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Optimization of Liquid-State Anaerobic Digestion by Defining the Optimal Composition of a Complex Mixture of Substrates Using a Simplex Centroid Design

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Abstract: This study aimed to define the optimal composition of three heterogeneous substrates of the anaerobic digestion process to maximize methane production. The investigated substrates were sewage sludge (SS), the organic fraction of municipal solid waste (OFMSW), and horse waste (HW). The optimal composition of these substrates was defined using the mixture design and, more specifically, the simplex-centroid mixture design. Customized methods and materials were employed to study the complex mixture design of these substrates. The findings revealed that the optimal mixture involved all three substrates with the composition 0.17 HW, 0.66 SS, and 0.17 OFMSW, which demonstrated the highest methane yield at $269 \text{ NmL} \cdot \text{gVS}^{-1}$. In addition, a mathematical model was developed to predict methane production based on a specific composition of co-substrates. The results were validated at the small pilot scale.

Keywords: liquid-state anaerobic digestion; organic fraction of municipal waste; horse waste; mixture design; optimization



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1. Introduction

Due to the scarcity and cost of fossil fuels, the energy sector has been compelled to embrace alternative methods of energy production [1,2]. New policies and shifts in behavior have been implemented to embrace renewable energy technologies, aiming to mitigate the adverse environmental effects associated with fossil fuel usage. The increasing demand for energy alternatives has motivated countries to enhance existing technologies, develop novel solutions, and explore diverse feedstocks capable of generating energy, such as agricultural waste, municipal waste, animal dung, sewage sludge, and industrial effluents [3]. These biodegradable resources can be converted to biogas, a valuable renewable energy resource, by the anaerobic digestion process [4].

Anaerobic digestion is a natural biological process with a series of stages, where biomass is decomposed and stabilized in the absence of O_2 [5]. Various types of anaerobic microorganisms are involved in the conversion of biomass into a renewable energy resource called biogas, primarily consisting of methane and carbon dioxide. This biogas serves as a viable substitute for fossil fuels and has applications in generating heat and electricity [6,7]. The anaerobic digestion (AD) process can be distinguished into various types depending on many factors. The total dry matter content determines whether the AD process is

liquid-state or solid-state. Liquid-state AD is when the dry matter is less than 15%, and solid-state AD is when the dry matter is more than 15% [8,9].

The liquid-state anaerobic digestion (LSAD) process remains the predominant choice for most biogas plants [10,11]. This technology offers numerous advantages due to a high water content, including the homogenization of the digester's contents, enhanced interaction between microorganisms and substrates, minimized diffusion issues, and reduced potential for inhibitory effects [12,13]. LSAD also has some drawbacks. These include the requirement for a large volume of water, an imbalance between the biogas produced and the volume of the reactor, and the need for a substantial amount of substrates to generate a significant volume of gas, resulting in high costs when purchasing and operating large reactors [14].

The anaerobic digestion of biowaste can be carried out on an individual substrate, known as mono-digestion, or by simultaneously processing a combination of substrates, referred to as co-digestion. Initially, AD was primarily intended for mono-digestion. However, research has revealed that co-digestion of multiple substrates can positively impact biogas production [15–17]. Co-digestion facilitates the efficient breakdown of various substrates, assists in odor and pathogen management, and promotes environmentally friendly practices [18]. The optimal proportion of substrates remains largely unexplored, especially when considering complex co-substrates.

Among the widely embraced statistical optimization approaches, response surface methodology (RSM) stands out. RSM utilizes mathematical and statistical techniques to enhance various processes. A subset of RSM, known as the mixture design, specializes in defining the optimal composition ratios of different ingredients of a mixture [19]. In most earlier studies, the selection of substrate proportions was random.

However, certain research endeavors have delved into strategies for enhancing AD by concentrating on the composition of co-substrates using the mixture design. Ref. [20] employed mixture design to investigate the interactions among three substrates and determine the optimal combination for maximizing biogas production using two sets of substrates. The initial set consisted of SS, cow dung, and garden waste, while the second set consisted of SS, cow dung, and fruit juice wastewater. Ref. [21] investigated the biogas production performance at 35 °C, focusing on methane yield as well as reducing the chemical and biological oxygen demand. They conducted these studies using a mixture of cow manure and cheesy whey. Ref. [22] applied a four-factor mixture simplex–centroid design to investigate a combination of four substrates, which included solid cattle slaughterhouse waste, manure, diverse crops, and municipal solid waste. They focused their study on two key responses: methane yield, and the specific methane production rate. Ref. [23] studied the interactions between two wastewater sludge samples, grease trap waste, and food processing waste originating from a meat processing plant. Ref. [24] investigated two mixture sets: the first consisted of poultry droppings, sugarcane bagasse, and press mud, while the second set consisted of poultry droppings, press mud, and sugar beet roots and tops. They applied the augmented simplex–centroid design to study the interactions between substrates. Ref. [25] used the mixture design to study the interactions between piglet manure, cow manure, and starch wastewater. Ref. [26] examined the co-digestion process of sheep's blood, cheese whey, and cow manure by monitoring biogas production. Ref. [27] evaluated the performance of the anaerobic digestion of bovine slaughterhouse wastewater streams and manure. They focused on methane yield and kinetics to evaluate the process performance. In their 2022 paper, [28] included an extra step in their study of the optimization of the anaerobic digestion process. They combined the determination of the optimal composition of two substrates—namely, cattle manure and damp grass—with two critical operational parameters: the immersion rate, and recirculation frequency. They accomplished this through the utilization of a mixed-response design and bootstrapping techniques.

The mixture design has been widely considered to be a promising and effective method to determine the proportions of variables in a mixture. Furthermore, this method enables users to minimize the number of experiments and identify the interactions between the

different ingredients, as well as their effects on the results [29]. Mixture design experiments have achieved widespread usage and demonstrated satisfactory results in determining the optimal compositions of multiple ingredients. Several papers have studied the anaerobic digestion of agricultural waste.

It has been reported in the literature that horse waste poses challenges due to its abundance, odor generation during storage, and potential water source contamination upon land application; therefore, farmers are subject to higher costs to handle this waste [30,31]. The horse stable in the commune of “Maisons-Laffitte” (France) produces horse waste composed of 99% wheat straw and 1% horse manure. In addition, the organic fraction of municipal solid waste produced in France is 20.1 million tons [32]. The high rate of production of these waste products in France and the proximity of their providers to SIAAP (the syndicate responsible for the sanitation of wastewater in the greater Paris area) have led to a collaboration that aims to explore innovative approaches to managing and adding value to these waste products alongside wastewater.

However, there is limited knowledge of the synergistic and antagonistic effects resulting from combining three complex and heterogeneous substrates such as horse waste, wastewater, and the OFMSW. In addition, no data have been reported on identifying the optimal mixture of these substrates. This knowledge gap underscores the need for further research to understand the interactions between these substrates and to optimize biomethane production.

Therefore, this was a unique study that aimed to define a methodology to find the best mixture of three complex and heterogeneous substrates, including horse waste, the organic fraction of municipal waste, and mixed sewage sludge, and to investigate their combined effects. The challenges posed by the complexity of these substrates, particularly in terms of handling, were resolved using an experimental design model at the laboratory scale. In addition, we created a mathematical model to predict the optimal combination of up to three substrates using the mixture design approach (more specifically, the simplex-centroid mixture design).

2. Materials and Methods

2.1. Substrates

Different types of waste were used in this study. Horse waste, composed of 99% wheat straw and 1% horse manure, was obtained from a horse stable in Maisons-Laffitte, France (Eq'invest). The organic fraction of municipal solid waste originated from a metropolitan household waste agency (Syctom, Paris, France). Solid substrates were homogenized and dried at a temperature of 105 °C for 24 h prior to the experiments. Then, they underwent size reduction by passing them through a shredder equipped with a 5 mm sieve. This aimed to prevent clogging in the ducts and hindering of the mixing process caused by the adherence of the particles to the helices.

Urban sewage sludge (mixed sewage sludge) was obtained from a municipal wastewater treatment plant (SIAAP, Achères, France). The inoculum was a digested sewage sludge originating from the same wastewater treatment plant (WWTP)—more specifically, from a full-scale anaerobic digester operating under mesophilic conditions.

The main characteristics of the substrates are given in Table 1 below.

Table 1. Characterization of the substrates used in this study.

	TS (%RM *)	VS (%TS)	VFA (g·kg ^{−1})	TA (g·kg ^{−1})	BMP ** (NmL·g _{MV} ^{−1})
Wheat Straw (99% of the Horse Waste)	50 ± 18	80.7	0.122 ± 0.007	-	266 ± 4
Horse Manure (1% of the Horse Waste)	21.1	68.7	3.6 ± 0.2	-	
OFMSW	45.7	64	41.8 ± 0.4	-	250 ± 11
Sewage Sludge	3.4 ± 0.3	79.1 ± 8	1.5	1.6 ± 0.2	242 ± 12

Notes: * RM: raw matter; ** BMP: biochemical methane potential.

Analytical Testing

Various analytical parameters were determined for all substrates at the beginning and end of the AMPTS experiments (see below), and twice a week for the semi-continuous pilot digesters. For the total solids (TS) content, the samples were subjected to a 24 h heating process at 105 °C, in accordance with the NFISO 11465 standard [33]. The volatile solids (VS) content was then determined by calcining the dried samples at 550 °C for 2 h, following the NF U44-160 standard [34]. The total alkalinity (TA) measurement was carried out by titration with a standard acid solution, in accordance with the NF EN ISO 9963-1 standards [35]. Finally, the volatile fatty acids (VFAs) produced during the AD process, such as acetate, propionate, and butyrate, were analyzed using ion-exclusion chromatography coupled with conductivity detection [36].

For the 10 L digesters, a flow meter (BPC μ Flow: standalone gas flow meter, Bioprocess Control, Sweden) measured the biogas flow every 30 min. A dual-wavelength infrared analyzer (SWG 100 Biogas Compact Analyzer, Gruter & Marchand, Nanterre, France) measured the quality of the biogas every 2 h. While biogas typically comprises methane (CH_4) and carbon dioxide (CO_2), this investigation only focused on methane production, and we explored how various operational conditions influence its generation.

2.2. Mixture Design Methodology

The mixture design approach enables the identification of the optimal combination of components with a minimal number of experiments [37]. In this study, a specific type of mixture design, the simplex–centroid design, was used. This approach discerns interactions among various components and assesses the impact of both individual components and their interactions on the measured experimental responses. Therefore, it can identify the optimal mixture that retains the desirable characteristics of each raw material while eliminating undesirable ones [38,39]. This tool allowed us to define the best composition of OFMSW, SS, and HW to produce the maximum yield of methane.

To define the best combination of these three substrates, an augmented simplex–centroid mixture design was used [40].

The experimental points based on the mixture design are represented in Figure 1. Points 1, 2, and 3 or A, B, and C correspond to horse waste, sewage sludge, and OFMSW alone, respectively. Points 4, 5, and 6 correspond to binary combinations positioned at the midpoints of the three sides of the triangle. Point 7 represents the central point of the centroid and was replicated five times in order to define the pure error and compare it to the lack of fit from the ANOVA in the mixture design. Finally, points 8, 9, and 10 are the three augmented points that represent ternary combinations and serve as control points.

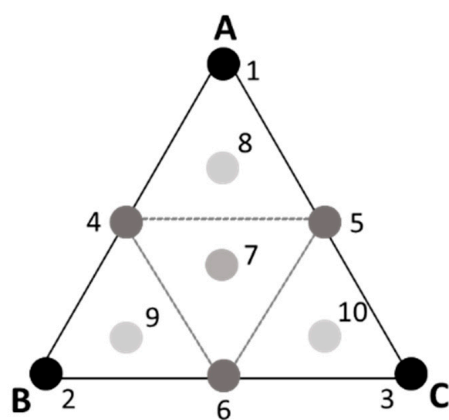


Figure 1. An overview of the augmented simplex–centroid mixture design for a three-component mixture (A: horse waste; B: sewage sludge; C: OFMSW. Points 1 to 10 correspond to different compositions of substrates, as defined in Table 2 below).

Table 2. Mix response design data for the laboratory-scale experiment.

Experimental Point	Composition of Substrates (Based on the Volatile Solids Content)		
	Horse Waste (HW)	Sewage Sludge (SS)	Organic Fraction of Municipal Solid Waste (OFMSW)
1	1.00	0.00	0.00
2	0.00	1.00	0.00
3	0.00	0.00	1.00
4	0.50	0.50	0.00
5	0.50	0.00	0.50
6	0.00	0.50	0.50
7	0.33	0.33	0.34
8	0.66	0.17	0.17
9	0.17	0.66	0.17
10	0.17	0.17	0.66

Based on Figure 1, each experimental point is associated with a specific composition of substrates and is represented in Table 2 below.

2.3. Experimental Setup

2.3.1. Digesters

AMPTS

Biochemical methane potential (BMP) tests provided an estimation of the biodegradability rate of the substrates and their biomethane potential. The experiments were conducted in an automatic methane potential test system (AMPTS II), designed and manufactured by Bioprocess Control (BPC, Lund, Sweden). This system can visualize methane production and kinetics.

The inoculum used was a digested sewage sludge taken from the WWTP (SIAAP, Achères, France). For each experimental point, a homogeneous mixture of the appropriate fraction of each substrate and volume of inoculum was prepared and added to the 500 mL bottle, leaving a 50 mL headspace. The digesters were then placed in a thermostatic water bath to create mesophilic conditions with a temperature of 37 °C (Aqualine AL 18, LAUDA, Lauda-Köninghofen, Germany).

During the anaerobic digestion process, the generated biogas was directed into a CO₂ trap, which captured CO₂ and only allowed the methane to be measured by the flowmeters. The methane production was monitored until the daily production of methane was less than 1% of cumulative production for three consecutive days [41].

Ten-Liter Digesters

Two 10 L digesters made of stainless steel (CSTR-10S, Bioprocess Control, Lund, Sweden) were operating in parallel under mesophilic conditions (37 ± 1 °C). The temperature was kept stable through a double-walled structure with thermostatically regulated water recirculation. The digesters were continuously mixed by internal propellers through mechanical stirring. The temperature and pH were also monitored in the system.

Initially, the digesters, presented in Figure 2, were fed with digested sewage sludge originating from a full-scale mesophilic anaerobic digester used to process municipal sludge at 37 °C. This sewage sludge served as an inoculum, providing the microorganisms responsible for the degradation of the substrates later on.

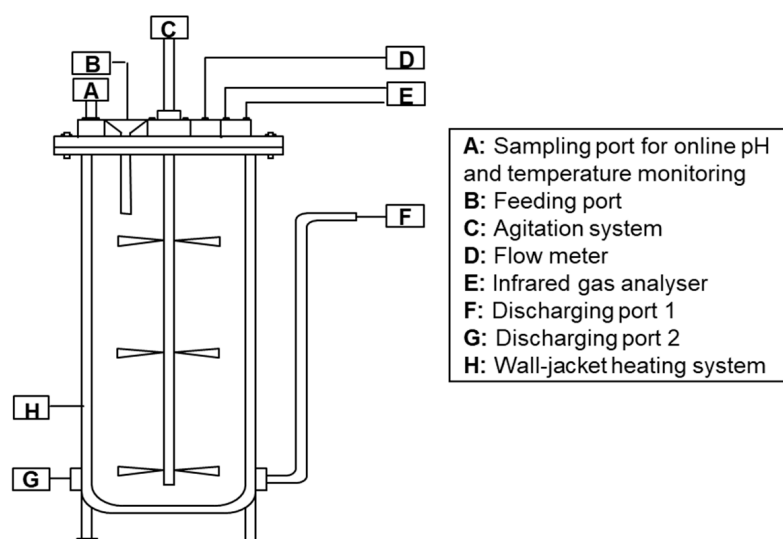


Figure 2. Experimental setup of the continuous stirred-tank reactor (CSTR-10S, Bioprocess Control, Lund, Sweden) [42].

2.3.2. Experimental Procedure

Definition of the Best Mixture of Substrates by AMPTS

The focus of this study was to define the best mixture of three solid and complex substrates. In order to define the best mixture, ten different compositions were tested. The method used to define the experimental points was mixture analysis design.

Every digester contained an equivalent amount of volatile solids. The inoculum-to-substrate ratio was equal to 2 (grams of volatile solids of inoculum per gram of volatile solids of substrates).

Blank assays, with only inoculum, were used to estimate the endogenous methane potential. This production was then subtracted from the methane potential of each experimental point to estimate the methane production of the studied substrates. In addition, positive control experiments were carried out using cellulose as a substrate to confirm the proper operation of the AMPTS system [43].

The complete experimental design included 10 experimental points with five replications of the central point and three replications for all of the other points. A total of 60 digesters were operated simultaneously, including the experimental points, blank assays, and positive controls in each AMPTS. Preliminary experiments were conducted to ensure the repeatability of the experiments within the digesters.

Validation at the Small Pilot Scale

After conducting the laboratory-scale experiments in the AMPTS digesters with a working volume of 450 mL, the study progressed to validating these findings on a small pilot scale using BPC digesters with a working volume of 10 L.

Both digesters were operated at the same OLR throughout the experiment. The OLR was determined based on two factors: the hydraulic retention time, and the composition of the substrates. It was increased by $0.5 \text{ kg}_{\text{VS}} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ each week, starting at 0.5 in the first week, and reaching $2.4 \text{ kg}_{\text{VS}} \cdot \text{m}^{-3} \cdot \text{d}^{-1}$ by the fifth week. After this, the OLR remained constant for three hydraulic retention times, each lasting 25 days. Both digesters were fed twice a week with a specific mixture of the substrates of organic matter (0.25 HW, 0.50 SS, 0.25 OFMSW), and an equal amount of surplus was removed to maintain a constant working volume of 9.5 L.

2.4. Statistical Analysis of the Model: Calibration and Validation

After conducting the experiments, the results were fitted to a special cubic polynomial model and were analyzed using least squares regression to estimate the coefficients of the model presented in Equation (1):

$$y = b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3 \quad (1)$$

where y represents the methane yield, b_1 is the magnitude of the effect of substrate x_1 on the methane yield, b_2 is the magnitude of the effect of substrate x_2 on the methane yield, b_3 is the magnitude of the effect of substrate x_3 on the methane yield, b_{12} is the magnitude of the effect of the interaction between the substrates x_1 and x_2 , b_{13} is the magnitude of the effect of the interaction between the substrates x_1 and x_3 , b_{23} is the magnitude of the effect of the interaction between the substrates x_2 and x_3 , and b_{123} is the magnitude of the interactive effect between the three substrates x_1 , x_2 , and x_3 . Equation (1) represents a complete model for a mixture design that corresponds to the three substrates. In the following parts of this paper, our selection of regressors kept only those that were significant.

The statistical analysis of the model was carried out using the data analysis tool from Excel. Fisher's test was conducted to investigate the significance of the model, with a confidence level of 95%. An F-value was calculated to determine the model's significance and the confidence interval of each value in the study. The coefficient of determination (R^2) determined how well the model fitted the experimental data and provided correlations between the observed and the predicted responses. The adjusted coefficient of determination (R_{adj}^2) defined the adequacy of the model. These indicators were independent of the number of experimental points, as they took into account the degrees of freedom of the model. Consequently, they provided information about the quality of the model and allowed for the comparison of different models regardless of the experimental points used.

Each experimental point underwent three repetitions, while the central point underwent five repetitions. These repetitions served to define the sum of squares and the degree of freedom in order to establish the significance of the model and residual error. The residual error encompassed both the model adjustment error and the experimental error. The experimental sum of squares was calculated by comparing the final methane yield from the five repetitions of the central point to their mean yield.

The responses of experiments 1 to 7 were fitted in the mixture design, considering all of the repetitions, in order to develop an equation to predict methane yield based on the combination of up to three substrates. Experiments 8, 9, and 10 were used to validate the model. These validation points played an important role in comparing the calculated methane production based on the model against the experimental results. This comparison confirmed that the model was representative of the experimental results and that it could accurately predict methane production based on the defined operational conditions. The model described the effect of the substrate alone or the interaction of two or three substrates on the methane yield. A negative coefficient indicated a negative effect of the associated substrate, or mix of substrates, on the methane yield. A positive coefficient indicated that the associated substrate, or mix of substrates, had a positive effect on the methane yield.

In addition, Student's t-test was performed, at a significance level of 95%, to define the significance of the regressors. Each coefficient is associated with a p -value. The regression coefficients of the model were defined by analysis of variance (ANOVA), and the selection of regressors was carried out based on the significance of each coefficient [44].

3. Results and Discussion

3.1. Experimental Methane Yield Results

This study focused on optimizing the anaerobic digestion process by determining the best combinations of three different substrates. This was achieved through the application of the simplex-centroid mixture design methodology. Based on the mixture design,

ten experiments were conducted, and the observed results of each experiment are presented in Table 3. Different combinations were studied, including the pure components (experiments 1, 2, and 3), binary mixtures (4, 5, and 6), and ternary mixtures (8, 9, and 10). The methane yield for each experiment represented the average of three repetitions, with the exception of the central point (7), which was based on the average of five repetitions.

Table 3. Different combinations of substrates based on the mixture design, and the observed responses of the experiments.

Experiments	Horse Waste	Sewage Sludge	OFMSW	Repetitions	Methane Yield (NmL·g _{VS} ^{−1})
1	1	0	0	3	266 ± 4
2	0	1	0	3	242 ± 12
3	0	0	1	3	250 ± 11
4	0.5	0.5	0	3	243 ± 6
5	0.5	0	0.5	3	255 ± 4
6	0	0.5	0.5	3	250 ± 13
7	0.33	0.33	0.33	5	266 ± 13
8	0.66	0.17	0.17	3	262 ± 22
9	0.17	0.66	0.17	3	269 ± 14
10	0.17	0.17	0.66	3	268 ± 12

Table 3 indicates the influence of different combinations of substrates on methane production. When comparing the mono-digestion of each substrate separately, it can be seen that HW yielded the highest methane output, with a value of 266 NmL·g_{VS}^{−1}, followed by the OFMSW at 250 NmL·g_{VS}^{−1} and SS at 242 NmL·g_{VS}^{−1}. The high production of methane for HW was due to its nutrient richness and proportion of organic material [30], which are beneficial for the microbial consortium responsible for organic matter degradation. Similarly, the OFMSW is rich in moisture and volatile solids; therefore, it also creates a good environment for the microorganisms [45]. On the other hand, SS is characterized by lower total solids and organic content, and it undergoes faster digestion, potentially leading to the accumulation of VFAs, pH drops and, consequently, process inhibition.

Different effects may be expected due to the interaction of several components. These can be neutral, additive, antagonistic, or synergistic. Therefore, in some cases, the addition of a co-substrate did not have any impact compared to mono-digestion—a condition referred to as “indifference” [46]. In other cases, the addition of a co-substrate resulted in an increase in available biodegradable organic matter, resulting in an additive effect that enhanced the methane yield [47]. Co-digestion may also induce a synergistic effect, which can either boost the methane yield of individual substrates or accelerate the degradation kinetics [47]. On the other hand, co-digestion can also result in antagonistic effects [48].

The anaerobic co-digestion of sewage sludge with one or several substrates has been widely adopted in different research papers to increase biogas production [49,50]. Among these substrates, the co-digestion of SS and the OFMSW has been the most used, due to the accessibility of the OFMSW and its high methane yield. Experimental point 6, which represented a combination of the OFMSW and SS, yielded methane production similar to that of the OFMSW alone but higher than the methane production of SS. This highlights the advantage of adding the OFMSW to SS, as the OFMSW is an easily degradable substrate and can increase biogas production by introducing solids into the mixture [51,52]. On the other hand, experimental point 4, with a composition of 0.5 HW and 0.5 SS, exhibited a biogas production of 243 NmL·g_{VS}^{−1}, similar to the production of the mono-digestion of SS, but less than the mono-digestion of HW. Therefore, the addition of HW to SS did not have a positive impact on methane production.

Experimental point 9, with a composition of 0.17 HW, 0.66 SS, and 0.17 OFMSW, showed the highest BMP, with a value of 269 NmL·g_{VS}^{−1}. When considering the confidence level of each point, Table 3 shows that points 7, 8, 9, and 10 had the highest methane yields. It can be concluded that the highest methane yields were achieved by the simultaneous

presence of all three substrates, which can be explained by the synergistic effect between them. Each substrate contributed to produce the highest methane yield: HW and the OFMSW are rich in nutrients and volatile solids, and SS hosts microorganisms capable of degrading organic matter. Moreover, HW and the OFMSW have a buffering capacity to balance the low pH and high VFAs that can be produced during the mono-digestion of SS. Economically, combining high-total-solids substrates with SS reduces the volume requirement for the digester, offering potential cost savings [53,54].

The importance of the synergistic effect will be emphasized in the following section and more precisely in the prediction model.

3.2. Calibration and Validation of the Prediction Model

3.2.1. Calibration of the Prediction Model

To ensure that the model was reliable, several statistical tests were used, such as the coefficient of determination R^2 and the adjusted R^2 to indicate whether the model represents a good fit for the data or not. In addition, a variance analysis (ANOVA) was also carried out in order to show the statistical significance of the regression model.

The first seven combinations of substrates were run according to the experimental design, and the following model was obtained, considering all interactions:

$$y = 266x_1 + 242x_2 + 249x_3 - 41x_1x_2 + 17x_2x_3 - 9x_1x_3 + 403x_1x_2x_3 \quad (2)$$

The analysis of variance of the fitted model and the respective coefficients of the regression model were obtained.

Based on Table 4, the F-value was 2153, which implied that the model was significant. In addition, the associated p -value was <0.0001 , indicating that the overall model was significant at 99.99%. Furthermore, the high values of the coefficient of determination R^2 and adjusted coefficient of determination R^2 (99.89% and 93.97%, respectively) show a correlation between the experimental and predicted values of the adapted model.

Table 4. Analysis of variance (ANOVA) of the fitted model (with all interactions).

	df	SS	MS	F	Significance F
Regression	7	1,545,588	220,798	2153	<0.0001
Residual	17	1743	102		
Total	24	1,547,331			
R^2	99.89%				
Adjusted R^2	93.97%				
Standard Error	10.13				

Note: SS: sum of squares, df: degrees of freedom, MS: mean square.

Table 5 shows the coefficients of the regression model and the associated p -value of each coefficient. The model coefficients x_1 , x_2 , x_3 , and $x_1x_2x_3$ were statistically significant, with p -values lower than 0.05, except for the coefficients that corresponded to the binary interactions, which showed p -values higher than 0.05. The coefficient corresponding to x_1x_3 had the highest p -value. Therefore, a new model was created using the same experimental points but without considering the interaction between factors 1 and 3. The model corresponded to the equation below, representing a mathematical prediction model without the x_1x_3 factor:

$$y = 265x_1 + 242x_2 + 248x_3 - 40x_1x_2 + 19x_2x_3 + 381x_1x_2x_3 \quad (3)$$

The analysis of variance and respective coefficients were estimated.

The F-value in Table 6 is 2641; therefore, the model was significant. In addition, the coefficient of determination R^2 was 99.89%, and the adjusted coefficient of determination R^2 was 94.3%. These high values validate the correlation between the experimental and model-predicted values.

Table 5. Estimated regression coefficients for the regression model (with all interactions).

	Coefficients	Standard Error	<i>t</i> Stat	<i>p</i> -Value
HW (x_1)	266	6	45	<0.0001
SS (x_2)	242	6	41	<0.0001
OFMSW (x_3)	249	6	43	<0.0001
x_1x_2	−41	29	−1	0.2
x_2x_3	17	29	0.6	0.6
x_1x_3	−9	26	−0.3	0.7
$x_1x_2x_3$	403	171	2	0.03

Table 6. Analysis of variance (ANOVA) of the fitted model (without the x_1x_3 factor).

	Df	SS	MS	F	Significance F
Regression	6	1,545,576	257,596	2641	<0.0001
Residual	18	1755	98		
Total	24	1,547,331			
R ²	99.89%				
Adjusted R ²	94.29%				
Standard Error	9.88				

Note: SS: sum of squares, df: degrees of freedom, MS: mean square.

Table 7 verifies that the coefficients x_1 , x_2 , x_3 , and $x_1x_2x_3$ had *p*-values lower than 0.05 and, therefore, were statistically significant, except for the coefficients x_1x_2 and x_2x_3 , each with a *p*-value higher than 0.05. In the next model, the factor x_2x_3 , with the higher *p*-value, was omitted:

$$y = 265x_1 + 243x_2 + 250x_3 - 42x_1x_2 + 421x_1x_2x_3 \quad (4)$$

Table 7. Estimated regression coefficients for the regression model (without the x_1x_3 factor).

	Coefficients	Standard Error	<i>t</i> Stat	<i>p</i> -Value
HW (x_1)	265	5	52	<0.0001
SS (x_2)	242	6	42	<0.0001
OFMSW (x_3)	248	5	49	<0.0001
x_1x_2	−40	28	−1	0.2
x_2x_3	19	28	0.7	0.5
$x_1x_2x_3$	381	156	3	0.03

The analysis of variance and coefficient of the model were studied.

Table 8 shows a high F-value of 3264; therefore, the model was significant. In addition, high values of R² and adjusted R² of 99.88% and 94.59%, respectively, proved the existence of a correlation between the experimental and predicted data.

Table 8. Analysis of variance (ANOVA) of the fitted model (without the x_2x_3 factor).

	Df	SS	MS	F	Significance F
Regression	5	1,545,532	309,106	3264	<0.0001
Residual	19	1800	95		
Total	24	1,547,331			
R ²	99.88%				
Adjusted R ²	94.59%				
Standard Error	9.73				

Note: SS: sum of squares, df: degrees of freedom, MS: mean square.

The coefficients of the regression model are associated with their *p*-values in Table 9. The coefficients x_1 , x_2 , x_3 , and $x_1x_2x_3$ were statistically significant, with *p*-values lower than 0.05. Conversely, the coefficient corresponding to x_1x_2 showed a *p*-value higher than 0.05.

Table 9. Estimated regression coefficients for the regression model (without the x_2x_3 factor).

	Coefficients	Standard Error	<i>t</i> Stat	<i>p</i> -Value
HM (x_1)	265	5	53	<0.0001
SS (x_2)	243	5	48	<0.0001
OFMSW (x_3)	250	5	54	<0.0001
x_1x_2	−42	27	−2	0.13
$x_1x_2x_3$	421	142	3	0.01

These results show the importance of the simultaneous presence of the three substrates to achieve a positive impact on biogas production, and that the presence of only two substrates does not have a significant impact on biogas production.

Therefore, the non-significant coefficients were eliminated from the model, and a new model without binary interactions was statistically analyzed.

Based on Table 10, the F-value was 3801, which implied that the model was also significant. Moreover, the *p*-value was <0.0001, indicating that the main effect of the regression was significant. The coefficient of determination R^2 and adjusted coefficient of determination R^2 had values of 99.89% and 94.85%, respectively, showing a slightly lower value for the adjusted R^2 . These values indicate a better correlation between the experimental and predicted values of the adapted model than the first model considering binary interactions.

Table 10. Analysis of variance (ANOVA) of the fitted model.

	Df	SS	MS	F	Significance F
Regression	4	1,545,298	386,324	3801	<0.0001
Residual	20	2033	102		
Total	24	1,547,331			
R^2	99.87%				
Adjusted R^2	94.85%				
Standard Error	10.08				

Note: SS: sum of squares, df: degrees of freedom, MS: mean square.

In Table 11, all of the model coefficients are statistically significant, with *p*-values lower than 0.05.

Table 11. Estimated regression coefficients for the regression model.

	Coefficients	Standard Error	<i>t</i> Stat	<i>p</i> -Value
HW (x_1)	261	5	55	< 0.0001
SS (x_2)	240	5	49	< 0.0001
OFMSW (x_3)	251	5	52	< 0.0001
$x_1x_2x_3$	341	137	2	0.02

Therefore, the mathematical model enabling the prediction of biogas production for the combination of the three investigated substrates is presented as follows in Equation (5):

$$y = 261x_1 + 240x_2 + 251x_3 + 341x_1x_2x_3 \quad (5)$$

In this regression model, the coefficients of HW and the OFMSW were 261 and 251, respectively, compared to 240 for SS. This difference suggests a greater contribution of HW and the OFMSW to methane production. This observation was likely due to the fact that HW and the OFMSW have a high C/N ratio, which is beneficial for the bacterial consortium responsible for degradation. Conversely, the lower coefficient of SS might be attributed to its rapid degradation, leading to a rapid decrease in pH and accumulation of VFAs, which can cause system inhibition. Co-digestion of SS with carbon-rich substrates has led to the limitation of VFAs' accumulation; similar findings were found by [55], who studied the co-digestion of chicken manure with agricultural waste. Moreover, the higher

coefficient of the simultaneous presence of all three substrates compared to their individual respective coefficients demonstrated that the combined presence of the three substrates was more beneficial than each substrate alone. This implies that adding HW and the OFMWS to SS enhanced the C/N ratio and provided buffering capacity to compensate for the pH fluctuations caused by SS. Co-digestion of several substrates balances the C/N ratio [56] compared to mono-digestion. Additionally, SS can contribute to creating a more homogeneous medium and provide additional microorganisms in order to degrade lignocellulosic substrates. Similar findings were found by [27], who studied the co-digestion of wastewater and manure from a bovine slaughterhouse and found that co-digesting multiple components is more advantageous than binary co-digestion. The authors of [57] also studied the co-digestion of the OFMSW, primary sludge, and thickened waste activated sludge, and they found that ternary mixtures yielded more methane than binary mixtures.

3.2.2. Validation of the Mathematical Prediction Model with Experimental Points

In order to make sure that the prediction model was representative of the experimental points, this study compared the methane yield found in the experiments with the predicted yield calculated from the model. The comparison of both yields is presented in Figure 3 below.

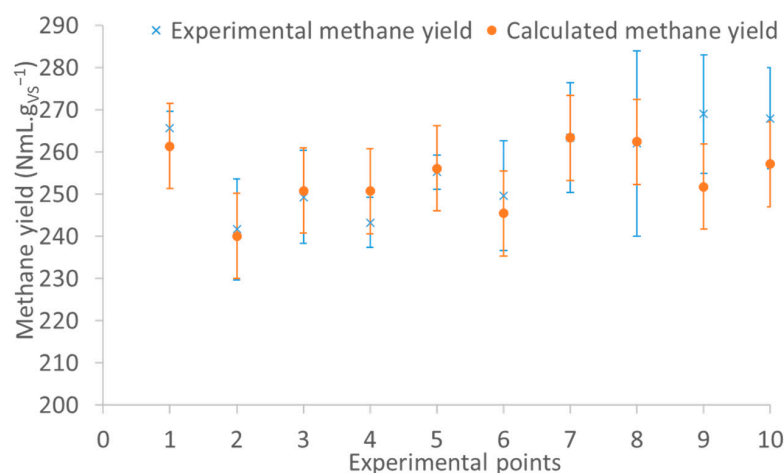


Figure 3. Comparison of the experimental and calculated methane yields.

In this figure, all of the repetitions of the experimental points were taken into consideration to obtain the maximum precision.

The figure suggests that the predicted methane yield (calculated from the prediction model) for each experimental data point fell within the acceptable range defined by the experimental methane yields and their respective standard deviations.

Therefore, the prediction model, presented in Equation (5), was validated with the experimental points, especially 8, 9, and 10. These results indicate that this model can be used to define the best mixture of substrates under the studied conditions to obtain a specific methane yield.

The specific values for the experimental versus calculated methane yields, along with their associated standard deviations, are presented in Table 12 below.

Table 12. Experimental versus calculated methane yields associated with each experimental point.

Experiments	Horse Waste	Sewage Sludge	OFMSW	Experimental Methane Yield (NmL.g _{VS} ⁻¹)	Calculated Methane Yield (NmL.g _{VS} ⁻¹)
1	1	0	0	266 ± 4	261 ± 10 *
2	0	1	0	242 ± 12	240 ± 10
3	0	0	1	250 ± 11	251 ± 10
4	0.5	0.5	0	243 ± 6	251 ± 10

Table 12. Cont.

Experiments	Horse Waste	Sewage Sludge	OFMSW	Experimental Methane Yield (NmL.g _{VS} ⁻¹)	Calculated Methane Yield (NmL.g _{VS} ⁻¹)
5	0.5	0	0.5	255 ± 4	256 ± 10
6	0	0.5	0.5	250 ± 13	246 ± 10
7	0.33	0.33	0.33	266 ± 13	263 ± 10
8	0.66	0.17	0.17	262 ± 22	262 ± 10
9	0.17	0.66	0.17	269 ± 14	252 ± 10
10	0.17	0.17	0.66	268 ± 12	257 ± 10

Note: * The standard deviation of the calculated methane yield was determined by the model.

3.2.3. Graphical Presentation of Optimal Zones

Figure 4 represents the optimal points based on the predicted model using MATLAB software (version R2018b).

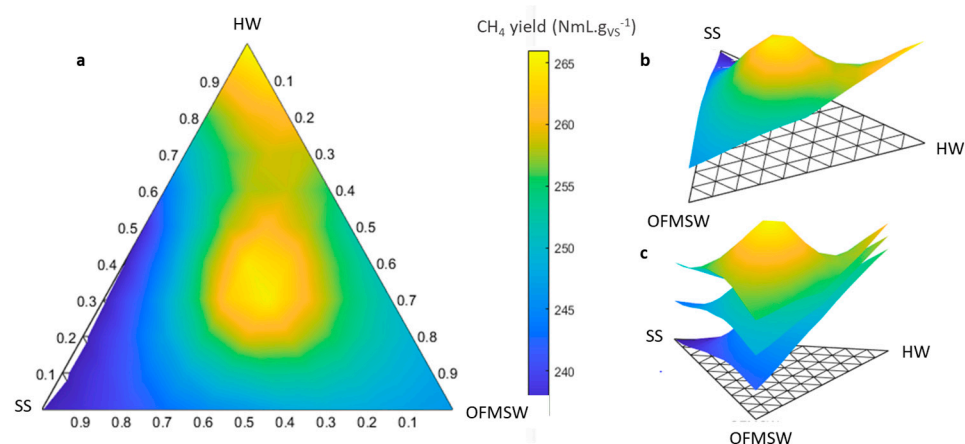


Figure 4. (a) Mixture surface plots in 2D, (b) 3D without considering the standard deviation, and (c) 3D considering the standard deviation of each experimental point, where the top plot displays the maximum values from the repetitions, the middle plot shows the average values from the repetitions, and the bottom plot illustrates the minimum values from the repetitions.

The 2D and 3D mixture surface plots presented in Figure 4 illustrate the impact of the mixture of different substrates on methane production, along with the synergies between them. Notably, the 3D plots demonstrate different levels of curvature, indicating the relationships and interactions between substrates and their impact on methane production. The aspect of the plots shows a positive interaction between the three substrates, indicating that the optimal methane production was concentrated in the central region of the ternary plot, suggesting the simultaneous presence of all three substrates. In addition, the area where only HW was present showed an important increase in methane production, whereas SS demonstrated the smallest contribution and exhibited an antagonistic effect when combined with HW, but a neutral effect when combined with the OFMSW. These observations were consistent with previous findings (Table 3).

3.3. Validation of the Optimal Mixture of Substrates at the Small Pilot Scale

Two digesters operating in semi-continuous mode, with a working volume of 9.5 L, were kept in identical conditions. In addition, stability indicators like the pH and VFAs were continuously monitored to verify the proper operation of the digesters. The methane yield was monitored for a period of 120 days, including 4 weeks of capacity increase and three HRTs, each of 28 days, to stabilize the microorganisms.

Laboratory experiments conducted on a small scale revealed that the highest methane production occurred when all three substrates were present. The selection of the substrate

composition, 0.25 HW, 0.5 SS, and 0.25 OFMSW, was determined by aiming for an intermediate composition between points 7 and 9. Therefore, these findings will serve to compare methane yields across various scales.

Figure 5a,c show that the biogas composition in both digesters was approximately 60% CH₄ and 40% CO₂, aligning with the existing literature [58].

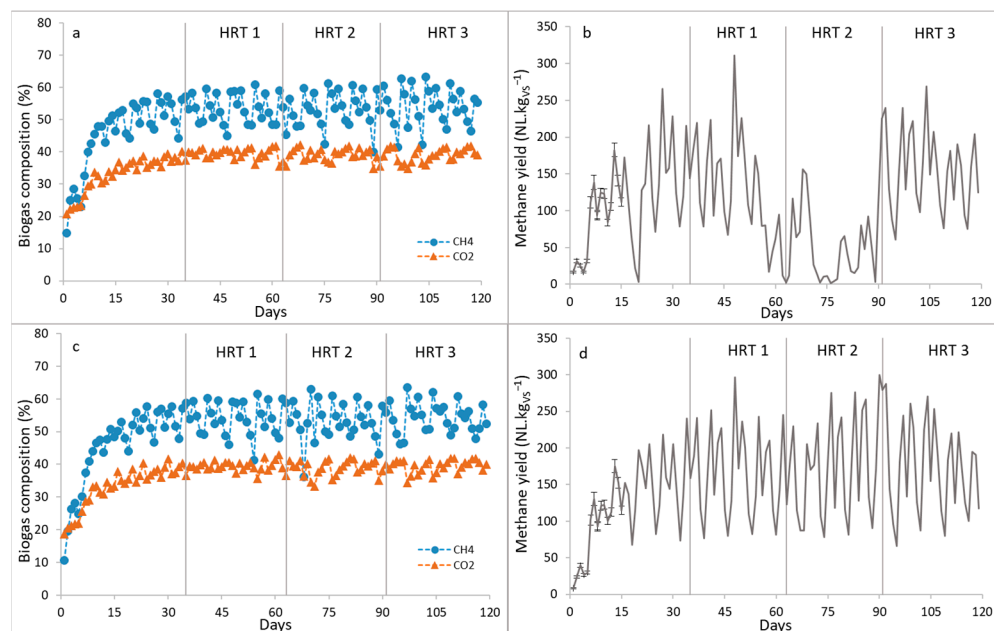


Figure 5. Composition of biogas for (a) digester 1 and (c) digester 2; methane yield for (b) digester 1 and (d) digester 2.

When considering the daily methane yield through the three HRTs, the average methane yields of digesters 1 and 2 were $156 \text{ NmL} \cdot \text{g}_{\text{VS}}^{-1}$ and $172 \text{ NmL} \cdot \text{g}_{\text{VS}}^{-1}$, respectively. The methane yield in digester 1 during the first and second HRTs was not considered in the calculation due to technical issues that resulted in reduced output from the digester during this specific period. The “boom and bust” behavior shown in Figure 5b,d was due to the twice-weekly feeding of the reactors.

The BMP observed in the prior experiment for the specified composition (0.25 HW, 0.5 SS, and 0.25 OFMSW) fell within the range of 266 to $269 \text{ NmL} \cdot \text{g}_{\text{VS}}^{-1}$, aligning with the respective BMPs of experimental points 7 and 9. Consequently, it can be deduced that the methane yield achieved at a 10 L scale corresponded to 59% and 66% of the BMP determined at the laboratory scale. When comparing this yield to previous studies [42] investigating the co-digestion of HW and SS, their findings showed 50% of the BMP when scaling up.

Evaluation of the Performance of the Small-Scale Pilot

To assess the stability of the anaerobic digestion process, certain parameters served as indicators of equilibrium, including the pH, VFAs, TA, and C:N ratio [59,60]. This investigation concentrated on examining the pH and VFAs.

The pH level represents the acidity or alkalinity of the examined medium, thereby influencing the bacterial activity [61,62]. Hence, maintaining an optimal pH level is crucial for maximizing methane production. To optimize methane production, it is essential to maintain the pH level between 6.5 and 8.5, with the most favorable values falling between 7 and 8 [63]. Figure 6a,c illustrate that the pH levels were between 7 and 8 throughout the experiment for both digesters, remaining in the optimal range for methane production. A stable pH within the range of 7 to 8 was also observed in [64] during the co-digestion of sewage sludge with horse waste, composed of 99% WS and 1% HM. This observation

underscores the importance of co-digestion in maintaining pH stability, highlighting the buffering capacity introduced by adding organic solid waste to the mixture.

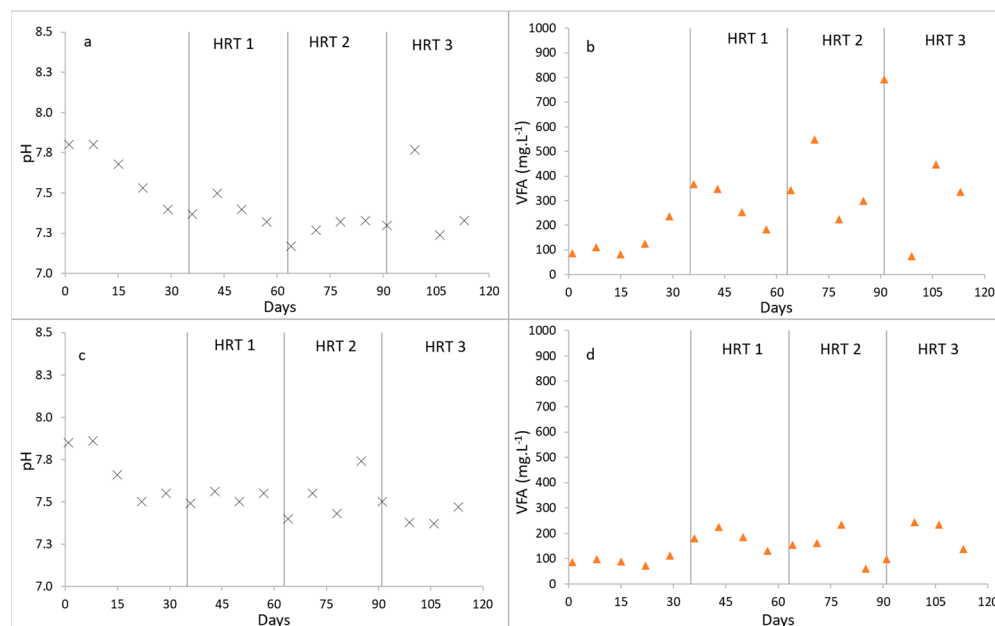


Figure 6. (a) pH for digester 1 and (c) digester 2; (b) VFAs for digester 1 and (d) digester 2.

Volatile fatty acids are a widely recognized factor influencing the anaerobic digestion process. These intermediate compounds arise during AD, and their accumulation occurs when production in the hydrolysis step outpaces uptake in the acetogenesis step [61]. This accumulation leads to a decrease in pH, thereby inhibiting the activity of methanogenic bacterial communities [65]. Figure 6b,d show VFA levels of less than 800 mg·L⁻¹ and 300 mg·L⁻¹ in digesters 1 and 2, respectively. When comparing the VFA levels to those found in the literature, these values did not exceed 1000 mg·L⁻¹ when co-digesting sewage sludge and horse waste [64]. Given that the inhibitory effects of VFAs are observed at concentrations exceeding 2000 mg·L⁻¹ [66], it can be concluded that the digesters were in a stable state.

These two parameters ensured that the digestion of 0.25 HW, 0.5 SS, and 0.25 OFMSW was undertaken in stable conditions in semi-continuous digesters with a volume of 10 L. The results suggest that scaling up the process did not affect its stability, thus validating the benefits of co-digestion with three complex substrates for optimizing methane production while maintaining stable conditions. In this study, the focus was on monitoring parameters directly influencing the stability of the process, namely, the pH and VFAs. However, verification of additional parameters, such as FOS/TAC, heavy metals, and ammonium, could provide a more complete understanding of the process and mitigate the potential risks of inhibition.

4. Conclusions

This study determined the best mixture of three complex and heterogeneous substrates, HW, OFMSW, and SS, using a mixture design methodology to mitigate the constraints arising from their various physicochemical characteristics. The most effective combination found at the laboratory scale was experimental point 9, with a composition of 0.17 HW, 0.66 SS, and 0.17 OFMSW, emphasizing the significance of the simultaneous presence of the three substrates. Laboratory experiments generated data that were used to model the responses into 2D and 3D surface plots illustrating the synergistic effects of the substrate combinations, and highlighting the importance of their compositions and interactions in methane production. The findings were then used to develop a predictive mathematical

model for the methane yield. Subsequently, the optimal mixture was validated at a larger scale to compare the results across different scales.

Although this study presents an initial phase towards more intricate investigations involving an expanded range of substrates, experimental parameters such as the frequency of liquid-phase recirculation and immersion rate, as well as the application of more sophisticated response designs, should be considered in the future.

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References

1. Muhammad, G.; Alam, M.A.; Mofijur, M.; Jahirul, M.I.; Lv, Y.; Xiong, W.; Ong, H.C.; Xu, J. Modern Developmental Aspects in the Field of Economical Harvesting and Biodiesel Production from Microalgae Biomass. *Renew. Sustain. Energy Rev.* **2021**, *135*, 110209. [\[CrossRef\]](#)
2. Shahir, S.A.; Masjuki, H.H.; Kalam, M.A.; Imran, A.; Fattah, I.M.R.; Sanjid, A. Feasibility of Diesel-Biodiesel-Ethanol/Bioethanol Blend as Existing CI Engine Fuel: An Assessment of Properties, Material Compatibility, Safety and Combustion. *Renew. Sustain. Energy Rev.* **2014**, *32*, 379–395. [\[CrossRef\]](#)
3. Kunatsa, T.; Xia, X. A Review on Anaerobic Digestion with Focus on the Role of Biomass Co-Digestion, Modelling and Optimisation on Biogas Production and Enhancement. *Bioresour. Technol.* **2022**, *344*, 126311. [\[CrossRef\]](#) [\[PubMed\]](#)
4. Van, D.P.; Fujiwara, T.; Leu Tho, B.; Song Toan, P.P.; Hoang Minh, G. A Review of Anaerobic Digestion Systems for Biodegradable Waste: Configurations, Operating Parameters, and Current Trends. *Environ. Eng. Res.* **2019**, *25*, 1–17. [\[CrossRef\]](#)
5. Wu, D.; Li, L.; Zhao, X.; Peng, Y.; Yang, P.; Peng, X. Anaerobic Digestion: A Review on Process Monitoring. *Renew. Sustain. Energy Rev.* **2019**, *103*, 1–12. [\[CrossRef\]](#)
6. Pellera, F.-M.; Gidarakos, E. Anaerobic Digestion of Solid Agroindustrial Waste in Semi-Continuous Mode: Evaluation of Mono-Digestion and Co-Digestion Systems. *Waste Manag.* **2017**, *68*, 103–119. [\[CrossRef\]](#)
7. Sun, C.; Cao, W.; Banks, C.J.; Heaven, S.; Liu, R. Biogas Production from Undiluted Chicken Manure and Maize Silage: A Study of Ammonia Inhibition in High Solids Anaerobic Digestion. *Bioresour. Technol.* **2016**, *218*, 1215–1223. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Chen, X.; Yan, W.; Sheng, K.; Sanati, M. Comparison of High-Solids to Liquid Anaerobic Co-Digestion of Food Waste and Green Waste. *Bioresour. Technol.* **2014**, *154*, 215–221. [\[CrossRef\]](#)
9. Shi, J.; Wang, Z.; Stiverson, J.A.; Yu, Z.; Li, Y. Reactor Performance and Microbial Community Dynamics during Solid-State Anaerobic Digestion of Corn Stover at Mesophilic and Thermophilic Conditions. *Bioresour. Technol.* **2013**, *136*, 574–581. [\[CrossRef\]](#)
10. Di Maria, F.; Barratta, M.; Bianconi, F.; Placidi, P.; Passeri, D. Solid Anaerobic Digestion Batch with Liquid Digestate Recirculation and Wet Anaerobic Digestion of Organic Waste: Comparison of System Performances and Identification of Microbial Guilds. *Waste Manag.* **2017**, *59*, 172–180. [\[CrossRef\]](#)
11. Shinnars, K.; Binversie, B.; Muck, R.; Weimer, P. Comparison of Wet and Dry Corn Stover Harvest and Storage. *Biomass Bioenergy* **2007**, *31*, 211–221. [\[CrossRef\]](#)
12. Abbassi-Guendouz, A.; Brockmann, D.; Trably, E.; Dumas, C.; Delgenès, J.-P.; Steyer, J.-P.; Escudé, R. Total Solids Content Drives High Solid Anaerobic Digestion via Mass Transfer Limitation. *Bioresour. Technol.* **2012**, *111*, 55–61. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Rocamora, I.; Wagland, S.T.; Villa, R.; Simpson, E.W.; Fernández, O.; Bajón-Fernández, Y. Dry Anaerobic Digestion of Organic Waste: A Review of Operational Parameters and Their Impact on Process Performance. *Bioresour. Technol.* **2020**, *299*, 122681. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Kothari, R.; Pandey, A.K.; Kumar, S.; Tyagi, V.V.; Tyagi, S.K. Different Aspects of Dry Anaerobic Digestion for Bio-Energy: An Overview. *Renew. Sustain. Energy Rev.* **2014**, *39*, 174–195. [\[CrossRef\]](#)
15. Lee, J.; Hong, J.; Jeong, S.; Chandran, K.; Park, K.Y. Interactions between Substrate Characteristics and Microbial Communities on Biogas Production Yield and Rate. *Bioresour. Technol.* **2020**, *303*, 122934. [\[CrossRef\]](#) [\[PubMed\]](#)

16. Maragkaki, A.E.; Vasileiadis, I.; Fountoulakis, M.; Kyriakou, A.; Lasaridi, K.; Manios, T. Improving Biogas Production from Anaerobic Co-Digestion of Sewage Sludge with a Thermal Dried Mixture of Food Waste, Cheese Whey and Olive Mill Wastewater. *Waste Manag.* **2018**, *71*, 644–651. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Vivekanand, V.; Mulat, D.G.; Eijssink, V.G.H.; Horn, S.J. Synergistic Effects of Anaerobic Co-Digestion of Whey, Manure and Fish Ensilage. *Bioresour. Technol.* **2018**, *249*, 35–41. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Yasar, A.; Nazir, S.; Tabinda, A.B.; Nazar, M.; Rasheed, R.; Afzaal, M. Socio-Economic, Health and Agriculture Benefits of Rural Household Biogas Plants in Energy Scarce Developing Countries: A Case Study from Pakistan. *Renew. Energy* **2017**, *108*, 19–25. [\[CrossRef\]](#)
19. Buruk Sahin, Y.; Aktar Demirtaş, E.; Burnak, N. Mixture Design: A Review of Recent Applications in the Food Industry. *Pamukkale Univ. J. Eng. Sci.* **2016**, *22*, 297–304. [\[CrossRef\]](#)
20. Rao, P.V.; Baral, S.S. Experimental Design of Mixture for the Anaerobic Co-Digestion of Sewage Sludge. *Chem. Eng. J.* **2011**, *172*, 977–986. [\[CrossRef\]](#)
21. Comino, E.; Riggio, V.A.; Rosso, M. Biogas Production by Anaerobic Co-Digestion of Cattle Slurry and Cheese Whey. *Bioresour. Technol.* **2012**, *114*, 46–53. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Pagés-Díaz, J.; Pereda-Reyes, I.; Taherzadeh, M.J.; Sárvári-Horváth, I.; Lundin, M. Anaerobic Co-Digestion of Solid Slaughterhouse Wastes with Agro-Residues: Synergistic and Antagonistic Interactions Determined in Batch Digestion Assays. *Chem. Eng. J.* **2014**, *245*, 89–98. [\[CrossRef\]](#)
23. Kashi, S.; Satari, B.; Lundin, M.; Horváth, I.S.; Othman, M. Application of a Mixture Design to Identify the Effects of Substrates Ratios and Interactions on Anaerobic Co-Digestion of Municipal Sludge, Grease Trap Waste, and Meat Processing Waste. *J. Environ. Chem. Eng.* **2017**, *5*, 6156–6164. [\[CrossRef\]](#)
24. Rahman, M.A.; Saha, C.K.; Ward, A.J.; Møller, H.B.; Alam, M.M. Anaerobic Co-Digestions of Agro-Industrial Waste Blends Using Mixture Design. *Biomass Bioenergy* **2019**, *122*, 156–164. [\[CrossRef\]](#)
25. Regalado, R.E.H.; Weide, T.; Baumkötter, D.; Wettwer, L.; Häner, J.; Brüggling, E.; Tränckner, J. Optimization and Analysis of Liquid Anaerobic Co-Digestion of Agro-Industrial Wastes via Mixture Design. *Processes* **2021**, *9*, 877. [\[CrossRef\]](#)
26. Aydrum, R.; Haji Agha Alizade, H.; Rasouli, M.; Shadidi, B. Simplex Centroid Mixture Design for Optimizing and Promoting the Anaerobic Co-Digestion Performance of Sheep Blood and Cheese Whey. *J. Renew. Energy Environ.* **2021**, *8*, 8–15. [\[CrossRef\]](#)
27. Sánchez, Z.; Poggio, D.; Castro, L.; Escalante, H. Simultaneous Synergy in CH₄ Yield and Kinetics: Criteria for Selecting the Best Mixtures during Co-Digestion of Wastewater and Manure from a Bovine Slaughterhouse. *Energies* **2021**, *14*, 384. [\[CrossRef\]](#)
28. Coutu, A.; Mottelet, S.; Guérin, S.; Rocher, V.; Pauss, A.; Ribeiro, T. Methane Yield Optimization Using Mix Response Design and Bootstrapping: Application to Solid-State Anaerobic Co-Digestion Process of Cattle Manure and Damp Grass. *Bioresour. Technol. Rep.* **2022**, *17*, 100883. [\[CrossRef\]](#)
29. Munhoz, V.M.; Longhini, R.; Souza, J.R.P.; Zequi, J.A.C.; Mello, E.V.S.L.; Lopes, G.C.; Mello, J.C.P. Extraction of Flavonoids from Tagetes Patula: Process Optimization and Screening for Biological Activity. *Rev. Bras. Farmacogn.* **2014**, *24*, 576–583. [\[CrossRef\]](#)
30. Hadin, Å.; Eriksson, O. Horse Manure as Feedstock for Anaerobic Digestion. *Waste Manag.* **2016**, *56*, 506–518. [\[CrossRef\]](#)
31. Romano, P.V.; Krogmann, U.; Westendorf, M.L.; Strom, P.F. Small-Scale Composting of Horse Manure Mixed with Wood Shavings. *Compos. Sci. Util.* **2006**, *14*, 132–141. [\[CrossRef\]](#)
32. ADEME. *Déchets Chiffres-Clés*, 2023rd ed.; ADEME: Montrouge, France, 2023; 84p.
33. NF ISO 11465; Soil Quality—Determination of Dry Matter and Water Content on a Mass Basis—Gravimetric Method. ISO: Geneva, Switzerland, 1993.
34. NF U44-160; Organic Soil Conditioners and Organic Material for Soil Improvement—Determination of Total Organic Matter—Calcination Method. AFNOR: Paris, France, 1985.
35. NF EN ISO 9963-1; Water Quality—Determination of Alkalinity—Part 1: Determination of Total and Composite Alkalinity. ISO: Geneva, Switzerland, 1994.
36. Shelor, C.P.; Dasgupta, P.K.; Liao, H. Conductometric Gradient Ion Exclusion Chromatography for Volatile Fatty Acids. *Anal. Chem.* **2016**, *88*, 12323–12329. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Fadil, M.; Fikri-Benbrahim, K.; Rachiq, S.; Ihssane, B.; Lebrazi, S.; Chraïbi, M.; Haloui, T.; Farah, A. Combined Treatment of *Thymus vulgaris* L., *Rosmarinus officinalis* L. and *Myrtus communis* L. Essential Oils against *Salmonella typhimurium*: Optimization of Antibacterial Activity by Mixture Design Methodology. *Eur. J. Pharm. Biopharm.* **2018**, *126*, 211–220. [\[CrossRef\]](#)
38. Handa, C.L.; De Lima, F.S.; Guelfi, M.F.G.; Georgetti, S.R.; Ida, E.I. Multi-Response Optimisation of the Extraction Solvent System for Phenolics and Antioxidant Activities from Fermented Soy Flour Using a Simplex-Centroid Design. *Food Chem.* **2016**, *197*, 175–184. [\[CrossRef\]](#)
39. Orives, J.R.; Galvan, D.; Coppo, R.L.; Rodrigues, C.H.F.; Angilelli, K.G.; Borsato, D. Multiresponse Optimisation on Biodiesel Obtained through a Ternary Mixture of Vegetable Oil and Animal Fat: Simplex-Centroid Mixture Design Application. *Energy Convers. Manag.* **2014**, *79*, 398–404. [\[CrossRef\]](#)
40. Goupy, J.; Creighton, L. *Introduction to Design of Experiments with JMP Examples*; SAS Publishing: Cary, NC, USA, 2007.
41. Hafner, S.D.; Fruteau De Lacroix, H.; Koch, K.; Holliger, C. Improving Inter-Laboratory Reproducibility in Measurement of Biochemical Methane Potential (BMP). *Water* **2020**, *12*, 1752. [\[CrossRef\]](#)
42. Naji, A.; Rechdaoui, S.G.; Jabagi, E.; Lacroix, C.; Azimi, S.; Rocher, V. Pilot-Scale Anaerobic Co-Digestion of Wastewater Sludge with Lignocellulosic Waste: A Study of Performance and Limits. *Energies* **2023**, *16*, 6595. [\[CrossRef\]](#)

43. Holliger, C.; Alves, M.; Andrade, D.; Angelidaki, I.; Astals, S.; Baier, U.; Bougrier, C.; Buffière, P.; Carballa, M.; De Wilde, V.; et al. Towards a Standardization of Biomethane Potential Tests. *Water Sci. Technol.* **2016**, *74*, 2515–2522. [\[CrossRef\]](#)
44. Ammar, A.H.; Zagrouba, F.; Romdhane, M. Optimization of Operating Conditions of Tunisian Myrtle (*Myrtus communis* L.) Essential Oil Extraction by a Hydrodistillation Process Using a 24 Complete Factorial Design. *Flavour Fragr. J.* **2010**, *25*, 503–507. [\[CrossRef\]](#)
45. Jiang, Y.; Heaven, S.; Banks, C.J. Strategies for Stable Anaerobic Digestion of Vegetable Waste. *Renew. Energy* **2012**, *44*, 206–214. [\[CrossRef\]](#)
46. Silvestre, G.; Fernández, B.; Bonmatí, A. Addition of Crude Glycerine as Strategy to Balance the C/N Ratio on Sewage Sludge Thermophilic and Mesophilic Anaerobic Co-Digestion. *Bioresour. Technol.* **2015**, *193*, 377–385. [\[CrossRef\]](#) [\[PubMed\]](#)
47. Xie, S.; Wickham, R.; Nghiem, L.D. Synergistic Effect from Anaerobic Co-Digestion of Sewage Sludge and Organic Wastes. *Int. Biodeterior. Biodegrad.* **2017**, *116*, 191–197. [\[CrossRef\]](#)
48. Silvestre, G.; Illa, J.; Fernández, B.; Bonmatí, A. Thermophilic Anaerobic Co-Digestion of Sewage Sludge with Grease Waste: Effect of Long Chain Fatty Acids in the Methane Yield and Its Dewatering Properties. *Appl. Energy* **2014**, *117*, 87–94. [\[CrossRef\]](#)
49. Mata-Alvarez, J.; Dosta, J.; Romero-Güiza, M.S.; Fonoll, X.; Peces, M.; Astals, S. A Critical Review on Anaerobic Co-Digestion Achievements between 2010 and 2013. *Renew. Sustain. Energy Rev.* **2014**, *36*, 412–427. [\[CrossRef\]](#)
50. Macintosh, C.; Astals, S.; Sembera, C.; Ertl, A.; Drewes, J.E.; Jensen, P.D.; Koch, K. Successful Strategies for Increasing Energy Self-Sufficiency at Grüneck Wastewater Treatment Plant in Germany by Food Waste Co-Digestion and Improved Aeration. *Appl. Energy* **2019**, *242*, 797–808. [\[CrossRef\]](#)
51. Capson-Tojo, G.; Rouez, M.; Crest, M.; Steyer, J.-P.; Delgenès, J.-P.; Escudíé, R. Food Waste Valorization via Anaerobic Processes: A Review. *Rev. Environ. Sci. Bio/Technol.* **2016**, *15*, 499–547. [\[CrossRef\]](#)
52. Nghiem, L.D.; Koch, K.; Bolzonella, D.; Drewes, J.E. Full Scale Co-Digestion of Wastewater Sludge and Food Waste: Bottlenecks and Possibilities. *Renew. Sustain. Energy Rev.* **2017**, *72*, 354–362. [\[CrossRef\]](#)
53. Borowski, S.; Boniecki, P.; Kubacki, P.; Czyżowska, A. Food Waste Co-Digestion with Slaughterhouse Waste and Sewage Sludge: Digestate Conditioning and Supernatant Quality. *Waste Manag.* **2018**, *74*, 158–167. [\[CrossRef\]](#) [\[PubMed\]](#)
54. Jain, S.; Jain, S.; Wolf, I.T.; Lee, J.; Tong, Y.W. A Comprehensive Review on Operating Parameters and Different Pretreatment Methodologies for Anaerobic Digestion of Municipal Solid Waste. *Renew. Sustain. Energy Rev.* **2015**, *52*, 142–154. [\[CrossRef\]](#)
55. Abouelenien, F.; Namba, Y.; Kosseva, M.R.; Nishio, N.; Nakashimada, Y. Enhancement of Methane Production from Co-Digestion of Chicken Manure with Agricultural Wastes. *Bioresour. Technol.* **2014**, *159*, 80–87. [\[CrossRef\]](#)
56. Castro-Molano, L.D.P.; Escalante-Hernández, H.; Lambis-Benítez, L.E.; Marín-Batista, J.D. Synergistic Effects in Anaerobic Codigestion of Chicken Manure with Industrial Wastes. *DYNA* **2018**, *85*, 135–141. [\[CrossRef\]](#)
57. Ara, E.; Sartaj, M.; Kennedy, K. Enhanced Biogas Production by Anaerobic Co-Digestion from a Ternary Mix Substrate over a Binary Mix Substrate. *Waste Manag. Res.* **2015**, *33*, 578–587. [\[CrossRef\]](#) [\[PubMed\]](#)
58. Calbry-Muzyka, A.; Madi, H.; Rüsche-Pfund, F.; Gandiglio, M.; Biollaz, S. Biogas Composition from Agricultural Sources and Organic Fraction of Municipal Solid Waste. *Renew. Energy* **2022**, *181*, 1000–1007. [\[CrossRef\]](#)
59. Battista, F.; Ruggeri, B.; Fino, D.; Erriquens, F.; Rutigliano, L.; Mescia, D. Toward the Scale-up of Agro-Food Feed Mixture for Biogas Production. *J. Environ. Chem. Eng.* **2013**, *1*, 1223–1230. [\[CrossRef\]](#)
60. Cuertos, M.J.; Gómez, X.; Otero, M.; Morán, A. Anaerobic Digestion and Co-Digestion of Slaughterhouse Waste (SHW): Influence of Heat and Pressure Pre-Treatment in Biogas Yield. *Waste Manag.* **2010**, *30*, 1780–1789. [\[CrossRef\]](#) [\[PubMed\]](#)
61. Aworanti, O.A.; Agbede, O.O.; Agarry, S.E.; Ajani, A.O.; Ogunkunle, O.; Laseinde, O.T.; Rahman, S.M.A.; Fattah, I.M.R. Decoding Anaerobic Digestion: A Holistic Analysis of Biomass Waste Technology, Process Kinetics, and Operational Variables. *Energies* **2023**, *16*, 3378. [\[CrossRef\]](#)
62. Zhang, L.; Loh, K.-C.; Zhang, J. Enhanced Biogas Production from Anaerobic Digestion of Solid Organic Wastes: Current Status and Prospects. *Bioresour. Technol. Rep.* **2019**, *5*, 280–296. [\[CrossRef\]](#)
63. Weiland, P. Biogas Production: Current State and Perspectives. *Appl. Microbiol. Biotechnol.* **2010**, *85*, 849–860. [\[CrossRef\]](#) [\[PubMed\]](#)
64. Naji, A.; Rechdaoui, S.G.; Jabagi, E.; Lacroix, C.; Azimi, S.; Rocher, V. Horse Manure and Lignocellulosic Biomass Characterization as Methane Production Substrates. *Fermentation* **2023**, *9*, 580. [\[CrossRef\]](#)
65. Guendouz, J.; Buffière, P.; Cacho, J.; Carrère, M.; Delgenes, J.-P. Dry Anaerobic Digestion in Batch Mode: Design and Operation of a Laboratory-Scale, Completely Mixed Reactor. *Waste Manag.* **2010**, *30*, 1768–1771. [\[CrossRef\]](#)
66. Karthikeyan, O.P.; Visvanathan, C. Bio-Energy Recovery from High-Solid Organic Substrates by Dry Anaerobic Bio-Conversion Processes: A Review. *Rev. Environ. Sci. Bio/Technol.* **2013**, *12*, 257–284. [\[CrossRef\]](#)

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