



Research Paper

Studies on the effect of pH and bacteria count on biogas generation using cattle rumen content at different retention time

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The study was undertaken to investigate the effect of pH and bacterial count using on biogas generation using Cattle rumen content at different retention time. The bacterial associated with the production as well as the pH of the slurry before and after the biogas production was determined. From the result, bacteria colony count range from (2.08×10^7 cfu/ml to 4.2×10^7 cfu/ml). Isolate identified from biogas generation of spent slurry are *Yersinia enterocolitica bacillus lintus*, *Bacillus lichinoformis*, *Bacillus firmus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Starphylococcus carpitis*, *Bacillus alvei*, *Bacillus pathenticus*, *Salmonella sp*, *Bacillus sp* and *Proteus vulgarius* the highest volume of biogas generation increase with time up to a maximum of (2240 cm³) at the end of

second week at rate of (93.3) per hour, while the least volume (620 cm³) was obtained in week one. The optimum pHs of the digester before and after biogas generation were 6.3 and 7.2 respectively. The most frequently encounter bacteria isolated from the spent slurry is *Bacillus sp.* which account for (49.8%). The study entails that bacterial isolated from spent slurry are essential for biogas generation. Government should train expert in the biogas generation and developed researcher on biogas using cattle rumen at different retention time.

Keywords: Biogas generation, bacteria count, cattle rumen

INTRODUCTION

The most fascinating features of any civilized communities are abundant availability of energy for domestic, agricultural, industrial purposes (Baki, 2004). Energy is the source of economic growth, energy consumption reflect the state of development of a nation. Renewable energy effectively uses natural resources such as sun light, wind, rain, tides and geothermal heat, which may naturally replenish. Renewable energy technologies range from solar power, wind power, hydroelectricity, microhydro, biomas and biofuels for transportation. In 2006, about 18 percent global final energy consumption come from renewable energy, with 13% coming from traditional biomass, like wood-burning. Hydro power was next largest renewable source providing 3% (Oyeleke *et al.*, 2003).

The production of biogas from renewable energy

source is becoming a prominent feature of most developed and developing countries of the world. Despite the variability of international opinion on this technology, it is agreed that it plays important role in the domestic and agricultural life of rural dwellers in countries like India, China, Korea and Malaysia. It is used for cooking, crop drying and soil fertilizer (Mena and Vijay, 2010). Biogas production is a complex biochemical reaction found to take place under the action of delicately pH sensitive microbes mainly bacteria in the presence of little or no oxygen. Three major groups of bacteria are (Hydrolytic, Acidogens/Acetogens and Methanogens) are responsible for breaking down the large complex polymer in biomas waste to form biogas at anaerobic condition and animal manure have been established as a major source of this gas (Bori *et al*, 2007). It has been reported

that seventeen fermentative bacteria species have played important role in the production of biogas (Ramasamy *et al.*, 1991). However, the natures of the feed stock determine the type and extent of fermentative bacteria present in the digester (Bori *et al.*, 2007). Biogas is produced when bacteria degrade biological material in the absence of oxygen, in a process known as anaerobic digestion (Garba and Atiku, 1992). Anaerobic treatment is used of biological process in the absence of oxygen for breakdown of organic matter and the stabilization of this material for conversion to methane and carbon dioxide gases and nearly stable residue (Cossidy *et al.*, 2008). Animal waste can be used as a source of nutrient, feed ingredient to microorganism and as a fuel energy source. They contain high level of organic matter that could be converted into energy as supplement for fossils. Animal wastes are abundant all over the world with Nigeria producing about 227,500 tons of fresh waste each day (Oyeleke *et al.*, 2003). That 1 kg of fresh animal waste produce about 0.03 cm³ of gas per day this shows theoretically that Nigeria can produce 6.8 million m³ of biogas daily, which in term of energy is equivalent to 3.9 million liter of petroleum. The use of biogas is capable of providing a special impetus in both rural and urban area. Biogas plant can be build by using material which is locally available in most developing countries (Baki, 2004).

Nigeria is abundantly blessed with different types of energy resources. The climate permits average solar radiation as high as 5.538 km (World Energy Council, 1993). Making the country operate mainly under mesophilic temperature at ambient condition. This energy need to be tapped especially as the energy supply of the country is grossly in adequate. Consequently, biogas via anaerobic digestion can be good source channel if properly harnessed as in the case of China and India. Moreover, the effluent of this process is a residue rich in essential inorganic element like, nitrogen and phosphorus needed for healthy plant growth known as biofertilizer which when applied to soil, enriches in which no detrimental effect on the environment (Bori *et al.*, 2007). This will further augment the inadequate supply of chemical fertilizer which is very expensive in spite of the fact that the country is a net food importer. This study investigates the effect of pH on bacteria count using abattoir waste at retention time. The study is also continuation of an earlier investigation on isolation and identification of common pathogen found in these animal wastes (Ofeofu and Uzoinma, 2005). The anaerobic digestion of municipal waste can have positive environmental value, since it can combine waste removal and stabilization with net fuel (Biogas) production. The solid or liquid residue can further be used as feed or biomass briquette for cooking (Baki, 2004). Biogas is product of decomposition of organic matter, such as sewage, animal product and agricultural, industrial and municipal sewage in the absence of oxygen (Handbook of biogas utilization, 2012). Biogas most upgraded to a purity standard to fuel vehicle and be distributed via the existing natural grid.

Large-scale biogas plants are able to produce sufficient gas to fuel engine to generate electricity. The energy available from biogas is about 6 kwh/m³. This corresponds to half a liter of diesel oil and 5.5 kg of firewood 1kg of human faeces generate about 50 liters of biogas 1 kg of cattle dung deliver 40 liter of biogas. Animal manure can be collected and delivered to anaerobic digester to stabilizes and optimize methane production the resulting can be used to fuel. The (U.S) Environmental Production Agency (EPA) estimate 8,200 dairy and swine operation could support biogas recovery system with the 13 million megawatts hours and display about 1,670 megawatts of fossil fuel fired generation collectively per year (Alseadi, 2001).

The purpose of this research is to justify on three reasons that is the higher cost and limited availability of conventional energy, identifying useful microorganism for biogas generation and environmental implication. The production of biogas using abattoir waste was carried out in other to provide supplement or rather potential substitute to the higher cost and limited convention source of energy in the nearest future to prove that isolated and identifying microorganism are not useful but basic ingredient in providing renewable energy and also to reduce the environment implicati3on such soil erosion, desert encroachment and health problem as a result of cutting tress and burning wood to produce energy.

The objectives for the study is to enumerate the total bacteria count in fresh cattle rumen waste, to produce biogas using cattle rumen waste from Sokoto abattoir waste and quantity yield at different retention time and to isolate and identified the microbe associated biogas generation.

MATERIALS AND METHODS

Sample collection

1200 g of Fresh rumen content of cattle was collected from central abattoir in Sokoto metropolis. A clean container with cover was used for the collection of the waste. The sample was collected immediately the Cattle were being slaughtered. The container was placed in a box and transported immediately to the energy centre laboratory at Usmanu Danfodiyo University, Sokoto (Raba *et al.*, 2010).

Slurry preparation

One hundred gram (100 g) of the sample were weighed and mixed with 200cm³ of distilled water in a beaker to give 1:2 for each of the four digesters in order to obtain homogeneous solution. The mixture inside each of the 3x4 digester were strived thoroughly in order to obtain a homogenous mixture. The initial pH of the mixture was determined (Raba *et al.*, 2010).

Determination of pH of the slurry

The pH value of the prepared slurry for the sample was

determined using Digital pH meter before and after digestion (Raba *et al.*, 2010).

Biogas experimental set up

Four set of 500 g capacity tin containing 100 ratios 200 of digester labeled A, B, C and D were used. Each set were replicated three times to give a triplicate sample of equal concentration of slurry poured into the digester. The digester were sealed with araldite adhesive to cover leakage and connected with delivery tube which convey the gas from the digester to a 1000cm³ cylinder and inverted into bowl containing water for gas collection using water displacement method. During the period of biogas production, daily record for the volume of biogas produce at about 12:00 daily was taken. The water within the measuring cylinder was constantly re-filled immediately when displaced after the reading (Rabah *et al.*, 2010).

Microbial analysis

Media preparation

All media are prepared according to the manufacture instruction; the media used were nutrient agar (NA). 7 g of (NA) was dissolved in 250 ml, of distilled water in clean conical flask. The media is then heated to obtain a homogenized suspension. It was then autoclave for 121°C, for 10 min and then allowed to cool to 45°C, the media is then poured into different sterile petri-dishes and allow to solidify (Cowan and Steel, 1983). Serial dilution of the fresh sample and the digested slurry sample were carried out up to 10⁶ tube 0.5 ml will be obtained using sterile syringe from the 10⁵ tube and inoculated onto already prepared nutrient agar plate by spread method of inoculation. The plate was replicated four times modified Mackintosh and fildes pattern of anaerobic jar will be used to incubate the tubes. The residual Oxygen (Co₂) in the anaerobic jar will evaluate by placing a kindled match stick, when quenched immediately the left over oxygen were exhausted. The jar will be incubated for a period of 24 h at 37°C. After incubation, plates will be then observed for colonies, in term of appearance number and colour (Oyeleke and Manga, 2008).

$$\text{Colony forming unit} = \frac{\text{Number of colonies}}{\text{Inoculants size} \times \text{dilution factor}}$$

$$\text{Percentage frequency of occurrence} = \frac{\text{Number of isolate}}{\text{Total Number of isolate}} \times 100$$

Bacteria colonies that emerge on the plate, was counted and recorded as colony forming unit per milliliter (cfu/ml) of the sample. The colonies will be also subculture repeatedly on fresh plate to obtain pure isolate.

Gram staining

Smear was made on sterile glass slide using distilled water and sterile wire loop. The smear was heat fixed and allow to air dry. The smear were flooded with crystal violet for 1 minute rise with water, followed by decolorized acetone for 30 seconds risen with water, the smear was then finally counterstained (Safranin) for 1 minute, risen with water and allow to air dry (Prescott. and Klein, 2008).

Microscopy

The back of the glass slide was wiped clean and a drop of oil (glycerin) was applied on the smear which will examine microscopically with x 100 objective lens for the observation of gram reaction and morphological characteristics of the bacterial cells. Gram-positive bacteria, appears purple in colour, while the gram-negative cell retained the counter staining colour of safranin and appear pink in colour. After gram staining and microscopy, the isolate sub-culture into universal bottle containing nutrient agar in a slant form for subsequent used in biochemical test (Oyeleke and Manga, 2008).

Biochemical test

The pure bacteria isolate after gram-stained were subjected to different biochemical test which included production of coagulase, catalase, urease oxidase methyl red, voges proskaeur, citrate utilization test, H₂S production and carbohydrate fermentation as described by Cheesbrough (2006). The bacteria isolate were identified by comparing their characteristics with those of known taxa using the scheme of Cowan and Steel, (1993).

Sugar fermentation test

A 24 hours old culture was stabbed into sterile tripe sugar ion agar slant (TSI) in a test tube ad incubated at 37°C for 24 h. It was observed for glucose, lactose, sucrose, gas production and motility, in positive test for glucose will be indicated by redness of the bottom of the test tube. While in lactose the media appeared yellow, for motility in the line of stabation of the medium would not be sharply define and rest of the medium would be cloudy (Cheesebrough, 2006).

Hydrogen sulphite test (H₂S)

The prepared medium was used to detect the production of H₂S from different test organisms. Each test organism was inoculated into test tube by stabbing the medium. The test tubes were incubated for 24 h at 37°C. A black colour along the line of stabbing indicates a positive reaction (Cheesebrough, 2006).

Catalase test

It used to differentiate those bacteria that produce enzymes catalase, such as staphylococcus and from the non catalase producing bacteria such as streptococcus. A drop of hydrogen peroxide (H_2O_2) will be placed on a slide and a 24 hours growth culture will be emulsified with the drop of H_2O_2 on the slide. Immediately it will be observed for the present of bubbles as indication for positive reaction absence of bubbles indicate negative (Cheesebrough, 2006).

Coagulase test

This test was carried out to differentiate between the pathogenic staphylococcus and non pathogenic staphylococcus. A drop of the water on a slide and a pure culture were then emulsified with the drop water on the slide to a suspension. A drop of blood was then mixed on the slide and it was immediately observed for agglutination, a positive test indicates plasma has under gone clotting (Oyeleke and Manga, 2008).

Urease test

This test applied for bacteria that can decompose urea by enzymatic reaction to produce ammonia. The test organism were inoculate into urea agar base medium base medium contained in each bottle, which then incubate at 37°C for 48 h. A positive test is indicated by a change in colour from yellow to pink as result of ammonia production (Cawan and Steel, 1993).

Motility test

The test isolate was inoculated into motility medium by making a stab with a needle to a depth 1-2 cm short of the bottom of tube. Incubate at 35°C for 24 h at the end of the period incubation the tube. The line of inoculation would not be sharply defined and the rest of the medium would be somewhat cloudy if the restricted to the line of inoculation which become sharply defined the rest of the medium remain clear (Cheesebrough, 2006).

Indole test

The organism was grown on 5 ml of nutrient broth for 24 h. After 24 h of incubation 0.5 ml; Kovac's reagent will be added and shaken gently. A positive reaction was indicated by the development of red colour in the reagent layer above the broth within a minute (Cawan and Steel, 1993)

Citrate test

Heavy inoculum of the test organisms were incubated into a sterile citrate medium with the aid of the sterile wire

loop. The inoculated test tubes will be incubated at 37°C for 72 h. A positive test was indicated by turbid and change of colour of the medium from light green to blue (Cheesebrough, 2006).

Methyl red (MR)

Heavy inoculum of the test organism was incubated into MR medium contained in each tube. The tubes will be then incubated at 37°C for 48 h. A drop of methyl red indicator was added to the incubated test tube, red colour indicated positive result (Cheesebrough, 2006).

Voges proskauer (VP)

Heavy inoculum of the test organism was inoculated into VP tubes. They were then incubated at 37°C for 48 h. After incubation period 9.5ml of 40% KOH, it is then agitated and allowed to stand few minutes; a red to pink colour signified a positive test (Cheesebrough, 2006).

RESULTS AND DISCUSSION

The result of the biogas produced by the samples and temperature was recorded in (Table 1). The rumen of cattle started producing in the first week increasing throughout the retention period of three weeks. The biogas produced within optimum temperature (optimum temperature 25- 30°C).

Identification of isolates and frequency of occurrence

Table 1 shows the bacterial isolates based on morphological and biochemical characteristics. The percentage frequencies of occurrence of the isolates in relation to all samples are shown in (Tables 2 and 3). The isolated bacteria were *Yersinia enterocolitica*, *Bacillus lintus*, *Bacillus lichinoformis*, *Bacillus firmus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Starphylococcus carpitis*, *Bacillus alvei*, *Bacillus pathenticus*, *Salmonella spp*, *Bacillus spp* and *Proteus vulgaris*. The result shows that *Bacillus sp*, (49.8%) are the predominant organisms isolated in the abattoir waste. Other organisms are (8.3%) *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Starphylococcus carpitis*. The result from the study shows that the colony counts of bacteria were high at 8 to 14 days and the least count was observed at the period of 15 to 21 days of retention time.

Further studies shows that *Bacillus* appears to overlap from one stage to another during biogas production suggesting a succession species of bacteria during the process of gas production. But some species such as *Bacillus* were found to be present throughout the process of gas production (Baki, 2004). The result obtained from this study indicates that *Bacillus* species were the common bacteria isolated and identified during the

Table 1. Species of bacteria isolated during biogas generation.

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Glucose	+	+	+	+	+	-	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	+	-	+	+	-	-	-	+	+	-	+	+
Catalase	-	+	-	-	-	+	+	-	-	-	+	-
Coagulas	-	+	+	+	+	+	+	+	+	+	-	+
Motility	+	+	+	+	+	+	-	+	+	+	+	+
Indole	-	-	-	-	+	-	+	-	-	-	-	-
Citrate	-	-	+	-	-	+	-	+	-	+	+	-
Urease	+	-	+	+	-	-	-	-	-	-	-	+
H ₂ S	-	-	-	-	-	-	-	+	-	-	-	+
Gas	-	+	+	-	+	-	-	-	-	-	-	-
MR	+	+	-	+	+	+	-	-	+	+	-	-
VP	-	-	+	-	-	-	+	+	-	-	+	+
Gram stain	+rod	+rod	+rod	+rod	-rod	+rod	+cocci	+rod	+rod	-od	+rod	+rod

Key:

A1= *Yersinia enterocolitica*
A2= *Bacillus lintus*
A3= *Bacillus lichinoformis*
A4 =*Bacillus firmus*
A5 =*Escherichia coli*
A6 =*Pseudomonas aeruginosa*
A7=*Staphylococcus capitis*
A8= *Bacillus alvei*
A9= *Bacillus pathenticus*
A10= *Salmonella spp*
A11= *Bacillus spp*
A12 =*Proteus vulgaris*

Table 2. Number and Percentage frequency of occurrence of bacteria isolated in abattoir waste

Isolated organism	Frequency of occurrence	Percentage of frequency%
<i>Yersinia enterocolitica</i>	1	8.3
<i>Bacillus spp</i>	6	49.8
<i>Citrobacter ferundii</i>	1	8.3
<i>Pseudomona Areoginosa</i>	1	8.3
<i>Staphylococcus capitis</i>	1	8.3
<i>Salmonella spp</i>	1	8.3
<i>Proteus vulgaris</i>	1	8.3
Total	12	100%

Table 3. The colony count of organism isolated.

Retention time in days	Abattoir waste (cfu/ml)
1-7	2.5x10 ⁷
8-14	4.2x10 ⁷
15-21	2.3x10 ⁷
22-29	2.08x10 ⁷

research, suggestion that the species play a vital role in the microbial activity for the production of biogas. It should be noted that *Bacillus lintus*, *Bacillus lichinoformis* and *Yersinia entrolitca* were isolated during the first week and were able to produced (620 cm³) of biogas while *Citrobacter ferundii*, *Bacillus firmus* and *Psudomonas aeroginosa* were isolated in the second week (14 days) and produced (2240 cm³) of biogas. *Staphylococcus*

capitis, *Bacillus alvis* and *bacillus attenticus* occure in the third week (21 days) and were able to produced (1740 cm³) of biogas. However, salmonella species, *Bacillus sp* and *Proteus Vulgaricus* were isolated from the fourth week (28 days) and produce (1420 cm³) of biogas (Figure 1). The ability of *Bacillus sp* to overlap during the production were probably due to the fact the organism can withstand the hash anaerobic condition or it's evolve

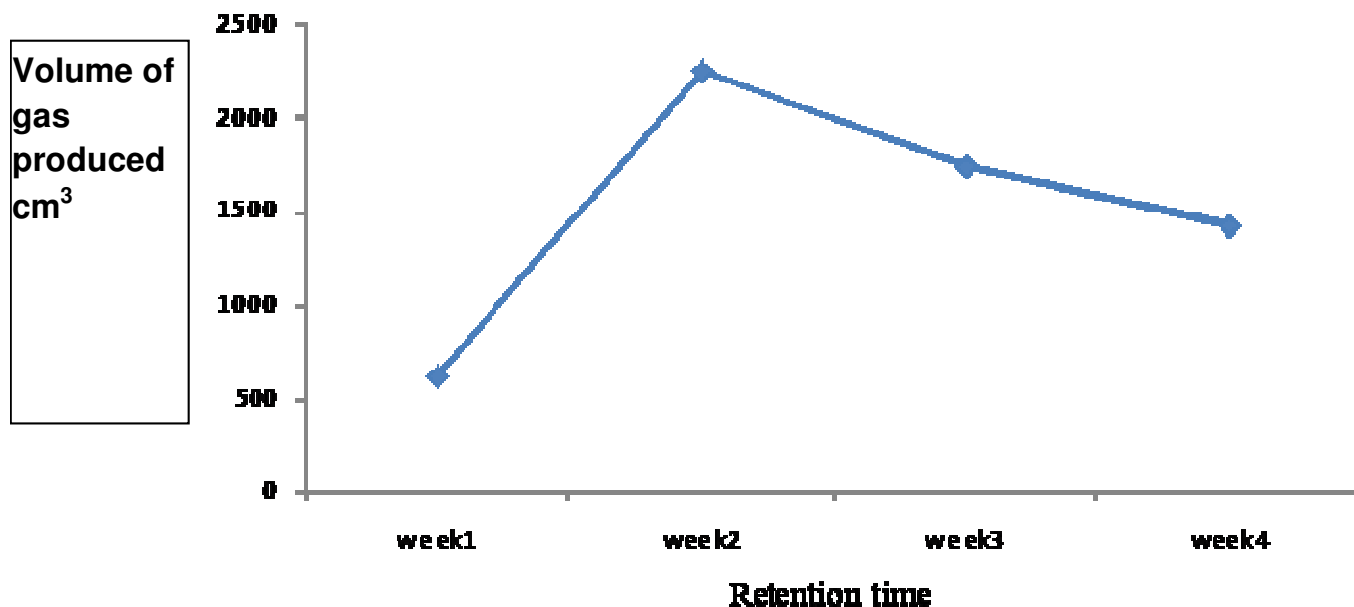


Figure 1. Average volume of biogas production from cattle rumen content waste.

during the biogas production (Baki, et al 2004). This finding were in line with that of (Oluyega *et al.*, 2006) in which *Bacillus*, *Yersinia*, and *Pseudomonas sp* were found to responsible for biogas production from cow dung. The pH of slurry appeared to decreasing in all the digester. This is not surprising as the decreasing in pH will be as result of anaerobic fermentation take place. pH is an important and factor that affects biogas production. It was reported that anaerobic bacteria require a natural environment (Garba and Atiku, 1992). And thus the pH ranging from 6.4-7.2 is required for optimum biogas production. Also, the decrease in pH may due to the action *acetogenic methanogen* as they break down sulphure containing organic and inorganic compound as well as the formation of fatty acid. It was reported by (Oyeleke *et al.*, 2003) that biogas produced is pH of 5 is greater than that of pH 10. Some microorganisms also evolve later in the process while others die off midway through the process. This may be explaining in term of shell fords law of tolerance that the occurrence of any organism in any environment is determine not only by availability of nutrient but also by various physiochemical factors. Therefore, as the medium tend to become acidic non acidic tolerance were replaced by acid tolerance organism.

Result from this work shows that biogas was produced from abattoir waste at different retention time. After the first week, there was sharp increase in volume of biogas produced in second week. However, from the third week to fourth week the volume biogas produce continue to

decline. Therefore it can be deduced that the increase in the second week indicated the acclimatization of biogas producing microorganism. After the hydrolysis of the waste in the first week by the hydrolyzing organism, the biogas production reached its peak in second week and the action of producing microorganism that tend to utilize some of the product of their action. This probably explained the continued decline in the volume of biogas produced in the third and fourth week.

Conclusion

The study was undertaken to investigate the effect of pH and bacteria count and biogas generation using cattle rumen content at different retention time. The biogas production from waste was carried out at the retention period of four week, the result obtain indicated that the higher biogas produce at the second week with the volume of (2240 cm³) while the least of the biogas produce associated with the biogas production was carried out and the result revealed the following microbe *Yersinia entocolitica bacillus lintus*, *Bacillus lichinoformis*, *Bacillus firmus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Starphylococcus carpitis*, *Bacillus alvei*, *Bacillus pathenticus*, *Salmonella spp*, *Bacillus spp* and *Proteus vulgaris*. The colony count and percentage of occurrence of the isolate was determined and *Bacillus sp* appears more than the other species of bacteria isolated. The pH of the slurry before and after

biogas production was determined, and there was significant change observed.

Recommendations

- (a) Government should train expert in the of biogas and developed researcher on biogas production using abattoir waste
- (b) It is therefore recommend that digested substrate still be used as a cheap source of bio fertilizer, since nitrogen, phosphorous, and potassium (NPK) still have the required value
- (c) Further research should be conducted particularly in the determination of pH and retention time in other to make conclusive remark

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