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A Study on Temperature Variations, PH Changes, and Biochemical Characterization of Bacteria in Cow Dung Substrates for Biogas Production

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ABSTRACT

This study aims to explore the potential of cow dung as a substrate for biogas production, focusing on the impact of temperature, pH variations on biogas yield and the identification of key bacteria involved in the biogas production. Biogas production was generated using the downward displacement method of water. Temperature readings were taken daily and pH of the cow dung substrate was measured before and after the digestion. Bacteria involved in biogas production were isolated and identified using Gram staining. The results showed that biogas production was significantly higher in weeks 3 and 4, with yields of (2360.00±183.60mL) and (3223.00±1377.00mL) at temperatures of 34.0±1.0°C and 36.5±1.5°C, respectively with no significant difference. However, the biogas yield in weeks 1 and 7, were (130.00±34.64 mL) and (70.00±20.00 mL) at temperatures of 26.5±1.0°C and 26.2±1.5°C, respectively, was significantly lower at($p < 0.05$) compared to weeks 3 and 4 (Table 1). The pH decreased slightly from 7.827±0.093 before digestion to 7.227±0.04 after digestion, but this change was not statistically significant. Gram staining identified Bacillus subtilis and Bacillus cereus as the primary bacteria involved in the biogas production process. In conclusion, the study showed that temperature significantly influences biogas production from cow dung, with optimal yields observed at higher temperatures and the presence of Bacillus subtilis and Bacillus cereus, contributes to the efficiency of the biogas production process. Further research should focus on optimizing temperature control during anaerobic digestion and exploring co-digestion with other substrates.

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Introduction

Biogas is a colorless, flammable gas produced via anaerobic digestion (fermentation) of animal, plant, human, industrial and municipal waste to produce methane (50-70%), Carbon dioxide (30-50%) and traces of other gases such as nitrogen, hydrogen, ammonia, hydrogen sulphide, water vapour (Less than 1%) [1]. Biogas can be produced from any biodegradable feedstock that is suitable for anaerobic digestion [2]. Production of biogas through anaerobic digestion (AD) of animal manure and slurries as well as of a wide range of digestible organic wastes convert these substrates into renewable energy. Anaerobic Digestion (AD) technology is well established, hence biogas is often categorized as a 'first generation' biofuel which has developed from a method for treatment of waste, to a process aiming at production of methane as an energy carrier [3,4]. Renewable natural gas (RNG) provides a clean, easily controlled source of renewable energy from organic waste materials, replacing fossil natural gas with a sustainable carbon neutral fuel option. Anaerobic digestion is the most commonly employed and technologically mature method; requiring only a low-oxygen environment for the naturally occurring breakdown of organic matter by bacteria [5].

The process of biogas production include hydrolysis, acidogenesis, acetogenesis and methanogenesis [6]. In hydrolysis, complex carbohydrate, fats and protein are first hydrolyzed to their monomeric form by enzymes and bacteria such as Bactericides, Clostridia and facultative bacteria such as Streptococci. In the second phase (Acid genesis) monomers are further degraded into short-chain acids such as acetic acid, propionic acid, butyric acid, carbonic acid, alcohols, hydrogen, and carbon dioxide. During acidogenesis, these short-chain acids are converted into acetate, hydrogen and carbon dioxide. In the last phase, methanogens convert the intermediate produced in methane and carbon dioxide. Almost one-third of methane formation is due to reduction of carbon dioxide by hydrogen [7]. Several factors that affect the production of biogas are the condition of the digester, pH, nutrients, temperature, the ratio C / N, and starter [3]. The condition in the anaerobic digester must be kept in equilibrium

and dynamic. The degree of acidity is maintained in the range of 6.6 to 7.6 for bacteria metanogenic can only work in above range of pH [8]. Adequate levels of nutrients such as nitrogen and phosphorus must be added in the system to ensure the availability of nutrients for bacterial growth [2]. The optimum temperature needed microorganisms to break down the material is 30-38°C for mesophilic and 49-57°C for thermophilic. The optimum ratio of C/N used in the process of biogas production is 25-30. Starter is very important part that supporting the production of biogas. It is used to accelerate the reform process of organic materials. Common starter used in biogas production are activated sludge or the content of rumen fluid [5].

The main challenge of the present world is to harness the energy source which is environmentally friendly and ecologically balanced. This need has forced the world to search for other alternate sources of energy. But unfortunately the new alternative energy sources like the solar, hydro, wind etc. require huge economical investment and technical power to operate, which seem to be very difficult for the developing countries like Nigeria [9]. Energy consumption in Nigeria has been increasing on a relatively high rate. On a global scale, Iwayemi, opined that the Nigerian energy industry is probably one of the most inefficient in meeting the needs of its customers [10]. This is most evident in the persistent disequilibrium in the markets for electricity and petroleum products. The dismal energy service provision has adversely affected living standards of the population and exacerbated income and energy poverty in an economy where the majority of the people live on less than $2 a day [11].

Development of biogas technology is a suitable alternative energy source that would be affordable and environmentally friendly that would help preserve the green forest thus achieving the 7th mandate of the Millennium Development Goal on environmental sustainability [6]. In addition to meeting the dire need for waste treatment options to enhance a clean environment, alternative processing technologies, such as anaerobic digestion, offer some potential for recovery of value from organic wastes (i.e., waste to wealth) by producing biogas [12]. Furthermore, millions of tonnes of wastes are released daily emit a lot of methane gas when exposed to the atmosphere, which is 320 times more harmful to human health than carbon dioxide [13]. This study is aimed to study biogas production from cow dungs and the effect of temperature variation, pH changes and biochemical characterisation of bacteria involved.

Materials and Methods

Study Area

Sokoto is a city located in the extreme northwest of Nigeria, near the confluence of the Sokoto River and the Rima River. Sokoto occupies 25,973 square kilometres with a population of 563,861 [14]. Using the exponential projection method as recommended by the National Population Commission (NPC) with a growth rate of 3%. The people in the study area are mainly the Hausa and the Fulani.

The area lies in the Sudan-Sahelian ecological zone which is semi-arid. It is characterized by three seasons – the cool and dry, the hot and dry and the hot and wet [15]. The area is influenced by the Tropical Continental air mass (cT) during the months of November to February. The hot season is experienced in the months of March to May, and it is associated with high temperature of up to 380C to 450C. The hot and wet season on the other hand usually starts from the month of May and lasts for about four months only. An annual rainfall of 500mm-800mm [17]. The vegetation consists of mostly short feathery grasses and some scattered trees most of which are deciduous in character, characterized by thorny species with a scatter of Acacia species [17]. The built-up environment is characterized by residential, agricultural, academic, commercial, religious, open spaces, among others.

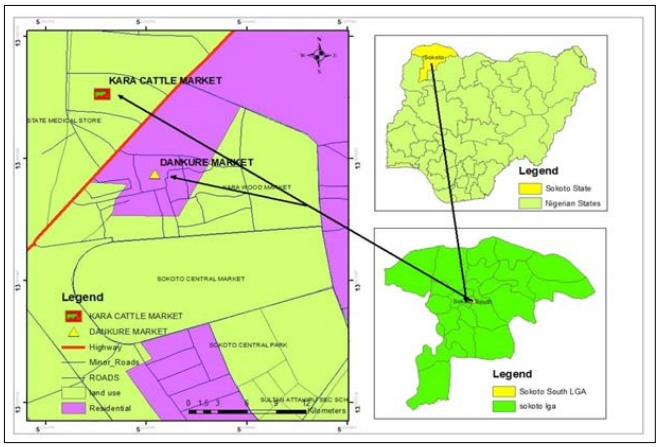

Figure 1: Map of Sokoto showing the study areas (Department of Geography Sokoto State University, Sokoto, 2023)

Sample Collection

Fresh samples of cow dungs was collected from the Kara market Sokoto in a clean polyethene. The samples was transported within 24 hours of collection to the Energy research centre, Usmanu Danfodiyo University Sokoto for laboratory analysis.

Sample Preparation

The fresh sample of the cow dungs was air dried under the sun after which is dried in an oven at 105oC. The sample were dried further dried at room temperature for a period of two weeks before grounded into powdered form using a pestle and mortar [18].

Experimental Design

A biogas plant was setup comprising of three (3) tins of 400g capacity as biogas digesters. A hole was made at the center of the lid of each of the three (3) tins and a hose pipe (1 inch) was connected to the hole of each digester and covered with epoxy steel gum to avoid leaking of the gas. The pipe conveyed the gas from the digester to a measuring cylinder (1000cm3 capacity) filled with water and placed in an inverted position in a basin filled with water (water displacement method). The cylinder was held firm by a retort stand. The gas produced from the digesters was conveyed through the hose pipe to the measuring cylinder which displaced the water downward. The volume of gas produced was measured by the amount of water being displaced from the measuring cylinder. Daily production temperature was recorded at 12:00 noon throughout the retention period of seven weeks [18].

Slurry Preparation

One hundred grams (100 g) of cow dungs were weighed using digital weighing scale (Ohaus Adventuer Pro; AV 4101 Model) and poured into three (3) empty tin of 400g capacity serving as digester, which was followed by the addition of 600ml to give (1:6 substrates to water ratio) of water in each digester. The mixtures were all stirred with a rod and continue to stir for five (5) minutes, until it's diluted to obtain homogeneity. All the digesters were sealed with a candle wax/epoxy gum (4 minutes) in order to block leakages to maintain anaerobic condition [18].

Determination of pH
The pH of substrates of cow dungs was measured before and after digestion using a digital pH meter (HANNA HI 8314). A slurry was prepared by mixing the feedstock with water, and the pH was measured after calibrating the meter with buffer solutions. The electrode was cleaned with distilled water between measurements for accuracy [18-20].

Determination of Temperature
Ambient temperature was monitored using a calibrated wall-mounted thermometer (Taylor Precision Products 5329 Indoor/Outdoor Thermometer) placed near the biogas digester. Readings were taken daily at 12:00 PM throughout the retention period. Data collected was analyzed to determine the relationship between ambient temperature and biogas yield [21,22].

Bacteriological Analysis
Bacteriological analysis was performed on digested substrates to assess the presence of pathogens or beneficial bacteria. Digested samples were refrigerated (4°C) for transportation to the laboratory, while undigested samples were sealed in sterile containers.

Media Preparation
Nutrient agar was prepared by dissolving 28 g of powder in 1 L of distilled water, sterilized by autoclaving at 121°C for 15–20 minutes, and poured into sterile petri dishes under aseptic conditions [23,24].

Inoculation
Samples were streaked onto agar using sterile loops and incubated at 37°C for 24 hours. Plates were sealed with parafilm and inverted to prevent condensation [25,26].

Incubation
The inoculated petri dishes was incubated at 37°C for 24 hours to allow it to grow [26]. Plates are placed upside-down (agar side up) to prevent condensation from forming on the agar surface. After incubation, the grown colonies were observed [27].

Isolation of Pure Culture
After the 24-hour incubation period, the petri dishes were removed from the incubator, and the bacterial colonies were examined and recorded. Each plate was observed carefully for distinct characteristics of the bacterial colonies, including colony size, shape, edge (margin), elevation, color, and opacity [27]. Further subculturing was performed until a pure culture was achieved [28].

Sub-Culturing
The selected colony was transferred to a new, sterile agar plate. The inoculation loop was used to streak the bacteria across the plate in a zig-zag pattern to support the growth of isolated colonies [27]. The streaked plates were incubated at 37°C for 24 hours. After this incubation period, plates were observed again and a single colony type was grown, confirming a pure culture [26].

Gram Staining
A small drop of distilled water was first placed on a clean glass slide. The bacteria were then mixed with the water to create a thin smear across the slide surface [26]. The slide is flooded with crystal violet to cover the smear, left for 30-60 sec, and then rinsed off with distilled water [29]. Lugol's iodine solution was applied to the smear for 30-60 sec to act as a mordant. The slide was then rinsed gently with distilled water [27]. A decolorizing agent, typically acetone, was used to wash the slide for a few seconds, differentiating the bacteria by cell wall type [26]. Finally, safranin was applied for 30–60 seconds and then rinsed off. Afterward, the slide was gently rinsed with distilled water and allowed to dry [25].

Microscopic Identification
Observations were then made regarding cell shape (e.g., cocci, bacilli), arrangement (e.g., chains, clusters), and Gram reaction, where Gram-positive bacteria appeared purple and Gram-negative bacteria appeared pink due to the differential staining process [25].

Biochemical Characterization
Catalase Test
Detects the production of catalase enzyme by bacteria, indicated by bubbling upon the addition of hydrogen peroxide [27].

Citrate Utilization Test
Evaluates the ability of bacteria to use citrate as the sole carbon source, indicated by a color change in Simmons citrate agar [26].

Urease Test (Urea)
Detects urease enzyme activity, where hydrolysis of urea produces ammonia, causing the medium to turn pink in color [25].

Indole Test (Indole)
Assesses the breakdown of tryptophan to indole, detected by adding Kovac's reagent, which turns red for positive results [29].

Carbohydrate Fermentation Tests (Glu., Lac., Fruc.)
Determines the ability of bacteria to ferment glucose, lactose, or fructose, indicated by acid or gas production in phenol red broth [30].

Hydrogen Sulfide Test (H2S)
Detects H2S production, visible as black precipitates in triple sugar iron (TSI) agar [27].

Gas Production (Gas)
Monitored during carbohydrate fermentation as bubbles in a Durham tube [26].

Statistical Analysis
The data obtained was summarized in weekly biogas production and ambient temperature using means and standard deviations. One-way Analysis of Variance (ANOVA) was employed to assess whether significant differences exist in biogas production and temperature across the seven weeks. Post-hoc tests, such as Tukey's Honest Significant Difference (HSD), are used to identify specific weeks with significant differences.

Results
Quantity of Biogas Generated from Cow Dungs in Seven Weeks
The study on biogas production from cow dung over seven weeks reveals significant variations in biogas output, closely linked to temperature changes. Weeks 3 and 4 recorded the highest biogas production, with quantities of 2360.00±183.60 mL and 3223.00±1377.00 mL, respectively, indicating that there is no significant difference in the quantity of biogas generated between these weeks at temperatures of 34.0±1.0°C and 36.5±1.5°C. In contrast, Weeks 1 and 7 had the lowest production, 130.00±34.64 mL and 70.00±20.00 mL, at lower temperatures of 26.5±1.0°C and 26.2±1.5°C, indicating significantly lower at $p<0.05$ as shown in (Table 1).

The results showed that weeks 2 to 5 had the highest temperatures, with week 4 reaching the peak at 36.5±1.5°C, followed by weeks 3 and 5, which maintained a steady 34.0±1.0°C. Week 2, with a temperature of 31.0±1.0°C, was lower than weeks 3 and 4. Weeks 1, 6, and 7: The temperatures in weeks 1 (26.5±1.0°C), 6 (29.0±1.0°C), and 7 (26.2±1.5°C) were significantly lower than those in the middle of the study period.

Table 1: Quantity of Biogas Generated from Cow Dungs in Seven Weeks

WEEKS	Biogas Quantity	Temperature (°C)
Week 1	130.00±34.64^{bc}	26.5±1.0^{ab}
Week 2	863.30±100.20^{a}	31.0±1.0^{b}
Week 3	2360.00±183.60^{a}	34.0±1.0^{ab}
Week 4	3223.00±1377.00^{a}	36.5±1.5^{b}
Week 5	1320.00±525.7^{b}	34.0±1.0^{b}
Week 6	410.0±138.9^{b}	29.0±1.0^{ab}
Week 7	70.00±20.00^{b}	26.2±1.5^{b}

Results are Expressed as Mean ± Standard Deviation, Means with the Same Letters are not Significantly Different (P<0.05)

(a) Figure 2: Cow Dungs Substrate

(b) Figure 3: Cow Dungs Substrate

(c) Figure 4: Weighing of Cow Dungs Substrate

(d) Figure 5: A Biogas Plant Setup

pH of Substrate (Cow Dungs) Before or After Digestion

The table presents the pH values of cow dung substrates before and after digestion showed that, the values before digestion are 7.827±0.093, and for after digestion, are 7.227±0.04. The statistical analysis indicates that there is no significant difference between the pH before and after digestion at the 5% significance level.

Table 2: pH of Substrate (Cow Dungs) before or After Digestion

Substrate	Before	After
Cow dungs	**7.827±0.093[a]**	**7.227±0.04[a]**

Results are Expressed as Mean ± Standard Deviation, means with the Same Letters are not Significantly Different (P<0.05)

Biochemical Characterisation and Isolation of Bacteria Involved in Biogas Production from Cow Dungs

In table 3, Both Bacillus subtilis and Bacillus cereus are Gram-positive, rod-shaped bacteria that form chains and produce spores. Both bacteria exhibited Gram-positive rod-shaped morphology with chain formation and spore production. The results showed that both species were catalase, citrate-positive and were negative for urea hydrolysis and indole production. In carbohydrate fermentation tests, both species fermented glucose and fructose (+) but were unable to ferment lactose (-). Gas production (+) during glucose fermentation was observed, particularly for Bacillus cereus. Additionally, both species were negative (-) for hydrogen sulfide production and the methyl red test. However, the positive Voges-Proskauer test indicated the production of acetoin during glucose fermentation

Table 3: Biochemical Characterization and Isolation of Bacteria Involved in Biogas Production from Cow Dungs

Substrates	Gram reaction	Cat.	Cit.	Urea	Indole	Glu.	Lac.	Fruc.	H2S
Cow dungs	Gram (＋) Rod in chain and with spores	＋	＋	－	－	＋	－	＋	－

Note: Cat. = Catalase, Cit. = Citrate, Glu. = Glucose, Lac. = Lactose, Fruc. = Fructose, Mr. = Methyl red, Vp = Voges-Pro

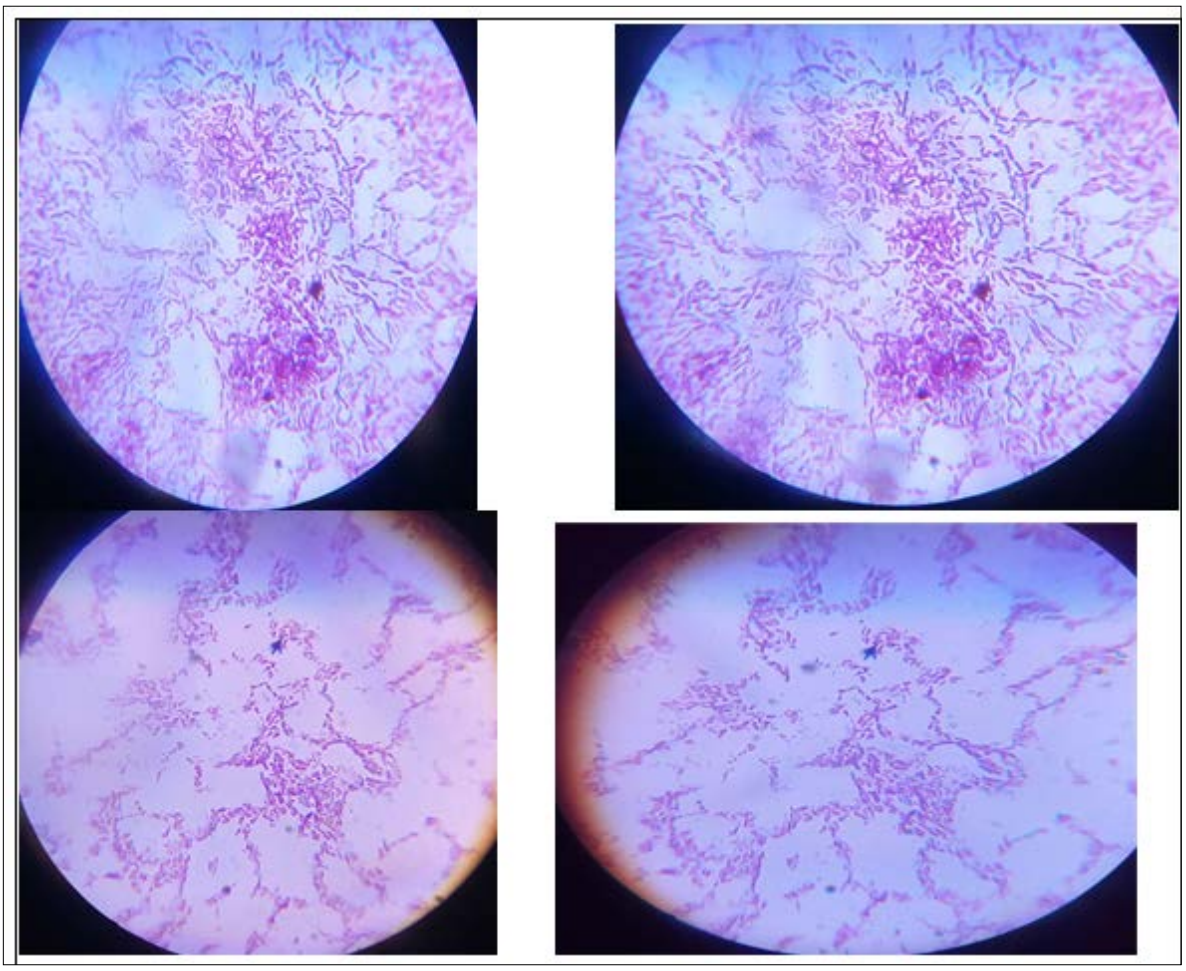

Figure 6: Microscopic Examination of Isolated Bacteria

Discussion

Biogas production, a crucial aspect of renewable energy generation, involves the anaerobic digestion of organic materials by microbial communities. The process yields biogas, primarily composed of methane and carbon dioxide, and a nutrient-rich digestate as byproducts. Factors such as substrate composition, microbial activity, and environmental conditions, particularly temperature, significantly influence the efficiency and quantity of biogas production [31,32].

The results of biogas production showed a dynamic pattern influenced by temperature changes over seven weeks. Biogas yield increased steadily from Week 1 (130.00 mL) to a peak in Week 4 (3223.00 mL), coinciding with a rise in temperature from 26.5°C to 36.5°C. This trend aligns with findings from Kangle, who reported that the microbial community involved in anaerobic digestion thrives in the mesophilic range (30–40°C), enhancing enzymatic activity and substrate breakdown. Ahn et al. similarly observed that optimal mesophilic conditions promote the efficiency of biogas production [32,33].

The sharp decline in biogas yield after Week 4, dropping to 1320.00 mL in Week 5 and further to 70.00 mL in Week 7, can be attributed to substrate depletion and the accumulation of inhibitory byproducts. This observation is consistent with studies by Banks. and Gunaseelan, which noted that the availability of digestible material is a limiting factor as digestion progresses, especially when substrates are not replenished [3,35]. Moreover, the temperature decrease to 26.2°C in Week 7 likely contributed to the reduced microbial activity and subsequent drop in biogas yield, as reported by Weiland, who emphasized the critical role of temperature in maintaining methanogenic efficiency [31].

The findings also corroborate research by Nasir, which highlighted that the initial phase of anaerobic digestion sees increased gas production due to microbial acclimatization and the availability of easily degradable substrates [36]. However, the later phases are characterized by diminishing returns as complex substrates dominate and metabolic inhibitors accumulate. These results suggest that strategies such as co-digestion or periodic substrate addition, as proposed by Ward et al., could help sustain biogas yield over longer durations [37].

The data from Table 2 shows a slight decrease in pH from 7.827 ± 0.093 (before digestion) to 7.227 ± 0.04 (after digestion), reflecting the biochemical changes during anaerobic digestion. This behavior aligns with findings in existing literature, which similarly describe pH dynamics in cow dung during biogas production.

The observed reduction in pH after digestion reflects typical biochemical processes in anaerobic systems. As noted by Angelidaki the acidogenesis phase produces volatile fatty acids (VFAs) such as acetic acid, propionic acid, and butyric acid. These VFAs contribute to a drop in pH during the early stages of digestion. However, as methanogenesis progresses, methanogenic archaea convert VFAs into methane and carbon dioxide, stabilizing the pH near neutral values.

Gerardi emphasizes that an ideal pH range of 6.8–7.5 is critical for methanogenic activity [39]. The near-neutral pH observed after digestion in this study (7.227 ± 0.04) suggests efficient conversion of VFAs and indicates a well-buffered system, as also described by Bhatt, who highlighted the importance of maintaining pH stability for optimal methane yield [40].

The results align with findings from studies on other substrates. Rao et al. reported a more significant pH drop in poultry waste substrates due to higher acid production and lower buffering capacity. In contrast, cow dung's pH stability underscores its suitability as a primary or co-substrate in anaerobic digesters.

Studies by Mshandete and Parawira compared cow dung with kitchen waste, finding that cow dung exhibited slower acid accumulation, maintaining better conditions for methanogens [42]. The similarity in pH behavior across studies reinforces the robustness of cow dung as a substrate for biogas production.

The table (3) indicates the presence of Bacillus subtilis and Bacillus cereus in cow dung, identified through gram staining and biochemical tests such as catalase, citrate, urease, and sugar fermentation assays. These findings align with previous research highlighting the importance of the genus Bacillus in biogas production. These species contribute significantly to the hydrolysis and acidogenesis stages of anaerobic digestion, essential for breaking down complex organic matter into simpler molecules. Both bacteria were Gram-positive, rod-shaped, and spore-forming, typical of the Bacillus genus. This aligns with Gunaseelan, who reported that Bacillus species are well-adapted to anaerobic environments due to their ability to form spores under stress [35].

Bacillus subtilis and Bacillus cereus have been frequently isolated from animal waste substrates used in biogas production [31]. Their enzymatic capabilities make them crucial for the hydrolysis phase, similar to findings by Ahn [33]. The absence of hydrogen sulfide (H2S) production in these isolates is significant, as H2S is a common contaminant in biogas. This aligns with Ward who suggested that bacterial communities in substrates like cow dung contribute to cleaner biogas by minimizing sulfur compounds [37,43].

Conclusion

The study confirms that cow dung substrates provide an effective and stable environment for biogas production, driven by optimal temperature conditions, pH stability, and robust microbial activity. The pH remained near neutral, decreasing slightly from 7.827 ± 0.093 before digestion to 7.227 ± 0.04 after digestion. The mesophilic range (20–40°C) was observed to support robust microbial activity, while thermophilic conditions (45–70°C) improved the degradation of complex organic materials and inactivated pathogens. The microbial diversity within cow dung, comprising hydrolytic, acidogenic, acetogenic, and methanogenic bacteria, is critical to the anaerobic digestion process. These results underscore the potential of cow dung as a renewable energy source while addressing waste management challenges, contributing to sustainable energy solutions. Future research could explore advanced microbial enhancement techniques and co-digestion strategies to further optimize biogas production systems.

Recommendations

1. Introduce microbial inoculants containing hydrolytic, acidogenic, and methanogenic bacteria to boost the digestion process.
2. Employ physical, chemical, or biological pre-treatments to enhance the breakdown of lignocellulosic materials in cow dung, making the organic matter more accessible to microbes.
3. Combine cow dung with complementary substrates such as food waste, agricultural residues, or wastewater sludge to improve the carbon-to-nitrogen ratio and enhance biogas yield.

4. Future research could focus on leveraging advanced microbial techniques, such as metagenomics, to further enhance the efficiency of biogas systems and explore the potential benefits of co-digestion strategies.

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