Supporting Information

Title

Exploiting day- and night-time metabolism of *Synechocystis* sp. PCC 6803 for fitness-coupled fumarate production around the clock.

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Methods

Batch Cultivation

Batch cultivation was performed in a Multi-Cultivator (MC1000-OD, PSI, Czech Republic), with light supplied by a "cool-white" LED panel (PSI, CZ), of which the output intensity was controlled. BG-11 supplemented with 10 mM TES-NaOH (pH = 8.0) was used for *Synechocystis* cultivation at 30 °C and bubbled with a mix (v/v) of 99 % N_2 and 1% CO_2 at a flow rate of ~150 ml min^{-1}. Pre-cultures ($OD_{730} \approx 2$) from shake flasks were used for inoculation of the Multi-Cultivator cultures, after dilution to an OD_{730} of 0.05 and a working volume of 60 ml. Continuous light was supplied at a fixed light intensity of 30 μmol photons m^{-2} s^{-1} after inoculation, and 120 μmol photons m^{-2} s^{-1} when OD_{730} reached 0.5. Samples were taken daily, where OD was recorded and supernatant was prepared.

Concerning photonfluxostat experiments[1], a cultivation approach that allows cell growth at different yet constant growth rates, all the cultivation conditions were the same as for batch cultivation except for the light intensity settings. Light intensity was 30 μmol photons m^{-2} s^{-1} after inoculation. When OD_{720} (measured through the build-in OD sensor of the Multi-Cultivator at 720 nm) was above 0.6, light intensity was automatically adjusted every 5 min to ensure light intensity per OD_{720} was constant. This light regime was maintained until maximum capacity of the LED panel was reached. Specifically, the light regimes applied were 32.5, 35, 37.5, 42.5, 45, 52.5, 55, 57.5, 70, 80, 90, 100 μmol photons m^{-2} s^{-1} OD^{-1}. Samples were taken every a few hours during this phase, where OD_{730} was measured and fumarate concentration was quantified.

Day-night Batch Cultivation

Using a custom-made software package as previously reported[1], a day-night regime was simulated by creating a dependency between light intensity and time described by the function:

$$I = A \sin\left(\frac{2\pi\tau}{P} + X_{offset}\right) + Y_{offset}$$

Where I being the desired intensity, A the amplitude of the regime, τ the time since the start of the regime (in seconds) and P the period of the regime (in seconds). Using the X_{offset} the start position in the regime can be adjusted, while the Y_{offset} changes the ratio between day and night. We parameterized the dependency such that the intensity oscillates between -1 and 1 (A = 1) in combination with a cut-off – setting negative values to zero. This effectively creates a sinusoidal regime oscillating between 0 and 1. We then set P to 86400 seconds to create a 24-hour period and in order to smoothly transit from continuous light to day-night, we started the regime at midday by setting the X offset to 1.570796. The Y offset was left to 0, giving us a 12h – 12h period with a peak at 1.

Since the regime oscillates between 0 and 1 we can interpret the output as percentage intensity, combined with the photonfluxostat regime we get a rhythm where at midday we get 100% of the intensity given by the photonfluxostat regime. The usage of the photonfluxostat allows one to create a region during which growth rate achieved at each stage of a day period is relatively stable over multiple days.

The cultivation conditions in the L/D experiments were the same as in the photonfluxostat cultivation except for the gas composition and light intensity settings. Instead of 99% N_2 mixed with 1% CO_2, 99% Air was used with 1% CO_2, to allow for photorespiration to happen. After inoculation the light intensity was set to 30 µmol photons m^{-2} s^{-1}. When the OD_{720} (measured through the build-in OD sensor of the Multi-Cultivator at 720 nm) was above 0.3

the photonfluxostat regime was started, in order to automatically adjust the light intensity to keep the ratio of light intensity over biomass constant at 100 µmol photons m^{-2} s^{-1} OD_{720}^{-1}. 1 day after inoculation the day-night regime was configured to start at midday 16:00, therefore every day thereafter dawn occurred at 10:00 and dusk at 22:00. Samples were taken daily immediately before dawn and immediately after dusk for OD measurement and fumarate quantification.

Prolonged Cultivation

We studied the genetic stability of our strains in populations maintained under turbidostat conditions[2]. In this continuous cultivation method, microbial populations are kept at a fixed biomass density by diluting the culture with fresh medium at the same rate as the populations grows. This feedback loop applies a strong selection pressure on cells to grow at the maximal specific growth rate achievable. The turbidostat setup used in this experiment is based on a modified Multi-Cultivator, equipped with additional pumps (Reglo ICC, ISMATEC, Germany) for the transfer of fresh medium to the cultures, and subsequently, to a waste container (*i.e.* as in a classical chemostat). The "pycultivator" software package[1] that controls the Multi-Cultivator and adjunct hardware, activates the pumps to dilute the cultures if the selected OD_{720} threshold is reached[1]. Cells from pre-cultures in shake flasks were inoculated at OD_{720} ~0.05 in 4 independent cylindrical vessels of the Multi-Cultivator, using the same conditions as specified before, except for the incident light intensity, which was fixed at 100 µmol photons m^{-2} s^{-1}. The OD_{720} was recorded every 5 min. When the threshold of $OD_{720} > 0.6$ was reached, cultures were diluted by 8% (v/v) with fresh BG11. Strain stability was assessed by monitoring growth rate and fumarate- and lactate-production at regular time points. Samples for exometabolite production were collected periodically throughout the cultivation period. The variation in production rate, expressed in percentage, was calculated relative to the one observed at the beginning of the cultivation experiment.

Fumarate quantification

To determine the extracellular fumarate concentration, at least a 500 µL aliquot was sampled at target time points. Cells were removed through centrifugation for 10 min at 15,000 rpm at 4 ºC. The resulting supernatant was then filtered (Sartorius Stedin Biotech, minisart SRP 4, 0.22 µm) for sample preparation. Fumarate concentration was measured by HPLC-UV/VIS (LC-20AT, Prominence, Shimadzu), with ion exclusion Rezex ROA-Organic Acid column (250x4.6 mm; Phenomenex) and UV detector (SPD-20A, Prominence, Shimadzu) at 210 nm wavelength. 10 µL of the HPLC samples was injected through an autosampler (SIL-20AC, Prominence, Shimadzu), with 5 mM H_2SO_4 as eluent at a flow rate of 0.15 ml min^{-1} and a column temperature of 45 ^oC. The retention time of fumarate is about 17 min in this system (Fig. S1).

Lactate quantification

Lactate was quantified through an enzymatic assay (L-Lactic Acid Assay Kit Megazyme) as previously reported[3].

Figures

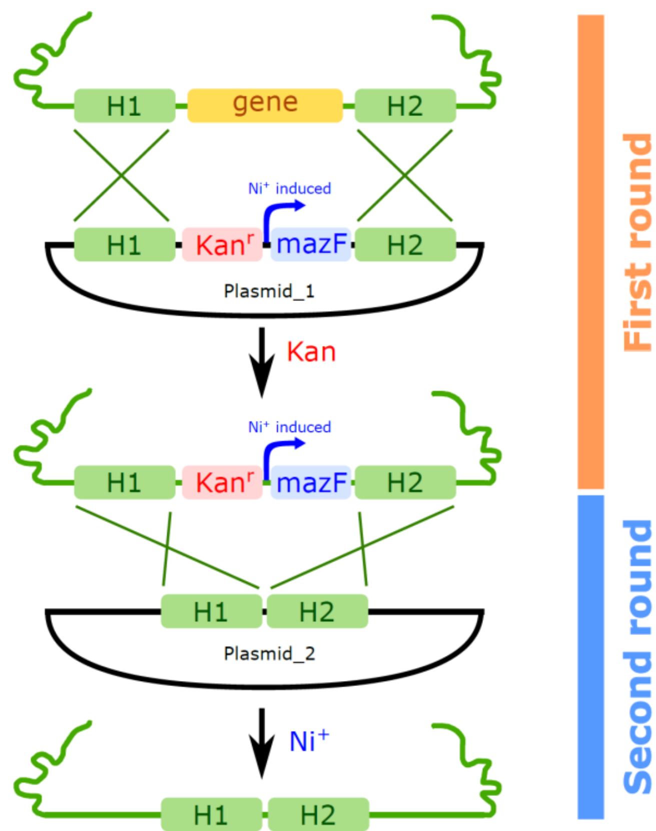

Figure S1 Schematic drawing of the *Synechocystis* markerless mutant construction via the *mazF* counter-selection method[4]. H1 and H2 indicate the upstream and downstream homologous regions of the target gene, respectively.

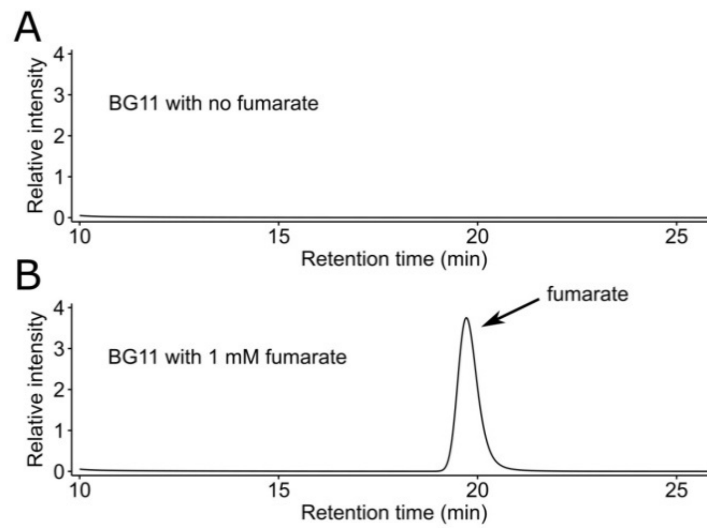

Figure S2 Fumarate assay using HPLC. (A) Elution curve for standard BG-11 with no fumarate addition. (B) Elution curve for standard BG-11 supplemented with 1 mM di-sodium fumarate.

Tables

Table S1 OptKnock predicted rates of fumarate production after further gene deletion in the *ΔfumC* background under night-time conditions (i.e. heterotrophic growth on glucose) and the predicted growth rate of such construct in day-time.

Gene(s)	Night-time Fumarate Yield (mmol gDW^{-1})	Photoautotrophic Growth Rate (hr^{-1})
ΔfumC	0.848	0.050182
slr1843	3.17	0.050175
slr1843, slr2132	9.23	0.050
slr1843, slr2132, slr0394	12.5	0*

* Engineering of such production strain would obviously require the usage of a conditional deletion system, for instance based on CRISPRi.

Table S2 Plasmids, strains and primers used in this study

Plasmids, strains, and primers#	Relevant characteristics	Reference
pFL-AN	BioBrick "T" vector with AvrII and NheI on each side	[5]
pWD42	$Amp^r Km^r$, containing selection cassette	[6]
pWD060	pFL-AN derivate, Amp^r, containing *fumC* gene upstream and downstream homologous regions	This study
pWD061	pFL-AN derivate, $Amp^r Km^r$, containing *fumC* gene upstream homologous region, selection cassette, and downstream homologous region	This study
pWD084	pFL-AN derivate, Amp^r, containing *zwf* gene upstream and downstream homologous regions	This study
pWD085	pFL-AN derivate, $Amp^r Km^r$, containing *zwf* gene upstream homologous region, selection cassette, and downstream homologous region	This study
Synechocystis sp. PCC6803	*Synechocystis* sp. PCC6803 wild type	D. Bhaya
ΔfumC	*Synechocystis* sp. PCC6803 *fumC* gene knock out mutant	This study
ΔfumC Δzwf	*Synechocystis* sp. PCC6803 *fumC* and *zwf* double genes knock out mutant	This study
SAA023	*Synechocystis* sp. PCC6803 L-lactate production strain	[7]
fumC-up-Fwd	GAGCAGACGTTCACATCG	This study
fumC-up-Rev	CAATCATCTGCTCTGGAACGtctagaCATACTGTCGGTTTCAAGGC	This study
fumC-down-Fwd	GCCTTGAAACCGACAGTATGtctagaCGTTCCAGAGCAGATGATTG	This study
fumC-down-Rev	CCTAGATTAGGACCTGTCAGC	This study
fumC-seq	AACCATTGTCCAAGGTCTGCG	This study
zwf-up-Fwd	TTCGCCTCAATCGCATTC	This study
zwf-up-Rev	GCGACGGCCATCTTTATTactagtTCTTGACGGAGTCCAGTG	This study
zwf-down-Fwd	CACTGGACTCCGTCAAGAactagtAATAAAGATGGCCGTCGC	This study
zwf-down-Rev	ATCTAACACTGCCAGCGT	This study
zwf-seq	TAGCCCAGTCTTATCAGG	This study

[#] primer sequences are given from 5'→3'

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