Study of the impacts of supplements on the specific methane production during anaerobic digestion of the West African Gamba and Guinea Grass

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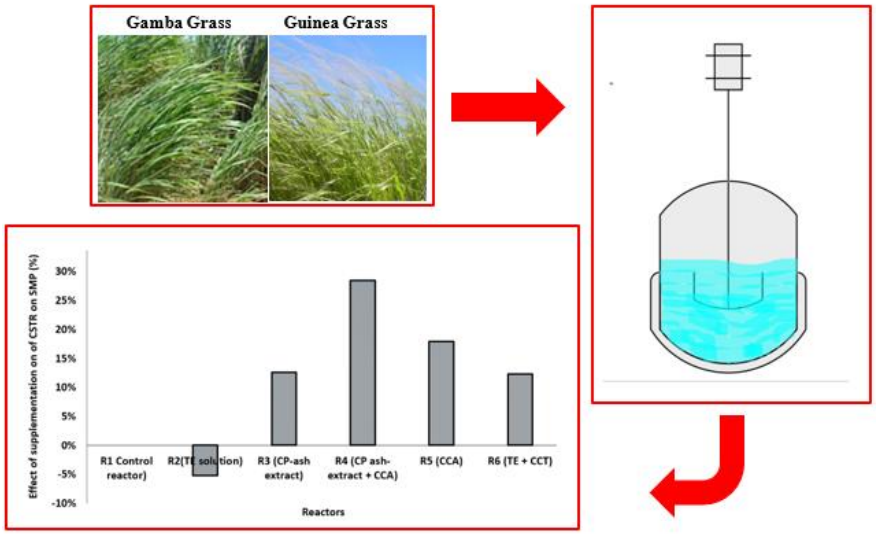
Abstract

Nutrient supplementation could improve the biomethane production of different biomass feedstocks during anaerobic digestion. In this study, the impact of nutrient supplementation on the anaerobic digestion of the West African Gamba and Guinea Grass for biomethane production is presented. This was undertaken in 6 separate continuous stirred tank reactors for a hydraulic residence time of 25 days under supplementation regime with trace elements (TE), cocoa pod (CP) ash-extract, and commercial cellulase from *Aspergillus niger* (CCA) or *Trichoderma reesei* ATCC 26921 (CCT). The results showed that TE inhibits the specific methane production (SMP) with about 5% lower SMP than the control. In contrast, the other supplements namely CP, CP+CCA, CCA and CCT+TE showed about 13, 28, 18 and 12% higher SMP than the control respectively. This study is the first demonstration of the impacts of different supplements on SMP during the anaerobic digestion of the West African Gamba and Guinea grass.

Graphical abstract



Keywords: Anaerobic digestion; Cellulase enzyme; ash-extract; co-supplementation; Gamba grass; Guinea grass; specific methane production

Highlights

Physicochemical characteristics obtained for Gamba and Guinea grass

Anaerobic digestion of Gamba and Guinea grass undertaken under supplementation regimes

Trace element supplements found to inhibit the SMP of the feedstock.

Combined supplements of CP, CCA and CCT found to enhance SMP of the feedstock

List of abbreviations

Abbreviations	Meaning
CSTR	Continuously stirred tank reactor
TE	Trace elements
CP	Cocoa pod ash-extract
CCA	Commercial cellulase from Aspergillus niger
CCT	Trichoderma reesei ATCC 26921
SMP	Specific methane production
R1	Feedstock only
R2	Feedstock + TE
R3	Feedstock + CP ash-extract
R4	Feedstock + CP ash-extract + CCA
R5	Feedstock + CCA
R6	Feedstock + CCT +TE
VFA	Volatile fatty acid
HRT	Hydraulic residence time
P:A ratio	Propionate: acetate ratio
P, N and S	Phosphorus, Nitrogen and Sulphur
Ni, Fe, Mo, W, Co, Se	Nickel, Iron, Molybdenum, tungsten, cobalt and selenium

1 Introduction

1.1 Background and motivation

Global warming has been attributed mostly to anthropogenic CO_2 emission from fossil-fuel energy sources. There is now a growing global commitment such as the 2015 Paris Agreement

(UNFCCC, 2015) to address this problem by diversifying energy sources to carbon neutral sources such as bioenergy. Bioenergy currently accounts for about a tenth of the primary global energy supply with biofuel production expected to rise by around 25% by 2024 (IEA, 2019). This inspired the need to understand and optimise bioenergy production from different sources. Typical sources include energy crops such as the Gamba grass (*Andropogon gayanus*) and Guinea grass (*Panicum maximum*) which have high lignocellulose content. The Gamba and Guinea grass are commonly available especially in the Sub-Saharan region of Africa. They are known to mature rapidly and are tolerant of low fertility and harsh weather conditions (Adedeji and Faluyi, 2006; Bello *et al*., 2016). This makes them a good choice as a feedstock for biomethane production. Like other Lignocellulosic biomass, they are made up of cellulose and hemicellulose tightly bound to lignin and as a result resists degradation (Horan *et al.*, 2018). Through anaerobic digestion (AD), which involves the degradation of organic materials by microorganisms in the absences of oxygen to produce biogas containing methane (CH_4) and carbon dioxide (CO_2), and digestate (Cabbai *et al.*, 2016; Karthikeyan *et al.*, 2016), these substrates could be utilized as a biomass feedstock. However, the use of energy crops alone as AD feedstock has been reported to be prone to instability and process failure due to lack of adequate minerals and nutrients required to enhance the activities of the microorganisms responsible for the degradation process (Wall *et al.*, 2013). Thus, most research on AD tends to focus on co-digestion, which provides the microbes with a spectrum of nutrients, vitamins and trace metals (Cabbai *et al.*, 2016; Nges & Björnsson, 2012; Shah, 2014). Co-digestion also appears to possess a greater potential for improving the settling of floating biomass and production of a high-quality digestate that can serve as a fertilizer. Thus, when using energy crops such as grass silage as a monosubstrate for anaerobic digestion, it is imperative to supplement the digestion process with any limiting nutrient to ensure a stable degradation process which will result in enhanced methane recovery from the biomass feedstock.

1.2 State-of-the-art

Many additives have been used as nutrient supplements during AD processes (Romero-Güiza *et al.* (2016), which include: (i) macronutrients (e.g. P, N and S) and trace elements (e.g. Ni, Fe, Mo, W, Co, Se), (ii) incineration ashes, (iii) compounds that can reduce ammonia inhibition through struvite formation. (iv) bioaugmentation using microbial inoculum with high hydrolytic or methanogenic activity, (v) addition of enzymes as a supplement to enhance the solubilisation of the biomass feedstock. Romero-Güiza *et al.* (2016) showed that the conversion of free ammonia, which is toxic to methanogens, to struvite ($MgNH_4PO_4.6H_2O$) could be achieved by supplementing the reactor with chemicals that have high ion exchange capacity such as bentonite, glauconite, phosphorite and zeolites, clay, and manganese oxides. The formed struvite is valuable as a slow-release fertilizer when applied to soil to steadily provide nutrients and enhance crop yield (Bationo *et al.*, 2011; Federation, 2017; Romero-Güiza *et al.*, 2016). In addition, trace element supplementation such as cobalt, nickel and molybdenum, during AD processes has been reported to enhance biogas and methane production from food waste by 42% at reduced residence time due to the formation of a thicker methanogenic fixed-film (Stronach *et al.*, 2012). Similarly, Cai *et al.* (2017) found that supplementing the AD of rice straw with Fe, Mn, Mo, and Se enhanced the degradation of VFA and methane generation. However, according to the NIIR (2005), only a small amount of trace elements supplement within the range of 10^{-9} mol/L to 10^{-6} mol/L is required during the AD process, because many trace elements are extremely toxic at higher concentrations (usually > 10^{-4} mol/L). The authors (NIIR, 2005) reported that the trace elements are added as soluble inorganic salts since they are taken up by cells as ions or ion chelates, and that chloride and nitrate salts are a more suitable sources of trace nutrients due to their high solubility in water. In addition, NIIR (2005) suggested that the sulphates of zinc (Zn), copper (Cu), and nickel (Ni) may be used, and that iron (III) may precipitate out of acidic media which makes iron (III) citrate preferable.

96 Molybdenum (Mo) supplements can be prepared from salts such as ammonium molybdate
97 $(NH_4)_6Mo_7O_{24}$ or sodium molybdate (Na_2MoO_4) and selenium (Se) can be sourced from
98 sodium selenite ($Na_2O_4.Se$) (NIIR, 2005).

99 Recent studies have also shown that biomass ash can not only be used as a supplement for
100 enhancing AD process but also can be used to remove hydrogen sulphide from biogas in small
101 and medium scale AD plants (Fernandez-Delgado Juarez *et al.*, 2018). Biomass ash is an
102 abundant waste material generated in large quantities in developing countries from the
103 traditional burning of wood as fuel for cooking. The impact of biomass ash due to its alkalis
104 and trace metal contents which are able to leach out from the ash when the digestion process is
105 operating at pH values (6.5 – 8), although it may also increase metal concentrations which may
106 be detrimental to the stability of the AD process (Romero-Güiza *et al.*, 2016).

107 Bioaugmentation involving the supplementation of AD process using specific biological
108 cultures and by-products by adding microbial inoculum with high hydrolytic or methanogenic
109 activity to enhance the digestion process have also been reported (Korres, 2013; Romero-Güiza
110 *et al.*, 2016). Other compounds and processes which have also been used as an additive to
111 improve the methane production from biomass include activated carbon, lactobacillus culture,
112 urea and cobalt-60 radiation (Korres, 2013; Nijaguna, 2006), as well as recycling of digested
113 slurry and filtrate back into the reactor (Korres, 2013).

114 Finally, research has also shown that the addition of enzymes during the anaerobic digestion
115 of lignocellulosic biomass could facilitate lignin degradation, which improves the hydrolysis
116 rate of cellulose and hemicellulose by allowing microbes to gain access to these polymers
117 (Horan *et al.*, 2018). According to Karthikeyan *et al.* (2016), the enzymes that degrade biomass
118 are produced by microorganisms present in the AD digestate. Research by Romano *et al.*
119 (2009) also showed that the treatment of wheat grass with enzymes increased its solubilisation

and enhanced the anaerobic digestion process. Specifically, the degradation process can be enhanced by the addition of a mixture of enzymes, which may comprise cellulase, and hemicellulose, pectin and starch-degrading enzymes (Karthikeyan *et al.*, 2016).

1.3 Aim and novel contributions of this study

The aim of this study is to quantify the impact of adding different supplements, namely trace elements (TE), cocoa pod (CP) ash-extract, and commercial cellulase from *Aspergillus niger* (CCA) or *Trichoderma reesei* ATCC 26921 (CCT), during the anaerobic digestion of the West African Gamba and Guinea grass in terms of the specific methane production. In achieving this aim, this study will deliver the following novel contributions:

a. Physicochemical characterisation of West African Gamba and Guinea grass
b. Anaerobic digestion of Gamba and Guinea grass to obtain biomethane in a lab-scale continuous stirred tank reactor (CSTR). The state-of-the-art review in Section 1.2 showed that there are no studies on the anaerobic digestion of Gamba and Guinea grass.
c. Anaerobic digestion of West and Guinea grass to obtain biomethane in a lab-scale continuous stirred tank reactor (CSTR) under supplementation regime with trace elements (TE), cocoa pod (CP) ash-extract, and commercial cellulase from *Aspergillus niger* (CCA) or *Trichoderma reesei* ATCC 26921 (CCT). Similarly, there is no previous study on the supplementation or co-supplementation of AD of Gamba and Guinea grass with trace elements, enzymes, or biomass ash-extract supplements.

2 Materials and methods

2.1 Materials

2.1.1 Preparation of biomass feedstock and inoculum

Gamba grass and Guinea grass were freshly harvested by cutting from an open grassland at Afikpo Nigeria. Afikpo is located on latitude 5° 53' 33.29" N and longitude 7° 56' 7.22" E (https://latitude.to/map/ng/nigeria/cities/afikpo accessed on 14/12/2019). The harvested feedstock was sun-dried for about 2 weeks and thereafter was cut to about 2 cm size and then ground to a powdered form (< 1 mm) using a food blender. Subsequently, the powdered forms of these grasses were securely sealed in air-tight cellophane bags in which they were conveyed to Newcastle University and then stored in a 4 °C freezer prior to their use. The total solid content (%TS) and volatile solid content (%VS) of each biomass were determined using methods 2540 B and 2540 G, respectively, as outlined in the standard methods for the examination of water and wastewater (APHA., 2005). Also, the cellulose, hemicellulose and lignin contents of each biomass were determined as detailed by Goel (2007) and Sharma (2008). From the powdered grass samples, the AD reactors feedstock was then prepared by mixing equal weights of the powdered Gamba (50%) and Guinea grass (50%) in terms of their TS contents. The inoculum was collected from one of the commercial mesophilic AD plants at the Cockle Park Farm owned by Newcastle University and which is located at Morpeth, Northumberland, North-East England. The physiochemical characteristics of the mixed biomass feedstock and inoculum are presented in Table 1.

159 Table 1 Physiochemical characteristics of biomass feedstocks and inoculum

Parameter	Inoculum	Biomass feedstock		
		Mixture	Gamba	Guinea
Total solids content (as %TS in wet weight)	2%	94%	91%	94%
Volatile solids content (as % VS in TS)	66%	94%	81%	89%
Moisture contents (%)	98%	6%	9%	6%
C/N ratio	n.d	n.d	36:1	36.4:2
ODM (%)	n.d	n.d	93%	96%
Ash (%)	n.d	n.d	10%	9%
NDF (%)	n.d	n.d	73%	70%
ADF (%)	n.d	n.d	54%	53%
ADL (%)	n.d	n.d	10%	10%
Hemicellulose (%)	n.d	n.d	19%	17%
Cellulose (%)	n.d	n.d	44%	43%

160 C/N ratio = carbon-to-nitrogen ratio

161 ODM = Organic dry mass

162 NDF = Neutral detergent fibre

163 ADF = Acid detergent fibre

164 ADL = Acid detergent lignin

165 n.d means not determined

166

2.1.2 Sources and preparation of supplements

The three (3) trace elements (TE), namely: molybdenum (Mb), nickel (Ni) and cobalt (Co), and anhydrous sodium carbonate (Na_2CO_3) used to adjust the pH in the reactors, were purchased from VWR and BDH, United Kingdom and were prepared to achieve the standard concentrations shown in Table 2. The cellulose from *Aspergillus niger* (0.8 U/mg) (CCA) was purchased from Sigma-Aldrich, UK and prepared by dissolving 100 g of the cellulase in 1 L of D.I water. Similarly, Cellulase from *Trichoderma reesei* ATCC 26921 (CCT) (aqueous solution ≥ 700 units/g), also from Sigma-Aldrich, UK was prepared by dissolving 50% of the cellulase solution in 50% of D.I water.

Table 2 Composition of the solution of the trace elements supplement (Co,Ni,Mb)

Reagent	**Chemical formula**	**M (g/mol)**	**TE**	**MTE (g/mol)**	**M:MTE ratio**	**WTE (g)**	**TE conc. (M/ml)**
Cobalt (III) Nitrate hexahydrate (VWR)	$CoN_2O_6.6H_2O$	291.03	Co	58.93	4.94:1	0.05	1.70×10^{-7}
Nickel (II) chloride hexahydrate (BDH)	$NiCl_2.6H_2O$	237.69	Ni	58.69	4.05:1	0.1	1.70×10^{-6}
Sodium molybdate dihydrate (VWR)	$Na_2MoO_4.2H_2O$	241.96	Mo	95.94	2.52:1	0.1	1.04×10^{-6}

M = Molecular mass

TE = Trace element

MTE = Molar mass of trace nutrient

M: MTE = Weight of salt that contains 1 g weight of trace nutrient

WTE = Weight of trace element (g) dissolved in 1 dm^3 D.I water

TE = Amount of trace element (mol) in 1 mL of solution

2.2 Methods

2.2.1 Continuous stirred-tank reactors (CSTR)

The continuous stirred-tank reactors (CSTR) consisted of six Quickfit® borosilicate culture vessels each of 5 litres capacity purchased from Sigma-Aldrich, United Kingdom. These vessels were covered with Quickfit® flat headplate which had parallel centre joints, ST/NS: 19/26, and a 10° side socket joint vacuum adapter with screw-thread (ST) connector for flexible tubing. The headplate seal was made air-tight using a white silicone sealant and a high vacuum grease purchased from VWR UK. Each reactor was also fitted with a 60 cm stainless steel stirring rob with 20 cm stirring bar passing through the center joint of the head plate with a water seal and clamped to a variable speed overhead stirrer engine. Each reactor was fully mixed by setting its own overhead stirrer at the speed of 120 rpm. All the reactors had a working volume of 4.5 L and were feed at an organic loading rate (OLR) of 1.15 gVS/L.d at a hydraulic retention time (HRT) of 25 days for a duration of 74 days. The complete setup for the CSTR is as shown in Figure 1.

Figure 1 CSTR setup with tubing connected to gas sampling bag, a variable speed overhead stirrer motor with stirring rod passing through Quickfit® flat head plate and a parallel centre joint (water seal)

Also fitted on each reactor was a 10° side socket joint vacuum adapter, black insulating mat over heater pad, k-type thermocouple inserted into the reactor using a red coloured rubber bung, and a control box fitted with Sestos temperature controllers. Heating for each reactor was provided using non-adhesive wire wound Silicon heating pad (190 x 415 mm, 230V), with 1M lead purchased from Holroyd Components Ltd United Kingdom. The heating pad was wrapped around the reactors by means of hooks and springs attached to them. A black insulating mat was also used to cover the heating pad to minimize heat loss. Each CSTR was identified based on the type of supplement added to it as detailed in Table 3.

209 Table 3 CSTR names, supplement type, volume and frequency of supplementation

CSTR	Feedstock (Gamba + Guinea grass)	Frequency of supplementation
R1	Inoculum + feedstock	Nil
R2	Inoculum + feedstock + 5 mL Trace Element solution (TE)	Daily
R3	Inoculum + feedstock + 5mL 20% Cocoa pod (CP) ash-extract	Daily
R4	Inoculum + feedstock + 5mL 20% CP ash-extract + 5 mL cellulase from *Aspergillus niger* (CCA)	Daily
R5	Inoculum + feedstock + 5 mL CCA	Daily
R6	Inoculum + feedstock + 3 mL cellulase from *Trichoderma reesei* ATCC 26921 (CCT) + 5 ml TE	Daily

210 The temperature inside each reactor vessel was monitored using a K-thermocouple probe on a
211 Sestos temperature controller inserted into the reactor mixture, which controlled output to the
212 heater pads. The pH in the reactors was also measured daily using a Thermo Scientific™ Orion
213 Star™ A326 pH/Dissolved Oxygen Portable Multiparameter Meter. Physico-chemical
214 parameters, such as: total solids (TS), volatile solids (VS), chemical oxygen demand,
215 ammonium nitrogen (NH_4^+-N), total Kjeldahl nitrogen (TKN), alkalinity and volatile acid
216 concentrations were measured weekly according to methods APHA 2540 B, APHA 5220B
217 open reflux, APHA 4500-NH_4 -N B&C, APHA 4500-N_{org}B, 2320[4] and 5560C respectively, in
218 accordance with the standard methods for the examination of water and wastewater (APHA.,
219 2005). Stability of the process was determined using the propionate-to-acetate ratio as reported
220 by Hill *et al.* (1987). Daily biogas production from each reactor was collected using a 10 L
221 Supel™-Inert Multi-Layer Foil Gas Sampling Bag fitted with a Thermogreen® LB-2 Septa
222 and a Push/Pull Lock Valve (PLV), which was connected to one of the outlets on the Quickfit®
223 reactor's head plate. The methane content (%) in the biogas was measured using a Carlo Erba
224 HRGC 5160 gas chromatograph equipped a flame ionization detector, an electron capture
225 (ECD) detector, and an on-column MFC injector with a split/splitless controller as described
226 by (Edward *et al.*, 2015).

2.2.2 Feeding conditions

Daily feeding of all the reactors were maintained at an organic loading rate (OLR) of 1.15 gVS/L.d from day 1 – 25, 26 – 50 and 51 – 74 which correspond to the 1st, 2nd and 3rd HRT cycles, respectively. However, daily feeding was suspended from day 60 – 74 when the CSTR were opened which exposed the reactors' contents to room air for about 5 hours to fix one of the CSTR that broke down. However, supplementation with CP ash extract continued from day 63 – 74.

2.3 Data analysis

The data obtained from the current study were analyzed using the statistical packages SPSS version 17.0, Microsoft Excel 2016. All the analyses were based on a 5% statistical significance level for all parameters tested and results are presented within ± 2 S.D. The correlation and regression analysis, analysis of variance and paired samples T-tests (2- tailed) were also used to determine the statistical significance of the differences between the mean values of the results obtained from different experiments carried out.

3 Results and Discussion

3.1 Volatile solids (VS) destruction

The mean volatile solids (VS) destruction achieved in reactors, R1, 2, 3, 4, 5 and 6 from day 1 – 74 were 64, 55, 59, 65, 65 and 67%, respectively (Figure 2). However, during the 2nd HRT cycle (day 26 – 50), the mean VS destruction achieved in reactors, R1, 2, 3, 4, 5 and 6 were 65, 65, 61, 59, 61 and 67%, respectively. A paired sample t-test showed that the differences between the VS destruction in the control CSTR (R1) and each of the supplemented CSTR

(R2, 3, 4, 5 and 6) at 95% confidence interval of the differences, showed that the differences in the VS destruction were not statistically significant ($p > 0.05$).

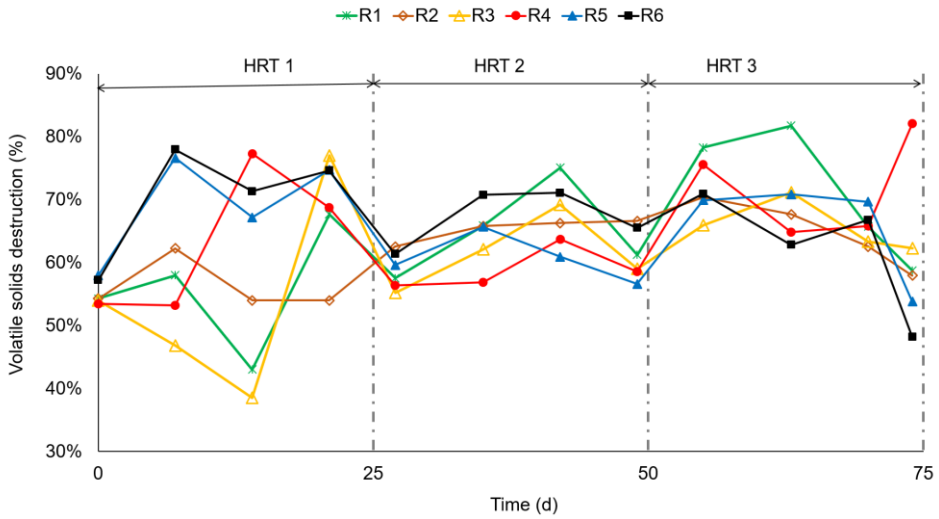


Figure 2 Volatile solids destruction in the CSTR. R1 (control), R2 (supplemented with trace elements (TE), R3 (supplemented with CP ash-extract), R4 (co-supplemented with cocoa pod (CP) ash extract and Cellulase from Aspergillus Enzyme (CCA), R5 (supplemented with Cellulase from *Aspergillus niger*) and R6 (co-supplemented with trace elements (TE) and Cellulase from *Trichoderma reesei* ATCC 26921) (CCT)

The results in Figure 2 suggest that neither the cocoa extract, enzymes, trace elements nor a combination of any two types of supplement increased VS destruction. These results are consistent with previous studies. For example, Lue-Hing (1998) reported that the addition of enzymes to AD digesters did not give improvement in the VS destruction, and Horan *et al.* (2018) reported that even though enzymatic pretreatment was found to promote hydrolysis of lignocellulosic biomass, it did not result in any significant increase in VS destruction compared to the untreated reactors. Unfortunately, the determination of VS does not account for volatile substances such as VFA, alcohols, esters, etc which could represent a considerable portion of the VS destruction or energy potential of the feedstock.

3.2 Effect of bicarbonate addition on pH of reactors

From day 1-74, the pH inside the CSTR varied as follows: R1 (6.32 - 7.70), R2 (6.54 - 7.70), R3 (6.54 - 7.70), R4 (6.55 - 7.70), R5 (6.31 - 7.70) and R6 (6.10 - 7.70), which correspond to mean pH value of 6.93, 6.94, 7.01, 7.00, 6.94 and 6.96, respectively (Figure 3). However, during the 2nd HRT cycle (day 26 – 50), during which the reactors attained a pseudo-steady-state condition of operation, CSTR R1, R2, R3, R4, R5 and R6 had mean pH values of 6.80, 6.84, 6.91, 6.90, 6.84 and 6.87, respectively. These mean pH values recorded in all the CSTR during the pseudo-steady-state period, were all within the pH range 6.8 – 7.4, which is regarded as the optimum range for anaerobic digestion (Grady Jr, 2011; Khanal, 2011; Nijaguna, 2006).

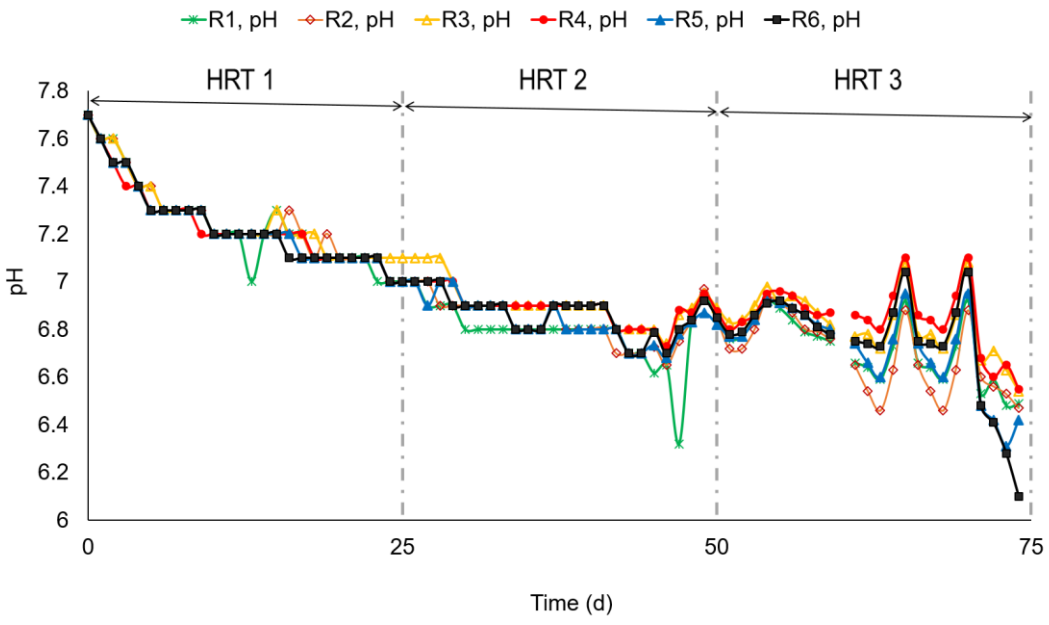


Figure 3 pH profile in R1, R2, R3, R4, R5 and R6.

The pH of all the reactors was regulated by dosing 1g of anhydrous sodium carbonate (Na_2CO_3) on day 43, 45, 47, 48, 52, 53, 54, 63, 64, 68 and 69, and that enabled the reactors to maintain the optimum pH (Figure 3), except for day 63 when 2g of the salt was added due to a decrease in the pH of all the reactor to pH < 6.8. On day 64, it was observed that the 2g of Na_2CO_3 added to the reactors on day 63, produced a negative pressure inside all the reactors which drained

281 the water in the shaft water-seal, thereby exposing the reactors' headspace to ambient air. This
282 pressure reduction inside the reactors probably occurred due to the rapid reaction of Na_2CO_3
283 with biogas CO_2, leading to removal of CO_2 as shown in 1.

$$Na_2CO_3 + H_2O + CO_2 \rightarrow 2NaHCO_3 \qquad 1$$

284 Oxygen from the air is toxic to methanogens (Wang *et al.*, 2010), and thus, its entrance into
285 the AD vessel headspace on day 63 led to a substantial decrease in the volume of biogas and
286 methane produced in each reactor (Figure), and also led to a subsequent accumulation of VFA
287 (Figure) and reduction in pH (Figure 3), implying that oxygen from air reached concentrations
288 within the digestate liquor that exerted toxic effects on the methanogens. Gerardi (2003), had
289 also reported that the development of low partial pressure conditions under the digester dome
290 due to the addition of Na_2CO_3 or calcium hydroxide during the AD process may cause the
291 collapse of the digester cover. This problem associated with the use of Na_2CO_3 as a source of
292 alkalinity in AD reactors and explains why sodium bicarbonate ($NaHCO_3$) is mostly preferred
293 as a buffering reagent for pH control (Grady Jr, 2011).

294 Although a comparison between the pH in the control reactor (R1) and each of R2, 3, 4, 5 and
295 6 showed that they were strongly and positively correlated (R^2-values > 0.7) in all cases, the
296 paired sample t-test (2-tailed) comparison between the pH in reactor, R1and R2, R1 and R3,
297 R1 and R4, R1 and R5 and R1 and R6, showed that the pH of the control reactor was
298 statistically significantly different from the pH of each of the supplemented reactors ($p < 0.05$).
299 This shows that pH inside the reactors was affected by supplementation and because pH is
300 closely related to the toxicity of many compounds in AD due to its ability to control the
301 movement of undissociated weak acids and bases which can penetrate the cell membrane of
302 microbes (de Jong & van Ommen, 2014). Thus, at low pH, VFA diffuses into the cell of AD
303 microbes and dissociate in their cytoplasm, causing an imbalance in the cellular homeostasis

(de Jong & van Ommen, 2014), and this could explain why all the CSTR (R1 – R6) started to fail around day 65 due to VFA accumulation (Figure).

3.3 Ammonia-N, Total Kjeldahl Nitrogen, Organic nitrogen and COD

The concentration of organic-N in CSTR R1, R2, R3, R4, R5 and R6 decreased from 577.5 – 175, 577.5 -157, 577.5 – 175, 577.5 – 176.4, 588.0 – 217.0 and 577.5 – 175.0 mg/L, respectively (Figure). The results indicate that during the AD process, a substantial part of the organic-N was converted to ammonium-N, especially during the pseudo-steady-state operation period (day 26 – 50) and beyond, and that may have been favoured by the prevailing mean pH-values which were effectively below pH 7 (Figure 3) in all the reactors. Similarly, from day 1 – 74, the concentration of ammonium-N decreased in CSTR R1, R2, R3, R4, R5 and R6, from 1400 – 39.2, 1400 – 84, 1400 – 39.2, 1400 – 61.6, 1400 – 63 and 1400 – 50.4 mg/L, respectively. This large net change in the ammonia-N concentration suggests that the amount of nitrogen available in the reactors was insufficient to meet the biosynthetic needs of the new biomass, and this could explain why free ammonia inhibition was not detected in the current study.

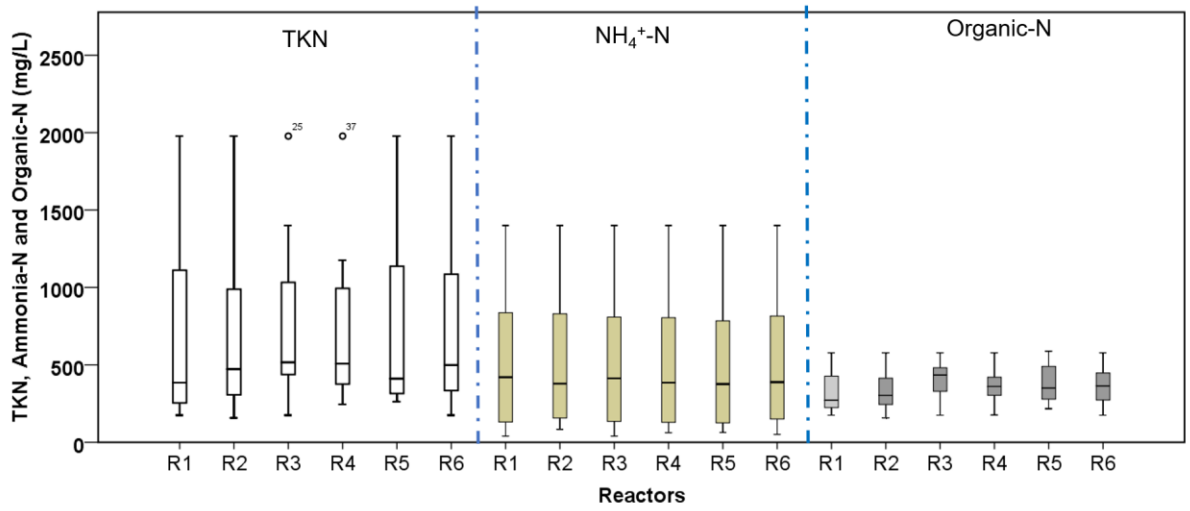


Figure 4 Ammonia-N, Total Kjeldahl Nitrogen, and Organic nitrogen. R1, R2, R3, R4, R5 and R6.

Correspondingly, Figure shows that the concentration of the TKN in CSTR R1, R2, R3, R4, R5 and R6 decreased from 1977.5 -175, 1977.5 – 157.5, 1977.5 – 175, 1977 – 245, 1977.5 – 262.5 and 1977.5 – 175 mg/L, respectively, due to the changes in ammonium-N and organic-N in the reactors. According to Wellinger (2013), the determination of the total Kjeldahl nitrogen (TKN) equates closely to the nitrogen concentration in an AD reactor, since nitrate and nitrite are very low, and reveals whether the available nitrogen is sufficient to support the growth of the AD microbes.

3.4 Volatile fatty acids

3.4.1 Total volatile fatty acids (TVFA) from day 1 - 74

The concentration of total volatile fatty acids in all reactors from day 1 – 74 are shown in Figure . According to Khanal (2011), the level of VFA indicates the health of an AD process, and for a healthy reactor, the VFA concentration in the low range of 50 – 250 mg HAc/L. Thus, in the current study, it can be concluded that the CSTR 1 – 6 were healthy from day 1 – 60 (Figure), although the VFA concentration during this period may have been influenced by the Na_2CO_3 supplement.

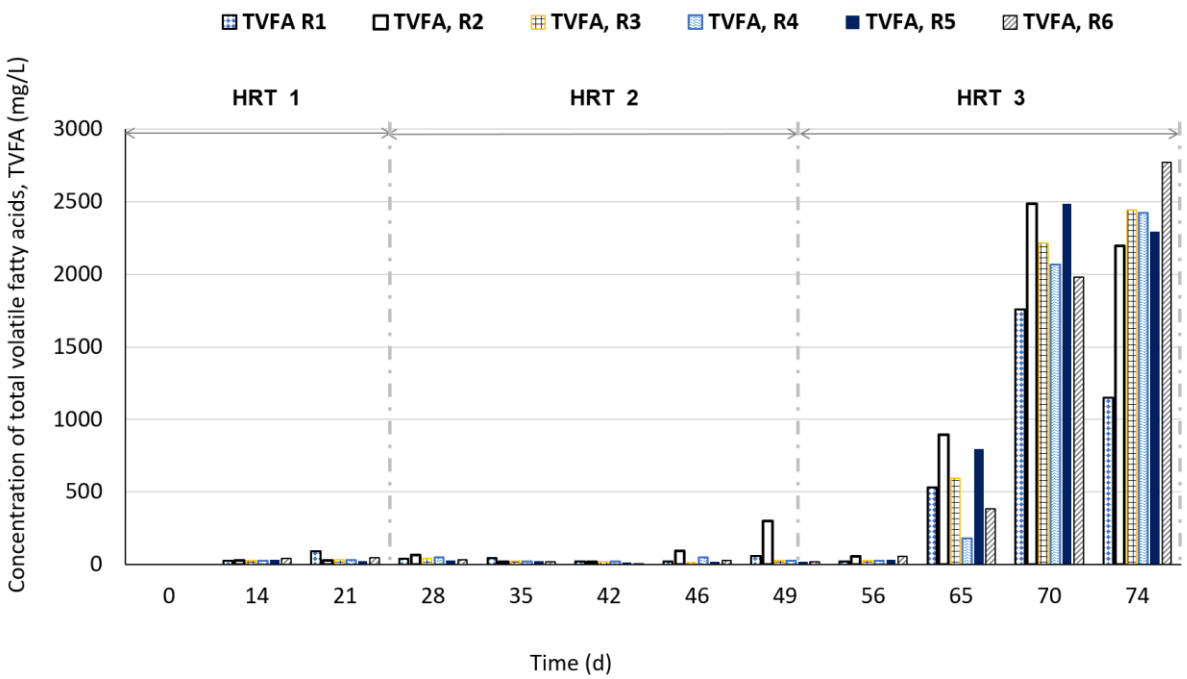


Figure 5 Concentration of total volatile fatty acids in all reactors from day 1 – 74. Details of the reactors classification and supplementation are shown in Table 3.

However, from day 60 – 75, unstable conditions caused by opening of all the reactors, caused both overloading and the exposure of the reactors to toxic compounds (oxygen), and the accumulation of VFA (Figure 5), which caused the pH to gradually decrease from the desired pH 7 to pH <6 within this period (Figure 3), resulting to failure of all the reactors and substantial decrease in the methane production (Figure). The VFA accumulation during this period suggests that opening the reactors may have caused a severe imbalance between the activities of the acidogenic and methanogenic microbes. It also depicts that the activities of the acetoclastic methanogens may have been hindered, resulting to further decrease in pH. According to de Jong and van Ommen (2014), increase in VFA concentration and lower pH increases the concentration of undissociated acetic acid, amplifies the toxic effects of the VFA on the AD system.

3.4.2 Checks for instability of the AD process using propionate-to-acetate ratio

The results from the stability checks using the P/A ratios measured from the total VFA concentrations from the individual reactors, R1-6 from day 1 - 74 are presented in Table .

Table 4 Checks for instability using the propionate to acetate (P/A) ratio

Hydraulic retention time (HRT)	Day	R1 P/A	R2 P/A	R3 P/A	R4 P/A	R5 P/A	R6 P/A
1st HRT	0	n.d	n.d	n.d	n.d	n.d	n.d
	14	n.d	n.d	n.d	1/2.1	1/1.6	0/0
	21	1/4.3	1/3.7	1/3.4	n.d	n.d	1/2.8
2nd HRT	28	n.d	n.d	n.d	1/6.6	0/0	1/4.9
	35	n.d	n.d	n.d	n.d	1/2.4	n.d
	42	n.d	n.d	n.d	n.d	1/1.2	n.d
	46	1/0.4	1/0.1	1/1.5	1/0.2	1/0.6	n.d
	49	1/1.4	1/0.1	1/2.0	1/5.5	n.d	n.d
3rd HRT	56	1/4.0	1/0.3	1/0.8	1/2.9	1/3.9	1/2.9
	65	1/4.2	1/4.5	1/0.7	1/1.5	1/4.4	1/0.2
	70	1/5.9	1/4.7	1/1.8	1/1.6	1/6.8	1/1.7
	74	1/1.8	1/1.6	1/3.6	1/1.6	1/1.8	1/3.8

n.d stands for not detected

According to Hill *et al.* (1987), P: A ratio of 1:1.4 or propionate concentration above 800 mg/ L indicate impending digester failure. During the 2nd HRT (day 26 – 50), when all CSTR attained pseudo-steady-state condition, the ratio P/A < 1:1.4 was prevalent in all the CSTR, R1 – R6, showing a good balance between propionate production and acetate utilization, except

for the periods where supplementation of Na_2CO_3 introduced partial pressures leading entrance of air and sudden VFA building up as shown in Table and Figure . Thus, the results in Table confirms that all the reactors were relatively stable within this 2nd HRT period, compared to 1st HRT (especially on day 21) and 3rd HRT (day 51 – 74) periods where the ratios P/A>1:1.4 were prevalent in all the CSTR (Table).

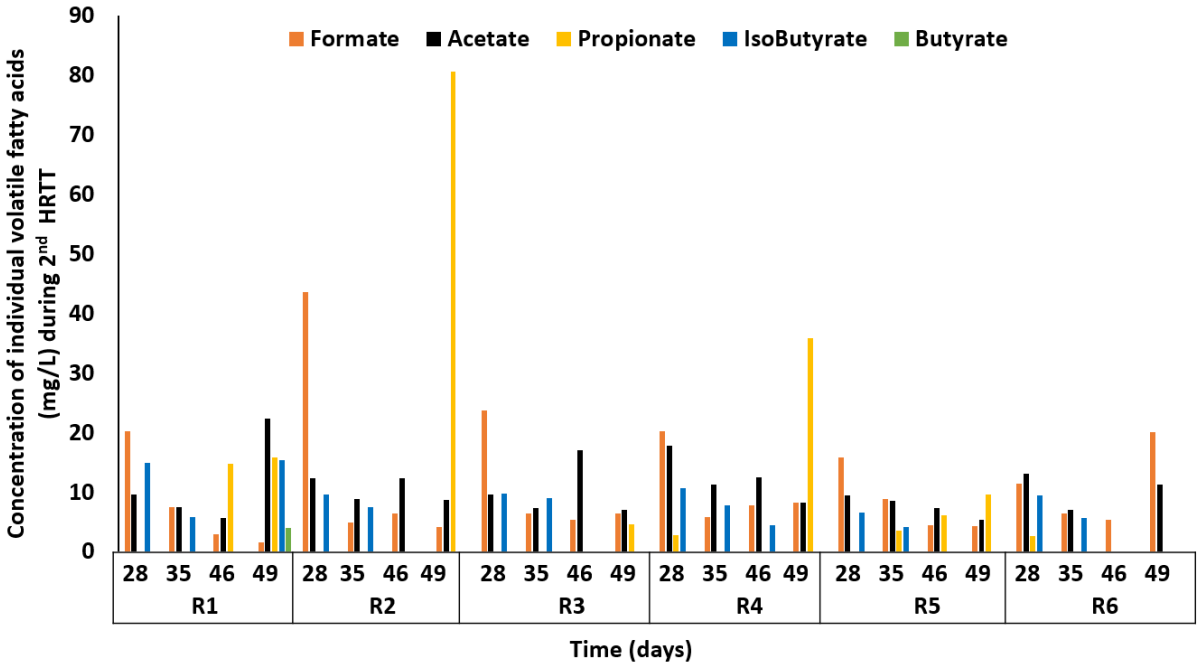


Figure 6 Concentration of individual volatile fatty acids in all the reactors from day 28 – 49 (within 2nd HRT cycle).

The concentration of the individual VFA in all the reactors during the 2nd HRT cycle (Figure), also indicates that during the pseudo-state condition of operation, their concentrations were effectively lower than 50 mg/L, except for day 49 where the concentration of propionate in reactor, R2 was 80 mg/L. These results could explain why all the CSTR were able to achieve stable operational conditions, and equally agree with previous studies which reported that concentration of total TVFA in AD reactors operating normally should be below 200 mg/L (Andreoli, 2007). However, from day 50 – 75, a P/A ratio greater than 1:1.4 dominated the

reactors, which lead to a decrease in pH (Figure 3) due to the presence of high concentration of VFA. The situation led to a daily decrease in biogas and methane production in all the reactors (Figure 7) and total failure of all reactors, possibly due to the entrance of air (oxygen) into the reactors as previously discussed in Section 3.2.

3.5 Methane production

3.5.1 Methane content (%) in biogas

The methane contents in the biogas from each of the CSTR, including the mean, minimum, and maximum values measured from day 1 – 74 and day 26 – 50 are shown in Table 1.

Table 5 Methane content (%) in biogas

Reactors	HRT (1 – 3) (Day 1 – 74)			HRT 2 (Day 25 - 50)		
	Mean	Min.	Max.	Mean	Min	Max
R1	59.2%	22.4%	67.8%	60.0%	56.0%	64.0%
R2	59.9%	21.4%	67.5%	61.0%	57.0%	66.0%
R3	59.9%	22.4%	65.9%	61.0%	56.0%	65.0%
R4	59.0%	20.4%	65.3%	60.0%	56.0%	64.0%
R5	58.5%	22.4%	65.6%	60.0%	55.0%	66.0%
R6	60.8%	19.1%	69.0%	62.0%	57.0%	66.0%

From the data presented in Table 5, the methane contents recorded from day 1 – 74 during the current study from CSTR (R1 – 6) were all within the ranges of methane contents typical of CSTR published from various studies (Cabbai *et al.*, 2016; Chan *et al.*, 2018; Wang *et al.*, 2010; Gerardi, 2003). The paired sample tests between the methane content in control reactor, R1 and R2, R1 and R3, R1 and R5, and R1 and R6 gave a p-value <0.05 which shows that supplementation significantly affected the percentage of methane contained in the biogas produced in each of the supplemented reactors. In contrast, a p-value > 0.05 was obtained in

the case of the comparison between control, R1 and R4, which means that that the percentage of methane produced in R4 was comparable to the methane produced in the control reactor, R1. Similarly, when the reactors attained a pseudo-steady-state condition during their operation which corresponds to Day 26 – 50 (HRT 2), the mean concentration of methane, as well as the minimum and the maximum methane contents (Table 1). Also, a paired samples t-test between the control (R1) and each of the supplemented reactors, showed that for Pair 1 (R1 and R2), Pair 2 (R1 and R3), Pair 3 (R1 and R6), gave a value of $p < 0.05$ (2 -tailed) which shows that the addition of TE to R2, CP ash-extract to R3, CP ash extract + CCA to R4 and CCA to R5, affected the SMP of each reactor significantly.

3.5.2 Specific methane production and volumetric methane production (day 1 – 74).

The SMP from the CSTR R1, R2, R3, R4, R5 and R6 from day 1 – 74 were 169.3, 157.4, 179.9, 204.3, 189.8 and 175.0 N $mLCH_4$/kgVS fed. Similarly, the VMP obtained from R1, R2, R3, R4, R5 and R6 were 211.7, 196.8, 224.9, 255.4, 237.2 and 218.8 N $mLCH_4$/L.d fed. Thus, except for reactor R2 which produced SMP that was 7% less than the SMP from the control reactor, R1, the SMP from R3, R4, R5 and R6 were 13, 28, 18 and 12% higher than that of the control reactor (R1). Similarly, the paired samples t-test between the control (R1) and each of the supplemented reactors, showed that for Pair 1 (R1 and R2), Pair 2 (R1 and R3), Pair 3 (R1 and R4), and Pair 4 (R1 and R5), the value of $p < 0.05$ (2 -tailed) which indicates that the supplementation of R2 with TE, R3 with CP ash-extract, co-supplementation of R4 with CP Ash-extract + CCA, supplementation of R5 with CCA and co-supplementation of R6 with CCT and TE increased the SMP and VMP of each reactor significantly.

3.5.3 Specific methane production and volumetric methane production (day 26 – 50)

The mean SMP in CSTR, R1, R2, R3, R4, R5 and R6 were 171.7, 162.7, 193.4, 220.6, 202.5 and 192.9 N $mLCH_4$/gVS.d, respectively. These results show that the SMP in the reactor, R2

was 5% less than the volume of SMP from the control reactor, R1. In contrast, the SMP in CSTR, R3, R4, R5 and R6 were 11, 22, 15 and 11%, respectively, higher than that of the control reactor, R1. Similarly, the VMP obtained from CSTR, R1, R2, R3, R4, R5 and R6 were 214.6, 203.4, 241.7, 275.7, 253.1, and 241.1 N mLCH_4/L.d. Similarly, the VMP in CSTR, R3, R4, R5 and R6 were 11, 22, 15 and 11%, respectively, higher than that of the control reactor, R1. These results demonstrate the supplementation of reactor R2 with trace elements solution, led to inhibition of the AD process, which supports the study by Cai *et al.* (2017) which reported that the addition of Co and Ni did not improve reactor performance during an AD process involving the mono-digestion of rice straw. However, their study revealed that a combination of Fe, Mn, Mo and Se enhanced methane production. Similarly, in an extensive review on the impacts of trace element supplementation on the performance of anaerobic digestion process, Choong *et al.* (2016), highlighted that trace element (Fe, Ni, Co) supplementation improves AD performance, especially when added at sub-optimal dosages. Similarly, Pobeheim *et al.* (2010) reported that supplementation using 0.6 mg/L Ni and 0.1 mg/L Co to the anaerobic digestion of maize silage improved the methane yield by 25% and 10%, respectively. Cai *et al.* (2018) also reported that when 0.01 mg/L Mo, 0.1 mg/L Se and 0.1 mg/L Mn were added to their AD of rice straw in the first 10 days, that the methane yield increased by 59.3%, 47.1% and 48.9%, respectively. The inhibition experienced in reactor R2, which was supplemented with trace elements solutions (Table 3) may have resulted due to the accumulation of some of trace elements in higher concentration, especially Ni was previously found be Jiang *et al.* (2017) to exhibit inhibitory effects on VFA degradation due to its toxic nature to methanogens at high concentration. When Nges and Björnsson (2012) encountered a similar inhibitory situation during their study on the anaerobic digestion of crop mixtures, they suggested that the trace elements Fe, Co, Mo and Ni, which they used in their study may have been chelated by phytic acid thereby preventing their absorption due to poor bioavailability. Their inference agrees with

the results obtained in the current study and Ortner *et al.* (2014) has also confirmed that between 30 – 70% of the trace elements added to an AD process is not directly bioavailable.

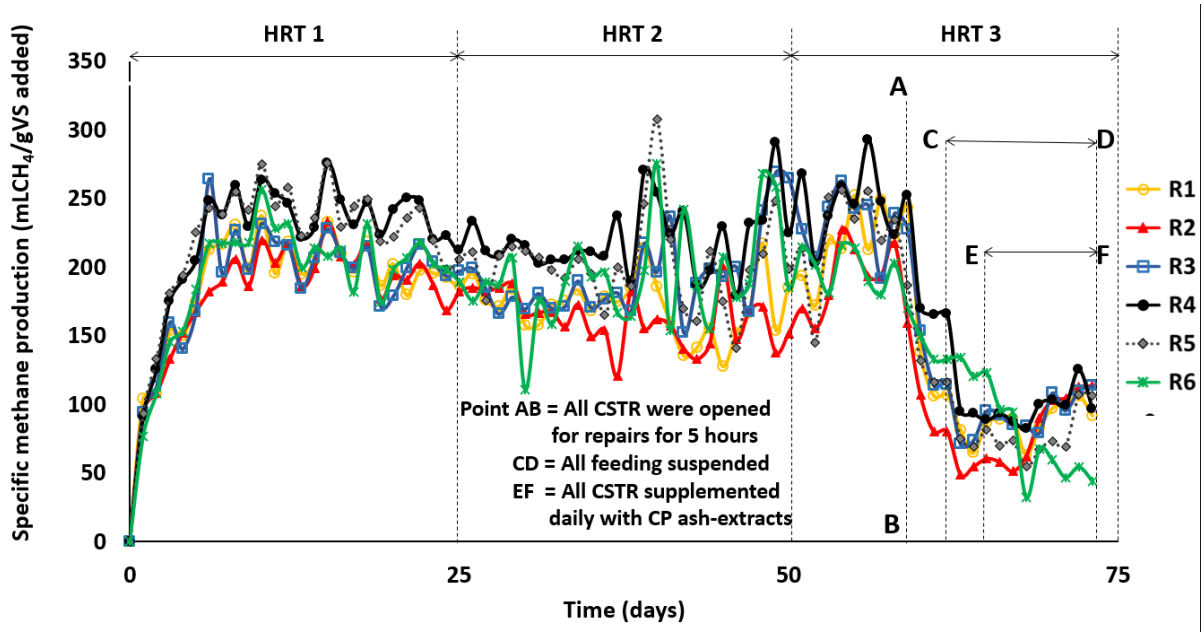
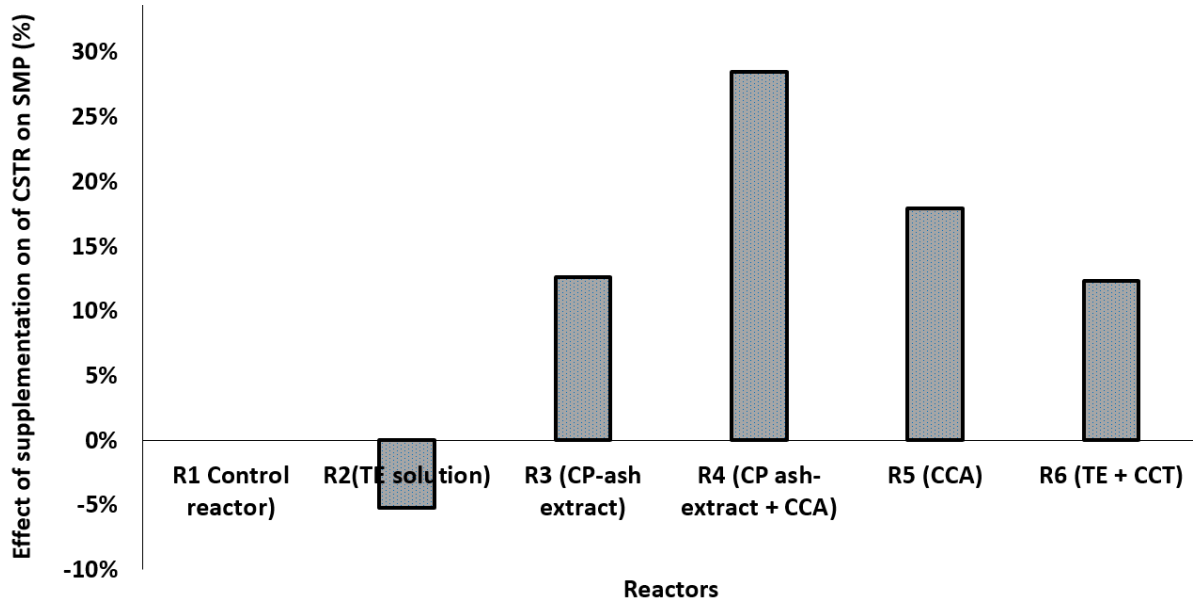


Figure 7 Specific methane production (SMP), volumetric methane production (VMP) and cumulative methane production in R1 (control), R2 (TE), R3 (CP ash-extract, R4 (5mL 20% CP ash-extract + CCP), R5 (CCA) and R6 (CCT + TE) from day 1 – 74.

During the pseudo-steady-state operation (HRT 2), the correlation between the SMP produced in R1 and R2, R1 and R3, R1 and R4, R1 and R5, and R1 and R6 had a coefficient of determination, R^2 = 0.23, 0.24, 0.22, 0.41 and 0.19 respectively, which indicates that the correlation between two different reactors conditions was positive but weak. A paired sample t-test (2-tailed) statistical comparison of either the SMP between R1 and R3, R1 and R4, R1 and R5, and R1 and R6 gave a p-value < 0.05. In contrast, the same t-test comparison between the SMP of R1 and R2, gave a p-value > 0.05. These results show that apart from the CSTR, R2 which encountered inhibition as a result of TE supplement, that the addition of other supplements or a mixture of supplements (Table 3), significantly enhanced the SMP from the biomass feedstock used in the current study. Considering the R^2-values from the statistical correlation tests, the results clearly showed that neither a comparison between reactors R1 and

459 R3, nor R2 and R3 were correlated in terms of SMP. In contrast, a comparison between the
460 SMP in R3 and R4 or R4 and R6 showed a positive but weak correlation. Comparison of the
461 paired reactors conditions indicates that supplementation affected the SMP from each CSTR
462 significantly (p-value < 0.05). Thus, supplementation of the reactors, R2, R3, R4, R5 and R6
463 increased SMP by -5%, 13%, 28%, 18% and 12%, respectively, compared to the control
464 reactor, R1. The effects of supplementation on SMP of paired reactors conditions (Table 3) are
465 presented in Figure 8.



466

467 Figure 8 Effects of supplementation on SMP of paired supplemented reactors conditions. The
468 reactors conditions are defined in Figure 2.

469 The comparison between the supplemented reactors only (Figure 8) also showed that the SMP
470 from R3 was higher than that of R2 by 16%. Equally, the SMP from R4 was 12% higher that
471 the SMP from R3, while the SMP from R4 was also 9% higher than that of R5. Similarly, the
472 SMP from R6 was 16% higher than the SMP of R2. These results indicate that the cellulase
473 from *Aspergillus niger* (CCA), cellulase from *Trichoderma reesei* (CCT) and cocoa pod ash-
474 extracts enhanced the SMP in all reactors containing these supplements, however, they produce

greater enhancement of SMP when added as combined supplements. As the enhancement appeared to be greater than the sum of the individual supplements, it appeared that CP ash-extracts increased the effect of cellulase from *Aspergillus niger*, and this may explain why reactor R4 achieved the highest SMP during pseudo-steady-state operation. In a study of the effects of 25 types of commercial enzymes on AD process it was found that enzymatic pre-treatment produced only minimal effects on the biogas yield from sludge and manure, and only about 10% for grass silage and concluded that the enzyme were probably degraded by microbes native in the AD reactor (Karthikeyan *et al.*, 2016). Thus, the results from the current study strongly suggest that CP ash-extracts may have increased the activity of CCT and could explain why reactor R4 achieved the highest SMP during the pseudo-steady-state condition between day 26 – 50 (Figure and Section 3.5.3), compared to other reactors.

On day 63, the biogas and methane production (SMP) in all the CSTR (R1-6) decreased from the mean values presented in Section 3.5.3 to mean SMP of 81.5, 48.8, 71.1, 94.8, 75.4 and 134.3 N mLCH_4/gVS.d, as a result of opening the reactors on day 60 (see Section 2.2.2 and Figure). Due to the failing situation of the reactors, feeding was suspended, and immediately followed up with daily supplementation of CP-ash extract to all the reactors from day 64 – 74 (Section 2.2.2). Interestingly, except for reactor R6, all other reactors, R1-5, started to recover progressively (Figure), and on day 74, the mean SMP had increased for these reactors to 91.5, 113.7, 113.4, 96.9 and 106.6 N mLCH_4/gVS.d, respectively. In contrast, reactor R6 (which had trace nutrient + cellulase from *Aspergillus niger*) did not recover (Figure). Also, due to CP ash-extract supplementation from day 70 – 74, the P/A ratio in reactors, R1, R2 and R5 decreased substantially (Table), which suggests an increase in the acetate utilization by the methanogens, as the decrease corresponded with increase in methane production in the reactors (Figure). In contrast, the P/A ratios increased in reactors, R3 and R6, but remained at the same

499 value in the reactor, R4 (Table). These results suggest that supplementation of ash-extracts
500 from cocoa pod can also help enhance the recovery of failed CSTR over time.

501 # 4 Conclusions

502 A comprehensive experimental investigation of the supplementation of the AD of mixed West
503 African Gamba and Guinea grass with different materials, namely TE solution, CP ash-extract,
504 CP ash-extract + CCA, CCA, CCT, CCT + TE was conducted. In this study, the results showed
505 that supplementation with TE solution (Ni, Co, Mo) leads to an inhibition of the AD process
506 resulting in a decrease in the specific methane production (SMP) from the supplemented reactor
507 by -5% compared to the control, indicating that the trace element solution used in this study
508 does not improve SMP. However, the co-supplementation with TE and CCT led to a
509 statistically significant increase in SMP (12%) compared to the control. The addition of CP
510 ash-extract supplement alone gave a statistically significant increase in methane production
511 (13%) compared to the control reactor, indicating that the natural trace nutrients and alkalinity
512 of the CP ash-extract had a positive effect on the AD process. This was considered to have
513 been due to pH stability provided by the alkaline characteristics of the CP-ash extract, and the
514 wide range of bioavailable trace elements present in the ash-extract that were less toxic than
515 the TE solution. Finally, co-supplementation with CP ash-extract solution and CCA resulted in
516 a statistically significant increase in SMP (28%) compared to supplementation with CP ash-
517 extract alone and CCA alone. This strongly suggests that CP ash-extracts increased the activity
518 of CCA especially during the pseudo-steady-state conditions.

519

520 **Acknowledgement**

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