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EFFECT OF SOME STRESSORS ON BIOLOGICAL AND BIOCHEMICAL PARAMETERS IN THE *Rd* GREEN MICROALGA

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Abstract

Photosynthetic microorganisms, in addition to the major planetary role in solar energy conversion and biogeochemical cycles, have a major contribution to a wide range of biotechnological applications. In this paper, we present our original results concerning the use of different concentrations of nitrate as stressor for enhancing the lipid content in our isolate, a green microalga named Rd. The effects on cellular growth, lipid, total protein, chlorophyll a and b, and carotenes content are also presented. The results are discussed with respect to applicative and basic research approaches for a deeper understanding of the mechanisms involved in the response of microorganisms to stress.

Key words: *green microalgae, lipids, nitrate, total proteins.*

INTRODUCTION

The ability of photosynthetic microorganisms to synthetize intracellular lipid droplets is an important topic both for fundamental research as well as for practical applications (Xin et al., 2010; Sharma et al., 2012; Borowitzka, 2013; Benemann, 2013; Ardelean, 2015; Ardelean et al., 2017; 2018; Lari et al., 2016; Moisescu et al., 2018; Velea et al., 2017; Sibi et al., 2016; Tale et al., 2017).

One strategy to increase the synthesis of lipids concerns the use of so-called stressors, such as the limitation in nitrogen or phosphorus availability, the presence of heavy metals, gamma irradiation at low doses which are not inhibitory for cellular growth, or treatment with exogenous hydrogen peroxide (Li et al., 2008; Choi et al., 2015; Ermavitalini et al., 2017a; 2017b; Sibi et al., 2016; Tale et al., 2017; Gomes et al., 2017).

In this context, the aim of this paper is to show the effect that different nitrate ($NaNO_3$) concentrations have on lipid, chlorophyll *a* and *b*, carotenes, and total protein content in *Rd* strain, a new isolated green microalga.

MATERIALS AND METHODS

The photosynthetic microorganisms. The *Rd* green microalga strain was previously selected by random mutagenesis (Ardelean et al., 2018), due to increased lipid content as well as a practical important characteristic, namely growth in liquid medium as microcolonies which significantly promote harvest by rapid gravitational sedimentation. The *Rd* strain was cultivated on four variations of BG_{11} medium, with different concentrations of nitrate ($NaNO_3$): classic BG_{11} (with 1.5 g/l $NaNO_3$), BG_0 (without $NaNO_3$), and the modified BG_{11} (with 0.03 or 0.375 g/l $NaNO_3$).

Lipid content was estimated using the phospho-vanillin method (Park et al., 2016). Microalgal paste was resuspended in 2:1 parts of chloroform: methanol (v/v) by manually shaking the tube vigorously for a few seconds or until the biomass was dispersed in the solvent system. Finally, a 0.73% NaCl water solution was added to produce a 2:1:0.8 system of chloroform: methanol: water (v/v/v). The phospho-vanillin reagent was prepared by dissolving 0.75 g vanillin in 125 ml distilled water and mixed with 500 ml of 85% phosphoric

acid solution. The final concentration of reagent was 1.2 mg/ml vanillin in 68% phosphoric acid. Algal oil was dissolved in chloroform (10 mg in 10 ml for a final concentration of 1 mg/ml), and different concentrations (10 to 150 µg) of standard lipid samples were prepared in clean glass vials. The vials were incubated at 90°C for 10 min to evaporate the chloroform. 0.1 ml of concentrated sulphuric acid was added to each vial and then heated at 90°C for 10-20 min. After cooling on ice for about 5 min, 2.4 ml of phospho-vanillin reagent was added and allowed to develop for 10 min, until the colour of the sample turned pink (Park et al., 2016).

The equation for etalon curve is the following y = 0.0034 x -0.006 (R^2 = 0.9826), using oil extracted from mixture of microalgae populations.

Nile red labelling of lipids has been done as previously described (Ardelean et al., 2018).

Chlorophyll *a* and *b* was extracted in 90% methanol and the concentration calculated as previously described (Ardelean et al., 2018).

Carotenoids were measured spectrophotometerically using the modified method of Mackinney (1941) as presented by Boyer (2006). Briefly, a known volume of culture was centrifuged at 4000×g for 10 min. The supernatant was decanted and the same volume of methanol was added to the pellet. The mixture was incubated in a water bath at 55°C for 15 min and then centrifuged at 4000×g for 10 min. The absorbance (A) of the extract was measured against a blank of free methanol at 650, 665, and 452 nm. Carotenoids were estimated as µg/ml of culture suspension using the following equation: Carotenoids (µg/ ml) = 4.2 A452 – [0.0246 (10.3 A665 – 0.918 A650)].

Total soluble proteins were estimated using the biuret method. After carotenoids extraction, residual cells were extracted using 1 N NaOH in a boiling water bath for 2 h. Serum bovine albumin was used to construct the calibration curve, the equation being the following: y = 0.0346 x -0.0004 (R^2 = 0.9998). This equation was further used to calculate the concentrations of total proteins in our experimental samples.

RESULTS AND DISCUSSIONS

In Figure 1 there is presented the quantity of lipids in the green microalga *Rd*, grown in classic BG_{11} medium (with 1.5 g/l $NaNO_3$) or in different variants of this medium: BG_0 (without $NaNO_3$) or modified BG_{11} (with either 0.03or 0.375 g/l $NaNO_3$).

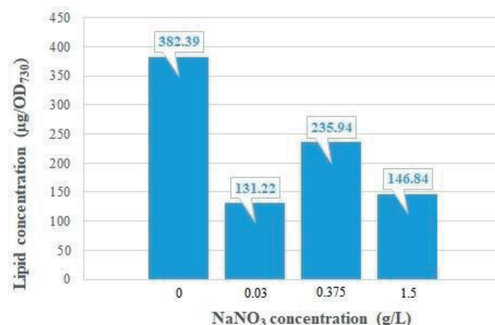

Figure 1. Lipid content of *Rd* green microalga grown in various $NaNO_3$ concentrations

The lipid content varies significantly when the green microalga *Rd* is grown in the same mineral medium, but with different $NaNO_3$ concentrations. The highest value was obtained for cells previously grown in BG_{11} and then transferred in BG_0, in order to induce nitrogen starvation.

These results are in general agreement with other reports concerning the increase in lipid content following nitrogen starvation (Li et al., 2008). However, we have no explanation for why at very low $NaNO_3$ concentration in the growing medium (0.03 g/l $NaNO_3$) the lipid content is lower (131.22 µg lipids/unit OD) than in the case of 0.375 g/l $NaNO_3$ (382.39 µg lipids/unit OD). It can be speculated that even at that very low $NaNO_3$ concentration, the metabolism is not sufficiently stimulated to change.

These empirical observations could be helpful not only for practical oriented research but also for fundamental research.

In Figure 2, there are presented cells of *Rd* strain grown in BG_{11} with 0.03 g/l $NaNO_3$ and labelled with Nile red for lipid inclusions, in both green and red fluorescence.

In Figure 3, in the *Rd* cells grown in BG_0 and labelled with Nile red, the lipids droplets are seen much clearer, their concentration being higher (i.e., 382.39 µg lipids/unit OD) as compared to the cells grown in BG_{11} (Figure 2) where the concentration of lipids is lower (i.e., 131.22 µg lipids/unit OD).

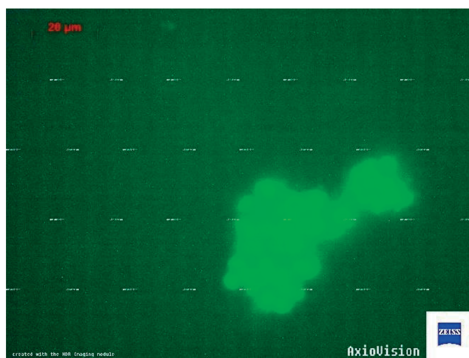

Figure 2. Microscope images of the same microscopic field showing *Rd* cells grown in BG_{11}, and labelled with Nile red for lipid inclusions

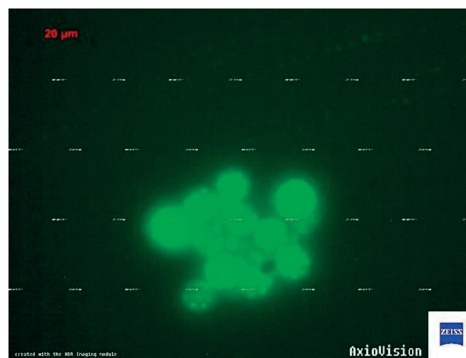

Figure 3. Microscope images of the same microscopic field showing *Rd* cells grown in BG_0, and labelled with Nile red for lipid inclusions.

The results concerning the content in chlorophyll *a* and *b*, total proteins and total carotenes in *Rd* grown in either BG_{11} or BG_0 are presented in Table 1.

Table 1. The content in chlorophyll *a* and *b*, total proteins and carotenes in *Rd* strain grown in BG_{11} or BG_0

Culture	Biomass (mg)	Lipids (µg/DO_{730})	Proteins	Chl *a*	Chl *b*	Carotenes
			(µg/ml)			
BG_0	0.20	382	5.1	4.8	1.0	2.6
BG_{11}	0.14	147	3.3	2.8	1.3	1.9

The high decrease in total protein content in *Rd* cells grown in BG_0, where the lipid content is highly increased suggests that, in the absence of exogenous $NaNO_3$, some of the proteins are mobilised and converted to lipids, which are deposited inside the cells as droplets.
However, low dose gamma irradiation seems to be less efficient in promoting lipid accumulation compared with $NaNO_3$ starvation (results not shown).

CONCLUSIONS

Different nitrogen concentration is able to induce changes in lipid composition of the green microalga *Rd* as compared with cells grown in classic BG_{11} medium. The highest lipid concentration was obtained under complete nitrogen starvation, when no nitrate was present in the extracellular medium (BG_0).
In these conditions (BG_0) other cellular components also exhibit different variations in their concentrations.

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