Heterotrophic growth of *Chlorella vulgaris* on crude glycerol

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Abstract

The heterotrophic growth of *C. vulgaris* using crude glycerol as the sole carbon source was studied in five 5L flasks. Air in each flask was provided at a rate of 300 L/hr and the contents were continuously stirred with a magnetic bar. Temperature, pH and initial inorganic nutrients (nitrogen, phosphorus, potassium and micronutrients) were kept the same in all flasks. The C/N ratio varied in each of the five flasks was set at 6, 13, 25.4, 61.5 and 118 respectively while the nitrogen initial concentration was equal to 45.4 mg/L in all flasks. The residual organic carbon was measured as a function of cultivation time. It was found that biomass growth rates as well the lipid and protein content were dependent on the C/N ratio. Lipid content was proportional to the C/N ratio while the protein content was inversely proportional to the C/N ratio. Carbon concentrations above 2500 mg/L inhibited the growth rate.

Keywords: microalgae, heterotrophic growth, glycerol, carbon

1. Introduction

Microalgae are a potential source of lipids, protein and other antioxidants. It is well known that microalgae possess advantages over conventional plants. They can grow, up to thirty times faster than conventional plants under favorable conditions and, depending on the particular species and growth conditions, are very high on lipids (10-60%) and protein (10-70%) on a dry biomass basis (Metsoviti *et al.*, 2019).

Their growth as autotrophic organisms is however limited by light availability: as biomass accumulates in the growth medium light shielding effects become prominent and lead to substantial decreases in the growth rates necessitating special bioreactor and illumination designs (Papapolymerou *et al.*, 2019). Heterotrophic growth is not limited by light shielding as the microalgae use a carbon source from the growth media (Perez- Garcia *et al.*, 2011; Devi *et al.*, 2012).

Microalgae are used in pharmaceutical and cosmetic industry, in biodiesel production, in wastewater management, as nutritional supplements for human nutrition and as feed for animals and fish (Pulz & Gross, 2004).

The present study was conducted in order to investigate the effect of C/N ratio on growth, lipid and protein content of *C. vulgaris* grown heterotrophically on crude glycerol, so that the biomass can be a potential source of fish food and algal biofuel.

2. Materials and Methods

The microalgae species *C.vulgaris* (SAG Strain Number: 211-11b) was obtained from the Experimental Phycology and Culture Collection of Algae from the University of Goettingen in Germany (EPSAG) and was grown in Basal Medium (= ES- ('Erddekokt + Salze') containing an initial concentration of atomic nitrogen equal to 45.4 mg/l (NH_4Cl = 0,307 gr/4,5L and KNO_3 = 0,90 gr/4.5L). Others nutrients were added as follows: 0.02 g/L K_2HPO_4 and 0.02 g/L $MgSO_4.7H_2O$, 30 mL. Other micronutrients were the following: 0.005 mg/L ZnSO4.7H2O, 0.01 mg/L $MnSO_4.4H_2O$, 0.05 mg/L H_3BO_3, 0.005 mg/L $Co(NO_3)_2.6H_2O$, 0.005 mg/L $Na_2MoO_4.2H_2O$, 0.000025 mg/L $CuSO_4.5H_2O$, 3.5 mg/L $FeSO_4.7H_2O$, 4 mg/L EDTA. Also, to provide other necessary micronutrients soil extract 30 ml of soil extract per liter growth medium was also added (SAG, 2007).

C. vulgaris was cultivated in 5 L glass circular flasks that were filled up to 4.5 L. In each experiment the culture medium was inoculated with a standard quantity (250 mL of *C. vulgaris* inoculum) which was prepared as follows: 1 L flask, containing the necessary culture medium, was inoculated with *C. vulgaris* culture directly obtained from EPSAG and cultivated in a sterile environment until it reached an absorbance reading of 0.5. The cultivation of the inoculum was done always under the same conditions namely, at a temperature of 25°C, under natural illumination and by using an orbital shaker at 60 rpm in order to prevent sticking of algae to the surfaces of the flask.

Crude glycerol was obtained from a local biodiesel manufacturing plant. Its composition is approximately 86% glycerine, 0.5% methanol, 4% free fatty acids and 7.5% H_2O. From this analysis the carbon content was calculated approximately so that the approximate C/N ratios would be estimated and then, after the growth media were made, the initial carbon content in each bioreactor was measured analytically and the exact C/N ratios were calculated.

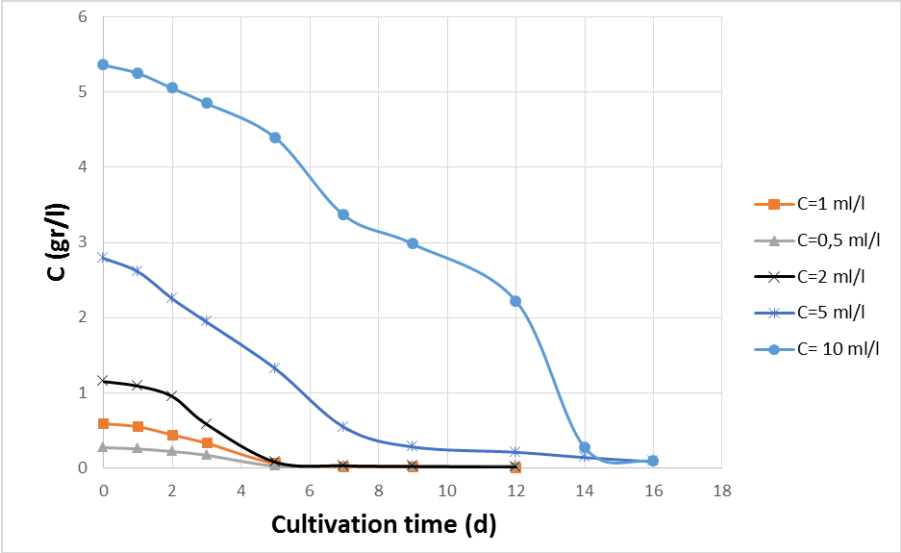
The bioreactors, the glass tubing and the culture medium were sterilized before use. Sterilization was performed as follows: The bioreactors and glass tubing was sterilized in an oven at 130 ^oC for two hours. The culture medium was also sterilized at 121 ^oC for 5-10

min. Air was passed through a 0,045 μm filter while the silicon tubing was sterilized by immersing in a H_2O_2 solution (15%) for several hours.
Air was continuously passed through the solution at 300 l/hr though a 2 mm glass tubing positioned at the tip of a magnetic bar and the air bubbles were dispersed with a magnetic bar at the bottom of the glass flasks at a rotational speed of 500 rpm.

3. Results and Discussion

Figure 1 shows the reduction in the initial carbon concentration as a function of cultivation time. It is noted that carbon decreases with time. This is due to the fact that the carbon in organic form found in the growth media is utilized by the *C. vulgaris* cells in order that they multiply and make proteins, lipids, carbohydrates and other compounds. The rate of decrease is dependent on the initial concentration of carbon since the initial nitrogen concentration is kept constant in all five bioreactors. Basically, all carbon is utilized within the first five days in the first three bioreactors where 0.5, 1 and 2 ml/L glycerol was added to the culture medium. In the bioreactors 4 and where 5 and 10 ml/L of glycerol carbon utilization ceases after approximately 9 and 14 days respectively.
As far as the nutrient content of the biomass produced, it was found that the lipid and protein contents were dependent on the C/N ratio. Lipid content was proportional to the C/N ratio while the protein content was inversely proportional to the C/N ratio in the algal biomass of *C. vulgaris* grown heterotrophically.

Figure 1. The decrease of the carbon concentration as a function of cultivation time for the amounts of glycerol added to the growth medium shown on the graph, namely, 0.5, 1, 2, 5 and 5 ml/L. For clarity, solid lines are drawn though the data.



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References
Devi M.P., Subhash G.V. and Mohan S.V. (2012), Heterotrophic cultivation of mixed microalgae for lipid accumulation and wastewater treatment during sequential growth and starvation phases: Effect of nutrient supplementation. *Renew. Energ.* **43**, 276-283.
Metsoviti, M.N., Katsoulas, N., Karapanagiotidis, I.T. and Papapolymerou, G. (2019), Effect of nitrogen concentration, two-stage and prolonged cultivation on growth rate, lipid and protein content of *C. vulgaris. J.Chem.Techn.Biotech*., **94**, 1466–1473.
Papapolymerou, G., Karayannis, V., Besios, A., Riga, A., Gougoulias, N. and Spiliotis, X. (2019), Scaling-up sustainable *C. vulgaris* microalgal biomass cultivation from laboratory to pilot-plant photobioreactor, towards biofuel. *G. NEST J*., **21**, 37-42.
Perez- Garcia O., Escalante F.M.E., de Bashan L.M.E., and Bashan Y. (2011), Heterotrophic cultures of microalgae: Metabolism and potential products.*Water Research*, **45**, 11-36.
Pulz O and Gross W. (2004), Valuable products from biotechnology of microalgae. *Applied Microbiology and Biotechnology*. **65** (6): 635–648.
SAG. Sammlung von Algenkulturen der Universität Göttingen. Culture Collection of Algae, Abteilung Experimentelle Phykologie und Sammlung von Algenkulturen (EPSAG), Universität Göttingen, Deutschland (2007) Available at: http://epsag.uni-goettingen.de