

Article

Factors That Affect Methane Yield Using Raw Olive Alperujo (Unhydrolyzed) as Substrate in BMP Assays

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Abstract: The olive alperujo (OA) corresponds to the solid waste generated in the olive oil extraction process using the two-phase centrifugation method. OA is produced in large quantities (800 kg OA/ton olives processed) and is characterized by its high moisture content, organic matter, and low pH. In Chile, the olive oil industry is recent, and one of its main challenges is to be able to manage OA to reduce the impact caused by its disposal. In this sense, its valorization as biogas by means of anaerobic digestion is an economically attractive option. For this, it is previously necessary to evaluate the biomethane potential (BMP) of the raw OA using batch assays. This study was focused on evaluating the factors that most affect the methane yield (MY) when using OA as substrate in BMP tests. First, a sweep analysis (Plackett–Burman) was applied to determine those factors that, according to the literature, would have an influence on the BMP tests. Among the factors studied, the most significant were preincubation, OA concentration, and agitation level. Subsequently, a 2³ factorial experimental design was applied to evaluate the effect of these factors on MY at different levels. Results show that the OA concentration was the most significant factor affecting MY.

Keywords: BMP assays; factorial experimental design; Gompertz model; methane yield; olive alperujo



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1. Introduction

The olive oil industry in Chile is recent, with its beginning set in the late 1990s [1]. This industrial sector is growing and stands out to produce high-quality extra virgin olive oil. The main cultivated varieties are Arbequina (57%), Arbosana (20%), Italian varieties Frantoio and Leccino (10%), and others such as Picual, Koroneiki, and Coratina [1]. At the national level, production has increased by 121% during the last decade, reporting 18,500 tons in 2019 [1]. Most of this production (60%) is exported to countries such as Brazil, the United States, China, Spain, etc. [1]. Chile is ranked 10th among the exporting countries of olive oil worldwide. The national total area of olive plantations in 2019 was 25,000 hectares, distributed between the Atacama Region and the Maule Region [1].

During the 2018/2019 season, according to information published by the International Olive Council, the world production of olive oil reached 3,217,000 tons [1]. Spain is the largest producer (45.3%), followed by Italy (10.8%) and Greece (8.4%) [1]. Chile contributes

0.6% of world production and is recognized for specializing in the production of high-quality extra virgin olive oil [1]. One of the main challenges facing the industry is the management of solid waste, with the aim of promoting its valorization [2,3]. Therefore, in 2013, the Association of Olive Oil Producers Chilean signed the first Clean Production Agreement, which was renewed in 2020 [1].

According to the type of olive oil extraction technology, the characteristics of solid waste generated vary [4,5]. The most used technology for the extraction of olive oil is the two-phase centrifugation system. This type of system allows reducing the consumption of water (almost 80%) and energy and, therefore, also the generation of wastewater [6]. However, it generates olive alperujo as solid waste, which corresponds to a mixture of the skin and pulp of the olive fruit (20%), the crushed stone (15%), and the oil mill wastewater [7]. The olive alperujo has high moisture content (65–75%), high content of organic matter (e.g., volatile solids/total solids ratio (VS/TS) > 70%), and pH around 5 [2,6,8,9]. According to Alburquerque et al. [4], 800 kg of olive alperujo is generated during the olive oil extraction process for each ton of processed fruit. The high organic matter content of this waste opens the possibility of using it to produce biogas (methane or CH₄) through the application of anaerobic digestion [2,7].

Biomethane potential (BMP) assays are discontinuous tests that allow knowing the anaerobic biodegradability of organic waste by quantifying methane generated [10,11]. The information provided by these assays is used to evaluate the feasibility of implementing the large-scale anaerobic digestion process [11,12]. However, the estimation of the CH₄ generated by complex solid wastes is not an easy task since the results of BMP assays are influenced by several factors (e.g., substrate concentration, the composition of the substrate, pH, temperature, substrate/inoculum (S/I) ratio, etc.) [10–17]. Currently, studies on olive alperujo are focused on evaluating the effect that different pretreatments (mainly thermal) have on the hydrolysis stage, and therefore the production, of biogas (e.g., methane yield) in BMP assays [2,6,8,9]. However, the incorporation of a thermal hydrolysis stage as a pretreatment to the anaerobic digestion process implies an additional increase in treatment costs for this type of substrate [6]. For this reason, the objective of this research was to determine the factors that most affect the methane yield when using raw olive alperujo (unhydrolyzed) as substrate in BMP tests. For this, two experimental statistical designs were carried out [18,19].

2. Materials and Methods

2.1. Olive Alperujo (Substrate) and Inoculum

The substrate raw olive alperujo used in this study was obtained from the Center of the Study of Processed Foods (CEAP by its acronym in Spanish) of Talca, Maule Region, Chile. This substrate is derived from the process of extracting olive oil from the Arbequina variety, the most used in Chile [1]. The raw olive alperujo sample was collected two weeks before the assays and kept refrigerated at 4 °C. For this study, the stones were not removed from the raw olive alperujo sample. This substrate was previously characterized in terms of its physicochemical parameters (e.g., pH, total solids (TS), volatile solids (VS), moisture content, total nitrogen (TN), total protein (TP), total lipids (TL), and total fiber (TF)). The VS/TS ratio was 1.0. Table 1 shows the physicochemical characteristics of raw olive alperujo.

For the BMP assays, an anaerobic inoculum from a lab-scale reactor treating synthetic wastewater at a temperature of 37 °C was used. This inoculum had 24.3 g TS L⁻¹ and 15.1 g VS L⁻¹ (Table 1). Its initial specific methanogenic activity (SMA) was determined using the protocol described by Soto et al. [20], obtaining a value of 0.6 g COD g VSS⁻¹·d⁻¹.

2.2. BMP Assays

The BMP assays were performed according to the protocols described by Angelidaki et al. [12] and Moody et al. [16]. Methane production was measured by liquid displacement of an alkaline solution (40 g NaOH L⁻¹) [21].

Table 1. Physicochemical characterization of the raw olive alperujo (substrate) and the inoculum used in this study (average value \pm standard deviation (SD)).

Parameter	This Study		References		
	Olive Alperujo	Inoculum	[2]	[4]	[8]
pH	4.9 \pm 0.1	7.0 \pm 0.1	4.6 \pm 0.1	5.3	4.9 \pm 0.2
Total solids (TS, g kg ⁻¹)	331.8 \pm 5.0	24.3 \pm 0.0 *	436.5 \pm 0.6 **	–	272.2 \pm 1.7
Volatile solids (VS, g kg ⁻¹)	322.7 \pm 5.2	15.1 \pm 0.0 *	415.8 \pm 6.9 **	–	234.6 \pm 2.5
Moisture content (%)	74.3 \pm 0.9	–	–	64.0	74
Total nitrogen (TN, g kg ⁻¹)	4.1 \pm 0.5	–	–	11.4	–
Total protein (TP, g kg ⁻¹)	25.4 \pm 2.9	–	–	71.5	–
Total lipids (TL, g kg ⁻¹)	45.4 \pm 5.4	–	–	–	–
Total fiber (TF, g kg ⁻¹)	605.7 \pm 9.9	–	–	–	–
VS/TS	1.0	0.6	1.0	–	0.9
Ash (g kg ⁻¹)	43.7 ***	–	–	67.4	–
Specific methanogenic activity (SMA, g COD gVSS ⁻¹ ·d ⁻¹)	–	0.6 \pm 0.0	–	–	–

* Concentration in g L⁻¹, ** mg L⁻¹, *** estimation from TN [4].

These assays were carried out in glass bottles of 0.12 L of total volume and 0.1 L working volume. Different volumes of inoculum, raw olive alperujo (substrate), and mineral medium (micronutrients and macronutrients) were placed in them depending on the type of assay. Due to the low nutrient content in the raw olive alperujo sample (Table 1), it was necessary to add a mineral medium to the assays. Some authors [12,14,22] indicate that this is important for properly carrying out the anaerobic test and is recommended for those substrates that present deficiencies in the nutrient content, as is the case in this study. The micronutrient solution [12] contained the following compounds (per liter): FeCl₃·4H₂O, 1 g; CoCl₂·6H₂O, 1 g; MnCl₂·4H₂O, 0.25 g; CuCl₂·2H₂O, 0.015 g; ZnCl₂, 0.025 g; H₃BO₃, 0.025 g; (NH₄)₆Mo₇O₂₄·4H₂O, 0.045 g; Na₂SeO₃·H₂O, 0.050 g; NiCl₂·6H₂O, 0.025 g; EDTA, 0.5 g; HCl 36%, 0.5 g; resazurin, 0.250 g. An aliquot the 0.4 mL of this solution was added for every 0.1 L of the volume of the liquid phase. The macronutrient solution [12] contained the following compounds (per liter): NH₄Cl, 85 g; KH₂PO₄, 37 g; CaCl₂·2H₂O, 8 g; MgSO₄·4H₂O, 9 g; Na₂S·9H₂O, 20 g; yeast extract, 20 g. An aliquot the 1 mL of this solution was added for every 0.1 L of the volume of the liquid phase. A blank control containing inoculum and water was used. All the assays were performed in triplicate.

Diluted HCl or NaOH solutions were used to adjust the initial pH value to 7.0 \pm 0.1. The pH buffering capacity was increased by adding 1 g of NaHCO₃ per gram of VS of the inoculum. Later, each bottle was bubbled with a mixture N₂:CO₂ (80:20%) for 1 min at a pressure of 1.5 Pa to remove air from the headspace [15]. Finally, the bottles were incubated in a chamber and kept at a temperature of 37 °C (Figure 1).

The duration of the assays was set at 30 days since this time would be sufficient to achieve the maximum degradation of the raw olive alperujo [2,6,17].

2.3. Calculations for BMP

For each assay, the methane yield (N mL CH₄ g VS⁻¹) was calculated according to ISO/DIS, 10,707 [23], while the maximum methane production rate in the experimental (R_{m obs}: N mL CH₄ g VS⁻¹·d⁻¹) was estimated from the maximum slope observed in each curve. These values were compared with those obtained by fitting the experimental data to the Gompertz model (1) [24].

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

where $M(t)$: accumulated methane production (N mL CH₄ g VS⁻¹); P_m : maximum methane potential (N mL CH₄ g VS⁻¹); R_m : maximum methane production rate (N mL CH₄ g VS⁻¹·d⁻¹); e : e number; λ : delay time (d); t : time (d).



Figure 1. System used for the quantification of methane production by liquid displacement: (1) sample; (2) biogas outlet; (3) trap for liquid; (4) Mariotte bottle with alkaline solution; (5) volume output; (6) probe to measure displaced liquid.

2.4. Statistical Analysis

A Plackett–Burman design was previously used to select the relevant quantitative factors affecting methane production. In this design, the evaluated factors were selected according to the literature and classified as low (−1) and high (+1) levels, as shown in Table 2. These factors were preincubation time (X_1), inoculum concentration (X_2), the addition of mineral medium (X_3), pH adjustment (X_4), particle size (X_5), substrate concentration (X_6), air purge from headspace with N₂/CO₂ (X_7), and agitation level (X_8). The preincubation consisted of the depletion of the residual biodegradable organic matter present in the inoculum over 6 days at 37 °C [25]. For the assays with pH adjustment, sodium bicarbonate (NaHCO₃) was added as a buffer in a proportion of 1 g NaHCO₃ per g VS of the substrate. For assays in which the particle size effect was studied, raw and ground (particle size lower than 1 mm) waste was used. The results obtained from the Plackett–Burman design were analyzed by the Minitab 14 tool with a confidence level of 95% (p -value ≤ 0.05), and the factors with the greatest effect (X_1 , X_6 , and X_8) were determined using a Pareto diagram.

Table 2. Factors and levels evaluated for the Plackett–Burman design.

Factors	Level		Unit
	−1	+1	
X_1 Preincubation	0	5	d
X_2 Inoculum concentration	2	26	g VS L ⁻¹
X_3 Mineral medium	No	Yes	–
X_4 pH adjustment	No	Yes	–
X_5 Particle size	Without milling	With milling	–
X_6 Substrate concentration	2	20	g VS L ⁻¹
X_7 Air purge with N ₂ /CO ₂	No	Yes	–
X_8 Agitation level	0	200	rpm

To evaluate the effects of the preincubation time (X_1), substrate concentration (X_2), and agitation level (X_3) on the methane yield (as response variable) in BMP assays, a 2³ factorial experimental design with a central point was applied [18,26]. Table 3 shows the evaluated factors and their levels (low (−1), central point (0), and high (+1)). For the application of this experimental design, the following conditions were established in the BMP tests: (1) inoculum concentration, 5 g VS L⁻¹; (2) mineral medium (micro and

macronutrient solutions) was added; (3) initial pH, 7.0 ± 0.1 ; (4) substrate particle size lower than 1 mm; (5) the headspace of all the bottles were purged with a mixture of $N_2:CO_2$ (80:20) to guarantee anaerobic conditions.

Table 3. Factors and levels evaluated by means of a 2^3 factorial experimental design.

	Factors	Level			Unit
		−1	0	+1	
X_1	Preincubation	0	3	6	d
X_2	Substrate concentration	2	11	20	g VS L ^{−1}
X_3	Agitation level	0	100	200	rpm

For the statistical analysis of the data obtained from the 2^3 factorial experimental design, the Jamovi 1.2.27 program was used (<https://www.jamovi.org/> (accessed on 8 October 2021)) [27]. In the first instance, this analysis considered the revision of the assumptions of homogeneity of the variances (Levene’s test if appropriate) and normality (Shapiro–Wilk test if appropriate). Once these assumptions were confirmed, the parametric type (ANOVA) method with a confidence level of 95% (p -value ≤ 0.05) was applied. In the case of factors with a p -value ≤ 0.05 , a post hoc test (Tukey test or Games–Howell test, as appropriate) was applied to determine the level of the factor studied that generates a significant statistical difference. Finally, the application of a linear regression model was evaluated in order to identify which are the factors most affecting the methane yield (as well as their interactions).

2.5. Analytical Methods

The TS, VS, and moisture content were determined according to the protocols described by standard methods [28]. The substrate was characterized in terms of (1) TP and TN according to the described by Chow et al. [29]; (2) TF according to the protocol AOAC 962.09 [30]; (3) TL according to the Soxhlet method [31]. The pH was evaluated using electrodes (Orion Star A215 brand equipment). Ash content was estimated from the TN content of the sample by means of the equation proposed by Albuquerque et al. [4] as follows:

$$\text{Ash (g kg}^{-1}\text{)} = \frac{\text{TN} - 0.6042}{0.0800} \quad (2)$$

To determine the effect of particle size, the raw olive alperujo samples were ground and sieved by means of an SM-400 mill and standard sieve (Figure 2) [10,11], until obtaining enough amount of sample, with a particle size of <1 mm, to carry out the assays.

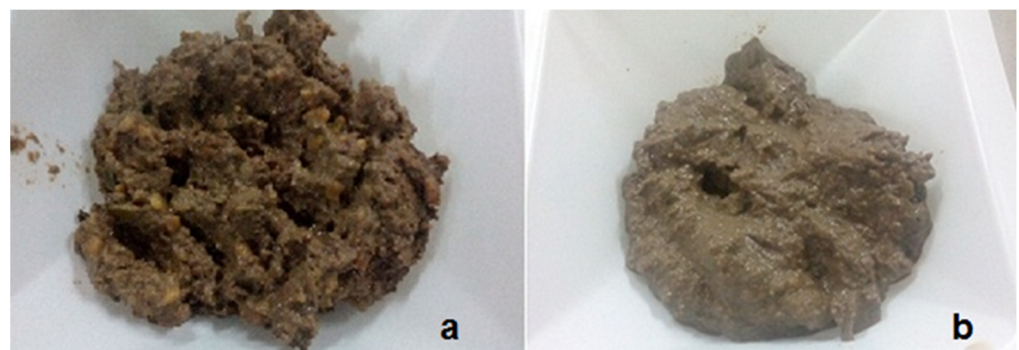


Figure 2. Olive alperujo: (a) without grinding and (b) with grinding.

3. Results

3.1. Preliminary Exploration: Plackett–Burman Design

Methane yield (N mL CH₄ g VS⁻¹) values obtained from the Plackett–Burman design are shown in Table 4. Average methane yield values ranged from 5.7 to 435.1 N mL CH₄ g VS⁻¹, and according to the statistical analysis, the factors that would have a greater effect (Pareto diagram) on the methane yield were substrate concentration (X₆) > preincubation time (X₁) > agitation level (X₈). Based on these results, a 2³ factorial experimental design was applied.

Table 4. Experimental matrix of the Plackett–Burman design and methane yield values obtained (average value ± SD).

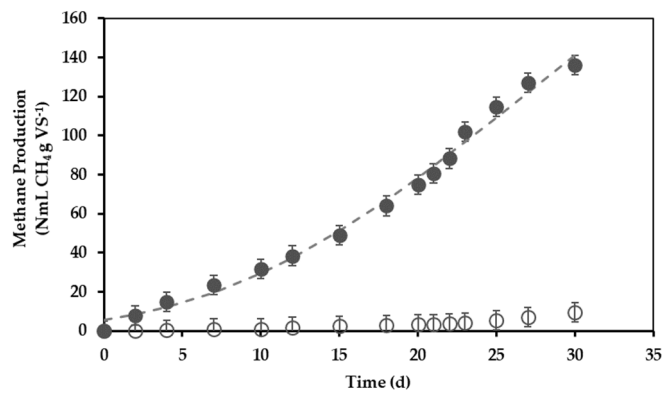
Assay	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	X ₈	Methane Yield (N mL CH ₄ g VS ⁻¹)
1	+	-	+	-	-	-	+	+	158.8 ± 11.3
2	+	-	-	-	+	+	+	-	6.6 ± 0.6
3	+	+	-	+	+	-	+	-	147.5 ± 15.8
4	-	-	-	-	-	-	-	-	169.3 ± 0.9
5	-	-	+	+	+	-	+	+	231.6 ± 189.6
6	-	+	+	+	-	+	+	-	26.5 ± 6.2
7	+	+	-	+	-	-	-	+	165.7 ± 32.8
8	-	-	-	+	+	+	-	+	5.7 ± 1.9
9	-	+	-	-	-	+	+	+	36.1 ± 2.2
10	+	-	+	+	-	+	-	-	36.1 ± 0.6
11	-	+	+	-	+	-	-	-	435.1 ± 121.9
12	+	+	+	-	+	+	-	+	23.4 ± 6.5

3.2. Effect of Preincubation Time, Substrate Concentration, and Agitation Level on Methane Yield: 2³ Factorial Experimental Design

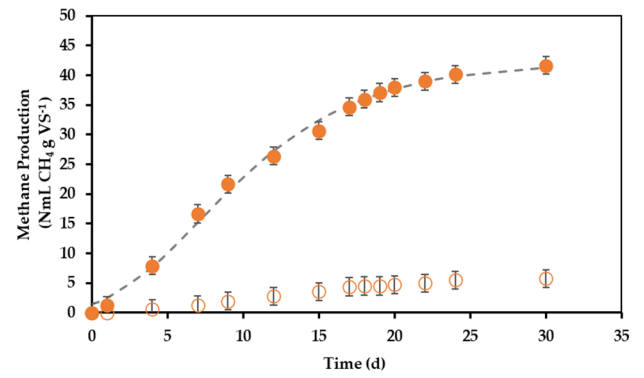
Figure 3 shows the behavior the methane production during the BMP assays. Except in the case of assays 1, 5, and 6 (Figure 3a,e,f, respectively), the variation in methane production at the end of the tests was lower than 1%, and therefore, the steady state was reached according to the criterium proposed by Holliger et al. [32]. Achieving this steady state took 15 d for the tests 2, 3, 4, and central point (Figure 3b–d,i, respectively) and 20 d for assays 7 and 8 (Figure 3g,h, respectively). In the case of tests 1, 5, and 6 (Figure 3a,e,f, respectively), the variation in methane production at the end of the experimental period was around 2%, which fulfilled the criterium of the steady-state achievement according to Fernández-Rodríguez et al. [8]; therefore, the methane yield values obtained in these assays were considered for the statistical analysis.

On the other hand, a lag phase was observed in all assays (Figure 3), except for test 1. In the assays 2, 3, 4, and central point, this lag phase lasted 4 d (Figure 3b–d,i) while in tests 5, 6, 7, and 8, it lasted up to 10 d (Figure 3e–h).

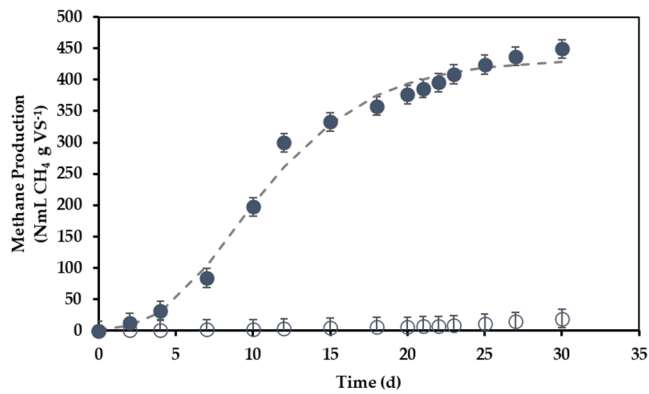
Table 5 shows the methane yield, maximum methane production rate, and the Gompertz model parameters values that were obtained for each BMP assay. The highest methane yield was obtained in test 7, with a value of 480.2 ± 57.4 N mL CH₄ g VS⁻¹, while in assay 2, the lowest methane yield was obtained, with a value of 41.7 ± 0.7 N mL CH₄ g VS⁻¹. In the test corresponding to the central point, the methane yield was 265.8 ± 10.6 N mL CH₄ g VS⁻¹, equivalent to 55% of the highest methane yield value obtained. The R_{m obs} values ranged from 2.6 to 37.8 N mL CH₄ g VS⁻¹·d⁻¹ (Table 5). It can be observed that in the assays with the highest substrate concentration tested (assays 1, 2, 5, and 6), R_{m obs} values were lower than 7.5 N mL CH₄ g VS⁻¹·d⁻¹. Conversely, in those assays with the lowest substrate concentration (assays 3, 4, 7, and 8) R_{m obs} values were higher than 23.0 N mL CH₄ g VS⁻¹·d⁻¹.



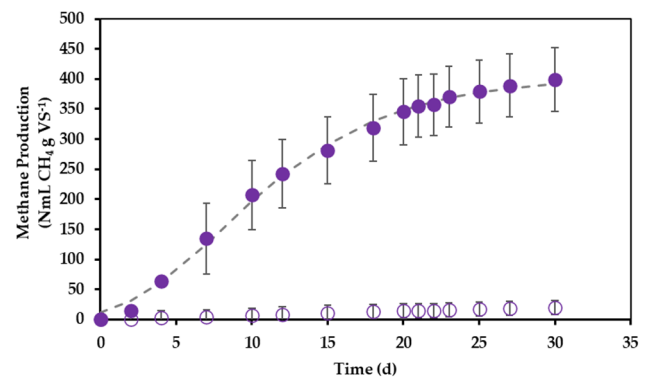
(a)



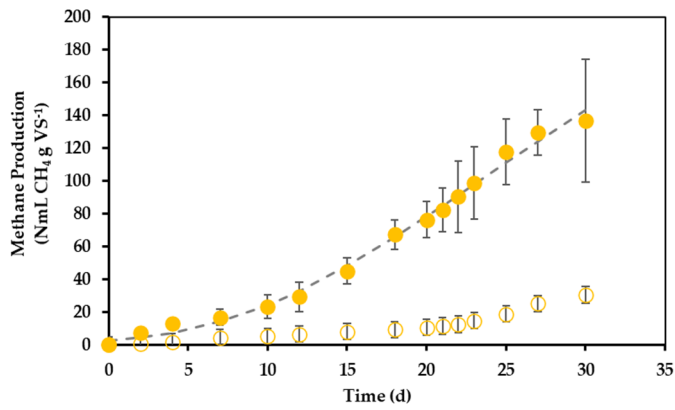
(b)



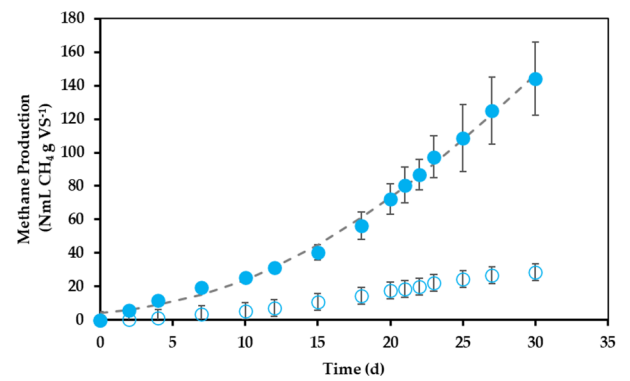
(c)



(d)



(e)



(f)

Figure 3. Cont.

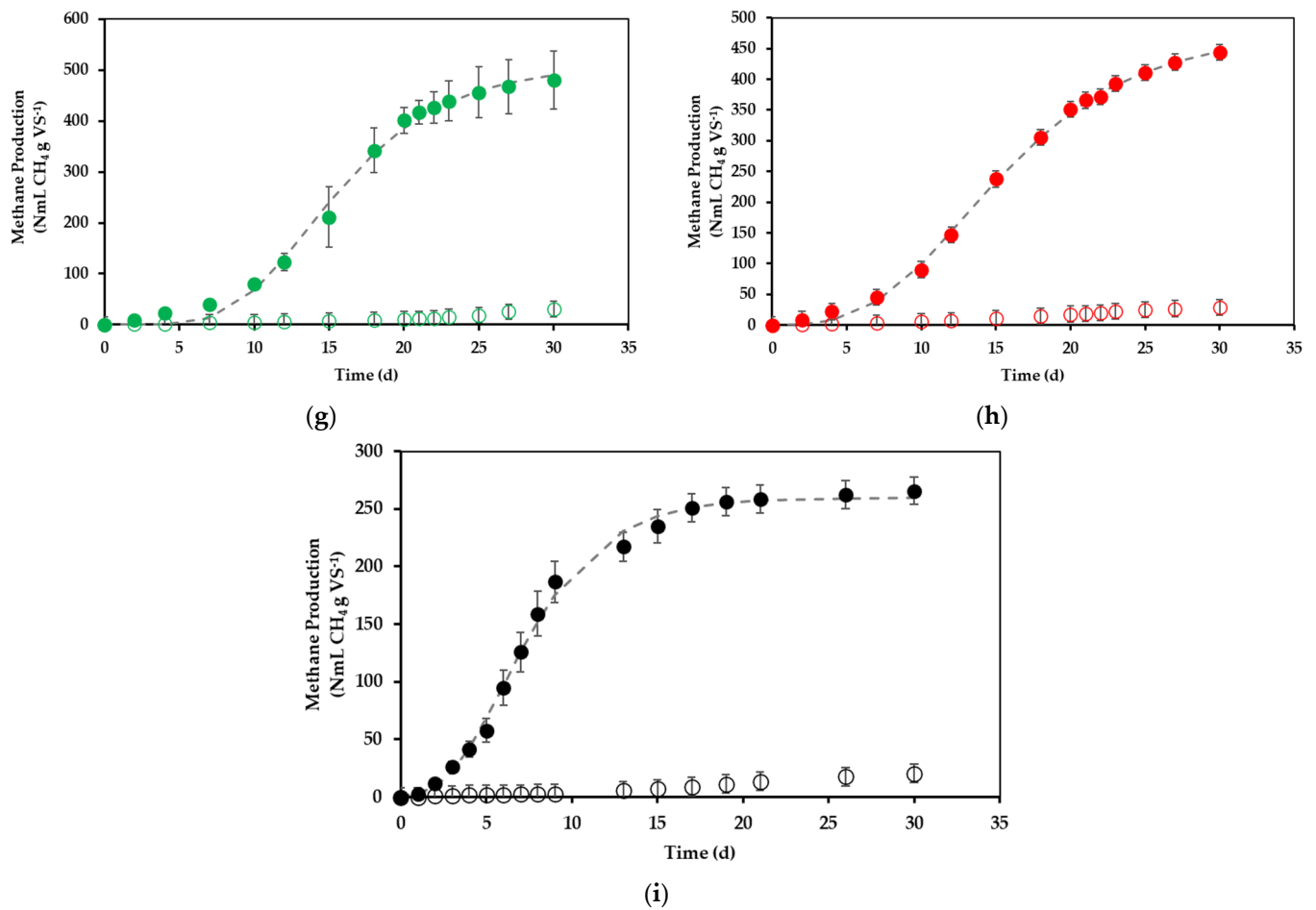


Figure 3. Behavior of the methane production during the incubation time for each of the conditions tested in the 2³ factorial design (average value ± SD): (●) samples of the raw olive alperujo; (○) control assay; (—) adjusted Gompertz model. Assays: (a) 1, (b) 2, (c) 3, (d) 4, (e) 5, (f) 6, (g) 7, (h) 8 and (i) central point.

Table 5. Results corresponding to the experimental conditions and the Gompertz model for a 2³ factorial design (experimental: average value ± SD; model: value obtained from the solver tool).

Assay	X ₁	X ₂	X ₃	X ₁ X ₂	X ₁ X ₃	X ₂ X ₃	X ₁ X ₂ X ₃	Experimental		Model		
								Methane Yield (N mL CH ₄ g VS ⁻¹)	R _m obs (N mL CH ₄ g VS ⁻¹ .d ⁻¹)	P _m (N mL CH ₄ g VS ⁻¹)	R _m (N mL CH ₄ g VS ⁻¹ .d ⁻¹)	λ (d)
1	+	+	+	+	+	+	+	136.0 ± 12.7	5.5 ± 0.4	338.1	6.4	7.9
2	-	+	+	-	-	+	-	41.7 ± 0.7	2.6 ± 0.0	42.1	2.6	1.2
3	+	-	+	-	+	-	-	449.3 ± 11.1	37.8 ± 0.8	433.6	33.0	3.9
4	-	-	+	+	-	-	+	399.0 ± 52.5	23.0 ± 3.0	403.2	23.6	1.7
5	+	+	-	+	-	-	-	136.8 ± 18.8	7.5 ± 1.1	269.1	6.6	8.2
6	-	+	-	-	+	-	+	144.1 ± 21.9	7.3 ± 1.0	461.9	8.0	11.8
7	+	-	-	-	-	+	+	480.2 ± 57.4	36.5 ± 4.4	509.7	36.7	8.5
8	-	-	-	+	+	+	-	443.8 ± 9.8	29.6 ± 0.6	476.7	27.6	6.5
CP	0	0	0	0	0	0	0	265.8 ± 10.6	32.2 ± 1.3	285.8	31.9	2.7

CP: central point.

The kinetic parameters of the Gompertz model (P_m, R_m, and λ) were estimated for each BMP assay (Table 5). The methane yield predicted by the model (P_m) matched relatively well to that obtained experimentally for assays 2, 3, 4, 7, 8, and central point. However, for assays 1, 5, and 6, the Gompertz model overestimated the methane yield by 150%,

96%, and 222%, respectively. The R_m values of the model were similar to those obtained experimentally ($R_{m\text{ obs}}$), ranging from 2.6 to 36.7 N mL CH₄ g VS⁻¹·d⁻¹.

The λ values varied between 1.2 and 11.8 d. For assays 1, 2, 3, 4, and central point, the average value of λ was 3.5 d (assays with agitation), while in tests 5, 6, 7, and 8, the average value of λ was 8.9 d. These values are similar to those obtained experimentally (Figure 3).

A normality test was performed using the Shapiro–Wilk statistic [27]. The results obtained were a p -value ≤ 0.05 for all the factors studied. Consequently, a parametric ANOVA test was used for the statistical analysis of the data. The results obtained from the analysis of variance indicated that the factors preincubation (X_1), substrate concentration (X_2), agitation level (X_3), and some of their interactions (X_1X_3 , $X_1X_2X_3$) affected the methane yield (Equation (2), R^2 : 0.99). The concentration of olive alperujo was the most significant factor (p -value less than 0.001).

$$\begin{aligned} \text{Methane Yield (N mL CH}_4 \text{ g VS}^{-1}) \\ = 271.9 + 23.4X_1 - 161.9X_2 - 26.0X_3 + 13.8X_1X_2 + 10.8X_1X_2X_3 \end{aligned} \quad (3)$$

Figure 4 shows that the higher the concentrations of olive alperujo (20 g VS L⁻¹), the lower the methane yield. This corroborates what was found in the statistical analysis and linear regression model Equation (3).

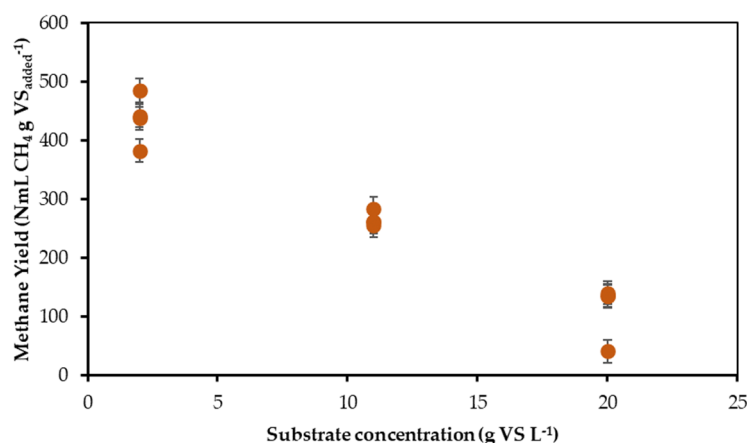


Figure 4. Influence of the olive alperujo concentration (g VS L⁻¹) on the methane yield (average value \pm SD).

4. Discussion

4.1. Raw Olive Alperujo Composition

The raw olive alperujo was characterized by its high content of solids, organic matter, fiber, moisture content, and low pH (Table 1). It was observed that a large part of the solids present in the olive alperujo corresponded to organic matter (VS/TS ratio of 0.97). It was also observed that the nitrogen content was low, with a VS/NT ratio of 78.7.

These results agree with those found by several authors [2,4,6] for this type of solid waste generated from olive oil extraction processes in two stages. In this regard, Albuquerque et al. [4] found that raw olive alperujo is characterized by high moisture content (64%), high C/N ratio (47.8 g g⁻¹), and slightly acidic pH values (5.3) but with low presence of nutrients (TN: 11.4 g kg⁻¹) (Table 1). Regarding the ash content, its average value was 67.4 g kg (range: 24–151 g kg⁻¹) [4] higher than the value of 43.7 g kg⁻¹ estimated in the present study.

Gallego et al. [33] indicated that the VS content of the waste is used as an indicator of the amount of organic matter that can be transformed into biogas. Moreover, these authors indicated that the optimal application of the anaerobic digestion process requires a VS/TS ratio greater than 0.6, moisture content around 80–90%, and a C/N ratio between 20 and

30. Such characteristics are fulfilled by the raw olive alperujo used in the present study; thus, its possible valorization as methane could be feasible.

4.2. Relevant Factors When Using Raw Olive Alperujo as Substrate in BMP Assays

According to the analysis of the results obtained from the Plackett–Burman design (Table 4), substrate concentration affects the methane yield [12,15,25,26,34]. In the case of the assays performed with the highest substrate concentration (20 g VS L⁻¹), an acidification process was observed. For this reason, several authors indicate that a key factor in BMP tests is to maintain an adequate substrate concentration that allows avoiding volatile fatty acid (VFA) accumulation but provides a reliable measure of the biogas generated [25,35,36]. The VDI 4630 [37] guide suggests that the solids content not exceed 10% in order to ensure an adequate mass transfer.

The preincubation time was also found to be a factor affecting the methane yield. In fact, some authors indicate that this factor can have a positive effect on the methane yield curve, reducing both the delay time and the duration of the test [12,25]. Agitation was the third factor that showed a significant effect on the methane yield. Vavilin et al. [38] indicate that the type and duration of agitation can affect the production of biogas in BMP tests. The agitation would favor the mixture between microorganisms/enzymes, substrate, intermediates, and nutrients, in addition to guaranteeing homogeneous conditions.

Several authors [25,39–41] found that the particle size of the waste plays an important role in anaerobic tests since it affects the rate of the hydrolysis stage, which generally limits the rate of the anaerobic process, and, therefore, the rate of methane production [25,40]. However, the analysis of data from the Plackett–Burman design applied showed that the decrease in the raw olive alperujo particle size did not affect the methane yield (Table 4).

4.3. Effects Preincubation Time, Substrate Concentration, and Agitation Level in Methane Yield by BMP Assays

The results obtained in terms of methane yield and production rate were similar to those obtained by other authors [2,6] when carried out BMP assays with this type of waste. Rincón et al. [6] reported a methane yield of 150 N mL CH₄ g VS⁻¹ and an R_m of 44 N mL CH₄ g VS⁻¹·d⁻¹ for a S/I ratio of 2 g VS g VS⁻¹, a temperature of 35 °C, and a stirring speed of 500 rpm, while Fernández-Prior et al. [2] reported methane yields between 150 and 366 N mL CH₄ g VS⁻¹.

The linear regression analysis (Equation (3)) performed in the present study indicates that the preincubation time (*p*-value: < 0.001), substrate concentration (*p*-value: < 0.001), agitation level (*p*-value: < 0.001), and some of the interactions (X₁X₃, *p*-value: 0.011; X₁X₂X₃, *p*-value: 0.028) have significant effects on the methane yield, with substrate concentration as the most influential factor. In fact, the average methane yield decreased from 443 to 115 N mL CH₄ g VS⁻¹ when the raw olive alperujo concentration increased from 2 to 20 g VS L⁻¹ (Table 5 and Figure 4). This increase in substrate concentration caused the methane production rate to decrease 5.6 times (Table 5). This implied that the substrate concentration had a statistically significant effect (*p*-value < 0.001) on the methane yield. In this regard, several authors [12,25,35] indicated that substrate concentration is a key factor in BMP assays since it can affect the activity of microorganisms and, therefore, biogas production. A priori, high concentrations of substrate would enhance the methane production rate, but they can also promote the accumulation of VFA and, thus, the inhibition of the methanogenic Archaea, as occurred in this study, in which a substrate concentration of 20 g VS L⁻¹ was tested [25]. Therefore, the substrate concentration would affect both the methane yield and its production rate. In this regard, Raposo et al. [25] recommend using substrate concentrations lower than 2 g COD L⁻¹ in order to avoid acidification episodes.

According to the linear regression analysis, the preincubation time had a positive effect on the methane yield but was much less than that of the substrate concentration. In fact, some authors [9,15,25,42] recommended a preincubation period of 2–7 days when carrying BMP assays with residues of vegetable origin that have high fiber content, as is the

case of the olive alperujo (Table 1). The preincubation of the inoculum allowed increasing the methane yield up to 14% and its production rate up to 53%. Positive effects on the process kinetics have been already reported in the literature describing the process, which were attributed to both an increase in the methane production rate and a reduction in the delay time [12,14,25]. Nevertheless, in the present research, the effect of preincubation on the delay time was not evident (Figure 3).

The level of agitation had an effect on the methane yield but not on its production rate. The results obtained (Table 5) show that the application of applying agitation during the BMP tests reduced the methane yield by 17%. Contrarily, Raposo et al. [25] found that the agitation applied manual, magnetic, orbital, or otherwise, favored methane yield since it promotes the contact between substrate and inoculum [10,11]. The discrepancy between our results and those observed by Raposo et al. [25] could be attributed to the fact that agitation also would promote the inhibitory effect found in the case of the assays carried out at an olive alperujo concentration of 20 g VS L⁻¹. In fact, the lowest methane yield (41.7 N mL CH₄ g VS⁻¹) was obtained under the conditions with the highest substrate concentration and agitation level.

5. Conclusions

The maximum yield of methane using raw olive alperujo as substrate in BMP assays was 480 N mL CH₄ g VS⁻¹. This occurred under the preincubation conditions of 6 d, a substrate concentration of 2 g VS L⁻¹, and without agitation.

When using raw olive alperujo as substrate in BMP assays, the main factor affecting the methane yield, was the substrate concentration. High olive alperujo concentrations promoted acidification episodes, which inhibited methanogenic Archaea and, therefore, decreased the methane yield.

The preincubation time and the agitation level also affected the methane yield but to a lesser extent. The preincubation of the inoculum increased the methane yield, while the agitation level worsened it, probably due to the promotion of the inhibitory effects caused by high substrate concentrations.

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