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Crypthecodinium cohnii Lipid Fractionation for the Simultaneous DHA and Biodiesel Production

Patricia Moniz, Gabriel Andrade, Alberto Reis, Teresa L. da Silva

Laboratório Nacional de Energia e Geologia, Unidade de Bionergia e Biorrefinarias, Estrada do Paço do Lumiar, 22 1649-038 Lisboa, Portugal
patricia.moniz@lneg.pt

In recent years, oil derived from microalgae has been considered as one of the most promising sources of high value added products and biofuels. The marine microalgae *Crypthecodinium cohnii* has the capacity to produce significant amounts of lipids, about 20% to 50% of its cellular dry weight, with a high proportion of docosahexaenoic acid (DHA), which is a ω-3 polyunsaturated fatty acid (PUFA) with globally recognized benefits in human health. However, most of the recent published studies on this matter only valorize *C. cohnii* PUFA fraction, neglecting the remaining microalgal lipid fraction, after DHA removal. This research explored the overall *C. cohni* lipid fractionation, to obtain a PUFA rich fraction (namely DHA) that can be used in pharmaceutical/nutraceutical/food purposes, and another lipid fraction, rich in saturated/monounsaturated fatty acids, which can be used for biodiesel purposes. In this way, all the microalgal lipid fractions can be used and valorized. *C. cohnii* lipid fractionating was carried out using urea complexes, at different temperatures. The best temperature that led to the highest DHA proportion in the noncrystallized phase was -30 °C, attaining 79.67 % w/w total fatty acids (TFA). The remaining lipid fraction was characterized and evaluated for biodiesel purpose. The results obtained in this research contributed to a better valorization of all *C. cohnii* lipid fractions, being possible to apply this approach to other microalgal oils.

1. Introduction

Crypthecodinium cohnii is a marine heterotrophic microalga that is widespread in tropical and temperate waters worldwide. In Nature, they appear to feed on decomposing macrophytes (marine macroalgae), especially of the Fucus spp. (Beam et al, 1982). C. cohnii cells are show in Figure 1.
C. cohnii microalga is of special interest among other similar microalgae as they accumulate a large amount of DHA, being the only PUFA accumulated at significant amounts (Mendes et al., 2009). This makes this species interesting for obtaining large quantities of DHA and simplifies its purification. This microalga has been used industrially to produce DHA for infant formula, among other supplements.
DHA is a long-chain ω-3 polyunsaturated fatty acid (PUFA) with 22 carbon atoms and 6 double bonds in its fatty acid chain. It is synthetized by algae and microalgae (including *C.cohnii*), becoming increasingly bioaccumulated in organisms in the food chain. The most common dietary sources are fish, crustaceans, water mollusks and their derivative products.
Multiple studies have been made on the DHA and other omega-3 fatty acids effects on human health. The consumption of omega-3 fatty acids, in particular DHA, appears to be related to a variety of positive health effects. The presence of fish, and therefore DHA, in the human diet, may correlate to less incidence of cardiovascular diseases and major depression (Horrocks, and Yeo, 1999). The consumption of fish and omega-3 fats and related products also appears to delay Alzheimer's disease and other types of dementia in adults (Horrocks, and Yeo, 1999). The proliferation of cancer cells and metastasis also appears to be reduced by a diet with high contents of DHA and other omega-3 fatty acids (Horrocks, and Yeo, 1999).

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DHA content in the microalgae *C. cohnii* can reach as high as 50% w/w of the total extractable lipids (Mendes et al., 2009). Multiple studies have been carried out to study the ideal growth conditions for DHA accumulation. Low carbon source concentrations increase the DHA proportion in microalgal lipids, but at the expense of slower growth rates (Jiang and Chen, 2000). Lipid production occurs during growth-limiting conditions, when the cells are stressed or during the stationary phase (Mendes et al., 2009). The percentage of DHA also decreases with the increase in lipid production.

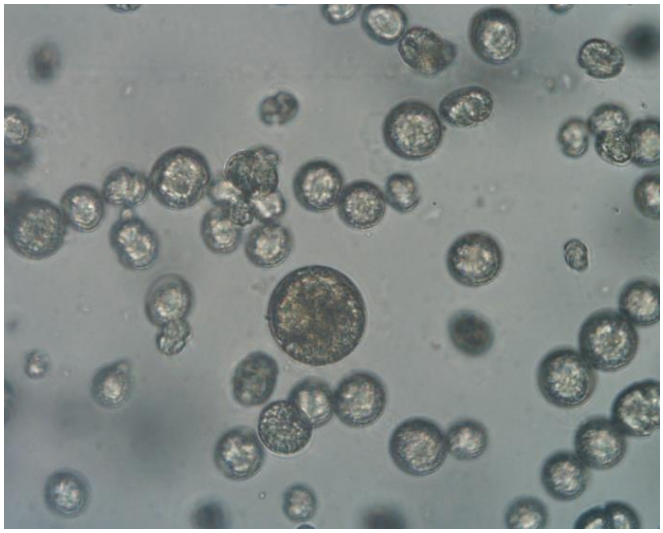

Figure 1 – Microscopic photograph of C. cohnii (at 1000x magnification).

In addition, alternative biodiesel sources have been pursued, including waste vegetable oils, sewage grease and microalgae oil (Singh and Singh, 2010). However, it is not realistically possible to meet the transport fuel needs with neither waste lipid alone. One promising alternative consists of considering the oils derived from microalgae as biodiesel. This biofuel is composed of long-chained alkyl esters, usually methyl esters, produced from vegetable oils, animal fats or lipids from microorganisms, with the purpose of being used as a greener substitute of conventional, petrol-based diesel fuel (petrodiesel).
There are several methods for concentration of PUFA, but only few are suitable for large-scale production.
Urea molecules readily form solid-phase complexes with saturated free fatty acids (FFA). In this way, PUFA and branched FFA may be separated from saturated FFA.
Urea complexation seems to be one of the most appropriate methods for PUFA enrichment. This process allows the handling of large quantities of material in simple equipment, requires inexpensive solvents such as ethanol or hexane, as well as milder conditions *(e.g.* temperature), the separation is more efficient than with other methods such as fractional crystallization or selective solvent extraction, and the cost is lower (Guil-Guerrero and Belarbi, 2001; Medina et al., 1995). Moreover, urea complexation protects the omega 3-PUFA from autoxidation (Shahidi and Wanasundara, 1998).
The present work aims at the *Crypthecodinium cohnii* oil fractionation, in order to obtain a ω-3 rich lipidic fraction for pharmaceutical and food purposes, and another saturated/monounsaturated rich lipidic fraction, for biodiesel purposes, using the rapid, inexpensive urea complexation method. It is known that the crystallization temperature strongly influences the efficiency of DHA purification process (Wanasundara and Shahidi, 1999). In this way, different crystallization temperatures were used in order to achieve the best concentration and purification process efficiency.

2. Materials and Methods

2.1 Growth conditions

Crypthecodinium cohnii ATCC 30772 culture was grown in a 7L-bioreactor (Fermac 360, Electrolab Biotech, Tewkesbury, UK) containing a growth medium using industrial glycerol (20 g/L) as carbon source, at 120 rpm and 27 °C, in the dark (Moniz et al., 2021). Biomass was collected at the stationary phase (5 days) by centrifugation at 7000 rpm using a Sigma centrifuge, (USA) for 15 min and then freeze dried and frozen for further experiments.

2.2 Saponification and transmetylation

A volume of 1190 mL of ethanol 96 % (by volume) and 25 g of KOH were added to 10 g of dry biomass. The mixture was incubated in an orbital shaker at 100 rpm and 20 °C, overnight. Afterwards, 107 mL of distilled water were added, followed by five hexane extractions (5x213 mL) in order to separate the unsaponifiable fraction. The hydroalcoholic phase, containing the soaps, was acidified to pH=1 by the addition of hydrochloride solution (1:1 by volume, HCl 37 % Merck, Darmstadt, Germany). Then the free fatty acids (FFA) were recovered by eight extractions (8x100 mL) with hexane. The organic phase, containing the FFA, was dried with anhydrous sodium sulphate, and the solvent was evaporated in a vacuum rotary evaporator at 35 °C. The FFAs were then ethylated according to Khozin-Goldberg et al. (1999), with modifications, by adding the ethylation mixture of 200 mL of ethanol (Merck, Darmstadt, Germany) and 4 ml of H_2SO_4 (Merck, Darmstadt, Germany) and heating at 80 °C for 1 hour. After cooling to room temperature, 100 mL of water and the same volume of hexane were added and the upper hexane layer, containing the ethyl esters, was removed and dried with anhydrous sodium sulphate. The solvent was evaporated in a vacuum rotary evaporator at 35 °C and 2 mL of hexane was added.

2.3 Fatty acid analysis

C. cohnii lipids were extracted according to the following protocol: The microalgal biomass collected after the broth centrifugation was freeze-dried (Heto PowerDry LL3000 Freeze Dryer, Thermo Scientific, USA, coupled with a vacuum pump from Vacuubrand, Germany). Approximately 100mg of freeze-dried biomass and/or algal oil derived from urea complexation process were transferred to a vial under nitrogen atmosphere and transmethylated at 80 ºC for 1h, with 2mL of a methanol/acetyl chloride mixture (95:5 v/v) and 0.2 mL of heptadecanoic acid (17:0) (5mg/mL petroleum ether, boiling point 80-100°C) as an internal standard. Afterwards, the vial contents were cooled diluted with 1mL water and the lipids were extracted with 2mL of n-heptane. The organic phase was separated from the aqueous phase, dried using sodium sulphate (Na_2SO_4) and placed in a vial adequate for gas chromatography analysis.
The methyl/ethyl esters were then analyzed by gas–liquid chromatography, on a Bruker Scion 436-GC (Germany) equipped with a flame ionization detector. Separation was carried out on a 0.32 mm × 30 m fused silica capillary column (film 0.32 mm) Supelcowax 10 (Supelco, Bellafonte, Palo Alto, CA, USA) with helium as a carrier gas, at a flow rate of 3.5 mL/min. The column temperature was programmed at an initial temperature of 200°C for 8 min, then increased at 4 °C/min to 240 °C and held there for 16 min. Injector and detector temperatures were 250 °C and 280 °C, respectively, and the split ratio was 1:50 for 5 min and then 1:10 for the remaining time. The column pressure was 13.5 psi. Peak identification and response factor calculation were carried out using known standards (GLC 459 and GLC 463, Nu-chek-Prep, USA) The quantities of individual fatty acids were calculated from the peak areas on the chromatogram using heptadecanoic acid (17:0) as the internal standard. Each sample was prepared in duplicate and injected twice (Taborda et al., 2020)

2.4 Urea complexation

Based on previous GC analysis of lipid fraction containing methyl esters, 150 µL of the fatty acids fraction and 50 mL of ethanol were added to 1.30 g of urea, in order to achieve an urea/fatty acid ratio of 3.5 (by mass), according to Mendes et al. (2007). This ethanol/urea ratio allowed a homogeneous and clear mixing of ethyl esters with urea. The mixture was heated at 60–65 °C and stirred until the solution became clear. The urea complexes were obtained by cooling the solution at different temperatures (-30, -18, 5 °C) overnight, followed by a filtration, in order to separate the urea complexing from the non-urea complexing fractions. A volume of 1 mL of distilled water at 60 °C was added to both fractions and vortexed, followed by the addition of 1 mL of hexane to extract the methyl esters. The hexane phases from both fractions (urea complexing and non-complexing) were collected and evaporated under nitrogen atmosphere for further GC analysis. Heptadecanoic acid (17:0) ester was added as internal standard, as previously described (Taborda et al., 2020).

3. Results

3.1 Fatty acids ethylation and profile characterization

After cell growth and collection, the fatty acids present in *C. cohnii* biomass were subjected to ethylation, in order to obtain the corresponding fatty acid ethyl esters (FAEE), that were further quantified.
Ethylated fatty acids were characterized and the ethyl esters obtained are shown in Figure 2.
DHA ethyl ester was the main fatty acid obtained (38.3%). Others fatty acids that showed significant concentrations were the ethyl esters 16:0 (21%), 14:0 (15%) and 18:1ω9 (11%).
The FAEE that resulted in the lowest percentages were those referring to fatty acids 10:0 (1.7%), 14:1 (0.36%), 16:1ω9 (0.94%) and 18:0 (1.2%).

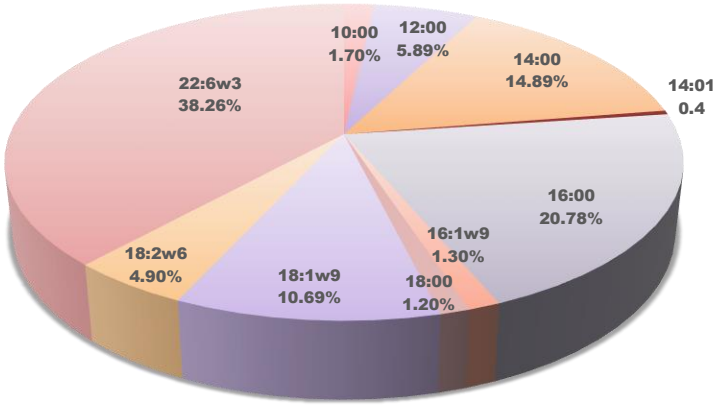

Figure 2 – Fatty acid ethyl esters/total fatty acids (w/w) present in C. cohnii oil.

3.2 Urea Complexation

Urea alone crystallizes in a tightly packed tetragonal structure with channels of 5.67 Å diameter. However, in the presence of long straight-chain molecules, it crystallizes in a hexagonal structure with channels of 8–12 Å diameter within the hexagonal crystals. the urea channels are sufficiently large to accommodate aliphatic chains. The presence of double bonds in the carbon chain increases the bulk of the molecule and reduces the likelihood of its complexation with urea, thus monoenes are more readily complexed as compared to dienes, which, in turn, are more readily complexed than trienes (Shahidi and Wanasundara, 1998; Zhu et al., 2000). This explains why a large fraction of saturated and monounsaturated fatty acids are incorporated in the urea complexes (Mendes et al., 2007).

3.3 . C cohnii lipid fractionation

Table 1 – DHA (% w/w) in the non-crystallized samples and in the crystallized samples after urea treatment

DHA/Total fatty acids (w/w)		
-30ºC	**-18ºC**	**5ºC**
Non-urea complexing fractions		
79.67%	79.45%	73.56%
Urea complexing fractions		
11.82%	10.81%	33.61%

3.3.1 Non-urea complexing fraction

As expected, the DHA proportion of total fatty acids in the non-urea complexing fractions was much higher than that found in the urea complexing fractions.

High concentrations of DHA were obtained in the non-crystallized samples ranging between 73.56% and 79.67 % purity.The fractions with the highest proportion of DHA (79.67 % and 79.45 % w/w of TFA), with a recovery yield (defined as the ratio between the amount of DHA before and after the urea treatment) of more than 90 % of total DHA, in both cases, were obtained at -30 °C and -18 °C, respectively (Table 1).

The saturated fatty acid, which was present in the highest proportion in the non-urea complexing fraction, was 12:0, in low percentage (0.3–2.2 % of total fatty acids). The percentages of 14:0, 16:0, 16:1ω9 and 22:5ω3 ethyl esters in the non-urea complexing fraction were less than 3 % of total fatty acids; 18:0 was absent. As a result, non-urea complexing fractions with high proportions of DHA were obtained.

Senanayake and Shahidi, (2000) concentrated DHA from the oil extracted from the microalga *C. cohnii* and reported a DHA enrichment from 47.4 to 97.1 % with a recovery yield of 32.5 % of the mass of the original algal oil. However, the present work presents an alternative fatty acid extraction, saponification and ethylation process.

3.3.2 Urea-complexing fraction

As expected, the saturated fatty acids EE were retained the.in urea complexing fraction, being 16:0 and 14:0 the dominant compounds: 16:0 was the ethyl ester that showed the higher content in the crystallized samples, accounting for 35% at -18 °C and 33% at 5 °C, followed by 14:0 ethyl ester that showed a concentration 24% and 22%, at the temperatures -18 °C and 5 °C, respectively.
As referred in Biodiesel Standards (2003), biodiesel needs to meet strict parameters to be commercialized in the European Union. The crystalized fraction obtained will be further studied in order to meet the parameters described in the EU Standard for biodiesel.

4. Conclusions

The current study, saponification and ethylation performed in *C. cohnii* biomass, followed by urea complexation in a sequential way, was an appropriate way for concentration and purification of DHA from *C. cohnii* biomass. High proportions of DHA are obtained in non-urea complexing fractions using this methodology.
Further studies will be carried out in order to reach higher DHA purity from non-urea complexing fractions. The EE retained in the urea complexing fraction are being evaluated for biodiesel production.

Nomenclature

DHA– Docosahexaenoic acid
EE – Ethyl esters
FAEE – Fatty acids ethyl esters
FFA – Free fatty acids
GC– Gas chromatography
PUFA– Polyunsaturated fatty acid
TFA– Total fatty acids

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References

Beam C, Himes M., 1982, Distribution of Members of the *Crypthecodinium cohnii* (Dinophyceae) Species Complex, The Journal of Protozoology, 29, 8-15.
European Committee for Standardization, EN 14214 “Automotive fuels - Fatty acid methyl esters (FAME) for diesel engines - Requirements and test methods, 2003. [Online]. Available: https:// ec.europa.eu/energy /intelligent/projects/sites/iee_projects/files/projects/documents/biofuel_marketplac biofuel_standards_f or_ transport _ in_the_eu.pdf. Accessed 29 01 2022.
Guil-Guerrero J.L., Belarbi E., 2001, Purification process for cod liver oil polyunsaturated fatty acids, Journal Am. Oil Chemistry Soc, 78, 477–484.
Horrocks L., Yeo K., 1999, Health benefits of docosahexaenoic acid (DHA), Pharmacological research, 40, 211-225.
Jiang Y., Chen F., 2000 Effects of medium glucose concentration and pH on docosahexaenoic acid content of heterotrophic *Crypthecodinium cohnii*, Process Biochemistry, 35, 1205-1209.

Khozin-Goldberg I., Bigogno C., Cohen Z., 1999, Salicylhydroxamic acid inhibits delta 6 desaturation in the microalga *Porphyridium cruentum*, Biochemistry Biophysics. Acta, 1439, 384– 394.

Mendes A., da Silva T.L., Reis A., 2007, DHA concentration and purification from the marine heterotrophic microalga *Crypthecodinium cohnii* CCMP 316 by winterization and urea complexation. Food Technology and Biotechnology; *45*, 38–44.

Mendes A., Reis A., Vasconcelos R., Guerra P., Lopes da Silva T., 2009, *Crypthecodinium cohnii* with emphasis on DHA production: a review, Journal of applied phycology*,* 21, 199-214.

Medina A.R., Giménez A., Camacho F., Pérez J.A., Grima E., Gómes A., 1995, Concentration and purification of stearidonic, eicosapentaenoic, and docosahexaenoic acids from cod liver oil and the marine microalga Isochrysis galbana, J. Am. Oil Chem. Soc, 72, 575–583

Moniz P., Silva, C., Oliveira A.C., Reis, A., Lopes da Silva T., 2021, Raw Glycerol Based Medium for DHA and Lipids Production, Using the Marine Heterotrophic Microalga *Crypthecodinium cohnii*, Processes, 9, 11.

Senanayake S.P., Shahidi F., 2000, Concentration of docosahexaenoic acid (DHA) from algal oil via urea complexation, Journal of Food Lipids, 7, 51–61.

Shahidi F., Wanasundara U.N., 1998, Omega-3 fatty acid concentrates: Nutritional aspects and production technologies, Trends Food Science Technology, 9, 230–240.

Singh P., Singh D., Biodiesel production through the use of different sources and characterization of oils and their esters as the substitute of diesel: a review, 2010, Renewable and sustainable energy reviews*,* 14, 200-216.

Taborda T.; Moniz P.; Reis A.; Lopes da Silva T., 2020, Evaluating low-cost substrates for *Crypthecodnium cohnii* lipids and DHA production, by flow cytomtery. Journal of Applied Phycology, 33, 263–274.

Wanasundara U., Shahidi F., 1999, Concentration of omega-3 polyunsaturated fatty acids of seal blubber oil by urea complexation: Optimization of reaction conditions, Food Chemistry, 65, 41–49.

Zhu M., Zhou P.P., Yu L.J., 2000, Extraction of lipids from *Mortierella alpina* and enrichment of arachidonic acid from the fungal lipids, Bioresource Technology, 84, 93–95.