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Isolation and Characterization of Methanogenic Bacteria from Cattle Rumen Content

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ABSTRACT

Methanogens are diverse group of archaebacteria that produce methane as a major product of their metabolism and a basic requirement in waste water treatment and biofuel generation. There are few experiments that have focused on the study of methanogenic bacteria; this paper describes the methanogens identified in the rumen contents of cattle. Fresh rumen were collected from three different slaughter houses in Ota, Ogun State and carefully transported to the laboratory for analysis. A compounded enrichment mineral medium was employed for culturing and presumptive identification of the characteristics methanogen isolates was based on cultural and phenotypic characteristics premised on standard methods. The total plate count ranged from 5.65×10^{11} to 4.0×10^{5} cfu/ml and 75% of the isolates are Gram negative bacteria while 25% are Gram positive bacteria. Also, 12 methanogens were identified in the rumen samples belonging to the genera *Methanococcus*, *Methanobacterium* and *Methanosarcina*. The mean difference shows that the *Methanococcus*was the predominant among the other genera. The molecular analysis of some selected isolates shows the presence of DNA in the characterized sample. Methanogens isolated from remote source like cattle rumen content can serve as catalyst in biofuel production to solve the recent and future world energy problem.

Keywords
Methanogens, Rumen, Biofuel and Isolates

INTRODUCTION

The increase in use of fossil fuels, and its non-renewable nature coupled with its attendant environmental challenges associated with processing and usage has given a need to research recent notion to provide energy and produce of essential reagent for the industry. One of these alternate to crude oil and fossil fuel potentially will be bioconversion of carbondioxide to methane through methanogenic bacteria (Bär et al., 2015). Methanogens or methanogenic bacteria are biocatalysts that have the ability to add to a solution or solve the later energy problem by generating methane as storable energy carrier. This prokaryotic organisms known as methanogens are archaeadomain , mostly found in wetlands where they cause marsh gas, also present in ruminant and tract of humans where they cause belching and flatulence respectively as a result of their methane composition (Mondav et al., 2014). This diverse group are distinguished by their capacity to produce methane which is a flammable gas (Blasco-Gómez et al., 2017). The methane gas can be used as vehicles for fuel, electricity or base chemical for synthesis. Methanogenicarchaea also play an indispensable role in the anaerobic treatment of wastewater aside for its use in biogas or biomethane production (Wang et al., 2017). These diverse groups of organisms are solely the organism on earth with the capacity of methane production, they are distinctive in terms of metabolism and energy conservation, they also show a high diversity in morphology and physiological parameters (Merlin et al., 2014). Due to the advantages of these groups of organism, the objective of this research is to isolate and characterize methanogens from the rumen contents of cattle.

MATERIALS AND METHODS

Study area: The fresh rumen contents were obtained from three different slaughter houses around Covenant University, Otta, Ogun State. The slaughter houses include: Cafteria 2 Abbattoir, Covenant University; Alafiaa Abattoir, Opposite Canaaland, Otta and Alubarika Abattoir Market, Igboloye, Otta.

Sample Collection: A clean sample bottle with cover was used for collection of samples and transported immediately to Covenant University Microbiology research Laboratory for further analysis. Each of the rumen fluid samples were collected right after the cattle was slaughtered and in a manner to avoid exposure to air.

Culture Media Preparation and Sterilization: A Compounded Enriched Mineral Medium was used for isolation of methanogens. The compositions of enriched mineral medium were according to the method of Ghosh et al. (2014)andManimegalai et al. (2014): for 1000ml, 0.2g sodium benzoate, 0.075g ammonium chloride, 0.04g di-potassium hydrogen phosphate, 0.01g magnesium chloride, 0.0001gresazurin, 0.15g sodium carbonate, 0.025g Sodium sulphide, 20g agar. The pH of the medium was 7.2. All reagents used were of analytical grade and measured using an analytical weigh balance (Model EK-1200A).

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Total Bacteria Count: One gram of fresh sample was weighed and homogenized to to 9ml of sterile water; serial dillutions to 10^6 was obtained. A ml of the 4^{th} to the 6^{th} dilution factors were inoculated into the prepared enriched mineral medium. All dillutions and inoculations were done in sterile syringes to eliminate/reduce air contact. Anaerobic jar was employed in incubating the inoculated plates, the remaining oxygen in the anaerobic jar was cleared using Oxoid gas pack. Incubated was for a period of 7days at 37^{o}C in an SM 9082 Uniscope incubator. Visible colonies on the plates were counted and recorded as colony forming units per milliliter (cfu/ml) of the sample. The colonies were also subculture repeatedly on fresh plates to obtain pure cultures.

Bacterial Characterization: The morphological and biochemical characteristics of the methanogens were determined via the Gram staining, Indole, Triple sugar ion, Starch hydrolysis, Citrate, Catalase, H_2S production, Motility and sugar utilization/ fermentation tests employing mannitol, glucose, frucose, sucrose, rhamnose, sorbitol, alditol, starchiose, arabinose, verbacose.

Culture preservation: Thioglyolate broth was used to maintain methanogens pure culture at -80^{o}C with addition of 20% (v/v) glycerol as cryoprotective agent for long term preservation. The stock cultures were sub-cultured in broth at 37^{O}c for 24-48 hours before use for further work.

DNA Isolation and Quantification: Bacterial genomic DNA was isolated using the protocol reported by Trindade, Marques, Lopes and Ferreira (2007). Cultures of single colonies grown on liquid medium were centrifuged for 5 min at 4600x g. In a 520 microliters of TE buffer (which consist of 10 mMTris-HCl, 1mM EDTA, pH 8.0), the resultant pellets was resuspended with an addittion of 15 µl of 20% SDS with 3 µl of Proteinase K (20 mg/ml) were added. Incubation of solution was done at 37 ºC for 1 hour, added to the solution was 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl, all were mixed together. Following the mixture of the solution was incubation at 65 ºC for 10 mins and placed on ice for 15 mins. Chloroform and isoamyl alcohol were added in ratio 24:1, it was then incubated on ice 5 min and centrifuged for 20 mins at 7200 x g. The aqueous phase of the solution was poured into a new tube, followed by addition of isopropanol (1: 0.6) and precipitation of DNA at –20 ºC for 16 h. DNA collection was done using centrifuge for 10 min at 7200 x g, followed by washing with 500 µl of 70% ethanol, dried in the incubator at 37^{o}C for 30 minutes and lastly dissolved in 50 µl of sterile distilled water as stock DNA.

Quantification of extracted DNA sample**;** The quantification and determination of DNA concentration and purity respectively was done on a Nanodrop Spectrophotometer, model 2000 from ThermoScientific®. DNA purity determination was done at 260/280nm wavelenght to ascertain the quantity of proteins left in the DNA extract. Following nanodrop quantification, 1.5% Agarose gel ran for 40min at a voltage of 120V was used to ascertain the presence of bacteria DNA, this was done under UV light. Genomic DNA was diluted to a factor of 1:50 dilution before used as template for PCR amplification.

Preparation of nucleic acid template for PCR amplification; Nucleic acid samples were thawed on ice and gently vortexed. One microlitre of stock nucleic acid sample was pipetted into fresh 0.65 ml tubes and diluted to a ratio of 1:20 (v/v) by adding 19 µl sterile distilled water. The resulting solution was vortexed slightly as working nucleic acid template and placed on ice for further analysis.

PCR amplification using 16S primers; The PCR analysis made use of 16s universal primers for bacteria identification. We used 27F 5'- AGAGTTTGATCMTGGCTCAG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′. primers (White et al., 1990). In a 25µL reaction volume containing 11.88ul of sterile distilled water, 5µL of 5x colorless GoTaq buffer, 1.5µL of MgCl2, 0.5µL of DNTPs, 0.12µL of Taq DNA polymerase, 1µL of each primer, and 4ul diluted genomic DNA. The PCR amplification conditions involved a cycle of initial denaturation at 94^{0}C, denaturation at 94^{0}C, primer annealing at 50^{o}C, extension at 72^{0}Cand a final extension at 72^{o}C for 5 min, 30secs , 30secs, 1.5 min and 7min respectively.

RESULTS AND DISCUSSION

The total colony forming units of the isolates generated are represented in table1, rumen sample A has the highest microbial load of 5.65×10^{11} and sample B with the lowest load of 4.0×10^5, a total of twelve isolates were obtained and coded as one to twelve. On the bases of Gram reaction (Fig.2), gram positive and gram negative bacteria were both present in the isolate which tallied with a research report from Manimegalai et al., (2014).

Table 1: Mean Total Microbial Plate Count

S/N	Rumen Samples	Total Plate Count (cfu/ml)
1	A	5.65×10^{11}
2	B	4.0×10^5
3	C	4.15×10^{10}

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Key: A= Cattle rumen fluid from cafeteria two
B= Cattle rumen fluid from Alafiaa Abattoir
C=Cattle rumen fluid from Alubarika Abattoir

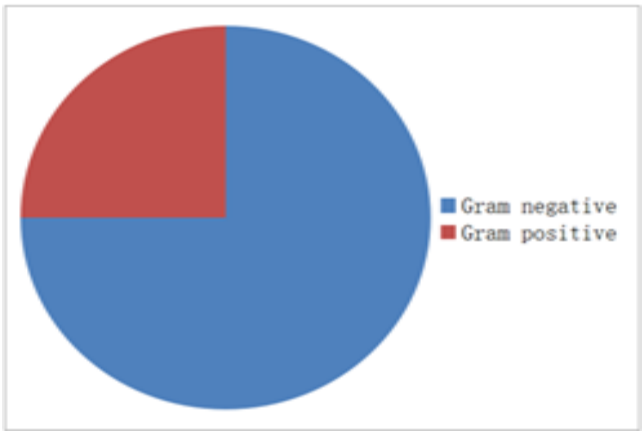

Figure 1: Gram stain reaction of isolates from rumen samples

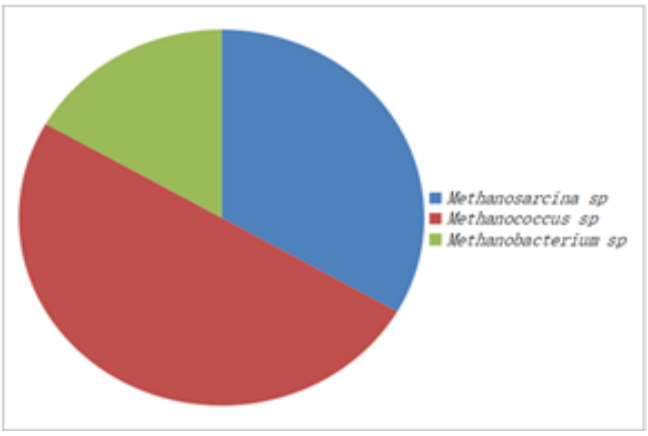

Figure 2: The genera of methanogenic isolates from rumen samples

The identification of bacteria isolates based on the morphology and biochemical traits indicates that isolated genera are of the genera; *Methanococcussp*, *Methanobacteriumsp* and *Methanosarcinasp*, with *Methanococcussp* been the most predomiant, as seen in figure3. The isolated genera of methanogens have a mesophilic temperature spectrum, the specie *Methanococcus* and *Methanosarcina*was isolated in a digestion regime carried out by Dahunsi et al., (2018). Methanogemic organisms are stable and resilient, but they are also complex and largely undefined, the result of our research are in accordance with other studies described in literature (Kushkevych et al., 2017). On the bases of biochemical study, all 12 1solates were indole positive, which indicates it activity on amino acids and carbondioxide to produce methane and other gases when triptophanase is present which is a function of methanogenic bacteria (Manimegalai et al., 2014). All isolates were positive to triple sugar indicating utilization of glucose and hydrogen production into media, they utilize sugar and produce hydrogen in the media, which was earlier reported during biogas production (Solara et al., 2002). They were all negative to catalase, mannitol and other sugars which could be as a result of it acidic and anaerobic environment. Isolate 1, 10 and 11were used for the DNA extraction and quantification with higher production of hydrogen sulphide and also positive to production of citrate indicating citritase enzyme presence by breaking down citrate to oxaloacetate and acetate and further oxaloacetate into pyruvate and carbondioxide which also produce ammonia with sodium citrate. Similarly, these products have been reported during biogas production (Solera et al., 2002). The result of quantification is in Table 2, shows that the quantities of DNA from these samples were within the required standard for polymerase chain reaction. Figure 3 indicates the presence of DNA in these isolates and figure 4 shows the separation of purified PCR products amplified on agarose gel electrophoresis to get more amplicons of these samples. These amplicons can be used for more molecular analysis.

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Table 2: DNA Quantification Result on Spectrophotometer

Sample ID	Nucleic Acid(ng/ml)	260/280	260/230
Isolate 1	758.3	2.11	1.89
Isolate 10	958.2	1.77	1.87
Isolate 11	1135	2.12	2.01

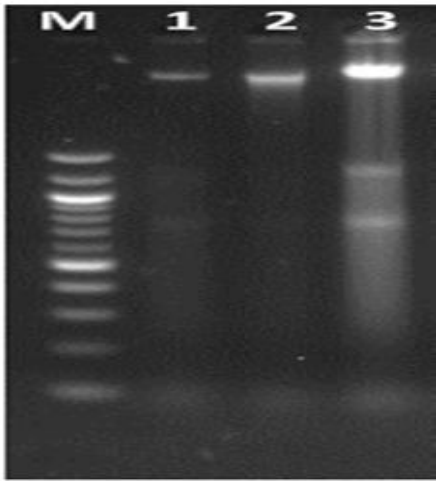

Figure 3: Presence of DNA with bands in rumen content

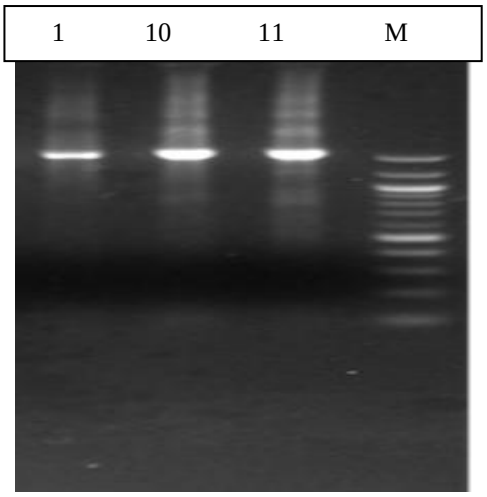

Figure 4: Agarose gel electrophoresis analysis of PCR amplified products of reactions performed with 16s primers (M=Molecular marker size)

CONCLUSION

The analysis of this study concluded that the selected isolates are methanogenic bacteria present in the rumen content of cattle, hence the study recommends further sequencing of isolates. The present study also suggests further optimization of physical and chemical parameters of the rumen fluid which can be utilized in biogas production.

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CONFLICTS OF INTEREST
The authors declare no conflict of interest.

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