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EFFECT OF LIGHT INTENSITY ON THE PRODUCTION OF PIGMENTS IN *NOSTOC* SPP.

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ABSTRACT: *Cyanobacteria, especially the Nostoc and Spirulina genera, have been used for centuries as food, and currently their biotechnological potential is notable for the presence of several compounds relevant to the market, such as pigments and antioxidants. Cyanobacteria are appealing because of the increasing demand for natural pigments and antioxidants over the synthetic ones. Because cyanobacteria are photosynthetic organisms, one of the factors that most influences their metabolism is the level of incident light. However, the relationship between light intensity and the synthesis of bioactive compounds is not well understood in all species. Therefore, the present study aimed to determine the influence of different light intensities on the yield of biomass and pigments present in Nostoc spp. isolates. Lower intensities were more advantageous in terms of the yield of phycobiliproteins and chlorophyll a. For the isolates examined in the present study, the content of light-absorbing pigments, such as chlorophyll a and phycobiliproteins, was higher when the light availability was low. When the light availability increased, the content of these pigments decreased as a strategy for prevention of photo-oxidative damage caused by the production of free radicals. However, with respect to carotenoids, after the content of light-absorbing pigments decreased, it increased again at higher irradiances, which reflects the function of these pigments as dissipaters of excess absorbed light energy and as antioxidants in the photosynthetic apparatus.*

KEYWORDS: Cyanobacteria, Pigments, Carotenoids, Phycobiliproteins, Chlorophyll.

INTRODUCTION

Cyanobacteria exhibit significant biotechnological potential. Cyanobacteria are currently produced in closed photo-bioreactors and in open tanks to be used as food and for the isolation of many products, such as drugs, fluorescent markers, biological pigments, enzymes, antioxidants and exopolysaccharides for use as gelling agents, emulsifiers, flocculants and moisturizers (Otero and Vincenzini, 2003; Tokusoglu and Unal, 2003; Cepoi et al., 2009). Cyanobacteria also have the ability to store reserve materials that are sources of lipids, proteins, vitamins and minerals (Rastogi and Sinha, 2009).

The pigments contained in the cells of cyanobacteria are currently used by the food, cosmetic and pharmaceutical industries. These pigments are added to other products, such as colorants or antioxidants, or used alone as drugs. In addition to carotenoids and chlorophyll, cyanobacteria possess another group of pigments not expressed in plants, the phycobiliproteins. These pigments include the blue pigment phycocyanin and the red pigment phycoerythrin. Additionally, these pigments represent the major constituents of red algae and cyanobacteria and may represent up to 60% of the total protein content of the cell (Viskari and Colyer, 2003).

Currently, the therapeutic effects of phycobiliproteins are well explored, including their antiplatelet, immunomodulatory (Jensen et al., 2001), antidiabetogenic (Soni et al., 2009), anti-hepatotoxic (Gallardo-Casas et al., 2010; Jensen et al., 2001; Chiu et al., 2006; Soni et al., 2009), anti-inflammatory and anti-carcinogenic effects, their use in the treatment of Parkinson's disease and Alzheimer's disease (Rimbau et al., 2001) and their effect in reversing the multiple drug resistance phenotype of several types of tumor cells (Morlière et al., 1998; Rodríguez et al., 2006). At least 11 major companies in the world are involved in the production and sale of phycobiliproteins (Sekar and Chandramohan, 2008). Carotenoids, on the other hand, have physiological functions in addition to their use as dyes. Carotenoids exhibit pro-vitamin A activity in addition to strengthening the immune system and reducing the risk of degenerative diseases, such as cancer, cardiovascular diseases, cataract and macular degeneration (Müller et al., 2003).

Obtaining pigments and natural antioxidants is important because of the increase in the current demand of consumers for natural products, combined with the fact that some dyes and synthetic antioxidants may trigger allergic and carcinogenic processes (Botterweck et al., 2000).

Nostoc is a genus of filamentous cyanobacteria that form macro- and microscopic colonies. *Nostoc* is common in both aquatic and terrestrial environments (Dodds et al., 1995). In China, *Nostoc* has great economic value and has been used as food for nearly two thousand years. The therapeutic and functional effects of *Nostoc* and its importance for biotechnology are increasingly recognized. Thus, China has been implementing strategic programs for *Nostoc* production due to the increased market demand and the concurrent decline of sources. However, almost all of the research articles on *Nostoc* have been published in Mandarin, so they are not well known outside China (Gao, 1998).

The distribution of the pigments in the different species and strains of cyanobacteria is extremely diverse, as is their production response to different variables. The luminous intensity is the main factor that can influence the composition of pigments and other biomolecules in cyanobacteria (Porter et al., 1978). Thus, the present study aimed to analyze the influence of light intensity on the production of pigments by two *Nostoc* sp. isolates.

MATERIAL AND METHODS

Culture conditions

Two isolates of edaphic algae from the genus *Nostoc*, namely *Nostoc* sp. F108 and *Nostoc* sp. F105, were selected from the Cyanobacteria Collection of the Laboratory of Physiology of Microorganisms of the Federal University of Viçosa (Universidade Federal de Viçosa).

The species were cultured at different light intensities from 10 to 150 µmoles of photons / m^2 / s. They were cultured in a semi-discontinuous process with agitation (110 rpm) and constant illumination in a liquid BG11 medium (Allen and Stanier, 1968, modified by Pontes, 1988), with $NaNO_3$ (1.5 g / L medium) as a nitrogen source.

The cells, which were obtained from stock cultures in BG11 solid medium, were initially activated for 7 days in BG11 medium. After the incubation period, the biomass was centrifuged, rinsed with sterile saline, transferred to BG11 broth in a 2% wet weight proportion (m / v) and incubated for 7 days at different light intensities under agitation (110 rpm) at room temperature.

To prevent cell aggregation at the center of the container, which is favored by rotational agitation, the aggregates were broken every day by exposing them to flows produced with a sterile syringe. After obtaining the biomass, the analyses of pigments and antioxidants were performed in triplicate.

Dry weight determination

The dry weight was determined according to Fiore et al. (2000). A 2-mL aliquot of the cell suspension was centrifuged at 10,000 *x* g, washed twice in sterile saline (0.85% NaCl), resuspended in the same volume of saline, transferred to aluminum crucibles previously dried at 105°C for 72 hours and weighed.

Carotenoids and chlorophyll a extraction and quantification

Carotenoids and chlorophyll *a* were extracted according to the procedure described by Morales et al. (2002). A 1-mL aliquot of wet biomass was centrifuged. The sediment was resuspended in the same initial volume of 100% methanol and kept in an amber container in the dark for 24 hours at 4°C. This process was repeated until the biomass was completely colorless. At the end of the extraction process, the collected supernatants were gathered, and the concentration of chlorophyll *a* and carotenoids was measured at 665 nm and 480 nm, respectively. The chlorophyll *a* content was determined according to Marker (1972), and the carotenoid content was determined according to Britton (1985).

Extraction and quantification of phycobiliproteins

A 5-ml aliquot of the culture was centrifuged at 10,000 *x* g for 15 minutes at 20°C. The biomass was then washed twice by resuspension in 20 mM sodium acetate buffer pH 5.5, subsequently discarding the supernatant. The final pellet was resuspended again in 5 mL of the same buffer (Tandeau de Marsac and Houmard, 1988). To break the cells, the suspension was subjected to a French Press® device, applying an 800-atm pressure under a flow of 3 to 4 drops per second.

In 1.9 mL of the extracts from broken cells, 100 µL of streptomycin sulfate solution (200 mg / mL) was added, and the solution was kept at 4 °C for 30 minutes. After this period, the solution was centrifuged at 18,000 *x* g for 10 minutes, and the supernatant was subsequently supplemented with 400 µL of 1 mM dithiothreitol solution.

The concentrations of phycocyanin (PC), allophycocyanin (Ap) and phycoerythrin (Pe) were obtained from absorbance data of each sample (640 to 565, 620 and 650 nm) (Tandeau de Marsac and Houmard, 1988).

Statistical analysis
The pigments' values in the different light intensities were compared by analysis of variance (ANOVA). Tukey's test was used at a significance level of 5%.

RESULTS
Among the cyanobacteria studied, *Nostoc* sp. F105 produced more biomass than *Nostoc* sp. F108 (Figure 1).

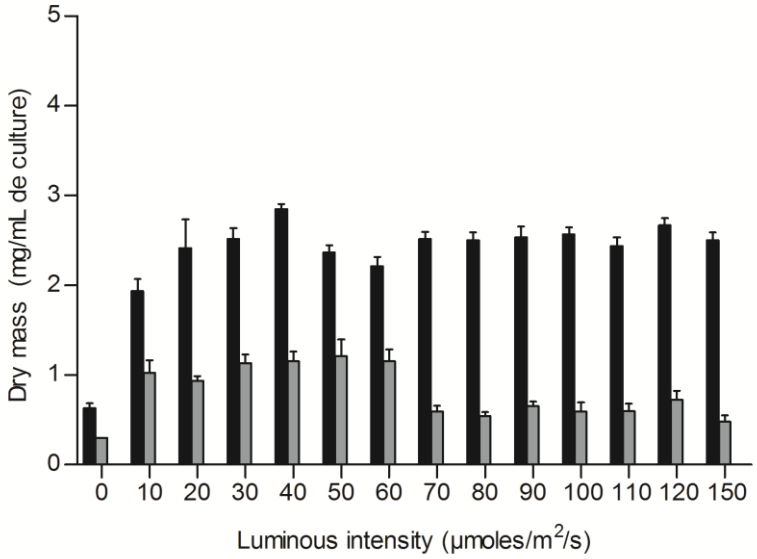


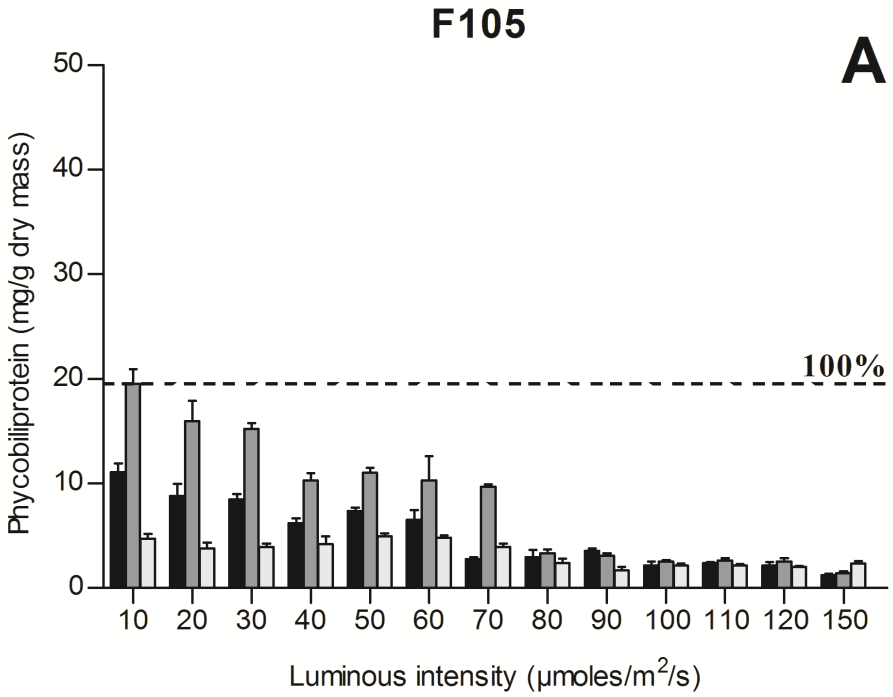
Figure 1: Dry mass per mL of culture for *Nostoc* sp F105 and F108 under different light intensities. The cultures were given under constant agitation of 110 rpm for seven days.

Comparison of the treatments with light intensities ranging from 10 to 150 µmoles / m^2 / s revealed that the production of biomass by *Nostoc* sp. F105 was greater than 10 µmoles/ m^2 / s and lower than 40 µmoles / m^2 / s. The production of biomass did not change between the other light intensities within this range (Figure 1).

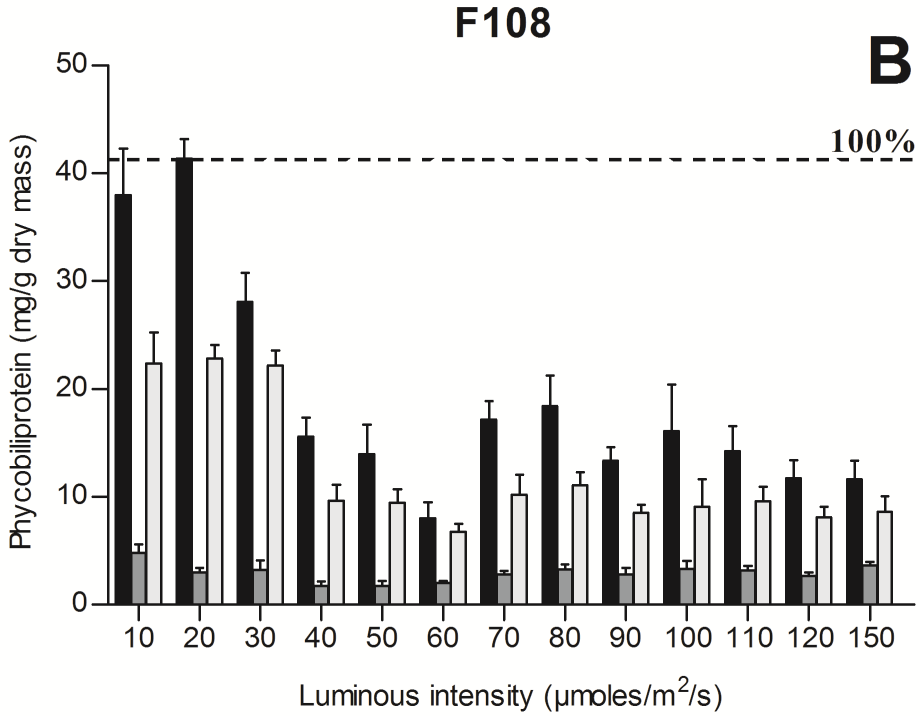
Comparison of the treatments with light intensities ranging from 10 to 150 µmoles / m^2 / s revealed that the production of biomass by *Nostoc* sp. F108 was greater than 50 µmoles / m^2 / s and lower than 150 µmoles / m^2 / s. For this cyanobacterium, a different trend was observed when it was compared to *Nostoc* sp. F105. The biomass production was lower when the light intensity was between 70 and 150 µmoles / m^2 / s and higher between 10 and 60 µmoles / m^2 / s (Figure 1). These findings demonstrate that the light intensity does not exert a direct influence on the production of biomass by *Nostoc* sp. F105. These results are in contrast to those of *Nostoc* sp. F108, for which the best intensity for biomass production was 50 µmoles / m^2 / s.

A decrease in the production of phycobiliproteins was observed when the cyanobacteria were cultured with increasing light intensities (Figure 2 and 3).

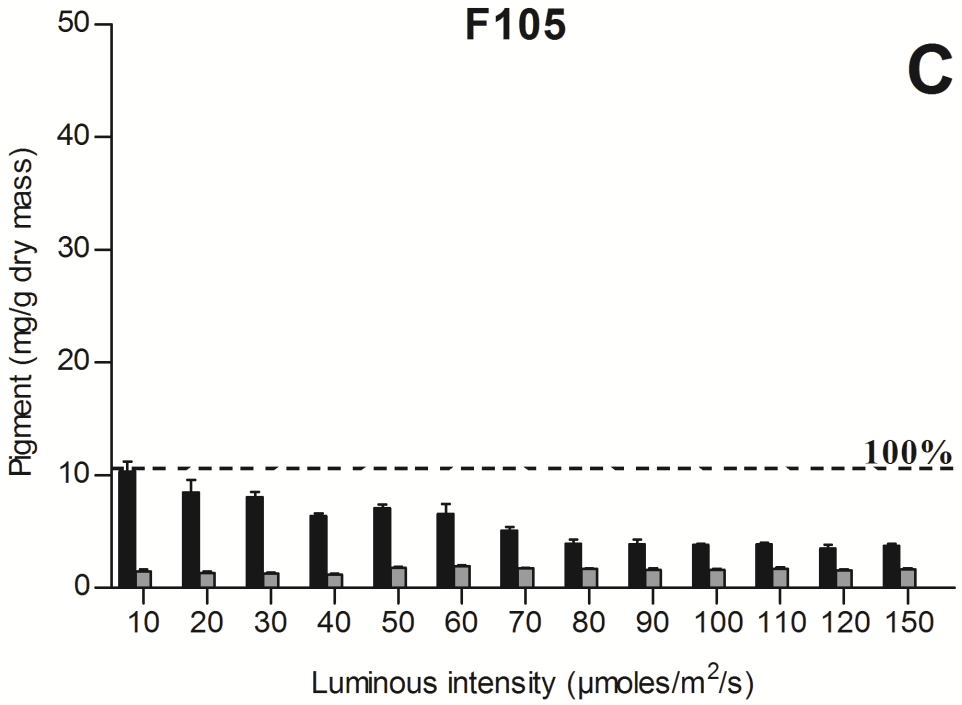
Figure 2: Content of Pigments for *Nostoc* sp F108 and 105, under different light intensities. The cultures were given under constant agitation of 110 rpm for seven days. Pc: Phycocyanin, Pe: Phycoerythrin, Ap: allophycocyanin.



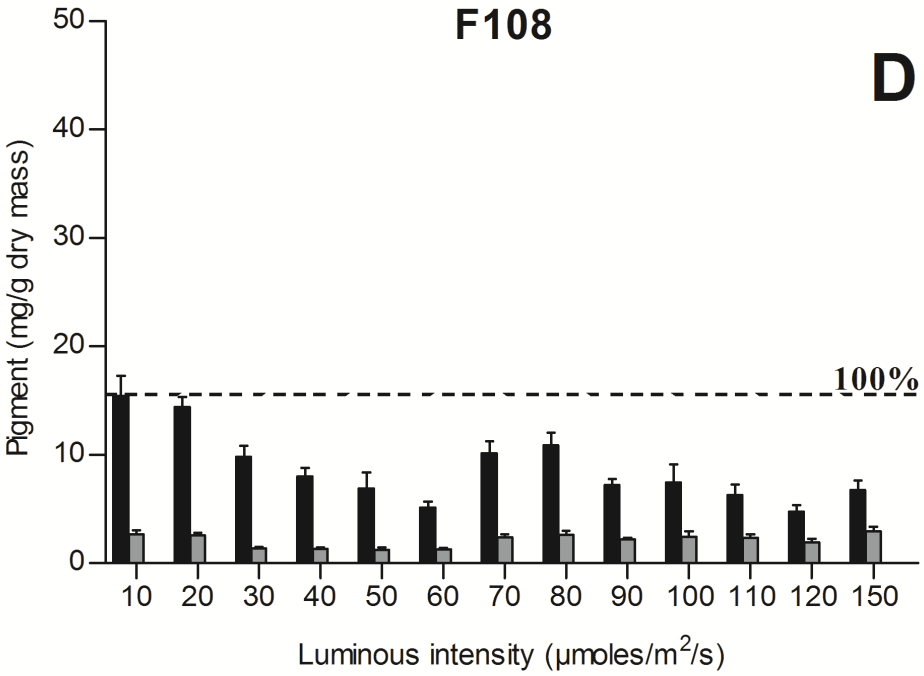
A – Content of Phycobiliproteins for *Nostoc* sp F108 under different light intensities.



B - Content of phycobiliproteins to *Nostoc* sp F105 under different light intensities.



C - Content of chlorophyll a to *Nostoc* sp F105 and F108 under different light intensities.



D - Content of carotenoids for *Nostoc* sp F105 and F108 under different light intensities.

For both cyanobacteria, the production of phycobiliproteins was higher between 10 and 30 µmoles / m^2 / s of light intensity. For *Nostoc* sp. F105, there was a progressive decrease in the production of phycobiliproteins from 30 µmoles / m^2 / s. From 80 µmoles / m^2 / s, no change

in the production of these pigments was observed. Regarding *Nostoc* sp. F108, there was a drastic reduction in the production of this pigment from 40 μmoles / m^2 / s.
When the phycobiliprotein types were analyzed, higher production of Pe and PC was observed in *Nostoc* sp. F105 and *Nostoc* sp. F108, respectively (Figure 2 and 3). Additionally, *Nostoc* sp. F108 produced more Ap per gram of dry weight (Figure 3). For *Nostoc* sp. F105, progressive decreases in the levels of Pe and PC were observed with the increase in light intensity (Figure 2). For *Nostoc* sp. F108, the highest value for PC was observed at 20 μmoles / m^2 / s. Between the light intensities of 40 to 150 μmoles / m^2 / s, variations in the content of PC were observed, and the lowest value was obtained at 60 μmoles / m^2 / s (Figure 3). For Ap, the higher values occurred between 10 and 30 μmoles / m^2 / s, and the lowest value was observed at 60 μmoles / m^2 / s.

PC, in particular, was elevated at 10 and 20 μmoles / m^2 / s, with no significant differences, followed by the level at 30 μmoles / m^2 / s intensity. The lowest values were observed at 60, 120 and 150 μmoles / m^2 / s, with no significant differences.

For F108, minor changes in the concentration of the red Pe pigment were observed with variations in light intensity, and the red Pe was always observed in lower concentrations than the blue pigment. This fact can be confirmed by the exclusively bluish color of the phycobiliprotein extracts produced by this isolate. The pigment Ap was the second most important pigment in terms of quantity, and its levels oscillated similarly to PC. At different light intensities, the levels of PC and Ap exhibited directly proportional variation.

When compared to F108, the predominant phycobiliproteins produced by *Nostoc* sp. F105 exhibited an inverse ratio (Figure 2). Pe was the major component for all intensities, with the exception of 100 μmoles / m^2 / s, followed by PC. The treatment of 10 μmoles / m^2 / s resulted in the highest quantity of all phycobiliproteins analyzed.

Regarding the PC / Pe ratio for *Nostoc* sp. F105, a smaller difference in the levels of these two pigments was observed. This peculiarity of combining the two pigments in significant proportions gave an intense pink / purple color to the extracts of phycobiliproteins from *Nostoc* sp. F105.

Figures 4 and 5 show the content of chlorophyll *a* and carotenoids for *Nostoc* spp. F105 and F108 according to the light intensity. These two pigments varied in a relatively similar way according to the light intensity.

For both isolates, the highest chlorophyll *a* value was obtained at 10 μmoles / m^2 / s. The carotenoid content according to the light intensity was similar for both organisms (Figure 5), and the carotenoid content was significantly lower than the chlorophyll *a* content for both microorganisms in all treatments.

A progressive decrease in the chlorophyll *a* content for *Nostoc* sp. F105 (Figure 3) *Nostoc* sp. F108 was between 10 and 80 μmoles / m^2 / s and between 10 and 60 μmoles / m^2 / s, respectively (Figure 4). At higher intensities, several increases and decreases in the chlorophyll *a* content were observed. For chlorophyll *a*, further increases were not sufficient to meet or overcome the higher initial levels of chlorophyll *a* (Figure 4). In contrast, at the majority of light intensities, the increase in the carotenoid content resulted in higher carotenoid levels than the initial levels for the two microorganisms.

DISCUSSION

Depending on the purpose, the intensity resulting in the highest biomass yield should not always be chosen for culture because this intensity may not match the intensity at which the pigment production is optimized.

With respect to light intensity, an interesting analysis can be performed by comparing the dry weight profile (Figure 1) with the carotenoid profile (Figure 5). For *Nostoc* sp. F108, the profiles are clearly opposite. At 70 μmoles / m^2 / s (Figure 1), there was a significant drop in the levels of dry weight, and coincidentally, the first increase in the carotenoid content (Figure 5). Similarly, at 50 μmoles / m^2 / s, the highest value for dry weight and the lowest value for carotenoids were observed. A similar comparison can be performed for *Nostoc* sp. F105, which exhibited a descending carotenoid profile for up to 40 μmoles / m^2 / s (Figure 5) and an ascending profile for dry weight (Figure 1). At this intensity, the cyanobacterium exhibited the highest dry weight yield but the lowest carotenoid value. These data suggest that for these isolates, carotenoids may represent a defensive function against oxidative stress, increasing in cellular concentration exactly when the cell decreases its growth due to higher irradiance.

Several studies have been conducted demonstrating that many cyanobacteria species are characterized by an optimum growth rate at low or intermediate irradiance, emphasizing that these progressive changes are different for each species or strain and must be identified by growth response curves to different intensities. As an example, the following species have been studied: *Spirulina platensis* (Baldía et al., 1991), *Anabaena* PCC7120 (Loreto et al., 2003), *Anabaena* PCC7120, *Pseudanabaena galeata* (Romo, 1994), *Anabaena variabilis* (Yoon et al., 2007).

Regarding the amount of biomass produced, *Nostoc* sp. F105 exhibited higher values for all intensities compared with *Nostoc* sp. F108. However, *Nostoc* sp. F108 initially appears to exhibit a higher biomass yield. This effect occurs as a result of a higher exopolysaccharide production, which, due to the hygroscopic characteristics of exopolysaccharides, causes an increase in the volume of biomass present in the culture medium, leading to a false impression of higher yield compared with *Nostoc* sp. F105.

In the present study, in side experiments, the comparison of the weight loss of the two isolates after lyophilization of the cell mass demonstrated once again the relationship between the largest production of exopolysaccharides and the higher hygroscopicity. After lyophilization, the yield in dry weight for *Nostoc* sp. F108 reached only 1%, whereas for *Nostoc* sp. F105, the yield in dry weight reached 8%.

This difference suggests that the profile of exopolysaccharide synthesis must be analyzed according to the biotechnological interest. When aiming to isolate intracellular compounds such as pigments, a strain that has a high production of extracellular polysaccharides may not be desirable.

The macroscopic aspect of the biomass from both isolates in liquid culture medium was significantly different. Unlike *Nostoc* sp. F108, a significant decrease in the biomass yield at the higher intensities tested was not observed for *Nostoc* sp. F105. This fact is explained by the architecture of the colonies, which are more aggregated in *Nostoc* sp. F105. This structure

would prevent the real perception of light intensity by the cells positioned inside the colony because aggregation is one of the factors that contributes to the self-shading of cells. The interference of this shading was minimized in the present study by breaking the aggregates every day by exposing them to flows produced with a syringe.

As noted, the lower intensities exhibited the best results for the production of phycobiliproteins regarding dry mass, corroborating the affirmation that because they function as accessory pigments in light absorption, phycobiliprotein synthesis is optimized at low light intensity. At high light intensities, phycobiliproteins are decreased due to the decreased synthesis and the increased degradation by proteases (Pojidaeva et al., 2004).

Rosales-Loaiza et al. (2008) hypothesized that high irradiance increases the incorporation of total carbon in the polysaccharide fraction more than in the protein fraction.

The primary response to higher light intensities is a decrease in the phycobiliprotein content to prevent the absorption of radiation in excess. However, at the highest intensities tested, the smallest decrease in the biomass yield for F105 compared to F108 (Figure 1) may occur due to the predominance of Pe, given that this pigment is less sensitive to photo-degradation than PC and would therefore play a key role in cell photo-protection (Aráoz and Häder, 1999). This fact is corroborated by the increase in fluorescence emission by Pe of the *Nostoc* spp., which is induced by solar energy and artificial UVB. As a result, a significant amount of energy is not transferred to photosystem II, thus inhibiting the photosynthetic apparatus and the oxidative damage of target molecules (Aráoz and Häder, 1999).

Regarding the susceptibility to oxidative damage, the photo-bleaching of phycobiliproteins, especially PC, occurred before and more quickly than for chlorophyll *a* in *Anabaena* cells subjected to oxidative stress (He and Hader, 2002).

The peak production of chlorophyll *a* at low light intensities is a phenomenon exhibited by many species of cyanobacteria, such as *Anabaena* (Martín-Trillo, 1995), *Spirulina subsalsa* (Tomaselli and Margheri, 1995), *Plectonema boryanum* UTEX 485 (Miskiewicz et al., 2000) and *Anabaena* PCC7120 (Loreto et al., 2003). In the present study, the higher chlorophyll *a* content at the intensity of 10 μmoles / m^2 / s reflects the role that this pigment plays in capturing light energy when the light availability is low. At low light intensities, the cells promote the increase of thylakoid membranes and phycobilisomes to capture the greatest quantity of radiant energy as possible (Tandeau de Marsac and Houmard, 1993). Therefore, an intensity of 10 μmoles / m^2 / s can be considered a low light intensity for the two organisms studied. In low light intensities, carotenoids function as antenna accessory pigments in light absorption, increasing the photosynthesis efficiency. This function occurs because carotenoids are close to the chlorophyll *a* molecules in the photosystems and transfer the absorbed energy.

In the present study, the decreased levels of chlorophyll *a* and carotenoids at the highest intensities tested indicate that the increase in the concentration of pigments was no longer necessary because light was becoming increasingly available. Thus, the increased availability of light energy caused a decrease in the content of chlorophyll *a* and carotenoids. This reduction represents a defense strategy against the photo-oxidative damage caused by an excess of irradiance, as this decrease in chlorophyll *a* and carotenoids causes the cell to absorb less light. Excess absorbed light energy leads to the formation of free radicals such as

singlet oxygen, which can oxidize key proteins of the photosynthetic apparatus, including the D1 protein of photosystem II, leading to photo-inhibition (Grasses et al., 2001; Backasch et al., 2005; Inoue et al., 2011).

After the decrease in the concentration of carotenoids and chlorophyll *a* reported above, the increase in the carotenoid content at higher intensities may reflect carotenoids' function as dissipaters of excess absorbed light energy and their function as the first line of defense against oxidative stress caused by the photosynthetic process (Steiger et al., 1999). Several studies have reported increased carotenoid content in cyanobacteria in response to increased irradiance as a strategy for preventing oxidative damage (Rucker et al., 1995; Nigoyi et al., 1997; Miskiewicz et al., 2000). In fact, this change seems to be the primary response in most of the cyanobacteria studied.

Zeaxanthin can suppress the excess excitation energy of chlorophyll *a* through charge transfer (Holt et al., 2005). Zeaxanthin mutants exhibit higher sensitivity to light, with decreased pigment concentrations and increased levels of free radicals. The presence of conjugated double bonds is responsible for the antioxidant activity of carotenoids (Sies and Stahl, 1995), which deactivate reactive oxygen species (ROS) and quench free radicals. Given the excess illuminance of photosynthetic organisms, carotenoids suppress singlet oxygen and chlorophyll *a* in an excited state, dissipating the energy as heat. β-carotene efficiently removes singlet oxygen originating in the reaction center of photosystem II (Telfer et al., 1995). Higher ROS content is observed in cells mutant for xanthophylls (Bianchi et al., 2010).

Thus, the increase in carotenoids may represent two different phenomena. At low irradiance, carotenoids function as accessory pigments, increasing the light absorption, whereas at high irradiance, carotenoids would act as dissipaters of the excess energy absorbed and as antioxidants.

However, the increased degradation of carotenoids by photo-oxidation with exposure to higher irradiances has already been reported, and this degradation may stimulate the de novo synthesis of carotenoids. Thus, the pool of carotenoids in the cyanobacterial cells may be the result of the degradation, the de novo synthesis or a combination of both processes. Nonetheless, the de novo synthesis might not always be sufficient to restore the initial carotenoid content (Steiger et al., 1999). In addition, other studies did not report an increased carotenoid content due to increased irradiance. When analyzing the production of pigments by *Anabaena*, Loreto et al. (2003) did not observe any change in the carotenoid content at different light intensities, suggesting that this cyanobacterium activates another acclimatization or control process to prevent the photo-oxidation of the pigments at high light intensities. Similar results were observed in *Synechococcus* PCC 7002, *Synechococcus* PCC 6301 and *Microcystis aeruginosa*. Their carotenoid content did not change as a result of the illuminance (Tandeau de Marsac and Houmard, 1993). In *Plectonema boryanum* UTEX 485, the concentrations of chlorophyll *a* and β-carotene decreased with the increase in irradiance from 150 to 750 μmoles/ m^2 / s (Miskiewicz et al., 2000).

In cyanobacteria, β-carotene is often the major carotenoid. In addition to β-carotene, the hydroxylated derivatives zeaxanthin and nostoxanthin, the keto derivatives, such as echinenone and canthaxanthin, and the glycosylated carotenoids mixol 2-glycosides and 2,2-oscillol di-glycosides are among the major carotenoids of cyanobacteria. Some species do not

express specific carotenoids due to the absence of genes, enzymes or biosynthetic pathways. *Nostoc punctiforme*, for example, expresses β-carotene and little or no zeaxanthin (Takaichi and Mochimaru, 2007).

The cultures must be handled at the appropriate light intensity, and the quantity and quality of the light should be optimized to obtain a pigment-enriched biomass (Rosales-Loaiza et al., 2008). The intensity of light that yields the highest biomass should not always be selected because many times, it may not match the intensity at which the pigment production is optimized. In these cases, a viable alternative is to promote the initial culture under the light intensity that most favors the growth and then transfer the biomass to the intensity that most favors the production of the pigments of interest.

CONCLUSION

The best light intensity for the culture of a certain cyanobacterium should be chosen based on response curves that provide information on the biomass yield and the bioactive compounds of interest. The *Nostoc* sp. 105 isolate provided a higher biomass yield than *Nostoc* sp. 108.

In the isolates examined in the present study, the content of light-absorbing pigments, such as chlorophyll *a* and phycobiliproteins, was higher when the light availability was low. When the light availability increased, the levels of these pigments decreased as a strategy for protection against the photo-oxidative damage caused by the production of free radicals. However, for carotenoids, after this decrease, levels increased again at higher irradiances, which reflects carotenoids' function as dissipaters of excess absorbed light energy and as antioxidants in the photosynthetic apparatus.

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