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Microbial Pretreatment for Biogas: Analyzing Dairy Rumen Anaerobic Bacteria Inoculum's Impact on Alfalfa Biomass and Energy Value

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Abstract: Lignocellulose is a complex and abundant biomass source, and finding ways to efficiently break it down is essential for various applications, including bioenergy production and waste management. Biogas production can be significantly enhanced by adding rumen fluid to the anaerobic digestion process, which contains a variety of microorganisms with the enzyme activity necessary to breakdown complex lignocellulosic materials. This study examined the influence of rumen anaerobic bacteria inoculum on alfalfa biomass biogas yield and quality. Inoculation experiments were performed, and the higher biogas yield from organic matter was gained in experiment (A), with a rumen fluid addition of 340 ± 3.2 L/kg$_{VS}$, compared to the utilization of a digestate alone in (B), 238 ± 1.2 L/kg$_{VS}$. The results demonstrated that a pretreatment temperature of 37 °C (experiment D) yielded the highest biogas production, 381 ± 3.9 L/kg$_{VS}$, and maintained a high methane content of 63.9 ± 1.9%. Notably, pretreatment at 25 °C resulted in only a 3% increase over the raw sample and a pretreatment at 50 °C (respectively, experiments C and E) showed no significant changes, emphasizing the sensitivity of pretreatment efficiency to temperature variations.

Keywords: anaerobic inoculum; bacteria; feedstock; anaerobic digestion; alfalfa; biomass; biomethane; rumen

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1. Introduction

Alfalfa biomass (AB) is an excellent feedstock for anaerobic digestion (AD) since it has an organic solids content of over 20%. However, the high concentration of fibers and lignocellulose, and the material's ability to layer, makes this feedstock problematic to digest in continuous stirred-tank bioreactors. Lignocellulosic biomass has a huge potential to be used as feedstock for the sustainable production of fuels and chemicals through fermentation [1]. Today, plant substrates, also called lignocellulosic biomass, are seen as one of the most promising materials that can replace fossil energy resources in the production of fuels and chemicals with reduced GHG emissions [2].

Alfalfa is a forage crop with one of the highest protein contents available, which makes it an ideal choice for livestock and other animals that require a protein-rich diet. This is especially important for animals in their growth stages [3]. Alfalfa is highly digestible, meaning animals can efficiently extract nutrients from it during digestion. This results in better utilization of the feed and improved animal growth and productivity [4]. The characteristics that make alfalfa an ideal choice for animal nutrition, including its high protein content and digestibility, also render it a favorable candidate for anaerobic digestion in biogas production processes. When used as a feedstock in biogas digesters, alfalfa can contribute to increased methane production due to its rich organic matter content, making it a sustainable and efficient resource for biogas generation.

The influence of inoculum used for anaerobic lignocellulosic biomass treatment varies in every specific case. In cellulolytic rumen bacteria, highly active cellulolytic and hemicellulolytic enzymes are combined in extracellular multienzyme complexes, or cellulosomes [5]. Recent research related to the degradation of lignocellulose in biogas processes has had a strong focus on the microorganisms involved, with the aim of further understanding and improving biodegradation [6]. These studies have, for example, evaluated the whole bacterial and archaea community by analyzing the 16 S rRNA genes [7].

Cellulosic compounds found in plants play a critical role in impeding the hydrolysis step in energy production using green waste, particularly under anaerobic conditions. The significance of the anaerobic fungi and microorganisms present in dairy rumen becomes evident as they possess a remarkable ability to produce a range of enzymes, including cellulolytic, hemicellulolytic, glycolytic, and proteolytic enzymes, which are essential for the biodegradation of lignocellulose [8–10]. In the rumen fluid of ruminants, the rumen serves as a natural bioreactor when the efficient breakdown of polysaccharides within plant cell walls occurs. This degradation process is performed by a diverse community of rumen microorganisms, encompassing bacteria, anaerobic eukaryotes, protozoa, and archaea [11]. Improving the performance of the microbial strains for an efficient conversion of sugars from complex substrates (hydrolysates produced from lignocellulosic biomass) is an important issue to be solved to support the large-scale implementation of these bioprocesses. Researchers have investigated the degradation of straw and cellulose during batch cultivation using materials from different full-scale biogas plants as the inoculum source [12]. The results showed similar biogas yields but differences in the degradation rate, as well as a correlation between degradation rate and the composition of the cellulose-degrading community. Employing a combination of two or more microbial species for bioprocessing biomass into biogas remains an underrated strategy for increasing processing efficiency [13]. A cutting-edge approach to revolutionizing biotechnological biomass usage involves synergizing multiple microbial species for bioprocessing, which might enhance the efficiency of the overall process. This co-cultivation approach can potentially alleviate some of the problems associated with lignocellulose biomass use. The general idea behind this concept is to take advantage of the specialized ability of several organisms and create a synergistic effect. Since multiple strains are used in a single process, a broader variation in beneficial characteristics can be selected. Optimization of a co-cultivation process could then be performed by selecting the right strains to be combined, instead of engineering one do-it-all strain [5].

Different inoculum sources, including cattle manure, sewage sludge, and acclimatized anaerobic sludge, were tested in the co-digestion process. Cattle manure was found to be the most effective inoculum, resulting in the highest biogas production. In their study, Bella K. et al. investigated the use of septage as a co-substrate for anaerobic digestion of cheese whey. The results suggest that septage is a suitable co-substrate and can enhance the digestibility of cheese whey [14].

The addition of rumen fluid to the anaerobic digestion process can significantly enhance biogas production by providing a diverse range of microorganisms that possess the necessary enzymatic activity to break down complex lignocellulosic materials. The optimal range for rumen fluid addition varies depending on the type of feedstock and operational conditions of the biogas plant, with the ideal range being between 25 and 50%. However, it is important to carefully monitor the process and avoid overloading the system with rumen fluid, as this can lead to unwanted process disruptions [15].

The aim of this work was to investigate the influence of a rumen anaerobic bacteria inoculum on yield and quality of biogas produced from alfalfa biomass.

Part of this research was presented in the Sciforum and published in Engineering proceedings under the title “The influence of dairy rumen anaerobic bacteria inoculum on biogas process” [16]. This means that our findings on the use of dairy rumen anaerobic bacteria as an inoculum in biogas processes is the sequel to our previous research.

2. Materials and Methods

The influence of the dairy rumen fluid inoculum selected for anaerobic treatment and pretreatment of the organic fraction of AB was studied in this work. Table 1 presents the feedstocks compositions and pretreatment conditions used in a series of experiments designed to assess the potential for biogas production under various conditions. The alfalfa biomass was immersed in rumen fluid and incubated in a temperature-controlled bath for a duration of three days. Containers during AB pretreatment were stirred hourly.

Table 1. Feedstocks used in the experimental setup.

Experimental Set	Alfalfa Biomass, Gram	Digestate, Gram	Rumen Fluid, Gram	Conditions
A	16	400	400	Raw feedstock
B	16	800	0	Raw feedstock
C	16	400	400	3 days pretreatment at 25 °C
D	16	400	400	3 days pretreatment at 37 °C
E	16	400	400	3 days pretreatment at 50 °C

In experiment A, a combination of 16 g of AB, 400 g of digestate, and 400 g of rumen fluid were used as the raw feedstock. In experiment B, a similar mixture of AB and digestate was employed, but without rumen fluid. These results were partially presented in the Sciforum and published in Engineering proceedings.

Experiments C, D, E involved a 16 g AB, 400 g digestate, and 400 g rumen fluid mix, but with a three-day pretreatment period at 25 °C, 37 °C, and 50 °C, respectively. These experiments allowed for the evaluation of the influence of varying pretreatment conditions on the biomethane potential of the feedstocks.

Dairy rumen fluid was taken from a dairy farm in southwestern Lithuania. Dairy cows were fed with the same Alfalfa biomass feed as used in other research. The rumen fluid was taken via oral stomach tubing, packaged in a 15 L airtight container, and stored at 37.0 ± 0.2 °C to be protected from environmental influences until the start of the experiment. The transportation period from the collection of rumen fluid to the start of the experiment took 2 h. Prior to the commencement of the experiment, the dairy rumen fluid was filtered through a 0.5 mm stainless steel mesh.

The chemical analysis of the feedstock was carried out on AB composition content. Total solids (TSs) and volatile solids (VSs) contents were determined gravimetrically via drying at 105 °C and subsequent ashing at 550 °C according to LST EN 13039:2012 standards [17].

The size of crushed AB varied from 0.10 to 1.00 mm.

A single-load biogas yield experiment was carried out on a biochemical biogas potential test bench (BBP) (Figure 1). The mesophilic temperature was maintained at 37.0 ± 0.2 °C during the experiment. To determine the potential biogas yield and production from AB, separate BBP experiments were conducted with triplicate samples for each experiment set.

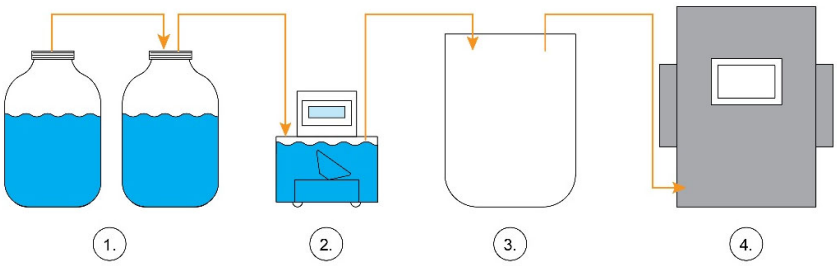

Figure 1. Technological research scheme. 1—a set of BBP bioreactors, 2—Ritter Miligascounter, 3—Tedlar biogas bag, 4—Awite Bioenergie GmbH AwiFlex biogas analyzer.

The volume of biogas from each bioreactor was monitored daily and the concentrations of methane (CH_4), carbon dioxide (CO_2), and hydrogen sulfide (H_2S) were monitored after the 35 days of experiment. The amount of gas formed was registered with RITTER MilliGascounters (RITTER, Bochum, Germany) (2). A 20 L volume Tedlar PVF gas sampling bag (DuPont, Wilmington, DE, USA) (3) was used for biogas collection. The collected biogas was analyzed with an Awite Bioenergie GmbH AwiFlex (Awite Bioenergie GmbH, Langenbach, Germany) biogas analyzer (4). Bioreactors were stirred hourly.

Variations in the physical and chemical properties of the same feedstock can be attributed to differences in their volatile solids content. To ensure consistency in our research findings, the biogas yield results were recalculated based on the raw (B_M) and pretreated biomass's volatile solids (B_{VS}). These calculations were performed using the following equations: $B_M = b_{dt}/m$; $B_{VS} = b_{dt}/m_{VS}$. Here, b_{dt} represents the volume of biogas produced during the time interval dt in liters, m is the mass of the sample in kilograms, m_{VS} is the mass of the sample's volatile solids in kilograms.

Methane is the most important component in biogas and it serves as an indicator of the energy value of biomass during anaerobic digestion (e_M). The energy harnessed from the biomass can be determined using the equation: $e_M = B_M \cdot e_b$, where e_b denotes the energy value of biogas and is contingent on the methane concentration within the biogas in MJ/L. The energy value of the biogas is determined using the equation: $e_b = 0.0353 \cdot C_{CH4}/100$, where C_{CH4} shows the methane concentration within the biogas, measured as a percentage.

To evaluate the effectiveness of the rumen fluid inoculum pretreatment on alfalfa biomass, the impact on volatile solids biogas yield (B_{VS}) was assessed using the following formula:

$$\text{PTE } (\%) = [(B_{VS}\_\text{pretreated} - B_{VS}\_\text{raw})/B_{VS}\_\text{raw}] * 100\%$$

This involved comparing the biogas production from pretreated alfalfa biomass (B_{VS}_pretreated) with that from untreated (raw) alfalfa biomass (B_{VS}_raw). The PTE represents the increase in biogas yield from volatile solids achieved through pretreatment compared to the raw feedstock. It provides a percentage value that indicates the improvement in biogas production due to the pretreatment process. This evaluation method allows for a clear assessment of the efficiency of pretreatment methods in enhancing the biogas yield from AB.

A one-way ANOVA method was used to check if there were significant differences between our results comparing biogas yield from raw feedstock with biogas yield of pretreated feedstock. Significant differences were reported at a *p*-value lower than 0.05.

3. Results and Discussion

The measured pH of the digestate and the rumen fluid inoculants was 7.3 and 6.1, respectively. The normal pH of dairy cow rumen fluid is typically between 6.0 and 7.0. However, it can fluctuate depending on the cow's diet and feeding schedule [18]. The pH of the digestate typically varies from 6.5 to 8.2 [19] with optimal values for anaerobic digestion producing methane of 6.8–7.2 [20].

The main feedstock for the experiment was crushed alfalfa biomass, which was evenly dried and homogeneous, with a TS content of 64.3% and a concentration of TS of 91.9%. The total and volatile solids content of AB can vary depending on factors such as the stage of growth, weather conditions, and location [21].

In the present study, the total solids and volatile solids tests were also conducted for both the digestate and rumen fluid. The digestate and rumen fluid inoculants had low total solids concentrations in this experiment, with a respective concentration of 4% and 1.8%. The volatile solids content in these inoculants was observed to be high, with respective values of 98.2% and 97.4%. It is important to note that the volatile and total solids experiments were conducted for sieved digestate and rumen fluid.

3.1. The Impact of Rumen Fluid Inoculation on Biogas Production

First, raw digestate was used as a reference to establish a baseline for digestate, which was 27.7 ± 0.52 L/kg_{VS}. A BBP experiment was also performed using 700 g of digestate and 100 g of rumen fluid to assess biogas production from rumen fluid alone, resulting in a biogas production of 11.24 ± 0.9 L/kg_{VS}.

The BBP experiment results (Figure 2) illustrate that the maximum biogas yield from AB volatile solids using digestate (experiment B) was 259 ± 1.4 L/kg_{VS}. The biogas yield from the AB with a combination of rumen fluid and digestate (experiment A) reached 370 ± 3.3 L/kg_{VS}. These findings suggest that the increase in biogas yield was due to the presence of highly active cellulolytic and hemicellulolytic enzymes, which are combined in extracellular multienzyme complexes known as cellulosomes [22].

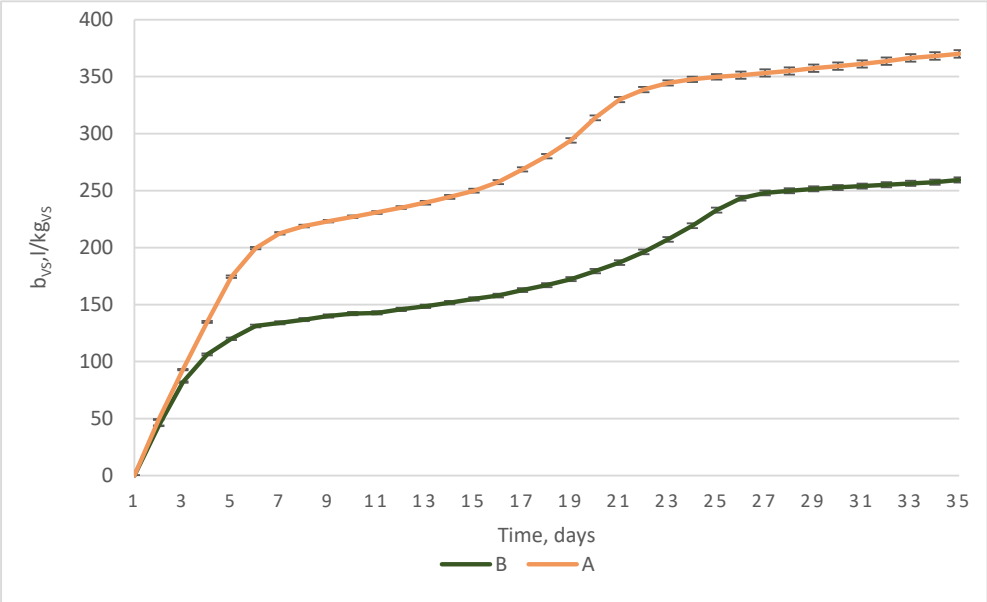

Figure 2. Biogas yield from alfalfa biomass and rumen inoculum. A—experiment A; B—experiment B.

The concentration of methane in the biogas was also dependent on the inoculum used in the research. The higher concentration of methane was gained from experiment A (63.2 ± 1.5%). The biogas gained from BM in common digestate (experiment B) had a lower concentration of methane, at 54.6 ± 1.1%. This finding supports the conclusions from the experiment conducted by Zheng et al. [23], which demonstrated that a ratio of 1:5 of rumen microorganisms to biogas slurry yielded high methane production and content, thereby establishing it as the optimal ratio.

The biomethane yield acquired during the experiment employing digestate stood at 142 ± 9 L_{CH4}/kg_{VS}, while the composition of digestate and rumen fluid yielded 234 ± 9 L_{CH4}/kg_{VS}, marking a significant increase of 62%. The experimental results from our research were complemented by Nagler et al. [24], who explained that the inclusion of rumen liquid enhances the degradation of complex lignocellulosic compounds by providing a diverse range of cellulolytic and hemicellulolytic microorganisms. This leads to an increase in biogas production and improved process stability. The authors suggest that the addition of rumen liquid could be a simple and effective strategy to enhance the performance of lignocellulose-degrading biogas plants.

Hakl et al. [25] performed experimental research on alfalfa biomass biomethane yield. In their experiment, approximately 250 to 390 L_{CH4}/kg_{VS} of lucerne forage was obtained. Comparisons of these research results suggest that optimizing the conditions of alfalfa digestion, such as feedstock characteristics, inoculum type, and operating conditions, will lead to improved biomethane yields.

Khadka et al.'s [26] study explained that the substrate to inoculum (S:I) ratios have a significant impact on biogas yields and process kinetics during the anaerobic digestion (AD) of food waste. Among the S:I ratios tested, a ratio of 1 to 1 resulted in the highest average biogas yields (674.40 ± 29.10 NmL/g_{VS}). However, when considering variability and comparisons with literature, the study suggests that the optimal S:I ratio for FW anaerobic digestion falls in the range of 1 to 2 (VS basis).

3.2. The Effect of Rumen Fluid Inoculation as a Pretreatment Method on Biogas Production

The pretreatment BBP experiments were designed to assess how different pretreatment conditions influenced the biomethane potential of the feedstocks, providing valuable insights into the temperature-dependent effects on biogas production. Experiments C, D, and E entailed a combination of 16 g of alfalfa biomass, 400 g of digestate, and 400 g of rumen fluid, with each experiment subject to a distinct three-day pretreatment at temperatures of 25 °C, 37 °C, and 50 °C, respectively. The alfalfa biomass was immersed in rumen fluid and incubated in a temperature-controlled bath for a duration of three days. The result was similar to experiment A, and the same composition of feedstock was also used in experiments without pretreatment.

The results of the BBP experiment illustrate that the highest biogas yield was 415 ± 4.2 L/kg_{VS}, and this result was achieved following pretreatment at 37 °C (experiment D) (Figure 3). In contrast, the biogas yield from the same feedstock composition without pretreatment was 370 ± 3.3 L/kg_{VS} (experiment A), indicating a notable pretreatment efficiency of 12%. Statistical analysis of the data from the A and D experiments showed that there was a statistically significant difference ($p < 0.05$). Notably, pretreatment at 25 °C resulted in a biogas yield of 381 ± 2.3 L/kg_{VS} (experiment C), representing only a 3% increase compared to the raw sample, emphasizing the temperature-dependent impact on the pretreatment's effectiveness. Statistical analysis of the data from the A and C experiments showed that there was statistically significant difference ($p < 0.05$). Conversely, pretreatment at 50 °C (experiment E) led to a biogas yield of 359 ± 3.2 L/kg_{VS}, indicating a slight decrease of 3% compared to the raw sample, highlighting the sensitivity of the pretreatment outcomes to temperature variations. Statistical analysis of the data from the A and E experiments showed that there was no statistically significant difference ($p > 0.05$).

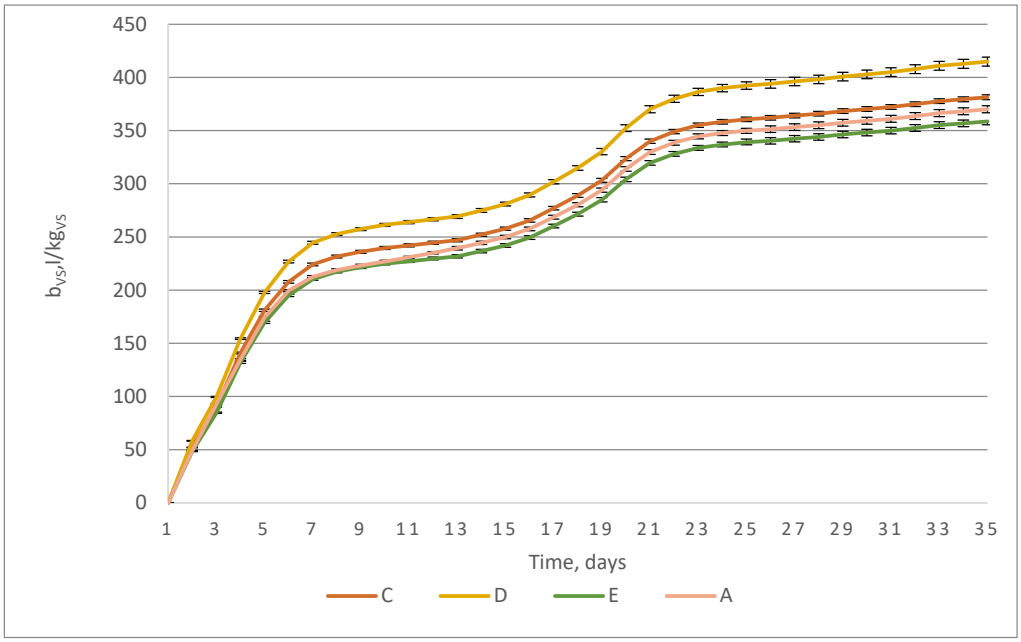

Figure 3. Biogas yield from alfalfa biomass vs. with rumen inoculum pretreatment. A—experiment A; C—experiment C; D—experiment D; E—experiment E.

AB digested without pretreatment (experiment A) generated biogas with a methane concentration of 63.2 ± 1.5% and H_2S concentrations of 146 ± 25 ppm. Notably, experiment C, conducted with pretreatment at 25 °C, demonstrated a higher methane content of 64.2 ± 1.2% and increased H_2S levels of 389 ± 38 ppm. Experiment D, performed at 37 °C, not only achieved the highest biogas yield but also maintained methane content of 63.9 ± 1.9% and reduced H_2S levels of 168 ± 45 ppm. Conversely, experiment E, conducted at 50 °C, displayed a slightly lower methane content of 59.5 ± 1.1% and H_2S concentrations of 280 ± 13 ppm. These results underscore the complex interplay between temperature, pretreatment properties, and biogas composition, offering important insights for optimizing biogas production processes.

Biological pretreatments involve the breakdown of complex lignocellulosic molecules using pure cultures (specific strains), microbial communities (consortia), or hydrolytic enzymes (such as cellulases, hemicellulases, and laccases) produced by microorganisms [27]. Hydrolytic bacteria are a vital component of the rumen microbial community. Their primary role is to break down complex carbohydrates, such as cellulose and hemicellulose, into simpler sugar molecules through a process called hydrolysis [28]. This is a crucial step in the anaerobic digestion of biogas plant feedstocks. The increased availability of simple sugars and the breakdown of complex barriers like lignin may be the result in higher gas production during anaerobic digestion [29].

3.3. The Effect of Rumen Fluid Inoculation on Biomass Energy Value

The experimental results of the 35-days BBP, as presented in Table 2, provide an overview of the biogas production and energy-related parameters across five distinct experiments using AB, a rumen fluid as an inoculum itself, and an inoculum for alfalfa pretreatment. Comparing experiments A and B, the higher biogas yield was gained in experiment A, because of the synergistic effects of the rumen fluid and digestate. Experiment B, utilizing only digestate and AB, yielded a lower biogas production, underlining the significant contribution of the rumen anaerobic bacteria inoculum in experiment A. This increase in biogas yield, attributed to the presence of highly active cellulolytic and hemicellulolytic enzymes, is substantiated by the higher methane concentration observed in experiment A (63.2%) compared to experiment B (54.6%).

Table 2. Biogas, biomethane yield, and biomass energy value.

Indicator	A	B	C	D	E
Biogas yield from biomass (B_M), L/kg	576 ± 4.8	403 ± 1.8	593 ± 4.5	645 ± 6	558 ± 4
Biogas yield from total solids (B_{TS}), L/kg_{TS}	370 ± 3.3	259 ± 1.4	381 ± 2.2	415 ± 4.2	359 ± 3.2
Biogas yield from organic matter (B_{VS}), L/kg_{VS}	340 ± 3.2	238 ± 1.2	350 ± 2.1	381 ± 3.9	330 ± 3.1
Methane concentration in biogas (C_{CH4}), %	63.2 ± 1.5	54.6 ± 1.1	64.2 ± 1.2	63.9 ± 1.9	59.5 ± 1
Biomethane yield B_{CH4vs}, L/kg_{vs}	234 ± 2	142 ± 6.7	245 ± 2	265 ± 6	213 ± 1
Energetic value of biogas (e_b), MJ/m^3	22.31 ± 0.76	19.27 ± 0.88	22.66 ± 0.32	22.56 ± 0.22	21 ± 0.18
Energy obtained from biomass (e_M), MJ/kg	12.84 ± 0.55	7.77 ± 0.35	13.44 ± 0.2	14.55 ± 0.14	11.71 ± 0.11
Energy obtained from dry matter (e_{TS}), MJ/kg	8.26 ± 0.29	5 ± 0.63	8.64 ± 0.35	9.36 ± 0.24	7.53 ± 0.18
Energy obtained from organic matter (e_{VS}), MJ/kg	7.59 ± 0.24	4.59 ± 0.58	7.94 ± 0.32	8.6 ± 0.23	6.92 ± 0.17

Furthermore, the impact of pretreatment at different temperatures (experiments C, D, E) on biogas yield is evident. Experiment D, conducted at 37 °C, achieved the highest biogas yield at 415 ± 4.2 L/kg_{VS}, emphasizing the temperature-dependent nature of pretreatment efficiency. This was accompanied by the maintenance of a high methane concentration (63.9 ± 1.9). These results highlight the significance of temperature control in optimizing biogas production and pretreatment.

In terms of energy-related parameters, the energy value of biogas (e_b) varied across the experiments, with the highest value of 22.66 ± 0.32 MJ/m^3 in experiment C. Energy obtained from biomass fresh matter (e_M), biomass energy from TS (e_{TS}), and biomass energy from volatile solids (e_{VS}) followed a similar trend, with experiment D consistently

leading in energy potential, underscoring the substantial potential of the pretreatment process at 37 °C.

In terms of pretreatment process efficiency (Table 3) experiment D with rumen fluid at 37 °C demonstrated the most significant increases, with a 60 ± 0.6% rise in biogas yield and an 87.2 ± 0.8% increase in biomethane production compared to alfalfa biomass and digestate as inoculum (experiment B). Inoculum C also proved effective, resulting in a 47 ± 0.5% increase in biogas yield and a 72.9 ± 0.7% increase in biomethane production.

Table 3. Rumen fluid pretreatment efficiency on biogas process.

Indicator	A	B	C	D	E
Biogas yield increase compared to digestate as inoculum %	+42.7 ± 2%	0%	+47 ± 0.5%	+60 ± 0.6%	+38.3 ± 0.4%
Biomethane increase compared to digestate as inoculum	+65.2 ± 2%	0%	+72.9 ± 0.7%	+87.2 ± 0.8%	+50.7 ± 0.6%
Pretreated biomass vs. biogas yield compared to raw, %	0%	−29.9 ± 1%	+3.1 ± 0.1%	+12.1 ± 0.1%	−3.9 ± 0.1%
Pretreated biomass vs. biomethane yield compared to raw, %	0%	−39.5 ± 2%	+4.7 ± 0.2%	+13.4 ± 0.1%	−8.6 ± 0.1%

The pretreatment process had varying effects on conversion to biogas compared to raw biomass. Experiment D had the highest increase of 12.1 ± 0.1% in biogas yield from pretreated biomass, while inoculum C resulted in a 3.1 ± 0.1% increase.

The data from experiments emphasize the pivotal role of the dairy rumen anaerobic bacteria inoculum and temperature-controlled pretreatment in enhancing biogas production, methane concentration, biogas energy value, and biomass energy value. These findings provide valuable insights for optimizing biogas processes and advancing renewable energy generation, aligning with sustainability and environmental goals.

4. Conclusions

In conclusion, the results of the BBP experiment provide valuable insights into the influence of various factors on biogas production and composition, with a focus on the role of dairy rumen anaerobic bacteria inoculum in alfalfa biomass pretreatment. The combination of rumen fluid and digestate (experiment A) enhanced biogas yield compared to usage of only digestate as an inoculum.

Pretreatment experiments (experiments C, D, and E) explored the temperature-dependent effects of pretreatment on biogas production. The results demonstrated that a temperature of 37 °C (experiment D) yielded the highest biogas production, respectively, 381 ± 3.9 L/kg_{VS}, and maintained a high methane content of 63.9 ± 1.9%. Notably, pretreatment at 25 °C resulted in only a 3% increase compared to the raw sample, and 50 °C (respectively, experiments C and E) showed no significant results, emphasizing the sensitivity of pretreatment efficiency to temperature variations.

In summary, this research underscores the complex relationship between temperature, pretreatment methods, and biogas composition, providing critical insights for optimizing biogas production processes. Further research may investigate temperature variations more deeply to determine the optimal range for maximizing biogas production while maintaining favorable composition. While we had no possibility to additionally study rumen microbial communities, studying the rumen microbial communities involved in anaerobic digestion under varying temperature and pretreatment conditions may be explored. The findings offer a pathway to enhancing the efficiency and sustainability of biogas production, which is crucial for renewable energy and environmental goals.

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References

1. DNV. *Energy Transition Outlook 2020—A Global and Regional Forecast to 2050*; DNV: Høvik, Norway, 2023.
2. Lindorfer, J.; Lettner, M.; Hesser, F.; Fazeni, K.; Rosenfeld, D.; Annevelink, B.; Mandl, M. *Technical, Economic and Environmental Assessment of Biorefinery Concepts*; IEA Bioenergy: Paris, France, 2019; ISBN 978-1-910154-64-9.
3. Hidosa, D.; Biru, S.K. Dry Matter Yield and Chemical Composition of Alfalfa (Medicago sativa) Varieties as Animal Feed in the South Omo Zone of South-western Ethiopia. *Acta Vet. Brno* **2021**, *3*, 2582–3183.
4. Cheng, F.; Brewer, C.E. Conversion of protein-rich lignocellulosic wastes to bio-energy: Review and recommendations for hydrolysis + fermentation and anaerobic digestion. *Renew. Sustain. Energy Rev.* **2021**, *146*, 111167. [CrossRef]
5. Miron, J.; Ben-Ghedalia, D.; Morrison, M. Invited review: Adhesion mechanisms of rumen cellulolytic bacteria. *J. Dairy Sci.* **2001**, *84*, 1294–1309. [CrossRef] [PubMed]
6. Shrestha, S.; Fonoll, X.; Khanal, S.K.; Raskin, L. Biological strategies for enhanced hydrolysis of lignocellulosic biomass during anaerobic digestion: Current status and future perspectives. *Bioresour. Technol.* **2017**, *245*, 1245–1257. [CrossRef] [PubMed]
7. Sun, L.; Liu, T.; Müller, B.; Schnürer, A. The microbial community Structure in industrial biogas plants influences the degradation rate of Straw and cellulose in batch tests. *Biotechnol. Biofuels* **2016**, *9*, 128. [CrossRef] [PubMed]
8. Weimer, P.J. Degradation of Cellulose and Hemicellulose by Ruminal Microorganisms. *Microorganisms* **2022**, *10*, 2345. [CrossRef] [PubMed]
9. Yıldırım, E.; Ince, O.; Aydin, S.; Ince, B. Improvement of biogas potential of anaerobic digesters using rumen fungi. *Renew. Energy* **2017**, *109*, 346–353. [CrossRef]
10. Cai, S.; Li, J.; Hu, F.Z.; Zhang, K.; Luo, Y.; Janto, B.; Boissy, R.; Ehrlich, G.; Dong, X. Cellulosilyticum ruminicola, a Newly Described Rumen Bacterium That Possesses Redundant Fibrolytic-Protein-Encoding Genes and Degrades Lignocellulose with Multiple Carbohydrate- Borne Fibrolytic Enzymes. *Appl. Environ. Microbiol.* **2010**, *76*, 3818–3824. [CrossRef]
11. Rajeswari, G.; Jacob, S.; Chandel, A.K.; Kumar, V. Unlocking the potential of insect and ruminant host symbionts for recycling of lignocellulosic carbon with a biorefinery approach: A review. *Microb. Cell Fact.* **2021**, *20*, 107. [CrossRef]
12. Mussatto, S.I.; Yamakawa, C.K.; van der Maas, L.; Dragone, G. New trends in bioprocesses for lignocellulosic biomass and CO_2 utilization. *Renew. Sustain. Energy Rev.* **2021**, *152*, 111620. [CrossRef]
13. Ferdeş, M.; Dincă, M.N.; Moiceanu, G.; Zabava, B.Ş.; Paraschiv, G. Microorganisms and enzymes used in the biological pretreatment of the substrate to enhance biogas production: A review. *Sustainability* **2020**, *12*, 7205. [CrossRef]
14. Bella, K.; Venkateswara Rao, P. Anaerobic co-digestion of cheese whey and septage: Effect of substrate and inoculum on biogas production. *J. Environ. Manag.* **2022**, *308*, 114581. [CrossRef] [PubMed]
15. Budiyono, B.; Widiasa, I.N.; Johari, S.; Sunarso, S. Increasing Biogas Production Rate from Cattle Manure Using Rumen Fluid as Inoculums. *Int. J. Sci. Eng.* **2014**, *6*, 31–38. [CrossRef]
16. Žalys, B.; Venslauskas, K.; Navickas, K.; Buivydas, E.; Rubežius, M. The Influence of Dairy Rumen Anaerobic Bacteria Inoculum on Biogas Production. *Eng. Proc.* **2023**, *37*, 83. [CrossRef]
17. *LST EN 13039:2012*; Soil Improvers and Growing Media—Determination of Organic Matter Content and Ash. Lithuanian Standards Board: Vilnius, Lithuania, 2012.
18. Penner, G.B.; Beauchemin, K.A.; Mutsvangwa, T. Severity of ruminai acidosis in primiparous holstein cows during the periparturient period. *J. Dairy Sci.* **2007**, *90*, 365–375. [CrossRef] [PubMed]
19. Ward, A.J.; Hobbs, P.J.; Holliman, P.J.; Jones, D.L. Optimisation of the anaerobic digestion of agricultural resources. *Bioresour. Technol.* **2008**, *99*, 7928–7940. [CrossRef] [PubMed]
20. Rao, M.S.; Singh, S.P. Bioenergy conversion studies of organic fraction of MSW: Kinetic studies and gas yield-organic loading relationships for process optimisation. *Bioresour. Technol.* **2004**, *95*, 173–185. [CrossRef] [PubMed]
21. Sanz-Sáez, Á.; Erice, G.; Aguirreolea, J.; Muñoz, F.; Sánchez-Díaz, M.; Irigoyen, J.J. Alfalfa forage digestibility, quality and yield under future climate change scenarios vary with Sinorhizobium meliloti strain. *J. Plant Physiol.* **2012**, *169*, 782–788. [CrossRef]
22. Bayer, E.A.; Lamed, R.; Himmel, M.E. The potential of cellulases and cellulosomes for cellulosic waste management. *Curr. Opin. Biotechnol.* **2007**, *18*, 237–245. [CrossRef]
23. Zheng, Y.; Wang, X.; Yang, F. Improving the Anaerobic Digestion of Switchgrass via Cofermentation of Rumen Microorganisms (Rumen Bacteria, Protozoa, and Fungi) and a Biogas Slurry. *Energy Fuels* **2019**, *33*, 1185–1195. [CrossRef]
24. Nagler, M.; Kozjek, K.; Etemadi, M.; Insam, H.; Podmirseg, S.M. Simple yet effective: Microbial and biotechnological benefits of rumen liquid addition to lignocellulose-degrading biogas plants. *J. Biotechnol.* **2019**, *300*, 1–10. [CrossRef]
25. Hakl, J.; Fuksa, P.; Habart, J.; Šantrůček, J. The biogas production from lucerne biomass in relation to term of harvest. *Plant Soil Environ.* **2012**, *58*, 289–294. [CrossRef]
26. Khadka, A.; Parajuli, A.; Dangol, S.; Thapa, B.; Sapkota, L.; Carmona-Martínez, A.A.; Ghimire, A. Effect of the Substrate to Inoculum Ratios on the Kinetics of Biogas Production during the Mesophilic Anaerobic Digestion of Food Waste. *Energies* **2022**, *15*, 834. [CrossRef]

27. Atelge, M.R.; Atabani, A.E.; Banu, J.R.; Krisa, D.; Kaya, M.; Eskicioglu, C.; Kumar, G.; Lee, C.; Yildiz, Y.; Unalan, S.; et al. A critical review of pretreatment technologies to enhance anaerobic digestion and energy recovery. *Fuel* **2020**, *270*, 117494. [CrossRef]
28. Oreaua, B.M.I.; Ernandeza, I.F.; Eyrona, C.P.; Illetb, L.M.; Ontyb, G.F. Original article Fibrolytic activities and cellulolytic bacterial community structure in the solid and liquid phases of rumen contents. *Control* **2001**, *41*, 187–194.
29. Camargo, F.P.; Rabelo, C.A.B.S.; Duarte, I.C.S.; Silva, E.L.; Varesche, M.B.A. Biogas from lignocellulosic feedstock: A review on the main pretreatments, inocula and operational variables involved in anaerobic reactor efficiency. *Int. J. Hydrog. Energy* **2023**, *48*, 20613–20632. [CrossRef]