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**Research Article**

# Effects of Microbial Consortia at Different Digestion Temperatures on Methane Production from a Keratinolytic Substrate

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***ABSTRACT:*** The effects of four microbial consortia at different digestion temperatures on methane production from chicken feather were evaluated. The result of the physicochemical analysis of chicken feather powder showed that the carbon and nitrogen content was 55.97% and 14.0% respectively with carbon-nitrogen ratio of 4:1. Chicken feather powder was biologically-treated with mixed broth cultures of *Geobacillus stearothermophillus and Bacillus licheniformis* at 37$^{o}$C and pH of 7.50 for 5 days under submerged fermentation to disrupt the disulphide and hydrogen bonds of α- and β-keratin proteins for easy hydrolysis prior to anaerobic digestion. Anaerobic fermentation lasted for an average of 28 days at four temperature regimes and pH of 6.8-7.0 using four microbial consortia isolated from cow dung and chicken feather dump-site soil samples. The biogas produced was purified to upgrade it to methane standard using chemical absorption and adsorption methods. The result of methane produced by the consortia under different temperature conditions show that consortium 4 which contained more of the methanogens has better methane-yielding ability than others. The highest yield of gas was 1313 cm3 at 55$^{o}$C using consortium 4 for biotreated substrate and 739 cm$^{3}$ for untreated substrate. The optimum temperature of activity of the consortia was 55$^{o}$C with the highest methane yield of 1313 cm$^{3}$/200gm while the maximum was 65$^{o}$C with decline in gas yield. The analysis of variance of the results on gas volumes from different treatments showed that methane yield was significantly (0≤0.0.05) dependent on the microbial consortia, nature of substrate and fermentation temperatures.

***Keywords:*** *Chicken feather, Microbial consortia, Anaerobic digestion, Temperatures, Keratinase, Methane*

## 1. INTRODUCTION

Methanogenesis is a complex, redox biochemical reaction during which polymeric organic substrates are decomposed into simple chemically-stabilized compounds essentially methane and carbon dioxide under symbiotic effects of obligate and facultative anaerobic microbes in anoxic condition [1]. Methane and carbon dioxide account for about 60-70% and 30-40% respectively of the entire biogas volume while hydrogen sulphide ($H_2S$) has up to 0.5 to 1.0%, traces of siloxanes may also be found [2].

Methane is a colourless and odourless gas that burns with a clear blue flame and has autoignition temperature of 650 to 750$^{o}$C, density of 1.214g/m$^{3}$and calorific value of 20 to 26MJ/m$^{3}$. Biogas is about 20% lighter than air and liquefies at a pressure of 47.4 kg/cm$^{2}$ at a critical temperature of -82$^{o}$C [3].

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http://www.mdcjournals.org/ijsar-...html

The demand for renewable fuels is increasing with growing concern about air quality, climate change, energy import dependence and the depletion of fossil fuels [4]. Biogas is one of the versatile fuels which can be used for electric power, heating and automobile fuel and therefore biomass for methane yield should have a good source of carbon and moderate nitrogen with carbon/nitrogen ratio of at most 30:1 [5]. Biogas is smokeless, hygienic, cheap, renewable and more convenient to use than solid fuels. Biogas can be purified to upgrade it to methane by chemical scrubbing process through which impurities such as water vapour, carbon dioxide and hydrogen sulphide are removed [6]; [7]; [8].

Anaerobic digestion (AD) of organic substrates occurs in three main stages which include hydrolysis during which complex organics are broken down into soluble components such as sugars, amino acids and fatty acids through the extracellular microbial enzymes (cellulase, xynalase, amylase, lipase, protease). Most of the bacteria in this group are strict anaerobes such as *Bacteriocides, Clostridia* and *Bifidobacteria* [9]. However some facultative group such as *Bacillus, Pseudomonas, Streptococci* and *Enterobacteriaceae* take part. The second stage is acidogenesis during which acidogenic bacteria metabolize the monomers to alcohols, lactic acid, acetic acids, ammonia and volatile fatty acids [10].

The third stage involves the acetogens which covert the higher volatile fatty acids into acetate and hydrogen. Members of this group are obligate hydrogen-producing bacteria which include *Acetobacterium woodii* and *Clostridium acetium* and finally, the methanogenesis during which these intermediate products are metabolized to methane, carbon dioxide and trace of other gases by the methanogens [11]. The methanogens produce methane from acetate, hydrogen or carbon dioxide. Methanogenic bacteria are strict anaerobes and require a lower redox potential for growth than other anaerobes. Only a few species are able to degrade acetate into methane and carbon dioxide and they include *Methanosarcina barkeri, Methanococcus mazei and Methanotrix soehgenii* while all methanogens are able to utilize hydrogen to form methane [12]. The methanogenic biogas production rate is sensitive to changes in feed stock material, PH, temperature, organic loading rate and hydraulic retention time. These groups of organisms in the microbial community work in syntrophy to produce methane.

Irrespective of the disputed contribution of man to global warming, the dramatic effects *per se* and the involvement of gases like methane and carbon dioxide are unquestionable. [13] reported that the biosphere is under intense pressure due to greenhouse gas emissions and therefore urgent action is needed to avert its total collapse by partial or complete replacement of fossil fuels. It has therefore become an important global goal to reduce the uncontrollable greenhouse gas emissions and one possibility is to increase the portion of renewable energy sources like biogas in keeping with the Kyoto Protocol [14].

Poultry feathers are generated in bulk quantities as bye products in poultry industry globally. The poultry industry in the U.S.A alone produces about 2-4 billion pounds of feathers annually which often end up in landfills to contaminate the underground water resources [15]. Feather is a very rich source of protein with β-keratin constituting 91% of feather protein [16]. Feed stocks of high protein may inhibit methanogenesis due to ammonia inhibition resulting from accumulation of too much nitrogen [2]. Keratins are insoluble structural proteins that are extensively cross-linked by disulphide bonds, hydrogen bonds and hydrophobic interactions and they are abundant in hairs, feathers, claws and horns [17]. The *α*-helix and *B*-sheet configurations of keratins are characteristically resistant to degradation by common proteases including trypsin, pepsin and papain [18],[19]. Typically each bird has up to 125 gram of feather and with 400 million chickens being slaughtered every week world-wide, the daily accumulation of feather waste reaches about 5 million tons [20].

The bulk of the feather waste is poorly recycled and has limited utility due to the chemically unreactive nature of keratins. The disposal of this waste is a global environmental issue leading to pollution of air, surface and underground water resources [21]. In recent years, feather treated with microbial keratinases is attracting wide attention with several applications. Diverse groups of microorganisms *like fungi (Doratomyces microspores, Alternaria radicina, Trichurus spiralis, Aspergillus spp.,etc), Actinomycetes (Streptomyces pactum, S. alvis, S. Fradiae, Thermoactinomyces candidus) and several bacterial species such as Bacillus licheniformis, Bacillus*

International Journals of Scientific and Allied Research (IJSAR)
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http://www.mdcjournals.org/ijsar-...html

*megatarium, Bacillus subtilis, Bacillus pumilus, Pseudomonas aeruginosa, Microbacterium spp, etc* [22], [23], [24] have been reported to produce keratinases.

Keratinase-treated feather is increasingly considered as a viable source of dietary proteins in food and feed supplements, as the enzyme-treated end product retained high nutritive value. Schmidt [25] reported that chicken feather is now in use in developed world not only as animal feed supplements but as raw material for making shoe soles, plastics, upholstery padding, hurricane-proof roofs, diapers etc. A mixture of fibre glass and feathers are already in use in boat construction to improve buoyancy. In the words of Schmidt for his admiration of chicken feather potentials "it is possible to make chicken salads out of chicken shits" Research is on-going on using feathers in the aerospace and aircraft industry. [27], reported that poultry feathers should undergo pretreatment by wet steaming and biological treatment to breakdown the disulphide bonds prior to biogas production.

Effective management of poultry feathers littering slaughterhouse environments through anaerobic digestion will not only yield methane which is a renewable, cheap and clean energy source but also helps to mitigate the environmental and ecological impacts of these wastes such as air pollution, water-borne infections and greenhouse gas emissions.

## II. MATERIALS AND METHODS

### 2.1 Source of materials

Chicken feather and cow dung used for the research work were sourced from Fort Cox College of Agriculture Poultry processing Centre Middle Drift district and University of Fort Hare cow ranch farm in Alice respectively both in Eastern Cape Province, South Africa.

The equipment, reagents and media used for the physicochemical, molecular and microbiological analysis were provided by SA-MRC Microbial Water Quality Monitoring Centre, Department of Biochemistry and Microbiology, University of Fort Hare, Alice, Eastern Cape, South Africa.

### 2.2 Experimental Design

#### 2.2.1 Isolation and identification of microorganisms

*Isolation Techniques*

The bacteria group used for biogas production were isolated according to the methods described by [30], [5]; [26]; [27].

*Procedure:*

One (1.0) gram of fresh cow dung and poultry dump-site sample was respectively dissolved in 99ml double-distilled water each and serial dilutions with double-distilled water were made up to $10^{-8}$ folds. An inoculum from $10^{-8}$ fold dilution was inoculated on nutrient agar medium by streak plate technique and incubated at 37$^{o}$C for 24 hours. After incubation different colonies were subcultured into fresh nutrient agar medium to purify the isolates.

International Journals of Scientific and Allied Research (IJSAR)
ISSN: 2437-1246
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http://www.mdcjournals.org/ijsar-...html

Selective cultivation using selective media was applied for fastidious organisms. Members of *Enterobacteriaceae* were isolated using Eosin-Methylene Blue agar, *Pseudomonas* species-Pseudomonas Selective medium, *Streptococcus* spp.-Streptococcus agar, Clostridium species-Brain heart infusion medium, *Cellulomonas* species- Bushnell Haas Medium (BHM) supplemented with 1% Carboxymethyl cellulose (CMC).

Keratinase-producing Bacilli were isolated based on the methods described by [28] and [16].The serially diluted feather dump-site soil sample($10^{-8}$) was heated at 80$^{o}$C for 20 minutes and inoculated on nutrient agar medium by feather baiting technique and incubated at 37$^{o}$C for 24 hours. Potential keratinolytic isolates were re-inoculated on Basal Mineral Medium containing 0.5% $NH_4Cl$, 0.5% NaCl, 0.3% $K_2HPO4$, 0.4% $KH_2PO4$, 0.1%$MgCl.6H_2O$, 0.1% yeast extract and 1% feather powder at pH 7.50. All the plates containing the different bacterial inocula were incubated at 37$^{o}$C for 24 to 48 hours.

*Selective isolation of Methanogens* Methanogens were isolated using the methods described by [29].

The Methanogens were isolated using SAB medium containing mineral salts supplemented with yeast extract, vitamins and essential amino acids (tryptophan and l-cysteine), 2.6% sodium chloride (NaCl) was added to one part of SAB Medium for the selective isolation of *Methanobrevibacter* spp and *Methanomicrobium* spp while 3.0% sodium chloride was added in another part for isolation of *Methanosarcina* spp., 1.0% methanol was added in another part for isolation of *Methanosarcina barkeri and Methanobacterium ruminantum.* The serially diluted sample ($10^{-8}$) was inoculated on the enriched agar plates using streak plate technique and placed in anaerobic jar containing anaerobic gas kit to maintain anoxic condition at incubation temperature of 40$^{o}$C for 5 days.

After incubation both the bacterial and Archaeal isolates were subjected to morphological, molecular, biochemical and enzyme-producing tests to determine their morphology, biochemical characters and ability to degrade organic matters (carbohydrates, proteins and lipids).

### 2.2.2 Morphological and Biochemical Characterization

*2.2.2.1Morphological/Biochemical characteristics*

The morphological characters of the isolates determined were Gram reaction, Shapes and Motility while the biochemical characters were sugar fermentation, citrate, indole production, Voges-proskauer, catalase tests and ability to produce hydrolytic enzymes (Amylase, protease, cellulase, lipase and xylanase). These characteristics were determined as described by [30] and Norrell and [31].

***Biochemical characterization of the isolates***

*Sugar fermentation and gas production*

One (1) loopful of the isolate was inoculated into each 50ml culture tube containing sterile peptone water broth with 10% D-glucose. Two drops of phenol red indicator solution were added into the broth and an inverted Durham tube was inserted in the culture tube. The broth was incubated for 24 hours at 37$^{o}$C. Production of acid which is a product of fermentation was indicated by the change of yellow colour to red. Presence of gas was indicated by appearance of gas bubble in the Durham tube. Negative result shows no change in colour or appearance of gas.

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http://www.mdcjournals.org/ijsar-...html

*Citrate utilization*

One loopful of the isolate was inoculated into Simmon`s citrate agar containing two drops of bromothymol blue indicator and incubated at 37°C for 24 hours, positive test was indicated by the appearance of growth with blue colour while negative result shows no change in colour. Positive test is indicative that the organism can utilize citrate as source of carbon.

*Indole production*

One loopful of the culture was inoculated into peptone water broth and incubated at 37°C for 48-96 hours. 0.50 ml of kovac`s reagent was added into the broth culture and shaked, the appearance of pink colour in the alcohol layer indicates positive indole production while non appearance of pink or red colour indicate negative indole production. If the isolate possess enzyme tryptophanase, it will degrade amino acid tryptophan to indole.

*Voges-proskauer test*

**Reagents:** 40% KOH (40ml of KOH and 60ml of distilled water, 0.3% creatine ( 0.3 gram in 100ml of ditilled water, 5% solution of α-naphthol in absolute alcohol (5 gm in 100ml of absolute alcohol) and Glucose phosphate broth.

One loopful of the culture was inoculated into 5ml glucose phosphate broth and incubated at 37°C for 48 hours. After incubation, 1ml 40% KOH containing 0.3% creatine and 3ml of 5% solution of α-naphthol in absolute alcohol and shaked, appearance of pink colour in 2-5 minutes indicates positive test.

Fermentation of carbohydrates by some bacteria results in the production of acetyl methyl carbinol (acetion). In the presence of alkali and atmospheric oxygen, acetion is oxidized to diacetyl which reacts with peptone of the broth to give a red colour.

*Catalase test*

One loopful of the isolate was smeared on clean glass slide with a drop of hydrogen peroxide solution. Prompt effervescence indicates catalase production. Catalase is an enyme which can breakdown hydrogen peroxide to liberate oxygen gas. Negative catalase produces no gas.

**2.2.3: Enzyme test**

*Test for cellulase*:

The isolate was inoculated on mineral medium containing 1.0% peptone, 1.0% carboxymethyl cellulose (CMC), 0.2% $K_2HPO_4$, 2.0% agar, 0.3% $MgSO_47H_2O$, 0.25% ( $NH_4)_2SO4$ and 0.2% gelatin per 100ml of distilled water at PH7.0 using streak plate technique and incubated at 37°C for 48 hours. After incubation, the cultures were flooded with 1.0% congo- red dye solution and examined for the appearance of clear zones around the colonies.

*Test for Xylanase*:

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International Journal of Scientific and Allied Research
[IJSAR]

International Journals of Scientific and Allied Research (IJSAR)
ISSN: 2437-1246
Volume 4, Issue 1 (March, 2017), 27- 44
http://www.mdcjournals.org/ijsar-...html

The isolate was inoculated on nutrient agar medium containing 0.5% xylan and incubated at 37°C for 48hours. After incubation the cultures was flooded with lugol iodine solution and examined for the appearance of yellow zones around the colonies. Appearance of clear zones indicates the breakdown of xylan by the organism to sugars but appearance of blue black colour indicates presence of xylan and absence of xylanase.

Test for Protease: The isolate was inoculated on skimmed milk agar plate and incubated at 37°C for 24 hours. After incubation, the cultures were examined for the appearance of clear zones around the colonies which indicates presence of protease.

Test for Amylase: The isolate was inoculated on starch agar plate by streaking and incubated at 37°C for 24 hours, The cultures were flooded with Gram`s Lugol iodine solution and examined for appearance of clear yellow zones around the colonies which indicates the conversion of starch to sugars by the amylase produced by the organism. Presence of blue black colour indicates absence of amylase activity.

*Test for Lipase*:

The isolate was inoculated on Tributyrin agar plate containing skimmed milk by streaking and incubated at 37°C for 24 hours. Appearance of clear zones around the colonies indicates presence of lipase enzyme.

**2.2.4 Determination of keratin-degrading ability of the isolates**

The keratin-degrading ability of the isolates was determined based on the methods described by [26] and [28].

Method: Keratinase enzyme was produced by inoculating 1.0 ml of broth culture of each isolate into 100ml of liquid mineral medium containing o.5% NHCl, 0.05% NaCl, 0.03% $K_2HPO4$, 0.04% $KH_2PO4$, 0.024% $MgCl_2$, 0.1% yeast extract and 1.0% raw feather powder at pH7.5 in Erlenmeyer flask. The flask was shaken at 150 rpm for 10 minutes and incubated at 37°C for 5 days. After incubation, the broth was centrifuged at 1000 rpm for 10 minutes at 45°C. The supernatant was collected and examined for keratinase activity based on the protein content of the digestate containing the enzyme protein.*3.2.3.1 Determination of the enzyme protein of digestate*

The protein content of the digestate was determined using Spectrophotometric method as described by [26].

100µl of the sample was added to 5ml of Bradford reagent and incubated for 5 minutes. The absorbance of the sample was determined with a spectrophotometer (model: RDL8025S, XINRUILIAN, U.S.A) at a wave length of 660nm using diluted Bovine albumin as standard protein (containing 5-100µgm/ml protein in a 100µl volume). The protein content of the sample was extrapolated from the standard curve of the absorbance of diluted bovine albumin against the protein content.

**2.2.5 Molecular identification of the isolates**

The processes involved in molecular identification of the isolates were: Extraction of the rganism genomic DNA, Polymerase chain reaction (PCR), Denaturing Gradient Gel Electrophoresis (DGGE), sequencing of the PCR products (amplicons) and BLAST (Basic local alignment search tools).

**2.2.5.1 Extraction of organism genomic DNA**

The genomic DNA of each of the isolate was extracted following the boiling method as described by [32].

MDC Journals.
International Journal of Scientific and Allied Research
[IJSAR]

International Journals of Scientific and Allied Research (IJSAR)
ISSN: 2437-1246
Volume 4, Issue 1 (March, 2017), 27- 44
http://www.mdcjournals.org/ijsar-...html

*Procedure*

Four hundred microlitres (400µl) of sterile distilled water was measured into 1.50ml eppendorf tube and 3 loopfuls of each of the isolate were added into the tube, covered and vortexed to dissolve. The tubes containing the isolates were put into the wells of Acublock heater (Dri-Block Techne, model: FDBO3DD R, Mbby Scientific Ltd. U.S.A) and boiled at 100$^{o}$C for 10 minutes. After boiling, the tubes were cooled in ice and centrifuged at 15,000 rpm for 5 minutes. The supernatant of each isolate was decanted into another set of epperndorf tubes as DNA extracts and stored at -20$^{o}$C for PCR (polymerase chain reaction) amplification.

2.2.5.2 **Polymerase chain reaction**

The 16S rRNA target region was amplified using Dream Taq$^{tm}$ DNA polymerase (Thermo Scientific$^{tm}$), Bacterial primers 16S-27F (5`-AGAGTTTGATCMTGGCTCAG-3`) and 16S-1492R (5`-CGGTTACCTTGTTACGACTT-3`), archaeal universal primers 16S-Archf2$^{b}$ (5`-TTCYGGTTGATCCYGCCRA-3`) and 16S-Archr1386-(5`GCGGTGTGTGCAAGGAGC-3`) following the methods described by [33]

***Reagents and materials****:* Mastermix (containing DNA polymerase, dntps and $Mgcl_2$), nuclease-free water and DNA template (DNA extract), 1.5ml eppendorf tubes, PCR tubes and Thermal cycler,

***Procedure*****:** A cocktail solution was first prepared by measuring 12µl of mastermix, 1µl of forward and reverse primers each, 6µl nuclease-free water each (according to the number of DNA extract samples) into 1.5ml eppendoff tube. After preparing the cocktail solution, 5µl of DNA template and 20µl of cocktail solution of were added into each Pcr tube. The pcr tubes containing the reagents were then loaded into the thermal cycler.

The thermal cycler was then programmed for polymerase chain reaction which runs in cycles starting with the initial heating at 94$^{o}$C for 3 minutes to activate the DNA polymerase, denaturation at 93$^{o}$C for 30 seconds to disrupt the hydrogen bonds between complimentary bases yielding single- stranded DNA, annealing at 58$^{o}$C for 1 minute to bind the primers to the complimentary part of the DNA template, initial elongation at 72$^{o}$C for 7 minutes for DNA polymerase to synthesize a new DNA strand complementary to DNA template strand by adding dntps that are complimentary to the template in 5` to 3` direction, final elongation at 72$^{o}$C for 7 minutes to ensure that any remaining single-stranded DNA is fully extended and final hold at 4$^{o}$C for indefinite time for short storage of the reaction.

As a rule-of-thumb, the DNA polymerase at its optimum temperature polymerizes a thousand bases per minute. If there is no limitation due to limiting substrates or reagent at each extension step, the amount of DNA target is doubled leading to the exponential amplification of the specific DNA fragment.

*2.2.5.3 Denaturant Gradient Gel electrophoresis*

The PCR products were gel-extracted using Zymo Research, Zymoclean$^{tm}$ Gel DNA Recovery kit [33].

To check if the PCR generated the anticipated DNA fragments called the amplicons, an agarose gel electrophoresis was employed for size separation of the PCR products. The sizes of the PCR products was determined with a DNA ladder a molecular weight marker which contains DNA fragments of known size, run on the gel alongside the PCR products.

***Reagents/materials:*** Agarose gel (prepared by dissolving 1.0gram of the powder in 100ml of TBE buffer with 10µl ethidium bromide and heated in microwave for 3 minutes at 100$^{o}$C), loading dye, DNA lather, Gel tank, Elecrophoresis machine and Transilluminator equipment.

***Procedure:*** 200ml of TBE buffer was first poured into the gel tank containing the gel mass then 5µl of DNA ladder mixed with the loading dye was added into the first well in the gel followed by the negative control (5µl

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International Journal of Scientific and Allied Research
[IJSAR]

International Journals of Scientific and Allied Research (IJSAR)
ISSN: 2437-1246
Volume 4, Issue 1 (March, 2017), 27- 44
http://www.mdcjournals.org/ijsar-...html

nuclease-free water) and then 5µl of the amlpicon (PCR product) each mixed with the loading dye and added into each of the sample-well of the agarose gel.

After the addition of materials, the gel tank was connected to the electrophoresis machine and run for 45 minutes. During electrophoresis, the amplicons in the gel matrix placed in the gel tank moved from negative pole to positive under the influence of the electrolytes supplied by the TBE buffer. After gel electrophoresis, the gel was placed in phototransilluminator equipment for documentation of the separated products which appear inform of bands. The height of the bands depends on the base pair of the target DNA on the ladder.

The principle of DGGE is based on the differing mobility on a gel of denatured DNA- fragments of the same size but with different nucleic acid sequences thus generating band patterns that directly reflect the genetic biodiversity of the sample. The numbers of bands correspond to the number of dominant species.

*2.2.5.4 Sequencing and BLAST*

The purified PCR products were sequenced in the forward and reverse directions on the ABI PRISM$^{tm}$ 350xl Genetic Analyser. Purified sequencing products (Zymo Research, ZR-96 DNA sequencing clean-up kit $^{tm}$ ) were analysed using CLC main workbench 7 followed by a BLAST search (NCBL) [33].

**2.3.Thermal pretreatment of chicken feather**

Raw chicken feather was hydrothermally-treated by subjecting each batch of 1.0kg to wet steam sterilization at 100$^{o}$C for 30 minutes to soften the disulphide and hydrogen bonds of α and β-keratin proteins using the autoclave. The pretreated feather was dried at 100$^{o}$C for 12 hours, cooled and milled to powder using disk mill (DIN 53492, German model)

**2.4. Proximate Analysis of the feedstock**

*Determination of the moisture content*: The moisture content of chicken feather powder triplicate samples was determined as described by [34] and [35]

Five (5) grams of each sample was weighed into a tarred moisture dish and dried in a preheated oven at 105$^{o}$C for 24 hours. Triplicate samples were subjected to the oven drying conditions. After drying, the dried samples were cooled in a dissector containing activated silica for 3 hours and reweighed. The oven dry weight of the sample each was noted and the moisture content calculated from the formula:

% moisture = (WS-WSd/WS) × 100.

Where WS= wt of sample before drying,

WSd = oven dry wt of the sample

*2.4.1 Determination of total solids (dry matter) content.*

The total solids content of each sample was determined from the oven dry weight of the samples as described by [35]. Known weight (5 gm each) of the duplicate sample was dried at 105$^{o}$C for 24 hours. After drying and cooling, the oven dry weight of the sample was recorded and calculated in percentage as stated:

% Total solids =WD/W S ×100, where WD= dried wt, WS= sample wt.

*Determination of the volatile solids and ash content.*

MDC Journals.
International Journal of Scientific and Allied Research
[IJSAR]

International Journals of Scientific and Allied Research (IJSAR)
ISSN: 2437-1246
Volume 4, Issue 1 (March, 2017), 27- 44
http://www.mdcjournals.org/ijsar-...html

The total solids and ash content of the triplicate samples were determined as described by [34].

The overnight dry weight of each sample was combusted at 550$^{o}$C in a muffle furnace for 1hour. The sample weight after combustion was calculated in percentage as ash content while the percentage volatile solids was calculated from the difference in weight of the total solids and ash content.

% volatile solids = (Wdm-Wash/ Wdm) ×100, where Wdm= total solids, Wash =wt of ash.

% ash = (Wash/Ws) ×100. Where Wash =wt of ash, Ws=wt of original sample.

Percentage of organic carbon= 58% × wt of volatile solids (dry organic matter*),* [36].

*2.4.2 Determination of Total Nitrogen:*

The nitrogen content of the feed stock was determined based on the method described by [37].

*Extraction of Nitrate*: Nitrate was extracted from 1.0 gram of dry organic matter in a 50ml beaker using 50ml 1M $NH_4Cl_2$ solution for 30 minutes, stirring every 10 minutes interval. During extraction, the nitrate was reduced to nitrite and forms a red-azo dye. The intensity of the red colour produced is proportional to the nitrate level in the sample. The nitrate level was determined using Palintest photometer.

Procedure: A round glass test tube was filled to 10 ml mark with the extract. One (1) Nitricol N-tablet was ground in a motar and mixed with the extract solution to dissolve. The solution was allowed to stand for 10 minutes to develop full colour. The nitrate nitrogen was determined at a wave length of 570 nm using the photometer. The nitrate calibration chart was used to find the nitrate nitrogen concentration in the sample.

**2.5. Formulation of Starter Cultures for Biogas production**

The microorganisms used to formulate the starter cultures for biogas production were selected based on their organic matter-degrading potentials. The organisms were combined in different ratios to form different consortia used for the anaerobic digestion of pretreated chicken feather to produce biogas at different temperature conditions. The starter cultures were formulated based on the methods described by [5].

*Digester Units:* Four digester units of 2-litre capacity each were designed, each one representing a consortium made of different organisms as follows:

**Table1: Starter cultures showing the composition of four microbial consortia**

| Consortium | Microbial composition |
|---|---|
| 1 | 1%hydrogens,1%acidogens, 1%methanogens |
| 2 | 1.5%hydrogens,0.75%acidogens,0.75%methanogens |
| 3 | 1.5%acidogens,0.75%hydrogens,0.75%methanogens |
| 4 | 1.5%methanogens,0.75%hydrogens, 0.75%acidogens |

**2.5.1 Biological treatment of Feedstock**

Chicken feather powder was biologically treated for easy hydrolysis of keratin proteins by microbial keratinases according to the method described by [26].

Thirty (30) ml of mixed broth culture of *Bacillus licheniformis* and *Geobacillus stearothermophilus* was added into 1500ml of chicken feather slurry in a 2-litre glass digester and subjected to submerged fermentation at 37$^{o}$C for 5 days. These organisms were proved to be keratinase-producing organisms which hydrolyse the disulphide-hydrogen bonds of β-keratin proteins producing amino acids.

**2.5.2 Formulation of organic slurry**

Each batch of organic slurry was composed of the following:

Chicken feather powder= 200gram per batch

Water = 1,500 cm$^3$

3% Consortium = 45 cm$^3$,

Feedstock : water ratio =1:7

**2.5.3 Anaerobic Digestion**:

Each batch of organic slurry containing 200 gram biologically-treated feather powder was subjected to anaerobic digestion to produce biogas at different temperature regimes (35$^{o}$C, 45$^{o}$C, 55$^{o}$C and 65$^{o}$C) using 3% of the respective consortium. The hydraulic retention time and pH for gas production was noted for each batch of feed stock at the end of digestion.

2.5.4 **Purification of biogas and measurement of methane volume:**

The raw biogas stream produced from anaerobic digestion of feed stock was subjected to purification processes as described by [8]. The scrubbing processes involved removal of water vapour, hydrogen sulphide and carbon dioxide with silica gel, activated clay and potassium hydroxide in scrubbing unit1, 2 and 3 respectively.

The volume of methane produced from each batch of chicken feather slurry was measured by downward displacement of water process as shown in the figure below. The volume of water displaced from a graduated glass bottle into a measuring cylinder equals the volume of methane gas produced per unit time under each treatment.

2.5.5 **Flammability test of biogas**

The biogas sample after purification was subjected to flammability test as demonstrated in the figure below

**2.5.6 Assessment of the Methane-producing potential of the bacterial Consortia**

The methane-producing potentials of the bacterial consortia were assessed based on the average volume of the gas produced by each consortium under different temperature conditions.

**2.6. Statistical analysis of data**

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International Journal of Scientific and Allied Research
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International Journals of Scientific and Allied Research (IJSAR)
ISSN: 2437-1246
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http://www.mdcjournals.org/ijsar-...html

The results obtained from different treatments with respect to the volumes of methane were subjected to one-way analysis of variance (ANOVA) according [38] to determine the significant difference in volume of gas produced by each consortium under various temperature conditions.

## 3. RESULTS AND DISCUSSION

The methane-producing ability of four microbial consortia from chicken feather at different temperatures was determined. The results of the molecular identification of the microorganisms used in biogas production based on 16S rRNA genes according to [33] showed that *Bacillus and Pseudomonas* species were the dominant bacterial species which are of the *Phylum, Firmicutes and Proteobacteria* respectively. This finding is in agreement with the report of [10] that Firmicutes and Proteobacteria could be found among the hydrolytic and acid-producing bacteria in a microbial community for anaerobic digestion. The dominant archaea were *Methanosarcina and Methanobrevibacter* species which are acetotrophic and hydrogenotrophic methanogens respectively according to [2]. Methanosarcina spp as acetotrophic methanogen is capable of metabolizing acetate to methane and carbon dioxide while Methanobrevibacter as a hydrogenotroph can convert hydrogen and carbon dioxide to methane [1; 2]

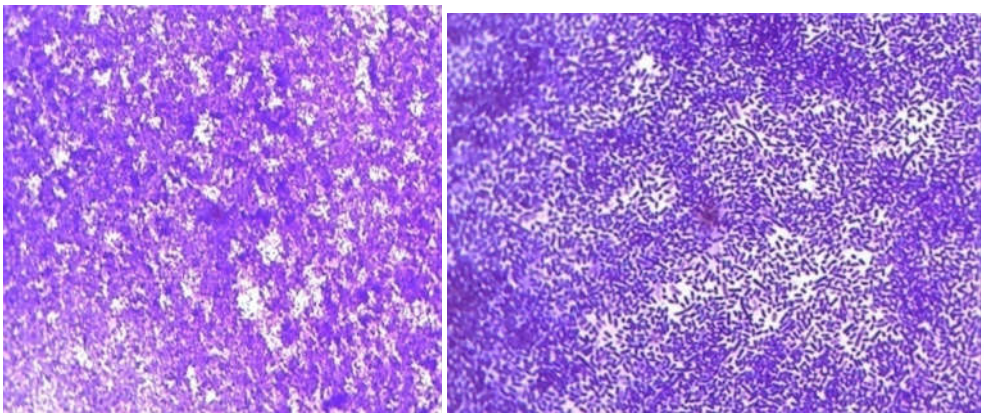


Figure 1: **Microscopic view of *Methanosarcina* and *Methanobrevibacter* species**

**Table 2: Enzyme-production test on the identified isolates according to Norrel and Messely (2003) and Sharma (2000)**

| Isolate | Amylase | Cellulase | Protease | Xylanase | Lipase |
|---|---|---|---|---|---|
| *Geobacillus stearothermophilus* | +++ | ++ | +++ | ++ | + |
| *Bacillus licheniformis* | ++ | ++ | +++ | + | Trace |
| *Escherichia coli* | + | + | + | + | Trace |
| *Bacillus cereus* | ++ | + | ++ | + | + |
| *Bacillus thuringiensis* | ++ | + | ++ | + | + |
| *Pseudomonas aeruginosa* | + | + | ++ | + | + |
| *Bulkholderia fungorum* | ++ | ++ | + | ++ | + |
| *Pseudomonas mendocina* | + | + | + | + | + |
| *Bacillus pumilus* | ++ | + | ++ | + | + |
| *Bacillus aerophilus* | ++ | + | ++ | + | + |

International Journals of Scientific and Allied Research (IJSAR)
ISSN: 2437-1246
Volume 4, Issue 1 (March, 2017), 27- 44
http://www.mdcjournals.org/ijsar-...html

| | | | | | |
|---|---|---|---|---|---|
| *Methanosarcina spp* | ++ | + | + | + | + |
| *Methanobrevibacter spp* | ++ | + | + | + | + |
| *Phanerochaete chrsosporium* | ++ | +++ | + | ++ | + |
| *Aspergillus niger* | ++ | ++ | + | ++ | ++ |
| *Bacillus licheniformis* | ++ | + | +++ | + | ++ |

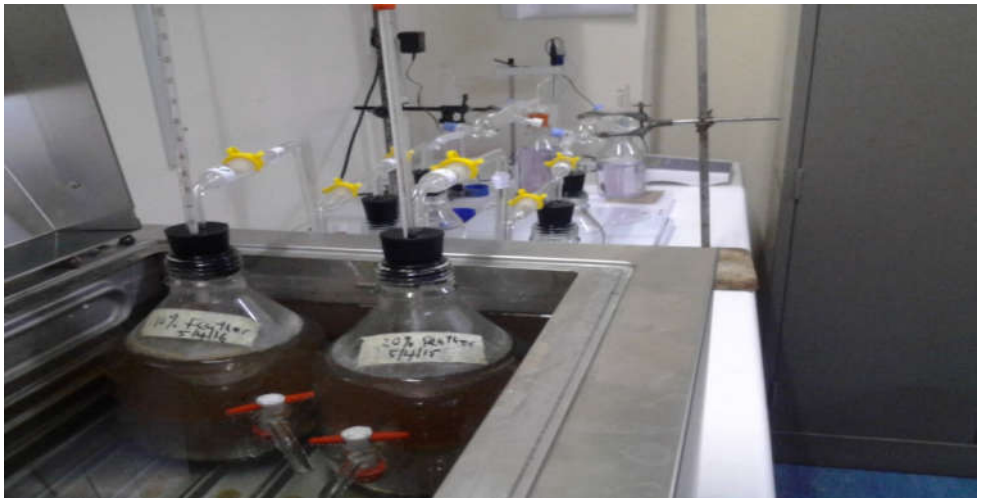
Chicken feather was hydrothermally-treated by steaming at 100$^{o}$C for 30 minutes to weaken the disulphide bonds (for easy hydrolysis) dried and milled into powder as recommended by [26].

The result of the proximate analysis of chicken feather powder showed that the volatile solids, carbon and nitrogen content of chicken feather sample were 96.50%, 55.97% and 14.0% respectively. The carbon content is good enough as gas yield largely depends on carbon but the carbon-nitrogen ratio of 4:1 implies that the nitrogen content of the chicken feather was too high and therefore using it for biogas production could result in ammonia inhibition of microbial consortia, sluggish fermentation and poor yield of methane as reported by [2]. Ideally the carbon-nitrogen ratio (C/N) of a feedstock for biogas production should fall within the range of 20 to 30:1 as reported by [5].

**Table 3: Results of the proximate composition of chicken feather**

| Parameter | Result |
|---|---|
| % Moisture | 2.50 ± 0.20 |
| %Dry matter | 97.50 ± 0.17 |
| % Ash | 1.50 ± 0.15 |
| %Volatile solids | 96.50 ± 0.25 |
| % Carbon | 55.97 ± 0.10 |
| %Nitrogen | 14.0 ± 0.150 |
| C/N Ratio | 4:1 |
| | |

Chicken feather slurry was biologically-treated using mixed broth culture of *Geobacillus Stearothermophilus* and *Bacillus licheniformis* to further disrupt the disulphide bonds of α and β-keratin after hydrothermal treatment as described by [26]. Starter cultures for biogas production was constituted based on the result of enzyme tests carried out on the isolates as described by [5] with little adjustment and used as inocula for anaerobic digestion in 1500cm$^3$ organic slurry. Anaerobic digestion was allowed to proceed in a 2 litre glass digester containing 1500cm$^3$ chicken feather slurry with 3% starter culture each at four temperature regimes (35$^{o}$C, 45$^{o}$C, 55$^{o}$C and 65$^{o}$C). Raw biogas stream was subjected to purification processes using chemical absorption and adsorption methods to remove water vapour, hydrogen sulphide and carbon dioxide to upgrade it to methane standard as recommended by [7;8]



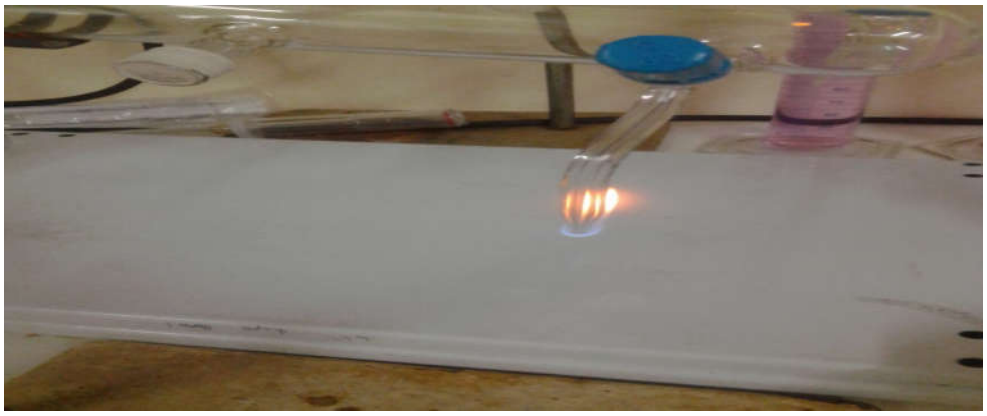


**Fig. 2: show the anaerobic digestion units and purified methane under flammability test**

International Journals of Scientific and Allied Research (IJSAR)
ISSN: 2437-1246
Volume 4, Issue 1 (March, 2017), 27- 44
http://www.mdcjournals.org/ijsar-...html

**Fig.3: Measurement of gas volume by downward displacement of water**

The result of the methane yield based on the volume of gas from each treatment at four temperature regimes showed that consortium 4 containing more of the methanogens has the highest yield of gas followed by consortium 3 at different temperatures.

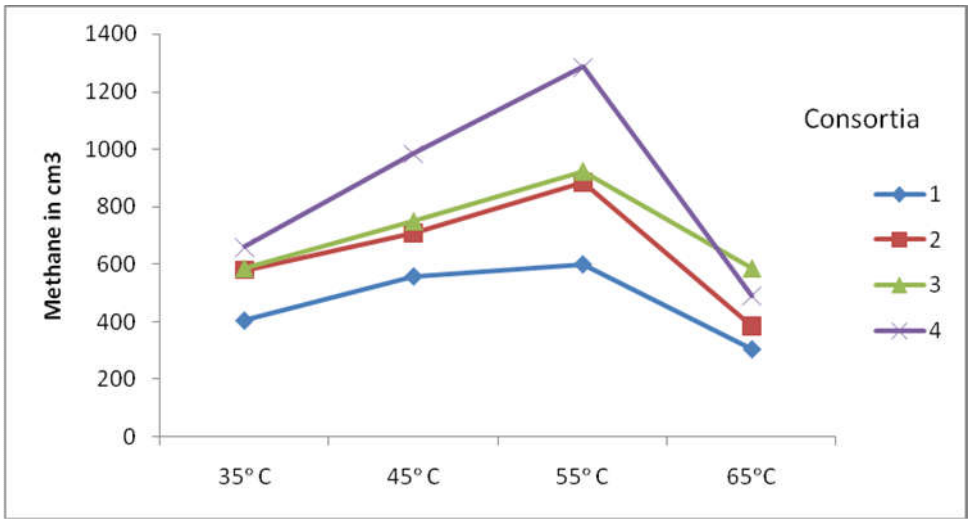



**Fig.4 Methane yield from different consortia against digestion temperature**

**Table 4: Result of average methane yield in $cm^3$ from 100% untreated chicken Feather at different temperature using four consortia**

| Consortium | 35 C | 45 C | 55 C | 65 C |
|---|---|---|---|---|
| 1 | 193±7.78 | 333±45.96 | 502±23.33 | 182±2.12 |

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International Journals of Scientific and Allied Research (IJSAR)
ISSN: 2437-1246
Volume 4, Issue 1 (March, 2017), 27- 44
http://www.mdcjournals.org/ijsar-...html

| | | | | |
|---|---|---|---|---|
| 2 | 239±52.32 | 498±17.68 | 543±57.28 | 223±24.75 |
| 3 | 295±21.21 | 549±61.52 | 605±29.70 | 280±14.14 |
| 4 | 439±46.67 | 687±24.04 | 739±45.96 | 378±38.9 |

**Table 5: Result of average methane yield in $cm^3$ from 100% treated chicken feather at different temperatures using four consortia**

| Consortium | 35°C | 45°C | 55°C | 65°C |
|---|---|---|---|---|
| 1 | 428±31.82 | 600±28.28 | 655±66.47 | 343±52.33 |
| 2 | 605±35.36 | 735±36.06 | 918±45.96 | 423±53.03 |
| 3 | 643±80.61 | 775±35.36 | 952±39.60 | 558±39.60 |
| 4 | 720±85.56 | 994±12.02 | 1313±38.18 | 540±84.85 |

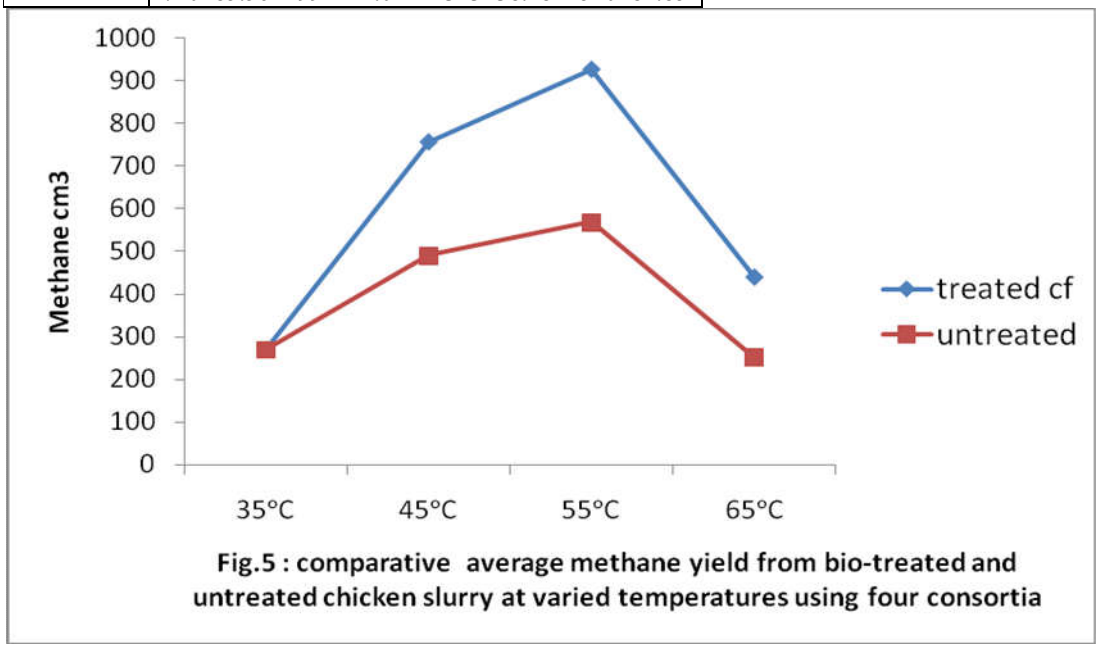


**Fig.5 : comparative average methane yield from bio-treated and untreated chicken slurry at varied temperatures using four consortia**

The highest yield of gas was 1313 cm3 at 55°C using consortium 4 for biotreated substrate and 739 $cm^3$ for untreated substrate. The result of gas yield obtained from the study is in agreement with the report of [2] and [5] that methanogens were the major key players in anaerobic digestion and usually determine the level of methane yield. The optimum temperature of activity at which gas yield was enhanced in all the treatments was 55°C while the maximum was 65°C with sharp decline in gas yield. The two-way analysis of variance (ANOVA) of the results on gas yield based on methane volumes from different treatments showed that methane production was significantly ($0 \leq 0.05$) dependent on the microbial consortia, nature of substrate and digestion temperature.

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International Journals of Scientific and Allied Research (IJSAR)
ISSN: 2437-1246
Volume 4, Issue 1 (March, 2017), 27- 44
http://www.mdcjournals.org/ijsar-...html

**Table 6: Result of two-way analysis of variance of the results on methane yield from chicken feather using four consortia at different digestion temperatures**

Dependent Variable: gas yield

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. | Partial Eta Squared |
|---|---|---|---|---|---|---|
| Corrected Model | 2805792.813[a] | 15 | 187052.854 | 13.226 | .000 | .861 |
| Intercept | 21758093.521 | 1 | 21758093.521 | 1538.494 | .000 | .980 |
| temperature | 1585746.562 | 3 | 528582.187 | 37.376 | .000 | .778 |
| Consortium | 1023607.063 | 3 | 341202.354 | 24.126 | .000 | .693 |
| temperature * Consortium | 196439.187 | 9 | 21826.576 | 1.543 | .175 | .303 |
| Error | 452558.667 | 32 | 14142.458 | | | |
| Total | 25016445.000 | 48 | | | | |
| Corrected Total | 3258351.479 | 47 | | | | |

Summary of test of variance between means showing the significant difference in gas yield from chicken feather among the temperatures and consortia : gas yield is significantly dependent on temperature and consortium ($p \leq 0.05$)/ at 95% confidence level

## CONCLUSION

The results of the studies have shown that it is possible to produce biogas from 100% chicken feather despite the recalcitrant nature of the feed stock. Pre-treatment of feathers by hydrothermal and biological methods prior to anaerobic digestion is very essential to disrupt the disulphide and hydrogen bonds of β-keratins for easy hydrolysis by microbial enzymes in order to improve biogas yield.

Future research should look at the effects of co-digestion of chicken feather which has high nitrogen with feed stock of lower nitrogen and high carbon content such as plant residues on biogas yield. Research interest should focus on the possibility of encoding the genes for methanogenesis in faster metabolizing organisms such as yeasts and *Escherichia coli* to enhance biogas production.

## CONFLICT OF INTEREST

The authors declare no personal, commercial or financial conflict of interest in the course of carrying out this research studies up to the point of publication.

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http://www.mdcjournals.org/ijsar-...html

## REFERENCES

[1] Naik SN, Goud VV, Dalai AK (2010). "Production of first and second generation biofuels: a comprehensive review", *Renewable and Sustainable Energy Reviews,* 14(2): 578-597.
[2] Fayyaz, AS, Qaisar M, Mohammad MS, Arshid P,Saeed AA. Ecology of anaerobic digesters, *The Science World Journal*, 2014; 183752-21
[3] Appels L, Baeyens J, Degreve J, Dewil, R (2008). Principles and potentials of anaerobic digestion of waste activated sludge, *Progress in Energy and Combustion Science,234* :755-25
[4] Rasi S, Lanteta J and Rintala J(2011). Trace compounds affecting biogas energy utilization, *Energy Conversion and Management*, 52:3369-3375.
[5] Gopinath LR, MerlinChristy P, Mahesh K, Bhuvaneswari R, Divya D (2014). Use of bacterial consortia for biogas production, *Journal of Environmental Science, Toxicology and food Technology, 8(3):80-6*
[6] Lastella G. Testa C, Comachia G, Notoornicok M, Voltasio F, Sharma VK (2002). Biogas and its purification, Energy Conservation and Management, 43:63-75.
[7] Kapdi SS, Vijay VK, Rajesh SK and Prasad, R (2005). Biogas Scrubbing, Compression and Storage-Indian perspective, *Renewal Energy, 30*: 1195-7.
[8] Wellinger A, Lindberg A (2005). Biogas upgrading and utilization, *International Energy Agency Bioenergy* , 24:1100-100.
[9] Gerardi MH, (2008). The microbiology of anaerobic digesters, In: Waste Microbiology, John Willey and Sons lnco. Newyork pp. 640-670.
[10] Metcalf S, EDDY G(2004). Waste water Engineering Management and Reuse, McGraw Hills International edition Newyork pp.983-1035
[11] Karakashev D, Batstone DJ, Angelladaki I (2005). Influence of environmental conditions on methanogenic compositions in anaerobic bioreactors, *Applied and Environmental Microbiology*, 7(1):331-338.
[12] Shink B (1997) Energetics of synthrophic cooperation in methanogenic degradation of biomass. , *Microbiology and Molecular Biology Reviews*, 61(2):262-280
[13] Ogbonna JC (2011). Renewable energy sources as better alternatives to fossil fuels, *Proceedings of the 19th Annual conference of FADIB held at IMT, Enugu Nigeria*,
[14] Illmer P, Reltschuler C, Wagner AO, Schwarzenauer T, Lin P(2014). Microbial succession during thermopile anaerobic digestion-the potentials of Methanosarcina spp., *Applied Microbiology and Biotechnology,* 85:849-860.
[15] Gorman J (2002). Industrial and economic Potentials of poultry feathers, *Science News*, 61(8):8-10
[16] Lakshmi PJ, Ch M, Kumari C and Lakshmi JJ (2013). Efficient degradation of feathers by keratinase-producing *Bacillus* spp *International Journal of Microbiology,* 2013:608321-608342.
[17] Mabrouk MEM (2008) Feather degradation by e new Streptomyces spp. MS-2, *World Journal of Microbiology and Biotechnology, 24*(10):2331-2338
[18] Thys RC, Lucas FS, Riffel A, Heeb P, Brandelli A (2004). Characterization of a protease from feather-degrading Microbacterium spp, *Letters In Applied Microbiology*, 39(2):181
[19] Riffel A, Lucas F, Heeb P, Brandelli A (2003) Characterization of a new keratinolytic bacterium that completely degrades native feather keratin, *Archives of Microbiology,* 179(4) 258-265.
[20] Han M, LUO W, Gu Q, Yu X (2012). Isolation and characterization of a keratinolytic protease from Pseudomonas aeruginosa C11, *African Journal of Microbiology Research*, 6:2211-2222.
[21] Lin X, Lee CG, Casale ES, Shih JCH (1992). Purification and Characterization of feather-degrading Bacillus licheniformis strain, *Applied and Environmental Microbiology*, 58(10):3271-3275.
[22] Deivasigamani B, Alagappan (2008).Industrial application of keratinase and soluble proteins from feather keratins, *Journal of Environmental Biology,* 29(6) 933-936.
[23] Suneetha V, Lakshmi VV (2005). Optimization of parameters for fermentative production of keratinase by feather-degrading microbes, *World Journal of Microbiology and Biotechnology,* 7(1):106-115.
[24] Wang JJ, Shih JCH (1999). Fermentative production of keratinase from Bacillus licheniformis FDB-29, *Journal of Industrial Microbiology and Biotechnology,* 22(6):608-616.
[25] Schidmit W (2001). Microcrystalline keratin from feathers to composite products, *Material Research Society* 20: 25-32.
[26] Pandian S, Jawahar S, Prabakaran P. Isolation and identification of feather-degrading bacteria, *European Journal of Experimental Biology,* 2012; 2(1): 274-8
[27] Mezes L, Nagy A, Galya B, Tamas J (2015). Poultry waste recycling possibility as soil nutrients, *Eurassian Journal of Soil Science*, 4(4)244-252
[28] Williams CM, Richer CS, Mackenzie JR, Jason CH Shih (1990) Isolation, identification and characterization of feather-degrading bacterium, *Applied and Environmental Microbiology,* 56(6):1509-1515
[29] Thakker CD, Ranade DR (2002). Alkalophilic Methanosarcina spp isolated from Lonar Lake, *Current Science,* 82:455-3.
[30] Sharma k. Manual of Microbiology Tools and Techniques, NewDelhi India,
[31] Norrel SA, Messley K.E (2013). Microbiology Laboratory Manual, 2nd edition, Prentice Hall, India.
[32] Maria IQ, Juan DDC, Manuel M, Maria JB, Pillorm DNA (2008). Extraction of DNA by boiling method *Clinical Vaccine Immunology*, 15 (2):293-3.

[33] Stephen FA, Thomas LM, Alejandro AS, Jinghui, Z, Zheng Z, Webb M, David JL (1997). Basic Technologies and Applications, Institute of Cell and Molecular Science, UK.
[34] Cioabla AE, Lonel L, Dumitrel GA, Popescu, F (2012). Factors affecting anaerobic digestion of agricultural residues, *Biotechnology Biofuels, 5* : 39-3.
[35] Manyi-loh CE, Mamphweli SN, Meyer EL, Okoh AI, Makaka G, Michael S (2015). Investigation into the biogas potential of cattle manure, Journal of *Clean Energy Technologies 3*(5):326-5
[36] Tinsley J, Nowakowski T (1959). Organic carbon content in the soil, *Journal of Food and Agriculture*, 10:14-12.
[37] Dioha IJ, Ikeme CH, Nafiu T, Soba NI, Yusuf MBS (2013) Carbon and Nitrogen content of various biogas feedstocks *International Resource Journal of Natural S*cience, 1(3):1-10
[38] Gelman A (2005) "Analysis of variance, why it is more important than ever", *The Annals of Statistics,* 33:1-53.
[39] Nagal S and Jain Pc (2010). Feather degradation by strains of Bacillus isolated from decomposing feathers, *Brazilian Journal of Microbiology*, 41(1):