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Effect of nitrogen addition on lipid productivity of nitrogen starved *Nannochloropsis gaditana*

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ABSTRACT

Microalgae are a sustainable source of lipids. A commonly used strategy for lipid accumulation in microalgae is a two-step batch cultivation, with a growth phase followed by a nitrogen starvation phase. A problem with this process is the decrease in photosynthetic efficiency during the nitrogen starvation phase, which leads to low lipid productivities. In this research, a new process strategy was studied with the aim to improve lipid productivity of the microalgae *Nannochloropsis gaditana*. The nitrogen concentrations were chosen to assure consumption of most part of the nitrogen during the night. An improvement of the photosystem II maximum quantum yield and an increase in the dry weight and TAG concentration was achieved from day 7 of nitrogen starvation onwards when the culture was fed with nitrogen each night compared to a culture without nitrogen addition. Consequently, the time-average TAG yield on light was also higher after 7 days of nitrogen starvation. However, since the maximal time-averaged triacylglycerol (TAG) yield on light was reached after 3 days of nitrogen starvation, the improved photosynthetic activity did not lead to an increase of the maximal time-averaged TAG yield on light. The culture with nitrogen addition had a higher protein concentration (1.1 compared to 0.7 $g\,L^{-1}$), showing that the added nitrogen was mainly used for protein production. A higher chlorophyll *a* content (2.0 compared to 0.8 $\mu g\,mg^{-1}$) showed improved photosystem and that a small part of nitrogen was used for chlorophyll *a*. Small nightly nitrogen additions during batch cultivation of nitrogen starved *N. gaditana* did result in improvement in photosystem II maximal quantum yield, biomass concentration, TAG production and a higher time-averaged maximal TAG yield on light, after 7 days of nitrogen starvation.

1. Introduction

Microalgae are a sustainable source of lipids, proteins and carbohydrates for food, feed and fuel applications [1]. Higher lipid productivities, however, are required to achieve an economically feasible process [2]. The microalgae *Nannochloropsis gaditana* is used as a model species because of its ability to accumulate large amount of lipids [3], the available genome sequence and annotation [4,5] and being a robust species shown by already being cultivated outdoors [6]. The lipids in *Nannochloropsis* are present in a polar lipid (PL) fraction and triacylglycerol (TAG) fraction. One of the often applied strategies to induce TAG accumulation is nitrogen starvation [3,7]. TAG accumulation is often applied as a two phase batch system, consisting of a growth phase followed by a nitrogen starvation phase, where TAG accumulates. A problem faced during nitrogen starvation is the decrease in photosynthetic efficiency and biomass productivity leading to low TAG productivities [7,8].

Different process strategies have been investigated to improve photosynthesis during nitrogen starvation. One of the cultivation strategies previously applied to improve TAG productivity was nitrogen limitation in *Neochloris oleoabundans* and *Acutodesmus obliquus*. In this strategy limited amount of nitrogen was fed to a continuous culture, to sustain cell growth and at the same time induce TAG accumulation [9,10]. This strategy, however, did not increase TAG yield on light compared to a conventional batch cultivation followed by nitrogen starvation. Repeated batch cultivations has also been tested for TAG

Abbreviation: TAG, triacylglycerol; PL, polar lipid; OD, optical density; DW, dry weight; r_{ph}, volumetric photon supply rate ($mol_{ph}\,L^{-1}\,day^{-1}$); $Y_{TAG/ph}$, time averaged TAG yield on light ($g_{TAG}\,mol_{ph}^{-1}$); $Y_{x/ph}$, time averaged biomass yield on light ($g_x\,mol_{ph}^{-1}$); PSII, photosystem II; F_v/F_m, photosystem II maximum quantum yield

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production. During this strategy part of the nitrogen starved culture is harvested and replaced by fresh cultivation medium containing nitrogen. Due to cell growth the nitrogen is depleted, TAG accumulates and at a certain point in starvation, part of the culture is harvested again and replaced by fresh medium containing nitrogen. For *Nannochloropsis* sp. it was shown that batch cultivation followed by nitrogen starvation led to higher lipid productivities compared to repeated batch cultivations [8]. It was hypothesized that the TAG yield on light was not improved in repeated batch because light energy was not used only for TAG accumulation but also for cell regrowth, after replenishing the nitrogen starved culture with fresh medium. Previous research also showed that nitrogen starved cultures of the microalgae species *Nannochloropsis oceanica*, *Chromochloris zofingiensis* and *Chlamydomonas reinhardtii* can increase their photosynthetic efficiency again after nitrogen resupply [11–14]. After nitrogen replenishment, TAG productivity decreases drastically because TAG is used as energy source for the production of biomass and possibly for nitrogen uptake. TAG has been reported to be degraded within hours after nitrogen resupply [13,14]. When nitrogen was resupplied to a dark culture of *Chlamydomonas reinhartii*, after three days of nitrogen starvation, it first induced starch degradation and later TAG degradation to support cell growth [13]. For a mixed culture it was shown that in a repeated batch cultivation the storage compound starch, was maximized when ammonium was supplied as nitrogen source at the start of the dark period rather than in the light period [15].

In this research a new cultivation strategy was tested to improve the TAG yield on light by adding small amounts of nitrogen during each night to a nitrogen starved culture. The nitrogen concentrations were chosen to assure consumption of most part of the nitrogen during the night. We hypothesized that nitrogen addition during the night reduces light loss during the day by maintaining photosynthetic efficiency. In this way, the energy available in the light period can be more efficiently used for TAG accumulation and lead to an increased TAG yield on light [8,12]. During nitrogen starvation microalgal cells degrade their photosystems. By addition of nitrogen, the cells are not nitrogen starved anymore and can direct their energy again to photosynthesis. The added nitrogen can be used for de novo protein synthesis and maintain or rebuild the photosystems. Energy necessary to assimilate nitrogen during the night could be provided by for example carbohydrate storage. A more efficient photosystem can more efficiently produce TAG and thus improve TAG yield on light.

2. Materials and methods

2.1. Strain, cultivation medium and pre-cultivation

The marine microalgae *Nannochloropsis gaditana* CCFM-01 was obtained from the Microalgae Collection of Fitoplancton Marino S.L. Precultures were maintained in 250 mL Erlenmeyer flasks containing 100 mL filter sterilized (pore size 0.2 μm) cultivation medium and incubated in an orbital shaker incubator (100 rpm), at 25 °C, with air in headspace and an incident light intensity of 30–40 μmol $m^{-2} s^{-1}$ with 18:6 h light dark cycle. The culture media was based on Breuer et al. 2012 [7] and contained: NaCl 445 mM; KNO_3 33.6 mM; Na_2SO_4 3.5 mM; $MgSO_4{\cdot}7H_2O$ 3 mM; $CaCl_2{\cdot}2H_2O$ 2.5 mM; K_2HPO_4 2.5 mM; NaFeEDTA 28 μM; $Na_2EDTA{\cdot}2H_2O$ 80 μM; $MnCl_2{\cdot}4H_2O$ 19 μM; $ZnSO_4{\cdot}7H_2O$ 4 μM; $CoCl_2{\cdot}6H_2O$ 1.2 μM; $CuSO_4{\cdot}5H_2O$ 1.3 μM; $Na_2MoO_4{\cdot}2H_2O$ 0.1 μM; Biotin 0.1 μM; vitamin B1 3.3 μM; vitamin B12 0.1 μM; and 10 mM $NaHCO_3$. The pH was adjusted with NaOH to 7.5. During pre-cultivation in Erlenmeyer flasks 100 mM 4-(2-hydroxyethyl) piperazin-1-ethanesulfonic acid (HEPES) was added as pH buffer. Prior to inoculation into the photobioreactor the cultures were transferred to 100 μmol $m^{-2} s^{-1}$ continuous light with 2.5% CO_2 enriched air. During cultivation in the photobioreactor KNO_3 was reduced to 7.02 mM, which is sufficient to reach a biomass concentration of 1.4 g L^{-1}.

2.2. Experimental and reactor setup

N. gaditana was cultivated in heat-sterilized flat panel photobioreactor with a light path of 20.7 mm and a working volume of 1.8 L (Labfors 5 Lux, Infors HT, Switzerland, 2010). The reactor was kept at 26 °C by a waterjacket. The pH was controlled at 7.5 by on demand addition of sulphuric acid (2.5% v/v) and at the start of the culture sodium hydroxide (1 M). The culture was mixed by filter sterilized air with 2% CO_2 with a flow rate of 1 L min^{-1}. The off gas was cooled in a condenser. The light was provided by 260 LED lamps with warm white spectrum (450–620 nm). The incident light intensity was measured with a LI-COR 190-SA 2π PAR (400–700 nm) quantum sensor (LI-COR, USA). The incident light intensity started at 63 μmol $m^{-2} s^{-1}$ and increased daily, by keeping the outgoing light intensity 30–40 μmol $m^{-2} s^{-1}$, up to 636 μmol $m^{-2} s^{-1}$ and kept constant during the nitrogen starvation period. A day: night cycle of 16: 8 h was used. The backside of the reactor was covered with a black cover to prevent interference of environmental light. The microalgae were cultivated in batch mode with two phases; growth phase and nitrogen starvation phase when all nitrogen was consumed. The growth phase was inoculated at biomass concentration of 0.1 g L^{-1}. Nitrogen runout at a biomass concentration of 1.8 g L^{-1} concentration and this time was considered the start of nitrogen starvation. From this moment on nitrogen was added during the night. Nitrogen addition was performed by adding a concentrated KNO_3 solution (0.29 M) at the start of each night (the total volume added was 17 and 23 mL for experiment 1 and 2). The amount of added nitrogen was tuned to be mostly consumed at the end of the night. Previously experiments showed that the nitrogen uptake rate per amount of biomass decreased with increasing time of nitrogen starvation. This knowledge was used to determine the amount of nitrogen added. The amounts nitrogen added was decreased over the course of the experiment (Table A.1 in appendix). The experiment was performed in duplicate photobioreactors. The control experiment had an identical setup and process conditions but without nitrogen addition in the night. Control experiments were also performed in duplicate.

2.3. Offline measurements culture

The optical density (OD) was measured daily in duplicate at 750, 680 and 480 nm using a UV-VIS spectrophotometer (Hack Lange DR-6000, light path 10 mm). The OD680 and OD480 were used as an estimate for, respectively, the chlorophyll and carotenoid content. To correct for scattering, the OD750 was subtracted and the result was divided by the OD750. From these samples the cell number, cell size, quantum yield and the dry weight-specific optical cross section were also measured. The cell number and size were measured with the Multisizer III (Beckman Coulter) using 50 μm aperture tube and after diluting the sample 100 times in ISOTON II diluent. The photosystem II (PSII) maximum quantum yield (F_v/F_m) was measured after 15 min dark adaptation at room temperature (AquaPen-C 100, PSI, Czech Republic; excitation light 455 nm, saturating light pulse: 3000 μmol $m^{-2} s^{-1}$). F_v/F_m ratio was calculated according to Benvenuti et al. 2015 [16].

The average dry weight-specific optical cross section (α_c in $m^2 g^{-1}$) was measured and calculated according to de Mooij et al. 2015 [17] using the absorbance from 400 to 750 nm with a steps size of 1 nm.

Biomass concentration (C_x in g L^{-1}) was measured from the moment of nitrogen starvation by measuring the dry weight of the culture. The dry weight was measured in triplicate as described by Kliphuis et al. 2012 [18] with the exception that 0.5 M ammonium formate was used to wash the filters.

Regularly, biomass samples were taken, at the start and end of the dark period, to determine the biomass composition. The biomass samples were centrifuged 5 min at 4255 g (Beckman coulter Allegra X-30R centrifuge) and washed twice with 0.5 M ammonium formate, stored at −20 °C and lyophilized. Since, the supernatant was reddish turbid and