The Journal of Engineering and Exact Sciences – jCEC, Vol. 06 N. 03 (2020)
journal homepage: https://periodicos.ufv.br/ojs/jcec
doi: 10.18540/jcecvl6iss3pp0228-0315
OPEN ACCESS – ISSN: 2527-1075

EVALUATION OF PHICOCYANIN PRODUCTION BY *Anabaena variabilis* USING DIFFERENT ORGANIC CARBON SOURCES

AVALIAÇÃO DA PRODUÇÃO DE FICOCIANINA POR *Anabaena variabilis* UTILIZANDO DIFERENTES FONTES DE CARBONO ORGÂNICO

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***Preliminary version of this work was published in: Anais do XIII Congresso Brasileiro de Engenharia Química em Iniciação Científica. São Paulo: Blucher, 2019. ISSN 2359-1757, DOI 10.5151/cobecic2019-PBIO51*

ARTICLE INFO

Article history:
Received 2020-07-31
Accepted 2020-09-04
Available online 2020-09-04

palavras-chave
Ficocianina
Crescimento celular
Suplementação

keywords
Phycocyanin
Cell growth
Supplementation

ABSTRACT

Phycocyanin (PC) is one of the phycobiliproteins, pigments found in cyanobacteria, which can be used as natural dyes, antioxidants and fluorescent markers. This study aimed to investigate the effect of supplementation of the basal culture medium ($BG11_0$) of the cyanobacterium Anabaena variabilis with different sources of organic carbon in concentration of 1 g L^{-1}, evaluating the production of biomass and PC. The best condition obtained was the medium supplemented with glucose that produced 75.36 mg g^{-1} PC, about 5 times greater than the control with 14.57 mg g^{-1} PC. All supplemented media also showed higher biomass production compared to the basal medium, again to stand out for the glucose medium, presenting 0.29 g L^{-1} of dry biomass.

RESUMO

A ficocianina (FC) é uma das ficobiliproteínas, pigmentos encontrados nas cianobactérias, que podem ser usadas como corantes naturais, antioxidantes e marcadores fluorescentes. Este estudo teve como objetivo investigar o efeito da suplementação do meio de cultura basal ($BG11_0$) da cianobactéria Anabaena variabilis com diferentes fontes de carbono orgânico em concentração de 1 g L^{-1}, de modo a avaliar à produção de biomassa e FC. A melhor condição obtida foi o meio suplementado com glicose que produziu 75,36 mg g^{-1} FC, cerca de 5 vezes maior que o controle com 14,57 mg g^{-1} FC. Todos os meios suplementados também apresentaram maior produção de biomassa em comparação com o meio basal, novamente para destaque para o meio com glicose, apresentando 0,29 g L^{-1} de biomassa seca.

1. INTRODUCTION

Cyanobacteria are prokaryotic, unicellular and photosynthetic organisms found in the presence of light, with unicellular, multicellular or filamentous forms and producers of a wide variety of secondary metabolites with biotechnological application (Whitton and Potts, 2012). They are classified as microalgae and belong to the phylum Chlorophyta because they have chlorophyll and photosynthetic pigments such as phycobiliproteins, responsible for the photosynthesis process (Olaizola, 2003).

In general, cyanobacteria perform photosynthesis under autotrophic medium, using light energy and CO_2 as a carbon source, mostly. Nevertheless, some strains may assimilate, depending on light, nutrients from organic compounds to maintain metabolism and can also grow in mixotrophic and heterotrophic environments (Prasanna et al., 2004; Skleryk et al., 2002). This advantage has been applied to increase the production of biomass, and secondary metabolites as hydrogen, exopolysaccharides and pigments (Manirafasha et al, 2016; Noreña-Caro and Benton, 2018). Cyanobacteria of the genus *Anabaena* produce a variety of products. The most studied are toxins, however, researchers have been dedicating to investigate the potential of these microorganisms in the production of bioactive further compounds, such as peptides, fatty acids, alkaloids and other metabolites (Jaiswal et al., 2008; Schrader and Dayan, 2009; Tan, 2007).

Among these metabolites, phycobiliproteins (PBP) stands out, they represent 50% of the total cellular protein of cyanobacteria. Phycobiliproteins are found in a complex structure called the tilacoidal membrane, which plays an important role in light capture and energy transfer for the photosynthesis reaction (Rastogi et al., 2015; Viskari and Colyer, 2003). The three main phycobiliproteins found in cyanobacteria are: phycoerythrin (EF, λmax= ~ 560 nm, red), phycocyanin (PC, λmax= ~ 615 nm, blue) and allophycocyanin (APC, λmax= ~ 650 nm, light blue). Since the more prominent color is blue, cyanobacteria received the name of blue algae (Aryee et al., 2018; Kumar et al., 2014; Stal, 2007).

Phycocyanin (PC) has been the most studied PBP, due to properties such as antioxidant, non-toxic and anti-inflammatory potential. It has applications in the food industry as a natural dye for products such as chewing gum, desserts, candies, cake decoration, jellies and ice cream; in the cosmetic industry applied in lipsticks and eyeliners and the medical industry used as a marker in clinical diagnostics, in photodynamic therapy of tumors and immunoassay in fluorescent tubes (Ilter et al., 2018; Lauceri et al., 2018).

The synthesis of this bioactive compound can be evaluated in order to increase its production on an industrial scale, by addressing parameters such as light, medium composition, pH, temperature and photoperiod. More specifically, about the composition, the carbon source influences the formation mainly of macronutrients, such as carbohydrates, lipids and proteins (Manirafasha et al., 2016; Pagels et al., 2019).

Among the media to cultivation of cyanobacterial biomass, the mineral medium $BG11_0$ is largely used, constituting a photoautotrophic culture. Nevertheless, the influence of mixotrophic environment, in which the carbon source is formed by inorganic and organic substances, has been evaluated in order to increase the productivity of biomass and products, like phycobiliproteins (Borsari et al., 2007; Rizzo et al., 2015).

This study aimed to evaluate the effect of supplementing basal culture medium of cyanobacteria *Anabaena variabilis* ($BG11_0$) using different organic carbon sources (glucose, lactose, fructose, sucrose and galactose), aiming to increase the production of biomass and phycocyanin (PC).

2. MATERIAL AND METHODS

2.1 Cyanobacterial cultivation and maintenance

The cyanobacteria strain used, *Anabaena variabilis* ATCC 29413, was kindly donated by the Cyanobacteria and Phytotoxin Laboratory, from Institute of Oceanography, Federal University of Rio Grande (Rio Grande, Brazil)

The cultivation of cyanobacteria was carried out in $BG11_0$ basal medium, which is prepared by adding 1 mL of each of the following solutions: K_2HPO_4 (30.0 g· L^{-1}), $MgSO_4.7H_2O$ (75.0 g·L^{-1}), $CaCl_2.2H_2O$ (36 g·L^{-1}), ferric ammonium citrate (6 g·L^{-1}), Na_2.EDTA (Ethylene Diamino Tetracetic Acid Na_2, 1 g·L^{-1}), citric acid (6 g·L^{-1}), Na_2CO_3 (20 g·L^{-1}) and trace metal solution. The trace metal solution contained $ZnSO_4.7H_2O$ (0.222 g·L^{-1}), $MnCl_2.4H_2O$ (1.81 g·L^{-1}), $Na_2MoO_4.2H_2O$ (0.39 g·L^{-1}), $CuSO_4.5H_2O$ (0.079 g·L^{-1}), $CoCl_2.6H_2O$ (0.04 g·L^{-1}) and H_3BO_3 (2.86 g·L^{-1}). The volume was completed with deionized water to 1 L and the final pH maintained between 7.4 - 7.6 (Rippka et al., 1979).

The culture was kept in 250 and 500 mL cotton-plugged Erlenmeyer flasks, in a photoperiod chamber, with temperature controlled at 30 ºC, luminosity of 1,553 lux provided by white LED lamps with 12 h light/dark photoperiod, in an aerobic environment. The medium was renewed every 14 days.

2.2 Cultivation conditions

The strain maintained in basal medium ($BG11_0$) was used as inoculum in the experiments carried out to test the effect of different sources of organic carbon for the production of biomass and phycocyanin by fermentation.

The assays were performed in Erlenmeyers of 125 mL, with a working volume of 50 mL, with an inoculum volume of 20% (v/v), which corresponded to an initial cell concentration of 0.11 g L^{-1}. It was prepared medium without adding the carbon source (control) and medium supplemented with 1 g L^{-1} of each sugar. The sugars analyzed were: glucose, lactose, fructose,

sucrose and galactose. Each condition was performed in triplicate under agitation of 150 rpm in an orbital shaker; to 30 ºC; with light intensity of 1,533 lux; and a photoperiod of 12 h light/dark.

2.3 Organic carbon quantification

The quantification of sugars was performed by HPLC (High Performance Liquid Chromatography). The sample was filtered through a 0.22 µm filter and injected into the Shimadzu chromatograph model LC-20A Pronience, equipped with SUPELCOGEL C-610H column and refractive index detector, with phosphoric acid solution (0.1%) used as the mobile phase, with a pump flow of 0.5 mL/min, oven temperature of 32 ºC and injection volume of 20 µL.

2.4 Cell growth analysis

Cell concentration was analyzed at 2, 4, 6 and 8 days of fermentation. The biomass was determined by measuring optical density in a spectrophotometer (Shimadzu UV mini-1240, Shimadzu, Kyoto, Japan) with a wavelength of 750 nm, and correlated with the dry cell mass concentration (g dry mass·L^{-1}) by a calibration curve (Hemlata e Fatma, 2009; Rizzo et al., 2015).

The experiments were carried out using a laminar flow chamber, previously cleaned with 70% alcohol and kept in ultraviolet light for 15 min, with previously sterilized materials, in order to avoid contamination. For each assay, 5 mL samples were taken, and subsequently centrifuged at 5000 g and washed with distilled water three times in order to eliminate interferences from the culture medium during absorbance measurements on the spectrophotometer.

2.5. PC production

Since phycobiliproteins are intracellular compounds, in the last day of assay, PC extraction was performed by collecting 10 mL of culture suspension in a Falcon tube of 15 mL for freezing and thawing cycles combined with ultrasound, with 5 freezing cycles at -15ºC for 1.5 h and thawing in the bath ultrasonic at 25ºC for 30 min, using the 0.1 M phosphate buffer pH 7.0 as the extracting solution (Cottas et al., 2020).

The extracted samples were analyzed by spectrophotometry (Shimadzu UV mini-1240) and the PC concentration (mg·mL^{-1}) was calculated according to the Equation 1 (Khazi et al., 2018).

$$C_{PC} = \frac{A_{615} - 0.474 \times A_{652}}{5.34} \quad (1)$$

where A615 and A652 are respectively the characteristic wavelengths of phycocyanin and allophycocyanin.

2.6 Statistical Analysis

Statistical analyzes for mean difference analysis of biomass concentration and phycocyanin content, using the 95% confidence Tukey test, were performed by Statistica® 8.0 Software.

3. RESULTS AND DISCUSSION

3.1 Organic carbon quantification

The consumption of carbon sources in different cultivation media over 6 days is shown in Table 1.

Table 1 - Carbon source consumption during 6 days in the cultivation of *Anabaena variabilis.*

Carbon Source/ g·L^{-1}	Time/ d				Consumption/ %
	0	2	4	6	
Glucose	1.00	0.81 ± 0.02	0.75 ± 0.04	0.43 ± 0.01	56.7
Lactose	1.00	0.66 ± 0.01	0.51 ± 0.01	0.47 ± 0.01	52.8
Fructose	1.00	0.68 ± 0.02	0.47 ± 0.02	0.34 ± 0.02	66.2
Sucrose	1.00	0.66 ± 0.02	0.60 ± 0.02	0.38 ± 0.01	61.7
Galactose	1.00	0.72 ± 0.01	0.61 ± 0.02	0.43 ± 0.02	56.9

Note: Values represent mean ± standard deviation, obtained by triplicate**.**

Table 1 shows that there was a consumption, in this increasing order of lactose, galactose, glucose, sucrose and fructose, indicating that the cyanobacterium of *Anabaena variabilis* can assimilate all sources of carbon tested.

These results corroborate the study by Chen et al. (2008), who studied supplementation to the culture medium of the strain of *Anabaena* sp. with different organic carbon sources at the initial concentration of 2 g L^{-1}, obtaining that fructose was the preferred source of organic carbon for this strain, with almost 100% of substrate consumption in 8 days of cultivation, besides the consumption of sucrose in about 70% as the third most consumed organic carbon source. Khetkorn et al. (2010) found similar consumption behaviors to the present study analyzing the uptake of different sugars by *Anabaena siamensis*. These authors used sugar concentrations of 0.5 g L^{-1}, obtaining 45% consumption for fructose, followed by sucrose and glucose with 25%. Both authors discover that galactose and lactose may not be used as an organic carbon source since they found the lowest consumption by using this sugar. In the current work, *A. variabilis* showed the smallest consumption with lactose

(52.8%), but yet, it was considerable, since the consumption varied from 52.8 % to 66.2%.

Studies show that cyanobacteria can be successfully used in mixotrophic cultivation since they have genes responsible for the assimilation of organic carbon sources. This characteristic is an important ecological peculiarity that can be applied in the integration of wastewater treatment or reuse of by-products that are originated from agroindustrial sector as they are largely consisted by sugar (Muñoz-Marín et al., 2020).

3.2 Growth of *A. variabilis*

Growth curves for the *A. variabilis* strain in basal medium and supplemented media with different carbon sources are shown in Figure 1.

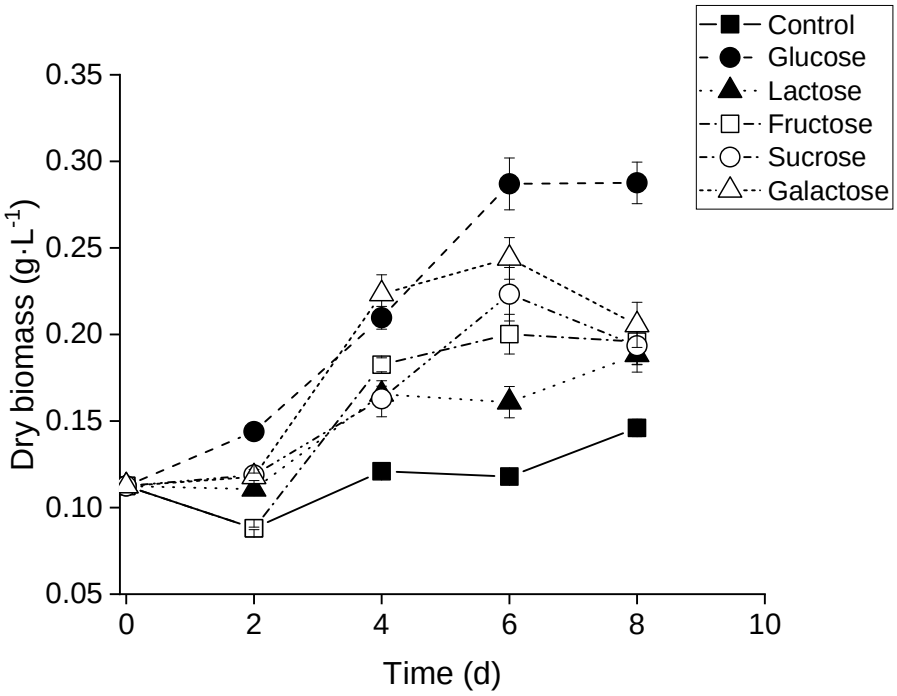

Figure 1 - Cell concentration obtained from *A. variabilis* under different culture media in 8 days. Values represent mean ± standard deviation, obtained by triplicate.

Figure 1 shows that all supplemented media favored biomass growth and cell concentration was superior to the biomass content observed for the control medium at the end of 8 days. In the medium supplemented with glucose, cell concentration achieved the highest magnitude (0.29 g L^{-1}), about 2 times higher than in the medium control (0.15 g L^{-1}). It is noteworthy that glucose was not the most consumed sugar (Figure 1), but resulted in higher biomass growth.

This result corroborates the fact that the mixotrophic culture promotes an increase in cell concentration, in relation to the photoautrophic culture. The most studied source of organic carbon used to increase cell biomass is glucose. Kovac et al. (2017) observed that the addition of glucose 1.5 g L^{-1} to the control medium increased the biomass concentration of cyanobacteria of the genus *Anabaena*, with the C2 strain obtained an increase of 50% while the C5 strain obtained an increase of about 200% compared to the control with 35 days of cultivation. In the work developed by Vargas et al. (2018), that applied the strain *Anabaena* sp. showed that the best biomass yield was obtained from a temperature of 32 °C and 2.1 g L^{-1} of glucose, reaching about 0.68 g L^{-1}, 1.3 times higher than the control.

The other culture media supplemented (lactose, fructose, sucrose and galactose) showed no significant difference for cell concentration, reaching approximately 0.20 g L^{-1} of dry biomass after 8 days of cultivation, 25% higher than the control medium.

Further works proposed to analyze the possibility of using these sugars for the production of cellular biomass and obtained varied results. Sahu et al. (2007) observed that the cell growth rate of *Anabaena* sp. the medium supplemented with 5 g L^{-1} of lactose was the highest, followed by the medium containing fructose. Khattar et al. (2015) observed that sucrose was the best carbon source for the growth of *Anabaena. fertilissima*, showing biomass concentration 30% higher in the presence of sucrose than the control cultures. Khetkorn et al. (2020) supplemented almost 11 g L^{-1} of fructose to the $BG11_0$ medium using *Anabaena* PCC 7120 and reached 2 times higher cell concentration than medium without fructose during 12 days.

The use of galactose as a source of organic carbon for the production of biomass for the genus *Anabaena* is rarely reported in the literature, although it shows a good conversion for cell growth. Other sources of carbon, such as mannose and xylose are reported, but they have caused a decrease in the concentration of cyanobacterial biomass (Sahu et al., 2007).

3.3 PC production

To determine the presence of phycocyanin in the extract of the strain of *Anabaena variabilis*, a spectral scan was performed, as shown in Figure 2.

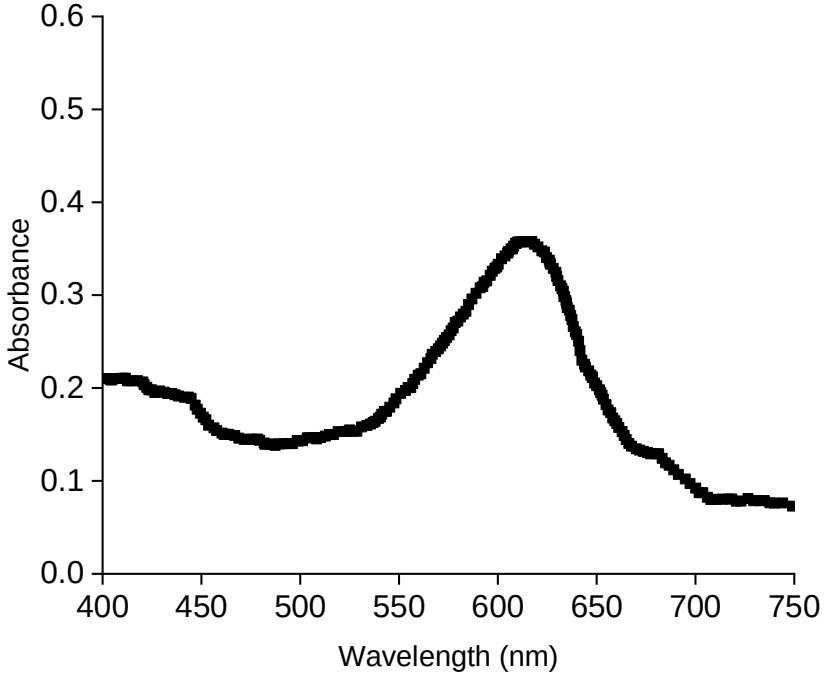

Figure 2 – Spectral scan of crude extract for *Anabaena variabilis*.

According to Figure 2, the presence of a peak in the region of 610-620 nm can be observed, a peak region characteristic of PC, indicating its presence in the crude extract (Rizzo et al., 2015).

The effect of sugar supplementation to the basal medium ($BG11_0$) on the production of phycocyanin by *Anabaena variabilis* is shown in Figure 3.

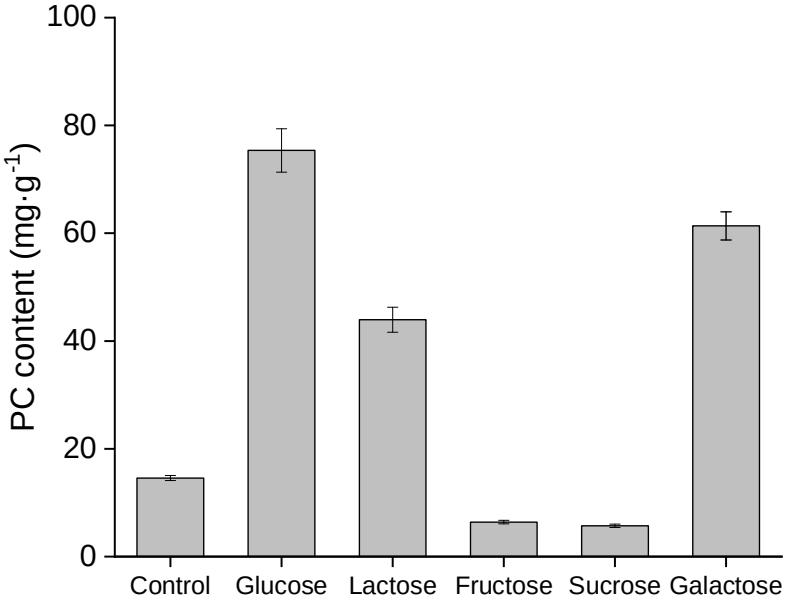

Figure 3 - Phycocyanin concentration obtained by supplementing the basal medium with different carbon sources after 8 days. Values are means ± standard deviation.

According to Figure 3, cultures containing glucose, lactose and galactose favored the increase of phycocyanin concentration, with a higher content in comparison to the assay using control medium. In contrast, cultivation in media containing fructose and sucrose hindered its production.

The highest concentration of PC was obtained for the medium containing glucose, reaching 75.36 mg g^{-1}, presenting an increase of 5.2 times in relation to the control medium. Other significant concentrations were obtained by galactose with 61.36 mg g^{-1} and lactose with 43.94 mg g^{-1}, respectively 4.2 and 3 times higher than the control medium.

The media supplemented with fructose and sucrose showed no significant difference, with concentrations in the order of 6 mg g^{-1} of PC, a value about 40% lower than the control medium. This result indicates that the consumption of these carbon sources does not indicate a higher production of phycocyanin (Figure 3) and biomass production (Figure 2), since fructose was the most consumed sugar, indicating that different metabolic routes were used by cyanobacteria in function of the carbon source.

The optimum source of organic carbon supplemented to the culture medium of cyanobacteria in order to obtain PBP, more specifically PC, is also variable in the literature. Khattar et al. (2015), also studied the synthesis of PBP by supplementing the culture medium for *Anabaena fertilissima*. Their work showed that better results were obtained when fructose and sucrose were added to the media, being 2.9 and 2.5 times higher than in the assay with glucose. Kaushal et al. (2017), that used *Nodularia sphaerocarpa*, found that the basal culture medium (Chu-10) plus 0.5% glucose was able to increase the PC concentration by about 1.9 times in relation to the medium control. In the work developed by Cottas et al. (2020), that used *Anabaena variabilis* and analyzed the supplementation of glucose and sodium nitrate isolated and simultaneously, proved that medium supplemented with only glucose 1 g L^{-1} enable to attain PC concentration of 77.68 mg·g^{-1} in 10 days of cultivation.

The synthesis of phycobiliproteins by cyanobacteria in the presence of lactose and galactose as organic sources of carbon is still little explored in the literature and the present work indicates positive results. All these sugars tested in the current study are present in agroindustrial residues or by-products and they are an alternative of low-cost feedstock, as cheese whey, sugarcane and soybean molasses or fruit processing residue. Kabariya and Ramani (2018) investigated dairy wastewater treatment by *Oscillatoria* sp., *Phormidium* sp. and its co-culture system and observed PC concentration of 0.13, 0.03 and 0.10 mg L^{-1}, respectively. According to Hung et al. (2005), dairy wastewater has high organic loading rate, with the main contributors being lactose.

Comparing this work with the literature, it is observed that the species of cyanobacteria is also a variable to be considered in the evaluation of PC production, and the species may present different metabolisms according to the composition of the culture medium and the type of source of supplemented organic carbon (Pagels et al., 2019), causing different production of metabolites.

The production of PC with only one variable, the carbon source, is a step towards future stages with the combination of several factors that influence the production of phycocyanin, such as the pH of the medium, supplementation of nitrogen sources, sources of phosphorus, photoperiod, intensity and quality of light.

4. CONCLUSION

The present work indicates that *Anabaena variabilis* grown in a mixotrophic medium has different assimilations to different sources of organic carbon, suffering changes in cell growth and phycocyanin production. It is concluded that the best condition is supplementation with $BG11_0$ medium with glucose, with cell concentration 2 times higher and favoring the production of PC about 5 times during 8 days of culture, compared to the control medium.

5. AKCNOWLEDGMENTS

The authors acknowledge CAPES, CNPq and FAPEMIG from Brazil for financial support.

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