Evaluating low-cost substrates for Cryptothecodinium cohnii lipids and DHA production, by flow cytometry

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Abstract
Cryptothecodinium cohnii growth was studied on pure carbon sources (glucose, acetate, glycerol) and low-cost complex carbon sources (sugarcane molasses, crude glycerol and vinegar effluent) for lipid and DHA production. Among the pure substrates, glucose induced the highest lipid content (14.75% w/w DCW) and DHA content (7.14 mg g⁻¹ DCW). Among the low-cost substrates, the highest lipid and DHA content were observed for the crude glycerol assay (14.7% w/w DCW and 6.56 mg g⁻¹, respectively). Molasses induced the highest proportion of DHA of total fatty acids (49.58% w/w TFA) among all the substrates studied. Flow cytometric analysis revealed that the vinegar effluent induced the highest proportion of C. cohnii cells with injured membrane (92.8%). These results foresee the possibility of using these low-cost substrates at a larger scale for C. cohnii DHA and biodiesel production, aiming at zero wastes and process costs reduction.

Keywords Cryptothecodinium cohnii · Pure substrates · Low-cost substrates · Lipids · DHA · Flow cytometry

Introduction
The marine microalga Cryptothecodinium cohnii, a heterotrophic non-photosynthetic dinoflagellate, accumulates significant amounts of lipids (20–50% of its cell dry weight) with a high fraction of docosahexaenoic acid (DHA), a ω-3 polyunsaturated fatty acid which is a component of neural and retinal tissues, a key fatty acid component in breast milk, and it is necessary for brain development in infants. This compound has well known benefits on the human health, having currently several nutritional and pharmaceutical applications, and a growing market size (Diao et al. 2018).

DHA can be obtained from marine fish sources; however, its production from microalgal sources shows benefits over DHA obtained from fish, since the pure microalgal oil is odourless, non-dependent on fish stocks, does not contain ocean-borne contaminants, and its vegetarian nature attracts young people (Lopes da Silva et al. 2019). Under certain cultivation conditions, C. cohnii cells accumulate less than 1% of the other type of PUFAs, which is a clear advantage for the downstream DHA purification process. On the other hand, the presence of more than 50–60% content of fatty acids with 16 and 18 carbon atoms (C16-C18) in C. cohnii total fatty acids makes this microalga a potential source for biodiesel production.

The carbon source is the most expensive component of fermentation media. In the late 1990s and early 2000s, several pure carbon sources were studied to grow C. cohnii, such as glucose (De Swaaf et al. 1999), acetic acid (De Swaaf et al. 2003), ethanol (De Swaaf et al. 2003) and pure glycerol (Hosoglu and Elibol, 2017a). However, despite these carbon sources induce high lipid and DHA productivities, they are expensive to be used at large scale (glucose 16 € kg⁻¹; ethanol 1.82 € kg⁻¹; acetic acid 0.45 € kg⁻¹, www.alibaba.com). In addition, ethanol and acetic acid are dangerous compounds that are difficult to handle and transport. On the other hand, the increasing environmental awareness about the circular economy rules has boosted the necessity to use low-cost or zero-cost wastes/byproducts/effluents as nutrients in media formulations for microbial growth. In fact, in recent years, substrates as food waste such as carob pulp syrup (Mendes et al. 2007), rapeseed meal hydrolysate mixed with crude molasses (Gong et al. 2015) and cheese whey with corn steep liquor (Hosoglu and Elibol 2017b) have been used in media formulations for C. cohnii ω-3 compounds production. Nevertheless, despite the low cost of these substrates, they often contain inhibitory compounds that affect the cell...
metabolism and reduce the process yield. Therefore, the cell physiological status monitoring during the bioprocess development is crucial, particularly when wastes/byproducts/effluents are used as substrates, to evaluate the cell stress response. Indeed, a high proportion of damaged/dead cells in the broth will have a detrimental impact on the process performance, since those cells do not participate in the biotransformation, thus reducing the process yield; moreover, if this information is obtained near real time during the process development, it can be used to change the process control strategy, in order to enhance the process efficiency. However, most of the published works reporting the use of low-cost substrates to grow *C. cohnii* uses conventional monitoring techniques to monitor the microalgae cultivations (i.e., optical density, dry cell weight, cell count) which do not give any information on cell status.

The present work evaluates *C. cohnii* ATCC 30772 growth and lipid production on the pure carbon sources most used (glucose, acetate and pure glycerol) and low-cost complex carbon sources containing these compounds (sugarcane molasses which contains sucrose, glucose and fructose; vinegar effluent which contains acetate and crude glycerol which contains glycerol). Flow cytometry (FC) was used to evaluate the microalgae cell stress response to the different substrates, monitoring the enzymatic activity, membrane integrity and ROS production.

### Materials and methods

*Crypthecodinium cohnii* (strain ATCC 30772) was obtained from the American Type Culture Collection (ATCC). The cultures were maintained in a medium composed of yeast extract (1.8 g L\(^{-1}\), Oxoid), sea salt (Oceanusiberia, Portugal) (23 g L\(^{-1}\)) and glucose monohydrate (9.9 g L\(^{-1}\)) (Acros Organics). These cultures were re-inoculated every 2 weeks.

Inocula were prepared from these cultures in shake-flask cultures (500-mL flasks, 150 rpm, 27 °C) containing 150 mL of medium with the following composition: yeast extract (2 g L\(^{-1}\)), sea salt (25 g L\(^{-1}\)) and glucose (20 g L\(^{-1}\)).

The carbon sources glucose (Scharlau), sodium acetate (Merck), pure glycerol (Labsolve), sugarcane molasses, vinegar effluent and crude glycerol were added to the culture medium at the same concentration (0.67 mol carbon atoms per litre). Crude glycerol was previously distilled to reduce the methanol concentration. The complexes substrates characterisation is displayed in Table 1. Volumes of 250 mL of sterile culture medium containing yeast extract (2 g L\(^{-1}\)), sea salt (25 g L\(^{-1}\)) and 0.666 mol of carbon per litre of each carbon source were transferred to 500-mL Erlenmeyers, inoculated with 10% v/v of inoculum and incubated in the darkness at 27 °C and 150 rpm to 8 days.

For all media, the initial pH was adjusted to 6.5 before sterilisation using NaOH and HCl solutions and autoclaved at 121 °C, for 20 min. For the medium containing glucose, the solution containing the salt and yeast extract was previously autoclaved separately from the glucose solution and eventually mixed under sterile conditions to avoid caramelisation. For the vinegar effluent assay, the medium was sterilised by filtration, in order to prevent acetic acid evaporation. The sugarcane molasses was previously hydrolysed to break down sucrose into glucose and fructose by decreasing the pH to 3 with HCl and storing it at 50 °C for 24 h.

### Growth parameters

The dry weight was determined by filtering 3 mL of culture through pre-weighed 0.45-μm nylon filters (Millipore, Germany) under vacuum, which were subsequently placed in an oven at 100 °C up to constant weight, over 18 h. The filtrate was collected and frozen at −18 °C for the later analysis of the carbon source concentration in the medium by high performance liquid chromatography (HPLC).

The carbon sources were analysed by HPLC (LaChrom Merck/Hitachi, Germany). Two different chromatographic columns were used: Aminex HPX-87P (Bio-Rad, USA) used to detect all carbon sources; sugarcane molasses was analysed using a SugarPack (Waters, USA). The chromatograms were analysed using the ChemStation for LC 3D Systems Rev. B.01.03 software (Agilent Technologies, USA).

The pH was determined using a Consort C3021 potentiometer (Consort, Belgium) which was calibrated regularly.

### Lipid quantification

Lipids were extracted according to the protocol of Lopes da Silva et al. (2006) with modifications: The microalgal biomass collected after the broth centrifugation was freeze-dried. Approximately 100 mg of freeze-dried biomass were transferred to a vial under nitrogen atmosphere and transmethylated at 80 °C for 1 h, with 2 mL of a methanol/acetyl chloride mixture (95:5 v/v) and 0.2 mL of heptadecanoic acid (17:0) (5 mg mL\(^{-1}\) petroleum ether, boiling point 80–100 °C) as an internal standard. Afterwards, the vial contents were cooled, diluted with 1 mL water and the lipids were extracted with 2 mL of n-heptane. The organic phase was separated from the aqueous phase, dried using sodium sulphate (Na\(_2\)SO\(_4\)) and placed in a vial adequate for gas chromatography analysis.

The methyl esters were then analysed by gas–liquid chromatography, on a Bruker Scion 436-GC (Germany) equipped with a flame ionisation detector. Separation was carried out on a 0.32 mm × 30 m fused silica capillary column (film 0.32 mm) Supelcowax 10 (Supelco, USA) with helium as carrier gas at a flow rate of 3.5 mL min\(^{-1}\). The column temperature was programmed at an initial temperature of 200 °C.
for 8 min, then increased at 4 °C min⁻¹ 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.

**Flow cytometry (FC)**

Flow cytometry (FC) analysis was performed in a BD FACSCalibur cytometer (Becton Dickinson, USA) equipped with a blue laser, FSC/SSC light scattering detectors and four fluorescence detectors FL1–FL4. For the analysis in the flow cytometer, 3 mL of culture were removed under sterile conditions and stored in Falcon tubes. These were subsequently subjected to ultrasound treatment for 15 s to disintegrate cellular aggregates. Samples were diluted in McIlvaine buffer (pH 4.0) for the simultaneous detection of cellular enzymatic activity and membrane integrity and were diluted in PBS buffer (pH 7.4) for ROS detection.

The fluorescent dye carboxyfluorescein diacetate (CFDA, Invitrogen) and propidium iodide (PI, Invitrogen) were used for the simultaneous detection of enzymatic activity and membrane integrity. CFDA is a non-fluorescent compound which penetrates the cells by passive diffusion. Once inside the cell, if esterases are active, they will hydrolyse CFDA to a fluorescent compound. Therefore, if a cell is stained with CFDA, it means that its enzymatic system (esterases) is active and its membrane is intact; if the cell is not stained with CFDA, it means that its enzymatic system is not active, or its cell membrane is not intact as permeabilised membrane allows the fluorescent compound to exit the cell. Thus, when using CFDA to evaluate the cell status, it is convenient to simultaneously use a dye for membrane integrity detection. Propidium iodide (PI) was used for membrane integrity detection. PI binds to DNA but cannot cross an intact cytoplasmic membrane. A double staining protocol was used to monitor simultaneously *C. cohnii* enzymatic activity and membrane integrity during all the microalgal assays. CFDA was detected in the FL1 detector, and PI in the FL3 detector.

Dihydrorhodamine 123 (DHR, Invitrogen) was used to evaluate *C. cohnii* oxidative stress response, by monitoring the reactive oxygen species (ROS) intracellular generation, as oxygen peroxide (H₂O₂). DHR is specifically responsive to H₂O₂ and passively diffuses across cell membranes. Once inside the cell, it can be oxidised, mainly by H₂O₂, to form cationic rhodamine 123 (Prado et al. 2012) which is a fluorescent compound, usually localised in the mitochondria, emitting a bright fluorescent signal with a maximum emission at 529 nm, detected in FL1 channel.

For control purposes, unstained samples were recorded to take into account the autofluorescence of the cells. For CFDA/PI double staining, the following amounts of dyes were sequentially added, per 495 μL of cell suspension, followed by 15 min incubation in the dark: 3 μL of CFDA 10 mg mL⁻¹ stock solution, followed by 2 μL of PI 1 mg mL⁻¹ stock solution. CFDA and PI were detected in the FL1 and FL2 channels respectively. For DHR staining, 3 μL of DHR (Invitrogen) stock solution (5 mM in DMSO) were added to 497 μL of cell suspension, and incubated at room temperature in darkness for 15 min before analysis. DHR fluorescence was detected at the FL1 channel. At least 2 replicates were made for each different dye used. The obtained cytograms were processed and imaged using the Flowing Software (version 2.5.0, Perttu Terho).

**Results**

Figure 1 shows the biomass profiles for *C. cohnii* cultivations on different substrates, as a semi-log plot. Acetate and pure glycerol assays attained the higher maximum biomass concentrations (7.03 and 6.33 g L⁻¹). Glucose assay attained the
lowest maximum biomass concentration (2.66 g L\(^{-1}\)) among the assays that showed cell growth. The assay containing the vinegar effluent did not allow cell growth. The assays containing the complex substrates, sugarcane molasses and crude glycerol, attained lower maximum biomass concentrations (3.91 and 5.05 g L\(^{-1}\), respectively) than the acetate and pure glycerol assays (7.03 and 6.33 g L\(^{-1}\), respectively), but higher than the glucose assay (2.66 g L\(^{-1}\)).

It was also observed that \textit{C. cohnii} cells entered the exponential phase immediately after the inoculation for all the assays, except for the glucose and vinegar effluent assays (Fig. 1).

The slow biomass increase observed after the exponential phase for glucose and molasses assays suggests oxygen-limiting conditions, as a result of a low oxygen transfer rate, at that stage (Fig. 1).

Figure 2 shows the substrate consumption profiles during all microalgae cultivations. There was no acetate consumption during the vinegar effluent assay; thus, it was not included in the graph. Glucose, acetate, glycerol and crude glycerol were completely exhausted after \(t = 180\) h, being acetate depletion faster comparing to other carbon sources. During the sugarcane molasses assay, glucose was completely exhausted after 96 h, but fructose was not consumed.

Figure 3 shows the kinetic data for \textit{C. cohnii} assays. The highest specific growth rate (\(\mu\)) was observed for the acetate assay (0.025 h\(^{-1}\)) followed by pure glycerol (0.019 h\(^{-1}\)), crude glycerol (0.018 h\(^{-1}\)), glucose (0.017 h\(^{-1}\)) and sugarcane molasses (0.013 h\(^{-1}\)).

The highest biomass volumetric rate (\(r_x\)) was observed for the acetate assay (0.031 g L\(^{-1}\) h\(^{-1}\)), followed by glycerol (0.025 g L\(^{-1}\) h\(^{-1}\)), sugarcane molasses (0.018 g L\(^{-1}\) h\(^{-1}\)), crude glycerol (0.022 g L\(^{-1}\) h\(^{-1}\)) and glucose (0.013 g L\(^{-1}\) h\(^{-1}\)) assays.

The highest substrate uptake volumetric rate (\(r_s\)) was observed for the acetate assay (0.125 g L\(^{-1}\) h\(^{-1}\)) and the lowest for the glucose assay (0.099 g L\(^{-1}\) h\(^{-1}\)).

**Flow cytometry (FC)**

Flow cytometric controls/CFDA-PI Previous flow cytometric controls were carried out using \textit{C. cohnii} cells at different physiological conditions, in order to evaluate the efficiency of the flow cytometric protocol in association with the CFDA/PI mixture and DHR staining procedures. These controls were then compared with data obtained during the microalgal cultivations.

Figure 4 shows the dot plots for FL1 (CFDA fluorescence intensity)/FL3 (PI fluorescence intensity) concerning the controls for \textit{C. cohnii} cells. Unstained cells (autofluorescence, Fig. 4a), exponentially growing cells (Fig. 4b), aged cells (Fig. 4c) and heat-treated cells (incubated in a water bath at 100 °C for 20 min, Fig. 4d) stained with CFDA/PI mixture were analysed by FC.

The quadrants were defined based on the unstained cells population (Fig. 4a). Concerning exponential growing cells stained with the CFDA/PI mixture (Fig. 4b), a major population B comprised 88.89% of \textit{C. cohnii} cells stained with CFDA, but not with PI (CFDA+, PI–). These cells have active esterases and intact membrane, considered as metabolically active cells (“healthy cells”).

Aged cells (collected during advanced stationary phase) dot plot revealed that 33.9% of \textit{C. cohnii} cells (subpopulation A) were not stained with CFDA, neither with PI (CFDA–, PI–), indicating that these cells have intact membrane but inactive...
C. cohnii plots FL1/FSC concerning the controls for 2008). The regions R1 and R2 were defined based on the cells signals give information on cell size, Lopes da Silva and Reis DHR fluorescence intensity detected in FL1 versus FSC (FSC stained with DHR for ROS detection. These plots show the 

35.9% of the cells showed active esterases (subpopulation C) meaning that these cells have damaged membrane, although stained with PI (97.6%, sum of subpopulations C and D), indicating that these cells, despite having active esterases, have also injured membrane; 3.9% of the cells were not 

esterases. The same plot also shows that 51.0% of the cells were stained with CFDA but not with PI (metabolic active cells, subpopulation B). Moreover, 5.9% of the cells (subpopulation C) were stained with CFDA and PI (CFDA+PI+), indicating that these cells, despite having active esterases, have also injured membrane; 3.9% of the cells were not stained with CFDA, but were stained with PI (CFDA–PI+, subpopulation D), thus having injured membrane and inactive esterases. This dot plot shows various C. cohnii subpopulations, demonstrating the cell physiological states heterogeneity that exists in microbial cultures. Heat-treated C. cohnii cells dot plot is shown in Fig. 4d). Most of the cells were stained with PI (97.6%, sum of subpopulations C and D), meaning that these cells have damaged membrane, although 35.9% of the cells showed active esterases (subpopulation C) and 61.7% have not enzymatic activity.

Flow cytometric controls—DHR Figure 4e–f show the dot plots FL1/FSC concerning the controls for C. cohnii cells stained with DHR for ROS detection. These plots show the DHR fluorescence intensity detected in FL1 versus FSC (FSC signals give information on cell size, Lopes da Silva and Reis 2008). The regions R1 and R2 were defined based on the cells autofluorescence (Fig. 4e). Exponential growing cells stained with DHR dot plot shows a single population E (99.7%) composed of cells low ROS production, as expected. Aged cells stained with DHR dot plot shows a major subpopulation E (99.3%, Fig. 4g) indicating that age did not induce ROS production. When the heat-treated cells were stained with DHR, a major population (DHR+, 88.2%, placed in R2 region, Fig. 4f) was detected, composed of cells with high ROS production, demonstrating that the heat-treatment induced intracellular ROS production.

These results demonstrated that FC, in association with the double staining CFDA/PI mixture and DHR staining, was an efficient method to differentiate C. cohnii cell physiological status concerning cellular enzymatic activity, membrane integrity and intracellular ROS production, which characterise the microalga stress response to adverse environments. This information is crucial when the microalga is grown on industrial effluents.

C. cohnii cell stress response

Enzymatic activity and membrane integrity (CFDA/PI) Esterase activity and membrane integrity are a measure of microbiological activity and viability and have also been associated with cell stress response (Amariei et al. 2020). Figure 5 shows C. cohnii cells subpopulations percentages during the cultivations on different substrates, pure and complexes. For glucose assay (Fig. 5a), the proportion of subpopulation B, composed of intact and metabolically active cells, attained 74.3% at t = 48 h, afterwards decreasing up to 44.1% at t = 92 h. These variations were accompanied by a concomitant increase in stressed cells (subpopulation A, composed of intact cells without enzymatic activity, which reached 44.0% at t = 92 h, and subpopulation C, composed of permeabilised cells with enzymatic activity, which reached 19% at t = 144 h). The decrease in subpopulation B (thus, C. cohnii cells with active esterases) may reflect the oxygen-limiting conditions that cells might have experienced during the exponential phase and early stationary phase (Fig. 1), when the cellular oxygen requirements are higher due to cell growth. As above referred, the oxygen availability in shake flasks cultures is short. Indeed Takaç et al. (2010) demonstrated that the excess of dissolved oxygen in the stationary phase of growth enhanced Candida rugosa esterase activity. The increase in subpopulation B percentage, followed by a plateau, observed during t = 92 h and t = 164 h, could be due to the higher oxygen availability as a result of the lower cellular oxygen requirements during the stationary phase, since growth slowed down at that stage. The lowest percentage of subpopulation B at the end of the cultivation (34.6% at t = 190 h) was probably due to the carbon exhaustion (Figs. 2 and 5a).

A decrease in subpopulation B (metabolic active cells) until t = 168 h was also observed for the molasses assay (Fig. 5b), accompanied by a steady increase in subpopulation C (stressed cells), which attained 83.5% at t = 48 h. Again these variations were attributed to the low oxygen availability in the medium, as observed for the glucose assay, although the cell stress response was stronger for the molasses assay than for
the glucose assay. Two factors might have contributed for this situation: as molasses could have increased the broth viscosity, the oxygen transfer rate could be even lower in the medium containing molasses than in the medium containing glucose. On the other hand, it is well known that sugarcane molasses contains anti-microbial compounds such as phenolics (Takara et al. 2007) which may explain the higher proportion of cells with injured membrane observed during this assay.

Cells percentages profiles observed during glycerol and crude glycerol experiments are depicted in Fig. 5c, d, respectively. It can be seen that both profiles are similar. At \( t = 24 \) h, both cultures displayed a higher proportion of metabolically active cells (subpopulation B, 88.0 and 85.1% for glycerol and crude glycerol, respectively). As the cultures developed, the proportion of subpopulation B decreased up to 37.1 and 21.1%, respectively, at \( t = 72 \) h, with the concomitant increase in the proportion of the stressed subpopulation A (intact cells without enzymatic activity) up to 56.8 and 74.3%, respectively, as a result of oxygen-limiting conditions. After \( t = 72 \) h, the proportion of subpopulation B increased in both cultures, reaching 40.0 and 60.0% at \( t = 189 \) h, for glycerol and crude glycerol, respectively. The subpopulation B proportion increase observed for the crude glycerol at \( t > 166 \) h, higher than that observed for pure glycerol assay, demonstrated that this substrate was not toxic for the alga.

Concerning the acetate assay, subpopulation B followed the same trend as the previous assays (Fig. 5e). After \( t = 87.2 \) h, an abrupt decrease in this subpopulation was observed, with a concomitant increase in the stressed cells subpopulations C and D. This was attributed to the marked medium pH increase observed for the acetate assay broth for \( t > 92 \) h, reaching pH = 9.4 at \( t = 189 \) h. Indeed, the acetate medium pH showed a higher variation during the acetate experiment, comparing to the other medium pH assays.

Since no algal growth was observed during the vinegar effluent cultivation, the assay was concluded at \( t = 96 \) h. At that time, the proportion of subpopulation B (intact cells with enzymatic activity) was only 4.4% (Fig. 5f). Most of the microalgal cells had intact membrane but showed no enzymatic activity (59.7%, subpopulation A); 33.1% of the cells have injured membrane and no enzymatic activity (subpopulation D) and 2.9% displayed enzymatic activity but their membrane

Subpopulation (C) composed of C. cohnii cells with permeabilised membrane and enzymatic activity; Subpopulation (D) composed of C. cohnii cells with intact membrane and without enzymatic activity. e-h FL1/FSC dot plots concerning C. cohnii cells flow cytometric controls for intracellular ROS detection. a C. cohnii cells autofluorescence detected in FL1 and FSC detectors. b C. cohnii exponential growing cells stained with DHR. c C. cohnii aged cells stained with DHR. d Heat-treated C. cohnii cells stained with DHR. Subpopulation (A) composed of C. cohnii cells with intact membrane and no enzymatic activity; Subpopulation (B) composed of C. cohnii cells with intact membrane and enzymatic activity (“healthy cells”); Subpopulation (C) composed of C. cohnii cells with permeabilised membrane and enzymatic activity; Subpopulation (D) composed of C. cohnii cells with intact membrane and without enzymatic activity. e-h FL1/FSC dot plots concerning C. cohnii cells flow cytometric controls for intracellular ROS detection. a C. cohnii cells autofluorescence detected in FL1 and FSC detectors. b C. cohnii exponential growing cells stained with DHR. c C. cohnii aged cells stained with DHR. d Heat-treated C. cohnii cells stained with DHR. e-h FL1/FSC dot plots concerning C. cohnii cells flow cytometric controls for intracellular ROS detection. a C. cohnii cells autofluorescence detected in FL1 and FSC detectors. b C. cohnii exponential growing cells stained with DHR. c C. cohnii aged cells stained with DHR. d Heat-treated C. cohnii cells stained with DHR. Subpopulation (A) composed of C. cohnii cells with intact membrane and no enzymatic activity; Subpopulation (B) composed of C. cohnii cells with intact membrane and enzymatic activity (“healthy cells”); Subpopulation (C) composed of C. cohnii cells with permeabilised membrane and enzymatic activity; Subpopulation (D) composed of C. cohnii cells with intact membrane and without enzymatic activity. e-h FL1/FSC dot plots concerning C. cohnii cells flow cytometric controls for intracellular ROS detection. a C. cohnii cells autofluorescence detected in FL1 and FSC detectors. b C. cohnii exponential growing cells stained with DHR. c C. cohnii aged cells stained with DHR. d Heat-treated C. cohnii cells stained with DHR. e-h FL1/FSC dot plots concerning C. cohnii cells flow cytometric controls for intracellular ROS detection. a C. cohnii cells autofluorescence detected in FL1 and FSC detectors. b C. cohnii exponential growing cells stained with DHR. c C. cohnii aged cells stained with DHR. d Heat-treated C. cohnii cells stained with DHR. Subpopulation (A) composed of C. cohnii cells with intact membrane and no enzymatic activity; Subpopulation (B) composed of C. cohnii cells with intact membrane and enzymatic activity (“healthy cells”); Subpopulation (C) composed of C. cohnii cells with permeabilised membrane and enzymatic activity; Subpopulation (D) composed of C. cohnii cells with intact membrane and without enzymatic activity. e-h FL1/FSC dot plots concerning C. cohnii cells flow cytometric controls for intracellular ROS detection. a C. cohnii cells autofluorescence detected in FL1 and FSC detectors. b C. cohnii exponential growing cells stained with DHR. c C. cohnii aged cells stained with DHR. d Heat-treated C. cohnii cells stained with DHR. e-h FL1/FSC dot plots concerning C. cohnii cells flow cytometric controls for intracellular ROS detection. a C. cohnii cells autofluorescence detected in FL1 and FSC detectors. b C. cohnii exponential growing cells stained with DHR. c C. cohnii aged cells stained with DHR. d Heat-treated C. cohnii cells stained with DHR.
was damaged. These subpopulations profile demonstrated the harsh conditions that cells experienced during this assay, and explains the growth inhibition.

**Reactive oxygen species (DHR)** Microbial cells produce ROS through oxygen reduction by the action of reducing agents such as NADH and NADPH, with the support of electron-transfer enzymes or through redox-active chemical species such as quinones and transition metals. Oxidative stress is a physiological response detected in microorganisms exposed to adverse conditions that occurs when the ROS generation exceeds the capacity of their antioxidant defences. Low concentrations of ROS facilitates signal transduction, enzyme activation and other cellular functions, but high concentration of ROS damages DNA, proteins or lipids and can lead to irreversible cellular damages (Amariei et al. 2020).

Figure 6 shows the percentage or cells that produced ROS during all the *C. cohnii* cultivations. The highest proportions of cells with ROS were observed for glycerol and crude glycerol assays, which attained 24.2 and 17.3% at $t = 45$ h, respectively. Again, crude glycerol assay displayed lower stressed cells proportion than pure glycerol assay. These results are consistent with the CFDA/PI results for the glycerol and crude glycerol cultivations (Fig. 5c, d) since the production of ROS coincided with the reduction of esterase activity (subpopulation A increase). The remaining cultures showed less than 7% of cells with ROS throughout the experiments.
Lipid and DHA production

Table 2 shows *C. cohnii* lipid production assessed at the end of all assays. The highest lipid and DHA content was observed for the glucose assay (14.7% and 7.15% w/w DCW, respectively) and for the crude glycerol assay (14.7% and 6.56% w/w DCW, respectively). The remaining assays yielded a lipid content around 12% (w/w DCW) and the DHA content varied between 2.23 and 5.51% w/w DCW, while the lipid productivity was highest for the acetate assay (3.89 mg L$^{-1}$ h$^{-1}$) and crude glycerol (3.19 mg L$^{-1}$ h$^{-1}$).

The highest DHA percentage of total fatty acids (TFA) was detected for the molasses assay (49.58% w/w TFA) followed by glucose assay (48.45%). Glycerol showed the lowest proportion of DHA (25.04% w/w TFA).

The lower lipid productivity observed for the glucose assay (1.89 mg L$^{-1}$ h$^{-1}$), despite the higher lipid content (14.7%), resulted from the lower biomass concentration observed at the end of this assay (2.4 g L$^{-1}$, Fig. 1). The highest DHA productivity was detected for the acetate and crude glycerol assays (1.64 and 1.43 mg L$^{-1}$ h$^{-1}$, respectively). The highest DHA concentration was observed for the acetate (31.18 mg L$^{-1}$ h$^{-1}$) and crude glycerol (26.96 mg L$^{-1}$ h$^{-1}$) assays.

Fatty acid profiles and lipid classes

Concerning the fatty acids profiles (Fig. 7a), the dominant fatty acids present in *C. cohnii* 30772 biomass collected at the end of the assays were the lauric (12:0), myristic (14:0), palmitic (16:0), oleic (18:1$\omega_9$) and DHA (22:6$\omega_3$). In most of the assays, the major fatty acid was DHA, comprising more than 40% w/w of total fatty acids (TFA), except for the pure glycerol assay, in which the microalgal biomass contained only 25% DHA w/w of TFA. The biomass produced at the end of the sugarcane molasses assay showed the highest DHA percentage (49.57% w/w TFA). The collected microalgal biomass from the assays containing the pure carbon sources (glucose, acetate, pure glycerol) had higher proportions of myristic acid (14:0) (> 18% w/w TFA), while the biomass collected from the assays containing complex carbon sources (sugarcane molasses and crude glycerol) contained 11.27 and 16.05% w/w of TFA of 14:0, respectively. For the assays containing sugars (sugarcane molasses and glucose), the palmitic acid (16:0) attained lower proportions (< 16%) comparing with the remaining assays which reached ~ 22% w/w of TFA. In all the assays, the oleic acid (18:0) proportion varied between 6 and 9% w/w TFA and the lauric acid (12:0) varied between 2.88 and 6.81% w/w TFA.

Concerning the unsaturation level of *C. cohnii* lipids, the collected biomass from the pure glycerol assay contained the

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**Table 2** *C. cohnii* lipid and DHA production using different substrates. Results are shown as mean ± standard deviation ($n = 2$)

| Substrate  | Lipid content (% w/w DCW) | Lipid productivity (mg L$^{-1}$ h$^{-1}$) | DHA in TFA (% w/w TFA) | DHA content (mg g$^{-1}$) DCW | DHA productivity (mg L$^{-1}$ h$^{-1}$) | DHA concentration (mg L$^{-1}$) |
|------------|---------------------------|------------------------------------------|------------------------|--------------------------------|----------------------------------------|--------------------------------|
| Glucose    | 14.70 ± 0.07              | 1.86 ± 0.09                             | 48.56 ± 2.28           | 7.14 ± 0.36                    | 0.90 ± 0.04                            | 16.34 ± 1.68                       |
| Molasses   | 11.12 ± 0.56              | 2.00 ± 0.10                             | 49.58 ± 2.48           | 5.51 ± 0.27                    | 0.99 ± 0.05                            | 19.56 ± 1.00                       |
| Glycerol   | 11.04 ± 0.50              | 2.77 ± 0.14                             | 25.04 ± 1.25           | 2.76 ± 0.14                    | 0.69 ± 0.03                            | 13.07 ± 0.72                       |
| Crude glycerol | 14.70 ± 0.73          | 3.19 ± 0.16                             | 44.66 ± 2.44           | 6.56 ± 0.33                    | 1.42 ± 0.07                            | 26.96 ± 1.07                       |
| Acetate    | 12.43 ± 0.62              | 3.89 ± 0.19                             | 42.12 ± 2.11           | 5.24 ± 0.26                    | 1.64 ± 0.08                            | 31.18 ± 1.60                       |
highest proportion of saturated fatty acids (SAT) (65.68% w/w of TFA) (Fig. 7b). In all the assays, the final biomass contained low percentages of monounsaturated fatty acids (MONO-UNSAT) (~8% w/w TFA). As expected, the biomass obtained from the sugarcane molasses assay showed the highest proportion of polyunsaturated fatty acids (PUFA) (50.84% w/w of TFA) since it contained the highest proportion of DHA, the major fatty acid.

Discussion

Cryptothecodinium cohnii growth on several pure and complex substrates has been previously reported, in order to reduce DHA production process costs. In this work, pure (glucose, glycerol and acetate) and complex substrates containing the pure carbon sources (sugarcane molasses, crude glycerol and vinegar affluent) were tested. Acetate and pure glycerol assays produced the highest biomass concentration. This result is supported by De Swaaf et al. (2003) who reported a superior performance of the acetic acid-grown C. cohnii 30772 cultures, relatively to the glucose grown cultures. This could be due to C. cohnii glucose metabolism which involves a number of steps—glucose uptake, glycolysis, transport of pyruvate into mitochondria, conversion of pyruvate through the citric acid cycle—contrarily to acetate assimilation, which directly feeds the pool of acetyl-CoA, which in turns feeds the citric acid cycle, crucial for energy production needed for cell growth and lipid synthesis.

Hosoglu and Elibol (2017a) reported better C. cohnii CCMP 316 growth and lipid production on glycerol rather than on glucose. Moreover, in the study in which C. cohnii was grown on different carbons sources including glucose and glycerol, Safdar et al. (2017) also reported the highest microalgal biomass concentration for the assay containing glycerol. Cryptothecodinium cohnii glycerol assimilation is comparable to glucose assimilation, since the pathway is similar, being pyruvate directly yielded by the conversion of 3-phosphoglycerol and further converted to acetyl-CoA, although glycerol metabolism saves a few steps in the glycolysis
pathway, comparing to glucose assimilation (Hilling 2014). This may explain *C. cohnii* better performance when grown on acetate and glycerol than on glucose.

The specific growth rate observed for *C. cohnii* cultivations on glucose (0.02 h⁻¹) was lower than that one reported by Swaaf et al. (1999) (−0.05 h⁻¹) who also cultivated *C. cohnii* ATCC 30772 cells in shake flasks, but used higher glucose concentrations (25–75 g L⁻¹), which can explain the higher reported specific growth rate. Saifdar et al. (2017) also reported a specific growth rate of 0.03 h⁻¹ for *C. cohnii* cultivations on glucose, developed in a 1-L fermenter. The higher specific growth rate reported by these authors is probably due to the use of the fermenter, which usually allows better microalgae performance due to higher mass transference and control of the medium pH, stirring and aeration rates ensured by these systems, avoiding drastic medium pH variations and mass transference limitations that exist in shake flasks cultivations, and negatively affect the cell growth.

The alga did not grow on vinegar effluent, probably due to the presence of polyphenols, known to be present in vinegars and to inhibit microbial cells (Sengun et al. 2019).

During the cultivations, all the substrates were exhausted, except for the molasses assay. It has been previously reported that *C. cohnii* CCMP 316 did not consume fructose when carob pulp syrup (containing glucose and fructose) was used as carbon source (Mendes et al. 2007). Nevertheless, Okuda et al. (2013) reported that D-fructose promoted the growth of strain D31, a related species of *C. cohnii*. In the present work, during the sugarcane molasses assay, glucose was completely exhausted at t = 96 h, but fructose was not consumed. Since molasses was previously hydrolysed to glucose and fructose, the microalgal glucose uptake might have inhibited the fructose assimilation, as it was reported for *Saccharomyces cerevisiae* and *Saccharomyces uvarum* (carlsbergensis) when grown on glucose and fructose (D’Amore et al. 1989).

As far as the authors know, this is the first work reporting the use of FC coupled with the stains CFDA and DHR, to analyse *C. cohnii* enzymatic activity and ROS production, as a measure of *C. cohnii* cells stress level, when grown on different carbon sources. Indeed, Prado et al. (2012) used DHR to studied ROS intracellular levels for the freshwater green microalga *Chlamydomonas moewusii* after 96 h of exposure to different concentrations of the herbicide paraquat.

The FC results (Figs. 5 and 6) showed variations in *C. cohnii* subpopulations proportions as a result of the environmental conditions they were experiencing, near real time. The most notorious fall in the proportion of intact cells with enzymatic activity (presumably “healthy cells”, subpopulation B) during *C. cohnii* cultivations was observed during the acetate assay after t = 96 h (Fig. 5 e), and might be related to the *C. cohnii* cell morphology change observed at that time, as a result of the medium pH increase. Indeed, according to Tuttle and Loeblish (1975), the optimal pH for *C. cohnii* (Seligo) strain growth is 6.6. The medium pH increase during the acetate assay resulted from the gradual CH₃COO⁻ anions removal, and their replacement by OH⁻ and other anions, resulting in the generation of NaOH, a stronger base than CH₃COONa, which was responsible for the medium pH increase, according to Chalima et al. (2019). At high medium pH values, the microalga cells tend to aggregate and, consequently, to precipitate, which explains the pronounced loss of enzymatic activity and membrane integrity for a significant proportion of *C. cohnii* cells during this assay, detected by FC analysis. In fact, aggregated cells are exposed to nutrient and oxygen starvation conditions due to nutritional diffusion limitations that exist inside the aggregates, reducing the cell viability. Therefore, when *C. cohnii* cells grow on acetate, the medium pH should always be maintained near the optimal pH (Ratledge et al. 2001; Chalima et al. 2019), which is not so crucial for the remaining studied substrates, since the cell viability did not fall, as it fell for the acetate assay. However, the medium pH maintenance requirement implies additional costs and equipment. Ratledge et al. (2001) have used an efficient pH-auxostat culture bioreactor system in which a low initial concentration of sodium acetate was used in the initial growth medium, and acetic acid was used to maintain a constant medium pH value and supply a further carbon source for growth.

Comparing the CFDA/PI and DHR results obtained by FC, it seemed that CFDA/PI double staining method is more sensitive to *C. cohnii* cell physiological states variations than DHR staining method. This observation was supported by the flow cytometric controls, which showed a stronger microalgal stress response to age when cells were stained with CFDA/PI than they were stained with DHR (Fig. 4). The FC results described in this work described *C. cohnii* behaviour when grown on different carbon sources, allowing understating the physiological response of the microalgae to the different environments.

Concerning *C. cohnii* lipid and DHA production, and comparing the pure with the complex substrates, glucose assay displayed higher lipid and DHA content than molasses assay (Table 2), possibly due to the presence of a higher proportion of cells from subpopulation B (intact cells with enzymatic activity) throughout the cultivation time course, contrarily to the molasses assay (Fig. 5a, b), in which a higher proportion of permeabilised cells (subpopulation C) might have negatively affected the microalgal lipid and DHA synthesis during that cultivation. Indeed, dead or stressed cells cannot participate in the biortransformation in the same way as metabolically active cells do.

Crude glycerol assay displayed higher lipid and DHA content than glycerol assay, possibly due to the higher proportion of metabolically active cells (subpopulation B) during the crude glycerol assay development, particularly during the stationary phase, when the cells produce storage lipidic materials.
Since the proportion of stressed cells was always high during the vinegar effluent assay time course (92.8%, sum of subpopulation A and D), no biomass and no lipids were produced by the microalgae. Contrarily, the acetate assay displayed a high proportion of metabolically active cells until the stationary phase, allowing cell growth and lipid synthesis.

Gong et al. (2015) studied DHA production by the marine dinoflagellate C. cohnii ATCC 30772, using the low-cost substrates rapeseed meal hydrolysate (RMH) and molasses, as alternative feedstock, added to a basal medium similar to that used in this work, in shake flasks. They found that, in the batch fermentations using media composed of diluted RMH (7%) and 1–9% waste molasses, the highest biomass concentration and DHA yield reached 3.43 g L$^{-1}$ and 8.72 mg L$^{-1}$, respectively. The algal biomass produced from RMH and molasses medium also contained (22–34%) DHA in total fatty acids. These results, obtained in similar conditions as the results described in the present work, are lower than those here reported.

The high proportion of SAT and PUFA in C. cohnii biomass, observed in all the assays, suggests that the lipid fraction containing PUFA ($\omega$-3 fatty acids) can be eventually separated from the remaining microalgal lipids (SAT+ MONO-UNSAT), to obtain a rich $\omega$-3 compounds fraction with applications in pharmaceutical/food areas, and the remaining fraction can be directed for bioenergy or biodiesel production. Such approach will take advantage of the various lipidic products synthesized by the microalgae, therefore maximizing the value derived from the whole process, with a desired minimal environmental impact as all fractions are valorised. In this way, the economics of the process may be greatly improved, as the high value-added product (DHA) may sustain the microbial biodiesel production.

Conclusions

Among the studied low-cost substrates, the highest lipid, DHA content and lipid productivity were observed for the crude glycerol, while molasses induced the highest proportion of DHA of total fatty acids (49.58% w/w TFA). As these substrates are widely available as sugar and biodiesel industries byproducts, they can be further converted and valorised towards the production of DHA, with an additional possibility of co-producing a lipidic fraction composed of the remaining saturated and monounsaturated fatty acids that can be directed towards biodiesel purposes. In this way, the overall process tends to produce zero waste. Moreover, the higher DHA proportions observed for the molasses and crude glycerol assays represents an advantage for the DHA purification step. The present work evaluated C. cohnii growth, lipid and DHA production on pure and low-cost complex sources using FC, to evaluate the microalga cell stress response. CFDA/PI double-staining method was more sensitive to C. cohnii cell physiological states variations than DHR staining method. This information, obtained near real time, allows underestimating the microalgae cell response to the environmental conditions, and also allows changing the process control strategy during the process development, facilitating the process scale-up step.

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