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Diazotrophic Cyanobacteria for Protein Production: Hotorespirometry to Assess the Effect of Light and Temperature

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Protein production, thriving on the chemical fixation of nitrogen in fertilizers and intensive use of agricultural and livestock practices, significantly compromises environmental sustainability. The need for alternative food resources to meet the raised demand due to the fast-growing global population has turned pressing. Diazotrophic cyanobacteria, capable of converting atmospheric nitrogen into bioavailable forms, offer promise in sustainable protein production, bypassing traditional inefficiencies. Such microorganisms' potential might be fully exploited if the production process is well-characterized and controlled. The primary objective of this study is to investigate the specific effects of light and temperature on the growth dynamics of *Nostoc* PCC 7120 by using photorespirometry. The study aims to retrieve kinetic parameters essential for predictive modeling in industrial applications. The optimal light intensity (376 µmol m^{-2} s^{-1}) was estimated, as well as the optimal temperature. The latter showed to be dependent on culture conditions: under nitrogen-fixing conditions, it drops from 30 to 27 °C, highlighting temperature-driven effects based on the nitrogen source.

1. **Introduction**

The relentless surge in the global population, expected to reach 10 billion people within the next 25 years, poses an imminent and pressing demand for alternative food sources, particularly proteins (Young et al., 2021). An optimal alternative food source should provide balanced nutrition, sustainability, cost-effectiveness, and safety while using innovative production methods, reducing waste, and ensuring accessibility (Torres-Tiji et al., 2020). It follows that the solution cannot rely on the further intensification of conventional food production methods, disregarding the environmental challenges linked to climate change, soil and water scarcity, as well as biotic and abiotic stress (Henchion et al., 2017). Microalgae, encompassing photosynthetic microorganisms such as cyanobacteria, are gaining attention as promising and high-nutrient food resources (Torres-Tiji et al., 2020). They are being considered for their potential adaptability to large-scale sustainable production, capitalizing on high biomass yields per unit area and the ability to thrive in non-arable land using non-potable or saline water. Moreover, they are noteworthy as producers of bioactive compounds, vitamins, and antioxidants (Torres-Tiji et al., 2020). In the search for viable alternatives, diazotrophic cyanobacteria could play an essential role. These microorganisms can convert atmospheric nitrogen into bioavailable forms. The biological nitrogen fixation (BNF) capability of cyanobacteria has been traditionally investigated for biological fertilizer production replacing chemical ones to increase crop production (Young et al., 2021). Nevertheless, the direct use of biomass as a protein source could circumvent the inefficiencies associated with traditional protein production from livestock or crops, thereby reducing dependence on nitrogen fertilizers and mitigating the extent of Haber-Bosch process application in the global nitrogen economy (Young et al., 2021). Since it is highly energy-consuming and GHG-producing (Razon, 2014), the environmental impacts would be clearly positive. Hence, protein production harnessing the cultivation of diazotrophic cyanobacteria eliminates the need for nitrogen fertilizers, providing biomass that potentially offers comparable or better nutritional values than plant-based ones. Despite these advantageous features, further advancements are required to fully integrate these microorganisms into the food production system. Some diazotrophic species are already consumed as food sources in some Eastern regions.

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For instance, the *Nostoc* genus is consumed in China. However, their cultivation predominantly depends on open batch systems or harvesting from natural environments (Mendes et al., 2022).
Given the process potential, optimizing nitrogen fixation and enhancing biomass productivity might ensure competitiveness. To optimize productivity, precise control and fine-tuning of process operating conditions are necessary. Mathematical models are instrumental in assessing diverse process variables to estimate process parameters, like growth rate and productivity, as well as in facilitating process scale-up to large-scale operations (Darvehei et al., 2018). Thus, proper values of operative variables must be investigated in order to quantify kinetic parameters and develop reliable growth models.
In this work, photorespirometry is proposed as a promising tool to investigate the kinetic aspect of BNF. Photorespirometry is a technique typically used to study microbial metabolism, but it also emerges as a successful tool to investigate phototrophy, based on measurements of oxygen evolution in solution due to photosynthetic activity when light is supplied (Sforza et al., 2019). The investigation focused on the heterocystous cyanobacterium *Nostoc* PCC 7120, which is one of the well-established nitrogen-fixing cyanobacteria. Through respirometry tests, the effect of temperature and light intensity on the growth of the diazotrophic species *Nostoc* PCC 7120 was investigated by varying one variable at a time.

2. Materials and methods

The diazotrophic heterocystous cyanobacterium *Nostoc* PCC 7120 (also *Nostoc* sp.), served from the UTEX Culture Collection of Algae at the University of Texas at Austin (US), was employed in this work. It was maintained and propagated in sterilized $BG11_0$ medium (Rippka et al., 1979), modified by removing nitrogen and substituting HEPES with 250 g L^{-1} of sodium hydrogen carbonate, to maintain the pH within the optimal interval of 6.5-7.5.

2.1 Photorespirometry tests

Photorespirometry was implemented to assess the effect of light intensity and temperature on the physiological responses of *Nostoc* sp. The photorespirometry setup consisted of a glass vessel filled with 110 mL of steady-stated pre-adapted culture, magnetic stirring ensuring culture homogeneity, a continuous PAR-spectrum LED light source, a temperature control system, and a N_2-bubbling stream to maintain oxygen concentration between 4 and 7 mg L^{-1}. The latter aspect guaranteed that the mass transfer effect did not interfere with the evaluation of the microorganism physiological responses, since the O_2 concentration is far from the extreme values of the gas solubility equilibrium, described by Henry's law. During the photorespirometry tests, the culture underwent light-dark cycles of 3-5 minutes. Each variable – light intensity and temperature – was investigated independently, facilitating a focused analysis of their individual effects on the cyanobacterium growth and each condition was tested at least in triplicate, by measuring the dissolved oxygen evolution with an oximeter (HD2109.1 DELTA OHM). The net oxygen production rate was calculated as the sum of the absolute value of the oxygen production rate (OPR) and the oxygen consumption rate (OCR). Light intensity was systematically adjusted to values in the range 50, 150, 200, 300, 500, 1000, 1500, 2000, 2500, and 3000 µmol m^{-2} s^{-1}, while maintaining a constant temperature of 27 °C, following a minimum of 3 light-dark cycles. On the other hand, the temperature effect was investigated by maintaining an irradiance of 550 µmol m^{-2} s^{-1} and varying the temperature between 14 and 45 °C. The temperature effect was examined in both $BG11_0$, i.e. under nitrogen-fixing conditions, and in the presence of combined nitrogen in culture medium (BG11), particularly sodium nitrate at a concentration of 1500 mg L^{-1}. Upon identifying the kinetic model describing the microorganism growth concerning the studied operating variable, parameter values were retrieved by fitting the kinetic model on photorespirometry data (Sforza et al., 2019). A modified Haldane kinetic model proposed by Bernard & Rémond (2012) and stated in Eq(1) was used to fit the dependence of growth rate (µ) on light intensity (I).

$$\mu = \mu_{max} \frac{I}{I + K_I \left(\frac{I}{I_{opt}} - 1\right)^2} \quad (1)$$

In Eq(1), the parameters include μ_{max}, K_I and I_{opt}, denoting the maximum growth rate (d^{-1}), the half-saturation constant (µmol m^{-2} s^{-1}) and the optimal light intensity (µmol m^{-2} s^{-1}), respectively.
Eq(2) describes the kinetic model used for temperature (T) (Bernard & Rémond, 2012),

$$\Phi(T) = \frac{(T - T_{max})(T - T_{min})^2}{(T_{opt} - T_{min})\left[(T_{opt} - T_{min})(T - T_{opt}) - (T_{opt} - T_{max})(T_{opt} + T_{min} - 2T)\right]} \quad (2)$$

where T_{max} and T_{min} are the maximum and the minimum temperature values, beyond which growth does not occur, while T_{opt} denotes the optimal one.

2.2 Batch experiments and growth rate

Batch experiments were performed to confirm photorespirometry outcomes concerning temperature. *Nostoc* sp. was cultivated in 5-cm-Quickfit® Drechsel Bottles for 9 days in at least two independent biological replicates. Experiments were carried out in a thermostated incubator at two different temperatures (24 °C and 30 °C). Light was supplied continuously at 100 µmol photons m^{-2} s^{-1}, while mixing was ensured by a magnetic stirrer and CO_2-air (5% v/v) mixture continuously bubbling at the bottom of the bottle (total gas flow rate of 1 L h^{-1}) for non-limiting CO_2 supply. Tests were performed in the presence (BG11) or not ($BG11_0$) of nitrate (NO_3) in the medium. Biomass concentration was quantified by measuring dry cell weight concentration (g L^{-1}). Growth rate (µ) was determined during exponential growth by interpolating the logarithm of dry cell weight over time.

2.3 Statistical analysis

The ANOVA and the Tukey's tests were implemented to assess the statistically significant differences between the mean values of experimental data, assuming a value of 0.05 as the reference p-value.

3. Results and discussion

Photorespirometric tests were carried out to assess the kinetic parameters specific for *Nostoc* PCC 7120 growth description under diazo-phototrophic cultivation. The following sections present and discuss the outcomes concerning the effect of light intensity and temperature.

3.1 Effect of light intensity on photorespirometry

The investigation by Lucato et al. (2024) reported and preliminarily discussed the oxygen production (OPR) and consumption (OCR) rates of *Nostoc* sp. under diazotrophic conditions at different light intensities. This study revisits and deepens the understanding derived from that dataset. The earlier work mainly outlined the trends within the experimental data, while the current investigation aims to delineate species-specific light-related kinetic parameters from the dataset. For further insights into the biology and physiology of the microorganism, reference is made to the aforementioned paper.

As depicted in Figure 1A, all tested conditions supported the cyanobacterium growth. Low light intensities (50 – 200 µmol m^{-2} s^{-1}) resulted in photolimitation. According to statistical analysis, an irradiance of 200 µmol m^{-2} s^{-1} proved sufficient to reach photosaturation, which persisted up to 1000 µmol m^{-2} s^{-1}. The experimental trend in the growth rate with increasing light intensity – rising to an optimal value, followed by a decline due to the inhibitory effect – is consistent with the behaviour observed in other microalgal species (Carvalho et al., 2011). The most suitable kinetic model to describe this behaviour aligns with the Haldane equation. The values of the parameters retrieved through the fitting of the experimental data are reported in Table 1.

Table 1: Summary of the fitted parameter values for light intensity.

Parameter	Value	Unit
μ_{max}	5.18	d^{-1}
K_I	93.9	µmol m^{-2} s^{-1}
I_{opt}	376.6	µmol m^{-2} s^{-1}

The observed trend is consistent with data obtained in continuous cultivation (Lucato et al., 2024), where photosaturation was observed around a light intensity of 550 µmol m^{-2} s^{-1}. To validate the chemostat data as a function of light intensity, the Lambert-Beer equation should also be included, to account for the effect of varying biomass concentration in the reactor as a result of the residence time set. In fact, when working with continuous chemostat photobioreactors, the cell concentration achieved at steady state results from the integrated effect of multiple variables, making it challenging to distinctly isolate the effect of light intensity. The cultivation environment in photobioreactors is more complex and, during steady-state cultivation, the microorganism can implement adaptive responses to the environment, especially regarding light, affecting the results. Photosynthetic microorganisms can optimize their pigmentary apparatus, ensuring efficient utilization of available light (Sanfilippo et al., 2019). Conversely, in the context of photorespirometry, rapid responses to environmental changes are assessed. Barbera et al. (2019) evidenced that species adaptation to different light intensities during the pre-treatment phase does not significantly alter parameter values. Hence, photorespirometry stands validated as a method for determining kinetic parameters, which prove useful for modeling aspects associated with microorganism growth. Once kinetic parameters are retrieved, kinetic models enable growth predictions within the cultivation system considering multiple integrated variables and the identification of optimized conditions.

3.2 Effect on temperature on photorespirometry

Photorespirometry tests as a function of temperature were carried out under both N-fixing conditions (using $BG11_0$) and in the presence of combined nitrogen in the culture medium (using BG11). The results are displayed in Figure 1B. The net oxygen production rate data in both scenarios exhibited a bell-shaped distribution with respect to temperature, highlighting distinct curve peaks. The trend obtained with $BG11_0$ is skewed towards lower temperatures, indicating an altered growth response. To analyze the temperature dependency of the growth rate, the Bernard-Rémond kinetic model was applied to fit the experimental photorespirometry data. The resulting values of temperature-related parameters, listed in Table 2, supported the previously described findings. While the minimum temperature remained consistent in both conditions, when N_2 was the only nitrogen source in the medium, the optimal and maximum temperatures were lower than for growth in BG11.

Table 2: Summary of the fitted parameter values for temperature.

Parameter	Unit	BG11	$BG11_0$
T_{min}	°C	14.79	14.94
T_{opt}	°C	30.35	27.07
T_{max}	°C	45.92	39.19

Batch experiments were also performed using BG11 and $BG11_0$ at different temperatures (24 °C and 30 °C) to evaluate the effect of temperature on growth rate. Batch results confirmed respirometry test outcomes (Table 3). Specifically, under diazotrophic conditions, no statistically significant difference in growth rate was evident between 24 °C and 30 °C, aligning with the trend observed in Figure 1BTable 2. However, at 30 °C, a higher growth rate was measured when nitrate was supplied with respect to growth in $BG11_0$ medium, further corroborating the earlier-established observation.

Table 3: Exponential growth rate obtained through batch experiments at different temperatures and culture conditions.

Culture condition	Temperature (°C)	Growth rate μ (d^{-1})
BG11	30	0.54 ± 0.02^a
$BG11_0$	30	0.46 ± 0.02^b
$BG11_0$	24	0.46 ± 0.03^b

The discrepancy at higher temperatures may support two hypotheses. The first is that at those temperatures, enzymes and proteins involved in the nitrogen-fixation metabolism or heterocysts structure exhibit heightened sensitivity, leading to decreased activity, which compromises growth. Temperature profoundly affects enzymatic reactions, protein structure – which regulates crucial cellular functions – and membrane functionality. However, a comprehensive understanding of its specific influences and underlying mechanisms remains elusive. Bauersachs et al. (2014) highlighted temperature-induced variations in the heterocyst glycolipid envelope. They reported an increased fractional abundance of diols along with a reduction in keto-ols as temperature increases and this alteration in composition is proposed to limit oxygen diffusion into the heterocysts. The latter are specialized cells exclusively dedicated to nitrogen fixation and present a structure ensuring favorable conditions for such a cellular process (Walsby, 2007). Changes in structure that alter gas diffusion, such as oxygen which has an inhibitory effect on the enzyme responsible for nitrogen fixation, affect the ability of the microorganism to fix nitrogen. In a separate investigation, Compaoré & Stal (2010) conclude that nitrogen fixation is primarily regulated by the availability of reducing equivalents within the heterocysts, suggesting a dynamic regulation of gas transport in and out of the heterocysts in response to temperature and oxygen concentration.

Alternatively, the reason might be attributed to the effect of temperature on gas solubility. In the case of diazotrophic cultivation, the available nitrogen is regulated by the equilibrium according to the Henry's law and, as the temperature rises, the gas solubility decreases. It is plausible that reduced solubility led to growth limitation due to the nutrient consumption kinetics. If this second hypothesis holds, it might suggest that the effect of temperature on nitrogen solubility should also be taken into account for kinetic growth modeling.

Photorespirometry has thus emerged as a valuable tool within the application of nitrogen-fixing cyanobacteria, revealing insights into the characteristic effect associated with the operating variable temperature. Although the mechanism leading to this response remains unclear, the retrieved kinetic parameters hold significant potential for application in predictive modeling.

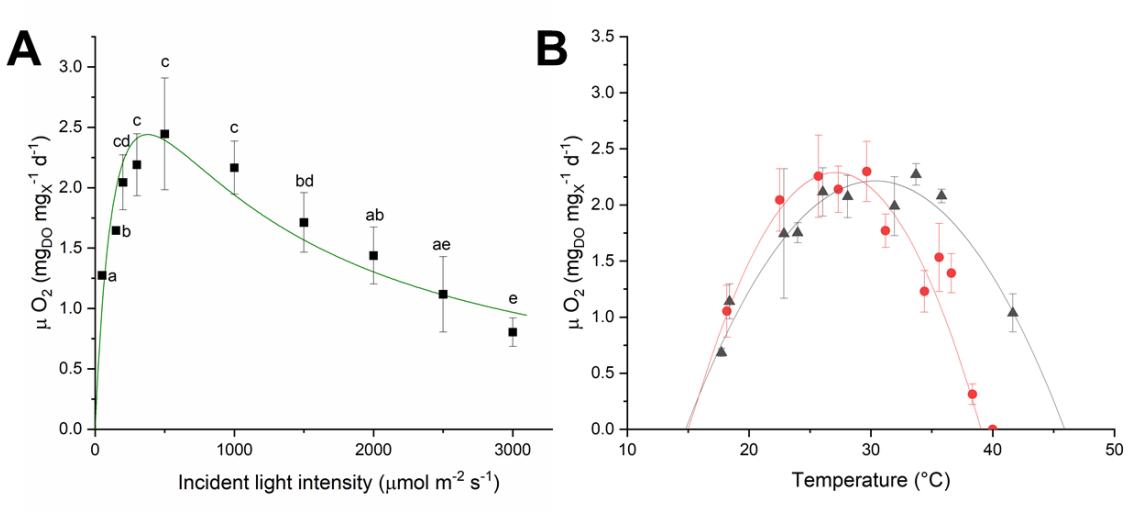

Figure 1: Net oxygen production rate at different incident light intensities (A) and temperatures (B). Red dots and grey triangles refer to BG11$_0$ and BG11, respectively. Fitted trends are depicted by continuous lines. Error bars represent the standard deviation. Data sharing the same letter do not show statistically significant differences (n ≥3).

Previous research emphasizes the need to find a compromise between conditions ensuring a high protein content in biomass and high biomass productivity, to achieve improved protein productivity (Lucato et al., 2024). In this regard, photorespirometry has proven to be an effective method for assessing growth- and biomass production-related aspects. Once the kinetic parameter values are retrieved, kinetic models describing the nitrogen-fixing cyanobacteria growth can be used to identify the optimal growth conditions. Accordingly, only the effect of operational variables on protein content remains to be studied. In this study, a nitrogen content around 8-10% of the biomass dry weight (data are not shown) was found, aligning with prior research (Lucato et al., 2024). These prior findings correlated such nitrogen content with protein content ranging between 30% and 50% of biomass dry weight, depending on the specific cultivation conditions.

4. Conclusions

In this study, the growth kinetics of the nitrogen-fixing cyanobacterium *Nostoc* PCC 7120 under different light intensities and temperatures have been investigated. Kinetic parameter values have been retrieved for both operating variables assessed by fitting the proper kinetic model to photorespirometric data. Although testing in more complex industrial cultivation systems to account for factors like photobioreactor design and light delivery is still necessary, photorespirometry is a quick method that allows for a reduction in the range of light intensity to be tested. This targeted approach not only saves time and resources but also translates to economic efficiency in industrial applications. Photorespirometry additionally unveiled temperature-dependent responses, differently impacting growth rates depending on nitrogen sources. High temperatures showed greater adverse effects under nitrogen-fixing conditions. These discrepancies pointed out potential mechanisms related to enzymatic and cellular activity, and gas solubility, which necessitate further investigation for comprehensive understanding. Findings underscored the significance of photorespirometry in revealing specific aspects associated with diazotrophic cultivation and provided essential kinetic parameters, which are useful for predictive modeling.

Nomenclature

BNF – Biological Nitrogen Fixation
μ – growth rate, d^{-1}
μ_{max} – maximum growth rate, d^{-1}
I – light intensity, μmol m^{-2} s^{-1}
K_I – half-saturation constant for light intensity, μmol m^{-2} s^{-1}
I_{opt} – optimal light intensity, μmol m^{-2} s^{-1}
PAR – Photosynthetically Active Radiation
T – Temperature, °C
T_{max} – maximum temperature, °C
T_{min} – minimum temperature, °C
T_{opt} – optimal temperature, °C
CSTR – Continuous Stirred-Tank Reactor
OPR – Oxygen Production Rate, mg_{DO} mg_X^{-1} d^{-1}
OCR – Oxygen Consumption Rate, mg_{DO} mg_X^{-1} d^{-1}

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