J Ind Microbiol Biotechnol (2008) 35:875–887
DOI 10.1007/s10295-008-0360-7

ORIGINAL PAPER

The use of multi-parameter flow cytometry to study the impact of *n*-dodecane additions to marine dinoflagellate microalga *Crypthecodinium cohnii* batch fermentations and DHA production

Teresa Lopes da Silva · Alberto Reis

Received: 19 March 2008 / Accepted: 17 April 2008 / Published online: 7 May 2008

Abstract The physiological response of *Crypthecodinium cohnii* batch cultivations and docosahexaenoic acid (DHA) production to *n*-dodecane additions were studied. Different *n*-dodecane concentrations [0, 0.5, 1, 2.5, 5, 10 and 20% (v/v)] were added to preliminary shake flask cultivations. The *n*-dodecane fraction that gave best results in terms of biomass and DHA production was 0.5% (v/v). The *n*-dodecane fractions of 2.5, 5, 10 and 20% (v/v) to *C. cohnii* preliminary shake flask cultures inhibited the microalgal growth and DHA production, although a high proportion of cells with intact cytoplasmic membrane was present in the end of these fermentations. After the addition of a pulse of *n*-dodecane (0.5% v/v) to *C. cohnii* exponential growing cells in a bioreactor, glucose uptake volumetric rate increased 2.5-fold, while biomass production volumetric rate increased 2.8-fold. The specific growth rate was increased 1.5-fold. The DHA % in biomass, DHA % of TFA and DHA concentration also increased (54, 22 and 58%, respectively), after the *n*-dodecane addition. At this *n*-dodecane fraction (0.5% v/v), multi-parameter flow cytometry demonstrated that *C. cohnii* cell membrane integrity was not affected. The results demonstrated that the addition of 0.5% of *n*-dodecane (v/v) to *C. cohnii* fermentations can be an easy and cheap way for enhancing the biomass and DHA production, avoiding the use of high speed rates (resulting in important power agitation costs) that affects the microalga proliferation and increases the bioprocess costs. A new strategy to improve the DHA production from this microalga in two-phase large-scale bioreactors is now in progress.

Keywords *Crypthecodinium cohnii* · *n*-Dodecane · DHA · Flow cytometry · Propidium iodide

T. Lopes da Silva (✉) · A. Reis
Instituto Nacional de Engenharia, Tecnologia e Inovação, Departamento de Biotecnologia, Unidade de Bioengenharia e Bioprocessos, Estrada do Paço do Lumair, 22, 1649-038 Lisbon, Portugal
e-mail: teresa.lopesilva@ineti.pt

Introduction

The therapeutic importance of ω-3 polyunsaturated fatty acids (PUFAs) has been demonstrated by extensive clinical and epidemiological studies. Docosahexaenoic acid (DHA) along with eicosapentaenoic acid (EPA), which are the most important ω-3 PUFAs, are known to provide health benefits associated with the consumption of certain marine fish and their oils. DHA (22:6ω3) accumulates in the membranes of human nervous, visual and reproductive tissues and is also the most abundant fatty acid in the grey matter of the brain. In addition, DHA is considered to be particularly important in infant nutrition and brain development and it is also involved in anticholesterolaemic and anti-inflammatory activities [3, 15]. The traditional source of PUFAs, fish oil, is a limited resource and its composition and quality are variable. Moreover, among others fish oil contains a large quantity of saturated and ω-6 fatty acids making concentration and purification time-consuming and expensive. The peculiar taste and odour limit the use of fish oil as a source of DHA. Considerable evidence has indicated that ω-3 fatty acids in fish oils actually derive from zooplankton that consumes algae. Therefore, microalgae are considered among the most promising sources of DHA.

Attempts have been made to produce DHA photoautotrophically by growing microalgae in photobioreactors, but it is difficult to achieve high biomass concentrations due to

two unsolved problems, namely light limitation and oxygen accumulation, in photoautotrophic cultures [22]. Heterotrophic growth of microalgae eliminates the requirement for light and offers the possibility of greatly increasing algal cell density and productivity on a large scale. Screening of microalgae for heterotrophic production of DHA is therefore of great significance.

DHA is the characteristic PUFA of marine dinoflagellates. *Crypthecodinium cohnii* is a non-photosynthetic, heterotrophic marine dinoflagellate in which 16:0 and DHA fatty acids predominate. Usually, nearly 25–60% of its constituent fatty acids is 22:6ω3 fatty acid and no other PUFAs are present in excess of 1% [26], making DHA separation and concentration easy and cheap. This characteristic makes the DHA production from this microorganism very attractive particularly for pharmaceutical applications since the inclusion of a PUFA as a drug component requires its purification to over 95% [24]. Mendes et al. [20] have reported a successful DHA concentration and purification process from *C. cohnii* cells, where a fraction contained 99.2% of DHA of the total fatty acids (TFA) was obtained.

Important parameters for optimal DHA production include growth rate, final biomass concentration, total lipid content and DHA proportion of the lipid, which depend on the proportion of metabolically active cells. Significant lipid accumulation, particularly triacylglycerols (TAGs), occurs during idiophase, after a culture nutrient other than the carbon source is depleted. The limiting nutrient is usually nitrogen. Therefore, industrial *C. cohnii* fermentations are usually a carbon-fed batch and progress in two stages. The first is the active growth phase during which the lipid content of the biomass is about 20%. Once the nitrogen source is depleted, carbon is continuously supplied to the fermenter. Since cell growth and division is halted due to the lack of nitrogen for de novo protein and nucleotide synthesis, the supplied carbon is converted into a storage lipid (TAG) rich in DHA [28].

The aeration conditions are of crucial importance for *C. cohnii* cells growth as the specific growth decreased when the cells are growing under restricted supply oxygen conditions [1]. Moreover, oxygen transfer is likely to be a limiting factor during a commercial-scale high-cell-density cultivation of *C. cohnii* cells, leading to the process productivity decrease. In such conditions, to maintain aerobic conditions, a very high stirrer speed had to be maintained during a large part of the process, resulting in the power input increase [3]. On the other hand, cell proliferation of dinoflagellates is negatively affected by mechanical agitation [26, 29], the way generally used to improve mass transfer in submerged fermentations.

It is possible to increase the oxygen transfer in microbial fermentations by adding an organic phase with a higher affinity to oxygen (oxygen vector) [23]. Whereas no more than the saturation concentration of oxygen can be dissolved in the aqueous phase from the aqueous phase, the supply of oxygen to the aqueous phase from the gas stream may be supplemented by equilibrium partitioning of dissolved oxygen from the organic phase to the aqueous phase. Several research groups have applied oxygen vectors to enhance oxygen supply and, as a consequence, to increase the biomass in different culture systems [11, 15, 21]. The main oxygen vectors used in biotechnology are hydrocarbons such as *n*-dodecane and *n*-hexadecane [4–6, 12, 13, 16, 27] perfluorocarbons [18, 19, 21] as well as vegetable oils [30].

Recently we reported the potential use of biphasic bioreactors for enhancement of *Chrypthecodinium cohnii* growth and DHA production [4]. The biomass concentration, DHA and TFAs production of the fermentation with *n*-dodecane were higher, compared to fermentation control with no *n*-dodecane. However, no physiological response in terms of *C. cohnii* cell viability to the *n*-dodecane presence was monitored. Active microbial lipid production is present in intact, metabolically active cells. Moreover, a high proportion of dead cells present in any part of the bioprocess will be detrimental as such cells do not contribute for the product formation, decreasing the process yield. It is therefore important to have information on cell viability in real time, with a high degree of statistical resolution. Multi-parameter flow cytometry is capable of providing such information, rapidly, with a high degree of accuracy, having a number of advantages over more traditional methods [10].

The present work studied the impact of *n*-dodecane additions to *C. cohnii* shake flask and bench bioreactor cultivations, using flow cytometry as a tool to monitor the microalga physiological response. The work was carried out as part of a study within the SAPIENS project POCTI/EQU/47689/2002 entitled "Enhancement of bubble and drop mass transfer processes using additives".

Materials and methods

Oxygen vector

n-Dodecane (p.a. Merck, Darmstadt, Germany; density 750 g l^{-1} at 20°C, oxygen solubility 54.9 $\times$ 10^{-3} g l^{-1} at 35°C and atmospheric air pressure) was added to the shake flasks medium culture in fractions of 0.5, 1, 2.5, 5, 10 and 20% (v/v). The fraction which gave best results in terms of DHA productivity was used in a bench bioreactor fermentation.

C. cohnii fermentations

Growth conditions

C. cohnii CCMP 316 was obtained from the Provasoli-Guillard Center for Culture of Marine Phytoplankton (CCMP) Culture Collection (Boothbay Harbour, ME, USA) and was maintained in axenic conditions by sub-culturing every 2 weeks in f/2 + NPM medium [7–9] supplemented with glucose (6 g l^{-1}). Cultures were grown on 500 ml of the f2 + NPM medium supplemented with glucose (15 g l^{-1}) in 2-l shake flasks at 120 rpm and 27°C in the dark. After 3 days (exponential growth phase), these cultures were used to inoculate the shake flasks and the bioreactor.

Shake flask experiments

In total, 250-ml shake flasks containing 60 culture medium were inoculated with 10% (v/v) of exponential growing cultures as described earlier.

Different *n*-dodecane concentrations (0.5, 1, 2.5, 5, 10 and 20% v/v) were added to the growth medium (glucose 15 g l^{-1}; red sea salt, 25 g l^{-1}; yeast extract 2 g l^{-1}) in the 250 ml-shake flasks, so that the final volume was 60 ml. A control experiment was carried out under the same conditions, but without *n*-dodecane.

C. cohnii growth was followed for 8 days, at 27°C, 120 rpm, in the dark.

Bioreactor experiments

The growth medium was the same as described previously, and the inoculum was 5% (v/v).

All experiments were carried out in a 5 l—cylindro-conical continuously stirred—bioreactor with 4.8-l working volume, equipped with two impellers, a marine turbine on the top and a six-bladed rushton turbine on the bottom.

The pH value was adjusted to 6.5 by adding a concentrate sodium hydroxide or acid solution of 1 M NaOH or 1 M HCl. The pH of the fermentation medium was measured with a Broadley James (Irvine, CA, USA) steam-sterilisable pH electrode. The dissolved oxygen tension (DOT) in the medium was measured with an AppliSens (Schiedam, The Netherlands) oxygen probe. The stirring rate was manually increased (200–600 rpm) whenever DOT was below 30%. The aeration rate was 1 vvm.

Dry cell weight measurements

Biomass, expressed as dry cell mass, was measured gravimetrically on 5-ml culture samples, from which cells were harvested, washed and dried for 18 h at 100°C. When *n*-dodecane was present, it was previously eliminated by centrifugation for 10 min and the cells were washed once with *n*-dodecane-free fresh medium and then re-suspended in fresh sterile medium before dry cell weight assay.

Determination of specific growth rate

Specific growth rate was determined by plotting the natural logarithm of biomass concentration against time. Readings within the exponential phase were then used to obtain correct values of the specific growth rate by linear regression.

Residual glucose concentration

The residual glucose and organic acid concentrations in filtered (0.2 μm) samples were determined using a Cecil HPLC system with an Aminex HPX-87P carbohydrate analysis column (Bio-Rad, Hercules, USA).

Fatty acid analysis

Fatty acids' extraction and preparation of methyl esters were carried out according to Lepage and Roy method [17] with modifications. Freeze-dried samples of *C. cohnii* (100 mg) were transmethylated with 2 ml of methanol/acetyl chloride (95:5 v/v) and 0.2 ml heptadecanoic acid (5 mg ml^{-1}, Nu-Check-Prep, Elysian, USA) as an internal standard. The mixture was sealed in a light-protected Teflon-lined vial under nitrogen atmosphere and heated at 80°C for 1 h. The vial contents were then cooled, diluted with 1-ml water and extracted with 2 ml of *n*-hexane. The hexane layer was dried over Na_2SO_4, evaporated to dryness under nitrogen atmosphere and re-dissolved in hexane, which contained the methyl esters. The methyl esters were then analyzed by gas–liquid chromatography, on a Varian (Palo Alto, CA, USA) 3800 gas–liquid chromatograph (USA), equipped with a flame ionisation detector. Separation was carried out on a 0.32 mm × 30 m fused silica capillary column (film 0.32 μm) Supelcowax 10 (Supelco, Bellafonte, Palo Alto, CA, USA) with helium as a 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 and 280°C, respectively, and 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 was carried out using known standards (Nu-Chek-Prep, Elysian, MN, USA). Each sample was made in duplicate and injected twice.

Flow cytometry

All cells are bound by the cytoplasmic membrane, which allows the cell to communicate selectively with its immediate environment. Propidium iodide (PI, Molecular Probes, P-1304) was used in this study in order to monitor *C. cohnii* cell membrane integrity. PI binds to DNA and cannot cross an intact cytoplasmic membrane. Flow cytometry measurements were made using a Coulter EPICS ELITE (Fullerton, CA, USA) flow cytometer with 488 nm excitation from an argon-ion laser at 15 mW. Samples taken from the culture were immediately diluted (at least 1:2000 v/v) with phosphate buffer solution (pH 7.0) and stained with PI. Samples were kept in a sonication bath for 10 s prior to analysis, in order to avoid problems associated with cell aggregation. PI stock solution was made up at 2 mg ml^{-1} in distilled water. The working concentrations of PI was and 10 μg ml^{-1}. All solutions were passed through a 0.2 μm filter, immediately prior to use, to remove particulate contamination. In addition, the control software was set on both the light scattering properties in the forward angle direction (FALS) signal and the right angle direction (RALS) signal. The optical filters were set-up so that PI fluorescence was measured at 630 nm.

Results and discussion

Flow cytometry controls

In order to demonstrate that it is possible, using multiparameter flow cytometry, to characterise the cell membrane integrity of individual cells of *C. cohnii* fermentations, it was necessary to establish a positively control. This was then used for comparison with data produced from cultivation experiments. Cells of *C. cohnii* grown for 4 days in shake flasks (control experiment) were exposed to ethanol 70% (v/v) for 1 min and then centrifuged and resuspended in buffer solution before stained with PI. Under these conditions, mean PI fluorescence intensity was greatly increased (mean = 900) when compared with the mean PI fluorescence intensity of untreated cells (mean = 7) (Fig. 1). The density plot (Fig. 2) shows one single PI positive sub-population, corresponding to ethanol treated *C. cohnii* cells with permeabilised membrane.

Yeung and Wong [29] also used successfully PI to confirm the viability of *C. cohnii* under shaking conditions.

Shake flask experiments

Figure 3a, b shows the biomass and residual glucose profiles of *C. cohnii* shake flask cultivations. Shake flask fermentations with 0, 0.5 and 1% showed typical batch

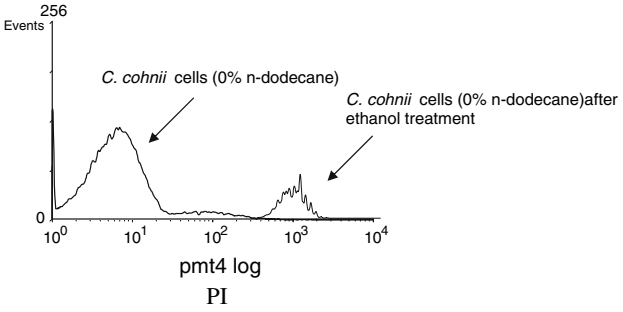

Fig. 1 Effect of ethanol (70%) on *C. cohnii* cell membrane integrity. The cells were stained with PI

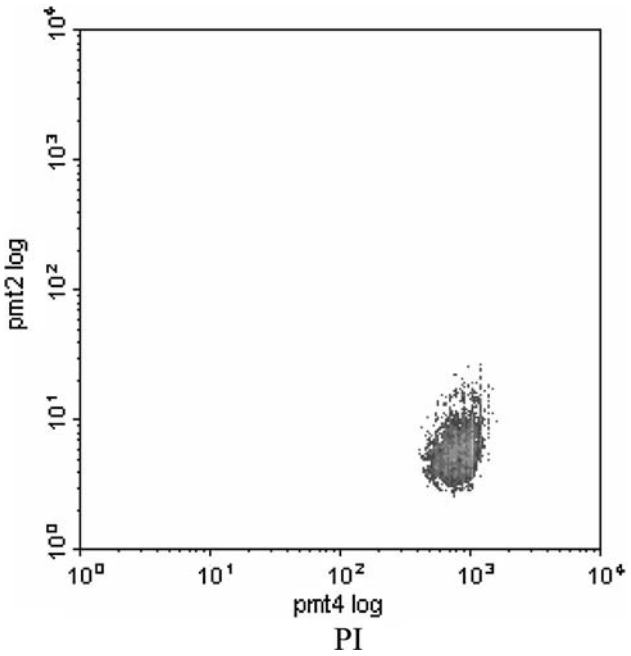

Fig. 2 Ethanol 70% (v/v) treated *C. cohnii* cells density plot. Only one sub-population can be distinguished, corresponding to cells with permeabilised membranes

fermentation curves with lag, exponential and stationary phases. Fermentations at 0 and 0.5% (v/v) attained the highest biomass concentrations (6.7 and 6.43 g l^{-1}, at $t = 6$ and 7 days, respectively, Fig. 3a), although the highest specific growth rate was observed for the fermentation at 0.5 (v/v) (0.4 day^{-1}, Table 1). Glucose was completely exhausted 6–7 days after the inoculation (Fig. 3b). The highest glucose uptake and biomass production volumetric rates were observed at 0.5% *n*-dodecane concentration ($r_{glucose} = 2.3$ g l^{-1} day^{-1}, $r_{biomass} = 1.1$ g l^{-1} day^{-1}, Table 1), suggesting that the *n*-dodecane addition, at that fraction, might have increased the oxygen availability, stimulating *C. cohnii* biomass production.

After a time period of ~3 days of microalga cultivation, the biomass concentration of the fermentation at 2.5, 5, 10

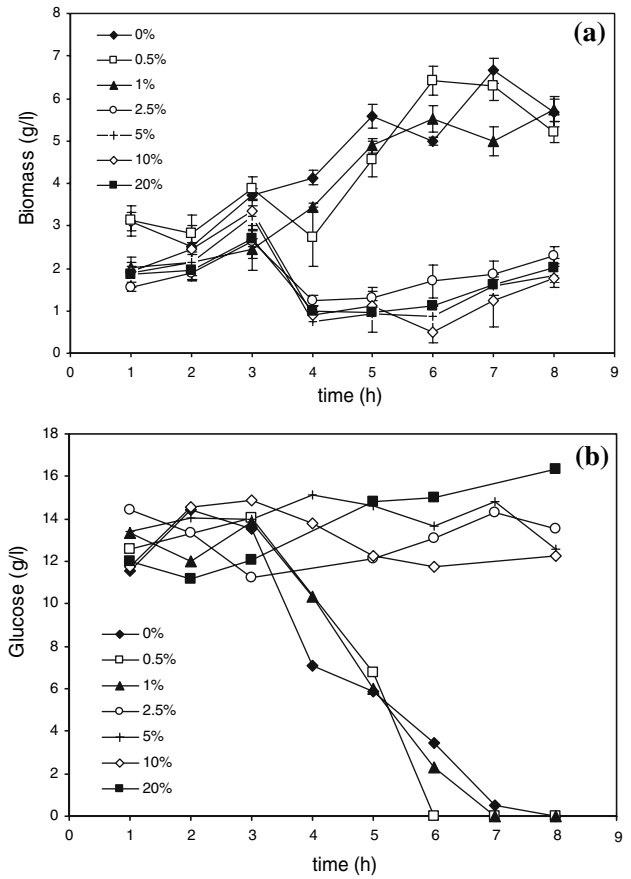

Fig. 3 Fermentation profiles for *C. cohnii* shake flask cultivations at 0 (control experiment), 0.5, 1, 2.5, 5, 10 and 20% (v/v) *n*-dodecane. **a** Biomass. **b** Residual glucose profiles

Table 1 Kinetic parameters of *C. cohnii* shake flask fermentations

	n-Dodecane additions (%v/v)						
	0	0.5	1	2.5	5	10	20
μ (day^{-1})	0.2	0.4	0.3	–	–	–	–
$r_{glucose}$ (g glucose l^{-1} day^{-1})	1.9	2.3	2.0	0	0	0	0
$r_{biomass}$ (g biomass l^{-1} day^{-1})	1.0	1.1	0.7	0	0	0	0

and 20% (v/v) *n*-dodecane dropped, but began to rise slightly 4–6 days after inoculation (Fig. 3a). Glucose was not consumed in these fermentations (Fig. 3b), indicating that the *n*-dodecane could have inhibited *C. cohnii* glucose uptake metabolic pathway. In this situation, other carbon sources (from the yeast extract) could have been consumed, allowing the slight biomass increase.

Flow cytometric results contributed for a better understanding of this behaviour. In Fig. 4, the proportion of intact cells (PI negative cells) is plotted against time.

The ratio of intact cells was high (>90%) during the time course of the shake flask cultivation at the lowest *n*-dodecane concentrations (0, 0.5 and 1%) even after the

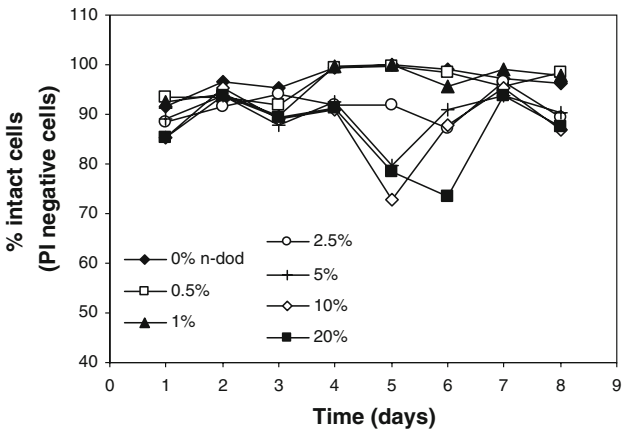

Fig. 4 Effect of *n*-dodecane on *C. cohnii* cell membrane integrity during the shake flask cultivations at different *n*-dodecane concentrations

glucose exhaustion (Fig. 3b), suggesting again that, after glucose depletion, other carbon sources (probably from the yeast extract) were consumed. However, in the fermentations at higher *n*-dodecane fractions (2.5, 5, 10 and 20%), the proportion of intact cells dropped approximately 4–6 days after the inoculation, being more pronounced in the fermentations at 10 and 20% (v/v), attaining ~73%, 5 and 6 days after the inoculation, respectively. Afterwards ($t = 7$ days), the proportion of intact (PI negative) cells increased, which was consistent with the biomass increase observed in the shake flask experiments at 2.5, 5, 10 and 20% *n*-dodecane (v/v) (Fig. 3a), suggesting that, even at these higher *n*-dodecane concentrations, *C. cohnii* cells could adapt to the organic solvent presence, by increasing their lag phase. Previously we reported that *n*-dodecane fractions higher than 1% (v/v) to *C. cohnii* shake flask cultures resulted in cell lysis, based on traditional optical microscope observations [4]. In the present work, multiparameter flow cytometry showed that, despite the higher *n*-dodecane concentrations that compromised the *C. cohnii* cell membrane integrity, the microalga was able to recover. Such information was obtained in near real time and with a high degree of statistical resolution.

Fatty acids analyses and DHA production

Table 2 shows *C. cohnii* fatty acid composition of cells cultivated in shake flasks at different *n*-dodecane concentrations. The major fatty acids were 12:0, 14:0, 16:0, 18:1ω9 and 22:6ω6. In all the cases, during the lag phase ($t = 4$ days), the total saturated fatty acids percentage (12:0, 14:0, 16:0, 18:0) was higher than the total unsaturated fatty acids percentage (18:1ω9, 22:5ω5 and 22:6ω3) and this tendency was inverted as the microalga attained the stationary phase, which was mainly due to the increase

Table 2 Influence of *n*-dodecane concentration on *C. cohnii* cellular fatty acid composition at different fermentation times

Fermentation time (days)	*n*-Dodecane fractions (v/v)									
	12:0	14:0	16:0	16:1ω7	18:0	18:1ω9	22:5ω3	22:6ω3	%Saturated	%Unsaturated
Fatty acids (w/w %) (0%)										
4	11.5 ± 0.8	26.9 ± 4.4	37.4 ± 3.2	0.2 ± 0.5	2.0 ± 0.6	12.7 ± 1.6	0.8 ± 0.9	8.5 ± 1.4	77.8	22.2
5	14.5 ± 1.3	31.7 ± 1.8	34 ± 0.7	0.0 ± 0.0	1.3 ± 0.1	9.2 ± 0.5	1.3 ± 0.1	8.0 ± 1.7	81.5	18.5
6	12.0 ± 2.1	27.9 ± 2.5	28.9 ± 0.7	0.9 ± 0.0	1.4 ± 0.2	11.5 ± 1.1	1.6 ± 0.1	15.8 ± 2.6	70.2	29.8
7	15.5 ± 0.2	21.2 ± 1.2	21.6 ± 0.7	1.1 ± 0.1	1.4 ± 0.2	9.8 ± 0.3	1.1 ± 0.1	28.3 ± 1.8	59.2	40.3
8	3.0 ± 1.0	12.0 ± 1.0	18.1 ± 0.3	0.2 ± 0.2	1.5 ± 0.0	8.6 ± 0.3	2.1 ± 0.3	54.5 ± 4.1	35.0	65.4
Fatty acids (w/w %) (0.5%)										
4	14.3 ± 6.2	26.3 ± 4.2	33.0 ± 6.8	0.0 ± 0.0	1.8 ± 0.3	12.5 ± 2.1	1.5 ± 0.3	10.5 ± 0.9	75.4	24.6
5	9.1 ± 1.8	23.3 ± 1.4	34.0 ± 2.3	0.2 ± 0.4	1.3 ± 0.6	12.5 ± 0.8	1.6 ± 0.2	18.0 ± 1.7	67.7	32.2
6	9.6 ± 1.6	25.3 ± 1.9	22.1 ± 1.2	1.1 ± 0.0	2.3 ± 0.6	12.3 ± 1.4	1.5 ± 0.3	26.7 ± 5.8	58.7	41.3
7	8.0 ± 2.2	22.0 ± 3.6	16.7 ± 1.4	0.8 ± 0.9	1.5 ± 0.1	10.5 ± 0.5	1.4 ± 0.1	39.2 ± 6.7	48.5	51.5
8	13.0 ± 1.7	12.6 ± 1.2	15.2 ± 0.7	0.5 ± 0.1	11.3 ± 0.0	9.8 ± 0.2	1.7 ± 0.2	63.2 ± 0.8	32.1	67.9
Fatty acids (w/w %) (1.0%)										
4	11.2 ± 1.9	24.8 ± 4.7	31.6 ± 4.6	0.2 ± 0.0	1.9 ± 0.1	13.3 ± 0.7	1.7 ± 0.2	15.3 ± 0.2	69.4	30.5
5	11.5 ± 0.3	25.3 ± 0.4	30.9 ± 0.2	1.0 ± 0.0	2.9 ± 0.0	14.4 ± 0.1	1.5 ± 0.0	12.5 ± 1.2	70.6	29.4
6	9.4 ± 0.5	23.3 ± 1.3	27.1 ± 1.0	0.7 ± 0.6	1.8 ± 0.2	14.3 ± 0.6	2.4 ± 0.2	20.9 ± 1.4	53.6	46.4
7	7.3 ± 0.5	25.8 ± 1.1	27.0 ± 0.4	0.7 ± 0.0	2.3 ± 0.1	15.0 ± 0.5	2.3 ± 0.2	19.7 ± 0.8	60.4	39.5
8	7.0 ± 0.8	18.6 ± 1.4	17.9 ± 0.5	0.5 ± 0.0	1.5 ± 0.0	9.5 ± 0.2	1.5 ± 0.1	43.5 ± 2.5	44.9	55.1
Fatty acids (w/w %) (2.5%)										
4	7.8 ± 0.0	15.2 ± 0.8	34.6 ± 0.4	3.1 ± 0.0	3.8 ± 1.2	19.3 ± 0.4	2.6 ± 0.2	13.5 ± 0.4	61.5	38.5
5	8.8 ± 1.0	14.3 ± 1.7	27.6 ± 1.6	2.4 ± 0.1	2.9 ± 0.1	14.7 ± 0.3	2.3 ± 0.6	27.0 ± 3.4	53.6	46.4
6	10.6 ± 3.7	13.6 ± 3.0	25.5 ± 0.5	2.5 ± 0.0	2.5 ± 0.3	13.1 ± 1.1	2.0 ± 0.1	30.1 ± 6.0	52.3	47.7
7	–	–	–	–	–	–	–	–	–	–
8	8.6 ± 0.0	9.4 ± 0.3	20.0 ± 0.5	0.0 ± 0.0	2.6 ± 0.1	12.8 ± 0.0	2.5 ± 0.8	44.6 ± 1.7	40.1	59.9
Fatty acids (w/w %) (5%)										
4	11.2 ± 1.4	16.1 ± 0.3	39.6 ± 1.8	0.0 ± 0.0	4.6 ± 0.3	20.4 ± 2.4	0.0 ± 0.0	6.6 ± 1.1	72.6	27.4
5	13.6 ± 1.4	17.7 ± 0.7	33.6 ± 0.9	4.3 ± 0.0	3.1 ± 0.2	16.6 ± 1.1	1.8 ± 0.2	9.2 ± 0.3	68.1	31.9
6	14.8 ± 1.9	16.6 ± 0.8	34.4 ± 0.8	1.5 ± 2.1	3.2 ± 0.1	18.8 ± 1.0	0.7 ± 1.0	10.0 ± 0.6	69.0	31.0
7	14.6 ± 2.2	15.8 ± 0.5	29.6 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	15.2 ± 3.2	0.0 ± 0.0	24.8 ± 2.5	60.0	40.0
8	12.8 ± 0.6	13.8 ± 1.4	24.4 ± 2.7	1.4 ± 0.2	1.1 ± 1.6	13.4 ± 0.3	0.8 ± 0.1	32.2 ± 2.3	52.1	47.9
Fatty acids (w/w %) (10%)										
4	12.9 ± 0.4	19.2 ± 0.2	41.2 ± 4.6	0.0 ± 0.0	0.0 ± 0.0	20.0 ± 2.4	0.0 ± 0.0	6.7 ± 0.3	73.4	26.6
5	–	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–	–	–
7	10.3 ± 1.3	12.0 ± 2.1	24.0 ± 1.4	2.1 ± 0.2	2.3 ± 0.3	14.0 ± 1.0	1.7 ± 0.3	33.5 ± 3.4	48.7	51.3
8	6.8 ± 2.6	10.6 ± 1.0	21.6 ± 1.2	0.0 ± 0.0	2.4 ± 0.0	14.0 ± 0.8	2.2 ± 0.3	42.5 ± 6.4	41.4	58.6
Fatty acids (w/w %) (20%)										
4	8.5 ± 1.5	16.8 ± 2.5	35.9 ± 1.5	3.1 ± 0.4	3.5 ± 0.4	17.1 ± 1.5	2.3 ± 0.7	12.9 ± 0.1	64.7	35.3
5	10.3 ± 2.3	17.6 ± 2.6	36.2 ± 0.1	0.0 ± 0.0	4.2 ± 0.4	18.7 ± 1.2	0.0 ± 0.0	12.9 ± 3.5	68.3	31.7
6	11.8 ± 0.8	13.6 ± 0.5	29.2 ± 1.6	0.0 ± 0.0	0.0 ± 0.0	16.7 ± 0.4	0.0 ± 0.0	28.7 ± 3.4	54.6	45.4
7	10.6 ± 0.2	15.7 ± 1.0	32.5 ± 4.4	2.8 ± 0.3	2.7 ± 0.1	17.3 ± 0.9	2.1 ± 0.0	16.7 ± 1.4	61.0	39.0
8	17.0 ± 0.2	23.9 ± 1.4	35.1 ± 1.3	0.0 ± 0.0	0.0 ± 0.0	16.9 ± 1.9	0.0 ± 0.0	34.2 ± 6.7	59.1	40.9

of 22:6ω3. The same behaviour has been described for *C. cohnii* ATCC 30556 [14]. These authors attributed the synthesis of more saturated fatty acids during the lag and exponential phases to the sufficient nutrient conditions. As the culture aged up to the early stationary phase, incomplete or starvation of some specific nutrients in the medium induced qualitative and quantitative changes in fatty acids, resulting in the increase of DHA.

On the other hand, the percentage of total saturated fatty acids from cells taken during the stationary phase ($t = 7$–8 days) increased as the *n*-dodecane fraction increased, which suggested an adaptation mechanism of the microorganism to the organic solvent presence. Indeed, it has been reported that apolar solvents cause an increase of the saturation degree of fatty acids in some solvent-tolerant bacteria strains, which changes the fluidity of the membrane and in this way compensate for the effects caused by solvents [25].

Figure 5 shows the DHA percentage in biomass (a), DHA percentage in total fatty acids (TFAs) (b) and DHA concentration in broth (c), during *C. cohnii* shake flask cultivations.

The DHA percentage in biomass and the DHA concentration increased during the time course of the shake flask fermentations with 0, 0.5 and 1% (v/v) of *n*-dodecane (Fig. 5a, c) as the nutrients became depleted in the broth and the microalgal cells produced storage lipids as a survival mechanism to starvation periods. In *C. cohnii*, such lipids are known to be rich in DHA [28]. In the fermentations with 2.5, 5, 10 and 20% *n*-dodecane (v/v), the DHA percentage in biomass and the DHA concentration remained almost unchanged (Fig. 5a, c). In all fermentations, the DHA in TFA increased during the time course, except at 20% (v/v) *n*-dodecane (Fig. 5b).

The DHA percentage in biomass, DHA percentage in TFA and DHA concentration in broth attained the maximum when the microalga was grown at 0.5% *n*-dodecane, reaching 5.5, 63.2 and 278.2 mg l^{-1}, respectively. Such DHA percentage in TFA is one of the highest ever reported for *C. cohnii* shake flask fermentations.

It was clear that the *n*-dodecane concentration at 0.5% had a positive effect on the microalgal DHA production, as compared to the control experiment (0% *n*-dodecane). In fact, the DHA percentage in biomass, the DHA percentage in TFA and the DHA concentration increased 39, 16 and 22%, respectively, compared to the control experiment (0% *n*-dodecane).

Higher *n*-dodecane concentrations [(1, 2.5, 5, 10 and 20% (v/v)] reduced the DHA percentage in biomass, DHA percentage in TFA and DHA concentration), relatively to the control experiment.

These results confirmed that *n*-dodecane low concentrations improve *C. cohnii* DHA production, which is in agreement with the results previously reported [4].

Light scatter measurements

Yeung and Wong [29] reported that dinoflagellates can be detected from the background on the basis of their intrinsic light scattering properties in FALS (forward angle light scatter) and RALS (right angle light scatter).

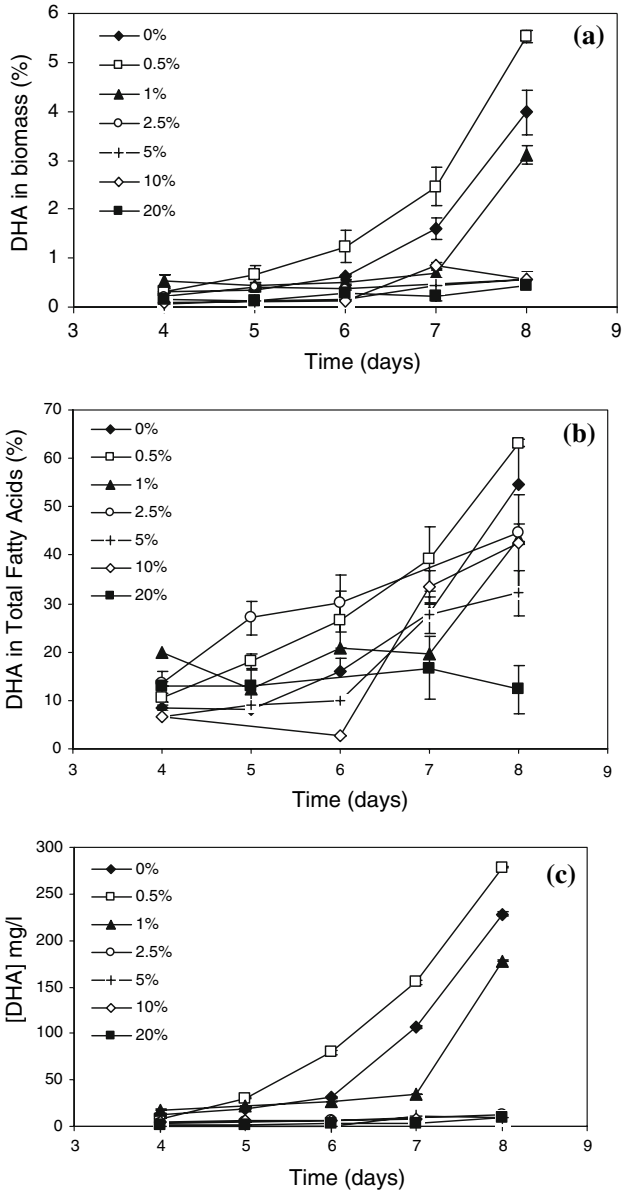

Fig. 5 DHA production (DHA percentage in biomass, DHA in TFA and DHA concentration) profiles during *C. cohnii* shake flask cultivations, at different *n*-dodecane concentrations

Dinoflagellates are unicellular microorganisms that are eukaryotes with a G_1-S-G_2-M cell cycle [2]. In a normal cell cycle of the heterotrophic microalga, motile G_1 cells will shed their flagella, encyst and execute the remaining cell-cycle phases [DNA synthesis (S), G_2 and mitosis (M)]. The cysts will subsequently produce either two, four, or eight daughter cells depending upon which cell-cycle pathway the individual cells have taken [29]. These different cell phases of *C. Cohnii* cell-cycle might explain the elliptical and broaden distribution with respect to FALS and RALS flow cytometric analyses, suggesting cell size and shape heterogeneity (Fig. 6a, e, f).

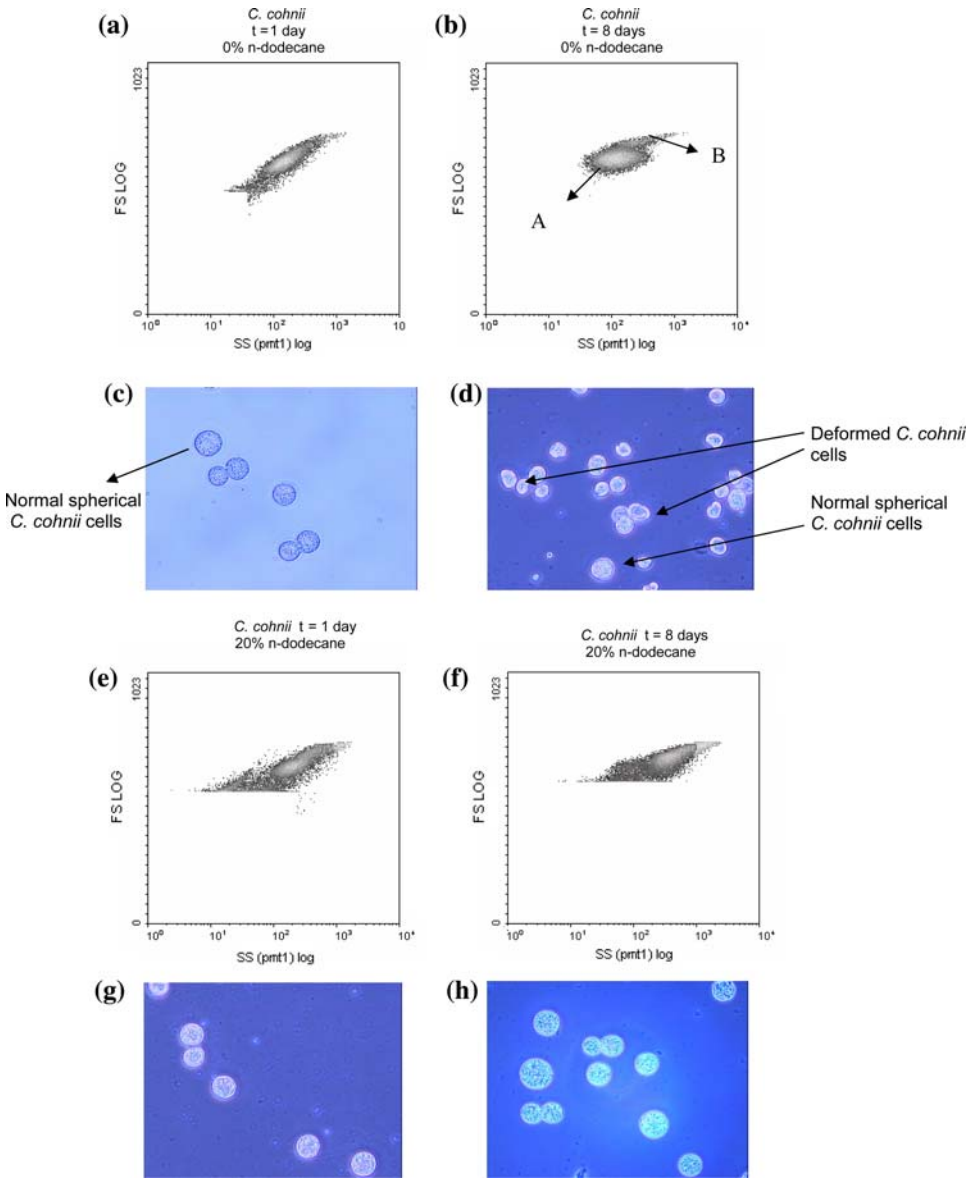

Fig. 6 Changes in the intrinsic light scattering FALS vs RALS (**a**, **b**, **e**, **f**) and traditional optical microscope observations (**c**, **d**, **g**, **h**), during *C. cohnii* shake flask cultivations, 1 and 8 days after the inoculation. Shake flask control experiment, (**a**–**d**) and shake flask cultivation with *n*-dodecane 20% (v/v), (**e**–**h**). **c**, **d**, **g**, **h** ×1,000

In addition, changes in light scatter were detected in *C. cohnii* cells 1 and 8 days after growing in shake flasks without *n*-dodecane (Fig. 6a, b). While the first (1 day after inoculation) generated an elliptical distribution (Fig. 6a), the second (8 days after inoculation) generated a bimodal clustering with respect to FALS (clusters A and B, Fig. 6b). Examination of samples using a traditional optical microscope showed morphological changes in *C. cohnii* cells during these shake flask fermentations (Fig. 6c, d). *C. cohnii* cells grown for 1 day, in the *n*-dodecane absence, depicted the usual spherical morphology (Fig. 6c), while a sample collected 8 days after the inoculation (in the *n*-dodecane absence), showed a few normal, spherical cells and a few deformed and smaller cells as they seemed shrunken, exhibiting an elliptical shape (Fig. 6d). The same was observed for the lowest *n*-dodecane concentrations (0.5, 1 and 2.5% v/v *n*-dodecane). A progressive reduction in the proportion of deformed *C. cohnii* cells was observed as the *n*-dodecane concentration increased. At 20% (v/v) no deformed *C. cohnii* cells were observed (Fig. 6g, h).

These two cell morphologies (normal and deformed cells) could explain the two sub-populations found in Fig. 6b, the smaller and deformed cells corresponding to sub-population (A) and the "normal" and bigger cells to sub-population (B), assuming that, in this case, FALS is related to relative cell size [29].

These morphological changes observed when *C. cohnii* cells were grown in the absence or low concentration of *n*-dodecane could be due to the higher cell densities attained under these conditions, leading to a high oxygen uptake rate, which could result in oxygen-limiting conditions. When the microalga was grown in the presence of higher *n*-

dodecane concentrations, the cell concentration was lower; thus, the oxygen availability in the broth should be higher due to a lower oxygen uptake rate. In addition, higher oxygen vector concentrations should result in higher oxygen availability in the broth.

Bioreactor experiments

The *n*-dodecane concentration which led to the highest DHA production (0.5% v/v) was further used in microalgal bioreactor batch fermentations, in order to enhance *C. cohnii* DHA production.

Figure 7a–c shows the fermentation profiles for a *C. cohnii* batch fermentation. The *n*-dodecane was added to *C. cohnii* mid-exponential growing cells, at a constant specific growth rate μ. Any change in the specific growth rate, after the *n*-dodecane addition, was attributed to the *n*-dodecane presence.

The exponential microalgal growth started after a lag phase of 66 h. At that time, residual glucose and biomass concentrations were 15 and 1.7 g l^{-1}, respectively. As biomass concentration increased, *C. cohnii* cells oxygen requirements increased, resulting in the DOT decreased (Fig. 7b, c). The speed rate then was step wise increased from 200 to 600 rpm in order to avoid oxygen-limiting conditions during this aerobic process. At $t = 88.6$ h, the speed rate was 600 rpm, the biomass was 7 g l^{-1} and DOT reached 29% of the oxygen saturation concentration in the broth. It is well known that mechanical agitation (high speed rates) inhibits dinoflagellates cell proliferation [29]. Therefore, in order to protect the microalga cells, the maximum speed rate used was 600 rpm. As DOT was below 30%, a pulse of *n*-dodecane was added ($t = 89.2$ h) to the exponential growing culture so that the oxygen transfer rate could increase due to the oxygen vector presence, without increasing the speed rate. After the *n*-dodecane addition, DOT increased 2.7-fold relatively to DOT before the oxygen-vector addition, reaching 78%, 6.7 h after the *n*-dodecane addition ($t = 95.3$ h). Thereafter, as the carbon source was consumed, the microalgal growth ceased, resulting in DOT increase (84% at $t = 119.2$ h). The biomass concentration remained unchanged (~6.7 g l^{-1}) during the next 5 h after the *n*-dodecane addition, following a biomass concentration increase reaching the maximum (~10 g l^{-1}), at $t = 99.4$ h. As glucose became depleted, biomass concentration began to drop, reaching 7.5 g l^{-1} at $t = 111$ h. The biomass concentration increase observed at the end of the fermentation (8.5 g l^{-1}, $t = 119.2$ h) and the concomitant DOT decreased (84% at $t = 119.2$ h) could be due to endogenous metabolism, as cell material resulting from cell lysis could serve as nutrients for cell growth.

Table 3 shows the kinetic data from the bioreactor microalga batch fermentation. Biomass production and

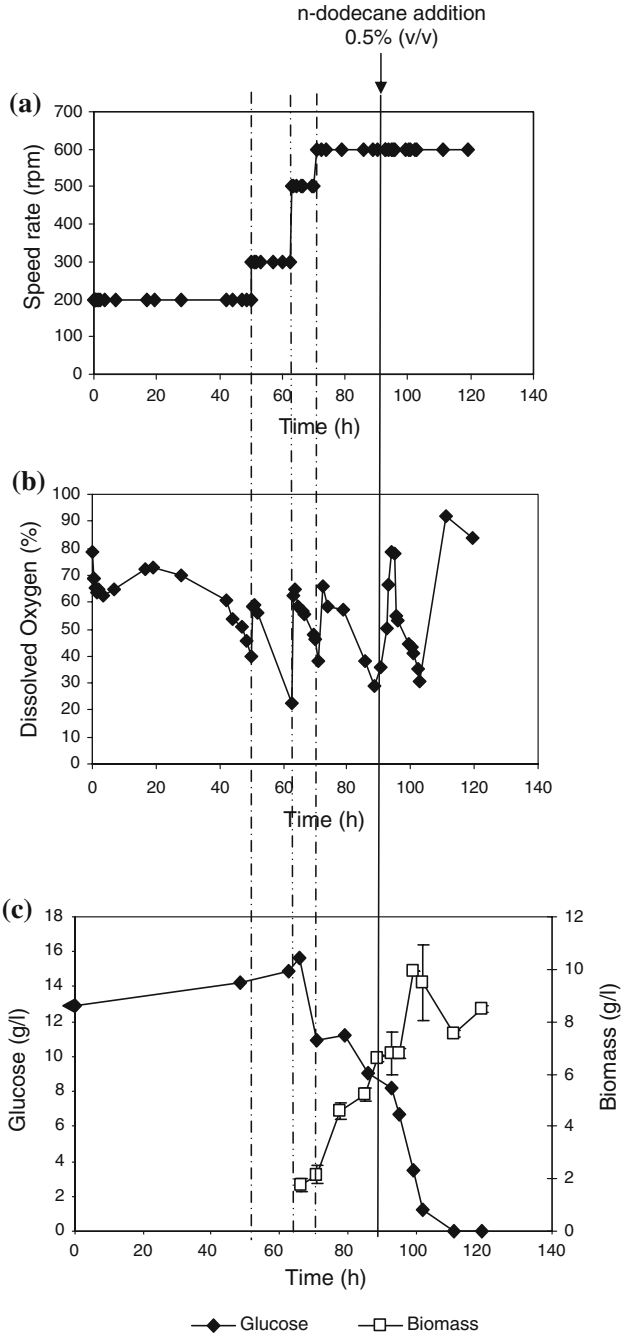

Fig. 7 Effect of *n*-dodecane addition on *C. cohnii* batch fermentation profiles. *Dash-dotted lines*, speed rate increase, continuous line and *n*-dodecane addition

Table 3 Kinetic parameters of the *C. cohnii* shake batch fermentations

	Before *n*-dodecane addition	After *n*-dodecane addition
$r_{x\ (g\ biomass\ l^{-1}\ h^{-1})}$	0.20	0.50
$r_{glucose\ (g\ glucose\ l^{-1}\ h^{-1})}$	0.26	0.74
μ (h^{-1})	0.06	0.09

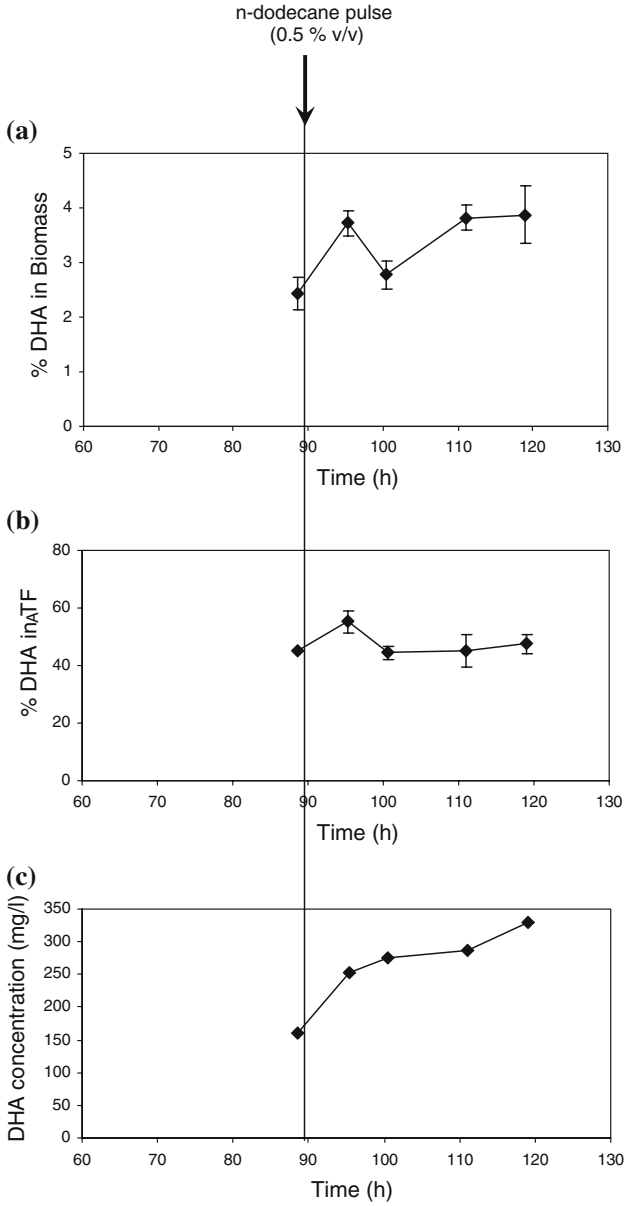

Fig. 8 Effect of *n*-dodecane addition on DHA production (DHA percentage in biomass, DHA in TFA and DHA concentration) during a *C. cohnii* batch fermentation

glucose uptake volumetric rates increased 2.5 and 2.8-fold, respectively, which could be due to the enhanced oxygen transfer rate after the oxygen-vector addition, enabling oxygen-sufficient conditions in the broth. In addition, the specific growth rate increased from 0.06 to 0.09 h^{-1}, after the oxygen vector addition, which could indicate that the culture was under oxygen-limiting conditions before the *n*-dodecane addition.

The DHA percentage in biomass, DHA percentage in TFA and DHA concentration profiles during the batch fermentation are depicted in Fig. 8a–c.

Table 4 Influence of *n*-dodecane addition (0.5% v/v) on *C. cohnii* cellular fatty acid composition at different batch fermentation times

Fatty acids (w/w %)										
Fermentation time (h)	Fermentation time after *n*-dod pulse (h)	12:0	14:0	16:0	16:1ω7	18:0	18:1ω9	22:5ω3	22:6ω3	%Saturated	%Unsaturated
88.6	–	2.5 ± 0.1	19.3 ± 0.0	24.7 ± 0.0	0.0 ± 0.0	1.0 ± 0.0	5.8 ± 0.0	1.3 ± 0.0	45.3 ± 0.0	47.2	52.8
89.2	0	*n*-dodecane addition									
95.3	6.1	1.6 ± 0.5	12.1 ± 1.5	22.0 ± 2.3	0.2 ± 0.0	1.6 ± 0.0	5.9 ± 0.2	1.2 ± 0.2	55.2 ± 4.8	37.4	62.6
100.5	11.3	3.4 ± 0.2	17.4 ± 0.8	25.9 ± 1.5	0.0 ± 0.0	1.7 ± 0.2	6.2 ± 0.2	1.0 ± 0.1	44.4 ± 2.3	48.5	51.5
111.0	21.8	4.3 ± 0.8	16.6 ± 1.9	25.7 ± 2.7	0.0 ± 0.1	2.6 ± 0.3	5.6 ± 0.3	0.9 ± 0.1	45.2 ± 5.6	47.9	52.1
119.2	30.0	4.3 ± 0.6	15.0 ± 1.8	25.2 ± 1.8	0.1 ± 0.0	3.0 ± 0.0	5.8 ± 0.1	0.7 ± 0.0	45.9 ± 4.2	47.5	52.5

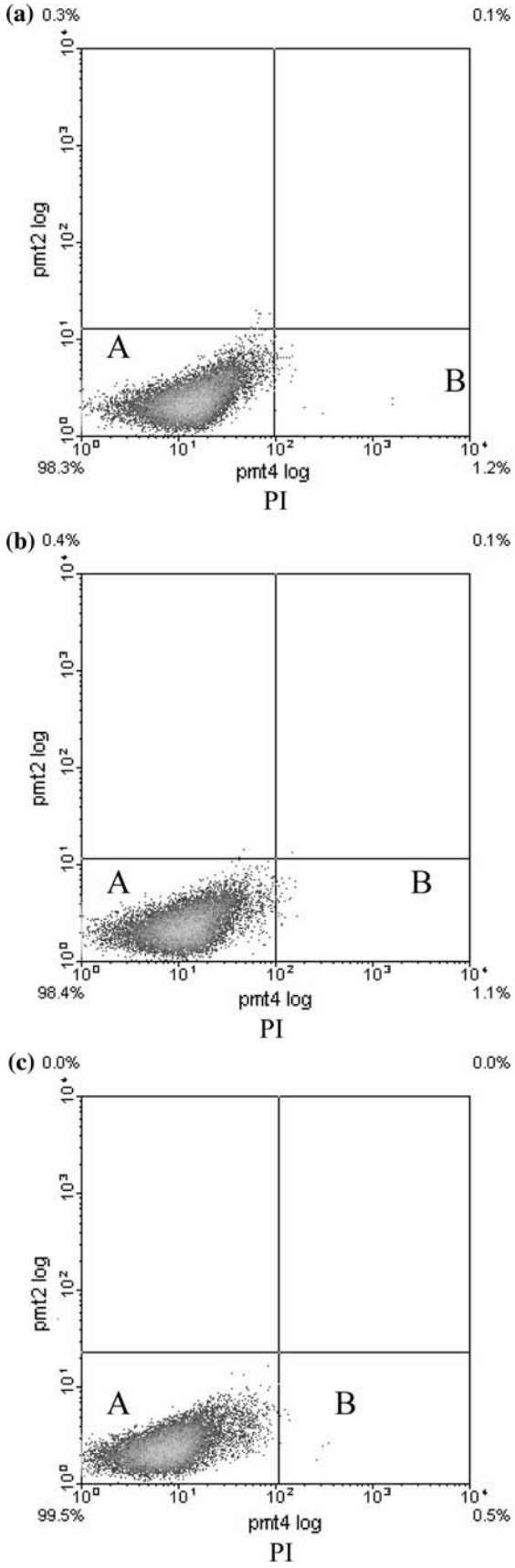

Fig. 9 Cell samples taken at 88.6, 95.3 and 119.2 h (just before the ► *n*-dodecane addition, 6.1 and 30 h after the *n*-dodecane addition, respectively) during a batch cultivation with *C. cohnii* cells stained with PI. Only one population of cells could be distinguished corresponding to intact cells, no PI stained

At $t = 88.6$ h, the DHA percentage in biomass was 2.4% (Fig. 8a). 6.1 h after the *n*-dodecane addition ($t = 95.3$ h), the DHA percentage in biomass increased 54%, reaching 3.7%. The DHA percentage in TFA also increased 22% after the *n*-dodecane addition, while the DHA concentration increased 58% (Fig. 8b, c). From Table 4, it can be seen that, at that fermentation time ($t = 95.3$ h), the unsaturated fatty acids increase was mainly due to the DHA percentage increase as the other unsaturated fatty acids (16:1ω7, 18:1ω9 and 22:5ω3) almost remained unchanged, which is consistent with the results previously reported [4]. These results suggest that, when *n*-dodecane was present, the DHA increase could be due to a selective lipid (namely TAG rich in DHA) extraction solvent rather than the oxygen transfer rate increase due to the oxygen vector presence. In the latter situation, the unsaturated fatty acids other than DHA should have increased with DOT increase, as the biosynthesis of these fatty acids involves oxygen-dependent desaturases, which did not happen.

At $t = 100.5$ h, the DHA percentage in biomass and DHA percentage in TFA decreased (Fig. 8a, b), as glucose became depleted in the broth (Fig. 7c). At the same time, the rate of increase of DHA concentration also dropped (Fig. 8c). In fact, significant lipid (TAG) accumulation has been reported in the idiophase after a culture nutrient other than the carbon source is depleted [28]. However, in this case, despite the microalgal growth had reached the stationary phase, the glucose exhaustion seemed to have halted the microalgal lipid production, thus, the DHA production.

The DHA percentage in biomass increased after the glucose exhaustion ($t = 111$ h) attaining a plateau ($\sim$3.8%) until the end of the fermentation, suggesting that *C. cohnii* cells could have uptaken other carbon sources than glucose (i.e., from the yeast extract) for storage lipids production as a survival strategy during starvation periods, as already stated. The DHA percentage of TFA remained unchanged until the end of the fermentation ($\sim$45%), while the DHA concentration slightly increased in the end of the fermentation, attaining 230 mg l^{-1}.

Flow cytometric analysis of *C. cohnii* cell samples demonstrated that during the course of the batch cultivation in bioreactor, the microalga cells did not take PI stain, indicating that all the cells had intact cytoplasmic membranes. Therefore, the *n*-dodecane addition at the studied

concentration (0.5% v/v) did not affect *C. cohnii* cell membrane integrity (Fig. 9a–c).

Conclusions

The *n*-dodecane concentration that gave best results in terms of biomass and DHA production, in *C. cohnii* shake flask cultivations, was 0.5% v/v.

The addition of *n*-dodecane (0.5% v/v) to exponential growing *C. cohnii* cells in a bioreactor batch fermentation resulted in an increase of the glucose uptake and biomass production volumetric rates as well as the specific growth rate. The DHA production (DHA % in biomass, DHA % of TFA and DHA concentration) also increased after the *n*-dodecane addition. At this *n*-dodecane fraction (0.5% v/v), *C. cohnii* cells cytoplasmic membrane integrity was not affected.

The results demonstrated that the addition of 0.5% (v/v) *n*-dodecane to *C. cohnii* fermentations can be an easy and cheap way of enhancing the biomass and DHA production, maintaining a high number of intact cells and avoiding the use of high speed rates (resulting in important power agitation costs) that affect the microalga proliferation, increasing the bioprocess costs.

A new strategy to improve the DHA production from this microalga in two-phase large-scale bioreactors is now in progress.

Multi-parameter flow cytometry was a useful tool for monitoring microalga physiological response during microalgal fermentations in real time, and with a high degree of statistical resolution.

Acknowledgment Teresa Lopes da Silva and Alberto Reis wish to acknowledge Fundação Calouste Gulbenkian for financial support, respectively; Post-Doctoral Grants 76875 e 76876, respectively. The authors also wish to acknowledge Doutora Helena Gaspar (INETIDTIQ) for the help and advice on HPLC analyses

References

1. Beach DH, Holz GG (1973) Environmental influences on the docosahexaenoate content of the triacylglycerols and phosphatidylcholine of a heterotrophic, marine dinoflagellate, *Crypthecodinium cohnii*. Biochim Biophys Acta 316:56
2. Bhaud Y, Guillebault D, Lennon JF, Defacque H, Soyer-Gobillard MO, Moreau H (2000) Morphology and behaviour of dinoflagellates chromosomes during the cell cycle and mitosis. J Cell Sci 113:1231–1239
3. de Swaaf M, Rijk TC, Meer P, Eggink G, Sijtsma L (2003) Analysis of docosahexaenoic acid biosynthesis in *Crypthecodinium cohnii* by ^{13}C labelling and desaturase inhibitor experiments. J Biotechnol 103:21–29
4. da Silva TL, Mendes A, Mendes R, Calado V, Alves SS, Vasconcelos JMT, Reis A (2006) Effect of n-dodecane on *Crypthecodinium cohnii* fermentations and DHA production. J Ind Microbiol Biotechnol 33:408–416
5. Galaction AI, Cascaval D, Turnea M, Folescu E (2005) Enhancement of oxygen mass transfer in stirred bioreactors using oxygen-vectors. 2. *Propionobacterium shermanii* broths. Bioprocess Biosyst Eng 27:263–271
6. Giridhar R, Srivastava A (2000) Productivity enhancement in L-sorbose fermentation using oxygen vector. Enzyme Microb Technol 27:537–541
7. Guillard RL (1960) A mutant of *Chlamydomonas moewusii* lacking contractile vacuoles. J Protozool 7:262–269
8. Guillard RL, Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* (*Hustedt*) and *Detonula confervacea* Cleve. Can J Microbiol 8:229–239
9. Guillard RL (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH (eds) Culture of marine invertebrate animals. Plenum Press, New York, pp 26–60
10. Hewitt CJ, Nebe-von-Caron G (2004) The application of multi-parameter flow cytometry to monitor individual microbial cell physiological state. In: Enfors SO (ed) Advances biochemical engineering/biotechnology physiological stress responses in bioprocesses, vol 89. Springer, New York, pp 197–223
11. Ho CS, Ju LU, Baddour R (1990) Enhancing penicillin fermentations by increased oxygen solubility through the addition of n-hexadecane. Biotechnol Bioeng 36:1110–1118
12. Jia S, Wang M, Kahar P, Park Y, Okabe M (1997) Enhanced of yeast fermentation by addition of oxygen vectors in air-lift bioreactors. J Ferm Bioeng 84:176–178
13. Jialong W (2000) Enhancement of citric acid production by *Aspergillus niger* using n-dodecane as an oxygen vector. Process Biochem 35:1079–1083
14. Jiang Y, Chen F (2000) Effects of temperature and temperature shift on docosahexaenoic acid production by the marine microalga *Crypthecodinium cohnii*. J Am Chem Soc 77:613–617
15. Jiang Y, Chen F, Liang S (1999) Production potential of docosahexaenoic acid by the heterotrophic marine dinoflagellate *Crypthecodinium cohnii*. Process Biochem 34:633–637
16. Lai L, Tsai T, Wang T (2002) Application of oxygen vectors to *Aspergillus terreus* cultivation. J Bios Bioeng 94:453–459
17. Lepage G, Roy CC (1986) Direct transmethylation of all classes of lipids in a one-step reaction. J Lipid Res 27:114–119
18. Lowe KC, Davey MR, Power JB (1998) Perfluorochemicals: their applications and benefits to cell culture. Trends Biotechnol 16:272–277
19. MacLean GT (1977) Oxygen diffusion rates in organic fermentation broths. Process Biochem 12:22–28
20. Mendes A, da Silva TL, Reis A (2007) DHA concentration and purification from the marine heterotrophic microalga *Crypthecodinium cohnii* CCMP 316 by winterization and urea complexation. Food Technol Biotechnol 45:38–44
21. Menge M, Mukherjee J, Scheper T (2001) Application of oxygen vectors to *Claviceps purpurea* cultivation. Appl Microbiol Biotechnol 55:411–416
22. Molina E, Fernandez J, Acien FG, Chisti Y (2001) Tubular photobioreactor design for algal cultures. J Biotechnol 92:113–119
23. Nielson DR, Daugulis AJ, Lellan PJ (2003) A novel method of simulating oxygen mass transfer in two-phase partitioning bioreactors. Biotechnol Bioeng 83:735–742
24. Ratledge C, Streekstra H, Cohen Z, Fichtali J (2005) Downstream processing, extraction, and purification of single cell oils. In: Cohen Z, Ratledge C (eds) Single cell oils. AOCS Press, Champaign, pp 202–219
25. Shinitsky M (1984) Membrane fluidity and cellular functions. In: Shinitsky M (ed) Physiology of membrane fluidity, vol II. CRC Press, Boca Raton
26. Tuttle RC, Loeblich AR (1975) An optimal growth medium for the dinoflagellate *Crypthecodimium cohnii*. Phycologia 14:1–8

27. Wei DZ, Liu H (1998) Promotion of L-asparaginase production by using n-dodecane. Biotechnol Tech 12:129–131
28. Wynn J, Behrens P, Sundararajan A, Hansen J, Apt K (2005) Production of single cell oils by dinoflagellates. In: Cohen Z, Ratledge C (eds) Single cell oils. AOCS Press, Champaign, pp 86–98
29. Yeung PKK, Wong JTY (2003) Inhibition of cell proliferation by mechanical agitation involves transient cell cycle arrest at G_1 phase dinoflagellates. Protoplasma 220:173–178
30. Zhao S, Kuttava SG, Ju LK (1999) Oxygen transfer characteristics of multiple-phase dispersions simulating water-in-oil xanthan fermentations. Bioprocess Eng 20:313–323