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Study on the optimal growing conditions of *Chlorella vulgaris* in bubble column photobioreactors

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An in-dept investigation on productivity and lipid yield of *Chlorella vulgaris*, a freshwater unicellular alga belonging to the Chlorophyceae class, in a wide range of growing conditions is presented. Various combinations of starvation are designed to turn the metabolism into an anabolic lipid accumulating phase, with the aim of defining the most suitable growing conditions for large scale biodiesel production.

The assays in regimen of mixotrophy, heterotrophy and autotrophy, conducted in the same conditions of irradiance, algal concentration and culture medium, show that *C. vulgaris* is able to increase its lipid content from ~6% (balanced-, autotrophically-grown biomass) to ~40% and highlighted the difference between the type (i.e., polar or nonpolar) of the lipids accumulated upon nitrogen and phosphorus starvation.

1. Introduction

The need to fulfill the ever increasing global energy demand caused the intensive use of fossil fuels like coal, petroleum and natural gas during the last century and nowadays they represent more than the 80% of the energetic resources. Owing to their exhaustibility and unsustainable environmental impact, both generated by their fossil origin, growing attention has emerged about renewable energy, with the aim of diversifying the energy resources to reduce the utilization of fossil fuels and hence limiting their negative effect.

In this context one of the most important renewable energy resources is biodiesel, which is produced from triglycerides by transesterification reactions (Li et al., 2008).

A great variety of biomasses have been investigated as feedstock for biodiesel production, in particular vegetable oils derived from soybean and rapeseed, corn, cottonseed, peanut, sunflower, safflower, coconut or palm. However, these feedstocks derived from superior plants show many cons. The competition between cultivar for energetic and agricultural goals causes the pauperization of primary food and on the other hand deforestation for the creation of new cultivable lands. Terrestrial culture are not so efficient in capturing solar energy, grow slowly, have low biomass yield and need fertilizers that produces N_2O emissions, reducing the greenhouse gasses saving.

Actually, one of the most promising feedstock for biodiesel production are unicellular algae (Demirbas, 2009; Pienkos, 2009). In fact, when compared with superior plants microalgae show higher photosynthetic efficiency, higher biomass productivities and faster growth rates. This aspect, together with an high intracellular lipid content, can

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potentially make a number of unicellular algae species among the most efficient producers of lipids of the planet.

Moreover unlike traditional oilseed crops, microalgae cultures do not need of herbicides or pesticides and can be performed in ponds or photobioreactors on non-arable lands including marginal areas unsuitable for agricultural purposes, minimizing damages caused to eco and food-chain systems (Chisti, 2007) and without compromising the production of food, fodder and other products derived from crops.

Furthermore, unicellular algae physiology can be manipulated to obtain certain desiderated effects. It is well known the possibility of addressing the algal metabolism to the accumulation of lipid in spite of cellular duplication and protein synthesis by imposing nutrient limiting conditions. Some microalgae are capable of use organic substrates in regimen of mixotrophy and heterotrophy.

Chlorella vulgaris has a great potential as a resource for biodiesel production due to faster growth and easier cultivation. However, lipids content in *Chlorella vulgaris* under general growth conditions is up to ~20% by weight of dry biomass (Illman et al., 2000; Spolaore et al., 2006), which cannot meet the standard industrial requirements.

In this study, an in-dept investigation on the growth rate and lipid yield of *Chlorella vulgaris* in a wide range of growing conditions is presented. Various combinations of starvation are designed to turn the metabolism into an anabolic lipid accumulating phase. In order to obtain matchable data, the assays are conducted in the same conditions of irradiance, algal concentration and culture medium, in regimen of mixotrophy and heterotrophy. Objective of the study was to define the most suitable growing conditions for large scale biodiesel production.

2. Materials and Methods

2.1 Operating conditions

The unicellular alga *Chlorella vulgaris* used in these experiments was cultivated in 500 mL bubbling tubes exposed to 2×36 W fluorescent daylight equal to 13000 lux in 12:12 circadian cycles at room temperature.All the experimentations last 72 h and were performed in duplicates.The condition of mixotrophy was induced by adding glucose to the culture broth in concentration of 6 gr/L and exposing culture to the normal circadian cycle. The condition of heterotrophy was induced by adding glucose to the medium in concentration of 6 gr/L and then maintaining the tubes in complete dark.

2.2 Medium

The microalgae cultures were performed in fresh water medium BG-11. The nutrient limiting conditions studied were: nitrogen limitation, nitrogen deprivation, simultaneous nitrogen limitation and phosphorus limitation, simultaneous nitrogen limitation and phosphorus deprivation.

The depleted conditions were obtained just by excluding the unwanted nutrient from the medium. Limiting conditions for nitrogen were obtained reducing the daily need, estimated by ionic chromatography (data not shown), to 1/8 of the daily need. Limiting conditions for phosphorus follow the same procedure: the daily amount of phosphorus was calculate by the Redfield ratio starting from nitrogen limiting conditions.

2.3 Analytical Methods

Biomass Concentration

The biomass concentration was determined using three different procedures: by cellular count in Burker chamber, spectrophotometrically at 686 nm using a linear correlation of optical density versus cellular count and by dry cell weight.

Glucose concentration estimation

The glucose concentration in broth medium was determined by Miller assay using DNSA.

Lipid content

Lipid analysis consisted in lipid extraction and gravimetric lipid quantitation.

To this aim from 1 gr of the dried biomass sample lipids were continuously extracted (by sohxlet) with 100 mL 2/1 (v/v) dichloromethane/methanol solution for 3,5 hours. The resulting solution was evaporated under vacuum and total lipids were determined gravimetrically. The lipids dried sample was then resuspendend in 30 mL hexane with 0.1 ml of distilled water added (to favour the swallow of polar lipids), carefully shaken (by a vortex mixer) and centrifuged. The supernatant solution was withdrawn and the solid residue treated other two times in the same manner. All the withdrawn hexane solutions were mixed, evaporated and dried under vacuum and the remaining substance was weighed as nonpolar lipids. The solid residue after centrifugation was dried under vacuum and weighed as polar lipids.

3. Results and Discussion

Firstly the effect of autotrophic, mixotrophic and heterotrophic growth conditions in optimal medium was compared. The obtained growth curves are reported in Figure 1.

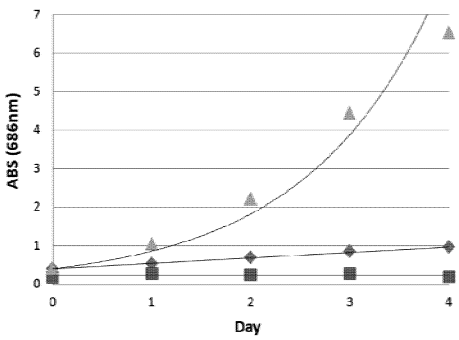


Figure 1. Growth curves for autotrophic (♦), mixotrophic (▲) and heterotrophic (■) conditions in optimal medium.

This preparatory evidence pointed out the need of light as signal for cellular duplication and the synergic effect of respiration and photosynthesis, developed in mixotrophic condition, on grow rate.

In the adopted experimental conditions in autotrophic regimen *Chlorella vulgaris* growth rate was equal to 1,4 cell 10^4/h, showing a 96 h biomass productivity of 0.27 gr/L with a lipid content of 6% dry weight. These data confirm that autotrophic conditions are insufficient to support a large scale biodiesel production, as previously reported in literature (Liang el al. 2009, Widjaja et al. 2009, Ogbonna et al 1998). Moreover the total lipid content yielded in autotrophic conditions is lower than in both mixotrophic and heterotrophic conditions (16.6% and 9% respectively). As a consequence starvation conditions were tested only in mixotrophic and heterotrophic growth regimen.

While nitrogen limitation was designed to improve the lipid production, phosphorus was assayed to change the lipid composition. The presence of phosphorus in the lipids used for the biodiesel production led to an high concentration of phosphorus in the final biofuel. This is deleterious for the quality of biodiesel.

3.1 Mixotrophic Growth Conditions

All the four nutrient limiting conditions performed in mixotrophy were analyzed for cellular growth, glucose consumption and biomass and lipid productivity.

As shown in Figure 2 the simple nitrogen limiting conditions consent the highest algal proliferation. On the contrary complete nitrogen deprivation is not compatible with cellular duplication as it is a main component of DNA and protein. The combined starvation of nitrogen and phosphorus consent a slight culture growth.

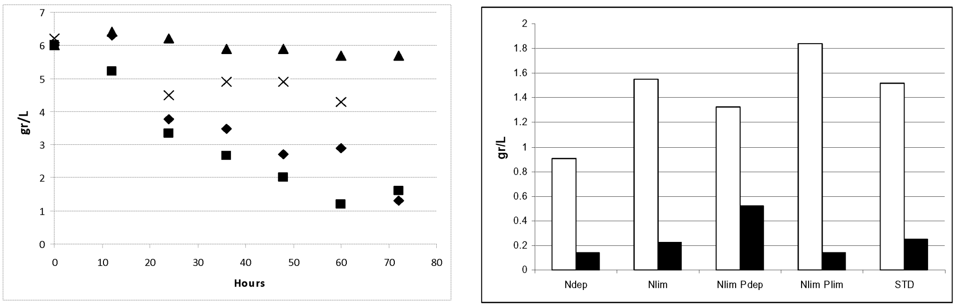


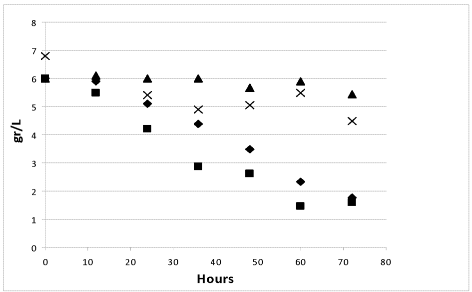
Figure 2. Mixotrophic growth condition. Left: glucose consumption for N-dep(▲); N-lim(×); N-lim P-dep(♦); N-lim Plim (■). Right: 72 h biomass and lipids productivity

The glucose consumption agrees with the precedent data. Nitrogen depleted cultures do not use glucose as energetic substrate and seems such as metabolically quiescent. On the other hand the combined limitations of nitrogen and phosphorus induces an higher respiratory activity than the simple nitrogen limitation.

The biomass productivity shows how the complete deprivation of nitrogen or phosphorus do not support an high productivity, reasonably for the impossibility to develop a variety of fundamental physiological processes and cellular structures. The double limitation instead give the highest productivity, showing an unbalanced growth. That means microalgae were accumulating substrates that increase the culture biomass but not the cells density. The lipid yield ranged from the 7,7% to the 39.4% of the dry weight (figure 2), evidencing the possibility to switch the anabolic activity from the protein and DNA synthesis to the lipid accumulation.

3.2 Heterotrophic Growth Conditions

All the four nutrient limiting conditions performed in mixotrophy were analyzed for cellular growth, glucose consumption and biomass and lipid productivity.
Such as in the mixotrophical conditions nitrogen depletion do not allow the algal reproduction. Differently from the mixotrophical conditions nitrogen starvation seems to be the limiting nutrient for the cellular reproduction, in spite phosphorus limitation or absence do not interfere. Glucose consumption follow the same pattern of mixotrophical conditions. Finally the biomass productivity was generally lower than mixotrophy as well as lipid the maximum lipid productivity was 31.68%



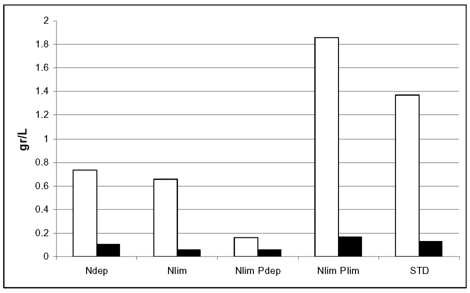


Figure 3. Heterotrophic growth condition. Left: glucose consumption for N-dep(▲); N-lim(×); N-lim P-dep(♦); N-lim Plim (■). Right: 72 h biomass and lipids productivity

3.3 Lipids characterization

The total lipids extracted from each trial were divided into the relative polar and nonpolar fraction. The results are reported in table 1 together with a comparison of lipid distribution obtained in optimal nutrient conditions (BG-11).

Table 1 Lipids characterization for the different nutrient limiting condition

Mixotrophy	N-dep	N-lim	N-lim P-dep	N-lim P-lim	BG-11
Total lipids (%)	16.4	14.2	39.4	7.7	16.6
Polar (%)	60.7	44.2	20.1	-	99.3
Nonpolar (%)	39.3	63.8	79.9	-	0.7
Eterotrophy	N-dep	N-lim	N-lim P-dep	N-lim P-lim	BG-11
Total lipids (%)	14.3	8.7	37.5	9.1	9
Polar (%)	36.9	36.9	7.26	24.67	36.9
Nonpolar (%)	63.1	63.1	92.74	75.33	63.1

The nitrogen deprivation, or limitation in mixotrophic growth conditions, causes an accumulation of polar lipids not suitable for biodiesel production. The same starvation in the heterotrophic growth causes a more balanced distribution of lipids. This is due to the different duplication attitude induced by the two growing regimens. In mixotrophic conditions, in fact, the cultures show an higher cellular density with cells of little size, so the biggest part of lipid content is represented by the cellular membranes.

Heterotrophic cultures instead show a not balanced growth and are formed by a few big cells, in which the lipids are stocked as triglycerides.
The phosphorus deprivation or limitation, appear to induce the synthesis of almost only nonpolar lipids in both mixotrophic and heterotrophic growth conditions.
The obtained results clearly show, considering both biomass and lipid productivitity and lipid nonpolar content that, for large scale biodiesel production from *Chlorella vulgaris* cultures the best option appears to be mixotrophic nitrogen limited and phosphorus deprivated growth conditions.

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