Optimization and Kinetics of Anaerobic Digestion Processes for Three-phase Olive Mill Wastewater: Evaluation of Inoculum Performance and Determination of the Most Appropriate Choice

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Abstract—**This study is focused on optimizing the anaerobic digestion process of liquid waste generated by three-phase olive oil mills based on the effect of inoculum type and substrate-inoculum ratio on biogas production. For this purpose, we tested two of the best-known inoculum types (cow manure, and sewage sludge) by analyzing the variability of biogas production as a function of different substrate-inoculum ratios (SIR) in nine reactors (R1 to R9). The results, analyzed using the one-factor ANOVA test and Fisher's LSD post hoc test via SPSS, revealed significant differences between the ratios studied, with Reactor 4 (R4), which operates with sewage sludge as inoculum, representing the most interesting results. In addition, the application of the Gompertz kinetic model has enabled us to understand the effect of the substrate-inoculum ratio on the specific parameters of this model. A change in the substrate-inoculum ratio has an impact on the maximum specific biogas production (Rm), the maximum biogas production potential (A), and the lag phase (λ). These results have important consequences for sustainable waste management in the olive oil industry, as they offer indications as to the optimum operating conditions for the anaerobic digestion of olive mill wastewater.**

Keywords—**three-phase olive mill wastewater, Anaerobic digestion, Kinetics, Substrate-inoculum ratio, cow manure, sewage sludge**

I. Introduction

Recovering olive oil mill waste has been the focus of recent scientific research, thanks to the rapid development of the olive sector, which generates a considerable amount of solid and liquid waste that can have a significant environmental impact if not properly managed.

Olive oil extraction today is usually based on a continuous two- or three-phase process, which can treat between 30 and 32 tons of olives per day, so the use of the three-phase process produces high quantities of olive mill wastewater (OMWW) due to the important production of hot water introduced during centrifugation [1].

Although several countries have drawn up laws concerning the separation, recycling, or reuse of waste, we now know that in many countries several olive oil mills transfer their waste to waterways or other environmental receptors, causing serious environmental impacts. Anaerobic digestion represents an innovative recovery solution that reduces these problems, contributing to a more sustainable and responsible management of this industry's residues by producing renewable energy from waste, which in turn reduces dependence on fossil fuels and greenhouse gas emissions. The final product of anaerobic digestion can be applied in moderate doses to the soil as a fertilizer, due to its high content of N, P, and K, which are essential plant nutrients, as shown in the Losák *et al.*'s study [2] and other previous research [3, 4].

Although OMWW has a concentration of organic matter 20 to 400 times higher than municipal wastewater, its biodegradability is limited, mainly due to the high concentration of polyphenols, inhibiting organic degradation. Other factors characterizing olive mill waste (OMW) are also susceptible to inhibiting anaerobic digestion, such as an acid pH, an unbalanced nutrient ratio (low nitrogen and phosphorus content), and a high lipid concentration [5].

To overcome these issues and optimize the conditions for anaerobic digestion of OMWW, various parameters have been studied, including dilution with water, the addition of a nitrogen source, or alkalinity correction with substances such as $NaHCO_3$, NaOH, or $Ca(OH)_2$. However, the use of chemicals creates environmental impacts, and diluting OMW with water generates large volumes of unwanted effluent [1].

The use of inoculum is an essential step in anaerobic digestion, providing an aqueous medium rich in bacteria capable of degrading organic matter in anaerobic conditions, and also helping to overcome reactor inhibition [6]. Choosing the right inoculum and optimum substrate-inoculum ratio (SIR) is essential to stimulate efficient anaerobic fermentation and achieve high biogas yields. Several studies have been carried out to identify the ideal inoculum and optimum ratio for anaerobic digestion of waste. A study conducted by [7], examined the effect of different inoculums on the anaerobic digestion of swine wastewater. Revealed that the addition of any type of inoculum improved the rate of methane production, with better production when using sewage sludge as inoculum. Another study published by the same researchers, to study the effect of SIR on the kinetics of anaerobic methane production from swine wastewater using sewage sludge as inoculum, showed high production with a 1:1 ratio, i.e., with increasing inoculum quantity [8]. Studies

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in this area are multiple, using several types of substrates and inoculums [9, 10].

According to our research, optimization, and kinetics of anaerobic digestion of OMW, in particular OMWW, by studying the substrate-inoculum ratio is still little studied. This study aimed to optimize anaerobic digestion conditions, by studying the effect of inoculum type on the biogas production process and determining the optimum SIR. Two different inocula were compared, in particular sewage sludge and cattle manure, while five SIR values were tested (0, 0.5, 1, 2, 4, based on total volume).

II. Materials and Methods

A. Sampling and Inoculum Preparation

The substrate used in this study was collected from three oil mills located in the eastern region of Morocco that crush olives using the three-phase system, to ensure a representative sample. The substrate sample was refrigerated at –16°C before being used, to maintain its constant characteristics throughout the experimental period. The choice of this type of waste was based mainly on the abundance and availability of this type of residue and its clear impact on the environment, to fill a gap in the literature and balance the available knowledge.

The first inoculum (cow manure) used in our experiments was obtained from a laboratory-scale mesophilic anaerobic reactor. The sample was collected from a dairy farm and transferred to the laboratory for inoculum preparation. Inoculum preparation was carried out by mixing 100 g of collected residue with 300 mL of distilled water in a 500 mL glass digester and then placing it in a water bath at mesophilic temperature for 15 days. A 500 mL bottle filled with water and connected to the digester by a tube was used to prevent the entry of oxygen and the exit of biogas.

The second inoculum (sewage sludge) was collected from a wastewater treatment plant in Kenitra City, Morocco, which uses the sewage sludge to produce renewable energy by anaerobic digestion, using mesophilic batch reactors. Sampling of the activated sewage sludge was carried out directly before each manipulation to ensure that the characteristics did not change.

B. Analysis of Total Solids, Volatile Fatty Acids, Volatile Solids, Alkalinity and Total Phenols

Characterization of the substrate and the two inoculums, as well as reactor influent and effluent flows to determine substrate removal efficiency, included determination of the following parameters: pH, temperature, Total Solids (TS), Volatile Solids (VS), Volatile Fatty Acids (VFA), total alkalinity (TA) and total phenols.

A WTW 197i pH meter was used to determine pH and temperature (the pH of cow manure was measured in a sterile distilled water suspension). Following standard APHA protocols [11], Total Solids (TS) were determined by exposing the sample to a drying process in an oven at 105 °C for 24 h, and Volatile Solids (VS) were measured by placing the dried sample in an oven at 550 °C for 2 h. The concentration of VS indicates the amount of organic matter present in the sample. Volatile fatty acid and alkalinity analyses were also carried out by standard APHA methods. Total phenolic compounds were determined using the Folin-Ciocalteu spectrophotometric method [12]. All analyses were performed in triplicate.

A summary of the characteristics of the three-phase olive mill wastewater (3POMWW) and the two inoculums used in the experiments is given in Table 1.

Table 1. Characteristics of the three-phase olive mill wastewater and the two inoculums

Parameters	Units	Substrate (3POMWW)	Cow manure	Sewage sludge
pH		5.10±0.34	7.1±0.44	7.1±0.45
Temperature	°C	20	35	37
VFA	g/L	32.5±1.30	1.17±0.11	0.64±0.01
TA	g/L	12±1.17	1.7±0.39	4.31±0.97
TS	%	6.00±0.16	4.00±0.02	8.16±0.21
VS	%	5.73±0.90	2.37±0.31	6.15±0.19
Total phenols	g/L	5.57±0.01	0.62±0.13	0.33±0.08

C. Experimental Set-up

The mesophilic anaerobic digestion process (35 °C) was carried out using a total of 18 reactors, including duplicates and controls. Sterile 30cL bottles with butyl rubber closures were used as batch reactors, with a work volume of 300mL. Based on the total volume, 50 mL of the substrate was added to each reactor, then 0, 25, 50, 100, and 200 mL of each inoculum were added to obtain SIR of 0, 0.5, 1, 2, 4, and total volume has been adjusted to 250 mL by adding sterile distilled water (Table 2). To prepare a suitable environment for biogas production by the bacteria, the pH was checked and adjusted to 7 if necessary, before starting the anaerobic digestion process using sodium hydroxide (NaOH) or sulfuric acid. Our study was divided into two experimental sets, the first using cow manure and the second with sewage sludge. The reactors were hermetically closed with rubber stoppers and parafilm, labeled, and placed in a water bath at mesophilic temperature.

Table 2. Experimental conditions for the various anaerobic digestion tests carried out

Reactors	SIR	Used quantity (based on volume)		
		Substrate (mL)	Inoculum (mL)	
R1 (Control)	0	50 (2.86g VS)		0
R2	0.5	50 (2.86g VS)	Sewage sludge	25
R3	1	50 (2.86g VS)		50
R4	2	50 (2.86g VS)		100
R5	4	50 (2.86g VS)		200
R6	0.5	50 (2.86g VS)	Cow manure	25
R7	1	50 (2.86g VS)		50
R8	2	50 (2.86g VS)		100
R9	4	50 (2.86g VS)		200

To maintain the bacteria in suspension, the reactors were manually agitated twice a day. A batch anaerobic digestion process was used, and daily biogas production was determined using the water displacement method, by measuring the volume of water displaced in the burette [13]. On completion of the test, the volumes of biogas produced were referred to as normal liters (0 °C to 1 atmosphere).

It should be noted that all the tests carried out in this study were duplicated, and the total duration of the tests was 30 days.

D. Kinetic Study

Cumulative biogas production values for the nine batch trials were fitted to the modified Gompertz equation, which assumes that the amount of biogas produced depends on the growth rate of the methanogenic bacteria [9–14].

To determine the following biogas production kinetics: maximum biogas production potential, A (N•mL/g VS); maximum biogas specific yield, Rm (N•mL/g VS/day); and lag phase, λ (days).

$$P(t) = A\exp\left\{-\exp\left[\frac{Rm\times e}{A}(\lambda - t)+1\right]\right\} \quad (1)$$

where $P(t)$ is the cumulative biogas production at time (N•mL/g VS) and e is a mathematical constant.

E. Statistical Analysis

The 1-factor ANOVA test and Fisher's LSD post-hoc test were used to study the effect of the factor (SIR) on the quantitative variable (biogas yield), by comparing the daily and cumulative biogas yields of the various SIR. SPSS version 21 software was used to perform statistical tests, with a confidence level of 95%.

III. Result and Discussion

A. Characteristics of the Substrate and Two Inoculums

Table 1 presents data on various parameters measured for the substrate and the two types of inocula. The data show that the pH of the substrate is different from that of the inoculums, and the two inoculums have the same pH value. The temperature measured in degrees Celsius (°C) was 20 °C for the substrate, 35 °C for cow manure and 37 °C for sewage sludge. The substrate has the highest volatile fatty acid concentration with 32.5±1.30 g/L, followed by Cow Manure with 1.17±0.11 g/L, and sewage Sludge with 0.64±0.01 g/L.

Alkalinity is a measure of a substance's ability to neutralize acids. It is expressed in grams per liter (g/L). Alkalinity values also vary, with 12±1.17 g/L for the substrate (3POMWW), 1.7±0.39 g/L for Cow manure, and 4.31±0.97 g/L for sewage Sludge. Total solids indicate the proportion of solids in each substrate relative to total volume, representing 6.00±0.16 % of the substrate (3POMWW), 4.00±0.02 % of Cow Manure, and 8.16±0.21 % of sewage Sludge. On the other hand, Volatile solids represent 5.73±0.90 % of the substrate (3POMWW), 2.37±0.31 % of the Cow Manure, and 6.15±0.19 % of the sewage Sludge. The concentration of phenols, expressed in grams per liter (g/L) is measured for each substrate. The substrate (3POMWW) has the highest concentration at 5.57±0.01 g/L, while the Cow Manure has a concentration of 0.62±0.13 g/L, and the Sludge has 0.33±0.08 g/L.

The results in Table 1 are coherent with most studies [14–17]. Although specific differences can be observed. Note that the physicochemical characteristics of the substrate can change according to several parameters, including the source, initial composition, environmental conditions, and storage time.

B. Daily and Cumulative Biogas Output

In the present study, which examined daily and cumulative biogas production in 9 reactors with different inoculums and SIR, several significant observations were made. Generally, it was clear that any variation in SIR had a direct impact on biogas production. Some reactors produced much more than others, showing that it is essential to adjust these ratios to maximize microbial productivity in the reactors. This importance was also reported in a study published by Salut Owamah *et al*. who studied the influence of SIR on the yield and kinetics of biogas from the anaerobic co-digestion of food waste and corn husks [6]. Other studies reinforce the validity of our results [18, 19].

Biogas production versus time graphs (Figs. 1–3) show a similar general tendency throughout the anaerobic digestion period. They are generally separated into three phases, an initial phase characterized by low production, a subsequent phase of accelerated growth, and finally a phase of stabilized production [19]. However, the duration of each phase differs from one ratio to another. We have observed that most reactors containing a higher proportion of substrate have a longer initial phase and a shorter growth phase. The long duration of the initial phase may be due to the growth period required for the methanogenic bacteria to react to the substrates. The short duration of the growth phase may be explained by the blockage of anaerobic digestion due to the high concentration of VFAs and phenols.

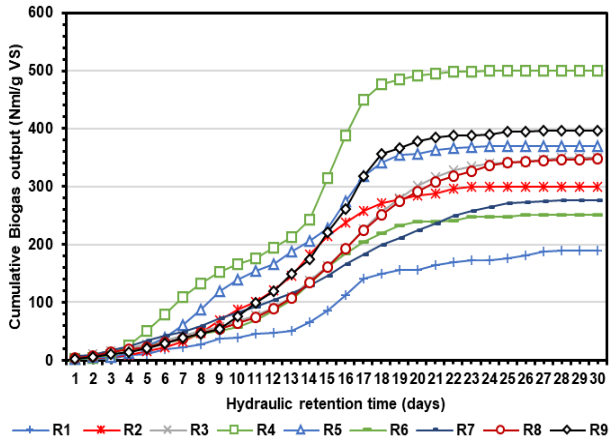

Fig. 1. Cumulative biogas output at different substrate-inoculum ratios.

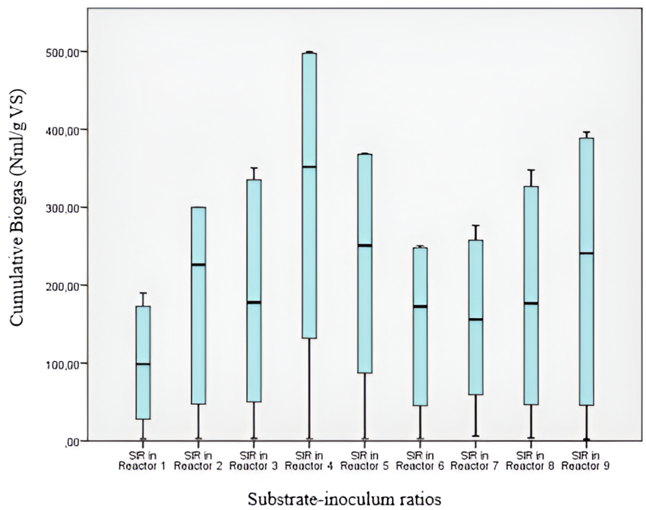

Fig. 2. Graphical representation of the distribution of cumulative Biogas yield data at various substrate-inoculum ratios (box plot).

In this respect, during the first nine days of anaerobic digestion, the volume of biogas produced in reactors R2, R3, R4, R5, R6, R7, R8, and R9 was significantly different from that produced in the control reactor R1, with higher volumes in reactors R4 and R5. This implies two important points: the addition of inoculum is essential for efficient biogas production, and the use of anaerobic sludge as inoculum guarantees better start-up performance than cow manure. Maximum production is generally observed on incubation days 15, 16, and 17 for all nine reactors, with high maximum production levels in reactors R4, R5, R9 (74.136 N•mL/g VS, 45.826 N•mL /g VS, 57.035 N•mL/g VS respectively), and a minimum level in reactor R7 (19.285 N•mL/g VS).

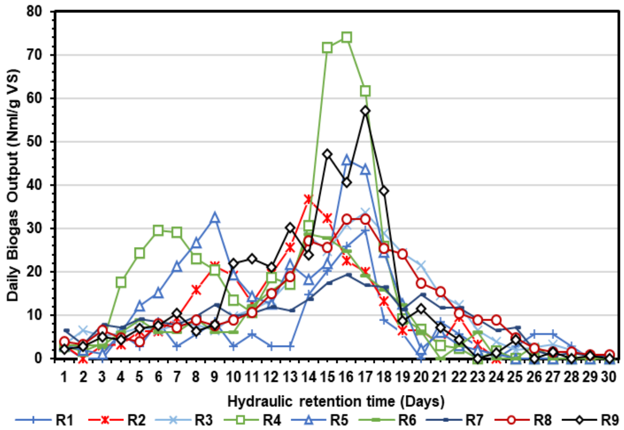

Fig. 3. Daily biogas output at different substrate-inoculum ratios.

The box plots in Figs. 2 and 4 represent the daily and cumulative biogas yield of the nine reactors over 30 days. They enable analysis of the distribution of data for each reactor.

Firstly, by examining the medians of each box, we observe that Reactor 4 (SIR2) has the highest value, and Reactor 1 (Control) has the lowest. By analyzing the boxes, we note a high dispersion of cumulative Biogas production data. This means that production values are generally close to the median, with little variability.

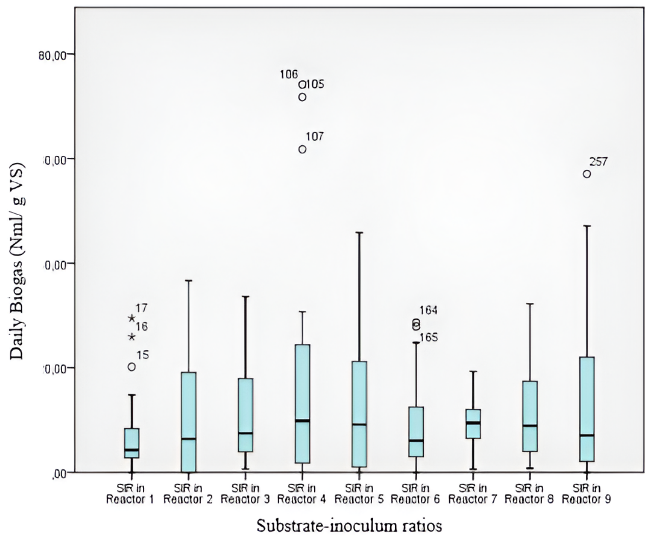

Fig. 4. Graphical representation of the distribution of daily Biogas yield data at various substrate-inoculum ratios (box plot).

C. Statistical Analysis

Statistical analyses were carried out to evaluate the effect of the SIR studied on daily and cumulative biogas yields. For this purpose, we performed a one-way analysis of variance (ANOVA) to determine if any significant difference existed between these factors. The test results revealed a statistically significant difference between the factors ($p < 0.05$), which clearly shows that substrate-inoculum ratios have an impact on biogas yield. (Tables 3–5).

To further analyze differences between factors, a Fisher' s LSD post hoc test was performed. This test identifies pairs of factors between which there are significant differences. The results showed significant differences in biogas production between most of the ratios studied. This finding shows that the choice of SIR can have a significant impact on biogas production. (Tables 4–6).

Table 3. One-way analysis of variance (ANOVA) to compare daily biogas yields at different substrate-inoculum ratios

Daily. Biogas	Sum of squares	ddl	Average square	F	Significance
Inter-Group	2172.260	8	271.532	1.893	0.041
Intra-Group	37429.255	261	143.407		
Total	39601.515	269			

Table 4. Fisher's LSD post hoc test for detailed analysis of differences between factors (Daily biogas)

	Reactor1	Reactor 2	Reactor 3	Reactor 4	Reactor 5	Reactor 6	Reactor 7	Reactor 8	Reactor 9
Reactor1		0.237	0.085	0.001*	0.035*	0.511	0.350	0.020*	0.027*
Reactor 2			0.586	0.032*	0.457	0.598	0.803	0.605	0.298
Reactor 3				0.000*	0.841	0.285	0.427	0.978	0.619
Reactor 4					0.160	0.008*	0.017*	0.103	0.266
Reactor 5						0.004*	0.321	0.820	0.767
Reactor 6							0.782	0.297	0.118
Reactor 7								0.444	0.047*
Reactor 8									0.600
Reactor 9									

Multiple Comparisons
Dependent variable: Daily. Biogas
LSD
*The average difference is significant at the 0.05 level.

Table 5. One-way analysis of variance (ANOVA) to compare cumulative biogas yields at different substrate-inoculum ratios

Cumulative. Biogas	Sum of squares	ddl	Average square	F	Significance
Inter-Group	815212.474	8	101901.559	5.654	0.000
Intra-Group	4703635.076	261	18021.590		
Total	5518847.550	269			

Table 6. Fisher's LSD post hoc test for detailed analysis of differences between factors (Cumulative biogas)

	Reactor1	Reactor 2	Reactor 3	Reactor 4	Reactor 5	Reactor 6	Reactor 7	Reactor 8	Reactor 9
Reactor1		0.022*	0.013*	0.000*	0.000*	0.176	0.115	0.018*	0.000*
Reactor 2			0.850	0.000*	0.181	0.346	0.473	0.933	0.215
Reactor 3				0.001	0.251	0.259	0.365	0.916	0.292
Reactor 4					0.022*	0.000*	0.000*	0.000*	0.017*
Reactor 5						0.023*	0.040*	0.210	0.923
Reactor 6							0.823	0.305	0.030*
Reactor 7								0.423	0.050
Reactor 8									0.247
Reactor 9									

Multiple Comparisons
Dependent variable: Cumulative. Biogas
LSD
*The average difference is significant at the 0.05 level.

A study carried out in 2012 by Xu and Li on anaerobic co-digestion of expired dog food and corn stalks revealed a positive correlation between substrate-inoculum ratios and maximum biogas production [20].

These results highlight the importance of choosing the right SIR in the biogas production process since this ratio can have a significant impact on total yield. They also provide a better understanding of how to optimize anaerobic digestion process conditions according to ratios.

A. Biogas Yields

The results in Table 7 show the overall and maximum biogas production yields in N•mL (normal liters) per gram of volatile dry matter (g VS) for different reactors. These reactors are labeled from R1 to R9 and are separated into three groups: the control group, the group of reactors operating using sewage sludge as inoculum, and the group of reactors operating using cow manure.

Table 7. Global and maximal biogas yield for the nine reactors

Groups	Reactors	SIR	Maximum biogas yield (NmL / g VS)	Global biogas yield (NmL / g VS)
Control	R1	0	29.472	15.875
Sewage sludge	R2	0.5	36.639	55.167
	R3	1	33.610	91.603
	R4	2	74.136	284.078
	R5	4	45.826	130.099
Cow manure	R6	0.5	28.652	27.0482
	R7	1	19.285	40.3681
	R8	2	32.234	85.2564
	R9	4	57.035	186.786

In the control group, the global biogas yield was 15.88 N•mL/g VS. Anaerobic digestion in the absence of inoculum, meaning there is no substrate-inoculum ratio (SIR=0) generally gives the lowest biogas value, which is the case in our study. The lowest SIR value indicates the absence of methanogenic microorganisms digesting the organic matter contained (substrate) resulting in low biogas production.

Global biogas yield increases with increasing SIR in the group of reactors using cow manure as inoculum. With increasing production of 27.05 N•mL/g VS, 40.37 N•mL/g VS, 85.26 N•mL/g VS, 186.79 N•mL/g VS. This is in line with the results of several studies, which have observed that higher inoculum concentrations lead to higher biogas yields [21, 22].

On the other hand, in the group of reactors that operate using sewage sludge as inoculum, reactor R4 gives a higher yield than reactor R5. A decrease in yield can be attributed to the inhibition of anaerobic digestion. This means that although the addition of inoculum enhances biogas production, it is necessary to know and use the right substrate-inoculum ratio for a specific substrate.

Bovina *et al.* reported that higher rates of OMWW rather than sewage sludge, lead to higher biogas yields [23].

A study by Benalia *et al.* showed that the highest quantities of biogas and methane were recorded when using a larger quantity of olive mill wastewater [24].

In this study, we found that the use of sewage sludge at a substrate-inoculum ratio of 2 (SIR2) generated a global production of 284.08 N•mL/g of volatile solids. The appropriate choice of inoculum and SIR resulted in an improvement in biogas production from OMWW of 94.42%. Gu *et al.* reported an improvement of 15.5% by selecting an appropriate inoculum for biogas production from corn stalks via SS-AD [25].

B. Disposal Efficiency

Fig. 5 shows the substrate removal efficiency for the nine reactors. Evaluation of the performance of each reactor could be determined by measuring characteristic substrate parameters before and after the anaerobic digestion process. This evaluation was based on the measurement of the percentage removal of volatile fatty acids, total solids, volatile solids, and phenols.

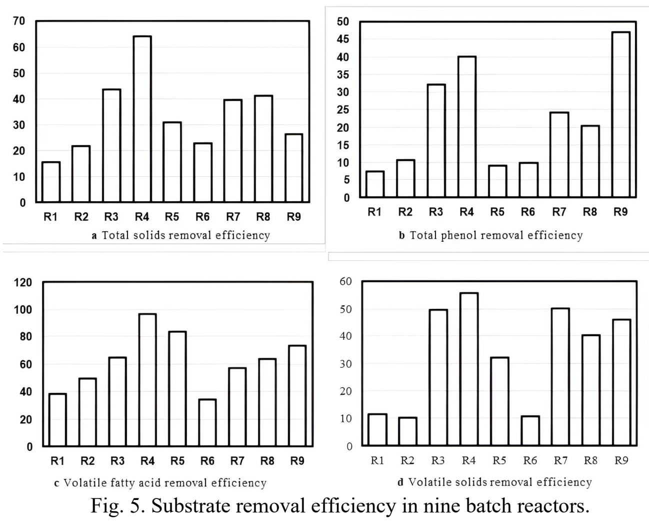

Fig. 5. Substrate removal efficiency in nine batch reactors.

Reactors R4 and R5 are the most effective at removing volatile fatty acids, with an impressive removal rate of 96.78% and 83.79% respectively. These reactors operate with a sewage sludge inoculum. In addition, reactor R9, which uses cow manure as inoculum, also showed significant VFA removal with 73.51%.

Regarding total solids and volatile solids removal, the best performance for TS was observed for reactors R4, R3, and R8 with rates of 64.16%, 43.65%, and 41.15% respectively. The best VS removal performance was achieved by reactors R4, R3, and R7 with percentages of 55.61%, 49.58%, and 50% respectively. In this case, it should be noted that the choice of inoculum did not have as significant an Impact as for VFAs.

Reactor R9, using cow manure as inoculum, gives the best results in terms of phenol removal, with a rate of 47.06%, followed by R4 and R3 with rates of 40% and 32.15% respectively.

However, the overall phenol removal efficiency is lower than that of the other parameters studied. Not surprisingly, this is because phenols are difficult to biodegrade [26, 27]. The concentration of phenols in our influents from the nine reactors was less than 2 g/L. Several studies have pointed out that the concentration of phenols should not exceed 2 g/L in order not to have an inhibitory effect on the methanation process [28, 29].

C. *Biogas Yields kinetics: Modified Gompertz model*

The kinetic results of the modified Gompertz model indicate different biogas production performances. Interpretation of these results is based on an analysis of the model's specific parameters. These parameters are the cumulative biogas production at time (P(t)), maximum biogas production potential (A), maximum biogas specific yield (Rm), and lag phase (λ). Fig. 6 shows theoretical results for adjusting cumulative biogas production versus time (day) for the different SIRs using the modified Gompertz model. Table 8 provides a brief overview of the kinetic parameter values obtained from the modeling curves.

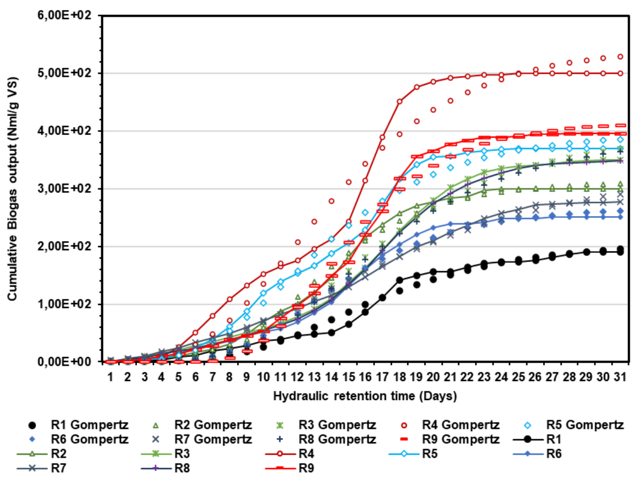

Fig. 6. Graphical representation of the results of the modified Gompertz model for different substrate-inoculum ratios.

The results show that maximum biogas production potential (A) varies positively with increasing cow manure substrate-inoculum ratio from 266.91 to 415.72 N•mL/g VS, but negatively with varying sewage sludge substrate-inoculum ratio. However, a decrease was observed after SIR2. This suggests that higher concentrations may have a negative influence on biogas production.

Concerning the relationship between SIR and lag phase (λ) for two types of inoculum, we have noticed that the change in SIR affects the lag phase. On the other hand, the two studies by Boulanger *et al.* 2012 [21] and Salut Owamah *et al.* 2021[6] reported that the lag phase (λ) increases with a decrease of SIR, which is explained by the reduced population of active methanogenic bacteria, resulting in a longer preparation phase.

As shown in Table 8, for both inoculum types, a change in the SIR has a direct impact on maximum specific biogas production (Rm). Reactors 4, 5, and 9 with the highest ratios give the maximum Rm values of 36.84, 28.22, and 38.30 N•mL/g VS/day respectively. On the other hand, the control (R1) with a low SIR gives the most minimal value with 13.18 N•mL/g VS/day.

Table 8. Kinetic parameters results

Reactors	A (NmL/g VS)	Rm (NmL/g VS/day)	Λ (d)	R^2	Error (%)
R1	205.84	13.18	7.47	0.98	2.75
R2	312.44	27.44	6.91	0.99	3.07
R3	389.46	24.74	7.65	0.98	4.95
R4	545.14	36.84	5.38	0.97	5.89
R5	394.32	28.22	5.40	0.98	4.47
R6	266.91	20.72	6.96	0.98	4.16
R7	327.34	14.76	4.43	0.99	5.11
R8	384.94	24.68	7.82	0.99	4.53
R9	415.72	38.30	8.54	0.99	3.53

IV. Conclusion

Examining the effect of inoculum type and substrate-inoculum ratio on the biogas production process, this study demonstrated that the use of sewage sludge as inoculum with SIR of 2 for anaerobic digestion of wastewater from three-phase olive oil mills is the most appropriate choice, for several reasons. According to our results, unlike cow manure, sewage sludge generates higher biogas values at lower concentrations, meaning that the use of sewage sludge does not require large quantities. On the other hand, our study showed that biogas yields and substrate removal efficiency varied according to inoculum type and substrate-inoculum ratios, demonstrating the need to use an inoculum and substrate-inoculum ratio adapted to each type of substrate.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: Inass Hamdi, Benaissa Attarassi; Methodology: Inass Hamdi, Abdelhafid Chafi; Formal analysis and investigation: Inass Hamdi, Khadija Lihi; Writing - original draft preparation: Inass Hamdi; Writing - review and editing: Inass Hamdi; Funding acquisition: Benaissa Attarassi, Nabila Auajjar; Resources: Inass Hamdi, Hameed Saleh Ali Yahya; Supervision: Benaissa Attarassi, Abdelhafid Chafi; all authors had approved the final version.

References

[1] M. Aboelfetoh, A. Hassanein, M. Ragab, M. El-kassas, and E. R. Marzouk, "Olive mill waste-based anaerobic digestion as a source of local renewable energy and nutrients," *Sustainability*, vol. 14, no. 3, pp. 14–02, 2022.

[2] T. Lošák, T. Válka, J. Elbl, A. Kintl, A. Keutgen, N. Keutgen, L Demková, J. Árvay, L. Varga, H. Hnátková, K Gondek, and M. Mierzwa-Hersztek, "Fertilization with magnesium- and

sulfur-supplemented digestate increases the yield and quality of kohlrabi," *Sustainability*, vol. 12, no. 14, pp. 57–33, Jul. 2020.
[3] F. Han, S. Yun, C. Zhang, H. Xu, and Z. Wang, "Steel slag as accelerant in anaerobic digestion for nonhazardous treatment and digestate fertilizer utilization," *Bioresour Technol*, vol. 282, pp. 331–338, Jun. 2019.
[4] Z. Wang, S. Yun, H. Xu, C. Wang, Y. Zhang, J. Chen, and B. Jia, "Mesophilic anaerobic co-digestion of acorn slag waste with dairy manure in a batch digester: Focusing on mixing ratios and bio-based carbon accelerants," *Bioresour Technol*, vol. 286, pp. 121–394, Aug. 2019.
[5] G. Manthos, D. Zagklis, V. Papavasileiou, N. A. Gkountou, Z. Saita, C. Zafiri, and M. Kornaros, "High-rate upflow anaerobic sludge blanket bioreactor for the treatment of olive mill effluents: Laboratory and pilot scale systems investigation," *Renew Energy*, vol. 217, pp. 119–215, Nov. 2023.
[6] H. I. Owamah, S. C. Ikpeseni, M. I. Alfa, S. O. Oyebisi, S. Gopikumar, O. David Samuel, and S. C. Ilabor, "Influence of inoculum/substrate ratio on biogas yield and kinetics from the anaerobic co-digestion of food waste and maize husk," *Environ Nanotechnol Monit Manag*, vol. 16, pp. 100–558, Dec. 2021.
[7] V. Córdoba, M. Fernández, and E. Santalla, "The effect of different inoculums on anaerobic digestion of swine wastewater," *J Environ Chem Eng*, vol. 4, no. 1, pp. 115–122, Mar. 2016.
[8] V. Córdoba, M. Fernández, and E. Santalla, "The effect of substrate/inoculum ratio on the kinetics of methane production in swine wastewater anaerobic digestion," *Environmental Science and Pollution Research*, vol. 25, no. 22, pp. 21308–21317, Aug. 2018.
[9] K. Dhamodharan, V. Kumar, and A. S. Kalamdhad, "Effect of different livestock dungs as inoculum on food waste anaerobic digestion and its kinetics," *Bioresour Technol*, vol. 180, pp. 237–241, Mar. 2015.
[10] B. Aylin Alagöz, O. Yenigün, and A. Erdinçler, "Enhancement of anaerobic digestion efficiency of wastewater sludge and olive waste: Synergistic effect of co-digestion and ultrasonic/microwave sludge pre-treatment," *Waste Management*, vol. 46, pp. 182–188, Dec. 2015.
[11] Standard Methods for the Examination of Water and Wastewater, 23rd ed., American Public Health Association., APHA, 2017.
[12] B. M. Esteves, C. S. D. Rodrigues, F. J. Maldonado-Hódar, and L. M. Madeira, "Treatment of high-strength olive mill wastewater by combined Fenton-like oxidation and coagulation/flocculation," *J Environ Chem Eng*, vol. 7, no. 4, pp. 103–252, Aug. 2019.
[13] M. Aboelfetoh, A. Hassanein, M. Ragab, M. El-Kassas, and E. R. Marzouk, "Olive mill waste-based anaerobic digestion as a source of local renewable energy and nutrients," *Sustainability,* vol. 14, no. 3, pp. 14–02, Jan. 2022.
[14] M. A. Dareioti, S. N. Dokianakis, K. Stamatelatou, C. Zafiri, and M. Kornaros, "Exploitation of olive mill wastewater and liquid cow manure for biogas production," *Waste Management*, vol. 30, no. 10, pp. 1841–1848, Oct. 2010.
[15] L. M. Fleyfel, N. K. V. Leitner, M. Deborde, J. Matta, and N. H. El Najjar, "Olive oil liquid wastes–Characteristics and treatments: A literature review," *Process Safety and Environmental Protection*, vol. 168, pp. 1031–1048, Dec. 2022.
[16] N. A. Oz and A. C. Uzun, "Ultrasound pretreatment for enhanced biogas production from olive mill wastewater," *Ultrason Sonochem*, vol. 22, pp. 565–572, Jan. 2015.
[17] P. G. Kougias, T. A. Kotsopoulos, and G. G. Martzopoulos, "Effect of feedstock composition and organic loading rate during the mesophilic co-digestion of olive mill wastewater and swine manure," *Renew Energy*, vol. 69, pp. 202–207, Sep. 2014.
[18] J. A. Rubio, L. I. Romero, A. C. Wilkie, and J. L. García-Morales, "Mesophilic Anaerobic co-digestion of olive-mill waste with cattle manure: effects of mixture ratio," *Front Sustain Food Syst*, vol. 3, pp. 429–565, Feb. 2019.
[19] F. M. Pellera and E. Gidarakos, "Effect of substrate to inoculum ratio and inoculum type on the biochemical methane potential of solid agroindustrial waste," *J Environ Chem Eng*, vol. 4, no. 3, pp. 3217–3229, Sep. 2016.
[20] F. Xu and Y. Li, "Solid-state co-digestion of expired dog food and corn stover for methane production," *Bioresour Technol*, vol. 118, pp. 219–226, Aug. 2012.
[21] A. Boulanger, E. Pinet, M. Bouix, T. Bouchez, and A. A. Mansour, "Effect of inoculum to substrate ratio (I/S) on municipal solid waste anaerobic degradation kinetics and potential," *Waste Management*, vol. 32, no. 12, pp. 2258–2265, Dec. 2012.
[22] F. Raposo, R. Borja, M. A. Martín, A. Martín, M. A. de la Rubia, and B. Rincón, "Influence of inoculum–substrate ratio on the anaerobic digestion of sunflower oil cake in batch mode: Process stability and kinetic evaluation," *Chemical Engineering Journal*, vol. 149, no. 1–3, pp. 70–77, Jul. 2009.
[23] S. Bovina, D. Frascari, A. Ragini, F. Avolio, G. N. Scarcella, and D. Pinelli, "Development of a continuous-flow anaerobic co-digestion process of olive mill wastewater and municipal sewage sludge," *Journal of Chemical Technology & Biotechnology*, vol. 96, no. 2, pp. 532–543, Feb. 2021.
[24] S. Benalia, G. Falcone, T. Stillitano, A. I. De Luca, A. Strano, G. Gulisano, G Zimbalatti, B. Bernardi, "Increasing the content of olive mill wastewater in biogas reactors for a sustainable recovery: Methane productivity and life cycle analyses of the process," *Foods*, vol. 10, no. 5, pp. 10–29, May 2021.
[25] Y. Gu, X. Chen, Z. Liu, X. Zhou, and Y. Zhang, "Effect of inoculum sources on the anaerobic digestion of rice straw," *Bioresour Technol*, vol. 158, pp. 149–155, Apr. 2014.
[26] N. Solomakou and A. M. Goula, "Treatment of olive mill wastewater by adsorption of phenolic compounds," *Reviews in Environmental Science and Bio/Technology,* vol. 20, no. 3, pp. 839–863, Jul. 2021.
[27] I. Hamdi, O. Elasri, K. Lihi, H. Hamdaoui, N. Auajjar, A. Chafi, and B. Attarassi, "Comparative evaluation of biogas yield and physicochemical properties of three-phase and traditional olive oil mill wastes – the most suitable choice for efficient anaerobic digestion," *Ecological Engineering and Environmental Technology*, vol. 24, no. 5, 2023.
[28] R. Borja, C. J. Banks, R. Maestro-Durán, and J. Alba, "The effects of the most important phenolic constituents of olive mill wastewater on batch anaerobic methanogenesis," *Environ Technol*, vol. 17, no. 2, pp. 167–174, Feb. 1996.
[29] P. M. Fedorak and S. E. Hrudey, "The effects of phenol and some alkyl phenolics on batch anaerobic methanogenesis," *Water Res*, vol. 18, no. 3, pp. 361–367, Jan. 1984.