European Journal of Applied Sciences – Vol. 10, No. 2
Publication Date: April 25, 2022
DOI:10.14738/aivp.102.11872.
Gani, M., Dabai, A. I., Jumare, F. I., Maiturare, H. M., & Muhammad, S. (2022). Comparative Study on the Optimisation of the Hydrolysis Step During Biogas Production from *Typha Grass* Using Conventional Microbiology and Metagenomics Tools. European Journal of Applied Sciences, 10(2). 51-63.

Comparative Study on the Optimisation of the Hydrolysis Step During Biogas Production from *Typha Grass* Using Conventional Microbiology and Metagenomics Tools

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ABSTRACT

The impact of climate change has increased by many orders of magnitudes in recent years. These changes have necessitated a global drive towards alternative sources of energy. Biogas has been re-echoed as one form of renewable energy that is sustainable because of the possibility of using several biomass sources to produce biogas. To date, two major factors hindered biogas production, the first been the biomass (having the appropriate C: N ratio) secondly enhanced hydrolysis to boost and sustained the yield of the biogas. The present study explored *Typha* grass as biomass for biogas production. The proximate composition was determined by the AOAC method. The biogas produced in the reactor was measured using water displacement method. The bacteria were isolated using standard bacteriology method and characterised by PCR amplification of the 16S rDNA gene. The metagenomics microbial community was determined by the Illumina amplicon sequencing method. The percentage of acid-insoluble lignin was determined using TAPPI (T222) method. The holocellulose content was determined according to DIN 2403. The α- Cellulose content of the substrate was determined in the 17.5% NaOH solutions using TAPPI 203 om-93 method. Typha grass had low lipid value of 2.05% and fiber 2.3% and an appreciable amount of lignin 47.05%, followed by holocellulose of 40.55%, hemicellulose 29.5% and cellulose was 11.5%. Biogas production started after 6 days and was optimum after 8 days with mean gas yield

of 180 mL and decreased to 25 mL after 12 days. The reactor had a bacterium count of 1.33E+08 CFU/g at day 8. The result of the PCR amplification indicates the presence of bacterial species such as *Acinetobacter* sp*., Pseudomonas putida*, *Pseudomonas veronii*, *Citrobacter* species and *Delftia* sp, in the reactor. The metagenomics data revealed that bacteria were the major microbial community detected within the reactor and the inoculum and it accounted for more than 99%. At the phyla level, unknow bacteria, *Firmicutes, Bacteroidetes* and *Proteobacteria* were the major group within the reactor and in the inoculum. Uncultured bacterium, unclassified, rumen, *Bacteroidetes* and Archaeon constitute the major genera in the reactor. The results suggest that *Typha* grass can be exploited as biomass for biogas production and the complex microbial especially uncultured bacterium are important in the production process.

Keywords: Biogas, Biomass, Lignocellulose, *Typha* grass, Metagenomics, Renewable energy

INTRODUCTION

Currently, energy production is focused on the processing of fossil fuels, which is associated with the increased production of greenhouse gases (Weiland 2010). Due to plans to increase the share of renewable energy (by 20%) in whole energy production in European Union by 2020 and a growing demand for energy, a viable alternative seems to be the use of biogas (which production doesn't adversely affect the environment or climate and is economically beneficial) (Yadvika *et al.,* 2004; Fountoulakis and Manios, 2009; Klimiuk *et al.* 2010; Weiland 2010). The biogas, which consists mainly of methane (60-70%) and carbon dioxide, is produced from the feedstock in the anaerobic digestion process, carried out by several groups of microorganisms (Yadvika *et al.* 2004; López *et al.* 2009; Weiland 2010; Li *et al*. 2011). The gas can be used as a vehicle fuel or energy or heat production purposes (Berglund and Börjesson 2006).

Typha (commonly referred to as 'cattail') is the only genus in the family *Typhaceae*, comprising of nearly 40 species and hybrids, is ubiquitous across wetland ecosystems throughout the globe. An abundance of wind-dispersed seeds allows *Typha* makes it capable of colonising wetlands across great distances, and its rapid growth rate, large stature, and aggressive clonal propagation can result in dense monotypic stands. These stands have considerable impact on local flora and fauna, biogeochemical cycling, and wetland hydrology, which correspondingly impact the function of wetland. Over recent decades, *Typha* distribution and abundance in wetland ecosystems around the world, particularly in North America, has increased due to anthropogenic-related disturbances to wetland hydrology and nutrient loads. In addition, vigorous non-native and hybrid taxa have exacerbated the rapid spread of *Typha*. *Typha* invasion and expansion in wetland ecosystems have required widespread management, albeit control is often short-lived or ineffective. Despite the negative impacts of *Typha* on natural systems, this plant can provide beneficial ecosystem services under certain conditions, including bioremediation in constructed wetlands to reduce nutrient loads and pollution, and providing feedstocks for biofuel needed to help offset global carbon dioxide (CO2) emissions (Bansal *et al*., 2019).

The production of biogas is composed of four stages: hydrolysis, acidogenesis (fermentation), acetogenesis and methanation. The first step is carried out by some groups of microorganisms (such as *Cellulomonas, Clostridium* etc.) that produce extracellular enzymes to decompose the feedstock compounds into smaller molecules (Weiland 2010; Li *et al.* 2011). During the acidogenesis step (fermentation) mainly the volatile fatty acids are produced. There are a few groups of bacteria with the ability to carry out this stage (for example *Acetobacterium, Clostridium* and the Candidate division - OD1) (Li *et al.* 2011, Wrighton *et al.* 2012). High numbers of OD1 have been connected with high methane production in some anoxic environments and found in a few anaerobic digesters (Guermazi *et al*. 2008; Peura *et al*. 2012).

Some bacteria (for example *Acetobacterium, Sporomusa, Clostridium)* convert volatile fatty acids into acetate and hydrogen during the acetogenesis (Weiland 2010; Li *et al*. 2011). In the last step - methanogenesis, methane is produced from acetate, CO2 and H2 or from methyl groups by a few groups (in order: acetoclastic, hydrogenotrophic or methylotrophic) of methanogens which are *Archaea* (Garcia *et al.* 2000; Demirel and Scherer 2008; Weiland 2010). There are five orders known so far among these *Archaea* which are, as follows: *Methanomicrobiales*, *Methanococcales, Methanobacteriales, Methanosarcinales* and *Methanopyrales* (Garcia *et al*. 2000). Hydrogenotrophic pathway of producing methane is characteristic for *Methanobacteriales, Methanococcales*, *Methanomicrobiales* and *Methanopyrales*. Order *Methanosarcinales* consists of two families containing acetotrophic (obligatory acetotrophic methanogens – family *Methanosaetaceae*) or acetotrophic as well as methylotrophic (family *Methanosarcinaceae*) *Archaea* (Garcia et al. 2000). Some species mediating the hydrogenotrophic pathway are related to low methane yield (Ziganshin et al. 2011).

Although it is observed that the predominant methane production is via the acetoclastic pathway in anaerobic digestion, there are some research studies which showed not only the dominance of acetoclastic but also hydrogenotrophic methanogens as well as the *Clostridia* or *Bacteroidetes* in some digesters varying in performance (Liu *et al*. 2002; McHugh *et al.* 2003; Rivière *et al.* 2009; Krause *et al.* 2008; Schlüter *et al.* 2008; Krakat *et al*. 2010; Wirth *et al*. 2012). Therefore, the present study explored the potential of microbial communities in anaerobic digesters with *Typha* grass for biogas production.

METHODOLOGY

Sample collection

The *Typha* grass was collected from Kware Lake in Kware local government area, Sokoto State, Nigeria. The sample was washed under tap water, chopped into smaller pieces and blended using a handheld blender. The rumen content from the cow was collected from Sokoto abattoir with the help of the Veterinary Dr. on duty.

Digester's set-up

The anaerobic digester to be employed for the biogas production was set up in 1-litre flasks and was sealed using silicon seal, tubing was connected to monitor the biogas yield by the water displacement method (Rabah *et al*., 2010). Cow rumen content was used as inoculum and was in a ratio of 1:9 to the *Typha* biomass.

Proximate Composition of the *Typha* biomass
Samples were analysed in triplicate as described in a previous method (AOAC, 2006).

Ash Content Determination
Two grams (2g) of each sample was weighed and dispensed into pre-weighed crucible (W_o). The sample was incinerated in lenton furnaces (England) for over night. The sample was cooled in a desiccator and the crucible and dry sample was weighed.

% Ash was calculated using equation 1.

$$\%\text{Ash} = \frac{W_2 - W_0}{W_1 - W_0} \times 100 \quad \text{..... 1}$$

Where W_o= Weight of crucible; W_1 =Weight of crucible + sample before incineration; W_2=Weight of crucible + sample after incineration

Crude Fibre Determination
In order to determine the crude fibre of the substrate, two gram (2g) each sample were weighed into a conical flask (W_0), after which 20 mL of 10% H_2SO_4 was added and boiled gently for 30 minutes and was filtered through a poplin cloth stretched over the Buchner funnel. It was rinsed with hot distilled water and the material was scraped back into the flask with a spatula. This is followed by the addition of 20 mL of 10% NaOH in the same flask and was boiled gently for 30 minutes, after which the mixture was filtered using a poplin cloth. The residue was washed thoroughly with hot distilled water and rinsed once with 10% HCl and twice with ethanol. It was then rinsed three times with petroleum ether and allowed to drain dry. The residue was scraped into a crucible and dried overnight at 105 ^{O}C in the oven (Gallenhamp Oven BS). It was cooled in desiccators and weighed as (W_1). It was ash at 550 ^{O}C for 90minutes in lenton furnaces (England) and cooled in desiccators and finally weighed as (W_2).

% crude fibre is calculated using equation 2.

$$\%\text{Crude fibre} = \frac{W_1 - W_2}{W_0} \times 100 \quad \text{---------- 2}$$

W_1 = Dry weight; W_2 = Ash weight; W_0 = Sample weight (AOAC, 2006).

Determination of Moisture Content
Two grams (2g) each of sample were weighed into empty dish earlier weighed as (W_0). The samples were weighed, and then dried at 105 ^{O}C overnight in an oven (Gallenhamp Oven BS) and the dried sample was weighed and designated W_2.

% moisture was calculated using equation 3

$$\%\text{ Moisture} = \frac{W_1 - W_2}{W_1 - W_0} \times 100 \quad \text{---------- 3}$$

W_0 = weight of dish, W_1 = Initial weight of sample + dish, W_2 = Final weight (AOAC, 2006).

Determination of Crude Lipid

The Percentage (%) of the crude fat was extracted with hexane using Soxhlet Extraction method (Parkouda, *et al*, 2008). The extraction flask of 250 ml was oven-dried at 105- 110^{o}C, allowed to cool in a desiccator and weighed. Two grams (2g) of the sample were weighed into a porous thimble and the mouth was covered with clean white cotton wool. N-hexane of 200 mL was added into the extraction flask. The covered porous thimble was placed into the condenser and the apparatus was assembled. The extraction was done for 5-6 hours, after which porous thimble was carefully removed and the N- hexane was collected in the top condenser for re-use. The extraction flask was removed from the water bath, oven-dried at 105- 110^{o}C for one, cooled in the desiccator, and weighed.

% crude lipid was calculated using equation 4.

$$\%\text{Crude lipid} = \frac{W_3 - W_2}{W_1 - W_0} \times 100 \quad \text{---------- 4}$$

Determination of Soluble Carbohydrate (Nitrogen Free Extractive)

The Nitrogen-Free Extractive (N. F. E) referred to as soluble carbohydrate is not determined directly but obtained as a difference between crude protein and the sum of ash, protein, crude lipid and crude fibre.

N. F. E. = 100 - (% Ash + % Crude fibre + % Crude Lipid + % crude protein)

Chemical composition Analysis of Typha Grass Biomass

The total solids content (also called the percent dry weight) was determined according to the Laboratory Analytical Procedure No.001 (LAP-001) from the National Renewable Energy Laboratory (NREL) (Ehrman, 1994). The extractives were removed from analysed samples by Soxhlet-extraction with ethanol-acetone (1:2 v/v) for 6 h and dried at 103 ± 2 °C for 24 h, according to procedure adapted from TAPPI standard T 204 om-97. The percentage of acid-insoluble lignin was determined according to TAPPI procedure (T224 om-88): a 1 g sample was treated with 72% sulfuric acid (15 mL) and stirred frequently for 2 h at room temperature. After 2 h, the sulfuric acid was diluted with distilled water (560 mL) to obtain the sulfuric acid at a 3% concentration. The solution was then boiled for 4 h, filtered with distilled water, dried at 103 ± 2 °C for 24 h, and finally weighed. The holocellulose content was determined according to DIN 2403. A mixture of 80 mL distilled water, 1 mL acetic acid (98%), 3 g sodium chloride, and 2 g of rubberwood sample was heated in a water bath at 70 °C for one hour. The mixture was stirred every 5 min during this time. Acetic acid (1 mL) and sodium chloride (3 g) were added each hour for the next 3 hours. After 4 hours, the samples were filtered, washed with methanol 3 times, and then dried at 103 ± 2 °C for 24 h and weighed. The α-cellulose content of rubberwood was determined as the residue insoluble in the 17.5% NaOH solution according to TAPPI 203 om-93. Aqueous NaOH solution (25 mL of 17.5%) was added to a flask containing a sample of holocellulose (1 g) and stirred at 20 °C for 40 min, and then 25 mL of distilled water was added. After 5 min, the residue was filtered, and then 40 mL of 10% acetic acid aqueous solution was added to the residue, filtered and washed with 1 L of boiling water. The residue (α-cellulose), was filtrated, dried at 103 ± 2 °C for 48 h and weighed. The percentage hemicellulose was calculated by subtracting the percent α-cellulose from holocellulose. Dry mass loss was calculated as the percentage of total solids loss after pretreatment. Lignin

degradation, cellulose loss and hemicellulose loss were defined as the percentage of cellulose, hemicellulose and lignin reduction.

Isolation of bacteria during biogas production

This was done using minimal salt medium as described by Abd-Elsalam and El-Hanafy (2009).

Pure culture DNA Isolation and PCR Amplification of 16S rDNA

DNA was isolated from the pure culture of each isolate using Genomic DNA isolation kit and PCR was performed as described below:

The 16S rRNA gene amplification was performed using the universal primer sets {(8f) (5'-AGAGTTTGATCMTGGCTCAG-3') and 907r (5'-CCGTCAATTCMTTTRAGTTT-3')} as previously described (Barghouthi, 2008). The amplification step was performed to amplify the isolated total bacterial DNA. The PCR reaction was performed in 0.2 mL thin wall PCR tubes with flat frosted caps (Thermo scientific Massachusetts, USA) in 25 µl PCR reaction volume containing 2 µl of template DNA, 1µl each of primer 27f and 907r, 12.5 µLTaq master Mix (containing - (20 µM) of deoxynucleoside triphosphates (dNTP), Taq DNA Polymerase and Taq buffer with $MgCl_2$) (New England Biolabs, UK) and 8.5µL of molecular grade water. The amplification of the 16S rRNA was performed in an Applied Biosystems GeneAmp 9700 PCR system (Applied Biosystems, CA, USA). The protocol for PCR amplifications was as follows: initial denaturation at 95°C for 4 min, followed by 28 amplification cycles of 30 s at 94 °C, 30 s at 56 °C and 1 min at 70 °C, followed by final extension at 70°C for 10 min. The PCR product was run on 1% agarose gel electrophoresis at 90V for 50 minutes to allow proper separation of the PCR bands

Metagenomic DNA extraction

Freshly obtained digestate samples from Digesters was used to extract total DNA using MoBio PowerSoil DNA isolation kit.

Bioinformatics and Statistical Analysis

Sequence data generated from Sanger sequencing was analysed using Finch TV software and Blast against National Center for Biotechnology Information - NCBI database. The Illumina sequence were analysed using CLC BIO Workstation.

RESULTS AND DISCUSSION

Typha had a low lipid value of 2.05% and fiber 2.3% and an appreciable amount of lignin 47.05%, followed by holocellulose of 40.55%, hemicellulose 29.5% and cellulose was 11.5% (**Figure 1**). The sort of values obtained in the research was within the values range previously reported (Bala *et al*., 2018). Typha has been suggested as a potential feedstock for biofuel production (Bansal *et al*., 2019). Previous study showed that Typha can be used for the production of bioethanol (Bala *et al*., 2018). Here, we have demonstrated that biogas can also be produced using Typha as a biomass feedstock for the reactor. The mean biogas yield started at day 6, peaked at day 8 and reduced at day 12 to 25 mL (**Figure 2**). The result suggests that the optimum observed at day 8 is either reduction in the amount of substrate required by the microorganisms for biogas production or a shift in the microbial community structure and abundance. The heterotrophic bacteria count was many folds higher in the reactor compared to the uninoculated control or rumen content of the cow (**Figure 3**). The reactor is known to harbour complex and diverse microbial groups (Krause *et al*., 2013; Koike *et al*., 2003). The low

count observed in the rumen might suggest loss of the microbial community due to exposure to non-rumen conditions or the inability of many rumens' microbial community to grow outside the rumen. This problem has hindered the successful isolation and characterisation of microorganisms from the rumen environment.

Acinetobacter sp*., Pseudomonas putida*, *Pseudomonas veronii*, *Citrobacter* species and *Delftia* sp were the culturable bacteria isolated from the reactor (**Table 1**). *Pseudomonas putida* is one of the best-studied species of the metabolically versatile and ubiquitous genus of *Pseudomonas*. These characteristics make *P. putida* strains attractive for environmental, biotechnological, and agricultural uses (Dos Santos *et al*., 2004; Espinosa-Urgel *et al*., 2000; Puchalka *et al*., 2008; Ramos *et al*., 1994; Timmis, 2002). *Citrobacter* species are commonly found in water, soil, food, and the intestinal tracts of animals and humans. *Pseudomonas veronii* is useful for bioremediation of contaminated soils, as it has been shown to degrade a variety of simple aromatic organic compounds (Nam *et al*., 2003; Onaca *et al*., 2007). *Delftia* sp. LCW*,* a strain isolated from a constructed wetland shows novel properties for the degradation of dimethylphenol isomers **(**Vásquez-Piñeros *et al*., 2018**).**

The metagenomic data revealed that bacteria were the major microbial community detected within the reactor and the inoculum and it accounted for more than 99%. At the phyla unknow bacteria, *Firmicutes, Bacteroidetes* were the major group within the reactor and together with proteobacteria they constitute the phyla detected in the inoculum. Uncultured bacterium, unclassified, rumen, Bacteroidetes and archaeon constitute the major genera in the reactor (**Figure 4**). The rumens of ruminant animals are shown have a complex microbial community that can cooperatively and completely digest the lignocellulosic biomass (Krause *et al*., 2013; Koike *et al*., 2003). Phyum Firmicutes detected in the reactor are known as the major cellulose degraders, although few express glycoside hydrolases (Hanreich *et al.,* 2013). This phylum represented a 32 % of the bacterial community in wet fermentation conditions and 25 % in dry fermentation conditions, respectively (Stolze *et al*., 2015). A small fraction of the reactor metagenome represents the phylum Bacteroidetes, which are previously been found to be involve in complex polysaccharides degradation (Qiao *et al*., 2013). It is the second-most abundant bacterial taxon in lignocellulose degrading bacteria cultures, and the third most abundant bacteria in lower termite Coptotermes gut (Do *et al*., 2014). Cellulose-degrading microbes are shown to be common adherent to plant cell walls. A lack of understanding of how to exploit this process may be one of the reasons that limited the rumen microorganism's inoculation (Wang *et al*., 2013). Novel xylanase genes have been cloned from uncultured strains in rumen fluid for use as a feed additive due to the high digestibility of the rumen toward cellulosic stuffs (Wang *et al.*, 2015). Besides, a novel uncultured environmental strain, 7WAY-8- 7(T), is capable of converting glucose into acetate and hydrogen (Qiu *et al*., 2014). The considerable ecological significance of uncultured bacteria in the rumen is supported by the positive interaction between strains R-25 and F. Their activities of carboxy-methylcellulose and xylanase can be increased by further co-culture (Fukuma *et al*., 2012). However, the remaining 32 clones were unclassified bacteria; therefore, as-yet-unknown species may be involved in substrate degradation (Zhao *et al*., 2016).

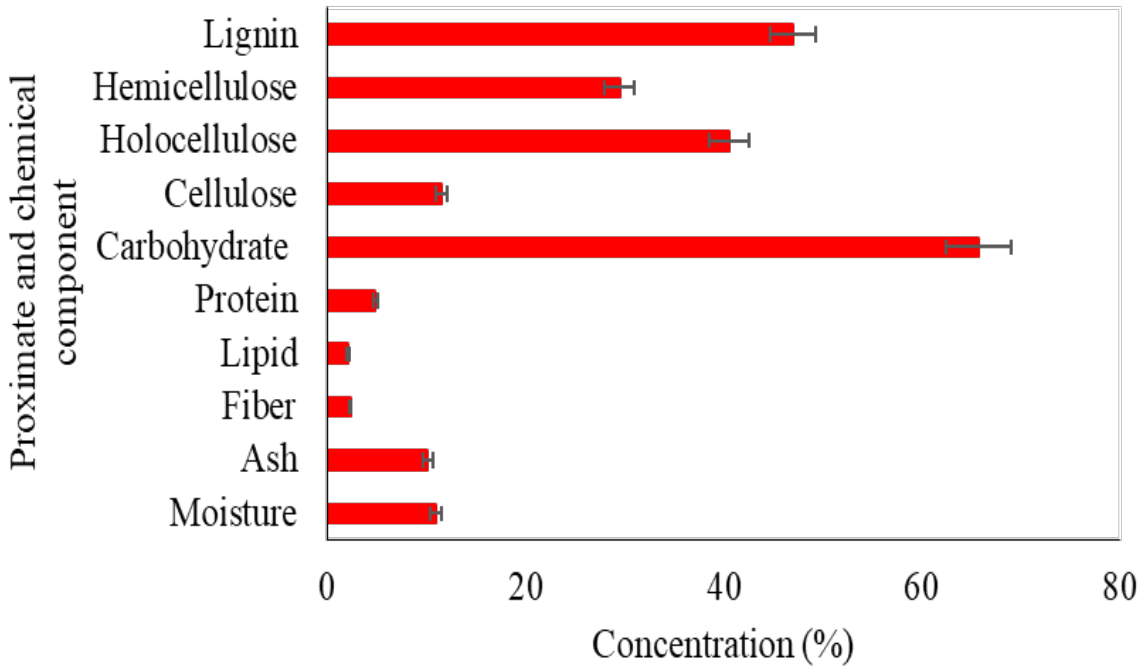


Figure 1: Proximate and chemical profile of Typha grass obtained from Kware Lake.

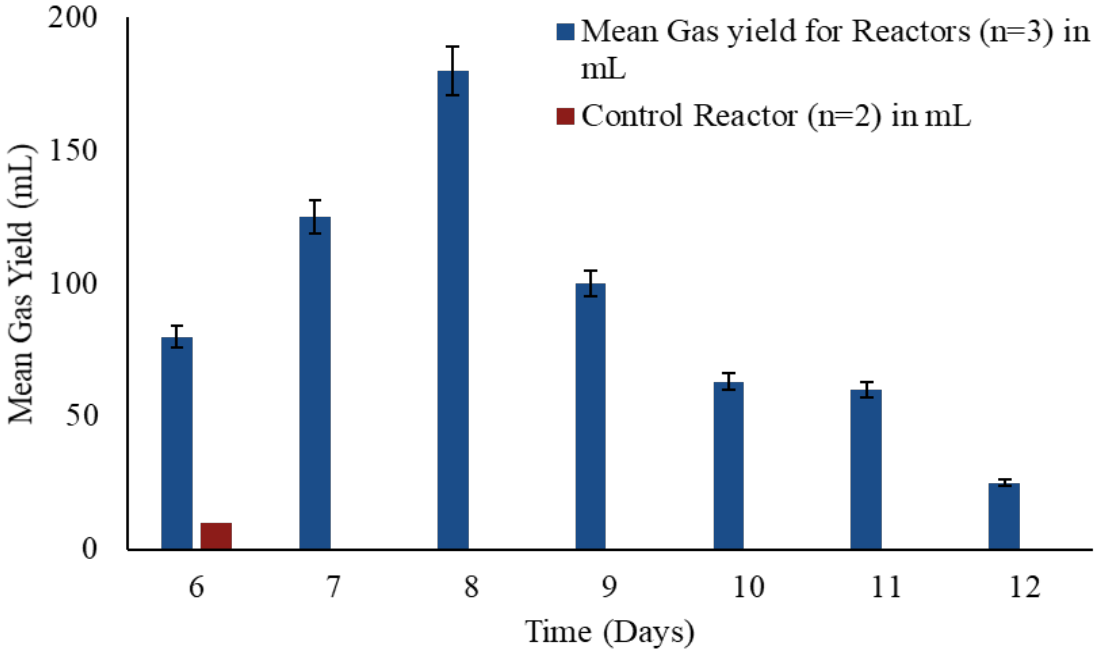


Figure 2: Biogas yield produced from Typha grass obtained from Kware Lake.

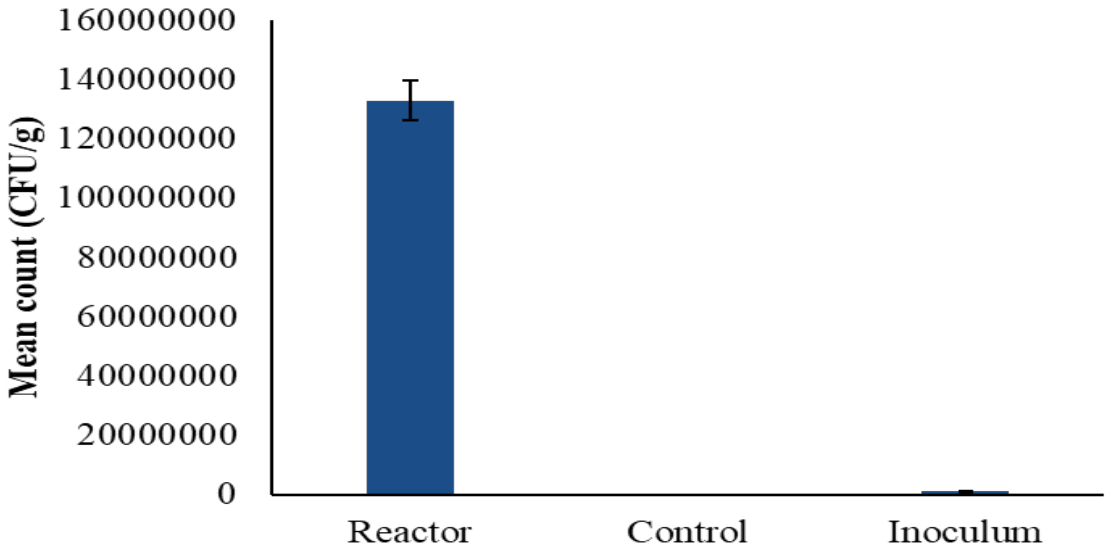


Figure 3: Heterotrophic bacteria count of Biogas yield producing reactor using Typha grass obtained from Kware Lake as biomass

Table 1: Best hit identity of amplified 16S gene products with entries in the GenBank database

Isolate	Best Hit	% Q. cov.	% Ident	Accession No.
C11_08	*Pseudomonas putida* strain XT811	100	99.41	MT424785.1
D11_11	*Citrobacter* sp. XT-36	97	99	KR063562.1
E11_14	*Citrobacter freundii* DNA	78	68	LC110442.1
F11_17	*Pseudomonas* sp. F026	85	84	KJ846478.1
G11_20	*Pseudomonas veronii* strain NSA97	86	70	KT302405.1
H11_23	*Acinetobacter baumannii* strain 309-14	97	99	MG557814.1
A12_03	*Acinetobacter* sp. strain PrPf067	97	99	MF948943.1
B12_06	*Klebsiella* sp. IPPW-8	97	99	FJ646640.1
C12_09	*Delftia* sp. strain D10	97	99	MF689014.1
D12_12	*Acinetobacter baumannii* strain Rajni04	99	81	MF595519.1

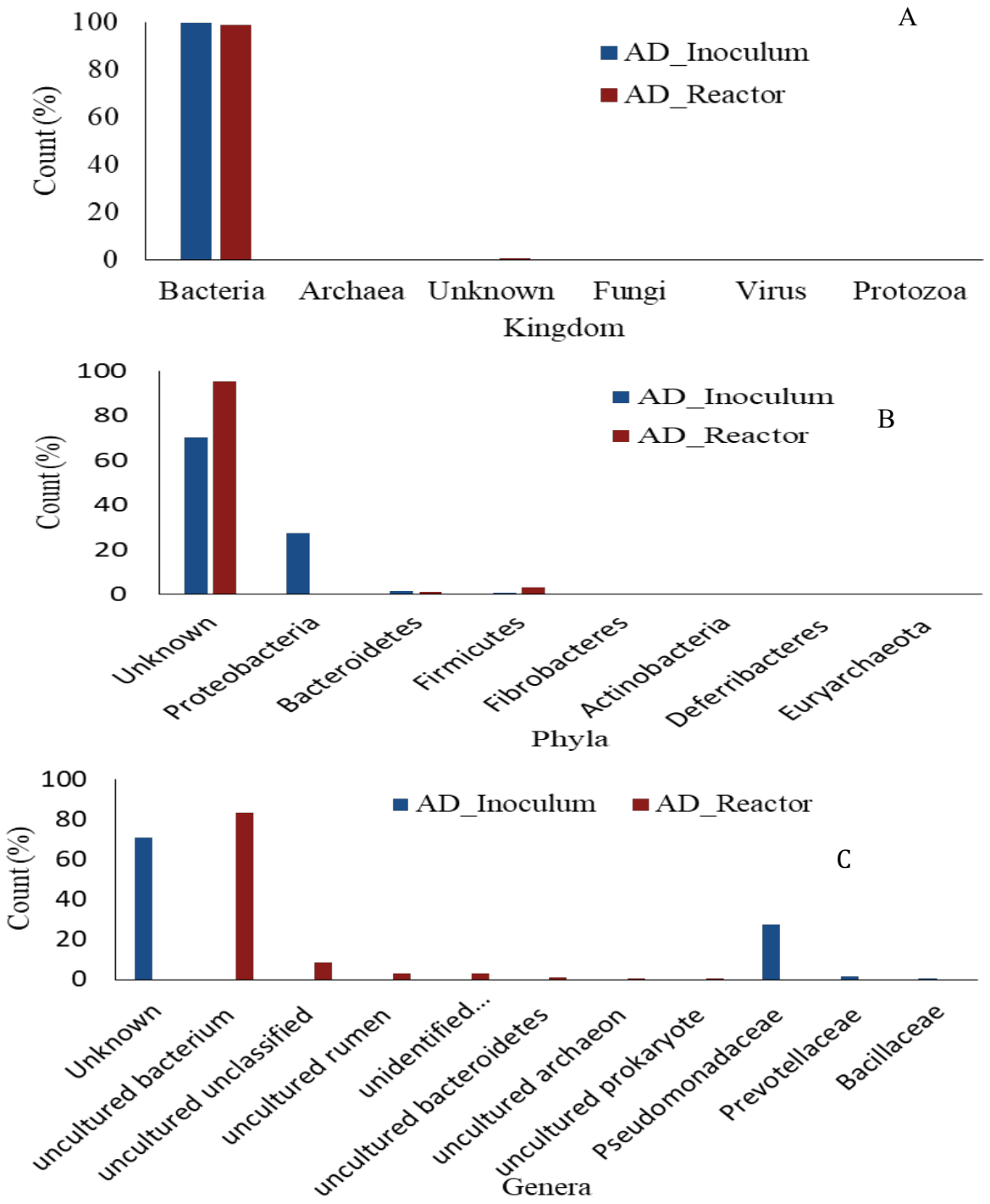


Figure 4: Metagenomic microbial community of Biogas yield producing reactor using Typha grass obtained from Kware Lake as biomass

CONCLUSION

Typha had low lipid value of 2.05% and fiber 2.3% and an appreciable amount of lignin 47.05%, followed by holocellulose of 40.55%, hemicellulose 29.5% and cellulose was 11.5%. Biogas production started after 6 days and was optimum after 8 days with mean gas yield of 180 mL and decreased to 25 mL after 12 days. The reactor had a bacterium count of 1.33E+08 CFU/g at

day 8. The bacteria species identified in the reactor includes *Acinetobacter* sp*., Pseudomonas putida*, *Pseudomonas veronii*, *Citrobacter* species and *Delftia* sp. The metagenomic data revealed that bacteria were the major microbial community detected within the reactor and the inoculum and it accounted for more than 99%. At the phyla unknow bacteria, firmicutes, Bacteroidetes were the major group within the reactor and together with proteobacteria they constitute the phyla detected in the inoculum. Uncultured bacterium, unclassified, rumen, Bacteroidetes and archaeon constitute the major genera in the reactor. The results suggest that Typha can be exploited as biomass for biogas production and the complex microbial especially uncultured are important in the production process.

ACKNOWLEDGEMENT

We thank the Tertiary Education Trust Fund (TetFund) Nigeria and the management of Sokoto State University, Sokoto Nigeria for funding this research work under the Institute Based Research component (**Project No. TETFUND/DESS/UNI/SOKOTO/PR/2016/VOL.1)**.

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