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African Journal of Biotechnology

Full Length Research Paper

Bioactive compounds from *Hagenia abyssinica* with activity against bean pathogenic bacteria

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Common bean (*Phaseolus vulgaris* L.) is a major food crop in Africa and its production is plagued by diseases that reduce the potential yield, thus threatening food security. This study evaluated compounds from *Hagenia abyssinica* for bioactivity against bean bacterial pathogens *Xanthomonus axonopodis* pv. *phaseoli* and *Pseudomonus savastanoi* pv. *phaseolicola*. One triterpenoid, 1,3,19-trihydroxy-2-oxo-12-ursen-28-oic acid (1) and two flavans namely, 3,3',4',5'-tetrahydroxyflavan (2) and 3,3',4',5,7-pentahydroxyflavan (3) were isolated from the bark of *H. abyssinica*. Structures of the compounds were elucidated based on nuclear magnetic resonance (NMR) and high-resolution electrospray ionization mass spectra (HRESIMS) data analysis. These compounds and the ethyl acetate extract were evaluated against the bean pathogens using agar disc diffusion method. The ethyl acetate crude extract showed activity against the two pathogens with minimum inhibitory concentrations of 1.25 mg/mL. Compound 1 showed good activity against *X. axonopodis* pv. *phaseoli* and *P. savastanoi* pv. *phaseolicola* with minimum inhibitory concentrations of 5 and 1.25 mg/mL, respectively, whereas compounds 2 and 3 showed modest activity. This study demonstrated that compound 1 and the ethyl acetate crude extract from *H. abyssinica* have good activity against the two bean pathogens and can be used in the development of biopesticides to control bean diseases.

Key words: Bean pathogens, bioactive compounds, *Hagenia abyssinica*, *Xanthomonus axonopodis* pv. *phaseoli, Pseudomonus savastanoi* pv. *phaseolicola*.

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is a major legume crop that is largely consumed among various communities in sub-Saharan Africa. It provides a cheaper alternative source of protein and household food security to the low-income earners in towns and the rural poor population (Gichangi et al., 2012). Often referred to as "the meat of

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the poor," beans provide a highly nutritious food, containing protein, fiber, complex carbohydrates, vitamins, and micronutrients (CIAT, 2019). Beans also provide income for millions of people, particularly in Africa. However, its productivity is constrained by bacterial infections such as common bacterial blight caused by *Xanthomonas axonopodis* pv. *phaseoli* and halo blight caused by *Pseudomonas savastanoi* pv. *phaseolicola*. There are no effective, economical and environmentally friendly strategies of managing these diseases, especially for resource-poor farmers. According to literature (Macharia et al., 2009), approximately 263 tons of synthetic pesticides are applied at an average rate of 0.82 kg/ha and of these pesticides, 8% were classified as highly hazardous compounds by the World Health Organization, 25% as carcinogens, while 43% are said to be possible carcinogens. These synthetic pesticides are used by farmers across the region. Moreover, some of these pesticides cause "collateral damage" to other flora or to fauna, and may persist in the environment for years. Therefore, there is need to find alternative ways of controlling these pathogens using extracts and compounds from natural sources. The main objective of this study was to improve food security through the production of biopesticides for the control of bean diseases. Due to climate change, emerging, re-emerging and endemic plant diseases continue to be a major challenge in food production leading to food insecurity especially in sub-Saharan Africa. The study identified the medicinal plant *Hagenia abyssinica* as the source of bioactive compounds with activity against pathogenic bacteria that affect beans. This plant has been reported to show antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Salmonella typhi* (Karumi et al., 2013; Wolde et al., 2016). The bioactive compounds can be used for standardization in the development of biopesticides formulations from the plant materials. In our continued search for molecules with activity against bean phytopathogens, we report here the isolation and elucidation of three antibacterial compounds from *H. abyssinica*.

MATERIALS AND METHODS

Plant

The bark of *H. abyssinica* was collected from Mt. Elgon Forest (1.1493° N, 34.5418° E), Kenya in March 2019. Prof. S. T. Kariuki, a taxonomist of the Herbarium at Egerton University, identified the plants. Voucher specimens were deposited at the Department of Biological Sciences, Egerton University, Kenya.

General experimental procedures

Nuclear magnetic resonance (NMR) spectroscopy experiments were performed with a Bruker Avance III spectrometer operating at 700 MHz (^{1}H) and 175 MHz (^{13}C). High-resolution electrospray ionization mass spectra (HRESIMS) were carried out using LTQ Orbitrap spectrometer (Thermo Scientific, USA) equipped with a HESI-II ion source. For column chromatography, Silica gel 60 (0.063 – 0.2 mm, Macherey-Nagel) and Sephadex LH–20 (18 – 111 µm, GE Healthcare) were used. Thin layer chromatography (TLC) was carried out on pre-coated silica gel 60 plates (0.20 mm, Macherey-Nagel). Semi-preparative high-performance liquid chromatography (HPLC) was done on Shimadzu LC-20AP pump equipped with DGU-20A5R degassing unit, Shimadzu SPD-M20A detector, Shimadzu SIL-20ACHT auto-sampler using LabSolutions software.

Extraction and isolation of the bioactive compounds

The bark material of *H. abyssinica* was air dried under shade to constant weight and ground to a fine powder using a Thomas-Wiley mill model 4. Two kilograms of the ground material were soaked in methanol at room temperature and the contents were then filtered through Whatman no. 1 filter paper. The filtrate was concentrated in vacuum at 60°C using Buchi Rotavapor R-205.

The concentrated crude methanol extract was suspended in distilled water and extracted sequentially with hexane followed by ethyl acetate. The ethyl acetate crude extract (200 g) was subjected to column chromatography using the solvent mixture of ethyl acetate, hexane, methanol (6:3:1) to yield four fractions after pooling together those with similar TLC profiles. Fraction 2 and 3 were purified on a reverse preparative HPLC using Gemini C18 column (10 × 250 mm, 10 µm particle size, Phenomenex). The mobile phase used was double distilled water (with 0.1% formic acid) (A) and HPLC grade methanol (B). Fraction 2 yielded compounds 1 (10.2 mg) and 2 (11.8 mg) while fraction 3 yielded compound 3 (12.1 mg).

Antibacterial assay

The test organisms used in this study were *X. axonopodis* pv. *phaseoli* and *P. savastanoi* pv. *phaseolicola*. The antibacterial assays of the extracts and pure compounds were performed using agar disc diffusion method as described by Kajaria et al. (2012) with a slight modification. The media used in this assay was nutrient agar (28 g/1000 mL of distilled water). A 24-h bacterial population of 1.5×10^8 CFU/mL (1.0×10^8 – 2.0×10^8 CFU/mL) was spread on the plate containing media and left to dry. All extracts were weighed and a 20 mg/mL concentration of the extracts and compounds were made using dimethyl sulfoxide (DMSO). Blank sterile disc of Whatman filter paper No. 1, of 6 mm in diameter was impregnated with 100 µL of the different extracts and plated against the test organisms. Chloramphenicol was used as the reference standard whereas DMSO was the negative control. The plates were incubated at 32°C overnight and zone of inhibition measured in millimeters.

RESULTS AND DISCUSSION

Compound 1 was obtained as a dark brown substance whose mass spectral data gave a molecular ion peak of m/z = 503.3362 that calculated for the molecular formula $C_{30}H_{47}O_6$, [M + H] (Figure 1). The 1D and 2D NMR spectral data of compound 1 are summarized in Table 1. Its ^{1}H-NMR spectrum showed characteristics signals of 7 methyl groups at δH 0.58 (H-24), 1.08 (H-25), 0.70 (H-26), 1.36 (H-27), 1.09 (H-29), 0.85 (H-30), and one

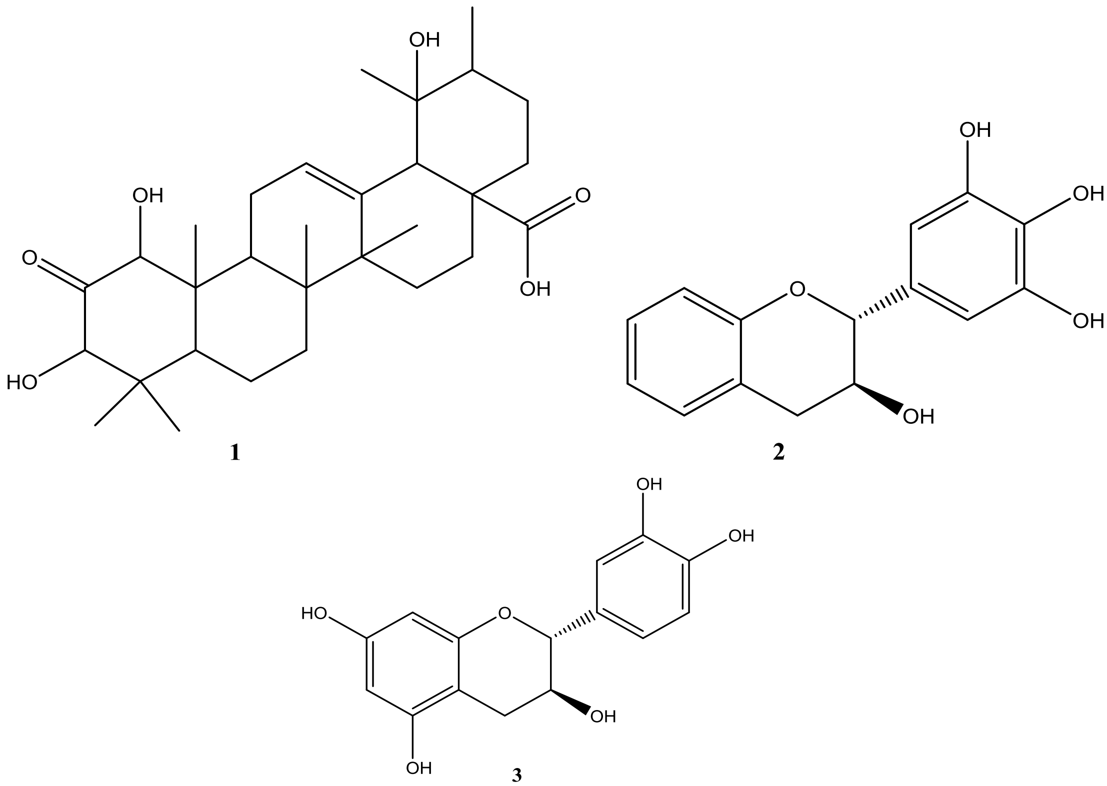

Figure 1. Structure of compounds 1, 2 and 3.
Source: Authors

olefinic proton at δH 5.16 (H-12) indicating that it is an ursen-12-en skeleton. Two oxymethine protons are observed at 4.08 (H-1), 4.00 (H-3) and an oxygenated quaternary carbon at C – 19 indicating the presence of hydroxyl groups in those positions.

The interpretation ^{13}C NMR spectra together with Heteronuclear Single Quantum Coherence (HSQC) showed that compound 1 had a total of 30 carbons which consisted of two carbons of a trisubstituted double bond, seven methyl groups, seven methylene groups, seven methine groups and nine quaternary carbons. The presence of a signal of a carbonyl group at δ 179.4 (C-28) suggested the compound as ursolic acid (Seebacher et al., 2003). Additionally, another carbonyl group is observed δ 211.7 (C-2). The HSQC spectrum was used to assign protons directly attached to carbon atoms shown in Table 1.

The Heteronuclear Multiple Bond Correlation (HMBC) between δH 0.69 (H-25) and δc 84.1 (C-1) placed the hydroxy group in position one. Similar correlations of the protons of the methyl groups in positions 23 and 24 with δc 80.6 (C-3) placed the other hydroxyl group at position 3. The third hydroxyl group was assigned position 19 through HMBC of δH 2.38 (H-18) and δH 1.09 (H-29) with C-19. There were also HMBC between two oxymethine protons δH 4.08 (H-1) and δH 4.00 (H-3) and the carbonyl carbon C-2 placing it at position 2. The HMBC spectrum also showed correlations of δH 2.38 (H-18) with C-12 and C-13 confirming the position of the double bond. These HMBC confirmed that compound 1 is 1,3,19-Trihydroxy-2-oxo-12-ursen-28-oic acid.

Compound 2 was obtained as yellowish powder. Its molecular formular of $C_{15}H_{14}O_5$ was determined from its HRESIMS m/z 275.075 [M + H] (Figure 1). In the proton NMR spectrum, the aromatic signals at δ 6.50 (H, d, 2.17 Hz), δ 6.71 (1H, d, 2.17 Hz), δ 7.40 (1H, d, 1.90 Hz) and δ 7.49 (1H, d, 8.11 Hz) is indicative of a 1',3',4',5' tetra-substitution in ring B. Correlation spectroscopy (COSY) showed that the two oxygenated methine protons δ 4.63 (H-3) and δ 5.28 (H-2) are adjacent to each other with a vicinal coupling constant of 7.52 Hz indicating they are *trans* to each other. The HMBC of δ 5.28 (H-2) with carbon atoms in ring B place it position 2 and also confirmed the positions 2' and 6'. The analysis of the NMR data suggested compound 2 to 3,3',4',5'-Tetrahydroxflavan. This was the first reported isolation of this compound.

Compound 3 was also obtained as a yellowish powder and its molecular formular of $C_{15}H_{14}O_6$ was determined from HREIMS at m/z 291.0824 [M+H] (Figure 1). The aromatic methine proton signals at δ 5.69 (1H, d, 2.13 Hz) and δ 5.89 (1H, d, 2.13 Hz) suggest *meta* coupled

Table 1. [1] H NMR (700 MHz) and ^{13}C NMR (175 MHz) spectral data of compounds **1 – 3.**

S/N	1 (DMSO)		2 (DMSO)		3 (DMSO)	
	^{13}C (δ)	^{1}H (δ)	^{13}C (δ)	^{1}H (δ)	^{13}C (δ)	^{1}H (δ)
1	84.1, CH	4.08	-	-	-	-
2	211.7, Cq	-	82.3, CH	5.28	81.5, CH	4.47
3	80.6, CH	4.00	67.6, CH	4.63	66.8, CH	3.81
4	45.2, Cq	-	29.2, CH_2	3.16, 4.47	28.6, CH_2	2.35, 2.65
5	51.0, CH	1.44	100.3, CH	7.53	156.6, Cq	-
6	18.3, CH_2	1.44, 1.62	119.7, CH	7.40	95.6, CH	5.89
7	33.0, CH_2	1.28	95.1, CH	6.50	156.9, Cq	-
8	41.2, Cq	-	96.4, CH	6.71	94.3, CH	5.69
9	47.4, CH	2.12	156.6, Cq	-	155.8, Cq	-
10	48.7, Cq	-	115.0, Cq	-	99.5, Cq	-
11	26.9, CH_2	1.95				
12	128.3, CH	5.16				
13	138.0, Cq	-				
14	41.6, Cq	-				
15	28.7, CH_2	1.69				
16	25.7, CH_2	1.40				
17	47.4, Cq	-				
18	53.6, CH	2.38				
19	72.1, Cq	-				
20	41.9, CH	1.26				
21	26.4, CH_2	1.61				
22	37.7, CH_2	1.49, 1.60				
23	17.0, CH_3	0.58				
24	29.2, CH_3	1.08				
25	12.3, CH_3	0.69				
26	16.9, CH_3	0.70				
27	24.4, CH_3	1.36				
28	179.4, Cq	-				
29	26.8, CH_3	1.09				
30	16.8, CH_3	0.85				
1’			131.8, Cq	-	131.0, Cq	-
2’			119.7, CH	-	115.0, Cq	6.72
3’			157.8, Cq	-	148.1, Cq	-
4’			146.2, Cq	-	145.3, Cq	-
5’			157.5, Cq	-	115.5, CH	6.68
6’			116.5, CH	7.49	118.9, CH	6.59

Source: Authors

pattern due to tetra-substitution in ring A. The other aromatic proton signals of δ 6.59 (1H, dd, 1.75, 8.15 Hz), δ 6.68 (1H, d, 8.15 Hz), and δ 6.75 (1H, d, 1.75 Hz) show both meta and para coupling patterns suggesting trisubstitution in ring B. The COSY and HMBC of the oxymethine protons δ 3.81 (H-3) and δ 4.47 (H-2) showed a similar pattern as in compound 2. Just like in compound 2, the vicinal coupling constant of 7.58 Hz between H-2 and H-3 indicates they are *trans* to each other. Therefore, compound 3 was deduced as 3,3’4’,5,7-Pentahydroxyflavan.

Ursane triterpenoids related to 1 have been reported to show antibacterial activity against human pathogenic bacteria (Jian-Jun et al., 2008) but to the best of our knowledge this is the first report on bean pathogens. It is also known that the introduction of functionalities into ring A like 1 improves biological activities (Li-Rong et al., 2016). Flavans like 2 and 3 have been reported to show antibacterial activities against a wide range of bacterial pathogens (Babe et al., 2018). The antibacterial activities of compounds 1 to 3 were tested against *X. axonopodis* pv. *phaseoli* and *P. savastanoi* pv. *phaseolicola*. The

Table 2. Inhibition zones (mm) and minimum inhibitory concentrations of compounds **1** – **3** and ethyl acetate crude extract.

Compound	Inhibition zone (mm)		Minimum Inhibitory Concentration (mg/mL)	
	X. axonopodis	*P. phaseolicola*	*X. axonopodis*	*P. phaseolicola*
1	16.33 ± 3.06	16.33 ± 1.15	5.00	1.25
2	6.33 ± 0.58	6	6	6
3	7.33 ± 0.58	6	6	6
*ETAOC extract	11.00 ± 1.00	10.67 ± 0.58	1.25	1.25
Chloramphenicol	20.00	40.00	-	-

*ETAOC = Ethyl acetate.
Source: Authors

results (Table 2) indicated that compound 1 was the most active with an inhibition diameter of 16.33 ± 3.06 mm for *X. axonopodis* pv. *phaseoli* compared to 20 mm for the reference standard chloramphenicol. Similar results were observed for *P. savastanoi* pv. *phaseolicola*. This compound had a minimum inhibition concentration of 5.0 and 1.25 mg/mL for *X. axonopodis* pv. *phaseoli* and *P. savastanoi* pv. *phaseolicola,* respectively. Compounds 2 and 3 showed modest activity.

Conclusion

This study demonstrated that the compounds isolated from *H. abyssinica* have activity against the bean pathogens *X. axonopodis* pv*. phaseoli* and *P. savastanoi* pv*. phaseolicola*. The results indicated that these compounds can be used as leads in the development of biopesticides to control bean diseases.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Harnessing the bio-stimulatory properties of heavy metals in improving biogas yield from agro-wastes: Two-phase logistic modeling of diauxic response

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This study aimed to harness the bio-stimulatory properties of two heavy metals, Zn (II) and Cd (II) at low concentrations in improving biogas yield from anaerobic digestion of cow dung (CD) and pig dung (PD). Proximate characteristics of the feedstocks were estimated by adopting standard methods. The batch fermentation experiment which was set-up in triplicates with equal weights of CD/PD and three concentrations: 0.02, 0.05 and 0.1 mM of the heavy metals in the reactors was conducted at ambient temperature range of 24 - 35 ± 2°C for 50 days. Results showed that the PD had remarkably higher volatile solids (55.50%) and lower carbon-to-nitrogen ratio (7.40%) whereas CD had higher carbon-to-nitrogen ratio (11.0%) but lower volatile solids (28.20%). Biogas yield increased with increase in concentration of the heavy metals except in 0.02 mM of Cd (II). The highest yield, 0.16 dm^3/g VS was recorded at 0.1 mM of Zn (II) which amounted to 33.65 dm^3 and 48.83% increase compared to the control. This was followed by 0.05 mM of Cd (II) with 0.15 dm^3/g VS (32.85 dm^3) and 45.29% increase. At higher concentration, 0.1 mM of Cd (II), a decline of 8.54% in biogas yield was recorded (0.14 dm^3/g VS). Diauxic response in biogas production was observed in all the treatments. The adapted model, two-phase logistic function model adequately described the diauxic behavior with very high accuracy as indicated by the relative coefficient (R^2) which was approximately 0.999 in all the tests. Diauxic biogas production responses from anaerobic digestion of lignocelluloses are ignored most often. This study has demonstrated the efficiency of two-phase logistic function model in the kinetic evaluation of diauxic biogas production from biomass with high accuracy.

Key words: Agro-wastes, heavy metals, biogas yield, diauxic response, logistic model.

INTRODUCTION

Generally, economic growth and development have been strongly correlated with a hike in energy demand and increasing greenhouse gas (GHG) emissions and consequently, climate change which now affects almost all nations. Sadly, approximately two-third of GHG emissions causing this climate change can be traced to the use of fossil fuels (Flavio et al., 2020). Transformation pathways toward a drastic reduction in GHG emissions

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are therefore apparently required in mitigating climate change to a manageable level (Edenhofer et al., 2013). Fortunately, in the portfolio of options available for the mitigation of climate change and global warming, renewable energy has been located (Shah et al., 2015). Improvement on access to efficient, sustainable and renewable energy is therefore urgently needed to promote and support the progress of nations.

Renewable energies are energy sources that are continually replenished by nature and derived directly from the sun (such as thermal, photo-chemical, and photo-electric), indirectly from the sun (such as photosynthetic energy stored in biomass, wind and hydropower), or from other natural movements and mechanisms of the environment (such as geothermal and tidal energy) (Rajha and Shilaja, 2019). Among all these renewable energy sources, biomass has been identified as one of the most dominant energies globally used which has the potentials to deliver a huge reduction in carbon emissions (Varma et al., 2017; Cheong et al., 2022; Rosi et al., 2022). It has been considered as one of the prevailing sources for next generation renewable energy that could provide a continuous and ecofriendly power generation (Chozhavendhan et al., 2019). A wide variety of biomass that have been considered suitable for production of biogas include wastes from crop production and animal husbandry (livestock manure), municipal solid wastes (MSW), sewage sludge, dedicated energy crops, industrial wastes from food processing etc. (Kulichkova et al., 2020; Szila´gyi et al., 2021). Biogas has been produced by anaerobic digestion (AD) of biomass (Stanley et al., 2022; Suhartini et al., 2021), a technology very efficient and well-established for the transformation of organic fractions into renewable bio-fuels, such as biomethane (Sayara and Sánchez, 2019; Almomani and Bhosale, 2020).

Studies have shown that AD is a very sensitive and complex biochemical process involving series of reactions in four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (García-Gen et al., 2015; Walter et al., 2016). The performance and stability of AD is influenced by several operational and environmental factors such as pH and alkalinity (Cetecioglu et al., 2022), temperature (Macias-Corral et al., 2017), organic loading rate (OLR) (Hegde and Trabold, 2019), C/N ratio (Choi et al., 2020), feedstock characteristics (Rajput and Sheikh, 2019; Spyridonidis et al., 2020), bioreactor design (Kreutz et al., 2021; Nsair et al., 2020), hydraulic retention time, trace element supplementation (Jadhav et al., 2022; Opurum et al., 2021a; Zhang et al., 2019; Tian et al., 2019) among others.

Findings have also shown that the percentage methane content of biogas is in the range of 50 to 75% (Kigozi et al., 2014; Anukam et al., 2019); this is relatively low when compared with natural gas whose methane content is approximately 90% (Mango et al., 1994). This has been identified as one of the major challenges in biogas production (Danmallam et al., 2020). Driven by this limitation, numerous researches have been geared toward improving biomethane yield. The approaches to achieve this ranges from co-substrate digestion (Ewunie et al., 2020), physical and chemical pretreatment to biological pretreatment (Amin et al., 2017). Harnessing the stimulatory characteristics of trace elements in the AD of organic materials for biogas production has also been established as one of the approaches to enhance biomethane yield (Alrawashdeh et al., 2020; Buia et al., 2020; Nguyen et al., 2019).

Biogas yield has been linked to the content of micro and macro nutrients which are responsible for the major metabolic processes that lead to biogas production (Matheri et al., 2016). Depending on the concentration, trace elements could exhibit stimulatory, inhibitory or even toxic effect on microorganisms, the key players in anaerobic fermentation and biogas production (Mudhoo and Kumar, 2013). A number of trace elements including Nickel, Zinc, Cadmium, Iron, Selenium, Cobalt etc., have been revealed to be vital for the growth and performance of microorganisms involved in anaerobic digestion (Swapnavahini et al., 2013; Arthur et al., 2022).

Trace elements have also been found to constitute the catalytic (active) center in a number of enzymes that play major roles in the biomethanation process (Dokulilová et al., 2018). Nickel for instance, is the catalytic (active) center of the enzyme, methyl-coenzyme M reductase (known as F_{430}) and many H_2-consuming hydrogenases as well as enzymes that catalyze acetate formation (Khan et al., 2021). Furthermore, it is associated with other enzymes, such as Ni - Fe hydrogenases and the carbon monoxide dehydrogenase/acetyl-CoA synthase in acetoclastic methanogenesis, as well as energy-converting hydrogenases and F_{420}-reducing hydrogenases during hydrogenotrophic methanogenesis and acetogenesis (Šafaric et al., 2020).

Metals such as Cd and Zn are significantly important to microorganisms associated with anaerobic digestion processes for their optimal growth and performance. Studies have shown that at a certain concentration, Cd promotes anaerobic digestion process, particularly acidogenesis (Yu and Fang, 2001). Kumar et al. (2006) investigated the effect of Ni(II), Zn(II) and Cd(II) on biogasification of potato waste and cow dung mixture at 50:50. Two different concentrations, 2.5 and 5.0 ppm of the heavy metals were used in the study. Results showed that at 2.5 ppm, the three heavy metals increased biogas production rate over the control, and the percentage increase was highest with Cd, followed by Ni and Zn. Zn is important in the functioning of enzymes involved in methanogenesis such as coenzyme M methyltransferase (Sauer and Thauer, 2000). Zn (II) is also associated with enzymes that affect phosphate, carbohydrate, protein metabolism, RNA and ribosome synthesis and regulation of redox potential of cells (hydrogenase, dehydrogenase formate, superoxide dismutase, etc.); it stabilizes membrane components, determining their reactivity

Table 1. The characteristics of the cow and pig dung.

Parameter (%)	Cow dung (CD)	Pig dung (PD)
Total solids (TS)	87.62	82.20
Volatile solids (VS)	28.20	55.50
C/N ratio	11.0	7. 40
Moisture content	12.43	17.18

Source: Authors.

(Golub et al., 2022). In the supplementation of pineapple pulp waste with urea and heavy metals, Nickel, Iron, Zinc, Copper and Cobalt were found to exert stimulatory effect, increasing production of biogas by 19 % compared to the control (Gopinathan et al., 2015).

In the contrary, Abdel-Shafy and Mansour (2014) investigated the effects of heavy metals, Cd, Hg and Cr on the anaerobic digestion of sludge for biogas production. The inhibitory effect on the biogas production and toxicity level of the metals was determined, and they appeared to be in the following ranking: Hg > Cd > Cr (III). Reports are scanty on the stimulatory effects of Cd and Zn on the anaerobic digestion of mixed pig dung and cow dung for biogas production. And given the fact that the threshold concentrations at which Cd and Zn stimulates or inhibits anaerobic digestion of animal manure have not been conclusively determined, this study therefore evaluated the bio-stimulatory properties of Zn^{2+} and Cd^{2+} at different concentrations in the improvement of biogas yield from mixed digestion of pig and cow dung.

Studies have shown that digestion of some complex multicomponent feedstocks for production of hydrogen, biogas, etc., exhibit diauxic behavior (Björkmalm et al., 2018; Buitrón et al., 2019), in which a diphasic mean cumulative biogas production curves are observed (Opurum et al., 2021b). The initial biogas production, after the lag phase is followed by a temporary cessation (second lag phase) in gas production, and after a plateau-phase, the biogas production resumed. Diauxic pattern of biogas production has been attributed to a number of reasons: acclimatization of microorganisms, the separate biodegradation of low and high complexity/multicomponent feedstocks or excessive production and accumulation of volatile fatty acids (Gomes et al., 2021).

Several kinetic models have been used in predicting the biogas production potential of different substrates, both in mono- and co-digestion experiments, these include Monod (Pererva et al., 2020), modified Gompertz model (Zhang et al., 2021), logistic model (Pramanik et al., 2019) among others. In these studies, the model equation was used to fit the experimental data, and a good fit has mostly been reported. In most of these existing models, the phenomenon of diauxic biogas production process was not quantitatively captured entirely. Logistic and modified Gompertz model have most commonly been used to fit biogas production curves. Without modification, however, a one growth phase model cannot represent diauxic behavior accurately. If the first phase of diauxic response is represented by the lag-phase or both phases are represented as one and the model ignores the plateau-phase, this would lead to a lower correlation coefficient (Gomes et al., 2021) and poor description of the process by the model.

To bridge this existing gap, therefore, this study aimed to adapt the commonly used logistic function model to fit biogas production curve with diauxic response. The performance of the adapted two-phase logistic function model was afterwards used to simulate biogas production curves from the anaerobic digestion of mixtures of pig and cow dung supplemented with Cd^{2+} and Zn^{2+}.

MATERIALS AND METHODS

Chemical reagents

Analytical grade heavy metal salts, Cadmium sulphate 8/3 - hydrate ($CdSO_4.8/3H_2O$) and Zinc nitrate hexahydrate ($Zn(NO_3)_2.6H_2O$) sourced from Sigma (Germany) were used in this study. A 10 mM stock solution of the salts was separately prepared in distilled water.

Feedstocks

The pig dung (PD) was collected from a local piggery farm and the cow dung (CD) from an abattoir, both in Owerri, Imo State Nigeria. The test samples were subjected to sun-drying for seven days and subsequently ground using an electric grinder after removing debris. The samples were sieved to a particle size of < 2 mm and stored in air-tight bags. The characteristics of the feedstocks used in this study are shown in Table 1.

Inoculum preparation

Rumen waste of cow (freshly slaughtered) was collected in an air-tight container. Sterile water was added to prepare slurry and subsequently filtered using triple-layered cheesecloth after vigorous stirring. The rumen liquor which served as the source of the methanogens was stored under anaerobiosis and used as the inoculum (Opurum et al., 2017).

Experimental design and batch fermentation

The experiment was performed in batch reactors (made of transparent PVC materials) of 10 L capacity with 8 L working volume. On the lid of the reactors were installed mercury-glass thermometers for temperature monitoring. Triplicate slurry in 1:1 ratio of the PD and CD was prepared with sterile water and the volume of which was adjusted to approximately 6 L. Afterwards, specified volumes (16, 40 and 80 ml) of each of the prepared 10 mM stock solution of the metal salts (Zn and Cd) were separately added to achieve the desired 0.02, 0.05, and 0.1 mM concentrations. The control reactor contained 1:1 ratio of PD/CD only. The preparations were fed once into the batch reactors properly labeled, and eight liter working volume was finally attained by inoculating each of feedstock-fed reactors with the prepared active microbial seed and hermetically sealed thereafter to forestall infiltration by air.

The outlet gas hose from the reactors was connected to the biogas harvesting system in accordance to the experimental design. Agitation of the reactor content was done manually on daily basis. The downward displacement method was adopted in the determination of the daily biogas yield by measuring the volume of water displaced. The AD lasted for 50 days under ambient temperature range of 24 - 35 ± 2°C.

Two-phase logistic function model and statistical studies

In recognition of the observed diauxic biogas production response, a two-phase logistic function model (Equation 1) was adapted from logistic function equation as described by Pramanik et al. (2019) and Opurum et al. (2021b) to study the two-step biogas production process. The performance of the adapted model, which was implemented using Sigma Plot version 10.0, was evaluated by simulating the diauxic biogas production curves.

$$Gy = \frac{B_{P1}}{1+\exp\left[\dfrac{4P_{R1}(\lambda_1 - t)}{B_{P1}}+2\right]} + \frac{B_{P2}-B_{P1}}{1+\exp\left[\dfrac{4P_{R2}(\lambda_2 - t)}{B_{P2}-B_{P1}}+2\right]} \quad (9)$$

where *Gy* - biogas yield (dm^3) with respect to time *t* (days), B_{P1} - maximum biogas potential of the substrate (dm^3) before the second lag phase, P_{R1}-maximum biogas production rate ($dm^3.d$) before the second lag phase, B_{P2} - maximum biogas potential of the substrate (dm^3) in the second phase,
P_{R2} - maximum biogas production rate ($dm^3.d$) in the second phase,
λ_1 - first lag phase (days), and
λ_2 - second lag phase (days), *t* - time (days).

Statistical difference in the means of the cumulative biogas yield from the various treatments were determined using Post-HocDuncan test implemented in IBM SPSS statistics software version 20.0.

RESULTS

Biogas production

The daily biogas production profiles from CD/PD with the 0.02, 0.05 and 0.1-mM concentrations of Zn are presented in Figure 1. Diauxic biogas production response with a two lag phases and two peaks of biogas yield were observed in all the reactors. The initial lag phase which witnessed very low and non-flammable biogas production, as indicated by flammability test, was considerably long and ranged between 11 and 17 days. However, after this adaptation period the process displayed an accelerated biogas production rate. The second lag phase observed between day 30 and 35 was shorter and also characterized by a remarkably decrease in biogas production, and afterwards the fluctuating gas production accelerated to attain the second biogas production peak.

The first peak of biogas production was recorded on days 21 and 24 in reactors with CD/PD only (control) and 0.02 mM of Zn concentration, and days 20 for 0.05 and 0.1 mM of Zn with biogas yield of 1.07, 1.60, 2.18 and 2.0 dm^3, respectively. The second peak was observed on day 36 in the reactors with 0.05 (2.01 dm^3) and 0.1 mM of Zn (1.91 dm^3), and on day 38 and 41 for reactors charged with CD/PD only (1.27 dm^3) and 0.02 mM (1.48 dm^3), respectively.

Similarly, Figure 2 presented the profile of biogas production in reactors supplemented with different concentrations of Cd. The initial lag phase lasted between 9 and 16 days, followed by increased biogas production and the second lag phase recorded between days 29 and 32. The first peak of biogas production was observed on days 23, 24 and 22 with 0.50, 1.98 and 2.32 dm^3 as the maximum daily biogas yield for reactors supplemented with 0.02, 0.05 and 0.1 mM of Cd, respectively. The second biogas production peaks were noted on days 43, 36 and 35 for reactors 0.02, 0.05 and 0.1 mM of Cd with maximum daily biogas yield of 0.92, 1.86 and 1.82 dm^3, respectively. Relative to the control, it is worthy of note that Cd and Zn supplementation of the feedstock had no influence on the lag phase.

The cumulative biogas yield and percentage increase

The mean cumulative biogas yield was the index for the assessment of stimulatory properties of the evaluated heavy metals. In the reactors supplemented with Cd, the highest cumulative yield in biogas was obtained at the concentration of 0.05 mM, with mean cumulative biogas yield of 32.85 dm^3 and 45.29% increase relative to the control reactor (Table 2). The biogas yield declined by 8.54% when the Cd concentration was increased to 0.1 mM, revealing that the stimulatory threshold has been exceeded. Biogas yield increased with increase in the concentration of Zn and the highest gas yield was at 0.1 mM (33.65 dm^3) with a 48.83% increase. Analysis of variance (ANOVA) showed at statistical difference ($P \le 0.05$) in reactors supplemented with 0.05 mM of Zn and 0.1 mM of Cd compared to the control.

Two-phase logistic function model

The two-phase logistic model was used to simulate the experimental data from the heavy metal-supplemented mixtures of CD/PD as shown in Figures 3 and 4. In all the reactors, the correlation coefficient (R^2) was above 0.999, an indication that the adapted model described the

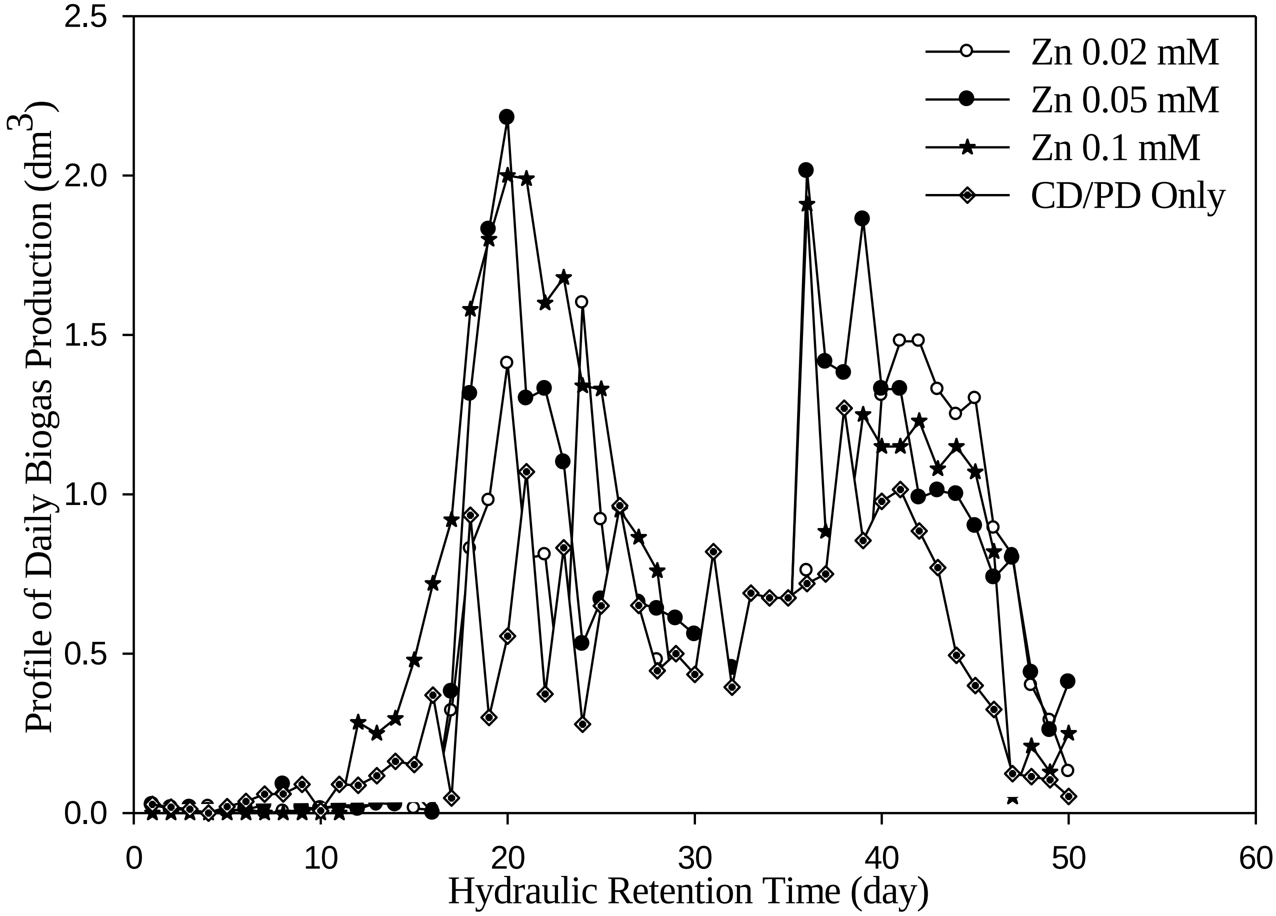

Figure 1. Daily biogas production profile from CD/PD with different concentrations of Zn.
Source: Authors.

diauxic biogas production process with a high accuracy.

Table 3 shows the kinetic parameters obtained using the two-phase logistic function model. The observed initial lag phase which lasted for 11 to 17 days was in congruent with the model determined initial lag phase (λ_1) which was estimated to be 13 to 18 days, but remarkably differed from λ_2 (27 - 37 days). The biogas production rate (*Rm*) in the second phase, Rm_2 $(dm^3.d)$ is two times that of the first phase, Rm_1 $(dm^3.d)$ in the reactor with 0.02 mM of Cd. Conversely, Rm_2 $(dm^3.d)$ was two times lower than Rm_1 $(dm^3.d)$ in the reactor with 0.1 mM of Cd. In all the reactors, the maximum biogas production potential (P_{b2} (dm^3) in the second phase was 2 to 3 times higher than that estimated in the first phase (P_{b1} (dm^3)).

DISCUSSION

Though the tested heavy metals improved biogas yield, the lag period was not affected by the heavy metal supplementation as very long lag period was observed in all the treatments, just as the control. This implies that the treatment had no influence on the hydrolysis stage of the anaerobic fermentation process. It could be that the tested metals (Cd and Zn) are neither part of the active center of the enzymes associated with hydrolysis stage nor cofactors to the hydrolyzing enzymes. This observation is in consistence with the report of Mudhoo and Kumar (2013). The authors carried out a review work on the effects of heavy metals as stress factors on AD processes and biogas production from biomass. The review report did not find any published data on the effects of heavy metals on the hydrolysis stage of AD process chemistry, and therefore recommended further studies.

The long lag phase was undoubtedly due to adaption of microbial community to the substrates and the characteristic presence of recalcitrant lignocelluloses in cow and pig dung (Ahmed et al., 2019). This observation calls for research attention to investigate possible approaches toward the minimization of lag period in the anaerobic digestion of animal manure for biogas production.

The results of this study have demonstrated the bio-

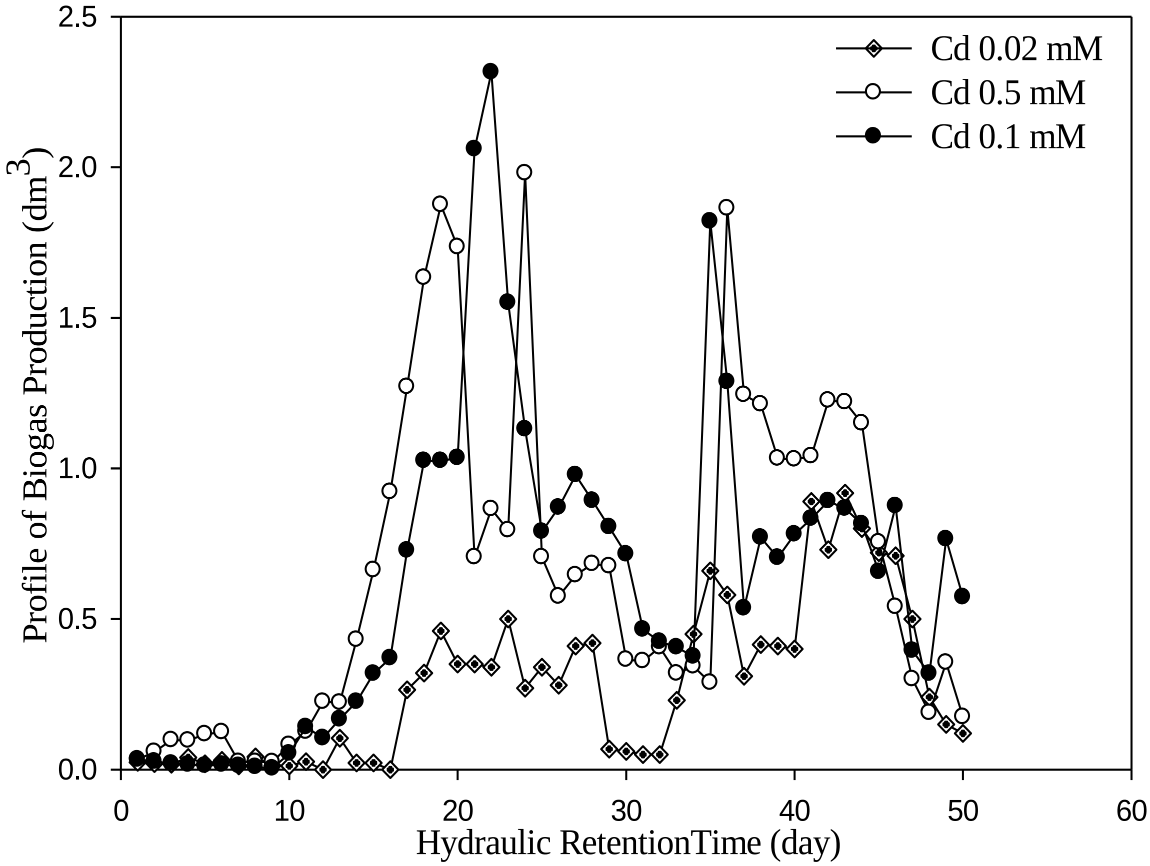

Figure 2. Daily biogas production profile from CD/PD with different concentrations of Cd.
Source: Authors.

Table 2. Mean cumulative biogas yield and the percentage increase.

Metal ion concentration (mM)	Mean cumulative biogas yield (dm^3)	Yield per g/VS (dm^3)	% Increase in biogas yield
Cd (II) 0.02	14.34	0.07	-
Cd (II) 0.05	32.85	0.15	45.29
Cd (II) 0.1	30.92	0.14	36.75
Zn (II) 0.02	22.48	0.10	-
Zn (II) 0.05	30.67	0.14	35.65
Zn (II) 0.1	33.65	0.16	48.83
PD/CD alone	22.61	0.10	-

Source: Authors.

stimulatory characteristics of two heavy metals, Zn^{2+} and Cd^{2+} at some of the tested concentrations in the improvement of biogas yield from the AD of livestock manure. In consonance with some previous studies, a good number of heavy metals such as Nickel, Cobalt, Iron, Zinc, etc., have been shown to exhibit stimulatory effect on AD for biogas production at low concentrations (Myszograj et al., 2018; Golub et al., 2022) and inhibitory effects at high concentration (Alrawashdeh et al., 2020). As the concentration of Cd increased from 0.05 to 0.1 mM the biogas yield declined by 8.54%. This is an indication that the stimulatory threshold has been exceeded.

A significant increase in biogas yield was recorded in reactors with 0.05 mM of Zn and 0.1 mM of Cd supplementation compared with the control reactor. The

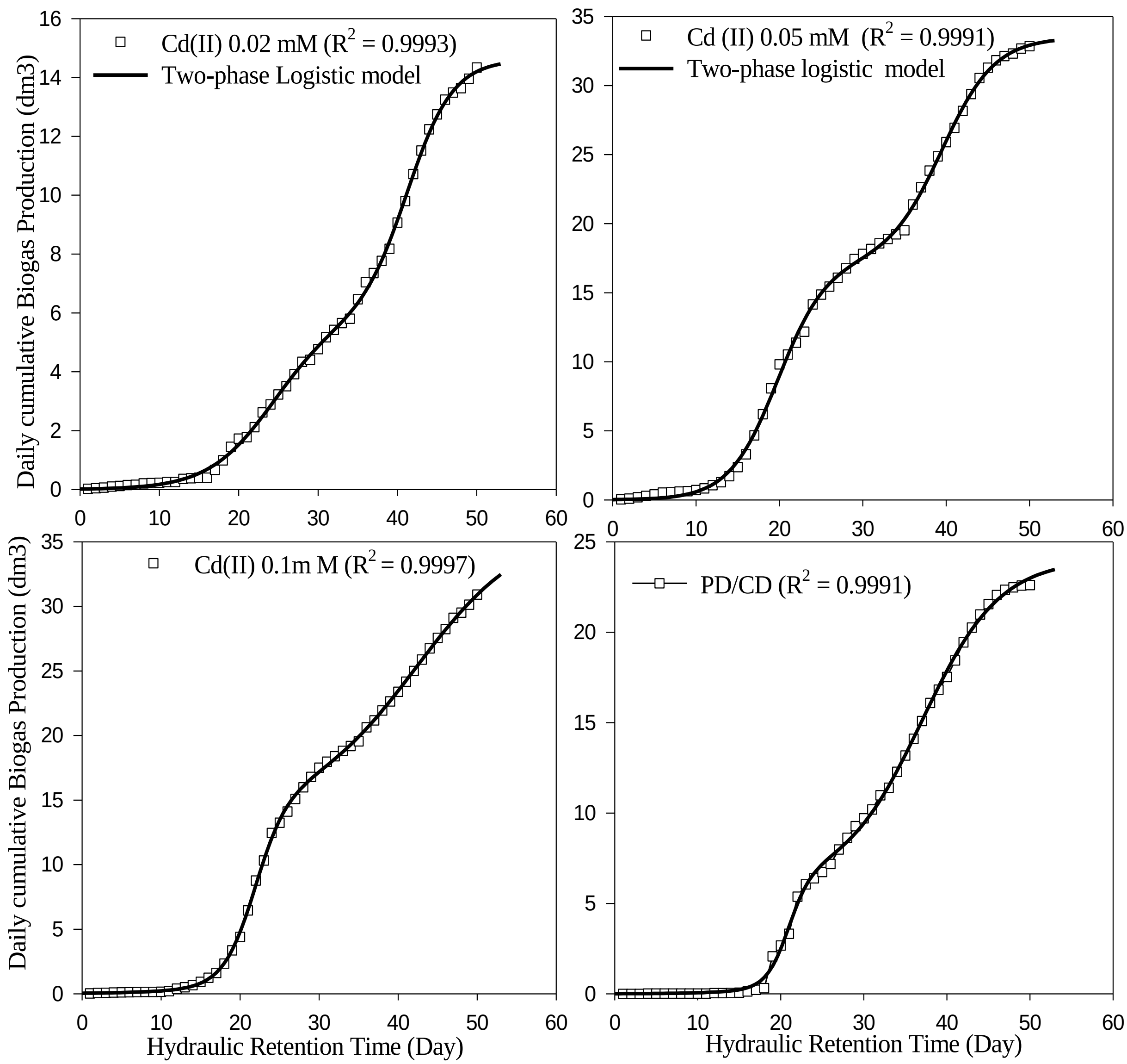

Figure 3. Mean cumulative biogas production curves from Cd supplementation fitted with two-phase logistic function model.
Source: Authors.

Table 3. Kinetic parameters estimated using two-phase logistic function model.

Treatment	Metal ion concentration (mM)	Diphasic logistic model parameters					
		Pb_1 (dm^3)	R_{m1}(dm^3.d)	λ_1 (days)	P_{b2} (dm^3)	R_{m2} (dm^3.d)	λ_2 (days)
	Cd (II) 0.02	5.86	0.36	16.11	14.61	0.76	35.56
PD/CD	Cd (II) 0.05	17.26	1.47	13.96	33.49	1.28	33.17
	Cd (II) 0.1	14.28	1.81	17.93	36.25	0.80	28.61
	Zn (II) 0.02	10.77	1.09	17.16	22.91	1.40	37.24
PD/CD	Zn (II) 0.05	11.74	1.70	17.04	31.56	1.40	31.39
	Zn (II) 0.1	19.15	2.02	16.08	34.25	1.28	33.19
PD/CD (Control)	Nil	6.15	1.17	18.31	24.04	0.95	27.61

Source: Authors.

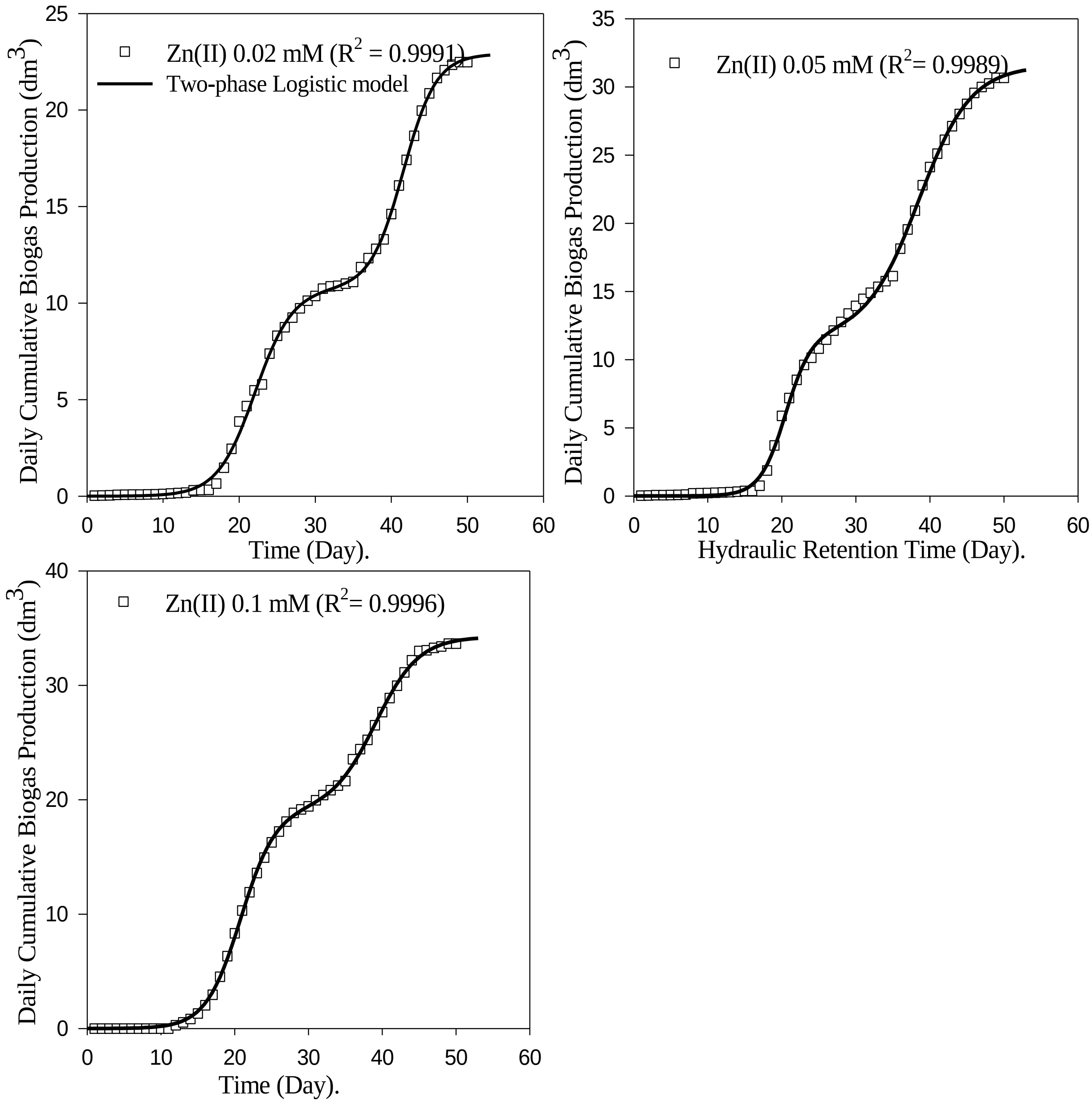

Figure 4. Mean cumulative biogas production curves from Zn supplementation fitted with two-phase logistic function model.
Source: Authors.

observed improvement in biogas yield could be attributed to enhanced process stability. Tian et al. (2019) investigated the impact of Zn addition on the Cd-containing AD process, biodegradation and the microbial communities. The results obtained showed that the addition of Cd together with Zn (Cd + Zn) increased the maximum daily and cumulative biogas yields, and brought forward the gas production peak compared with the Cd-added group. Zn addition was reported to have promoted the activity of coenzyme M and increased the abundance of Methanothermobacter.

However, contrary to the recorded stimulatory characteristics of Zn and Cd in anaerobic digestion (AD) for biogas production in this study, Abdel-Shafy and Mansour (2014) and Dokulilová et al. (2018) in their separate studies have reported that Cd and Zn exhibited inhibitory effects on anaerobic digestion of sludge at a concentration of 400 mg Zn^{+2}.

The AD process was diauxic in all the reactors with two biogas production peaks. After the initial lag phase, gas production commenced and shortly attained the first peak of biogas production which is an indication that the necessary stages (hydrolysis, acidogenesis, acetogenesis and methanogenesis) of AD have been triggered off. The bio-digestion process at the acidogenic stage may have resulted in a high production and accumulation of volatile fatty acids (VFA) which inhibited the methanogenic activities and hence, the declined biogas production. The second peak may be attributed to the reduction in the volatile fatty acids concentration that now gave way for effective methanogenesis and biogas production. In the AD of collagen-based substrates for biogas generation reported by Gomes et al. (2019), diauxic response in gas production was observed in some batches, and the authors attributed it to excessive production and accumulation of acetic acid and propionic acid. Two-step AD was previously reported by Kim and Kim (2017). In their study, agricultural by-products with a high or medium level of carbohydrate and low fat (Cheese whey, Cabbage and Skim milk) exhibited a single step digestion process whereas low carbohydrate and high fat level (Bean curd and, Perilla seed) showed two step digestion process.

The diauxic curves were modeled using the adapted model, two-phase logistic function model and a good fit was achieved as indicated by the high coefficient (R^2) which was $>$ 0.999. Anaerobic digestion of complex and lignocellulosic organic wastes most often result in diauxic response and the modeling has mostly been carried out using modified Gompertz model, logistic function model, etc. The two-phase logistic function model brings solution to modeling of diauxic behaviors in biogas production process by anaerobic digestion. The diauxic response in microbial growth could be adapted in mathematical terms using segmented regression or the sum of two functions (Gomes et al., 2021), and modeling of diauxic patterns have been undertaken by several researchers (Björkmalm et al., 2018; Kim and Kim, 2018). Mischan et al. (2015) evaluated the goodness of fit of three models: Model I - monophasic logistic model, Model II - diphasic logistic model segmented regression and Model III - diphasic logistic sum of functions to the observed growth data of the trunks of *Eucaliptus grandis*. The results obtained showed a better fit of the logistic diphasic sum as compared with segmented regression and monophasic logistic models.

Conclusion

Results from this study have shown that bio-stimulatory characteristics of Zn (II) and Cd (II) at low concentrations could be harnessed in improving biogas yield from livestock manure. The peak of cumulative biogas yield was recorded at 0.05 mM Cd (II), giving 0.15 dm^3/g VS (45.29%), however, the yield declined by 8.54% at 0.1 mM Cd (II), with 0.14 dm^3/g VS (36.75%) yield. Biogas yield from the livestock manure increased with increase in the concentration of Zn (II) addition, the highest was at 0.1 mM Zn (II) concentration, with the yield of 0.16 dm^3/g VS which amounted to 48.83% increase relative to the control.

Plots of the daily cumulative biogas yield against hydraulic retention time indicated diauxic behavior in all the treatments. Diauxic responses in anaerobic digestion of complex lignocellulosic agricultural wastes for biogas production occur in most occasions and are often times ignored. Simulation of diauxic biogas production pattern with the most commonly used models such as logistic and modified Gompertz model have not properly described the anaerobic digestion process and fit the biogas production curves accurately. The suitability of the adapted model, two-phase logistic function model with high accuracy in simulation (as indicated by the R^2) of diauxic biogas production curves has been demonstrated in this study.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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