Isolation and Identification of Microorganisms Associated With Substrate Used For Biogas Generation

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Abstract: **Microorganisms play a vital role in processing organic matter and returning the chemical elements into active cycle. In these, decomposers are effective in dismantling the complex organic matter through a sequential breakdown and release of energy. Biogas is one such process which occurs in the absence of oxygen and several groups of microorganisms involved in degrading the organic compounds only releases methane gas, to attain biogas with high methane concentration, it is important to treat and maintain the suitable microorganisms involved within the digester. Therefore, the research was carried out to isolate and characterized microorganisms associated with biogas generation from cow dung and kitchen wastes before anaerobic digestion and to determine the proximate composition of the substrates. The proximate composition of the substrates were determined according to official method of association of official analytical chemist (2000). The result of proximate composition of the substrates to be use in this research shows that moisture content of cow dung was 7.23 ± 0.18 and kitchen 8.1 ± 0.16, ash contents 2.73 ± 0.3 and 3.24 ± 0.1, crude protein content 14.19 ± 0.6 and 17.1 ± 0.2, crude fiber content 1.08 ± 0.08 and 2.02 ± 0.7, fat content 0.63 ± 0.02 and 0.8 ± 0.04, carbohydrate content 7.0 ± 0.6 and 8.2 ± 0.7 respectively. Microbiological analysis were carried out using standard microbiological procedures. Total heterotrophic bacteria and fungi count indicate that cow dung had 7.46 x 10^{-5} (cfu/ml) kitchen waste 5.82 x 10^{-5} (cfu/ml). For fungi count 6.04 x 10^{-5} (cfu/ml) and 4.93 x 10^{-5} (cfu/ml), respectively. The result revealed that 12 heterotrophic bacteria which includes *Pseudomonas spp, Escherichia coli, Bacillus spp, Salmonella spp, Staphylococcus aureus, Serratia spp, Shigella spp, Micrococcus spp, Proteus vulgaris, Citobacter spp, Kelbsiella spp* and 4 fungi which includes; *Fusarium spp, Mucor spp, Aspergillus spp* and *Penicillium spp* where isolated from the substrates.**

I. INTRODUCTION

The most fascinating features of any civilized communities are the abundant availability of energy for domestic, agricultural and industrial purposes (1). Biogas is produced when bacteria degrade biological materials in the absence of oxygen, in a process known as anaerobic digestion (2). Anaerobic treatment is the use of biological processes, in the absence of oxygen, for the breakdown of organic matter and the stabilization of these materials by conversion to methane and carbon dioxide gases and a nearly stable residue (3). Animal wastes and kitchen wastes can be used as sources of nutrient, feed ingredients to microorganisms and as fuel energy source, they contain high level of organic matter that could be converted into energy as supplement for fossils. Animal wastes are abundant all over the world with Nigeria producing about 227,500 tons of fresh waste each day (4).

Environmental pollution is one of the serious problems facing humanity and all other living thing today. Economic growth and heavy consumption of natural resources are responsible for the global warming, acid rain and the destruction of the ozone layer (5). However, biogas is one of the renewable energy sources and the continuous increase of the world's energy needs, extinction of fossil-based energy sources and changes caused by the destroyed natural balances requires the more effective use of fossil – based and renewable energy sources.

Biogas has provided an economically viable and sustainable means of meeting the thermal energy needs throughout developing world with a warm, stable climbed and easy availability of plant materials and animal wastes (cow dung, poultry droppings, pig excreta among others) occasioned by the current positive shift in agricultural policies of government, Nigeria is an advantageous position for adopting and popularizing biogas. At present, much of the dung produced by about million of herds of cattle in Nigeria is either wasting away as waste. This section of the work aimed at determining the proximate composition of the substrates, isolation and characterization microorganisms associated with the substrates to be used in biogas generation.

II. MATERIALS AND METHODS

This section described the methodology adopted to analyze the key parameters of the cow dung and kitchen wastes, which will be later used as an indicator to predict the methane generation potential of the substrates.

Source of Waste Use

The cow dung used in this work was obtained from Abattoir Dump Site from Ndibe Beach, Afikpo, Ebonyi State, Nigeria while the kitchen wastes (vegetable lefts over, plantain peels and left over garri and rice was obtained from different eatery centers in Akanu Ibiam Federal Polytechnic, Unwana, Ebonyi State).

Preparation and Measurement Of Waste

Fifty kilogram of cow dung were sun dried for 20 days while the kitchen wastes were milled into a coarse form followed by

sun drying for 20 days. The slurry was prepared by measuring 50 liters ($0.05m^3$) of cow dung and poured into a pre-treatment drum. One hundred liters ($0.1m^3$) of water was added to the cow dung inside the pre-treatment drum (i.e. in ratio 1:2, cow dung to water). The same procedure was applied to kitchen waste and stirred manually.

Proximate Analysis

Determination of moisture content, ash content, crude protein, fat content, crude fiber and carbohydrate content were carried out using the standard procedure of (6).

Microbial Screening from the Slurry

One gram (1 g) of each of the slurry was aseptically weighed and added into 90 ml of sterile distilled water to form aliquot.

Preparation of Media and Reagents

All the media and reagents used were prepared according to the manufacturer's instructions.

Sterilization of Materials

The media used for this work were prepared and sterilized at 121^{o}C for 15 minutes under 15 lb pressure with an autoclave while glassware were sterilized at 180^{o}C for one hour using hot air oven.

Enumeration of Total Heterotrophic Bacteria

Total heterotrophic bacteria in the substrates were enumeration by spread plate technique using nutrient agar. A tenfold serial dilution of the slurry was carried out by transferring1ml each of the aliquot into test tubes containing 9 ml of sterile distilled water arranged serially in the order 10^{-1}–10^{-10}. The amount of 0.1 ml at 10^{-5} dilution was spread over nutrient agar media using sterile spreader. The plates were incubated at 37^{o}C for 24–48 hours for the appearance of colonies. Discrete colonies were sub-cultured in nutrient broth and streaked over nutrient media plates. The pure bacteria colonies obtained were primary identified using morphological analysis. The total heterotrophic bacteria were calculated using formular:

Cfu/ml = No. of Colonies x Dilution Factor/Volume of Inoculums.

Enumeration of Total Heterotrophic Fungi

A 0.1 milliliter aliquot of the serially diluted samples at 10^{-5} dilution were introduced into culture plates containing sterile Sabruraud Dextrose Agar (SDA), with chloramphenicol at concentration of 0.05mg/ml to inhibit bacteria growth. The samples were uniformly spread on the surface of the medium with a sterile glass rod. Incubation was carried out in an inverted position at 28^{o}C for 5 days for the development of fungi colonies.

Preparation of Stock Cultures (Slants)

Ten milliliter (10 ml) of sterile molten nutrient media were poured aseptically into sterile MacCantney bottles and were kept at slanting position to set. Pure cultures of 24 hours bacteria isolates were streaked on surface of the nutrient agar slant and labeled accordingly. All slants were incubated at 37^{o}C for 24 hours. The slants were stored at 4^{o}C for further use.

Also plates for corn meal agar were prepared and allowed to set. Pure fungi isolates were planted on the solid media and incubated at room temperature for 5 days and transferred into refrigerator for storage at 4^{o}C for further use.

Characterization and Identification of the Bacterial Isolates

The isolates were subjected to morphological and biochemical tests and were identified by comparing their characteristics with those of known taxa. Gram staining, motility test, sugar fermentation test (glucose, lactose and mannitol), catalase test, oxidase test, citrate test, urease test, coagulase test, indole test, methyl red tes, citrate utilization test, hydrogen sulphide production test and voges proskaeur test were carried out as done by (7).

Characterization and Identification of the Fungal Isolates

The colonial and microscopic characteristics of the fungi isolates were determined using the lactophenol cotton blue staining method and the slide culture test.

Lactophenol Cotton Blue Staining

A solution of lactophenol cotton blue was prepared. Using a straight wire, a fragment of a fungal isolate was placed on a clean grease-free slide. Two drops of the lactophenol cotton blue solution were added and the stain allowed to penetrate. The slide was then viewed under the microscope. The isolates were identified following the description of (9).

Slide Culture Test

A fragment of the aerial mycelia was inoculated on a slide containing sterile prepared sabouraud dextrose agar with a sterile inoculating loop. The slide was thereafter incubated at 28^{o}C for 24 hours, then stained with lactophenol cotton blue dye, covered with a coverslip, and examined under the x100 objective lens of the microscope.

III. RESULTS

Table 1: Initial Characteristics of Proximate Composition of the substrates.

Proximate Composition (mg/100g)%	Cow Dung	Kitchen
Moisture content	7.23±0.18	8.1±0.16
Ash content	2.73±0.3	3.24±0.1
Crude protein content	14.29±0.6	17.1±0.2
Crude fiber content	1.08±0.08	2.02±0.7
Fat content	0.63±0.02	0.8±0.04
Carbohydrate content	7.0±0.6	8.2±0.7

Data are represented as the mean of ± standard deviation of three replicates.

Table 2: Total Heterotrophic Bacteria and Fungi Count

Samples	Bacteria Count (cfu/ml)	Fungi Count (cfu/ml)
Cow dung	7.46×10^{-5}	6.04×10^{-5}
Kitchen waste	5.82×10^{-5}	4.93×10^{-5}

Table 3: Morphological and Biochemical Characteristics of the Bacterial Isolates

Code	Morphology	Gran Reaction	Motility	Oxidase	Catalase	Indole	Citrase	Urease	V.P	M.R	Coagulase	H_2S	Glucose	Lactose	Manitol	Organism
1	Cocco bacilli	–	+	+	+	–	+	–	–	–	–	–	–	–	–	*Pseudomonas spp*
2	Single rod	–	+	–	+	+	–	–	–		–	–	+	+	+	*Escherichia coli*
3	Straight rod	–	+	+	+	+	+	+	+	–	–	+	+	–	–	*Bacillus spp*
4	Straight rod	–	–	–	+	–	–	+	+	–	+	–	+	+	+	*Salmonella spp*
5	Cocci		–	–	+	–	+	+	–	+	+	–	–	+	+	*Staphylococcus aureus*
6	Straight rod	–	+	–	–	–	+	–	+	–	–	–		+	+	*Serratia spp*
7	Straight rod	–	–	–	+	+	+	–	+	+	–	–	–	+	+	*Shigella spp*
8	Cocci		–	+	+	–	+	–	–	+	–	–	+	–	–	*Micrococcus spp*
9	Straight rods	–	+	–	+	+	+	–	+	–	–	–	+	+	+	*Proteus vulgaris*
10	Straight rods	–	–	–	+	–	–	+	+	–	+	–	+	+	+	*Salmonella spp*
11	Straight rods	–	+	–	+	–	+	–	–	+	–	–	+	+	+	*Citobacter spp*
12	Straight rods	–	–	–	+	+	+	–	+	–	–	–	+	+	+	*Klebsiella spp*

Table 4: Morphological Characteristics of the Fungi Isolates

Code	Colour of Hypae	Macroscopic Features	Probable Organism
1.	Pink wooly	Narrow septate hyphae, conidiophores occur singly and in groups, multicellular creasent shaped conidia	*Fusarium spp*
2.	White fluffy	Aseptate, broad hyphae with large spherical head without rhizoid	*Mucor spp*
3.	Back velvety	Septate and broad, hyphae, with large head entirely covered with chains of conidia	*Aspergillus spp*
4.	Greenish velvety	Steptate hyphae, with conidiophores developing into branced phalides bearing chains with brush like appearance	*Penicillium spp*

IV. DISCUSSION

The initial characteristic of the proximate composition of the substrates to be used for biogas generation were analyzed the result showed. The moisture content of cow dung is 7.23 ± 0.18 and kitchen 8.1 ± 0.16, ash contents 2.73 ± 0.3 and 3.24 ± 0.1, crude protein content 14.19 ± 0.6 and 17.1 ± 0.2, crude fiber content 1.08 ± 0.08 and 2.02 ± 0.7, fat content 0.63 ± 0.02 and 0.8 ± 0.04, carbohydrate content 7.0 ± 0.6 and 8.2 ± 0.7 respectively. This research reveal a total of 12 (twelve) heterotrophic bacteria isolated from the substrate and 4 (four) fungi isolated from the substrate respectively. 2 (two) out of the 12 (twelve) bacteria were found to be gram positive bacteria, which includes; *Staphylococcus aureus* and *Micrococcus spp*. The remaining 10 (ten) bacteria were found to be gram negative bacteria which includes; *Pseudomonas* spp, *Escherichia coli*, *Bacillus* spp, *Salmonella* spp, *Serratia* spp, *Shigella* spp, *Proteus vulgaris*, *Citrobacter* spp and *Klebsiella* spp, these bacteria isolated were different species which reveals that the substrate contains different range of specie of bacteria. The heterotrophic fungi isolated were *Fusarium* spp, *Mucor* spp, *Aspergillus* spp, *Penicillium* spp. These findings were in line with (9), which reported that the substrates involved in the production of biogas is a combination of different groups of organism ranging from bacteria to fungal which is useful in the degrading process of the substrate to give methane gas. There is another report of using *proteus vulgaris* for improved biogas production by (10). Heterotrophic isolates have the potential to metabolize methane and takes part in methane production. Cow dung produces gas, which is as a result of collaboration with other microbes as reported by (11).

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