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Subject/Topic:

OPTIMIZATION OF BIOGAS PRODUCTION FROM ANAEROBIC CO-DIGESTION OF POULTRY MANURE AND COCOA POD HUSKS: EFFECT OF CARBON-TO-NITROGEN RATIO AND TEMPERATURE REGIME

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DEDICATION

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First and foremost, I dedicate this work to God Almighty, whose infinite grace, protection, and strength have guided me throughout this journey and enabled me to bring this work to fruition.

This thesis is lovingly dedicated to my family, whose unwavering support and encouragement have been the cornerstone of my academic journey:

- **To my father, AKO Brou Félix,**
- **To my mother, DIBY Agbrou Anne Marie,**
- **To all my siblings,**
- **And to my uncle, N'GUESSAN Albert,**

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ABSTRACT

The growing challenges of organic waste accumulation, energy insecurity, and environmental degradation in West Africa underscore the need for sustainable waste-to-energy solutions. Poultry manure (PM) and cocoa pod husks (CPH), two abundantly available agricultural residues in the region, exhibit complementary characteristics for biogas production: high nitrogen and buffering capacity in PM, and high carbon and energy potential in CPH. This study investigated the optimization of biogas production from the co-digestion of poultry manure (PM) and cocoa pod husks (CPH), with a particular focus on the effect of carbon-to-nitrogen (C/N) ratio and digestion temperature on the biogas yield. Substrate characterization was performed to determine physicochemical and nutritional properties. Batch anaerobic digestion assays were conducted under mesophilic (37 °C) and thermophilic (55 °C) conditions with C/N ratios of 20, 25, 30, and 35 using the ANKOM gas production system over 30 days. Cellulose was included as a positive control to confirm inoculum microbial activity and system reliability. Biogas yields were monitored daily, and the cumulative production was statistically analysed using two-way ANOVA followed by Tukey's HSD test at a 95% confidence level. As a result, CPH displayed higher volatile solids (84.06%) and energy content (HHV: 19,987 kJ/kg) but had a high C/N ratio (51.16) and lignin content (23.71%), limiting its biodegradability. PM, though rich in nutrients (e.g., N, P, Ca, Mg) and alkalinity (pH 8.70), exhibited high ash content (25.84%) and low C/N ratio (16.07), raising the risk of ammonia inhibition. The mono-digestion trials yielded 244.65 ± 8.48 mL/g VS for CPH and 210.73 ± 27.12 mL/g VS for PM under mesophilic conditions (37°C). Co-digestion significantly improved biogas yields, with the highest cumulative yield (348.65 ± 10.44 mL/g VS) recorded at C/N 25 under mesophilic conditions and contributing to 65.45% improvement over PM and 42.51% improvement over CPH. Statistical analysis confirmed a significant effect of C/N ratio ($p = 0.001$), as well as its interaction with temperature ($p = 0.031$) on biogas yield, while temperature alone had no significant impact ($p = 0.259$). Thermophilic digestion improved performance for carbon-rich conditions (C/N 30 and 35), while mesophilic conditions provided greater stability at lower C/N ratios (C/N 20 and 25). The co-digestion of PM and CPH demonstrates strong synergistic effects, yielding higher and more stable biogas production than the mono-digestion. Optimal performance was then achieved at a C/N ratio of 25 under mesophilic conditions, demonstrating that proper adjustment of substrate mixing ratios and digestion temperature can substantially improve the efficiency of biogas systems. This study contributes to knowledge on the anaerobic co-digestion of nitrogen-rich and lignocellulosic residues in West Africa, especially in Togo, providing experimental evidence to support waste-to-energy strategies, climate change mitigation, and sustainable agricultural development.

Key-words: Anaerobic Co-digestion, Poultry manure, Cocoa pod husk, Carbon-to-nitrogen ratio, Mesophilic digestion, Thermophilic digestion, Biogas optimization.

RÉSUMÉ

Face aux défis croissants liés à l'accumulation des déchets organiques, l'insécurité énergétique et la dégradation environnementale en Afrique de l'Ouest, des solutions durables de valorisation énergétique s'imposent. Le fumier de volaille (FV), riche en azote et doté d'une bonne capacité tampon, et les coques de cabosses de cacao (CCC), riches en carbone et à fort potentiel énergétique, offrent des caractéristiques complémentaires favorisant la production de biogas. Cette étude a donc porté sur l'optimisation de la production de biogaz à partir de la co-digestion du fumier de volaille (PM) et des coques de cabosses de cacao (CCC), en mettant particulièrement l'accent sur l'effet du rapport carbone/azote (C/N) et de la température de digestion. Une caractérisation des substrats a été réalisée afin de déterminer leurs propriétés physico-chimiques et nutritionnelles. Des essais de digestion anaerobie en mode discontinu ont été conduits en conditions mésophiles (37 °C) et thermophiles (55 °C), avec des rapports C/N de 20, 25, 30 et 35, à l'aide du système ANKOM de production de gaz pendant 30 jours. La cellulose a été utilisée comme témoin positif afin de confirmer l'activité microbienne de l'inoculum et la fiabilité du système. Les rendements en biogaz ont été suivis quotidiennement, et la production cumulative a été analysée statistiquement à l'aide d'une ANOVA à deux facteurs, suivie du test HSD de Tukey au seuil de confiance de 95 %. Les résultats ont révélé que les CCC présentaient une teneur élevée en solides volatils (84,06 %) et un pouvoir calorifique supérieur de 19 987 kJ/kg, mais leur forte teneur en lignine (23,71 %) et leur rapport C/N élevé (51,16) limitaient leur biodégradabilité. Le FV, riche en nutriments (N, P, Ca, Mg) et alcalin (pH 8,70), montrait une teneur élevée en cendres (25,84 %) et un faible rapport C/N (16,07), favorisant le risque d'inhibition ammoniacale. Les essais de mono-digestion ont produit 244,65 ± 8,48 mL/g VS pour les CCC et 210,73 ± 27,12 mL/g VS pour le FV en conditions mésophiles (37 °C). La co-digestion a nettement amélioré les rendements en biogaz, avec un maximum de 348,65 ± 10,44 mL/g VS obtenu avec le rapport C/N de 25 en condition mésophile, soit une amélioration de 65,45 % par rapport au FV et de 42,51 % par rapport aux CCC. L'analyse statistique a confirmé un effet significatif du rapport C/N ($p = 0{,}001$) ainsi que son interaction avec la température ($p = 0{,}031$), tandis que la température seule n'a pas montré d'impact significatif ($p = 0{,}259$). La co-digestion du FV et des CCC a donc montré un fort effet synergique, permettant une production de biogaz plus élevée et plus stable que la mono-digestion. Les meilleures performances ont été obtenues avec un rapport C/N de 25 en condition mésophile. Cela prouve qu'un bon équilibre entre les substrats et la température de digestion peut considérablement améliorer l'efficacité des systèmes de production de biogaz. Cette étude apporte ainsi des résultats concrets pour soutenir la valorisation énergétique des déchets agricoles, la lutte contre le changement climatique et la promotion de l'agriculture durable en Afrique de l'Ouest, particulièrement au Togo.

Mots-clés : Co-digestion anaérobie, Fumier de volaille, Coques de cabosses de cacao, Rapport C/N, Digestion mésophile, Digestion thermophile, Optimisation du biogaz.

ACRONYMS AND ABBREVIATIONS

AC	: Ash Content
AcoD	: Anaerobic co-digestion
AD	: Anaerobic Digestion
ADF	: Acid Detergent Fiber
ADL	: Acid Detergent Lignin
ANOVA	: Analysis of Variance
BMP	: Biochemical Methane Potential
BMFTR	: Bundesministerium für Forschung, Technologie und Raumfahrt (German Federal Ministry of Research, Technology, and Space)
CCC	: Coques de Cabosses de Cacao
C/N	: Carbon-to-Nitrogen Ratio
CPH	: Cocoa Pod Husk
CoA	: Coenzyme A
CoM	: Coenzyme M
CSTRs	: Continuously Stirred Tank Reactors
DBFZ	: Deutsches Biomasseforschungszentrum gemeinnützige GmbH (German Biomass Research Center)
DIN	: Deutsches Institut für Normung (German Institute for Standardization)
DM	: Dry Matter
EN	: Europäische Norm (European Standard)
ESA	: École Supérieure d'Agronomie
FAO	: Food and Agriculture Organization
FM	: Fresh Matter
FV	: Fumier de Volaille
GHGs	: Greenhouse Gases
GLM	: General Linear Model
GSP	: Graduate School Programme
HC3	: Heteroscedasticity-Consistent Covariance Estimator, version 3 (robust standard errors for small samples).
HHV	: Higher Heating Value
HRT	: Hydraulic Retention Time
HSD	: Honestly Significant Difference
ISO	: International Organization for Standardization
LCA	: Life Cycle Assessment
LECO	: Laboratory Equipment Corporation (instrument manufacturer)
LHV	: Lower Heating Value

LT2B : Laboratoir des Technologies de la Biomasse et des Bioénergies (Laboratory of biomass and bioenergy technologies)
MC : Moisture Content
MSW : Municipal Solid Waste
NDCs : Nationally Determined Contributions
NDF : Neutral Detergent Fiber
OLR : Organic Loading Rate
PCI : Pouvoir Calorifique Inférieur
PCS : Pouvoir Calorifique Supérieur
PFRs : Plug Flow Reactors
pH : potential of Hydrogen
PM : Poultry Manure
SD : Standard Deviation
SDGs : Sustainable Development Goals
S/I : Substrate-to-Inoculum Ratio
STP : Standard Temperature and Pressure
TAN : Total Ammonia Nitrogen
TEA : Techno-Economic Analysis
TGA : Thermogravimetric Analysis
TS : Total Solids
UASB : Upflow Anaerobic Sludge Blanket
VFAs : Volatile Fatty Acids
VS : Volatile Solids
VDI : Verein Deutscher Ingenieure (Association of German Engineers)
WASCAL : West African Science Service Center on Climate Change and Adapted Land Use
WBG : World Bank Group
WLS : Weighted Least Squares (variance-weighted regression method used in Welch-type ANOVA)
XLSTAT : Microsoft Excel-based Statistical Analysis Software

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INTRODUCTION

1. Background and Rationale

Togo, like many West African nations, faces interconnected environmental and energy challenges. The country's strong dependence on fossil fuels, combined with limited waste management infrastructure, particularly for organic residues, exacerbates greenhouse gas (GHG) emissions, environmental degradation, and public health risks. In 2018, Togo's GHG emissions were estimated at approximately 21 million tons, with the agriculture and energy sectors being the most significant contributors (Agbossou et al., 2022). Among the country's key economic sectors, poultry farming and cocoa cultivation generate large volumes of organic residues, including poultry manure (PM) and cocoa pod husks (CPH). If properly valorised, these by-products could serve as important resources for renewable energy generation and nutrient recycling, thereby addressing both energy security and soil fertility. However, the absence of structured waste management systems leads to open dumping, burning, or uncontrolled decomposition in nature, which causes water and air pollution, GHG emissions, and economic losses for farmers.

Poultry manure is a nitrogen-rich resource that, when improperly managed, for instance by disposing of it in the environment without adequate treatment, can lead to significant environmental issues, including water and air pollution (Kabelitz et al., 2021; Kiss et al., 2023; Preuss & You, 2023). The untreated disposal of poultry manure can lead to soil and water pollution by introducing excess nutrients, such as nitrogen and phosphorus, which contribute to the eutrophication of lakes and the proliferation of harmful aquatic plants like water hyacinth and algal blooms (Kiss et al., 2023; Rosemarin et al., 2020). Additionally, during the decomposition of poultry manure discarded in the environment, hazardous gases such as ammonia (NH_3) and hydrogen sulfide (H_2S) are released, contributing to localized air pollution. This decomposition process also emits greenhouse gases, including methane (CH_4), carbon dioxide (CO_2), and nitrous oxide (N_2O), which contribute to global climate change (Boakye-Yiadom et al., 2024; Jiang et al., 2023; Savina, 2025). On the other hand, cocoa pod husks, which are carbon-rich byproducts abundantly produced in cocoa farms, are often either open-burned or left to decompose directly in the plantations. Open burning releases significant amounts of greenhouse gases, such as carbon dioxide (CO_2) and carbon monoxide (CO), as well as harmful pollutants like fine particulate matter, which can degrade air quality and pose respiratory health risks to the farmers and nearby communities (Kone et al., 2020; Mwafulirwa et al., 2024; Tovar et al., 2024). Additionally, when the cocoa pod husks are left to decompose on the ground, they create a moist environment that encourages the growth and spread of fungal pathogens, particularly Phytophthora palmivora, the causative agent of cocoa pod rot. This disease can negatively impact the yields, resulting in substantial economic losses for farmers (Mboua et al., 2020; Ouattara et al., 2021; Perrine-Walker, 2020). Also, this uncontrolled decomposition of the cocoa pod husks emits methane (CH_4) and nitrous oxide (N_2O), contributing to climate change. However, while poultry manure and cocoa pod husks pose environmental risks when mismanaged, their high organic content makes them ideal feedstocks for renewable energy production, particularly through the anaerobic co-digestion processes that can simultaneously convert multiple organic substrates into biogas. In this regard, studies have demonstrated that combining cocoa pod husks and poultry manure in anaerobic digestion systems can effectively produce biogas, offering a sustainable solution for waste management and energy generation (Acosta et al., 2021; Atmowidjojo et al., 2023; Kadam et al., 2024; Mora-Cortés et al., 2020).

Anaerobic digestion is a biological process that breaks down organic substrates in an oxygen-free environment. This process produces biogas, a renewable energy source, typically consisting of 55-65% methane (CH_4) and 35-45% carbon dioxide (CO_2), which can be used for clean cooking, heating, or electricity generation (Francisco López et al., 2024). Another valuable byproduct of this process is the digestate, a nutrient-rich material that remain after digestion, and can be used as an effective organic fertilizer (Gadirli et al., 2023; Kunz et al., 2022; Neri et al., 2023). Combining poultry manure and cocoa pod husks in anaerobic co-digestion offers a sustainable solution for agricultural waste management. Poultry manure is rich in nitrogen, while cocoa pod husk is rich in carbon. When combined, these substrates can create a more balanced feedstock that could enhance microbial activity and biogas production. Research has shown that co-digesting multiple organic materials not only increases biogas yield but also produces high-quality digestate suitable for soil amendment (Atmowidjojo et al., 2023; Jasińska et al., 2023). Beyond waste valorisation, this strategy could reduce dependence on imported fossil fuels, provide decentralized rural energy solutions, and mitigate the environmental burden of unmanaged residues in West Africa. Nevertheless, the influence of key operational factors must be rigorously evaluated in order to identify the optimal conditions for improved process performance and maximize biogas yield.

2. Problem statement

Although anaerobic co-digestion has demonstrated potential for biogas production, its efficiency and stability depend critically on operational parameters, particularly the carbon-to-nitrogen (C/N) ratio of the substrate mixture and the digestion temperature regime. These factors directly influence microbial activity, the organic matter degradation, and ultimately the quantity and quality of biogas produced (Bardi & Oliaee, 2021; Hu & Shen, 2024; Lee et al., 2024).

Poultry manure is a nitrogen-rich substrate with a naturally low C/N ratio, typically ranging between 5 and 10. When digested alone, it can lead to ammonia accumulation, which inhibits the methanogenic microorganisms and destabilizes the digestion process (Tawfik et al., 2023). In contrast, cocoa pod husks are carbon-rich, with a high C/N ratio, typically from 31 to 33

(Antwi et al., 2019; Darwin et al., 2016), and tend to exhibit low biodegradability and limited microbial activity when digested alone (Hermansyah et al., 2020). These complementary characteristics create a valuable opportunity: combining poultry manure and cocoa pod husks in anaerobic co-digestion can potentially create a balanced substrate mixture with an optimal C/N ratio, enhancing microbial synergy and improving biogas yield (Atmowidjojo et al., 2023; Naidu et al., 2024). Furthermore, maintaining the proper C/N ratio is crucial, as an excessively high C/N ratio can lead to rapid nitrogen depletion by acidogenic bacteria, thereby stalling methanogenesis. Conversely, a low C/N ratio promotes ammonia formation, which can be toxic to methanogens and reduce process efficiency (Suhartini et al., 2021). Hence, identifying and maintaining a balanced C/N ratio is essential for stable and high-performance anaerobic digestion. In addition to substrate composition, digestion temperature also plays a critical role. Anaerobic digestion can be carried out under mesophilic or thermophilic conditions (Wang et al., 2019). Thermophilic digestion typically accelerates hydrolysis, which can allow rapid biogas production. However, it demands higher energy input and is more prone to process instability, particularly in systems with low C/N ratios that exacerbate ammonia inhibition. On the other hand, mesophilic digestion offers greater microbial stability, lower energy requirements, and is generally more suitable for small-scale or rural biogas systems, which are common in developing regions like Togo (Steiniger et al., 2023; Yang et al., 2024).

Despite the potential of poultry manure and cocoa pod husks as complementary substrates, their combined use in anaerobic co-digestion remains underexplored. Most studies have focused on co-digesting these materials with other residues, such as cocoa leaves (Suhartini et al., 2021), cocoa bean shells (Atmowidjojo et al., 2023), cow manure (Hermansyah et al., 2020), corn stover (Yu et al., 2023), banana waste (Khatun et al., 2023), wheat straw (Zhan et al., 2022), and cassava effluents (Kayaba et al., 2024), and so on, under various conditions. Furthermore, very few investigations have been conducted under West African agro-climatic conditions, where both substrates are locally abundant, focusing on the optimization of critical parameters, such as the C/N ratio or temperature. This research gap poses a barrier to the technical optimization and regional deployment of biogas systems. Understanding how poultry manure and cocoa pod husks interact under varying C/N ratios and temperature regimes is essential to designing efficient, cost-effective, and scalable anaerobic co-digestion systems tailored to the needs of rural communities in Togo. Without such knowledge, the broader adoption of biogas technology for waste valorisation and energy access in the region remains constrained.

3. Research questions

1. What are the physicochemical properties of poultry manure and cocoa pod husks that influence their biogas potential?
2. What is the biogas potential of poultry manure and cocoa pod husks under mesophilic conditions?

3. What C/N ratio and temperature regime optimize biogas production during their co-digestion?

4. Research hypotheses

1. The physicochemical characteristics of poultry manure and cocoa pod husks are favourable for anaerobic digestion.
2. Co-digestion of poultry manure and cocoa pod husk enhances biogas production compared to mono-digestion.
3. An optimal C/N ratio and temperature condition exist that significantly maximize biogas yield during co-digestion.

5. Objectives of the study

The main objective of this study is to investigate the anaerobic co-digestion of poultry manure and cocoa pod husks under varying C/N ratios and temperature regimes to optimize the biogas production. The specific objectives are to:

1. Assess PM and CPH suitability for anaerobic digestion by characterizing their physicochemical properties.
2. Quantify the biogas potential of PM and CPH in mono-digestion under mesophilic conditions.
3. Assess the effects of varying C/N ratios and temperature regimes on the biogas yield during the co-digestion process.

6. Structure of the thesis

This thesis is organized into five main sections. The present Introduction (Section 1) provides the background, research problem, questions, hypotheses, and objectives. Section 2, which is Chapter 1, reviews the literature on anaerobic digestion, substrate characteristics, process parameters, and co-digestion strategies. Section 3, also Chapter 2, presents the materials, methods, and experimental design used to evaluate biogas production. Section 4, representing Chapter 3, reports and discusses the experimental results, with emphasis on the influence of C/N ratio and temperature. Finally, Section 5 concludes the study, outlines key implications, and proposes perspectives and recommendations for future research and implementation.

CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

The global transition toward sustainable energy systems has heightened interest in anaerobic digestion (AD) as a versatile technology for converting organic waste into renewable energy. In Sub-Saharan Africa, and particularly in Togo, agricultural residues such as poultry manure (PM) and cocoa pod husks (CPH) are abundant yet underutilized biomass resources. Their mono-digestion is often constrained by nutrient imbalances, process instability, or limited biodegradability. This chapter reviews the foundations of AD with a focus on co-digestion strategies, substrate characteristics, and key operational parameters. Special attention is given to PM and CPH as co-substrates, the optimization of carbon-to-nitrogen (C/N) ratios, and temperature regimes, which form the scientific rationale for this study.

1.2. Biogas Production Through Anaerobic Digestion

1.2.1. Overview of Anaerobic Digestion

Anaerobic digestion (AD) is a complex biological process in which organic matter is decomposed by a consortium of specialized microorganisms in oxygen-free environments, characterized by a redox potential less or equal to 200 mV (Gadirli et al., 2023). This multi-stage biochemical process involves a succession of microbial groups, each carrying out specific metabolic functions, that work synergistically to convert complex organic compounds into simpler molecules (Bhatt & Tao, 2020; Neri et al., 2023). The process leads to the production of biogas, a renewable energy source primarily composed of methane (CH_4) and carbon dioxide (CO_2), alongside digestate. This nutrient-rich byproduct can be utilized as an organic fertilizer to enhance soil fertility and promote sustainable agricultural practices.

The anaerobic digestion process occurs naturally in various anaerobic environments such as wetlands, swamps, rice paddies, and within the digestive tracts of ruminant animals (e.g., cows, goats, and sheep), where it plays a fundamental role in the natural recycling of organic matter (Vögeli et al., 2014). In these ecosystems, anaerobic microbial activity plays a crucial role in maintaining ecological balance by breaking down dead plant material and animal waste, thereby preventing the accumulation of organic pollutants (da Cunha-Santino & Júnior, 2023). In engineered systems, the anaerobic digestion process is carefully designed and operated under controlled conditions to optimize the degradation of biodegradable organic materials. This is achieved in airtight reactor tanks, known as digesters, which maintain controlled environmental conditions that allow microbial communities to carry out the digestion process efficiently (Song et al., 2023a; Z. Wu et al., 2023). Modern anaerobic digestion systems are highly versatile and can accommodate a diverse array of feedstocks, including livestock manure, crop residues, food waste, agro-industrial effluents, and municipal sludge (Janesch et al., 2021). This flexibility has positioned AD as a cornerstone technology within circular bioeconomy models and sustainable waste management strategies worldwide.

1.2.2. Microbial Stages of the Anaerobic Digestion Process

The anaerobic digestion (AD) process is typically divided into four interdependent biochemical stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, as illustrated in Figure 1.1. Each phase is carried out by distinct groups of microorganisms working in syntrophy and is defined by specific microbial activities, unique metabolic pathways, and characteristic intermediate products (Kouas, 2018; Sharma et al., 2023). A defining characteristic of anaerobic digestion is its sequential and synergistic microbial activity: the metabolites produced during one phase serve as substrates for the subsequent phase, creating a self-sustaining and continuous chain of reactions within a single biological system (Tshemese et al., 2023). Although conceptually distinct, these stages occur simultaneously and interactively in practical applications, especially within single-stage reactors, where all microbial groups coexist and function in a dynamic balance. This results in narrow limits and high demands for specific environmental and operating conditions to degrade complex substrates (Kostopoulou et al., 2023). Therefore, a detailed understanding of each phase, the properties of the involved microbial communities, and the variables influencing their activity is essential for optimizing, modelling, and controlling anaerobic digestion processes.

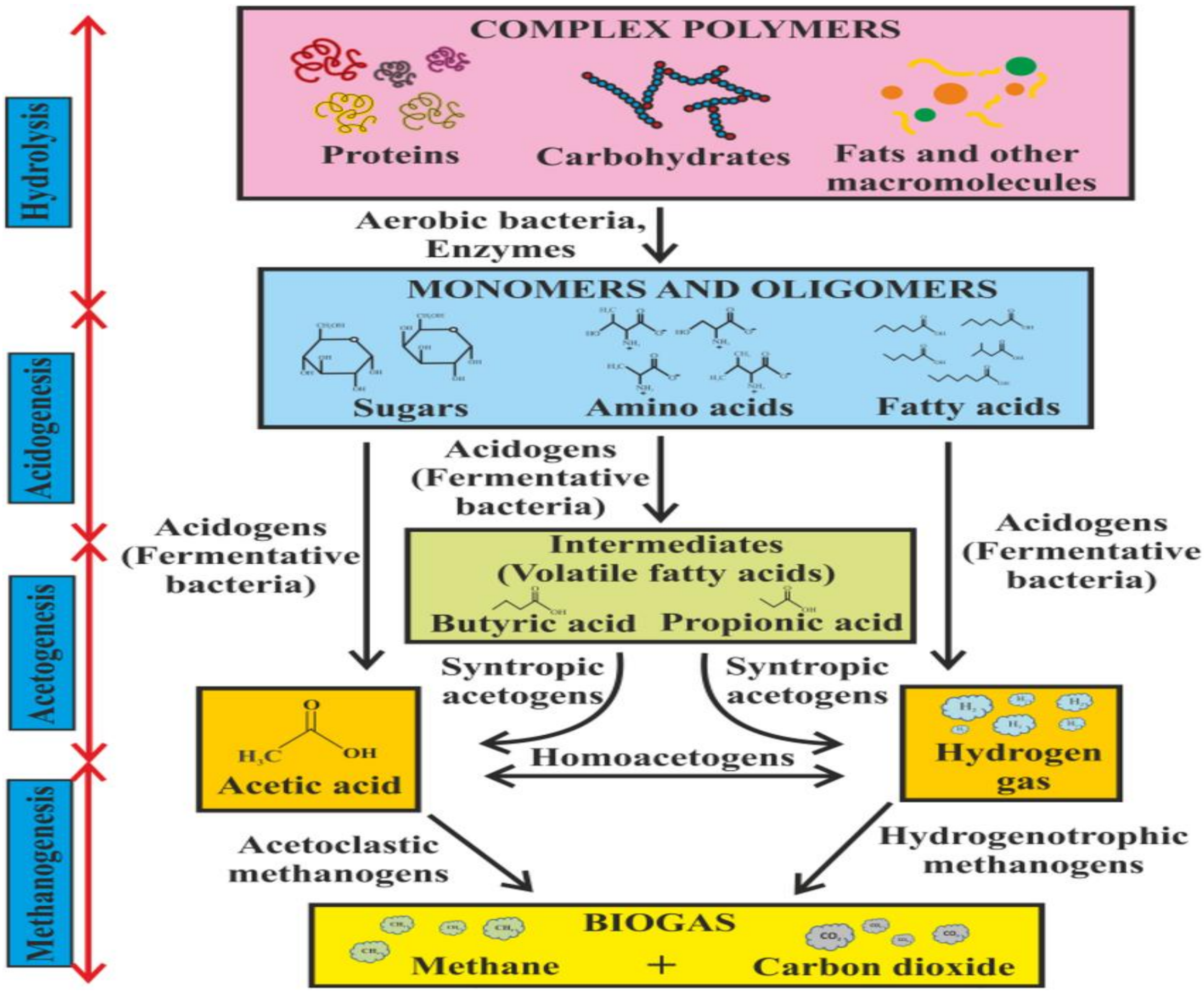

Figure 1.1. Characteristic process phases during anaerobic digestion (Sharma et al., 2023).

1.2.2.1. Hydrolysis

The hydrolysis phase of anaerobic digestion breaks down high-molecular-weight compounds, such as lipids, carbohydrates, and proteins, into simpler monomers and water-soluble organic substances. This process involves the action of extracellular enzymes (hydrolases) that are secreted by hydrolytic bacteria, mainly Bacteroides, Clostridia, and Bifidobacteria, and sometimes Streptococci and Enterobacteriaceae. Depending on the composition and bioavailability of the substrates, different amounts of sugars, amino acids, glycerin, and long-chain fatty acids are produced during the process (Lim et al., 2020). During hydrolysis, the substrate's composition affects both the distribution of intermediate products and the hydrolysis rate. Easily degradable, dissolved organic compounds, such as those in municipal sewage sludge or animal manure, can directly proceed to subsequent fermentation stages. Conversely, agricultural residues and biowaste contain complex and resistant materials, making hydrolysis the rate-limiting step of the process (Nayeri et al., 2024). The efficiency of hydrolysis is also influenced by the type of substrate and the concentration of hydrolytic microorganisms, which produce the enzymes necessary for breaking down polymers. As hydrolysis progresses, complex macromolecules are broken down into soluble compounds that microorganisms can absorb and metabolize during the later stages of digestion.

1.2.2.2. Acidogenesis

During the acidogenesis phase of anaerobic digestion, which is known as the fermentation stage, available hydrolysis products (e.g., amino acids, lipids, and glucose) are primarily converted, through a fermentation process, into short-chain organic acids (e.g., butyric, propionic, and acetic acids), alcohols, hydrogen, carbon dioxide, ammonia, and hydrogen sulphide (Zheng & Li, 2024). Acidogenic bacteria, including Streptococcus, Escherichia, Staphylococcus, Pseudomonas, Bacillus, Sarcina, Desulfovibrio, Lactobacillus, and others, primarily conduct this process. It proceeds via multiple metabolic pathways, with its outcomes strongly influenced by environmental factors such as temperature and hydrogen partial pressure. The latter directly affects the oxidation state of the products. If it is too high, it will result in products with a higher amount of carbon (Tshemese et al., 2023).

1.2.2.3. Acetogenesis

During the acetogenesis phase, various metabolic products of previous degradation stages are mainly broken down into acetic acid (acetate), hydrogen, and carbon dioxide, under the action of acetogenic bacteria, the most active being Clostridium, Syntrophomonas wolfeii, and Syntrophomonas wolinii (Agudelo-Patiño et al., 2024; Tshemese et al., 2023). This third phase of anaerobic digestion is considered critical to the process. Corresponding to the positive free enthalpy $\Delta G°$, many of the acid-forming reactions are endergonic under standard conditions and therefore do not occur spontaneously (Table 1.1). Thus, to shift the state of equilibrium and yield exergonic reactions, the resulting hydrogen must be consumed continuously (Kunz et al., 2022). Acetogenic bacteria, therefore, depend on a close symbiotic relationship with hydrogen-utilizing archaea during the methanogenesis process. For example, hydrogen produced by the oxidation of butyric acid can be used directly for hydrogenotrophic methane formation. To enable direct hydrogen exchange (interspecies hydrogen transfer) between the microorganisms involved, a small interbacterial distance and a narrow range of hydrogen partial pressure are required to create thermodynamically favourable conditions for both acid formation and hydrogen-utilizing methane formation. (Weinrich & Nelles, 2021).

Table 1.1. Stoichiometry and free enthalpy of relevant degradation pathways during acetogenesis (Weinrich & Nelles, 2021).

Reactant	Reaction	ΔG°
Propionate	$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2$	76.5
Butyrate	$CH_3[CH_2]_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2$	48.3
Valerate	$CH_3[CH_2]_3COO^- + 2H_2O \rightarrow CH_3COO^- + CH_3CH_2COO^- + H^+ + 2H_2$	48.3
Capronate	$CH_3[CH_2]_4COO^- + 4H_2O \rightarrow 3CH_3COO^- + 2H^+ + 4H_2$	97.7
Lactate	$CH_3CHOHCOO^- + 2H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 2H_2$	-4.0
Ethanol	$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$	9.6
Glycerol	$C_3H_8O_3 + 2H_2O \rightarrow CH_3COO^- + HCO_3^- + 2H^+ + 3H_2$	-73.1

1.2.2.4. Methanogenesis

During the methanogenesis phase, obligate anaerobic bacteria convert acetic acid, hydrogen, and carbon dioxide to methane, water, and carbon dioxide. This final stage of the anaerobic digestion process occurs under strictly anaerobic conditions and is characterized by exothermic reactions (Menzel et al., 2020). In principle, there are many pathways for methane formation (Table 1.2). Methane can be formed through the reduction of carbon dioxide with formate or through the disproportionation of methanol or various methylamines (Weinrich & Nelles, 2021).

Table 1.2. Stoichiometry and free enthalpy of relevant degradation pathways during methanogenesis (Weinrich & Nelles, 2021).

Reactant	Reaction	ΔG°
Acetate	$CH_3COO^- + 2H_2O \rightarrow CH_4 + HCO_3^-$	-31.0
Hydrogen	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-135.5
Formate	$HCOO^- + 3H_2 + H^+ \rightarrow CH_4 + 2H_2O$	-134.2
Methanol	$CH_3OH + H_2O \rightarrow CH_4 + H_2O$	112.5
Acetate oxidation	$CH_3COO^- + 2H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+$	104.5

According to their metabolic pathways, two types of obligate anaerobic bacteria are involved in this stage: the acetoclastic methanogenic archaea, with Methanosarcina and Methanosaeta active species, and the hydrogenotrophic methanogenic archaea, with Methanospirilum, Methanobacterium formicicum, Methanoplanus, and Methanobrevibacterium as the dominant species (Tshemese et al., 2023). Acetoclastic methanogenic archaea convert acetate into methane, while hydrogenotrophic methanogenic archaea convert hydrogen and carbon dioxide into methane. Both reactions are exothermic (Kunz et al., 2022). The pathways for methane formation via acetate or carbon dioxide are shown in Figure 1.2.

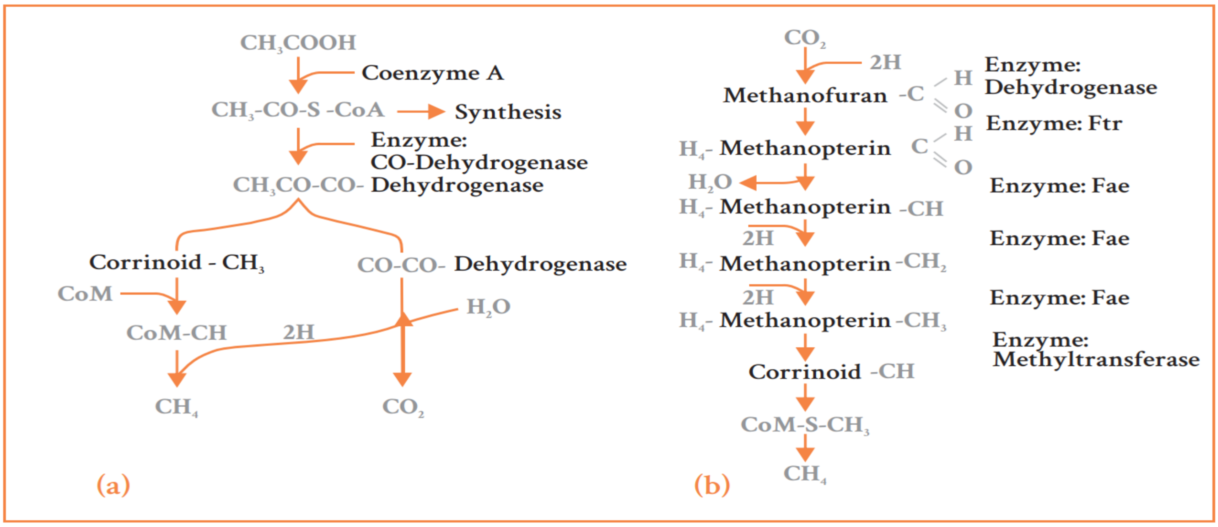

Figure 1.2. Methane formation pathway: (a) Formation of methane by acetate; (b) methane formation by carbon dioxide. CoA = coenzyme A; CoM = coenzyme M (Kunz et al., 2022).

1.2.3. Key Parameters Affecting Biogas Yield

A range of operational and substrate-related parameters influences biogas yield and process stability in anaerobic digestion. Understanding these parameters is crucial for optimizing biogas production, particularly when working with substrates of varying characteristics, such as poultry manure and cocoa pod husk.

1.2.3.1. Substrate Characteristics

The physicochemical composition of substrates is a fundamental determinant of the efficiency and stability of the anaerobic digestion process. Parameters such as moisture content, volatile solids (VS), biodegradability, lignocellulosic content, and the presence of inhibitory compounds (including heavy metals, ammonia, and antibiotics) significantly influence microbial activity and methane yield. Substrates with high moisture and readily biodegradable organic fractions, such as food waste or animal slurry, promote rapid digestion and higher gas yields (Yaser et al., 2022). In contrast, dry and lignocellulosic materials, such as cocoa pod husks, are structurally resistant due to their high cellulose, hemicellulose, and lignin content, which limits microbial access and hydrolysis efficiency (Antwi et al., 2019; Hermansyah et al., 2020). Therefore, pretreatment strategies are often necessary to enhance digestibility. Additionally, substrates rich in nutrients and free from toxic compounds are preferred, as imbalances in micronutrient availability or the presence of inhibitors can adversely affect microbial consortia responsible for bioconversion (Manyi-Loh & Lues, 2023). In co-digestion systems, the complementary nature of substrates can be leveraged to balance nutrient deficiencies and enhance biodegradability (Mora-Cortés et al., 2020), as observed in mixtures of nitrogen-rich poultry manure and carbon-rich agricultural residues, such as cocoa pod husks.

1.2.3.2. Carbon-to-Nitrogen Ratio (C/N)

The carbon-to-nitrogen (C/N) ratio is one of the most critical indicators for optimizing microbial metabolism during anaerobic digestion. An appropriate C/N balance ensures that there is adequate carbon for microbial energy needs and sufficient nitrogen for protein synthesis and microbial growth (Konkol et al., 2023). The optimal C/N ratio typically ranges between 20 and 30 (Ahmad et al., 2024); deviations from this range may lead to process inefficiencies. A low C/N ratio can result in nitrogen excess and the formation of free ammonia (NH_3), which can be toxic to methanogenic archaea and reduces process performance and biogas yield (Morozova et al., 2020). Conversely, high C/N substrates, may lead to nitrogen limitation, slowing microbial activity and hindering gas production (Schultz et al., 2025). Therefore, co-digestion of carbon-rich and nitrogen-rich substrates offers a strategy to achieve a balanced C/N ratio, enhancing process stability and methane yield. Multiple studies have demonstrated improved biogas performance through the optimization of the C/N ratio in co-digestion systems (Kadam et al., 2024; Suhartini et al., 2021).

1.2.3.3. Operational Temperature

Temperature is a major factor controlling the metabolic rate of anaerobic microorganisms and the overall kinetics of the digestion process. Anaerobic digestion is typically conducted under mesophilic (30-40 °C) or thermophilic (50-60 °C) conditions, each offering specific advantages (Wang, 2014). Thermophilic digestion accelerates hydrolysis and enhances pathogen removal, often leading to higher biogas production rates. However, it is energy-intensive and more prone to process instability due to ammonia accumulation and narrow microbial tolerance ranges (Murillo-Roos et al., 2022). In contrast, mesophilic digestion offers greater operational stability, resilience to load fluctuations, and lower energy demand, making it more suitable for decentralized or rural systems common in low-income regions (Labatut et al., 2014). The choice between mesophilic and thermophilic regimes must consider substrate characteristics, system design, energy availability, and resilience to potential ammonia inhibition, particularly relevant in systems digesting poultry manure (Hu & Shen, 2024; Steiniger et al., 2023).

1.2.3.4. pH and Alkalinity

Maintaining an optimal pH environment is essential for the sequential microbial processes involved in anaerobic digestion, particularly methanogenesis. Most methanogens thrive within a narrow pH range of 6.8 to 7.5, and deviations from this interval can inhibit their metabolic function (Marić et al., 2024). The accumulation of volatile fatty acids (VFAs) during acidogenesis can cause pH to drop, leading to acidification and system failure if not adequately buffered (He et al., 2024). Alkalinity, provided by buffering agents such as bicarbonates and ammonium ions, especially abundant in poultry manure, plays a crucial role in maintaining pH stability. Systems with poor buffering capacity are more susceptible to pH shocks, particularly under high organic loading or imbalanced substrate conditions (Francisco López et al., 2024). Proper substrate selection, co-digestion strategies, and system monitoring are therefore required to manage pH and ensure steady-state operation.

1.2.3.5. Organic Loading Rate and Hydraulic Retention Time

Organic loading rate (OLR) and hydraulic retention time (HRT) are interconnected parameters that significantly influence the digestion kinetics and stability of anaerobic digestion systems. The OLR represents the amount of substrate added to the biodigester within a given period. It refers to the amount of organic matter introduced into the digester per unit volume per day (g VS/L/day), as expressed in Equations 1.1 or 1.3 (Kunz et al., 2022). High OLR can increase biogas production but may also lead to the accumulation of volatile fatty acids (VFA) and ammonia, causing acidification and process inhibition. Conversely, low OLR can result in suboptimal reactor utilization and poor energy recovery (Prasanna Kumar et al., 2024; Wu et al., 2025). HRT defines the mean time that the substrate remains inside the biodigester, which is the ratio between the biodigester volume and the feeding flow rate, as determined using Equation 1.2 (Kunz et al., 2022). Shorter HRTs reduce reactor size but risk incomplete digestion, while longer HRTs ensure complete degradation at the expense of larger system volumes (Shi et al., 2017). Optimizing both organic loading rate and hydraulic retention time is essential to ensure microbial stability, maximize methane yield, and prevent system overloading or washout of microbial populations. Tailoring these parameters to the substrate composition, especially in co-digestion systems involving high-nitrogen poultry manure and fibrous cocoa pod husks, can enhance process efficiency and energy output.

$$\mathrm{OLR} = \frac{\mathrm{Q} \times \mathrm{Sc}}{\mathrm{V}} \tag{1.1}$$

$$\mathrm{HRT} = \frac{\mathrm{V}}{\mathrm{Q}} \tag{1.2}$$

$$\mathrm{OLR} = \frac{\mathrm{Sc}}{\mathrm{HRT}} \tag{1.3}$$

Where: OLR: Organic loading rate (kg/m^3/day), Q: Flow rate (m^3/day), Sc: Substrate concentration (kg/m^3), V: Reactor volume (m^3), and HRT: Hydraulic retention time (day).

1.2.3.6. Substrate-to-Inoculum Ratio (S/I) and Inoculum Quality

The substrate-to-inoculum (S/I) ratio is a key design parameter, particularly in biochemical methane potential (BMP) tests and batch digestion setups, as it affects microbial seeding density and process kinetics (Khadka et al., 2022). An optimal S/I ratio ensures efficient microbial colonization, balanced acidogenesis and methanogenesis, and prevention of substrate overload. Excessive substrate concentrations (high S/I ratios) can cause rapid acidification and methanogen inhibition, while excessively low S/I ratios may lead to underutilized systems and lower biogas productivity (Valentin et al., 2024).

Additionally, the origin and quality of the inoculum significantly influence digestion outcomes. Inoculum sourced from active, well-acclimated digesters typically contains a robust and diverse microbial community capable of handling a range of substrates (De Vrieze et al., 2015; Yangin-Gomec et al., 2020). This microbial diversity is particularly important when dealing with lignocellulosic and recalcitrant biomass, such as cocoa pod husks, which require effective hydrolytic and methanogenic consortia to enhance substrate biodegradability and methane conversion efficiency. Several studies have demonstrated that inoculum acclimation, microbial diversity, or prior exposure to similar substrate types significantly improve digestion performance, both in terms of methane yield and process stability (Atmowidjojo et al., 2023; Jasińska et al., 2023). Various inocula have been successfully used in anaerobic digestion studies, depending on substrate characteristics and research context. Cow dung or cattle slurry is one of the most common and effective inocula, especially in rural and agricultural systems, due to its balanced microbial community and buffering capacity. It has been used successfully in co-digestion studies involving fibrous materials, including cocoa wastes (Hermansyah et al., 2020). Anaerobic sludge from municipal wastewater treatment plants is another widely used inoculum, valued for its high microbial activity and consistency, particularly in laboratory-scale biochemical methane potential (BMP) tests. However, its adaptation to lignocellulosic substrates may be limited without acclimatization (Lallement et al., 2021; Oliva et al., 2025). More substrate-specific inocula include digestate from operating biogas plants, especially those processing agro-industrial or food waste. These inocula are often well adapted to similar feedstock compositions and have demonstrated improved digestion performance when co-digested with cocoa residues and animal manure (Atmowidjojo et al., 2023). Pig manure has also been employed, although its high nitrogen content can pose a risk of ammonia inhibition (Nordgård et al., 2017). In research targeting the hydrolysis of recalcitrant plant fibers, rumen fluid from ruminants has shown promise due to its naturally high concentration of cellulolytic and ligninolytic microorganisms. However, ethical and logistical constraints often limit its use to laboratory investigations (Meyer et al., 2022; Yu et al., 2024). Overall, the selection and adaptation of inoculum should be guided by the nature of the substrate mixture. For the co-digestion of poultry manure and cocoa pod husks, inocula derived from manure-based digesters or agro-waste treatment plants are especially appropriate. These not only ensure microbial compatibility but also enhance the system's resilience to common digestion inhibitors, such as free ammonia and volatile fatty acids.

1.2.4. Main products of anaerobic digestion: Biogas and Digestate

Anaerobic digestion yields two primary products of significant environmental and economic value: biogas and digestate. Biogas is a combustible gas mixture primarily composed of CH_4 (50-70%) and CO_2 (30-50%), with minor traces of hydrogen sulphide (H_2S), ammonia (NH_3), hydrogen (H_2), and water vapor. The methane content is crucial as it determines the calorific value of the biogas, typically ranging from 20 to 25 MJ/m^3, making it a versatile renewable energy carrier. Biogas can be used directly for heat, cooking, or electricity generation, or upgraded into biomethane (greater that 95% CH4) for injection into natural gas grids or as a vehicle fuel (Francisco López et al., 2024). Aside from its energy potential, biogas production significantly reduces greenhouse gas emissions by capturing CH_4 that would otherwise escape into the atmosphere from decaying organic matter.

On the other hand, digestate, the solid-liquid residue remaining after digestion, is a nutrient-rich biofertilizer containing nitrogen, phosphorus, potassium, and trace minerals in plant-available forms. Digestate enhances soil fertility, improves soil structure, and reduces the need for synthetic fertilizers, thereby contributing to sustainable agricultural practices (Badagliacca et al., 2024). The digestate can be separated into solid and liquid fractions. The solid portion is often used for composting or direct land application, while the liquid phase may be used for fertigation or subjected to further treatment to recover nutrients. The characteristics and quality of digestate largely depend on feedstock composition, digestion conditions, and the retention time. In co-digestion systems involving nitrogen-rich and carbon-rich substrates, such as poultry manure and cocoa pod husks, the digestate can be enriched with a balanced nutrient profile suitable for use as a bio-based fertilizer, especially in degraded or nutrient-deficient soils (Gadirli et al., 2023; Neri et al., 2023).

Both products highlight the dual value of anaerobic digestion as a renewable energy solution and a resource recovery pathway that aligns with the principles of a circular economy.

1.2.5. Benefit of Biogas Production Technology

Anaerobic digestion has gained global recognition as a sustainable waste-to-energy technology, offering an integrated solution for managing agricultural residues, livestock waste, and a wide range of other organic materials (Scarlat et al., 2018; Yudi & Teguh, 2025). This technology offers numerous benefits, including environmental, energy, and socio-economic advantages (Vögeli et al., 2014). Its environmental significance lies primarily in its capacity to capture and utilize methane (CH_4), a greenhouse gas over 25 times more potent than carbon dioxide (CO_2), thereby playing a vital role in climate change mitigation (Daniel-Gromke et al., 2015; El Mashad et al., 2023). Furthermore, biogas, the main output of AD, is a renewable and flexible energy carrier that can be used for electricity generation, heating, or upgraded to biomethane for integration into natural gas networks. This enhances energy security, reduces reliance on fossil fuels, and supports the development of decentralized, low-carbon energy systems (Yudi & Teguh, 2025). In addition to its environmental and energy advantages, AD offers significant socio-economic benefits. It significantly reduces the volume of organic waste, thereby lowering waste disposal costs and easing pressure on landfill infrastructures (Huang, 2024). The process transforms waste into valuable resources: biogas for energy, and digestate as a biofertilizer, contributing to soil fertility and supporting sustainable agricultural practices. The expansion of anaerobic digestion technology stimulates local job creation in areas such as plant construction, operation, feedstock logistics, biogas upgrading, and digestate management, contributing to rural development, skills enhancement, and income generation (Scarlat et al., 2018). By offering a clean alternative to traditional biomass fuels (firewood), AD technologies also help to reduce deforestation, preserve ecosystems, and lower pollution associated with organic waste and fossil fuel use.

1.3. Existing Anaerobic Digestion Technologies

Anaerobic digestion (AD) technologies have evolved into a diverse array of engineered systems that support the efficient transformation of organic matter into biogas and digestate. These technologies are classified based on system configuration, feedstock type, operational mode, solids content, and temperature regime (Aworanti et al., 2023).

From an operational standpoint, AD systems are either batch or continuous. Batch digesters are simpler and more affordable, commonly used in small-scale or rural settings, but they offer intermittent gas production and require longer retention times. Continuous systems include continuously stirred tank reactors (CSTRs) and plug flow reactors (PFRs). Up-flow anaerobic sludge blanket (UASB) reactors offer more stable and efficient digestion, making them suitable for larger-scale applications; however, they require stricter process control and monitoring (Gadirli et al., 2023; Kunz et al., 2022).

Anaerobic digesters are also differentiated based on solids content into wet (low solids) and dry (high solids) systems. Wet digesters operate effectively with feedstocks containing less than 15% total solids, making them ideal for liquid manure, sewage sludge, and slurry. In comparison, dry systems handle feedstocks with solids content between 15-40%, such as crop residues, food waste, or cocoa pod husks, offering better energy density and lower water use (Wang et al., 2023). Depending on the temperature range, digesters are categorized into mesophilic (35-40°C) and thermophilic (50-60°C) systems. While mesophilic digesters offer process stability and energy efficiency, thermophilic systems enable faster reaction kinetics, better pathogen destruction, and higher biogas output; however, they are more sensitive to disturbances and require more energy for heating (Lee et al., 2024).

Technological advancements have led to the development of two-stage or two-phase digestion systems, in which the hydrolytic-acidogenic and acetogenic-methanogenic stages are separated into distinct reactors. This separation enables improved microbial specialization, enhanced stability, and increased methane yields, particularly for complex or inhibitory substrates (Simeonov et al., 2025). In low-income and rural regions, small-scale and low-cost digesters, such as fixed-dome, floating-drum, and tubular plastic designs (Figure 1.3), are increasingly promoted due to their affordability, simplicity, and adaptability to local organic waste streams. These systems are particularly relevant in sub-Saharan Africa, where livestock manure and agricultural residues, such as cocoa pod husks and poultry droppings, are abundant, and energy access remains limited (Vögeli et al., 2014). Selecting an appropriate AD technology thus requires a context-specific approach that considers substrate availability, technical expertise, climatic conditions, economic constraints, and intended end uses of the biogas and digestate.

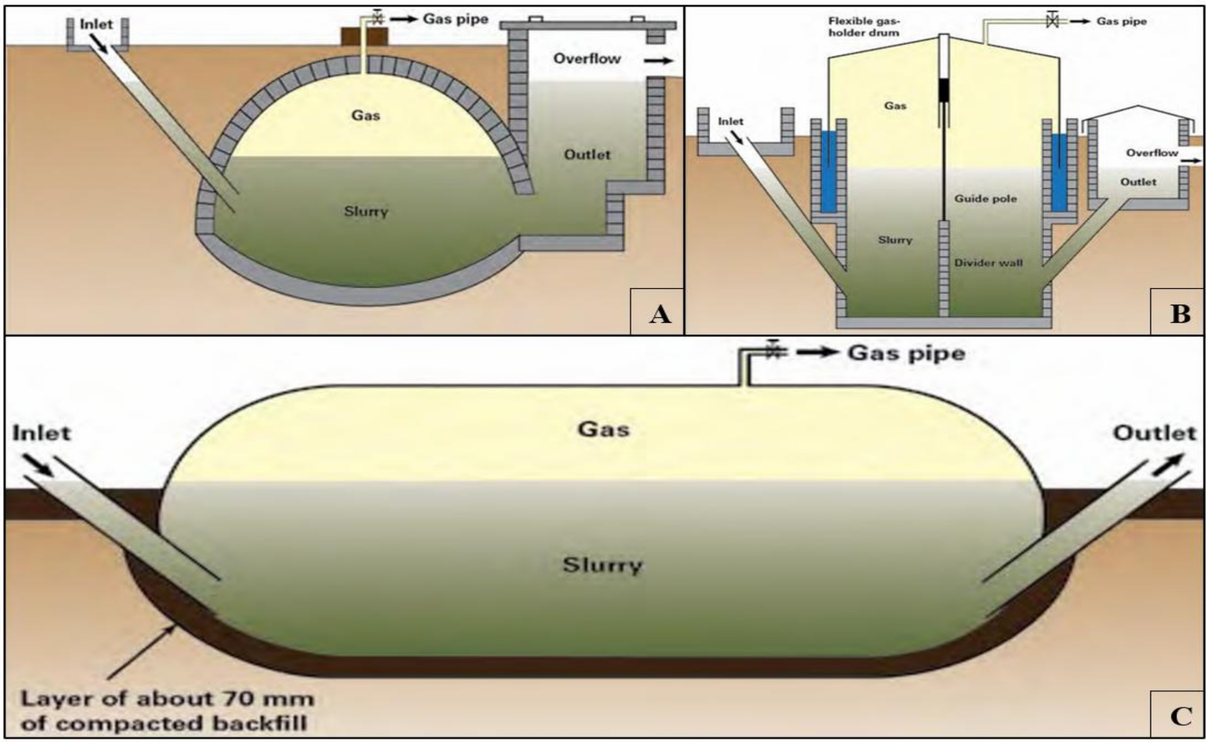

Figure 1.3. Most developed biodigester technologies in developing countries: Fixed-dome (A), floating-drum (B), and tubular plastic (C) digesters (Vögeli et al., 2014).

1.4. Biochemical Methane Potential (BMP) Test

The Biochemical Methane Potential (BMP) test is a standardized laboratory method used to evaluate the anaerobic biodegradability and methane or biogas production potential of organic substrates under controlled conditions. It serves as a critical tool in assessing the technical feasibility of substrates for anaerobic digestion, guiding reactor design, co-digestion strategies, and process optimization (Filer et al., 2019; Koch et al., 2020).

The BMP test provides quantitative data on the specific methane or biogas yield, typically expressed in millilitres of methane or biogas per gram of volatile solids added (mL CH_4/g VS or mL Biogas/g VS), and helps determine the degradation rate, kinetics, and lag phase associated with a given substrate (Llanos-Lizcano et al., 2024). The test is conducted in airtight, small-scale batch reactors incubated under mesophilic (35-38 °C) or thermophilic (50-55 °C) conditions, depending on the intended digestion regime. The reactors are inoculated with anaerobic sludge from an active digester and fed with a defined quantity of the test substrate, maintaining an appropriate substrate-to-inoculum ratio (S/I), typically between 0.5 and 1.0 on a volatile solids basis, to prevent system overload or substrate limitation (VDI 4630, 2016). A blank control containing only inoculum is run in parallel to account for baseline gas production. During the test, biogas production is monitored over time (often 20-40 days), and methane content is analysed. The cumulative methane or biogas yield is corrected for standard temperature and pressure (STP) and normalized to the VS content of the substrate.

For quality assurance, BMP protocols must ensure proper sealing of reactors, regular agitation to avoid stratification, and monitoring of pH, VFAs, and gas composition throughout the test period. The German VDI 4630 standard and the ISO 11734 protocol are widely used for ensuring methodological consistency in BMP testing. In addition to determining methane potential, BMP tests can reveal inhibitory effects caused by ammonia, sulphur compounds, or recalcitrant lignin, providing valuable insights into substrate compatibility and the necessary pretreatment conditions. When assessing the co-digestion of substrates such as poultry manure and cocoa pod husks, BMP tests enable a comparative evaluation of mono- and co-digestion scenarios, allowing for the identification of synergistic effects or nutrient imbalances. Thus, the BMP test remains an indispensable method in the early-stage evaluation of feedstocks and the development of efficient, tailored anaerobic digestion systems.

1.5. Agricultural Wastes as Feedstock for Anaerobic Digestion

Agricultural wastes represent one of the most promising and underutilized feedstock categories for anaerobic digestion (AD), offering both environmental and economic benefits through waste valorisation, renewable energy generation, and nutrient recovery, as illustrated in Figure 1.4 (Adnane et al., 2024; Alengebawy et al., 2024). These wastes are primarily composed of crop residues (e.g., straw, stalks, husks, leaves), animal manure, agro-industrial by-products, and slaughterhouse wastes, all of which contain significant amounts of biodegradable organic matter suitable for microbial degradation under anaerobic conditions (Zielińska & Bułkowska, 2024). Their high availability, especially in rural and agrarian economies like those of West Africa, provides a decentralized and low-cost biomass source for biogas production. However, the digestibility of agricultural wastes varies widely depending on their composition. Lignocellulosic materials such as cereal straw, corn stover, and cocoa pod husks contain complex polymers (cellulose, hemicellulose, and lignin) that limit microbial access and enzymatic breakdown, often requiring pretreatment methods, such as thermal, alkaline, or biological processes, to enhance hydrolysis and improve methane yields. Conversely, animal manures, including those from poultry, cattle, and pigs, are rich in nitrogen and easily degradable organic matter but may pose risks of ammonia inhibition, primarily when digested alone (Díaz-González et al., 2022; García Álvaro et al., 2024). The co-digestion of different agricultural residues with complementary characteristics (carbon-rich crop waste and nitrogen-rich manure) offers a strategic pathway to balance nutrient ratios (particularly the C/N ratio), dilute inhibitors, and improve buffering capacity and biogas yield (Jasińska et al., 2023; Kadam et al., 2024). Utilizing these residues also contributes to sustainable agricultural practices by minimizing open-field burning and nutrient losses, reducing greenhouse gas emissions, and producing digestate as a soil conditioner to enhance soil fertility. When adequately managed, agricultural waste can be transformed from an environmental liability into a valuable resource that supports clean energy access, climate resilience, and circular economy objectives in both developed and developing regions.

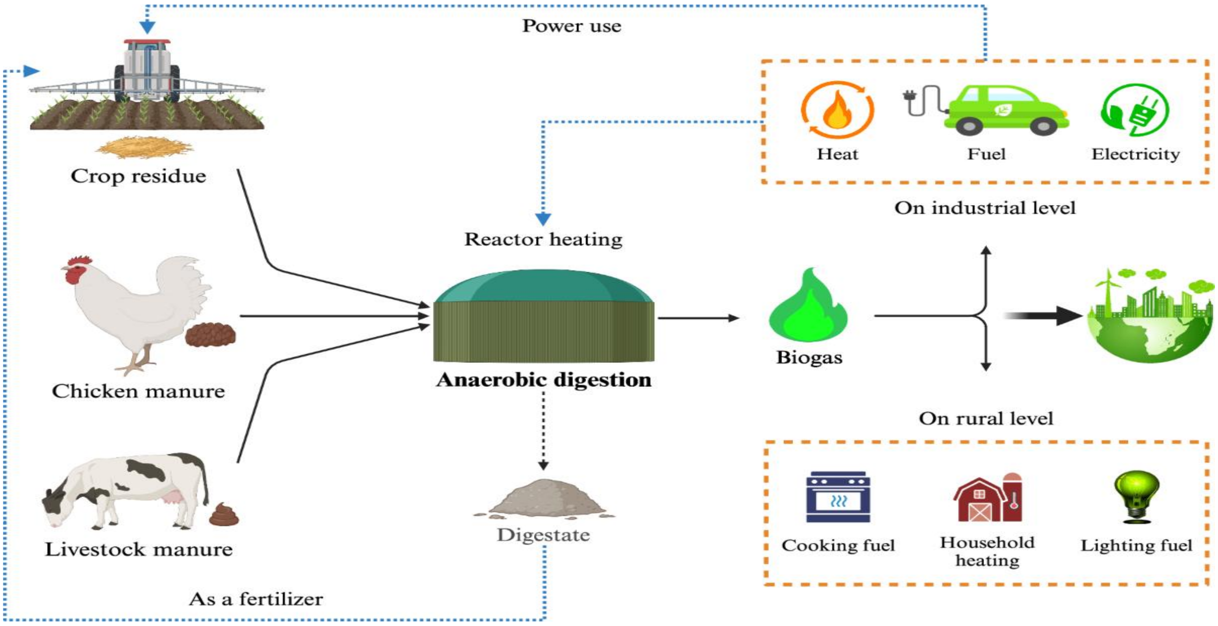

Figure 1.4. Biogas production from agricultural waste and biogas utilization (Alengebawy et al., 2024).

1.6. Poultry Manure as a Substrate for Anaerobic Digestion

Poultry manure is a widely available and nutrient-rich organic waste with high potential for biogas production through anaerobic digestion (AD). It is composed of feces, bedding materials (like sawdust, wood chips, rice husks, and straw), feed residues, feathers, and uric acid excretions, resulting in a substrate that is particularly high in nitrogen, volatile solids (VS), and essential micronutrients such as phosphorus and potassium (Kadam et al., 2024; Tawfik et al., 2023). Due to its high biodegradability and relatively low lignin content, poultry manure is quickly metabolized during AD, offering short digestion times and significant biogas yields, often ranging from 200-617mL/g VS (Al-Zoubi et al., 2024; Jasińska et al., 2023; Jurgutis et al., 2020; Li et al., 2013; Michailidou et al., 2024), depending on system conditions and manure characteristics. However, one of the primary limitations of using poultry manure alone lies in its low carbon-to-nitrogen (C/N) ratio, typically between 5 and 10, which can lead to the accumulation of free ammonia (NH_3) during digestion. Elevated NH_3 concentrations are toxic to methanogenic archaea and can result in process inhibition, pH disturbances, and reduced methane yield (Tawfik et al., 2023; Yang et al., 2024). A summary of chicken manure characteristics is shown in Figure 1.5. Additionally, poultry manure often exhibits a high buffering capacity due to the presence of bicarbonates and ammonium ions, which may help stabilize pH levels but can also mask the early signs of acid accumulation and system imbalance (Song et al., 2023b).

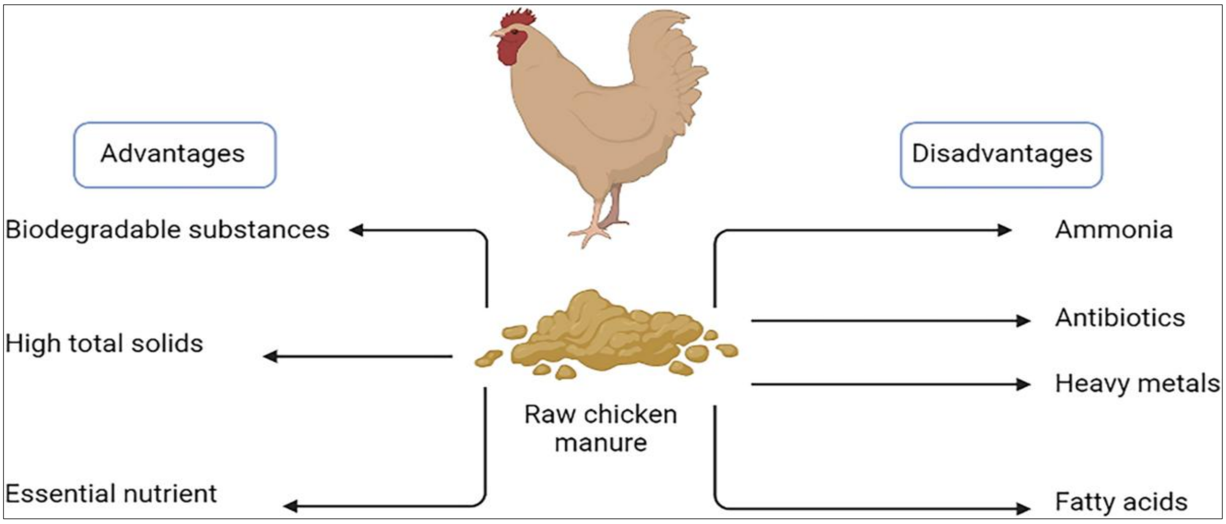

Figure 1.5. Characteristics of poultry manure as feedstock for anaerobic digestion (Tawfik et al., 2023).

Co-digestion with carbon-rich substrates, such as crop residues, agro-industrial waste, or lignocellulosic biomass like cocoa pod husks, has been widely recommended to adjust the C/N ratio and dilute inhibitory compounds, thereby enhancing both microbial stability and methane yield (Jasińska et al., 2023; Kadam et al., 2024). Moreover, poultry manure provides an excellent source of essential trace elements (like iron, cobalt, nickel, etc.) that support enzymatic pathways in methanogenesis, especially when combined with poorly buffered or nutrient-deficient substrates. Pretreatment is generally not required due to the high biodegradability of the material, but sieving or dilution may be necessary to reduce solids and ensure proper digester flow and mixing. From a management perspective, utilizing poultry manure in biogas systems reduces environmental burdens associated with nutrient leaching, greenhouse gas emissions, and odors typically linked to raw manure storage or land application. AD also converts pathogenic organisms into safer residues, contributing to improved public health and hygienic waste disposal (Boakye-Yiadom et al., 2024; Burch et al., 2018). Overall, poultry manure represents a technically feasible and energetically valuable substrate for anaerobic digestion, especially when integrated into co-digestion schemes that balance its chemical limitations and maximize its fertilizing potential through the recovery of digestate.

1.7. Cocoa Pod Husk as a Substrate for Anaerobic Digestion

Cocoa pod husk (CPH), as shown in Figure 1.6, is the primary by-product of cocoa processing, constituting approximately 70-80% of the total weight of the cocoa fruit (Ouattara et al., 2021). In cocoa-producing countries such as Côte d'Ivoire, Ghana, Nigeria, and Togo, CPH is generated in large quantities and is often left to decompose on the plantation or openly burned, leading to nutrient loss, greenhouse gas emissions, and increased risk of fungal infestations such as Phytophthora palmivora, the causative agent of black pod disease (Anoraga et al., 2024). However, CPH possesses significant biochemical potential for biogas production due to its high content of carbohydrates, particularly cellulose and hemicellulose, along with moderate levels of protein and lipids (Atmowidjojo et al., 2023; Bugarin et al., 2025). Its high carbon-to-nitrogen (C/N) ratio, generally ranging from 35 to 55, makes it ideal as a co-substrate with nitrogen-rich materials, such as poultry manure, to achieve a balanced nutrient profile and reduce ammonia inhibition in anaerobic digestion (Hennessey-Ramos et al., 2024; Schultz et al., 2025). Despite this potential, the lignocellulosic structure of CPH renders it relatively resistant to microbial degradation, making the hydrolysis phase the rate-limiting step during digestion. This limitation can be addressed through pretreatment methods, such as mechanical shredding, thermal hydrolysis, alkaline soaking, or enzymatic treatment, which improve surface area, break down lignin, and increase substrate availability for fermentative microorganisms (Mora-Cortés et al., 2020).

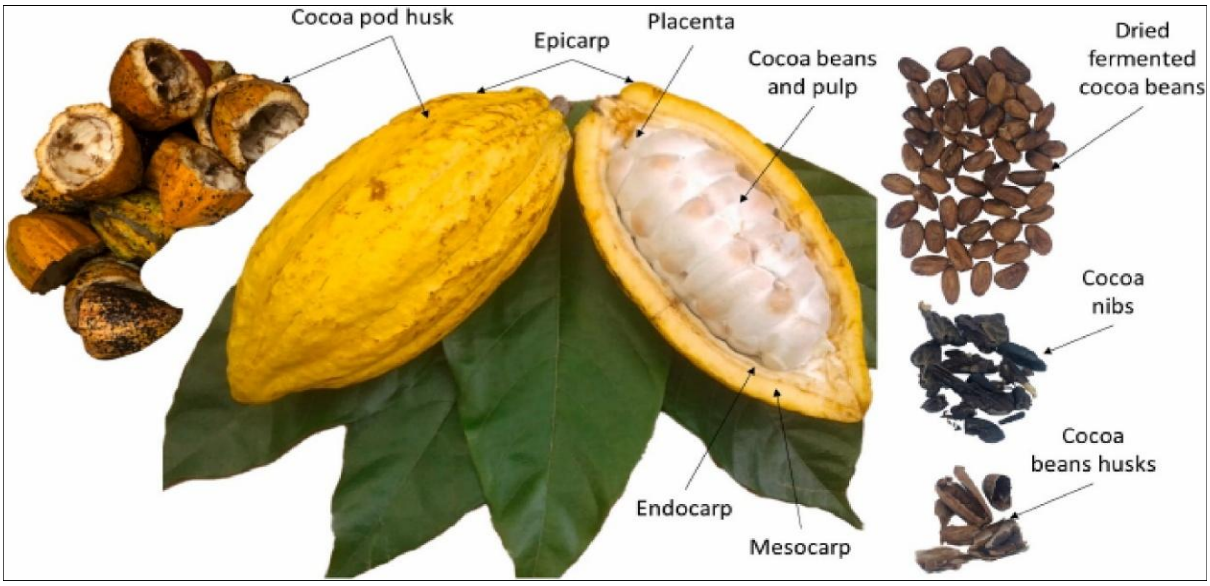

Figure 1.6. Parts of the cocoa fruit (Anoraga et al., 2024).

Studies have demonstrated that CPH, when properly pretreated or co-digested, can yield substantial volumes of biogas, with biogas potentials reported between 150-450 mL/g VS under mesophilic conditions (Antwi et al., 2019; Atmowidjojo et al., 2023; Hennessey-Ramos et al., 2024). Additionally, CPH contains micronutrients such as potassium, calcium, and magnesium, which enhance the agronomic value of the resulting digestate. However, excessive lignin, polyphenols, or high total solids content can inhibit digestion if not appropriately managed (Kone et al., 2020; Soares & Oliveira, 2022). In rural contexts, CPH is readily available at the point of harvest, making it a cost-effective and decentralized resource for small- and medium-scale biogas systems. Valorising this waste through anaerobic digestion not only contributes to sustainable waste management and clean energy generation but also addresses soil fertility and public health concerns associated with unmanaged cocoa waste. Integrating CPH into co-digestion schemes, particularly with poultry manure, offers a synergistic pathway to optimize methane yield, nutrient recovery, and circular bioeconomy practices in cocoa-producing regions.

1.8. Anaerobic Co-Digestion: Concept and Benefits

Anaerobic co-digestion (AcoD) is the simultaneous anaerobic digestion of two or more organic substrates within the same reactor, designed to leverage the complementary characteristics of different feedstocks to improve process efficiency, biogas yield, and operational stability (Chow et al., 2020; González et al., 2022). The core principle of co-digestion involves balancing the nutrient content, particularly the carbon-to-nitrogen (C/N) ratio, and optimizing the physicochemical and biological conditions for microbial activity. This approach mitigates limitations often associated with mono-digestion, such as ammonia inhibition from nitrogen-rich materials (e.g., poultry manure) or low biodegradability of lignocellulosic biomass (e.g., cocoa pod husks) (Ibro et al., 2022; Jasińska et al., 2023; Suhartini et al., 2021)

One of the key benefits of AcoD is the optimization of the substrate mix, where high-nitrogen wastes can be diluted by carbon-rich residues, resulting in a C/N ratio typically between 20 and 30, which is ideal for stable methanogenesis. Additionally, co-digestion can dilute inhibitory substances, improve buffering capacity, enhance microbial diversity, and increase volumetric biogas productivity (Aworanti et al., 2023; Kadam et al., 2024; Rabii et al., 2019). These synergistic effects extend to the biochemical methane potential (BMP) of the mixture, which often surpasses that of individual substrates due to improved hydrolysis kinetics and microbial syntrophy. Anaerobic co-digestion also increases the flexibility of digester feedstock supply and enables better management of seasonal or regionally available biomass (Miller et al., 2021). Furthermore, co-digestion promotes resource efficiency and waste management integration, allowing different sectors (e.g., agriculture, food processing, municipal services) to treat organic residues collaboratively (Macias-Corral et al., 2017).

From an environmental standpoint, Anaerobic co-digestion reduces greenhouse gas emissions by diverting biodegradable wastes from open dumping, lagoons, or uncontrolled decomposition. It contributes to improved sanitation and nutrient recycling through digestate production, which serves as an organic fertilizer with improved nutrient balance compared to mono-digested residues. In terms of economic and energy performance, Anaerobic co-digestion offers higher biogas production rates when substrates are mixed in an optimal ratio, increased energy output per unit reactor volume, and improved cost-effectiveness by maximizing the use of digester infrastructure and reducing downtime (Tan et al., 2021). In developing regions such as sub-Saharan Africa, where multiple organic waste streams coexist and energy poverty persists, co-digestion provides a compelling pathway for decentralized, low-cost biogas systems tailored to local needs. As research continues to optimize substrate combinations, inoculum ratios, and digestion conditions, AcoD is increasingly recognized as a cornerstone of sustainable biogas technology and circular bioeconomy strategies.

1.9. Co-Digestion of Poultry Manure and Cocoa Pod Husk

The co-digestion of poultry manure (PM) and cocoa pod husk (CPH) has emerged as a promising approach to address the individual limitations of these substrates while maximizing their combined potential for biogas production. Poultry manure is rich in nitrogen, readily biodegradable, and contains high concentrations of essential micronutrients that support methanogenesis; however, its low carbon-to-nitrogen (C/N) ratio (typically 5-10) often leads to ammonia accumulation, which can inhibit methanogenic archaea and reduce process stability (Song et al., 2023b; Yang et al., 2024). On the other hand, cocoa pod husk is a lignocellulosic biomass with a high C/N ratio (35-55) and low nitrogen content. While its high carbon content is advantageous, its recalcitrant structure, dominated by cellulose, hemicellulose, and lignin, limits microbial accessibility and slows down the hydrolysis phase of anaerobic digestion (Antwi et al., 2019; Hermansyah et al., 2020).

When combined in anaerobic co-digestion, PM and CPH exhibit complementary chemical characteristics, enabling a balanced C/N ratio that enhances microbial synergy, prevents ammonia toxicity, and improves methane yield. Few studies have highlighted the synergistic benefits of co-digesting PM and CPH, reporting higher biogas yields and improved process stability compared to the mono-digestion of either substrate. For instance, Dahunsi et al. (2019) observed a 68% increase in biogas production when pretreated CPH was co-digested with poultry manure, underlining the potential of this combination. These outcomes are attributed to better buffering capacity, dilution of inhibitory compounds, improved degradation of fibrous materials, and enhanced microbial diversity within the digester.

In addition to improved energy yields, the digestate generated from PM-CPH co-digestion is rich in nutrients. It can be used as an organic fertilizer, particularly suitable for cocoa plantations, where enrichment in potassium and organic matter is beneficial. Furthermore, co-digestion reduces environmental hazards associated with the uncontrolled disposal of poultry manure (like groundwater contamination, odor emissions) and open burning or rotting of cocoa husks, which release greenhouse gases and promote fungal growth. While research on this specific substrate combination remains limited, particularly under West African agroclimatic conditions, the available evidence supports the technical feasibility and environmental relevance of PM-CPH co-digestion. Further experimental research is needed to optimize critical parameters, such as mixing ratios, retention times, temperature regimes, and pretreatment strategies, to fully harness the energetic and agronomic value of these abundantly available wastes.

1.10. Research Gaps and Justification for the Present Study

Despite extensive research on anaerobic digestion (AD) and co-digestion strategies, significant research gaps persist, particularly regarding the combined valorisation of poultry manure (PM) and cocoa pod husk (CPH) under West African agroclimatic conditions. While PM and CPH are individually well-documented substrates, their co-digestion remains underexplored. Most existing studies focus on either mono-digestion or co-digestion with conventional crop residues or food waste (Atmowidjojo et al., 2023; Mora-Cortés et al., 2020), while the synergistic potential of PM-CPH combinations remains insufficiently quantified. Moreover, the influence of operational parameters such as temperature regime (mesophilic and thermophilic), substrate mixing ratio, and C/N balance on biogas yield and process stability in PM-CPH systems has not been systematically assessed. Another critical gap lies in the lack of region-specific data regarding the biogas and biochemical methane potential (BMP) of locally sourced CPH and PM, including variations due to feedstock handling and environmental conditions. Without this data, efforts to scale biogas production technologies in cocoa-growing regions such as Togo, Côte d'Ivoire, or Ghana remain speculative.

This study aims to fill these gaps by assessing the biogas potential from the Anaerobic co-digestion of poultry manure and cocoa pod husk in Togo, with a focus on optimizing biogas yield by evaluating the effect of different C/N ratios on biogas production under both mesophilic and thermophilic conditions. By conducting controlled BMP assays and characterizing the substrates, this research will generate context-relevant scientific data that can guide the deployment of small- to medium-scale biogas systems tailored to West African realities. The study is further justified by the abundance and low cost of the target feedstocks, which are often considered waste and pose environmental management challenges. Valorising them through anaerobic co-digestion aligns with circular economy principles and supports sustainable agriculture, rural energy access, and climate resilience in low-income contexts.

1.11. Conclusion

The literature confirms the synergistic potential of co-digesting poultry manure with carbon-rich lignocellulosic substrates like cocoa pod husks. However, the optimization of operational parameters, particularly the C/N ratio and temperature, remains underexplored in West African contexts. Addressing these gaps through experimental validation is crucial for advancing biogas production technology as a means to achieve energy access, effective waste management, and climate resilience in the region.

CHAPTER 2: MATERIALS AND METHODOLOGY

2.1. Introduction

This chapter presents the materials and methods used to investigate the anaerobic co-digestion of poultry manure (PM) and cocoa pod husks (CPH), with the aim of evaluating the effects of carbon-to-nitrogen (C/N) ratio and temperature regime on biogas production. It describes the study context, the collection and pre-treatment of substrates, and their physicochemical characterization (pH, proximate, fiber, ultimate, and energy analyses). The chapter also outlines the experimental design of the Biochemical Methane Potential (BMP) tests conducted under mesophilic and thermophilic conditions at different C/N ratios, together with the control setups, and concludes with the statistical methods applied to ensure the reliability of the results.

2.2. Study Area and Experimental Context

As illustrated in Figure 2.1, Togo is a West African country covering approximately 56,600 km², with Lomé as its capital city (Agboka et al., 2024). The country exhibits a diverse intertropical climate gradient, ranging from the humid, sub-equatorial Guinean zone in the south to the drier Sudanian zone in the north (WBG, 2021). This climatic variability has a strong influence on agricultural practices and the availability of residues, making Togo a representative case study for bioenergy research and feedstock diversification.

In the southern part of the country, areas such as Kpalimé and Agou benefit from high rainfall levels, ranging from 1,000 to 1,600 mm annually (Agboka et al., 2024), and are well-known cocoa-producing regions, contributing approximately 15,000 tons of cocoa in 2023 (FAOSTAT, 2023). These regions generate significant quantities of cocoa pod husks (CPH) as agricultural waste. Conversely, the northern regions are more arid and dominated by livestock and cereal farming, resulting in the production of organic residues, including livestock waste and crop residues. Across the country, particularly in peri-urban areas, poultry farming is widespread, contributing large volumes of poultry manure (PM). A key source for this study was the poultry farm of the School of Agronomy (École Supérieure d'Agronomie, ESA), University of Lomé, which supplied the PM samples used in the experiments.

To ensure alignment with local biomass resource availability, all feedstocks were sourced and then pretreated at the laboratory of biomass and bioenergy technologies (LT2B), University of Lomé, Togo. Subsequent in-depth analyses, including pH, proximate, ultimate, fiber analysis, and all biochemical methane potential (BMP) tests, were performed at the technical scale lab for bioenergy, University of Rostock, Germany, providing standardized, high-precision data for this study.

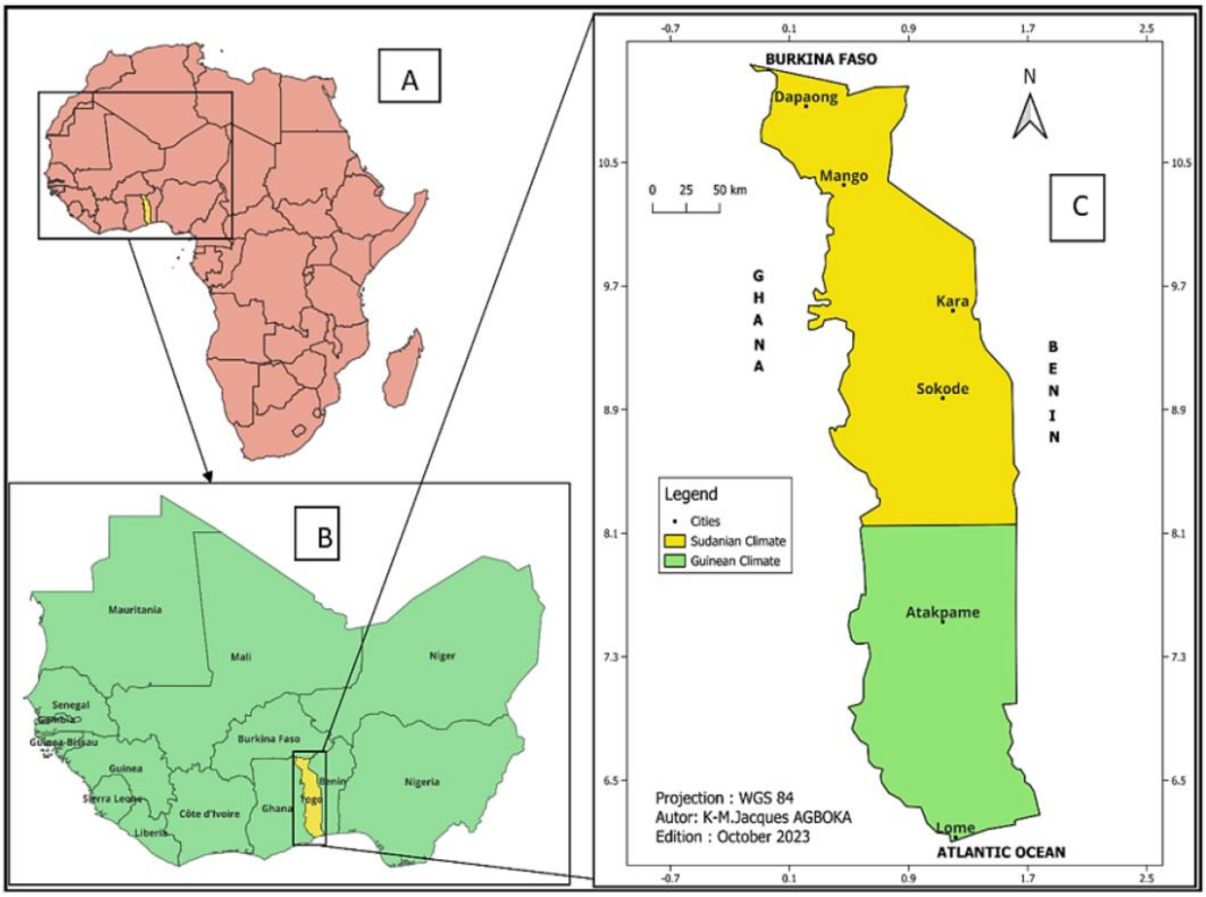

Figure 2.1. Location of the study area A: Togo in Africa B: Togo in West Africa C: Climatic zones of Togo (Agboka et al., 2024).

2.3. Study Material

2.3.1. Substrates: Cocoa Pod Husk (CPH) and Poultry Manure (PM)

CPH were collected on February 13, 2025, from a cocoa plantation located in Agou, a town approximately 106 kilometers from Lomé, the capital of Togo. The collection process involved gathering empty cocoa pods, after the beans had been extracted, and placing them into polypropylene bags to facilitate transportation to the University of Lomé. However, PM was sourced from the farm of the School of Agronomy (École Supérieure d'Agronomie, ESA) at the University of Lomé. At this facility, manure is typically removed from the poultry houses and stored in multiple polypropylene bags, which are then piled in an open area designated as the farm's manure dumping zone. The sampling, conducted on February 15, 2025, involved collecting manure from various bags and thoroughly mixing the samples to obtain a composite sample representative of the farm's overall output. The resulting sample was then transferred to a clean plastic bag for storage before laboratory analysis and experimentation. Pictures of the collected substrate are displayed in Figure 2.2. Also, an illustration of the collection step is displayed in APPENDIX A: Substrate Collection.

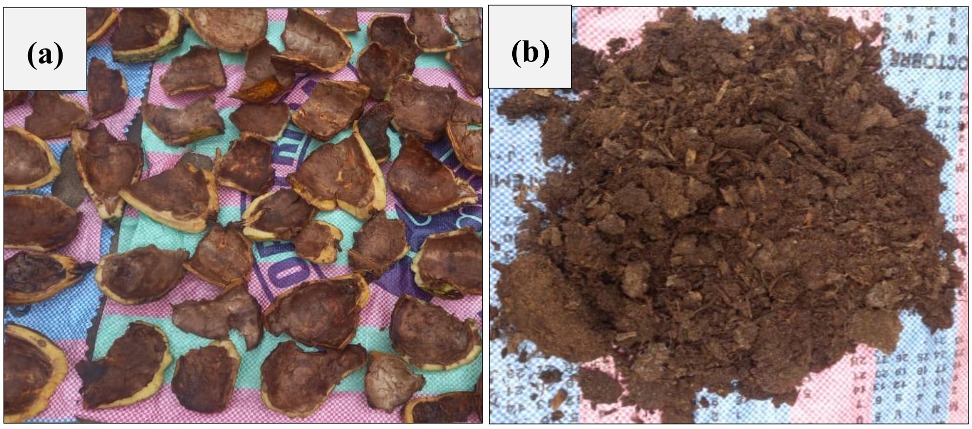

Figure 2.2. Collected study substrate: (a) cocoa pod husks and (b) poultry manure.

2.3.2. Inoculum

The inoculum used in this study was collected on May 13, 2025, from the Hofladen Hof Postma biogas plant located in Lambrechtshagen, Germany. This plant operates a continuously stirred mesophilic anaerobic digester, treating a mixture of cow manure and straw as primary feedstock. After collection, the inoculum was transported to the laboratory and left to stabilize at ambient temperature for one week to allow for the degassing phase, during which residual easily degradable compounds are consumed. This step ensured a biologically active yet stable inoculum, minimizing background gas production during BMP testing.

2.4. Substrate Pre-treatment and Preparation

Before laboratory analyses, the collected samples were first sun-dried for five days at the University of Lomé. This preliminary drying phase aimed to reduce the moisture content and preserve the samples from microbial decomposition before any laboratory treatments. Following sun-drying, the poultry manure samples were manually sorted to remove undesirable or non-biodegradable materials such as plastic fibers, feathers, and small stones. This step was essential to ensure uniformity and prevent interference during digestion and analytical processes. After natural sun-drying, both dried samples (PM and CPH) were placed in a drying oven at 105 °C for 24 hours on March 24, 2025, at the Laboratory of Biomass and Bioenergy Technologies (LT2B) of the University of Lomé, Togo. This controlled drying was performed to achieve uniform and minimal moisture content across all samples, thereby ensuring standardized moisture levels and enhancing the accuracy and comparability of subsequent physicochemical and biochemical analyses (Florentino de Carvalho et al., 2024). The completely dried samples were then ground using an electric grinder to obtain a uniform particle size of approximately 1 mm, which is ideal for subsequent characterization and anaerobic digestion testing. The obtained ground samples were stored in plastic bottles and sealed to ensure preservation and facilitate their transportation. To ensure clear identification and traceability, each pre-cleaned and dried plastic bottle was labelled with detailed information, including the sample's name, type, origin, owner, final destination, and purpose of analysis.

This labelling was accompanied by a photographic reference for each sample, as shown in Figure 2.3. This three-step pretreatment (sun-drying, oven-drying, and grinding) of the substrates is illustrated in APPENDIX B: Substrate Pretreatment.

Figure 2.3. Study substrate after pretreatment and packaging: (a) Cocoa pod husk and (b) Poultry manure.

2.5. Physicochemical Characterization

Comprehensive physicochemical characterization of the substrates was performed to assess their suitability for anaerobic digestion and to provide baseline data for formulating balanced feedstock mixtures. The analyses included pH measurement, proximate analysis, fiber composition, and ultimate analysis, which collectively describe the substrates' chemical environment, organic matter availability, structural carbohydrate fractions, elemental composition, and energy potential. The specific methodologies and procedures applied for each analysis are detailed in the following sub-sections.

2.5.1. pH Measurement

The pH of each substrate was determined according to the German standard DIN EN 15933:2020 (DIN, 2022). Approximately 5 g of oven-dried, finely ground sample was mixed with 50 mL of distilled water, establishing a 1:10 (V/W) solid-to-liquid ratio. The suspension was stirred continuously for 10 minutes to achieve complete homogenization and allow stabilization. After equilibration, the pH of the suspension was measured at room temperature (approximately 25 °C) using WTW pH 3310, a calibrated digital pH meter. Each substrate was analysed in triplicate, and the mean value was reported as the representative pH.

2.5.2. Proximate analysis

The proximate analysis was performed to quantify the fundamental physical properties of the substrates that influence their anaerobic digestion performance, particularly their organic matter availability and biodegradability (Llanos-Lizcano et al., 2024). The parameters assessed included moisture content (MC), total solids (TS), volatile solids (VS), and ash content (AC). These indicators are critical for evaluating substrate suitability for designing balanced feedstock mixtures during biochemical methane potential (BMP) testing. All analyses were carried out on the oven-dried samples, obtained following substrate pretreatments, at the Technical Scale Lab for Bioenergy, University of Rostock (Germany), prior to BMP testing. Measurements were conducted using the LECO TGA701 Thermogravimetric Analyzer, a high-precision instrument that enables automated determination of MC, TS, VS, and AC by applying a controlled thermal profile. Approximately 5 g of finely ground sample (less than 1 mm particle size) was placed in ceramic crucibles, and the TGA701 subjected the material to sequential heating under defined atmospheres. The procedure consisted of three main stages:

- Moisture determination: Heating to approximately 105 °C under inert conditions to evaporate free water.
- Volatile solids determination: Subsequent heating to approximately 550 °C under nitrogen to volatilize the organic fraction without oxidation, leaving ash excluded.
- Ash content determination: Introduction of oxygen and maintenance of combustion conditions to oxidize residual organics, leaving only the inorganic fraction.

The analyzer continuously recorded mass loss throughout the process, enabling precise calculation of each parameter. All analyses were conducted in triplicate to ensure reproducibility. Data acquisition and processing were carried out using LECO software, with results further validated against standardized Excel-based templates integrated into laboratory protocols.

2.5.3. Fiber analysis

Fiber analysis was performed, using the BEHR CF6, to quantify the structural carbohydrate fractions of the substrates, especially cellulose, hemicellulose, and lignin. These fractions are critical indicators of the biodegradability of lignocellulosic biomass under anaerobic digestion. High lignin content, in particular, is widely recognized as a limiting factor for methane production, owing to its structural rigidity and microbial recalcitrance, which hinder enzymatic hydrolysis and subsequent fermentation. The analyses were conducted following the German standard DIN EN ISO 13906:2008-11 (DIN, 2008), using an established in-house protocol developed by the German Biomass Research Center (DBFZ) and documented in its report "Biomass Energy use" (Liebetrau & Pfeiffer, 2020). This methodology enables sequential determination of:

- Neutral Detergent Fiber (NDF): representing cellulose, hemicellulose, and lignin fractions collectively,
- Acid Detergent Fiber (ADF): representing cellulose and lignin fractions, and
- Acid Detergent Lignin (ADL): representing the lignin fraction alone.

From these values, cellulose, hemicellulose, and lignin content were calculated using equations 2.1, 2.2, and 2.3, respectively. The analysis thereby provides a detailed characterization of the structural fiber components, which are essential for predicting substrate degradability and for interpreting variations in biogas yield during biochemical methane potential (BMP) testing.

$$\text{Cellulose} = \text{ADF} - \text{ADL} \tag{2.1}$$

$$\text{Hemicellulose} = \text{NDF} - \text{ADF} \tag{2.2}$$

$$\text{Lignin} = \text{ADL} \tag{2.3}$$

2.5.4. Ultimate analysis

Ultimate analysis was performed at an external accredited laboratory to firstly determine the elemental composition of the substrates, a key factor in evaluating their nutrient balance and energy potential for anaerobic digestion. The parameters quantified included carbon (C), hydrogen (H), nitrogen (N), and sulphur (S), in accordance with the German standard DIN EN ISO 21663:2021-03. Then, oxygen (O) content was calculated by difference following the DIN 51733:2016-04 procedure. From these data, the carbon-to-nitrogen (C/N) ratio was derived, which represents a critical indicator for optimizing substrate mixing ratios and maintaining microbial balance in co-digestion systems. In addition to the primary elemental analysis, the nutrient composition of the substrates was assessed, including iron (Fe), calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), and phosphorus (P), following DIN 22022-2:2001-02. These macro- and micronutrients are important for supporting microbial metabolism and enzymatic activity during anaerobic digestion. Furthermore, the energy content of poultry manure and cocoa pod husks was determined by measuring both the higher heating value (HHV) and the lower heating value (LHV), in compliance with DIN EN ISO 21654:2021-12. These values provide an additional indicator of the substrates' calorific potential and complement the biochemical methane potential (BMP) tests in evaluating their overall bioenergy yield.

2.6. Experimental Design for BMP Tests

The biochemical methane potential (BMP) tests were performed in accordance with the VDI 4630 standard (VDI 4630, 2016), at the Technical Scale Lab for Bioenergy, University of Rostock (Germany). The experiments aimed to determine the individual biogas potential of poultry manure (PM) and cocoa pod husk (CPH) and evaluate the influence of carbon-to-nitrogen (C/N) ratio and operating temperature (mesophilic: 37 °C; thermophilic: 55 °C) on biogas yield during anaerobic co-digestion.

2.6.1 Experimental Setup

The BMP essays were conducted using the ANKOM Gas Production System, which provides automated, high-resolution monitoring of biogas generation via pressure sensors installed on each digestion bottle. Each reactor had a total volume of 500 mL and a working volume of 400 mL. Reactors were loaded at a substrate concentration (SC) of 10 g VS/L and a substrate-to-inoculum (S/I) ratio of 0.5 on a volatile solids (VS) basis. The required mass of volatile solids (Q_{VS}) was calculated using the following equation 2.4:

$$Q_{VS} = \frac{SC \times V_r}{1000} \tag{2.4}$$

Where: Q_{VS}: Mass of volatile solids (g), SC: Substrate concentration (10g VS/L), and Vr: Working volume of the reactor (400 mL).

After loading, reactors were hermetically sealed and incubated in thermostatically controlled water baths for 30 days, with continuous pressure recording. To avoid sedimentation and scum formation, magnetic stirring was applied daily for 5 minutes. All experimental conditions were conducted in duplicate, and the mean values were reported. The experimental setup is shown in Figure 2.4. Some pictures of the experiment setting up are presented in APPENDIX G: Some Lab Manipulation Photos for BMP Tests Set Up.

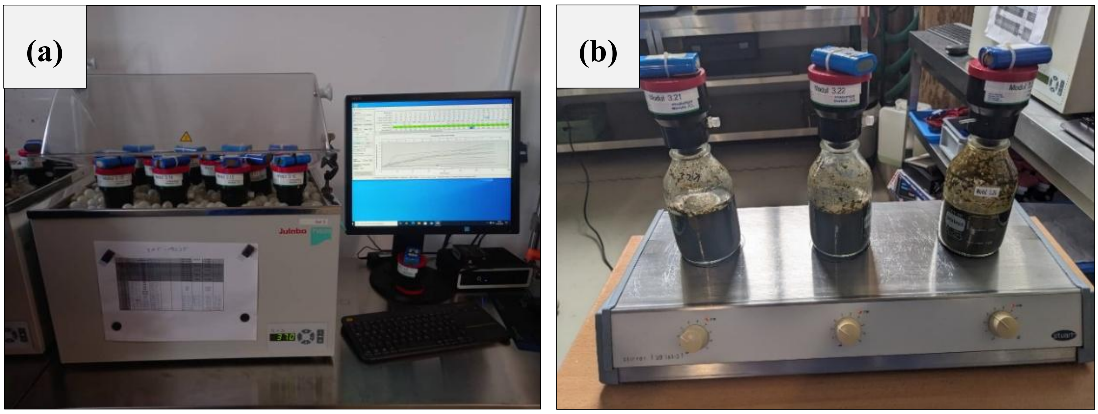

Figure 2.4. BMP experiments setting up: (a) digesting reactors with measuring module incubated, and (b) magnetic stirring of digestion reactors.

2.6.2 Mono-digestion Tests

Mono-digestion essays were conducted to evaluate the baseline biogas potential of PM and CPH individually under mesophilic conditions (37°C), reflecting the common practice outlined in VDI 4630 standard for BMP protocols. Although performing the BMP test under mesophilic condition is not mandated by the standard, this approach was used to ensure comparability with widely adopted literature benchmarks.

2.6.3 Co-digestion Tests

Co-digestion essays were designed to assess the combined effect of C/N ratio and temperature on biogas production yield. Four C/N ratios were targeted (20, 25, 30, and 35), chosen based on the optimal range (20-30) reported by Wang et al. (2014) for microbial balance and enhanced biogas production. Consequently, substrate mixtures were formulated by combining PM and

CPH according to their VS content and elemental carbon (C) and nitrogen (N) concentrations. The required mixing ratios were calculated using a modified substrate balance approach adapted from the equation used by Haider et al. (2015) in their work (equations 2.5) and equation 2.6.

$$C/N = \frac{(Q_{VS_{PM}} \times C_{PM}) + (Q_{VS_{CPH}} \times C_{CPH})}{(Q_{VS_{PM}} \times N_{PM}) + (Q_{VS_{CPH}} \times N_{CPH})} \quad (2.5)$$

With: $Q_{VS} = Qvs_{PM} + Qvs_{CPH} = \frac{SC \times V_r}{1000}$ (2.6)

Where: $Q_{VS_{PM}}$ and $Q_{VS_{CPH}}$, the amounts of volatile solids from poultry manure and cocoa pod husk, respectively (g), C_{PM} and C_{CPH}, the carbon content of poultry manure and cocoa pod husk, respectively (%), N_{PM} and N_{CPH}, the nitrogen content of poultry manure and cocoa pod husk, respectively (%), Q_{VS}, the amounts of volatile solids added in the reactor (g), SC (gVS/l), the substrate concentration (10g VS/L), and Vr (mL), the net volume of the reactor (400 mL).

By applying Equations 2.5 and 2.6 simultaneously, the substrate mixing ratios were precisely adjusted to meet the target C/N ratios while maintaining a constant organic loading rate. Each formulation was tested under both mesophilic (37 °C) and thermophilic (55 °C) conditions, enabling a two-factorial experimental design to assess main and interaction effects of the C/N ratio and temperature conditions on biogas yield.

2.6.4 Control Tests

Two types of controls were included at each temperature regime:

- **Blank tests (inoculum only):** to account for background biogas production. Biogas from blanks was subtracted from experimental values to isolate substrate-derived yields.
- **Positive control tests (inoculum + microcrystalline cellulose):** to verify inoculum activity and system reliability, as recommended by the VDI 4630 (2016) Standard. Consistent yields from microcrystalline cellulose, typically around 745 mL/g VS ± 10% VDI 4630 (2016), Confirm the microbiological integrity of the inoculum and proper system functioning.

2.6.5. Data Collection and Biogas Volume Calculation

Biogas production was monitored in real-time using the ANKOM Gas Production System, which records headspace pressure increases every 30 minutes via wireless sensors. The cumulative biogas volume was calculated using the ideal gas law, considering the measured pressure differential, reactor headspace, and temperature through equation 2.7. Then, the net specific biogas yield (also known as biogas potential or BMP) corrected for blank inoculum production, was calculated using equation 2.8.

$$dV = \left(\frac{V_0 \times v_0}{R \times T}\right) dp \tag{2.7}$$

$$V_{SS}(t) = \frac{V_C(t) - V_{IS}(t) \times Q_{VS}(I)}{Q_{VS}(S)} \tag{2.8}$$

With: dV: Biogas volume (mL), dp: Pressure change (Pa), V_0: Headspace volume (mL), v_0: Molar volume of the gas (assumed to behave as an ideal gas, at pressures lower than 150 kPa, with a molar volume of 22.4 L/mol), R: Universal gas constant (8314.4626 $L.Pa.K^{-1}.mol^{-1}$), and T: Temperature (K), $V_{SS}(t)$: Substrate-specific biogas yield at time t (mL/gVS), $V_C(t)$: Cumulative biogas at time t (mL), $V_{IS}(t)$: Inoculum-specific biogas yield at time t (mL/gVS), $Q_{VS}(I)$: Mass of inoculum (gVS), and $Q_{VS}(S)$: Mass of substrate (gVS).

All calculations were performed using Microsoft Excel with ANKOM's data export format. The continuous pressure logging and automatic normalization capabilities of the ANKOM system allowed for high-resolution kinetic profiling and robust comparisons between treatments.

2.6.6. Overview of Experimental Design

In total, 28 BMP tests were conducted: 4 mono-digestion tests (2 substrates × 1 temperature × 2 replicates), 16 co-digestion tests (4 C/N ratios × 2 temperatures × 2 replicates), 4 blank tests (2 temperatures × 2 replicates), and 4 positive controls (2 temperatures × 2 replicates).

This factorial design enabled a comprehensive evaluation of the effects of substrate type, C/N ratio, and operating temperature, as well as their interactions, on biogas yield. A schematic overview of the experimental design is presented in Figure 2.5.

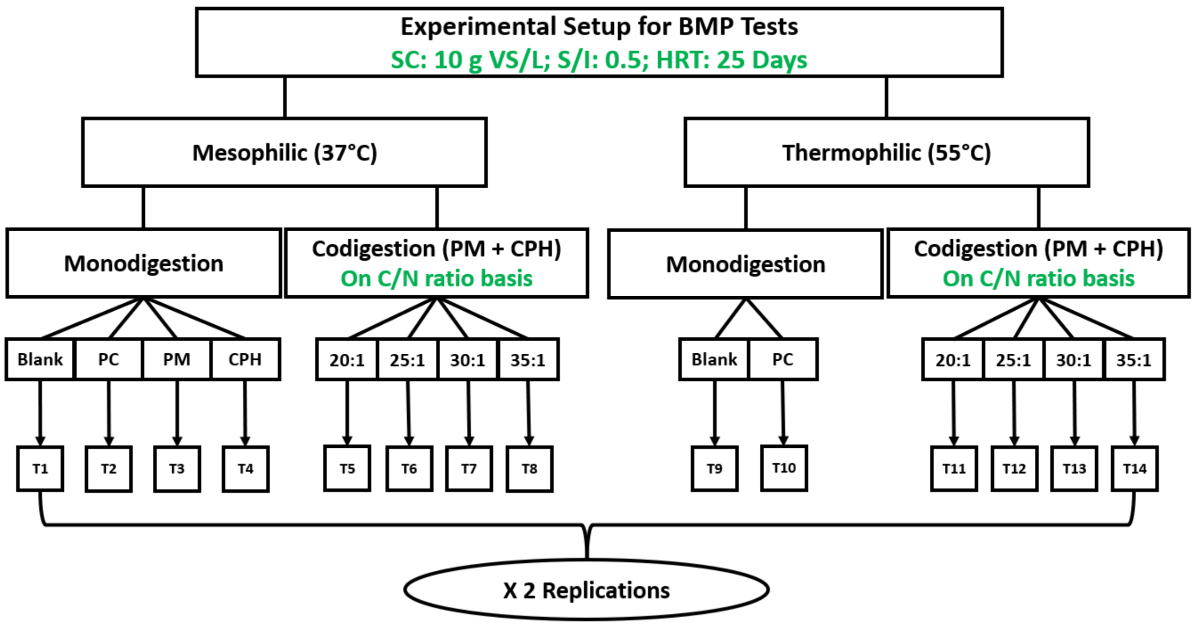

Figure 2.5. Experimental setup for biochemical methane potential (BMP) tests.

2.7. Statistical Analysis Methods

All Biochemical Methane Potential (BMP) data were subjected to statistical analysis to evaluate the effects of C/N ratio, temperature regime, and their interaction on cumulative biogas yield. The applied statistical analysis comprised descriptive statistics, assumption testing, analysis of variance (ANOVA), robust validation analyses under heteroscedasticity, and post-hoc multiple comparisons.

Descriptive statistics, including mean and standard deviation (SD), were first calculated for each treatment group to summarize central tendency and variability. Data visualization was performed through line and bar plots with error bars (mean ± SD) to illustrate temporal patterns and treatment differences. A two-way ANOVA was then applied to evaluate the main effects of C/N ratio (20, 25, 30, and 35) and temperature (mesophilic at 37 °C and thermophilic at 55 °C), as well as their interaction effect. The corresponding null and alternative hypotheses are presented in APPENDIX F.6. Tested hypotheses for ANOVA Prior to analysis, residual diagnostics were conducted: normality was verified using the Shapiro-Wilk test while homogeneity (equality of variances) of variances was assessed using Levene's test (Brown-Forsythe modification). Where assumptions were met, ANOVA results were interpreted directly. However, given the observed violation of homogeneity of variances, two heteroscedasticity-robust approaches were additionally implemented to validate the ANOVA results:

- **Welch-type factorial ANOVA:** Implemented as a weighted least squares model with inverse-variance weights, this method adjusts test statistics by down-weighting groups with higher variances, providing reliable inference under heteroscedasticity (Wilcox, 2022).
- **General Linear Model (GLM) with HC3-robust standard errors:** This approach provides heteroscedasticity-consistent variance estimates, particularly reliable for small sample sizes. HC3 is particularly recommended for small to moderate sample sizes because it offers better Type I error control compared to other HC estimators (Long & Ervin, 2000).

While applying both methods is not compulsory, their combined use was deliberate, to ensured convergent validation of results through two complementary robustness strategies: variance weighting (Welch) and robust covariance estimation (HC3). This approach strengthened the reliability of inference and helped identify effects that may be sensitive to the choice of heteroscedasticity correction. Finally, to detect pairwise differences between treatment means, a Tukey's Honestly Significant Difference (HSD) post-hoc test was performed at a 95% confidence level ($\alpha = 0.05$), controlling for Type I error across multiple comparisons.

All analyses were conducted using XLSTAT (version 2025) for descriptive statistics, classical ANOVA, and Tukey HSD, while Python/Statsmodels (version 0.14.4) was used to perform Welch-type ANOVA and HC3-robust GLM validation.

2.8. Conclusion

The adopted methodology enabled the generation of reliable and reproducible data to evaluate the interactive effects of temperature and substrate C/N ratio on anaerobic co-digestion performance. The use of a well-structured factorial design, combined with rigorous physicochemical characterization and statistical evaluation, provides a robust framework for interpreting the resulting biogas yields. This chapter lays the experimental foundation for analysing and discussing the observed outcomes in the subsequent chapter.

CHAPTER 3: RESULTS AND DISCUSSION

3.1. Introduction

This chapter presents and interprets the results obtained from the experimental investigation, which include the physicochemical characterization of the substrate, the BMP experiments conducted under varying C/N ratios and temperature conditions, and the statistical analysis. The findings are analysed to evaluate the anaerobic digestion suitability of poultry manure (PM) and cocoa pod husk (CPH), determine their individual and combined biogas potential, and assess the synergistic effects of co-digestion on the biogas production process performance. Special attention is given to the influence of substrate composition and operational conditions on biogas yield and process stability. Statistical analyses (ANOVA, Welch-type ANOVA, and HC3-robust GLM) are incorporated to evaluate the significance of treatment effects and interactions. A comparative analysis with recent peer-reviewed studies is employed to contextualize the results, validate the observations, and provide explanations for the observed trends. The goal is to identify optimal co-digestion conditions that maximize biogas production and ensure robust and scalable process performance. The entire detailed results are presented in the appendix.

3.2. Results

3.2.1. Physicochemical Characterization of Substrates

3.2.1.1. pH Values and Proximate Composition

The pH measurements revealed distinct differences between the two substrates. As shown in Table 3.1, Cocoa pod husk (CPH) exhibited a slightly acidic pH of 6.89 ± 0.04, whereas poultry manure (PM) was strongly alkaline, with a pH of 8.70 ± 0.02.

The proximate analysis results are also summarized in Table 3.1. The total solids (TS) content was slightly higher in poultry manure (PM) (96.34 ± 0.48% FM) than in cocoa pod husk (CPH) (94.82 ± 0.93% FM), while the corresponding moisture content (MC) was lower in PM (3.66 ± 0.48% FM) than in CPH (5.18 ± 0.93% FM). Volatile solids (VS), representing the biodegradable organic fraction, were considerably higher in CPH (84.06 ± 0.97% FM) compared to PM (70.50 ± 0.26% FM). Consequently, the VS/TS ratio was also greater in CPH (0.89 ± 0,04) than in PM (0.73 ± 0,32). Despite this difference, both substrates, however, displayed VS/TS values above 0.70, which is generally considered suitable for anaerobic digestion. In contrast, the ash content (AC), indicative of inert mineral matter, was significantly higher in PM (25.84 ± 0.26% FM) compared to CPH (10.76 ± 0.97% FM), which may impact its overall biodegradability and biogas yield.

Table 3.1. pH and proximate characteristics of cocoa pod husk and poultry manure.

Parameter	Unit	cocoa pod husk (CPH)		poultry manure (PM)	
pH	-	6,89	± 0,04	8,70	± 0,02
MC	%FM	5,18	± 0,93	3,66	± 0,48
TS	%FM	94,82	± 0,93	96,34	± 0,48
VS	%FM	84,06	± 0,97	70,50	± 0,26
AC	%FM	10,76	± 0,97	25,84	± 0,26
VS/TS	-	0,89	± 0,04	0,73	± 0,32

3.2.1.2. Fiber Composition

The structural fiber composition, presented in Figure 3.1, indicates that cocoa pod husk (CPH) had a markedly higher lignin content (23.71 ± 0.48% TS) than poultry manure (PM) (9.90 ± 0.53% TS). However, the cellulose content was relatively similar for both substrates, with 26.59 ± 0.70% TS for CPH and 25.02 ± 0.65% TS for PM. PM showed a higher hemicellulose content (12.15 ± 0.79% TS) than CPH (8.32 ± 0.61% TS).

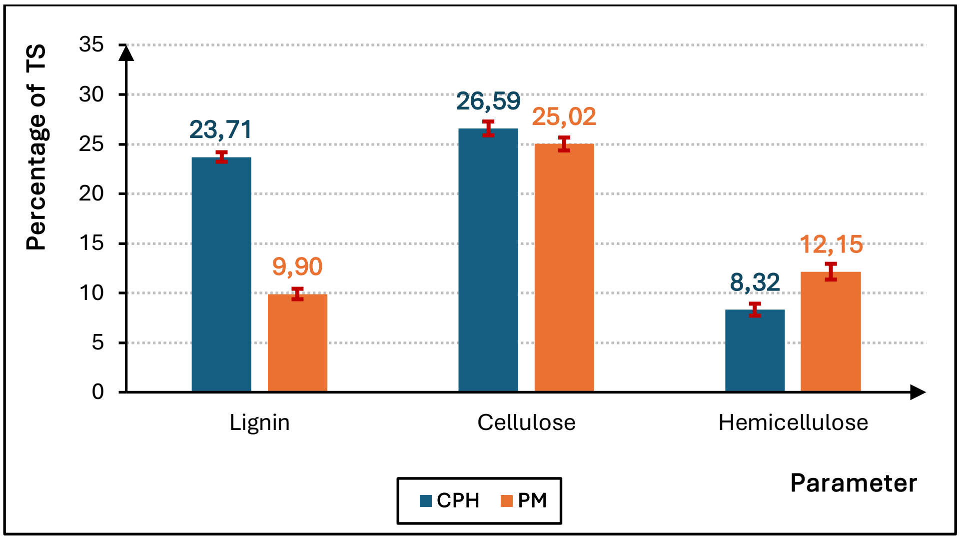

Figure 3.1. Fiber composition of cocoa pod husk and poultry manure.

3.2.1.3. Ultimate Analysis and Nutrient Composition

Table 3.2 presents the elemental composition of the two substrates. Cocoa pod husk (CPH) was richer in carbon (48.60 ± 0.10% TS) and exhibited a higher C/N ratio (51.16) compared to poultry manure (PM), which had a C/N ratio of 16.07, due to its higher nitrogen content (2.19 ± 0.06% TS) versus 0.95 ± 0.14% TS in CPH.

PM also contained relatively higher levels of nutrients and trace elements, including sulfur (S), iron (Fe), calcium (Ca), magnesium (Mg), sodium (Na), and phosphorus (P), all of which are important for microbial metabolism and enzymatic activity during digestion. CPH, on the other hand, had higher values for hydrogen (H), oxygen (O), and potassium (K), contributing to the energy density.

Table 3.2. Elemental composition and heating value of cocoa pod husk and poultry manure.

Parameter	Unit	cocoa pod husk (CPH)		poultry manure (PM)	
Carbon (C)	%TS	48,60	± 0,10	35,15	± 0,27
Hydrogen (H)	%TS	5,34	± 0,08	4,41	± 0,07
Oxygen (O)	%TS	38,25	± 0,05	30,05	± 0,53
Nitrogen (N)	%TS	0,95	± 0,14	2,19	± 0,06
Sulfur (S)	%TS	0,10	± 0,01	0,35	± 0,03
Iron (Fe)	%TS	0,017	± 0,003	0,26	± 0,09
Calcium (Ca)	%TS	0,84	± 0,01	3,76	± 0,19
Potassium (K)	%TS	4,15	± 0,02	2,53	± 0,15
Magnesium (Mg)	%TS	0,41	± 0,01	0,48	± 0,09
Sodium (Na)	%TS	0,003	± 0,003	0,46	± 0,06
Phosphorus (P)	%TS	0,14	± 0,01	1,49	± 0,07
C/N	-	51,16	-	16,07	-

As shown in Figure 3.2, the higher heating value (HHV) and lower heating value (LHV) of CPH were 16987 ± 6.56 kJ/kg and 15824 ± 22 kJ/kg, respectively, while those of PM were lower, at 13187 ± 50.51 kJ/kg and 12227 ± 65.51 kJ/kg. This confirms the greater energetic potential of CPH.

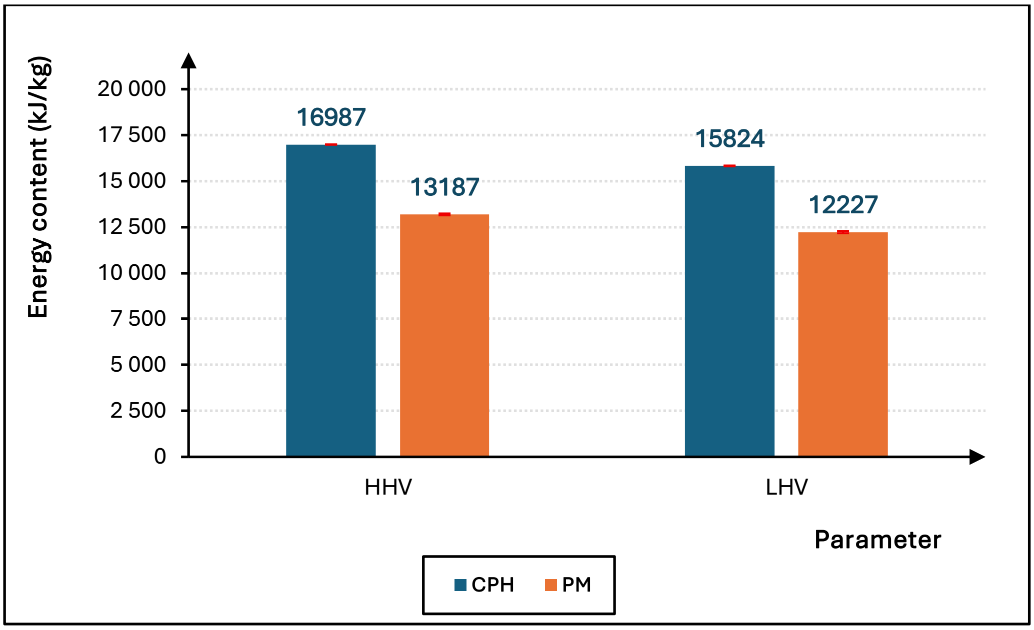

Figure 3.2. Energy content of Poultry manure and cocoa pod husk.

3.2.2. Mono-Digestion Performance

Mono-digestion experiments were conducted under mesophilic conditions (37 °C) for 30 days using batch reactors to evaluate the biogas potential of cocoa pod husk (CPH) and poultry manure (PM).

Figure 3.3 illustrates the cumulative biogas production profiles of the experiments. The cumulative biogas yields from the positive control (cellulose) reached 705.46 ± 13.98 mL/g VS, while the blank inoculum produced 136.69 ± 6.90 mL/g VS. Among the tested substrates, CPH recorded the highest cumulative yield, reaching 244.65 ± 8.48 mL/g VS. At the same time, PM produced 210.73 ± 27.12 mL/g VS. Weekly biogas production analysis, shown in Figure 3.4, revealed that approximately 80.6% of CPH's total biogas yield was generated within the first seven days, followed by a sharp decline and stabilization after day 21. In contrast, PM exhibited a slower initial biogas release, with 64.2% of its total yield produced during the first week, and a more gradual production pattern extending into the third week.

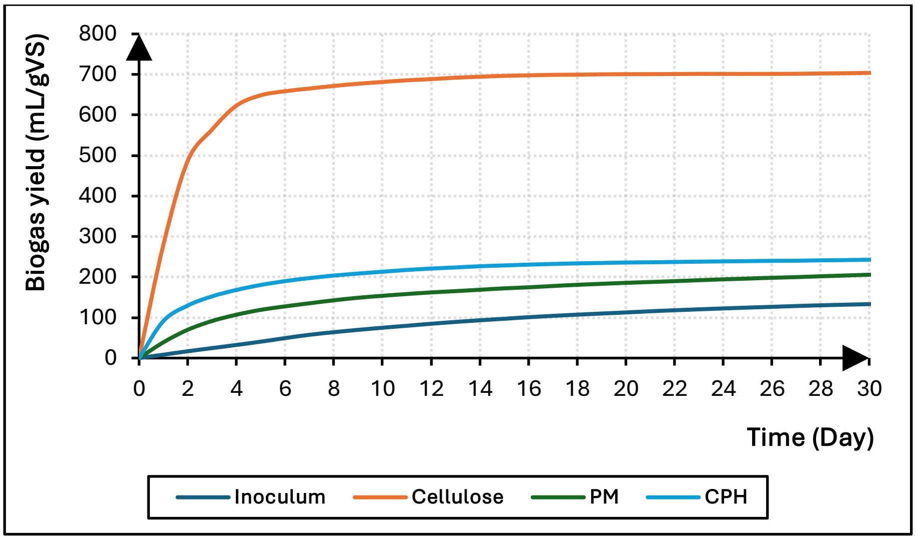

Figure 3.3. Cumulative biogas production profile.

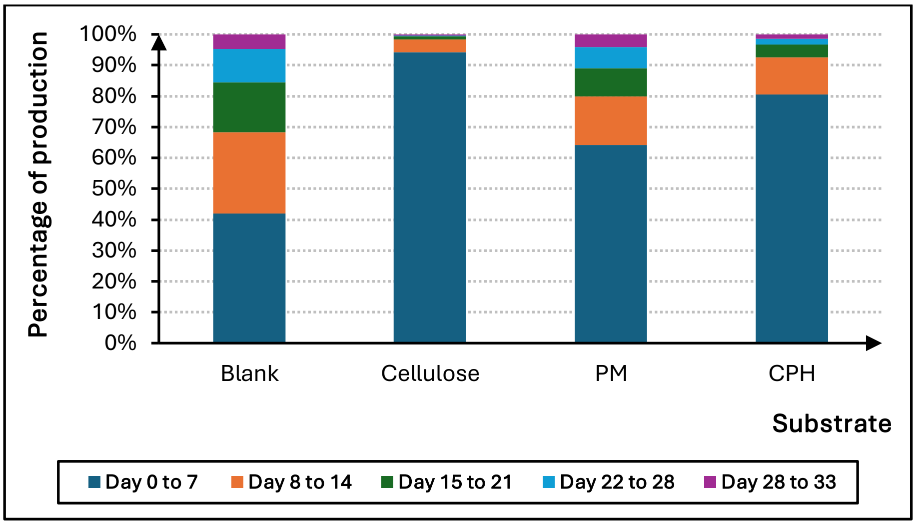

Figure 3.4. Weekly biogas production percentage per substrate.

Daily biogas production trends, presented in Figure 3.5, revealed that CPH displayed a sharp peak during the initial phase. In contrast, PM showed a more sustained daily production rate, with more minor fluctuations over time. Both substrates exhibited typical batch digestion profiles, with rapid hydrolysis and acidogenesis during the early phase (the first seven days), followed by a deceleration of gas production as substrates were depleted. The weekly production analysis (Figure 3.4) further confirmed these trends. The majority of biogas from CPH was produced during the first two weeks, whereas PM exhibited a more evenly distributed production pattern over the entire digestion period compared to CPH.

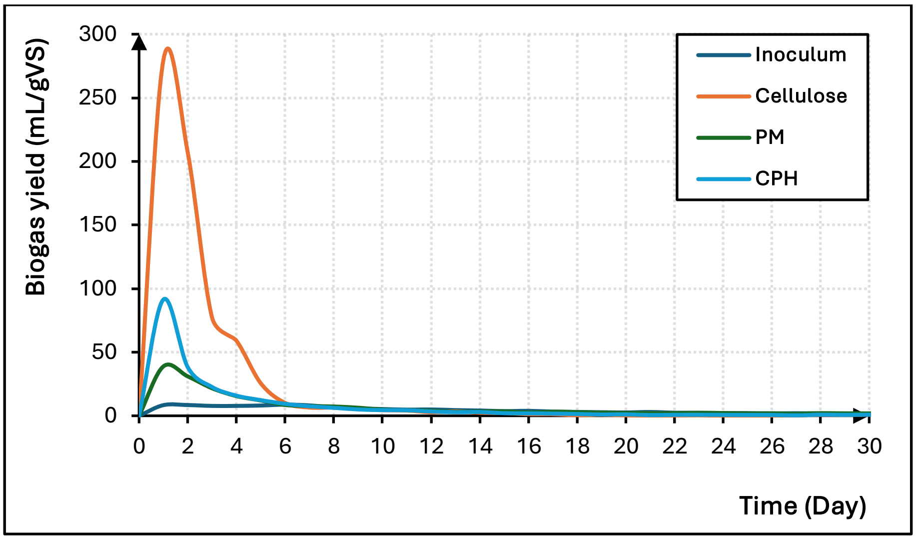

Figure 3.5. Daily biogas production yield.

3.2.3. Co-Digestion Performance

Co-digestion experiments were conducted over 30 days to investigate the interactive effects of substrate nutrient balance, expressed as carbon-to-nitrogen (C/N) ratio, and temperature regime on biogas production from poultry manure (PM) and cocoa pod husk (CPH). The trials were carried out under mesophilic (37 °C) and thermophilic (55 °C) conditions across four C/N ratios (20, 25, 30, and 35).

3.2.3.1. Daily Biogas Production Trends

As depicted in Figure 3.6 and Figure 3.7, daily biogas production profiles varied substantially across treatments, reflecting distinct kinetic patterns influenced by both temperature and substrate C/N ratio. Under mesophilic conditions, biogas production exhibited a rapid onset and peak within the first three days, accounting for the majority of the total gas generated. This early increase, particularly pronounced in the C/N 25 and C/N 30 treatments, peaked on Day 2 at 93.16 mL/g VS and 80.46 mL/g VS, respectively. By contrast, the C/N 35 treatment consistently showed the lowest daily production. As digestion progressed, daily yields across all mesophilic treatments declined gradually. Under thermophilic conditions, the daily biogas production trend was more irregular and less stable. Although early gas production was observed across all C/N treatments, the curves exhibited frequent fluctuations, mid-phase depressions, and several negative yield values, particularly in C/N 20 (Days 13-29) and C/N 35 (Days 15-17). Despite these challenges, thermophilic treatments at C/N 25 and 30 maintained relatively robust and sustained gas production throughout the digestion period.

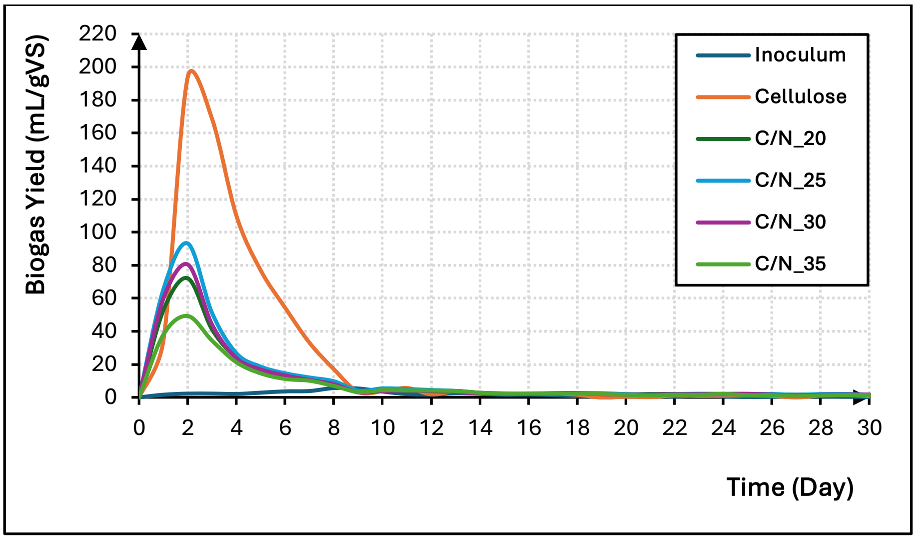

Figure 3.6. Mesophilic Daily Biogas Yield (mL/g VS).

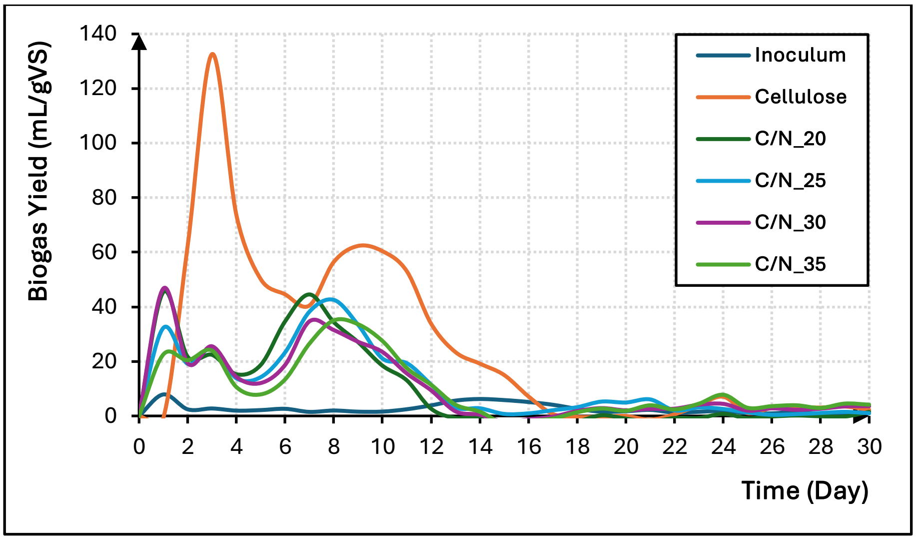

Figure 3.7. Thermophilic Daily Biogas yield (mL/g VS).

3.2.3.2. Cumulative Biogas Yields

Figure 3.8 and Figure 3.9 present the cumulative biogas production trends, which serve as integrative indicators of overall substrate biodegradability and system performance. Under mesophilic digestion, the highest cumulative biogas yield was achieved at C/N 25, reaching 348.65 ± 10.44 mL/g VS, followed by C/N 30 (306.02 ± 18.62 mL/g VS), C/N 20 (293.21 ± 35.43 mL/g VS), and C/N 35 (234.15 ± 13.05 mL/g VS). These results reflect the importance of achieving an optimal nutrient balance for microbial metabolism, with C/N 25 providing the most favourable stoichiometric conditions. A similar trend was observed under thermophilic conditions, where C/N 25 again yielded the highest production

(336.21 ± 15.92 mL/g VS), followed by C/N 30 (314.15 ± 17.89 mL/g VS), and notably, C/N 35 (296.98 ± 10.86 mL/g VS), which outperformed C/N 20 (275.02 ± 13.71 mL/g VS). The superior performance of C/N 35 over C/N 20 in thermophilic digestion contrasts with the mesophilic profile. The positive control (cellulose) yielded 725.43 ± 51.88 mL/g VS (mesophilic) and 760.15 ± 15.51 mL/g VS (thermophilic).

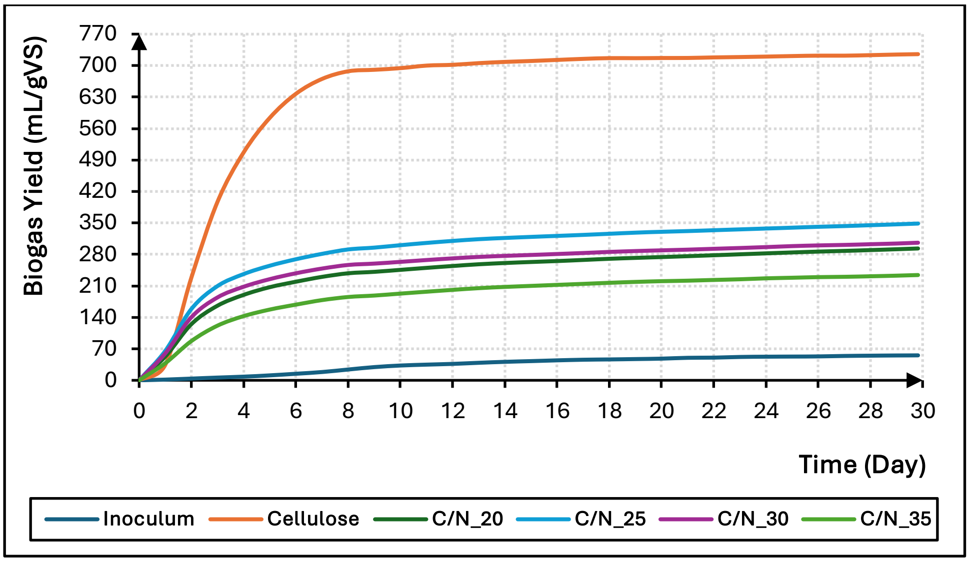

Figure 3.8. Mesophilic Cumulative Biogas Yield (mL/g VS).

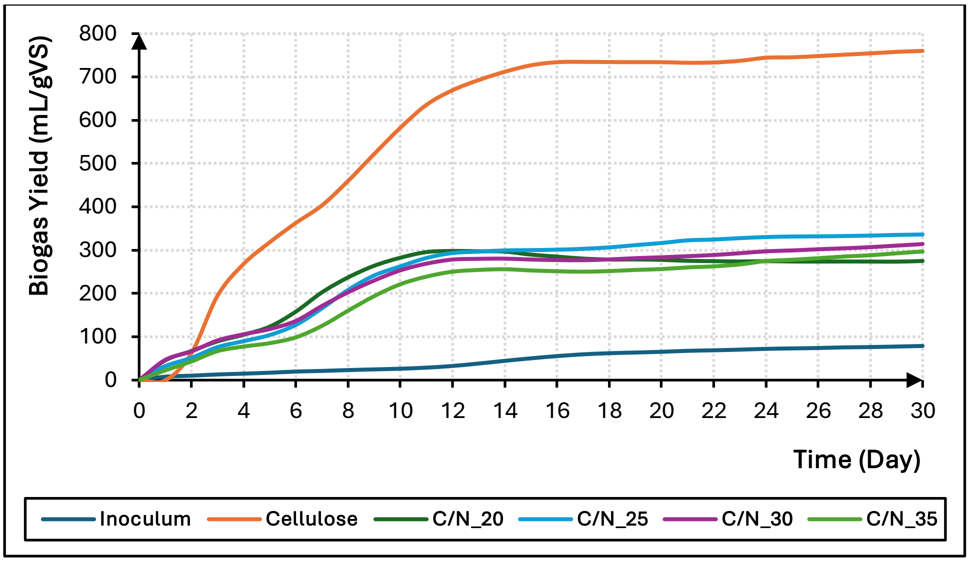

Figure 3.9. Thermophilic Cumulative Biogas yield (mL/g VS).

3.2.3.3. Statistical Analysis Results

A two-way analysis of variance (ANOVA) was conducted to evaluate the effects of C/N ratio, temperature regime, and their interaction on cumulative biogas yield. Prior to model fitting, assumption diagnostics were performed. As presented in Table 3.3, the Shapiro-Wilk test confirmed that residuals were normally distributed ($W = 0.972$, $p = 0.865$), satisfying the normality assumption. However, Levene's test indicated a strong violation of the homogeneity of variance ($F = 1.11\times10^{29}$, $p < 0.001$), implying heteroscedasticity across groups.

Table 3.3. Normality and Homogeneity Assumptions Test results.

Test	DF	W statistic	p-value	Interpretation
Shapiro-Wilk Test (Normality)	8	0,972	0.8652	Residuals are normally distributed
Levene's Test (Homogeneity of variance)	8	$1{,}11x10^{29}$	<0.001	Homogeneity of variances is violated

As displayed in Table 3.4, the two-way ANOVA revealed that the C/N ratio had a highly significant effect on cumulative yield ($F = 16.17$, $p = 0.001$). The temperature main effect was not significant ($F = 1.48$, $p = 0.259$). A statistically significant interaction between C/N ratio and temperature was observed ($F = 4.96$, $p = 0.031$). These findings suggest that nutrient stoichiometry, particularly the C/N ratio, plays a crucial role in determining digestion performance, with its effect varying according to the operating temperature regime.

Given the assumption violation, results were validated using two heteroscedasticity-robust approaches (Table 3.4):

- Welch-type ANOVA (variance-weighted WLS): All three effects were statistically significant (C/N ratio: $F = 68.75$, $p < 0.001$; Temperature: $F = 38.51$, $p = 0.0003$; Interaction: $F = 12.37$, $p = 0.002$). This suggests that variance heterogeneity masked part of the temperature effect in the classical model.
- HC3-robust GLM (heteroscedasticity-consistent SE): Confirmed the significance of C/N ratio ($F = 37.58$, $p < 0.001$) and the interaction ($F = 6.18$, $p = 0.018$), but not the temperature main effect ($F = 0.26$, $p = 0.624$). This pattern mirrors the classical ANOVA, suggesting that the contribution of temperature is primarily expressed through its interaction with nutrient balance.

Taken together, the convergence of results across all three methods underscores the robustness of the C/N effect and the C/N-Temperature interaction, while the independent role of temperature remains less certain and sensitive to the chosen correction.

Table 3.4. Analyses of variance results.

Effect	ANOVA	Welch-type ANOVA	HC3-robust GLM
C/N Ratio	F = 16.17 p = 0.001	F = 68.75 p < 0.001	F = 37.58 p < 0.001
Temperature	F = 1.48 p = 0.259	F = 38.51 p = 0.0003	F = 0.26 p = 0.624
C/N × Temperature	F = 4.96 p = 0.031	F = 12.37 p = 0.002	F = 6.18 p = 0.018

As shown in Table 3.5, the Tukey Honest Significant Difference (HSD) post-hoc test identified five statistically significant pairwise differences ($p < 0.05$). In particular:

- C/N 25 (mesophilic) produced significantly higher yields than C/N 20 (thermophilic) and C/N 35 (mesophilic).
- Both C/N 25 and C/N 30 (under either temperature regime) significantly outperformed C/N 35 (mesophilic), which consistently yielded the lowest values.

Across all analyses, nutrient balance (C/N ratio) emerged as the dominant determinant of biogas yield. Its effect was significantly modulated by temperature regime, confirming an interaction between substrate composition and thermal conditions. While the Welch-type test suggested a stronger independent temperature effect, the conservative HC3 approach and the classical ANOVA indicate that temperature primarily acts through interaction effects rather than as a stand-alone driver. Importantly, C/N 25 under mesophilic conditions was consistently identified as the optimal configuration, achieving the highest cumulative biogas yields from poultry manure and cocoa pod husk mixtures. These findings reinforce the central role of substrate composition, particularly nutrient balance, in modulating anaerobic digestion efficiency and support the identification of C/N 25 as the optimal condition under mesophilic operation for maximizing biogas recovery from PM and CPH mixtures. All results from the statistical analysis (Assumption tests, ANOVA, Welch-type ANOVA, HC3-robust GLM, and Post-hoc) are displayed in Appendix G.

Table 3.5. Tukey HSD Post-hoc Test results.

Group 1	Group 2	Mean difference (mL/g VS)	Adjusted p-value	Significant $p < 0.05$
C/N 25 (Mesophilic)	C/N 35 (Mesophilic)	114.506	0.002	Yes
C/N 25 (Mesophilic)	C/N 20 (Thermophilic)	73.633	0.027	Yes
C/N 25 (Thermophilic)	C/N 35 (Mesophilic)	102.062	0.004	Yes
C/N 30 (Thermophilic)	C/N 35 (Mesophilic)	80.006	0.017	Yes
C/N 30 (Mesophilic)	C/N 35 (Mesophilic)	71.873	0.031	Yes

3.3. Discussion

3.3.1. Assessment of Substrate Suitability for Biogas Production

3.3.1.1. pH Implication

pH is a key determinant of anaerobic digestion performance and stability, directly influencing the activity of methanogenic archaea, which typically thrive between pH 6.8 and 7.4 (VDI 4630, 2016). In this study (Table 3.1), cocoa pod husk (CPH) presented a slightly acidic pH (6.89), consistent with its lignocellulosic origin and the presence of phenolic acids commonly reported in cocoa-based residues (Meza-Sepulveda et al., 2024). By contrast, poultry manure (PM) exhibited a strongly alkaline pH (8.70), attributable to its high ammoniacal nitrogen content and buffering compounds such as bicarbonates and phosphates (Kacprzak et al., 2023; Swelum et al., 2021). This marked difference indicates a potential buffering synergy during co-digestion: the alkalinity of PM could counteract the acidity of CPH, stabilizing the mixture within the optimal methanogenic window. Such synergy has been demonstrated in other co-digestion systems, for example, when acidic agro residues were combined with manure to prevent pH inhibition under mesophilic digestion (Dahunsi et al., 2019). The ANOVA results further support this synergy: although temperature alone did not significantly affect yield, the C/N × temperature interaction was significant, suggesting that the buffering and nutrient balance provided by PM enhanced the stability of CPH digestion under different thermal regimes. While pH affects the buffering capacity of the system, the actual biodegradability of the substrates is more closely related to their organic and fiber composition, as explored in the following sections.

3.3.1.2. Volatile Solid and Organic content

Volatile solids (VS) represent the biodegradable organic fraction of feedstocks and are thus a strong predictor of biogas potential. Here, CPH showed a higher VS content (84.06% FM, VS/TS = 0.89) than PM (70.50 ± 0.26% FM, VS/TS = 0.73) (Table 3.1), indicating greater intrinsic biodegradability. This aligns with literature values where lignocellulosic by-products, such as cocoa husk, typically contain around 80-90% VS/TS (Antwi et al., 2019; Nakhate et al., 2022), compared to 65-75% for poultry or cattle manure (Jurgutis et al., 2020; Wang et al., 2014). However, PM contained a higher ash fraction (25.84% FM) than CPH (9.60% FM), indicating more inert material that can dilute the effective organic load. High ash content is often linked with lower biogas yields per unit of total solids and may also introduce trace inorganic elements that could inhibit microbial activity at excessive concentrations (Llanos-Lizcano et al., 2024). Studies on chicken manure digestion under mesophilic conditions similarly reported ash fractions above 20%, which reduced methane yields per unit TS (Wijaya et al., 2020). Despite this, the nutrient-rich composition of PM enhances microbial activity, making it valuable in co-digestion even when its specific organic fraction is lower.

3.3.1.3. Fiber Composition and Digestibility

The lignocellulosic profile of the substrates strongly influences their digestibility. Fiber analysis (Figure 3.1) confirmed the high lignin content of CPH (23.71% TS), almost 2.5 times that of PM (9.90% TS). Lignin is known to impede microbial hydrolysis due to its recalcitrant aromatic structure, explaining why mono-digestion of CPH is typically inefficient unless pretreatment is applied (Dewi et al., 2025; Rahim et al., 2019). By comparison, PM contained more hemicellulose (12.15% TS), which hydrolyses faster than cellulose and lignin, thereby contributing to rapid initial biogas release during hydrolysis and acidogenesis (Dahunsi et al., 2019). These findings suggest that CPH, although energy-rich and having high organic content, may perform poorly in mono-digestion unless pretreated. However, co-digestion with nutrient-rich and more degradable substrates, such as PM, can offset this limitation by improving hydrolysis kinetics and enhancing nutrient availability. Moreover, the physical pretreatment applied in this study (drying and grinding) also enhanced biodegradability by reducing particle size and partially weakening the lignin matrix. Similar pretreatments of CPH have been shown to improve methane yields by 15-25% under mesophilic digestion (Díaz-González et al., 2022; Ouattara et al., 2021).

3.3.1.4. Ultimate Analysis and Nutrient Balance

Elemental compositions, especially carbon and nitrogen concentrations, are essential for microbial metabolism in anaerobic systems. According to the results (Table 3.2), CPH had a much higher carbon content (48.60 ± 0.10% TS) than PM (35.15 ± 0.27% TS), while PM was significantly richer in nitrogen (2.19 ± 0.06% TS) compared to CPH (0.95 ± 0.14% TS). As a result, the calculated C/N ratio was 51.16 for CPH and 16.07 for PM. Both values fall outside the recommended range of 20-30 for optimal biogas production (Shahbaz et al., 2020; Wang et al., 2014). Such imbalances are known to cause inhibition: nitrogen deficiency (as in CPH) slows microbial growth, while excess nitrogen (as in PM) risks ammonia toxicity, especially at thermophilic temperatures (Haider et al., 2015). Therefore, the co-digestion provides a rational solution, as confirmed by the ANOVA results: C/N ratio was highly significant ($p < 0.001$), underscoring nutrient balance as a dominant factor for yield. Combining PM and CPH in adjusted ratios could then balance the C/N ratio, optimize nutrient availability, and enhance biogas yield while minimizing inhibition risks. These findings justify the experimental design strategy of co-digestion at various C/N ratios (18-35) to identify optimal nutrient conditions for stable and efficient digestion.

In addition to carbon and nitrogen, trace elements such as phosphorus (P), calcium (Ca), sodium (Na), and magnesium (Mg) play supportive roles in microbial enzyme function and methanogenesis (Somak & Sagar, 2023). PM was significantly richer in these elements, notably phosphorus (1.49 ± 0.07% TS) compared to CPH (0.14 ± 0.01% TS), which supports nucleic acid and ATP synthesis. While beneficial in moderate amounts, these minerals, particularly sodium and sulphur, can become inhibitory if concentrations exceed microbial tolerance thresholds. However, the levels recorded here remain within generally accepted limits for anaerobic digestion systems (Czatzkowska et al., 2020; Liebetrau & Pfeiffer, 2020).

3.3.1.5. Energy Content and Biomethane Potential

The energy characterization (Figure 3.2) further underlined the complementary roles of the substrates. CPH exhibited higher HHV (16,987 kJ/kg) and LHV (15,824 kJ/kg) than PM (13,187 kJ/kg and 12,227 kJ/kg, respectively). These values align with reports of cocoa husk biomass showing HHVs between 15,000 and 19,000 kJ/kg (Martínez-Ángel et al., 2015; Salcedo-Puerto et al., 2025), while poultry manure HHVs are typically 11,000-14,000 kJ/kg depending on bedding material and moisture (Quiroga et al., 2010; Topcu et al., 2022). While

CPH therefore represents the energy-dense substrate, PM contributes nutrient stability and buffering capacity, making their combination particularly advantageous. This synergy was validated in the BMP trials, where co-digestion mixtures balanced energy density with microbial nutrient requirements, ultimately leading to significantly higher yields than mono-digestion.

3.3.2. Mono-digestion Performance: Biogas potential assessment

The mono-digestion performance of cocoa pod husk (CPH) and poultry manure (PM) revealed important insights into their biodegradability and suitability for anaerobic digestion under mesophilic conditions.

3.3.2.1. Experimental System Validation and Inoculum Integrity

The positive control with cellulose provided a strong validation of the experimental system. Biogas yields were 705.46 ± 13.98 mL/g VS in mesophilic mono-digestion trials, 725.43 ± 51.88 mL/g VS in mesophilic co-digestion trials, and 760.15 ± 15.51 mL/g VS in thermophilic co-digestion trials. These values closely align with the German guideline VDI 4630 (2016), which reports a standard methane potential for cellulose of 671-820 mL/g VS (745 ± 10%) under mesophilic conditions. The results confirm the enzymatic activity and microbiological integrity of the inoculum, ruling out inhibitory conditions or operational faults. Thus, the observed differences in yields between poultry manure (PM) and cocoa pod husk (CPH) can be confidently attributed to substrate-specific properties rather than methodological artifacts.

3.3.2.2. Biogas Potential of Cocoa Pod Husk (CPH) and Poultry Manure (PM)

As shown in Figure 3.10, CPH achieved a higher cumulative yield (244.65 ± 8.48 mL/g VS) than PM (210.73 ± 27.12 mL/g VS). This is consistent with its higher volatile solids content (84.06% FM, VS/TS = 0.89) and energy density (HHV = 16,987 kJ/kg). However, despite its higher organic fraction, CPH exhibited an early production peak followed by a rapid plateau (Figure 3.5). This profile reflects its high lignin content (23.71% TS), which limits microbial hydrolysis and prevents complete conversion of structural carbohydrates. Lignin recalcitrance has been identified as a significant constraint in lignocellulosic digestion (Liebetrau & Pfeiffer, 2020; Nakhate et al., 2022). The initial burst of gas production likely arose from easily hydrolysable fractions (soluble sugars and hemicellulose), partly enhanced by the applied physical pretreatment (drying and fine grinding). However, without chemical, biological, or advanced pretreatment, the lignin-rich fraction remained resistant, leading to incomplete utilization of CPH's theoretical potential.

The total biogas yield for CPH obtained in this study (244.65 ± 8.48 mL/g VS) is lower than values reported elsewhere. For example, Hennessey-Ramos et al. (2024) recorded a yield of 314.86 ± 4.45 mL/g VS under a 35-day mesophilic (35°C) batch digestion, using inoculum from an active anaerobic digester with an S/I ratio of 1/3. Moreover, Antwi et al. (2019) reported an even higher value of 357 mL/g VS, recorded under the same conditions (mesophilic at 38°C, inoculum from an active biodigester, and a S/I ratio of 1/3). These discrepancies likely arise from geographic variation in CPH composition, the absence of advanced pretreatment in the present study, and differences in inoculum type, especially the feeding material of the active anaerobic digester where the inoculum was sourced. Notably, the absence of chemical or thermal pretreatment in this study likely limited the biodegradation of lignin-rich sections, which are known to hinder the complete hydrolysis of lignocellulosic materials like CPH.

By contrast, PM demonstrated a more sustained gas production profile, consistent with its moderate volatile solids content (VS of 70.50% and VS/TS ratio of 0.73) and high inorganic fraction. As shown in Figure 3.10 PM's lower cumulative yield (210.73 ± 27.12 mL/g VS) compared to CPH may be due to the dilution effect caused by the high ash content (25.84%), which reduces the proportion of organic matter available for conversion. Moreover, PM's relatively low C/N ratio (16.07) may have contributed to increased ammonia levels during digestion. A low C/N ratio may lead to excessive nitrogen loading, which is known to inhibit methanogenesis through free ammonia toxicity, especially under conditions of poor buffering (Duan et al., 2018; Haider et al., 2015; Wang et al., 2014). Despite these challenges, PM maintained a stable biogas production rate over time, which could be attributed to its rich supply of essential nutrients such as phosphorus, magnesium, and calcium. These micronutrients are important for microbial growth and enzyme function, as noted by (Zhu et al., 2023).

The 210.73 ± 27.12 mL/g VS yield for PM was lower than many reported values. For instance, Al-Zoubi et al. (2024) reported 285 mL/g VS under a 30-day mesophilic digestion (37°C), considering a S/I ratio of 1/2. Michailidou et al. (2024) observed yields ranging from 363 to 400 mL/g VS, under mesophilic conditions, with cattle manure as inoculum, using the AMPTS III system. Even higher yields were recorded by Jurgutis et al. (2020) (508 mL/g VS), using the AMPTS II system with an active digestate as inoculum at an S/I ratio of 1/2, and Li et al. (2013) (617 mL/g VS), considering a substrate concentration of 3g VS/L and an S/I ratio of 1/2, both under mesophilic conditions. The relatively lower performance in the present study is most plausibly explained by the use of wood chips as bedding material in the PM source. This increased lignocellulosic and ash content, thereby diluting the degradable fraction. Wood-based bedding materials are known to introduce recalcitrant fibers, increase the C/N ratio imbalance, and reduce overall methane conversion efficiency unless pretreated. Additional factors, such as substrate pretreatment and storage conditions, as well as microbial inoculum adaptation, may also have contributed to the relatively modest biogas recovery observed in this experiment.

Furthermore, the greater variability of PM yields (±27.12 mL/g VS) compared to CPH (±8.48 mL/g VS) likely reflects heterogeneity in manure composition and transient microbial stress from ammonia fluctuations. However, the fact that PM maintained stable production beyond day 14 highlights its buffering strength and microbial adaptability, justifying its role as a stabilizing co-substrate in this study.

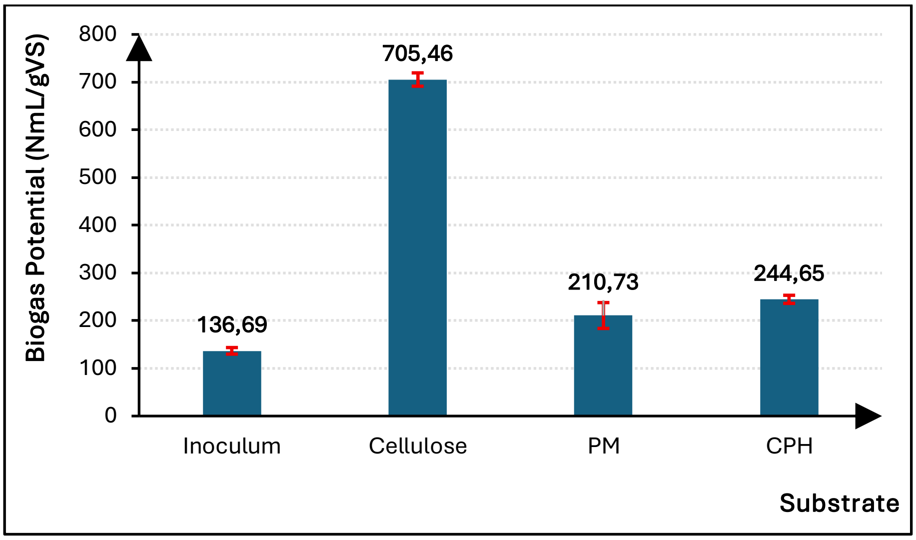

Figure 3.10. Biogas potential per substrate.

Overall, the mono-digestion results underscore the contrasting yet complementary characteristics of cocoa pod husk (CPH) and poultry manure (PM). CPH is energy-dense due to its high volatile solids and calorific value, yet its high lignin content renders it structurally recalcitrant and poorly degradable without pretreatment. Conversely, PM is rich in essential nutrients and offers favourable buffering capacity, but its lower organic content and biogas yield per unit mass limit its effectiveness as a standalone substrate. This duality highlights the strategic potential of co-digestion, where combining carbon-rich, lignified biomass (CPH) with nitrogen-rich, readily degradable organic matter (PM) can address the limitations inherent in each substrate. Such synergy enables:

- Balancing the carbon-to-nitrogen (C/N) ratio, improving microbial metabolic equilibrium,
- Enhancing hydrolysis efficiency, particularly by leveraging PM's enzymatic and nutrient profile to stimulate microbial attack on lignocellulosic material,
- Diluting inhibitory compounds, such as free ammonia or phenolics,
- And stabilizing process performance, especially in decentralized, low-tech anaerobic systems.

These results provide a quantitative baseline for determining optimal substrate mixing ratios in subsequent co-digestion experiments.

3.3.3. Co-digestion performance assessment: effect of varying C/N ratio under mesophilic and thermophilic conditions

3.3.3.1. Enhanced Biogas Production through Co-Digestion

The co-digestion of PM and CPH (Figure 3.8 and Figure 3.9) resulted in a clear enhancement of biogas yield compared to mono-digestion trials (Figure 3.3), thereby confirming the synergistic benefits of combining nitrogen-rich and carbon-dense substrates. The observed improvement across all tested C/N ratios reflects a biochemical complementarity between substrates. PM, with its low C/N ratio, high nitrogen content, and strong buffering capacity, offsets the nitrogen deficiency and acidic tendency of lignocellulosic CPH, which is characterized by high carbon and phenolic content. This nutrient synergy created a balanced environment for microbial growth and enzymatic activity, stabilizing pH and facilitating efficient methanogenesis. In addition to balancing nutrients, co-digestion likely minimized the accumulation of inhibitory compounds commonly encountered in mono-digestion. Free ammonia (typically from nitrogen-rich manure), volatile fatty acids (VFAs), and phenolics (from cocoa residues) are known to disrupt microbial metabolism when present in excess. By combining substrates, these risks were diluted, allowing the microbial consortia to operate within tolerable ranges. The two-way ANOVA results corroborated this synergy, showing that the C/N ratio had a highly significant effect ($p < 0.001$). At the same time, temperature alone was not significant, but the C/N × temperature interaction was significant ($p = 0.031$). This statistical evidence supports the interpretation that nutrient balancing, not temperature alone, was the principal driver of the enhanced biogas yields observed.

These findings are consistent with previous reports. Awais et al. (2016) demonstrated that co-digesting poultry manure with crop residues under mesophilic conditions (37 °C, 30 days, S/I ratio 0.5) significantly improved methane yields compared to mono-digestion, due to better nutrient equilibrium. Similarly, Dahunsi et al. (2019) observed a significant improvement when poultry manure was co-digested with alkaline-pretreated CPH under mesophilic BMP assays, highlighting the role of both nutrient balance and pretreatment in overcoming lignin recalcitrance. Moreover, in the present study, the physical pretreatment of substrates (oven-drying and fine grinding) likely amplified the synergy by increasing surface area, partially disrupting the lignin-cellulose matrix, and accelerating hydrolysis during the early stages.

Overall, the results reinforce co-digestion as a scalable strategy for valorising agricultural residues, especially in decentralized or rural settings. In such contexts, mono-digestion systems are often constrained by substrate imbalances or accumulation of inhibitors, while co-digestion offers a low-cost, practical means of enhancing both productivity and stability without the need for advanced process control.

3.3.3.2. Quantitative Improvement Rate Compared to Mono-Digestion

To quantitatively assess the impact of co-digestion, the biogas yields obtained from the various co-digestion scenarios were compared to the mono-digestion benchmarks of PM and CPH under mesophilic conditions (PM: 210.73 ± 27.12 mL/g VS and CPH: 244.65 ± 8.48 mL/g VS). As presented in Table 3.6, the results revealed substantial improvements across all co-digestion scenarios, with the most pronounced enhancements at C/N 25 and C/N 30, confirming these as the most favourable nutrient balances.

Table 3.6. Percentage of biogas yield improvement compared to mono-digestion.

Condition	Cumulative Yield (mL/g VS)	% Improvement vs. PM	% Improvement vs. CPH
Mono-digestion: PM	210.73 ± 27.12	-	-
Mono-digestion: CPH	244.65 ± 8.48	16,10 %	-
Co-digestion C/N 20	293,21 ± 35.43	39,14 %	19,85 %
Co-digestion C/N 25	348,65 ± 10.44	65,45 %	42,51 %
Co-digestion C/N 30	306,02 ± 18.62	45,22 %	25,08 %
Co-digestion C/N 35	234,15 ± 13.05	11,11 %	-4,29 %

The C/N 25 condition produced the most significant enhancement, with yields 65.45% higher than PM and 42.51% higher than CPH, confirming this ratio as the optimal configuration for nutrient balance and microbial activity. This observation is entirely consistent with the ANOVA results, which showed a highly significant main effect of C/N ratio ($p < 0.001$) on yield. By contrast, C/N 35 yielded only a modest 11.11% improvement over PM and underperformed relative to CPH (-4.29%), reflecting nitrogen limitation and the inhibitory influence of lignin-rich CPH fractions. This illustrates how imbalanced nutrient availability and recalcitrant organic matter constrain digestion efficiency, even when co-digestion is applied.

These findings are in line with several recent studies Dahunsi et al. (2019) reported up to 68% improvement in methane yield when poultry manure was co-digested with alkaline-pretreated CPH under mesophilic BMP conditions (37 °C, 30 days, S/I ratio 0.5), highlighting the role of pretreatment in further enhancing synergy. Similarly, Rahman et al. (2023) observed a 16% increase when poultry manure was co-digested with kitchen waste under mesophilic digestion, confirming that nutrient balancing through co-digestion consistently improves yields across diverse organic substrates.

Overall, the quantitative analysis confirms that nutrient stoichiometry balance, particularly at C/N ratios of 25-30, maximizes synergistic interactions, enabling higher cumulative yields than either PM or CPH alone. The improvement rates further emphasize that substrate ratio optimization is central to designing efficient co-digestion systems, especially in decentralized biogas applications where feedstock variability is high.

3.3.3.3. Effect of C/N Ratio on Modulating Co-Digestion Kinetics and Yield

The carbon-to-nitrogen (C/N) ratio is a key process parameter in anaerobic digestion, influencing microbial metabolism, enzymatic activity, and overall process stability. In this study, varying the C/N ratio (20, 25, 30, 35) significantly affected both the kinetics and cumulative yield of biogas during co-digestion. The ANOVA results confirmed the C/N ratio as the most influential factor ($p < 0.001$), underscoring its central role in determining digestion efficiency.

Among all tested configurations (Figure 3.11), C/N 25 consistently produced the highest yields under both mesophilic (348.65 ± 10.44 mL/g VS) and thermophilic (336.21 ± 15.92 mL/g VS) conditions. This reflects an optimal balance between readily available carbon (energy source) and nitrogen (protein and cofactor synthesis). At this ratio, the microbial community was neither carbon-limited nor subject to nitrogen overload, supporting efficient hydrolysis, acidogenesis, and methanogenesis. These findings align with the widely reported optimal C/N window of 20-30 for mixed organic substrates stability (Shahbaz et al., 2020; Wang et al., 2014). At C/N 20, yields were lower (293.21 ± 35.43 mL/g VS at mesophilic and 275.02 ± 13.71 mL/g VS at thermophilic), and process instability was particularly evident under thermophilic conditions (Figure 3.7). The excess nitrogen from PM likely elevated free ammonia (NH_3) levels, which readily diffuses across microbial membranes and disrupts methanogenesis, especially at higher temperatures (Haider et al., 2015). The irregular daily production observed in this treatment is consistent with ammonia-induced inhibition reported in manure-rich digestion systems under thermophilic conditions by Duan et al. (2018). At the opposite end, C/N 35 produced the lowest mesophilic yield (234.15 ± 13.05 mL/g VS), reflecting nitrogen limitation that constrains microbial protein synthesis and slows metabolic rates. The high CPH proportion also introduced a greater lignin load, further limiting hydrolysis (Dewi et al., 2025). Interestingly, this limitation was partially offset under thermophilic digestion (296.98 ± 10.86 mL/g VS), where elevated hydrolytic activity facilitated partial breakdown of lignocellulosic fractions. A similar trend was observed by David et al. (2018), who reported improved methane recovery from straw-rich substrates at 55 °C compared to 37 °C. The intermediate C/N 30 configuration produced relatively high yields (mesophilic: 306.02 ± 18.62 mL/g VS; thermophilic: 314.15 ± 17.89 mL/g VS) and maintained stable gas production. This suggests that a broad C/N window (25-30) is suitable for PM and CPH co-digestion, providing resilience against moderate variations in substrate composition.

Collectively, these results reinforce the C/N ratio as a critical control parameter for optimizing co-digestion. Maintaining a balanced ratio not only maximizes yield but also minimizes risks of ammonia toxicity or acidification, enhances microbial diversity, and improves digester buffering capacity. These findings are consistent with earlier studies, including those by Wang et al. (2014) and Ibro et al. (2022), who emphasized the importance of adjusting substrate proportions to maintain nutrient equilibrium. From a practical perspective, targeting a C/N ratio of 25-30 provides the best balance between productivity and process stability, making it especially relevant for decentralized biogas production systems where feedstock variability is high, and operational monitoring may be limited.

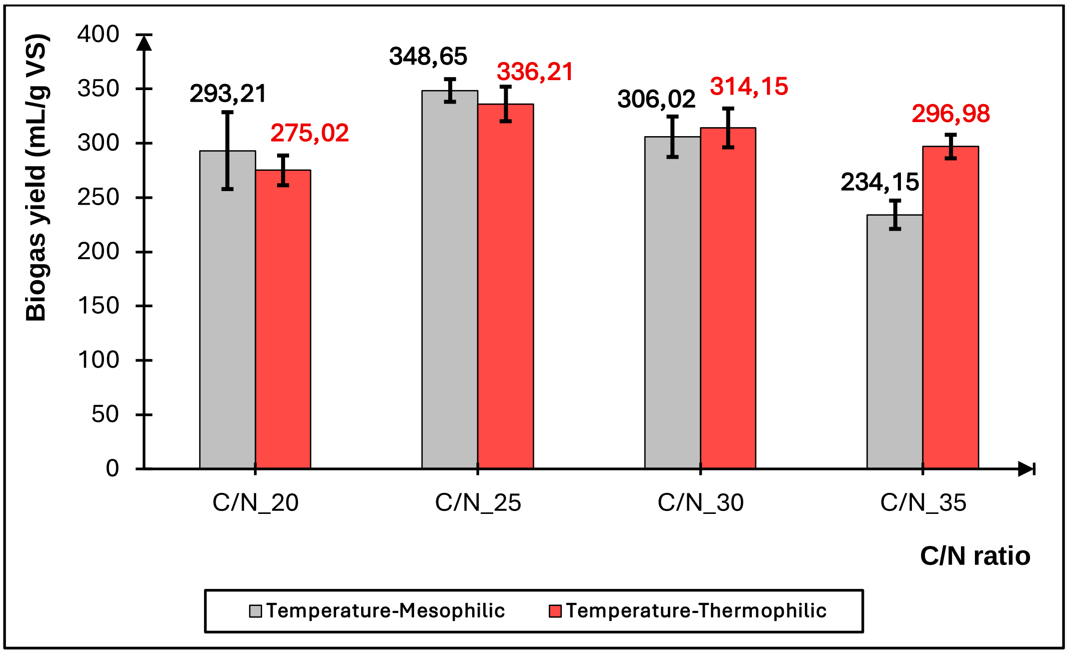

Figure 3.11. Changes in biogas yield in terms of C/N ratio and temperature conditions.

3.3.3.4. Effect of Temperature Regime on Co-Digestion Kinetics and Yield

Temperature is a critical operational parameter in anaerobic digestion, shaping microbial community structure, hydrolytic enzyme activity, and the tolerance threshold for inhibitory compounds such as free ammonia. In this study, the effect of mesophilic (37 °C) and thermophilic (55 °C) regimes was assessed across different C/N ratios during the co-digestion of PM and CPH. The ANOVA results indicated that temperature alone was not statistically significant ($p = 0.259$), yet the interaction between temperature and C/N ratio was significant ($p = 0.031$). This confirms that the influence of temperature is strongly dependent on nutrient balance rather than being an independent driver of biogas performance.

As illustrated in Figure 3.11, mesophilic digestion consistently outperformed thermophilic digestion at lower C/N ratios (20 and 25). At C/N 25, the overall optimum, cumulative yield reached 348.65 ± 10.44 mL/g VS under mesophilic conditions, compared to 336.21 ± 15.92 mL/g VS under thermophilic conditions. The superior mesophilic performance reflects greater microbial stability, stronger buffering capacity, and reduced susceptibility to ammonia inhibition. Elevated temperatures favour the dissociation of ammonium (NH_4^+) to free ammonia (NH_3), which diffuses readily across microbial membranes and disrupts methanogenesis (Labatut et al., 2014; S. Wang et al., 2019). Similar patterns were reported by Orlando & Borja (2020), who found that poultry manure digestion was more stable under mesophilic BMP conditions than under thermophilic operation due to ammonia stress. Conversely, thermophilic digestion provided advantages at higher C/N ratios (30 and 35), where the substrate mix was more carbon-rich and structurally recalcitrant. At C/N 35, yield increased by approximately 27% under thermophilic conditions (296.98 ± 10.86 mL/g VS) compared to mesophilic (234.15 ± 13.05 mL/g VS). This indicates that elevated temperatures enhanced the breakdown of lignocellulosic fractions, consistent with reports by David et al. (2018) and Singh et al. (2023), who observed improved hydrolysis of straw and crop residues at 55 °C compared to 37 °C in BMP tests.

In addition, biogas production kinetics further revealed the differential impact of temperature. Under mesophilic conditions, daily gas production peaked rapidly (93.16 mL/g VS at C/N 25; 80.46 mL/g VS at C/N 30 on Day 2), followed by a smooth decline, indicative of well-buffered microbial activity and process stability (Figure 3.6). In contrast, thermophilic profiles (Figure 3.7) were less stable, characterized by mid-phase drops, daily fluctuations, and even negative yields at C/N 20 and C/N 35. These irregular dynamics suggest episodes of microbial inhibition, likely due to the accumulation of ammonia and acidification. Despite these challenges, thermophilic digestion at C/N 25 and C/N 30 maintained relatively stable and sustained production, demonstrating that, with adequate nutrient balance, thermophilic conditions can remain viable. However, the superior overall stability of mesophilic operation, especially under nitrogen-rich or unbalanced conditions, suggests that mesophilic digestion is better suited for decentralized and rural applications, where simple operation and resilience are priorities, and continuous process monitoring may not be feasible.

3.3.3.5. Interactive Effects Between C/N Ratio and Thermal Regime

The two-way ANOVA revealed a significant interaction between C/N ratio and temperature ($F = 4.96$; $p = 0.031$), confirming that the effect of temperature on biogas yield is strongly dependent on substrate nutrient composition rather than acting independently. This finding highlights that thermal regime and C/N ratio must be considered jointly when optimizing anaerobic digestion performance. As illustrated in Figure 3.12, thermophilic digestion outperformed mesophilic digestion only at higher C/N ratios (30 and 35), where nitrogen deficiency and lignocellulosic recalcitrance constrained mesophilic performance. Elevated temperatures accelerated hydrolysis, enabling partial breakdown of carbon-rich, lignin-heavy fractions, consistent with reports that thermophilic regimes improve the degradation of structurally complex feedstocks (David et al., 2018; Singh et al., 2023). Conversely, under nitrogen-rich conditions (C/N 20), thermophilic digestion produced lower yields and unstable kinetics, consistent with ammonia inhibition. Elevated temperatures favor the shift of the NH_4^+/NH_3 equilibrium toward free ammonia, which disrupts methanogenic activity at relatively low concentrations (Czatzkowska et al., 2020; Wang et al., 2014). This explains the irregular production patterns and episodes of microbial inhibition observed in the C/N 20 at thermophilic treatment.

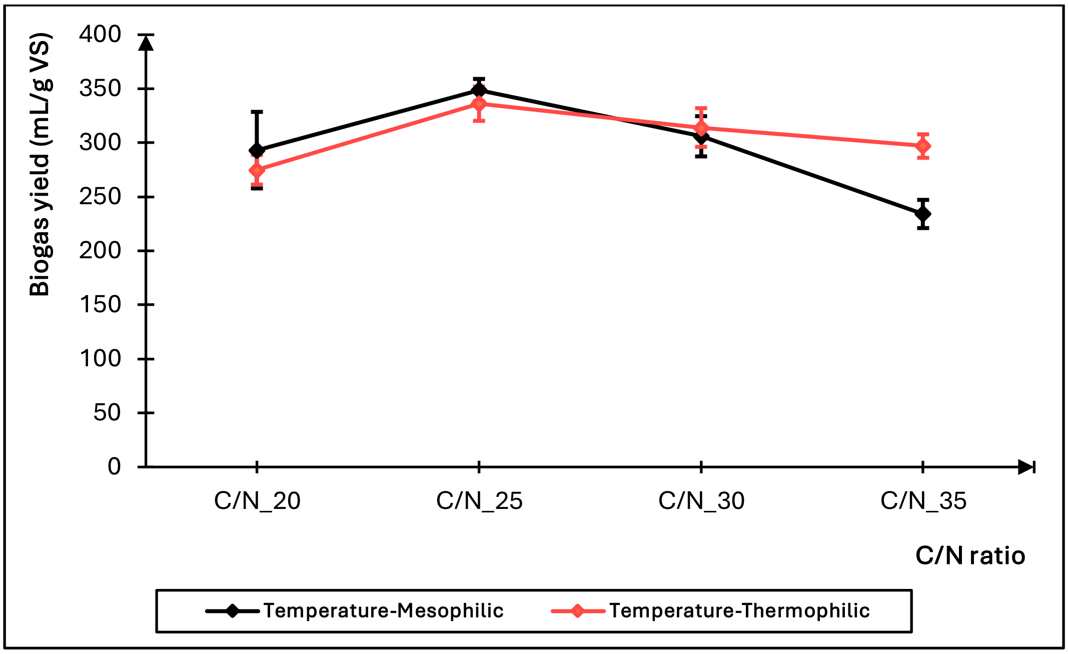

Figure 3.12. Interactive effect between C/N ratio and thermal regime on biogas yield.

The comparative analysis reveals that mesophilic co-digestion at a C/N ratio of 25-30 provides the optimal balance between productivity and stability, characterized by rapid early gas production and gradual, sustained yields with minimal variability. These characteristics are particularly relevant for decentralized and rural biogas systems, where operational simplicity, low risk of inhibition, and minimal monitoring requirements are critical. At the same time, thermophilic operation may be advantageous for carbon-rich, lignocellulosic mixtures (C/N ≥ 30), provided that buffering and process stability can be maintained. From a practical standpoint, these results advocate for a context-specific approach to temperature management in co-digestion:

- Mesophilic conditions are more suitable for nitrogen-rich or moderately balanced substrates (C/N 20-30), where stability and robustness are priorities.
- Thermophilic conditions can be beneficial for carbon-rich, lignin-heavy feedstocks (C/N ≥ 30), where enhanced hydrolysis offsets nitrogen limitation.

3.3.4. Practical Implications for Sustainable Waste Management

The co-digestion of poultry manure (PM) and cocoa pod husk (CPH), as demonstrated in this study, provides a practical pathway for advancing sustainable waste management, particularly in agriculture-dominated regions with abundant organic residues. The results highlight not only enhanced biogas yields and improved process stability but also the potential to address environmental, energy, and agricultural challenges simultaneously.

3.3.4.1. Valorisation of Agricultural Residues for Energy Access and Decentralized Biogas Solutions

Both PM and CPH are locally abundant but underutilized resources in cocoa-producing and poultry farming countries, such as Togo. Poultry manure is commonly land-applied or disposed of untreated, contributing to nutrient leaching, odor emissions, and pathogen dissemination (Swelum et al., 2021). Cocoa pod husk, on the other hand, is a lignocellulosic by-product that often accumulates during cocoa processing and is traditionally discarded by open burning or dumping, creating environmental burdens (Ouattara et al., 2021). The present study demonstrates that mesophilic co-digestion at C/N 25-30 produces consistently high and stable biogas yields, confirming the statistical significance of C/N ratio as the dominant process parameter (ANOVA, $p < 0.001$). This finding has important implications for energy access in rural communities, where biogas systems can:

- Provide clean cooking fuel to substitute firewood or charcoal, reducing indoor air pollution.
- Generate electricity for households or micro-enterprises via small-scale combined heat and power (CHP) units.
- Supply thermal energy for agro-processing applications such as drying and heating.

Beyond energy, the resulting digestate is a nutrient-rich by-product containing nitrogen, phosphorus, potassium, calcium, and magnesium. Its reuse as an organic fertilizer supports soil fertility, crop yields, water retention, and agroecological intensification, while reducing reliance on costly synthetic fertilizers. This dual energy-agriculture synergy promotes circular resource flows and enhances the economic resilience of smallholder farmers. Importantly, the use of locally available feedstocks and the minimal need for external pH or thermal control under mesophilic operation make the technology low-cost and accessible for resource-constrained users, thereby advancing energy equity and inclusivity.

3.3.4.2. Climate and Environmental Co-Benefits

The co-digestion of poultry manure (PM) and cocoa pod husk (CPH) generates multiple climate and environmental co-benefits that extend well beyond renewable energy production. Anaerobic digestion (AD) prevents uncontrolled methane emissions from unmanaged manure heaps, a significant source of greenhouse gases in agricultural systems. By replacing traditional biomass fuels such as firewood and charcoal, AD also reduces deforestation, indoor air pollution, and black carbon emissions, thereby contributing directly to climate change mitigation. Furthermore, the stabilized digestate produced through PM-CPH co-digestion reduces dependency on synthetic fertilizers, enhances soil organic matter, and improves soil health and resilience. These outcomes are consistent with climate-smart agriculture strategies and support national greenhouse gas reduction commitments under the Paris Agreement and nationally determined contributions (NDCs) (FAO, 2022).

The broader co-benefits of PM-CPH co-digestion align closely with several Sustainable Development Goals (SDGs). As illustrated in Figure 3.13, the process simultaneously advances:

- SDG 7 (Affordable and Clean Energy): by expanding access to biogas for cooking, electricity, and agro processing.
- SDG 12 (Responsible Consumption and Production): by promoting waste valorisation and nutrient recycling within a circular economy framework.
- SDG 13 (Climate Action): by capturing methane emissions and displacing fossil fuel use.
- SDG 2 (Zero Hunger) and SDG 15 (Life on Land): by enhancing food security, restoring soil fertility, and supporting sustainable land management through digestate application.
- SDG 3 (Good Health and Well-being): indirectly, through reductions in smoke-related illnesses associated with firewood and charcoal use.

In conclusion, the performance demonstrated in this study validates co-digestion as a comprehensive waste-to-energy solution that integrates climate mitigation, energy access, and sustainable agricultural practices. Its scalability and adaptability to diverse feedstocks make it highly relevant for agriculture-based economies in sub-Saharan Africa, where integrated strategies are essential to meeting both energy transition goals and sustainable development objectives.

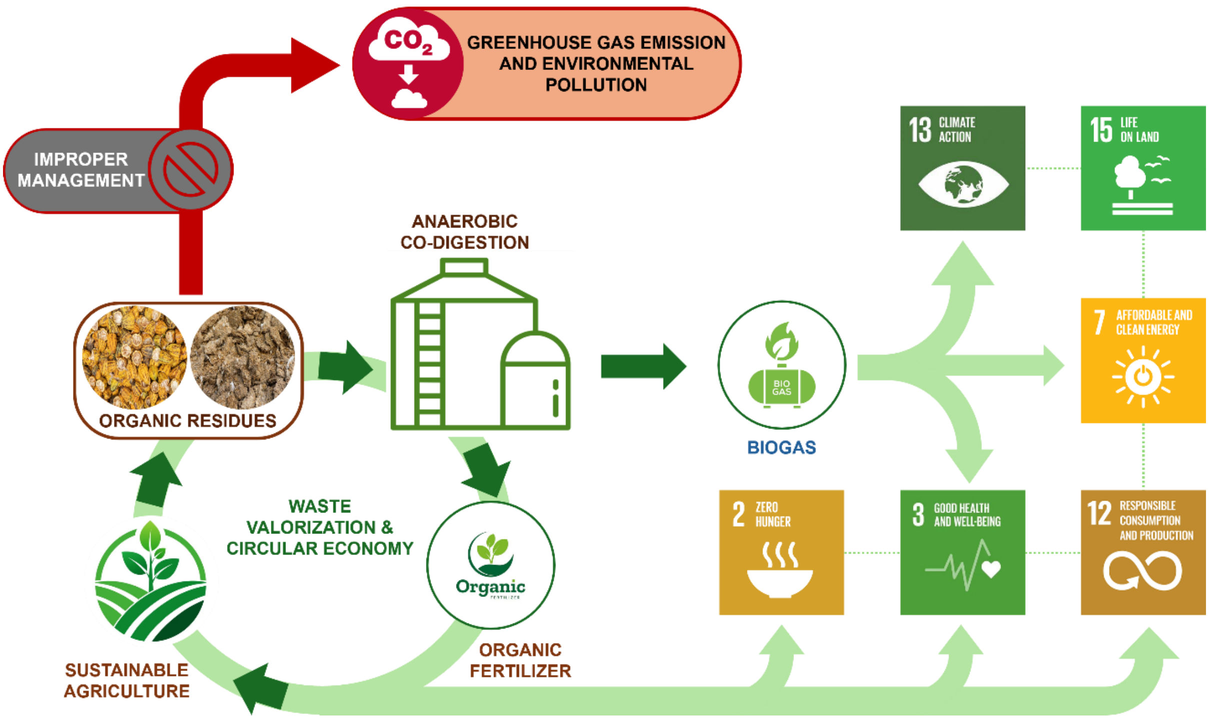

Figure 3.13. Schematic representation of the benefits of poultry manure (PM) and cocoa pod husk (CPH) co-digestion and their interconnections with Sustainable Development Goals (SDGs).

3.4. Conclusion

The results demonstrate that co-digestion of PM and CPH markedly enhances biogas yield and process stability compared to mono-digestion. C/N ratio was identified as the most influential factor ($p < 0.001$), with optimal performance observed at C/N 25 under mesophilic conditions (348.65 ± 10.44 mL/g VS). While temperature alone was not significant, the interaction between C/N ratio and temperature was significant ($p = 0.031$), confirming that substrate nutrient balance modulates the effect of thermal regime. Compared to literature values, the yields align with global reports, though bedding materials reduced PM's performance, and CPH's was constrained by lignin recalcitrance. Practically, the study demonstrates that mesophilic co-digestion at a C/N ratio of 25-30 offers the best balance of productivity, stability, and accessibility, making it suitable for decentralized rural applications. The findings contribute to knowledge on waste-to-energy solutions in West Africa and highlight co-digestion as a strategy with strong synergies across energy access, climate mitigation, and sustainable agriculture.

CONCLUSION AND PERSPECTIVES

1. Conclusion

The growing challenges of organic waste accumulation, energy insecurity, and environmental degradation in West Africa highlight the urgent need for sustainable waste-to-energy solutions. Poultry manure (PM) and cocoa pod husks (CPH), two widely available agricultural residues, exhibit complementary characteristics: PM is rich in nitrogen and buffering capacity, while CPH provides high carbon content and energy potential. This study focused on optimizing biogas production from the anaerobic co-digestion of PM and CPH by investigating the effects of substrate carbon-to-nitrogen (C/N) ratio and temperature regime. Substrate physicochemical properties were characterized, and batch biochemical methane potential (BMP) assays were carried out using the ANKOM gas production system over 30 days under mesophilic (37 °C) and thermophilic (55 °C) conditions at C/N ratios of 20, 25, 30, and 35. Biogas yields were monitored daily, and cumulative production was statistically evaluated using two-way ANOVA with Tukey's HSD post-hoc test at a 95% confidence level.

The physicochemical characterization of the substrates revealed that CPH is carbon-rich with a high volatile solids content and calorific value but limited biodegradability due to its high lignin fraction. In contrast, PM is nitrogen-rich and nutritionally balanced but contains high ash content and a low C/N ratio that may induce ammonia inhibition. The complementary substrate properties demonstrated high complementarity and potential synergy when co-digested. Mono-digestion trials confirmed the moderate biogas yields of both substrates, with CPH yielding 244.65 ± 8.48 mL/g VS and PM yielding 210.73 ± 27.12 mL/g VS. In contrast, co-digestion significantly enhanced biogas production across all tested C/N ratios, with the highest yield (348.65 ± 10.44 mL/g VS) achieved at a C/N ratio of 25 under mesophilic conditions, which contributed to 65.45% improvement over PM and 42.51% improvement over CPH. The two-way ANOVA showed that the C/N ratio had a statistically significant effect on biogas yield ($p = 0.001$), while temperature had no independent effect. However, a significant interaction was found between C/N ratio and temperature ($p = 0.031$), indicating that temperature effects are substrate-dependent and vice versa.

These findings directly support the initial research hypotheses. The first hypothesis, that the physicochemical characteristics of PM and CPH are favourable for anaerobic digestion, was confirmed by their complementary nutrient profiles. The second hypothesis, that co-digestion enhances biogas production compared to mono-digestion, was validated by the substantial yield improvements observed across all C/N ratios. Finally, the third hypothesis, that an optimal C/N ratio and temperature regime exist to maximize yield, was supported by the identification of a C/N ratio of 25 under mesophilic conditions as the most effective configuration, with thermophilic operation proving advantageous only under carbon-rich conditions. Together, these results demonstrate that optimization of co-digestion parameters is essential for designing efficient biogas production systems in the West African context.

Overall, the findings demonstrate that co-digestion of PM and CPH at optimal C/N ratios (25–30) under mesophilic conditions provides a stable, efficient, and scalable strategy for enhancing biogas production. This integrated approach provides a practical pathway to address waste management challenges, enhance renewable energy access, and promote nutrient recycling in poultry farming and cocoa-producing regions of West Africa.

2. Perspectives

While this study demonstrates the effectiveness of co-digesting poultry manure (PM) and cocoa pod husk (CPH) under varying C/N ratios and temperature regimes, it also highlights several avenues for further investigation to deepen scientific understanding, enhance practical applicability, and support the scaling of anaerobic digestion (AD) technologies in resource-constrained settings. Thus, based on the insights and limitations of this study, the following perspectives are proposed:

- **Qualitative Assessment of Biogas Produced from the Co-digestion of PM and CPH:** Future studies should measure methane (CH_4), carbon dioxide (CO_2), hydrogen sulfide (H_2S), etc., concentrations to better assess the energy content and end-use suitability of the produced biogas.
- **Anaerobic Co-digestion of PM and CPH: Investigation of Pretreatment Strategies for Biogas Production Enhancement:** Given the high lignin content of cocoa pod husks, investigations into mechanical, thermal, alkaline, or enzymatic pretreatments are needed to enhance biodegradability and maximize gas yield, while evaluating energy balance and cost-effectiveness.
- **Assessment of the Agronomic Potential of PM and CPH through the Anaerobic Co-digestion Process:** Field trials are necessary to evaluate the nutrient availability, potential heavy metal risks, and effects on soil fertility and crop productivity of digestate derived from PM–CPH co-digestion.
- **Techno-Economic Feasibility and Environmental Impact Assessment of Anaerobic Co-digestion of PM and CPH:** A comprehensive techno-economic analysis (TEA) and life cycle assessment (LCA) will provide critical insights into the economic viability, environmental performance, and policy implications of deploying PM-CPH biogas production systems at scale.

These perspectives provide a roadmap for advancing anaerobic co-digestion as a practical, scalable, and climate-smart solution for Togo and the broader West African region.

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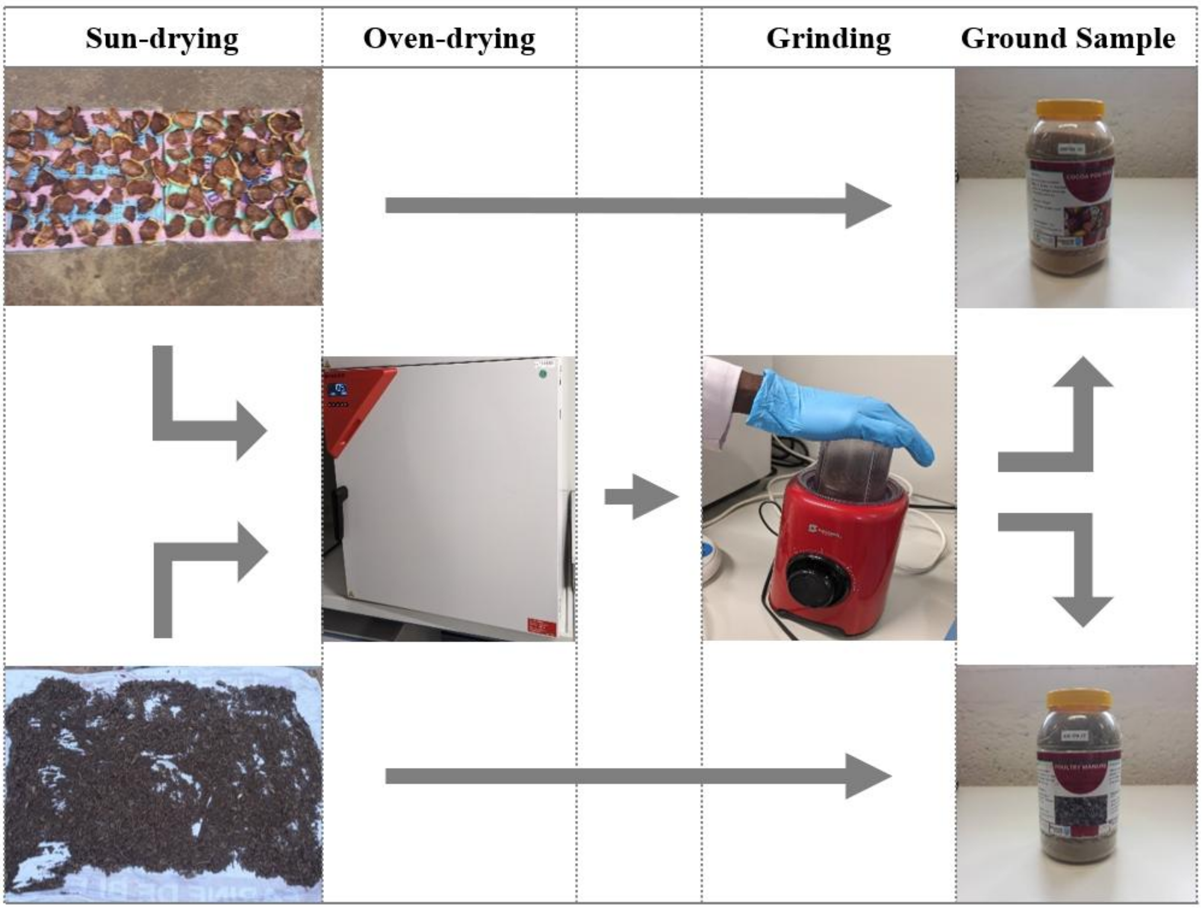
APPENDIXES

APPENDIX A: Substrate Collection

APPENDIX B: Substrate Pretreatment



APPENDIX C: Substrate Proximate, Fiber, and Ultimate Analysis Results

APPENDIX C.1. Proximate Analysis Results

Parameters	Unit	Meas.V1	Meas.V2	Meas.V3	Average	STDEV
Poultry Manure						
TS	%(FM)	96,48	95,81	96,73	96,34	0,48
MC	%(FM)	3,80	3,13	4,05	3,66	0,48
VS	%(FM)	70,46	70,26	70,78	70,50	0,26
AC	%(FM)	25,80	25,60	26,12	25,84	0,26
Cocoa Pod Husk						
TS	%(FM)	93,93	94,75	95,78	94,82	0,93
MC	%(FM)	4,29	5,11	6,14	5,18	0,93
VS	%(FM)	83,12	84,01	85,05	84,06	0,97
AC	%(FM)	9,82	10,71	11,75	10,76	0,97

APPENDIX C.2. Fiber Analysis Results

Parameter	Unit	Meas.V1	Meas.V2	Meas.V3	Average	STDEV
Poultry Manure						
Lignin	%TS	10,33	9,31	10,06	9,90	0,53
Cellulose	%TS	25,61	24,32	25,13	25,02	0,65
Hemicellulose	%TS	11,85	11,55	13,05	12,15	0,79
Cocoa Pod Husk						
Lignin	%TS	23,49	24,26	23,38	23,71	0,48
Cellulose	%TS	27,26	26,64	25,87	26,59	0,70
Hemicellulose	%TS	7,72	8,31	8,93	8,32	0,61

APPENDIX C.3. Ultimate Analysis Results

Parameter	Unit	Meas.V1	Meas.V2	Meas.V3	Average	STDEV
Cocoa Pod Husk						
Carbon (C)	%TS	48.70	48,50	48,60	48,60	0,10
Hydrogen (H)	%TS	5.26	5,41	5,34	5,34	0,08
Oxygen (O)	%TS	38.30	38,20	38,25	38,25	0,05
Nitrogen (N)	%TS	1.09	0,81	0,95	0,95	0,14
Sulfur (S)	%TS	0.10	0,09	0,10	0,10	0,01
Iron (Fe)	%TS	0.02	0,02	0,02	0,02	0,00
Calcium (Ca)	%TS	0.84	0,85	0,84	0,84	0,01
Potassium (K)	%TS	4.13	4,15	4,17	4,15	0,02
Magnesium (Mg)	%TS	0.42	0,42	0,40	0,41	0,01
Sodium (Na)	%TS	0.003	0,006	0,001	0,003	0,003
Phosphorus (P)	%TS	0.15	0,14	0,13	0,14	0,01
HHV	kJ/kg	16993	16980,00	16988,00	16987,00	6,56
LHV	kJ/kg	15846	15802,00	15824,00	15824,00	22,00
Poultry Manure						
Carbon (C)	%TS	34,90	35,43	35,12	35,15	0,27
Hydrgen (H)	%TS	4,34	4,47	4,42	4,41	0,07
Oxygen (O)	%TS	30,60	29,55	30,00	30,05	0,53
Nitrogen (N)	%TS	2,20	2,13	2,24	2,19	0,06
Sulfur (S)	%TS	0,33	0,39	0,33	0,35	0,03
Iron (Fe)	%TS	0,23	0,36	0,19	0,26	0,09

Calcium (Ca)	%TS	3,56	3,78	3,94	3,76	0,19
Potassium (K)	%TS	2,36	2,58	2,65	2,53	0,15
Magnesium (Mg)	%TS	0,38	0,54	0,52	0,48	0,09
Sodium (Na)	%TS	0,53	0,43	0,42	0,46	0,06
Phosphorus (P)	%TS	1,46	1,57	1,44	1,49	0,07
HHV	kJ/kg	13238	13186	13137	13187	50,51
LHV	kJ/kg	12292	12161	12228	12227	65,51

APPENDIX D: Mono-Digestion BMP Results

Time	**Average Cumulative Biogas yield (mL/gVS)**				**Average Daily Biogas yield (mL/gVS)**			
(Day)	**In.**	**Cel.**	**PM**	**CPH**	**In.**	**Cel.**	**PM**	**CPH**
0	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
1	8,37	279,76	38,85	91,41	8,37	279,76	38,85	91,41
2	16,82	486,77	69,83	129,62	8,44	207,01	30,98	38,22
3	24,57	563,95	91,45	152,26	7,76	77,18	21,62	22,64
4	32,36	622,98	106,96	168,10	7,79	59,03	15,51	15,84
5	40,44	648,55	119,01	180,42	8,07	25,57	12,05	12,32
6	49,21	658,67	127,54	189,81	8,78	10,12	8,52	9,39
7	57,38	665,19	135,22	197,30	8,17	6,52	7,68	7,49
8	63,82	671,62	142,52	203,60	6,44	6,43	7,31	6,30
9	69,56	677,02	148,85	208,59	5,74	5,40	6,33	5,00
10	74,86	681,43	153,68	213,05	5,30	4,41	4,83	4,45
11	79,69	685,58	158,07	217,41	4,83	4,15	4,39	4,36
12	84,65	688,44	162,03	220,84	4,97	2,86	3,96	3,43
13	89,12	691,95	165,19	223,67	4,47	3,51	3,16	2,83
14	93,26	694,35	168,57	226,63	4,14	2,40	3,38	2,96
15	96,94	696,53	172,05	228,67	3,67	2,18	3,48	2,04
16	100,80	697,82	174,64	230,59	3,86	1,29	2,59	1,92
17	104,09	699,04	177,85	232,29	3,30	1,22	3,21	1,70
18	107,11	699,48	180,76	233,70	3,02	0,44	2,91	1,41
19	109,91	700,31	183,25	234,69	2,80	0,84	2,49	0,99
20	112,53	700,68	185,61	235,82	2,61	0,36	2,36	1,13
21	115,50	700,77	187,67	236,39	2,98	0,10	2,06	0,57
22	117,93	701,00	189,81	237,12	2,42	0,23	2,14	0,74
23	120,31	701,45	192,11	237,94	2,39	0,45	2,30	0,81
24	122,46	701,34	194,29	238,64	2,15	-0,10	2,18	0,71
25	124,56	701,27	196,15	239,27	2,10	-0,07	1,86	0,63
26	126,43	701,41	198,12	239,94	1,87	0,14	1,97	0,67
27	128,45	701,65	199,77	240,29	2,03	0,24	1,65	0,35
28	130,23	702,41	201,85	241,15	1,77	0,76	2,08	0,86
29	131,70	703,02	203,76	241,85	1,47	0,61	1,91	0,69
30	133,01	703,87	205,65	242,69	1,31	0,85	1,89	0,85
31	134,31	704,30	207,32	243,32	1,30	0,43	1,66	0,62
32	135,56	705,10	209,16	244,20	1,25	0,80	1,84	0,89
33	136,69	705,46	210,73	244,65	1,13	0,36	1,58	0,45

Substrate	**Biogas Yield (NmL/gVS)**	**STDEV**
Inoculum	136,69	6,90
Cellulose	705,46	13,98
PM	210,73	27,12
CPH	244,65	8,48

APPENDIX E: Co-Digestion BMP Results

APPENDIX E.1. Mesophilic (37°C) Co-Digestion results

Time	**Mesophilic Average Cumulative Biogas Yield (mL/g VS)**						**Mesophilic Average Daily Biogas Yield (mL/g VS)**					
(Day)	**In.**	**Cel.**	**C/N_20**	**C/N_25**	**C/N_30**	**C/N_35**	**In.**	**Cel.**	**C/N_20**	**C/N_25**	**C/N_30**	**C/N_35**
0	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
1	1,57	34,41	52,78	65,39	59,87	38,02	1,57	34,41	52,78	65,39	59,87	38,02
2	3,82	228,30	124,86	158,55	140,33	87,26	2,25	193,89	72,08	93,16	80,46	49,24
3	6,07	396,86	165,92	209,61	184,34	121,55	2,25	168,56	41,06	51,06	44,02	34,29
4	8,12	506,80	190,00	236,29	208,08	142,79	2,04	109,93	24,08	26,67	23,74	21,23
5	10,95	583,63	207,12	254,85	224,97	157,29	2,83	76,84	17,11	18,56	16,88	14,51
6	14,58	637,66	219,38	269,38	238,18	168,42	3,63	54,03	12,26	14,53	13,22	11,13
7	18,41	670,53	230,15	281,37	248,66	178,47	3,84	32,87	10,78	11,99	10,48	10,05
8	24,04	687,43	238,09	291,08	256,60	185,34	5,63	16,91	7,93	9,71	7,93	6,88
9	29,38	690,68	241,29	295,18	259,59	188,56	5,34	3,25	3,21	4,09	3,00	3,21
10	32,88	694,37	245,72	300,58	263,46	192,91	3,50	3,69	4,43	5,40	3,87	4,35
11	34,84	700,00	250,13	305,63	267,54	197,08	1,96	5,63	4,41	5,06	4,08	4,17
12	36,55	701,79	254,31	310,05	271,24	201,17	1,71	1,78	4,17	4,41	3,70	4,10
13	38,97	705,53	258,13	313,94	274,49	204,99	2,42	3,74	3,82	3,89	3,24	3,82
14	41,09	708,23	260,90	316,63	276,80	207,77	2,13	2,70	2,77	2,68	2,31	2,77
15	42,68	710,26	263,20	319,01	278,83	209,99	1,58	2,03	2,30	2,38	2,03	2,22
16	44,38	712,63	265,25	321,22	280,93	212,29	1,71	2,37	2,05	2,21	2,10	2,30
17	45,76	715,02	267,73	323,44	283,14	214,53	1,38	2,39	2,47	2,23	2,21	2,23
18	46,43	716,63	270,31	325,95	285,57	216,87	0,67	1,61	2,59	2,50	2,43	2,34
19	47,26	716,47	272,32	328,44	287,50	218,88	0,83	-0,15	2,01	2,50	1,93	2,01
20	48,22	716,89	274,16	330,36	289,10	220,47	0,96	0,42	1,84	1,92	1,60	1,59
21	50,05	717,04	276,12	331,91	290,63	221,62	1,83	0,15	1,97	1,55	1,53	1,15
22	50,47	718,19	278,15	333,85	292,52	223,24	0,42	1,15	2,02	1,94	1,88	1,62
23	51,93	719,05	280,29	335,75	294,31	224,73	1,46	0,86	2,14	1,89	1,80	1,49
24	52,55	720,08	282,39	337,60	296,34	226,83	0,63	1,02	2,10	1,85	2,03	2,10

25	52,80	721,23	284,42	339,54	298,48	228,12	0,25	1,16	2,03	1,95	2,14	1,30
26	53,18	722,15	286,28	341,49	300,05	229,50	0,38	0,92	1,86	1,94	1,57	1,37
27	54,18	722,10	287,87	343,08	301,17	230,20	1,00	-0,05	1,59	1,59	1,12	0,70
28	54,80	723,24	289,64	345,01	302,71	231,40	0,63	1,14	1,77	1,93	1,54	1,20
29	55,22	724,62	291,59	346,95	304,28	232,77	0,42	1,38	1,94	1,94	1,57	1,37
30	55,47	725,43	293,21	348,65	306,02	234,15	0,25	0,81	1,62	1,70	1,74	1,38

Biogas yield _ 37°C		
Substrate	**mL/g VS**	**STDEV**
Inoculum	55,47	5,56
cellulose	725,43	51,88
C/N-20	293,21	35,43
C/N-25	348,65	0,44
C/N-30	306,02	18,62
C/N-35	234,15	13,05

APPENDIX E.2. Thermophilic (55°C) Co-Digestion results

Time	**Cumulative Biogas yield (mL/g VS)**						**Daily Biogas yield (mL/g VS)**					
(Day)	**In.**	**Cel.**	**C/N_20**	**C/N_25**	**C/N_30**	**C/N_35**	**In.**	**Cel.**	**C/N_20**	**C/N_25**	**C/N_30**	**C/N_35**
0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
1,00	7,88	-0,63	45,44	32,29	46,73	22,56	7,88	-0,63	45,44	32,29	46,73	22,56
2,00	10,30	61,97	67,09	51,36	66,07	42,99	2,42	62,60	21,65	19,08	19,34	20,42
3,00	13,03	194,59	89,49	76,15	91,59	66,92	2,73	132,62	22,40	24,79	25,52	23,93
4,00	14,98	268,17	104,77	89,93	105,86	77,49	1,95	73,58	15,28	13,78	14,28	10,57
5,00	17,13	318,29	123,47	104,15	117,93	85,39	2,14	50,12	18,70	14,22	12,06	7,90
6,00	19,74	362,85	158,13	127,44	136,61	98,78	2,61	44,56	34,66	23,29	18,68	13,39
7,00	21,22	403,33	202,67	165,65	171,27	125,28	1,48	40,48	44,54	38,21	34,66	26,50
8,00	23,21	459,77	237,06	208,19	202,75	160,30	1,99	56,44	34,39	42,54	31,48	35,02

9,00	24,77	522,08	264,09	241,57	229,92	193,90	1,56	62,31	27,03	33,39	27,17	33,60
10,00	26,36	582,47	282,52	262,63	253,37	221,37	1,60	60,39	18,43	21,05	23,44	27,47
11,00	28,82	635,54	295,57	281,97	268,94	238,92	2,45	53,07	13,06	19,34	15,58	17,55
12,00	32,68	669,35	298,20	293,45	278,33	250,33	3,86	33,81	2,63	11,48	9,38	11,41
13,00	38,30	692,74	297,47	296,63	280,01	254,46	5,62	23,39	-0,73	3,18	1,68	4,13
14,00	44,50	711,92	296,78	299,44	280,46	256,01	6,21	19,18	-0,69	2,81	0,45	1,55
15,00	50,32	726,92	289,72	300,17	278,41	253,24	5,82	15,00	-7,06	0,73	-2,05	-2,77
16,00	55,43	734,08	285,28	301,12	277,17	251,37	5,11	7,15	-4,44	0,94	-1,24	-1,87
17,00	59,52	734,80	280,59	303,20	276,91	250,42	4,10	0,73	-4,69	2,09	-0,26	-0,95
18,00	62,14	734,45	278,41	306,42	278,72	251,77	2,61	-0,35	-2,18	3,21	1,81	1,35
19,00	63,70	734,14	278,64	311,67	281,59	254,48	1,56	-0,31	0,23	5,25	2,87	2,72
20,00	65,41	734,13	277,65	316,60	283,63	256,37	1,72	-0,02	-1,00	4,93	2,05	1,89
21,00	67,63	732,77	275,48	322,66	286,23	260,26	2,22	-1,35	-2,16	6,06	2,59	3,90
22,00	68,84	733,35	274,74	324,68	288,89	262,64	1,21	0,58	-0,74	2,03	2,66	2,38
23,00	70,36	737,27	273,98	327,79	292,99	267,13	1,52	3,92	-0,76	3,10	4,10	4,49
24,00	72,20	744,32	274,73	330,34	297,42	274,90	1,83	7,05	0,75	2,55	4,43	7,77
25,00	73,21	745,35	273,92	331,58	299,38	277,89	1,01	1,03	-0,81	1,24	1,96	2,99
26,00	74,15	748,30	273,72	332,00	302,14	281,48	0,94	2,94	-0,20	0,42	2,76	3,59
27,00	75,59	751,39	273,88	332,67	304,41	285,38	1,44	3,09	0,16	0,67	2,27	3,90
28,00	76,33	754,46	273,77	333,74	307,05	288,33	0,74	3,08	-0,11	1,07	2,64	2,95
29,00	77,50	757,91	273,57	335,22	310,54	292,85	1,17	3,44	-0,21	1,49	3,50	4,52
30,00	78,90	760,15	275,02	336,21	314,15	296,98	1,40	2,25	1,46	0,99	3,60	4,13

	Biogas yield	
Substrate	**mL/g VS**	**STDEV**
Inoculum	78,90	3,56
cellulose	760,15	15,51
C/N-20	275,02	13,71
C/N-25	336,21	15,92
C/N-30	314,15	17,89
C/N-35	296,98	10,86

APPENDIX F: Statistical Analysis

APPENDIX F.1. Normality and Homogeneity Assumptions Test results

Test	DF	W statistic	p-value	Interpretation
Shapiro-Wilk Test (Normality)	8	0,972	0.8652	Residuals are normally distributed
Levene's Test (Homogeneity of variance)	8	$1{,}11x10^{29}$	<0.001	Homogeneity of variances is violated

APPENDIX F.2. Classical ANOVA

Source	DF	Sum of Squares	Mean Square	F-value	p-value	Partial η^2	Significant
C/N ratio	3	13356.21	4452.07	16.167	0.001	0.666	Yes
Temperature	1	406.63	406.63	1.477	0.259	0.020	No
C/N ratio vs Temperature	3	4093.42	1364.47	4.955	0.031	0.204	Yes
Residual	8	2202.97	275.40	-	-	-	-

DF: Degree of freedom

APPENDIX F.3. Welch-Type (WLS) Two-Way ANOVA

Source	DF	F	p-value	η^2	Significant
C/N Ratio	3	68,75	<0.001	0.963	Yes
Temperature	1	38,51	0.0003	0.828	Yes
C/N × Temp	3	12,37	0.0023	0.823	Yes

APPENDIX F.4. General Linear Model (GLM) with HC3-robust standard errors

Source	DF 1	DF 1	F	p-value	Significant
C/N Ratio	3	8	37,58	<0.001	Yes
Temperature	1	8	0,26	0.624	No
C/N × Temp	3	8	6,18	0.018	Yes

APPENDIX F.5. Tukey's Honestly Significant Difference (HSD) post-hoc test

Analysis of the differences between the categories (C/N ratio vs Temperature) with a confidence interval of 95% Biogas yield (mL/g VS).

Group 1 vs Group 2	Mean Difference mL/g VS	Standardized difference	Critical value	Adjusted p-value	Significant	Lower bound (95%)	Upper bound (95%)
C/N 25 (Mesophilic) vs C/N 35 (Mesophilic)	114,506	6,900	3,957	0,002	Yes	48,840	180,171
C/N 25 (Mesophilic) vs C/N 20 (Thermophilic)	73,633	4,437	3,957	0,027	Yes	7,968	139,298
C/N 25 (Mesophilic) vs C/N 20 (Mesophilic)	55,440	3,341	3,957	0,110	No	-10,226	121,105
C/N 25 (Mesophilic) vs C/N 35 (Thermophilic)	51,672	3,114	3,957	0,147	No	-13,994	117,337
C/N 25 (Mesophilic) vs C/N 30 (Mesophilic)	42,633	2,569	3,957	0,287	No	-23,033	108,298
C/N 25 (Mesophilic) vs C/N 30(Thermophilic)	34,500	2,079	3,957	0,493	No	-31,165	100,165
C/N 25 (Mesophilic) vs C/N 25 (Thermophilic)	12,443	0,750	3,957	0,992	No	-53,222	78,109
C/N 25 (Thermophilic) vs C/N 35 (Mesophilic)	102,062	6,150	3,957	0,004	Yes	36,397	167,728
C/N 25 (Thermophilic) vs C/N 20 (Thermophilic)	61,190	3,687	3,957	0,070	No	-4,476	126,855
C/N 25 (Thermophilic) vs C/N 20 (Mesophilic)	42,997	2,591	3,957	0,280	No	-22,669	108,662
C/N 25 (Thermophilic) vs C/N 35 (Thermophilic)	39,229	2,364	3,957	0,364	No	-26,437	104,894
C/N 25 (Thermophilic) vs C/N 30 (Mesophilic)	30,190	1,819	3,957	0,626	No	-35,476	95,855
C/N 25 (Thermophilic) vs C/N 30 (Thermophilic)	22,057	1,329	3,957	0,865	No	-43,609	87,722
C/N 30 (Thermophilic) vs C/N 35 (Mesophilic)	80,006	4,821	3,957	0,017	Yes	14,340	145,671
C/N 30 (Thermophilic) vs C/N 20 (Thermophilic)	39,133	2,358	3,957	0,366	No	-26,532	104,798
C/N 30 (Thermophilic) vs C/N 20 (Mesophilic)	20,940	1,262	3,957	0,890	No	-44,726	86,605
C/N 30 (Thermophilic) vs C/N 35 (Thermophilic)	17,172	1,035	3,957	0,955	No	-48,493	82,837
C/N 30 (Thermophilic) vs C/N 30 (Mesophilic)	8,133	0,490	3,957	0,999	No	-57,533	73,798
C/N 30 (Mesophilic) vs C/N 35 (Mesophilic)	71,873	4,331	3,957	0,031	Yes	6,207	137,538
C/N 30 (Mesophilic) vs C/N 20 (Thermophilic)	31,000	1,868	3,957	0,601	No	-34,665	96,666
C/N 30 (Mesophilic) vs C/N 20 (Mesophilic)	12,807	0,772	3,957	0,990	No	-52,858	78,472

C/N 30 (Mesophilic) vs C/N 35 (Thermophilic)	9,039	0,545	3,957	0,999	No	-56,626	74,704
C/N 35 (Thermophilic) vs C/N 35 (Mesophilic)	62,834	3,786	3,957	0,062	No	-2,832	128,499
C/N 35 (Thermophilic) vs C/N 20 (Thermophilic)	21,961	1,323	3,957	0,867	No	-43,704	87,626
C/N 35 (Thermophilic) vs C/N 20 (Mesophilic)	3,768	0,227	3,957	1,000	No	-61,897	69,433
C/N 20 (Mesophilic) vs C/N 35 (Mesophilic)	59,066	3,559	3,957	0,083	No	-6,600	124,731
C/N 20 (Mesophilic) vs C/N 20 (Thermophilic)	18,193	1,096	3,957	0,941	No	-47,472	83,859
C/N 20 (Thermophilic) vs C/N 35 (Mesophilic)	40,873	2,463	3,957	0,325	No	-24,793	106,538

APPENDIX F.6. Tested hypotheses for ANOVA

Effect	Null hypotheses (H_0)	Alternative hypotheses (H_1)
Main Effect of C/N Ratio	There is no significant difference in biogas yield between the different C/N ratios.	At least one C/N ratio level results in a significantly different biogas yield.
Main Effect of Temperature	There is no significant difference in biogas yield between mesophilic and thermophilic temperatures.	The mean biogas yield differs significantly between the two temperature levels.
Interaction Effect (C/N Ratio × Temperature)	There is no interaction between C/N ratio and temperature on biogas yield.	There is a significant interaction between C/N ratio and temperature, such that the effect of one factor depends on the level of the other.

APPENDIX G: Some Lab Manipulation Photos for BMP Tests Set Up