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Induction of triacylglycerol production in *Chlamydomonas reinhardtii*: Comparative analysis of different element regimes

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HIGHLIGHTS

- All element regimes, except S deprivation, were associated with decrease in total biovolume.
- Macro- and microelement composition of *C. reinhardtii* greatly differed.
- Overall TAG output under N, Mg, S or P deprivation was most pronounced.
- FAME profiles of N, S and P deprived cells reflect the requirements of EN 14214.
- Dramatic morphological changes were observed under different element regimes.

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ABSTRACT

In this study, impacts of different element absence (nitrogen, sulfur, phosphorus and magnesium) and supplementation (nitrogen and zinc) on element uptake and triacylglycerol production was followed in wild type *Chlamydomonas reinhardtii* CC-124 strain. Macro- and microelement composition of *C. reinhardtii* greatly differed under element regimes studied. In particular, heavy metal quotas of the microalgae increased strikingly under zinc supplementation. Growth was suppressed, cell biovolume, carbohydrate, total neutral lipid and triacylglycerol levels increased when microalgae were incubated under these element regimes. Most of the intracellular space was occupied by lipid bodies under all nutrient starvations, as observed by confocal microscopy and transmission electron micrographs. Results suggest that sulfur, magnesium and phosphorus deprivations are superior to nitrogen deprivation for the induction triacylglycerol production in *C. reinhardtii*. On the other hand, FAME profiles of the nitrogen, sulfur and phosphorus deprived cells were found to meet the requirements of international standards for biodiesel.

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1. Introduction

Recent increases in oil prices have encouraged much research into the use of alternative sources to accommodate the fuel requirements of modern civilization, and various organisms and organic wastes have been proposed as sustainable and environmentally friendly sources for the production of usable forms of energy such as biohydrogen, biodiesel and bioethanol. Among the liquid biofuels, biodiesels are typically formed from alkyl-esters of long chain fatty acids, and transesterification with alcohols is a widely used method to convert lipid molecules into their alkyl-esters (Tsai et al., 2013). While oil crops such as soybean, jatropha and oil palm are particularly suitable for use in biodiesel production, unicellular algae have received considerable attention in the recent decade as rapidly proliferating, oil-rich biofuel sources with sufficient biomass output to fully replace petroleum-derived fuels (Chisti, 2007). Microalgae derived biodiesel is a second generation biofuel and does not require arable land for stock production, which prevents potential conflicts between biodiesel production and the cultivation of edible plants. In addition, microalgae possess several advantages over land crops, such as year-round growth and harvesting capacity, substantially higher biomass per area and lipid content maxima, and resistance to pests and pathogens that commonly infest crop plants (Chisti, 2007).

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Various microalgal species have been proposed for enhanced biodiesel production, including *Scenedesmus obliquus*, *Nannochloropsis oculata*, *Chlorella* sp., *Neochloris oleoabundans* (Breuer et al., 2012). In addition, common laboratory species are widely utilized as a platform for developing new strategies that would assist in reaching higher yields. *Chlamydomonas reinhardtii* is one of the most frequently used model organisms and has long been the center of physiological, biochemical and genetic studies for division *Chlorophyta.* As methods of gene transfer were developed fairly early for *C. reinhardtii*, several genetic studies have already been conducted to increase the biomass and biodiesel production of this species (Rosales-Mendoza et al., 2012). While *C. reinhardtii* is not widely regarded as the most suitable organism for biodiesel production at large scales, this species is nonetheless preferred in this field due to its ease of handling and capacity for non-photosynthetic growth (Rupprecht, 2009).

Despite having numerous advantages over crop plants, microalgae are associated with a number of technical, biological and economical hindrances that prevent algal biodiesels from effectively replacing petroleum-derived fuels (Chisti, 2007). The predominant biological problem is the low lipid contents displayed by many microalgal species. Under stress conditions, most microalgae direct their metabolism to the production of non-polar lipids, and especially TAG, which are used as storage molecules and rapidly degraded for energy production when optimal conditions arise. One of the most widely used stress conditions is nutrient starvation, which strongly induces TAG production of microalgae (Bölling and Fiehn, 2005). However, the exact mechanisms by which nutrient deficiencies direct the *Chlamydomonas* metabolism towards increased TAG production are not sufficiently explored. The present study aims to decipher the biochemical response patterns associated with the deficiency or over supplementation of several vital and trace elements by extensive quantitative and qualitative analyses, with emphasis on lipid biosynthesis. Effects of nitrogen deficiency on algal metabolism have previously been elucidated to great detail, and are utilized as a point of comparison for the effects of other element deficiencies and overabundances. Impacts of different element absences (N, S, P and Mg) and overabundances (N and Zn) on the microalgal metabolism have been followed by the evaluation of lipid quantities, time-based fluctuations in ion concentrations in the organism, alterations in synthesis pathways and changes in cell morphology and growth rates.

2. Methods

2.1. Culturing conditions

The wild type mt(-) 137c strain CC-124 was obtained from the Chlamydomonas Resource Center (www.chlamy.org). Cells were grown at 23 °C under continuous light (150 μmoles photons m^{-2} s^{-1}) in liquid cultures on a rotary shaker (120 rpm). Standard Tris–Acetate–Phosphate (TAP) medium was prepared as described previously. Starting cell densities were approximately 3×10^4 cells ml^{-1} in all groups. For N starvation studies, cells were centrifuged at 2500 rpm for 3 min at room temperature, and the pellets were washed twice in TAP medium without N (TAP-N). The pellets were then resuspended in TAP-N medium and the cells were grown under constant light exposure on a rotary shaker. The same procedure was applied for other element manipulations. Initial pH values in all media were set to 7 prior to algal cell inoculation, pH values of the media were checked every 24 h and found not to deviate more than 8% from the initial throughout the 10-day incubation period. Cell growth and size were monitored by haemocytometer cell counts using Lugol's solution (Sigma) and Image-J to perform volumetric calculations (Collins, 2007). Total cell biovolume was calculated using the equation "$B = CV$", in which B is the total biovolume, C is the number of cells, and V is cell volume. For relative dry weight measurements, a volume of medium containing 1×10^9 cells was centrifuged at 3000 rpm for 5 min; and the pellet was air-dried for 5 min, weighed, incubated at 80 °C for 48 h and re-weighed. Cells from all experimental groups were harvested at the 1st, 3rd, 5th, 7th and 10th days.

2.2. Elemental analyses

To determine metal content in algae and media, ICP mass spectrometry was utilized. Approximately 5×10^7 cells were collected for each treatment group, washed with 2 mM EDTA twice, ashed in crucibles at 600 °C for 12 h and dissolved in HNO_3. Samples were filtered prior to analysis. Elemental analysis was performed by an XSeries2 ICP-MS (Thermo Scientific, US-MA). All measurements were performed using a fully-quantified calibration method. Correlation coefficients were over $R^2 = 0.99$ for each element. Plasma power was set to 600 and 1400 W for cool and hot plasma applications, respectively. Isotopes with the fewest number of polyatomic interferences were chosen for m/z detection in mass spectrometry. An internal standard solution (10 ppb Bi) was used throughout the measurement period, measurements were repeated when internal standard concentration went beyond the tolerance range (±20%) or an unaccountably high deviation was observed between two reads of the same sample.

Concentrations of C, H, N, S and O were measured using a Flash 2000 organic elemental analyzer (Thermo Scientific, US-MA). 3–4 mg of oven-dried algal biomass was analyzed in tin capsules, vanadium pentoxide (V_2O_5) was added as an oxidation catalyst prior to sealing to increase the reliability of measurements. BBOT (2,5-Bis[5-tert-butyl-benzoxazol-2yl] thiophene) was used as a standard for all measurements. A manufacturer-provided protocol for algae was utilized as the measurement method.

2.3. Quantification of total protein and neutral lipids

Frozen cell pellets were re-suspended in lysis buffer (50 mM Tris–HCl pH 8.0, 2% SDS, 10 mM EDTA, and protease inhibitor mix), subjugated to sonication (3510E-DTH, Branson) for 1 min at 60% power (≈7 W/pin) and centrifuged at 13,000*g* at 4 °C. The supernatant was then used for protein determination with Bradford method.

Neutral lipid staining was performed using Nile Red as described by Elsey et al. (2007). Approximately 29.3×10^4 cells ml^{-1} were stained with 22 μl of 7.8×10^{-5} M Nile Red (Invitrogen) dissolved in acetone, left to incubate on a shaker for 15 min under darkness and washed twice. Relative fluorescence intensity of Nile Red staining was quantified on a fluorescence spectrometer (SpectraMax M5, MDS Analytical Technologies) using 490 nm excitation and 585 nm emission wavelengths. Total lipid levels were also confirmed gravimetrically.

2.4. Fourier transform infrared spectroscopy (FTIR)

A 1.3 ml sample was concentrated and 30 μl of which was then deposited on a 96 well silicon microplate and oven-dried for 45 min to form homogeneous thin films (Dean et al., 2010). FTIR spectra were recorded using a Nicolet 6700 Research FT-IR Spectrometer (Thermo Scientific). The bands were assigned to specific molecular groups on the basis of biochemical standards and published studies as previously described (Movasaghi et al., 2008). FTIR peak values were of particular interest which were attributed to ester group (C=O) vibration of triglycerides (1744 cm^{-1}), carbohydrate bands (1200–950 cm^{-1}) and amide I absorption (1652 cm^{-1}). FTIR spectra levels of amide I band obtained from

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