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Simultaneous growth and neutral lipid accumulation in microalgae

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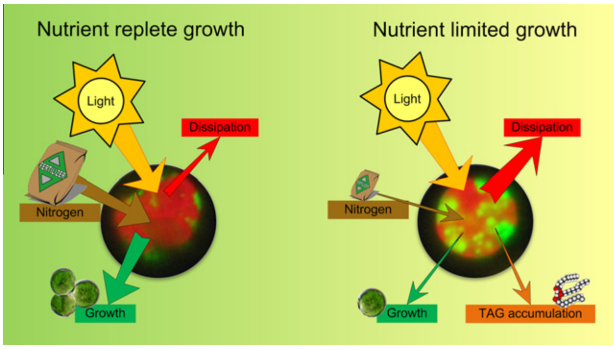
HIGHLIGHTS

- ▶ Nitrogen limitation in turbidostats causes a metabolic energy imbalance in microalgae.
- ▶ This energy imbalance allows simultaneous cell replication and TAG accumulation.
- ▶ *Neochloris oleoabundans* used TAG as an energy sink when exposed to excess light energy.
- ▶ Excess light energy was predominantly dissipated rather than stored in TAG.

GRAPHICAL ABSTRACT



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ABSTRACT

In this paper the hypothesis was tested whether TAG accumulation serves as an energy sink when microalgae are exposed to an energy imbalance caused by nutrient limitation. In our continuous culture system, excess light absorption and growth-limiting nitrogen supply rates were combined, which resulted in accumulation of TAG (from 1.5% to 12.4% w/w) in visible lipid bodies in *Neochloris oleoabundans*, while cell replication was sustained. A fourfold increase in TAG productivity showed that TAG indeed served as an energy sink. However, the bulk of excess energy was dissipated leading to a significantly reduced biomass productivity and yield of biomass on light. This demonstrates that when aiming at industrial TAG production, sustaining efficient light energy use under nutrient stress is an important trait to look for in potential production organisms.

Abbreviations: α_{DW}, absorption cross section of the dry biomass ($m^2\ g^{-1}$); CLSM, confocal laser scanning microscopy; C_X, biomass concentration ($g\ L^{-1}$); D, dilution rate (d^{-1}); DR, degree of reduction (mol electrons per g biomass^{-1}); δ_{pbr}, light path of the photobioreactor (m); F_N, nitrogen supply rate ($g\ L^{-1}\ d^{-1}$); HL, high light intensity; LL, low light intensity; μ, specific growth rate (d^{-1}); MW$_N$, molecular weight of nitrogen ($g\ mol^{-1}$); PFD$_{av}$, average photon flux density in the system ($\mu mol\ m^{-2}\ s^{-1}$); PFD$_{blank}$, photon flux density leaving the photobioreactor containing only growth medium ($\mu mol\ m^{-2}\ s^{-1}$); PFD$_{in}$, photon flux density entering the algal culture ($\mu mol\ m^{-2}\ s^{-1}$); PFD$_{out}$, photon flux density leaving the algal culture ($\mu mol\ m^{-2}\ s^{-1}$); PFD$_{out,pbr}$, photon flux density leaving the photobioreactor ($\mu mol\ m^{-2}\ s^{-1}$); PNR, photon nitrogen uptake ratio: photon absorption rate over the nitrogen uptake rate in the system ($mol\ \gamma\ mol\ N^{-1}$); f_N, mass fraction of nitrogen in biomass ($g\ g^{-1}$); r_γ, light uptake rate ($mol\ L^{-1}\ d^{-1}$); r_N, nitrogen consumption rate ($g\ L^{-1}\ d^{-1}$); r_{TAG}, volumetric TAG productivity ($mg\ L^{-1}\ d^{-1}$); r_x, volumetric biomass productivity ($g\ L^{-1}\ d^{-1}$); % r_x, relative volumetric biomass productivity (–); TAG, triacylglycerol; TFA, total fatty acids; $V_{24\ h}$, volume of overflow collected over 24 h (L); V_{pbr}, working volume of the photobioreactor (L); $Y_{x,E}$, Yield of biomass on light ($g\ mol^{-1}$); $Y_{TAG,E}$, yield TAG on light ($g\ mol^{-1}$).

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1. Introduction

1.1. Background

Microalgae are very promising for the production of sustainable biofuel. They can produce large amounts of triacylglycerides (TAGs) (Hu et al., 2008) that have a fatty acid composition comparable to vegetable oils and thus are easily converted into an effective and clean fuel for diesel engines (Haik et al., 2011). Furthermore, microalgae can potentially be grown year round on non-arable land using salt, brackish or waste stream water. Much higher areal yields can be achieved than with traditional agricultural crops (Wijffels and Barbosa, 2010), thus microalgae provide some major advantages over commonly grown oleaginous plants such as palm, soy or rapeseed.

1.2. Current research focus

Most microalgae only produce TAGs when cultivated under adverse growth conditions. Therefore, studies on TAG production in algae are usually done in a two-step, batch wise process. First, algae are grown under optimal conditions until sufficient biomass is obtained. Then TAG accumulation is triggered by arresting the growth using for example nutrient deprivation, of which nitrogen deprivation is the most commonly used method. Often, these studies are not so much focused on the biological mechanism of TAG accumulation, but on the alga with the highest TAG content. Very high lipid contents (up to 60% total fatty acids (TFA) w/w) have been reported (Sheehan et al., 1998) and articles claiming to have found a new, better production organism based on its lipid content appear regularly.

Although a very high TAG content results in more efficient and cheap downstream processing, it is often overlooked that to obtain these contents, algae have to be exposed to unfavourable environmental conditions that limit growth for a relatively long time. The highest TAG formation rate is obtained in the first hours to days of nutrient depletion, after which the formation rate gradually decreases. Producing algae with contents of 50% TAG (w/w) or more takes considerably longer, in the order of 1 or 2 weeks of nutrient depletion, depending on the light conditions (Griffiths and Harrison, 2009; Rodolfi et al., 2009). Such prolonged stress periods typically lead to reduced overall biomass and TAG productivities and, correspondingly, to a reduced overall light use efficiency. Therefore producing biomass with a high TAG content demands more ground area and reactor material to be used for each litre of oil produced per year, compared to harvesting in the earlier stages of nutrient depletion (at a slightly lower TAG content).

To be able to make a fair comparison between different production systems and organisms, it is important to not only look at the TAG content but also at the yield of biomass and TAG on light and the average production rate of TAG over the complete production period. This should also include the down-time required to start-up a new batch, and the growth phase to produce biomass. The ideal TAG production process will rely on an optimal balance between an efficient light conversion, a high TAG production rate and a TAG content that allows for economical downstream processing. Such optimization requires detailed understanding of the underlying mechanisms of TAG accumulation.

1.3. A hypothesis for the mechanism of TAG accumulation based on energy fluxes

Although some advances have been made in unravelling the genetic and biochemical processes of TAG accumulation in microalgae, a widely accepted explanation for this phenomenon is yet to be found (Liu and Benning, 2012; Merchant et al., 2012). Attempts have been made to increase TAG content by process optimisation, medium optimisation and also by genetic modification, revealing the ever recurring pattern that increasing TAG content seems to be countered by a loss in growth. This leads to a rather black and white approach in which TAG accumulation is seen as a part of secondary metabolism that can only be induced by extreme external factors that eventually completely halt growth, such as zero nitrogen.

An alternative hypothesis is that TAG accumulation is triggered by an energy imbalance experienced by the algal cell when exposed to an external stress factor, as was also suggested by Hu et al. (2008). For example, a nutrient shortage will disturb the anabolic processes in the cell in need of these nutrients. Consequently, the energy demand for anabolism will fall behind with the energy supply through photosynthesis. This leads to overreduction of the photosynthetic machinery, which in turn can cause formation of damaging reactive oxygen species (Ledford and Niyogi, 2005). Under these conditions, microalgae are known to dissipate energy as heat and fluorescence (Kolber et al., 1988). Highly reduced compounds such as TAG, which do not contain the limiting nutrient, serve as an alternative energy sink and allow the cell to continue harvesting light energy and at the same time decrease the formation rate of reactive oxygen species.

This hypothesis applies to the production of TAG in lipid producing microalgae, but also to overproduction of various other compounds upon environmental stress, such as pigments or starch. Lamers et al. (2008) already hypothesized that β-carotene accumulation in *Dunaliella salina* is similarly caused by an energy imbalance, in which the pigments serve as an electron sink. However, in this case the accumulated metabolites also function as a sunscreen, protecting the cells by absorbing part of the excess irradiation, giving it a dual protective function.

1.4. TAG accumulation in a continuous production system

If indeed this hypothesis is valid, TAG accumulation is not necessarily coupled to a complete halt of growth as is often suggested. It should be possible to create an energy imbalance by reducing the nutrient supply to the cells at constant light input, while simultaneously allowing cell division to continue. The energy imbalance should then induce a similar response of TAG accumulation as is observed in classical nutrient depletion experiments.

To test our hypothesis a system was designed in which turbidostat operation was combined with a separate continuous nitrogen supply, which can be regarded as a distinctive operating feature of this set-up. Turbidostat operation ensured that the amount of light absorbed by the culture was always the same and in this way a fixed energy intake could be imposed on the culture. Next, an energy imbalance was created by decreasing the nitrogen supply rate below the minimum value necessary to sustain nutrient replete, light limited growth.

The effect of two light energy uptake rates and several nitrogen supply rates on growth and TAG accumulation in *Neochloris oleoabundans* was tested in the dedicated turbidostat set-up. *N. oleoabundans* was used because it is known to accumulate up to 56% TFA (w/w) of its dry weight under nitrogen deprivation, in which up to 80% of total lipids consisted of TAG. Also, its growth rate under nitrogen replete conditions is substantial: up to 2.2 d^{-1}. It can be cultivated in fresh as well as in salt medium, and at elevated pH it shows substantial growth rates (Gouveia et al., 2009; Pruvost et al., 2009; Santos et al., 2012). This makes *N. oleoabundans* a very versatile and suitable model organism to study the trade-off between growth and TAG accumulation.

The present work describes how growth and TAG accumulation can occur simultaneously using the aforementioned experimental