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ORIGINAL PAPER

Neochloris oleabundans UTEX #1185: a suitable renewable lipid source for biofuel production

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Abstract Energy crises, global warming, and climatic changes call for technological and commercial advances in manufacturing high-quality transportation fuels from unconventional feedstocks. Microalgae is one of the most promising sources of biofuels due to the high yields attained per unit area and because it does not displace food crops. *Neochloris oleabundans* (Neo) microalga is an important promising microbial source of single-cell oil (SCO). Different experimental growth and lipid production conditions were evaluated and compared by using optical density (540 nm), dry-weight determination, and flow cytometry (FC). Best Neo average biomass productivity was obtained at 30°C under conditions of nitrogen-sufficiency and CO_2 supplementation (N+/30°C/CO_2), with an average doubling time of 1.4 days. The second and third highest productivities occurred with N-sufficient cultures without CO_2 supplementation at 26°C (N+/26°C) and at 30°C (N+/30°C), with doubling times of 1.7 and 2.2 days, respectively. Microbial lipid production was monitored by flow cytometry using Nile red (NR), a lipophilic fluorochrome that possesses several advantageous characteristics for in situ screening near real time (at line). Results showed maximum lipid content (56%) after 6 days of nitrogen depletion under nitrogen starvation without CO_2 supplementation (N−/30°C), followed by N−/30°C/CO_2 and N−/26°C conditions with 52% lipid content, after 5 and 6 days of N starvation, respectively. The adequate fatty acid profile and iodine value of Neo lipids reinforced this microalga as a good source of SCO, in particular for use as biodiesel.

Keywords *Neochloris oleabundans* · Lipid · Oil · Fatty acid · Biodiesel · Flow cytometry · Nitrogen starvation

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Introduction

Renewable energy is needed to displace petroleum-derived transport fuels, which contribute to global warming and are of limited availability.

Biodiesel from microalgae seems to be the only renewable biofuel that has the potential to completely displace petroleum-derived transport fuels without adversely affecting the supply of food and other crop products [5, 6].

Microalgae are the most primitive form of plants, and the high yields attained per unit area make microalgae one of the most promising sources of biofuels. While the mechanism of photosynthesis in microalgae is similar to that of higher plants, microalgae are generally more efficient converters of solar energy because of their simple cellular structure. In addition, because cells grow in aqueous suspension that can also be recycled, they have more efficient access to water, CO_2, and other nutrients. For these reasons, microalgae are capable of producing 30 times the amount of oil per unit area of land than terrestrial oilseed crops [18]. In addition, microalgae cultivation does not displace any food crop, does not compete for fertile soil, requires less water than oilseed crops, can be undertaken on nonarable

and degraded land using nonpotable water, and can be harvested daily.

A microalgae screening was previously done by the authors in order to test their growth characteristics, oil content, fatty acid profile, and acid value, which are important parameters for biodiesel quality. *Neochloris oleabundans* was revealed to have the best performance potential [15].

However, lipid production by microalgae is regulated by environmental factors, and to optimize them, a control is needed [4, 9], which can be done by using flow cytometry (FC). The advantages of this technique for culture-lipid evaluation over the more conventional gravimetric techniques are presented. Using lipid-specific fluorochromes, it is possible to achieve above average lipid content for any individual microbial cell.

Traditional analysis of lipid content in biological samples has been performed by solvent extraction and gravimetric determination [2], and further analysis and characterization is carried out by GC or HPLC [9]. These steps are time consuming and generate high amounts of waste (organic solvents), which is nocive to the environment. In addition, adequate amounts of biomass must be cultured for the extraction and derivatization. The amount of sample needed and preparation time, however, can be greatly reduced if the lipid content of algal cells is measured in situ. Lipid measurement has been previously proposed using Nile red (NR), a lipid-soluble fluorescence probe that possesses several characteristics advantageous to in situ screening. Its fluorescence is produced in highly hydrophobic environments and is quenched in hydrophilic ones. Flow cytometry simultaneously measures autofluorescence and the fluorescence of individual cells stained with specific dyes. De la Jara et al. [8] assessed the lipid composition of *Crypthecodinium cohnii* cells using flow cytometry coupled with NR. In this way, it is possible to monitor total cellular lipid content, in situ and in real time, and with a high degree of statistical resolution (>50,000 cells in minutes) during microalgal growth.

In this work, *Neochloris oleabundans* was evaluated as a potential source for biofuel production. The optimization of the growth environment, especially in terms of N and C sources (nitrate and CO_2) as well as temperature, was undertaken, and the lipid content was determined in near-real time (at line) by flow cytometry. Fatty-acid profile and iodine values were determined as important parameters to evaluate biodiesel final quality. To the best of our knowledge, after an extensive survey, the use of flow cytometry to monitor the effects of different nutrient limitations (carbon, nitrogen, or a combination of both) as well as temperature changes on lipid content of microalgae cultures is reported for the very first time.

Materials and methods

Microalga

The microalga *Neochloris oleabundans* (Neo) UTEX #1185 was obtained from UTEX culture collection (University of Texas, Austin, TX, USA). It is a freshwater microalga that belongs to the *Chlorophyceae* class and *Chlorococcaceae* family [3].

The microalga was grown in 1-L glass bubble column bioreactors with continuous stirring by bubbling filtered air (volumetric flux: 5.4 L/h) in Bristol medium [16]. The tested culture parameters were temperature (26 and 30°C), CO_2 [without or with supplementation: 0.04 and 5% (v/v), respectively], and nitrate (with or without), and all combinations of these variables. The cultures were continuously illuminated with six fluorescent lamps (Philips TL-DM 36W/54-765) at an irradiance level of 150 μE m^{-2} s^{-1} for 18 days.

Growth evaluation

Growth was evaluated over time in terms of optical density (OD) (540 nm) (Hitachi U-2000, spectrophotometer), DW (dry weight) (Whatman GF/C 1.2 μm), and N (nitrate) medium concentration. All samplings were performed at least in duplicate.

Doubling time (days) under different experimental conditions was calculated using the formula $t_d = \ln 2/\mu_{max}$, where μ_{max} is the maximum specific growth rate, calculated as the maximum slope from the plot of ln OD or ln DW versus culture time.

Lipid content

Lipid content was evaluated by multiparameter flow cytometry and by traditional analysis [2]. Samplings and lipid evaluations were performed in duplicate.

Microalgal cells were stained with NR (Sigma) according to de la Jara et al. [8] with modifications. Flow cytometry was optimized to the microalga *Neochloris oleabundans.* Microalgal cells were stained with 5 mM NR [9]. Thirty microliters of a working solution of NR and acetone (0.03 mg mL^{-1}) was added to 1 mL of a cell suspension containing 8.3×10^{-6} cells corresponding to 600–800 events/s. This mix was gently vortexed and incubated for 2 min at 37°C in darkness. NR florescence was determined using a FACScan flow cytometer (Becton-Dickinson Instruments) equipped with a 488-nm argon laser. Upon excitation by the 488-nm argon laser, NR exhibits yellow and red fluorescence when dissolved in lipids, which are detected in the FL2 and FL3 channels. Nonstained cells were used as an autofluorescence control. The total fluorescence

was determined as the sum of the normalized fluorescence intensity of the NR-stained cells detected in both FL2 and FL3, relative to the autofluorescence.

Lipid content versus fluorescence intensity was determined as a correlation between the NR fluorescence intensity and lipid content assayed by the Bligh and Dyer extraction method [2], by analyzing at least four samples in duplicate, with different lipid contents.

Lipid characterization

Fatty-acid composition

In order to determine the Neo fatty-acid composition in biomass, freeze-dried samples (~150 mg) were chemically derivatized using the borum trifluoride method described in the EN ISO 5509 [11]. The organic phase obtained was analyzed by gas chromatography according to EN 14103 [12]. Derivatization was carried out in duplicate, and each duplicate was injected twice in order to achieve statistical significance ($n = 4$).

Iodine value

The oil from Neo was characterized in terms of iodine value according to the European Standard EN 14111 [10].

Nitrate (nitrogen source) analysis

Nitrate concentration in the culture medium was evaluated spectrophotometrically at 220 nm after subtracting the absorbance reading at 275 nm to remove any trace of dissolved organic matter present in the culture medium according to Standard methods [1]. Samples were filtered first to remove possible interference from suspended particles, i.e., cells. Potassium nitrate at different concentrations was used for calibration purposes.

Results and discussion

Microalgal biomass growth data of *Neochloris oleabundans* under the six experimental conditions are depicted in Fig. 1. Culture curves showed the fastest growth for N+/30°C/CO_2 (nitrate-sufficient medium with CO_2 enrichment at 30°C). Comparing this culture with N−/30°C/CO_2 culture (nitrogen-free medium, 30°C, with CO_2 supplementation), it can be observed that the former attained a 10-fold higher growth after 6 days.

The CO_2 enrichment in the inlet air flow enhanced algal growth as expected, which can be seen by comparing the culture curves for N+/30°C/CO_2 with N+/30°C and N−/30°C/CO_2 with N−/30°C, showing that cultures under air bubbling were C-limited.

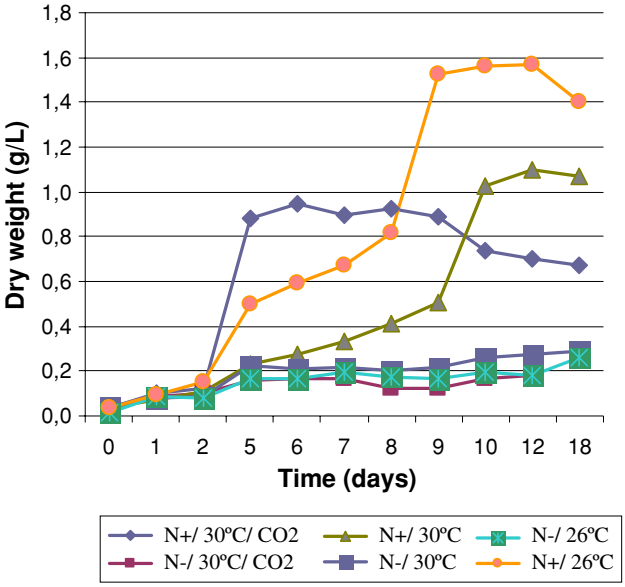

Fig. 1 Average dry weights of *Neochloris oleabundans* under different growth conditions over time. Growth conditions: N+/30°C/CO_2: nitrate-sufficient medium, 30°C, with CO_2 enrichment; N−/30°C/CO_2: nitrate-limited medium, 30°C, with CO_2 enrichment; N+/30°C: nitrate-sufficient medium, 30°C, without CO_2 enrichment; N−/30°C: nitrate-limited medium, 30°C, without CO_2 enrichment; N+/26°C: nitrate-sufficient medium, 26°C, without CO_2 enrichment; N−/26°C: nitrate-limited medium, 26°C, without CO_2 enrichment. Standard deviations (not shown) were below 10% for all situations

In terms of temperature influence on Neo biomass productivity, Fig. 1 curves showed that Neo grew faster at 26°C than 30°C, irrespective of supplemented CO_2.

Specific growth rates are represented in Fig. 2 and Table 1. Higher (steeper) slopes mean higher specific growth rates, which were observed in the cultures with nitrate supply. In Fig. 2 as expected, the highest specific growth rate for N+/30°C/CO_2 culture can be observed, followed by N+/26°C and N+/30°C. Culture under nitrogen-starvation revealed very poor growth, as expected for a N-limited environment, highlighting the importance of N as a building block for protein synthesis, and thus, biomass production. On the other hand, cultures exposed to higher C concentrations (CO_2 supplementation) achieved the fastest growth rates and higher biomass concentrations. Again, C is the most important building block for biomass production, irrespective of the cellular constituents where incorporated (protein, carbohydrate, lipid, or others).

Table 1 shows the results of the doubling time exhibited for each culture according to different experimental conditions. The shortest average doubling time was 1.4 days for the N+/30°C/CO_2 culture, followed by the N+/26°C and N+/30°C cultures (1.7 and 2.2 days, respectively). Cultures

Table 1 Average biomass growth data and biomass and lipid productivities for *Neochloris oleabundans* cultures under different growth conditions

Growth conditions	Average doubling time (days)	P_X (g L^{-1} day^{-1})	P_{LIP} (mg L^{-1} day^{-1})
N+/30°C/CO_2	1.4	0.15	37.66
N−/30°C/CO_2	1.7	0.03	14.42
N+/30°C	2.2	0.09	14.31
N−/30°C	3.9	0.04	13.22
N+/26°C	3.9	0.13	38.78
N−/26°C	5.0	0.03	10.67

Experiments were conducted at least in duplicate. Standard deviations (not shown) were below 10% for all situations

P_X Average biomass volumetric productivity (on a DW basis) calculated for the time interval corresponding to the maximum biomass concentration, P_{LIP} average lipid volumetric productivity calculated for the time interval corresponding to the maximum lipid content. Growth conditions: N+/30°C/CO_2: nitrate-sufficient medium, 30°C, with CO_2 enrichment; N−/30°C/CO_2: nitrate-limited medium, 30°C, with CO_2 enrichment; N+/30°C: nitrate-sufficient medium, 30°C, without CO_2 enrichment; N−/30°C: nitrate-limited medium, 30°C, without CO_2 enrichment; N+/26°C: nitrate-sufficient medium, 26°C, without CO_2 enrichment; N−/26°C: nitrate-limited medium, 26°C, without CO_2 enrichment

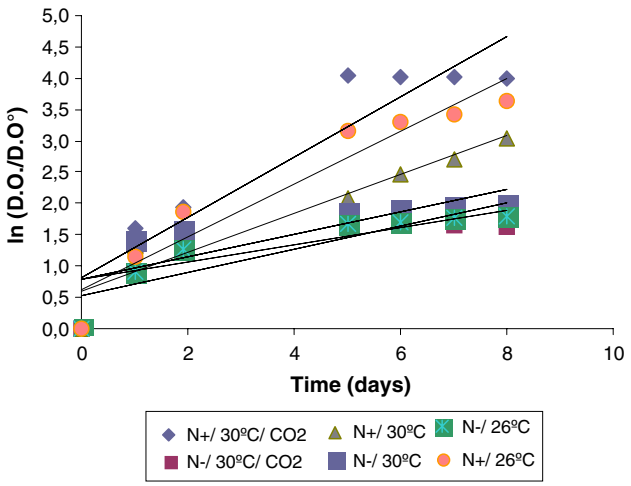

Fig. 2 *Neochloris oleabundans* microalgal biomass growth rates under different growth conditions (determined by normalized optical density at 540 nm). DO^0 Optical density at time zero. Growth conditions: N+/30°C/CO_2: nitrate-sufficient medium, 30°C, with CO_2 enrichment; N−/30°C/CO_2: nitrate-limited medium, 30°C, with CO_2 enrichment; N+/30°C: nitrate-sufficient medium, 30°C, without CO_2 enrichment; N−/30°C: nitrate-limited medium, 30°C, without CO_2 enrichment; N+/26°C: nitrate-sufficient medium, 26°C, without CO_2 enrichment; N−/26°C: nitrate-limited medium, 26°C, without CO_2 enrichment. Standard deviations (not shown) were below 10% for all situations

under nitrogen starvation showed the longest average doubling time (3.9 and 5 days) corresponding to the slowest biomass productivity.

Nitrogen consumption in the culture medium is presented in Fig. 3, and the faster consumption of nitrogen for

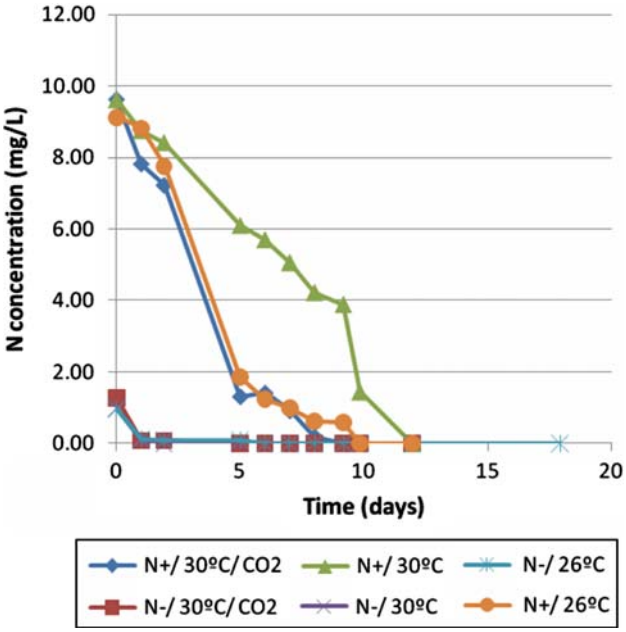

Fig. 3 Average nitrogen concentration in culture medium for the different growth conditions. N+/30°C/CO_2: nitrate-sufficient medium, 30°C, with CO_2 enrichment; N−/30°C/CO_2: nitrate-limited medium, 30°C, with CO_2 enrichment; N+/30°C: nitrate-sufficient medium, 30°C, without CO_2 enrichment; N−/30°C: nitrate-limited medium, 30°C, without CO_2 enrichment; N+/26°C: nitrate-sufficient medium, 26°C, without CO_2 enrichment; N−/26°C: nitrate-limited medium, 26°C, without CO_2 enrichment. Values are shown for duplicates. Standard deviations (not shown) were below 10% for all situations

the N+/30°C/CO_2 culture is to be noted, similar to N+/26°C with an abrupt decrease in N concentration, followed by N+/30°C, showing the close correlation between biomass concentration and N consumption. This decrease in N concentration accompanied the exponential growth of the culture during the first 5 days (Figs. 1, 3). The initial N concentration for N-limited cultures was marginal (~1 mg/L) reflecting the residual N content of the inoculum medium; this became completely exhausted during the first day of the experiment. The assumptions of complete N starvation during the experiment for N-limited samples were correct.

There was a strong linear correlation between the normalized fluorescence intensity of the total NR-stained cells detected in both FL2 and FL3 and the microalgal lipid content determined gravimetrically by the Bligh and Dyer method ($r^2 = 0.97$). This correlation was used to determine the lipid content of the microalgal cells collected during the growth experiments, which were stained with NR and analyzed by flow cytometry. In this way, the cellular lipid content was monitored during the microalgal growth in real time by using the stained fluorescence intensity of the NR cells, which was a quicker and easer method of lipid detection compared to traditional gravimetric methods. In addition, flow cytometry coupled with NR avoids the sample

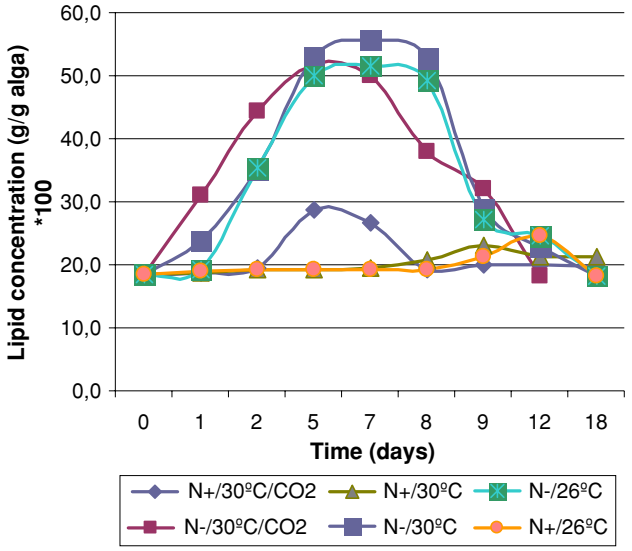

Fig. 4 *Neochloris oleabundans* lipid content (average values) detected by flow cytometry coupled with NR for different experimental conditions. N+/30°C/CO_2: nitrate-sufficient medium, 30°C, with CO_2 enrichment; N−/30°C/CO_2: nitrate-limited medium, 30°C, with CO_2 enrichment; N+/30°C: nitrate-sufficient medium, 30°C, without CO_2 enrichment; N−/30°C: nitrate-limited medium, 30°C, without CO_2 enrichment; N+/26°C: nitrate-sufficient medium, 26°C, without CO_2 enrichment; N−/26°C: nitrate-limited medium, 26°C, without CO_2 enrichment. Standard deviations (not shown) were below 10% for all situations

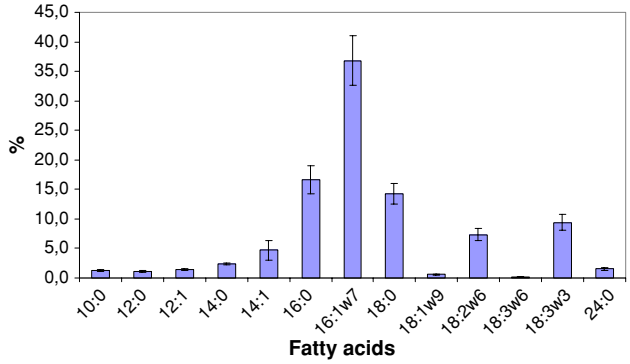

Fig. 5 *Neochloris oleabundans* fatty-acid profile. Average values are calculated based on duplicate samples injected twice ($n = 4$). *Error bars* indicate standard deviation

freeze-drying step as well as the use of large amounts of organic solvents used in the traditional lipid detection methods, saving time and energy. This method allows monitoring of lipid production during microalgal growth, in situ and in real time, therefore allowing changes to be made in the growth conditions to maximize lipid production. It can also be used to screen microalgal strains for lipid production.

Figure 4 shows the higher lipid content for the cultures under nitrogen starvation. N−/30°C culture reached the maximum lipid content (56%) after 6 days of nitrogen shortage, comparable with results from other authors [21], followed by the N−/30°C/CO_2 and N−/26°C cultures with 52% lipid content, after 5 and 6 days, respectively.

Nitrogen-limited growth can stimulate the cells to produce more lipids per cell since protein biosynthesis is limited [19]. Lipid contents for the nitrogen-starved cultures were around 50% higher than the corresponding nitrogen-sufficient cultures (23, 29, and 25%).

The best growth conditions for high biomass productivity were markedly different from those leading to maximum lipid productivity, which is expected for a secondary metabolite (not associated with growth). The authors suggest a two-stage process for lipid production. Neo should be grown in nitrogen-sufficient Bristol medium in order to attain maximum biomass productivity as quickly as possible (growth stage). Then the biomass should be separated and recovered from the remaining culture medium by centrifugation for further final reinoculation in the same Bristol medium, without nitrate, in order to optimize the lipid synthesis (lipid production stage).

The Neo cellular fatty-acid profile is present in Fig. 5, showing 18:1 ω9 (oleic) as the main fatty acid present, followed by 16:0 (palmitic) and 18:0 (stearic). Linolenic acid (18:3 ω3) proportion was below 12%, which meets the requirements of the European Standard EN 14214 for biodiesel production [13].

Iodine value of the extracted oil from Neo biomass was 72, which is an interesting result because it could meet the biodiesel quality specifications, which require a value below 120 [13].

Conclusions

Algae are considered a promising potential feedstock for next-generation biofuels because some species contain high amounts of oil that can be extracted, processed, and refined into transportation fuels using currently available technology. Other benefits are the fast growth rates, cultivation on nonarable and degraded land avoiding competition for fertile soil, nondisplacement of any food crop, less water demand than oilseed crops, use of nonpotable water, and daily harvesting.

Neochloris oleabundans has the best growth in nitrogen-sufficient culture medium and CO_2 supplementation, at 30°C, with a doubling time of 1.4 days, yielding an average biomass volumetric productivity of 0.15 g L^{-1} day^{-1}. Even under these experimental conditions, an average lipid volumetric productivity of 37.66 mg L^{-1} day^{-1} was obtained. This was near the maximum lipid productivity obtained in this experiment (38.78 mg L^{-1} day^{-1}), corresponding to nitrogen-sufficient culture medium at 26°C without CO_2

supplementation (Table 1). These values fall in the same order of magnitude as data published by Rodolfi et al. [17] in which 30 selected microalgae strains yielded lipid productivities in the range 17.4–61.0 mg L^{-1} day^{-1}.

Flow cytometry coupled with NR can be an important tool to evaluate, monitor, and optimize microalgae growth and lipid production conditions, making optimization studies faster and more reliable. The sample amount and preparation time can be greatly reduced and shortened when the lipid content of the algal cells is measured in situ, near real time (at line). Such a technique can be particularly useful when used in scaled-up microalgal lipid production processes, allowing changes to process-control strategy that lead to maximum lipid productivity as previously described by da Silva et al. [7].

Neochloris oleabundans microalgal biomass lipid content can reach 56% (DW) after 5 days of N depletion under N starvation at 30°C, with an adequate fatty-acid profile and iodine value for biodiesel production specifications, allowing this microalga to serve as an important raw material for biofuel production, particularly for biodiesel.

The extraction, separation, and concentration of value-added chemicals other than Neo oil, such as carotenoids, is now in progress to fully exploit the biorefinery concept [20].

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