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ABSTRACT

The fresh water microalga *Neochloris oleoabundans* was investigated for its ability to accumulate lipids and especially triacylglycerols (TAG). A systematic study was conducted, from the determination of the growth medium to its characterization in an airlift photobioreactor. Without nutrient limitation, a maximal biomass areal productivity of 16.5 g m^{-2} day^{-1} was found. Effects of nitrogen starvation to induce lipids accumulation was next investigated. Due to initial *N. oleoabundans* total lipids high content (23% of dry weight), highest productivity was obtained without mineral limitation with a maximal total lipids productivity of 3.8 g m^{-2} day^{-1}. Regarding TAG, an almost similar productivity was found whatever the protocol was: continuous production without mineral limitation (0.5 g m^{-2} day^{-1}) or batch production with either sudden or progressive nitrogen deprivation (0.7 g m^{-2} day^{-1}). The decrease in growth rate reduces the benefit of the important lipids and TAG accumulation as obtained in nitrogen starvation (37% and 18% of dry weight, respectively).

1. Introduction

Owing to some of their intrinsic capacities, photosynthetic microorganisms are found as one of the more promising primary resources. Compared to plants, higher areal productivities can be achieved due to higher photosynthetic conversion efficiencies. Considering the increasing worldwide energy demand, photosynthetic microorganisms are thus often discussed as a solution to set up sustainable future energy production systems. Because photosynthetic growth imposes a consumption of inorganic carbon in the form of dissolved carbon dioxide, carbon neutral production can be achieved. In addition, depending on applied culture conditions and cultivated species, high energy compounds can be produced such as hydrogen, lipids for biodiesel application, sugars for biomass fermentation or gasification (Benemann, 2004; Maria L. Ghirardi, 2000; Melis, 2002; Scragg et al., 2002; Spolaore et al., 2006; Tsukahara and Sawayama, 2005).

Because of the variety of energy vectors, the optimal use for energetic purpose of algae is still under debate. For example, although great breakthroughs were achieved during last decade (Melis and Melnicki, 2006), H_2 production using green algae seems at the moment far from industrial application because of too low productivities. This is mainly due to the fact that the specific metabolism leading to H_2 release is not yet clearly elucidated. On the contrary, lipids production for biodiesel application appears as a rather mid-term solution. Lipids accumulation in microalgae is indeed well known as stated by many examples of their uses in aquaculture for fish or mollusks feeding (Borowitzka, 1997; Richmond, 2004a). Although numerous studies on the use of microalgae in aquaculture exist, application in biodiesel production is less documented despite the specific problems involved (reviews can be found in Chisti (2007) or Hu et al. (2008)).

Biodiesel must comply with some specifications for engine application such as cetane number, viscosity, flash and solidifying points (Miao and Wu, 2006). Lipids issued from a vegetal source (soybeans, rapeseeds, palm, jatropha, etc.) have to be transesterified leading to fatty acid methyl esters (FAME or biodiesel) that fulfill diesel characteristics (Miao and Wu, 2006). For prolonged storage, biodiesel must present a low sensitivity to oxidation that is correlated to the unsaturated nature of fatty acids measured by its iodine value. Polyunsaturated fatty acids (PUFA) are, in this context, not ideal for biodiesel application. Neutral lipids, mainly TAG, have to be preferred to phospholipids or glycolipids because of their higher percentage of fatty acids and lack of phosphate. Contrary to glyco- or phospholipids which have an important structural role in cells, TAG synthesis is usually attributed to carbon and energy storage. TAG cellular accumulation corresponds to a shift in metabolism under unfavorable environmental conditions. Nitrogen starvation is known to trigger TAG accumulation to the detriment of cell division. Lipids process productivity depends on both lipids content in cell and biomass accumulation. Optimal culture conditions are different for cell lipids content and biomass productivity. Then, maximizing lipids productivity is therefore not trivial.

In this study, *Neochloris oleoabundans* was retained to investigate lipids production, especially when nitrogen deprivation is applied. Although poorly documented, this strain is known to accumulate TAG, which makes it interesting for biodiesel application (Tornabene et al., 1983). A recent study has confirmed its interest for biodiesel production with a total lipids content up to 40% obtained in nitrate starvation conditions and a total lipids productivity of 0.133 kg m^{-3} day^{-1} (Li et al., 2008). The present systematic study will give additional information by conducting a deep investigation in a flat-panel PBR. In addition to standard growth, different conditions leading to nitrogen deprivation were applied to induce lipids accumulation. Because nitrogen starvation influences the TAG/total lipids ratio, total lipids and TAG productivities were considered separately. This study also highlights the specific physiological responses that could affect PBR production for further optimization. Light transfer conditions, as a relevant parameter of PBR, were discussed in this purpose.

2. Methods

2.1. Algal strain and cultivation conditions

Experiments were conducted with *N. oleoabundans* (strain 1185 from the culture collection of the University of Austin, Texas). The medium described later was a modified bold basal medium (BBM). Cultivation conditions were pH 7.5 and $T = 25$ °C in all experiments.

2.2. Airlift photobioreactor description

A flat-panel airlift PBR was used for experiments. The light supplying device was placed in front of the PBR perpendicularly to its optical surface. The light source was composed of a set of eight daylight fluorescent tubes (Sylvanialuxline FHO 24W/TS/860) placed horizontally and parallel to the front side of the PBR with the same height as the PBR. Air was injected at the bottom for culture mixing with a constant air flow rate of 0.5 L min^{-1}. PBR consisted of three parts: one central where air was injected (riser) and two lateral for culture recirculation (downcomer). This ensured good mixing condition and prevented cells sedimentation.

PBR volume was 1 L with a depth of culture $L_z = 30$ mm (perpendicular to the optical surface). The illuminated surface to volume ratio of the reactor was equal to 33.3 m^{-1}. PBR was built in transparent polymethyl methacrylate (PMMA) except for the rear side that was in stainless steel for reactor cooling by ambient air blowing (fan). PBR was set with a complete loop of common sensors and automations for microalga culture, namely temperature, pH and gas injections (CO_2 and air). pH was regulated by automatic injection of CO_2 and temperature by ambient air blowing. Before all experiments, the PBR was sterilised 30 min with a 5 mM peroxyacetic acid solution.

Continuous cultures were realized in chemostat mode under continuous light illumination. A KNF Stepdos® pump (Stepdos pump 08/RC, KNF Neuberger) was used for medium injection while the culture was harvested by overflowing. A constant dilution rate was applied and daily controlled (for batch cultivation, the dilution rate was null).

The incident light flux q_o or photons flux density (PFD) was measured in the 400–700 nm waveband (photosynthetically active radiation, PAR) for different distances between PBR and tubes using a flat cosine quantum sensor (Li-190SA, Li-COR, Lincoln, NE). The incident light flux was obtained by averaging sensor measurements for 12 different locations on the PBR front side. A variation less than 10% was observed showing a homogeneous illumination of the PBR's optical surface. A PFD value of $q_o = 270$ μmole m^{-2} s^{-1} was applied in all experiments.

2.3. Analytical methods

2.3.1. Dry weight and elementary analysis

The algal dry weight was determined by filtration through a pre-dried and pre-weighed glass-fiber filter (Whatman GF/F). The filter were dried 24 h at 105 °C, cooled in a dessiccator and then weighed again. Results given are the average of three measurements. For elementary analysis, biomass samples were concentrated by centrifugation (6′ at 3600*g*) and lyophilized. Elementary analysis was performed by the CNRS Central Analysis Service (Vernaison, France).

2.3.2. Pigment content

Pigment concentration was determined using a spectrophotometric method. Pigments were extracted with methanol (99.9%) during 30 min in the dark at 45 °C. Samples were centrifugated (13000 rpm, 5 min) before measurements. Absorption spectrum was collected in the range 400–750 nm on a spectrophotometer Jenway 6500. Chlorophyll-*a* (CChl-*a*), chlorophyll-*b* (CChl-*b*) and photoprotective carotenoids (CPPC) concentrations were determined according to the equations of Ritchie (2006) for chlorophylls and Strickland and Parsons (1968) for carotenoids:

$$[\text{Chl-}a]\mu\text{g/ml} = -8.0962 \times A_{652} + 16.5169 \times A_{665}$$
$$[\text{Chl-}b]\mu\text{g/ml} = 27.4405 \times A_{652} - 12.1688 \times A_{665}$$
$$[\text{Carotenoids}]\mu\text{g/ml} = 4 \times A_{480}$$

Absorbencies at 480, 652 and 665 nm were corrected from turbidity by subtracting absorbencies at 750 nm. Pigment content was obtained by dividing by biomass concentration. Results given were an average of three measurements.

2.3.3. Nitrate, sulfates and phosphate concentrations

These three mineral nutrients concentrations were measured using anionic chromatography (DIONEX-120, IonPac AS14A anionic column with SRS). Eluant was a solution of 8 mM Na_2CO_3 and 1 mM $NaHCO_3$ with a flow of 1.0 ml/mn at RT.

2.3.4. Total lipids and TAG

Total lipids were measured following the modified protocol of Folch as proposed by Christie (1989). A microalgae volume chosen for obtaining finally 5–10 mg of lipids was concentrated by centrifugation (6 mn at 3600*g*) and transferred into a glass tube. All following centrifugations were done 2 mn at 3600*g* if not precised. After being centrifuged, supernatant was eliminated and extraction started with 2 ml of methanol for 1 h. Then 4 ml of chloroform were added and mixed for 2 h. After being centrifuged, supernatant containing lipids was transferred into another tube and residue was extracted a second time during 30 mn with 3 ml of a mixture of methanol/chloroform (1/2). After being centrifuged, supernatant was pooled with the first one and washed with 2.25 ml of a 0.88% KCl solution. Separation in two phases was accelerated by centrifugation and a second washing was done with 0.75 ml of methanol and 0.75 ml of 0.88% KCl solution. After centrifugation, the lower layer was transferred into another glass tube and centrifuged 6 min at 3600*g* for complete elimination of water and solids. Solvent was evaporated under nitrogen at 40 °C, dissolved with 1.5 ml of chloroform, transferred into a 4 ml pre weighted vial and dried until constant weight.

Besides TAG, total lipids include chlorophylls, carotenoids, glyco and phospholipids, and sterols. Biodiesel requires fatty acids, which means that molecules without fatty acids like pigments, sterols and hydrocarbons are not suited for biodiesel production.

In addition phosphate or phosphorus are, respectively, considered as problems for transesterification step and for engine. All these considerations lead to the conclusion that TAG content is more relevant than total lipids content for biodiesel production. TAG content was thus measured by hexane extraction. A microalgae volume chosen for obtaining finally 5–10 mg of TAG was centrifuged at 3600*g* for 10 mn and supernatant discarded as much as possible. Centrifugation was repeated till nearly complete water elimination. Hexane was added and sonication used for helping transferring in the soxhlet'thimble. Soxhlet extraction was performed during 6 h. Extract was transferred in a pre-weight-vial and dried until constant weight.

3. Results and discussion

3.1. Determination of culture medium for obtaining light-limited condition in PBR

In photoautotrophic condition, highest biomass productivity for a given PBR geometry is obtained for the so-called light-limited growth condition where growth is only limited by light (Cornet and Dussap, 2009; Cornet et al., 1998; Pruvost et al., 2008). It implies that all other nutrients such as minerals and inorganic carbon source are provided in excess. Because *N. oleoabundans* is a rather unknown species, its culture medium was firstly defined to be in the light-limited growth condition except for nitrogen limitation experiments.

Measurement of the elementary composition of *N. oleoabundans* was first used to estimate macronutrients consumption. Coefficients of the biomass elements have been normalized to the carbon. An overall stoichiometry characterizing the yields of conversion of substrates into products has been established based on the conservation of elements (C, H, O, N, S, P) (Roels, 1983):

$$CO_2 + 0.148HNO_3 + 0.014H_2SO_4 + 0.012H_3PO_4 + 0.751H_2O \rightarrow CH_{1.715}O_{0.427}N_{0.148}S_{0.014}P_{0.012} + 1.437O_2 \quad (1)$$

From the elemental composition of biomass, the value of its C-molar mass is 23.45 g C-mol^{-1}.

This equation was used to define medium composition valid up to a given maximal biomass concentration in terms of macronutrients, namely nitrates, phosphates and sulfates. Concentration of trace elements was the same as in bold basal medium (BBM) as used by Tornabene et al. (1983) for *N. oleoabundans*. This medium is given in Table 1 as well as an example of modified BBM designed for a biomass concentration up to 1.5 kg m^{-3} before any occurrence of mineral limitation with nitrate and sulfate concentrations equal to 3-fold the standard ones (noted 3N3S in Table 1). This medium was used for experiments in continuous mode presented in the next section. Using stoichiometry to determine medium composition does not guarantee an optimal composition: exometabolites and trace nutrients were not included in Eq. (1), chemical interactions can appear between nutrients, a too high calculated concentration of a given nutrients can inhibit growth, etc. It was thus retained to conduct a batch culture in PBR with macronutrients concentrations in high excess (18N9S4P), secondary nutrients concentrations having been multiplied by 5 compared to standard BBM. As shown in Fig. 1, a biomass concentration up to more than 6 kg m^{-3} was achieved. Because any limitation or excess in macro or secondary nutrients would have prevented to reach such high biomass concentration, it was concluded that medium formulation BBM 3N3S would allow to reach light-limited condition in standard continuous production. It must be noticed that standard BBM composition allows a very low maximal biomass concentration, around 0.5 kg m^{-3}, due to a too low nitrate concentration. This was already observed by Tornabene et al. (1983) who increased initial BBM nitrate concentration up to three times.

Table 1
Growth medium for *Neochloris oleoabundans* and adaptation for PBR production.

Standard composition of bold basal medium (BBM)	Modified composition of bold basal medium 3N3S
$MgSO_4{\cdot}7H_2O$ = 0.3 mM	$MgSO_4{\cdot}7H_2O$ = 0.91 mM
$NaNO_3$ = 2.94 mM	$NaNO_3$ = 8.82 mM
KH_2PO_4 = 0.43 mM	Id
K_2HPO_4 = 1.29 mM	Id
NaCl = 0.43 mM	Id
$CaCl_2{\cdot}2H_2O$ = 0.17 mM	Id
$ZnSO_4{\cdot}7H_2O$ = 30.7 μM	Id
$MnCl_2{\cdot}4H_2O$ = 7.3 μM	Id
MoO_3 = 4.9 μM	Id
$CuSO_4{\cdot}5H_2O$ = 6.3 μM	Id
$CoNO_3{\cdot}6H_2O$ = 1.7 μM	Id
H_3BO_3 = 0.185 mM	Id
EDTA = 0.171 mM	Id
KOH = 0.553 mM	Id
$FeSO_4{\cdot}7H_2O$ = 18 μM	Id
H_2SO_4 = 10.2 μM	Id

3.2. Continuous production of *N. oleoabundans* in an airlift photobioreactor

Biomass productivity of *N. oleoabundans* was determined by conducting continuous culture in PBR. Feeding medium was BBM 3N3S as described in Section 3.1. Analyses were carried out under steady-state conditions. In continuous mode, the biomass daily volumetric productivity r_x is obtained for a given daily dilution rate D by measuring the biomass concentration X inside the PBR:

$$r_x = D \cdot X \quad (2)$$

Macronutrients concentrations were monitored in order to check that productivities were only light-limited (no minerals limitation). In addition, experimental mass balance for biomass and the main macronutrients gave the yields of conversion of each of those substrates into biomass. The knowledge of the yield coefficients, in particular for nitrogen, is of great interest to set up the operating conditions leading to nitrogen culture starvation. An estimate of these macronutrient yields of conversion were given by the stoichiometric equation assuming biomass as the sole product of photosynthetic growth with no secondary exo-metabolites production. Experimental results are summarized in Table 2. A good agreement was observed, confirming that photosynthetic growth was mainly directed towards biomass formation.

Results of biomass productivities are given in Fig. 2 for an incident light flux q_o = 270 μmole m^{-2} s^{-1}. A classical evolution was observed with a decrease of biomass concentration with the increase of dilution rate D. A maximal value of biomass volumetric productivity was obtained for an optimum dilution rate of about 0.6 day^{-1}. As described by Richmond (2004a,b), this corresponds to an "optimal cell density" (or biomass concentration) of 0.9 kg/m^3. Maximal value of biomass volumetric productivity in the operated conditions was r_x = 0.55 kg m^{-3} day^{-1}. For a flat-panel geometry having a depth of culture L = 0.03 m, a daily areal productivity of r_s = 16.5 g m^{-2} day^{-1} ($r_s = L_z{\cdot}r_x$) was obtained similar to those of other species usually cultivated as *Arthrospira platensis* or *Chlorella vulgaris* (Richmond, 2004a).

Using the stoichiometry in Eq. (1), these productivities give a good estimate of carbon fixation rate assuming biomass as the major product of photosynthetic carbon fixation by neglecting secondary metabolites released in the medium. For *N. oleoabundans* cultivated in the conditions described above, this gives 1 kg m^{-3} day^{-1} and 31 g m^{-2} day^{-1} for CO_2 volumetric and areal fixation productivities, respectively.

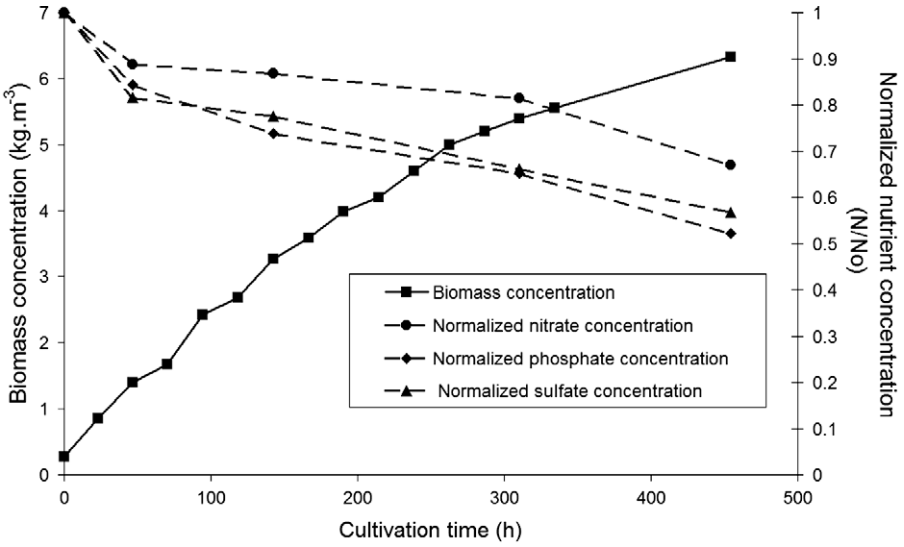

Fig. 1. Time course of biomass and macronutrients concentrations (light-limited growth, batch culture, q_o = 270 μmole m^{-2} s^{-1}).

Table 2
Mass balance on macronutrients obtained in a continuous culture for molar yield coefficients determination (q_0 = 270 μmole m^{-2} s^{-1}, X = 0.9 kg m^{-3}).

Anion (–)	Concentration in feeding medium (kg/m^3)	Concentration in PBR (steady state) (kg/m^3)	Mass yield coefficient (g of nutrients/g of DW)	Molar yield coefficient (mol mol^{-1} biomass)
Nitrate	0.509	0.160	0.384	0.145
Sulphate	0.113	0.074	0.043	0.013
Phosphate	0.198	0.147	0.056	0.017

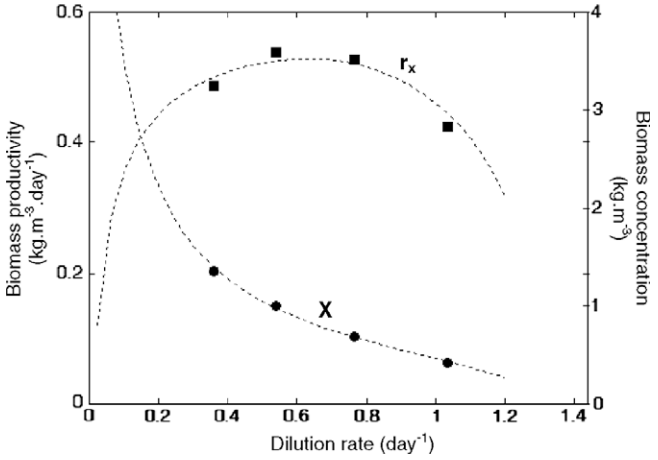

Fig. 2. Biomass concentration and biomass volumetric productivities obtained in continuous mode (light-limited growth, q_o = 270 μmole m^{-2} s^{-1}).

3.3. Induction of lipids production by *N. oleoabundans*

Influence of nitrate starvation on batch culture of *N. oleoabundans* was investigated. Two airlift PBR were used. The first one (PBR1) was operated in continuous mode at the dilution rate D = 0.72 day^{-1} for an incident light flux q_o = 270 μmole m^{-2} s^{-1}. The steady-state output was used to inoculate the second one (PBR2) in order to get constant composition and physiological state of *N. oleoabundans* at the beginning of all experiments. Two protocols of nitrate starvation under batch conditions were investigated. In all experiments, time-evolution of biomass and nitrate concentrations in the PBR as well as total lipids and pigment contents were measured.

3.3.1. Effects of a progressive nitrate starvation

A first series of experiments was carried out using three different initial nitrate concentrations and the same initial biomass concentration. Since the residual nitrate concentration in the harvest of PBR1 was known, nitrate concentration in the culture medium in PBR2 was adjusted accordingly. The higher initial concentration (N_o = 26 mM) was leading to a non-limiting nitrate concentration at the end of the batch culture, resulting in a standard growth similar to the one presented in Fig. 1 (data not shown). Lipids content remained almost unchanged all along the culture running. With respect to the biomass dry weight, total lipids content was 23%, TAG content was 3% (13% of total lipids). Chlorophyll-*a*, chlorophyll-*b* and carotenoids contents were found equal to 3.5%, 1% and 0.3%, respectively. The two lower nitrate concentrations (N_o = 1.45 mM and N_o = 3.37 mM) were determined so that a progressive nitrate starvation was induced during the culture. Results are given in Fig. 3a and b. The effect of nitrate starvation on growth is perfectly shown with a sudden stop of growth after the total nitrate consumption. Depending on the initial nitrate concentration, various maximal biomass concentrations were obtained. As expected and already reported elsewhere (Hu et al., 2008; Li et al., 2008; Scragg et al., 2002; Tornabene et al., 1983), in addition to stopping cell division, nitrate starvation triggered total lipids accumulation. The highest content was obtained for N_o = 1.45 mM, with an accumulation of up to 37% of dry weight. At the end of the culture, TAGs content was measured and was found to represent 50% of total lipids in mass, thus around 18% of the dry weight. This value has to be compared to the TAG content in standard growth conditions that is around 3% of the dry weight. This clearly indicates that lipids accumulation was mainly found in the TAG pool (Hu et al., 2008).

Due to the central role of nitrogen, important modification in cell physiology occurred with nitrate starvation. In addition to

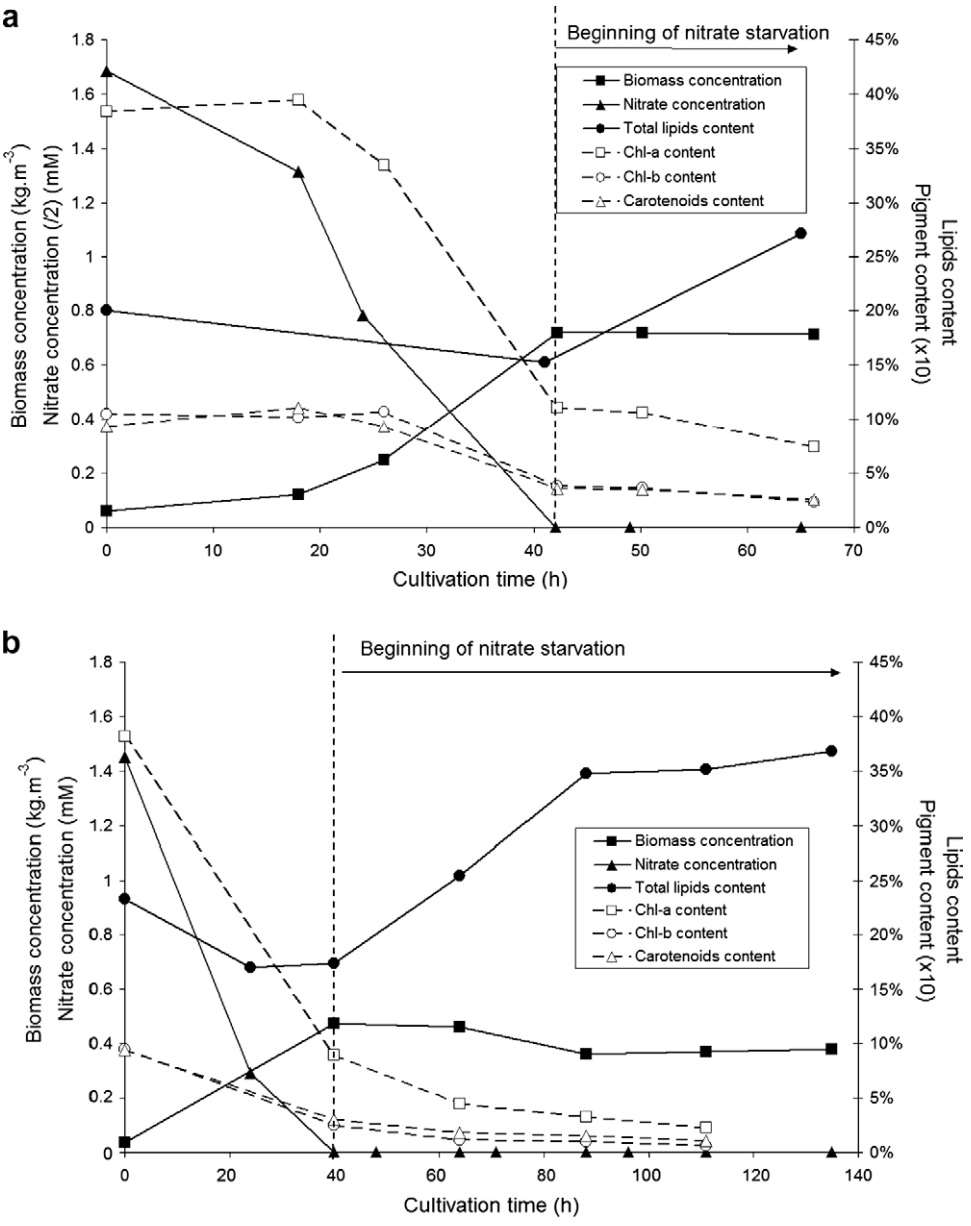

Fig. 3. Time course profiles during progressive nitrate starvation with N_o = 3.37 M (a) and N_o = 1.45 M (b) (batch culture, q_o = 270 μmole m^{-2} s^{-1}).

lipids accumulation, a great decrease in pigments content was observed. This change started before the total nitrate starvation, indicating a progressive modification of the physiological response at a low but non-null value of nitrate concentration. During the progressive establishment of nitrate deprivation, pigments were firstly and significantly affected with a decrease up to more than 10-fold of the initial pigments content (for N = 1.45 mM, chlorophyll-*a* content decreases from 3.8% to 0.3% of dry weight). This was already observed by Li et al. (2008) for *N. oleoabundans.*

Comparison of the two experiments with nitrate starvation (N_o = 1.45 mM and N_o = 3.37 mM) shows a great difference in terms of kinetics. These kinetics are obviously correlated to the remaining nitrate concentration in the medium that differs in both cases. A relationship also appears with biomass concentrations that are finally reached in PBRs. This was also the case for total lipids content. The faster kinetic of lipids accumulation was observed for the lower biomass concentration obtained (N_o = 1.45 mM), which also led to the higher lipids content. This fact emphasized a role of light in the process. Since the same incident light flux was used in all experiments, a lower biomass concentration supposed a higher light available per cell, which certainly affected the metabolic response.

As light received per cell influences cell response, the great variation in pigments content during nitrate starvation makes complex the light effect analysis. Light attenuation is a function of biomass concentration but also of pigments content (Berberoglu et al., 2008; Cornet et al., 1994; Cornet et al., 1995; Pottier et al., 2005). To illustrate that, light attenuation profiles were determined at two different key-times, for initial and final cultivation time for the experiment with N_o = 1.45 mM. The method described in Pottier et al. (2005) was used. This fully predictive radiative model allows to introduce any pigments content to determine corresponding optical properties for a given species. Calculation was conducted for pigments content at initial and final (t = 111 h) cultivation times giving values of mass absorption coefficients E_a = 453 m^2/kg and E_a = 68 m^2/kg, respectively. This decrease is directly explained by the reduction in pigments content. To calculate irradiance profiles using the two-flux radiative model

as described in Pottier et al. (2005), mass scattering coefficient E_s and backward scattering fraction b are also requested. Those values are based on the shape and size of cells. The purpose being here only to illustrate the effect of a variation of pigment content on light available, values already published for *Chlamydomonas reinhardtii* were used in first assumption ($E_s = 868\ m^2/kg$; $b = 0.01728$). As for *N. oleoabundans*, *C. reinhardtii* is of spherical shape (exact determination of *N. oleoabundans* optical properties will be the aim of future works). Optical properties being known, irradiance profile G along the depth of culture z can be determined for a given incident light flux q_o and biomass concentration. Experiment values were used, $q_o = 270\ \mu mole\ m^{-2}\ s^{-1}$ with $X = 0.04\ kg\ m^{-3}$ for $t = 0$ and $X = 0.46\ kg\ m^{-3}$ for $t = 111$ h. Results are given in Fig. 4. Although a higher biomass was obtained at the end of cultivation, almost identical light attenuation was observed. This is explained by the high decrease in pigment content and the very low value of corresponding mass absorption coefficient. An additional attenuation profile is given, where pigments content is supposed to remain constant during the cultivation. This shows that culture conditions would have turned from almost transparent culture to full attenuation during a normal batch growth (Pruvost et al., 2008). This is not the case when nitrogen starvation was applied. Because initial nitrate concentration led to different biomass concentration, which in turns affected light attenuation also modified by pigments evolution, this has certainly an influence that should have to be more investigated in future works. Light transfer inside the culture, light absorbed by the cell and light use for metabolic intracellular reactions are indeed all related, the photons energy harnessed being a function of light available and of the pigments content of antennae. As a result, light-dependant lipid production is difficult to optimize.

3.3.2. Effects of a sudden nitrate starvation

In a second series of experiments, nitrate starvation was applied at the beginning of the cultivation. Algae harvested from PBR1 were centrifuged and then re-suspended in a nitrate-free medium. Algae volumes from PBR1 were adjusted to obtain different initial biomass concentration in PBR2. Those concentrations were chosen to be around the maximal ones obtained after total nitrate consumption in previous experiments ($X_o = 0.26\ kg\ m^{-3}$, $X_o = 0.46\ kg\ m^{-3}$). When progressive nitrate starvation was applied, the pigments decrease was found at the earlier stage of nitrate limitation with a stop in biomass growth when nitrate starvation occurred. As shown in Fig. 5, in the case of sudden deprivation, a decrease of pigments content was also observed but with a parallel increase in biomass concentration although cells were in a nitrate-free medium (data are only given for $X_o = 0.26\ kg\ m^{-3}$, same evolution for $X_o = 0.46\ kg\ m^{-3}$). When pigments content tends to very small values, biomass growth decreases significantly. It was also observed that lipids accumulate immediately as soon as cells were placed in the nitrate-free medium. There was a simultaneous production of biomass and associated lipids. In parallel to the reduction of growth rate, lipids were progressively over-accumulated. This was not observed in the case of progressive nitrate deprivation where lipids accumulation was only found when nitrate starvation was achieved but without biomass production. This indicates that total lipids accumulation seems to be mainly related to the nitrate concentration in the medium, biomass growth being supported at least partly by an intracellular storage of nitrogen which is progressively remobilized for growth. In addition, as observed in all experiments, when pigments content was dropped at very low value (<0.5% of the dry weight), growth was almost null (same was observed by Li et al., 2008).

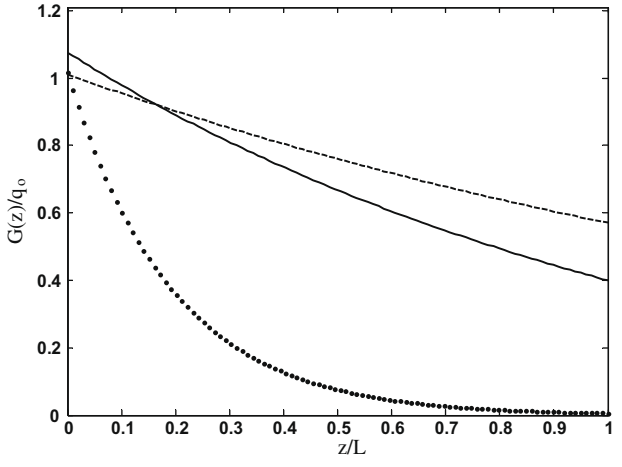

Fig. 4. Example of light attenuation profiles (solid line: $t = 0$ h, $X = 0.04\ kg\ m^{-3}$ – dotted line: $t = 111$ h, $X = 0.37\ kg\ m^{-3}$ – dashed line: same as dotted line, but assuming no pigments degradation, see text for details).

3.4. Estimation of productivities with N. oleoabundans

Previous experiments allowed to estimate productivities achieved with *N. oleoabundans* for the conditions investigated. Total lipids volumetric productivity was deduced knowing the lipid content w_L (% lipids/biomass) and biomass productivity:

$$r_L = r_X \cdot w_L \tag{3}$$

Biomass volumetric productivity in continuous mode has already been defined (Eq. (2)). For a batch culture, the mean biomass volumetric productivity can be estimated from the culture duration t_c before harvesting:

$$\overline{r_X} = \frac{X - X_o}{t_c} \tag{4}$$

where X_o is the initial biomass concentration.

Because biomass growth is not constant with time in batch mode, the biomass productivity is a function of the culture duration. Results are presented for total lipids in Fig. 6 (same tendencies for TAG production, data not shown). This illustrates that increasing total lipids content only is not sufficient in batch mode to ensure a higher total lipids productivity, total lipids productivity being the global result of cell total lipids content, biomass growth, and time of cultivation. There is an optimal duration for biomass harvesting in order to obtain maximal productivity. In the conditions investigated, maximal lipids productivities were not obtained for the maximal total lipids content (37% after 4 days) but after 40–50 h of cultivation corresponding to 25% in total lipids content. The lower lipids productivity was obtained in the case of a progressive starvation with the higher initial nitrate concentration ($N_o = 3.22$ M) that resulted, however, in a high biomass concentration of about $1\ kg\ m^{-3}$ in dry weight. For other three experiments, it must be pointed out that similar maximal total lipids productivities in the range of 60–70 $g\ m^{-3}\ day^{-1}$ were achieved, although very different protocols of nitrate starvation were applied.

A summary of best productions is given in Table 3. This shows that in the case of *N. oleoabundans*, as probably for other strains, various production strategies can be defined. Considering TAG production, although nitrate starvation is necessary to induce TAG accumulation (18% of dry weight), this can be compensated in terms of productivity by working at maximal biomass productivity because of TAG natural content in *N. oleoabundans* as obtained in continuous mode in light-limited condition (3% of the dry weight). Considering total lipids leads to a different conclusion: a higher productivity was indeed achieved in light-limited continuous production explained by the high natural total lipids content of

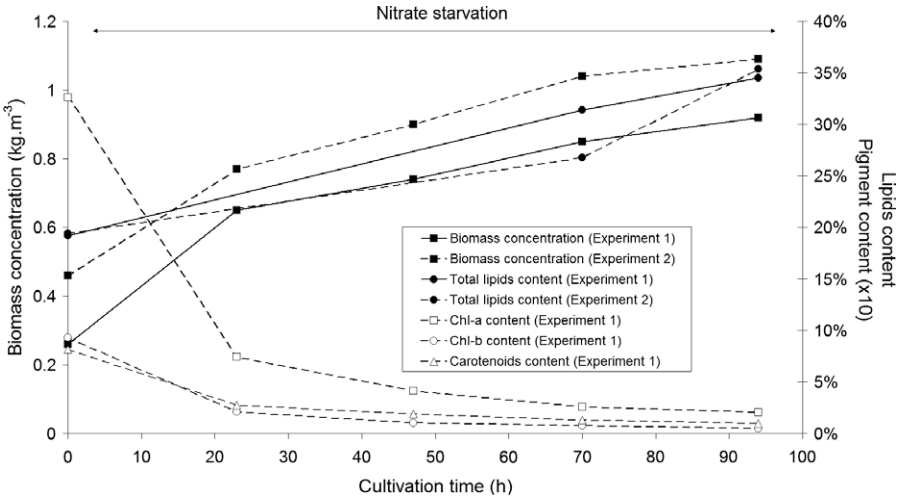

Fig. 5. Time course profiles during sudden nitrate starvation with initial biomass concentration X_o = 0.26 kg m^{-3} (Experiment 1) and X_o = 0.46 kg m^{-3} (Experiment 2) (batch culture, q_o = 270 μmole m^{-2} s^{-1}).

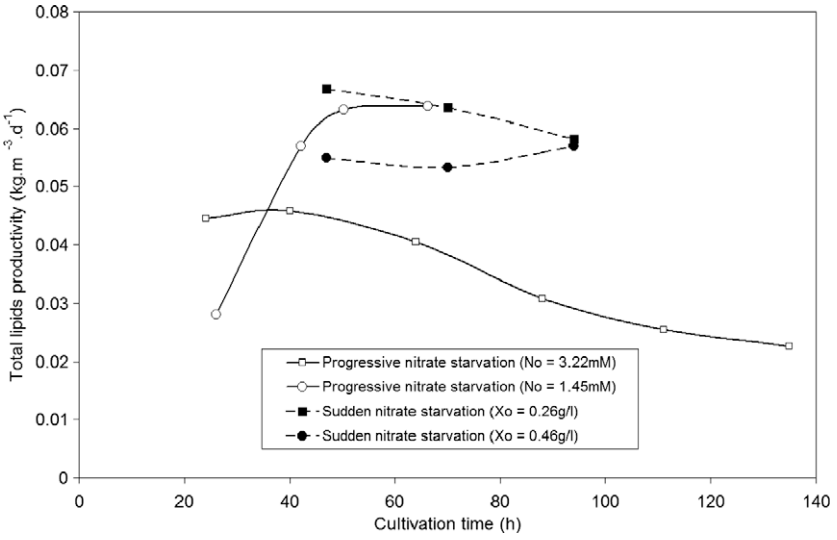

Fig. 6. Lipids productivity as a function of cultivation time for batch production protocols.

Table 3
Total lipids and TAG productivities for batch with nitrate starvation and continuous production in light-emitted condition.

	Volumetric productivities (total lipids), g m^{-3} day^{-1}	Areal productivities (total lipids), g m^{-2} day^{-1}	Volumetric productivities (TAG), g m^{-3} day^{-1}	Areal productivities (TAG), g m^{-2} day^{-1}	Maximal lipid content
Progressive nitrate starvation (N_o = 1.45 M)	r_L = 65	r_L = 2	r_L = 23	r_L = 0.7	37% (total lipids) 18% (TAG)
Continuous production (without mineral limitation)	r_L = 126	r_L = 3.8	r_L = 16.5	r_L = 0.5	23% (total lipids) 3% (TAG)

N. oleoabundans (23% of the dry weight). In this case, areal productivities of 3.8 g m^{-2} day^{-1} and 0.5 g m^{-2} day^{-1} are achieved for total lipids and TAG, respectively (biomass productivity of 16.5 g m^{-2} day^{-1}).

Those results can be compared to those of Li et al. (2008), although they provided only volumetric productivities that were highly dependent on the PBR (or culture vessel) geometry. A maximal lipids productivity of 133 g m^{-3} day^{-1} was obtained with nitrate starvation for a PFD of 360 μmole m^{-2} s^{-1}. This is close to the value of 126 g m^{-3} day^{-1} obtained in this study for a PFD of 270 μmole m^{-2} s^{-1}, but without nitrogen limitation. In our study, this important production is explained by the systematic optimization of growth conditions to allow light-limited condition to be obtained. In case of high natural lipids content as in *N. oleoabundans*, increasing only biomass production gives satisfactory results. Obviously, interest of such production protocol depends also on several other factors whose discussion is out of the scope of this study (extraction yield being probably affected by lipids content, suitable lipids quality for biodiesel application, practical feasibility of batch or continuous production). This is thus not a definitive

conclusion that can be generalized to any biofuel production by microalgae but this has to be kept in mind for setting future production strategies of this primary resource at industrial scale.

4. Conclusion

The growth of the microalga *N. oleoabundans* was investigated without nutrient limitation and with progressive or sudden nitrate starvation. Both biomass and lipids productivities confirm the potential of this rather unknown strain for biodiesel application. For the conditions investigated, rather similar productivities were observed in terms of TAG whatever tested protocols. Considering total lipids led to a higher productivity without mineral limitation, as explained by the natural high content of *N. oleoabundans* in total lipids. Those results show that in the case of *N. oleoabundans*, as probably for other strains, various lipids production strategies can be defined.

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