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Vegetal oil from microalgae: species selection and optimization of growth parameters

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Vegetal oil from microalgae seems to be the only renewable biofuel with the potential to completely replace petroleum-derived transport fuels. Microalgae can be potentially employed for the production of biofuels in an economically effective and environmentally sustainable manner. In order to fully exploit this potential, one challenge is to increase the growth rate and another one is to improve the lipid content of microalgal strains. To these aims we have screened three microalgal species (*Scenedesmus quadricauda, Chlamydomonas reinhardtii, Nannochloropsis salina*) for their biomass productivity, growth kinetic parameters, maximum biomass concentration and lipid content under autotrophic conditions. Among the different species analyzed we selected *N. salina* for further studies in view of its high biomass productivity and high lipid content. We first cultivated *N. salina* in bottles agitated by air flowing through a frit. We also changed culture conditions, in order to evaluate the effect of nitrogen content in the culture medium on kinetics growth parameters and lipid accumulation. The concentration of biomass in the culture was also enhanced by increasing the mole fraction of CO_2 in bubbling air.

1. Introduction

Production of vegetal oil and biodiesel using microalgae biomass appears to be the only alternative that could replace petroleum-derived transport fuels in significant amounts. Microalgae are a highly diverse group of unicellular organisms comprising eukaryotic protists and prokaryotic cyanobacteria, that can grow rapidly due to their simple structure. Microalgae are photosynthetic microorganisms which convert sunlight, water and CO_2 to sugars, which can be afterwards converted also in lipids and triacylglycerols (TAGs). These molecules can be potentially employed for the production of biofuels in an economically effective and environmentally sustainable manner (Chisti 2007). In order to develop a cost-effective second generation feedstock for biodiesel production, there is still the need to improve both the growth rate and the lipid content of microalgal strains. On the one hand, genetic engineering should allow to increase as much as possible the value of μ_{max} (the growth rate constant). On the other hand, we need to select the species capable of accumulating the largest amount of lipids and optimize culture conditions. In algal culturing aimed to achieving high yields, it is important to ensure the adequate supply of basic nutrients: light, carbon dioxide, water and inorganic nutrients. In culture media nutrients are usually supplied in excess to avoid that they become the rate-limiting factor. The biochemical composition of microalgae can be modified through environmental manipulations, including nutrient availability. Several studies showed that cell lipid content can vary as a result of changes in growth conditions or nutrient concentration (Converti et al., 2009; Rodolfi et al., 2009). The

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most efficient approach to increase lipid content in algae seems to be nitrogen deficiency. However, in these conditions, productivity is generally reduced in comparison with nutrient-sufficient growth (Rodolfi et al., 2009) and nitrogen deprivation is often associated to a reduction in biomass yield and decline of growth rate. In this respect, it is important to investigate the best conditions to obtain maximal biomass productivity, as well as the induction of lipid synthesis through nutritional limitation/deprivation. The goal is a two-phase strategy, already successfully experimented (Rodolfi et al., 2009), where a first nutrient sufficient biomass production step is followed by a lipid production phase under nitrogen deprivation. Phototrophic microalgal growth also requires a supply of carbon dioxide as a carbon source. According to previous studies, the supply of carbon to microalgal mass culture systems is one of the principal difficulties and limitations must be solved (Benemann et al., 1987). In this work, we screened three microalgal species (*S. quadricauda, C. reinhardtii, N. salina*) for their biomass productivity and lipid content, and we evaluated the lipid accumulation of these species during different growth phases . We also changed culture conditions, in order to determine the effect of nitrogen content in culture medium and the percent of CO_2 content in bubbling air, on growth kinetics and lipid accumulation.

2. Materials and methods

2.1 Microalgae and media composition

Three microalgae species were used in this study, specifically *N. salina* 40.85 (obtained from SAG-Goettingen), *S. quadricauda* (obtained from CNR-Florence) and *C. reinhardtii*, and were screened for their growth characteristics. *C. reinhardtii* and *S. quadricauda* are freshwater species, and were grown in BG11 medium (Rippka et al., 1979). *N.salina*, a marine species, was cultured in sterilized sea salts 22g/L solution enriched with f/2 Guillard solution as described by Guillard and Ryther (1962).

2.2 Growth analysis

Growth experiments were done in 1.0 L-Erlenmeyer flasks in duplicate. The growth temperature was 24±1°C, with artificial lighting under a continuous photon flux density of 130±10μE m^{-2} s^{-1}. Algal growth was measured by daily changes in optical density and cells number. In the logarithmic growth phase cells number was related to optical density. For dry weight (DW) determinations cells were harvested with a 0.22 μm filter. DW was measured gravimetrically upon drying the filters at 100°C for 4 h in a laboratory oven. Biomass concentration was related to cell concentration. The specific growth rate was calculated by the slope of logarithmic phase in terms of biomass of cells. In order to study the effect of CO_2 on growth, a number of experiments were conducted in 0.25-L glass bubbled tubes, agitated by air flowing (with different percentage of CO_2) through a ceramic frit. For the nitrogen-starvation experiments, cultures in the early stationary phase were centrifuged at 5000 g and resuspended in nitrogen depleted medium.

2.3 Analytical methods

Lipid content was determined by staining the algal cell suspension with Nile Red dye at final concentration of 2.5 μg/mL, for 10 minutes at 37°C (Chen 2009). The fluorescence

was measured using a spectrofluorometer (OLIS DM45), with excitation wavelength at 488 nm and emission wavelength in the range of 500 and 700 nm. The relative fluorescence of Nile Red for the lipids was obtained after subtraction of the autofluorescence of algal cells and the self-fluorescence of Nile Red. Total lipids were extracted from dried cells using ethanol-hexane (2.5:1 vol/vol) as solvent (Molina Grima et al., 1994) in a soxhlet apparatus for 10 hr. The lipid mass was measured gravimetrically after solvent removal by a rotary evaporator. The fluorescence intensity of cells stained by NR and the gravimetric ratio of cellular lipid was found linearly.

3. Results and discussion

3.1 Screening of species

The first part of this study examines the growth parameters and lipid accumulation characteristics of some microalgal species, in order to determine the which one could be potentially used for large scale oil production. Three microalgal species were screened for their kinetic growth and lipid production. A summary of results is reported in tab 1.

Table 1 Specific growth rate (μ) of microalgae screened. Maximum concentration data refer to the biomass amount obtained in stationary phase. The lipid concentration is the maximum observed for each species.

Species	μ (days^{-1})	Max. biomass conc. (g/L)	Max. lipid conc. (%DW)
S. quadricauda	0.18	0.42±0.01	30±0.01
C. reinhardtii	0.13	0.74±0.01	20±0.01
N. salina	0.49	0.72±0.01	69±0.01

All species were initially grown under conditions suggested by the literature, in media with minimal composition, to determine the best performance about biomass production and lipid accumulation. Two freshwater species were screened. Under laboratory conditions, *C. reinhardtii* (fig.1A) shows a very slow growth rate of 0.13 days^{-1}, which correspond to a doubling time of 5.33 days. This species reaches the stationary phase at concentration of 0.74 g/L DW. Lipid content remains constant during growth, with a fluorescence intensity about 1500 (for 2millions of cells), corresponding to 20% DW ratio of lipids, according to data reported by Spolaore 2006. *S .quadricauda* (fig.1B) show a growth rate of 0.18 days^{-1}, corresponding to a doubling time of 3.85 days The maximum concentration in stationary phase was and 0.42 g/L DW. *S. quadricauda* reaches a considerable amount of lipids, corresponding to 30% DW, in the early stationary phase. *N. salina* (fig.1C), a marine species, seems to be the best lipid producers, showing the best combination of specific growth rate (0.497 days^{-1}, corresponding to a doubling time of 1.39 days), and maximum lipid content. In fact, the lipid content of the algal cells changed with the growth phase. Cells sampled in the stationary phase show an increased fluorescence intensity, with respect to cells from cultures in the early logarithmic phase. Moreover, the lipid content continues to increase during stationary phase, up to a fluorescence intensity corresponding to a lipid ratio of 69% DW. This could be due to accumulation of lipids as storage products when growth becomes limited (Boussiba 1987). Interestingly, while no significant lipid accumulation was observed for freshwater species, lipid content increased in the stationary phase in the case of *N. salina*. It is noteworthy that the main difference in the culture media of

marine and freshwater species was the nitrate content, which was higher for freshwater culture media. In fact, nitrogen is generally far more abundant in freshwater than in seawater (McClintock and Baker, 2001). There are evidences that nitrogen limitation is responsible of lipid accumulation in microalgae. Thus, it is possible that the increased lipid content in *N. salina* is caused by low nitrate concentration in culture media, which becomes limiting in stationary phase. Nevertheless, although we could expect the lipid content to increase in other species as well by culture conditions optimization, we retained *Nannochloropsis* for further work since not only is accumulating lipids but also shows by far the best growth rate.

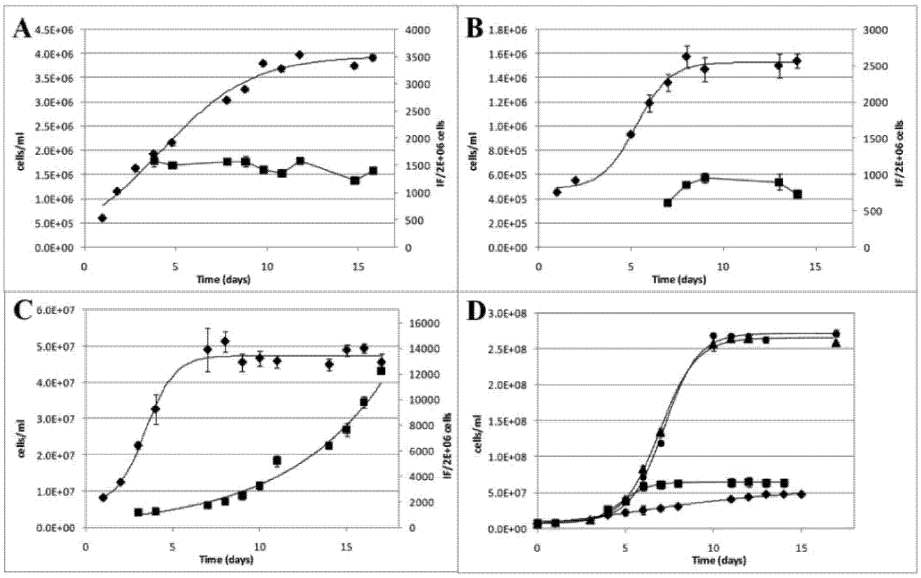

Fig.1 Growth curves (♦) and IF of Nile Red staining (■) of C. reinhardtii (1A), S. quadricauda (1B) and N. salina (1C), in 1-L flasks, under autotrophic conditions and minimum media composition. In fig. 1D is reported the effect of additional CO_2 and nitrate source on cell growth of N. salina, in bottles bubbled. The following added concentration were respectively: (♦) air-0.075g/L $NaNO_3$; (■) CO2 5%-0.075g/L $NaNO_3$; (▲) CO2 5%-1.5g/L $NaNO_3$; (●) CO2 5%-2g/L $NaNO_3$.

3.2 Optimization of growth parameters

N. salina was selected for the following experiments performed in bottles bubbled with CO_2 enriched air. Algal biomass consists of 40% to 50% carbon, which suggests that about 1.5 to 2.0 kg of CO_2 is required to produce 1.0 kg of biomass (Mazzucca Sobczuck et al, 2000). According to previous studies, the supply of carbon to microalgal mass culture systems is one of the main difficulties and limitations must be solved (Benemann et al., 1987): CO_2 it must not reach the upper concentration that produces inhibition and, on the other hand, must never fall below the minimum concentration that limits growth. To be available to the culture, carbon dioxide must be transferred from a gas phase to a liquid phase. Thus, the amount of CO_2 that could be fixed by algae is a function of the volumetric mass transfer coefficient and the driving force (i.e., the difference between the partial pressure of CO_2 in the gas and the concentration in solution). Considering typical transfer mass coefficient of bubbling and airlift reactors

(Chisti and Jauregui-Haza, 2002), it is possible to calculate that the CO_2 concentration in air is limiting for algae growth. To overcome this limitation, following experiments were performed with 5% CO_2 in bubbling air, that is not limiting for algae growth. In order to determine if nitrate content of marine culture medium is limiting for growth of *N. salina*, three different concentration of sodium nitrate (0.075, 1.5 and 2 g/L) were used all in the presence of 5% CO_2. As reported in fig.1D, the CO_2 content in air is limiting for growth. In fact, in the presence of 5% of CO_2, the culture reached a higher cells concentration in stationary phase (about 64 millions of cells / ml) as compared with culture bubbled with air (about 48 millions of cells). However, the largest effect is played by nitrogen availability. In fact, a specific growth rate of 0.599 $days^{-1}$ and maximum cells concentration of about 270 millions of cells/ml (corresponding to 2.87g/L DW) were obtained with nitrogen enriched medium. This is more that 4 times higher than in N poor medium. The data show that no difference in the maximum biomass concentration was obtained with 1.5 or 2g/L of $NaNO_3$, suggesting that 1.5g/L of inorganic nitrogen is more than enough to support growth.

3.3 N-limitation

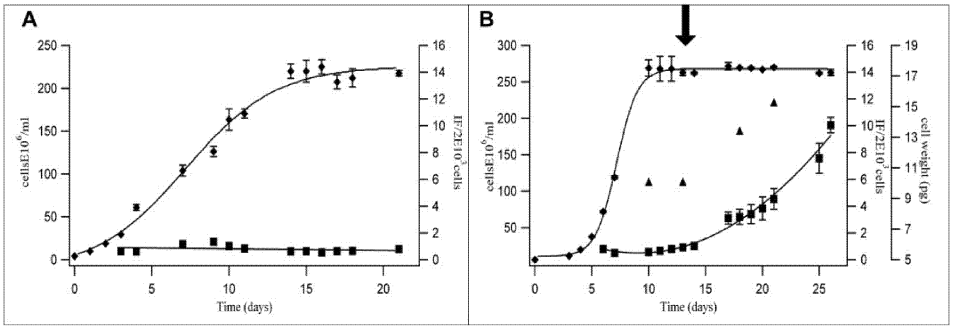

Fig.2 Growth curves (♦) and IF of Nile Red staining (■) of N. salina, under autotrophic conditions and 1.5g/L of $NaNO_3$ (2A). In fig. 2B is reported the effect of N-limitation on growth (♦) and IF (■); the arrow indicate the centrifugation and resuspension of culture in nitrogen depleted medium. In fig. 2B the increase of weight per cell (▲) during time is also reported.

When nitrogen is present in excess, the fluorescence intensity do not increases during growth (fig.2A). These results also suggested that, in standard conditions, the lipid accumulation in late growth phase is determined by N limiting concentration (fig.1C). In fact, when cells collected by centrifugation, in the early stationary phase, were re-inoculated into N-deficient medium, the lipid content increased until a fluorescence intensity about 11000, corresponding to a lipid ratio of 63±1% DW (fig.2B). These results showed that N-limitation could induce considerable lipid accumulation in *N.salina*, as observed also by Rodolfi et al. (2009). The higher concentration of nitrates during the earlier phase of growth, is able to stimulate the biomass increase. On the contrary, under nitrogen deficiency, microalgae start accumulating lipids, whit a net increase in cells DW (up to a biomass concentration of 4.05g/L DW), while cells number remained constant (fig. 2B). These results suggest that in these conditions algae continue to fix carbon which is only used for lipid biosynthesis (fig. 2B).

4. Conclusions

S.quadricauda, C. reinhardtii, N. salina were cultivated under autotrophic conditions. *N. salina* was selected for its biomass concentration (0.74 g/L DW), growth kinetic parameters (μ= 0.497 $days^{-1}$) and lipid content (69%). In high nitrogen content media and with an adequate supply of CO_2, *N. salina* reached a concentration 4 times higher (2.87g/L DW) and the growth rate increased up to 0.599 $days^{-1}$, while the lipid content remained at low level. The nitrogen starvation induced an increase of lipid content, associated with an increase of DW of cells, up to a biomass concentration of 4.05g/L DW, suggesting that further CO_2 was fixed into the lipid moiety (about 70% DW).

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