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IDENTIFICATION AND CHARACTERIZATION OF MICROBIAL COMMUNITY OF ANAEROBIC DIGESTED POULTRY LITTER

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ABSTRACT

Livestock farming have resulted in the release of excessive wastes which, contains abundant organic matter and microbial population. The need to develop an alternative and sustainable methods to minimize the waste generated and it effects on the environment led to the application of anaerobic digestion (AD) for the treatment of waste and generation of methane gas. The study focused on investigation of the microbiome involve in AD of poultry litter and was conducted using poultry litter as organic substrate under batch conditions at hydraulic retention time (HRT) of fifty-six (56) days in fifteen (15) liter fabricated digesters at 37^{O}C. The pH, total solid (TS), moisture content (MC) total ammonia nitrogen, volatile solid (VS) was assessed before and after digestion while the microbial community diversity was analyzed using 16S rRNA amplicon-based next-generation sequencing (NGS). The results indicated a pH of 7.91±0.04 before digestion and 7.33±0.06 after digestion and a TS value of 56.40±0.6% before digestion and 6.30±0.34% after digestion. A collective biogas yield of 5.21±21.00 bars were recorded. The characterization of biogas analyzed with non-dispersive infrared (NDIR) gas analyzer (gas board 3100p) revealed a percentage methane content of 50.31±1.33. The microbial community indicate *Bacteroidetes* (46.37%), *Firmicutes* (48.37%), *Proteobacteria* (8.17%), as the most dominant phylum. This study suggests the importance of molecular analysis as a fundamental tool to gain insight and deeper understanding of anaerobic digester performances.

Keywords: Anaerobic digestion; biogas; microbial community; retention time; substrate; systems

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INTRODUCTION

Poultry industry is one of the steadily growing agricultural sectors in Nigeria which is as a result of low financial investment required for startup. The evolutional growth of poultry farming in Nigerian has resulted in increased poultry waste amassing with the resultant environmental impacts [1]. These wastes are by-products of droppings, bedding materials such as straws, sawdust, wood shavings or rice hulls among others, dead birds, hatchery wastes, feathers processing waste water and bio-solids generated during and after production periods [2]. Poultry litter (PL) nutritional composition constitute of nitrogen (N), phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur(S), manganese (Mn), copper (Cu), zinc (Zn), chlorine (Cl), boron (B), iron (Fe), and molybdenum (Mo). It also contains inherent microorganisms that can potentially be beneficial and harmful to the environment. Many of the beneficial organisms play an important role in the ecological nutrient cycles associated with carbon, nitrogen, phosphorus, Sulphur and other elements which are linked to the nutritional composition of PL while the harmful pathogenic microorganisms affect the environment and human health [3]. These wastes pose serious environmental pollution problems through microbial infection, offensive odours, promotion of flies and rodent breeding, release of greenhouse gases among others [3 ,4]

Estimates by Musa *et al*. [5] reported about 932.5 metric tonnes of commercial poultry manure waste produced annually in Nigeria. The waste left without proper disposal and management constituent hazard to the environment. Anaerobic digestion (AD) as an alternative source of energy generation and waste treatment is a biological process that naturally occur when microorganisms break down organic matter in the absence of oxygen. It is a collection of processes (metabolic interactions) among various groups of microbes to decompose biodegradable materials in an oxygen depleted environment [6]. The process produce biogas consisting of methane (CH_4), carbon dioxide (CO_2), trace gases like hydrogen sulfide (H_2S) and hydrogen (H_2). Anaerobic digestion is widely used as a source of renewable energy. The biogas can be used directly as fuel, in combined heat and power gas engines or upgraded to natural gas-quality bio-methane. The entire process of anaerobic digestion for biogas production and waste treatment consists of four reaction which includes hydrolysis, acidogenesis, acetogenesis and methanogenesis [3,6]. Although AD can be considered to take place in three stages all reactions occur simultaneously and are inter-dependent. Each of the stage is linked with different population consortium of microbial community diversity [6]. Disruption at any step affects the populations of the other stages and causes an imbalance in the process which can result in accumulation of intermediate products, signifying that the microbial community is under

stress [7]. Most of these problems occur as a result of inadequate operational and process control and a lack of understanding of the structural community diversity of AD system [8]. To increase uptake of the technology, an understanding of the microbial ecology is required. Therefore, analysis of the microbial community will reveal the community with superior function which is indispensable for AD monitoring, waste treatment and biogas production.

MATERIALS AND METHODS

Study Site

The anaerobic digestion of poultry litter to produce biogas was carried out at the Department of Microbiology laboratory, Federal University of Technology Minna, Niger State, Nigeria.

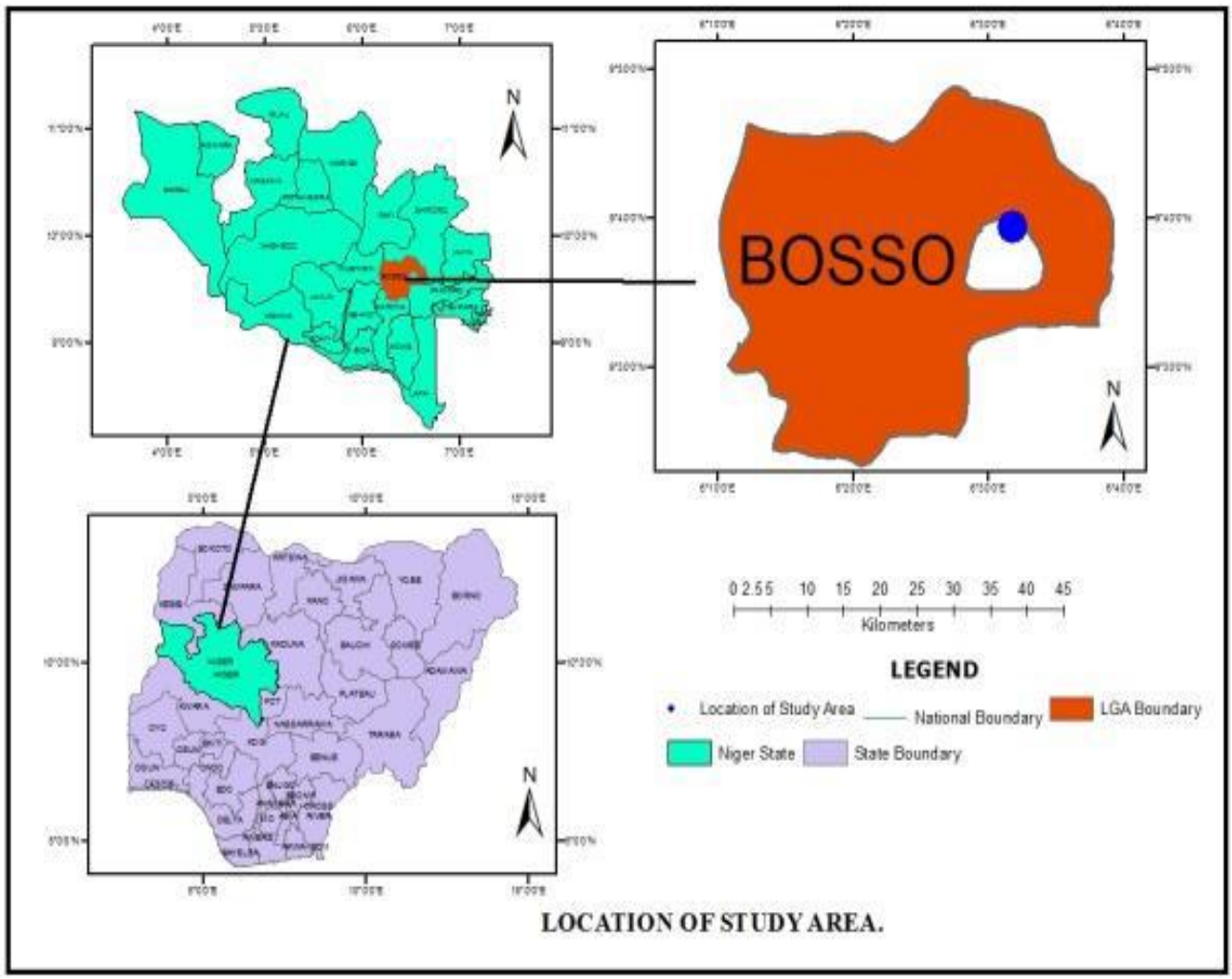


Plate I: Geographical location of study area [9]

Sample Collection

The fresh poultry litter used in this study was obtained from Premium poultry farm located at Kuje Federal Capital Territory Abuja, Nigeria. The samples were collected in a sterile container and transported to the Microbiology laboratory, Federal University of Technology Minna, Niger State, Nigeria.

Anaerobic Digester Design

A fifteen-liter semi-continuous capacity aluminum fabricated digester (Plate 1) was used for the study. Its dimension consists of a height (H) of 46cm and diameter (D) of 28cm. It has cast, internal gas re-injecting agitating mechanism to stimulate mixing within the digester. It has an attached thermometer to read the average temperature within the digester as well as an attached substrate collector (H-26cm, D-17.5cm). The substrate collector has an inlet to collect the substrate which feed the digester and an outlet to remove the digested slurry. The digester also has an attached gas collector (H-17.5cm, D-15cm) to collect the biogas, pressure gauge to measure the pressure within the reactor and highly resilient adhesive and plastic seals to prevent leakages.

Plate 1 Digester design

Experimental Design

To investigate the microbial community diversity of anaerobic digestion of poultry litter, nine (9) kilograms of the poultry litter was weighted into a sterile container containing three liters (3) of water in a ratio of 3:1 of waste to water. The combination was homogenously mixed together by stirring continuously. The mixture was fed into the fifteen-liter fabricated semi-continuous digesters through the inlet and sealed properly to prevent air from entering. Anaerobic digestion of the organic poultry litter was allowed for a period of fifty-six (56) days under mesophilic condition.

Within the retention time biogas production and composition were monitored and recorded using non-dispersive infrared (NDIR) gas analyzer (gas board 3100p) and pressure gauge at seven (7) days interval for 56 day while the microbial community was identify using 16S rRNA amplicon-based next-generation sequencing (NGS).

Operational parameter analysis

The operational parameter of the poultry litter was determined before and after anaerobic digestion. Parameters such as pH was determined using pHep pocket-sized pH meter (HANNA Instruments). Volatile solids (VS), Total solids (TS) and moisture content was measured according to standard APHA methods [10] while ammonium concentration was analyzed using the method described by Lin *et al.* [11]

Microbial community diversity analysis [12].

Thirty grams (30 g) aliquot of the sample was collected using aseptic techniques and dispensed into 20 ML of sterile LB broth. The added mixture was incubated for twenty hours (24h) before the total community DNA was extracted using the Qiagen Dneasy Blood and Tissue Kit (cat. 69506). The growth from the broth were pelletized in a well labelled 1.5mL microcentrifuge tubes, 200µL Buffer AL (lysis buffer to break open cells) was added to each of the tubes and mixed by vortexing. The tubes were then incubated at 56°C for 10 minutes after which 200µl of ethanol (96–100%) was added and mixed thoroughly by vortexing. The mixture was pipetted into a DNeasy Mini spin column in a 2 ml collection tube and centrifuged at 6000 x g (8000 rpm) for 1 min. The flow-through and collection tube were discarded. The spin columns were placed in new 2 ml collection tubes.500µl of Buffer AW1(wash solution buffer) was added to the spin column and centrifuged for 1 minute at 6000 x g. The process was repeated with the addition of 500µl of Buffer AW2 (wash solution buffer) and centrifuged for 3 minutes at 20,000 x g (14,000 rpm). The flow-through and collection tube were discarded and the spin columns were carefully removed to avoid contact with the flow-through. The spin columns were then transferred into new 1.5 ml or 2 ml microcentrifuge tubes of which 200µl of Buffer AE was added to the centre of the spin column for elution of the genomic DNA. The eluent was then Incubated for 1 min at room temperature and centrifuge for 1 min at 6000 x g. DNA quality and concentration were checked by running 2µl of the diluted DNA sample on 1% agarose gel. Accurate DNA

quantification was carried out using a NANODROP®2000 spectrophotometer (Thermo Scientific Inc.)

Genomic DNA samples were PCR amplified using a universal primer pair for 341F and 785R (Table 1) targeting the V3 and V4 region of the 16S rRNA gene. PCR was carried out in a total volume of 25µl containing 100ng of genomic DNA, 2.5µl of 10× PCR buffer, 1µl of 50mM $MgCl_2$, 2µl of 2.5mM dNTPs (Thermo Scientific), 0.1µl Taq polymerase (Thermo Scientific), 1µl of DMSO, 1µl each of forward and reverse primer and 11.3µl of H_2O. Touch-down PCR was used for amplification as follows: initial denaturation step of 5mins at 94°C, followed by 9 cycles each consisting of a denaturation step of 20sec at 94°C, annealing step of 30sec at 65˚C, and an extension step of 72°C for 45sec, this was followed by another 30 cycles each consisting of a denaturation step of 20sec at 94°C, annealing step of 30sec at 55˚C, and an extension step of 72°C for 45sec. Resulting amplicons were gel purified, end repaired and illumina specific adapter sequence were ligated to each amplicon (NEBNext Ultra II DNA library prep kit).

Following quantification, the samples were individually indexed (NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1), and another AMPure XP bead-based purification step was performed.

Amplicons were then sequenced on illumina's MiSeq platform, using a MiSeq v3 (600 cycle) kit. For each samples 20Mb of data (2x300bp long paired end reads) were produced for each sample. The BLAST-based data analysis was performed using an Inqaba in-house developed data analysis pipeline and the Galaxy platform (http://galaxyproject.org/) .

Table 1 Primers used in PCR amplification of 16S rRNA genes

Target	Primer	Sequence (5'--3')
V3-V4	S-D-Bact-0341-b-s-17	CCTACGGGNGGCWGCAG
	S-D-Bact-0785-a-A-21	TTACCGCGGCKGCTG

* [12]

Data Analysis

Data generated were analyzed using Analysis of variance (ANOVA) with multiple error terms to test for significant difference between means at significant level of ($P<0.05$)

RESULTS AND DISCUSSION

Operational Parameter of the poultry litter

Table 2 shows the results of the operational parameters of the poultry litter before and after digestion within a retention time of fifty-six (56).

Table 2 Operational parameter of the substrate before and after digestion

Parameters	Initial	Final
pH	7.91 ± 0.04^{c}	7.33 ± 0.06^{b}
TS (%)	56.40 ± 0.6^{c}	6.30 ± 0.34^{b}
VS (%)	64.70 ± 0.7^{c}	50.7 ± 1.80^{b}
NH_4^+-N (mg/l)	0.35 ± 0.02^{b}	0.56 ± 0.03^{b}
MC (%)	27.70 ± 0.50^{b}	91.8 ± 2.25^{a}

Values are Mean±SEM of triplicate determinations. Superscript with different alphabets across a row are significantly different at $p<0.05$. ALK: Alkalinity, TS: Total solid, VS: Volatile solid, OM: Organic matter, TC: Total carbon Alkalinity, NH4+-N=Ammonia-Nitrogen, COD=Chemical oxygen dissolved, MC: Moisture content

A pH value of 7.91±0.04 was recorded before digestion while 7. 33±0.06 pH was observed after digestion of poultry litter. Total solid (TS) and volatile solid (VS) were observed to have a percentage of 6.30±0.34 and 50.7±1.80 respectively after digestion. The result also showed a significant ($p<0.05$) reduction in pH value from the initial pH. The reduction is probably due to the stages of anaerobic digestion process, substrate composition or the activities of microorganisms. The pH of a substrate has a strong influence on microbial activities and the process of anaerobic digestion [13]. A low pH in the digester inhibits the activity of microbes involved in the digestion process particularly methanogenic bacteria [13]. The pH value for methanogenic bacteria is within neutral to slightly basic. Methanogens are more sensitive to pH. The pH recorded in this study is within the pH range for efficient digestion necessary to activate the growth of methanogens and for biogas production [13].

Ammonium Nitrogen (NH_4^+-N) increase from 0.35±0.02 to 0.56±0.03 mg/l. NH_4^+-N contribute to the vital nutrients for microbial growth and replication [14]. The result obtained may presumably be accredited to substrate composition. Poultry litter consists of high level of organic nitrogen concentration which when used as substrate for anaerobic digestion may result in high concentration of total ammonium ion plus free ammonia [15]. High level of NH_4^+-N as reported by Tada *et al*., [16] can be toxic to anaerobic microorganism and can inhibit their activity and biogas production. In this finding the recorded concentration is not inhibitory to

anaerobic digestion process and is in good agreement with that reported by Oleszkiewicz and Poggi Varaldo [17] who observed an increase from the initial of 0.359g/kg to 0.82g/kg with a biogas yield of 302L/kg.

Total and volatile solid are important operational parameters that help determine the characterization and composition of a substrate. The amount of biogas generated depends on the quantity of total and volatile solid. TS and VS is the amount of solids present in the waste and its degradability. The study recorded a reduction in TS and VS. The percentage reduction of TS and VS maybe due to active performance of microorganisms digesting the substrate resulting from sufficient availability of moisture content concentration as indicated in the final concentration when compared to the initial (Table 2). The total solids and volatile solid in this study are within the range for biogas production when compared with Dupade *et al.* [18]

Biogas Production (bars)

The collective amount of biogas produced from the poultry litter within a retention time of fifty-six (56) days at 37^{O}C is shown in Figure 1.

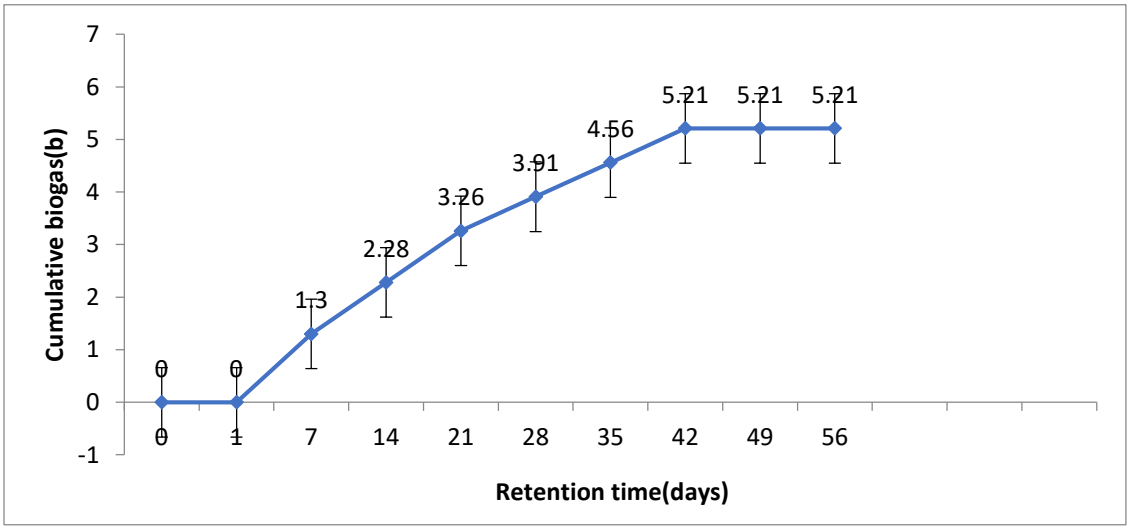


Figure 1 Cumulative biogas production yield

The anaerobic digestion of poultry litter indicates a gradual increase from day one to day 56 . At day forty-two, the methane content was 5.21 bars which continued until day 56. The recorded progressive increase in the quantity of gas produced may be attributed to microbial adaptation to the substrate and biodegradation activities of the microorganisms present in the digester [19; 20] while parallel amount was observed from day 42-56 may have resulted from depletion in substrate and total solid reduction which according to Sadaka *et al.* [21] and *Leh-*Togi *et al.*, [22] implies complete substrate utilization by the microorganisms and subsequent increase in biogas yield.

Characterization of Methane Gas

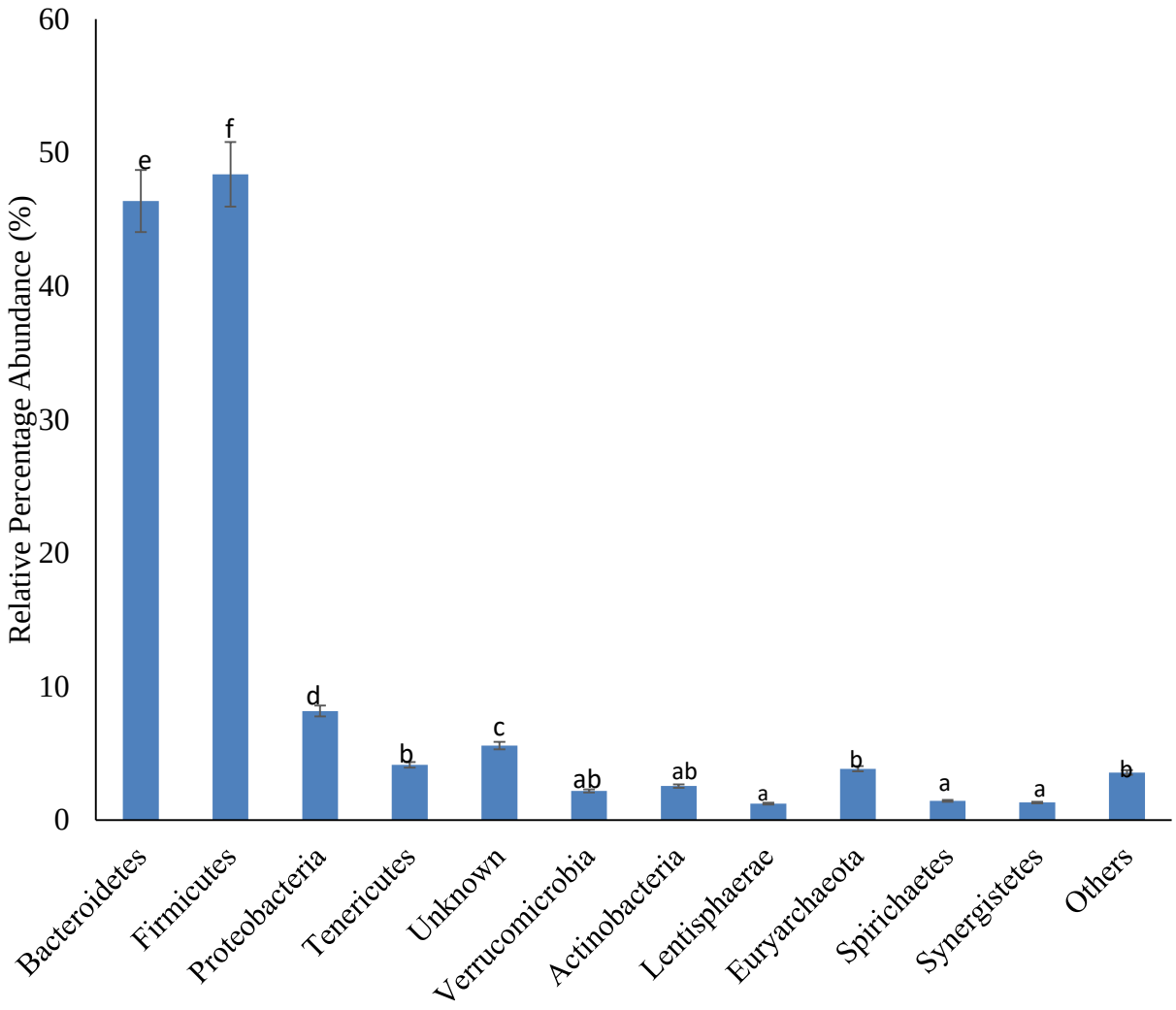
T able 3 Characterization of methane gas (%) from NDIR gas analyzer

Component	D3
CH_4	$50.31\pm1.33_d$
CO_2	$16.68\pm0.70_c$
H_2	$1.29\pm0.04_a$
O_2	$2.87\pm0.25_{ab}$
H_2S	$1.11\pm0.11_a$

Values are Mean±SEM of triplicate determinations. Different subscripts along the column respectively are significantly different at $p<0.05$

The characterization of gas generated from NDIR gas analyzer detected CH_4, CO_2, H_2, O_2 and H_2S gases (Table 3). The analysis revealed an average percentage content of CH_4, CO_2, H_2, O_2 and H_2S value as 49.61±1.14, 20.84±1.33, 2.66±0.64, 5.19±0.21, 1.44±0.10 respectively. The percentage composition of methane recorded in the digesters agrees with that reported by Vishwanath [23] but is not in consonance with that of Demirbas *et al*., [24] who reported that biogas is made-up of CH_4 (55-75%), CO_2 (25-45%), H_2S (0-1%), and O_2 (0-2%). The gas characterized reveals consistency with data obtained from previous study.

Microbial Community Diversity



Top microbial taxanomic phyla distribution

Bars with different alphabets within each phylum are significantly different at $p<0.05$

Figure 2 Frequency of Occurrence (% Abundance) of Microbial distribution at Phylum level at Day 56

The major microbial phylum identified and their frequency at day 56 is shown in Figure 2. The study recorded relative abundance of 46.37%, 48.37% and 8.17%, for *Bacteroidetes, Firmicutes* and *Proteobacteria*. *Firmicutes, Bacteroidetes* and *Proteobacteria* were the most dominant phylum which were also observed in the findings of Guo *et al.*, [25]. As indicated in the result *Firmicutes* had the highest occurrence (%) with *Clostridia* and *Mollicutes* as the highest Class. As reported by Westerholm *et al.*, [26] the predominance of *Firmicutes* maybe attributed to

substrate availability which promote the growth of members within the phylum. *Firmicutes* produce endospores which are resistant to environmental stress thereby surviving other phylum upon deprivation of nutrient as observed in Figure1 from day 42-56 corresponding biogas yield resulting from depletion of substrate. The result showed no significant difference between the relative abundance of Phylum *Bacteroidete*s and *Firmicute*s. The abundance of *Bacteroidete*s maybe due to their ability to withstand the fermentation process and produce organic acids as metabolic product. Therefore, their presence in anaerobic digestion is related to high level of volatile fatty acids production [26,27].

The phylum *Euryarchaeota* recorded a 3.85% relative abundance in the microbial community which include the Order belonging to *methanosarcinales* (2.61%), *Methanomicrobiales* (1.96%) *Methanomicrobiales* (0.83%), *Methanopyrales* (0.01%) and *Methanococcales* (0.01%). *Methanosarcinales* had the highest percentage richness. Their presence in an AD system indicates methane production. *Methanosarcinales* can produce methane using acetolactic, hydrogenotrophic, and methylotrophic pathway at high acetate concentration [27] thus, they are regarded to be more competitive than other order due to the fact that they can withstand increasing concentration of AD inhibitory agents such as ammonia, hydrogen sulphide and high volatile fatty acids. These agents allow for the growth of *Methanosarcinales* during AD operation [28] thus their presence in an AD system can be used to monitor the process since it suggests accumulation of Acetate.

CONCLUSION

The study identified and characterized the microbial community of anaerobic digestion of poultry litter using next generation sequencing. The research revealed phylum *Firmicutes* and *Bacteroidetes* as the most dominant bacteria while *Euryachaeaota* as the major Archaea in the digester. The presence of the order *methanosarcinale* indicate high level of acetate and its conversion to methane. The findings therefore reveals that the presence of some particular microbial phylum in an AD system can indicate biogas production and can also be used to monitor and optimize the AD process.

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