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Fermentation Wastes from *Chrypthecodinium cohnii* Lipid Production for Energy Recovery by Anaerobic Digestion

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Abstract: Wastes generated during the cultivation of marine microalga *Crypthecodinium cohnii* and after the lipid extraction process, were energetically valorized into biogas production through anaerobic digestion (AD). The tested wastes were extracted microalgae (Ae) with hexane (AeH) using supercritical extraction methods (AeS) and the supernatant obtained after culture medium centrifugation (M). The digestion of the algae biomass in the admixture with the supernatant medium (AeH+M+I and AeS+M+I) provided a higher methane content and a higher methane yield (582 and 440 L CH_4/kg VS) than the substrates Ae and M, individually digested (155 and 96 L CH_4/kg VS, respectively). Flow cytometry monitoring processes during AD indicated that the yield of the accumulated biogas was influenced by the operating conditions. The mixture of AeH+M+I was the only assay with a proportion of cells with less damaged membranes after AD, providing the highest methane yield and productivity (582 L CH_4/kg VS and 31 L CH_4/kg VS.d, respectively) and the highest energetic potential of 5.8 KWh/kg VS of all the substrates. From the results, AD integration to lipid production by *C. cohnii* to recover energy from the generated wastes enhanced the sustainability of the entire process and promoted the practice of zero waste.

Keywords: marine microalgae; *Crypthecodinium cohnii*; wastes; flow cytometry; anaerobic digestion; biogas/methane

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1. Introduction

The heterotrophic marine dinoflagellate microalga *Crypthecodinium cohnii* produces significant amounts of lipids (20–50% cell dry weight) with a high proportion of docosahexaenoic acid (DHA), a ω-3 polyunsaturated fatty acid, necessary for brain development during pregnancy and childhood [1]. Furthermore, this compound has been recognized benefits on human health, improving vision, psoriasis, cancer prevention, heart health and inflammatory response reduction [2]. Currently, DHA has several applications in the nutritional, pharmaceutical and cosmetic industries, foreseeing a growing market size [3]. A few companies have commercialized DHA-rich oil obtained from heterotrophic microalgal oil, such as DSM and Lonza [3].

Several methods can be used to extract the intracellular microalgal lipids, namely, the Soxhlet lipid extraction method that is widely applied in the food industry as it uses hexane. Supercritical extraction (SCE), by resorting to supercritical carbon dioxide (CO_2), is a cleaner method of lipid extraction by way of no residues being present in the extracted lipids.

In addition to obtaining lipids, the microalgal oil production process generates wastes such as fermentation broth supernatant and microalgal biomass leftovers, obtained after lipid extraction which are usually neglected. Because they contain carbon, nitrogen, and other nutrients, they could be useful as substrates for the biogas/methane production. The anaerobic digestion process (AD) transforms organic matter into biogas (mainly methane and carbon dioxide) and preserves nutrients (N and P) in the digestate, through the action of a microbial community, under anaerobic conditions. Biogas can be applied to electric

and thermal energy supply or converted into biomethane and injected into the natural gas network [4]. In this way, it can be regarded as an energy-carrying gas and useful for the *C. cohnii* lipid production process, reducing the energy demand of the overall process and fulfilling the circular economy principles [4]. A solid stream of digestate can be stabilized (composted) and converted into a product for agriculture or animal feed while the liquid fraction can be recovered as water irrigation or, even, as "water" directed towards the production of e-methane through the electrolysis process, taking advantage of the power-to-methane concept [5]. AD is a well-known process with recognized advantages associated with mitigating climate change, economic benefits as well as the diversification of opportunities [6]. It has been suggested as the most environmentally sustainable option [7]. Diverse studies have been carried out regarding the energy recovery of algal biomass, highlighting the inherent advantages of the joint digestion of algal biomass and other organic effluents [8–11].

During the bioprocess evolution, microorganisms are often exposed to adverse conditions such as nutrient limitation, pH gradients, osmotic stress, inhibitor presence, etc., which may affect cellular functions and viability, and thus the process efficiency. Usually, these conditions damage the cell membrane, thus compromising the cellular integrity, which may result in cell dormancy or death. If a high proportion of dormant or dead cells are present during the process development, this will inevitably lead to a reduction in bioprocess yield. Therefore, it is essential to monitor the cell viability throughout any bioprocess, to evaluate the cell physiological status throughout the process development [12].

The main objective of the present research was to take advantage of all wastes from *C. cohnii* microalgae fermentation and lipid extraction process, foreseeing zero wastes during the OmegaFuel project (PTDC/EAM-AMB/30169/2017) [13,14]. In an innovative approach, it was intended to evaluate the integration of microalgae lipid production with the energetic and agricultural valorization of the generated wastes by AD. The definite goal was to understand the benefits of digesting the different wastes simultaneously, taking advantage of the substrate complementarity concept, to make the overall system simpler and cheaper and, consequently, more sustainable. Since the wastes (biomass leftovers containing solvents and fermentation broths with salt) used as substrates for biogas production contain compounds that may inhibit the anaerobic microbial cells of the inoculum, flow cytometry was used to evaluate and understand the impact of these substrates on the consortium of cells.

This is the first work that reports the valorization of wastes generated during the *C. cohnii* lipid production process, for biogas production through AD, accomplishing the circular economy principles, which represents an important improvement of the microalgal lipid production process.

2. Materials and Methods

C. cohnii was the focus of the study in the OmegaFuel project regarding its ability to produce DHA and biofuels. *C. cohnii* biomass was produced in a 7 L fed-batch bioreactor. At the end of fermentation, the culture medium was centrifuged, and the biomass was taken for further intracellular lipid extraction [14].

2.1. Substrates and Inoculum

Several wastes were generated during the *C. cohnii* lipid production step, which were used as substrates for AD: microalgae (Ae) after lipid extraction using hexane (AeH) and supercritical extraction methods (AeS), and the supernatant obtained after culture medium centrifugation (M). This medium was composed of yeast extract (corresponding to 0.11 g/L nitrogen), sea salt (25 g/L), and glycerol (19.8 g/L) [14]. The proportion used to prepare both mixtures (AeH+M and AeS+M) was based on the quantities of each residue obtained at the end of the cultivation process (M) and after the lipid extraction steps (AeH or AeS): 8 g Ae/L M (ratio of 0.008:1, *m*/*v*).

Biological solids (1.3 ± 0.0 g VSS/L) were collected in an anaerobic digester plant at Quinta do Conde (Portugal) and used as the inoculum (I, 30% *v*/*v*) in the AD process at a substrate/inoculum ratio value of 2.3 expressed in volatile solids.

2.2. Anaerobic Digestion Experimental Set-Up

The experiment was carried out in triplicate under batch conditions, using 70 mL glass units, with useful volume of 40 mL and leaving 30 mL of headspace. The reactors were sealed, and the headspace of each unit was flushed with nitrogen at the beginning of the assay to ensure anaerobic conditions. The test units were incubated within the constant mesophilic range of temperature (37 ± 1 °C) and maintained until it was possible to positively confirm the initial assumptions, that is, to verify the possibility of using AD to valorize the remaining wastes from the microalgae fermentation and lipid extraction processes, as well as to assess the benefits of digesting the different wastes together. According to this, the experiments lasted about a month.

2.3. Analytical and Chromatograph Methods

Performance of the process was monitored by analytical characterizations of all samples and by the volume and quality of the obtained biogas. Chemical oxygen demand (COD), volatile solids (VS), volatile suspended solids (VSS), total nitrogen (Kjeldahl, TN), ammonium (NH_4^+-N), and pH, were assayed according to standard methods [15].

Analytical measures of the described parameters were performed at the beginning ($_{in}$) and at the end of the assay experimental time (data not shown). Results are presented as a percentage of the removal of each parameter. The biogas production was monitored daily with a pressure transducer, expressed under standard conditions of temperature and pressure (STP: 0 °C, 1 bar) defined by IUPAC (International Union of Pure Applied Chemistry). The methane content of the biogas collected in each unit headspace was measured by injecting 0.25 mL of the gas sample into a gas chromatograph (GC Thermo Electron Corporation Trace GC Ultra), equipped with a thermal conductivity detector and a Carboxen®-1010 PLOT Capillary GC Column (L × I.D. 30 m × 0.32 mm, average thickness 15 µm). The injector and detector temperatures were 200 and 230 °C, respectively. The column temperature profile was: isot 35 °C for 7.5 min, ramp 24 °C/min, 5 min isot. Helium was utilized as the carrier gas (1.5 mL/min). Quantification of each gas produced was performed by comparing the obtained graphical peak areas with the pattern of an injected gas mix at the beginning of each analysis.

All values of the biogas and methane yields are presented under STP conditions and divided by the mass of volatile solids (L CH4/kg SV_{in}) or by the organic matter content (L CH4/kg COD_{in}) of the substrate fed at the beginning of the assay. The primary energy yield (kWh/kg VS_{in} and kWh/kg COD_{in}) of the tested mixtures was calculated using the lower methane heating value (LHV) of 9.97 kWh/m^3 CH4 [16].

2.4. Flow Cytometric Analysis

Flow cytometry was used to evaluate the physiological status of the anaerobic microbial consortium of cells, used to inoculate all samples, to understand the response of these cells to the different operational conditions of the AD experiment, using membrane integrity as a cell viability marker [12]. This information complements data given by traditional methods used to monitor anaerobic digestion cultivations, such as biogas and methane productions, which does not provide any information on cell physiological status. Two fluorescent dyes were used: SYBR Green I (SYBR) and propidium iodide (PI) [17]. SYBR is a nucleic acid-specific stain that penetrates all cells, and PI is a stain indicator of membrane integrity, as intact membranes exclude this dye. In this way, it was possible to distinguish the microbial consortium of cells from the medium particles.

Samples were sonicated for 15 s to disintegrate cellular aggregates and ensure the analysis of individual cells. For staining, 2 µL of SYBR Green I solution (1:30 dilution of SYBR Green I commercial stock (from Invitrogen) solution made in dimethyl sulfoxide)

and 2 µL of PI (from Invitrogen, stock solution of 1 mg mL^{-1} prepared with water) were added simultaneously to 500 µL of sample and incubated for 30 min in the dark.

Samples taken from the vials were analyzed at the beginning and the end of the experiment using a Cytoflex Beckman-Coulter flow cytometer, equipped with a blue laser, FSC/SSC light scattering detectors and five fluorescence detectors.

All analyses were carried out in duplicate, and each analysis was stopped after more than 5000 events were detected. The data were analyzed using the CytExpert 2.5 software.

The proportion of cells with damaged membrane (permeabilized cells) was calculated according to Equation (1):

$$\Delta(\text{SYBR/PI}) = \%\ (\text{SYBR/PI})_f - \%\ (\text{SYBR/PI})_0 \quad (1)$$

where % $(SYBR/PI)_f$ is the percentage of permeabilized cells detected at the end of the AD process; % $(SYBR/PI)_0$ is the percentage of permeabilized cells detected at the beginning of the AD process; and Δ(SYBR/PI) is the difference between the above two parcels.

In this way, an increase in the number permeabilized cells, reflecting the cells' stress response to each assay condition, was assessed throughout the AD process.

3. Results

3.1. Chemical Composition of Substrates and Inoculum

There was a considerable difference between the chemical composition of AeH and M (Table 1). Algae clearly had a higher content than the substrate M, which was practically devoid of nitrogen. Given these characteristics, M could complement and somehow work as a liquid medium when both substrates AeH and M are digested together. Biological solids, used as inoculum for the assays, held the highest concentration of nitrogen present in its ionized form (NH_4^+) suggesting the occurrence of organic material degradation.

Table 1. Substrates and inoculum: chemical composition.

Substrates and Inoculum	COD (g/L)	VS (g/L)	TN (g/L)	NH_4^+-N (mg/L)
AeH+M	96.6 ± 5.6	37.3 ± 0.7	0.17 ± 0.0	0.56 ± 0.0
AeS+M	103.6 ± 2.2	37.6 ± 1.2	0.15 ± 0.0	0.56 ± 0.0
AeH	1638.3 ± 90	413.6 ± 30	17.4 ± 0.4	14.0 ± 0.0
M	96.5 ± 0.0	37.3 ± 0.2	0.17 ± 0.0	0.28 ± 0.0
I	20.0 ± 0.6	5.1 ± 0.2	0.45 ± 0.0	462.0 ± 4.0

COD—chemical oxygen demand; VS—volatile solids; TN—total nitrogen.

The mixtures containing the defatted microalgae biomass—AeH+M and AeS+M—showed an identical chemical composition in terms of COD, VS, and nitrogen, indicating that the extraction process did not seem to influence the energy potential of the remaining algal biomass, as shown in Table 1. Comparatively, the medium (M) with similar concentrations constituted a very interesting potential in terms of energy recovery. Therefore, organic flows above 96 g COD/L that came from *C. cohnii* lipid production as by-products were available and susceptible to energy valorization.

3.2. Anaerobic Digestion of Microalgae Fermentation Wastes

Biogas production was observed in all the operational conditions tested, with no lag phase evidenced in any of them, as shown in Figure 1. The experiments can be described in three distinct phases: an initial phase (the first 7 experimental days), with a steeper slope than the others; an intermediate phase (the following 12 days) where stabilization of gas production was noticed and, finally, a terminus phase of the experiment in which an increase in biogas production was observed, mainly in the case of AeH+M+I and AeS+M+I. The comparison between these two units showed that the first had supremacy in terms of the ability to generate more accentuated increases in gas production than the other and this

can be associated with a faster microbial consortium development that in the final stage is more adapted to the substrate in digestion. Effectively, in the case of AeH+M+I, it was possible to verify the similarity between gas production volume obtained in the first seven days and that generated in the last seven experimental days.

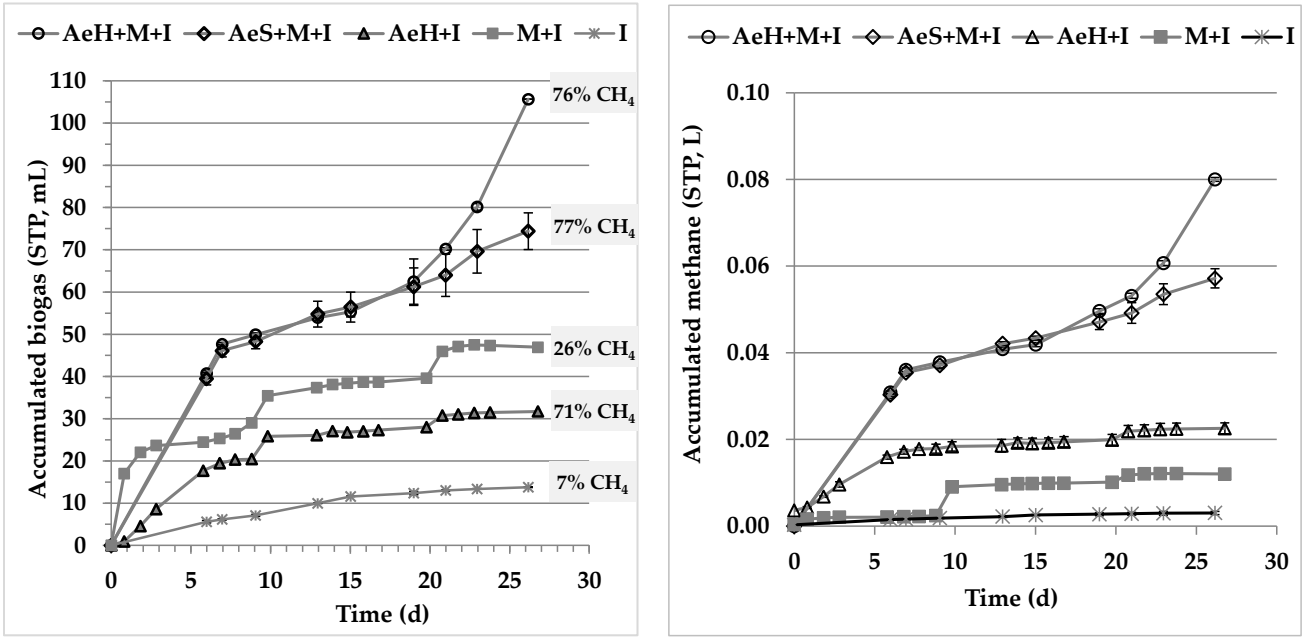

Figure 1. Anaerobic digestion of wastes from fermentation: (**a**) cumulative biogas production; (**b**) cumulative methane production. STP—standard temperature and pressure. The values presented for the percentage of methane refer to the last day of the experiment.

The highest gas productions were recorded in the units digesting the defatted microalgae biomass mixed with the culture medium—AeH+M+I and AeS+M+I—followed by the medium alone, M+I, with accumulated biogas volumes of 106, 74, and 48 mL, respectively. Regarding the quality of the produced biogas at the end of the experiment, it was noted that both mixtures provided a crucial concentration of methane (76–77%), while the collected biogas from the digestion of medium alone was very poor in methane (26%).

Both mixtures, AeH+M+I and AeS+M+I, with COD_{in} concentrations of 27 and 22 g/L, respectively, showed the highest removals in proportions of 68–61% (Table 2), resulting in a methane yield of 75 and 65 L CH_4/kg COD_{in}, respectively (Figure 2a). The highest yields of methane production, in terms of volatile solids, were also obtained with the mixtures AeH+M+I and AeS+M+I, resulting in 582 and 440 L CH_4/kg VS_{in}, respectively (Figure 2b). It was interesting to observe that despite the high production of ammonium levels in these mixtures, an inhibitory effect on the anaerobic digestion process was not observed. Under these conditions, this fact can be explained by the low ammonium concentrations determined, 0.06 g/L at the beginning, increasing to 0.18 g/L (data not shown) at the end of the process (Table 2). On the other hand, M+I with 30 g COD_{in}/L and 63% of COD removal, showed a low methane yield of 0.02 L CH_4/kg COD_{in}. In fact, the reduced biogas volume of M+I and its low quality may be related to the nitrogen involved, whose content of 0.60 g/L and 0.17 g/L NH_4^+-N_{in} at the beginning of the process may have caused an imbalance and thus inhibition of the process.

Table 2. Performance of anaerobic digestion of microalgae fermentation wastes.

Mixture	pH		COD		Volatile Solids		Total Nitrogen		Ammonia Nitrogen	
	pH_{in}	pH_f	COD_{in} (g/L)	COD_r (%)	VS_{in} (g/L)	VS_r (%)	TN_{in} (g/L)	TN_r (%)	NH_4^+-N_{in} (g/L)	NH_4^+-N_r (%)
AeH+M+I	9.4	7.25	26.7 ± 0.0	68	3.4 ± 0	42	0.15 ± 0.000	*−97*	0.06 ± 0.002	*−181*
AeS+M+I	9.4	7.26	22.0 ± 0.0	61	3.2 ± 0	40	0.16 ± 0.010	*−90*	0.06 ± 0.002	*−99*
AeH+I	7.63	7.75	12.9 ± 0.0	*−8*	5.1 ± 1	*−59*	0.59 ± 0.004	25	0.23 ± 0.000	N.d.
M+I	7.46	7.75	29.8 ± 0.4	63	12.3 ± 1	48	0.60 ± 0.000	25	0.17 ± 0.000	N.d.
I	7.68	7.19	6.4 ± 1.8	27	1.6 ± 0	75	0.26 ± 0.004	67	0.04 ± 0.001	*−22*

COD—chemical oxygen demand; VS—volatile solids; TN—total nitrogen. N.d.—Not determined.

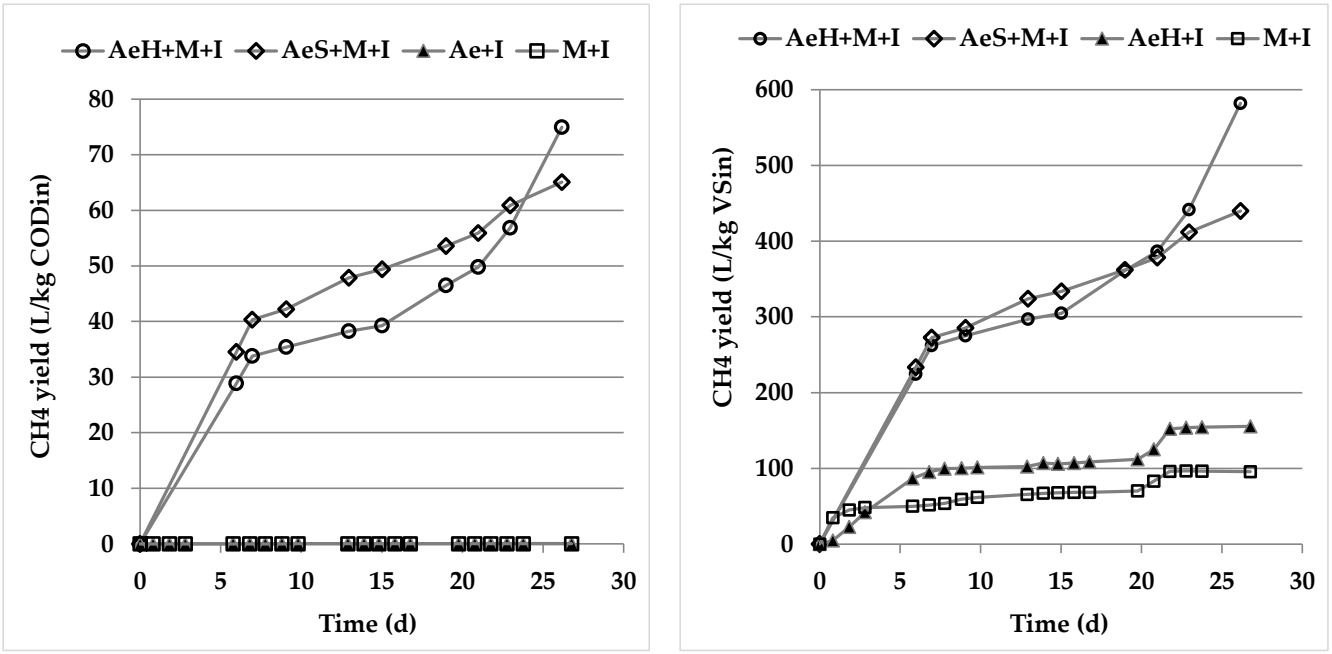

Figure 2. Anaerobic digestion of wastes from fermentation: (**a**) cumulative methane yield expressed as L CH_4/g COD added at the beginning of the AD; (**b**) cumulative methane yield expressed as L CH_4/g VS added at the beginning of the AD.

Despite the relevant proportion of methane (71%) present in the biogas provided by the digestion of extracted algae with hexane AeH+I, the volume of gas obtained in this assay was the lowest of all experiments performed (Figure 1). It becomes evident, accordingly, that the joint digestion of the algae with the culture medium, taking advantage of the substrate complementarity concept [8], is much more interesting as it allows doubling/tripling of the production of biogas and further promoting the gas quality. Biogas volumes of 35 mL (71% CH_4) and about 100 mL (76% CH_4) were accumulated in AeH+I and AeH+M+I anaerobic units, respectively. In comparison, this can be justified by the difference between the organic material initially available in AeH+I which was about half that of the existing organic material in AeH+M+I (13 vs. 27 g COD/L, Table 2) and, conversely, with the initial nitrogen levels which were about four times higher than those recorded in the AeH+M+I.

As expected, the digestion mixture AeH+M+I from the fermentation wastes displayed the highest methane productivity of the whole experiment, 31 L CH_4/kg VS_{in}.d and 4.0 L CH_4/kg COD_{in}.d, followed by the digestion mixture AeS+M+I. These data indicate that the sustainability of the lipids production, from the heterotrophic marine microalga *C. cohnii*, could be promoted by energetic valorization of the remaining materials through integration of the anaerobic digestion techniques. An energetic potential of 4.4–5.8 KWh/Kg VS_{in} and

0.65–0.75 KWh/Kg COD_{in} (Table 3) can be retrieved and applied to the lipid production process itself.

Table 3. Methane productivity and energy potential estimated at the end of the anaerobic digestion for microalgae fermentation wastes and mixtures used as feedstock.

Mixture	Methane Productivity		Energy Content	
	(L CH_4/kg VS.d)	(L CH_4/kg COD.d)	(KWh/Kg VS)	(KWh/Kg COD)
AeH+M+I	31.4	4.04	5.80 ± 0.00	0.75 ± 0.00
AeS+M+I	11.2	1.66	4.39 ± 0.26	0.65 ± 0.04
AeH+I	9.53	0.006	1.10 ± 0.06	0.0007 ± 0.00
M+I	3.32	0.002	0.24 ± 0.01	0.0002 ± 0.00

3.3. Flow Citometry

Figure 3 shows the evolution of permeabilized cells of the anaerobic microbial consortium during AD, shown as Δ(SYBR/PI), calculated according to Equation (1). Therefore, the lower the difference values of Δ(SYBR/PI), the lower the percentage of permeabilized cells at the end of the anaerobic process. A negative Δ(SYBR/PI) value means that the percentage of permeabilized cells at the end of the assay (t_f) was lower than that at the beginning of the experiment (t_0), which indicates that the microbial consortium of cells was exposed to favorable conditions during the AD process, such that the proportion of permeabilized cells decreased during the assay, relatively to the percentage of permeabilized cells detected at the beginning of the assay. This fact happened for the assay which used AeH+M+I as substrate (−8.3%).

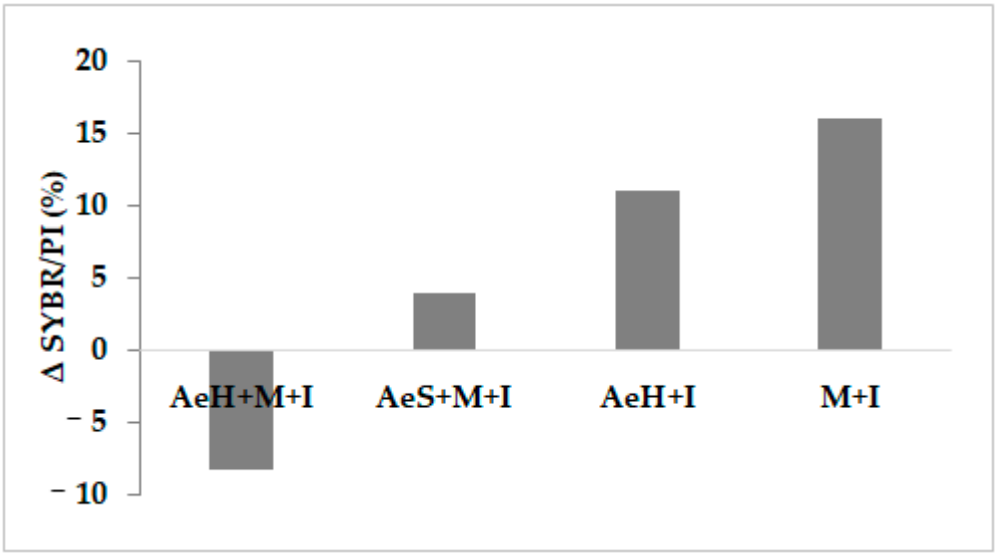

Figure 3. ΔSYBR/PI calculated for all the assays.

In contrast, a Δ(SYBR/PI) positive signal indicates that the percentage of permeabilized cells increased throughout the AD process, relatively to the beginning of the assay. This means that the microbial consortium cells were exposed to adverse conditions that induced the cell membrane permeabilization. This was observed for assays AeS+M+I (+4%), AeH+I (+11%) and M+I (+16%).

4. Discussion

Cultivation of marine microalgae requires a high sodium chloride content (0.5–1 M); however, substrates with salinity values of 0.4 M can already affect methane production during anaerobic digestion, and above 0.5 M are toxic [18,19]. The culture medium super-

natant (M) used in this work came from the fermentation of the marine microalgae *C. cohnii*, whose culture medium contains sea salt (25 g/L) [14], that is, the medium has a salinity of about 0.4 M. Indeed, as shown in Figure 1, the digestion of the culture medium (M) produced some biogas volume, 48 mL, but with low quality (26% CH_4), and all yields and methane productivity values obtained from the anaerobic digestion of this residue were also the lowest of all the tests, probably due to the concentration of sea salt. The option of mixing of microalgae biomass leftovers was a good approach to overcome the negative impact of this parameter, making it possible to obtain a biogas production of 74 and 106 mL (76–77% CH_4) during the anaerobic digestion of the global wastes.

In this work, AeH+I or M+I showed the highest content of nitrogen and ammonia (Table 2), thus producing the lowest biogas volumes of all the substrates tested by anaerobic digestion, 32 and 48 mL, respectively. The mono-digestion of microalgae has been shown to be difficult [6]. The high content in nitrogen, usually as proteins, leads to low C:N ratios. During the anaerobic digestion process, acidogenic bacteria and methanogens can be inhibited by high levels of ammonia released by the degradation of these proteins [20]. On the other hand, as proposed by some authors [18,20], the co-digestion of microalgae with carbon-rich feedstock can be a cost-effective and efficient approach to avoid ammonia inhibition. In this case, we used the remaining culture medium (M) as a carbon-rich feedstock, in terms of COD and VS, for the AD of the two extracted microalgae (AeH and AeS), and consequently, biogas production was highly increased by up to 106 mL (76% methane) for AeH+M+I and 74 mL (77% methane) for AeS+M+I. Although at lower percentages, up to 62%, other authors also recorded significant increases in methane production using this approach, the co-digestion of microalgae with high-carbon wastes [21–23].

The results from flow cytometric analysis were in accordance with the results obtained for methane production and can be explained by the presence of available nutrients in the media resulting from the extracted alga residues, which contain proteins and carbohydrates that can be readily uptaken by the microbial consortium during anaerobic digestion. Therefore, it seems that the assayed AeH+M+I (Δ(SYBR/PI) = −8.3%) contained a higher nutrient availability in the culture medium than the other assays, which not only favored biogas production, but also protected the cells against nutrient limitation, a condition that often induces cell membrane damage [24]. It should be noted that no hexane or hexane traces were present in the extracted algal biomass (AeH), in the assayed AeH+M+I and AeH+I since it was previously evaporated. The lower Δ(SYBR/PI) value observed for AeS+M+I (Δ(SYBR/PI) = +4%), relatively to AeH+M+I (Δ(SYBR/PI) = +11%) may be explained by the less efficient alga extraction with hexane (AeH), compared to the supercritical alga extraction (AeS), resulting in biomass leftovers with a higher nutrient content that could be consumed by the microbial consortium, resulting in the highest methane yield and productivity (Figure 2 and Table 3, respectively). Indeed, it is well known that supercritical extraction is much more efficient than traditional solvent extraction methods [25]. This means that less nutrients remain in the microalgal biomass leftovers, after supercritical extraction, compared to the biomass leftovers resulting from hexane extraction. The highest difference Δ(SYBR/PI) observed for the M+I assay (16%), was attributed to lower nutrient availability due to the fermentation broth (M), since it corresponded with the exhausted medium collected at the end of the microalgal biomass production step. Additionally, the high fermentation broth salinity value of 0.4 M (25 g/L) might have had a negative impact on the consortium of cells involved in the M+I assay, resulting in the cell permeabilization of 16% of the total cell population. The presence of microalgal biomass residues in the remaining assays (AeH+M+I, AeS+M+I, AeH+I) might have mitigated the negative effect of the salt present in M since the salt present in the microalgal biomass was removed during the extraction step.

There have been some works reporting anaerobic co-digestion of microalgal biomass- and organic-rich feedstocks in batch condition tests. The mesophilic co-digestion of a mixture of 75% OMSW (olive mill solid waste) and 25% *Dunaliella* salina generated a methane yield of 330 L CH_4/kg VS added [22]. Hermann et al. [6] reported that the co-

digestion of 15% *Arthrospira platensis* with 85% seaweed achieved a biochemical methane yield of 311 L CH_4/kg VS and a maximum specific methane production rate of 29 L CH_4/kg VS.d. The co-digestion of mixtures of algae bloom (*Microcystis* spp.) and lake water with corn straw achieved 325 L CH_4/kg VS [21]. *Tetraselmis suecica* biomass co-digested with glycerol increased the methane yield rate from 174 to 438 L CH_4/kg VS in relation to its mono-digestion [26]. The synergetic effect was observed in a mixture containing 63% undigested sewage sludge and 37% wet algae slurry, in which a CH_4 yield of 408 L/Kg VS was reached under mesophilic conditions [23]. All these values were considerably lower than those obtained in the present work for the digestion of the AeH+M+I mixture, 582 L CH_4/kg VS, and lower than those obtained with the AeS+M+I mixture, 440 L CH_4/kg VS (Figure 2).

The selection of biomass for the AD process among other operational conditions decides the composition of the biogas. To be economically feasible, the overall process should guarantee the maximal concentration of methane in the biogas content, so the calorific value of the biogas is increased. The extraction of lipids from microalgae biomass prior to the processing of gaseous bioenergy will results in an increase in the production yield and the biorefinery approach of microalgae biomass has a zero-waste discharge [27]. The concept of the *C. conhii* biorefinery achieved with a high-energy content, 4.4–5.8 KWh/Kg VS, revealed in the biogas produced (Table 3) after anaerobic digestion of both mixtures containing the lipids extracted *C. cohnii* biomass leftovers and the supernatant of the culture medium (AeS+M+I and AeH+M+I). The overall approach offers solutions that address societal needs both in terms of products and processes [28].

5. Conclusions

The anaerobic digestion process can be successfully applied to the energetic valorization of *C. cohnii* defatted biomass and the culture medium supernatant, the main wastes generated during the microalgal lipid production process. AD integration with the lipid production step avoids wastes, using the biomass leftovers as AD feedstocks.

The results indicate that the process used for lipid extraction had an influence on the energy recovery through anaerobic digestion. Concerning the substrates used for AD, the joint digestion of algae with the culture medium was more interesting than the individual digestion of each one, insofar as the combination of the two wastes, taking advantage of the substrate complementarity concept, promoted a zero-waste practice and provided a greater volume of biogas of better quality.

Given the interest of the results presented here, the authors consider that the proposal of this work deserves larger scale trials. Thus, based on the achieved data, they intend to proceed with the research on the energetic valorization of the tested wastes generated from *C. cohnii* microalgae fermentation and the lipid extraction process, and evaluate the impact on the microbial consortium of cells by the anaerobic digestion process. It will be important to design AD assays on a larger scale to consolidate all the results obtained in the present work.

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