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The Biorefinery of the Marine Microalga *Crypthecodinium cohnii* as a Strategy to Valorize Microalgal Oil Fractions

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Abstract: *Chrypthecodinium cohnii* lipids have been almost exclusively used as a source of Docosahexaenoic acid (DHA). Such an approach wastes the remaining microalgal lipid fraction. The present work presents a novel process to produce *C. cohnii* biomass, using low-cost industrial by-products (raw glycerol and corn steep liquor), in a 7L-bioreactor, under fed-batch regime. At the end of the fermentation, the biomass concentration reached 9.2 g/L and the lipid content and lipid average productivity attained 28.0% (*w*/*w* dry cell weight) and 13.6 mg/L h, respectively. Afterwards the microalgal biomass underwent a saponification reaction to produce fatty acid (FA) soaps, which were further converted into FA ethyl ester (FA EE). *C. cohnii* FA EE mixture was then fractionated, using the urea complexation method at different temperatures, in order to obtain a polyunsaturated fatty acid ethyl ester (PUFA EE) rich fraction, that could be used for food/pharmaceutical/cosmetic purposes, and a saturated fatty acid ethyl ester (SAT EE) rich fraction, which could be used as biodiesel. The temperature that promoted the best separation between PUFA and SAT EE, was −18 °C, resulting in a liquid fraction with 91.6% (*w*/*w*) DHA, and a solid phase with 88.2% of SAT and monounsaturated fatty acid ethyl ester (MONOUNSAT), which could be used for biodiesel purposes after a hydrogenation step.

Keywords: *Crypthecodinium cohnii*; low-cost substrates; glycerol; corn steep liquor (CSL); biorefinery; lipids; urea complexation; Docosahexaenoic acid (DHA); biodiesel

Citation: Moniz, P.; Martins, D.; Oliveira, A.C.; Reis, A.; Lopes da Silva, T. The Biorefinery of the Marine Microalga *Crypthecodinium cohnii* as a Strategy to Valorize Microalgal Oil Fractions. *Fermentation* **2022**, *8*, 502. https://doi.org/10.3390/fermentation8100502

Academic Editors: Abdelrahman Zaky and Abd El-Fatah Abomohra

Received: 12 August 2022
Accepted: 21 September 2022
Published: 30 September 2022

1. Introduction

The beneficial role of polyunsaturated fatty acids (PUFAs) in human health is well known [1,2]. These compounds include ω-3 lipids, which play important roles in the human body as components of the phospholipids that form the structures of cell membranes. Since human bodies do not make essential fatty acids, it is recommended to take them up through the diet [3,4].

Docosahexaenoic acid (DHA) is a ω-3 fatty acid (22:6ω3) which is well known for its benefits regarding several diseases, as it may help to prevent or improve chronic conditions, such as heart disease, certain cancers, Alzheimer's disease, depression and inflammatory conditions like rheumatoid arthritis [5,6].

Fatty fish species, such as salmon, menhaden, herring, mackerel, and sardine are rich in ω-3 PUFAs, including DHA [4,7,8]. However, the ongoing declining global fish stocks cannot sustain the supply of ω-3 fatty compounds. Moreover, the quality of fish oil is highly variable, depending on season, fish species, and catching site location. In addition, usually fish oils present a disagreeable smell which is not attractive for consumers. Another issue is the fact that they may be contaminated by toxic compounds, such as heavy metals (such as mercury, cadmium) and polychlorobiphenyls (PCBs), making them unsuitable to be used in food and feed, in infant formulas, or in pharmaceutical formulations. Furthermore, as marine fish oil is composed of fatty acids with different lengths and degrees of unsaturation, further expensive DHA concentration and purification steps may be required, before application [9].

Despite PUFAs being found in fish oils, the maximum quantities of triacylglycerols of PUFA in such products are generally 18–25%, which is low. To enrich the PUFA levels in foods, eicosapentaenoic acid (EPA, 22:5ω3) and DHA from fish oils are usually purified and stabilized as ethyl esters (EEs) and are sold as dietary supplements [10–12].

Oil from several microalgal species is rich in PUFA, cholesterol free, contaminant free, and tastes good. The fatty acid profile of the heterotrophic marine microalga *C. cohnii* is unique, since the DHA proportion can be up to 65% of total fatty acids (TFAs) [13]. Other polyunsaturated fatty acids (PUFAs) remain below 1% of TFA content, which requires the DHA purification step, since most PUFAs contains intermediate fatty acids that hamper the DHA fractionation and concentration [14,15].

Published works reporting *C. cohnii* DHA production and concentration do not consider the remaining lipid fraction composed of saturated (SAT) and monounsaturated (MONOUNSAT) fatty acids after PUFA removal [16,17], this fraction neglected. However, this lipidic fraction, obtained after DHA extraction, can also be considered for biodiesel production. In the approach purposed in the present work, similar to a biorefinery, all the microalgal lipidic fractions are valorized and have potential commercial uses [18], the lipidic fraction rich in PUFA possibly being directed for food/pharmaceutical purposes, and the other saturated/monounsaturated fraction directed for biofuels production. In this way, there is no waste resulting from the microalgal lipid fractionation.

Several methods have been used to concentrate/purify PUFA produced from oleaginous microalgae, which include molecular distillation [19], fractional crystallization [20], solvent extraction [21], urea complexes [10,11,16], simulated moving bed chromatography [22], supercritical fluid chromatography [23] and preparative HPLC [24]. Molecular distillation uses high temperature under vacuum to eliminate impurities, which can result in the degradation of the fatty acids by oxidation, polymerization and production of trans-isomers of ω-3 fatty acids [14], while chromatographic methods are expensive, particularly at large scale.

Urea complexation is considered one of the most efficient and cost-effective methods for PUFA concentration, suitable for large-scale applications [25–27]. Urea molecules readily form solid-phase complexes with SAT and MONOUNSAT, but not with PUFA. This is because pure urea crystallizes as a tightly packed tetragonal structure with channels of 5.67 Å of diameter. SAT has straight chain molecules forming a hexagonal crystal with urea in 8–12 Å channel diameter [16]. Monoenes are more readily complexed as compared to dienes, which, in turn, are more readily complexed than trienes. In this way, PUFAs are easily separated from the remaining fatty acids This method requires inexpensive solvents, such as methanol, ethanol or hexane, uses mild conditions, and the separation is more efficient and cheaper than other methods, such as fractional crystallization or selective solvent extraction [28]. Importantly, urea complexation protects the PUFA from autoxidation [12].

The feedstock used in media formulations for microbial growth usually determines the bioprocess costs. In this way, it is crucial to use low-cost substrates, to reduce the overall costs [29]. Nevertheless, there are still many published studies that use expensive nutrients, such as glucose [15,30,31] and yeast extract [15,31], to produce DHA from *C. cohnii*.

The present work reports *C. cohnii* growth on a low-cost culture medium containing raw glycerol as the carbon source, and starch industry derived corn steep liquor (CLS) as the nitrogen source, developed in a 7L bioreactor, under the fed-batch regime, in order to enhance microalgal biomass and lipid production. Afterwards, the microalgal biomass underwent a saponification reaction to produce FA soaps, which were further converted into FA ethyl ester (FA EE). Urea was added to this EE mixture, at different temperatures, in order to promote two lipidic fractions, according to the FA EE saturation degree: a PUFA EE rich fraction, with potential uses in the food, pharmaceutical or cosmetic industries, and a saturated/monounsaturated fraction, with potential application in the biodiesel industry.

This is the first work reporting on a *C. cohnii* biomass biorefinery to obtain two lipidic fractions with potential commercial applications. The method here reported is simple,

inexpensive, valorizes all microalgal lipidic fractions avoiding waste generation during the overall process, and can be applied to other oleaginous microalgae.

2. Materials and Methods

2.1. C. cohnii Starter Cultures

Crypthecodinium cohnii ATCC 30772 was purchased from American Type Culture Collection. The microalgal cultures (starters) were maintained in a medium composed of yeast extract (YE) (1.8 g/L, Oxoid), sea salt (OceanusIberia, Queluz, Portugal) (23 g/L) and glucose monohydrate (9.9 g/L) (Acros Organics, Geel, Belgium) which were monthly re-inoculated.

2.2. Bioreactor Cultivations

2.2.1. Inoculum

Inocula were prepared from the starter cultures, by inoculating 500 mL Erlenmeyers containing 150 mL of growth media containing yeast extract 2 g/L, sea salt 25 g/L and glucose (20 g/L). These cultures were incubated for 6 days, at 120 rpm, 27 °C.

2.2.2. Bioreactor Experiments

The 7L-bioreactor (Electrolab, FerMac 360 EMC) was equipped with two Rushton turbines and contained 2700 mL of the growth medium growth with the following composition: sea salt 25 g/L, yeast extract 0.5 g/L and 4.59 g/L of corn steep liquor. The pH and temperature were set at 6.5 and 27 °C, respectively. The aeration rate was set at 1 vvm, and the agitation rate was adjusted according to dissolved oxygen (DO) tension readings, being manually adjusted so that the DO never decreased below 30%. In this way, avoidance of oxygen limiting conditions was expected.

After a batch growth period, pulses containing glycerol, CLS and YE were added to the culture (20 g/L, 1.83 g/L, and 0.5 g/L of glycerol, CSL and YE, respectively) as final concentrations, in order to extend the active microalgal growth phase and induce the intracellular lipid accumulation, so that higher lipid productivities could be achieved.

2.3. Analytical Methods

2.3.1. FAEE Quantification

C. cohnii lipids were analyzed as TFA. The method was the same as described by Moniz et al. [32], except that ethanol replaced methanol in the acetyl chloride solution, since ethanol is less toxic than methanol and the final DHA EE, after the EE, was intended for food/pharmaceutical and cosmetic purposes. Microalgal biomass collected after the broth centrifugation was freeze-dried. Approximately 100 mg of freeze-dried biomass were transferred to a vial under nitrogen atmosphere and trans-ethylated at 80 °C for 1 h, with 2 mL of an ethanol/acetyl chloride mixture (95:5 *v*/*v*) and 0.2 mL of heptadecanoic acid (17:0) (5 mg/mL petroleum ether, boiling point 80–100 °C) as an internal standard. Afterwards, the vial contents were cooled, diluted with 1 mL water, and the lipids were extracted with 2 mL of n-heptane. The organic phase was separated from the aqueous phase, dried using sodium sulfate (Na_2SO_4) and placed in a vial adequate for gas chromatography analysis. The EE were then analyzed by gas–liquid chromatography, on a Bruker Scion 436-GC (Munich, Germany) equipped with a flame ionization detector. Separation was carried out on a 0.32 mm × 30 m fused silica capillary column (film 0.32 mm) Supelcowax 10 (Supelco, Bellefonte, PA, USA) with helium as carrier gas, at a flow rate of 3.5 mL/min. The column temperature was programmed at an initial temperature of 200 °C. For FAEE analysis after urea complexation, 0.2 mL of heptadecanoate ester (as internal standard) was added to the samples.

2.3.2. Flow Cytometry

Flow cytometry (FC) analysis was performed in a Cytoflex Beckman-Coulter flow cytometer, equipped with a blue laser, FSC/SSC light scattering detectors and five fluorescence detectors, using the protocol described in Moniz et al. [32].

2.3.3. *C. cohnii* Biomass Saponification and EE Fractionation

Figure 1 shows *C. cohnii* biomass saponification and EE separation. The method was based on that reported by Mendes et al. [16] and Lopes da Silva et al. [33], with improvements. In this work, the EE fractionation by urea addition at different temperatures (25 °C, 4 °C and −18 °C) was performed. In this way, this procedure was faster and simpler than that previously reported.

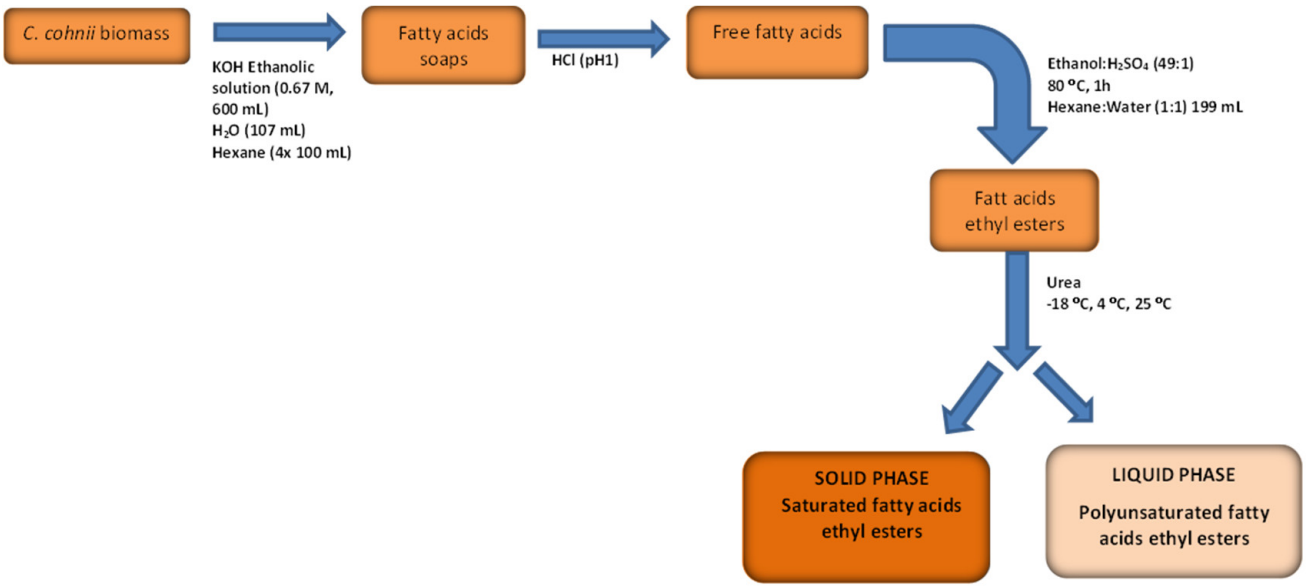

Figure 1. *C. cohnii* biomass saponification and fatty acid ethyl esters fractionation using urea complexation at different temperatures.

Biomass Saponification

An amount of 600 mL of a KOH 0.4 M solution were added to a tube containing 5 g of freeze-dried biomass, previously milled with a pestle in a mortar. The tubes were incubated at 20 °C, under nitrogen atmosphere, at a constant stirring rate of 100 rpm, overnight. Afterwards 107 mL of distilled water were added, followed by the addition of 100 mL of hexane to promote phase separation. After agitation, the upper organic phase, containing the unsaponifiable matter was removed and transferred to another vessel. The saponifiable hydroalcoholic phase, containing the fatty acid soaps, was then acidified to a pH 1 by adding an HCl 5 M solution. The free fatty acids (FFAs) were then recovered after four extractions with 50 mL of n-hexane. Afterwards, the organic solvent was evaporated in a Büchi rotary evaporator R-200, in a water bath at 30 °C, to avoid lipid degradation. The recovered hexane was used in further extractions. The FFA residue obtained after the hexane removal were filtered by membrane Whatman N° 1 to remove the impurities and hexane traces.

Ethylation

The FFAs were converted to fatty acid ethyl ester (FA EE) which were further quantified and fractionated. An amount of 204 mL of a solution of absolute ethanol and H_2SO_4 95–97% (49:1) was added to the FA EE and the mixture was incubated at 80 °C, for 1 h. After cooling, 100 mL of n-hexane and distilled water mixture (1:1) was added, in order to promote the phases separation and extraction of the FA EE present in the sample. The organic solvent was removed from the FAEE phase in the Büchi rotary evaporator R-200, followed by nitrogen continuous stream evaporation. The FAEE phase was resuspended in 2 mL hexane and stored at −18 °C for further GC analysis and fractionation.

Urea Crystallization and Fractionation

An amount of 5 mL of a solution containing 1.60 g of urea dissolved in 50 mL ethanol at 60 °C and 750 rpm, was added to 150 µL of the FA EE obtained in the previous step, in a tube (the ratio urea/FAEE was 4.0, according to Mendes et al. [16]). Afterwards, the tubes were stored overnight at different temperatures (−18 °C, 4 °C and 25 °C).

After storage, two fractions were formed. The liquid phase, containing PUFA EE was separated from the solid phase, containing the mono and saturated ethyl esters (MONO EE and SAT EE, respectively) retained in the urea complexes, by vacuum filtration. After filtration, the solid phases were resuspended in 1 mL hexane, in order to recover the MONO EE and SAT EE. This procedure was carried out three times.

2.3.4. Estimation of Solid Phase Properties Based on its FA EE Profile

The EE profiles of the solid phases, obtained after urea complexation at −18 °C, 4 °C and 25 °C, were used to estimate some parameters that attested the biodiesel quality.

In the first approach, the equations developed for FAME [34,35] were used to estimate the iodine value (IV), saponification value (SV), cetane number (CN), long-chain saturation factor (LCSF) and cold filter plugging point (CFPP) for the FAEE, since the structural differences between these fatty acid alkyl esters are imparted by the alcohols used in their production:

$$IV = \sum(254 \times D \times N)/M \quad (1)$$

$$SV = \sum(560 \times N)/M \quad (2)$$

$$CN = 46.3 + (5458/SV) - (0.225 \times IV) \quad (3)$$

$$LCSF = (0.1 \times C16) + (0.5 \times C18) + (1 \times C20) \quad (4)$$

$$CFPP = (3.1417 \times LCSF) - 16.477 \quad (5)$$

where N is the percentage of each FA EE, D is the number of double bonds, M is the molecular mass of each FA EE.

3. Results and Discussion

3.1. C. cohnii Fed-Batch Fermentation

Figure 2 shows the biomass and substrate concentrations, DO, TFA and DHA percentages, and subpopulation profiles during *C. cohnii* fed-batch cultivation. The culture entered the exponential phase at t = 24 h, with a specific growth rate (µ) of 0.07/h, attaining the stationary phase at t = 72 h, and reaching a biomass concentration of 4.0 g/L (Figure 2a). Lower *C. cohnii* specific growth rates have been reported when the microalga was grown on glycerol, compared to other carbon sources.

De Swaaf et al. (2003) [36] reported a specific growth rate of 0.05/h for a *C. cohnii* culture grown in a 2L bioreactor working under fed-batch mode continuously fed with ethanol. Cui et al. (2018) [37] also reported a specific growth rate of 0.05/h for *C. cohnii* growth on glucose, in 250 mL shake flasks. However, Berzins et al. (2022) [38] reported *C. cohnii* specific growth rates of 0.05 0.02 and 0.05/h when the microalga was grown on glucose, glycerol and ethanol, respectively, in 1 L shake flasks. Therefore, the specific growth rate reported in this work was higher than that reported by Berzins et al. (2022) [38] for glycerol. This difference might be due to the different systems used to grow the microalga, since cells grown in shake flasks [38] are usually exposed to adverse conditions, such as oxygen limitation and/or uncontrolled medium pH, which are overcome in a bioreactor (as used in this work), due to a higher mass transference (as a result of a better aeration, agitation and mixing) and medium pH control.

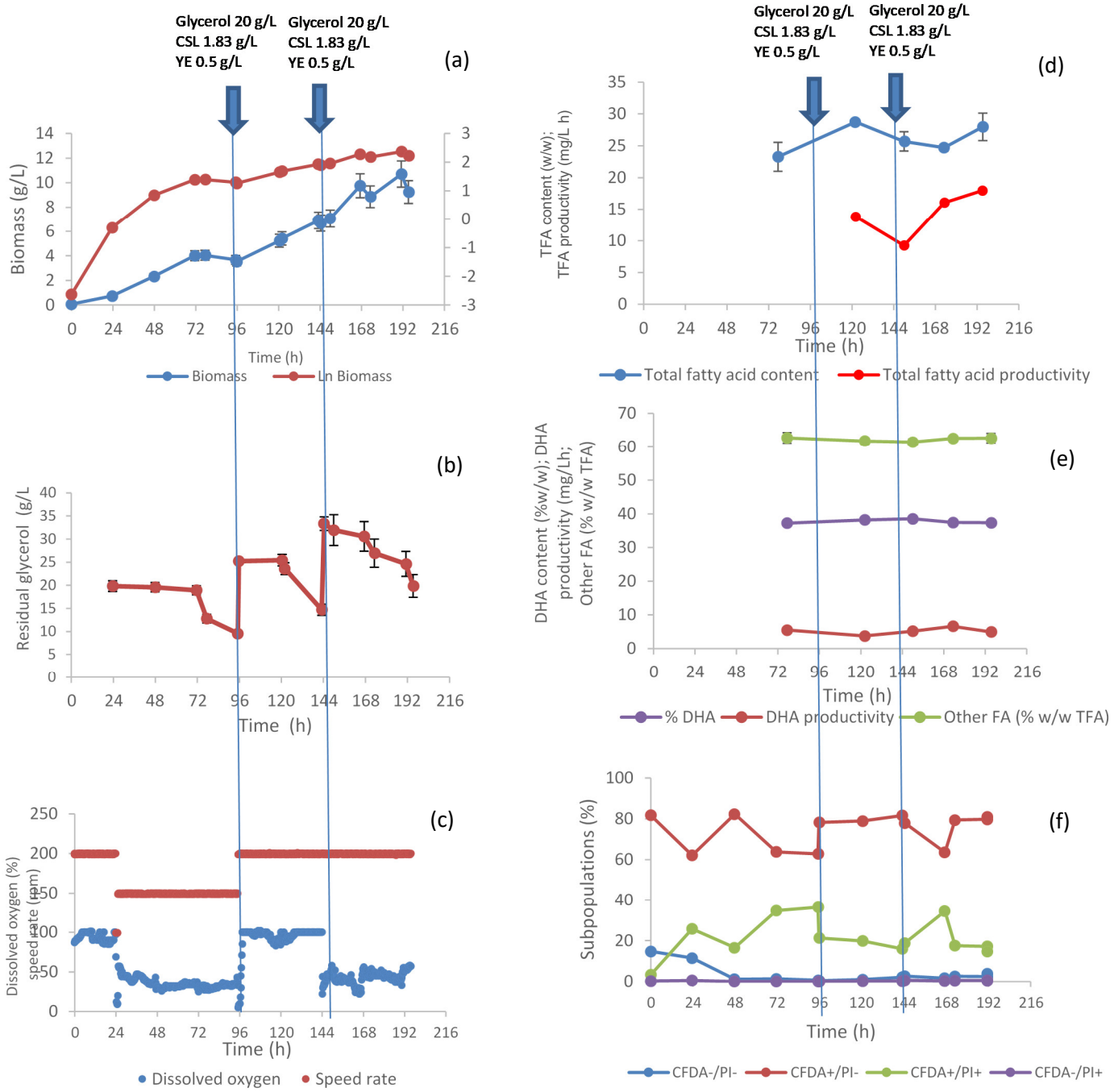

Figure 2. *C. cohnii* profiles during a fed-batch fermentation in a 7L bioreactor. (**a**) Biomass concentration and natural logarithmic; (**b**) Residual glycerol concentration; (**c**) DO and speed rate; (**d**) TFA content and productivity; (**e**) DHA content, DHA productivity and other fatty acids percentage; (**f**) *C. cohnii* subpopulation cells stained with CFDA and PI and analyzed by FC. Subpopulation CFDA+/PI− included cells with intact membranes with enzymatic activity; Subpopulation CFDA+/PI+ included cells with injured membranes and enzymatic activity; Subpopulation CFDA−/PI+ included cells with injured membranes and no enzymatic activity. Biomass, residual glycerol concentrations, TFA and DHA content values are the average value of two independent replicates with a standard deviation ($n = 2$) represented as error bars. Biomass productivity (g/L h) was calculated as follows: $X_t - X_0/(t_t - t_0)$, where X_t is the biomass concentration at the instant t, X_0 is the biomass concentration at t_0 (inoculation time); the same formula was used to calculate TFA and DHA productivities.

At t = 96 h the glycerol concentration attained 9.6 g/L (Figure 2b). In order to extend the microalgae growth, a pulse containing glycerol, CSL and YE (final concentration of 20 g/L, 1.83 g/L and 0.5 g/L, respectively) was manually added. Indeed, the biomass concentration slightly increased up to 6.7 g/L at t = 144 h, while the glycerol concentration attained 14.7 g/L. A final nutrient pulse was added at that time, in order to ensure glycerol (carbon) excess conditions, to promote the intracellular lipid synthesis. At the end of the cultivation, the glycerol concentration was 19.8 g/L and the biomass concentration attained 9.2 g/L.

The DO percentage was maintained above 30% by adjusting the speed rate, to prevent oxygen limiting conditions (Figure 2c). In fact, beyond carbon excess conditions, *C. cohnii* cells require oxygen excess conditions, since the microalga is an obligate aerobic microorganism [39].

The TFA content attained 23.3% (*w*/*w*) at the end of the batch phase, increasing after the nutrient pulse addition. At the end of the assay (t = 195 h), the TFA content was 28.0% (*w*/*w*), corresponding to the lipid productivity of 18.0 mg/L h, the highest attained during this cultivation. The TFA average productivity was 13.5 mg/L h. The nutrient pulse additions increased the TFA from 23.3% to 28.0% (*w*/*w*) (Figure 2d).

The DHA proportion in TFA was almost constant over the assay, around 38% (*w*/*w* TFA (Figure 2e). The DHA productivity was also stable during the cultivation (~5 mg/L h). The remaining fatty acids proportion also remained stable (~60% *w*/*w* TFA) (Figure 2e).

Moniz et al. [32] used biodiesel derived glycerol, previously distilled, and CSL from the starch industry, as carbon and nitrogen sources, respectively, in the medium composition to grow *C. cohnii* ATCC 30772, in a 7L-bioractor, working under batch regime. At the end of the fermentation, the authors reported a biomass concentration, lipid content, lipid productivity and DHA productivity of 5.3 g/L, 11.0% (*w*/*w*), 4.0 mg/L h and 1.6 mg/L h, respectively. In the present study, the biomass concentration, lipid content, lipid productivity and DHA productivity were 9.2 g/L, 28.0% (*w*/*w*), 13.5 mg/L h and 5.1 mg/L h, respectively, at the end of the cultivation (Table 1). As expected, under fed-batch regime, the lipid productivity increased 63% relative to the lipid productivity reported for *C. cohnii* batch cultivation, which highlights the benefits of using fed-batch cultivations for lipid production, since the addition of nutrient pulses improves microalgal biomass and lipid production, compared to batch cultivations.

Table 1. Low-cost substrates used to grow *C. cohnii* for lipid and DHA production.

Strain	Low-Cost Substrate	Cultivation System	Biomass Concentration (g/L)	Lipid Content (% *w*/*w*)	Lipid Productivity (mg/L h)	DHA Productivity (mg/L h)	Reference
C. cohnii CCMP 316	Carob pulp syrup	2L-bioreactor	42.0	9.2	38.5	18.5	[39]
C. cohnii ATCC 30772	Rapeseed meal hydrolysate + waste molasses	250 mL shake flasks	2.9	27.7	4.7	0.5	[40]
C. cohnii CCMP 316	Cheese whey + CSL	250 mL shake flaks	-	28.7	-	-	[41]
C. cohnii ATCC 30772	Raw glycerol + CSL	7L-biorector/batch	5.3	11.0	4.0	1.6	[25]
C. cohnii ATCC 30772	Raw glycerol + CSL	7L-bioreactor/ fed-batch	9.2	28.0	13.1	5.1	This work

Flow cytometry wa used to monitor *C. cohnii* cultures, as it allows rapid, simultaneous and quantitative measurements related to cell morphology and physiology [40,41]. The flow cytometric analysis revealed that most of the *C. cohnii* cells (>60%) maintained their membranes intact (subpopulation CFDA+, PI−) during the cultivation (Figure 2f). The use of flow cytometry to monitor *C. cohnii* growth on low-cost substrates, as biodiesel derived glycerol and CLS, was crucial to understand the impact of these feedstocks on the microalga cell status. In this work, the high proportion of healthy cells throughout the cultivation (always higher than 60%) demonstrated that they were not exposed to harsh conditions during the cultivation. These results demonstrated the efficient use of

crude glycerol and CSL as a carbon and nitrogen source, respectively, to produce DHA and lipids from *C. cohnii* cells, since most of them maintained their enzymatic systems and intact membranes.

Taborda et al. [42] used, for the first time, raw glycerol to grow *C. cohnii*. The authors studied several pure carbon sources (glucose, acetate and glycerol) and complex substrates (sugarcane molasses, raw glycerol, and industry vinegar effluent) in culture medium for *C. cohnii* ATCC 30772 growth and lipid production. They concluded that the complex substrate that led to the highest lipid and DHA content and productivity was raw glycerol [14.7 (*w*/*w*), 3.19 mg/Lh, 44.7% DHA of TFA (*w*/*w*), 1.43 mg/Lh, respectively], which showed better results even than pure glycerol. As expected, *C. cohnii* lipid production reported in this work was higher than that reported by Taborda et al., who carried out their microalgal cultivations in 1L shake flasks, [42], for raw glycerol,. Hosoglu and Elibol (2017) [43] concluded that the highest *C. cohnii* CCMP 316 biomass and lipid content (7.3 g/L and 36.5% (*w*/*w*), respectively) were attained when pure glycerol was used as carbon source, when compared to glucose. Berzins et al. [38] studied *C. cohnii* CCMP 316 growth and DHA production using glucose, ethanol and glycerol as carbon sources, and showed mathematical modeling results which demonstrated that glycerol had the best experimentally observed carbon transformation rate into biomass, reaching the closest values to the theoretical upper limit. The authors concluded that crude glycerol was readily consumed by *C. cohnii*, making this feedstock an attractive substrate for DHA production from this microalga, which corroborated the results reported in the present work.

Importantly, Moniz et al. [32] have demonstrated that using low-cost carbon and nitrogen sources, such as biodiesel derived glycerol and CSL from the starch industry, for *C. cohnii* lipid production, can represent up to 84% in cost savings, relative to conventional carbon and nitrogen sources (glucose and yeast extract) used for *C. cohnii* lipid production. In fact, the culture medium cost can account for up to 30% of the total production costs in commercial fermentations [44]. This means that efforts must be made to reduce the medium cost, by using low-cost substrates as industrial byproducts. This approach not only contributes to reducing the overall bioprocess costs, but also contributes to a circular economy-based society, which is based on three principles: eliminate waste and pollution, circulate products and materials (at their highest value), and regenerate Nature [45].

Nevertheless, there are still only a few works reporting the use of low-cost substrates for *C. cohnii* growth and lipid/DHA production (Table 1).

Mendes et al. [46] used carob pulp syrup as carbon source to grow *C. cohnii* CCMP 316, and reported final biomass concentrations of 42.0 g/L, 9.2% *w*/*w* of lipids, 38.5 mg/L h of lipid productivity and 18.5 mg/L h DHA productivity, after 104 h of growth (Table 1). These results are higher than those reported in the present study. However, Mendes et al. [46] used yeast extract as nitrogen source, which may explain their higher results in terms of lipid and DHA productivities, since yeast extract, a very expensive product, is a complex mixture that contains all the nutrients required for heterotrophic growth.

Rapeseed meal hydrolysate and waste molasses were also used in the culture medium used to grow this microalga in shake flasks for DHA production, resulting in lower results (2.9 g/L biomass concentration, 27.7% (*w*/*w*) lipid content, 4.7 mg/L h lipid productivity, and 1.5 mg/L h DHA productivity [47] (Table 1).

Isleten-Hosoglu and Elibol [48] used cheese whey and CSL to grow *C. cohnii* CCMP 316 and reported a similar lipid content (27.8% *w*/*w*) to the one reported in the present work (28.0 *w*/*w*).

Overall, the microalga lipid content varied from 9% to 28% (*w*/*w*) when *C. cohnii* was cultivated on low-cost substrates (Table 1).

3.2. C. cohnii FAEE Fractionation

The *C. cohnii* biomass production step was followed by biomass saponification and lipid fractionation using the urea complexation method, which has been successfully

used to concentrate PUFA in diverse lipidic materials, including vegetable and fish microalgal oils [11,16,27,33].

A major concern of this work was the use of non-toxic and environmentally friendly chemicals, since the liquid fraction, rich in PUFA, was proposed to be directed for human consumption. Therefore, the FFA obtained after the saponification step was converted into EE before the addition of urea (Figure 1), since ethanol is less toxic than methanol [49]. Indeed, several authors have enriched PUFA EE, rather than PUFA methyl ester (ME), from lipidic samples [11,50,51]. The main sources of EPA and DHA are currently commercial formulations based on oral ethyl ester [10]. Conversely, Wanasundara and Shahidi (1999) [52] and Kaliban et al. [53] converted the FFA, obtained after the saponification, into an ME mixture, which might compromise the adequacy of the DHA ME concentrated fraction for human consumption. Other authors have concentrated PUFA compounds from lipidic samples as FFA using the urea complexation method [27], but these compounds are highly prone to oxidation, requiring a further esterification step to convert them into triacaylaglycerols.

Figure 3 and Table 2 show the FA EE profiles before and after *C. cohnii* biomass saponification and urea complexation at different temperatures.

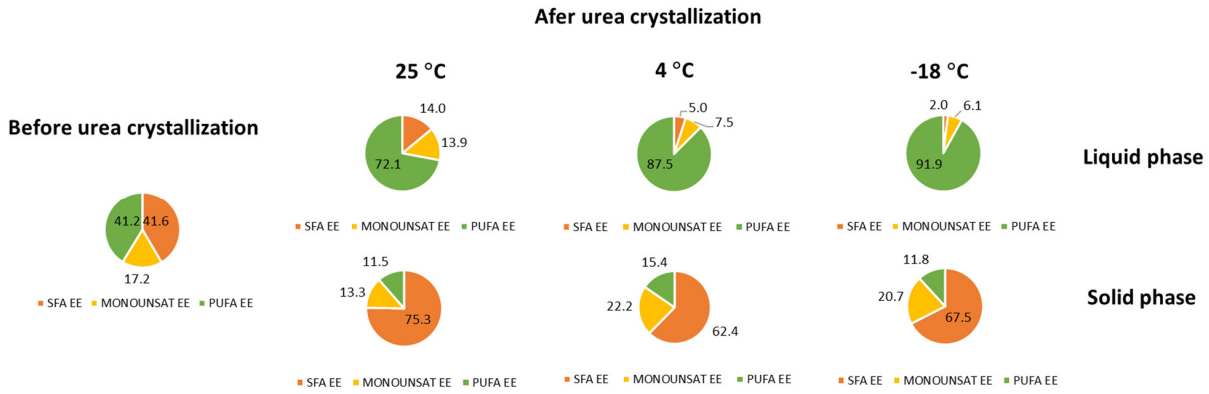

Figure 3. SAT, MONOUNSAT and PUFA EE percentages before and after urea crystallization.

Table 2. *C. cohnii* FA EE percentages (*w*/*w*) before and after urea crystallization.

	EE Profile before Urea Crystallization	EE Profile after Urea Crystallization					
		Liquid Phase			Solid Phase		
FAEE		25 °C	+4 °C	−18 °C	25 °C	+4 °C	−18 °C
10:0	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0
12:0	4.0 ± 0.2	3.0 ± 0.2	1.8 ± 0.1	1.2 ± 0.3	3. ± 0.18	4.8 ± 0.2	4.6 ± 0.2
14:0	17.4 ± 0.5	7.6 ± 0.4	2.4 ± 0.1	0.7 ± 0.0	27.7 ± 1.9	25.4 ± 1.3	27.2 ± 1.4
14:1ω5	0.7 ± 0.0	0.9 ± 0.0	0.0 ± 0.0	1.0 ± 0.0	0.1 ± 0.0	0.6 ± 0.4	0.0 ± 0.0
16:0	19.2 ± 0.0	3.4 ± 0.2	0.8 ± 0.0	0.0 ± 0.0	41.9 ± 2.1	30.5 ± 1.5	34.3 ± 1.7
16:1ω9	2.7 ± 0.1	2.5 ± 0.1	2.1 ± 0.1	2.0 ± 0.1	1.7 ± 0.8	3.0 ± 1.0	2.6 ± 0.0
18:0	0.7 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 0.1	1.1 ± 0.0	1.2 ± 0.0
18:1ω9	13.8 ± 0.3	10.5 ± 0.5	5.3 ± 0.03	3.2 ± 0.2	11.6 ± 0.6	19.0 ± 0.9	18.0 ± 0.9
18:2ω6	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0
22:5ω3	0.4 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:6ω3	40.6 ± 0.3	71.8 ± 3.6	87.2 ± 4.4	91.6 ± 4.6	11.4 ± 0.6	15.2 ± 0.8	11.8 ± 1.1
SAT	41.6 ± 0.5	14.0 ± 0.4	5.0 ± 0.2	2.0 ± 0.1o	75.1 ± 2.5	61.7 ± 2.0	67.31 ± 2.2
MONOUNSAT	17.2 ± 0.3	13.9 ± 0.5	7.5 ± 0.3	6.1 ± 0.2	13.3 ± 0.6	22.65 ± 1.0	20.65 ± 0.9
PUFA	41.0 ± 0.3	72.1 ± 3.6	87.5 ± 4.4	91.9 ± 4.6	11.4 ± 0.6	15.94 ± 0.8	11.82 ± 0.6

The temperature that allowed the highest PUFA EE enrichment in the liquid phase was −18 °C, resulting in an increase from 41.3% (before urea complexation) to 91.9% (after urea complexation) (Figure 3, Table 2).

The proportion of SAT EE and MONOUNSAT EE in the solid phase was higher at 25 °C (75.3% + 13.3% = 88.8%), although no significant changes in SAT EE, MONOUNSAT EE and PUFA EE percentages occurred for the other temperatures.

The dominant PUFA EE in the liquid fraction was DHA EE, being above 72% in all fractions, at different temperatures (Table 2). DHA EE percentage increased from 40.6% to 91.6%, after urea complexation at −18 °C, which corresponded to an increase of 125%. A significant decrease in PUFA EE and DHA EE percentages were observed as the temperature increased from −18 °C to 25 °C (91.9%, 87.5% and 72.1% for PUFA EE, and 91.6%, 87.2% and 71.8% for DHA EE percentage, at −18 °C, 4 °C and 25 °C, respectively (Table 2, Figure 3). This was accompanied by an increase in SAT EE and MONOUNSAT EE, which increased from 2.0% to 14.0%, and 6.1% to 13.9%, respectively, when the temperature ranged from −18 °C to 25 °C.

The dominant SAT EEs in the solid fraction were palmitic (16:0) and myristic (14:0) acids. The proportion of palmitic acid increased up to 41.9% in the solid phase, after urea complexation at 25 °C, while the myristic acid (14:0) only increased up to 27.7%. The EE mixture before the urea complexation contained a low percentage of stearic acid EE (18:0, 0.7%) and this EE was completely retained by the urea molecules in the solid phase, since no 18:0 was detected in the liquid phase.

Part of the MONOUNSAT EE (14:1ω5, 16:1ω, 18:1ω9) was retained in the urea molecules, and another part was also present in the liquid phase (Table 2). Toumi et al. [27], who fractioned *Chlorella sorokiniana* lipids using the urea complexation method, reported a similar distribution of MONOUNSAT FFA in the liquid and solid phases after urea complexation, but Senanayake and Shahidi [17] stated that these compounds were mostly retained in the urea (solid) fraction.

The highest PUFA EE enrichment obtained in the liquid phase, at the lowest temperature (−18 °C), was attributed to the lower urea solubility in ethanol at lower temperatures, which promoted more efficient SAT EE retention in the urea phase [15]. This liquid phase, containing 91.6% of DHA, may be used in food, pharmaceutical and cosmetic industries. This result was higher than that described by Toumi et al. 2022 [27], who used a urea/FFA ratio of 3:1, at 6 °C, reporting a *C. sorokiniana* liquid fraction containing 81% PUFA, 11% MONOUNSAT and 7.5% SAT FFA. Kabilan et al. [53] concentrated *Thraustochytrium* sp. DHA methyl ester from 45% of TFA to 82% of TFA in the liquid phase, after urea addition, at 25 °C, at a urea/FAME of 3.3. The better result obtained in the present work (the DHA percentage increased from 40.6%, to 91.6% in the liquid phase) can be attributed to the lower temperature at which the sample with the urea was incubated (−18 °C), and/or to the higher urea/FA EE ratio used in this work (4:1). In another study *C. cohnii* oil was enriched in DHA from 47.7 to 97.1% of the TFA fraction by using an urea:fatty acid ME ratio of 3:1 at 4 °C [46]. The liquid fraction comprised 98.2% of PUFA, whereas the urea complexing solid fraction comprised 66.9% of SFA and MUFA. Although Mendes et al. (2007) [46] reported a higher PUFA ME enrichment, the use of methanol is a disadvantage if the final product is directed for human consumption.

Figure 4 shows the recovery yield (RY), defined as the ratio between the initial EE mixture weight, before the urea addition step, and the weight of each EE fraction obtained after the urea addition, at different temperatures. It can be seen that, as the temperature was dropped, the RY decreased for the liquid phase (attaining 12.1%, 11.5% and 18.6% at −18 °C, 4 °C and 25 °C, respectively). The opposite was observed for the solid phase, wherein RY increased, as the temperature was lower (115.8%, 88.3% and 56.5% at −18 °C, 4 °C and 25 °C, respectively). It was also observed that the liquid phase RYs were lower than the solid phase RYs, which was attributed to losses during the filtration step. This result was supported by Toumi et al. [27], who reported a solid phase yield 3-fold higher than the liquid phase yield. Setyawardhani et al. [28] studied the effect of the temperature (−15 °C, 5 °C and 30 °C) on urea crystallization with corn oil, aiming at PUFA concentration, having concluded that urea crystallization, at lower temperatures, produced higher PUFA

concentration. However, this condition led to lesser yield. These results also supported those reported in the present work.

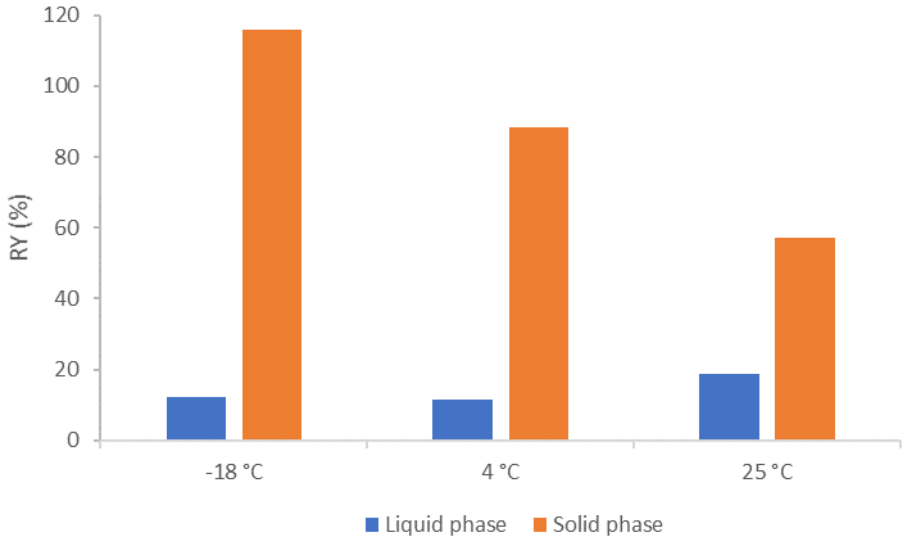

Figure 4. EE fractions recovery yields (RY).

3.3. Theoretical Estimation of FAEE Solid Phase Quality, as Biofuel

Some FA EE properties as biofuel (Table 3) were estimated from the fatty acid composition of the solid phases that resulted from the urea complexation at −18 °C, 4 °C, and 25 °C. The cetane number (CN), a measure of the readiness of the fuel to auto-ignite when injected into an engine, was within the biodiesel specification, as well as the iodine value (IV), a parameter related to the fatty acid alkyl ester unsaturation level. The IV value was always lower than the limit value (120 g I2/100 g), which was indicative of good oxidation stability of these FA EE mixtures. In addition, in all cases, the proportion of linolenic acid (18:3) was below the level limit (12% *w*/*w*). However, a considerable amount of polyunsaturated alkyl esters (≥4 double bonds) was still present in the FA EE solid phases (>11%). According to Ramos et al. (2009) [54], biodiesel oxidation stability decreased with increase of PUFA ME content. Therefore, an additional reactional step (e.g., hydrogenation reaction) should be considered to decrease the level below 1% (*w*/*w*) in *C. cohnii* solid fraction.

Table 3. Theoretical estimate of FAEE quality.

	Estimated Values Solid Phase				
	Temperature			**Limits EN 14214 (Europe)**	**Limits ASTM D6751 (USA)**
Parameter	25 °C	4 °C	−18 °C		
Iodine value, IV (g I2/100 g)	59.8	85.6	69.4	<120	-
Saponification value, SV (mg KOH/g)	189	201	202	-	<370
Cetane number, CN	61	55	58	>51	>47
LCSF (% *w*/*w*)	5	4	4	-	-
CFPP (°C)	−0.9	−5.1	−4.0	(Class C)	<5
Polyunsaturated (≥4 double bonds) alkyl esters (% *w*/*w*)	11.4	15.2	11.8	<1	-
C18:3 (% *w*/*w*)	Not det.	Not det.	Not det.	<12	-

Not det.—Not detected.

Since the optimal temperature for PUFA EE enrichment was −18 °C, and considering that the quality of the solid phase, in terms of biodiesel purposes, obtained at −18 °C was similar to the remaining solid fractions obtained at 4 °C and 25 °C, the EE mixture fractionation should be carried out at −18 °C. Under these conditions, the liquid fraction

rich in DHA EE (>90% *w*/*w*) may be directed to food, pharmaceutical and cosmetic industries [55], although additional studies are needed to confirm its suitability for human consumption [27]. The resultant solid phase may be directed to biodiesel purposes, after a hydrogenation step.

4. Conclusions

This work presents a novel process for *C. cohnii* biomass production using low-cost nutrients (crude glycerol and CSL), followed by microalgal lipid fractionation, to obtain a lipid fraction rich in DHA EE, that can be used in food, pharmaceutical and cosmetic industries, and another lipid fraction, rich in SAT EE, that may be used as biodiesel, after a hydrogenation step. This approach, never reported before, valorizes all microalgal lipid fractions and contributes to reducing the overall costs of microalgal lipid heterotrophic production, thus accomplishing the three principles of the circular bioeconomy.

Since this study represents an innovative and environmentally friendly process for DHA and biodiesel industrial production from *C. cohnii*, increasing the potential revenue generated by the whole process, large-scale *C. cohnni* fermentations are now in progress, to evaluate the practical and economic feasibility of the microalgae lipids fractionation scale-up.

However, despite several studies considering the urea complexation method as an efficient and economic approach for fractionation and purification of oils, further studies are needed to ensure the technical, environmental, and economic sustainability of this process applied to microalgae lipids.

Author Contributions: Conceptualization, T.L.d.S. and A.R.; Methodology, P.M., D.M., A.R. and T.L.d.S.; Data analysis, P.M., A.C.O. and T.L.d.S.; Writing—original draft preparation, T.L.d.S.; Writing—review and editing, all the authors; Supervision, P.M., A.R. and T.L.d.S.; Project administration, T.L.d.S.; Funding acquisition, T.L.d.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financed by national funds through FCT-Fundação para a Ciência e a Tecnologia, I.P., within the scope of the project PTDC/EAM-AMB/30169/2017.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank the Biomass and Bioenergy Research Infrastructure (BBRI)—LISBOA-01-0145-FEDER-022059, which is supported by Operational Programme for Competitiveness and Internationalization (PORTUGAL2020), by Lisbon Portugal Regional Operational Programme (Lisboa 2020) and by North Portugal Regional Operational Programme (Norte 2020) under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). The authors acknowledge Maria do Céu Penedo for the HPLC analysis.

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

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