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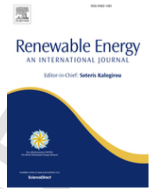

Hybrid two-stage culture of *Halamphora coffeaeformis* for biodiesel production: Growth phases, nutritional stages and biorefinery approach

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ABSTRACT

The growth, lipid accumulation and bioproducts of the marine diatom *Halamphora coffeaeformis* were evaluated in a hybrid two-stage culture using seawater enriched with nutrients and without vitamins. The influence of dissolved and internal nutrients on growth and lipid accumulation was also analyzed. Total lipid content increased in the declining phase up to 33.4% ash-free dry weight, due to an increase in neutral lipids, which reached 87% of total lipids. The observed delay in triacylglycerol (TAG) accumulation could be explained by the accumulation of large internal pools of nitrogen in *H. coffeaeformis*. TAG, frustules (silica-containing cell wall) and soluble exopolysaccharides are analyzed and proposed as bioproducts of commercial interest. A biorefinery approach for the economic and environmentally sustainable production of biodiesel from the hybrid two-stage culture of *H. coffeaeformis* is suggested.

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1. Introduction

Microalgal biomass and oil constitute promising renewable feedstocks for the emerging chemical technology and biotechnology industries, mainly for biodiesel production. Compared with plants, optimized microalgal cultures produce more oil per hectare and have a shorter production cycle [1–4]. Moreover, microalgae avoid food-versus-fuel conflicts since they do not require the use of farmland. In particular, diatoms are unicellular or colonial microalgae, included within the Ochrophyta division based on the presence of fucoxanthin and chlorophyll *a* and *c*, chloroplasts within periplasmic endoplasmic reticulum and chrysolaminarin reserve [5]. They belong to the Bacillariophyceae class because of their unique type of siliceous cell wall or frustule [6]. There are about 10^5 species, which are identified by the species-specific morphology of their frustules [7]. Due to the resistance of their cell walls, diatoms constitute an important rock-forming microfossil group; thus, they are useful biostratigraphy markers of environmental changes and hydrocarbon exploration [8]. Centric diatoms are essentially planktonic microalgae, which are found in all open water masses, while pennate diatoms are mostly benthic organisms that grow on sediments or attached to rocks or macroalgae [9]. Some species of diatoms present advantageous features for large-scale production: they are tolerant to hostile environments and can accumulate high levels of lipids, particularly triacylglycerols (TAG), which are the most suitable feedstock for biodiesel production [3]. From an ecological point of view, diatoms are responsible for 20–25% of total terrestrial primary production and approximately 40% of annual marine biomass production, which make the most dominant group of organisms sequestering carbon from the atmosphere [10].

In this study the marine benthic diatom *Halamphora coffeaeformis* proved to have appropriate characteristics for its potential use in biodiesel production due to its high TAG content and robustness [11]. However, the commercial viability of biodiesel from microalgae is still marginal [12]. Thus, some strategies suggested for improving the process economy can be summarized as follows: 1) increasing biomass and lipid productivity; 2) reducing the resource requirements and operating costs of algae cultivation systems; and/or 3) encouraging additional income from biomass through application of the biorefinery concept [12]. Regarding the first point, lipid accumulation in microalgae is associated with situations of environmental stress, which inevitably produces a tradeoff between optimal growth and lipid accumulation, thus diminishing the overall productivity of the process [13]. In general, nutrient limitation [14] and more specifically, nitrogen deficiency, is a culture strategy used to induce lipid ac-

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cumulation in microalgae [12]. For this purpose, an inoculum of cells in the exponential growth phase must be transferred to a culture with limited or no nitrogen, a condition generally achieved in hybrid cultures [15]. Diatoms are advantageous because they naturally accumulate TAG in response to nutrient depletion during their stationary phase of growth [16,17]. However, this response is species specific and includes limitation or starvation of nitrogen, phosphorus or silicate [14]. Moreover, diatoms are characterized by accumulating large internal pools of nitrate [18], silicate [19] and phosphate [20], reaching internal concentrations of up to 10^3 times that of dissolved silicate (Si) [19] and 10^3 to 10^4 times that of dissolved nitrate [18]. These pools can be used as reservoirs to hold cell division when dissolved nutrients are depleted; however, this nutritional reserve could also delay lipid accumulation. It is therefore necessary to assess the kinetics of both dissolved and intracellular nutrients in order to design a large-scale diatom culture for biodiesel production.

Major economic bottlenecks that affect microalgal biofuel production include: culture, harvest, oil extraction and refining processes [14]. Benthic species, such as the one used in this study, form a biofilm that avoids the need to centrifuge large volumes of water, thus reducing harvest costs [11,21] which typically represent up to 20 or 30% of operating costs [22]. On the other hand, culture media used on a laboratory scale are too costly for industrial use. For instance, the f/2 medium [23], which is widely used for laboratory-scale diatom cultures, contains vitamins (cobalamine, thiamine and biotin) representing approximately 50% of the cost of the medium. However, most benthic species require only one or two vitamins [24] and some do not require any at all [25]. Reducing resource requirements by using a medium without vitamins may therefore be a viable growth strategy in the case of those species unaffected by their absence.

The biorefinery concept involves maximizing biomass value by producing energy carriers and valuable co-products [4] and has focused on improving both the economic viability and environmental sustainability of microalgal biofuels [13]. At present, one of the main industrial applications of diatoms is the exploitation of diatomaceous earth, also known as diatomite, and consisting of fossilized diatom frustules made up of amorphous opaline silica (SiO_2.nH2O). Diatomite has been used in 300–500 commercial applications relating to filtration, abrasion, adsorption, food additives, insecticides, biosensors, nanotechnology and medical implants [26–28]. Frustules from clonal cultures like the ones analyzed in the present study show compelling advantages over diatomite in terms of both sustainability and quality [29]. However, their economic competitiveness will depend on eventual production costs. An additional distinctive feature of some species of diatoms is their capacity to produce exopolysaccharides (EPS) [21], which have potential applications in the pharmaceutical industry and in agronomy for the controlled delivery of products [30], or for bioremediation due to their adsorbent capacity [31].

The main goal of this study was to assess the growth and lipid accumulation of the benthic diatom *H. coffeaeformis* in a hybrid two-stage culture using seawater enriched with nutrients and without vitamins. The kinetics of dissolved and intracellular nutrients and the feasibility of producing potentially valuable bioproducts were also determined. The combined data enable us to propose specific growth requirements and to shed light on the relationships among the previously mentioned features in connection with sustainable biodiesel production from *H. coffeaeformis* cultures under a biorefinery approach.

2. Methods

2.1. Algal strain and culture conditions

Halamphora coffeaeformis (C. Agardh) Levkov was isolated from Bahía Blanca Estuary (38° 45′ S, 62° 22′ W). Non-axenic cultures were grown and maintained in f/2 medium [23], which was prepared with sterile seawater with a salinity of 30, at 15 ± 1 °C temperature, 60 μmol photons m^{-2} s^{-1} of light intensity and 12:12 h light/darkness photoperiod. Light was supplied by cool-white fluorescent Phillips tubes. This strain is maintained in stock cultures at the Laboratorio de Estudios Básicos y Biotecnológicos en Algas (LEBBA), CERZOS-CONICET, Bahía Blanca, Argentina.

2.2. Two-stage culture conditions

Cultures were carried out in a hybrid two-stage process according to Martín et al. [11]. In short, an inoculum of *H. coffeaeformis* was cultured in photobioreactor (PBR) during 8 days, and transferred to an indoor PVC raceway pond in order to obtain a biomass rich in TAG. The experiment in the raceway pond was performed with 100 L of culture at a depth of 0.3 m. In the present study the culture was carried out without vitamins. Seawater was supplemented with $NaNO_3$ (N), K_2HPO_4 (P), Na_2SiO_3 (Si), and trace metals according to f/2 medium [23]. For harvesting, the paddle wheels were stopped on day 32 in order to autoflocculate the suspended cells. After about 2 h, the cell-free supernatant was removed by siphoning and flocculated cells were collected by scraping. The harvested pellet was washed with distilled water, centrifuged (10 min at 3600 g) and kept at −80 °C.

2.3. Growth: chlorophyll and biomass measurements

2.3.1. Chlorophyll, cell density and volume

Five milliliters of living microalgal cells were taken daily in triplicate for *in vivo* fluorometric chlorophyll *a* (Chl *a*) detection. Chl *a* fluorescence intensity (Chl *a*-FI) was measured in arbitrary fluorescence units (au). Excitation wavelength was set at 430 nm and emission wavelength was scanned from 600 to 750 nm (spectrum mode with excitation and emission slits set at 5 nm) using a spectrofluorometer (Schimadzu RF-5301PC). Emission wavelength peak was selected at 680 ± 5 nm. Cell density was determined by counting three replicate samples by means of a Sedgwick-Rafter chamber. Two mL samples were taken daily to measure cell size. The length and width of 20 individual cells were recorded. Individual cell volume (V, μm^3) was calculated using the formula for the cymbelloid form according to Eqs. (1) and (2) [32]:

$$V = 4/6 \cdot \pi \cdot b^2 \cdot a \cdot \beta/360 \quad (1)$$

$$\sin \beta/2 = c/(2 \cdot b) \quad (2)$$

where *a* = apical axis (length); *b* = transapical axis (width); *c* = pervalvar axis on dorsal side; *β* = angle between two transapical sides.

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

Triplicated samples (20 mL) were filtered through pre-dried and pre-weighed glass-fiber filters (Whatman GF/F), which were dried at 100 °C for 12 h, cooled in a desiccator, and weighed until constant

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weight (DW). Then, 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 percentages of organic matter and frustules (ash weight) were estimated from these data.

2.4. Nutrient analysis

Samples of 10 mL were taken periodically, then filtered onto Whatman GF/F (0.7 µm) and frozen at −20 °C until analysis. The filtrate was used for dissolved nutrient determination and the filters containing the cellular pellet for internal nutrient analysis. Internal nutrients were determined after extraction with boiling, distilled and deionized water [18]. Nutrient concentrations (dissolved and internal) were determined via colorimetric assays in order to measure phosphate (PO_4^{3-}; P) [33]; nitrate (NO_3^-; N) and silicate (SiO_4^{4-}; Si) [34]. Absorbances were measured with a Varian Cary 60 UV/Vis Spectrophotometer at 543 nm for N, 885 nm for P and 810 nm for Si. Dissolved nutrients were referenced as µM and internal nutrient concentrations were normalized to both cell number and volume (mmol L cell^{-1} = mM) according to Collos et al. [35].

2.5. Lipid evaluation

2.5.1. Total lipid extraction

Samples for lipid content were taken at five time points (three at the stationary growth phase and two during the declining growth phase). Each sample was centrifuged (10 min at 3600 g), washed with distilled water, lyophilized and kept at −20 °C until lipid gravimetric analysis. Lipid extraction was performed according to a modified Folch's method [36] 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. Then, the mixture was poured into a 15-mL centrifuge tube for lipid extraction, and the supernatant was placed in a separatory funnel with 4 mL NaCl 0.9% (*m*/*m*) to create a biphasic system; this procedure was carried out for three times. Extracted lipids were removed and evaporated to dryness under nitrogen and kept at −20 °C. All chemicals used were analytical grade.

2.5.2. Lipid fractionation and fatty acid methyl ester analysis

Lipid fractionation into neutral lipids (NL), glycolipids (GL) and phospholipids (PL) was performed using a silica cartridge Sep-Pack (SP) of 1000 mg (J. T. Baker Inc., Phillipsburg, N. J.) according to Popovich et al. [37]. Briefly, this procedure included five steps: (a) adsorbent conditioning with 30 mL of chloroform, (b) sample loading, 1 mL of chloroform/oil solution containing 20 mg of oil; (c) elution of NL from the adsorbent bed with 15 mL of chloroform/acetic acid (9:1, v/v), (d) GL recovery by elution with 20 mL acetone/methanol (9:1, v/v) and (e) phospholipids recovery by elution with 20 mL of methanol. Each fraction was collected into a conical vial and evaporated to dryness under nitrogen. Two replicates of each lipid fraction were made.

The fatty acid derivation was done according to Popovich et al. [37]. FAMEs were analysed by gas chromatography with an HP Agilent 4890D gas chromatograph, 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 lm) (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; then, the temperature was increased up to 240 °C with a speed of 4 °C/min, holding it isothermal for 15 min. The detection limit of the chromatographic method was set to 0.01%. The HP 3398A GC Chemstation Software (Hewlett Packard, 1998) was used for chromatographic analysis. FAMEs identification was performed by comparison with standard certificate material, Supelco FAME 10 mix 37 (Bellefonte, PA, USA), according to AOCS Official Method Ce 1b-89. Four replicates of each FAME analysis were done.

2.5.3. Characterization of neutral lipid bodies (LBs) with Nile Red

Samples for neutral lipid body (LB) detection via Nile Red (NR) fluorescence were taken at five time points (three at the stationary growth phase and two during the declining growth phase). 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 [38]. The mixture was agitated vigorously in a vortex mixer. Intracellular LBs containing neutral lipids were detected by epifluorescence using a TCS SP2 SE microscope with a 475 nm band-excitation filter and a 580 nm band-emission filter.

2.6. Protein and carbohydrate content

Samples for biochemical composition were taken at the end of the raceway pond culture. Each sample was centrifuged (10 min at 3600 g), washed with distilled water, lyophilized and kept at −20 °C until analysis. For protein and carbohydrate quantification, triplicate samples of 10 mg biomass were immersed in 5 mL ultrapure water. The samples were placed in an ultrasonic bath (40 KHz, 160 W) and sonicated for 30 min. Then, protein and carbohydrates were quantified according to Bradford [39] and Dubois et al. [40], respectively.

2.7. Exopolysaccharides (EPS)

Soluble exopolysaccharides (S-EPS) were recovered from the culture supernatant by centrifugation at 3500*g* for 15 min. Total carbohydrates were dosed by the phenol–sulphuric acid assay [40], using glucose as standard.

2.8. Frustule analysis

In order to evaluate the structural integrity of the frustules from the harvested biomass and the residue post lipid-extraction, different samples were treated as follow: 1) a sample of harvested biomass was washed with distilled water to eliminate salts (untreated biomass); 2) a sample of harvested biomass was cleaned with 20 vol hydrogen peroxide, boiled for 30 min and washed with distilled water until pH 7.0 (H_2O_2-biomass); 3) a sample of residue obtained from lipid-extraction was washed with distilled water to extract the solvents (untreated residue); 4) a sample of residue was mixed with 20 vol hydrogen peroxide, boiled for 30 min and washed with distilled water until pH 7.0 (H_2O_2-residue). All treatments were dried at 60 °C until the water was fully evaporated. The samples were analyzed using a scanning electron microscope LEO, EVO-40XVP (CC-CONICET, Bahía Blanca).

2.9. Biodiesel properties

The biodiesel properties, such as kinematic viscosity (KV), specific gravity (SG), cloud point (CP), cetane number (CN), iodine value (IV), and higher heating value (HHV), were calculated from the fatty acid profile of neutral lipid fraction. Average degree of unsaturation (ADU) computed from fatty acid profile was calculated according to Eq. (3) [41].

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$$ADU = \sum M \cdot Y_i \quad (3)$$

where M is the number of carbon-carbon double bonds in each fatty acid and Y_i is the mass fraction of each fatty acid.

Biodiesel properties were calculated from the relationships between biodiesel unsaturation and other fuel properties according to Eqs. (4)–(9) [42].

$$KV = -0.6316\, x + 5.2065 \quad (4)$$

$$SG = -0.0055\, x + 0.8726 \quad (5)$$

$$CP = -13.356\, x + 19.994 \quad (6)$$

$$CN = -6.6684\, x + 62.876 \quad (7)$$

$$IV = 74.373\, x + 12.71 \quad (8)$$

$$HHV = 1.7601\, x + 38.534 \quad (9)$$

where $x = ADU$.

3. Results and discussion

3.1. Physicochemical variables

Temperature values in the raceway pond showed an increasing trend throughout the culture period, varying between 11.1 °C and 21.6 °C. This range is similar to the average spring-summer temperatures of the Bahía Blanca Estuary [43], highlighting the importance of using a native species that has already been optimized under the local climatic conditions. Dissolved oxygen (DO) values were relatively stable at around 1.77 ± 0.88 mg L^{-1} (Fig. 1a). The pH values increased from 7.65 on day 0 up to 8.69 on day 11 in agreement with the culture growth and later exhibited a slightly decreasing trend until day 32 in correspondence with both stationary and declining phases (Fig. 1b; Fig. 2). The salinity values showed an increasing trend throughout the culture period, ranging from 30 to 49.2 (Fig. 1b), which indicates high evaporation. The tolerance of this species to high salinities represents an advantage for open-door cultures because high salinities may limit culture contamination [12].

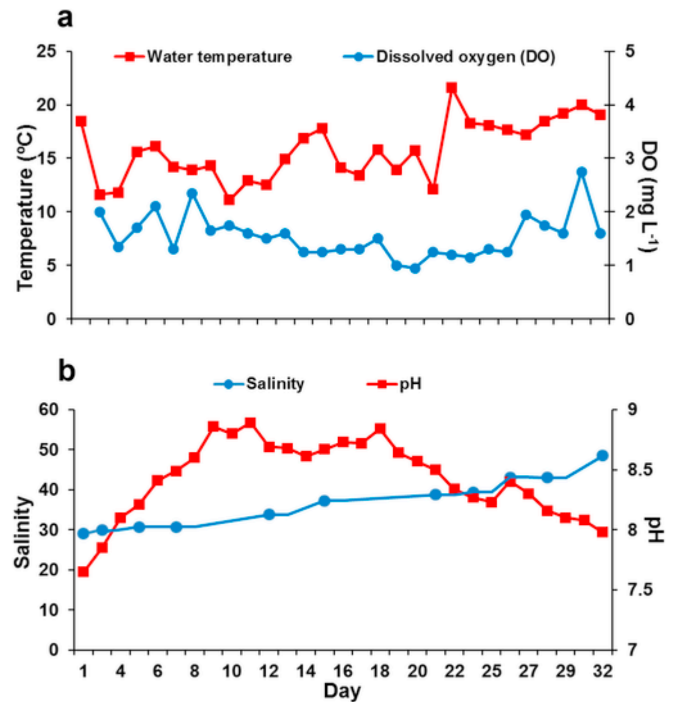

Fig. 1. Physicochemical variables in raceway pond. **a**. Water temperature and dissolved oxygen (DO). **b**. Salinity and pH.

3.2. Growth phases

Fig. 2 shows the time course of biomass (dry weight) concentration and chlorohyll *a* fluorescence intensity of *H. coffeaeformis* growing in raceway pond. These two variables showed the same trend, allowing the characterization of the species‘ growth curve. The cells presented a short lag-phase, followed by a six-day exponential growth phase during which dry weight (DW) and Chl *a* fluorescence intensity (Chl *a*-FI) values increased by almost three- and two-fold, respectively. The stationary growth phase occurred between days 10–22 and was characterized by relatively stable values of DW and Chl *a* -FI. From day 22 onwards, both biomass and Chl *a*-FI values showed a decreasing trend, indicating a declining growth phase. However, until day 32 the cells did not show signs of potential crash-

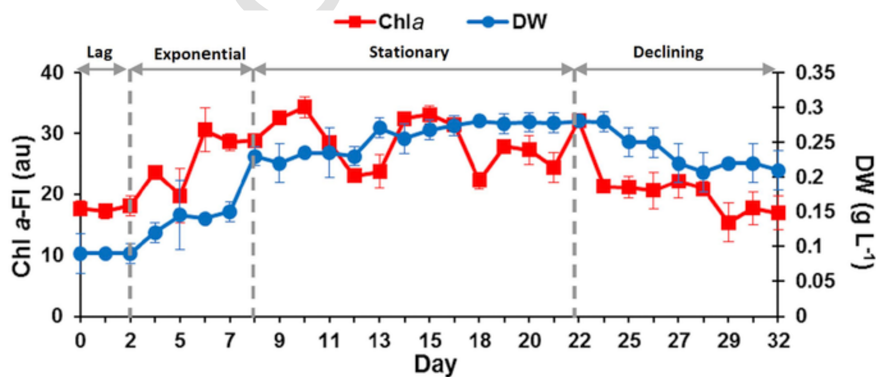

Fig. 2. Time course of biomass (dry weight) concentration and chlorohyll *a* fluorescence intensity of *H. coffeaeformis* in raceway pond.

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ing. In addition, the absence of thiamine (B_1), biotin (B_7) and cobalamine (B_{12}) in the culture medium did not affect the growth of *H. coffeaeformis* compared to its growth in a complete f/2 medium [11]. The use of a medium without vitamins would therefore be an adequate strategy to decrease the resource requirements of *H. coffeaeformis* cultivation systems.

3.3. Lipid content and composition

Fig. 3a shows the lipid accumulation kinetics in the *H. coffeaeformis* culture. In the stationary growth phase (days 10–22), total lipid content ranged between 10 and 17% (AFDW), with a maximum value of neutral lipids of 9.89% (AFDW). Total lipid content increased in the declining growth phase up to 33.41% (AFDW) due to an increase in neutral lipids, which reached 29.15% (AFDW) and 87% with respect to total lipids. In addition, the minimum value of phospholipids (0.57% AFDW) was attained on day 32. Observing the evolution of the lipid accumulation kinetics under an epifluorescence microscope, neutral lipids appeared as small lipid bodies (LBs) at the beginning of the stationary growth phase (Fig. 3b); they then increased in size towards the beginning of the declining growth phase (day 29) and reached maximum size on day 32 (Fig. 3c). This behavior might indicate that the species studied accumulates neutral lipids in pre-existing LBs. A similar LB dynamics was reported by Wong and Franz [44] in *Phaeodactylum tricornutum*. In contrast, these authors observed that *Tetraselmis suecica* accumulated numerous small LBs.

With respect to neutral lipid composition, the saturated fatty acids (SFA) accounted for 30.36% of total fatty acids, palmitic (C16:0) and myristic (C14:0) acids being the main ones. Monounsaturated fatty acids (MUFA) represented 34.52% of fatty acids, the main one (26.04%) being palmitoleic acid (C16:1n7). The neutral fraction also presented 35.12% of polyunsaturated fatty acids (PUFA), the dominant one (23.63%) being eicosapentaenoic acid (EPA, C20:5n-3). The proportion of PUFA in diatoms usually decreases in the stationary phase of growth [11,37,45]. However, in this study a high proportion of PUFA was observed in the declining growth phase. This trend is consistent with that observed in *Skeletonema marinoi* during its declining growth phase [46], suggesting a regulation of PUFA pathways dependent on the growth conditions. The total fatty acid profile of neutral lipids in *H. coffeaeformis* is shown in Supplementary material.

3.4. Nutritional status of H. coffeaeformis hybrid culture

Fig. 4 shows dissolved nutrient kinetics (nitrate, phosphate and silicate) in the photobioreactor (PBR) and in the raceway pond. Internal nutrient kinetics in the raceway pond is also presented in order to evaluate the effect of cell nutritional status on lipid accumulation. In all figures the limiting dissolved nutrients for diatom growth [47] are indicated. In the raceway pond, the dissolved N showed a gradual decrease and reached its limiting value on day 22 ($N < 10.2$ μM), corresponding with the start of declining growth phase (Fig. 4a). Meanwhile, P and Si decreased exponentially and reached their limiting values in the first days of culture ($P < 8.9$ μM on day 7 and $Si < 22$ μM on day 2). The main resources for microalgal growth are nutrients, together with light and CO_2. On the other hand, dissolved N limitation has been used in microalgae cultures to stimulate lipid accumulation [12]. However, in batch cultures of diatoms, the accumulation of TAG in response to N limitation is more variable [13,14]. This may be because diatoms have the ability to luxury uptake nutrients during dissolved nutrient-replete conditions and store them in

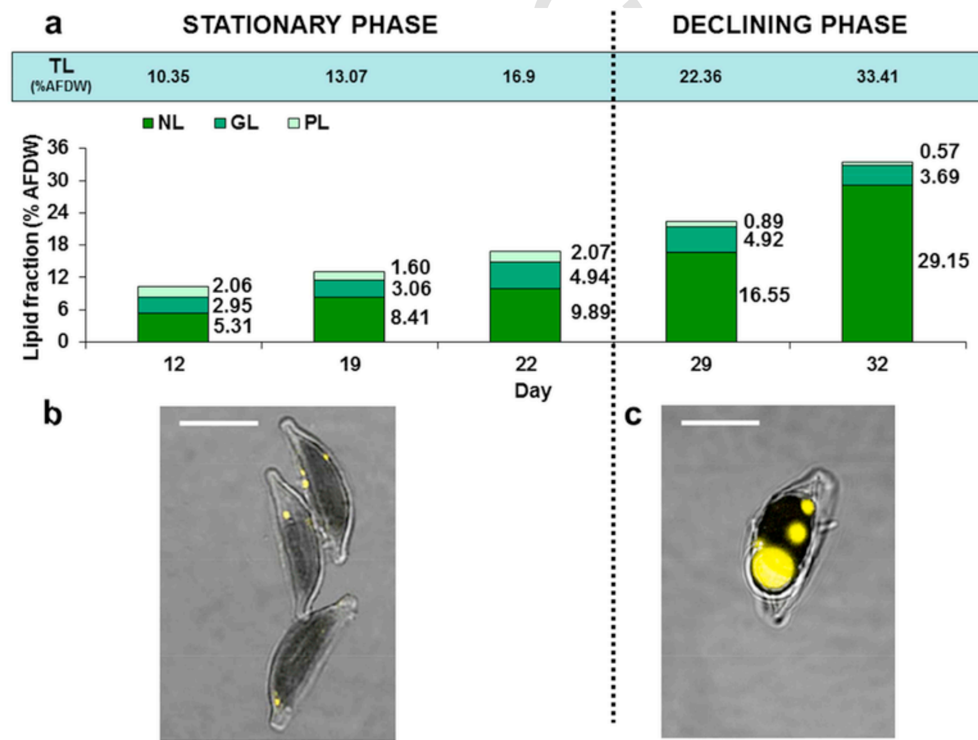

Fig. 3. *Halamphora coffeaeformis'* lipid accumulation in stationary and declining phases. **a**. Total lipid content (TL) as a percentage of ash-free dry weight biomass (% AFDW) and lipid fractions (in % AFDW). Neutral lipids (NL), glycolipids (GL) and phospholipids (PL). **b-c**. Nile Red stained cells showing neutral lipid droplets in stationary and declining phases, respectively. Scale bars: 20 μm.

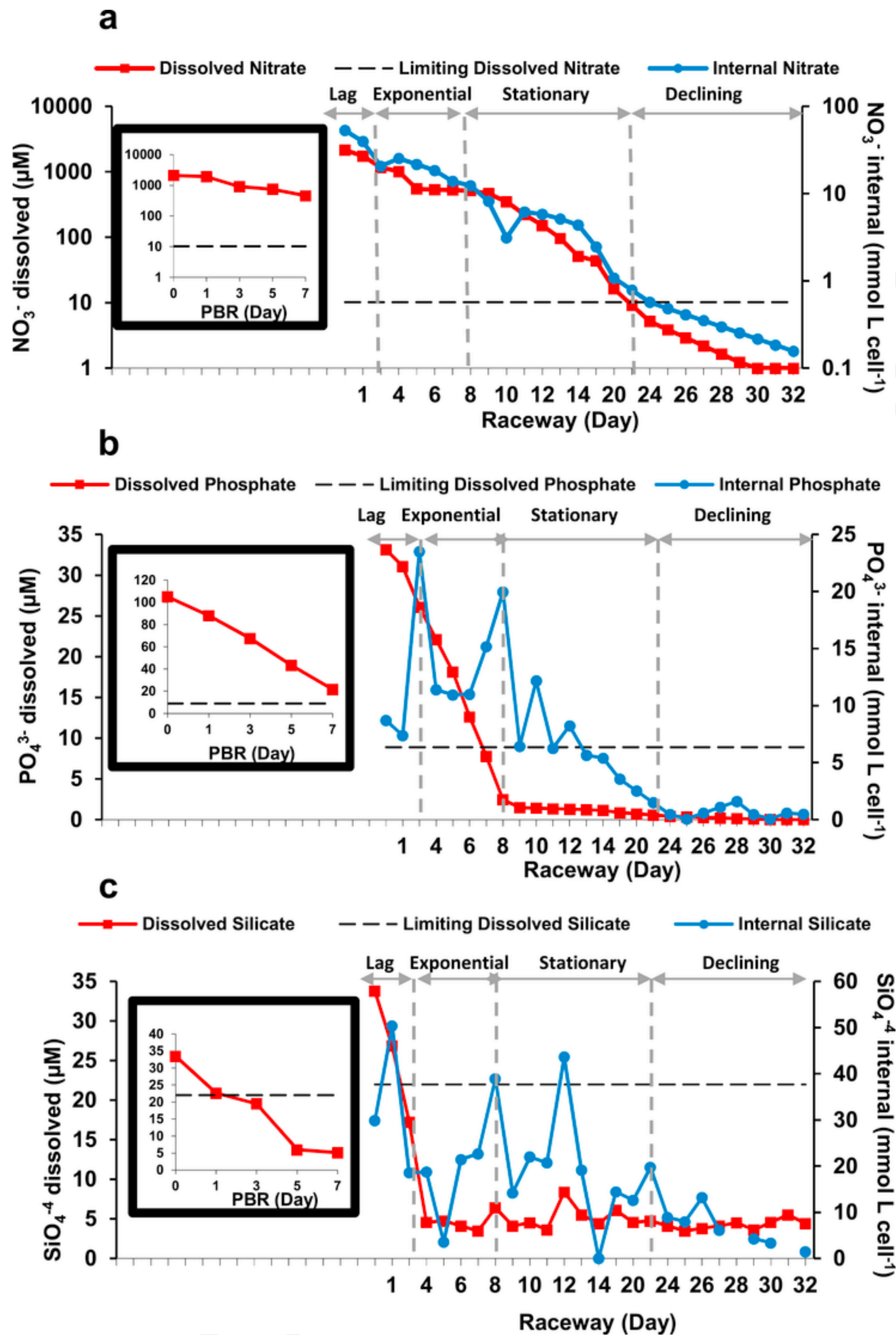

Fig. 4. Dissolved and internal nutrient kinetics in raceway pond. Insert: dissolved nutrient kinetics in PBR. **a.** Nitrate. **b.** Phosphate. **c.** Silicate.

large intracellular pools generating an additional reserve [18–20]. In the present study it is noteworthy that although P and Si achieved limiting values rapidly, and dissolved N did so on day 22, neutral lipid concentration peaked at day 32. Furthermore, the intracellular nitrate pool at the beginning of the culture was 53.07 mM N, which was 10^4 times higher than the dissolved nitrate (Fig. 4a), in agreement with results for the diatom *Skeletonema costatum* [18]. This reserve was used throughout the culture and reached minimum values during the declining growth phase, when the neutral lipid accumulation was maximal. Thus, both sources of N (dissolved plus internal) had to be depleted to induce the maximum accumulation of neutral lipid.

The preconditioning of diatom cells affects their subsequent nutritional behavior [18], a concept relevant to hybrid two-stage cultures where the nutritional status of cells transferred to the raceway pond reflects their growth conditions in the photobioreactor. In general, depending on the preconditioning of diatoms, two nutritional trends can be distinguished: 1) when nutrient-deficient cells are exposed to a nu-

trient-rich environment, they are still able to grow and to store excess nutrients in large intracellular pools through luxury uptake pathways; 2) when nutrient-sufficient cells are exposed to a nutrient-rich environment, it takes longer for the available nutrients to diminish and the use of internal nutrients will depend on the concentrations of dissolved nutrients [35]. It is important to note that luxury uptake indicates the accumulation of a non-limiting nutrient above the levels required to maintain the current growth rate [48]. In this study, the cells growing in the PBR at harvest time presented limitation of dissolved Si and P and availability of nitrate (up to 456.6 μM) (Fig. 4 a–c; PBR). When the cells were transferred to the raceway pond, which was rich in dissolved nutrients, they rapidly used both dissolved P and Si and showed an increase of internal P and Si, in agreement with trend 1 (Fig. 4b and c). However, the cells did not show signs of nitrate accumulation in the raceway pond, in correspondence with trend 2. Moreover, the cells consumed the internal N pool until day 30, coinciding with the decrease in dissolved N. This might be because the cells presented a saturated internal N pool. According to Dortch [18] and Collos [35], dissolved nitrogen concentrations higher than 100 μM are necessary to saturate the internal N pool. In the present study, this value was greatly exceeded both in PBR and the raceway pond. Thus, the accumulation of intracellular nitrogen in *H. coffeaeformis* may explain the observed delay in TAG accumulation, which reached a maximum value on day 32. Moreover, this internal N pool might help to understand the variable effect of dissolved N on TAG accumulation in diatoms. For the purpose of designing large-scale cultures of *H. coffeaeformis* for biodiesel production, a possible strategy would therefore be to transfer sufficient N-cells (with a saturated internal pool of N) to a raceway pond without dissolved N. Under this nutritional scenario, the cells would use the intracellular accumulated N to grow briefly, thus optimizing TAG accumulation.

3.5. Bioproducts

The biochemical composition of *H. coffeaeformis* biomass harvested on day 32 showed 24.62% of cell walls or frustules and 75% of organic matter consisting of 25.20% total lipids, 18.54% proteins, 16.02% carbohydrates and 15.62% other components. The optimum moment to obtain a good lipid feedstock for biodiesel production was day 32, both in terms of lipid quantity and composition (see section 3.3, Fig. 3). At this stage the high TAG content ensures a high transesterification yield and the lowest proportion of phospholipids is obtained, which should be below 10 mg L^{-1} according to the European standard (EN 14214) [12]. As to the fatty acid profile in the neutral fraction, the high palmitoleic fatty acid content improves the biodiesel properties (calculated from fatty acid profile), according to the standards established in ASTM D6751-08 (US). On the other hand, although the relatively high PUFA content increases the Iodine Value (IV) to 149.6 g I_2/100 g (European standard (EN 14214) <120), it improves the cold flow properties of biodiesel. For example, the calculated cloud point of biodiesel from *H. coffeaeformis* (−4.6 °C) was better than that from soybean (0 °C), a conventional feedstock [42].

With respect to the remaining bioproducts after lipid extraction, approximately 50% of the biomass corresponded to organic matter and 25% to the frustules. The latter consist of two overlapping thecae (epitheca and hipotheca), each made up of the valve and several girdle bands bridging the circumference of the cell. The structural integrity of diatom frustules is an important requirement in diatom bionanotechnology, a newly evolving interdisciplinary field [28]. Fig. 5 depicts scanning electron microscopy images of frustules from both harvested biomass and residues after lipid extraction. Harvested biomass samples (untreated biomass) show cells with integral frustules

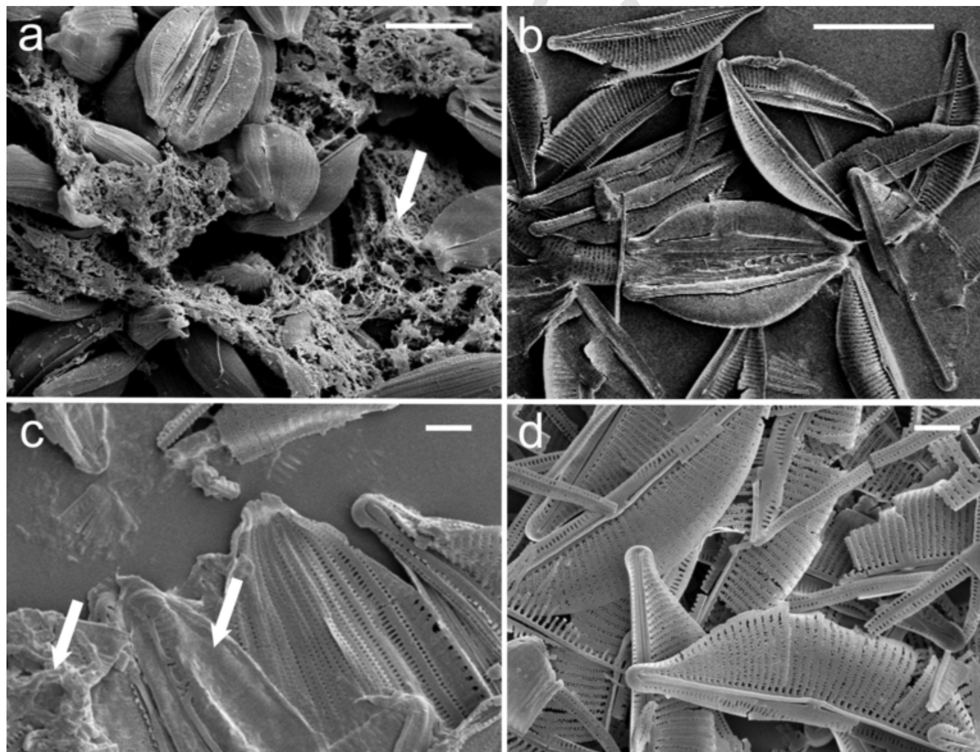

Fig. 5. Scanning electron microscopy images of frustules. **a-b**. Harvested biomass. **a.** Untreated biomass. The arrow indicates B-EPS. **b.** H_2O_2-biomass. c-d. Residues after lipid extraction. **c.** Untreated residue. The arrows indicate organic matter. **d**. H_2O_2-residue. Scale bars: a, b = 10 μm; c, d = 2 μm. Magnifications: a–b: X 3000; c–d: X 8700.

masked by bound exopolysaccharides (B-EPS) (Fig. 5a). When this biomass was treated with hydrogen peroxide (H_2O_2-biomass), the cells presented integral frustules free of B-EPS (Fig. 5b). Untreated residue samples (post-lipid extraction) showed moderately damaged frustules covered by organic matter (Fig. 5c). Finally, when these samples were treated with hydrogen peroxide (H_2O_2-residue), the cells had the same appearance as the samples shown in Fig. 5c, but they were free of organic matter (Fig. 5d). Thus, frustules obtained after lipid extraction for biodiesel production would have a limited use in nanotechnology due to the damage caused to their microstructure. However, they are potentially useful for abrasion, adsorption and insecticides, where structural integrity of frustules is not required.

Exopolysaccharides (EPS) are a further possible bioproduct. They mainly consist of glycoproteins and heteropolysaccharides [21,41] and can be classified as soluble EPS (S-EPS) or freely soluble in the aquatic environment, or bound EPS (B-EPS), associated with cell aggregates [21] (see Fig. 5a). Soluble EPS production in culture represents a source of valuable bioproducts because these substances present interesting bio-active properties for the pharmaceutical industry and in agronomy [49–51]. The concentration of soluble EPS (S-EPS) in the supernatant of the *H. coffeaeformis* raceway pond was 27.97 mg L^{-1}, exceeding the value of *N. cincta* (18 mg L^{-1}) [21], and those reported for several diatom species [49,52–55]. The highest concentrations of EPS cited in the literature in some diatom species were observed during the stationary growth phase in association with culture aging and nutrient deficiency [21,53,54,56]. In the present study, the culture lasted up to the declining growth phase, when both dissolved and internal nutrients reached their minimum values, this perhaps being the reason for the high S-EPS value observed. An additional factor to take into account is that S-EPS are easy to recover from the culture supernatant, as reported by Ref. [21].

3.6. Conclusions

In view of the present findings, a biorefinery scheme based on a hybrid two-stage culture of *Halamphora coffeaeformis* for biodiesel production is proposed (Fig. 6). The biomass is recovered by autoflocculation and the supernatant could be used as a source of soluble exopolysaccharides, a bioproduct with potential added value. The biomass could be subjected to lipid extraction processes to obtain biodiesel by transesterification. The remaining organic matter (approximately 50% of biomass) together with the frustules (26% of the biomass), could become a substrate for anaerobic digestion (AD). In fact, we suggest that AD could serve as a strategy to transform post-lipid extraction organic residues into methane simultaneously with frustule cleaning. Depending on the integrity of the frustules, these could represent an innovative source of amorphous silicate with potential industrial applications. In addition, AD produces liquid effluents rich in nutrients, which can be recycled for new cultures [57]. Although these hypotheses are supported in the literature [58], further studies are required to fully substantiate the concepts. In particular, Sialve et al. [58] have proposed that AD of microalgal residual biomass is an alternative pathway for improving the sustainability of the microalgal biodiesel process. In addition, the high nutrient storage capacity of *H. coffeaeformis* makes it a potential candidate for wastewater bioremediation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.renene.2017.10.086.

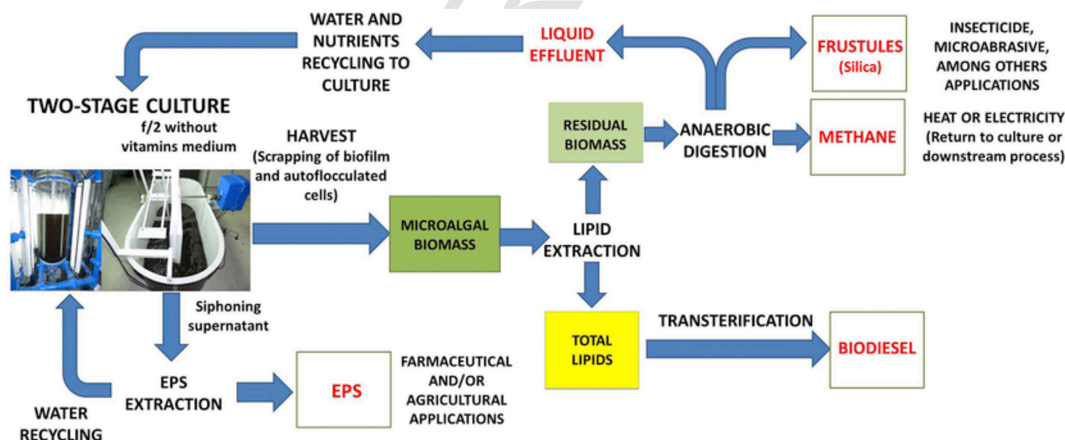

Fig. 6. Schematic diagram of proposed process of *H. coffeaeformis* biorefinery. EPS: exopolysaccharides.

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