EVOLUTION OF CONTROL PARAMETERS IN BIOCHEMICAL METHANE POTENTIAL TESTS OF OLIVE MILL SOLID WASTE (OMSW), THERMAL PRE-TREATED OMSW AND ITS CO-DIGESTION with *Dunaliella salina*

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ABSTRACT

The aim of the present work was to compare the mesophilic anaerobic digestion of untreated olive mill solid waste (OMSW), soft hydrothermal pre-treated OMSW (SHP OMSW) and a co-digestion mixture of 95% OMSW and 5% microalga *Dunaliella salina* (Co-OMSW). During the co-digestion experiment, the volatile fatty acid accumulation decreased in comparison with that obtained for OMSW and SHP OMSW, reducing the slight inhibition observed during the OMSW and SHP OMSW experiments. Final values of methane yield of $380 \pm 1 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}_{\text{added}}$ for the OMSW, $424 \pm 2 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}_{\text{added}}$ for the SHP OMSW and $491 \pm 1 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}_{\text{added}}$ for the co-OMSW were determined. Two mathematical models, first-order kinetics and modified Gompertz model, were employed to fit the experimental data with the aim of elucidating the anaerobic biodegradation and obtain the kinetic constants. Both models allowed for adequately fitting the experimental results of methane production with time. The kinetic constant, k , of the first-order model increased by 12% for the Co-OMSW compared with the values achieved for OMSW and SHP OMSW. The modified Gompertz model revealed that the maximum methane production rate, R_m , for the Co-OMSW and SHP OMSW increased by 34.7% and 10.3% compared to the value obtained for OMSW.

KEYWORDS: olive mill solid waste, soft hydrothermal pre-treatment, anaerobic co-digestion, microalgae; kinetics.

INTRODUCTION

The valorization of waste is the most viable alternative to reduce the large amount of waste generated in agriculture and the agro-industrial sector every year. This recycling strategy not only avoids disposal cost and environmental issues, but also brings a valorization pattern to the agricultural and agro-industrial sectors (Muchagato et al., 2018). The valorization of by-products is a trend that is in progress and has been increasing its value in the last decade (Borges et al., 2019, Meini et al., 2019). The olive oil industry is one of the main agro-economic activities in Spain and this is a sector that is booming worldwide. More and more countries are starting to produce olive oil, although the Mediterranean area contributes more than 80% to the worldwide production. Spain and Italy are the two leaders in olive oil production, while

Greece holds the third place (Carbone et al., 2018). During the 90s, a new extraction system for olive oil, the two-phase system, expanded rapidly in Spain. This innovative system contributed to an improvement in the quality of the product and a decrease in water consumption during the process (del Pozo et al., 2018). Even so, during two-phase olive oil processing a huge amount of highly polluting waste (800 kg of olive mill solid waste for one ton of processed olives) is generated in a short period of time (November-February) every year. The olive mill solid waste (OMSW) is underutilized and often ends up polluting the environment (del Pozo et al., 2018). OMSW is a lignocellulosic by-product with high water content and it is characterized as a highly polluting waste, not only because of the large amount that is generated every year but also due to its composition. OMSW is characterized as a by-product with acidic pH and high electrical conductivity which is very rich in organic matter since it has a high content of sugars, polyalcohols, pectins, lipids and aromatic compounds which give it a phytotoxic and antimicrobial character (Muktadirul Bari Chowdhury et al., 2013). Anaerobic digestion (AD) is a biological process that breaks down organic materials in the absence of oxygen into methane and carbon dioxide, mainly. The AD of OMSW is a widely studied process (Borja et al., 2002; Rincón et al., 2007; Christoforou and Fokaides, 2016). There is also information about the use of different pre-treatments on OMSW or lignocellulosic wastes before AD where methane production has been improved: thermal pre-treatments on OMSW (Rincón et al., 2013), ultrasound pre-treatments (Rincón et al., 2014), microwave pre-treatments (Rincón et al., 2016) and hydrothermal carbonization (Volpe et al., 2016; Codignole et al., 2018). Another widely studied alternative to improve methane yield during the AD of OMSW is the co-digestion of this substrate with microalgae. The nitrogen-rich co-substrates such as microalgae help to balance the carbon/nitrogen (C/N) ratio in the co-substrate mixture, as well as macro and micronutrients, pH, inhibitors/toxic compounds and also accelerating the hydrolysis process. As a result of the co-digestion mixture of OMSW with different microalgae an improvement in biogas production was previously reported (Fernández-Rodríguez et al., 2014; Fernández-Rodríguez et al., 2019).

The biological methane potential (BMP) test has proved to be a suitable method to obtain maximum methane production and provide valuable information for optimizing the design and functioning of an anaerobic digester (Holliger et al., 2016; Xu et al., 2019). Literature related to the BMP assays is extensive, and shows that this test has been used to evaluate a wide variety of substrates. However, there is scarce information and very few reports on the evolution or temporal variation in the parameters of a BMP assay and there is no information on the evolution of BMP test parameters

comparing a substrate, its co-digestion with another co-substrate and the pre-treatment of that same substrate. This type of information can help improve the implementation of a full scale AD plant as well as provide important information about the evolution of the AD process.

This work aims to give information about the evolution of parameters during the BMP test of raw OMSW, soft hydrothermal pretreated OMSW (SHP OMSW) and co-digestion of 95% OMSW and 5% microalga *Dunaliella salina* (Co-OMSW). The parameters soluble chemical oxygen demand (sCOD), pH, alkalinity, ammonium, volatile fatty acids (VFA) and methane production were evaluated over time. At present, no previous available literature has reported on the comparative evolution of different parameters during the AD of this raw substrate, its AD previously thermally pre-treated (SHP OMSW) or a co-digestion mixture of the same substrate with microalga *Dunaliella salina*.

MATERIALS AND METHODS

Substrates and inoculum

Three different substrates were used: (i) OMSW, (ii) OMSW, pre-treated with a soft hydrothermal pre-treatment at 121°C, 30 minutes 1.1 bar (SHP OMSW) and (iii) the co-digestion mixture of 95% OMSW and 5% microalga *Dunaliella salina* (95% OMSW-5% *D. salina*).

The pre-treatment at 121 °C and 1.1 bars during 30 min was chosen based on previous results obtained from different soft hydrothermal pre-treatments carried out on OMSW (Fernández-Rodríguez et al., 2020). 500 g of OMSW were introduced into a 1L autoclaved bottle and then it was autoclaved under the conditions selected. The sample was then cooled to room temperature and stored at 4 °C for less than 24 h until use.

The unicellular, marine green algae *Dunaliella salina* is a halophilic flagellate microalga able to tolerate and acclimate to a wide range of salts (Hadizadeh et al., 2020). The possibility of cultivating it up in the brines of table olive, the main wastewater produced in the olive sector (Rincón-Llorente et al., 2018), would help to reduce the need of fresh water and would opt for a very low-cost in its cultivation system (Hadizadeh et al., 2020). The co-digestion mixture assayed was chosen based on previous AD

studies which observed a decrease in the methane production when the percentage of *D. salina* increased in the mixtures OMSW-*D. salina* (Fernández-Rodríguez et al., 2014).

Table 1 shows the main characteristics of the substrates and the inoculum used during the experiments. OMSW was collected from the Experimental Olive Oil Mill Factory located in the ‘Instituto de la Grasa (CSIC)’, Seville (Spain). Olive stone pieces were removed using a 2 mm mesh. *Dunaliella salina* (*D. salina*) was provided as a lyophilized biomass by University of Huelva, Huelva (Spain). The main characteristics of the *D. salina* are presented in Table 1.

Anaerobic reactors were inoculated with biomass obtained from an industrial up-flow anaerobic sludge blanket reactor for wastewater from a brewery located in Seville (Spain). The inoculum was chosen due to its high methanogenic activity, which was determined in previous anaerobic assays (Fernández-Rodríguez et al., 2019).

Biochemical methane potential (BMP) tests

The tests were carried out in a multi-batch reactor system with an effective volume of 200 mL. The reactors were continuously agitated by magnetic bars at 440 rpm and placed in a thermostatic water bath at mesophilic temperature (35 ± 2 °C).

The inoculum to substrate ratio was 2 (VS basis). Each reactor containing 150 mL of inoculum and the amount of substrate needed to give the required inoculum to substrate ratio. Finally, 150 µL of a trace element solution were added. The composition of trace element solution is given elsewhere (Fernández-Rodríguez et al., 2019).

Reactors with inoculum and trace element solution but without substrate addition were used as blank controls. The methane production of the blank controls reactors was removed from the final results of the methane yield of the three experimental groups.

The reactors were sealed and the headspace of each flask was flushed with nitrogen at the beginning of the assay. The produced biogas was passed through a 3N NaOH solution to capture CO₂; the remaining gas was assumed to be methane. The biogas volume was expressed at standard pressure and temperature conditions (273K, 1bar). The AD experiments were run for a period of c.a. 34 days until the accumulated gas production remained essentially unchanged. 10 reactors of each experiment were placed

and 7 of them were sacrificed for carrying out different analyses in order to evaluate the evolution of the different parameters.

Analytical methods

All analyses were performed according to the Standard Methods of APHA (APHA, 1998). The following parameters were measured: total chemical oxygen demand (COD), soluble chemical oxygen demand (sCOD), total solids (TS), volatile solids (VS), Total alkalinity (TA), pH, Total Kjeldahl Nitrogen (TKN) and volatile fatty acids (VFA). Soluble parameters were determined after sample centrifugation (Eppendorf, 10000 rpm, 10 min) and filtration (glass fiber filter 47 mm).

TS and VS were determined according to the standard methods 2540B and 2540E, respectively (APHA, 1998); COD was determined by the method described in detail elsewhere (Raposo et al., 2008), while sCOD was determined using the closed digestion and the colorimetric standard method 5220D (APHA, 1998). pH was determined using a pH-meter model Crison 20 Basic. TA was determined by pH titration to 4.3 (APHA, 1998). TKN was determined using a method based on the 4500-Norg B of Standard Methods (APHA, 1998).

Individual VFA from C2 to C7 including iso-C4, iso-C5 and iso-C6 were analyzed using a Gas Chromatograph (Shimadzu GC 2010) equipped with a flame ionization detector (FID) and a capillary column filled with Nukol (polyethylene glycol modified by nitroterephthalic acid). Prior to injection, 900 μL of the sample were mixed with 150 μL of H_3PO_4 (1:2, v:v) to adjust the pH to below 2.0 and 150 μL of a solution of crotonic acid (2000 mg L^{-1}) as internal standard. This mixture was centrifuged to remove any solids and transferred to a 1500 μL gas chromatography vial; the sample injection volume was 1 μL . The temperatures of the injector and detector were maintained at 200 $^{\circ}\text{C}$ and 250 $^{\circ}\text{C}$, respectively, while the column temperature was increased from 120 to 160 $^{\circ}\text{C}$ at an increasing rate of 10 $^{\circ}\text{C min}^{-1}$.

RESULTS

Table 2 shows the pH evolution of the three experiments. The pH trend did not show relevant fluctuations in any of the experiments. During the AD of OMSW, the minimum pH value was 7.6 at the beginning of

the experiment and the maximum value, 8.1, was reached at the end of the experiment. When the AD of SHP OMSW and co-OMSW were carried out, the pH values were between 7.5 and 8 (Table 2).

The evolution of the TA values throughout the OMSW experiment ranged from 4349 ± 66 mg $\text{CaCO}_3 \text{ L}^{-1}$ to 5551 ± 62 mg $\text{CaCO}_3 \text{ L}^{-1}$, during SHP OMSW experiment TA values ranged from 3341 ± 74 mg $\text{CaCO}_3 \text{ L}^{-1}$ to 5534 ± 67 mg $\text{CaCO}_3 \text{ L}^{-1}$ and from 4182 ± 68 mg $\text{CaCO}_3 \text{ L}^{-1}$ to 5693 ± 82 mg $\text{CaCO}_3 \text{ L}^{-1}$ for the co-digestion experiment (Table 2).

VFA content is one of the most widely used parameter to control the stability of the AD process. VFA concentration is the main factor affecting the intermediate alkalinity (Yu et al., 2018). The variation in VFA throughout time is summarized in Figure 1. Throughout the SHP OMSW experiment, the VFA concentration was at its highest on day 4. The concentration of iso-butyric acid, butyric acid, valeric acid and caproic acid in the SHP OMSW experiment reached values of 625 mg L^{-1} , 477 mg L^{-1} , 466 mg L^{-1} and 484 mg L^{-1} , respectively. The total VFA concentration reached on day 4 was 2052 mg L^{-1} . The pre-treatment accelerated the solubilization (hydrolysis) of OMSW and reduced the particle size so that a shortened hydrolytic stage could be observed. The total VFA concentrations observed during the AD of OMSW were significantly higher than those obtained for the SHP OMSW experiments. The OMSW VFA accumulation started on day 2 and was at its highest on day 6. Then the VFA concentration decreased until 0 on day 8. The maximum VFA peak was observed two days later than that observed during the SHP OMSW AD. The highest VFA accumulation during OMSW AD was on day 6 and mainly consisted of acetic acid (1710 mg L^{-1}), iso-butyric acid (825 mg L^{-1}), valeric acid (448 mg L^{-1}) and caproic acid (251 mg L^{-1}), reaching a total VFA concentration of 3226 mg L^{-1} . During the co-digestion experiment, the main VFA concentrations were caproic acid (747 mg L^{-1}), iso-butyric acid (578 mg L^{-1}) and valeric acid (421 mg L^{-1}) on day 6, but similar values were reached on day 8 (iso-butyric acid= 460 mg L^{-1} and caproic acid= 747 mg L^{-1}). The VFA accumulation started on day 2 and was observed until day 8 (Figure 1).

Figure 2 shows the evolution of sCOD throughout the trials. The organic matter solubilization was quite different throughout the three experiments. In the co-digestion experiment, a constant concentration of soluble organic matter was observed. The contribution of microalgae to the system added a more easily biodegradable organic matter than the lignocellulosic OMSW, maintaining the values of sCOD almost constant at around $5000 \text{ mg O}_2 \text{ L}^{-1}$. Only a decrease in the soluble organic matter concentration was

observed between days 8 and 10, in which, sCOD values of 3864 mg O₂ L⁻¹ and 4328 mg O₂ L⁻¹ were reached, respectively (Figure 2). The sCOD concentration in the OMSW and SHP OMSW experiments often fluctuates considerably. The initial sCOD value in the OMSW experiment was 2203 mg O₂ L⁻¹, and 2771 mg O₂ L⁻¹ for the SHP OMSW experiment, reaching values of 5455 mg O₂ L⁻¹ and 5309 mg O₂ L⁻¹ for the AD of SHP OMSW and OMSW, respectively.

The effect of pre-treatment (SHP OMSW) and anaerobic co-digestion of OMSW and the microalgae *D. salina* in a mixing ratio of 95 and 5%, respectively, were investigated in comparison with the AD of raw OMSW. The methane yields for the three experiments are shown in Figure 3. After 34 days of AD the cumulative methane production showed the same variation tendency in each experiment. The methane production in the entire test grew exponentially in the first 8 days and then it was stabilized. Despite being a difficult biodegradable substrate, the methane production was immediate in each of the three experiments, possibly because the bacteria used an inner fraction that was easily degradable, thus, the hydrolysis step might have begun with this more available substrate (Cirne et al., 2007). In all three cases the methane yield became stable from the 22nd day after the experiment had started. Finally, higher methane productions were observed for the co-digestion than for individual substrates, OMSW and SHP OMSW. Final values of 380±1 mL CH₄ g⁻¹ VS_{added} for the OMSW, 424±2 mL CH₄ g⁻¹ VS_{added} for the SHP OMSW and 491±1 mL CH₄ g⁻¹ VS_{added} for the co-digestion mixture were determined.

Estimation of model parameters by kinetic modeling

First-order kinetic model

In order to study the process kinetics and estimate the process performance in the AD and co-digestion of the three cases studied, the following first-order kinetic model was used:

$$G = G_m \cdot [1 - \exp(-k \cdot t)] \quad (1)$$

where G is the cumulative specific methane production (mL CH₄ g⁻¹ VS_{added}), G_m is the ultimate methane production (mL CH₄ g⁻¹ VS_{added}), k is the specific rate constant (days⁻¹) and t is the digestion time (days). This kinetic model is normally applied to assess the kinetics of the batch AD processes for different types of biodegradable substrates (Li et al., 2012; Scarcelli et al., 2020). This model is based on the assumption that methane production is proportional to the amount of substrate and not limited by microbial cell mass (Wang et al., 2017).

Table 3 summarizes the kinetic parameters obtained from Eq. (1) for the co-digestion mixture and the two substrates studied alone. Values situated after $\pm$ represent the standard deviation of each parameter. As can be seen, deviations between the experimental G_m values (Figure 3) and the theoretical ones (Table 3) lower than 7.7% were obtained for all the cases studied. In addition, the low values of the standard deviations and the high determination coefficient values prove the appropriate fit of the experimental results to the proposed model.

As can be seen in Table 3, the ultimate methane production increased by 8.5% and 24% when the OMSW was thermally-pre-treated and co-digested with *D. salina* (95% OMSW-5% *D. salina*) respectively, compared to the value obtained for untreated OMSW.

Modified Gompertz Kinetic Model

On the other hand, the Modified Gompertz Kinetic Model is a sigmoid function that is considered as a type of mathematical model for a time series (Amiri et al., 2017). Therefore, it can be the one of the best functions for predicting the biogas production in a batch-mode AD process. Many researchers have studied the application of different kinetic models and found that the Modified Gompertz model has one of the best fit to the data from biogas or methane production as a function of time under anaerobic processes conducted in batch mode. In addition, the Modified Gompertz model was calibrated and examined using many experimental data (Amiri et al., 2017; Donoso-Bravo et al., 2010; Donoso-Bravo et al., 2016; Li et al., 2011).

In the Modified Gompertz model, the cumulative methane production is related to the digestion time through the following equation:

$$B = B_m \cdot \exp [-\exp [(R_m \cdot e / B_m) \cdot (\lambda - t) + 1]] \quad (2)$$

where B is the cumulative methane production at time t (mL CH₄ g⁻¹ VS_{added}); B_m is the maximum methane production or methane yield potential (mL CH₄ g⁻¹ VS_{added}); R_m is maximum methane production rate (mL CH₄ g⁻¹ VS_{added} · d⁻¹); λ is the lag time (d); t is the digestion time (d) at which the cumulative methane production is calculated; and finally, e is the exp (1)=2.7183. The parameters B_m , R_m and λ were calculated for each one of the runs studied using the non-linear regression approach with the software SigmaPlot 11.0. Table 4 shows the values for the model parameters obtained from the Modified Gompertz model for the three substrates assayed. Similarly to what occurred with the experimental

maximum methane production values, the theoretical maximum methane production was 8 and 23% higher when the OMSW was thermally pre-treated and when it is co-digested with *D. salina* compared to the value obtained for untreated OMSW. Therefore, a considerable increase in the biodegradability of the substrate was observed when the OMSW was thermally pre-treated or co-digested with *D. salina*, especially in this last case, compared to the raw OMSW.

In addition, the differences between measured and predicted methane yields were found to be only 6.7, 10.1 and 12.1% for the untreated OMSW, thermally pre-treated OMSW and OMSW co-digested with *D. salina*, respectively. The high values for the determination coefficients (R^2) and the low values of the standard errors of estimates (Table 4), again show the excellent fit of the experimental results to the Modified Gompertz Model.

The maximum methane production rate, R_m , for the co-digestion of 95% OMSW-5% *D. salina* and thermally pre-treated OMSW increased by 34.7% and 10.3% compared to the values obtained for raw OMSW.

DISCUSSION

The pH values of the three experiments remained relatively constant in all the runs within the range described as optimal for the growth of methanogenic Archaea (Yuan et al., 2016).

In all three cases, the increase in alkalinity in the system was progressive, reaching the highest TA value at the end of the experiment (Table 2). This increase in the TA values coincides with the ammonium accumulation in the system due to the degradation and stabilization of the organic matter that is being hydrolyzed. During the hydrolysis stage of the AD process, the complex organic matter was degraded into simpler soluble molecules that can be used as substrates for microbial metabolism by hydrolysis and acidogenesis. Then the substrates were consumed in the acetogenesis and methanogenesis phases (Mao et al., 2019). Throughout the three experiments, the final ammonium concentration was below the limit established as toxic for the evolution of the AD process (Polizzi et al. 2018). The TA values were also relatively stable, and within the optimal range for the AD process, indicating good process stability and self-buffering capacity during the three experiments carried out (Fannin et al., 1987).

OMSW is a lignocellulosic substrate mainly composed of three types of polymers: cellulose, hemicellulose and lignin. When the raw OMSW was subjected to AD, the hydrolytic bacteria took longer

to decompose the untreated or raw OMSW into simpler substances, and a large accumulation of VFA was observed on the 6th day of the experiment, indicating that the AD process is not as direct as in the case of using the pre-treatment, that helped to break down these lignocellulose polymers. Both SHP OMSW and OMSW AD produced a VFA accumulation above the limit established by Siegert and Banks (2005). Siegert and Banks (2005) reported the inhibition of cellulolytic activity and, therefore, in the rate of cellulose hydrolysis when the VFA concentration was equal to or greater than 2 g L⁻¹, regardless of the system pH. During the co-digestion experiment, although most of the organic matter came from the OMSW, the contribution of the microalgae helped to smooth the hydrolytic stage, making it more evenly-spaced over time, thus improving the AD process. The VFA accumulation decreased in comparison with that obtained for OMSW. The total VFA maximum concentration obtained during the anaerobic co-digestion was lower than those observed during the OMSW and SHP OMSW experiments and did not exceed the limit established by Siegert and Banks (2005) as toxic.

This constant concentration of dissolved organic matter led to a synergism between the nutrients and bacteria involved in the anaerobic digestion process. During the co-digestion of water hyacinth and banana peels, Barua et al. (2019) observed a synergism that balanced the nutrients and the existence of adaptable and dynamic microbial community. The sCOD concentration observed during co-digestion was higher than those observed in the SHP OMSW and OMSW AD experiments. Similar results were reported by Yin et al. (2016) for co-digested activated sludge and food waste. They observed that sCOD was higher in the co-digestion than in the mono-digestion of substrate with and without pre-treatment. The sCOD concentration in the OMSW and SHP OMSW experiments often fluctuates considerably. The initial sCOD value in the OMSW experiment was 2203 mg O₂ L⁻¹, and 2771 mg O₂ L⁻¹ for the SHP OMSW experiment. This small difference is due to the effect of the thermal pre-treatment on the substrate, which helped to break the lignocellulose fibers and solubilized organic matter (Pagliaccia et al., 2019). The effect of thermal pre-treatment was also observed over time, since in the SHP OMSW experiment two maximum peaks of organic matter solubilization were observed, the first one on day 2 and the second one on day 14 after the start of the experiment (figure 1). On the other hand, during the AD of the OMSW, only a maximum peak of organic matter solubilization was observed and it was delayed over time, since it was observed on day 8. These fluctuations in organic matter solubilization did not allow a synergism between nutrients and the bacteria community. During OMSW AD hydrolysis was observed to be the rate-limiting step. The hydrolysis step is the main bottleneck during a lignocellulose

substrate AD process due to the low biodegradability of the substrate (Chiu and Lo, 2016). Thermal pre-treatment helped to break cellulose, hemicellulose and lignin fibers (Rincón et al., 2013) but it was still the hydrolytic bacteria that caused an imbalance in the AD process. However, during co-digestion, an improvement in the hydrolysis step was observed, with the co-substrate acting as a catalyst for hydrolytic enzymes and a synergism was observed to balance the nutrients with the bacterial community. Several authors have also observed an improvement in enzymatic activity during the anaerobic co-digestion of organic waste and microalgae due to the C/N balance as well as macro and micronutrients (Chiu and Lo 2016).

A 21% and 4% increase in methane yield was achieved for co-digestion and SHP OMSW compared to the raw OMSW, respectively. The methane yield increase obtained in the SHP OMSW compared to raw OMSW (4%) was similar to the one reported by Rincón et al. (2013) for the BMP test of thermally pre-treated OMSW compared to the raw OMSW (5%). Rincón et al. (2013) reported a methane yield of $373 \pm 4 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}_{\text{added}}$ for the BMP test of raw OMSW and $392 \pm 14 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}_{\text{added}}$ for the BMP test of pre-treated OMSW at 120 °C for 180 min.

The maximum daily methane production of $48 \pm 8 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}_{\text{added}}$ was achieved on the 7th day for the OMSW BMP test and 58 ± 2 and $76 \pm 5 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}_{\text{added}}$ on day 5 for the SHP OMSW and co-digestion mixture, respectively. Fernández-Rodríguez et al. (2014) also obtained an improvement in methane production through the co-digestion OMSW-microalga *Dunaliella salina*. However, a lower methane yield was achieved with the mixture 75% OMSW-25% *D. salina* ($330 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}_{\text{added}}$), improving OMSW methane production by only 2.8% (Fernández-Rodríguez et al., 2014).

The ultimate methane production obtained by the First-order kinetic model increased by 8.5% and 24% when the OMSW was thermally-pre-treated and co-digested with *D. salina* (95% OMSW-5% *D. salina*) respectively, compared to the value obtained for untreated OMSW.

It was reported that BMP tests of mixtures of source selected organic fraction of municipal solid wastes and sewage sludge showed a methane production increase from 18% to 47% compared to the AD of single sewage sludge (Cabbai et al., 2013). The kinetic constant, k , increased by 12% when the OMSW was co-digested with *D. salina* (95% OMSW-5% *D. salina*) compared with the values achieved for untreated OMSW and thermally pretreated OMSW (0.17 days^{-1} in both cases). In the same way, no significant differences were observed by Donoso-Bravo et al. (2010) in the kinetic constant of this first-

order model during batch AD tests of untreated secondary sludge (from an urban wastewater treatment plant) and thermally pre-treated secondary sludge at 175 °C for 30 minutes (0.22 and 0.18 days⁻¹, respectively). By contrast, a reduction of around 50% was observed in the first-order kinetic constants of BMP tests of wheat straw (from 0.10 to 0.05 days⁻¹) and sugarcane bagasse (from 0.083 to 0.048 days⁻¹) when these wastes were subjected to an autoclaving pre-treatment (at 121 °C, 60 minutes) compared to their respective untreated wastes (Bolado-Rodríguez et al., 2016). This decrease was attributed to the presence of inhibitory compounds for AD after thermal pre-treatment. On the other hand, the co-digestion of a hardly biodegradable substrate, i.e. sugar bagasse (SB), and easily biodegradable waste, i.e. fruit vegetable waste (FVW), at a ratio 70% FVW-30% SB allowed for increasing the kinetic constant from 0.0023 days⁻¹ (100% SB) to 0.022 days⁻¹ (70% FVW-30% SB) (Vats et al., 2019). This increase is much higher than that observed in the present work when the OMSW ($k=0.17$ days⁻¹ for 100% OMSW) was co-digested with *D. salina* at a ratio (95% OMSW-5% *D. salina*) ($k=0.19$ days⁻¹). By contrast, the co-digestion of *Salvinia molesta* (SM), one of the free-floating aquatic weeds (that can grow very rapidly) with rice straw (RS) at different mixture ratios (from 40:60 to 0:100, SM:RS) at different initial pH values (6-8) gave the same first-order kinetic constant values (0.01 days⁻¹) regardless of the concentration of SM and RS in the mixture and initial pH (Syaichurrozi et al., 2018).

The maximum methane production rate, R_m , obtained by the Modified Gompertz Kinetic Model for the co-digestion of 95% OMSW-5% *D. salina* and thermally pre-treated OMSW increased by 34.7 and 10.3% compared to the values obtained for raw OMSW. Therefore, the thermal pre-treatment and co-digestion of this substrate with *D. salina* improved the rate of the AD process of OMSW, accelerating the methane production rate. In this way, Donoso-Bravo et al. (2016) found that the maximum methane production rate of untreated OMSW and thermally pre-treated OMSW at 148 °C for 30 minutes were 28.1 and 42.7 mL CH₄ (g VS·d)⁻¹, respectively, showing an increase of 51% when the substrate was pre-treated. In any case, the R_m values achieved in the present work were always higher than those reported by Donoso-Bravo et al. (2016). In the same way, the modified Gompertz model applied to the batch AD of the microalga *Chlorella sp.* (grown in digested swine waste) and to thermally pre-treated *Chlorella sp.* revealed an increase in the R_m values from 30.5 to 37.5 and to 44.5 mL CH₄ (g VS·d)⁻¹ when the temperature of pre-treatment increased from 70 to 90 and 120 °C, respectively (Wang et al., 2015). These values were always higher than those obtained for untreated *Chlorella sp.* (25.1 mL CH₄ (g VS·d)⁻¹) (Wang et al., 2015).

On the other hand, the R_m values found in the present work either for untreated OMSW or a mixture of 95% OMSW-5% *D. salina* were always much higher than those found during the anaerobic co-digestion of 40% *Salvinia molesta* and 60% rice straw ($2.1\text{--}2.6 \text{ mL CH}_4 (\text{g VS}\cdot\text{d})^{-1}$) regardless of the initial pH of the mixture (6-8) (Syaichurrozi et al., 2018).

Similarly to what occurred in the present work, the anaerobic co-digestion of mixed microalgae (*Scenedesmus* sp. and *Chlorella* sp.) (MA) – food waste (FW) at a ratio of 20% MA-80% FW showed a R_m value of $44.3 \text{ mL CH}_4 (\text{g VS}\cdot\text{d})^{-1}$, which was 15% higher than that obtained for 100% FW ($38.6 \text{ mL CH}_4 (\text{g VS}\cdot\text{d})^{-1}$) and 266% higher than that achieved for 100% MA ($12.1 \text{ mL CH}_4 (\text{g VS}\cdot\text{d})^{-1}$) (Zhen et al., 2016).

CONCLUSIONS

The performance of AD of OMSW, thermally pre-treated OMSW and the co-digestion of 95% OMSW-5% *D. salina* were evaluated in this research and the corresponding biological degradations and kinetics of the processes were analyzed through model simulation. The introduction of the microalga *D. salina* improved OMSW digestion performance, maintaining relatively constant soluble organic matter concentrations, reducing the VFA accumulation. The co-digestion reached the highest methane yield of $491 \pm 22 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}$ at a ratio of 95% OMSW-5% *D. salina*, achieving 1.29-fold increase in comparison to that of the single OMSW ($380 \pm 28 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}$). The first-order and the modified Gompertz models showed a good fit to the experimental results in the three scenarios studied, and, thus, could describe the kinetics of the digestion of OMSW, thermally pre-treated OMSW and the co-digestion of 95% OMSW-5% *D. salina*. The highest values for the kinetic constant (k) and the maximum methane production rate (R_m) achieved in the co-digestion of 95% OMSW-5% *D. salina* revealed the robustness of the co-digestion of this mixture compared to the single digestion of raw OMSW or thermally pre-treated OMSW.

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Table 1. Characteristics of the inoculum, olive mill solid waste (OMSW), and microalga *Dunaliella salina* used in the experiments. Where TS: total solids, VS: volatile solids, COD: total chemical oxygen demand, sCOD: soluble chemical oxygen demand, TKN: Total Kjeldahl nitrogen, TA: total alkalinity, nd: not determined. Values represent means $\pm$ standard errors, n=3.

	Values	Values	Values
Parameters	Inoculum	OMSW*	<i>D. salina</i> **
TS (g kg ⁻¹)	27.7 $\pm$ 1.8	231.5 $\pm$ 2.3	887.9 $\pm$ 7.3
VS (g kg ⁻¹)	20.9 $\pm$ 1.7	201.8 $\pm$ 2.6	472.3 $\pm$ 8.1
COD (g O ₂ kg ⁻¹)	nd	325.1 $\pm$ 0.4	272 $\pm$ 8
sCOD (g O ₂ kg ⁻¹)	nd	144.4 $\pm$ 4.1	nd
TKN (g kg ⁻¹)	nd	nd	7. 9 $\pm$ 0.7
pH	7.1 $\pm$ 0.2	4.7 $\pm$ 0.1	8.22 $\pm$ 0.2 (1:20)***
TA (g CaCO ₃ kg ⁻¹)	nd	2.7 $\pm$ 0.0	nd

*Concentrations expressed as: weight/weight of wet sample. **Concentrations expressed as: weight/weight of lyophilized sample. *** (w:v) using distilled water.

Table 2. pH and total alkalinity (TA) evolution in the batch anaerobic digestion experiments of olive mill solid waste (OMSW), soft hydrothermal pre-treated OMSW (SHP OMSW), and co-digestion of 95% OMSW and 5% *Dunaliella salina*. Values represent means $\pm$ standard errors, n=3.

Substrate	Day	pH	TA (mg CaCO ₃ L ⁻¹)
OMSW	2	7.59	4349 $\pm$ 13
	4	7.81	4481 $\pm$ 58
	6	7.81	4364 $\pm$ 02
	8	7.79	4528 $\pm$ 71
	11	7.88	4040 $\pm$ 54
	14	7.86	4216 $\pm$ 47
	21	8.08	5551 $\pm$ 00
	34	8.32	5190 $\pm$ 16
Co-digestion mixture (95% OMSW-5% <i>Dunaliella</i> <i>salina</i>)	2	7.49	4495 $\pm$ 12
	4	7.68	4182 $\pm$ 61
	6	7.63	4370 $\pm$ 28
	8	7.77	4818 $\pm$ 11
	11	7.89	4812 $\pm$ 95
	14	7.89	4799 $\pm$ 21
	21	8.13	5148 $\pm$ 52
	34	8.36	5693 $\pm$ 20
SHP OMSW	2	7.46	3828 $\pm$ 21
	4	7.71	4608 $\pm$ 17
	6	7.59	3341 $\pm$ 46
	8	7.71	4595 $\pm$ 34
	11	7.54	4998 $\pm$ 47
	14	7.82	4609 $\pm$ 65
	21	7.94	4986 $\pm$ 30
	34	8.30	5534 $\pm$ 76

*NF: not founded

Table 3. Kinetic parameters obtained from the first-order kinetic model in the batch anaerobic digestion experiments of OMSW, soft hydrothermal pre-treated (SHP OMSW), and co-digestion of 95% OMSW and 5% *Dunaliella salina*. Values represent means $\pm$ standard errors.

Substrate	G_m (mL CH ₄ g ⁻¹ VS _{added})	k (days ⁻¹)	R ²	S.E.E.	Error (%)
OMSW	364 $\pm$ 6	0.17 $\pm$ 0.01	0.9778	22.09	4.1
SHP OMSW	395 $\pm$ 6	0.17 $\pm$ 0.00	0.9811	22.42	6.3
Co-digestion 95%OMSW-5% <i>D. salina</i>	451 $\pm$ 5	0.19 $\pm$ 0.00	0.9862	22.21	7.7

where G_m is the ultimate methane production (mL CH₄ g⁻¹ VS_{added}), k is the specific rate constant (days⁻¹), R²: coefficient of determination, S.E.E.: Standard error of estimate and Error was defined as the difference between measured and predicted methane yield values.

Table 4. Parameters of the Modified Gompertz model for the three substrates studied olive mill solid waste (OMSW), soft hydrothermal pre-treated OMSW (SHP OMSW), and co-digestion of 95% OMSW and 5% *Dunaliella salina*. Values represent means $\pm$ standard errors.

Substrate	B_m (mL CH ₄ g ⁻¹ VS)	R_m (mL CH ₄ g ⁻¹ VS d ⁻¹)	α (d)	R ²	S.E.E.	Error (%)
OMSW	355 $\pm$ 6	40.6 $\pm$ 2.1	8 $\cdot$ 10 ⁻⁴	0.9631	28.73	6.7
SHP OMSW	383 $\pm$ 6	44.9 $\pm$ 2.7	6 $\cdot$ 10 ⁻¹¹	0.9688	29.11	10.1
Co-digestion 95%OMSW- 5% <i>Dunaliella</i> <i>salina</i>	437 $\pm$ 7	54.7 $\pm$ 4.1	1 $\cdot$ 10 ⁻⁸	0.9751	30.16	12.1

B_m is the maximum methane production or methane yield potential (mL CH₄ g⁻¹ VS_{added}); R_m is maximum methane production rate (mL CH₄ g⁻¹ VS_{added} $\cdot$ d⁻¹), α is the lag time (d), R²: coefficient of determination, S.E.E.: Standard error of estimate and Error was defined as the difference between measured and predicted methane yield values.

Figure 1. Total volatile fatty acids (total VFA) evolution in the batch anaerobic digestion experiments of olive mill solid waste (OMSW), soft hydrothermal pre-treated OMSW (SHP OMSW), and co-digestion of 95% OMSW and 5% *Dunaliella salina*. Values represent means $\pm$ standard errors, n=3.

Figure 2. Soluble Chemical oxygen demand (sCOD) evolution in the batch anaerobic digestion experiments of olive mill solid waste (OMSW), soft hydrothermal pre-treated OMSW (SHP OMSW), and co-digestion of 95% OMSW and 5% *Dunaliella salina*. Values represent means $\pm$ standard errors, n=3.

Figure 3. Methane yield obtained in the batch anaerobic digestion experiments of olive mill solid waste (OMSW), soft hydrothermal pre-treated OMSW (SHP OMSW), and co-digestion of 95% OMSW and 5% *Dunaliella salina*. Values represent means $\pm$ standard errors, n=3.

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