Integration of enzymatic pretreatment and sludge co-digestion in biogas production from microalgae

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13 **Abstract**

14 Integration of microalgae-based systems with conventional wastewater treatment plants provides
15 an effective alternative to waste stream management. In this work, alkaline and enzymatic
16 pretreatments of a microalgal culture mainly constituted by *Chlorella* sp. and *Scenedesmus* sp.
17 and cultivated in wastewater from an industrial winery wastewater treatment plant were assessed.
18 Microalgal enzymatic pretreatments were expected to overcome algal recalcitrancy before
19 anaerobic digestion. pH-induced flocculation at pH 10 and 11 did not enhance microalgal
20 harvesting and solubilisation, achieving a performance similar to that of natural sedimentation.
21 Enzymatic hydrolysis of algal biomass was carried out using three commercial enzymatic
22 cocktails (A, B and C) at two enzymatic doses (1% and 2% (v/v)) over 3 h of exposure time at 37
23 °C. Since pretreatments at a 1% dose for 0.5 h and 2% dose for 2 h achieved higher solubilisation,
24 they were selected to evaluate the influence of the pretreatment on microalgal anaerobic
25 digestibility. Biochemical methane potential tests showed that the pretreatments increased the
26 methane production of the raw algal biomass 3.6- to 5.3-fold. The methane yield was 9-27%
27 higher at the lower enzyme dose. Hence, microalgae pretreated with enzymes B and C at a 1%
28 dose were co-digested with waste activated sludge (WAS). Even when the enzyme increased the
29 methane yield of the inoculum and the WAS, the methane yield of the raw microalgae and WAS
30 mixture was not significantly different from that obtained when algae were enzymatically
31 pretreated. Nonetheless, co-digestion may achieve the goals of a waste recycled bio-circular
32 economy.

33

34 **Keywords**

35 Alkaline pretreatment - Enzymatic hydrolysis - Solubilisation - Waste activated sludge - Methane
36 yield

37

38 **1. Introduction**

Microalgae-based wastewater treatment is a cost-effective alternative to conventional wastewater treatment plants based on processes that require carbon and energy consumption. Microalgae contribute to nitrogen and phosphorus removal from wastewater and CO_2 fixation through photosynthesis, producing biomass and oxygen. Harvested microalgal biomass can be further valorised through anaerobic digestion for organic matter stabilisation and its bioconversion to energy.

Harvesting is a crucial step for biomass recovery, but it is considered a bottleneck in microalgal production due to small sizes on cells (1-30 µm) and their low concentration in water (~1 g L^{-1}) (Postma et al., 2017; Van Haver and Nayar, 2017). Microalgal cell recovery costs are estimated to contribute at least 20- 30% of the total production costs (Singh and Patidar, 2018). Centrifugation, filtration, and chemical flocculation are energy-intensive and resource-demanding harvesting techniques that render microalgal biomass production economically infeasible. Alkaline flocculation is a harvesting method that induces microalgal concentration by increasing the pH of the medium (Branyikova et al., 2018; Wan et al., 2015), thus avoiding the addition of chemical flocculants. Microalgal cells possess a negative surface charge that arises from deprotonated carboxylate, phosphate, and hydroxyl functional groups, and mutual repulsion between anionic microalgae creates stable suspensions in water (Bilal et al., 2018; Brady et al., 2014). Protonation and deprotonation of functional groups at microalgal surfaces rely on the culture conditions and microalgal growth phase (González-Fernández et al., 2013). Flocculation via pH adjustment is stimulated by H^+ when changing the H^+/OH^- ratio in the medium (Brady et al., 2014). When the pH increases to 9.5-11, some naturally available cations present in the medium, such as Mg^{2+} or Ca^{2+}, can precipitate and form positively charged precipitates that can interact with the negatively charged microalgal surface, allowing microalgal cells to flocculate through charge neutralisation and/or by a sweeping mechanism (Brady et al., 2014; Muylaert et al., 2015). Normally, the concentration of magnesium in water is adequate for production of microalgal flocculation when the pH of the medium is increased by the addition of a base (Vandamme et al., 2016). Afterwards alkaline flocculation, the supernatant can be recovered and reused after pH neutralization, and the quality of the harvested microalgae must be check to assess its feasibility to be employed in a defined future use (Barros et al., 2015; Li et al., 2020). Despite pH-induced flocculation is more expensive compared to gravity-based sedimentation, it

is an interesting method to pre-concentrate microalgal biomass due to its simplicity, low cost, and low energy consumption (Li et al., 2020). In this study, alkaline flocculation was assessed for microalgal concentration.

Anaerobic digestion is a technique widely employed for biomass-to-energy conversion. Generally, anaerobic digestion of microalgae is restrained to the inherent nature of microalgae. The recalcitrancy of microalgae biomass is strongly influenced by its cell wall structure and composition which is specie-dependant, and could limit biomass hydrolysis (Soto-Sierra et al., 2018). Although lignin is not present in the microalgal cell wall, it is characterized by a rigid structure of organic compounds with low biodegradability that protects the cells from the environment. Hence, pretreatment is a mandatory step for microalgal hydrolysis during anaerobic digestion, thus improving the accessibility of anaerobic microorganisms to microalgal components and increasing their digestibility for biogas production. Several pretreatment methods have been studied for microalgal cell wall disruption (Kendir and Ugurlu, 2018; Passos et al., 2014). Compared with thermal, mechanical, and thermochemical pretreatments, enzymatic hydrolysis is a biological treatment that digests microalgal cell walls, making them permeable and liberating intracellular compounds or improving their accessibility to microorganisms (Gerken et al., 2013). On the other hand, thermal and ultrasound pretreatments break or deform microalgal cell walls without digestion (Ometto et al., 2014). Enzymatic pretreatment is performed under mild conditions. This process reduces or eliminates toxic compound formation, requires low energy consumption, and keeps downstream processing costs low (Mahdy et al., 2016; Zabed et al., 2019). Enzymatic pretreatment is more specific in cell wall hydrolysis due to enzyme specificity to a certain substrate. In this sense, the appropriate enzyme(s) could be selected according to the target microalgal species. Some studies reported the use of pectinases to degrade *S. obliquus* (Ometto et al., 2014), and proteases for degradation of proteins in *Porphyridium cruentum* (Kendir Çakmak and Ugurlu, 2020) and *Chlorella vulgaris* (Mahdy et al., 2016). Meanwhile, carbohydrases are among the typically employed enzymes for microalgae enzymatic pretreatment before anaerobic digestion. For instance, cellulase was used for the degradation of the cellulose inner wall layer of the marine microalgae *Nannochloropsis* sp. (Maffei et al., 2018), and other carbohydrase enzymes were used for C*hlorella vulgaris* and *Scenedesmus* sp. hydrolysis (Mahdy et al., 2015b). As other authors have argued, a higher soluble chemical oxygen

demand (sCOD) is obtained when using enzymatic cocktails due to a greater variety of enzymes that could interact with the microalgal cell wall (Carrillo-Reyes et al., 2016; Ometto et al., 2014), thus contributing to enhanced digestibility for biogas production. In this work, enzymatic pretreatments were tested using three different catalyst cocktails and diverse dosages.

In addition to microalgal pretreatment, biogas production can be upgraded by co-digestion of microalgae with other carbon-rich substrates, such as waste activated sludge (WAS) (Beltrán et al., 2016a; Thorin et al., 2017). Co-digestion of both substrates contributes to balancing the C/N ratio, avoids ammonia inhibition from the degradation of lipid-rich substrates, enhances nutrient availability for anaerobic microorganisms, and promotes the integration of wastewater treatment plant (WWTP) facilities and microalgae-based systems, thus improving the economic feasibility of wastewater treatment (Solé-Bundó et al., 2019).

This work is developed within a circular economy project in a winery company aimed at microalgal cultivation for mitigation of CO_2 produced in the winery company. This study evaluates the efficiency of alkaline and enzymatic pretreatment methods for microalgal concentration and solubilisation as well as algal biomass valorisation through co-digestion with waste activated sludge generated by the company. This study provides useful information since it is the first to address the effects of co-digestion of microalgae enzymatically pretreated as a co-substrate to activated sludge for waste streams valorisation in WWTPs.

2. Materials and methods

2.1. Substrates

Microalgal biomass was cultivated in 9 L column photobioreactors (PBRs). Samples were taken from the reactors when microalgae reached the exponential growth phase. PBRs were fed with secondary effluent from a WWTP of a winery company (hereafter referred to as the company). Inlet wastewater characterisation throughout a year is presented in Table S1. PBRs were located inside a greenhouse chamber in which illumination was naturally provided, and the temperature was approximately 20 ± 10 °C. Microscopic examination identified *Chlorella* sp. and

126 *Scenedesmus* sp. (Fig. S1) as the main microalgal species present in the culture (Zeiss, AixoCam
127 ERc 5 s).

128 The WAS used in anaerobic co-digestion assays was obtained from the aerobic biological
129 reactors of the company WWTP. A defined WAS and microalgal mixture (WAS:RM) composed of
130 93% WAS and 7% microalgae on a volatile solids (VS) basis was used in biochemical methane
131 potential tests (BMP)-set 2 experiments (explained below). The proportion of the mixture was
132 established according to the daily volume production of both substrates by the company.

133 **2.2. Set-up for pH-induced flocculation through pH adjustment**

134 A flux diagram of the performed experiments is presented in Fig. 1a. A volume of 1000 mL of
135 microalgal biomass from the PBRs was added into a 1 L glass graduated cylinder to assess the
136 effect of the alkaline pretreatment on microalgal concentration and solubilisation. Microalgae were
137 flocculated by adjusting the pH of the culture medium to pH 10 (PBR-10) and pH 11 (PBR-11) by
138 addition of 5 N sodium hydroxide (NaOH) and stirring with a magnetic stirrer until the pH was
139 adjusted to the desired value (pH-adjusted treatments, n = 3). Additionally, controls of microalgae
140 biomass without pH adjustment (PBR-10-C and PBR-11-C) were employed (n = 1) to compare
141 the effect of natural sedimentation. The initial soluble chemical oxygen demand (sCOD), initial
142 and final total suspended solids (TSS) and initial and final volatile suspended solids (VSS) in the
143 supernatant were determined from the pH-adjusted treatments and the controls. A 1 mL sample
144 of the supernatant was withdrawn from the middle of the clarified zone at the initial time and at
145 diverse exposure times during the 7 days after pH adjustment to measure the optical density
146 (OD_{680}) and calculate the clarification efficiency (CE) according to Eq. 1:

147 $$\mathrm{CE}\ (\%) = \mathrm{OD_i} - \mathrm{OD_t} \ / \ \mathrm{OD_i} \qquad (1)$$

148 where OD_i is the initial OD_{680} before adjusting the pH of the culture, and OD_t is the OD_{680} of the
149 culture at time t after adjusting the pH to the desired value. Solids clarification in the supernatant
150 is an indirect measurement of the concentration of solids in the thickened zone, allowing
151 experimental measurement over time without distorting the sample.

After 7 days of flocculation, the microalgal pellet (concentrated microalgal biomass) was separated from the supernatant, and the pH of the supernatant was measured and neutralized to pH 7 by adding 2 N hydrochloric acid (HCl). Final TSS, VSS, and sCOD were determined from the neutralized supernatant.

Figure 1

2.3. Enzymatic pretreatment of microalgal biomass

Three enzymatic commercial preparations were applied to hydrolyse the microalgal biomass and increase microalgal digestibility: enzyme A, enzyme B, and enzyme C (a description of the enzymes is shown in Table S2). Enzyme A (Passos et al., 2016) and enzyme B are multi-enzymatic preparations composed of diverse enzymes. Enzymes A, B, and C were tested at two doses of 1% and 2% (v/v) to identify the following pretreatment methods (enzyme name and dose): A1, B1, C1, A2, B2, and C2. Thus, the pretreatment methods applied to the microalgal biomass (M) were identified as M-A1, M-B1, M-C1, M-A2, M-B2, and M-C2. A volume of 100 mL of microalgal solution was placed into Erlenmeyer flasks (250 mL), and the enzyme was added. Enzymatic hydrolysis was conducted at 37 °C under orbital agitation (100 rpm). The pH was not previously fixed or controlled during the pretreatment. Two sets of enzymatic hydrolysis (EH) experiments were carried out (Fig. 1a and 1b). In EH-set 1, samples were removed from all the trials to measure the total soluble organic matter released by analysing the sCOD of the filtrate at the initial time and over an exposure time of 0.5, 1, 2, and 3 h after enzyme addition. According to the results obtained, in EH-set 2, the enzymatic pretreatments were performed at the optimal exposure time in both doses. All trials were carried out in triplicate. To evaluate the effect of the pretreatment on microalgal solubilisation, a control reactor (RM) containing the raw microalgal culture without enzymatic pretreatment and exposure to 37 °C was used. The effect of the enzymatic pretreatment on the hydrolysis efficiency was determined by comparing the increase in sCOD after the pretreatment and the sCOD concentration in the control.

2.4. Biochemical methane potential (BMP) tests

Two sets of BMP tests were performed: BMP-set 1 and BMP-set 2 (Fig. 1a and 1b). The aim of BMP-set 1 was to evaluate the methane yield of the selected enzymatically pretreated microalgal biomass. BMP-set 2 assessed the co-digestion efficiency of a mixture of WAS and enzymatically pretreated microalgal selected in BMP-set 1. Anaerobic batch assays were performed according to a previously described procedure (Martín-González et al., 2010) with consideration of suggestions from other authors (Angelidaki et al., 2009; Holliger et al., 2016). BMP assays were carried out in triplicate under mesophilic conditions (37 °C). BMP-set 1 tests were carried out using 120 mL glass bottles (80 mL working volume), and raw microalgae without pretreatment (RM) were used to compare the effect of the pretreatments on biogas production. BMP-set 2 tests were performed in 900 mL aluminium bottles (600 mL working volume), and different controls were used: WAS without pretreatment (WAS), WAS and raw microalgal mixture without pretreatment (WAS:RM), WAS with enzyme addition (WAS-enzyme-dose), and inoculum with enzyme addition (I-enzyme-dose).

The anaerobic sludge employed as inoculum in the biochemical methane potential (BMP) assays was collected from the anaerobic digesters of the Riu Sec WWTP (Sabadell, Barcelona). To guarantee the consumption of the organic matter contained in the inoculum, it was pre-incubated at 37 °C for 12 days. All BMP bottles were filled with inoculum, substrate, and tap water until the working volume was reached. Subsequently, the bottles were flushed with pure N_2 to ensure anaerobic conditions, closed, and incubated in a temperature-controlled chamber. Biogas production and accumulation in the headspace of the bottles were measured with an SMC pressure switch manometer (1 bar, 5% accuracy) until biogas generation ceased. Bottles containing only the same amount of inoculum (blank) in the trials were used to analyse the background biogas production of the inoculum. Net biogas production was determined by subtracting the biogas production of the blank from the gross biogas production of the sample bottles. Moreover, crystalline cellulose was employed as a reference substrate to evaluate the activity of the inoculum (control). The reactors were shaken manually every time a gas sample was taken. Periodically, biogas composition was determined through gas chromatography. The generated biogas was expressed as the volume of methane generated per mass of VS of the

208 added substrate (NmL CH_4 g VS^{-1}) expressed under standard pressure and temperature (273.15
209 K and 1.0133 bar).

210 **2.5. Analytical techniques**

211 Hach Lange cuvettes (LCK 314, 114, and 014), and the spectrophotometer DR 3900 (Hach Lange
212 GmbH, Düsseldorf, Germany) were employed for sCOD determination using filtered supernatant
213 (GF/A glass microfibre filters, Whatman, GE Healthcare, USA). TSS, VSS, TS, and VS were
214 determined according to the procedures defined in Standard Methods (APHA, 2008). pH was
215 measured by a pH meter (Crison, Spain).

216 Carbon dioxide and methane content in the biogas were analysed using a gas chromatograph
217 (Hewlett Packard 5890, Agilent Technologies, Mississauga, Canada) equipped with a thermal
218 conductivity detector and a Supelco Porapack Q column (3 m × 3.2 mm) (Pennsylvania, USA).
219 Helium was the carrier gas (338 KPa); and the oven, injector, and detector temperatures were
220 70, 150 and 180 °C, respectively. Samples were injected with a 100 µL syringe (VICI PS Syringe
221 A-2, 0.74 mm × 0.13 mm × 50.8 mm).

222 Before volatile fatty acids (VFAs) determination, samples were centrifuged (10 min, 8000 rpm,
223 Beckman Coulter, Avanti J20 XP, USA) and then filtered (0.45 µm nylon syringe filter). VFAs were
224 analysed by a Dionex 3000 ultimate high-performance liquid chromatography (Barcelona, Spain)
225 equipped with a UV/visible detector (210 nm). The chromatographic separation was performed
226 using an ICE-COREGEL 87H3 column (7.8 × 300 mm, Transgenomic, USA), heated at 40 °C,
227 employing 0.006 mM of H_2SO_4 as a mobile phase at a flow rate of 0.5 mL min^{-1}.

228 **2.6. Data analysis**

229 The experimental data were analysed statistically, and differences were considered significant at
230 p values below 0.05. When the null hypothesis was rejected (significance level < 0.05), post hoc
231 comparisons were performed. All statistical calculations were carried out using R software
232 (version 3.6.3).

233 The modified Gompertz equation (Nielfa et al., 2015) was employed to model biomethane
234 production and calculate kinetic parameters for anaerobic degradation according to Eq. 2:

$$P_{net}(t) = P_{max} \cdot \exp\left\{-\exp\left[\frac{R_{max} \cdot e}{P_{max}}(\lambda - t) + 1\right]\right\} \quad (2)$$

where $P_{net}(t)$ is the net cumulative methane yield (NmL CH_4 g VS^{-1}) at time t, P_{max} is the methane yield potential (NmL CH_4 g VS^{-1}), R_{max} is the maximum methane production rate (NmL CH_4 g VS^{-1} d^{-1}), t is the digestion time (d), and λ represents the lag phase (d). The hydrolysis rate of the anaerobic digestion was evaluated according to Eq. 3, adjusting the experimental data to a first-order kinetic model by the least squares method (Martín Juárez et al., 2018):

$$B(t) = B_0\left(1 - \exp^{-K_H \cdot t}\right) \quad (3)$$

where B(t) is the cumulative methane yield at time t (NmL CH_4 g VS^{-1}) obtained experimentally, B_0 is the ultimate methane yield (NmL CH_4 g VS^{-1}), K_H is the hydrolysis rate constant (d^{-1}), and t is the digestion time (d). The values of the above parameters were estimated by an algorithm developed in MATLAB R2015a (MathWorks Inc. Natick, MA, USA).

3. Results and discussion

3.1. Microalgal concentration through alkaline flocculation

When the pH of the solution was adjusted to 10 (Fig. 2), the clarification efficiency in PBR-10 increased by 4.4% (96.8%) compared to the untreated control (PBR-10-C, 92.5%). Comparable results were obtained when adjusting the pH to 11 (Fig. 2). The clarification efficiency in PBR (PBR-11) increased by 5.3% compared with the control without pH adjustment (PBR-11-C) (96.7% and 91.6%, respectively). When adjusting the pH to 11, high clarification efficiencies were achieved after 2 days; however, it took at least 6 days to reach similar clarification efficiencies when adjusting the pH to 10. The evolution of the clarification during alkaline pretreatment at each pH is shown in Fig. S2. Overall, after 7 days of pretreatment at pH 10 and pH 11, equivalent efficiencies were attained compared with the controls under natural sedimentation, while slight differences were observed at shorter exposure times.

Figure 2

According to our results and in agreement with other authors, the absence of flocculation of *Chlorella vulgaris* biomass at up to pH 10.2-10.5 was reported (Smith and Davis, 2012; Vandamme et al., 2012), suggesting that natural sedimentation was the main mechanism involved in PBR-10 over the 7 days. Moreover, VSS reduction in the supernatant (Fig. 3) was higher in the untreated controls. The results under the tested conditions indicated that adjusting the pH to 10 and 11 had a slight or negligible effect on microalgal biomass flocculation compared with the controls. Contrary to our results, other authors reported >95% recovery of *Dunaliella viridis* after 24 h of adjusting the pH of the culture medium to 10 (Mixson et al., 2014) and 90% recovery of *Chlorella vulgaris* as pH increased to 10 (Branyikova et al., 2018). Ummalyma et al. (2016) obtained a 94% flocculation efficiency of the freshwater microalgae *Chlorococcum* sp. at pH 12. Diverse results could be explained by the differences in the medium composition (Mg^{2+} and Ca^{2+} content) since the amount of base needed to induce flocculation depends on the buffering capacity of the culture and the concentrations of Ca^{2+} and/or Mg^{2+} (García-Pérez et al., 2014; Muylaert et al., 2015; Vandamme et al., 2012).

Figure 3

Dissolved organic carbon in the supernatant increased by 1.5-fold at the end of the alkaline pretreatment for PBR-11 (Table S3). This fact could be associated with the presence of dissolved organic matter excreted by microalgal cells in the supernatant, also referred to as algal organic matter (AOM) (Barros et al., 2015), rather than sCOD from cell wall solubilisation. *Chlorella* sp. and *Scenedesmus* sp. are characterized by the high recalcitrance and robustness of their cell walls (González-Fernández et al., 2012), and as reported by other authors, alkaline pretreatment of *Chlorella* biomass was ineffective in biomass solubilisation (Bohutskyi et al., 2014). In addition, dissolved organic matter has a negative charge that also interacts with hydroxides, decreasing the available magnesium in the medium and requiring a higher dose of NaOH to form precipitates and a higher pH to achieve the same flocculation efficiency (Barros et al., 2015). For instance, Vandamme et al. (2016) reported that a longer cultivation time of *Chlorella vulgaris* leads to

289 greater excretion of AOM to the media, which mainly contains polysaccharides that interfere with
290 and inhibit alkaline flocculation, thus increasing the dose of base addition.

291 Thus, the lower recovery efficiencies in our study could be limited by the medium composition
292 (the content of Mg^{2+} and Ca^{2+}) as well as the presence of AOM excreted by the microalgal
293 biomass. Overall, the results indicate that pH adjustment of the microalgal solution to pH 10 and
294 11 neither enhances microalgal harvesting nor its solubility. When comparing alkaline flocculation
295 with other harvesting techniques, such as bio-flocculation, some authors reported >98%
296 clarification after the co-pelletization of the algal biomass with filamentous fungi (Hom-Diaz et al.,
297 2017) and 90% harvesting efficiency with use of a bacterial strain (Wan et al., 2013). Although
298 these results showed higher flocculation efficiencies, additional time and costs were required for
299 microorganism (fungal or bacterial) production.

300 ## 3.2. Solubility and anaerobic digestibility of enzymatically pretreated microalgal
301 biomass

302 ### 3.2.1. Enzymatic pretreatment of microalgal biomass

303 Microalgal biomass (0.36 ± 0.07 g VS L^{-1}) from the PBR was enzymatically pretreated to evaluate
304 the effect of the pretreatment on biomass solubility. Enzymatic pretreatments were performed at
305 37 °C, combining the action of temperature with the catalytic activity of the enzyme. Enzymatic
306 hydrolysis was tested using three enzymes (A, B, and C) and two enzymatic loads (1% and 2%
307 v/v) over exposure times of 0.5, 1, 2, and 3 h (EH-set 1, Fig. 1a and 1b). The selection of enzyme
308 A was due to the effective organic matter solubilisation of microalgal biomass grown in open
309 ponds for wastewater treatment, as reported by Passos et al. (2016). Enzymes B and C were
310 employed due to their availability within the winery industry, as they are also applied to other
311 industrial processes, as well as their similarity in composition to enzyme A.

312 Microalgal biomass without an enzymatic treatment displayed the lowest sCOD concentrations
313 and was fairly constant over time (Table S4). In all cases, at higher enzyme doses, higher sCOD
314 was released as a result of microalgal biomass solubilisation (Fig. 4a and 4b). When comparing
315 all of the enzymatic pretreatments at the lower dose of the enzyme (Fig. 4a) and at the same
316 exposure time, COD solubilisation was negligible ($p > 0.05$). However, significant differences in

317 sCOD were found at 0.5 h when comparing pretreatments B1 and C1 ($p < 0.05$) (Fig. 4a). At the
318 higher dose (Fig. 4b), significant variations in sCOD were identified when comparing the diverse
319 pretreatments at each exposure time ($p < 0.05$) (Table S4). Furthermore, when analysing
320 pretreatments individually, significant differences were identified in sCOD at different exposure
321 times for pretreatments A2 and B2 ($p < 0.05$) (Table S4). Pretreatments A2 and B2 released
322 greater sCOD after 2 h, and sCOD subsequently decreased. On the other hand, the sCOD
323 concentration in pretreatment C2 remained fairly constant from the first hour.

324

325 Figure 4

326

327 While pretreatments at the 1% dose exhibited faster COD solubilisation, pretreatments at the 2%
328 dose attained greater solubilisation after 2 h of hydrolysis. According to these results, 0.5 h and
329 2 h were set as the optimum exposure times for the enzymatic pretreatments at 1% and 2%
330 doses, respectively (EH-set 2). At the 1% dose and 0.5 h exposure time, sCOD increased by 138-
331 to 159-fold, and at the 2% dose and 2 h exposure time, sCOD improved by 257- to 311-fold.

332 Higher sCOD after enzymatic hydrolysis indicates effective microalgal cell wall degradation and
333 removal of recalcitrant compounds. Comparison with other studies is not proper since the effect
334 of the pretreatment depends on the microalgal species and the conditions applied. Enzymatic
335 pretreatments were carried out at 37 °C in this study since mesophilic anaerobic digestion (37 °C)
336 was applied after the hydrolysis treatment. Moreover, studies typically treated pure microalgal
337 species. For instance, Mahdy et al. (2015b) stated that the differences in hydrolysis efficiency of
338 *Chlorella vulgaris* and *Scenedesmus* sp. were due to their diversity in the cell wall and intracellular
339 composition. Cell wall composition varies among species and growth conditions. In this work,
340 selection of enzymes agreed with the microalgal cell wall composition. In this sense, cellulase
341 hydrolyses cellulose, and glucohydrolase and xylanase degrade hemicellulose. Pectinliase and
342 poligalacturonase are responsible for the degradation of pectin, and protease catalyses the
343 breakdown of proteins. *Chlorella vulgaris* possesses a robust polymeric cell wall structure
344 constituted by hydrolysable (xylose, mannose, galactose, glucose, fucose, arabinose, rhamnose

and uronic acids) and resistant (glucosamine) compounds (Gerken et al., 2013). Pectin was also identified in *C. vulgaris* (Gerken et al., 2013) and *Scenedesmus* sp. The cell wall consists of carbohydrates composed of cellulose and hemicellulose (González-Fernández et al., 2012) in the presence of sporopollenins (Carrillo-Reyes et al., 2016). Ometto et al. (2014) tested sCOD released by three microalgal species after enzymatic pretreatment (24 h, 50 °C) using five different enzymes and doses, showing that pectinases generated higher solubilisation of *S. obliquus* biomass. Similarly, Maffei et al. (2018) reported cell damage, changes in cell morphology, and release of microalgal intracellular components after enzymatic pretreatment of *Nannochloropsis* sp. with cellulase and mannanase. Passos et al. (2016) likely obtained high solubilisation of the algal biomass when applying enzyme A and cellulase at a 1% dose (t = 6 h, 37 °C). Due to the synergetic effect on the diverse macromolecules of the algal biomass, those researchers highlighted the use of the enzymatic cocktail (enzyme A) over the sole enzyme (cellulase), and moreover, they stated that the enzymes glucohydrolase and xylanase may have had an effect once the organic matter was hydrolysed by cellulase (Passos et al., 2016).

3.2.2. Anaerobic digestion of enzymatically pretreated microalgal biomass

To further test the effect of the enzymatic pretreatment on algal biomass anaerobic digestibility, BMP tests of the enzymatically pretreated microalgal biomass were performed under the previously defined optimal conditions of 1% and 2% enzyme doses at exposure times of 0.5 h and 2 h, respectively (BMP-set 1, Fig. 1a and 1b). The microalgal biomass contained 0.44 g VS L^{-1}.

The net methane yield obtained in all trials in BMP-set 1 is shown in Fig. 5a and 5b. Differences between methane yields achieved after pretreatments M-B1 and M-C1 (640.9 ± 19.7 and 652.0 ± 13.8 NmL CH_4 g VS^{-1}, respectively) were not statistically significant ($p > 0.05$) (Fig. 5a and Table 1), and biogas production amounts for both pretreatments were 5.2- and 5.3-fold higher than that obtained by the untreated biomass (RM), respectively (differences were statistically significant, $p < 0.05$). Although sCOD was reduced by 91% in M-A1 (Table 1), methane production was 43-46% lower (447.5 ± 40.0 NmL CH_4 g VS^{-1}) compared with M-B1 and M-C1 ($p < 0.05$). Similarly, M-B1 and M-C1 presented a greater methane production rate (4.3 mL d^{-1}) than M-A1 (3.0 mL d^{-}

374 [1]). However, the bioconversion process for M-A1 (K_H = 0.194 d^{-1}) was more than 2-fold higher
375 than that of the other pretreatments at the 1% dose (Table 1). For methane productivity, reactors
376 M-A1, M-B1, and M-C1 achieved 90% methane production after 23, 19 and 15 days, respectively.
377 The different outputs could be associated with the assorted enzyme composition of the enzymatic
378 cocktails and their interaction with the microalgal biomass.

379

380 Figure 5

381

382 Surprisingly, when the enzyme dose (2%) was increased, methane production for pretreatments
383 M-A2, M-B2, and M-C2 decreased by 9%, 27%, and 16%, respectively (Fig. 5b and Table 1)
384 compared with the pretreatment with the same enzyme at the lower dose (1%). Although higher
385 solubilisation was achieved with pretreatments at the 2% dose and t = 2 h, anaerobic digestibility
386 was lower than that with pretreatments at the lower dose (Table 1). Bearing in mind that the
387 microalgal concentration was the same in all pretreatments, one hypothesis is that the excess
388 enzyme in the pretreatments at the 2% dose might not interact with the microalgal biomass, thus
389 inhibiting anaerobic microorganisms and reducing methane yield. In contrast to digestion of raw
390 microalgae, the methane yield increased sharply when microalgae were pretreated with the three
391 enzymes at the 2% dose ($p < 0.05$). Moreover, significant statistically differences were found
392 between the M-A2 and M-C2 pretreatments.

393

394 Table 1

395

396 Methane production increased faster during the first 15 days for all pretreatments. For biogas
397 composition, no differences were identified among the trials (Table 1). At the end of the BMP
398 tests, pH values of the digestates between 7.2 and 7.7 suggest the stability of the process.

399 Moreover, the concentration of VFAs was negligible in all cases. Fig. S3 shows that the
400 relationship between methane yield and solubilisation increases after pretreatment.

401 The reduction in sCOD in BMP-set 1 was similar and higher than 90% for all trials. Nonetheless,
402 the differences obtained in the methane yield for all of the trials are not consistent with their
403 respective solubility increases after the enzymatic pretreatment. This fact suggests that the
404 solubilized organic matter is not totally converted into methane. Consequently, it was not possible
405 to identify a direct relationship between the reduction in sCOD and the methane yield. The
406 compromise between a low enzymatic dose applied at a short exposure time to achieve a high
407 methane yield represents the most favourable strategy for addressing the economic feasibility
408 and applicability of the treatment (Fig. S3). Based on these outcomes, the enzymatic
409 pretreatments of microalgae with enzyme B1 (M-B1) and C1 (M-C1) at t = 30 min were selected
410 as the proper treatments for co-digestion studies (BMP-set 2) (Fig. 1a and 1b).

411 **3.3. Co-digestion of enzymatically pretreated microalgal biomass and waste activated**
412 **sludge**

413 According to the results from the above experiments, two optimal enzymatic pretreatments
414 (enzyme B and enzyme C at a 1% dose (v/v) at t = 0.5 h) were selected to further study the co-
415 digestion of the pretreated microalgal biomass with WAS as a co-substrate (BMP-set 2, Fig. 1a
416 and 1b). This set of experiments contained 17.3 ± 0.1, 4.3 ± 0.1, and 8.5 ± 0.3 g VS L^{-1} inoculum,
417 WAS, and gravity-concentrated microalgae, respectively.

418 As shown in Fig. S4 (net methane yield) and Fig. 6 (cumulative methane production), the methane
419 production obtained when co-digesting enzymatically pretreated microalgae (M-B1 and M-C1)
420 with WAS (WAS:M-B1 and WAS:M-C1) was similar to values obtained when using raw
421 microalgae in the mixture (WAS:RM). Compared with WAS:RM, the net methane yield was 2%
422 and 7% lower for WAS:M-B1 and WAS:M-C1 (Fig. S4), respectively; however, this difference was
423 not statistically significant (p > 0.05). Similarly, VS reduction was similar in the three reactors
424 (Table 2). The methane yield of WAS:RM increased faster during the first days, causing a poor fit
425 for the lag phase (λ) parameter of the Gompertz model (Table 2). Compared with WAS:RM, the
426 hydrolysis rate (K_H) decreased for WAS:M-B1 (0.064 d^{-1}). However, the R_{max} values showed a

higher methane production rate for this mixture (Table 2). This result could be explained by the effect of the previous microalgal enzymatic hydrolysis contributing to a greater bioavailability of the substrate. The major net methane yield of all the trials was obtained for WAS mono-digestion (179.3 g CH_4 g VS^{-1}) (Fig. S4 and Table 2). Although the difference was not statistically significant ($p > 0.05$), the methane yield of WAS and raw microalgae co-digested (WAS:RM) was 7% lower than that obtained by WAS. Nevertheless, the co-digestion of WAS:RM improved the K_H by 11% and the VS reduction by 27% compared with the mono-digestion of WAS. According to our results, enzymes applied to the microalgal biomass did not enhance methane production when co-digested with WAS. The values of VFAs accumulated in the reactors at the end of the BMP tests (Table 2) could indicate that biomasses were also converted to volatile compounds other than methane. Some studies showed an enhancement in methane yield after co-digestion of sewage sludge and raw microalgal biomass, attributing this result to higher nutrient availability, enhanced alkalinity, and a balanced C/N ratio to avoid ammonia inhibition (Beltrán et al., 2016b; Olsson et al., 2014; Solé-Bundó et al., 2020). However, Kim and Kang (2015) also observed a decline of 9% in methane accumulation when microalgae (*Chlorella* sp.) were co-digested with WAS compared with WAS mono-digestion. Caporgno et al. (2015) stated that there was no synergy between microalgae (25% *Selenastrum capricornutum*) and sludge (75% of a blend of primary and secondary sludge) co-digestion and obtained lower methane than sludge alone. In the same way, a mixture of microalgae (*Ankistrodesmus*, *Chlorella*, *Coelastrum*, *Scenedesmus opoliensis*, *Scenedesmus quadricauda, Scenedesmus* sp., among others) and sludge (37% and 63% on a VS basis, respectively) showed low digestibility, obtaining a lower methane yield compared with the sludge alone, as reported by Olsson et al. (2018). In addition, Wang et al. (2013) observed comparable methane yields of WAS alone and WAS co-digested with raw microalgal biomass (*Chlorella* sp.). Diverse outputs after microalgae and sludge co-digestion could be related to the specific features of both substrates (microalgal composition is strain-specific) as well as to the different proportions of WAS and microalgae employed in the mixtures. The results of co-digestion assays differed slightly from those of WAS mono-digestion. Pretreated and untreated microalgal co-digestion with WAS seemed neither to have a synergetic effect nor a toxicity effect on biogas production, indicating that both substrates could be digested together, thus avoiding the costs associated with separated digestion processes (Elalami et al., 2019).

Figure 6

Table 2

Typically, studies report sludge co-digestion with untreated co-substrates such as the organic fraction of municipal solid waste and agro-industrial and fatty wastes as co-substrates. Nonetheless, few studies have assessed the co-digestion of WAS and previously treated co-substrates such as microalgal biomass. An increase of 12% in methane production was achieved when co-digesting a mixture of 75% secondary sludge and 25% microalgae (C. *vulgaris*) when both substrates were thermally pretreated (120 °C, 40 min) (Mahdy et al., 2015a). Compared with untreated biomasses, Scarcelli et al. (2020) reported a slight increase in methane production when a thermal pretreatment (65 °C, 4 h) was applied to a WAS (60%) and microalgal (*Chlorella* sp., 40%) mixture. Similarly, Zhang et al. (2018) studied the co-digestion of microalgae (*Chlorella* sp.) pretreated with an enzymatic cocktail of cellulase, xylanase, and pectinase for lipid extraction using energy grass (*Pennisetum* hybrid) as a co-substrate. As far as the authors are concerned, this study assesses the co-digestion of WAS and enzymatically pretreated microalgae for the first time.

To test the effect of the enzymes on the WAS, the same dose of enzyme applied to the microalgae was directly added to the WAS in the BMP reactors WAS-B1 and WAS-C1. The results showed that enzymes B1 and C1 weakly reduced the net methane yield of WAS by 5% (WAS-B1) and 2% (WAS-C1), respectively (Fig. S4 and Table 2), but these differences were not statistically significant ($p > 0.05$). Nonetheless, a greater VS reduction took place in the WAS-B1 and WAS-C1 reactors (30% and 27%, respectively) compared with WAS (19%), suggesting that the enzymes could contribute to major solubility and further degradation of the organic matter in the

WAS. Furthermore, the hydrolysis rate of the secondary sludge slightly increased in the reactors to which the enzymes were added (Table 2).

To verify that the enzymes did not negatively affect the inoculum, the influence of the enzymes on the inoculum in biogas production was tested. The results in Fig. 6 indicated that I-B1 and I-C1 exhibited 33% and 42% increases in methane production compared with the blank reactor (I), respectively. This fact suggests that enzymes B and C did not inhibit the anaerobic microorganisms present in the inoculum.

4. Conclusions

This study assessed the pretreatment of microalgal biomass for solubility enhancement and further anaerobic digestion of pretreated microalgae as well as its co-digestion with WAS.

First, similar results were obtained when harvesting microalgae by natural sedimentation and alkaline pretreatment at pH 10 and 11. Similarly, microalgal solubility was not improved by pH adjustment. Hence, this pretreatment was rejected for algal biomass harvesting prior to valorisation.

Second, enzymatic hydrolysis pretreatments were performed to enhance the solubility of the microalgal biomass. The optimal pretreatment conditions were t = 0.5 h and t = 2 h for 1 and 2% doses, respectively. Compared with raw microalgal biomass, the enzymatic pretreatment highly enhanced the solubility and the biogas yield of the algal biomass at both doses, showing efficient solubilisation and anaerobic digestibility of the biomass. Although the organic matter solubilisation registered was higher for pretreatments at the higher dosage, the methane yield markedly increased for microalgal biomass pretreated with the lower enzyme dosage. When co-digesting microalgae with WAS, comparable methane yields were obtained for enzymatically pretreated and untreated algal biomass. Since the previous enzymatic treatment of the microalgae did not enhance the methane yield, it can be neglected to reduce costs. Overall, even though co-digestion with microalgae under the studied conditions did not improve energy production, co-digestion is

509 a promising and economically feasible alternative for diverse waste stream treatments via the
510 integration of WWTP facilities and microalgae-based systems.

511 Based on the obtained outcomes, future research should include pilot-scale studies to verify these
512 results, assess the start-up of the reactor, and the influence of substrates variability along seasons
513 in the process performance. Moreover, the evaluation of hydrogen production potential should be
514 considered in future studies as an important avenue to improve energy conversion from algal
515 biomass.

516

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522

523 **Competing interest statement**

524 We declare that no conflict of interest exists in the submission of this manuscript.

525

526 **Author contribution**

527 Romina Avila: experimental work, data interpretation, discussion of results and writing the main
528 manuscript. Teresa Vicent and Paqui Blánquez: conception and experimental design, supervision
529 of the experimental work, review, and edition of the manuscript. Elvira Carrero: photobioreactor
530 experimental work.

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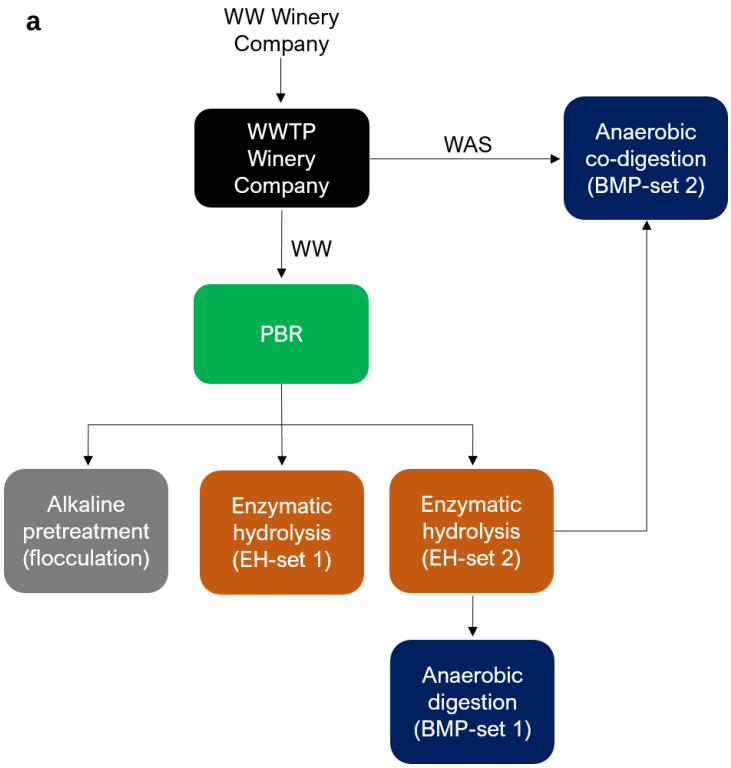
a
WW Winery Company
WWTP Winery Company
WAS
Anaerobic co-digestion (BMP-set 2)
WW
PBR
Alkaline pretreatment (flocculation)
Enzymatic hydrolysis (EH-set 1)
Enzymatic hydrolysis (EH-set 2)
Anaerobic digestion (BMP-set 1)

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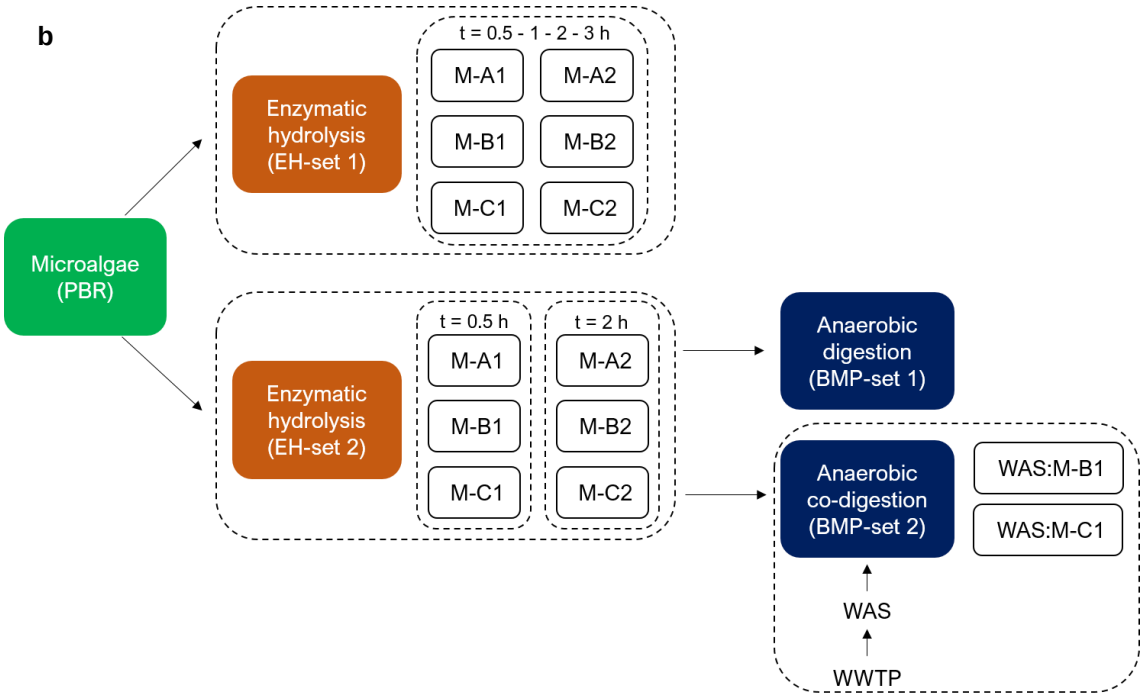
b
Microalgae (PBR)
Enzymatic hydrolysis (EH-set 1)
t = 0.5 - 1 - 2 - 3 h
M-A1
M-A2
M-B1
M-B2
M-C1
M-C2
Enzymatic hydrolysis (EH-set 2)
t = 0.5 h
t = 2 h
Anaerobic digestion (BMP-set 1)
Anaerobic co-digestion (BMP-set 2)
WAS:M-B1
WAS:M-C1
WAS
WWTP

726

727 **Figure 1.** (a) Flux diagram of the performed experiments. (b) Schematic description of the
728 experimental set-up for enzymatic pretreatments and biochemical methane potential tests, and
729 their respective nomenclature. References: WW = wastewater. WWTP = wastewater treatment
730 plant. WAS = waste activated sludge. PBR = photobioreactor. EH = enzymatic hydrolysis. BMP
731 = biochemical methane potential test. M = microalgae. A1, B1, and C1 refer to pretreatments with
732 the enzymes at a 1% dose while A2, B2, and C2 refer to pretreatments with the enzymes at a 2%
733 dose.

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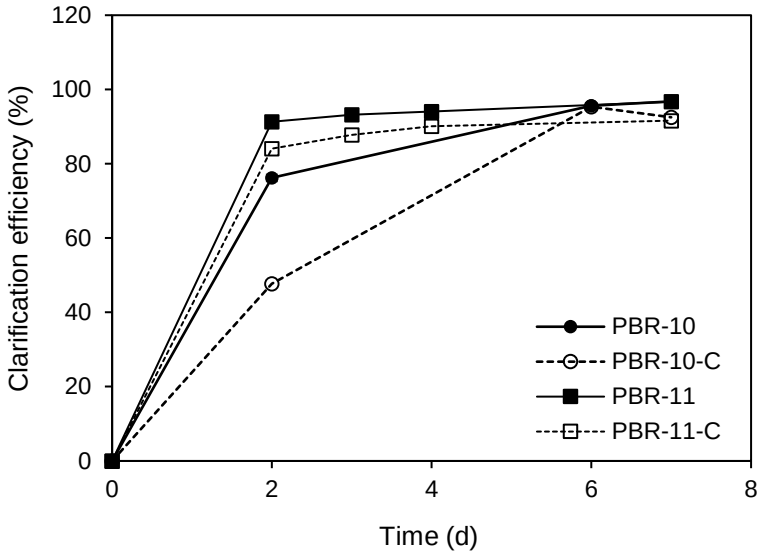

735

736 **Figure 2.** Clarification efficiency (%) after the alkaline pretreatment at pH 10 (PBR-10) and pH 11
737 (PBR-11), and their respective untreated controls (PBR-10-C and PBR-11-C). Error bars in PBR-
738 10 and PBR-11 represent the standard deviation of the mean (n = 3).

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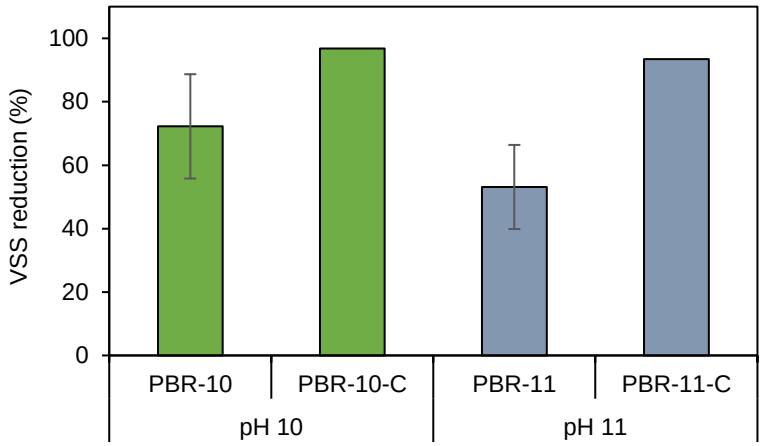

741 **Figure 3.** VSS reduction in the supernatant of PBR-10 and PBR-11 after pH-adjustment and
742 neutralization, and their respective untreated controls (PBR-10-C and PBR-11-C). Error bars in
743 PBR-10 and PBR-11 represent the standard deviation of the mean (n = 3).

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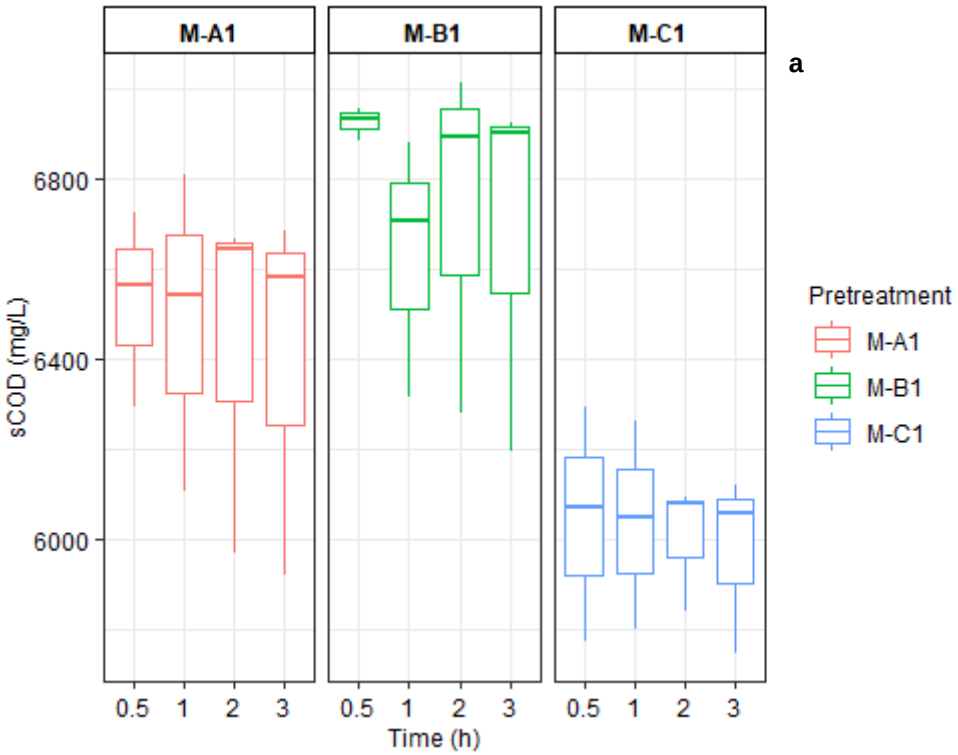

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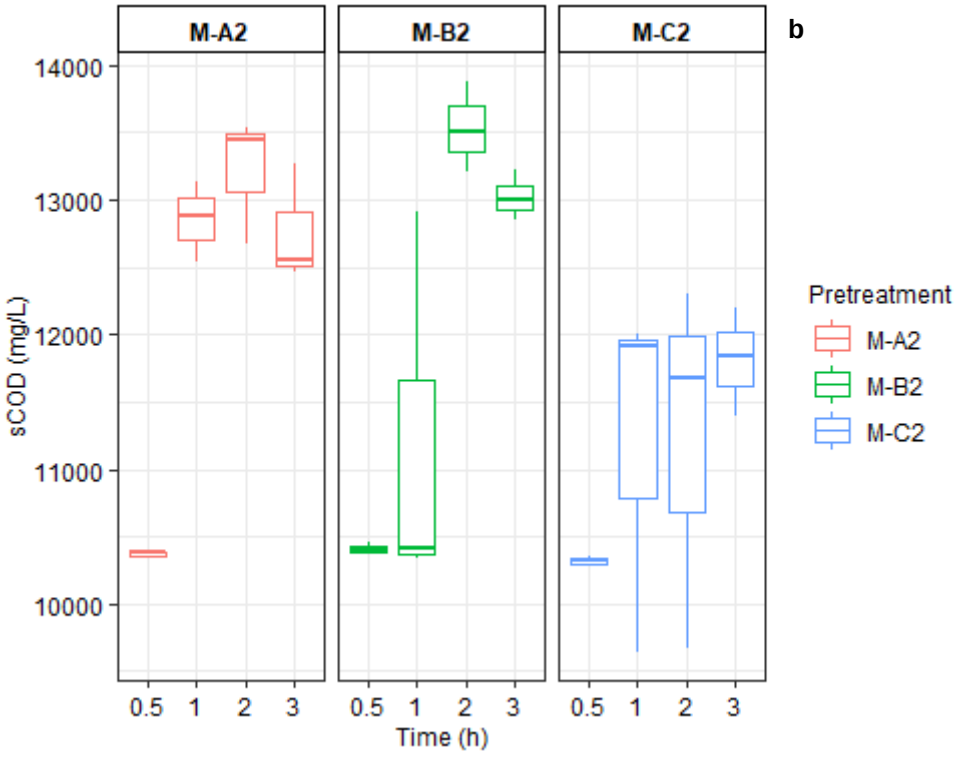

747 **Figure 4.** Soluble chemical oxygen demand (sCOD) released after enzymatic pretreatments of
748 microalgal biomass with enzymes A, B, and C, at doses of (a) 1% v/v (M-A1, M-B1, and M-C1),

749 and (b) 2% v/v (M-A2, M-B2, and M-C2) in EH-set 1. Boxplots represent the median value, and
750 the 25^{th} and 75^{th} percentile at each time.

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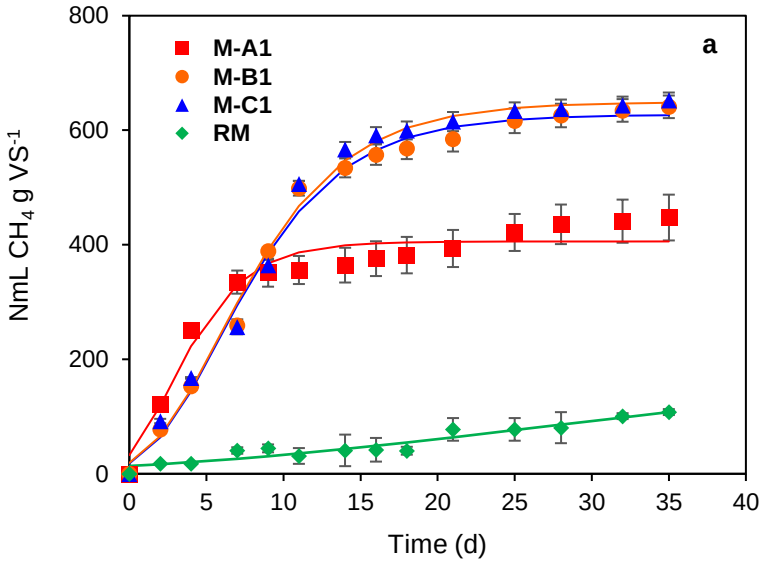

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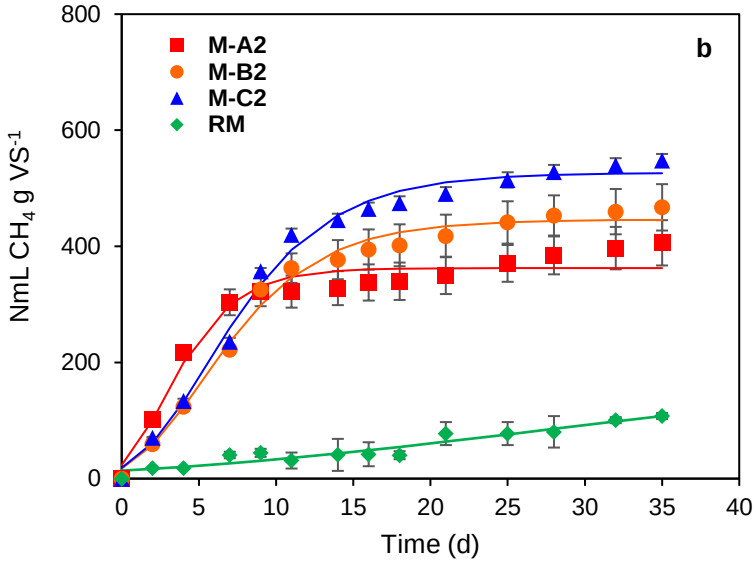


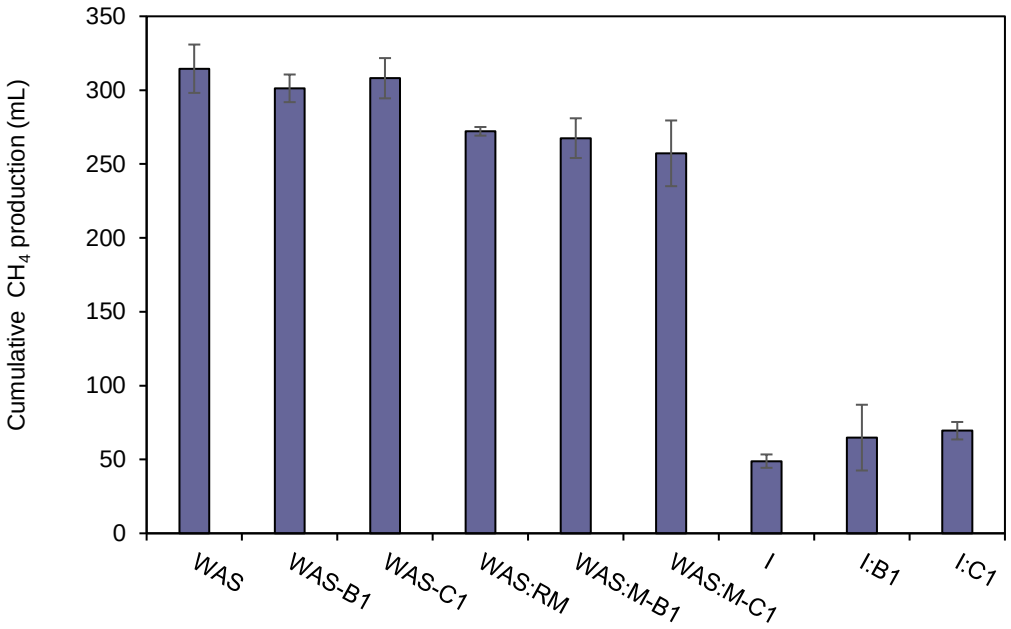
753

754 **Figure 5**. Net methane yield of microalgal biomass enzymatically pretreated at (a) a 1% dose v/v
755 with enzyme A, B, and C (M-A1, M-B1, and M-C1) for 0.5 h; and at (b) a 2% v/v dose with enzyme
756 A, B, and C (M-A2, M-B2, and M-C2) for 2 h, in BMP-set 1. RM refers to microalgal biomass
757 without pretreatment. Dots represent the experimental data while continuous lines correspond to
758 the fitting by the Gompertz model. Error bars represent the standard deviation of the mean (n =
759 3).

760 **Table 1.** Parameters from the enzymatic hydrolysis of microalgae (EH-set 2) and biochemical methane potential tests (BMP-set 1).

Trial	Enzymatic pretreatment (EH-set 2)	Biomethane potential test (BMP-set 1)								
					Gompertz model				Hydrolysis rate	
	$sCOD_f$* (mg L^{-1})	sCOD reduction (%)**	Net experimental CH_4 yield (NmL CH_4 g VS^{-1})	Methane content (%)	P_{max} (NmL CH_4 g VS^{-1})	R_{max} (NmL CH_4 g VS^{-1} d^{-1})	λ (d)	r^2	K_H (d^{-1})	r^2
RM	52.8 ± 2.7	18 ± 0	124.2 ± 7.9	73 ± 4	232.5 ± 179.1	3.2 ± 0.7	1.1 ± 0.8	0.920	0.008	0.907
M-A1	5858.7 ± 1449.8	91 ± 0	447.5 ± 40.0	69 ± 1	405.8 ± 10.7	53.7 ± 9.4	-0.2 ± 0.7	0.955	0.194	0.974
M-B1	6163.3 ± 1308.0	95 ± 1	640.9 ± 19.7	69 ± 8	627.1 ± 9.9	50.9 ± 3.2	1.2 ± 0.4	0.993	0.092	0.975
M-C1	6085.3 ± 60.0	91 ± 2	652.0 ± 13.8	68 ± 10	649.1 ± 11.6	51.6 ± 3.7	1.2 ± 0.5	0.991	0.092	0.969
M-A2	13096.0 ± 1119.4	90 ± 3	406.1 ± 39.0	70 ± 1	362.6 ± 9.1	50.9 ± 8.8	0.0 ± 0.6	0.959	0.194	0.972
M-B2	12950.0 ± 500.3	90 ± 1	467.2 ± 39.0	70 ± 7	446.0 ± 8.3	38.1 ± 3.1	0.8 ± 0.5	0.989	0.103	0.982
M-C2	11546.7 ± 1012.0	92 ± 2	548.0 ± 11.2	70 ± 8	526.7 ± 9.1	43.1 ± 3.0	0.9 ± 0.4	0.991	0.096	0.980

761 References: RM = raw microalgae (without pretreatment). Enzymatically pretreated microalgae with enzymes A, B, and C at a 1% dose = M-A1, M-B1, and M-C1. Enzymatically pretreated microalgae
762 with enzymes A, B, and C at a 2% dose = M-A2, M-B2, and M-C2.
763 (*) Soluble chemical oxygen demand at the end of the enzymatic pretreatment (0.5 h for 1% dose, and 2 h for 2% dose).
764 (**) Soluble chemical oxygen demand reduction was calculated considering sCOD values at initial and final time of the BMP tests.
765



766

767 **Figure 6.** Cumulative methane production in BMP-set 2 for the following trials: WAS; WAS with
768 enzyme addition (WAS-B1 and WAS-C1); mixture of WAS and raw microalgae (WAS:RM);
769 mixture of WAS and enzymatically pretreated microalgae (WAS:M-B1 and WAS:M-C1); inoculum
770 (I); and inoculum with enzyme addition (I-B1 and I-C1). Error bars indicate the standard deviation
771 of the mean (n = 3). References: RA = raw microalgae without pretreatment. WAS = waste
772 activated sludge. I = inoculum. B1 = enzyme B at a 1 % dose. C1 = enzyme C at a 1% dose. M-
773 B1 = microalgae pretreated with enzyme B at a 1% dose (t = 0.5 h). M-C1 = microalgae pretreated
774 with enzyme C at a 1% dose (t = 0.5 h).

775 **Table 2.** Experimental and calculated parameters from the biochemical methane potential tests of WAS (waste activated sludge) and microalgae co-digestion
776 (BMP-set 2).

777

Trial	VS reduction (%)	Net experimental CH_4 yield (NmL CH_4 g VS^{-1})	Methane content (%)	VFAs (mg L^{-1})		Gompertz model				Hydrolysis rate	
				Acetic acid	Propionic acid	P_{max} (NmL CH_4 g VS^{-1})	R_{max} (NmL CH_4 g VS^{-1} d^{-1})	λ (d)	r^2	K_H (d^{-1})	r^2
WAS:RM	26 ± 2	166.0 ± 2.2	71 ± 2	34.1 ± 2.0	107.1 ± 17.9	164.7 ± 5.6	8.2 ± 0.8	-1.4 ± 1.7	0.984	0.080	0.993
WAS:M-B1	24 ± 3	162.6 ± 10.0	69 ± 10	38.5 ± 7.9	152.4 ± 4.6	158.3 ± 4.4	9.6 ± 0.9	0.9 ± 0.7	0.988	0.064	0.994
WAS:M-C1	24 ± 10	154.9 ± 16.5	70 ± 3	34.8 ± 7.2	131.5 ± 18.6	154.4 ± 5.1	7.8 ± 0.6	0.4 ± 0.7	0.989	0.055	0.992
WAS	19 ± 3	179.3 ± 11.0	70 ± 2	56.0 ± 4.6	158.3 ± 61.2	179.4 ± 6.5	8.4 ± 0.8	-1.4 ± 0.8	0.985	0.072	0.994
WAS-B1	30 ± 12	170.4 ± 6.3	70 ± 2	20.1 ± 3.7	77.1 ± 12.9	167.1 ± 6.1	8.5 ± 0.9	-1.5 ± 0.9	0.980	0.085	0.992
WAS-C1	27 ± 1	175.0 ± 9.2	68 ± 3	24.9 ± 1.7	104.7 ± 26.0	171.3 ± 6.2	8.7 ± 0.9	-1.3 ± 0.9	0.982	0.081	0.994

778 References: WAS:RM = mixture of waste activated sludge (WAS) and raw microalgae without pretreatment (RM). WAS:M-B1 and WAS:M-C1 = mixture of WAS and enzymatically pretreated microalgae
779 with enzyme B at a 1% dose and enzyme C at a 1% dose, respectively. WAS-B1 and WAS-C1 = WAS with addition of enzyme B at a 1% dose and enzyme C at a 1% dose, respectively.