We recently assessed the metabolism of *Synechocystis* sp PCC6803 through a constraints-based reconstruction and analysis approach and identified its main metabolic properties. These include reduced metabolic robustness, in contrast to a high photosynthetic robustness driving the optimal autotrophic metabolism. Here, we address how these metabolic features affect biotechnological capabilities of this bacterium. The search for growth-coupled overproducer strains revealed that the carbon flux re-routing, but not the electron flux, is significantly more challenging under autotrophic conditions than under mixo- or heterotrophic conditions. We also found that the blocking of the light-driven metabolism was required for carbon flux re-routing under mixotrophic conditions. Overall, our analysis, which represents the first systematic evaluation of the biotechnological capabilities of a photosynthetic organism, paradoxically suggests that the light-driven metabolism itself and its unique metabolic features are the main bottlenecks in harnessing the biotechnological potential of *Synechocystis*.

**Introduction**

The development of renewable energy sources has received significant interest in recent years owing to the depletion of fossil fuels, ever-increasing demand for energy, and concerns over climate change. A promising source of renewable energy is the recycling of CO2 into usable fuels and fine chemicals by photosynthetic organisms using solar energy. There are, however, increasing concerns over the methods currently in use for producing biodiesel from crops and biomass. The problems include high production costs and a reduction in the amount of land available for growing edible crops. These issues highlight the need for a new generation of biofuel technology.1,2 Cyanobacteria possess several properties, which make them promising candidates for sustainable bioenergy generation. They are the only prokaryotes capable of carrying out oxygenic CO2-fixation photosynthesis with higher efficiency than vascular plants.3-5 The cultivation of cyanobacteria is simple, inexpensive and it does not compete directly with agricultural crops for land or water. In addition, they are a source of natural high-value products, such as carotenoids, lipids, and vitamins.6,7 They are also amenable to genetic manipulation.8,9 These features have motivated recent engineering efforts of cyanobacteria for producing valuable chemicals and biofuel-like compounds from the main biosynthetic building blocks, establishing a proof of concept of direct biofuel production from oxygenic photosynthesis.10-18 However, with a few exceptions19,20 the productivity has been very low compared with heterotrophic organisms.21

We recently reconstructed a genome-scale metabolic model of the cyanobacteria *Synechocystis* sp PCC6803 (iJN678).22 The model was used to study in detail the photosynthetic process under different light and inorganic carbon conditions as well as under genetic perturbations. The systems analysis identified two main states of the photosynthetic apparatus:
while enforcing the maximal growth rate;
thus, revealing growth-coupled designs.
By repeating this process many times,
a list of different knockout designs was
obtained. The number of knockouts was
varied between 3 and 25, and the number
of repeats was between 5 × 10^6 and 5 ×
10^7. The knockout search was performed
under both autotrophic and mixotrophic
conditions, employing a light-limited state
(LLS) where the photon uptake rate was
fixed to 30 mmol.gDW⁻¹.h⁻¹ and a car-
bon-limited state (CLS) where the pho-
ton uptake rate was fixed to 100 mmol.
gDW⁻¹.h⁻¹. The HCO₃⁻ uptake rate was
set to 3.7 mmol.gDW⁻¹.h⁻¹ in all cases and
for mixotrophic conditions, the glucose
uptake rate was set to 1 mmol.gDW⁻¹.h⁻¹.
Heterotrophic conditions were analyzed
by setting the photon uptake rate to zero.
The iJN678 model includes 678 genes,
which results in a very large search space
and to make the search more manage-
able, a pre-processing step for reducing the
number of target genes was performed. By
A CO₂ limited state (CLS) and a light-
limited state (LLS). In order to explore the impact of these
unique metabolic features and to obtain
a better understanding of the opportu-
nities and bottlenecks offered by cya-
obacteria in biotechnology, we present
here the first analysis of the metabolic
engineering capabilities of Synechocystis
using iJN678. Employing an approach
analogous to those previously used for
in silico-driven metabolic engineering in
heterotrophic organisms,25 we analyzed
how the electron and carbon flux can be
funneled to the overproduction of both
native (fumarate, ethanol, sucrose, and
H₂) and non-native compounds (l-lactate
and 1-butanol).

Figure 1. A depiction of the central metabolism of Synechocystis. Native and non-native experimentally overproduced metabolites in Synechocystis
are represented by black and grey squares, respectively and the metabolites analyzed in this study are indicated by red lines. The carbon partition-
ing (in %) to sugar, lipids and terpenoid biosynthesis together with the predicted carbon flux distribution (normalized to the CO₂ uptake rate) under
autotrophic conditions is also shown. The non-native metabolites are 1-butanol (1-BUT), lactate (LAC), isobutyraldehyde (IBAL). The abbreviations for
the native metabolites are given in Nogales et al. 22

The above metabolites were chosen as
representatives of key points in the metab-
olism and/or because they have already
been overproduced in Synechocystis (Fig. 1).
Growth-coupled production designs were
attempted since they represent a stable
phenotype, allowing for an easy selec-
tion of the overproducing strains.26 Flux
balance analysis (FBA)24,25 was used to
predict flux values in both the wild type
and the mutants. The search for mutants
was performed using a randomized ver-

tion of the strategy described in Nogales
et al.26 Using gene-protein-reaction asso-
ciations, which specify via Boolean rules
the gene product(s) catalyzing a reaction,
a mutant was created by randomly knock-
out of a fixed number of genes and there-
fore, disabling flux through the affected
reaction(s). An FBA was performed to
determine the maximum growth rate of
the mutant, followed by another FBA to
determine the maximum product rate.
Finally, a third FBA was performed to
determine the minimum product rate,
while enforcing the maximal growth rate;
thus, revealing growth-coupled designs. By
repeating this process many times, a list of different knockout designs was
obtained. The number of knockouts was
varied between 3 and 25, and the number
of repeats was between 5 × 10⁶ and 5 ×
10⁷. The knockout search was performed
under both autotrophic and mixotrophic
conditions, employing a light-limited state
(LLS) where the photon uptake rate was
fixed to 30 mmol.gDW⁻¹.h⁻¹ and a car-
bon-limited state (CLS) where the pho-
ton uptake rate was fixed to 100 mmol.
gDW⁻¹.h⁻¹. The HCO₃⁻ uptake rate was
set to 3.7 mmol.gDW⁻¹.h⁻¹ in all cases and
for mixotrophic conditions, the glucose
uptake rate was set to 1 mmol.gDW⁻¹.h⁻¹.
Heterotrophic conditions were analyzed
by setting the photon uptake rate to zero.
The iJN678 model includes 678 genes,
which results in a very large search space
and to make the search more manage-
able, a pre-processing step for reducing the
number of target genes was performed. By
CO2), however, the theoretical maximal moles of carbon of metabolite/mole of ≈ predicted to be almost identical (Table 1). Under autotrophic conditions, we found to 5% of the maximal growth achieved under each growth condition. In Table 1, the yields were found - - - 0.292 None found - - - 0.878 None found - - - 0.017 mmol gDW-1 h-1 under the CLS (Table 1). Since, pyruvate decahydroxylation and alcohol dehydrogenase II genes from the obligatory ethanol producing Zymomonas mobilis have previously been introduced in cyanobacteria for overproducing ethanol,14,27 our results suggest that the production rate in these recombinant strains could be improved by blocking PTAr and/or ACKr. Taken together, the results obtained under autotrophic conditions and the low yields found experimentally strongly suggest that a re-routing of the carbon flux is more difficult to achieve in photosynthetic organisms than in heterotrophs, such as E. coli.27

To give additional support to this hypothesis, and to investigate whether the autotrophic metabolism itself and/or the overall metabolic network of Synechocystis is responsible for this phenomenon, we searched for overproducing mutants under heterotrophic conditions with glucose as the sole carbon and energy source. Several growth-coupled knockouts were identified.

**Results**

Under autotrophic conditions, we found that the photosynthetic states determined the theoretical maximum production of the metabolites under consideration. In the LLS, the yield per carbon of molecule of metabolites with neutral or positive oxidation states, e.g., fumarate, sucrose, and lactate, was significantly higher (Fig. 2; Table 1). Under the LLS, the yields were predicted to be almost identical (≈0.95 moles of carbon of metabolite/mole of CO2), however, the theoretical maximal production rate of both 1-butanol and ethanol increased significantly suggesting that the production of the reduced compounds is limited by the light availability under the LLS. The maximum production of H2 was much higher in the CLS, which is consistent with the excess of light and the photolytic origin of the electrons used to reduce the protons. The search for knockout mutants for overproducing fumarate, ethanol, 1-butanol, sucrose, or L-lactate under autotrophic conditions was very challenging. For fumarate, a maximal production rate of 0.043 mmol gDW−1 h−1 in the LLS and of 0.069 mmol gDW−1 h−1 in the CLS were achieved with a deletion of the gene slr0018, which encodes for fumarase (FUM) (Table 1). These predicted production rates are significantly lower than those reported in computational studies with heterotrophic bacteria,29 but similar to the in vivo yields found for other overproduced metabolites in cyanobacteria.30 While cyanobacteria have successfully been engineered to produce ethanol, 1-butanol, sucrose, and L-lactate under autotrophic conditions, by expressing heterologous enzymes,31,33,34 our search did not reveal any growth-coupled mutants overproducing these metabolites. However, mutant strains able to produce small amounts of metabolites at their maximum growth rates were identified in some cases. For instance, the deletion of either slr2132, which encodes for phosphate acetyltransferase (PTAr), or slr1299 encoding for acetate kinase (ACKr), resulted in a theoretical maximum production of ethanol of 0.010 mmol gDW−1 h−1 under the LLS and 0.017 mmol gDW−1 h−1 under the CLS (Table 1).
for all the metabolites analyzed. In addition, very high yields were predicted, ranging from 75% of the maximum production rate for fumarate and succrose to more than 95% for ethanol, 1-butanol, and lactate (Table 2; Fig. 2). In fact, the maximum yields for ethanol, lactate, and fumarate were 1.9; 1.85 and 1.14 mmol/ mmol of glucose, respectively, in the same range as those predicted in silico for E. coli and by using a similar number of knockouts. These findings indicate that it is the light-driven metabolism, rather than the metabolic network itself, that is responsible for the lack of success in obtaining growth-coupled mutants under autotrophic conditions.

*Synechocystis* is able to grow mixotrophically with the auto- and heterotrophic metabolism occurring concurrently. We simulated this condition in order to analyze the effects of the simultaneous presence of glucose and light on the production yields. The mixotrophic metabolism behaved similarly to the heterotrophic metabolism and provided much more flexibility for re-routting the carbon flux, compared with the autotrophic metabolism. High yielding growth-coupled knockouts were found for all the metabolites, in both the LLS and the CLS (Table 3; Fig. 2). However, these yields, ranging from 20–50% of the maximum production under the LLS and from 10–40% under the CLS, were markedly lower than those found under heterotrophic conditions. The mixotrophic mutants were found to share several interesting features: First, many equivalent overproducing mutants were predicted in the two photosynthetic states but the excess of light in CLS led to a significant decrease in the number of overproducing mutants. Under the CLS, the AEF pathways are essential for growth due to their role in redox balancing and they cannot be blocked simultaneously. This could indicate that the essentiality of the AEF pathways under the CLS limits the biotechnological potential of *Synechocystis* in this state. Second, the blocking of key photosynthetic reactions, including photosystems I (PSI) and II (PSII), the cytochrome b6f (CBFC) or ferredoxin NADP+ oxidoreductase (FNOR), as well as several AEFs (leading to reduced photosynthetic robustness) was required to couple the production of the target metabolite to growth. Third, in most of the cases, the overproducing mutants were non-viable under autotrophic conditions and glucose was used as the sole carbon source. Consistently and with few exceptions, the theoretical maximum production and the growth rates of the mixotrophic mutants were within the range predicted for the heterotrophic condition (Fig. 2). Fumarate was a notable exception and several mutants were found to be in the range corresponding to the LLS and the CLS mixotrophic states.

![Figure 2. Production envelopes for wild-type and knockout *Synechocystis* strains.](image_url)

The production envelopes for each metabolite is shown as a function of the biomass production rate of the wild-type *Synechocystis* network under heterotrophic (black lines), autotrophic LLS (dark green lines), autotrophic CLS (light green lines), mixotrophic LLS (red lines) and mixotrophic CLS (blue lines), as well as the growth-coupled deletion mutants identified (dots). The number of growth-coupled knockouts found in each condition is shown in brackets.
the photosynthetic processes do indeed limit the possibilities in re-routing of the carbon flux and consequently the overproduction of the target metabolites.

Photohydrogen production has previously been reported in Synechocystis and other cyanobacteria. In order to explore how the electron flux can be re-routed under different growth conditions, we extended our analysis to search for H₂ overproducing mutants. Several mutants with high production rates were found in all the growth conditions. The yields of H₂ were highest under autotrophic and mixotrophic conditions. The overproducing mutants found under heterotrophic conditions were beyond the scope of this study and the conclusions may not apply to other metabolites or compounds of interest. In addition, it must be taken into account that we have mainly explored the growth-coupled biotechnological capabilities of Synechocystis and that alternative production strategies were beyond the analysis of the Synechocystis network under different growth condition presented here and the comparison of the results with those obtained previously with heterotrophic bacteria, offers a general view of the biotechnological capabilities of this cyanobacterium.

Several conclusions can be inferred from this study. First, we have found that the re-routing of the carbon flux in Synechocystis under autotrophic conditions is significantly more challenging than under hetero- or mixotrophic conditions.

Table 2. Properties of the growth-coupled overproducer designs under heterotrophic conditions

| Metabolite | Maximum production rate | Number of knockouts | Growth rate | Production rate | BPCY |
|------------|-------------------------|---------------------|-------------|----------------|------|
|            | (mmol gDW⁻¹ h⁻¹)        |                     | (h⁻¹)       | (mmol gDW⁻¹ h⁻¹) |      |
| Fumarate (4C) | 1.462                   | 4                   | 0.045       | 0.719          | 0.0324 |
| Ethanol (2 C)   | 1.901                   | 2                   | 0.035       | 1.218          | 0.0426 |
| 1-Butanol (4C)  | 0.951                   | 4                   | 0.036       | 0.559          | 0.0199 |
| Sucrose (12 C)  | 0.443                   | 5                   | 0.062       | 0.053          | 0.0033 |
| Lactate (3 C)   | 1.867                   | 3                   | 0.023       | 1.444          | 0.0366 |
| H₂             | 10.502                  | 7                   | 0.019       | 3.195          | 0.062 |

LLS, light limiting state; CLS, carbon limiting state; BPCY, biomass-product coupled yield. Numbers inside parenthesis represent the wild type growth rate.

Table 3. Properties of the growth-coupled overproducer designs under mixotrophic conditions

| Metabolite | Maximum production rate | Number of knockouts | Growth rate | Production rate | BPCY |
|------------|-------------------------|---------------------|-------------|----------------|------|
|            | (mmol gDW⁻¹ h⁻¹)        |                     | (h⁻¹)       | (mmol gDW⁻¹ h⁻¹) |      |
| Fumarate (4C) | 2.351                   | 6                   | 0.091       | 0.487          | 4.607 |
| Ethanol (2 C)   | 3.092                   | 10                  | 0.099       | 0.0441         | 7     |
| 1-Butanol (4C)  | 1.546                   | 5                   | 0.038       | 0.591          | 0.0222 |
| Sucrose (12 C)  | 0.732                   | 5                   | 0.03        | 0.295          | 0.0767 |
| Lactate (3 C)   | 3.085                   | 6                   | 0.029       | 1.008          | 0.0291 |
| H₂             | 18.554                  | 11                  | 0.112       | 2.697          | 33.452 |

LLS, light limiting state; CLS, carbon limiting state; BPCY, biomass-product coupled yield. Numbers inside parenthesis represent the wild type growth rate.
The total fixed CO2 is funneled to sugar distribution, in which up to 80–85% of conditions. The constrained carbon flux that, in contrast to the carbon flux, the electron flux can be manipulated more easily.

Growth-coupled production is an attractive strategy in metabolic engineering. It is achieved by reducing the metabolic robustness of the host organism by deleting competing pathways, while the biosynthetic pathway of the target metabolite remains as the sole carbon and/or electron sink in the network. This way, the overproduction of the target compound is required for the organism to grow. The presented results strongly suggest that, while the high photosynthetic robustness required for optimal autotrophic metabolism allows flexible re-routing of the electron flux, it might also act as a non-desirable electron and carbon sink.

Combined with low metabolic robustness inherent to cyanobacteria networks, this may hamper the possibilities for re-routing the carbon flux, thus, limiting the biotechnological capabilities of Synechocystis.

Disclosures of Potential Conflicts of Interest

The authors declare that they have no conflict of interest.

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