Nutrient Based Bacterial Growth Dynamics And Comparative Biogas Yield Using Cow Dung And Cow Intestinal Exudates

Ajunwa, O.M.[1], Adeleke, A.J.[1], Audu, J.O.[2*], Tsahyel, J.[1], Adedeji, B.A.M.[1]

[1.] Department of Microbiology, Modibbo Adama University of Technology, Yola, Adamawa state, Nigeria
[2.] Department of Laboratory Technology, Modibbo Adama University of Technology, Yola, Adamawa state, Nigeria
*Corresponding Author: j3suwa@yahoo.com, +60102122159

Abstract: Biogas is a product of anaerobic digestion of organic matter with microbial aid. Nutrient based bacterial growth dynamics for cow dung and cow intestinal exudates was studied when Mineral salt medium (MSM) was supplemented differently with acetate (5% v/v), and methanol (5% v/v) as carbon sources. Time-based growth patterns according to media used indicated growth successions from MSM (pH 4) to MSM/acetate to MSM/methanol when sampled at 1, 10, 20, and 30 days retention period. Total viable bacterial counts were conducted using nutrient agar and showed highest growth of 9.6×10^{10} CFU/g for cow dung was obtained when sampled on the 20th day, and the highest growth of 7.2×10^9 CFU/g for cow intestinal exudates was also obtained when sampled on the 20th day. Bacterial counts for cow dung and cow intestinal exudates sampled were higher on the 1st day and reduced on the 30th day (4.5×10^7 to 3.4×10^4 – cow dung; 2.7×10^7 to 3.1×10^3 – cow intestinal exudates). Biogas yield for cow dung had highest value of 51ml on the 17th day, while gas yield for cow intestinal exudates was highest (48ml) on the 16th day. Cumulative gas yields for cow dung (901ml) was higher than cumulative yield for cow intestinal exudates (871ml) after total retention period. Bacteria isolated include *Enterobacter* species, *Bacillus cereus, Escherichia coli, Klebsiella pneumoniae, Staphylococus* specie*, Citrobacter freundii, Proteus vulgaris, Salmonella choleraesius, Salmonella typhi, Bacillus* species*, Staphylococcus aureus, Yersinia pestis,* and *Clostridium* spp., and were all distributed throughout the digestion times sampled (1st, 10th, 20th and 30th days). Gas produced was flammable when tested with laboratory fabricated bioreactors and burners.
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1. Introduction

Global energy demand is rapidly on the increase. Contrastingly, fossil fuel reserves are decreasing, thus throwing up issues of instability, economic insecurity and unfavourable costs (Asikong *et al*., 2014). Together with these issues, there is also the concern of maintaining the integrity of the environment especially in the face of blatant and indiscriminate ooze out of aero-altering gases in form of fumes from energy engines. These disadvantageous effects of fossil fuels in the wake of global consciousness on its relationship with climate change, have led to the development of renewable alternative energy sources (Ofoefule *et al*., 2010, Rabah *et al*., 2010, Wirth *et al*., 2012).

Renewable energy sources are a function of the availability of natural resources like sunlight, wind, water, biomass and others. According to Rabah *et al.* (2010), in 2006, about 18% of global energy consumption was obtained from renewable sources. Essentially, sources of renewable energy are of valued importance with respect to their environmental safety and reduction of overall cost of energy. Biomass sources are a major form of renewable energy with its technological application becoming a source of

present day and future energy needs especially in cooking, heating and electricity generation for lighting and powering turbines (Okoroigwe *et al*., 2014). The impacts of renewable energy with respect to biomass application are essentially felt in the environmental friendliness and relatively lower costs (Mc Kendry, 2002).

A favourable candidate of the biomass resource application is the biogas technology which can be devoloped using a variety of organic wastes (Angelidaki and Ellegaard, 2003; Weiland, 2003; Santosh *et al*., 2004; Wirth *et al*., 2012). Biogas is the direct application of energy conserved within biomass units of natural organic components with it becoming an important feature of the renewable energy resources in developing countries in the world, as it has been successfully evaluated in countries like Korea, Malaysia, India and China (Meena and Vijay 2010). Coupled with these facts, the biogas technology also helps serve as a viable means of waste treatment and environmental protection method especially in the third world (Arvanitoyannis *et al*., 2007). According to Ofoefule *et al*. (2010), biogas can be described as a colourless, flammable gas produced through the

process of anaerobic digestion of animal, plant, domestic and industrial wastes. The chemical composition of the gas is variable; and dependent on several factors involving decomposing feed stock and digestion conditions like temperature and pH (Anunputtikul and Rodtong, 2004; Khalid and Naz, 2013). However, Maishanu *et al*. (1990) explained that biogas was a combination of gases in various proportions with the main ones being methane (50-70%), Carbon dioxide (20-40%) and the remaining percentage occupied by traces of other gases such as Nitrogen, Hydrogen, water vapour, Hydrogen sulphide and ammonia among others.

A critical component of biogas generation and yield depending on biomass feedstocks, is the microbial life responsible for the biodegradation of the organic matter (Khalid and Naz, 2013). Microorganisms require anaerobic digestion systems for the bioconversion and biodegradation of the biomass feedstock, yielding biogas in the process (Garba and Atiku, 1992; Asikong *et al*., 2014). Microbial diversity within biogas production systems have been a subject of study as they participate in gradually degrading complex molecules into a mixture of gases (Bayer *et al*., 2004; Darke *et al*., 2002; Cirne *et al*., 2007), with special and direct focus on the unique microbial consortium responsible for the different stages of biogas production. Biogas generation from organic matter as feed stock basically follows the Wood/Ljungdahl pathway and entails stage by stage bioprocess involving the hydrolysis stage, acidogenesis/acetogenesis stage and the methanogenesis stage duly characterized by the production of various products of hydrolysis, acetic acid, and methane gas (among other gases) respectively (Ofoefule *et al.,* 2010; Wirth *et al.*, 2012)

Metabolically speaking, the microbiology of the system with respect to the different stages of biogas generation can vary potentially with respect to the products of the stages, and can be manipulated for optimum gas yield, thus a defined understanding of the structure and function of the micro-communities is important (Wirth *et al.*, 2012). Also, as stated earlier, the nature of the feedstock within the digester is very important in determining microbial activity and simultaneous gas yield. The variability in feed stock has been hypothesized to affect microbial growth dynamics and also and also with immediate effects on biogas generation (Weiland, 2010)

This work aims to assess bacterial growth dynamics in correlation with the different stages of digestion, by mimicking the different stages with the aid of acetate and methanol supplemented mineral salt media. The effect of the different substrates on gas yield was also assessed in a bid to draw a nexus between bacterial growth and biogas yield. Cow intestinal exudates and cow dung were comparatively evaluated as substrates for biogas production and the time based biogas yield based on a 30-day digestion period was carried out using laboratory scale bioreactors. The bacterial species isolated with respect to the substrates were identified based on standard biochemical and morphological properties to determine their distribution and spread within the digester system over the different times sampled.

2. Materials and methods
2.1. Sample collection/preparation

Fresh cow dung was used in this study, and was obtained from the school farm of the Modibbo Adama University of Technology (MAUTECH), Yola. Cow intestinal exudates used were collected in the form of rumen extracts from the abattoir in Yola, Adamawa state. The samples were collected in clean unused polyethylene bags and transported to the laboratory for further conditioning and analysis. The fresh cow dung samples and the cow intestinal exudates were separately made into a slurry with equal volumes of distilled water in the ratio 1: 2 according to the method of Rabah *et al*. (2010), and subsequently charged into the bioreactors

2.2. Bioreactor configuration

Two (2) different sizes of laboratory scale bioreactors with 15 liter and 20 liter capacity were fabricated. The 15 liter bioreactor was fabricated with aluminum and an orifice was created on the reactor as a sampling point for microbiological analysis at different fermentation stages (Figure 1). The 15 liter reactor was connected to gas tubing which was subsequently connected to a 50ml water displacement - gas measuring system. A second digester was constructed to carry out flammability check. It was designed as a closed system with a 25 liter capacity, and was connected to a burner, which was controlled by a gas valve (Figure 2).

Figure 1: Laboratory scale (15L) biogas fermentor for assessment of gas yield

Figure 2: A 25 Liter biogas fermentor for flammability tests

2.3. Media preparation

Three different laboratory compounded media Mineral salt medium (MSM), Acetate based mineral salt medium (Acetate – MSM), and Methanol based mineral salt medium (Methanol – MSM) were prepared with modifications according to the method of Ogbulie *et al.* (2001). MSM was compounded with the following: 2g $NaNO_3$, 1g KH_2PO_4, 0.5g KCl, 0.5g $MgSO_4$, 0.01g $FeSO_4$, 25g Agar powder, and 1liter distilled water. Acetate (40% v/v) and methanol (40% v/v) was incorporated into the mineral salt medium in the ratio of 1:10 with the medium for the culture of metabolically active biogas producing microorganisms.

2.4. Bacterial growth assessment

Cow intestinal exudates and cow dung were obtained differently from the sampling orifice of the 15 L bioreactor serially diluted in sterile distilled water and then introduced in 0.5 ml volume per plate for the three different media assessed (MSM, MSA/acetate, and MSM/methanol). The samples were taken at different times during the fermentation; 1st day, 10th day, 20th day, and 30th day. The plates were incubated anaerobically at 37°C for 72h, and the loads on the different media at the different sampling times were used to determine their nutrient preferences and growth pattern

2.5. Bacterial identification

Nutrient agar (NA) plates were prepared and used to determine bacterial presence and total viable count within the fermentation system. Serial dilution of the samples obtained from the fermentation system at different fermentation times were carried out up to 10^7. After dilution, 0.5ml of the 10^5 and 10^6 tubes were inoculated onto freshly prepared nutrient agar plates by spread plate method. The plates were incubated anaerobically for 72h at 37°C. After incubation, emerging colonies were counted and the colony forming units per milliliter (CFU/ml) were determined. The values were converted to their logarithmic form for easy graphical representation. Pure culture of the bacterial colonies were also obtained by aseptic techniques and subjected to standard microscopic and biochemical characterization. Characteristics of isolates were compared with that of known microbial identities (Barrow and Feltham, 1993).

2.6. Biogas measurements

This was carried out with the aid of the liquid-displacement gas measurement system constructed on the bioreactors. Distilled water was the liquid introduced into the system and as the gas was produced, the water was being displaced and forced out through the needle provided at the end of the displacement system. The gas produced was measured by reading the calibration and was read in milliliters.

2.7. Biogas flammability

The flammability of biogas produced was determined by constructing a burner onto the 20L bioreactor (Figure 5) and the gas produced was tested for flammability. The flammability of the biogas produced by using the different substrate (cow dung and cow intestinal exudates) was determined.

3. Results

Assessment of the total viable bacterial count as colony forming units per gram (CFU/g) in the cow dung and cow intestinal exudates substrates at the different sampling times - 1st, 10th, 20th, and 30th days (Table 1) showed that there was highest bacterial load on the 20th day for both substrates (9.6 x 10^{10} – cow dung; 7.2 x 10^9 – cow intestinal exudates). The total viable bacterial count for cow dung was however relatively higher than that of the cow intestinal exudates at the different days sampled. The lowest values in bacterial load for the two substrates were observed when sampled on the 30th day.

Table 1: Biogas-associated total viable bacterial count from cow dung and cow intestinal exudates

Sampling time (Days)	Total Viable Bacterial Count in Cow dung (CFU/g)	Total Viable Bacterial Count in Cow intestinal exudates (CFU/g)
1	4.5 x 10^7	2.7 x 10^7
10	6.3 x 10^8	6.1 x 10^4
20	**9.6 x 10^{10}**	**7.2 x 10^9**
30	3.4 x 10^4	3.1 x 10^3

Assessment of growth patterns of the bacterial community within the biogas production units for both substrates was determined using different media for classical nutrient preference and growth conditioning. Figure 3 shows the time and nutrient based growth of biogas associated bacteria from the cow dung substrate. It was observed that there was a growth succession trend that was nutrient dependent. The bacterial growth pattern as observed on MSM showed the highest observed load with the logarithmic (log) value of 7.65 on the 1st day and a gradual reduction at the different days sampled, with the lowest log value of 1.6 on the 30th day. Growth in MSM/Acetate media showed that there was a gradual increase in bacteria with acetate-dependent metabolism. A log value of 2.54 was observed on the 1st day and a subsequent increase with highest value (7.32) obtained on the 30th day. A similar increase in bacterial growth in MSM/Methanol media over time showed an observed log value of 2.18 on the 1st day and the highest log value of 7.64 on the 30th day.
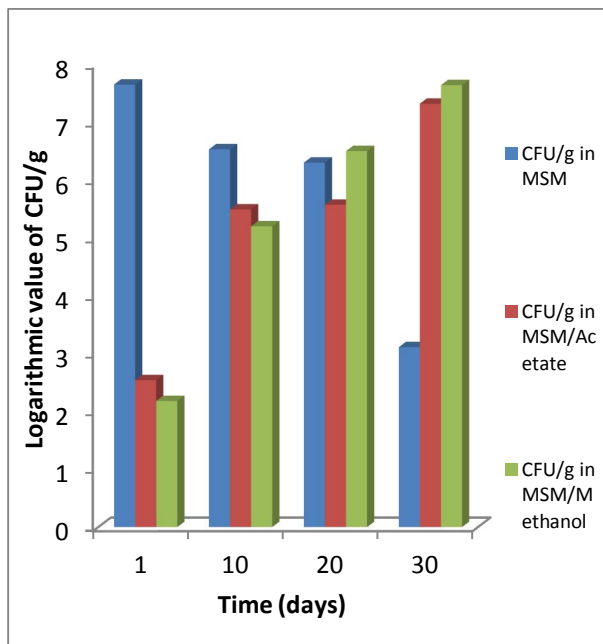

Figure 3: Time and Nutrient based growth of biogas associated Bacteria from cow dung

The bacterial growth patterns within the cow intestinal exudates substrate (Figure 4) also showed a similarity with the bacterial growth dynamics of the cow dung substrate with slight variation in the value of the growth pattern in MSM/Acetate which had highest log value of 5.61 obtained from samples of the 20th day and a reduction in value (4.58) on the 30th day sampled. Logarithmic values of the CFU/g for

bacterial growth in MSM showed a gradual decline from 7.63 on the first day till it reached 1.6 on the 30th day. Bacterial growth in MSM/methanol media was highest on the 30th day (8.78) and the lowest on the 1st day (2.30).
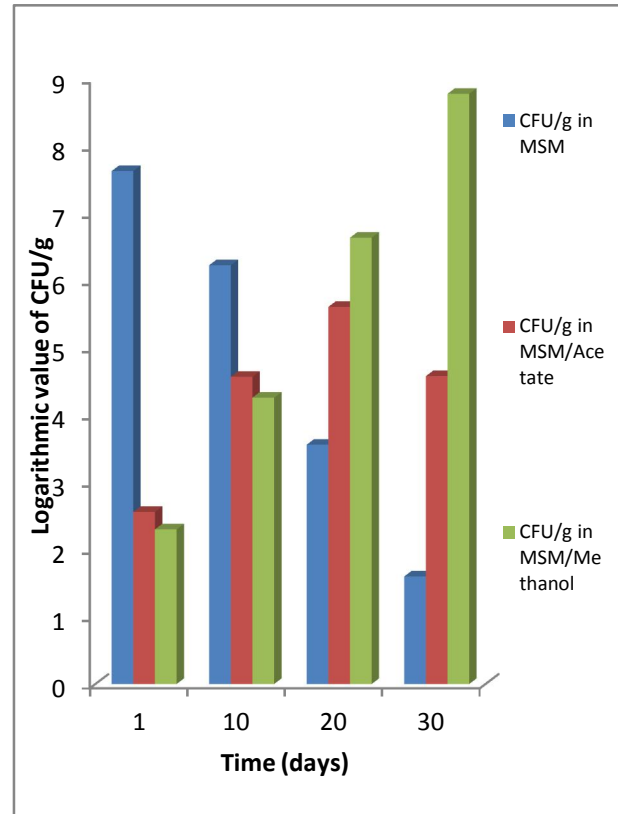

Figure 4: Time and Nutrient based growth of biogas associated Bacteria from cow intestinal exudates

The comparative biogas yields of the two different substrates over a 30 day period (Figure 5) showed a gradual increase in gas yield from substrates. The gas produced was measured in milliliters and the values showed a peak gas yield (51ml) for cow dung on the 17th day, while a peak value of 48ml was observed for the cow intestinal exudates on the 16th day. A steady decline in gas yields from the highest values was observed afterwards, thus resulting in the lowest value being observed on the 30th day. In comparison, gas yield from the cow dung substrate was observed to be higher than the yield from cow intestinal exudates. The cumulative gas produced as determined was higher in the cow dung substrate (901ml) than in the cow intestinal exudates substrates (871ml).
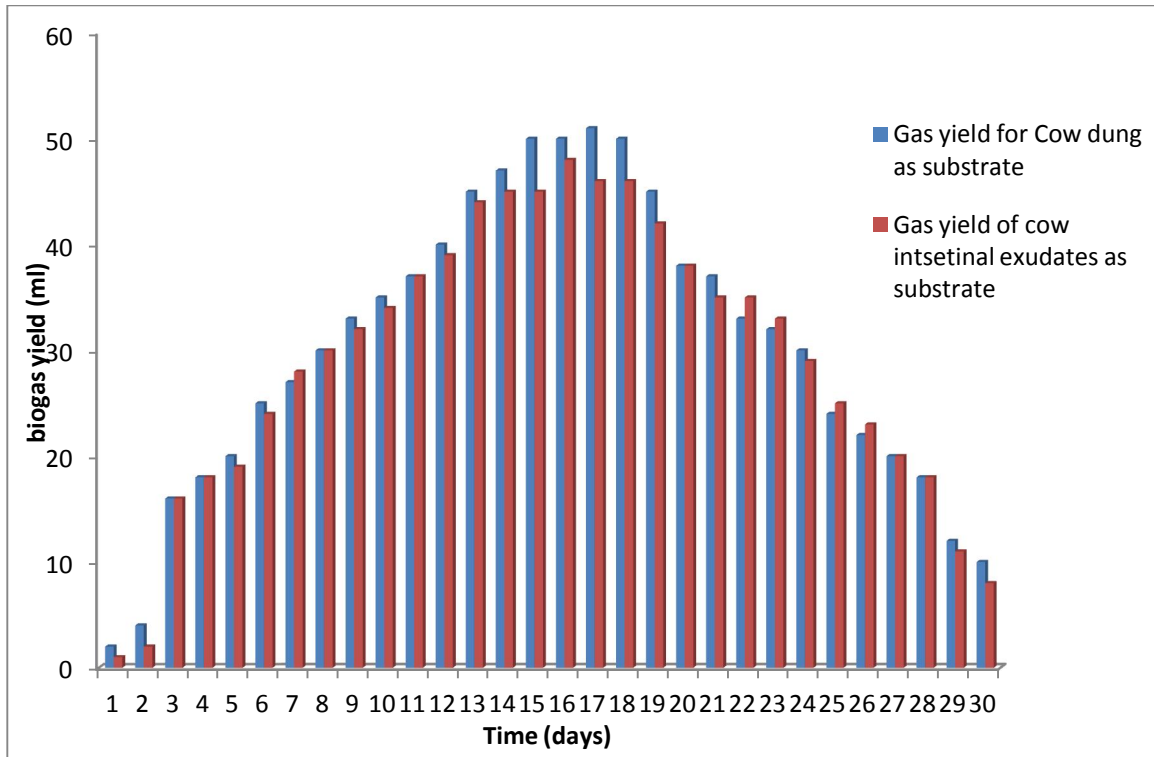
Figure 5: Comparative gas yield for cow dung and cow intestinal exudates as substrates for 30 days retention time

Table 2: **Characteristics of the isolates obtained from the cow dung and cow intestinal exudates.**

Isolate Code	Gram reaction and Morphology	O₂ Preference	Spore production	Motility	Growth on MAC	Growth on MSA	Indole	Catalase	Citrate utilisation	Oxidase	M R	Coagulase	VP	Urease	Nitrate reduction	Glucose	Lactose	Mannitol	xylose	maltose	Probable organism
K06	Gram positive cocci in clusters	Aerobic	-	-	-	+	ND	+	-	-	ND	+	+	+	+		+	+	-	+	*Staphylococcus aureus*
F09	Gram positive large bacilli	Strictly Anaerobic	+	+	-	-	-	-	-	-	-	--	-	-	+	+	ND	ND	ND		*Clostridium* sp
J07	Gram positive large bacilli in chains	Aerobic	+	+	-	-	+	+	+	+	-	+	+	-	-	+	-	-	-	-	*Bacillus* sp
B20	Gram positive bacili	Aerobic	+	+	-	-	+	+	+	+	-	+	+	-	+	-	-	-	+		*Bacillus cereus*
A02	Gram negative bacilli	Facultative anaerobic	-	+	+	-	+	+	-	-	+	-	-	+	+	+	+	+	+		*Escherichia coli*
C03	Gram negative bacilli	Facultative anaerobic	-	-	+	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	*Salmonella choleraesius*
I11	Gram negative bacilli	Facultative anaerobic	-	+	+	-	-	+	+	-	-	-	-	ND	+	+	+	+	+		*Enterobacter* sp
R10	Gram negative bacilli	Strictly anaerobic	+	+	-	-	-	-	-	-	ND	-	--	-	+	+	ND	ND	ND		*Clostridium* sp
Q13	Gram negative bacilli	Facultativaanaerobic	-	-	+	-	-	+	-	-	+	-	-	-	+	+	+	+	-		*Yersinia pestis*
H08	Gram positive large bacilli	Aerobic	+	+	-	-	+	+	+	+	-	+	+	-	+	-	-	-	-		*Bacillus* sp
R05	Gram positive cocci in clusters	Aerobic	-	-	-	+	ND	+	-	-	ND	+	+	+	+		+	+	-	+	*Staphylococcus* sp
A18	Gram negative bacilli	Facultative anaerobic	-	+	+	-	-	-	-	-	-	-	-	ND	+	+	+	+	+		*Enterobacter* sp
E02	Gram negative bacilli	Facultative anaerobic	-	+	+	-	+	+	-	-	+	-	-	+	+	+	+	+	+		*Escherichia coli*
G25	Gram negative bacilli	Facultative anaerobic	-	+	+	-	-	+	+	-	+	-	-	+	+	+	+	+	+		*Citrobacter freundii*
H17	Gram negative bacilli	Facultative anaerobic	-	+	+	-	+	+	+	-	-	+	+	-	+	+	+	+	+		*Proteus vulgaris*
R16	Gran negative bacilli	Facultative anaerobic	-	-	+	-	+	+	+	-	-	ND	+	ND	+	+	+	+	+		*Klebsiella pneumoniae*
J19	Gram negative bacilli	Facultative anaerobic	+	-	+	-	+	+	-	-	+	-	-	+	-	+	+	+	+		*Yersinia pestis*
Q33	Gram positive bacilli	Aerobic	+	+	-	-	+	+	+	+	-	+	+	-	+	-	-	-	-		*Bacillus* sp
B12	Gram negative bacilli	Facultative anaerobic	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	+		*Salmonella typhi*

Key: MAC – MacConkey agar ; MSA – Mannitol salt agar ; ND- Not determined; VP – Voges Proskaeur

4.   Discussion

Results shown above depict a typical biogas production system, however with variables that are largely dependent on factors of different inclinations. With respect to bacterial growth, the highest values were obtained on the 20th day for both cow dung and cow intestinal exudates. This is in line with results as obtained by Ofoefule *et al*. (2010) which showed a value of growth at the rate of 10^7 cells correlating with the 20th to 22nd day ably determined in the periods of peak gas production. Comparing the total viable bacterial counts of the two substrates, the cow dung substrate had higher values than the cow intestinal exudates. This could be attributed to the amount of organic matter present within the cow dung as compared to the intestinal exudates. According to Ofoefule *et al.* (2010), the dung from cattle as ruminant animals evidently contain high concentrations of microbial flora due to the presence of nutritive parts like crude protein, crude fibre, crude fat, ash, and other valuable components. Godi *et al*. (2013) also corroborated this fact by comparing cow dungs and biogas yields from different cow breeds sampled. Results obtained showed the proportional nutrient volume as determined by calorific values in calories per meter cubed.

The presence of high nutritive components and total viable bacteria does not however classically differentiate the bacteria based on nutrient preferences. The aim of this is to determine the distribution of bacteria species within the substrates and their growth dynamics in succession; so as to be able to plan a robust bioaugmentation or biostimulation system subsequently. Biogas production within a controlled bacterial system is governed by three key biochemical stages: hydrolysis, acidogenesis/acetogenesis and methanogenesis. These three stages are directly a consequence of the bacteria flora present within the system. Monitoring the growth of bacteria species (in colony forming units per gram) using three different media for supporting bacterial growth mimicking the 3 biochemical stages on the 1st, 10th, 20th and 30th days of sampling it would be observed that the various media harboured varying degrees of bacterial loads. However, there seemed to be a trend as observed with the two substrates, bacterial growth in media for detecting hydrolytic bacteria was highest on the first day of sampling for both cow dung and cow intestinal exudates but gradually reduced in a steady fashion until the 30th day of sampling. The reverse was however the case for the bacterial growth in acetogenic- and methanogenic-supporting media as their bacterial growths gradually increased; with the methanogenic-supporting media having highest bacterial load as determined on the 30th day when determined in the two substrates. In this regard, it could be inferred that bacterial growth dynamics within the biogas system was highly nutrient dependent, and occurred in a succession style. The hydrolytic bacteria were gradually succeeded over time by the acetogenic and methanogenic species within the substrates.

Comparing biogas yields by the two substrates over a 30 day period, it was observed that cow dung produced more biogas than the cow intestinal exudates. This could also be as a result of the higher bacterial load of cow dung as compared with cow intestinal exudates. Between the 12th and 19th days, biogas production was highest for the two substrates. This fact is also in line with the studies of Rabah *et al*. (2010) who observed the highest volumes of gas produced on the second week of substrate retention.

Bacterial diversity within biogas systems are an essential part of biogas producing communities. Bacteria isolated from the two substrates were biochemically identified as *Bacillus cereus*, *Bacillus* spp *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Proteus vulgaris*, *Salmonella choleraesius*, *Salmonella typhi*, *Staphylococcus aureus*, *Yersinia pestis* and *Clostridium* spp, *Staphylococcus* spp, *Enterobacter* sp. These are organisms classically associated with the gastrointestinal tracts of ruminant animals and have good potentials in facilitating biogas production because of the hydrolytic effects of their metabolism on substrates. They are typical members of biogas microbial communities associated with anaerobic systems due to their obligate/facultatively anaerobic nature of metabolism depending on the specie involved. Based on taxonomic profiles of biogas micro-communities usin short-read next generation DNA sequencing, Wirth *et al.* (2012) proved the presence (at varying degrees of abundance) of *Clostridium* species, *Enterobacter* species, *Bacillus* species, *Escherichia coli*, *Staphylococcus* species, and a host of other bacteria belonging to the Phyla Firmicutes, Bacteriodetes, Tenericutes and Actinobacteria, thus supporting the scientific observations of this study.

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