedures should be carried out on any waste prior and during its digestion anaerobically to make the process fast and efficient, (Ranalli, 2007). Suitable pretreatment methods for maximizing biogas production are discussed below.

2.6.1 Chemical Pretreatment

The alkaline hydrolysis (AH) hydrolysates can be beneficially used in AD to enhance biogas production (Salimon, Abdalla, and Salih, 2011; Salehian, Karimi, Zilouei, and Jeihanipour, 2013; Taherdanak and Zilouei, 2013; Zhang, Su, Baeyens and Tan, 2014). A combination of sodium hydroxide and potassium hydroxide catalyze hydrolysis of biological material (protein, nucleic acids, carbohydrates, lipids, etc.) into a sterile aqueous solution consisting of small peptides, amino acids, sugars as well as soaps (Franke-White and Insam, 2013). Gousterova et al. (2005) found out that microbial hydrolysates which are products of hydrolysis step, contained predominantly of low molecular peptides and amino acids including essential ones while AH produced predominantly peptides of higher molecular weight. Considering that the hydrolysates are

composed of a mixture of single amino acids, small peptide and fatty acids which are growth nutrients for microorganisms, usage in AD is very attractive (Kaye, Weber, Evans, and Venezia, 1998). Considerable amounts of ammonia may be formed during the alkaline hydrolysis of proteins. The ammonia is not formed by the decomposition of any amino acid as such, but is obtained from the alkali-labile groups that exist in the protein (Warner and Cannan, 1942; Marmelstein, Moreno and Fielder 2017). Coupled with AD, an alkaline hydrolysis could produce significant amounts of energy.

Among the chemical pretreatments, alkali pretreatment has been typically used in lignocellulosic material with high lignin content where the major objective has been to disrupt the lignin structure in the biomass improving the susceptibility of the remaining polysaccharides (cellulose and hemicellulose) for other treatments (Taherdanak and Zilouei, 2013). Alkaline hydrolysis makes cellulose to swell and increase in internal surface area. It also leads to a decrease in the degree of polymerization and crystallinity as well as particle solvation of hemicellulose, destroying the structural linkages between lignin and carbohydrates by saponification of intermolecular ester bonds The lignin structure is disrupted by breaking the glycosidic ether bonds and reduction in waste volume (Taherdanak and Zilouei, 2013; Wunna, Nakasaki, Auresenia, Abella, and Gaspillo, 2017). This means that AH can be applied on rumen and intestinal waste to break down the predominant lignocellulosic fibers.

Alkaline hydrolysis pretreatment at ambient temperatures inactivates pathogens and is therefore used as an alternative for treatment and disposal of infectious wastes (Kaye et al., 1998; Dixon et al., 2011). The advantage of using AH for inactivation of disease agents in abattoir wastes are the combining of sterilization and digestion steps into one

operation. The waste volume and weight is reduced by approximately 97%. The process totally destroys pathogens including prions and has a benefit of lower emission of odour (Dixon et al., 2011; Franke-White and Insam, 2013). Alkaline hydrolysis destroys all representative classes of potentially infectious agents and the sterile product of AH could be released into a sanitary sewer. For release into the sanitary sewers, an acid should be added into the hydrolysates at the end of the treatment to achieve a pH range of 8 or less.

Chemicals that can effectively be used in AH are sodium hydroxide, potassium hydroxide, calcium hydroxide, hydrazine and ammonium hydroxide (Taherdanak and Zilouei, 2013). Sodium hydroxide has been found to be the most effective in alkaline hydrolysis when a concentration of 5% w/w or more is used at temperatures of 70-100℃, for at least 60 minutes at pH 13 (Zhou, Zhang, and Dong , 2012; See also Salehian et al., 2013; Sun, Liu, Cao and Wu, 2017). Findings by Kovacs et al., (2014) support pasteurization of wastes (applying 70℃ for minimum 1 hour to tests using 5% v/v or more sodium hydroxide. Franke-White and Insam (2013) hints that the Swedish law and the European commission regulation (EC) no. 1774/2002 and no. 208/2006 require biogas plants that use animal waste to pasteurize the incoming substrate at 70 ℃ for at least 60 minutes prior to digestion to ensure hygienically acceptable end product. Even when using a thermophillic AD process it is advisable to use an additional heat treatment at the end of the process to fully inactivate pathogens capable of surviving the AD (i.e. the spore formers).

Several other chemicals have been known to potentially increase biogas. Chemicals such as ammonium hydroxide and hydrogen peroxide are used to degrade contents of substrate such as lignin, cellulose and hemicellulose significantly. Optimal treatment by

ammonium hydroxide and hydrogen peroxide have been achieved by concentrations of 4 and 3% respectively (Tulun and Bilgin, 2019).

2.6.2 Biological pretreatment

Aerobic microorganisms with efficient cellulotic activities have been used to improve lignocellulosic biomass for biogas production during fermentation. Mutschlechner, Illmer and Wagner (2015) used *Trichoderma viride* fungus in an aerobic upstream process prior to anaerobic digestion. Its use led to a threefold increase in the yield in the increase of methane and total gas

2.6.3 Size reduction

Mechanical size reduction is inexpensive and quite effective on the rate of biogas production from different substrates. Biogas yield increases with a reduction in particle size. Similarly, methane content is inversely proportional to particle size (Mshandete et al., 2006; Nalinga and Legonda, 2016). Pretreatment of waste into fine particles produces higher methane yields in biogas. Particle size below 2mm gives the best yields of biogas and methane content (Kuglarz, Karakashev and Angelidaki, 2013).There is a particle size whose digestion proportion reaches an optimum therefore produces the highest yield of biogas and corresponding methane within a short hydraulic retention time. Further reduction in size beyond this optimum produces insignificant increments in biogas and methane yields at a similar hydraulic retention time (Dlabaja and Malatak, 2013).

2.6.4 Bioaugmentation

Protein degradation augmentation

Biogas production in low C/N substrates is not efficient enough without addition of substrate treatment before or during the process (Hubman and Plowman, 1997; Kovacs et al., 2013). At the right pH, optimum biogas production is achieved at C/N ration 30:1 (Totok, Suswa, Hapsoro, and Anggara, 2017). Though stable biogas production over longer periods can be achieved when the natural biogas producing community is adapted to unusual substrate, significant increase in biogas production and improvement in methane content for C/N ratios lower than 30:1 are achieved through bioaugmentation for protein digestion. Addition of selected protein degrading strains leads to effective AD without acclimation (Kovacs et al., 2015).Some of the pure strains identified as protein digesters include *Bacillus coagulans*, *Bacillus subtilis* and *pseudomonas fluorescens* (Kovacs et al., 2015). The bacteria added to the system are expected to help the biogas producing community to cope with the stressful task of utilizing the protein-rich substrates. *Bacillus subtilis* is known to use glutamine as the best source of nitrogen. In the absence of glutamine, alternative sources such as ammonia can be used. Ammonia utilization can involve the uptake of the gas or the ammonium ion (Detsch and J*ö*rg, 2003). The soil dwelling gram positive bacterium *Bacillus subtilis* actively seeks and utilizes amino acids and have been proved to survive in mesophilic AD systems (Moses et al., 2011; Murunga, 2017). *Bacillus subtilis* produce natural components including cyclic lipopeptides, polypeptides, protein enzymes and non-peptide products (Wang et al., 2015).

Hydrogen augmentation

Escherichia coli is a known hydrogen (H_2) producing bacterial strain that can survive in an AD environment (*Á*cs et al., (2015). Addition of a hydrogen producing bacterium using different substrates at constant operational parameters has an impact of increasing biogas produced. *Escherichia coli* convert sugars to a mixture of products by fermentation. The major soluble products are acetate, ethanol and formiate with smaller amounts of succinate. In addition, hydrogen and carbon dioxide are produced in substantial amounts (Clark, 1989). Reactors inoculated with *Escherichia coli* have good digestion of volatile organic acids/total acids and recover more rapidly leading to a pronounced increase in biogas yield and methane content. Therefore, hydrogen producing bacteria enhance the hydrolyzing efficiency (*Á*cs et al., (2015; Kovacs et al., 2013). It has been established that the availability of dihydrogen is a limiting factor for hydrogenetrophic methanogenesis under both mesophilic and thermophillic conditions. When hydrogen producers are added to biogas systems, significant intensification of biogas is observed (Kovacs et al., 2013). Some bacteria such as *Calicellulosyruptor saccharilyticilus* may be good hydrogen producers, with cellulotic activity and are suitable when cellulose containing biomass is used (Bagi et al., 2007). The study reported an increase of at least 160-170% biogas production after adding hydrogen producers. In another study, biogas production in both thermophillic and mesophilic temperature range was improved by adding hydrogen (H_2) producing species (Kovacs et al. (2013)). When freshly grown cultures of *Enterobacter coagulans* are added in a liquid medium at controlled pH of 7.0 to the reactors directly at a concentration of 5% (V/V), more than 30% increase in biogas volume and methane mole fraction can be achieved (Kovacs et

al., 2013; Pessuto, Scopel, Perondi, Godinho, and Dettmer, 2016). Microbial analysis of spent slurry from digesters with high biogas yields reveals the presence of *Bacillus licheriformis*, *Escherichia coli* and Clostridium species (Onwuliri, Onyimba and Nwaukwu, 2013).

Escherichia coli has an ability to respond to the condition in its surrounding by developing a capability to utilize predominant nutrients apart from its most preferred glucose diet (Alpert, Sheel, Engs, Loh and Blaut, 2009). Chalova, Sirsat, O'Brian, Crandall and Ricke (2009) observed that *Escherichia coli* can not only synthesize all of the amino acids from inorganic compounds but it is also capable of transporting intact amino acids into the cell from extracellular environment. Non-multiplying and growing cultures of rumen bacteria called *Entodinium caudatum* can engulf *Escherichia coli* specifically labeled with individual c-amino acids and incorporate the amino acids into protozoal protein without conversion into any other amino acid (Coleman, 1967). Rumen waste can be used as an inoculum in order to reduce proteins in the reactor. As an adaptation, *Escherichia coli* has been found to synthesize and assimilate ammonia which is inhibitory to methanogenesis (Mikami, Yonda, Tatsukami, Aoki and Uenda, 2017).

2.7 Identification and confirmation of *Bacillus subtilis* and *Escherichia coli*

Bacillus subtilis and *Escherichia coli* can be identified through isolation and culture of the bacteria. The culture characteristics, morphology observation, biochemical test, 16s rRNA PCR amplification, sequence analysis and homology analysis assist in identification (Lupindu, 2017; Zhenxiang, Weina and Chang, 2018). According to Zhenxiang et al., (2018) *Bacillus subtilis* bacteria is rod shaped, gram positive, while

Indole and methyl red tests are negative. The circular morphology of this bacterium is rough, opaque, fuzy or slightly yellow with jagged edges (Lu, Guo and Liu, 2018).

Lupindu, 2017 suggests that the suspected *E.coli* isolate can be confirmed biochemically by use of a traditional method called Indole, Methyl Red, Voges-Proskauer and Citrate utilization IMViC tests. *Escherichia coli* is Gram negative (Niemi, Mentu, Siitonen and Niemela (2003).

2.8 Abattoir waste as substrate

A representative sample of all generated liquid streams in the abattoir facility should be used for accurate information. Ware, 2016 has shown that digestion of combined waste streams is viable with no decrease in methane. The liquid waste comprising of blood from the bleeding process, intestinal waste, rumen waste and wash water suspension can be mixed together according to their generation proportion from an average animal weight for use as substrate.

All samples should be mixed and blended thoroughly. Samples should be filtered to create a representative specimen with a uniform particle size. A particle size less than 8mm is preferred for high efficiency of biogas production (Ranalli, 2007).

2.9 Starting up the digester

Inoculum is required for seeding of the digester to provide a consortium of microbes to undertake activities from hydrolysis, acidogenesis, acetogenesis and methanogenesis. No commercial seeding material is available and no special measures for pure cultures fermentation are applied (Suryawanshi et al., 2013). The required cause of fermentation must be controlled by the cultivation conditions which include: temperature, substrate

composition, sludge loading rate and retention time (Suryawanshi et al., 2013). Ranalli (2007) recommends that if seeding material (digester sludge) from comparable processes is available, the startup process can easily be initiated by adding seed sludge (20-30% v/v) together with small amount of substrate into the digester. Suryawanshi et al. (2013) suggests an inoculum comprising of secondary sludge to primary sludge in the ratio 1:10 where primary is substrate and secondary sludge is effluent from a digester using similar substrate. An inoculum/substrate (I/S) ratio of 1.0 is regarded optimum in continuous systems (Sri Bala, Kalyanaraman, Porselvam, and Thenasekeran, 2012). Additional reactor volume is required for I/S greater than 1.0 with insignificant incremental benefit in terms of biogas produced from substrate. Similarly, for I/S ratio below 1.0, there is decrease in reactor volume with a high decrement in terms of bioenergy generated. While secondary sludge may be highly concentrated with facultative anaerobes, primary sludge contains facultative anaerobes and methane forming anaerobes. Therefore, anaerobic digester cannot be successfully seeded with primary sludge alone. If no primary seeding material is available, a reactor that has been warmed up to 35^{o}C can be seeded using fresh cattle manure/dung or rumen fluid (Budiyono, Widiasa, Johari and Sunarso, 2009; Suryawanshi et al.,2013; Ozbasgram, Ince, O., Ince, B., Harms and Kleinsteuber, 2018). Intestinal fluid containing fresh cattle dung and rumen fluid can be used in inoculation of reactors. Rumen fluid and fresh cow dung can be prepared for use as inoculum by diluting to disperse the solid material and filtering in a filter cloth. To ensure that solid content is dominated by bacteria, the solution can be filtered using a 50 micron cartridge filter (Budiyono et al., 2011). Lignocellulose biodegradability occurs by biological means (Saritha, Arora and Lata, 2012). In cellulotic rumen bacteria, highly active cellulotic and

hemicellulotic enzymes are combined in extracellular multi enzyme complexes called cellulosines (Artizi, Edward and Morais, 2016). During start up, loading the digester should proceed slowly. While doing so careful, monitoring of pH is essential until the digester attain a range of pH 6.8-7.2. For this purpose, different alkalis or acids could be added to maintain desired levels. Alkalis provide buffering capacity to neutralize acids within the digester (Suryawanshi et al., 2013). High biogas yield is observed after attaining digester stability in the second week after inoculation for anaerobically digested abattoir waste (Onwuliri et al., 2013).

2.10 Digester operation

Biogas production in the laboratory can easily be determined through positive liquid displacement (Ranalli, 2007; Ware, 2016). Start up, monitoring, biogas collection and control of parameters should be observed for a successful operation of the digester (Suryawanshi et al., (2013). Up to 10 % of the digester should be left empty as head space and substrate should not fill it to accumulate biogas (Samer, 2012).

CHAPTER 3. MATERIALS AND EXPERIMENTS

3.0 Equipment and materials

Refer to Appendix A for details of research equipment and materials.

3.1 Sampling

Abattoir waste was obtained from the Talai slaughterhouse being about 1.5km away from Moi University, main campus whose global positioning system (GPS) coordinates are 0^{o} $30^{'}$ $40.187^{''}$ N, 35^{o} 17' $3.178^{''}$ E. Five sheep slaughtered at the abattoir were randomly selected and used in determining the average waste stream proportions. Each of the three waste components was put in a plastic container with known empty weight then transported to the laboratory for weighing in grams using an accurate scale. The difference between weight of container with waste and empty container as in Table 3.1 was used to calculate the weight of waste.

Table 3.1: Measurements for determination of weights of various abattoir wastes

Item	Blood (g)	Rumen solids (g)	Intestinal waste (g)
Empty container	42.5	63.4	63.4
Sheep1 waste in container	842.5	2397.1	801.4
Sheep 2 waste in container	1151.4	3815.0	1293.7
Sheep 3 waste in container	1585.8	4085.0	2013.1
Sheep 4 waste in container	1128.5	3837.3	1047.6
Sheep 5 waste in container	1400.7	3983.9	1135.0

The difference between weight of container having wastes and that of empty container was the weight of waste. The total weight of the blood, rumen solids and intestinal wastes for the five sheep considered were 5896.4, 17799.3 and 5973.8g respectively. The average weight of each component was achieved by dividing the total weight of a waste

component for the sheep under consideration by five (number of sheep). This average weight figures for each stream of waste (blood (BW), rumen waste (RW) and intestinal waste (IW) were obtained by dividing total stream weight by number of sheep considered (in this case 5). The produced mixed waste ratio (PMWR) used as substrate was given by the ratio (BW: RW: IW) representing 1179.28:3559.86:1194.76 translating to the average weight of blood, rumen waste and intestinal wastes ratio of 1:3.02:1.01 that can also be translated into percentage form for convenience. This ratio was used to constitute mixed abattoir waste substrate based on weight.

3.2 Material preparation

Mixed abattoir waste was used as substrate. Samples of waste streams consisting of blood, rumen and intestinal waste were filled separately in three different plastic containers, covered with lids and transported to the laboratory within 1 hour for pretreatment, blending and further experimentation.

3.2.1 Waste pretreatment

Intestinal and rumen wastes were each separately diluted with tap water in the ratio 1:1 by weight. The content was then stirred vigorously and filtered using a 4 mm stainless steel wire meshed sieve (Blau-Metall, Germany) to achieve finer particles (Adelekan and Bamgboye, 2009; Nalinga and Legonda, 2016). Blood was filtered using 2mm sieve (Blau-Metall, Germany) to remove large insoluble particles before pasteurization. Filtration was carried out as in Figure 3.1.

Figure 3.1: Filtering wastes

3.2.2 Heat treatment of the wastes

The blood, rumen fluid and intestinal fluid wastes were separately poured into 1 litre Erlenmeyer flasks, covered with aluminium foil and placed in a thermostatic water bath (Model WB10, Polyscience, China) and heated at 70 ℃ for 100 minutes. Afterwards, the samples were cooled to 45 ^{o}C. A calibrated mercury thermometer was used to confirm water bath temperatures at all times.

3.2.3 Blending of abattoir waste streams

The heat treated blood, rumen and intestinal fluids were mixed in the abattoir stream compositions obtained in section 3.1 to constitute a 5kg substrate in a 20L bucket. The resultant mixed waste (MW) substrate was neutralized by adding 1M sulfuric acid and 1M sodium hydroxide to a pH 7.0 during which a 3 minute vigorous stirring was done for homogeneity. The pH of samples was measured using a pH meter (model HI98127, HANNA instruments, Romania). Part of the waste was fed directly into conical flask digesters and the remaining preserved in 1 litre conical flasks, covered with aluminium foil and refrigerated below 4 ^{o}C for subsequent tests. The pH of mixed abattoir waste was measure as presented in Figure 3.2.

Figure 3.2: Substrate pH measurement

3.2.4 Inoculum used in biogas production

Two inoculums: cow dung and rumen fluids were used in this experimentation. Both Cow dung and rumen fluids were made separately by diluting fresh portions of cow dung and rumen solids with tap water each in the ratio of 1:1 based on volumes. The resultant solutions were filtered through a 50µm stainless steel wire meshed sieve (Blau-Metall, Germany).

3.3 Physicochemical characteristics of mixed abattoir waste substrate

3.3.1 Determination of total solids (TS)

The procedure in APHA (1999) was followed in determination of total solids at 103-105 ℃.

Preparation of evaporating dish: Since only total solids were measured, the dish was heat cleaned at 103 -105 ℃ for 1 hour. The dish was stored and cooled in a desiccator until needed. The evaporating dish was weighed before use.

Sample analysis: The volume for analysis was chosen from a series of trials using volumes of 1.0, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8 and 3ml. The 1.5-2.4ml volume sample was adequate to yield a residue between 2.5 - 200mg and hence 2ml was adopted for use.

A well mixed sample was pipetted to a pre-weighed evaporating dish. Stirring of the sample was done using a magnetic stirrer during transfer from the storage container to the evaporating dish. For homogeneous samples, pipetting was done from the approximate midpoint of the container. The sample was then evaporated to dryness on a drying oven. When evaporating in a drying oven, the temperature was lowered to approximately 98 ℃ to prevent spattering. The evaporated sample was dried for at least 1 hour in an oven at 103 – 105 ℃. Afterwards, the dish was cooled in a desiccator to balance temperature, and weighed. The cycle of drying, cooling, desiccating and weighing was repeated until a constant weight was obtained. When weighing of dried samples was not done immediately then change in weight due to air exposure and/or sample degradation was minimized by covering the evaporating dishes. Weighing was done using a high precision weighing balance. The sample was done in duplicate.

Calculation of total solids: Total solids were calculated using Equation 3.1.

$$\text{Mg total solids/L} = \frac{A-B}{\text{Sample volume ,ml}} \times 1000 \quad \text{.....Equation 3.1}$$

Where:

A = Weight of dried residue + Dish (mg) = 11.647×1000 mg

B = Weight of Dish (mg) = 11.556×1000 mg

3.3.2 Determination of total suspended solids (TSS)

Total suspended solids were determined using the standard APHA (1999) procedure. This involved:

Preparation of glass filter paper: The filter paper was cut to fit in the filter funnel-sunction assembly and was inserted in filtration apparatus. The vacuum was applied and the filter paper washed three times using 20ml of reagent grade water. Sunction was continued to remove all traces of water before turning off the vacuum and discarding the washings. The filter paper was removed from filtration apparatus and transferred to an inert aluminum weighing dish then dried in the oven at 103 – 105 ℃ for 15 minutes. The filter paper was cooled in a desiccator to balance temperature and weighed. The cycle of drying, cooling, desiccating and weighing was repeated until a constant weight was obtained. The dried filter paper with a constant weighed was stored in a desiccator until needed.

Selection of filter and sample sizes: A volume sample was determined as described in section 3.4.1 while a filter paper through which filtration process took place in less than 10 minutes was preferred. A 2ml volume of sample was used to yield 0.2 -200mg of dried residue.

Sample analysis: Filtering apparatus and filter paper were assembled before beginning sunction of wet filter. A small volume of distilled water was used to seat the filter paper. The sample was stirred using a magnetic stirrer. While stirring, 2ml of sample were pipetted into the seated filter paper. For homogeneous samples, pipetting was done from the approximate midpoint of container but not in vortex. A pipetting point was chosen such that it was at the middepth and midway between wall and vortex of the liquid being stirred. The sample filtered through the filter paper under influence of the sunction applied. The filter paper was washed four successive times using 10ml of distilled water allowing complete drainage between washings. After filtration was completed, sunction

was done for 3 minutes. The filter paper was carefully removed from filtration apparatus and transferred to an aluminum weighing dish. The residue was dried for at least 1 hour at 103 -105 ℃ in an oven. The residue was then cooled in a desiccator to balance temperature and subsequently weighed. The cycle of drying, cooling, desiccating and weighing was repeated until a constant weight was obtained. Weighing was done using a high precision weighing balance. A repeat of the same was done using another sample and the results were noted. The total suspended solids were calculated by Equation 3.2. Average of the two outcomes was used in the calculation of the residue weight.

$$\text{Mg TSS /L} = \frac{A-B}{\text{Sample volume ,ml}} \times 1000 \quad \text{.....Equation 3.2}$$

Where:

A = Weight of filter + Dried residue, mg

B = Weight of filter, mg

3.3.3 Calculation of total dissolved solids (TDS)

Total dissolved solids were determined by subtracting total suspended solids from total solids using the formula (APHA, 1999). Total solids are given by equation 3.3.

$$TS = TSS + TDS \quad \text{.....Equation 3.3}$$

This was converted to equation 3.4 before use.

$$TDS = TS - TSS \quad \text{.....Equation 3.4}$$

Where; TS = Total solids, TSS = total suspended solids and TDS = Total dissolved solids

3.3.4 Determination of Chemical Oxygen Demand (COD)

The procedures for determining chemical oxygen demand in method 5220 D; closed reflux, colorimetric method in APHA (1999) was followed.

Preparation of reagents

Digestion solution was prepared by adding 10.216g of potassium dichromate ($K_2Cr_2O_7$), primary standard grade, previously dried at 103 ^oC for 2 hours and 167 ml conc sulfuric acid (H_2SO_4) to 500ml distilled water The contents were given time to dissolve , cooled to room temperature and diluted to 1000ml using distilled reverse osmosis water. The sulfuric acid reagent was prepared by adding silver sulfate (Ag_2SO_4), reagent or technical grade, powder, to conc. H_2SO_4. The mixture was left to stand 2 days to dissolve Ag_2SO_4.Potassium hydrogen phthalate (KPH) standard was made by lightly crushing and then drying potassium hydrogen phthalate ($HOOCC_6H_4COOK$) to constant weight at 120 ^oC. 425 mg of the crushed KPH were dissolved in distilled water and diluted to 1000ml. KHP has a theoretical COD of 1.176 mgO_2/mg and this solution has a theoretical COD of 500μgO_2/ml. This solution is stable when refrigerated for up to 3 months in the absence of visible biological growth.

Treatment of samples

Suitable volumes of sample and reagents were measured as indicated in Appendix A, Tables 6.1(a) and (b).

Measurement of COD by Hanna Method

Substrate and digestate sample were made using dilution factors ranging from 10 to 100. The sample to be tested was diluted using reverse osmosis (RO) water. High range reagents had an expected COD range of 0 to 1500mg/L mg/L (ppm) $O_{2.}$ A tit pipette was used to dose 1ml of dilute sample into a vial that contained ready to use high range reagents. The lid was closed tight and the vials inverted upside down three times to mix the contents. Standards were prepared by using potassium hydrogen phthalate (KHP) where a milligram of KHP is equal to 1.175 mg/L COD. Five standards from potassium hydrogen phthalate solution with COD equivalents from 10 to 900mg O_2/L were prepared. The same reagent volumes, vial size and digestion procedure as for sample were used. Closed reflux (colorimetric system) digestion was achieved by placing the vials with samples and standards in the Hanna COD incubator (HI-839800 thermo reactor) having 25 vial capacities and was preset at 150^{o}C for two hours, allowed to cool before measurements were done. The cooled samples and standards were inverted several times and solids were allowed to settle before measuring absorbance. Solids that adhered to the container wall were dislodged by gentle tapping and settling. Scratched or blemished glassware were discarded. The dilution ratio 1:100 was adopted since vials in which ratios were lower than 100 turned bluish green in colour signifying sensitivity failure. The absorbance of cooled vials was measured using a uv-vis spectrophotometer at 600nm wavelength. Unopened vials were inserted through access door into light path of spectrophotometer set at 600nm. The absorbance readings for each vial were made. Optically matched culture vials were used for greater sensitivity; the absorbance was measured for samples that had a pale orange-brownish colour similar to the standard

samples. A calibration curve of measured absorbance against standard concentration was prepared using the five standards from potassium hydrogen phthalate solution with COD equivalents from 10 to 900mg O_2/L for the chosen lot of vials. The measured absorbance for the samples was fitted on the curve equation to extrapolate/ the COD concentrations of substrate and digestate. The COD was determined using Equation 3.5.

COD (x), $mgL^{-1} = [\frac{(\boldsymbol{Absorbance}\times\mathbf{1000})-\mathbf{0.884}}{\mathbf{0.191}}] \times \boldsymbol{DF}$.....Equation 3.5

Where DF is dilution factor or dilution ratio used.

The absorbance values for the COD tests were read at ʎ = 600 nm. Standard COD concentration and absorbance data generated the standard COD curve in Figure 3.3.

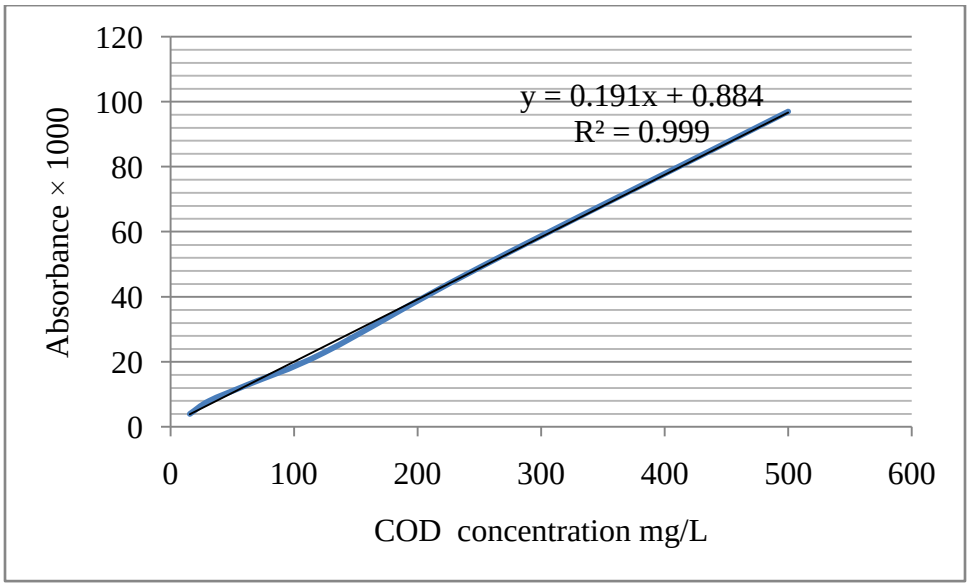

Figure 3.3: Standard chemical oxygen demand and absorbance curve

The R^2 value for this curve was above at 0.9999 therefore above the recommended 0.95 value hence suitable for use in estimation of unknown concentration. The substrate with dilution factor below 100 failed tests as they turned blue indicating the COD range was above that of the standard reagents used. The dilution factor of 100 was suitable to be

used for COD analysis of this sample and gave absorbance of 0.380 and 0.382 from the two samples of mixed abattoir waste substrate analyzed giving a mean value of 0.381. The COD value for the substrate was estimated by superimposing the mean absorbance of the mixed waste substrate on the standard curve equation 3.5.

3.3.5 Determination of nitrogen content

Total Nitrogen (TN) was determined using the Total Protein (TP) protocol. In this method nitrogen available was oxidized to nitrates which were quantified by absorbance using uv-vis spectrometer at λ = 540 nm. A Beckman uv-vis spectrophotometer was used. The nitrogen was then calculated from the molecular formula of nitrates, (Nydahl, 1978).

Preparation of digestion reagent solution (DR-sol) and standard curve

A total of 8g of Potassium peroxidisulfate ($K_2S_2O_8$) special grade for Nitrogen analysis were dissolved in 100ml of reverse osmosis (RO) water, 0.9g of special grade sodium hydroxide (NaOH) were dissolved in 100ml of RO water. Then the two solutions were mixed to form digestion reagent. Ten milliliter sample and 5ml DR-sol were pipetted into PP digestion bottle. This was followed by autoclaving the treatment at 121^{o}C for 90 minutes. The bottle was cooled down to room temperature then pH confirmed to be 2 as required. Standard curve for nitrate-nitrogen test was obtained by measuring the absorbance of standard reagent potassium hydrogen phthalate diluted to known concentrations at λ = 540 nm. A plot of absorbance against concentrations (mgL^{-1}) was made. The ultra violet light (UV) absorbance of the sample was used to estimate the nitrate concentration through interpolation. The ratio of nitrogen mass to the nitrate molecular weight gave the total nitrogen concentration (mgL^{-1}) in the samples using Equations 3.6 and 3.7.

Nitrate (x) $mgL^{-1} = \frac{Absorbance - 0.010}{0.019} \times DF$ ……...….Equation 3.6

Nitrogen $mgL^{-1} = \frac{14}{62} \times Nitrates\ mg/L$, ……..….Equation 3.7.

Where DF is the dilution factor.

Total Nitrogen (TN) was determined using the Total Protein (TP) protocol. In this method nitrogen available was oxidized to nitrates which were quantified by absorbance using a Beckman uv-vis spectrophotometer at ʎ = 540 nm. The nitrogen was then calculated from the molecular formula of nitrates, (Nydahl, 1978). The standard concentrations and absorbance generated the standard curve in Figure 3.4 from which nitrates were estimated. This standard curve does not pass through the origin as it only works for the concentration range taken to plot the graph, It will not work below this concentration.

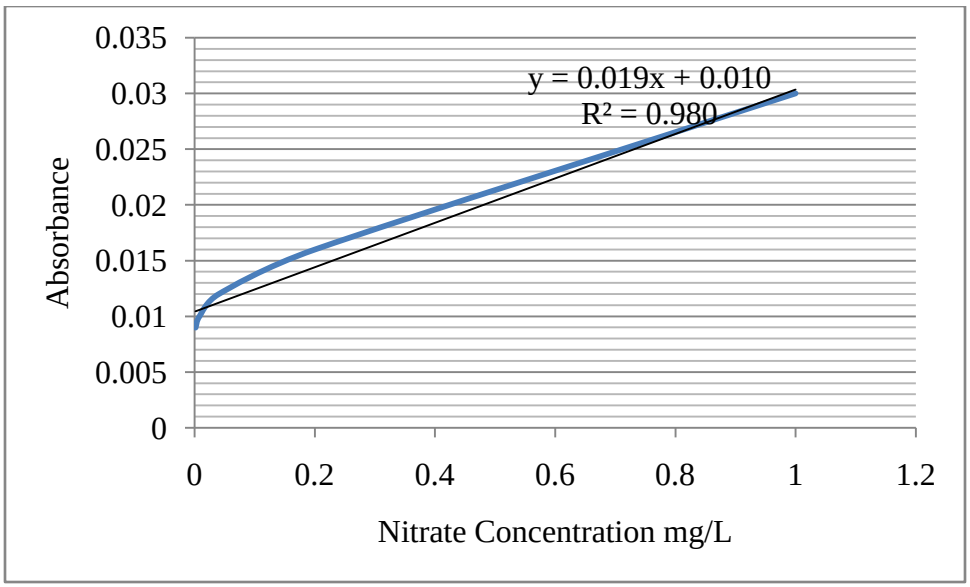

Figure 3.4: Standard Nitrate concentrations and absorbance curve

The equation of the curve in Figure 4 was used in estimation of unknown nitrate concentration as the value of R^2 was 0.95 or higher. The R^2 value of 0.980 meant that this curve could be used to estimate unknown values of concentration. The standard curve relationship is given by Equation 3.8.

$y = 0.019x + 0.010$ ……Equation 3.8

In which y represent absorbance and x the nitrate concentration mgL^{-1}.

The two samples of mixed abattoir substrate gave a mean absorbance of 0.710 at a dilution factor (DF) 100.

This characterization was significant to the general objective in two ways: first it was an indication of the organic potential of the mixed abattoir waste to produce biogas and lastly the depletion in total solids and chemical oxygen demand was used in complimenting increase in biogas production of the experiments against controls.

3.4 Bacterial strains used in the study

The study used two bacteria strains: *Bacillus subtillis* and *Escherichia coli.* *Bacillus subtillis* was used as a protein degrading bacteria in mesophilic conditions. The bacterium was isolated from soils with decaying organic matter comprising of leaves near plant routes in a flower garden. *Escherichia coli* were used as hydrogen generating bacteria under mesophilic conditions. This bacterium was isolated from Moi University sewage sludge. The bacterial species were chosen based on the ease of isolation and cultivation on inexpensive media.

3.5 Isolation and growth of bacterial strains

3.5.1 Media preparation and Isolation of *Bacillus subtillis*

Preparation of yeast extract agar media

The *Bacillus subtillis* was cultured on peptone yeast extract agar. The peptone yeast extract agar was prepared by adding 22g of peptone yeast extract agar to 1L of distilled water. The solution was mixed and autoclaved using ALLAMERICAN 25X-2, USA machine at 121 ℃ for 15 minutes. The media was cooled to 50 ℃ after 1 hour and poured into sterile petri dishes.

Isolation of *Bacillus subtillis* and plating

The *Bacillus subtillis* was isolated from soils with decaying matter (logs and leaves) and grown on peptone yeast extract agar according to Bacillus isolation method (Brown, 2013). In a microfuge tube, a loop full amount of soil was suspended in 2 drops of distilled water. It was agitated by stirring till clear, mixed well and placed on a heating block at 80 ℃ for 10 minutes. A loop was used to streak a sample of this heat treated soil onto a peptone-yeast extract plate.

It was then incubated aerobically in dark at 30 ℃ for 2 days. The samples were then examined under a high power microscope lens. A well isolated colony was picked and restreaked onto a fresh plate of the peptone yeast extract agar plat and incubated at 30 ℃ as before.

Bacillus subtilis characterization

In this study, identification of this bacteria was conducted mainly through conventional methods such as observing bacterial colony characteristics, morphology and biochemical test for comprehensive judgment. When the isolate was restreak on ordinary nutrient agar and incubated at 37 ^{o}C for 48h, the morphology of the circular colony of this bacteria was rough, opaque, fuzzy white/slightly yellow with jagged edges as suggested by Lu et al., (2018).

Biochemical test

Gram staining was done as the only biochemical test for identification of *Bacillus subtilis*. Gram staining detects a fundamental difference in the cell wall composition of bacteria. A bacterial smear was prepared from a pure culture. A drop of saline, distilled water, Phosphate-Buffered Saline fat (PBS), pH 7.1 was put on a clean glass slide. Using a sterile loop, an isolated colony was touched and mixed in the water drop. Mixing was done until just a slightly turbid bacterial solution was observed (light bacteria is the best, excess bacteria will not stain properly). The mixture was left to air dry and heat fixed to avoid overheating. The slide was allowed to cool before it was flooded with crystal violet which was allowed to remain on the slide for 60 seconds. The crystal violet was washed off with running tap water. The slide was then flooded with Gram's iodine, and allowed to remain on the slide for another 60 seconds. The Grama's iodine was washed off with running tap water. Decolorizing solution was applied until the solvent flowed colorless from the slide (approximate 5 to 10 seconds). Excessive decolorization was avoided since it could result in a false gram-negative reading. Also, too little decolorization could give a false positive result. Rinsing was done immediately with running tap water. Counter

staining with safranin for 60 second was done. Finally, rinsing with tap water was done and the slide allowed to air dry for observation. Identified *Bacillus subtilis was then* grown on freshly prepared peptone agar solution in 250ml conical flasks and incubated at 37 ^{o}C for subsequent use.

3.5.2 Isolation of *Escherichia coli*

Standard procedures for isolation, purification, quality control and confirmation of *E.coli* isolates were used (Lupindu, 2017).

Procedure

A total of 40g of MacConkey agar was suspended in 950ml distilled water and heated to boiling while being stirred to dissolve the medium completely. Autoclaving of the MacConkey agar media was done at 15 Lbs pressure (121 ℃) for 15 minutes. The media was then cooled to 45 ℃, mixed well before pouring into sterile petridish plates. Raw sewage sludge was then inoculated on cooled MacConkey media in sterile petridish using a loop. Incubation was done for 24 hours aerobically at 37 ℃. Since the expected doubling time for *E.coli* at 37 ^{o}C incubation was 17-18 min therefore 60-80 *E.coli* cell generations would be produced in 18-24h incubation. To validate the accuracy of the bacteria isolates, two uninoculated media plates were incubated simultaneously with inoculated media plates. Pink, round medium sized colonies were picked as *E.coli* suspect colonies in inoculated media plates. the media plates without inoculation had no microbial growth after incubation an indication that the sampling equipment had not been contaminated. Restreaking colonies of *E coli* was done on fresh sterile MacConkey plate and incubated at 37 ℃ for two days as before.

Biochemical confirmation of *E.coli* isolates

The suspected *E.coli* isolate was confirmed biochemically by use of a traditional method called Indole-Methyl red-Voges-Proskauer-Citrate utilization (IMViC) tests.

Indole test

Peptone water was put in a tube containing tryptophan and was inoculated with the bacteria isolate. The mixture was incubated overnight at 37 ^{o}C. Then, a few drops of Kovac's reagent were added to the mixture.

Methyl Red test

The bacterium isolate was inoculated into glucose phosphate (MRVP) broth, which contained glucose and a phosphate buffer and incubated at 37 ^{o}C for 48h. Four drops of methyl red reagent were added to the tube.

Voges-Proskauer test

The isolated bacterium was inoculated into glucose phosphate (MRVP) broth in a tube and incubated for 72 hours. This was followed by addition of 15 drops of alpha naphthol to the test broth then shaking. Then five drops of 40% potassium hydroxide (KOH) were added to the broth before shaking well. The tube was then allowed to stand for 15minto see a positive red discoloration.

Citrate utilization test

A loopful of bacteria was streak onto a citrate agar slant without stabbing the butt and incubated at 37 ^{o}C for 24 hours with a loose cap.

Gram Stain

Gram staining was another biochemical test for identification of *E.coli*. A bacterial smear was prepared from a pure culture. A drop of saline, distilled water, Phosphate-Buffered Saline fat (PBS), pH 7.1 was put on a clean glass slide. Using a sterile loop, an isolated colony of *E.coli* was touched and mixed in the water drop. Mixing was done until just a slightly turbid bacterial solution was observed (light bacteria is the best, excess bacteria will not stain properly). The mixture was left to air dry and heat fixed to avoid overheating. The slide was allowed to cool before it was flooded with crystal violet which was allowed to remain on the slide for 60 seconds. The crystal violet was washed off with running tap water. The slide was then flooded with Gram's iodine, and allowed to remain on the slide for another 60 seconds. The Grama's iodine was washed off with running tap water. Decolorizing solution was applied until the solvent flowed colorless from the slide (approximate 5 to 10 seconds). Excessive decolorization was avoided since it could result in a false gram-negative reading. Also, too little decolorization could give a false positive result. Rinsing was done immediately with running tap water. Counter staining with safranin for 60 second was done. Finally, rinsing with tap water was done and the slide allowed to air dry for observation. *Escherichia coli* were then grown on freshly prepared peptone agar solution in 250ml conical flasks and incubated at 37 ^{o}C for subsequent use as in the case of *Bacillus subtilis*.

3.6 Sterilization of equipment, surface and media for bacteria isolation

Standard sterilization methods in laboratory practice were used (Cheesbrough, 2006). All equipment's were sterilized prior to use or assembling. Cultures media and isolates for disposal were autoclaved at 121 ℃ for 20 minutes. Glassware and plastic ware were

boiled at 100 ℃ for 20 minutes before use. Disposable waste, cotton wool, plastic and gloves were burned in an incinerator after use. Microscope slides, pipette, overalls, and re-usable gloves were soaked overnight in 1000ppm chlorine disinfectant as bench surfaces and spillages were wiped using 2500ppm and 10,000ppm chlorine solutions respectively. Inoculating loops were flamed in a Bunsen burner till they were red hot and cooled before use.

3.7 Bioaugmentation

Solutions of freshly prepared bacterial strains of *Bacillus subtilis* and *Escherichia coli* were used in the bioaugmentation experiments. The research used the acceptable inoculum to substrate ratio volume of 0.25 – 1.0 (Sri Bala et al., 2012; Suryawanshi et al., 2013). Total inoculum comprised of rumen fluid, fresh intestinal fluid and the culture solutions of the microorganisms. Where no *Bacillus subtilis* or *Escherichia coli* microbes were required the cultured microorganism solutions were sterilized by autoclaving. The abattoir waste solution used in the research was black in colour eliminating the need to provide for a dark environment for the anaerobic digestion process.

3.7.1 Acclimatization of strains

The bacterial strains were acclimatized overnight to digester temperature (kept at 37 ^{o}C) before inoculation (Kovacs et al., 2015). The contents of the digester were then mixed by shaking the flasks for uniform distribution of the introduced species before sealing for anaerobic digestion to take place.

3.8 Experiment design

Three different experiments were set up as in sections 3.9.1, 3.9.2 and 3.9.3. In the first experiment percentages of 0, 20 and 50 rumen fluid inoculum were used to investigate the influence of rumen fluid inoculum on the digestion of substrate formed by mixing abattoir waste streams of blood, rumen solid and intestinal waste in their production ratios. In the second experiment, bioaugmentation was done by using *Bacillus subtilis* and *Escherichia coli* singly and in combination adopting the rumen fluid inoculum with the highest performance from experiment 1. In the last experiment, Box Behnken experimental design as described in Montgomery (2013) was used to investigate optimum range conditions of mesophilic temperature, possible percentage of rumen fluid inoculum in cow dung and hydraulic retention time (HRT) for biogas production from mixed abattoir waste.

3.8.1 Experiment 1: Effect of rumen fluid on anaerobic digestion of abattoir waste

Various percentages of rumen fluid in cow dung were used to investigate effect of using rumen fluid as inoculum for abattoir waste digestion. Three 250ml conical flask digesters were labeled A, B and C, each fed at once by 190 ml of pretreated mixed abattoir waste. Inoculum was composed of fresh liquid cow dung and fresh rumen fluid. The two fluids gave a total of 60ml. Therefore the inoculum substrate ratio was kept at 24%. The inoculum was obtained by diluting the wastes separately with tap water in the ratio 1:1 based on mass and filtering through a 50µm stainless steel wire meshed laboratory test sieve (Blau-Metall, Germany). Digester A, B and C had 0%, 20% and 50 % respectively of rumen fluid as inoculum. The reactors were closed using rubber corks and sealed using silicon adhesive and were placed in a 3 liter analog water bath (Avishkar International

Ltd, India) that was set at 35^{o}C for 30 days. The experiment was performed in triplicate and results analysed. The total solids (TS) and the chemical oxygen demand (COD) for digestate was analysed to determine the extent of digestion in each case. The contents of each reactor were as in the Table 3.2 and the experiment set up as in Figure 6.1 in the Appendix B.

Table 3.2: Biogas production experiment using varying rumen inoculum percentage

Reactor	**A**	**B**	**C**
Substrate volume, ml	190	190	190
Rumen fluid inoculum, ml	0 (0%)	12(20%)	30(50%)
Cow dung fluid in inoculum, ml	60	48	30

Total volume for inoculum used in each flask in the experiment was 60ml. Table 3.3 gives volume of measured volume of rumen fluid and its equivalent percent based on total inoculum volume.

3.8.2 Experiment 2: Effect of bioaugmentation on biogas production from abattoir waste

Bacillus subtilis and *Escherichia coli* were used to investigate effect of bioaugmentation utilizing rumen inoculum on biogas production from abattoir waste in four 500ml conical flasks labeled D, E, F and G for *Bacillus subtilis*, *Escherichia coli*, *Bacillus subtilis* and *Escherichia coli*, and none of the microbes respectively. Each reactor was fed with 360 ml of the pretreated mixed abattoir waste substrate, 120ml inoculum composed of rumen fluid and cow dung fluid in the ratio 1:1 (50% rumen inoculum). The reactors were closed using rubber corks, sealed using silicon adhesive and were placed in a 5 liter digital water bath (Model WB10: Polyscience, China) water bath that was set at 37 ^{o}C

for 26 days. The experiment was performed in triplicate. The total solids (TS) and the chemical oxygen demand (COD) for digestate was analysed to determine the extent of digestion in each case. The design was as shown in Table 3.3 and experiment set up as in Figure 6.3 in Appendix D.

Table 3.3: Experiment design for biogas production using *B.subtilis* and *E.coli*

Reactor	**D**	**E**	**F**	**G**
Substrate volume, ml	380	380	380	380
Rumen fluid inoculum, ml	60	60	60	60
Cow dung fluid inoculum, ml	60	60	60	60
B.subtilis in peptone agar solution, ml	18	18(sterile)	18	18(sterile)
E.coli in peptone agar solution, ml	18(sterile)	18	18	18(sterile)

3.8.3 Experiment 3: Optimizing conditions for biogas production from abattoir waste

Three variable Box-Behnken experimental design was used in optimization of temperature, percent of rumen fluid by volume in inoculum and hydraulic retention time for biogas production from mixed abattoir waste. The 250 ml conical flasks were used as digesters where the substrate was 190ml and total inoculum of 60 ml in each case. The pretreated substrate was digested anaerobically under different levels of mesophilic temperature range, percent rumen fluid in the inoculum and retention time. The ranges of anaerobic digestion temperature, percent of rumen fluid in inoculum and HRT investigated through experimental design were 35-40 ℃, 20-100% and 12-18 days respectively (Ambar et al., 2017; Budiyono, Widiasa, Johari, and Sunarso, 2014; Ezekoye et al., 2011;Uzodinma et al., 2007; Suryawanshi, 2013).

The Box-Behnken experiment design used was economical and involved fewer runs. A total of 15 runs were conducted. Each run had three factor level combinations. Three center-runs made up a total of 15 required runs in the Box-Behnken design (Montgomery, 2013). Table 3.4 shows the various level combinations for each run of the Box-Behnken experimental design while the set up was as shown in Figure 6.5 in Appendix F.

Table 3.4: Box-Behnken experiment design for optimization of biogas production from abattoir waste

	Natural variables			Coded variables			Biogas yield
Run	T(^{o}C)	PR%	HRT(days)	x1	x2	x3	y(ml)
1	35	20	15	-1	-1	0	-
2	35	100	15	-1	1	0	-
3	40	20	15	1	-1	0	-
4	40	100	15	1	1	0	-
5	35	60	12	-1	0	-1	-
6	35	60	18	-1	0	1	-
7	40	60	12	1	0	-1	-
8	40	60	18	1	0	1	-
9	37.5	20	12	0	-1	-1	-
10	37.5	20	18	0	-1	1	-
11	37.5	100	12	0	1	-1	-
12	37.5	100	18	0	1	1	-
13	37.5	60	15	0	0	0	-
14	37.5	60	15	0	0	0	-
15	37.5	60	15	0	0	0	-

Where T stands for temperature, PR for percentage of rumen fluid in inoculum and HRT for hydraulic retention time in the table.

Choice and control of operating conditions in the Box-Behnken design

Digestion temperature (℃), x_1

Natural optimal digestion temperature values 35 ℃, 37.5 ℃ and 40 ℃ were used (Uzodinma et al., 2007). The 35 ℃ and 40 ℃ temperatures were controlled using 3 litre analog thermostatic water baths from Avishkar International Ltd, India. The temperature of 37.5 ℃ was controlled using a high accuracy 5 litre digital thermostatic water bath model WB10 from Polyscience, China. Temperature measurements in all thermostatic water baths was done after every four hours using a standard mercury thermometer to check for any deviations. The real and coded design parameter of temperature was related in the Equation 3.9.

Temperature (T) range: 35-40^{o}C, X_1 (Coded T) = $\frac{(\boldsymbol{T-37.5})}{\boldsymbol{2.5}}$……Equation 3.9

Percent rumen fluid in inoculum (RI), x_2

The variable, X_2 was regulated by varying composition of rumen fluid in inoculum from 20%to 100%while reducing cow dung. The percent rumen fluid in inoculum (PR%) settings were kept at 20%, 60% and 100% of the 60.0 ml of substrate consisting of rumen fluid and liquid cow dung.

The real and coded design parameter of percent rumen fluid in inoculum (RI %) were related in the Equation 3.10.

RI range: 20%-100%, X_2 (Coded PR) = $\frac{(\boldsymbol{RI-60})}{\boldsymbol{40}}$……Equation 3.10

Where RI = Rumen Inoculum

Hydraulic retention time (HRT), x_3

Each run in the Box Behnken experiment design lasted for the design retention time. Counting of days commenced on the startup date. Natural values of the digester hydraulic retention time were set at 12, 15 and 18 days to collaborate with the findings by (Ezekoye et al., 2011; Getahun et al., 2014). The real and coded design parameter of temperature is related in the Equation 3.11.

HRT range: 12-18 days: X_3 (Coded HRT) = $\frac{(\boldsymbol{HRT-15})}{\mathbf{3}}$ ……..Equation 3.11

Where HRT = Hydraulic retention time

3.9 Reactor volume considerations

Considerations of volume measurements suggested by other research works was adhered to for successful operation of biogas reactors. The volume of treated substrate in each reactor was measured and recorded to ensure that reactor head space was at least 10% total reactor volume (Suryawanshi et al., 2013) and inoculum/substrate ratio was 20-30% volume per volume with a ratio of fresh rumen fluid-fresh cow dung fluid in selected ratios (Budiyono et al., 2011; Suryawanshi et al., 2013).

3.10 Digester mixing

The digester contents were continuously mixed by regular shaking at least three times per day to mix up the contents throughout the experiment (Keanoi et al., 2014; Getahun et al., 2014; Rusin, 2017). This was necessary to overcome caking, mix up the digester contents and facilitate gas escape from the liquid digestate. The difference between shaking and stirring is that practically, shaking achieves the same results more quicker.

3.11 Biogas measurement

Measurements were done by water displacement from conical flasks (Ranalli, 2007). The gas from the digester was connected to a 350 ml conical flask filled with water. The gas displaced water equal to the volume of water that exited the flask via a delivery tube leading to a 250 ml. Care was taken to keep the collection flask cold to minimize evaporation error by keeping flasks below 20 ℃. The water in the 350 ml conical flasks contained methyl orange indicator for meniscus clarity. During start up, biogas volume readings were done twice per day and thereafter recording was done once a day. A record of the volume of biogas produced daily was kept for the selected hydraulic retention times for the experiments.

3.12 Measurement of biogas composition

Methane composition was analyzed using gas chromatography with flame ionization detector (FID) and nitrogen carrier gas (Chamarthi, Bhale, Reddy and Mouli 2013). Standard methane gas was analyzed followed by analysis of the sample biogas collected from reactors A, B, C, D, E, F and G. The areas with peak times closer to that of standard methane gas were compared with the standard peak area to compute methane composition in the sample. The FID method and nitrogen carrier gas could only analyze methane proportion as opposed to the use of Thermal Conductivity detector (TCD) and helium as carrier gas which gives a whole range of gaseous products present in a sample. Flame ionization detector (FID) and hydrogen carrier gas were used in gas chromatograph test and therefore the only peak produced in any run corresponded to methane gas. The quantified biogas based on methane composition enabled a relative comparison of methane quantity for the various runs to be made. The use of thermal

conductivity detector (TCD) and helium as carrier gas is recommended where all the gaseous components of the biogas are to be known.

For practical purposes, volume fractions and mole fractions are interchangeable in gases. It also makes no difference whether one views volume fractions or pressure fractions (Griffith, 2015). When gases mix at the same temperature and pressure, they diffuse till the mole fractions even out (Key and Ball, 2014). Therefore for purpose of methane analysis in set up, gas was drawn from each of the three repeated runs on the 14^{th} day of each run into a single balloon. The balloon was then tightly tied for the gas to be used later as chromatography sample. The same was repeated for all set ups. The methane composition was determined by extrapolation on the standard methane curve obtained using the same equipment at similar operating conditions.

3.13 Data analysis

Data from the Box-Behnken experimental design was analysed by response surface method (RSM) using a quadratic model on design-expert-fashion 12 (Montgomery, 2013). The output of the analysis gave conditions of temperature, percentage rumen inoculum and hydraulic retention time for optimum biogas production.

3.14 Digestate characterization

Only total solids and chemical oxygen demand was analysed for the purpose of estimating extent of the waste digestion in reactors utilizing *Bacillus subtilis*, *Escherichia coli*, *Bacillus subtilis* + *Escherichia coli* and none of the microbes.

The procedure for measurement of total solids described in section 3.3.1 was repeated on each digestate and mean values of residues calculated from a 2 ml pipetted sample

volume. The mean residues values were 55, 43, 42, 34, 35, 26.5 and 42 mg respectively for digesters A, B, C, D, E, F and G respectively from which total solids were calculated using equation 3.1.

The samples for digestate from all reactors mentioned in the preceding paragraph were analyzed for COD using method described in 3.3.4

After digestion the pH measurements were done on the digestate of each reactor using a hand held meter (model HI98127 from HANNA instruments, Romania) measuring pH and temperature at the same time.

3.15 Statistical analysis and data presentation

The experiment for each run was done three times. The mean value for gas produced on each day was calculated from which a cumulative figure for each day was obtained. The mean values for biogas produced were obtained using equation 3.12, where a1, a2, a3 are gases volumes produced on that day from the three runs.

$$(x_i) = \frac{a1 + a2 + a3}{3} \quad \text{.....Equation 3.12}$$

Standard deviation (SD) was calculated for each set of data obtained. As a rule for accepting data, standard deviation less than 2 implies variation of data from the calculated mean and thus hypothetical true value is very small thus acceptable (Montgomery and Runger, 2003). Any point with standard deviation greater than 2 was repeated.

The cumulative gas for each day was calculated by adding the daily average gas produced to those of previous days for the run, using equation 3.13.

$y_i = = x_1 + x_2 + \ldots + x_i$ .....Equation 3.13

Where x_1, $x_2 \ldots x_i$ is the average gas produced on the each day from equation 3.12.

The standard deviation was calculated using equation 3.14.

$SD = \sqrt{\left[\frac{\sum(xi-x)^2}{n} \right]}$ .....Equation 3.14

Where xi is the mean daily biogas produced while x is production in each experiment on that day and n is the number of data points.

The standard deviation for each data point was calculated and tabulated. As a rule for accepting data, 99.8% standard deviation less than 3 implies variation of data from the calculated mean and thus hypothetical true value is very small thus acceptable (Montgomery and Runger, 2003). Standard deviation error bars were used to predict reliability of mean data values and their statistical significance.

In optimization part, the experiment for each run was done in duplicate. The gas for each run was measured and mean values for an individual run calculated for each day using equation 3.15.

Mean value ($\bar{a}_n$) = $\frac{a1 + a2}{2}$ .....Equation 3.15

The cumulative gas for each day was calculated by adding the mean values of gas produced for that day and previous days as in equation 3.16.

Cumulative gas for the nth day = $\bar{a}_1 + \bar{a}_2 + \ldots + \bar{a}_n$ .....Equation 3.16

Response surface methodology involving the use of quadratic equation as desired for optimization problems was used, (Montgomery, 2013). Design expert version 12 software was used to analyze the data using the two way ANOVA. A statistical report describing the suitability and reliability of the data and model have been provided in the analysis section.

Biogas potential

Since the total solids were 64000 mgL^{-1} liquid waste, then total solids in 190 ml of substrate digested in 0, 20 and 50% rumen inoculum were given by equation 3.17 while those in digesters using *B.subtilis*, *E.coli*, *B.subtilis* + *E.coli* and without any of the microbes were given by equation 3.18.

Total solids digested (solids in 190 ml waste) = $\frac{190}{1000} \times 64000$ mg = 12160mg.

For a measured biogas volume of R mls,

Biogas potential = $\frac{R}{12160}$ $mlmg^{-1}TS$…….……...Equation 3.17

The substrate total solid calculated was 64000 mgL^{-1} Therefore in 380ml of substrate digested by bioaugmentation using *Bacillus subtilis*, *Escherichia coli*, *Bacillus subtilis* + *Escherichia coli* was given by;

Total solids = $\frac{380}{1000} \times 64000$ mg = 24320mgTS.

Biogas potential = $\frac{R}{24320}$ $mlmg^{-1}TS$……Equation 3.18

T test

Is a test of statistical significance difference between two groups of data (Montgomery and Runger, 2003). There are two possible hypotheses. The null hypothesis is that there is

not a significant difference between the two as observed differences may be due to chance and sampling error. The alternative hypothesis is that there is a significant difference between the two groups; the observed differences are most likely not due to chance or sampling error. The t value was calculated using equation as shown in equation 3.19. The T-Distribution tables in Beyer (2017) and Glen (n.d) are the source of T critical (at any given degree of freedom).

$$t = \frac{|(\boldsymbol{x1}-\boldsymbol{x2})|}{\sqrt{(\boldsymbol{y1}+\boldsymbol{y2})}} \quad \text{Equation 3.19}$$

Where $y_1 = \frac{(s1)\text{^}2}{n1}$ and $y_2 = \frac{(s2)\text{^}2}{n1}$

x_1 = the mean of sample 1

s_1 = standard deviation of sample 1

x_2 = the mean of sample 2

s_2 = standard deviation of sample 2

n_2 = sample size in sample 2

The critical T-value is set at p vale of 0.05. In a t-test, the degree of freedom is the sum of the sample sizes of both groups minus 2. The alternative hypothesis holds when the calculated t-value is larger than the T-critical value read from standard tables.

CHAPTER 4. RESULTS AND DISCUSSION

4.0 Composition and physicochemical properties of mixed abattoir waste

Abattoir waste streams observed were blood, intestinal waste and rumen solids. These streams were mixed in production ratios to obtain mixed waste which was characterized for total solids, total suspended solids, chemical oxygen demand and total nitrogen. The results are summarized in table 4.1.

Table 4.1: Summary of composition and physicochemical properties of abattoir waste

Streams	**Percentage composition (%)**
Blood	19.88
Intestinal waste	20.08
Rumen solids	60.04
Physiochemical properties	**Concentration (mg/L)**
Total solids	64000.00
Total suspended solids	36000.00
Chemical oxygen demand	199013.61
Total Nitrogen	831.92

These results suggested that the rumen solids were the dominant waste produced as compared to blood and intestinal wastes. Rumen waste formed 60.04% of the average slaughter waste from sheep. This was followed in abundance by intestinal waste and blood in almost equal proportions at 20.08% and 19.88% respectively. Rumen and intestinal wastes gave a total of 80.12% total organic waste. The figures obtained in this study compares favourably with the findings of Klintenberg et al (2014) in which it was recommended that the most optimum mixture of slaughterhouse waste was relatively large amounts of stomach and intestine content. It is by now generally accepted that rumen solids is the most abundant waste in abattoirs. Such substrate mixture reflecting

the actual ratio of waste generated in the abattoir process could be applied as substrate for biogas production in anaerobic digestion

Characterization of physicochemical properties makes it clear that abattoir waste is rich in organic matter content. The pH of 7.0 means that mixed abattoir waste is a neutral solution. Therefore adjustment of pH for commencement of anaerobic digestion process is not necessary. High nitrogen possibly from blood protein may cause generation of ammonia that has been identified as an agent of inhibiting the biogas production. This result ties well with previous studies wherein the high chemical oxygen demand and total nitrogen values could mainly be attributed to blood while the total solids and total suspended solids to liquid manure. Tritt and Schuchardt (1992) found that blood was a high contributor of organic loads with up to 375,000 mg/l COD. In other studies, blood and partially digested plants were found to contribute highly to COD, total solid and total nitrogen (Bazrafshan, Mostafapour, Farzadkia, Ownagh and Mahvi, 2012; Mittal, 2014; Yaakub, Mohamed, Al-Gheethi and Kassim, 2018). The total organic carbon analyzer was not available and thus it was not possible to investigate total organic carbon. Therefore the carbon/nitrogen (C/N) ratio of the mixed abattoir waste could not be estimated. The physicochemical properties measured represent nutrients to be used by microbes during anaerobic digestion. The high values of total solids and COD is a clear indication that mixed abattoir waste is a good substrate for biogas production. The concentration of each property is expected to reduce during aerobic digestion as is converted into biogas and byproducts of digestion. The higher the depletion of nutrients, the more gas is produced.

4.1 Enhancing biogas production from abattoir waste using rumen inoculum

4.1.1 Statistical analysis and biogas yield

Biogas was measured on a daily basis for the experimentation period where each set of experiment was repeated three times. Refer to Appendix B for the raw data for daily biogas yield from abattoir waste using rumen inoculum. Standard deviation (SD) for biogas produced each day was analysed using equation 11 in section 3.16 and found to fall within $\pm$2SD (Montgomery and Runger, 2003), hence acceptance of results. The mean biogas volume production results were as summarized in table 4.2 while analysed standard deviations for each data point were as presented in Table 4.3 where A, B and C represent 0,20 and 50% rumen inoculum respectively.

Table 4.2: Daily mean biogas yield (mls) using rumen inoculum of varying percentages

Day	0% rumen inoculum (A)	20% rumen inoculum (B)	50% rumen inoculum (C)
1	1.83	4.07	9.20
2	6.97	158.33	185.90
3	33.53	201.43	208.47
4	45.43	145.57	151.87
5	66.07	87.07	97.57
6	69.40	77.77	46.13
7	97.10	45.27	47.63
8	45.70	36.8	38.07
9	39.87	14.8	9.63
10	28.13	5.27	6.73
11	18.87	6.67	4.13
12	23.83	16.93	5.80
13	19.13	5.00	8.13
14	12.23	3.73	3.33
15	10.33	2.00	0.47
16	11.57	0.53	2.70
17	7.90	1.20	1.97
18	5.27	1.00	0.70
19	4.93	0.00	1.33
20	7.80	1.87	0.33
21	9.73	0.00	1.33
22	11.47	1.93	1.00
23	3.40	0.00	1.80
24	11.13	1.87	0.67
25	5.13	0.00	1.87
26	8.47	0.53	0.80
27	5.93	1.07	1.13
28	7.40	0.47	0.53
29	5.53	0.87	0.80
30	3.87	0.33	0.33

Where A, B and C represent, 0, 20 and 50% Rumen inoculum solution in cow dung solution (v/v) respectively.

Table 4.3: Analysed standard deviations for daily biogas produced using rumen inoculum

	Standard deviation Values		
DAYS	0%(A)	20%(B)	50%(C)
1	0.17	0.09	0.57
2	0.33	0.21	0.37
3	0.41	0.39	0.19
4	0.08	0.73	1.32
5	0.09	0.36	1.82
6	0.86	0.31	0.31
7	0.70	0.43	0.24
8	0.14	0.00	0.82
9	0.25	0.16	0.40
10	0.84	0.09	0.25
11	0.09	0.41	0.19
12	0.05	0.68	0.98
13	0.52	0.57	0.59
14	0.05	0.25	0.81
15	0.47	0.43	0.52
16	0.05	0.41	0.08
17	0.08	0.09	0.26
18	0.21	0.00	0.50
19	0.09	0.00	0.47
20	0.33	0.66	0.47
21	0.41	0.00	0.52
22	0.34	0.66	1.41
23	0.33	0.00	1.30
24	0.74	0.09	0.94
25	0.94	0.00	1.32
26	1.09	0.75	1.13
27	0.75	0.75	0.81
28	0.43	0.66	0.75
29	0.77	0.62	0.57
30	0.50	0.47	0.47

Where A, B and C represent, 0, 20 and 50% Rumen inoculum solution in cow dung solution (v/v) respectively.

4.1.2 Biogas potential and methane content

Analysis of methane percentage in biogas

Using the standard methane parameters of 99.50% gas, volume of 3.00 µl and a measured area of 1263.00 units, the chromatograph produced peak area and time plot as shown in Figure 4.1. The standard linear curve of the form in Equation 4.1 was achieved from a one point plot of methane percentage against peak area in Figure 4.2 .

$y = 0.078x$ ……..Equation 4.1

Where x represented the peak area while y was the methane percent. The equation of the curve can be used in estimation of unknown concentration if the value of R^2 is 0.95 or more. This value should be close to 1 as possible. The value of 1 in this case means curve was suitable for interpolation usage. Samples collected from set ups A (0% rumen inoculum), B (20% rumen inoculum) and C (50% rumen inoculum) were tested using same equipment set at same conditions to obtain respective peak area, peak time and volume used. See Appendix C on methane composition analysis of biogas produced from abattoir waste using rumen inoculum.

Chromatograph report for standard methane gas

Methane content in standard sample

Printing time: Sun Sep 29 10:35:06 2019

Injection time: Sat Sep 28 21:00:00 2019

GC model 3420A, MRC-UK

Column- HP-PLOT/Q Agilent

Column Temp- 150

Injector Temp- 200

Split ratio- 100:1

Detector -FID

Detector Temp- 250

Nitrogen (carrier gas) flow rate 200mls/min

Air flow- 300mls/min

Hydrogen flow- 40mls/min

Sample volume- 3mls

Standard methane gas chromatograph peak area and time

File opened: C:\Users\PC\Desktop\Peak-ABC\program\Mathane Standard 3.hw

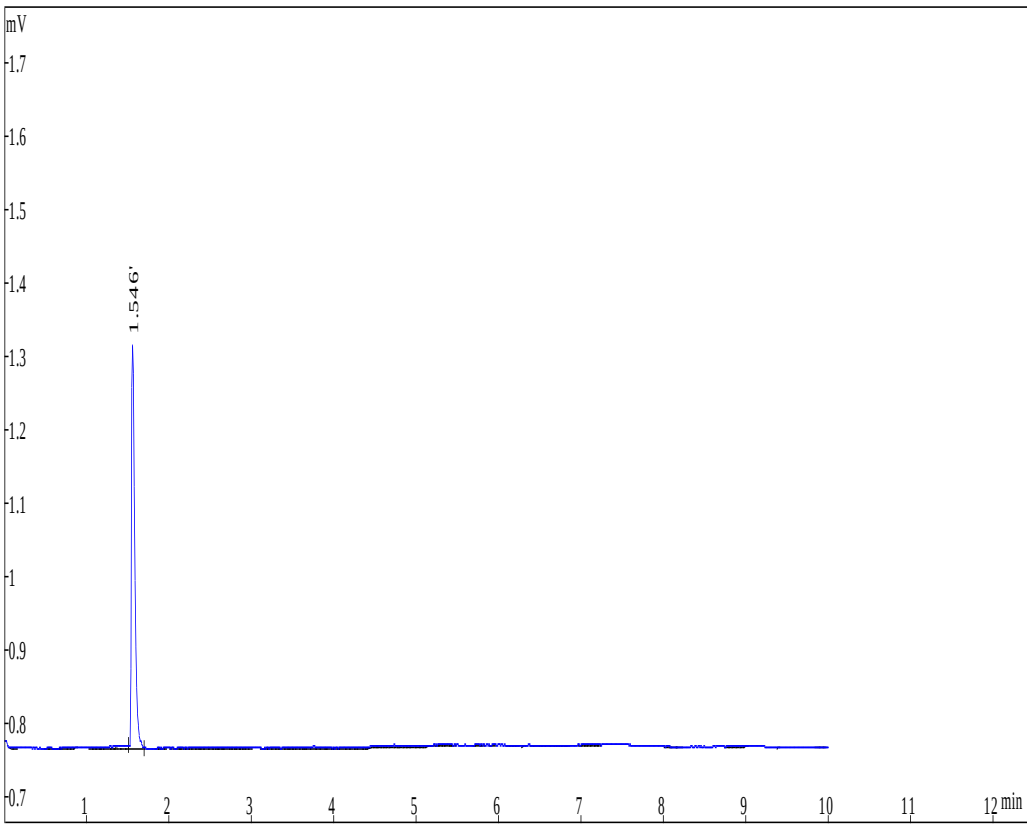

Figure 4.1: Standard chromatograph peak time and area for methane

---

Rank Time Name Area% Area

---

1 1.546 100 1263

Total 100 1263

RESULTS

Standard sample represents 3mls, 99.50 % methane.

Area = 1263.00

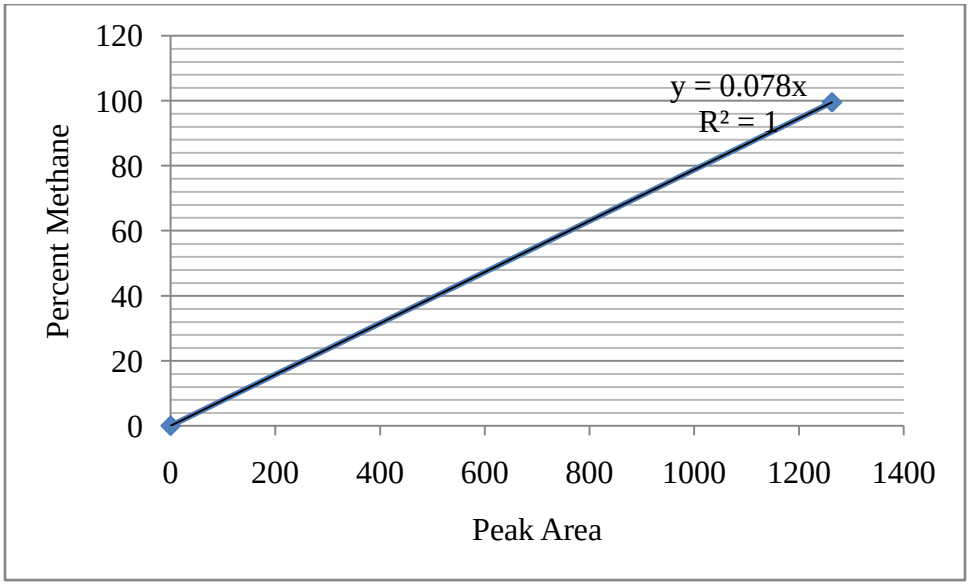

Figure 4.2: Standard methane percentage against peak area plot

The standard curve was obtained by plotting methane percent against peak area in a one point plot. The standard linear curve was of the form y = 0.078x whereby x is the peak area while y is the methane percent. The equation of the curve can be used in estimation of unknown concentration if the value of R^2 is 0.95 or more. This value should be close

to 1 as possible. The value of 1 in this case means this curve can be used to estimate unknown values of concentration. The equation was converted into another equation that was used for calculating the methane percentage of biogas which can be given using Equation 4.2.

Methane percentage in biogas = 0.078 × Peak area × VF…….....Equation 4.2

Where VF is the volume factor and is given in Equation 4.3.

$$VF = \frac{\textbf{Standard methane volume injected in the chromatograph,}}{\textbf{Sample biogas volume injected in the chromatograph,mls}}$$……....Equation 4.3

4.1.3 Biogas potential

A summary of mean biogas yield, biogas potential and methane percentage is provided in table 4.4.

Table 4.4: Quality of biogas produced using different rumen inoculum percent

Reactor	Yield ml (30day)	Potential ml/mgTS	Percent Methane
0%RI	627.97	0.052	48.91
20%RI	820.37	0.068	54.13
50%RI	840.37	0.069	60.12

4.1.4 Digestate characteristics

The total solids were calculated using equations 1 in section 3.3.1 while COD were calculated using equation 5 in section 3.3.4. The calculated mean total residue solids from reactors using 0%, 20% and 50% rumen inoculum were 55.0, 43.0 and 42.0mg which represented 27500, 21500 and 21000mgl^{-1} respectively. The mean absorbance for digestate from reactors that used 0, 20 and 50% rumen inoculum were 0.166, 0.145 and 0.142 which were equivalent to chemical oxygen demand of 86448.17, 75453.40 and

73882.72 mgl^{-1} respectively. The digestate from reactors using 0, 20 and 50% rumen inoculum had a pH of 7.6, 7.2 and 7.1 respectively.

4.1.5 Digestion of abattoir waste using rumen inoculum

All digesters experienced biogas production on the first day of the experiment and started increasing on the second day. However, gas production in digesters with rumen inoculum increased more sharply on the second day to reach optimum on day 3 with 50% rumen inoculum recording slightly more gas than reactor with 20% rumen inoculum. The production then started to decline sharply in the two reactors with rumen inoculum till day 13, recording below 2ml as from day 15 and gas becoming insignificant as from day 18. The reactor with 0% rumen inoculum (A) had the lowest increase in gas production taking 7 days to produce its peak biogas. Gas production continued in a decreasing unsteady manner and still produced above 2ml of biogas 30 days later. This rumen inoculum can be used to effectively reduce the retention time in digestion of mixed abattoir waste. The cumulative production with standard error bars in achieved was as shown in Figure 4.3.

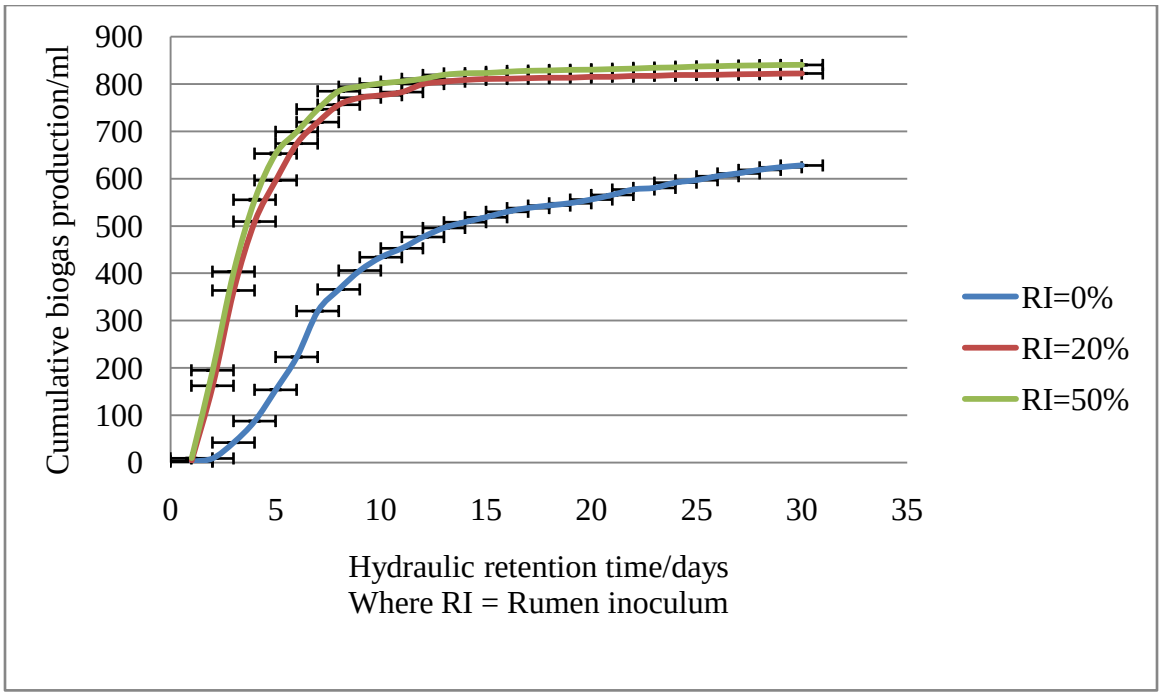

Figure 4.3: Cumulative biogas produced using different rumen inoculum percentages

The vertical error bars for the standard deviation are invisible whereas the horizontal ones are visible on scatter plot. Small error bars suggest high reliability of mean values in representing data sets. However, the small vertical error bars make it difficult to optically conclude about statistical significance of the data sets. Checking of statistical significance can preferably be done using P-test. However, P test requires more than thirty data points whereby each data point has got a calculated value of standard deviation. Where there are less than thirty data points which means dealing with insufficient data points such as in this case calls for use of T values over P values.

T_1 represented T test for the difference between the biogas volume means of reactor using 0% and 20% whereas T_2 was that between 0% and 50% rumen inoculum. T critical ($\propto = 0.05$) at degree of freedom 4 was 2.776 whereas T_1 and T_2 were found to be 230.277 and 251.599 respectively. The null hypothesis was rejected in both cases since

T_1 and T_2 were larger than T critical. The alternate hypothesis that the differences in the means of biogas volume for the reactors using 20 and 50% rumen fluid inoculum were statistically significant was adopted. The difference between volume of biogas produced using 20 and 50% rumen inoculum was similarly significant as T_3 was 129.679. It therefore means that the choice of interval range for rumen inoculum is justified.

Cumulatively, using 20 and 50% rumen inoculum achieved the highest gas production within the first 5 days and had at least more than 98% of their total respective cumulative for the 18 days of active gas production. This was more than total 30 day cumulative for biogas from reactor that did not use rumen inoculum. Reactor with 50% rumen inoculum had the highest cumulative at 840.37 ml followed by 20% rumen inoculum at 822.37ml and lastly 627.97ml for the reactor without rumen inoculum for the 30 days of the experimentation. From these results it is clear that rumen fluid inoculum accelerate anaerobic digestion of abattoir waste when used together with cow dung fluid as opposed to when cow dung fluid is used solely as the inoculum. A similar conclusion was reached in other studies (Seon, Creuly, Duchez, Pons and Duscap, 2003; Jin, Xu and Yang 2018). Jin et al (2018) found that rumen contain microbes that carry out hydrolysis and acidogenesis. It also has microbes that maintain methanogenesis step through the two known methanogenic pathways including hydrogenotrophic and acetoclastic methanogens that give higher biogas and maintain methane content at about 60%. The reason for this is that methanogens are dominated by *methanobacterium* and *methanosarina* that co-occur for methane production during anaerobic digestion. Budiyono et al (2014) found that, rumen fluid of ruminant animal when used as inoculum cause biogas production rate and efficiency to double in comparison to substrate without

rumen fluid inoculums. Increasing rumen content also increases biogas production. Rumen fluid content of 50% gave the best performance for biogas and methane content than that with 20%. The rapid growth of the microbes controlling the four biogas production steps enabled high gas production to be achieved. Initially reactor with 50% rumen inoculum had high concentration of microbes and thus experienced higher production of gas than B. Digester C realized 555.43 mls of gas in 4 days corresponding to 66.11% of the 30 day cumulative gas and had produced over 95% of the total by the 9^{th} day of the experiment while 20% rumen fluid attained it on the 11^{th} day. The decline in gas production after the 5^{th} day in reactor with 50% rumen fluid corresponded to the depletion of substrate in the digester. After 6 days of the experiment, growth of microbes in reactor with 20% rumen inoculum could have increased activity and thus gas production rise to start leading the reactor with 50% rumen inoculum whose substrate concentration was declining fast. After 30 days, reactors using 20 and 50% rumen inoculum produced 194.40 and 212.40 mls more gas than reactor without rumen inoculum. This was an increase of 30.96% and 33.82% respectively. Other studies show that the use of inoculum with between 20-50% rumen fluid can significantly increase biogas from anaerobic digestion of other wastes and can achieve hydraulic retention times in the range of multi stage digestion systems (Budiyono et al, 2014). The good digestion achieved in reactor with 20% and 50% rumen inoculum was indicated by the higher cumulative average biogas which was 822.37 mls and 840.37 mls with biogas potential 0.068 and 0.069 ml/mgTS respectively compared to the reactor that did not use rumen inoculum which produced an cumulative average biogas of 627.97 mls with a biogas potential of 0.052 ml/mgTS. The corresponding methane percentage for reactor

with 20 and 50% rumen inoculum were 54.13 and 60.22% respectively as compared 48.91% methane for reactor that did not use rumen inoculum. These values when expressed as total methane of the biogas produced suggest that more quality biogas could be produced from abattoir waste as percentage of rumen inoculum increases from 0 to 50% implying that a balance between microbes responsible for hydrolysis and acidogenesis with those for acetogenesis and methanogenesis seems to be achieved as rumen inoculum increases in the experiment. It also indicates that rumen inoculum contain microbes that can digest abattoir waste more as compared to use of cow dung fluid. Rumen inoculum when used together with cow dung fluid supply the microbes for hydrolysis, acidogenesis and acetogenesis and excellent methanogenesis. Rumen fluid content of 50% gave the best performance for biogas and methane content than that with 20% The contribution of rumen inoculum is twofold. First, quantity of microbes increase with the increasing homogenous volume. Secondly, rumen fluid contain more carbohydrates necessary for adjusting the C/N ratio arising from protein in the blood waste to the required range and hence a better digestion. Experiment using 20% and 50% operated in the optimal range of pH for biogas production which was 7.3 and 7.2 given that the optimal range is between 6.8 and 7.4 (Ranalli, 2007). The digestate of reactor without rumen fluid had a pH of 7.6 which could be caused by ammonia production explaining why the process was slow and prolonged. These results were complimented by the percent depletion of total solid and COD which were 57.03 and 56.56%, 66.41 and 62.09% lastly, 67.19 and 62.88% respectively for reactors with 0, 20 and 50% rumen fluid respectively. The quantity of methane calculated from the percentage and biogas quantity was 307.14, 445.15 and 505.23 mls respectively and the comparative increase in

methane quantity in reactors with 20 and 50% rumen fluid over the reactor with no fluid inoculum was 44.93 and 64.50% respectively. Previous studies on rumen microbial composition and metabolism suggest that bacteria and protozoa are predominant microbes in the rumen being fibrolytic, amylolytic and proteolytic types which preferentially digest structural carbohydrates, non-structural carbohydrates and proteins respectively (Cronje and Boomker, 2000; Dijkstra, Forbes and France, 2005). Fungi hydrolyse diets without fibre and help break down digesta particles while rumen archae are mostly autotrophic methanogens and produce methane. Most of the hydrogen produced by bacteria, protozoa and fungi is used by these methanogens to reduce carbon dioxide to methane (Hobson and Stewart, 1997; McCabe, Antille, Birf, Spence and BFernana, 2014).

The results show that use of rumen fluid together with fresh cow dung fluid does seem to impact the anaerobic digestion of mixed abattoir waste and quality of biogas produced. As discussed this is due to the fact that rumen fluid contain microbes that can carry out the four steps of anaerobic digestion in an effective manner.

4.2 Effectiveness of biogas production from abattoir waste using bioaugmentation

4.2.1 Identification of *Bacillus subtilis*

Characteristics of microbes indicated here are useful for identification purposes.

Morphology of *Bacillus subtillis*

The microorganism appeared as rod shaped organism with endospores (fairly rectangular rods, often occurring in pairs or chains with a mottled appearance with endospores). Bacillus colonies appeared typically white and dry or pasty looking, but some formed very mucoid colonies (that dripped on to the lid of the plate).

Biochemical test

With gram stain, *Bacillus subtilis* appeared purple in color, hence gram positive. Gram-negative cells are decolorized by the alcohol-acetone solution and take a pink to red color when counter stained with safranin. Gram-Positive cells retain the crystal violet and remained purple to dark blue. Figure 4.4 (a) is a photograph of *B.subtilis* smear as seen under a light microscope.

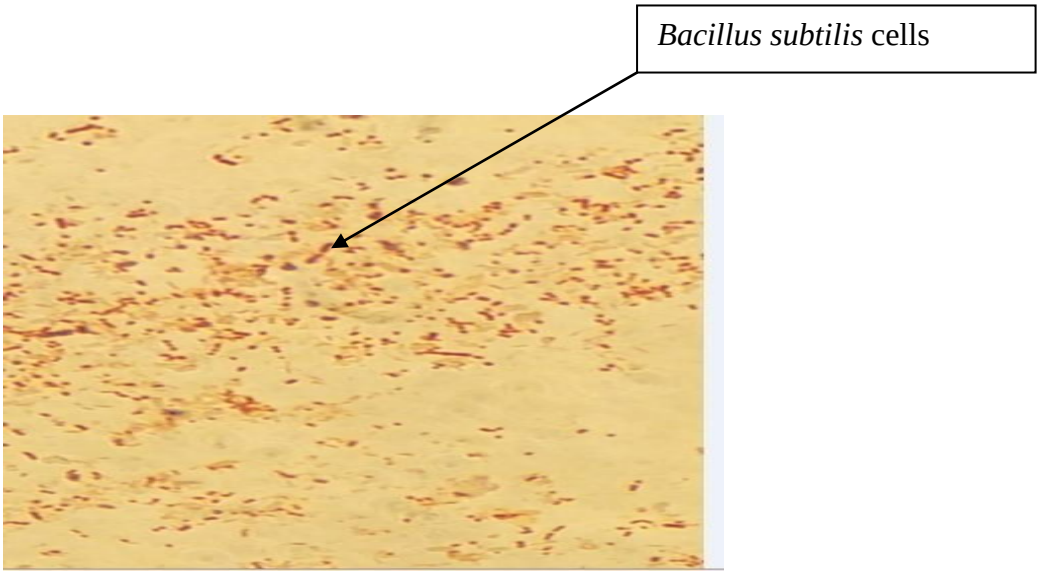

Figure 4.4(a): Gram stained *Bacillus subtilis* cells

4.2.2 Identification of *Escherichia coli*

Biochemical tests

The *E.coli* isolate gave the following results in IMViC and Gram tests.

Indole test

They formed a red/pink coloured ring at the top which indicated a positive reaction for *E.coli.*

Methyl Red test

A red colour developed, indicating a positive reaction that occurs when bacteria have produced enough acid to neutralize the phosphate buffer. Yellow discoloration could have occurred to methyl red negative bacteria. The *E.coli* was methyl red (MR) positive.

Voges-Proskauer test

After 1 hour, there was no colour change; the isolate was categorized as Voges-Proskauer (VP) negative confirming it to be *E.coli*. *E.coli* is VP negative.

Citrate utilization test

The slant remained green, a feature for negative test. *E.coli* is citrate negative. Citrate agar media contains a pH indicator called bromthymol blue. The agar media changes from green to blue at an alkaline pH. Citrate in the media breaks down to oxaloacetate and acetate due to action of enzyme citritase. Oxaloacetate is further broken down to pyruvate and carbon dioxide (CO_2). Production of sodium carbonate (Na_2CO_3) from sodium citrate changes the media into alkaline pH, hence the colour change from green to

blue. The only enterobacteriaceae member that show the same pattern as *E.coli* is *Proteus vulgaries*, but *proteus spp.* are lactose-negative, motile and show swarming behavior.

Gram test

A gram stain process left *E. coli* colony with pink colour and thus it was gram negative. Figure 4.4(b) is a photograph *of E.coli* smear as seen under a light microscope.

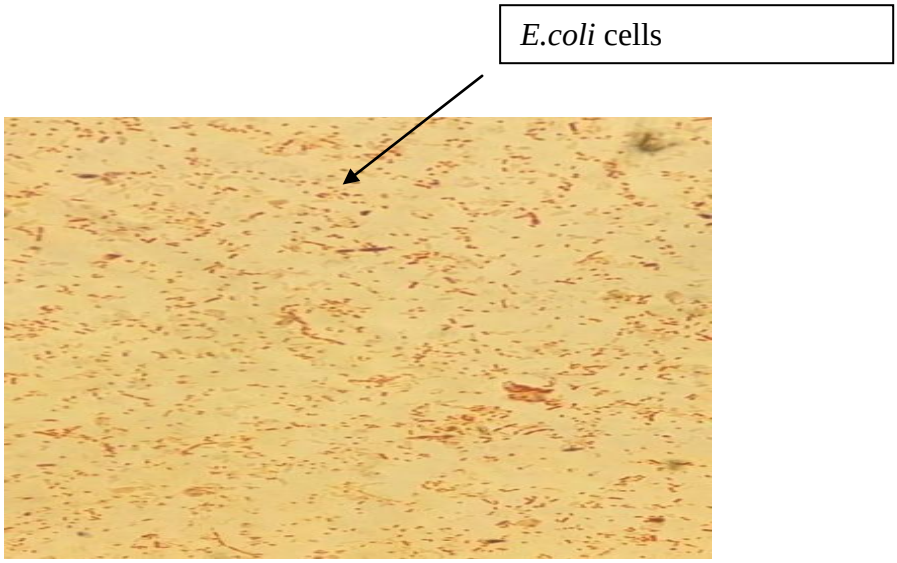

Figure 4.4(b): Gram stained E.coli bacteria as seen under light microscope

4.2.3 Biogas yield

Biogas was measured on a daily basis for the experimentation period of 25 days where each set of experiment was repeated three times. Refer to Appendix D for the raw data for daily biogas yield from abattoir waste using bioaugmentation and rumen inoculum. Standard deviation (SD) for biogas produced each day was analysed using equation 11 in section 3.16 and found to fall within $\pm 2SD$ (Montgomery and Runger, 2003), hence acceptance of results. The daily mean volume of biogas were as in table 4.5 while standard deviations were presented in table 4.6 and.

Table 4.5: Daily mean biogas yield (mls) using 50% RI and bioaugmentation

Day	***B.subtilis*(D)**	***E.coli*(E)**	***B.subtilis*+*E.coli*(F)**	**None(G)**
1	19.73	16.70	18.60	22.00
2	246.93	204.8	247.27	252.07
3	434.33	414.83	482.87	353.60
4	357.67	273.93	421.67	300.07
5	257.33	268.33	257.20	230.93
6	183.87	194.93	206.53	157.33
7	146.53	163.47	189.30	136.80
8	78.40	104.13	109.27	52.00
9	44.00	97.23	39.07	46.20
10	33.93	80.93	20.80	43.60
11	22.53	27.40	13.40	33.33
12	16.47	8.07	5.93	21.20
13	8.93	3.47	2.27	14.20
14	1.80	1.53	1.07	2.87
15	0.00	0.00	0.40	1.13
16	0.00	1.20	0.80	1.13
17	0.00	2.03	0.00	0.33
18	0.00	0.00	0.00	0.80
19	0.00	0.00	0.00	1.47
20	0.00	0.00	0.00	0.67
21	0.00	0.00	0.00	0.00
22	0.00	0.00	0.00	1.20
23	0.00	0.00	0.00	1.00
24	0.00	0.00	0.00	0.00
25	0.00	0.00	0.00	0.40

Table 4.6: Analysed standard deviations for biogas yields from impact of bioaugmentation

	Standard deviation Values			
DAYS	D	E	F	G
1	0.04	0.54	0.43	0.82
2	0.33	1.84	0.53	0.66
3	0.94	0.94	0.84	1.56
4	0.62	0.74	0.77	1.56
5	0.41	0.50	1.70	0.77
6	0.82	0.96	1.15	0.47
7	0.74	0.61	0.57	0.57
8	0.99	0.66	1.52	1.41
9	0.71	0.52	1.32	0.59
10	1.64	0.54	1.02	0.85
11	1.00	1.00	1.02	0.47
12	1.11	1.51	0.82	0.85
13	0.53	0.38	0.24	0.42
14	0.85	0.38	0.60	0.38
15	0.00	0.00	0.57	0.09
16	0.00	0.00	0.57	0.19
17	0.00	0.08	0.00	0.47
18	0.00	0.00	0.00	0.57
19	0.00	0.00	0.00	0.19
20	0.00	0.00	0.00	0.47
21	0.00	0.00	0.00	0.00
22	0.00	0.00	0.00	0.03
23	0.00	0.00	0.00	0.09
24	0.00	0.00	0.00	0.00
25	0.00	0.00	0.00	0.57

Where D, E, F and G represent *Bacillus subtilis*, *Escherichia coli*, *Bacillus subtilis* + *Escherichia coli*, and none of the microbes respectively.

4.2.4 Methane content of biogas

See Appendix E for chromatograph results of methane composition analysis for biogas produced using bioaugmentation and rumen inoculum. Biogas from reactors inoculated with *B.subtilis*, *E.coli*, *B.subtilis* + *E.coli* and none of the microbes were found to contain 62.24, 62.71, 66.92 and 60.37% methane respectively.

4.2.5 Digestate characteristics

The total solids were calculated using equations 1 in section 3.3.1 while COD were calculated using equation 5 in section 3.3.4. The calculated mean total residue solids from reactors using *Bacillus subtilis*, *E.coli*, *Bacillus subtilis* plus *E.coli* and without bioaugmentation were found to be 34.0, 35.0, 26.5 and 42.0mg which represented 17000, 17500, 13250 and 21000mgl-1 respectively. The equivalent calculated COD values for digestate from reactors that used *Bacillus subtilis*, *E.coli*, *Bacillus subtilis* plus *E.coli* and none of the microbes for bioaugmentation were 60793.72, 61317.28, 42469.11 and 61317.28mgl^{-1} respectively. Similarly, digestate from reactors using *B.subtilis, E.coli, B.subtilis* + *E.coli* and rumen inoculum only had a pH of 7.1, 7.2, 7.2 and 7.2 respectively

4.2.6 Biogas potential

Table 4.7 has a summary of mean biogas yield, biogas potential and respective methane content.

Table 4.7: Quality of biogas produced using 50% rumen inoculum and bioaugmentation

Reactor	Yield ml (25day)	Potential ml/mgTS	Percent Methane
B.subtilis(D)	1852.47	0.076	62.24
E.coli(E)	1863.00	0.077	62.71
B.subtilis+ *E.coli*(F)	2016.43	0.083	66.92
None (G)	1674.33	0.069	60.37

Biogas was produced from mixed abattoir waste using a fresh rumen-cow dung fluids where rumen fluid set at 50% denoted as D, E, F and G for bioaugmentation using *B.subtilis*, *E.coli*, *B.subtilis* + E.coli and none of the microbes respectively. Biogas was measured on a daily basis for the experimentation period where each set of experiment was repeated three times.

Biogas production started slowly on the first day and increased significantly on the second day in all the four digesters inoculated with *B.subtilis, E.coli, B.subtilis +E.coli* and none of the microbes. There was very high gas production between the second and fifth day with peak on the third day for each experiment. Digester using rumen inoculum only led the others in gas production for the first two days after which it was overtaken by the reactors bioaugmented using *B.subtilis*, *E.coli*, *B.subtilis +E.coli*. These three reactors started producing more gas from third to eighth day. Only reactor with E.coli continued producing more gas up to the 10^{th} day. There was a rapid decline in biogas production in the three reactors using bioaugmentation recording zero gas production on the 15^{th} day. However, digesters inoculated with *E.coli* produced some gas between the 16^{th} and 17^{th} day stopping on the 18^{th} day completely. The decline of biogas production in reactor with rumen inoculum and without any bioaugmentation was slow and took 21 days to reach zero after which negligible gas was.

The temperature of 37 ^{o}C was conducive not only for the microorganisms in the inoculum consisting of 50% rumen fluid but also to the introduced *Bacillus subtilis* and *Escherichia coli* which grew very fast to sustain the high gas production in the first five days from the abattoir waste. The *B.subtilis*, *E.coli* and *B.subtilis +E.coli* introduced worked together with the consortia in inoculum to produce more biogas within a very short time depleting the substrate

The cumulative gas curves with standard error bars are shown in Figure 4.5.

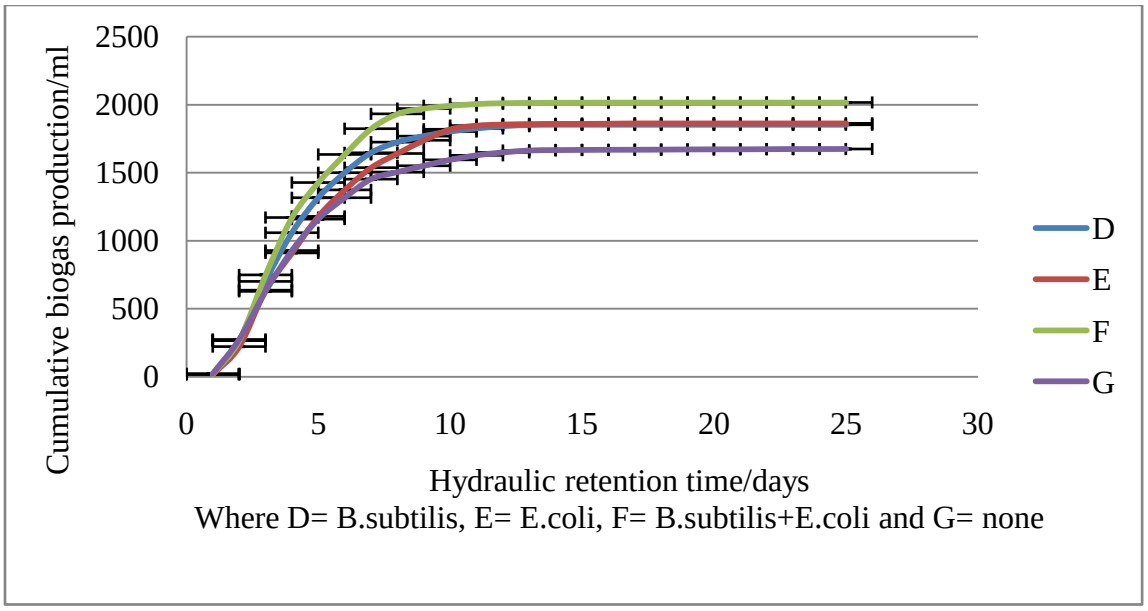

Figure 4.5: Mean cumulative biogas produced using bioaugmentation

The vertical error bars for the standard deviation are invisible whereas the horizontal ones are visible on scatter plot. Small error bars suggest high reliability of mean values in representing data sets. However, the small vertical error bars make it difficult to optically

conclude about statistical significance of the data sets. Due to less than thirty data points, T test is preferable in checking for statistical significance.

The values of T in the t-test were done using equation 19 in section 3.16, where T_4, T_5, represented t test for the difference between the biogas volume means of reactor using *Bacillus subtilis* and *Escherichia coli* over volume mean from reactor with no bioaugmentation respectively while T_6 and T_7 were tests for volume means produced by *Bacillus subtilis* + *Escherichia coli* over volumes produced by *Bacillus subtilis* and *Escherichia coli* as single species respectively. T critical ($\propto = 0.05$) at degree of freedom 4 was 2.776 whereas T_4 and T_5 were 41.684 and 69.874 respectively. The null hypothesis was rejected in both cases since T_4 and T_5 were larger than T critical. The alternate hypothesis that the differences in the means of biogas volume for the reactors using *Bacillus subtilis* and *Escherichia coli* were statistically significant from the mean volume of biogas produced from reactor without bioaugmentation. The difference between mean volume of biogas produced using *Bacillus subtilis* + *Escherichia coli* and *Bacillus subtilis* and *Escherichia coli* when used as single species were similarly significant as T_6 and T_7 were 44.648 and 92.113 respectively. Thus null hypothesis rejection and adoption of alternate hypothesis as in the case of T_4 and T_5.

The highest cumulative biogas was produced by digester bioaugmented using *B.subtilis* + *E.coli* that took a short hydraulic retention time of 16 days to reach optimum of 2016.43 ml. About 99.5% of this volume was achieved on the 11th day. Reactor using *E.coli* came second attaining an optimum of 1863.00 ml gas on the 17th day with 99.5% of this volume coming between the 11th and 12th days. This was closely followed by reactor using *B.subtilis* which produced an optimum of 1852.47 ml on the 14th day with 99.5% of this volume being achieved between the 12th and 13th day. Digester without bioaugmentation gave the least achieving a volume of 1674.33 after 25 days with 99.5% of this volume coming out between the 13th and 14th day as shown in Table 14 and Figure 15. The biogas potential and methane content in Table 13 followed a similar trend with a combination of *B.subtilis* and *E.coli* having the largest potential of 0.083 ml/mgTS and a methane content of 66.92%. This was followed by *E.coli* and *B.subtilis* with a biogas potential of 0.077 and 0.076 ml/mgTS and methane percent of 62.71 and 62.24% respectively. Digester with no bioaugmentation had the lowest biogas potential of 0.069 ml/mgTS and 60.37% methane in biogas. These results were confirmed by total solids and COD depletion percentages calculated from substrate and digestate characteristics in sections 4.1 and 4.3.5 which were 79.30 and 78.66% for *B.subtilis* + *E.coli*, 72.66 and 69.20 for *E.coli*, 73.43 and 69.45% for *B.subtilis* then lastly 67.19 and 63.14% respectively for reactor without bioaugmentation. The stoppage in biogas production was probably due to depletion of substrate and not inhibition as digestate pH was 7.1, 7.2, 7.2 and 7.2 for reactors using *B.subtilis, E.coli, B.subtilis* + *E.coli* and without bioaugmentation respectively. The digestate pH for all the reactors was within the suggested optimum for anaerobic digestion which is 6.7-7.4 (Ranalli, 2007). A

combination of *B.subtilis* and *E.coli* was used to bioaugment for protein hydrolysis and acetogenesis and gave high volume of biogas each day of the experiment. Reactor with *B.subtilis* led reactor using *E.coli* till the sixth day when it started lagging. Initially *Bacillus subtilis* hydrolysed proteintious substrate alongside natural consortia in inoculum improving hydrolysis. The available methanogens consumed the intermediate by products enabling the acetogens to survive well and the acetogenic inhibition might have occurred on day 5 consequently reducing biogas production in reactor using *B.subtilis*. Therefore four stages of hydrolysis, acidogenesis, acetogenesis and methanogenesis were in equilibrium for the first five days. Hydrolysis was a bit slower in reactor with *E.coli* which stabilized when reactor started producing more gas. The *E.coli* sustained acetogenesis consequently methanation was sustained and therefore reactor with started leading in biogas production over that with *B.subtilis* alone. This trend was observed until day 12 when reactor with *B.subtilis* again produced more biogas than that with *E.coli*. Reduced concentration of substrate ensured that hydrolysis and acidogenesis were also slowing down and this enabled acetogens to recover in time to supply nutrients to methanogens thereby continued biogas production for this reactor using *B.subtilis*. This could be attributed to the balanced digestion that depleted the intermediates faster in reactor with *E.coli*, giving reactor with *B.subtilis* (with higher acid concentration) the chance to lead once more after stabilization. At the prevailing conditions, most digestable substrate had been depleted by the day 15 thereby reactors using bioaugmentation started producing negligible gas. Digester without any bioaugmentation took 21 days to produce zero biogas implying that hydrolysis and the natural recovery of acetogens in consortia was a bit slower making the process to take longer at temperature 37 ^{o}C. On the 10th day

this reactor started producing more gas than all the other three digesters using bioaugmentation an indication that it requires a longer hydraulic retention time.

The increase of biogas in reactor using *B.subtilis* plus *E.coli* over that using none of the microbes was 20.47% while that for *E.coli* and *B.subtilis* were 11.27 and 10.64%. The quantity of methane generated was 1349.39, 1168.29, 1152.98 and 1010.79 mls in set ups using *B.subtilis* plus *E.coli*, *E.coli*, *B.subtilis* and none of the microbes for bioaugmentation respectively. Therefore, the comparative increase in methane quantity in reactors using *B.subtilis* plus *E.coli*, *E.coli* and *B.subtilis* over that using no bioaugmentation was 33.50, 15.58 and 14.07 % respectively. The results demonstrate two things. First, bioaugmentation using either protein digesting bacteria or hydrogen generating bacteria have an impact on anaerobic digestion of abattoir waste and quality of biogas produced. Secondly, if both protein digesting and hydrogen producing bacteria are used at the same time digestion of abattoir waste and quality of biogas are enhanced further. In line with previous studies the use of *Escherichia coli* in digester E gave results that were similar to other findings (Jayalakshmi et al, 2007); (Bagi et al, 2007). In another study Victor, Shajin, Roshni and Asha (2014) reported *Escherichia coli* as a modest range enhancer of biogas production in which tremendous gas production took place within short period of time. The use of *Bacillus subtil*is in digester D gave results that are in line with previous studies (Sonakya, Raizada and Kalia, 2001). We have verified that using *Bacillus subtilis* in anaerobic digestion of abattoir waste improves the process and quality of biogas. The simultaneous use of *Bacillus subtilis* and *Escherichia coli* gave the highest biogas potential and, methane content. This might be due to protein digestion and optimized acetogenesis step working together to create a pronounced impact. These

findings support the notion bioaugmentation optimizes biogas production from abattoir waste and that combining *Bacillus subtilis* and *Escherichia coli* in coaugmentation produces more biogas that is rich in methane content as compared to when single species of microbe is used. Overally, a better digestion is achieved with the two microbes working together on abattoir waste.

4.3 Optimization of temperature, percentage rumen inoculum and hydraulic retention time

The optimisation experiment was done in duplicate. The results were reported as cumulative mean biogas per day in table 4.8. See Appendix F for raw data on biogas yield for optimization of biogas production conditions from abattoir waste.

Table 4.8: Mean biogas production using Box-Behnken experiment design

	Natural variables			Coded variables			Biogas yield
Run	T(^{o}C)	PR%	HRT(days)	x1	x2	x3	y(ml)
1	35	20	15	-1	-1	0	815.90
2	35	100	15	-1	1	0	813.90
3	40	20	15	1	-1	0	838.40
4	40	100	15	1	1	0	829.40
5	35	60	12	-1	0	-1	818.65
6	35	60	18	-1	0	1	826.30
7	40	60	12	1	0	-1	838.00
8	40	60	18	1	0	1	836.80
9	37.5	20	12	0	-1	-1	835.90
10	37.5	20	18	0	-1	1	810.00
11	37.5	100	12	0	1	-1	824.60
12	37.5	100	18	0	1	1	826.80
13	37.5	60	15	0	0	0	826.00
14	37.5	60	15	0	0	0	818.05
15	37.5	60	15	0	0	0	823.30

The design expert output for average biogas produced in the designed experiment is presented in Table 4.9, Figures 4.6 and 4.7.

Fit summary: Warning: The Cubic model is aliased.
Response 1: Biogas yield

Sequence model sum of squares *[Type 1]*
Table 4.9: Design expert output for fitted model

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Mean vs Total	1.022E+07	1	1.022E+07			
Linear vs Mean	321.92	3	107.31	1.42	0.2884	
2FI vs Linear	21.19	3	7.06	0.0699	0.9744	
Quadratic vs 2FI	**805.12**	**3**	**268.37**	**432.28**	**< 0.0001**	**Suggested**
Cubic vs Quadratic	1.72	3	0.5725	0.8257	0.5884	Aliased
Residual	1.39	2	0.6933			
Total	1.022E+07	15	6.815E+05			

Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Table 4.9 (Continued)

Lack of fit tests

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Linear	828.02	9	92.00	132.70	0.0075	
2FI	806.84	6	134.47	193.95	0.0051	
Quadratic	**1.72**	**3**	**0.5725**	**0.8257**	**0.5884**	**Suggested**
Cubic	0.0000	0				Aliased
Pure Error	1.39	2	0.6933			

Lack of fit tests: Want the selected model to have insignificant lack-of-fit.

Model summary statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	8.68	0.2796	0.0831	-0.1520	1326.34	
2FI	10.05	0.2980	-0.2285	-1.0955	2412.60	
Quadratic	**0.7879**	**0.9973**	**0.9925**	**0.9734**	**30.60**	**Suggested**
Cubic	0.8327	0.9988	0.9916		*	Aliased

- Case(s) with leverage of 1.0000: PRESS statistic not defined.

Focus on the model maximizing the **Adjusted R^2** and the **Predicted R^2**.

Analysis of variance (ANOVA)
Response 1: Biogas yield

Table 4.9 (Continued)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1148.22	9	127.58	205.50	< 0.0001	significant
A-Temperature	185.28	1	185.28	298.44	< 0.0001	
B-RI	7.03	1	7.03	11.33	0.0200	
C-HRT	129.60	1	129.60	208.76	< 0.0001	
AB	0.0225	1	0.0225	0.0362	0.8565	
AC	0.0025	1	0.0025	0.0040	0.9519	
BC	21.16	1	21.16	34.08	0.0021	
A^2	715.10	1	715.10	1151.84	< 0.0001	
B^2	48.97	1	48.97	78.87	0.0003	
C^2	109.34	1	109.34	176.11	< 0.0001	
Residual	3.10	5	0.6208			
Lack of Fit	1.72	3	0.5725	0.8257	0.5884	not significant
Pure Error	1.39	2	0.6933			
Cor Total	1151.33	14				

Factor coding is **coded**. Sum of squares is **Type III – Partial.**

Fit statistics

Std. Dev.	0.7879	**R^2**	0.9973
Mean	825.47	**Adjusted R^2**	0.9925
C.V. %	0.0955	**Predicted R^2**	0.9734
		Adeq Precision	43.7883

The **Predicted R^2** of 0.9734 is in reasonable agreement with the **Adjusted R^2** of 0.9925; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 43.788 indicates an adequate signal. This model can be used to navigate the design space.

The **Model F-value** of 205.50 implies the model is significant. There is only a 0.01% 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 A, B, C, BC, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The model terms AB and AC are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 0.83 implies the Lack of Fit is not significant relative to the pure error.

Table 4.9 (Continued)

Fit summary

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.2884	0.0075	0.0831	-0.1520	
2FI	0.9744	0.0051	-0.2285	-1.0955	
Quadratic	**< 0.0001**	**0.5884**	**0.9925**	**0.9734**	**Suggested**
Cubic	0.5884		0.9916		Aliased

Coefficients

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	837.73	1	0.4549	836.56	838.90	
A-Temperature	4.81	1	0.2786	4.10	5.53	1.0000
B-RI	0.9375	1	0.2786	0.2214	1.65	1.0000
C-HRT	4.02	1	0.2786	3.31	4.74	1.0000
AB	-0.0750	1	0.3940	-1.09	0.9377	1.0000
AC	0.0250	1	0.3940	-0.9877	1.04	1.0000
BC	2.30	1	0.3940	1.29	3.31	1.0000
A^2	-13.92	1	0.4101	-14.97	-12.86	1.01
B^2	-3.64	1	0.4101	-4.70	-2.59	1.01
C^2	-5.44	1	0.4101	-6.50	-4.39	1.01

The coefficient estimate represented the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design was the overall average response of all the runs. The coefficients were adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicated multi-colinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Final Equation in Terms of Actual Factors

The final equation is given as;

Biogas Yield = 837.73333 + 4.81250Temperature + 0.937500RI + 4.02500HRT – 0.075000Temperature*RI + 0.025000Temperature*HRT + 2.30000RI*HRT – $13.91667Temperature^2 – 3.64167RI^2 – 5.44167HRT^2$……Equation 20

P-values for the model terms of the quadratic polynomial equation and the model coefficients in equation 21 indicate that temperature, percentage of rumen fluid inoculum and hydraulic retention time were influential terms affecting biogas production from abattoir waste using rumen inoculum. The only influential factor interaction term is that for percentage of rumen inoculum and hydraulic retention time.

Final Equation in Terms of Coded Factors

The final equation is given in coded factors as;

Biogas Yield = 837.73 + 4.81A + 0.9375B + 4.02C – 0.0750AB + 0.0250AC + 2.3BC – $13.91667A^2 – 3.64B^2 – 5.44C^2$ ……Equation 21

The equation in terms of coded factors was used to make predictions about the response for given levels of each factor. By default, the high levels of the factors were coded as +1 and the low levels as -1. The coded equation was useful for identifying the relative impact of the factors by comparing the factor coefficients.

Table 4.9 (Continued)

Statistical report

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	815.90	816.38	-0.4750	0.750	-1.206	-1.280	0.436	-2.218	3
2	813.90	814.35	-0.4500	0.750	-1.142	-1.188	0.391	-2.058	1
3	838.40	837.73	0.6667	0.333	1.036	1.046	0.054	0.740	13
4	829.40	829.44	-0.0375	0.750	-0.095	-0.085	0.003	-0.148	11
5	818.65	819.14	-0.4875	0.750	-1.237	-1.329	0.459	-2.302	6
6	826.30	825.85	0.4500	0.750	1.142	1.188	0.391	2.058	4
7	838.00	837.73	0.2667	0.333	0.415	0.377	0.009	0.267	15
8	836.80	837.73	-0.9333	0.333	-1.451	-1.705	0.105	-1.206	14
9	835.90	835.91	-0.0125	0.750	-0.032	-0.028	0.000	-0.049	12
10	810.00	809.56	0.4375	0.750	1.111	1.144	0.370	1.982	5
11	824.60	824.13	0.4750	0.750	1.206	1.280	0.436	2.218	2
12	826.80	827.24	-0.4375	0.750	-1.111	-1.144	0.370	-1.982	8
13	826.00	825.99	0.0125	0.750	0.032	0.028	0.000	0.049	9
14	818.05	817.56	0.4875	0.750	1.237	1.329	0.459	2.302	7
15	823.30	823.26	0.0375	0.750	0.095	0.085	0.003	0.148	10

The statistical report in table 4.9 above indicates that there were no outliers in the data points hence results can be used for analysis.

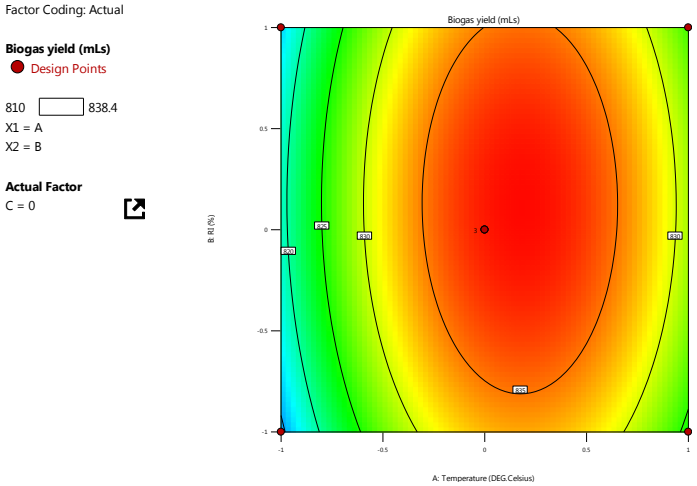

Figure 4.6: Contour plot

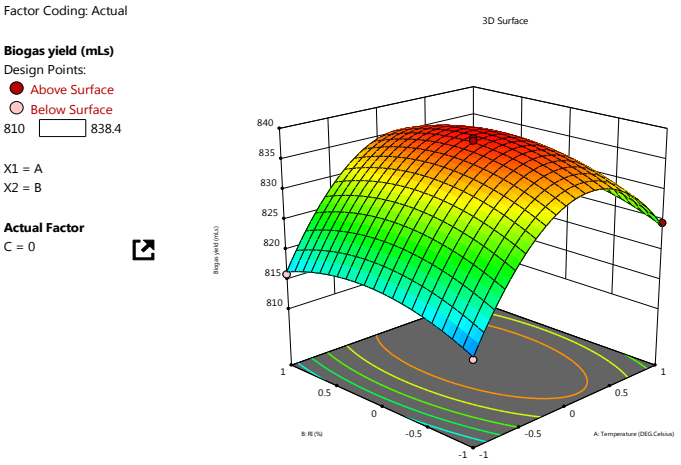

Figure 4.7: Response surface plot

4.3.1 Stationary point

Montgomery (2013) gives the solution to this point as $x_s = \begin{bmatrix} x1 \\ x2 \\ x3 \end{bmatrix}$

x_1 (temperature, ^{O}C), x_2 (Percent of rumen fluid in inoculum, V/V %), x_3 (hydraulic retention time, HRT in days)

Where $x_s = -\frac{1}{2} B^{-1}b$, Where x_s is the stationary point, $B = \begin{bmatrix} a & b & c \\ b & d & e \\ c & e & f \end{bmatrix}$

Where $a = \beta_{11}$, $b = \beta_{12}/2$, $c = \beta_{13}/2$, $d = \beta_{22}$, $e = \beta_{23}/2$, $f = \beta_{33}$, $b = \begin{bmatrix} m \\ n \\ p \end{bmatrix}$

$m = \beta_1$, $n = \beta_2$, $p = \beta_3$, Where $\beta_0 = 837.7333$, $\beta_1 = 4.81250$, $\beta_2 = 0.937500$, $\beta_3 = 4.02500$, $\beta_{11} = -13.91667$, $\beta_{12} = -0.075000$, $\beta_{22} = -3.64167$, $\beta_{13} = 0.025000$, $\beta_{23} = 2.30000$ and $\beta_{33} = -5.44167$

Using excel the matrix inverse of B is;

$$B^{-1} = \begin{bmatrix} -0.07186 & 0.000737 & -0.00000930902 \\ 0.000737 & -0.29424 & -0.062181338 \\ -0.0000093 & -0.06218 & -0.196908055 \end{bmatrix}$$

Again using Excel matrix multiplication, $B^{-1}b = \begin{bmatrix} -0.34516 \\ -0.52259 \\ -0.85089 \end{bmatrix}$

And, $x_s = -\frac{1}{2} B^{-1}b = \begin{bmatrix} x1 \\ x2 \\ x3 \end{bmatrix} = \begin{bmatrix} 0.172582 \\ 0.261293 \\ 0.425447 \end{bmatrix}$

Coded factors into Actual factors

$x_1 = \frac{T-37.5}{2.5}$ = 0.172582, T = 37.93^{O}C, $x_2 = \frac{RI\%-60}{40}$ = 0.261293, RI% = 70.45%

$x_3 = \frac{HRT-15}{3}$ = 0.425447, HRT = 16.28 days

4.3.2 Digestate pH

The digestate pH for each run was measured and given in Table 4.10.

Table 4.10: Digestate pH obtained from experiment using bioaugmentation to enhance biogas

Run	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
pH	7.3	7.2	7.0	7.1	7.4	7.3	7.0	7.1	6.8	6.9	6.8	6.8	7.0	7.0	7.0

4.4 Optimum temperature, percentage of rumen inoculum and hydraulic retention time for biogas production from abattoir waste

Using the coded factors, Design expert version 12 produced curves of biogas yield against each factor for the results. The curves indicated that there is a point within the optimum range for each factor corresponding to the maximum biogas yield. The yield of biogas increases sharply as temperature rises from 35.00 to 37.50 ℃ after which it reaches a maximum then starts falling as temperature is increased to 38.75 ℃. The fall in the yield again starts falling sharply as temperature is raised to 40.00℃ though more gas is obtained at 40.00 ℃ as compared to 35.00 ℃. The AD process is carried out by microbes and therefore sensitive to temperature. The activity of microbes increases with temperature to reach a maximum since temperature affects their activeness. The activity of microbes increases with increase in temperature and every microbe as a unique temperature at which their maximum activity is achieved. More biogas is produced at 40

℃ as the microbes are more active relative to growth and metabolism as compared to those at 35 ℃. This optimum temperature has to be close to the temperature of a live ruminant animal, natural host where these microbes are known to be very effective methane producers. Similar findings were reported by Ezekoye et al. (2011) and Uzodinma et al. (2007). The biogas yield vs percent rumen in inoculum curve show that the yield is lowest at 20% rumen inoculum and increases moderately to reach a maximum between 60 and 80%. Thereafter, it starts to fall when 100% rumen inoculum is used. It is also evident that more gas is achieved at 100% rumen inoculum compared to when it is 20%. Rumen inoculum has a composition of several microbes responsible for carrying out the four steps of biogas generation from hydrolysis to methanogenesis. The biogas yield vs the hydraulic retention time indicate that the yield is increased as the retention time is increased from 12.00 to 15.00 days holding temperature and percentage of rumen inoculum constant and reaches a maximum as the retention time approaches 16.50 days after which the increase in yield become insignificant and drops to zero when approaching 18 days for this substrate. Since HRT affects the extend of degradation, a reduction in biogas is expected as substrate gets depleted to a certain point. Figure 4.8 is a design expert graphical representation of relationship between biogas production from mixed abattoir waste with the three factors (temperature, percentage rumen inoculum and hydraulic retention time) investigate

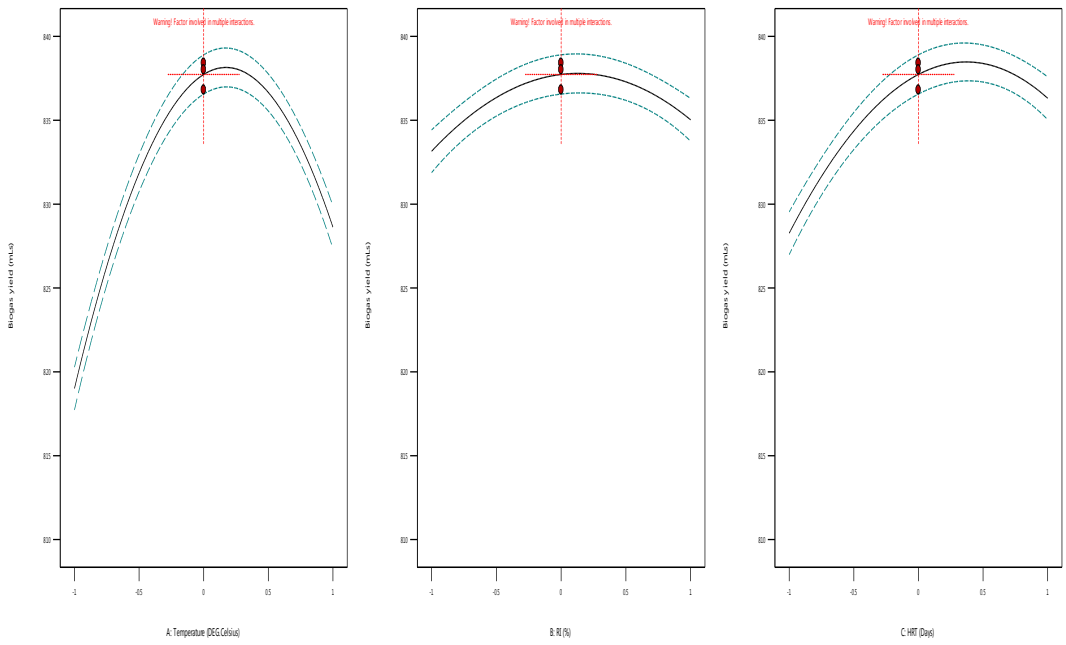

Figure 4.8: All factor curves (Design Expert output) for biogas production

Digestion and consequently biogas production stopped due to substrate depletion and not from inhibition as indicated by digestate pH. The measured pH fell in the optimum range of 6.7-7.4. Our findings on the optimum conditions of temperature, percentage of rumen inoculum and hydraulic retention time at least hint that optimum biogas from the anaerobic digestion of abattoir waste mixed in production proportions can be achieved when the parameters are set at re 37.93 ^{O}C, 70.45% and 16.28 days respectively. In line with previous studies, the results suggest that a percentage between 60 and 80% has sufficient composition that can break down the mixed abattoir effectively as suggested by other studies (Ranalli, 2007; Suryawanshi et al., 2013; Wang, 2014). This finding is similar to that by Budiyono et al. (2009). Furthermore, the desirable carbon hydrogen ratio favoring digestion can be achieved by using 70.45% rumen inoculum to digest mixed abattoir waste. Klintenberg et al. (2014) has suggested that using the right ratio of rumen fluid inoculum is important in optimizing biogas from production from mixed

abattoir waste and that a longer hydraulic retention time may cause inhibition of anaerobic digestion of proteineous wastes. In this study, using 70.45% rumen fluid inoculum at a temperature of 37.93 ^{O}C can give optimum digestion of mixed abattoir waste in a hydraulic retention time of 16.28 days. Hydraulic retention time shorter than 20 days for a mesophilic system should be considered optimum. These results go beyond previous studies, showing that the optimum hydraulic retention time for a mesophilic system is around 30 days (Jayaraj et al., 2014). The results confirm that a given system of digestable wastes with all other parameters fixed could have its own optimum hydraulic retention time (Balmant and Ordonez, 2013). This is particularly important when investigating biogas production from wastes that encounter biogas production inhibition under longer hydraulic retention times such as abattoir waste. We speculate that this shorter hydraulic retention time might be due to robust hydrolysis, acidogenesis, acetogenesis and methanogenesis steps under the influence of optimum temperature and microbes contained in the rumen inoculum. As discussed rumen has microbes well adapted to carry out the four steps of anaerobic digestion mentioned accurately if the digester prevailing conditions can be same as those found in the stomach of a live ruminant animal that is a perfect naturally occurring anaerobic digester. .

CHAPTER 5. CONCLUSION AND RECOMMENDATIONS

Major streams forming abattoir wastes generated during slaughter of livestock are rumen solids, intestinal waste and blood which form 60.04, 20.08 and 19.88% respectively by weight. Mixing the streams in these percentages will form a waste that has very high organic content suitable for use in anaerobic digestion with total solids, COD and nitrogen content being 64000.00, 199013.61 and 831.92 mg/l respectively. Rumen inoculum of 20 and 50% (v/v) can be used to achieve a significant increase in biogas potential and methane content over use of 0% rumen inoculum. Rumen inoculum of 20 and 50% achieve production potentials of 0.068 and 0.069 ml/mgTS with methane content being 54.13 and 60.12% respectively compared to 0% rumen inoculum which attains a potential and methane content of 0.052 ml/mgTS and 48.91% respectively. Similarly, combining *Bacillus subtilis* and *Escherichia coli* significantly improves biogas yield and methane content compared to when each microbe is used separately during digestion of abattoir waste. The combined power of the microbes and rumen inoculum achieves a potential of 0.083 ml/mgTS and a methane content of 66.92%. Separately *E.coli* and *Bacillus subtilis* achieve a potential of 0.077 and 0.076 ml/mgTS and methane content of 62.71 and 62.24% respectively. Optimum levels of temperature, percent rumen inoculum and hydraulic retention are 37.93℃, 70.45% and 16.28 days respectively.

This study recommends mixing rumen solids, intestinal wastes and blood in production ratios for use in anaerobic digestion whose optimisation is by using a combination of *E.coli*, *Bacillus subtilis* and 70.45% rumen inoculum at temperature of 37.93℃. Further work to optimize microbe mixing ratios and charging intervals is suggested.

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APPENDIX

Appendix A

Equipment and materials

The equipment and materials used for the research are given in tables 6.1(a) and (b).

Table 6.1(a): Research equipment details

Equipment	Model	Source
3 L water bath	AV-562	Avishakar, India
5 L water bath	WB10	Polyscience, China
Autoclave	25X-2	All American, USA
COD reactor	HI 839800	Hanna instruments, Romania
Gas chromatograph	3420A	MRC-UK
Incubator	DFI-150	Marc, USA
Laminar flow cabinet	BBS-DDS	Marc, USA
pH meter	HI 98127	Hanna instruments, Romania
Spectrophotometer	DU 720	Beckman Coulter, USA
Wire meshed laboratory test sieve	ISO 3310	Blau-metall, Germany

Table 6.1(b): Research materials

Material	Grade	Source
Alpha naphthol	Analytical	Gelsup supplies, Kenya
Citrate agar		Gelsup supplies, Kenya
Crystal violet	Analytical	Gelsup supplies, Kenya
Gram's iodine		Gelsup supplies, Kenya
Glucose phosphate broth		Gelsup supplies, Kenya
MacConkey agar		Gelsup supplies, Kenya
Peptone agar		Gelsup supplies, Kenya
Peptone yeast extract agar		Gelsup supplies, Kenya
Phosphate saline fat buffer		Gelsup supplies, Kenya
Potassium hydroxide	99.50%	Gelsup supplies, Kenya
Potassium hydrogen phthalate	99.90%	Gelsup supplies, Kenya
Potassium peroxidisulfate	99.90%	Gelsup supplies, Kenya
Safranin		Gelsup supplies, Kenya
Sodium hydroxide	99.50%	Gelsup supplies, Kenya
Sulfuric acid	99.50%	Gelsup supplies, Kenya
Tryptophan	Analytical	Gelsup supplies, Kenya

Table 6.1(c): Recommended reagent quantities for chemical oxygen demand tests (adapted from APHA 1999)

Digestion Vessel	Sample ml	Digestion solution ml	Sulfuric reagent ml	Total value ml
Culture tubes:				
16×100mm	2.5	1.5	3.5	7.5
20×150mm	5.0	3.0	7.0	15.0
20×150mm	10.0	6.0	14.0	30.0
Standard 10mm ampules	2.5	1.5	3.5	7.5

Appendix B

i. Experiment set up for biogas production from abattoir waste using rumen inoculum

Figure 6.1: Biogas production using varying rumen inoculum percentages

ii. Raw data for daily biogas production from abattoir waste using rumen inoculum

The experiment was done in triplicate and raw data for biogas produced from abattoir waste was as shown in tables 6.2(a), 6.2(b) and 6.2(c). In these Tables, A= 0% rumen inoculum, B= 20% rumen inoculum and C= 50% rumen inoculum

Table 6.2(a): Daily biogas produced using rumen inoculum in first experiment

Day	A	B	C
1	1.9	4.0	10.0
2	6.5	158.6	185.8
3	34.0	200.9	208.6
4	45.1	146.6	150.0
5	66.0	86.8	95.0
6	70.2	78.2	46.0
7	98.0	45.0	47.8
8	45.6	36.8	39.0
9	39.8	14.8	10.2
10	28.4	5.2	6.4
11	18.8	7.2	4.0
12	23.9	16.0	7.0
13	19.0	4.6	8.6
14	12.2	3.8	2.2
15	11.0	2.6	1.2
16	11.6	1.0	2.6
17	7.8	1.2	2.0
18	5.0	1.0	0.0
19	5.0	0.0	2.0
20	7.4	1.0	1.0
21	9.8	0.0	0.0
22	11.6	1.2	3.0
23	3.0	0.0	0.0
24	11.2	1.8	2.0
25	3.8	0.0	2.8
26	9.6	1.6	0.0
27	5.4	0.0	1.8
28	8.0	1.4	0.0
29	4.8	0.0	1.2
30	3.2	1.0	0.0

Table 6.2(b): Daily biogas produced using rumen inoculum in second experiment

Day	A	B	C
1	1.6	4.2	8.8
2	7.2	158.3	185.5
3	33.0	201.8	208.2
4	45.8	145.1	152.8
5	66.2	87.1	98.7
6	69.8	77.5	45.8
7	96.3	45.6	47.3
8	45.7	36.8	37.0
9	39.6	14.6	9.3
10	29.0	5.4	6.8
11	18.8	6.6	4.4
12	23.8	17.2	5.8
13	19.2	5.4	7.8
14	12.3	4.0	3.8
15	10.0	1.6	0.0
16	11.5	0.0	2.8
17	7.9	1.2	2.1
18	5.5	1.0	1.0
19	4.8	0.0	1.0
20	8.2	2.6	0.0
21	9.2	0.0	2.8
22	11.0	1.8	0
23	3.4	0.0	3.0
24	10.2	2.0	0.0
25	5.8	0.0	2.8
26	7.0	0.0	0.0
27	7.0	1.6	1.6
28	7.0	0.0	0.0
29	5.2	1.2	1.2
30	4.4	0.0	0.0

Table 6.2(c): Daily biogas produced using rumen inoculum in third experiment

Day	A	B	C
1	2.0	4.0	8.8
2	7.2	158.1	186.4
3	33.6	201.6	208.6
4	45.4	145.0	152.8
5	66	87.3	99.0
6	68.2	77.6	46.6
7	97.0	45.2	47.8
8	45.8	36.8	38.2
9	40.2	15.0	9.4
10	27.0	5.2	7.0
11	19.0	6.2	4.0
12	23.8	17.6	4.6
13	19.2	5.0	8.0
14	12.2	3.4	4.0
15	10.0	1.8	0.2
16	11.6	0.6	2.7
17	8.0	1.2	1.8
18	5.3	1.0	1.1
19	5.0	0.0	1.0
20	7.8	2.0	0.0
21	10.2	0.0	1.2
22	11.8	2.8	0.0
23	3.8	0.0	2.4
24	12.0	1.8	0.0
25	5.8	0.0	0.0
26	8.8	0.0	2.4
27	5.4	1.6	0.0
28	7.2	0.0	1.6
29	6.6	1.4	0.0
30	4.0	0.0	1.0

Appendix C

Methane composition analysis curves for biogas produced using rumen inoculum

Methane content in biogas sample A (0% rumen inoculum used) report

Printing time: Sun Sep 29 10:41:41 2019

Injection time: Sat Sep 28 18:12:58 2019

GC model 3420A, MRC-UK

Column- HP-PLOT/Q Agilent

Column Temp- 150

Injector Temp- 200

Split ratio- 100:1

Detector -FID

Detector Temp- 250

Nitrogen (carrier gas) flow rate 200mls/min

Air flow- 300mls/min

Hydrogen flow- 40mls/min

Sample volume- 3mls

File opened: C:\Users\PC\Desktop\Peak-ABC\program\sample A - Makokha.hw

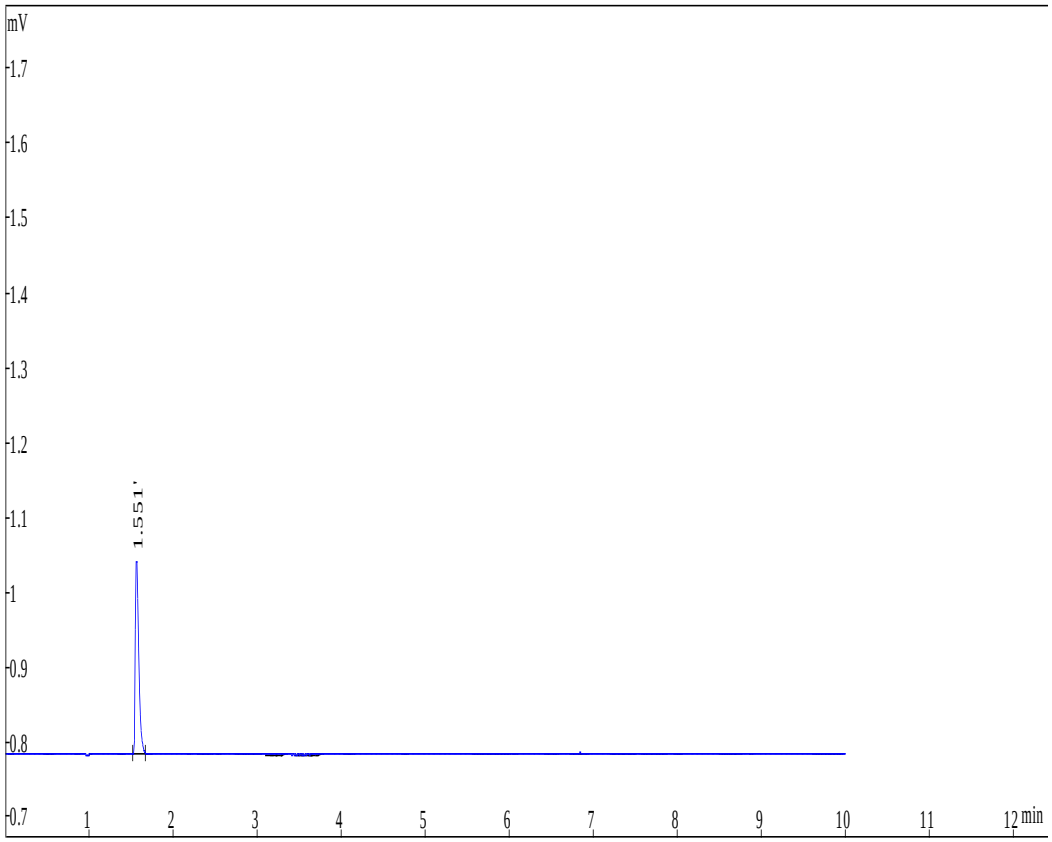

Figure 6.2(a): Chromatograph peak area and time for Methane in biogas in reactor A

Rank	Time	Name	Area%	Area
1	1.551		100	627
Total			100	627

RESULTS

Sample A represents 3mls, 100%

Area = 627

Methane content in sample biogas B (20% rumen inoculum used) Report

Printing time: Sun Sep 29 10:48:11 2019

Injection time: Sat Sep 28 18:26:28 2019

GC model 3420A, MRC-UK

Column- HP-PLOT/Q Agilent

Column Temp- 150

Injector Temp- 200

Split ratio- 100:1

Detector -FID

Detector Temp- 250

Nitrogen (carrier gas) flow rate 200mls/min

Air flow- 300mls/min

Hydrogen flow- 40mls/min

Sample volume- 3mls

File opened: C:\Users\PC\Desktop\Peak-ABC\program\Sample B - Makokha.hw

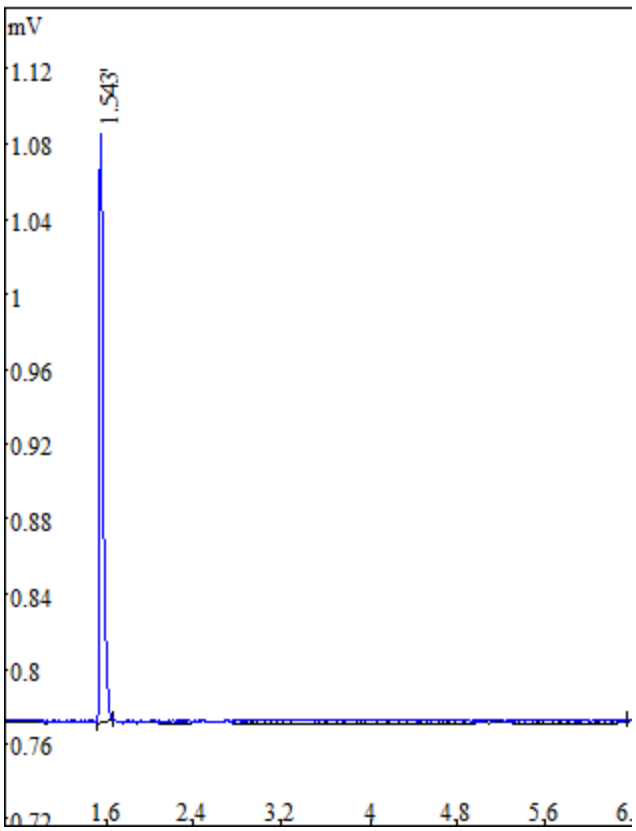

Figure 5.2(b): Chromatograph peak area and time for Methane in biogas in reactor B

Rank	Time	Name	Area%	Area
1	1.543		100	694
Total			100	694

RESULTS

Sample B represents 3mls, 100%

Area = 694

Methane content in Sample biogas C (50% rumen inoculum used) Report

Printing time: Sun Sep 29 10:53:32 2019

Injection time: Sat Sep 28 18:58:59 2019

GC model 3420A, MRC-UK

Column- HP-PLOT/Q Agilent

Column Temp- 150

Injector Temp- 200

Split ratio- 100:1

Detector -FID

Detector Temp- 250

Nitrogen (carrier gas) flow rate 200mls/min

Air flow- 300mls/min

Hydrogen flow- 40mls/min

Sample volume- 1.5mls

File opened: C:\Users\PC\Desktop\Peak-ABC\program\Sample C- Makokha(20190928 18;58;59).hw

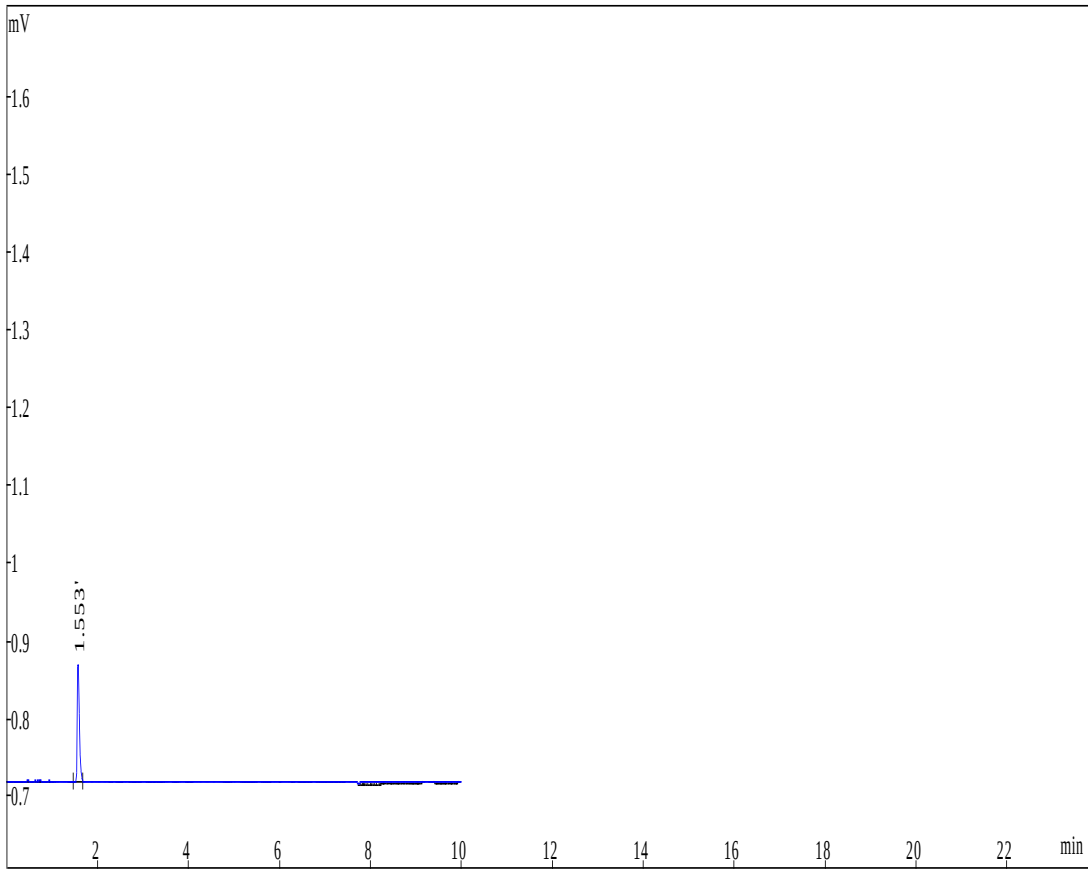

Figure 6.2(c): Chromatograph peak area and time for Methane in biogas in reactor C

Rank	Time	Name	Area%	Area
1	1.553		100	386
Total			100	386

RESULTS

Sample C represents 1.5mls, 100%

Area = 386

Appendix D

i. Experiment set up for biogas production from abattoir waste using bioaugmentation

Figure 6.3: Biogas production using *B.subtilis* and *E.coli*

ii. Raw data for daily biogas yield from abattoir waste using bioaugmentation

The experiment was done in triplicate and raw data for biogas produced from abattoir waste was as shown in tables 6.3 (a), (b) and (c). In these experiments, 50% rumen inoculum was used in each case. The letters represent reactors inoculated with different bacteria as follows; D= *B.subtilis*, E= *E.coli*, F= *B.subtilis* + *E.coli* and G = none of the microbes.

Table 6.3(a): Daily biogas produced using bioaugmentation in first experiment

Day	D	E	F	G
1	20.0	16.0	18.0	22.2
2	247.0	206.0	246.0	253.0
3	433.0	413.5	482.6	352.8
4	356.8	273.0	420.6	302.2
5	256.8	268.2	254.8	229.0
6	182.9	195.4	205.2	158.0
7	145.5	162.6	188.8	136.0
8	77.0	103.4	109.8	54.0
9	45.0	96.8	38.8	45.6
10	33.8	81.8	21.8	44.8
11	23.6	28.2	14.8	34.0
12	16.0	10.2	7.0	20.0
13	12.0	4.0	2.6	13.6
14	1.2	1.0	1.2	5.4
15	0.0	0.0	1.2	3.4
16	0.0	1.2	0.0	1.4
17	0.0	2.1	0.0	1.0
18	0.0	0.0	0.0	1.2
19	0.0	0.0	0.0	1.2
20	0.0	0.0	0.0	1.0
21	0.0	0.0	0.0	0.0
22	0.0	0.0	0.0	1.4
23	0.0	0.0	0.0	1.0
24	0.0	0.0	0.0	0.0
25	0.0	0.0	0.0	0.0

Table 6.3(b): Daily biogas produced using bioaugmentation in second experiment

Day	D	E	F	G
1	19.6	17.3	18.8	20.9
2	247.3	206.2	248.0	251.6
3	435.0	415.5	484.0	353.5
4	358.2	274.8	422.0	298.5
5	257.8	267.8	258.4	231.9
6	184.9	193.6	206.4	157.0
7	147.2	163.9	189	137.2
8	79.0	104.0	110.8	51.0
9	43.5	98.0	40.8	47.0
10	32.0	80.0	19.4	43.0
11	22.8	26.0	12.4	33.0
12	18.0	7.0	5.8	21.8
13	11.8	3.2	2.1	14.5
14	3.0	1.8	1.0	1.6
15	0.0	0.0	0.0	2.6
16	0.0	1.2	1.2	1.0
17	0.0	2.0	0.0	0.0
18	0.0	0.0	0.0	0.0
19	0.0	0.0	0.0	1.6
20	0.0	0.0	0.0	0.0
21	0.0	0.0	0.0	0.0
22	0.0	0.0	0.0	1.2
23	0.0	0.0	0.0	1.0
24	0.0	0.0	0.0	0.0
25	0.0	0.0	0.0	1.2

Table 6.3(c): Daily biogas produced using bioaugmentation in third experiment

Day	D	E	F	G
1	19.6	16.8	19.0	22.9
2	246.5	202.2	247.8	251.6
3	435.0	415.5	482.0	354.5
4	358.0	274.0	422.4	299.5
5	257.4	269.0	258.4	231.9
6	183.8	195.8	208.0	157.0
7	146.9	163.9	190.1	137.2
8	79.0	105.0	107.2	51.0
9	43.5	96.9	37.6	46.0
10	36.0	81.0	21.2	43.0
11	21.2	28.0	13.0	33.0
12	15.4	7.0	5.0	21.8
13	3.0	3.2	2.1	14.5
14	1.2	1.8	1.0	1.6
15	0.0	0.0	0.0	2.6
16	0.0	1.2	1.2	1.0
17	0.0	2.0	0.0	0.0
18	0.0	0.0	0.0	1.2
19	0.0	0.0	0.0	1.6
20	0.0	0.0	0.0	1.0
21	0.0	0.0	0.0	0.0
22	0.0	0.0	0.0	1.0
23	0.0	0.0	0.0	1.0
24	0.0	0.0	0.0	0.0
25	0.0	0.0	0.0	0.0

Appendix E

Methane composition analysis curves for biogas produced using bioaugmentation and rumen inoculum

All the digesters in this set up used 50% rumen inoculum.

Methane content in sample biogas D (*B.subtilis* used) Report

Printing time: Sun Sep 29 10:58:18 2019

Injection time: Sat Sep 28 20:37:06 2019

GC model 3420A, MRC-UK

Column- HP-PLOT/Q Agilent

Column Temp- 150

Injector Temp- 200

Split ratio- 100:1

Detector -FID

Detector Temp- 250

Nitrogen (carrier gas) flow rate 200mls/min

Air flow- 300mls/min

Hydrogen flow- 40mls/min

Sample volume- 0.5mls

File opened: C:\Users\PC\Desktop\Peak-ABC\program\Makokha sample D.hw

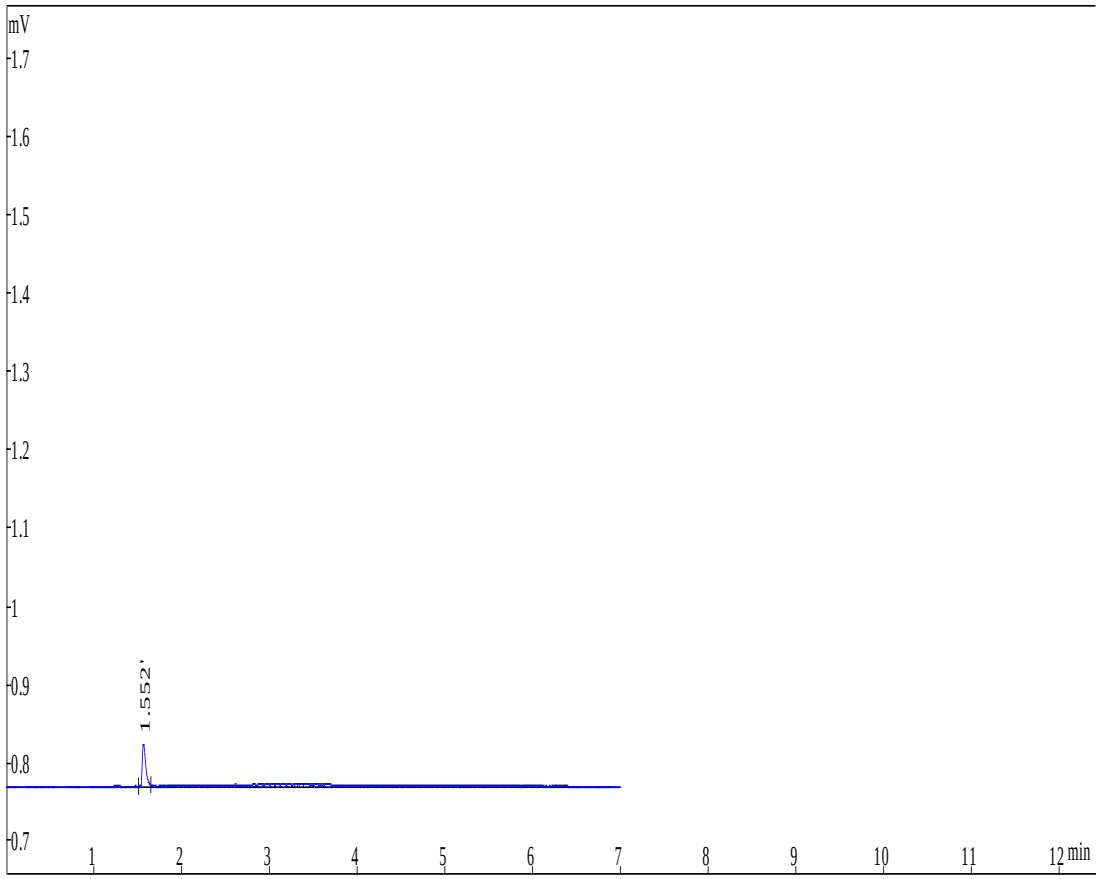

Figure 6.4(a): Chromatograph peak area and time for Methane in biogas in reactor D

Rank	Time	Name	Area%	Area
1	1.552		100	133
Total			100	133

RESULTS

Sample D represents 0.5mls, 100%

Area = 133

Methane content in sample biogas E (*E.coli* used) Report

Printing time: Sun Sep 29 11:00:48 2019

Injection time: Sat Sep 28 20:29:17 2019

GC model 3420A, MRC-UK

Column- HP-PLOT/Q Agilent

Column Temp- 150

Injector Temp- 200

Split ratio- 100:1

Detector -FID

Detector Temp- 250

Nitrogen (carrier gas) flow rate 200mls/min

Air flow- 300mls/min

Hydrogen flow- 40mls/min

Sample volume- 1ml

File opened: C:\Users\PC\Desktop\Peak-ABC\program\Makokha sample E.hw

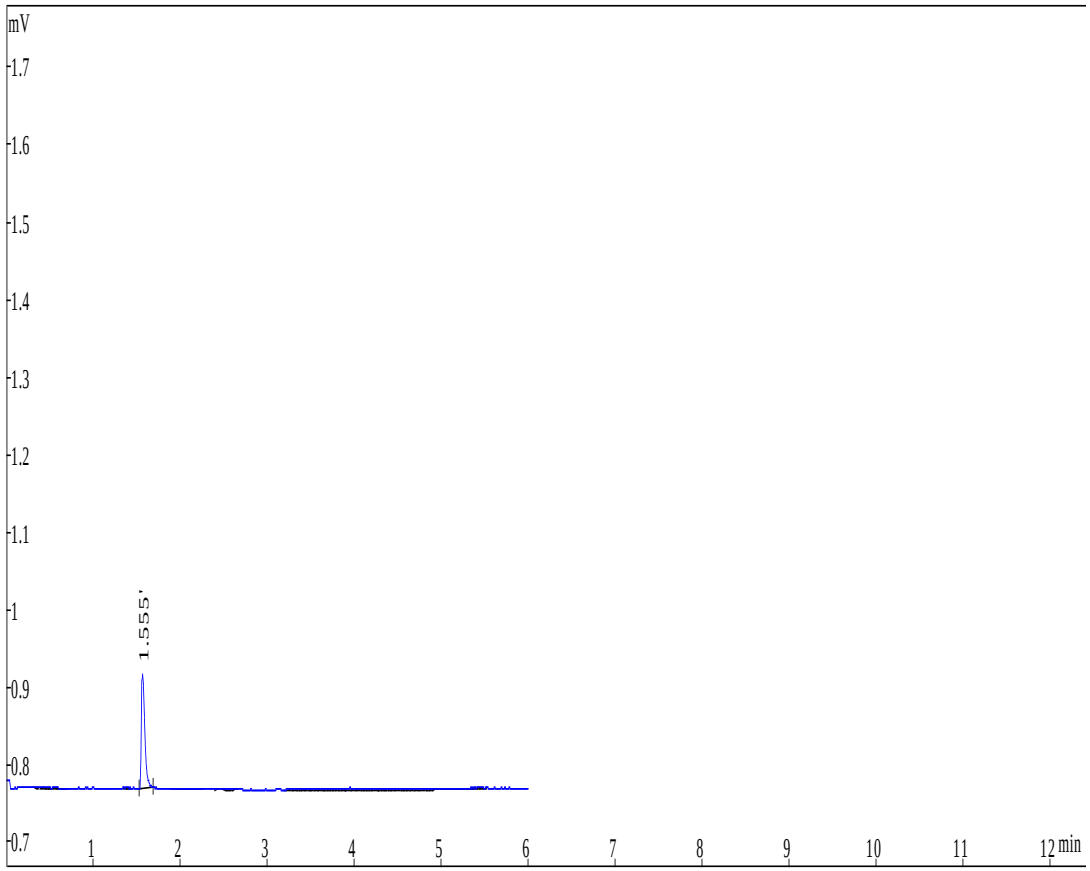

Figure 6.4(b): Chromatograph peak area and time for Methane in biogas in reactor E

Rank	Time	Name	Area%	Area
1	1.555		100	268
Total			100	268

RESULTS
Sample E represents 1ml, 100%

Area = 268

Methane content in Sample biogas F (Use of *B.subtilis* and *E.coli*) Report

Printing time: Sun Sep 29 11:07:54 2019

Injection time: Sat Sep 28 20:10:06 2019

GC model 3420A, MRC-UK

Column- HP-PLOT/Q Agilent

Column Temp- 150

Injector Temp- 200

Split ratio- 100:1

Detector -FID

Detector Temp- 250

Nitrogen (carrier gas) flow rate 200mls/min

Air flow- 300mls/min

Hydrogen flow- 40mls/min

Sample volume- 3mls

File opened: C:\Users\PC\Desktop\Peak-ABC\program\Makokha sample F.hw

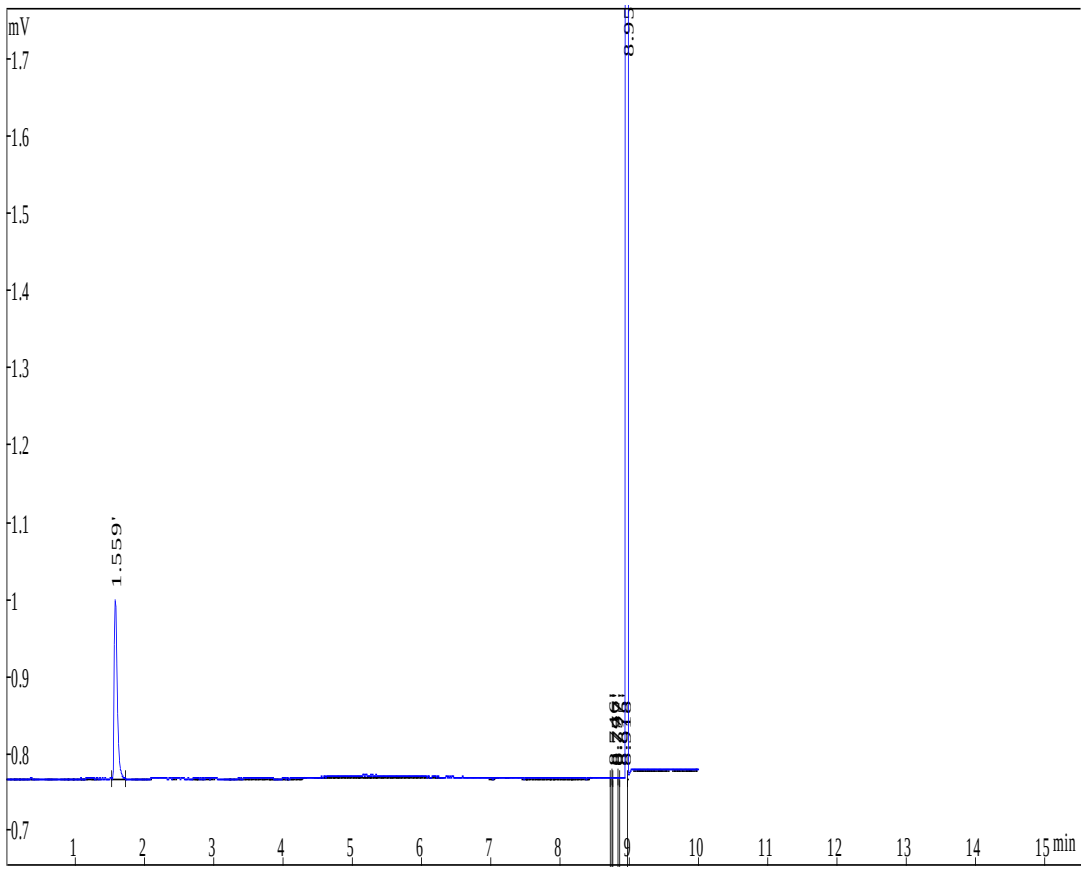

Figure 6.4(c): Chromatograph peak area and time for Methane in biogas in reactor F

Rank	Time	Name	Area%	Area
1	1.559		100	858
Total			100	858

RESULTS

Sample F represents 3mls, 100%

Area = 858

Methane content in sample biogas G (No bioaugmentation) Report

Printing time: Sun Sep 29 11:28:37 2019

Injection time: Sat Sep 28 20:21:16 2019

GC model 3420A, MRC-UK

Column- HP-PLOT/Q Agilent

Column Temp- 150

Injector Temp- 200

Split ratio- 100:1

Detector -FID

Detector Temp- 250

Nitrogen (carrier gas) flow rate 200mls/min

Air flow- 300mls/min

Hydrogen flow- 40mls/min

Sample volume- 1 ml

File opened: C:\Users\PC\Desktop\Peak-ABC\program\Makokha sample G.hw

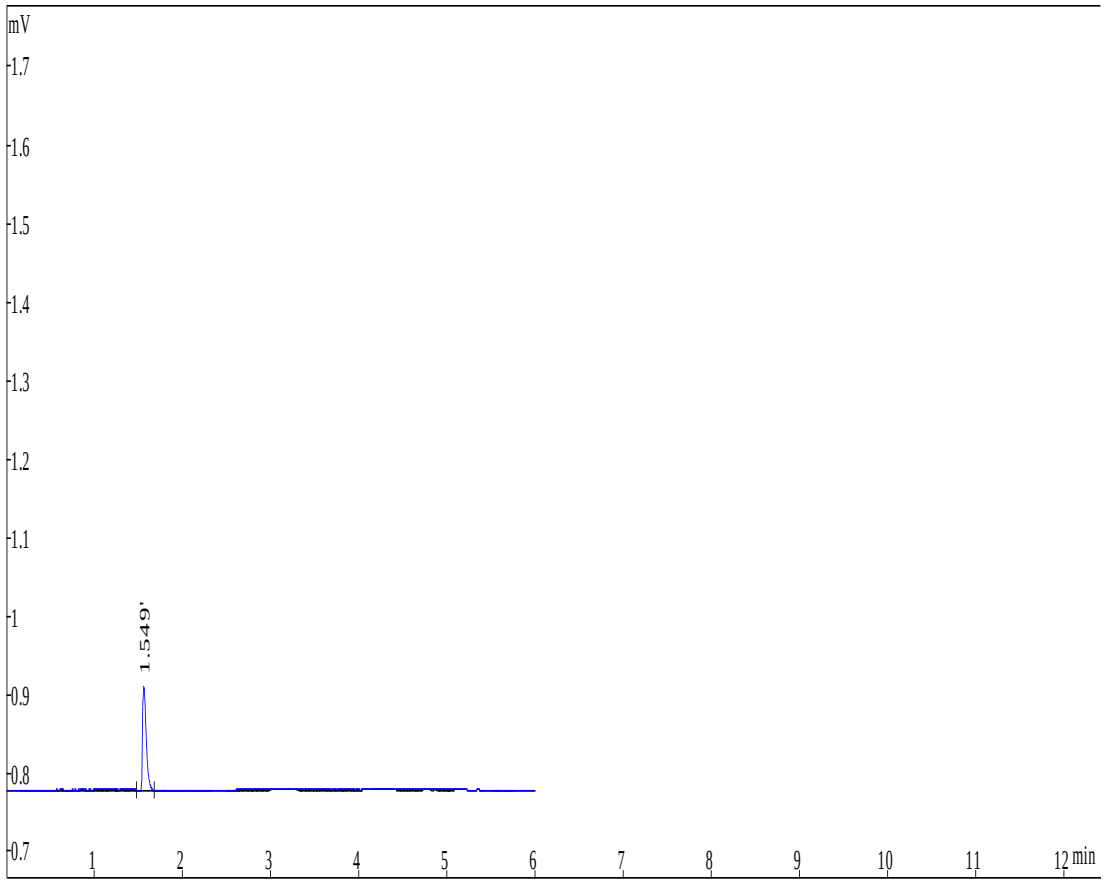

Figure 6.4(d): Chromatograph peak area and time for Methane in biogas in reactor G

Rank	Time	Name	Area%	Area
1	1.549		100	258
Total			100	258

RESULTS

Sample G represents 1ml, 100%

Area = 258

Appendix F

ii. Experiment set up for optimisation of biogas production from abattoir waste

Figure 6.5: Production of biogas using the Box-Behnken experiment

ii. Raw data for biogas yield for experiments in optimisation of biogas production from abattoir waste

The order of the runs in the Box-Behnken design was determined using design expert fashion 12 software. The experiment was done in duplicate and raw data for biogas produced from abattoir waste was as shown in tables 6.4(a) and (b).

Table 6.4(a): Daily biogas yield for first optimisation experiment

	Run							
Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1	4.6	5.2	7.0	6.2	5.8	6.4	6.8	7.0
2	105.0	110.0	260.0	116.4	54.5	91.9	120.5	244.8
3	285.2	229.4	181.8	192.8	147.8	136.6	210.7	207.6
4	190.0	213.4	177.9	260.7	162.0	219.2	196.0	206.0
5	93.6	86.0	80.0	98.4	86.6	98	122.3	110.8
6	46.8	5.02	45.8	65.3	69.0	56.2	71.8	23.3
7	13.0	28.8	40.1	42.3	51.2	22.6	46.6	5.4
8	6.8	14.0	12.5	32.3	45.4	30.9	22.7	10.5
9	7.4	11.5	3.4	6.6	60.6	17.0	8.3	3.0
10	10.0	15.0	1.6	2.4	63.5	49.4	2.0	3.8
11	15.0	19.7	1.0	1.0	49.2	55.9	4.0	1.0
12	20.0	18.0	1.2	1.0	14.4	32.6	2.5	0.8
13	9.2	13.4	1.2	0.8	7.8	0.0	1.0	1.2
14	4.0	7.0	1.4	1.0	2.8	0.0	2.6	0.0
15	1.4	1.2	1.0	1.0	0.0	0.0	0.0	0.0
16	1.2	1.8	1.8	1.6	0.0	0.0	1.2	0.0
17	1.0	0.0	1.2	1.8	0.0	0.0	0.0	1.6
18	1.8	1.1	1.0	1.0	0.0	0.0	1.0	0.0
19	0.0	0.0	1.0	0.0	1.0	1.0	1.0	4.0
20	1.2	0.0	1.2	1.0	0.0	2.0	1.2	1.2
21	0.0	0.0	0.0	0.0	0.0	2.0	0.0	1.0
22	1.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0
23	0.0	1.5	1.0	0.0	0.0	2.0	0.0	0.0
24	0.0	0.0	0.0	0.0	0.0	5.0	0.0	1.0
25	1.2	1.0	0.0	0.0	1.2	1.0	0.0	0.0
26	0.0	0.0	0.0	0.0	0.0	2.0	0.0	0.0

Table 6.4(a) continued

				Run			
Day	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1	8.4	7.6	8.0	8.0	8.5	8.4	9.0
2	204.6	203.4	221.9	208	215.7	195	198
3	182	209.3	274.2	263	258.8	249.3	259.3
4	192.8	179.4	167.2	158.8	176	138.8	174.4
5	128.2	93.9	81.8	94.6	70.2	95.0	89.5
6	68.5	66.6	31.4	65.5	39.4	79.3	51.4
7	20.6	21.8	13.6	14.9	10.5	17.0	24.6
8	10.5	14.8	10.5	11.4	15.6	16.0	11.2
9	4.6	11.6	7.8	4.3	10.1	12.7	12.6
10	4.0	6.0	8.8	2.0	2.8	4.5	1.6
11	1.8	3.2	2.6	1.4	16.6	13.2	1.9
12	0.0	5.7	1.6	1.6	6.8	4.2	2.7
13	1.0	0.0	0.0	0.0	4.6	0.0	1.8
14	2.5	0.0	0.0	0.4	2.8	1.8	0.0
15	1.2	0.0	0.0	2.0	0.0	0.0	0.0
16	0.0	0.5	0.0	0.0	0.0	0.0	0.0
17	1.0	0.0	0.0	0.0	0.0	1.0	1.0
18	0.0	1.2	0.0	0.0	0.0	0.0	0.0
19	0.0	2.0	0.8	0.0	0.0	0.0	0.0
20	1.0	0.0	1.0	1.4	0.0	0.0	0.0
21	0.0	0.0	1.0	0.0	0.0	0.0	0.0
22	0.0	0.0	1.0	0.0	0.0	0.0	0.0
23	0.0	1.2	0.0	0.0	0.0	0.0	0.0
24	0.0	0.0	0.0	0.0	0.0	0.0	0.0
25	0.0	1.9	0.0	0.0	0.0	1.2	0.0
26	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 6.4(b): Daily biogas yield for second optimisation experiment

	Run							
Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1	5.6	4.8	7.4	5.4	5.3	7.0	6.2	7.2
2	106.0	109	261	116.2	54.0	92.2	120.2	245.2
3	286.2	229.2	182.0	191.8	148.6	137.1	210.2	207.8
4	190.8	213.1	178.2	259.7	162.6	210.0	195.5	205.4
5	89.8	87.0	80.0	98.0	86.4	109.7	121.8	110.4
6	49.8	52.0	45.8	65.2	68.8	70.5	71.3	23.5
7	13.8	28.8	39.1	42.0	60.2	60	46.4	5.4
8	6.8	14.0	11.6	32.3	55.4	33.8	22.3	10.5
9	7.4	11.5	3.4	6.6	55.6	30.9	8.1	3.0
10	10.2	15.0	1.6	2.4	53.5	49.6	2.6	3.8
11	15.0	19.7	1.0	1.0	41.4	17.2	9.0	1.0
12	20.0	18.0	1.2	1.0	18.2	2.6	2.5	0.8
13	9.0	14.3	1.2	0.8	6.8	1.7	0.0	1.2
14	4.0	7.0	1.4	1.0	2.8	0.0	0.0	0.0
15	1.4	1.2	1.0	1.0	1.5	0.0	0.0	0.0
16	0.0	1.8	1.8	1.6	0.0	1.0	0.0	0.0
17	1.0	0.0	1.2	1.8	0.0	1.0	0.0	1.6
18	0.0	1.1	1.0	1.0	0.0	0.0	0.0	0.0
19	0.0	0.0	0.0	0.0	0.0	1.0	1.0	4.0
20	1.2	2.8	1.2	1.0	0.0	0.0	1.2	1.2
21	0.0	1.6	0.0	0.0	0.0	0.0	0.0	1.0
22	1.0	0.0	0.0	0.0	1.0	1.0	1.8	0.0
23	0.0	1.5	1.0	0.0	0.0	2.0	0.0	0.0
24	0.0	0.0	0.0	0.0	0.0	5.0	1.0	1.0
25	1.2	1.0	0.0	0.0	0.0	1.0	0.0	0.0
26	0.0	2.0	0.0	0.0	0.0	2.0	0.0	0.0

Table 6.4(b) continued

	Run						
Day	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1	8.0	7.5	8.1	8.4	8.1	9.4	8.6
2	204.4	202.4	221.9	208.2	215.5	195.4	197.8
3	182.8	206	274.2	263.2	258.6	249.5	259.1
4	193.4	179.4	166.2	159.2	175.6	139	174.2
5	128.0	99.7	80.8	93.8	70	95.2	90.3
6	68.3	66.6	31.4	64.7	39.2	79.8	51.2
7	20.4	21.8	13.6	15.1	13.4	17.5	25
8	10.3	14.8	9.6	11.6	15.4	16.6	11.6
9	4.6	10.2	7.8	4.3	9.6	13.1	12.2
10	4.0	6.0	8.8	2.0	2.2	4.5	1.6
11	1.8	3.2	2.6	1.4	16.6	13.2	1.9
12	0.0	5.7	1.6	1.6	6.8	3.4	2.7
13	1.0	1.0	0.8	0.0	4.6	0.0	1.8
14	2.5	3.2	1.0	0.4	2.8	1.8	0.0
15	1.2	1.0	1.0	2.0	0.0	0.0	0.0
16	0.0	0.5	0.0	0.0	0.0	0.0	0.0
17	1.0	0.0	0.0	0.0	0.0	0.0	1.0
18	0.0	0.0	0.0	0.0	0.0	0.0	0.0
19	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20	1.0	0.0	0.0	1.4	0.0	0.0	0.0
21	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22	0.0	0.0	1.0	0.0	0.0	0.0	0.0
23	0.0	1.2	0.0	0.0	0.0	0.0	0.0
24	0.0	0.0	0.0	0.0	0.0	0.0	0.0
25	0.0	1.9	0.0	0.0	0.0	1.2	0.0
26	0.0	0.0	0.0	0.0	0.0	0.0	0.0