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Improved biofertilizer properties of digestate from codigestion of brewer's spent grain and palm oil mill effluent by manure supplementation

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Abstract

The biofertilizer quality of anaerobically codigested agroindustrial residues from brewer's spent grain and palm oil mill effluent was evaluated after supplementation with different livestock manure in order to validate its use as organic fertilizer. Manure supplementation assay was performed using different animal manure-inoculum to demonstrate the influence of inoculum-type on the nutrient status, plant growth promoting bacteria (PGPB) and other plant growth promoting attributes of the resultant digestate. In addition to elevated nutrient levels ($K > P > Ca > Mg > S > N$), the plethora of essential microbial groups (phosphate solubilizers > diazotrophs > auxin producers) that enhance nutrition and promote plant growth was evinced in the supplemented digestate compared to the control. On the other hand, environmental risk assessment revealed a notable yet inadequate reduction in indicator bacteria and putative pathogens ($> 3.0 \log \text{CFU mL}^{-1}$) with potentially toxic elements within publicly available requirements. The preponderance of PGPB with excellent biofertilizing attributes observed in this study could be leveraged upon by plants thus substantiating its potential for use as organic fertilizer. However, the presence of pathogens highlights the importance of post-treatment hygienization to eliminate its biosafety risk.

Keywords: PGPB, Manure supplementation, Biofertilizer, Environmental risk, Digestate

Introduction

Nigeria like many other developing countries generates humongous amounts of untreated wastes from several agricultural and industrial operations, including brewer's spent grain (BSG) and palm oil mill effluent (POME) [1–3]. The breakdown of these organic waste streams has resulted in widespread contamination and deterioration of the ecosphere. However, with the burgeoning population and increasing demand for energy and sustainable environment, interest in waste valorization (treatment/stabilization) with simultaneous generation of renewable energy continues to intensify. Anaerobic digestion (AD), a

widely used technology in many countries presents a suitable option for the production of biogas from organic wastes [4, 5]. It involves a controlled biological process whereby biomass (including varying types of organic materials, wastewater treatment sludge, catering and food processing wastes, energy crops, livestock manure and biodegradable plant residues) is transformed by the activity of certain bacteria in an anaerobic environment at suitable temperatures into a desired methane rich biogas, yielding a plant nutrient rich residue (digestate) as by-product [6]. The transformation processes vary between 15 and 30 d at mesophilic temperatures (35–42 °C) and 10–20 d at thermophilic temperatures (45–60 °C). However, some materials require longer times to be sufficiently degraded thus their hydraulic retention time (HRT) which is the average process time of the ingestate in the

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bioreactor, may be as long as 60–80 d after which most or all of the energy content of the biomass is digested with diminishing biogas production, yielding the resultant residue termed digestate [7].

Besides temperature and HRT, other operating conditions such as the digester (bioreactor) design, feedstock concentration, moisture content, nutrient content, C/N ratio and pH can equally affect the AD process [8]. Since the AD process is very complex involving different groups of microorganisms with various environmental requirements, any adjustment in one or more of these operating conditions will influence the growth and performance of the microorganisms and hence the yield and quality of the biogas and digestate [9]. Certain waste/residues with complementary properties can be codigested anaerobically to achieve elevated biogas yields [10–12]. The biogas generated from AD processes has been utilized as a source of renewable energy in many developed countries with potentials for heating, electricity and vehicle fuel. This is as a result of the high greenhouse gas emissions and other environmental impacts associated with the utilization of fossil fuels [13, 14].

Besides biogas, the digestate has been investigated [15–17] and identified with soil fertilizing properties, improving soil respiration. Qi et al. [18] reported that the fertilizer properties of mesophilic and thermophilic anaerobic digestates contain varying degree of nutrients and plant growth promoting bacteria (PGPB), while Alfa et al. [15] reported that the use of digestate from AD is already a practice that has led to improved soil management and less toxic chemical consumption in cropping systems. The digestate which is the remains of the AD process is mostly a semi-solid, fibrous solid and liquid mass containing both organic and inorganic matter. The microbial communities digest most of the organic matter during the AD process [9], converting them into inorganic compounds. Also, Qi et al. [18] reported that the inorganic nutrients in the digestate are present in plant-utilizable forms at a markedly higher level compared to the feedstock, due to the mineralization of organic matter found in the feedstock during AD. For example, organic nitrogen in the feedstock is converted to bioavailable nutrients (ammonium-N and nitrates) and is beneficial if the digestate is intended for use as biofertilizer. Certain bacteria under anaerobic conditions have been identified with the ability to not only convert organic N in feedstock to inorganic plant-utilizable forms. Qi et al. [18] evaluated the plant growth-promoting properties of bacterial species and these bacteria were proposed to occupy the rhizosphere of many plant species exerting beneficial effects on plant growth through direct and/or indirect mechanisms. These mechanisms include solubilization of immobilized phosphate in mineral ions into plant-utilizable forms, production of siderophores (iron-chelating agents) which can solubilize

iron from minerals or organic compounds under iron-limiting conditions to make iron accessible to plants, synthesis of phytohormones like indole acetic acid for enhanced cell division and root development in plants as well as production of antibiotics and enzymes that combat/suppress phytopathogens.

The recent elevated global application of inorganic fertilizers on soil, coupled with the pressurizing need for an increase in food production to meet the demand of the increasing world population has played a role in addressing notable problems including extreme global soil quality decline. Other concerns arise from the limited bioavailability of nutrients [9] and the relatively high cost of purchase of inorganic fertilizers. These problems urgently suggest the compelling need for the use of organic amendment material (digestate) as a sustainable alternative. The biofertilizer property of any digestate is the usefulness of such digestate in promoting plant growth [15, 19]. The microorganisms in the digestate may be the normal flora of the original feedstock for the AD process and/or an inoculation of allochthonous microorganisms from anaerobic sludge to augment the microbial constituent and activity as well as improve the performance of the AD process. Different studies have recorded the use of diverse livestock manure as feedstock for AD and production of biofertilizers [9, 15, 18]. Several authors [20–22] have also reported the enhancement of biomethane production through bioaugmentation using hydrolytic and acid-degrading bacteria from different sources (peat, soil, anaerobic sludge) but no literature exists on the enhancement of biofertilizer quality of digestate by inoculum addition (through supplementation with microbial community such as those present in livestock manure). The digestate however is not innocuous as it contains heavy metals as well as pathogens (including antibiotic-resistant bacteria) that may be inimical to soil organisms, plants and humans at large [18, 23–25]. This study aimed at evaluating the relative effects of supplementation with different livestock manure on the biofertilizer quality of the resultant digestate obtained from anaerobic codigestion of BSG and POME by focusing on the plant growth promoting attributes such as phosphate solubilization, nitrogen fixation, auxin (indole-3-acetic acid, IAA) production, bioload of PGPB and nutrient profile of the digestate. In addition, the quantities of potentially toxic elements (PTEs) and indicator bacterial loads of the whole digestate were also assessed to establish its suitability for use as soil conditioner and organic fertilizer.

Materials and methods

Batch AD

The AD was performed in a lab-scale batch system using amber borosilicate glass serum bottles (100 mL capacity)

(Wheaton 223,766, USA) and 20 mm aluminium crimp seal with PTFE/Butyl septa for headspace vial (Wheaton W224224, USA) as reactors [12]. The experimental design of Cater et al. [20] was adopted for the anaerobic codigestion and manure supplementation assay. BSG and POME used as feedstock and co-substrate were obtained from Champions Brewery Plc and an open pond at Domita Farms, respectively, in Uyo, Nigeria. Livestock manure (cow dung, swine slurry and poultry droppings) used as inoculum was collected from Domita Farms, Uyo, Nigeria. The substrate/inoculum mixture characteristics prior to feeding the reactors are presented in Table 1. The digesters were fed with 55 mL POME + 10 g BSG + 5 g livestock manure except for the control (without livestock manure) and placed in a water bath at mesophilic temperature (40 °C) and reaction time of 30 d. Briefly, there were four (4) reactors (A–D) with the following composition: Reactor A (10 g BSG + 55 mL POME + 5 g cow dung); Reactor B (10 g BSG + 55 mL POME + 5 g pig Slurry); Reactor C (10 g BSG + 55 mL POME + 5 g poultry dropping); Reactor D (10 g BSG + 55 mL POME) – control. Digestates were simultaneously discharged from the digesters following exhaustion of biogas production after 30 d and analysed for the presence of potential PGPB, indicator bacteria and potential pathogens, soil macro- and micronutrients (N, P, K, Mg, and Ca) as well as heavy metals (Mn, Zn, Cu, Pb and Ni).

Microbiological analyses

Isolation of potential PGPB and evaluation of biofertilizing characteristics of the digestate

The potential PGPB were isolated and enumerated on selected media. They were characterized and identified following standard procedures described by Holt et al. [26]. These bacteria were also tested for phosphate solubilizing activity, diazotrophic nitrogen-fixing efficiency and IAA production to determine the biofertilizing potential of the anaerobic digestate.

Phosphate solubilizing potential of bacterial isolates

To enumerate the total phosphate solubilizing bacteria (PSB), 1 mL each of appropriate serially diluted sample

was seeded into sterile petri dishes and about 20 mL of Pikovskaya’s medium was pour-plated in replicate under aseptic condition following previously reported methods [27]. The plates were incubated at 28 ± 2 °C for 7 d. The representative colonies were enumerated and reported as colony forming unit (CFU) by multiplying with the reciprocal of the dilution factor. Bacterial growth was observed as the qualitative evidence of phosphate utilization.

For phosphate solubilisation efficiency, National Botanical Research Institute’s phosphate growth medium was prepared by adding 10 g glucose, 5 g of insoluble Ca₃(PO₄)₂ as a source of phosphate, 5 g MgCl₂, 0.25 g MgSO₄, 0.2 g KCl, 0.1 g (NH₄)₂SO₄ and 15 g agar in 1 L of water [9]. To each plate, a loopful of the test isolate was spot-inoculated unto the surface of the agar plate. The plates were incubated for 7 d at 37 °C before observation for a visible halozone formation around the inoculated colony. The ability of the microbial isolate to solubilize insoluble phosphate was expressed by its solubilisation index using Eq. (1) [9].

$$\text{Solubilization index (SI)} = \frac{\text{Colony diameter} + \text{Halozone diameter}}{\text{Colony diameter}} \tag{1}$$

Isolation of nitrogen-fixing bacteria from digestate

Digestate samples were serially diluted for the enumeration of diazotrophic bacteria. In sterile petri plates, 1 mL of appropriate dilution was pour-plated using Burk’s nitrogen free medium [9]. The medium contained in a litre; 0.2 g MgSO₄, 0.80 g K₂HPO₄, 0.2 g KHPO₄, 0.13 g CaSO₄, 0.00145 g FeCl₃, 0.000253 g Na₂MoO₄, 20 g sucrose and 15 g agar. Plates were incubated at 30 °C for 7 d. The potential to fix atmospheric nitrogen was indicated by the ability to grow after incubation.

Nitrogen fixation efficiency was determined using Jensen’s nitrogen deficient medium [C₁₂H₂₂O₁₁ (20 g), K₂HPO₄ (1 g), MgSO₄·7H₂O (0.5 g), FeSO₄·H₂O (0.1 g), NaCl (0.5 g), Na₂MoO₄ (0.005 g), CaCO₃ (2.0 g), agar

Table 1 Physicochemical characteristics of experimental mixtures

Bioreactor	Initial (feed stock)			Final (digestate)			Cumulative biogas yield (mL g ⁻¹ VS)	Methane yield (mol %)
	TS (%)	VS (%)	pH	TS (%)	VS (%)	pH		
A	25.4 ± 0.15 ^d	8.1 ± 0.10 ^d	7.3 ± 0.10 ^b	9.6 ± 0.10 ^a	3.8 ± 0.21 ^a	5.6 ± 0.06 ^b	310	89.9
B	21.6 ± 1.00 ^b	7.3 ± 0.10 ^b	6.9 ± 0.10 ^a	13.5 ± 0.10 ^c	4.6 ± 0.10 ^b	5.3 ± 0.10 ^a	194	75.7
C	23.4 ± 0.10 ^c	7.9 ± 0.10 ^c	7.4 ± 0.10 ^b	11.2 ± 0.10 ^b	3.8 ± 0.10 ^a	5.4 ± 0.10 ^a	241	81.1
D (control)	19.0 ± 1.00 ^a	6.9 ± 0.10 ^a	6.8 ± 0.10 ^a	17.1 ± 0.10 ^b	6.6 ± 0.10 ^c	5.2 ± 0.10 ^a	41	59.3
BSG alone	19.0 ± 0.08	14.2 ± 0.03	7.1 ± 0.01	15.3 ± 0.11	9.5 ± 0.17	6.1 ± 1.00	35	44.4
POME alone	15.9 ± 0.15	4.9 ± 0.01	5.6 ± 0.10	13.9 ± 0.06	4.0 ± 0.09	5.0 ± 0.12	27	37.5

Similar superscript letters mean not significantly different in mean (p > 0.05), while different superscript letters mean significantly different (p < 0.05); A–D indicate bioreactors with different waste composition (n = 3)

(15.0 g) in 1 L]. The isolated colonies were streaked on the nitrogen free agar as described by Vimal et al. [28] and incubated for 48 h ($28 \pm 2^\circ\text{C}$). Bacterial growth was observed as the qualitative evidence of N_2 -fixation. Too dark?

Indole-3-acetic acid (IAA) production assay

Spot test for auxin (IAA) production was performed with a little modification in the methods of Khamna et al. and Hamza et al. [29, 30]. Pure isolates were cultivated on Tryptone Soya Agar medium (Oxoid, UK) supplemented with $\text{C}_6\text{H}_{12}\text{O}_6$ (10 g), K_2HPO_4 (0.5 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g), NaCl (0.1 g) and yeast extract (1.0 g) in 1 L volume. Following incubation for 48 h at $28 \pm 2^\circ\text{C}$, emerging colonies were smeared on filter paper saturated with Salkowski's reagent (0.5 M $\text{FeCl}_3 + 40\%$ H_2SO_4). Production of IAA was denoted by the appearance of pink colour. In addition, the plates were observed for visible halozones around the colonies for determination of IAA index using Eq. (2) [31].

$$\text{IAA production index} = \frac{\text{Colony diameter} + \text{Cavity diameter}}{\text{Cavity diameter}} \quad (2)$$

Isolation of indicator bacteria and potential pathogens

The standard plate count method was performed to quantify indicator bacteria and potential pathogens in the digestates. Samples from digesters were serially diluted in phosphate buffered saline ($\text{pH} = 7.4$) to isolate and enumerate the predominant indicator bacteria and selected potential pathogenic species. Sampling was conducted at the initial time (t_0) and final time (t_f) in all the digesters. The samples were handled under aseptic conditions in a biosafety cabinet. Following a ten-fold serial dilution, 1 mL of aliquot from 10^{-5} dilution were poured in triplicates on some selective media (Mannitol Salt agar, Thiosulphate Citrate Bile Salt Sucrose agar, Salmonella Shigella agar, MacConkey agar, Eosin Methylene Blue agar and Schaedler agar) for the enumeration, isolation, characterization and identification of *Staphylococcus*, *Vibrio*, *Salmonella* species, total coliforms, coliforms and anaerobes respectively. All media used in this study were products of Oxoid, UK. The plates were incubated aerobically at $37 \pm 2^\circ\text{C}$ according to manufacturer's specifications and observed after 24 h except for the Schaedler agar plates. For the estimation and isolation of total anaerobic bacteria, Schaedler agar plates were incubated anaerobically using the gaspak anaerobic system with resazurin indicator strip (pink) which turns colourless upon oxygen elimination to ensure the integrity of the anaerobiosis. All isolates were identified based on their morphological and biochemical characteristics

[26, 32]. Identified species were preserved at -4°C by freezing pure cultures in sterilized skimmed milk with glycerol (10%) for further analyses.

Analytical methods

The light (Ca, Mg, Na, K) and heavy (Pb, Cd, Cu, Zn, Ni, Cr, Hg) metals contents in the analyzed wastes and resultant digestate were determined using microwave plasma-atomic emission spectrometer according to the guidelines of Standard Method 3120 [33]. Buchi Kjeldahl apparatus was used for measuring Total Kjeldahl nitrogen according to European standard protocol of Association of Official Analytical Chemists while total phosphate was determined following the methods of Katakai et al. [34]. The total solids (TS) and volatile solids (VS) of substrate and digestates were determined following standard procedures described by the Standard Methods [33] while pH was measured using a portable pH meter.

Statistical analyses

All experiments were performed in triplicates. The digestion process performance data of each reactor were expressed as the mean \pm standard deviation of the samples during the period of operation. An analysis of variance by SPSS (Statistical Package for the Social Science, version 20.0) was employed in this study to test the significance of the results, and $p \leq 0.05$ was considered statistically significant. In addition, coefficient of variation, correlation analysis and comparative description of physicochemical data were carried out using SPSS statistical software at 95% level of confidence for each test.

Results and discussion

Effect of manure supplementation on AD performance

The cumulative biochemical methane potential and process performance indicators (pH, TS and VS removal) were used to establish the effect of manure supplementation on the performance of the AD process. Some specific characteristics of individual BSG and POME as well as the AD process performance data are presented in Table 1. From the study, addition of livestock manure resulted in extensive loss in TS and VS contents of the waste mixtures. Following manure seeding, the initial TS and VS contents in waste mixture for reactor A was 25.4 and 8.1%, 21.6 and 7.3% for reactor B, 23.4 and 7.9% for reactor C and 19.0 and 6.9% for reactor D (control), respectively. The TS content of manure supplemented reactors was higher than that of the control. However, these TS and VS concentrations reduced drastically ($p < 0.05$) at the end of the experiment to 9.6 and 3.8% (reactor A), 13.5 and 4.6% (reactor B), 11.2 and 3.8% (reactor C) and 17.1 and 6.6% (reactor D – control), respectively. All manure supplemented reactors gave a higher TS and VS removal efficiency

indicating the degradative capacity of microbial consortia in manure-based samples in utilizing the organic fraction of the waste. Recently, other authors have reported similar reduction in TS and VS contents during AD [11, 14]. The pH value of the bioreactors ranged between 6.8 to 7.4 at the beginning and 5.2 to 5.7 at the end of the digestion process. This reduction in pH supports previous findings and may be a consequence of the accumulation of short chain fatty acids which is inimical to methane production [13]. The cumulative biogas and methane yield from reactors A to D of the experimental mixtures were 310 mL g⁻¹ VS and 89.9 mol%, 194 mL g⁻¹ VS and 75.7 mol%, 241 mL g⁻¹ VS and 81.1 mol%, 41 mL g⁻¹ VS and 59.3 mol%, respectively (Table 1). There was a statistically significant increase ($p < 0.05$) in cumulative biogas and methane yield between the control (non-supplemented) and the supplemented reactors which may have been a function of improved methanogenesis in inoculum supplemented reactors. This increase strongly correlated ($r = 0.96$) with the amount of total anaerobic bacteria in the feedstock. Relative to other inocula, significant biogas and methane yield were obtained with cow dung manure addition. The results (Table 1) reveal that for the initial (feedstock), the level of TS and VS in bioreactor A was significantly higher than that obtained for bioreactor B, C and D ($p < 0.05$) while TS and pH in the control were significantly less than that of other groups ($p < 0.05$). For pH, there was no significant difference between that of bioreactor D and B while A and C were significantly higher than that of other reactors ($p < 0.05$). Also, there was significant difference ($p < 0.05$) between the initial and final TS, initial and final VS, and initial and final pH across all experimental sets. Similarly, Cater et al. [21] recorded significantly elevated biogas and methane production in biogas reactors through manure supplementation. Generally, codigestion improved pH, TS, and VS characteristics with proportionate increase in biogas and biomethane production when compared to single substrate (mono) digestion of either BSG or POME (Table 1). Statistical differences between the results were confirmed using one-way analysis of variance.

Influence of manure supplementation on PGPB populations in anaerobic digestate and their biofertilizing attributes

Anaerobic digestates were estimated for the presence of cultivable PGPB populations. A comprehensive investigation of the biofertilizing abilities and plant growth promoting characteristics (Table 2) of the estimable indigenous bacterial populations from anaerobic digesters were also carried out. The concentration of PGPB in digestate samples are shown in Fig. 1. From the result, the total PSB (ranging from 1.6–2.5 log CFU

mL⁻¹) was significantly higher than the nitrogen fixing bacteria (NFB, 0.5–1.4 log CFU mL⁻¹) across all bioreactors. Supplementation through the addition of livestock manure (as inoculum) significantly increased the populations of these PGPB groups ($p < 0.05$) which differed significantly across all the reactors compared to the control in the following order of abundance: reactor C > reactor A > reactor B > reactor D (control). The plant growth promoting characteristics of the bacterial isolates are presented in Table 2. The digestate was observed to be a repository of PGPB with remarkable biofertilizing traits as the density of these microbial groups are essential for plant growth. Manure supplementation improved the richness of these microbial groups (Fig. 1) which was a desirable characteristic in the digestate. This assertion supports previous findings by other authors [9, 15, 19, 33, 35]. Apart from the (control reactor) lacking livestock manure amendment, the elevated levels and abundance of PGPB groups (PSB > NFB) in the anaerobic digestate under evaluation was different across the bioreactors in the sequence; reactor A > reactor C > reactor B > reactor D (control) with a strong positive relationship between NFB and PSB ($r = 0.955$) indicating the consequence of supplementation or bioaugmentation using different livestock manure inocula. The significant increase in the populations of PGPB groups can be attributed to inoculum addition which served as a source of these beneficial bacteria while supplying additional nutrients for their proliferation. It may be said that the biofertilizing quality of anaerobic digestate is a function of the populations of PGPB as well as the nutrient concentration in such digestate [19]. PGPB can encourage plant growth with either of the following mechanisms: bioprotection (suppress plant disease), biofertilization (improve nutrient availability and acquisition) and bio-stimulation (produce phytohormones) [36].

Effect of AD on microbial load profile of feedstock and digestate

The results of the microbial load profile of the feedstock and digestate is presented in Fig. 2. It is noteworthy that *Vibrio* species were completely eliminated at the termination of the AD process. Since all bioreactors were maintained at the same temperature (40 °C) for 30 d and manure addition was the only changing factor, the subsequent decline in initial concentration of mesophilic bacteria during AD may be a function of nutrient (substrate) limitation and reactor (feedstock/inoculum) composition rather than the direct effect of temperature and reaction time [38]. Therefore, supplementation with manure had significant influence on the reduction ratios of the studied microbial groups.

At the end of digestion, a general decrease in microbial levels was observed for all bacterial groups across all the

Table 2 Plant growth promoting properties of bacterial isolates from mesophilic digestate

Isolate code	Nitrogen fixation	Phosphate solubilization	IAA production	Identified bacteria
A1	+	+	-	<i>Clostridium</i> sp
A2	-	+	+	<i>Bacillus</i> sp
A3	-	+	+	<i>Staphylococcus</i> sp
A4	+	+	-	<i>Bacillus</i> sp
A5	-	+	+	<i>Lactobacillus</i> sp
A6	-	+	+	<i>Salmonella</i> sp
A7	+	+	+	<i>Enterobacter</i> sp
A8	-	+	+	<i>Citrobacter</i> sp
B1	+	+	+	<i>Clostridium</i> sp
B2	-	+	+	<i>Staphylococcus</i> sp
B3	+	-	-	<i>Salmonella</i> sp
B4	-	+	+	<i>Pseudomonas</i> sp
B5	-	+	+	<i>Enterobacter</i> sp
B6	+	-	+	<i>Bacillus</i> sp
C1	-	+	+	<i>Lactobacillus</i> sp
C2	-	+	+	<i>Micrococcus</i> sp
C3	+	-	+	<i>Bacillus</i> sp
C4	+	+	-	<i>Enterobacter</i> sp
C5	+	+	+	<i>Clostridium</i> sp
C6	+	+	+	<i>Bacillus</i> sp
C7	-	-	-	<i>Salmonella</i> sp
D1	+	+	+	<i>Bacillus</i> sp
D2	-	+	+	<i>Staphylococcus</i> sp
D3	+	+	+	<i>Enterobacter</i> sp
D4	-	+	+	<i>Staphylococcus</i> sp
D5	-	+	+	<i>Salmonella</i> sp

Key: (+) indicates production activity, (-) indicates non-production activity, A, B, C, D represents reactors and source of isolates

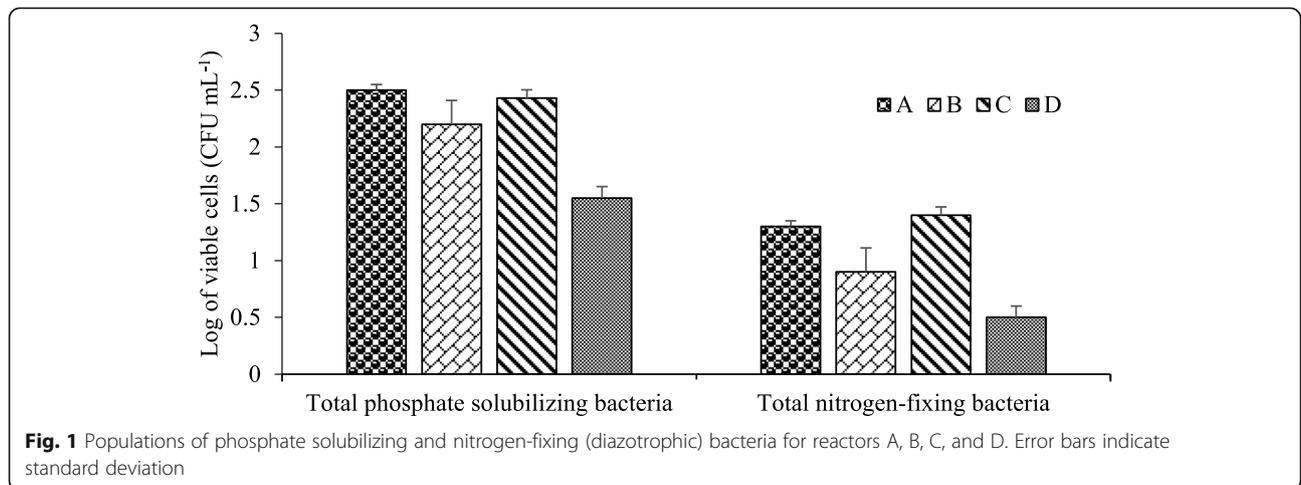
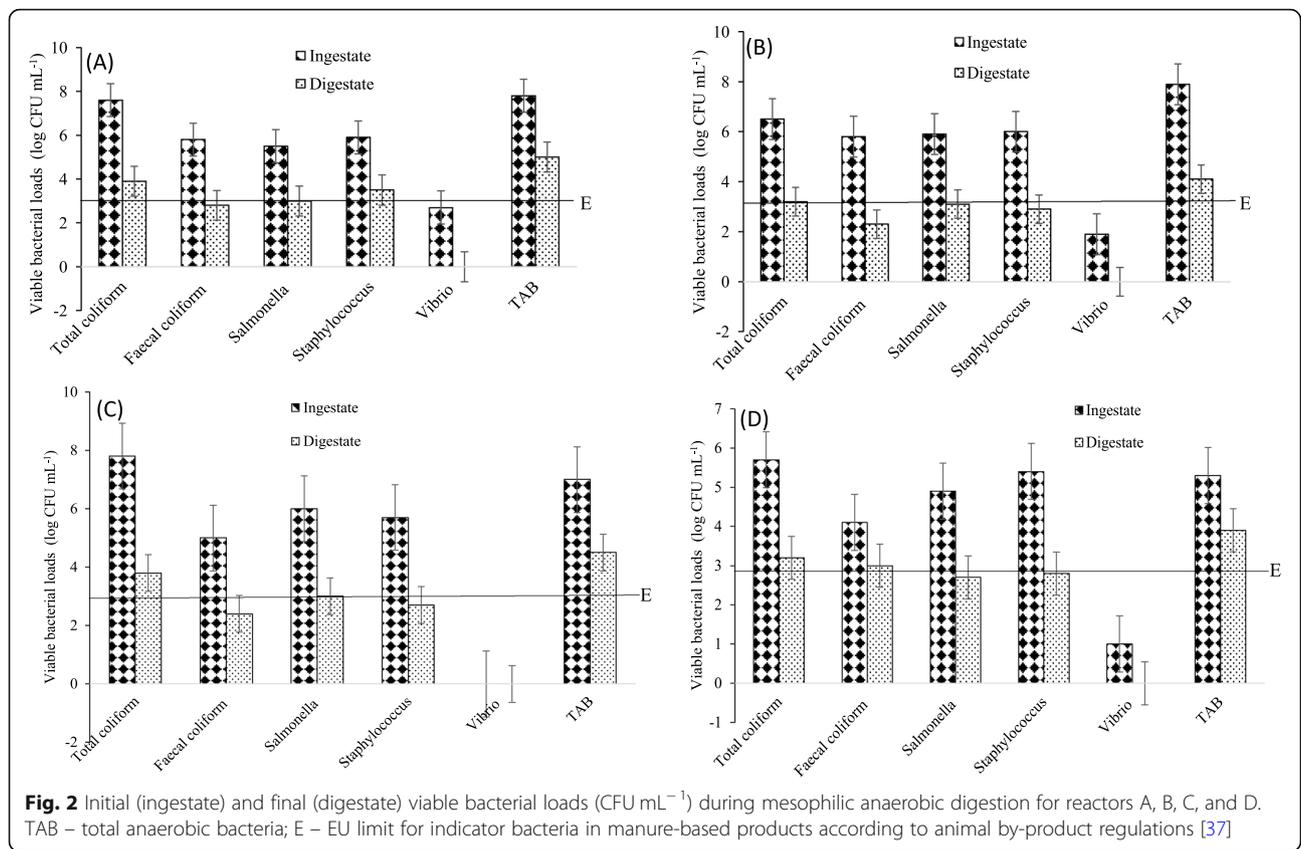


Fig. 1 Populations of phosphate solubilizing and nitrogen-fixing (diazotrophic) bacteria for reactors A, B, C, and D. Error bars indicate standard deviation



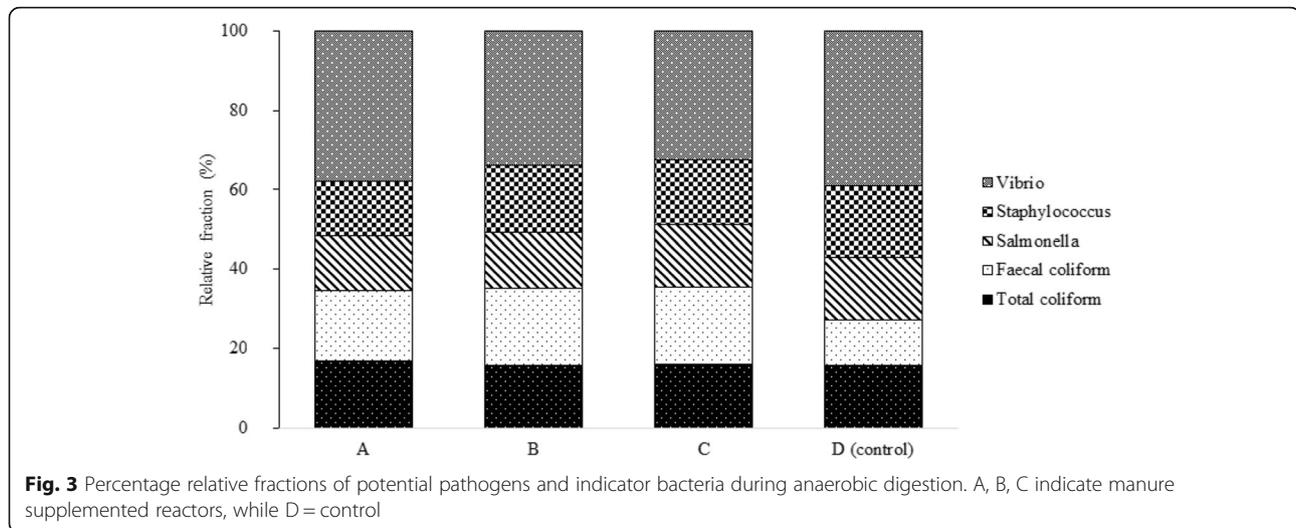
reactors suggesting microbial decay. Averagely across all digesters (Table 3), anaerobic treatment resulted in undetectable levels (99.5% reduction) of *Vibrio* species with simultaneous decline in the levels of total coliforms (45%), faecal coliforms (49%), *Salmonella* count (42%) and *Staphylococcus* count (46%) and their relative fractions as illustrated in Fig. 3. This marked reduction was statistically significant ($p < 0.05$) ranging from 0.4 to 1.0 log CFU mL⁻¹ thus substantiating the earlier report of Alfa et al. [15] who recorded similar trends for coliform count and fungal count. However, Qi et al. [39] recorded a 100% reduction in all indicator and pathogenic bacteria. These results indicate that laboratory-scale

Table 3 Reduction ratios (%) of potential pathogens and indicator bacteria in digestate samples

Bacterial groups	Reactors			
	A	B	C	D (control)
Total coliforms	44.4 ± 0.10 ^b	46.2 ± 1.00 ^c	49.3 ± 1.00 ^d	40.0 ± 1.00 ^a
Faecal coliforms	47.2 ± 1.00 ^b	59.2 ± 1.00 ^c	60.0 ± 1.00 ^c	29.3 ± 1.00 ^a
<i>Salmonella</i>	36.0 ± 1.00 ^a	41.2 ± 0.81 ^b	49.2 ± 1.12 ^c	40.0 ± 0.40 ^b
<i>Staphylococcus</i>	36.4 ± 0.50 ^a	50.0 ± 1.00 ^b	50.0 ± 5.00 ^b	46.0 ± 4.00 ^b
<i>Vibrio</i>	99.6 ± 0.58 ^a	99.3 ± 1.15 ^a	99.3 ± 1.15 ^a	99.6 ± 0.58 ^a

Similar superscript letter means not significantly different in mean ($p > 0.05$), while different superscript letters mean significantly different ($p < 0.05$); A–D indicate bioreactors with different waste composition

reactors may be less efficient in pathogen reduction than full-scale biogas plants. Cote et al. [40] studied efficiency of low temperature (psychrophilic) anaerobic treatment in reducing viable populations of indicator microorganisms in pig slurries. These authors reported 1.62 to 4.23 log CFU mL⁻¹ reduction in populations of indicator organisms thus contradicting the results of this study. This disparity may be attributed to the differences in the treated waste composition, environmental factors, prevailing operating digester condition and digester design. Despite the apparent reduction observed, some indicator bacterial loads were still above the European Union permissible limit of 3.0 log CFU mL⁻¹ for land application of digestate in agriculture [23], indicating that the incubation temperature of 40 °C and reaction time of 30 d were insufficient to completely eliminate these potential pathogens in the resultant digestate. Total coliforms load significantly correlated with faecal coliforms ($r = 0.924$, $p < 0.01$) and *Salmonella* loads ($r = 0.655$, $p < 0.01$) while other bacterial groups had no significant relationship with total coliforms ($p > 0.05$). The level of faecal coliforms was not significantly correlated with *Salmonella* ($r = 0.506$, $p > 0.05$), *Staphylococcus* ($r = 0.334$, $p > 0.05$) and *Vibrio* ($r = -0.222$, $p > 0.05$). There was a significant positive relationship between *Salmonella* and *Vibrio* count ($r = 0.708$, $p < 0.01$).



The detection, persistence and survival of indicator bacteria and potential pathogens above the permissible limit after AD have also previously been documented by other authors [16, 41]. Notably, Qi et al. [18] recorded $> 3.0 \log \text{CFU mL}^{-1}$ in *E. coli*, *Salmonella* and *Enterococcus* loads in mesophilic and thermophilic anaerobic digestates. Similarly, Resende et al. have isolated elevated levels of Enterobacteriaceae, non-fermenting Gram-negative rods and Gram-positive cocci in digestate after 60 d [24]. This calls for concern as previous studies [23–25] have encountered similar pathogens following AD, most of which have been implicated in foodborne infections. Hence the use of raw and/or untreated livestock manure poses greater risk to consumers of fresh produce than digestate as the survival of pathogens after AD largely depends on the temperature and reaction time of the substrates in the bioreactors. To overcome this, hygienization and further post-treatment of anaerobic digestate can be obtained at elevated temperatures over an extended retention time [42]. Though not entirely adequate, the reduction in pathogen levels obtained in this study may in part be due to the long reaction time of 30 d. Apart from the effect of temperature on pathogenic organisms, efficient mixing and organic matter stabilisation are additionally important factors controlling the inactivation rate or destruction of pathogens during AD of biowastes [43]. Moreover, the reduction ratio in viable bacteria largely depends on the bacterial species and the initial bacterial load in the feedstock. In this study, the bacterial species encountered at the end of the AD process were facultative anaerobes, strict anaerobes and endospore-forming mesophiles which are not readily destroyed during the mesophilic AD process as they become hardy, resisting the prevailing milieu in the digesters.

Nutrients and light metals concentrations before and after AD

The concentrations of primary (N, P, K) and secondary (Ca, Mg, S) plant nutrients are presented in Table 4. From the result, the concentration of N was detected at higher levels compared to other nutrients ranging from 2.5 to 5.0 g kg^{-1} in the ingestate and 2.7 to 5.10 g kg^{-1} in the digestate. However, the increase in nutrient concentration of all digestate samples was statistically significant ($p < 0.05$). The relative abundance in primary and secondary macronutrients in the samples were as follows; $\text{N} > \text{K} > \text{P} > \text{Ca} > \text{Mg} > \text{S}$ showing an increasing trend as follows: Reactor C > Reactor B > Reactor A > Reactor D (control).

Nutrient analysis of ingestate (raw substrates) and digestate (effluent) revealed an increase in concentrations of N, P, S, K, Ca and Mg (Table 4). Among these elements, sulphur had the highest variation relative to its mean with the coefficient of variation, $CV = 48\%$ followed by potassium ($CV = 39\%$) and magnesium ($CV = 36\%$). In contrast, calcium, nitrogen and phosphorus had the least variability with respect to their mean values and CV ranging from 12 to 23%). Across the samples, statistically significant differences were obtained for all the elements analysed indicating a strong influence of the AD process on the nutrient contents of the digested and undigested waste mixture. From the present study, AD was identified as an excellent option for nutrient recovery and recycling thus contributing to reducing agricultural cost via the use of inorganic fertilizer. This may in part be due to organic matter decomposition by bacterial consortia during the AD process. Accordingly, organically bound nutrients become mineralized into readily available forms during digestion. Evidently, AD tends to increase the contents of readily available nitrogen in the form of ammonium-N,

Table 4 Nutrient and light metal concentration of feedstock (t_0) and digestate (t_f)

Concentration	Initial concentration (t_0)				Final concentration (t_f)			
	A ₀	B ₀	C ₀	D ₀	A _f	B _f	C _f	D _f
Total N (g kg ⁻¹)	5.00 ^b	3.67 ^b	4.67 ^b	2.50 ^b	5.10 ^c	3.80 ^b	4.80 ^d	2.70 ^a
Ammonium N (g kg ⁻¹)	3.5 ^b	2.0 ^a	2.8 ^c	1.1 ^a	3.9 ^b	2.8 ^c	3.2 ^d	1.98 ^a
Phosphorus (mg kg ⁻¹)	1066 ^b	1086 ^b	1129 ^b	650 ^a	1133 ^c	1116 ^c	1311 ^d	665 ^a
Sulphur (mg kg ⁻¹)	342 ^b	427 ^c	468 ^d	90 ^a	341 ^b	450 ^c	491 ^d	91.3 ^a
Potassium (mg kg ⁻¹)	1321 ^b	1290 ^b	1301 ^b	400 ^a	1322 ^c	1315 ^b	1334 ^d	411.8 ^a
Calcium (mg kg ⁻¹)	571 ^d	545 ^b	561 ^c	433 ^a	578 ^c	570 ^b	580 ^d	430.2 ^a
Magnesium (mg kg ⁻¹)	625 ^b	621 ^b	623 ^b	2161 ^a	625 ^c	621 ^b	626 ^c	223.5 ^a

Similar superscript letter means not significantly different in mean ($p > 0.05$), while different superscript letters mean significantly different ($p < 0.05$); Total N – total nitrogen; A–D (Reactors)

and a strong positive correlation between total nitrogen content and total NFB population ($r = 0.960$) was observed. This corroborated the reports of Qi et al. [18] who recorded a significant increase in raw dairy manure from 5.3 g kg⁻¹ NH₄-N to 12.2 g kg⁻¹ in thermophilic anaerobic digestates. Moller and Muller [44] reported an increase in concentrations of NH₄-N by 45 to 80% following AD. Likewise, Coelho and other authors recorded an increase in concentration of macronutrients [33, 39]. Like nitrogen, phosphorus may be present in organic or inorganic form in anaerobic digestate. In this study, the total phosphorus concentration in the digestate samples ranged between 1116 and 1310 mg kg⁻¹ in inoculum amended reactors and was 1.78 times higher than contents measured in control bioreactors (Table 4). This difference was found to be significant ($p < 0.05$) alluding to the influence of manure addition on the nutrient property of anaerobic digestate. Potassium, another key nutrient to be supplied by soil conditioner, is often found in inorganic form. The potassium contents measured in the control and manure-supplemented bioreactors showed significant differences ($p < 0.05$) with values ranging from 400 to 1332 mg kg⁻¹. This amount may be considered high when compared to potassium levels (36.75 mg L⁻¹) reported by Quintanar-Orozco et al. [19] in digestate (biofertilizer) derived from *Opuntia heliabravoana* cladodes. Potassium is a macroelement that is of fundamental importance and plays a vital role in the water balance of plants, activation of enzymes and participates in photosynthetic processes among other functions. It is noteworthy to state that there exists a strong positive association between potassium and calcium contents ($r = 0.999$) as well as magnesium and calcium ($r = 0.998$). The concentrations of Ca and Mg were higher in the livestock manure amended bioreactors and were respectively 1.33 and 2.79 times significantly higher than the reactors with no livestock manure amendment (control). These levels are however lower than those reported by Albuquerque et al. [35]. Altogether, Qi et al. [18] have stated that N, P, K nutrients contribute most to the

fertilizing properties of organic soil amendment as these are the primary plant nutrients. These essential elements (NPK) are required by plants in higher quantities than other secondary (Ca, Mg, S) and micronutrients (Pb, Ni, Zn, Cu, Cr, Hg, Cd).

Potentially toxic elements concentrations of feedstock and digestate

The potentially toxic element concentration of the various feedstock compositions (reactors A to D) and digestate are compared in Table 5. There was a general reduction in heavy metal concentrations in the digestate samples although it was not significant at $p = 0.05$. Compared to other elements, zinc was the most abundant across all the samples ranging between 15 and 57 mg kg⁻¹. However, chromium, mercury and cadmium were below the detection level of <0.01 mg kg⁻¹ in all samples.

The recycling of nutrients present in biomass feedstock may also pose the risk of toxic metals in the digestate meant for environmental application. The toxic and harmful effects of these metals are not limited to plants and microbes but include humans also. This concern therefore limits the safe utilization of digestate in soils and crops. Generally, studies piloted by several researchers [9, 33, 34, 39] revealed a decreasing trend in the concentrations of PTEs in digestate samples within the recommended limits [45] of publicly available specifications (PAS-110). This decreasing trend was in conformity with the concentrations of Pb, Ni, Zn and Cu obtained in this study and was in line with the PAS-110 standard for the safe application of digestate on land. Moreover, Cr, Hg and Cd concentrations were below the detection levels of <0.01 mg kg⁻¹ each (Table 4). The reduction in metal concentration of the digestate samples may be a result of the role of PGPB in metal hyperaccumulation and sequestration during the digestion process [46].

The PTE concentrations revealed a high degree of variability ($CV = 0-81\%$) suggesting a significant

Table 5 Heavy metal concentrations in feedstock (t_0) and digestate (t_f)

Concentration (mg kg ⁻¹)	Initial concentration (t_0)				Final concentration (t_f)				PAS110
	A ₀	B ₀	C ₀	D ₀	A _f	B _f	C _f	D _f	
Lead	4.0 ± 1.00 ^b	4.3 ± 0.12 ^b	2.6 ± 0.10 ^a	2.3 ± 0.00 ^a	BDL	3.6 ± 0.10 ^c	BDL	2.3 ± 0.10 ^b	200
Nickel	10.5 ± 0.10 ^b	11.3 ± 0.55 ^b	12.5 ± 1.00 ^b	6.3 ± 0.55 ^a	2.3 ± 0.10 ^a	4.4 ± 0.10 ^d	3.5 ± 0.20 ^b	5.3 ± 0.00 ^c	50
Zinc	49.2 ± 1.10 ^b	51.3 ± 0.85 ^c	57.9 ± 1.01 ^d	45.5 ± 0.55 ^a	15.2 ± 0.32 ^a	27.2 ± 1.00 ^b	29.3 ± 0.63 ^c	40.1 ± 0.15 ^d	400
Copper	3.7 ± 0.10 ^b	5.4 ± 0.10 ^c	4.0 ± 1.00 ^b	2.5 ± 0.20 ^a	0.9 ± 0.08 ^b	3.1 ± 0.10 ^c	BDL	BDL	200
Chromium	BDL	BDL	BDL	BDL	ND	ND	ND	ND	100
Mercury	BDL	BDL	BDL	BDL	ND	ND	ND	ND	1.0
Cadmium	BDL	BDL	BDL	BDL	ND	ND	ND	ND	1.5

Similar superscript letter means not significantly different in mean ($p > 0.05$), while different superscript letters mean significantly different ($p < 0.05$); PAS110 – Publicly available specification according to British Standard Institute [44]; BDL – Below detection level; ND – Not determined; A – D (Reactors). Minimum detection limit – 0.01 mg kg⁻¹

influence ($p < 0.05$) of the waste feedstock on the heavy metal characteristics of the digestate. The concentration of these metals was generally lower in the digestate than in the undigested raw feedstock although this difference was however statistically not significant ($p > 0.05$). Besides being potentially harmful to microbes, plants and humans, plant micronutrients are necessary for plant growth and development but are needed in minute quantities.

Conclusions

Using different livestock manure as inoculum, the effect of manure supplementation on the biofertilizer quality of anaerobic digestate was investigated. Results indicated that manure addition enhanced the biofertilizing properties of the resultant digestion residue with poultry manure giving the highest result in terms of its PGPB and nutrient composition. Besides serving as a depository of numerous beneficial microbial groups and plant nutrients, the digestates also harboured notable indicator bacteria and potential pathogens with miniscule amount of potentially toxic elements. However, as a biological soil amendment or conditioner, evaluation of the biosafety risk and post treatment (hygienization) of any digestate should be encouraged before its land application as potential contamination of fresh produce by pathogenic bacteria through the application of anaerobic digestate is of concern.

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Authors' contributions

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Availability of data and materials

No such sources of data or materials were used for this study.

Competing interests

None.

Author details

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