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METHANE PRODUCTION FROM VIVARIUM WASTE USING RUMINAL FLUID AS INOCULUM

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Abstract

This study aimed to evaluate the efficiency of anaerobic biodigesters in processing vivarium residues as substrates, using ruminal fluid as an inoculum for methane production and organic matter reduction, thereby mitigating the environmental impacts associated with the improper disposal of these wastes. The experiment utilized 2L Duran® flasks, operated in duplicate as batch reactors, fed with pine bedding (R1), corn cob bedding (R2), and sugarcane bedding (R3). The reactors were maintained under static conditions at approximately 37 °C. The highest accumulated methane production was observed in reactor R1, fed with pine bedding, yielding 12.56 L-CH_4, with a maximum daily production of 0.0067 L-CH_4/g-VS/d and a methane yield of 256 mL-CH_4/g-VS. In comparison, reactors R2 and R3 produced 5.24 L-CH_4 and 6.83 L-CH_4, with methane yields of 70 mL-CH_4/g-VS and 120 mL-CH_4/g-VS, respectively. Statistical analysis confirmed the superior performance of R1 (p-value < 0.05). Additionally, the consumption of volatile acids in R1, along with a final pH of 7.2, created favorable conditions for methanogenic microorganisms. In contrast, reactors R2 and R3 experienced medium acidification, which likely inhibited methane production. These findings demonstrate that waste generated at laboratory animal breeding facilities holds potential as a substrate for methane production when processed using anaerobic digestion technologies.

Keywords: *cage bedding waste, batch reactor, waste treatment, environment pollution.*

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Introduction

Waste generated by animal husbandry activities, such as livestock farming or laboratory animal breeding, can pose significant environmental and public health risks if not properly managed. For example, livestock residues (including manure, urine, and washing water), commonly used as fertilizers, often contain potentially harmful compounds, such as high concentrations of organic matter, suspended solids, nutrients, and pharmaceutical substances, which may contaminate soil, surface water, and groundwater (Tullo *et al*., 2019; Victorin *et al*., 2019).

Samoraj *et al*. (2022) disclose the challenge of standardizing the composition of residues from animal husbandry due to substantial variations in their physicochemical properties, influenced by factors such as the type and number of animals, diet, housing systems, and breeding conditions (Victorin *et al*., 2019). For instance, Nurdiawati *et al*. (2019) reported nutrient concentrations of 14.70 g-N/kg, 12.50 g-P/kg, and 2.38 g-K/kg in poultry manure, whereas Hossain *et al*. (2021) found 45.20 g-N/kg, 16.84 g-P/kg and 21.24 g-K/kg in the same type of compost. These findings demonstrate that materials of the same type can exhibit highly variable compositions.

In the case of animal research laboratories or *vivariums*, residues may contain infectious agents, pathogens, tested compounds, and chemical substances such as chlorine, underscoring the critical importance of proper treatment (Qiao *et al*., 2022). Cage bedding represents a significant waste stream from animal laboratory activities, consisting primarily of excreta and materials such as paper pulp, coconut husks, wood shavings, wheat straw, or rice straw, which are used for packaging and maintaining cage hygiene (Mohamed *et al*., 2018; Patel *et al*., 2021).

Anaerobic digestion presents an alternative for treating this type of waste. Animal bedding residues typically consist of manure, fermentable carbohydrates, and fibers, materials with high potential for biogas production. This process involves microbial communities breaking down organic matter in an oxygen-free environment, resulting in the production of gases primarily composed of methane and carbon dioxide (Neshat *et al*., 2017).

Victorin *et al*. (2019) investigated the use of animal bedding collected from a dairy farm as a substrate of anaerobic digestion to produce methane. Their analysis revealed that the feedstock consisted of approximately 34% manure, 41% fermentable carbohydrates, and 15% lignin, indicating that about 75% of the composition was suitable for bioconversion. Also analyzing beddings from a dairy farm, Sanchis-Sebastiá *et al*. (2020) observed a manure content varying from 26 to 41%, and about 20% of fermentable carbohydrates. The authors concluded that the residence time in the cage affects the organic fraction of the manure and reduces the content of fermentable carbohydrates in the straw, interfering with the potential for biogas production.

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In another study, Neshat *et al*. (2017) emphasized that lignocellulosic biomass (present in cage beddings), when subjected to physical, chemical, or biological pretreatment, can mitigate carbon deficiency in manure, providing an effective strategy to enhance the anaerobic digestion efficiency of animal waste. Building on this, previous studies have explored livestock bedding as a substrate for biological methane production (Riggio *et al*., 2017; Sanchis-Sebastiá *et al*., 2020; Victorin *et al*., 2019). Nevertheless, no studies to date have specifically examined the potential of laboratory animal bedding for this purpose.

Therefore, the objective of this study was to evaluate the potential of vivarium waste digestion for obtaining biomethane, using ruminal fluid as the inoculum, and to assess the reduction in organic matter concentration through batch reactor experiments.

Materials and methods

Vivarium Waste

The research employed three batch reactors, each fed with a different type of substrate: (Reactor 1 - R1) pine beddings, (Reactor 2 – R2) corn cob beddings, and (Reactor 3 – R3) sugarcane bagasse beddings. The residues of animal laboratory cages were obtained in the Central Animal House of the Federal University of Alagoas - BIOCEN/UFAL.

Substrates contained feces of rats and mice, being collected directly from the dirty cages after handling the animals, packed in 1 L beaker, and transported in thermal boxes to the Environmental Sanitation Laboratory - LSA/CTEC for characterization (Table 1). A crusher was used to reduce the size of the particles and obtain the most homogeneous consistency possible, maintaining a concentration of 10% of total solids in the reaction medium.

Table 1. Characterization of vivarium waste

Reactor	Waste	pH	COD (g/L)	TS (g/L)	TVS(g/L)	TFS(g/L)
R1	Pine bedding	8.92	14.98	61.22	55.84	53.78
R2	Corn cob bedding	8.80	14.52	39.04	34.58	44.56
R3	Sugarcane bagasse bedding	8.70	12.68	65.65	57.52	81.22

Note: COD (Chemical Oxygen Demand); TS (Total Solids); TVS (Total Volatile Solids); TFS (Total Fixed Solids)

Inoculum

Bovine ruminal fluid was used as the inoculum due to the specific microbiota in the gastrointestinal tract of these animals, which actively contributes to the digestion of food residues

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(Wang *et al*., 2021). The ruminal fluid was sourced from a slaughterhouse in a city in the state of Alagoas, collected directly from the bovine rumen immediately after slaughter (Sunarso *et al*., 2010). It was then filtered through a nylon sieve to remove solid fractions, retaining only the liquid portion (rumen fluid) of the gastric content.

The inoculum samples were obtained from three different animals to reduce the risk of compromised microbiota and minimize the influence of the animals' diets. After collecting, the ruminal fluid was stored in plastic bottles and transported to the laboratory in insulated containers. The ruminal fluid used had a pH of 7.2, total solids (TS) concentration of 2.02 g/L, total volatile solids (TVS) concentration of 1.53 g/L, and a chemical oxygen demand (COD) of 2.18 g COD/L.

The collection of vivarium residues and ruminal fluid did not involve contact with live animals or interfere with the handling of animals in the vivarium or the cattle slaughtering procedures.

Nutritional medium

Besides the substrate (cage bedding waste) and inoculum, a nutritional solution was added to the reaction medium, presenting the following composition (mg/L): CH_4N_2O = 125; $NiSO_4 \cdot 6H_2O$ = 1; $FeSO_4 \cdot 7H_2O$ = 5; $FeCl_3 \cdot 6H_2O$ = 0.5; $CaCl_2 \cdot 6H_2O$ = 47; $CoCl_2 \cdot 2H_2O$ = 0.08; SeO_2 = 0.07; KH_2PO_4 = 85; $KHPO_4$ = 21.7; and $Na_2HPO_4 \cdot 2H_2O$ = 33.4 (Del Nery, 1987).

Reactors

The experiment was conducted in duplicate for each of the three batch reactors under non-continuous hydraulic flow conditions. The reactors consisted of 2000 mL Duran® flasks, with 1000 mL allocated for the reaction medium and the remaining 1000 mL reserved for headspace. During the preparation of the reaction medium, 8% (v/v) of inoculum was added based on the total reactor volume (Macedo *et al*., 2012). Anaerobic conditions were established by replacing the atmospheric air in the headspace with nitrogen gas via bubbling. The flasks were sealed hermetically using butyl rubber stoppers and plastic caps to prevent gas leakage.

The reactors were kept at approximately 37 °C using a heating system developed specifically for the maintenance of the reaction, built in a 120 L thermal box and a 50 L lower reservoir equipped with thermostat-controlled heating. The water recirculation between the reaction bed and the reservoir was carried out by a Resun® submersible pump of 1,200 L/h. The experiment was conducted for 60 days with methane production monitoring and evaluation of organic load removal through the analysis of COD (estimated) and total solids.

Physical-chemical and chromatographic analysis

During the experimental period, the volatile fatty acid (VFA), alcohol concentrations, and the biogas methane content were measured using a gas chromatograph equipped with a flame-

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ionization detector (FID) and a Supelcowax 10 column (30 m high × 0.25 mm id × 0.25 µm film thickness), a thermal conductivity detector (TCD), argon as the carrier gas, and the column was packed with a Supelco Carboxen 1010 Plot (30 m×0.53 mm id) (Tibúrcio Neto *et al*., 2024). The pH, COD and solids concentrations were measured at the beginning and end of the experimental period, according to the procedures described in the Standard Methods (APHA, 2017).

Statistical analysis

The standard deviation and the coefficient of variation were used as the average composition of the replicas of the reactors. The Boltzmann sigmoid, based on the sum of the squares of the residues, was applied to adjust the experimental data obtained through the average production of methane of the batch reactors (Florentino *et al*., 2010). The Boltzmann non-linear model allowed the maximum methane rate calculation from experimental data.

Welch's t-test was used to analyze the differences between the reactors. This modified version of the traditional t-test is suitable for samples with unequal variances and small sample sizes. It was chosen because each experimental group (reactors R1, R2, R3) had only two independent observations for the accumulated CH_4 variable, making the data prone to natural variability between samples (West, 2021). Unlike the standard t-test, which assumes equal variances between groups, Welch's t-test adjusts variance estimates for each group individually, offering greater robustness when variances may not be equal. This approach is particularly relevant in this study, as each reactor was fed with different substrates, creating distinct experimental conditions and potentially different variances in the results.

Results and discussion

Methane production

The data presented were obtained as the average of the duplicates of accumulated methane values (Table 2). The initial pH of the substrates was not standardized at the start of the reactors, as all values ranged from 7.32 to 8.9, within the optimal range for methane production (7.5 to 8.5). Within this range, methanogenic archaea grow more slowly than acetogenic microorganisms (Xavier, 2009).

Reactors R2 and R3 exhibited the lowest final pH values of 4.97 and 5.01, respectively (Table 2). This reduction is likely associated with the significant production of acetic, butyric, and propionic acids, as shown later in Table 4. The increase in these acids may be related to the characteristics of the residues used (sugarcane chips and corn cobs), which contain high amounts of carbohydrates.

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Table 2. Accumulated methane production in the reactors

Reactors	R1.1	R1.3	R2.1	R2.2	R3.1	R3.3
Accumulated $L_{CH4}/L_{headspace}$	12.60	12.52	5.33	5.07	6.43	7.22
Average $L_{CH4}/L_{headspace}$	12.56		5.20		6.83	
SD±	0.0566		0.1792		0.5556	
Initial pH	8.93		7.70		8.88	
Final pH	7.20		4.97		5.01	

The accumulated methane production in reactor R1 was 12.56 L-CH_4, higher than R3 (6.83 L-CH_4) and R2 (5.20 L-CH_4) (Table 2). The Welch t-test results (Table 3) indicate that R1 achieved significantly higher methane production compared to R2 and R3. Significant differences were observed between R1 and R2 ($p = 0.006$) and between R1 and R3 ($p = 0.042$), highlighting the superior performance of R1 in methane generation. Conversely, R2 and R3 were statistically similar ($p > 0.05$).

Table 3. Welch's t-test for comparison between reactors

Compared reactors		Accumulated_CH_4		
		Statistic	df	p-value
R1	R2	54.112	1.19	0.006
R1	R3	14.45	1.02	0.042
R2	R3	-3.91	1.21	0.125

These findings emphasize the potential of R1 (pine bedding) as the most efficient system for biogas production. The final pH of 7.20 in R1 (Table 2) likely contributed to its superior performance, as pH levels around 7 support methanogenic archaea. In contrast, the lower pH values in R2 and R3 probably inhibited critical enzymes involved in methanogenesis by facilitating the diffusion of molecular acids or free ammonia (Qiu *et al*., 2023).

The methane production rate was determined by the ratio between the maximum methane production rate (L-CH_4/d) and the initial biomass concentration (g-VS). Reactor R1, fed with pine litter, showed the highest production of 0.0067 L-CH_4/g-VS/d and a yield of 256 mL-CH_4/g-VS. R3 presented the second-highest methane production rate of 0.003 L-CH_4/g-VS/d and a yield of 146 mL-CH_4/g-VS. R2 reactor produced 0.000047 L-CH_4/g-VS/d and a yield of 70 mL-CH_4/g-VS (Table 4).

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Table 4. Maximum production rate, maximum time for CH_4 production and CH_4 yield

Reactor	CH_4 Yield	Maximum rate CH_4		
	(mL-CH_4/g-VS)	Volume (L)	Time (days)	R^2
R1	256	0.328	38	0.9594
R2	70	0.003	26	0.9948
R3	146	0.131	33	0.9938

Victorin *et al*. (2019) reported higher methane yield values (501–504 mL-CH_4/g-VS) compared to those obtained in the present study. Their work involved the co-digestion of washing liquid and hydrolysate derived from animal bedding from dairy farms, with varying proportions of manure (14.4–42.5%) and fermentable carbohydrates (36.5–54.2%), for about 40 days. Digested sludge from an anaerobic digester at a municipal wastewater treatment plant was used as inoculum. The authors highlighted that fractionating animal bedding ensures consistent processing regardless of the manure's content or composition, improving conversion efficiency and fostering synergies between biogas and bioethanol production.

However, the methane yield values were comparable to those reported by Riggio *et al*. (2017) (192–239 mL-CH_4/g-VS), who studied the batch anaerobic digestion of various types of spent livestock bedding from sheep, goats, horses, and cows, using liquid inoculum from an UASB reactor treating sugar industry wastewater. The study was conducted for 60 days. The authors highlighted that the long-term accumulation of nitrogen and potassium in the leachate was a primary concern when using this type of substrate.

Reactor R1 reached maximum methane production on the 38th day of the experiment, presenting the highest production rate according to the Boltzmann linear fit, of 0.33 L-CH_4 (Figure 1A). Reactor R2 (Figure 1B) reached maximum production on the 26th day and obtained the lowest accumulated production and the lowest CH_4 production rate, of 0.0035 L-CH_4, lower than that recorded in reactor R3 (Figure 1C), which presented a production rate of 0.13 L-CH_4 on the 33rd day of the operational phase.

Methane, hydrogen, and carbon dioxide gases were monitored, as the biogas composition can contain up to 70% methane and 45% carbon dioxide (Mafaciolli, 2014). Reactors 1, 2, and 3 presented 35.42, 12.71, and 15.23% of methane in the composition of the biogas produced, respectively.

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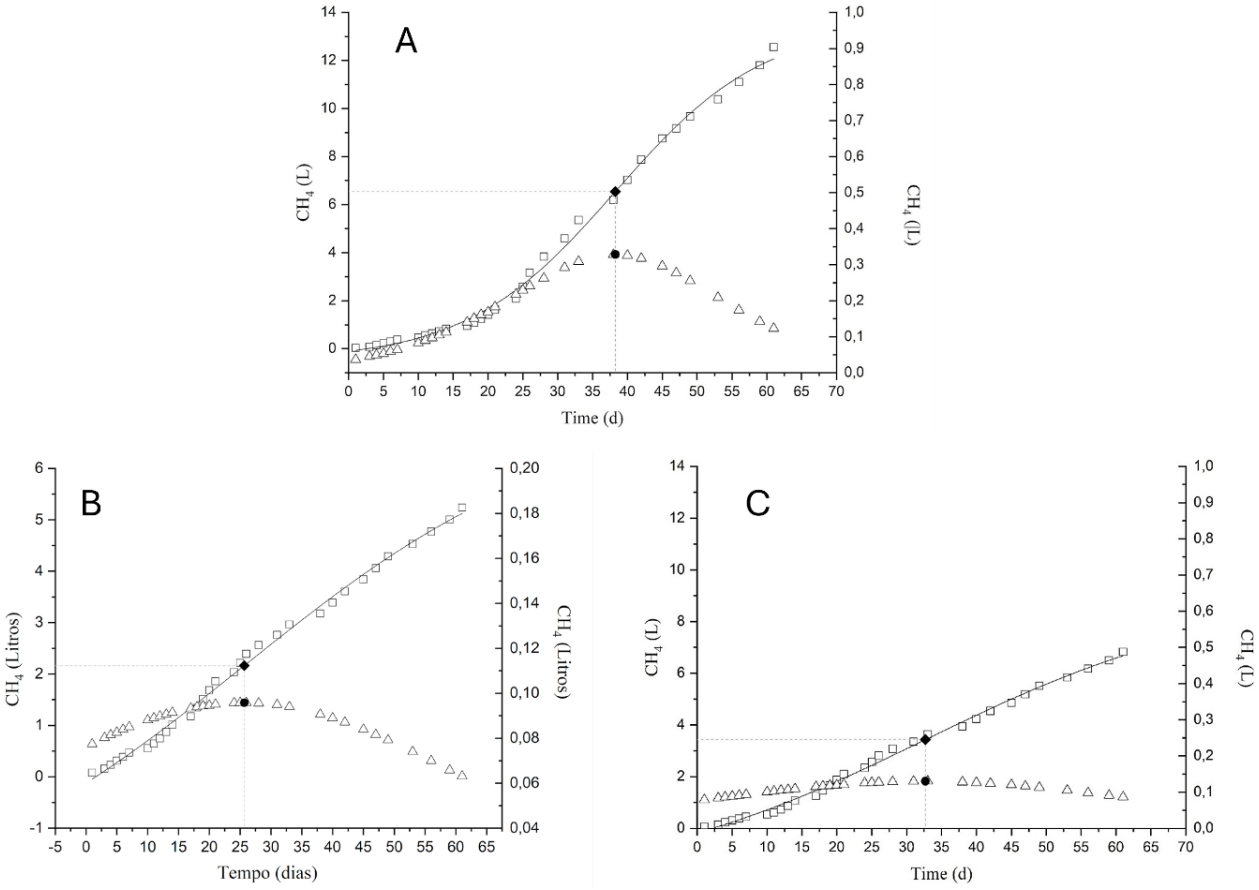

Figure 1. Accumulated CH_4 concentration (□), Inflection point (♦), Derivative of the function (Δ), Maximum CH_4 production rate (●) over time in R1(A), R2 (B) and R3(C)

Solids consumption efficiency and metabolite production

The consumption of solids in the reactor is a key factor in evaluating the anaerobic digestion process, as it can serve as an indicator of efficiency in methane production. Reactor R2 (corn cob litter substrate) achieved the highest TS and TVS conversions (86.80% and 86.40%, respectively) (Table 5). However, it also presented the lowest average methane yield (70 mL-CH_4/g-VS).

Prasanna Kumar *et al*. (2024) explain that higher initial substrate concentrations and total solids in reactors can inhibit methanogenic microorganisms due to elevated organic loading or high concentrations of NH_3 and volatile fatty acids (VFAs). This may explain the performance of R2, which had the highest initial solids concentrations (155,423 mg-TS/L and 121,927 mg-TVS/L)

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and a significant increase in acetate, butyrate, and propionate levels (Table 5). The accumulation of these VFAs likely caused acidification, as corroborated by the reactor's low final pH of 4.97 (Table 2), suppressing methane production (Qiu *et al*., 2023).

Table 5. Initial and final concentration of total solids – ST; total volatile solids, their respective consumption efficiencies, and alcohol and volatile organic acids

Reactor	Phase	TS		TVS		Acetic acid	Butyric acid	Propionic acid	Ethanol
		mg/L	consumption efficiency	mg/L	consumption efficiency	mg/L			
R1	Initial	60980	74.92	49109	76.69	722.34	31.59	77.75	66.12
	Final	15294		11446		56.97	18.19	522.15	66.33
R2	Initial	155423	86.80	121927	86.40	1215.64	55.25	90.53	65.66
	Final	20518		16579		6291.69	1354.37	2224.84	68.69
R3	Initial	58990	46.75	47551	52.63	690.99	47.02	81.08	65.60
	Final	31414		22525		8300.09	1278.30	1648.87	111.79

The highest increase in acid concentrations was observed in R3 (sugarcane bagasse litter), which had a final pH of 5.01. However, R3 exhibited lower solids removal efficiencies (TS = 46.75% and TVS = 52.63%). Similar to R2, the substantial increase in concentrations of acetic acid, butyric acid in R3 probably resulted in the presence of VFAs in their undissociated forms, which are more toxic to microorganisms, negatively impacting CH_4 production (Liotta *et al*., 2014).

In contrast, R1 showed a decrease in concentrations of acetic acid and butyric acid. Besides that, Reactor R1, which utilized pine bedding as a carbon source, achieved significant reductions in TS (74.92%) and TVS (76.69%), indicating efficient conversion of organic matter into biogas. This aligns with the experimental results, where R1 recorded the highest methane concentration in biogas. The decline in acetic acid (from 722.34 to 56.97 mg/L) and butyric acid (from 31.59 to 18.19 mg/L) suggests that acetogens and methanogens effectively consumed VFAs. This maintained a neutral pH of 7.20 and supported the establishment of a mature microbial community with balanced metabolic activity (Cai *et al*., 2021).

Despite the observed increase in propionate levels across all three reactors, its influence varied between systems. In reactors R2 and R3, where CH_4 production was lower, the accumulation of all VFAs, including propionate, likely acted as an additional inhibitory factor. Conversely, in reactor

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R1, propionate may have served as a carbon source rather than an inhibitor. Liu *et al*. (2024) highlight that, under specific conditions, propionate can function not only as an inhibitory compound but also as an effective carbon source for methane production. When multiple nutrients are present, propionate can contribute up to 35% of methane output. This occurs through its conversion into acryloyl CoA via metabolic pathways involving propionate and acetate, which are subsequently transformed into methane through hydrogen and acetate pathways. Over time, propionic acid fermentation can reshape microbial community structures, enhancing the system's tolerance to propionate and improving the overall resilience of the anaerobic digestion process (Liu *et al*., 2024).

COD removal efficiency

In general, high COD removal efficiency is typically associated with greater consumption of volatile solids, resulting in increased methane production (Tibúrcio Neto *et al*., 2024). In the present study, COD removal (R2 = 84.92%, R1 = 81.08%, and R3 = 51.93%) (Table 6) were directly proportional to the reductions in total solids (R2 = 86.80%, R1 = 74.92%, and R3 = 46.75%) (Table 5). As previously discussed, despite R2 achieving the highest COD removal rate, reactor acidification hindered its methane production performance.

Table 6. Initial and final concentrations and COD removal efficiency.

Reactors	*Estimated COD		
	Initial (g/L)	Final (g/L)	Removal efficiency (%)
R1	82.74 ±22.97	15.66 ± 3.49	81.08
R2	152.94 ± 81.10	23.06 ±1.25	84.92
R3	66.20 ±4.61	31.82 ±0.75	51.93

**Estimated COD calculated by theoretical and approximate COD cell mass conversion factor of the order of 1.42 gCOD/gSTV*

In general, the COD removal efficiencies observed in this study were higher compared to those reported in other studies. For instance, Wei *et al*. (2019), while evaluating the anaerobic co-digestion of sewage sludge (SS) and cow manure (CM), reported COD removal rates ranging from 47.2% to 57.1%, with methane production varying between 352.3 and 470.3 mL-CH_4/g-VS.

Santos *et al*. (2024) investigated the anaerobic co-digestion of goat manure (GM) and cheese whey (CW) in batch reactors. The inoculum utilized in their study was sludge sourced from a UASB reactor treating domestic sewage. Their results similarly demonstrated that the highest COD

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removal rate (63.73%) occurred in the reactor with the greatest solids reduction (58.33%), which employed a GM/CW substrate ratio of 50/50. However, akin to the findings of the present study, this reactor did not achieve the highest methane production, yielding 0.192 L-CH_4 over approximately 78 days. The reactor with the highest cumulative methane production (0.294 L-CH_4 over 94 days) utilized a GM/CW substrate ratio of 0/100 and achieved a COD removal rate of 50.32% and a VS removal rate of 31.37%.

Finally, the results indicate that pine bedding was the most effective substrate among those evaluated. However, its performance could potentially be enhanced by applying techniques such as material fractionation (Victorin *et al*., 2019), recycling a portion of the fibers, or employing pre-treatment methods to degrade lignocellulosic components (Neshat *et al*., 2017).

Conclusion

Anaerobic digestion (AD) emerges as a sustainable approach to waste treatment, addressing environmental issues such as water and soil contamination while facilitating renewable energy production through methane generation. Its implementation in animal facilities can lower operational costs, support localized energy generation, and provide a foundation for innovative treatment of lignocellulosic waste, thereby advancing a circular and sustainable economy.

This study highlighted the effectiveness of using cage beddings as a substrate, demonstrating the degradation of organic matter and its conversion into biogas, predominantly methane. Notably, the R1 reactor, fed with pine litter, achieved a COD removal of 81.08%, a TS reduction of 74.92%, and accumulated methane of 12.56 L-CH_4, confirming anaerobic digestion as an efficient and sustainable solution.

Patent

This experiment resulted in the deposit of a patent entitled: “methane production with vivarium waste using ruminal fluid as inoculum” under protocol nº BR 10 2019 020899 6 A2, by the Federal University of Alagoas - UFAL with the INPI.

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