Synthesis of omega-3 and fatty acids for biodiesel production by the marine microalgae *Crypthecodinium cohnii* and study of green methods for their extraction

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Abstract

In this study, the microalgae *Crypthecodinium cohnii* was cultivated under the fed-batch regime, in order to produce high concentrations of docosahexaenoic acid (DHA) and fatty acids (FA) for biodiesel production. Three assays were performed, using glucose, sugar cane molasses and crude glycerol as carbon sources. In the assay I, the fatty acid concentration obtained was reduced (13.5% (m/m)), possibly by oxygen limiting conditions. In trial II, the hydrolysis of molasses was not complete, resulting in a poor medium in carbon source and not allowing the growth of biomass. Furthermore, a yeast contamination occurred. In test III a biomass concentration of 18.65 g/L was obtained, with an average yield of 0.114 g/(L.h) and a DHA concentration of 7.52 % (m/m). The biomass from trial III was used to study the accelerated solvent extraction (ASE) and supercritical extraction (SCE) methods in the recovery of lipids accumulated by the microalgae. To study the ASE method, an experimental planning was performed, varying the factors temperature and extraction time, in order to maximize the yield in lipids, TFA, DHA and FA for biodiesel production. Using hexane as solvent, the condition that obtained the highest yields was 40 °C and 11 minutes. The yields in lipids, TFA, DHA and FA for biodiesel production were 26.35, 21.37, 8.18 and 13.05 g/100 g of dry and ash free biomass, respectively. When using ethanol, the highest yields were obtained at 140 °C and 11 minutes - 18.96, 7.28 and 11.56 g/100 g of dry and ash free biomass in TFA, DHA and FA for biodiesel production, respectively. For the study of the SCE method, 5 assays with different pressure and temperature conditions were performed. The highest yield in lipids was obtained at 300 bar and 41.3 °C - 22,40 g/100g dry and ash free biomass. The highest yields in TFA, DHA and FA for biodiesel production - 18.39, 7.08 and 11.20 g/100g dry and ash free biomass, respectively - were obtained at 300 bar and 60 °C. In the study of the methods for the lipids recovery, it was possible to quantify the total pigments synthesized by *C. cohnii*. The highest total pigment content - 121.37 µg/g dry and ash free biomass - was obtained in the ASE method with hexane, at 115 °C and 19 minutes. Finally, physical parameters of the biodiesel fraction from trial I were estimated and confirmed to be within the limits defined by the European Standard EN 14214.

Keywords: *Crypthecodinium cohnii*, docosahexaenoic acid, biodiesel, accelerated solvent extraction, supercritical extraction.

1. Introduction

The marine microalgae *Crypthecodinium cohnii* is a non-photosynthetic heterotrophic dinoflagellate that can be found in tropical and temperate waters worldwide [2]. It is an oleaginous organism capable of accumulating significant amounts of lipids (20-50% of its dry weight), with a high fraction of docosahexaenoic acid (DHA), a polyunsaturated fatty acid (PUFA) of the omega-3 group, thus facilitating its purification and industrial use. Depending on cultivation conditions, about 70% of the lipids are neutral - DHA-rich triglycerides (from 30 to 45% of the total fatty acids). Several studies have shown that DHA plays a key role in human development during pregnancy and lactation. In addition, there is a correlation between fish intake - and therefore DHA - and cognitive development, lower incidence of cardiovascular disease and depression, and lower risk of visual diseases. Thus, there is a need for increased production of dietary supplements such as algae oil, which are already on the market by several industries [9].

Growth of *C. cohnii* has been studied, in order to optimize biomass and lipid accumulation, and

can occur in large-scale bioreactors or small flasks, under fed-batch or batch conditions [14].

Biodiesel is the name given to long-chain alkyl esters derived from renewable raw materials such as vegetable oils, animal fat or industrial waste (e.g. recycled oils), composed of triglycerides. It is produced with the purpose of being an environmentally friendly alternative to petroleum-based fuel [1] [13]. Recently, microalgae have emerged as a promising feedstock since they have the ability to produce significant amounts of lipids. In addition, they have a higher specific growth rate than land-based crops, achieving high fermentation yields, and the advantage of being arable [3].

Flow cytometry is an effective technique that allows detailed evaluation of the development of cultures, both their growth and other physiological characteristics, such as enzyme and respiratory activities, intracellular pH and cell membrane integrity [16]. Usually, fluorescent dyes are also used that selectively accumulate in compartments or organelles of the cell, or change their properties through chemical reactions. By absorbing the light from the laser and re-emitting it at a higher wavelength, they allow analysis of the other characteristics in addition to cell density [5] .

Accelerated solvent extraction (ASE) is an ecologically friendly technique for extracting lipids and other compounds of interest widely used in industry. It consists of a fast and efficient method that uses solvents pressurized at high temperatures, above their boiling point and below their critical point. In this way, the solvent remains in the liquid state during the process [7]. It is necessary for the pressure to be high so that the solvent is forced through the fixed bed of the extractor and with close contact, facilitating its flow, and for the boiling point of the solvent to be relatively low, ensuring that it remains in the liquid state. Pressures are typically between 70 and 140 bar [8]. Regarding the temperature, it usually varies between 25 and 200 ºC, depending on the type of compound to be removed and the solvent used [10]. High temperatures increase the solubility of bioactive compounds and the diffusion rates of solvents. The extraction time consists of the product of the number of cycles by the time of each cycle, where one cycle corresponds to one passage of fresh solvent through the extraction cell. This method is efficient because it is extremely faster than traditional methods, with extraction being complete after 5 to 30 minutes. Furthermore, it consumes a low amount of solvent compared to other extraction methods (20-25 mL).

Supercritical extraction (SCE) is a separation technique based on the dissolving or solubilizing power of supercritical fluids, which consist of fluids that are above their critical point. The critical point is defined as the pressure and temperature value at which the pure substance exhibits thermophysical properties intermediate between a gas and a liquid [11]. The extraction process comprises two distinct steps. The first consists of the extraction of the compound of interest, in which a continuous and uniform flow of the chosen supercritical fluid is fed to the extractor, which contains a fixed bed of solid particles. After dissolution of the compound of interest in the fluid, they leave the extractor, to be separated - the second stage. This is the stage where the fluid will be removed, through depressurization, which decreases its density. In this way, the solubilizing power of the fluid decreases and the compound of interest precipitates [6]. This method has been widely used as an alternative to traditional solvent extraction methods as it has several advantages. The fact that it operates at moderate temperatures allows the separation of substances sensitive to thermal degradation. On the other hand, several inexpensive and non-toxic fluids can be used, the most common being carbon dioxide, which is still non-corrosive, non-flammable, chemically relatively inert and environmentally friendly.

2. Materials and methods

2.1. Growth conditions

Initially, cells of *Crypthecodinium cohnii* (*American Type Culture Collection* 30772) were maintained in starter cultures, in 250 mL erlenmeyers, containing 150 mL of medium composed of sea salt (23 g/L), yeast extract (1.8 g/L) and glucose (9 g/L). This medium was sterilized in an autoclave (Uniclave 88, A. J. Costa LDA, Portugal) at 121°C for 20 minutes, and then inoculated with a cell suspension of *C. cohnii* (10% v/v). These cultures were kept in the dark at 25 °C and re-inoculated monthly in the same medium. The inoculum cultures (culture that is inoculated to the bioreactor) were prepared in 500 mL erlenmeyers, containing 135 mL of culture medium (composed of 2 g/L yeast extract, 25 g/L sea salt and 20 g/L of the carbon source, according to the assay performed) and 15 mL of starter. They were incubated at 27 °C and 110 rpm (Unitrom Infors incubator, Switzerland) for 7 days prior to the start of fermentation. The inoculum medium is composed of 2 g/L yeast extract, 25 g/L sea salt, and 20 g/L of the carbon source, according to the assay performed. To prevent bacterial contamination, a solution (1 mL per liter of inoculum medium) consisting of three antibiotics was added: chloramphenicol, penicillin G, and streptomycin, at concentrations of 5, 62, and 100 mg/L, respectively.

2.2. Fermentation

The three assays were performed in a 7 L bioreactor (Electrolab Biotech, model FerMac 360), under

the fed-batch regime. Whenever cell growth slowed down, 'nutrient pulses' were introduced, consisting of 300 mL solutions of 10-fold concentrated culture medium (or carbon source only). The bioreactor contained an initial volume of 2.7 L of culture medium, to which 300 mL of inoculum was added. The culture medium, previously centrifuged to remove possible residues, was composed of 27 g/L sea salt, 0.5 g/L yeast extract, 5.5 g/L corn steep liquor, and carbon source. The culture medium in trial I had 20 g/L glucose, in trial II 20 g/L hydrolyzed molasses, and in trial III 24.52 g/L crude glycerol, in order to equal 20 g/L pure glycerol. In the fermenter was coupled a rotor, an air inlet rotameter (flow rate of 1 g/L.h), an air outlet opening, a thermometer, a heating mantle (being 27 °C the set-point) and a pH, temperature and dissolved oxygen controlled module. When there was an increase or decrease of the set pH value (6.5), the system automatically introduced 2.5 M HCl or 2.5 M NaOH, respectively. When DO dropped below 30%, agitation was increased in order to maintain dissolved oxygen readings above this value. During the three trials, samples were taken and analyzed each time under a microscope, by spectrophotometry and flow cytometry. The samples and the final biomass were centrifuged and freeze-dried and the supernatant was used for subsequent determination of nitrogen and residual carbon source.

2.3. Optical density

In order to follow the growth of the microalgae, the optical density of all collected samples was read in triplicate in the UV-Vis spectrophotometer (Thermo Spectronic, model Genesys 20) at 470 nm. With the properly corrected optical density values, the respective dry weights (g/L) were determined for each instant, using the previously established correlation between absorbance and dry weight (DW) - equation 1.

$$DW(g/L) = (1.0889 \times OD) - 0.2497 \quad (1)$$

2.4. Flow Cytometry

Flow cytometry was used to determine the autofluorescence of the cells, as an additional method to control the growth of the microalgae, and also to analyze the physiological state of the cells, namely their membrane integrity and enzyme activity. PI and CFDA dyes were used for these analyses, respectively. To measure autofluorescence, the sample was diluted with PBS solvent in a cytometer tube, so that the number of events per second was 200 to 500, for a total volume of 500 µL. To measure the physiological state, another tube was used, with the same volume of sample, 3 µL of CFDA and 2 µL of PI, and the remaining PBS were added to make up the 500 µL. All measurements were performed in triplicate, and the stained samples were analyzed after 15 minutes of incubation in the dark, to ensure that the dyes penetrated the cells. The autofluorescence of the cells is given by the number of cells per unit volume and was used to determine the dry weight (DW) of the sample using the previously established correlation - 2.

$$DW(g/L) = (9 \times 10^{-7} \times Cells/mL) - 0.4133 \quad (2)$$

2.5. Residual carbon source

To determine the concentration of the carbon source in the fermentation medium, the collected samples were centrifuged, in order to collect the supernatant. The supernatant was filtered and injected into a high performance liquid chromatography column. Before injecting the samples, correlations were established, relating peak areas to the concentration of the respective carbon sources in the culture medium, by HPLC analysis of solutions of known concentration. The glucose content was evaluated in real time, through glucose rapid detection strips, which in contact with the sample taken from the reactor, show a shade corresponding to a certain range of glucose concentration. In the fermentation in which the carbon source was glycerol, a kit was used to determine its concentration in the medium, following the procedure described in it.

2.6. Ash and moisture quantification

In order to correct the weight of the biomass used in the quantification of lipids in the samples, the percentage of moisture and ash present in them were determined. For this, about 100 mg of freeze-dried biomass was weighed in duplicate into previously weighed porcelain crucibles. They were then placed for 24 hours in an oven at 100 °C and weighed again after cooling in the desiccator to room temperature. For ash quantification, the crucibles were then placed in a muffle furnace at 550 °C for 3 hours and cooled in the desiccator to be weighed. The percentage of moisture was then calculated using the equation 3 and the percentage of ash using the equation 4.

$$Moisture\,(\%) = \frac{w_{crucible+biomass} - w_{crucible}}{w_{biomass}} \times 100 \quad (3)$$

$$Ash\,(\%) = \frac{w_{crucible+ash} - w_{crucible}}{w_{biomass}} \times 100 \quad (4)$$

2.7. Fatty acid profile

Gas chromatography was used to determine the fatty acid profile of the samples taken throughout the fermentations and of the final biomasses. It was also used in the analysis of all the oils extracted from the biomass in trial III. In the direct transterification of biomass, 100 mg of freeze-dried was used. For the determination of fatty acids from the extracted oil, 15 to 20 mg of oil were used. All tests

were performed in duplicate. The biomass was previously submitted to milling, which was performed in a ball mill, placing in each mortar approximately 0.5 g of freeze-dried biomass. The grinding was performed at 25 Hz for 3 minutes and 30 seconds. The transterification proceeded as follows: to each sample, placed in a test tube, 0.2 mL of heptadecanoic acid was added as internal standard (C17:0, 5 mg/mL) and 2 mL of methanol/acetyl chloride solution (19:1 v/v), previously prepared in an ice bath - the reaction is strongly exothermic. The tubes with the samples and reagents were placed under inert atmosphere, closed and placed in the dark. The reaction proceeded for one hour at 80 °C. After cooling the tubes to room temperature, 1 mL of distilled water and 2 mL of n-heptane were added to separate the aqueous and organic phases. The organic phase was collected into vials using pasteur pipettes, and passed under a filter of hydrophilic cotton and anhydrous sodium sulfate to trap suspended particles and remove aqueous phase that may have been co-extracted. The samples were analyzed on a gas chromatograph. The fatty acids present were identified according to their retention times compared to those of known standards. Their quantification was determined using the areas of their peaks relative to the area of the internal standard, according to the equation 5.

$$w_{FAi} = \frac{A_{FAi}}{A_{(17:0)}} \times FR_{FAi} \quad (5)$$

where w_{FAi} is the mass of the fatty acid, A_{FAi} is its peak area, $A_{(17:0)}$ is the peak area of the internal standard, and FR_{FAi} is the response factor of the fatty acid.

2.8. Soxhlet

The lipid extraction from the biomass prepared in the assay III was performed by the conventional Soxhlet method and testing 3 solvents: hexane, ethanol and acetone. The biomass prepared in assays I and II was also subjected to Soxhlet extraction with hexane, in order to quantify the total lipids present in the biomass. For each assay, 0.5 g of freeze-dried biomass, previously ground in a ball mill, was weighed in duplicate into a cellulose cartridge. The cartridge was placed in a Soxhlet extractor tube and about 140-150 mL of solvent was used in each distillation flask. The extraction time was 6 hours. After extraction, the solution with the lipid extract was filtered under vacuum, using sequentially filters of 0.2 and 0.1 µm of porosity, in order to remove any biomass that might be in suspension. Next, the solution with extract was concentrated in a rotary vacuum evaporator in order to completely evaporate the solvent and thus be able to gravimetrically quantify the extracted lipids. The concentrated lipid extract was placed under inert atmosphere and covered from light. After 1 hour of drying in the oven at 30 °C, for complete evaporation of any solvent remaining in the concentration flask, the extract was placed in the desiccator in order to stabilize at constant weight. The equation 6 allowed to calculate the percentage of lipids obtained in each extraction, being $w_{biomass}$ the mass of the biomass corrected (removing the moisture and ash previously determined).

$$Lipids\ (\%) = \frac{w_{flask+lipids} - w_{flask}}{w_{biomass}} \times 100 \quad (6)$$

2.9. Accelerated solvent extraction - ASE

The accelerated solvent extraction (ASE) method was studied as a potential method for the extraction of lipids from the biomass of *C. cohnii*, prepared in Assay III. The ASE extraction study was started with some preliminary trials, in order to choose which conditions to implement in the experimental design. For this, some solvents (hexane, ethanol and acetone), numbers of cycles (1 and 2) and duration of cycles (3, 6 and 26 minutes) were tested. A Thermo Scientific Dionex ASE 150 apparatus was used. The preliminary tests were performed using about 250 mg of freeze-dried biomass, previously ground in a ball mill (same conditions as described above). To avoid clogging of the bed, the biomass was mixed with diactomaceous earth (about 0.5 g) in the first assays performed and in the remaining with glass beads (about 5 g) of 0.3 mm diameter, since these are reusable after washing, unlike the first filling. Hydrophilic cotton and cellulose filters were placed at the ends of the extraction cell (5 mL internal volume) in order to avoid dragging solid particles by the solvent. After extraction, each solution containing the lipid extract was filtered and the extract concentrated.

2.10. Experimental planning

After the preliminary tests it was possible to define the conditions for designing the experimental planning. Thus, an experimental plan was defined for the solvents hexane and ethanol, in order to determine the ranges of temperature and extraction time that maximize the yields in lipids, total fatty acids, DHA and fatty acids for biodiesel production. A methodology based on a response surface was used, with a distribution for two Doehlert [4] factors. A minimum limit of 40 °C and a maximum limit of 140 °C were set for the temperature. For the extraction time factor, 2 and 20 minutes were selected as minimum and maximum limits, respectively. Only 1 extraction cycle was considered. With this experimental domain the Doehlert distribution was then applied, determining the conditions for the tests to be performed. Seven different conditions were defined - table 1 - and 14 tests were performed, since

they were performed in duplicate.

Table 1: Temperature and extraction time conditions tested in the experimental planning.

Test	Temperature (°C)	Time (min)
1	90	11
2	140	11
3	40	11
4	115	19
5	65	3
6	115	3
7	65	19

2.11. Supercritical extraction

To perform the supercritical extraction tests, the apparatus available at the LNEG (National Laboratory for Energy and Geology) was used. The unit is connected to a bottle of liquefied carbon dioxide which has an extension pipe, ensuring that the CO_2 comes out in a liquid state. This passes through a non-return valve and is cooled in a coil and immersed in an ice bath, to ensure that it enters the pump completely liquefied. After leaving the pump, the pressurized fluid is heated up to working temperature and then enters the extraction cell. This consists of an ASI 316 stainless steel vessel, with an internal volume of 5 cm^3, in which the freeze-dried and ball-milled biomass (about 1.2 g) is placed. The bed was filled with glass beads (approximately 5 g) of about 0.3 mm diameter, in order to avoid clogging (formation of a cake). The fluid exits the extraction cell and is expanded to near atmospheric pressure, causing precipitation of the extracted compounds. The compounds are trapped in the three-way valve, the cotton previously inserted in a U-tube (13), immersed in ice, and the tubing in between. To recover them in their entirety, several washes are made with an appropriate solvent (hexane). Four extracts are collected over the extraction time. The first, second and third extracts are taken sequentially after 30 minutes and the fourth is collected after 3 hours. The 4 U-tubes and corresponding cotton wadding used for the collection of each extract are washed with the solvent in order to recover the precipitated lipids. Each extract is vacuum filtered with a 0.2 µm filter and concentrated in the rotary vacuum evaporator mentioned earlier. After concentration, the extract, under inert atmosphere and covered from light, is placed in the oven at 30 °C for 1 hour to allow better drying of the hexane. Then the extracts are placed in the desiccator for complete drying and accurate weighing.

2.12. Evaluation of the biodiesel fraction

The oil from the microalgae was characterized in terms of several evaluation indicators for biodiesel. This was done by separating the monounsaturated and saturated fraction from the polyunsaturated one, by urea complexation [15], and then some parameters were calculated through the fatty acid profile obtained from the biodiesel fraction. By calculating the average unsaturation - AU - the parameters were estimated, using the equations 8, 9, 10 and 11, previously determined and present in the literature [12]. The average unsaturation - equation 7 - is based on the fatty acid profile, where N is the number of double bonds of the unsaturated fatty acid and C_i is its concentration in the total fatty acids, in mass fraction.

$$AU = \sum N \times C_i \tag{7}$$

$$Viscosity = -0.6316AU + 5.2065 \tag{8}$$

$$Specific\ gravity = 0.0055AU + 0.8726 \tag{9}$$

$$cetane\ number = -6.6684AU + 62.876 \tag{10}$$

$$iodine\ number = 74.373AU + 12.71 \tag{11}$$

2.13. Quantification of pigments

To quantify the pigments present in each lipid extract, UV/Visible spectrophotometry was used after volume measurement. The spectrum between 380 and 700 nm was plotted and the Lamber-Beer law was used to determine the concentration in the extract and, consequently, the pigment mass - equation 12. A corresponds to the absorbance of the solution at the wavelength of the absorption maximum of the compound, l to the optical path, ε to the absorptivity of the compound and C to its concentration.

$$A = \varepsilon \times C \times l \tag{12}$$

3. Results

3.1. Fermentations

3.1.1 Assay I

In the first experiment, glucose was used as the carbon source. The culture showed exponential growth until 66 hours, reaching a concentration of 6.65 g/L. From then on, pulses were inoculated whenever there was no glucose in the medium or the optical density decreased. At t = 166.6 hours, it was decided to end the fermentation, since the optical density began to decrease and the medium contained carbon - 12.7 g/L. A final biomass concentration of 18.7 g/L was reached. The percentage of total lipids in the final biomass, obtained by soxhlet, was 16.8%. In the previous sample analyzed, t = 144 hours, the concentration of FA was 16.5%, and it is possible to conclude that the cells started to consume their own lipids instead of the glucose available in the medium. This event was possibly due to oxygen deficiency, which inhibited lipid synthesis.

3.1.2 Assay II

In the second trial, previously hydrolyzed cane molasses was used as a carbon source. As it was detected by HPLC, after the fermentation, that the hydrolysis of molasses was not efficient, the concentration of glucose in the medium was always reduced, preventing the growth of biomass. In addition, yeast contamination appeared after 119 hours. Thus, the fermentation was terminated at this point, reaching a concentration of algae and yeast biomass of 16.8 g/L, with only 7.5% total lipids and 2.5% of DHA.

3.1.3 Assay III

In the last experiment, crude glycerol was used as the carbon source. The graphic representing the evolution of dry weight is shown in the figure 1. It can be seen that until 67.25 hours of fermentation, the biomass growth was exponential, with a specific growth rate of 0.043 h^{-1}. From that moment until 95.25 hours the growth slowed down, reaching a dry weight concentration of 8.01 g/L.

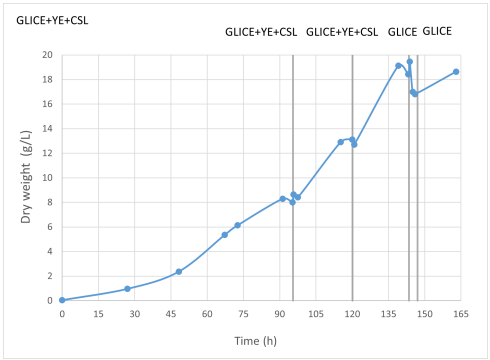

Figure 1: Dry cell weight.

It was found that at this instant the carbon source concentration was zero. In order to prolong cell growth, a pulse was inoculated with glycerol, yeast extract and corn extract. The graph of the figure 2, obtained later by HPLC, allows us to conclude that there was no glycerol in the medium at that moment. A 4th and final pulse of glycerol was inoculated at time t = 147h, since the glycerol quantification method detected a low glycerol concentration and a small decrease in optical density was observed. However, it can be seen from the figure 2, that there was an error in the procedure, in that at this time the residual glycerol was 18.51 g/L. Since the concentration of the carbon source was too high (30.87 g/L), the biomass did not grow as expected due to inhibition by the substrate, ending the fermentation after 164 hours, with a dry weight concentration of 18.65 g/L. The average biomass productivity was calculated at this instant - 0.114

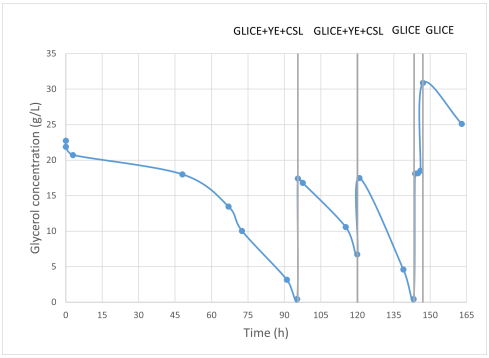

Figure 2: Residual glycerol concentration.

g/(L.h). The figure 3 shows the evolution of the dissolved oxygen percentage and the stirring speed over time, recorded by the control module. When dissolved oxygen readings were below 30%, the agitation speed was increased and varied between 100 and 300 rpm.

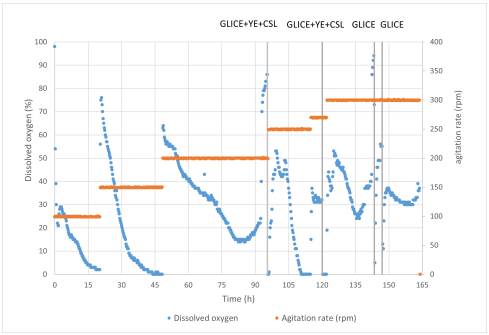

Figure 3: DO percentage and agitation rate.

Three samples were collected during the stationary phase, in addition to the final one, to analyze the evolution of the fatty acid concentration and its composition. From 96 hours, the percentage of fatty acids in biomass varied between 15.02 and 18.80 and DHA between 5.22 and 7.52. The concentration of DHA in the TFAs was increasing throughout the trial, being 40.02% in the final biomass. The evolution of TFA and ADH concentration in the biomass is represented in figure 4. Figure 5 shows the evolution of the percentage of cells in each subpopulation. The percentage of cells with permeabilized membrane and no enzymatic activity (yellow curve) was zero throughout the fermentation. The subpopulation with permeabilized membrane and enzymatic activity (gray curve) started as the second dominant subpopulation, with about 24.4% of the cells, and decreased over time, ending the assay with only 6.5%. Cells with intact membrane and enzyme activity were always the dominant subpopulation, ranging from 52.9 to 83.4%. After the inoculation of the last pulse, the percentage of these cells in

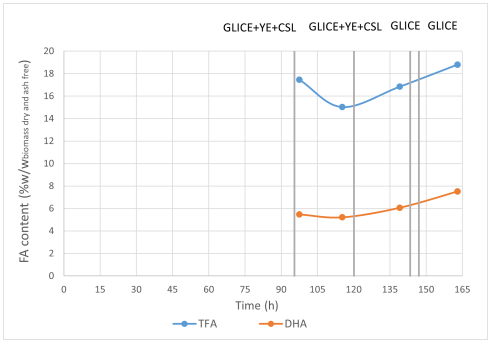

Figure 4: TFA and DHA content.

the medium decreased, and the percentage of cells with intact membrane but without enzymatic activity increased, reaching the highest of the whole fermentation. These results suggest that the enzymatic activity of the cells was compromised by the excessive concentration of glycerol in the medium.

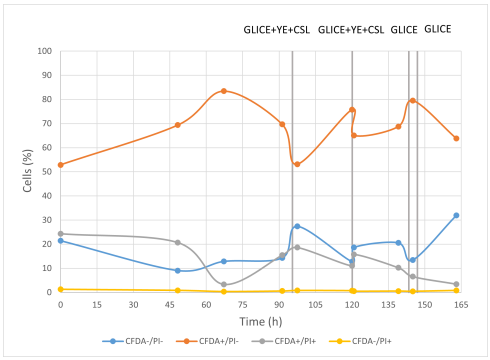

Figure 5: Percentage of cells in each subpopulation.

3.2. Lipid extraction

Of the three microalgae culture trials performed, it was found that trial III was the one that allowed to obtain the highest amount of biomass, which presented high lipid and fatty acid content. For these reasons, this was the biomass used to perform the lipid extraction studies.

3.2.1 Soxhlet

In order to evaluate the yields of the green ASE and SCE methods, the Soxhlet extraction, the conventional method, was performed first. Tests were performed in duplicate with hexane, ethanol and acetone. The yield in total lipids, in total fatty acids (TFA), DHA and fatty acids for biodiesel was determined. Table 2 presents the results obtained.

It is observed that the lipid yield is considerably higher for ethanol and acetone. In fact, due to their polarity, these solvents extract other non-lipid compounds, so the extract obtained with these solvents

Table 2: Results obtained by Soxhlet extraction. The yields are presented in g/100g dry biomass and ash-free.

Solv.	Lipids yield	TFA yield	DHA yield	Biodie. yield
Hex.	20.6±06	18.5±0.9	7.0±0.5	11.4±0.5
Et.	29.7±0.4	21.9±0.1	8.4±0.1	13.4±0.1
Acet.	26.4±0.4	16.0±0.2	6.0±0.3	9,9±0.1

does not correspond only to the total lipids of the biomass and is therefore overestimated. Analyzing the values of the table 2, it is concluded that the solvents hexane and ethanol are the most efficient solvents in the extraction of DHA and fatty acids for biodiesel, the compounds of interest of this study. The results obtained for acetone show that this solvent extracts a high amount of non-lipid compounds, showing a less efficient extraction of fatty acids.

3.2.2 Accelerated solvent extraction - ASE

ASE was the green method studied with most emphasis in this work, and an experimental planning based on a response surface according to the Doehlert distribution was performed in order to determine the ranges of temperature and extraction time that maximize the yield in lipids, TFA, DHA and biodiesel. The first tests consisted in the analysis of hexane, acetone and ethanol as solvents, since only 2 experimental matrices were going to be performed. For this, tests were performed, in duplicate, at 120 °C, with 2 extraction cycles, each of 3 minutes. The same conditions were repeated with hexane, using glass beads instead of diactomaceous earth, since these can be reused. The results obtained are shown in table 3.

Table 3: Preliminars results obtained by ASE.The yields are presented in g/100g dry biomass and ash-free.

Solv.	Lipids yield	TFA yield	DHA yield	Biodi. yield
Hex.	19.16	16.12	6.26	9.76
Et.	28.1±0.1	19.4±1.6	5.0±0,2	14.4±1.8
Acet.	23.4±0.1	14.4±0.3	5.5±0.1	8.8±0.1
w/be.	21.9±0.3	19.1±0.5	7.4±0.2	11.6±0.3

Analyzing the table, it is possible to conclude that acetone is the solvent that allows obtaining less satisfactory yields, as verified in Soxhlet. Regarding hexane, similar results were obtained, particularly using glass beads. Thus, it was decided that the

two solvents to be studied would be hexane and ethanol and that glass beads would be used instead of diactomaceous earth. Then it was defined that the experimental planning would consist of trials with only 1 cycle, and the extraction time interval to be studied would be from 2 to 20 minutes. Regarding the temperature, the range to be studied was defined as 40 to 140 °C. Table 4 presents the tests performed and the responses obtained for the yield in lipids, in TFA, in DHA and fatty acids for biodiesel, using hexane as the solvent. Fixing the

Table 4: Experimental planning matrix and responses obtained, using hexane as solvent. The yields are presented in g/100g dry biomass and ash-free.

Test	°C	min	lipids	TFA	DHA	Biodie.
1	90	11	21.82	18.70	6.95	11.61
2	90	11	22.18	19.02	7.28	11.61
3	140	11	21.09	19,87	7.64	12.09
4	140	11	22.48	20.95	8.02	12.80
5	40	11	27.58	22.16	8.43	13.58
6	40	11	25.12	20.57	7.92	12,52
7	115	19	22.04	17.74	6.75	10.87
8	115	19	23.74	18.26	6.92	11.21
9	65	3	20.85	19.28	7.28	11.87
10	65	3	21.75	19.30	7.36	11.82
11	115	3	20.91	17.15	6,52	10.52
12	115	3	20.85	17.57	6.73	10.73
13	65	19	23.58	17.23	6.47	10,58
14	65	19	23.38	17.66	6.85	10.70

extraction time at 11 min, the value of the average extraction yield in lipids of 26.4 g/100g of biomass, obtained at 40°C decreases by about 18% when the temperature increases to 90°C. With the increase in temperature to 140°C the value of extraction practically does not change. This extraction profile is observed in the remaining answers. For lower extraction times (3 min) the temperature change from 65°C to 115°C shows a slight decrease of 0.5 g/100g biomass corresponding to a 2.3% decrease in lipid extraction. The remaining answers decrease as well. It should be noted that, for all conditions, lipid yields higher or similar to those obtained in the soxhlet method with hexane were obtained. The lowest lipid yield - 20.88 g/100g biomass - was obtained at a temperature of 115°C and a time of 3 minutes; however, based on the soxhlet yield, a recovery of 101.3% was obtained. The highest lipid yield obtained - 26.35 g/100g biomass - presents a lipid recovery rate of 128.9%. This conclusion is quite satisfactory in that the ASE method is a more sustainable process than the conventional one, since it presents a shorter extraction time and uses smaller volumes of organic solvents. In the experimental planning matrix using ethanol as solvent, the highest yields were obtained for 140°C and 11 minutes: 18.96, 7.28 and 11.56 g/100g biomass, for TFA, DHA and biodiesel, respectively. Although higher yields were obtained in soxhlet with this solvent, the recovery rates based on this method were high: 86.7 percent for TFA, 86.9 percent for DHA, and 86.5 percent for biodiesel.

3.2.3 Supercritical extraction - SCE

In addition to the sustainable ASE method, the SCE with carbon dioxide was studied. Five trials with different pressure and temperature conditions were performed in order to evaluate the effect of varying them. The cumulative yield in lipids is shown in figure 6.

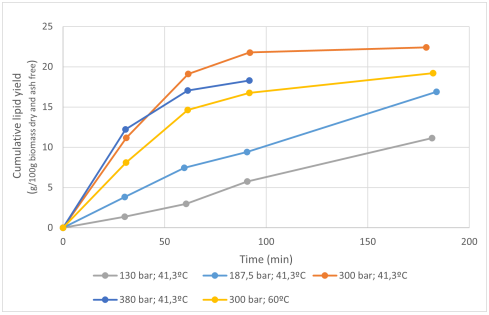

Figure 6: Cumulative lipid yield over the extraction time for the SCE conditions performed.

Observing the graphic, it is possible to conclude that, fixing the temperature at 41.3 °C, the yield increases by varying the pressure from 130 to 187.5 bar, and from 187.5 bar to 300 bar. However, by increasing the pressure from 300 to 380 bar the yield decreases. It can be seen that the progression of lipid yield over extraction time has distinct profiles. For the lowest pressures, the curve shows a linear shape, with an almost proportional increase in yield over time. The other three curves have two zones: a linear and a plateau. The existence of these two zones is due to the fact that the SCE process comprises two phases. The first one consists in the extraction of the most accessible compounds. In the second stage, the solute that was free has been depleted, and the extraction of the intracellular compounds begins. First those that are fragmented are extracted, and then those that are intact. Thus, it is concluded that the tests at 41.3 °C and 130 and 187.5 bar, did not reach the second stage of extraction, requiring more than 3 hours for this to happen. At the condition of 300 bar and 41.3 °C, the lipid yield was the maximum observed, 22.40 g

per 100 g of biomass. In comparison with the soxhlet method, 108.7% of the lipids were recovered, a very positive result. It can be seen that at 300 bar, increasing the temperature from 41.3 °C to 60 °C results in a decrease in the slope of the 1st phase curve, concluding that the solubility of total lipids in the solvent is lower at 6o °C, resulting in a lower yield of lipids. There is also a decrease in yield when the pressure is increased from 300 to 380 bar at 41.3 °C, but for different reasons, since the slope of the curve corresponding to the first phase is similar. At this temperature, the pressure variation to high values results in the decrease of the lipid yield, since in the phase that is controlled by diffusion, the solvent has more difficulty in diffusing into the pores of the solid matrix. The extracts were then analyzed by gas chromatography, in order to calculate the yields in TFA, DHA and biodiesel, and are summarized in table 5.

Table 5: Yields obtained for the ESC trials, in g per 100g dry and ash-free biomass .

	Conditions	TFA	DHA	Biodie.
1	187..5 bar, 41.3°C	11.97	4.42	7.47
2	300 bar, 41.3ºC	15.38	5.87	9.39
3	130 bar, 41.3°C	9.69	2.59	7.04
4	300 bar, 60°C	18.39	7.08	11.20
5	380 bar, 41.3°C	15.64	6.02	9.53

Looking at the table, it can be concluded that higher yields of TFA correspond to higher yields of DHA and FA for biodiesel production. Keeping the temperature at 41.3 °C and increasing the pressure from 187.5 to 300 bar, the yield in TFA increased 28.5%, in DHA 32.8% and in biodiesel 25.7%. Increasing to 380 bar, the observed yields were similar, concluding that at 41.3 ºC, maximum solubility for fatty acids is reached at pressures near 300 bar, not benefiting from higher pressures to extract these compounds. Observing the results of tests 2 and 4, it can be seen that by increasing the temperature from 41.3 to 60 °C, at a fixed pressure of 300 bar, the yields in TFA, DHA and biodiesel increase by 19.6, 20.6 and 19.3%. Although a higher yield in total lipids was observed for 41.3 °C, it is concluded that at this temperature carbon dioxide has a higher solubilizing power of other lipids, however, the solubility of fatty acids is lower. Compared to the soxhlet method with hexane, very satisfactory recovery rates were obtained for test 4: 99.6% for TFA, 101.2% for DHA and 98.7% for biodiesel.

3.3. Pigments

All lipid extracts obtained by the soxhlet, ASE and SCE methods had a yellow-orange hue, which is stronger the higher the concentration of pigments. In order to determine this, the extracts were analyzed by UV/Visible spectrophotometry. The highest pigment content - 121.37 µg/g of dry, ash-free biomass - was obtained using the ASE method with hexane at 115 °C and 19 minutes.

3.4. Evaluation of the biodiesel fraction

As mentioned above, it was done the separation of the fraction for biodiesel from the oil extracted from the biomass produced in trial I by urea complexation method. The method used proved to be very efficient, in that a fraction rich in saturated and monounsaturated fatty acids was obtained and had the ability to reduce the percentage of DHA in the TFA from 51.60 to 8.359%. In order to assess whether this oil was suitable for biodiesel, the parameters were determined by calculating the AU. The value obtained for the AU was 0.68861 and the estimated values for the properties are presented in the table 6.

Table 6: Values obtained for biodiesel parameters from the AU and their comparison with those required in the standard EN 14214

Parameter	Value	Required value
Viscosity (mm^2/s)	4.77	3.5-5.0
Specific gravity	0.876	0.86-0.9
Cetane number	56.51	Min. 51
Iodine number	63.92	Max. 120

Observing the table, it can be seen that the 4 estimated parameters are within the established regulations. Thus, taking into account these properties, it is foreseen the possibility of using the obtained oil for biodiesel production.

4. Conclusions

The main objective of the present work was to produce high cell concentration cultures of the microalgae *C. cohnii*, with high intracellular lipid content. Tests were performed with three carbon sources: glucose, and industrial by-products: cane molasses and crude glycerol. The assay with crude glycerol resulted in a final biomass concentration of 18.7 g/L, with a lipid and DHA content of 20.6 and 7.5% (w/w), demonstrating that it can be used as an alternative to glucose. The biomass obtained in trial III was used to study greener lipid extraction techniques: ASE and SCE. The ASE study concluded that, using hexane as solvent, this method benefits from lower temperatures (40-45 °C) and higher extraction times (13-19 minutes) to maximize the total lipid yield. Regarding TFA, DHA and FA for biodiesel production, the maximization of its yield benefits from temperatures in the same range,

however, it does not need high extraction times, showing a maximum yield in the range of 2-12 minutes. When comparing this process with the Soxhlet method with hexane, it was concluded that it was possible to obtain higher yields, showing itself to be a very promising method. The experimental planning allowed concluding that using ethanol as solvent, the variation of the extraction time causes a greater increase in the yield in TFA, DHA and FA for biodiesel production than the increase in temperature, being that with intermediate times (10-15 minutes) and a minimum temperature of 60 °C, the yield is maximum. Although it does not prove to be as efficient a solvent as hexane in this technique, it also proves to be a solvent with potential use in this method. The SCE tests performed allowed the conclusion that at low temperatures (41.3-60 °C) and higher pressures (300-380 bar), the yields of lipids, TFA, DHA and FA for biodiesel production are higher than those obtained by Soxhlet extraction. These findings are very positive, since this method has environmental advantages. It was possible to conclude that the ASE and SCE methods also present a high extraction power of the pigments synthesized by the *C. cohnii*. Physical parameters of the biodiesel fraction were estimated and confirmed to be in accordance with the limits stipulated by the European standard.

References

[1] A. Atabani, A.S.Silitonga, I.Anjum, T.M.I.Mahlia, H.H.Masjuki, and S.Mekhilef. A comprehensive review on biodiesel as an alternative energy resource and its characteristics. *Renew. Sustain. Energy Rev.*, 16:2070–2093, 2012.

[2] C.A.Beam and M. Himes. Distribution of members of the crypthecodinium cohnii (dynophyceae) species complex. *J. Protozool*, 29(1), 1982.

[3] Y. Chisti. Biodiesel from microalgae. *Biotechnology advances*, 25:294–306, 2007.

[4] D. H. Doehlert. Uniform shell designs. *Journal of the Royal statistical society series*, pages 231–239, 1970.

[5] D. F. e N. Valentinyi e L.Rasz e P.Mizsey. Evaluation of microalgae-based biorefinery alternatives. *Clean Technol.Environ.Policy*, pages 501–5015, 2016.

[6] R. M. C. e Pedro Calado Simões e Alberto Reis e Teresa Lopes da Silva e Vitor Hugo Martins e Yolanda Sanchez Vicente. Supercritical fluid extraction of lipids from the heterotrophic microalga crypthecoidinium cohnii. *Eng. Life Sci.*, 10:158–164, 2010.

[7] M. M. e S.D Sarker. Accelerated solvent extraction for natural products isolation. *Natural Products Isolation. Methods in Molecular Biology*, 864:75–87, 2012.

[8] I. Efthymiopoulos, P. Hellier, N. Ladommatos, A. E. 1, and B. Mills-Lamptey. Factores affecting the efficiency of pressurized solvent extraction of oil from spent coffee grounds. *Detritus*, pages 1–9, 2019.

[9] F. Industries. Microalgae n-3 pufas production and use in food and feed industries. *Marine Drugs*, 19:1–29, 2021.

[10] L. Jaime, I. Rodrıguez-Meizoso, A. Cifuentes, S. Santoyo, S. Suarez, E. Ibanez, and F. J. Senorans. Pressurized liquids as an alternative process to antioxidant carotenoids' extraction from haematococcus pluvialis microalgae. *Elsevier*, 43:105–112, 2010.

[11] T. J.Bruno, C. N. de Castro, J.-F. P.Hamel, and A. M. Palvra. Supercritical fluid extraction of biological products. *Recovery Processes for Biological Materials*, pages 302–353, 1993.

[12] S. kongruang e S. Roytrakul e M. Syriariyann. Renewable biodiesel production from oleaginous yeast biomass using industrial wastes. *Edp science*, 2020.

[13] S.N.Naik, V. Goud, P.K.Rout, and A.K.Dalai. Production of first and second generation biofuels: A comprehensive review. 14:578–597, 2010.

[14] M. D. Swaaf, J. Pronk, and L. Sijtsma. Fedbatch cultivation of the docosahexaenoic acid producing marine alga crypthecodinium cohnii on ethanol. *Applied microbiology and biotechnology*, 61(1):40–43, 2003.

[15] R. G. e. A. R. T. Lopes da Silva, A.R. Santos. Valorizing fish canning industry by-products to produce -3 compounds and biodiesel. *Environmental Technology Innovation*, pages 74–81, 2018.

[16] T.Lopes and C. Silva. The dark side of microalgae biotechnology: a heterotrophic biorefinery platform directed to w-3 rich lipid production. *Microorganisms*, 7, 2019.