Bulgarian Journal of Agricultural Science, 24 (Supplement 1) 2018, 150-155

GROWTH PARAMETERS, PROTEIN AND PHOTOSYNTHETIC PIGMENT CONTENT OF *CHLORELLA VULGARIS* CULTIVATED UNDER PHOTOAUTOTROPHIC AND MIXOTROPHIC CONDITIONS

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Abstract

VELICHKOVA, K. AND I. SIRAKOV, 2018. Growth parameters, protein and photosynthetic pigment content of *Chlorella vulgaris* cultivated under photoautotrophic and mixotrophic conditions. *Bulg. J. Agric. Sci.*, 24 (Suppl. 1): 150–155

The purpose of this study was the determination of growth parameters, chlorophyll, carotenoid and protein content of the green microalgae *Chlorella vulgaris* cultivated under different mixotrophic and photoautotrophic conditions. Microalgae cultivation was initiated in a laboratory bioreactor of 500ml Erlenmeyer flask containing 250 ml nutrition media BBM. The cultures were maintained at room temperature (25-27ºC) on a fluorescent light with a light:dark photoperiod of 12 h:12 h. The strains were checked for 96 hours growth period in photoautotrophic variants with carbon dioxide (2%, v/v), mixotrophic – CO_2 + 3g.l^{-1} glucose, mixotrophic – CO_2 + 3g.l^{-1} lactose. In the present study we found that *C. vulgaris* showed better growth in mixotrophic conditions with CO_2 and glucose. Higher content of chlorophylls, carotenoid and protein was obtained in the photoautotrophic culture.

Key words: Chlorella vulgaris; biomass; mixotrophic; pigment; photoautotrophic; protein

Introduction

The microalgae cultivation stemmed from their use for the provision of vitamins, coloring materials including carotene, chlorophyll, various pharmaceutical substances, energy products (biofuel, methane, bioethanol, biohydrogen), as animal feed and as a food in the human diet (Ogbonna et al., 1997; Agwa et al., 2012). Algae cultivation requires light, carbon source, growth medium and nutrients. Some algae species can use organic sources from municipal and industrial wastewaters as carbon source through heterotrophic metabolism (Blair et al., 2013; Sirakov and Velichkova, 2014). Microalga can grow heterotrophically with an organic carbon source instead of using a continuous flow of carbon dioxide and light in the same nutrition media as in phototrophic cultures (Morales-Sanchez et al., 2013). The advantages of the phototrophic cultivation is that microalgae fixes carbon dioxide and produces oxygen, contributing to the reduction of carbon emissions to the atmosphere (Gouveia and Oliveira, 2009). Mixotrophic cultivation uses simultaneously inorganic and organic compounds as carbon source (Dragone et al., 2010). Carbon sources (like lactose, glucose, galactose) from industrial dairy waste may use for algae cultivation (Abreu et al., 2012). Cultivation of microalgae with nutrients from wastewater, such as nitrogen and phosphate, can decrease the cost of the raw materials and also provide some environmental benefits (Sirakov et al., 2013; Velichkova, 2014). Also if the algae have the capability to grow on heterotrophic mode, organic carbon sources can stimulate the cell growth rate and increase the lipid content of the biomass (Heredia-Arroyo et al., 2011). Mixotrophic cultivation in comparison with photoautotrophic cultures has been associated with lower energy costs due to its relatively lower requirements for light intensities (Garcia et al., 2005). Mixotrophic organisms have the possibility of living under both conditions autotrophic and heterotrophic based on the available light intensity and organic compound concentration (Mata et al., 2010). Also for mixotrophic culture conditions the organic sources might be less costly considering the high carbon cost (Liang et al.,

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2009). Mixotrophic cultivation of microalgae provides higher biomass and lipid productivities than cultivation under photoautotrophic conditions (Bhatnagar et al., 2011).

C. vulgaris is a photosynthetic microalgae with a fast growth rate used for food and biofuel due to its high protein and lipid contents (Phukan et al., 2011; Seyfabadi et al., 2011). Chlorella showed great potentials as future bioenergy producers due to their endurance, high growth rate, and high oil content, and it can be cultured both under autotrophic and heterotrophic conditions (Miao and Wu, 2004, 2006; Liang et al., 2009). The researches show that *C. vulgaris* grown on exogenous sugars like glucose medium may provide a higher microalgal biomass (Dvorakova-Hladka, 1966; Liang et al., 2009; Abreu et al., 2012).

The purpose of this study was cultivation of the green microalgae *Chlorella vulgaris* under different mixotrophic and photoautotrophic conditions and determination of growth parameters, chlorophyll, carotenoid and protein content.

Materials and Methods

Chlorella vulgaris (SKU: 100-CVC00-50) was delivered from Algae depot – USA (www.algaedepot.com). The strain was cultivated in BBM medium which chemical compositions is $NaNO_3$ – 10.0 g, $MgSO_4$. $7H_2O$ – 3.0 g, NaCl – 1.0 g, K_2HPO_4 – 3.0 g, $KH_2PO_4.3H_2O$ – 7.0 g, $CaCl_2.2H_2O$ – 1.0 g (stocks per 400 ml); $ZnSO_4.7H_2O$ – 8.82 g, $MnCl_2.4H_2O$ – 1.44 g, MoO_3 – 0.71 g, $CuSO_4.5H_2O$ – 1.57 g, $Co(NO_3)_2.6H_2O$ – 0.49 g (trace elements solution per litre); $EDTANa_2$ – 5.0 g, $FeSO_4.7H_2O$ – 4.98 g.

Algae cultivation was initiated in bioreactor from 500ml Erlenmeyer flask containing 250 ml of BBM nutrition media. The experiment was conducted in photoautotrophic variants with carbon dioxide (2%, v/v), mixotrophic – CO_2 + 3g.l^{-1} glucose, mixotrophic – CO_2 + 3g.l^{-1} lactose. Three luminescent lamps Sylvania Aqua Star – 18w, 10 000 K were placed at a distance of 30 mm from flasks. Light regime was adjusted at 12:12 h light:dark cycle in an illumination incubator until the end of experiment. The temperature was kept between 25 and 27^{o}C. The strains were checked for 96 hours growth period.

Growth measurements

Optical densities of microalgae cultures were measured at 0, 24, 48, 72 and 96 hours after the start of the experiment in three replicates. The sample with volume one ml was appropriately diluted with deionized water and the average value was recorded by absorbance at 680 nm with the help of spectrophotometer DR 2800 (Hach Lange).

The cultures were determined gravimetrically and growth was expressed in terms of dry weight (mg/l) (Rao et al., 2007). The cultures were harvested by centrifugation at 3.000 x g for 10 min and the cells were washed with distilled water. The pellet was freeze dried. The dry weight of algal biomass was determined gravimetrically and growth was expressed in terms of dry weight (mg.l^{-1}).

Chlorophyll and carotenoid content

The isolation of pigments from algae cells included the following procedures: harvesting 2 ml of microalgae cells by centrifugation at 10000 rpm, two times for 3 min and discarding the supernatant, suspension of cells in 2 ml methanol/water 90:10 v/v and mixing of Vortex for 1 min., heating of the suspension for half an hour in a water bath at 60ºC, cooling of the samples at room temperature, centrifugation of suspension (10000 rpm for 3 min) and discarding the supernatant with dissolved pigments. The absorbance of the pigments extract (665, 652 nm for chlorophyll content (a+b) and 470, 666nm for carotenoids content) was recorded by using spectrophotometer DR 2800 (Hach Lange). The chlorophyll content was computed (mg.l-1) according Porra et al. (1989) and carotenoid content was computed (mg. l-1) according Lichtenthaler (1987).

Protein content

Crude protein content was calculated by converting the nitrogen content, identifi ed by Kjeldahl's method, using an automatic Kjeldahl system (Kjeltec 8400, FOSS, Sweden).

Data analyses were conducted by using Analysis of Variance ANOVA (MS Office, 2010).

Results and Discussion

C. vulgaris cultivated under mixotrophic conditions synthesize compounds characteristic of both photosynthetic and heterotrophic metabolism. Microalgae growth was characterized with optical density and dry weight under different cultivation conditions (Figure 1, 2).

The best optical density was measured in the cultivation of Chlorella in mixotrophic conditions with carbon dioxide and glucose, which was 58.3% better than photoautotrophic cultivation (Figure 1). When comparing the optical densities of the two mixotrophic conditions, a 13.7% better development of the strain with carbon dioxide and glucose sources was observed. Every 24 hours, the optical density of the mixotrophic conditions increased in triplicate compared to photoautotrophic.

Maximum dry biomass (1.3 g.l^{-1}) of C.*vulgaris* was obtained in mixotrophic conditions with carbon dioxide and glucose, in comparison to its dry weight in photoautotrophic conditions (0.25 g.l^{-1}) (Figure 2). At the end of the experi-

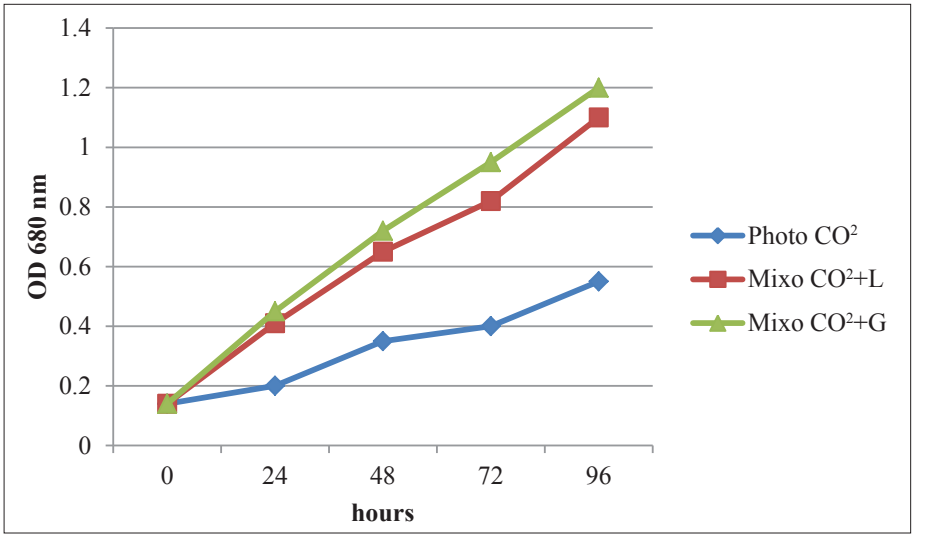

Fig. 1. Optical density of mixotrophic and photoautotrophic mode of *C. vulgaris*

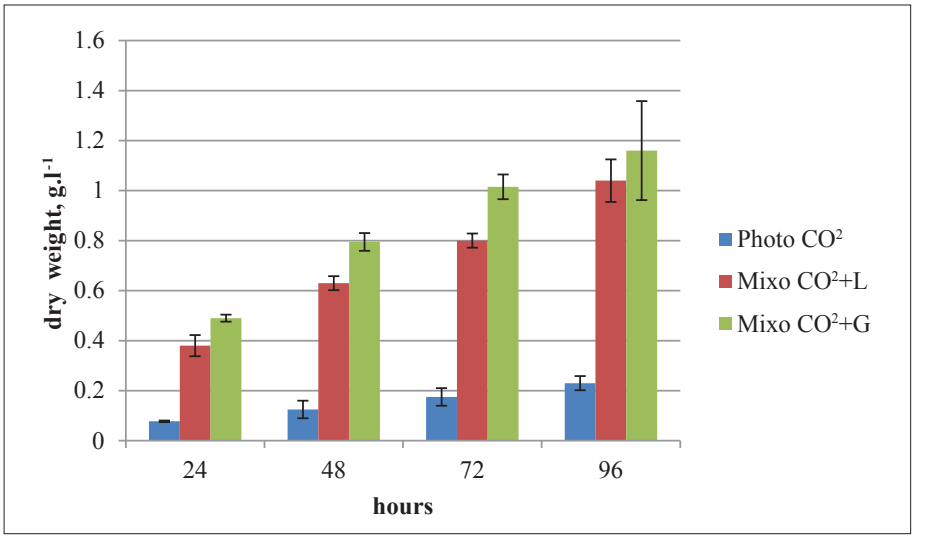

Fig. 2. Effect of photoautotrophic and mixotrophic mode on the dry weight (g.l^{-1}) of *C. vulgaris*

ment, the biomass in the mixotrophic cultivation with carbon dioxide and glucose was 80.8% higher than photoautotrophic. This is due to the fact that the energy density of the carbon source is higher compared to carbon dioxide and therefore the cell masses obtained under the mixotrophic conditions are higher (Perez-Garcia et al., 2011).

The difference between biomass is only 15.4% higher in the culture with carbon dioxide and glucose under the two mixotrophic conditions. These results are confirmed by another authors study, which reported that mixotrophic *C. vulgaris* growth in glucose yielded higher biomass content and productivity than cells grown under photoautotrophic conditions. Light source and organic carbon source has been considered as the most efficient process for the production of microalgal biomass (Lee et al., 1996). Reduced light energy used for CO_2 fixation in mixotrophic cultures leads to energy is used for carbon assimilation. Mixotrophy provides higher energetic efficiency than other cultivation modes because the amount of energy dissipated is minimal (Lalucat et al., 1984). According Shi et al. (1999) glucose can be considered the best

organic carbon substrate for the growth of Chlorella. Cultivation of *C. vulgaris* with glucose influences metabolic carbon assimilation pathways, cells' size, quantity of storage materials (starch, lipids, protein) and cellular contents of chlorophyll, RNA, vitamins (Perez-Garcia et al., 2011). Therefore glucose is one of the most used carbon sources for most living cells and is used as a carbon and energy source in many heterotrophic cultures of microalgae (Vazhappilly and Chen, 1998; Jiang and Chen, 2000; Cheng et al., 2009).

The protein content of photoautotrophic and mixotrophic microalgal cells were compared in Figure 3.

Cultivation of *C. vulgaris* using CO_2 as carbon source led to the highest protein content (26.4%). The highest protein content obtained in our study was significantly higher with 39% than that found in *C. vulgaris* cultivated in mixotrophic with CO_2 and lactose, and with 30.7% in CO_2 and glucose as carbon source. According to some authors, the level of protein increases in mixotrophic culturing (Abreu et al., 2012). Other authors report a higher amount of protein under phototrophic conditions (Orus et al., 1991; Bajwa et al., 2016).

The amount of total pigments (chlorophyll and carotenoid) in *C. vulgaris* cultured under photoautotrophic and mixotrophic conditions were also determined (Table 1).

As summarized in Table 1, the maximum chlorophyll (a+b) pigment content (8.4 $g.l^{-1}$) was obtained in the photoautotrophic culture. In both mixotrophic conditions, the content of chlorophyll is twice as low (48.2%) as compared to the phototrophic cultivation of the strain, and the differences were statistically proven ($p \leq 0.05$) (Table 1). These results are confirmed by other authors (Kong et al., 2013; Xiong et al., 2010).

The formation of photosynthetic apparatus in Chlorella may be disturbed by the presence of organic substrates (Yang

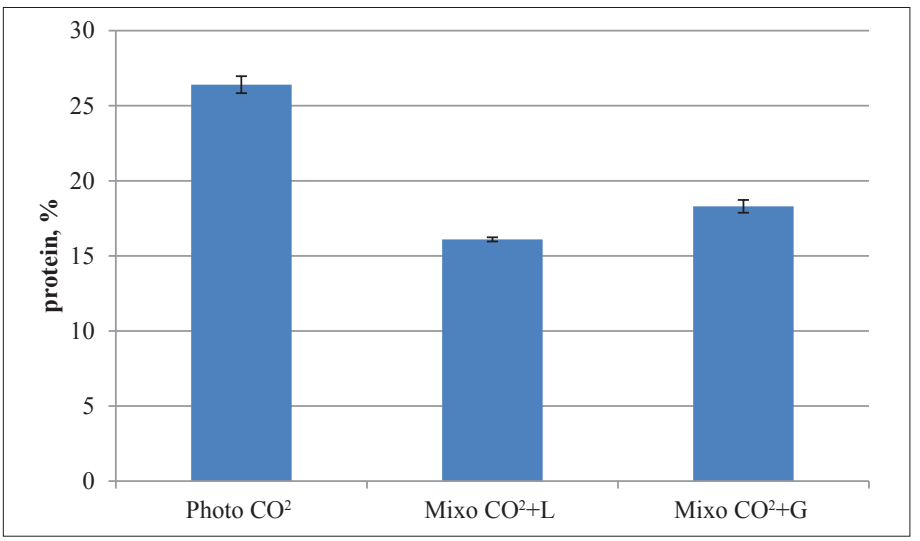

Fig. 3. Effect of photoautotrophic and mixotrophic mode on the protein content of *C. vulgaris*

Table 1
Effect of photoautotrophic and mixotrophic mode on the photosynthetic pigment content ($g.l^{-1}$) of *C. vulgaris*

Growth condition	Chlorophyll (a+b)				Carotenoid			
	24	48	72	96	24	48	72	96
Photoautotrophic CO2	1.74±0.09a	3.31±0.15a	6.25±0.07a	8.4±0.14a	0.27±0.02a	0.64±0.05a	1.25±0.07a	2.4±0.14a
Mixotrophic CO2+L	0.51±0.12b	1.36±0.22b	2.26±0.08b	4.35±0.35b	0.11±0.02b	0.27±0.02b	0.5±0.03b	0.7±0.03b
Mixotrophic C O2+ G	0.62±0.08b	1.6±0.28b	2.7±0.14b	4.5±0.42 b	0.15±0.02b	0.33±0.03b	0.59±0.01c	0.85±0.06b

*Mean ± standard error in the same column followed by different letters represent significant differences ($p \leq 0.05$).

et al., 2000), resulting in a decreased production of photosynthetic pigments when compared with that obtained under photoautotrophic conditions. The higher content of chlorophylls obtained in the photoautotrophic culture confirms such observation when compared to mixotrophic cultures. Such observations with enhancement of chlorophyll biosynthesis by photoautotrophic Chlorella strains compared with that resulting from mixotrophic cells have been reported by other authors (Ip et al., 2004; Kong et al., 2011).

Among the different nutritional modes tested, the highest carotenoids content (2.4 $g.l^{-1}$) was also found in the photoautotrophic culture in 96 hours. This value dropped to 70.8 % when cells were grown in mixotrophic medium supplemented with CO_2 and lactose and with 64.6% in CO_2 and glucose, respectively. These results are consistent with those of Liu et al. (2009) who found lower amount of carotenoids in mixotrophic cells when compared to cells grown on photoautotrophic culture.

Conclusions

In the present study *C. vulgaris* showed better growth in mixotrophic conditions with CO_2 and glucose. Higher content of chlorophylls, carotenoid and protein was obtained in the photoautotrophic culture. Mixotrophic cultivation of *C. vulgaris* using glucose and lactose can reduce the costs of microalgae biomass production and so contribute to a better economic impact and lower energy costs.

Acknowledgements

Financial support was provided by the Faculty of Agriculture, Trakia University, Project № 4E/14.

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