Effect of pre-treatments on the production of biofuels from *Phaeodactylum tricornutum*

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Abstract

Several characteristics make *Phaeodactylum tricornutum* potential candidate for biofuels production such as methane and biodiesel. For this reason, some alternatives are evaluated in this manuscript to improve the conversion of this microalgae into methane.

One of these alternatives is the addition of sewage sludge to *P. tricornutum* for anaerobic co-digestion. Although the co-digestion resulted in lack of synergy, the absence of inhibition indicated that both substrates could be co-digested under certain circumstances, for example if microalgae are cultivated for wastewater treatment purposes.

The extraction of lipids using organic solvents has been evaluated for biodiesel production but also as a pre-treatment for anaerobic digestion. The results revealed that

the type of solvent influences lipid and biodiesel yields. The high polarity of the mixture methanol/hexane increased the lipid and the biodiesel yields from 10±1 to 53±2 $g_{Lipids}/100\ g_{VS}$ and from 7±1 to 11±1 $g_{Biodiesel}/100\ g_{VS}$ compared with hexane. However, none of these solvents affected the composition of biodiesel. Regarding the methane production after the extraction, it yielded 257±8 and 180±6 mL_{CH4}/g_{VS} from lipid-extracted *P. tricornutum* using hexane and methanol/hexane respectively. The methane production from the raw microalga was 258±5 mL_{CH4}/g_{VS} in the same experiment. The difference in methane production, mainly after the extraction with methanol/hexane, was a consequence of the changes in the composition of the microalgae after extraction. The extraction did not influence the biodegradability.

The ultrasonic pre-treatment prior anaerobic digestion completely disrupted the microalgae cells, but the solubilisation of the organic fraction was scarce (<9.5%). The methane production from pre-treated samples was barely 10-11% higher than the obtained from non pre-treated samples, indicating that the refractory nature of the organic fraction in *P. tricornutum* is the main obstacle for the methane production.

Keywords:

Co-digestion, Lipid extraction, Methane, *Phaeodactylum tricornutum*, Substrate to Inoculum ratio (SIR), Ultrasonic pre-treatment.

1. INTRODUCTION

In a wide variety of microalgae species considered as promising feedstocks for renewable biofuels, the seawater *Phaeodactylum tricornutum* presents several advantages: cultivation at commercial scale, high biomass productivities and

accumulation of lipids [Silva Benavides et al., 2013; Bellou et al., 2014; Song et al., 2014; Davis et al., 2015; Vinayak et al., 2015]. Unfortunately, the scarce literature about methane production from *P. tricornutum* reports that the species present low degradability during anaerobic digestion (AD) [Zamalloa et al., 2012; Frigon et al., 2014; Zhao et al., 2014].

The low degradability of microalgae is usually attributed to several causes, one of which is the low C:N. The low ratio lead AD to fail due to the high concentration of ammonia generated, but it can be overcome by mixing microalgae and carbon-reach substrates. Several substrates have been co-digested with microalgae [Schwede et al., 2013; Wang et al., 2013; Olsson et al., 2014; Prajapati et al., 2014; Caporgno et al., 2016]. Sewage sludge has shown synergy when co-digested with some microalgae species [Wang et al., 2013; Olsson et al., 2014; Mahdy et al., 2015]. The over-sized digesters in wastewater treatment plants (WWTP) [Mata-Alvarez et al., 2014] and the possibility of recycling nutrients by growing *P. tricornutum* in wastewaters [Davis et al., 2015] are additional reasons to investigate the co-digestion of this species and sewage sludge.

The low degradability can be also consequence of strong cell walls, which hamper the microorganisms attack. Pre-treatments, such as the ultrasonic, can break the cell walls, release internal compounds and increase the methane production [González-Fernández et al., 2012; Alzate et al., 2014]. However, the effects of pre-treatment on the degradability of *P. tricornutum* have not been evaluated yet. Furthermore, organic solvents used for the extraction of lipids in the biodiesel production process can act as pre-treatment increasing the degradability of the microalgae waste [Alzate et al., 2014; Ramos-Suárez and Carreras, 2014; Caporgno et al., 2016]. Although some authors reported the AD *P. tricornutum* after lipid-extraction [Frigon et al., 2014; Zhao et al.,

2014], the extraction was not always performed using organic solvents. More information about the influence of the solvents is necessary.

This communication attempts to show some preliminary results about the possibility of coupling the AD of *P. tricornutum* with other processes like the sewage sludge treatment in WWTP. For this reason, the first option considers the possibility of co-digest microalgae and sewage sludge. The influence of the substrate to inoculum ratio (SIR) was evaluated for both substrates, and then, different mixtures of substrates were co-digested. Furthermore, the possibility of increasing the degradability of microalgae was evaluated by applying an ultrasonic pre-treatment at varying intensities.

A second option considers the lipid-extraction for biodiesel production and as pre-treatment for AD. The effects of different solvents on biodiesel yields and composition, and methane productions from lipid-extracted microalgae have been evaluated in these experiments.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Microalgae, inoculum and sewage sludge

The marine microalgae *Phaeodactylum tricornutum* Bohlin (strain CCAP 1055/5) were obtained from the Culture Collection of Algae and Protozoa (CCAP). The cultivation was started in 200 mL culture and scaled up through 4 L cultures, using seawater (37 g/L salinity) filtered through 0.22 μm, enriched with Walne's medium [Walne, 1970] and autoclaved. Cultures were kept at 22±2 °C, illuminated (16:8 light: dark cycle) with cool daylight fluorescents (Osram L30W/865) to give an irradiance of 100-140 $\mu E/m^2s$, and aerated with air. For 300 L culture, a vertical bag was used as photobioreactor.

Seawater was filtered through four filter cartridges with 25, 10, 5 and 1 μm pore sizes and treated with UV light to eliminate biological contamination, and then enriched with 0,3 mL/L of the commercial fertilizer Codafol 14-6-5 and 107 μM Na_2SiO_3. Cultures were kept at 22±2 °C, illuminated (16:8 light: dark cycle) with cool daylight fluorescents (Philips TLD 58W/865) to give an irradiance of ca. 200 $\mu E/m^2s$ at the culture surface, and aerated with air. Microalgae were then concentrated to approximate 70 g_{TS}/L in a continuous centrifuge. Microalgae were stored in a freezer at -20 ºC until utilisation.

The sewage sludge consisted of a primary and secondary-sludge blend (65:35 v/v), collected from the municipal WWTPs in Reus (Tarragona, Spain). Regarding the inoculum, it consisted of digested sludge taken from an anaerobic semi-continuous plant as described in a previous work [Caporgno et al., 2015].

2.2 Experimental procedure

2.2.1 Biomass processing

The first experiments consisted in the co-digestion of microalgae and sewage sludge. For co-digestion, mixtures of both substrates containing 25%, 50% and 75% sewage sludge on a VS basis were fed into the reactors.

In the following experiments, the lipids from microalgae were first extracted and converted into biodiesel, and the remaining microalgae was converted into methane. The lipid extraction was performed using hexane and methanol/hexane in ratio (2:3 v:v), following the procedure detailed in [Caporgno et al., 2016]. Since the microalgae were dried using freeze-drying equipment (FT33-A Freeze Drier, Armfield Inc.) prior extraction, the dried microalgae were digested to evaluate the effects of drying on AD.

The ultrasonic pre-treatment was carried out using an ultrasonic device (UP200S Hielscher Ultrasonics GmbH, Germany) at 24 kHz working frequency and 93 W ultrasonic power. The samples were disintegrated at room temperature in a water bath to avoid heating the sample. Three energy inputs were evaluated, 21 MJ/kg_{TS}, 36 MJ/kg_{TS} and 52 MJ/kg_{TS}. The Disintegration degree (Dd) was measured by the soluble COD increase:

$$Dd = \frac{(\mathrm{SCOD} - \mathrm{SCOD}_0) \cdot 100}{\mathrm{TCOD}_0 - \mathrm{SCOD}_0} \quad \text{(Eq. 1)}$$

where SCOD is the soluble COD; $SCOD_0$ represents the values of soluble COD before the disintegration treatment; $TCOD_0$ represents the values of total COD before the disintegration treatment.

Microalgae samples were observed under a light microscope (ZEISS Axio Scope.A1, with ProgRes® SpeedXT core 3 camera) to evaluate the effects of ultrasonic pre-treatment on the microalgae cells.

2.2.2 Anaerobic digestion experiments

Batch reactors were set up at 33ºC following the procedure described in [Angelidaki et al. 2009]. The effects of the SIR were evaluated using raw microalgae and sewage sludge as substrates; the SIRs were set at 1:4, 1:2 and 1:1 $VS_{Substrate}$:$VS_{Inoculum}$, where VS is the volatile solid content in substrates and inoculum. Based on the results, the SIR was decided at 1:2 $VS_{Substrate}$:$VS_{Inoculum}$ for the experiments using lipid-extracted and ultrasonic pre-treated microalgae.

The first order hydrolysis model was used to determine the hydrolysis constant, k_h ($days^{-1}$) [Caporgno et al., 2016]. The theoretical methane potential was calculated based on the biochemical composition of the substrates, and assuming the specific methane

yields of 1014 mL_{CH4}/g_{VS}, 496 mL_{CH4}/g_{VS}, 415 mL_{CH4}/g_{VS} for lipid, protein and carbohydrate respectively [Caporgno et al., 2016]. The biodegradability was defined by the following equation:

$$\text{Biodegradability}(\%) = \frac{\text{measured methane production}}{\text{theoretical methane potential}} \times 100 \qquad \text{(Eq. 2)}$$

2.2.3 Analytical techniques

Total solids (TS), volatile solids (VS) and chemical oxygen demand (COD) were analysed according to standard methods 2540B, 2540E and 5220D respectively [Rice et al., 2012]. The soluble COD (SCOD) was measured following the same procedure that for COD, but the sample consisted of the supernatant after centrifugation. The biochemical composition of microalgae was determined according to the Lowry method for protein determination [Lowry et al., 1951], phenol-sulphuric acid method for sugars determination [Dubois et al., 1956] and the Bligh and Dyer method for lipids determination [Bligh and Dyer, 1959]. The characteristics of the inoculum, the sewage sludge and the microalgae are summarised in Table 1.

The biogas production, biogas composition, volatile fatty acid and concentration (VFA) and ammonia concentration were measured as described in [Caporgno et al., 2015]. The lipid extraction yields were quantified gravimetrically, and the biodiesel yields and composition were determined as described in [Olkiewicz et al., 2014].

3. RESULTS AND DISCUSION

3.1. Raw microalgae digestion

3.1.1 Influence of the Substrate to inoculum ratio (SIR)

The SIR influenced the AD of sewage sludge and microalgae differently, as it can be seen in Table 2. The final methane production from sewage sludge was unaffected by the SIR increase from 1:4 to 1:2, but the SIR increase up to 1:1 resulted in a high methane production. The values agree with previous results [Caporgno et al., 2015]. On the other hand, *P. tricornutum* resulted in similar methane production for all the SIR evaluated. These values are around 20-30% lower than the reported in the literature [Zamalloa et al., 2012; Frigon et al., 2014; Zhao et al., 2014], but the differences can be attributed to differences in microalgae caused by the cultivation conditions [Silva Benavides et al., 2013] or to the differences in the inocula.

Although the highest methane production was obtained at SIR of 1:1 with both substrates, the kinetic parameter k_h showed opposite results (Table 2). The k_h depends on the methane production throughout the first days of the experiment, which was negatively influenced by the SIR increase. Similar effects were reported during the AD of other microalgae species under comparable conditions [Alzate et al., 2014; Zhao et al., 2014; Caporgno et al., 2016]. The decreased values of k_h at high SIR can be attributed to the abruptly change in the concentration of substrate after feeding the reactors. The high substrate concentration after feeding caused a stress response in bacteria, requiring their adaptation [Caporgno et al., 2016]. There is a SIR threshold which leads to inhibition; the VFA produced during hydrolysis are not efficiently converted into methane at this SIR, the pH decreases and the methane production stops [Zhao et al., 2014]. The low k_h but the high methane production at the end of the experiment (Table 2) indicate that the VFA accumulated at 1:1, but their concentration was not high enough to cause inhibition and the methane production continued afterwards. Nevertheless, SIR higher than 1:1 could lead to inhibition. Regarding

192 sewage sludge digestion at SIR 1:1, the stress response was not observed because the
193 inoculum was acclimatised to this substrate, as described in section 2.1.1. Based on the
194 results, the SIR 1:2 was set in further experiments. The comparison between both
195 substrates indicates that microalgae digestion produced more methane than sewage
196 sludge throughout the first days of the experiment, in spite of their low methane
197 production at the end of the experiment. Since the same amount of VS was loaded in
198 reactors with the same SIR, these differences are caused the characteristics of the
199 organic fraction. Microalgae have a readily degradable organic fraction easily converted
200 into methane; nonetheless, the major part of the organic fraction resisted degradation
201 during the experiment. On the contrary, the organic fraction from sewage required more
202 time for degradation at the beginning, but the degradation is high.
203 At the end of the experiment, the pH ranged between 7.20 and 7.42. The ammonium
204 nitrogen increased from 510 mg/L to 760 mg/L when the SIR increased from 1:4 to 1:1,
205 but the levels were lower than the threshold for inhibition [Caporgno et al., 2016];
206 furthermore, VFA were not detected, confirming the absence of inhibition. The methane
207 content on biogas, analysed several times during the experiments, ranged between 69%
208 and 74% and it was independent of the substrate.
209
210 *3.1.2. Influence of the co-digestion with sewage sludge*
211 The methane production curves during Aco-D are shown in Figure 1a. At the end of the
212 experiment, the highest and the lowest yields were obtained from sewage sludge and *P.*
213 *tricornutum* respectively. The higher the addition of sewage sludge, the higher the
214 methane production, but no synergy was observed during co-digestion [Caporgno et al.,
215 2015; 2016]. The methane production from the mixtures could be calculated based on

the relative fractions of microalgae and sewage sludge, and their methane productions. These calculated productions were quite similar to the values measured in the experiments, thus they could be caused by the experimental variation. These results are in agreement with the results reported during the co-digestion of other microalgae species and sewage sludge [Caporgno et al., 2015; 2016; Mahdy et al., 2015].

Regarding the k_h, the highest k_h was observed in reactors with microalgae and the lowest k_h in reactors with sewage sludge, 0.35 (0.93) and 0.27 (0.98) respectively (in brackets the values of R^2). During co-digestion, the values of k_h were proportional to the composition of the mixtures. All reactors exceeded the 80% of their final methane production after the first week.

The C:N were 6.06 and 13.88 during *P. tricornutum* and sewage sludge digestion respectively (Table 1). Both substrates have the C:N ratio lower than the considered as optimal for anaerobic digestion, which is in the range 20-25 [Prajapati et al., 2014]. The addition of sewage sludge in co-digestion did not increased the C:N significantly. Opposite results are reported in the literature regarding the influence of the C:N on co-digestion; synergy was reported after mixing microalgae and substrates with low C:N [Olsson et al., 2014; Prajapati et al., 2014; Caporgno et al., 2015; 2016] but also no synergy when microalgae were mixed with carbon-reach substrates [Schwede et al., 2013]. The increased methane production in–co-digestion has been attributed to a favoured nutrients availability for microorganisms [Olsson et al., 2014; Prajapati et al., 2014] or the more stability in the process too [Schwede et al., 2013]. It is evident that the C:N ratio is not be the primary agent in the *P. tricornutum* digestion. Additionally, the benefits of mixing microalgae and sewage sludge may be hidden by the addition of

239 the synthetic medium according to the methodology or due to the characteristics of the
240 microalgae.
241 On the other hand, the absence of inhibition during co-digestion suggest the possibility
242 of coupling microalgae cultivation in WWTP for wastewater treatment and their
243 conversion into methane, as reported by other authors [Caporgno et al., 2015; 2016;
244 Mahdy et al., 2015]. This option become even more interesting when considering the
245 availability of over-sized digesters in WWTP [Mata-Alvarez et al., 2014]. The sewage
246 sludge blend utilised in the experiments represents the sludge generated in WWTP, thus
247 minor modification would be required in the facility.
248
249 **3.2. Microalgae pre-treatments before anaerobic digestion**
250 *3.2.1. Lipid extraction*
251 Table 3 summarises the lipid and biodiesel yields, and the composition of the biodiesel
252 obtained from *P. tricornutum* using hexane and methanol/hexane. The highest lipid
253 extraction yields were obtained using methanol/hexane due to the polar characteristics
254 of this mixture [Hernández et al., 2014; Ryckebosch et al., 2014]. Hexane, as other non-
255 polar solvents, extracted mainly non-polar lipids and part of lipids remains in
256 microalgae after the extraction (Table 1) [Frigon et al., 2014; Zhao et al., 2014]. The
257 mixture of methanol/hexane extracts polar lipids, but some non-lipid components are
258 extracted too. These non-lipid components increase the extraction yield but most of
259 them fail transesterification [Ehimen et al., 2009; Hernández et al., 2014; Caporgno et
260 al., 2016]. As can be seen in Table 1, the lipid yield obtained using methanol/hexane
261 exceeded the lipid content in raw microalgae determined following the Bligh and Dyer
262 method due to the presence of non-lipid components. The Bligh and Dyer method uses a

polar mixture but includes the addition of water which removes the non-lipid components.

Regarding the biodiesel yields, the yield obtained using methanol/hexane was slightly higher compared to using hexane, but similar to the yield obtained from the lipids extracted with the Bligh and Dyer method (9±2 $g_{Biodiesel}/100g_{VS}$). The result demonstrates that the major part of the compounds extracted with methanol/hexane fails transesterification. On the other hand, the composition of the biodiesel was not affected by the solvents; the fatty acid profiles were dominated by palmitic (C16:0), palmitoleic (C16:1) and eicosapentaenoic (C20:5), in agreement with the profiles reported in the literature for the same microalgae species [Silva-Benavides et al., 2013; Frigon et al., 2014].

The methane production curves from lipid-extracted microalgae are presented in Figure 1b; raw microalgae were also digested for comparison purposes. It is worth mentioning that raw microalgae produced 258±5 mL_{CH4}/g_{VS}, a bit less than during the experiments presented in section 3.1. These experiments were not performed simultaneously, thus differences can be caused by changes in the inocula used. After extraction with hexane, the methane production was similar to the obtained from raw microalgae. Comparable results were reported using hexane and supercritical CO_2 to extract lipids from *P. tricornutum* before AD [Frigon et al., 2014; Zhao et al., 2014]. Regarding the extraction using methanol/hexane, a large decrease on the methane production was observed compared to raw microalgae. Since the same amount of VS was the same in all reactors (SIR 1:2), the differences in the methane production were caused by changes in the composition and characteristics of the substrates. The microalgae composition resulted almost unaffected by hexane due to the low extraction yield, but opposed results were

observed by using methanol/hexane (Table 1). The changes in the composition decrease the theoretical methane production, due to the extraction of the lipids. The biodegradability (Eq. 1) was 39%, 42% and 38% in raw microalgae and extracted with hexane and methanol/hexane respectively. The results show that the biodegradability was almost unaffected, and lipid-extracted microalgae are still difficult to digest.

As can be seen in Figure 1b, the reactors with raw microalgae had the highest production of methane throughout the first days of experiments, whereas reactors with microalgae extracted using methanol/hexane had the lowest. The extraction process can either extract easily-degradable components or slow down the degradation of some components, but further study would be necessary to confirm the effect of solvents. Regarding the effects of drying, only the methane production at the beginning of the experiment was affected (Figure 1b). Although freeze-drying can modify microalgae composition and characteristics [Cordero-Esquivel et al., 1993], the detrimental effects of drying on AD are mainly attributed to heating [Olsson et al., 2014], which is avoided in the freeze-drying method.

The values of pH, concentration of ammonia and VFA at the end of the experiments confirmed stability in all the reactors.

3.2.2. Ultrasound

The Dd (Eq. 1) gives useful information about the organic compound solubilisation after pre-treatment. The Dd increased by 3.8%, 7.2% and 9.5% after energy inputs of 21 MJ/kg_{TS}, 36 MJ/kg_{TS} and 52 MJ/kg_{TS} respectively. The stronger the energy input, the higher the disintegration level; however, the solubilisation of the organic fraction was slightly affected. Figure 2 shows the changes in the microalgae structures throughout

311 the experiment. Even after applying the lowest energy input (Figure 2c), the pictures
312 reveal structural changes on the cells and the release of the inner content. When
313 compared to the microalgae structure before pre-treatments (Figure 2a), the cell wall
314 structure completely disappeared in all the pre-treated samples (Figure 2c-e). Some
315 chloroplasts remained close, like aggregates (Figure 2c), due to the release of the inner
316 content of the cells [González-Fernández et al., 2012]. *P. tricornutum* has an atypical
317 weakly silicified cell wall compared with other diatoms [De Martino et al., 2007], being
318 easily disrupted at low energy input. As it has been recently reported by
319 Chantrasakdakul et al., some organics and polymers released during the pre-treatment
320 may act as flocculants. The flocs constitute the pellet after centrifugation and, as a
321 consequence, the solubilisation of organic compounds (solubilised COD or solubilised
322 solids) can be low or decrease in spite of the high cell disruption [Chantrasakdakul et
323 al., 2015].
324 As shown in Figure 1c, the methane production was unaffected by the changes in the
325 energy input, 287±11 mL_{CH4}/g_{VS}, 284±9 mL_{CH4}/g_{VS} and 285±4 mL_{CH4}/g_{VS} were
326 achieved after applying 21, 36 and 52 MJ/kg_{TS} respectively. These values represent
327 barely 10% increase in the methane production over the raw microalgae used as a blank
328 experiment (258±12 mL_{CH4}/g_{VS}). The lower-energy pre-treatment was strong enough to
329 break *P. tricornutum* cells and to increase the methane production.
330 The highest energy input decreased the daily methane production during the first days
331 (Figure 1c); the first day is an exception, but it is characterised by high standard
332 deviation in the methane production. However, no major differences were observed by
333 the end of the experiment. The increases in the energy input can lead to re-flocculation
334 [González-Fernández et al., 2012; Chantrasakdakul et al., 2015], reducing the methane

335 production rate. The energy input can also affect the characteristics of the biomass,
336 increasing the solubilisation but decreasing the methane production [González-
337 Fernández et al., 2012; Alzate et al., 2014].
338 The biodegradability increased from 39% to around 43% after the pre-treatment. Some
339 microalgae cells were recovered from reactors fed with raw microalgae; the pictures
340 revealed that the cells had similar appearance before (Figure 2a) and after digestion
341 (Figure 2b). Although this showed the high resistance of cells to the microorganisms
342 attack, the degradability of pre-treated microalgae demonstrated that the low methane
343 production is a consequence of the low degradability of the organic fraction in *P.*
344 *tricornutum*.
345
346 **4. CONCLUSIONS**
347 In spite of the co-digestion of *Phaeodactylum tricornutum* and sewage sludge does not
348 shows synergy, the absence of inhibitory effects suggest that the process can be
349 beneficial under certain circumstances such as the microalgae cultivation for wastewater
350 treatment or over-sized digesters in WWTP.
351 The anaerobic digestion of *P. tricornutum* can be also coupled to the biodiesel
352 production process. The lipid extraction using organic solvents do not affect the
353 biodegradability of microalgae, but it influences the lipid and biodiesel yields. The
354 proper selection of the solvents for extraction of valuable compounds from *P.*
355 *tricornutum* can affect the quality of the extract and the methane production of the waste
356 when digested.

The ultrasonic pre-treatment confirms that the main obstacle of the *Phaeodactylum tricornutum* anaerobic digestion is the refractory nature of the organic fraction, since the pre-treatment disrupts the microalgae cells but it does not enhance the degradability.

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Figure captions

Figure 1. The methane production curves from *P. tricornutum* (a) during co-digestion with sewage sludge, (b) after lipid-extraction using hexane and methanol/hexane, and (c) after ultrasonic pre-treatment applying 21, 36 and 52 MJ/kg_{TS}. Batch reactors at 33ºC and SIR of 1:2 $VS_{Substrate}$:$VS_{Inoculum}$.

Figure 2. Light micrographs of *P. tricornutum* (a) raw, (b) recovered from digestate, and after ultrasonic pre-treatment applying (c) 21, (d) 36 and (e) 52 MJ/kg_{TS}.