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Biodiesel and omega-3 lipids production from the marine microalgae *Crypthecodinium cohnii*

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In the present work the marine microalgae *Crypthecodinium cohnii* ATCC 30772 was cultivated using a by-product from the biodiesel industry - crude glycerol - to establish a protocol to obtain cultures of high cell density, in batch and fed-batch regime, with the objective to produce, simultaneously, docosahexaenoic acid (DHA) and other lipids with potential application as biodiesel. Three experiments (I, II and III) were performed in a 7 L bench bioreactor. The purpose of experiment I was to study the growth phases of the microalgae (latent, exponential, and stationary). In experiment II, a concentrated nutrient medium was used to extend the exponential growth phase of the microalgae. However, in this assay, the microalgae growth ceased possibly due to substrate inhibition and, on the other hand, the particles in the medium interfered with the quantification of the biomass concentration. Finally, in experiment III, the concentration of the feeding medium was half reduced, and cell growth was monitored by flow cytometry (cells/mL), in addition to optical density control, correlations were established between these readings and algal biomass concentration. The highest biomass concentration was obtained in experiment II, with the value of 20.82 g/L. The highest productivity was also obtained in this assay, 1.2 g/(L.h). In the experiment III, it was not possible to obtain lipid content for technical reasons. However, in experiment II it was possible to obtain the highest value of lipids in this study, 26.75% w/w dry biomass. The maximum DHA content was 53.06% w/w total fatty acids, and maximum DHA productivity of 0.086 g/(L.h). Physical parameters of a biodiesel fraction from experiment II were also estimated, and all met the limits defined by the European Standard EN 14214, to be used as fuel. Flow cytometry was successful used to quantify cell growth and to analyse the viability of *C. cohnii* cells throughout the assays.

Keywords: *Crypthecodinium cohnii*; docosahexaenoic acid (DHA); crude glycerol; fed-batch regime; biodiesel; flow cytometry.

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1. Introduction

Currently, the energy demand worldwide is strongly dependent on fossil fuels and their derivatives consumption, which contributes for the greenhouse effect (GHE) increase, and the fossil reserves exhaustion, in a near future [1]. As a result of the global energy requirements imbalance, a massive pressure is being applied on the purchase prices levels, coupled with environmental problems and political concerns [2]. An attempt to meet the increasing energy demand in a sustainable way consists of using renewable bio-base resources to produce materials and energy [3].

Similar to common petroleum refinery where fossil fuels are the input to produce energy, biorefineries are green energy systems that use as an input biomass to produce several products, including biofuels, using a combination of technology and processes [1]. The sources of biomass are diverse, from agriculture, forestry and aquaculture, including industry and households wastes such as wood, forest residues, organic residues, both animal and plant derived, agricultural crops, and aquatic biomass (algae and seaweeds) [3].

Microalgae are one of the earliest forms of life and have some peculiarities that make them the most studied microorganisms for microbial biorefineries. The ecological habitats where microalgae can be found are diverse, which make them able to thrive in numerous extreme environments in terms of pH and temperature [4]. Furthermore, many microalgae are single-cell organisms, and they can grow fast in raceways as continuous culture systems, or in bioreactors. In addition, the process productivity can be improved by changing the growth and harvesting conditions [4]. There are many autotrophic microalgae based biorefineries reported in literature [5][6][7]. However, autotrophic microalgae cultivation systems present a few bottlenecks: (i) the microalgae growth is light-depending, requiring expensive and specific equipment design; (ii) for an efficient conversion, the culture media are usually treated in large volumes, in photobioreactors, in which light penetration into the dense cultures is hindered due to the self-shading effect; (iii) as a result, the microalgae cell concentration in the culture is usually low, due to the inefficient light penetration, aggravated by the light shading effect. In addition, microalgae grown under autotrophic conditions usually produce low amounts of intracellular products, such as lipids and pigments, due to the low biomass concentrations and productivities. Moreover, autotrophic microalgae growth is affected by temperature and light availability; hence, this technology is not suitable in areas of high latitude, where most seasons have low temperature and fewer daylight hours, as the European Northern countries [8].

On contrary, heterotrophic microalgae use organic compounds as carbon and energy sources to grow and do not use light as an energy source. Indeed, these microorganisms show several benefits over the autotrophic microalgae such as: (a) they can grow in cheaper conventional bioreactors, requiring less sophisticated equipment, and are, therefore, easily scaled-up; (b) they do not require light to grow, which reduces the equipment requirements and costs; (c) the cultures attain higher, denser cell concentrations and intracellular product productivities than autotrophic cultures; (d) the algal biomass composition can be tailored by changing the type of organic substrate in the medium; (e) heterotrophic microalgae can remove organic carbonaceous, nitrogen, and phosphorus compounds from the wastes more efficiently than autotrophic growth [8].

At a commercial scale, there are no reports on the biorefinery concept applied to heterotrophic microalgal biomass, despite its potential to obtain lipids for biodiesel or bioenergy production, and ω-3 fatty acids, which have several applications for pharmaceutical, nutraceutical and food purposes.

Crypthecodinium cohnii (*C. cohnii*) is a marine dinoflagellate, heterotrophic and unicellular microalgae that is part of a species complex, with numerous isolated species siblings morphologically very analogous, that can be found worldwide in temperate and tropical waters [9]. Some strains of *C. cohnii* grow axenically in an organic medium, however, in nature they are often phagotrophic strains present among macrophytes, predominantly Fucus spp., or other decaying seaweeds, having a typical myzocytosis food uptake mechanism [10].

C. cohnii is an oleaginous organism, and it is known by the ability to produce and accumulate lipids with a high fraction of docosahexaenoic acid (DHA), a polyunsaturated fatty acid (PUFA), part of ω-3 group (22:6). The peculiarity of this microalgae is related with the fact that no other PUFAs are produced in significant amount, which facilitates the downstream DHA purification step, and makes attractive the industrial production [11].

The total lipid content accumulated can exceed more than 20% of biomass dry cell weight (DCW), depending on the growth conditions [12], [13]. Fatty acids (FAs) biosynthesis culminates in the formation of C16 or C18 saturated FAs in a first phase, subsequently they are converted to monounsaturated FAs and 22:6 ω-3 (de novo synthesis) through a series of desaturases and elongases resulting in an extended range of PUFAs 70% are neutral lipids and characterized as triacylglycerols (TAGs) rich in DHA (over 30% of total fatty acid content), the remaining are polar lipids [13][14].

In addition to DHA, *C. cohnii* fatty acid profile reports include lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0), 18:2ω6 (linoleic acid) and 18:2ω9 (oleic acid) as common fatty acids [12][14][15]. The distribution of these fatty acids between the neutral or polar fractions is dependent on the strain of *C. cohnii* and the respective cultivation stage, mainly at nutrient limitation [16].

Several efforts have been made to study the optimal growth conditions for lipid accumulation. Considering the carbon source, studies demonstrate that the microalgae is able to grow in different media composition, containing ethanol, pure glycerol, acetic acid (acetate), glucose, sucrose, dextrose, galactose or mannose [16][17][18][19]. Positive results were also obtained with other complex sources, such as rapeseed meal hydrolysate mixed with waste molasses (two agro-industrial by-products) [20], carob pulp syrup [21], cheese whey with corn steep liquor [22] and crude glycerol [23].

Crude glycerol ($C_3H_8O_3$) is the main by-product of biodiesel production and with the increase of interest in this biofuel worldwide combined with sustainability issues. Crude glycerol price has decreased being essential to manage its production, in order to have a zero-waste disposal [24].

Comparing to hydrocarbons obtained in traditional petrochemical refineries, glycerol is highly functionalized molecule that can be directly used as an alternative feedstock for numerous processes, including for the production of DHA and other lipids by heterotrophic microalgae, reducing the cost of biodiesel commercial production [25].

Da Silva *et al.* used low-carbon sources substrates (sugarcane molasses, vinegar effluent and crude glycerol) for *C. cohnii* ATC 30772 lipids production in 500 mL shake-flasks cultures. Crude glycerol produced the highest lipid and DHA content (14.7% w/w DCW and 6.56 mg/g, respectively) [23].

Algae and microalgae, including *C. cohnii*, are direct producers of two PUFAs, DHA and EPA (eicosapentaenoic, 20:5 n-3), and are currently being used and formulated for fish farms and for human consumption, and are currently in the market by different industries [26]. Microalgae have the peculiarity of producing singular PUFAs that are easily extracted and purified. In addition, they are cholesterol free, taste good and are odourless [14].

Emphasis on omega-3 supplementation for human health is growing, since DHA is incorporated into phospholipids membrane in the brain, playing a vital role in prenatal and postnatal development, cognitive development, learning and memory; it reduces the risk of neuropsychiatric disorders as well as visual injuries, since it is also present in high concentrations in the retina [14][27]. EPA and DHA appear to be involved in antiangiogenic activities, with antitumoral and anticancer properties [28]; they also prevent diabetic complications and obesity conditions [29].

So far, a few wild-type microorganisms are qualified to produce PUFAs containing more than 20 carbon atoms, and this group includes fungi, marine microalgae, and bacteria.

Lipid accumulation is a dynamic process that depends on the microorganism and the growth conditions.

Therefore, some strategies involve separating the cultivation into two phases: biomass production and lipid accumulation. *C. cohnii* starts to accumulate oil in the stationary phase or during growth-limiting conditions when cells are under stress. It has been reported that *C. cohnii* DHA production is negatively affected by increasing lipid concentration, thus maximum growth and maximum DHA accumulation occur for different culture media [14].

Fatty acid alkyl esters (FAAEs), commonly known as biodiesel, is an ecologically friendly fuel that is an alternative to diesel fuel or extender. Biodiesel is derived from renewable feedstocks, such as animal fats, vegetable oils (edible and non-edible oils) or waste (recycled oils) [30][31][32]. These feedstocks are composed of TAGs containing a LC-PUFA chemically bound to a glycerol backbone.

The common process to convert TAG into biodiesel is through a reaction between TAG and an alcohol in the presence of a catalyst at high temperature - transesterification reaction - resulting in a mixture of FAAEs and glycerol

Recently, microalgae have emerged as the third generation of biodiesel feedstock, as a replacement of other renewable fuels that struggle for land use and with the food industry.

Microalgae show benefits over other feedstock due to their harvestability as they can double their biomass in 24 hours, having higher productivity and fermentation yields. The main limitation to commercial development is microalgae cultivation, and it has been addressed by many researchers, also to improve downstream processing, conversion, and extraction techniques [33].

Biodiesel fuel in FAME form is now manufactured in many countries. In the United States the relevant standard for biodiesel characterization is ASTM D6751, while in Europe EN 14214 is a separated standard valid to the automotive sector and EN 14213 for heating purposes [34].

Batch processes are more attractive given their robustness and simplicity, yet the only way to achieve high-cell-density is to follow a fed-bath regime, which, despite being more complex, allows the monitoring of the strains' metabolism [35]. In addition, high-cell-density cultures result in cost savings in terms of product recovery compared to low-cell-density batch cultures.

C. cohnii growth has been achieved in large scale conventional fermenters and in fed-batch regimes, obtaining high cell densities; using acetic acid as carbon source, Ratledge *et al.* performed fed-batch cultivations in 3.5 L and 5 L fermenters obtaining 20-30 g/L (DCW) biomass concentrations [17]; Swaaf *et al.* in 2 L bioreactors obtained 83 g/L (DCW) and 109 g/L (DCW) in pure ethanol and pure acetic acid feedstocks [18][36].

Microalgal culture monitoring by Flow Cytometry (FC) is an effective technique that provides information on cells physiological states, instead of the traditional methods to monitor culture developments, such as DCW, optical density and cell counting [11]. This is especially important when cells are growing in media containing industrial wastes, since this condition may reduce the process performance due to the presence of inhibitory compounds that affect cells metabolism [15][18].

In this study FC was used to monitor *C. cohnii* cell physiological response (membrane integrity and enzymatic activity), as high percentage of injured or dead cells present in the cultivation will reduce the process efficiency; additionally, cell counting was performed as a measure of *C. cohnii* biomass concentration. Moreover, this study aims to establish a protocol to optimize *C. cohnii* ATCC 30772 biomass and lipid production, using a 7 L benchtop bioreactor, operating in fed-batch mode, and using crude glycerol as carbon source.

2. Materials and Methods

2.1. *C. cohnii starter cultures*

In this study *Crypthecodinium cohnii* (ATCC 30772) cells were maintained as static cultures (150 mL in 250 mL shake flasks) on a standard medium composed of sea salt (23 g/L) and yeast extract (1.8 g/L) and a carbon source (9 g/L), at 25 ºC in the dark. Glucose and glycerol were the two carbon sources used. All medium components were heat sterilised (Uniclave 88, Portugal or A. J. Costa LDA., Portugal) at 121 ºC for 20 min, apart from glucose, to avoid its caramelisation (Maillard reaction). Also, to prevent bacterial medium contaminations, a filtered solution (0.2 µm) containing three antibiotics (chloramphenicol (5 mg/L), penicillin G (62 mg/L) and streptomycin (100 mg/L)), were added to the sterilised medium. These cultures were re-inoculated in the same medium monthly and were used to as seed for further experiments.

2.2. *Carbon source*

Crude glycerol (previously distilled), a by-product of biodiesel industry, was the carbon source used in inoculum cultures and the media culture of the bioreactor experiments (I, II, III). It was kindly supplied by Iberol (Alhandra, Portugal) and its composition was analysed: glycerol – 83.8% w/w (Ea 6-51 (AOCS)), water – 9.90% w/w (EN ISO 12937), methanol 0.007 % w/w (EN 14110/2003) and ashes- 6.54% w/w (NP 1688).

2.3. *Inoculum*

The inocula for the 7 L bioreactor experiment were prepared in shake-flask cultures (500 mL flasks, at 150 rpm) containing 135 mL of medium and 10% v/v of the starter culture (15 mL). The medium for these cultures contained yeast extract (0.5 g/L), crude glycerol (23.94 g/L, to obtain 20 g/L of glycerol in the medium), corn steep liquor (CSL, 4.59 g/L) and sea salt (25 g/L). The pH was adjusted to 6.5 using NaOH (5 mM) and HCl (5 mM), and all media were autoclaved (Uniclave 88, Portugal) at 121 ºC for 20 min. The pH determination was performed using a Mettler probe and a Consort C3021 potentiometer (Consort, Belgium), calibrated regularly. 1 mL of the antibiotic solution was added to the inocula (1 mL/L), and culture media used in the 7 L bioreactor. The inocula were grown for 6-7 days, in an agitated incubator (Unitrom Infors, Switzerland) in the dark, at 27 ºC and 150 rpm.

2.4. *Cultivation in bioreactor*

Experimental assays (I, II and III) were carried out in a 7 L bioreactor, with 5 L of working volume (FerMac 310 bioreactor, Electrolab Biotech, United Kingdom), coupled with a controller module composed by a stirrer, dissolved oxygen, temperature, pH probes, and foam formation sensor (FerMac 360 bioreactor, Electrolab Biotech, United Kingdom). Before inoculation, the bioreactor, with the culture media was sterilized in the autoclave (Uniclave 88, Portugal or A. J. Costa LDA., Portugal), at 121 ºC, for 30 min. Under asepsis conditions, 297 mL of the inoculum, and 3 mL of the antibiotic solution were added to 2700 ml of culture medium, making up the total volume of 3000 mL. By automatic addition of 2.5 M NaOH or 2.5 M HCl, the pH was regulated to the pH set-point (6.5). The anti-foaming agent, polypropylene glycol, was manually added whenever necessary, in a 1:10 concentration.

DO in the medium was controlled by the stirring and aeration rates, assuring that the microalgae growth was not limited by oxygen. Initially the speed rate was set at 300 rpm, and was adjusted according to DO readings (kept above 30% of air saturation); the temperature set-point was 27 ºC. To verify possible contaminations, all samples collected from the bioreactor were observed, as well as the starter and pre-inoculum cultures before bioreactor inoculation. The observations were performed under visible light using the Olympus BX60 fluorescence microscope (Olympus Corporation, Japan), with mercury fluorescence illuminator and Nomarski/DIC Prism for transmitted light [37].

The samples collected (10 mL) were centrifuged (Sigma 2-16K, Sartorius, Germany) in falcon tubes during 10 min, at 7000 rpm and 4 ºC. The supernatant was kept in another falcon tube, and the pellet (biomass) was stored in a freezer (-18 ºC).

Experiment I was carried out as a preliminary assay to study *C. cohnii* batch growth in culture medium containing crude glycerol and corn steep liquor; afterwards *C. cohnii* was grown in a 7 L bioreactor under fed-batch mode (assays II and III). At the end of the exponential phase, the bioreactor was feed with a fresh concentrated nutrient solution that was pumped using a peristaltic pump (Watson Marlow 520 Du, United Kingdom), adjusting the rate feeding according to DO readings, biomass, and residual glycerol concentrations.

2.5. *Analysis of biomass concentration*

For experiments I and II, the two methods used to quantify the biomass concentration were optical density (OD) readings, and DCW determination. A previous correlation between OD and DCW was established.

To measure the DCW, 1 mL of sample duplicates were collected in Eppendorfs (1.5 mL) previously dried in a 100 ºC oven (Memmert, Germany) for 18h, following 30 min cooling, in a low humidity atmosphere of a desiccator, and weighted. After samples centrifugation (Beckman Avanti J-25I, EUA), at 5 min, 7000 rpm and 20 ºC, the pellet was washed once with distilled water to remove the medium salt. Afterwards, the biomass pellets were placed in the oven, at 100 ºC for 24h, cooled and weighed.

In experiment III, the biomass concentration was evaluated by cell number/volume assessed by flow cytometry, in triplicate, to eliminate the interference of the particles present in the growth medium due to the corn steep liquor (CLS) and crude glycerol presence. A previous correlation between the cell number/volume and DCW, and cell number/volume and OD was established. To all samples taken throughout the experiment, their OD was discounted by the OD and the dry weight of the cell-free medium.

2.6. *Flow Cytometry*

Experiment I and II were carried out using a BD FACScalibur™ cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), equipped with an argon laser and a red diode laser which radiation emission is 488 nm and 635 nm, respectively; this equipment contained, in addition, photomultipliers as fluorescence detectors - FL1 (green: 530 ± 30 nm), FL2 (yellow: 585 ± 42 nm), FL3 (orange: > 670 nm) and FL4 (red: 600 ± 16 nm) [38], that from stained samples

with CFDA and PI, cell enzymatic activity and cell membrane integrity was examined using Flowing Software 2.5.0 tools.

For experiment III, a CytoFLEX flow cytometer (Beckman Coulter Life Sciences, USA) was used, with five channels from the 488 nm (blue) laser, three from the 638 nm (red) laser, five from the 405 nm (violet) laser. The instrument includes 13 band pass filters which can be repositioned as needed [39], the photomultipliers used in the study were FSC-A and SSC-A for auto-fluorescence; FITC-A and PC5.5-A for CFDA and PI dyed samples, respectively. Data was analysed CytExpert 2.4 software (Perttu Terho). Previous flow cytometric controls were carried out using *C. cohnii* cells at different growth conditions, to evaluate the efficiency of the flow cytometric protocol in association with the CFDA/PI mixture, to identify different cell physiological status. These controls were then compared with data obtained during the microalgal cultivations [40].

All culture samples were sonicated (Transsonic T 660/H, Elma, Germany) for 10 s, at 35 kHz, to remove cell aggregates, and ensure individual cell analysis.

Samples were diluted in McIlvaine buffer (pH 4.0) previously filtered in a 0.2 mm membrane (TPP Syringe filter 22, Switzerland), to remove the particles in suspension.

For *C. cohnii* enzymatic activity and membrane integrity detection, 3 μL of CFDA (10 mg/mL stock solution) and 2 μL (1 mg/mL stock solution) were added to 500 μL cell suspension, incubated for 15 min in the darkness. Before cell analysis, the sample was always mixed in the vortex to avoid cell deposition in tube bottom.

2.7. *Nitrogen Source and Total Nitrogen Amount Determination*

The total nitrogen concentration in the sample supernatant were quantified according to the modified Kjeldahl method adapted from the standard 4500-Norg [41]. This determination was also carried out for nitrogen content quantification in the corn steep liquor (CSL), a nutrient used in the medium formulation as nitrogen source, to define the appropriate amount to add to the growth medium. CLS was gently supplied by COPAM, Companhia Portuguesa de Amidos SA, S. João da Talha, Portugal. After collected from the industrial plant, the CSL was left uncovered, for a few weeks, so that volatile toxic compounds could be released. This substrate contains 0.035 g nitrogen/g.

The total nitrogen content of each sample, as a percentage, was calculated using (1). V is the HCl (0.1 N) volume in mL spent in the sample titration, and m is the mass, in grams, of the tested sample.

$$\text{Nitrogen} = \frac{0{,}14 \times \text{V}}{\text{m}} \quad \textbf{(1)}$$

2.8. *Ash and Moisture Content Determination*

Moisture and ash content of biomass samples was sequentially determined, to further correct the biomass weight used for FA analysis. 100 mg of lyophilized sample were accurately weighed (triplicate samples) in previously weighed calcined crucibles, which were placed in an oven at 100 ºC for 24 h; thereafter, the crucibles containing the biomass were cooled in a desiccator and weighted. The moisture content was calculated by the difference between the mass of the crucible with biomass and the empty crucible (both after oven at 100 ºC), divided by the total mass of biomass. Afterwards, the crucibles were placed in a muffle furnace at 550ºC for 1 h, cooled in a desiccator and weighed to determine the ash content (difference between the mass of the crucible with ash and the empty crucible divided by the total mass of biomass).

2.9. *Identification and quantification of lipids as Total Fatty Acids (TFA)*

Gas chromatography (GC) was used to determine *C. cohnii* lipid content and fatty acid profiles. To achieve that, a transesterification reaction was applied to *C. cohnii* freeze-dried biomass to transform the mono-, di- or triglycerides into the respective FAMEs. The procedure protocol used was an adaptation of the described by Lepage and Roy (1986) [42]. The samples were analysed in a gas-liquid chromatograph (SCION GC 436 from Bruker, Germany), equipped with a flame ionization detector (FID). The separation of the compounds was performed on a fused silica capillary column (Supelcowax 10, SUPELCO, USA), 30 m long, 0.32 mm internal diameter and 0.25 μm film thickness. Helium was used as carrier gas at a flow rate of 1.6 mL/min. Each sample was processed in duplicate, and each duplicate was injected once.

The FAMEs were identified by comparison with the retention times of the components of standard 461 (Nu-Chek-Prep, Elysian, MN, USA). The quantification of each of the fatty acids was performed according to (2), where m FAi is the mass of fatty acid i, A FA_i is the area of the fatty acid peak, A (17:0) is the area of the peak corresponding to the internal standard and RF FA is the response factor of the fatty acid FA_i.

$$\text{m FAi} = \frac{\text{A FA}_\text{i}}{\text{A (17:0)}} \times \text{RF FA} \quad \textbf{(2)}$$

2.10. *HPLC*

To determine the glycerol content, samples were filtered and injected (LaChrom L-7200 (Hitachi, Japan) in a HPLC column, Aminex™ HPX-87P (Bio-Rad, California, USA). This Agilent Chromatographer was equipped with a diode array detector (DAD) and a refractive index detector (RI). The software used to analyse the chromatograms was the Chromeleon™ Chromatography Data System (CDS) software. (ThermoFisher Scientific™, USA).

3. Results and discussion

3.1. *Cultivations of C. cohnii ATCC 30772*

Table 1 shows the kinetic parameters calculated for *C. cohnii* ATCC 30772 batch (experiment I) and fed-batch cultivations (experiments II and III).

Table 1. Kinetic parameters calculated for *C. cohnii* growth in batch and fed-batch experiments.

Kinetic parameter	Batch I	Fed-batch II	Fed-batch III	
			DCW	FC
Specific growth rate, μ_{max} (h^{-1})	0.034	*0.059; 0.024	***0.059; 0.026	***0.015; 0.047
Maximum biomass concentration (g/L)	4.34	20.82	9.63	6.56
Maximum productivity in biomass $P_{X\ max}$ (g/(L.h))	0.513	1.2	0.37	0.33
Average productivity in biomass, $P_{X\ average}$ (g/(L.h))	0.059	0.07	0.0325	0.00023
Total lipid content (TFA) (% $w_{TFA}/w_{biomass}$)	24.61	11.54 - 26.75	-	-
TFA yield, $Y_{TFA/S}$ ($w_{TFA}/w_{glycerol}$)	0.00108	-	-	-
Volumetric productivity in TFA, P_{TFA} (g/(L.h))	0.015	0.00429	-	-
DHA content in biomass (% $w_{DHA}/w_{biomass}$)	8.20	**7.38	-	-
DHA Volumetric productivity (g/(L.h))	0.005	**0.086	-	-
DHA content in TFA (% w_{DHA}/w_{TFA})	33.36	31.16-53.06	-	-
DHA yield $Y_{DHA/S}$ ($w_{DHA}/w_{glycerol}$)	0.00036	-	-	-
Biomass yield, $Y_{X/S}$ ($w_{biomass}/w_{glycerol}$)	0.00424	-	-	-
Substrate consumption volumetric rate, $-r_S$ (g/(L.h))	0.18875	-	-	-

*Cultivation time 0 h <t<17.5 h and 29.3 h <t <137 h, respectively.
** Maximum obtained at t = 131.17 h.
***0 h<t<42 h and 66.0 h < t < 114.0 h, respectively.

A correlation between *C. cohnii* dry cell weight (DCW in g/L), cell number (cells/mL from flow cytometry, FC) and the optical density at 470 nm (OD_{470}), was established.

In batch experiment, after the inoculation the culture showed a linear growth, achieving a specific growth rate (μ_{max}) of 0.034 h^{-1} (R^2 = 0.991) (Table 1). The absence of exponential phase was observed, indicating that the microalgae growth could be limited by some nutrient. The microalgae growth ceased at t = 70.33 h, reaching at that time the maximum biomass concentration of 4.34 g/L, possibly as a result of oxygen limitation, as DO readings after

t = 48 h were below 30%. The highest biomass productivity ($P_{x\ max}$) was achieved at t = 52.62 h, with the value of 0.513 g/(L.h), while the average biomass productivity ($P_{x\ average}$) was 0.059 g/(L.h) for the total batch time (73.75 h). Safdar *et al.* [43] also grew *C. cohnii* ATCC 30555 on pure glycerol in 1 L fermenter for lipid production and reported a biomass concentration of 15.1 g/L DCW, and a μ_{max} of 0.72 d^{-1} (0.03 h^{-1}), similar to this work. Hosoglu and Elibol [44] reported a final cell dry weight around 5.7-7.3 g/L DCW and a $P_{x\ max}$ of 1.0 g/L.h (0.042 g/(L.h)) for *C. cohnii* CCMP 316 cultivated in pure glycerol, in batch (2 L bioreactor,) higher than that obtained in this study, which may be explained by the higher glycerol purity used by those authors, since it should not contain inhibitor compounds. Other works reported *C. cohnii* biomass productivities between 0.72-1.0 g/(L.h) when cultivated in different carbon sources, namely glucose, ethanol, carob pulp and acid acetic [17] [21][45][46].

The speed rate was maintained, in the 3 experiments, at low levels (250-300 rpm), since this microalgae is particularly affected by the shear stress present in turbulent environments.

In batch experiment, the glycerol concentration decreased throughout the fermentation, but was not depleted, reaching a final concentration of 11.97 g/L. The total lipid content (measured as TFA) in this experiment was obtained only from the biomass collected at the end of the batch, attaining 24.61% ($w_{TFA}/w_{biomass}$), corresponding to a TFA concentration of 1.10 g/L, a TFA yield of 0.00108 $w_{TFA}/w_{glycerol}$ and a TFA productivity of 0.015 g/(L.h) (Table 1).

To improve *C. cohnii* cell growth and lipid production, further fed-batch cultivations were carried out. The fed-batch mode has the advantage of extending the microalgae exponential growth phase, allowing to achieve higher cell densities, which cannot be obtained using batch cultures. However, the crude glycerol and CSL contained a high proportion of particles that might have interfered in the biomass quantification, especially during the feeding stage, since a concentrated solution containing glycerol and CSL was feeding the bioreactor, which might have given overestimated biomass concentration results.

In experiment II, afterwards the feeding was started (at t = 102.75 h), the biomass concentration increased up to 20.82 g/L, at the end of the cultivation (t = 209.1 h), corresponding to an average productivity of 0.07 g/L.h (Table 1). Px max was 1.2 g/L.h at t = 191.1 h, when the biomass concentration attained 13.57 g/L. The μ_{max} was calculated for the cultivation time 0 h <t<17.5 h and 29.3 h <t <137 h, giving 0.059 h^{-1} and 0.024 h^{-1}, respectively. The specific growth rate decrease at t = 24 h could be due to the speed rate increase, as above referred (Yeung *et al.* [7]), since *C. cohnii* growth is affected by shear stress. The TFA content varied between 11.54% w/w and 26.75% w/w during the assay, with an average productivity of 0.00429 g/(L.h) (Table 1). As expected, after the batch phase (t = 102.75 h), *C. cohnii* started accumulating lipids, and the maximum TFA content (26.75% w/w DCW) was reached at t = 137.08 h, when the microalga entered the stationary phase.

During experiment III, the biomass concentration quantification was performed by two methods: dry cell weight and by cell counting, using flow cytometry, since this technique allows discriminating the microalgae cells population from the background. Comparing the biomass concentration values obtained by the two methods, they are quite disparate, although the flow cytometry results should be, in principle, more reliable. The biomass concentration results obtained by the two methods reached a maximum, after the feeding start, of 9.63 g/L and 6.56 g/L, for DCW and FC, respectively (Table 1), when the residual nitrogen and carbon were in excess in the broth. As expected, the biomass concentration values were higher when determined by dry cell weight, comparing to those obtained by FC. The batch phase was extended compared to the other two trials, up to t = 114.0 h, corresponding to a biomass concentration of 6.10 g/L (measured by DCW) with a maximum biomass productivity of 0.37 g/(L.h) for t = 136.75 h and the maximum biomass concentration was reached at t = 155.0 h of 9.63 h/L. The specific growth rate was 0.059 h-1 (calculated for the period of 0 h<t<42 h) and 0.026 h-1 (calculated for the time period of 66.0 h<t<114.0 h). On the other hand, according to the FC results, the specific growth rate was 0.015 h-1 (0 h<t<42 h) and 0.047 h-1 (66.0 h < t < 114.0 h), and the maximum biomass productivity was 0.33 g/(L.h) at t = 122.75 h. Unfortunately, it was not possible to quantify the lipids in this trial.

3.2 *Flow Cytometry*

Flow cytometry (FC) was used to investigate cell viability throughout cultivation. In experiment I, FC results revealed that the microalgae cells could adapt to the growth conditions, since at the end of the batch subpopulations of cells in stress were less than 15.86%, confirming that most of the cells were metabolically active. In fed-batch experiment II FC confirmed that cells were healthy in batch phase, and as soon as feeding started, subpopulations with permeabilised cytoplasmic membranes, remained at low percentages, less than 20%, demonstrating that the crude glycerol, used as carbon source in the medium formulation for *C. cohnii* growth, did not significantly affect the microalgae metabolism. However, in experiment III results suggested that *C. cohnii* membrane integrity and enzymatic system were seriously affected during the period 72 h < t < 144 h, leading to the microalgal growth stop, probably due to oxygen limitation (unfortunately no DO readings could be obtained during this assay).

3.3 *Lipid Quantification*

The fatty acid composition for *C. cohnii* saponifiable lipid fraction was analysed to evaluate its potential as DHA and biodiesel sources. The results obtained at the end of the batch are shown in Table 2, and the fraction of biodiesel obtained at experiment II, in fed-batch that was used to estimate physical parameters of *C. cohnii* SFA+MUFA is also present.

Table 2. Fatty acid composition, as percentage of total fatty acids (%w/w TFA), obtained for assay I and II concerning *C. cohnii* ATCC 30772, grown in batch and fed-batch mode. Data represent the mean of two analyses (two independent samples, injected once).

Time (h)		Batch (The end at t = 73.75 h)	Fed-Batch I (At t = 168.08 h)
FAs (%w/w TFA)			
Capric	**10:0**	1.65±0.13	0.34±0.03
Lauric	**12:0**	9.82±0.73	4.11±0.55
Myristic	**14:0**	20.29±1.12	11.66±14.28
Palmitic	**16:0**	16.36±0.23	5.65±0.91
Stearic	**18:0**	0.84±0.15	0.98±0.15
Oleic	**18:1w9**	13.01±0.78	20.77±3.46
Linoleic	**18:2w6**	1.98±0.37	3.36±0.52
DHA	**22:6w3**	33.36±1.13	53.06±8.66
Others		2.68	0.06
Saturated fatty acids (SFA)		48.96	22.74
Polyunsaturated (≥2 double bonds) (PUFA)		35.34	56.42
Monounsaturated (1 double bond)(MUFA)		15.69	20.84

In batch experiment, DHA (22:6w3) was the dominant fatty acid in *C. cohnii* biomass collected at the end of the cultivation (33.36% w/w TFA) (Table 2). Other abundant FAs were the saturated fatty acids (SFAs) 12:0 (9.82% w/w TFA), 14:0 (20.29% w/w TFA), 16:0 (16.36% w/w TFA), and the monounsaturated fatty acid 18:1w9 (13.01% w/w TFA). The FAs 10:0, 18:0 and 18:2w6 and were present in smaller amounts (<1.98% w/w TFA). Safdar *et al.* [43] obtained a similar profile in fatty acids, especially the DHA proportion, which was also much higher than the remaining FA. SFAs have the highest percentage, 48.96% w/w TFA, followed by PUFAs with 35.34% w/w TFA and finally MUFAs, with 15.69% w/w TFA. For this alga, other authors have also reported similar results [15][16].

Biodiesel to be marketed in the European Union needs to comply within certain parameter values, namely for density, kinematic viscosity and cetane number properties. The fraction selected met the limits defined by the European Standard EN 14214, to be used as fuel.

4. Conclusions

In this work, three assays were performed: one in batch and two in fed-batch mode. Regarding one of the mentioned objectives, *C. cohnii* grew on crude glycerol demonstrating that this substrate can be used as an alternative to commercial glucose, carbon source that is widely used in media formulations for microbial growth, including this microalgae, as described in the literature. This approach will result in the process costs decrease, as well as environmental benefits, since an industrial by-product is reused, according to the circular economy rules.

Flow cytometry was useful in monitoring cell growth as well as the microalgae physiological state assessment throughout the assays; moreover, it revealed that the cells did not show high levels of stress, which confirms that crude glycerol did not drastically affect their metabolic activity, at least in terms of enzyme activity and cell membrane integrity.

In experiment II, compared to experiment I, the biomass concentration increased 5-fold, reaching 20.82 g/L, an expected increase given that the objective of the fed-batch implementation was exactly to prolong the growth phase. However, considering that a concentrated nutrient solution containing crude glycerol and CLS was added to the broth, it is thought that part of this biomass could be due to particles present in the medium, although the OD and dry weight of the medium without cells were subtracted from the OD of the samples taken throughout the experiment.

Indeed, the particles interference in the biomass concentration quantification was particularly severe during the fed-batch fermentations, since under these conditions it is more difficult to subtract the OD/DCW values from the medium without cells, since the addition of the medium was continuous. Thus, during assay III attempts were made to overcome this problem, by cell counting using FC, which was considered to give more reliable results.

During the exponential phase, cells use carbon for cell division. When entering the stationary phase, they use the excess carbon for lipid production, which was verified in experiment II. Nevertheless, in experiment I, a DHA content of 8.20% ($w_{DHA}/w_{biomass}$) in biomass was obtained, higher than the maximum value obtained in experiment II, 7.38% ($w_{DHA}/w_{biomass}$), most probably due to substrate inhibition as a result of the uncontrolled nutrient addition after the batch phase.

Regarding the physical parameters determined for a biodiesel fraction from experiment II, all are within the limits stipulated by the EU Standard, which envisages the possibility of using this microalgae as a source of biodiesel, beyond the well-known source of the DHA.

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