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Short communication

Photonfluxostat: A method for light-limited batch cultivation of cyanobacteria at different, yet constant, growth rates

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ABSTRACT

The growth rate and physiology of photoautotrophic bacteria are dependent on the incident light color and intensity. Here we report a widely applicable and straightforward method for light-limited batch cultivation of phototrophic bacteria at different, yet constant, growth rates. We illustrate its usage with *Synechocystis* sp. PCC6803, a model cyanobacterium used as a chassis for sustainable cell-factories and capable of turning CO_2 into commodity products. The cultivation method we developed resembles a 'photonfluxostat'. It enables the setting of the growth rate of phototrophs during batch cultivation by adjustment of the illumination intensity ('photon dosing'). Using this method to study the growth-rate response of *Synechocystis*, we found that while the cell volume increased, the chlorophyll *a* content and the PSI/PSII ratio decreased, as growth rate increased. This method allows for a quantitative and controlled study of the light-dependent physiology of phototropic bacteria, a highly relevant group of bacteria for modern biotechnology.

1. Introduction

Growth rate is an important characteristic of the physiological state of a bacterial population. Understanding how cells achieve balanced growth by coordinating the expression of thousands of genes for a variety of cellular processes while competing for resources under fluctuating environmental conditions, is still one of the most fundamental challenges in cell physiology [1]. From an evolutionary point of view, it is very intriguing to understand how and why cells have different metabolic strategies (*e.g.* with respect to catabolic efficiency) at different growth rates [2]. Such phenotypic traits have now been observed across all domains of life [3]. From an application point of view, control of microbial growth rate by modulation of nutrient availability (*e.g.* for medium optimization) can have a huge impact on product yield in industrial settings [4–6]. Therefore, detailing the correlation of growth rate with various cellular responses is of paramount importance for better understanding of cell physiology.

Cyanobacterial research has attracted considerable attention recently. Not only are cyanobacteria used as model organisms for understanding basic cellular processes, such as oxygenic photosynthesis [7,8], the circadian clock [9], and various stress responses [10], but also for the design of green cell factories for the sustainable production of compounds of interest [11,12]. This latter aspect is very pertinent as it aims to contribute to the transition towards a bio-based economy that would make our societies much more sustainable [13]. Dissecting the specific growth rate-dependent responses of cyanobacteria should be further explored, to allow further optimization of such sustainability applications. Unlike chemotrophic organisms, cyanobacteria use (sun)light as their primary energy source during autotrophic growth. Experiments in which growth rate is systematically varied as a function of light availability will help increase our insight into cyanobacterial physiology.

So far, in microbial physiology, the chemostat [14] and the turbidostat [15] have been the most widely applied cultivation devices in studies that involved the systematic variation of growth rate, both for chemotrophic and photoautotrophic organisms. For photo(auto)trophic organisms this implies the use of a constant incident light intensity, which, if intended to be non-limiting, will restrict workable cell densities to very low values, since light has to travel a considerable distance (*i.e.* several cm) through most planes of the culturing vessel. Occasionally, dynamic illumination regimes have been used, for instance to simulate a natural diel regime [16] and to maximize photosynthetic activity [17]. Recently, the turbidostat was used to characterize growth of *Synechocystis* sp. PCC6803 (hereafter *Synechocystis*) in a flat-panel photobiorector, where the effects of varying light quantity and color on cell growth were explored [18]. In addition, using a similar set up, the parameters related to the growth rate of *Synechocystis* with respect to circadian rhythms were systematically investigated [19]. The

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successful application of these cultivation strategies indeed broadens our understanding of cyanobacterial cell physiology, and inevitably, also raises relevant new questions. Turbidostats inevitably take longer than standard batch cultivations and require many additional control units [15]. Thus, the number of conditions and strains that can be investigated are limited. In this contribution we describe how to overcome this limitation of current cultivation methods.

Light supply, because of its inherent physical properties, and unlike conventional nutrients such as carbon or nitrogen sources, can be accurately manipulated dynamically with respect to color and intensity. This results in additional ways to achieve steady state exponential growth, for instance, *via* the possibility of a dynamic adjustment of the incident light intensity. We have explored this property to devise a simple and convenient cultivation strategy, which combines the flexibility of batch culturing with the ability of the chemostat to obtain stable physiological states (*i.e.* 'steady-states'). It allows for light-limited batch cultivation of cyanobacteria at different, yet constant, growth rates. The method can be understood in terms of the Herbert-Pirt equation, a classical relation in bacterial physiology. We applied this new cultivation method to study the relationship in *Synechocystis* between growth rate and relevant physiological parameters such as average cell size, chlorophyll *a* (Chl *a*) content and PSI/PSII ratio. The results obtained indicate that average cell size positively correlates with growth rate, whereas the cellular contents of Chl *a* and the PSI/PSII ratio, do not. These results illustrate the feasibility and advantages of this new method as an easier mean, in comparison with continuous cultivation systems, to obtain controlled phototrophic growth in the lab. Moreover, our method can be directly applied for the physiological characterization of other photo(auto)trophs in the future.

2. Mathematical framework

In microbiology, the Herbert-Pirt equation [20,21] expresses a relation between the specific uptake rate (q_s) of a limiting nutrient, the biomass-yield ($Y_{x/s}$) of the organism on this nutrient, the organism's specific growth rate (μ) and the maintenance requirement (m_s) of the cells for the growth-limiting nutrient to maintain cellular integrity,

$$q_s = \frac{1}{Y_{x/s}}\mu + m_s, \tag{1}$$

with q_s and m_s in $\frac{\text{mol substrate}}{\text{hr}\times\text{gram biomass}}$, $Y_{x/s}$ in $\frac{\text{gram biomass}}{\text{mol substrate}}$ and μ in hr^{-1}.

Eq. (1) expresses a steady-state flux relation: the specific substrate uptake rate (q_s) equals the substrate-uptake rate leading to growth, which amounts to $\frac{1}{Y_{x/s}}\mu$ moles of substrate per unit time per gram biomass, and the rate required for cellular maintenance, which is growth-rate independent, equals m_s moles of substrate per unit time per unit biomass.

We can extend this relation to the uptake of photons [22], which we study in this work. We choose to denote photons as substrate by *h*, such that Eq. (1) becomes.

$$q_h = \frac{1}{Y_{x/h}}\mu + m_h, \tag{2}$$

The total uptake rate of photons by a culture then equals $J_h = l\times q_h \times X$ with assumed *l* as a constant for photon loss specific to the cultivation set-up (absorption by medium, scattering, *etc.*) and *X* as the amount of biomass in grams, such that,

$$\begin{aligned} &\frac{J_h}{X} = l\times q_h = \frac{l}{Y_{x/h}}\mu + l\times m_h \\ &\Rightarrow \frac{J_h}{X} = q_h' = \frac{1}{Y'_{x/h}}\mu + m'_h \end{aligned} \tag{3}$$

This equation relates the biomass specific photon uptake rate, $\frac{J_h}{X}$, by a culture (*e.g.* cyanobacteria), controlled by the light-intensity (I_h) set by the experimentalist, to the specific growth rate of the organism, μ, the apparent (*i.e.* corrected for losses by scattering, absorption by medium components, *etc.*) photon yield, $Y'_{x/h}$, and the apparent (*idem*) maintenance requirement of photons, m'_h.

We use Eq. (3) to describe how the growth rate depends on the specific photon uptake rate by the culture $\frac{J_h}{X}$. We tailor this by setting light intensity, I_h in μmol photons $\text{m}^{-2}\ \text{s}^{-1}$, that we expose the culture to; hence, we assume that they are proportional (*i.e.* $J_h \propto I_h$). So, by setting the incident light intensity, corrected for the biomass density in the reactor, we also set the growth rate – as long as the culture is light-limited.

3. Practical implementation

The practical implementation of the mathematical framework presented above requires a cultivation set-up in which biomass density (here, optical density at 720 nm, OD_{720}) can be monitored frequently (within a few minutes); and subsequently, light intensity (I_h) can be modulated such that $q'_h = \frac{J_h}{\text{OD}_{720}} = Constant$. We decided to implement this new cultivation regime, here termed "photonfluxostat", in a Multi-Cultivator (MC1000-OD, Photon Systems Instruments, Czech Republic, Fig. S1) controlled remotely by a newly developed computer program. This device enables multiple parallel cultivations (up to 8) to run simultaneously, testing different strains and/or conditions independently under photonfluxostat conditions. The newly developed software was written in Python. It is completely open-source and made available at *https://gitlab.com/mmp-uva/pyCultivator* along with extensive documentation. We wrote a set of software packages to monitor and control a Multi-Cultivator. Its built-in OD sensor is capable of measuring at 680 nm and 720 nm and can be used *via* a serial link, which also supports changes in various settings such as light intensity and gas flow. Software configurations are stored in a XML file to enable automatic triggering of the software.

Generally, once all measurements are stored, the software calculates whether adjustments need to be made to the light settings. The light state can be configured to be conditional on time, to simulate day/night regimes, and the light intensity can be configured to be dependent on the measured OD values. The new light intensity is calculated such that $q_h' = constant$, while changes are only made when the difference between current and desired intensity is >1 μmol photons $\text{m}^{-2}\ \text{s}^{-1}$.

4. Results and discussion

We used the cultivation set-up described above to test specific growth-rate dependent responses of *Synechocystis*. We ran a total of 39 completely independent (parallel) photonfluxostat cultivations at different growth rates by varying the light intensity per OD_{720}. The OD_{720} was monitored every 5 min and the light flux (J_h, μmol photons $\text{m}^{-2}\ \text{s}^{-1}$) was adjusted such that the $q_h' = \frac{J_h}{X}$ (*i.e.* μmol photons $\text{m}^{-2}\ \text{s}^{-1}\ \text{OD}_{720}^{-1}$) remained constant. The photonfluxostat regime was maintained until the maximum irradiation capacity of the LED panel, equipped with "cool-white" LED (PSI, CZ), was reached. This means, depending on the photonfluxostat light regime imposed, which ranged from 20 up to 200 μmol photons $\text{m}^{-2}\ \text{s}^{-1}\ \text{OD}_{720}^{-1}$, that at least an OD_{720} of 1.5 was reached. Cultivations began with an initial OD_{720} of around 0.05, illuminated with a constant light intensity until OD_{720} reached 0.6, except for the light regime at 20 μmol photons $\text{m}^{-2}\ \text{s}^{-1}\ \text{OD}_{720}^{-1}$, for which we waited until OD_{720} 0.825. At this point, the different photonfluxostat regimes were imposed by adjusting the light intensity to the actual OD_{720} as explained above, and as exemplified for one light regime in Fig. 1A. Samples for offline measurements were collected at $\text{OD}_{720} \approx 1$.