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Biorefinery Approach from *Nannochloropsis oceanica* CCALA 978: Neutral Lipid and Carotenoid Co-Production Under Nitrate or Phosphate Deprivation

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Abstract

The large-scale culture of microalgae has become a focus of interest for the biorefinery industry, combining the production of biofuels with that of other value-added bioproducts and thus increasing the profitability of the process. In order to evaluate its potential as biodiesel feedstock under a biorefinery approach, the biomass, pigment, nutrient, total lipid and lipid fraction kinetics of the Argentinian *Nannochloropsis oceanica* CCALA 978 strain were analysed. The species was first cultivated under optimal conditions (f/2 medium) and then transferred to nutritional stress conditions (N-deprivation or P-deprivation media). Under complete f/2 medium, *N. oceanica* presented maximum growth rate (μ) and chlorophyll-*a* values and minimum values for lipid and carotenoid content. The high nutrient consumption rate (N or P) in this exponential phase was associated with increasing cell density. Under both nitrate and phosphate deprivation, μ was drastically reduced but biomass production reached its highest values, indicating a phase of metabolite accumulation. Maximum values of total lipids (ca. 39% dw) and carotenoids ($1100 \times 10^{-3} \pm 15$ μg mL^{-1}) were obtained under nitrate deprivation. In addition, total lipid composition was characterized by 92% neutral lipids, 0.7% phospholipids, a high percentage of C16:1 (ca. 37% of total fatty acids) and minimum values of PUFA (5.9% of total fatty acids). Biodiesel properties inferred from the fatty acid methyl ester profile of neutral lipids meet the standards established by EN 14214 and ASTMD 6751-08. Although N and P deficiency both produced an increase in neutral lipids in the studied strain, under a biorefinery approach, nitrate deprivation is considered to be a better method of stress induction than phosphate deprivation for the purpose of co-producing neutral lipids for biodiesel and value-added pigments.

Keywords Marine microalga biomass · Nutrient kinetics · Lipid fractions · Pigments · Biodiesel feedstock

Introduction

Biodiesel derived from microalgae is considered a promising alternative source of energy in the framework of emergent technologies [1]. Specifically, oleaginous microalgae can produce high amounts of neutral lipids (NL), the main feedstock for biodiesel transesterification [2]. Even though the production of biodiesel and its blends is technically feasible [3, 4], the culture of microalgae on a large scale is still not economically profitable. Strategies used to lower costs and increase yields include the use of flue gases, the utilization of wastewater for the nutrient supply and the production of valuable co-products [5]. In particular, some species of microalgae are able to synthetize polyunsaturated fatty acids, natural dyes, carotenoids, enzyme polymers, peptides, toxins and sterols with profitable applications in different industrial sectors [6].

Microalgae biorefineries are of interest since they combine the production of biofuels and other value-added products, increasing the viability of the process [7]. However, highly

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diverse biorefinery scenarios arising from various factors affect microalgal culture (for example, microalgal strain, cell size, optimal environmental factors, stress factors, culture units). A viable strategy requires the selection of a species with the capacity to produce, within the same environmental scenario, suitable neutral lipids for biodiesel and valuable metabolites [8]. Obtaining biomass that contains all the products to be extracted in the same culture phase offers a significant advantage since harvesting can represent up to 20 to 30% of the culture costs [9].

Nannochloropsis is a microalgal genus belonging to the Eustigmatophyceae class whose species are characterized by their robustness and plasticity. Most are marine species and can grow under different environmental conditions [10–15]. As with other microalgae species, the biochemical composition of *Nannochloropsis* varies according to environmental factors and culture strategies [1]. Several studies worldwide have shown the promising potential of this species as a candidate for biodiesel production due to its ability to accumulate large amounts of neutral lipids, mainly triglycerides (TAG) [16–18]. Furthermore, *Nannochloropsis* species produce valuable products, such as carotenoids, eicosapentaenoic acid (EPA) and proteins [7, 19]. The potential of *Nannochloropsis* cultures has been studied under two scenarios: (1) biodiesel production and EPA as a nutraceutical biocompound [20, 21] and (2) biodiesel production and carotenoids for food [7, 14]. Contrary to TAG production, the synthesis of PUFA is more active in cells growing under the exponential phase. Respect to second scenario, the synthesis of carotenoids in some *Nannochloropsis* strains has been reported together with lipid accumulation when cells are exposed to nutrient limitation [7]. Carotenoids can be used in food, feed, cosmetics, biomaterials, nanostructures and the pharmaceutical industry; however, most of the recent attention has been focused on their function as antioxidants. The global carotenoid market was estimated to be worth ~ 1.24 billion USD in 2016, projected to increase to ~ 1.53 billion USD by 2021, at a compound annual growth rate (CAGR) of 3.78% from 2016 to 2021 [22]. This latter scenario therefore adds to the strategic interest in enhancing knowledge of biorefineries based on *Nannochloropsis* species.

In particular, with regard *Nannochloropsis oceanica*, Dong et al. [23] reported on the synthesis of total lipids and accessory pigments in *N. oceanica* IMET1 under long-term nitrogen starvation, while Solovchenko et al. [14] evaluated the fatty acid and carotenoid profiles in *N. oceanica* CCALA 804 under nitrogen deficiency. Thus, the effect of N or P deprivation on the simultaneous production of neutral lipids for biodiesel and carotenoids as a value-added co-product in the Argentinian *N. oceanica* CCALA 978 strain was systematically evaluated. For this, the strain was cultivated under optimal and nutritional stress conditions (N-free or P-free media) in order to obtain basic information on the following aspects: (1) biomass and pigment production, (2) nutrient kinetics, (3) total lipids, neutral lipids and polar lipids, (4) fatty acid methyl ester (FAME) profiles and (5) oil quality for biodiesel production. The results of this study are expected to provide valuable input for large-scale cultures of *N. oceanica* under a biorefinery approach.

Materials and Methods

Algal Strain and Culture Conditions

Nannochloropsis oceanica CCALA 978 (Culture Collection of Autotrophic Organisms, Institute of Botany, Academy of Sciences of the Czech Republic) was isolated from the Southwestern Atlantic Ocean coast (65° 01′ W, 43° 18′ S, Argentina) and provided by CRIAR, Instituto de Biología Marina y Pesquera Almirante Storni, San Antonio Oeste, Río Negro province, Argentina. The molecular and phylogenetic identification of the species was carried out by Bongiovani et al. [24]. The culture medium was prepared with natural seawater from Bahía Blanca Estuary (Argentina) and adjusted to a salinity value of 30. The seawater was filtered by cellulose acetate membrane (0.45-μm size), sterilized and enriched with f/2 nutrients according to Guillard [25] as follows: $NaNO_3$ (880 μM), NaH_2PO_4 (36 μM), trace metal ($CuSO_4 \cdot 5H_2O$ (0.039 μM), $ZnSO_4 \cdot 7H_2O$ (0.076 μM), $CoCl_2 \cdot 6H_2O$ (0.042 μM), $MnCl_2 \cdot 4H_2O$ (0.91 μM), $Na_2MoO_4 \cdot 2H_2O$ (0.026 μM)), TRIS (1.65×10^6 μM), vitamin B12 (3.69×10^{-4} μM), biotin (2.05×10^{-3} μM) and vitamin B1 (0.29 μM).

Nannochloropsis oceanica cells were cultured in 2-L Erlenmeyer flasks at 25 ± 1 °C with continuous bubbling of air (500–700 cm^3 min^{-1}) enriched with CO_2 (1%) during 4–5 h every day. Light was supplied by cool white Philips fluorescent tubes in a 16:8-h light–dark photoperiod at an average of 100 μmol photons m^{-2} s^{-1}, measured by a photoradiometer (LICOR, LI-192SB 2π Model).

Cultures with Complete f/2 Medium (Culture Step 1)

Cultures were grown in triplicate in 2 L of complete f/2 medium in batch mode (culture step 1). The cultures were harvested in the exponential phase by centrifugation (3500*g*) on day 7. A pellet from one culture was washed three times with distilled water (3 volumes of distilled water) and kept at − 20 °C until lipid analysis.

Cultures with Nitrate or Phosphate Deprivation (Culture Step 2)

The second pellet from cells in the exponential phase of step 1 was resuspended in f/2 medium under nitrate deprivation [f/2

(N-)] in order to evaluate the effect of the absence of nitrogen. The third pellet from step 1 was resuspended in f/2 medium under phosphate deprivation [f/2 (P-)] in order to evaluate the effect of the absence of phosphorous. Finally, the cultures were centrifuged on day 21 and the biomass was washed three times with distilled water (3 volumes of distilled water) and kept at − 20 °C until lipid analysis.

Determinations of Growth, Dry Cell Weight and Biomass Productivity

The cell density (no. cell mL^{-1}) was determined daily by counting three replicate samples in a Neubauer haemocytometer under an optical microscope Leica DM 2000. The specific growth rate (μ) was estimated during the period of exponential growth by a least squares fit to a straight line of logarithmically transformed data [25]. The dry weight (DW) was estimated by a modified gravimetric method according to Chini Zittelli et al. [26]. Samples of 5 mL were filtered through pre-conditioned and pre-weighed filters (Munktell GF/F). The filters with samples were then washed three times with 20 mL distilled water and dried overnight at 60 °C until constant weight. Biomass productivity (mg mL^{-1} day^{-1}) was calculated as the difference between initial and final dry weight biomasses divided by the duration of the cultivation period. Culture samples were taken in triplicate every 2 or 3 days.

Chlorophyll-*a* and Total Carotenoid Measurements

For the chlorophyll-*a* (Chl *a*) and total carotenoid (Car) content determinations, 4-mL samples were taken every 2 or 3 days and filtered through GF/F filters (Munktell MGF). The filters were treated with 5 mL absolute methanol three times and kept overnight in the dark at − 18 °C [27]. The extract was clarified by filtration and the Chl *a* and Car contents were determined spectrophotometrically with a spectrophotometer Shimatzu UV-Visible 1603 at 665 and 480 nm, respectively. The Chl *a* and Car contents were estimated according to the following equation: Chl *a* (μL mL^{-1}) = 13.9 ($A_{665} - A_{750}$) [28] and Car (μL mL^{-1}) = 4 ($A_{480} - A_{750}$) [29]. The absorption spectra of *N. oceanica* CCALA 978 were recorded and normalized to cell density. Determinations were carried out in triplicate.

Nutrient Analysis

For the determination of dissolved nitrate and phosphate concentrations, 4-mL samples were taken every 2 or 3 days and filtered through 0.7-μm-pore GF/F filters. The cell-free supernatant was kept at − 20 °C until nutrient analysis. Phosphate concentration was determined using the orthophosphate method according to Murphy and Riley [30]. Nitrate concentration was determined using the Cd/Cu column reduction method according to Strickland and Parsons [31]. The absorbance signals of nitrate and phosphate were read at 543 nm and 885 nm, respectively. Absorbance was measured using a spectrophotometer SHIMATZU UV-VIS model UV-1603.

Lipid Analysis

Neutral Lipid Kinetics

For neutral lipid kinetics, 5-mL samples in triplicate were taken every 2 or 3 days and frozen at − 20 °C until analysis. The determination of neutral lipids was done by the spectrofluorometric method with Nile Red [27]. The fluorescence intensity (FI) was measured using a spectrofluorometer RF-5301PC Schimadzu at excitation/emission wavelengths of 480/570 nm, respectively. The relative fluorescence intensity (RFI) was calculated as the subtraction of both the fluorescence of microalgal cell suspensions and the self-fluorescence of Nile Red. Samples were taken in triplicate.

Total Lipid Extraction

Total lipid (TL) extraction was performed according to a modified Folch method [32] assisted with ultrasound [33]. Freeze-dried samples in quadruplicate of 150–200 mg of biomass were treated with 4 mL of chloroform:methanol (2:1 v/v), vortexed thoroughly for 1–2 min and ultrasonicated during 15 min at room temperature. The mixture was then centrifuged at 3600*g* for 5 min and the supernatant was recovered and collected in a funnel separator; this procedure was repeated three times. The supernatant was washed twice with 20% NaCl 0.9% (w/w) and allowed to stand until a biphasic system was achieved. The lower phase containing the extracted lipids was recovered into a glass flask, evaporated to dryness under a nitrogen stream and kept at − 20 °C until lipid fractionation.

Lipid Fractionation

Total lipids were fractionated into neutral lipids (NL), glycolipids (GL) and phospholipids (PL) using a silica Sep-Pack cartridge of 1000 mg (J. T. Baker Inc., Phillipsburg, N. J.) according to the protocol described by Berge et al. [34]. The procedure consisted of the following steps: (i) conditioning of the silica cartridge eluting 30 mL of chloroform, (ii) sample loading (25–30 mg of total lipid extract diluted in 5 mL of chloroform); (iii) recovery of NL from the adsorbent bed by elution with 15 mL of a solution of chloroform:acetic acid (9:1 v/v); (iv) GL recovery by elution with 20 mL of a solution of acetone:methanol (9:1 v/v) and finally (v) PL recovery by elution with 20 mL of methanol. Each fraction was collected into a pre-weighed conical glass vial, evaporated until dryness

under a clean nitrogen stream and weighed. Two replicates of each lipid fraction were made.

Methyl Ester Derivation and Fatty Acid Analysis

The fatty acid derivation was carried out according to a modified method of Morrison and Smith [35]. An aliquot of total lipid and each lipid fraction (about 25–30 mg) was weighed in a conical thermo-resistant flask. Two millilitres of KOH-methanol solution (10% w/v) was added and shaken vigorously in a vortex. The air was purged under nitrogen stream and the flask was hermetically sealed. The flask with lipid–KOH:methanol solution was heated for 45 min in a water bath at 80 °C. The unsaponifiable material was then extracted using 2 mL of petroleum ether, twice. The upper phase was discarded. Concentrated HCl was added to the lower phase and shaken in a vortex tube during 1 min. Petroleum ether (2 mL) was aggregated and shaken vigorously in a vortex, recovering the FA in the upper phase. This step was repeated twice. The ether-lipid extract was dried under a nitrogen stream. A solution of BF_3–methanol (10% v/v, Sigma Aldrich) was added and the samples were incubated at 80 °C for 30 min in a water bath. Then, fatty acid methyl esters (FAME) were extracted twice with petroleum ether (2 mL), the upper phase was recovered and the esters were evaporated to dryness under a nitrogen stream. Finally, hexane (chromatography grade) was added to a final volume of 1 mL for total and neutral lipids and 0.5 mL for glycolipids and phospholipids. FAME were analysed by gas chromatography with a gas chromatograph HP Agilent 4890D, equipped with a flame-ionization detector at a temperature of 260 °C, a split/splitless injector and a capillary column SP-2560 (100 m, 0.25 mm and 0.2 μm) (Supelco Inc., Bellefonte, PA). The carrier gas was high purity hydrogen at 18 cm s^{-1}. The GC oven was initially held at 140 °C for 5 min after which the temperature was increased at 4 °C/min to 240 °C and held isothermal for 15 min. The detection limit of the chromatographic method was set at 0.01%. The HP 3398A GC Chemstation Software (Hewlett Packard, 1998) was used for chromatographic analysis. FAME identification was performed by comparison with standard certificate material, Supelco FAME 10 mix 37 (Bellefonte, PA, USA), according to the AOCS Official Method Ce 1b-89. Four replicates of TL and each lipid fraction were made. All employed solvents were of analytical grade.

Biodiesel Quality from Fatty Acid Profiles

Different biodiesel properties were estimated on the basis of the fatty acid profiles of total and neutral lipids. Average degree of unsaturation (ADU), iodine value (IV), cetane number (CN), cloud point (CP), specific gravity (SG), kinematic viscosity (KV) and higher heating value (HHV) were calculated according to Hoekman et al. [36]; cold filter plugging points were estimated according to Talebi et al. [37]. The algorithms were as follows:

$$
\begin{aligned}
ADU &= \sum MY_i \\
IV &= 74.373\ ADU + 12.71 \\
CN &= -6.6684\ ADU + 62.876 \\
CP &= -13.356\ ADU + 19.994 \\
SG &= 0.0055\ ADU + 0.8726 \\
KV &= -0.6316\ ADU + 5.2065 \\
HHV &= 1.7601\ ADU + 38.534 \\
CFPP &= (3.14171417 \times LCSF) - 16.477
\end{aligned}
$$

where Y_i is the percentage fraction of each fatty acid (FA) component, M is the number of carbon–carbon double bonds in each FA component and LCSF is the long-chain saturated factor.

Statistical Analysis

The differences in mean values of total lipid and lipid fractions and between mean value percentages of fatty acids were assessed by two-way ANOVA, followed by Fisher's test in order to identify the sources of detected significance. In all cases, comparisons that showed $p < 0.05$ were considered significant. All statistical analyses were carried out with Infostat Software [38].

Results and Discussion

Growth, Biomass Production, Nutrient and Pigment Kinetics of *N. oceanica* CCALA 978

The growth rate, biomass production and productivity and nutrient kinetics of *N. oceanica* growing first in a complete medium (f/2) (culture step 1) and then under nutrient deprivation [f/2 (N-) or f/2 (P-)] (culture step 2) are shown in Fig. 1. Under complete f/2 medium, *N. oceanica* CCALA 978 presented an exponential growth phase until day 7 with a μ of 0.46 ± 0.02 day^{-1} and a maximum cell density of ca. 35×10^6 cells mL^{-1} (Fig. 1a). Maximum biomass production and productivity were ca. 320 mg L^{-1} (day 7) and 37.8 mg L^{-1} day^{-1}, respectively (Fig. 1b, c). Under nutrient deprivation, μ was drastically reduced to 0.02 day^{-1} and 0.003 day^{-1} for f/2 (N-) and f/2 (P-), respectively. Cell density under f/2 (N-) increased slightly on day 16 reaching an average of ca. 43×10^6 cells mL^{-1}, whereas it remained stable with ca. 35×10^6 cells mL^{-1} under f/2 (P-) (Fig. 1a). Biomass production reached its highest values under nutrient deprivation (Fig. 1b) and was significantly higher under f/2 (N-) (ca. 980.0 ± 100.0 mg L^{-1}) than under f/2 (P-) (ca. 800.0 ± 40 mg L^{-1}) ($p < 0.05$) (Fig. 1b). Biomass productivity under

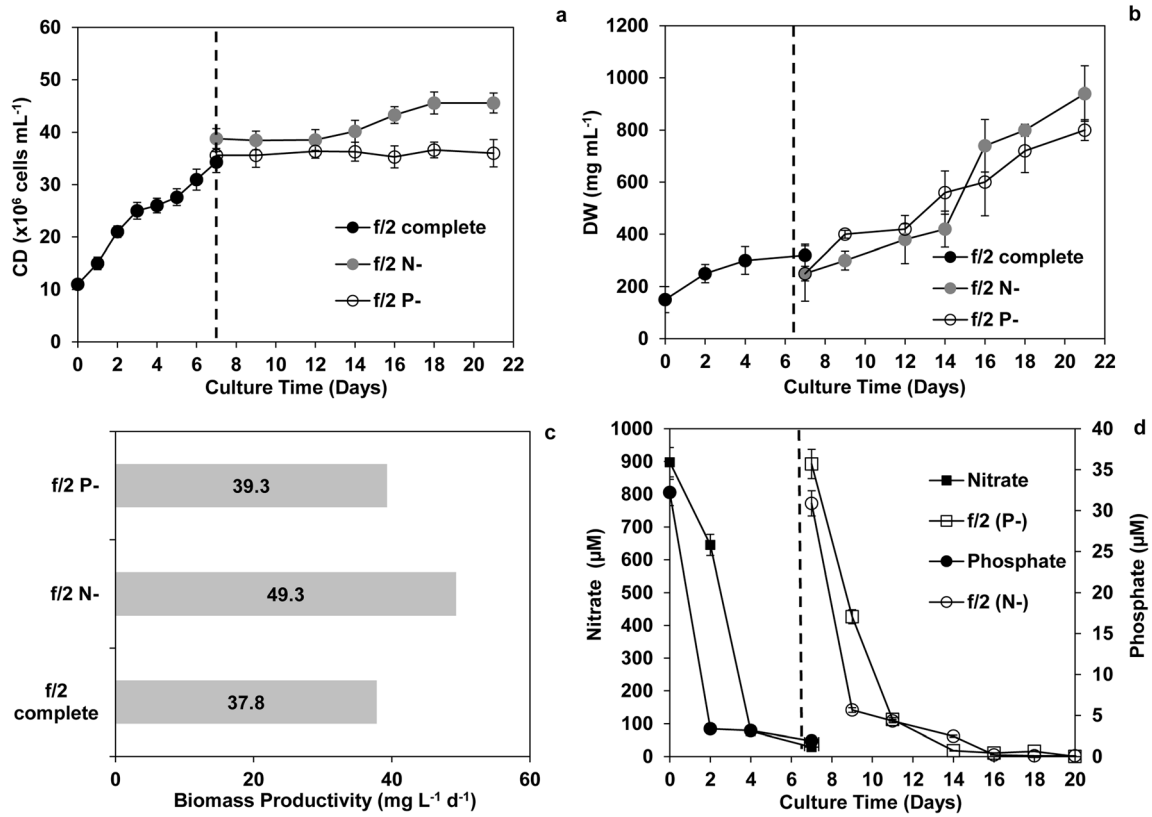

Fig. 1 Growth, biomass production and nutrient kinetics of *N. oceanica* CCALA 978 under complete f/2 and nutrient deprivation conditions. **a** Growth curves; **b** biomass production (DCW: dry cell weight); **c** biomass productivity; and **d** nitrate and phosphate concentrations. In **a**, **b**, **d**, the dashed line indicates the beginning of the nitrate or phosphate deprivation phase. Data are expressed as the average ± standard deviation of three replicates. CD, cell density; DCW, dry cell weight; f/2 (N-), nitrate deprivation; f/2 (P-), phosphate deprivation

f/2 (N-) reached ca. 49 ± 2.1 mg L^{-1} day^{-1} (Fig. 1c) and was significantly higher ($p < 0.05$) than under complete f/2 and f/2 (P-) ($p < 0.05$), without significant differences between complete f/2 and f/2 (-P) ($p > 0.05$). The growth rate of *N. oceanica* CCALA 978 with complete f/2 was within the same range as that for other species growing in complete medium, such as *N. salina* (0.49 day^{-1}, [11]) and *Nannochloropsis* sp. (0.33 day^{-1} [10]), but was lower than for the *N. oceanica* CCALA 804 (0.83 day^{-1}) and *Nannochloropsis* sp. (0.76 day^{-1}) strains studied by Pal et al. [13] and Griffiths et al. [12], respectively. The differences among the values reported could be due to variations in the environmental conditions employed (such as continuous light, photoperiod, light intensities). When the species studied in the present work was deprived of nitrate or phosphate, there was a marked increase in biomass production, particularly under nitrogen deficiency, indicating a metabolite accumulation stage.

Nutrient kinetics under complete f/2 medium and under both nitrate and phosphate deprivation were also analysed (Fig. 1d). Under complete f/2, phosphate presented a consumption rate of 14.5 µM day^{-1} in 2 days and that of nitrate 117 µM day^{-1} in 4 days, leading to a ca. 30-fold and ca. 15-fold decrease in nitrate and phosphate content, respectively. It is noteworthy how the luxury uptake of both macronutrients in the first days of culture step 1 contributed to cell growth and consequently to increased cell density (Fig. 1a). Under nutrient deprivation conditions (culture step 2), the consumption rates of nitrate and phosphate (155.9 µM day^{-1} and 5.3 µM day^{-1}, respectively, over 5 days) were significantly lower than under complete f/2 ($p < 0.05$), resulting in a ca. 8-fold and ca.7-fold decrease in the initial levels, respectively. These trends indicate that the absence of a nutrient (N or P) affects the absorption of available nutrients (N or P), causing a decrease in cell growth.

With respect to the pigment kinetics of *N. oceanica* CCALA 978, chlorophyll-*a* content increased up to ca. 900×10^{-3} µg mL^{-1} on day 4 (culture step 1), while under nutrient deprivation, it dropped to 400×10^{-3} µg mL^{-1} and 200×10^{-3} µg mL^{-1} in f/2 (N-) and f/2 (P-), respectively (Fig. 2a). Carotenoid content increased 5-fold and 1.6-fold under f/2 (N-) and f/2 (P-) conditions, respectively, compared with complete f/2 (Fig. 2b), with an increase in carotenoid content of up to ca. $1100 \times 10^{-3} \pm 15$ µg mL^{-1} under nitrate deprivation (Fig. 2b). The UV–Vis pigment absorption spectra of *N. oceanica* under control and nitrate deprivation conditions are shown in Fig. 2c. Under control conditions, the spectrum exhibited (1) two peaks typical for Chl *a*, one at 430 nm and one at 663 nm; and (2) a peak at 473 nm, which represent

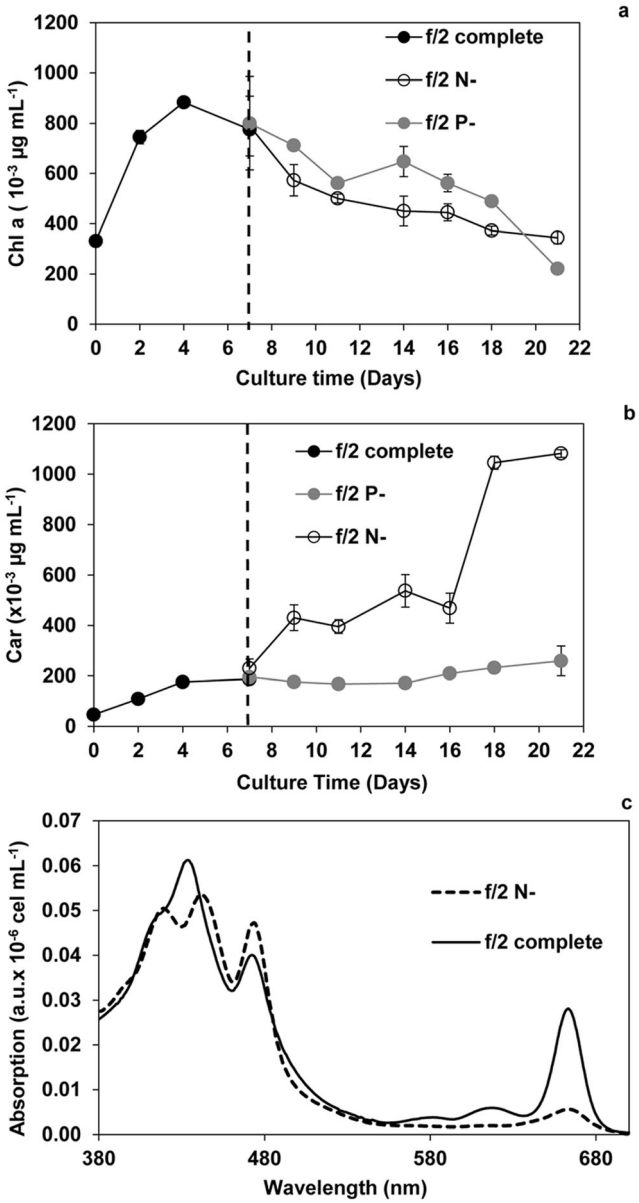

Fig. 2 Pigment kinetics of *N. oceanica* CCALA 978. **a** Chlorophyll-*a* (Chl *a*) concentration kinetics; **b** carotenoid concentration kinetics (Car) and **c** absorption spectrum of pigments under nitrate deprivation and control conditions. In **a**, **b**, the dashed line indicates the beginning of the nitrate or phosphate deprivation phase. Data are expressed as the average ± standard deviation of three replicates. f/2 (N-), nitrate deprivation; f/2 (P-), phosphate deprivation. Spectra were normalized to the cellular density

the sum of contribution from some carotenoids. Under nitrate deprivation, the signals between 400 and 500 nm were more diverse, with a higher contribution in the absorption at 473 nm, while the Chl *a* peak at 663 decreased with respect to control conditions. Thus, the Chl *a*/Car ratios (663/473) were 0.70 and 0.12 under control and nitrate deprivation conditions, respectively, suggesting an increase in carotenoids when N is lacking. Primary photosynthetic carotenoids are associated with thylakoid membranes and are synthesized under favourable growth conditions, whereas secondary carotenoids are not involved in photosynthesis and are produced under stress culture conditions (for example, nitrogen starvation, high light intensities and high salinity) [39]. The lack of N in the studied strain could thus be related to the production of secondary carotenoids. The genus *Nannochloropsis* is a source of valuable carotenoids, such as violaxanthin (Vio), β-carotene, zeaxanthin (Zea), antheraxanthin, vaucheriaxanthin (Vau) and its fatty acid esters (VE) [7, 14]. In particular, increased Zea content in *N. oceanica* IMET1 under nitrogen deprivation and increased VE content in *N. oceanica* CCALA 804 under both nitrogen starvation and high light intensity have been reported by Dong et al. [23] and Solovchenko et al. [14], respectively. In contrast, Vio and β-carotene content decreased under N-free medium [14]. Zeaxanthin has been associated with photoprotection and thermal dissipation of absorbed light in *N. oceanica* IMET1 [23]. Furthermore, among several tested species, *Nannochloropsis granulata* showed the highest antioxidant properties under the stationary phase with limiting nutrients [40]. Even though no pigment composition was reported in the present study, the high carotenoid levels in the studied strain suggest its potential as a source of antioxidant compounds. For *Nannochloropsis* sp., Ma et al. [41] reported increased carotenoid production at low light intensity (50 µmol m^{-2} s^{-1}). In fact, this is an advantage for outdoor mass cultivation where the light intensity can be attenuated inside the pond with increasing cell densities. On the other hand, there was no significant increase in carotenoids under f/2 (P-) condition in the strain studied. It is important to note that the effect of phosphate deficiency on pigment behaviour in *N. oceanica* has not been reported in the literature yet. Therefore, all new information is relevant to establish which nutrient is the most effective for triggering and enhancing carotenogenesis in the studied strain.

Neutral Lipid Accumulation Kinetics

The neutral lipid (NL) kinetics of *N. oceanica* expressed as the relative fluorescence intensity of neutral lipids (RFI-NL) under both complete f/2 and nutrient deprivation are shown in Fig. 3 a and b. The RFI-NL increased 3-fold from day 2 to day 7 (until ca. 800 au) under complete f/2, coinciding with a sharp decrease in nutrient concentrations and an increase in cell density (Fig. 1d). Under nutrient deprivation conditions (Fig. 3b), RFI-NL values increased significantly, indicating an accumulation of neutral lipids. Under f/2 (N-), the RFI-NL values (RFI-NL > 6500 au) showed an 8-fold increase in 7 days (from day 7 to day 14 of culture), whereas the RFI-NL values under f/2 (P-) increased 8-fold in 11 days (from day 7 to day 18). Thus, the main difference between the results of the two nutrient deprivation conditions was in the length of the period of neutral lipid accumulation. The significant increase in biomass observed in *N. oceanica* CCALA 978 under

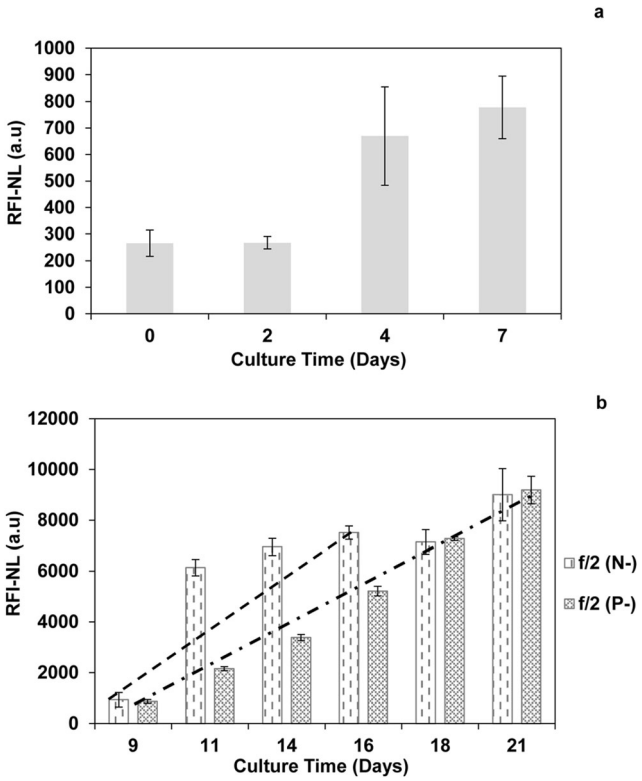

Fig. 3 Neutral lipid accumulation kinetics of *N. oceanica* CCALA 978. **a** Complete medium condition; **b** nutrient deprivation conditions. In **b**, lines indicate the accumulation time of NL under the two nutrient deprivation conditions (dashed line: f/2 (N-); dash-dotted line: f/2 (P-)). Data are expressed as the average ± standard deviation of three replicates

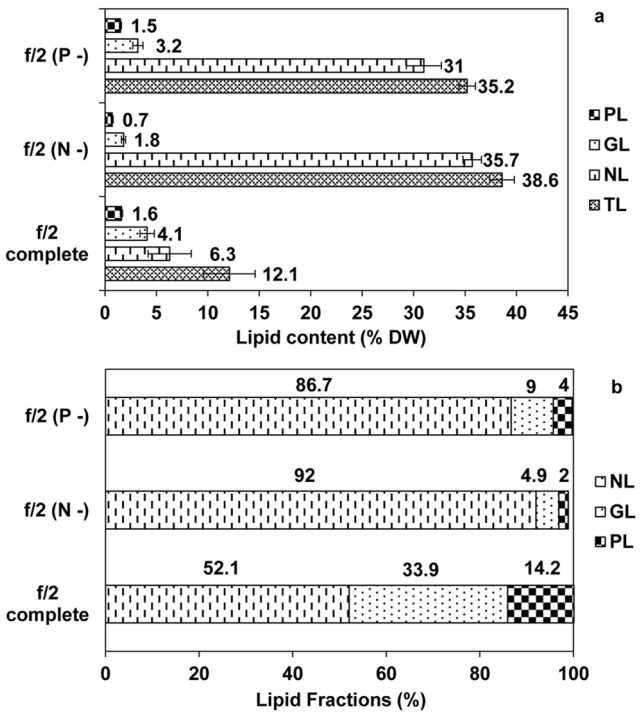

Fig. 4 Lipid content of *N. oceanica* CCALA 978 under control and N- and P- conditions. **a** Total lipid and lipid fraction content (expressed as a percentage of dry weight, % dw); **b** relative proportion of lipid fractions (expressed as % of total lipids). Data are expressed as average ± standard deviation of three replicates. f/2 (N-), nitrogen deprivation; f/2 (P-), phosphorous deprivation; TL, total lipids; NL, neutral lipids; GL, glycolipids; PL, phospholipids

nutrient deprivation (Fig. 1b) can be partially explained by neutral lipid accumulation. Moreover, the RFI-NL kinetics were positively correlated with the biomass kinetics under nutrient deprivation ($r = 0.82$ and $r = 0.96$ for f/2 (N-) and f/2 (P-), respectively, $p < 0.05$). The carotenoid kinetics also showed an important increase when N was lacking. Thus, nitrate deprivation is a good stress strategy for producing neutral lipids for biodiesel and value-added pigments in *N. oceanica* CCALA 978 under a biorefinery approach.

Lipid Content

Figure 4 a shows the content of total lipids (TL) and lipid fractions (neutral lipids (NL), glycolipids (GL) and phospholipids (PL)) of *N. oceanica* CCALA 978 as a percentages of dry weight (% dw). Under complete f/2 medium, the TL were ca. 12% dw, whereas they increased significantly ($p < 0.05$) under nutrient deprivation conditions due to accumulation of neutral lipids. In particular, TL reached their maximum level (ca. 39% dw) under f/2 (N-), the neutral lipid content being ca. 35.7% dw (Fig. 4a). In terms of lipid fractions, TL were composed of ca. 52% NL and ca. 48% polar lipids under complete f/2, whereas under f/2 (N-), NL represented ca. 92% of TL (Fig. 4b). These results are in agreement with those obtained with other *Nannochloropsis* species, where neutral lipids, TAG in particular, increased under nitrate deprivation [16, 42, 43]. This behaviour ensures an adequate stock of lipids suitable for biodiesel production by transesterification [44]. Both glycolipid and phospholipid contents decreased significantly under f/2 (N-), representing the lowest values (1.8% dw (GL) and 0.7% dw (PL)); there were no significant differences ($p > 0.05$) between f/2 (P-) and complete f/2 media (Fig. 4a). According to Zezza et al. [45], the phosphorus found in biodiesel comes from the phospholipids contained in the oil used as feedstock. High phosphorus levels have been shown to damage the catalytic converters used in emission control systems and the low phosphorus content in the oil of the studied strain under nitrate deprivation therefore assures good biodiesel quality. Moreover, a maximum level of 10 mg kg^{-1} of phosphorus in biodiesel samples has been established by the ASTM 6751 standard.

The lipid content of the studied species is similar to those found in other *Nannochloropsis oceanica* strains. For example, Iwai et al. [46] reported similar TAG content in *N. oceanica* (*Nannochloropsis* sp. NIES-2145) under phosphorus and nitrogen deprivation. Dong et al. [23] indicated values of ca. 37% dw of TL on day 16 of culture and a maximum of ca. 59% dw of TL on day 31 under natural nitrogen depletion (20 °C under 14:10 dark:light photoperiod,

100 μmol photons m^{-2} $s^{-1}$and continuous CO_2 bubbling). Based on our results, it is important to highlight that the increase in biomass production observed in *N. oceanica* CCALA 978 under phosphorus and nitrogen deprivation was due in part to neutral lipid accumulation rather than an increase in cell density. In addition, the accumulation of neutral lipids was accompanied by an increase in the carotenoid production when the strain grew under nitrogen deprivation. On the other hand, the lack of P would not exert the same effect on carotenogenesis in the studied strain.

Lipid Classes and Fatty Acid Profiles

Table 1 shows the main fatty acids (saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA)) corresponding to total lipids (TL), neutral lipids (NL), glycolipids (GL) and phospholipids (PL) in *N. oceanica* CCALA 978 under complete f/2 medium and nutrient deprivation conditions. The fatty acid profiles showed strong variations among these conditions. As is to be expected given that LN makes up ca. 52% of TL (the rest being polar lipids), the total lipid profile under complete f/2 reflected an equal contribution of each lipid fraction. In the TL profile, the dominance of SFA followed by MUFA was therefore a reflection of the neutral lipid profile, while PUFA (ca. 26%) were the largest component of polar lipids. The dominance of PUFA in PL was due to the high percentage of eicosapentaenoic acid (C20:5n3) (EPA) (ca. 41% in PUFA-GL and ca. 28% in PUFA-PL). *N. oceanica* also produced arachidonic (ARA), linoleic (LA) and linolenic (ALA) acids, particularly in phospholipids. This composition is common during the active growth phase in *Nannochloropsis* species, as occurred during the culture step 1 with complete f/2, where the cells presented high percentages of PUFA (for example, EPA and DHA).

Under nutrient deprivation, TL profiles mainly revealed the contribution of neutral lipids (NL > 80% of TL, Fig. 4b). Under f/2 (N-), MUFA were the dominant class due to the high percentage of C16:1 (ca. 37%), followed by SFA with a high percentage of C16:0 (ca. 34%). A significant decrease ($p < 0.05$) in EPA was detected in NL under f/2 (N-). Under f/2 (P-), the SFA class was dominant due to the high concentration of C16:0 (ca. 44%), followed by MUFA with a high concentration of C16:1 (ca. 28%). Thus, our results indicate three important features of *N. oceanica* CCALA 978 as follows: (1) PUFA derived mainly from the polar fraction under optimal growth conditions; (2) f/2 (N-) produced a significant increase in MUFA ($p < 0.05$) for both TL (ca. 55%) and NL (ca.54%) fractions due to the dominance of C16:1 and the minimum PUFA value and (3) f/2 (P-) produced a significant increase ($p < 0.05$) in SFA due to the dominance of C16:0. Fatty acid profiles obtained in this work are in agreement with those reported in the literature for other strains of *N. oceanica* growing under different conditions [14, 23, 47, 48]. However, the neutral lipid fatty acid profile of the studied strain under f/2 (N-) presented the best balance among the lipid classes necessary for high-quality biodiesel production (MUFA (54.1%) > SFA (40.24%) > PUFA (5.96%)).

Biodiesel Quality from Fatty Acid Profiles

High-quality biodiesel production requires, among other factors, the use of oils with high neutral lipid content and with a suitable fatty acid profile [49]. Fatty acids with a carbon chain length ranging from C16 to C18, such as palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0) and oleic (C18:1) acids, ensure adequate biodiesel properties as they provide a reasonable balance between cetane number, cold flow, oxidative stability and combustion properties [50]. Since the maximum level of neutral lipids was obtained with f/2 (N-), the biodiesel quality was inferred from the total lipid and neutral fraction profiles under nitrate deprivation (Table 2). The estimated parameters were compared with those cited for several strains of *N. oceanica* by Ma et al. [44] and with the US and European Standards.

The iodine value, cetane number, kinematic viscosity and specific gravity of the biodiesel produced from the studied species meet the standards established by EN 14214 and U.S. (ASTMD 6751-08) and are similar to those estimated for other *N. oceanica* strains [44]. Cloud point and cold filter plugging point were also estimated for *N. oceanica* oils, though there are no specifications relating to these properties owing to seasonal differences between geographical regions. The cloud point of the studied strain was higher (8.6 °C and 9.9 °C in TL and NL, respectively) than those reported by Ma et al. [44] for the *N. oceanica* IMET1 and *N. oceanica* 805 strains; but it was lower than the one indicated in *N. oceanica* CCMP531. Poor cloud points arise in the presence of long-chain saturated fatty acids [36]. According to *N. oceanica* CCALA 978 profiles, the amount of palmitic acid in the neutral lipids was relatively high, which would contribute to a poor cloud point. However, the cold filter plugging point (CFPP), which measures the temperature at which fuel can no longer pass through a filter in a given time [51], was adequate for temperatures below 0 °C.

From an environmental viewpoint, one of the most attractive characteristics of biodiesel is its high biodegradability; however, this characteristic makes it susceptible to oxidative and thermal degradation. Some of the factors affecting oxidative stability during storage and commercialization are PUFA presence [50], light, high temperatures, humidity, enzymes, metallic elements and pigments [52]. With respect to PUFA

Table 1 Fatty acid profiles of *N. oceanica* CCALA 978 under complete medium and nutrient deprivation. N-, nitrogen deprivation; P-, phosphorous deprivation. Data are expressed as the average ± standard deviation of four replicates. Different letters for each fatty acid class among treatments indicate significant differences ($p < 0.05$)

FA	TL			NL			GL			PL		
	f/2 complete	f/2 (N-)	f/2 (P-)	f/2 complete	f/2 (N-)	f/2 (P-)	f/2 complete	f/2 (N-)	f/2 (P-)	f/2 complete	f/2 (N-)	f/2 (P-)
C14:0	6.12 (0.21)	3.86 (0.02)	3.57 (0.09)	5.60 (0.25)	4.86 (0.02)	3.49 (0.02)	10.15 (0.15)	5.60 (0.13)	4.06 (0.04)	3.43 (0.56)	3.43 (0.56)	6.25 (0.03)
C16:0	34.99 (0.44)	32.54 (0.01)	42.63 (0.84)	45.07 (0.12)	33.68 (0.45)	43.96 (0.08)	5.45 (0.06)	41.10 (0.32)	34.97 (0.07)	23.20 (0.14)	35.03 (0.23)	25.59 (0.12)
C18:0	2.24 (0.04)	0.62 (0.02)	3.47 (0.50)	6.09 (0.13)	0.94 (0.03)	2.96 (0.03)	1.02 (0.02)	7.88 (0.82)	2.58 (0.02)	1.38 (0.01)	8.88 (0.36)	1.70 (0.09)
C16:1	22.75 (0.38)	37.07 (0.02)	30.01 (0.58)	25.48 (0.18)	36.945 (0.65)	28.21 (0.08)	17.70 (0.09)	16.11 (0.84)	16.88 (0.03)	17.09 (0.13)	12.62 (0.82)	18.49 (0.09)
C18:1	7.29 (0.01)	18.18 (0.02)	12.45 (0.20)	8.37 (0.10)	17.11 (2.55)	13.64 (0.05)	0.91 (0.04)	12.47 (0.97)	5.96 (0.03)	11.51 (0.30)	19.90 (0.12)	11.05 (0.06)
C18:2n6t	–	–	–	–	–	–	–	–	2.55 (0.02)	–	–	–
C18:2n6c	2.82 (0.04)	1.36 (0.02)	2.34 (0.23)	2.14 (0.08)	1.62 (0.10)	2.12 (0.01)	1.55 (0.002)	2.77 (0.23)	3.35 (0.06)	7.80 (0.11)	17.99 (0.18)	6.24 (0.09)
C18:3n6	0.17 (0.04)	0.17 (0.01)	–	–	0.45 (0.03)	0.12 (0.01)	–	3.92 (0.98)	2.88 (0.02)	–	–	–
C18:3n3	–	–	–	–	–	–	–	–	–	–	–	1.45 (0.01)
C20:3n6	0.20 (0.03)	0.29 (0.02)	0.13 (0.02)	–	0.25 (0.03)	0.10 (0.00)	–	–	1.47 (0.02)	–	–	–
C20:4n6	2.75 (0.13)	0.84 (0.00)	0.69 (0.06)	0.93 (0.02)	1.33 (0.03)	0.75 (0.01)	1.74 (0.02)	2.86 (0.49)	1.40 (0.10)	8.23 (0.12)	–	8.03 (0.03)
C20:5n3	19.74 (0.76)	4.55 (0.00)	3.59 (0.08)	5.33 (0.15)	2.01 (0.53)	3.63 (0.06)	40.66 (0.26)	5.29 (1.00)	15.77 (0.03)	27.95 (0.24)	2.15 (0.09)	20.54 (0.08)
SFA	44.28 (a)	37.53 (b)	50.66 (c)	57.75 (a)	40.24 (b)	51.43 (c)	37.45 (a)	54.58 (b)	44.67 (c)	27.42 (a)	47.34 (b)	34.19 (c)
MUFA	30.04 (a)	55.25 (b)	42.46 (c)	33.85 (a)	54.1 (b)	41.85 (c)	18.61 (a)	28.58 (b)	27.90 (c)	28.60 (a)	32.52 (b)	29.54 (c)
PUFA	25.68 (a)	7.21 (b)	6.91 (c)	8.40 (a)	5.96 (b)	6.72 (c)	43.94 (a)	14.84 (b)	27.43 (c)	43.98 (a)	20.14 (b)	36.27 (c)

TL, total lipids; *NL*, neutral lipids; *GL*, glycolipids; *PL*, phospholipids; *SFA*, saturated fatty acids; *MUFA*, monounsaturated fatty acids; *PUFA*, polyunsaturated fatty acids

Table 2 Biodiesel properties of *N. oceanica* CCALA 978 calculated from fatty acid profiles of TL and NL under nitrogen deprivation. **a**, biodiesel properties of *N. oceanica* IMET1 [44]; **b**, biodiesel properties of *N. oceanica* 805 [44]; **c**, biodiesel properties of *N. oceanica* CMP531 [44]

	0054L (f/2 N-)	NL (f/2–N)	Biodiesel[a]	Biodiesel[b]	Biodiesel[c]	US (ASTMD 6751-08)	Europe (EN14214)
AUD	0.85	0.75	1.24	1.25	0.72	–	–
Iodine value (g I_2/100 FAME)	75.93	68.5	104.85	105.67	66.58	–	Max 120
Cetane number	57.20	57.9	54.61	54.54	58.05	Min 47	Min 51
Cloud point (°C)	8.64	9.9	3.45	3.30	10.32	Report	Country-specific
Kinematic viscosity (40 °C $mm^2 s^{-1}$)	4.66	4.73	4.42	4.42	4.75	1.9–6.0	3.5–5.0
Specific gravity (kg L^{-1})	0.88	0.88	0.879	0.879	0.877	0.85–0.90	–
HHV (Mj kg^{-1})	40.03	39.8	–	–	–	–	–
CFPP	− 5.30	− 4.41	–	–	–	–	Depends on the location and time of the year
Linolenic acid (%)	0.17	0.45	1.67	0.91	0.52	–	Max 12
≥ 4 db (%)	5.39	3.34	14.15	14.46	5.42	–	Max 1

content, the European EN Standard 14214 requires a maximum of 12% linolenic acid methyl ester and a maximum of 1% fatty acid methyl ester with four or more double bonds (CEN 2003) [53]. These specifications are necessary to prevent the oxidation of the double bonds and thus ensure an adequate oxidative stability of the biodiesel. The linolenic acid content of *N. oceanica* CCALA 978 was very low (0.12–0.45%); even though PUFA with more than 4 double bonds exceeded the standard, the EPA content in the studied species under f/2(N-) was the lowest in comparison with other values in *N. oceanica* strains [23, 47]. Synthetic antioxidants have been used to prevent oxidation of biodiesel and other fuels (e.g., butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate and tertiary butylhydroquinone) [54]. Unfortunately, most of these antioxidants exhibit poor biodegradability and they are usually expensive. On the other hand, tocopherols, ascorbic acid, flavonoids and carotenoids are readily biodegradable, non-toxic natural antioxidants, able to improve the oxidative stability of fuels [52]. Thus, the high carotenoid levels in the studied strain suggest its potential as a source of antioxidant compounds. Moreover, lipids and carotenoids may simultaneously be extracted by using supercritical carbon dioxide as has been reported for other microalgae [55]. This extraction technique is particularly suitable when thermo-labile compounds are present since it is easy to separate them from the extract and to avoid the use of toxic solvents [3, 56].

Conclusions

Nannochloropsis oceanica CCALA 978 cultures under nutritional stress conditions (N-free or P-free media) showed a significant increase of neutral lipid content. However, the neutral lipid accumulation period was shorter under nitrate deprivation. Moreover, the carotenoid kinetics also showed an important increase when N was lacking. Therefore, a biorefinery approach encourages testing the production of neutral lipids with natural antioxidants from cultures of *N. oceanica* CCALA 978 in order to obtain biodiesel and blends with reduced oxidative vulnerability. In addition, under these conditions, the oils produced would ensure biodiesel with low phosphorus content, as well as CN, KV and SG meeting the quality conditions established by EN 14214 and US 404 (ASTMD 6751-08) standards.

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