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Análisis de la producción de biomasa de *Nostoc muscorum* en sistema hidropónico

Analysis of *Nostoc muscorum* biomass production in a hydroponic system

Análise da produção de biomassa de *Nostoc muscorum* num sistema hidropônico

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Resumen

Nostoc es un género de cianobacterias filamentosas con aplicaciones biotecnológicas en nutrición humana, biomedicina, biofertilización y producción comercial de biocombustibles. Sin embargo, su baja tasa de crecimiento en medio líquido por su naturaleza perifítica y su tendencia a formar biofilms, limita su producción a gran escala. Por lo tanto, el objetivo de este estudio fue analizar la producción de biomasa de *Nostoc muscorum* en un sistema hidropónico modificado. Para ello, se realizaron cultivos de *N. muscorum* por triplicado, en un sistema hidropónico bajo condiciones semicontroladas de temperatura (29 ± 13°C), intensidad lumínica (32 ± 54 µmol/m^2/s) y fotoperiodo (12 horas), durante 23 días en un invernadero. La temperatura, el pH, la conductividad eléctrica y la producción de biomasa seca, fueron monitoreados en días alternados. Los resultados arrojaron que la producción máxima de biomasa seca fue de 0.2276 ± 0.0114 g/m^2/día, y la productividad promedio fue de 0.4149 ± 0.0207 g/m^2/día. A su vez, la producción máxima de biomasa de *N. muscorum* se obtuvo el día trece con 0.3185 ± 0.0159 g/m^2/día. El análisis estadístico de correlación de variables ambientales no arrojó diferencias significativas, por lo que la temperatura, el pH y la conductividad eléctrica no afectaron la producción de biomasa de *N. muscorum*. Consecuentemente, el crecimiento algal fue influenciado por la fisiología de la especie. El soporte empleado en el sistema hidropónico permitió la adherencia y el desarrollo de la capa mucilaginosa de la cianobacteria sin requerir períodos de desecación como en los cultivos convencionales. El sistema hidropónico proporcionó un flujo continuo de nutrientes que podría prevenir el ataque de bacterias y hongos oportunistas, generando una alta tasa de crecimiento. De este modo, este sistema hidropónico representa una alternativa viable para la producción de biomasa de *N. muscorum* en condiciones de invernadero a gran escala.

Palabras clave: *biomasa; cianobacteria; crecimiento algal; hidroponía*

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Abstract

Nostoc is a genus of filamentous cyanobacteria with biotechnological applications in human nutrition, biomedicine, biofertilization and commercial production of biofuels. However, the low growth rate in liquid medium due to its periphytic nature and its tendency to form biofilms, limits its large-scale production. Therefore, the aim of this study was to evaluate the biomass production of Nostoc muscorum in a modified hydroponic system. Cultures of *N. muscorum* were made by triplicate, in a hydroponic system under semicontrolled conditions of temperature (29 ± 13 °C), light intensity (32 ± 54 µmol/m^2/s) and photoperiod (12 hours), for a total of 23 days inside a greenhouse. Temperature, pH, conductivity and dry biomass production were monitored on alternating days. The results showed that the maximum dry biomass production was 0.2276 ± 0.0114 g/m^2/day, and the average productivity was 0.4149 ± 0.0207 g/m^2/day. The maximum biomass production of *N. muscorum* was achieved on day thirteen with 0.3185 ± 0.0159 g/m^2/day. The correlation statistical analysis of environmental variables did not show significant differences; thus, temperature, pH and electrical conductivity did not affect the biomass production of *N. muscorum*. Consequently, the algal growth was influenced by the species physiology only. The support used in the hydroponic system allowed the adhesion and development of the algae mucilaginous layer without requiring drying periods as in conventional crops. The hydroponic system provided a continuous flow of nutrients hat could prevent the attack of opportunistic bacteria and fungi, generating a high growth rate of *N. muscorum*. The hydroponic system represents a viable alternative for the production of *N. muscorum* biomass under greenhouse conditions at large scale.

Keywords: *algal growth; biomass; cyanobacteria; hydroponics*.

Resumo

Nostoc é um gênero de cianobactérias filamentosas com aplicações biotecnológicas em nutrição humana, biomedicina, biofertilização e produção comercial de biocombustíveis. Entretanto, sua baixa taxa de crescimento em meio líquido, devido à sua natureza perifítica e sua tendência a formar biofilmes, limita sua produção em larga escala. Portanto, o objetivo deste estudo foi analisar a produção de biomassa de *Nostoc muscorum* num sistema hidropônico modificado. Para isso, foram realizadas culturas de N. muscorum em triplicata num sistema hidropônico sob temperatura (29 ± 13°C) e intensidade da luz (32 ± 54 µmol/m^2 /s) semicontroladas com fotoperíodo de 12 horas durante 23 dias numa estufa. A temperatura, o pH, a condutividade elétrica e a biomassa seca foram monitorados em dias alternados. Os resultados mostraram que a produção máxima de biomassa seca foi de 0,2276 ± 0,0114 g/m^2 /dia e a produtividade média foi de 0,4149 ± 0,0207 g/m^2 /dia. No entanto, a produção máxima de biomassa de *N. muscorum* foi obtida no dia treze com 0,3185 ± 0,0159 g/m / dia. A análise estatística da correlação de variáveis ambientais não mostrou nenhuma diferença significativa, de modo que a temperatura, o pH e a condutividade eléctrica não afetaram a produção de biomassa de *N. muscorum* e o crescimento foi influenciado pela fisiologia da espécie. O suporte utilizado permitiu a adesão e desenvolvimento da camada mucilaginosa das algas sem a necessidade de períodos de secagem como nas culturas convencionais. O sistema hidropônico proporcionou um fluxo contínuo de nutrientes que impede o ataque de bactérias e fungos oportunistas, gerando uma alta taxa de crescimento. Portanto, este sistema hidropônico representa uma alternativa viável para a produção de biomassa de *N. muscorum* em larga escala sob condições de estufa.

Palavras chave: *biomassa; cianobactérias; crescimento de algas; hidroponia*

Introduction

Cyanobacteria, also known as blue-green algae, are an unique group of photosynthetic prokaryotes with worldwide distribution, characterized by their great morphological diversity, pigmentation and oxygenic photosynthesis (Wehr *et al.,* 2015). The production of cyanobacteria and their cultivation systems have interested researchers given their commercial importance (Hultberg *et al*., 2013). They have been widely studied because of their enormous biotechnological potential, mainly due to the ability to synthesize different biomolecules for nutritional, pharmaceutical and industrial applications (Nowruzi *et al.,* 2018); including the production of food, fuel and oxygen on Mars (Verseux *et al.,* 2016). Given the versatility of employing cyanobacteria, several studies have focused on their production and economic yield (Barone *et al*., 2019).

Among cyanobacteria, the most interesting genera, from a biotechnological point of view, are *Spirulina*, *Anabaena* and *Nostoc* (Wehr *et al.,* 2015). *Nostoc* genus is used for medical applications and human nutrition in countries like China, Japan, India and Colombia (Liao *et al.,* 2015; Guo *et al.,* 2016; Benítez *et al.,* 2018). This cyanophycea produces active antiviral substances against the human immunodeficiency virus (HIV), and also antibiotics that can control cryptococcosis, a secondary fungal infection typical of the acquired immunodeficiency syndrome (AIDS) (Raja *et al.,* 2016; Lotfi *et al.,* 2017). In addition to nutritional and medical applications, *Nostoc* had too been used as biofertilizer in different crops such as rice, canola, tomato, corn, wheat, peas, and oats. As biofertilizer, *Nostoc* can contribute to nitrogen fixation, phytohormones synthesis and production of hygroscopic polysaccharides that help plant growth, and, it can also prevent erosion and

improve soil properties (Dhar *et al.,* 2015; Ranjan *et al.,* 2016). Moreover, the species *Nostoc muscorum* synthesizes polymers such as the polyhydroxybutyrate thermoplastic that is used for clinical and industrial applications (Haase *et al.,* 2012; Ansari and Fatma, 2016); and, unsaturated fatty acids of pharmaceutical interest like α-linolenic acid (18:3) (Kim *et al.,* 2015). Additionally, the ultrastructure of *N. muscorum* can be exploited for the production of based-hydrogen fuel (Shah *et al*., 2003; Singh *et al.,* 2017).

The cultivation of microalgae is a globally emerging industry with great commercial potential (Hultberg *et al*., 2013). Traditionally, the main alternatives for cultivating microalgae are raceway pond systems and photobioreactors. However, *N. muscorum* tends to present a low growth rate and an abnormal growth pattern when in liquid medium without any support; also, the traditional cultivation systems are still expensive and need complex technological developments and technical expertise to be cost efficient and environmentally sustainable (Zhang *et al.,* 2017). The species *N. muscorum* does not have an optimal growth in liquid medium because the agitation tends to adhere the cells to each other, form biofilms, and migrate towards the part where there is no culture medium; consequently, the cyanophycea undergoes depigmentation and rapid cell death due to the lack of nutrients (Diao and Yang, 2014; Rusydi *et al.,* 2015; Lotfi *et al.,* 2017). Moreover, the mucilaginous envelope of *N. muscorum* cannot be formed properly, thus, allowing opportunistic bacteria and fungi to degrade it (Rusydi *et al.,* 2015; Cui *et al.,* 2017). Therefore, a successful strategy for increasing the biomass production in cultivation systems of filamentous cyanobacteria that do not have a good adaptation to liquid conditions, e.g. *N. muscorum*, requires the optimal utilization of a solid substrate (Barone *et al*., 2019). The use of an inert support allows the adhesion of periphytic microalgae, thus, an optimal growth and biomass production (Vorndran and Lindberg, 2016; Cui *et al.,* 2017). Additionally, for a high growth rate, a culture system that allows the exchange of heat and nutrients is necessary (Alatorre-Cobos *et al.,* 2014).

Hydroponic systems have shown to be cost-effective and environmentally sustainable in previous studies with cyanobacteria *Calothrix* sp.*, Anabaena laxa* and *Anabaena azollae* (Alatorre-Cobos *et al.,* 2014). The hydroponic method is mainly used as an alternative for wastewater treatment aimed at a more effective reduction of nutrients (Bawiec *et al*., 2019). A great support for the purification processes in such systems are microalgae due to their use of inorganic forms of nitrogen and phosphorus, and the ability to uptake heavy metal ions, organic pollutants, and coliform bacteria (Abdel-Raouf *et al*., 2012; Ye *et al*., 2016). Together with their space efficiency, low cost and cleanliness, hydroponic systems have become increasingly popular as an ecofriendly method for wastewater treatment; which happens to be an ideal nutrient solution for the development of cyanobacteria used for the production of biofuels (Singh *et al.,* 2017). On the other hand, hydroponic systems have also been studied for co-cultivation of plants and microalgae. Zhang *et al*., (2017) and Barone *et al*., (2019) showed that the hydroponic co-cultivation system was beneficial for the growth of both plants and microalgae, resulting in a biostimulant effect for crop biomass provided by algal photosynthesis, while crop root respiration and exudation acted as sources of carbon that increased microalgal biomass. Furthermore, hydroponic systems with cyanobacteria have been used in the process of priming rice and wheat plants (Babu *et al*., 2015; Bidyarani *et al*., 2015). This is mainly supported by studies on biofilm formation (Prasanna *et al*., 2018), and colonization of root tissues (Bidyarani *et al*., 2015). Bharti *et al*., (2019) also studied co-culturing as a promising option for seedlings development before planting in pots or field, finding that hydroponic co-cultivation of chrysanthemum with cyanobacteria provided a continuous supply of oxygen gradients, carbohydrates, and nitrogenous compounds, that had an effect on the proliferation of roots and growth of cuttings, and observed a synergistic behaviour of both parts in terms of biomass accumulation.

Hence, in order to use hydroponic systems for exploiting the biotechnological potential of cyanobacteria, there is a need for monitoring their growth first. The aim of this study was to analyze the growth of *N. muscorum* in a modified hydroponic system, as an alternative for biomass production.

Materials and methods

Preliminary tests

The biomass production of two *Nostoc* species was evaluated:

Nostoc ellipsosporum (Göttingen University B1453-79): obtained from the Institute of Plant Biochemistry and Photosynthesis (IBVF-cicCartuja) of the University of Seville (Spain). Filamentous cyanobacterium; with trichomes (chain of cells) that grows by intercalar cell division. Reproduction by formation of hormogons, random breakage of the trichome, and, sometimes by germination of acinets. In the absence of combined nitrogen, trichomes have heterocysts, some also acinets. Cellular division in one plane.

Nostoc muscorum (code number 026): obtained from the collection of freshwater algae in the Botany Department of the Federal University of São Carlos (UFSCar, Brazil). Cells are filamentous, green-brown colored, and can form spores under desiccation conditions. Heterocysts (5-10% of cells) appear when transferred to nitrogen free media. Cells possess a motile stage in its life cycle through the use of hormogones.

Culture medium: The hydroponic solution composition was 0.64 g/l of calcium nitrate (Calcinit®), 0.4 g/l of potassium nitrate (K Crista®), 0.08 g/l of monoammonium phosphate (MAP® Crista), 0.34 g/l of magnesium sulfate, 0.015 g/l of chelated iron in EDDHMA (M48® Rexolin Yara), and 0.02 g/l of micronutrients (Mg, S, B, Cu, Fe, Mo, Mn) (KSC Roullier mix®). This solution was diluted in the following volume/volume percentages: 30% (H30%), 40% (H40%), 50% (H50%), and 60% (H60%).

A test was developed to determine the optimal concentration of hydroponic solution for the *N. ellipsosporum* culture with the following treatments: BG-11 (UTEX) (Vaara *et al*., 1979), H30%, H40%, H50% and H60%, each by triplicate in 100 ml Erlenmeyer flasks, with an initial cellular concentration of 3.4 x 10^5 cells/ml each. Optical density was measured at 650 nm on days: 0, 2, 3, 4, 5 and 8. The controlled laboratory conditions were as follow: 32.4 ± 0.1 µmol.photons/m^2/s light intensity, 12 hours of photoperiod, and 23°C temperature.

Lastly, for the selection of the strain with the highest biomass production yield, a comparison was made between *N. ellipsosporum* and *N. muscorum* in the H50% culture medium by triplicate in 100 ml Erlenmeyer flasks, with an initial cellular concentration of 2.8 x 10^4 cells/ml. Optical density data was measured at 650 nm on days: 1, 4, 6 and 7. The controlled laboratory conditions were as follow: 32.4 ± 0.1 µmol. photons/m^2/s light intensity, 12 hours of photoperiod, and 23°C temperature.

Nostoc muscorum culture in a modified hydroponic system

N. muscorum inoculum was grown in 1 l Erlenmeyer flasks, with 500 ml of hydroponic solution until cellular density reached 2 x 10^6 cells/ml. Later, the cell culture was transferred to the hydroponic system inside a greenhouse located at the Algae Biotechnology Laboratory of the Botany Department at the Federal University of São Carlos (UFSCar, Brazil), 21°58'59.60'' S - 47°52'47.79'' W, 854 masl. Microalgae grew under the following conditions: 895 ± 18 µs/cm electrical conductivity, 23°C temperature, pH 7, 32.4 ± 0.1 µmol.photons/m^2/s light intensity, and 12 hours photoperiod.

Experiments were carried out in an inert material hydroponic system (Figure 1) composed by a feeding tank (10 l), a positive flow pump and a box with inclined support covered by a pore size 5 µm cloth. The system was disinfected with 70% ethanol. 10 l of the hydroponic solution was used to feed the system, which was inoculated with 6.3 ± 3.6 x 10^4 cells/ml of *N. muscorum*. The following parameters were monitored on alternate

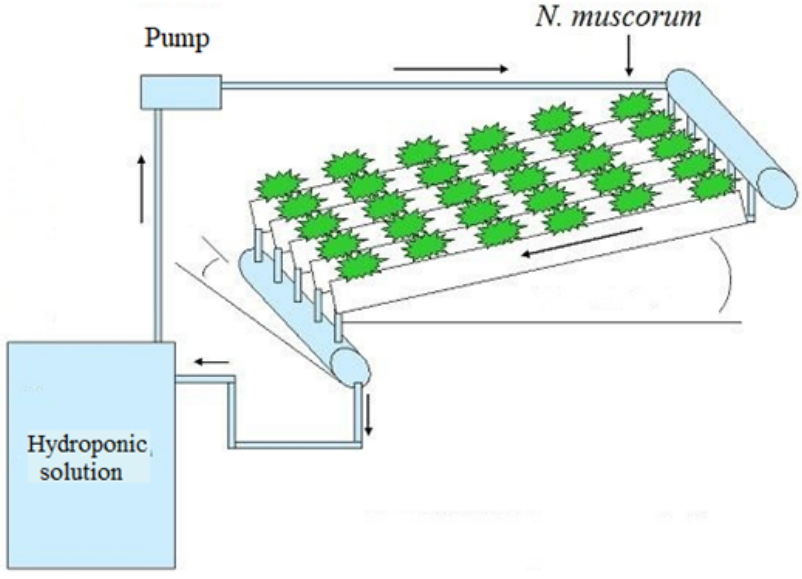

Figure 1. Hydroponic system for *N. muscorum* culture. Modified from Lenzi *et al*., (2011).

days: pH, temperature, electrical conductivity, and light intensity (measured at 10 am everyday); using a portable pH meter (HI98108 – Hanna, Hungary), conductivity meter (CDC401 – Hach, USA) and a light meter (0713700 – Phywe, Germany). The pH was regulated with the addition of 9.4 x 10^{-4} M $NaHCO_3$.

As days went by, the *N. muscorum* cells covered the whole cloth, making it necessary to harvest the biomass in order to avoid "self-shading". The harvest of biomass was done by scraping on the following days: 6, 8, 13, 16, 20, 22 and 23. The algae biomass collected from the hydroponic system was dried in a convection oven (315SE – Fanem, Brazil) at 37°C for 48 to 72 hours, depending on the biomass humidity, and until reaching a constant weight. Dry biomass was weighed in an analytical balance with 0.0001 g resolution (TE214S – Sartorius, Germany). To verify cell adhesion of *N. muscorum* on the cloth, 2 ml samples of the hydroponic solution were taken during the sampling days. These samples were fixed with one drop of Lugol's solution, stored in microcentrifuge tubes and analyzed by light microscopy.

Statistical analysis

Mean values of triplicates were calculated using Microsoft Excel 2017 including standard deviation (SD) values as depicted in the error bars of the Figures 3, 4, 5, 6 and 7. Data of the strains growth and biomass production were analyzed using the analysis of variance test, one-way ANOVA, with a confidence level of 95%. Correlations were also calculated in Microsoft Excel 2017 at P value of 5%, using a linear analysis of mixed effects, for the factors: pH, temperature, electrical conductivity and biomass production of *N. muscorum*.

Results

Preliminary tests

To determine the optimal concentration of hydroponic solution for the *Nostoc* culture, five treatments (BG-11, H30%, H40%, H50% y H60%) were evaluated, obtaining the following optical density values: BG-11 0.051 ± 0.001 OD_{650}; H30% 0.044 ± 0.001 OD_{650}; H40% 0.045 ± 0.001 OD_{650}; H50% 0.069 ± 0.001 OD_{650}; and H60% 0.054 ± 0.001 OD_{650}. The ANOVA showed no significant differences among the treatments of the diluted hydroponic solution and the BG-11 reference medium (P value 0.784). Nevertheless, the H50% treatment displayed the highest value of optical density during the experiment.

The biomass production of two *Nostoc* species was evaluated in the H50% treatment according to their optical density values. The ANOVA exhibited significant differences between the strains (P value 0.012), indicating that the strain of *N. muscorum* had a significant greater growth with an OD_{650} of 0.087 ± 0.001. On the other hand, *N. ellipsosporum* reached an OD_{650} of 0.066 ± 0.001.

Nostoc muscorum culture in a modified hydroponic system

A high growth rate of *N. muscorum* was observed in the hydroponic system with a porous cloth under semicontrolled environmental conditions (Figure 2). Figu-

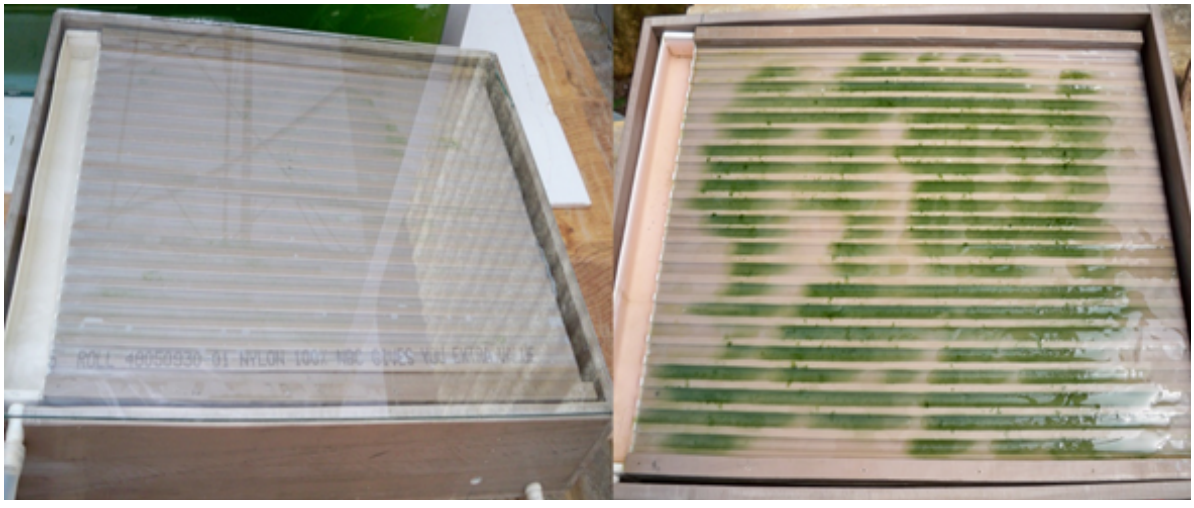

Figure 2. Appearance of *N. muscorum* growth in a hydroponic system with a solid substrate. Left: Day 1. Right: Day 4.

res 3 and 4, indicate the temperature and luminosity variations in the greenhouse. Moreover, for further analysis of *N. muscorum* growth, the dry biomass production (Figure 5), pH (Figure 6) and electrical conductivity (Figure 7) were daily measured. Only a modified hydroponic system was evaluated in the present work.

The obtained average biomass productivity was 0.4149 ± 0.0207 g/m^2/day, and the maximum daily production was 0.2276 ± 0.0114 g/m^2/day. The maximum biomass production of *N. muscorum* was obtained on day 13 with 0.3185 ± 0.0159 g/m^2/day (Figure 5). This result was obtained after seven days of high temperature and luminosity, as shown in Figures 3 and 4. The total biomass produced by the culture system was 0.0053 ± 1.0689 g/m^2/day on day 23.

Correlation analysis by mixed effects allowed to identify that there was no dependence between pH (P value 0.3360), temperature (P value 0.7504), electrical conductivity (P value 0.6633), and *N. muscorum* biomass production factors. Therefore, the algal growth was only influenced by the physiology of the species under the evaluated culture conditions.

Discussion

Nostoc muscorum is a cyanophycea with great biotechnological potential due to the products that can be obtained from its biomass. However, conventional liquid cultures are not efficient for biomass production of *N. muscorum*, as well as for other filamentous cyanobacteria (Yu, *et al.*, 2009; Cui *et al.*, 2017). Diao and Yang (2014) and Rusydi *et al.*, (2015), found that conventional liquid culture generates disintegration of colonies of *Nostoc* species, mainly associated with bacteria and fungi propagation. At the same time, periodic exposures to desiccation could cause malformation of the exopolysaccharide layer, because the *Nostoc* sheath can have its structure modified and become the nutritive base for microbial growth (Rossi and De Philippis, 2015; Widder *et al.*, 2016).

In this way, the viability of biomass production of *N. muscorum* cultivated in a hydroponic system is demonstrated, presenting several advantages:

1. It does not require drying periods like conventional cultures implemented by Cui *et al.,* (2017). This is possible because the substrate (cloth) acts as a support for the adherence and development of the algae mucilaginous layer, allowing the continuous flow of nutrients and preventing a dominant growth of opportunistic bacteria and fungi, resulting in a high rate of algal growth (Rossi and De Philippis, 2015).

2. *N. muscorum* culture in a hydroponic system maintains the textural elasticity for colony sheath formation, which the algae use to prevent cell disintegration and to generate proteins resistant to drought and UV rays (Rossi and De Philippis, 2015).

3. The continuous flow of oxygen provided by the pump in the hydroponic system depresses bacterial growth (Widder *et al.,* 2016). In fact, during the active growth of *N. muscorum* in the hydroponic system, the disintegration of colonial filaments was significantly delayed, suggesting a reduction in the opportunistic microorganism activities.

Furthermore, according to the monitored parameters, the maximum algae growth occurred after several days of high luminosity and temperature (Figures 3 and 4). These results could rely on the fact that *N. muscorum* adapts very well to environmental conditions with high exposure to UV light, unlike other cyanobacteria (Singh *et al.,* 2011; Rosales-Loaiza *et al.,* 2016). Ge-

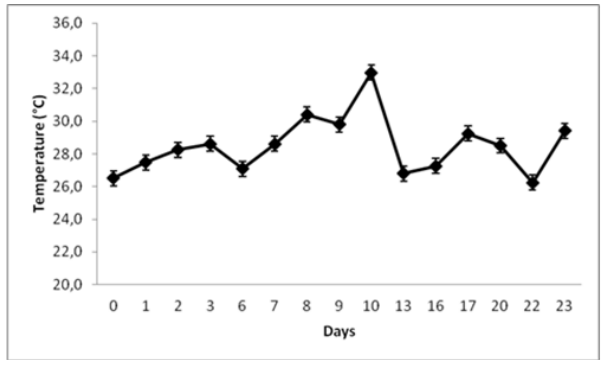

Figure 3. Temperature fluctuations in the greenhouse where biomass production of *N. muscorum* on a solid substrate within the hydroponic system was helded

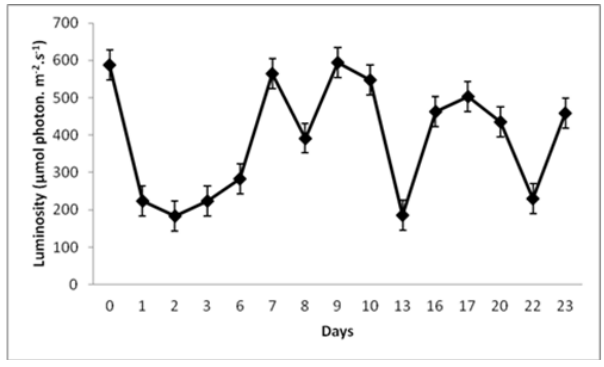

Figure 4. Luminosity fluctuations in the greenhouse where biomass production of *N. muscorum* on a solid substrate within the hydroponic system was helded

nerally, the temperature change from 15°C to 30°C does not affect morphological development, even in conventional liquid culture conditions. This *Nostoc* UV light insensitivity is generated by the presence of Mycosporin and Scytonemin type amino acids in the sheath (Ferroni *et al.*, 2010). In this study, *N. muscorum* tolerated a greater intensity of light (391.8 µmol. photons/m^2/s) and temperature (33°C) in the hydroponic culture system, as shown in Figures 1 and 2. The obtained biomass values, 0.4149 ± 0.0207 g/m^2/day surpass the results from Cui *et al.,* (2017) in conventional culture systems. Since the water flow in the hydroponic system allowed the release of heat in the form of steam, that was condensed in the same system, water loss and consequent changes in electrical conductivity were minimized (Figure 7). These two features can reduce water and monitoring costs in the maintenance of cyanobacteria growth.

The decrease in the pH value of the hydroponic solution during the cyanobacteria growth could be due to the excretion of organic acids and metabolic products associated with phosphorus solubilization in the environment (Haase *et al.,* 2012). This ability confers an ecological advantage to the species compare to other

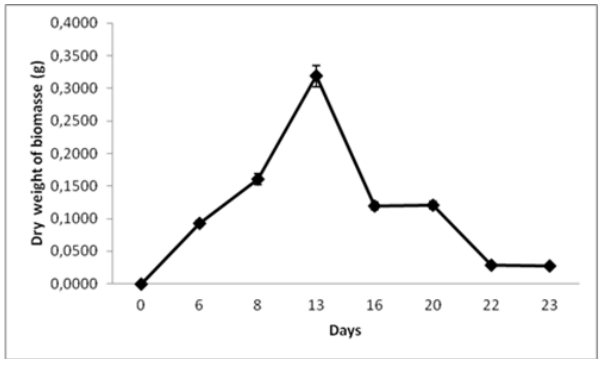

Figure 5. Biomass production of *N. muscorum* on a solid substrate within the hydroponic system.

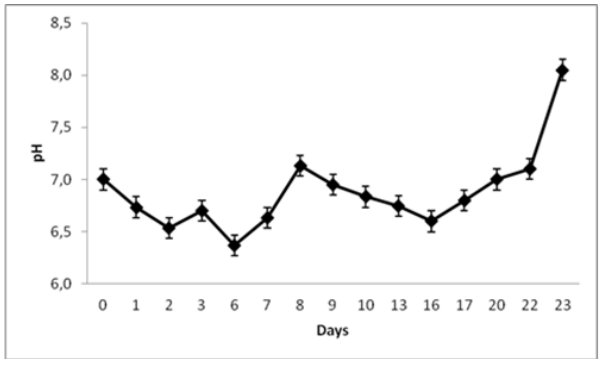

Figure 6. pH fluctuations during the biomass production of *N. muscorum* on a solid substrate within the hydroponic system.

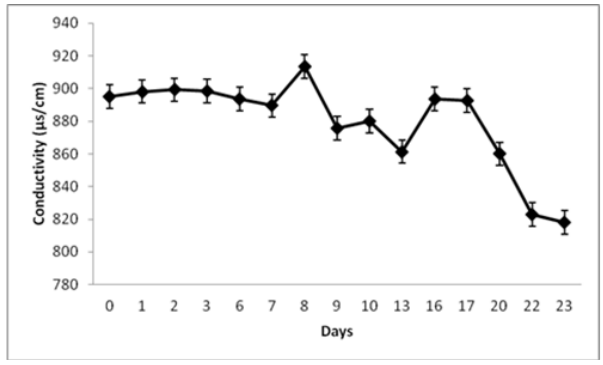

Figure 7. Conductivity fluctuations during the biomass production of *N. muscorum* on a solid substrate within the hydroponic system

microalgae because of its possible applications in biofertilizer production (Dhar *et al.,* 2015; Flores *et al.,* 2015).

Furthermore, microscopic analysis of the hydroponic solution showed the absence of cells in the fluid, which indicates that *N. muscorum* completely adhered to the substrate using its mucilaginous layer of exopolysaccharides (Singh *et al.,* 2011; Haase *et al.,* 2012)

Finally, the hydroponic system with solid support can be manufactured with common elements and requires simple monitoring and harvesting, representing an economical alternative using available technology for cyanobacteria culture. Besides, this hydroponic system is more affordable than other farming systems because it does not need automatic desiccation as in conventional crops. In conclusion, algal biomass production in a hydroponic system with solid support proved to be a viable alternative for the growth of peryphytic filamentous cyanobacteria such as *N. muscorum* in an indefinite culture medium with nutrients excess (hydroponic solution), compared to the BG-11 medium which is a defined one.

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