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Simultaneous production assessment of triacylglycerols for biodiesel and exopolysaccharides as valuable co-products in *Navicula cincta*

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ABSTRACT

The marine benthic diatom *Navicula cincta* was cultured in order to evaluate its capacity to produce both neutral lipid-triacylglycerols (TAG) for biodiesel, and exopolysaccharides (EPS), as valuable co-products, under the same environmental conditions. The species was cultured without applying stress conditions, except for the ones naturally created by the culture age. Peaks of neutral lipid accumulation were estimated by fluorimetry by Nile Red. Consequently, lipids were extracted and fractionated into neutral and polar fractions and the fatty acid profile of each fraction analysed by GC. The stationary phase began on day 6, when phosphate and silicate reached limiting values for diatom growth. Total lipids and lipid fractions did not show differences between harvesting time points, reaching total lipid up to 41% of ash-free dry weight (AFDW) and TAG the dominant fraction (ca. 90% of total lipids). Particularly noticeable was the storage of palmitoleic acid (ca. 54% of total fatty acid methyl esters) and a lower level of polyunsaturated fatty acids, which may impart overall favourable properties to a biodiesel fuel, especially cold flow and oxidative stability. The maximum concentrations of EPS corresponded to soluble fraction, which was most significant when the cultures reached the stationary phase and when the medium was almost phosphate and silicate depleted. The characterization of soluble EPS indicated the presence of *N*-glycopeptides. According to the present results, a hypothetical scheme of demonstrative cultures under a biorefinery approach is proposed for *N. cincta*.

1. Introduction

Diatoms are important microalgae in the planet because they represent one of the major biomass and oxygen producers in the oceans [1]. Furthermore, they play a key role in the biogeochemical cycling of silica because they are surrounded by a highly structured silica cell wall [2]. There are more than 200 genera of living diatoms comprising approximately 100,000 species [3]. Despite their abundance and diversity, few diatom species are used for the production of biotechnologically relevant products [4,5]. Examples of some application include the following: (1) the use of silica from their cell walls, for technological applications in nanotechnology, pollution bioremediation, abrasive products, insecticides and (2) the production of intracellular metabolites, such as lipids, particularly eicosapentaenoic acid (EPA), and amino acids, for pharmaceutical and cosmetic applications [5,1]. However, the major biotechnological focus of diatoms has been on aquaculture, due to the ability of certain species to synthesize omega 3-polyunsaturated fatty acids (Ω3-PUFAs), e.g. EPA and docosahexaenoic acid (DHA) [6,7]. In addition, some species of diatoms can accumulate high triacylglycerol levels (TAG) as a carbon storage metabolite than can be easily transesterified to biodiesel. However, the competitiveness of biotechnologically relevant products from diatoms will depend on their cost of production.

Recent studies have indicated that economically feasible production of biodiesel by microalgae requires the use of a biorefinery approach. For example, the utilization of high added value components of the harvested biomass (e.g. β-carotene, PUFAs, astaxanthin, C-phycocyanin) contributes to both economic success and environmental sustainability [8,9]. However, the synthesis of these biomolecules is not at random. A biorefinery approach to biodiesel production requires an appropriate selection of microalgal species, as well as the optimisation of culture conditions to stimulate simultaneously the production of TAG and valuable co-products in a synchronized mode. Regarding diatoms, the

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production of lipids [11,12] and TAG [12–15] related to biodiesel production has been studied in some species. Although there is not a common pattern for all diatoms species, in general, the TAG production increases naturally during stationary growth phase [16,17] and when diatom cultures are imposed to starvation of nitrogen and silicate [12, 10] or phosphate and nitrate [14]. On the other hand, the production of EPA [18] and DHA [19] as valuable co-products has been highlighted. These long chain Ω-3 PUFAs provide significant health benefits, particularly in reducing cardiac diseases such as arrhythmia, stroke and high blood pressure [20]. However, and contrary to TAG production, the synthesis of PUFAs is more active in cells growing in exponential phase [16], under optimum levels of nutrients. Therefore, based on biorefinery concept, the production of TAG and valuable Ω-3 PUFAs in diatoms would not present the same environmental scenario.

Additionally, some diatom species are capable of producing exopolysaccharides (EPSs) during the stationary growth phase [21] or when the cells are either exposed to N or P limitation [22–24]. The composition of EPS is complex, mainly consisting of glycoproteins [25–27] and heteropolysaccharides that contain substantial amounts of uronic acid and sulphate residues [28]. The EPSs in benthic diatoms are related to their motility, ability to nutrient capture, substrate adhesion, biofouling, avoidance of desiccation, biofilm formation and bioestabilization of sediments [29–31]. Quantity and quality of EPS are strictly dependent on the sludge origin and extraction method used. Although classification of extracellular carbohydrate exudates can vary according to the extraction protocol employed, the benthic diatom EPS are often classified as soluble EPS (S-EPS) and bound EPS (B-EPS) [32,33]. The first, are readily soluble in the aquatic environment, while the second are associated with cells forming dense aggregates [32]. According to the degree of association, the bound EPS can be termed loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) [33].

Being polyanions, EPS exopolymers serve as biosorbing agents by accumulating nutrients from the surrounding environment and also play a crucial role in biosorption of heavy metals. This biadsorbent capacity has raised considerable interest in bioremediation assays [34,35]. The presence of anionic groups in EPS polymers is also amenable for interpolyelectrolyte complex (IPEC) production [36]. IPECs resulting from the interaction of polyanionic and polycationic polymers have multiple applications, for instance in controlled delivery of pharmaceutical or agronomic products [36,37]. Thus, the EPS could turn out to be a commodity by product in the biodiesel production from diatoms.

Therefore, the principal goal of the present study is to quantify and characterize the biochemical composition of lipids and EPS in the Argentinian *Navicula cincta* strain without applying stress conditions, except those naturally elicited by the culture ageing. Nile Red fluorescence was used to detect peaks of neutral lipid accumulation and consequently, to select the harvesting time in order to characterize and quantify lipids in the biomass. This baseline information will be useful to evaluate the potential of *N. cincta* to produce simultaneously TAG for biodiesel and EPS as a valuable co-product. In this way, strategies for large-scale cultures under a biorefinery approach can be established.

2. Materials and methods

2.1. Algal strain isolation and culture conditions

N. cincta was isolated from Bahía Blanca Estuary (South Atlantic coast, 38°45′–39°40′ S and 61°45′–62°30′ W) by micropipette technique and successive washes with sterile culture medium. The Bahía Blanca Estuary is located in a temperate climate region with annual average temperature of 20 °C. The estuary is eutrophic and turbid and presents an annual average salinity of 32 [38]. According to these environmental conditions, non-axenic cultures were established in Guillard's standard f/2 medium [39] at 20 °C and 32 of salinity. Light was supplied by cool-white fluorescent lamps in a 12:12 h light–dark cycle in order to get an average irradiance of 23 ± 2 μmol photons m^{-2} seg^{-1}; which was measured with a photoradiometer (LICOR, LI-192SB 2π Model). The f/2 medium was prepared with aged and filtered (0.45 μm Millipore) seawater from Bahía Blanca Estuary, autoclaved and the pH was adjusted to 8.0. For experiments, six batch cultures were carried out in 2-L Erlenmeyer flasks, which were filled with 0.9 L of f/2 and inoculated (1×10^5 cell mL^{-1}) with cells from mid logarithmic phase cultures in order to reach working volume of 1.2 L. The cultures were shaken by hand twice a day. Cell density, dissolved nutrient concentration, EPS and fluorometric intensities of chlorophyll *a* and Nile Red were measured. When, the combination of these data indicated peaks of neutral lipid accumulation, Erlenmeyer flasks were harvested. Volumes and inocula were estimated in order to have sufficient biomass for performing measurements of total lipids, lipid fractions, fatty acid profiles and EPS composition.

2.2. Morphological study

The species was identified through light microscopic examination of cells treated with 30% hydrogen peroxide according to [40]. Identification of the species *N. cincta* was confirmed using scanning electron microscopy LEO, EVO-40XVP (CC-CONICET, Bahía Blanca).

2.3. Growth, chlorophyll and biomass measurements

2.3.1. Growth and chlorophyll

Cell density (cells mL^{-1}) was determined by counting three replicate samples of 1 mL by means of a gridded Sedgwick-Rafter chamber under an optical microscope Leica DM 2000. Growth rate (k) was estimated during the period of exponential growth by least squares fit to a straight line of logarithmically transformed data [41]. Doubling time (t_d, days) was also calculated as t_d: ln 2/k.

Three millilitres of living microalgal cells by triplicate were taken every two days for 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.

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

Triplicate samples of pellets were resuspended and washed with 10 mL distilled water by centrifugation up to negative chlorination reaction. The samples were dried at 100–105 °C to a constant weight, cooled down in a vacuum desiccator, and weighed to obtain the biomass dry weight (DW). These oven samples were then ashed in a furnace at 450 °C 8 h, cooled in a vacuum desiccator, and weighed to obtain the AFDW.

2.4. Lipid analysis

2.4.1. Neutral lipid detection and kinetics

During growth, 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}) was added to a 5 mL cell suspension [42]. The mixture was vigorously agitated in a vortex mixer. Epifluorescence to detect the presence of intracellular bodies containing neutral lipids was detected 20 min after staining by means of a Leica DMIRE2 Confocal 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 subtraction of microalgal

cell autofluorescence and self-fluorescence of Nile Red and it was measured in arbitrary units (au).

2.4.2. Total lipid extraction

To extract lipids, cells were harvested at two time points of stationary growth phase according to Nile Red kinetics. Biomass was harvested by centrifugation (10 min at 3600 *g*), washed with distilled water and lyophilized. Lipid extraction was performed according to a modified Folch's method [43] assisted with ultrasound [44]. Duplicate freeze-dried samples of 150–200 mg of biomass were treated with 4 ml of chloroform: methanol (2:1 v/v), homogenized with a magnetic stirrer and ultrasonicated for 15 min at room temperature. Then, the mixture was centrifuged at 3000 g for 15 min and the supernatant was recovered and collected in a funnel separator three times. The supernatant was shaken with 4 ml of NaCl 0.9% (w/w) to obtain a biphasic system two times. The lower phase (containing the extracted lipids) was recovered into glass flask, evaporated to dryness under nitrogen stream and kept at −20 °C until lipid fractionation.

2.4.3. Lipid fractionation

Lipid fractionation into neutral lipids (NL) and polar lipids (PL) was performed using a silica cartridge Sep-Pack (SP) of 1000 mg (J.T. Baker Inc., Phillipsburg, N. J.) according to [13]. Glycolipids and phospholipids were put together in the same vial and denominated as polar lipids. Each fraction was collected into a conical glass vial and dried under nitrogen stream. Two replicates of each lipid fraction were made.

2.4.4. Methyl ester derivation and fatty acid methyl ester (FAME) analysis

For FAME profile determination, an aliquot of each lipid fraction (about 25–30 mg) was analysed according to [13] by methyl ester derivation and GC analysis.

2.4.5. Iodine value

The iodine values of *N. cincta's* oils were calculated according to AOCS recommended practice Cd 1c-85 [45]. This method estimates the grammes of halogen absorbed by 100 g of the fat.

2.5. Lipid productivity

The neutral lipid productivity was obtained according to [46]. Accumulation time was estimated from neutral lipid time course by using Nile Red fluorescence intensities.

2.6. Nutrient analyses

Five millilitre samples for dissolved nutrient determination were taken every two days and frozen until analysis. One hour before nutrient determination, the samples were defrosted and filtrated by Whatman GF/F (0.7 μm). Nutrient concentrations were determined via colorimetric assays in order to measure phosphate (PO_4^{3-}; P) [47]; nitrate (NO_3^-; N) and silicate (SiO_4^{4-}; Si) [48]. Absorbance was measured using a Spectronic 20 spectrophotometer at 543 nm for N, 885 nm for P and 810 nm for Si.

2.7. Determination of exopolysaccharide (EPS) concentration and composition

For EPS kinetics, five millilitres of samples were taken every two days. Soluble and bound EPS were analysed using a sequential extraction procedure. Soluble carbohydrates were recovered from the culture supernatant by centrifugation at 3500 *g* for 15 min (S-EPS). Loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) were obtained by extraction of the pellet, according to [29]. Namely, LB-EPS were solubilized by agitation in milliQ water for 1 h at 30 °C. The cellular pellet was further extracted with 1 ml 100 mM Na_2EDTA for 3 h at 4 °C to obtain TB-EPS [33]. Total carbohydrates were dosed by the phenol–sulphuric acid assay [49], using glucose as standard. Protein and uronic acid contents were determined by [50,51] using, respectively, bovine albumin and glucuronolactone as standards.

Monosaccharide composition was determined by gas chromatography (GLC) after hydrolysis of the product with 2 M trifluoroacetic acid (90 min, 120 °C) followed by conversion of the monosaccharides to their alditol acetates. GLC was carried out on a Hewlett–Packard 5890A gas chromatograph fitted with a fused-silica capillary column (30 m × 0.25 mm i.d.) WCOT-coated with a 0.20 μm film of SP-2330 operating isothermally at 220 °C. When necessary, GLC–MS analyses were carried out on a Shimadzu GC-17A gas–liquid chromatograph equipped with the SP-2330 capillary column interfaced to a GCMSQP 5050A mass spectrometer (Kyoto, Japan). Mass spectra were recorded over a mass range of 30–500 Da.

For the detection of aminosugars, the products were hydrolysed in 2 M trifluoroacetic acid (240 min, 120 °C). Anlayses were carried out in a Dionex HPAEC apparatus equipped with a pulse amperometric detector. A CarboPac PA20 column was used and eluted isocratically with 200 mM NaOH.

Infrared spectra were recorded with a Thermo Scientific Nicolet IS50 FTIR spectrophotometer, equipped with an ATR accessory, at 4000–500 cm^{-1}; 32 scans were taken with a resolution of 4 cm^{-1}.

2.8. Statistical analysis

Mean values of lipid classes (n = 4) and fatty acids (n = 4) between day 10 and day 14 were compared using a Fisher's least significant difference (LSD) test with a probability threshold of 0.05.

3. Results

3.1. Morphology, growth rate and chlorophyll a kinetics

The exponential growth phase in *N. cincta* was characterized by the proliferation of single cells with two chloroplasts lying along each side of girdle (Fig. 1a). The valves were linear-lanceolate or rhombic-lanceolate, with rounded ends, 22–28 μm long, 5.5–8 μm wide and with a central raphe (Fig. 1a). The striae were radial in the centre and gently convergent at the apices, with 10–11 striae in 10 μm (Fig. 1b). The stationary phase was associated with dense diatom aggregates, rather than with single cells (Fig. 1c). Epifluorescence microscopy revealed that during this phase most Nile Red-stained cells showed conspicuous yellow-gold fluorescent droplets occupying a large proportion of their cytoplasm (Fig. 1d and e).

Exponential growth phase occurred between days 2 and 6 (Fig. 2a), being the average growth rate (k) 1.42 ± 0.09 div day^{-1} and the doubling time (t_d) 16.8 ± 2.4 h. Stationary growth phase was sustained until day 14 with an average cell density of 300 ± 35 × 10^3 cells mL^{-1}. The Chl *a*-FI values showed a similar trend to cell density (Fig. 2a).

3.2. In situ neutral lipid kinetics

Neutral lipid accumulation in *N. cincta* was analysed by spectrofluorimetry following the NR-RFI kinetics (Fig. 2b). In addition, the relationship between NR-RFI and cell density was used in order to estimate time points of maximum neutral lipid accumulation. The NR-RFI values showed an increasing trend from day 8 to day 14, while the NR-RFI: cell density ratio increased 1.58-fold between day 8 and day 10. Thus, considering that the first maximum NR-RFI: cell density ratio was achieved on day 10, this time point was selected for a first harvest. Then, the second harvesting time was chosen on day 14 considering a decreasing trend of the NR-RFI: cell density ratio. This time point was regarded as the end of the experience.

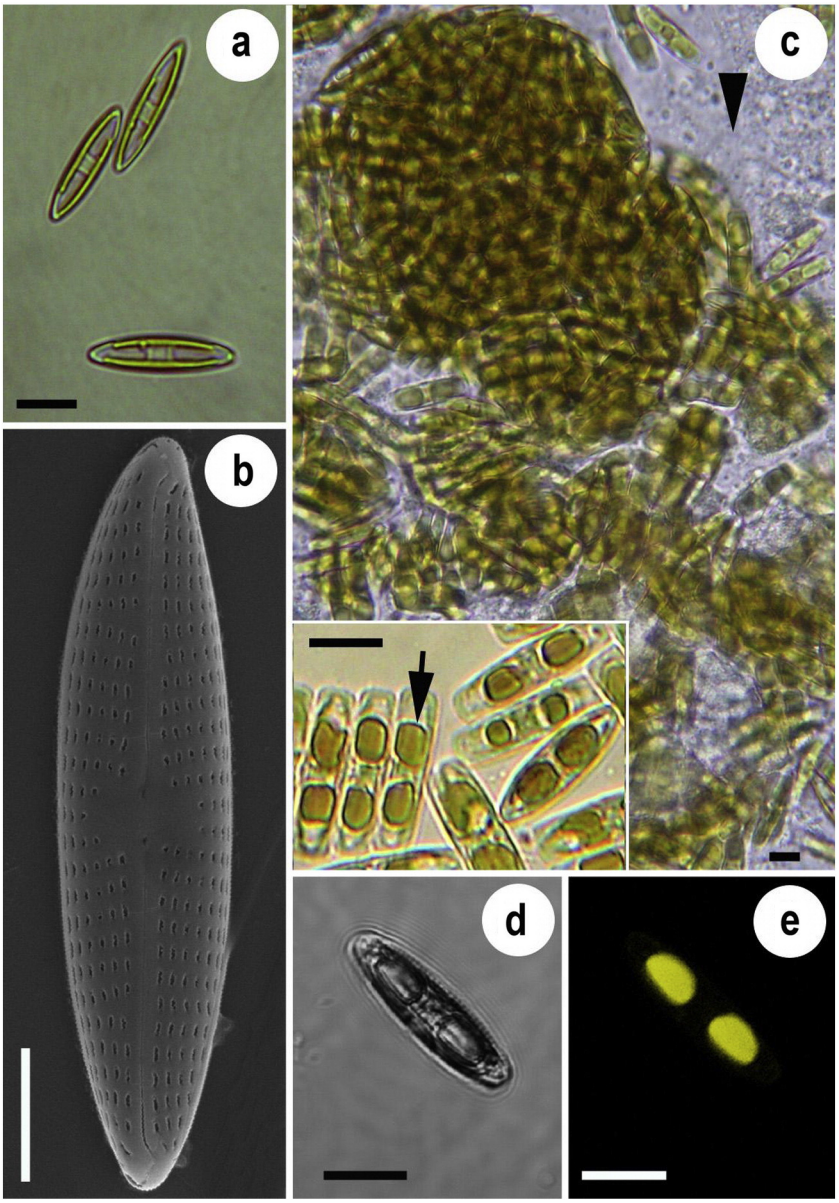

Fig. 1. a–e Morphological observations of *N. cincta* cells in culture (a) Single cells in exponential phase with parietal chloroplasts. (b) Detail of silica external valve view. (c) Cell aggregates in stationary phase. The arrowhead indicates the EPS. (c insert) Note lipid droplets stained with Sudan IV (arrow). (d–e) Nile Red stained cells in stationary phase showing neutral lipid droplets with phase contrast microscopy (d) and epifluorescent microscopy (e). (a, c) Light microscopy. (d) Scanning electron microscopy. Scale bars = 10 μm.

3.3. Total and fraction lipid contents

Table 1 shows the contents of both total and fraction lipids as percentages of ash-free dry weight biomass (% AFDW) as well as the percentages of neutral and polar lipids with respect to total lipids (% TL) on days 10 and 14. The lipid content ranged from ca. 38–41% AFDW on days 10 and 14, respectively. Neutral lipid content reached values up to ca. 35% AFDW, and represented the main fraction of total lipids, The lipid classes (saturated [SFA], monounsaturated [MUFA] and polyunsaturated fatty acids [PUFA]) and the main FAMEs of neutral lipids are shown in Fig. 3a and b, respectively. A comparison between days 10 and 14 indicated that the fatty acid composition of neutral lipids on day 10 presented significantly ($p \leq 0.05$) more SAFs (ca. 29%) and MUFAs (ca. 60%). This pattern was due to the higher percentages of palmitic acid (C16:0) and palmitoleic acid (C16:1n−7) on day 10. Furthermore, neutral lipids on day 10 presented significantly ($p < 0.05$) less PUFAs (ca. 11%), with the EPA (C20:5n3) reaching 8%. In addition, the total fatty acid profiles of both neutral and polar lipids in *N. cincta* on both days are shown in Online Resource 1.

3.4. Nutrient kinetics

The dissolved nutrient concentrations decreased exponentially during the first 6 days of growth (Fig. 4). In this period, the consumption rates (μM d^{-1}) for each nutrient were: 2.1 (μM d^{-1}) for phosphate, 81 μM d^{-1} for nitrate and 21.9 μM d^{-1} for silicate. Minimum concentrations of phosphate (6.3 μM) and silicate (5.1 μM) were reached around the 8th day of incubation, while minimum nitrate concentration (169.6 μM) was reached recently on 14th day. The minimum values of phosphate, nitrate and silicate were about 20%, 22% and 3%, with respect to the initial concentrations, respectively. Fig. 4 shows that *N. cincta*'s cultures were subjected to limiting phosphate and silicate values for diatom growth [52] from day 6, while nitrate was never limiting.

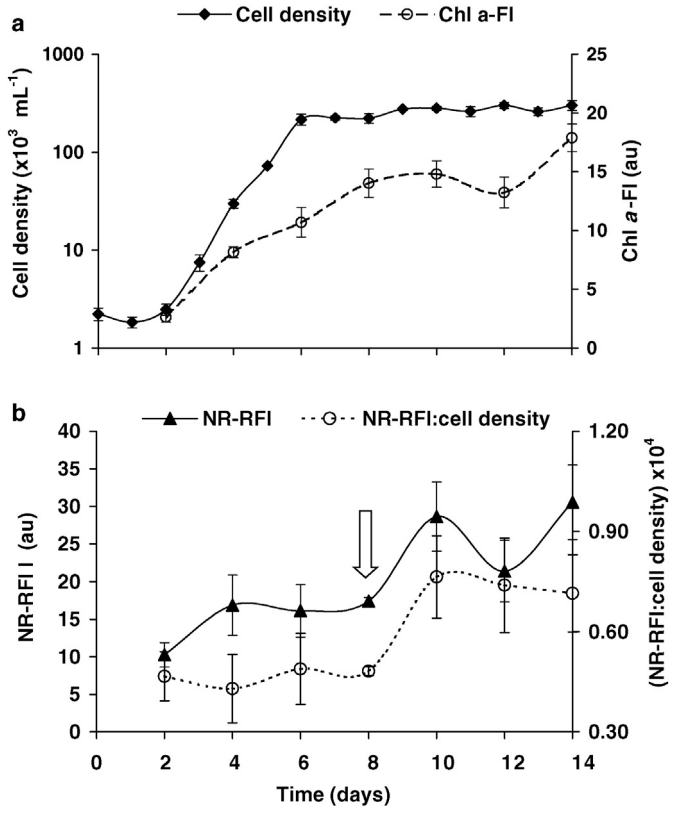

Fig. 2. *N. cincta* growth and neutral lipid accumulation. Growth curve and time course of fluorescence intensity of chlorophyll *a* (Chl*a*-Fl) (a). Time course of relative fluorescence intensity of neutral lipids (NR-RFI), and NR-RFI:cell density ratio (b). The arrow indicates the beginning of neutral lipid accumulation. The data are expressed as the average ± standard deviation of two or three independent samples.

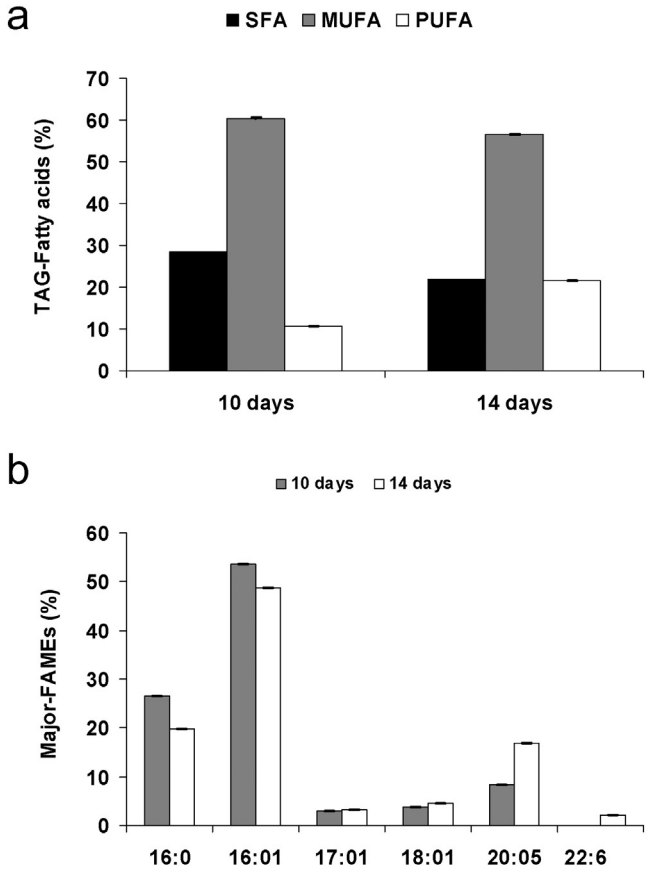

Fig. 3. Neutral lipid (TAG) composition of *N. cincta*. Fatty acid classes (a) and main fatty acid methyl esters (FAMEs) (b). SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. FAME values are expressed as the average of four independent samples.

3.5. EPS quantity and composition

Fig. 5 shows the temporal dynamics of the EPS fractions extracted from *N. cincta*'s cultures. The contents of soluble EPS (S-EPS) were low (≈8–12 mg L^{-1}) on the first days of incubation, i.e. during the period of the initial growth phases. When the stationary growth phase was established and the culture medium depleted of phosphates, a higher increase in S-EPS content was verified. This took place around the 8th day of incubation. Tightly bound EPS (TB-EPS) fraction showed higher production during exponential growth phase; while it decreased significantly in the stationary phase. In turn, loosely bound EPS (LB-EPS) fraction did not exhibit a defined pattern; however, the highest levels were observed on day 14.

General characterization and neutral monosaccharide composition of the products is depicted in Table 2. Uronic acids were negligible, according to the detection of the colorimetric method. All the products contained significant amount of proteins, being the carbohydrate:protein ratio 1:1 for S-EPS and LB-EPS and 1:2 for TB-EPS. LB-EPS was heavily contaminated with residual salts from culture medium, leading to an overestimation of its yield. In all cases, monosaccharide composition suggests great heterogeneity of the products. S-EPS and TB-EPS were xylose, mannose and *N*-acetylglucosamine enriched, while in LB-EPS the content of aminosugars was much lower and consisted mainly of galactose, glucose and rhamnose (Table 2, Online Resource 2). The absence of uronic acids was also confirmed by Dionex HPAEC.

Table 1
Total lipid and lipid fractions-neutral and polar — (as percentage of ash-free dry weight biomass = % AFDW) of *N. cincta*. In addition, relative proportions of lipid fractions (in percentage of total lipid = %). Values are means of two independent samples.

Day	Total lipids (% AFDW)	Neutral lipids (% AFDW)	Polar lipids (% AFDW)	Neutral lipids (% TL)	Polar lipids (% TL)
10	37.9 ± 2.2	34.1 ± 2.7	3.8 ± 2.7	90.0 ± 2.8	10.0 ± 2.8
14	40.8 ± 2.2	34.6 ± 3.8	6.2 ± 3.2	84.7 ± 2.5	15.3 ± 2.5

4. Discussion

Potential advantages of some microalgae for biofuels include their ability: 1) to grow at high rates (e.g. 1–3 div d^{-1}); 2) to accumulate large TAG levels; 3) to inhabit in saline waters; and 4) to produce value-added co-products. In addition, techno-economic analyses suggest that for profitable use of microalgae in biofuel process, the species should have non-resistant cell walls to lipid extraction and possess harvesting advantages (e.g. autoflocculation). In the present study, the marine benthic diatom *N. cincta* growing under a standardized culture medium (f/2) at 20 °C and at very low light intensity (23 ± 2 μmol photons m^{-2} seg^{-1}), required ca. 17 h to duplicate a generation. This fact indicates an adequate growth rate in comparison with other *Navicula* species cultured at the same temperature [53,54]. In addition, its robustness was evident from the ability of *N. cincta* to grow at very low irradiances in comparison with other benthic marine diatoms (cultured to 600 μmol m^{-2} s^{-1}) [55]. This feature is advantageous for a sustainable use of the species as fuel resource, especially when larger scale cultures may cause a shadow effect due to biomass accumulation. Another interesting aspect of this species is its behaviour. It can proliferate in the bottom of the culture forming a biofilm, as well as grow in suspension by the effect of aeration, autoflocculating when air is suspended. These advantages are major to achieve an easy inexpensive

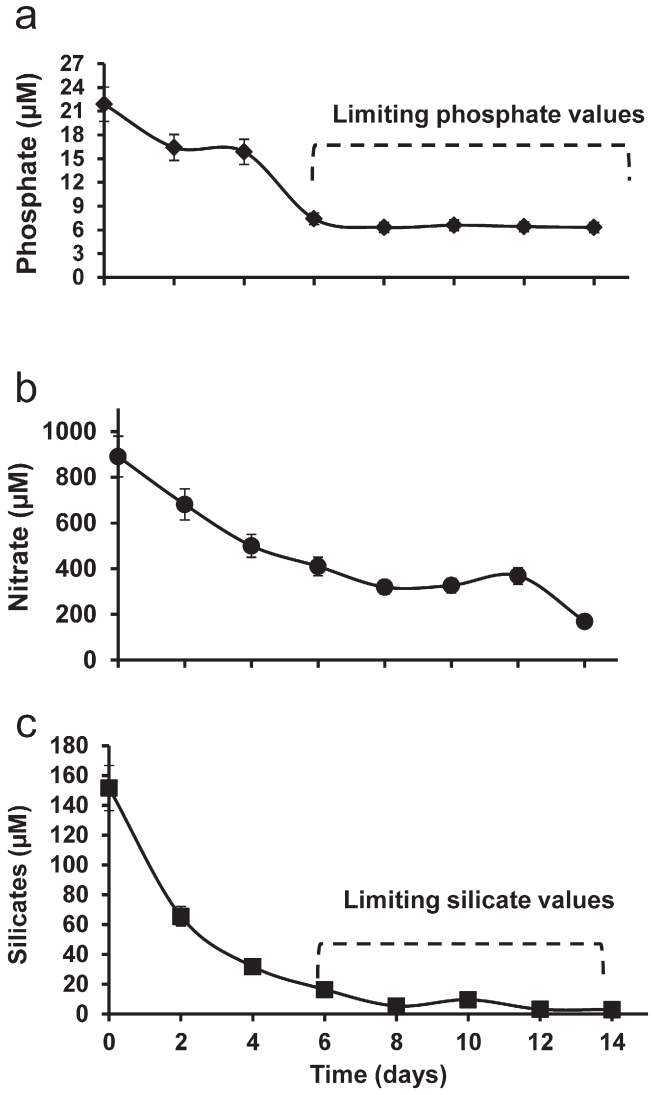

Fig. 4. Time course of phosphate (a), nitrate (b) and silicate (c) consumption of N. cincta cultures. Limiting phosphate and silicate values are shown. Nutrient limiting values for diatom growth (P < 8.9 μM; N < 10.2 μM; Si < 22 μM) according to [52].

biomass harvest. For example, gravity sedimentation together with siphoning off the supernatant is widely accepted as a viable harvesting method in wastewater treatment processes [9].

For industrial purposes, interesting microalgal strains are those that produce 20% or more of oil relative to their biomass in dry weight [56]. Particularly when diatom's biomass is evaluated, it is important to consider the high ash content attributed to the silica component in their cell wall. Thus, the total lipid content reported in the specie studied varied between ca. 38% and 41% of AFDW after 10 and 14 days of growth, respectively. These values are similar to those obtained in three *Navicula* strains under nitrogen deficiency (32.4%–38.2% of AFDW). These strains were suggested for fuels-from-algae technology development [53]. However, triacylglycerol determination is the clearest signal of the amount of suitable substrate for biodiesel production by transesterification [17]. At present, there are still a few studies reporting TAG content (% AFDW) in marine diatoms, which would allow a better comparison when diatom strains are screened for feasible biodiesel production. In this study, the neutral lipids were 90% and 84.7% of the total lipids on days 10 and 14, respectively. These values indicate a high accumulation of neutral lipids with respect to polar lipids with ageing of culture. In comparison with other marine diatoms growing in batch cultures, the TAG content of *N. cincta* was much higher than the one in *Navicula jeffreyi* (total lipid content of 6.6% dw and TAG proportion with respect to total lipid of ca. 57%), which was cultured without forcing oil synthesis by using chemical or physical stimuli [54]. TAG content was also higher than those reported for *Thalassiosira pseudonana* (14–18% dw) and *Phaeodactylum tricornutum* (14% dw), which were cultured under nitrate or silicate starvation [12]. In the present study, stress condition was obtained naturally when the cells were in transition to stationary phase. In fact, the neutral lipid accumulation monitored via Nile Red (NR-RFI: cell density ratio) indicated that neutral lipids started to accumulate approximately 2 days after cultures entered in stationary phase, i.e. when growth stopped. Thus, *N. cincta* showed neutral lipid productivities of 9.95 mg TAG L^{-1} $d^{-1}$and 4.22 mg TAG L^{-1} d^{-1} on days 10 and 14, respectively. These values were even higher that the total lipid productivity values of other benthic diatoms as *Nitzschia* LBK-017 (2.13 mg LT L^{-1} d^{-1}) [57] and *Navicula* sp. (3.67 mg LT L^{-1} d^{-1}) [58]. It is important to remark that the TAG productivity of *N. cincta* must be overcome to build sustainable large scale cultures for biofuel production.

According to ranges of half-saturation constants for phosphate (Ks–P: 0.01–8.9 μM), orthosilicic acid (Ks–Si: 0.2–22 μM) and nitrate (Ks–N: 0.02–10.2 μM) uptake in diatoms [52], we can suggest that the minimum levels of phosphate (ca. 6 μM) and silicate (ca. 5 μM) reached in the present study might be involved in the TAG accumulation in *N. cincta*. In fact, an important decrease of P and Si with respect to N was indicated in the context of both Redfield et al. [59] and Brzezinski [60] ratios (N:Si:P of 16:16:1), with N:Si and N:P ratios up to ca. 117 and 60, respectively. In addition, nitrate did not reach limiting values. As reported by [14], the depletion of dissolved phosphate might be an early trigger for lipid accumulation in *P. tricornutum*, but the accumulation rate would only be magnified upon nitrate depletion. Diatoms store

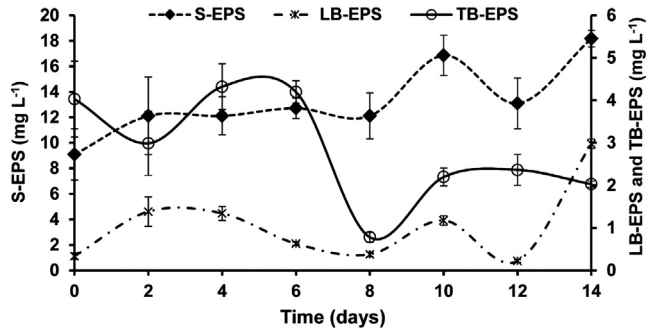

Fig. 5. Time course of soluble EPS (S-EPS), loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) in *N. cincta* cultures.

Table 2
General characterization and neutral monosaccharide composition of soluble EPS (S-EPS), loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) products. Data are expressed as average ± SD of two replicates. a. Yield expressed as mg of product L-1; b. Yield expressed as % of initial freeze-dried cell biomass; c. Traces (less than 1%).

	S-EPS	LB-EPS	TB-EPS
Yield	10.9[a]	70.7[b]	2.4[b]
Carbohydrates:protein ratio	1:1	1:1	1:2
Monosaccharide composition (molar %)			
Fucose	10	12	5
Arabinose	7	Tr.[c]	13
Xylose	23	7	26
Mannose	26	2	13
Galactose	4	40	6
Glucose	4	33	12
N-acetylgalactosamine	4	Tr	9
N-acetylglucosamine	22	6	16

[a] Yield expressed as mg of product L^{-1}.
[b] Yield expressed as % of initial freeze-dried cell biomass.
[c] Traces (less than 1%).

significant amounts of nitrate internally [61]. Therefore, one way to increase naturally the accumulation of lipids in *N. cincta* may be by decreasing the initial concentration of nitrate in the culture medium. Considering that in the present study the initial concentration of nitrate was ca. 900 μM and that the nitrate consumption rate was 21.9 μM d^{-1} throughout 6 days of exponential growth, we suggest that cultures of this strain oriented to biodiesel production should begin with nitrate concentrations not higher than 100 μM in order to reach limiting values <10.2 μ [52], which may produce a higher lipid accumulation.

In general, the fatty acid composition in diatoms depends on the growth phase (culture age), temperature and nutrient status [62–64]. Phosphate limitation has been indicated as a factor associated with a higher amount of SFAs and MUFAs and lower values of PUFAs in marine diatoms [65]. These authors indicated that both a drop in the cellular ATP level and in the rate of phosphorylation-linked metabolic pathways may direct the fatty acid biosynthesis pathways to more energetically economical routes, yielding mostly SFAs and MUFAs. Then, we suggest that the limitation of P levels reported in our study contributed to *N. cincta*'s oil quality, which presented a dominance of MUFAs followed by SFAs. In addition, a significant effect of the culture period was also detected on lipid class content. Hence, the fatty acid composition of the biomass harvested on day 10 displayed the best profile in terms of biodiesel quality, reaching the highest levels of SFAs and MUFAs, due to the highest levels of both palmitic (26.5%) and palmitoleic (53.6%) acids. As reported by [66], fatty acid profiles enriched in palmitoleic acid may impart overall favourable properties to biodiesel, especially cold flow. However, oils with high enrichment of palmitoleic acid are currently commercially unavailable. Therefore, the *N. cincta*'s oil enriched in palmitoleic and palmitic acids in combination with low levels of high-melting saturated species of fatty acids, such as C_{20} and C_{22}, exhibits a combination of improved fuel properties with emphasis in cold flow issue.

Regarding oxidative stability, the European EN 14214 standard [67] limits linolenic acid's methyl ester (ALA) for vehicle use to 12% (w/w) and the methyl esters with four and more double bonds to a maximum of 1% (w/w). These limits are essential to avoid autoxidation by the presence of double bonds in the chains of many unsaturated FAMEs, which cause problems during fuel storage [68]. The oils extracted from *N. cincta* presented ALA contents within specifications. Although the calculated iodine values for *N. cincta*'s oil (57.87 g I_2/100 g and 53.77 g I_2/100 g on days 10 and 14, respectively) were well below the allowed [67] (<120 g I_2/100 g), unsaturated fatty acids, especially EPA, should be present at lower levels in order to achieve fuel property enhancement in terms of oxidative stability. In the present study, the lowest level of EPA was reached on day 10, when the NR-RFI:cell density was maximum. Consequently, it would be necessary to take into account fatty acid variations along the stationary growth phase in *N. cincta*'s cultures in order to obtain a good oil quality for biodiesel production. Moreover, if the purpose is to characterize biodiesel quality, other specifications should be taken into account in the standards, including acid values, free and total glycerine and heteroelements [66].

The data presented in this study demonstrate that *N. cincta* was an effective producer of exopolysaccharides. The maximum concentrations of EPS corresponded to soluble fraction whose yield (9–18 mg L^{-1}) fell within the average yields reported in literature for other diatom species (0.6–26 mg L^{-1}) [23,32,26]. S-EPS release was most significant when the cultures reached the stationary phase and when the medium was almost phosphate and silicate depleted. A possible explanation for EPS secretion is that it may happen because of an overflow metabolism, which has been defined as an excess amount of carbon dioxide fixed relative to growth requirements [70]. This behaviour agrees with the response of other diatoms under an increase in the N/P ratio above the classical Redfield ratio of 16 [65,69]. However, the type and amount of polysaccharide excreted and the effects of nutrient limitation are often highly species-specific. The light–dark cycle is another factor that affects the EPS production, being S-EPS independent of the light, and bound EPS highly light-dependent [32]. According to these authors, bound EPS may be transformed to soluble EPS in the dark or when the light availability decrease due to the size of the aggregates. In the present study, S-EPS were relatively constant and their levels increase at the end of the experience. In addition, T-EPS declined from day 6 until the end of the experience, which might explain the observed increase in S-EPS. On the other hand, the T-EPS decrease was in agreement with the beginning of the stationary phase, when the cell aggregation was notable and light availability may be limiting. Cell aggregation revealed a decrease in motility of the cells with the consequent less production of mucilaginous trails associated with bound fractions. Moreover, the composition of ATR-IR spectra of S-EPS and TB-EPS was similar, suggesting that soluble products in the culture solution would derive from partial solubilization of T-EPS, as reported by [26,27].

The comparison between the composition of EPS and mucilage fractions with previous literature data is difficult since there is not a unique extraction procedure [26]. Thus, differences in reported monosaccharide composition may actually respond to variation in the extraction protocols. Another important issue concerning to EPS's determination is the contamination with intracellular biopolymers leaking out of damaged cells. Even a mild procedure as room temperature aqueous extraction was claimed to provoke cell rupture [71]. As a consequence, products are usually enriched in chrysolaminaran [72]. In our case, room temperature extraction of cells increased glucose content in LB-EPS, even to a further extent than in the hot water extracted TB-EPS, indicating membrane damage by the hypotonic extraction solvent. In addition, prolonging acid hydrolysis can result in the destruction of the more labile sugar components (i.e., arabinose) as it was reported by [21]. Accordingly, no arabinose could be detected in products analysed by HPAEC-PAD, but the presence of aminosugars was revealed. On the other hand, no uronic acids appeared, in agreement with colorimetric determinations. Carbohydrate:protein ratio and the identification of *N*-acetylglucosamine and *N*-acetylgalactosamine suggests the presence of proteoglycans and/or glycoproteins in the EPS. The IR spectroscopy also showed the presence of carbohydrate and protein bands. In general, signals were poorer in LB-EPS, but this is not surprising since this product was heavily contaminated with salts. Assuming a glycoproteic composition for these products, both are xylose and mannose rich, similarly to the informed for *Craspedostauros australis* and *P. tricornutum* [25,73]. This fact, together with the presence of significant percentages of *N*-acetylglucosamine, suggests the presence of *N*-glycoproteins. Taking into account that attached *N*-glycans are responsible for a number of important biological recognition processes, such as intracellular sorting, cell adhesion, host-pathogen interaction and immune response, further characterization of glycoproteins in EPS could result in by-products of pharmaceutical applications.

5. Conclusions

The present results indicated that *N. cincta* under the environmental conditions tested can be proposed as a valuable microalga for biodiesel production for the following reasons: 1) it has a relatively small size, which increases its growth rate; 2) it grows in seawater, a resource that does not compete with freshwater for human consumption; 3) it can grow at low levels of light radiation, which is particularly important for the achievement of sustainable energy cultures; 4) it can naturally store high contents of TAG in a short time (10 days); 5) its TAG's fatty acid profile is adequate for a good quality biodiesel; 6) its production of bound EPS in culture promotes the biofilm formation, which may improve the biomass harvest by making the downstream processes in biofuel production less expensive; and 7) its production of soluble EPS in culture represents a valuable co-product, which can be obtained by ethanol precipitation from aqueous phase. This fact also contributes increasing profitability of biodiesel production processes. In addition, we suggest that cell walls rich in silica (25% of biomass estimated by

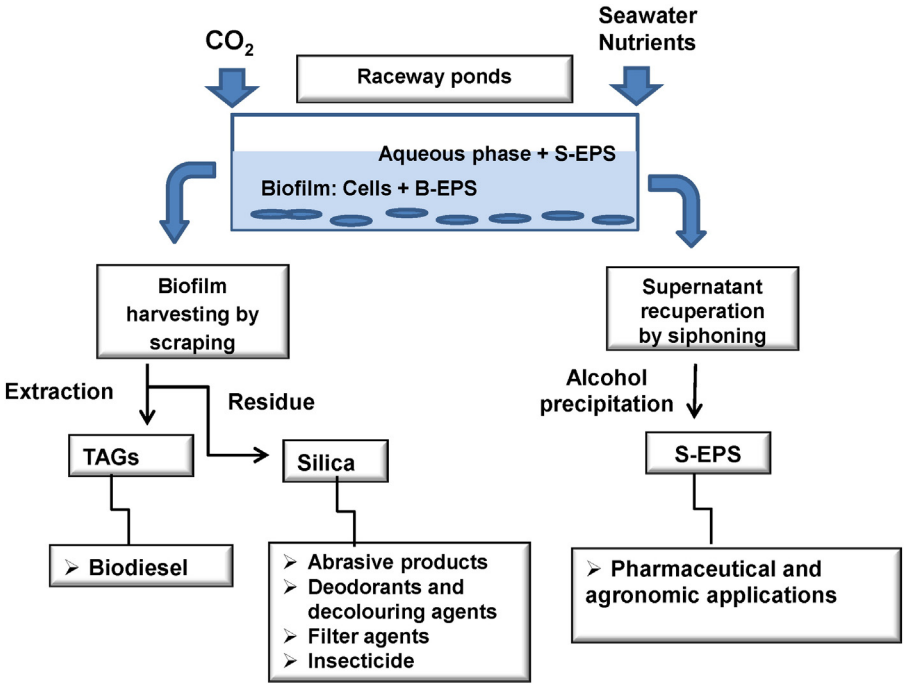

Fig. 6. Hypothetical scalable bioprocesses in *N. cincta*'s culture under a biorefinery approach. Under the same environmental scenario (stationary phase and limitation of P and N), apart from TAG for biodiesel, the product portfolio includes S-EPS and silica. Additional processes as biofilm formation and carbon sequestration may contribute to a sustainable procedure.

AFDW) represent a valuable residue, which may be used in different industries according to their quality. We consider that the synchronous production of TAG for biodiesel and S-EPS as valuable co-products in marine benthonic diatoms lends an interesting perspective to sustainable production of biofuels. Then, this study provides the bases of our next objective, which is to transfer the biorefinery approach in *N. cincta* cultures from laboratory to pilot scale production. As a first approximation, a hypothetical culture scheme of *N. cincta* in raceway pond is presented (Fig. 6), keeping in mind the added advantages of this system for CO_2 sequestration.

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