Optimization of light and nutrients supply to stabilize long-term industrial cultivation of metabolically engineered cyanobacteria: a model-based analysis

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Abstract

Metabolically engineered cyanobacteria are promising photosynthetic cell factories to produce valuable compounds in view of a bio-based industry. However, when the producer population is affected by a production burden, it usually experiences genetic instability leading to cells that lose the production capability, here defined as retro-mutants, which in long-term take over the culture. Here we show that, by exploiting differences in nutrient and light use between these two phenotypes, in a continuous culture the operative conditions can be set to specifically select the producers. A mathematical model-based analysis used to investigate the effect of kinetic parameters shows that, in specific combinations of their values, a continuous stirred-tank reactor (CSTR) can be operated to favor the producer's growth. The feasibility of the approach proposed is discussed in the context of literature data. Based on overall mass and energy balance analysis, a new approach to stabilize the producer phenotype in long-term industrial cultivation is proposed.

Keywords: Cyanobacterial cell factories, genetic instability, mathematical modeling, metabolic engineering

1. Introduction

Cyanobacteria are versatile microorganisms able to exploit solar energy to convert CO_2 and water into biomass and compounds valuable for a bio-based industry [1,2]. In addition, cyanobacteria are promising cell factories, due to the simple protocols required for genetic modification, related to their capability of natural transformation and homologous recombination exploitable by metabolic engineering techniques [3,4]. The latter consist in the manipulation of the microbial metabolism through genetic engineering and synthetic biology tools, mostly by insertion of heterologous genes and overexpression and/or deletion of native genes, to direct the carbon and energy flux towards the formation of a product of interest [5]. In the last decade(s) this approach was successful for the heterologous production of a broad range of products of interest, such as ethanol [6,7], ethylene [8,9], isoprene [10,11], 1-butanol [12], 2-3 butanediol [13], limonene [14], and for the enhanced accumulation of native compounds of commercial value, such as pigments [15], polyhydroxybutyrate (PHB) [16], glycogen [17], cyanophycin [18], and fumarate [19] in cyanobacteria. Despite the potential of these biotechnological platforms, industrial applications of engineered cyanobacteria are still limited, and only few companies have developed engineered cyanobacteria based-processes for the industrial production of chemical compounds such as ethanol (for an overview see Ref. 20[20]), butanol (Phytonix Corporation, North Carolina), organic acids, sugars and amino acids (Proterro Inc, New Jersey; Photanol, The Netherlands).

One of the issues that needs to be addressed for the spreading of such engineered cyanobacteria at industrial scale is the drop in productivity due to retro-mutation events, namely the mutational suppression of the feature of interest, which has been observed in several cyanobacterial strains [8,21–25]. Indeed, when homoplasmy of the metabolically engineered cyanobacteria and full segregation of the modified genotype are achieved, the maintenance of the mutation of interest in the engineered strain during prolonged cultivation is often not verified. The possibility of losing the modified trait depends on the metabolic engineering approach used: in case of gene deletion, the loss of function of the target trait is reasonably null, because the chance of re-evolving the deleted trait is unlikely in short evolutionary time-scale; in case of gene insertion (by chromosome integration or expression vector), this becomes possible. When the metabolic engineering is aimed at improving the biomass growth, it is unlikely that the resulting strain is affected by a metabolic burden and consequently the chance of retro-mutation event is reduced. On the other hand, the chance of mutational loss-of-function increases when the production of the target compound is a metabolic burden to the producer cell, which uses part of the resources to obtain the target molecule [26–29]. Indeed, producer cells that

carry a genetic modification not associated with a competitive fitness advantage usually experience a metabolic burden related to product synthesis (i.e., the production burden), leading to genetic instability and degenerated/abortive production phenotype[30]. When the production of the target metabolite causes a fitness impairment, the cells that randomly lose their production capability and retrieve the wild type (WT) growth phenotype, hereafter referred to as retro-mutant cells, have a selective advantage against the producer cells. Hence, the retro-mutants will take over the population determining the drop of productivity. Recently, metabolic engineering strategies aiming at reducing the genetic instability related to the synthesis of target metabolites have been developed. One of these strategies relies on coupling the synthesis of the product of interest to the biomass formation, so that the production becomes mandatory for the microbial growth [31–33]. Another strategy consists in linking the end-product with an intermediary metabolite essential to the growth of the producer cells (i.e., metabolic addiction) and in engineering feedback control genetic circuits conferring a competitive growth advantage to the overproduction strain (i.e., feedback genetic circuits) [34,35].

Recently, it has been demonstrated that, in the presence of a production burden affecting the growth rate of the producers, the occurrence of a retro-mutation event will lead, after a certain number of generations during the cultivation, to the take-over of the culture by the retro-mutants, irrespective of the continuous or semi-continuous cultivation mode adopted [36]. This result was achieved assuming that the production burden affecting the growth of the producers is independent of the growth conditions adopted (e.g., concentration of nutrients, light availability, etc.). However, some differences in the growth performances between the producer and the retro-mutant strains at different growth conditions might exist. In the case of metabolically engineered cyanobacteria, the culturing conditions favoring the growth of the producers with respect to the retro-mutants could be exploited in a continuous stirred-tank reactor (CSTR) to give an advantage to the producer population, similarly to what previously suggested for selecting a microbial mutant with respect to its parental strain [37]. In fact, in a CSTR working at steady state, microbial growth is stabilized as well as other physiological acclimation traits [38]. The microbial selection in continuous culture, based on the establishment of specific growth conditions favoring the producers, would thus represent a potential way to stabilize the long-term cultivation of metabolically engineered cyanobacteria, overcoming their intrinsic genetic instability and ultimately increasing their effective application as photosynthetic cell factories at industrial scale.

In this work, an assessment of the variables and operative conditions in continuous cultures that may stabilize the cyanobacterial producer phenotype were explored through a mathematical modeling

analysis. This analysis is based on the hypothesis that possible differences in the exploitation of nutrients and light may occur in a population of metabolically engineered cyanobacteria, as a side-effect of the specific mutation introduced. It is well known that the modification of a metabolic pathway can affect the overall metabolism of the engineered strain [39], which may consequently show differences with respect to the WT in nutrient uptake or, in case of photosynthetic microorganisms, in light exploitation. Nowadays, however, in the literature there is still a general lack of studies reporting extensive characterization of metabolically engineered cyanobacteria, which are often characterized exclusively for the specific modified trait or only partially in terms of mass and energy balances. This makes difficult a fine-tuning of the operative parameters of continuous culturing that could allow the microbial selection of the producers with respect to the retro-mutants. Here we propose a mathematical model-based methodology that, by exploring different scenarios of nutrients and light utilization possibly displayed by the engineered cyanobacteria and the parental strains, is able to identify the operative conditions inside a CSTR to stabilize the long-term growth of the producer phenotype affected by a production burden, despite the random appearance of retro-mutants. For this work, we derived inspiration from previous studies on the competition among different species for the same substrate grown in a continuous cultivation system [37,40,41]. Our investigation relies on the fact that if the producer strain has characteristics sufficiently different either in nutrient uptake and exploitation or in light utilization compared to the retro-mutant, then it can be positively selected under nutrient-limitation or specific light conditions, respectively. If these differences are experimentally observed in metabolically engineered cyanobacteria suffering from a production burden grown under different nutrient concentrations and light intensities, then the set of operative conditions deduced in our study for nutrients and light growth parameters can be exploited to stabilize their growth overcoming their genetic instability, ultimately preventing the retro-mutants to outcompete the producers during long-term cultivation periods. The feasibility of this approach was discussed in the context of the current literature, reporting data about the nutrient and light utilization ability of engineered cyanobacteria.

2. Mathematical models

The genetic instability of metabolically engineered microorganisms is a well-known issue that limits their adoption in industrial processes [26,28]. The instability usually consists in the loss of the trait inserted for the production of the compound of interest by the engineered strain, which retrieves the WT growth phenotype and here is referred to as the retro-mutant strain.

In Battaglino *et al.* [36], it has been shown that the appearance of retro-mutants in a metabolically engineered population is completely determined by two intrinsic properties of the engineered strain. Precisely, the number of microbial generations after which half of the population is retro-mutated depends only on the burden on the growth caused by the production of the compound of interest and by the probability that a beneficial mutation occurs, whatever the cultivation conditions are. This result has been achieved simulating the semi-continuous serial batch transfer and the continuous chemostat and turbidostat cultivation systems, among which the chemostat is appealing for an industrial scale-up [42,43]. However, in Battaglino *et al*.[36] just a proportionality factor, represented by the metabolic burden, has been used to model the differences in the metabolism of the engineered and the retro-mutant strains overlooking possible complex responses to factors such as nutrients concentration and light availability, deeply discussed in Section 3.

In the following, a CSTR operated in chemostat mode has been studied by means of the population dynamics model [36,44]:

$$\begin{cases} \frac{\mathrm{d}p}{\mathrm{d}t} = \mu_p(1-m)p - Dp \\ \frac{\mathrm{d}w}{\mathrm{d}t} = \mu_w w + \mu_p m p - Dw \end{cases} \quad (1)$$

where $p(t)$ and $w(t)$ are, respectively, the engineered and the retro-mutant biomass densities at time instant t, μ_p and μ_w are the growth rates of the producer and retro-mutant populations, respectively, m is the specific mutation rate of the considered strain and D is the chemostat dilution rate. Different constitutive relations were considered for the growth rates, in order to analyze possible metabolic changes occurring in the engineered strain. Physical variables and parameters used in this work are listed in **Table 1**.

Table 1. Physical quantities used in this paper.

Symbol	Quantity	Unit
p	Biomass density of the engineered strain (subscript for the engineered strain)	mg L^{-1}
w	Biomass density of the retro-mutant strain (subscript for the retro-mutant strain)	mg L^{-1}
μ	Growth rate	d^{-1}

m	Specific mutation rate	-
D	Dilution rate	d^{-1}
ρ	Production burden	-
μ_{max}	Maximum growth rate of the retro-mutant strain	d^{-1}
c	Nutrient concentration	mg L^{-1}
k	Half-saturation constant (nutrient)	mg L^{-1}
I	Light intensity	μmol photons m^{-2} s^{-1}
k_i	Half-saturation constant (light)	μmol photons m^{-2} s^{-1}
i_{opt}	Optimal light intensity	μmol photons m^{-2} s^{-1}
α	Ratio of the half-saturation constants (light)	-
β	Ratio of the optimal light intensities	-

2.1. Proportional growth rates

A common approach to model the differences in the metabolism of the engineered and the retro-mutant strains is to assume proportional growth rates [28,45],

$$\mu_p = (1-\rho)\mu_w, \quad (2)$$

being $\rho \in (0,1)$ the production burden.

In Battaglino *et al*. [36] it has been proven that if the two strains were related according to (2), then in a continuous (as well as in a semi-continuous) cultivation system the engineered strain would eventually disappear leading to the loss of productivity. To check the stability of the growth of the engineered strain in a CSTR operated in chemostat mode, it is sufficient to analyze (1) at steady-state assuming a positive biomass density of the engineered strain (i.e., $p > 0$). Thus, the first equation of system (1) can be rewritten as

$$D = \mu_w(1-\rho)(1-m) \quad (3)$$

that substituted in the second equation of the system leads to

$$\frac{p}{w} = \frac{(1-\rho)(1-m)-1}{(1-\rho)m} \tag{4}$$

This ratio is negative, but this is physically impossible, because the biomass densities p and w must be non-negatives. This fact proves that the model (1) with the constitutive relation (2) at steady-state admits only solutions with $p = 0$, and so that the appearance of the retro-mutant strain cannot be avoided, whatever the operative conditions adopted for the chemostat system are. Actually, it is possible to guarantee a long-term stable growth of the engineered strain only when $\mu_p\,(1 - m) > \mu_w$.

2.2. Nutrient-limited growth

The different metabolism of the two strains can be modelled with more detail by assuming different parameters in the law governing the growth rates. In the case of a nutrient-limited growth, the Monod model[46] can be written as

$$\mu_w(c) = \mu_{\max}\frac{c}{c+k_w}, \qquad \mu_p(c) = (1-\rho)\mu_{\max}\frac{c}{c+k_p} \tag{5}$$

where c is the nutrient concentration in the culture broth, $\mu_{\max}$ is the maximum growth rate of the retro-mutant strain, $\rho \in (0,1)$ is the production burden in saturating nutrient conditions and k_w and k_p are the half-saturation constants for the retro-mutant and the engineered strains, respectively.

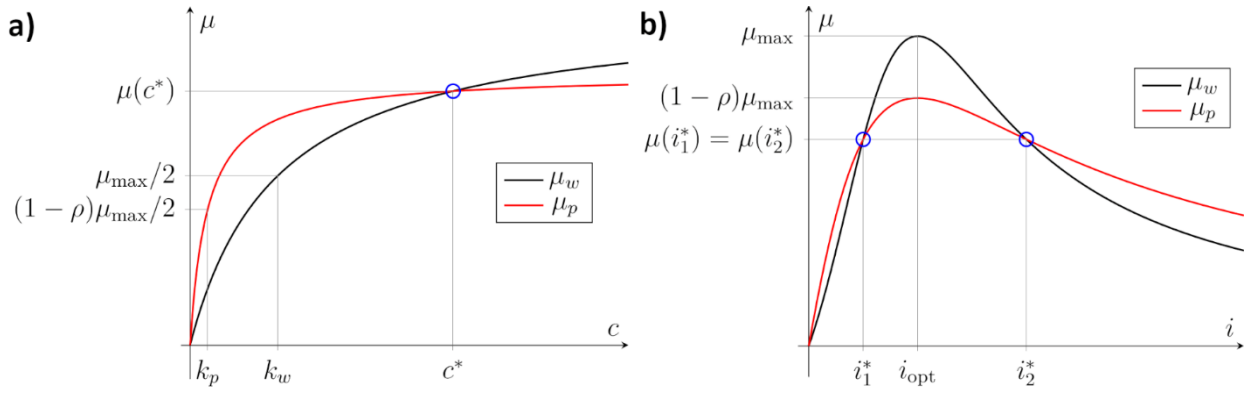

Fig. 1. Trends of the growth rates of the producers and the retro-mutants with different nutrient concentrations and light intensities. (**a**) Plot with the response to the nutrient concentration c according to

the Monod model[46] (eq.5); (**b**) plot with the response to the light intensities *i* according to the model by Bernard, et al [47] (eq. 8).

In order to stabilize the permanence of the engineered strain in the bioreactor, the nutrient concentration should be such that, despite the production burden, the condition $\mu_p\ (1 - m) > \mu_w$ is verified. As visible in **Fig. 1a**, this inequality is possible if the half-saturation constants for the two strains are sufficiently different. Indeed, this occurs if a critical nutrient concentration $c^* > 0$ at which the growth rates are equal exists, namely

$$c^* = \frac{(1-m)(1-\rho)k_w - k_p}{1-(1-m)(1-\rho)} > 0 \quad (6)$$

from which the condition $k_p < (1 - m)\ (1 - \rho)\ k_w$. It is worth noting that the mutation rate *m* is a very small quantity, in the range of $10^{-8} \div 10^{-5}$ for prokaryotic organisms [48,49] , with respect to the production burden, in the range of $0.15 \div 0.40$ [8,25,28,50]. Thus, the latter inequality can reasonably be approximated by

$$k_p < (1 - \rho)k_w \quad (7)$$

From the first equation of system (1), at steady-state there is a positive biomass density of the engineered strain $p > 0$ only if $D = \mu_p\ (1 - m)$. Thus, being μ_p a monotonically increasing function of *c* and being *m* value very small, if condition (eq. 7) is verified, it is possible to guarantee a long-term stable growth of the engineered strain in the chemostat CSTR when the dilution rate *D* is kept lower than the critical growth rate $\mu_p(c^*)$, since for that values $\mu_p > \mu_w$ (**Fig. 1a**).

2.3. Light-limited or inhibited growth

Photosynthetic microorganisms are photoautotrophs that require a specific light level to reach the maximum growth rate, referred to as saturation light level. Indeed, their metabolism is strictly dependent on the light intensity. A low light intensity, below the saturation level, limits the growth of the microorganisms (i.e., light-limitation), whereas a high light intensity, above the saturation level, inhibits their growth (i.e., photoinhibition). A plethora of models, mostly equivalent from a qualitative viewpoint, that take into account both the limiting and the inhibiting effects of light have been proposed in the literature [47,51,52]. In this work, the model by Bernard et al. [47] has been chosen because

of the explicit biological interpretability of its parameters. Thus, for photosynthetic microorganisms in saturating nutrient conditions,

$$\mu_w(i) = \mu_{\max} \frac{i}{i + k_w\left(\frac{i}{i_{\mathrm{opt},w}} - 1\right)^2}, \qquad \mu_p(i) = (1-\rho)\mu_{\max} \frac{i}{i + k_p\left(\frac{i}{i_{\mathrm{opt},p}} - 1\right)^2} \tag{8}$$

where i is the available light intensity within the reactor, $\mu_{\max}$ is the maximum growth rate of the retro-mutant strain, $\rho \in (0,1)$ is the production burden in optimal light conditions and the couples (k_{i_w}, $i_{\mathrm{opt},w}$) and (k_{i_p}, $i_{\mathrm{opt},p}$) are the half-saturation constants and the optimal light intensity parameters for the retro-mutant and the engineered strains, respectively.

Since the stability condition is studied by analyzing the system of equations (1) at steady-state, the culture in this growth phase can be considered photo-acclimated to the light intensity experienced within the photo-bioreactor, which is assumed to be run under constant and continuous illumination (e.g., artificial LED illumination). It means that the constitutive relations (8) describe, at each light intensity, the growth rate at steady-state of the strains photo-acclimated to that specific light intensity.

A well-known issue that affects the industrial scale-up of photo-bioreactors is the heterogeneity of light intensity within the CSTR. However, several papers can be found in the literature that deal with this issue and suggest strategies to take into account the light attenuation profile in estimating the average light intensity experienced within CSTRs of different geometries[53,54] Because of the increased number of parameters with respect to the Monod model, the analysis of the stability of the engineered strain in system (1) with constitutive relations (eq. 8) is split in two steps. First, the two strains are assumed to have different half-saturation constants, but the same optimal light intensity. Then, the optimal light intensities are assumed different in the two strains, as well.

2.3.1. Strains with different half-saturation constants and same optimal light intensity

Similarly to what discussed previously in section 2.2, the stable permanence of the engineered strain in the chemostat CSTR is possible if the available light intensity is such that, despite the production burden, $\mu_p (1 - m) > \mu_w$. This inequality is effectively approximated by $\mu_p > \mu_w$, which simplifies the computation maintaining the practical relevance.

Fig. 1b shows the growth rates of the two strains in response to different light intensities when their optimal light intensities are equal (i.e., $i_{\mathrm{opt}} = i_{\mathrm{opt},w} = i_{\mathrm{opt},p}$), suggesting that an operative light intensity

range in which the latter inequality holds can exist only if there are critical light intensities i^* at which the growth rates are equal.

The critical light intensities i^* solve the second-order polynomial equation

$$\underbrace{(\sigma-\alpha)\frac{k_w}{i_{\text{opt}}^2}}_{a} i^{*2} + \underbrace{\left(-\rho - 2(\sigma-\alpha)\frac{k_w}{i_{\text{opt}}}\right)}_{b} i^* + \underbrace{(\sigma-\alpha)k_w}_{c} = 0, \quad (9)$$

where the ratio between the half-saturation constants is denoted by $\alpha = k_{i,p}/k_{i,w}$ and the complement of the production burden by $\sigma = 1 - \rho$. The coefficients of the polynomial from the higher degree to the lower are denoted for simplicity as a, b and c. Thus, the critical light intensities exist only if $b^2 \geq 4ac$, that is equivalent to the condition

$$\sigma - \alpha \geq -\frac{i_{\text{opt}}}{4k_w}\rho \quad (10)$$

From (eq. 10), it can be deduced that b has a negative value, because a non-negative b would lead to the inequality

$$\sigma - \alpha \leq -\frac{i_{\text{opt}}}{2k_w}\rho \quad (11)$$

which cannot hold true under condition (eq. 10). Since the sign of a and c is always the same (i.e., $ac > 0$) and $b < 0$, it is trivial to see that $-b \pm \sqrt{b^2 - 4ac} > 0$. Thus, the critical light intensities exist and are positive if and only if a is positive. Indeed, $a > 0$ is equivalent to $\sigma - \alpha > 0$, which implies (10) and is equivalent to

$$k_p < (1-\rho)k_w \quad (12)$$

The critical light intensities are denoted by $i_1^* < i_{\text{opt}} < i_2^*$, as indicated in **Fig. 1b**. As discussed previously in section 2.2, at steady-state there is a positive biomass density of the engineered strain $p > 0$ only if $D = \mu_p (1 - m)$. Thus, being m value very small, if condition (eq. 12) is verified, then it is possible to guarantee a long-term stable permanence of the engineered strain in the chemostat CSTR when the dilution rate D is kept lower than the critical growth rate $\mu_p(i_1^*)$ or $\mu_p(i_2^*)$, depending on the supplied light intensity, since for that values $\mu_p > \mu_w$ (**Fig. 1b**).

2.3.2. Strains with different half-saturation constants and different optimal light intensities

If the producers and retro-mutants have different optimal light intensities, the ratio between these intensities can be introduced as $\beta = i_{opt,p}/i_{opt,w}$. In this case, the critical light intensities i^*, which induce the same growth rate in the engineered and the retro-mutant strains, solve the second-order polynomial equation

$$\left(\sigma - \frac{\alpha}{\beta^2}\right)\frac{k_w}{i_{opt,w}^2} i^{*2} + \left(-\rho - 2\left(\sigma - \frac{\alpha}{\beta}\right)\frac{k_w}{i_{opt,w}}\right) i^* + (\sigma - \alpha)k_w = 0 \qquad (13)$$

Differently from the previous case, a simple condition for the existence of positive solutions to (13) cannot be found analytically. Indeed, a numerical investigation shows that the existence and the sign of the critical light intensities depend on k_{i_w} and $i_{opt,w}$, as well as on σ, α and β. Moreover, in this case it is possible to have zero, one or two critical light intensities.

The different solutions are illustrated in **Fig. 2**, where the trends of the growth rates of the producers and retro-mutants are reported for three values of β. The kinetic parameters deduced from Cordara et al. [55] refer to the WT *Synechocystis* sp. PCC6803 and were used for the retro-mutant strain. Moreover, it has been assumed a production burden $\rho = 0.15$ and a ratio between the half-saturation constants $\alpha = 1$. When β is sufficiently lower than 1 (**Fig. 2a**), namely the optimal light intensity of the engineered strain is shifted towards left, two critical light intensities $i_1^* < i_2^*$ appear such that the stability condition $\mu_p > \mu_w$ is fulfilled for light intensities ranging from i_1^* to i_2^*. For values of β around 1 (**Fig. 2b**), the growth rate of the engineered strain is lower than that of the retro-mutant strain at any available light intensity. Finally, when β is sufficiently greater than 1 (**Fig. 2c**), namely the optimal light intensity of the engineered strain is shifted towards right, a single critical light intensity i^* appears such that the stability condition $\mu_p > \mu_w$ is verified for light intensities higher than i^*.

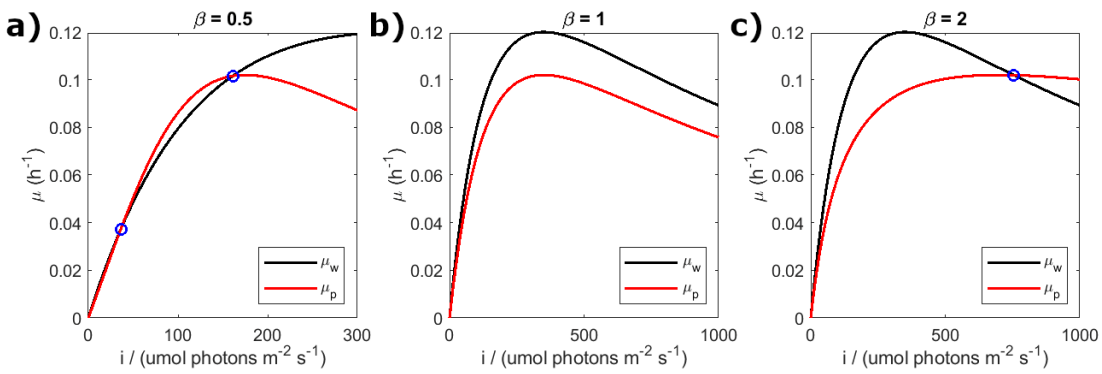

Fig. 2. Trends of the growth rates of the producers and the retro-mutants with different optimal light intensities. Three different ratios of optimal light intensities have been tested: (**a**) $\beta = 0.5$, (**b**) $\beta = 1$, (**c**) $\beta = 2$. The plots have been obtained with kinetic parameters experimentally measured in the WT *Synechocystis* sp. PCC6803 ($\mu_{max} = 0.12$ h^{-1}, $k_w = 100$ μmol photons m^{-2} s^{-1}, $i_{opt,w} = 350$ μmol photons m^{-2} s^{-1}) [55], assuming $\rho = 0.15$ for its corresponding generic producing strain and $\alpha = 1$.

A complete representation of the light conditions able to guarantee the stable growth of an engineered strain taken as a case study is shown in **Fig. 3**. This diagram has been calculated using kinetic parameters experimentally measured in the *Synechocystis* sp. PCC6803 WT [55] and assuming a $\rho = 0.15$ for its corresponding generic producing mutant. For each couple of values of β and i, **Fig. 3** reports the highest value α^* of the parameter α such that the stability condition $\mu_p > \mu_w$ is fulfilled at the given light intensity i. Thus, for a given value of the parameter α, the couple β and i satisfies $\mu_p > \mu_w$ if and only if they fall in a region where $\alpha^* > \alpha$. In the white regions, with i ranging from about 160 μmol photons m^{-2} s^{-1} to 750 μmol photons m^{-2} s^{-1} with the adopted parameters, the inequality cannot be satisfied, independently of the values of α and β. In the case of $\alpha = 1$, the region with $\alpha^* > 1$ recalls what has been qualitatively described in the previous paragraph with reference to **Fig. 2**. It is worth noting that by changing the considered species or the values of the parameters, the diagram reported in **Fig. 3**, including the amplitude of the white region, would be different.

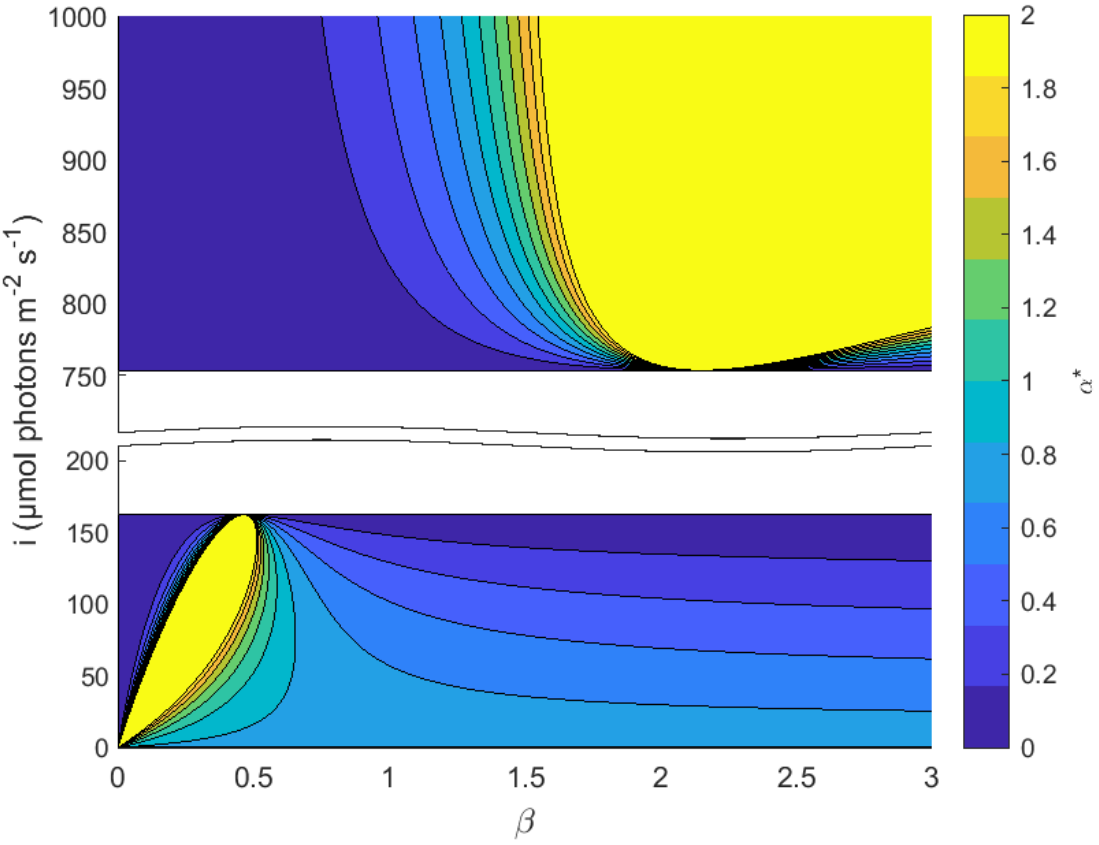

Fig. 3. **Example of diagram of the light intensity intervals in which $\mu_p > \mu_w$ for given values of α and β.** Each point in the diagram corresponds to a specific value for the parameter β and a specific light intensity i, which satisfy the inequality $\mu_p > \mu_w$ if and only if the parameter α is lower than or equal to the value α^* identified by the colormap in that point. The scale of the colormap has been arbitrarily saturated at 2. In the white regions, the inequality can never be verified. This diagram has been obtained using kinetic parameters experimentally measured in the WT *Synechocystis* sp. PCC6803 (k_w = 100 µmol photons m^{-2} s^{-1}, $i_{opt,w}$ = 350 µmol photons m^{-2} s^{-1}) [55], assuming ρ = 0.15 for its corresponding generic producing strain.

3. Discussion

As shown in the mathematical modeling analyses reported in section 2.2 and 2.3, differences in nutrient or light utilization by the engineered producing and retro-mutant strains may be exploited to properly set the culturing operative conditions aimed at guaranteeing the maintenance of the producer phenotype in long-term cultivations in a CSTR system. If the engineered producing strain is able to use a nutrient or light source differently from the retro-mutant, the resources can be externally provided in specific conditions appropriate to select and maintain the producing population and wash out from the CSTR the eventually occurring retro-mutants. Comprehensive analysis on the utilization

of macro-nutrients and light by metabolically engineered cyanobacteria in comparison with the retro-mutant ones, or at least with the WT, is generally lacking in the literature. Indeed, common approaches of mutant characterization often neglect the overall mass and energy balance for the nutrient- and light-dependent growth of the strain, while just focusing on the specific trait subjected to mutation. Nevertheless, a number of indications of possible differences on the kinetic growth parameters of engineered cyanobacteria compared to the WT is present in the literature, apparently supporting our thesis.

In the following, a detailed analysis of the open literature has been performed. The analyzed studies concern photosynthetic microorganisms engineered for the production of both native and heterologous compounds. Precisely, the analysis was focused on producing strains showing a dependence of the growth rate on a certain nutrient concentration or light-intensity, indicating a possible variation of their metabolic burden under determined growth conditions. Also strains engineered to produce a higher biomass were taken into account. This last case is not suitable to validate our hypothesis, since these mutants are not affected by a production burden with respect to the WT, thus in a continuous cultivation system the producers will be naturally selected and the retro-mutation, even if possible, will not result in a predominance of the retro-mutant population. Nonetheless, the selected papers related to this last kind of mutants show evidence that the engineering of photosynthetic microorganisms may introduce differences in the kinetic growth parameters of the mutant with respect to the parental strain that can be found out only through an exhaustive characterization of the strains during the mutant screening at lab-scale. Based on the information available, here we report a quantitative elaboration or a qualitative discussion of the significant data present in the literature.

3.1. Mutants with different nutrient uptake and exploitation: working in a nutrient-limited chemostat CSTR

As a consequence of what discussed in section 2.1 and demonstrated in section 2.2, to stabilize the growth of the producer phenotype, the producing and retro-mutant populations should have a different half-saturation constant for a particular nutrient, as a resulting side-effect of the introduced mutation. More precisely, the half-saturation constant of the producer must be lower than that of the retro-mutant multiplied by the complement of the production burden ($k_p < (1 - \rho)\ k_w$). To assess experimentally the existence of such condition, the producer and the retro-mutant strains should be grown at different concentrations of a certain nutrient. To corroborate the relevance of our proposed

mathematical model, a detailed survey of the current literature was carried out aimed at searching for experimental data demonstrating the occurrence of different kinetic parameters between the engineered and the parental strains.

An example of a different use of phosphorus between the WT and a cyanophycin engineered strain affected by a production burden is present in the study by Trauttman *et al* [18]. To induce the accumulation of cyanophycin (multi-L-arginyl-poly-L-aspartate), a nitrogen and carbon reserve polymer in cyanobacteria and a product of biotechnological interest as source of amino acids and polyaspartic acid, a *Synechocystis* sp. PCC6803 mutant (BW86) was engineered to express a constitutively active version of a single transduction protein involved in the arginine biosynthesis that is the precursor of cyanophycin [18]. The growth of the mutant and WT strains under phosphate starvation was subsequently evaluated, attesting the presence of a production burden in the engineered mutant and different growth parameters in the two strains [56]. This phenomenon is justified by the regulation of the cyanophycin production, which is well known to be boosted under phosphorus depletion [18], suggesting that the regulation of phosphorus uptake and utilization is linked to the metabolic pathways of cyanophycin production. To quantify the different phosphorus exploitation by the engineered BW86 strain and the WT, we based our calculation of the half-saturation constants on the original data shown in Trautmann *et al*. [56], reporting the initial and final biomass concentration obtained under several phosphorus concentrations for both strains. On our data elaboration (**Fig. S1** and **Fig. S2**, Supporting Information), the growth rate was calculated considering the final biomass concentration provided in the original experimental data (Table 1 in Trautmann *et al*. [56]). It should be noted, however, that the growth rate we calculated may not correspond to that of the exponential phase. From the elaboration of these data, the half-saturation constant for phosphorus appears to be different between the BW86 strain and the WT (**Table 2**). A discussion of the measurement conditions under which the observed differences are statistically relevant is provided in the Supporting information (**Fig. S3**). These differences suggest that a change in the metabolism of a nutrient may occur in mutants designed to over-accumulate native molecules of biotechnological interest. As discussed previously, this characteristic can be exploited by managing the operating conditions of the chemostat CSTR, so to limit the possible prevalence of the retro-mutant population. Precisely, since the inequality $\rho < (k_w - k_p) / k_w$, obtained by inverting (eq. 7), seems to be verified for the mutant BW86 ($\rho = 0.10$, $(k_w - k_p) / k_w = 0.44$), the engineered culture can be stably maintained working with a dilution rate $D < 0.31$ d^{-1} (elaboration reported in **Fig. S2** of Supporting Information and **Table 2**). Beside the dilution rate, another operative condition to be accounted for in the design

of the CSTR cultivation system is the supply of the nutrient, which should be selected in the proper range of concentration. Assuming a CSTR, in particular, it should be recalled that the concentration of the nutrient relevant in the reaction term of the balance is that one at the outlet. To select it, the equations for the biomass (eq. 1) should be solved along with the mass balance for the nutrient, which is linked to the former through the species-specific biomass yield term.

Table 2. Values of kinetic parameters obtained for the wild type (WT) and engineered BW86 strain as a function of phosphorus retrieved from data by Trauttman *et al*.[56]. The coefficient of determination (R^2) refers to the fitting from which the kinetic parameters are retrieved.

Parameter	Value	Unit	R^2
$\mu_{\max}$ (WT)	0.40	d^{-1}	0,843
k_w (WT)	0.32	mg L^{-1}	
$(1-\rho)\mu_{\max}$ (BW86)	0.36	d^{-1}	0,944
k_p (BW86)	0.18	mg L^{-1}	

Other papers corroborate, even though only in a qualitative way, the hypothesis that different kinetic parameters as a function of a specific nutrient may exist between the mutant and the parental strains. Recently, several *Synechocystis* sp. PCC6803 mutants have been engineered by gene deletion and over-expression to over-accumulate poly-β-hydroxybutyrate (PHB) [24], a native carbon-storing polymer in cyanobacteria. These strains showed lower growth rates compared to the WT in the tested growth conditions, suggesting that a production burden due to the modulation of the PHB pathway is present. On the other hand, a different exploitation of nitrogen and phosphorus nutrients between the engineered strains and the WT also appeared, even though the data collected in this study are not sufficient to estimate the value of the kinetic parameters for these two nutrients in the different strains. Nevertheless, the differences in the consumption of these two macro-nutrients displayed by the engineered strains and the WT during the batch cultivation experiments [24] suggest that different half-saturation constants for nitrogen and phosphorus may occur in the strains.

Other *Synechocystis* sp. PCC6803 mutants for the over-production of PHB have been obtained via transconjugation with expression vectors carrying *pha* genes for PHB synthesis [57]. In this work, some differences at level of CO_2 utilization between the WT (KZ_WT) and the mutant strain (KZ_EC) might be present. Indeed, the biomass growth of the KZ_EC mutant was lower compared to that of the KZ_WT with a CO_2 supply of 0.04 %, thus indicating the presence of a metabolic burden in this

growth condition, but the two strains showed similar biomass increase with a CO_2 supply of 2 %. From a modeling point of view, this means that, as the growth rate ratio changes under different carbon concentrations, a different half-saturation constant for carbon should be expected between the WT and the engineered strain. Hence, based on the possible difference of kinetic parameters for producer and retro-mutant pairs, with the producer affected by a burden, it is suggested that under a controlled supply of carbon source, the predominance of the producer strain in a chemostat CSTR could be achieved. Unfortunately, data from Hondo *et al.* [57] are not sufficient to retrieve the values of the parameters for CO_2 kinetics, and it is not clearly proved if managing the concentration of CO_2 makes it possible to compensate the slower kinetic due to the burden in the producer strain.

In the work by Gupta *et al.* [58], the distinct over-expression in the cyanobacterium *Synechococcus* sp. PCC7002 of two bicarbonate transporters, SbtA and BicA respectively, increased the growth rate of the two mutants and their accumulation of glycogen as storage molecule. Also this study revealed a difference in CO_2 utilization between the engineered strains and the WT. Even though the engineered strains do not seem to experience a production burden, because they show a higher biomass productivity at any tested CO_2 concentration compared to the WT, the different kinetic parameters for CO_2 suggest that an overall balancing of the metabolism in the engineered strains over-expressing the SbtA and BicA transporters might had occurred. An analogue behavior with respect to the supplied CO_2 was observed in the study by Kamennaya *et al.* [59], in which a bicarbonate transporter (BicA) and its point-mutational version ($BicA_{T485G}$) were separately over-expressed in the cyanobacterium *Synechocystis* sp. PCC6803 leading to the generation of two mutants with a higher biomass productivity compared to the WT. Also in this case, growth experiments, performed under different percentages of CO_2 supplied, highlighted different half-saturation constants between the mutants and the WT.

In summary, the strategy of managing the nutrient supply, as well as setting the proper dilution rate and operating conditions in a chemostat, seems to be a key approach to overcome the instability of producer strains affected by a production burden in the perspective of an industrial long-term cultivation of relevant producer strains. Moreover, we would like to point out that our study has interesting insights also for scientists that need to properly set up experiments at lab-scale for screening the engineered strains. In this perspective, it is evident the importance of addressing the mass balance of the overall growth of the selected mutants, with particular attention to the side-effects of the mutation on the nutrient exploitation, since this feature could be exploited for the maintenance of the desired phenotype in long-term industrial cultivation. A particular focus might be addressed to

nutrients whose allocation is somehow involved in the formation of the product of interest by the engineered strain. Indeed, these nutrients might display a relevant effect on the growth rate of the producer with respect to the parental strain (e.g. phosphorus for the cyanophycin over-accumulating *Synechocystis* mutants[18,56]).

3.2. Mutants with different light exploitation: working with specific light intensities in a chemostat CSTR

If the approach of controlling nutrient supply to stabilize the producer population is a general methodology for all the engineered microorganisms, the photosynthetic nature of cyanobacteria opens a new range of possibilities, related to the effect of different light intensities on the operating performances of an industrial CSTR. Similarly to what discussed for the utilization of macro-nutrients, the engineered strains might show a dependency of the growth performance on the light intensity supplied as a side-effect of a mutation introduced to produce a molecule of interest. These differences can be exploited to maintain the desired phenotypic trait in long-term cultivations, as described in section 2.3, based on possible differences in α and β values, as described in section 2.3.2. Similarly to what previously highlighted for nutrients, also the information about light utilization capabilities of the producer strains is not well represented in the literature. Nevertheless, some evidence of the existence of different kinetic parameters with respect to light supply between the producers and the parental strain can be found. An interesting example was reported by Chaves *et al.* [11], where an isoprene synthase (IspS) enzyme fused in different configurations with a highly expressed native protein was introduced in *Synechocystis* sp. PCC 6803, resulting in several mutants with increased production of isoprene. The authors, performing light saturation curves of photosynthesis through oxygen evolution measurements (i.e., photosynthesis versus irradiance (PvI) curves), observed different oxygen evolution rates for the five mutants analyzed, with respect to the WT, resulting in a half-saturation constant ranging from 145 to 200 µmol photons m^{-2} s^{-1} for the formers, instead of 100 µmol photons m^{-2} s^{-1} for the latter. It is important to note that this experiment was carried out without photo-acclimating the cells to each measuring light intensity used in the oxygen evolution test. Although the analysis of these data shows that metabolic differences between the engineered and the retro-mutant strains might exist, for a reliable quantitative measurement of the kinetic parameters related to the light, it would be necessary to perform PvI curves only on cells previously photo-acclimated to each testing light intensity in combination with growth rate measurements, as done for instance in the works by Cordara *et al.*[55] or by Gupta *et al.*[58]. Indeed, it

was demonstrated that when cells are photo-acclimated at light intensities differing by one order of magnitude, the subsequent PvI curves obtained are different among these cultures, leading to different values of the kinetic parameters[60]. In case of availability of kinetic growth parameters properly collected highlighting a sufficiently different light utilization between the engineered strain suffering from a burden and the parental strain, then a set of operative parameters that guarantee the predominance of the producer strain in a chemostat CSTR may be looked for according to our approach.

In general, most of the papers reporting mutations of the photosynthetic apparatus, aiming at increasing the photo-conversion and biomass production, show that the resulting engineered strains are not affected by a production burden. This is the case of two studies by Dall'Osto *et al.* [61] and Kirst *et al.* [62] aimed at limiting the over-absorption of sunlight by the photosystems of single cells to increase the light penetration in the inner regions of the photobioreactor and, thus, enhance the biomass productivity of the whole culture. In these studies, the strain engineering procedure, consisting in random mutagenesis and selection in the microalga *Chlorella vulgaris* [61] and targeted deletion in the cyanobacterium *Synechocystis* sp. PCC6803 [62], generated pigment deficient mutants with a truncated antenna system (i.e., respectively, reduced light harvesting complex II and phycobilisome), which are not affected by a production burden. Nevertheless, in these papers the analysis of the microbial growth behavior highlighted that this engineering strategy may lead to different light half-saturation constants for the mutant and the WT, supporting the feasibility of the modeling approach here proposed.

An example of differences in both the half-saturation constant and the optimal light intensity between the engineered strain and the parental strain is present in the work by Gupta *et al.* [58] mentioned above in section 3.1. In this study the glycogen accumulating *Synechococcus* sp. PCC7002 mutants that over-express the bicarbonate SbtA (A) and BicA (B) transporters, not affected by a production burden, fortuitously showed a different growth rate compared to the WT strain when grown at different light intensities, thus suggesting a possible alteration of their capability of light usage and tolerance. As the growth experiments shown in this paper required several days, a photo-acclimation of the cells can be reasonably assumed, thus allowing to retrieve properly the kinetic growth parameters. Even though these engineered strains do not present a burden compared to the parental strain, both of them showed a greater difference in the optimal light intensity i_{opt} when compared to the WT (elaboration reported in **Table 3 and Fig. S4 in** Supporting Information), leading to β equal to 0.60 and 0.83 for mutants A and B, respectively. As the values of half-saturation constant are not

particularly different from the WT (**Table 3**), α has values near to 1 (0.99 and 0.93 for mutant A and B, respectively). Another example of differences in the kinetic parameters related to the light exploitation is present in the work by Thiel *et al.* [63], where a production burden in the engineered strain is not evident anyway. This study evaluated the possibility to re-direct the photosynthetic electron flux towards the accumulation of intracellular carbon sink compounds (sucrose, glycogen and PHB) in *Synechocystis* sp. PCC6803 by deleting the flavodiiron protein Flv3 from the parental sucrose producing strain (i.e., S01, carrying an over-expression of the sucrose permease CscB). The results in this study suggest that the deletion of Flv3 in the double-mutant strain (S01:Δ*flv3*) determined a higher half-saturation constant k_p and $i_{opt,p}$ compared to the parental strain (S01) (elaboration reported in **Fig. S5** and **Table S1**, Supporting Information). However, it should be noted that in this case the parameters were retrieved from PvI curves obtained from oxygen evolution measurements, which showed to be affected by acclimation phenomena to light intensity. For this reason, we suggest to measure kinetic parameters from growth curves, preferably from cultures cultivated in continuous reactors, that allow the acclimation of the cells to the light intensity provided [38].

In summary, even though the papers discussed in this section are not fully applicable to our approach, because they do not show cases of mutants suffering from a production burden, they provide evidence that differences in α and β values between the mutant and parental strains may occur, suggesting that they can be exploited also for mutants with burdens. The different growth characteristics should be accounted for the cultivation of promising producer strains showing a metabolic burden, by setting the proper operative conditions in a chemostat CSTR to possibly push the maintenance of the phenotype of interest in long-term cultivation. It should be noted that this approach can also be extended to non-photosynthetic microorganisms, as the model by Bernard *et al.* [47] here used is equivalent to the Haldane one for substrate inhibition [64], which is generally valid for heterotrophic organisms. Accordingly, the approach here proposed for the case of light utilization can be extended also to other cases, such as that of toxic substrates.

Besides knowing the trophic mode of the microorganism of interest, it is clear that an exhaustive characterization of the growth behaviour of the mutant strain is of pivotal importance to better define the real feasibility of using metabolically engineered photosynthetic microorganisms as microbial cell factories in the industrial sector.

Table 3. Values of kinetic parameters obtained for the wild type (WT) and the SbtA-over-expressing (A) and BicA-over-expressing (B) strains as a function of light intensity. Elaboration carried out on data published by

Gupta *et al.* [58] . The coefficient of determination (R^2) refers to the fitting from which the kinetic parameters are retrieved.

Parameter	Value	Unit	R^2
$\mu_{\max}$ (WT)	0.7	µmol mg(Chl)$^{-1}$ h^{-1}	
k_w (WT)	29.9	µmol photons m^{-2} s^{-1}	0.999
$i_{\mathrm{opt},w}$ (WT)	457	µmol photons m^{-2} s^{-1}	
$\mu_{\max,\mathrm{A}}$ (A)	0.8	µmol mg(Chl)$^{-1}$ h^{-1}	
k_{pA} (A)	29.5	µmol photons m^{-2} s^{-1}	0.983
$i_{\mathrm{opt},pA}$ (A)	273	µmol photons m^{-2} s^{-1}	
$\mu_{\max,\mathrm{B}}$ (B)	0.8	µmol mg(Chl)$^{-1}$ h^{-1}	
k_{pB} (B)	27.9	µmol photons m^{-2} s^{-1}	0.998
$i_{\mathrm{opt},pB}$ (B)	379	µmol photons m^{-2} s^{-1}	

Conclusions

The issue of retro-mutation in long-term industrial cultivation of metabolically engineered cyanobacteria was assessed, with the aim of identifying the proper operative conditions in a continuous reactor able to specifically select and maintain the producer population in the system. Based on a mathematical model approach, it was demonstrated that the critical issue represented by the occurrence of retro-mutation in metabolically engineered cyanobacteria can be overcome by exploiting specific traits of the engineered strains related to nutrient or light utilization. According to the specific kinetic features, proper operating conditions can be set in a CSTR at steady state to sharpen the differences in growth capabilities of the producer strain with respect to the retro-mutant populations, resulting in a selection of the strain of interest that guarantees its long-term permanence in continuous culturing systems. The proposed approach relies on an exhaustive knowledge of the overall mass and energy balances of the engineered strain in terms of nutrients and light exploitation that need to be performed *a priori* during the selection phase of the producer mutants at lab-scale, as it can affect the long-term stability of the production during the scale-up.

The method presented in this paper, tailored for the continuous cultivation of metabolically engineered cyanobacteria, can be easily extended to any metabolically engineered microorganism showing different uptake and exploitation of nutrients, or characterized by a substrate inhibition

kinetic, as well as to systems where growth kinetics with co-limiting factors occurs, for which an analytical condition for the long-term stability could not be obtained.

Author contributions

AA, ES and AB conceived the original idea. AA performed the mathematical analysis. BB and CP searched and critically analyzed the literature. ES elaborated and discussed the literature data. BB drafted section 1, AA drafted section 2, BB and ES drafted section 3. CP critically revised the biological part of the manuscript. AB critically revised the final version of the manuscript. All authors discussed the results and contributed to the final manuscript.

Supporting Information

Elaboration of experimental data about growth as a function of nutrient and light between different mutant strains

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