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Photosynthetic aspects and lipid profiles in the mixotrophic alga *Neochloris oleoabundans* as useful parameters for biodiesel production

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ABSTRACT

Since fossil fuels are expected to run out within few decades, attention has increasingly been focused on renewable energy sources, including microalgae. *Neochloris oleoabundans* (Chlorophyta) has a capability to accumulate lipids, in particular triacylglycerols (TAG), useful for biodiesel production; furthermore, it can grow mixotrophically. The present work deals with two fundamental steps of mixotrophic cultivation with glucose (late exponential – 6 days – and late stationary – 14 days – phases of growth), focusing on the relationship between photosynthesis and lipid production. Results confirmed that the use of glucose induces a high biomass productivity, which is associated to a rapid cell replication until day 6 followed by cell enlargement until day 14. At day 6, mixotrophic cells contained numerous stromatic starch grains, while at day 14 lipids were highly accumulated and starch tended to reduce. Photosynthetic pigment and protein content decreased under mixotrophy. The degree of photoinhibition under high light was not significantly affected by mixotrophic cultivation at both experimental times. The creation of a reducing environment due to the photosynthetic activity, together with alterations of N:C ratio, promoted the lipid synthesis. Neutral lipids increased under mixotrophy and oleic acid was the major component, while linolenic acid decreased; these aspects match requirements for biodiesel production.

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1. Introduction

The world energy demand is rapidly increasing because of the continuous rise of human population, urbanization and modernization [1]. At present, energy is mainly supplied by fossil fuels (about 80%), while minor inputs derive from renewable sources (13.5%) and nuclear power (6.5%) [1]. The large use of fossil fuels raises many issues, in terms of both environmental pollution and geopolitical aspects. Moreover, the depletion time for the fossil fuel reserves is calculated in few decades [2]. Research efforts worldwide aim at increasing and improving energy supply by renewable, clean sources, instead of non-renewable. Among renewable energy sources, lipid-rich microalgal biomass is proposed as a useful biofuel feedstock [3–7]. *Neochloris oleoabundans* (syn. *Ettlia oleoabundans*) is a green unicellular alga largely studied for its capability to accumulate lipids, especially triacylglycerols (TAGs), inside the cytoplasm [8–11]. *N. oleoabundans* is characterised by a high lipid content under different growth conditions, such as N-starvation, high-light exposure, pH variations, and mixotrophy [8,10–14]. Moreover, it was shown that, in *N. oleoabundans*, N-starvation has a negligible impact on the qualitative lipid profile [8,10]. However, in general, the proportion of lipid classes that differ with respect to the length of C-chain or degree of unsaturation can considerably change depending on environmental conditions [15]. Among culture conditions useful for both biomass and lipid production, the algal cultivation realised in the presence of organic carbon sources (glucose, acetate, organic acids, etc.) and light exploits a metabolic condition, called mixotrophy, shared by many algal species, *N. oleoabundans* included [11,14,16–20]. It has been recently demonstrated that *N. oleoabundans* can grow mixotrophically in the presence of pure glucose, but also of a glucose-containing apple waste product (AWP). In both cases, biomass production increased and lipids

Abbreviations: ALA, linolenic acid; AWP, apple waste product; Car, carotenoid; Chl, chlorophyll; DW, dry weight; FAME, fatty acid methyl ester; F_0, minimum fluorescence in the dark-adapted state; F_M, maximum fluorescence in the dark-adapted state; F_M', maximum fluorescence in the light-adapted state; F_S, steady state fluorescence; F_V, variable fluorescence ($F_M - F_0$); MUFA, monounsaturated fatty acid; PSII, photosystem II; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TAG, triacylglycerol; TEM, transmission electron microscopy; Y(NO), quantum yield of non-regulatory thermal dissipation and fluorescence; Y(NPQ), quantum yield of regulatory thermal dissipation; Y(PSII), quantum yield of PSII photochemistry.

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accumulated inside the alga [11,14,18]. In particular, growth rates under mixotrophic cultivation were higher than under autotrophy, leading to 6–7 times higher cell density and biomass productivity [11,14,18]. The biomass increase, observed when either pure glucose or AWP were added, was supported by a good photosynthetic activity of photosystem II (PSII), even if accompanied by different photosynthetic pigment patterns [11,14,18]. Only when the PSII maximum quantum yield decreased, lipids started to accumulate [14,18]. In fact, a decrease in F_V/F_M ratio reflects a damage to PSII, symptomatic of an unbalanced accumulation of reducing power, which in turn promotes the synthesis of lipids [21,22]. It is known that there is a close link between energy carrier accumulation, due to a feedback inhibition of photosynthate buildup, and decrease in the rate and photon efficiency of photosynthesis [22]. This confirms the great complexity of the relationship between photosynthesis and lipid synthesis, especially in the case of mixotrophy, as already highlighted by the complex behaviour of the photosynthetic apparatus of *N. oleoabundans* grown in the presence of glucose or AWP [14,18]. In this perspective, the link between these metabolic pathways (photosynthesis and lipid synthesis) under mixotrophic growth conditions deserves further investigations. Moreover, lipid quality, which is basic information for any bioenergetic applications, still remains poorly characterised under such conditions, especially for microalgae belonging to *Neochloris* genus [20].

In the present work, in order to better exploit the biotechnological potential of *N. oleoabundans*, we provide an in-depth characterisation of morpho-physiological aspects of the alga at two crucial steps (late exponential and late stationary phases of growth) during the lipid synthesis process induced by the mixotrophic cultivation with glucose. It was reported, in fact, that the alga replicates rapidly, but does not produce lipids, when a sufficient combination of nutrients is available in the culture medium (i.e. up to the late exponential phase of growth), while it slows replication, with a concomitant lipid synthesis induction, when nitrogen becomes limiting [14,18,23,24]. Moreover, we included a comparison of lipid production and quality in *N. oleoabundans* cultivated mixotrophically in the presence of AWP, in order to test if the lipid profile remains unchanged upon cultivation with different mixotrophic substrates.

2. Materials and methods

2.1. Culture conditions

Axenic cultures of *N. oleoabundans* UTEX 1185 (Sphaeropleales, Neochloridaceae) (syn. *E. oleoabundans*) were grown and maintained in liquid BM brackish medium in static conditions inside a growth chamber (24 ± 1 °C temperature, 80 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ PAR and 16:8 h of light:darkness photoperiod; light was supplied by cool-white fluorescent Philips tubes) [18,25]. For experiments, algae were inoculated in BM medium containing 2.5 $g\ L^{-1}$ of glucose at a cell density of $0.5–0.7 \times 10^6$ cells mL^{-1} in 500 mL flasks (300 mL of total culture volume); flasks were maintained under continuous shaking at 80 rpm, as reported in Giovanardi et al. [18]. Controls in BM medium containing 0 $g\ L^{-1}$ of glucose were set up as well and cultivated in the same culture conditions described above (initial cell density: $0.5–0.7 \times 10^6$ cells mL^{-1}; stirring; 24 ± 1 °C temperature; 80 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ PAR and 16:8 h of light:darkness photoperiod) [18]. Experiments lasted 14 days and were performed at least in triplicate. For analyses (growth measurements excluded), aliquots of cells were collected after 0 (inoculation), 6 (late exponential phase) and 14 days (late stationary phase) of cultivation.

To compare the effect of a different mixotrophic substrate on the lipids produced by the alga, *N. oleoabundans* was also cultivated for 28 days in static conditions in the presence of a diluted apple waste product (AWP; 1:20 dilution in BM medium), as reported in Baldisserotto et al. [14]. For details on AWP preparation and composition see Giovanardi et al. [11]. Aliquots of cells were harvested for lipid analyses at 28 days of cultivation, when lipids were accumulated [14]. Controls in BM medium without AWP were done in parallel [14] and experiments were performed in triplicate.

2.2. Analyses on growth

2.2.1. Growth parameters of microalgae

Control and glucose-treated cells were periodically counted with a Thoma haemocytometer under a light microscope (Zeiss, Mod. Axiophot, Jena, Germany); cell densities were plotted on a logarithmic scale to obtain the growth kinetics. Cell biomass ($\mu g_{DW}\ 10^{-6}$ cells), biomass concentration ($g_{DW}\ L^{-1}$) and biomass productivity ($g_{DW}\ L^{-1}\ d^{-1}$) were calculated on the basis of dry weight of samples collected after 0, 6 and 14 days of cultivation. For dry weight determination, cell samples were treated as reported in Popovich et al. [10].

2.2.2. Nitrate quantification in the culture media

For nitrate concentration analysis, autotrophic and mixotrophic culture media were harvested by centrifugation (2000 *g*, 10 min) after 0, 6 and 14 days of cultivation. Nitrate was quantified colorimetrically using a flow-injection autoanalyser (FlowSys, Systea, Roma, Italy).

2.3. Transmission electron microscopy (TEM)

After 6 and 14 days of cultivation, control and glucose-treated cells were harvested by centrifugation (500 *g*, 10 min) and prepared for transmission electron microscopy as reported in Baldisserotto et al. [25]. Sections were observed with a Hitachi H800 electron microscope (Electron Microscopy Centre, University of Ferrara). Images were employed to calculate the cell volume.

2.4. Analysis of photosynthetic parameters

2.4.1. Photosynthetic pigments extraction and quantification

Aliquots of cell suspensions from both autotrophic controls and glucose-treated cultures were harvested by centrifugation at 8000 *g*, 10 min. Then, pellets were extracted with absolute methanol at 80 °C for 10 min under a dim green light to avoid photo-degradation [14]. The extracts were clarified by centrifugation and analysed with an UV/Vis spectrophotometer (Pharmacia Biotech Ultrospec® 2000) (1 nm resolution). For chlorophyll *a* (Chl*a*), chlorophyll *b* (Chl*b*) and carotenoid (Car) quantification, the extracts were measured at 666 nm (Chl*a*), 653 nm (Chl*b*) and 470 nm (Car) and the equations proposed by Wellburn [26] were applied. Pigments were expressed as percentage of total dry weight (% DW) by dividing pigment content by biomass concentration. Pigment content was also expressed on a cell basis, as $nmol_{pigment}\ 10^{-6}$ cells.

2.4.2. Pulse amplitude modulated fluorimetry (PAM) analyses

For analyses, aliquots of cells from glucose-treated cultures and their controls were collected by centrifugation (8000 *g*, 5 min); then, pellets, drop by drop, were put onto small pieces of wet filter paper (Schleicher & Schuell) [27]. After 15 min of dark adaptation PSII maximum quantum yield $[F_V/F_M = (F_M - F_0) / F_M]$ was measured with a pulse amplitude modulated fluorimeter (ADC-OS1-FL, ADC Bioscientific Ltd., Herts, UK). Furthermore, induction/relaxation curves of fluorescence parameters were obtained by applying standard protocols [28]. In detail, the dark-adapted pellets were illuminated with a halogen lamp through a fibre-optic system for 5 min at an irradiance of 1100 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ and a saturation pulse was applied every minute. After the induction phase, pellets were returned to darkness for relaxation and a saturation pulse was applied after 1, 2 and 5 min. In particular, induction/relaxation kinetics were recorded for the actual PSII quantum yield $Y(PSII) = (F_M' - F_S) / F_M'$ [29], the quantum yield of the regulated energy dissipation $Y(NPQ) = (F_S / F_M') - (F_S / F_M)$ and the combined yield of fluorescence and

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