



Cellular Bioenergetics in *Spirulina platensis* towards Growth and Phycocyanin Production Under Different Photon Flux Densities Using the Modified Zarrouk's Medium

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ABSTRACT

From all the pigments found in *Spirulina platensis*, phycocyanin has been found to have a diverse application in various fields, and has a high market demand, calling for a need to increase production and easy isolation methods. In general, phycocyanin production in cells depends on the light conditions, among other factors during the cultivation period. The focus of this study was to look at the effect of different light intensities on phycocyanin production in *Spirulina platensis*. Other cellular biochemical parameters, including chlorophyll content and protein, were explored under the different treatments. An experimental design containing 4 different light intensities of 20, 150, 300 and 600 $\mu\text{mol photons m}^2/\text{s}$ was administered with 3 replicates. The results obtained from the study showed that high phycocyanin content was obtained from a low light intensity treatment. Chlorophyll results were a bit in contrary to the results obtained for phycocyanin, with high chlorophyll content obtained in high light intensity treatments. Protein and biomass accumulation also followed the same trend, where they were observed to be higher in high light intensities, with the maximum biomass achieved at 600 $\mu\text{mol photons m}^2/\text{s}$ and maximum protein content achieved at 300 $\mu\text{mol photons m}^2/\text{s}$. Due to the commercial potential of phycocyanin to humans, its low cost downstream cultivation and processing of *Spirulina platensis* will be of economic advantage to the relevant stakeholders to fulfil the rampant demands and affordability of the blue phycocyanin pigment to both first and third World countries, hence the need of producing phycocyanin using the modified Zarrouk's media which has cheaper if not affordable ingredients.

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Introduction

Cyanobacteria and microalgae have been documented as a source of high-value products such as vitamins, essential minerals, fatty acids, polysaccharides and pigments. Natural pigments (chlorophylls, carotenoids and phycobiliproteins) have an important role in the photosynthetic and pigmentation metabolism of microalgae and cyanobacteria (Mulders et al. 2014), and also exhibit a wide range of biological activities like anti-cancer, antioxidant and anti-inflammatory (Raposo et al. 2013). The use of microalgae for the food, pharmaceutical, nutraceuticals and cosmetic market is increasingly relevant. Natural pigments of microalgae and cyanobacteria are a good alternative to synthetic colorants, and have the potential to be competitive with the same components from other sources and the ease of cultivation.

Currently, the pigments produced by cyanobacteria and microalgae and used commercially are carotenoids (β -carotene) from unicellular alga *Dunaliella bardawil*; astaxanthin from green algae *Haematococcus pluvialis*; phycobiliproteins from cyanobacteria and red algae. *Spirulina*, stand out in this context by presenting biomass with excellent nutritional characteristics. Besides the high protein content, containing all the essential amino acids (Shi et al. 2009) there are several compounds and natural pigments with functional properties. Among the phycobiliproteins (PBP) derived from *Spirulina*, phycoerythrin (PE) and allophycocyanin (AP) can be found in small amounts, but the most abundant is phycocyanin (PC), a brilliant blue pigment that, depending on its purity, finds different important applications (Gualtieri and Barsanti, 2006).

Among the PBP's, phycocyanin has been the subject of active researches because of its potential for application as a natural pigment (food colorant, cosmetic) (Eriksen, 2008), having therapeutic properties including anti-inflammatory, neuroprotective, antioxidant (Romay et al. 2003), anti-cancer activities and its fluorescence properties (Kronick, 1986). Phycocyanin, has recently drawn attention in biotechnological aspects due to its fluorescence specific-properties and physiological functions and is used as fluorescent reagent for clinical diagnosis and immunochemistry (Sio et al, 2013 and Romay et al. 2003). In addition, phycocyanin has significant antioxidative, anti-inflammatory, and radical scavenging properties (Romay et al. 1988). Thus, it is a potential therapeutic or protective agent for the treatment of oxidative stress-induced diseases. However the dependence on light by *Spirulina* as an energy source, is a key factor to enhance biosynthesis of phycocyanin by manipulating photon flux densities to the culture.

Therefore, the aim of this research study is on the provision of different photon flux densities, to determine the photon flux density for *Spirulina platensis* towards increasing phycocyanin production by using the modified Zarrouk's medium, which will consist of substitute and modified ingredients to enhance massive production even for poor subsistence farmers

Materials and Methods

The experiment was conducted in a laboratory at the Central and Northern Arava Research station, Yair (Hazeva) under controlled growth conditions. *Spirulina platensis* was grown for 11 days at four different photon flux densities (20 $\mu\text{mol photons m}^2/\text{s}$, 150 $\mu\text{mol photons m}^2/\text{s}$, 300 $\mu\text{mol photons m}^2/\text{s}$ and 600 $\mu\text{mol photons m}^2/\text{s}$) and samples were taken daily for biomass, chlorophyll, phycocyanin, nutrients and protein determination.

Spirulina platensis cultivation

Spirulina platensis was obtained from the culture collection of the microalgae laboratory at the R&D station. *S. platensis* stock cultures were grown in Zarrouk medium at 25-28°C with aeration using CO₂ enriched air flow at 0.5 vvm (gas volume/medium volume/minute) under 16-hour light/8-hour dark light cycle. In preparation of the experiment an

initial 18 L culture was grown in polyethylene bags with 17 L minimal medium (Table 1) and 1 L inoculum of a stock culture for 7 days. Growth conditions were a 16-hour light/8-hour dark photoperiod, 25-28°C, at an irradiance of 150±10 $\mu\text{mol photons m}^2/\text{s}$ provided through a vertical light bank containing 16 bulbs of 54W white fluorescent light (T5 HO 54W/865 CDL, Electra) and continuous agitation was induced by insufflation of a CO₂ enriched air flow (aeration flow of 0.5vvm). Daily samples of 50 ml were extracted from the culture to determine growth rate. The culture bag was harvested during mid-end exponential growth phase. Before harvesting the biomass and nutrients of this cultures were measured and then cells were harvested on a 22 μm mesh and washed well with distilled water. The concentrated wet biomass was collected from the mesh and an equal amount, determined by weight, was used as an inoculum for each treatment of the experiment.

In total 12 polyethylene bags with 1.4 L minimal medium each were prepared. The bags were positioned 25 cm from the vertical light bank and grown under the same growth conditions mentioned above except for the light.

Experimental design

Cultures was grown under 4 different irradiances (Table 2), with three replications per treatment. The irradiance was measured on the bag surface using a LI-250A light meter (Nebraska, USA) and a Li-Cor quantum sensor. A control treatment of 150 (B) $\mu\text{mol photons m}^2/\text{s}$, a low light treatment of 20 (A) $\mu\text{mol photons m}^2/\text{s}$ using black shade net and black material on the surface of the light bank, and two high light treatments of 300 (C) and 600 (D) $\mu\text{mol photons m}^2/\text{s}$ using additional 54W white fluorescent bulbs (17W Daylight E27 150mA PF=0.5, 220-240V 50/60Hz, Midea), positioned 10 cm in front the culture was set up.

Over a 11 day period samples were taken every 24 hours from each of the 12 bags in order to determine biomass, nutrients, protein, chlorophyll and phycocyanin concentrations. A 50 mL of the cultures was used to analyze the biomass and nutrients on the same day, whereas samples for chlorophyll and protein (in total 10 mL) and for phycocyanin (40 mL) were shortly treated according to the protocols (see below) and then stored at 4°C and -20°C, respectively until final analysis.

Table 1. Composition of minimal medium for cultivation of *S. platensis*.

Chemical	Chemical Composition	Quantities for 1 L of medium (g/L)	Quantities for 17 L of medium (g/L)
Sodium Chloride	NaCl	1.0	17.0
di-Potassium hydrogen phosphate	K ₂ HPO ₄	0.1	1.7
Iron Sulphate Heptahydrate	FeSO ₄ * 7H ₂ O	0.04	0.68
Magnesium Sulphate Heptahydrate	MgSO ₄ * 7H ₂ O	0.1	1.7
Potassium Nitrate	KNO ₃	1.0	17.0
Sea Salt	NaCl and natural trace elements	1.0	17.0
Sodium Hydrogen Carbonate	NaHCO ₃	2.0	34.0

Table 2. Summary of the four (4) irradiance treatments (A, B, C, D)

Treatment	Light intensities ($\mu\text{mol photons m}^2/\text{s}$)	Light source/condition	Distance between culture bags and light source (cm)
A	20	Black shade net covering light bank	25
B	150	Light bank	25
C	300	Light bank + 3 additional bulbs	25 and 10
D	600	Light bank + 6 additional bulbs	25 and 10

Parameter Measurement Determination

Biomass

For total biomass determination 50 mL from each individual culture was used. The samples were centrifuged at 4000 rpm for 10 minutes at $21 \pm 2^\circ\text{C}$, and the supernatant was then transferred to a new tube and used for nutrient analysis. The algae pellet was washed twice with DDW and resolved in 50 mL DDW. The algae were then filtered onto a GF-F filter (diameter 4.5 cm, Satorius) using a vacuum pump at 1.2 bars suction pressure (Model: MP3 2R 24 VAC 9W IP54), microwaved for 4 minutes on medium heat and left to cool before weighing with an analytical balance (BSM - 220.4). Before use the GF-F filters were prepared by rinsing with DDW and drying in a microwave. The weight of the empty, dry filter was measured. The following equation was then used to determine the Biomass (BM) (mg/mL) per daily sample:

$$\text{BM} = \frac{(\text{DFW} + \text{C}) - (\text{DFW})}{\text{C}} \times 1000$$

DFW: Dry filter weight (g)

C : 50 mL culture

The average biomass of the 3 replicates for each sample under the 4 different irradiance treatments was used to construct growth curves exploring algae growth (mg/mL) over time (days).

Nutrient analysis

Nutrient analysis was conducted using the supernatant obtained from the biomass samples. Nutrient test kits (Reflectoquant) were used to analyze concentrations of phosphate (Analytical Test Kits, 2012), nitrite (Analytical Test Kits, 2013), nitrate (Analytical Test Kits, 2016) and ammonium (Analytical Test Kits, 2018) in the samples. The nutrient concentrations was analyzed using 5 mL samples with the Analytical Test Kit protocol through a reflectometric method, with test strips and a reagent provided specific to each test. Daily checks of potential hydrogen (pH), temperature and salinity of the sample were conducted (Eutech PC TESTr 35-01X441504 / Oakton 35425-00). The nutrient concentration values were recorded daily, and used to explore how the concentration changed over time during culture growth.

Chlorophyll Extraction and Analysis

For extraction of chlorophyll, 10 mL of culture was harvested in 15 mL tubes each day and centrifuged at 4000 rpm for 6 minutes at 21°C . The supernatant was discarded and the remaining algae pellet was washed with DDW to remove excess chemicals. The algae pellet was then dissolved in 10 mL methanol (CH_3OH) and kept in the dark at 4°C until analysis. At the end of the experiment, all samples were analyzed for chlorophyll concentration. Therefore, the tubes were centrifuged at 4000 rpm for 5 minutes at 21°C . The supernatant was removed and a volume of 200 μL from each supernatant was transferred and stored in an Eppendorf reaction tube for further analysis. Another 200 μL sample was placed directly into a 96 well plate. This was inserted into a spectrophotometer plate reader (UV-Vis Spectrophotometer: Gen5™ Data Analysis Software; Optional Gen5 Secure for 21 CFR Part 11 compliance) and the absorbance of the samples at 665 nm wavelength was measured. The optical density

readings obtained from the spectrophotometer were then used to calculate the chlorophyll content using the formula:

$\text{Chl in mg/mL} = \text{OD } 665 \text{ nm} \times 13.9$ (coefficient factor specific for *Spirulina* after Vonshak (1997a).

The algae pellets from each sample were kept and dried overnight under a chemical hood at room temperature and used for the protein content determination Vonshak (1997b).

Protein Extraction and Analysis

Total protein content for each sample was determined using the Bradford protein assay protocol (Bradford, 1976). Firstly, the dried algae pellets obtained from the chlorophyll extractions were dissolved in 4 mL of 0.5 M NaOH buffer and protein was denatured at 100°C . The tubes were centrifuged at 4000 rpm for 5 minutes at 21°C . A sample of diluted 50 μL (1:10, 5 μL sample :45 μL DDW) was pipetted into a 96 well plate and mixed with 150 μL of 1:4 diluted Bradford reagent. A serial dilution of known bovine serum albumin (BSA) concentrations were added to the plate and was used for creating a standard curve. The 96 well plate was then inserted into a spectrophotometer and absorbance was read at 595 nm. The optical density values were related to the BSA standard curve, and used to calculate total protein (mg/mL).

Phycocyanin Extraction

For phycocyanin extraction, 40 mL of the cultures were extracted daily from each of the 12 bags. The samples were centrifuged at 4000 rpm for 10 minutes at 21°C . The supernatant was discarded, and the wet biomass transferred evenly into 2 Eppendorf tubes. The tubes were spun at 6000 rpm for 2 minutes at 21°C . The supernatant was removed and the remaining wet biomass was then stored at -20°C until analysis. For phycocyanin concentration analysis 1 mL of phosphate buffer (88mL 0.2M NaH_2PO_4 , 12mL 0.2M Na_2HPO_4 and 100mL DDW) was added to each tube and the frozen wet biomass was defrosted. The cells were broken using a bullet blender (Bullet blender 24, Next-Advance) and beads at 150 rpm three times. The resulting cell solution was centrifuged at 6000 rpm for 2 minutes at 21°C . A 200 μL sample of the supernatant was then pipetted into a 96 well plate. The 96 well plate was then inserted into the spectrophotometer and absorbance was measured at 615 nm and 652 nm. The optical density readings were converted to phycocyanin concentration using the equation by Bennett and Bogorad (1973):

$$\text{Phycocyanin (mg/mL)} = [A_{615} - 0.474 (A_{652})] / 5.34$$

Statistical Analysis

Statistical package Genstat (version 18) was used to generate a one-way Analysis of Variance (ANOVA) analyzing the influence of different photon flux densities (20, 150, 300 and 600 $\mu\text{mol photons m}^2/\text{s}$), on the growth rate of *S. platensis*, and the production of phycocyanin. Cultures that were cultivated at irradiance of 150 $\mu\text{mol photons m}^2/\text{s}$ served as the control group. Three replicates were conducted at all 4 irradiance treatments, and were used in statistical analysis. A post hoc test Tukey's HSD (Honestly significant difference) was used to compare biomass and phycocyanin contents between irradiance treatments with a probability value of $P < 0.05$ considered significant.

Results

Biomass

In the experiment, cyanobacteria *Spirulina platensis* were cultured using various light intensities that is (20, 150, 300 and 600 $\mu\text{mol photons m}^2/\text{s}$). The initial biomass concentration in all experimental treatments was 0.59 ± 0.14 mg/mL. As shown in Figure 1, the highest biomass concentration was obtained in the culture with, 600 $\mu\text{mol photons m}^2/\text{s}$ light intensity and it amounted to 2.15 ± 0.53 g mg/mL. In terms of biomass concentration, the culture using 300 $\mu\text{mol photons m}^2/\text{s}$ lighting appeared to be the second highest with a value of 1.83 ± 0.083 mg/mL. The lowest biomass concentration of 0.85 ± 0.032 mg/mL was noted in the culture lit by 20 $\mu\text{mol photons m}^2/\text{s}$. The experiment results are consistent with a study by Lee et al. (2012).

Phycocyanin content

The influence of light intensity was investigated by incubating the cultures at different light intensities (20 $\mu\text{mol photons m}^2/\text{s}$, 150 $\mu\text{mol photons m}^2/\text{s}$, 300 $\mu\text{mol photons m}^2/\text{s}$ and 600 $\mu\text{mol photons m}^2/\text{s}$) for an eleven-day duration. Light intensities were adjusted to desired levels

by changing the distance/orientation/angle of culture bags from the light source and/or by changing the number/power of fluorescent tubes/bag. The other culture conditions (e.g. temperature, light- dark period and pH) were kept optimally constant.

Results of the effect of light intensity on phycocyanin content of *S. platensis* grown on minimum medium are plotted in Figure 2 and Table 1. There was significant ($P(0.049) < 0.05$), differences between the four different light treatments. The phycocyanin content decreased with increasing light intensity. The highest phycocyanin content was found at a light intensity of 20 $\mu\text{mol photons m}^2/\text{s}$. At light intensities of 300 $\mu\text{mol photons m}^2/\text{s}$ and 600 $\mu\text{mol photons m}^2/\text{s}$, the phycocyanin content decreased respectively to approximately 2.2 % and 1.7 %, from the initial phycocyanin content from the culture bag which had 8.7% and 6.6% phycocyanin content respectively. It is clear that light has an important role in affecting phycocyanin formation since phycocyanin is an important photosynthetic accessory pigment (Bogorad, 1975).

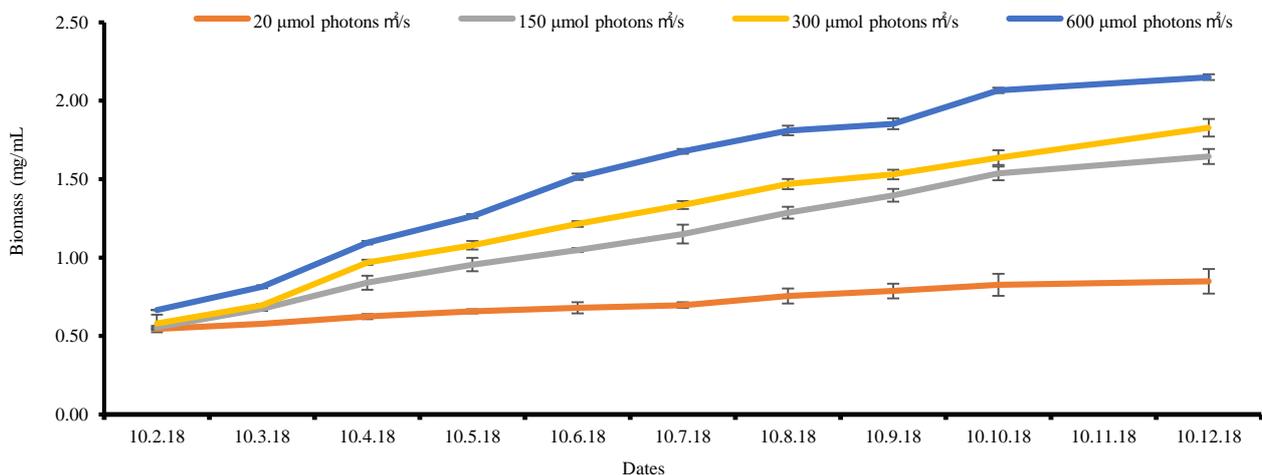


Figure 1. Growth rate (mean \pm standard error) of *Spirulina platensis* cultivated under different photon flux densities during a 11day incubation period, represented as dry weight (mg/mL), with measurements calculated from three technical replicates

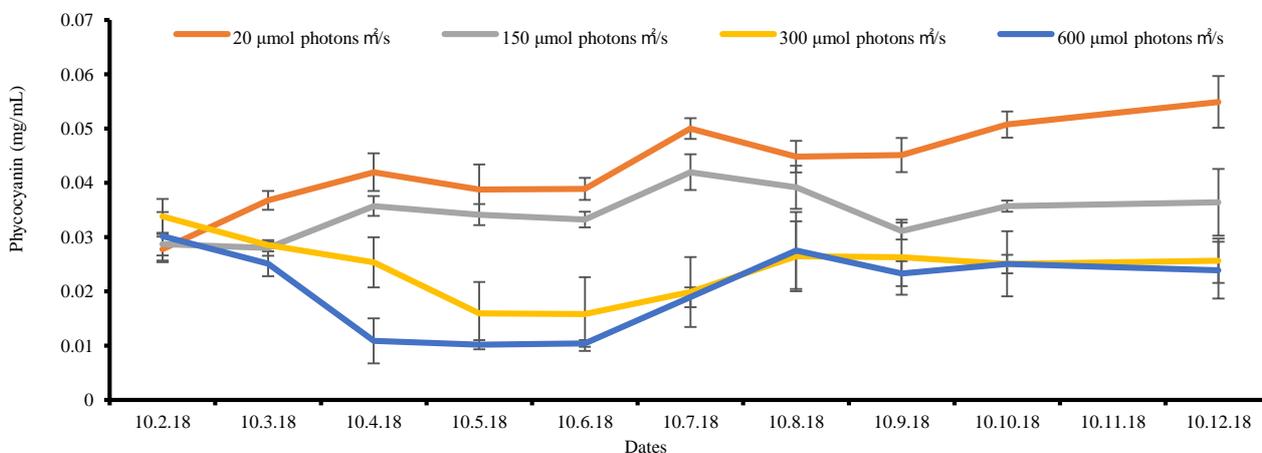


Figure 2. Phycocyanin yield (mean \pm standard error) under four different photon flux densities Yield was expressed as (mg/mL) phycocyanin per (mg/mL) dry biomass

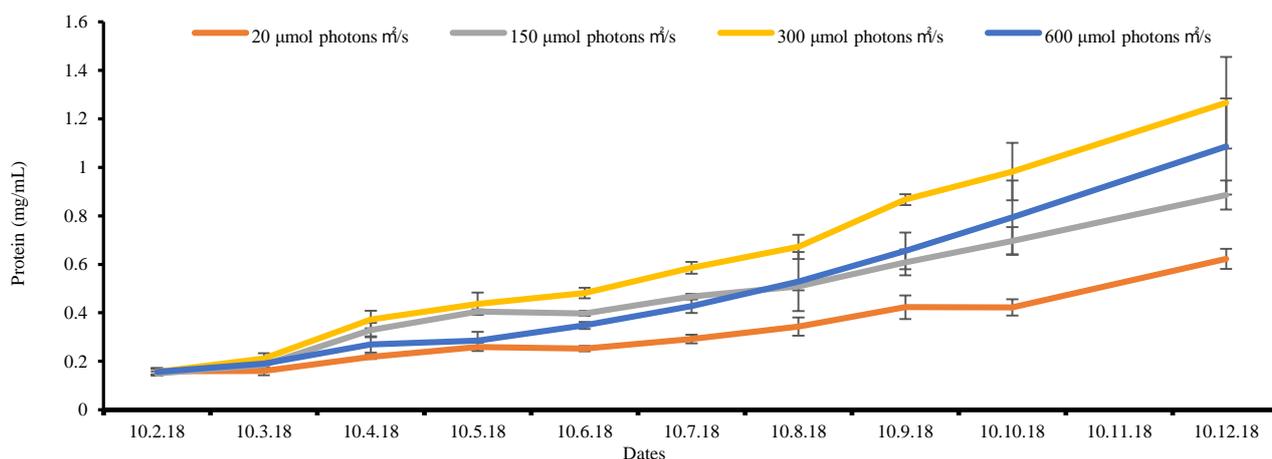


Figure 3. Effect of different photon flux densities on the protein content of *Spirulina platensis*. Error bars indicate (mean \pm standard error)

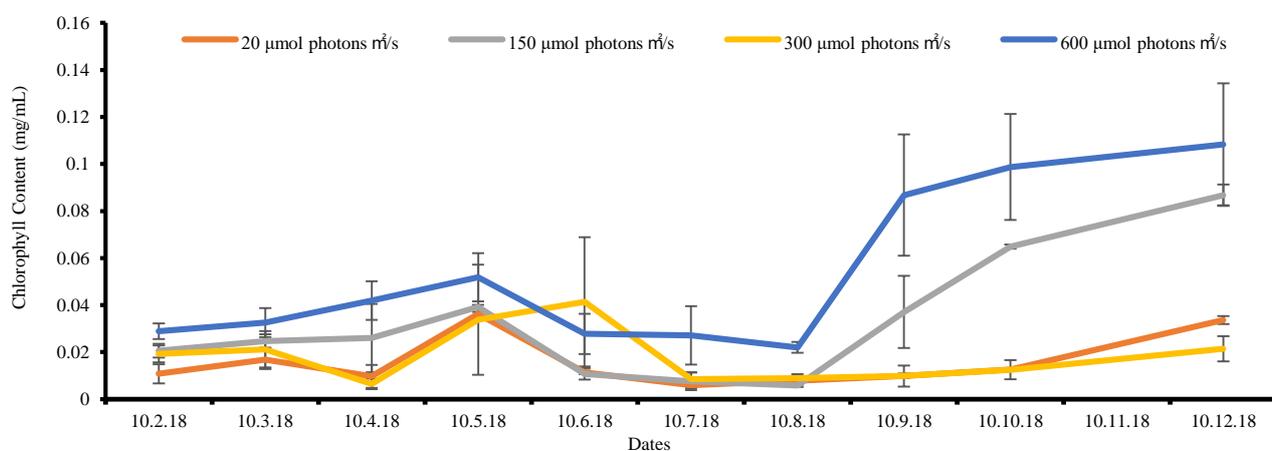


Figure 4. The chlorophyll content (mg/mL) of *Spirulina platensis* under four different photon flux. Error bars indicate (mean \pm standard error)

Protein Content

Total protein content after 11 days of growth under all conditions studied showed statistically significant difference ($P(0.01) < 0.05$) (Figure 3). According to (Belay et al. 2008) *Spirulina platensis* has a high protein content, equivalent to 60-70% of the total mass. The values found in the present study ranged from 20% to 75% under the conditions evaluated.

Chlorophyll Content

The chlorophyll content of *S. platensis* increased over time in all runs for the different light intensities that were administered under this study. It is known that light penetration decreases as the cell density of the cultures increases due to self-shading, and that chlorophyll production increases as the cell density rises, so that the photosynthetic efficiency of the algae would be improved (Rodrigues et al., 2011). Thus, the increase of the biomass production and consequently the increase of cell density causes a chlorophyll increase over time. Although the chlorophyll content in cultures under 300 $\mu\text{mol photons m}^{-2}/\text{s}$ light intensity was lower than in cultures with 150 $\mu\text{mol photons m}^{-2}/\text{s}$, the chlorophyll produced was significantly higher in the cultures with the high light intensity. It seems that higher light intensity results to higher cell density, which intensifies the self-shading effect. Therefore, it was assumed that the chlorophyll

production is regulated by the combination of light intensity, cell concentration and culture age. This, in turn suggests that more chlorophyll could be produced even in higher light intensities due to the higher biomass production. Rodrigues et al. (2011) obtained also a high chlorophyll content (up to 2.28 %) using 156 $\mu\text{mol photons m}^{-2}/\text{s}$ light intensity. However, Danesi et al. (2004) reported an increase of up to 29% in total chlorophyll production, when the light intensity of the culture decreased at some point from 60 to 24 $\mu\text{mol photons m}^{-2}/\text{s}$.

Discussion

The experiment results in the four different photon flux densities to which the cyanobacteria cultivations were administered, in terms of maximum cell concentration, chlorophyll, protein and total phycocyanin content are expressed and outlined in the above chapter. Maximum growth of *Spirulina platensis* occurred with the use light intensity of 600 $\mu\text{mol photons m}^{-2}/\text{s}$, at 26°C, where 2.15mg/mL of cell concentration was achieved, and minimum occurred with the use of light intensity of 20 $\mu\text{mol photons m}^{-2}/\text{s}$, achieving 0.83 mg/mL. In the intermediate light intensity (150 $\mu\text{mol photons m}^{-2}/\text{s}$), the maximum cell concentration was about 1.65 mg/mL.

The influence of illumination on cell growth was evident showing a clear relation between the cell

concentration and the light intensity (Jensen, 1993). In fact, once the nutrient and temperature requirements have been satisfied, so that these do not limit the growth, the light intensity and its duration define the growth rate and the production yield. The cell concentration interferes in this phenomenon, as there are few cells per unit of volume, at the beginning of cultivation; each cell receives a quantity of light energy higher than the minimum required for the photosynthesis (Samuelsson et al., 1986). Thus, an increase in cell concentration occurs, as the photosynthesis rate is higher than the one of respiration. At the end of cultivation, due to the mutual shadow, a reduction in energy provided to the cell occurs. This can be explained for the cell growth by the so-called photosynthetic compensation point (Abdulqader et al., 2000).

The chlorophyll contents obtained confirmed the influence of different photon flux densities on the content of this pigment. In lower light intensities, higher contents occurred, as the cells need to optimize capture of small amount of available light. However, these levels were achieved due to a very low growth, and the total chlorophyll was less than the ones obtained in high light intensities (600 $\mu\text{mol photons m}^2/\text{s}$). Under intermediate light intensities, at 150 $\mu\text{mol photons m}^2/\text{s}$, the chlorophyll contents in *Spirulina platensis* were close to the ones indicated in literature (Belay, 2008)

Cell productivity was influenced in an evident manner by the illumination, where, in greater light intensities, higher values were detected. On the other hand, in regard to chlorophyll productivity, higher productivities were identified in intermediate values of light intensity, this means, in the central point, at 300 $\mu\text{mol photons m}^2/\text{s}$, in accordance with the results of Aldea et al. (2002) and indicated by Kebede and Ahlgren, 1996). In fact, the maximum total chlorophyll productions occur in intermediate light intensity levels, in which the gain in investment in new chlorophyll equilibrates cost of synthesis.

The highest protein content was observed in photon flux density of 300 $\mu\text{mol photons m}^2/\text{s}$ of nearly 60%. These results are in accordance with Ballot et al. (2009), who stated that *S. platensis* contains 60-70% protein and about 50, 000 kg of protein per hectare could be produced annually. Oliveira et al. (2009), observed the accumulation of protein in cultivations under different light intensities, and the protein content in *S. platensis* was 64.35%, which is in the same range of the values found in the present study (Figure 3).

Light intensity of 25 $\mu\text{mol photons m}^2/\text{s}$ (=1850 lux) has been reported to be optimal for phycocyanin production in cyanobacteria *Synechococcus* NKBG 042902 (Altschul et al., 1990 and Amato et al., 1997), *Spirulina subsalsa* and *S. maxima* (Tomaselli et al., 1997), *Synechocystis* (Khatoun et al., 2018)) and *Anabaena* NCCU-9 (Hemlata and Fatma, 2009), of which these results are in agreement with the findings of this research paper. Cyanobacteria are known to prefer low light intensities and stimulate phycobiliprotein synthesis because of their low specific maintenance energy and pigment composition (We et al., 2005; Grossman et al., 1993). For all photosynthetic organisms, including cyanobacteria, light (primary energy source) is an essential factor or requirement for growth and development, and the intensity, quality and duration of light plays a critical role in

the growth and physiology of cyanobacteria, enabling them to carry out all the necessary metabolic processes.

The antenna sizes of both PS I and PS II are larger in cyanobacteria growing in low light, (Habib et al., 2008). PS II antenna size is affected to a greater extent. This has been independently shown by, Cevallos et al. (2008) and Diraman et al. (2009) for higher plants. In the case of cyanobacteria, the size of Phycobilisomes (antenna for PS II) increases under low light irradiance (Bao et al., 2012), and. This possibly give the reason why under low light intensities there was a high phycocyanin content and percentage per cell. Richmond (1992) and Rafiqul et al. (2004) reported that an increase in light intensity causes an increase in the concentration of gas vesicles and trichome length and ultimately a decrease in phycobiliprotein.

Conclusion

Three key findings are presented in this work. First, a noticeable difference between the optimal light intensity for cell growth (600 $\mu\text{mol photons m}^2/\text{s}$) and phycocyanin synthesis (20 $\mu\text{mol photons m}^2/\text{s}$) is identified. Second, high light intensity is demonstrated to be the primary factor causing the decrease of intracellular phycocyanin content, while it has also significant effect on total protein and chlorophyll production. Finally, although high light photon flux densities can enhance cell growth, it is demonstrated to suppress intracellular phycocyanin accumulation in a long-term operation.

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