Bacteriological and Physicochemical Assessment of Digestates after Anaerobic Digestion

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Abstract: The demand for an alternative to inorganic fertilizer is steadily increasing due to the drawbacks associated with its usage. The utilization of digestates has gained significant recognition recently due to their remarkable benefits even after being applied to the soil. Hence, this investigation aimed to assess the bacteriological profile of digestates, effluents, and feedstock originating from various waste streams, as well as the isolates' ability to enhance plant growth. Conventional methods were employed in the analyses. Data analysis was conducted using the Microsoft Excel package, and basic descriptive statistics were employed to interpret the acquired data. The results unveiled that the heterotrophic bacterial counts (expressed as log10 cfu/g or ml) in the digestates, effluents, and feedstock samples ranged from 4.80±0.14 (effluent samples) to 6.09±0.01 (cow dung sample). The coliform counts (log10 cfu/g or ml) obtained from the samples varied from 4.24±0.34 (effluent samples) to 5.67±0.03 (cow dung). The bacterial species isolated from the digestates, effluents, and feedstock samples comprised *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Serratia marcescens*, and *Klebsiella oxytoca*. Among these isolates *B. subtilis* exhibited the highest frequency of occurrence (27.8%), while *P. aeruginosa* demonstrated the lowest occurrence (11.1%). Regarding the plant growth promoting characteristics (rhizobacterial properties) of the bacterial isolates, all isolates exhibited ammonia production, while *Bacillus*, *Klebsiella*, and *Serratia* demonstrated nitrogen-fixation capability. Overall, *Bacillus* and *Klebsiella* were positively associated with at least three out of the four tests for plant growth promotion in the study. The findings of this study revealed the importance of these bacterial isolates from the digestates as organic biofertilizer for plant growth enhanced performance, thus guaranteeing food security and safety.

Key word: Anaerobic digestion, bacterial isolates, digestates, plant growth

INTRODUCTION

As a result of the global consensus on reducing dependence on fossil fuels and addressing climate change, there is a growing recognition of the need to develop renewable energy technologies (Mathiesen *et al.*, 2011). These new energy solutions must prioritize environmental sustainability throughout their lifecycle, minimizing greenhouse gas emissions, preserving natural ecosystems, and safeguarding food production. Anaerobic digestion (AD) has emerged as a viable option, offering the production of biogas as a renewable energy source. Anaerobic digestion involves a systematic process comprising four distinct stages namely, hydrolysis, acidogenesis, acetogenesis, and methanogenesis. This process not only generates biogas, but also facilitates the conservation of essential plant nutrients present in the initial biomass feedstock, allowing for their recycling as fertilizers for soil enrichment (Möller, 2009; Gunnarsson *et al.*, 2010). Anaerobic digestion is a complex process that relies on various types of microorganisms with diverse environmental requirements (Leung and Wang, 2016).

Biogas technology presents several advantages, including the potential to mitigate global CO_2 emissions, substitute synthetic nitrogen fertilizers, adhere to recycling principles to address limited nutrient resources (such as phosphorus), and reduce fertilizer costs for farmers (Cordell *et al.*, 2009). Farmers commonly utilize manure to supplement fertility programs due to its high nitrogen and phosphorus content, which are crucial nutrients for crop growth. Moreover, manure serves as a source of organic matter, enhancing soil porosity, water retention capacity, and erosion prevention. However, it is essential to note that manure can also contribute to environmental pollution and pose risks to human health if not managed properly (Petersen *et al.*, 2007). Excessive accumulation of phosphorus-based nutrients in farmland resulting from the prolonged application of manure is a particular concern for watershed protection.

In the AD process for biogas production, waste biomass is introduced into a sealed digester tank, where it undergoes heating and agitation. In the absence of oxygen, anaerobic microorganisms metabolize the biomass, proliferating and generating biogas. So during, this process, AD captures methane (CH_4) and carbon dioxide (CO_2) from manure and food by-products, effectively reducing greenhouse gas emissions associated with agricultural nutrients management. The residual by-products derived from the digester are referred to as digestate.

Digestate, the residual semi-solid substance obtained after the extraction of biogas through anaerobic digestion (AD), serves as a valuable source of organic matter and nutrients, particularly nitrogen and phosphorus, which are essential for optimal plant growth (Khalid *et al.*, 2011). Numerous factors influence biogas yield, digestate quality, and the growth of anaerobic microorganisms within the digester. These factors encompass pH levels, carbon-to-nitrogen (C/N) ratio, microbial composition, nutrient content, temperature, hydraulic retention time, moisture content, digester design, and feedstock concentration (Khalid *et al.*, 2011).

Increasingly, digestate finds application as a soil conditioner and/or amendment in various settings such as urban gardens, farmlands, and recreational areas, or as a means to cultivate energy crops on brownfield and/or marginal land, as well as in sports turf production (WRAP, 2013). Consequently, the field application of digestate has been recognized as a sustainable practice in alignment with the European Union standards for Good Agricultural and Environmental Conditions (GAEC) (European Union, 2000). However, it is crucial to handle and manage digestate appropriately as it has been found that digested slurries can be significant sources of ammonia (NH_3), methane (CH_4), and nitrous oxide (N_2O) emissions (Nkoa, 2014), which can have potential implications for local and regional climates, as well as human health. The author's comprehensive review highlights that digestates should only be considered as organic amendments or fertilizers when properly managed. Inadequate disposal of unstable digestate on land may result in the release of residual biogas, potentially exposing individuals to non-methanic volatile organic compounds, hazardous air pollutants, and odorous compounds, thereby posing health risks (Palmiotto *et al.*, 2014).

On the other hand, the establishment of AD power plants generates a significant volume of digestate as a byproduct. As a matter of fact, digestate's macro and micronutrient content makes it suitable for use as an organic fertilizer in place of mineral fertilizer (Risberg *et al.*, 2017), the large volume and low dry matter content of digestates pose challenges and expenses related to their management, storage, and application onto the soil. Furthermore, the storage, transport, and application of substantial quantities of digestate contribute to methane (CH_4) and ammonia (NH_3) emissions, exacerbating global warming potential and soil acidification, respectively (Longhurst *et al.*, 2019). Hence, the use of digestate as a fertilizer without appropriate treatment raises environmental concerns. Additionally, the nutritional value of digestates can be enhanced by combining them with other nutrient sources such as ashes (Bougnom *et al.*, 2012; Isagba *et al.*, 2022). This study aimed to assess the bacteriological and physicochemical properties of digestates, effluent, and cow dung in terms of their suitability as soil conditioners, specifically evaluating their potential for promoting the growth of plant-beneficial rhizobacteria (PGPR).

MATERIALS AND METHODS

Collection of samples: Samples were obtained from the Anaerobic Digester (AD) plants of National Centre for Energy and Environment (NCEE), University of Benin and Okomu Oil Company, both in Edo State, Nigeria. All experiments were performed in triplicates.

Enumeration and isolation of the samples: The method by Willey *et al.* (2008) was employed. A ten-fold serial dilution of the samples were prepared aseptically in sterile physiological saline. An aliquot of 0.1 ml was plated using the pour plate technique. Plates were cultured at 28±2^{o}C for 24 h. The number of colony forming unit per milliliter (cfu/ml) was calculated.

Phenotypic identification of isolates: Bacterial isolates were characterized using cultural, morphological and biochemical methods (Holt *et al*., 1994).

Rhizobacterial potential of bacterial isolates

Screening for Indole Acetic Acid (IAA) production: This was determined by reaction of liquid culture of rhizobacterial isolates grown in 500 mg/l L-tryptophan placed in tryptic soy broth (1 g/l MES hydrate, pH 6) and Salkowki's reagent according to Patten and Glick (2002), Kumar *et al.* (2012) and Ngoma *et al.* (2013). Development of pink colour after incubation at room temperature indicated IAA production and a colourless solution was observed for the control.

Screening for ammonia production

Freshly grown bacterial cultures were inoculated in 10 ml nutrient broth and incubated at 30°C for 48 h in a rotator shaker (Orbitron Rotator II, model 260250). After incubation, 0.5 ml of Nessler's reagent was added to each tube. The development of a yellow to brown colour indicated a positive reaction for ammonia production. The solution remained clear and colourless with no precipitate formed for the control (Kumar *et al*., 2012).

Screening for nitrogen fixation activity: A 24hr cultures of bacterial isolates grown on nutrient agar was streaked on a Jensen's Nitrogen free medium. Growth on nitrogen deficient medium confirms the ability to fix nitrogen (Weselowski *et al*., 2016).

Screening for phosphate solubilization activity: Rhizobacteria cultures was spotted in triplicates of separately on the top of Pikovskya's agar plates and incubated at 30°C for 3 days. A zone of clearance around the colonies after 3 days was scored as positive for phosphate solubilization (Ngoma *et al*., 2013; Gupta *et al*., 2014; Doilom *et al.,* 2020).

Physicochemical properties of digestates: Physicochemical properties of the digestates determined in this study were pH, moisture content, carbon content, total nitrogen, dry matter, fat, crude fibre, crude protein, ash content, non-fatty extract (carbohydrate), volatile solid, available phosphorus, and potassium content (AOAC, 2005). Nutrient elements (Na, Mg, Ca, Mn,) were also determined. For heavy metal analysis, nitric acid (HNO_3) digestion method was employed to digest metals in the samples (AOAC, 2005).

Data analysis: The data generated during this study were all subjected to various descriptive and inferential statistics and this statistical analysis was done using Microsoft excel package 2019 (Ogbeibu, 2015).

RESULTS

In this study, the microbiological properties of digestates and feedstock were evaluated to determine their potential as soil amendments for sustainable food production and the promotion of a green economy. The assessment included the enumeration of heterotrophic bacterial and coliform counts (expressed as log10 cfu/g or ml) in the various samples. The findings revealed a range of values for heterotrophic bacterial counts, with the lowest being 4.80±0.14 (in the case of effluent samples) and the highest being 6.09±0.01 (in the cow dung sample). Similarly, the coliform counts varied, ranging from 4.24±0.34 (in effluent samples) to 5.67±0.03 (in cow dung). Notably, the digestates consistently exhibited lower heterotrophic and coliform counts compared to the cow dung used as feedstock as depicted in Figure 1a and b). The species of bacteria isolated from the digestates, effluent and feedstock samples included *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Serratia marcescens* and *Klebsiella oxytoca* (Figure 2). The distribution of bacterial isolates revealed that *B. subtilis* was isolated from all samples (being most predominant), followed by *S. marcescens* and *E. coli*, which was present in all samples except for effluent and water hyacinth respectively. The isolate, *P. aeruginosa* had the least distribution as it was only present water hyacinth and effluent samples, but absent in the others. The frequency of occurrence of the identified bacterial isolates revealed that *B. subtilis* (27.8 %) had the highest percentage frequency of occurrence, while *P. aeruginosa* (11.1 %) had the least occurrence.

The physicochemical and nutritional parameters evaluated for the digestates, revealed that the pH ranged from 6.49 ± 0.02 – 7.15 ± 0.18. The moisture (%) and dry matter contents (%) ranged from 86.37 ± 0.21 – 98 ± 1.34 and 1.04 ± 0.09 – 13.22 ± 0.09 respectively. The nitrogen content (%) ranged from 0.02 ± 0.01 – 0.52 ± 0.00. (Table 1). The digestates were found to be rich in both micro and macro nutrients needed by plants for growth and productivity. The phosphorus contents (mg/kg) of the digestates ranged from 427 ± 1.428 – 763.53 ± 0.37, while the magnesium and potassium contents (mg/kg) were found to range between 173 ± 0.00 – 374.63 ± 0.97 and 2633 ± 3.50 – 3544.53 ± 0.37 respectively. Several of the physicochemical parameters of the digestates (1 and 2) were found to be statistically different ($p<0.05$) from each other with only a few exceptions. The crude fibre, nitrogen, crude protein, ash and volatile solids contents were found to be statistically significantly different. The heavy metal contents (mg/kg) of the digestates revealed low values for nickel, chromium, lead and cadmium (Table 2). The digestates were found to be high iron content (mg/kg) which ranged from 654.11 – 2113. The plant growth promoting characteristics (rhizobacterial properties) of the bacterial isolates obtained in the study revealed that all isolates were capable of ammonia production, while *Bacillus*, *Klebsiella* and *Serratia* were capable of nitrogen fixation. Overall, *Bacillus* and *Klebsiella* were found to be positive to at least three of the four tests for plant growth promotion in the study (Table 3). This further revealed that all bacterial isolates except for *Serratia marcescens* obtained from the digestates had the capacity to promote the growth of plant by 75 % of the PGPR tests in the study. The only isolates with the ability for phosphate solubilization were *Bacillus subtilis* and *Pseudomonas aeruginosa* (Figure 3) and *B. subtilis* had the highest solubilization index of 1.75 mm.

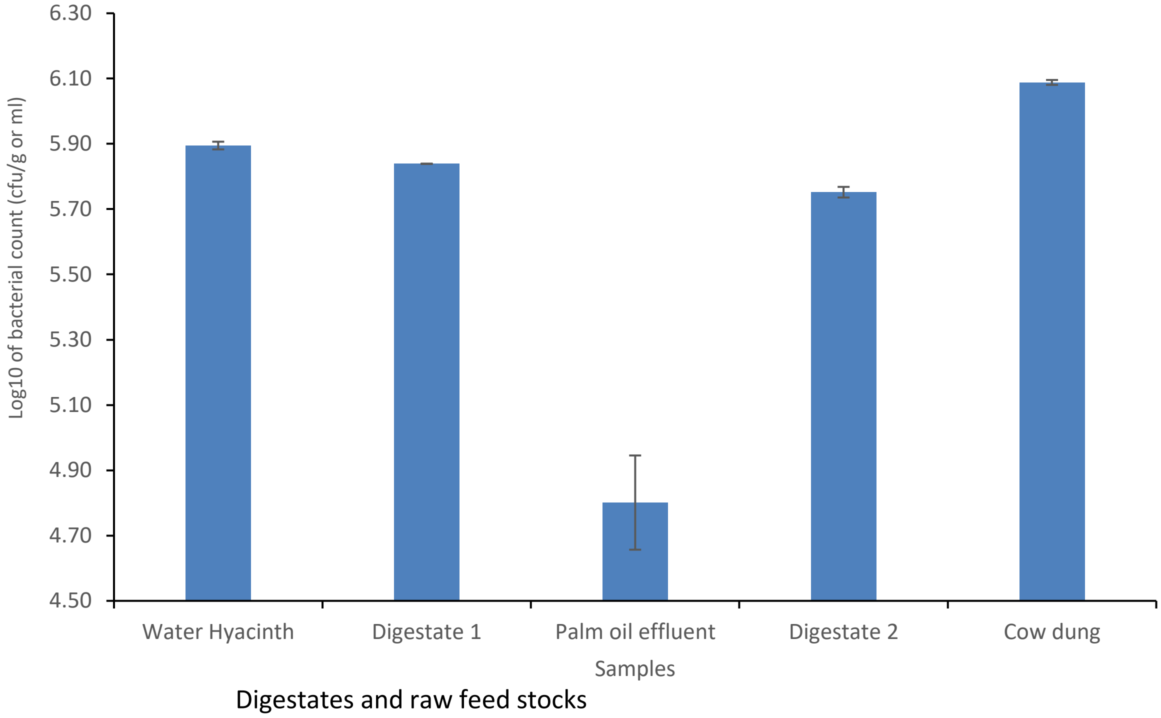

Figure 1a: Heterotrophic bacterial counts of digestates and raw feed stock samples

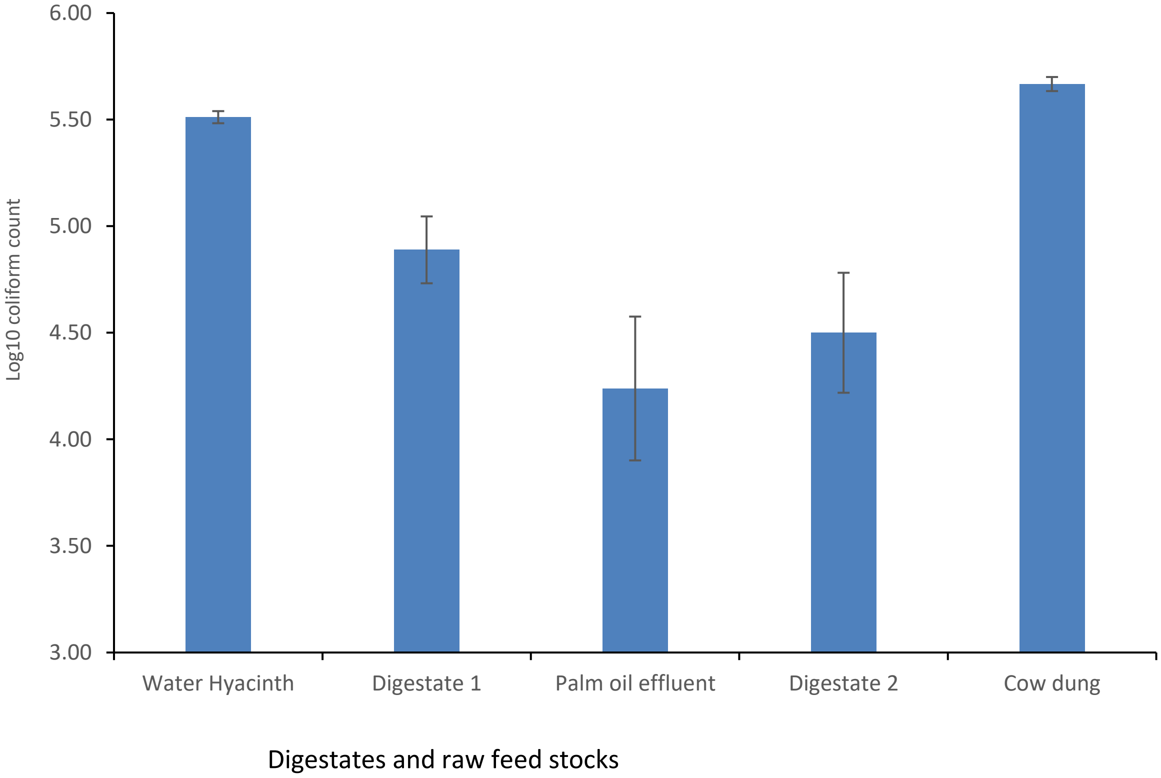

Figure 1b: Coliform counts of digestates and raw feed stock samples

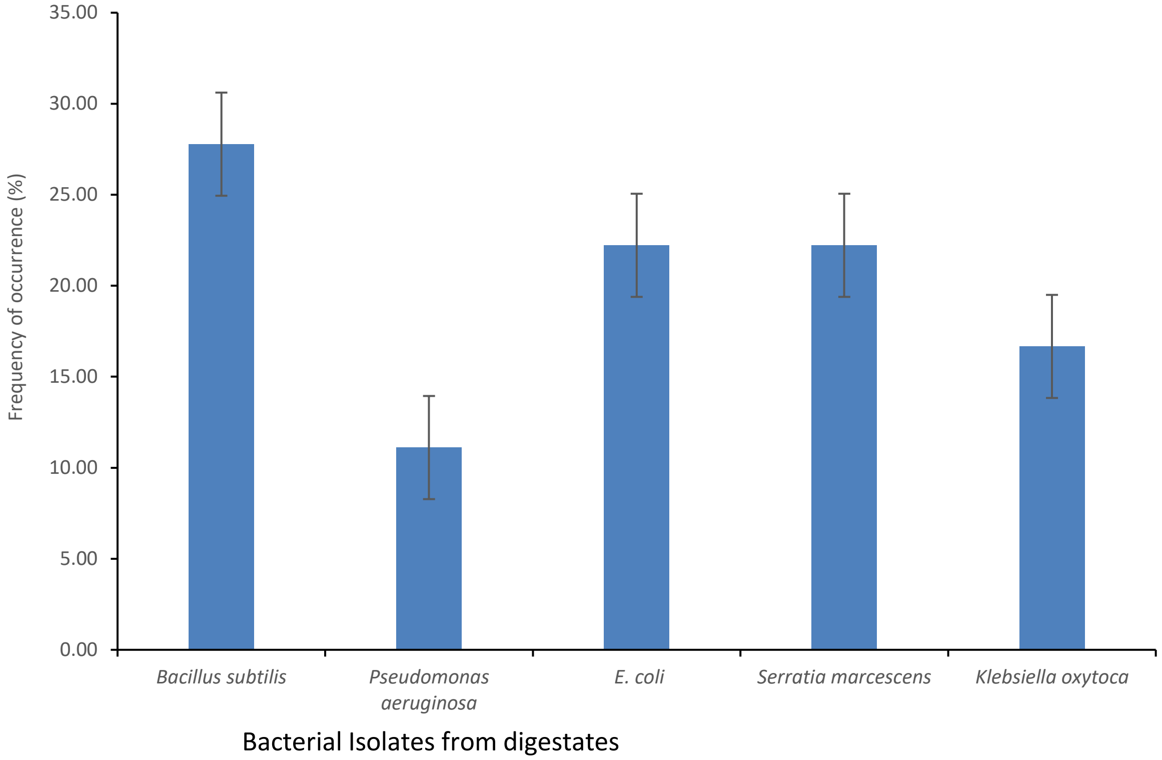

Figure 2: Identified bacterial isolates and their frequency of occurrence from the digestates

Table 1: Physicochemical properties and nutrient composition of the digestates

Parameters	Digestate 1	Digestate 2
pH	7.15 ± 0.18	6.49 ± 0.02
Moisture (%)	86.37 ± 0.21	98 ± 1.34
Dry matter (%)	13.22 ± 0.09	1.04 ± 0.09
Fat (%)	0.43 ± 0.00	0.00 ± 0.00
Crude fibre (%)	6.53 ± 0.03	0.37 ± 0.02
Nitrogen (%)	0.52 ± 0.00	0.02 ± 0.01
Crude protein (%)	3.27 ± 0.00	0.12 ± 0.01
Ash content (%)	0.71 ± 0.01	0.07 ± 0.01
NFE (%)	2.34 ± 0.01	0.57 ± 0.05
VS (%)	12.26 ± 0.26	1.01 ± 0.01
Na (mg/kg)	37.12 ± 0.15	14.1 ± 0.18
K (mg/kg)	3544.53 ± 0.37	2633 ± 3.50
P (mg/kg)	763.53 ± 0.37	427 ± 1.428
Ca (mg/kg)	82.25 ± 0.25	32.60 ± 0.08
Mg (mg/kg)	374.63 ± 0.97	173 ± 0.00
Mn (mg/kg)	19.31 ± 0.26	8.50 ± 0.28

NFE - Non Fatty Extract (Carbohydrate), VS - Volatile Solid

Table 2: Heavy metal content of the digestates

Heavy Metals	Digestate 1	Digestate 2
Zinc	47.3± 0.28	36.5± 0.34
Copper	24.3± 0.11	21.2± 0.01
Nickel	5.93± 0.46	2.13± 0.19
Chromium	15.7± 0.06	3.77± 0.89
Lead	1.54± 0.31	0.85± 0.08
Cadmium	0.47± 0.00	0.27± 0.15
Iron	2113± 0.28	654.13± 0.64

Table 3: Plant growth promoting characteristics (rhizobacterial properties) of bacterial isolates from digestates and effluents

Isolates	Nitrogen Fixation capacity	Ammonia production	Indole Acetic acid production	Phosphate solubilization	% Growth promoting capacity
Bacillus subtilis	+	+	-	+	75
Pseudomonas aeruginosa	-	+	-	+	50
E. coli	+	+	+	-	75
Serratia marcescens	+	+	-	-	75
Klebsiella oxytoca	+	+	+	-	75

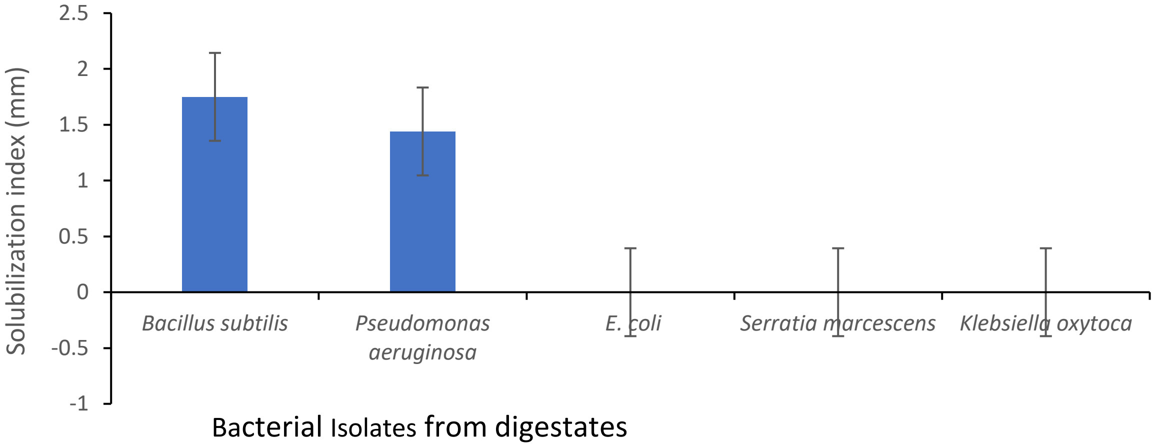

Figure 3: Phosphate solubilization index of bacterial isolates from digestates and effluent

DISCUSSION

The study was to assess the suitability of two digestates derived from the anaerobic digestion of cow dung mixed with water hyacinth and palm oil effluent, respectively, as organic soil amendments. The aim was to compare their effectiveness to the untreated substrates currently used as feedstock for anaerobic digestion, namely cow dung, water hyacinth, and palm oil effluent. A greater number of microbial isolates were obtained from the feedstocks (cow dung and water hyacinth) compared to the digestates, likely due to the environmental pressures exerted during the anaerobic digestion process. Some microorganisms present in the feedstock may not have survived under the unfavourable conditions of anaerobic digestion. The identification of these isolates revealed a higher cultivable microbial diversity in both cow dung and water hyacinth compared to the digestates. However, certain genera, such as *Escherichia* and *Pseudomonas*, were isolated specifically from the cow dung samples. Some species within these genera are known to be pathogenic. As reported by Carbone *et al.* (2002), animal faeces naturally contain *E. coli* as a pathogen that is excreted in the faecal matter. Therefore, the use of untreated manure carries a higher risk of containing pathogens compared to digestate (Adesemoye and Kloepper, 2009). *Bacillus subtilis* was found to be predominantly present in all the samples. *Bacillus* species are known for their role in biological control against bacterial pathogens, as many of them contribute to plant health. (Sondang *et al.*, 2021). The antibiotics produced by these bacteria effectively inhibit the growth of plant pathogens and the diseases they cause (Choudhary and John, 2009). These findings is further supported by Zhou *et al.* (2009), who documented the inhibitory role of *Bacillus* sp. in suppressing the growth of pathogenic microorganisms. The research conducted by Parida *et al.* (2016)

demonstrated that *B. subtilis* plays a crucial role in inducing resistance to leaf blight in rice plants. *Bacillus. subtilis* is widely utilized as a biofertilizer due to its ability to bind atmospheric nitrogen, solubilize phosphorus and potassium, produce growth-promoting substances, and suppress the growth of pathogens (Ahmad *et al.*, 2018). As cellulolytic bacteria, *Bacillus* species significantly contribute to the degradation of cellulose during fermentation processes. Isnawati and Trimulyono (2018) emphasized that a greater diversity of bacteria working in synergy results in more enzymes participating in the fermentation process, leading to enhanced fermentation rates. *Pseudomonas* and *Bacillus* genera have the potential to bind nitrogen, increase the solubility of phosphorus and potassium (as biofertilizers), produce indole acetic acid (IAA) compounds as growth stimulants (biostimulants), and suppress the growth of pathogens (biocontrol). *Pseudomonas* and *Bacillus* species interact synergistically with other microorganisms. Souza *et al.* (2015) highlighted the significance of plant growth-promoting bacteria (PGPB), which enhance plant growth and protect plants from diseases through various mechanisms. The isolates obtained from cow dung, water hyacinth, palm oil effluent, and digestates were further assessed for their ability to enhance soil fertility. All isolates demonstrated one or more soil-enhancing attributes. However, the ability to fix atmospheric nitrogen was more pronounced in most isolates compared to their abilities to solubilize phosphate and produce indole acetic acid (IAA). It is worth noting that the quality of digestate depends on the substrate used during the anaerobic digestion process (Mata-Alvarez *et al.*, 2000). In this study, shared genera were identified in both the cow dung and digestate samples. The presence of plant growth-promoting bacteria (PGPB) in digestate is advantageous as these organisms promote environmentally friendly plant growth compared to chemical fertilizers. A long-term study conducted by Odlare *et al.* (2011) comparing barley yields using digestate and compost found that the highest barley yield was achieved with digestate, highlighting its potential as a valuable long-term fertilizer. Therefore, in addition to making nutrients available for plants, the presence of PGPB in digestate can also enhance plant nutrient uptake. A noticeable reduction in volatile solids (VS), potassium, and phosphorus content was observed in digestate 2 compared to digestate 1. The higher dry matter and volatile solids content in digestate 1, as opposed to digestate 2, can be attributed to the utilization of organic matter present in the cow dung used to prepare digestate 1 by anaerobic bacteria during the biogas production process (Moller, 2009). The same principle applies to macro and micronutrients. The pH fluctuations during the anaerobic digestion (AD) process can have a significant impact on the microbial communities involved. According to Chen *et al.* (2010), anaerobic bacteria thrive within a pH range of 6.5 to 7.5, which is optimal for biogas production. As established in previous studies (Alburquerque *et al.*, 2012; Astals *et al.*, 2012), the type of substrate used in biogas production influences the nutrient composition and quality of the resulting digestate. The substrates utilized in this study provided the necessary macronutrients required by anaerobic bacteria. The digestates exhibited significantly lower levels of heavy metal content, which is advantageous for environmental purposes when the digestate is intended for use as a fertilizer. In the European Union, the European Commission has set limits for heavy metals in digestate through the Fertilizer Regulation (EC) No 2003/2003. According to Annex II of the regulation, the maximum allowable concentrations for heavy metals in digestate were as follows: cadmium (Cd): 1.0 mg/kg dry matter, lead (Pb): 50 mg/kg dry matter, mercury (Hg): 0.5 mg/kg dry matter, chromium (Cr): 1000 mg/kg dry matter, nickel (Ni): 100 mg/kg dry matter, copper (Cu): 1000 mg/kg dry matter and zinc (Zn): 2500 mg/kg dry matter (EU, 2000). The metals content from the digestates in this research were within the limits. During the anaerobic digestion process, various physical, chemical, and microbial processes can affect the concentration of heavy metals (Xie *et al.*, 2015). High concentrations of heavy metals such as copper, nickel, zinc, chromium, and cadmium pose environmental risks to humans, fish, and invertebrates (Obiakara-Amaechi *et al.*, 2022). The bio-magnification of heavy metals along the food chain amplifies these risks (√Yi *et al.*, 2011). Moreover, the accumulation of heavy metals can harm plants by affecting their growth and cellular metabolism (Madu *et al.*, 2011, Oyem and Oyem, 2024).

CONCLUSION

In this study the digestates exhibited culturable microbial communities with soil fertility enhancing attributes. The heavy metal content in both digestates was found to be low. However, it is important to note that both digestates also contained pathogenic microorganisms, which could potentially harm plants and cause diseases in humans. This highlights the need for additional treatment measures to reduce the presence of pathogenic organisms in the digestates. Based on the findings, digestate 1 showed more potential as a soil amendment fertilizer compared to digestate 2 Therefore, the digestate produced from the combination of cow dung and water hyacinth holds promise as a biofertilizer. Nonetheless, further research is necessary to explore the suitability of digestates from different substrates as biofertilizers.

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