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Two-stage biohydrogen and biogas production using agro-food waste biomass

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SOMMARIO ESTESO

Il costante aumento della popolazione mondiale ha portato ad una richiesta sempre maggiore di energia. La maggior parte dell'energia utilizzata nel mondo è ricavata dallo sfruttamento dei giacimenti di petrolio. La combustione degli idrocarburi ricavati da quest'ultimo rilasciano però CO_2 che contribuisce all'effetto serra. Un ulteriore problema, dal punto di vista ambientale, è rappresentato dalla gestione dei rifiuti. A fronte di queste problematiche, negli ultimi anni, diversi studi sono rivolti alla ricerca di diverse metodologie per la conversione degli scarti per la produzione di energia pulita e sostenibile dal punto di vista ambientale. L'idrogeno potrebbe rappresentare una soluzione agli idrocarburi per via del suo elevato potere calorifico e poiché la sua combustione non rilascia la CO_2. L'idrogeno ed il metano possono essere prodotti a partire dai rifiuti agro-alimentari. In questo modo è possibile gestire sia lo smaltimento di quest'ultimi, sia ottenere prodotti a elevato contenuto energetico. Questo è possibile attraverso due processi metabolici condotti da diversi microrganismi, che sono la dark fermentation (DF) e la digestione anaerobica (DA) rispettivamente per produrre idrogeno e metano. Questi due processi possono essere anche combinati per ottenere rese maggiori di gas attraverso il processo di digestione anaerobica a doppio stadio. Il presente lavoro di tesi si focalizza sullo studio di DF e AD di diverse biomasse, provenienti dal settore agro-alimentare, per produrre idrogeno e metano. Gli scarti agro-alimentari hanno un elevato contenuto di lignina che rallenta e talvolta inibisce i processi fermentativi. Dunque, per consentire la completa fermentazione sono stati eseguiti pretrattamenti chimici e fisici. Le biomasse utilizzate sono: scarti di latte, scarti vegetali, letame, scarti zuccherini industriali e vinacce. In particolare, sulle vinacce i pretrattamenti sono stati finalizzati alla rimozione dei polifenoli attraverso i seguenti step: macinazione, ultrasuoni, estrazione di polifenoli attraverso una soluzione di etanolo all'80% e degradazione lignina attraverso un trattamento alcalino con NaOH 3M per 24 ore. I processi fermentativi sono stati condotti al 6% di secco con un rapporto substrato-inoculo 1:1 in condizioni mesofile e alimentazione batch. Nel processo di DF il pH è 7 per controbilanciare la possibile caduta di pH a seguito della formazione di acidi grassi volatili e l'inoculo è pretrattato termicamente a 80 °C per inibire la popolazione metanigena. La produzione di gas giornaliera è stata misurata quantitativamente mediante spiazzamento di acqua e qualitativamente mediante analisi con gas cromatografia. Le prime analisi hanno rilevato che le configurazioni contenenti letame in combinazione con scarti di latte o scarti vegetali non sono state in grado di produrre idrogeno attraverso il processo di DF. Questo sia nel caso di un trattamento termico per inoculo e metano, sia nel caso di un pretrattamento acido a pH 4 per il letame. Un'ulteriore analisi è stata condotta per valutare la produzione di idrogeno da parte di sistemi che utilizzano il letame come biomassa. La prima configurazione costituita dal 50% letame e 50% scarti vegetali, così come la seconda contenete 50% letame e 50% scarti zuccherini, non hanno

prodotto idrogeno. Successivamente è stata testata una configurazione di co-digestione costituita per il 50% di da scarti di latte e 50% da scarti vegetali, che ha prodotto 50 mL/gVS di idrogeno e di ulteriori 100 mL/gVS di metano, attraverso un processo a doppio stadio. La configurazione contenente 5% latte e 95% letame ha portato alla produzione di 250 mL/gVS di metano attraverso un singolo stadio di digestione anaerobica. Il letame in combinazione con altre biomasse è stato sottoposto più volte ad un processo di dark fermentation non garantendo mai però una produzione di idrogeno superiore a 1 mL/gVS. La configurazione composta da 50% e i restanti 50% da vegetali è stata testata anche con un rapporto di solidi volatili di biomassa e inoculo pari a 2:1, sempre attraverso un processo di doppio stadio, portando una produzione di 30 mL/gVS di idrogeno e 90 mL/gVS di metano, leggermente minore di metano rispetto all'esperimento precedente in cui il rapporto era pari a uno. Per quanto riguarda i trattamenti sulle vinacce, combinando quelli fisici a quelli chimici, non si è riusciti a rendere queste biomasse dei substrati utilizzabili per la dark fermentation. Le produzioni di idrogeno sono risultate praticamente uguali a zero. Per valutare l'efficacia dei trattamenti sono state effettuate le analisi COD. Le analisi hanno evidenziato che il pretrattamento più efficace è stato la macinazione in una soluzione di etanolo all'80%, con un valore di 509 mg/L. In conclusione, si è osservato come il letame non sia risultato un substrato ottimale per la produzione di idrogeno, ma la sua combinazione con altri substrati può portare a produrre fino a 250 mL/gVS di metano attraverso la digestione anaerobica. Viceversa, la configurazione di scarti di latte e vegetali ha prodotto 50 mL/gVS di idrogeno ed ha permesso la realizzazione di un processo di doppio stadio per 100 mL/gVS di metano. Questo lavoro di tesi potrebbe in futuro essere implementato attraverso lo studio e ottimizzazione delle condizioni operative per cercare di ottenere rese maggiori di idrogeno e metano. Cambiare ad esempio il contenuto di solidi totali e lavorare in condizioni di temperatura e pH differenti, monitorandone costantemente i cambiamenti.

ABSTRACT

To address climate change and the increasing energy demand, several studies have been conducted over the past to find ways to produce energy from renewable sources. Hydrogen is an excellent energy carrier, as it has a high calorific value and, unlike hydrocarbons, does not release CO2 during combustion. Another environmental issue is waste management. One way to tackle both this problem and the sustainable production of energy-rich gases is through metabolic processes carried out by various microorganisms. These processes include dark fermentation and anaerobic digestion, which can be conducted individually or in a combined process called two-stage anaerobic digestion. This thesis focused on experimenting with different types of biomasses derived from agro-industrial waste to achieve high yields of hydrogen and methane. The biomasses included milk waste, vegetable waste, cow manure, industrial sugar waste, and vinasses. Agro-food waste has a high lignin content, which slows down and sometimes inhibits fermentation processes. In order to allow complete fermentation, various physical and chemical pretreatments were studied to degrade lignin and other high molecular weight compounds such as polyphenols, to make them more accessible for consumption by microorganisms. The inoculum used to set up the systems was taken from mesophilic cow manure digestate and it was treated thermically, in the case of dark fermentation, to inhibit methanogenic microorganisms. The analyses included daily sampling of gas produced in bags connected to the production system and analysis of its composition using a gas chromatograph. The processes were tested with a total solids content of 6% and a volatile solids ratio of biomass to inoculum of one. The dark fermentation of the dairy and vegetable waste configuration resulted in yields of up to 50 mL/gVS, while the anaerobic digestion of manure and milk produced 300 mL/gVS. The two-stage process yielded 50 mL/gVS and 100 mL/gVS of methane. The pretreatments for the vinasses consist of grinding and ultrasound for mechanical treatment. Chemical treatment instead in extraction in an 80% ethanol solution to remove polyphenols or lignin degradation in 3M NaOH. These were not sufficient to make vinasses usable biomass for hydrogen production. The effectiveness of polyphenol extraction through pretreatments was evaluated by measuring COD, where mixing in an 80% ethanol solution proved to be the most effective treatment. In conclusion, manure is not suitable for hydrogen production but is effective for methane production when combined with milk waste in a single-stage anaerobic digestion process. On the other hand, configurations containing milk and vegetable waste allow the production of both hydrogen and methane through a two-stage process. The next steps are focused on finding operating conditions that allow for higher yields of hydrogen and methane. Specifically, experimenting with the process at temperatures different from 35 °C and at varying pH levels, monitoring daily fluctuations, and adjusting them by adding acidifying or basic agents.

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Nomenclature

- **CE** = Circular Economy
- **H2 =** Hydrogen
- **DF** = Dark Fermentation
- **MEC** = Microbial electrolysis cell
- **VFAs** = Volatile fatty acids
- **AD** = Anaerobic digestion
- **CO_2** = Carbon dioxide
- **CBE** = Circular bioeconomy
- **AWs** = Agro-industrial wastes
- **CO** = carbon monoxide
- **CH_4 =** Methane
- **C** = Carbon
- **O** = Oxygen
- **SCWG** = Supercritical water gasification
- **PSI** = Photosystem I
- **HRT =** hydraulic retention time
- **ORL =** Organic loading rate

1. Introduction

1.1 Introduction

The concept of circular economy (CE) it's about a mechanism that tries to avoid the excessive use of energy, natural resources, and waste production by closing loops of utilities and materials flows. This is possible through the reduction of raw material inputs, reduction of waste outputs, and the recycling of products in the production system when they reach the end of their life cycle. [1] Sustainability has become one of the major issues of our century, and the circular economy has been taken as one of the ways to achieve it. [2] This is because the world population, and consequently the consumption of energy and raw materials, are continuously increasing, raising the issue of fossil fuel depletion. [3]

The global energy demand is continuously increasing due to ongoing innovations in the scientific field and the exponential growth of human civilization. Oil and its derivatives currently represent the most widely used energy source on the planet. The inordinate use of fossil fuels has led the last decade to be distinguished by rising pollution levels and the resulting impact on public health. As a result, global society has strived to promote renewable energy to replace conventional energy resources. Considering the various alternative energy sources, biomass could play a key role as a renewable energy source with enormous potential in the production of biofuels, biohydrogen and biogas. [4]

Hydrogen (H_2) has a high heating value (~120 kJ/g) and generates the least pollution compared to other fuels. If this is derived from fossil fuels, it has a negative impact on the environment since it releases carbon dioxide. Synthesis of hydrogen from waste biomass through fermentation, on the other hand, is a low-cost and environmentally friendly approach. [5]

Three processing methods for biological hydrogen production from agro-industrial waste biomass are dark fermentation (DF), photofermentation, and microbial electrolysis cells (MEC). Dark fermentation is very favorable since it involves primary fermentation and hydrolysis of organic substrates to form gaseous compounds. This results in the simultaneous disposal of waste and formation of products including hydrogen, thus becoming one of the most widely used methods for small-scale production using this type of biomass. [6]

In dark fermentation also volatile fatty acids (VFAs) are formed. It is possible to couple this process with anaerobic digestion (AD), realizing a two-stage anaerobic digestion, to increase the amount of energy produced cause in AD, syntrophic microorganisms convert VFAs to methane through acetogenic and methanogenic processes. VFAs are typically degraded to acetate, hydrogen, and carbon dioxide (CO_2) in acetogenesis. The methanogens can utilize hydrogen to reduce CO_2 into methane during methanogenesis, which is the primary pathway of methane (CH_4) production [7] [8].

1.2 Circular Bioeconomy

The circular economy concept argues that the current global economy induces an inordinate consumption of natural resources. For this reason, that system allows waste to be used to generate a product of interest repeatedly or at least used to extract valuable substances and save energy. [9] In the case where inputs are derived from renewable resources, then the concept of CE extends to that of circular bioeconomy (CBE). Bioeconomy refers to general economic activities around biological processes and products. Combining the concepts of the bioeconomy with those of the CE, especially

those involving the use of biotechnology, is called the circular bioeconomy. Since the 1990s, both concepts of CE and CBE have attracted particular interest in international politics. [10] In the 2000s, the Lisbon Agenda marked significant economic changes, making the concept of bioeconomy very popular in Europe. In the USA, it is also widespread and focused on the biosynthetic sector. In China, important studies have been conducted on waste recycling to promote the circular economy. The bioeconomy includes, in addition to the traditional agro-food sectors, the chemical and energy industries. This highlights how diverse this concept is across different disciplines. Moreover, unlike the circular economy, the bioeconomy not only emphasizes resource efficiency to increase productivity and reduce waste but also takes social and ecological dynamics into account. For this reason, the input materials are represented by biological resources, as well as the processes to convert them. Among the key social aspects, human health and nutrition are emphasized, with efforts to avoid the degradation of arable land. [2]

1.2.1 Circular Economy in the agriculture field

Agro-industrial wastes (AWs) are all the leftovers from agriculture and livestock and represent the most common residue produced by human activities.[9] The principle of circularity in agriculture is to optimize the use of all biomasses, with the primary goal of closing the materials loop, thereby reducing resource use and discharges to the environment. The lack of resources, combined with climate change and increased demand for food, are the main features of today's society. The circular economy is a possible solution to achieve sustainable development of agriculture. [11]

The anaerobic co-digestion of food waste, as well as wastewater treatment, are effective methods to utilize microbial biotechnologies for generating biohydrogen and biomethane, enabling the transition to a circular economy. The CE is supported by business models that promote the development of bio-based products, like biohydrogen, offering economic benefits to eco-friendly industries. [12]

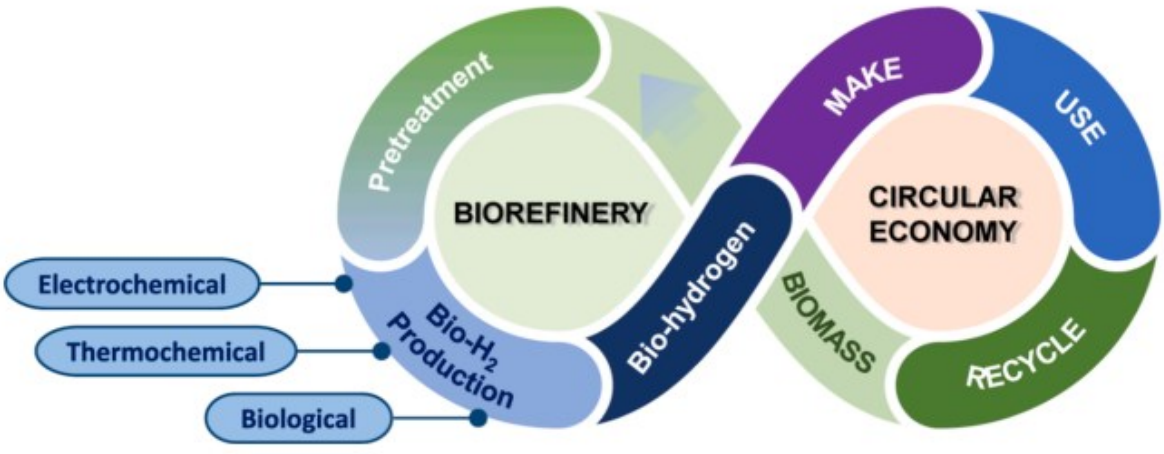

Figure 1: Circular Economy Concept [12]

1.3 Waste biomass

Biomass is one of the cleanest widespread energy sources, and its conversion is an effective method for reducing carbon dioxide emissions; therefore, it contributes to mitigate climate change, since biomass is a secondary raw material for energy production, and it is recyclable and reusable. For its use to be part of the circular economy concept, however, it is important that the supply chain of biomasses is efficient: from cultivation to transportation and storage. If the distribution network is not optimal and an industry does not have its biomass production, accessibility to biomass and competition with fossil fuels would be a non-negligible problem. [3] This is because the high-energy products derived from processes using biomass are sold at a low price. For this reason, in order for these methodologies to become widely adopted, it is crucial to achieve a significant cost reduction by optimizing the entire supply chain. [13]

For the energy valorization of organic waste, the process conventionally used is anaerobic digestion as it allows the production of high-value-added products such as methane. In addition, these products can further undergo dark fermentation to produce biohydrogen and other market-competitive products such as volatile fatty acids, carboxylic acids, and alcohols. [10]

1.3.1 Lignocellulosic biomass

An important factor of lignocellulosic biomass is its abundant availability. Countries with strong agricultural economies could utilize bioethanol and biogas produced from these waste biomasses as a sustainable energy source. Currently, most of this waste biomass is incinerated to make room for new crop lands, but this approach not only fails to produce bioethanol, which can also be sold to pharmaceutical industries, but also increases CO2 emissions into the atmosphere. Lignocellulosic biomasses are composed of lignin, hemicellulose, and cellulose.[14] The conversion of lignocellulosic biomass into usable raw materials for clean energy production is crucial to the realization of the concept of sustainability. Lignin is composed of aromatic units formed by carbon-carbon bonds (C-C) and carbon-oxygen bonds (C=O). The focus of several research projects has been the study of catalysts capable of converting lignin into high value-added chemicals. Especially, important studies have been conducted on breaking C-C and C=O bonds while trying to maintain aromatic functions. [3] Cellulose, on the other hand, is a high-density polysaccharide that has a crystalline structure, which makes its degradation complex. Hemicellulose, in contrast, is not crystalline. [14]

Lignocellulosic biomass could be a feedstock for biohydrogen production. This type of biomass is nothing more than a plant-based material derived from agricultural or forestry waste, energy crops, or garden waste. The production of biohydrogen from lignocellulosic biomass traditionally requires three stages: pretreatment, hydrolysis, and fermentation. hydrolysis is carried out using enzymes. To prepare the biomass for hydrolysis, pretreatments are performed that involve the use of alkalis and acids, which are not environmentally sustainable but are economically viable. [10] Through hydrolysis, polysaccharides can be converted into simple sugars that are more accessible to microorganisms, ensuring higher yields of biogas or bioethanol. Instead of acids and bases, ionic liquids can also be used; however, they have the disadvantage of being more expensive than the previous approach. [14]

1.3.2 Microalgal biomass

Microalgal biomass is very valuable for producing biodiesel, this is because microalgae are easy to cultivate and have the advantage of not exploiting arable land.[15] Additionally, microalgae are also a source of nutritional products. The main challenge with this type of biomass is the economic aspect, as the biomass yield from cultures is very low. Some types of biomasses have a robust cell wall, while others have a fragile one. When intracellular products have high economic value, costly downstream processes can be used for their recovery. However, for biofuel production, it is crucial to optimize these processes to reduce costs. A key feature of microalgae is their high growth rate. Despite this characteristic, they currently have a very limited market, primarily in the food sector, where Spirulina is prominent due to its high protein content. Each microalgal strain has a unique composition, making it suitable for the development of specific products of interest. Microalgae consist of lipids, carbohydrates, and proteins, and by altering the composition of the growth medium, it is possible to enhance the production of a particular component. In the food sector, the goal is to work with a low C/N ratio in the growth medium to promote protein development, whereas for biofuel production, high C/N ratios are used to maximize lipid production.[16]

The production of biofuel from microalgae is done through the extraction of lipid cells containing triacylglycerides, which through a process of transesterification can be converted into biodiesel. [15] Microalgae also found use for biohydrogen production. They can be grown through open systems called open pounds or in closed systems, used to manage the contamination problem, called photobioreactors. [10].

1.3.2 Total and volatile solids

Biomass, in general, is composed of a certain percentage of water and a percentage of total solids. Total solids represent the overall quantity of solids within the biomass and can be measured by exposing the biomass to a temperature of 105°C in an oven overnight. By knowing the weight of the biomass before placing it in the oven and then weighing the biomass after the overnight treatment, the water fraction of the biomass is obtained by calculating the difference between the two weights. Total solids are composed of inert solids and volatile solids. The measurement of the latter is obtained by exposing the total solids in an oven at 550°C for 6 hours. After 6 hours, what remains in the oven are only the inert solids. By weighing the obtained inert solids and knowing the weight of the total solids before placing them in the oven, the amount of volatile solids contained in the biomass can be determined by calculating the difference. Dark fermentation can be conducted in three different ways: in wet conditions if the total solids content within the system is less than 10%, in semi-wet conditions if the total solids content is between 10% and 20%, and in dry conditions if the total solids content is greater than 20%.[17]

2. Strategies for biohydrogen production

2.1 Thermochemical hydrogen production

Biomass, especially lignocellulosic biomass, can be converted to biohydrogen through thermochemical processes. By using high temperatures in a safely maintained environment, the

intermolecular bonds in the biomass can be broken, resulting in the release of energy. Among the most widely used techniques are gasification and pyrolysis. [18]

Carbon monoxide (CO) and methane (CH_4) are also produced in these two processes, which can potentially undergo steam reforming and water gas shift reactions to increase the biohydrogen yield. Unlike purely biological methods, these techniques do not require a pretreatment phase; however, they do necessitate the use of a catalyst and incur high operating costs, which are two significant factors to consider when planning for industrial scale-up. [19]

2.1.1 Pyrolysis

Pyrolysis is one of the most used processes for the degradation of lignocellulosic biomass to obtain high-value-added products. There are different types of pyrolysis, which are distinguished based on various operating conditions, such as residence time, temperature, and biomass size. [22]

The first type of pyrolysis is slow pyrolysis, which occurs at a temperature between 300 °C and 700 °C and a residence time of more than 300 seconds. The production of syngas ranges between 20% and 30%. The feedstock size must be in a range that space from a minimum of 2 mm to a maximum of 50 mm. The second type of pyrolysis is intermediate pyrolysis, which occurs at a temperature of around 500 °C and has a much lower residence time compared to the first type, specifically less than 4 seconds. The processing time ranges from 30 seconds to 25 minutes. The syngas yield is the same as in the first type. To conclude, there is fast pyrolysis, which operates with a residence time between 0.5 and 10 seconds. For this reason, the reactor configuration is often a fluidized bed reactor. Temperatures range between 450 °C and 800 °C, and the syngas yield is approximately 20%. [22]

If an increase in biohydrogen yield is desired, catalytic pyrolysis can be used. The use of a catalyst reduces operating costs since the operating temperatures are low. However, the disadvantages include the high cost of catalysts and the formation of coke. The most used catalysts include alkali metals, zeolites, iron oxide, activated carbon, and magnetite. [18],[19]

2.1.2 Gasification

Gasification is an economical technique for converting agricultural residues, animal waste, lignocellulosic biomass, etc. into biohydrogen and other high-value products. This process involves the application of a gasifying agent, commonly air or steam, at high pressure (up to 33 bar) inside a reactor called a gasifier, maintained at temperatures above 700°C in the presence of the gasifying agent. A drying pretreatment is carried out to remove moisture, which is necessary to prevent agglomeration issues. This treatment is achieved by heating the feedstock between 100°C and 200°C. [18],[19]

The products of gasification are CH_4, CO, and H_2. The best gasifying agent for producing syngas is steam: if used instead of air, it is possible to double the yield, achieving a production of 30-40%. When the biomass to be used has a very high-water content, thermochemical gasification becomes inefficient because the amount of heat required for thermal pretreatment becomes too high. [19] In such cases, supercritical water gasification (SCWG) involves the conversion of biomass into gaseous compounds and water above its critical point (374°C and 22.1 MPa). Water is the reaction medium, and for this reason, biomasses with high moisture content are suitable. [20]

The SCWG can also be conducted using catalysts. A study reported that by using KOH as a catalyst, it was possible to achieve a maximum biohydrogen yield of 80% at a temperature of 600°C. [19]

2.2 Microbial electronic cell

An interesting system to produce biohydrogen, which uses organic compounds as raw materials, is the microbial electrolysis cell (MEC). The discovery of this technology dates to 2005 by two research groups, one at Penn State University and another at Wageningen University. MECs consist of an anode, where bacteria transfer electrons, and a cathode, where the production of chemical compounds occurs. For the operation of an MEC, an external voltage of approximately 0.2-0.8 V is required because the production of hydrogen from organic substrates, such as acetate, is not spontaneous under standard conditions. An advantage of MECs over conventional electrolysis is that they require lower energy input. Additionally, they allow production of high-purity hydrogen gas, albeit at a lower production rate [21],[22].

The anode, where the microorganisms are located, requires biologically assisted conditions, an appropriate culture medium, and therefore optimal pH and temperature for their growth. In the anode, organic compounds are oxidized by microorganisms, producing CO_2, protons, and electrons, which are then transferred through an electrical circuit to the cathode, where hydrogen production takes place. There is also a membrane whose function is to help to maintain the purity of generated hydrogen [22].

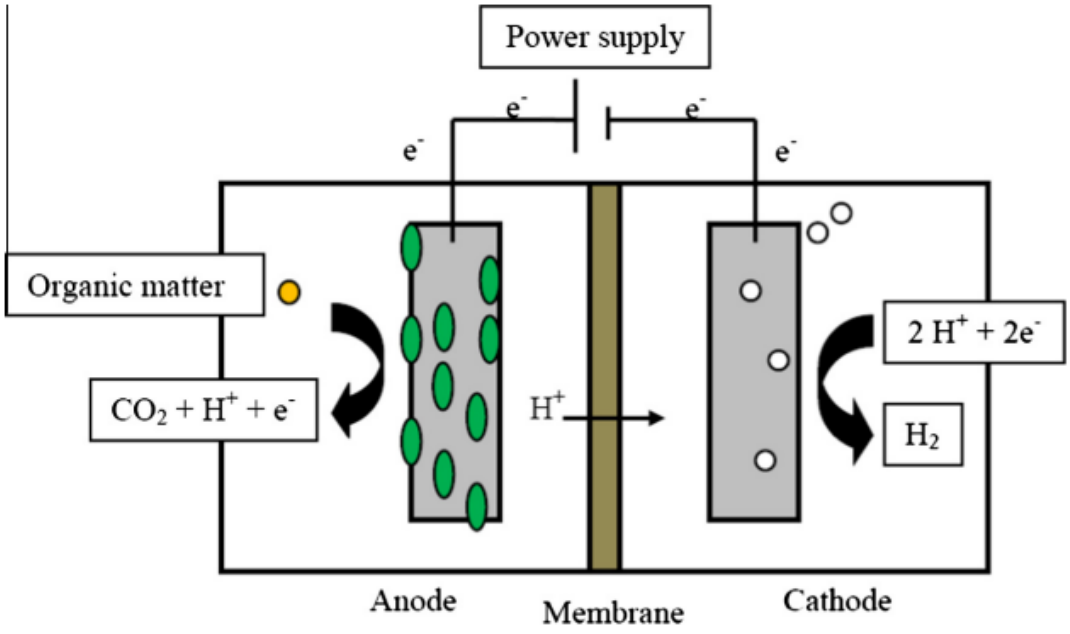

Figure 2: Schematic of the operation of a MEC [21]

The most used membrane is the proton exchange membrane, which contains $–SO_3$ functional groups that allow only protons (H+) to pass through. [21]

2.3 Biohydrogen production through light-dependent methods

Light-dependent processes can harness sunlight to generate clean and renewable energy through photosynthesis. The production of biohydrogen is carried out by microorganisms such as microalgae, cyanobacteria, and anoxygenic photosynthetic bacteria. The first two, which are oxygenic, convert light energy into water and hydrogen. Bio-photolysis can be either direct or indirect. In the former,

H_2O is split when light is absorbed by photosystem II (PSII), and the electrons generated from the splitting are used to reduce ferredoxin (Fd), which then donates electrons to reduce protons with the help of the hydrogenase enzyme. Direct bio-photolysis allows to production hydrogen with a high degree of purity (98%). [19], [23]

Indirect bio-photolysis consists of two sequential stages: first, CO_2 is fixed into carbohydrates, and then these organic compounds undergo catabolism, producing electrons that enter the plastoquinone chain and subsequently photosystem I (PSI). By absorbing sunlight, PSI transfers these electrons to Fd and then to the hydrogenase and nitrogenase enzymes. [23]

2.3.1 Photofermentation

Photofermentation is a method for producing bio-hydrogen that utilizes sunlight to degrade organic acids. The bacteria used for this approach are purple non-sulfur bacteria and Rhodobacter. Sunlight is harnessed by these bacteria to produce ATP through cyclic phosphorylation, which provides the necessary energy for the synthesis of bio-hydrogen. Some studies have shown that Rhodobacter can utilize a wide range of substrates, including simple sugars, organic compounds, and industrial waste. The following equation shows how these bacteria synthesize hydrogen:

$$16ATP + N_2 + 16H_2O + 10H^+ + 8e^- + light \rightarrow 16ADP + 2NH_4^+ + 16pi + H_2 \quad (1)$$

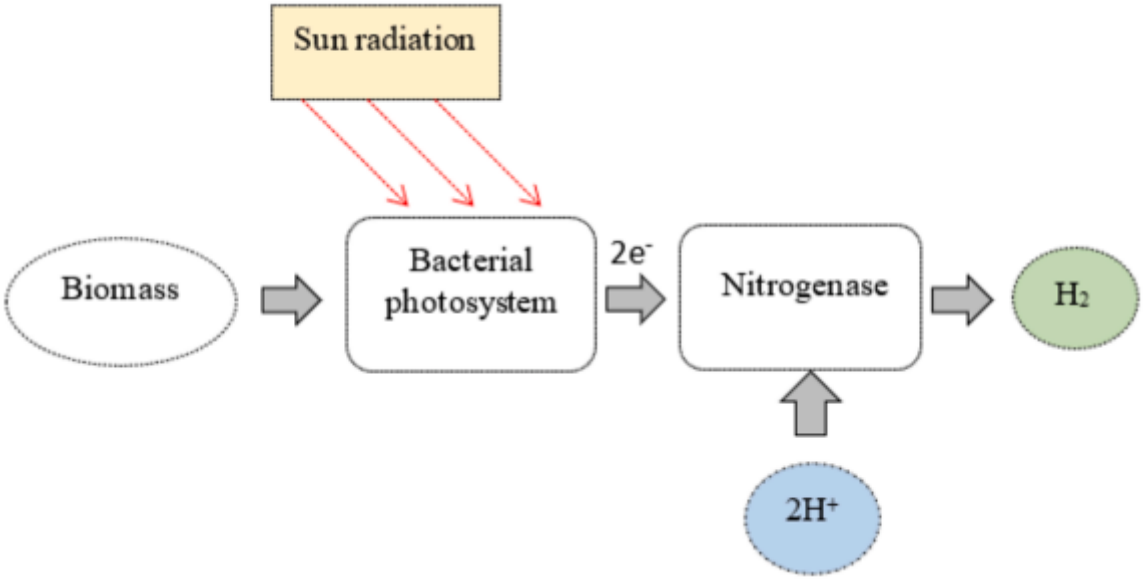

Figure 3: Schematic of the photofermentation process [24]

If there is a nitrogen deficit in the environment, then organic acids are used together with sunlight to synthesize hydrogen. [24]

However, with the latter, depending on the color of the wastewater, issues may arise regarding the penetration of sunlight or the presence of heavy metal ions harmful to the microorganisms, making pretreatments necessary. The limited availability of organic acids is the main limitation of this method. [19],[24]

3. Dark Fermentation

3.1 Dark Fermentation

Dark fermentation is a process through which organic substrates can be converted into biofuels using anaerobic microbes. Among the microbial species known for this type of fermentation are Clostridium, Bacillus, and Enterobacter. The main producers of biohydrogen are spore-forming obligate anaerobes, followed by non-spore-forming obligate anaerobes, and finally facultative anaerobes. [25]

Agro-food waste, due to its high carbon content, is a suitable substrate for biohydrogen production through dark fermentation. [6] Hydrogen production can be influenced by biomass loading, although an excess may lead to issues with reduced mass and heat flow. Compared to hydrogen production processes that rely on light, dark fermentation shows greater potential. Several process variables influence the final product yield, including pH, hydraulic retention time (HRT), gas partial pressure and VFA concentration. [19]

Hydrogen production is due to butyrate and acetate fermentation, whose reactions are reported in Equations (2) and (3):

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2COOH + 2H_2 + 2CO_2 \quad (2)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad (3)$$

3.2 Factor influencing the process

3.2.1 Inoculum

The bacteria capable of producing hydrogen through the dark fermentation process are diverse. Clostridium species operate under mesophilic conditions and are gram-negative, rod-shaped, strict anaerobes capable of sporulation. Some of them can degrade cellulose via a complex of enzymes called the cellulosome. [26] Facultative anaerobic bacteria are capable of functioning, albeit not optimally, even under aerobic conditions. This category includes enterobacteria, which produce hydrogen through pyruvate formate-lyase and formate hydrogen-lyase. At temperatures between 30 °C and 45 °C, enterics and mesophilic clostridia can operate, but due to the use of NADH as an electron donor, it is difficult to achieve yields higher than 2 moles of hydrogen per mole of glucose consumed. [27] The Bacillus strains, on the other hand, can consume O_2 within the culture medium and can also produce H_2. This capability has paved the way for co-cultures, as the removal of O_2 allows bacteria like Clostridia to proliferate and facilitate the bioprocess. [28]

3.2.2 Iron and sulfide concentration

Most of the substrates used for dark fermentation contain sulfate (SO_4^{2+}), which can be reduced to sulfide (S^{2-}) by sulfate-reducing bacteria. Its formation can also occur through the degradation of proteins by anaerobic bacteria. Several studies have concluded that sulfide is an inhibitor for biohydrogen-producing microorganisms, as these microorganisms require metals for their metabolism, and these metals are depleted through the formation of insoluble metal sulfides. The formation of these insoluble compounds, however, allows for the control of high sulfide concentrations through the addition of Fe^{2+}. [29]

3.2.3 pH

One of the main parameters that influence dark fermentation is pH. Microorganisms are highly sensitive to pH changes and adapt to them by altering various metabolic processes. However, extreme pH values can lead to protein denaturation and nucleic acid degradation. Within the culture medium, the pH constantly changes during dark fermentation, as most of the microorganisms involved produce volatile fatty acids, which could lower the pH value to the point of complete inhibition of the process. [30] In the case study of *Clostridium* using glucose as a substrate, a pH below 5 leads to the formation of butanol and acetone. For agro-industrial waste, the optimal pH ranges between 4.5 and 7, but this value changes depending on the substrate used. Therefore, it becomes essential to determine the optimal pH based on the substrate being used. [31]

3.2.4 Temperature

A key parameter for DF is the operational temperature. This process can be conducted across different temperature ranges, with the most studied being the mesophilic (35-37°C) and thermophilic (65°C) ranges. Biohydrogen production is influenced by temperature, and in particular, some studies have found that higher yields of this gas can be achieved under thermophilic conditions. In the thermophilic range, the main by-product is acetic acid, while in the mesophilic range it is butyrate. [32] The higher yields in the thermophilic range can be attributed to the fact that hydrolysis is favored at higher temperatures, making it possible to obtain more gas when using lignocellulosic biomass. The choice of the temperature range at which to operate should be made after conducting a cost analysis to support the DF process and assessing how much gas can be obtained under specific operational conditions. [31]

3.2.5 H_2 partial pressure

A high partial pressure of hydrogen inhibits the dark fermentation (DF) process because it decreases the mass transfer from the liquid phase to the gas phase (and therefore into the headspace). It leads to a high concentration of hydrogen in the liquid, causing hydrogenases to slow down their production. Reducing the partial pressure can positively influence DF. In the anaerobic digestion (AD) process, the reduction occurs because methanogens use hydrogen to form CH_4. Other techniques to reduce the partial pressure of hydrogen include using a vacuum pump, which increases the cost of the process, or using a hydrogen-permeable membrane. [31]

3.2.6 HRT and ORL

HRT is a key parameter because it is involved in the inhibition of the activities of hydrogen consumers in DF. Reducing this parameter, combined with an optimal pH value, allows the elimination of methanogens and thus promotes hydrogen production. [31] This parameter corresponds to the contact time between the substrate and the microorganisms. Its variation also impacts the organic loading rate (OLR), another parameter that measures the mass of organic material applied daily per unit volume of the bioreactor. HRT and OLR are inversely proportional. There are no precise optimal values for these two parameters to use in a process, as they depend on the type of substrate being used, particularly its biodegradability. [33]

3.3 Anaerobic digestion

A method for the conversion of waste biomass for energy production is anaerobic digestion. Among the products of AD are biogas and inorganic compounds. This process consists of several steps,

including hydrolysis, where the breakdown of complex organic materials into simple oligomers and monomers occurs, facilitated by enzymes produced by microorganisms. Subsequently, the oligomers and monomers are converted into VFAs, CO_2, H_2, and other organic substances in the step known as acidogenesis. The next step is acetogenesis, where the organic molecules produced during acidogenesis are converted into acetate, CO_2, and H_2. The final step is called methanogenesis, where biogas production occurs. Some bacteria use acetate to produce methane, while others use H_2 and CO_2 to produce it [34].

The AD process is widely used for the treatment of agro-food waste and solid waste. Among the main issues related to this process is foam formation caused by the reduction of the sludge's surface tension due to VFAs and the accumulation of other surface-active materials. Foam reduces mass transfer and consequently biogas production, in addition to creating problems in the bioreactor. The accumulation of VFAs, in addition to causing foam formation, leads to the acidification of the system and thus a reduction in pH, which is a crucial parameter for the proper functioning of the process. If the substrate used is rich in proteins, among the products of digestion we find NH4+-N, which causes the accumulation of acetate and propionate, leading to a decrease in pH. [35]

The factors influencing the process include the OLR, which, if high, induces the accumulation of VFAs. Another factor is temperature; AD can occur in three different ranges: psychrophilic (below 20 °C), mesophilic (20–45 °C), and thermophilic (55–70 °C). The thermophilic range is optimal for the acidogenesis phase, but it inhibits the activity of methanogens. On the other hand, the mesophilic range provides better stability at the expense of a reduced production rate. Finally, we have pH, which influences the growth of microorganisms. The optimal pH value is around 7.5. The initial pH value of the process affects the reaction products and the order in which they are generated. The accumulation of volatile fatty acids, CO_2, and NH_4+-N contributes to the continuous variation of this factor [34].

3.3.1 Two-stage anaerobic digestion

An additional approach that can be used for energy production from waste biomass is two-stage anaerobic digestion. This process consists of two stages, where the first involves dark fermentation for hydrogen production, and the second one is the AD process for biogas production. The advantage compared to processes operated individually is an increased substrate consumption, resulting in higher biogas production. Important factors are the process parameters that influence its operation. These are the same as those found in DF and AD. [36]

The four stages of AD involve the activity of different microorganisms for each stage, which are combined within a single reactor to facilitate methane production. The issue is that each stage has optimal conditions that differ from the others, which is why efforts are made to shift the methanogenesis stage to the second stage. [37]

In single-stage AD, H_2 is utilized by methanogenic bacteria. In contrast, in the two-stage process, it is possible to separate the production of H_2 and CH_4 into two different stages, ensuring the recovery of both gases. Alternatively, H_2 can be used to consume CO_2 at the expense of CH_4 production, thereby increasing its yield. [36]

4. Pretreatment methods for inoculum and biomass

4.1 pretreatment of lignocellulosic biomass in AD process

Pretreatments are necessary to increase the biodegradability of biomass, leading to an enhanced biogas yield in an anaerobic digestion process. For instance, after hydrolysis, some materials may remain undegraded, and proper pretreatment can help break these materials down into substances that are usable by microorganisms. Lignocellulosic biomasses are composed of cellulose, hemicellulose, and lignin. Several studies have shown that lignin, when present in modest amounts, negatively impacts biogas production. However, various pretreatments aimed at degrading the biomass are available to ensure that the anaerobic digestion (AD) process proceeds smoothly. A pretreatment must not only be effective but also economical and environmentally sustainable. [38]

4.1.1 Mechanicals treatment

Mechanical treatments aim to reduce the size of the biomass, thereby increasing its specific surface area and accessibility. The main methods are milling, grinding, and extrusion. These treatments are not particularly suitable for biomass with high moisture content due to the high energy consumption required. In contrast, plant-derived biomasses are the most subjected to these mechanisms. [39] Mechanical pretreatments involve the use of external forces to reduce the size of the biomass. Milling is the most used treatment. This mechanism operates through mills that run continuously. The particle size can be reduced to as low as 120 mm. Among the various treatment alternatives, we also have microwaves. Using a power of up to 1200 W with a frequency of 2450 MHz for 40 minutes, it was possible to increase biogas production by up to 78%. Such high biogas yields allow for a positive energy balance, meaning that the energy spent on the process is less than the energy obtained. [38] Most of the pretreatments are aimed at obtaining biogas and biofuels; for this reason, an enzymatic hydrolysis process or a fermentative process follows these treatments. Mechanical pretreatments do not disrupt the action of enzymes because they only reduce the size and do not alter the chemical composition of the biomass. [40]

4.1.1.1 Ultrasound treatment

Ultrasounds are waves that create pressure gradients in the solution through which they propagate. The frequency of these waves ranges from 20 to 1000 kHz. The continuous rarefaction and compression of these waves in the liquid solution create bubbles that can coalesce, increasing in size, or can collapse due to the wave. The collapse of the bubbles locally increases the temperature and generates highly reactive free radicals.[41] Low-frequency ultrasound is used to pretreat lignocellulosic biomass in order to degrade lignin. Ultrasonic pretreatment also enhances the efficiency of delignification when combined with specific solvents, including ethanol. [42]

4.1.2 Thermal treatment

Thermal pre-treatments at temperatures above 160 °C allow for the solubilization of lignin, as well as the formation of phenolic compounds that are harmful to methanogenic microorganisms. [39] This treatment requires high energy expenditure but has the advantage of breaking down complex organic compounds into simpler ones that can be easily utilized by microorganisms. Thermal treatment is influenced by two parameters: temperature and treatment time. To implement this methodology, hydrothermal treatment, thermal hydrolysis, and similar methods are used. The former achieves good biomass degradation, with operational parameters of 150°C for 20 minutes resulting in a 63% higher

yield compared to untreated biomass. Thermal hydrolysis, on the other hand, involves the combined action of temperature and pressure in the presence of water, allowing for biogas yields up to 87% higher than untreated biomass. [38]

4.1.3 Chemical Treatment

Chemical pre-treatments are simple and fast and enable the degradation of complex structures in lignocellulosic biomasses. Among these, acid treatments, alkaline treatments, and ionic liquid treatments stand out. Acid treatments are very effective at solubilizing lignin but have the drawback of also generating undesirable by-products that can inhibit the process. Additionally, using acids in reactor configurations requires corrosion-resistant materials, which are very costly. Alkaline treatments are generally combined with other pre-treatments. This treatment allow for the solubilization of lignin but are slower compared to acid treatments. They are consumed by microorganisms, which means that the concentration in the digester must be high. [39]

4.1.3.1 Alkaline treatment

Alkaline pretreatment has the advantage of being cost-effective as it only requires the use of a base to degrade the components of lignocellulosic biomass, specifically lignin and hemicellulose. The mechanism underlying this treatment is the breaking of intermolecular bonds. Its effectiveness is influenced by several factors, including the choice of alkaline reagent. The most common ones are sodium hydroxide, calcium hydroxide, and ammonia. NaOH is the most widely used due to its low cost and availability, and it is generally used in concentrated solutions at 10% w/v. [39] Alkaline treatment makes cellulose more accessible to enzymes, thereby facilitating the enzymatic hydrolysis necessary for producing products like biodiesel. It also reduces the amount of enzymes needed for hydrolysis, significantly lowering costs since alkaline reagents are much cheaper than enzymes. Among the drawbacks of this treatment is the fact that if one is solely interested in the removal of lignin, there will inevitably also be a loss of cellulose and hemicellulose, as there are no selective bases targeting only lignin. Furthermore, it is advisable to adjust the pH of the treated biomass, as the high values reached during alkaline pretreatment could degrade enzymes or inhibit microbial activities of interest. [42]

4.1.3.2 Acid treatment

Acid pretreatment allows for the isolation of cellulose from hemicellulose and lignin through the addition of an acid. The acids used include sulfuric acid in concentrations ranging from 0.4% to 2% w/v with a biomass loading of 10%. [38] Other types of acid include phosphoric acid, which can be used at concentrations up to 8 mol/L. The issue with inorganic acids is the production of degradation products, which are undesirable for applications related to the fermentation of lignocellulosic biomass for bioethanol or biogas production. For this reason, the use of organic acids, including acetic, formic, and citric acid, is becoming more common. The efficiency of this pretreatment is influenced by several factors, including temperature, treatment time, and acid concentration. Like alkaline pretreatment, the advantage of this approach is to increase the accessibility of cellulose to enzymes or microorganisms. Among the disadvantages are the corrosion of materials caused by acids, the need to neutralize them, and their environmental impact. [42]

5. Materials and methods

5.1 Experimental campaign

The goal of the experimental campaign was to obtain hydrogen through a Dark Fermentation (DF) process, using various agro-food waste. Specifically, the aim was to find the appropriate pre-treatment for the inoculum and biomass, as well as an optimal pH value, to achieve a suitable configuration for obtaining a good yield of bio-hydrogen (bio-H_2). Once a configuration capable of producing enough hydrogen was identified, a two-stage process was implemented, transitioning to anaerobic digestion to utilize the remaining substrate for biogas production. Regarding the final experimental campaign, various pre-treatments were applied to test certain lignocellulosic biomasses for bio-hydrogen production. Different methods were investigated to determine which one was the most advantageous in terms of hydrogen yield.

5.2 Composition of biomass and inoculum

The biomass used was of various types. Among the different types of waste used as substrates in the first experimental campaign were: cow manure, sourced from a farm located near the province of Turin. Additionally, vegetable waste was used, consisting of three-quarters commercial frozen minestrone and one-quarter apple waste, also found commercially. Diluted milk waste from a dairy industry, also located near Turin, was used as well. In the subsequent trials, sugary waste from the company Sedamyl was used as a substrate, and finally, two type of vinasses. The inoculum used comes from the digestate of cow manure. The *table 1* provides a summary of the composition of all the biomass and inoculum used:

Name	C (%)	N (%)	S (%)	H (%)	VS/TS (%)	TS (%)	C/N ratio
Cow Manure	61.22	3.88	0.77	5.78	91.60	25.50	15.77
Vegetable waste (¾ vegetables, ¼ apple)	41.65	1.52	0.21	6.34	85.00	10.90	27.48
Sedamyl's sugar waste	44.51	1.09	0.07	7.58	99.90	32.10	40.83
Waste milk	53.74	2.90	0.34	6.46	96.70	0.51	18.53
Vinasse 1	14.10	1.15	/	/	98.10	57.90	12.26
Vinasse 2	14.45	1.14	/	/	98.00	52.90	12.73
Inoculum	34.58	3.01	0.33	4.42	73.20	4.30	11.48

Table 1: Characteristics of biomasses

5.3 Experiment setup

5.3.1 Total and volatile solids analysis

The tests conducted in the experimental campaign of this thesis were performed under wet conditions, with a total solids content of 6%. An additional constraint in the system, beyond having 6% total

solids, involves the ratio of volatile solids between the biomass and the inoculum. Specifically, this ratio was maintained at 1:1. In the experimental campaign concerning dark fermentation and the two-stage anaerobic digestion, glass bottles with a volume of 250 mL were used as reactor configurations. In the second part, were used bottles with a volume of 125 mL. In either case, the operational volume of the system is 80% of the total volume, while the remaining 20% is occupied by the headspace, where gaseous products from the fermentation process accumulate. By combining the information about the total volume of the bottle and the percentage of total solids that must be present in the system, it is possible to determine the volume of total solids that should be added to the system. A simplifying assumption was adopted for these experiments: the density of everything present in the system is equal to that of water. At this point, using the density and the volume of total solids to be added to the system, it was possible to calculate their mass. The total solids present in the system are composed of the total solids of the biomass and the total solids of the inoculum. By applying the condition that the ratio of volatile solids between the inoculum and the biomass is equal to 1:1, it was possible to determine, through these steps, the mass of the inoculum and the mass of biomass to be added to each reactor configuration.

$$\frac{VS_{inoculum}}{\%\frac{VS}{TS}inoc} + \frac{VS_{biomass}}{\%\frac{VS}{TS}biom} = TS_{inoculum} + TS_{biomass} = TS_{tot} \qquad [5.1]$$

$$TS_{inoculum} = \frac{V_{inoculum}}{\%\frac{VS}{TS}inoc} \rightarrow m_{inoculum} = \frac{TS_{inoculum}}{\%TS_{inoculum}} \qquad [5.2]$$

$$TS_{biomass} = \frac{VS_{biomass}}{\%\frac{VS}{TS}biom} \rightarrow m_{biomass} = \frac{TS_{biomass}}{\%TS_{biomass}} \qquad [5.3]$$

In the case where there is more than one biomass, the procedures for determining the masses to be included in the system are similar; the difference is that the VS of the biomass are composed of the sum of the VS of the substrates.

5.3.2 First experiment

The first experiment focused on studying dark fermentation in four different configurations, repeated in duplicate, resulting in a total of eight reactors. The first configuration studied, was the inoculum used as blank. Its production was subtracted by the other one to calculate the net H_2 production of biomasses. The other configuration studied involved manure and vegetable waste, with 50% of the total solids from biomass consisting of manure and the remaining 50% from vegetable waste. Another configuration had 100% of the total solids from biomass made up of manure, and finally, in the last configuration, 10% of the total solids were milk waste, while the remaining 90% consisted of cow manure.

Once the percentages of total solids for the biomasses were set, using formulas 5.1, 5.2, and 5.3, it was possible to calculate the quantities to insert into the different configurations. Subsequently, a thermal pretreatment was conducted on the biomasses derived from animals, such as cow manure and dairy industry waste, as well as on the inoculum itself. The pretreatment involved placing these substances in an oven for 1 hour and 15 minutes at a temperature of 80°C. This was done to inhibit

methanogenic bacteria while allowing spore formation of hydrogen-producing bacteria, to avoid competition between the two for the consumption of the substrate and to optimize the process towards hydrogen production. Under optimal operational conditions of temperature and pH, the hydrogen-producing bacteria can then resume reproduction and restart their life cycle, allowing the process to function correctly. Plant waste, on the other hand, was not thermally treated because the presence of methanogenic bacteria is usually associated with waste derived from animals. For this reason, the decision was made not to treat them, also considering the sustainability of the process in economic and environmental terms, since thermal pretreatment requires a significant amount of energy.

Figure 4: Oven for thermal treatment

The next step was to insert the correct quantities of biomass and inoculum, for each configuration made in duplicate, into different glass laboratory beakers using a properly calibrated electronic balance. At this point, measuring the pH is important using a device called a pH meter.

This digital device, by measuring the concentration of [H+] ions, can provide the pH value of the compound being analyzed. The pH scale ranges from 0 to 14, where a pH of 7 is neutral and corresponds to the measurable pH value of distilled water. A pH below 7 is acidic, while a pH above 7 is basic. The measurement of hydrogen ion concentration is possible through the difference in electrical potential between a reference electrode and the pH meter's electrode. To correctly calibrate a pH meter and obtain a reliable pH reading, buffer solutions with a known pH value are used. [43]

Figure 5: pH meter

The pH value of the inoculum and cow manure, after several measurements, was found to be basic, which is why the pH values of the configurations were also basic. However, the optimal pH value for dark fermentation is around 6.2, which falls within an acidic range. Among the fermentation products, there are volatile fatty acids that lower the pH to levels where the process is inhibited. For this reason, efforts were made to maintain a pH range that would delay pH-related inhibition as much as possible. To reach this pH, 3 M hydrochloric acid (HCl) was added. Below there is a table summarizing the pH values before the addition of HCl, the amount of HCl added for each configuration which means that to obtain the amount of acid for each bottle, the value must be divided by two and in the end, the final pH.

Once the optimal pH value was obtained, the next step was to manually fill the bottles with the appropriate volume. For this experiment, 250 mL bottles were used, with an operational volume equal to 80% of the total volume. Therefore, the key quantity was 200 mL. Assuming that the density of the system is comparable to that of water, and using an electronic balance, two bottles of 200 g of solution each were filled for each configuration. The *table 2* shows the actual quantities placed in each bottle, as manual filling can be subject to errors.

Reactor	Real working volume [mL]	Initial pH	HCl 3M added [mL]	pH after HCl
Blank inoculum	200.10	8.20	4.50	7.19
Blank inoculum	200.00	8.20	4.50	7.19
50%Manure+50%Vegetables	200.00	7.93	3.00	7.20
50%Manure+50%Vegetables	200.00	7.93	3.00	7.20
100% Manure	199.90	8.16	5.00	7.22
100% Manure	200.30	8.16	5.00	7.22

5% Milk + 95% Vegetables	200.50	8.11	5.00	6.98
5% Milk + 95% Vegetables	199.90	8.11	5.00	6.98

Table 2: Volume and pH of the bottles from the first experiment

Once the reactors were filled, the oxygen inside the bottles was removed. Hermetically sealed caps were used, with two threaded holes on the top capable of accommodating plugs. In one of the two holes, a red plug was screwed in to prevent air, and therefore oxygen, from entering or escaping, while in the other hole, a perforated plug was screwed in with a black tube properly connected to a nitrogen cylinder. Nitrogen was then flushed into the bottle for about forty-five minutes to inert the entire volume of the bottles and remove the oxygen. This is done because dark fermentation is an anaerobic process, and the presence of oxygen would reduce hydrogen yield or completely inhibit the process. After forty-five minutes, the plug connected to the nitrogen cylinder was removed, and an additional plug with a tube connected to plastic bags was attached. The plastic bags have a valve that can be opened or closed depending on how it is intended to be used. When closed, the bag collects the gases produced by the fermentation, while when opened, it is possible to extract these gases. In Figure 6 are showed the components used to perform these steps:

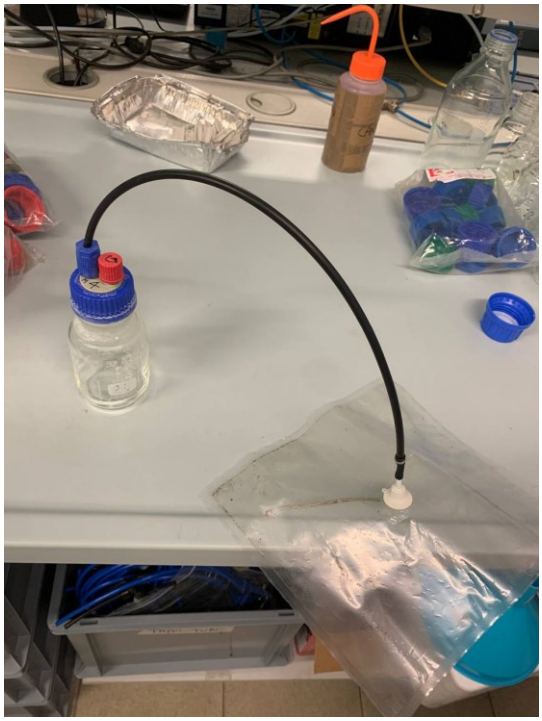

Figure 6: bottle with bag

The final step was to incubate the reactors at 35°C in a water bath. The bath was then properly covered to minimize water evaporation as much as possible. This ensured a water level that allowed for the complete immersion of the bottles, preventing unwanted thermal gradients. The sealed bottles do not have an independent stirring system. For this reason, the bottles were manually shaken daily to promote mass and heat transfer, avoiding reliance solely on diffusion and conduction, respectively. The system, in general, can be considered as a batch process with manual mechanical stirring. The figure 7 shows the temperature-controlled bath. *Table 3* shows the OLR and the C/N ratio of the various configurations.

Figure 7: Isothermal bath

Configuration	OLR (kgVS/m3 day)	C/N ratio
50% Manure+50%Vegetables	12.00	21.56
100% Manure	16.28	15.77
5% Milk + 95% Vegetables	12.22	17.58

Table 3: OLR and C/N ratio of configurations from the first experiment

5.3.2 Second experiement

In the second experiment, three different configurations were used in duplicate, for a total of six reactors. The biomasses used and their composition were the same as those used in the first experiment. The first configuration consisted of inoculum, with manure making up 50% of the total solids of the biomass, and the remaining 50% made up of vegetable waste. In the second configuration, the total solids of the biomass were made up entirely of cow manure. Lastly, the third configuration consisted of 5% of the total solids from dairy industry waste, with the remaining 95% being cow manure.

As in the first experiment, thermal treatment at 80°C for one hour and fifteen minutes in an oven was applied to the dairy waste and the inoculum. However, a different treatment was applied to the manure. The treatment used was an acid treatment. Using a pH meter and adding 3M hydrochloric acid, the pH of the manure was lowered to a value of 4 to ensure the elimination of methane-producing bacteria. The *table 4* shows the initial pH values, the amount of HCl added, and the pH after the addition of the acid, to achieve the optimal pH value for the process. *Table 5* shows the OLR and the C/N ratio of the various configurations.

Reactor	Real working volume [mL]	HCl 3M added in manure [mL]	pH after HCl
50%Manure+50%Vegetables	199.90	3.00	7.19
50%Manure+50%Vegetables	197.70	3.00	7.19
100% Manure	201.80	4.50	7.20
100% Manure	194.80	4.50	7.20
5% Milk + 95% Vegetables	202.70	3.50	7.22
5% Milk + 95% Vegetables	202.20	3.50	7.22

Table 4: Volume and pH of the bottles from the second experiment

Configuration	OLR (kgVS/m3 day)	C/N ratio
50% Manure+50%Vegetables	12.00	21.56
100% Manure	16.28	15.77
5% Milk + 95% Vegetables	12.22	17.58

Table 5:OLR and C/N ratio of configurations from the second experiment

5.3.3 Third experiment

The third test involved the experimentation of six different configurations, replicated in duplicate for a total of twelve bottles, three of which were subjected to a dark fermentation process, while the remaining three underwent anaerobic digestion. The experiment was investigate the hydrogen yields for DF and the methane yields for AD, to potentially implement a two-stage process to further increase gas yields. The biomass used for this test includes cow manure, vegetable waste, and dairy industry milk waste, whose elemental composition is the same as in tests one and two. Additionally, a new substrate has been used, consisting of a sugary waste from the Sedamyl company, whose characteristics are summarized in the *table 7*. The first configuration for the DF was set up with 50% of the total solids from cow manure and the remaining 50% from vegetable waste. The second configuration had 50% of the total solids from cow manure and the remainder from sugary waste from the Sedamyl company. The third and final DF configuration consisted of 5% dairy waste and 95% vegetable waste. As for the AD, the first configuration was set with inoculum used as blank. Its production was subtracted by the other one to calculate the net CH_4 production of biomasses. The second AD configuration consisted of 100% of the total solids from cow manure, while the third and final one consisted of 5% dairy waste from the dairy industry and the rest from cow manure. For the three DF configurations, a heat treatment was applied to the inoculum, the dairy waste, and the cow manure in an oven at 80°C for one hour and 15 minutes, to inhibit the methane-producing bacteria. The vegetable and sugary waste, on the other hand, did not undergo heat treatment. Subsequently, following the same procedures used for the previous tests, the reactors were assembled, and after a pH adjustment, they were degassed to eliminate the oxygen present in the system and finally placed in the isothermal water bath. The pH value reached is of 6.5 for all the bottles. For the AD configurations, no heat treatments were applied, as the methanogens are needed to sustain the anaerobic digestion process. At this point, only a pH adjustment was made to achieve a range that would be optimal for the AD process. Also for these bottles the pH reached is of 6.5. *Table 6* shows the OLR and the C/N ratio of the various configurations.

Configuration	OLR (kg VS/m3 day)	C/N ratio

50%Manure+50%Vegetables	16.00	21.55
50%Manure+50%Sedamyl	16.28	17.37
5%Milk+95%Vegetables	16.30	21.56
100% Manure	1.97	15.77
5% Milk+ 95%Manure	1.48	10.20

Table 6:OLR and C/N ratio of configurations from the third experiment

	Indicative composition v/v:	Average composition of the sugar fraction
Sedamyl sugar waste	-15% fatty acids derived from the enzymatic hydrolysis of phospholipids present in wheat (mainly lysophosphatidylcholine); -8% fiber; -1% non-starch polysaccharides; -75% soluble carbohydrates	-Polysaccharides 0.5%; -Trisaccharide 1%; -Maltose 2%; -Dextrose >95%; -Fructose 0.5%;

Table 7:Sedamyl sugar waste characteristics

5.3.4 Fourth experiment

The fourth experiment focused on the study of dark fermentation of 5 different configurations. The first configuration consists of 5% of the total solids from dairy waste biomass and 95% from vegetable waste. Additionally, in this configuration, a volatile solids ratio between the biomass and the inoculum of 2:1 was maintained. The second configuration is the same as the first, except that the volatile solids ratio between the inoculum and biomass is 1:1 this time. The third configuration consists of 10% dairy waste and 90% manure. Meanwhile, the fourth and fifth configurations consist of 10% dairy waste and 90%, in the first case of vinasse 1 and in the second case of vinasse 2. In *table 8*, we find the pH values before and after adjustment with HCl.

Reactors	Real working volume [mL]	Initial pH	HCl 3M added [mL]	pH after HCl
5%Milk+95%Vegetables 2:1	199.80	6.85	1.50	6.11
5%Milk+95%Vegetables 2:1	200.30	6.84	1.50	6.17
5%Milk+95%Vegetables 1:1	198.90	7.04	3.00	6.29
5%Milk+95%Vegetables 1:1	201.20	7.10	3.00	6.33
10% Milk + 90% Manure	200.00	8.32	4.50	6.49
10% Milk + 90% Manure	199.90	8.30	4.50	6.45

10% Milk + 90% Vinasse 1	199.70	7.03	3.00	6.36
10% Milk + 90% Vinasse 1	200.20	7.09	3.00	6.34
10% Milk + 90% Vinasse 2	200.00	6.60	1.00	6.35
10% Milk + 90% Vinasse 2	200.40	6.68	1.00	6.32

Table 8 : Volume and pH of the bottles from the fourth experiment

The manure, milk, and inoculum were subjected to a heat treatment for one hour and fifteen minutes, while the plant waste was not treated. The vinasse was shredded to make it more accessible to microorganisms for consumption. *Table 9* shows the OLR and the C/N ratio of the various configurations.

Configuration	OLR (kgVS/m3 day)	C/N ratio
50%Manure+50%Vegetables 2:1	12.14	21.62
50%Manure+50%Vegetables 1:1	11.83	21.62
10% Milk + 90% Manure	10.28	16.93
10% Milk + 90% Vinasse 1	12.56	14.12
10% Milk + 90% Vinasse 2	12.56	18.33

Table 9:OLR and C/N ratio of configurations from the fourth experiment

5.3.5 Fifth experiment

The fifth test focused on exploring an effective treatment for vinasse to increase hydrogen yield after a Dark Fermentation process. Vinasses are lignocellulosic biomass, and lignin itself is difficult for microorganisms to digest. Additionally, the presence of polyphenols, with their high molecular weight, makes them substances not particularly suitable for digestion. For this reason, an approach was sought to break down the biomass cell walls as much as possible, thus promoting the release of polyphenols, and subsequently removing them using a solution composed of 80% ethanol (EtOH) by volume. [44]

The configurations used for this experiment are three for each type of vinasse, meaning three for vinasse 1 and three for vinasse 2. The three configurations are the same for each type of vinasse and were duplicated, resulting in a total of twelve reactor of 125 mL, with a working volume of 100 mL. The first two configurations consist of 10% total solids from milk waste biomass and 90% consists of vinasse subjected to pretreatment in an 80% ethanol (EtOH) solution, with ultrasound treatment first for five minutes and then for ten minutes. The ultrasound treatment was carried out by alternating five seconds of operation with five seconds of rest to control heat transfer phenomena. For this reason, five minutes of effective treatment required ten minutes of total operation. In terms of solution quantity, 30 mL of solution was used for each gram of vinasse to be treated. The third configuration consist of 10% total solids from milk waste biomass and 90% of vinasse mixed with H2O. *Table 10* and *table 11* summarize all the operating conditions that were adopted:

Reactors	Real working volume [mL]	Initial pH	HCl 3M added [mL]	pH after HCl
10%Milk+90%Vinasse 1 Ultrasound 5’	99.60	8.15	2.00	6.15
10%Milk+90%Vinasse 1 Ultrasound 5’	100.50	8.21	2.00	6.10
10%Milk+90%Vinasse 1 Ultrasound 10’	100.10	8.26	2.50	6.18
10%Milk+90%Vinasse 1 Ultrasound 10’	99.90	8.23	2.50	6.13
10%Milk + 90% Vinasse 1 crushing with H_2O	100.00	7.98	2.50	6.16
10%Milk + 90% Vinasse 1 crushing with H_2O	99.70	8.02	2.50	6.20
10%Milk + 90% Vinasse 2 Ultrasound 5’	100.20	8.02	2.50	6.20
10%Milk + 90% Vinasse 2 Ultrasound 5’	100.70	8.10	2.50	5.96
10%Milk + 90% Vinasse 2 Ultrasound 10’	99.20	8.18	2.50	5.87
10%Milk+90% Vinasse 2 Ultrasound 10’	100.40	8.23	2.50	6.00
10%Milk+90% Vinasse 2 crushing with H_2O	100.00	7.69	2.00	6.24
10%Milk+90% Vinasse 2 crushing with H_2O	99.80	7.77	2.00	6.25

Table 10: Volume and pH of the bottles from the fifth experiment

Configuration	OLR (kgVS/m3 day)	C/N ratio
10%Milk+90%Vinasse 1 Ultrasound 5’	12.56	18.34
10%Milk+90%Vinasse 1 Ultrasound 10’	12.56	18.34
10%Milk+90%Vinasse 1 crushing with H_2O	12.56	18.34
10%Milk+90% Vinasse 2 Ultrasound 5’	12.56	18.33

10%Milk+90% Vinasse 2 Ultrasound 10’	12.56	18.33
10%Milk+90% Vinasse 2 crushing with H_2O	12.56	18.33

Table 11:OLR and C/N ratio of configurations from the fifth experiment

5.3.6 Sixth experiment

The sixth and final experiment was conducted with the objective, as in the previous experiment, of testing pretreatments for the valorization of vinasse as a substrate for dark fermentation. In addition to the methods previously used, an alkaline treatment was employed to degrade the complex structures of lignin, making the substrate more accessible to microbial consumption. The alkaline treatment involved mixing the biomass with water, and then, through the addition of 3M NaOH, achieving a pH of 12.5. During the NaOH addition, the suspension was kept under agitation to ensure maximum homogeneity and maintain a uniform pH level. After 24 hours, solid-liquid extraction of the vinasse from the NaOH solution, and the complex organic substances extracted from it, was carried out using a vacuum pump and filtration. Following a rinse with 300 mL of distilled water, the substrate was returned to a condition suitable for use in fermentation. The first configuration used was 10% dairy waste and 90% vinasse 1 treated with ultrasound in an 80% EtOH solution. The second configuration, on the other hand, consisted of 10% dairy waste and 90% vinasse 1 treated with the alkaline method. The third and fourth configurations are the same as the first and second, the only difference being that the vinasse used is vinasse 2. *Table 12* and *table 13* summarize all the operating conditions.

Reactors	Real working volume [mL]	Initial pH	HCl 3M added [mL]	pH after HCl
10%Milk+90%Vinasse 1 Ultrasound 5’	199.50	7.54	1.50	6.30
10%Milk+90%Vinasse 1 Ultrasound 5’	199.90	7.63	1.50	6.30
10%Milk+90%Vinasse 1 Alkaline treatment	200.30	8.35	2.00	5.90
10%Milk+90%Vinasse 1 Alkaline treatment	200.40	8.51	2.00	6.05
10%Milk+90%Vinasse 2 Ultrasound 5’	200.00	7.47	1.50	6.09
10%Milk+90%Vinasse 2 Ultrasound 5’	199.80	7.52	1.50	6.15
10%Milk+90%Vinasse 2 Alkaline treatment	199.70	8.29	2.00	5.73
10%Milk+90%Vinasse 2 Alkaline treatment	200.20	8.32	2.00	5.67

Table 12: Volume and pH of the bottles from the sixth experiment

Configuration	OLR (kgVS/m3 day)	C/N ratio

10%Milk+90%Vinasse 1 Ultrasound 5’	8.30	18.33
10%Milk+90%Vinasse 1 Alkaline treatment	8.30	18.33
10%Milk+90%Vinasse 2 Ultrasound 5’	8.30	18.33
10%Milk+90%Vinasse 2 Alkaline treatment	8.30	18.33

Table 13:OLR and C/N ratio of configurations from the sixth experiment

5.4 Analysis performed

Following the dark fermentation processes, anaerobic digestion, or after the treatments, various analyses were performed to monitor the efficiency of the processes in terms of product yields and beyond.

5.4.1 Measurement of gas quantity

One of the analyses performed was the measurement of the amount of gas accumulated in the gas-bags during the fermentation process. The analysis was carried out daily using a syringe with a known capacity of 60 mL. The syringe allowed for the extraction of gas from the valves of the bags in an open position. The daily gas production was an indicator to assess whether the process was proceeding correctly, as higher gas production corresponds to greater substrate consumption.

5.4.2 Gas Chromatography

Another analysis performed to evaluate the experiments was gas chromatography. This instrument can analyze the composition of a gas flow, known as the mobile phase, which is injected into the chromatographic column. Inside the column, the components are separated, and based on their retention time, a computer directly connected to the column can accurately and precisely determine the components being analyzed. Depending on the area under these curves, it is possible to estimate the concentration of the component of interest that passed through the column. Naturally, the larger the area, the higher the concentration of the component. [45]

Gas chromatography was primarily used to identify the composition of hydrogen, methane, and carbon dioxide present in the gas produced in the bags during the DF and AD processes. For the first process, a high concentration of hydrogen in the gas was the target, as it indicated a high yield. For AD, a high concentration of methane was the key objective to determine whether the process was proceeding correctly.

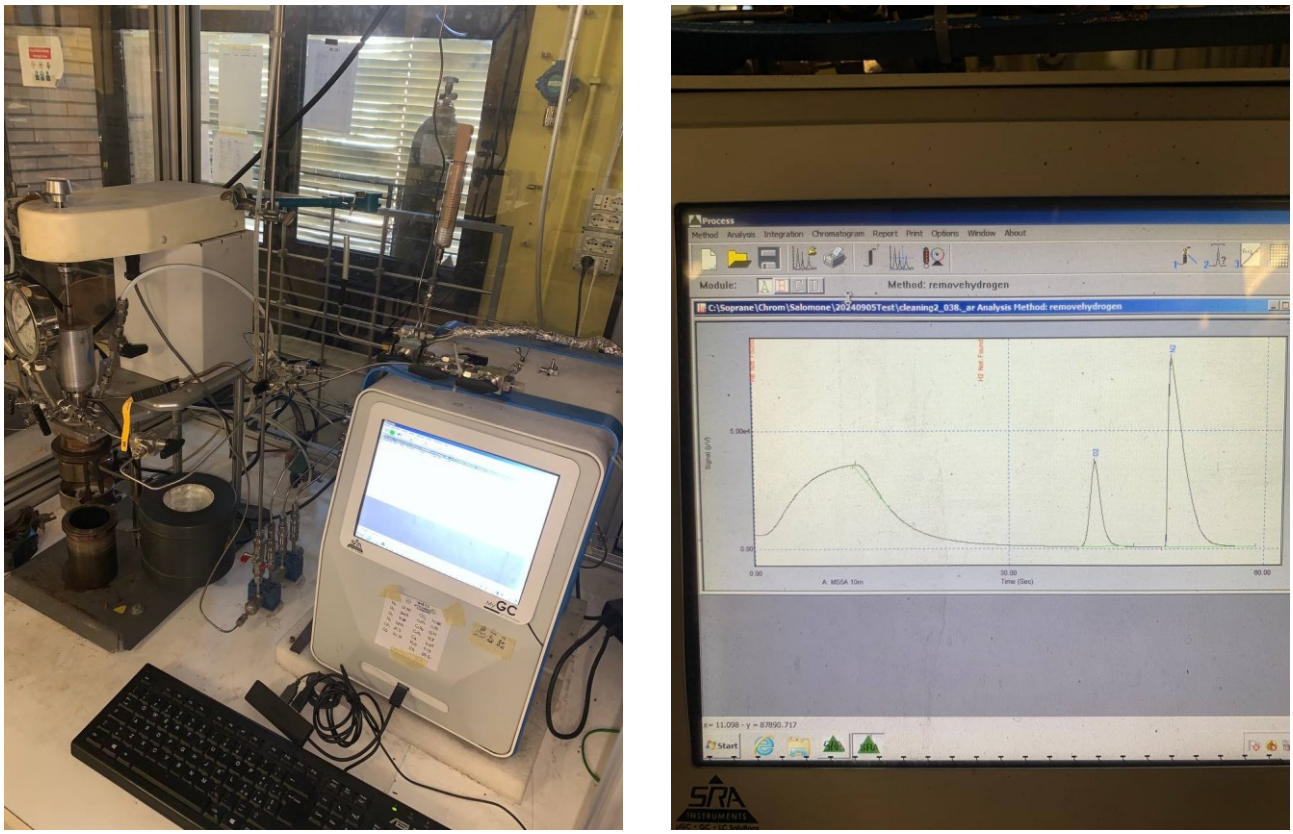

Figure 8: Gas Chromatograph and chromathograph peak

5.4.3 Analysis of the elemental composition: CHNSO

The CHNSO analysis allows for the examination of the elemental composition of various organic components. Its use was employed to analyze the composition of biomass to understand the C/N ratio and properly configure the experiments. Additionally, it was used for the analysis of digestates at the end of the DF and AD processes. The goal is to understand how the elemental composition has changed to determine how much these elements were utilized by microorganisms to produce the target gases. The net carbon balance at the end of the process must be maintained: the carbon introduced into the system should equal the carbon at the end of the process plus the carbon found in the produced gases.

To set up a CHNSO analysis, several steps are required. Specifically, a balance and other components are needed to prepare the organic substances for analysis. For this purpose, aluminum foils are used, which are shaped into cylinders with a mold to hold powders. Inside these aluminum cylinders, 20 mg of the substance to be analyzed, previously subjected to a thermal process in an oven at 550°C for 6 hours, is placed, followed by another 20 mg of tungsten oxide. The practical use of tungsten oxide is to enhance combustion within the analyzing equipment. Once the correct quantities are placed in the aluminum cylinder, the cylinder is sealed by pressing into a mold that gives it the shape of a pellet. The pellet is now ready to be analyzed.

5.4.4 Analysis of total solids and volatile solids

The analysis of total solids and volatile solids in biomass and digestates from DF and AD processes is crucial for accurately setting up the calculations for the amounts to be used in experimental setups. To obtain these data, several steps must be taken. The first step is to weigh aluminum containers using an electronic balance. Then, approximately 5 grams of biomass, from which the desired data is to be obtained, is placed into the container. After determining the weight of the sample plus the container, the container is placed in an oven at a temperature of 105°C overnight. During this process, the water contained in the biomass evaporates completely, leaving a dry residue in the container. The container is then reweighed to determine the weight of the dry residue. Using the dry weight, the percentage of total solids in the biomass can be calculated. The following formulas summarize this procedure:

$$Container\ weight\ +\ dry\ weight\ -\ container\ weight\ =\ dry\ weight$$

$$TS\% = \frac{dry\ weight}{Weight\ of\ biomass\ before\ thermal\ treatment} * 100 \qquad [5.4]$$

Once the total solids percentage is obtained, it is possible to proceed with determining the percentage of volatile solids relative to the total solids. To do this, ceramic containers capable of withstanding very high temperatures are used. The process begins by weighing these containers, which are then filled with enough dry residue obtained from the previous treatment. At this point, the ceramic container with the dry residue is weighed. The containers are then placed in an oven at 550°C for a total of 6 hours. Such a high temperature is sufficient to evaporate all the volatile solids contained in the total solids, leaving only inert ash in the container. By recovering the containers after the thermal treatment and weighing them, the weight of the containers plus the ash can be determined. Using the following formulas, it is then possible to calculate the percentage of volatile solids relative to the total solids in the system:

$$Container\ weight + ash\ weight - container\ weight = ash\ weight$$

$$dry\ weight\ in\ ceramic\ container - ash\ weight = volatile\ solid\ weight$$

$$\frac{VS}{TS}\% = \frac{volatile\ solid\ weight}{Weight\ of\ biomass\ before\ thermal\ treatment} * 100 \qquad [5.5]$$

Figure 9:Containers

5.5.5 COD analysis

The COD analysis was performed on the liquid extracts obtained by filtering the solutions in which vinasse was immersed following mechanical and chemical treatments aimed at extracting polyphenols. The analysis was carried out to determine which method was more effective in extracting these high molecular weight molecules from the vinasse.

A standard kit with a barcode recognized by a commercial spectrophotometer was used to measure this parameter. Through an intrinsic relationship of the equipment, the spectrophotometer can link the wavelength measured in the kit to a concentration, which is then displayed for reading. The steps for preparing these kits for analysis in the spectrophotometer are standard and provided on the kit's packaging. The kit contains test tubes with a solid deposit. The first step is to shake the test tubes to resuspend the deposit. Once a homogeneous suspension is achieved, 2 mL of a 1:5000 diluted solution of the sample and water are added to the test tube.

To prepare this dilution, 10 microliters of the solution were analyzed, and 49.99 mL of distilled water was mixed in a 50 mL Falcon tube. From this Falcon tube, 2 mL were taken and added to the test tube in the kit. Once the solution is added, the suspended solids in the test tube will react with the organic compounds in the 2 mL, resulting in an exothermic reaction. The next step is to place the reacted test tubes in a heating block with holes to hold the tubes at a temperature of 130°C for two hours. After two hours, the tubes were removed and cooled to 60°C. At this point, the tubes were analyzed using the spectrophotometer.

6. Results

In this chapter, all the results of the analyses conducted in the various experiments listed in Chapter 5 have been presented.

6.1 First experiment results

In *table 14*, it is possible to observe the highest amount of gas produced daily by the DF process in the various reactors, collected in the gas-bags. The gas quantity is reported as mL/day.

Configurations	Highest amount collected in gas-bags (mL)
Blank inoculum first reactor	21
Blank inoculum second reactor	30
50% Manure + 50% Vegetables first reactor	215
50% Manure + 50% Vegetables second reactor	240
100% Manure first reactor	65
100% Manure second reactor	70
10% Milk + 90% Manure first reactor	48
10% Milk + 90% Manure first reactor	53

Table 14:Highest gas production of the first experiment

From the data reported in the table, it is possible to observe that configuration number two, consisting of 50% manure and 50% Vegetables, is the one that produced the most biogas. Using the gas chromatograph, it was possible to analyze the composition of this gas, and in *Figure 10, 11, 12* and *13*, we can observe the trend of hydrogen, methane, carbon dioxide and biogas cumulate concentrations over time.

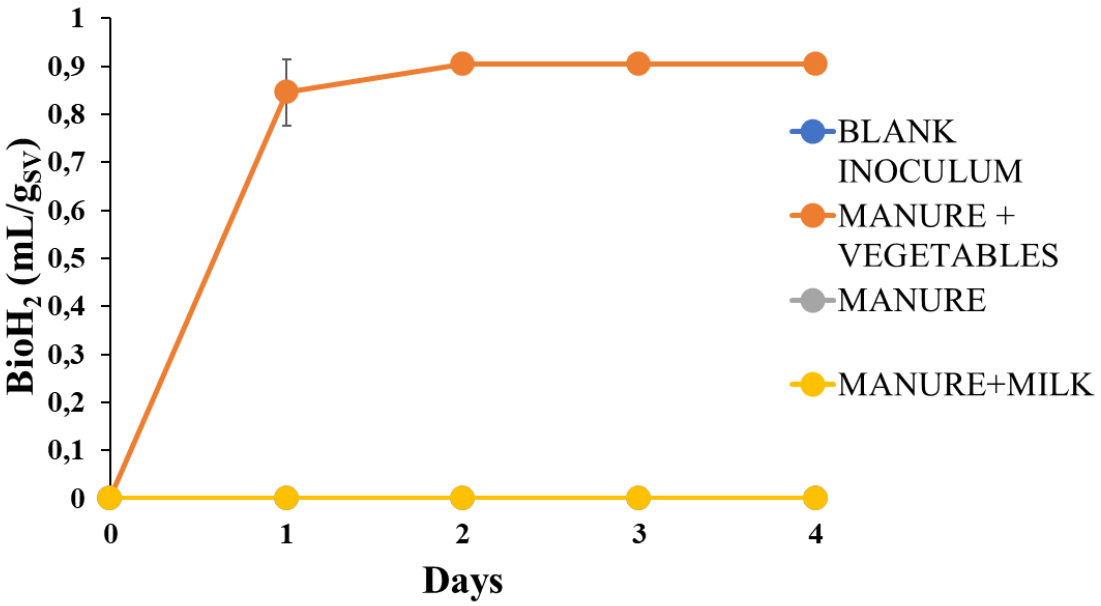

Figure 10:Bio-H_2 producted by fermentation

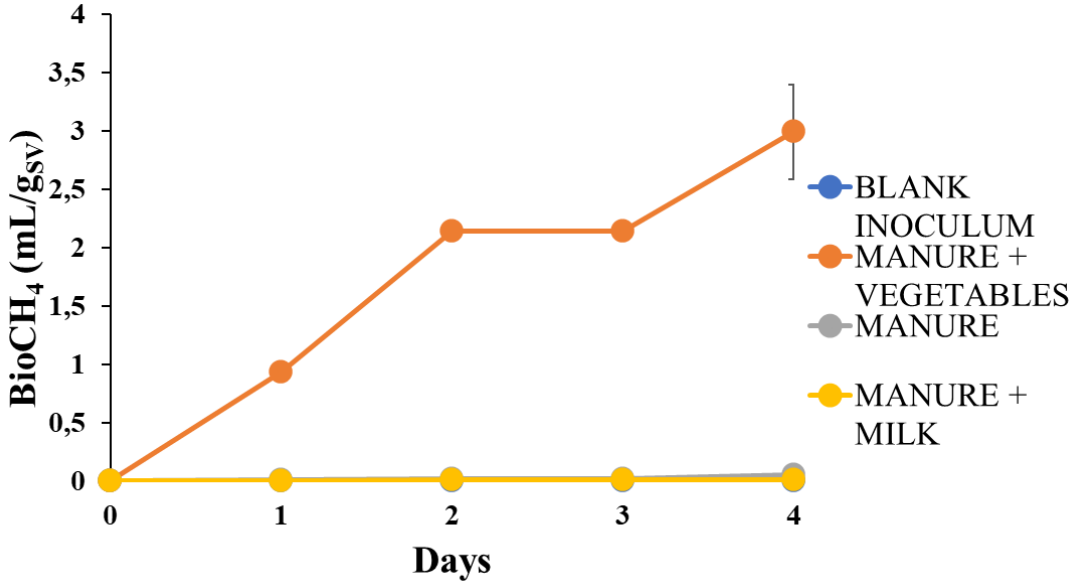

Figure 11:Bio-CH_4 producted by fermentation

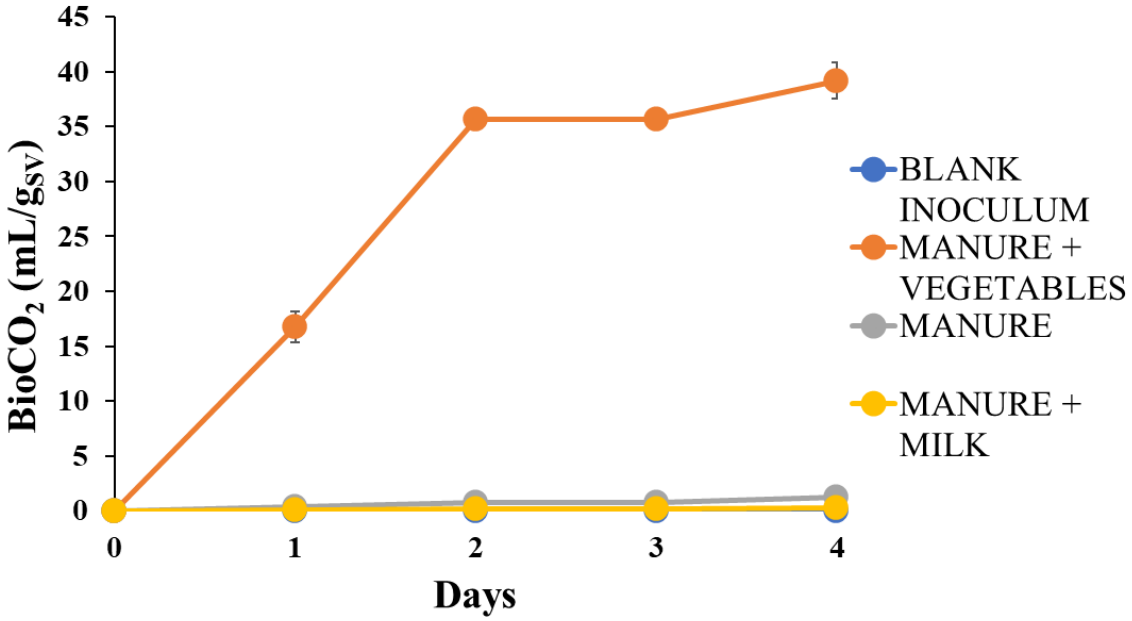

Figure 12:Bio-CO_2 producted by fermentation

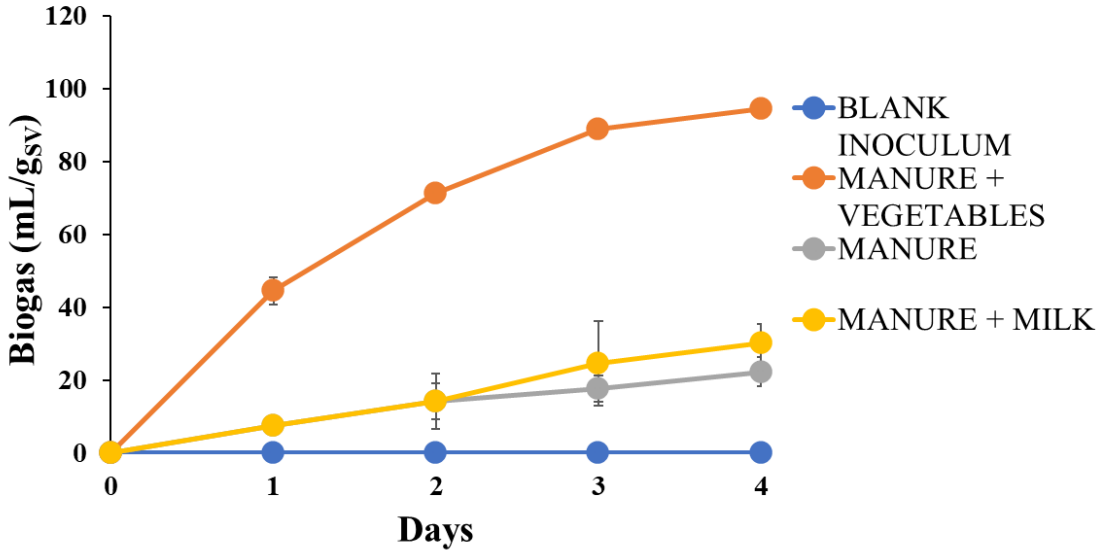

Figure 13: Biogas producted by fermentation

In *table 15*, it is possible to observe the maximum yield in terms of gas composition obtained in the different compositions.

Configurations	H_2 (%)	CH_4 (%)	CO_2 (%)
Blank Inoculum	0.00	8.56	4.46
50% Manure + 50% Vegetables	1.90	15.16	70.72
100% Manure	0.00	0.77	11.00
10% Milk + 90% Manure	0.00	0.09	1.29

Table 15:Maximum yieds compositions of gas produced by different configurations (1° experiment)

We can observe that, despite thermal treatment, the inoculum alone, referring to reactors one and two, was not able to produce hydrogen. Moreover, the combination of plant-based and animal-based waste, as in the case of 50% manure and 50% vegetables, resulted in higher hydrogen production compared to all other configurations, although with still relatively low yields, only 0.9 mL/gVS. In general, for these configurations, all of which contain manure, the results show that the dark fermentation process did not produce hydrogen. Additionally, methane production on the last day suggests that methanogens survived the thermal treatment. The highest methane concentration in the gas was observed on the fourth day of the experiment, in the configuration composed by 50% of manure and 50% of vegetables with a value of 3 mL/gVS. These results obtained do not align with the results obtained in the study conducted by Xiao Wu et al., (2009) where with a similar reactor configuration and an initial pH of 7.5, combining manure with other agro-industrial waste, they were able to achieve 135 mL/gVS of hydrogen.

Subsequently, the pH of the digestate was also measured after the fermentation process to determine whether acidogenesis occurred, with the production of volatile fatty acids, which lower the system's pH. The *table 16* presents the results.

Configuration	pH after the process
Blank Inoculum	8.01
50% Manure + 50% Vegetables	6.62
100% Manure	7.05
10% Milk + 90% Manure	7.03

Table 16: pH values of the configurations from the first experiment after DF

The final pH measurement of the digestates showed a significant deviation for the first configuration compared to the value of 7.19 measured before the experiment. Configurations number two and three present lower values of pH after the process respect the initial value of 7.2 measured before the experiment. This pH decrease can be attributed to the fermentation products accumulated in the system during the four days of the experiment. The last configuration had a pH value approximately equal to the one measured at the beginning of the process, which was 6.98. After the pH analysis, the total solids and volatile solids analysis of the digestates was also conducted, and the results are summarized in the *table 17*:

Configurations	TS (%)	Standard Deviation TS (%)	VS/TS (%)	Standard Deviation VS/TS (%)
Blank Inoculum	6.49	0.32	89.51	8.05
50% Manure + 50% Vegetables	8.97	0.29	90.45	2.06
100% Manure	10.25	0.92	86.96	0.51
10% Milk + 90% Manure	10.77	2.43	81.60	3.43

Table 17: Total solids and volatile solids values of the configurations from the first experiment after DF

Additionally, in *table 18* the results of the CHNSO analysis are presented, which was conducted to understand how the elemental composition of the system changed during the process.

Configurations	N (%)	Standard Deviation N (%)	C (%)	Standard Deviation C (%)	H [%]	Standard Deviation H (%)	S [%]	C/N ratio
Blank Inoculum	2.54	13.44	34.68	7.78	0.85	16.26	0.07	13.68
50% Manure + 50% Vegetables	2.84	4.24	37.30	14.28	13.65	1.97	1.90	13.13
100% Manure	2.24	1.41	35.41	19.37	29.98	0.94	1.91	15.80
10% Milk + 90% Manure	2.43	19.09	35.84	8.69	15.20	1.64	3.32	14.77

Table 18:CHNSO analysis of digestates from first experiment

6.2 Second experiment results

From the first experiment, it was concluded that the thermal treatment was not sufficient to achieve a good result in the inhibition of methanogenic bacteria, which negatively impacted the DF process, resulting in poor hydrogen production and a low amount of gas. For this reason, a different approach was used in the second experiment to treat the manure, which, as mentioned in Chapter 5, was treated with hydrochloric acid until a pH of 4 was reached. The *table 19* shows the highest amount collected in gas-bags for each configurations:

Configurations	Highest amount collected in gas-bags (mL)
50% Manure + 50% Vegetables first reactor	387
50% Manure + 50% Vegetables second reactor	356

100% Manure first reactor	39
100% Manure second reactor	62
10% Milk + 90% Manure first reactor	72
10% Milk + 90% Manure first reactor	50

Table 19: Highest gas production of the second experiment

From the daily gas production, it can be observed that, even in this second experiment, the configuration that produced the most gas was that containing 50% manure and 50% vegetables. However, this type of acid treatment led to a decrease in gas production for the other configurations. In *Figure 14, 15, 16* and *17* are reported the cumulative production of hydrogen, methane, and carbon dioxide in mL/gVS.

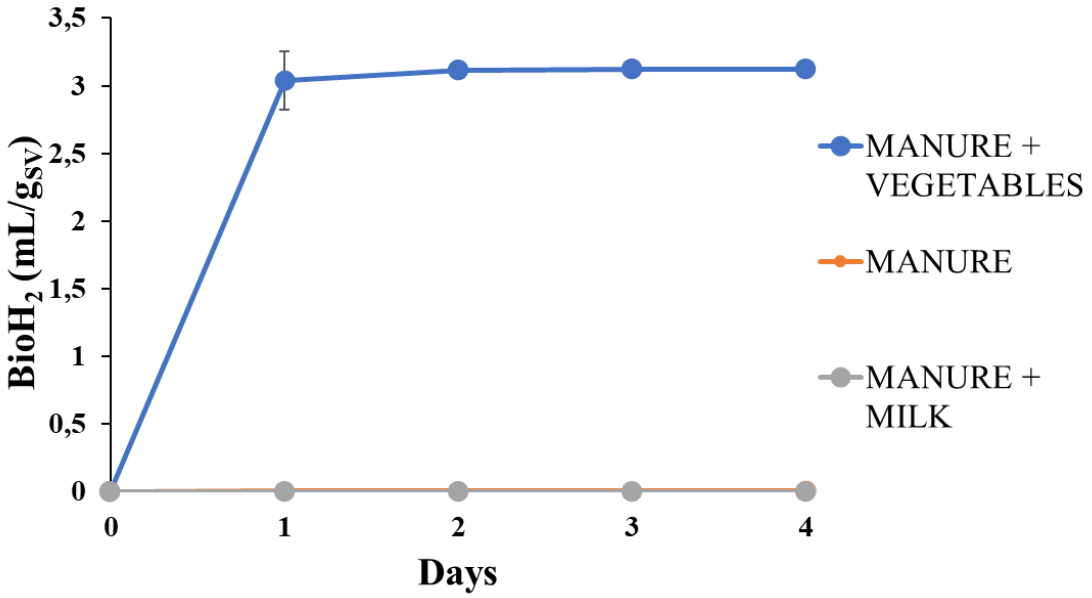

Figure 14: Bio-H_2 cumulative concentration second experiment

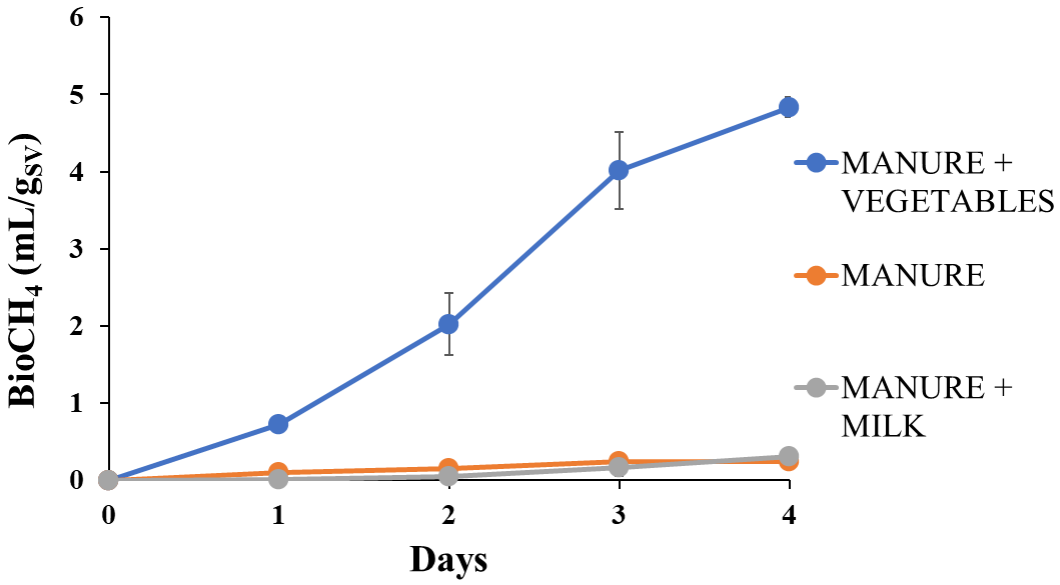

Figure 15: Bio-CH_4 cumulative concentration second experiment

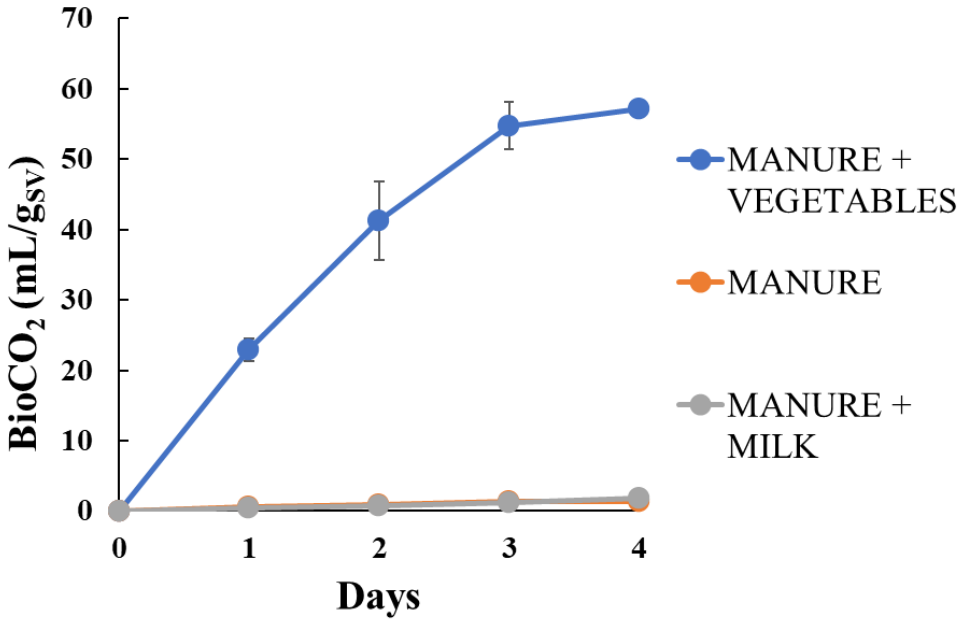

Figure 16: Bio-CO_2 cumulative concentration second experiment

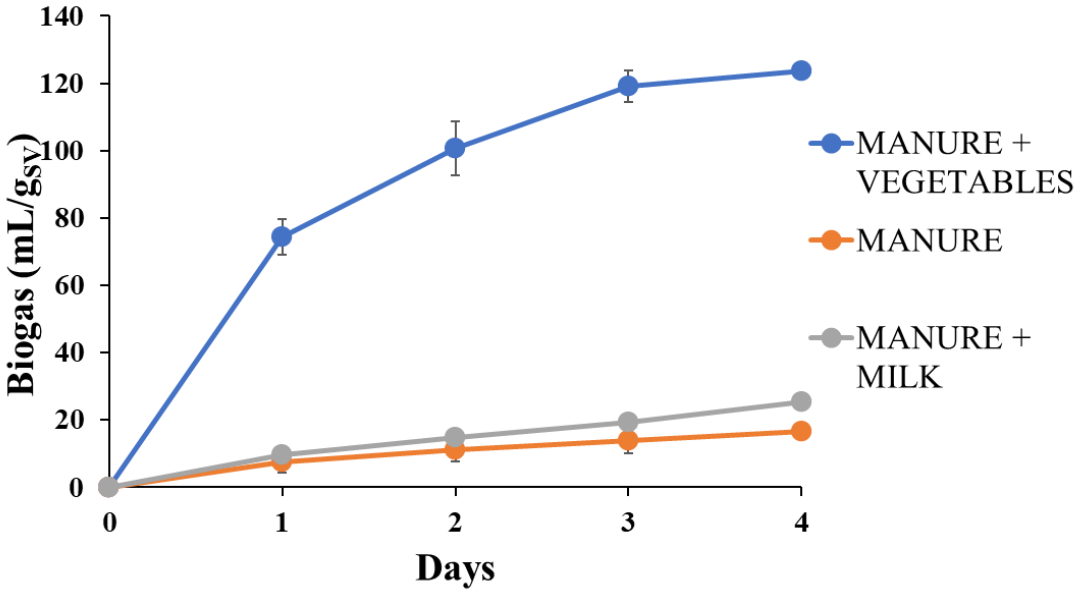

Figure 17: Biogas cumulative concentration second experiment

The results shows that the maximum total biogas production achieved is around 130 mL/gVS. The higher amount of hydrogen was producted by the first configuration, containing 50% manure and 50% vegetables, with a yield of 3 mL/gVS. Moreover, its production stops after the first day, giving way to methane production, indicating the presence of methanogenic bacteria. Manure alone is not capable of producing hydrogen, nor is manure combined with milk waste. The results do not align with the results obtained in the study conducted by Xiao Wu et al., (2009) where with a similar reactor configuration and an initial pH of 7.5, combining manure with other agro-industrial waste, they were able to achieve 135 mL/gVS of hydrogen.

In *table 20*, it is possible to observe the maximum yield in terms of gas composition obtained in the different compositions.

Configuration	H_2 (%)	CH_4 (%)	CO_2 (%)
50% Manure + 50% Vegetables	4.08	17.48	73.08
100% Manure	0.05	2.77	12.22
10% Milk + 90% Manure	0.00	2.55	10.37

Table 20: Maximum yieds compositions of gas produced by different configurations (2° experiment)

From the perspective of hydrogen percentage composition, the new treatment for the manure resulted in a hydrogen production three times higher than in the first experiment. However, the concentration is still low in terms of hydrogen production. The final pH values of the digestates after the fermentation process are reported in *table 21*. *Table 22* and *23* report VS/TS, and CHNSO analysis.

Configuration	pH after the process
50% Manure + 50% Vegetables	7.09
100% Manure	7.05

10% Milk + 90% Manure	7.03

Table 21: pH values of the configuration from the second experiment after DF

The pH values of the digestates are all lower than the initial values. This decrease in pH can be attributed to the fermentation products accumulated in the system.

Configuration	TS (%)	Standard Deviation TS (%)	VS/TS (%)	Standard Deviation VS/TS (%)
50% Manure + 50% Vegetables	10.28	0.88	87.90	2.82
100% Manure	11.47	0.29	90.44	0.76
10% Milk + 90% Manure	7.82	1.90	86.63	1.84

Table 22:Total solids and volatile solids values of the configuration from the second experiment after DF

Configurations	N (%)	Standard Deviation N (%)	C (%)	Standard Deviation C (%)	H [%]	Standard Deviation H (%)	S [%]	Standard Deviation S (%)	C/N ratio
50% Manure + 50% Vegetables	2.84	2.12	37.69	16.26	4.76	6.71	0.62	1.13	13.25
100% Manure	2.55	14.85	36.48	1.97	4.68	23.54	0.59	0.21	14.27
10% Milk + 90% Manure	2.33	6.36	35.28	9.40	4.53	9.12	0.58	0.35	15.10

Table 23:CHNS analysis of digestates from second experiment

6.3 Third experiment results

Through the first two experiments, the results shown that the configurations with manure, except when combined with vegetables, were not particularly suitable for a dark fermentation process. For this reason, in the third experiment, three different configurations were adopted for dark fermentation. Additionally, three configurations were studied for anaerobic digestion to investigate whether manure could produce methane.

6.3.1 Anaerobic digestion results

The anaerobic digestion process was carried out for a total of 34 days, and the highest amount of gas produced and collected in gas bags are reported in *table 24*:

Reactors	Highest amount collected in gas-bags (mL)
Blank Inoculum first reactor	83
Blank Inoculum second reactor	40
100% Manure first reactor	57
100% Manure second reactor	46
5% Milk 95% Manure first reactor	70
5% Milk + 95% Manure second reactor	85

Table 24: Highest gas production of the third experiment AD

In *Figure 21, 22, 23* and *24* are reported the cumulative production of hydrogen, methane, and carbon dioxide in mL/gVS

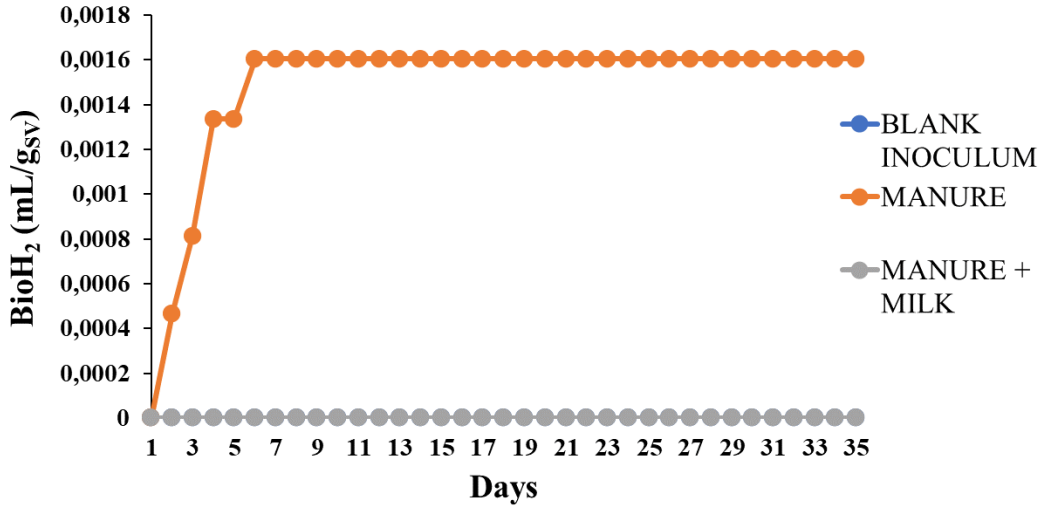

Figure 18: Bio-H_2 cumulative concentration produced by AD (3° experiment)

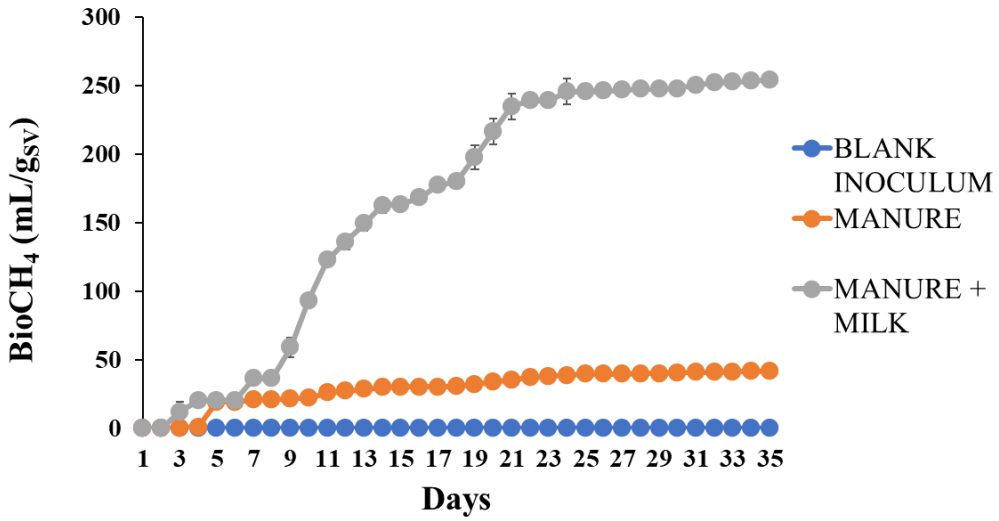

Figure 19: Bio-CH_4 cumulative concentration produced by AD (3° experiment)

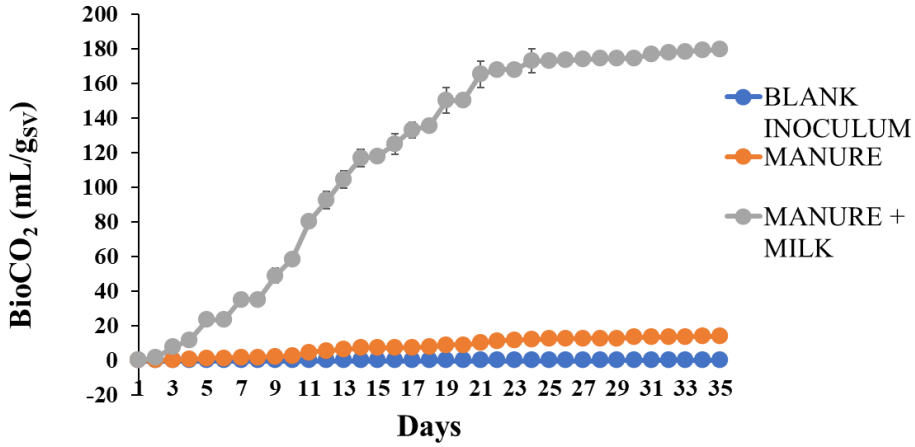

Figure 20: Bio-CO_2 cumulative concentration produced by AD (3° experiment)

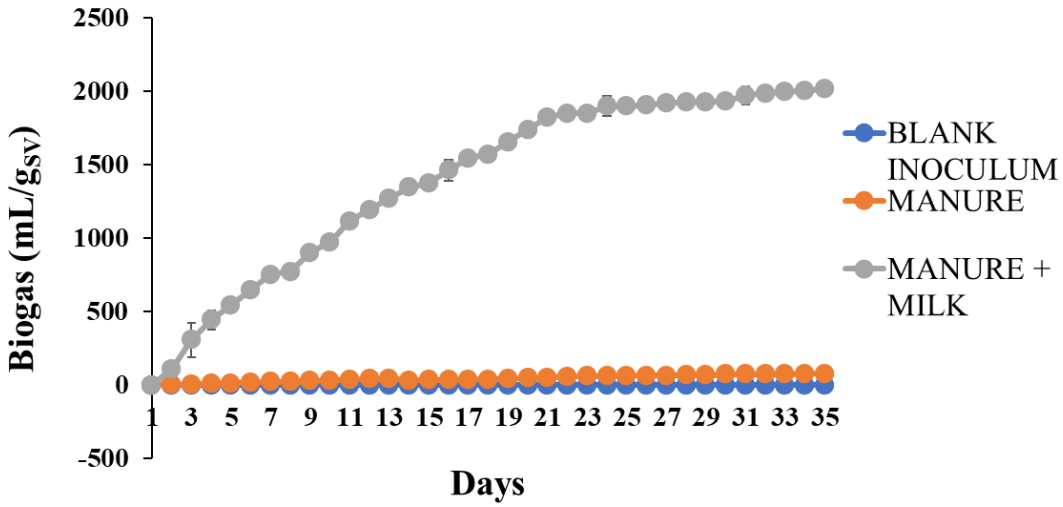

Figure 21: Biogas cumulative concentration produced by AD (3° experiment)

From the anaerobic digestion test, we can see that the amount of hydrogen produced is practically negligible for all configurations, as it is consumed by methanogenic bacteria to produce methane. For the configuration containing 100% manure, there was a production of 50 mL/gVS of methane. However, with the presence of another substrate, such as milk waste, it is possible to achieve productions of over 250 mL/gVS. The results obtained are slightly different from the 77 mL/gVS of methane obtained in the study conducted by L. Kalsum et al. (2020) where in similar conditions, the manure was used as substrate.

Furthermore, for this anaerobic digestion experiment, after twelve days, a pH check was conducted to see if the operating parameters had significantly changed from the optimal conditions. The caps were unscrewed, and the following pH measurements were taken using a pH meter. HCl was used to adjust the parameter. All data are reported in the *table 25*:

Reactors	pH post 12 days of AD	HCl 2M added [mL]	pH after HCl

Blank Inoculum first reactor	7.89	1	7.32
Blank Inoculum second reactor	7.81	1	7.56
100% Manure first reactor	7.42	/	7.42
100% Manure second reactor	7.47	/	7.47
5% Milk 95% Manure first reactor	7.3	/	7.3
5% Milk + 95% Manure second reactor	7.39	/	7.39

Table 25: pH values of the reactors from the third experiment after twelve days of AD

In *table 25*, it is possible to observe the maximum yield in terms of gas composition obtained in the different compositions.

Configuration	H_2 (%)	CH_4 (%)	CO_2 (%)
Blank Inoculum	0.25	2.19	3.22
100% Manure	0.02	52.67	34.23
5% Milk 95% Manure	0.00	46.61	17.82

Table 26: Maximum yieds compositions of gas produced by different configurations (3° experiment AD)

The analysis of TS and VS/TS of the anaerobic digestion digestates are also reported in *table 26*.

Configuration	TS (%)	Standard Deviation TS (%)	VS/TS (%)	Standard Deviation VS/TS (%)
Blank Inoculum	2.72	0.06	65.27	2.47
100% Manure	3.13	0.89	59.97	0.49
5% Milk 95% Manure	2.04	0.18	53.60	0.99

Table 27:Total solids and volatile solids values of configurations from the third experiment after AD

Configuration	N (%)	Standard Deviation N (%)	C (%)	Standard Deviation C (%)	H (%)	Standard Deviation H (%)	S (%)	Standard Deviation S (%)	C/N ratio
Blank Inoculum	2.85	15.56	29.50	0.91	3.69	9.40	0.61	19.37	10.35
100% Manure	2.30	12.73	24.66	2.61	3.02	0.46	0.65	18.60	10.72
5% Milk 95% Manure	2.72	2.83	27.96	2.68	3.42	12.37	0.67	11.74	10.28

Table 28: CHNS analysis of digestates from third experiment AD

6.3.2 Dark fermentation results

The highest amount of gas produced and collected in gas bags are reported in *table 29*:

Configuration	Highest amount collected in gas-bags (mL)
50% Manure + 50% Vegetables first reactor	484
50% Manure + 50% Vegetables second reactor	460
50% Manure + 50% Sedamyl first reactor	405
50% Manure + 50% Sedamyl second reactor	420
5% Milk + 95% Vegetables first reactor	780
5% Milk + 95% Vegetables second reactor	865

Table 29: Highest gas production of the third experiment DF

Compared to previous experiments, the gas productions are higher. The configuration that produced the most gas was the third one, made up of 5% milk and 95% vegetables. In fact, after the dark fermentation process, the digestate from these configurations was used as a substrate for anaerobic digestion, following the addition of an adequate amount of fresh inoculum. This made it possible to initiate a two-stage process to further valorize food waste and obtain high value-added products. The *figure 25, 26, 27* and *28* show the trends of cumulative hydrogen, methane, carbon dioxide, and biogas for the processes of dark fermentation (DF)

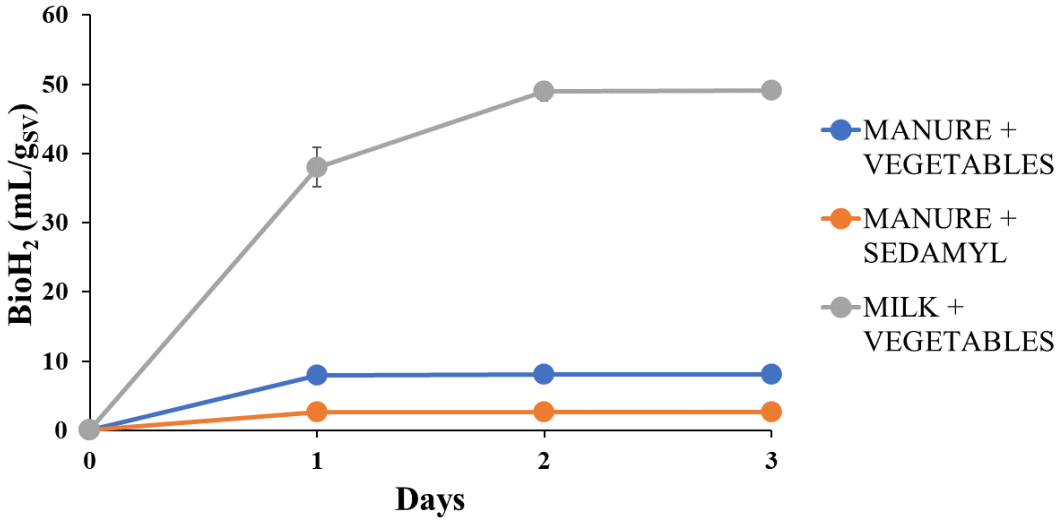

Figure 22: Bio-H_2 cumulative concentration produced by DF (3° experiment)

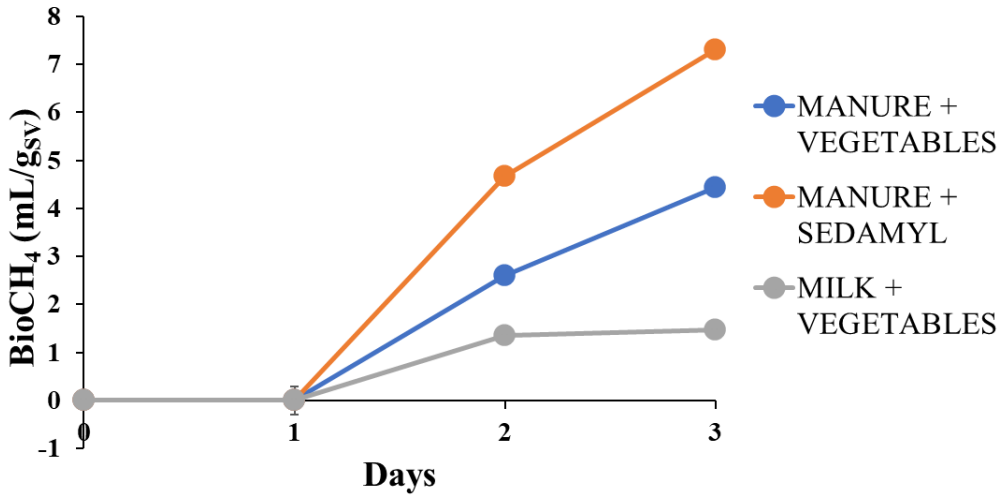

Figure 23:Bio-CH_4 cumulative concentration produced by DF (3° experiment)

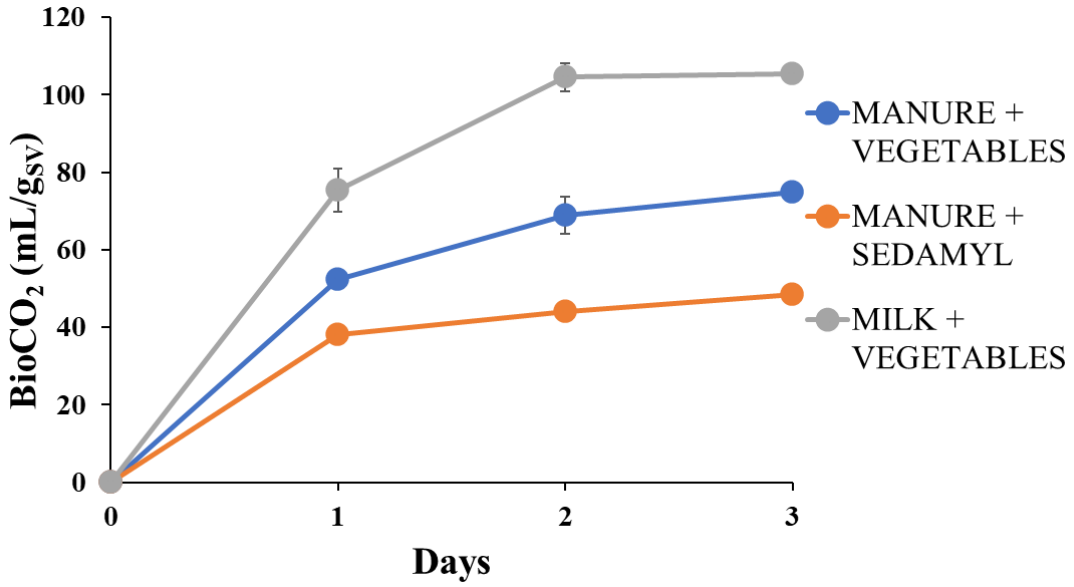

Figure 24:Bio-CO_2 cumulative concentration produced by DF (3° experiment)

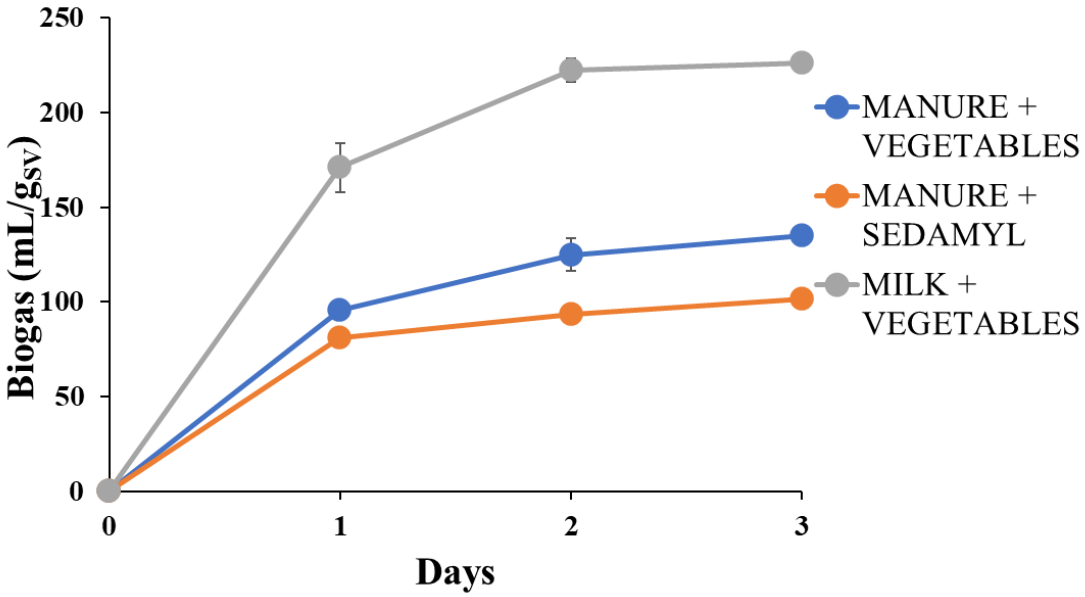

Figure 25:Biogas cumulative concentration produced by DF (3° experiment)

In *table 30*, it is possible to observe the maximum yield in terms of gas composition obtained in the different compositions.

Configuration	H_2 (%)	CH_4 (%)	CO_2 (%)
50% Manure + 50%Vegetables	8.28	18.06	4.46
50% Manure + 50% Sedamyl	3.17	37.82	55.57
5% Milk + 95% Vegetables	22.25	3.12	56.60

Table 30: Maximum yieds compositions of gas produced by different configurations (3° experiment DF)

The results shows that configuration containing milk and vegetables waste produced 50 mL/gVS of hydrogen. As for the other two configurations, except for the first day of the experiment, they began producing methane. A similar result was obtained in the study of Weronika Cieciura-Włoch et al. (2013), where 52 mL H_2/gVS was produced through the DF of plant waste and an inoculum taken from a wastewater treatment plant, with an OLR of 17 kgVS/m^3d.

The results of the TS and VS analyses are also shown in *table 31*:

Configuration	TS (%)	Standard Deviation TS (%)	VS/TS (%)	Standard Deviation VS/TS (%)
50% Manure + 50%Vegetables	4.85	0.11	74.95	1.90
50% Manure + 50% Sedamyl	3.18	0.10	63.09	3.82
5% Milk + 95% Vegetables	3.35	0.74	79.12	1.26

Table 31: Total solids and volatile solids values of the configurations from the third experiment after DF

Configuration	pH final
50% Manure + 50%Vegetables	6.85
50% Manure + 50% Sedamyl	6.67
5% Milk + 95% Vegetables	5.98

Table 32: pH values of the configurations from the third experiment after DF

Configuration	N (%)	Standard Deviation N (%)	C (%)	Standard Deviation C (%)	H (%)	Standard Deviation H (%)	S (%)	Standard Deviation S (%)	C/N ratio
50% Manure + 50%Vegetables	3.31	17.68	30.08	1.64	4.13	0.39	0.56	2.47	9.07
50% Manure + 50% Sedamyl	2.95	27.58	27.03	4.66	3.77	0.62	0.58	5.16	9.14
5% Milk + 95% Vegetables	3.24	19.80	32.62	1.68	4.35	9.97	0.57	9.05	10.06

Table 33:CHNS analysis of digestates from third experiment DF

6.3.3 Two-stage results

For third configuration made up of 5% milk and 95% vegetables, processed through dark fermentation, it was possible to operate a two-stage process. By analyzing the total solids and volatile solids of the digestate from this configuration, the exact amount to be added to the new system was determined, along with the amount of fresh inoculum to be added to operate in a system used in previous cases, with a working volume of 200 mL. The NaOH was used to adjust the system's pH. All data are summarized in *table 34*:

Confuguration	Initial pH	NaOH 3M added	pH after NaOH
Digestate of 5% Milk + 95% Vegetables first reactor	6.56	1,50	7.10
Digestate of 5% Milk + 95% Vegetables second reactor	6.67	1.00	7.01

Table 34 : Initial pH of the configurations subjected to the two-stage process.

At this point, after proper degassing, the anaerobic digestion process took place in the system for 21 days. In *Table 35* the highest amount of gas collected gas-bags is reported. In *Figure 30*, *31* and *32* are reported the cumulative production of methane, carbon dioxide and biogas in mL/gVS:

Confuguration	Highest amount of gas collected in gas-bags (mL)
Digestate of 5% Milk + 95% Vegetables first reactor	286
Digestate of 5% Milk + 95% Vegetables second reactor	253

Table 35: Highest gas production of the third experiment two-stage AD

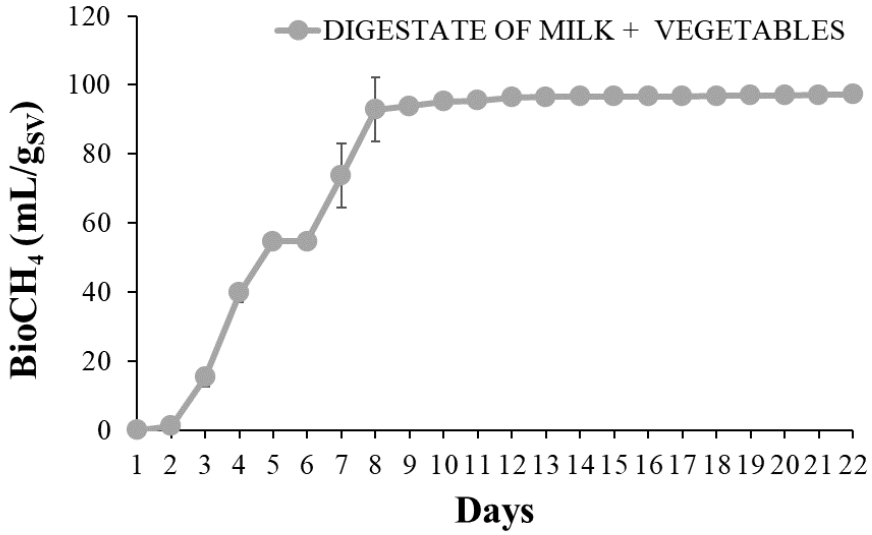

Figure 26: Bio-CH_4 produced by two stage AD (3° Experiment)

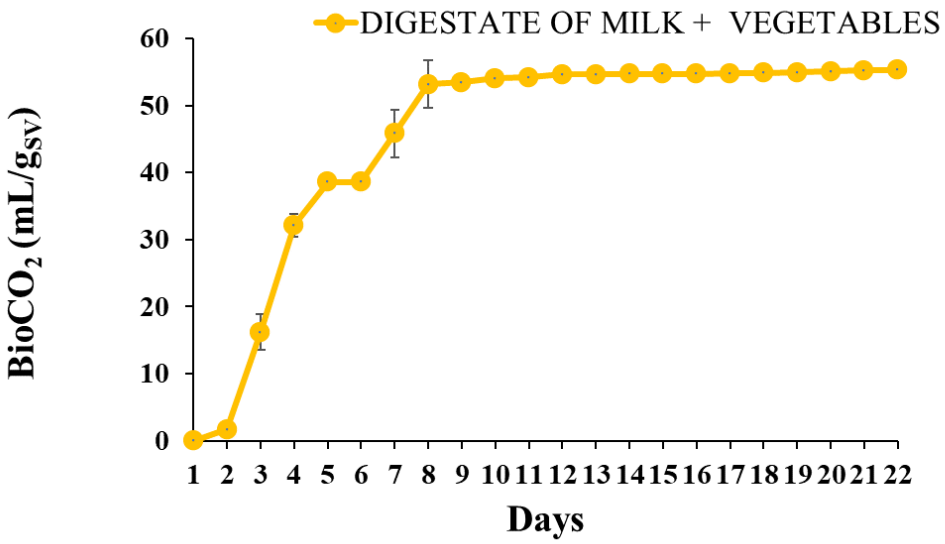

Figure 27:Bio-CO_2 produced by two stage AD (3° Experiment)

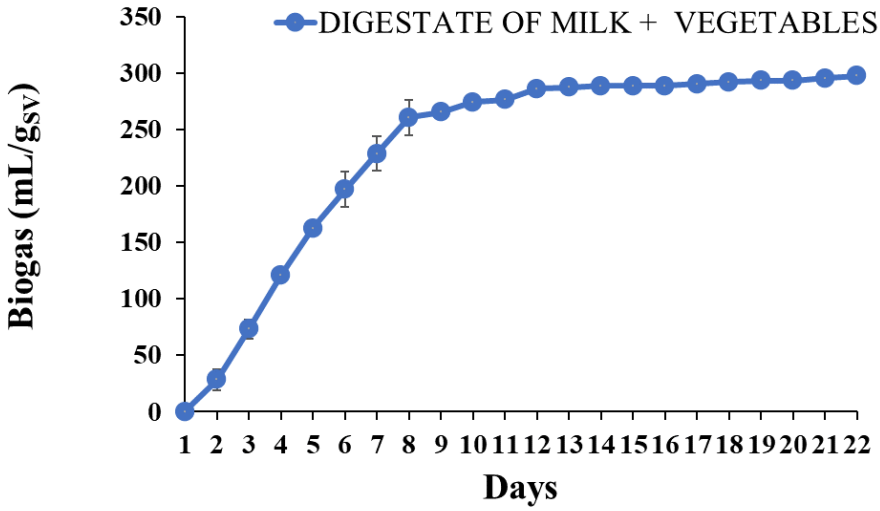

Figure 28: Biogas produced by two stage AD (3° Experiment)

From the results, it can be observed that biogas production mainly occurred in the first days of the experiment. Hydrogen production was not reported because it was zero. With the two-stage process, it was possible to obtain 100 mL of methane per gram of volatile solids present in the digestate. In the study of Shen F et al. (2013), plant waste was processed using two-stage anaerobic digestion in systems with an OLR of 1-5 kg VS/m³d, yielding 198–546 mL CH_4/gVS, which is therefore higher than in this thesis study.

In *table 34*, it is possible to observe the maximum yield in terms of gas composition.

Configuration	H_2 (%)	CH_4 (%)	CO_2 (%)
Digestate of 5% Milk + 95% Vegetables	0.00	59.68	32.42

Table 36: Maximum yields compositions of gas produced (3° experiment two-stage AD)

The VS/TS analysis of the digestate at the end of the two-stage process is reported in table 35. In table 36 the final pH at the end of the process is reported.

Configuration	TS [%]	Standard Deviation TS (%)	VS/TS [%]	Standard Deviation VS/TS (%)
Digestate of 5% Milk + 95% Vegetables	2.82	0.25	67.70	2.06

Table 37: Total solids and volatile solids values of the configuration from the third experiment after double-stage

Configuration	pH final
Digestate of 5% Milk + 95% Vegetables	7.13

Table 38:pH values of the configuration from the third experiment after double stage

Configuration	N (%)	Standard Deviation N (%)	C (%)	Standard Deviation C (%)	H (%)	Standard Deviation H (%)	S (%)	Standard Deviation S (%)	C/N ratio

Digestate of 5% Milk + 95% Vegetables	2.89	0.71	29.60	1.47	3.80	21.00	0.68	4.73	10.22

Table 39: CHNS analysis of digestates from third experiment two-stage AD

6.4 Fourth experiment results

Following the results obtained in the third experiment, it was decided to further experiment with the configuration composed of milk waste biomass and plant waste. For this combination of biomasses, a configuration was also tested where the ratio of volatile solids of biomass to inoculum was 2:1, in addition to the standard configuration with a ratio of 1:1. Additionally, manure mixed with milk was tested one last time to see if the results were consistent with previous experiments. Finally, two new lignocellulosic biomasses were tested: vinasse 1 and vinasse 2.

6.4.1 Dark fermentation results

Dark fermentation was tested for four days, and the highest amount of gas collected in gas-bags measured in mL is reported in *table 40*:

Configuration	Highest amount of gas collected in gas-bags (mL)
5% Milk + 95% Vegetables 2:1 first reactor	260
5% Milk + 95% Vegetables 2:1 second reactor	245
5% Milk + 95% Vegetables 1:1 first reactor	723
5% Milk + 95% Vegetables 1:1 second reactor	745
10% Milk + 90% Manure first reactor	95
10% Milk + 90% Manure second reactor	107
10% Milk + 90% Vinasse 1 first reactor	97
10% Milk + 90% Vinasse 1 second reactor	214

10% Milk + 90% Vinasse 2 first reactor	247
10% Milk + 90% Vinasse 2 second reactor	240

Table 40: Highest gas production of the fourth experiment DF

In *Figure 33, 34, 35* and *36* are reported the cumulative production of hydrogen, methane, carbon dioxide and biogas in mL/gVS:

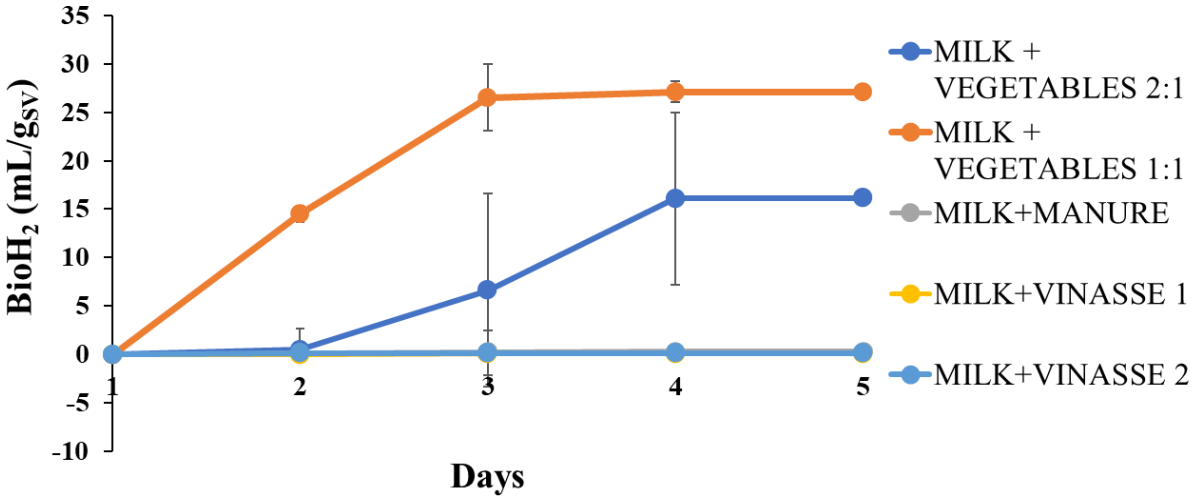

Figure 29: Bio-H_2 cumulative concentration DF fourth experiment

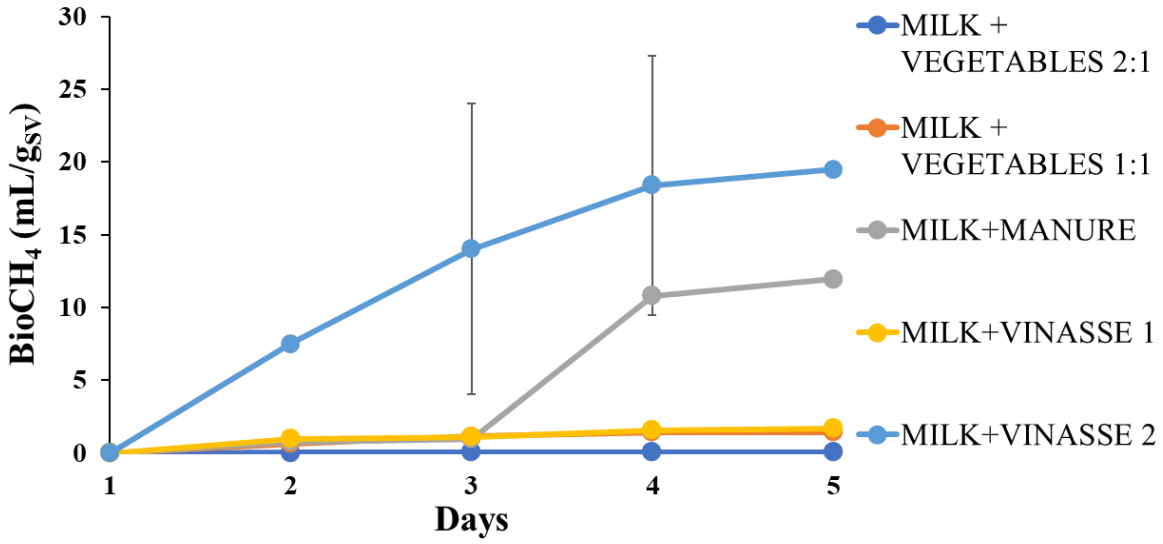

Figure 30: Bio-CH_4 cumulative concentration DF fourth experiment

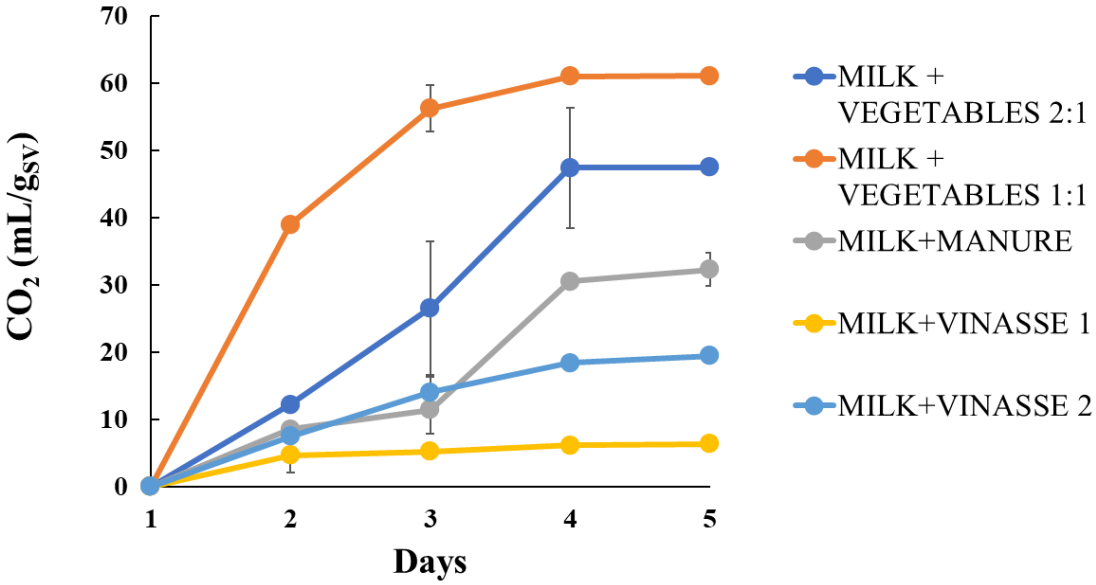

Figure 31: Bio-CO_2 cumulative concentration DF fourth experiment

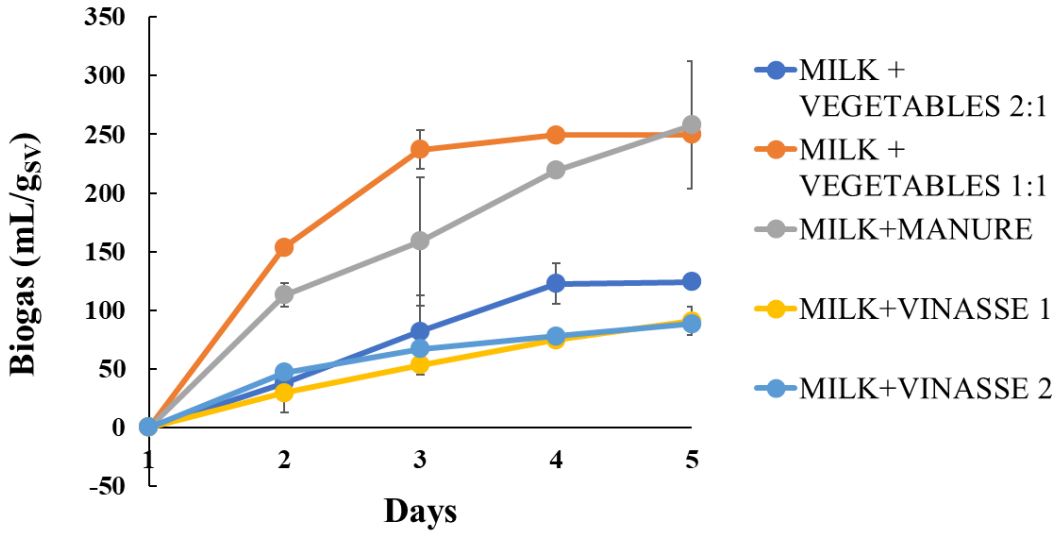

Figure 32: Biogas cumulative concentration DF fourth experiment

In *table 41*, it is possible to observe the maximum yield in terms of gas composition obtained in the different configurations.

Configuration	H_2 (%)	CH_4 (%)	CO_2 (%)
5% Milk + 95% Vegetables 2:1	23.14	0.09	51.11
5% Milk + 95% Vegetables 1:1	14.43	2.36	38.12
10% Milk + 90% Manure	0.23	16.14	31.47
10% Milk + 90% Vinasse 1	0.01	3.27	15.94

10% Milk + 90% Vinasse 2	0.27	12.49	40.82

Table 41: Maximum yields compositions of gas produced (4° experiment DF)

The first two configuration produced the higher amount of hydrogen, while the manure mixed with milk did not produce hydrogen. The configuration with 5% milk and 95% vegetables with a ratio of volatile solid of biomass and inoculum 1:1 produced 25 mL/gVS of hydrogen, while the same configuration but with a ratio of 2:1 produced 15 mL/gVS of hydrogen. The configurations four and five containing milk and vinasse 1 and 2 did not produce hydrogen. The results of the TS and VS analyses are reported in *table 42*. In *table 43* and *44* are reported pH and CHNSO analysis.

Configuration	TS [%]	Standard Deviation TS (%)	VS/TS [%]	Standard Deviation VS/TS(%)
5% Milk + 95% Vegetables 2:1	4.18	0.46	87.34	3.73
5% Milk + 95% Vegetables 1:1	7.20	0.74	94.27	0.59
10% Milk + 90% Manure	5.51	0.76	83.98	3.03
10% Milk + 90% Vinasse 1	3.14	0.99	87.13	4.72
10% Milk + 90% Vinasse 2	3.06	1.14	88.08	2.15

Table 42:Total solids and volatile solids values of configurations from the fourth experiment after DF

Configuration	pH final
5% Milk + 95% Vegetables 2:1	4.91
5% Milk + 95% Vegetables 1:1	5.48
10% Milk + 90% Manure	7.07
10% Milk + 90% Vinasse 1	7.21
10% Milk + 90% Vinasse 2	7.47

Table 43:pH values of configurations from the fourth experiment after DF

Configuration	N (%)	Standard Deviation N (%)	C (%)	Standard Deviation C (%)	H (%)	Standard Deviation H (%)	S (%)	Standard Deviation S (%)	C/N ratio
5% Milk + 95% Vegetables 2:1	3.25	0.00	43.32	7.46	5.45	0.70	0.80	23.82	13.32
5% Milk + 95% Vegetables 1:1	3.56	19.09	44.47	1.43	6.30	0.45	0.55	4.95	12.47
10% Milk + 90% Manure	2.61	4.95	32.17	2.00	3.82	3.11	0.52	5.94	12.30

10% Milk + 90% Vinasse 1	2.62	3.18	36.12	4.03	4.33	3.81	0.49	4.10	13.81
10% Milk + 90% Vinasse 2	2.65	4.24	39.05	2.50	4.62	2.84	0.46	5.65	14.73

Table 44:CHNS analysis of digestates from fourth experiment DF

6.4.2 Two-stage results

At this point, a two-stage anaerobic digestion process was adopted for the first two configurations, made up of 5% milk and 95% vegetables. The pH was adjusted using 3M NaOH. Additionally, to improve the carbon-nitrogen ratio of the system, two configurations were used where manure was added alongside the digestate. The the *table 45* summarize all the data:

Configuration	Real Working Volume	Initial pH	NaOH 3M added	pH after NaOH
Digestate of 5% Milk + 95% Vegetables 1:1 first reactor	199.90	6.60	1.00	7.00
Digestate 5% Milk + 95% Vegetables 1:1 second reactor	197.70	6.57	1.00	7.00
30 % Digestate of 5% Milk + 95% Vegetables 1:1 + 70% Manure first reactor	201.80	7.57	0.00	7.57
30 % Digestate of 5% Milk + 95% Vegetables 1:1 + 70% Manure second reactor	197.80	7.52	0.00	7.52
Digestate of 5% Milk + 95% Vegetables 2:1 first reactor	202.70	6.03	3.00	7.48
Digestate 5% Milk + 95% Vegetables 2:1	202.20	6.11	3.00	7.40

second reactor				
30 % Digestate of 5% Milk + 95% Vegetables 2:1 + 70% Manure first reactor	200.00	7.30	0.00	7.30
30 % Digestate of 5% Milk + 95% Vegetables 2:1 + 70% Manure second reactor	199.90	7.20	0.00	7.20

Table 45: Volume and pH values of the bottles subjected to the two-stage process from the fourth experiment

The two-stage AD process was tested for a total of 17 days. The highest amount of gas production of the various configurations is reported in mL in *table 46:*

Configuration	Highest amount of gas collected in gas-bags (mL)
Digestate of 5% Milk + 95% Vegetables 1:1 first reactor	122
Digestate 5% Milk + 95% Vegetables 1:1 second reactor	152
30 % Digestate of 5% Milk + 95% Vegetables 1:1 + 70% Manure first reactor	83
30 % Digestate of 5% Milk + 95% Vegetables 1:1 + 70% Manure second reactor	23
Digestate of 5% Milk + 95% Vegetables 2:1 first reactor	146
Digestate 5% Milk + 95% Vegetables 2:1 second reactor	174
30 % Digestate of 5% Milk + 95% Vegetables 2:1 + 70% Manure first reactor	20
30 % Digestate of 5% Milk + 95% Vegetables 2:1 + 70% Manure second reactor	88

Table 46: Highest gas production of the fourth experiment two-stage AD

In *Figure 41, 42*, and *43* are reported the cumulative production of methane, carbon dioxide and biogas in mL/gVS:

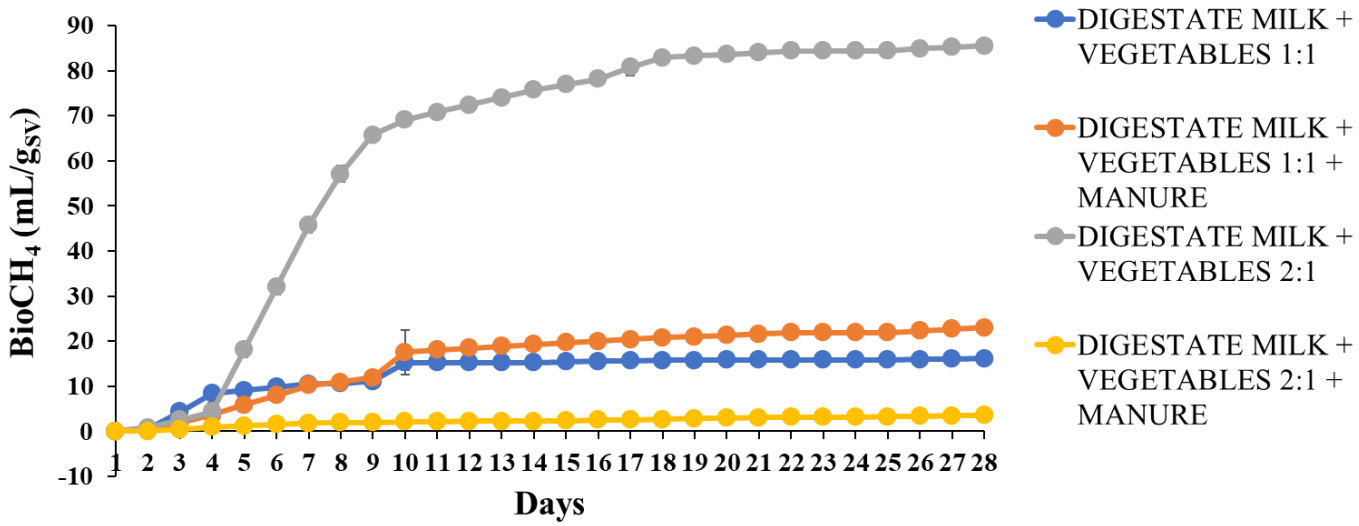

Figure 33: Bio-CH_4 cumulative concentration two-stage AD fourth experiment

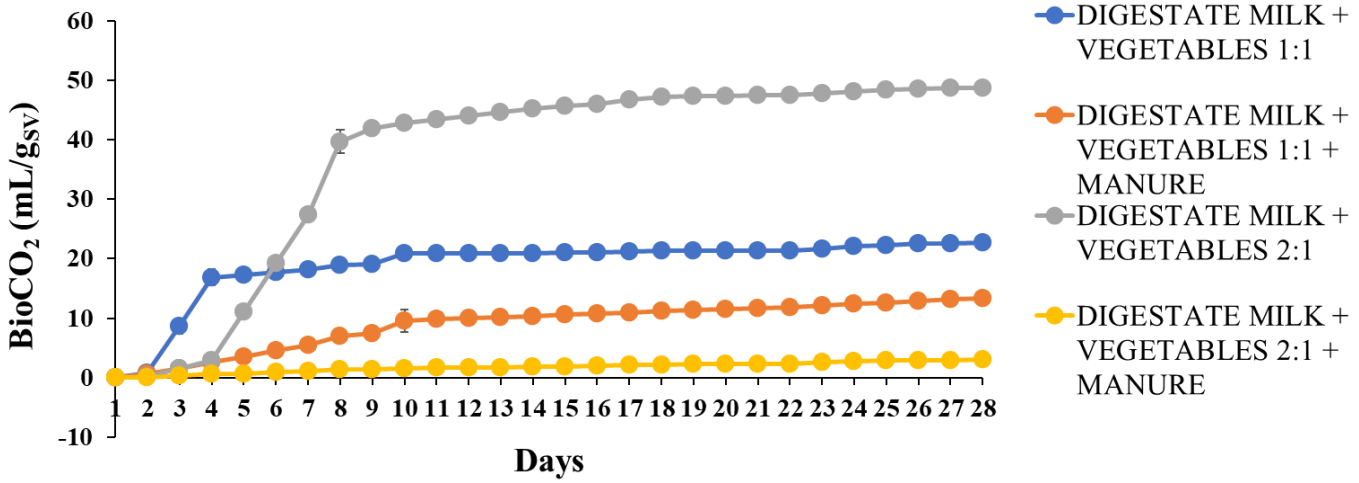

Figure 34: Bio-CO_2 cumulative concentration two-stage AD fourth experiment

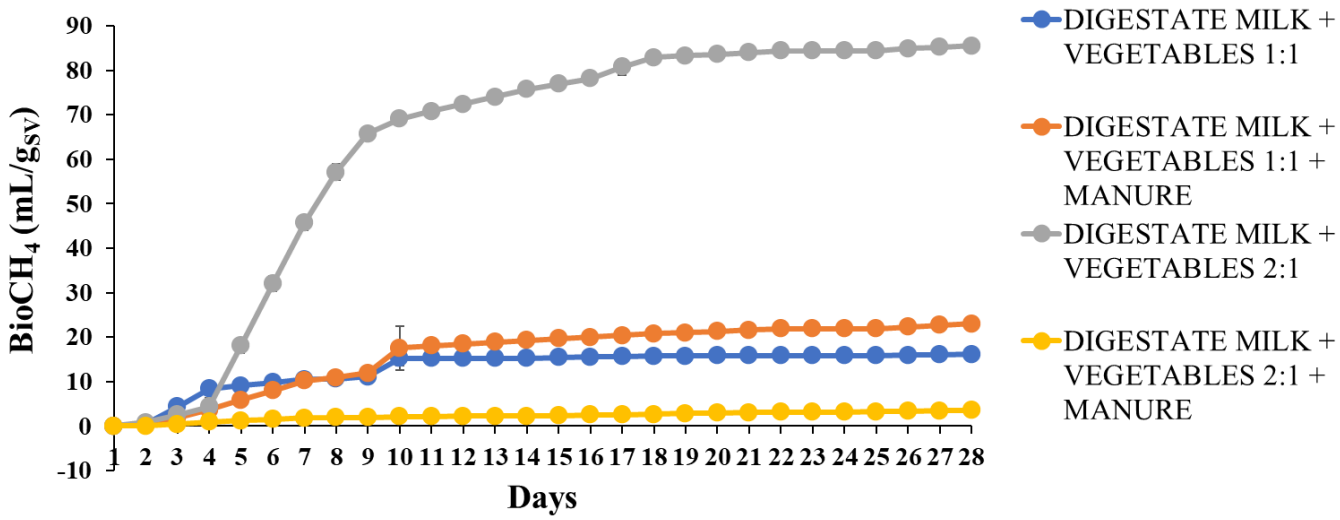

Figure 35:Biogas cumulative concentration two-stage AD fourth experiment

From the results, it can be observed that the highest biogas production is attributed to the configuration containing only milk and plant digestate, without manure, with a biomass-inoculum volatile solids ratio of 2:1. The production is around 270 mL/gVS, while the production of methane is of 85 ml/gVS. The same configuration but with a ratio of 1:1 produced 23 mL/gVS of methane. The configurations that produced the least amount of biogas are those containing manure. In *table 47*, it is possible to observe the maximum yield in terms of gas composition obtained in the different configurations.

Configurations	H_2 (%)	CH_4 (%)	CO_2 (%)
Digestate of 5% Milk + 95% Vegetables 1:1	0.31	16.13	30.16
30 % Digestate of 5% Milk + 95% Vegetables 1:1 + 70% Manure	0.02	48.93	22.09
Digestate of 5% Milk + 95% Vegetables 2:1	0.00	59.58	35.25
30 % Digestate of 5% Milk + 95% Vegetables 2:1 + 70% Manure	0.00	16.15	9.22

Table 47:Table 38: Maximum yields compositions of gas produced (4° experiment two-stage AD)

The *table 48* report the final pH values. In *table 49* and *50* report TS-VS and CHNSO analysis of the digestate from the two-stage process.

Configurations	pH final
Digestate of 5% Milk + 95% Vegetables 1:1	7.46

30 % Digestate of 5% Milk + 95% Vegetables 1:1 + 70% Manure	7.55
Digestate of 5% Milk + 95% Vegetables 2:1	7.20
30 % Digestate of 5% Milk + 95% Vegetables 2:1 + 70% Manure	7.36

Table 48: pH values of configurations from the fourth experiment after double stage

Except for bottles 5 and 6, the pH values are slightly higher than the initial values.

Configurations	TS [%]	Standard Deviation TS (%)	VS/TS [%]	Standard Deviation VS/TS (%)
Digestate of 5% Milk + 95% Vegetables 1:1	5.54	0.54	90.91	2.01
30 % Digestate of 5% Milk + 95% Vegetables 1:1 + 70% Manure	5.93	0.29	84.54	4.16
Digestate of 5% Milk + 95% Vegetables 2:1	3.81	1.70	82.67	4.39
30 % Digestate of 5% Milk + 95% Vegetables 2:1 + 70% Manure	4.97	1.48	83.79	7.26

Table 49: Total solids and volatile solids values of configurations from the fourth experiment after double stage

Configurations	N (%)	Standard Deviation N (%)	C (%)	Standard Deviation C (%)	H (%)	Standard Deviation H (%)	S (%)	Standard Deviation S (%)	C/N ratio
Digestate of 5% Milk + 95% Vegetables 1:1	3.07	16.26	36.18	4.87	4.42	4.31	0.50	1.55	11.76
30 % Digestate of 5% Milk + 95% Vegetables 1:1 + 70% Manure	2.64	9.19	31.37	1.49	3.74	2.42	0.49	0.77	11.86
Digestate of 5% Milk + 95% Vegetables 2:1	2.63	2.12	34.05	1.64	4.37	3.28	0.49	2.051	12.92
30 % Digestate of 5% Milk + 95% Vegetables 2:1	2.60	9.17	30.15	2.12	3.64	0.42	0.54	2.051	11.57

+ 70% Manure									

Table 50: CHNS analysis of digestates from fourth experiment after double stage

6.5 Fifth experiment results

Based on the results of the fourth experiment, it was observed that the configurations with vinasse are not particularly suitable for hydrogen production through the DF process. One of the main reasons is that this type of substrate is a lignocellulosic biomass, making it difficult for microorganisms to digest. Additionally, its polyphenol content, which consists of high molecular weight molecules, does not help, as these, like lignin, are not easily digestible by microorganisms. For this reason, treatments were studied in the fifth experiment to make this substrate suitable for hydrogen production. Three different treatments were performed for the two types of vinasses, for a total of twelve bottles, and dark fermentation was tested for a total of four days.

6.5.1 Dark fermentation results

The highest gas production in mL of the various configurations is reported in *table 51*:

Configurations	mL produced at day 1
10% milk + 90% vinasse 1 treated for 5 min. with ultrasound first reactor	17
10% milk + 90% vinasse 1 treated for 5 min. with ultrasound second reactor	70
10% milk + 90% vinasse 1 treated for 10 min. with ultrasound first reactor	14
10% milk + 90% vinasse 1 treated for 10 min. with ultrasound second reactor	85
10% milk + 90% vinasse 1 crushed with a mixer in H_2O first reactor	15

10% milk + 90% vinasse 1 crushed with a mixer in H_2O second reactor	31
10% milk + 90% vinasse 2 treated for 5 min. with ultrasound first reactor	20
10% milk + 90% vinasse 2 treated for 5 min. with ultrasound second reactor	15
10% milk + 90% vinasse 2 treated for 10 min. with ultrasound first reactor	20
10% milk + 90% vinasse 2 treated for 10 min. with ultrasound second reactor	13
10% milk + 90% vinasse 2 crushed with a mixer in H_2O first reactor	30
10% milk + 90% vinasse 2 crushed with a mixer in H_2O second reactor	14

Table 51:Daily gas production of the fifth experiment

The third day of the experiment, the reactors were opened to check the pH. The check was done to verify if the working conditions were still optimal for dark fermentation. The *table 52* shows the results of the pH check:

Configuration	Starting pH	HCl 2 M added (ml)	pH after HCl
10% milk + 90% vinasse 1 treated for 5 min. with ultrasound first reactor	5,31	0	5,31

10% milk + 90% vinasse 1 treated for 5 min. with ultrasound second reactor	5,34	0	5,34
10% milk + 90% vinasse 1 treated for 10 min. with ultrasound first reactor	5,45	0	5,45
10% milk + 90% vinasse 1 treated for 10 min. with ultrasound second reactor	5,46	0	5,46
10% milk + 90% vinasse 1 crushed with a mixer in H_2O first reactor	6,3	0,5	5,6
10% milk + 90% vinasse 1 crushed with a mixer in H_2O second reactor	6,5	0,5	5,6
10% milk + 90% vinasse 2 treated for 5 min. with ultrasound first reactor	5,76	0	5,76
10% milk + 90% vinasse 2 treated for 5 min. with ultrasound second reactor	5,3	0	5,3
10% milk + 90% vinasse 2 treated for 10 min. with ultrasound first reactor	5,6	0	5,6
10% milk + 90% vinasse 2 treated for 10 min. with ultrasound second reactor	5,4	0	5,4
10% milk + 90% vinasse 2 crushed with a mixer in H_2O first reactor	6,26	0,5	5,6
10% milk + 90% vinasse 2 crushed with a mixer in H_2O second reactor	6,4	0,5	5,4

Table 52: pH value of the bottles from fifth experiment after three days of DF

In *Figure 44*, *45* and *46* are reported the cumulative production of hydrogen, methane and biogas in mL/gVS:

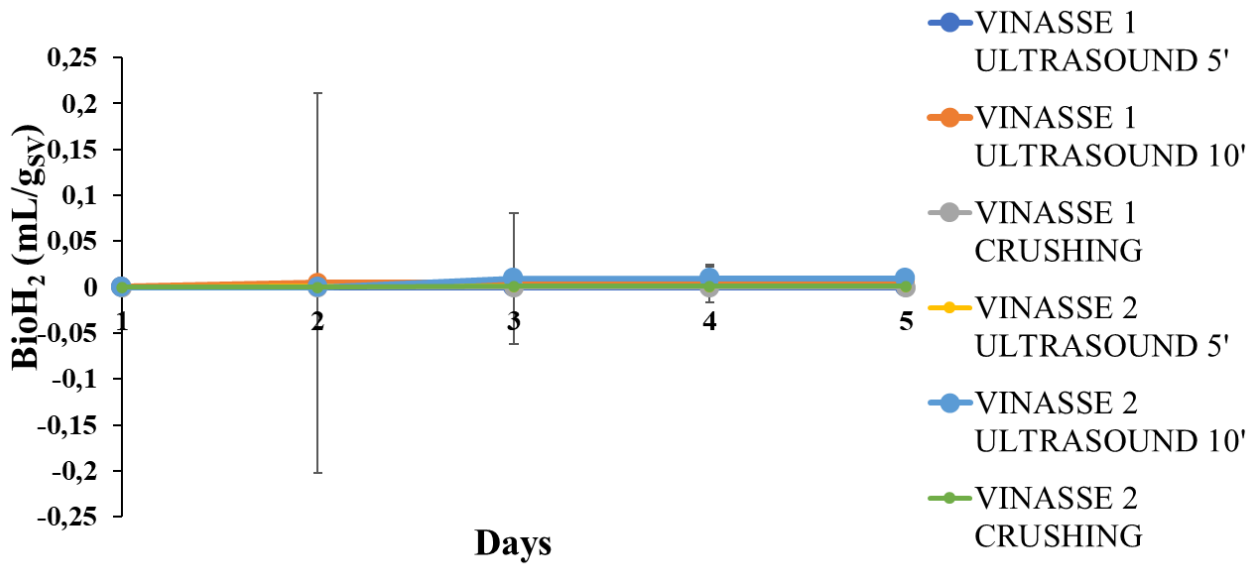

Figure 36:Bio-H_2 cumulative concentration DF fifth experiment

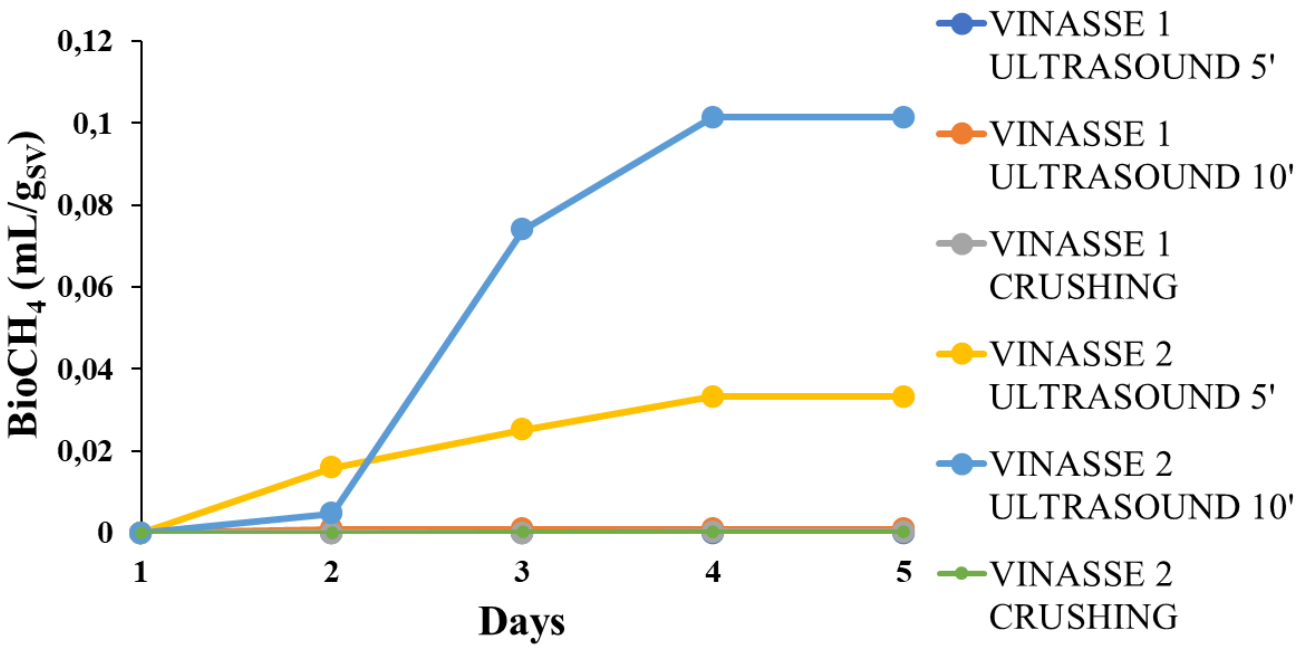

Figure 37:Bio-CH_4 cumulative concentration DF fifth experiment

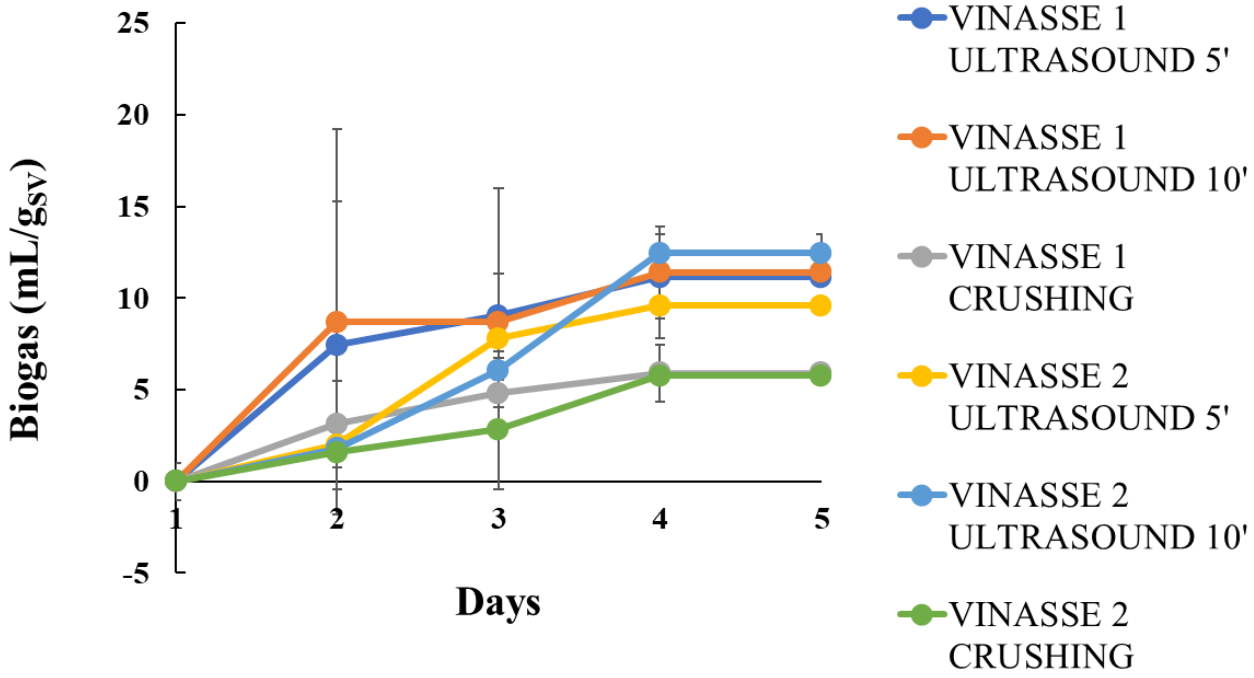

Figure 38: Bio-CH4 cumulative concentration DF fifth experiment

The results show that the production of hydrogen is about 0 mL/gVS. In contrast, in the study of Shen F et al., 14.8 mL/gVS of hydrogen was obtained using grape pomace as a substrate in a 250 mL reactor with a pH of 5.2-7.2 [50].

In *table 50*, it is possible to observe the maximum yield in terms of gas composition obtained in the different configurations.

Configuration	H_2 (%)	CH_4 (%)	CO_2 (%)
10% milk + 90% vinasse 1 treated for 5 min. with ultrasound	0.00	0.00	0.10
10% milk + 90% vinasse 1 treated for 10 min. with ultrasound	0.05	0.01	1.97
10% milk + 90% vinasse 1 crushed with a mixer in H_2O	0.00	0.00	3.73
10% milk + 90% vinasse 2 treated for 5 min. with ultrasound	0.06	0.00	0.80
10% milk + 90% vinasse 2 treated for 10 min. with ultrasound	0.22	0.00	1.61
10% milk + 90% vinasse 2 crushed with a mixer in H_2O	0.10	0.02	2.14

Table 53:Maximum yields compositions of gas produced (5° experiment DF)

In *table 54*, *55* and *56* are reported in order TS-VS, pH and CHNSO analysis:

Configuration	TS [%]	Standard Deviation TS (%)	VS [%]	Standard Deviation VS/TS (%)
10% milk + 90% vinasse 1 treated for 5 min. with ultrasound	5.82	1.06	90.47	6.34
10% milk + 90% vinasse 1 treated for 10 min. with ultrasound	5.50	0.21	94.53	1.27
10% milk + 90% vinasse 1 crushed with a mixer in H_2O	4.51	1.98	87.55	0.13
10% milk + 90% vinasse 2 treated for 5 min. with ultrasound	3.27	0.94	89.05	4.53
10% milk + 90% vinasse 2 treated for 10 min. with ultrasound	4.73	0.50	91.55	4.37
10% milk + 90% vinasse 2 crushed with a mixer in H_2O	2.56	0.28	88.62	5.32

Table 54:Total solids and volatile solids values of configurations from the fifth experiment after DF

Configuration	pH final
10% milk + 90% vinasse 1 treated for 5 min. with ultrasound	5.53
10% milk + 90% vinasse 1 treated for 10 min. with ultrasound	5.22
10% milk + 90% vinasse 1 crushed with a mixer in H_2O	5.89
10% milk + 90% vinasse 2 treated for 5 min. with ultrasound	5.16
10% milk + 90% vinasse 2 treated for 10 min. with ultrasound	4.98
10% milk + 90% vinasse 2 crushed with a mixer in H_2O	5.49

Table 55:pH values of configurations from the fifth experiment after DF

Configuration	N (%)	Standard Deviation N (%)	C (%)	Standard Deviation C (%)	H (%)	Standard Deviation H (%)	S (%)	Standard Deviation S (%)	C/N ratio

10% milk + 90% vinasse 1 treated for 5 min. with ultrasound	3.23	19.09	45.09	2.82	5.67	2.24	0.39	1.06	13.93
10% milk + 90% vinasse 1 treated for 10 min. with ultrasound	3.20	3.67	41.67	6.57	5.25	5.65	0.93	4.31	13.02
10% milk + 90% vinasse 1 crushed with a mixer in H_2O	3.73	5.37	42.45	7.37	5.46	0.95	0.44	5.94	11.38
10% milk + 90% vinasse 2 treated for 5 min. with ultrasound	3.93	5.37	2.33	2.85	3.10	3.71	0.29	4.58	59.53
10% milk + 90% vinasse 2 treated for 10 min. with ultrasound	3.42	17.68	3.68	2.01	4.73	6.15	0.37	11.10	10.74
10% milk + 90% vinasse 2 crushed with a mixer in H_2O	3.77	15.56	3.63	8.04	4.88	1.00	0.46	11.73	9.637

Table 56:CHNSO analysis of digestates from fifth experiment after DF

For this experiment, a COD analysis was also performed, and the results are summarized in *table 54*:

Configuration	Concentration [mg/L]
Barbera not mixed treated with ultrasound for 5 minutes	275.00
Barbera not mixed treated with ultrasound for 10 minutes	211.00
Barbera mixed treated with ultrasound for 5 minutes	221.00
Barbera mixed treated with ultrasound for 10 minutes	259.00
Barbera mixed with water	4.70
Barbera mixed with EtOH	518.00
Nebbiolo not mixed treated with ultrasound for 5 minutes	242.00

Nebbiolo not mixed treated with ultrasound for 10 minutes	77.60
Nebbiolo mixed treated with ultrasound for 5 minutes	218.00
Nebbiolo mixed treated with ultrasound for 10 minutes	254.00
Nebbiolo mixed with EtOH	257.00
Nebbiolo mixed with H_2O	7.06

Table 57: COD analysis results

From the table, it can be observed that in both vinasses, the best treatment turns out to be simple mixing in an 80% v/v EtOH solution. Moreover, in both cases, it can be seen that without premixing, the concentration is higher with ultrasound treatment for 5 minutes. However, when mixing is done before the ultrasound treatment, the concentration is higher if the treatment is performed for a longer duration. Mixing with only water, on the other hand, appears to be less effective. The results shown do not take into account the fact that the solutions are diluted 1:5000.

6.6 Sixth experiment results

Based on the results obtained in the fifth experiment, it was decided to proceed with testing a different treatment, the alkaline one. In addition, the configuration with the ultrasound treatment in an EtOH solution performed for 5 minutes was also tested again. Fermentation was carried out for a total duration of 6 days

6.6.1 Dark fermentation results

The results of the highest gas productions from the various configurations are summarized in *table 58:*

Configuration	Highest gas production collected in gas-bags (mL)
10% milk + 90% vinasse 1 treated for 5 min. with ultrasound first reactor	62
10% milk + 90% vinasse 1 treated for 5 min. with ultrasound	54

second reactor	
10% milk + 90% vinasse 1 treated with NaOH first reactor	51
10% milk + 90% vinasse 1 treated with NaOH second reactor	46
10% milk + 90% vinasse 2 treated for 5 min. with ultrasound first reactor	40
10% milk + 90% vinasse 2 treated for 5 min. with ultrasound second reactor	51
10% milk + 90% vinasse 2 treated with NaOH first reactor	50
10% milk + 90% vinasse 2 treated with NaOH second reactor	55

Table 58: Highest gas production of the sixth experiment

In *Figure 48, 49* and *50* are reported the cumulative production of hydrogen, methane and biogas in mL/gVS:

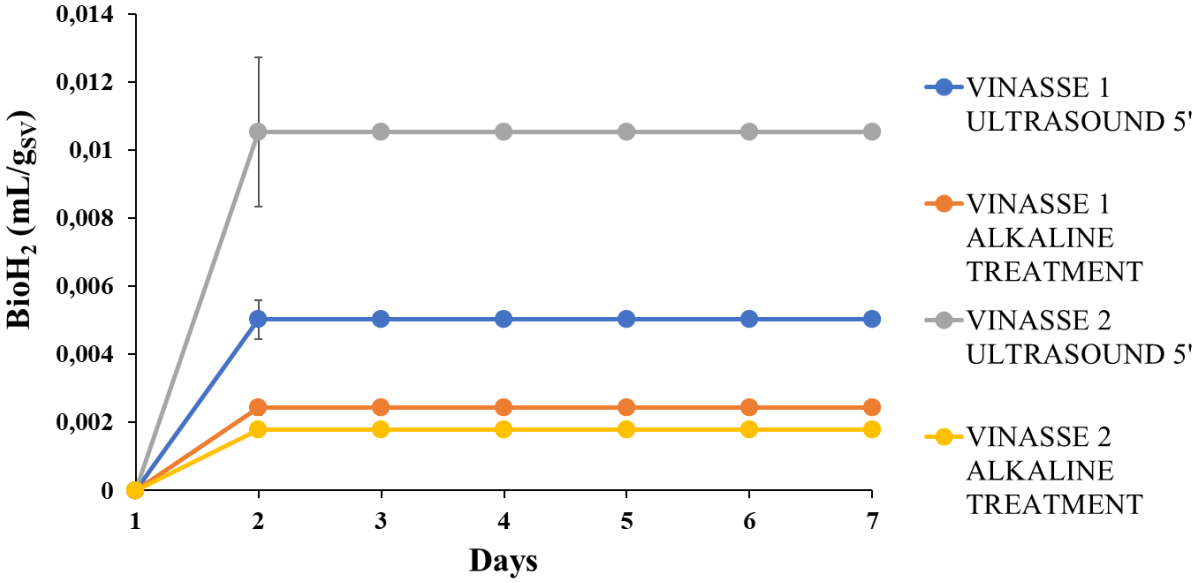

Figure 39:Bio-H_2 cumulative concentration DF fifth experiment

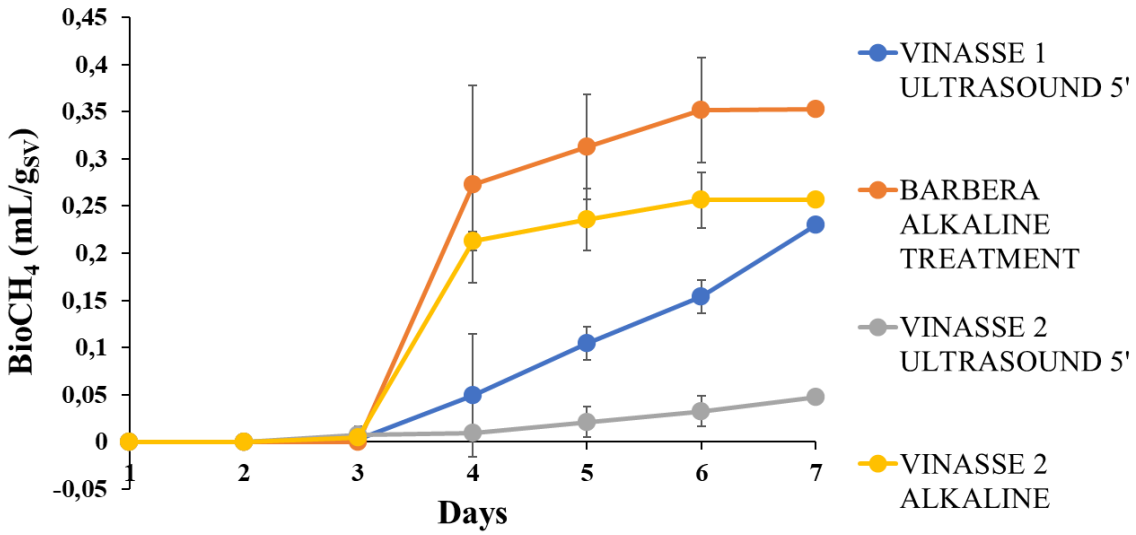

Figure 40:Bio-CH_4 cumulative concentration DF fifth experiment

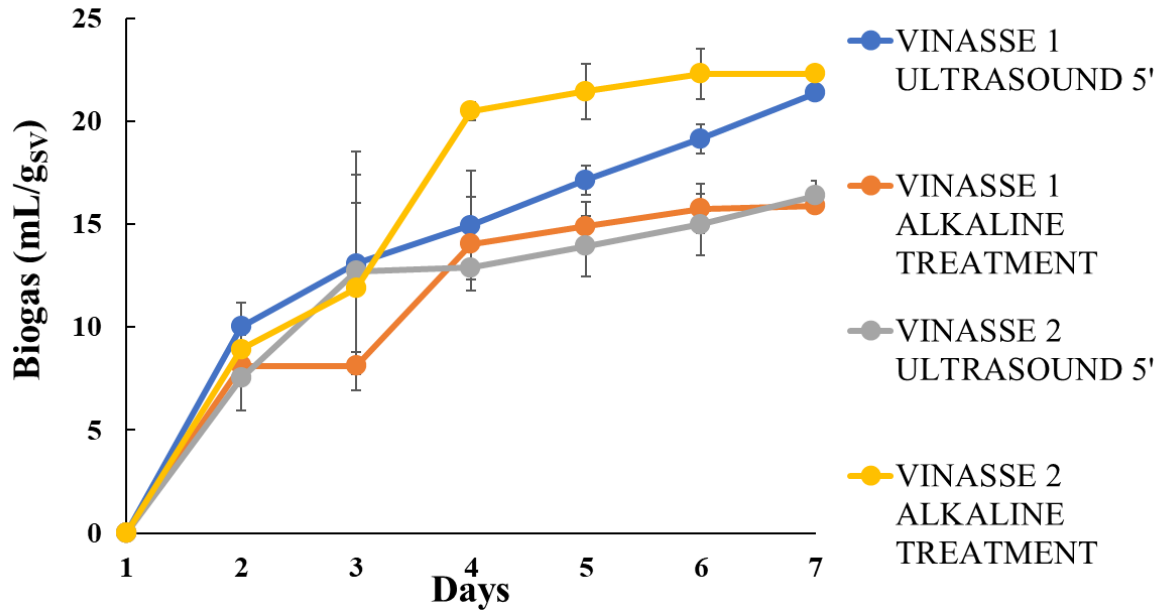

Figure 41:Biogas cumulative concentration DF fifth experiment

As can be observed from the results, during the dark fermentation process hydrogen was not produced. Also, methane was not produced. In contrast, in the study of Shen F et al. (2013), 14.8 mL/gVS of hydrogen was obtained using grape pomace as a substrate in a 250 mL reactor with a pH of 5.2-7.2 [50].

In *table 59*, it is possible to observe the maximum yield in terms of gas composition obtained in the different configurations.

Configurations	H_2 (%)	CH_4 (%)	CO_2 (%)
10% milk + 90% vinasse 1 treated for 5 min. with ultrasound	0.05	2.48	10.46
10% milk + 90% vinasse 1 treated with NaOH	0.03	4.61	11.39
10% milk + 90% vinasse 2 treated for 5 min. with ultrasound	0.14	1.09	9.16
10% milk + 90% vinasse 2 treated with NaOH	0.02	2.41	4.69

Table 59:Maximum yields compositions of gas produced (5° experiment DF)

In *table 60*, it is possible to observe the results of the TS-VS analysis of the digestates. The *table 61* and *62* shows pH and CHNSO analysis.

Configurations	TS (%)	Standard Deviation	VS (%)	Standard Deviation

		TS (%)		TS/VS (%)
10% milk + 90% vinasse 1 treated for 5 min. with ultrasound	2.90	0.07	86.95	2.60
10% milk + 90% vinasse 1 treated with NaOH	2.82	0.13	89.27	3.15
10% milk + 90% vinasse 2 treated for 5 min. with ultrasound	3.74	0.10	89.12	6.88
10% milk + 90% vinasse 2 treated with NaOH	2.16	0.30	80.26	4.97

Table 60: Total solids and volatile solids values of configurations from the sixth experiment after DF

Configurations	pH final
10% milk + 90% vinasse 1 treated for 5 min. with ultrasound	6.47
10% milk + 90% vinasse 1 treated with NaOH	6.33
10% milk + 90% vinasse 2 treated for 5 min. with ultrasound	6.12
10% milk + 90% vinasse 2 treated with NaOH	5.93

Table 61: pH values of configurations from the sixth experiment after DF

Configurations	N (%)	Standard Deviation N (%)	C (%)	Standard Deviation C (%)	H (%)	Standard Deviation H (%)	S (%)	Standard Deviation S (%)	C/N ratio
10% milk + 90% vinasse 1 treated for 5 min. with ultrasound	3.30	8.49	38.00	4.85	4.84	5.21	0.54	1.66	11.51
10% milk + 90% vinasse 1 treated with NaOH	3.34	0.71	33.31	1.22	4.49	11.31	0.53	1.21	9.96
10% milk + 90% vinasse 2 treated for 5 min. with ultrasound	3.27	18.38	42.70	2.60	5.25	2.52	0.49	5.94	13.06
10% milk + 90% vinasse 2 treated with NaOH	3.59	4.95	35.38	0.60	4.69	0.84	0.78	2.61	9.84

Table 62: CHNS analysis of digestates from sixth experiment after DF

6.7 comparison with the literature

The first two experiments do not align with the results obtained in the study conducted by Xiao Wu et al.,[46] where with a similar reactor configuration and an initial pH of 7.5, combining manure with other agro-industrial waste, they were able to achieve 135 mL/gVS of hydrogen. In the third experiment, it was possible to obtain 50 mL/gVS of methane from the manure-only configuration through the anaerobic digestion process, which slightly differs from the 77 mL/gVS obtained in the study conducted by L. Kalsum et al. [47]. In the third experiment, it was possible to achieve a production of 50 mL H_2/gVS through the DF process in a system with an OLR of 16 kgVS/m^3d, using a configuration containing dairy waste and plant waste. A similar result was obtained in the study of Weronika Cieciura-Włoch, where 52 mL H_2/gVS was produced through the DF of plant waste and an inoculum taken from a wastewater treatment plant, with an OLR of 17 kgVS/m^3d [49]. In the third and fourth experiments, the two-stage anaerobic digestion process conducted on configurations containing dairy and plant waste resulted in 80-100 mL CH_4/gVS in systems with an OLR of 1-2 kg VS/m^3d. In the study of Shen F et al., plant waste was processed using two-stage anaerobic digestion in systems with an OLR of 1-5 kg VS/m^3d, yielding 198–546 mL CH_4/gVS, which is therefore higher than in this thesis study [50]. For the last two experiments, despite the different pretreatments performed on the vinasses, it was not possible to obtain hydrogen through the dark fermentation process. In contrast, in the study of Shen F et al., 14.8 mL/gVS of hydrogen was obtained using grape pomace as a substrate in a 250 mL reactor with a pH of 5.2-7.2 [50].

7. Conclusion

The following study investigated the hydrogen and methane yields from various lignocellulosic biomasses derived from the agro-food industry. The first experiment highlighted that, despite a thermal treatment of 1 hour and 15 minutes for the inoculum and manure in an oven at 80°C, the manure configurations combined with other wastes, including vegetables and milk, did not allow for hydrogen production through the DF process. The second experiment demonstrated that the same configurations, using an acid treatment instead of thermal, did not result in hydrogen production exceeding 3 mL/gVS. These hydrogen yields, in both the first and second experiments, were low, leading to the conclusion that manure is not capable of producing hydrogen through the DF process. Conversely, in the third experiment, manure combined with milk waste resulted in 250 mL/gVS of methane being produced over 34 days through an anaerobic digestion process. In experiment three, the milk and vegetable waste configuration produced 50 mL/gVS of hydrogen through DF over 3 days, followed by an additional 100 mL/gVS of methane through a two-stage AD process. Another trial using the same configuration, but with a volatile solids ratio of biomass

to inoculum of 2:1, produced 17 mL/gVS of hydrogen and 80 mL/gVS of methane through the two-stage process. The final two trials showed that vinasses, despite different pretreatments, was unable to produce hydrogen through the DF process when combined with milk waste. Future developments involve researching and optimizing various operating conditions to achieve higher yields of hydrogen and methane. The first operational parameter concerns the total solids content in the system. Adjusting the percentage could be explored to see if better results can be achieved. Another parameter is pH. All operational systems were started with a pH of around 7, staying slightly below the optimal level for DF to prevent the process from self-inhibiting due to fermentation byproducts. Therefore, the optimal pH value can be targeted, with daily monitoring and correction as needed using a base. Further studies could focus on using different pretreatments for both the inoculum and biomass to try to achieve higher hydrogen and methane yields, same for vinasses.

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