

Article

Steam Explosion and Vibrating Membrane Filtration to Improve the Processing Cost of Microalgae Cell Disruption and Fractionation

Esther Lorente ¹, Monika Hapońska ², Ester Clavero ¹ , Carles Torras ^{2,*}  and Joan Salvadó ²

¹ Catalonia Institute for Energy Research, IREC, Marcel·lí Domingo 2, 43007 Tarragona, Catalonia, Spain; elorenteroyo@gmail.com (E.L.); ester.clavero@gmail.com (E.C.)

² Chemical Engineering Department, Universitat Rovira i Virgili, Av. Països Catalans 26, 43007 Tarragona, Catalonia, Spain; monika.haponska@gmail.com (M.H.); joan.salvado@urv.cat (J.S.)

* Correspondence: carlestorrasfont@gmail.com; Tel.: +34-977-202-444

Received: 8 February 2018; Accepted: 20 March 2018; Published: 22 March 2018



Abstract: The aim of this study is to explore an innovative downstream route for microalgae processing to reduce cost production. Experiments have been carried out on cell disruption and fractionation stages to recover lipids, sugars, and proteins. Steam explosion and dynamic membrane filtration were used as unit operations. The species tested were *Nannochloropsis gaditana*, *Chlorella sorokiniana*, and *Dunaliella tertiolecta* with different cell wall characteristics. Acid-catalysed steam explosion permitted cell disruption, as well as the hydrolysis of carbohydrates and partial hydrolysis of proteins. This permitted a better access to non-polar solvents for lipid extraction. Dynamic filtration was used to moderate the impact of fouling. Filtration enabled two streams: A permeate containing water and monosaccharides and a low-volume retentate containing the lipids and proteins. The necessary volume of solvent to extract the lipids is thus much lower. An estimation of operational costs of both steam explosion and membrane filtration was performed. The results show that the steam explosion operation cost varies between 0.005 \$/kg and 0.014 \$/kg of microalgae dry sample, depending on the cost of fuel. Membrane filtration cost in fractionation was estimated at 0.12 \$/kg of microalgae dry sample.

Keywords: cell disruption; costs; dynamic membrane filtration; fractionation; microalgae; steam explosion

1. Introduction

Around 10 years ago, the idea of using microalgae as a very efficient photosynthetic crop to provide energy was re-adopted [1], following the results obtained in earlier studies [2]. Microalgae appeared as a good alternative to produce transportation fuels in the context of energy crisis and climate change.

Cost barriers in the several stages of mass production of energy vectors appeared. This resulted in having to re-address improvements in culture, harvesting, cell disruption, lipid extraction, and final production.

The production of biofuels from microalgae results in a variety of returns. These include a high lipid content, no competition for arable lands, and the use of a variety of water qualities, including wastewaters during the cultivation period [3]. However, it has become clear that the option to produce only fuel from microalgae is not economically viable [4].

Researchers have learned from the preliminary results that, apart from reducing the costs of microalgae production, benefits have to be obtained from all fractions while also looking for other side paybacks in order to have an economically feasible production [5].

To achieve a positive economic balance, several matters should be taken into account such as CO₂ capture [6,7], water quality improvement [8,9], procurement of commodities [10,11], and high-added value products [12,13].

The process unit operations needed in order to proceed to microalgae biorefining depend very much on the strain and products to be sought (i.e., commodity or high-added value products), but a typical sequence is: Culture in open ponds or photobioreactors [14,15], dewatering [16–21], cell disruption [22–27], and fractionation [13,20,28].

In the present study, the focus is on the operation of cell disruption by using steam explosion, and secondly, in the process of fractionation.

Although steam explosion has been in use from the beginning of the 20th century, it has only been used in a few cases for microalgae biorefining, and therefore a comparison of the results can hardly be performed [22,29]. As regards the results of previous work [26], steam explosion is used at relatively mild conditions to break the cell walls and produce the hydrolysis of carbohydrates.

Depending on the strain cell wall characteristics, cell disruption can be a cost-intensive operation and several procedures have been reported at the laboratory level, including the use of ultrasounds, microwave, or high pressure [25,30]. Steam explosion is proposed in this work as an innovative technique for this application and is easy to scale-up with pilot plant results because of the nature of the equipment and because it is widely used industrially [22,24]. Steam explosion has given the best results when compared with other methods for cell disruption such as ultrasonication, microwave, and autoclave [29]. Beyond breaking the cell wall, if a low concentration of acid is used, steam explosion can hydrolyse the poly-saccharides in the cell and produce sugars in a first stage of fractionation [29]. The main energy input for the steam explosion process is heat, thus reducing the cell disruption costs considering other techniques like sonication and that residual heat can be used. It should be stated that steam explosion is a commercial high-throughput available technology. A pilot plant with a capacity of 2 Tm/h has already been operated successfully with lignocellulosic materials from 1991 [31].

Membrane filtration and solvent extraction are methods to be used for fractionation [24,28]. In a first unit operation, membrane filtration can be used to obtain two streams: a retentate containing lipids and proteins and a permeate containing water with the hydrolyzed monosaccharides [24]. As in microalgae dewatering, fouling is a main drawback. To overcome this problem, dynamic filtration provides an adequate solution [32]. Also, the use of ultrafiltration membranes (instead of microfiltration) increases permeability [20]. In a second unit operation, sugars could be concentrated using nanofiltration membranes [33]. To recover non-polar lipids from the retentate stream of the first operation, a hexane extraction is used. In our previous work [24], the microalga *Nannochloropsis gaditana* was selected to investigate the fractionation strategy for lipids and carbohydrates recovery. In this study, we intend to validate the selected fractionation path when different common microalgae species were used: *Chlorella sorokiniana* [34], *Nannochloropsis gaditana* [35], and *Dunaliella tertiolecta* [36]. They are representative of different types of species of freshwater and marine strains. They have also been chosen because they represent different levels of strength in their cell walls. *N. gaditana* and *C. sorokiniana* are two species with recalcitrant cell walls, whereas *D. tertiolecta* lacks a cell wall. The cell wall of *N. gaditana* is primarily cellulose (75%) [37]. This inner cellulose layer is protected by an algaenan layer which is assumed to be primarily responsible for the wall's recalcitrance to breakage [37]. Besides, the *C. sorokiniana* cell wall contains little glucose [38] and therefore its cell wall may lack cellulose. On the other hand, the presence of algaenan in the *C. sorokiniana* cell wall may depend on the physiological state of the culture [39].

The study about the use of steam explosion will provide a basis of cost comparison with those technologies that use electrical power to operate.

2. Materials and Methods

2.1. Microalgae Samples

A semi-closed photobioreactor, with a 3050 L capacity and placed outdoors, was used for growing *Nannochloropsis gaditana* Lubián (strain CCMP1775, Provasoli—Guillard National Center for Marine Algae and Microbiota). A more detailed description of the photobioreactor is given in Nurra et al. [26]. Cultures were performed between May and July, when the mean temperature ranged from 27 °C to 33 °C. The medium for the *N. gaditana* culture consisted of seawater enriched with 0.3 mL/L of Codafof 14.6.5 (Coda Sustainable Agro Solution S.A.). This plant fertilizer contains, in *w/w*, 14% nitrogen, 6% P₂O₅, 5% K₂O, 0.1% Fe, 0.05% Zn, 0.05% Mn, 0.05% Cu, and 0.001% Mo.

Chlorella sorokiniana (strain CCAP 211/8k) and *Dunaliella tertiolecta* (strain CCAP19/6B) were grown indoors in column photobioreactors (300 L, 50 cm diam.) aerated with air and illuminated with Philips MASTER TLD 58 W/865 fluorescents giving an irradiance at the photobioreactor surface of 300 µmol photon/m²/s. *C. sorokiniana* was cultured at 22 ± 3 °C in tap water enriched with the following nutrients: NaNO₃ (5.8 mM), K₂HPO₄·3H₂O (0.092 mM), KH₂PO₄ (0.28 mM), Na₂EDTA (0.045 mM), FeCl₃·6H₂O (17.9 µM), ZnSO₄·7H₂O (1.69 µM), MnCl₂·4H₂O (4.48 µM), Na₂MoO₄·2H₂O (0.10 µM), CuSO₄·5H₂O (0.17 µM), and CoCl₂·6H₂O (0.06 µM). *D. tertiolecta* was cultured at 20 ± 3 °C in artificial seawater prepared with tap water and 37 g·L⁻¹ of Aquaforest Reef Salt[®] enriched with NaNO₃ (4.4 mM), Na₂HPO₄·2H₂O (0.04 mM), and the same micronutrient concentrations as in *C. sorokiniana*. Phosphate was fed-batch to increase the concentration of the culture to 3.2 µM to avoid precipitation, presumably with magnesium and calcium ions.

All the cultures were harvested some days after the stationary phase of growing was reached, except for the cultures of *D. tertiolecta* used in the steam explosion treatment without acid, which were harvested at the end of the log phase.

A continuous centrifuge (Clara 20 High Flow, Alfa-Laval, Lund, Sweden) was used to concentrate the microalgal biomass samples. The centrifuge was operated at 9060 rpm, using a counter pressure of 4 bar. A Seepex progressive cavity pump (BN series) was used to feed the sample with 1000 L/h of a nominal flow rate. After concentration, the samples *N. gaditana* and *C. sorokiniana* were frozen at −80 °C. For defrosting the samples, they were placed at 4 °C for two days, prior to the steam explosion procedure. *D. tertiolecta* was harvested and concentrated just before the biorefinery process to avoid extra actions that might break its naked cells.

2.2. Steam Explosion

The equipment for the steam explosion of microalgae consisted of a 16 L reactor, operated in batch, and a collection vessel. The generation of steam was achieved with an electric boiler (Boreal, 380 V/82 kW) and thermally isolated high-pressure pipes were used to conduct the steam to the reactor. This was regulated by two valves placed in series, which were used to control the entrance of steam into the reactor. In the upper part of the reactor, there was a valve (2" diameter) for feeding the sample. In the bottom of the reactor, a flash valve allowed a fast decompression to the collecting tank at atmospheric pressure. The tank consisted of a cylinder with a capacity of 100 L and a diameter of 50 cm. It had two valves, one for steam release and another for the collection of sample in liquid phase.

In each experiment, 4 kg of microalgae was introduced into the reactor, which had been preheated. Some samples were previously impregnated with sulphuric acid at a concentration of 5% (*w/w*, wet sample basis) by mixing for 2 h at room temperature. The steam explosion pre-treatments were conducted at 150 °C (which corresponds to a saturated steam pressure of 4.7 bar) with a retention time of 5 min. The selection of the experimental conditions, which includes temperature, time, and acid concentration, was performed in a previous study [29]. After reaction and before the fractionation experiments, the exploded samples were collected and neutralized (to pH 5).

2.3. Filtration

A Vibratory Shear Enhanced Processing (VSEP, serie L, New Logic Research, Inc., Emeryville, CA, USA) system was used to perform dynamic membrane filtration experiments. A detailed description of this filtering system can be found elsewhere [19]. Approximately 6.0 kg of microalgal sample was used for each experiment, with a transmembrane pressure of 5 bar and a vibrational frequency of 55.4 ± 0.1 Hz.

Experiments were performed with PE5, a commercial polymeric membrane (Nanostone, Eden Prairie, MN, USA), manufactured from polyether-sulfone and with a molecular weight cut-off (MWCO) of 5000 Da. The filtration area was 0.0446 m^2 .

Water flux measurements were performed in order to determine the permeability of virgin membranes. After that, the steam exploded microalgae biomass was filtered and measurements of permeability vs. time were conducted during the experiment. The permeability with pre-treated algae was determined at the fixed time of 60 min. Finally, after cleaning, the system water permeability was measured again. The last step allowed for the determination of the irreversible fouling resistance of membranes. Also, two factors could be calculated, i.e., the irreversible fouling factor (IF), which is determined as the ratio of water permeabilities before and after the experiment, and total fouling factor (TF), consisting of the ratio between virgin membrane permeability with water and microalgae sludge permeability. In all cases, permeability was calculated from measurements of permeate mass weight progress with time. Permeate output was driven to a vessel placed on a scale, which was connected to a computer. An own-made software was recording and calculating permeability in real time to assess experimentation. Permeability was determined as follows. For water, measurements were performed at three different transmembrane pressures between the recommended range given by the manufacturer to ensure that a linear correlation between both parameters was achieved. For microalgae sludge, flow rate measurements were being performed with an interval of 10 s.

2.4. Lipid Extraction

The lipids from microalgal samples were extracted by contacting the same volume of sample and of *n*-hexane (20 mL). The extraction conditions were 60°C and agitation at 800 rpm, for 2 h. After the contact time, separation was achieved by centrifugation at 4000 rpm for 10 min. The mixture partitioned into three fractions: organic phase, aqueous phase, and residual solid. To extract and quantify lipids, the top hexane phase was recovered and was then heated to complete dryness in the oven (at 70°C).

2.5. Analytical Techniques

2.5.1. Light Microscope

A Zeiss Axio Scope A1 (Carl Zeiss Light Microscopy, Jena, Germany) microscope, equipped with Nomarski interference contrast optics, was used to check the effects of the steam explosion technique on cell morphology. A digital camera JENOPTIK ProgRes Speed Xcore 3 was used to obtain the light micrographs. Objective magnifications from 10 to 100 were used.

2.5.2. Dry Matter and Ash Content (TGA)

Thermogravimetric analyses (TGA), with a LECO instrument (TGA701), were performed in order to determine the dry ash free (DAF) weight of the samples which allows us to verify the mass balance during the steam explosion and membrane filtration processes. The samples were dried in a nitrogen atmosphere at 105°C to constant mass, for the dry matter content determination. After that, the atmosphere was changed to oxygen and the temperature was increased up to 550°C , in order to determine the ash content.

2.5.3. Total Lipid Extraction with Bligh and Dyer Method

The Bligh and Dyer method was used to extract the lipids from the fresh and steam exploded microalgal biomass. This method is the most commonly used at the analytical level for the quantitative extraction of lipids from microalgae [40].

2.5.4. Analytical Acid Hydrolysis

In order to determine the total extractable sugars, analytical acid hydrolysis experiments were conducted with the fresh microalgal samples, following a standard procedure (ASTM D1106-84). Although this method was originally used with lignocellulosic materials, microalgal biomass has also been previously analyzed [41]. The process consists of sulphuric acid hydrolysis in two stages. In the first stage, the freeze dried algal biomass sample (300 mg) is placed in contact with 72% (*w/w*, wet basis) sulphuric acid in a water bath at 30 °C, for 1 h. In the second stage, the sample is diluted to a concentration of 4% (*w/w*, wet basis) sulphuric acid and placed in an autoclave at 120 °C, for 45 min. After hydrolysis, filtration is performed using glass fiber filters in order to separate the acid insoluble residues from the hydrolysate. Finally, HPLC (high performance liquid chromatography) analyses were performed to quantitatively determine the sugar contents.

2.5.5. Monosaccharides Analysis

HPLC analyses were conducted in order to identify and quantify the monosaccharides present in the microalgal samples in solution. A Biorad Aminex HPX-87H column (300 mm × 7.8 mm) at 50 °C was used, with a refraction index detector. Additionally, the mobile phase was a 5 mM solution of sulphuric acid with a flow rate of 0.5 mL/min. The identification of monomeric sugars was achieved by a comparison of retention times with those of the standards. The integration of peaks in the chromatograms allowed the quantification, using a calibration curve, which was previously prepared with the standards.

2.5.6. Protein Analysis

Two different methods were used for protein analysis, namely solubilization and hot NaOH. To quantify the proteins released by the steam explosion treatment, the solubilization method was used. In this method, proteins were suspended by mixing 0.2 mL of sample in 1 mL 0.1 N NaOH. After 1 h of incubation at room temperature, samples were centrifuged at 4000 rpm for 10 min. Protein in the supernatant was precipitated with trichloroacetic acid (TCA) to avoid interfering substances. Following Barbarino and Lourenço [42], proteins were precipitated with 25% TCA at the ratio of 2.5:1 (TCA:homogenate) and centrifuged at 4000 rpm. Pellets were consecutively re-suspended in 10% and 5% TCA and finally solubilized in 0.1 N NaOH for the Bicinchoninic acid protein assay (BCA kit, Sigma-Aldrich, St. Louis, MO, USA). Color development was measured as absorbance at 562 nm using a microplate reader (INFINITE M200 PRO, Tecan, Männedorf, Switzerland). Absorbance values were read against a standard curve generated with a protein standard (bovine serum albumin), and percentage protein was calculated on a dry weight basis.

Since cell disruption was not expected using the solubilization method, a stronger method (hot NaOH) that allowed cell wall disruption was also applied to the concentrated culture and the steam exploded sample to evaluate the effects of steam explosion. In this procedure, 0.5 mL samples were extracted with 0.5 mL 2 N NaOH with 0.5% β -mercaptoethanol (*v/v*) at 90 °C for 10 min and centrifuged at 4000 rpm. Proteins were precipitated with TCA and solubilized in 0.1 N NaOH for the Bicinchoninic acid assay, as explained previously. Both extraction methods were performed in triplicate.

2.5.7. Particle Size Distribution

A Malvern Mastersizer 2000 piece of equipment with the Hydro 2000 MU module for liquid samples was used for particle size distribution measurements. A blue laser light was used. The medium consisted of 500 mL of demineralized water and sludge sample drops were added without further treatment until obtaining an appropriate obscuration level (as stated by the equipment instructions).

Two different levels of sonication: 6 kHz and 24 kHz, were used in the measurements, in order to check if aggregation had occurred with particles present in the sludge.

2.5.8. Optical Density

Absorbance measurements at 750 nm were performed to estimate the turbidity of the permeate, which can confirm total particle or oil rejection after membrane filtration. Absorbance was measured using a microplate reader (INFINITE M200 PRO, Tecan), and 96 well plates were used for the absorbance determinations. The optical density (OD750 nm) values were obtained by dividing the raw values over the path-length, and using as a reference the OD750 nm of filtered (0.45 µm) seawater.

3. Results and Discussion

3.1. Steam Explosion Treatment of Studied Strains

A steam explosion experiment was performed for each microalgae sample, at 150 °C, for 5 min and using 5% sulphuric acid to impregnate the samples. An additional experiment was performed with *D. tertiolecta*, to analyze the effect of steam explosion without acid impregnation, since this microalga has no cell wall. By comparing the dry ash free weight values of the samples before and after the steam explosion treatment, good balance closures (>97%) were obtained for all the experiments.

3.1.1. Cell Morphology

The examination of cell morphology by light microscopy showed that *C. sorokiniana*, *N. gaditana*, and *D. tertiolecta* had experienced high levels of cell disruption after the steam explosion pretreatment (Figure 1). Original samples consisted of isolated cells, except for *C. sorokiniana*, which contained both single cells and cell aggregates, hence the bimodal distribution in Figure 2B. Sonication dispersed cells and most aggregates were disintegrated. Accordingly, after sonication, the peak centered in ca. 3 µm, matching the *C. sorokiniana* cell size, was much higher, and the peak centered at ca. 20 µm which corresponds to aggregates almost disappeared.

Although *C. sorokiniana* appeared slightly damaged after thawing, with the cytoplasm slightly shrunken and retracted from the smooth cell wall, it was the less injured of the three species after steam explosion. *C. sorokiniana* cells showed three different patterns of disruption. Cells could be totally disrupted, algal material appearing as granulated aggregates. Cells could also maintain their unity but have granular cytoplasm and wrinkled margins. In this condition, cells had a low contrast appearance, which reveals that shapes may be flatter, probably due to a thinner and softer cell wall. More often, *C. sorokiniana* cells maintained their unity and high contrast appearance with smooth margins, but the cellular content was homogeneous except for a central depression, and no intracellular organelles (like chloroplast or pyrenoid) could be detected. After thawing, *N. gaditana* cells had the same morphology as live cells. However, after steam explosion treatment, algal material was mostly unevenly distributed in aggregates. They correspond to particles of different sizes. Some of them presented a yellow-brown color and could correspond to chloroplast remains. In a few cases, cells were detected, but then they appeared with granular cytoplasm and wrinkled margins as the intermediate disruption pattern of *C. sorokiniana*. It should be noted that the cell disruption effect of steam explosion was not apparently enhanced by freezing because *N. gaditana*, the cell walled species whose morphology appeared more altered after thawing, was less affected by steam explosion. Naked cells of *D. tertiolecta* were strongly sensitive, even to the centrifugation process. After centrifugation, cells lost their internal structure or were totally disrupted. The steam explosion treatment further disintegrated the algal material and

formed granulated aggregates. The same kind of cell debris was observed in the treatments with and without acid.

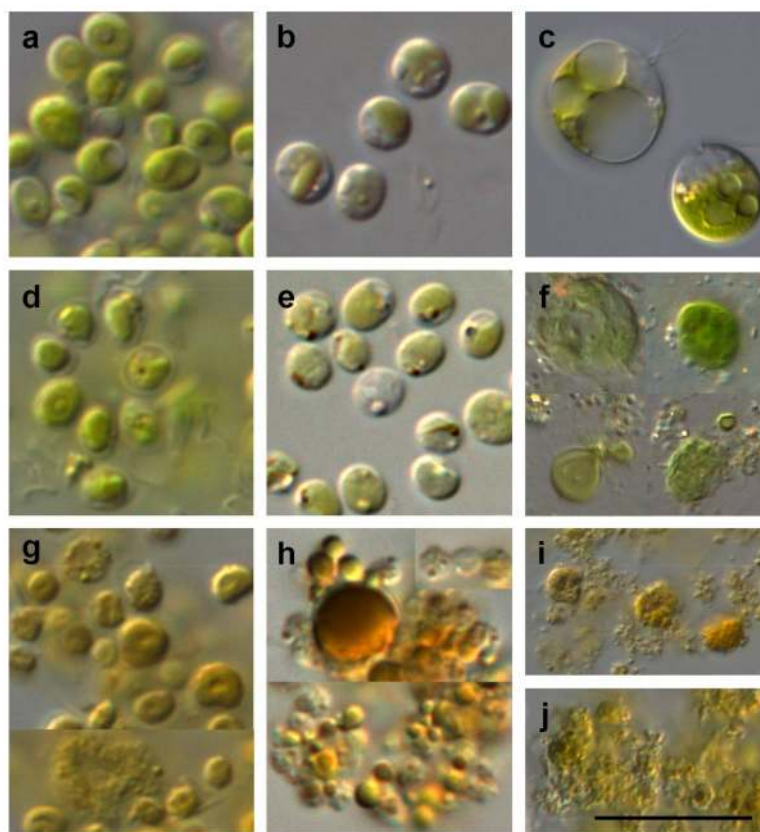


Figure 1. Light micrographs of *Chlorella sorokiniana* (a,d,g), *Nannochloropsis gaditana* (b,e,h), and *Dunaliella tertiolecta* (c,f,i,j) before and after steam explosion. (a,b,c) Live cells; (d,e) Thawed material; (f) *D. tertiolecta* after centrifugation; (g,h,i) Algal material after steam explosion with acid; (j) *D. tertiolecta* after steam explosion without acid. Scale bar corresponds to 10 μm in (a–f) and to 20 μm in (g–j).

3.1.2. Particle Size Distribution

Morphological characterization by means of microscopy was confirmed by the results obtained from particle size distribution (Figure 2).

Steam explosion produces aggregates when used with *N. gaditana* and *C. sorokiniana*. These aggregates disappear after filtration, probably due to the pump effect and the stress this caused. This effect is observed in almost all cases where these species were used. But this aggregation effect does not occur with *D. tertiolecta*, where the particle size distributions are always similar. Nevertheless, a smooth shift of the unique existent peak occurs, indicating some mass aggregation as the microscopy images show. The mean size ranges from 3 μm to 30 μm , whereas the size of the nominal microalgae cell is around 15 μm . The sample regarding the filtration retentate is the one with a smaller mean particle size due to the disaggregating role of the pump. The samples related to steam explosion treatment performed with acid have mean particle sizes which are slightly smaller than those performed without acid. Concerning *D. tertiolecta*, it is interesting to note the ability of sonication to break the microalgae cells. This only happens with this species and is probably due to the fact that *D. tertiolecta* does not have a cell wall. With other species, sonication only breaks aggregates. This is only observed with the sample after being harvested, but not with samples after steam explosion and membrane filtration. The reason for this is that at those stages, cells are almost totally unstructured, in agreement with microscopy images.

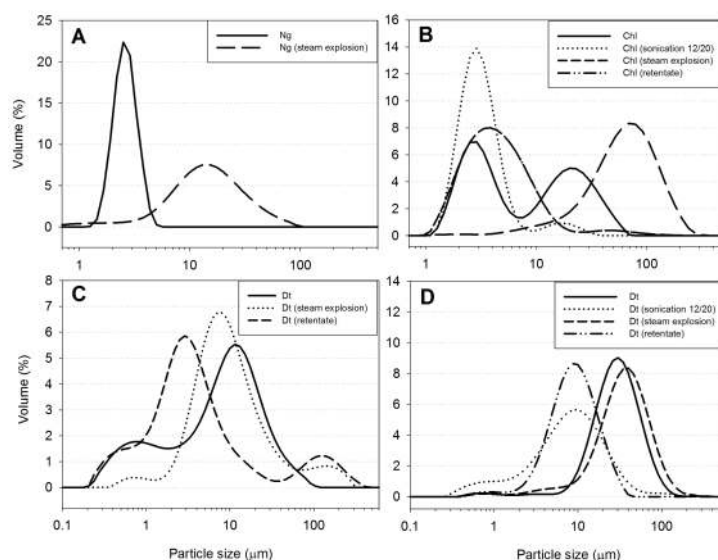


Figure 2. Particle size distribution results. (A) *Nannochloropsis gaditana* (B) *Chlorella sorokiniana* (C) *Dunaliella tertiolecta* (steam explosion with acid) (D) *D. tertiolecta* (steam explosion without acid). In all cases except those indicated, sonication was 0/12. All plots were obtained from an average of three measurements.

3.1.3. Lipid, Sugar, and Protein Contents

Table 1 shows the results of the steam explosion experiments. The amount of lipid extracted (by Bligh and Dyer and *n*-hexane), sugar, and protein contents are indicated. For the purpose of comparison, the values of lipid, total sugar content, and proteins from the fresh untreated samples are also included.

Table 1. Results of lipid, sugar, and protein analysis of steam explosion experiments (150 °C, 5 min and 5% *w/w*. H₂SO₄ except sample *D. tertiolecta* (II) with no acid). Values are expressed as the mean and the standard deviation is indicated in brackets.

		Lipids		Sugar	Protein	
		Bligh & Dyer	Hexane		Hot NaOH	Solubilization
<i>Nannochloropsis gaditana</i>	Untreated	22.2% (0.4)	2.1% (0.3)	18.8% (0.8)	17.3% (0.8)	1.4% (0.1)
	Steam exploded	22.3% (0.1)	17.6% (0.2)	12.9% (0.6)	8.4% (0.6)	9.1% (0.4)
<i>Chlorella sorokiniana</i>	Untreated	13.0% (0.2)	0.6% (0.0)	23.5% (1.3)	19.2% (0.3)	2.2% (0.0)
	Steam exploded	11.8% (0.1)	4.8% (0.2)	18.6% (0.9)	9.2% (0.1)	10.7% (0.1)
<i>Dunaliella tertiolecta</i> (I)	Untreated	26.6% (0.8)	2.8% (0.7)	26.1% (2.2)	14.5% (0.5)	12.0% (0.3)
	Steam exploded	29.7% (3.2)	10.6% (0.1)	19.2% (0.8)	2.6% (0.0)	5.1% (0.3)
<i>Dunaliella tertiolecta</i> (II)	Untreated	11.4% (1.2)	1.6% (0.1)	25.8% (2.4)	10.5% (0.0)	5.9% (0.1)
	Steam exploded No acid	11.9% (0.1)	2.1% (0.0)	8.6% (0.6)	4.8% (0.1)	4.4% (0.4)

By comparing the total lipid contents, as determined by the Bligh and Dyer method, of the untreated and steam exploded samples, we can observe that similar values are obtained in all the cases. This is because the Bligh and Dyer method yields the highest lipid recoveries, because it is a stronger method. But the use of *n*-hexane was considered as organic solvent for lipid isolation from microalgae to avoid the use of chloroform, which presents environmental and health risks, especially when it is used at an industrial scale. The experiments performed with the untreated microalgae samples showed the low extraction capability of *n*-hexane, with a maximum of 2.8% (*w/w*, DAF basis) lipid yield in the case of *D. tertiolecta*. But the amount of lipid extracted with *n*-hexane improved with the application of the steam explosion technique. Among the three microalgae species studied, *N. gaditana* yielded the maximum amount of lipid recovery of the steam exploded sample (at 150 °C, with 5% sulfuric acid),

with 17.6% (*w/w*, DAF of untreated microalga basis). It signifies 79% of the total lipid as obtained by the Bligh and Dyer method. For *C. sorokiniana*, the amount of lipid extracted after steam explosion (at 150 °C, with 5% sulfuric acid) was only 4.8% (*w/w*, DAF of untreated microalga basis), representing 41% of the total amount of lipids of this microalga. In the case of *D. tertiolecta*, the extraction of lipids with *n*-hexane greatly enhanced due to the use of acid in the steam explosion process. A lipid yield of 2.1% (*w/w*, DAF of untreated microalga basis) was obtained when steam explosion was applied without acid impregnation, whereas this value increased to 10.6% (*w/w*, DAF of untreated microalga basis), as a consequence of using 5% sulphuric acid in the steam explosion experiment. This result is in agreement with our previous study [29], and shows the importance of carbohydrate hydrolysis to achieve a higher lipid extraction yield from microalgal sludge, using *n*-hexane as the solvent.

Concerning carbohydrates, the total sugar content of the untreated microalga, obtained by analytical acid hydrolysis, was determined for each microalgae species and the specific values are presented in Table 1. These values can be compared with the measured concentration of sugar in the solution of the steam exploded samples, which are also included in Table 1. For the steam explosion experiments performed with acid impregnation, a high percentage, between 70% and 80%, of the total sugar content of the microalga was found in solution after steam explosion. Contrary to this, the experiment performed with *D. tertiolecta* without the use of acid resulted in a low sugar concentration, representing 33% of the total sugar content of the untreated sample.

The protein concentration of the untreated microalgal samples ranged between 10% and 19% of DAF in the three species (Table 1). These values are in the range reported for species of the same genera in the stationary phase of culture.

The protein contents of *D. tertiolecta* detected after solubilization with dilute NaOH or after extraction at a high temperature were similar (Table 1). Thus, proteins were already available for solubilization in the harvested cultures of this naked microalgae species, meaning that it was not necessary to apply a disruption treatment. On the other hand, the protein contents detected after solubilization with dilute NaOH of both *N. gaditana* and *C. sorokiniana* were much higher after steam explosion. This rise in the detected protein revealed the cell disruption effect of steam explosion. However, the number of proteins detected after extraction at high temperature was lower in the steam exploded material than in the untreated sample for the three species. This protein loss may be explained by the occurrence of protein hydrolysis during steam explosion. The color reaction that is measured in the bicinchoninic acid assay is due to the reduction of Cu^{2+} to Cu^{+} by the oxidation of aromatic residues and peptide bonds in the protein in the reaction solution. Therefore, a lighter coloration may evidence a reduction in the number of peptide bonds due to protein hydrolysis.

3.2. Fractionation of Steam Exploded Samples by Means of Membrane Filtration

According to the results of a previous study [24], the fractionation strategy followed in the present work consists of filtrating the exploded sample with a membrane set-up and then extracting the retentate and permeate streams with solvent. The filtration was performed with dynamic filtration, which allowed for a much better permeability with just a little more energy compared to conventional cross-flow filtration. This was because fouling is highly reduced. Not only are less pores blocked, but, primarily, the cake molding over the surface of the membrane that occurs in conventional filtration is hardly produced in dynamic filtration. Therefore, vibrating filtration highly reduces microalgae attachment on the membrane surface. A PE5 membrane (MWCO = 5000 Da) was used, since it exhibited the best performance in the filtration experiments regarding permeability and irreversible fouling.

3.2.1. Rejection

Table 2 presents the results of the filtration experiments including the total weight and DAF percentage and the lipids, sugars, and protein content of each of the different streams. From the values of the DAF percentages, it can be observed that different concentrations of the retentate streams were attained (from 3% to 10% DAF). This mainly depended on the concentration of the starting material.

Table 2. Results of total mass balance, and lipid, sugar, and protein analysis of filtration experiments.

	<i>Nannochloropsis gaditana</i>			<i>Chlorella sorokiniana</i>		
	Steam Exploded Sample	Retentate	Permeate	Steam Exploded Sample	Retentate	Permeate
Total weight (g)	6000	2400	3600	6000	2240	3760
DAF percentage	5.1 (0.1)	10.1 (0.2)	1.8 (0.05)	2.7 (0.02)	5.8 (0.08)	0.9 (0.01)
Lipid (g/L)	9.2 (0.3)	22.7 (0.5)	0.07 (0.01)	1.3 (0.05)	3.9 (0.09)	0.05 (0.01)
Sugar (g/L)	6.8 (0.3)	5.9 (0.2)	6.0 (0.2)	5.1 (0.1)	5.2 (0.2)	4.9 (0.1)
Protein (g/L)	4.7 (0.2)	5.65 (0.15)	n.d.	2.92 (0.04)	4.8 (0.14)	n.d.

	<i>Dunaliella tertiolecta</i>			<i>Dunaliella tertiolecta</i> (No Acid)		
	Steam Exploded Sample	Retentate	Permeate	Steam Exploded Sample	Retentate	Permeate
Total weight (g)	6000	2290	3710	6000	2400	3600
DAF percentage	1.7 (0.01)	3.2 (0.04)	0.9 (0.01)	1.6 (0.01)	3.0 (0.02)	0.7 (0.01)
Lipid (g/L)	1.8 (0.02)	3.5 (0.08)	0.07 (0.01)	0.34 (0.03)	1.3 (0.01)	0.07 (0.01)
Sugar (g/L)	3.3 (0.2)	2.9 (0.1)	3.2 (0.2)	1.4 (0.1)	1.3 (0.1)	1.2 (0.1)
Protein (g/L)	0.89 (0.05)	1.46 (0.06)	n.d.	0.71 (0.06)	1.13 (0.07)	n.d.

The amount of lipid extracted with *n*-hexane and the proteins obtained with the solubilization method from the steam exploded and the permeate and retentate are included in Table 2. These values are expressed as a concentration of each stream, to allow for a better comparison. The permeate streams have a negligible content of lipids and no proteins. This result was also confirmed by optical density measurements. OD750 nm of permeates were like that of filtered (0.45 µm) seawater (Table 3). Therefore, it was assumed that lipid rejection was obtained in all the experiments. The absence of lipids and proteins in the permeate implies that the membrane PE5 is suitable for rejecting lipids and proteins from different microalgae species. On the other hand, the concentration of lipids and proteins in the retentate streams is much higher than that of the steam exploded sample before filtration.

Table 3. Optical density at 750 nm after filtration of steam exploded microalgae. Raw values are compared to filtered (0.45 µm) seawater (blank). Values are expressed as the mean and the standard deviation is provided in brackets.

	OD _{750nm}	
	Blank	Permeate
<i>Nannochloropsis gaditana</i>	0.081 (0.001)	0.091 (0.003)
<i>Chlorella sorokiniana</i>	0.081 (0.001)	0.101 (0.002)
<i>Dunaliella tertiolecta</i>	0.083 (0.001)	0.085 (0.001)
<i>Dunaliella tertiolecta</i> (no acid)	0.083 (0.001)	0.083 (0.000)

Concerning the sugar analysis, approximately the same values of concentration were obtained for the steam exploded sample and retentate and permeate streams, for the different microalgae species. This means that the employed membrane (PE5) is unable to retain sugars.

3.2.2. Permeability

Regarding the performance of the membrane in using dynamic filtration, Figure 3 shows membrane permeabilities including water permeability with the new (unused) membrane and after the experiment, for the different microalgae species studied. The permeabilities of steam-exploded biomass were measured. With them, the total fouling of materials was calculated. Concerning the permeability for the water of new PE5 membranes, the values between 30.4 L/h/m²/bar (for *D. tertiolecta* exploded without acid) and 90.8 L/h/m²/bar (for *N. gaditana*) were obtained. In an ideal system where a liquid that does not provide fouling is used and virgin membranes perfectly manufactured are used, the same permeabilities would be obtained. But in laboratory or pilot-scale scenarios, both conditions hardly occur. As checked earlier with the help of a scanning electron microscope, membrane thicknesses differ within the same sample. Following Darcy's law, this makes the permeability change accordingly.

If enough surface of membrane is used, a mean permeability value with a low deviation is normally obtained. But this is not the case with a pilot unit as the one used in this work.

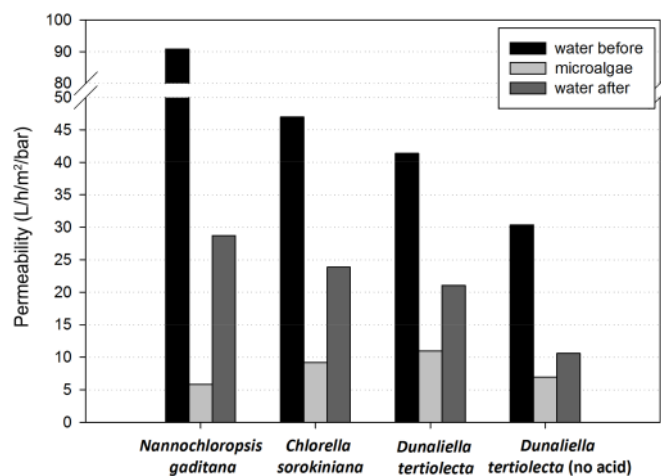


Figure 3. Water and sample permeabilities for the different microalgae samples.

In terms of pretreated microalgae filtration, the *N. gaditana* sample resulted in a microalgae permeability of 5.84 L/h/m²/bar, the lowest value among the samples. With *D. tertiolecta* exploded without acid, a microalgae permeability of 6.93 L/h/m²/bar was obtained, and with *C. sorokiniana*, a permeability of 9.18 L/h/m²/bar was reached. The best membrane performance was obtained when filtrating the sample of *D. tertiolecta* exploded with acid, with the permeability value of 10.97 L/h/m²/bar.

The total fouling factor (TF) of PE5 was the highest for *N. gaditana*, with the value of 15.55. In the case of *C. sorokiniana*, TF was lower with the value of 5.12 and with *D. tertiolecta* exploded without acid, where TF = 4.39. The best performance in terms of TF was obtained with *D. tertiolecta* exploded with acid, where the value of 3.77 was given.

3.2.3. Irreversible Fouling

To calculate irreversible fouling, membrane permeability with water before and after the experiment was measured (the system was cleaned before performing the water permeability measurements after the experiment). PE5 with *N. gaditana* resulted in the permeability of 28.7 L/h/m²/bar, *C. sorokiniana* performed with the value of 23.85 L/h/m²/bar, *D. tertiolecta* exploded with acid gave the value of 21.07 L/h/m²/bar, and finally, *D. tertiolecta* exploded without acid performed with the value of 10.61 L/h/m²/bar. Therefore, the experiment with the lowest irreversible fouling factor of 1.96 was *D. tertiolecta* exploded with acid, while *N. gaditana*, *C. sorokiniana*, and *D. tertiolecta* exploded without acid resulted in IF = 3.16, IF = 2.86, and IF = 1.97, respectively.

Figure 4 presents the exploded microalgae permeability profiles vs. time for dynamic filtration with *N. gaditana* and *C. sorokiniana*. In the filtration of *C. sorokiniana*, a steady state was reached after 30 min of the experiment with the permeability value of 9.5 L/h/m²/bar. On the contrary, in the filtration of *N. gaditana*, the plateau was not reached, even though the experiment lasted longer than *C. sorokiniana*. After 130 min of filtrating, the value of permeability with *N. gaditana* was 4.2 L/h/m²/bar and still decreasing.

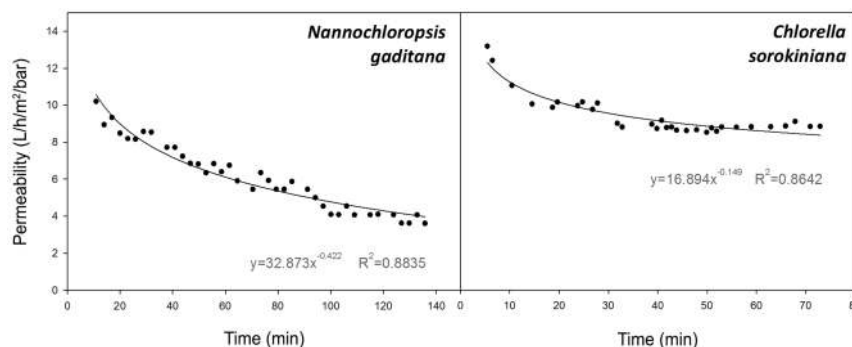


Figure 4. Permeability profiles vs. time of filtration experiments performed with (a) *Nannochloropsis gaditana* and (b) *Chlorella sorokiniana*.

4. Process Costs

One of the biggest drawbacks in the profitability of a bio-refinery of microalgae is to reduce harvest and fractionation costs [43,44]. It is necessary to have information that allows a careful calculation of costs to evaluate the different stages of a given process and that is what is discussed below.

4.1. Steam Explosion Costs

The energy consumption associated with the steam explosion operation was calculated by using the analysis provided by Sui and Chen [45]. Once a continuous stable operation is reached (e.g., the reactor steel has been heated to the operating temperature), the different energy topics considered are heating the steam, the air in the reactor, and the microalgal sludge. The other energy losses considering the expansion to atmospheric pressure are those from steam, water, air, and dry materials. We considered that the good thermal insulation of the reactor allowed us to ignore other heat losses. The experiments performed with microalgae sludge show that the humidity of the sample is the factor that most influences the steam consumption, in complete agreement with the conclusions of the above-mentioned paper. Considering a humidity of 80% in the sample, the required steam is 1.2 kg/kg dry sample. If the humidity is 85%, the steam needed is 1.6 kg/kg dry sample.

The cost of the steam strongly depends on the cost of the fuel used to produce it (TLV 2018), but it can be greatly reduced if the heat needed is produced on-site [46]. The vaporization enthalpy experiences a very minor change with the steam pressure.

To proceed with the first stage of fractionation, we consider the production of saturated steam at 5 barg (159.2 °C) from water at 20 °C. If the cost of fuel oscillates from 1.65 \$/GJ to 4.5 \$/GJ, the cost of the steam oscillates from 4.4 \$/metric Ton to 12.2 \$/metric Ton. Assuming that it is needed, for 1.2 kg of steam/kg of dry microalgae sample, the cost of the energy associated with the steam explosion operation oscillates between 0.005 \$/kg of dry sample to 0.014 \$/kg of dry sample.

A comparison with other techniques such as High Pressure Homogenization, Ultrasonication, Microwave treatment, Laser treatment, High Speed Homogenization, Bead Milling, and Pulse Electric Field gives a range of disruption cost between 14.68 and 0.006 \$/kg dry sample [47]. The high range of cost is also due to different levels of humidity in the sample to be treated. Another aspect is that all the energy involved in those technologies is electrical, which implies a higher energy cost compared to the cost of steam production. A good energy integration in the plant using residual heat will allow for the reduction of the initial sample humidity and help reduce the cost of steam production.

4.2. Dynamic Membrane Filtration Operational Costs

Vibrational membrane filtration lowers the separation operational cost compared to conventional filtration or centrifugation. The reduction of fouling causes high flux rates that means more throughput

capacity per monetary unit of capital invested. For the same reason, the membranes need less replacement and less cleaning and maintenance, which also reduces the operational cost.

In membrane filtration, microalgae can be almost fully rejected, whereas centrifugation only recovers between 80% and 90% of the biomass [48].

Considering an available commercial dynamic membrane filtration setup with a membrane area of 140 m² [49], a microalgae sludge permeability of 2 L/h/m²/bar, one year of operation (1800 h), and a final stream with a lipid concentration of 100 g/L, a yield of 6 tones/year of lipids (dry basis) could be produced. The permeability value used corresponds to 25% of the mean value of the experimental permeability measured. It is a conservative value considering that the final concentration is higher than the one reached in the experiments. The required power of the equipment is about 47 kW [49], which means that around 84,000 kWh/year would be consumed. Considering an energy cost basis in Spain of 0.097 \$/kWh [50], an electrical cost of 8100 \$/year would be necessary. It must be noted that the power cost in Spain is quite high compared to other neighboring countries. If this cost is normalized per unit of product, the cost would be about 0.08 \$/kg microalgae (dry basis). Other operational costs are membrane replacement, maintenance, and cleaning. With current research results demonstrating that much cheaper membranes can be used for this application [17], the membrane replacement cost can be assumed to be half the electricity cost [51], and the total operation cost should be around 0.12 \$/kg microalgae (dry basis).

Bibliographical data estimates that electrical centrifugation costs, with a sludge in similar conditions and with a similar yield, are higher than 0.6 \$/kg microalgae (dry basis) [52].

To sum up, accumulated operating costs from cell disruption and membrane filtration are around 0.13 \$/kg dry microalgae (0.01 steam explosion +0.12 membrane filtration). Filtration contributes to more than 90% of the cost and it is where the research attention should be focused.

5. Conclusions

Steam explosion has the potential to become a broad-spectrum microalgae cell disruption, as well as pre-fractionation, treatment. It provided proper availability of organic compounds and carbohydrate hydrolysis into sugars with all the various kinds of used microalgae and it is particularly effective when the strains have recalcitrant cell walls.

The use of steam explosion, besides breaking the cell wall, partially hydrolyzes proteins.

With all the tested strains, dynamic membrane filtration offers an excellent performance regarding permeability by rejecting lipids.

The sequence of steam explosion, dynamic membrane filtration, and solvent extraction as downstream unit operations in a microalgae biorefinery clearly allows for the reduction of process costs. All the mentioned technologies for all the stages are already commercially available.

Acknowledgments: This study was funded by the Spanish Ministry of Economy and Competitiveness (grant number CTQ2014-56285-R). The research was also supported by the European Regional Development Funds (ERDF, FEDER Programa Competitividad de Catalunya 2007–2013) and by the project “Fuels from Biomass” funded by Excma. Diputació de Tarragona. M. Hapońska is grateful to the Universitat Rovira i Virgili (URV) for her PhD scholarship.

Author Contributions: E.L. designed part of the experiments, performed the experiments, analyzed the data, and wrote the paper. M.H. designed part of the experiments, performed the experiments, analyzed the data, and wrote the paper. E.C. designed part of the experiments performed part of the experiments, analyzed the data, and wrote the paper. C.T. conceived and designed part of the experiments, performed part of the experiments, analyzed the data, and wrote the paper. J.S. conceived and designed part of the experiments, analyzed the data, and wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Chisti, Y. Biodiesel from microalgae. *Biotechnol. Adv.* **2007**, *25*, 294–306. [[CrossRef](#)] [[PubMed](#)]

2. Sheehan, J.; Dunahay, T.; Benemann, J.; Roessler, P. *A Look Back at the US Department of ENERGY'S Aquatic Species, Program: Biodiesel from Algae*; National Renewable Energy Laboratory (NREL): Golden, CO, USA, 1998.
3. Zhu, L.; Ketola, T. Microalgae production as a biofuel feedstock: Risks and challenges. *Int. J. Sustain. Dev. World* **2012**, *19*, 268–274. [[CrossRef](#)]
4. Zhu, L. Biorefinery as a promising approach to promote microalgae industry: An innovative framework. *Renew. Sustain. Energy Rev.* **2015**, *41*, 1376–1384. [[CrossRef](#)]
5. Wijffels, R.H.; Barbosa, M.J.; Eppink, M.H.M. Microalgae for the production of bulk chemicals and biofuels. *Biofuels Bioprod. Biorefin.* **2010**, *4*, 287–295. [[CrossRef](#)]
6. Aslam, A.; Thomas-Hall, S.R.; Mughal, T.A.; Schenk, P.M. Selection and adaptation of microalgae to growth in 100% unfiltered coal-fired flue gas. *Bioresour. Technol.* **2017**, *233*, 271–283. [[CrossRef](#)] [[PubMed](#)]
7. Zhao, B.; Su, Y. Process effect of microalgal-carbon dioxide fixation and biomass production: A review. *Renew. Sustain. Energy Rev.* **2014**, *31*, 121–132. [[CrossRef](#)]
8. Gupta, P.L.; Lee, S.M.; Choi, H.J. Integration of microalgal cultivation system for wastewater remediation and sustainable biomass production. *World J. Microbiol. Biotechnol.* **2016**, *32*, 139. [[CrossRef](#)] [[PubMed](#)]
9. Suresh Kumar, K.; Dahms, H.U.; Won, E.J.; Lee, J.S.; Shin, K.H. Microalgae—A promising tool for heavy metal remediation. *Ecotoxicol. Environ. Saf.* **2015**, *113*, 329–352. [[CrossRef](#)] [[PubMed](#)]
10. Lupatini, A.L.; Colla, L.M.; Canan, C.; Colla, E. Potential application of microalga spirulina platensis as a protein source. *J. Sci. Food Agric.* **2017**, *97*, 724–732. [[CrossRef](#)] [[PubMed](#)]
11. Sanchez Rizza, L.; Sanz Smachetti, M.E.; Do Nascimento, M.; Salerno, G.L.; Curatti, L. Bioprospecting for native microalgae as an alternative source of sugars for the production of bioethanol. *Algal Res.* **2017**, *22*, 140–147. [[CrossRef](#)]
12. Reyes, F.A.; Mendiola, J.A.; Ibañez, E.; Del Valle, J.M. Astaxanthin extraction from haematococcus pluvialis using CO₂-expanded ethanol. *J. Supercrit. Fluids* **2014**, *92*, 75–83. [[CrossRef](#)]
13. Uquiche, E.; Antilaf, I.; Millao, S. Enhancement of pigment extraction from *B. braunii* pretreated using CO₂ rapid depressurization. *Braz. J. Microbiol.* **2016**, *47*, 497–505. [[CrossRef](#)] [[PubMed](#)]
14. Cuello, J.L.; Hoshino, T.; Kuwahara, S.; Brown, C.L. Scale-up-bioreactor design and culture optimization. In *Biotechnology for Biofuel Production and Optimization*; Elsevier: Amsterdam, The Netherlands, 2016; pp. 497–511.
15. Thomassen, G.; Eiguren Vila, U.; Van Dael, M.; Lemmens, B.; Van Passel, S. A techno-economic assessment of an algal-based biorefinery. *Clean Technol. Environ. Policy* **2016**, *18*, 1849–1862. [[CrossRef](#)]
16. Ersahin, M.E.; Ozgun, H.; Dereli, R.K.; Ozturk, I.; Roest, K.; van Lier, J.B. A review on dynamic membrane filtration: Materials, applications and future perspectives. *Bioresour. Technol.* **2012**, *122*, 196–206. [[CrossRef](#)] [[PubMed](#)]
17. Hapońska, M.; Clavero, E.; Salvadó, J.; Torras, C. Application of ABS membranes in dynamic filtration for *Chlorella sorokiniana* dewatering. *Biomass Bioenergy* **2016**, *111*, 224–231. [[CrossRef](#)]
18. Kim, J.; Yoo, G.; Lee, H.; Lim, J.; Kim, K.; Kim, C.W.; Park, M.S.; Yang, J.W. Methods of downstream processing for the production of biodiesel from microalgae. *Biotechnol. Adv.* **2013**, *31*, 862–876. [[CrossRef](#)] [[PubMed](#)]
19. Nurra, C.; Clavero, E.; Salvadó, J.; Torras, C. Vibrating membrane filtration as improved technology for microalgae dewatering. *Bioresour. Technol.* **2014**, *157*, 247–253. [[CrossRef](#)] [[PubMed](#)]
20. Rossignol, N.; Vandanjon, L.; Jaouen, P.; Quéméneur, F. Membrane technology for the continuous separation microalgae/culture medium: Compared performances of cross-flow microfiltration and ultrafiltration. *Aquac. Eng.* **1999**, *20*, 191–208. [[CrossRef](#)]
21. Zhao, F.; Chu, H.; Zhang, Y.; Jiang, S.; Yu, Z.; Zhou, X.; Zhao, J. Increasing the vibration frequency to mitigate reversible and irreversible membrane fouling using an axial vibration membrane in microalgae harvesting. *J. Membr. Sci.* **2017**, *529*, 215–223. [[CrossRef](#)]
22. Cheng, J.; Huang, R.; Li, T.; Zhou, J.; Cen, K. Physicochemical characterization of wet microalgal cells disrupted with instant catapult steam explosion for lipid extraction. *Bioresour. Technol.* **2015**, *191*, 66–72. [[CrossRef](#)] [[PubMed](#)]
23. Grimi, N.; Dubois, A.; Marchal, L.; Jubeau, S.; Lebovka, N.I.; Vorobiev, E. Selective extraction from *Microalgae nannochloropsis* sp. Using different methods of cell disruption. *Bioresour. Technol.* **2014**, *153*, 254–259. [[CrossRef](#)] [[PubMed](#)]
24. Lorente, E.; Hapońska, M.; Clavero, E.; Torras, C.; Salvadó, J. Microalgae fractionation using steam explosion, dynamic and tangential cross-flow membrane filtration. *Bioresour. Technol.* **2017**, *237*, 3–10. [[CrossRef](#)] [[PubMed](#)]

25. Mendes-Pinto, M.M.; Raposo, M.F.J.; Bowen, J.; Young, A.J.; Morais, R. Evaluation of different cell disruption processes on encysted cells of *haematococcus pluvialis*: Effects on astaxanthin recovery and implications for bio-availability. *J. Appl. Phycol.* **2001**, *13*, 19–24. [\[CrossRef\]](#)
26. Nurra, C.; Torras, C.; Clavero, E.; Ríos, S.; Rey, M.; Lorente, E.; Farriol, X.; Salvadó, J. Biorefinery concept in a microalgae pilot plant. Culturing, dynamic filtration and steam explosion fractionation. *Bioresour. Technol.* **2014**, *163*, 136–142. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Yap, B.H.J.; Dumsday, G.J.; Scales, P.J.; Martin, G.J.O. Energy evaluation of algal cell disruption by high pressure homogenisation. *Bioresour. Technol.* **2015**, *184*, 280–285. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Safi, C. *Microalgae Biorefinery: Proposition of a Fractionation Process*; Université de Toulouse: Toulouse, France, 2013.
29. Lorente, E.; Farriol, X.; Salvadó, J. Steam explosion as a fractionation step in biofuel production from microalgae. *Fuel Process. Technol.* **2015**, *131*, 93–98. [\[CrossRef\]](#)
30. Lee, A.K.; Lewis, D.M.; Ashman, P.J. Disruption of microalgal cells for the extraction of lipids for biofuels: Processes and specific energy requirements. *Biomass Bioenergy* **2012**, *46*, 89–101. [\[CrossRef\]](#)
31. Heitz, M.; Capek-Ménard, E.; Koeberle, P.G.; Gagné, J.; Chornet, E.; Overend, R.P.; Taylor, J.D.; Yu, E. Fractionation of *populus tremuloides* at the pilot plant scale: Optimization of steam pretreatment conditions using the stake II technology. *Bioresour. Technol.* **1991**, *35*, 23–32. [\[CrossRef\]](#)
32. Verwijst, T.; Baggerman, J.; Liebermann, F.; van Rijn, C.J.M. High-frequency flow reversal for continuous microfiltration of milk with microsieves. *J. Membr. Sci.* **2015**, *494*, 121–129. [\[CrossRef\]](#)
33. Malmali, M.; Stickel, J.J.; Wickramasinghe, S.R. Sugar concentration and detoxification of clarified biomass hydrolysate by nanofiltration. *Sep. Purif. Technol.* **2014**, *132*, 655–665. [\[CrossRef\]](#)
34. Kumar, K.; Das, D. Growth characteristics of *chlorella sorokiniana* in airlift and bubble column photobioreactors. *Bioresour. Technol.* **2012**, *116*, 307–313. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Chua, E.T.; Schenk, P.M. A biorefinery for nannochloropsis: Induction, harvesting, and extraction of epa-rich oil and high-value protein. *Bioresour. Technol.* **2017**, *244*, 1416–1424. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Francavilla, M.; Kamaterou, P.; Intini, S.; Monteleone, M.; Zabaniotou, A. Cascading microalgae biorefinery: Fast pyrolysis of *dunaliella tertiolecta* lipid extracted-residue. *Algal Res.* **2015**, *11*, 184–193. [\[CrossRef\]](#)
37. Scholz, M.J.; Weiss, T.L.; Jinkerson, R.E.; Jing, J.; Roth, R.; Goodenough, U.; Posewitz, M.C.; Gerken, H.G. Ultrastructure and composition of the nannochloropsis gaditana cell wall. *Eukaryot. Cell* **2014**, *13*, 1450–1464. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Takeda, H. Taxonomical assignment of chlorococcal algae from their cell wall composition. *Phytochemistry* **1993**, *34*, 1053–1055. [\[CrossRef\]](#)
39. Kodner, R.B.; Summons, R.E.; Knoll, A.H. Phylogenetic investigation of the aliphatic, non-hydrolyzable biopolymer algaenan, with a focus on green algae. *Org. Geochem.* **2009**, *40*, 854–862. [\[CrossRef\]](#)
40. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Phys.* **1959**, *37*, 911–917. [\[CrossRef\]](#)
41. Templeton, D.W.; Quinn, M.; Van Wychen, S.; Hyman, D.; Laurens, L.M.L. Separation and quantification of microalgal carbohydrates. *J. Chromatogr. A* **2012**, *1270*, 225–234. [\[CrossRef\]](#) [\[PubMed\]](#)
42. Barbarino, E.; Lourenço, S.O. An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae. *J. Appl. Phycol.* **2005**, *17*, 447–460. [\[CrossRef\]](#)
43. Hoffman, J.; Pate, R.C.; Drennen, T.; Quinn, J.C. Techno-economic assessment of open microalgae production systems. *Algal Res.* **2017**, *23*, 51–57. [\[CrossRef\]](#)
44. t Lam, G.P.; Vermuë, M.H.; Eppink, M.H.M.; Wijffels, R.H.; van den Berg, C. Multi-product microalgae biorefineries: From concept towards reality. *Trends Biotechnol.* **2017**, *36*, 216–277. [\[CrossRef\]](#) [\[PubMed\]](#)
45. Sui, W.; Chen, H. Multi-stage energy analysis of steam explosion process. *Chem. Eng. Sci.* **2014**, *116*, 254–262. [\[CrossRef\]](#)
46. Carrere, H.; Antonopoulou, G.; Affes, R.; Passos, F.; Battimelli, A.; Lyberatos, G.; Ferrer, I. Review of feedstock pretreatment strategies for improved anaerobic digestion: From lab-scale research to full-scale application. *Bioresour. Technol.* **2016**, *199*, 386–397. [\[CrossRef\]](#) [\[PubMed\]](#)
47. 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. [\[CrossRef\]](#) [\[PubMed\]](#)
48. Chen, C.-Y.; Yeh, K.-L.; Aisyah, R.; Lee, D.-J.; Chang, J.-S. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. *Bioresour. Technol.* **2011**, *102*, 71–81. [\[CrossRef\]](#) [\[PubMed\]](#)

49. New Logic Research, Inc. Vsep Products. Available online: <http://www.vsep.com/products/index.html> (accessed on 23 October 2017).
50. Ministerio de Energía, Turismo y Agenda Digital de España. Energía y Emisiones. Available online: http://www.minetad.gob.es/es-ES/IndicadoresyEstadisticas/DatosEstadisticos/IV.%20Energ%C3%ADa%20y%20emisiones/IV_12.pdf (accessed on 23 October 2017).
51. New Logic Research, Inc. Vsep Case Studies and Application Notes. Available online: http://www.vsep.com/downloads/case_studies_application_notes.html (accessed on 23 October 2017).
52. Dassey, A.J.; Theegala, C.S. Harvesting economics and strategies using centrifugation for cost effective separation of microalgae cells for biodiesel applications. *Bioresour. Technol.* **2013**, *128*, 241–245. [[CrossRef](#)] [[PubMed](#)]



© 2018 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).