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Oil assessment of *Halamphora coffeaeformis* diatom growing in a hybrid two-stage system for biodiesel production

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ABSTRACT

The lipid content, composition and productivity of the marine benthic diatom *Halamphora coffeaeformis* were studied in order to evaluate its potential as feedstock for biodiesel production. Cultures were carried out in two stages: I) in photobioreactors (PBRs) to increase the biomass as inoculum for larger volumes, and II) in raceway ponds to increase naturally the triacylglycerol (TAG) content during the stationary growth phase. Biomass concentrations of 0.64 g L^{-1} and 0.23 g L^{-1} were reached in the PBR and the raceway pond, respectively. Total lipid content was 54.4 (±11.6) % ash free dry weight (AFDW) in the raceway pond on day 19 (harvest day), with a neutral lipid content of 34% AFDW. The TAG productivity in the raceway pond was 1.2 mg L^{-1} d^{-1}. The indicators of biodiesel, calculated from fatty acid profile composition, showed that *H. coffeaeformis* oil was of good quality, according to international standards. Some hypothetical aspects are proposed in order to improve lipid productivity and net energy ratio in processes at larger scales.

1. Introduction

Fossil reserve depletion and increasing damage to the environment make microalgae potentially interesting because they are capable of producing biomass as a promising clean energetic resource [4,22]. The Aquatic Species Program, carried out by the US Energy Department, has recommended diverse species of diatoms as potential feedstock sources for biodiesel production [38]. They were chosen on account of their high growth rates, their high lipid content and their tolerance to hostile environments. In spite of these recommendations, up to now, only a few species have been studied with bioenergetic aims [12,23].

Some species of diatoms can accumulate high levels of triacylglycerol (TAG) as a carbon storage metabolite that can be easily transesterified to biodiesel. In addition, diatoms mainly store saturated fatty acids (SFA) and monoinsaturated fatty acids (MUFA), which yield higher energy on oxidation when compared with polyunsaturated fatty acids (PUFA) with the same number of carbons. In particular, palmitoleic acid (C16:1) is the predominant fatty acid in marine diatoms [23]. Considering that biodiesel quality is directly influenced by the properties of the fatty acid profiles it is made from, the use of feedstock with high MUFA levels, such as palmitoleic acid, would provide good quality biodiesel [20,23]. On the other hand, the employment of benthic diatoms could reduce harvesting costs due to their capacity to form biofilms as both the centrifugation of large culture volumes and the addition of flocculants are avoided. This feature is advantageous since the harvesting and dewatering processes are a significant bottleneck that increases biodiesel production costs between 20% and 30% [4]. In addition, the diatoms have a characteristic siliceous cell wall, composed of two valves that together form the frustule. Moreover, because of their siliceous composition ($SiO_2 \cdot nH_2O$), the frustules are very well preserved in fossil deposits (e.g diatomite), and they have numerous industrial uses (e.g. filtration, insulation, fine abrasion, absorption, pesticide, food additive) [21,11]. In particular, the cell wall structure, which is molded into species-specific

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nanometer scale structures, is especially interesting. Therefore, its applications have been proposed in materials science and nanotechnology [27,6,18,48]. All these characteristics indicate the potential that the frustules might have as valuable co-products in biodiesel production processes from diatom oil, contributing to the sustainability of the bioenergetic cultures. In addition, diatoms have been cultivated in large-scale outdoor systems for aquaculture for decades in order to produce value-added products (e.g. eicosapentaenoic and docosahexaenoic acids and isoprenoids) and they are amenable to omic and genetic manipulation approaches [12].

The Energy Return On Investment (EROI) or the Net Energy Ratio (NER) measure the net gain of a system as a function of energy (in terms of biomass produced or energy generated) and they are used to evaluate the feasibility of microalgal culture at large scales [19,23,3]. In general, the NERs for microalgal biomass production in open raceway ponds are estimated to be > 1 indicating that these culture systems are economically feasible [16]. However, they exhibit various advantages and limitations. One of the major limitations is low productivity as compared with photobioreactors. Besides, the environmental factors cannot be fully controlled. Therefore, the raceway ponds are typically used at the commercial scale for the cultivation of robust microalgae and cyanobacteria, e.g. species that can tolerate high salinity values [35]. Photobioreactors (PBRs) are high-cost systems that exhibit higher final biomass concentration and higher biomass productivity than in ponds [19]. They are generally used for culturing microalgae for high-value products, such as pharmaceuticals that cannot be grown as monocultures in open systems [35]. So, hybrid two-stage production systems of microalgae for biofuels have been proposed [3,17]. This method combines distinct growth stages in PBRs and in open ponds. The first stage is in a PBR where controllable conditions minimise contamination from other organisms and favour continuous cell division to maintain a continuous supply of consistent inoculum. The second production stage in a raceway pond aims at exposing the cells to nutrient stress, which enhances lipid synthesis [4].

Although there is no common pattern for all diatoms species, TAG production generally increases when diatom cultures are subjected to starvation of nitrogen and silicate [5], phosphate and nitrate [45] or naturally by nutrient depletion associate with culture ageing in the stationary growth phase [26,1,15]. The latter strategy might simplify culture processes, thus avoiding the operating costs of transferring the culture from a medium with nutrients to one without them. This natural stress condition has been studied at laboratory scale [26,1,15,32]. Nevertheless, the efficiency of this kind of stress at greater volumes has not been studied extensively.

In view of the above mentioned features, the objective of this research was to evaluate lipid quantity, quality and the productivity of the marine benthic diatom *Halamphora coffeaeformis* through its natural ageing. The purpose was to analyze the feasibility of employing this species for biodiesel production at larger scales. The approach consisted of the following steps: 1) to obtain a functional inoculum with a high biomass by means of a cylindrical PBR; 2) to get TAG-rich biomass, which is apt for biodiesel production, using an indoor raceway pond. In order to carry out these steps, culture experiments at laboratory scale were performed, in the first place, using two culture media so as to choose the most appropriate one for each stage.

2. Methods

2.1. Algal strain and culture conditions

H. coffeaeformis (C.Agardh) Levkov was isolated from the inner zone of Bahía Blanca Estuary (38° 45′ S, 62° 22′ W), at the mouth of an untreated sewage outlet. Unialgal non-axenic cultures were established in f/2 medium [28], prepared with sterile seawater with a salinity of 30. This strain is maintained in culture at the Laboratorio de Estudios Básicos y Biotecnológicos en Algas (LEBBA), CERZOS–CONICET, Bahía Blanca, Argentina. Stock cultures were kept at 15 ± 1 °C and under 60 μmol photons m^{-2} s^{-1} of light intensity provided by cool white Phillips L-35 fluorescent lamps under a cycle of 12:12 h light/darkness. A radiometer LI-COR model LI-192SB was used to measure the photosynthetically active radiation (PAR).

2.2. Growth rate and biovolume determinations

Experiments were carried out in batch cultures in order to define the appropriate medium to employ in PBR and raceway systems. Continuous air bubbling (500–700 cm^3 min^{-1}) containing 0.30 cm^3 min^{-1} of CO_2 was used. Erlenmeyer flasks of 250 mL capacity were filled with 100 mL of either SWES (seawater + soil extract + salts) or f/2 media and inoculated with 2700 cells mL^{-1}. Three replicates of the cultures were done. Two culture media with a salinity of 30 were prepared from aged filtered (0.45 μm Millipore) seawater from Bahía Blanca Estuary. The SWES medium was made according to [33] and the f/2 medium was prepared according to [28]. The SWES medium has nitrate, phosphate and silicate concentrations that represent 2-fold, 5-fold and 14-fold their concentrations in the f/2 medium, respectively. The cell concentration was determined by counting three replicate samples by means of a Sedgwick-Rafter chamber. Growth rate (k) was estimated during the period of exponential growth by a least squares fit to a straight line of the logarithmically transformed data [10]. Experiments lasted 11 days in order to determine the beginning and duration of both logarithmic and stationary phases.

Two mL samples were taken daily to measure the size of cells growing in the SWES and f/2 media. The length and width of 20 individual cells were recorded. The cell volume of each individual cell was calculated using the formula for cymbelloid form [13].

2.3. Characterization of neutral lipids with Nile Red

Duplicated samples were collected and analysed every two or three days for cellular neutral lipid presence and neutral lipid kinetics via Nile Red (NR) fluorescence. Five microliters of Nile Red (9-diethylamino-5H-benzo[a] phenoxazine-5-one, Sigma) in acetone (1 mg mL^{-1}) were added to a 5 mL cell suspension [34]. The mixture was agitated vigorously in a vortex mixer. Intracellular bodies containing neutral lipids were detected by epifluorescence by using a TCS SP2 SE microscope with a 475 nm band-excitation filter and a 580 nm band-emission filter. For neutral lipid kinetics, duplicate samples were analysed with a RF-5301 PC Schimatzu spectrofluorometer, reading at an excitation wavelength of 480 nm and an emission wavelength of 580 ± 10 nm. The relative fluorescence intensity of neutral lipids (NR-RFI) was attained after the subtraction of microalgal cells autofluorescence and self-fluorescence of Nile Red. This intensity was measured in arbitrary units (au).

2.4. Photobioreactor (PBR) culture

A cylindrical PBR (Figmay) of 0.25 m diameter, 0.70 m height was used in order to obtain a functional inoculum for a raceway pond of 100 L. The SWES medium (see Section 2.3) was employed considering the results obtained at laboratory scale. The culture was carried out over 11 days, in a room at 20 ± 2 °C. The light supplying device provided by cool white Phillips L-35 fluorescent

lamps was placed around the PBR so as to provide 100 μmol m^{-2} s^{-1} under a cycle of 12:12 h light/darkness. The culture was supplied with 1% CO_2 in air and maintained under continuous stirring by means of a system of central paddles at 30 rpm. The stirring ensured good mixing condition and prevented cell sedimentation. Daily temperature, pH and DO measurements *in situ* were recorded with a multiparameter digital meter CONSORT C562. One sample of 1.5 L was taken on day 11, centrifuged (10 min at 3600 g), washed with distilled water, lyophilized and kept at −20 °C until lipid gravimetric analyses. The remaining culture was harvested and transferred to a raceway pond.

2.5. Indoor raceway pond culture

A PVC indoor raceway pond of 1.6 m length, 0.5 m width and 0.5 m height was used in order to obtain a biomass rich in TAG. The culture was maintained at the 0.3 m level, which is the volume of 100 L of culture. The lighting system was placed over the pond, which consisted of lamps with daylight-type fluorescent lights (Philips, 36W, TLD 965) so as to provide 100 μmol m^{-2} s^{-1}, with 12:12 L:D cycle. The stirring was produced by PVC paddle wheels attached to a rotator axle driven by an electric motor (37 W, 12 V and 5 A). Additional turbulence was provided by air-bubbling enriched with 1% CO_2 from a tube placed at the bottom of the pond. Aged filtered (0.45 μm Millipore) seawater was sterilized with 5% sodium hypochlorite, neutralized with 0.15 g L^{-1} sodium thiosulfate and UV irradiated. Then, seawater was supplemented with $NaNO_3$, K_2HPO_4, Na_2SiO_3, vitamins and micronutrient solution according to f/2 medium. This culture medium was inoculated with the biomass from the PBR. Temperature, pH and DO were measured daily *in situ* with a multiparameter digital meter CONSORT C562.

The lipid contents were analysed at two time points (exponential and late stationary growth phases) according to both Nile Red and nutrient kinetics. The first sample of 1.5 L was taken on day 7, centrifuged (10 min at 3600 g), washed with distilled water, lyophilized and kept at −20 °C until lipid gravimetric analyses. The second sample was taken on day 19 in correspondence with the final harvesting of the pond, and this received the same treatment as the first sample. For harvesting, the paddle wheels were stopped in order to autoflocculate the suspended cells. After about 2 h, the cell-free supernatant was removed by siphoning and both flocculated cells and those that formed a biofilm were collected by scraping with a spatula. This pellet was centrifuged (10 min at 3600 g) to remove the remaining water and washed with distilled water.

2.6. Dry weight (DW) and ash-free dry weight (AFDW) determinations

Triplicate samples of pellets were resuspended and washed with deionized water until a negative reaction for chloride ions (with $AgNO_3$) was obtained. The samples were dried in an oven at 100–105 °C, cooled in a desiccator and finally weighed. This process was repeated until a constant weight (DW). Afterwards, these samples were ashed in a muffle furnace at 450 °C for 8 h, cooled in a vacuum desiccator, and weighed to obtain the ash-free dry weight (AFDW). The percentage of organic matter and silica content of the cell wall were estimated from these data.

2.7. Lipid extraction

2.7.1. Folch method

Samples of *H. coffeaeformis* from the PBR and the raceway pond were taken on different days as mentioned in Sections 2.4 and 2.5, respectively. Lipid extraction was performed according to a modified Folch method [9] assisted with ultrasound. Duplicate freeze-dried samples of 200 mg of biomass were treated with 3.5 mL chloroform:methanol (2:1, v/v), vortexed thoroughly for 30 s and ultrasonicated for 10 min, three times at ambient temperature. The chloroform:methanol solution (3.5 mL) in the vial was poured into a 15-mL centrifuge tube for lipid extraction between each interval. The mixture was placed and shaken in a separatory funnel with 4 mL 0.9% NaCl (m/m) to create a biphasic system two/three times. Extracted lipids were removed and evaporated to dryness under nitrogen and kept at −20 °C. All chemicals used were analytical grade.

2.7.2. Soxhlet extraction

On day 19, an extraction of lipids by the Soxhlet method from the raceway pond biomass was also performed in order to establish the most efficient procedure for analysing lipid content rich in TAG. Sub-samples of ca 0.7 g of dry biomass were settled in a cellulose cartridge. The extraction was performed during 5 h in a Soxhlet apparatus with *n*-hexane. During this period, about 10 solvent vaporation/condensation/percolation cycles per hour were observed in the extraction chamber. After extraction, the solvent was evaporated under reduced pressure and the lipid fraction was dried to constant weight in an oven at 60 °C.

2.8. Lipid fractionation and fatty acid methyl esters analysis

Lipid fractionation into neutral lipids, glycolipids and phospholipids was performed by using a silica Sep-Pack cartridge (SP) of 1000 mg. Fatty acid profile was determined by methyl ester derivation and GC analysis. All the procedures mentioned above were carried out according to [32].

2.9. Lipid productivity

Total lipids and TAG productivities were estimated with equations according to [7]. Accumulation time was estimated from neutral lipid kinetics by using Nile Red fluorescence intensities.

2.10. Nutrient analyses

Samples of five ml were taken from the PFR and the raceway pond every two days for dissolved nutrient determination, then filtered with Whatman GF/F (0.7 μm) and frozen at −20 °C until analysis. Nutrient concentrations were determined via colorimetric assays in order to measure phosphate (PO_4^{3-}; P) [30]; nitrate (NO_3^-; N) and silicate (SiO_4^{4-}; Si) [43]. Absorbance was measured by using a Varian Cary 60 UV/Vis Spectrophotometer at 543 nm for N, 885 nm for P and 810 nm for Si.

2.11. Quality of oil for biodiesel

Cetane number (CN), iodine value (IV), kinematic viscosity, specific gravity, higher heating value (HHV) and the cloud point were calculated from the average degree of unsaturation (ADU) according to [14]. Saponification value (SV), cold filter plugging point (CFPP) and long chain saturation factor (LCSF) were estimated according to [44].

2.12. Statistical analysis

The differences in length, width and biovolume ($n = 20$) and the growth rate (k) of cells growing in both culture media, the mean values of lipid contents and lipid classes ($n = 3$), as well as the mean percentages ($n = 4$) of fatty acids were assessed with ANOVA and Student's t-test, being statistically different at a significance level of 5%.

3. Results and discussion

3.1. Identification of the species and selection of culture medium at laboratory scale

The quantity and quality of lipids in microalgae is species-specific [15]. Thus, in the present study, the species isolated was properly identified as *H. coffeaeformis* (Agardh) Levkov, according to the characteristics of its cell wall observed with a scanning electron microscope (Fig. 1a). This species has previously been named as *Amphora coffeaeformis* [24]. Moreover, most of the literature related to lipid production only identified *Amphora* at the genus level [8,38,39,47].

In the present study, cultures of *H. coffeaeformis* in f/2 and SWES media were firstly carried out at a small scale in order to select the most appropriate medium to develop the objectives proposed in each culture system (PBR and raceway pond). For this purpose, the measurements of cell size and growth rate were evaluated (Table 1; Fig. 2a). The maximum growth rate (k) of *H. coffeaeformis* in SWES medium (0.7 div $d^{-1} \pm 0.04$) was significantly ($p < 0.05$) higher than the rate obtained in f/2 medium (0.4 div $d^{-1} \pm 0.02$). Both values are in the range of 0.2–0.9 div d^{-1} obtained by Scholz and Liebezeit [37] for 25 species of marine benthic diatoms. More precisely, the maximum growth rates ranged between 0.4 and 0.7 div d^{-1} for four species of *Amphora* (*Amphora angusta, Amphora arenaria, Amphora graeffii* and *Amphora exigua*). The cultures of *Halamphora coffeaeformis* harvested on day 11 presented concentrations of 0.8×10^6 cells mL^{-1} and 0.3×10^6 cells mL^{-1} grown in SWES and f/2 media, respectively (Fig 2a). In f/2 medium the stationary growth phase was reached on day 4, whereas this phase was achieved on day 8 in SWES, suggesting that the cultures present nutritional stress faster in f/2 medium. In addition, the cells showed a biovolume significantly ($p < 0.05$) lower in f/2 (1092 μm^3) than in SWES (1341.5 μm^3) (Fig. 2a; Table 1). Cell size is a key determinant of microalgal metabolism [36]. Small cells are more proficient (i.e., they have a smaller half-saturation constant) than large cells because they have a greater surface to volume (S/V) ratio, which increases the exchanges of gases and solutes across the cell surface. In our study, *H. coffeaefformis* cells grown in f/2 presented larger neutral lipid droplets in the stationary phase (Fig. 1b and c) than those in cells grown in SWES at the same period of time (Fig. 1d and e). Thus, we suggest that smaller cells growing in f/2 may present a better uptake of the dissolved nutrient accelerating the nutritional stress and consequently, increasing the neutral lipid accumulation.

Considering all these features we propose: 1) a first stage of culture in PBR with SWES. This medium optimizes both the growth rate and cell density, and it extends the exponential phase that consequently improves the production of a physiologically active inoculum; and 2) a second stage of culture in a raceway pond with f/2. This medium allows an early stationary phase, where neutral lipids are naturally accumulated in higher proportion related to the cell size.

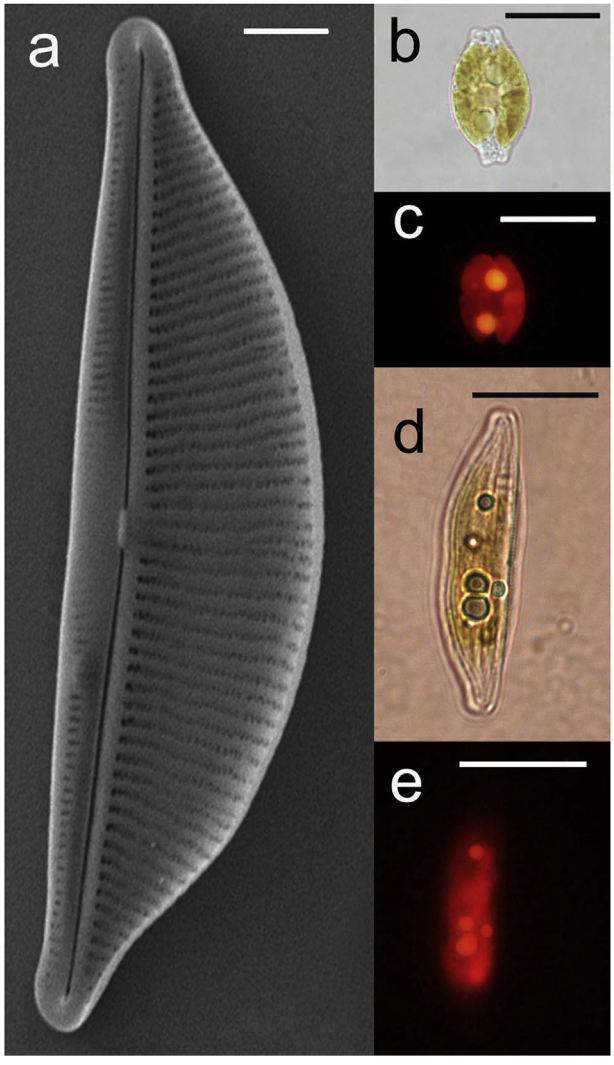

Fig. 1. *H. coffeaeformis* micrographs. (a) Detail of external valve view of silica. Semi-lanceolate valve showing biseriate dorsal striae and short ventral striae. Number of striae in 10 μm: 19. Scanning electron microscopy. (b–c) Cell growing in f/2 medium. (d–e) Cell growing in SWES medium. (b–e) Nile Red stained cells in stationary phase showing neutral lipid droplets. Light microscopy (b and d) and epifluorescent microscopy (c and e). Scale bars: (a) = 2 μm; (b–e) = 20 μm.

3.2. Physicochemical variables, biomass production and neutral lipid kinetics in PBR and raceway pond

PBR and raceway pond temperatures were relatively stable, being around 20.4 ± 1.15 °C. Besides, the dissolved oxygen (DO) showed a decreasing trend throughout both experiences, ranging from 15.9 mg L^{-1} to 9.7 mg L^{-1} and from 12.4 mg L^{-1} to 3.1 mg L^{-1} in PBR and raceway pond, respectively. The pH values in both culture systems were between 8.1 and 8.4, being close to the optimal pH range from 8.2 to 8.7 that is indicated for marine microalgal cultures [2].

Fig. 2b shows both the biomass production and the neutral lipid kinetics (NR-RFI values) in PBR and raceway pond, respectively. In the PBR, rapid sustained growth was observed, reaching values of cell density and biomass production of 3.7×10^6 cells mL^{-1} and 0.64 ± 0.13 g L^{-1} of dry weight (DW) on day 11 (Fig. 2b), respectively. The NR-RFI values in PBR were low (<75 au) and relatively stable throughout the experience. The biomass was harvested on day 11 in order to obtain a dense physiologically active inoculum. In the raceway pond the cells started to enter the exponential growth phase after a lag-phase of five days. Thus, both cell density and biomass production reached their highest values (0.65×10^6 cells mL^{-1} and 0.23 ± 0.01 g L^{-1} of DW, respectively) on day 11. The NR-RFI kinetics followed a similar trend to the one observed for PBR until day 15 (NR-RFI < 100 au); from this time point onwards, the NR-RFI kinetics showed a significantly increasing trend until day 19 (ca. 100 to 800 au), when the biomass was harvested. Therefore, the raceway pond provided a biphasic operational system, where the first phase allowed the growth of the inoculum and the second one induced neutral lipid accumulation. The latter phase occurred when the cells were in the late stationary

Table 1
Linear dimensions (μm) and volume (μm^3) of *H. coffeaeformis* in two culture media. Differences were not significant ($p > 0.05$) for groups with the same superscript.

Culture media	Length (μm)	Width (μm)	Volume (μm^3)
	Min-max (mean ± SD)	Min-max (mean ± SD)	Min-max (mean ± SD)
f/2	15.12–51.66 (31.5 ± 2.2)^a	6.3–12.5 (7.5 ± 0.6)^c	833–1258.36 (1092 ± 128.9)^d
SWES	11.34–54.18 (35.5 ± 2.0)^b	5–12.6 (8.0 ± 1.0)^c	991.78–1701.71 (1341.5 ± 238.4)^e

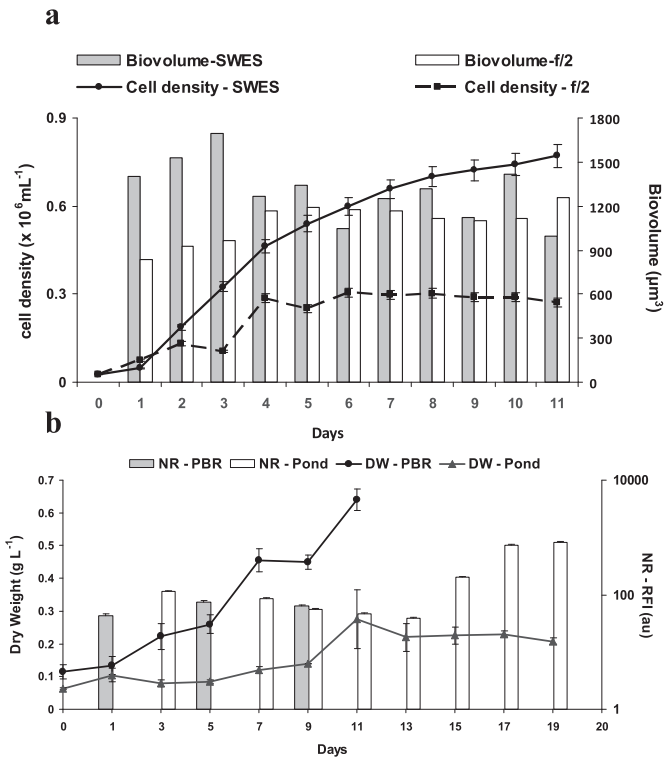

Fig. 2. (a) Growth curves and biovolume variation of *H. coffeaeformis* in two culture media. (b) Biomass (dry weight) variation in photobioreactor (DW-PBR) and raceway pond (DW-Pond); kinetics of relative fluorescence intensity of neutral lipids (NR-RFI) in the photobioreactor (NR-PBR) and the raceway pond (NR-Pond) cultures. The data are expressed as the average ± standard deviation of two or three replicates.

growth phase. These results confirm the behaviour of some species of diatoms, where the neutral lipid accumulation is triggered by nutritional limitation due to the culture ageing in the stationary phase [26,1,32].

3.3. Nutrient kinetics in PBR and raceway pond

Fig. 3 shows the nutrient kinetics in PBR and raceway pond, respectively, indicating the limiting values for diatom growth according to Sarthou et al. [36]. In the PBR, nitrate (N) and phosphate (P) concentrations never reached limiting values (Fig. 3a and b), while silicate (Si) concentrations dropped below the limiting value from day 5 (Fig. 3c). The consumption rates of these nutrients were 269.8 μM N d^{-1}, 12.3 μM P d^{-1} and 21.7 μM Si d^{-1}. In the raceway pond, all nutrients reached limiting values for diatom growth [36]. The limiting value for N appeared as from day 17 (Fig. 3d), while limiting values for P and Si were observed from day 7 (Fig. 3e and f). The consumption rates of these nutrients were 72.1 μM N d^{-1}, 3.7 μM P d^{-1} and 21.2 μM Si d^{-1}. Hence, nitrogen might be the major neutral lipid trigger since the cells started to show high NR-RFI values when it was depleted.

Biofuel production in tandem with wastewater treatment seems to be one of the areas with the most plausible commercial application of microalgae in the short term [4]. It could be a strategy that may help to reduce the production cost of algae and might contribute to environmental sustainability [41]. According to our results, *H. coffeaeformis* growing in the raceway pond was able to achieve 93% elimination of nitrogen and a complete removal (100%) of phosphorus at the final harvest, when the neutral lipids reached their maximum levels.

3.4. Lipid content and productivity of cultures in PBR and raceway pond

In order to analyse the lipid content of each culture system, the harvest points were selected according to data from growth, nutrient and NR-RFI kinetics. Total lipid (TL) content varied in both culture systems (Fig. 4a). In the PBR on day 11, the percentage of total lipids was 23.7 (±0.4) % AFDW and the total lipid content per cell was 30.9 pg cell^{-1} in cells growing exponentially, when nutrients had not reached their limiting levels and NR-RFI < 75 au. Thus, the biomass harvested on this day represented a dense and physiologically active inoculum. In the raceway pond two harvest points were selected in order to analyse the increase of lipid content. Hence, the total lipid content on day 7 was 12.2 (±0.5) % AFDW in cells growing exponentially, when Si and P had reached their limiting values and NR-RFI < 100 au. Nevertheless, TL increased significantly on day 19 up to 34.6 (±5.1) % AFDW and 83.6 pg cell^{-1} in cells under stationary growth phase, when N had reached its limiting value and NR-RFI at its maximum (ca. 800 au) (Fig. 4a). The increase of total lipids was due to a significant increment of neutral lipids (34% AFDW), which accounted for 86% of total lipids (Fig. 4b). Thus, the neutral lipid accumulation on day 19 doubled the amount obtained on day 7, indicating the day 19 suitable for obtaining biomass for biodiesel production. In addition, when the Soxhlet method was applied to the final biomass, the lipid content increased up to 54.4 (±11.5) % AFDW. This method was more efficient than the Folch method, which is attributed to a greater extraction of neutral lipids.

Fig. 4c shows the productivity of biomass (BP), total lipids (TLP) and TAG (TAGP) in the two culture systems. The BP in the PBR [43.7 (±12.4) mg DW L^{-1}d^{-1}] was significantly higher ($p > 0.05$) than the one obtained in the raceway pond [13.2 (±0.3) mg DW L^{-1}d^{-1}]. The latter value transformed into biomass areal yield was equivalent to 44.1 (±1.1) g m^{-2} d^{-1}, which was higher than the upper limit (25 g m^{-2} d^{-1}) reported for diatoms and other species of microalgae [12]. The areal yield obtained in *H. coffeaeformis* is equivalent to the values of 45–50 g m^{-2} d^{-1} reported by Sheehan et al. [38] for *Amphora* sp. grown in open ponds. In addition, the biomass areal yield from *H. coffeaeformis* was more than two-fold the value (17 g m^{-2} d^{-1}) established to have an EROI > 1 [23], which represents a positive energy balance. However, recent studies indicate that an EROI should be > 3 to be economically viable [3].

On the other hand, the total lipid productivity in the PBR was more than two-fold the value obtained in the raceway pond (Fig

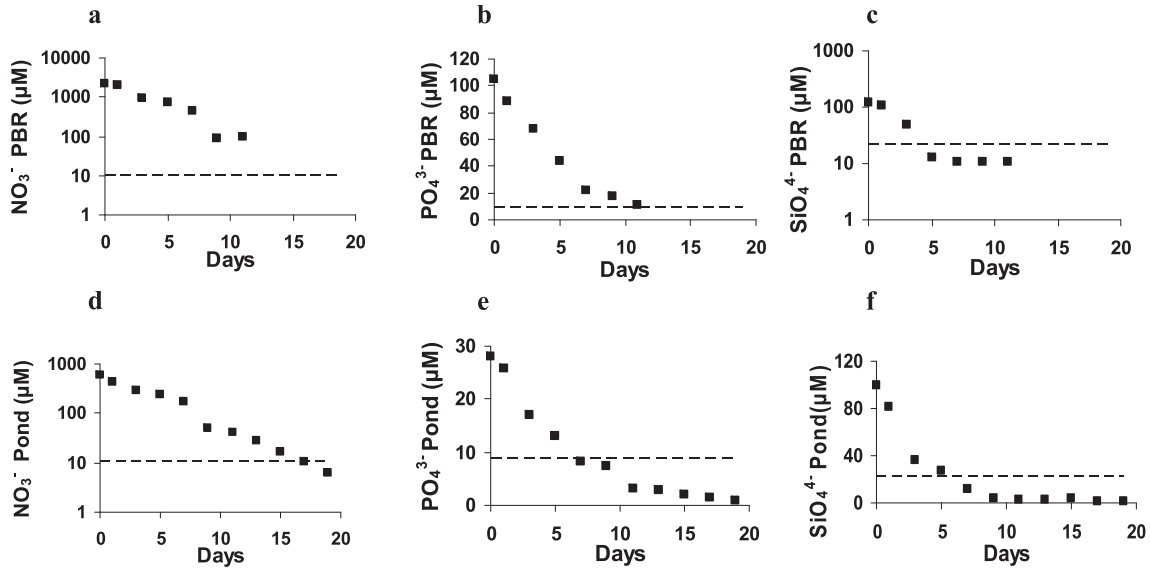

Fig. 3. Kinetics of dissolved nutrient concentrations in *H. coffeaeformis* cultures in PBR (a–c) and raceway pond (d–f). (a, d) Nitrate. (b, e) Phosphate. (c, f) Silicate. Limiting values of nutrients for diatom growth (P < 8.9 µM; N < 10.2 µM; Si < 22 µM) according to [36] are shown (dashed lines).

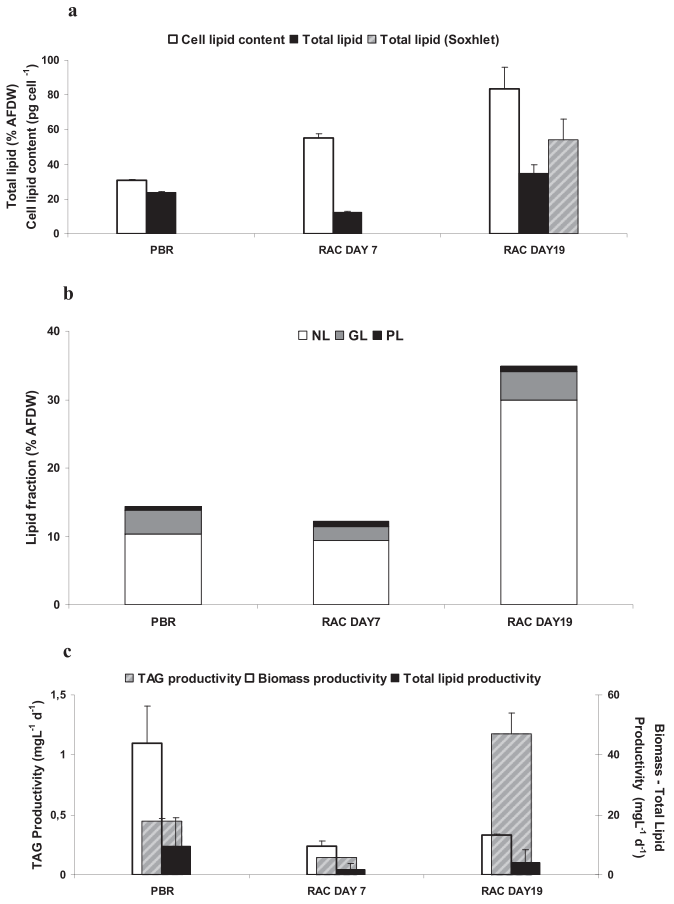

Fig. 4. (a) Total lipid content (in percentage of ash free dry weight biomass = % AFDW) and cell lipid content (in pg cell^{-1}) of *H. coffeaeformis* in different culture systems. (b) Lipid fractions (in % AFDW) of *H. coffeaeformis* (neutral lipid [NL], glycolipid [GL] and phospholipid [PL]. Values are the means of two replicates. (c) Biomass productivity (in mgL^{-1}d^{-1}) and lipid productivities (Total and TAG productivities, in mgL^{-1}d^{-1}).

4c). This was due to a higher biomass production in the PBR. However, TAG productivity is a better indicator when the biomass is destined for biodiesel production [15,7]. Thus, the TAG productivity of *H. coffeaeformis* in the raceway pond, which was calculated within the neutral lipid accumulation period, was close to three-fold (1.2 mg L^{-1}d^{-1}) the value obtained in the PBR (0.45 mg L^{-1}d^{-1}).

During the stationary growth phase most diatoms are capable of sustaining relatively high biomass-specific carbon fixation rates for conditions of nutrient limitation in contrast with other microalgae [27]. In addition [40], found a higher lipid content in dense cultures of *Phaeodactylum tricornutum* in ponds (>0.6 g L^{-1} of dry weight) than the one in less dense cultures. Although in the present study the cells accumulated high values of neutral lipids from day 15 in the raceway pond, greater production of biomass would be necessary to increase the TAG productivity.

As reported by Valenzuela et al. [45]; the depletion of dissolved phosphate might be an early trigger for lipid accumulation in *P. tricornutum*, but the accumulation rate would only be magnified when nitrate is depleted. Diatoms store significant amounts of nitrate internally [25]. In the present study, nitrogen values in the raceway pond remained high for a longer period than the one for the other nutrients (Fig. 3). Hence, considering a consumption rate of 30.81 µM N d^{-1}, the second phase in the raceway pond should be initiated with a concentration of approximately 300 µM in order to reduce TAG accumulation times and consequently to improve their productivities.

3.5. Fatty acid composition and its implications in biodiesel quality

The fatty acid composition in *H. coffeaeformis* was similar for cells grown in PBR as for those in the raceway pond (Supplementary material) and it was revealed that myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1n7), oleic (C18:1 n-9c) and eicosapentaenoic (EPA, C20:5 n-3) acids were the major components (Fig. 5a). Thus, SFA and MUFA were the dominant classes in the PBR and raceway pond, respectively (Fig. 5b); however, the PUFA presented the lowest value in the raceway pond on day 19. This reduced amount of PUFA was consistent with other studies of fatty acid composition in diatoms during the late stationary phase [32,23]. On day 19 in particular, the MUFA percentage in the neutral lipid fraction was significantly higher (α = 0.05) than the SFA

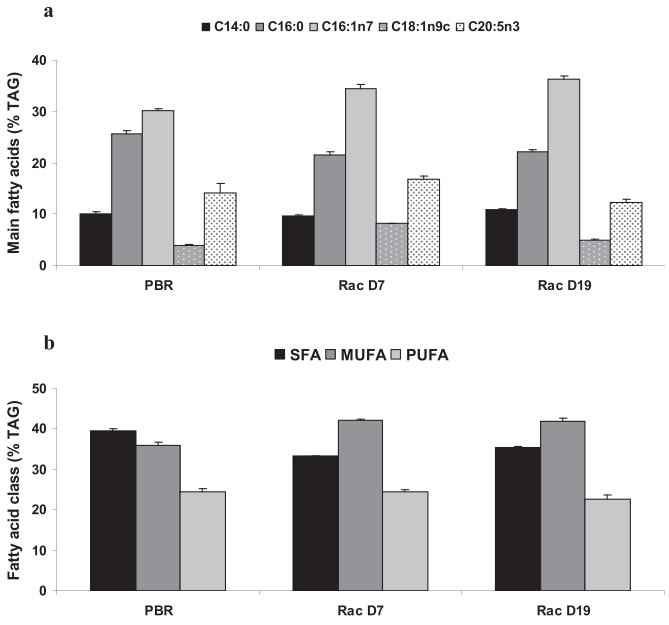

Fig. 5. Neutral lipid (TAG) quality of *H. coffeaeformis*. (a) Main fatty acids (in % of TAG fraction). (b) Fatty acid classes (in % of TAG fraction). SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

characteristics of biodiesel produced by transesterification [20]. In general, the use of feedstocks with high MUFA levels, such as C16:1 and C18:1 fatty acids, is almost ideal [23]. A practical way of predicting biodiesel characteristics is based on certain properties that are calculated from fatty acid compositions [14]. found that several fuel properties – including viscosity, specific gravity, cetane number, iodine value and low-temperature performance metrics – are highly correlated with the average unsaturation of the FAME profiles of vegetable oils. The biodiesel properties of 10 microalgal strains were predicted by determining their fatty acid profiles [42] based on these equations. According to these authors, the best strain was the diatom *P. tricornutum*, with a fatty acid profile that would confer suitable biodiesel properties of higher cetane number (55.10), a lower iodine number (99.2 gI_2/100 g) and a relatively low cloud point (4.47 °C) (Table 2). In comparison, the fatty acid composition of *H. coffeaeformis* on day 19 in the raceway pond showed that the oils are of good quality, according to the parameters established in EN14213-14214 (Europe) and ASTM D6751-08 (US) standards for cetane number (CN), iodine value (IV), kinematic viscosity and specific gravity (Table 2). Additionally, the highest heating value (HHV) was in the range of conventional biodiesel values, and the cloud point (CP) was relatively low (2.42 °C), taking into account that other biodiesels from tallow and palm can reach up to 13 and 14 °C, respectively [14]. Nevertheless, the *H. coffeaeformis* CP was close to that of biodiesel from soybean

Table 2
Biodiesel properties calculated from fatty acid composition of *H. coffeaeformis* grown in the raceway pond and harvested on day 19. a: Biodiesel properties for 10 microalgae from Song et al. [42]. b: Biodiesel properties for 11 microalgae from Talebi et al. [44]. c: Biodiesel properties for diverse vegetable oils and fat from Hoekman et al. [14]. d: Biodiesel properties for soybean and canola oils in Talebi et al. [44].

	H. coffeaeformis	Algal biodiesel[a]	Algal biodiesel[b]	Biodiesel[c]	US (ASTM D6751-08)	Europe (EN 14214)
Kinematic viscosity (40 °C mm^2 s^{-1})	4.375	4.15–5.07		4–5	1.9–6.0	3.5–5.0
Specific gravity (kg L^{-1})	0.88	0.87–0.88		0.87–0.89	0.85–0.90	–
Cloud point (°C)	2.422	−2.38 to 17.02		−4–14	Report	–
Cetane number	54.102	51.7–61.4	43.77–62.33	45–55	>47	>51
Iodine value (g I_2/100 g)	110.56	29.3–134.0	57.56–150.44	18.5–152.8	–	<120
HHV (MJ/kg)	40.85	38.9–41.5		38–41	–	–
Average unsaturation	1.316	0.22–1.68		0.6–1.6	–	–
LCSF	3.47		2.16–9.19	1.4–3.1[d]	–	–
SV (mgKOH)	213.19		152.99–194		–	–
CFPP	−5.57		−9.71 to 12.41	−12 to 13	–	Depends upon the location and time of year

percentage, being the palmitoleic acid (ca. 36%) the predominant fatty acid-MUFA.

The proportions of fatty acids in oils directly influence the oil (0 °C ± 2) and lower than the one from *P. tricornutum* oil [42]. Additionally, the *H. coffeaeformis* cold filter plugging point (CFPP) was much lower (−5.57) than CFPP from *Amphora* sp. (12.41)

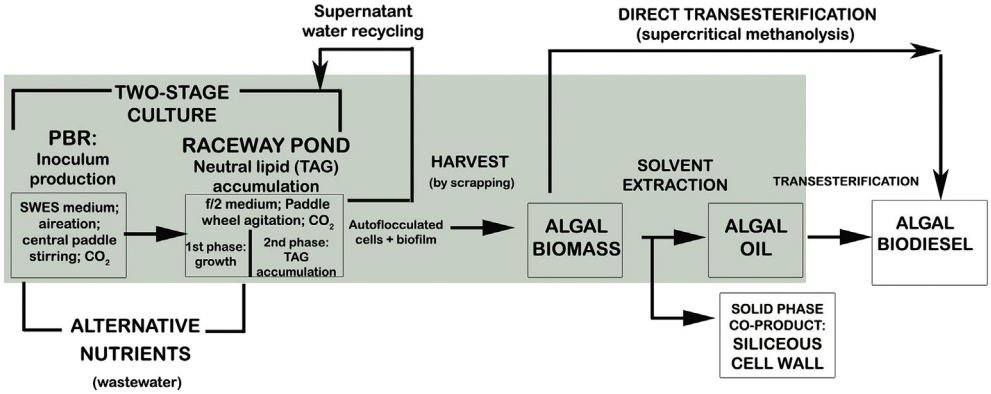

Fig. 6. Schematic representation of two-stage culture of *H. coffeaeformis* and biodiesel production. Grey box: the present study. Additional tools as alternative nutrient use (e.g., wastewater with nutrient levels similar to those of SWES and f/2 media used in PBR and raceway pond, respectively), water recycling, direct transesterification and co-product recovery are shown in order to improve the EROI.

estimated by Talebi et al. [44].

According to the present results, a scheme of *H. coffeaeformis* culture for the purpose of biodiesel production is presented (Fig. 6). Furthermore, some hypothetical tools are proposed in order to improve the EROI in larger scale processes. The following proposals can be considered: (1) its potential to support eutrophic waters, which was the main characteristic of its isolation site, encourages the use of this species for bioremediation and energy production simultaneously; (2) its growth capacity at high salinities contributes to reducing contamination risk, which is a major constraint in open raceway ponds; (3) its benthic habit promotes biofilm formation, which may improve the biomass harvesting costs by making the downstream processes cheaper; (4) the silica component of its cell-wall represented 25% of the harvested biomass, which is indicative of a potential co-product with promising biotechnological applications. Furthermore, this baseline study allows us suggesting that the culture of *H. coffeaeformis* in larger scale processes for biodiesel production could be sustained with seawater enriched with alternative sources of nutrients (e.g., wastewater), which should present nutrient levels similar to the conventional culture media used in this study.

It is important to highlight that the biomass rich in neutral lipids obtained in the raceway pond in this study provided enough feedstock to carry out analyses of biodiesel production. The direct supercritical methanolysis method has been proposed recently as an alternative energy-efficient technology for obtaining biodiesel from conventional feedstock [46], as well as it being an economical route for microalgal biodiesel production [31]. Our preliminary results of supercritical fatty-acid recovery from *Neochloris oleoabundans* biomass for biodiesel production [29] encouraged us to produce biodiesel by means this method in *H. coffeaeformis.*

Finally, it has been envisaged that algal biofuel development will have a regional character and so it will be necessary to develop strains optimally adapted to different environments [12]. This study provides baseline data of *H. coffeaeformis* culture in indoor pilot-scale systems for biodiesel production. This source of primary information, particularly the one related to biomass productivity and lipid yield values, helps to focus on process and scale-up assumptions in this species. Moreover, from an economic perspective, the proximity to a seawater source and the marginal land surrounding the Bahía Blanca Estuary, in tandem with the tools mentioned previously, would contribute to reducing the cost of bioenergetic cultures of *H. coffeaeformis.*

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.renene.2016.01.078.

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