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Research Article

Biogas Production from Cow-Dung at Low Temperature by Integration of Microbial Electrolysis System

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Abstract

Biogas, a sustainable energy source produced from anaerobic digestion of organic materials, holds significant potential in Nepal due to its abundant agricultural resources. This study investigates the production of biogas from cow dung integrating a microbial electrolysis cell (MEC) system to enhance performance of biogas production under low-temperature (15 ± 0.5°C). The biogas production process was assessed using a pilot-scale MEC system optimized at 2.5 V and 1:5 dilution. Performance metrics included biogas yield, methane content, and COD reduction. The MEC integration significantly improved biogas production, with a 20-fold increase at 2.5 V compared to the control reactor at 15°C. Scaling up to an 8.5 L pilot system demonstrated a fourfold increase in biogas production. Optimal conditions of 2.5 V and 1:5 dilution resulted in an 84.21% COD reduction at 15°C, increasing to 88.28% at the pilot scale. Molecular identification of microbial consortia isolated from biogas production medium were specifically *Bacillus cereus, Bacillus paramycoides, Bacillus licheniformis* and *Priestia flexa* which play crucial role in organic matter degradation and electron transfer in MEC operation at low temperature. Hence, integration of MEC technology substantially enhances biogas production and organic waste treatment efficiency, particularly at low temperature.

Keywords: Anaerobic digestion, Biogas, COD, Cow-Dung, MEC, Methane

Introduction

A viable source of renewable energy in Nepal is domestic biogas technology. Domestic biogas technology is widely used in Nepal's peri-urban and rural regions, and it may be scaled up to take the place of solid fuel cook stove (Lohani et al., 2022). In Nepal, wood is the most common fuel for cooking, followed by LPG, biogas, and other fuels including kerosene and electricity. Traditional indoor burning on inefficient indoor stoves or open fireplaces has caused numerous serious public health issues as well as environmental issues. Homes with inadequate ventilation expose occupants to a variety of air contaminants, including deadly carbon monoxide and particles smaller than 2.5 mm, which have been linked to cataracts, cardiovascular problems, respiratory illnesses and cancer (Gross et al., 2017).

Typically, biogas is composed of 50–70% methane, 30–40% carbondioxide, 5–10% hydrogen, 0-3% nitrogen, and trace amounts of hydrogen sulfide. Methane is the major energy carrier in biogas, which

has a greater heating value of around 38 $MJNm^{-3}$ and is produced by anaerobic decomposition of organic substrates (Gross et al., 2017). Anaerobic digestion (AD) helps to dramatically minimize the possibility of seepage of nitrates into groundwater, releasing nitrates and pathogens into surface water, and emitting aromas from storage lagoons. The digested organic substrates may be used as a beneficial fertilizer because of the increased nitrogen availability and decreased pathogen survival. According to widespread consensus, AD occurs in three steps that follow each other: the hydrolysis or solubilization phase, the acidogenesis or acid-producing phase, and the methanogenesis or methane-producing phase (Weiland, 2010). One of the crucial factors of AD is temperature because it has a great impact on microbial communities, thus varying the efficiency and stability of the digestion process. Methane-producing bacteria perform optimally at temperature ranging from 30-40°C or from 48-60°C (Baweja, 2018). Nepal, with diverse altitudes and climates across its regions, may experience impacts on biogas production in its cooler areas. Lowering temperature during winter season decreases the biogas production because low temperature result in reduced activity and growth rate of methanogens. To minimize the effect of low temperature, microbial electrolysis cell (MEC) can be integrated with AD to enhance the biogas production (Liu et al., 2016). An external voltage is applied between the electrodes to surpass the thermodynamic barrier. Integrating a MEC-AD system enhance performance through several key benefits: boosting methane production, stabilizing processes more effectively, achieving thorough removal of organic matter, efficiently producing methane from hydrogen ions and volatile fatty acids (Carrillo-Peña et al., 2024). This research attempts to enhance production of biogas using electro-methanogenesis technology.

Materials and Methods

Sample collection

Sample of fresh cow-dung was collected from a cow-farm in Kirtipur, Kathmandu.

Physiochemical analysis of sample

For pH analysis, sample was mixed with distilled water, 1:10 dilution and measured pH (HI2002-02 Edge® pH Meter, HANNA) (Estefan et al., 2013). The Association of Official Analytical Chemists' (AOAC, 2000) guidelines were followed in determining the total solid (TS) and moisture content for which sample was weighed and heated at 105 °C overnight. The dried sample was kept in muffle furnace at 550 °C for 2 h to determine ash content and volatile solid (VS) content. For determination of chemical oxygen demand (COD), sample was digested using digestion solution and catalyst solution and 1000 mg/L potassium acid phthalate was used as standard solution as described by Pisal, (2003). The reducing sugar of sample was determined spectrophotometrically using DNSA method (Miller et al., 1961). The ammonical-nitrogen was determined using Nesslers reagent and ammonium chloride (1000 mg/l) as standard solution. For determination of phosphorus and trace elements, sample was digested using H_2SO_4-Salicylic acid–H_2O_2 assay method. The phosphorus was determined by spectrophotometer using potassium dihydrogen phosphate as standard solution while trace elements were determined using atomic absorption spectroscopy (Pisal, 2003).

Biogas production in microbial electrochemical cell (MEC)

For biogas production, inoculum was prepared by using indigenous microorganisms present in cow-dung. About 100 mg of cow-dung was inoculated into 100 ml of anaerobic nutrient broth medium and subjected to anaerobic conditions for 7 days at 28 °C to enrich microbes present in cow-dung indigenously. After two to three subcultures, the culture was utilized as the inoculum in MEC system with or without voltage supply. A pair of carbon felt of dimension 10 cm × 3 cm × 1.5 cm were inserted into the 2 l three neck round bottom flask to make a biological reactor referred as microbial electrochemical cell (MEC) anaerobic reactor (Liu et al., 2016). The voltage supplied was fixed at 1.5 V, 2 V, 2.5 V and 3 V. The experimental control was constructed in common reactor similar to MEC-anaerobic reactor without application of voltage. Before digestion, anaerobic conditions were maintained by eliminating oxygen by the bubbling of nitrogen gas for 10 minutes. The gas volume was measured using measuring cylinder by downward displacement of water and 1 M KOH solution. The experiments were carried out at 15 °C and 28 °C. The sample was diluted at 1:2, 1:5 and 1:10 ratios. The reactors were operated in batch mode for 15

days. Samples after 15 days of digestion were taken and processed for determination of COD, reducing sugar and pH.

Experimental design for scale up process

Large scale digestion was carried out in a carboxy jar of 10 l size. Treated carbon felt electrodes having dimension of 24 cm × 4 cm × 1.5 cm were kept at 6 cm distance. The sample was diluted at 1:5 ratio, and digester was kept at 15°C with 2.4 V supply for 15 days. Measurement of biogas was done by downward displacement of KOH. For analysis of composition, gas was collected in a plastic bag and analyzed by infrared syngas analyzer. The presence of possible different gases like methane, CO_2, N_2, H_2 etc. was analyzed using gas analyzer. Samples after 15 days of digestion were taken from the digester and processed for determination of COD, reducing sugar and pH.

Molecular identification of microorganism

Isolation of microorganism

About 0.1 ml sample after digestion was spread on the agar plate of DSMZ 825 Methanobacterium II media (MMII). The plates were incubated at 37 °C for 24 h in an anaerobic jar. After incubation, the different colonies of bacteria were selected and isolation of pure culture was performed. Liquid culture of bacteria was prepared by inoculating pure colony of bacteria into MMII broth culture tube. The culture tubes were sealed, kept in anaerobic jar and incubated at 37 °C for 24 h (Lozano et al., 2009).

Molecular characterization of microorganism

The genomic DNA of isolated microorganism was extracted using SDS-based technique from the broth culture of isolates (Sambrook & Russell, 2001). The amplification of genomic DNA (gDNA) was carried out by using 16S rRNA primers. The forward primer, U1971-C070-27F-01, 5'-AGAGTTTGATYMTGGCTCAG-3', and reverse primer, U1971-C070-515R-02, 5'-TTACCGCGGCKGCTGGCAC-3' were used. The PCR conditions were initial denaturation (94 °C for 3 min), final denaturation (94 °C for 30 sec), annealing (64 °C for 40 sec), extension (72 °C for 1 min) and final extension (72 °C for 5 min). After completion of 28 cycles of PCR, gDNA was run in 2% gel-electrophoresis at 60 V for 60 minutes and was visualized under gel documentation (Hathway et al., 2021). The amplicons were subjected to sequence analysis. The acquired 16S rRNA gene sequences were input into the MUSCLE algorithm for multiple sequence alignment and phylogeny tree was constructed using Neighbor-joining (NJ) method with bootstrap value 1000 using MEGA v.11 software.

Data analysis

Statistical analysis of data and visualization of generated data was done using GraphPad Prism 8.0.2 (GraphPad Software, 2024), JASP v 0.17.3 (JASP Team, 2024), and Microsoft office excel (Microsoft Corporation, 2018). The associations between two categorical values were evaluated using chi-square test. Student's t-test was used for both paired and independent sample for determination of level of significance. Correlations between two datasets were accessed by Pearson correlation coefficient test and the level of significance was set at p value <0.01.

Results and Discussion

Physiochemical characteristics of cow-dung substrate

The physical and chemical characteristics of cow-dung were shown in Table 1. The pH of sample was found to be 7 and moisture content was 85±0.01%. Total solid (TS) and volatile solid (VS) were found 14.705±0.445 % and 78.905±0.389 % respectively. Our analysis showed the similarity with the study conducted by Gautam et al. (2009); Rawat et al. (2019); and Singh et al. (2021) in which TS and VS were found to be within range of 15-24% and 80-88 % respectively. The total chemical oxygen demand (COD) of cow-dung was found to be 81.91±1.15 mg/g. The reducing sugar content of cow dung was found to be 105.29±4.07 mg/g, indicating a substantial concentration of easily degradable carbohydrates. The amount of ammonia (0.0054±0.0001 mg/g) and phosphorous (0.015±0.001 mg/g), however, in manure is greatly affected by moisture, temperature, pH of manure as well as different microbiological activities (Bleizgys & Naujokienė, 2023). On analysis of trace elements and heavy metals, i.e.; iron (0.22 mg/g), zinc (0.0053 mg/g) and manganese (0.27 mg/g) were found. However, copper and nickel were below the

detection limit of the atomic absorption spectroscopy (AAS).

Table 1: Physicochemical parameters of cow-dung.

Characteristics of sample	Concentration
Total solid	14.705 ± 0.44 %
Volatile solid	78.905 ± 0.38 %
Ash content	21.095 ± 0.38 %
Moisture Content	85.295 ± 0.445 %
Total reducing sugar	105.29 ± 4.07 mg/g
Ammoniacal nitrogen	0.0054 ± 0.0001 mg/g
Chemical oxygen demand	81.91 ± 1.15 mg/g
Phosphorus	0.015 ± 0.001 mg/g
Iron	0.22 mg/g
Copper	Below detection limit
Nickel	Below detection limit
Zinc	0.0053 mg/g
Manganese	0.27 mg/g

Optimization of biogas production at low temperature

Biogas production at low temperature is a crucial topic as production rate deeply falls as temperature decreases. This might be due to reduction in microbial growth and activity at lower temperature. To enhance the biogas production at low temperature, microbial electrolysis cell can be used which provides additional energy to the microbial consortium, particularly the electro-active bacteria, which can facilitate the degradation of organic substrates into the biogas more efficiently under low temperature conditions.

Voltage optimization

To maximize the biogas production, different voltages i.e., 1.5 V, 2 V, 2.5 V & 3 V were applied, in which highest biogas production was observed from the MEC with 2.5 V, which was found to be 464 ± 8.49 ml after 15 days. The MEC with 1.5 V, 2 V & 3 V also showed biogas production of 108.5 ± 12.02 ml, 251 ± 15.56 ml and 346 ± 5.66 ml respectively while control reactor showed least biogas production i.e., 20 ± 5.66 ml in 15 days (Figure 1). The MEC with 2.5 V produced biogas more than 20 times higher than control. The volume of gas produced in MEC reactor upon application of different voltage was found to be statistically significant, which further suggested that when voltage was altered, the amount of gas produced in reactor was also altered significantly. In a related study, Liu et al. (2016) reported that when the cathodic potential was 0.90 V, the methane production was 5.3–6.6 times higher than that of the control group at 10 ºC. The applied voltage drives process of electromethanogenesis, as methanogenic microbes utilize electrons and protons producing methane which is typically coupled with the reduction of CO_2, where the applied voltage reduces CO_2 to methane at the cathode. This reduction works efficiently at certain voltage threshold (Blasco-Gómez et al., 2017). On top of that methanogens are sensitive to the applied voltage. Different methanogens might have optimal voltage ranges for their activity (Siegert et al., 2015). The increased gas generation at low temperatures may be caused by the MEC system's improved microbial metabolism in a number of ways, including increased activity of key enzymes. However, research on the primary methane generation pathway, the structure of the functioning microbial population, and the activity of a crucial enzyme in MES was rarely conducted at low temperatures (Wang et al., 2022).

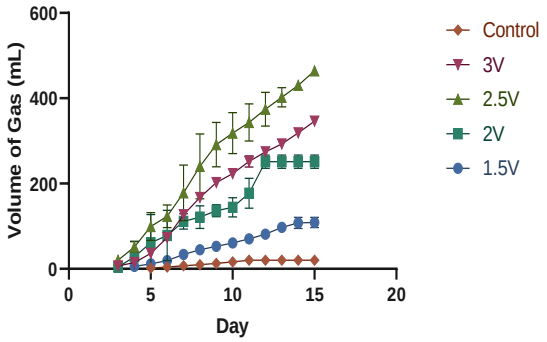

Figure 1: Biogas production from MEC with application of different voltages at 15 ºC and 1:10 dilution.

Dilution optimization

Three dilutions (1:10, 1:5 and 1:2) were used at optimized voltage of 2.5 V at 15 °C in which the MEC of 1:5 dilution showed the highest biogas production of 607 ± 24.04 ml whereas 1:10 and 1:2 dilution produced 464 ± 8.49 ml and 228 ± 16.97 ml biogas respectively. The control system without voltage supply with dilution of 1:10, 1:5 and 1:2 produced 20 ± 5.66 ml, 44.5 ± 3.54 ml and 72.5 ± 12.02 ml respectively after 15 days (Figure 2). The volume of gas produced in MEC reactor upon application of different dilution was statistically

significant (p<0.0001), which further suggested that alteration in dilution of substrate results in the alteration of biogas production significantly also indicated by strong positive Pearson's correlation coefficients across dilutions. Lower dilutions is likely led to substrate inhibition or mass transfer limitations, reducing gas production, whereas higher dilutions might have provided insufficient substrate for optimal microbial activity, as observed in previous studies by Siegert et al. (2015) and Zhang et al. (2019).

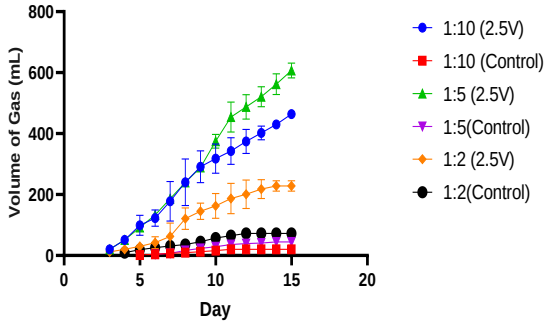

Figure 2: Biogas production by 1:10, 1:5 and 1:2 dilution at 15 °C and 2.5 V.

Scaling up biogas production

The biogas production was scaled up to pilot scale of working volume 10 L at 15 °C, in which MEC produced biogas of 3333 ± 29.69 ml and control produced 779.5 ± 20.51 ml after 15 days (Figure 3). The MEC produced 4 times more biogas as compared to control. This significant increase in biogas production is likely due to the applied voltage (2.5 V as optimized in lab scale) in the MEC, which is found to enhance the electrochemical reactions and microbial activity, leading to more efficient substrate utilization and higher biogas yields (Villano et al., 2010). The pilot scale also has higher and improved mass transfer and substrate availability, which further supports microbial activity and this leads in increase in overall gas production (Blasco-Gómez et al., 2017; Siegert et al., 2015).

Comparison of biogas collection using KOH and water displacement method

The total collected biogas volume was observed to be higher in MEC with water displacement which was found to be 741.5 ± 13.44 ml as compared with KOH displacement i.e., 607 ± 24.04 ml, indicating that 18% of total biogas thus produced was CO_2 in MEC reactor at 15 °C, 2.5 V and 1:5 dilution (Figure 4). The biogas produced in control reactor with water and KOH displacement was found to be 95.5 ± 2.12 ml and 44.5 ± 3.54 ml respectively, suggesting presence of 53% CO_2 in total biogas composition. The statistically significant difference shows that the choice of displacement solution has a significant impact on the biogas collection. According to Ahn & Logan (2010) and Mourad et al. (2022), KOH resulted a more accurate reflection of methane content but overall lower total biogas volume was collected comparing to that of water displacement method.

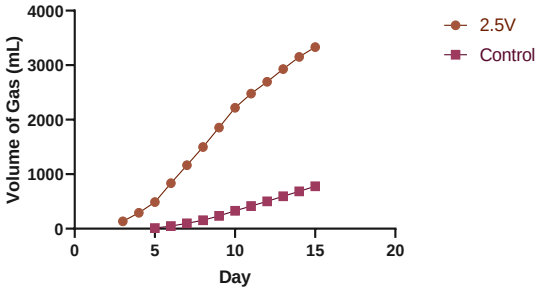

Figure 3: Biogas production in pilot scale of working volume 8.5 l at 15°C, 1:5 dilution and 2.5 V.

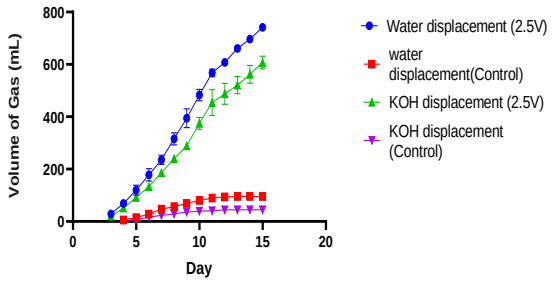

Figure 4: Comparison of biogas collected by water and KOH displacement at 15°C, 2.5V and 1:5 dilution.

Reduction in pH, COD and reducing sugar after digestion

The COD reduction results (Figure 5a) indicated that MEC system significantly enhanced treatment efficiency compared to control, with the most effective conditions found to be at 2.5 V and a 1:5 dilution. At this optimized voltage condition, the 81.59% COD reduction at 15 °C was observed in MEC, which increased to 88.28% when applied to pilot scale. This COD reduction is more than double that of control setup, which signifies MEC's performance in organic matter degradation. The

COD reduction data showed statistical significance with different voltages and dilutions, with *p*-values nearly equal to zero, confirming that 2.5 V and a 1:5 dilution were optimal for maximizing treatment efficiency as stated by Li et al. (2021); Logan et al. (2006); Mandal & Das (2018). There was significant reduction in soluble reducing sugar using MEC under various conditions, with the highest reduction (56.03%) observed at 2.5 V and 15 °C (Figure 5b). In a similar study, Feng et al. (2010) identified 2.5 V as optimal for enhancing organic matter degradation. According to Prathiba et al. (2022), microbial activity and electron transfer efficiency are also influenced by substrate concentration.

A strong correlation was seen between voltage and gas production (r = 0.878), as well as COD reduction (r = 0.854), suggesting that optimized voltage enhance metabolic activity of microorganisms and efficiency of degradation of organic substrate. Ding et al. (2016); Marmanis et al. (2022) further validates our findings. Dilution levels also significantly affected MEC performance, with correlations between dilution and gas production (r = 0.851) and COD reduction (r = 0.972). The pH levels remained relatively stable during the anaerobic digestion process, with only minimal fluctuations observed across various conditions as shown in Figure 5c. The pH values consistently remained between 6.7 and 7.0, indicating that the changes were statistically insignificant for voltage optimization as well as for dilution optimization.

Analysis of biogas composition

The analysis of biogas composition showed that the MEC system produced a significantly higher methane content than that of control reactor with lower CO_2 level in MEC compared to control reactor as shown in Table 2. The statistically significant differences in methane and CO_2 composition in those reactors (p = 0.02) indicated that MEC also helps in enhancing methane production and reducing CO_2 content. Thus, we can suggest that the MEC system improves the overall efficiency and quality of biogas production by increasing the methane to CO_2 ratio. Wellinger (2013) and Li et al. (2019) suggests that the raw biogas is normally composed of methane (50–75%), carbon dioxide (25–50%), and smaller amounts of nitrogen and other trace elements which also depends on the feedstock used. The higher production of methane in MEC reactor may be attributed to the favorable condition for efficient electron transfer processes that favor methanogenesis over other competing microbial processes (Wang et al., 2021; Zhao et al., 2021).

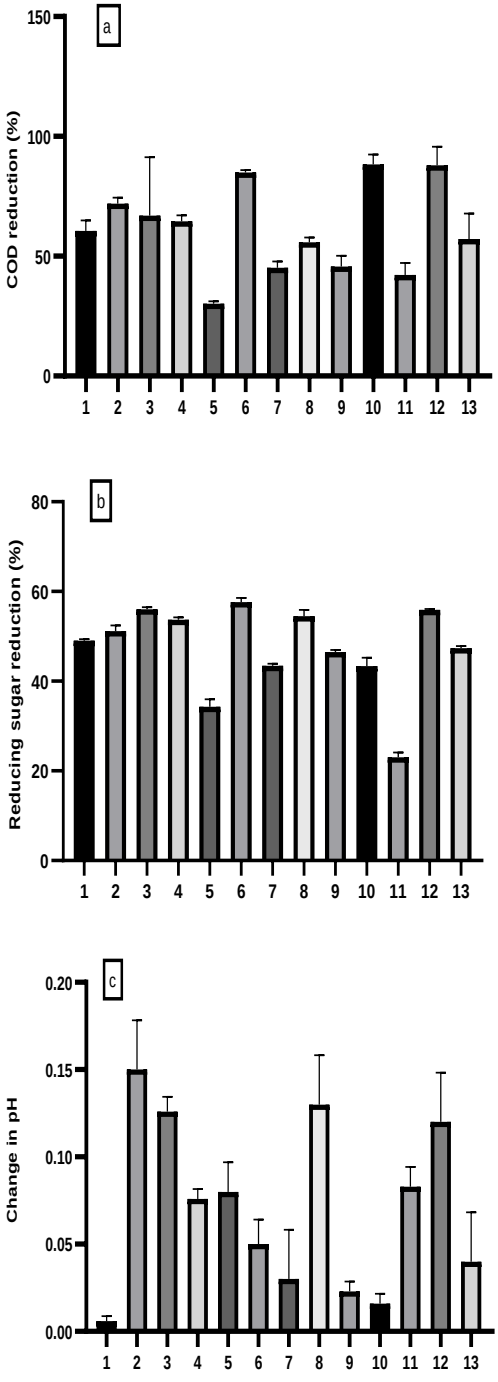

Figure 5: Reduction in a) Chemical oxygen demand b) Reducing sugar and c) Change in pH after 15 days of digestion under different conditions (1. MEC 1.5V (1:10), 2. MEC 2V (1:10), 3. MEC 2.5V (1:10), 4. MEC 3V (1:10), 5. Control (1:10), 6. MEC 2.5V (1:5), 7. Control (1:5), 8. MEC 2.5V (1:2), 9. Control (1:2), 10. MEC 2.5V (1:5, 8.5L), 11. Control (1:5, 8.5L), 12. MEC 2.5V (1:5,28°C) and 13. Control (1:5, 28°C)).

Table 2: Biogas composition in MEC and control setup and their p-value.

Gas	MEC	Control	Percentage of difference	*p*-value
CH_4	82.45±1.58	72.66±1.58	11.86	0.02
CO_2	11.35±1.25	20.68±1.59	45.15	0.02
H_2	4.67±0.64	4.41±0.64	5.67	0.71
Other	1.55±0.30	2.25±0.66	31.18	0.30

Isolation and molecular identification of microorganisms

From the sample after digestion in MEC, four different bacteria (MK1, MK2, MK3, and MK4) were isolated. The genomic DNA of four isolates were extracted and amplified by PCR using 16S rRNA primer. The amplified DNA was run on gel electrophoresis and visualized under gel documentation in which size of the amplified DNA of the four isolates was found to be 516 bp, which falls just above 500 bp on the ladder as shown in Figure 6.

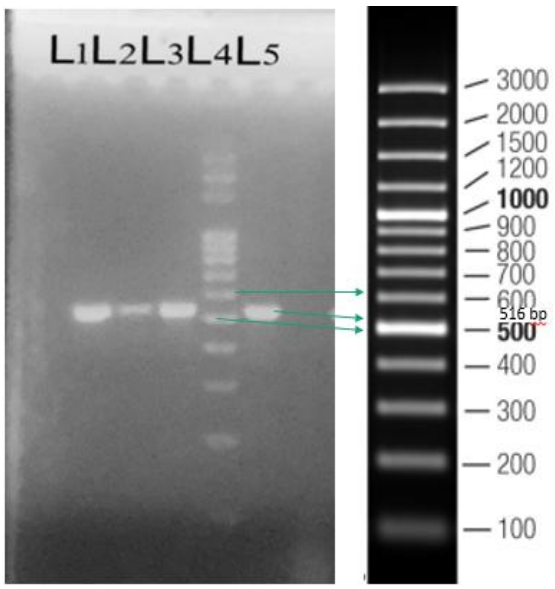

Figure 6: Gel electrophoresis of PCR products; L1, L2, L3 and L5 represents amplified DNA of isolate MK1, MK2, MK3 and MK4 respectively and L4 indicates Generuler 100 bp ladder (0.1 µg/µl).

The sequences of isolates MK1, MK2, MK3 and MK4 showed the evolutionary relationship with *Bacillus cereus*, *Bacillus paramycoides*, *Priestia flexa* and *Bacillus licheniformis* respectively based on 16S rRNA sequences (Figure 7). Li et al. (2023); Senés-Guerrero et al. (2019); Siddharth et al. (2024); Sun et al. (2023) reported that *Bacillus* species were frequently isolated from anaerobic environments, including biogas reactors and wastewater treatment plants. The close relationship of isolate MK3 with *Priestia flexa*, a relatively lesser-known member of the Bacillaceae family, which can degrade mucin and is better acclimatization in the human GI environment (Deswal et al., 2023) suggests that this strain may also play a specialized role in the anaerobic digestion process, potentially contributing to specific biochemical pathways that support the microbial community's stability and efficiency. *Bacillus* species, commonly enriched in cow dung due to its nutrient-rich environment (Behera & Ray, 2021; Bhatt & Maheshwari, 2019), play a crucial role during operation of MEC at low temperature. These microbes are essential to carry out decomposition of complex organic compounds into a much simpler forms necessary for sustaining microbial processes within the MEC, even at low temperatures i.e. 15 °C (Bazina et al., 2023). These species produces cellulases and proteases which are necessary for degradation of organic matter, crucial at low-temperature environments where microbial metabolic rates generally decrease (Li et al., 2019). *Bacillus* species have also been observed to facilitate transfer of extracellular electron, an essential process in MECs, by forming biofilms on the anode that enhance electron transfer from the oxidation of organic compounds at an anode to cathode (Chen et al., 2019; Yu et al., 2023). The ability of extracellular electron transfer is particularly significant at low temperatures, where the efficiency of electron transfer ability of other various species often diminishes, but *Bacillus* species can sustain this process by adopting different changes in membrane structure at low temperature (Beranová et al., 2010; Deng et al., 2023).

Conclusion

The study demonstrates the significant potential of microbial electrolysis cells (MEC) in enhancing biogas production from cow dung under low-temperature conditions (15 ± 0.5°C). The optimized voltage of 2.5 V led to a substantial increase in biogas production. Dilution optimization revealed that 1:5 dilution produced the highest biogas yield. Additionally, scaling up to a pilot reactor (10 l) confirmed the efficacy of MEC. The MEC also showed significant reduction of COD and reducing sugars. The MEC system enhanced biogas quality, increasing methane and reducing CO_2 content. The

microbial community identified four major strains of bacteria showing close evolutionary relationship with *Bacillus cereus*, *Bacillus paramycoides*, *Bacillus licheniformis*, and *Priestia flexa*, which play crucial roles in organic matter degradation and electron transfer, vital for MEC operation at low temperatures. These findings effectively illustrated the potential of MEC technology to enhance both the quantity and quality of biogas production from cow dung.

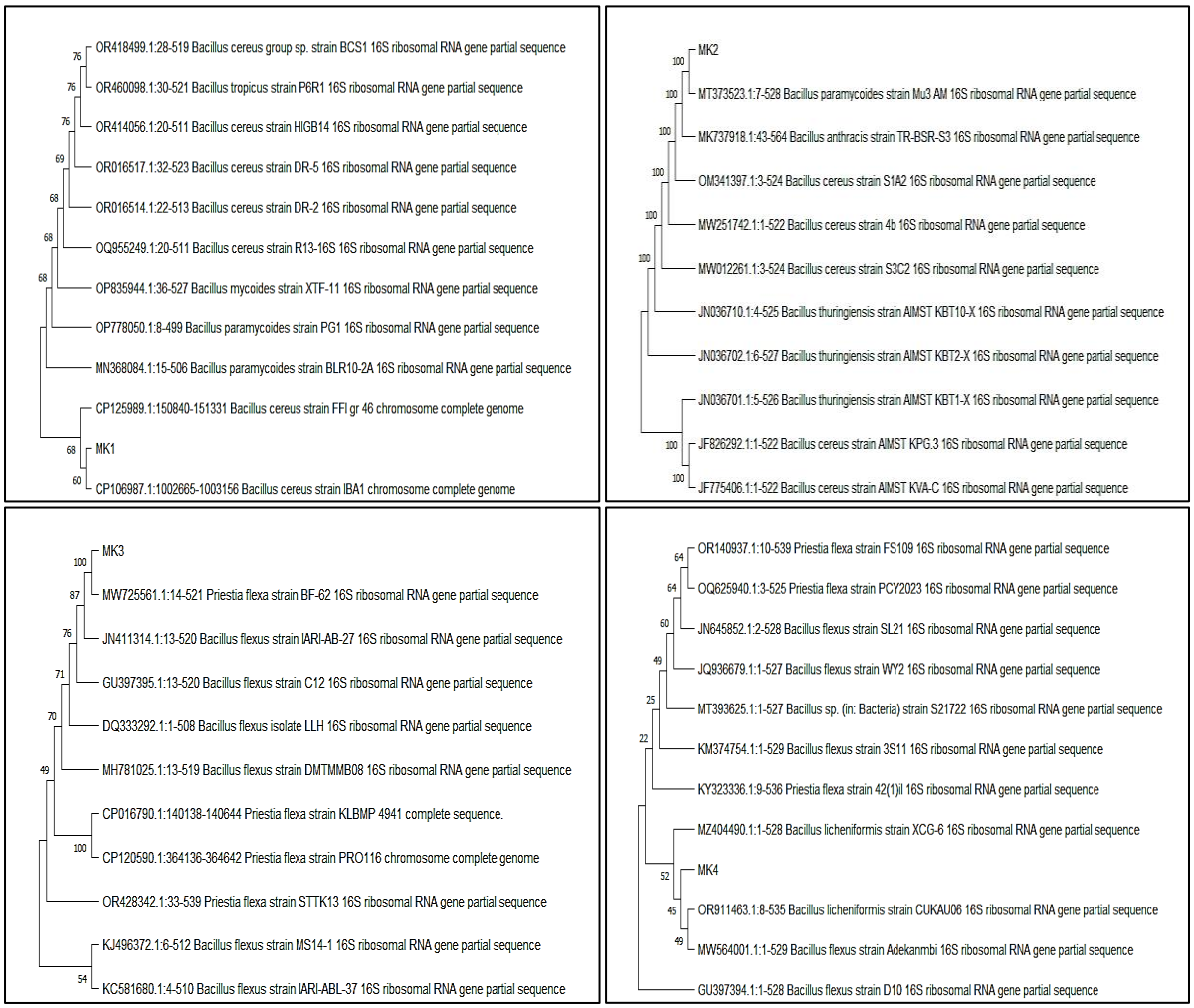

Figure 7: Phylogenetic tree of isolates MK1, MK2, MK3 and MK4.

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