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Optimal Ratio for Anaerobic Co-Digestion of Lignocellulosic Biomass using Cow Manure and Azolla Aquatic Ferns to Enhance Biogas Production.

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ABSTRACT

Rice straw (RS), a lignocellulosic biomass, poses challenges in anaerobic digestion (AD). Due to its high lignin content, which restricts biodegradability and methane production. Therefore, this research aims to improve and enhance RS digestion and biogas production by co-digesting RS with cow manure (CM) and azolla aquatic ferns (AZ). Four experimental sets were conducted. 1st trial of mono-substrate digestion [T1 (100% RS), T2 (100% CM), T3 (100% AZ)]; 2nd trial of RS-CM mixtures [T4 (75% RS + 25% CM), T5 (50% RS + 50% CM), T6 (25% RS + 75% CM)]; 3rd trial of RS-AZ mixtures [T7 (75% RS + 25% AZ), T8 (50% RS + 50 % AZ) and T9 (25% RS + 75% AZ)]; 4th trial is a ternary mixture[T10 (RS:CM:AZ = 1:1:1)]. The performance was evaluated based on daily and cumulative biogas production, methane percentage, calorific value, digestate chemical analysis, and statistical analysis. Notably, T10 yielded superior biogas production compared to treatments T1 through T9, with increases of 49.04%, 43.87%, 28.16%, 23.00%, 15.05%, 18.38%, 9.83%, and 6.28%, respectively, except for T3, which exceeded it by 1.64%. Among all treatments, T3 showed the highest methane concentration, while others ranged between 44.72% and 73.14%. The ANOVA results (R^2 = 1.000, F = 28,428,240.610, $p < 0.001$) confirmed that the digestion mixtures had a highly significant and reliable effect on biogas production (daily and cumulative), while the results for methane percentage (R^2 = 0.915, F = 23.894, $p < 0.001$) likewise indicated a highly significant and reliable effect.

Keywords: Azolla aquatic ferns, Rice straw, Biogas production, Chemical composition, Digestate.

1. Introduction

Climate change is one of the most critical environmental challenges currently facing the world, with developing countries being particularly vulnerable to its adverse impacts. Despite the fact that Egypt only accounts for roughly 0.57% of greenhouse gas emissions worldwide (Selim, 2009), it is expected to suffer significant consequences due to its climatic and geographic conditions. Burning crop residues, though a simple method for managing agricultural waste, releases harmful pollutants and greenhouse gases such as CO_2 and methane, contributing to air pollution and global warming. This practice poses significant environmental risks, highlighting the urgent need for sustainable waste management strategies to protect both ecosystems and human health (Kumar et al., 2025). Agricultural residues pose a major challenge to effective waste management (Malik et al., 2024). Crop residues can include stalks, straw, and plant trimmings. Some residues are left on the field to retain soil organic content and moisture, as well as prevent erosion. However, higher crop yields result in an increase in the amounts residues, andremoving a portion of these can be sustainable. Under Egyptian conditions, the total area harvested for the rice crop amounts to 642501 ha (FAO, 2023), with total emissions (CH_4) from burning crop residues estimated to be 0.9598 kilotons (FAO, 2022). Utilizing readily available biomass in an environmentally friendly manner is a

practical alternative for achieving carbon neutrality goals, as this solution addresses current needs related to energy supply, environmental protection, and the development of a low-carbon economy (Wang et al., 2025).

Anaerobic digestion of organic waste and residues combines both sustainable treatment and renewable energy production. Some substrates, such as lignocellulosic materials, are resistant to anaerobic digestion and can be converted into biogas, although only to a low extent. The low susceptibility of these materials to conversion into biogas is attributed to their composition and structure. Lignocellulose is the complex and rigid matrix of plant cells; it is resistant to enzymatic attack because of the tight association between lignin, cellulose, and hemicellulose. Cellulose and hemicellulose can be degraded in biogas processes. However, lignin cannot be degraded under anaerobic conditions (Fernandes et al., 2009). Cow manure co-digests a variety of lignocellulosic materials, including rice straw, to increase methane production and secure the fermentation reactor's steady operation (Li et al., 2015). Lignocellulosic biomass, rich in cellulose, hemicellulose, and lignin, is a renewable resource, but its complex structure poses challenges for anaerobic digestion. Its rigid, crystalline structure hinders the breakdown of the biomass and reduces biogas production, particularly the methane yield. To overcome this, costly and energy-intensive pretreatment processes are required. However, improper pretreatment can damage the cellulose structure or produce inhibitory compounds, further reducing the efficiency of the digestion process (Xu et al., 2019). Grinding rice straw is essential, as it increases the surface area of the material, which contributes to the more efficient breakdown of lignocellulosic components such as cellulose and hemicellulose, which are typically difficult to degrade due to their complex structure. Grinding contributes to a more homogeneous substrate mixture, allowing for better mixing and contact with anaerobic sludge or inoculum. This uniformity ensures consistent digestion and biogas production rates. Studies suggest that optimizing particle size (3 mm for rice straw) can yield better results without excessive energy consumption (Luo et al., 2021). 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. Reaction chambers containing alkaline salts such as $Ca(OH)_2$, NaOH, and KOH were used to remove CO_2 and H_2S from biogas produced from cow manure. Water vapour was removed by passing the gas through a silica gel bed. The methane concentration after the purification process was also studied, as it is directly proportional to the impurity removal efficiency. The biogas flow rate and the concentration of the alkaline solution were varied, and both parameters affected the biogas composition. The highest methane concentration (94.80%) was achieved at the lowest biogas flow rate (0.54 m^3/h) and the highest solution concentration (1N). However, H_2S removal was not possible because its concentration in the biogas was only 2 ppm (Katariya and Patolia, 2023).

Regarding the use of azolla for biogas production, the suitability of azolla (Azolla pinnata) for biogas slurry enrichment was conducted to determine the optimum ratio of cow manure and azolla for biogas slurry enrichment, to identify the best material to prepare quality organic manure from the enriched slurry and also to assess the crop response to enriched organic manure. The results indicated that mixing of cow manure and azolla in a proportion of 1: 0.5 produced the highest volume of gas (0.29 m^3 kg^{-1} TS) in 20 days of hydraulic retention time (HRT). But the proportion of 1: 1 favoured in terms of N (3.44 percent) content in slurry, followed by 3.23 per cent in 1: 0.75 ratio. These treatments were designated as enriched slurry I (3.44 percent N, 0.74 percent P and 2.93 percent K) and enriched slurry II (3.23 percent N, 0.77 percent P and 2.91 percent K). Addition of azolla increased pH from 7.1 to 7.8 but decreased the total solids of slurry from 5.40 percent in cow manure alone to 2.68 per cent in the ratio of 1: 1 (Bishnu, 2012). Aquatic fern is used as a basis of green manure and decomposed organic material (Razavipour et al., 2018). 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 operational effectiveness and process stability of anaerobic digestion systems are determined by several physicochemical factors, including the carbon-to-nitrogen

(C/N) ratio, temperature , and pH (Mao et al., 2015). 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, enhances crop productivity, and reduces dependence on synthetic fertilizers, thereby promoting sustainable agricultural practices (Doyeni et al., 2021). In line with these findings, Erdal and Yaylac (2021) demonstrated that the application of biogas digestate (BD) under greenhouse conditions not only increased wheat dry weight but also posed no risk of heavy metal accumulation, further confirming the potential of BD as an environmentally safe and effective soil amendment for sustainable crop production.

This research addresses rice, the second most important crop in Egypt, which produces high emissions from burning residues. Addressing this problem is challenging due to its high lignin content, which causes a long hydraulic retention time, 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 rice straw to produce biogas. Therefore, this current research aims to highlight the use of azolla as a non-conventional resource for biogas production mixed with rice straw, compared to the conventional cow manure method.

Hence, the objectives of the current work can be explained in the following points:

- Determine the optimum mixture of the used substrates (rice straw, azolla and cow manure), that gives the highest methane production.
- Evaluate the digestion performance using azolla as a non-traditional source compared to traditional cow manure.
- Select the appropriate fermentation mixture rich in nutrients to use as a biofertilizer for the soil.

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 rice straw mixed with cow manure and azolla ferns.

2.1. Materials

2.1.1. Anaerobic unit

The biogas production unit, made of stainless steel, is rectangular in shape, with dimensions of 1400 × 770 × 500 mm and 1.5 mm wall thickness. It was designed to be suitable for laboratory-scale reactors (10 reactors), as shown in Fig.1. The unit incorporates a perforated stainless steel mesh screen, measuring 1380 × 750 mm, mounted 80 mm above the base to facilitate water flow, thus providing optimal conditions for the fermentation process. It also features openings for positioning the reactors. Two electric heaters (3 kW, 220 V) were used to maintain a temperature of 40 ± 3 °C in a controlled mesophilic conditions, as described by Yan et al. (2023). Each heater was equipped with a thermostat to regulate the desired temperature. A water pump is used to circulate water around the fermenters to achieve the desired temperature uniformity. The pump specifications are as follows: 90 W power, maximum water flow rate of 2500 l/h, model of LBP-D2500, 220 V/50 Hz. The water surface was covered with pieces of white corkboard to maintain an appropriate temperature for the fermentation process and minimize water evaporation. The unit was fully isolated to reduce heat losses. The unit was equipped with both an inlet and a water outlet. It connected to a water tank for its water supply, while the outgoing water was recycled within a closed system. Valves are installed to control the water flow.

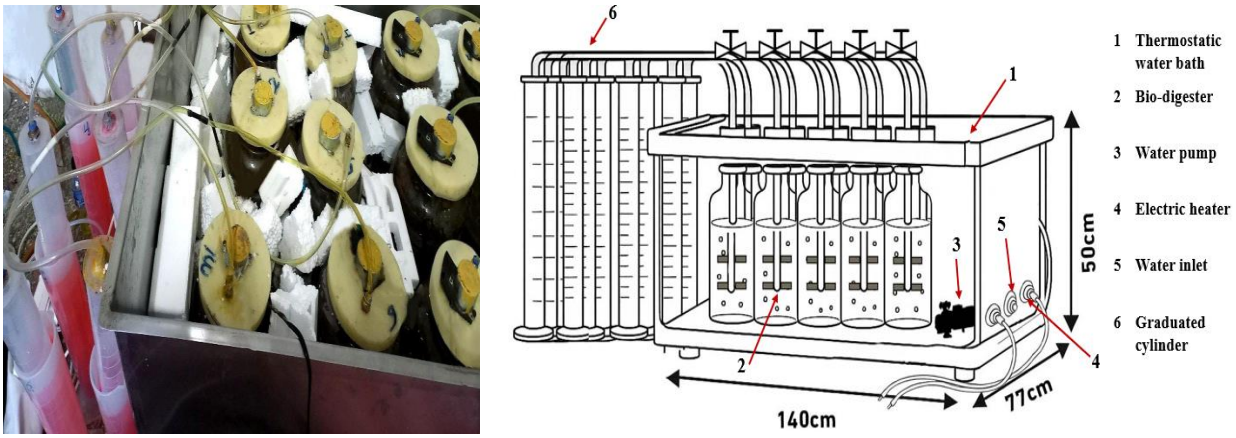


Fig. 1. Anaerobic unit

2.1.1.1. Digester

As clarified in Fig. 2, the used digester consisted of a glass jar with 250 mm x 195 mm dimensions and 3 mm wall thickness (5L volume). It was equipped with a stirrer shaft made of stainless steel, 200 mm in length. The shaft was provided with two stainless-steel blades of 20 mm width and 100 mm length. The stirrer was powered by a 4.5 W electric motor to provide homogeneity of mixtures. For 15 minute every four hours, mechanical stirring was maintained at a speed of 35 ± 1 rpm to ensure substrate homogeneity and uniform digestion temperature. The glass jar was covered tightly with a Teflon cap to prevent gas leakage.

The biogas outlet was connected to the gas collection chamber and measured using the water displacement method, where the gas pressure generated by the digester displaced an equivalent volume of water into the calibrated graduated cylinder as recommended by (Adelekan and Bamgboye, 2009). The biogas outlet was connected to a control valve. A polypropylene graded cylinder 500 ml was placed upside down in another polypropylene cylinder 1000 ml that was filled with water and connected to the gas outlet. Temperature plays a crucial role in microbial activity.

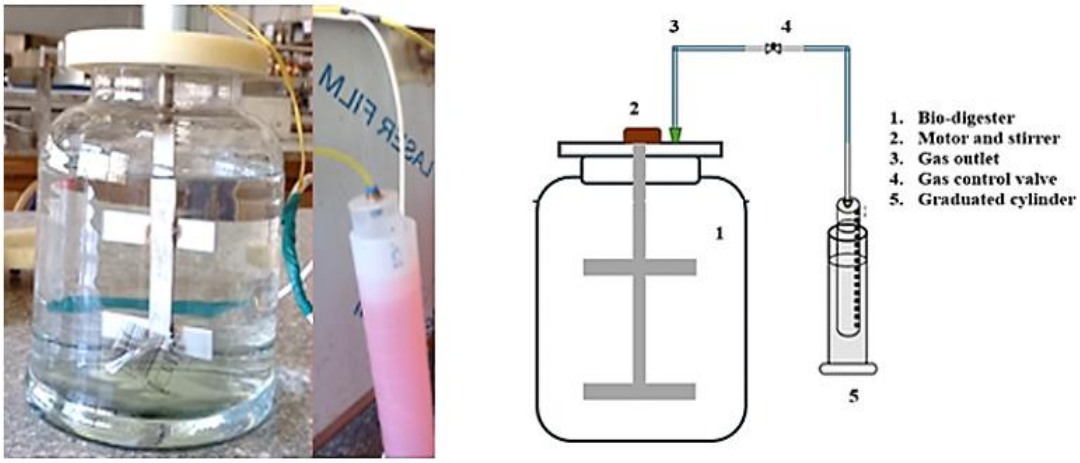


Fig. 2. Digester unit

2.1.2. Substrates

Various substrates were used to prepare the fermentation mixtures as follows:

2.1.2.1. Rice Straw (RS)

The rice straw was obtained from Banayous farm (30.5983° N, 31.4999° E), Zagazig, Al Sharqia Governorate, Egypt. The straw was chopped into small pieces (3mm length), which is compatible with Luo et al. (2021). Rice

straw chopping is an essential pretreatment step that improves substrate accessibility, enhances bigas production, and improves anaerobic digestion (Dai et al., 2019).

2.1.2.2. 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. Samples were taken from each species, and the chemical properties were measured as shown in Table 1.

Table 1. Chemical properties of the used substrates

Substrate	Components					
	VS, %	Ash, %	pH	T.C, %	T.N, %	C/N
RS	74.30	25.70	7.2	46.20	1.0	46.20
CM	72.66	27.34	7.3	47.30	2.05	23.07
AZ	69.90	30.10	6.9	45.92	2.39	19.21

2.2. Methods

Experiments were conducted in controlled conditions of mesophilic temperature and pH ranging from 6 - 8 to promote stable digestion, high microbial diversity, and biogas yield (Yan et al., 2023). The pH was measured using a digital pH meter, model 915, category 13-636-916.2, with an accuracy of ±0.01 pH units. Rice straw, cow manure, and azolla were used separately and together as a mixture. The components of each substrate or mixture are homogenized carefully before being placed in the fermenter. After all the digesters are filled to the actual volume of 4L, tightly closed, and all connections are checked, they are placed in the water bath of the anaerobic unit.

2.2.1. Experimental conditions

The experiment for digestion performance was divided into four trials as illustrated in Fig. 3 under the following operating conditions:

1st trial of using 100% of each substance [T1 (100% RS), T2 (100% CM) and T3 (100% Az)],
2nd of rice straw mixture with cow manure [T4 (75% RS + 25% CM), T5 (50 % RS + 50 % CM) and T6 (25% RS + 75% CM)],
3rd trial of rice straw mixture with azolla [T7 (75% RS + 25% Az), T8 (50% RS + 50 % Az) and T9 (25% RS + 75% Az)],
4th trial is a mixture of all substrates (RS, CM and AZ) in equal proportions [T10 (1:1:1)].

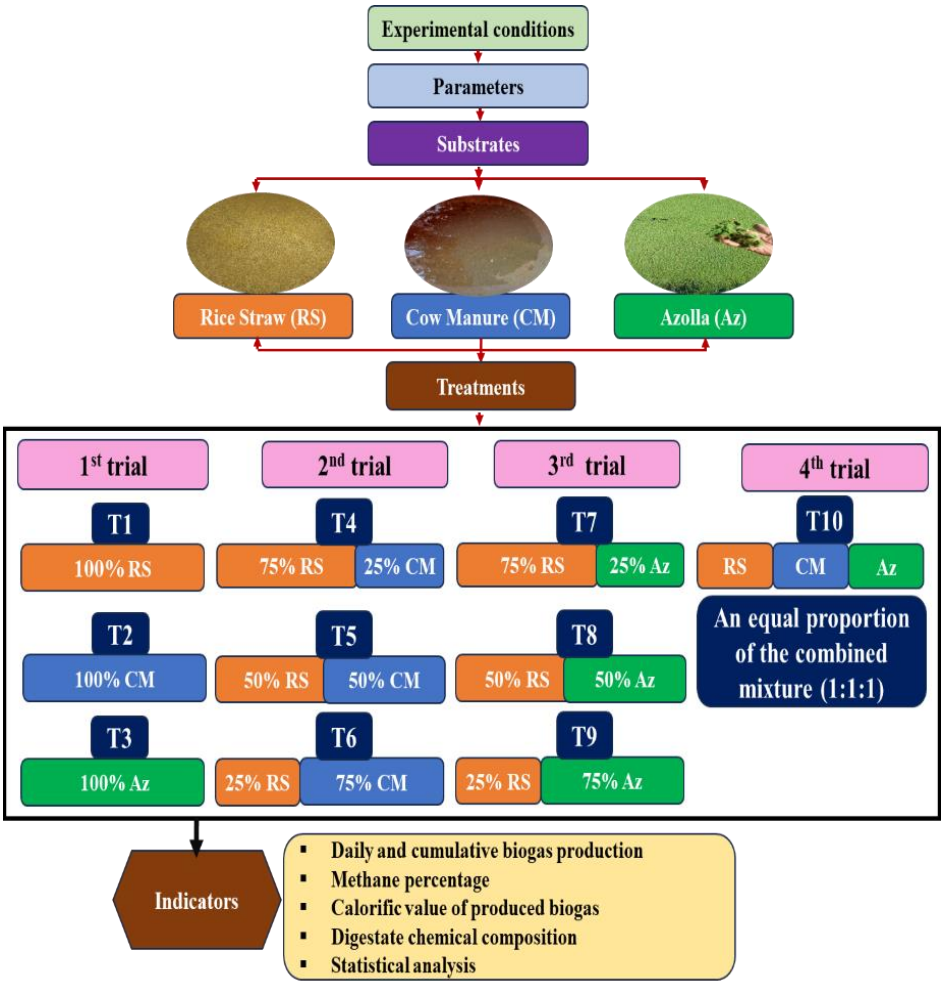


Fig. 3. Experimental conditions

To prepare the slurry media for the mixtures, water was added to the various prepared raw materials (v/w) to achieve a target total solids concentration of 8% prior to loading into the digesters. The required water was calculated based on the methodology , as cited from (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:

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

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).

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

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

Treatment	Components					
	VS, %	Ash, %	pH	T.C, %	T.N, %	C/N
75% RS + 25% CM	73.89	26.11	7.1	46.47	1.26	36.81
50 % RS + 50 % CM	73.48	26.52	7.2	46.75	1.52	30.65
25% RS + 75% CM	73.07	26.93	7.3	47.025	1.78	26.30
75% RS + 25% Az	73.20	26.80	7.0	46.13	1.34	34.23
50% RS + 50 % Az	72.10	27.90	7.1	46.06	1.69	27.17
25% RS + 75% Az	71.00	29.00	6.8	45.99	2.04	22.51
Combined mixture	72.10	27.90	7.1	46.35	1.80	25.62

2.2.2. Measurements and determinations

To evaluate the performance of the digestion under the effect of the previous treatments, the following indicators were taken into account:

3. Daily and cumulative biogas production

The amount of gas emitted was measured daily (l/day) using the water displacement. The cumulative biogas production for each treatment was calculated to select the best conditions for maximum production through the hydraulic retention time (HRT). Following that, the observed volumes were normalised to 1013 mbar and 0 °C using the correction method described 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).

4. Methane percentage

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

5. Calorific value of biogas

At standard temperature and pressure (STP), calorific value and density of biogas were 50 MJ/kg (equivalent to 36 MJ/m³) and 0.72 kg/m³, 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/m³), CG: Cumulative biogas production under standard circumstances (m³) an d CH_4: The methane percentage (%).

6. Total nitrogen

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

7. 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:

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

8. 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. Effects of various mixtures on biogas production (daily and cumulative)

The analysis of variance (ANOVA) revealed highly significant differences among the digestion mixtures treatments, showed Table 3. The results indicated that the statistical model explained the entire variability in the data ($R^2 = 1.000$, Adjusted $R^2 = 1.000$). The F-test value for the treatments (Mixed treatment) was 28,428,240.610, which was statistically significant at the 0.001 level ($p < 0.001$), demonstrating a clear and substantial effect of the treatments on the studied variable. Furthermore, the experimental error was found to be very small (Mean Square Error = 0.900), reflecting the accuracy and reliability of the experiment. The observed statistical power reached 1.000, suggesting that the probability of failing to detect differences among treatments, if they exist, was nearly zero. In conclusion, all tested treatments exerted a significant effect on the dependent variable, which enhances the reliability of the findings.

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 Power[b]
Corrected Model	230268748.942[a]	9	25585416.549	28428240.610	.000	255854165.492	1.000
Intercept	5628788893.328	1	5628788893.33	6254209881.48	.000	6254209881.48	1.000
Mixed treatment	230268748.942	9	25585416.549	28428240.610	.000	255854165.492	1.000
Error	18.000	20	.900				
Total	5859057660.270	30					
Corrected Total	230268766.942	29					

a. R Squared = 1.000 (Adjusted R Squared = 1.000)

b. Computed using alpha = .05

Results in (Figure 4) indicated that the fermentation results revealed variations in the onset of biogas production among the three treatments, T1 (100% rice straw), T2 (100% cow manure), and T3 (100% azolla), with some showing earlier responses than others. Biogas production in treatment T1 was first detected on the fifth day of fermentation, whereas treatments T2 and T3 exhibited earlier gas release, beginning on the second day of the process. Biogas production was additionally observed to commence between the third and fourth days in the remaining trials. Under standard conditions, the maximum daily biogas yields for T1, T2, and T3 were 509.59, 622.24, and 884.92 milliliters per day (ml/day) on the thirty-third, fifteenth, and seventeenth days, in sequential order. The impact of substrate properties on microbial activity is reflected in the differences in biogas production timing and yield among treatments T1, T2, and T3. Its high carbon-to-nitrogen ratio (C/N = 46.2), which restricts microbial efficiency, is probably the cause of T1's delayed onset. This conclusion is corroborated by Ngan et al. (2020), who underlined the necessity of pre-treating rice straw to improve biodegradability. These results suggest that azolla (T3) not only accelerates the onset of biogas production but also enhances peak yield, likely due to its balanced nutrient profile. Nitrogen-rich aquatic biomass may speed up anaerobic digestion, according to the excellent results of azolla-based therapies, especially when paired with RS. This is consistent with the findings of de la Lama-Calvente et al. (2023), who discovered that nitrogen bioavailability is essential for enhancing methane production. While Ngan et al. (2020) focused primarily on the limitations of rice straw, their findings indirectly support the superior performance of nitrogen-rich substrates, such as cow manure and azolla. The greatest daily biogas yields for T4, T5, and T6 were 648.26, 628.99, and 707.93 ml/day on the twenty-seventh, twenty-first, and nineteenth days of fermentation, in sequential order. The greatest daily biogas produced was 691.61, 698.62, 777.80, and 787.11 ml/day for T7, T8, and T9 on the twenty-first, seventeenth, and nineteenth day of fermentation, in sequential order accordingly. It was 787.11 ml/day for T10 on the seventeenth day of fermentation. 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 8638.15, 9514.19, and 17236.50 (ml) for T1, T2, and T3. It was clear that the cumulative biogas production was 12178.63, 13052.73, and 14400.94 (ml) for T4, T5, and T6. It was 13835.24, 15285.59, 15887.20 (ml) for T7, T8, and T9, and 16952.53 (ml) for T10, in sequential order, reflecting a progressively increasing biogas production over time across the various mixtures. The findings indicate that co-digestion of rice straw (RS) with either cow manure (CM) or azolla (AZ) in co-digestion systems leads to a marked improvement in biogas production compared to mono-substrate setups. Specifically, treatments involving RS blended with CM (T4–T6) or AZ (T7–T9) achieved superior daily and total biogas yields relative to the mono-substrate treatment (T1), suggesting that combining substrates enhances nutrient balance and stimulates microbial efficiency during anaerobic digestion. This finding aligns with Muhayodin et al. (2021), who reported that co-digestion of RS with CM improves biogas yield due to better nutrient availability and buffering capacity. Similarly, Ona & Agogo (2022) found that mixing RS with cow dung led to higher gas output than using RS alone. In line with de la Lama-Calvente et al. (2025), who showed that nitrogen bioavailability improves the efficiency and kinetic performance of anaerobic co-digestion, the superior performance of T6 and T9, which contain higher proportions of CM and Az, respectively, indicates that nitrogen-rich substrates speed up microbial metabolism; however, prudence is necessary because Yang et al. (2025) pointed out that too much nitrogen can cause ammonia inhibition, which could destabilize the digestion process. According to Sawasdee et al. (2023), who noted comparable patterns in pilot-scale co-digestion of RS and food waste, the slow drop in biogas output following peak days for all treatments is indicative of normal retention dynamics in anaerobic systems. Similar to the study's most productive treatments (T9), treatment T10, which mixed (RS), (CM), and (AZ) in equal amounts, showed a noticeably high biogas yield.

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 T3 was 3468 parts per million (ppm) at the beginning of the biogas's appearance, then 2530 ppm in the second measurement of the produced biogas, and gradually decreased with each measurement of the proportions of the biogas components until it reached 73ppm at the end of the experiment. The progressive reduction in hydrogen sulfide (H_2S) concentration serves as a reliable indicator of system stabilization during anaerobic digestion. In treatment T3, H_2S levels declined sharply from 3,468 ppm at the start to just 73 ppm by the end of the experiment. This initial spike in H_2S likely stemmed from the rapid decomposition of sulfur-rich

compounds during the early transitional phase, prior to the establishment of fully anaerobic conditions. As Krayzelova et al. (2015) noted, elevated sulfide concentrations are typical in the initial stages of digestion, particularly in nitrogen-rich environments where sulfate-reducing bacteria are active. The subsequent decline in H_2S suggests that the microbial community successfully adapted to the substrate conditions, leading to reduced production of inhibitory byproducts and fostering a more stable and efficient anaerobic environment. This stabilization was accompanied by notable improvements in both biogas yield and methane content, consistent with observations reported by Zheng and Li (2024). The biogas yield from T10 exceeded those of T1, T2, T3, T4, T5, T6, T7, T8, and T9 by 49.04%, 43.87%, 28.16%, 23.00%, 15.05%, 18.38%, 9.83%, and 6.28% respectively, underscoring the superior efficiency of T10 in biogas generation across most comparisons. T3 surpassed T10 in biogas production by 1.64%, suggesting a slight enhancement in yield efficiency.

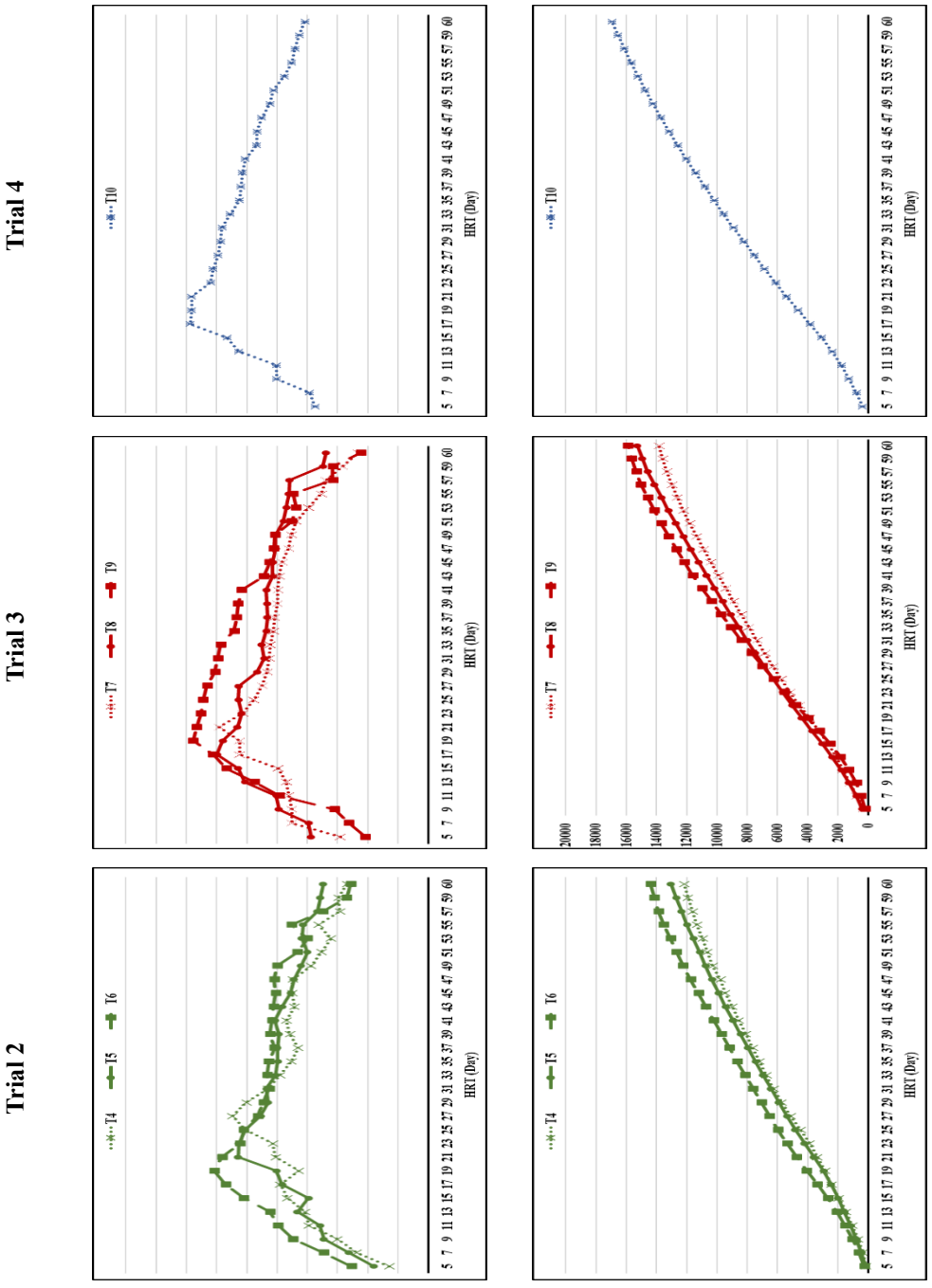
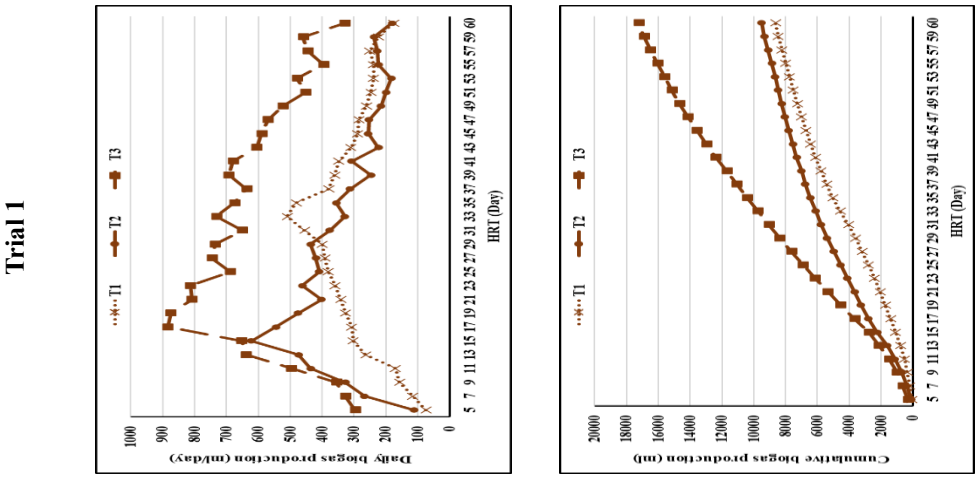


Fig. (4): Effect of different experimental trials of on daily and cumulative biogas production.



3.2. Effect of various pretreatments on methane percentage

The statistical analysis of variance (ANOVA) showed that the model was highly effective in explaining variability among treatments, accounting for 91.5% of the total variation (R^2 = 0.915, Adjusted R^2 = 0.877), (Table 4). Treatments had a significant effect on the dependent variable ($F = 23.894$, $p < 0.001$), confirming that differences were due to treatment effects rather than random error. The relatively small error variance (MSE = 11.379) and the highly significant intercept ($F = 11779.590$, $p < 0.001$) further demonstrate the reliability of the design, while the maximum statistical power (1.000) eliminates the risk of Type II error. Overall, the results confirm that the treatments exerted clear and measurable impacts on the studied variable.

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

Source	Type III Sum o Squares	Df	Mean Square	F	Sig.	Noncent. Parameter	Observed Power
Corrected Model	2447.122[a]	9	271.902	23.894	.000	215.050	1.000
Intercept	134043.610	1	134043.610	11779.590	.000	11779.590	1.000
Treatments	2447.122	9	271.902	23.894	.000	215.050	1.000
Error	227.586	20	11.379				
Total	136718.319	30					
Corrected Total	2674.709	29					

a. R Squared =0 .915 (Adjusted R Squared = .877)

b. Computed using alpha = .05

The results illustrated in Figure 5 present the average of methane content for different Trials. They were 44.72, 68.18, 81.18, 60.65, 63.86, 68.77, 66.75, 70.31, 73.14, and 70.88 % for T1, T2, T3 T4, T5, T6, T7, T8, T9, and T10 in the same order as the mixtures listed. 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. The appearance of CH_4 starting the second week, on the other hand, revealed that the presence of solely CO_2 during the first fermentation phase was indicative of the progressive creation of anaerobic conditions as residual oxygen was consumed.

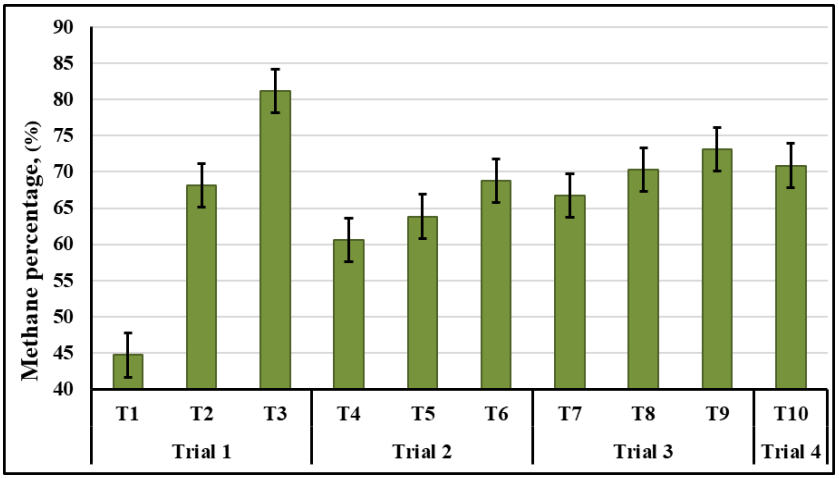


Fig. 5: Effect of different treatments on percentage of methane in biogas production. At a p-value of 0.05, different letters indicate statistically significant differences, and the error bars represent the standard error of biogas production.

3.3. Comparative assessment of calorific value (MJ) of produced biogas under various mixtures

Figure 6 shows the calorific value of the biogas produced in each Trial under the examined experimental conditions. According to the results obtained, for T1, T2, T3, T4, T5, T6, T7, T8, T9, and T10 the resulting biogas had calorific values of 0.139, 0.233, 0.503, 0.265, 0.300, 0.356, 0.332, 0.386, 0.418, and 0.432 MJ, in sequential order. The calorific value of biogas produced from T3 exceeded those of T1, T2, T4, T5, T6, T7, T8, T9, and T10 by 72.4%, 53.7%, 47.3%, 40.4%, 29.2%, 34.0%, 23.3%, 16.9%, and 14.1%, respectively. Similarly, biogas from T10 demonstrated calorific values that were 67.8%, 46.0%, 38.6%, 30.5%, 17.6%, 23.1%, 10.6%, and 3.2% higher than those of mixtures T1, T2, T4, T5, T6, T7, T8, and T9, 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.

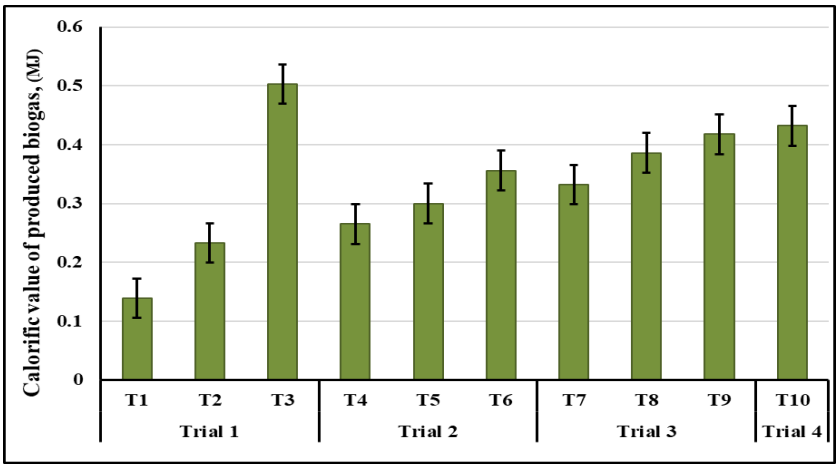


Fig. 6: Effect of different experimental mixtures on the calorific value of the produced biogas. At a p-value of 0.05, different letters indicate statistically significant differences, and the error bars represent the standard error of biogas production.

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

The data presented in Fig. (7) demonstrate distinct variations in biogas production rates across the tested mixtures. T1, T2, T3, T4, T5, T6, T7, T8, T9, and T10 yielded 0.359, 0.0396, 0.072, 0.051, 0.054, 0.060, 0.057, 0.063, 0.066, and 0.071 m^3 of gas per m^3 of digested raw material per day, respectively.

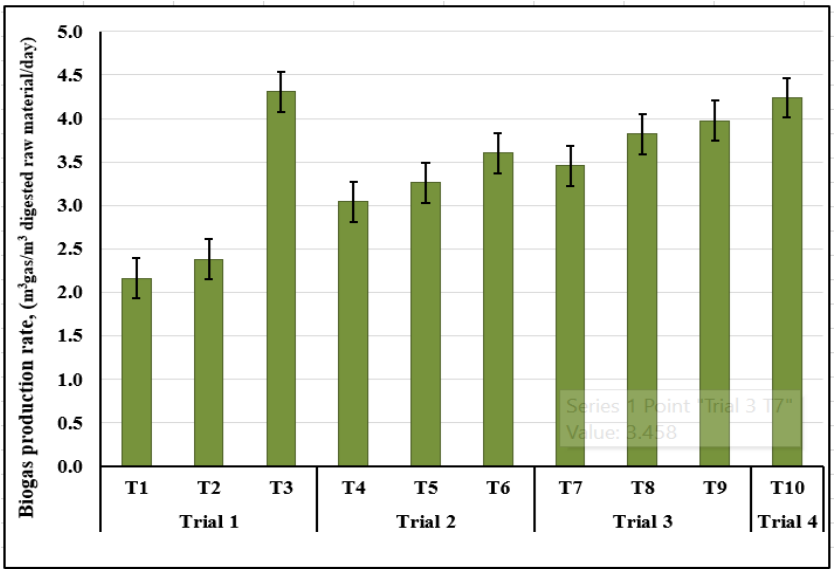


Fig. 7: Effect of different experimental mixtures on biogas production rate. At a p-value of 0.05, different letters indicate statistically significant differences, and the error bars represent the standard error of biogas production.

3.5. Digestate chemical composition characteristics under different treatments

The chemical composition of residual digestate of various mixtures includes estimations of the following, as (Table 5) illustrates: 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 6.3 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% – 46.99%), nitrogen levels ranged from 1.21% to 2.99%, potassium levels ranged from1.25 % to 2.60% and phosphorus levels ranged from 1.01% to 1.99%. Therefore, the residual digestate derived from various mixtures demonstrated potential as a valuable organic fertilizer. As shown in Table 5, T9 exhibited the highest concentrations of nitrogen (2.41%) and potassium (1.84%) among the rice straw-based formulations. Additionally, the effluent slurries from T8 and T9 presented the most favorable carbon-to-nitrogen (C/N) ratios, which are considered optimal for the mineralization of organic matter. These findings suggest that the effluents from T8 and T9 are particularly well-suited for application in sandy soils, enhancing nutrient availability and supporting sustainable agricultural practices. The chemical analysis of the residual digestate further confirmed the environmental value of the co-digestion process. This makes it suitable for agricultural applications, particularly in sandy soils with limited buffering capacity. The moderate variation in total carbon (42.60%–46.99%) and elevated nitrogen concentrations (up to 2.41% in T9) indicate a balanced nutrient profile supportive of soil fertility enhancement. Additionally, potassium and phosphorus levels also remained within beneficial ranges, confirming the digestate's suitability as a biofertilizer.

Table 5. Chemical composition of the different digestion mixtures (Residual digestate)

Treatement No.	Constituents								
	TS, %	VS, %	Ash, %	pH	T.C, %	T.N, %	P, %	k, %	C/N
1	5.71	72.55	27.45	6.3	45.25	1.21	1.01	1.3	37.39

2	4.55	68.12	31.88	6.8	44.89	2.25	1.98	2.6	19.95
3	3.42	65.6	34.4	7.5	42.60	2.99	1.88	1.7	14.24
4	5.58	71.58	28.42	6.8	44.95	1.72	1.48	1.29	26.13
5	4.75	70.88	29.12	6.9	44.55	2.01	1.65	1.37	22.16
6	4.2	69.55	30.45	6.8	43.61	2.15	1.87	1.54	20.28
7	4.91	70.82	29.18	7.4	46.99	2.12	1.63	1.25	22.16
8	4.52	68.5	31.5	6.7	45.45	2.33	1.75	1.48	19.50
9	4.16	67.56	32.44	6.4	43.50	2.41	1.99	1.84	18.04
10	4.03	66.32	33.68	7.4	43.17	1.98	1.97	1.81	21.80

5. Conclusions

This study confirmed that co-digesting rice straw with azolla and cow manure under controlled anaerobic conditions significantly enhances biogas production and methane content while keeping the fermentation process stable. Optimized mixtures—particularly T3, T6, T9, and T10 exceeded others in both cumulative biogas production and methane content. These results confirm the beneficial and advantageous effect of nutrient-rich co-substrates in stabilizing fermentation and improving energy production from high-lignocellulose biomass. 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 rice straw digestion alone. Rice straw can be utilized as a non-traditional energy source by mixing rice straw 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—such as cotton stalks, which pose significant environmental challenges—is of particular interest due to their structural complexity, which is comparable to that of rice straw, 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:

RS :Rice straw

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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