INFLUENCE OF THE CELL WALL OF *Chlamydomonas reinhardtii* ON ANAEROBIC DIGESTION YIELD AND ON ITS ANAEROBIC CO-DIGESTION WITH A CARBON-RICH SUBSTRATE

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ABSTRACT

The aim of this study was to investigate the influence of the cell wall of the microalga *Chlamydomonas reinhardtii* (*C. reinhardtii*) on its anaerobic digestion (AD) by comparing the AD of *C. reinhardtii* 6145 and of its mutant without cell wall *C. reinhardtii* cw15, assessing simultaneously the influence of the cell wall on anaerobic co-digestion of these strains with a carbon-rich substrate, olive mill solid waste (OMSW). The OMSW is the main by-product from two-phase olive oil manufacturing process. Biochemical methane potential (BMP) tests of the sole substrates and different mixtures (OMSW-microalgae) (85%-15%, 75%-25%, 50%-50%) were carried out. The results showed that the cell wall was not hindrance and became an advantage by releasing intracellular biomass to be degraded slowly. The influence of the substrate composition in the mixtures of OMSW and *Chlamydomonas reinhardtii* (6145 and cw15) was assessed through the calculation of performance and kinetic parameters by using the Transference Function model. The mixture 50% OMSW-50% *C. reinhardtii*

6145 allowed the highest methane yield (542 ± 4 mL CH₄/g VS) and also resulted in one of the highest maximum methane production rates, R_{max} (129 ± 3 mL CH₄/(g VS·d).

KEYWORDS

Microalgae, cell wall, olive mill solid waste, anaerobic co-digestion, kinetics.

1. INTRODUCTION

Green microalgae are considered to be a promising source of sustainable energy and/or high-value added products such as carotene, vitamins or fatty acids (Baudeflet et al., 2017). Chlorophyta has been the focus of taxonomists, whose studies have led to a better understanding of its cell wall. Chlorophyta is the most prominent phylum with great interest in biotechnology and pharmaceuticals industries (Baudeflet et al., 2017). The polyphyletic genus *Chlamydomonas*, within Chlorophyta family comprises biflagellate unicellular green alga which swims along a helical trajectory by the synchronous beating of its flagellar pair (Baudeflet et al., 2017). The cell wall of *Chlamydomonas* consisting in a five-layered cell wall with no cellulose but is mainly composed of proteins linked by covalent bonds (Voigt and Frank, 2003).

The anaerobic digestion (AD) of microalgae in order to produce biogas is currently an alternative with low operational costs compared to other processes, with a wide range of biogas yields being reported, due to different factors: microalgae composition, cell wall structures or operational cultivation (Frigon et al., 2013). Among the bibliography is the object of consensus that microalgae as substrates for AD are directly related to cell wall digestibility, which depends on the content of the cellulose, hemicellulose and other hardly biodegradable biopolymers (Jankowska et al., 2017). Microalgae cell wall presents a high resistance to enzymatic hydrolysis and limits the availability for AD (Jankowska et al., 2017). For this reason, several physical, chemical or biological pre-

treatments have been evaluated in order to increase the biodegradability of the cell wall and, hence, enhance the methane production after AD (Jankowska et al., 2017). Mussnug et al. (2010) showed that the hydrogen production of *C. reinhardtii* as a pre-treatment increased its biogas production potential by up to 123% (587 mL biogas/g VS). Passos et al. (2013) showed that a thermal pre-treatment increased the methane yield by up to 7 - 61% (170 - 272 mL CH₄/g VS). These results suggest that even in the case of a weak cell wall (like in *C. reinhardtii*) a pre-treatment is needed in order to enhance the AD process. However, Mahdy et al. (2014) achieved a methane yield of 263.1 mL CH₄/g COD in the AD of *C. reinhardtii* and this study concluded that the use of carbohydrases as a pre-treatment, which increased the solubilization of the organic matter, did not significantly increase the methane potential after AD. This could be explained by the lack of cellulose in the cell wall and because the released organic matter came from already easily bioavailable organic matter in the raw material. By contrast to this, when using proteases, the methane yield increased slightly (up to 311.6 mL CH₄/g COD). This can also be explained based on cell wall composition, which is mainly composed of glycoproteins (Mahdy et al., 2014).

Besides the cell wall, a major issue concerning the anaerobic digestion of any biomass is the C/N ratio of the substrate. A low C/N ratio, with containing a high level of nitrogen, produces an intolerant concentration of ammonia for the correct activity of the microbial community within the anaerobic sludge, while a high C/N ratio, containing a low level of nitrogen, is related to a low pH substrate, poor buffering capacity and the volatile fatty acid accumulation (González-Fernández et al., 2011). Several authors have observed that the optimum C/N ratio of the substrate for AD with regards to higher pollutant and CO₂ removal must be between 20:1 and 30:1 (Fernández-Rodríguez et al., 2014; Xu et al., 2017). Since microalgal biomass presents a high nitrogen content, with

C/N ratios ranging from 5 to 12 (Fernández-Rodríguez et al., 2014; Klassen et al., 2015), an additional source of carbon must be added in order to reach the optimal ratio. To this respect, co-digestion with high carbon biomass, like olive mill solid waste (OMSW), presents several improvements such as enhancing the C/N ratio, balancing the macro and micronutrient compositions, neutralizing the pH and reducing the effect of inhibitors/toxic compounds (Hartmann and Ahring, 2005; Li et al., 2018; Ferreira et al., 2018).

The OMSW is the main waste generated in the two-phase olive oil extraction process. This by-product is produced in an amount of 800 kg/t of olives processed. Its characteristics (high moisture, low pH, high content in organic matter, presence of inhibitory compounds such as poly-phenols, etc.) make it a very pollutant waste (de la Lama et al., 2017).

The potential of using microalgae as co-substrate of carbon-rich by-products has recently been reported in several studies. González-Fernández et al. (2011) reported the lowest methane yield values when the microalgae percentage was increased in relation to a carbon-rich substrate, due to the highly resistant cell wall, stating that a pre-treatment method is required to improve microalgae digestibility. Another approach for anaerobic co-digestion of lignocellulosic residues with microalgae showed a synergistic effect during anaerobic co-digestion of both substrates, achieving the best methane production when the microalgae biomass was decreased and the lignocellulosic waste increased (Fernández-Rodríguez et al., 2014). Similar results were reported by Zhang et al. (2019) that obtained an increase in methane production of up to 47% with the lowest percentage of microalgae biomass during its co-digestion with potato processing waste. The aim of this study was to determine the influence of non-rigid cell wall microalgae (*C. reinhardtii*) on an anaerobic digestion and co-digestion of these microalgae with a

high carbon lignocellulosic biomass (OMSW). To this aim, two strains of *C. reinhardtii* were evaluated, 6145 and cw15. *C. reinhardtii* cw15 is a mutant specimen developed by Hyams and Davies (Hyams and Davies, 1972), by using N-methyl-N'-nitrosoguanidine (NNG) as mutagen which produces a phenotypic change where the cell wall is almost completely reduced. To the best of our knowledge, the influence of the cell wall of microalgae of the same species on their anaerobic digestion and anaerobic co-digestion of these with a carbon-rich waste such as OMSW have not been previously reported.

2. MATERIALS AND METHODS

2.1. Olive mill solid waste

The two-phase OMSW was collected from the Experimental Olive Oil Mill Factory located in the 'Instituto de la Grasa (CSIC)', Seville (Spain). The OMSW was sifted through a 2 mm mesh with the purpose of removing olive stone pieces. The main characteristics of the OMSW used in the experiments were: pH: 4.9, total solids (TS): 256.7 ± 4.4 g/kg, volatile solids (VS): 226.2 ± 4.6 g/kg, chemical oxygen demand (COD): 345.3 ± 6.3 g O₂/kg and C/N ratio: 31.4 ± 0.1 .

2.2 Microalgae cultivation

The chlorophytes *Chlamydomonas reinhardtii* 6145 and cw15 were grown at 25 °C in a liquid medium. The microalgae were grown in the medium as described by Harris (1989). The algal biomass was cultivated under 12:8 light:dark cycles in an AGP-700-ESP incubator chamber (Rdiber S.A., Barcelona, Spain) with illumination provided by 6 fluorescent lamps (36 W). To ensure that the algal culture was composed mainly of *Chlamydomonas reinhardtii*, microscope observations were carried out periodically. The cells from the culture were concentrated by centrifugation (5 min at 3500 rpm).

Finally, microalgal biomass was placed in liquid nitrogen (-196 °C) and stored at -80 °C. Table 1 shows the composition of both strains.

2.3. Microalgae chemical composition

The following parameters were determined to characterize the microalgal chemical composition: total solids and volatile solids were determined according to Standard Methods 2540B and 2540E (APHA, 1998), respectively; total lipids were determined by the Bligh and Dyer method (Bligh and Dyer, 1959); carbohydrates were quantified by Dubois method (Dubois et al., 1956). The concentrations of chlorophylls and carotenoids were measured at 663.2, 646.8, and 470 nm according to the standard method 10200H (APHA, 1998), and pigment concentrations were determined using the method described in detail elsewhere (Lichtenthaler, 1987). Fatty acids were extracted with Soxhlet equipment, with a mixture of chloroform and methanol (2:1) and were quantified spectrophotometrically at 628 nm by the reaction with sulfophosphovanillin (Ahlgren et al., 1992). Carbon and nitrogen contents were determined through an Elemental Analyzer LECO CHNS-932 (Leco Corporation, St Joseph, MI, E.E.U.U.).

2.4. Inocula for anaerobic digestion

The anaerobic sludge was obtained from an industrial up-flow anaerobic sludge blanket (UASB) reactor treating brewery wastewater in Sevilla (Spain). The main characteristics of the inoculum used were: pH: 7.43; total solids (TS): 23.9 ± 1.1 g/L; volatile solids (VS): 18.7 ± 1.4 g/L; chemical oxygen demand (COD): 31.9 ± 1.1 g O₂/L.

2.5. 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 contained 192 mL of inoculum, the amount of substrate needed to give the required inoculum to substrate ratio and, 192 μ L of trace element solution were added.

The composition of the trace elements solution was: $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 2000 mg/L; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 2000 mg/L; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 500 mg/L; $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, 90 mg/L; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 50 mg/L; H_3BO_3 , 50 mg/L; ZnCl_2 , 50 mg/L; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 38 mg/L, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 50 mg/L, $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$, 194 mg/L and EDTA 1000 mg/L. Two reactors with inoculum and trace element solution but without substrate addition were used as blank controls. The methane production due to biomass decay and the possible presence of residual substrate in the inoculum was subtracted by performing these blank controls. Therefore, the methane produced by the blank controls was subtracted from the reactors with substrate.

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 CO_2 ; the remaining gas was assumed to be methane. Thus, methane production was determined by liquid displacement. The anaerobic digestion experiments were run for a period of c.a. 29 days until the accumulated gas production remained essentially unchanged, i.e. on the last day production was lower than 2% of the accumulated methane produced. Each experiment was carried out in triplicate.

OMSW was co-digested with different percentages of both microalgal strains in order to compare the effect of cell wall on anaerobic co-digestion with OMSW. The co-digestion mixtures studied were the same for both microalgae, i.e. 85% OMSW-15% *C.*

reinhardtii; 75% OMSW-25% *C. reinhardtii*; 50% OMSW-50% *C. reinhardtii*. The sole substrate OMSW and the single anaerobic digestion of *C. reinhardtii* 6145 and *C. reinhardtii* cw15 were also tested in order to show the effect of the cell wall on anaerobic digestion.

2.6. 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), pH, ammonium concentration (N-NH_4^+) and elemental C and N. 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 (APHA, 1998), respectively; 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 analyzed using a pH-meter model Crison 20 Basic. Total alkalinity (TA) was determined by pH titration to 4.3 (APHA, 1998). Total ammonium was determined using a method based on 4500- N_{org} B of Standard Methods (APHA, 1998). Free ammonia concentration was calculated according to the following formula (Østergaard, 1985):

$$\frac{[\text{NH}_3]}{[\text{TNH}_3]} = \left(1 + \frac{10^{-\text{pH}}}{10^{-\left(0.09018 + \frac{2729.92}{T(\text{K})}\right)}} \right)^{-1} \quad (1)$$

where $[\text{NH}_3]$ is the concentration of free ammonia, $[\text{TNH}_3]$ is the total ammonia concentration and $T(\text{K})$ is the temperature measured in degrees kelvin.

Individual volatile fatty acids (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) as an internal standard. This mixture was centrifuged to remove any solids and transferred to a 1500 μL gas chromatography (GC) 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 $^{\circ}\text{C}/\text{min}$. Total phenols concentration was quantified by spectrophotometry through the Folin-Ciocalteau method (de la Lama et al., 2017).

2.7. Statistical analysis

Data are presented as means $\pm$ standard deviation of the means, and statistical significances were assessed by analysis of variance (ANOVA). A p value of less than 0.05 was considered statistically significant.

2.8. Kinetic study

The Transference Function (TF) model was applied to fit the experimental data of methane production during BMP tests (eq. 2). The transference function (Reaction curve-type model) (RC), used mainly for control purposes, considers that any process might be analyzed as a system receiving inputs and generating outputs (Donoso-Bravo et al., 2010). The TF model was successfully applied by several authors for biomethanization of different organic wastes (Donoso-Bravo et al., 2010; Li et al., 2012). The TF model is given by the following expression:

$$B = B_{max} * \left(1 - \exp\left[-\frac{R_{max}(t-\gamma)}{B_{max}}\right]\right) \quad (2)$$

where B (mL CH₄/g VS_{added}) is the cumulative specific methane production, B_{max} (mL CH₄/g VS_{added}) is the ultimate methane production, R_{max} is the maximum methane production rate (mL CH₄/(g VS_{added}·d)), t (d) is the digestion time and γ (d) is the lag time.

Error (%), determination coefficient (R^2) and standard error of estimate were calculated to evaluate the goodness-of-fit and the accuracy of the results. Error was defined as the percentage difference between the experimental and the predicted or theoretical methane yield coefficient. The kinetic parameters for each experiment and mathematical adjustment were determined numerically from the experimental data obtained by non-linear regression using the software Sigma-Plot (version 11).

3. RESULTS AND DISCUSSION

3.1. Chemical composition of *C. reinhardtii*

Several studies have recommended the application of pre-treatments on microalgae in order to enhance their biodegradability and, therefore, their bioconversion into methane (Passos et al., 2014). However, it is known that methane production strongly depends on the microalga type (Mussnug et al., 2010). As discussed previously, *C. reinhardtii* does not present cellulose in its cell wall, which consists instead of a five-layered extracellular matrix composed of carbohydrates and polypeptides (Voigt and Frank, 2003).

In order to test the effect of this type of cell wall in AD processes, two different types of *C. reinhardtii* were used, i.e. a wild type (6145) and a mutant type (cw15). cw15 strain type is a mutant that differs from the 6145 type in the absence of cell wall by using N-methyl-N'-nitrosoguanidine (NNG) as mutagen which produces a phenotypic change (Hyams and Davies, 1972).

The chemical composition of the microalgae depends on growth conditions, harvesting time and conservation technology (Wang and Park, 2015). Consequently, as both strains have been cultivated under the same conditions, i.e. harvested at same time and conserved with the same technology, they presented similar chemical characteristics with no significant differences ($p < 0.005$).

As shown in Table 1, the lipid contents were 20% and 21% on VS basis for *C. reinhardtii* 6145 and *C. reinhardtii* cw15, respectively. The protein and carbohydrate contents were 50% and 16% on VS basis for both cases, respectively. The chlorophyll contents were 212 and 209 $\mu\text{g/L}$ for *C. reinhardtii* 6145 and *C. reinhardtii* cw15, respectively. And the C/N ratio for both strains was 5.2. Similar results were obtained by Mahdy et al. (2014), which showed a concentration of lipids, protein and carbohydrates of 126.0 mg/g_{dry weight}, 647.6 ± 8.0 mg/g_{dry weight} and 226.4 ± 21.9 mg/g_{dry weight}, respectively, for a *C. reinhardtii* wild type.

The low C/N ratio of both strains, which implies a high N content, suggests that an anaerobic co-digestion with a high carbon co-substrate like OMSW will result in higher methane production.

3.2. Nitrogen and volatile fatty acids inhibition

Due to the high nitrogen content of microalgae (5.2 C/N ratio) it was expected that methane production would be inhibited by one of its main methanogen inhibitors, the free ammonia, which can be toxic at concentrations as low as 2000 mg/L (Yenigün and Demirel, 2013). Although Wang et al. (2012) pointed out the 4.4 C/N ratio as the limit ratio to have ammonium inhibition, pH and ammonium were measured at the end of each BMP test and free ammonia was calculated on the base of temperature, pH and

total ammonium concentration, in order to confirm that any inhibitory effect appeared was not related to ammonia toxicity (Table 2).

At the end of the study, the pH ranged between 7.66 and 8.12 and the total ammonium concentration ranged between 1.67 ± 0.07 g/L (100% *C. reinhardtii* cw15) and 0.94 ± 0.02 g/L (85% OMSW-15% *C. reinhardtii* 6145). These results showed that in no case the minimum concentrations for inhibitory effects were reached and, due to the pH range, it was expected that the predominant form of inorganic nitrogen was ammonium (Hansen et al., 1998). Even so, free ammonia was also calculated by using eq.1. The results ranged between 187.0 ± 7.8 mg/L (100% *C. reinhardtii* cw15) and 81.9 ± 6.3 mg/L (75% OMSW-25% *C. reinhardtii* cw45). Hansen et al. (1998) concluded that the value found for the inhibition of the biogas process was 1100 mg/L of free ammonia. The free ammonia values calculated in these experiments were drastically lower than the inhibitory limit; hence, free ammonia was not the cause of methanogenesis inhibition in any case.

The free ammonia content at the end of the experiments of the different co-digested mixtures were lower than in the AD of 100% *C. reinhardtii* 6145 and *C. reinhardtii* cw15. These results are consistent with others previously reported, i.e. Jianlong and Jing (2005) showed a reduction of 40% total ammonia when the initial concentration was 70-250 mg/L using an expanded granular bed reactor initiated by feeding the anaerobic sludge from a brewery wastewater treatment plant.

When comparing both microalgal strains no significant differences were observed for total ammonia except in the mixture 50% OMSW-50% *C. reinhardtii*, where the experiment with the wild type 6145 presented a concentration 33% higher than the same mixture with the mutant type cw15. By contrast, the smallest difference observed for the free ammonia was found in the 50% OMSW-50% *C. reinhardtii* where the wild type

6145 mixture presented a content only 4% higher than the same mixture with the mutant type cw15. In the single microalga experiments and in the mixture 85% OMSW-15% *C. reinhardtii*, the mutant type cw15 presented concentrations which were 10 and 12% higher, respectively, than observed for the wild type 6145. The mixture 75% OMSW-25% *C. reinhardtii* showed the greatest difference between both microalgae, with 21% higher concentration found in the mixture with the wild type 6145.

VFA were also tested in order to evaluate whether there was any inhibitory accumulation due to the unbalance of acidogenic and methanogenic reactions. VFA accumulation is the main consequence of process overloading which can acidify the reactor and hence inhibit the process. Table 3 showed the individual fatty acids determined. The single OMSW and the co-digested mixtures only presented acetic acid, the smaller VFA, which means that the acidogenic step was not inhibited. Moreover, the acetic acid concentrations were lower than 425 ppm for these cases, which suggest that no significant accumulation had taken place and that the methanogenic microorganisms were not totally inhibited by VFA. Franke-Whittle et al. (2014) reported that acetic acid and butyric acid concentrations of 2400 and 1800 mg/L respectively, resulted in no significant inhibition of the activity of methanogens, while a propionic acid concentration of 900 mg/L resulted in significant inhibition of the methanogens. By contrast, a much higher concentration of acetic acid was found in the single digestion of *C. reinhardtii* (1221.1 ± 16.0 ppm and 1074.3 ± 1.8 ppm for *C. reinhardtii* 6145 and *C. reinhardtii* cw15, respectively). Moreover, in these single microalga digestions, propionic acid was found. These results suggest that the acidogenic and the methanogenic steps showed certain inhibition, due to the accumulation of VFA while the methane productions decayed to zero. Siegert and Banks (2005) reported similar results, concluding that biogas production using paper as substrate was reduced to half

when the concentration of initial VFA was 1000 mg/L, indicating inhibition of the hydrolysis process.

3.3 Biodegradability

An important parameter to reflect anaerobic digestion efficiency is the biodegradability of the substrate. Many authors pointed out the low biodegradability of the cell wall of microalgae due to the rigid cell wall mainly composed of cellulose, hemicellulose and other hardly biodegradable biopolymers (Jankowska et al., 2017). In contrast, the insolubilization of the cell wall of *C. reinhardtii* is mainly due to covalent bonds between glycoproteins (isodityrosine and dityrosine, mainly), protease resistant isopeptide bonds and oligosaccharide side chain cross-link. Thus, as shown in Figure 1, the biodegradability (VS basis) of both strains was greater than 88%. These results were higher than those previously observed by Mahdy et al. (2014) who reported biodegradability of 75% using *C. reinhardtii* and similar to the 86% of the *C. vulgaris*.

Figure 1, also shows the biodegradability of the single digestion of OMSW and the different mixtures that were under study. As shown, OMSW presented the lowest biodegradability (56.7%) due to the presence of non-easily-biodegradable compounds such as cellulose, hemicellulose and lignin which could represent up to the 30% of the dry weight (de la Lama et al., 2017).

The biodegradability of the mixture 75% OMSW-25% *C. reinhardtii* was similar for both species (65-67%). The mixtures 85% OMSW-15% *C. reinhardtii* presented similar values and the highest biodegradability (89.06% and 81.60% for the wild type 6145 and the mutant type cw15, respectively). By contrast, the mixtures 50% OMSW-50% *C. reinhardtii* showed greater differences between microalga strains, which were 81.28% for cw15 and 61.12% for 6145. Similar biodegradability values were found by

Fernández-Rodríguez et al. (2014) who reported a maximum biodegradability of 73% when *Dunaliella salina* was co-digested with OMSW at a ratio of 1:1.

Furthermore, as the OMSW presented lower biodegradability than the microalgae, it was expected that the higher the microalga percentage in the mixture the higher the biodegradability would be. However, results showed that the highest biodegradability was obtained with the lowest microalga percentage, which suggests a synergic effect between both substrates and a better C/N balance in the mixture 85% OMSW – 15% *C. reinhardtii*.

3.4. Effect of cell wall on anaerobic digestion

Figure 2a shows the mean values for the accumulated methane productions during the experimental time (29 days) for the two different microalgae tested, *C. reinhardtii* 6145 (with cell wall) and *C. reinhardtii* cw15 (the mutant without cell wall). As can be seen, the methane production increased after a short lag phase and an inflection point on the 5th day of digestion time for *C. reinhardtii* 6145 and *C. reinhardtii* cw15, respectively, up to a maximum value at the end of the digestion period. During the first four days of digestion both microalgae exhibited the same methane yield, but after 5 days the trend in the variation of methane production with time was somewhat different. At the end of the experiment, the maximum methane production reached by the microalga without cell wall was 381 ± 37 mL CH₄/g VS, while the microalga *C. reinhardtii* 6145 with cell wall gave a final methane production of 351 ± 39 mL CH₄/g VS. Therefore, the methane yield for the *C. reinhardtii* cw15 only increased by 8.5% in relation to the value obtained for *C. reinhardtii* 6145. Thus, the differences in these methane yields were not statistically significant. However, the shape of the curves of methane production with time revealed that the mutant without cell wall, *C. reinhardtii* cw15, was more bioavailable.

The experimental methane yield obtained for *C. reinhardtii* cw15 in the present work (381 ± 37 mL CH₄/g VS) was virtually identical to the value reported by Mussgnug et al. (2010) for *C. reinhardtii* (387 mL CH₄/g VS) and higher than that reported by Frigon et al. (2013) in this case using *Chlamydomonas* sp.-AMLS1b3 (333 mL CH₄/g VS) and *Chlamydomonas debaryana*-AMB1 (302 mL CH₄/g VS) as substrates for anaerobic digestion. The lower methane yields observed in the reported studies can most likely be attributed to the fact that different *Chlamydomonas* strains were used in the mentioned studies and/or that strict phototrophic cultivation conditions were applied in them. Therefore, this work clearly shows that methane yield from microalgal biomass is highly variable and dependent on the strain used as substrate for digestion as well as the growth conditions applied to generate the biomass (Mussgnug et al., 2010).

On the other hand, by comparing the two species of *C. reinhardtii* used in this work (*C. reinhardtii* 6145 and *C. reinhardtii* cw15), it can be seen that the species with a high degree of decomposition and low amount of indigestible residues or cell wall (*C. reinhardtii* cw15) showed somewhat higher methane yield (381 mL CH₄/g VS) compared to the species with a lower degree of decomposition and higher amount of indigestible residues (*C. reinhardtii* 6145) (351 mL CH₄/g VS). Consequently, these results indicate that without a pre-treatment, the accessibility to cell disintegration is most likely a major factor for the efficiency of fermentative methane production. As a consequence, the species with a very weak cell wall composed basically of protein with no cellulose or hemicellulose (*C. reinhardtii* cw15) generated a higher methane yield compared to the species *C. reinhardtii* 6145 with a more rigid cell wall containing some biopolymers.

It has also been reported that the enzymatic hydrolysis of *C. reinhardtii* with carbohydrase (Viscozyme) and protease (Alcalase) resulted in high carbohydrates and

protein solubilization of this biomass. Despite the high carbohydrate solubilization, methane production was not improved after this pre-treatment, while the addition of protease increased methane production only by 1.17-fold (Mahdy et al., 2014). By contrast, a novel, one-stage combined cultivation/anaerobic fermentation strategy including inherently progressing nitrogen starvation conditions to generate improved microalgal biomass substrates allowed for increasing the C/N ratio of *C. reinhardtii* biomass up to levels of 24-26. This nitrogen limitation treatment resulted in a 65% increase in biogas yield for *C. reinhardtii* (698 mL biogas/g VS) when compared to biomass grown under replete conditions (Klassen et al., 2015). Finally, to evaluate integrative biorefinery concepts, biosolar hydrogen production in *C. reinhardtii* prior to anaerobic digestion resulted in an increase of biogas production to 123% compared to the fermentation of fresh untreated *C. reinhardtii* (Mussnug et al., 2010).

3.5. Effect of different *C. reinhardtii* 6145-OMSW and *C. reinhardtii* cw15-OMSW mixtures on methane production

Figure 2b shows the variation in the mean values of the accumulated methane production with digestion time for the single OMSW substrate and different *C. reinhardtii* 6145-OMSW and *C. reinhardtii* cw15-OMSW mixtures tested.

For the co-digestion of OMSW with *C. reinhardtii* cw15 the maximum methane yield was achieved for the mixture 50% OMSW-50% *C. reinhardtii* cw15 (451 ± 36 mL CH₄/g VS added), which was 18% higher than that obtained for the single anaerobic digestion of 100% *C. reinhardtii* cw15 and 32% and 31% higher than that achieved for the mixtures 75% OMSW-25% *C. reinhardtii* cw15 and 85% OMSW-15% *C. reinhardtii* cw15 (341 ± 15 and 342 ± 47 mL CH₄/g VS), respectively. Different effects could be observed when comparing the results from the co-digestion 85% OMSW-15%

C. reinhardtii cw15 and 75% OMSW-25% *C. reinhardtii* cw15 with this single microalga, since neither of these two mixtures improved the amount of methane obtained in relation to the anaerobic digestion of the sole microalga.

During the co-digestion of OMSW with *C. reinhardtii* 6145 (cell wall microalgae), the highest methane production was reached for the mixture 50% OMSW-50% *C. reinhardtii* 6145 (542 ± 38 mL CH₄/g VS), which was 54% higher than that achieved for the single digestion of *C. reinhardtii* 6145. Despite the C/N values considered optimal in the literature varies between 20 to 30, other mixtures such as processed industrial food waste (IFW) with sewage sludge showed optimum methane yield at a C/N ratio of 15 (Benn and Zitomer, 2018), which is very similar to the C/N ratio of the mixture 50% OMSW – 50% *C. reinhardtii* 6145 (18.3) tested in the present work (Table 4). On the other hand, anaerobic co-digestion experiments of algal biomass and paper waste also demonstrated that the highest methane yield obtained in the above-mentioned mixture was attributed to the fact that microalgae were not only a nitrogen source supplier in this co-digestion, but also supplied nutrients to the digester microflora after the degradation of algal biomass (Yen and Brune, 2007).

The methane yield obtained for the digestion of the sole OMSW was 415 ± 34 mL CH₄/g VS added while the final methane yield for the single microalga (*C. reinhardtii* 6145) was 351 ± 39 mL CH₄/g VS added. The methane yield values obtained during the co-digestion of *C. reinhardtii* 6145 were 308 ± 3 mL CH₄/g VS added for the mixture 85% OMSW-15% *C. reinhardtii* 6145 and 421 ± 16 mL CH₄/g VS added for the mixture 75% OMSW-25% *C. reinhardtii* 6145. Therefore, only the co-digestions of 50% OMSW-50% *C. reinhardtii* 6145 and 75% OMSW-25% *C. reinhardtii* 6145 showed enhancement in the methane yield of 54% and 20%, respectively, with respect to the value obtained for the single microalga (351 ± 39 mL CH₄/g VS added).

The results obtained suggest that the cell wall of this microalga had no negative effect during its anaerobic co-digestion with OMSW. In fact, the methane productions obtained during the co-digestion of the microalga with cell wall (*C. reinhardtii* 6145) with OMSW was significantly better than those obtained during the co-digestion of OMSW with the mutant microalga without cell wall (*C. reinhardtii* cw15).

3.6. Study of possible synergic effects

The experimental methane yields observed for each co-digestion mixture (Figure 2b) were compared to calculated methane yields based on the OMSW, *C. reinhardtii* 6145 and *C. reinhardtii* cw15 methane yields separately according to equations (3) and (4).

$$\begin{aligned} \text{Calculated methane yield (mL CH}_4\text{/g VS}_{\text{added}}) = \\ \% \text{ OMSW} \cdot (415) + \% \text{ C. reinhardtii 6145} \cdot (351) \end{aligned} \quad (3)$$

$$\begin{aligned} \text{Calculated methane yield (mL CH}_4\text{/g VS}_{\text{added}}) = \\ \% \text{ OMSW} \cdot (415) + \% \text{ C. reinhardtii cw15} \cdot (381) \end{aligned} \quad (4)$$

where 415, 351 and 381 are the experimental methane yields (mL CH₄/g VS_{added}) obtained for OMSW, *C. reinhardtii* 6145 and *C. reinhardtii* cw15, respectively. % OMSW, % *C. reinhardtii* 6145 and % *C. reinhardtii* cw15 are the percentages of OMSW, *C. reinhardtii* 6145 and *C. reinhardtii* cw15, respectively, in each co-digestion mixture.

Experimental BMP values were higher than the calculated methane yields for some of the co-digestion mixtures tested (Table 4). 5.5% for the co-digestion mixture 75% OMSW-25% *C. reinhardtii* 6145, 41.5% for the co-digestion mixture 50% OMSW-50% *C. reinhardtii* 6145 and 13.3% for the co-digestion mixture 50% OMSW-50% *C. reinhardtii* cw15. Therefore, according to the increase in the BMP values, the biodegradability of the above-mentioned co-digestion mixtures was also much higher

than the biodegradability of the sole substrates. The synergy effects of the OMSW and *C. reinhardtii* 6145 and of OMSW and *C. reinhardtii* cw15 co-digestions with the above percentages were clearly shown with these results.

3.7. Kinetics of the single anaerobic digestion and co-digestion processes

Table 5 shows the main performance and kinetic parameters obtained from the application of the Transference model to the experimental data of methane production-time corresponding to the different BMPs of all the mixtures and single substrates carried out. As highlighted, the high R^2 values as well as the low values of errors and standard errors of estimates indicated that the experimental data correctly fit the proposed model.

The calculated lag times (λ) were found to be zero in all cases, because the easiest and most available biodegradable components of all the substrates were quickly consumed in all the anaerobic digestion processes studied (Li et al., 2012).

The R_{max} values presented a somewhat different behavior and trend to that observed for B_{max} for the different experiments carried out. The highest R_{max} value was found for the mixture 85% OMSW-15% *C. reinhardtii* 6145 with 139 ± 5 mL CH₄/(g VS·d), mixture that also showed the highest biodegradability value (89%). This R_{max} value was 2.2% and 110.6% higher than that obtained for 100% OMSW (136 ± 8 mL CH₄/(g VS·d) and 100% *C. reinhardtii* 6145 (66 ± 5 mL CH₄/(g VS·d), respectively. High R_{max} values were also obtained for the mixtures 85% OMSW-15% *C. reinhardtii* cw15 (133 ± 16 mL CH₄/(g VS·d)) and 50% OMSW-50% *C. reinhardtii* 6145 (129 ± 3 mL CH₄/(g VS·d)). By contrast, the lowest R_{max} value was found for the mixture 50% OMSW-50% *C. reinhardtii* cw15 (88 ± 3 mL CH₄/(g VS·d)). This decrease in R_{max} for the mentioned

mixture is a good indication that compounds in this mixture might have a lower initial availability for its anaerobic biodegradation.

On the other hand, the R_{max} values obtained in the present work were always higher than those obtained during the co-digestion of OMSW and *Dunaliella salina*, for which the maximum value (48.1 mL CH₄/(g VS·d)) was obtained for the mixture 75% OMSW-25% *D. salina*. This fact reveals the higher anaerobic biodegradability of *C. reinhardtii* 6145 and *C. reinhardtii* cw15 compared to *D. salina* either alone or in mixtures with OMSW (Fernández-Rodríguez et al., 2014).

Finally, taking into account both the B_{max} and R_{max} values as well as the synergy effect observed, the mixture 50% OMSW-50% *C. reinhardtii* 6145 can be considered as the optimum one among the different mixtures tested in the present work.

4. CONCLUSIONS

It has been demonstrated that the cell wall of the microalga *C. reinhardtii* 6145 had no negative effect during its anaerobic co-digestion with OMSW. The methane production obtained during the co-digestion of this microalga with cell wall with OMSW was better than that obtained during the co-digestion of OMSW with the mutant microalga without cell wall (*C. reinhardtii* cw15).

The mixture 50% OMSW-50% *C. reinhardtii* 6145, increased the methane yield compared to the anaerobic digestion of the sole substrates and other co-digestion mixtures percentages. Anaerobic co-digestion of 85% OMSW-15% *C. reinhardtii* 6145 resulted in the highest maximum methane production rate.

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REFERENCES

- Ahlgren, G., Gustafsson, I.B., Boberb, M., 1992. Fatty acid content and chemical composition of freshwater microalgae. *J. Phycology*, 28 (1), 37-52. DOI: 10.1111/j.0022-3646.1992.00037.x
- APHA–AWWA–WPCF, 1998. Standard Methods for the Examination of Water and Wastewater, 20th Edition, American Public Health Association, American Water Works Association and Water Environmental Federation, Washington DC.
- Baudelet, P.H., Ricochon, G., Linder, M. and Muniglia, L., 2017. A new insight cell walls of Chlorophyta. *Algal research*. 25, 333-371. DOI: 10.1016/j.algal.2017.04.008
- Benn, N., Zitomer, D., 2018. Pretreatment and anaerobic co-digestion of selected PHB and PLA bioplastics. *Front. Environ. Sci.*, 5(JAN) doi:10.3389/fenvs.2017.00093
- Bligh, E.G. and Dyer, W.J., 1959. A rapid method of total lipid extraction and purification.. *Can. J. Biochem.* 37(8): 911-917. DOI: 10.1139/o59-099.
- de la Lama, D., Borja, R., and Rincón, B., 2017. Performance evaluation and substrate removal kinetics in the semi-continuous anaerobic digestion of thermally pretreated two-phase olive pomace or “Alperujo”. *Process Saf. Environ. Prot.* 105, 288-296. DOI: 10.1016/j.psep.2016.11.014
- Donoso-Bravo, A., Perez-Elvira, S.I. and Fernández-Polanco, F., 2010. Application of simplified models for anaerobic biodegradability tests. Evaluation of pre-treatment processes. *Chem. Eng. J.* 160, 607-614. DOI: 10.1016/j.cej.2010.03.082
- Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F., 1956. Colorimetric method for determination of sugar and related substances. *Anal. Chem.* 28(3), 350-356. DOI: 10.1021/ac60111a017
- Fernández-Rodríguez, M.J., Rincón, B., Feroso, F.G., Jiménez, A.M. and Borja, R., 2014. Assessment of two-phase olive mill solid waste and microalgae co-digestion to improve methane production and process kinetics. *Bioresour. Technol.* 157, 263-269. DOI: 10.1016/j.biortech.2014.01.096
- Ferreira, J. S., Volschan, I., Jr., and Cammarota, M. C., 2018. Enhanced biogas production in pilot digesters treating a mixture of sewage sludge, glycerol, and food waste. *Energy and Fuels*, 32(6), 6839-6846. doi:10.1021/acs.energyfuels.8b00742
- Frigon, J.C., Matteu-Lebrun, F., Hamani Abdou, R., McGinn, P.J., O’Leary, S.J.B. and

- Guiot, S.R., 2013. Screening microalgae strains for their productivity in methane following anaerobic digestion. *Appl. Energy*. 108, 100-107. DOI: 10.1016/j.apenergy.2013.02.051
- González-Fernández, C., Molinuevo-Salces, B. and García-González, M.C., 2011. Evaluation of anaerobic codigestion of microbial biomass and swine manure via response surface methodology. *Appl. Energy*. 88, 3448-3453. DOI: 10.1016/j.apenergy.2010.12.035
- Hansen, K.H., Angelidaki, I. and Ahring, B.K., 1998. Anaerobic digestion of swine manure: inhibition by ammonia. *Water Res.* 32(1), 5-12. DOI: 10.1016/S0043-1354(97)00201-7
- Harris, E. H., 1989. The *Chlamydomonas* Sourcebook: A Comprehensive Guide to Biology and Laboratory Use. Academic Press. San Diego, CA, pp. 25-31. DOI: 10.1126/science.246.4936.1503-a
- Hartmann, H. and Ahring, B.K., 2005. Anaerobic digestion of the organic fraction of municipal solid waste: influence of co-digestion with manure. *Water Res.* 39, 1543-1552. DOI: 10.1016/j.watres.2005.02.001
- Hyams, J. and Davies, D.R., 1972. The induction and characterisation of cell wall mutants of *Chlamydomonas reinhardtii*. *Mutation Research* 14, 381-389. DOI: 10.1016/0027-5107(72)90135-2
- Jankowska, E., Sahu, A.K. and Oleskiewicz-Popiel, P., 2017. Biogas from microalgae: Review on microalgae's cultivation, harvesting and pretreatment for anaerobic digestion. *Renewable and Sustainable Energy Reviews* 75, 692-709. DOI: 10.1016/j.rser.2016.11.045
- Jianlong, W. and Jing, K., 2005. The characteristics of anaerobic ammonium oxidation (ANAMMOX) by granular sludge from an EGSB reactor. *Process Biochem.* 40(5) 1973-1978. DOI: 10.1016/j.procbio.2004.08.001
- Klassen, V., Blifernéz-Klassen, O., Hoekzema, Y., Mussnang, J.H. and Kruse, O., 2015. A novel one-stage cultivation/fermentation strategy for improved biogas production with microalgal biomass. *J. Biotechnol.* 215, 44-51. DOI: 10.1016/j.jbiotec.2015.05.008.
- Li, L., Kong, X., Yang, F., Li, D., Yuan, Z. and Sun, Y., 2012. Biogas production potential and kinetics of microwave and conventional thermal pretreatment of grass. *Appl. Biochem. Biotechnol.* 166, 1188-1191. DOI: 10.1007/s12010-011-9503-9
- Li, L., Li, Y., Sun, Y., Yuan, Z., Lv, P., Kang, X., Zhang, Y. and Yang, G., 2018. Influence of the Feedstock Ratio and Organic Loading Rate on the Co-digestion Performance of Pennisetum hybrid and Cow Manure, *Energy and Fuels*, vol. 32, no. 4, pp. 5171-5180
- Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol.* 148, 350-382. DOI: 10.1016/0076-6879(87)48036-1
- Mahdy, A., Mendez, L., Ballesteros, M. and González-Fernández, C., 2014. Enhanced methane production of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* by hydrolitic enzymes addition. *Energy Convers. Manage.* 85, 551-557. DOI: 10.1016/j.enconman.2014.04.097
- Mussnang, J.H., Klassen, V., Schlüter, A. and Kruse, O., 2010. Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. *J. Biotechnol.* 150, 51-56. DOI: 10.1016/j.jbiotec.2010.07.030
- Østergaard, N., 1985. Biogasproduktion i det termofile temperaturinterval. STUB rapport nr. 21. Kemiteknik. Dansk Teknologisk Institut. Taastrup (in Danish).
- Passos, F., García, J. and Ferrer, I., 2013. Impact of low temperature pretreatment on the anaerobic digestion of microalgal biomass. *Bioresour. Technol.* 138, 79-86. DOI: 10.1016/j.biortech.2013.03.114
- Passos, F., Astals, S. and Ferrer, I., 2014. Anaerobic digestion of microalgal biomass after ultrasound pretreatment. *Waste Manage. (Oxford)*. 34, 2098-2103. DOI:

10.1016/j.wasman.2014.06.004

- Raposo, F., de la Rubia, M. A., Borja, R. and Alaiz, M., 2008. Assessment of a modified and optimised method for determining chemical oxygen demand of solid substrates and solutions with high suspended solid content. *Talanta* 76(2), 448-453. DOI: 10.1016/j.talanta.2008.03.030
- Siebert, I. and Banks, C., 2005. The effect of volatile fatty acid additions on the anaerobic digestion of cellulose and glucose in batch reactors. *Process Biochem.* 40, 3412-3418. DOI: 10.1016/j.procbio.2005.01.025
- Voigt, J. and Frank, R., 2003. 14-3-3 proteins are constituents of the insoluble glycoprotein framework of the *Chlamydomonas* cell wall. *Plant Cell* 15, 1399-1413. DOI: 10.1105/tpc.010611
- Xu, J., Wang, X., Sun, S., Zhao, Y. and Hu, C., 2017. Effects of influent C/N ratios and treatment technologies on integral biogas upgrading and pollutants removal from synthetic domestic sewage. *Sci. Rep.* 7, 10897. DOI: 10.1038/s41598-017-11207-y
- Wang, X., Yang, G., Feng, Y., Ren, G. and Han, X., 2012. Optimizing feeding composition and carbon-nitrogen ratios for improved methane yield during anaerobic co-digestion of dairy, chicken manure and wheat straw. *Bioresour. Technol.* 120, 78-83. DOI: 10.1016/j.biortech.2012.06.058
- Wang, M. and Park, C., 2015. Investigation on anaerobic digestion of *Chlorella* sp. and *Micractinium* sp. grown in high-nitrogen wastewater and their co-digestion with waste activated sludge. *Biomass Bioenergy* 80, 30-37. DOI: 10.1016/j.biombioe.2015.04.028
- Yen, H., Brune, D. E., 2007. Anaerobic co-digestion of algal sludge and waste paper to produce methane. *Bioresour. Technol.* 98(1), 130-134. doi:10.1016/j.biortech.2005.11.010
- Yenigün, O. and Demirel, B., 2013. Ammonia inhibition in anaerobic digestion: A review. *Process Biochem.* 48(5-6), 901-911. DOI: 10.1016/j.procbio.2013.04.012
- Zhang, L., Zheng, P., Tang, C. and Jin, R., 2008. Anaerobic ammonium oxidation for treatment of ammonium-rich wastewaters. *J. Zhejiang Univ. Sci. B* 9(5), 416-426. DOI: 10.1631/jzus.B0710590
- Zhang, Y., Caldwell, G.S., Zealand, A.M. and Sallis, P.J., 2019. Anaerobic co-digestion of microalgae *Chlorella vulgaris* and potato processing waste: Effect of mixing ratio, waste type and substrate to inoculum ratio. *Biochem. Eng. J.* 91-100. DOI: 91-100. doi:10.1016/j.bej.2018.12.021

Figure 1. Biodegradability of the different tests carried out. Biodegradability was calculated by the difference between the initial Volatile Solids (VS) and the final VS on a percentage basis.

A: *Chlamydomonas reinhardtii* 6145 (Ch.r.6145); B: *Chlamydomonas reinhardtii* cw15 (Ch.r.cw15); C: olive mill solid waste (OMSW); D: 85% OMSW-15% Ch.r.6145; E: 85% OMSW-15% Ch.r.cw15; F: 75% OMSW-25% Ch.r.6145; G: 75% OMSW-25% Ch.r.cw15; H: 50% OMSW-50% Ch.r.6145; I: 50% OMSW-50% Ch.r.cw15.

Figure 2a. Accumulated methane of single microalgae anaerobic digestion. *Chlamydomonas reinhardtii* 6145 (Ch.r. 6145) and *Chlamydomonas reinhardtii* cw15 (Ch.r. cw15).

Figure 2b. Accumulated methane of single olive mill solid waste (OMSW) digestion and different co-digestion mixtures.

*Ch.r.: *Chlamydomonas reinhardtii*, OMSW: olive mil solid waste

Table 1. Chemical characteristics of the different microalgal strains *Chlamydomonas reinhardtii* 6145 (*Ch.r.* 6145) and *Chlamydomonas reinhardtii* cw15 (*Ch.r.* cw15) before anaerobic digestion.

Parameter	Ch.r.6145	Ch.r.cw15
Total Solid (g/L)	57.6 ± 5.4	57.4 ± 2.2
Volatile Solids (g/L)	57.1 ± 2.4	56.2 ± 3.3
Chemical Oxygen Demand (g O ₂ /L)	49.4 ± 0.0	58.1 ± 8.7
H (%)	6.73 ± 0.23	6.86 ± 0.42
C/N	5.2 ± 0.0	5.2 ± 0.1
Proteins (%)	50±1	49±1
Lipids (%)	20±1	21±1
Carbohydrates (%)	16±1	16±1
Chlorophylls (µg/L)	212±20	209±12
Carotenoids (µg/L)	154±14	155±17

Table 2. Chemical composition of the Biochemical Methane Potential (BMP) test effluents.

Substrate	pH	Volatile Solids (g/L)	Soluble Phenols (ppm)	Soluble Sugars (ppm)	sCOD (gO ₂ /L)	COD (gO ₂ /L)	Total N-NH ₄ ⁺ (g/L)	Free N-NH ₄ ⁺ (mg/L)
100% <i>Ch. r.</i> 6145	8.02 ± 0.04	17.63 ± 0.59	347 ± 51	17.42 ± 1.33	6.53 ± 0.57	23.38 ± 1.48	1.62 ± 0.08	170.59 ± 8.42
100% <i>Ch. r.</i> cw15	8.05 ± 0.02	17.58 ± 1.42	311 ± 54	18.44 ± 4.67	6.27 ± 0.80	25.82 ± 4.13	1.67 ± 0.07	187.02 ± 7.84
100% OMSW	7.91 ± 0.16	22.88 ± 2.43	311 ± 40	11.27 ± 4.67	4.32 ± 1.04	27.76 ± 1.57	1.14 ± 0.12	95.43 ± 10.05
85%OMSW-15% <i>Ch.r.</i> 6145	7.97 ± 0.06	17.84 ± 1.11	275 ± 20	8.94 ± 1.80	3.12 ± 0.42	24.66 ± 1.42	0.94 ± 0.02	89.24 ± 1.90
85%OMSW-15% <i>Ch.r.</i> cw15	8.00 ± 0.11	18.89 ± 0.77	258 ± 25	7.18 ± 1.50	3.12 ± 0.51	25.01 ± 2.27	0.99 ± 0.02	100.03 ± 2.02
75%OMSW-25% <i>Ch.r.</i> 6145	7.90 ± 0.04	21.37 ± 0.50	219 ± 19	7.82 ± 0.97	3.48 ± 0.84	26.78 ± 1.51	1.23 ± 0.11	100.81 ± 9.02
75%OMSW-25% <i>Ch.r.</i> cw15	7.83 ± 0.15	21.73 ± 1.45	232 ± 22	7.76 ± 1.46	3.01 ± 0.36	27.14 ± 4.17	1.16 ± 0.09	81.92 ± 6.36
50%OMSW-50% <i>Ch.r.</i> 6145	7.94 ± 0.11	22.24 ± 1.50	281 ± 33	14.83 ± 1.75	5.12 ± 0.87	28.51 ± 0.47	1.74 ± 0.03	155.15 ± 2.67
50%OMSW-50% <i>Ch.r.</i> cw15	8.06 ± 0.03	18.98 ± 0.65	250 ± 5	13.98 ± 1.86	5.06 ± 0.27	26.83 ± 4.94	1.30 ± 0.23	148.59 ± 26.29

**Ch.r.* 6145: *Chlamydomonas reinhardtii* 6145, *Ch.r.* cw15: *Chlamydomonas reinhardtii* cw15, OMSW: olive mill solid waste, sCOD: soluble chemical oxygen demand, COD: chemical oxygen demand

Table 3. Volatile fatty acids of the different effluents measured by gas chromatography.

Substrate	Acetic Acid (ppm)	Propionic Acid (ppm)
100% <i>Ch.r.</i> 6145	1221.1 ± 16.0	30.5 ± 1.6
100% <i>Ch.r.</i> cw15	1074.3 ± 1.8	14.6 ± 1.7
100% OMSW	81.0 ± 11.6	NF
85% OMSW-15% <i>Ch.r.</i> 6145	58.9 ± 0.7	NF
85% OMSW-15% <i>Ch.r.</i> cw15	44.3 ± 3.0	NF
75% OMSW-25% <i>Ch.r.</i> 6145	44.8 ± 2.8	NF
75% OMSW-25% <i>Ch.r.</i> cw15	42.1 ± 1.5	NF
50% OMSW-50% <i>Ch.r.</i> 6145	102.4 ± 1.1	NF
50% OMSW-50% <i>Ch.r.</i> cw15	425.0 ± 30.0	NF

*NF: not founded, *Ch.r.* 6145: *Chlamydomonas reinhardtii* 6145, *Ch.r.* cw15: *Chlamydomonas reinhardtii* cw15 and OMSW: olive mill solid waste

Table 4. Calculated methane yield values obtained from equations (3) and (4), experimental data obtained from Biochemical Methane Potential (BMP) tests of the different co-digestion mixtures.

Substrate	C/N ratio	B_{max} calculated (mL CH ₄ /g VS)	B_{max} experimental (mL CH ₄ /g VS)	Methane yield improvement (%)
85%OMSW-15% <i>Ch.r.</i> 6145	27.5	405	308	---
85%OMSW-15% <i>Ch.r.</i> cw15	27.5	410	342	---
75%OMSW-25% <i>Ch.r.</i> 6145	24.8	399	421	5.5%
75%OMSW-25% <i>Ch.r.</i> cw15	24.8	406	341	---
50%OMSW-50% <i>Ch.r.</i> 6145	18.3	383	542	41.5%
50%OMSW-50% <i>Ch.r.</i> cw15	18.3	398	451	13.3%

* *Ch.r.* 6145: *Chlamydomonas reinhardtii* 6145, *Ch.r.* cw15: *Chlamydomonas reinhardtii* OMSW: olive mill solid waste and B_{max} : ultimate methane production

Table 5. Kinetic parameters obtained from the Transference function model applied to the different Biochemical Methane Potential (BMP) assays of single substrates and mixtures carried out.

Substrate	B_{max} (mL CH ₄ /g VS)	R_{max} (mL CH ₄ /g VS·d)	λ (d)	R^2	S.E.E.*	Error** (%)
100% <i>Ch.r.</i> 61445	383 ± 9	66 ± 5	0	0.9695	30.43	9.1
100% <i>Ch.r.</i> cw 15	364 ± 4	67 ± 2	0	0.9937	12.76	4.3
100% OMSW	388 ± 4	136 ± 8	0	0.9825	21.49	6.5
85% OMSW–15% <i>Ch.r.</i> 6145	298 ± 2	139 ± 5	0	0.9938	9.58	2.9
85% OMSW–15% <i>Ch.r.</i> cw 15	295 ± 6	133 ± 16	0	0.9404	29.67	13.4
75% OMSW–25% <i>Ch.r.</i> 6145	404 ± 7	91 ± 7	0	0.9722	28.33	3.8
75% OMSW–25% <i>Ch.r.</i> cw 15	295 ± 6	101 ± 10	0	0.9514	26.99	13.4
50% OMSW–50% <i>Ch.r.</i> 6145	529 ± 4	129 ± 3	0	0.9962	14.14	2.3
50% OMSW–50% <i>Ch.r.</i> cw 15	438 ± 4	88 ± 3	0	0.9943	14.50	2.8

* *Ch.r.* 6145: *Chlamydomonas reinhardtii* 6145, *Ch.r.* cw15: *Chlamydomonas reinhardtii* cw15, B_{max} : ultimate methane production, R_{max} : maximum methane production rate, S.E.E.: Standard error of estimate, λ : calculated lag times

**Error $((B_{max\ experimental} - B_{max\ model})/B_{max\ experimental}) \cdot 100$