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Anaerobic Digestion of Microalga *Chlorella prototheocoides* and Metagenomic Analysis of Reddish-Colored Digestate

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Abstract: Microalga *Chlorella prototheocoides* materials were assessed as substrates for anaerobic digestion (AD) aiming at the simultaneous production of biogas/methane and pigments: whole autotrophic (AA) and heterotrophic algae (H); extracted heterotrophic microalgae from lipid production (HExt); and pretreated heterotrophic microalgae through enzymatic (HPEnz), autoclave (HPA), and ultrasound (HPU) processes. AA was more suitable for AD than H, as it was more efficiently converted into methane (279 vs. 180 L CH_4/kg VSin). In comparison, the pretreatment of heterotrophic microalgae had a positive effect on AD, with registered methane yield increases from 263 to 290 L CH_4/kg VSin (HPU, HPA, HExt). Reddish pigmentation developed in H and HPU units due to the presence of purple non-sulfur bacteria (PNSB). This phenomenon and the changes in microbiota structure during AD were confirmed by metagenomic analysis. At the end of the process, the relative abundance of *Clostridiales* and *Bacillales* increased, enhancing the hydrolysis of compounds in acetate. Consistently, *Methanosaeta* became the comparatively dominant methanogen, meaning that methane was produced through the acetoclastic methanogenesis pathway. The obtained results indicate for AD biorefinery feasibility—regarding the simultaneous production of biogas/methane—a digestate flow and pigments (bacteriochlorophyll *a* and carotenoids).

Keywords: *Chlorella prototheocoides*; microalgae residues; microalgae pretreatments; anaerobic digestion; microbial communities; *Rhodobacter*; *Rhodopseudomonas*

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1. Introduction

Renewable energy resources are efficient and sustainable, contributing to the decrease in global warming. In this category, bioenergy can be produced using organic wastes, helping them to enter the regional circular economy and thus increasing the efficiency of locally available resources. Anaerobic digestion (AD) is a process that provides a bioenergy carrier gas as it can efficiently deliver biogas/biomethane from organic wastes and has all the advantages of renewable energy while reducing greenhouse gas (GHG) emissions [1,2]. Presently, in Europe, municipal waste management includes AD of organic waste streams (domestic and industrial) as a frequent practice, as it is now a mature technology for producing biogas [3].

Combining sustainable microalgae cultivation with an anaerobic digestion of remaining microalgae to obtain biodiesel and biogas is a smart strategy to make specific biofuels and manage wastes properly. The microalga *Chlorella prototheocoides* can grow heterotrophically or autotrophically, with demonstrated improved productivity in biomass and oil, as well as high environmental sustainability [4]. The oil content accumulated in microalgae differs from one species to another and depends on the cultivation mode used. Heterotrophic cultivation of microalgae presents several advantages over the autotrophic mode, the most notable being a higher oil productivity. Heterotrophic microalgae growth can still be

combined with autotrophic microalgae cultivation to absorb the produced carbon dioxide and to avoid its emission to the atmosphere [5]. The biodiesel from microalgae is a third-generation biofuel; therefore, it does not compete with food production, as is the case with edible vegetable oils. The biodiesel production process is quite old and is based on extracting the oil from microalgae biomass and then making it react with alcohol to allow the transesterification into fatty acid methyl esters (FAMEs). The cellular debris from microalgae, after oil extraction, is a substrate adequate to convert into biogas through the AD process [6]. However, microalgae cells can resist biodegradation and the organic matter inside them will not be available for the anaerobic microorganisms to convert it, thus making hydrolysis the limiting anaerobic digestion step. There is some research on different pretreatment methods to increase the methane yield of microalgal biomass which highlight mechanical methods, such as ultrasound and hydrothermal ($T > 100\ ^{\circ}C$, $P > 1$ bar) methods, and biological, enzymatic methods [7]. The pretreatment choice depends on the microalgae genera as they have considerably different cell walls. In the case of *Chlorella* sp., the cell wall is medium robust, as its structure consists of only two layers compared with other genera which have three or four layers, and its composition is made of carbohydrate polymers with hemicellulose, galactose, and rhamnose [8]. Moreover, the cultivation medium, e.g., with or without glucose, and the environmental conditions, e.g., with light or in the absence of light, used to grow *Chlorella* cells may impact the cell wall structure and composition [8]. Hence, testing simple pretreatments should be enough to release intracellular soluble organic matter and increase the efficiency of AD. Although the microalgae theoretical methane yield was estimated to be in the range of 0.48–0.80 L CH_4/g VS [9], experimental results until now have only achieved the first figure. Much attention has been given to autotrophically grown *Chlorella* sp. [7]; therefore, more experimental data will be needed on the application of pretreatments, particularly for the heterotrophically grown microalga.

The AD process starts with breaking down organic matter through a set of biochemical reactions accomplished by bacteria and archaea, producing an energy-carrying gas consisting of carbon dioxide and methane [3]. At least three groups of microorganisms are involved in the AD process: primary fermenting bacteria, anaerobic oxidizing bacteria, and methanogenic archaea. Bacteria degrade biomass primarily to acetate and hydrogen. The acetoclastic and hydrogenotrophic methanogens are responsible for the conversion of the acetate or the hydrogen into carbon dioxide and methane, respectively [10].

Recently, most studies have used molecular analysis to investigate the composition and structure of microbiota in anaerobic digesters and to correlate them with the use of different substrates [11–13]. Although the results still depend on factors such as DNA extraction, primers, and amplification region, the application of next generation sequencing (NGS), which includes metagenome analysis, has been shown to be of great value in investigating the specific genomes, cultured and uncultured, that are present in an environmental community. In addition, these studies have contributed to increasing the genome repository in the collections of public databases [14].

In an earlier study, a reddish pigmentation was observed inside anaerobic digester units that were kept under light and AD anoxygenic conditions. That population of bacteria was identified as "purple non-sulfur bacteria" (PNSB) after analyzing the nucleotide sequences of the 16S rRNA genes [15]. Other authors have also spotted PNSB in the strictly anaerobic sewage sludge digester and in waste lagoons. They observed a pigmented bloom of PNSB with an intense red color in lagoons, and the bloom was associated with a significant reduction in odor [16]. These bacteria are photoheterotrophic organisms, i.e., they can use organic carbon compounds under light. At low levels of sulfide (H_2S), below 0.5 mM, purple non-sulfur bacteria are able to oxidize sulfide to sulfur (S^0), thiosulfates ($S_4O_6^{2-}$), or sulfate (SO_4^{2-}). They were named PNSB because when sulfur is formed it is deposited extracellularly, and so when observed under the microscope they do not show sulfur globules, as compared with PSB [16]. Another study used PNSB as a bioaugmentation strategy in AD, which resulted in the enhancement of the overall performance of their

indigenous methanogenic culture through the attained upgrade to both the organic load removal efficiency and the yielded biogas quantity and quality [17].

PNSB has bacteriochlorophyll *a* and *b* and carotenoid pigments, the latter including spirilloxanthin, spheroidene, lycopene, and rhodopsin [18]. These pigments can give them colors ranging between purple, red, brown, and orange, depending on the relative content. Lycopene is an important carotenoid with applications as food additives, drugs, and cosmetics due to its anti-oxidative, anti-cancer, and anti-inflammatory activities, but its extraction from plants suffers from seasonality. The proposed alternative for the sustainable production of lycopene has been through the growth of PNSB. The accumulation of carotenoids during the growth of PNSB can be exploited for biotechnology applications [19,20]. Moreover, there is evidence that the application of PNSB in plant growth has benefits due to the accumulation of polyphosphate, pigments, and vitamins and the production of plant-growth-promoting substances (PGPSs) [21].

The main purpose of this study is the application of microalgae for energy purposes with the simultaneous production of energy carrier gas, digestate, and pigments, using microalgae materials of *Chlorella protothecoides* as AD process substrates, within the biorefinery concept framework. As far as the authors are aware, the production of these products simultaneously and during the same digestive anaerobic process/anaerobic reactor has never been the subject of any investigation by other researchers.

Thus, it is intended to comparatively evaluate the applicability of the AD process using different forms of algal substrates, such as autotrophic and heterotrophic microalgae, in their original state and after being subjected to amendment actions. Due to the inherent advantages of algal production under heterotrophic conditions and since these operational settings are less studied than autotrophic ones, we aimed to evaluate the AD process using the algal heterotrophic residues as substrates, obtained after lipid extraction from the production of biodiesel, and heterotrophic algae previously treated through different processes (enzymatic, autoclave, and ultrasound).

The importance of this study was further reinforced by, on the one hand, evaluating the structure of the microbiota and, on the other hand, assessing the presence of anoxygenic PNSB, relating this to the quantity and quality of the biogas generated.

2. Materials and Methods

The biomass from microalga *Chlorella protothecoides* was used in this work and was cultivated under autotrophically and heterotrophically conditions as previously described [4].

Whole heterotrophic microalgae tested with enzymatic, thermal, and ultrasound pretreatment methods were suspended in Millipore water, 12 g/L (m/v), before further use in anaerobic digestion.

2.1. Microalgae Pretreatments

2.1.1. Lipid Extraction with Hexane

Prior to initiating lipid extraction, a sample of heterotrophic algae was ground in a vibratory disk mill for two and a half minutes at 1500 rpm (final fineness: <20 µm, RETSCH RS 200). The extraction of lipids was performed with hexane in a Soxhlet apparatus and lasted for 10 h; it was followed by evaporation with the purpose of eliminating almost all hexane adsorbed to the extracted biomass. The extracted alga was placed in the oven for 1 h at 130 °C and then placed in the desiccator until room temperature was reached. The extracted alga was suspended in Millipore water, 12 g/L (m/v), before further use in anaerobic digestion. A sample of extracted algae was further designated as HExt.

2.1.2. Enzymatic Pretreatment

The heterotrophic algae were pretreated with lysozyme at a concentration of 100 mg/mL, incubating at 37 °C at 100 rpm for the duration of 16 h [22]. A sample of pretreated algae with enzyme was further designated as HPEnz.

2.1.3. Thermal Pretreatment

The heat pretreatment was performed using the autoclave at 120 °C and 1.2 bar for 30 min. Samples of pretreated heterotrophic algae were further designated as HPA.

2.1.4. Ultrasound Pretreatment

The ultrasound treatment was realized 3 times for 5 min at 20 kHz output on ice (ultrasound Vibra-Cell VC505, Sonics, Newtown, CT, USA). Samples of heterotrophic pretreated algae were further designated as HPU.

2.2. Substrates and Inoculum

Whole autotrophic (AA) and heterotrophic (H) microalgae and different substrates obtained from the latter were tested as AD substrates: a residue of heterotrophic algae obtained after lipid extraction (HExt) and different pretreated heterotrophic algae by using enzymatic (HPEnz), autoclave (HPA), and ultrasound (HPU) processes.

Sludge (1.3 ± 0.0 g VSS/L) was collected in a municipal anaerobic digester plant (SIMARSUL, Sesimbra, Portugal).

2.3. Anaerobic Digestion Experimental Setup

Anaerobic digestion was carried out under batch conditions using 70 mL glass vials, with 40 mL of useful volume. Each vial contained 70% (*v/v*) of substrate and 30% (*v/v*) of homogenized inoculum. The experiment was run in triplicate and units were flushed with nitrogen and sealed at the beginning of the assay to ensure anaerobic conditions. Incubation was at mesophilic conditions (37 ± 1 °C) and maintained constant for 45 days of the experiment.

2.4. Analytical Methods

Samples were collected at the beginning (IN) and at the end (OUT) of the experimental time for analytical characterization. Chemical oxygen demand (COD), total and volatile solids (TS, VS), and pH were assayed according to standard methods [23].

The volume of the obtained biogas was monitored daily, and the methane content was measured by gas chromatography [11].

2.5. Optical Microscopy

The effect of the pretreatments on cells of *Chlorella protothecoides* and the pigmentation clusters formed in the digestate were observed under an optical microscope (400× magnification, Olympus BX51, Tokyo, Japan).

2.6. Absorbance Spectrometry

To assess the effect of pretreatment methods and oil extraction, an aliquot of microalgae culture medium was collected and was diluted in water (1:10). The absorbance spectra were measured within a range of 190 and 290 nm (Shimadzu UV—2401PC).

The presence of photosynthetic pigments, such as bacteriochlorophyll *a* and carotenoid pigments, was also assessed by absorbance spectrometry. An aliquot of the sample was collected from the HPU unit at the end of AD. Then, the absorption spectrum of supernatant was measured within a range of 380–900 nm (Shimadzu UV—2401PC).

2.7. Metagenomic Analysis: Next-Generation Sequencing (NGS) of 16S rRNA Gene Amplicons

DNA extraction and gene sequencing were performed as described by Eusébio et al. [11] for samples collected at IN and OUT of units H and HPU. Library construction was performed using the Illumina 16S Metagenomic Sequencing Library preparation protocol [24]. The generated DNA fragments (DNA libraries) were sequenced with MiSeq Reagent Kit v3 in the lllumina MiSeq platform using 300 bp paired-end sequencing reads. The bioinformatics analysis of the generated raw sequence data was carried out as described by Eusébio et al. [11].

All the raw data were deposited in the NCBI Sequence Read Archive (SRA) database with the accession numbers SRR23216834–SRR23216838 (Bioproject PRJNA927270).

3. Results

3.1. Chemical Composition of Substrates and Inoculum after Pretreatment Processes

Although the organic load of the whole autotrophic alga (AA) is lower than the heterotrophic alga (H) (Table 1), AA has a more favorable pH for the AD process than H of 7.0 vs. 5.9, respectively. The neutral pH is preferable for the proper development of the AD process, whereas an acidic pH provides conditions for increasing the acid formation and creating an imbalance between the population that produces and consumes them. In an extreme situation, the excessive accumulation of acids causes a decrease in pH and a blockage of the AD process.

Table 1. Substrate and inoculum chemical composition: pH, COD, TS, and VS.

Substrate	pH	COD (g/L)	TS (g/L)	VS (g/L)
H	5.87	22.5 ± 0.2	8.40 ± 0.1	8.0 ± 0.0
HExt	5.76	9.70 ± 0.2	5.30 ± 0.3	5.0 ± 0.3
HPEnz	5.61	16.1 ± 0.0	7.20 ± 0.1	6.9 ± 0.0
HPA	5.63	23.4 ± 0.2	10.2 ± 0.1	9.7 ± 0.1
HPU	6.23	23.8 ± 0.0	8.80 ± 0.2	8.4 ± 0.2
AA	7.04	16.4 ± 0.0	9.50 ± 0.2	7.6 ± 0.0
I	–	17.6 ± 0.4	12.5 ± 0.1	9.1 ± 0.0

The substrates HPA and HPU have the highest organic loads (23–24 g/L COD, 9–10 g/L TS, and 8–10 g/L VS, Table 1), followed by HPEnz and, finally, HExt with the lowest concentrations, corresponding to about half of the higher ones. In fact, as HExt is a residue that results from a process that removes a substantial part of its organic potential (the lipid fraction), it would be expected that this would be the comparatively most diluted substrate. A pH value of 6.2 was registered in the HPU, which was the most favorable when compared to the other substrates with slightly lower values (5.6–5.8).

Under microscope observations, the structure of the materials to be digested revealed the extent the of cell wall disruption compared with the whole cells. It can be observed that H (Figure S1a) presents defined cell walls with slight changes due to the algae preservation method (freeze-dried after being stored in the freezer), as was expected. The structure of HExt (Figure S1b) shows extensive disruption with cellular burst and the agglutination of the cellular components. As for the heterotrophic alga pretreated with the enzyme (HEnz, Figure S1c), it is possible to observe some cellular disruption, although much less extensive than the previous case. The microscopic observations of the cellular structure of the algae after the pretreatment either with autoclave or with ultrasound show the identified zones with the cellular disruption and the intrasomatic part of the cells (Figure S1d and Figure S1e, respectively). The autotrophic algae (Figure S1f), which were not subjected to any pretreatment, show whole cells with the characteristic green coloration, and few empty cells, which may also be the result of the preservation method.

To assess the amount of dissolved organic compounds such as protein, carbohydrates, and DNA resulting from the applied pretreatment, the absorption spectra were measured at wavelengths from 190 to 290 nm, as shown in Figure S2. The greatest cellular disruption resulted from autoclave pretreatment, followed by ultrasound and untreated algae. The extracted algae were suspended in water, after lipid extraction; therefore, the release of components into the medium was moderate. In the case of algae pretreated with lysozyme, the resulting spectrum corresponds to the low cellular disruption observed at the microscope and presents the lowest intensity among all pretreatments. According to other research [25] involving cellulase, protease, and pectinase, in addition to lysozyme, the multiple-enzyme combination showed better results than the single-enzyme treatment. On the other hand, the costs of using multiple enzymes are higher.

3.2. Anaerobic Digestion of Microalga Chlorella Protothecoides

Biogas production was observed from the beginning in all the substrates tested, with no lag phase evidenced, as shown in Figure 1a. After the first week of the trial, the difference in the biogas production capacity of the tested substrates began to be noticed, and after 25 days it was possible to observe three distinct levels of accumulated biogas volume, namely: 125, 118, and 110 mL (HPA, AA, and HPEnz, respectively); 94 mL (HExt); and 59 mL (H and HPU). However, from this point onwards, the biogas volume collected in HExt increased the most, evolving positively over time until it equaled the volume recorded in HPA at 35 days of the trial (144 mL). The successive increases in the biogas volume in this unit suggest that the material being digested—the extracted heterotrophic alga (HExt)—was degraded in a more phased and gradual way throughout the experiment, compared to the others, but achieved the highest biogas volume (168 mL).

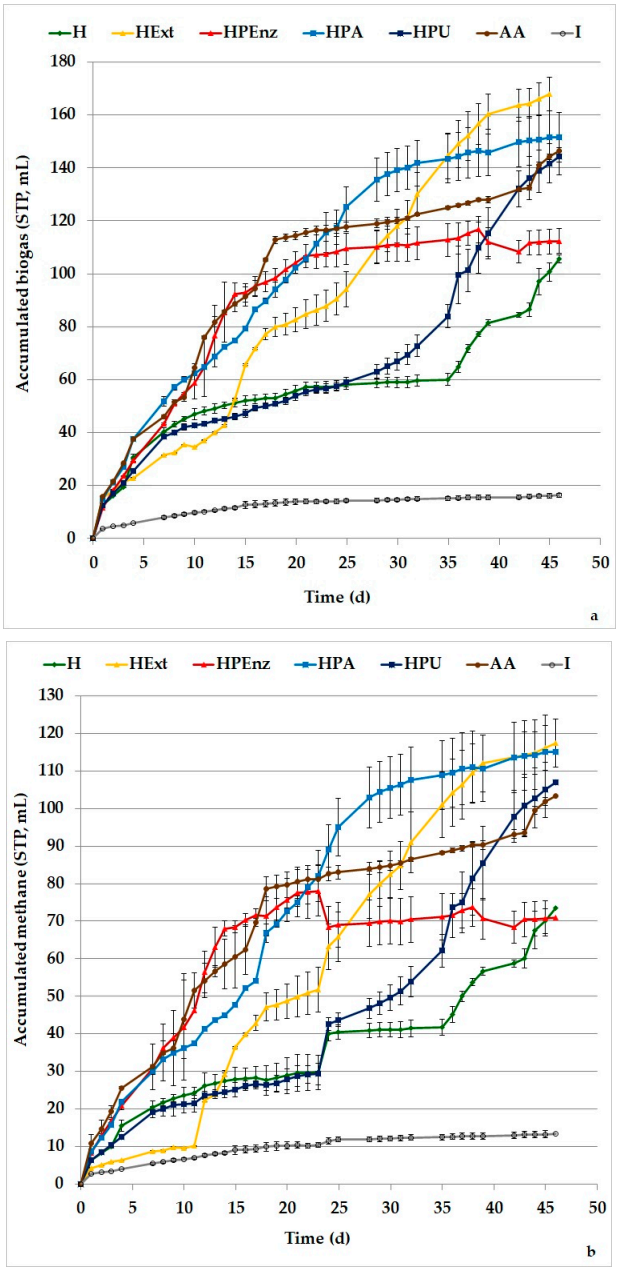


Figure 1. Volume of gas produced during anaerobic digestion of algae: (**a**) accumulated biogas; (**b**) accumulated methane. STP—standard temperature and pressure.

It is interesting to note the occurrence of a "restart" process observed in several units. For AA, the sharp increase in the biogas volume during the first 17 days of the test (6.3 mL/d, Figure 1a) was followed by prolonged small increments and, finally, about 25 days after, a further increase was observed in the biogas volume (4.0 mL/d). The other cases concern H and HPU units, which presented similar behavior to each other during the first 28 days of operation. However, on the following days, the HPU unit showed successive increases in the biogas volumes that end up being equal to those obtained in AA and close to the HPA production by the end of the experiment. These observations can be understood as a process of microbial consortium adaptation to the material being digested. In the case of HPU and AA, the microbial development in HPU occurred at a later stage than in AA. Nevertheless, the AA showed a higher biogas production than H.

The behavior comparison of whole autotrophic and heterotrophic algae (AA vs. H) showed that the former reached a gas production of 113 mL in the first 18 days and that amount was never reached by H until the end of the run. It should be noted, however, that both AA and H showed marked increases in the volume of biogas in the test's final phase, which indicates that the gas potential production of these substrates may be higher than those recorded.

This study showed that the lipid-extracted *Chlorella protothecoides* provided the best accumulated methane production (HExt, 117 mL, Figure 1b) due to the important action of prior grinding before extracting the oil, and that a good evaporation of the solvent guaranteed the absence of solvent residues in the algal material. In addition, the pretreatment effect on methane production showed that either the autoclave (HPU, 115 mL) or ultrasound (HPA, 107 mL) processes are great methods to disrupt the walls of microalgal cells, allowing aqueous solubilization of organic matter, as referred to by other authors [26]. Cho et al. [27] reported the highest accumulated methane production of 121 mL obtained with the microalgae biomass pretreated using the autoclave method. Ayala-Parra et al. [28] demonstrated the impact of ultrasound pretreatment on methane production, increasing the sonication time and improving the anaerobic degradability of algal biomass.

All the above-mentioned methods originated substrates that provided the highest methane volumes compared with the enzyme pretreatment (HPEnz, 78 mL). Effectively, the units digesting the heterotrophic algae pretreated by an enzymatic process were the only ones that showed a decrease in biogas production in the assay's final stage, indicating the exhaustion of the process (Figure 1a). Microalgae like *Chlorella* sp. contain complex cell walls that are composed of an outer layer, which may be resistant to the AD process [29]. In this study, considering that lysozyme was the only enzyme tested, and the worst results were obtained for that assay (HPEnz), it appears that the effect of the enzymatic action on the substrate was advantageous since the process required less time, about 20 days, to supply an identical biogas volume to that recorded in H units at the end (Figure 1a). As for the volume of collected methane under the different test conditions (Figure 1b), there is a behavior quite like that observed during the biogas production, except for HExt. The fact that HExt had limited methane production at the beginning of the experiment (Figure 1) can be understood as an adjustment of the balance of the microbial population to the absence of lipids into which, only after about 10 days, the archaea were able to convert the intermediate products in the methane. The superiority in biogas volume of HExt over HPA was not confirmed for the resulting methane as, in the experiment's final period, the same volume (115 mL CH_4) was obtained in both trial units.

The volume of gas recorded in the inoculum units (I) resulted from some traces of degradable material mixed with the used sludge, which demonstrated the activity and ability of the microorganisms present in this sludge to be efficiently used as inoculum in the AD tests. The quality of the respective inoculum is further confirmed by the fact that no lag phase was evidenced at the experiment startup (Figure 1a,b) as well as by the positive evolution in the methane amount recorded over time, and, consequently, any limitation observed throughout the different assays' conditions cannot be related to the quality of the applied inoculum.

The differences found in terms of gas production between units digesting whole autotrophic and heterotrophic algae (AA and H) are confirmed in the highest removals of the organic loads by AA compared with H (53 vs. 23% COD, 67 vs. 39% VS). Although they started from similar organic matter concentrations in the two assays (32–33 g/L COD, 9–10 g/L VS, Table 2), AA units presented a much more easily digested and converted material than H and were therefore more suitable for the AD process.

Table 2. Mixtures' chemical composition: COD, TS, VS, and pH.

Batch Condition	CODin (g/L)	COD Removal (%)	TSin (g/L)	VSin (g/L)	VS Removal (%)	pH Initial	pH Final
H	32.7 ± 0.0	23	11.6 ± 0.1	10.2 ± 0.1	39	6.81	6.72
HExt	21.3 ± 0.0	25	11.6 ± 0.3	10.1 ± 0.4	52	7.09	6.85
HPEnz	29.1 ± 0.0	14	10.0 ± 0.0	8.60 ± 0.1	53	6.85	6.95
HPA	27.0 ± 0.0	53	12.1 ± 0.1	10.5 ± 0.1	35	6.98	6.80
HPU	26.3 ± 0.0	25	11.7 ± 0.1	10.2 ± 0.1	40	6.88	6.72
AA	32.1 ± 0.0	53	11.6 ± 0.0	9.30 ± 0.0	67	7.21	6.87
I	11.4 ± 0.0	43	5.10 ± 0.1	1.10 ± 0.0	7	7.38	6.99

As for the pretreated heterotrophic algae, the highest COD removal (53%, HPA) was obtained with the autoclave method in the unit that presented the highest biogas/methane production (Table 2). Likewise, it was confirmed that the lowest removal (14%, HPEnz) was obtained for the enzymatic pretreatment, corresponding with the lowest gas production (Figure 1). Consequently, the decrease in gas in the HPEnz experiment can be understood as a disruption process that may be associated with a change in AD bacterial consortium and/or with the ineffectiveness of the enzymatic pretreatment on the algae cells in releasing their content. The pH of the solutions before the anaerobic process (Table 2) is higher than the initial values obtained in the substrates (Table 1). Values closer to neutrality were obtained in all digesters with substrates due to the addition of sludge for inoculation. There were no major changes in the pH values after digestion; however, there was a slight decrease in the values at the end of the experiment, except for HPEnz where a slight rise was detected.

Although HPEnz presents a good methane yield per COD removed (440 L CH_4/kg CODr, Table 3), the gas production was only observed in the first 25-day period of the test, probably due to a microbial population imbalance that prevented the conversion of the remaining load. This result is confirmed by the lowest COD removal (14%, Table 2) obtained for the enzymatic pretreatment.

Table 3. Methane yield per VS and COD at the beginning, and per COD removed, at the end of the anaerobic digestion of microalgae.

Mixture	Methane Yield		
	(L CH_4/kg CODin)	(L CH_4/kg CODr)	(L CH_4/kg VSin)
H	56	242	180
HExt	138	550	290
HPEnz	61	440	206
HPA	107	418	276
HPU	102	411	263
AA	81	153	279

The comparison of methane yields from whole autotrophic and heterotrophic algae revealed that the former (AA) was more effective in relation to the supplied food than H. However, when the removal of organic matter was considered, the situation was inverted, meaning that H was more efficient than AA in converting the available material to methane. Examining the H behavior in relation to the gas volumes recorded (Figure 1), most of the

gas production was carried out in the final phase of the experiment, and this period may have been insufficient for the complete consumption of all the substrate still available and resulted in a low COD removal.

The highest methane yields, in terms of COD and VS fed at the beginning of AD, were in the HExt, HPA, and HPU units (Table 3), corresponding to the highest methane volume achieved, as mentioned before. The highest methane yield of 290 L CH_4/kg VSin obtained for the lipid extraction process can be considered as an optimal pretreatment method if the tested algal materials do not hold any solvent residues. The extracted microalga in our study showed a higher methane yield than the value reported by Bohustskyi et al. [30] of 250 L CH_4/kg VS obtained in a semi-continuous stirred tank.

The application of ultrasonic pretreatment has been reported to easily disrupt algae cell walls, improve substrate solubility, and enhance the crude protein digestibility [31,32]. Ayala-Parra et al. [28] reported an improved methane yield of 327 L CH_4/kg VS using sonication pretreatment of microalga *Chlorella protothecoides*. This value is higher than the 263 L CH_4/kg VS attained in this study, yet those authors performed the AD using a basal medium for microbial growth supply, making a comparison between the methane yield values difficult.

The comparison of the behavior of the whole heterotrophic algae units (H) with those that digested the pretreated algae reveals that the application of any pretreatment is advantageous in terms of methane production.

3.3. Reddish Pigmentation in AD Units

The development of different gradations of reddish coloration in the AD units was verified throughout the experiment, and the intensity of color, observed visually over time in each digester, was described as follows: "reddish absent", "light reddish", "middle reddish", and "strong reddish" (Table 4). Differences in the coloring of the units at the end of AD can be seen in Figure S3.

Table 4. Appearance of a reddish coloration following the biogas production.

Day	H	HExt	HPEnz	HPA	HPU	AA	I
1–25	x	x	x	x	x	x	x
25–35	▲	x	■	●	●	x	■
35–50	▲	x	■	●	●	x	■

Legend for pigmentation: x—reddish absent; ■—light reddish; ●—middle reddish; ▲—strong reddish.

No coloration was detected in the HExt and AA units, indicating that the microorganisms responsible for this pigmentation, if present in the medium of these units, were not able to express themselves and therefore were not visually detected. It appears that the presence of lipids in the algae is related to the production of the red pigments. All other units with different operating conditions show the presence of the color, which only became evident after about 25 days of the experiment startup, indicating that these microorganisms needed at least about a month to develop and to produce the pigmentation.

The color found in HPEnz—light reddish—was identified as being like that found in the units containing inoculum (I), showing that these bacteria originated from the sludge that was used to inoculate the anaerobic digesters, and, given that all units contained the same inoculum, they all had the potential to produce the reddish pigmentation in their medium. The expected low biogas production observed in the inoculum units can be explained by the scarcity of adequate amounts of carbon source, which does not happen in the case of the HPEnz. The biogas-deficient performance of HPEnz cannot be related to the amount of organic matter available in these units, which started the digestion process with one of the highest concentrations (29 g/L COD, Table 2). On the other hand, considering the constant maintenance of the color of the medium, from its appearance (≈day 25) until the end of the run, the decrease in the volumes and quality of the biogas produced, in

the final experimental section of the HPEnz units, cannot be related to any harmful effect resulting from the presence of the reddish pigmentation. HPEnz behavior is more linked to the insufficient enzymatic action of lysozyme on the cell wall, which did not make the cell content available for use by the AD bacterial consortium.

The HPA and HPU units present similarities regarding the tone (middle reddish) and maintenance of the medium throughout the assay. However, at the same time of color detection (day 25–28), HPA starts a phase of slight increases in biogas production, while in comparison HPU revealed a period of a very noticeable volume increments (Figure 1a). These different unit behaviors are distinguishable by the amount of organic matter they should have available at the time of color emergence. It is plausible to consider that after about a month, HPA had already converted a large part of the initial organic load while HPU, with a production much lower than that of the HPA, uses the load kept in "reserve" and converts it into sharp increases in the volume of biogas/methane (Figure 1). The presence of the reddish pigmentation was not considered a negative effect as it did not prevent methane production and may even be associated with a positive action on the organic material conversation process. In the present situation of HPA and HPU, there is effectively a temporal coincidence between the observation of the increase in gas production and the detection of the color in the means of these units. However, it should be noted that there is a trade-off between high red pigment production and low biogas yield (H vs. HPA and HPU). These types of microorganisms that produce red pigmentation were studied by Hammam et al. [17] to enhance the overall performance of the indigenous methanogenic culture in the anaerobic digestion process. The strong reddish color was only observed in H units, where a period mediated by the color appearance and the increase in biogas production (from around day 25 to 35) seems to be necessary for the population to adapt and use the remaining organic load, which can be understood as a positive effect of reddish bacteria.

3.4. Molecular Characterization of Microbial Communities

A molecular characterization was conducted for inoculum and samples collected at the beginning (IN) and at the end (OUT) from H and HPU units to observe changes in the microbial populations during the experiment.

After NGS, the samples created between 600,188 and 837,438 raw sequence reads, corresponding to sample H (OUT) and to the inoculum (I), respectively. Table 5 shows a total of 1,074,985 sequences (1,068,111 bacterial, 6850 archaeal, and 24 unassigned) which were recovered and analyzed. A total of 3224 operational taxonomic units (OTUs) that comprised libraries were detected in each sample.

Table 5. Sequencing data results and microbiota diversity index.

Units	Number of Sequences	OTUs	Shannon–Wiener Index	
			Bacteria	Archaea
I	258,364	1248	8.52	2.50
H (IN)	236,493	65	1.64	0.00
H (OUT)	199,089	558	4.03	4.44
HPU (IN)	192,070	727	3.89	2.68
HPU (OUT)	188,969	626	4.44	3.81
Total	1,074,985	3224	-	-

OTUs—Operational Taxonomic Units.

The sludge used for inoculation had the highest Shannon–Wiener diversity index value (8.52) for bacteria, and a suitable archaeal diversity index value (2.50), suggesting that it is a reliable source of diverse bacterial and archaea populations for the AD process (Table 5).

In both experiments, H and HPU, samples OUT exhibited higher diversity indices than samples IN, which is confirmed by the Shannon–Wiener index. The higher increase

observed either in the OTUs or in the diversity index values for H units, during the anaerobic digestion period, indicates that there was a lower microbial acclimation to digest the heterotrophic algae than in the HPU units, which is consistent with the higher methane yields obtained in these samples (Table 3). Moreover, there was not any type of inhibition of the microbial growth in these units, and this is consistent with the reactor's satisfactory performance.

During the analysis of microbial diversity, it is appropriate to guarantee that the number of readings reached a satisfactory value, so that more sequencing does not significantly increase species diversity, since the rarefaction curves are an estimate of species richness. Figure S4 indicates that, at similarity levels of 97%, the rarefaction curve grows rapidly at the beginning, when the most common OTUs were found, and then stabilizes, becoming asymptotic, since only the rarest species remain to be sequenced. Sequencing was sufficient for all samples, and the sequence dataset had complete sample diversity in this analysis, and sufficient sequence depth was achieved.

The relative abundance of groups of bacteria and archaea was determined in terms of the percentage of the total number of sequences in each sample. Table 6 shows that, as in other anaerobic digestion processes [33–35], the bacteria domain predominates over the archaea domain in all studied units, accounting for more than 98.3% of the relative abundance. The relative abundance of the archaeal domain increased during AD and was especially pronounced in the digestion of ultrasound pretreated heterotrophic algae (HPU), which confirmed the highest methane yield obtained for this substrate.

Table 6. Relative abundance of bacteria and archaea domains.

Units	Relative Abundance (%)	
	Bacteria	Archaea
I	98.31	1.69
H (IN)	100.0	0.00
H (OUT)	99.70	0.30
HPU (IN)	99.90	0.09
HPU (OUT)	99.13	0.87

The compositions of bacterial communities with a relative abundance greater than 2%, in at least one sample, show 11 major phyla (Figure 2). A majority of about 82% were assigned to Bacteroidetes, Firmicutes, Proteobacteria, Spirochaetae, and Synergistetes, which are known to efficiently convert complex compounds into methane-containing biogas [36]. Proteobacteria, Firmicutes, and Bacteroidetes are known to be abundant in anaerobic digestion processes [34,37].

The inoculum shows the predominance of *Bacteroidales* (11%, Figure 3a), *Anaerolineales* (12%, Figure 3b), and *Clostridiales* (4%, Figure 3c), and the presence of members of all classes of the phylum Proteobacteria (Figure 3d), with dominance attributed to α-Proteobacteria (9%), β-Proteobacteria (11%), and δ-Proteobacteria (9%). The predominant populations in H (IN) units were *Pseudomonales* (77%, Figure 3d). After AD, a change was observed in the microbiota composition. Although the predominance of Proteobacteria remained, this phylum decreased to 59% (Figure 2) and became mainly constituted by *Rhizobiales* (39%, Figure 3d). Simultaneously, *Bacteroidales* (11%, Figure 3a) increased together with *Clostridiales* (10%, Figure 3c) in the bacterial populations, which is in line with what has been detected by other authors as the main bacterial populations present in anaerobic digesters [38]. The role of most members of these microbial populations is crucial in acid and acetogenesis since they can convert amino acids, sugars, and alcohols into volatile fatty acids.

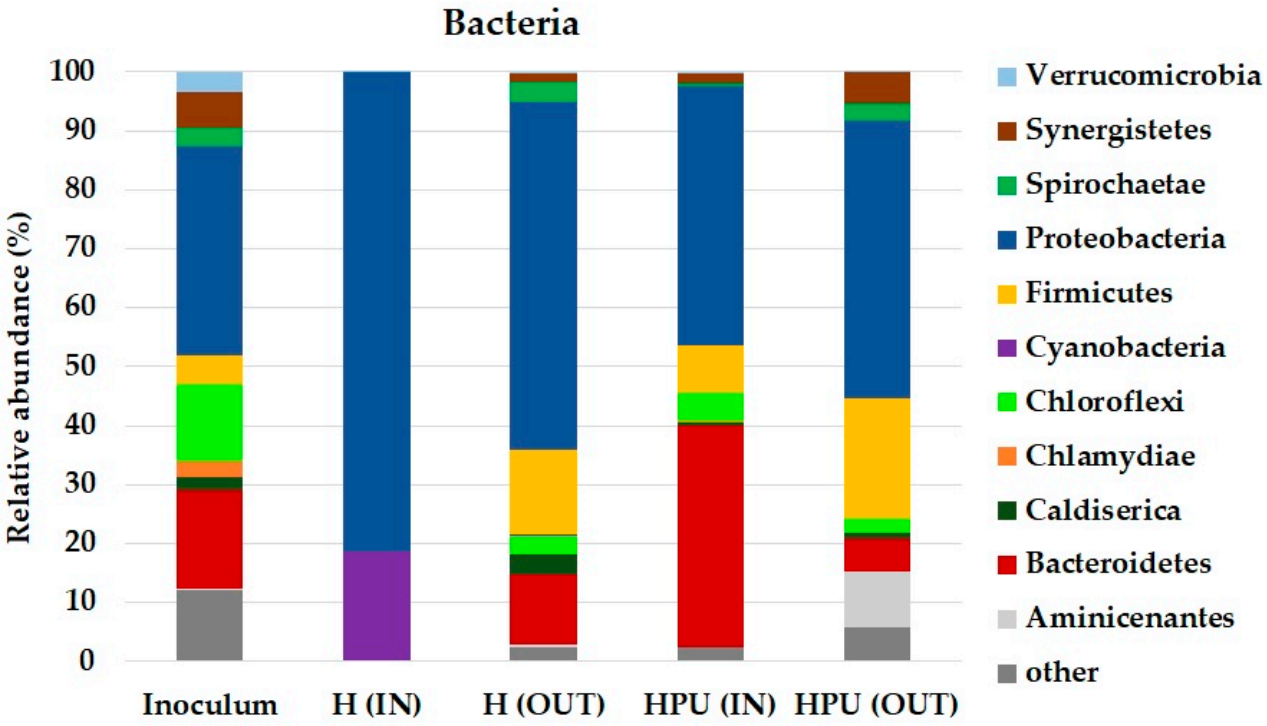
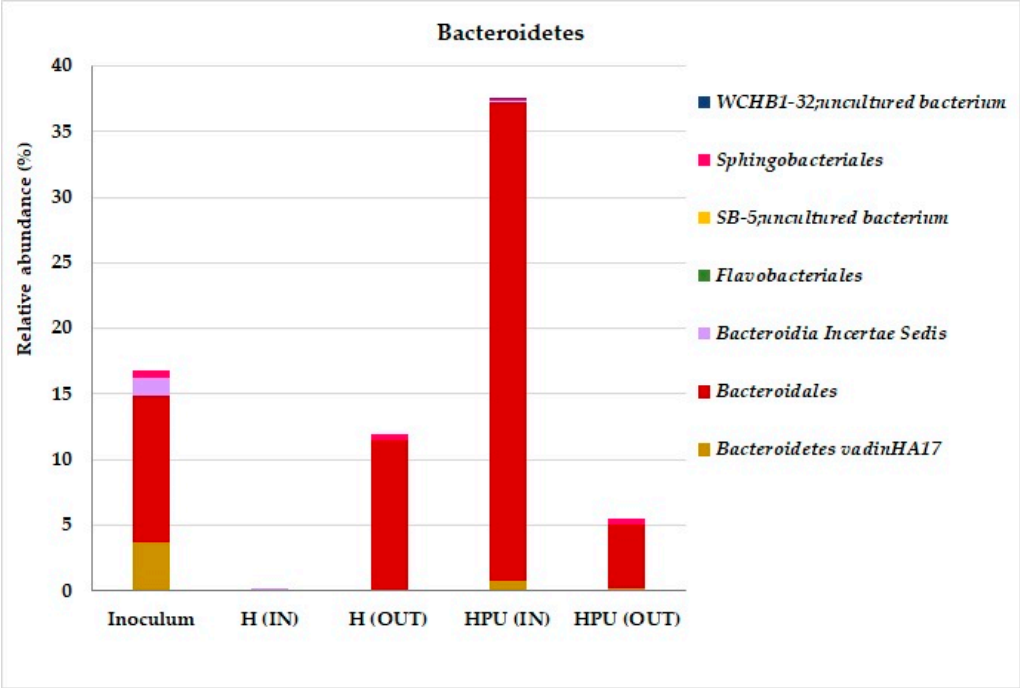


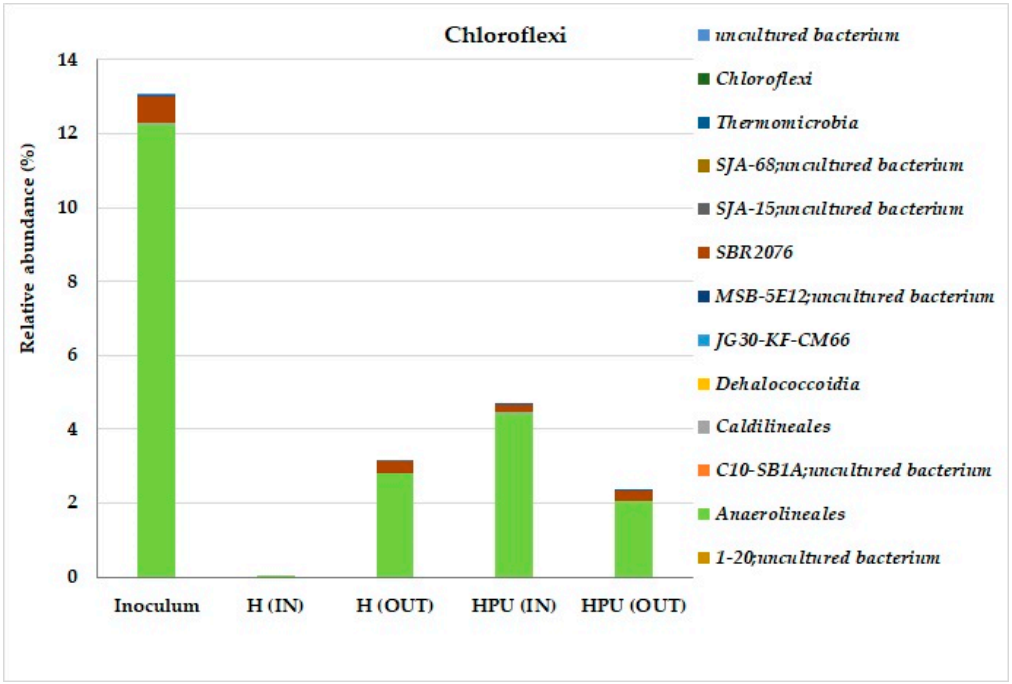
Figure 2. Relative abundance of bacterial communities and taxonomy at phylum level. "Other" are phyla that were not considered due to their low presence (0.01–2%) in the composition: Acidobacteria; Actinobacteria; Armatimonadetes; Atribacteria; BRC1; Candidatus Berkelbacteria; Chlorobi; Cloacimonetes; Deferribacteres; Deinococcus-Thermus; Elusimicrobia; Fusobacter; Gemmatimonadetes; Gracilibacteria; Hydrogenedentes; Latescibacteria; Lentisphaerae; Microgenomates; Nitrospirae; Omnitrophica; Parcubacteria; Planctomycetes; SR1 (Absconditabacteria); Saccharibacteria; TA06; TM6 (Dependentiae); Tenericutes; Thermotogae; WS6; WWE3; and unidentified bacteria.

A quite different behavior was seen in the HPU units, after AD, in which Proteobacteria showed a slight increase from 44% to 47%, and since these microorganisms are described as secondary degraders of polysaccharides [39], it suggests the availability of more complex substrates in this unit. *Bacteroidales* decreased from 37% to 4.9% (Figure 3a), and *Clostridiales* increased from 7% to 9% (Figure 3c), whose order consisted mainly of members of the family *Ruminococcaceae*. These populations can hydrolyze compounds such as cellulose, starch, and proteins into acetate, improving the methane yield [40,41]. Moreover, it is important to highlight that *Bacillales*, which became part of the composition of this microbiota (7.5%, Figure 3c), are known as good carbohydrate-utilizing species [40]. The other populations, more specifically Bacteroidetes, known as proteolytic bacteria, became almost non-existent, suggesting that members of these two phyla, Bacteroidetes and Firmicutes, might compete for the same resources and energy.

Methylotrophic, hydrogenotrophic, and acetoclastic are the three major pathways of methanogenesis [42]. In an anaerobic digester, the methane production depends on the methanogens and the substrates available for the process. Figure 4 shows the changes that occurred in the archaeal structure and composition during AD in the H and HPU units.

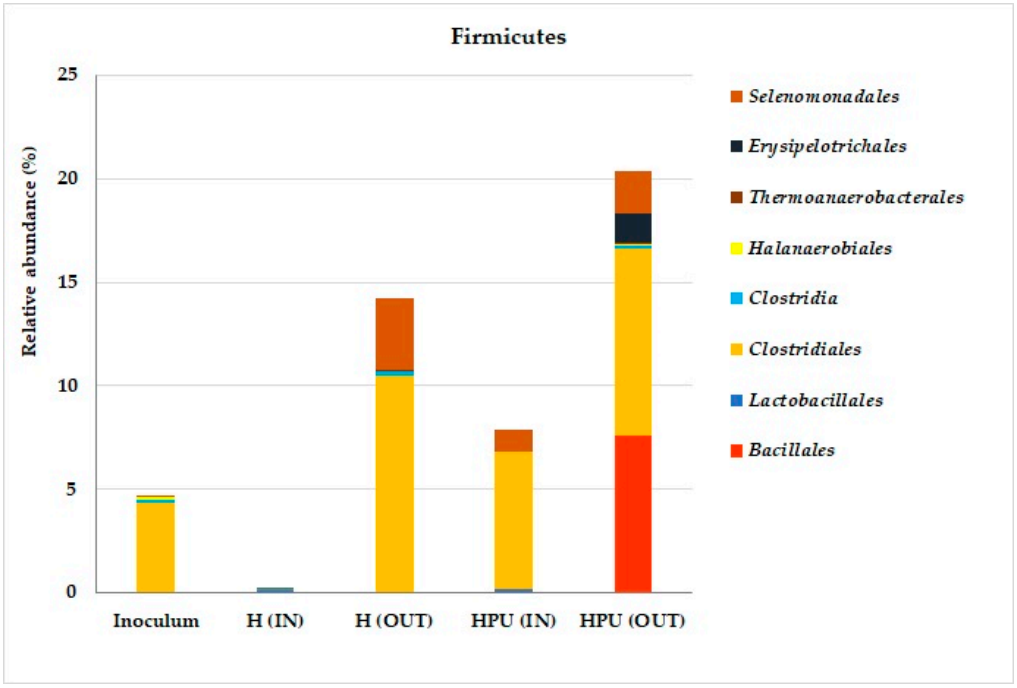


(**a**)

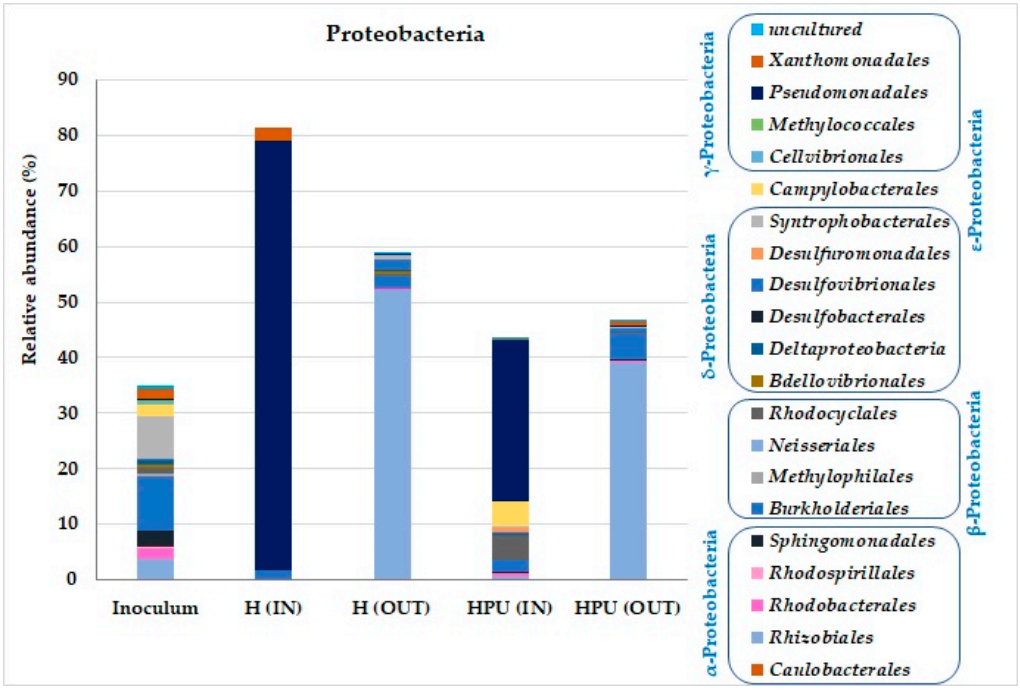


(**b**)

Figure 3. *Cont.*



(**c**)



(**d**)

Figure 3. Relative abundance of bacterial communities at the four main order levels: (**a**) Bacteroidetes; (**b**) Chloroflexi; (**c**) Firmicutes; and (**d**) α-Proteobacteria, β-Proteobacteria, δ-Proteobacteria, ε-Proteobacteria, and γ-Proteobacteria. The relative abundance is a percentage of the total number of sequences in each unit.

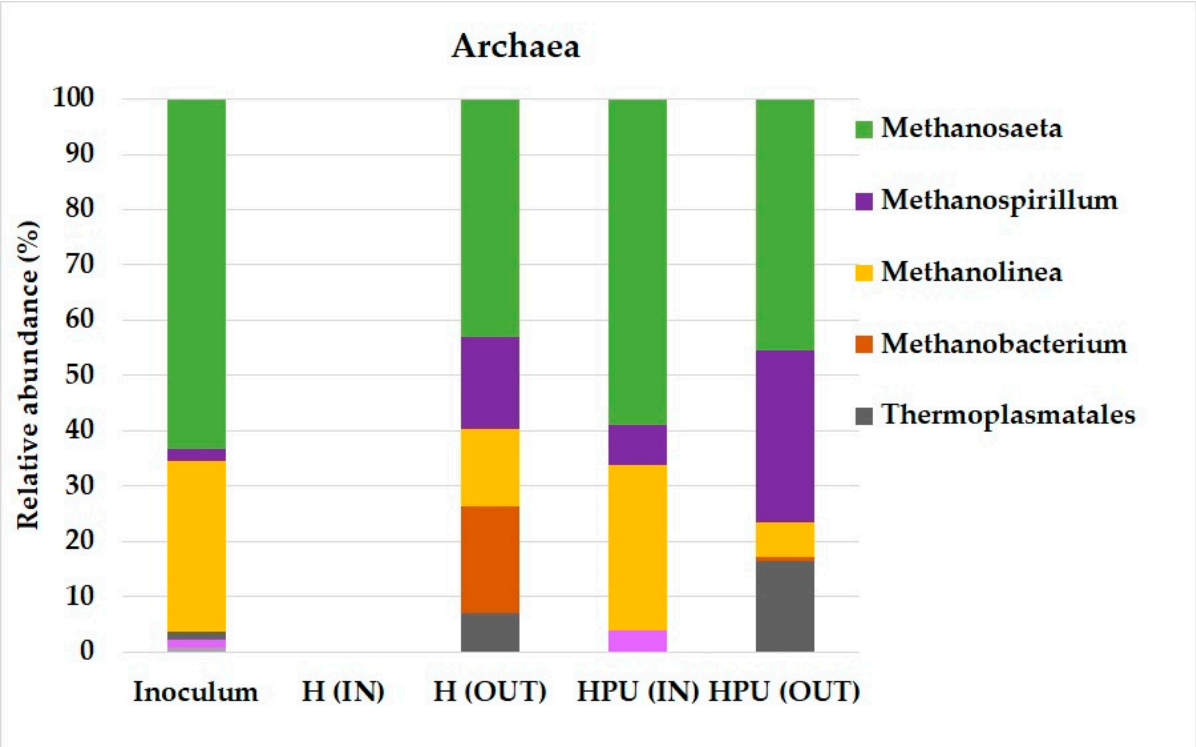


Figure 4. Relative abundance of archaeal communities and taxonomic classification at genus level.

All archaeal sequences were assigned to the phylum Euryarchaeota. Like the bacteria domain, the archaea domain also shows lower diversity (0.0 and 2.68, Table 5) in the samples for H (IN) and HPU (IN) units, respectively. The inoculum shows a predominance of *Methanosaeta*, with a relative abundance of 63% of the total archaeal sequences, and *Methanolinea* (31%). Despite no archaeal sequences being detected at the beginning of the AD in H units, *Methanosaeta*, an obligate acetoclastic methanogen, became the comparatively dominant genus at the end of the anaerobic digestion (43% of archaeal sequences, Figure 4), and kept the dominance in HPU units (45%), meaning that the methane produced was formed through acetoclastic methanogenesis as the main pathway [43–45]. However, the relative abundance of the hydrogenotrophic methanogens—that preferentially use H_2/CO_2 instead of organic acids as substrate, in both H (17% *Methanospirillum* and 19% *Methanobacterium*) and HPU units (31% *Methanospirillum*)—shows that a good balance has been set up in the population, capable of avoiding acidification of the medium and efficient in using all available substrates to convert them into methane. The acclimation behavior in HPU units shown by the microbial diversity index of methanogens (Table 5) explains the higher cumulative methane yield (263 L CH_4/kg VSin) obtained in this assay compared to H units (180 L CH_4/kg VSin).

3.5. Identification of Rhodobacter and Pigments in Anaerobic Digestion of Heterotrophic Algae

A sample was collected at the end of AD to identify the reddish-pigmented cells developed in H and HPU units. The pigmented cells and typical reddish-like clusters were seen under an optical microscope (Figure 5).

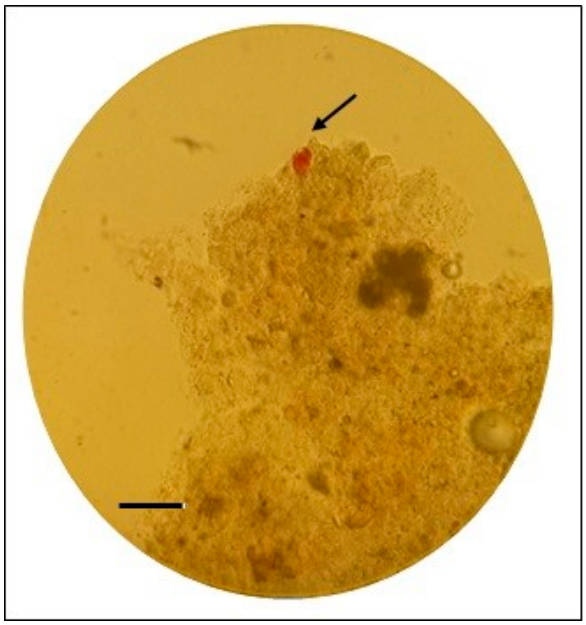


Figure 5. A liquid sample of culture medium observed under an optical microscope. Bar, 5 μm. The black arrow shows the reddish-like clusters that contain pigmentation.

The analysis of the photosynthetic pigments was performed by spectrometry, and the absorption spectrum is presented in Figure 6. As expected, the absorbance maxima of whole cells were found at 862, 806, 592, 528, and 490 nm, corresponding to the presence of characteristic pigments of PNSB, as bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series [46,47].

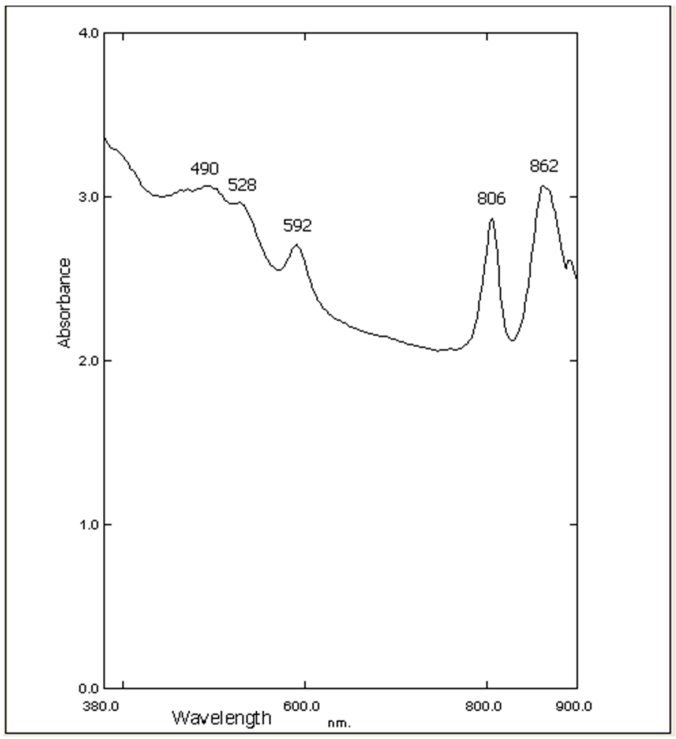


Figure 6. Absorption spectrum of the whole-cells sample. The wavelength (nm) of the absorption maxima is shown at the top of the peaks.

According to the literature, the color change resulted from an increase in phototrophic purple non-sulfur bacteria (PNSB) populations in anaerobic conditions [47,48]. PNSB are found among the *Rhodospirillales* order, including the genus *Rhodospirillum*, the *Rhizobiales* order, which includes *Rhodopseudomonas* and *Rhodomicrobium*, and the *Rhodobacteraceae* family including *Rhodobacter* [49].

Concerning the source of PNSB in our study, 16S rRNA gene sequences were found to be affiliated with *Rhodobacter* and *Rhodopseudomonas*, which were detected with a relative abundance of 0.48% and 0.26%, respectively, of the total bacterial sequences in the inoculum (Figure 7).

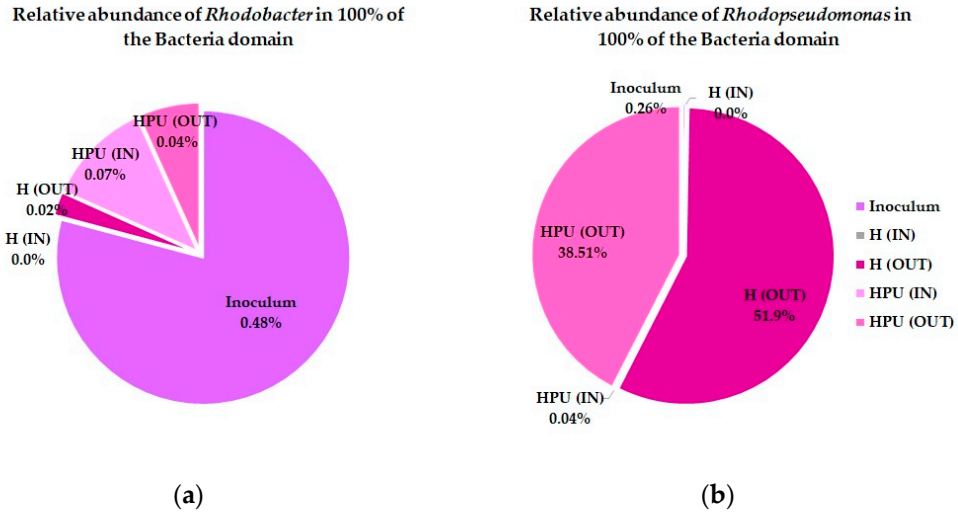


Figure 7. Relative abundance (%) in total bacteria sequences, in each sample collected from inoculum, H, and HPU units, for the taxonomic classification of bacterial reads at (**a**) *Rhodobacter* and (**b**) *Rhodopseudomonas* genus level.

At the end of AD, *Rhodobacter* populations were not detected with extremely low relative abundance values in H (OUT) with 0.02% and in HPU (OUT) with 0.04% (Figure 7a) of total bacterial sequences. Thus, as previously mentioned, the changes observed in the samples collected after AD, from both H (OUT) and HPU (OUT), which revealed a dominance in α-Proteobacteria (Figure 3d), may be related to the high relative abundance values of *Rhodopseudomonas* populations (38.5% and 52%, respectively, Figure 7b), which are responsible for the production of the reddish pigmentation in the digestates. In addition, the prevalence of these microorganisms, which produce nitrogenase, the key enzyme for hydrogen generation, under nitrogen-limited conditions, and in a photoheterotrophic mode using organic carbon as a source of electrons [50], is consistent with the simultaneous development of the hydrogenotrophic methanogens *Methanospirillum* and *Methanobacterium* in both H and HPU units during the AD process.

The fact that the process is carried out in transparent glass vials, in daylight, and the presence of PNSB in the inoculum may explain the favorable conditions for the growth of *Rhodobacter* and *Rhodopseudomonas* populations and the production of reddish pigments in AD. According to Lu et al. [49], the primary driving force for PNSB to obtain energy is the light, since they have light-dependent metabolism units as the pigments. In addition, the formation of a pigment system needs a reductive environment to prevent damage, so an anaerobic digester level provides good operating conditions [51]. In our study, these conditions were conducive to the development of PNSB, and it was observed that the biogas/methane yields were not much affected by this phenomenon. The highest value of 290 L CH_4/kg VSin obtained with HExt showed only a small decrease to 263 L CH_4/kg VSin in HPU units (Table 3), which means that it is possible to produce valuable pigments and simultaneously carry out the AD to produce high methane yields.

4. Conclusions

Autotrophic algae (AA) looks to be more suitable for the AD process than heterotrophic algae (H), as AA were more easily digested and converted to methane: 53% COD removal and 104 mL methane from AA; and 23% COD removal and 73 mL methane from H. Ultrasound and autoclave pretreatments and lipid extraction processes applied to the heterotrophic algae had a positive effect on biogas/methane production obtained in AD with these substrate processes by reaching greater volumes than those recorded in H (107, 115, and 117 mL, respectively), and also higher methane yields (263 to 290 L CH_4/kg VSin). The poorest behavior was recorded in HPEnz where the final gas volumes approached those of H.

The visible reddish pigmentation in the digestate of *Chlorella* materials after AD, preserved under light and anoxygenic conditions, was attributed to bacteriochlorophyll *a* and to carotenoid pigments of the spirilloxanthin series, characteristic of the purple non-sulfur bacteria (PNSB). Microbial identification showed that populations of Proteobacteria found in the inoculum remained predominant after anaerobic digestion in H and HPU units. The main changes observed after AD were the high increase in the relative abundance of *Clostridiales* and *Bacillales* responsible for the hydrolysis of complex compounds in acetate. *Methanosaeta*, predominant in the inoculum, became dominant in both H and HPU units until the end of AD, meaning that methane was produced through acetoclastic methanogenesis. The microbial characterization also showed the presence of PNSB—*Rhodobacter* and *Rhodopseudomonas*—in the inoculum, yet at the end of the AD process, *Rhodopseudomonas* was predominant in both samples, H (OUT) and HPU (OUT), with a high relative abundance of 38.5% and 52%, respectively. The obtained results show that PNSB did not prevent methane production, so it was possible to simultaneously produce biogas and photosynthetic pigments using the same AD unit applied for the energetic valorization of waste/materials supplied by *Chlorella protothecoides*.

The results from this first study can be regarded as a promising indicator for the improvement of a more sustainable AD process established on the biorefinery concept. Further research should focus on the enhancement of biogas/methane yields using the application of autoclave and/or ultrasound pretreatments, either on the autotrophically grown *Chlorella protothecoides* (AA) or on the extracted heterotrophically grown microalga (HExt). More research is needed to better understand the effect of PNSB on the digestion of microalgae materials under anoxygenic conditions and to determine the conditions for their development as well as the advantages and drawbacks of the competition for the same substrates.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13053325/s1, Figure S1: The effect of pretreatments on *Chlorella protothecoides* cells seen under an optical microscope. Bar, 5 μm; (a) H—heterotrophic algae; (b) HExt—extracted heterotrophic algae; pretreated heterotrophic algae: (c) HEnz—enzymatic; (d) HPA—autoclave; (e) HPU—ultrasound; (f) AA—autotrophic algae. Figure S2: UV spectrum of untreated algae and pretreated algae. Figure S3: Pictures at the end of the anaerobic digestion to see differences in coloration in units. H—heterotrophic algae (4–6); HExt—extracted heterotrophic algae (7–9); pretreated heterotrophic algae: (HPEnz—enzymatic (10–12); HPA—autoclave (13–15); HPU—ultrasound (16–18)); AA—autotrophic algae (19–21); I—inoculum (1–3). Figure S4: Alpha rarefaction curves for the occurrence with which OTUs were detected at each sample from inoculum (I) and samples from anaerobic digestion: H—heterotrophic algae; HPU—pretreated heterotrophic algae. OTU—operational taxonomic unit. The sample names can be looked up in the NCBI bioproject with accession code PRJNA927270.

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References

1. Paul, R.; Silkina, A.; Melville, L.; Suhartini, S.; Sulu, M. Optimisation of Ultrasound Pretreatment of Microalgal Biomass for Effective Biogas Production through Anaerobic Digestion Process. *Energies* **2023**, *16*, 553. [CrossRef]
2. O'Connor, S.; Ehimen, E.; Pillai, S.C.; Power, N.; Lyons, G.A.; Bartlett, J. An investigation of the potential adoption of anaerobic digestion for energy production in irish farms. *Environment* **2021**, *8*, 8. [CrossRef]
3. Náthia-Neves, G.; Berni, M.; Dragone, G.; Mussatto, S.I.; Forster-Carneiro, T. Anaerobic digestion process: Technological aspects and recent developments. *Int. J. Environ. Sci. Technol.* **2018**, *15*, 2033–2046. [CrossRef]
4. Santos, C.A.; Nobre, B.; da Silva, T.L.; Pinheiro, H.M.; Reis, A. Dual-mode cultivation of *Chlorella protothecoides* applying inter-reactors gas transfer improves microalgae biodiesel production. *J. Biotechnol.* **2014**, *184*, 74–83. [CrossRef]
5. Santos, C.A.; Ferreira, M.E.; Lopes da Silva, T.; Gouveia, L.; Novais, J.M.; Reis, A. A symbiotic gas exchange between bioreactors enhances microalgal biomass and lipid productivities: Taking advantage of complementary nutritional modes. *J. Ind. Microbiol. Biotechnol.* **2011**, *38*, 909–917. [CrossRef]
6. Kim, J.Y.; Jung, J.M.; Jung, S.; Jung, S.; Park, Y.K.; Tsang, Y.F.; Lin, K.Y.A.; Choi, Y.-E.; Kwon, E.E. Biodiesel from microalgae: Recent progress and key challenges. *Prog. Energy Combust. Sci.* **2022**, *93*, 101020. [CrossRef]
7. Oliveira, M.C.; Bassin, I.D.; Cammarota, M.C. Microalgae and Cyanobacteria Biomass Pretreatment Methods: A Comparative Analysis of Chemical and Thermochemical Pretreatment Methods Aimed at Methane Production. *Fermentation* **2022**, *8*, 497. [CrossRef]
8. Carvalho, J.C.; Magalhães, A.I.; Melo Pereira, G.V.; Medeiros, A.B.P.; Sydney, E.B.; Rodrigues, C.; Aulestia, D.T.M.; Vandenberghe, L.P.d.S.; Soccol, V.T.; Soccol, C.R. Microalgal biomass pretreatment for integrated processing into biofuels, food, and feed. *Bioresour. Technol.* **2020**, *300*, 122719. [CrossRef] [PubMed]
9. Sialve, B.; Bernet, N.; Bernard, O. Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. *Biotechnol. Adv.* **2009**, *27*, 409–416. [CrossRef]
10. Ciccoli, R.; Sperandei, M.; Petrazzuolo, F.; Broglia, M.; Chiarini, L.; Correnti, A.; Farneti, A.; Pignatelli, V.; Tabacchioni, S. Anaerobic digestion of the above ground biomass of Jerusalem Artichoke in a pilot plant: Impact of the preservation method on the biogas yield and microbial community. *Biomass Bioenergy* **2018**, *108*, 190–197. [CrossRef]
11. Eusébio, A.; Neves, A.; Marques, I.P. Complementary substrates-brewery wastewater and piggery effluent—Assessment and microbial community profiling in a hybrid anaerobic reactor. *Appl. Sci.* **2021**, *11*, 4364. [CrossRef]
12. Zhang, L.; Loh, K.C.; Lim, J.W.; Zhang, J. Bioinformatics analysis of metagenomics data of biogas-producing microbial communities in anaerobic digesters: A review. *Renew. Sustain. Energy Rev.* **2019**, *100*, 110–126. [CrossRef]
13. Soares, L.A.; Rabelo, C.A.B.S.; Sakamoto, I.K.; Delforno, T.P.; Silva, E.L.; Varesche, M.B.A. Metagenomic analysis and optimization of hydrogen production from sugarcane bagasse. *Biomass Bioenergy* **2018**, *117*, 78–85. [CrossRef]
14. Oulas, A.; Pavloudi, C.; Polymenakou, P.; Pavlopoulos, G.A.; Papanikolaou, N.; Kotoulas, G.; Arvanitidis, C.; Iliopoulos, L. Metagenomics: Tools and insights for analyzing next-generation sequencing data derived from biodiversity studies. *Bioinform. Biol. Insights* **2015**, *9*, 75–88. [CrossRef]
15. Goginyan, V.; Eusébio, A.; Neves, A.; Harutyunyan, B.; Hovhannisyan, R.; Andreasyan, N.; Marques, I.P. Purple non-sulphur photosynthetic bacteria in brewery wastewater during anaerobic digestion. In Proceedings of the 5th International Conference WASTES: Solutions, Treatments and Opportunities, Costa da Caparica, Portugal, 4–6 September 2019; pp. 87–89.
16. Madigan, M.T.; Jung, D.O. An Overview of Purple Bacteria: Systematics, Physiology, and Habitats. In *The Purple Phototrophic Bacteria*; Hunter, C.N., Dalda, F., Thurnauer, M.C., Beatty, J.T., Eds.; Springer: Urbana, IL, USA, 2019; Volume 28, pp. 1–15.
17. Hammam, M.S.; Abdalla, K.Z. Reinforcement of methanogenesis in anaerobic digesters through the application of a purple non-sulfur bacteria bio-augmentation scheme. *Int. J. Environ. Sci. Technol.* **2019**, *16*, 8161–8174. [CrossRef]
18. George, D.M.; Vincent, A.S.; Mackey, H.R. An overview of anoxygenic phototrophic bacteria and their applications in environmental biotechnology for sustainable Resource recovery. *Biotechnol. Rep.* **2020**, *28*, e00563. [CrossRef]

19. Li, M.; Xia, Q.; Lv, S.; Tong, J.; Wang, Z.; Nie, Q.; Yang, J. Enhanced CO_2 capture for photosynthetic lycopene production in engineered *Rhodopseudomonas palustris*, a purple nonsulfur bacterium. *Green Chem.* **2022**, *24*, 7500–7518. [CrossRef]
20. Soon, T.K.; Al-Azad, S.; Ransangan, J. Isolation and characterization of purple non-sulfur bacteria, Afifella marina, producing large amount of carotenoids from mangrove microhabitats. *J. Microbiol. Biotechnol.* **2014**, *24*, 1034–1043. [CrossRef]
21. Sakarika, M.; Spanoghe, J.; Sui, Y.; Wambacq, E.; Grunert, O.; Haesaert, G.; Spiller, M.; Vlaeminck, S.E. Purple non-sulphur bacteria and plant production: Benefits for fertilization, stress resistance and the environment. *Microb. Biotechnol.* **2020**, *13*, 1336–1365. [CrossRef]
22. Al-Zuhair, S.; Ashraf, S.; Hisaindee, S.; Darmaki, N.A.; Battah, S.; Svistunenko, D.; Reeder, B.; Stanway, G.; Chaudhary, A. Enzymatic pre-treatment of microalgae cells for enhanced extraction of proteins. *Eng. Life Sci.* **2017**, *17*, 175–185. [CrossRef]
23. American Public Health Association. *Standard Methods for the Examination of Water and Wastewater*, 20th ed.; APHA-American Wastewater Association: Washington, DC, USA, 2000.
24. Illumina. Illumina 16S Metagenomics Sequencing Protocol. 2017. Available online: https://emea.support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html. (accessed on 1 December 2019).
25. Lee, S.Y.; Cho, J.M.; Chang, Y.K.; Oh, Y.K. Cell disruption and lipid extraction for microalgal biorefineries: A review. *Bioresour. Technol.* **2017**, *244*, 1317–1328. [CrossRef] [PubMed]
26. Atelge, M.R.; Atabani, A.E.; Banu, J.R.; Krisa, D.; Kaya, M.; Eskicioglu, C.; Kumar, G.; Lee, C.; Yildiz, Y.Ş.; Unalan, S.; et al. A critical review of pretreatment technologies to enhance anaerobic digestion and energy recovery. *Fuel* **2020**, *270*, 117494. [CrossRef]
27. Cho, S.; Park, S.; Seon, J.; Yu, J.; Lee, T. Evaluation of thermal, ultrasonic and alkali pretreatments on mixed-microalgal biomass to enhance anaerobic methane production. *Bioresour. Technol.* **2013**, *143*, 330–336. [CrossRef] [PubMed]
28. Ayala-Parra, P.; Liu, Y.; Sierra-Alvarez, R.; Field, J.A. Pretreatments to enhance the anaerobic biodegradability of Chlorella protothecoides algal biomass. *Environ. Prog. Sustain. Energy* **2018**, *37*, 418–424. [CrossRef]
29. Passos, F.; Hom-Diaz, A.; Blanquez, P.; Vicent, T.; Ferrer, I. Improving biogas production from microalgae by enzymatic pretreatment. *Bioresour. Technol.* **2016**, *199*, 347–351. [CrossRef]
30. Bohutskyi, P.; Ketter, B.; Chow, S.; Adams, K.J.; Betenbaugh, M.J.; Allnutt, F.T.; Bouwer, E.J. Anaerobic digestion of lipid-extracted Auxenochlorella protothecoides biomass for methane generation and nutrient recovery. *Bioresour. Technol.* **2015**, *183*, 229–239. [CrossRef] [PubMed]
31. Günerken, E.; D'Hondt, E.; Eppink, M.H.M.; Garcia-Gonzalez, L.; Elst, K.; Wijffels, R.H. Cell disruption for microalgae biorefineries. *Biotechnol. Adv.* **2015**, *33*, 243–260. [CrossRef] [PubMed]
32. Schwede, S.; Kowalczyk, A.; Gerber, M.; Span, R. Influence of Different Cell Disruption Techniques on Mono Digestion of Algal Biomass. In Proceedings of the World Renewable Energy Congress, Linköping, Sweden, 8–13 May 2011; Volume 57, pp. 41–47. [CrossRef]
33. Krause, L.; Diaz, N.N.; Edwards, R.A.; Gartemann, K.H.; Krömeke, H.; Neuweger, H.; Pühler, A.; Runte, K.J.; Schlüter, A.; Stoye, J.; et al. Taxonomic composition and gene content of a methane-producing microbial community isolated from a biogas reactor. *J. Biotechnol.* **2008**, *136*, 91–101. [CrossRef]
34. Nelson, M.C.; Morrison, M.; Yu, Z. A meta-analysis of the microbial diversity observed in anaerobic digesters. *Bioresour. Technol.* **2011**, *102*, 3730–3739. [CrossRef]
35. Schlüter, A.; Bekel, T.; Diaz, N.N.; Dondrup, M.; Eichenlaub, R.; Gartemann, K.H.; Krahn, I.; Krause, L.; Krömeke, H.; Kruse, O.; et al. The metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter analysed by the 454-pyrosequencing technology. *J. Biotechnol.* **2008**, *136*, 77–90. [CrossRef]
36. Theuerl, S.; Klang, J.; Heiermann, M.; De Vrieze, J. Marker microbiome clusters are determined by operational parameters and specific key taxa combinations in anaerobic digestion. *Bioresour. Technol.* **2018**, *263*, 128–135. [CrossRef]
37. Gannoun, H.; Omri, I.; Chouari, R.; Khelifi, E.; Godon, J.J.; Hamdi, M.; Sghir, A.; Bouallagui, H. Microbial community structure associated with the high loading anaerobic codigestion of olive mill and abattoir wastewaters. *Bioresour. Technol.* **2016**, *201*, 337–346. [CrossRef]
38. Buhlmann, C.H.; Mickan, B.S.; Jenkins, S.N.; Tait, S.; Kahandawala, T.K.A.; Bahri, P.A. Ammonia stress on a resilient mesophilic anaerobic inoculum: Methane production, microbial community, and putative metabolic pathways. *Bioresour. Technol.* **2019**, *27*, 70–77. [CrossRef]
39. Hülsemann, B.; Zhou, L.; Merkle, W.; Hassa, J.; Müller, J.; Oechsner, H. Biomethane potential test: Influence of inoculum and the digestion system. *Appl. Sci.* **2020**, *10*, 2589. [CrossRef]
40. Gao, R.; Cao, Y.; Yuan, X.; Zhu, W.; Wang, X.; Cui, Z. Microbial diversity in a full-scale anaerobic reactor treating high concentration organic cassava wastewater. *Afr. J. Biotechnol.* **2012**, *11*, 6494–6500. [CrossRef]
41. Guo, W.; Li, Y.; Zhao, K.; Xu, Q.; Jiang, H.; Zhou, H. Performance and Microbial Community Analysis of Anaerobic Digestion of Vinegar Residue with Adding of Acetylene Black or Hydrochar. *Waste Biomass Valorization* **2020**, *11*, 3315–3325. [CrossRef]
42. Goswami, R.; Chattopadhyay, P.; Shome, A.; Banerjee, S.N.; Chakraborty, A.K.; Mathew, A.K.; Chaudhury, S. An overview of physico-chemical mechanisms of biogas production by microbial communities: A step towards sustainable waste management. *3 Biotech* **2016**, *6*, 72. [CrossRef] [PubMed]
43. Cho, S.K.; Im, W.T.; Kim, D.H.; Kim, M.H.; Shin, H.S.; Oh, S.E. Dry anaerobic digestion of food waste under mesophilic conditions: Performance and methanogenic community analysis. *Bioresour. Technol.* **2013**, *131*, 210–217. [CrossRef]

44. Wang, M.; Zhang, X.; Zhou, J.; Yuan, Y.; Dai, Y.; Li, D.; Li, Z.; Liu, X.; Yan, Z. The dynamic changes and interactional networks of prokaryotic community between co-digestion and mono-digestions of corn stalk and pig manure. *Bioresour. Technol.* **2017**, *225*, 23–33. [CrossRef]
45. Wang, P.; Wang, H.; Qiu, Y.; Ren, L.; Jiang, B. Microbial characteristics in anaerobic digestion process of food waste for methane production–A review. *Bioresour. Technol.* **2018**, *248*, 29–36. [CrossRef]
46. Okubo, Y.; Futamata, H.; Hiraishi, A. Characterization of phototrophic purple nonsulfur bacteria forming colored microbial mats in a swine wastewater ditch. *Appl. Environ. Microbiol.* **2006**, *72*, 6225–6233. [CrossRef] [PubMed]
47. Soto-Feliciano, K.; Jesús, M.D.; Vega-Sepúlveda, J.; Rios-Velázquez, C. Isolation and characterization of purple non-sulfur anoxyphototropic bacteria from two microecosystems: Tropical hypersaline microbial mats and bromeliads phytotelmata. In *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*, 2nd ed.; Méndez-Vilas, A., Ed.; Formatex Research Center: Badajos, Spain, 2010; Volume 1, pp. 109–116. ISBN 9788461461950.
48. Zhang, D.; Yang, H.; Huang, Z.; Zhang, W.; Liu, S.-J. *Rhodopseudomonas faecalis* sp. nov., a phototrophic bacterium isolated from an anaerobic reactor that digests chicken faeces. *Int. J. Syst. Evol. Microbiol.* **2002**, *52*, 2055–2060. [CrossRef] [PubMed]
49. Lu, H.; Zhang, G.; He, S.; Zhao, R.; Zhu, D. Purple non-sulfur bacteria technology: A promising and potential approach for wastewater treatment and bioresources recovery. *World J. Microbiol. Biotechnol.* **2021**, *37*, 161. [CrossRef] [PubMed]
50. Craven, J.; Sultan, M.A.; Sarma, R.; Wilson, S.; Meeks, N.; Kim, D.Y.; Hastings, J.T.; Bhattacharyya, D. *Rhodopseudomonas palustris*-based conversion of organic acids to hydrogen using plasmonic nanoparticles and near-infrared light. *RSC Adv.* **2019**, *9*, 41218–41227. [CrossRef] [PubMed]
51. Lu, H.; Zhang, G.; Dong, S. Quantitative study of PNSB energy metabolism in degrading pollutants under weak light-micro oxygen condition. *Bioresour. Technol.* **2011**, *102*, 4968–4973. [CrossRef]