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Photobiochemical changes in *Chlorella* g120 culture during trophic conversion (metabolic pathway shift) from heterotrophic to phototrophic growth regime

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Abstract

Physiological and photobiochemical changes and growth in the heterotrophic strain *Chlorella vulgaris* g120 were studied during trophic conversion from heterotrophic to phototrophic growth regime. After the exposure of the *Chlorella* g120 culture to light, it revealed a significant activity of the electron transport (450–700 μmol e^{-} m^{-2} s^{-1} as measured by chlorophyll fluorescence) and high PSII photochemical yield F_v/F_m between 0.7 and 0.8. Fast fluorescence induction kinetics showed that PSII electron acceptors in the plastoquinone pool remained partly oxidized, indicating no downregulation of PSII electron transport. The data further revealed that high photobiochemical activity is lost in futile (protective) processes of non-photochemical quenching and respiration which indicate that surplus energy is dissipated in these processes. Pigment analysis showed low chlorophyll content − 0.35–1.15% as compared with exclusively phototrophic strain *Chlorella vulgaris* R-117. Nevertheless, the carotenoid content in g120 was relatively high − 0.20–0.33% of dry weight which resulted in a considerably high ratio of carotenoid/chlorophyll − 0.31–0.44. This strain probably does not possess the fully competent photosynthetic apparatus and can only partially adapt to phototrophy. We show that the heterotrophically grown g120 strain can undergo metabolic shift from heterotrophic to phototrophic growth regime. It might be an interesting strain from biotechnological point of view as a source of carotenoids, especially lutein.

Keywords *Chlorella* · Chlorophyll fluorescence · Heterotrophy · Photosynthesis · Respiration · Trophic conversion

Introduction

The majority of microalgae strains grow phototrophically (in inorganic media with addition of carbon dioxide) obtaining their energy and reducing power via the absorption of light for reduction of CO_2 and the oxidation of water, with the release of oxygen (for a review see, e.g. Masojídek et al. 2013). Mixotrophic cultivation (in light with addition of CO_2 and organic compounds) was introduced for *Chlorella* in Japan in the 1960s, replacing phototrophic culturing (e.g. Endo et al. 1977). The advantage of this growth regime is a possibility to increase the culture concentration (> 30 g L^{-1}), in contrast to phototrophic cultivation in ponds where the cellular concentration is at least one order lower.

Some microalgae (e.g. *Chlorella*, *Arthrospira*, *Haematococcus*) can grow not only phototrophically or mixotrophically but also under heterotrophic conditions (Endo and Shirota 1972; Endo et al. 1977; Endo and Sansawa 1994; Doucha and Lívanský 2012) as they possess active transporters of some organic substrates across the

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membrane. Heterotrophic growth of microalgae, i.e. in the dark in the presence of organic substrates like glucose, acetate, glycerol, lactate and other organic substrates, has to be carried out axenically in fermenters. As compared with phototrophic growth, heterotrophic cultivation has several advantages including high growth rate, the elimination of light limitation, high biomass density, high degree of process control, and lower costs for growth and harvesting (Chen et al. 1997; Barclay et al. 2013; Liu and Hu 2013). However, the content of pigments in heterotrophically grown biomass is significantly lower (2–4 times) as compared with those from phototrophic growth (Sergejevová and Masojídek 2012; Benavente-Valdés et al. 2016; Mohammad Mirzaie et al. 2016). A chlorophyll (Chl) content between 1 and 1.8% of dry weight (DW) was reported in heterotrophically grown *Chlorella* while in phototrophically grown cells can reach 2.5–3.5% of DW (Endo et al. 1974; Malapascua et al. 2019).

Several microalgae strains (e.g. *Chlorella*, *Haematococcus*, *Chlorococcum*) are also able to undergo a metabolic pathway shift (trophic conversion from heterotrophic to phototrophic growth regime). In the late 1990s, a cultivation strategy called 'sequential heterotrophy-dilution-photoinduction' was developed (Ogbonna et al. 1997). Later, this procedure was scaled-up using *Chlorella* cultures to proof the feasibility of this approach in indoor and outdoor trials for biomass production (Fan et al. 2012; Wan et al. 2015). Firstly, the *Chlorella* culture was cultured heterotrophically to achieve high cell densities. After glucose consumption, the culture was diluted and exposed to light for phototrophic growth. Using this strategy, the quality of the *Chlorella* biomass may be closer to that of cultivated phototrophically. Sequential, two-step, heterotrophic-phototrophic cultivation procedure (transition from heterotrophy to phototrophy) was also tested for other microalgae species, e.g. *Haematococcus* (Hata et al. 2001; Wan et al. 2015). The conversion from phototrophy to heterotrophy was also used in the microalga *Auxenochlorella protothecoides* to produce biomass with high lipid content for biofuels (Bohutskyi et al. 2014). Nevertheless, not much is known and published about the nature of photobiochemical changes that accompany the trophic conversion (metabolic shift) from heterotrophy to phototrophy in microalgae cultures.

A possible solution for improving microalgal scale-up is to combine both modes of microalgal cultivation into an efficient two-stage production. Recently, this process was managed in pilot and industrial scale—a two-stage procedure was developed as heterotrophically *Chlorella vulgaris* cells grown in fermenters (1st stage) were used to directly inoculate an outdoor flat-panel and tubular PBRs (2nd stage) (Barros et al. 2019). This approach relies on heterotrophic growth to obtain highly concentrated inoculum for outdoor bioreactors operating under phototrophy.

Successful cultivation requires continuous monitoring of a culture's physicochemical variables, (pH, temperature, dissolved oxygen concentration, nutrient status, etc.) as well as microscopic examination to detect contamination by other microorganisms. Following the application of chlorophyll (Chl) fluorescence measurements in field crops, this technique started to be applied for monitoring photosynthetic performance of microalgae mass cultures as to detect various unfavourable effects and to optimize culture growth since the 1990s (Knoppová et al. 1993; Vonshak et al. 1996; Torzillo et al. 1996, 1998; Masojídek et al. 1999). Since then, Chl fluorescence techniques have become one of the most useful approaches used for monitoring of microalgae mass cultures due to its non-invasiveness, ease of use and sensitivity (Maxwell and Johnson 2000; Masojídek et al. 2000, 2011a, b; Vonshak et al. 2001; Strasser et al. 2004; Baker 2008; Malapascua et al. 2014; Babaei et al. 2017). Some variables, like the maximum quantum yield of PSII (F_v/F_m), electron transport (ETR) and non-photochemical quenching (NPQ) measured by fluorescence quenching technique have been used to correlate photosynthesis and growth (e.g. Torzillo et al. 1996, 1998; Kromkamp et al. 1998; Ralph and Gademann 2005; Enríquez and Borowitzka 2011; Masojídek et al. 2011b; Malapascua et al. 2014; Silva Benavides et al. 2017). While fluorescence quenching analysis gives information on the balance between photosynthetic electron transport and the Calvin-Benson cycle, fast fluorescence induction kinetics (the so-called OJIP test) provides information on the reduction of the photosynthetic electron transport chain (Malapascua et al. 2014). Measurements of photosynthetic oxygen evolution give similar information as Chl fluorescence techniques; one important advantage of oxygen exchange concerns the possibility to monitor dark respiration (Figueroa et al. 2003).

Understanding the nature of photobiochemical changes that accompany the trophic conversion (metabolic shift) from heterotrophy to phototrophy in microalgae cultures is a prerequisite to optimize the process. The aim of this work was to study changes in photosynthetic activity, biomass composition and growth (and biomass composition) of the laboratory cultures of *Chlorella* g120 during the trophic conversion process.

Materials and methods

Microalgae culturing

The green microalga *Chlorella vulgaris* g120 (registered as *Chlorella vulgaris* BEIJ., 1996/H 14, CCALA 30001, Culture Collection of Autotrophic Organisms, Institute of Botany, Třeboň, Czech Republic) is a natural, non-GMO strain (cell size between 3 and 5 μm) fast-growing when cultured

heterotrophically. After the transfer from agar, the axenic cultures were firstly grown in the presence of glucose in 250-mL Erlenmeyer flasks in the dark at laboratory temperature (25 °C) for 7 days to reach biomass density between 5 and 10 g biomass L^{-1}. The heterotrophic medium contained glucose (0.43 M), urea, macroelements and other salts of micro- and trace elements (Doucha and Lívanský 2012). The cells were harvested by centrifugation and washed twice to remove glucose. Then, to carry out phototrophic growth, the cultures were transferred to an inorganic medium containing the following compounds (in mg L^{-1}): KNO_3, 2021; KH_2PO_4, 340; $MgSO_4 \cdot 7H_2O$, 989; ferric-sodium chelatonate, 18.4; $CaCl_2$, 16; H_3BO_3, 1.9; $MnCl_2 \cdot 4H_2O$, 7.3; $ZnSO_4 \cdot 7H_2O$, 7; $CuSO_4 \cdot 5H_2O$, 2; $CoSO_4 \cdot 7H_2O$, 1.4; $(NH_4)_6Mo_7O_{24}$, 0.08; and NH_4VO_3, 0.06; pH 7.4 (Šetlík et al. 1972; Zachleder and Šetlík 1982). To reduce bacteria and fungi growth, autoclaved medium was used throughout this study. The population of bacteria and fungi in all cultures was microscopically checked daily. The *Chlorella* cultures of various initial biomass densities of about 0.2, 0.5, 1.0 g DW L^{-1} were grown in vertical glass columns (working volume 330 mL; internal diameter 33 mm) that were submerged in temperature-controlled bath (28–29 °C) and mixed by bubbling with a mixture of air + 1% CO_2 (v/v) (Fig. 1). Photosynthetically active radiation (PAR) of 120 μmol photons m^{-2} s^{-1} was provided continuously by a panel of horizontally placed, high-frequency cool fluorescent tubes (36 W/830 Lumilux, Osram, Germany). Light intensity reaching the surface of cultivation columns was measured as described below. Sterile distilled water was added to compensate the evaporation between samplings.

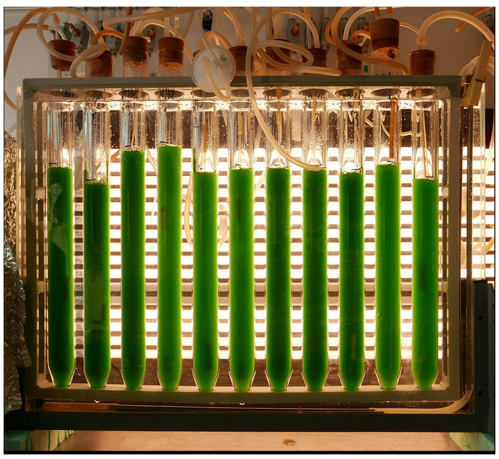

Fig. 1 Laboratory cultivation of *Chlorella* in glass columns that were placed in temperature-controlled bath with back illumination by high-frequency fluorescent tubes. The columns were mixed by bubbling where tubing was led through a rubber stopper to push the filtered mixture of air+CO_2 from the bottom and another tubing in the stopper is an exhaust. The total volume of columns is 0.5 L (inner diameter is 3.7 cm and length 46 cm), but working volume used in experiments was 330 mL

Photosynthesis measurements

Photosynthetic activity of samples taken from tested cultures was measured using three techniques—saturation pulse analysis of fluorescence quenching to record rapid light-response curves, fast fluorescence induction kinetics (OJIP) and photosynthetic oxygen production and respiration. All records were analysed to calculate photosynthesis variables.

Photosynthesis measurements were carried out using standardized procedures at temperatures corresponding to growth values. Prior to measurements the microalgae samples were dark-acclimated for 10 min to keep the same 'light' history (the photoacclimation status of cells). The samples taken from the culture columns were measured ex situ after dilution to 0.2 to 0.3 g DW L^{-1} (corresponding to 5 to 7 mg Chl L^{-1}) with the growth medium and dark adapted. In this way, light reabsorption problems in dense cultures were prevented by dilution and sufficient illumination was available to fully reduce the plastoquinone pool (closure of PSII reaction centres).

Rapid light-response curves

Rapid light-response curves (RLCs) of microalgae samples taken from the cultures were measured in a light-protected measuring chamber with mixing (3-mL glass cuvette with light path of 10 mm) using a pulse-amplitude-modulation fluorimeter (PAM-2500, H. Walz, Germany). A series of stepwise increasing irradiance intensities (red LEDs; 0–2000 μmol photons m^{-2} s^{-1}) were applied in 20-s intervals to obtain the steady-state fluorescence level F' and then a saturating pulse (> 10,000 μmol photons m^{-2} s^{-1}, 0.6-s duration) was triggered to reach the maximum F_m'. At each step, the actual PSII photochemical quantum yield in the light, Y_{II} was determined as $(F_m' - F')/F_m'$ where F_m' is the maximum fluorescence level and F' is the steady state fluorescence in the light-adapted state at respective irradiance level. Analysis of RLCs was used to estimate changes of the relative electron transport rate through PSII, rETR which was calculated by multiplication of the actual photochemical efficiency Y_{II} and the intensity of photosynthetically active radiation E_{PAR}, $rETR = Y_{II} \times E_{PAR}$ (e.g. Hofstraat et al. 1994; Ralph and Gademann 2005; White et al. 2011). In order to determine $rETR_{max}$ and the irradiance saturating photosynthesis, light response curves were fitted to the non-linear least-squares regression model by Eilers and Peeters (1988) using PamWin_3 software. The so-called Stern-Volmer non-photochemical quenching NPQ (= $[F_m - F_m']/F_m'$) is, in principle, inversely related to photochemistry (actual PSII photochemical yield Y_{II}). It indicates an increased futile heat dissipation of absorbed energy and is considered a safety valve protecting PSII reaction centres from damage by excess irradiance (Bilger and Björkman 1990).

The minimum and maximum fluorescence levels (F_0, F_m) were determined using a weak modulated light (< 0.15 μmol photons m^{-2} s^{-1}, frequency of 0.5–1 kHz) in the dark-adapted samples (actinic irradiance = 0, first step of RLC). The maximal PSII quantum yield was calculated as the ratio of variable and maximal fluorescence, $F_v/F_m = (F_m - F_o)/F_m$; it indicates the capacity of the system to absorb light through the reaction centres and the light-harvesting complex and expresses the maximum quantum efficiency of primary photochemistry (Strasser et al. 2004). The fluorescence nomenclature follows Schreiber et al. (1986) as later elaborated by van Kooten and Snel (1990) and Kromkamp and Forster (2003).

Fast fluorescence induction kinetics

Curves of fast Chl fluorescence induction kinetics (OJIP kinetics) were measured ex situ by a portable fluorimeter (AquaPen AP-100, P.S.I. Ltd., Czech Republic). The OJIP kinetics was followed in diluted (0.2 to 0.3 g DW L^{-1}) samples, dark-adapted for 10 min. The samples were transferred to a 3-mL measuring cuvette (light path of 10 mm) which was mounted in a light-protected holder in front of the detector (adjustable measuring light pulses, ~2.5 μs) while red LEDs served as high-intensity actinic light from both sides of the cuvette (up to 3000 μmol photons m^{-2} s^{-1}), perpendicular to the detector. The OJIP kinetics was measured in the time range between 50 μs and 1 s when the signal rises rapidly from the origin (*O*) to highest peak (*P*) via two inflexions—*J* and *I* (Strasser and Srivastava 1995). The *O* point (50 μs) of the fluorescence induction curve represents a minimum value (designated as constant fluorescence yield F_0) when PQ electron acceptors (Q_A and Q_B) of the PSII complex are oxidized. The inflexion *J* occurs after ~2–3 ms of illumination and reflects the dynamic equilibrium (queasy-steady state) between Q_A and Q_A^-. The *J*–*I* phase (at 30–50 ms) corresponds to the closure of the remaining centres, and the *I*–*P* (ends at about 300–500 ms) reflects full reduction of the plastoquinone pool (equivalent to maximum fluorescence level F_m) (Strasser et al. 2004; Goltsev et al. 2016). From the fluorescence levels at the *J* and *I* points, the variables V_j and V_i were calculated as follows: $V_j = (F_{2ms} - F_0)/(F_m - F_0)$ and $V_i = (F_{30ms} - F_0)/(F_m - F_0)$.

Light measurements

Photosynthetically active radiation (PAR) was measured either with the LI-190SA quantum sensor (cosine corrected up to 80° angle of incidence) or using the US-SQS/B spherical micro quantum sensor (scalar irradiance; Walz) coupled to the LI-250 Light Meter (Li-Cor, USA). All readings were taken as 15-s average values.

Oxygen production and respiration

Activity of respiration and photosynthetic oxygen production was measured polarographically using a temperature-controlled chamber with adjustable illumination, temperature control and mixing (DW2/2 connected to a control unit Oxygen Monitoring System Oxylab+; Hansatech, UK). Samples taken from experimental cultures were adapted for 10 min in the dark at desired temperature. The first step (2 min in the dark) provided the value of the dark respiration and then maximum oxygen production activity was measured at the saturating irradiance of 1000 μmol photons m^{-2} s^{-1} (red LED light source) estimated with a spherical quantum sensor inside the measuring chamber. The oxygen production and respiration were calculated in mmol O_2 mg^{-1} Chl h^{-1}.

Biomass density and pigment analysis

Biomass density was measured as dry weight (DW in g L^{-1}) by filtering 5 mL of culture samples on pre-weighed glass microfiber filters (GC-50). The filters with the cell sediment were washed twice with deionized water, dried in oven at 105 °C for 3 h, then they were transferred to a desiccator to equilibrate to laboratory temperature and weighed (precision of ± 0.01 mg).

The specific growth rates $\mu = (\ln X_2 - \ln X_1)/\Delta t$ [day^{-1}] of microalgae cultures were calculated in the period between 8 and 48 h of trials where *X* is biomass density.

Chl and carotenoid (Car) concentration was determined spectrophotometrically in methanol extracts. The samples of 500 μL were collected in 2-mL Eppendorf tubes and centrifuged at 13,000 rpm for 3 min (centrifuge Minispin, Eppendorf). The pellet was resuspended in 500 μL of 100% methanol, and the amount of 100 μL of sea sand was added to the tube. The cells were broken by intensive abrasion with sea sand for 2 min using a vortex mixer, then the tubes were cooled down in an ice bath and centrifuged at 10,000 rpm for 1 min. If necessary, the extraction was repeated several times until the pellet was colourless. The absorbance of the combined supernatants from all extraction steps was measured at 665 and 750 nm using a high-resolution spectrophotometer (UV 2600 UV-VIS, Shimadzu, Japan, slit width of 0.5 nm) and the concentrations of Chl were calculated according to Wellburn (1994).

Statistical analysis

Comparison between the cultures was conducted in Sigma Plot 11.0. One-way ANOVA and post hoc test were conducted for every binary combination of systems. *P* values lower than 0.05 were considered to be significantly different. Data were recorded in triplicate ($n = 3$). All experiments were conducted in triplicate as well as data records ($n = 3$).

Results

The growth of *Chlorella* g120 culture was studied at biomass densities of about 0.2, 0.5 and 1 g L^{-1} (Fig. 2) after the transfer from heterotrophic to phototrophic growth conditions. The *Chlorella* g120 culture showed similar growth trends at various biomass densities during 48 h, but different growth rates of 0.015, 0.009 and 0.003 h^{-1} for the cultures of 0.2, 0.5 and 1 g DW L^{-1}, respectively, which were calculated between 8 and 48 h of trial starting after the lag phase. A 20% decrease of biomass density was observed in the dense culture (1 g DW L^{-1}) at the beginning of experiment (0–8 h), which may be caused by partial cell lysis after transfer of heterotrophic culture to light; in other cultures, it was not visible. On the other hand, the growth of the cultures increased significantly in the second half of the experiment, namely at lower biomass densities.

Changes in Chl and Car content were monitored in heterotrophic/phototrophic culture during the experiment (Fig. 3). The g120 culture contained 0.26–0.35% Car and 0.61–0.90% Chl in DW (Fig. 3, panels a, b). The ratio of Car/Chl in the *Chlorella* g120 culture was between 0.31 and 0.44. As concerns the changes during the experiment, an increase of pigment content was observed in the cultures, especially at the highest biomass density. The content of Chl increased about 3-fold while that of Car by about one-half.

Photosynthetic activity was estimated as electron transport rate using fluorescence RLC measurements (Fig. 4). The course of all curves was similar in all cultures (0.2, 0.5 and 1 g DW L^{-1}), only the maxima were significantly decreased 24 h after the start of experiment which probably reflected increased cell density causing self-shading (Fig. 5). Three variables—the maximum of relative electron transport rate ($rETR_{max}$), the maximum quantum yield of PSII (F_v/F_m) and non-photochemical quenching (NPQ) were calculated from

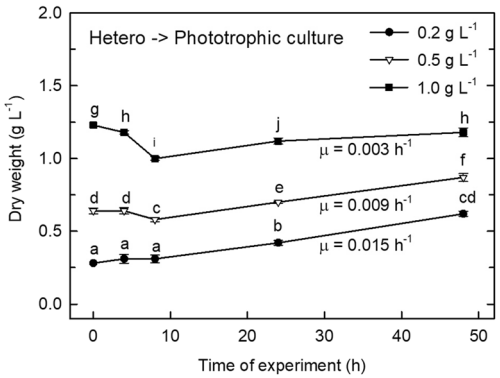

Fig. 2 Changes in DW during growth of the heterotrophic-phototrophic *Chlorella* g120 cultures with various starting biomass density (0.2, 0.5 and 1 g L^{-1}). Specific growth rates (μ) were calculated in the period between 8 and 48 h of the trial and are shown for the cultures with various biomass density. The results represent the mean value of three measurements ($n = 3$). Values with similar letter did not differ significantly from each other (P value > 0.05)

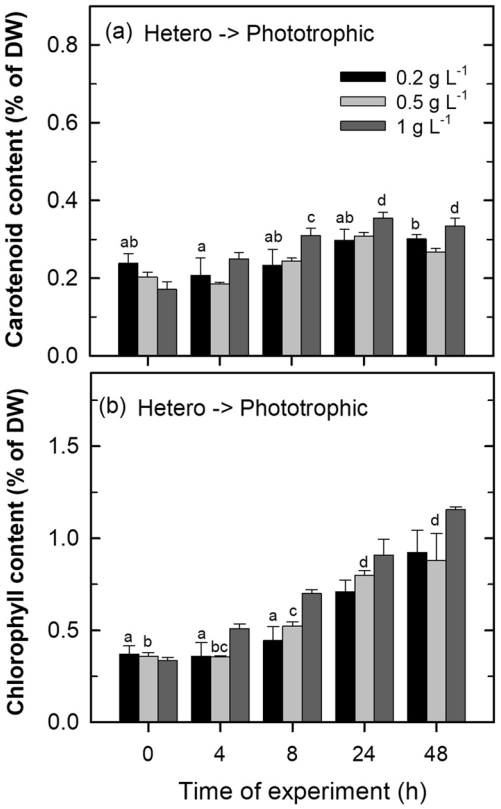

Fig. 3 Changes in (a) total Car and (b) total Chl content in *Chlorella* g120 cultures with various starting biomass density (0.2, 0.5 and 1 g L^{-1}). The results represent the mean value of three measurements ($n = 3$). Values with similar letter did not differ significantly from each other ($P > 0.05$)

RLCs (Fig. 5). The F_v/F_m ratio was between 0.69 and 0.79, slightly increasing up to after 8 h of experiment and it started to decrease. But basically the changes in the course of the trial were small showing that the cultures were not stressed during the experiment. The rETR activities of the cultures measured at 0, 8 and 24 h of the experiment were between 400 and 680 μmol e^- m^{-2} s^{-1}. At the beginning of the experiment, when the cultures of different biomass density were exposed to light, the $rETR_{max}$ activity was similar (P value > 0.05) (Fig. 4b). After 4 h, the $rETR_{max}$ values of the cultures were maximal increasing by about 20%. Then, the rETR activity of all cultures started to decrease. On the contrary, NPQ (calculated at $rETR_{max}$) significantly increased between 8 and 24 h of the experiment, and it decreased in the lower density cultures. Surprisingly, the high NPQ could be observed after 48 h in the highest biomass density cultures. This can be a partial explanation of absorbed energy fate in the cells—non-photochemical energy dissipation. One explanation could be the fact that this culture had a higher respiration rate that could have kept PQ pool reduced. Moreover, this culture stopped to grow because respiration rate was higher than oxygen evolution rate. Another potential source of NPQ build-up may relate to state-transition mechanisms (Cardol et al. 2011).

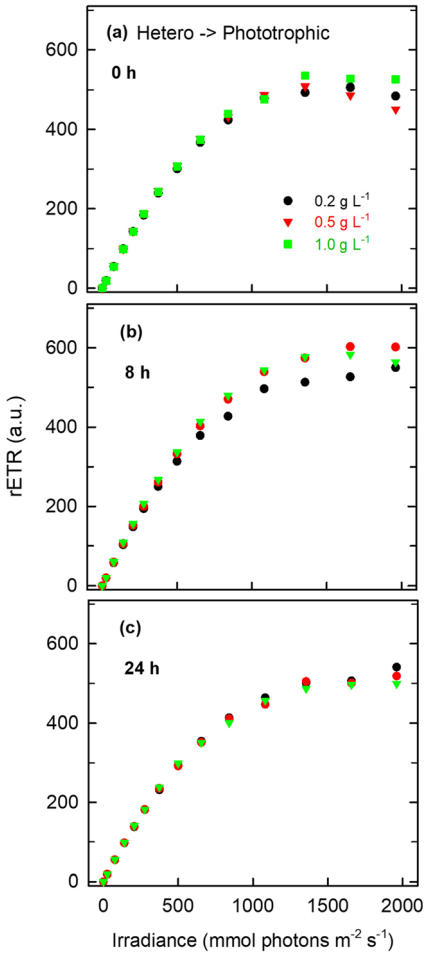

Fig. 4 Changes of rapid light-response curves of rETR monitored in the *Chlorella* g120 cultures with various starting biomass density (0.2, 0.5 and 1 g L^{-1}) after (a) 0 h, (b) 8 h and (c) 24 h. Light-response curves are shown as colour symbols without error bars as these obscure data point measured at the same irradiance

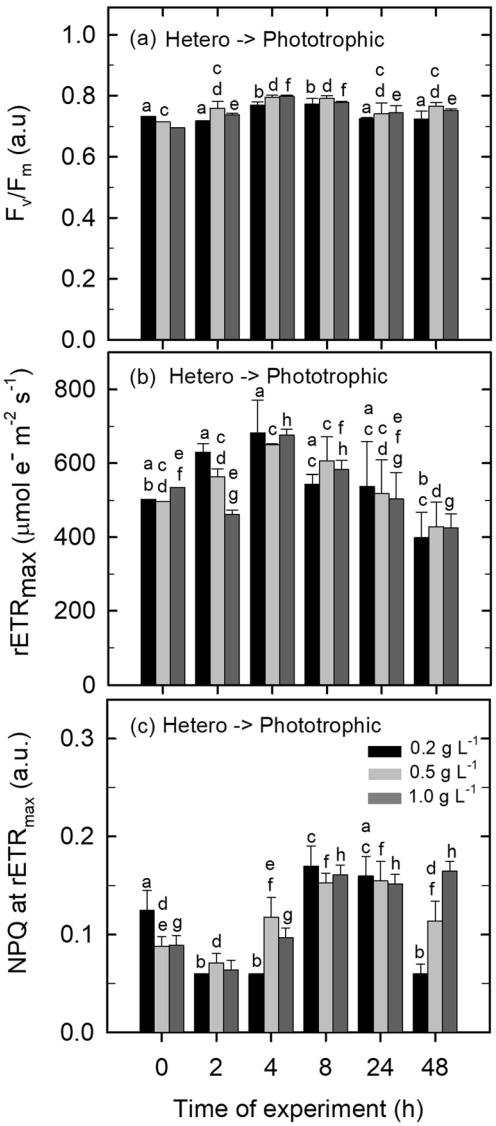

Fig. 5 Changes of variables (a) F_v/F_m, (b) rETR and (c) NPQ calculated from rapid light-response curves (see Fig. 4) in the *Chlorella* g120 culture with various initial biomass concentration. The results represent the mean value of three data ($n = 3$). Values with similar letter did not differ significantly from each other ($P > 0.05$)

Measurements of photosynthetic oxygen evolution and respiration also suggested the way of absorbed energy spending via respiration of cells. Photosynthetic oxygen production and respiration were measured in the well-growing culture of 0.5 g DW L^{-1} and normalized per Chl amount. It was found that the photosynthetic activity in the culture significantly increased after 8 h and reached twofold activity after 24 h and remained high even after 48 h (Fig. 6). The maximum of respiration activity was found after 8 h and then it was decreasing although photosynthetic oxygen still increased. Relatively high respiration activity (oxygen consumption) provides an evidence for energy dumping.

Rapid fluorescence induction kinetics recorded in the *Chlorella* g120 cultures using various initial biomass concentrations showed different courses of OJIP curves (Fig. 7). The important point of observation was that when the cultures of different initial biomass densities were exposed to light, we did not find any dramatic change in the induction curves which suggested that biomass densities were not substantially photostressed at the irradiance intensity used in the experiments. It is important to point out that in the cultures of 0.5 and 1 g DW L^{-1} we observed a slight increase at induction curve inflexion (Fig. 7). The fluorescence induction curves were analysed to calculate the inflexion points *J* and *I*. The *J* variable is measured after ~ 2–3 ms of illumination and reflects the equilibrium between Q_A and Q_A^- and the point I at 30–50 ms corresponds to the closure of the remaining centres (Strasser et al. 2004; Goltsev et al. 2016). After the exposure to light, all the cultures showed higher variables V_i and V_j which decreased during first until 4 h of experiment by about 10–20%. Then, they started to increase slowly as the cultures became denser (Fig. 8). The trends of the V_j and V_i variables were reflected by changes in growth curves (Fig. 2).

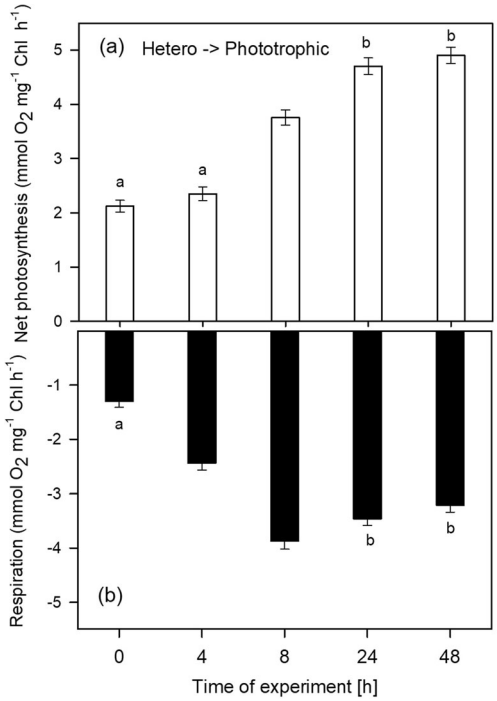

Fig. 6 Changes of (a) net photosynthesis and (b) respiration activity measured in the *Chlorella* g120 cultures (0.5 g DW L^{-1}) during first 8 h of experiment. The results represent the mean of three measurements ($n = 3$). Values with similar letter did not differ significantly from each other ($P > 0.05$)

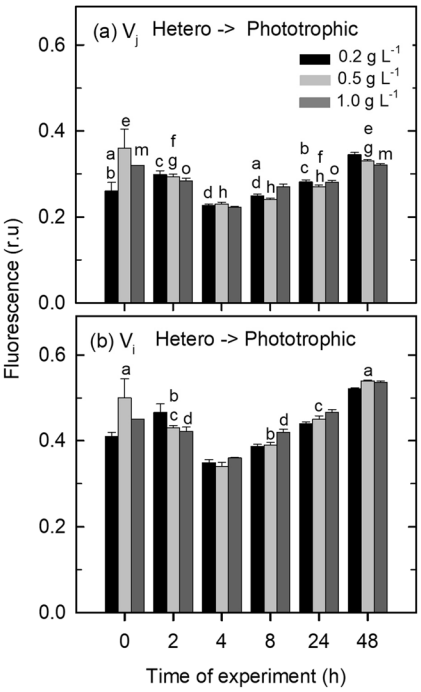

Fig. 8 Changes of the (a) V_j and (b) V_i variables calculated from rapid fluorescence induction curves (see Fig. 8) of the heterotrophic/phototrophic *Chlorella* g120 cultures with various biomass concentration (0.2, 0.5 and 1 g L^{-1}) after 48 h of experiment. The results represent the mean value of three measurements ($n = 3$). Values with similar letter did not differ significantly from each other ($P > 0.05$)

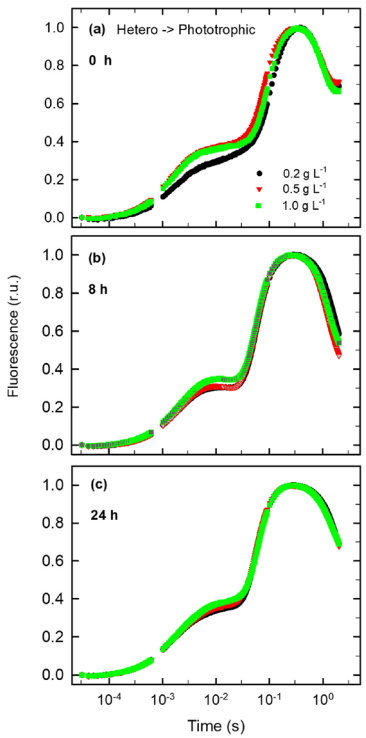

Fig. 7 Rapid Chl fluorescence kinetics of cultures with various biomass concentrations (0.2, 0.5 and 1 g L^{-1}) in the heterotrophic/phototrophic *Chlorella* g120 cultures after (a) 0 h, (b) 8 h and (c) 24 h

Discussion

The behaviour of heterotrophic production strain *Chlorella* g120 was studied in laboratory cultures as concerns physiological and photobiochemical changes during the trophic conversion, i.e. the adaptation from heterotrophic cultivation using an organic substrate to phototrophic growth in inorganic media. The idea was to obtain a bulk of biomass from the heterotrophic cultivation that is rapid—but resulting in lower pigmentation—and then to apply the second step, the exposure of the culture to phototrophic growth, in order to improve biomass quality (e.g. increase pigment content). We employed several techniques to evaluate early changes of the variables reflecting changes of physiology and photosynthetic activity that can be correlated with growth. Such data as to follow photobiochemical changes during trophic conversion have been scarce in literature (e.g. Jaffri 2011) although several authors have studied this trophic conversion in cultures of various microalgae (e.g. Ogbonna et al. 1997; Fan et al. 2012; Bohutskyi et al. 2014).

According to our results, the heterotrophic *Chlorella* g120 could acclimate to phototrophic growth rapidly within 4 h, and during next 48 h starts to grow increasing the biomass concentration by 1.7–2.2 times in the cultures with suitable biomass density (0.2–0.5 g DW L^{-1}). On the contrary, in denser cultures (~ 1 g DW L^{-1}) no relevant biomass density rise was

observed; most likely, these were photolimited when exposed to irradiance of 120 μmol photons m^{-2} s^{-1} (Fig. 2) and cell respiration contradicted the production. Our results are in agreement with the data in literature as the trophic conversion in heterotrophic cultures could recover photosynthetic activity within 24–48 h (Vernotte et al. 1992; Zheng et al. 2012).

The laboratory experiments showed that heterotrophic *Chlorella* g120 can undergo metabolic shift to phototrophic growth although the cells contain much less Chl (about 0.5% in DW) than phototrophic *Chlorella* R-117 (Malapascua et al. 2019). It has implied that *Chlorella* g120 may not possess the fully competent photosynthetic apparatus, namely light-harvesting function is decreased due to Chl deficiency. Similar phenomenon was described for the microalga *Dunaliella* (Melis et al. 1999). The colour of *Chlorella* g120 culture was always milky yellowish, paler as compared with phototrophic *Chlorella* cultures. This was due to much lower Chl content in biomass resulting in higher Car/Chl ratio. Important to note is that the averaged ratio of Car/Chl in the *Chlorella* g120 was high, 0.63, while it was found only about 0.2 in phototrophic *Chlorella vulgaris* R-117 (Malapascua et al. 2019). Nevertheless, the *Chlorella* g120 culture increased both Chl and Car concentration in biomass when exposed to phototrophic growth which indicated a certain degree of photoadaptation. Previous data showed that in *Chlorella* g120 culture the main carotenoid species were lutein (78%), violaxanthin (7%), other xanthophylls (12%) and beta-carotene (3%) calculated per total carotenoid amount (Zaťková et al. 2011). Comparing *Chlorella* g120 with fully phototrophic strain *C. vulgaris* R-117 (Malapascua et al. 2019), it was found that carotenoid content in biomass was similar (0.2–0.5% of DW), but only a half of Chl was present in the former one. Lutein-dependent non-photochemical quenching may be mobilized in *Chlorella* g120 as in microalgae this pigment was described to be associated with the light-harvesting complexes (Lin et al. 2015; Leuenberger et al. 2017).

If we consider which variables might be taken as reliable indicators of culture activity/adaptation, the maximum PSII photochemical yield, F_v/F_m and the relative electron transport rate, rETR, V_j and V_i measured by Chl fluorescence techniques as well as photosynthetic oxygen evolution are good markers. The relatively high values of F_v/F_m (around 0.7 and above) and rETR (between 400 and 680) showed that the cultures were not under significant photo-stress. At the start of cultivation when the cultures were exposed to light, we could observe a certain overreduction of the PQ pool manifested by the increase of V_j and V_i. It was a way how the culture was getting acclimated and started to grow (Fig. 8). Similar trend was also found in case of electron transport rate (Fig. 5b). Comparing the ETR measurements with photosynthesis measurements (i.e. photosynthetic oxygen evolution and respiration) and NPQ estimation (Figs. 5b, c and 6a, b), we have found a certain subsequence of events. First, after 4 h of the trial, ETR maxima were reached while NPQ was still low; then rETR started to decrease while NPQ was increasing as well as respiration. Although measurement of electron transport rate has become a fast and widely used tool in the study of photosynthesis, it can provide only indirect information on energy dissipation (NPQ), but cannot measure the dark respiration activity that may play an important role in the light acclimation via excess energy dissipation. Its role is evident as the *Chlorella* g120 cultures have high respiration rate (oxygen consumption calculated per Chl), contrariwise comparable with photosynthetic oxygen production (Fig. 6); it is probably an inherited feature of heterotrophic strains. Respiration serves as an energy sink protecting the photosynthetic apparatus against surplus irradiance but in this case limiting biomass growth and production. The cultures should not be exposed to high irradiance due to a possibility of photoinhibition, or we need to seed high cell density. High respiration rate (reduced PQ pool) may favoured state 1 to state 2 transition with NPQ rise (qT) (Cardol et al. 2011). There also exist other unspecified mechanisms, e.g., reaction centre quenching (Ivanov et al. 2008) that may also play a significant role in photoprotection and contribute to NPQ, especially if this strain has modified light-harvesting antennae. Our data are in agreement with observation in reduced antenna-size mutant of *C. vulgaris* which showed a decrease in Chl content (Shin et al. 2016). This strain had a decreased expression of peripheral light-harvesting PSII antenna proteins. The electron transport rate of this mutant was significantly higher and also showed reduced non-photochemical quenching (NPQ), compared with those of the wild type.

As concerns a possible mechanism of reaction centre quenching, it was described that PSII reaction centres can be reversibly switched over from photochemical energy transducers (that convert light into ATP and NADPH) to efficient, non-photochemical energy quenchers that protect the photosynthetic apparatus from photodamage (Ivanov et al. 2008). Reaction centre quenching (the accumulation of reduced Q_A) in the photosystem II reaction centres complements photoprotection through antenna quenching and may represent a general response of photoautotrophs. Since the evolution of reaction centres preceded the evolution of light-harvesting systems, reaction centre quenching may represent the oldest photoprotective mechanism.

We found some discrepancy between the rETRmax and net photosynthesis as the former first increased then decreased over the cultivation period; however, the net photosynthesis measured by oxygen evolution increased and remained high even after 48 h (Fig. 5 vs. Fig. 6). This discord seems to be rather complex to explain, but as mentioned above, the behaviour of the heterotrophic *Chlorella* g120 after exposure to light (during the shift from heterotrophy to phototrophy) and acclimation processes is probably rather complex. In our opinion,

the ETR firstly raised as the culture acclimated and then decreased as they became light-limited due to biomass density increase. After the acclimation of the culture to light when the maximum electron transport activity was reached (the maximum of F_v/F_m and $rETR_{max}$), the culture overcame a lag phase and started to grow. Photosynthetic oxygen production was increasing and remained constant between 24 and 48 h, while ETR started to decline. Photosynthetic activity reflects the underlying biophysical, biochemical and metabolic processes that regulate the whole process (Falkowski and Raven 1997). At light saturation, photosynthesis is limited on the acceptor side of PS II, generally by the capacity of enzymatic processes in the Calvin-Benson cycle (Sukenik et al. 1987). It is conceivable that cultures improved their photosynthetic performance by enhancing their enzymatic activities with the cultivation time. There also exist other possible sources for the discrepancy when estimating the rates of electron transport and net photosynthesis (measured as O_2 evolution). The primary reason may originate from the fact that the ETR variable reflects the gross PSII-dependent electron transport while photosynthetic oxygen production represents net photosynthesis (ETR minus losses). It is possible that a part of electrons is utilized in oxygen consumption and other processes (i.e. respiration, cyclic electron flow around PSII and/or the Mehler reaction), which can play a significant role. For example, the discrepancy between ETR and photosynthetic oxygen evolution found in some microalgae was usually attributed to the Mehler reaction—the reduction of O_2 by PSI (Flameling and Kromkamp 1998).

As the photosynthetic antennae in *Chlorella* g120 is not as well developed as in phototrophic strains, not all the energy, even under relatively week light, can probably be used for photosynthesis. At some moment after acclimation, the amount of transported electrons needed to maintain photosynthetic production is being in excess, and even smaller population of the functioning reaction centres can be sufficient to maintain oxygen production.

At the moderate irradiance of 120 μmol photons m^{-2} s^{-1} used in our laboratory cultivation trials, the optimum starting biomass density was between 0.2 and 0.5 g DW L^{-1}. In this case, we did see none or short lag phase when culture was exposed from dark to light at denser heterotrophic culture probably invested electron transport to respiration (Fig. 2).

We think that the capability of these cultures with reduced Chl content (about 0.5–1%, vs 2.5% in phototrophic strains) is not able to develop as high NPQ. Indeed, the NPQ values reported in Fig. 5 were below 0.2 which is rather low value considering that it was measured when at the maximum of light-response curves, i.e. at high rETR. We are to believe that respiration is one of the most important valves to dissipate excess of energy. High respiration rate is also the reason that reduces the growth of this strain compared with phototrophic *Chlorella* (Malapascua et al. 2019). However, other reasons that is the organization of the antenna cannot be ruled out. This point needs further investigation. As mentioned above, it is possible that also other mechanisms can participate in energy dissipation (and NPQ). A part of electrons can be utilized in oxygen consumption and other processes (i.e.; respiration, cyclic electron flow around PSII and/or the Mehler reaction) which can play a significant role.

Circumstantial evidence as well as results of our laboratory analyses of pigment-protein complexes has suggested that a certain part of light-harvesting antennae is naturally truncated or missing in *Chlorella* g120 cells (Masojídek et al. 2017). The strain g120 presents some important features to be considered as the so-called 'reduced antenna-size strain' (Melis et al. 1999; Shin et al. 2016), a feature desirable for mass culture growth. It has been speculated that small antenna-size strains perform better when grown under higher light as they may saturate at higher irradiance intensity (e.g. Gordon and Polle 2007); it is supposed that larger volume of culture can be irradiated as the light penetrates deeper due to reduced antennae. This may represent a certain technological advantage as truncated photosystem antennae can increase the photon use efficiency in microalgae cultures as it would avoid photoinhibition in cultures, minimize the wasteful dissipation of absorbed sunlight and diminish mutual cell shading permitting a greater light penetration through the culture (Melis et al. 1999). Nevertheless, per contra, the g120 strain presents a much higher respiration rate and non-photochemical energy dissipation.

Conclusions

The heterotrophic *Chlorella* g120 culture can undergo metabolic pathway shift to phototrophic growth regime but its photoadaptation is slow and incomplete, as its photosynthetic apparatus is probably conserved. The data also suggest that this strain does not possess the fully competent photosynthetic apparatus that cannot be restored; it might be characterized as a small-size light-harvesting antenna strain. *Chlorella* g120 revealed high electron transport activity, but this was not fully reflected by increased growth rate.

The important point was to clarify the physiological changes in cells during metabolic shift from heterotrophy to phototrophy. We have monitored various aspects of physiological and photosynthesis changes. The correlation between photosynthetic activity and culture growth is not as straightforward (as in fully competent phototrophic strains) since a part of absorbed energy is used for futile processes as NPQ and respiration which serves as protection against excess energy and this fact obstructs high growth rate.

The results suggest that high photobiochemical activity is lost in futile (protective) processes of non-photochemical quenching and respiration which indicate that surplus energy is dissipated

in this process and protects the photosynthetic apparatus against overreduction. A part of electrons might also be utilized in other processes (i.e.; cyclic electron flow around PSII and/or the Mehler reaction) which can play a significant role.

From the biotechnological point of view, strains with reduced light-harvesting antennae are desirable as to facilitate light penetration to deeper layers of the culture and its efficient utilization, provided that respiration rate is not too high thus vanishing the technological advantage. *Chlorella* g120 might be an interesting strain as it makes it possible to induce biomass enrichment in carotenoids upon transfer to phototrophic regime. Then it is important to mention that the ratio of Car/Chl in g120 is much higher than in other phototrophic Chlorella strains which makes the biomass use favourable as a source of carotenoids, especially lutein.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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