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Enhancing Methane Production from Lignocellulosic Waste through Optimized Operational Strategies in Anaerobic Co-Digestion: Cotton Stalks as a Case Study

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ABSTRACT

Cotton stalks (CS) are a lignocellulosic biomass that pose challenges for anaerobic digestion (AD) due to their high lignin content, which leads to long digestion periods, inhibits hydrolysis, limits biodegradability, and produces low methane yields. One successful method to overcome these limitations is co-digestion, which involves mixing cotton stalks with different substrates. For this reason, a thermostatic stainless-steel water bath and a ten-batch system of biodigesters were designed, established, and implemented at the Biochemistry Laboratory, Water and Soil Science Department, Faculty of Technology and Development, Zagazig University (Egypt). Azolla (AZ) and cow manure (CM) were used for different mixing ratios with cotton stalks to evaluate how different substrate mixtures influence biogas production and its methane content. Ten experimental mixtures were conducted: mixture1 (100% CS), mixture2 (100% CM), mixture3 (100% AZ), mixture4 (75% CS + 25% CM), mixture5 (50% CS + 50% CM), mixture 6 (25% CS + 75% CM), mixture7 (75% CS + 25% AZ), mixture8 (50% CS + 50 % AZ), mixture 9 (25% CS + 75% AZ) and mixture10 (CS:CM:AZ = 1:1:1). Experiments were performed under controlled conditions with daily biogas measurements and methane concentration, calorific value estimation, and chemical analysis of influent and effluent throughout digestion. Notably, mixture 10 yielded superior biogas production compared to mixtures 1 through 9, with increases of 56.13%, 35.92%, 43.45%, 32.21%, 21.65%, 21.12%, 13.54%, and 7.91%, respectively, except for mixture 3, which exceeded it by 13.85%. Among all mixtures, mixture 3 showed the highest methane concentration, while others ranged between 37.85% and 71.49%. The statistical analysis of both cumulative biogas and methane production showed a highly significant effect at $p < 0.001$. The R^2 reached 1.0 for cumulative biogas production and 96.9% for methane production, indicating clear and well defined differences among the treatments

Keywords: *Cotton stalks, Azolla, Cow manure, Biogas production..*

1. INTRODUCTION

Yearly, the world generates increasing amounts of waste, resulting in serious environmental and economic consequences. Among these wastes, biomass particularly underutilized lignocellulosic materials represent a valuable yet frequently discarded resource. Instead of allowing it to decompose naturally, harnessing its potential as a renewable energy source could help mitigate waste accumulation while contributing to sustainable energy solutions (Ahmed et al., 2020). Harnessing plentiful, eco-friendly biomass presents a practical pathway toward realizing carbon neutrality objectives, as it meets current demands for energy conservation, environmental sustainability, and a low-carbon economy (Wang et al., 2025). Bioenergy plays a crucial role in enhancing both food and energy security (Goodman, 2020). Many organic wastes, such as cotton stalks and rice straw, are often burned or discarded, causing environmental and economic harm. However, these substances may be useful bioenergy sources. Biogas can be efficiently produced through anaerobic digestion (AD) of agricultural residues like rice straw, sugarcane bagasse, and leftover fruits and vegetables. Effective anaerobic digestion (AD) hinges on key parameters—including the feedstock's chemical profile, degradability, and geographic accessibility—which collectively support its role as a sustainable approach to both waste valorization and clean energy generation. Biogas, an efficient and environmentally friendly technology, is a mixture of gases produced by the anaerobic decomposition of organic matter. It consists primarily of 50-70% methane (CH_4) by volume, 30-50% carbon dioxide (CO_2), and trace amounts of other gases such as hydrogen sulfide (H_2S) and water vapour (H_2O). The concentrations of CO_2, H_2S, and water vapour in biogas can affect the performance and lifespan of energy conversion systems, so removing these components before use is essential for improving biogas quality. Anaerobic fermentation is a type of biochemical pathway that transforms organic materials into biogas and nutrient-rich byproducts through a series of metabolic stages. The anaerobic digestion process comprises four fundamental stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, each driven by distinct.

microbial communities and metabolic activities. Hydrolysis marks the initial phase of anaerobic digestion and is widely regarded as the slowest due to the complex conversion of macromolecular substrates into simpler monomeric forms. This phase is

instrumental in influencing both the biodegradation rate and the operational effectiveness of the system (Zhang et al., 2014). Digestate, a by-product of anaerobic digestion, is rich in essential nutrients like potassium, phosphorus, and nitrogen, making it an effective natural fertilizer (biogas fertilizer). Using biogas fertilizer in agriculture improves soil fertility, increased crop yields, and reduced reliance on synthetic fertilizers, supporting sustainable farming (Doyeni et al., 2021). Lignocellulosic biomass, rich in cellulose, hemicellulose, and lignin, is a renewable resource but presents challenges in anaerobic digestion (AD). Its structural complexity forms a resistant crystalline matrix, restricting substrate biodegradation and diminishing biogas yield, particularly methane content (Sawatdeenarunat et al., 2015 and Xu et al., 2019). Elevated carbon relative to nitrogen content (C/N ratio) causes nutritional imbalances, restricting microbial activity (Eduok et al., 2018). Limited surface area reduces enzyme access, affecting degradation efficiency (Xu et al., 2019). Costly and energy-intensive pre-treatments are required to overcome biomass recalcitrance. However, improper processing can disrupt the cellulose matrix or generate inhibitors, reducing digestion efficiency (Xu et al., 2019 and Ahmed et al., 2019). Regarding the use of azolla for biogas production, azolla pinnata is an aquatic fern rich in nitrogen and proteins that has shown promise as a feedstock for anaerobic digestion and biogas production. Anaerobic digestion of Azolla biomass has been shown in recent research to greatly increase methane yield and lower chemical oxygen demand (COD), with kinetic modelling offering insights into ideal operating conditions (Kumar et al., 2020). Additionally, it has been demonstrated that co-digesting Azolla with activated sludge or food waste increases biogas generation and process efficiency, especially when hydraulic retention times and organic loading rates are adjusted (Mir et al., 2019). These findings highlight Azolla as a sustainable biological resource that can be integrated into waste management systems to simultaneously generate clean energy and improve the quality of digestate as organic fertilizer. The azolla is widely used as an organic fertilizer for rice and other crops due to its ability to fix atmospheric nitrogen, due to the presence of a symbiotic cyanobacterium, Anabaena azollae. It is also used for other purposes, such as food production, animal feed, biogas production, and heavy metal absorption. These diverse applications of the azolla-anabaena system make it an ideal and environmentally friendly option for sustainable agriculture (Santhiya and Jeeva, 2022). The carbon-to-nitrogen (C/N) ratio, temperature, and pH are some of the physicochemical variables that affect anaerobic digestion systems' operational efficiency and process stability(Mao et al., 2015). Temperature plays a critical role in microbial activity, with AD typically proceeds under either mesophilic conditions, ranging from 30 to 40 °C, or thermophilic regimes, spanning 50 to 60 °C Shi et al., (2018). Although thermophilic anaerobic digestion offers higher reaction rates, it often suffers from reduced process stability , and the accumulation of inhibitory substances, notably ammonia and volatile fatty acids, can significantly hinder biogas production efficiency and escalate the energy demands of the system (Liu et al., 2018). Conversely, low temperatures slow down microbial activity, negatively affecting gas generation and overall process efficiency (Rodríguez-Jiménez et al., 2022). As noted by Eduok et al. (2018) and Li et al. (2021), co-digestion offers operational advantages by optimizing nutrient balance, boosting biogas production, and reducing the need for intensive pre-treatment processes. Furthermore, mixing lignocellulosic biomass with low C/N substrates (food waste) improves microbial activity, stabilizes pH, and dilutes inhibitors, leading to higher methane yields and reactor stability Kriswantoro et al., (2024). It has been reported that cow manure co-digests a variety of lignocellulosic materials, including rice straw (Li et al., 2015), wheat straw (Wang et al., 2012), maize straw (Zou et al., 2016), cotton stalk (Wang et al., 2021), soybean straw, and sunflower stalks (Kovačić et al., 2018) to increase methane production and secure the fermentation reactor's steady operation.

According to Luo et al. (2021) and Muhayodin et al. (2021), grinding lignocellulosic biomass is crucial for increasing the area contact of the materials and promoting more effective degradation of lignocellulosic components like cellulose and hemicellulose, which are otherwise difficult to break down due to their rigid structure. This is the initial stage of anaerobic digestion (AD), which is crucial for enhancing methane production. Grinding helps partially destabilise the structure of lignin, making cellulose and hemicellulose more accessible for AD enzymatic action, according to Xin et al. (2018) and Mothe and Polisetty (2021). Digestate, a by-product of anaerobic digestion, is high in vital elements including potassium, phosphorus, and nitrogen. It can be used as a natural fertilizer (biogas). By increasing crop yield, boosting soil fertility, and lowering reliance on synthetic fertilizers, the use of biogas fertiliser in agriculture supports sustainable farming methods (Doyeni et al., 2021).

This study investigates the enhancement of anaerobic digestion for lignocellulosic biomass, particularly cotton stalks. Addressing this problem is challenging due to its high lignin content, which causes a long hydraulic retention period, hinders hydrolysis, and limits biodegradability, resulting in small amounts of methane. Therefore, the research aims to convert this waste into energy and produce a beneficial biofertilizer for the soil.

Previous studies have not yet mentioned using azolla ferns with cotton stalks to produce biogas. Therefore, this current research aims to highlight the use of azolla as a non-conventional resource for biogas production mixed with cotton stalks, compared to the conventional cow manure method, to improve nutrient balance, stabilize the process, and boost methane yield.

Therefore, the following points explain the current work's objectives:

Identify the best combination of the utilized substrates (cotton stalks, azolla, and cow manure) that produces the highest methane production.

Evaluate the digestive performance of traditional cow manure with that of azolla, a non-traditional source.

Select the right nutrient-rich fermentation mixture to apply as a soil biofertilizer.

2. Materials and Methods

The experiments were conducted on the lab-scale digesters during the winter season at the Department of Water and Soil Science, Faculty of Technology and Development, Zagazig University, Egypt, to determine the optimal conditions for increasing methane production from cotton stalks mixed with cow manure and azolla ferns.

2.1. Materials

2.1.1. Anaerobic unit

The biogas production system was constructed from stainless steel in a rectangular configuration, measuring 1400 × 770 × 500 mm with a wall thickness of 1.5 mm. It was specifically designed to accommodate ten laboratory-scale reactors, as illustrated in Fig. 1. Inside the unit, a perforated stainless steel mesh (1380 × 750 mm) was installed 80 mm above the bottom to enhance water circulation, thereby ensuring favorable conditions for fermentation. The structure also includes designated openings for digester placement. To sustain mesophilic conditions, two electric heaters (3 kW, 220 V) were employed, maintaining the temperature at 40 ± 3 °C, following the approach outlined by Yan et al. (2023). Each heater element was fitted with a thermostat to ensure precise temperature regulation. To maintain uniform thermal conditions around the digesters, a water pump was employed, operating at 90 W with a maximum flow capacity of 2500 l/h (model LBP-D2500, 220 V/50 Hz). The water surface was insulated using white corkboard sheets, which helped stabilize the fermentation temperature and reduce evaporation. The entire unit was thermally insulated to minimize heat loss. It was designed with both inlet and outlet connections, allowing integration with a water tank for supply, while the discharged water was recirculated within a closed-loop system. Flow control was achieved through the installation of valves.

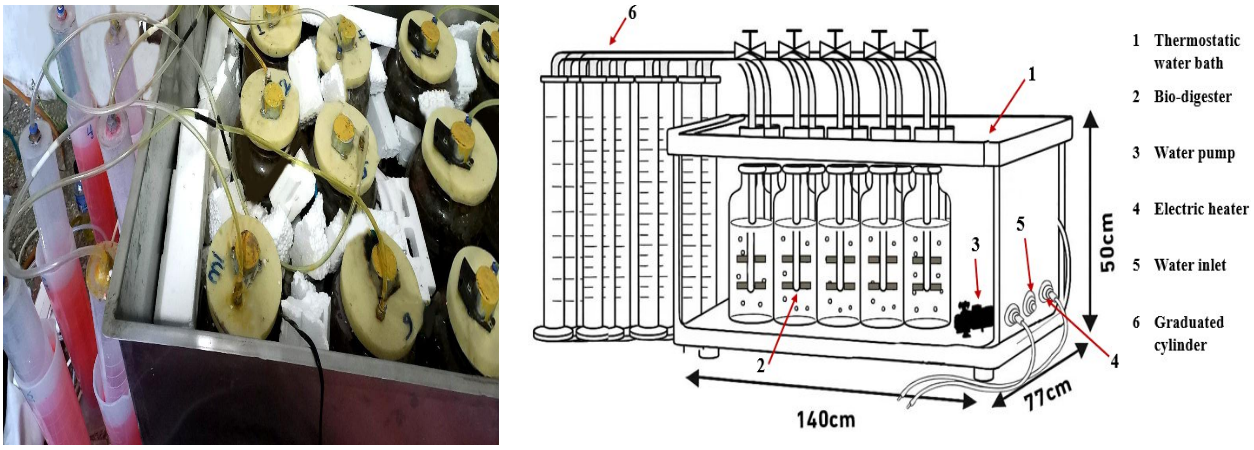


Fig. 1. Anaerobic unit

2.1.1.1. Digester

As illustrated in Fig. 2, the digester was designed using a glass vessel with dimensions of 250 × 195 mm, a wall thickness of 3 mm, and a total capacity of (5L volume). A stainless-steel stirrer shaft, 200 mm in length, was installed inside the jar and fitted with two blades measuring 20 mm in width and 100 mm in length. Mixing was driven by a 4.5 W electric motor to ensure proper blending of the contents. Mechanical agitation was applied for 15 minutes at four-hour intervals, operating at a speed of 35 ± 1 rpm, thereby maintaining uniform substrate distribution and stable digestion temperature. To prevent gas leakage, the glass jar was sealed securely with a Teflon cover. The biogas outlet was linked to a gas collection chamber, and the produced gas volume was quantified using the water displacement technique. In this method, the pressure generated within the digester forced an equivalent amount of water into a calibrated graduated cylinder, following the procedure described by Adelekan and Bamgboye (2009). A control valve was attached to the outlet to regulate gas flow. For measurement, a 500 ml polypropylene cylinder was inverted inside a 1000 ml polypropylene cylinder filled with water and connected to the gas outlet. Maintaining appropriate temperature is essential, as it directly influences microbial activity

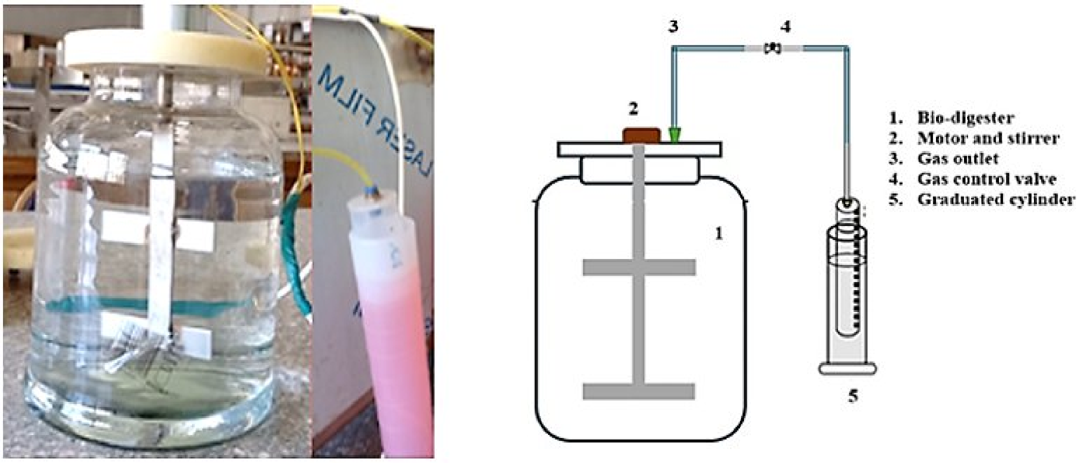


Fig. 2. Digester unit

2.1.2. Substrates

The following raw materials (Fig. 3) were used to prepare the fermentation mixtures.

Cotton Stalks (CS)

The cotton stalks were obtained from a farm located in one of the villages of San El Hagar (30.97694° N, 31.88000° E), El Husseiniya Center, Al Sharqia Governorate, Egypt. The cotton stalk was chopped into small pieces (3mm length), which is compatible with Luo et al. (2021). Cotton stalk chopping is an essential pretreatment step that improves substrate accessibility, enhances bigas production, and improves anaerobic digestion (Dai et al., 2019).

Cow Manure (CM)

Fresh cow manure was gathered from Banayous farm (30.5983° N, 31.4999° E), Zagazig, Al Sharqia Governorate, Egypt.

2.1.2.3. Azolla (AZ)

Fresh azolla (Salviniaceae M.) was obtained from Bani Amer (30.59094°N, 31.56597°E), Zagazig, Al Sharqia Governorate, Egypt.

Cotton stalks **b. Azolla** **c. Cow manure**

Fig.3. Raw materials used for anaerobic co-digestion.

This research's objective was to assess the influence of various organic substrate mixtures on overall biogas yield and methane concentration. Ten treatments were formulated using cotton stalks, cow manure, and Azolla in various combinations, as shown in table (1). A Completely Randomized Block Design (CRBD) was employed to structure the experimental trials, incorporating three replicates per treatment to confirm statistical reliability and account for potential variability. This design helps control experimental error and provides reliable comparisons among the treatments under the same conditions.

Table 1. The various cotton stalks digestion mixtures included

Mixture No.	Contents
1	100% Cotton stalks
2	100% Cow manure
3	100% Azolla
4	75% Cotton stalks + 25% Cow manure
5	50 % Cotton stalks + 50 % Cow manure
6	25% Cotton stalks + 75% Cow manure
7	75% Cotton stalks + 25% Azolla
8	50% Cotton stalks + 50 % Azolla
9	25% Cotton stalks + 75% Azolla
10	1 Cotton stalks: 1 Cow manure: 1 Azolla

2.2. Methods

The experiments were carried out under controlled mesophilic conditions to ensure stable digestion, enhance microbial diversity, and maximize biogas production (Yan et al., 2023). Each of the experiments had a pH range of 6.9-7.3. The pH was measured using a digital pH meter, model 915, category 13-636-916.2, with an accuracy of ±0.01 pH units. Cotton stalks, cow manure, and azolla were utilized both individually and in combination as substrates. Each material or mixture was thoroughly homogenized before loading into the digesters. Once the digesters were filled to a working volume of 4 L, they were securely sealed, and all connections were verified. Subsequently, the units were positioned within the water bath of the anaerobic system.

To formulate the slurry medium, water was incorporated into the pretreated raw materials (v/w) to adjust the total solids content to 8% before loading into the digesters. The amount of water required was determined following the procedure outlined by Elfar (2022).

$$Y = \frac{X\,(Ts_{man} - Ts_{dig})}{Ts_{dig}}$$

where: Y: Amount of required dilution water (L), X: Amount of raw material added (kg), TS_{rm}: Total solids of raw materials (%) and TS_{dig}: Total solids content of fermentation materials (%).

The total solids (TS, %) were determined by drying in an oven at 105 °C for 24 hours (APHA,2023) and calculated using the following equation:

$$TS = \frac{W_D}{W_W} \times 100$$

where: W_D: The sample's weight after drying (g) and W_W: The sample's weight before drying (g).

The C/N ratio was computed by dividing the total amount of organic carbon by the total amount of nitrogen, following the methodology,as cited from Abdellatif et al., (2021).

$$C/N = \frac{Q_1(C_1 \times (100 - M_1) + Q_2(C_2 \times (100 - M_2) + \dots}{Q_1(N_1 \times (100 - M_1) + Q_2(N_2 \times (100 - M_2) + \dots}$$

Where Q_1 and Q_2= Quantity of material to be added (Kg), C_1 and C_2 = The organic carbon content in each substrate (%), N_1 and N_2 = The nitrogen content in each substrate (%) and M_1 and M_2 = The moisture content of material (%).

The chemical analysis of the digestion mixtures was conducted as presented in Table 2.

Table 2. Chemical analysis of the various digestion mixtures (Influent slurry).

Mixture No.	Contents					
	VS, %	Ash, %	pH	T.C, %	T.N, %	C/N
1	72.04	27.96	7.0	44.8	1.1	40.72
2	72.66	27.34	7.3	47.30	2.05	23.07
3	69.90	30.10	6.9	45.92	2.39	19.21
4	72.22	27.78	7.0	45.42	1.33	33.96
5	72.37	27.63	7.1	46.05	1.57	29.23
6	72.51	27.49	7.2	46.67	1.81	25.75
7	71.53	28.47	7.0	45.08	1.42	31.69
8	71.00	29.00	7.0	45.36	1.74	25.99
9	70.45	29.55	6.9	45.64	1.90	24.02
10	71.38	28.62	7.0	45.89	1.84	24.91

2.2.2. Measurements and determinations

To assess the efficiency of the digestion process under the applied treatments, the following parameters were considered:

Daily and cumulative biogas production

The daily biogas production (l/day) was determined using the water displacement technique. For each treatment, cumulative biogas production was calculated to identify the optimal conditions for maximum yield across the hydraulic retention time (HRT). Subsequently, the recorded volumes were standardized to 1013 mbar and 0 °C, applying the correction procedure outlined by Abdellatif et al. (2020).

$$V_{tr} = \frac{V_f\,[273.15(P_1 - P_2 - P_3)]}{[273.15 + T]\,1013}\ \mathrm{m}^3$$

Where V_{tr} = Volume of dry gas under standard circumstances (milliliters), V_f = Volume of wet gas in millilitres at temperature T and pressure P, T = Temperature of wet gas (°C), P_1 = Pressure of air at temperature T (millibar), P_2 = Pressure of wet gas at temperature T (millibar), P_3 = The water's saturation steam pressure at temperature T, (millibar) and 1013 = absolute pressure in (millibar).

Methane percentage

The methane content (CH_4, %) of the produced biogas was measured using a GA 5000 portable gas analyzer, Geotechnical Instruments, UK.

Calorific value of biogas

At standard temperature and pressure (STP), calorific value and density of biogas were 50 MJ/kg (equivalent to 36 MJ/m^3) and 0.72 kg/m^3, respectively. The calorific value (Hu) under standard conditions was determined using the following equation presented by Abdellatif et al. (2020):

$$Hu = 36\ CG \times CH_4$$

where: 36: The calorific value of methane at standard conditions (MJ/m^3), CG: Cumulative biogas production under standard circumstances (m^3) and CH_4: The methane percentage (%).

Biogas production rate

The biogas production rate was calculated using the following equation to quantify the volumetric production over time as reported by El-Hadidi et al., (2016):

BPR= Bp / Dv (m^3 /m^3 /day)

Where BPR = Biogas production rate, (m^3 gas/m^3 digester/day), Bp = Biogas production (m^3 /day) and Dv = Effective digester volume (m^3).

Total nitrogen

Total nitrogen (TN, %) was determined for each treatment using the standard method using Kjeldahl (AOAC, 2023).

Volatile solids

Volatile solids (VS, %) were estimated by burning the dry raw material samples for 2 hours at 600°C in a digital muffle furnace according to the standard process described by described by APHA (2023), it was computed by the following equation:

$$VS = 100 - ash\ \%$$

Statistical analysis

All treatments were replicated three times and distributed according to a randomized complete block design (RCBD). The effects of the different treatments were statistically evaluated using the statistical analysis program (SPSS).

3. Results

Under the laboratory experimental conditions (digestion temperature 40 ±3 ºC, total solid 8%, stirring speed 35 ± 1 rpm, The system was subjected to intermittent stirring, with a duration of 15 minutes every four hours, while maintaining a hydraulic retention time of 60 days, the results obtained were shown as follows:

3.1. Effect of different mixtures of (cotton stalks and cow manure) and (cotton stalks and azolla) on daily and cumulative biogas production

Table (3) shows that treatments had a highly significant effect on the cumulative variable ($p < 0.001$), as indicated by ANOVA results, and the model explained almost all the variability ($R^2 = 1.0$). Clear differences between treatments were confirmed by the extraordinarily high F-value that resulted from the very large treatment Mean Square and minimal error term. The low error variance indicates strong consistency among replicates, and the observed power (1.0) shows excellent sensitivity in identifying the treatment. Results in Fig. (4), indicated that small quantities of biogas were released from the second and seventh days of fermentation time for the mixture 2 (100% cow manure) and mixture 1 (100% cotton stalks), respectively.

Table 3. The statistical analysis of factors affecting cumulative biogas production.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Noncent. Parameter	Observed Powerb
Corrected Model	272925850.889a	9	30325094.543	151625472.716	.000	1364629254.445	1.000
Intercept	4066237280.514	1	4066237280.514	20331186402.570	.000	20331186402.570	1.000
treatments	272925850.889	9	30325094.543	151625472.716	.000	1364629254.445	1.000
Error	4.000	20	.200				
Total	4339163135.403	30					
Corrected Total	272925854.889	29					
a. R Squared = 1.000 (Adjusted R Squared = 1.000)							
b. Computed using alpha = .05							

Biogas also started to release between the fourth and fifth days for the mixture 4 (75% cotton stalks +25% cow manure), mixture 5 (50% cotton stalks + 50% cow manure), mixture 6 (25% cotton stalks + 75% cow manure). The delay in biogas production may be due to the sluggish microbial activity at the start of fermentation and because of nitrogen limitation, mixtures with very high C/N ratios (such as 40.72) for mixture 1 may degrade more slowly. Under standard conditions, the maximum daily biogas yields for mixes 1, 2, 4, 5 and 6 were 362.57, 622.24, 419.97, 483.43 and 538.09 milliliter per day (ml/day) on the forty-first, fifteenth, thirty-fifth, twenty-ninth, and twenty-third days, in sequential order.

Following the peak, biogas production started to gradually decline during the remaining days of the retention time for all treatments. Furthermore,the cumulative biogas production was 6512.82, 9514.19, 8395.10, 10064.92 and 11632.43 milliliter (ml) for mixes 1, 2, 4, 5 and 6 reflecting a progressively increasing biogas production over time across the various mixtures. These findings indicate that the biogas production from mixture 6 exceeded those of mixtures 1, 2, 4, and 5 by 44.01%, 18.20%, 27.83%, and 13.47%, respectively, underscoring the superior efficiency of mixture 6 in biogas generation. The results illustrated in Figure (5) show that the biogas was started to release from the second and seventh days of fermentation time for mixture 3 (100% azolla) and mixture 1(100% cotton stalks) it was clear that the cumulative biogas production was 6512.82, 17236.50, 11710.16, 12836.17, 13671.93 and 14847.51 milliliter (ml) for mixtures 1,3, 7, 8, 9 and 10 in sequential order. The greatest daily biogas produced at the standard conditions was 362.57, 884.92, 600.43, 673.73, 646.21 and 696.01 ml/day for mixtures 1, 3, 7, 8, 9, and 10 On the forty-first, seventeenth, twenty-ninth, twenty-fifth, twenty-first, and nineteenth days of fermentation, in sequential order. On the other side, The cumulative biogas output corresponding to mixtures 1, 3, 7, 8, 9, and 10 measured 6512.82 ml, 17236.50 ml, 11710.16 ml, 12836.17 ml, 13671.93 ml, and 14847.51 ml in sequential orderAccordingly, the biogas yield from mixture 10 exceeded those of mixtures 1, 7, 8, and 9 by 56.13%, 21.12%, 13.54%, and 7.91% respectively, highlighting its comparatively enhanced performance and the biogas yield from mixture 3 surpassed that of mixtures 1, 7, 8, 9, and 10 by 62.21%, 32.05%, 25.52%, 20.67%, and 13.85% in sequential order, underscoring its relatively superior performance across most comparisons. The observed variation in biogas production could be linked to constraints in substrate concentration, which may have influenced microbial activity and, consequently, the overall yield. It was found that the amount of hydrogen sulphide (H_2S) in mixture 3 was 3468 parts per million (ppm) at the beginning of the biogas's appearance, then 2530 ppm in the second measuring of the produced biogas and gradually decreased with each measurement of the proportions of the gas components until it reached 73ppm at the end of the experiment. This could be attributed to rapid decomposition of organic matter due to low carbon-to-nitrogen (C/N) ratio.

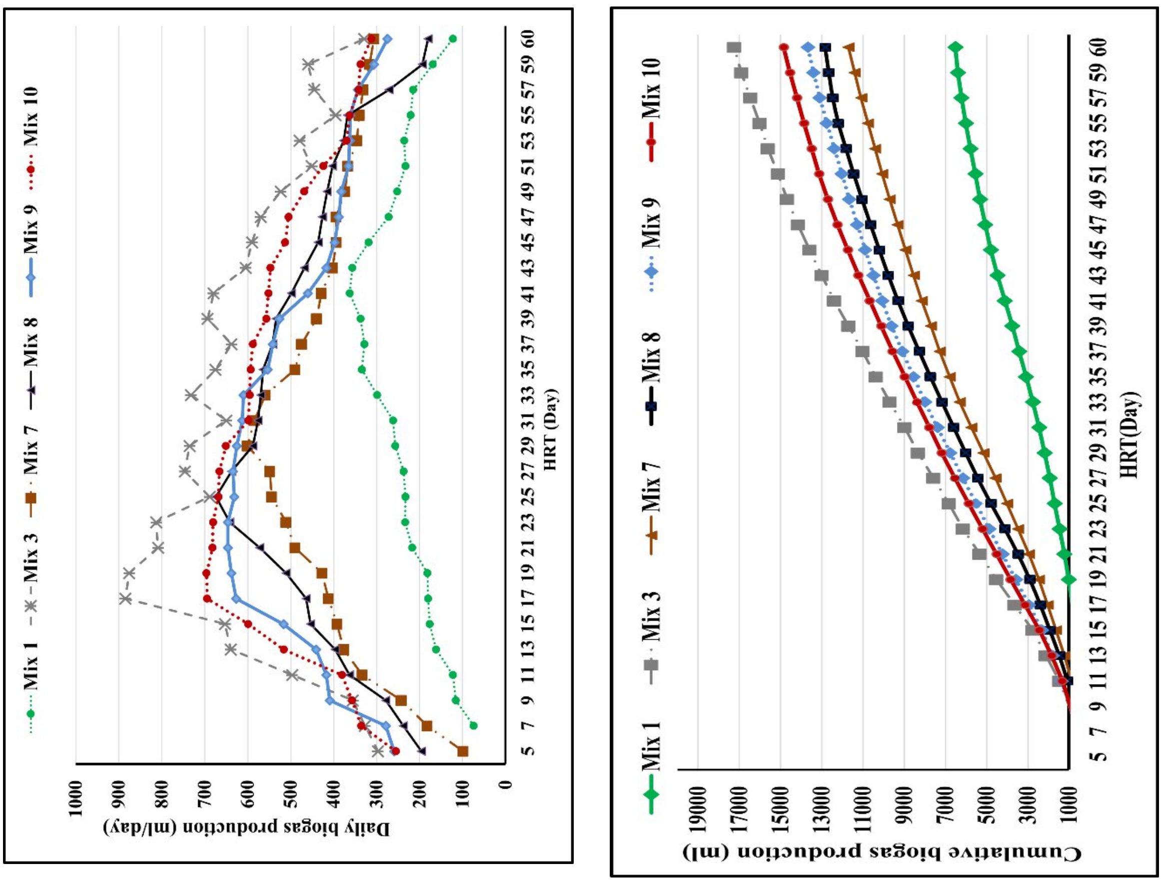


Fig. (5): Effect of different experimental mixtures of cotton stalks and azolla on daily and cumulative biogas production.

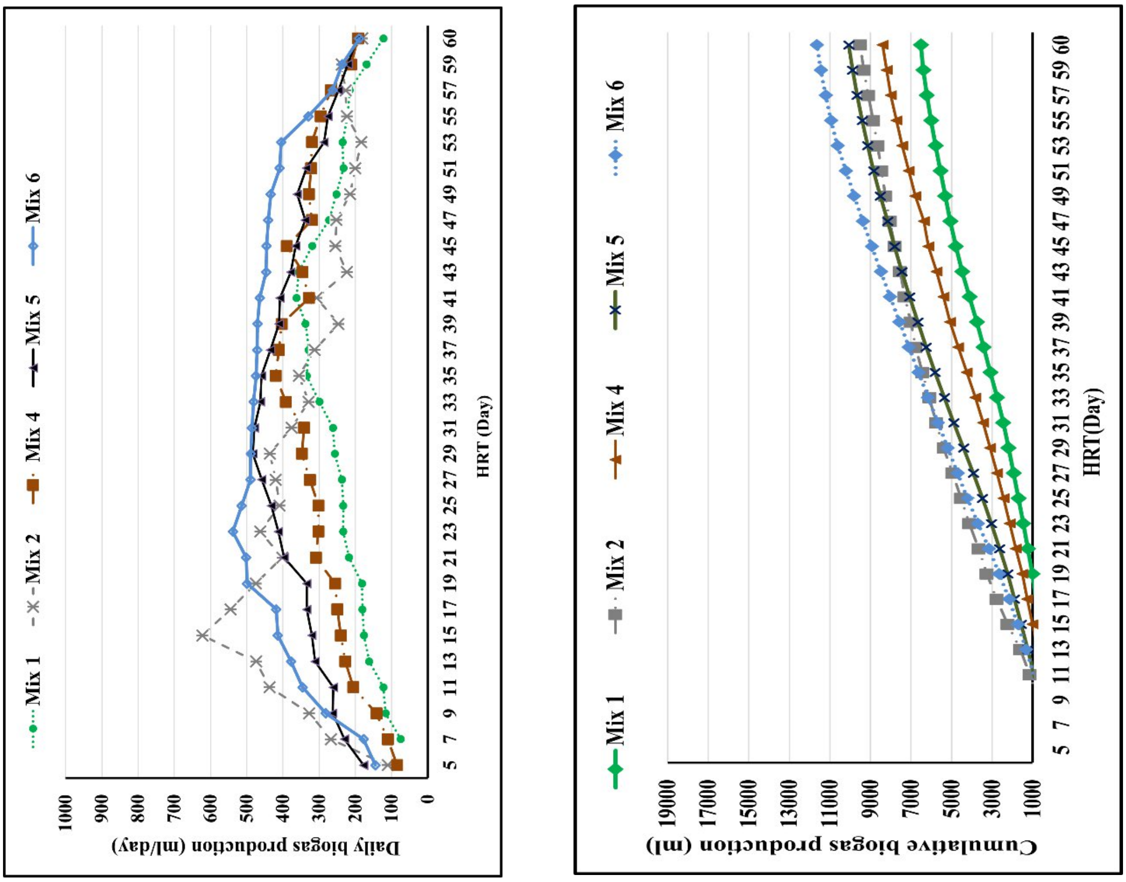


Fig. (4): Effect of different experimental mixtures of cotton stalks and cow manure on daily and cumulative biogas production

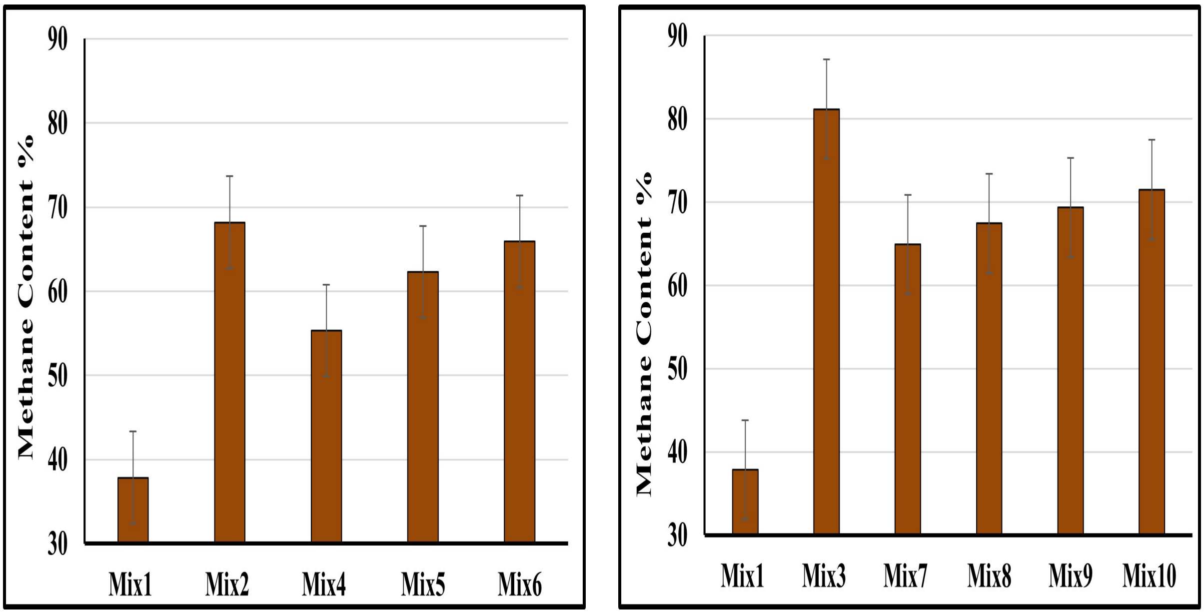
3.2. Effect of various mixtures on methane percentage

Table 4 demonstrated that Real differences between treatments are confirmed by the ANOVA results, which demonstrate a highly significant effect of treatments on methane ($p < 0.001$). A strong fit is indicated by the model's explanation of 96.9% of the variability ($R^2 = 0.969$). These findings highlight the crucial role of selecting and optimizing feedstock mixtures to enhance biogas quality, particularly in terms of methane content.

Table 4. The statistical analysis of factors affecting methane production.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Noncent. Parameter	Observed Powerb
Corrected Model	3521.845a	9	391.316	68.652	.000	617.867	1.000
Intercept	124397.617	1	124397.617	21824.143	.000	21824.143	1.000
treatments	3521.845	9	391.316	68.652	.000	617.867	1.000
Error	114.000	20	5.700				
Total	128033.462	30					
Corrected Total	3635.845	29					
a. R Squared = .969 (Adjusted R Squared = .955)							
b. Computed using alpha = .05							

The results illustrated in Fig. (6) present the average of methane content for different mixtures of cotton stalks and cow manure. They were 37.85, 68.18, 55.33, 62.30 and 65.91% for mixtures 1, 2, 4, 5, and 6 in the same order as the mixtures listed. while the average methane contents for different mixtures of cotton stalks and azolla were 37.85, 81.18, 64.19, 67.45, 69.34 and 71.49 for mixtures 1, 3, 7, 8, 9 and 10 respectively. According to the raw data, during the first week of fermentation, only CO_2 was created as opposed to CH_4. In the second week, anaerobic conditions took over, and combustible gas generation started when the digester's O_2 was depleted.The gradual increase in methane concentration observed during the co-digestion process reflects a significant enhancement in the overall quality of the produced biogas. This observation aligns with the findings of Qian et al. (2025), who demonstrated that co-digestion approaches can substantially boost methane output by improving the synergistic interactions between different substrates. In this context, the addition of azolla—recognized for its high nitrogen content—likely contributed to optimizing the carbon-to-nitrogen (C/N) ratio, thereby creating more favorable conditions for microbial activity and enhancing methanogenic performance. This interpretation is further supported by Ebrahim et al. (2024), who emphasized azolla's potential in recycling agricultural residues and increasing nitrogen availability, reinforcing its value as a co-substrate in anaerobic digestion systems.

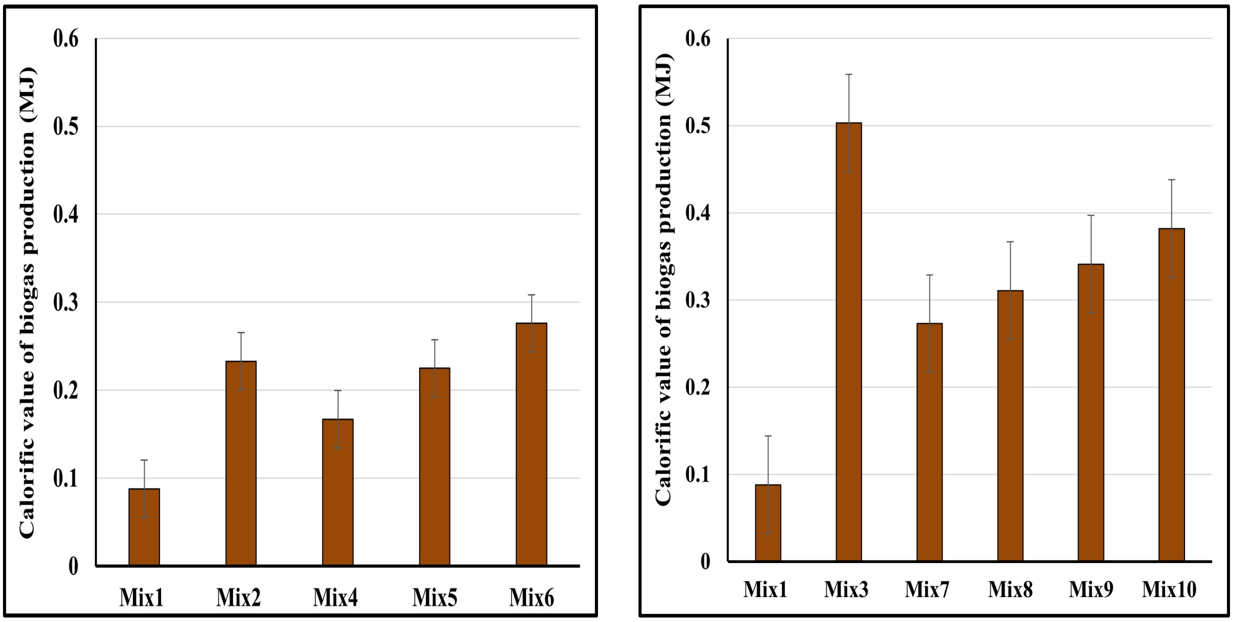


Mixing cotton stalks with cow manure **Mixing cotton stalks with azolla**

Fig. 6: Effect of different experimental mixtures on percentage of methane in biogas production.

3.3. Assessment of the calorific value of produced biogas (MJ)

Fig. (7) shows the calorific value of the biogas produced in each Trial under the examined experimental conditions. According to the results obtained, for mixes 1, 2, 4, 5, and 6, the resulting biogas had calorific values of 0.088, 0.233, 0.167, 0.225, and 0.276 MJ, in sequential order. Additionally, the findings showed that the calorific values of biogas produced from mixes 3, 7, 8, 9, and 10 were 0.503 MJ, 0.273 MJ, 0.311 MJ, 0.341 MJ, and 0.382 MJ, in sequential order. The calorific value of biogas produced from mixture 3 exceeded those of mixtures 1, 2, 4, 5, 6, 7, 8, 9, and 10 by 82.50%, 53.66%, 66.79%, 55.26%, 45.12%, 45.72%, 38.17%, 32.20%, and 24.05%, respectively. Similarly, biogas from mixture 10 demonstrated calorific values that were 76.96%, 39.00%, 56.28%, 41.09%, 27.74%, 28.53%, 18.58%, and 10.73% higher than those of mixtures 1, 2, 4, 5, 6, 7, 8, and 9, respectively. These variations in energy yield are likely attributed to differences in methane concentration among the produced biogas samples, as methane is the primary determinant of calorific value.

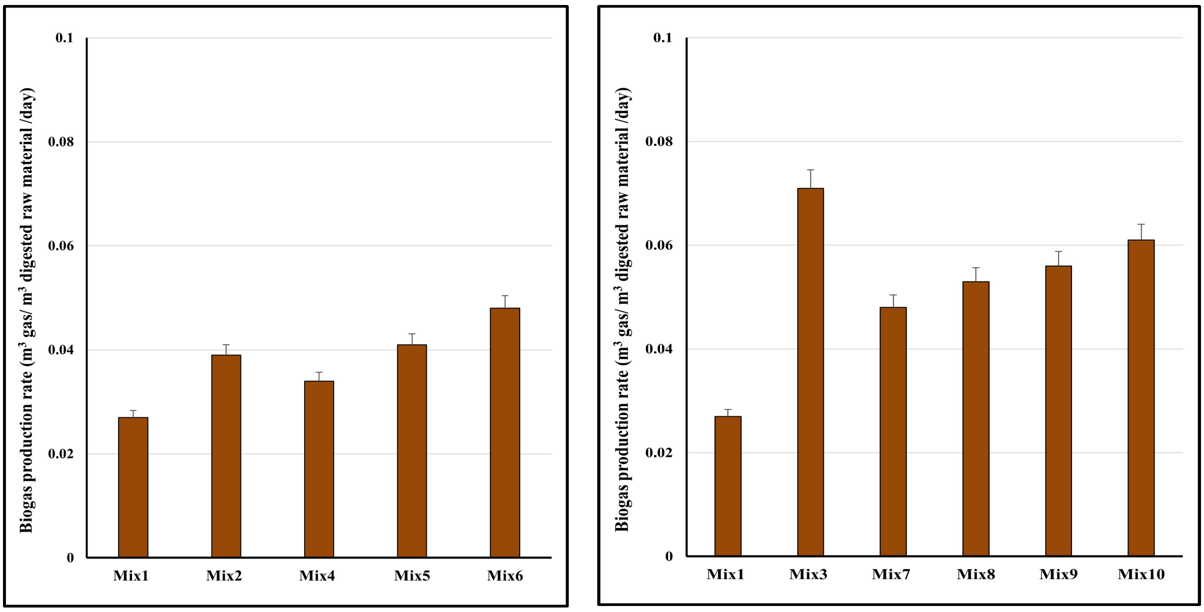


Mixing cotton stalks with cow manure **Mixing cotton stalks with Azolla**

Fig. 7: Effect of different experimental mixtures on the calorific value of the produced biogas.

3.4. Biogas production rate (m^3 gas/m^3 digested raw material/ day)

The data presented in Fig. (8) demonstrate distinct variations in biogas production rates across the tested mixtures. Mixtures 1, 2, 4, 5, and 6 yielded 0.027, 0.039, 0.034, 0.041, and 0.048 m^3 of gas per m^3 of digested raw material per day, respectively. In comparison, mixtures 3, 7, 8, 9, and 10 exhibited production rates of 0.071, 0.048, 0.053, 0.056, and 0.061 m^3/m^3/day, respectively.



Mixing cotton stalks with cow manure **Mixing cotton stalks with azolla**

Fig. 8: Effect of different experimental mixtures on biogas production rate.

3.5. Digestate chemical composition characteristics under different mixtures

The chemical composition of the effluent slurry obtained from different mixtures was assessed for several parameters, as presented in Table (5). These included measurements of Total Solids (TS), ash content,Volatile Solids (VS), Total Carbon (T.C.), pH, Nitrogen (N), Phosphorus (P), Potassium (K), and the Carbon-to-Nitrogen (C/N) ratio. The pH values, which range from 6.0 to 7.5, are neutral to slightly acidic and can be applied to soil. There was moderate variation in the total carbon content (42.60% – 44.90%), nitrogen levels ranged from 1.19% to 2.99%, potassium levels ranged from1.04% to 2.60% and phosphorus levels ranged from 0.05% to 1.98%. Therefore, the residual digestate derived from various mixtures demonstrated potential as a valuable organic fertilizer. As shown in Table 5, mixture 9 exhibited the highest concentrations of nitrogen (1.97%) and potassium (1.53%) among the cotton stalks-based formulations. Furthermore, mixture 10 exhibited the most favorable C/N ratios, which are considered optimal for effective organic matter mineralization. This suggests that the digestates from these mixtures are particularly advantageous for sandy soils, where they can improve nutrient availability and contribute to sustainable agricultural practices.The chemical assessment of the residual digestate reinforces the environmental benefits of the co-digestion process, confirming its suitability for agricultural use, especially in sandy soils with low buffering capacity. Additionally, the potassium and phosphorus concentrations remained within beneficial ranges, further supporting the digestate's role as a biofertilizer.

Table 5. Chemical composition of the different digestion mixtures (effluent slurry).

Treatement No.	Constituents								
	TS, %	VS, %	Ash, %	pH	T.C, %	T.N, %	P, %	k, %	C/N
1	6.20	70.80	29.20	6.0	43.30	1.19	0.05	1.04	36.38
2	4.55	68.12	31.88	6.8	44.89	2.25	1.98	2.60	19.95
3	3.42	65.60	34.40	7.5	42.60	2.99	1.88	1.70	14.24
4	5.95	71.70	28.30	6.7	44.89	1.52	1.01	1.42	29.53
5	5.00	71.00	29.00	6.8	44.55	1.63	1.15	1.81	27.33
6	4.84	70.89	29.11	6.8	44.00	1.87	1.39	1.38	23.52
7	4.43	69.35	30.65	6.4	44.90	1.66	0.96	1.20	27.04
8	4.25	68.52	31.48	7.3	43.80	1.90	1.11	1.37	23.05
9	4.19	68.00	32.00	7.4	44.40	1.97	1.42	1.53	22.53
10	4.10	67.40	32.60	7.5	44.20	1.99	1.34	1.77	22.21

5. Conclusions

The findings of this research demonstrate that mixing cotton stalks with azolla and cow manure under controlled anaerobic conditions markedly improves both biogas yield and methane concentration, while maintaining process stability. Mixtures 9 and 10 achieved the highest cumulative biogas production and methane yield. These outcomes highlight the positive influence of nutrient-rich co-substrates in stabilizing fermentation and enhancing energy recovery from lignocellulosic feedstocks. Chemical analysis of the residual digestate further focused on the environmental benefits of the anaerobic fermentation process, which has the possibility of being a suitable soil conditioner. Lastly, results support co-digestion as an effective strategy to overcome the limitations of cotton stalks digestion alone. Cotton stalks can be utilized as a non-traditional energy source by mixing cotton stalks with cow manure or azolla in suitable ratios. In conclusion, while the current study was carried out on a laboratory scale. The utilization of other high-lignocellulosic agricultural residues, which pose significant environmental challenges—is of particular interest due to their structural complexity, which is comparable to that of cotton stalks, and their wide availability in many agricultural regions. Upcoming studies may consider the incorporation of nano catalyst additives into the substrate mixtures to investigate their potential in enhancing anaerobic digestion efficiency. Exploring the role of nano-scale materials—such as metal oxides or carbon-based nanoparticles—could offer promising pathways to accelerate microbial activity, improve methane yield, and optimize biogas quality. Findings emphasize the importance of substrate selection in optimizing energy recovery and nutrient recycling, positioning co-digestion as a practical method for sustainable bioenergy production and waste management.

List of abbreviations:

CS: Cotton Stalks

AZ: Azolla

CM: Cow Manure

AD: Anaerobic Digestion

C/N: Carbon-to-Nitrogen ratio

CRBD: Completely Randomized Block Design

MJ: Megajoule

Ml: milliliter

ppm: parts per million

VS: Volatile Solid

T.C: Total Carbon

T. N: Total Nitrogen

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