Serial Anaerobic Digestion of Agricultural Substrate

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Seriell Anaerob Nedbrytning av Jordbrukssubstrat

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Abstract

As the global demand for sustainable energy rises, optimizing biogas production and minimizing post-digestion GHG emissions are crucial. This study compared two serially connected continuously stirred anaerobic reactors (CTRSs) with different volume configurations (65:35% and 35:65%) with a conventional single-step reactor (B1). The study aimed to investigate the effects of system configuration on biogas production, degradation, process efficiency, and stability, while also evaluating the possibility of mitigating post-digestion methane emission during storage and improving digestate quality. All systems were fed with agricultural substrate (manure and crop residue), with an overall organic loading (OLR) for the study of 2.2 L/day, with a retention time of 55 days. The study was repeated for a 1.3 hydraulic retention time (72 days) at a mesophilic temperature of 37°C.

Methane production was highest in the 65:35% serial configuration (C-system), producing 2.3-4.8% more specific methane than the single system and the 35:65% serial configuration (D system). In both serial systems, 70-90% of the methane was produced by the first reactors, with a small quantity, 20-30 % from the second reactors. Process stability was high for all reactors despite an initial volatile fatty acids (VFA) accumulation in the first 30 days, indicating a temporary microbial inhibition, which later decreased due to improved VFA conversion and microbial adaptation.

The serial C system had the highest volatile solids (VS) reduction (86%), indicating a higher substrate degradation, followed by the single-stage B1 (85%) and the D system (84%). Ammonium levels increased across all systems, showing mineralisation of proteins, with the serial systems having slightly higher levels than the single stage B1.

These findings suggest that serial digestion could enhance methane production and degradation efficiency, specifically with a larger to smaller volume configuration. However, its effectiveness depends on retention time, system configuration, and microbial adaptation with implications for improving digestate quality and reducing residual methane emissions during post-digestion storage.

Table of contents

Abbreviations

Abbreviation	**Description**
AD	Anaerobic Digestion
TS	Total Solids
VS	Volatile Solids
VFA	Volatile Fatty Acids
HRT	Hydraulic Retention Time
CSTR	Continuous Stirred Tank Reactor
OLR	Organic Loading Rate
SMP	Specific Methane Production
C/N	Carbon to Nitrogen Ratio
VMP	Volumetric Methane Production
GHG	Greenhouse gas
SPD	Single-phase Digestion
SAD	Serial Anaerobic digestion
HPLC	High Performance Liquid Chromatography
TPAD	Two-phase Digestion

List of tables

List of figures

1. Introduction

The increasing global demand for sustainable waste management, nutrient recovery, and renewable energy production has promoted the anaerobic production of biogas as an environmentally friendly and effective way to achieve this demand (Caruso et al., 2019; Holl et al., 2022; Mao et al., 2015; Mata-Alvarez et al., 2014) Anaerobic digestion (AD) involves transforming organic materials into biogas and digestate through a series of microbial processes. This process retains mineral nutrients in the digestate, making it suitable as a biofertilizer for agricultural use, along with the production of a renewable source of energy (Abdeshahian et al., 2016; Rouhollahi et al., 2020). AD technology is widely applied in treating diverse organic waste across various sectors, such as municipalities, households, wastewater treatment, agro-industrial facilities, and agriculture (Caruso et al., 2019; Mao et al., 2015; Mata-Alvarez et al., 2014).

In the agricultural sector, AD presents great opportunities. It provides local energy, reduces greenhouse gas emissions (GHG), improves waste management, and promotes nutrient recycling and biofertilizer provision, which minimizes agriculture's dependence on mineral fertilizer (Mao et al., 2015). Agricultural waste, such as manure and crop residues, contributes to a significant part of the organic waste produced globally, and to the overall GHG emissions, yet it is presently underutilized for biogas production (Sharma et al., 2024). Although produced in significant quantities, these substrates are frequently managed inadequately. Manure emits methane and nitrous oxide during storage (Ahlberg-Eliasson et al., 2017), while crop residues are incinerated or left to decompose, resulting in further emissions (Zhao et al., 2024). The integration of agricultural waste into biogas systems contributes to the climate objective and sustainability goals (Abdeshahian et al., 2016; Khan & Martin, 2016).

Nevertheless, various challenges inhibit the degradation efficiency of such substrates and thus the efficiency and economy of the systems, limiting the expansion. Manure is characterised as a poor substrate for biogas with a low methane yield due to high water content and prior partial breakdown before digestion (Ahlberg-Eliasson et al., 2017; Nwokolo et al., 2020). Crop residues, while having a high biogas potential, can be rich in lignocellulose fibre, a complex organic material that is difficult to degrade by anaerobic microorganisms. Co-digestion of manure and crop residue can help increase their biogas potential and improve nutrient balance.

Most conventional agricultural biogas plants are designed with one or two continuously stirred tank reactors (CSTR). If two reactors are used, the most common setup is to have a comparably larger first reactor and a smaller postdigester. Agricultural biogas system typically faces limitations such as insufficient breakdown of organic matter, potentially related to a relatively short retention time, which affects the overall degradation efficiency, biogas production, and organic matter content in digestate (Ahlberg-Eliasson et al., 2017; Feng et al., 2017). Incomplete degradation results can result in a surge of methane emissions during digestate storage and utilization (Romio et al., 2021). Connecting two reactors in series gives a prolonged retention time. It can improve the breakdown of substrate during digestion, and as a consequence, result in lower residual methane production during storage and handling of the digestate. The ideology of operating two or more CSTRs in sequence presents a strategy to improve the degradation efficiency of recalcitrant substrates like lignocellulose fibres and enhances the overall gas production of agricultural substrates (Feng et al., 2017; Kaparaju et al., 2009; Perman et al., 2022).

However, the optimal volume distribution and retention time of such a setup are not precise, as different studies have shown varying results in this regard. For example, Boe and Angelidaki (2009) demonstrated that serial CSTR configuration with volume distributions 90:10 and 80:20 achieved an 11% increase in methane production compared to a single-stage reactor. Similarly, Kaparaju et al. (2009) reported that a volume distribution of 50:50 and 70:30 enhances methane production by 13-17% compared to the single-stage reactor. The study also showed that the 50:50 and 70:30 % volume configurations enhanced methane productions compared to the 13:87, which yielded no improvement and produced lower methane than the single stage system

This study investigates the effectiveness of serial digestion in enhancing the treatment of agricultural feedstocks, with a focus on the co-digestion of manure and crop residue. The objective was to compare two volume distributions, 65:35% and 35:65%, in terms of anaerobic digestion performance during serial processes versus that in a one-step CSTR. It aims to evaluate how these configurations affect methane production and degradation efficiency. The study also aims to evaluate the nutrient composition of digestate. It is hypothesized that a volume distribution of 35:65% will yield a higher methane output and degree of degradation compared to a 65:35% distribution. This was hypothesized due to the performance of the 35:65% distribution in a previous experiment. The rationale was that a higher organic loading in the first reactor would facilitate the degradation of the most accessible substrate, leading to increased biogas production. The second reactor with a larger volume would give a prolonged time for microbial degradation of recalcitrant compounds, compared to reactors with smaller and larger volumes. If confirmed, this could, therefore, be considered a more environmentally sustainable biogas system for agricultural biogas plants.

2. Literature Review

AD system design plays a crucial role in optimizing biogas yield and process stability. An AD system can be set up in different designs, and several factors can limit its operation. They can be operated with one organic waste or a combination of substrates. This chapter presents an overview of AD, including core principles, environmental benefits, commonly operated AD designs, including serial anaerobic digestion, biochemical processes involved in AD, and the operation parameters that influence the process efficiency and stability.

2.1 Biochemical Processes in Biogas Production

Anaerobic digestion consists of four sequential stages:hydrolysis, acidogenesis, acetogenesis, and methanogenesis, each characterized by specific microorganisms (Figure 1). The biochemical stages are interrelated, with the byproduct of one phase acting as a substrate for the subsequent phase. The stages of anaerobic digestion operate in a dynamic equilibrium, where the obstruction of one reaction subsequently impacts the other reactions. They co-occur in an anaerobic setting, yielding biogas and digestate as byproducts. (Satpathy & Pradhan, 2023).

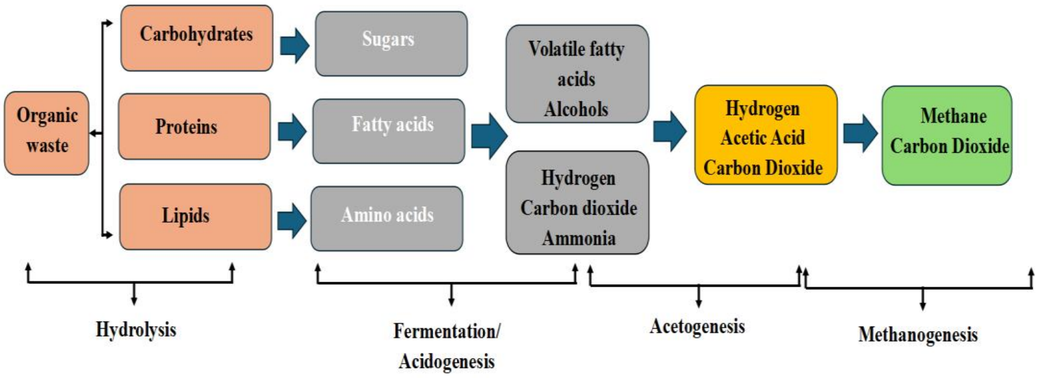


Figure 1:The four phases of anaerobic digestion of organic waste (Satpathy & Pradhan, 2023)

2.1.1 Hydrolysis

During hydrolysis, highly complex organic substances, including carbohydrates, proteins, and lipids, are broken down by microorganisms into basic, water-soluble compounds such as simple sugars, fatty acids, amino acids, and peptides.

Hydrolytic microorganisms, including facultative bacteria and anaerobic fungi, decompose this complex organic matter (Nwokolo et al., 2020). Microorganisms excrete extracellular enzymes, including cellulase, amylase, and proteinase, which facilitate biodegradation. This phase prepares the substrate for subsequent degradation during fermentation, as fermentative bacteria are unable to assimilate complex polymers directly (Schnürer et al., 2016).

2.1.2 Fermentation/Acidogenesis

At the acidogenesis stage, acidogenic bacteria transform the products from hydrolysis, such as sugars, amino acids, and lipids, into organic acids, volatile fatty acids (VFAs), alcohols, lactic acids, carbon dioxide (CO_2), and hydrogen gas (H_2). Facultative anaerobes, including bacteria, are predominant and responsible for fermentation and the initiation of acidogenesis (Nwokolo et al., 2020). The fatty acids (long-chain fatty acids) released from hydrolysis are not utilized in the phase but in the acetogenesis stage (Schnürer et al., 2016).

2.1.3 Acetogenesis

In the acetogenesis stage, products from fermentation, particularly VFAs longer than acetic acid, such as propionic and butyric acid, are converted to acetate, H_2, and CO_2. Syntrophic acetogens, in collaboration with methane-producing microorganisms, which are obligate anaerobes, are responsible for this process. (Nwokolo et al., 2020).

2.1.4 Methanogenesis

This is the final stage in anaerobic digestion. Methane-producing microbes, called methanogens, convert the acetate, hydrogen, and carbon dioxide produced in the previous stage to methane. Methanogens are archaea that are obligate anaerobic microorganisms. Acetotrophic and methanogenic microbes facilitate the conversion of acetate into methane (CH_4) through decarboxylation, whereas hydrogenotrophic methanogenic bacteria generate methane by reducing CO_2 with H_2 (Nwokolo et al., 2020; Schnürer et al., 2016).

2.2 Anaerobic Digestion of Agricultural Substrate

2.2.1 Mono-Digestion

Mono-digestion involves the digestion of one type of organic waste. Mono digestion of the substrate, depending on its composition, may present challenges, such as nutritional imbalance for microbes, an imbalanced C/N ratio, low biodegradability, and operational constraints, including process instability (Ma et al., 2020). Mono-digestion of agricultural substrates such as manure or crop residues can pose similar challenges. Manure is a protein- and nitrogen-rich substrate with a good buffering capacity; however, it has a low TS/VS content and sometimes a low C/N ratio, which can make it a poor substrate for mono-digestion. It also has a low total solids/volatile solids (TS/VS) content, which limits the quantity that can be added to reactors without negatively affecting degradation time. This can result in low system efficiency due to a slow degradation (Abdeshahian et al., 2016; Caruso et al., 2019). Additionally, the low energy content (low methane potential) of manure compared to food waste and other organic wastes further impacts the process efficiency and economy.

Crop residues are rich in complex organics, with typical slow degradation in AD. They mainly comprise cellulose, hemicellulose, and lignin, which influence their degradation. Cellulose is the most abundant and easily degradable, followed by hemicellulose, while lignin is highly recalcitrant to degradation. Lignin binds cellulose and hemicellulose to form a complex structure, impeding the decomposition of crop residues, especially under anaerobic conditions. (Murphy et al., 2011; Nwokolo et al., 2020). Moreover, the high TS content and sometimes low nitrogen content of the crop might pose challenges during mono-digestion (Mansour et al., 2024). In contrast, mono-digestion of manure, such as swine manure, might be problematic due to high ammonium concentration, which mono-digestion of nitrogen-rich substrates can lead to ammonia/ammonium inhibition. This can reduce biogas yield due to microbial inhibition, and co-digestion can limit these challenges (Ma et al., 2020). These limitations in the single substrate justify the need for co-digestion to help complement these challenges.

2.2.2 Substrate Composition and the Role of Co-digestion

The organic composition of substrates plays a critical role in anaerobic digestion, influencing digester performance, methane yield, and the overall quality of both biogas and digestate. Different substrates exhibit varying total solids (TS) and volatile solids (VS) fractions. Factors such as VFA concentrations, C/N ratios, and distinct proportions of carbohydrates, proteins, and lipids significantly impact gas production, process efficiency, and system stability (Nwokolo et al., 2020).

Substrates serve as the primary nutrient source for microbial communities, supporting their growth, reproduction, and metabolic processes. Maintaining a balanced substrate composition is essential for optimizing biodegradation and ensuring stable process performance (Mata-Alvarez et al., 2014). Any imbalance in these components can lead to inefficiencies in digestion, reduced methane production, and potential instability in the system.

Co-digestion can help address these challenges posed by imbalanced substrate compositions, this can help enhance nutrient synergy, stabilize pH and improve biogas yield.

Co-digestion involves the digestion of two or more organic wastes simultaneously. Co-digestion of substrate creates a nutrient and pH balance that can enhance biogas production in an AD system (Ma et al., 2020; Mata-Alvarez et al., 2014).By co-digestion, it is possible to resolve problems related to C/N imbalance, substrate degradability, pH regulation, and moisture content balance, among others. Combining manure and agricultural solid waste allows both substrates to synergistically benefit each other, improving digestion efficiency, gas production, and the overall characteristics of the digestate. (Ahlberg-Eliasson et al., 2017; Feng et al., 2017; Nwokolo et al., 2020). Co-digestion of animal manure with other feedstocks has been shown to significantly improve biogas yields, with increases ranging from 25% to as much as 400% compared to the mono-digestion of individual substrates (Rabii et al., 2019).

2.3 Anaerobic Digestion Designs

Biogas production can be operated in various modes and designs, including continuous, batch, plug-flow, and upflow anaerobic digestion systems. It can be designed as a single-phase, two-phase, or serially connected system. These various designs can be operated in continuous and batch systems.

2.3.1 Single-phase vs Two-phase

Single-phase (SPD) involves all the biochemical stages (hydrolysis to methanogenesis) occurring in one tank. Single-phase design is the most commonly and widely commercialised scale design compared to serial anaerobic digestion (SAD) and Two-phase anaerobic digestion (TPAD) due to its simpler design, lower capital and operational cost (Boe & Angelidaki, 2009; Kaparaju et al., 2009). Two-phase anaerobic digestion (TPAD), also known as two-stage AD, is a design in which the methanogenesis process (second stage) is physically separated from the hydrolysis and fermentation processes (first stage) and is carried out in two separate tanks. The primary or first stage is typically operated at a shorter HRT, while the second stage has a longer HRT to facilitate methanogenesis (Boe & Angelidaki, 2009). The primary stage is heated to facilitate the functioning of hydrolytic and acidogenic bacteria, while the second stage usually also requires heating and mixing to maintain optimal temperature and microbial contact, as the heat generated is not enough to sustain the temperature condition on its own (Bchir et al., 2011).

In some TPAD designs, thermophilic (50-60°C) temperatures are applied in the first stages, and mesophilic (50-60°C) temperatures are applied to optimize microbial activity and system stability (Angelidaki et al., 2011). TPAD has been shown to enhance the biodegradability of recalcitrant substrate, gas yield, and methane production, as well as give better process stability and higher organic loading rate (OLR) compared to the conventional SPD system (Feng et al., 2017). Despite these advantages, two-phase systems have complex and expensive operational and maintenance costs. In AD systems, there is a syntrophic relationship between microbes, where they mutually benefit from each other, with one microbe producing metabolites that the other microbe can consume. The bacteria produce metabolites for methanogens to consume for methane production, for process stability and efficiency. However, in TPAD, there is an interference in this syntrophic relationship between the bacteria and methanogens, which can result in product inhibition in the primary stage (Boe & Angelidaki, 2009).

2.3.2 Serial Anaerobic Digestion (SAD)

Instead of running two reactors in series with phase separation, the concept can be applied to two reactors where all biological steps are run in both reactors simultaneously. Serial Anaerobic Digestion (SAD) is the sequential operation of two or more reactors in series, operating under similar conditions, with both reactors undergoing all the biochemical processes involved in biogas production. These systems consist of a first digester, connected to a second or more digester with HRT split between the reactors relative to their different volume proportions. SAD has been shown to improve the degradation of resistant and complex materials, stabilize the digestion process, and enhance methane production efficiency compared to Single- and two-phase systems (Boe & Angelidaki, 2009; Feng et al., 2017). The connection of two reactors in series reduces “short-circuiting”, i.e., a period during which a portion of the organic material in the feedstock remains in the reactor for a duration shorter than the designated retention time (Boe & Angelidaki, 2009). For instance, in a 30-day hydraulic retention time (HRT) configuration, daily feeding and ejection will yield a digestate mixture comprising both thoroughly digested older substrate and partially digested recently introduced feedstock. SAD can also provide a stable environment that promotes microbial functioning. Serial configuration is beneficial for the digestion of fibrous-rich agricultural feedstocks, such as crop residues, where complete breakdown is crucial to the system's efficiency. SAD systems with volume distribution of 50-70% in the first digester and 30-50% in the second have been reported to increase biogas production by 13-18.7% compared to a single-step reactor (Boe & Angelidaki, 2009; Feng et al., 2017; Y. Q. Li et al., 2017; Perman et al., 2022)

Economically, SAD, compared to one reactor, has the potential to increase overall methane production as well as improve digestate quality and reduce digestion management costs by prolonging digestion to ensure complete degradation and efficient nutrient recovery (Boe & Angelidaki, 2009; Kaparaju et al., 2009). The economic downside of SAD is the costs involved in building a second digester and the operational costs of two reactors instead of one. Environmentally, SAD has the potential to contribute to lower greenhouse gas emissions related to digestate storage and utilization by reducing the residual methane potential in digestate as compared to running a single reactor (Perman et al., 2022). This helps mitigate the climate challenges related to methane emissions.

2.3.3 Batch Digestion

In a batch anaerobic digestion system, the reactor is initially loaded with both the substrate and inoculum, after which it is sealed, preventing any further addition of feedstock throughout the digestion process. This system operates on a discontinuous feeding cycle, typically requiring reloading every 50 to 60 days (Patel et al., 2021). Batch systems are predominantly utilized for dry digestion, maintaining a total solids (TS) content of approximately 20–30%. To enhance substrate degradation efficiency and optimize biogas yield, mechanical stirring is often employed to ensure uniform mixing and prevent stratification within the reactor (Singh et al., 2021). Once the retention period is complete, the digester is fully emptied before a new cycle begins. While batch digestion facilitates substantial breakdown of organic matter and yields high initial biogas production, gas generation declines over time as the available biodegradable material is gradually depleted (Wang et al., 2015).

As a non-steady-state system, batch reactors exhibit temporal variations in substrate conversion rather than spatial differences. At any given time, reactions progress uniformly across the reactor’s volume (Waldron et al., 2020). Several key factors influence the efficiency of anaerobic digestion in batch systems, including the inoculum-to-substrate ratio, inoculum activity, and the duration of the acclimation period. These parameters significantly impact reaction kinetics, methane yield, and overall process stability (Valentin et al., 2024). A batch system is easier to operate, requires less investment, and has a higher degree of degradation than a continuous system. However, it requires a longer retention time and consumes a smaller feed volume, as batch reactors have a specific amount they can hold for the set digestion. There is no continuous gas flow as it decreases over time (Zhou & Wen, 2019).

2.3.4 Continuous Digestion (SAD)

In a continuous anaerobic digestion system, the reactor is regularly supplied with fresh substrate while simultaneously discharging the digested material, ensuring a stable and consistent biogas production rate. The frequency of feeding and ejection typically ranges from 1 to 10 times per day, continuing until the HRT is reached (Svensson et al., 2018). The retention duration is related to the substrate type and is influenced by temperature, which affects the rate of degradation (Patel et al., 2021). In contrast to batch systems, the inoculum remains within the digester, sustaining microbial activity and facilitating uninterrupted gas production (Kakuk et al., 2017). This system is predominantly utilized for wet digestion and represents the most employed biogas production method (Anaya-Reza et al., 2024). Reactor designs may incorporate continuous or intermittent stirring to enhance mixing efficiency and prevent the formation of stratification. Some configurations involve unidirectional flow, where minimal stirring is applied, allowing the substrate to enter from one side and exit from the other after digestion is complete (Anaya-Reza et al., 2024; Kakuk et al., 2017). The most applied reactor technology is the CSTR, the continuously stirred tank reactor. Continuous systems provide significantly higher and stable biogas yields and require a shorter retention time. However, they have complex operation and control requirements and are expensive to set up compared to batch operations (Zhou & Wen, 2019).

2.3.5 Total Solids and Volatile Solids

TS and VS are key parameters influencing gas production and overall process efficiency in anaerobic digestion. TS represents the total dry matter content in a substrate and is typically measured by drying a sample at 105°C. An optimal TS concentration is crucial for efficient digestion, as both excessively high and low TS levels can negatively impact biogas production (Kakuk et al., 2017). TS content between 9-13% can enhance the gas production rate per unit volume (Yavini et al., 2014).

VS refers to the organic fraction of the substrate's dry matter (TS). VS constitutes a significant portion of TS and represents the biodegradable fraction of the substrate. A high VS content can lead to greater gas production and increased methane production when the substrate is easily degradable. This is because a substrate with high VS per unit TS can allow a high OLR without causing a short HRT, hence improving the overall methane and gas production per volume reactor (Ahlberg-Eliasson et al., 2017; Schnürer et al., 2016)). Notably, TS and VS measurements can be influenced by the presence of VFA and other volatile compounds within the substrate, resulting in lower recorded values during heating analysis (Schnürer et al., 2016).

2.3.6 Carbon-To-Nitrogen Ratio(C/N)

The C/N ratio affects the degradability of substrates by microorganisms and plant operation. A well-distributed C/N ratio is essential for efficient substrate degradation and microbial functioning (Abdeshahian et al., 2016). For optimal degradation in an AD process, this ratio has been suggested to be set between 15 and 25 (Schnürer et al., 2016), though higher ratios out of this range have also been reported without any process failures (Risberg et al., 2013). A higher C/N ratio poses a risk of nitrogen limitation to microbes. Higher ratios can also lead to process inhibition with a high fraction of easily degradable carbon substrate, which can cause rapid acidification. Ammonium/ammonia is released during the degradation of proteins. On the other hand, excess nitrogen can lead to high ammonia levels and inhibit methanogens (Rajagopal et al., 2013). A low C/N ratio can lead to ammonia inhibition, followed by VFA accumulation, resulting in reduced methane yields (Mao et al., 2015). The degradation of the excess C/N ratio can be balanced by adding nitrogen or carbon-rich substrates in co-digestion to help improve methane yields (Mao et al., 2015; Mata-Alvarez et al., 2014).

2.3.7 Fats, Proteins, And Carbohydrates

Lipids and protein-rich substrates have the most significant methane potential. However, degradation of lipids, such as fats and slaughter waste, may also lead to the production of toxic fatty acids, resulting in a decrease in pH. The breakdown of lipids can also lead to foaming due to the release of long fatty acids (Rasit et al., 2015). High amounts of fats/lipids will affect the microbial community and its functioning and may also lead to process inhibition in the digester. Protein digestion releases ammonium, which can increase the process's alkalinity due to the shift caused by ammonia volatilization. High fractions of ammonium enhance the digestate value as a fertilizer. However, it might inhibit the activities of the methanogens and cause process instability (Nwokolo et al., 2020; Rasit et al., 2015). In addition, a substrate rich in proteins can also result in a high fraction of hydrogen sulphide, which impacts gas quality and overall process (Ahlberg-Eliasson et al., 2017).

Carbohydrates (lignocellulose) rich substrates are grouped into mono, di, and polysaccharides. Monosaccharides and disaccharides are easily and quickly broken down. A high content of easily degraded carbohydrates can lead to an increase in fatty acids, as well as instability in the digestion process, while a low content provides no challenges (Zuo et al., 2015). Polysaccharides, on the other hand, exhibit low solubility and, in the case of lignocellulose, are recalcitrant to degradation (Nwokolo et al., 2020). Variation in composition and structure leads to different degradation rates, but they are, in general, slower than for di-and monosaccharides.

2.4 Evaluation of process efficiency

The efficiency of the AD process can be determined and quantified in different ways, such as specific methane and volumetric gas production, degree of degradation (VS reduction), and nitrogen mineralization *(Schnürer & Jarvis, 2018)*

2.4.1 Volumetric Gas and Specific Methane Production (SMP)

Volumetric gas represents the daily amount of gas produced per digester volume. SMP refers to the amount of methane produced per the quantity of organic material (VS) fed into the digester per day (SMP). Volumetric gas reflects how the digester volume is used efficiently, while SMP indicates the efficiency of the substrate's biodegradability over the digestion period (Schnürer et al., 2016). Volumetric gas is expressed as m^3 CH_4/ m^3 reactor volume and day, while SMP is expressed as m^3CH_4/kg VS day.

2.4.2 Degree of Degradation (VS reduction) and Nitrogen Mineralization

The degree of degradation evaluates the VS proportion of the substrate degraded during the period of digestion. It involves comparing the VS of the substrate out to the VS of the substrate into the digester. A higher VS reduction indicates an effective and efficient process, and vice versa, which correlates with the HRT (Schnürer et al., 2016).

Nitrogen mineralisation is the conversion of organic nitrogen to ammonium during digestion. It measures the difference between the total ammonium nitrogen concentrations in the digestate and the raw substrate. Higher mineralisation represents a higher protein breakdown, providing information on nitrogen conversion efficiency as well as the potential risk of ammonia inhibition (Schnürer & Jarvis, 2018).

2.5 Operational Parameters

2.5.1 Organic Loading Rate (OLR)

The OLR defines the ratio of the incoming organic matter flow to the active volume and duration of the reactor (Eq. 1). The OLR of the feeding substrate can be determined based on VS or chemical oxygen demand (COD). COD indicates the quantity of oxygen necessary for decomposing all organic compounds in a substrate. It is primarily utilised for diluted substances, such as wastewater (Schnürer et al., 2016). The type of substrate in terms of VS, COD, and degradability influences the OLR of the Feedtsock (Holl et al., 2022). OLR on VS typically fluctuates between 2 and 6 g VS/L per day, though this is contingent upon the HRT (Orhorhoro et al., 2018). A higher OLR reduces HRT and vice versa (Schnürer et al., 2016).

$$\boldsymbol{OLR} = \frac{Q_S \times VS_{in} \times \mathrm{TS}_{in}}{V_R \times day} \qquad \textbf{Eq. 1}$$

Whereby Qs is the flow into the reactor per day, VS_{in} is the VS content of the feedstock, TS_{in} is the dry matter content of the feedstock, and V_R i is the active reactor volume (Orhorhoro et al., 2018; Schnürer et al., 2016).

2.5.2 Hydraulic Retention Time (HRT)

The HRT defines the retention time of the waste material in the reactor. It is calculated based on the reactor volume ratio to the fed substrate flow (Eq. 2). HRT affects the efficiency of the degradation of waste fractions in biogas production. A higher retention time will ensure efficient and well-degraded substrate, while a decrease in retention will limit the material's degradation time. An HRT between 10-25 days is considered a short HRT range, and a more extended HRT range is between 30- 60 days (Anaya-Reza et al., 2024; Kaparaju et al., 2009). HRT is also dependent on the material used in the system. Substrates high in sugar and starch are highly hydrolysable and quickly decompose; hence, they require shorter retention durations. Slowly hydrolysable substrates such as fibre, lignin, and cellulose-dense plant material require extended durations for microbial degradation (Schnürer et al., 2016).

$$HRT = \frac{\boldsymbol{V_R}}{\boldsymbol{Q_S}} \qquad \textbf{Equ- 2}$$

Whereby Q_s is the volume flow into the reactor per day, and V_R is the active reactor volume (Orhorhoro et al., 2018; Schnürer et al., 2016).

2.5.3 Temperature

An optimal and constant temperature is required for the stable and optimal functioning of microbes, as well as for the biogas system. Temperature fluctuations can affect biogas production. Different organic matter exhibits varying responses to different temperature variations; therefore, the temperature used in a system may depend on the substrate in use (S. Wang et al., 2019). Temperature is categorised into psychrophilic (<20°C), mesophilic (20-45°C), and thermophilic (>50°C) classifications. Reduced temperatures yield increased microbial diversity, and the process exhibits diminished sensitivity to temperature variations. Thermophilic and mesophilic temperatures enhance gas production and degradation compared to non-heated systems, by accelerating hydrolysis and acidogenesis (Q. Li et al., 2020). Elevated temperatures also benefit microbes and their overall activity. Elevated temperatures facilitate the degradation rate, allowing increased organic loading rate and shorter HRT. Despite this, thermophilic temperatures have a lower microbial diversity compared to mesophilic temperatures, which can result in a less stable and efficient process. Higher temperatures increase the inhibition risk as they shift the equilibrium between ammonium and ammonia, increasing ammonia levels (Angelidaki et al., 2011). Psychrophilic temperatures exhibit inferior process performance relative to mesophilic and thermophilic temperatures (Holl et al., 2022). Thermophilic temperatures facilitate the sanitisation of materials by eliminating pathogens and lowering the viscosity of feedstock (Schnürer et al., 2016; R. Singh et al., 2023).

2.6 Monitoring and Stability Indicators

Various parameters influence the degradability of waste in a biogas system. These parameters affect the process efficiency, methane yield, and optimization. The microbial community in a biogas system requires different conditions to thrive and function effectively. Parameters such as temperature, pH, alkalinity, VFA, and ammonia/ammonium concentration are monitored to assess and maintain process stability of AD.

2.6.1 pH and Alkalinity

A stable and optimal pH is essential for the effective functioning of microbes and the biogas system. The different microbial groups have an optimal pH for their functioning and growth. The optimal pH between 7.0 and 8.5 is considered ideal for bio-methanation; however, it depends on the system's substrate type and operating parameters (Schnürer et al., 2016). Acidic conditions lower gas production and slow down the process performance. Higher pH levels can also disrupt biogas production and microbial balance. A high alkalinity can maintain a stable pH even during acidification, resulting in a more robust system in acid accumulation. Elevated and consistent alkalinity is necessary to maintain optimal and neutral pH levels. Ammonia released during the degradation of protein-rich substrate and volatile fatty acids can increase alkalinity. Excessively high or low alkalinity can adversely impact the bio-methanation process by inhibiting its progression (Schnürer et al., 2016).

2.6.2 Ammonia/Ammonium-Nitrogen

Ammonia and Ammonium are synthesized via amino acid fermentation, resulting from the hydrolysis of protein-rich materials. Elevated levels may result from system instability, signifying a reduction in the methane production rate and an increased volatile fatty acid content in effluents. Ammonium aids in pH buffering within reactors by enhancing process alkalinity and improving digestate quality. Ammonium-nitrogen proportions depend on the type of organic matter substrates, degree of degradation, temperature, pH, and microbial activity (Schnürer et al., 2016). A prolonged retention time results in an increased conversion of nitrogen in the substrate to ammonia. A substrate with higher nitrogen content will result in increased release of ammonia/ammonium. Elevated pH and temperature can enhance ammonia volatilisation, resulting in toxicity to methanogens and diminishing overall process efficiency, as ammonia is comparably toxic. Concentrations ranging from 53 mg/l to 1450 mg/l are deemed toxic and can induce ammonia inhibition (Rajagopal et al., 2013).

2.6.3 Volatile Fatty Acids (VFA)

VFAs are short-chain fatty acids, including acetic, propionic, butyric, and others. They are produced during the acidogenesis phase, which is the breakdown of substrates by acidogenic bacteria. VFA gives a good indication of the process stability of anaerobic digestion. The accumulation of VFA indicates an imbalance in the microbial steps and might lead to instability and failure by reducing the pH levels and suppressing microorganism/enzyme activity (Zuo et al., 2015). VFA composition varies depending on the type of organic matter used. Fats produce high VFA concentrations compared to protein and carbohydrates due to their high energy content (Liu et al., 2018).

3. Methods and Materials

3.1 Substrate Composition

Liquid manure and crop residues were obtained from the biogas treatment facility for the Swedish University of Agricultural Sciences (SLU) at Lövsta, Uppsala. The liquid manure, derived from diverse animals including cattle and pigs, was harvested from the farm at Lövsta and subsequently stored in the cooling room at 3.5 °C post-collection and during usage. The manure was blended with a kitchen blender to reduce its particle size before mixing with crop residue for feeding. The crop residue comprised diverse materials, such as flour, wheat, grass, and silage from the farm. It was preserved in a freezer at -20 °C until use to inhibit degradation and mould proliferation. The feed was prepared daily by combining manure, crop residue, and water in a specific ratio, similar to the method used at the biogas plant at Lövsta. An iron ($Fe^{2+/3+}$) chloride additive($FeCl_3$) was incorporated into the prepared feed to trap sulfide, to form iron sulfide, and reduce the amount of free hydrogen sulfide (H_2S) and sulfide ($S^{2-)}$ remaining. This enhances the overall quality of gas production. This was also executed in the full-scale facility. On wet weight, the total feedstock mixture had a VS and TS content of 12.3 and 14%, repsectively. The TS of the manure and crop residue was 6.5% and 75%, and the VS content was 4.6% and 69.4% respectively. The characteristics of the feedstock are shown in Table 1.

The **VS** of the mixed feedstock was calculated by

$$VS\ (\%) = \left(\frac{T_{VS}}{T_{WW}} \times 100\right) \qquad \text{Eq 3}$$

Where T_{VS} = Sum of total VS in grams of manure and crop residues in feed stock

T_{ww}= Total amount of feed stock in wet weight

The **TS** of the mixed feed stock was calculated by

$$TS\ (\%) = \left(\frac{T_{TS}}{T_{WW}} \times 100\right) \qquad \text{Eq 4}$$

Where T_{VS} = Sum of total TS in grams of manure and crop residues in feed stock

T_{ww}= Total amount of feed stock in wet weight

Table 1: Characteristics of Crop residue and Manure substrate

Parameters	Manure	Crops Residue	Mix Feedstock
Dry Matter Content, TS (% of wet weight)	6.4	75.0	14
Volatile Solids, VS (% of wet weight)	4.6	69.4	12.3
VS of Feedstock (g)/day	9.8	111.1	
TS of Feedstock (g)/day	13.6	159.7	
Total Nitrogen (Kg/Ton ww)	3.1	19.5	4.7
Organic Nitrogen (Kg/Ton)	1.6	18..5	3.5
Ammonium Nitrogen (Kg/Ton)	1.6	1..0	12
Tot-Carbon (Kg/Ton)	26.2	342.4	62.6
Tot-C/Tot-N	8.3	17.5	8.0
Total Phosphorus (Kg/Ton)	0.5	5.3	1.0
Total Potassium (Kg/Ton)	2.4	109	3.1
Total Magnesium (Kg/Ton)	0.5	2.30	0.7
Total Calcium (Kg/Ton)	1.1	1.89	1.0
Total Sodium (Kg/Ton)	0.4	0.17	0.3
Total Sulphur (Kg/Ton)	0.4	1.45	0.5

3.2 Experimental Setup

The experiment used laboratory-scale Continuous Stirred-Tank Reactors (CSTRs) (Belach Bioteknik, Stockholm, Sweden). Five reactors were utilised: four were arranged in series (C1, C2, D1, D2), and one was used as a control reactor (B1). Each reactor had a total volume of 10 litres and was equipped with a mechanical propeller to ensure continuous agitation and homogenisation of the substrate. The propeller was operated at a speed of 90 rpm for C2, D2, and B1 and 150 rpm for C1 and D1. The speed for C1 and D1 was adjusted to control foaming, prevent surface accumulation of the substrate, and ensure efficient degradation. External silicon heating mats, regulated for precise temperature control, were affixed to each glass reactor vessel. Gas production was monitored daily using an automated gas quantification system.

Two of the serially connected CSTRs, designated D1 and D2, were arranged in a smaller-to-larger configuration (Figure 2). D1 and D2 had 5 L and 3.5 L active working volumes, respectively. The active volume of D2 was reduced to adjust for the reactor's volume in the laboratory, to accommodate the necessary headspace in each reactor, and secure controlled inflow in D2. The correct combined working volume for the D1 and D2 systems was 14.5 L, having an actual volume of 5 and 9.5 L, respectively. This reflects a volume ratio of 35:65, based on their correct actual volumes. A second set of serially connected reactors, C1 and C2, was arranged in a larger-to-smaller configuration. C1 and C2 had 7 L and 2.5 L active working volumes, respectively. Also, here the active volume of C2 was reduced as compared to the real case to fit the reactor's volume in the laboratory, to accommodate the necessary headspace in each reactor, and control the inflow in C2. The size of C2 was decreased to avoid increasing the volume of C1 further, which would have left insufficient headspace. The correct combined working volume for the C1 and C2 setup was 10.5 L, with the actual volumes of 7 L and 3.5 L, respectively. This reflects a volume ratio of 65:35, based on their actual volumes. The control reactor operated independently, with an active working volume of 5 L. A schematic representation of the experimental setup is provided in Figure 2.

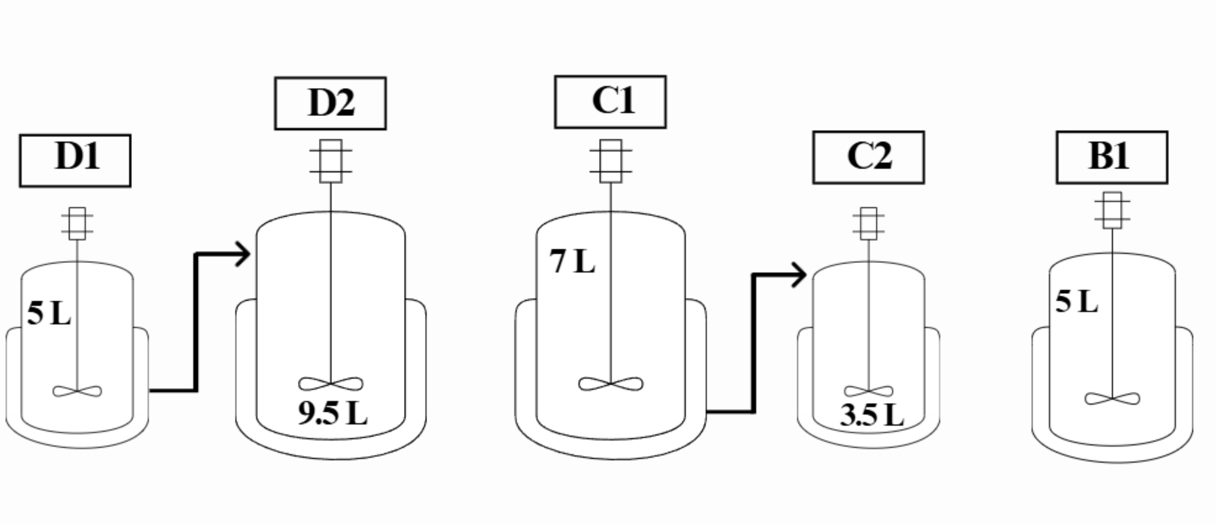


Figure 2: Lab-scale experimental set-up for serial digestion with 35:65% (D1+D2), 65:35% volume distribution (C1+C2), and a one-step (B1) with their correct volume shown.

The inoculum for the reactors was sourced from a full-scale co-digestion biogas facility in Lövsta, Uppsala, which operates a one-step biogas process. The full-scale process operates at 37 °C with a hydraulic retention time of 55 days. The co-digestion process consisted of 84% liquid manure and 16% crop residue on a wet weight basis. The laboratory-scale experiment utilized parameters derived from the full-scale model, including temperature, additive incorporation, HRT, and proportions of feedstock, all of which were scaled down to the laboratory scale. Reactor D1, C1, and B1 received 518 µL per 6.3 VS/day , 385µL per 4.6 VS/day, and 178µL per 2.2 VS/day of iron additive, respectively. Volumes were adjusted to correspond with the total capacities of the laboratory-scale reactors, and feeding rates were determined based on these revised volumes. Reactors were manually filled and emptied once daily, six days a week, resulting in an overall OLR of 2.2 gVS/L across the entire system. However, this was compensated for over 7 days. Reactors D1, C1, and B1 received fresh, mixed diluted substrates with OLRs of 6.3, 4.6, and 2.2 gVS/L per day. D2 and C2 received residues from D1 and C1 with OLRs of 2.3 and 3.1 g VS/L per day. The control reactor B1 receives the same OLR used in operation at Lövsta. The process parameters are presented in Table 2.

Table 2: Process parameters for the laboratory-scale test reactors for an OLR of 6 days.

System	Reactor	HRT (days)	Feeding Rate (g/day)	OLR (gVS/(L/d))	Correct Working Volume (L)	Working Volume in lab(L)	Temperature (°C)	Volume Proportion (%)
Two-step	Whole System	55	-	2.2	14.4	8.5	37	100
	D1	19	306	6.3	5	5		35
	D2	36	113.4	2.3	9.4	3.5		65
Two-step	Whole system	55	-	2.2	10.7	9.5		100
	C1	36	226.7	4.6	7	7		65
	C2	19	153.5	3.1	3.7	2.5		35
Single step	B1	55	106.1	2.2	5	5		100

3.3 Analytical Methods

TS and VS were measured according to Swedish standard methods (SS_28113_SV.Pdf , n.d.). Agri Lab AB in Uppsala analysed the concentrations of total nitrogen, carbon, C/N ratio, phosphorus, potassium, magnesium, calcium, sodium, sulphur, ammonium nitrogen, and organic nitrogen in both the liquid manure and the crop residue, as well as digestate.

The VFA content (lactic acid, acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate) of the feedstock and digestates was determined by High Performance Liquid Chromatography (HPLC) analysis. Samples were pretreated by (1) Centrifugation of frozen samples (11,000rpm for 15min); (2) transfer of 700µl of supernatant and 70µl of 5M sulphuric acid into new 2ml; (3) Freezing of the sample at -20°C; (4) Centrifugation (11000 rmp for 15min); (5) Filtering of supernatant through a 0.2µm membrane into a glass vial.

The chromatographic separation was performed using a Shimadzu 2050 series HPLC system fitted with an ion exclusion column (Rezex ROA organic Acids H+, 200 ×7.80 mm). A mobile phase was operated under isocratic conditions containing 5 mM sulphuric acid with a 0.6 mL/min flow. VFA detection was done with a UV detector set to 210 nm, since carboxylic acids absorb well in this range.

CO_2 composition was measured daily using a saccharometer filled with 7M sodium hydroxide. Gas production was monitored daily using an automated gas quantification system (Belach Bioteknik, Stockholm, Sweden) and calibrated using a RITTER Drum-type TG0.5 (RITTER Apparatebau GmbH & Co. KG, Bochum, Germany). The measured gas volumes were used to set a calibration value for the gas counter on the biogas reactors. Gas composition (CH_4, CO_2, H_2S, H_2, O_2) was analyzed twice a week using the Biogas 5000 (Geotech Instruments, Coventry, UK). Gas volume was normalized for standard temperature and pressure. The calibration value for gas volumes was calculated using volumes measured from the gas meter for each reactor. The pH and VFA to alkalinity ratio (FOS/TAC) was measured weekly with a potentiometric titrator meter at 15-35 °C, using an electrode (Titralab, Hach, AT1000, Germany).

3.4 Mathematical Calculations and Statistical Test

Statistical analyses were performed to evaluate the differences in biogas production and pH for all system setups and between the individual reactors. A paired t-test was used to compare the means of the related reactors/ systems. All statistical calculation was conducted using Minitab with a significance level of 0.05, and all mathematical calculations were conducted with Excel.

Equations 4 and 5 were used to calculate the specific methane production (SMP) for single and serial systems. Equation 4 gives the formula used to calculate the actual SMP for the individual systems D1, D2, C1, C2, and B1 per their respective actual gVS received per week.

$$SMP = \left(\frac{\mathrm{V}\times\mathrm{MC}}{\mathrm{gVS}}\right) \div 100 \qquad \text{Eq.-4}$$

Where SMP= specific methane production (mLCH$_4$/g VS)
V = Volume of gas in NL
MC = Methane content in %
gVS = Mass of ingoing volatile solids for each reactor

Equation 5 gives the formula used to calculate the total SMP for the whole D1+D2, C1+ C2 system based on the total VS added to the first reactors.

$$SMP = \frac{TVMC}{gVS} \qquad \text{Eq-5}$$

Where SMP= specific methane production (mLCH$_4$/g VS)
TVMC = Sum of the volume of methane from the serial system for each reactor (D1+D2; C1+C2) in mL CH$_4$
gVs = Mass of volatile solids added to the first reactor

Equations 6 and 7 calculated the volumetric methane potential (VMP) for single and serial systems. Equation 6 provides the formula used to calculate the actual VMP for the individual systems, D1, D2, C1, C2, and B1, based on their respective actual volumes.

$$VMP = \frac{MC}{V} \qquad \text{Eq-6}$$

Where VMP= Volumetric methane production (mLCH$_4$)
MC = volume of methane from each reactor in mLCH$_4$
V = Volume of individual reactor in L

Equation 7 provides the formula used to calculate the actual VMP for the entire D1+D2, C1+C2, and B1 systems, based on their respective total actual volumes.

$$VMP = \frac{TMC}{V} \qquad \text{Eq-7}$$

Where VMP= Volumetric methane production (mLCH_4)

TMC =Sum of volume of methane from the serial system for each reactor (D1+D2; C1+C2) in CH_4

V = Total volume of the serial system in L

The **degree of digestion (VS reduction)** was calculated using **equation 8**

$$Degree\ of\ digestion = \left(1 - \left(\frac{TS_{\ out} \times VS_{\ out}}{TS_{\ in} \times VS_{\ in}}\right)\right) \times 100 \qquad \text{Eq-8}$$

Where

$TS_{\ in/out}$ = Total solids in the mix of feedstock and in the digestate (% of wet weight)

$VS_{\ in/out}$ = Volatile Solids in the mix of feedstock and in the digestate (% of Total solids)

Nitrogen Mineralization (ML) of reactors was calculated using Equation 9

$$ML = \left(\frac{(NH_4^+ - N)_{digestate} - (NH_4^+ - N)_{substrate}}{(Org - N)_{substrate}}\right) \times 100 \qquad \text{Eq-9}$$

Where NH_4^+-N = Total Ammonium-Nitrogen

Org-N =Organic Nitrogen

4. Results

4.1 Effects of Serial and Single-Step Systems on Methane Production

The single and serial systems were run for 72 days, with day 55 as the first HRT day, and continuously fed once daily for 6 days. The OLRs of the reactors are presented in Table 2. The SMP ranged from 264.5 to 272 mL CH_4/g VS for both the serial systems (D1+D2; C1+C2) and the single system (B1) (Table 3, Figure 3), with no significant difference ($p>0.05$). VMP ranged from 590.4 to 606 mL CH4/L (Table 3, Figure 4), with D1 having the highest value, though it had the lowest SMP compared to C1 and B1. VMP was calculated based on the actual volume configuration of reactors(Table 2). Even though the SMP differences were not significant, the C1 + C2 system produced, on average, 2.8% more methane than the D1 + D2 system and 2.1% more than the single system (calculated for the whole period of operation). The single system B1 had 0.8% higher SMP than the serial system D1 + D2.

Table 3: Analytical data for gas production and digestate for all systems for the period studied: D1+D2 (serial system) with 35%:65% volume configuration, C1+C2 (serial system) with 65%:35% volume configuration, and B1 (single-step system) based on the average weekly gas production. Volume configurations were based on the actual volumes. Standard deviations are indicated within brackets (n=11 for VMP, SMP, CO2 Content, CH4 Content; n=3 for VS_{in}, VS reduction, and ML reduction).

Reactor	pH	VS in Digestate (%)	VS reduction (%)	VMP ($mLCH_4/L$)	SMP ($mLCH_4/g$ VS)	CH4 Content (%)	ML (%)	Methane Production (%)
D- system		4.5	84 (±0.03)	590.4 (±56.5)	264.4 (±25.3)	52.3 (±6.6)		100
D1	7.6 (±0.2)	5.8 (±0.3)	75 (±0.02)	1458 (±100.3)	226.7 (±15.6)	53.1 (±0.8)	6.9	70
D2	7.7 (±0.2)	4.5 (±0.6)	35 (±0.2)	128.6 (±45.4)	101.7 (±35.9)	48.0 (±6.7)	20.7	30
C-system		4.2	86 (±0.04)	606 (±37.7)	272 (±16.9)	51.5 (±3.7)		100
C1	7.6 (±0.1)	5.2 (±0.6)	79 (±0.04)	860.2 (±66.5)	252.6 (±19.5)	51.9 (±0.5)	18.4	90
C2	7.7 (±0.1)	4.2 (±0.6)	26 (±0.3)	125.4 (±35.8)	28.7 (±8.2)	46.5 (±4.0)	23	10
B1	7.5 (±0.2)	4.4 (±0.1)	85 (±0.01)	594.1 (±46.1)	266.5 (±20.7)	51 (±0.4)	20.7	100

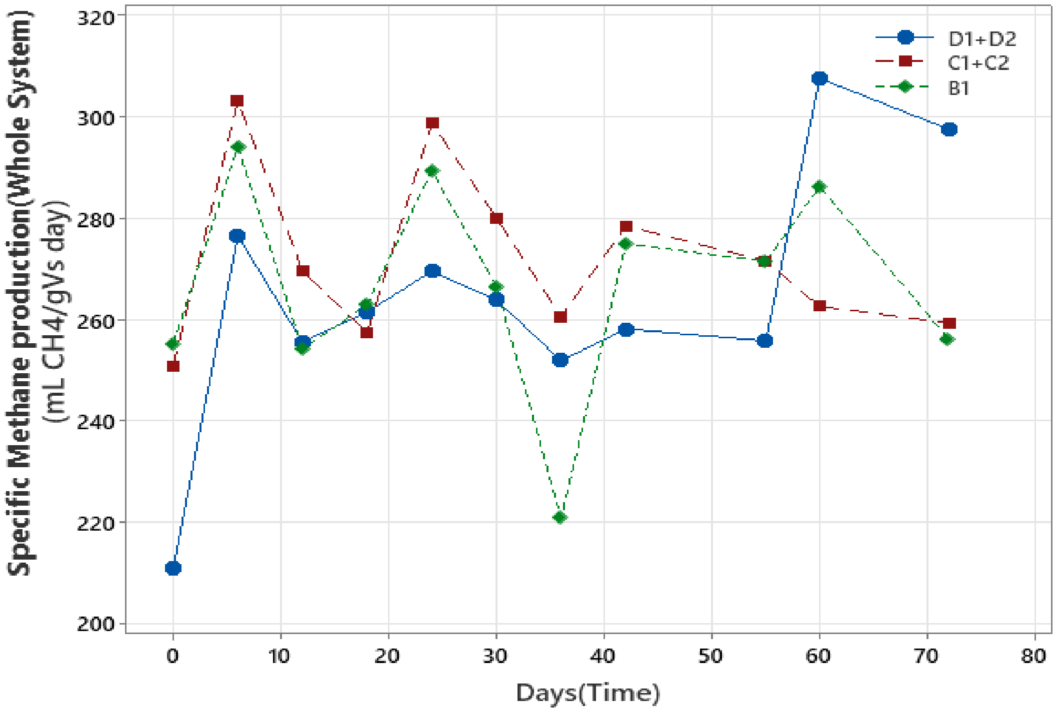


Figure 3: Specific methane production for whole serial systems for the period studied: D1+D2 (serial system) with 35%:65% volume configuration, C1+C2 (serial system) with 65%:35% volume configuration, and B1 (single-step system), based on the average weekly gas production

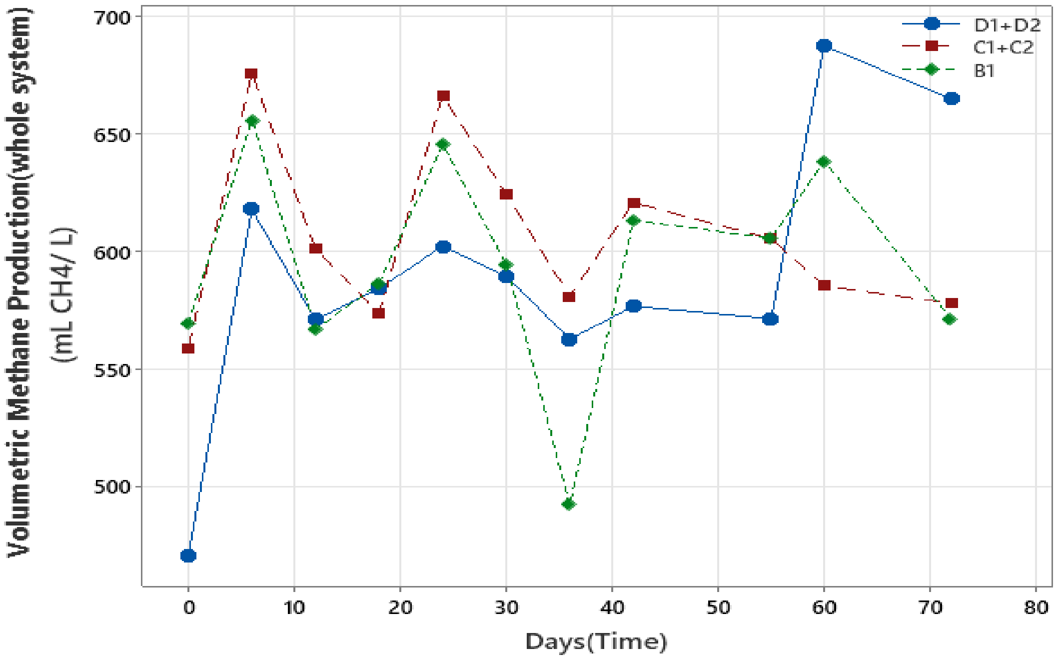


Figure 4: Volumetric methane production for whole serial systems during the studied period: D1+D2 (serial system) with a 35%:65% volume configuration, C1+C2 (serial system) with a 65%:35% volume configuration, and control B1 (single-step system) calculation based on the average weekly gas production. Volume configurations are based on the reactor's actual volumes.

SMP for the first reactors (D1; C1; B1) varied from 226.7 to 266.5 mLCH_4/g VS, while SMP for the second reactors (D2; C2) was 101.7 and 28.7 mL CH_4/g VS, respectively (Table 3, Figure 5). Reactor B1 produced 5.5% and 17.6% more methane per gram of VS than C1 and D1, respectively, and C1 produced 11.4% more than D1. D1, however, had the highest VMP, producing 69% methane than C1and B1, producing 59% less VMP, respectively (Table 3, Figure 6).

D2 had a higher SMP and VMP than C2. SMP differences among D1 and C1, D1 and B1, as well as D2 and C2 were statistically significant ($p<0.05$), whereas the comparison between C1 and B1 was insignificant ($p=0.306$). The VMP differences were statistically significant ($p< 0.05$) for D1, C1, and B1 ($p = 0.00$) but not for D2 and C2 ($p = 0.9$).

Most of the methane production (SMP) from the serial reactors was derived from the initial reactors, with 70% from D1 and 90% from C1, while smaller amounts, 30% and 10%, from the second reactors, D2 and C2, respectively (Table 3). This could be as a result of the fresh feedstock D1 and C1 received, and the second receiving the partially degraded substrate. The methane content (%) in the D system was higher (52.3%) than for the C system (51.5%) and the control B1 (51%). Individually, among the first reactors, the D1 had a higher methane content (53.1%) than the C1 and B1 reactors. Between the second reactors, D2 had a higher methane content (48%) than C2 (46.5%) (Table 3). The methane production between the first reactors, C1 and D1, was significant ($p<0.05$), while the production between C2 and D2 was insignificant ($p = 0.5$), indicating a clear difference between the systems.

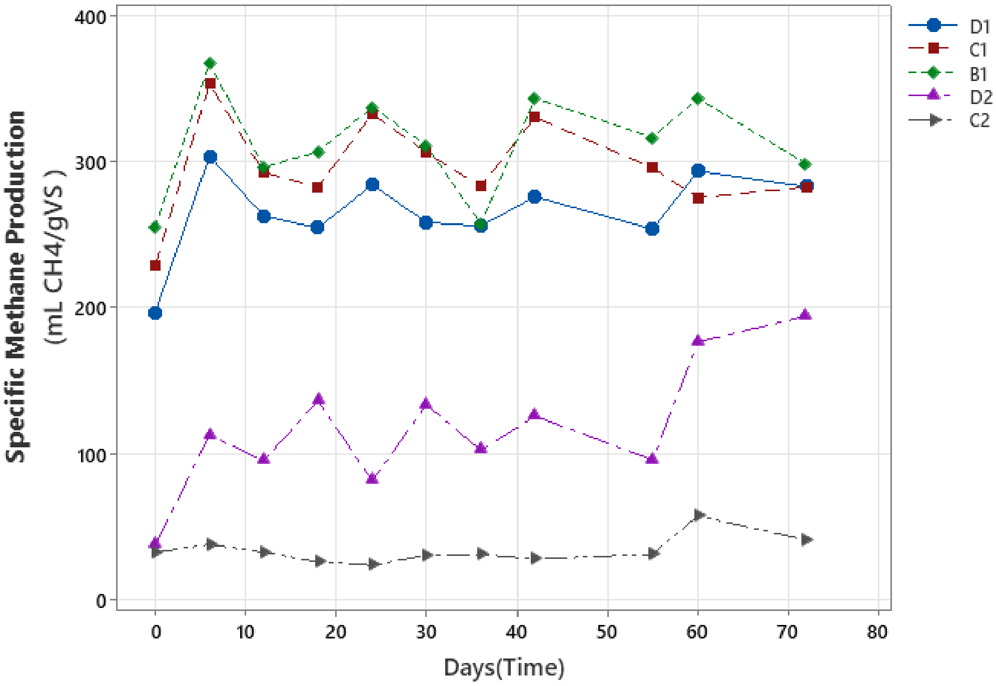


Figure 5: Specific methane production for individual reactors (D1; C1; B1, D2; C2) for the period studied: D1+D2 (serial system) with 35%:65% volume configuration, C1+C2 (serial system) with 65%:35% volume configuration, and B1 (single-step system) based on weekly average productions.

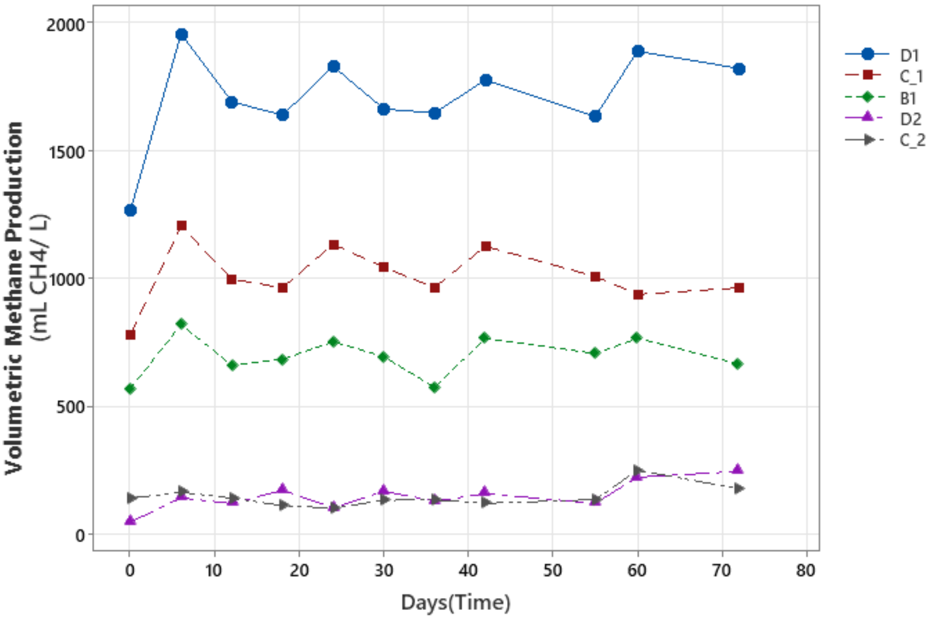


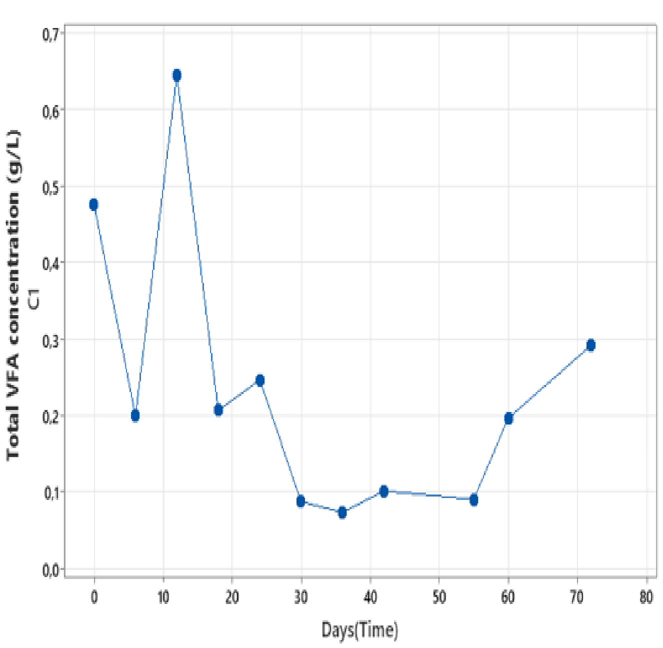
Figure 6:Volumetric methane production for individual reactors (D1, C1, B1, D2, and C2) for the period studied: D1+D2 (serial system) with 35%:65% volume configuration, C1+C2 (serial system) with 65%:35% volume configuration, and B1 (single-step system) based on weekly average production.

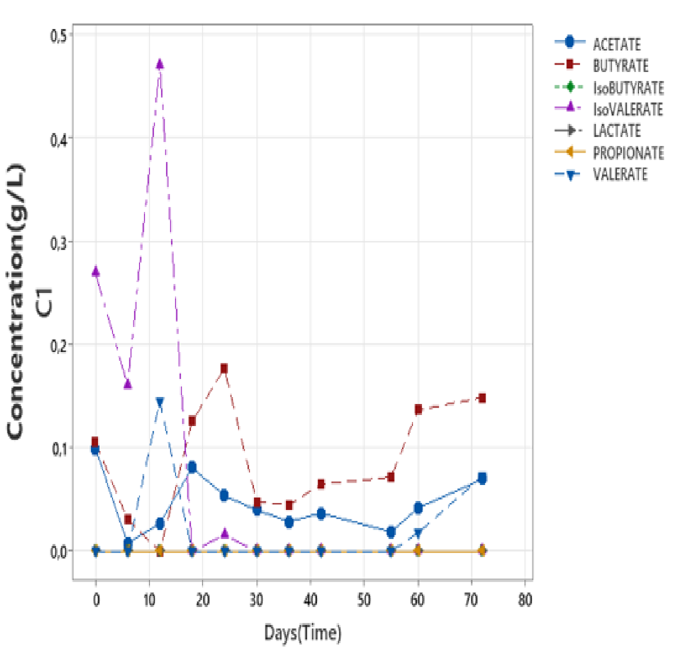
4.2 Degree of Digestion

The C1 of the serial configuration C exhibited a greater VS reduction (86%) than the single-stage (B1) and D1 of the serial configuration D (Table 3). The VS reduction differences between D1 and C1, and between B1 and C1, were not statistically significant ($p > 0.05$). However, the difference was significant between D1 and B1 ($p = 0.03$), with B1 exhibiting a higher reduction. Although C2 had a higher reduction than D2, the difference was not statistically significant ($p = 0.7$).

4.3 Total VFAs and pH

Lactate, acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate, interesting VFAs in an anaerobic digestion process, were analyzed weekly across the reactors. VFAs are crucial intermediates in the AD process; an imbalance or buildup could indicate process instability and impact methane production by influencing methanogenic activity. Among the first reactors (D1, C1, B1), B1 reached a VFA concentration (2.4 g/L) (Figure 7; Figure 9). Isovalerate was the most dominant VFA in C2 (2.7 g/L), D2 (1.6 g/L), B1 (2.3 g/L), and C1 (0.4 g/L). D1 had the lowest isovalerate concentration (0.04 g/L) and was the only reactor with detectable propionate concentration (0.03 g/L), a VFA known to inhibit methanogenesis at higher concentrations and early signs of process instability. D2 was the only reactor with lactate concentrations (0.4 g/L) and isobutyrate concentrations (2.3 g/L). By day 30, allthe VFA concentrations decreased to between 0 and 0.27 g/L in all reactors. The second reactors of the serial systems (D2, C2) had the highest VFA concentration (Figure 8) for all reactors used. All reactors remained stable individually during the experiment, even though there was an early VFA accumulation, which may have been caused by a temporary instability.





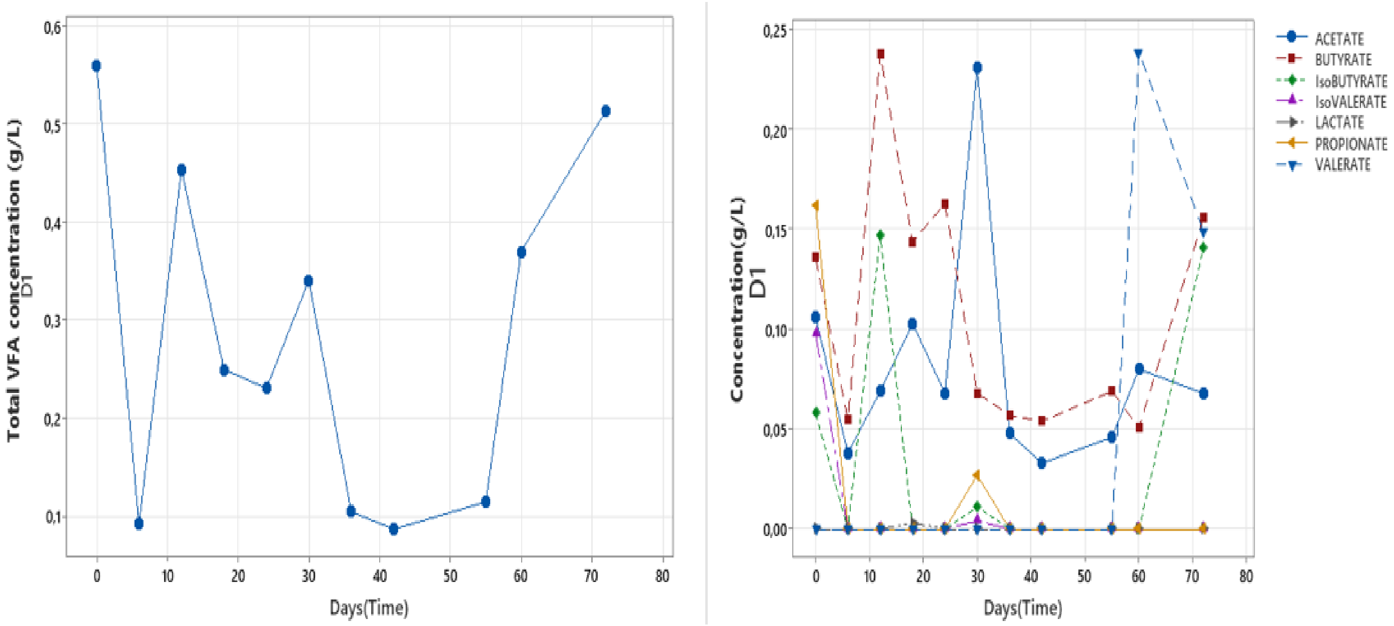
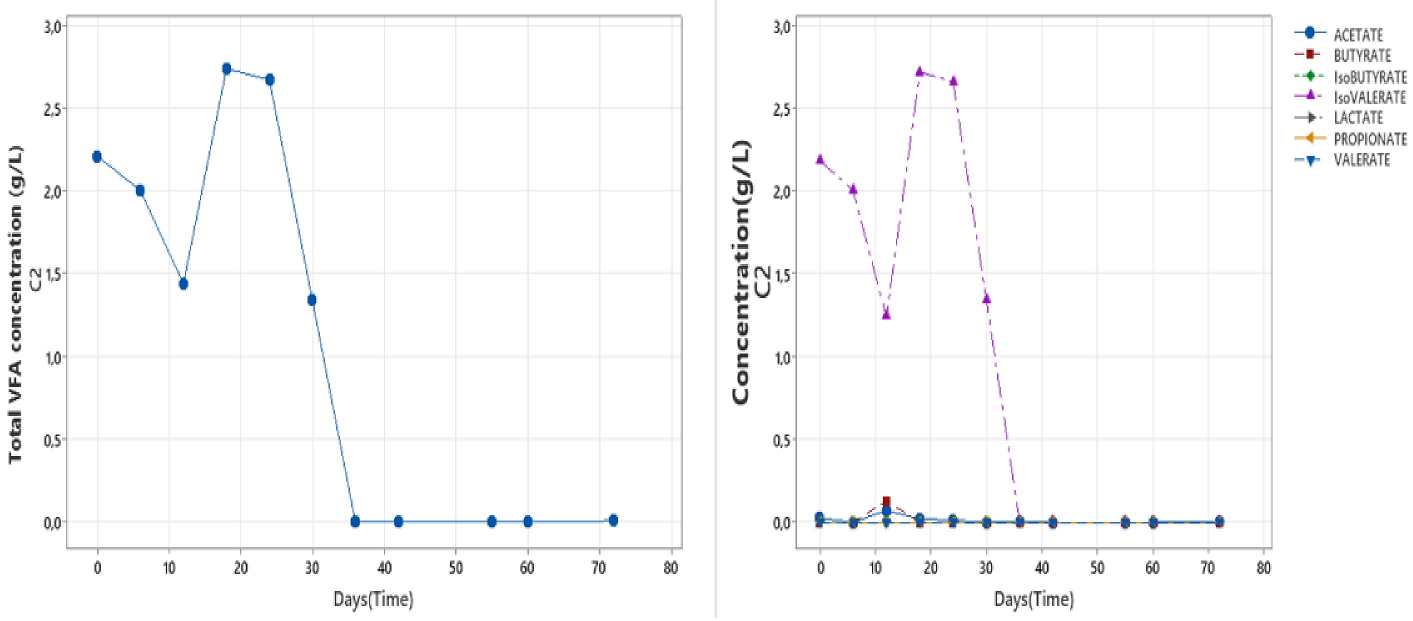


Figure 7: Weekly total VFA concentration and concentration per individual VFA type in first reactors D1 and C1 (serial system).



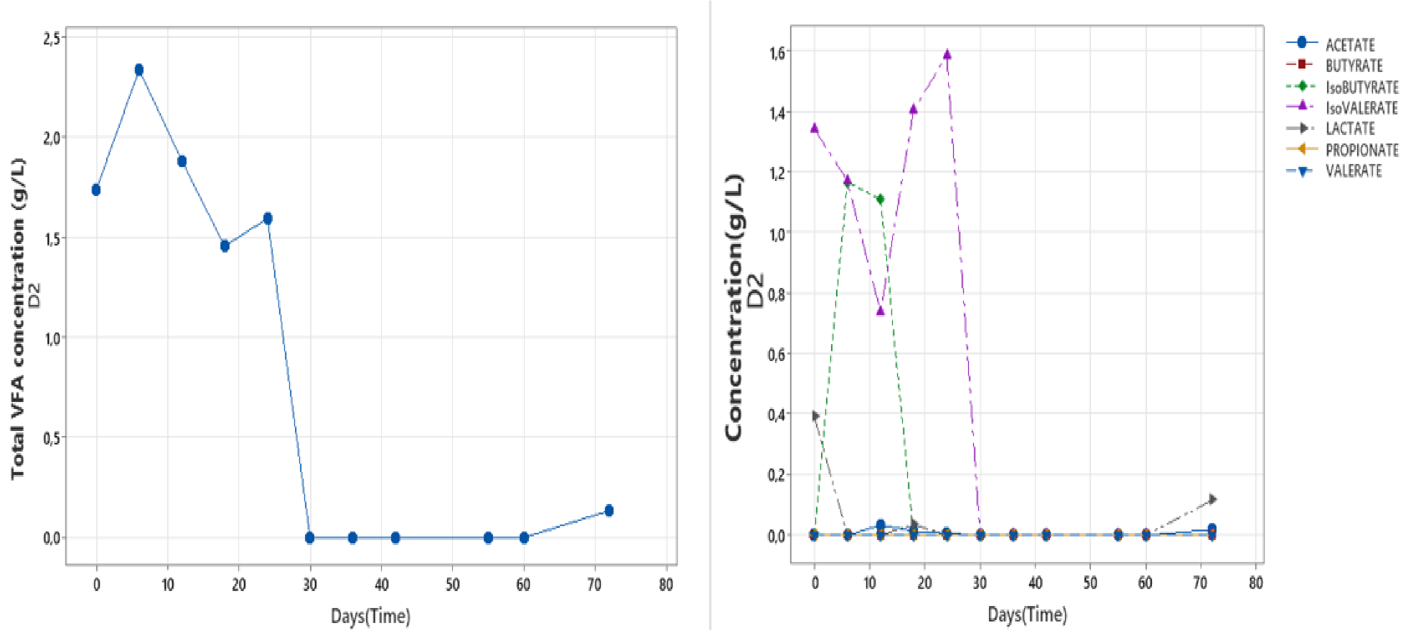


Figure 8: Weekly total VFA concentration and concentration per individual VFA type in second reactors C2 and D2 (serial system).

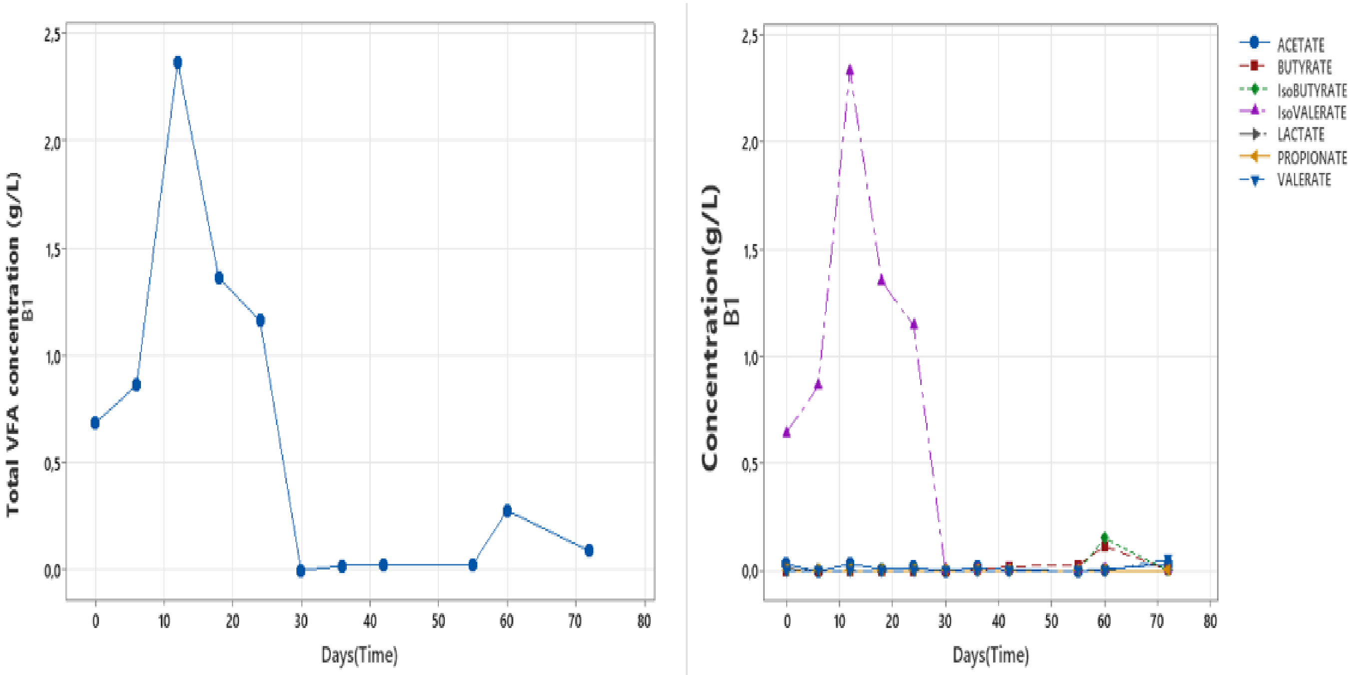


Figure 9: Weekly total VFA concentration and concentration per individual VFA type in B1 (single-step system).

Figure 10 illustrates the pH trends during the 55-day monitoring period. The pH in the first reactors of the serial system (D1 and C1) ranged from 7.4 to 7.8 and was slightly higher compared to the control B1 (7-7.7), though it was not statistically significant ($p > 0.05$). For the second reactors of the serial systems, C2 exhibited higher pH (7.7-7.8) compared to D2 (7.1-7.8), though this difference was also not statistically significant ($p = 0.7$). Despite minor differences in pH among systems, all reactors remained within the optimal pH range (7.0-8.0), which indicates stable operation conditions.

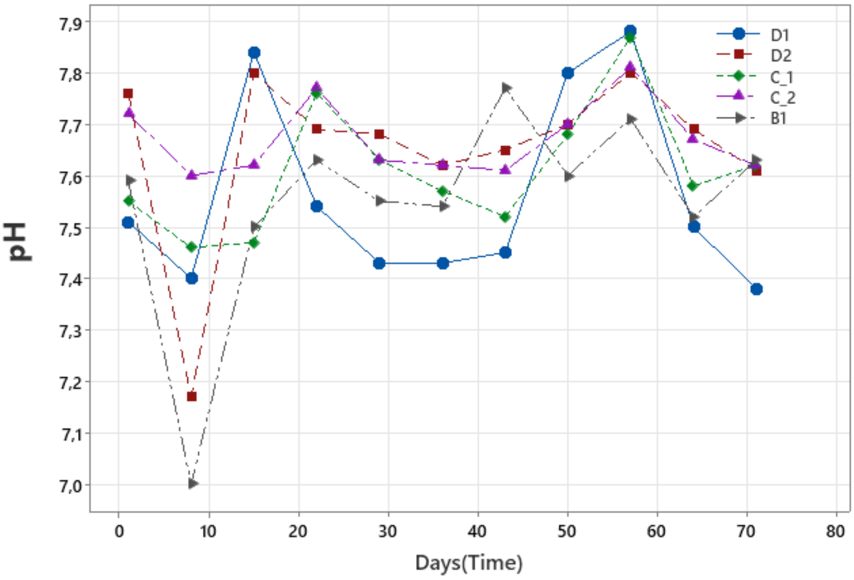


Figure 10: pH concentration in serial and single-step reactors for the period studied: D1+D2 (serial system), C1+ C2 (serial system and B1 (single-step system).

4.4 Nutrient composition in systems

Table 4 shows the nutrient composition from all reactors. Ammonium concentrations (NH_4^+-N) were higher in all digestates (1.9-2.5 kg/ton) compared to the feedstock (Table 1). This increase shows mineralization of organic nitrogen during the AD, where complex nitrogen compounds are converted to plant-available ammonium (Bareha et al., 2018). Mineralization (ML) was highest (20.7%) for the single-stage reactor (B1), compared to the first reactors (D1; C1) of the serial configurations, and between D1 and C1, C1 had the highest ML (18.6%). C2 had the highest ML (23%) compared to D2.

C1 and C2 had a slightly higher concentration than D1 and D2, and the control B1 showed a similar range, which may be due to system variability rather than performance difference, as C2 and D2 receive substrate from C1 and D1, respectively.

The total carbon content ranged from 21.7 to 32.3 kg/ton across all reactors. The C/N ratio was similar, ranging from 4.7 to 5.0 kg/ton with slight variations across the systems. The serial systems C and D resulted in a slightly lower total carbon than the single system (B1), although the serial configuration remained consistent. This reflects a more efficient conversion of organic matter.

Macro nutrients such as potassium (33.1-3.3 kg/ton), magnesium, calcium, phosphorus (0.9 kg/ton), and other mineral content were maintained in all systems with slight variations. Macronutrients do not degrade and are conserved in AD processes. Phosphorus in the organic form can undergo mineralisation and contribute to the overall availability in the digestate.

Table 4: Chemical composition of digestate from various reactors D1, D2, C1, C2 and B1.

Parameter	**D1**	**D2**	**C1**	**C2**	**B1**
Dry Matter (%)	7.5	5.6	6.8	5.3	5.9
Total Nitrogen (Kg/Ton)	4.8	4.7	4.9	4.7	4.8
Organic Nitrogen (Kg/Ton)	3.0	2.3	2.7	2.2	2.5
Ammonium Nitrogen (Kg/Ton)	1.8	2.4	2.3	2.5	2.4
Total Carbon (Kg/Ton)	32.3	22.8	28.6	21.7	24.3
Tot-C/Tot-N (Kg/Ton)	6.8	4.9	5.8	4.7	5,.0
Total Phosphorus (Kg/Ton)	0.9	0.9	0.8	0.7	0.8
Total Potassium (Kg/Ton)	3.1	3	3.2	3.1	3.3
Total Magnesium (Kg/Ton)	0.6	0.6	0.6	0.5	0.5
Total Calcium (Kg/Ton)	1.1	1.2	1,0	1.1	1.1
Total Sodium (Kg/Ton)	0.3	0.3	0,3	0.3	0.3
Total Sulphur (Kg/Ton)	0.4	0.4	0.4	0.4	0.4

5. Discussion

5.1 Biogas Production and System Performance

The study indicated that serial digestion with a configuration of 65:35% (C1+C2) improved biogas production compared to the serial configuration of 35:65% (D1+D2) and a conventional single-step CSTR reactor (B1). The C1+C2 showed a slightly higher SMP (2.8-2.1%) compared to serial D1+D2 and the conventional one-step system (B1), indicating improved process efficiency and biogas production with a larger first-reactor volume. A higher substrate-to-methane conversion indicates improved efficiency. The improved performance can be associated with a larger reactor volume and longer retention time in the first reactor, which allows a more stable breakdown, reduced VFA accumulation, and more complete biomass conversion, compared to the Serial system D and the control.

The first reactors produced most of the methane (D1=70%; C1=90%), with a small amount from the second reactors (D2=30%; C2=10%), with a retention time of 36 days for D2 and 19 days for C2. The methane production of the first reactors (D1; C1) may have been affected by their HRTs, as D1 had a shorter HRT of 19 days. A shorter retention time may lead to less complete degradation and hence a lower methane production. The HRT of D2 may have improved the methane production of the reactors, as a longer HRT allows a more complete degradation and organic conversion efficiency. A longer HRT and complete degradation in C1 could have resulted in C2 producing less methane compared to D2. C1, compared to D1, showed a better reduction of VS and total carbon, resulting in less biomass conversion for C2. The second reactors received substrates that are partially degraded from the first reactors, which accounts for their low SMPs. Interestingly, despite their different HRTs, the SMP for the two serial systems was similar, indicating that both systems C and D were able to handle the OLRs efficiently. This shows the system's robustness and ability to handle higher VS without impacting the methane yield of the systems.

The 65:35% (C-system) configuration outperformed the 35:65% configuration (D system), which could be attributed to the lower OLR (4 gVS/d) and higher OLR (6.3 gVS/d), and difference in HRTs, of the first reactors of the systems, which could have affected the process stability and overall performance. This could have resulted in D1 having a higher VMP and a lower SMP, which may be due to it receiving a higher OLR and having a lower biomass conversion efficiency. A higher OLR and shorter can also lead to early-stage acid formation and VFA accumulation, mainly occurring within the first 10- 20 days, which could have been aggravated and affected D1's performance and stability in the beginning.

These findings coincide with previous studies, which emphasized that serial digestion performance depends heavily on the system's design ratio, OLR and HRT, where a sufficiently large volume and HRT distribution of the first reactors and a relatively small volume in the second reactors are required to maintain a stable process (Kaparaju et al., 2009). Kaparaju et al (2009) showed that volume distribution of 13/87 or 30/70 produced less methane and showed less stability compared to balanced and well-proportioned systems, which was also dependent on the OLR and the HRT under which the study was operated. A well-proportioned serial system typically allocates 50-70% of the total volume to the first reactor with an HRT that allows efficient hydrolysis, acidogenesis, and initial methane production. The second reactor supports VFA conversion and stabilized effluent. This balance between the operational parameters and the volume ratios is crucial for optimizing microbial activity, improving biodegradation, and ensuring overall system efficiency.

Furthermore, the slightly higher methane content of serial system D compared to the C system and the control could indicate the differences in organic matter breakdown in the various systems. The breakdown of protein and lipid-rich substrates is known to yield higher methane content than carbohydrates (Q. Li et al, 2020). D1's higher methane (VMP) could be due to higher OLR than C1 and B1, even though they had the same feedstock. Regardless of the higher methane and VMP, the lower SMP shows a lower substrate to methane conversion and potential microbial stress and VFA accumulation in the early stages of the process. Higher methane content in D2 than in C2 could be due to a longer retention time in D2 (36 days), which allows for more complete material decomposition, such as degradation of proteins or fat, and enhanced VFA conversion and microbial adaptability (Guo et al., 2024).

For a full-scale application, a larger-to-smaller configuration will support process stability and optimization, especially when feedstock quality and quantity are considered (Kaparaju et al., 2009). According to Kapraju et al (2009), such configurations better promote hydrolysis and acidogenesis as they allow sufficient time for degradation and reduce the risk of process instability before the second reactor receives the first reactor's digestate. This helps ensure a stable and optimal process in the second digester. Walter et al. (2022) confirm that shifting from parallel to serial operations maintained process stability and enabled targeted microbial activity with efficient VFA degradation in the second reactor.

The findings of this study are similar to previous studies, as serial systems outperform single-stage reactors (Boe & Angelidaki, 2009; Kaparaju et al., 2009;

Li et al., 2017; Perman et al., 2022). Nonetheless, the findings of this study between the performance of the serial D system and the single step contradict (Table 3) with previous studies. In these studies by Boe & Angelidaki (2009) and Kaparaju et al. (2009), the volume distribution ranged from large to small for the serial systems, and the volume was 5-7.2 L for the single-stage reactor. In these studies, serial systems consistently outperformed single-stage setups. This discordance may be due to the shorter operational time in this study (1.3 HRT, 72 days) compared to the at least 3 HRTs (>100 days) under which these studies were conducted, which is also a key limitation of this study. The OLR used in these studies is also a key factor in the differences in results between this study and other studies. An HRT repeated at least 3 times gives credible results for the system's complete and concrete performance (Parajuli et al., 2022). A single HRT can be considered a stabilization phase during which the system adjusts to the experimental setups and feedstock composition.

5.2 Degree of degradation

The degree of degradability in the serial digestion C system was slightly higher than in the serial digester D and the control B1, as shown by the higher VS reduction and mineralisation in C2 compared to D2 and B1 (Table 3). This aligns with previous studies that showed VS reduction was higher in the serial system than in the single-step system (Li et al., 2017; Perman et al., 2022). However, it contradicts this study regarding the single reactor having a higher degree of degradation than the serial system D. The HRT difference between these studies and this study is a key factor in the contradictions. Perman et al. (2022) conducted the study for 55 days, with 35 days for the first reactor and 20 days for the second. Li et al (2017) also conducted their study under an HRT of 20 days for 140 days. The earlier studies were repeated for at least 3 retention times (165-210 days), which allowed more time for degradation and accurate results or performance of the various systems, while the present study was limited to one HRT (72 days). Among the serial configurations, the C system illustrated higher substrate degradation and conversion than the D system. This could be due to the D1 system's shorter retention time (19 days) compared to C1 (36 days), which gave it less time to complete degradation, even though overall HRT is the same. This may have limited its ability to ensure an effective digestion process in D1, therefore affecting the overall digestion efficiency (Boe & Angelidaki, 2009; Kaparaju et al., 2009; Boe & Angelidaki, 2009; Kaparaju et al., 2009).

The nutrient analysis and mineralisation show that all systems had similar NH_4^+-N, which indicates comparable organic nitrogen mineralization levels across all systems. Organic nitrogen is converted into easily and readily accessible forms. Despite the lower VS degradation in the first reactors of the serial systems compared to the control as a single reactor, the second digesters (D2=35%; C2=26%) contributed to the additional breakdown of remaining organic matter, indicating further degradation beyond the first digestion. The additional digestion also confirmed the carbon reduction and decrease in C/N in the digestate, indicating a more stabilized and mineralized digestate. ML levels also support this trend, as they show that the second reactors of the serial configurations add a measurable contribution to the degradation of the feedstock beyond the first reactors.

An advantage of serial systems lies in ensuring complete degradation through extending the average material retention time over single-step systems, even though B1 outperforms the serial system D. This occurs as the second digesters provide extra time for degradation and help control potential short-circuiting often associated with conventional one-step CSTR. This is especially relevant to this study as it addresses the challenges of incomplete degradation and recalcitrant feedstock like crop residues, which have high lignocellulose content and slow breakdown. The continued breakdown in the second reactor suggests a more stable and better degradation of the recalcitrant substrate, which can potentially reduce the methane emissions during post-digestion storage and improve nutrient recycling and recovery. It also helps combat GHG emissions associated with post-digestion storage and enhances nutrient recycling through complete material decomposition.

Lower residual methane potential and improved VS reduction have been proven for serial systems, regardless of the volume configuration (Kaparaju et al., 2009; Perman et al., 2022). The current results cannot yet conclusively confirm, as they are limited to a single HRT. The long-term benefits that the trends observed in the C system indicate that a serial configuration can offer performance and environmental advantages over the single-step digestion in the future.

5.3 Impacts of VFAs on systems

The accumulation of VFAs observed during the first 30 days suggests a temporary process instability and imbalances that can influence microbial activity and the methane production of various reactors. The subsequent decrease in VFA concentration after day 30 indicates the adaptation of microbes to the OLR and improved conversion efficiency, as reported by Q. Li et al. (2020). Q. Li et al 2020 investigated the effects of mesophilic and thermophilic temperatures on the methanogenic degradation of VFAs under different OLRs using digested sludge derived from food waste and sludge as substrate. This research showed that elevated OLR resulted in VFA accumulations and process instability, leading to system failure at 1.5g/VS/day under mesophilic temperatures and 2g/VS/day under thermophilic temperatures. In contrast, the reactors in this study were operated at an OLR of between 2.2 and 6.3g/VS/L/day. The processes remained stable after the initial VFA accumulation, especially in the first reactors of the serial systems, which received up to 6.3g/VS/L/day OLR. This shows that serial systems can provide a good buffering effect and enhanced microbial adaptations without a process failure even at high OLR. According to Q. Li et al. (2020), acetate and butyrate were degraded efficiently with specific microbial growth rates compared to propionate, which showed accumulation under thermophilic temperatures and resulted in process inhibition. Elevated VFA concentrations are known to impact methane production during AD, potentially accounting for the lower methane production in C2 compared to D2 (Liu et al., 2018).

The detection of propionate in D1 may suggest early signs of process instability, toxicity, poor conversion efficiency, and inhibition of methanogens. However, these levels were low and below the threshold to cause methanogen inhibition. Liu et al., (2018), reported that propionate concentration between 5-8g/L can substantially inhibit microbial activity using sewage sludge, which is significantly higher than what was observed in D1 (Figure 5). The lower SMP in D1 may be attributed rather be a consequene of the shorter HRT, leving less time for degradation.

The presence of isovalerate and other branched VFAs like isobutyrate in reactor D2, C1, C2, and B1 can be attributed to the degradation of protein-rich substrates, as they are byproducts of amino acid breakdown (Li et al., 2020). The dominance of isovalerate in these reactors indicates that protein breakdown was proficient in these reactors (C2, D2, and B1) or a slow VFA conversion. However, the overall SMP trends between C2 and D2 (Table 3) show a less efficient VFA to methane conversion despite the potential protein breakdown. Lactate accumulation in D2 may indicate a shift in metabolic pathways or inadequate methanogenic activity. Lactate is produced when microbes are under stress or when hydrogen-consuming methanogens are not well developed (Detman et al., 2018; Soubes et al., 1989). This might be associated with the constant variation in methane production in D2 (Figure 5).

VFA concentrations are linked to pH levels (Nativ et al., 2021) . In this study, the higher pH in C2 (7.7-7.8) compared to D2 may have lowered methanogenic activity and VFA accumulation (Table 3, Figure 5). Extreme pH values have been shown to increase VFA accumulation and microbial activity, influencing biogas production, though pH values were within optimal ranges (Bahira et al., 2018(Bahira et al., 2018.

6. Conclusion

The serial digestion with a volume distribution of 65:35% (C-system) performed better than the serial system with a 35:65% volume distribution (D-system) and a conventional one-step system at a retention time of 55 days and during an operational time of 72 days (1.3 HRT). Most of the methane produced in the serial reactors was derived from the first reactors, with a small additional amount from the second reactors.

The study shows that serial digestion can enhance methane production and process efficiency from agricultural waste, particularly manure and crop residues, compared to conventional single-step digestion. Beyond energy yield, serial digestion also contributes to environmental sustainability by reducing post-digestion methane emissions during storage. This can be achieved through allowing complete degradation and enhanced methane production, which contributes to the production of more renewable energy. Complete degradation can potentially reduce the residual methane in the digestate, minimising post-digestion methane emissions during storage. VS reduction and ML was notably higher in serial system C than in both serial system D and the control (B1). However, the control B1 outperformed the serial system D in SMP and VS reduction, refuting previous studies. This is likely due to the short operational time (72 days; 1.3 HRT) compared to other studies (>100). Although residual methane was not tested in this experiment, the observed feedstock decomposition suggests that serial digestion can promote energy recovery and environmental sustainability. However, efficiency and effectiveness depend highly on the volume proportions, whether it is a small-to-large or a large-to-small volume distribution, and the retention of the entire experiment.

VFA concentrations and pH impair process stability and overall biogas production. Early VFA accumulation may have led to process instability in the first few days of operation. This accumulation further dropped due to potential improved VFA conversion and microbial adaptations enhancing process stability. Different VFA types and concentrations show organic conversion efficiency, microbial activity, and performance per reactor. These observations stress that the reactors were operated for a to short time. A longer operational period may sallow for improved biogas yields and a better VFA conversion.

Further studies should allow multiple retention times, evaluation of the substrate's residual methane potential, possibly large-scale trials, and comparisons.

References

Abdeshahian, P., Lim, J. S., Ho, W. S., Hashim, H., & Lee, C. T. (2016). Potential of biogas production from farm animal waste in Malaysia. *Renewable and Sustainable Energy Reviews*, *60*, 714–723. https://doi.org/10.1016/J.RSER.2016.01.117

Ahlberg-Eliasson, K., Nadeau, E., Levén, L., & Schnürer, A. (2017). Production efficiency of Swedish farm-scale biogas plants. *Biomass and Bioenergy*, *97*, 27–37. https://doi.org/10.1016/J.BIOMBIOE.2016.12.002

Anaya-Reza, O., Altamirano-Corona, M. F., Basurto-García, G., Patricio-Fabián, H., García-González, S. A., Martinez-Hernandez, E., & Durán-Moreno, A. (2024). Wet anaerobic digestion of organic fraction of municipal solid waste: experience with long-term pilot plant operation and industrial scale-up. *Bioprocess and Biosystems Engineering*, *47*(2), 235–247. https://doi.org/10.1007/S00449-023-02958-2/TABLES/7

Angelidaki, I., Karakashev, D., Batstone, D. J., Plugge, C. M., & Stams, A. J. M. (2011). Biomethanation and Its Potential. *Methods in Enzymology*, *494*, 327–351. https://doi.org/10.1016/B978-0-12-385112-3.00016-0

Bahira, Baki, A. S., & Bello, A. (2018). *Effect of Varying pH on Biogas Generation using Cow Dungjournal/drjbb*. *4*(3), 28–33. https://doi.org/10.26765/DRJAFS.2018.7198

Bareha, Y., Girault, R., Jimenez, J., & Trémier, A. (2018). Characterization and prediction of organic nitrogen biodegradability during anaerobic digestion: A bioaccessibility approach. *Bioresource Technology*, *263*, 425–436. https://doi.org/10.1016/J.BIORTECH.2018.04.085

Bchir, F. S., Gannoun, H., Herry, S. El, & Hamdi, M. (2011). Optimization of Spongiochloris sp. biomass production in the abattoir digestate. *Bioresource Technology*, *102*(4), 3869–3876. https://doi.org/10.1016/J.BIORTECH.2010.11.036

Boe, K., & Angelidaki, I. (2009). Serial CSTR digester configuration for improving biogas production from manure. *Water Research*, *43*(1), 166–172. https://doi.org/10.1016/J.WATRES.2008.09.041

Caruso, M. C., Braghieri, A., Capece, A., Napolitano, F., Romano, P., Galgano, F., Altieri, G., & Genovese, F. (2019). Recent Updates on the Use of Agro-Food Waste for Biogas Production. *Applied Sciences 2019, Vol. 9, Page 1217*, *9*(6), 1217. https://doi.org/10.3390/APP9061217

Detman, A., Mielecki, D., Pleśniak, Ł., Bucha, M., Janiga, M., Matyasik, I., Chojnacka, A., Jędrysek, M. O., Błaszczyk, M. K., & Sikora, A. (2018). Methane-yielding microbial communities processing lactate-rich substrates: A piece of the anaerobic digestion puzzle. *Biotechnology for*

Biofuels, *11*(1), 1–18. https://doi.org/10.1186/S13068-018-1106-Z/FIGURES/6

Feng, L., Wahid, R., Ward, A. J., & Møller, H. B. (2017). Anaerobic co-digestion of cattle manure and meadow grass: Effect of serial configurations of continuous stirred tank reactors (CSTRs). *Biosystems Engineering*, *160*, 1–11. https://doi.org/10.1016/J.BIOSYSTEMSENG.2017.05.002

Guo, H., McIntyre, M., Visser, A., Kuipers, H., van Lier, J. B., & de Kreuk, M. (2024). Performance and microbial community composition of full-scale high-rate cascade sludge digestion system via pie-shaped reactor configuration. *Bioresource Technology*, *402*, 130771. https://doi.org/10.1016/J.BIORTECH.2024.130771

Holl, E., Steinbrenner, J., Merkle, W., Krümpel, J., Lansing, S., Baier, U., Oechsner, H., & Lemmer, A. (2022). Two-stage anaerobic digestion: State of technology and perspective roles in future energy systems. In *Bioresource Technology* (Vol. 360). Elsevier Ltd. https://doi.org/10.1016/j.biortech.2022.127633

Kakuk, B., Kovács, K. L., Szuhaj, M., Rákhely, G., & Bagi, Z. (2017). Adaptation of continuous biogas reactors operating under wet fermentation conditions to dry conditions with corn stover as substrate. *Anaerobe*, *46*, 78–85. https://doi.org/10.1016/J.ANAEROBE.2017.05.015

Kaparaju, P., Ellegaard, L., & Angelidaki, I. (2009). Optimisation of biogas production from manure through serial digestion: Lab-scale and pilot-scale studies. *Bioresource Technology*, *100*(2), 701–709. https://doi.org/10.1016/j.biortech.2008.07.023

Khan, E. U., & Martin, A. R. (2016). Review of biogas digester technology in rural Bangladesh. *Renewable and Sustainable Energy Reviews*, *62*, 247–259. https://doi.org/10.1016/J.RSER.2016.04.044

Li, J., Wang, H. Z., Yi, Y., Gou, M., Nobu, M. K., Chen, Y. T., & Tang, Y. Q. (2020). Response of Isovalerate-Degrading Methanogenic Microbial Community to Inhibitors. *Applied Biochemistry and Biotechnology*, *191*(3), 1010–1026. https://doi.org/10.1007/S12010-020-03234-9/METRICS

Li, Q., Liu, Y., Yang, X., Zhang, J., Lu, B., & Chen, R. (2020). Kinetic and thermodynamic effects of temperature on methanogenic degradation of acetate, propionate, butyrate and valerate. *Chemical Engineering Journal*, *396*, 125366. https://doi.org/10.1016/J.CEJ.2020.125366

Li, Y. Q., Liu, C. M., Wachemo, A. C., Yuan, H. R., Zou, D. X., Liu, Y. P., & Li, X. J. (2017). Serial completely stirred tank reactors for improving biogas production and substance degradation during anaerobic digestion of corn stover. *Bioresource Technology*, *235*, 380–388. https://doi.org/10.1016/J.BIORTECH.2017.03.058

Liu, H., Han, P., Liu, H., Zhou, G., Fu, B., & Zheng, Z. (2018). Full-scale production of VFAs from sewage sludge by anaerobic alkaline fermentation to improve biological nutrients removal in domestic wastewater. *Bioresource Technology*, *260*, 105–114. https://doi.org/10.1016/j.biortech.2018.03.105

Ma, G., Ndegwa, P., Harrison, J. H., & Chen, Y. (2020). Methane yields during anaerobic co-digestion of animal manure with other feedstocks: A meta-analysis. *Science of The Total Environment*, *728*, 138224. https://doi.org/10.1016/J.SCITOTENV.2020.138224

Mansour, M., Ince, O., Uzun, O., Ozbayram, E. G., Miraloglu, H. I., & Ince, B. (2024). Enhanced Anaerobic Mono-digestion and Co-digestion of Crop Residues by NaOH Alkali Pre-treatment: Digestion Performance and Microbial Community Dynamics. *Waste and Biomass Valorization*, *15*(5), 3003–3015. https://doi.org/10.1007/S12649-023-02350-X/FIGURES/6

Mao, C., Feng, Y., Wang, X., & Ren, G. (2015). Review on research achievements of biogas from anaerobic digestion. *Renewable and Sustainable Energy Reviews*, *45*, 540–555. https://doi.org/10.1016/J.RSER.2015.02.032

Mata-Alvarez, J., Dosta, J., Romero-Güiza, M. S., Fonoll, X., Peces, M., & Astals, S. (2014). A critical review on anaerobic co-digestion achievements between 2010 and 2013. *Renewable and Sustainable Energy Reviews*, *36*, 412–427. https://doi.org/10.1016/J.RSER.2014.04.039

Murphy, J., Braun, R., Weiland, P., & Wellinger, A. (2011). *Biogas from Crop Digestion Task 37-Energy from Biogas*.

Nativ, P., Gräber, Y., Aviezer, Y., & Lahav, O. (2021). A simple and accurate approach for determining the vfa concentration in anaerobic digestion liquors, relying on two titration points and an external inorganic carbon analysis. *ChemEngineering*, *5*(2), 15. https://doi.org/10.3390/CHEMENGINEERING5020015/S1

Nwokolo, N., Mukumba, P., Obileke, K., & Enebe, M. (2020). Waste to energy: A focus on the impact of substrate type in biogas production. *Processes*, *8*(10), 1–21. https://doi.org/10.3390/PR8101224

Orhorhoro, E. K., Ebunilo, P. O., & Sadjere, G. E. (2018). Effect of Organic Loading Rate (OLR) on Biogas Yield Using a Single and Three-Stages Continuous Anaerobic Digestion Reactors. *International Journal of Engineering Research in Africa*, *39*, 147–155. https://doi.org/10.4028/WWW.SCIENTIFIC.NET/JERA.39.147

Parajuli, A., Khadka, A., Sapkota, L., & Ghimire, A. (2022). Effect of Hydraulic Retention Time and Organic-Loading Rate on Two-Staged, Semi-Continuous Mesophilic Anaerobic Digestion of Food Waste during Start-Up. *Fermentation 2022, Vol. 8, Page 620*, *8*(11), 620. https://doi.org/10.3390/FERMENTATION8110620

Patel, C., Ks, P., & Chitra, M. R. (2021). Issue 12 www.jetir.org (ISSN-2349-5162). In *JETIR2112006 Journal of Emerging Technologies and Innovative Research* (Vol. 8). https://www.researchgate.net/publication/359173352

Perman, E., Schnürer, A., Björn, A., & Moestedt, J. (2022). Serial anaerobic digestion improves protein degradation and biogas production from mixed food waste. *Biomass and Bioenergy*, *161*, 106478. https://doi.org/10.1016/J.BIOMBIOE.2022.106478

Rabii, A., Aldin, S., Dahman, Y., & Elbeshbishy, E. (2019). A Review on Anaerobic Co-Digestion with a Focus on the Microbial Populations and the Effect of Multi-Stage Digester Configuration. *Energies 2019, Vol. 12, Page 1106*, *12*(6), 1106. https://doi.org/10.3390/EN12061106

Rajagopal, R., Massé, D. I., & Singh, G. (2013). A critical review on inhibition of anaerobic digestion process by excess ammonia. *Bioresource Technology*, *143*, 632–641. https://doi.org/10.1016/J.BIORTECH.2013.06.030

Rasit, N., Idris, A., Harun, R., & Wan Ab Karim Ghani, W. A. (2015). Effects of lipid inhibition on biogas production of anaerobic digestion from oily effluents and sludges: An overview. *Renewable and Sustainable Energy Reviews*, *45*, 351–358. https://doi.org/10.1016/J.RSER.2015.01.066

Risberg, K., Sun, L., Levén, L., Horn, S. J., & Schnürer, A. (2013). Biogas production from wheat straw and manure – Impact of pretreatment and process operating parameters. *Bioresource Technology*, *149*, 232–237. https://doi.org/10.1016/J.BIORTECH.2013.09.054

Romio, C., Vedel, M., Kofoed, W., Møller, H. B., Lohani, P., & Siciliano, A. (2021). Digestate Post-Treatment Strategies for Additional Biogas Recovery: A Review. *Sustainability 2021, Vol. 13, Page 9295*, *13*(16), 9295. https://doi.org/10.3390/SU13169295

Rouhollahi, Z., Ebrahimi-Nik, M., Ebrahimi, S. H., Abbaspour-Fard, M. H., Zeynali, R., & Bayati, M. R. (2020). Farm biogas plants, a sustainable waste to energy and bio-fertilizer opportunity for Iran. *Journal of Cleaner Production*, *253*, 119876. https://doi.org/10.1016/J.JCLEPRO.2019.119876

Satpathy, P., & Pradhan, C. (2023). Biogas as an alternative to stubble burning in India. *Biomass Conversion and Biorefinery*, *13*(1), 31–42. https://doi.org/10.1007/S13399-020-01131-Z

Schnürer, A., & Jarvis, Å. (2018). *Microbiology of the biogas process*.

Schnürer, A., Urer, €, Bohn, I., & Moestedt, J. (2016). *Protocol for Start-Up and Operation of CSTR Biogas Processes*. 171–200. https://doi.org/10.1007/8623_2016_214

Sharma, P., Bano, A., Singh, S. P., Varjani, S., & Tong, Y. W. (2024). Sustainable Organic Waste Management and Future Directions for Environmental Protection and Techno-Economic Perspectives. *Current Pollution Reports*, *10*(3), 459–477. https://doi.org/10.1007/S40726-024-00317-7/FIGURES/5

Singh, B., Singh, N., Čonka, Z., Kolcun, M., Siménfalvi, Z., Péter, Z., & Szamosi, Z. (2021). Critical Analysis of Methods Adopted for Evaluation of Mixing Efficiency in an Anaerobic Digester. *Sustainability 2021, Vol. 13, Page 6668*, *13*(12), 6668. https://doi.org/10.3390/SU13126668

Singh, R., Hans, M., Kumar, S., & Yadav, Y. K. (2023). Thermophilic Anaerobic Digestion: An Advancement towards Enhanced Biogas Production from Lignocellulosic Biomass. *Sustainability 2023, Vol. 15, Page 1859*, *15*(3), 1859. https://doi.org/10.3390/SU15031859

Soubes, M., Muxí, L., Zunino, L., & Fernandez, A. (1989). Microbial degradation of lactate under methanogenic conditions. *MIRCEN Journal of Applied Microbiology and Biotechnology*, *5*(2), 193–198. https://doi.org/10.1007/BF01741843/METRICS

Svensson, K., Paruch, L., Gaby, J. C., & Linjordet, R. (2018). Feeding frequency influences process performance and microbial community composition in anaerobic digesters treating steam exploded food waste. *Bioresource Technology*, *269*, 276–284. https://doi.org/10.1016/J.BIORTECH.2018.08.096

Valentin, M. T., Ciolkosz, D., & Białowiec, A. (2024). Influence of inoculum-to-substrate ratio on biomethane production via anaerobic digestion of biomass. *Environmental Microbiology Reports*, *16*(6), e70009. https://doi.org/10.1111/1758-2229.70009

Waldron, C., Pankajakshan, A., Quaglio, M., Cao, E., Galvanin, F., & Gavriilidis, A. (2020). *Model-based design of transient flow experiments for the identification of kinetic parameters †*. https://doi.org/10.1039/c9re00342h

Walter, A., Hanser, M., Ebner, C., Insam, H., Markt, R., Hupfauf, S., & Probst, M. (2022). Stability of the Anaerobic Digestion Process during Switch from Parallel to Serial Operation—A Microbiome Study.

Sustainability (Switzerland), *14*(12), 7161. https://doi.org/10.3390/SU14127161/S1

Wang, B., Strömberg, S., Li, C., Nges, I. A., Nistor, M., Deng, L., & Liu, J. (2015). Effects of substrate concentration on methane potential and degradation kinetics in batch anaerobic digestion. *Bioresource Technology*, *194*, 240–246. https://doi.org/10.1016/J.BIORTECH.2015.07.034

Yavini, T. D., Chia, A. I., & John, A. (2014). Evaluation of the Effect of Total Solids Concentration on Biogas Yields of Agricultural Wastes. In *Int. Res. J. Environment Sci. International Science Congress Association* (Vol. 3, Issue 2). www.isca.me

Zhao, K., Tian, X., Lai, W., & Xu, S. (2024). Agricultural production and air pollution: An investigation on crop straw fires. *PLOS ONE*, *19*(5), e0303830. https://doi.org/10.1371/JOURNAL.PONE.0303830

Zhou, H., & Wen, Z. (2019). Solid-State Anaerobic Digestion for Waste Management and Biogas Production. *Advances in Biochemical Engineering/Biotechnology*, *169*, 147–168. https://doi.org/10.1007/10_2019_86

Zuo, Z., Wu, S., Qi, X., & Dong, R. (2015). Performance enhancement of leaf vegetable waste in two-stage anaerobic systems under high organic loading rate: Role of recirculation and hydraulic retention time. *Applied Energy*, *147*, 279–286. https://doi.org/10.1016/J.APENERGY.2015.03.001