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Use of agricultural residues of rice straw and sheep manure as substrates for biogas production by anaerobic co-digestion

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Agricultural activities related to rice processing and sheep farming generate solid waste such as rice straw, and manure that can be processed to generate renewable energy. An efficient way to transform this waste into biogas and methane is efficient through anaerobic co-digestion, which represents a viable alternative for energy production. The objective of this research was to produce biogas with potential methane content, through the anaerobic co-digestion of rice straw (RS) residues, which provide carbon, and sheep manure (SM), which mainly provides nitrogen. The methodology included the physicochemical characterization of the substrates, followed by the pretreatment of the rice straw in alkaline sodium hydroxide solution (8 and 10%) in order to optimize the co-digestion process during 15 days. The doses of manure applied in co-digestion in relation to rice straw were 0%, 30%, 50%, 70% and 100%. Under these conditions, completely random combinations were made with 56 experimental units for the determination of the biochemical potential, which lasted 15 days. Biogas measurement was performed by volume displacement. The results indicated that a mixture of 50% pretreatment of the RS was more efficient than the other mixtures, with a maximum accumulated production of 1801 mL of total biogas. The technique of pretreatment of lignocellulosic substrates is appropriate in the processes of co-digestion of agricultural waste for the generation of biogas.

**Keywords**: anaerobic co-digestion, sheep manure, rice straw, lignocellulose pretreatment

* 1. Introduction

Currently, energy generation must consider environmental sustainability, in this context the transformation of second generation biomass or renewable biomass as part of the supply chain can be effective in renewable energy self-sufficiency (Vaccari, et al. 2022). Agricultural activity in Peru is vital because it is part of the self-consumption in several regions of the country, in 2021 the annual production of rice straw was 2.25 million tons (MINAGRI, 2021a), and the production of sheep was 109,423,09 units (MIDAGRI, 2021b). The Camaná valley in the Arequipa region stands out for its production of rice straw, which represents 14.2% of the production in tons at the national level. However, rice straw is often burned in the open air, leaving the soil bare and exposed to erosion, burning rice straw provides particulate matter and emits gases into the atmosphere (Seglah et al. 2020). Sheep manure often ends up polluting water due to runoff or infiltration, as well as polluting adjoining soils due to nutrient loading. Instead, these residues can be used in the generation of non-conventional energy; for example, biogas production uses various organic residues, its production comprises a mixture of gases made up mainly of methane (CH4), carbon dioxide (CO2), water vapor and trace elements (Lin et al. 2021). Anaerobic digestion consists of decomposing organic matter from the substrate, bacterial action is required, which is very sensitive and could be inhibited by unfriendly conditions and by the presence of oxygen, other determining factors are the concentrations of oxygen demand, water content and total solids (Koniuszewska et al. 2020).

Four defined stages occur in this process: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Lin et al. 2021). Hydrolysis requires the activity of fermenting bacteria, under strict anaerobic and facultative conditions, to transform and break down high molecular weight components such as lipids, fats, proteins, among others, producing soluble short-chain compounds (Lin et al. 2021). Acetic, propionic or butyric acid, hydrogen (H2) and carbon dioxide (CO2) are also generated (Reyes, 2018). In the second stage of acidification, molecular hydrogen and acetate are formed, which can be metabolized by methanogenic microorganisms, however the presence of butyrates, propionates, are transformed into hydrogen and acetate, by the action of acetogenic bacteria due to the fact that organic compounds act as receptors and electron donors, mainly producing volatile fatty acids (VFA) (Reyes, 2018). Methanogenesis ultimately produces carbon dioxide and methane, by means of methanogenic bacteria (Gharbi et al. 2022). Rice straw is an organic material that is difficult to biodegrade (Ngan et al. 2020), due to its polymeric composition of lignin, cellulose and hemicellulose. The mechanical pretreatment increases the specific area of the raw material, which is used by microorganisms that degrade bonds and complex compounds to simpler ones (Negi, et al. 2018). In the hydrolytic phase a size of less than or equal to 3 mm is recommended (Luo et al. 2021). Chemical pretreatment is effective, acids and bases are used, among the alkaline agents calcium hydroxide Ca(OH)2, magnesium hydroxide Mg(OH)2, potassium hydroxide (KOH) and sodium hydroxide stand out (NaOH) (Li et al 2020). One of the purposes is to improve not only the quality of biogas with higher methane yield, but also to increase the reaction rate and anaerobic degradability. For example, the chemical pretreatment of the lignocellulosic biomass of wheat straw has been studied, and it was determined that a proportion of chicken manure, in a proportion of 50% with the treated wheat straw, was efficient in an anaerobic co-digestion process (Zahan and Othman 2019). Zheng et al. (2018), used agricultural residues (grass, manure, and milk) in batch reactors that tested mono digestion and co-digestion of dairy manure with grass and achieved a methane yield greater than 39% for a cosubstrate ratio of 2:2. Likewise, Pohl et al. (2019) used rapeseed oil and wheat straw in an anaerobic co-digestion process and concluded that an optimal C/N ratio in the mixture under thermophilic conditions (55 °C); they produced a higher methane yield (18 %), due to an improvement in the organic rate expressed as volatile solids (2.5 g/L.d).

Wijaya, et al (2020), developed the biochemical methane potential (BMP) test of cattle and chicken manure, with rice straw and hornwort, under mesophilic conditions (35 °C) for working volumes of 350 mL and a inoculum to substrate ratio of 1:1, producing 3773.33 mL of biogas with 66.68 % CH4. Qiang et al. (2019) studied the effect of rice straw pretreated with an alkaline solution using microwaves and the use of pig manure as a co-substrate in an anaerobic co-digestion process and found that a high content of solid manure in the combination increased biogas yields by 25 %. Tran et al. (2021) also investigated the optimal C/N ratio when applying rice straw with pig manure, to increase methane production, treated rice straw with bioliquids and generated 78-84% biogas. This was due to the hydrolytic efficiency of the biodegradable carbohydrates, with a 1:1 mixing ratio (expressed as volatile solids).

In order to take advantage of the agricultural residues of rice straw and sheep manure that are generated in agricultural activities in Camaná (Arequipa, Peru) and Lima, the main objective of the research was to apply both cosubstrates in the production of biogas. The anaerobic co-digestion process was used, for which the following were evaluated: a) pretreatment conditions of rice straw in alkaline solution b) specific methanogenic test for the inoculum (SMA) and c) mixture proportions by BMP.

* 1. Materials and methods

**2.1 Collection of substrates, sample and inoculum**

The rice straw was obtained from the company "Agro Estrella" E.I.R.L, located in the province of Camana in the Arequipa Region, sample quartering method was applied, described by Wellinger, Murphy and Baxter (2013, p. 58), for the collection of 2 Kg of sample. The sheep manure was obtained from the breeding module for research and training of the sheep production system (RIGORANCH), of the Universidad Nacional Agraria La Molina, 2 Kg of sample were also collected using the described procedure. The test inoculum corresponded to the activated sludge extracted from the upflow reactor (UASB) of the Universidad Nacional de Ingeniería. The cosubstrates and the inoculum were stored at 4 °C until further analysis. The analysis of variance of a factor (ANOVA), is used to evaluate the effect of the biogas generation treatments ("y"), the statistical model used is:

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| $$y\_{ij}=t\_{i}+k+e\_{ij}$$ | (1) |

Where: yij is the amount of biogas produced (observed) for each treatment, k is the general mean, ti is the effect of the treatment and εij is a random error. The amount of substrate used in the reactors is based on the values of volatile solids, both in sheep manure, untreated rice straw and treated rice straw, defined by equation 2 (Contreras et al. 2012):

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| --- | --- |
| $$Pi= \frac{m\_{i}c\_{i}}{m\_{s}c\_{s}}$$ | (2) |

Where: *Pi* is the inoculum/substrate ratio; *mi* is the mass of the inoculum (g); *ms* is the mass of the substrate (g), *ci*are the volatile solids of the inoculum (%) and *cs* are the volatile solids of the substrate (%). In this research, an inoculum/substrate ratio of 0.5 was chosen for tests in batch reactors (Cárdenas et al, 2019, p.102).

**2.2 Physicochemical analysis and Alkaline pretreatment of lignocellulosic material**

Aliquots of each substrate and the inoculum were analyzed to determine the content of volatile solids (APHA 2540 E), total solids (APHA 2540B), pH (potentiometric method) in the chemistry laboratories of the Cesar Vallejo University. The Walkley and Black (1934) method was used to analyze organic carbon and the Kjeldahl method was used to analyze nitrogen, in charge of the Soil Analysis Laboratory of the La Molina National Agrarian University. On the other hand, the rice straw was crushed and sieved to produce a particle size between 1 and 3 mm, to generate a higher biomass specific area (Luo et al. 2021). An alkaline treatment was then carried out with 400 mL of sodium hydroxide (8% and 10%) in triplicate. Then, 30 grams of rice straw were placed in each jar and mixed. Samples were incubated for 72 hours at room temperature and then filtered. The cake was oven dried for 24 hours at 105°C and stored for later use at 4°C. The analysis of the lignocellulosic components before and after the treatment was carried out in the chemical laboratory of the Universidad Nacional Agraria La Molina.

**2.3 Measurement of the specific methanogenic activity of the inoculum (SMA) and Biochemical Methane Potential Test (BMP)**

The SMA of the inoculum was measured following the methodology proposed by Soto (1992). For this purpose, six 500 mL bottles were used, three containers with the inoculum and acetic acid as standard substrate, and the other 3 bottles contained only the inoculum without acid. Each digester was connected to a trap filled with a 0.625 N of NaOH solution to separate the CO2 formed in the biogas, and the gas volume was measured by water displacement. The test was carried out at mesophilic temperatures controlled by a 50 W thermostat. The following equation was applied to calculate the SMA:

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| $$SMA= \frac{m}{I+Vsample}$$ | (3) |

Where: “*m”* is the maximum slope of the methane production curve; *“I”* is the concentration of the inoculum in g VS/L; *Vsample* is the total volume of the sample in the bottle expressed in mL.

To evaluate the efficiency in the production of biogas composed mainly of methane, the method described by Filer et al. (2019) with some modifications. Figure 1, shows the procedure followed. For this, three proportions of pretreated rice straw (PRS) and sheep manure (SM) were mixed for anaerobic co digestion (SM/PRS: 70/30; 50/50; 30/70). In addition, pure substrates were used for anaerobic mono digestion (100% PRS and 100% SM). Control blanks were also included, triplicates were made for each test, and samples were measured daily for 15 days. The effective volume of each replicate was 450 mL in each reactor under mesophilic conditions (35 °C). A 2N alkaline solution trap was incorporated to capture the CO2 generated in the reactors; the volume of the gas was measured by displacement of water. The biochemical potential of methane varied depending on the composition of the nutrients provided by the difference between the mixing ratios (Tian et al. 2023).



*Figure 1. Biochemical test for methane by anaerobic digestion of rice straw and sheep manure. The anaerobic co digestion group (70%SM, 50%SM and 30%SM) and mono digestion (SM, PRS) were formed, as well as quality controls.*

**2.4 Analysis of data**

The Kruskal-Wallis test was applied to analyze the significant differences in biogas production applying different doses of co-substrates. Analysis of variance (ANOVA) was also used to evaluate the optimal dose of co-substrates for effective biogas production.

* 1. Results and Discussion
		1. Physicochemical characterization of substrates and inoculum

A characterization of the substrates was carried out prior to the anaerobic co-digestion process. According to table 1, the treated and untreated rice straw and sheep manure presented acceptable pH values above 7.4. Although the inoculum had a pH of 6.43, this value was found to be within an acceptable range (6 – 8) suggested by Filer et al. (2019). Lower pH values can cause less methane production. Regarding the C/N ratio, the inoculum and the sheep manure presented better values, due to a higher nitrogen content, while the untreated rice straw presented the highest carbon content (38.28). This value decreased during its alkaline pretreatment, remaining optimal for the experiment.

*Table 1. Characterization of rice straw with and without treatment, sheep manure and inoculum*

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| --- | --- | --- | --- | --- | --- |
| Parameter | Unit | Rice Straw | Sheep Manure | Pre-treated Rice Straw | Inoculum |
| Total Solids (TS) | % | 77.45 | 67.88 | 76.44 | 82.7 |
| Volatile Solids (VS) | % | 56.93 | 14.35 | 43.7 | 41.84 |
| pH | - | 7.44 | 8.64 | 7.86 | 6.43 |
| Moisture | % | 4.37 | - | - | - |
| Ash | % | 23.02 | - | - | - |
| Organic Carbon (OC) | % | 44.79 | 21.9 | 20.01 | 29.72 |
| Nitrogen (N) | % | 1.17 | 1.9 | 0.65 | 2.78 |
| C/N | % | 38.28 | 11.08 | 30.78 | 10.7 |
| Extractive | % | 7.27 | - | - | - |
| Lignin | % | 34.97 | - | - | - |
| Cellulose | % | 44.44 | - | - | - |
| Holocellulose | % | 34.5 | - | - | - |
| SMA | g COD/g VS.d | - | - | - | 0.8776 |

**3.2 Specific Methanogenic Activity (SMA) of the Inoculum**

To determine the methanogenic activity, a triplicate test was carried out based on the VS present in the anaerobic sludge, for 28 days and produced 0.62857 gCOD/g VS.d, a value considered in the range established for granular sludge (Parra-Orobio et al. 2018). Figure 2a shows the collection of the inoculum composed of the activated sludge extracted from the upflow reactor (UASB); while figure 2b shows the production of CH4 expressed in ml as a function of digestion time under aerobic conditions. As a result, a significant slope was produced (m= 7.45; R=0.9991)



*Figure 2. Inoculum: a) Inoculum collection and b) Specific methanogenic activity test*

**3.3 Effect of alkaline pretreatment**

Table 2a shows the results of the pretreatment of rice straw, a higher dose of NaOH (10 %) produced a lower content of lignin (17.03 %), holocellulose (20.97 %), cellulose (12.6 %) and hemicellulose (8.4 %). It was confirmed that the action of the alkaline solution breaks the bonds of this type of molecule, releasing polymers with shorter chains improving the efficiency of enzymatic hydrolysis (Negi, et al 2018). One-factor analysis of variance (ANOVA) confirmed the effect of the application of the alkaline solution on the rice straw samples (Table 2b). Lignin degradation was demonstrated by a p-value less than 0.05 (p=0.034) and was accompanied by the massive discovery of cellulose (Thomas et al. 2022) evidenced by a lower p-value (p = 0.012).

*Table 2. Results of pretreatment of rice straw with alkaline solution*

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| a) Alkaline pretreatment of rice straw | b) Pre-treatment of rice straw. ANOVA |
| Test | NaOH (8 %) | NaOH (10 %) |   |   | Sum of squares | gl | Root Mean Square | F | p-value. |
| Lignin (%) | 21.2 ± 5.9 | 17.0 ± 11.3 | Lignin | Between groups | 622.1 | 2 | 311.05 | 6.2 | 0.034 |
| Holocellulose (%) | 25.5 ±7.1 | 20.9 ± 9.6 |  | Within groups | 298.4 | 6 | 49.74 |  |  |
| Cellulose (%) | 20.1 ± 3.3 | 12.6 ± 5.0 |  | Total | 920.5 | 8 |  |  |  |
| Hemicelulosa (%) | 9.6 ± 7.8 | 8.4 ± 5.8 | Holoce-llulose | Between groups | 928.8 | 2 | 464.42 | 6.8 | 0.028 |
| Ceniza (%) | 59.8 ± 1.5 | 65.3 ± 0.8 |  | Within groups | 405.4 | 6 | 67.57 |  |  |
| Humedad (%) | 81.2 ± 1.1 | 80.9 ± 0.8 |  | Total | 1,334.2 | 8 |  |  |  |
| Extractivo (%) | 18.0 ± 6.1 | 19.1 ± 6.2 | Cellulo-se | Between groups | 742.7 | 2 | 371.36 | 10.0 | 0.012 |
|  |  |  |  | Within groups | 222.3 | 6 | 37.05 |  |  |
|   |   |   |   | Total | 965.0 | 8 |   |   |   |

**3.4 Biochemical production of methane (BPM)**

Figure 3 shows the process followed in the BPM as well as the results obtained. Figure 3a shows the collection of the sheep dung substrate and figure 3b-3c shows the milling and sifting process of rice straw. The mixture of both substrates was used in the BPM. Figure 3d shows the preparation of the mixtures of the substrates and Figure 3e shows the proportions prepared for SM (70%, 50%, 30%). Figure 3f shows the results of anaerobic mono digestion of sheep manure and rice straw substrates pretreated with alkaline solution, biogas production did not exceed 150 mL per rice straw, for 15 days. However, in figure 3g-3h, it can be seen that the 50% SM/SRS pretreated mixture was optimal, generated 240 mL of biogas on day 15 and an accumulated total of 1801 mL of biogas, which exceeded more than 100% that produced in mono digestion. This indicates that co-digestion of rice straw with nitrogen-rich substrates in a suitable mixture is capable of balancing the C/N ratio; improving biogas and methane yield (Mothe et al. 2021).



*Figure 3. Stages in the investigation: a) manure collection b) milling of rice straw, c) sieving of precursor material, d) and e) pretreatment of rice straw with NaOH solutions, f) Production of biogas by mono digestion and g) co-digestion of the mixtures; h) accumulated biogas.*

Table 3a shows the results of statistical significance (p = 0.011), calculated by means of the Kruskal-Wallis test, on the biogas production values (mL); and table 3b confirms that the optimal dose corresponded to a proportion of 50 % sheep manure/50 % treated rice straw, calculated by means of the Kruskal Wallis Post-Hoc test, which presented the highest average range (52.8 %).

*Table 3: Kruskal – Wallis test to evaluate the optimal dose in the production of biogas*

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| a) Kruskal-Wallis test | b) Kruskal-Wallis rank tests |
| Test statistics a,b | Biogas production | Mixture | N | Mean range | Mean range |
| Kruskal–Wallis H | 13.087 | 100 % SM | 15 | 27.47 |  |
| gl | 4 | 100 % RS | 15 | 29.67 |  |
| Asymptotic sig. | 0.011 | 70 % SM/ 30 % RS | 15 | 38.2 | 38.2 |
| a. KGrusKgal-Wallis test | 30 % SM/70 % RS | 15 | 41.87 | 41.87 |
| b. Grouping Variable: Mix | 50 % SM/50 % RS | 15 |   | 52.8 |

* 1. Conclusion

Rice straw and sheep manure residues have been used to produce biogas through an anaerobic co-digestion process enhanced by the pretreatment of rice straw with an alkaline solution. It was possible to reduce the content of lignin, holocellulose, cellulose, discovering a greater efficiency in the acceleration of anaerobic co-digestion compared to mono digestion of each substrate. A substrate ratio of 1:1 increased biogas production by at least 100%, demonstrating that co-digestion of rice straw with nitrogen-rich substrates is effective in balancing the C/N ratio and improving biogas yield. The results have improved the understanding of methane production through anaerobic co-digestion of sheep manure and rice straw, and serve as support for future research that seeks to optimize the yield of residual biomass transformation processes.

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