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Biotechnological Optimization of Light Use Efficiency in *Nannochloropsis* Cultures for Biodiesel Production

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In the last years new renewable energy sources started to be exploited to compensate exhausting fossil fuels and minimize anthropogenic factors on climate change. Microalgae have reemerged as potential next-generation feedstock for biofuels and they are considered very promising on the long term, since they have a potentially high productivity per area and they can be grown on marginal land without competing with food crops. Our work is focused on the seawater microalga *Nannochloropsis gaditana*, which combines a fast growth rate with a strong accumulation of lipids and therefore yields great potential for these kinds of applications.
Solar radiation provides all the energy supporting algae growth and lipids production and for this reason the available radiation must be exploited with the highest possible efficiency to optimize productivity and make their cultivation on a large scale competitive. Investigation of the bases affecting light use efficiency is thus seminal to elucidate the connection between light and the lipids/biomass productivity.
To this aim we investigated the influence of different illumination regimes, nutrients availability and batch/ continuous cultivation on *N. gaditana* cultures productivity and light use efficiency. This information will be exploited for optimization of growing systems but also to design *Nannochloropsis gaditana* genetic manipulations. Strains with altered composition of the photosynthetic apparatus and modified regulation of photosynthesis are being selected and characterized to increase *Nannochloropsis gaditana* productivity in photobioreactors.

1. Introduction

The production of biodiesel using microalgae biomass represents one interesting alternative to replace petroleum-derived transportation fuels. Among the several thousand different algae species, we need to select those characterized by a high growth rate and a high lipid content (Sforza et al., 2010). After the screening of different species, *Nannochloropsis gaditana* emerged as a promising candidate for these applications thanks precisely to its high growth rate in a wide range of light irradiations and the ability to accumulate large amounts of lipids, especially in condition of nitrogen deprivation (Simionato et al., 2011; Simionato et al., 2013b).
Algae are photosynthetic organisms and thus solar light provides all the energy supporting their metabolism (Rodolfi et al., 2009). Solar light is a very abundant resource but it is also distributed on the whole earth surface and therefore highly diluted. As a consequence, algae growing in an outdoor pond/photobioreactor are commonly limited by light availability. For this reason, in order to develop any sustainable system for large scale algae cultivation, it is fundamental that algae use light with the highest possible efficiency to produce biomass (Simionato et al., 2013a). One of the major issues in growing algae in a photobioreactor (or pond) is the fact that cultures have a high optical density and thus light is strongly inhomogeneously distributed (Zou and Richmond, 2000; Carvalho et al., 2011). Cells on the surface are more exposed and easily subjected to excess illumination, with an oversaturation of photosynthesis leading to energy dissipation as heat. These cells not only absorb most of the available energy but also use it with low efficiency since a large fraction is dissipated. The remaining cells in the deeper layers,

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instead, only receive a negligible amount of light, which reduces photosynthetic rate and biomass accumulation. Because of this inhomogeneous distribution, global photosynthetic productivity of algae growing in photobioreactors and ponds is reduced and far lower than the one achievable in laboratory conditions (Melis, 2009).

The improvement of algae photosynthetic productivity in a large scale cultivation system is an objective requiring a multidisciplinary approach. In fact a deeper understanding of the molecular mechanisms influencing light use efficiency in algae must be combined with the development of improved growing systems. Several complementary strategies should be investigated to reach a productivity sufficient to be economically and energetically competitive. As described in Sforza et al. (2012), for instance, an optimized alternation of light/dark cycles has good potential to increase photosynthetic efficiency. In fact, flashes of intense light can be exploited efficiently for photochemistry since they produce reduced electron transporters that can be processed during the following dark period. These data suggest that the optimization of the mixing rates, creating an appropriate alternation of light and dark phases within a photobioreactor, can greatly improve photosynthetic efficiency of the whole culture (Richmond et al., 2003; Chen et al., 2011).

The improvements in photobioreactor design should go in parallel with genetic engineering approaches which can also contribute to increase light use efficiency. Photosynthetic organisms evolved large antennae systems to increase the light harvesting efficiency and consequently maximize light absorption in their natural environments (Kirk, 1994). In the photobioreactor growing conditions, however, such a good light harvesting ability is detrimental, since, as discussed above, it strongly increases culture optical density (Li et al., 2009).

Different works from literature demonstrated that light distribution in a photobioreactor can be improved, for instance, using algae mutants with a reduced antenna size (Mussgnug et al., 2005). These mutations in fact bear a double positive effect: first, when a cell is exposed to a strong irradiation it harvests less light, reducing the damage on the photosynthetic apparatus and the need to activate energy dissipation mechanisms. Secondly, light is better distributed in the culture and thus more energy is available for cells in the internal parts of the mass culture, increasing their photosynthetic rate and, consequently, biomass accumulation (Melis, 2009).

Previous works in this direction are all focused on the model green alga *Chlamydomonas reinhardtii* which, although being a valuable model organism, is not suitable for biofuels production and cultivation in large scale systems. For this reason we decided to generate *Nannochloropsis gaditana* mutants with altered photosynthetic apparatus regulation through a random mutagenesis with Ethyl Methane Sulfonate (EMS). EMS mutagenesis is recognized as a powerful technology in mutation breeding: this alkylating agent causes a high frequency of nucleotide substitutions, as detected in different plant genomes (Talebi et al., 2012).

In the past years, EMS has been used to induce the over-production of metabolites in microalgae, including astaxanthin, carotenoids and eicosapentaenoic acid (EPA), an important polyunsaturated fatty acid (PUFA) for the prevention of several human diseases (Doan and Obbard, 2012). In our case an additional advantage is the fact that obtained strains will be more easily testable in outdoor photobioreactors avoiding issues with the use of transgenic organisms.

2. Materials and Methods

2.1 Microalgae growth

Nannochloropsis gaditana from CCAP, strain 849/5, was grown in sterile F/2 medium (Guillard and Ryther, 1962), using sea salts 32 g/L from Sigma Aldrich, 40 mM TRIS/HCl pH 8, Sigma Aldrich Guillard's (F/2) marine water enrichment solution. Cells were grown in Erlenmeyer flasks with 100 µmoles photons m^{-2} s^{-1} (µE) of illumination and agitation at 100 rpm. Temperature was set at 22 ± 1 °C. Cultures were then treated with an antibiotic cocktail of Ampicillin (100 µg/mL), Streptomycin sulphate (100 µg/mL) and Kanamycin sulfate (100 µg/mL) (all from Sigma Aldrich) for 48 h to obtain axenic cultures.

2.2 EMS mutagenesis and mortality determination

The microalgae suspension in the late exponential growth phase at a concentration of 2 x10^7 cells/mL were mutagenized using 70 mM EMS (Ethyl Methane Sulfonate) for 1 h in darkness at room temperature with mild agitation. Following incubation, treated cells were centrifuged at 5000 g for 8 min to separate cell pellets which were then washed four times with sterile F/2 medium to remove excess EMS. After EMS treatment, cells were then re-suspended in sterile F/2 medium and plated on agar F/2 dishes. The amount corresponding to ≈ 1500 cells was plated to obtain ≈ 150 colonies sufficiently separated. Plates were cultured at 22 ± 1 °C, under illumination at 20 µmoles photons m^{-2} s^{-1}, until the algae colonies were emerged.

The number of colonies obtained following EMS treatment was compared to the number of colonies obtained following the treatment with water (control procedure): this led to establish the percentage of mortality induced by the EMS treatment. In the mortality determination procedure the same initial number of cells was plated in F/2 agar plate, both for EMS treated cells and for those water treated.

2.3 Mutants selection and screening

Twenty days after EMS treatment, the obtained colonies were visually selected for altered coloration, an indication of altered photosynthetic apparatus. After this first retention, colonies were analysed by *in vivo* fluorescence based screening which allowed to identify the ones affected in regulation of photosynthetic apparatus. Fluorescence kinetic curves were recorded with a video-imaging apparatus: FluorCam FC 800 (Photon Systems Instruments) (saturating light was set at 400 µmoles photons m^{-2} s^{-1}). Colonies showing significant differences with respect to the wild type were retained for further analysis.

3. Results and Discussion

A *Nannochloropsis gaditana* culture in the late exponential growth phase was the starting material to perform EMS treatment. Conditions were set to induce 90 % cells mortality and thus ensure a suitable mutation rate. Following exposure to EMS, surviving cells were cultured in F/2 medium for 20 days prior to isolation of target mutants. 7 x 10^3 EMS treated colonies were visually analysed to select around 150 strains (2 %) with an altered pigmentation (Figure 1a).

Selected strains were thus re-cultured in F/2 agar plates and subjected to a further screening by measuring *in vivo* chlorophyll (Chl) fluorescence, to select those mutants with altered photosynthetic apparatus composition and regulation. Fluorescence measurements are an useful tool to gain indirect information on photosynthetic efficiency. Light absorbed by chlorophyll molecules can, in fact, follow three pathways: it can be used to drive photosynthesis (photochemistry), it can be dissipated as heat or it can be re-emitted as photons (fluorescence). Although a relatively little amount of energy is re-emitted as fluorescence (1-2 % of the light absorbed), its measure can be exploited to monitor the efficiency of the other two processes. These analyses have become one of the most powerful and widely used techniques to investigate photosynthetic apparatus and its regulatory mechanisms.

We here exploited *in vivo* fluorescence to test photosynthetic functionality of the selected mutants to identify mutants affected in photosynthetic apparatus (Table1). We employed a video-imaging apparatus to measure simultaneously multiple colonies on agar plates (Figure 1b). Different parameters were monitored and first of all we measured fluorescence of dark-adapted cells (*Fo*). This value, normalized on the area of the colony, yields a *Fo/Area* parameter, which provides an estimation of the Chl content of the colony.

Differences in this parameter are present when a colony has a reduced Chl content per cell, as expected for a mutant strain with reduced antenna systems. For this reason, all colonies with a significant alteration in chlorophyll (Chl) fluorescence per unit area (µm^2) were retained as potentially interesting mutants (e.g. mutants number 68, 124 and 133 in Figure 2a).

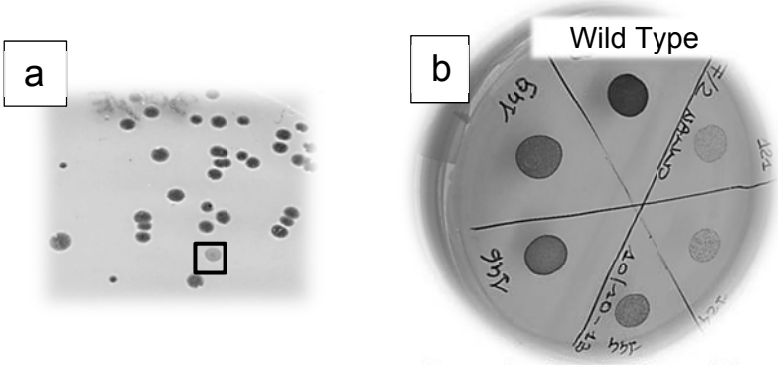

Figure 1: In (a) is presented a F/2 agar plate surface, showing the Nannochloropsis gaditana colonies, grown following EMS exposure. In the black square is shown a selected mutant colony, thanks to its pale color gradation, with respect to the other ones grown on the same agar plate. In (b) is displayed a F/2 agar plate, showing five mutants visually selected thanks to their pale color gradation and re-cultured to increase their dimensions for the in vivo fluorescence based screening. It is clearly evident the difference in the color gradation of the selected mutants, in relation to the wild type. Note that all spots were inoculated with similar cellular concentration (quantified by OD_{750}).

In parallel to this possibility it is important to underline that differences in this parameter can be due also to mutations affecting growth rate independently from the photosynthetic apparatus. In this case, in fact, the colony will contain less cells and thus appear as paler. To reduce the number of false positives, it was important to inoculate different strains as homogenously as possible (Figure 1b). Even more importantly we exploited additional video-imaging measurements further to select bona fide antenna mutants. These in fact are expected to have a higher saturation point of photosynthesis because of their reduced light harvesting ability. Saturation of photosynthesis can be monitored by measuring the quantum yield of Photosystem II (ΦPSII), which estimates the proportion of the light absorbed, exploited for photochemistry (Maxwell and Johnson, 2000). When cells are exposed to strong illumination, this value decreases because photosystems are oxidized and thus in a “closed” state, not available for photochemistry (Figure 2b).

The decrease in this parameter can be exploited as an indication of the photosynthesis saturation in light adapted cells and we expect mutants impaired in light harvesting to have higher values of this parameter with respect to the wild type. As shown in Figure 2b, we found that some mutants with a reduced *Fo/Area* also showed a decreased saturation of photosynthesis, thus suggesting that these are genuine antenna mutants (e.g. mutants numbers 68, 124 and 133). Twelve mutants, showing these characteristics, were identified and are currently under further investigation to verify their effective reduction of Chl content per cell, as well the detailed alteration in photosynthetic apparatus (antenna less mutants in Table 1).

The same fluorescence measurements, after exposition to strong illumination, can also be exploited to evaluate the ability of the mutants to activate regulatory mechanism of photosynthesis, quantifiable by the Non Photochemical Quenching parameter (NPQ). This allows estimating cells efficiency in dissipating energy as heat, when absorbed in excess by the photosynthetic apparatus. Mutants presenting an altered NPQ response were positively selected, yielding in the selection of nine additional mutants cataloged in the NPQ phenotype class of Table 1 (e.g. mutants number 34, 99 and 100 in Figure 2c). These strains are potentially interesting for a growth in a photobioreactor because the reduction of heat dissipation mechanisms, which is detrimental in a natural environment, can instead reduce energy waste in a photobioreactor, where most of the cells are exposed to limiting light.

Finally the same measurements of quantum yield of Photosystem II (Φ_{PSII}) allowed the identification of mutants where this parameter was lower or showing a stronger decrease than wild type upon light exposure. These seven mutants (selected as Φ_{PSII} phenotype mutants in Table 1, e.g. mutants numbers 59 and 62 in Figure 2b) are affected in photosynthetic apparatus assembly and/or have increased light sensitivity. These mutants are likely not suitable for a large scale cultivation since these defects are most likely resulting in a reduced productivity. Nevertheless their deeper investigation can provide new insights on photosynthetic apparatus composition and assembly in a group of algae relatively poorly characterized such as *Nannochloropsis* genus.

Table 1: Classification of the selected mutants on the basis of the most pronounced photosynthetic parameter through which they differ from the wild type.

Category	Phenothype
Antenna less	Less *Fo/Area* than wild type; Lower saturation of photosynthesis; Different degrees of chlorophyll reduction (pale color); Interesting for biofuels.
NPQ phenotype	NPQ values lower than wild type; Interesting for biofuels.
Φ_{PSII} phenotype	Strong alterations in Φ_{PSII}; Light sensitivity, impaired photosystems assembly; Not interesting for biofuels, interesting for basic investigations.

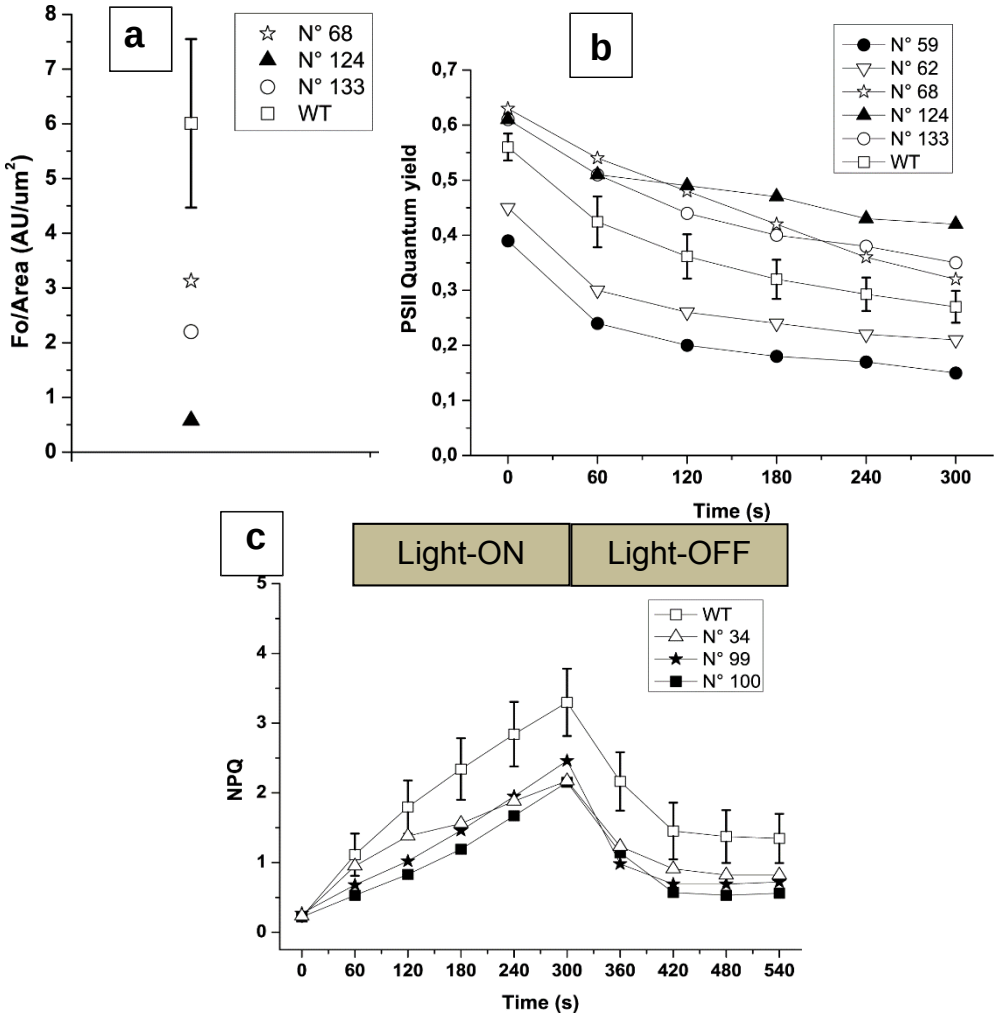

Figure 2: In this picture are presented some of the selected mutants, retained on the basis of their altered photosynthetic parameters. In picture (a) is shown the Fo/Area value for Wild Type (WT) and three mutants (68, 124, 133). In picture (b) is shown the Photosystem II quantum yield trend for dark adapted WT and five mutants (59, 62, 68, 124, 133), at different time point after having switched on the light. In picture (c) is shown the NPQ trend for WT and three mutants (34, 99, 100), during a first period in the light and a second one in the dark, to show the mutants recovery ability. From these pictures is clearly evident the huge gap between the wild type and the selected strains, for all the presented photosynthetic parameters. Mutants number 68, 124 and 133 belong to the antenna less mutant category; Mutants number 59 and 62 belong to the Φ_{PSII} phenotype class, showing an important alteration in photosystem II quantum yield trend and appearing as light sensitive. Mutants number 34, 99 and 100 belong to the NPQ phenotype class. (The presented WT values were obtained from the analyses on seven different colonies unaffected in photosynthetic apparatus regulation).

4. Conclusions

In this work we screened a *Nannochloropsis gaditana* random mutants collection, obtained following treatment with Ethyl methane sulfonate (EMS), looking for phenotypes which may have a positive effect on photosynthetic performances of this alga growing in a large scale system. The rationale of this effort is that wild type algae isolated in nature have regulatory mechanisms of photosynthesis which are optimized to survive and thrive in their natural environment. Instead, in order to achieve an optimal productivity in the artificial environment of photobioreactors there is thus the need to select improved strains.

The target is to find mutants which are not impaired in the basic reactions of photosynthesis but where photosynthetic apparatus composition and regulation is altered and allow a better exploitation of light in all the photobioreactor volume. For instance, the mutants with a reduced chlorophyll content per cell should reduce self-shading and then increase homogeneity in light distribution with a beneficial effect on

productivity. Also mutants with reduced heat dissipation mechanisms should be more productive since most of the culture volume is light limited. In this work we managed to select 21 mutants presenting potentially interesting photosynthetic properties. These will be, in the near future, the subject of further investigations to evaluate other parameters, like the growth rate, looking for a few candidates which can indeed provide a higher productivity on a large scale.

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