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Enhancement of Biogas Production and Archaeal Diversity during Anaerobic Digestion of Bacterially Pretreated Coconut (*Cocos nucifera L.*) Coir

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Abstract

Growing demand for renewable energy and sustainable waste management has increased interest in anaerobic digestion of lignocellulosic biomass. However, efficient conversion of such substrates remains limited by their complex structure, particularly the recalcitrant lignin–cellulose matrix, which restricts microbial accessibility and results in low hydrolysis efficiency. Coconut coir, a widely available agricultural waste, is particularly resistant to biodegradation, thereby limiting its potential as a feedstock for high-yield biogas production. This study aimed to enhance biogas production from coconut coir lignocellulosic biomass pretreated with cellulose-degrading bacteria for 150 days using standard protocols. Initial screening of bacteria from poultry feces revealed very high cellulose hydrolytic capacities, ranging from 9 to 11 mm. The identified bacteria included *Bacillus megaterium*, *Bacillus licheniformis*, *Lactobacillus plantarum*, *Bacillus cereus*, and *Bacillus subtilis*. Coconut coir was pretreated with a consortium of these bacteria, buffered with plantain peel ash solution, and inoculated with aged cow dung for anaerobic digestion. A control digester without bacterial pretreatment was also maintained. The total biogas yield in the control digester at the end of 150 days digestion was 67,105 mL, whereas the pretreated substrate produced 88,355 mL. The mean methane content was 45 ± 25% in the control and 56 ± 17% in the pretreated substrate, and indicated a significantly higher methane yield in the pretreated substrate ($p < 0.05$). Sequencing of PCR products amplified using universal 16S rRNA primers generated 244, 240 sequences, of which 236, 200 (97%) were identified as bacterial, 2,443 (1%) as archaeal, and 5,597 (2.3%) remained unclassified. The bacterial pretreatment of coconut coir enhanced both biogas volume and methane yield and influenced the abundance and diversity of the methanogenic archaeal community. These findings suggest that the use of cellulose-degrading bacteria from poultry feces and an organic buffering agent can significantly improve biogas production and methane content, providing a sustainable approach to biomass exploitation.

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1.0 Introduction

Lignocellulosic biomass derived from plants is a valuable and renewable carbon source with the potential to replace non-renewable fossil fuels. Its use in biogas generation has gained considerable attention because it provides fermentable sugars from cheap and widely available plant substrates (Eduok *et al*., 2018; Paul & Dutta, 2018). However, the complex structure and composition of lignocellulose, characterized by the presence of cellulose, hemicellulose, lignin, and trace amounts of ash, pectin, and proteins, hinder its biodegradability in anaerobic systems, a phenomenon referred to as biomass recalcitrance (Baruah *et al*., 2018; Beluhan *et al*., 2023; Mulat *et al*., 2018; Poszytek *et al*., 2016). Furthermore, cellulose microfibrils embedded within lignin–carbohydrate complexes (LCCs) increase biomass recalcitrance (Pu *et al*., 2013). Therefore, pretreatment of lignocellulosic biomass using physical, chemical, or biological methods either individually or in combination is necessary to enhance permeability, break cellulose–hemicellulose–lignin linkages, and expose fermentable cellulose for microbial conversion (Eduok *et al*., 2018; Gaur *et al*., 2017).

Among these methods, biological pretreatment using (i) pure, isolated cellulolytic enzymes or enzyme complexes or (ii) microbial consortia shows promise for efficient substrate degradation (Ferdes *et al*., 2020). Microbial consortia, in particular, offer enhanced stability under varying environmental conditions (Ferdes *et al*., 2020; Ali *et al*., 2019), leading to significant improvements in lignocellulosic biomass degradation and subsequent biogas production efficiencies (Li *et al*., 2020; Ali *et al*., 2019). For instance, biological pretreatment with mixed microbial cultures increased the degradation efficiencies of hemicellulose, cellulose, and lignin to 44%, 35%, and 39%, respectively (Li *et al*., 2020), whereas pretreatment with a bacterial consortium resulted in a high degree of hydrolysis of lignocellulosic content in sawdust and increased methane yield by more than 60% (Ali *et al*., 2019; Ali *et al*., 2020). Microbial consortia, including cellulose-degrading bacteria and fungi such as white rot fungi and various *Clostridium* species, produce ligninolytic and cellulolytic enzymes crucial for biomass breakdown (Poszytek *et al*., 2016), thereby playing a pivotal role in hydrolysing complex organic matter during anaerobic digestion.

Several studies have assessed anaerobic cellulose-degrading bacteria and their enzymatic capabilities to understand hydrolytic mechanisms and improve degradation rates, particularly from plant litter, composting material, and soil microbial communities capable of degrading diverse lignocellulosic substrates such as rice straw, wheat straw, corn stalk, paper, cotton, and cassava residues (Wang *et al*., 2011; Guo *et al*., 2011; Poszytek *et al*., 2016). In addition, although several reports have demonstrated enhanced methane production following biological pretreatment, comparatively little information exists regarding the microbial ecological mechanisms underlying these improvements, particularly the response of methanogenic archaeal communities during anaerobic digestion. Since archaea are directly responsible for methane formation, understanding shifts in archaeal diversity, abundance, and community composition is essential for elucidating the mechanisms through which biological pretreatment improves digester performance. Such information remains limited for highly recalcitrant substrates such as coconut coir.

Therefore, this study aimed to isolate and characterize cellulose-degrading bacteria from poultry faeces and evaluate their effectiveness as biological pretreatment agents for coconut coir lignocellulosic biomass. The study further assessed the influence of bacterial pretreatment on anaerobic digestion performance through analyses of biogas production and changes in the

structure, abundance, and diversity of archaeal communities. This work provides insights by utilizing poultry faeces-derived cellulolytic bacteria as pretreatment agents, investigating the anaerobic digestion potential of the relatively understudied coconut coir substrate, and elucidating microbial community shifts associated with enhanced methane production following biological pretreatment.

2.0 Materials and Methods

2.1 Isolation, screening and identification of cellulose degrading bacteria

Fresh cow dung and poultry feces were collected into sterile 25 L plastic containers from Animal Science Department, and transported to the Microbiology laboratory for processing and analysis. To isolate, screen and characterize cellulose-degrading-bacteria from poultry feces, we used the culture-dependent microbiological enrichment technique. Precisely 1 g of poultry feces was inoculated onto minimal salt medium (0.2% KH_2PO_4, 0.2% K_2HPO_4, 0.2% NH_4NO_3, 0.05% NaCl, 0.05% $MgSO_4.7H_20$, 0.001% $Fe_2SO_4.7H_2O$, 0.001% $MnSO_4.H_20$, pH 7.0) supplemented with 1% cellulose powder, incubated at 37 ± 2℃ and 100 rpm for 7 days in a shaker incubator (Kallenharm Company Inc. England). Thereafter, cultures were serially diluted, and a 0.1 mL aliquot from the 10^{-5} dilution was spread-plated on carboxymethyl cellulose (CMC) agar composed of peptone 10.0g, carboxymethyl cellulose 10.0g, K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.3g, $(NH_4)2SO_4$ 2.5 g, gelatine 2.0 and agar 15g. The plates were incubated at 37 ± 2 °C for 72 hours. Discrete bacterial colonies that grew on the plates were selected and purified by subculturing on fresh medium. The pure isolates were characterised, identified and screened for cellulose-degrading potential (Rawway *et al*., 2018). Each bacterial isolate was inoculated onto carboxymethyl cellulose (CMC) agar plates and incubated at 37 ± 2 °C for 72 hours. After incubation, the plates were flooded with 0.1% (w/v) Congo red solution and allowed to stand for 15 minutes at room temperature (27 ± 2 °C), followed by washing with 1 M NaCl. The appearance of a clear hydrolytic zone surrounding the colonies was taken as evidence of cellulose degradation. In addition, we qualitatively estimated the cellulose-degrading potential of the positive isolates by calculating hydrolytic capacity, the ratio of diameter of clearing zone and colony (Gupta *et al*., 2012).

A loopful of the pure bacterial isolates with hydrolytic capacity above 8.0 were inoculated onto CMC broth and incubated for 10 days at 27 ± 2 °C. The turbidity of bacterial suspension was adjusted using CMC broth. Density of the bacterial cell suspension was compared to the McFarland turbidity standard of 0.5 known to provide an optical density comparable to the density of a bacterial suspension with a 1.5 x 10^8 CFU/mL was prepared as previously described by Gayathiri et al. (2018). Pure cultures of the cellulose-degrading bacterial isolates with hydrolytic capacity above 8.0 were identified based on their colonial, morphological and biochemical characteristics compared with known taxa described in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

2.2 Mass cultivation and standardization of cellulose-degrading bacteria

Cellulose-degrading bacterial isolates that exhibited high hydrolytic activity were mass-cultivated in carboxymethyl cellulose (CMC) broth. A loopful of pure culture was inoculated into sterile CMC broth and incubated for 10 days at room temperature to obtain sufficient biomass for pretreatment. The bacterial suspension was standardized to 0.5 McFarland turbidity standard, corresponding to approximately 1.5 × 10^8 CFU/mL (Gayathiri *et al*., 2018). The McFarland standard was prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) with 9.95 mL of 1% sulfuric acid (H_2SO_4). The turbidity of the bacterial suspension was visually matched against the standard under light illumination using a white background with contrasting black lines, and

adjusted using sterile CMC broth where necessary.

2.3 Pretreatment of coconut coir

Coconut coir, the fibrous material between the shell and outer coat of a coconut (*Cocos nucifera L.)* was obtained from Municipal solid waste dumpsite. The coconut coir was air-dried; milled to particle size between 0.5 and 2 mm to increase the surface area. The milled coconut coir was subjected to solid state fermentation with a near absence of free moisture from water.

Briefly, the substrate was divided into two parts; one part made of 6 kg of coconut coir was enriched with 500 mL of the standardized cellulose degrading bacteria (0.5 McFarland; ~1.5×10^8 CFU/mL) in 16 L of water, whereas the other part, contained 6 kg of coconut coir moistened with 16 L of water served as the control. The substrate was separately and manually mixed in 60 L plastic containers every 48 h, allowed to stand for 28 days at greenhouse temperature of 29 ± 2 ºC and fed into the pilot scale anaerobic reactors. The 28-day pretreatment period was selected based on a study by Eduok *et al*. (2018), which reported that effective microbial colonization and lignocellulosic degradation in similar substrates typically occur within a 3 to 4 week incubation period under mesophilic conditions, allowing sufficient time for hydrolysis and microbial adaptation.

2.4 Inoculum, buffering and anaerobic digester set-up

Cow dung was used as the inoculum because of the rich methanogenic microbial community and its well-established suitability for anaerobic digestion and biogas production (Gaur and Suthar, 2017). Prior to inoculation, the seed inoculum (cow dung) was incubated at 37 ± 0.2 °C for 25 days to stabilize methanogenic activity and ensure that biogas production was primarily derived from the degradation of the substrate (Eduok *et al*., 2018).

Six batch anaerobic digesters with a capacity of 25 L each were fabricated from cylindrical plastic drums. Each reactor was fitted with an upper outlet for gas collection and a bottom outlet for sampling. The experiment was conducted in triplicate for both control and treated setups as follows: control reactors contained 17 kg of coconut coir, 3 kg of cow dung, and 12 L of water, while treatment reactors contained 17 kg of coconut coir pretreated with cellulose-degrading bacteria, 3 kg of cow dung, and 12 L of water.

Plantain (*Musa paradisiaca L*.) peel was collected from a municipal solid waste dumpsite, sun-dried, and incinerated. Subsequently, 80 g of plantain peel ash was dissolved in 8 L of distilled water and used as a buffering agent to maintain a stable pH during anaerobic digestion. The initial pH of the buffer solution was 11.40. The substrates were thoroughly mixed to form slurries and then fed into triplicate digesters. The digesters were filled to 60% of their total volume, leaving 40% headspace for biogas accumulation. The digesters were tightly sealed to maintain anaerobic conditions and manually agitated once daily to prevent scum formation and ensure uniform digestion. The reactors were maintained under mesophilic conditions in a greenhouse at an ambient temperature of 29 ± 2 °C throughout the experimental period.

2.5 Measurement of biogas volume and composition

The biogas volume was measured at three days interval for 150 days by connecting each digester to a graduated reverse cylinder device containing water as a barrier solution and the liquid displacement measured A portable biogas analyser (IRCD4, Shi'an Tech Instrument and Co., Bejing, China) was used for measurement of biogas composition. The biogas analyser was calibrated by the manufacturer. Prior to calibration, 100% nitrogen gas was used to flush the gas analyser. The measurement ranges were 0–100% (CH_4), 0 to 100% (CO_2), 0 to 25% (O_2) and 0 to 1,000 ppm (H_2S).

2.6 Molecular characterization of samples

2.6.1 Microbial DNA extraction and gradient centrifugation

Microbial biomass and diversity were determined as previously described (Eduok, 2013). A zymo-spintm iv-hrc spin filter was first prepared by snapping off the base, inserted into a collection tube, and spun in a microcentrifuge at 8,000 g for 3 minutes. Genomic DNA was extracted from the samples by transferring 0.25 g of each sample into different ZR BashingBeadTM Lysis Tubes. A total of 750 µL of lysis solution was added to each tube and vortexed for 5–10 minutes. The resulting mixture was placed in a bead beater equipped with a 2 mL tube holder and centrifuged at 10,000 g for 1 minute. The supernatant was then transferred to a ZymoSpin™ IV Spin Filter fitted into a collection tube and centrifuged at 7,000 g for 1 minute. Subsequently, 1,200 µL of DNA binding buffer was added to the filtrate, and 800 µL of this mixture was loaded onto a Zymo-Spin™ IIC column in a new collection tube, followed by centrifugation at 10,000 g for 1 minute. The flow-through was discarded, and 200 µL of DNA pre-wash buffer was added to the column and centrifuged under the same conditions. Next, 500 µL of DNA wash buffer was added, and the column was centrifuged again at 10,000 g for 1 minute. The Zymo-Spin™ IIC column was then transferred to a sterile 1.5 mL microcentrifuge tube, and DNA was eluted by adding 100 µL of elution buffer, followed by centrifugation at 10,000 g for 30 seconds. The eluted DNA was further purified using a Zymo-Spin™ IV-HRC Spin Filter placed in a clean 1.5 mL Eppendorf tube and centrifuged at 8,000 g for 1 minute. The resulting filtrate known as the filtered DNA was considered the genomic DNA.

2.6.2 Polymerase chain reaction and next generation sequencing

The extracted DNA samples were amplified by polymerase chain reaction using a 96 well thermal cycler (2ETM UK). Labelled Eppendorf tubes with sample codes and primer name on the top and sides were used for amplification. The amplifications were done using a universal primer pair 341F and 785R - targeting the V3 and V4 region of the 16S rRNA gene. Resulting amplicons were gel purified, end repaired and Illumina specific adapter sequence was ligated to each amplicon (NEBNext Ultra II DNA library prep kit). Following quantification, the samples were individually indexed (NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1), and another AMPure XP bead-based purification step was performed. Amplicons were then sequenced on Illumina's MiSeq platform, using a MiSeq v3 (600 cycle) kit to generate paired-end reads (2 × 300 bp), following the manufacturer's protocol.

2.6.3 Bioinformatics and sequence data analysis

Raw sequencing reads were processed using CLC Genomics Workbench version 7.5.1. Quality filtering was performed to remove low-quality reads, ambiguous bases, and adapter contamination based on Phred score thresholds. Paired-end reads were merged, and chimeric sequences were removed prior to downstream analysis. Operational taxonomic units (OTUs) were clustered at 97% sequence similarity, and taxonomic assignment was performed using BLAST against the NCBI nucleotide database. Sequence depth was normalized across all samples prior to diversity analysis. Alpha diversity was assessed using Shannon and Simpson diversity indices, while species richness was estimated using observed OTUs. Sequencing coverage was evaluated using Good's coverage estimator to assess sampling completeness. Beta diversity analysis was performed using Bray–Curtis dissimilarity and visualized using Principal Coordinates Analysis (PCoA) to compare microbial community structure across treatments. All processed sequences were deposited in the NCBI Sequence Read Archive (SRA) under accession number KJLG00000000.

2.7 Statistical analysis

The differences between treatments were assessed using one-way analysis of variance (ANOVA) in Statistica software® version 22 (StatSoft, Tulsa, OK, USA). In addition, correlation analysis was performed to evaluate the relationships between selected variables. All experiments were conducted in triplicate (n = 3),

and results are presented as mean ± standard deviation (SD). Statistical significance was determined at $p < 0.05$, and results are reported as either significant ($p < 0.05$) or not significant ($p > 0.05$), as appropriate.

3.0 Results and discussion

3.1 Cellulose degrading bacteria from poultry feces

The breakdown of coconut coir lignocellulosic biomass by microorganisms from poultry feces was evaluated by measuring the hydrolytic capacity, which ranged from 5 to 11 mm and 25 to 57 mm in diameter clear zones (Table 1). The variable cellulose breakdown potentials demonstrated by the clear zones around the bacterial isolates suggest that poultry feces are a rich source of cellulolytic bacteria. A total of 9 cellulose degrading bacteria were isolated, with five exhibiting high cellulose hydrolytic capacity (HC) that ranged from > 9 to 11 mm, while 4 showed a moderate HC of 5 to 7 mm. This variation in HC is attributed to differences in the cellulase enzymes produced by these isolates. The observed range of HC values is consistent with previous studies reporting ranges between 4.3 and 9.8 (Gupta *et al*., 2012), although higher HC value between 5 and 29 mm have been reported for cellulolytic aerobic bacterial isolates from agricultural waste dump site (Maravi and Kumar, 2020). The 5 bacterial isolates with HC > 8.0 were selected, identified and used in substrate pretreatment. The isolates and their similarity index were *Bacillus megaterium* (90.54%), *Bacillus licheniformis* (97.6%), *Lactobacillus plantarum* (74.53%), *B. cereus* (96.93%) and *B. subtilis* (96.14%). This result is consistent with previous studies, which have reported the use of various *Bacillus* sp. including *Bacillus megaterium* (Pinotti *et al*., 2020), *Bacillus licheniformis* (Maravi and Kuma, 2020; Huang *et al*., 2012), *B. cereus* (Vimal *et al*., 2016; Patagundi *et al*., 2014) and *Bacillus subtilis* (Rawway *et al*., 2018; Patagundi *et al*., 2014) for cellulase production. *Lactobacillus plantarum* RI 11 is a versatile bio-transformation agent for lignocellulosic biomass because it produces extracellular cellulolytic and hemicellulolytic enzymes concomitantly (Zabidi et al., 2020).

Table 1: Cellulose hydrolytic capacity of bacteria from poultry feces

Isolate code	Diameter of clearing zone (mm)	Diameter of colony (mm)	Hydrolytic capacity
PDI	25	4.86	5.14
PD2	28	5.56	5.04
PD3	30	5.36	5.60
PD4	40	4.34	9.22
PD5	41	5.96	6.88
PD6	43	3.95	10.89
PD7	40	4.59	9.71
PD8	53	4.89	11.2
PD9	57	5.41	10.54

Key: < 2.0 = Low cellulose hydrolytic capacity, 2.1 – 4.9 = Moderate cellulose hydrolytic capacity; 5.0 – 8.9 = High cellulose hydrolytic capacity, ≥ 9 = Very high cellulose hydrolytic capacity.

3.2 Distribution of Microbial Sequence Reads in Digestates and Inoculum

Table 2 presents the distribution of sequence reads obtained from the control digestate, pretreated digestate, poultry feces, and cow dung samples following microbial community analysis. A total of 244,240 sequence reads were generated across all samples. Bacterial sequences were the predominant microbial group, accounting for 236,200 reads (96.71%) of the total sequences, whereas archaeal sequences represented 2,443 reads (1.00%). Unclassified sequences accounted for 5,597 reads (2.29%). Among the analyzed samples, the pretreated

digestate recorded the highest number of bacterial sequences reads (90,682), followed by cow dung (57,909), poultry feces (46,760), and the control digestate (40,849). The predominance of bacterial sequences suggests intensive bacterial involvement in the hydrolysis and fermentation stages of anaerobic digestion, particularly during lignocellulosic biomass degradation. Similar dominance of bacterial communities during anaerobic digestion has been reported in previous studies, where bacteria were identified as the primary drivers of substrate decomposition and volatile fatty acid production (Ziganshin *et al*., 2013; De Vrieze *et al*., 2015).

Archaeal sequence reads were comparatively lower than bacterial reads, with the control digestate containing slightly higher archaeal abundance (1,242 reads) than the pretreated digestate (1,119 reads). Poultry feces and cow dung contained relatively few archaeal sequences. This observation agrees with the known functional roles of archaea in anaerobic digestion, where methanogenic archaea are specialized microorganisms involved mainly in the terminal methanogenesis stage, while bacteria dominate the earlier stages of substrate hydrolysis and acidogenesis (Liu and Whitman, 2008). The pretreated digestate also exhibited a higher proportion of unclassified sequences (3,289 reads) compared to the other samples, which may indicate the enrichment of uncultured or poorly characterized microorganisms during pretreatment and digestion. This finding suggests that bacterial pretreatment may have influenced microbial community structure and enhanced microbial activities associated with substrate degradation and biogas generation.

Table 2: Distribution of sequence reads in digestates, poultry feces and cow dung

Microbial Group	Control Digestate	Pretreated Digestate	Poultry Feces	Cow Dung	Total sequence reads (%)
Bacteria	40849	90682	46760	57909	236200 (96.71)
Archaea	1242	1119	21	61	2443 (1.00)
Unclassified	65	3289	2181	62	5597 (2.29)
Total	**42156**	**95090**	**48962**	**58032**	**244240**

3.3 Pretreatment effect on microbial community in the digestates

Biogas production during anaerobic digestion is mediated by syntrophic microbial communities involved in hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Rossi *et al*., 2022; Wang *et al*., 2018). A total of 17 bacterial and three archaeal phyla were identified in both control and pretreated digesters (Figures 1 and 2). Firmicutes, Bacteroidetes, Proteobacteria, Synergistetes, and Actinobacteria were detected in both digesters, whereas Acidobacteria (75%) and Cyanobacteria (2%) were detected only in the digesters containing pretreated coconut coir. The microbial community structure differed considerably between treatments, indicating that bacterial pretreatment influenced substrate utilization patterns and microbial metabolic activity during anaerobic digestion.

The abundance of Firmicutes increased from 6% in the control digesters to 59% in the digesters containing pretreated substrate. This increase suggests enhanced hydrolytic and acidogenic activity following pretreatment, since members of Firmicutes are known to produce extracellular enzymes such as cellulases, proteases, and lipases involved in lignocellulose degradation and volatile fatty acid (VFA) production (Yi *et al*., 2014). Similarly, Bacteroidetes abundance increased from 3% in the control digesters to 31% in the pretreated digesters. Members of this phylum are metabolically versatile fermenters that convert complex organic substrates into VFAs, hydrogen, carbon dioxide, and ethanol (Blachier, 2023; Loughrin *et al*., 2023). The enrichment of both Firmicutes and Bacteroidetes therefore indicates that pretreatment improved the biodegradability of coconut coir by increasing the

availability of fermentable substrates required for downstream methanogenesis.

Actinobacteria also showed a substantial increase in abundance in the pretreated digesters (40%) compared to the control digesters (0.1%). This phylum contains important lignocellulose-degrading organisms capable of decomposing recalcitrant plant biomass (Lewin *et al*., 2016; Theuer *et al*., 2020), suggesting that pretreatment enhanced degradation of the lignified coconut coir structure. In contrast, Spirochaetes were more abundant in the control digesters (99.91%) than in the pretreated digesters, while Planctomycetes and Verrucomicrobia, detected at 3% and 29%, respectively, in the control digesters, were absent in the pretreated systems. These differences suggest that pretreatment enriched microorganisms associated with rapid hydrolysis and fermentation, while reducing populations adapted to more recalcitrant substrate conditions.

Proteobacteria abundance was higher in the control digesters (32%) than in the pretreated digesters (20%). However, the persistence of this phylum in both systems indicates continued syntrophic acetogenic activity during digestion. Genera such as Syntrophobacter, Syntrophus, and Smithella are known to oxidize VFAs into hydrogen and carbon dioxide, which are subsequently utilized by hydrogenotrophic methanogens for methane production (Theuer *et al*., 2020; Zhao *et al*., 2019). This highlights the importance of syntrophic bacterial interactions in maintaining stable anaerobic digestion processes.

Three archaeal phyla, namely Euryarchaeota, Candidatus Thermoplasmatota, and Crenarchaeota, were identified in the digesters (Figure 2). Euryarchaeota was the dominant archaeal phylum, accounting for 83% of the archaeal community in the control digesters and >99% in the digesters containing pretreated substrate. Members of Euryarchaeota comprise the major methanogenic archaea involved in hydro-genotrophic and acetoclastic methanogenesis (Sun *et al*., 2015; Ritari *et al*., 2012). The higher abundance of this phylum in the pretreated digesters corresponded with increased methane production, with the pretreated systems producing 88,355 mL total biogas and a mean methane content of 56 ± 17%, compared to 67,105 mL and 45 ± 25% methane in the control digesters. These findings suggest that pretreatment enhanced hydrolysis and acidogenesis, thereby improving the availability of methanogenic substrates and strengthening syntrophic interactions between fermentative bacteria and methanogenic archaea.

Crenarchaeota was absent in the digesters containing pretreated substrate, whereas Candidatus Thermoplasmatota was detected in both treatments. Some members of Crenarchaeota have been associated with sulfate reduction and hydrogen sulfide generation under anaerobic conditions (Offre *et al*., 2013). However, hydrogen sulfide concentrations remained low and comparable in both systems, with mean H_2S concentrations of 0.01 ± 0.02% and 0.01 ± 0.01% recorded for the control and pretreated digesters, respectively (Table 3). The observed microbial community shifts indicate that bacterial pretreatment enhanced the hydrolytic, fermentative, and methanogenic phases of anaerobic digestion, resulting in improved methane recovery from coconut coir. These findings further highlight the potential applicability of biological pretreatment strategies for improving the large-scale anaerobic digestion of highly lignified agricultural residues.

3.4 Effect of pretreatment on biogas volume

The production of biogas from microbial mediated anaerobic breakdown of coconut coir lignocellulosic biomass was evaluated under greenhouse conditions. The control digesters produced biogas between day 15 and 21 with no further biogas production until it was augmented with fresh inoculum on day 30. In contrast, the

digesters with pretreated substrate started to produce biogas on day 3. Thus, the biological pretreatment not only increased the biogas production but also shortened the fermentation period of the coconut coir. The daily biogas volume in the digesters containing pretreated coconut coir ranged from 0 to 3,965 mL gVS d^{-1} which is about 2 times lower than the control digesters (Figure 3). The pretreated substrate showed an initial increase in biogas volume during the first 40 days (Figure 3), suggesting the breakdown and release of easily metabolizable organic materials. The subsequent reduction in volume between Days 40 and 150 was due to the exhaustion of degradable organic matter.

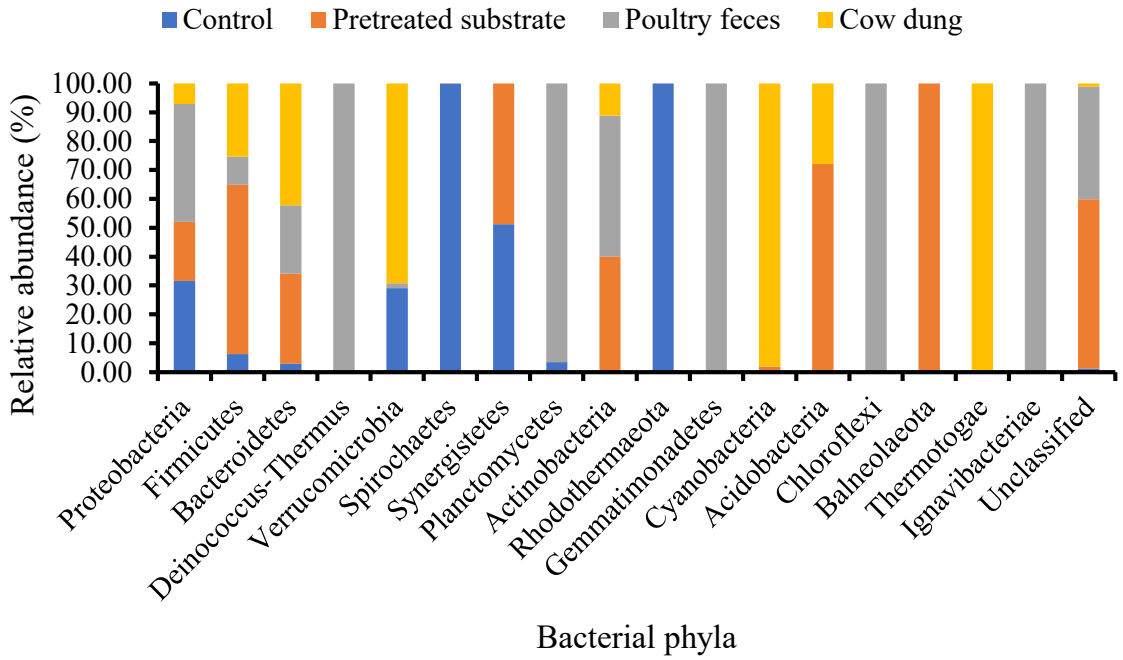

Figure 1: Relative abundance (%) of dominant bacterial phyla identified in digestates, poultry feces, and cow dung samples based on 16S rRNA gene metagenomic analysis

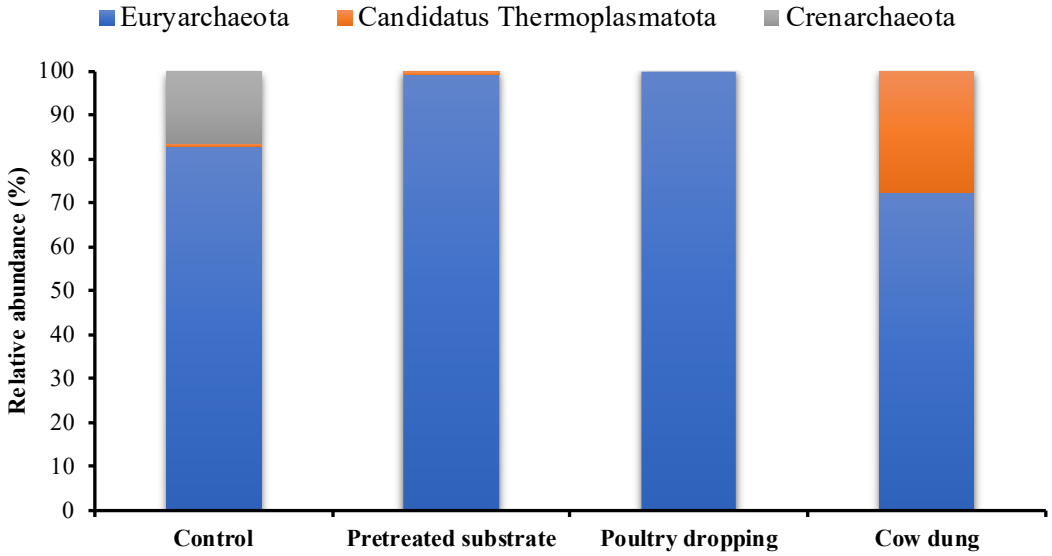

Figure 2: Relative abundance (%) of dominant archaeal phyla detected in poultry feces, cow dung inoculum, and digestates from anaerobic digestion of untreated and biologically pretreated coconut coir based on 16S rRNA gene metagenomic analysis.

This pattern is consistent with other studies, such as those on pretreated sawdust (Eduok *et al*., 2018 and food waste (Achinas and Euverink, 2019), which also showed an initial increase in biogas volume at the onset of anaerobic digestion.

The digesters fed with pretreated coconut coir produced a total biogas volume of 88,355 mL

which was 1.32 times higher than the control digesters (Figure 4). These findings suggest that bacterial pretreatment improved substrate hydrolysis and enhanced the availability of fermentable compounds. This pretreatment improved hydrolysis, the rate-limiting step in the breakdown of coconut coir lignocellulosic biomass by cellulose degraders, thereby enhancing acidogenesis, acetogenesis, and methanogenesis (Ali *et al*., 2019). The cumulative biogas volume in the digesters increased with retention time to 0.067 m^3 and 0.088 m^3 (Figure 5) in the control and pretreated substrate digesters, respectively. This increase is attributed to the conversion of volatile solids to monosaccharides, polysaccharides and amino acids over time. Similar increases in cumulative biogas volume have been reported (Adamu, 2014; Ezekoye *et al*., 2011).

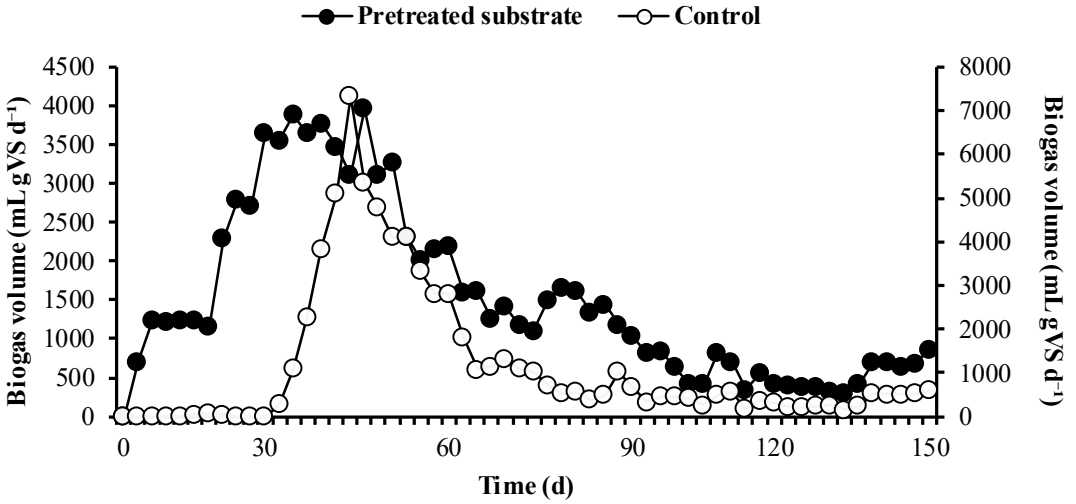

Figure 3: Daily biogas production during anaerobic digestion of untreated and biologically pretreated coconut coir.

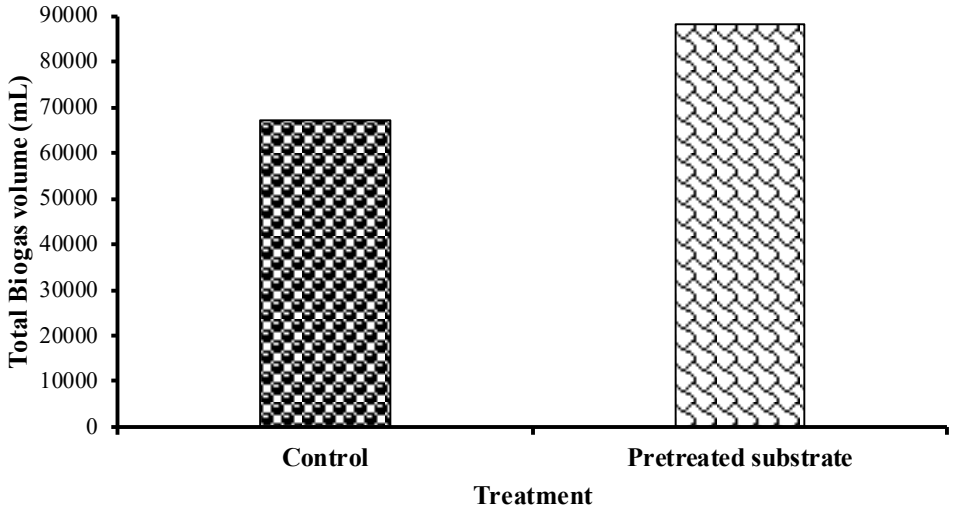

Figure 4: Total biogas production from anaerobic digestion of untreated and biologically pretreated coconut coir lignocellulosic biomass

3.5 Effect of pretreatment on biogas composition

The composition of biogas typically consists of 50-85% methane (CH_4), 25-50% carbon dioxide (CO_2), 1-2% hydrogen sulfide (H_2S), and small quantities of oxygen (O_2) and nitrogen (Atelge *et al*., 2020). A comparison between pretreated substrate and control digesters showed that the pretreated digesters produced a higher methane content range of 0 - 80%, which was approximately 1.08 times higher than the control digester that ranged from 0 - 74%. The untreated substrates (control) had a low initial methane content and indicates a longer lag phase for hydrolytic fermentation and methanogenesis

relative to the pretreated substrate. The result was consistent with previous study by Ozturk (2013), which showed that early biogas produced from whey had low methane content. However, at day 131, the methane yield in pretreated substrate digesters improved to 80%, whereas the control digesters recorded a lower value of 74% (Figure 6).

Daily methane composition in the control and pretreated digestate showed a strong positive correlation ((r = 0.80) between the samples. The mean methane content was 45 ± 25% for the control and 56 ± 17% for the pretreated substrate (Table 3). Carbon dioxide content followed the same trend in pretreated substrates compared to control (Figure 7). The results suggest that pretreatment with cellulose-degrading bacteria increased methane and carbon-dioxide contents, although there was a low positive correlation (r = 0.34) between carbon-dioxide in the pretreated and control digesters.

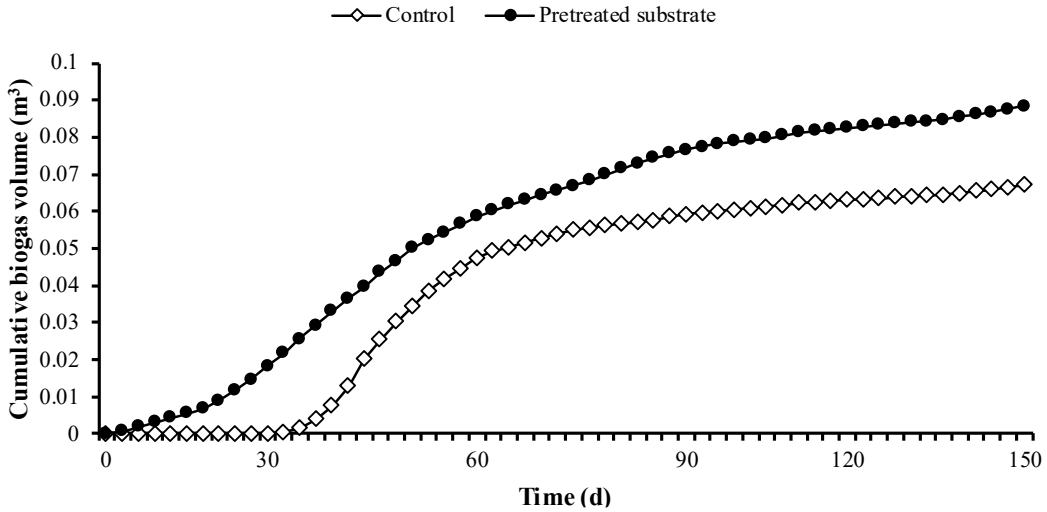

Figure 5: Cumulative biogas production during anaerobic digestion of untreated and biologically pretreated coconut coir lignocellulosic biomass

Hydrogen sulphide (H_2S) concentration is a critical parameter in biogas quality assessment because of its toxicity, corrosiveness, and negative impact on downstream utilization systems. Under anaerobic condition, sulphates and organic sulphur compounds in the feedstock create hydrogen sulphide (H_2S), which has an inhibitory effect on methanogens and methane output (Vu *et al*., 2022). In this study, the H_2S composition in the pretreated substrate digester ranged from 0 to 0.039%, which was approximately 2.81 times lower than that observed in control, where values ranged from 0 to 0.110% (Fig. 8).

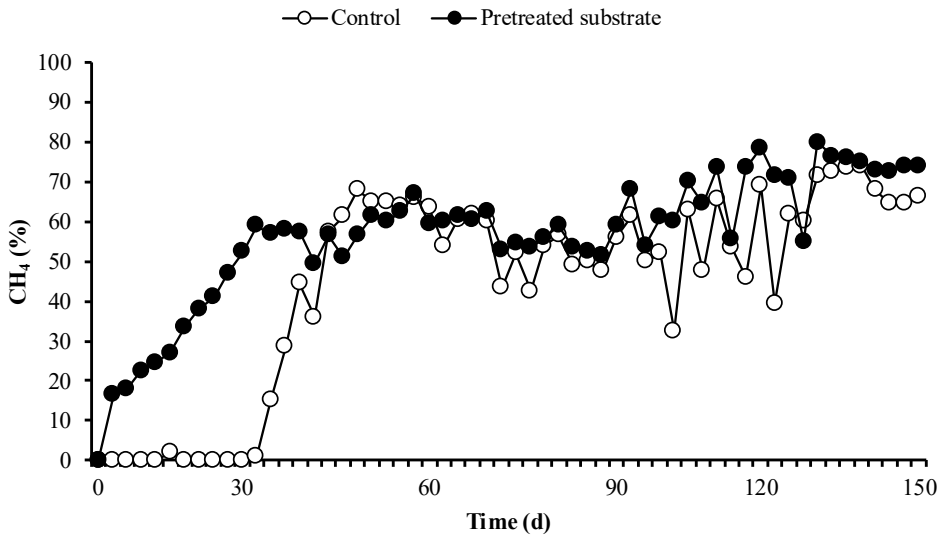

Figure 6: Daily variation in methane (CH_4) content during anaerobic digestion of untreated and biologically pretreated coconut coir lignocellulosic biomass

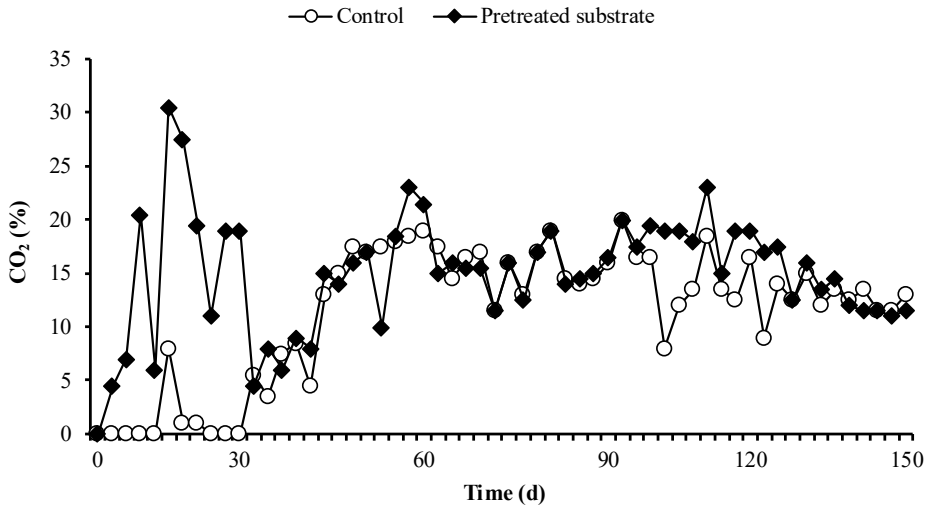

Figure 7: Daily variation in carbon dioxide (CO_2) content during anaerobic digestion of untreated and biologically pretreated coconut coir lignocellulosic biomass.

This reduction suggests that pretreated substrate may have contributed to some level of sulphide mitigation, possibly through altered microbial activity, improved substrate balance, or enhanced sulphur assimilation pathways. Despite this apparent difference in maximum values, statistical analysis indicated no significant difference ($p > 0.05$) between control and pretreated substrate. This lack of significance is further supported by the mean H_2S concentrations, which were identical at 0.01%, although with slightly different variability (±0.02 for control and ±0.01 for pretreated substrate) (Table 3). The relatively high standard deviation observed in control suggests greater fluctuation in H_2S production compared to the more stable profile in pretreated substrate. Temporal analysis showed that H_2S concentrations in both treatments peaked on Day 36, indicating a similar pattern of sulphide generation during the digestion process. This peak may correspond to a phase of intensified protein degradation and sulphur-containing amino acid metabolism, leading to increased activity of sulphate-reducing bacteria. After this peak, the likely decline in H_2S could be attributed to substrate depletion or shifts in microbial community dynamics. Oxygen dynamics revealed a rapid transition from initial aerobic conditions to anaerobiosis in all digesters, with notable differences between treatments. Oxygen (O_2) concentrations in the reactor containing pretreated substrates ranged from 0 to 19.7%, representing a marginal 1.03-times reduction compared to the control, which ranged from 0 to 20.25% (Fig. 9). Peak O_2 concentration occurred earlier in the pretreated substrate on Day 3 at 19.7%, whereas the control reached its peak on Day 12 at 20.25%, indicating faster oxygen depletion in the pretreated system. This early decline suggests that pretreatment with cellulose-degrading bacteria enhanced initial microbial respiration through accelerated hydrolysis of lignocellulosic biomass into readily utilizable substrates, thereby stimulating facultative aerobes to rapidly scavenge residual oxygen. Such rapid oxygen consumption is critical for establishing the strictly anaerobic conditions required for efficient methanogenesis Angelidaki *et al*., 2011). Although the mean O_2 concentrations were relatively low in both systems, the pretreated substrate recorded a slightly lower average of 2.30 ± 4.12% compared to 2.61 ± 3.32% in the control (Table 3), supporting more efficient oxygen removal. The higher variability observed, particularly in

pretreated substrate, reflect dynamic microbial activity and transient microaerophilic niches during the early digestion phase. Similar fluctuations in oxygen levels during start-up have been reported in anaerobic digestion systems and are often associated with reactor loading conditions, mixing, and microbial succession (Zhang *et al*., 2014). The accelerated depletion of oxygen in pretreated substrate facilitated an earlier onset of strictly anaerobic metabolism, promoting the activity of obligate anaerobes, including methanogens.

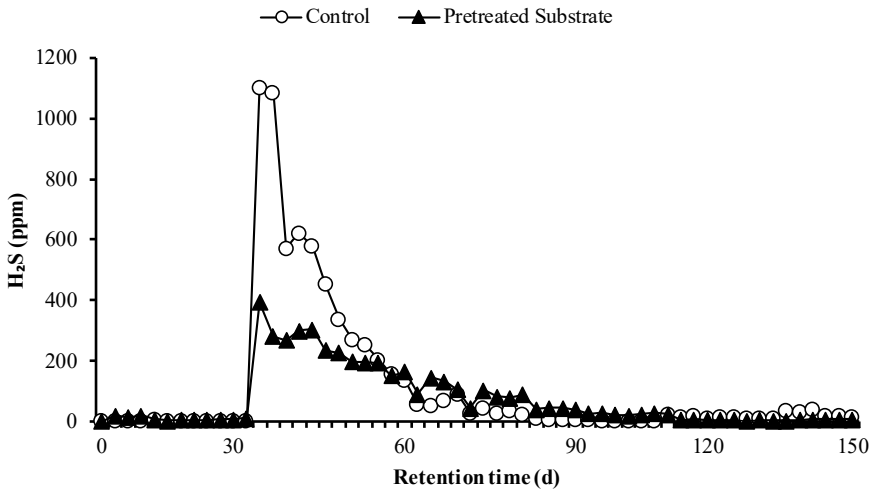

Figure 8: Daily variation in hydrogen sulphide (H_2S) concentration during anaerobic digestion of untreated and biologically pretreated coconut coir lignocellulosic biomass

Table 3: Mean biogas composition at 150 days anaerobic digestion of coconut coir

Biogas composition	Control	Pretreated substrate
Methane (%)	44.52 ± 25.4	56.38 ±16.54
Carbon-dioxide (%)	11.41 ± 6.24	15.01 ± 5.54
Hydrogen sulphide (ppm)	111.36 ± 238.8	72.86 ± 97.5
Oxygen (%)	2.61± 3.32	2.30 ± 2.12

This is consistent with the enhanced methane yields observed in the same treatment, illustrating the role of effective pretreatment strategies in improving reactor performance. Cellulose-degrading bacteria are known to enhance hydrolysis, which is the rate-limiting step in anaerobic digestion, thereby increasing substrate accessibility and stimulating overall microbial activity (Wu *et al*., 2016).

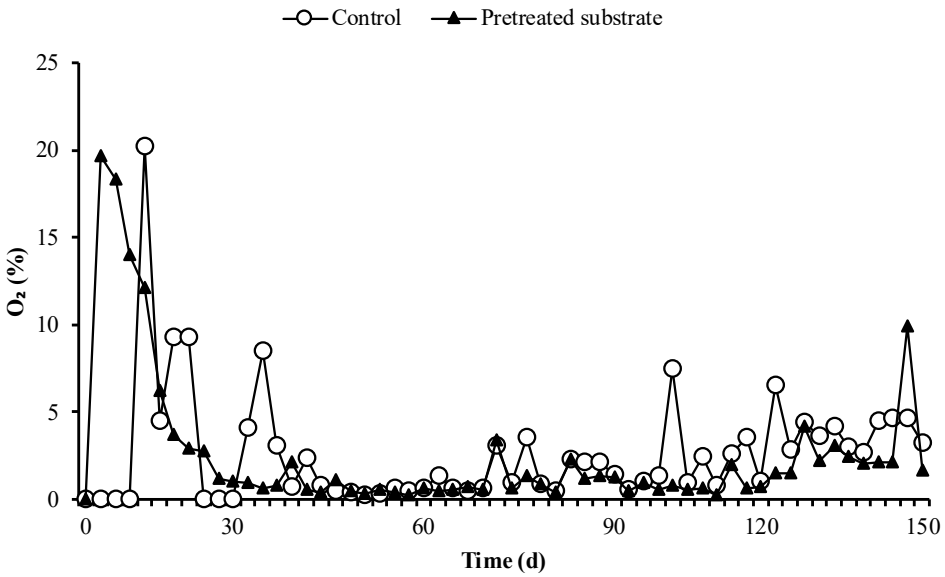

Figure 9: Daily variation in oxygen (O_2) content during anaerobic digestion of untreated and biologically pretreated coconut coir lignocellulosic biomass

4.0 Conclusion

Our study demonstrates that poultry feces are a rich source of cellulolytic bacteria with potential for degrading coconut coir lignocellulosic biomass. The use of plantain peel ash as a buffering agent contributed to stable digester performance and supported sustained biogas production with relatively high methane content. The pretreatment of coconut coir appeared to influence the archaeal community structure and diversity during anaerobic digestion. In particular, a higher relative abundance of Euryarchaeota was observed in digesters with pretreated substrate compared to the control, while Crenarchaeota was detected only in the control digestate. The control digestate also exhibited greater archaeal diversity, which was associated with lower biogas volume and methane content. In contrast, enrichment of Euryarchaeota in the pretreated system was associated with improved biogas yield and methane content. These findings suggest that bacterial pretreatment may have contributed to improved substrate hydrolysis and may have indirectly influenced archaeal community dynamics during anaerobic digestion. This provides a useful basis for exploring biological pretreatment strategies for lignocellulosic biomass to enhance biogas production. Future studies should investigate reactor optimization, enzyme activity profiling, and pilot-scale validation of the pretreatment strategy to better understand its scalability and practical application in large-scale biogas systems.

Declarations

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Competing interests

The authors declare that they have no competing interests, regarding the research, authorship or publication of this manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

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