Omega-3 Compounds Extraction from Microalgae *Crypthecodinium cohnii* Using Greens Solvents and Supercritical Fluids

Filipe Rosa
DEQ, Instituto Superior Técnico, Lisbon
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Abstract

In this study, marine microalgae *Crypthecodinium cohnii* was cultivated in a bench bioreactor under a fed-batch regime. Four experiments were carried out with the goal of obtaining a biomass high yielding in lipids and fatty acids, especially docosahexaenoic acid (DHA). Carbon sources included glycerol and cane molasses. Accumulated pigments were also analysed. Humidity and ashes content was evaluated in order to remove their influence in the results. On Experiment I, molasses was used as a carbon source. However, a contamination led to its ending and the culture was discarded. On Experiment II, Experiment I was repeated. However, high carbon concentration led to microalgae inhibition. This experiment yielded a biomass with low lipidic content. On Experiment III, glycerol was used as a carbon source. Nevertheless, equipment failure led to pH increase and the fermentation ended abruptly, yielding also a biomass with low lipidic content. Experiment IV was a reproduction of Experiment III and yielded a biomass with 26.58%(m/m), 25.45%(m/m) and 9.21%(m/m) of lipids, fatty acids and DHA and 61.37 $\mu g/g_{BM}$ of pigments. Since this biomass was the one with most yield in desired compounds, it was used for the experimental planning using accelerated solvent extraction (ASE). This consisted in studying the influence that temperature and extraction time would have on extraction yield and was done using green solvents. The best extraction conditions, for both solvents, were 2 minutes and 200°C, yielding 24.5 $g/100g_{BM}$ in fatty acids and 9.2 $g/100g_{BM}$ in DHA, for ethyl acetate, and 26.2 $g/100g_{BM}$ in fatty acids and 9.0 $g/100g_{BM}$ in DHA, for 2-methyltetrahydrofuran. Moreover, supercritical CO_2 extraction studies were also carried out with the aim of evaluating the influence of cell disruption mechanisms (high pressure homogenization and ball milling) and drying processes (convection and freeze drying) on the yield of total lipids, total fatty acids and DHA.

Keywords: *Crypthecodinium cohnii*, docosahexaenoic acid, fatty acids, accelerated solvent extraction, supercritical extraction, pigments.

1. Introduction

Microalgae are unicellular and generally photosynthetic microorganisms. Their economic interest comes from their ability to synthetize and accumulate important compounds such as lipids and pigments. Besides this, they can be implemented in bioremediation strategies, biofuel production and carbon capture strategies.[1] Also, they present many advantages over traditional biomasses, such as being able to be cultivated in land not suited for agriculture, fast biomass production and not competing with the food chain. However, there are some disadvantages such as high water requirement and costly extractions.[2]

Microalgae *Crypthecodinium cohnii* is a heterotrophic (non-photosynthetic) marine dinoflagellate that inhabits brackish waters all over the world. It is noteworthy due to its high capacity of storing lipids (over 20% dry weight) with a high concentration of docosahexaenoic acid (DHA) – over 30% of total fatty acids (TFA). DHA is a polyunsaturated fatty acid (PUFA) and it is defined as 22:6ω3 according to the fatty acid nomenclature.[5] It has numerous benefits to health, namely in the formation and maintenance of foetal nervous system, improving intellectual capacity, maintaining visual acuity, lowering hypertension and stroke risks, preventing depression and cognitive decline, lowering metastasis size and occurrence (on oncological patients) and has an anti-inflammatory effect, helping reduce the pain caused by arthritis.[3]

Besides this, FAME obtained through transesterification of the mono, di and triglycerides present in microalgal oil can be used to produce biodiesel.[2]

Since *C. Cohnii* is heterotrophic, its culture is obtained through aerobic fermentation on a dark bioreactor. The culture medium is composed of a carbon source (e.g., glucose, molasses or glycerol) with a concentration between 5-40 g/L, preferably 20 g/L; nitrogen source (e.g. yeast extract or corn steep liquor) with a concentration between 0-10 g/L, preferably 2 g/L and 17.8 g/L of sea salt.

Optimum growth conditions are 27°C, pH of 6.6, dissolved oxygen greater than 10% and low agitation.[4]

In order to obtain the compounds of interest from a solid matrix, an extraction technique must be used. Soxhlet extraction is a conventional method used as standard technique, due to its high extraction yields without as much waste solvent as in other conventional methods. Besides this, Soxhlet apparatus allows for constant contact of fresh solvent with the sample. Its main disadvantage is using high temperatures (close to solvent boiling points), which can degrade thermolabile components.[5]

Accelerated solvent extraction (ASE) is a newer technique which allows the usage of high temperature and pressure liquids to perform an extraction. The high pressure maintains the solvent in a liquid state, even in a temperature above its boiling point. ASE consists of injecting solvent in an extraction cell, which remains there for a static period (5-15 minutes, usually). After this, the solvent is flushed to a collection bottle and the sample is washed with an inert gas. This is called a static cycle and extractions can have many cycles, with the advantage of providing fresh solvent to the sample. The sample is usually mixed with a dispersing agent to prevent channelling and clogging. The usual working temperatures range from room temperature to 200°C. This is the most important factor because its increase leads to greater extraction efficiencies. However, higher temperatures can cause degradation of thermolabile compounds. Working pressure ranges from 35-200 bar and its increase leads to better infiltration of the solvent in the sample. Solvent choice is done by the “like dissolves like” rule but economical and environmental aspects must be taken account. Choosing green or generally recognized as safe solvents (GRAS) makes this a green technique.[6]

Supercritical fluid extraction (SFE) presents many advantages over conventional methods because the solvent is a supercritical fluid (SF). SF is a fluid whose pressure and temperature are above the critical point. Therefore, their physicochemical and transport properties are different from their normal state and, most of the times, more advantageous on extraction yield, e.g., lower viscosity and higher diffusivity, resulting in higher mass transfer rates. Also, density can be adjusted by temperature and pressure and, therefore, solubility of compounds, making it possible to adjust the extract composition by changing these parameters. Besides, if a gas is used as a SF, returning to standard temperature and pressure (STP) will allow for a solvent-free extract. Carbon dioxide is a preferred SF because it is not harmful to health and environment and has moderate critical conditions (31.2°C and 72.8 bar) which allows the extraction of thermolabile compounds free of solvent.[7]

Cellular wall disruption is a necessary step before extraction of intracellular compounds, as lipids and pigments. Ball milling consists of using a cell with biomass and spheres that are rapidly agitated causing cell wall breakage. High pressure homogenization (HPH) consists of pumping a cell suspension at a high pressure through a valve, onto an impact ring, causing cell disruption by sudden pressure change, impact and cavitation.[8]

Water removal from the biomass is imperative, to prevent degradation of compounds and because it negatively impacts extraction processes. Freeze drying consists of freezing the sample and remove the water by sublimation, through vacuum. Convective drying consists of using ovens (40-55°C) to evaporate the water.[9]

2. Materials and Methods

2.1. *C. Cohnii* Fermentation

Microalgae *C. Cohnii* (American Type Culture Collection 30772) cultures are maintained in starter medium, composed by 23 g/L of sea salt, 1.8 g/L of YE and 9 g/L of glucose, at 25°C, in the dark, on 250 mL Erlenmeyer flasks. These are filled with 90 mL of medium, previously sterilized in an autoclave (Uniclave 88, A. J. Costa LDA, Portugal), and 10 mL of a 10%(v/v) cell suspension. These cultures are reinoculated monthly to ensure cell and nutrient maintenance.

The inoculum cultures medium is composed of 25 g/L of sea salt, 2 g/L of YE and 20 g/L of glucose equivalents. This medium is autoclaved and 135 mL of it are added to 500 mL Erlenmeyer flasks along with 15 mL of starter culture. To prevent contaminations, 1 mL per litre of medium of antibiotic is added, composed of 5, 62 and 100 mg/L of chloramphenicol, penicillin G and streptomycin, respectively. These are then incubated (incubator, Unitrom Infors, Switzerland) for 7 days, at 27°C and 110 rpm.

Reactor medium is composed of 27 g/L of sea salt, 0.5 g/L of YE, 5.5 g/L of CSP and 20 g/L of glucose equivalent. This medium was centrifuged (model 6-16KS, Sigma) and autoclaved, to remove suspended particles and sterilize it. All pH levels were brought to 6.5 using NaOH and HCl.

On Experiments I and II, cane molasses (Sidul Açúcares, Portugal) was used, after diluting it in water (1:1 v/v) and hydrolysing it, by bringing its pH to 3 (HCl 5M) and autoclaving it, at 121°C for 1 hour. On Experiments III and IV, glycerol (Iberol, Portugal) was used.

2.2. Fermentation

All experiments were carried out in a 7 L bench bioreactor (model FerMac 360, Electrolab Biotec), without ever exceeding 3 L, in a fed-batch regime. The bioreactor and all its components are washed and autoclaved. To start the fermentation, 2.7 L of reactor medium and 300 mL of inoculum are added, along with the antibiotic aforementioned and an antifungal solution (8 mg of Fosfomycin in 4 mL of DMSO). If foam occurred, PEG was added. If nutrients were low, 300 mL of a 10-time concentrated solution of nutrients was added, henceforth named “pulse”.

The bioreactor was equipped with a rotor, a four blade Rushton turbine, acid and base pumps, pH and DO electrodes, a thermometer, heating mantle and cooling system, a rotameter (for air flow regulation) and sterile feeding tube and collection vial.

The temperature was kept at 27°C, the pH at 6.5 (HCl or NaOH 2.5M), DO above 30%, turbine rotation at 150 rpm and air flow at 1 g/(L.h). The pH electrode was calibrated with pH 4 and 7 buffer solutions and calibration was regularly checked. The DO electrode was calibrated by pumping nitrogen in the system (0% DO) and then air at maximum speed rotation (300-400 rpm, 100% DO). Higher speeds were not allowed due to dinoflagellate sensitivity to damage.

Samples were taken two times a day and the following analysis were made.

2.3. Optical density

Optical density, OD, allows to follow cellular growth. It was measured in triplicates, to ensure accuracy, in a UV-Vis spectrophotometer (model Genesys 20, Thermo Spectronic), at 470 nm, the wavelength of maximum absorption of the culture. Dilutions were made in order to keep its value between 0.2 and 0.6 and absorbance of all mediums was taken in order to correct the OD. Dry weight, DW, could be calculated by a previous determined correlation (Equation 1).

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

2.4. Flow cytometry

Flow cytometry (cytometer, model CytoFLEX Beckman Coulter Life Sciences, USA) was used to measure the autofluorescence, cell membrane integrity, enzyme activity (using PI and CFDA dyes, respectively) and dry weight. Autofluorescence was measured by diluting the sample (in PBS) in a way that there were 200 to 500 events/s and total volume was 500 µL, inside a cytometer tube. PI and CFDA were added (2 e 3µL, respectively), in another tube, maintaining the 500 µL total volume and the incubation time was 15 minutes (in the dark). The autofluorescence value was used to determine the DW, using previously determined correlations (Equation 2).

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

2.5. Residual carbon source quantification

Residual carbon source concentration was determined by liquid chromatography (HPLC, model 1260 Infinity II, Agilent) with an Aminex HPX-87H or HPX-87P column. Glycose, glycerol, fructose and saccharose standard solutions were prepared in order to relate their peak areas to concentration. Samples were centrifuged and supernatant was recovered and filtered to be analysed. Glycose concentration, when used, was roughly estimated in real time by using rapid detection strips (Combur^3 Test®, Roche).

2.6. Culture post-treatment

Culture from the bioreactor was centrifuged, supernatant was discarded and was washed distilled water. It was centrifuged again and supernatant was also discarded. The cake was then freeze or convection dried and was ready to be further analysed.

2.7. Ash and moisture content

Ash and moisture content was determined in order to correct the lipid and pigment content in the biomass. To do this, constant weight crucibles with 100-250 mg of dried biomass (BM) were kept at 103°C for 24 hours, in duplicate. They were cooled in a desiccator and were weighed. Then they were

taken to a kiln at 550°C for 3h, cooled in a desiccator and weighed. The content was obtained through Equations 3 and 4.

$$\%Moisture = \frac{w_{crucible+BM} - w_{crucible}}{m_{BM}} \quad (3)$$

$$\%Ash = \frac{w_{crucible+ash} - w_{crucible}}{w_{BM}} \quad (4)$$

2.8. Fatty acid profile

Fatty acid profile was determined by gas chromatography (GC, model SCION-436-GC, Buker, Germany). To do this, 15-20 mg of oil (approximately 100-160 mg of biomass) were added to a tube, along with 200 µL of heptadecanoic acid (C17:0, 5 mg/mL) as an internal standard and 2 mL of a 19:1 v/v mixture of methanol and vinyl chloride, prepared in an ice bath. Nitrogen was added to the tubes and they were closed and put in a thermostatic bath at 80°C for 1 hour, in the dark. After cooling, 1 mL of water and 2 mL of heptane are added. The organic phase (upper part) is extracted and filtered through hydrophile cotton and anhydrous sodium sulphate, to remove particulate and aqueous phase that could be co-extracted. The fatty acids were identified through their retention times, using standards, and their quantification was done by correlating their area peaks to the internal standard (Equation 5).

$$m_{FA_i} = \frac{A_{FA_i}}{A_{C17:0}} \times RF_{AG_i} \quad (5)$$

where m_{FA_i} is the mass of the fatty acid, A_{FA_i} and $A_{C17:0}$ are the peak areas of the fatty acid and internal standard and RF_{AG_i} is the response factor of the fatty acid.

2.9. Biomass pre-treatment

In order to help release active compounds from the solid matrix, biomass was subjected to pre-treatments. One of them was ball milling (ball mill, model MM400, Retsch), using 0.5 g of dried biomass in each mortar, with 3 steel spheres, for 3.5 minutes at 25 Hz. The other method was the use of high-pressure homogenization. Culture from the bioreactor, before centrifugation, was submitted to one cycle of HPH at 600 bar in a GEA Lab Homogenizer PandaPLUS 2000 and freeze dried.

2.10. Soxhlet extraction

Soxhlet extraction was performed in a Soxhlet apparatus using 0.5 g of pre-treated biomass inside a cellulose cartridge covered with hydrophile cotton and folded to prevent biomass leakage, using 160 mL of hexane as solvent, in duplicate. Extraction was carried out for 6h at approximately 80°C (above solvent boiling point). The extract solution (lipids, fatty acids and pigments) post-treatment is explained in 2.13.

2.11. Accelerated Solvent Extraction

ASE allows for similar results to Soxhlet in shorter times with fewer solvent and is a green technique. Dionex ASE 150 (Thermo Scientific) was used to perform the extractions. 250 mg of pre-treated biomass were weighed and mixed with 4 g of 3 mm glass beads, to prepare the extraction bed and prevent clogging and channelling.

Characterization extractions were performed with 2 static cycles, 3 minutes each, at 120°C, using hexane as solvent. Experimental planning extractions were performed with 1 cycle at the temperature and time of extraction predetermined, using two green solvents (ethyl acetate and 2-methyltetrahydrofuran). Other conditions (60% of wash volume, 30 s purge time and 1 minute of pre-heating) were the same. A similar extract solution to Soxhlet is obtained, which post-treatment is explained in Section 2.13.

Experimental planning was used to maximize DHA and TFA extraction. Previous studies [10] showed that using more than 1 extraction cycle and high extraction times don't lead to noticeable improvements. Therefore, temperature ranged from 40-200°C (equipment range) and time varied between 2-20 minutes. A surface response methodology of two factors (time and temperature) was used, according to a Doehlert distribution of the experimental domain [11]. 7 tests were performed, with one replicate for each test, and the conditions used are shown in Table 1.

Table 1 – Extraction conditions obtained in the experimental planning.

Tests	Temperature (˚C)	Time (min)
1-2	120	11
3-4	200	11
5-6	40	11
7-8	160	19
9-10	80	3
11-12	160	3
13-14	80	19

2.12. Supercritical fluid extraction

SFE allows for direct fractioning, solvent-free extract and is also a green

technique. It was carried in the apparatus available at LNEG (National Laboratory for Energy and Geology). Extraction bed consisted of 1.2 g of pre-treated biomass mixed with 5 g of 3 mm glass beads, to prevent clogging and channelling, inserted in a 5 cm^3 ASI 316 extraction vessel, which is attached to the apparatus. Liquefied CO_2 exits the cylinder and flows through an ice-cold coil to ensure it reaches the pump liquefied. It is then pumped through a non-return valve, to build up the working pressure. It is then heated up in a thermostatic bath at working temperature, allowing for its transition to the SC estate, after what it enters the extraction vessel. After exiting the cell, the fluid is expanded to near atmospheric pressure and interest compounds precipitate in a U-tube whose ends are filled with cotton. It also precipitates in the near tubing and expansion valve so they are washed with a liquid solvent (hexane). The U-tube and the cotton are washed afterwards, with hexane, to ensure full recovery. 4 samples are taken at 30, 60, 90 and 180 minutes of extraction and the washing procedure is repeated. The obtained extract solution, similar to the Soxhlet one, is then treated according to 2.13.

2.13. Lipid quantification

All extract solutions are filtered through 0.2 mm PTFE filters and solvent is then removed in a rotary vacuum evaporator. The extracts are then placed under inert atmosphere, covered from light and put in an oven at 30°C for 1h, to allow full solvent evaporation. They are then transferred to a desiccator where they stay for 12 h, allowing for precise gravimetric quantification, using Equation 6.

$$\%TL(mm) = \frac{w_{flask+lipids} - w_{flask}}{w_{BM}} \quad (6)$$

After this, the lipids are analysed through gas chromatography, as said in 2.8.

2.14. Pigments quantification

Pigments were quantified through spectrophotometry UV-Vis (Hitachi U-2000) using the maximum wavelength of absorption (453-458 nm) and Lambert-Beer Law (Equation 7).

$$A = \varepsilon \times l \times C \quad (7)$$

where A is absorbance, ε is the compound absorptivity, l is the optical path and C is the concentration. Since the ε used was β-carotene, results come in equivalents of β-carotene.

Pigments were also quantified through HPLC (model Series 1100, Hewlett Packard) in the same wavelength, which allowed to detect carotenes presence.

3. Results and discussion

3.1. Fermentation: Experiment I

Experiment I used cane molasses as a carbon source. A contamination led to its ending and the culture was discarded.

3.2. Fermentation: Experiment II

Experiment II was a retrial of Experiment I. Miscalculations led to a high carbon concentration which inhibited the microalgae. 60% of the bioreactor was purged and culture medium without carbon was added along with new inoculum. Little biomass was yielded with 0.2±0.2%(m/m) and 4.2±0.2%(m/m) humidity and ash content, respectively.

3.3. Fermentation: Experiment III

Experiment III used glycerol as a carbon source. Bioreactor malfunction led to pH increase and immediate cease of the fermentation. Little biomass was yielded with 1.3±0.1%(m/m) and 5.3±0.0%(m/m) humidity and ash content, respectively.

The fermentation was followed by cytometry and spectroscopy. With correlations 1 and 2, these values were converted in DW. Results are in Figure 1.

“Gli+YE+CSL” means pulses of glycose, YE and CSL. Using the corrected OD, specific growth rate was 0.019 h^{-1} and maximum cell concentration was 9.19 g/L. Residual carbon was evaluated by HPLC and results are in Figure 2. When glycose levels tended to zero, pulses of nutrients were added.

Cell populations, according to membrane integrity and enzymatic activity, were analysed by cytometry and results are in Figure 3. Cells with intact membrane and enzyme activity (orange curve) were the most present throughout all the fermentation. Cells with broken membrane and enzyme activity (grey curve) represented about 23% of the population, in the end, which can reveal cell breakage by increased agitation.

This fermentation yielded a large quantity of biomass with high lipidic content and 2.6±0.2%(m/m) and 3.8±0.1%(m/m) humidity and ash content, respectively.

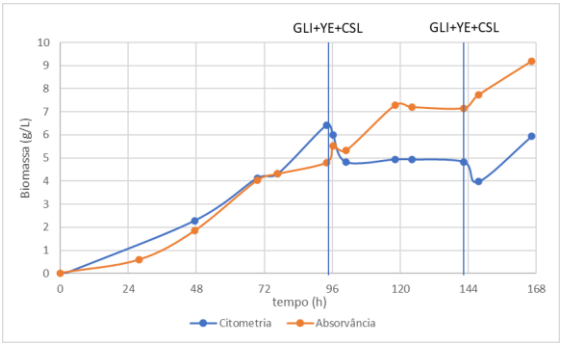

Figure 1 – DW evolution through cytometry and spectroscopy.

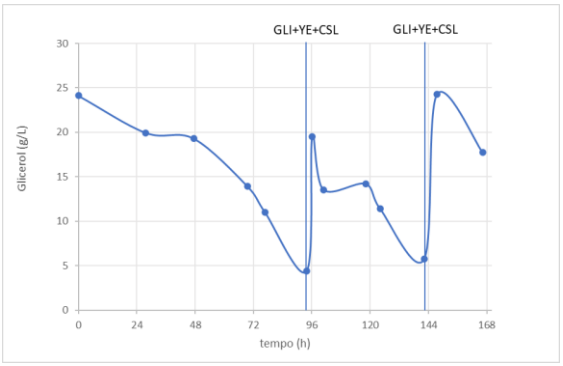

Figure 2 – Residual carbon concentration.

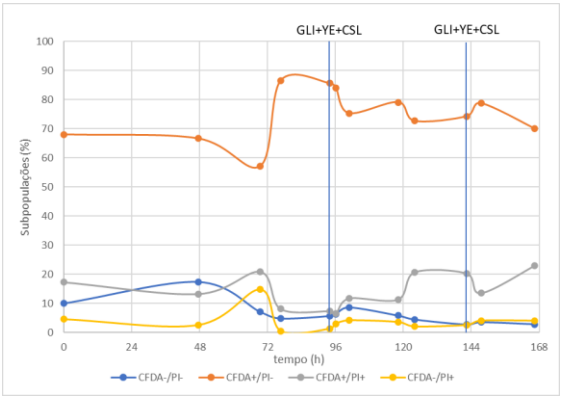

Figure 3 – Cytometry results for cell subpopulations.

3.4. Previous fermentations

HPH treated biomass was obtained by previous fermentations. This biomass was then dried through freeze drying, FD, and oven drying, OD. FD biomass had 3.6±0.2%(m/m) and 14.8±0.1%(m/m) humidity and ash content, respectively. OD biomass had 8.2±0.5%(m/m) and 14.5±0.1%(m/m) humidity and ash content, respectively. Higher humidity in oven dried biomass means that freeze drying is more effective than convective drying. The high ash content is due to salt presence since this culture was not washed in order to be put in the HPH equipment.

3.5. Soxhlet extraction

Table 2 – Soxhlet extraction results in g/100g$_{BM}$.

BM	%TL	%TFA	%DHA
Exp. II	7.6±2.7	5.9±0.1	2.0±0.0
Exp. III	12.3±0.1	9.2±0.2	3.7±0.1
Exp. IV	26.6±1.6	25.5±0.6	9.2±0.3
HPH+FD	22.0±3.8	16.5±1.6	6.4±0.7
HPH+OD	18.6±0.8	15.0±0.7	5.9±0.2

Soxhlet extraction was used as a standard and results are in Table 2. It is possible to conclude that biomass from Experiment IV is the highest yielding biomass in all three parameters. Also, freeze drying yields better results than oven drying.

Pigment extraction results are in Table 3.

Table 3 – Soxhlet extraction results in µg/g$_{BM}$.

BM	Pigments
Exp. II	–
Exp. III	28.9±7.0
Exp. IV	67.3±0.7
HPH+FD	64.4±0.2
HPH+OD	60.2±0.00

Again, freeze drying leads to better results than oven drying and Experiment IV biomass yielded better results.

3.6. Accelerated Solvent Extraction (ASE)

The response surfaces were only calculated to DHA and TFA because ethyl acetate and 2-methyltetrahydrofuran extract lipid-protein complexes, as well as some carbohydrates. The results are according to the model show in Equation 8.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \quad (8)$$

In this equation, Y is the response to each experience, β_0 is the response value in the centre of the experimental domain, β_1 is the temperature contribution, β_2 is the time contribution, β_{12} the interaction between the two factors, β_{11} and β_{22} the quadratic adjust (no physical value) and X the experimental factors. Model effectiveness was tested by Fisher test.

Ethyl acetate results

Response surfaces for DHA and TFA are in Figure 4. Both graphs have the same conformation, which indicates DHA concentration in TFA does not vary.

For all temperatures, increasing extraction time from 2 to 11 minutes leads to a lower yield of DHA and TFA, increasing again from 11 until 20. For example, for AGT, keeping T=120°C and increasing time from 2 to 11 minutes leads to a 6.7% yield decrease (24.0 to

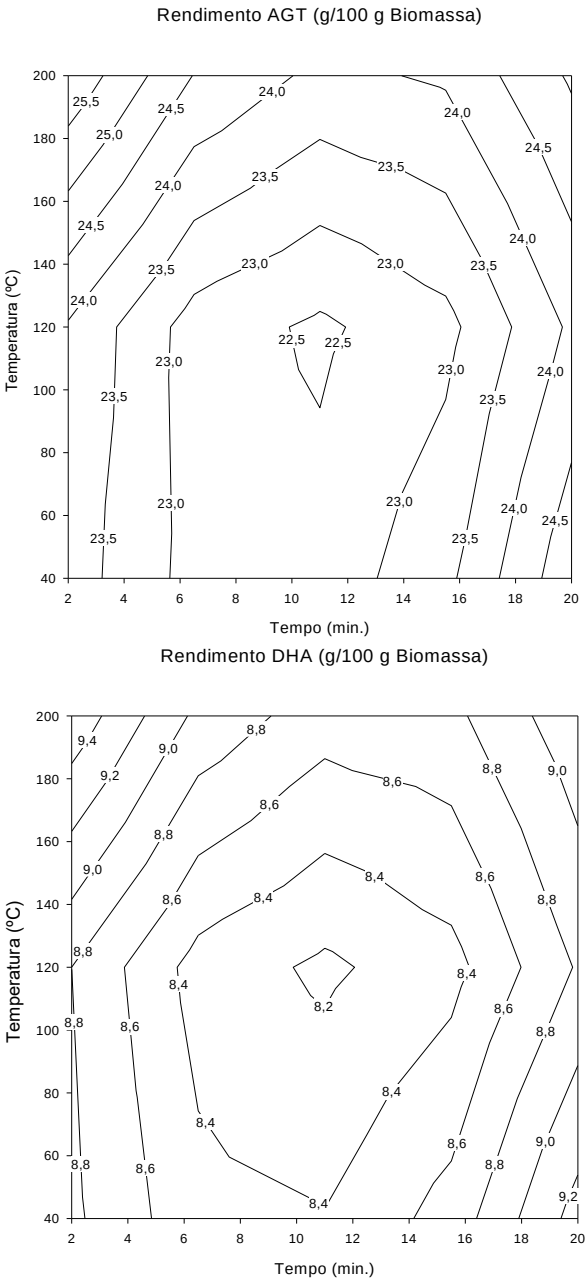

Figure 4 – Response surface for TFA/AGT and DHA. Results in g/100g$_{BM.}$

Table 4 – Model parameters results.

Model parameters	DHA	TFA
β_0	8,16	22,41
β_1	0,14	0,59
β_2	0,01	0,07
β_{12}	-0,21	-0,48
β_{11}	0,39	0,87
β_{22}	0,65	1,61
Effectiveness of parameters	2,85	3,95
Significance level (α), F(5,8)	0,91	0,96
Lack of fit	1,78	1,10
Significance level (α), F(1,7)	0,78	0,67

22.5 wt%). Increasing time to 20 minutes will increase the yield to its original value, which shows a symmetry for t=11 min.

For every extraction, temperature increase from 40°C to 110°C for TFA a 120°C for DHA leads to a yield decrease. Keeping t=11 min, for DHA, this represents a 2.4% reduction (8.4 to 8.2 wt%). By increasing the temperature to 200°C, yield increases by 6.1% (8.7 wt%).

The most important influence is from the temperature since there is a bigger range of values (for AGT at 40°C and 2 minutes, temperature increase to 200°C leads to a 25.5 wt% yield, from 24.0 wt%, while time increase to 20 minutes only leads to a 24.5% increase). Also, there are more isoresponse curves tending to the horizontal than the vertical, which means the variations are more pronounced by ordinates (temperature) than abscissas (time).

The higher yields for both TFA and DHA were obtained at 2 minutes and 200°C, 25.5 and 9.2 wt%, respectively. Also, good results were obtained for 40°C and 20 minutes. This means that it should be investigates if it is more economical and environmentally sustainable using a higher temperature for a short period of time or a lower temperature for a longer period.

Model parameters results are presented in Table 4.

β_1 and β_2 are both positive and close to zero, which means both time and temperature and a small positive influence in extraction yield. β_{12} is negative which shows that the interaction factor is not relevant.

Effectiveness significance levels are 0.91 and 0.96 for DHA and THA, respectively, which means great adjust of the model to the results. Lack of fit significance levels of 0.78 and 0.67 for DHA and THA, respectively, reveal there were some experimental errors.

2-methyltetrahydrofuran results

Response surfaces for DHA and TFA are in Figure 5.

TFA are practically invariable (25.0-26.2 wt%). A symmetry for t=11 min occurs.

Dividing the DHA response surface in quadrants, upper right and lower left quadrants are also invariable (8.0-8.4 wt%)

On the upper left quadrant (T=120°C and t=2 mins), temperature increase to 200°C leads to a 5.9% increase (8.5 to 9 wt%) and time increase to 11 minutes leads to a 6.25% decrease (8.5 to 8 wt%).

On the lower right quadrant (T=40°C and t=11 mins), temperature increase to 120°C doesn't

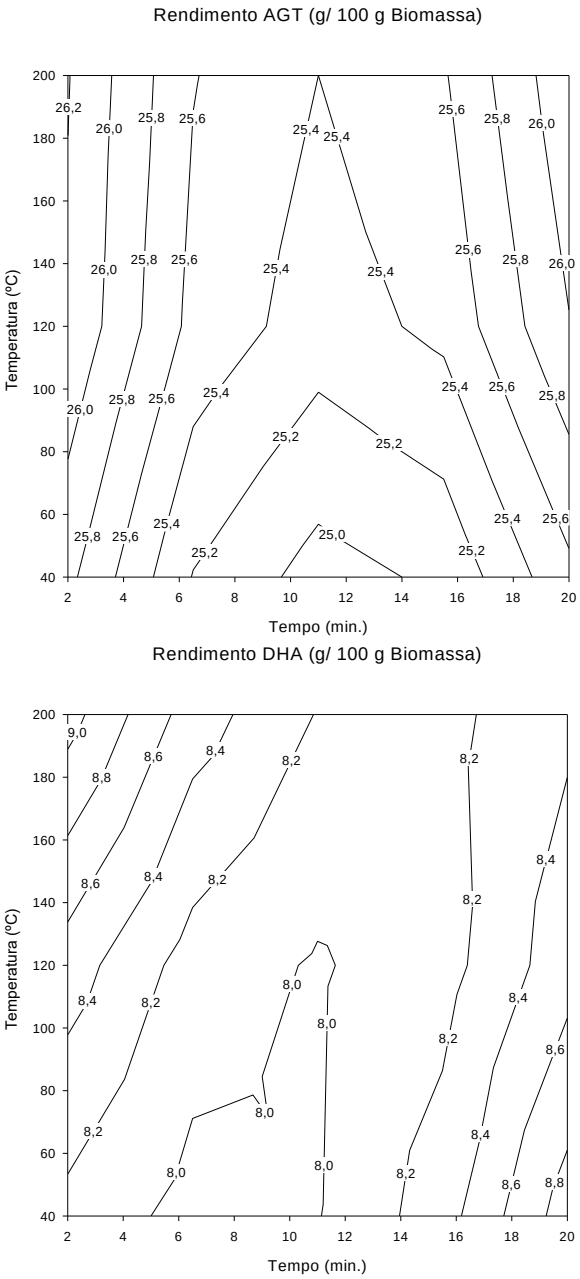

Figure 5 – Response surface for TFA/AGT and DHA. Results in g/100g$_{BM}$.

Table 5 – Model parameters results.

Model parameters	DHA	TFA
β_0	7,98	25,3
β_1	0,1	0,24
β_2	0,01	-0,09
β_{12}	-0,37	0,06
β_{11}	0,11	-0,14
β_{22}	0,53	0,78
Effectiveness of parameters	4,63	1,93
Significance level (α), F(5,8)	0,97	0,81
Lack of fit	5,21	2,32
Significance level (α), F(1,7)	0,94	0,83

affect the yield and time increase to 20 minutes leads to a 10% increase (8.0 to 8.8 wt%). Time effect is more relevant here, shown by several vertical isoresponse curves.

Model parameter results are shown in Table 5.

β_1 and β_2 are close to zero, which means both time and temperature present a small influence in extraction yield. β_{12} is also close to zero which shows that the interaction factor is not relevant.

Effectiveness significance levels are 0.97 and 0.81 for DHA and THA, respectively, which means the model is well adjusted to the results. Lack of fit significance levels of 0.94 and 0.81 for DHA and THA, respectively, reveal there were little experimental errors.

ASE pigment extraction revealed time had practically no influence on extraction yield and temperature had a big negative influence, due to pigment thermolability. Best extraction conditions were 2 minutes and 40°C, yielding 90 and 85 µg/g$_{BM}$ for ethyl acetate and 2-MeTHF. This reveals fast and low energy requiring extractions can be performed to extract pigments. Also, DHA and TFA have good extraction results for these conditions, so all interest compounds can be extracted in these mild conditions.

3.7. Supercritical fluid extraction

SFE with supercritical CO_2 is considered a green technique. SFE was used to study the influence of biomass pre-treatment techniques (HPH and ball milling) and drying methods (freeze, FD, and oven drying, OD). Total lipids extraction yield cumulative curve results are in Figure 6. Extractions were carried out at 41°C and 300 bar for 180 minutes.

First of all, it is possible to observe that a cell disruption method is indispensable to perform the extraction. The lowest yield, about 11%, is for untreated biomass and only about 50% of the lipids were extracted. Comparing ball milling to HPH, results were similar, with ball milling having a 2.2% higher yield than HPH.

Comparing drying methods, FD has better results than OD because oven drying is carried at higher temperatures and therefore lipid degradation is more probable.

All extractions curves have the same profile, where three distinct zones are clearly observed. Firstly, a linear zone, then a transition zone and finally a baseline zone. In the linear zone, readily available components are extracted, i.e., compounds that are in the cell

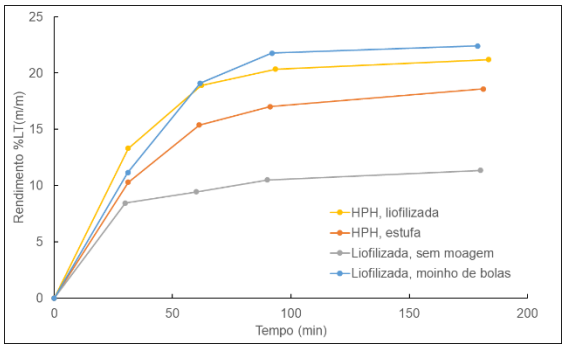

Figure 6 – SFE cumulative curve results for TL.

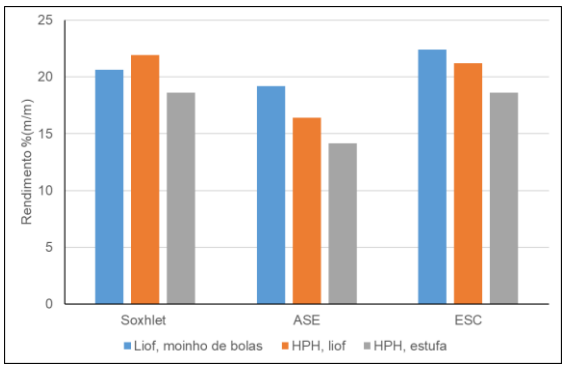

Figure 7 – TL yield comparison for the different extraction methods.

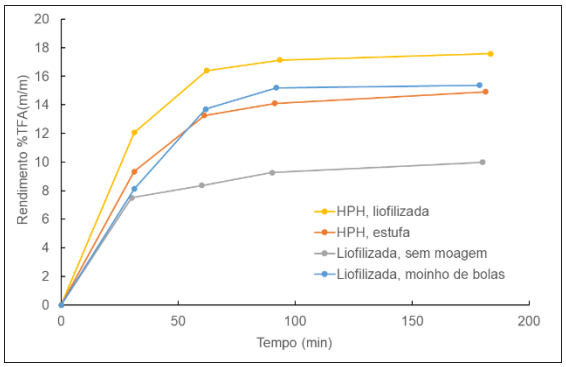

Figure 8 – SFE cumulative curve results for TFA.

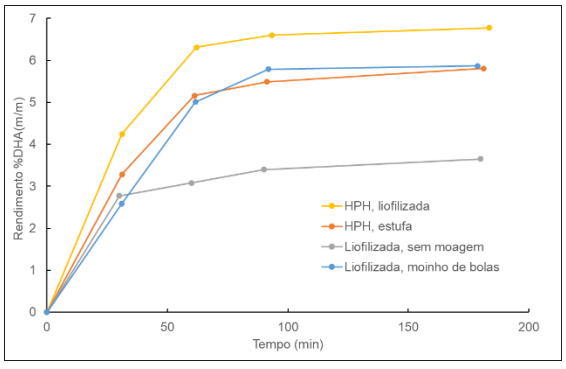

Figure 9 – SFE cumulative curve results for DHA.

exterior after cell breakage. The mechanism that defines the kinetics of the process here is solute solubility in the SF. After this, intracellular compound from broken cells start and kinetics are controlled by external mass transfer. Finally, the third extraction part corresponds to intracellular extraction from intact cells. In this part, internal mass transfer controls the kinetics of the process.

Soxhlet, ASE and SFE methods comparison for the pre-treatments is shown in Figure 7.

SFE presents yields similar to Soxhlet extraction and better than ASE for all biomass pre-treatments.

TFA and DHA results for all biomass pre-treatments are presented in Figure 8 and Figure 9.

HPH treated biomass has better results for TFA and DHA (14-15% higher) than ball milled biomass, so HPH is more efficient for these compounds extraction.

Similar to lipids, TFA and DHA extraction is more efficient for freeze drying than oven drying.

Comparing the TFA and DHA extraction yields for the three extraction methods, for the different biomasses, SFE presents lower yields than Soxhlet and ASE for freeze dried ball milled biomass. However, HPH and freeze-dried biomass has better extraction yields (5-6%) than Soxhlet, for both TFA and DHA. HPH and oven dried biomass had similar results to Soxhlet.

Pigments obtained from SFE follow the same trend as the lipids, for all biomasses.

4. Conclusions

The main goal of this work was to study the usage of green solvents in lipid extraction (fatty acids, specifically DHA) of marine microalgae *Crypthecodinium cohnii*. The influence of cell disruption mechanisms (ball milling and high-pressure homogenization) and drying techniques (freeze and oven drying) in extractions yields was also studied.

Besides, low-cost carbon sources influence on the microalgae development and lipid accumulation was also evaluated by using two industry subproducts: cane molasses and glycerol. To do this, 4 fermentations were carried out. Even after technical problems on three of the fermentations, it was possible to prove that a high-yielding lipid, TFA and DHA microalgae could be produced by a low-cost industrial subproduct carbon source.

Soxhlet extraction was used as a standard technique and revealed that for TL, HPH treated freeze dried biomass yielded more than ball milled biomass, although results were close. For TFA and DHA, the contrary was observed. As for HPH treated oven dried, it

yielded the less for all components, which indicates that oven drying leads to thermal decomposition. Pigments yield conclusions were the same.

ASE was used to verify the influence of time and temperature in extraction yield, using green solvents. Generally, time and temperature have a little positive influence, time having almost no influence. DHA in TFA for ethyl acetate is almost invariable. TFA yield is almost the same for every condition using 2-methyltetrahydrofuran, meaning DHA content in the extract can be controlled by changing the factors. This said, mild conditions yielded satisfying results, for both solvents. On the other hand, pigment yield is highly dependent on temperature, due to its thermolability.

Supercritical fluid extraction results were similar to Soxhlet extraction and conclusions regarding the pre-treatments and drying methods were the same.

Therefore, it is shown in this work that marine microalgae *C. cohnii* presents high industrial perspectives, since it can grow using low-cost substrates, yielding high values of fatty acids and important value-added compounds as DHA and carotenes. Also, biobased solvents presented results very similar, or even better, to traditional solvents, contributing to what was said.

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