Molecular Detection of Biogenic Bacteria During Biogas Production Using Domestic Feed Stock

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ABSTRACT

The search for a sustainable and environmental friendly energy has resulted to the development of an alternative renewable energy such as biogas. The objective of this work was to identify the presence of methanogenic bacteria in a short-term operated biogas reactor fed with kitchen waste by PCR-amplification of the methyl coenzyme M reductase (*mcrA*). Household organic waste was obtained from Bells University of Technology canteen while pig manure that served as the inoculum was obtained from a pig farm. Three replicate samples were taken every week and analysed for changes in the physico-chemical parameters to determine their effects on the rate of biogas production. Results obtained showed that the % moisture and carbon contents, before and after fermentation, were reduced from 88% to 80% and 16.7% to 12.5%, while % nitrogen content increased from 1.20% to 1.70%. The mean weekly biogas production ranged from 27.4 ml to 147 ml at 4 weeks and 6 weeks, respectively. Methyl coenzyme M reductase (mcrA) gene, which codes for the enzyme that catalyse the terminal step in methane production during the anaerobic fermentation of biomass, was amplified both in the methanogenic bacteria and transformed *E. coli* with the fragment size of 500 bp.

INTRODUCTION

Economic growth and industrialization in developing countries have led to a tremendous increase in the demand for energy worldwide including Nigeria. Up to date, Nigeria solely depends on fossils for generation of power and energy. The current dwindling of fossil fuel coupled with low pricing of crude oil, environmental degradation, and pollution have resulted in the search for an alternative source of renewable energy to meet the increasing demand for power and energy. Hence, the need for a search for an alternative means of energy has become imperative.

Biogas, a combustible gaseous fuel generated from anaerobic microbial degradation of organic matter is one of the most efficient and effective sources of renewable energy currently available (Mao *et al*., 2015; Bacenetti *et al.,* 2013; Rehl and Mueller, 2011). Biogas is principally a mixture of methane (CH_4) and carbon dioxide along with other trace gases (Wirth *et al.,* 2012). In the last decades, successful researches have been conducted on the use of energy crops, animal faeces, and organic waste for the production of biogas as an alternative to fossil fuel (Tavi and

Tiwari 2013; Kumar *et al*., 2013). The degradation of the organic materials requires a combined action of different groups of microorganisms (aerobic and anaerobic) with different metabolic capacities sensitive to variations in the operating conditions applied during the fermentation process. Thus, the anaerobic digestion process, if improperly managed, would become unstable and result in reduced biogas production (Mao *et al*., 2015). The composition of the active biogas producing microbial consortium, the Eubacteria, and Archaea depends on various factors, such as substrate ingredients, temperature, pH, mixing, or the geometry of the anaerobic digester (Hovarth *et al*., 2016; He *et al.*, 2007; Wirth *et al*., 2012). Study of the microbial community is very crucial for the optimization, control, and in-depth knowledge of the performance of the process for a better large scale biogas production. Recent developments in molecular biology techniques such as metagenomics approaches have provided a valuable tool for improved understanding of this complex microbiological system, which in turn could help optimize and control the process in an effective way (Hovarth *et al*., 2016; Li *et al.*, 2015; Wirth *et al.*, 2012). Revelation of compositions of microbial communities, based on the generation of 16 S rRNA gene clone libraries and Sanger sequencing of the 16 S rDNA amplicons, next generation sequencing (NGS) such as 454 pyrosequencing and Illumina sequencing techniques, have been developed (Klocke *et al.*, 2007; Mc Hugh *et al.,* 2003), but the use of the mcrA gene, which codes for one of the key enzymes in methanogenesis, the α-subunit of methyl-coenzyme M reductase occurring uniquely in methanogens has greatly improved the understanding of the microbial community involved in anaerobic biogas production (Schlüter, *et al.,* 2013; Krause *et al.,* 2008; Kröber *et al.,* 2009). Therefore, the general objective of this work was to identify the presence of methanogenic bacteria in a short-term operated biogas reactor fed with kitchen waste by PCR-

amplification of the methyl coenzyme M reductase (*mcrA)*.

MATERIALS AND METHODS
Digester Design:

A laboratory size digester made of stainless steel was employed. The digester dimensions were 3.8 L volume, thickness (0.8 mm), height (27 cm), and diameter (17 cm). A tap for sample collection and an outlet for daily measurement of temperature were fitted to the digester. The evolved gas left the fermenter through flexible neoprene tubing connected to the top and a port for gas sampling was installed through a silicone rubber septa.

Sample Collection and Pre-Treatment:

Organic household waste which consisted of potato, rice, carrot, apple, water melon peels, and pineapple peels were collected from food canteen situated at Bells University of Technology at Ota, Ogun State, Nigeria. Pig dung which served as inoculum was collected from a nearby farm at Ota, Ogun state, Nigeria. Extraneous materials like toothpick, tissue papers, and bones were removed from the organic waste and homogenization using a household blender. The sample was incubated in a closed container for three days with daily stirring for inoculum development. The inoculum was inoculated in to the kitchen waste and water at the ratio of 0.5:1:1 respectively, for batch fermentation (Lama *et al.,* 2012).

Determination of Slurry Physical Parameters:

During fermentation, the pH was monitored using pH meter (model M501 Rev Jenway CE350EU). Ambient and slurry temperatures were monitored twice daily at 9.00 am and 4.00 pm with the aid of an inbuilt mercury- in-bulb thermometer (*model 08500-40*, Cole-Parmer, Chicago, IL, USA). The percentage moisture content of the fermenting sample was monitored by drying to constant weight at $105^{\circ}C$ for 12 hrs. This step was repeated after the anaerobic digestion. The percentage ash and nitrogen

were determined before and after the fermentation according to AOAC (1990).

DNA Extraction and PCR Amplification of Community *mcrA* :

The community DNA was isolated from the slurry samples after digestion using the DNA extraction fodder kit, Transgen Biotech, China, according to manufacturer's instructions. The extracted community DNA quantity and purity was done using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Washington, USA) as described previously by Wirth et al., (2012). Amplification of the Methyl coenzyme reductase (*mcrA*), MCRF and MCRR was done using two sets of primers (mcr forward primer 5' GGTGGTGTMGGATTCACACARTAYGCWA CAGC-3', reverse primer 5'-TTCATTGCRTAGTTWGGRTAGTT-3') and the method previously described by Luton *et al.,* (2002) was adopted for the amplification. A 50µl PCR reaction containing; 100 nM primer, 0.2 mM dNTPs, 1X Colorless Taq Reaction Buffer which contained 1.5 mM $MgCl_2$ (Transgen biotech, China), and 1.25U GoTaq polymerase was used for the PCR. Template concentrations were approximately 10 ng per reaction tube. The PCR conditions were as follows: 95°C (5 min), 35 cycles of 95°C (1 min), 49°C (1 min), 72°C (3 min), and a final extension 72°C (10 min). The programme included a slow ramp in temperature (0.1°C s^{-1}) between the annealing and extension steps of the first five cycles of the protocol to assist in the initial formation of product because of the degenerate nature of the primers (Luton *et al*., 2002). PCR products were resolved on a 1.5% agarose gel, and the band of expected size was excised. The extracted DNA (using QIAquick gel extraction kit, Qiagen, Valencia, CA), was cloned into T1 cloning vector (Transgen Biotech, China), and then transformed into *Escherichia coli* top ten cells (Transgen Biotech, China). Plasmid extraction of the transformed *E. coli* was done using QIAquick (Qiagen, Valencia, CA) plasmid extraction kit following the manufacturer's instruction.

RESULTS

Determination of Slurry Physical Parameters:

Results obtained from the determination of the slurry physical parameters during the anaerobic digestion showed that the moisture content decreased from 88% to 80% at the end of the anaerobic digestion while there was an increase in the nitrogen content from 1.2% to 1.7%. The pH of the slurry decreased from 6.5 to 5.5 during the first week of the experiment followed by no further increase or decrease of pH at the 3rd and 4th week of the anaerobic digestion. The final pH at the end of the experiment was 5.0 as shown in (Fig. 1). The ash content of the slurry remained constant throughout the anaerobic digestion (Fig. 1).

Determination of the Daily Biogas Production:

Measurement of the daily gas production showed that there was a non-linearity in biogas production for the 42 days period of incubation (Fig. 2). Gas production started on the 4th day of fermentation and continued up to the 17th day. A breakage in gas production was observed on the 18th, 33rd, 34th, 37th, and 38th day of the anaerobic digestion and a final stop in production was observed from the 40th day to the end of the experiment (Fig. 2).

Effect of Temperature on Biogas Production:

Results obtained from the determination of effect of temperature on biogas production showed that there was a gradual change in biogas production in relation to temperature change and incubation time. In the first week of fermentation, production of biogas was very low (44.3 ml) at 30°C. However, as the temperature increased from 32°C to 36°C, gas production gradually increased from 82 ml to 142 ml. The highest gas production of 147.1 and 142.8 ml were achieved in week 4 and 5, respectively at 36°C (Table 1). Gas production decreased with time and finally stopped in the 6th week of fermentation at 27.4 ml/36°C (Fig. 3).
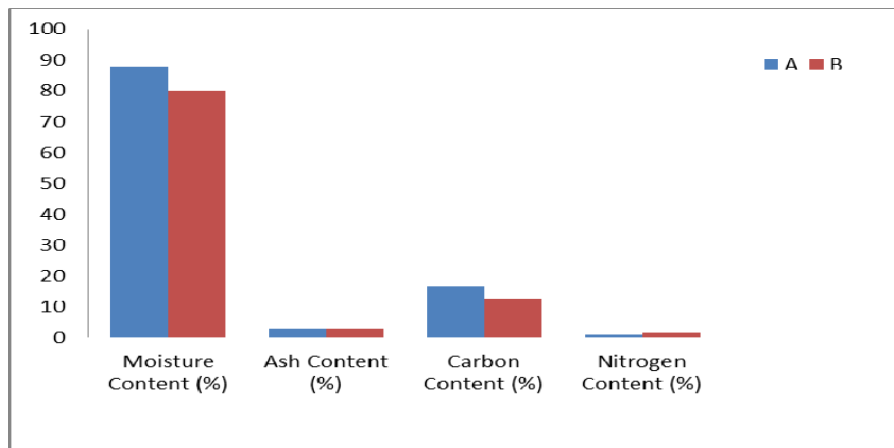
Fig. 1: Proximate analysis of Compositional feedstock before and after digestion. A, before digestion; B, after digestion.
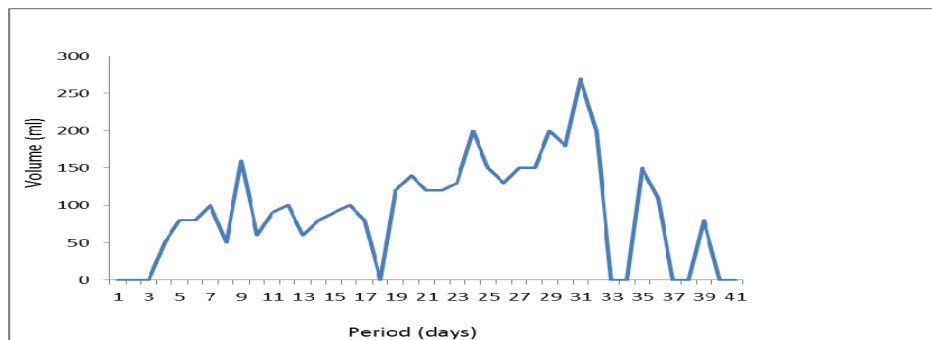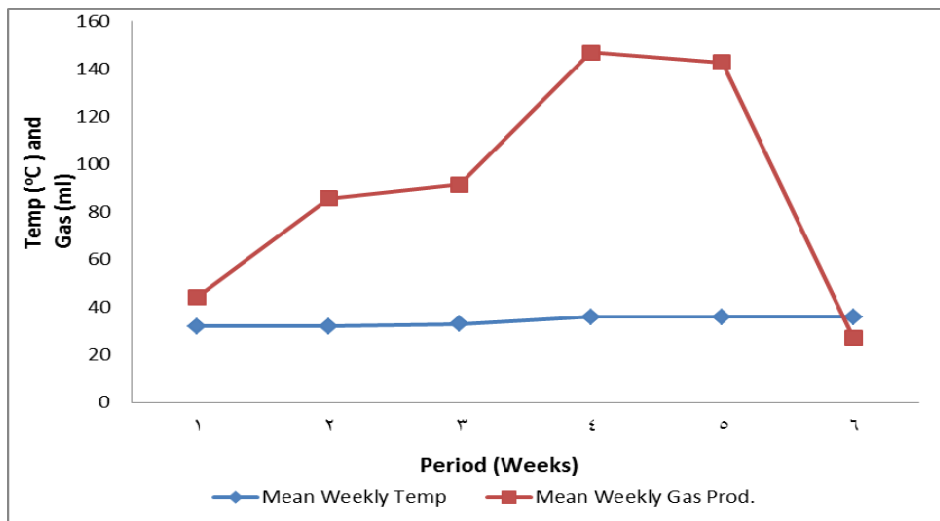

Fig. 2: Daily biogas production.

Fig. 3: Variation in Gas production and Temperature.

Table 1: Mean weekly temperature and biogas production over a period of 6 weeks.

Period (weeks)	Mean Temp.(^{o}C)	Mean Gas production (ml)
1	30	44.3
2	32	85.7
3	33	92.8
4	36	147.1
5	36	142.8
6	36	27.4

Amplification of the mcrA Gene:

The primer set used in the amplification of the McrA gene from the methanogens gave products of the expected size (500 bp) from all the samples analysed (Plate 1). Similarly, the same expected size of the product was obtained from the transformed *E. coli* cells after plasmid extraction when resolved on 1.5% agarose gel (Plate 2).
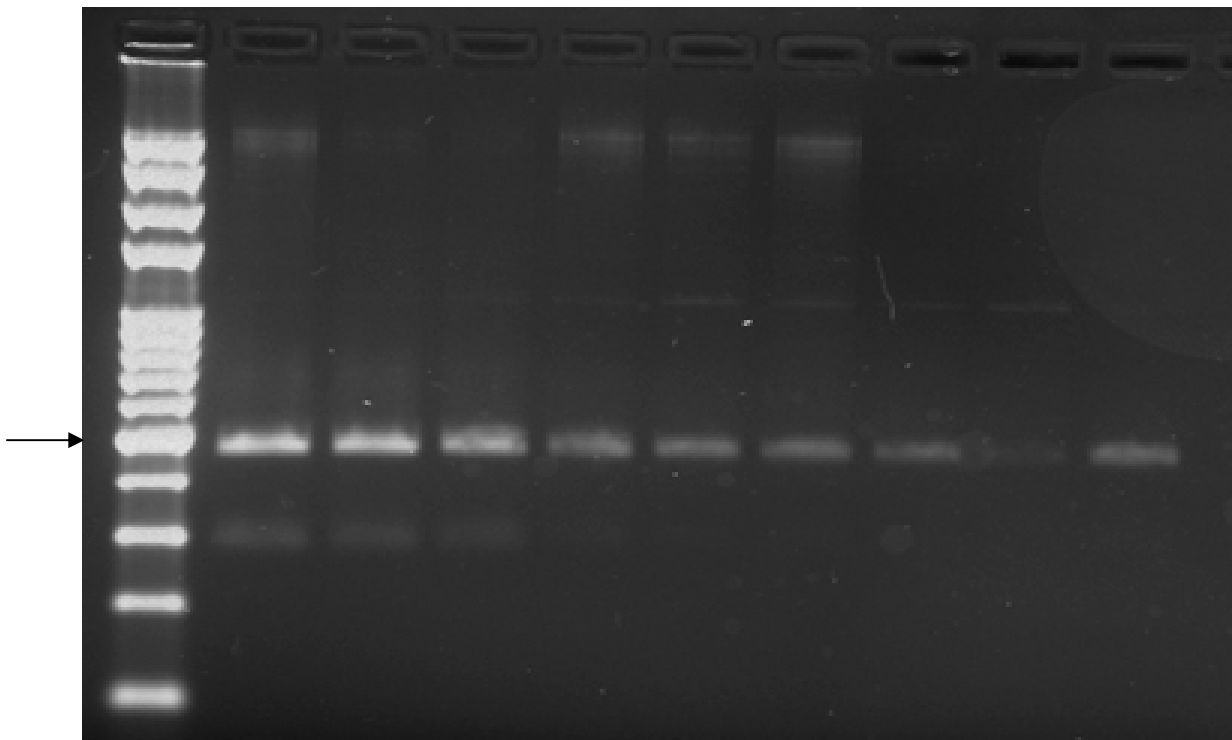

Plate 1: mcrA PCR products of the Methanogenic bacteria present in the slurry. Arrow shows the expected base size (500 bp) of the amplified mcrA gene from the community. Lane M is the molecular marker, lane 1 to 9, the amplified mcrA gene from the different samples anayzed.
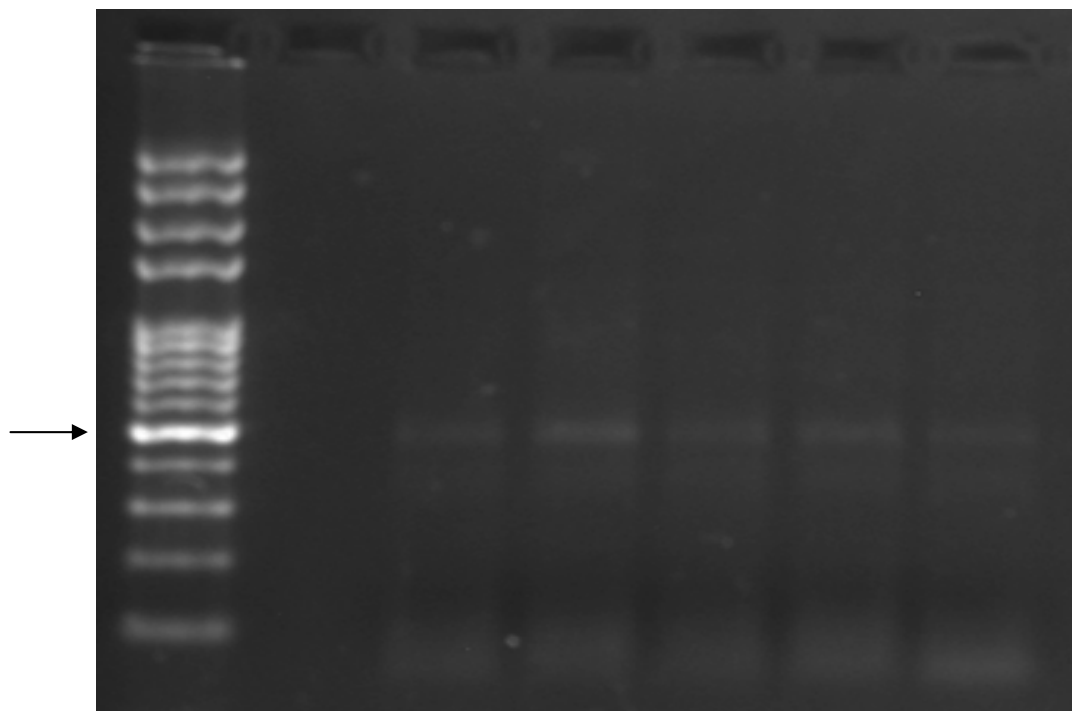
Plate 2: PCR products of transformed *E. coli*. Arrow shows the expected base size (500 bp) of the amplified mcrA gene from the plasmid vector. Lane M is the molecular marker, lane 1 to 5, the amplified mcrA gene from the transformed *E. coli* cell.

DISCUSSION

The production of biogas to replace the use of fossil fuel has received much attention over the years though it's still at an infant stage in Nigeria. The fermentation carried out for a period of six weeks showed the production of biogas by the microbial community present in the anaerobic digester. During the process, there was no production of biogas until the fourth day of fermentation, suggesting that there was a lag phase before the commencement of fermentation by the microorganisms. Similar observation was made by Tavi and Tawari (2013) who observed biogas production on the third day of fermentation. The volume of biogas produced in this study, is an evidence that all the feedstock used were good substrates and the homogenization of the feedstock also helped to increase the surface area which allows better digestion by the microbial community. The generation of biogas depends on several factors such as, pH, temperature, moisture, ash, nitrogen contents, and microbial activities (Ezekoye *et al.,* 2014; Elijah *et al.,* 2009), and these parameters were at optimal level during the anaerobic digestion excluding the moisture content which was above the optimal moisture content for favourable biogas production. The samples were found to have high moisture content (80-88%), and this may have reduced the cumulative volume of gas produced. Reports from previous works (Ezekoye *et al.,* 2014; Ubwa *et al.,* 2013; Ezekoye *et al.*, 2011; Maishanu and Sambo, 2007) showed that lower moisture of 4.1-9.1% favours production of higher volume of biogas compared to higher moisture observed during the anaerobic fermentation. This could have contributed to the low biogas production of 5.39 L within 6 weeks as against 10.33 L recorded by Ubwa *et al.,* (2013).

The samples have ash contents of 2.7% before and after digester. It is noteworthy that the stable ash content value after digestion may be due to return of minerals from dead microorganisms into the mixture. The C/N ratio (16.7:1.20) for the digester feedstock was very ideal for gas production in the substrate utilized and was

similar to the values (14.12:1.99 and 12.64:2.19) reported previously by Ezekoye *et al.,* (2011) and (2014), respectively.

Ambient and slurry temperature has been shown to affect the rate of anaerobic digestion and biogas production (Pramod *et al.,* 2012; Kuo and Lai, 2010; Climent *et al.,* 2007), thus a holistic knowledge of the optimal temperature for better biogas production is very crucial. In this study, the ambient temperature fluctuated between 30°C-35°C due to temperature gradient between the digester and its immediate environment (Tavi and Tawari, 2013). This implies that seasons could affect the rate of heat loss or gain from the digester which in turn affects the microbial activity and gas production (Ezekoye *et al.,* 2014; Kim *et al.,* 2003). The mesophilic system (30-35°C) used for this study is reported to be beneficial as it is more stable than the thermophilic system and less prone to system crash (Xiaojiao *et al.,* 2014; Tavi and Tiwari, 2013) although the thermophilic process offers higher production rate of methane, faster kinetics and pathogen removal (Kardos *et al.,* 2011). The stoppage of gas production after 6 weeks of incubation is normal as 80-90% of total gas is usually obtained within 3-4 weeks under optimal condition (Chael *et al.,* 2008).

The detection of the *mcrA* gene, a gene specific to methanogens, is an indication that this group of organisms known for methane production under anaerobic condition was very active during the fermentation process. The PCR products separated by agarose gel electrophoresis had an estimated fragment size of 500 bp in all the samples. This is similar to the results of Morris *et al.,* (2012) who reported fragment sizes of 460-490 bp, thus asserting the presence of methanogens in the bioreactor even at lower pH (5.5). Thus, this indicates that methanogens can survive in slightly acidic environments and methanogenesis can still occur. This agrees with previous findings by Smith (1996) and Zeikus (1999) who isolated methanogens from a slightly acidic environment of pH 5.0. Due to the

slightly acidic nature of the digestate, the groups of methanogens likely to be dominant in this study are methanogens which decompose acetates, *Methanosarcina barkeri* and *Methanosarcina* sp. Previous studies reported that this group of methanogens was isolated from environments of approximately pH 5.0 (Smith, 1996).

Results from direct PCR of randomly picked white clones showed that transformation was successful and reaffirmed the presence of *mcrA* genes in the transformed *E. coli* cells. This method is effective for the molecular characterization of methanogens; however, a potential pitfall of this method is that, it favours the selection of a particular order of methanogens thereby giving incomplete information for the characterization of the methanogenic community present in the bioreactor. The characterization of methanogens using a next generation sequencing techniques such as 454 pyrosequencing or Illumina sequencing to elucidate the total microbial community composition and/or to monitor the microbial succession in the fermentation slurry during anaerobic digestion would be a focus for further investigation.

REFERENCES

A.O.A.C. 1990. In Helrich K. (Ed.), *Official methods of analysis.* (15th ed.). Arlington, VA, USA

Bacenetti, J., Negri, M., Fiala, M. and Gonzalez-Garcia, S. (2013). Anaerobic digestion of different feed stocks: impact on energetic and environmental balances of biogas process. *Sci. Total Environ.,* 463: 541–51.

Chael, J., Celis, L. B., Alatriste-Mondragón, F. and Razo-Flores, E. (2008). Decreasing methane production in hydrogenogenic UASB reactors fed with cheese whey. *J. Biomass Bioenergy,* **63:**101–108.

Climent, M., Ferrer, I., Baeza, M. D., Artola, A., Vazquez, F. and Font, X. (2007). Effects of thermal and

mechanical pretreatments of secondary sludge on biogas production under thermophilic conditions. *Chem. Eng. J.,* **133**: 335-342.

Elijah T. I. Ibifuro, A. M. and Yahaya, S.M. 2009. The study of cow dung as co-substrate with rice husk in biogas production. *J. Environ. Microbiol.,* 4(9): 861-866.

Ezekoye, V. A., Ezekoye, B. A. and Offor, P. O. (2011). Effect of Retention Time on Biogas Production from Poultry. Droppings and Cassava Peel. *Nig. J. Biotechnol.* **22** (11): 53-59

Ezekoye, V. A., Onah, D. U., Offor, P. O. and B. A. (2014). Characterization of Biogas Produced from Rice Husks and Algae using a Metal Fixed-Dome Biodigester. *Global J. Sci. Front. Res.* **14** (1): 234-240.

He, R., Liu, X. F., Yuany, X., Yan, Z. Y., Liaoy, Z. and Li, X. D. (2007). Review on enhancing biogas production by additives. *China Biogas*, 5: 005

Horvath, I. S., Tabatabael, M., Karimi, K. and Kumar, R. (2016). Recent updates on biogas production – a review. *Biofuel Res. J.,* **3** (2): 394-402.

Kardos, I, Juhász, A., palkó, G. Y., Oláh, J. Barkács, K. and Záray, G. Y. (2011). Comparing of mesophilic and thermophilic anaerobic fermented sewage sludge based on chemical and biochemical tests. *J. Appl. Microbiol.,* **4** (1): 21-28.

Kim, J., Park, C., Kim, T-H., Lee, M., Kim, S., Kim, S-W et al. (2003). Effects of various pretreatments for enhanced anaerobic digestion with waste activated sludge. *J. Biosci. Bioeng.*, 95: 271–5

.Klocke, M., Mähnert, P., Mundt. K., Souidi, K. and Linke, B. (2007). Microbial community analysis of a biogas-producing completely stirred tank reactor fed continuously with fodder beet silage as mono substrate. *Syst. Appl. Microbiol.,* 30: 139–151.

Krause L, Diaz, N. N., Edwards, R. A., Gartemann, K. H., Krömeke, H., Neuwger, H., Pühler, A., Runte, K. J., Schlüter, A., Stoye, J., Szczepanowski, R., Tauch, A. and Goesmann, A. (2008). Taxonomic composition and gene content of methane producing microbial community isolated from a biogas reactor. *J. Biotechnol.,* **136**: 91–101.

Kröber, M., Bekel, T., Diaz, N. N., Goesmann, A. and Sebastian, J. (2009). Phylogenetic characterization of a biogas plant microbial community integrating clone library 16S-rDNA sequences and metagenome sequence data obtained by 454-pyrosequencing. *J. Biotechnol.,* **142**: 38–49.

Kumar, V. K., Sridevi, V., Rani, K., Sakunthala, M. and Kumar, C. S. (2013). A review on production of biogas, fundamentals, applications and its recent enhancing techniques. *Elixir Chem. Eng.,* **57**: 14073-14079

Kuo, W, C. and Lai, W. L. (2010). Treatment of kitchen waste using a mobile thermophilic anaerobic digestion system. *Renew. Energy.* 35: 2335-2339.

Lama, W. Jose, L. Giogio, E and Luiz, O. 2012. Production of Biogas from Food waste. *J. Appl. Therm. Eng.,* 29(10): 2061-2071.

Li, C., Moertelmaier, C., Winter, J. and Gallert, C. (2015). Microbial Community Shifts during Biogas Production from Biowaste and/or Propionate. *Bioeng.,* 2: 35-53

Luton P. E, Jonathan M. Wayne, Richard J. Sharp and Paul W. Riley. 2002. The *mcrA* gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill. *Microbiol. J.,* (148); 3521–3530

Maishanu, S. M. and Sambo, A. S. (2007). Biogas Technology. *Nig. J. Solar Energ.,* **7**(12): 23-30.

Mao, C., Feng, Y., Wang, X., and Ren, G. (2015). Review on research achievements of biogas from anaerobic digestion. *Renew. and Sust. Energ. Rev.,* **45**: 540-555.

McHugh, S., Carton, M., Mahony, T. O. and Flaherty, V. (2003). Methanogenic population structure in a variety of anaerobic bioreactors. *FEMS Microbiol. Lett.,* 219: 294–304.

Morris, R. Anne, S. Ujwal, B. Colleen, K and Daniel, Z. 2012. Methyl coenzyme M reductase (*mcrA*) gene abundance correlates with activity measurements of methanogenic H_2/CO_2-enriched anaerobic biomass. *J. Microbiol. Biotechnol.,* 7(1); 77-84.

Rehl, T. and Müller J. (2011). Life cycle assessment of biogas digestate processing technologies. *Res.Conserv. R.,* 56: 92–104.

Smith. P. 1996. "The microbial ecology of sludge methanogenesis. *J. Develop. Indust. Microbio.,* (7):156–161.

Tavi, J. and Tiwari, K. J. (2013). Daily production of biogas system from animal waste. *J. Sci. dir.,* 28(4) 360-375.

Ubwa, S. T., Ashamaye, k. and Oshido, B. (2013). Preparation of Biogas from plants and animal waste. *Int. J. Sci. Technol.,* 2(6): 2049-2060

Wirth, R., Kovács, E., Maróti, G., Bagi, Z., Rákhely, G. and Kovács, K. (2012). Characterization of a biogas-producing microbial community by short-read next generation DNA sequencing. *Biotechnol.Biofuels,* 5: 41

Xiaojiao Wang, X. L., Li, F. and Yang, G. (2014). Effects of temperature and carbon– nitrogen (C–N) ratio on the performance of anaerobic co-digestion of dairy manure, chicken manure and rice straw focusing on ammonia inhibition. *PLoS One* 7265.

Ziekus, H.K. 1999. Isolation of methanogens from acidic soil. *J. Microbiol.,*54: 532–571.

Zielinska, M., Cydzik-Kwiatkowska, A., Zielinski, M. and Debowski, M. (2013). Impact of temperature, microwave radiation and organic loading rate on methanogenic community and biogas production during fermentation of dairy wastewater. *Biores. Technol.,* 129: 308–314.