Anaerobic digestion of effluents and subproducts from a biorefinery manufactury for biogas production

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Abstract

This study intends to assess the viability of anaerobic digestion as a sustainable approach for treating wastewater and subproducts originating from the biodiesel refining industry. The biorefinery plants generate large amounts of glycerol, soaps, and free fatty acids as primary subproducts. The experimental work used 500 mL digesters inoculated with thermophilic sludge, typically characterized by its ability to digest substrates with a high organic and lipid content. Two series of experimental tests were carried out, based on the Taguchi's experimental design methodology: the first with thermophilic and the second with mesophilic inoculum. The statistical analysis identified one of the five parameters, parameter D, as the most influential factor considering the interactions between substrates. The experimental results corroborate the potential of anaerobic technologies to produce considerable quantities of biogas from the digestion of these feedstocks, reporting COD removal efficiencies up to 80% and a maximum production volume of biomethane verified for experiment X5 over 42 days with 300 mL useful volume and a I/S ratio of 1. In the mesophilic series, the absence of an acclimatization period for the inoculum, coupled with potential organic overload, resulted in nearly six-fold lower biomethane production compared to the thermophilic reactors. The mathematical model exhibited limitations in accurately predicting reactor behavior, particularly during the exponential phase, especially for cocktail mixtures containing substrate D and the foremost contributing factor is the absence of additional calibration and validation of the model's constants. Nevertheless, the model proved to be a valuable tool for the estimation of cumulative biomethane potential levels once the system reaches its plateau phase.

Keywords: Anaerobic Digestion, Biogas, Glycerin, Sustainability, Mathematical Modelling.

1. Introduction

Rapid industrialization and population growth have led to increased waste generation and energy demand [2]. These challenges stem from the significant growth in energy demand, shifting from traditional biomass to fossil fuels. Dispatchable energy sources, requiring minimal preprocessing, are essential in this context. This need for dispatchable energy sources presents a significant hurdle in replacing fossil fuels, as they offer practical advantages during peak demand scenarios. Substantial efforts have been directed toward developing renewable energy sources, with biofuels at the forefront. Biofuels, derived from biological feedstocks, are viewed as sustainable energy alternatives. However, as of 2022, biofuels represent a relatively small share, constituting less than 8% in the industrial sector and 4% in the transportation sector, which remains largely dominated by oil. This work explores the potential of converting biorefinery wastewater and subproducts into energy via anaerobic digestion, aligning with Waste-to-Energy (WTE) sustainability goals. It includes lab experiments to measure biomethane potential and calibrating kinetic constants for a predictive mathematical model of the system's behavior.

2. Background

Anaerobic Digestion:

Anaerobic digestion is a sequence of biological processes through which bacteria and yeast convert organic matter into biogas in the absence of oxygen. The reaction takes place in a digester, that can either be fed continuously or in batch. The temperature regime is usually mesophilic (20-45°C) but in some cases, it might justify having thermophilic conditions (45-75°C) although it increases the heating demand of the plant [29]. One of the biggest advantages of this technology is the flexibility concerning the feedstock since anaerobic digestion can receive as an input a variety of substrates starting with the simpler form of sugars like glucose till more complex examples such as sewage sludge, animal manure, and kitchen waste. Contrarily to other biological processes, AD makes it possible to valorize both alcohols and fatty acids, converting them into biogas through a series of sequential steps where

initially the lipids are broken down and converted into volatile fatty acids, hydrogen, and acetate to be used in methanogenesis for biogas production.

Biogas is mainly composed of methane (CH_4) and carbon dioxide (CO_2), typically around 50-70% and 30-50%, respectively [29]. Yet, depending on the feedstock used for the digestion, some other impurities have been reported to exist at low concentrations in biogas such as H_2S, NH_3, H_2, N_2, O_2, CO, siloxanes, and water vapor. Biogas can be used as a fuel and burnt directly in furnaces, producing flue gas that can be expanded in a turbine and converted into electrical energy or it can be upgraded to biomethane so that impurities and the CO_2 are removed, increasing the percentage of methane up to 99%, turning it into an incredible replacer for fossil natural gas with the possibility of grid injection or to be used as a greener fuel for combustion.

Mathematical Model:

Optimizing digester design and prediction remains challenging. Mathematical models offer a solution to mitigate uncertainty, reduce experimental costs, and improve efficiency. These models simulate biological reactions and kinetic effects in anaerobic environments. Lab-scale experiments provide input data for model training and constant adjustment.

The Anaerobic Digestion Model No. 1 (ADM1), developed by the International Water Association (IWA) Task Group, is a preferred model for co-digestion scenarios. It consists of ordinary differential equations, balancing mass in the process and it can be often generalized by Equation 1.

$$\frac{dS_i}{dt} = \frac{q_{in} \cdot S_{in}}{V} - \frac{q_{out} \cdot S_{out}}{V} + \sum \rho_j \nu_{i,j} \qquad (1)$$

Where S_i is the soluble concentration of substrate, S_{in} is the initial concentration of the soluble substrate, S_{out} is the final concentration of the soluble substrate, q_{in} and q_{out} are the inflow and outflow, respectively, V is the volume of the digester, and $\sum(\rho_j\ \nu_{i,j})$ the sum of the biochemical rate coefficients ($\nu_{i,j}$) and kinetic rates (ρ_j) [6, 12].

An adaptation of ADM1 was developed by students at students at the Instituto Superior Técnico - Lisboa, including modifications to account for the processing of mixtures of aggregated organic matter as an input parameter. This model accepts an initial COD concentration of undifferentiated organic matter, quantified in grams per liter of O_2, and depending on the specific biochemical rate coefficients, the organic matter undergoes disintegration into carbohydrates, proteins, lipids, inert components, and a newly introduced compound termed 'alcohols [27, 4].

Incorporating glycerol as a co-substrate into the model required the introduction of a novel parameter accounting for both glycerol and methanol. This adaptation was grounded in the premise that both compounds undergo decomposition into VFAs. It was further supported by the minimal methanol concentration within the reactors, which rendered it inconsequential in the context of kinetic considerations. To refine the model for preliminary biochemical methane potential estimation, a new kinetic rate constant ($k_{hyd,alc}$) was introduced, along with an additional biochemical rate coefficient to accommodate alcohol formation. In addition, carbon dioxide (CO_2) was considered as a product of lipid hydrolysis and an essential component in methanogenesis. The model was subsequently calibrated and executed using a set of kinetic rate constants drawn from pertinent literature, in conjunction with the standard biochemical rate coefficients inherent to the model.

3. Microbial Thresholds

The study aims to evaluate the viability of anaerobic digestion for plant effluents and subproducts. Substrates containing glycerin and soapstock subproducts are found to have favorable COD content, and their performance can be improved through composition optimization. A series of experimental assays were conducted to investigate the effects of different substrate combinations.

In the context of biodiesel production, glycerol, a predominant byproduct, and the use of natural gas in transesterification have spurred interest in harnessing anaerobic digestion for biogas production from glycerol. Glycerol, with a high COD content ranging from 1.0 to 2.4 $g{\cdot}L^{-1}$, is considered a suitable co-substrate for anaerobic processes [7, 22]. Administering glycerol at concentrations higher than 1% (v/v) led to a significant decrease in pH, inhibiting methane production [14]. Applying glycerol at 3% (v/v) inhibited microbial activity, but stability was maintained with 1% (v/v) glycerol [16]. For semi-batch studies on co-digestion of pig manure with glycerol, it was found that 1% (v/v) pure glycerol concentration ensured stable performance inside the reactor, while concentrations of 1.75% and 3.5% (v/v) led to system collapse [28]. A drastic decrease in methane production and pH instability was observed when using 3% (v/v) glycerol in co-digestion, while 1% (v/v) glycerol resulted in increased removal efficiency and improved methane production [24]. To ensure optimal system performance, an a% (v/v of useful volume) glycerol concentration was employed as a reference in the experiments, subject to confidentiality.

In anaerobic digestion, pH strongly affects microbial activity. Microorganisms have varying enzymatic performance at different pH levels. Neutral pH (around 7) is optimal for efficient enzymatic reactions. Acidogenic bacteria can adapt to a wide pH range (4.0 to 8.0), while methanogens prefer pH values between 6.5 and 7.5. To maximize biogas production, maintaining pH within the range of 6.8 to 7.4, starting at pH 7, is

recommended [30, 10].

Maintaining a balance between organic load and microbial diversity is essential for efficient anaerobic digestion. The I/S ratio drives biochemical reactions, and acclimatizing the inoculum is crucial for efficiency. To prevent organic overload and system failure, the I/S ratio should be equal to or greater than 1 for less biodegradable substrates [5]. Additionally, co-digestion offers the advantage of supplying various nutrients, supporting optimal microbial growth through nutrient solutions containing macronutrients and micronutrients.

4. Experimental Work

Experimental design:

As an exploratory essay, experimental results are essential to be compared against the literature. One of the objetives of this work was to study the effect of different cocktails to be used as a substrate in a digester. Owing to the number of parameters (P = 8) and levels (L = 2) with two extra levels for two of the parameters, a direct and intuitive experimental design would require around 128 assays to study each change individually, turning it into an expensive trial. For that reason, Taguchi's method, a fractional factorial design approach, was adopted to draw the experimental map of the conjugations to short the number of experiments.

Taguchi's orthogonal array method is extensively utilized in biotechnology to enhance experimental efficiency by identifying optimal combinations of controllable design factors that minimize performance variability [18]. This method relies on orthogonal arrays, systematic matrices that facilitate comprehensive coverage of all possible combinations, thus significantly reducing the required number of experiments [1]. For each parameter, all levels of the other parameters can be efficiently tested [9]. Only five substrates were selected either due to their cost implications for the company or low commercial revenues. For instance, in a case with five parameters and two levels, only eight experiments are needed according to Taguchi's method [9].

In accordance with the findings in Section 3, the system's ability to digest increased quantities of substrate A is contingent on the inoculum's adaptation to the anaerobic environment and culture medium. Given the initial exploration at a 1% (v/v) fixed percentage, an investigation into the impact of parameter A at higher concentrations, specifically 3% and 5% (v/v), has been initiated, resulting in two additional assays.

Similarly, recognizing the distinctive chemical properties of substrate B, experiments involving elevated concentrations of this substance have been introduced to assess the toxicity of its components. Consequently, the design matrix now includes two additional experiments featuring 3% and 5% (v/v) of parameter B, replacing two others that were deemed less pertinent, as their outcomes could be obtained from control and blank experiments.

Table 1: Taguchi's orthogonal array used for this experiment - adapted. Five parameters (A, B, C, D, and E) and four levels (level 1: not present; level 2: present at a fixed concentration; level 3 and level 4: present at higher fixed concentrations).

Taguchi: Adjusted for P = 5, L = 4 (1,2,3,4)

Run	P1 - A	P2 - B	P3 - C	P4 - D	P5 - E
X1	2	2	2	2	2
X2	2	2	2	1	1
X3	2	1	1	2	2
X4	2	1	1	1	1
X5	1	2	1	2	1
X6	1	2	1	1	2
X7	1	3	2	2	1
X8	1	4	2	1	2
X9	3	2	2	2	2
X10	4	2	2	2	2

Taking into consideration the Taguchi's matrix for the experimental design and based on the literature data on inhibitory concentrations and advisable ratios, ten combinations (X1-X10) were created plus four controls (C1-C5) and one blank assay (Blank).

BMP Expectations:

In the initial phase of the study, the maximum theoretical methane volume achievable from various substrates, including glycerol, oleic acid, glucose, proteins, and methanol was calculated using the chemical reactions based on the degradation of organic molecules into CH_4 and CO_2. Methane production was modeled in anaerobic conditions, considering substrate degradation. The calculations were based on 1 gram of each substrate, and the resulting methane volumes were determined using the ideal gas equation at standard conditions (303 K and 1 atm). Table 2 presents projected biomethane yields for experimental assays.

Secondly, as mentioned in Section 2, a mathematical model was chosen to describe both the process and biological behavior of a reactor during anaerobic digestion. When compared to theoretical calculations, the dynamic model comes with great advantages since it considers not only side reactions as the kinetics and microbial behavior, making it much more realistic and accurate. The model was adapted to include an additional input representing an alcohol group (glycerol and methanol), resulting in four inputs measured in $kgCOD{\cdot}m^{-3}$. For reasons explained previously, the values estimated by the model are lower than the ones suggested by the theoretical chemical equations, yet, these results come with considerable imprecision when compared to reality. Because of that, a third and more accurate approach was via laboratory experiments with 500 mL anaerobic reactors, working at batch regime, that was loaded with the different organic cocktails

understudy.

Table 2: Theoretical biogas and methane volume production in liters of the experiments.

	Units	X1	X2	X3	X4	X5
V_{CH_4}	L	6.26	3.18	4.81	1.73	1.34
Yield CH_4		65%	61%	67%	61%	71%
	Units	**X6**	**X7**	**X8**	**X9**	**X10**
V_{CH_4}	L	4.23	3.77	5.63	6.19	9.10
Yield CH_4		71%	67%	68%	60%	60%

Experimental Set-up:

The sixteen batch digesters operated at an average room temperature of 32.4°C for up to 42 days, following the setup proposed in the literature [15]. The setup included two glass bottles, one with 500 mL and the other with 1 L of a 2% NaOH solution, along with intravenous catheters, solution administration sets, and a graduated cylinder, as shown in Figure 1. During testing, challenges arose, such as valve irregularities causing variations in bubble generation speed, overflow of the NaOH solution, and reflux back into the reactor, potentially lowering pH levels. The final configuration, as depicted in Figure 1, featured a reactor bottle atop a support structure to act as a non-return valve. In some cases, a 1 L glass bottle replaced the measuring cylinder to prevent solution spillage and data loss. One-third of the valve apertures remained open to control the bubbling rate, ensuring an extended reaction period between carbon dioxide (CO_2) and sodium hydroxide (NaOH).

Figure 1: Experimental setup - reactor bottles (on the top of the table), inverted bottle filled with NaOH solution (packed in colorful nylon nets), measuring cylinders or empty bottles as recovery system.

In each digester, it was added a substrate cocktail, inoculum stored at 4°C for two weeks, adjusted the pH to 7 with NaOH, and occasionally included a nutrient source. The substrate cocktails were mixed for homogeneity, and the pH was regulated to 7 with NaOH. Despite the ideal anaerobic reactor environment, certain microorganisms, such as *Bacillus subtilis*, *Clostridium*, *Lactobacillus*, and *Methanosarcina*, can tolerate small oxygen amounts. This oxygen presence can prevent ammonia formation and enhance early-stage growth of these facultative microorganisms, as reported in the literature [20, 26]. To facilitate this, the reactors were not flushed with inert gas. Instead, they were sealed the bottles with butyl rubber stoppers and connected to a gas collection system via a solution administration set and a 14G intravenous catheter.

The experiments were shut down upon observing that the daily methane production had diminished to less than 1% of the cumulative total production up to that point, following the stopping criteria established by Holliger in one of his works, as demonstrated in Equation 2. All experiments were stopped based on this criterion, except for experiment C1, which was prematurely concluded due to an accidental return of the sodium hydroxide solution occurring inside the reactor [8].

$$\frac{V_{CH_4,i} - V_{CH_4,i-1}}{V_{CH_4,i-1}} < 0.01 \tag{2}$$

Based on the information discussed in Sections 2 and 3, a set of variables were fixated for the experimental design shown in Table 3.

Table 3: Fixed variables used for the experiment.

Fixed Variables	Value	Units
Temperature	30-35	°C
I/S	1	v/v
Reactor Volume	500	mL
Working Volume	300	mL
Head Space	40	%
Manual Stirring	2	min
Starting pH	7	

Five parameters were studied: the effect on anaerobic digestion of glycerol, soaps, methanol, bagasse, and free fatty acids. For a 500 mL reactor with a useful volume of 300 mL and an I/S of 1, meaning 150 mL of inoculum added to each reactor, a total of sixteen experiments were conducted and are described in Table 4, where it is visible that substrates A and B are varied from 0% to 5% (v/v), the feedstock D, when administered is added at a fixed concentration of 4% (v/v), the same for the compound F at 30% (v/v), C at 0.2% (v/v) and E at 1% (v/v). Regarding the wastewater from the extraction unit, this one will be used as a solvent to make up the 300 mL of working volume. The initial and final COD of each sample were determined by COD cuvette tests LCK914 - [5-60 $gO_2 \cdot L^{-1}$] and LCK014 - [1,000-10,000 $mgO_2 \cdot L^{-1}$] from Hach. The procedure was followed according to the protocol provided within the kits using a digester model LT200 - Hach and a portable spectrophotometer model DR2800 - Hach. The pH values of each mixture was also registered at the beginning and end of the experiments. The pH was determined by using color-fixed pH test strips from Macherey-Nagel.

Table 4: Substrate combinations for the assays in % (v/v).

	A	B	C	D	E	F	G	Inoculum	V_{work}
Assay	% (v/v)							mL	mL
X1	1	1	0.2	4	1	30	13	150	300
X2	1	1	0.2	-	-	30	18	150	300
X3	1	-	-	4	1	30	14	150	300
X4	1	-	-	-	-	30	19	150	300
X5	-	1	-	4	-	30	15	150	300
X6	-	1	-	-	1	30	18	150	300
X7	-	3	0.2	4	-	30	13	150	300
X8	-	5	0.2	-	-	30	15	150	300
X9	3	1	0.2	4	-	30	12	150	300
X10	5	1	0.2	4	-	30	10	150	300
C1	1	-	-	-	-	-	49	150	300
C2	-	1	-	-	-	-	49	150	300
C3	-	-	-	-	-	30	20	150	300
C4	-	-	-	4	-	-	46	150	300
C5	-	-	-	-	1	-	49	150	300
Blank	-	-	-	-	-	-	50	150	300

Biogas production measurement:

An indirect approach was employed to measure biogas production due to the experimental setup's simplicity. The method involved biogas washing through CO_2 and H_2S absorption in an alkaline solution. The biogas primarily consists of methane and carbon dioxide, with traces of hydrogen sulfide. When this biogas contacts a NaOH solution, CO_2 reacts with NaOH to form sodium carbonate (Na_2CO_3). In cases of sodium hydroxide exhaustion, a secondary reaction between CO_2 and Na_2CO_3 may produce sodium bicarbonate ($NaHCO_3$) [17].

Methane volume was quantified using a liquid displacement method, with the drained solution volume corresponding to the methane produced. This approach is valid because a 99.7% removal of H_2S and CO_2 was confirmed with a 2% NaOH solution. Although higher concentrations like 4% and 10% NaOH can achieve 100% removal efficiency, they were not used to prevent equipment corrosion [17, 19]. Additionally, the carbonate solution with excess NaOH was titrated using a 0.5 M HCl solution to verify the alkaline solution's effectiveness. The neutralization occurs in two steps, with the first step involving the formation of sodium chloride (NaCl) and sodium bicarbonate ($NaHCO_3$) at a pH of 8.3, suitable for phenolphthalein as an indicator, which changes color in the pH range of 8.0-10. The second step is the neutralization of bicarbonate with HCl at a pH between 3-4, aligning with the changing region of the methyl orange indicator [13].

In terms of calculations, following the methodology described by Crossno, the moles of CO_2 absorbed by the washing solution can be determined using Equation 3. Here, ΔV represents the difference between the volumes of HCl utilized in the initial and subsequent endpoint titrations. After establishing the moles of CO_2, the application of the "Law of Ideal Gases" permits the estimation of the corresponding volume of carbon dioxide within the biogas. Consequently, this allows for an estimation of the methane yield of the biogas produced [11].

$$n_{CO_2}\ (\text{mol}) = \Delta V\ (\text{L}) \times MW_{HCl}\ (\frac{\text{mol}}{\text{L}}) \qquad (3)$$

The calculation of biogas volume at time (i) involves summing the methane and carbon dioxide volumes produced from time (i-1) to time (i). Assuming the cleaning solution functions as expected, the volume of gas within the inverted bottle represents the methane volume produced and is equal to the volume displaced into the cylinder.

The experiment was carried out over a period of up to 42 days, and the volume collected in the cylinder was measured and titrated every two days. To ensure a consistent substrate solution and to prevent the formation of fat layers at the reactor's surface while facilitating the release of gases, the reactors were stirred manually every two days for two minutes. The room temperature was kept between 31.6°C and 33.3°C using an air conditioning system. Temperature levels were constantly monitored via a TR300 Amprobe data logger. Two additional direct measurements were performed on each reactor using an SKF thermal camera model TKTI 10, and the values proved that the temperature of the bottles was concordant with the room temperature.

5. Results

Taguchi's Statistical Analysis:

The experimental trial was structured according to Taguchi's matrix methodology to systematically curtail the number of reactor assemblies under study, thereby precluding direct pairwise comparisons among individual assays. Subsequently, an initial statistical analysis was performed employing the MiniTab 21 Statistical software to determine the primary influencing factors, such as feedstock selection, that showed greater influence and augmented system variability.

Figure 3 reveals the rank attributed to each parameter studied in this experiment concerning Signal-to-Noise ratio (S/N) and means. In this context, the substrates under study are called parameters, the signal is related to the mean value of the output results for the different trials with identical input conditions and noise represents the standard deviation between the results obtained from the different combinations. The criterion "Larger the better" was adopted for this experiment since the main goal of this work was to optimize biomethane production. Thus, it is possible to see that, parameter A is the most significant factor of the system, followed by parameters D, B, C, and E[1]. This implies that the manipulation of parameter A introduces variability into the system, which is quantified by a corresponding delta value. In instances where

[1]All the parameters are disclosed on the Confidential File.

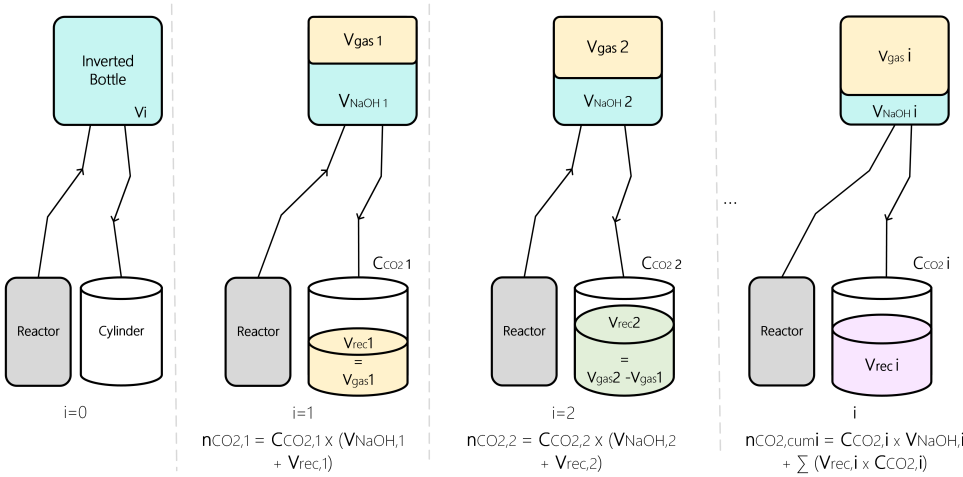

Figure 2: Schematic illustration of the CO_2 calculation reasoning.

a parameter is correlated with a heightened degree of variability, it is prudent to select lower values of this factor for subsequent experiments rather than opting for higher values to assure system stability.

Through a concise analysis of the S/N ratio graph, it is possible to identify the optimal levels for each parameter which are as follows: 5% (level 4) for parameter A, 1% (level 2) for parameter B, 0% (level 1) for parameter C, 4% (level 2) for D and 0% (level 1) for E. Yet, the flatness of the slopes shown for parameters C and E evidence that the system is barely affected by the variation of these factors, at least for the levels studied during this experiment. Such large variability observed for parameters A, B, and D indicates that these three factors should be further evaluated in subsequent studies, with an emphasis on modeling their behaviors.

In Taguchi methods analysis, the S/N ratio is a crucial indicator used to assess the performance of a process or system. The S/N ratio is typically used to optimize processes by maximizing it for desired outcomes or minimizing it for undesired outcomes.

Signal-to-Noise

Level	A	B	C	D	E
Delta	11.8295	5.2115	2.5469	6.4581	2.0409
Rank	1	3	4	2	5

Means

Level	A	B	C	D	E
Delta	2.4267	2.1299	1.3213	1.9380	1.2304
Rank	1	2	4	3	5

Figure 3: Response tables for Signal-to-Noise for "Larger the better criteria" [top] and Means [bottom], from MiniTab.

For the context of this problem, employing the "Larger the better" criteria for the optimal levels of each parameter, enables the software to estimate, that the maximum volume of biomethane possible to be produced would be 13.54 L. This value closely aligns with the result obtained in experiment X5, indicating that the experimental process is performing well in terms of the response variable under study.

It is suggested that a robust and comparative analysis is performed to assess the robustness of this optimal combination to variations or changes in factors and to compare the optimal combination to other factor settings to quantify the improvement and assess the statistical significance. Further experiments could be conducted to study the sensitivity of the system and to ensure that the process remains robust.

Experimental Data Analysis:

· **Thermophilic regime**

The cumulative production of both methane and carbon dioxide of the cocktail experiments (X1-X10) is presented in Figure 4 and Figure 5, respectively. The results for the controls can be found in Figure 6. The results in Table 5 indicate significantly higher CH_4 yields in the control experiments, ranging from 66% to 77% (v/v). This improvement in anaerobic digestion efficiency is likely due to the microorganisms' ability to adapt quickly to a simpler culture medium without oxygen. In contrast, complex mixtures with potential indigestible compounds prolong the adaptation period. Therefore, an acclimatization phase prior to feeding the inoculum with complex mixtures could enhance biogas production and prevent reactor failure, bringing the yields closer to the literature range of 50-80% (v/v) [23].

Experiment X6 yielded the lowest COD removal efficiency, with only 16% removed and 14% of that matter converted to methane. This indicates an unsuitable cocktail mixture for X6. Most experiments, except X1, X5, and control experiments C2 and C3, also had low COD removal, likely due to system overload caused by an inaccurate initial I/S ratio of 1. To prevent such issues in future studies, it's advisable to use an I/S ratio of 2, ensuring stable and optimal reactor conditions for improved performance and reliable results.

The 42-day temperature profile did not achieve thermophilic conditions, with a maximum of 33.3°C, significantly below the required range. Further analysis

of the microbial population might reveal partial mortality and adaptation to mesophilic conditions, aligning with the adaptability of facultative thermophiles. This partial microbial population loss not only reduces biodiversity but also contributes to the imbalanced I/S ratio, potentially explaining the low COD removal efficiencies observed.

Table 5: Experimental results of the thermophilic experiments plus controls. Volumes for methane and carbon dioxide are presented normalized. The absolute values can be found on the Confidential File.

	Start		End				
Assay	**COD** $g \cdot L^{-1}$	**pH**	**COD** $g \cdot L^{-1}$	**pH**	$\eta COD_{removal}$ %	V_{CH_4} L/L	V_{CO_2} L/L
X1	127.6	7.0	25.6	5.5	80	0.13	0.64
X2	81.8	7.0	45.6	6.0	44	0.04	1.00
X3	147.2	7.0	57.0	5.0	61	0.14	0.85
X4	60.4	7.0	31.6	6.5	48	0.05	0.60
X5	108.8	7.0	18.0	8.0	83	1.00	0.73
X6	61.8	7.0	52.1	-	16	0.04	0.66
X7	108.0	7.0	60.4	7.0	44	0.08	0.40
X8	167.6	7.0	61.2	-	63	0.09	0.62
X9	141.2	7.0	60.9	5.0	57	0.10	0.69
X10	187.9	7.0	86.4	5.0	54	0.29	0.69
C1	46.0	7.0	16.3	13.0	65	0.03	0.14
C2	29.0	7.0	18.6	7.0	36	0.19	0.29
C3	36.8	7.0	10.1	-	73	0.11	0.09
C4	85.5	7.0	15.2	8.0	82	0.13	0.16
C5	47.2	7.0	25.0	8.0	47	0.20	0.24
Blank	24.2	7.0	12.9	8.0	47	0.11	0.16

Experiments X2, X4, and X6 had methane yields below 21%, suggesting underperformance of methanogenic microorganisms. These experiments lacked feedstock D, identified as a positive influencer of biomethane production. Experiment X8 also lacked feedstock D but increased substrate B fivefold. While this mitigated some issues, X8's methane yield at 29% still falls significantly short of ideal biogas composition and literature values. The control group's absence of substrate D promoted favorable conditions for carbon digestion and increased methane production due to the simple cocktail composition and reduced organic loading. In contrast, experiments X2, X4, X6, and X8, with complex substrates and high organic loading, may have caused system saturation and collapse. These inhospitable conditions restricted microorganisms to basic fermentation, leading to alcohol and carbon dioxide formation, which can inhibit microbial activity at high levels [3].

For pH levels, all experiments began at pH 7 but experienced fluctuations during the digestion process. Sharp deviations from the optimal pH range could lead to operational failures. In X6, X8, and C3, final pH measurements were precluded by external factors, potentially leading to inconclusive results. Experiments X1, X3, X9, and X10 ended with a pH of 5 due to non-metabolized substances. X2 and X4 also showed pH declines, possibly due to feedstock A. Control C1 had an unexpected pH of 13 due to a solution leakage. In contrast, X5, C4, and C5 had a final pH of 8, possibly due to feedstock D alongside substrate C, leading to increased ammonia production. Another reason for the pH increase might be the efficient conversion of LCFAs and VFAs into methane, as these experiments had high methane production levels. Unfortunately, the pH of experiment X8 was not measured due to external circumstances, preventing a direct correlation with substrates.

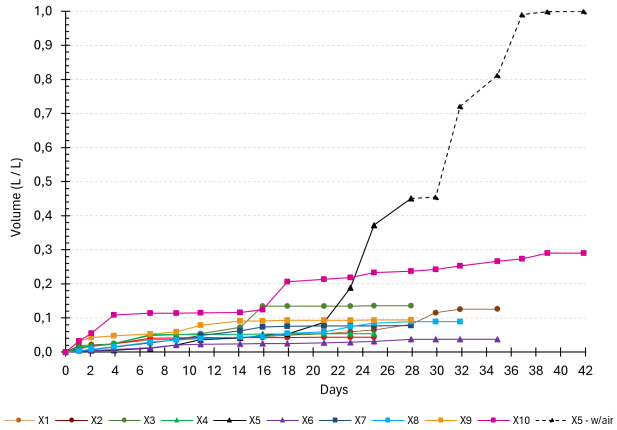

Figure 4: Cumulative CH_4 production for the thermophilic experiments - normalized.

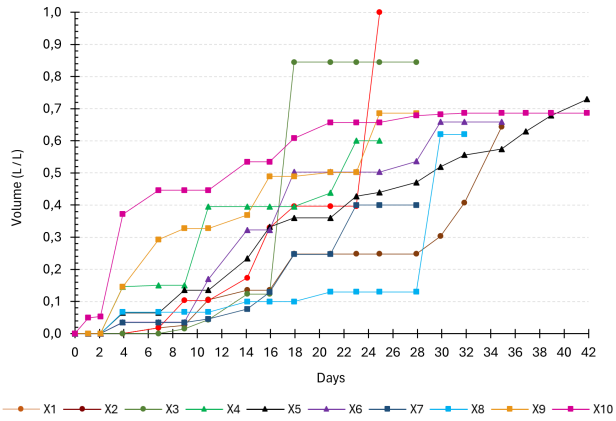

Figure 5: Cumulative CO_2 production for the thermophilic experiments - normalized.

In experiment X5, an unexpected methane production increase occurred around day 36 after an initial stabilization phase in anaerobic digestion. The cause was an operational issue with the reactor's needle attachment, which began to loosen around day 28, potentially allowing oxygen entry. Some microorganisms can switch to aerobic metabolism in the presence of oxygen. When the system was resealed, anaerobic conditions were restored, restarting the digestion process and creating a new baseline up to day 42. Between days 28 and 30, the reactor may have briefly operated as an open system, allowing external air entry, supported by the flat slope between days 28 and 30, showing zero methane production. Upon fixing and resealing the system, excess air raised internal pres-

sure, resulting in a sharp spike in methane production. However, this spike may not represent actual methane production but rather air displacement to restore equilibrium, supported by the nearly linear behavior of the carbon dioxide production curve over 42 days, indicating consistent methane production levels.

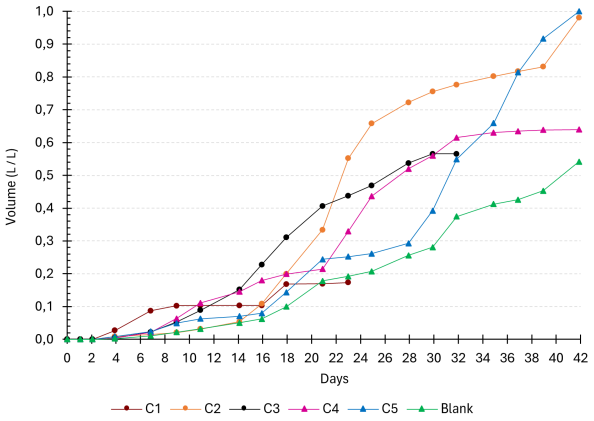

Figure 6: Cumulative CH_4 production for the thermophilic control experiments - normalized.

Experiments with substrate C, within a similar initial COD concentration range, exhibited lower carbon dioxide production and higher methane yields than those without it. This suggests that adding substrate C to the anaerobic reactor could promote methanogenic pathways, in line with existing literature.

· **Mesophilic regime**

The mesophilic trials included the most promising cocktail mixtures, specifically X3, X5, and X10. See Figure 7 for cumulative methane output for the mesophilic reactors. Contrary to expectations, mesophilic methane production was lower than thermophilic production due to the origin of the mesophilic inoculum, sourced from a lower COD wastewater facility. The superior thermophilic performance stemmed from an inoculum adapted to higher COD loads, although some thermophilic microbes may have shifted to mesophilic pathways. A visual difference in reactor configuration between thermophilic and mesophilic experiments was observed at the beginning of the mesophilic experiment, as seen in Figure 8 comparing experiments X3 and X5. Notably, a visible phase separation was apparent in mesophilic conditions, potentially indicating challenges for microorganisms in substrate access and digestion.

While some experiments achieved COD removal efficiencies of up to 80%, none reached a final effluent COD concentration below 1,500 mg·L^{-1}, the legal limit for wastewater discharge according to SIMTEJO regulations [25]. To meet this limit, a COD removal efficiency exceeding 98% is required, surpassing reported literature efficiencies, which are below 91% [21]. Large-scale applications will necessitate an integrated approach, potentially involving a secondary reactor or additional stages in a wastewater treatment plant, to achieve the required COD reduction and meet regulatory standards.

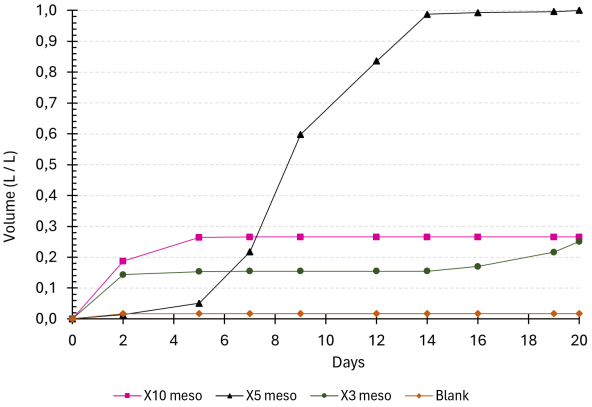

Figure 7: Cumulative CH_4 production for the mesophilic experiments - normalized.

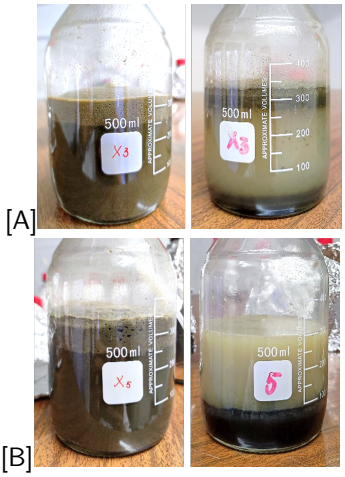

Figure 8: [A] - Reactor X3 thermophilic - viscous solution with an emulsified soap stopper on top [left]. Reactor X3 mesophilic - phase separation with fat floating on top and solids deposit on the bottom [right]. [B] - Reactor X5 thermophilic - viscous solution with an emulsified soap stopper on top, larger than in X3 [left]. Reactor X5 mesophilic - phase separation with solids deposit on the bottom [right].

Dynamic model fitting and validation:

The model calibration process used Microsoft Excel's Solver tool to minimize differences between model predictions and experimental cumulative methane production volumes up to a specific day. Biochemical coefficients for seven substrates were considered, but the focus was on six substrates (sludge, A, B, C, E, and F). Substrate D, too complex, was excluded from the optimization, which was conducted for experiments X2, X4, X6, and X8, as they did not contain substrate D. The Solver tool was used for extensive computational work to find the best constants and coefficients matching laboratory results. The optimization outcomes, determined by Solver, are detailed in the Appendix, with further experiment-specific discussions to follow.

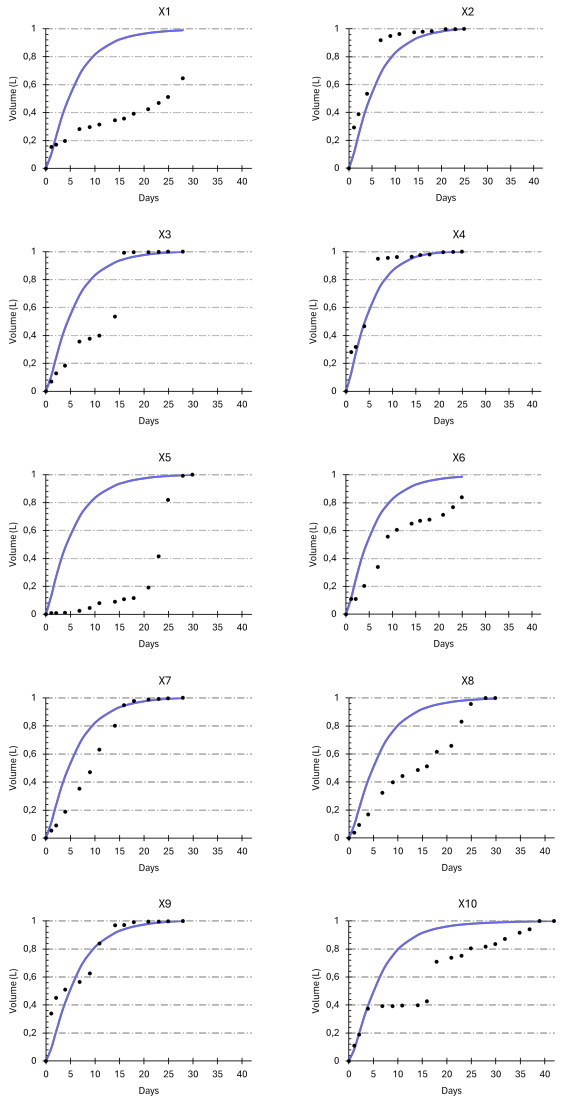

Figure 9: Fitting of the model predictions for the cumulative volume of methane produced experimentally. Model - blue solid line. Experimental data - black dots.

In this optimization step, we began with the coefficients and constants in Table A.1-1. Data from sixteen cocktail reactors over 42 days was integrated into an Excel spreadsheet. Each experiment had a separate column to calculate the absolute deviation between observed and model-derived methane production at each time point (i). A shared cell calculated the mean deviation across all columns. The Solver tool was then used to minimize this global mean deviation by adjusting the model's constants and coefficients based on input data from X0 to X5 concentrations (in kgCOD·m^{-3}). Initially, the optimization focused on kinetic rate equations (ρ_j) and biochemical rate coefficients ($\nu_{i,j}$) for compound degradation, detailed in Table Table A.1-2.

Figure 9 shows cumulative methane production based on the new model constants and coefficients. It's important to note that the model doesn't closely match the experimental data. This is expected due to the absence of additional optimization and sensitivity analysis in the modeling process and the intentional omission of substrate D from the simulation.

For experiments X3, X8, and X10, the model aligns closely with experimental data at the curve's end, but discrepancies occur in the exponential phase. The model accurately estimates final cumulative methane production for these experiments. In contrast, for experiments X1 and X5, where substrate D has a significant organic load contribution, the model falls short, as substrate D is deliberately excluded from the simulation. Experiments X2, X4, X7, and X9 exhibit near-perfect alignment between the model and experimental data, suggesting that in the absence of substrate E, substrate D has minimal influence on the system. However, when both feedstocks, E and D, are introduced into the reactors simultaneously, as in experiments X1 and X3, the model's accuracy is compromised.

6. Conclusion

The study investigates waste valorization technologies to address company challenges. Anaerobic digestion emerged as the most promising method. Experimental trials were conducted with company-specific feedstock, and the resulting data was used to adjust a numerical model for biomethane production.

The experimental trial was conducted systematically, following Taguchi's matrix methodology, identifying key factors, with parameter A having the most impact. The "Larger the better" criterion guided the optimization for maximum biomethane production, leading to specific parameter values that should be explored further.

This study corroborates the viability of producing substantial quantities of biogas through the anaerobic digestion of wastewater and sub-products derived from the biodiesel refining industry. Remarkably, an average volume of 1.7 L of biomethane was generated from only 150 mL of feed mixture, resulting in an output approximately eleven times greater than the input volume. The observed biogas quality exhibited an average CH_4 yield of 46%, closely aligning with the 50% value reported in the literature for untreated feedstocks. While certain experiments achieved COD removal efficiencies of up to 80%, none met the legal discharge limit. This implies the need for additional treatment stages or reactor adjustments to meet regulatory standards.

The mesophilic experiments yielded lower-than-expected methane production due to the inoculum's poor adaptation to high COD loads. Conversely, despite operating outside the optimal temperature range, the thermophilic experiments outperformed, likely owing to the thermophilic microbial consortium's adaptability to higher COD concentrations and increased lipid content.

The calibration of the dynamic model was a crucial step, involving the optimization of kinetic rate equations and biochemical rate coefficients. Substrate

D was omitted from the simulation due to complexity, and the optimization process took approximately 8 hours. However, for experiments without substrate E, D, and/or C, the model exhibited high accuracy and proved to be a valuable tool for predicting system behavior. Nevertheless, in most experiments, the model successfully predicted the maximum and final biomethane volumes attainable by each system, thus serving as a useful tool for estimating cumulative biomethane production levels once the anaerobic system reaches its plateau phase.

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