






## Research Article

# Lignolytic Isolates from Mixed Waste Digester and a Metagenomic Analysis of the Microbial Community

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## Abstract

This research showed the requisite necessity of lignolytic microorganisms derived from waste feed-stock in the environment, industry, (medicine, pharmacy, textile) and metabolic process confirming their degradability and gas producing ability. This study aimed at the isolation and identification of lignolytic microorganisms from mixed waste using molecular characterization and a metagenomic approach on waste derived microbiome and their potentials in gas production and waste conversion. The organisms of interest are the ligninolytic and gas producing microbes (methanogens). Metagenomics of the cow dung substrate was also done. Samples (Fruit and vegetable residue+wood waste+animal waste) were collected from AMAC Relocation market, Lugbe, F.C.T. and analysed at the International Institute for Tropical Agriculture (IITA) Oyo state. Mineral solution containing  $CuSO_4$  was used to identify cellulose degraders. Their degradative ability was confirmed by the formation of a clear zone. Twenty-four anaerobic bacteria were isolated from mixed waste using molecular characterization out of which six bacteria species were identified as *Methanothermobacter thermautotrophicu* CCSD, *Methanobacteriaceae archaeon* KR-H07 A06, *Methanobacteriaceae archaeo* RK-H07, and *Candidatus methanoperedenaceae* GB50, *Methanothermobacter thermautotrophicus* NPK, *Candidatus methanoperedenaceae* GB37. Biogas yield increased for most of the days when compared from (3.0 to 8.0 mL), (5.0 - 24.0 mL) on day 30. All the methanogens identified produce gas. A total of 180 bacterial species were found in the substrate in which *Bacteroidota* (26.91%), *Fermicutes* (25.87%) and *Proteobacteria* (7.24%) predominated. Seventy (70.0%) were anaerobes while 30% were aerobes. Clostridium were the highest having (28.8%) amongst the anaerobic bacterial community while *Acidobacteriota* was the least (0.01%). *Escherichia coli* was the highest aerobic species having (14.5%) while *Streptomyces* was the least (0.01%).

## 1. Introduction

Globally, agriculture produces an average of 23.7 million tons of food every day while also producing 21% of the world's greenhouse gas emissions. Field waste is present after harvesting crops and include stems, leaves, stalks, and waste after processing crops includes seeds, peels, husks. Microorganisms play an important role in reducing this waste to value added products; methanogens play integral role in such functions. Microorganisms are of great benefits to human, as they have a wide range of potentials in the human body as probiotics. They are also used in industrial processes and waste management strategies. Ruminant animals like sheep, cattle and deer feed on grass and leaves as they have special digestive systems. This system relies on microbes to help them break down cellulose from plant matter and other cellulolytic foods, since vertebrates don't have the necessary enzymes to do that themselves, methanogens breakdown this cellulose and release methane gas into the environment [1]. Methanogens can be used to digest organic matter and thus produce biogas. In sewage treatment plant for example, anaerobic digestion of solid waste is carried out by methanogens [2]. The gasses produced by this process mainly methane and carbon dioxide are harmful to the environment [3]. Biogas can be used as fuel for cooking or to power lighting and other devices. This use is seen especially in rural areas that have a lot of cattle that produce plenty raw materials as Cowdung is one of them. Enzymes produced by microorganisms have more demand in the recent years due to its numerous applications in biotechnology. (compounds, dye decolourization, Cosmetic industry, lignin degradation, bioethanol production and diagnostic kits [4]).

## 2. Materials and Methods

Description of Study Location Samples were collected from two sampling points; 1. Cow Dung, Mixed fruit and vegetable residue and Saw Dust from AMAC Market and Cattle Ranch, Lugbe, Federal Capital Territory. 2. Mixed fruit and Vegetable residue from Odi and Yenagoa environs, Bayelsa state. 3. Cow dung from slaughter house, Ibadan, Oyo state. Description of study location 1 One (1). The Federal Capital Territory is the capital of Nigeria, Abuja. It is within the middle belt region of the country with a total land area of 7,315 km<sup>2</sup> (2,824 sq mi), a total population of 2,238,800 and a density of 190/km<sup>2</sup> (500/sq mi). It has a coordinate of 9° 4' 0" N 7° 29' 0" E. The AMAC, Lugbe Relocation Market is located along airport road in the upcoming Lugbe Area of the F.C.T. The Ultra-Modern Market boast over 900 types of shops including Warehouses, Cold Rooms, and Meat shops, Slaughter House, Wood Shade and Cattle Ranch from where the samples were collected. Plate 3.1 and 3.2 shows the description of the two sampling points. Description of sampling point one (1) Samples were collected at the Cattle Ranch and the AMAC Lugbe relocation Market. Animal dung was collected from the cattle ranch (Cow droppings) in the early hours of the day from the cattle. Fruit and vegetable residue were also collected from house refuse at life camp, Abuja.

Description of Study location (2). Yenagoa is a Local Government in Bayelsa State, Nigeria 'and is the Capital of Bayelsa State in the Niger Delta regions of Nigeria. Its headquarters are in the town of Yenagoa (the State capital) in the South of the area at a latitude of 4° 55' 29" N 6° 15' 15" E and it has a coordinate; 5° 02' N 6° 20' E. It has a total area of 1,698 km<sup>2</sup> (656 sq mi). Tombia Market is located at Tombia round about Yenagoa, Bayelsa State. Description of sampling point 2. Fruits and vegetable residue was collected from their vendors at their selling tent in the Market. And also, sawdust was collected at the saw mill while Cowdung was also collected from the residential area of cattle rearers in Odi community of Bayelsa State.

## 3. Study Design and Scope

### 3.1. Scope of Study

This study focuses on the microbiota in mixed-waste (fruits and vegetable waste, saw-dust and cow dung waste).

### 3.2. Study Design

In this study, five reactors were used (Anaerobic digesters) for the three set up which include 1. Control 2. Mixed substrate with cow dung, mixed fruit and vegetable residue and Saw dust. 3. Single substrate. The process efficiency for each of the set up and comparisons were made. Sampling process and sample collection.

### 3.3. Sampling Process

Cow dung sample was collected at Tipa garage airport road, Ahmadu Bello Way, Lugbe, Abuja. Other non-wood substrate as mixed-fruit and vegetable waste of each of the samples was collected.

### 3.4. Determination of microbiological parameters was done

The media that were used in the study are, Nutrients agar, Minimal Salt Media (MSML) PDA (Potatoe Dextrose Agar) and MSA (Mannitol Salt Agar) using the method of Akintokun, 2017. For bacterial screening Dilutions 10<sup>-1</sup>, 10<sup>-3</sup>, 10<sup>-5</sup> and 10<sup>-7</sup> of the samples (upon serial dilution) were plated on Nutrient agar and nutrient-gelatin agar (hydrolytic bacteria media). The plates were incubated for 48 hrs and colonies were observed, there was no significant number of colonies after 48 hrs, therefore the plates were further incubated for 48 more hrs (96 hrs in all) which showed a high number of colonies with distinct clear zones.. Colonies on plates were counted, colony forming units per gram (CFU g<sup>-1</sup>) of bacterial growth between 30 - 300 colonies were enumerated. The colonies formed were further sub cultured on nutrient agar and minimal salt medium, colonies were observed after gram staining using cultural, morphological and biochemical methods.

### 3.5. Anaerobic Count (TVC)

The colonies formed were sub cultured and identified using a microscope as described [5]. Dilutions 3, 5 and 6 were plated on the Msmi media using spread plate method. Plates were incubated at 28° C for 48 hrs. Colonies on plate were counted, colonies with clear zones

around them were selected and sub cultured on Msm media for purification. Media Preparation Nutrient agar used for primary isolation was prepared according to manufacturer's instruction and sterilized by autoclaving at 121° C for 15 minutes. Media was plated on Petri-dishes and allowed to solidify before use. Minimal salt media (Mslm) was prepared for purification of isolates.

### 3.6. Media Preparation

Nutrient agar used for primary isolation was prepared according to manufacturer's instruction and sterilized by autoclaving at 121° C for 15 minutes. Media was plated on Petri-dishes and allowed to solidify before use. Minimal salt media (Mslm) was prepared for purification of isolates (Methanogens) using the following components with their concentrations.

Cl (13.08), NaCl (10.27), Sodium acetate (20.0), resazurin (0.003), Na<sub>2</sub>CO<sub>3</sub> (1.51) and Na<sub>2</sub>S. 9H<sub>2</sub>O (1.78); 10 mL/litre each of the trace element solution. Laboratory Sample Preparation 1 g of each of the sample was dissolved using a vortex mixer in 100 mls of distilled water. Serial dilution was carried out by taking 1 ml into the first tube containing 9 mls of distilled water coupled with mixing using a vortex mixer to homogenize the suspension. Dilutions 101, 103, 105 and 107 was inoculated on the plates containing nutrient agar media. Biochemical. Test for the Identification of Biodegrading Bacteria (ligninolytic) Oxidase Test Tested bacteria colonies were smeared in the filter paper previously saturated with freshly prepared oxidase reagent. Positive oxidase test was recorded as the development of a blue purple color within 10 seconds. Catalase Test Hydrogen peroxide was added to the microscopic slide containing the isolates, this was done to induce catalase activity. The appearance of bubbles on the slide indicates catalase activity and suggest the presence of catalase positive-bacteria Identification of Methanogens As methanogenic bacteria are extremely sensitive towards oxygen, they require reducing conditions, and pre reduced media conditions, these are essential for their growth and development as described by Derza et al [6] where microbial were grown on serum vials or Basal medium (35.5 mL) stoppered with aluminum seal. In this work, anaerobic jar was used to grow methanogenic cells.

### 3.7. Methanogenic Sample Enrichment

Samples were inoculated into sodium bicarbonate media incubated at 37° C for 15days. The growth of methanogens was observed using morphological and Biochemical test.

### 3.8. Metagenomics

Samples were sequenced on the Sequel IIE system by PacBio (www. pacb.com). Raw sub-reads were processed through the SMRTlink (v11.0). Circular Consensus Sequences (CCS) algorithm to produce highly accurate reads (> QV40). These highly accurate reads were then processed through vsearch (<https://github.com/torognes/vsearch>) and taxonomic information was determined based on QIMME2. Report generation command used: `$create_vsearch_single_sample_pdf_report_pacbio.py create_vsearch_single_sample_pdf_report_pacbio.py`.

## 4. Results And Discussion

Tentative identification of isolates with lignolytic and methanogenic properties based on performance using biochemical test and molecular characterization

Biochemical test and microscopy result interpreted revealed the presence of isolates positive for Citrase, sucrose and glucose with rods observed under the microscope at mesophilic and thermophilic temperature (35-45° C). This is typical of the genus methanocella as it was further confirmed using molecular characterization. Microscopy of some isolates revealed the presence of cocci and were positive for mannitol and mannose at thermophilic and slightly mesophilic temperature. Genus Methanococcus. Methanomicrobium was also identified as microscopy revealed short rods with the isolates positive to mannose, citrase and sucrose. Methanoregular was identified as microscopy revealed the presence of rods, cocci, short and tiny rods and isolates were positive to mannose test which is typical of the genus methanoregular. Isolates with short rods under the microscope and positive for citrase were observed which is typical of the genus Methanomicrobacterium. Short rods isolate positive for Glucose, mannose, sucrose and mannitol were also identified as methanolinear which is typical of its genus. This were all further confirmed by molecular characterization. Ligninolytic microorganisms were found present as they have utilized the glucose and produced enzymes during the biochemical test. Formation of clear zone was used to screen lignolytic microorganisms using selective aforementioned mineral solution.

### Final identification using molecular characterisation

*Methanothermobacter thermautotrophicus* CCSD, *Methanobacteriaceae archaeon* KR-H07 A06, *Methanobacteriaceae archaeon* RK-H07, and *Candidatus methanoperedenaceae* GB50, *Methanothermobacter thermautotrophicus* NPK, *Candidatus methanoperedenaceae* GB37 were molecularly characterized from the isolates that were tentatively identified as shown in figure as shown in Figure 1.

## 5. Results obtained from metagenomic analysis

A total of 180 bacterial species were found in the NSFCS of which Bacteroidota (26.91%), Firmicutes (25.87%) and Proteobacteria (7.24%) predominated. Seventy (70.0%) of the NSFCS were anaerobes. Clostridium was the highest (28.8%) amongst the anaerobic bacterial community, while Acidobacteriota was the least (0.01%). Escherichia coli was the highest aerobic species (14.5%), while Streptomyces was the least (0.01%).

Gas producing ability of the methagenomic isolates in consortium Figure 1 shows results obtained from the inoculation (seeding) of cow dung substrate using the identified methagenomic species during gas production. Figure 1: Effects of Microbial seeding on gas yield from cow dung

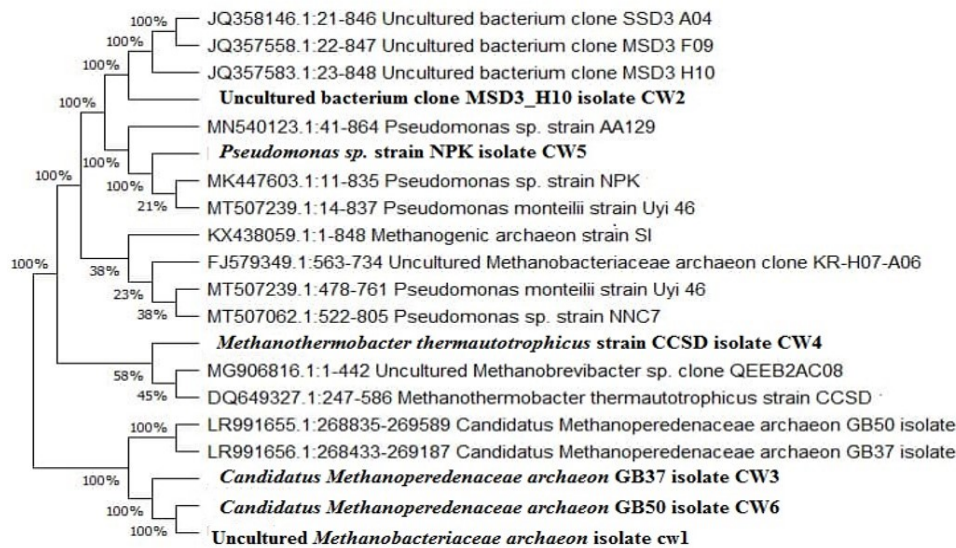


Figure 1: Molecular characterization of the isolates obtained

## 5.1. Applications

Ligninolytic microorganisms and methanogens (gas producing microorganisms) are largely essential in;

## 5.2. Medicine

Enzymes produced by these microorganisms during their metabolic activities (biodegradation) are used in producing diagnostic kits.

## 5.3. Industry

**Food industry:** Bioethanol production, probiotics

**Textile industry:** Degradation of natural rubber, dye decolourization.

**Pharmaceutical:** Oxidizing phenolic and non-phenolic compounds.

**Probiotics Antibiotics:** Conclusion and Recommendation. Most of the ligninolytic and gas producing (methanogens) microorganisms (Methanocella, Methanococcus, Methanomicrobium, Methanoregular, Methanomicrobacterium, Methanolinea, and Ligninolytic Methanolinea) isolated in this work produce enzymes. Some of these enzymes such as manganese per-oxide are key contributors in ligninolytic system. They also act as oxidizing agents, which breaks phenolic lignin and production.

## 6. Conclusion and Recommendation

Most of the ligninolytic and gas producing (methanogens) microorganisms (Methanocella, Methanococcus, Methanomicrobium, Methanoregular, Methanomicrobacterium, Methanolinea, and Ligninolytic Methanolinea) isolated in this work produce enzymes. Some of these enzymes such as manganese per-oxide are key contributors in ligninolytic system. They also act as oxidizing agents, which breaks phenolic lignin and produce free radicals that have a tendency to disintegrate involuntarily, this has a great application potential and ample opportunities in diverse areas such as Alcohol, pulp and paper, biofuel, agriculture, cosmetic, textile, and food industries. Nigerian Government should prioritize and promote research and funding in the areas of bioremediation, biodegradation (probiotics) and other enzyme related-industrial processes which will reduce cost and enhance a sustainable environment if technically applied especially in countries like Nigeria where oil spillage and other indiscriminate waste disposal has become the order of the day.

### Declaration of Interest

There is a high interest in this work as its processes are consequential in industry, environment, probiotics and pharmaceutical applications. All these areas have shown high interest in ligninolytic and gas producing microorganisms. Our interest in this work is basically to harness the potentials of these microbes in different fields of endeavor in science.

### Acknowledgment

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## Article Information

**Disclaimer (Artificial Intelligence):** The author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.), and text-to-image generators have been used during writing or editing of manuscripts.

**Competing Interests:** Authors have declared that no competing interests exist.

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