



## Research paper

Anaerobic conversion of *Chromolaena odorata* (Siam weed) to biogas

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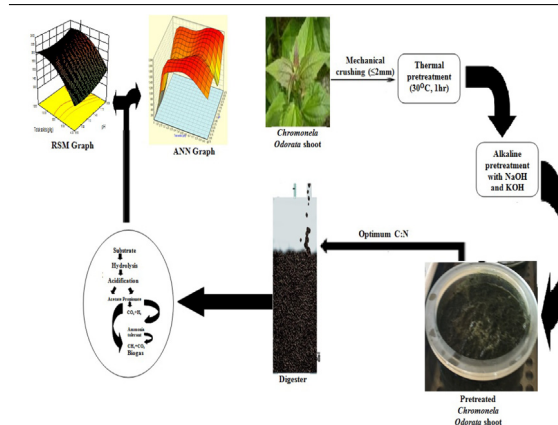
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## HIGHLIGHTS

- Biogas was produced from the digestion of *Chromolaena odorata* shoot.
- Thermo-alkaline pretreatment enhanced enormous biogas yield from the substrates.
- Over 49.20% gas yield was obtained with pretreatment over the untreated substrates.
- The optimal condition for maximal biogas yield were established.
- A positive energy balance was obtained which can be increased with higher loading.

## GRAPHICAL ABSTRACT



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## ABSTRACT

This study evaluated the anaerobic mono-digestion of two different samples of *Chromolaena odorata*. Combinations of mechanical and thermo-alkaline pretreatments were applied to one of the two samples and labeled as “X” while the second had no thermo-alkaline treatment and was labeled as “Y”. The Central Composite Design was used to design the pretreatments. The physicochemical characteristics of the substrates were carried out using standard methods after appropriate pretreatments. From the experimental set-ups, the most probable actual biogas yields in experiments “X” and “Y” were 0.3554 m<sup>3</sup>/kg Total Solid (TS)fed and 0.1803 m<sup>3</sup>/kg TSfed with the desirability of 99 and 100%, respectively. Further shown in the result is a 49.2% higher experimental (actual) biogas yield in experiment “X” over “Y”. Gas chromatographic analysis revealed the CH<sub>4</sub> and CO<sub>2</sub> content of both experiments to be 65 ± 1.5%; 21 ± 3% and 53.5 ± 2.5%; 26 ± 3%, respectively. Combination of different pretreatment methods enhanced enormous biogas yield from the digested substrates. Optimization of the generated biogas data was carried out using the Response Surface Methodology (RSM) and the Artificial Neural Networks (ANNs). The coefficient of determination (R<sup>2</sup>) for RSM was lower compared to that of ANN. This shows that ANNs

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model gives higher accuracy than RSM model. Further utilization of this weed for biogas production is encouraged by the results from this study.

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## 1. Introduction

Lignocellulosic biomass abundant and are obtained from several sources like agricultural, municipal, industrial and others. Their high fermentable carbohydrate contents make them suitable candidates for biogas generation (Alfa et al., 2012, 2013a,b). However, the inherent characteristics of lignocellulosic biomass such as cellulose crystallinity, non-accessible surface area, the high degree of cellulose polymerization, and presence of lignin and hemicellulose make them resistant to microbial degradation (Menon et al., 2016; Naran et al., 2016) and this has also hindered their commercial usage. The need, therefore, arises for substrate pretreatment in order to alter such inherent properties and various methods have been investigated which include, but are not limited to, mechanical (ultrasound, high pressure and lysis), thermal (different temperature ranges), chemical (ozonation, alkali, dilute acids) and biological (use of bacteria, fungi and enzymes) pretreatments.

Thermal pretreatment has mostly been applied in the treatment of waste activated sludge while its application to other substrates and lignocellulosic biomass are still under investigation (Liu et al., 2012; Dahunsi et al., 2016a,b, 2017a,b). Recent studies investigated the effect of alkaline treatment on biogas production from sunflower stalks and sorghum forage at laboratory scale and reported that the treatment enhanced methane production (Sambusiti et al., 2013). However, chemical pretreatment is not suitable for highly biodegradable substrates due to the tendency for volatile fatty acids (VFA's) accumulation and subsequent failure of the methanogenesis step. This then makes this pretreatment method suitable for lignocellulosic biomass since they are not easily degraded (Modenbach and Nokes, 2012). Use of combined pretreatments, is well reported as an efficient approach to improve on the limitations of single treatment procedures (Yuan et al., 2016). The most common of them i.e. thermo-chemical pretreatment has mainly been applied in the digestion of municipal wastewater treatment plant sludge (Zhang et al., 2016; Zhao et al., 2016).

*Chromolaena odorata* (Siam weed) originated in subtropical and tropical America and is found to be abundant in the diverse agricultural systems of the region. It has equally colonized other continents such as Asia, Africa, and Oceania. In most locations, *C. odorata* grows very rapidly and ultimately dominates newly cleared and abandoned farmlands through its roots, seed production and by wind dispersal (Koutika and Rainey, 2010; Amiolemen et al., 2012). It currently poses a threat to agricultural systems as a stubborn weed and has constituted a major concern as no particular control measure has been sought for it prior to this research. Though the co-digestion of *C. odorata* and poultry manure for biogas generation has been documented (Dahunsi et al., 2016b), the mass and energy balances, as well as the economic feasibility of pretreatments application, are major areas which previous researchers failed to address in the establishment of *C. odorata* as a viable biogas resource in mono-digestion, which the current study addressed.

## 2. Materials and methods

### 2.1. Collection of sample and pretreatment

The shoot of *C. odorata* was collected from the Teaching and Research Farms of Landmark University, Omu-Aran, Kwara State,

Nigeria. Since anaerobic digestion cannot take place in the absence of adequate microbial flora, bovine rumen contents from freshly slaughtered cattle were collected from the slaughterhouse of Landmark University's Cafeteria and used as inoculum in the study. The biomass was pretreated using two different methods. The first sample labeled 'X' was pretreated using mechanical and thermal-alkaline (NaOH) methods earlier documented for pretreating other lignocellulosic biomass (Dahunsi et al., 2017b,c). In carrying out this, the biomass was first crushed to a mesh size of  $\leq 20$  mm with the aid of a hammer mill and the crushed sample was taken through thermal treatment at 80 °C for one hour using the CLIFTON, 88 579 water bath (Nickel-electro Ltd., England). Before determining the appropriate temperature and duration of thermal treatment, an experimental design was done using the Central Composite Design (CCD) according to the method of Dahunsi et al. (2017b).

A two-factor design was adopted as follows: (i) the pretreatment time and (ii) the pretreatment temperature for *C. odorata* shoot. A time variation between 50 and 70 min and pretreatment temperatures of between 70 °C and 200 °C were considered. This was then followed with chemical treatment using 3 g NaOH/100 g TS at 55 °C for a 24 h period and at a solid loading of 35 g TS L<sup>-1</sup> since NaOH is usually the choice alkali in most thermo-chemical pretreatments. The same mechanical procedure was employed for the pretreatment of the second sample labeled 'Y' except that thermo-alkaline treatment was not used. After the pretreatment of the two samples, digestions were carried out using the 25-L digesters already described and used in the previous studies (Dahunsi et al., 2016a,b). In each digestion system is an air-tight digestion tank with an inbuilt mechanical stirrer meant for efficient mixing of substrate and to enhance adequate microbial distribution. Gas collection apparatus was also in place in form of a liquid displacement connected to the digestion tank (Dahunsi and Oranusi, 2013).

### 2.2. Experimental design

The experiment involving the anaerobic digestion of the two samples of *C. odorata* was designed using the Central Composite Design (CCD) (Montingelli et al., 2016). The five-level-five-factors factorial design used generated fifty different experimental accommodating five process parameters in anaerobic digestion process i.e. Temperature (°C), pH, Retention time (days), Total solids (g/kg) and Volatile solids (g/kg) represented as  $Q_1$ ,  $Q_2$ ,  $Q_3$ ,  $Q_4$  and  $Q_5$  respectively (Table 1).

### 2.3. Biochemical and residual methane potential tests

Prior to digestion, the biomethane potential and residual methane tests were carried out as the need arose to evaluate the methane production potential of *C. odorata* shoot at standard temperature and pressure. The protocols already established (Dahunsi et al., 2016a,b) were used which involved a 30-day anaerobic batch digestion using 2 flasks (250 mL each) for the experiment and the third one as blank all in triplicate. According to the standard reported (Ghasimi et al., 2016a,b), an inoculum to substrate ratio of 3 was adopted. The produced gas was continuously collected via a hose attached to each flask while the methane content was chromatographically determined.

**Table 1**

Factors and their levels for response surface study for biogas generation from the anaerobic digestion of *Chromolaena odorata* shoot.

Variable	Symbol	Coded factor levels				
		−2	−1	0	1	2
Temperature (°C)	Q <sub>1</sub>	30	32.5	35	37.5	40
pH	Q <sub>2</sub>	6.0	6.5	7.0	7.5	8.0
Retention time (days)	Q <sub>3</sub>	20	22.5	25	27.5	30
Total solids (g/kg)	Q <sub>4</sub>	4	6	8	10	12
Volatile solids (g/kg)	Q <sub>5</sub>	4	6	8	10	12

#### 2.4. Digestion process

The method earlier used by Dahunsi et al. (2017a,b) was employed in carrying out the digestion of the pretreated biomass. Temperature readings were done twice daily and the average recorded while the average pH measurement was done on a weekly basis. Weekly evaluation of microbial dynamics and physiochemical analyses of digesting feedstock and digestates were all carried out. Determination and quantification of the methane and carbon dioxide contents of the produced biogas were done using an HP 5890 Gas Chromatography (Avondale, USA) to which was attached a Hayesep Q column (13 m x 0.5 m x 1/800) and a flame ionization detector with hydrogen as the carrier gas and the equipment operated at 40 °C.

#### 2.5. Analytical procedures

There is usually the need to adequately characterize substrates before their conversion to biofuel (Fierro et al., 2016). In this respect, chemical analyses were carried out to determine and quantify the elements/nutrients compositions of the fermenting feedstock, inoculum, and digestates prior to the digestion of the two treated samples of *C. odorata*. Important physical factors like temperature and pH were also determined. Evaluation of the different parameters was done partly in the Environmental Engineering laboratory and also in the Soil mechanics/Geotechnical laboratory of Landmark University, Omu-Aran, Nigeria. The Palintest<sup>(R)</sup> Photometer 7500 (PHOT.1.1.AUTO.75) advanced digital-readout colorimeter (Camlad, Cambridge, United Kingdom) previously used (Dahunsi et al., 2016a,b, 2017a,b,d,e,f) with a standard protocol was also used in the determination of Total Carbon, Total Nitrogen (TN), Total Phosphorus (TP), Phosphates (PO<sub>4</sub>), Sulphates (SO<sub>4</sub>) Potassium (K), Sodium (Na), Magnesium (Mg), Calcium (Ca), Nitrates (NO<sub>3</sub>), Ammonium (NH<sub>4</sub>), Iron (Fe), Copper (Cu), Zinc (Zn), Aluminium (Al) and Manganese (Mn) with an absorbance of 0.5 and wavelength of 450 nm. All samples were analyzed in triplicates.

For the determination of Chemical Oxygen Demand (COD) of the samples, the standard method of the American Public Health Association for analyzing water and wastewaters (American Public Health Association, 2012) was adopted. Volatile fatty acids (VFAs) were determined by using a Clarus 580GC (PerkinElmer, USA) gas chromatography (GC) with an attached flame ionization detector. Total solids (TS) and volatile solids (VS) contents of the substrates were determined using the SFS 3008 protocol of the Finnish Standard Association (1990). The total phenolic contents of the samples were determined using a microtube test (Spectroquant, Merck) which was immediately followed by a 4-amino antipyrine colorimetric measurement (Monlau et al., 2012).

Extraction of soluble sugars (sucrose and inulin) was carried out using a mild acid hydrolysis protocol followed by the anthrone method for further quantification (Monlau et al., 2015). Similarly, quantification of structural carbohydrates i.e. glucose, xylose and arabinose and uronic acids i.e. galacturonic and glucuronic acids

were done using a strong acid hydrolysis protocol earlier described by Monlau et al. (2015). The Klason method was used for lignin content determination while for cellulose and hemicelluloses contents, the determination was based on the monomeric sugar content (Barakat et al., 2015).

#### 2.6. Energy balance and efficiency assessment of thermo-alkaline pretreatment application

Assessment of the balance between energy generation and consumption was carried out in this study so as to document the economic feasibility of applying the thermo-alkaline pretreatment in the AD of *C. odorata* shoot. In doing this, the cost of obtaining heat (Thermal) energy and chemicals (NaOH) was compared with the additional energy obtained after digestion. By this, it was determined whether the extra biogas yield obtained after the digestion of *C. odorata* shoot was enough to cover the cost of heat energy and NaOH. The thermal energy required (TER) in kWh t<sup>−1</sup> TS for the pretreatment of one ton TS of *C. odorata* shoot at 55 °C was computed using the equation below:

$$TER = \frac{m \times Sh * (T_{final} - T_{initial})}{3600} \quad (1)$$

where (1000 kg) = mass of the mixture of *C. odorata* shoot and water (kg); *Sh* = specific heat of water which is 4.18 kJ kg<sup>−1</sup> C<sup>−1</sup>; *T<sub>initial</sub>* (°C) = the initial temperature of substrate i.e. 25 °C; *T<sub>final</sub>* (°C) = the final temperature of substrate i.e. 55 °C. For the cost of NaOH, the United States cost was used.

#### 2.7. Microbiological assessment

##### 2.7.1. Aerobic bacterial, fungal and anaerobic bacterial enumeration

The aerobic organisms implicated during the digestion of both samples of *C. odorata* were enumerated and identified using standard methods for total aerobic plate enumeration. The analyses were done in the Microbiology laboratories of Biological Sciences Departments of Covenant and Landmark Universities, Nigeria. Media that were used are nutrient agar, eosin methylene blue (EMB) agar, peptone water, and MacConkey agar. Refrigerated samples collected weekly from the specialized sampling port on each digester were used for the analyses in triplicates. The presumptive isolates were phenotypically characterized and further confirmed with the aid of appropriate rapid API kits (BioMerieux) following the previous protocols (Dahunsi et al., 2016a,b, 2017a,b,c). Samples meant for fungal evaluation were initially cultured on Potato dextrose agar. For the identification, both microscopic and macroscopic features of the hyphal mass, the morphology of produced spores and the dynamics of the fruiting bodies were considered according to the method of Tsuneo (2010). However, it is believed that these methods did not detect all the organisms in the microbial diversity of the samples

The Reinforced Clostridia medium and blood agar were both used for culturing anaerobes mostly members of the *Clostridium* genera and other facultative anaerobes. The culturing was done in an anaerobic condition at 37 °C between 5 to 7 days. Developed colonies were further grown on the Brain Heart Infusion agar for ease of counting and were subsequently recorded according to the method of Ayandiran et al. (2014). The presumptive isolates were characterized morphologically and biochemically were further confirmed using appropriate rapid API kits (Ayandiran and Dahunsi, 2017).

### 2.7.2. Enumeration of methanogen (Archaea)

In our previous studies on the anaerobic digestion of different lignocelluloses, a mineral-rich basal medium was compounded and had been found efficient in the evaluation of methanogenic bacteria (Dahunsi et al., 2016a,b, 2017a,b,c). The medium contained several minerals and trace elements including  $\text{NH}_4\text{Cl}$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{NaHCO}_3$ ,  $\text{Na}_2\text{S}$ , cysteine-HCl, sodium-thioglycolate, and sodium resazurin dye all compounded and prepared under anaerobic environment using neutral pH double distilled water. Another supplement solution containing  $\text{NaHCO}_3$ , cysteine-HCl,  $\text{FeSO}_4$  in  $\text{H}_2\text{SO}_4$ , vitamins and trace elements was added to the basal medium earlier prepared using hydrogen gas as the hydrogen donor. Before the addition, both the basal medium and the supplement solution had been separately autoclaved and to the mixture was added filter sterilized  $\text{NaHCO}_3$  and cysteine-HCl. Nitrogen gas was used to expel dissolved oxygen from all the liquid media until the resazurin dye became colorless.

### 2.8. Optimization and statistical data analysis

After the digestion processes were completed, the response surface methodology (RSM) was statistically used to analyze all the generated data in order to fit the polynomial equation already generated via the 'Design-Expert software' (version 9.0.3.1). Multiple regressions were used to fit the coefficient of the polynomial model as a way of correlating both the independent factors and the respective response. The fit quality of the model was evaluated using tests of significance and analysis of variance (ANOVA) and the fitted quadratic equation obtained afterward is shown below:

$$Y = b_0 + \sum_{i=1}^k b_i X_i + \sum_{i=1}^k b_{ii} X_i^2 + \sum_{i < j} b_{ij} X_i X_j + e \quad (2)$$

where:  $Y$  = the response variable;  $b_0$  = intercept value;  $b_i$  ( $i = 1, 2, k$ ) = the first order model coefficient;  $b_{ij}$  = the interaction effect;  $b_{ii}$  = the quadratic coefficients of  $X_i$  while  $e$  = the random error.

The data obtained from the CCD were equally analyzed using the artificial neural networks (ANNs) accordingly as earlier reported (Dahunsi et al., 2016a,b). The optimum ANN structure and the higher coefficient of determination ( $R^2$ ) were determined using the mean square error (MSE) approach. Three-Dimensional curvature surface plots and relative importance were equally used to conduct variable analysis in order to study the effects of variables towards the biogas yield. Validation of the RSM and ANNs models was done using same digesters in triplicates and the predicted conditions by the software were applied. Thereafter, the deviations of actual from the observed values were plotted appropriately (Dahunsi et al., 2016a).

## 3. Results

### 3.1. Effects of thermo-alkaline pretreatment on the chemical composition of *C. odorata* shoot

As shown in Table 2, the results of the chemical analyses of the structural parameters carried out on the raw *C. odorata* shoot, the thermo-alkaline treated and untreated substrates used in the digestions are presented. In the thermo-alkaline pretreated experiments, there was solubilization/reduction in the concentrations of the structural components especially those of cellulose, hemicelluloses, and klason lignin when compared with the result obtained from the untreated experiment. At the end of the thermo-alkaline pretreatment, the concentration of cellulose was reduced by 56%. For the hemicelluloses and klason lignin, reductions of 44.29 and 56% were obtained respectively. Similarly, 38.16% reduction was recorded for uronic acids while a 50% increase was recorded for soluble sugars as a result of the solubilization of the structural components of *C. odorata* shoot.

### 3.2. Efficiency and stability of digestion

The methane potential tests showed production of gas from the 3rd and 6th days respectively for the pretreated and untreated set-ups. The recovered biogas was analyzed chromatographically and result showed the Methane ( $\text{CH}_4$ ) and carbon dioxide ( $\text{CO}_2$ ) contents to be  $65 \pm 1.5\%$ ;  $21 \pm 3\%$  and  $53.5 \pm 2.5\%$ ;  $26 \pm 3\%$  for both experiments. Several factors responsible for digester stability and performance were evaluated in this study. The anaerobic digestion process in this study was effective in the further degradation of all the structural components of the substrates. At the end of digestion, cellulose compositions in the digestates of both pretreated and untreated experiments were reduced by 26 and 21.01% respectively as against their initial values prior to digestion. For hemicelluloses and lignin, reductions of 43 and 30%; 27 and 20.5% were recorded respectively. Reductions in the concentrations of uronic acids were 64.18 and 56% respectively while 8.85 and 3% increases in soluble sugars contents were recorded after digestion both experiments respectively. Slightly alkaline pH was recorded throughout the digestion process for the two substrates in line with the values considered for experimental design i.e. 6.5 to 8 though with an initial fall in the early days of the experiment (Fig. 1). The same trend was observed for the temperature of all the digesters which remained at the mesophilic range all through the experiment.

The chemical analyses of both pretreated and untreated samples of *C. odorata* shoot shows that almost all the measured parameters recorded increase in values except for few such as total and volatile solids (which usually are converted to other intermediate products) and carbon and calcium which are essentially taken up by the microbes for efficient metabolism and subsequent bioconversion of substrates. Both the raw *C. odorata* shoot and the rumen contents were shown to have low C/N ratio with values ranging from 6 to 8. However, samples "X" and "Y" which were fed into the digesters recorded moderately high C/N ratio with "X" (20/1) being higher than "Y" (18/1). Chemical Oxygen Demand (COD) reduction of 55% was recorded for both experiments at the end of the digestions.

### 3.3. Dynamics of Volatile Fatty Acids (VFAs) and Ammonia

Table 2 further shows very low concentrations of VFAs in both pretreated and untreated substrates prior to digestion. The prominent VFAs were acetate and propionate with values (0.11 and 0.15 g COD/g VS) and (0.12 and 0.13 g COD/g VS) for the pretreated and untreated substrates respectively (Fig. 2). The initial ammonia ( $\text{NH}_3$ ) concentration was also low in both substrates (2.01 and 2.21 mg/g VS) respectively (Fig. 3). As the digestion progressed in both experiments, VFAs and  $\text{NH}_3$  accumulation were recorded through at with low level especially at the middle of the digestions (Days 9th to 14th) which indicate that there was an imbalance between the hydrolysis/acidogenesis and the subsequent acetogenesis/methanogenesis stages of digestions. The peak of total volatile fatty acids (TVFAs) and  $\text{NH}_3$  accumulation were observed between the 12th and 15th days before further reductions were recorded for the rest of the experiment.

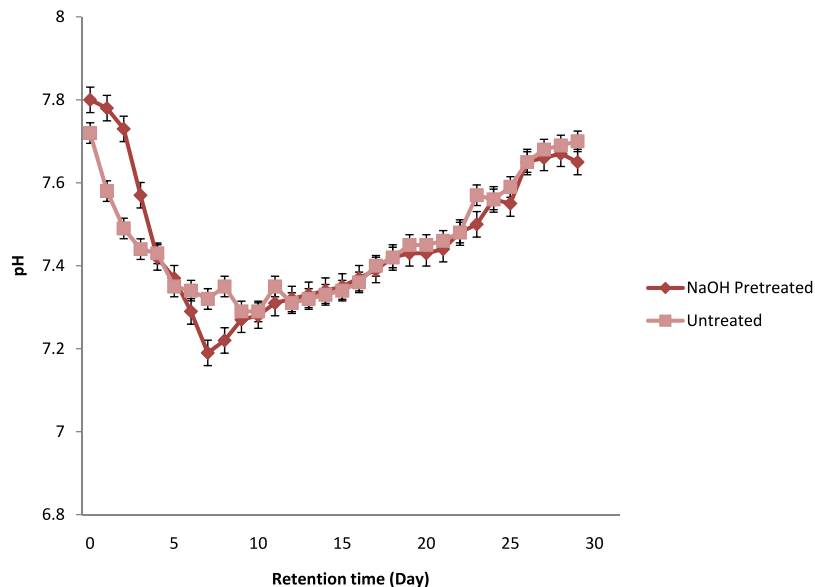
### 3.4. Optimization of pretreatment and biogas generation

According to the design of the thermo-alkaline pretreatment experimental employed in this study, pretreatment at  $80^\circ\text{C}$  for 60 min was optimum and also gave the highest biogas yield of  $3.565 \text{ m}^3/\text{kg TS}$  fed out of all the tested experimental runs (Table 3). Biogas generation in experiment "X" started between the 2nd and 5th days of digestion while that of experiment "Y" commenced between the 5th and the 7th days in almost all and progressive

**Table 2**  
Physical and chemical characteristics of *Chromolaena odorata* shoot and cattle rumen content.

Parameters	<i>C. odorata</i> shoot only	Rumen content	Pretreated <i>C. odorata</i> shoot + Rumen content		Untreated <i>C. odorata</i> shoot + Rumen content	
			Before digestion	After digestion	Before digestion	After digestion
pH	6.54 ± 0.22	7.91 ± 0.02	7.80 ± 0.12	7.65 ± 0.10	7.72 ± 0.11	7.70 ± 0.11
Total Solids (g/kg)	103.54 ± 0.21	90.52 ± 0.11	120.64 ± 0.11	80.09 ± 1.02	111.11 ± 0.10	81.02 ± 1.02
Volatile Solids (g/kg)	90.05 ± 0.01	80.44 ± 1.12	89.70 ± 0.02	42.38 ± 3.72	98.04 ± 0.02	69.88 ± 3.72
Ash Content %	4.44 ± 0.02	5.56 ± 1.02	5.30 ± 0.01	8.62 ± 1.02	5.72 ± 0.01	6.27 ± 1.00
Moisture Content %	89.32 ± 0.11	90.48 ± 3.02	87.35 ± 2.01	90.9 ± 2.32	91.52 ± 1.01	96.8 ± 0.02
Total Carbon (g/kg TS)	230.51 ± 2.02	265.21 ± 0.10	549.22 ± 5.22	298.00 ± 2.22	425.11 ± 6.02	341.40 ± 1.20
Total Nitrogen (g/kg TS)	28.21 ± 0.02	48.00 ± 2.02	29.00 ± 0.22	38.00 ± 0.21	23.20 ± 0.02	27.30 ± 0.20
C/N	8/1	6/1	20/1	8/1	18/1	13/1
Acetate (g COD/g VS)	0.06 ± 0.12	1.04 ± 0.10	0.11 ± 1.10	0.002 ± 0.01	0.06 ± 0.10	0.001 ± 0.10
Propionate (g COD/g VS)	0.08 ± 0.10	1.07 ± 0.02	0.15 ± 0.03	0.003 ± 0.02	0.09 ± 0.11	0.004 ± 0.10
TVFAs (g COD/g VS)	0.17 ± 0.02	2.44 ± 0.10	1.21 ± 0.10	0.09 ± 0.10	1.23 ± 0.10	0.07 ± 0.11
Ammonia (mg/g VS)	0.09 ± 0.11	4.97 ± 1.01	2.01 ± 1.10	1.15 ± 0.02	2.21 ± 0.02	1.18 ± 0.10
COD (g COD/g VS)	187.21 ± 0.02	168.21 ± 1.12	202.26 ± 1.40	90.91 ± 0.14	246.12 ± 1.00	109.88 ± 0.10
Cellulose (% VS)	30.15 ± 1.10	12.30 ± 0.10	17.55 ± 1.10	13.02 ± 1.02	39.88 ± 1.10	31.50 ± 1.02
Hemicelluloses (% VS)	15.82 ± 0.10	7.71 ± 1.10	11.08 ± 1.10	06.35 ± 1.10	19.89 ± 0.10	13.93 ± 1.10
Klason lignin (% VS)	35.77 ± 2.10	17.17 ± 1.12	22.18 ± 1.00	16.23 ± 0.01	49.87 ± 2.10	39.65 ± 0.05
Uronic acids (% VS)	3.51 ± 1.10	1.67 ± 1.11	2.82 ± 1.10	1.01 ± 0.10	4.56 ± 1.10	2.01 ± 0.10
®Soluble sugars (% VS)	2.05 ± 1.01	4.02 ± 2.10	8.14 ± 0.11	8.93 ± 1.10	4.11 ± 1.02	4.23 ± 0.10
Phenols (mg L <sup>-1</sup> )	0.003 ± 0.01	4.71 ± 2.10	0.005 ± 0.01	04.11 ± 0.10	1.91 ± 0.01	07.31 ± 0.10
Total Phosphorus (g/kg TS)	4.12 ± 0.01	6.30 ± 0.02	4.26 ± 0.12	5.62 ± 0.11	3.60 ± 0.11	3.97 ± 0.01
Potassium (g/kg TS)	5.69 ± 1.00	7.20 ± 0.11	6.6 ± 0.11	7.40 ± 0.02	4.6 ± 0.01	5.05 ± 0.01
Phosphate (g/g TS)	1.98 ± 0.12	3.00 ± 0.02	2.10 ± 0.11	2.70 ± 0.10	2.04 ± 0.01	2.10 ± 0.20
Sulphate (g/kg TS)	91.09 ± 1.01	134 ± 2.00	106.00 ± 6.10	107.00 ± 2.02	96.00 ± 2.00	96.74 ± 1.02
Calcium (g/kg TS)	339.31 ± 5.01	80.00 ± 0.10	400.0 ± 2.42	168.00 ± 4.09	309.4 ± 0.42	211.10 ± 2.03
Magnesium (g/kg TS)	61.29 ± 0.11	96.00 ± 0.10	56.00 ± 2.02	82.00 ± 1.40	37.60 ± 0.02	42.20 ± 1.10
Manganese (g/kg TS)	0.14 ± 0.01	1.18 ± 0.22	0.018 ± 0.04	0.024 ± 0.10	0.013 ± 0.01	0.014 ± 0.10
Iron (g/kg TS)	0.68 ± 0.10	1.18 ± 0.11	0.80 ± 0.03	1.14 ± 0.01	0.42 ± 0.01	0.48 ± 0.01
Zinc (g/kg TS)	24.21 ± 0.11	38.00 ± 0.02	26.00 ± 0.03	33.00 ± 0.01	21.50 ± 0.02	25.30 ± 0.01
Aluminium (g/kg TS)	0.38 ± 1.00	0.80 ± 0.11	0.46 ± 0.10	0.62 ± 0.02	0.28 ± 0.12	0.33 ± 0.12
Copper (g/kg TS)	3.21 ± 1.02	4.80 ± 0.10	3.30 ± 0.12	3.90 ± 0.12	2.04 ± 0.10	2.08 ± 0.11

N = 120; COD = Chemical Oxygen Demand; TVFAs = Total volatile fatty acids; C/N = Carbon/Nitrogen ratio.



**Fig. 1.** pH dynamics during the anaerobic digestions.

gas production continued till between the 18th and 24th days when reduction in quantity was observed until the end of the experiments (Fig. 4).

From the experimental set-ups, the most probable actual biogas yields in experiments “X” and “Y” were 0.3554 m<sup>3</sup>/kg TSfed and 0.1803 m<sup>3</sup>/kg TSfed with the desirability of 99 and 100% respectively. Further shown in the table is a 49.2% higher experimental biogas yield in experiment “X” over “Y”. Gas chromatographic analysis revealed the CH<sub>4</sub> and CO<sub>2</sub> content of both experiments to be 65 ± 1.5%; 21 ± 3% and 53.5 ± 2.5%; 26 ± 3% respectively.

### 3.5. Microbial dynamics

The microbiological analyses of the cattle rumen content used as inoculums reveal the presence of aerobic bacteria which include species of *Bacillus*, *Enterococcus* and *Proteus* and *Pseudomonas aeruginosa*. The mean total aerobic plate count (TAPC) was 4.1 × 10<sup>12</sup> CFU/ml. Fungal isolates in the rumen content are *Aspergillus niger*, *Aspergillus flavus*, species of *Mucor*, *Rhizopus* and *Penicillium* with total fungal count (TFC) of 2.0 × 10<sup>10</sup> cfu/ml. The isolated anaerobes include species of *Fusobacterium mortiferum*,

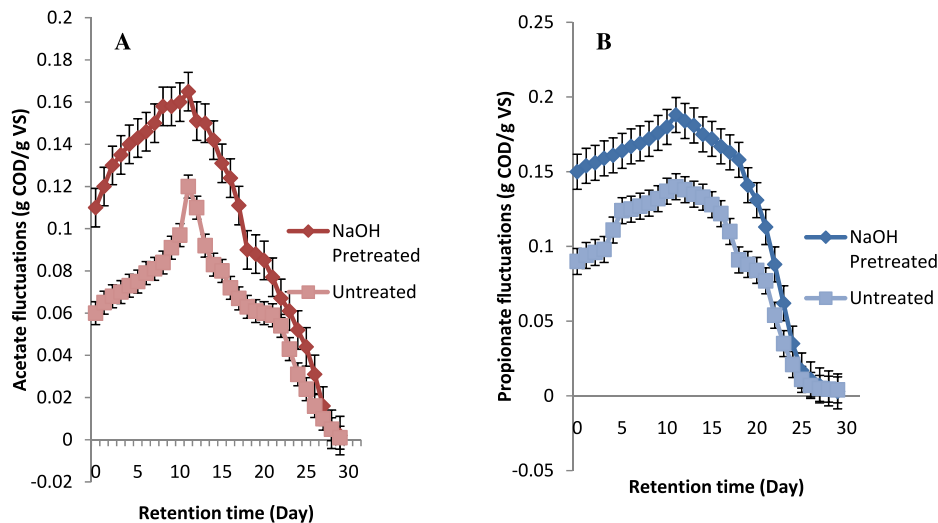


Fig. 2. Fluctuations in concentration of acetate (A) and propionate (B) during the anaerobic digestions.

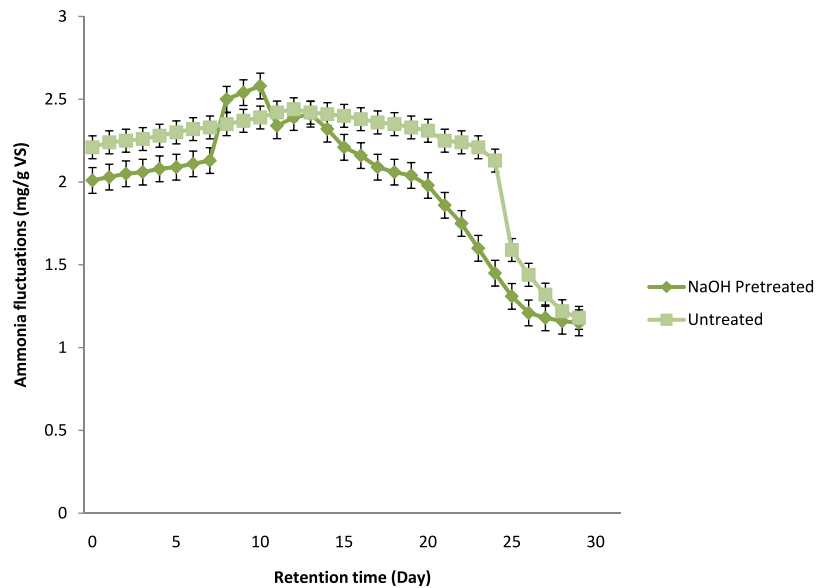


Fig. 3. Fluctuations in concentration of ammonia during the anaerobic digestions.

**Table 3**  
Experimental design of *Chromolaena odorata* shoots pretreatment prior to digestion.

Sample	Pretreatment temperature (°C)	Pretreatment time (Min)	Total biogas produced ( $10^{-4} \text{ m}^3/\text{kg TSfed}$ )
UCO	0	0	1811.23 ± 1.13
CO <sub>70, 70</sub>	70	70	3166.22 ± 2.03
CO <sub>80, 60</sub>	<b>80</b>	<b>60</b>	<b>3565.70</b> ± 1.01
CO <sub>90, 60</sub>	90	70	3122.71 ± 1.10
CO <sub>100, 60</sub>	100	60	3192.21 ± 2.03
CO <sub>110, 60</sub>	110	70	3526.13 ± 1.05
CO <sub>120, 60</sub>	120	60	3232.31 ± 1.03
CO <sub>130, 50</sub>	130	50	2975.51 ± 1.11
CO <sub>140, 70</sub>	140	70	3183.21 ± 1.10
CO <sub>150, 50</sub>	150	50	3123.24 ± 0.31
CO <sub>160, 70</sub>	160	70	1609.21 ± 1.33
CO <sub>170, 50</sub>	170	50	2550.01 ± 1.05
CO <sub>180, 50</sub>	180	50	2219.21 ± 1.12
CO <sub>190, 60</sub>	190	60	2031.40 ± 1.12
CO <sub>200, 50</sub>	200	50	1991.03 ± 2.02

Note: CO = *Chromolaena odorata*; UCO = Untreated *Chromolaena odorata*.

*Bacteroides fragilis*, *Clostridium clostridioforme*, *Clostridium histolytica*, *Clostridium* species, *Gemella morbillorum* and *Porphyromonas assacharolyticum*. The total plate count (TPC) of anaerobes was  $4.7 \times 10^{14}$  cfu/ml. Six different genera of methanogen namely *Methanococcus*, *Methanosarcinales*, *Methanosaeta*, *Methanobacteriales*, *Methanomicrobiales* and *Aminobacteria* spp were identified. The TPC of methanogens was  $5.3 \times 10^{14}$  cfu/ml.

Several aerobic and anaerobes bacteria, fungi and methanogens were implicated at different stages of the digestion in both experiments “X” and “Y”. Aerobes include *Bacillus polymyxa*, *Bacillus megaterium*, *Bacillus circulans*, *Bacillus licheniformis*, *Bacillus stearothermophilus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Proteus mirabilis*. Anaerobes implicated are *Bacteroides fragilis*, *Clostridium clostridioforme*, *Clostridium histolytica* and *Clostridium* spp while species of *Methanococcus*, *Methanosarcinales*, and *Methanosaeta* were identified as the methane producers in both digestions. Fungi of the genera *Aspergillus*, *Mucor*, *Rhizopus*, and *Penicillium* were also identified at the early stages of digestion. The population of aerobes and fungi reduced drastically from the second day of digestion until the end of the second week, the facultative anaerobes reached their

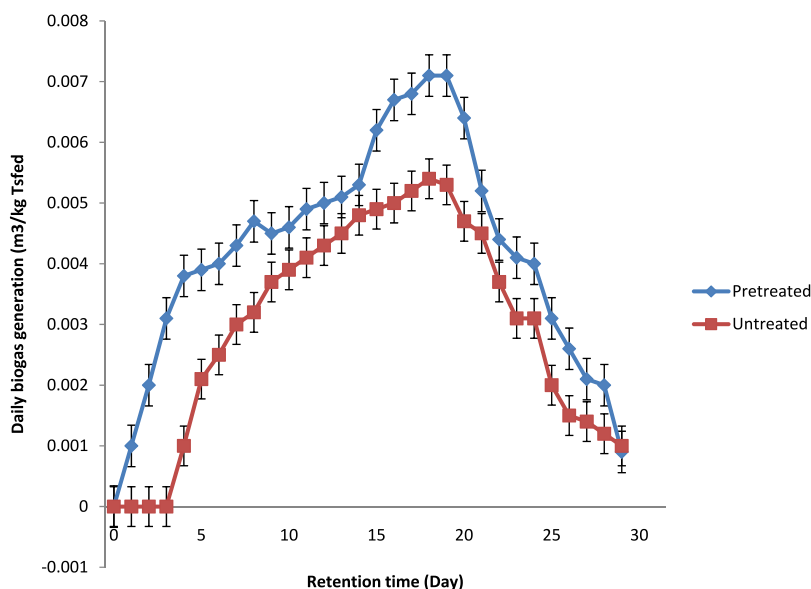


Fig. 4. Average daily biogas generation from the digestions.

Table 4

Stoichiometry and mass balance for one ton of substrate (*Chromolaena odorata*) shoot from the anaerobic digestion experiments.

Parameter	Experiment X	Experiment Y
<b>Input</b>		
<i>C. odorata</i> shoot + Rumen content (kg)	1000	1000
Volatile solids (VS) (kg)	897	980.4
<b>Output</b>		
Methane (CH <sub>4</sub> ) (%)	65	53.5
Carbon dioxide (CO <sub>2</sub> ) (%)	21	26
Digestate (kg VS)	425	699
<b>Cumulative output</b>		
	511	779
*Mass balance	43.03	21
<sup>§</sup> % Volatile solids (VS) consumption	53	29

\* = (Input-output)/input (%) <sup>§</sup> = (Input-Digestate)/Input (%).

highest population by the fourth week before decline set in while methanogens reached their highest population during the last week of digestion when the pH was stable at the alkaline range.

### 3.6. Stoichiometry and mass balance

Table 4 shows the mass balances of both pretreated and untreated *C. odorata* shoot with respect to VS consumption. In this computation, “*C. odorata* shoot” was used as the input variable whereas the trio of “CH<sub>4</sub>”, “CO<sub>2</sub>” and the “anaerobic digestate” were the output variables. In the long run, mass balances of 43.03 and 21 were recorded for experiments X and Y respectively. Also, a 51.17% higher mass balance was reported in experiment X over Y. As per VS degradation/consumption, values of 53 and 29% removal were recorded for both experiments respectively. Here, there was 45.28% higher VS consumption in experiment X over Y.

### 3.7. Process optimization

In the optimization studies for both “X” and “Y”, large F-values were recorded with corresponding low p-values resulting in the significance ( $p < 0.05$ ) of a good number of the model terms. In both studies, the F-values of 2.96 and 3.35 for the models having low p-values of 0.0489 and 0.0331 is an indication of significance. For experiment “X”, the model terms  $Q_4$ ,  $Q_3Q_4$  and  $Q_4^2$  are the

significant ( $p < 0.05$ ) while for “Y”,  $Q_1$ ,  $Q_1Q_2$ ,  $Q_2Q_5$ , and  $Q_4Q_5$  were the significant terms. The “goodness of fit” of the models was checked using the coefficient of determination ( $R^2$ ) and the F-values 0.16 and 3.36 with their corresponding terms of 0.9727 and 0.1591 imply non-significance for both experiments hence the goodness of the fit.

The adequacy of the models in the prediction of biogas form samples of *C. odorata* shoot was determined using the ‘Adequate Precision’ ratio and values of 7.607 and 8.009 were obtained which validated the adequacy of the models. The relationship between all the five separate variables (in coded form) used in the modeling study and that of the biogas yield ( $U$ ) is given in the single regression equation shown as follow:

$$U = 3545.80 - 168.76Q_1 + 158.38Q_2 + 215.05Q_3 + 297.09Q_4 + 12.61 + 110.61Q_1Q_2 + 389.41Q_1Q_3 + 174.68Q_1Q_4 + 233.59Q_1Q_5 - 220.72Q_2Q_3 - 246.93Q_2Q_4 - 207.79Q_2Q_5 + 44.81Q_3Q_4 + 28.35Q_3Q_5 - 143.87Q_4Q_5 + 151.63Q_1^2 + 160.46Q_2^2 + 64.84Q_3^2 + 26.97Q_4^2 - 44.20Q_5^2$$

where  $U$  = Biogas yield (m<sup>3</sup>/kg TSfed).

In solving the above equation, the optimal value for each independent variable used in the modeling and optimization of biogas generation from shoot of *C. odorata* and the obtained values are:  $Q_1 = 37.20$  °C,  $Q_2 = 7.50$ ,  $Q_3 = 27.95$  days,  $Q_4 = 11.97$  g/kg and  $Q_5 = 8.50$  g/kg. Applying these values, the most desired biogas yield predicted by RSM was 0.3565 m<sup>3</sup>/kg TSfed and 0.3555 m<sup>3</sup>/kg TSfed for ANNs in experiment “X” while for experiment “Y”, the RSM predicted yield was 0.1811 m<sup>3</sup>/kg TSfed while that of ANNs was 0.1800 m<sup>3</sup>/kg TSfed. Overall, there was a 49.20% increase in predicted biogas yield in experiment “X” over “Y”. From the validation experiments, the average biogas yield of 3.493 and 1.806m<sup>3</sup>/kg TSfed was obtained from “X” and “Y” respectively.

## 4. Discussion

Application of thermo-alkaline pretreatment brought about the high degradation/solubilization of important structural components of *C. odorata* shoot as seen in this study. The combination of thermal (Heating) and NaOH treatment coupled with the experimentally designed optimal pretreatment conditions enhanced the

solubilization/structural changes observed in the substrate after pretreatment. Prior to this research, several studies have reported the use of thermal and alkaline treatments on different biomasses with the resultant effects of lignin solubilization. In particular, Naran et al. (2016) reported enormous lignin breakdown and subsequent reduction when alkaline-thermal (NaOH) pretreatment was applied. Other researchers: (Monlau et al., 2012; Dahunsi et al., 2016a,b) obtained similar results.

Another effect of the thermo-alkaline pretreatment in this study was the solubilization of cellulose and hemicelluloses and this eventually led to higher biogas yield in the pretreated experiment. These results are similar to those obtained by previous studies that employed various pretreatment methods: thermo-alkaline, alkaline thermo-mechanical, dilute acids, ionic liquids, steam explosion, ammonia fiber expansion, thermal, and the Fenton process (Dahunsi et al., 2016a,b, 2017a,b,c; Espinoza-Acosta et al., 2014; Zou et al., 2016). The result of higher soluble sugar concentration recorded in the thermo-alkaline pretreated experiment can compare favorably with those of Monlau et al. (2012). These soluble sugars play crucial roles during the hydrolysis and majorly the acidogenesis stages of the AD as they enhance pronounced microbial activities, population, and diversity and the resultant release of intermediate products (acids) which are the raw materials for the acetogenesis and methanogenesis stages. Phenol production is another veritable proof of structural breakdown in the pretreated experiment and this is evident in the recorded concentrations. Such a trend has been reported when alkaline pretreatment was applied (Monlau et al., 2012). Considering the C/N ratio of the digested substrates, the thermo-alkaline pretreatments was effective in increasing the ratios the values of 20 and 18 obtained for both experiments were higher than the 17 obtained by Degueurce et al. (2016) in the anaerobic digestion of spent cow beddings.

The pH values reported in this study agree with values obtained in the previous studies (Dahunsi et al., 2016a,b, 2017a,b,c). There is the need to always and constantly maintain a suitable pH in anaerobic systems as this will enhance proper microbial activities and increased gas yield (Zonta et al., 2013; Mao et al., 2015). A pH of between 6.5 and 8.0 has been recommended for efficient anaerobic digestion during which methanogenesis is at its best (Zonta et al., 2013). Earlier research has also reported high microbial diversity and population at alkaline pH (Pang et al., 2008). The temperature of the digesters in both studies was mesophilic. Temperature directly affects the microbial activities in a digester so it is necessary to maintain a stable temperature range for efficient digestion (Jain et al., 2015; Dahunsi et al., 2016a,b, 2017a,b). The mesophilic temperature maintained throughout this research is such that has been reported to encourage better stability of bioconversion as well as higher richness in bacteria population (Kwietniewska and Tys, 2014).

*C. odorata* shoot was revealed to be bulkier than the inoculum especially in terms of solid contents which is likely due to partial digestion of the rumen content in the animal's bowel which must have had reducing the effect on those important parameters. The characteristics of the *C. odorata* shoot are comparable with those of lemongrass earlier utilized in biogas generation (Dahunsi et al., 2016a,b). Also, the high nutrient and elemental composition of the plant could be due to its high capacity for bioaccumulation of nutrients and metals from the rhizosphere (Koutika and Rainey, 2010). The high COD reduction (55%) is well comparable with other reported studies on COD treatment (Dahunsi et al., 2016a,b). The results of the stoichiometry and mass balance carried out indicates better and more pronounced substrate interactions, VS consumption and mass balance in experiment "X" over "Y". This could also be associated with the corresponding pretreatment administered in "Y" prior to digestion.

Elevated/higher nutrient levels were recorded in both digestates obtained in this study than their initial levels prior to digestion and elements that recorded this phenomenon are nitrogen, phosphorus, potassium, magnesium, manganese, iron, zinc, aluminum, and copper. This nutrient and microbiologically rich digestates, therefore, possess great potentials to increase the nutrient status of soil especially the depleted ones when applied as biofertilizers or soil conditioner. The use of such digestate as fertilizer after safety certification to ensure it does not contain harmful substances could also enhance plant growth and general wellbeing most especially in Sub-Saharan Africa where most nations are encumbered with issues of soil nutrient depletion and toxicity to soil microflora via the over-application of chemical fertilizers (Alfa et al., 2014a; Westphal et al., 2016). Total carbon and calcium contents of the digestates were reduced majorly because they were utilized for metabolism and synthesis of the microbial cell wall. The observed COD reduction in both experiments could be due to higher microbial diversity and population as the applied pretreatment procedures made the substrates malleable to quick and efficient microbial/enzymatic breakdown of organic matter.

The Nitrogen levels in the two substrates were low and therefore prevented the common occurrence of ammonia inhibition in anaerobic digestions. Similar results were recently obtained from the anaerobic co-digestion of food waste and spent animal beddings (Zhang et al., 2016; Riggio et al., 2017). Several aerobic and anaerobic organisms were implicated in experiments "X" and "Y". Most of the organisms have their source in the inoculum and were revealed to be similar to those reported in some recent anaerobic mono and co-digestion studies (Alfa et al., 2014b; Dahunsi et al., 2016a,b, 2017a,b,c). The facultative anaerobic microorganisms which were dominated by the genera *Clostridium* have been implicated in past anaerobic digestions where they are known for converting acids to acetone and other products that serve as raw materials for the methanogenesis stage. The high population and diversity of these anaerobes as recorded in this study contributed immensely to biomass degradation and higher biogas yield, especially in the thermo-alkaline pretreated substrate. The reported methanogens are similarly popular in the AD as they convert acetone and other products of acetogenesis to CH<sub>4</sub>, CO<sub>2</sub>, and others. Presence of a rich microbial population and diversity has been reported to enhance substrate degradation and improved biogas yield (Dahunsi et al., 2016a,b, 2017a,b,c).

The concentration of VFAs reported in this study is in agreement with those reported in earlier studies (Zhang et al., 2016; Riggio et al., 2017) and the high microbial diversity/population especially the *Clostridia* group may have contributed to the observed pronounced acetogenesis/methanogenesis stage. This is simply because these bacteria are capable of utilizing amino-acids in order to produce intermediate products like acetate, propionate, and ammonia (Riggio et al., 2017). Ammonia caused an appreciable level of buffering of the process thereby keeping a neutral pH. This further ensured the stability of the digestion and led to high methane yields which were more pronounced in experiment "X". The 49.20% higher gas generation in experiment "X" over "Y" was as a result of higher C/N ratio and the combination of mechanical, thermal and chemical pretreatments before digestion and this has been proposed in several studies especially for lignocellulosic biomass (Dahunsi et al., 2016a,b, 2017a).

The F-values from both experiments with their corresponding p-values coupled with the obtained R<sup>2</sup> values portrays the significance of the regression model. A value of  $\geq 4$  is usually recommended for the 'adequate precision' in order for a model to have a good fitting. The values obtained in the optimization studies show significant fitting and suitability of the models which was further corroborated by all the significant model terms with p-values <0.05. Non-significant lack-of-fit values were obtained



**Table 5**  
Energy and economic evaluation for the digestion of *Chromolaena odorata* shoot.

Energy parameters	Experiment X	Experiment Y
Produced electrical and thermal energy from combined heat and power (CHP)	2755	1248
Produced thermal energy (kWh t <sup>-1</sup> TS)	1887	685
Produced electrical energy (kWh t <sup>-1</sup> TS)	1675	697
<i>Thermal balance</i>		
*Thermal energy gain (kWh t <sup>-1</sup> TS)	1202	–
Thermal energy requirement (kWh t <sup>-1</sup> TS)	812	–
Thermal energy requirement with 80% of heat recovery (kWh t <sup>-1</sup> TS)	241	–
#Net thermal energy (kWh t <sup>-1</sup> TS)	390	–
Net thermal energy with 80% of heat recovery (kWh t <sup>-1</sup> TS)	812	–
<i>Electrical balance</i>		
§Electrical energy gain	978	–
Energy for mixing during pretreatment	–	–
Net electrical energy	978	–
<i>Economic evaluation</i>		
Cost of NaOH (€ t <sup>-1</sup> TS)		

\* = difference of thermal energies produced by the pretreated experiment minus the untreated; # = difference between the thermal energy gain and the thermal energy requirement for the thermo-alkaline pretreatment; § = difference of electricity energies produced by pretreated experiment minus the untreated.

from the optimization studies and this also validated the model's accuracy. The configuration of the entire RSMs 3-D graphs derived from solving the model regression equation reveals moderate relationship among all the variables ( $Q_1$ ,  $Q_2$ ,  $Q_3$ ,  $Q_4$  and  $Q_5$ ) while maximum/ pronounced interactions were shown by the ANNs plots. This shows that the ANNs model gave room for better interactions between the variables than the RSM.

The previous researchers (Dahunsi et al., 2016a,b, 2017a,b,c,d) have reported similar interactions which led to better yield prediction. Further analyses were carried out to estimate the accuracy and predictive abilities of both RSM and ANNs models using applicable parameters such as the mean squared error (RSME),  $R^2$  values and the biogas yield prediction. The RSME obtained for RSM (2.11 and 5.40) were lower than those of ANN (148.15 and 65.22) whereas the  $R^2$  for RSM (0.8680 and 0.8677%) were lower than ANNs' (0.9484 and 0.9562%). In all, RSMs prediction of gas was higher while ANNs' accuracy was higher in terms of  $R^2$  value and lower error values. ANNs is, therefore, a better model for predicting biogas yield and in the modeling of the anaerobic digestion of *C. odorata* is therefore recommended for the optimization and prediction of biogas yield of this substrate.

Equation (1) was used to evaluate the TER as a way of assessing both the energy balance and economic feasibility of applying the thermo-alkaline pretreatment employed in this study. In computing these balances, the combined heat and power (CHP) system with thermal and electrical efficiencies of 50% and 35% was used as shown in Table 5. This system has been employed in a previous research that used thermo-alkaline pretreatment for peanut hull and for the co-digestion of *Tithonia diversifolia* (Mexican Sunflower) shoots and poultry droppings (Dahunsi et al., 2017d). In the bid to recover the investment into thermal energy and NaOH procurement for the pretreatment process, the possibility that the profit obtained from the sale of the extra thermal and electrical energies will be sufficient to cover the cost so as to avoid loss of energy and resources.

The thermal energy required for raising the temperature of 35 g TS L<sup>-1</sup> mixture of *C. odorata* shoot and water from 25 °C to 55 °C was therefore determined using taking the specific heat of water to be 4.18 kJ kg<sup>-1</sup> °C<sup>-1</sup> while heat loss was neglected (Dahunsi et al., 2017b,d). For the thermo-alkaline pretreated experiment, the 1202 kWh t<sup>-1</sup> TS thermal energy gain at 35 g TS L<sup>-1</sup> solid

loading was higher than the 812 kWh t<sup>-1</sup> TS thermal energy required for the pretreatment. This gives a net thermal energy of 390 kWh t<sup>-1</sup> TS which can be increased by the use of heat exchanger during digester heating and/or for pretreatment. Earlier researchers (Zabranska et al., 2006; Dhar et al., 2012) reported that this technology is potent as to boost the thermal energy recovery for up to 80%. Another method of achieving this is via the use of full thermal energy integration which allows for the assessment of the economic feasibility of thermo-alkaline pretreatment application (Perez-Elvira and Fdz-Polanco, 2002; Fdz-Polanco et al., 2008).

In the electrical energy assessment, only the electric energy used during substrate mixing was accounted for while that consumed during mechanical grinding was neglected since this procedure was employed in both the pretreated and untreated experiments. This method had earlier been used (Menardo et al., 2012; Dahunsi et al., 2017b,d). From experiment with thermo-alkaline pretreatment, the net electrical energy obtained at a solid loading of 35 g TS L<sup>-1</sup> was 978 kWh t<sup>-1</sup> TS. The possibility is high that the net thermal and electrical energies obtained in this study can be directly injected into the national or regional energy grid or at best sold for a fixed price in order to incur additional economic benefit. As for NaOH, the United States price of 335 dollars ton<sup>-1</sup> was used.

## 5. Conclusion

Siam weed from this study has been shown to be a veritable candidate for biogas generation. The quantity and quality of produced biogas were higher compared to values obtained for other succulent plants and waste materials in earlier digestion procedures. Judging by the high value of  $R^2$  obtained, both RSM and ANN models demonstrated high efficiency in the biogas production prediction from the mono-digestion of the shoot of *C. odorata*. The major issue with Siam weed is its stubborn nature which has made it a peculiar weed of crop plants across many agricultural ecosystems. However, the high biogas-yielding ability of the plant according to this study suggests that it should not only be considered as a weed but also as an energy crop. Similarly, a positive energy balance was obtained for the thermo-alkaline pretreatment employed which further substantiated the economic feasibility of the pretreatment and biogas generation from the weed. Further utilization of this weed especially in co-digestion with other high energy-yielding substrates is encouraged.

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## Abbreviations

AD, anaerobic digestion; CRD, central composite design; TS, total solids; VFAs, volatile fatty acids; VS, volatile solids.

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