Photoautotrophic production of succinate via the oxidative branch of the tricarboxylic acid cycle influences glycogen accumulation in *Synechocystis* sp. PCC 6803

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Abstract

Cyanobacteria are interesting biocatalysts for the sustainable production of value-added compounds, but a functional link of biocatalytic efficiency and cell physiology is missing. Especially the role and structure of the tricarboxylic acid (TCA) cycle in cyanobacteria is not fully understood. Succinate dehydrogenase (SDH) is an essential enzyme linking the TCA cycle to the quinone pool and its substrate succinate is an important platform chemical. We utilized *Synechocystis* sp. PCC 6803 as a host organism for the photoautotrophic production of succinate via the oxidative branch of the TCA cycle. In *Synechocystis* sp. PCC 6803 SDH is described to be encoded by three open reading frames (ORF). Four SDH deletion mutants were created and characterized regarding the formation of succinate. *Synechocystis_*Δ*sll1625* was the best performing strain accumulating 420 mg L^{-1} succinate during cultivation in a 1.8L-photobioreactor under constant illumination. Furthermore, deletion of the SDH resulted in a non-bleaching phenotype and concomitant accumulation of glycogen and succinate during growth at 5% CO_2. This substantially influenced cell physiology of the mutant, reflected in decreased cell numbers and higher cell volumes. Thus the TCA cycle seems to have a key role in controlling carbon fluxes from CO_2 into biomass, storage compounds like glycogen, and other carbon sinks.

Keywords: cell physiology, cell volume, cyanobacteria, photobiotechnology, solar cell-factories, succinate-dehydrogenase

Abbreviations: **TCA**, tricarboxylic acid; **SDH,** succinate dehydrogenase; ***Synechocystis***, S*ynechocystis* sp. PCC 6803; **ORF**, open reading frame; **CDW**, cell dry weight; **HPLC**, high-performance liquid chromatography; $\mathbf{Y_{succ/bm}}$, yield of succinate on final biomass; **ANOVA**, analysis of variance

1 Introduction

The use of CO_2 as a cheap and abundant inorganic carbon source moves more and more into the focus of biotechnology. In this context cyanobacteria are highly interesting organisms. They are prokaryotes performing an oxygenic photosynthesis utilizing water as electron donor. A number of promising products synthesized by cyanobacteria from CO_2 have been published in the recent years and it is widely accepted, that cyanobacteria are versatile biocatalysts [1-4]. However, looking at the very low space-time-yields achieved for most of the products it is also obvious, that this fairly young research field is still far from becoming state-of-art in bioprocess development. *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) is one of the most intensively investigated cyanobacterial strains, but still many questions regarding metabolic regulation and phenotypic variability and their impact on productivity and space time yields remain unanswered [5]. Concepts known from engineering organo-heterotrophic organisms utilizing carbohydrates as carbon and energy source are transferred directly to cyanobacteria, which in contrast rely on light for energy, CO_2 for carbon, and H_2O for electron supply. Thus, both types of organisms define different system boundaries for cell engineering which are still largely unknown for cyanobacteria. The TCA cycle as a central part of carbon metabolism is a good example in this context. It is described to be not essential for the cell under photoautotrophic growth conditions and for a while was assumed to be incomplete due to the absence of α-ketoglutarate dehydrogenase [6, 7]. Yet, alternative shunt pathways have been identified in *Synechocystis* that close the TCA cycle, but still a bifurcated flux distribution favoring the reductive branch of the TCA cycle was observed under photoautotrophic conditions [7-9]. Nevertheless, *Synechocystis* exhibits a metabolic plasticity allowing a cyclic carbon flux of the TCA cycle upon genetic modification [10]. In addition, a detailed characterization of most TCA cycle enzymes is missing and kinetic parameters are only available for isocitrate and malate dehydrogenase [6]. Several TCA

cycle intermediates play an important role in the chemical industry and a deeper understanding of this part of the central carbon metabolism in *Synechocystis* is of special interest. Succinate for example can be used as a precursor for numerous chemicals such as 1,4-butanediol or tetrahydrofuran [11]. Thus, the photoautotrophic production of succinate is an excellent example to obtain insights into the regulation of the central carbon metabolism and the impact of genetic modifications of TCA cycle enzymes on the cell physiology. *Synechocystis* is known to secrete succinate in the absence of sugars by fermentation in dark anoxic conditions [12]. Here, we investigated if it is also possible to use the oxidative branch of the TCA cycle for succinate production under photoautotrophic growth conditions and how this influences cell physiology. Deletion of SDH in *Synechocystis* resulted in the formation of succinate to a final titer of 97 mg L^{-1}. This increased to 230 mg L^{-1} during cultivation in an enriched CO_2 atmosphere. In addition, significant changes in the cell physiology of the mutant strain were observed if cells were cultivated in the presence of 5% CO_2. Although measurements of the optical density indicated no differences between the wild type and the mutant strain, the cell number and cell volume were remarkably altered in the respective cultures. Furthermore, accumulation of intracellular glycogen was heavily influenced and the mutant revealed a non-bleaching phenotype in contrast to the wild type.

2 Materials and methods

2.1 Chemicals

All chemicals used in this work were obtained in the highest purity available from Carl Roth GmbH + Co. KG (Karlsruhe, Germany), Merck KGaA (Darmstadt, Germany), Sigma-Aldrich (St. Louis, USA) and Th. Geyer GmbH & Co. KG (Renningen, Germany).

2.2 Bacterial strains and cultivation conditions

E. coli DH5α served as cloning strain and was cultivated in LB medium at 37 °C, supplemented either with 50 µg mL^{-1} kanamycin or 34 µg mL^{-1} chloramphenicol. In this

work the substrain *Synechocystis* sp. PCC 6803_moscow (originating from the laboratory of S. Shestakov, Moscow State University) was used as model organism and is described as a motile substrain of *Synechocystis* sp. PCC 6803 [13]. It was grown in YBG11 medium containing 50 mM HEPES supplemented either with 50 µg mL^{-1} kanamycin or 14 µg mL^{-1} chloramphenicol [14]. For solid medium cultivations BG11 was used [15]. Liquid pre-cultures were inoculated with *Synechocystis* from an agar plate. Main cultures were inoculated with liquid pre-cultures to an initial OD_{750} of 0.05. Cultivation was carried out in a Multitron shaker (Infors, Bottmingen, Switzerland), equipped with LED panels at 30 °C, 150 rpm, 75 % relative humidity and continuous illumination at 50 µE m^{-2} s^{-1}. The CO_2 concentration during cultivation was either at 0.04 % (ambient conditions) or increased to 5 %. Next to shaking flask experiments, *Synechocystis* strains were cultivated in a 1.8 L Labfors 5 Lux flat panel photobioreactor (Infors AG, Bottmingen, Switzerland). Cultivation was carried out in YBG11 medium containing 50 mM HEPES at 30 °C, with a continuous illumination of 50 µE m^{-2} s^{-1} and a constant aeration with 1 L min^{-1} synthetic air, enriched with 2 % of CO_2. The reactor was inoculated to an initial OD_{750} of 0.1 from liquid pre-cultures as described above.

2.3 Construction of *Synechocystis* knockout mutants

For the deletion of subunit B of the SDH encoded by *sll0823* a plasmid was constructed, based on the single vector-double recombination method described in [16]. The homologous regions 1000 bp up- and downstream of the ORF were amplified from the genome of *Synechocystis.* Furthermore, the *nptI_sacB* cassette was amplified from the pDSlux plasmid and fused to a 500 bp spacer sequence. All fragments were cloned into a pUC18 backbone resulting in the final plasmid pUC18_Δ*sll0823*. Selection of the final deletion mutants was performed according to Viola *et al.* [16].

For the construction of *sll1625* (second ORF encoding subunit B) and *slr1233* (encoding subunit A of SDH) knockout mutants, the genes were replaced by a kanamycin or

chloramphenicol resistance cassette, respectively. Analog to the procedure described above for knock-out of *sll0823* respective flanking regions were amplified. Subsequently they were fused with the antibiotic resistance cassette and cloned into a pUC18 backbone. The resulting plasmids called pUC18_Δ*sll1625* and pUC18_Δ*slr1233* have been verified by sequencing (Eurofins, Ebersberg, Germany) prior to transformation.

Transformation of *Synechocystis* cells with either one of the integrative plasmids pUC18_Δ*sll0823,* pUC18_Δ*sll1625,* or pUC18_Δ*slr1233* was performed using naturally competent cells. Cells of a 50 mL culture were harvested by centrifugation (5 min, rcf 3,214, room temperature) in the early growth phase at an $OD_{750} \approx 0.5$ and resuspended in fresh medium to an OD_{750} of 2.5. 500 µL of this cell suspension was mixed with 5 µg of respective plasmid DNA and incubated at 30 °C under continuous illumination of 50 µE m^{-2} s^{-1}. After 3 h the cell suspension was gently mixed and subsequently incubated for another 3 h [17]. After these 6 h total incubation time, cells were plated on BG11 agar plates and incubated at 30 °C and continuous illumination of 50 µE m^{-2} s^{-1}. After three days, the respective antibiotic was added to the agar plate to select for the knockout mutants.

To verify complete segregation of the mutation throughout the multiple chromosome copies, genomic DNA of the respective mutants was isolated and analyzed via PCR. For each deletion two different PCRs were performed using primers binding either inside or outside the gene of interest (for respective agarose gels and primer sequences see Figure S1 and S2 and Table S1).

2.4 Determination of optical density, cell dry weight, cell number and cell volume

Planktonic cell growth of *Synechocystis* strains was followed spectrophotometrically at a wavelength of 750 nm, ensuring no overlap with chlorophyll absorptions. Cell dry weight (CDW) was determined by collecting 2 mL of the respective culture at different time points during cultivation. The cells were harvested by centrifugation, the respective

pellets were dried at 60°C for seven days, and weighted subsequently. Cell numbers and cell sizes were determined by using a coulter counter (Multisizer 3, Beckman Coulter Life Science, Brea, USA) following the procedure given by the company. Samples were diluted in a ratio of 1:50 in ISOTON® II Diluent (Beckman Coulter Life Science, Brea, USA) and analyzed using a 20 µm capillary.

2.5 Determination of intracellular glycogen content

Intracellular levels of glycogen were determined according to a modified version of the method described by Gründel *et al*. [18]. A 2 mL aliquot of the respective culture was centrifuged (rcf 17,000, 10 min, 4 °C) and the pellet was stored at −80°C. To isolate glycogen, the pellet was resuspended in 500 µl 30% w/v KOH and incubated in a thermoshaker at 95°C for 2 h (600 rpm). Precipitation of glycogen was achieved by the addition of 1.5 ml of ice-cold ethanol and overnight incubation at −20°C. After centrifugation (rcf 17,000, 10 min, 4 °C), the glycogen pellet was washed twice with ethanol (first step: 75 % ethanol, second step: 98 % ethanol). Excessive ethanol was evaporated. The pellet was resuspended in 100 µL of ultra-pure water and glycogen was hydrolyzed enzymatically to glucose by addition of 5 U mL^{-1} amyloglucosidase and incubation at 55 °C for 15 min. The amount of glycogen was determined by high-performance liquid chromatography (HPLC) in a Dionex Ultimate 3000 system (Thermo Fisher Scientific, Waltham, USA) equipped with a refractive index detector and an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad Laboratories GmbH, Hercules, CA). H_2SO_4 in a concentration of 5 mM was used as eluent with a flow rate of 0.8 mL min^{-1}. The column temperature was set to 65 °C, and a volume of 20 µL was injected. Quantification was based on calibration curves prepared with standard concentrations of glycogen and enzymatic hydrolyzation.

2.6 Determination of intracellular α-ketoglutarate and succinate concentrations

Extraction of α-ketoglutarate / succinate from *Synechocystis* cells was performed according to the protocol of Eisenhut *et al*. [19]. 50 mL of cultures with a cell concentration of about $2 \cdot 10^8$ cells mL^{-1} were centrifuged at 5000 g and 4 °C for 10 minutes. Supernatant was discarded and the cells were washed two times with 20 mM NaCl. Subsequent pellets were resuspended in 1 mL MeOH and incubated at 70 °C for 15 minutes under continuous shaking (2000 rpm). 666 µL of chloroform were added, followed by a second step of incubation at 37 °C and 1000 rpm for 5 minutes and the addition of 666 µL Milli-Q-water. The mixture was shaken at 2000 rpm for one minute and after 5 minutes of centrifugation (4 °C, 17.000 g) the polar phase was concentrated by a factor of 50 (Speed Vac and resuspension in 50 µL H_2O). Both acids were then analyzed via HPLC as described for extracellular succinate below.

2.7 Determination of extracellular succinate levels

Succinate was quantified by HPLC on a Dionex Ultimate 3000 system (Thermo Fisher Scientific, Waltham, USA) equipped with a RI detector and a HyperREZ XP Carbohydrate H^+ column (300x7.7 mm, 5 µM). 5 mM H_2SO_4 was used as eluent with a flowrate of 0.75 mL min^{-1}. The column temperature was set to 25 °C and a volume of 20 µL was injected. Samples were centrifuged for 10 min at rcf 17,000 and the supernatant was transferred to HPLC vials. Quantification was based on calibration curves prepared with standard concentrations of succinic acid.

2.8 Determination of nitrate in culture supernatant

Nitrate concentrations in the culture supernatant were quantified by HPLC in a Dionex Ultimate 3000 system (Thermo Fisher Scientific, Waltham, USA) equipped with a UV detector (225 nm) and an Acclaim Trinity P2 column (100x3.0 mm, 3 µm). Ammonium formiate (100 mM, pH 3.65) and water were mixed in a ratio of 80:20 and used as eluent with a flow rate of 0.6 mL min^{-1}. The column temperature was set to 30 °C, and a volume of

20 µL was injected. Quantification was based on calibration curves prepared with standard concentrations of nitrate.

3 Results

3.1 Strain development – Knock out of the succinate dehydrogenase activity

This study focused on the oxidative route of the TCA cycle and the impact of SDH activity in *Synechocystis*. SDH, catalyzing the oxidation of succinate to fumarate, was deleted to allow for the accumulation of succinate in the culture supernatant. In *Synechocystis* the two catalytically active subunits of SDH (A and B) have been identified [20]. They are encoded by one (subunit A) and two (subunit B) ORFs, respectively. These three ORFs have been selected as deletion targets. Respective gene deletions and complete segregation throughout the complete chromosomes have been verified using PCR and gel electrophoresis (Figure S1 and S2).

The ORFs *sll0823* and *sll1625* both encode for subunit B of the SDH and the corresponding gene products are described to have a similar function [21]. Thus for complete deletion of subunit B, the double knockout mutant *Synechocystis*_Δ*sll0823*_Δ*sll1625* was created. The construction of a triple deletion mutant (*Synechocystis*_Δ*sll0823*_Δ*sll1625*_Δslr*1233*) was not successful. No colonies having a completely segregated deletion of all three ORFs could be identified. The other four knockout mutant strains were characterized and investigated regarding the accumulation of succinate.

3.2 Proof of concept – Photoautotrophic production of succinate

To verify whether the deletion of the SDH subunits resulted in accumulation and secretion of succinate, *Synechocystis* wild type and the four knockout mutant strains were grown in shake flasks at ambient CO_2 concentration and constant illumination of 50 µE m^{-2} s^{-1}. Growth was determined by measuring the OD at 750 nm. *Synechocystis* wild type grew with an exponential rate of 0.05 h^{-1}. Based on OD measurements single and double knockout mutants had essentially the same growth rates and all strains reached the same

final OD_{750} of about 9. In Figure 1A results are exemplarily shown for *Synechocystis* wild type, *Synechocystis_*Δ*sll1625,* and *Synechocystis_*Δ*slr1233* (data for the other mutants *Synechocystis_*Δ*sll0823* and *Synechocystis_*Δ*sll0823_*Δ*sll1625* Figure S3, supplemental section).

In addition to the growth profile, the culture supernatants of the wild type and all knockout strains were analyzed for succinate production. Wild type cells as well as *Synechocystis_*Δ*sll0823* (Figure S3B) did not accumulate a significant amount of succinate in contrast to *Synechocystis_*Δ*sll1625* and *Synechocystis_*Δ*slr1233* (Figure 1B-D). For *Synechocystis_*Δ*slr1233*, a final product concentration of 270 µM was determined. The highest succinate titer of 820 µM was found for *Synechocystis_*Δ*sll1625.* It was about three times higher compared to *Synechocystis_*Δ*slr1233*. The additional deletion of the second ORF encoding subunit B (*Synechocystis_*Δ*sll0823_*Δ*sll1625*) did not change the maximal succinate concentration reached (Figure S3C).

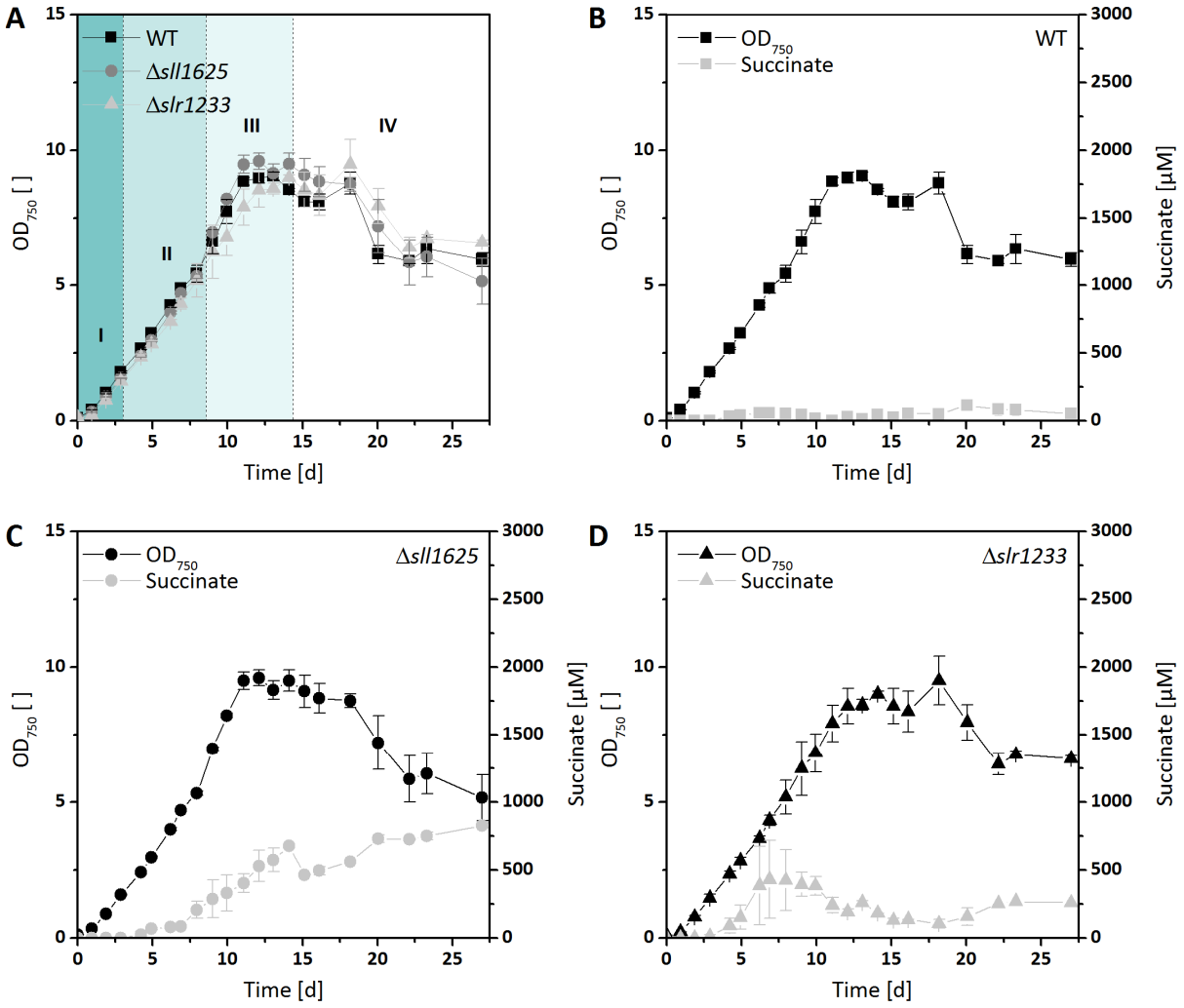

Figure 1: **A**: Growth curves including the different growth phases (I: exponential phase, II: linear phase, III: late phase, IV: stationary phase, according to Schuurmans *et al.* [22]) of *Synechocystis* wild type (■), *Synechocystis_Δsll1625* (●) and *Synechocystis_Δslr1233* (▲) knockout mutants, **B** – **D**: Growth curves (black) and succinate concentration (grey) in culture supernatant of *Synechocystis* wild type (**B**,■/■), *Synechocystis_Δsll1625* (**C**,●/●) and *Synechocystis_Δslr1233* (**D**,▲/▲), cultivation was carried out in ambient concentrations of CO_2 (0.04%). Mean values and standard deviation of biological duplicates are shown. . OD_{750}: optical density at 750 nm; WT: *Synechocystis* sp. PCC 6803; *Δsll1625: Synechocystis* sp. PCC 6803_*Δsll1625; Δslr1233: Synechocystis* sp. PCC 6803_*Δsll1233*

3.3 CO_2 limits succinate production

In our system CO_2 is the only carbon source available for the photoautotrophic production of succinate by *Synechocystis*. To elucidate the impact of CO_2 on product formation cultivation of *Synechocystis* wild type, *Synechocystis_*Δ*slr1233,* and *Synechocystis_*Δ*sll1625* was carried out in an enriched CO_2 atmosphere of 5% (v/v) (Figure 2). Under these conditions growth rates calculated from OD measurements increased to 0.067 h^{-1} and higher final OD_{750} values of 13 were reached compared to growth under ambient conditions (compare Figure 1A and 2A). In addition, the OD_{750} remained constant after 10 days for *Synechocystis_*Δ*sll1625* while it decreased for the other two strains as observed before under ambient conditions. Remarkably, the decreasing OD seems to correlate to bleaching of wild type and *Synechocystis_*Δ*slr1233* cells, when CO_2 concentrations were enhanced (Figure 3). This observation will be further addressed in section 3.4.

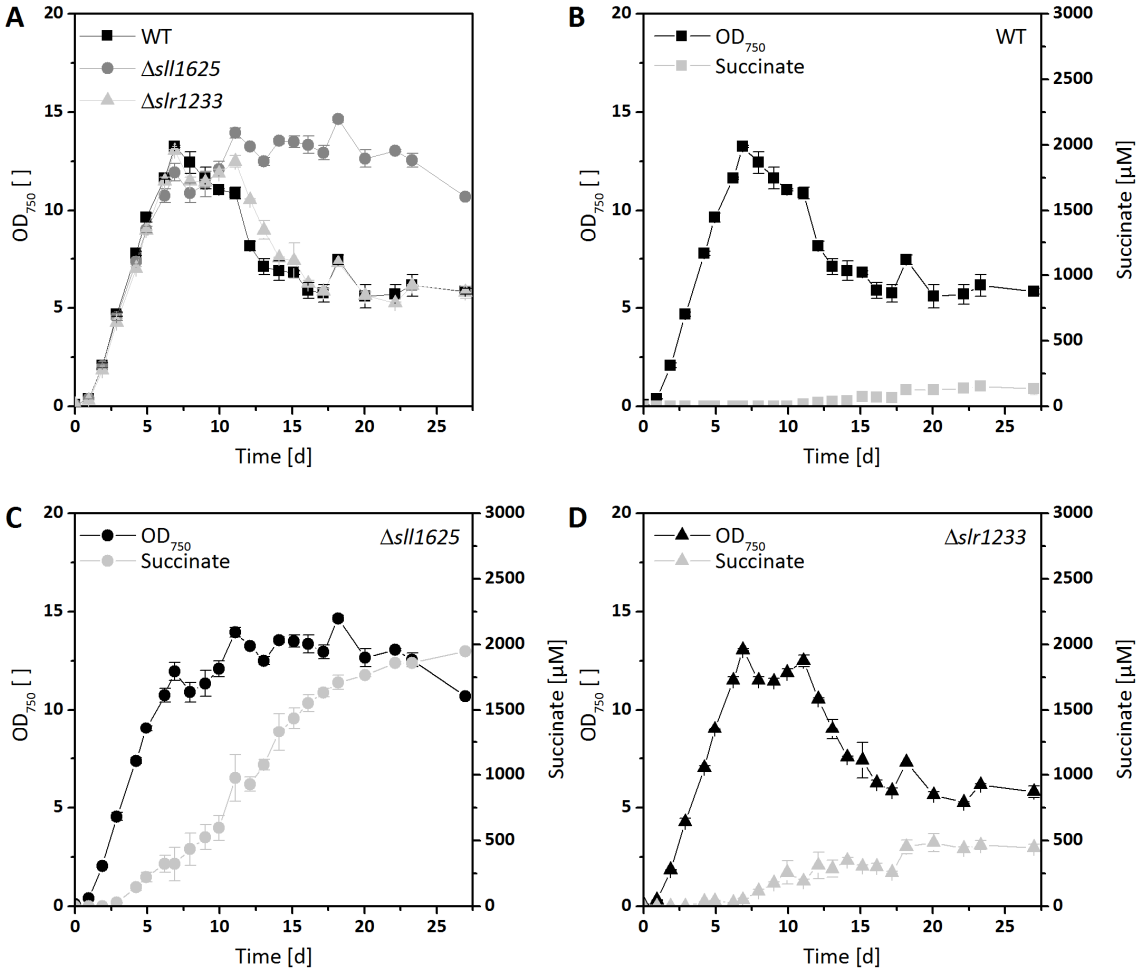

Figure 2: **A**: Growth curves of *Synechocystis* wild type (■), *Synechocystis_*Δ*sll1625* (●) and *Synechocystis_*Δ*slr1233* (▲) knockout mutants, **B** – **D**: growth curve (black) and succinate concentration (grey) in culture supernatant of *Synechocystis* wild type (**B**, ■/■), *Synechocystis_*Δ*sll1625* (**C**, ●/●) and *Synechocystis_*Δ*slr1233* (**D**, ▲/▲). Organisms have been cultivated in an enriched CO_2 (5%) atmosphere. Mean values and standard deviation of biological duplicates are shown. OD_{750}: optical density at 750 nm; WT: *Synechocystis* sp. PCC 6803; Δ*sll1625: Synechocystis* sp. PCC 6803_Δ*sll1625;* Δ*slr1233: Synechocystis* sp. PCC 6803_Δ*sll1233*

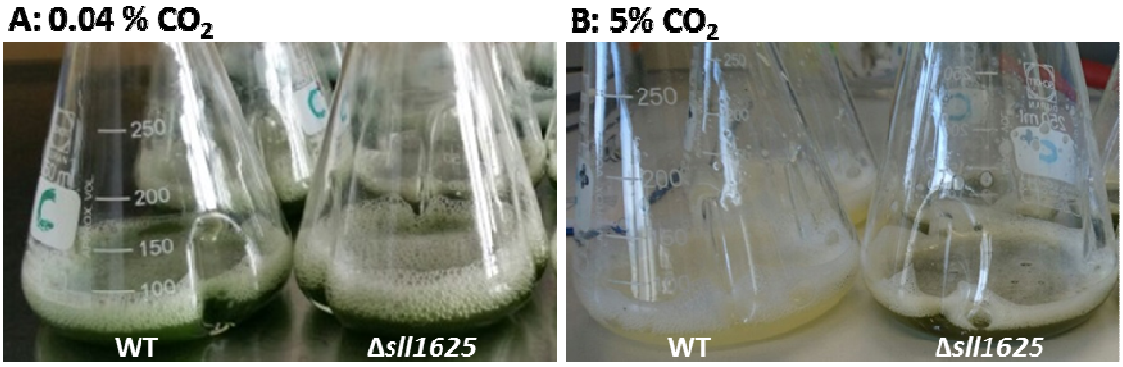

Figure 3: *Synechocystis* cultures after 25 d of cultivation in shaking flasks in an ambient **(A)** and enriched CO_2 (5%) atmosphere (**B**). Exemplarily wildtype and Δ*sll1625* are shown. Cultivation was carried out under constant illumination with 50 µE m^{-2} s^{-1}. WT: *Synechocystis* sp. PCC 6803; Δ*sll1625*: *Synechocystis* sp. PCC 6803_Δ*sll1625*

Furthermore, *Synechocystis*_Δ*sll1625* accumulated the highest extracellular succinate titer of 2 mM, which corresponds to a yield of succinate on final biomass ($Y_{succ/bm}$) of 91 mg g^{-1} compared to 54 mg g^{-1} under ambient conditions. The other mutant *Synechocystis*_Δ*slr1233* only produced 500 µM of succinate. Thus *Synechocystis*_Δ*sll1625* was identified as the best-performing knockout mutant at both concentrations of CO_2 and was further characterized.

3.4 Deletion of the SDH influences cell numbers and cell volumes

To further elucidate the observed decrease in OD and cell the correlations between OD, cell number and cell volume were closely investigated under either ambient (0.04 %) or enriched (5 %) concentrations of CO_2 for wild type and *Synechocystis*_Δ*sll1625.* In addition, the correlation factor between OD and CDW was determined by drying and weighing the respective cultures (see also materials and methods).

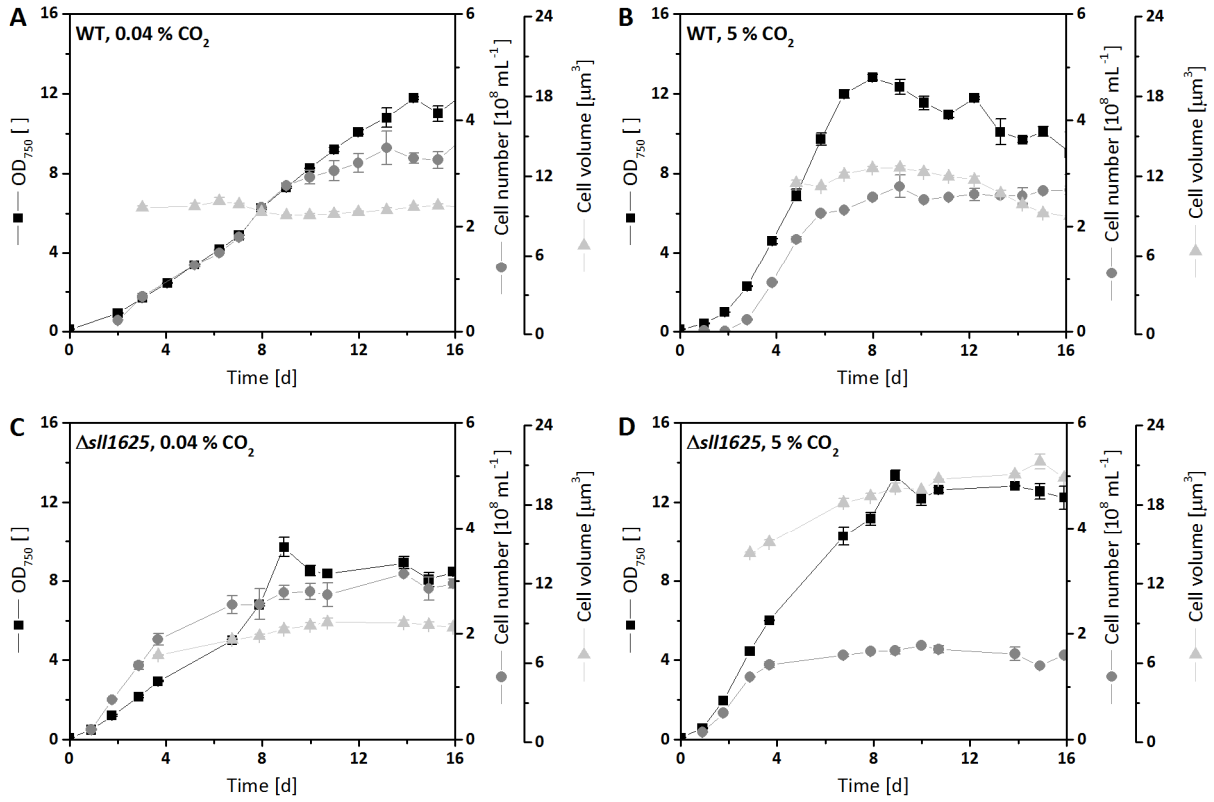

Figure 4: Determination of OD_{750} (■), cell number (●) and cell volume (▲) in *Synechocystis* cultures. **A**: *Synechocystis* wild type cultivated under ambient concentrations of CO_2 (0.04%), **B**: *Synechocystis* wild type cultivated in an enriched CO_2 (5%) atmosphere, **C**: *Synechocystis_Δsll1625* cultivated under ambient concentrations of CO_2 (0.04%), **D**: *Synechocystis_Δsll1625* cultivated in an enriched CO_2 (5%) atmosphere. Mean values and standard deviation of biological duplicates are shown. OD_{750}: optical density at 750 nm; WT: *Synechocystis* sp. PCC 6803; Δ*sll1625*: *Synechocystis* sp. PCC 6803_Δ*sll1625*

During cultivation under ambient conditions, no significant alterations of wild type and the *Synechocystis_Δsll1625* knockout mutant were observed (Figure 1A, 4A and 4C). The increase in cell number correlated with the increase in OD_{750} and both cultures reached a final cell number of about $3 \cdot 10^8$ cells mL^{-1}. Furthermore, the cell volume was relatively constant at a value of 2 μm^3.

In contrast, cultivation in an enriched (5%) CO_2 atmosphere resulted in tremendous differences (Figure 4B and D). The cell numbers of both strains stayed constant during the stationary phase while the cell volume correlated to the OD_{750}. Both decreased in the wild type cultures, while they stayed constant for the recombinant strain. This observation indicates an important connection between OD values and cell size.

Determining optical density as key parameter for cell growth may thus not be sufficient in cyanobacteria due to the interrelation of cell numbers and cell volumes. While the wild type cells continued cell division until day 6 (Figure 4B), division activity in the recombinant strain ceased already after 4 days, and cells only increased in volume (Figure 4D). Considering these findings, succinate production mainly occurred in the "non-dividing phase", where cells only increased in volume (Figure 1C and 2C).

The correlation between OD and CDW was determined. Both concentrations of CO_2 were tested and samples were analyzed at different phases of the cultivation. The resulting correlation factor for each strain did not change significantly over time and was identical for both conditions. However, there was a small difference between both strains resulting in a factor of 0.17 g L^{-1} for the wild type and 0.21 g L^{-1} for *Synechocystis_*Δ*sll1625*. According to an analysis of variance (ANOVA), the difference between the two correlation factors is significant [23]. Most likely, the higher correlation factor for *Synechocystis_*Δ*sll1625* resulted from the increased cell volume, as the cell concentration was lower in comparison to the wild type. However, the reasons for the divergent cell volumes were not yet identified. Possibly the cell volume is linked to the accumulation of glycogen as an intracellular storage compound which may also be different for the wild type and the knockout mutant.

3.5 Deletion of *sll1625* alters glycogen accumulation

In general, cultivation in shaking flasks is used for first screening and general characterization of a biological system. The sample volume is limited as is the number of

parameters to be followed, especially during long-term experiments. Cultivation in a lab-scale reactor system increases culture volume, the range of analyses can be extended, and in addition, process parameters can be controlled and monitored. For that reason, *Synechocystis* wild type and *Synechocystis_Δsll1625* were cultivated in a 1.8L-FPR. For technical reasons cultivations were performed at 2% CO_2. Control experiments in shake flasks showed the same growth and production behavior in 2% as in 5% CO_2 (see Figure S4, supplemental section).

During cultivation OD_{750}, cell number and cell volume were determined. The culture supernatant was analyzed for the presence of succinate and nitrate. Finally, the intracellular concentration of glycogen, succinate, and α-ketoglutarate were measured.

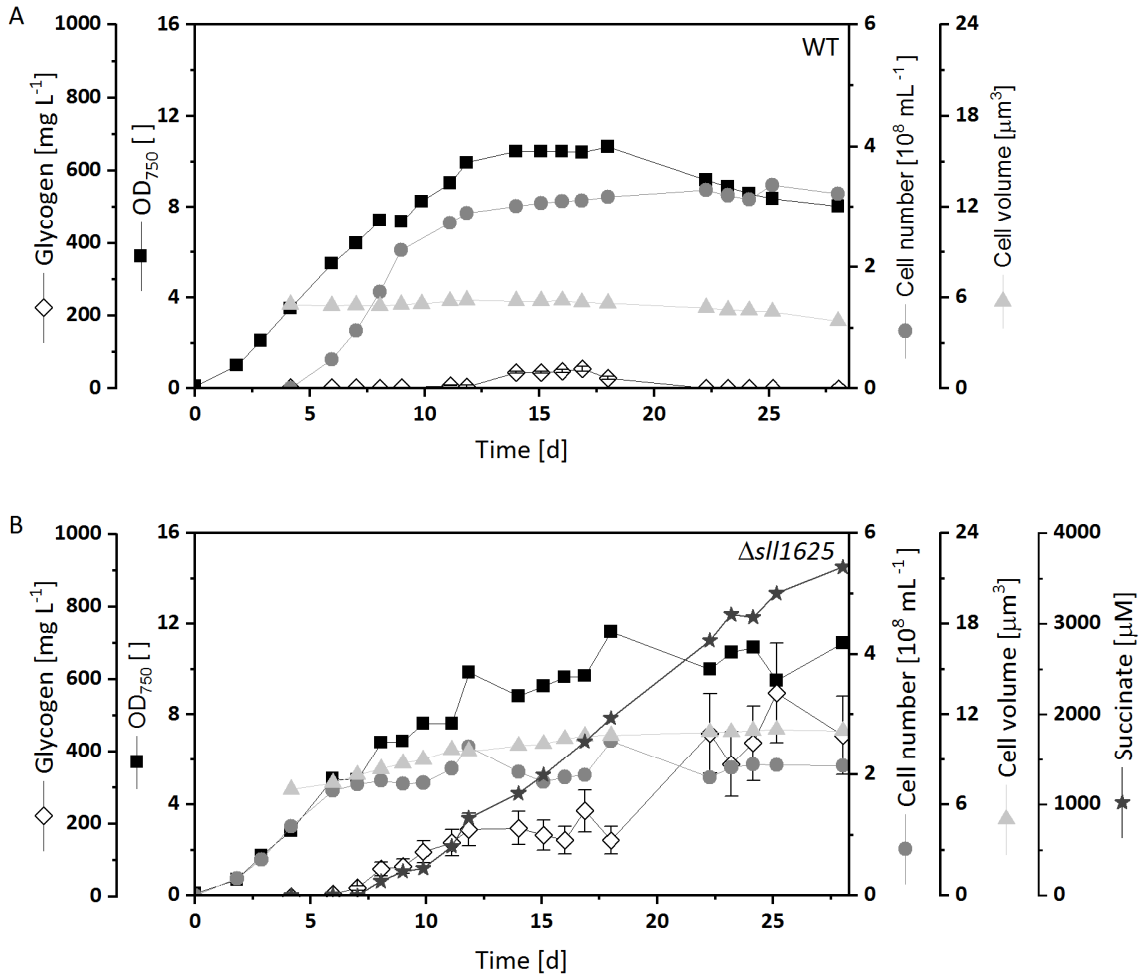

Figure 5: Cultivation of *Synechocystis* wild type (**A**) and *Synechocystis_*Δ*sll1625* (**B**) in a lab-scale FPR. OD_{750} (■), cell number (●), cell volume (▲), intracellular glycogen content (◇), and concentration of succinate in culture supernatant (*) were measured at different time points of the cultivation. Results from one reactor experiment are shown. Glycogen determination was performed in duplicates. OD_{750}: optical density at 750 nm; WT: *Synechocystis* sp. PCC 6803; Δ*sll1625*: *Synechocystis* sp. PCC 6803_Δ*sll1625*

As shown in Figure 5, growth, cell numbers and cell volumes were comparable to the results obtained during shaking flask experiments. Remarkably, the amount of succinate produced was 1.75fold higher although the final biomass concentration was slightly

reduced, calculating to a yield of 183 mg g_{CDW}^{-1}. Analysis of the intracellular glycogen amount showed significant differences between the two strains. *Synechocystis* wild type accumulated about 50 $mg_{glycogen}$ L^{-1} during 14 days of cultivation. After day 17 the synthesized glycogen was completely degraded again. In *Synechocystis*_Δ*sll1625* glycogen was accumulated until the end of the cultivation (day 28). During the first 18 days the intracellular glycogen quantities in the two strains were reflected in the changes of the cell volumes. A higher glycogen concentration correlated with an increased cell volume and resulted in double-sized cells of *Synechocystis*_Δ*sll1625* compared to the wild type. Towards the end of the *Synechocystis* _Δ*sll1625* cultivation (days 18 to 28), the cell volume seems to be constant, while glycogen is still increasing. Finally the mutant produced nearly ten times more glycogen, while the cell volume doubled.

Deletion of SDH in combination with elevated CO_2 concentration significantly influenced cell physiology and glycogen accumulation. These results show that the intracellular carbon distribution and the carbon fluxes in *Synechocystis* are highly flexible and support the findings on metabolic plasticity reported previously [10].

Apart from the observation, that elevated CO_2 concentrations had an effect on glycogen accumulation, *Synechocystis*_Δ*sll1625* revealed a non-bleaching phenotype under these conditions (Figure 3). Bleaching is often connected to nitrogen starvation. Thus the supernatant was analyzed for the presence of nitrate, which was supplied as nitrogen source (see supplemental Figure S5). *Synechocystis* wild type assimilated about 15.5 mM of nitrate during the first 10 days of cultivation and afterwards no more was consumed. In contrast, nitrate assimilation of *Synechocystis*_Δ*sll1625* proceeded until day 17 of the cultivation and only 13.5 mM were consumed. Towards the end of the cultivation there were still significant amounts of nitrate detectable in the medium (2 mM for the wild type and 4 mM for the mutant supernatants). However, correlating nitrate consumption to biomass formation without taking the glycogen fraction into account (where no nitrogen is

bound) nitrate consumption was the same for both strains. Furthermore, the intracellular concentration of α-ketoglutarate was determined at the end of the cultivation, as this metabolite plays an important role in sensing the nitrogen status in *Synechocystis* [24]. However, α-ketoglutarate concentrations were in the same range for both strains (330 µM for the wild type and 390 µM for *Synechocystis*_Δ*sll1625*). These findings indicate that bleaching of the wildtype culture is not due to a nitrogen limitation. Finally, intracellular succinate concentrations were determined to be 4 mM for *Synechocystis*_Δ*sll1625*, which is 17-fold higher as compared to the wild type. Although this is a minor concentration regarding the overall culture volume, it is a fairly high amount for an intracellular metabolite, which may impact regulation of the TCA cycle.

4 Discussion

Synechocystis has been shown to be a versatile biocatalyst for the synthesis of value added compounds directly from CO_2. TCA cycle derived products, like succinate, play an important role in this context. The photoautotrophic production of succinate was already demonstrated for *Synechococcus* as well as for *Synechocystis*. The studies for *Synechococcus* focused on the oxidative branch of the TCA cycle and production of succinate was realized by expressing genes encoding for the α-ketoglutarate decarboxylase and the succinate semialdehyde dehydrogenase [25]. To restore severe growth inhibition of the recombinant strain, genes encoding for a phosphoenolpyruvate carboxylase and a citrate synthase from *Corynebacterium glutamicum* needed to be co-expressed, resulting in a final succinate titer of 430 mg L^{-1} [25]. The production of succinate using S*ynechocystis* is only reported via the reductive arm of the TCA cycle. *Synechocystis* is known to secrete succinate in the absence of sugars by fermentation under dark anoxic conditions [12]. An additional overexpression of the gene coding for the

phosphoenolpyruvate carboxylase and a cultivation temperature of 37 °C resulted in the highest reported titer of 1.8 g L^{-1} under fermentative conditions [26, 27].

Here, we show for the first time the impact of SDH inactivation on succinate synthesis utilizing the oxidative branch of the TCA under constant illumination.

4.1 SDH as engineering target

SDH is a well described enzyme and numerous review articles are available [20, 28]. The enzyme consists of a peripheral domain, exposed to the cytoplasm, and an integral domain that functions as a membrane anchor, which is schematically shown in Figure 6. The peripheral part, which contains the succinate binding site, is composed of a flavoprotein (*sdhA*) with one covalently bound FAD and an iron-sulfur protein (*sdhB*) containing three iron-sulfur clusters [20].

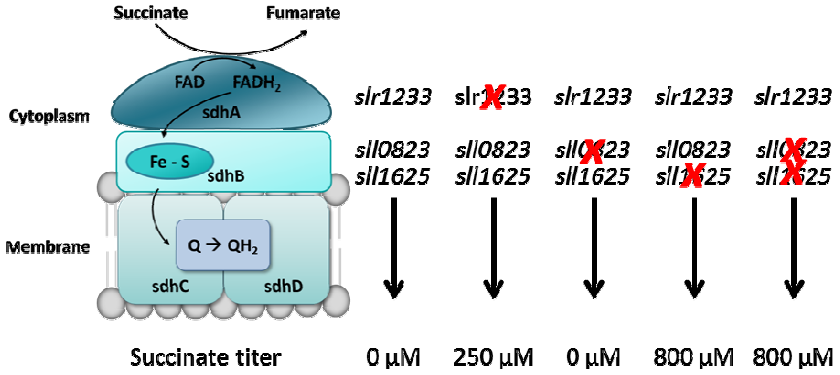

Figure 6: Organisation and function of the different SDH subunits as described in the literature [20]. On the right the various deletion mutants obtained in this study and the effect on the extracellular succinate titers are listed. Succinate titers given are based on initial experiments with ambient CO_2 concentrations. sdhA, sdhB, sdhC, sdhD: subunits A, B, C, D of the succinate dehydrogenase; FAD(H_2): flavin adenin dinucleotide (reduced form); Q(H_2): quinone (reduced form); Fe-S: iron-sulphur

In *Synechocystis* two genes homologous to *sdh*B (*sll0823* and *sll1625*), and one to *sdhA* (*slr1233*) were identified [20]. Cooley *et al*. was the first to investigate the function of the

SDH subunits in *Synechocystis* sp. PCC6803 and came to the conclusion that the *sll0823* and *sll1625* genes were functionally similar [21]. They looked at intracellular succinate concentrations and reported that these were decreasing upon deletion of either of the two B subunits. In our work deletion of *sll1625* (*sdhB*) resulted in enhanced intracellular succinate concentrations. Additionally, we detected succinate accumulation in the repective supernatants. Remarkably, *Synechocystis*_Δ*sll1625* produced significantly higher amounts than S*ynechocystis*_Δ*sll0823*. The combined deletion of *sll0823* and *sll1625* did not impact succinate titers (Figure 6). These findings indicate that the functional products of *sll1625* and *sll0823* behave differently under the here applied cultivation conditions. The experiments conducted in our study differ strongly from the work of Cooley *et al.* in terms of growth phase and cell densities at which respective concentrations have been determined [21]. Succinate determination was conducted towards the end of the stationary phase at an OD of 11, whereas Cooley *et al*. measured at the mid exponential phase at a significantly lower OD. At this time point in cultivation it was not possible with our analysis method to detect any succinate in the supernatant. From our observation regarding cell division and growth it seems as if significant succinate synthesis occures only in the stationary phase (no cell division), which may explain these contradictory findings.

Remarkably, the deletion of subunit A resulted in 3times lower succinate titers compared to *Synechocystis*_Δ*sll1625* carrying a deletion of subunit B. Based on the sequence analysis performed by Kaneko *et al.* subunit A may be the catalytically active part of SDH responsible for succinate oxidation [29]. Thus, it would have been expected that the deletion of this subunit impairs overall SDH function and results in similar or even higher succinate titers as determined for the subunit B deletion mutant. Also regulation mechanisms, affecting the carbon flux distribution towards the TCA cycle need to be considered in this context. Possibly the deletion of the catalytically active subunit A

impacts overall TCA cycle activity. Alternatively regulation may occur already on the transcriptional level of the SDH subunits and other enzymes of the TCA cycle.

We did not succeed to create a triple mutant in which all three ORFs were deleted, indicating that this mutation is lethal to *Synechocystis*. It is obvious, that more research is needed to unveil the function of this highly important enzyme in *Synechocystis* which links the TCA cycle to the PQ pool. The organization of the cyanobacterial TCA cycle and its alternative shunts, as well as the detailed characterization of its enzymes is still largely unknown [6].

4.2 Knockout of *sll1625* influences cell physiology and glycogen synthesis

During the initial characterization of *Synechocystis*_Δ*slr1233*, *Synechocystis*_Δ*sll0823*, *Synechocystis*_Δ*sll1625*, and *Synechocystis*_Δ*sll0823*_Δ*sll1625*, *Synechocystis*_Δ*sll1625* was identified as the best performing strain regarding succinate production rate and final titer. This strain was characterized in more detail. Beside the elevated formation of succinate, the deletion of *sll1625* significantly influenced the cell physiology of *Synechocystis*. Typically OD values are used as a measure for biomass concentrations. However, OD measurements are based on the turbidity of a culture and the turbidity is influenced not only by the amount but also by the volume and the morphology of the cells in a culture. For the succinate producing *Synechocystis*_Δ*sll1625* we showed that the cell number decreased while the cell volume and the intracellular glycogen concentration increased under high carbon conditions. This is in accordance to a study of Holland *et al*., who investigated the correlation of cell number, optical density, and cell diameter under low and high carbon conditions in different mutants of *Synechocystis,* like *Synechocystis* Δ*glgC*, which is deficient of glycogen synthesis [30]. Similar findings have been reported from David *et al.* for a recombinant *Synechocystis* producing 1,2-propanediol [31]. These examples reflect the flexibility of the cyanobacterial cell size and emphasize the link between glycogen accumulation, cell volume, and optical density. Glycogen is stored in the

form of granules in the cells and on average one glycogen granule has a diameter of about 42 nm and a molecular weight of 10^7 Da [32, 33]. Taking the cell volume, the cell number, and the glycogen concentration into account the volume of the glycogen granules in cells of *Synechocystis*_Δ*sll1625* sums up to about 4.5 μm^3, which correlated well with the measured increase in cell size towards the end of the cultivation (Figure 5).

As discussed above, the *sll1625* knockout mutant accumulated more glycogen as compared to the wild type. It was already shown, that genetic modifications may result in a higher carbon flux towards the TCA cycle [34]. Possibly, this is also the case for the deletion of the SDH due to regulating mechanisms that connect to the intracellular levels of the TCA cycle intermediates. An elevated flux through the TCA cycle will increase the amount of NADPH that is formed during the decarboxylation of isocitrate to α-ketoglutarate, and the oxidation of succinic-semialdehyde to succinate. In this case glycogen synthesis might function as an electron sink as it is known, that *Synechocystis* forms glycogen to store excessive energy or carbon [30, 35, 36]. In contrast to the production of 1,2-propanediol, the formation of succinate in *Synechocystis*_Δ*sll1625* is not depending on the degradation of glycogen [37]. Thus interrupting the glycogen synthesis as a pathway competing for carbon may be a strategy to enhance product titers. Understanding the interplay of storage compound accumulation, and product and biomass formation is an important aspect to develop further metabolic engineering approaches.

4.3 Secretion of succinate prevents bleaching

In addition to the differences already described, *Synechocystis*_Δ*sll1625* revealed a non-bleaching phenotype at elevated concentrations of CO_2. Typically, bleaching is connected to nitrogen depletion and the degradation of phycobilisomes to generate intracellular nitrogen [38]. Here we observed, that cells bleached despite the fact that at least 2 mM residual nitrate were still available in the medium. At a first glance the nitrate utilization appears to be enhanced in case of the wild type. However, if the nitrate consumption rate

is correlated to the biomass amount without considering the glycogen fraction, the rates are the same for both, wild type and the *sll1625* knockout mutant. The differences in nitrate amounts are related to the phenomenon, that in the wild type cultures we have more and smaller cells, whereas in the cultures of the deletion mutant cell numbers are less and the cell volume and the intracellular glycogen concentration are increased.

α-Ketoglutarate is a key metabolite in the regulation of the intracellular nitrogen cycle [39]. In *Synechocystis*, nitrogen excess is connected to low levels of α-ketoglutarate resulting in an inactive form of NctA, which has been identified as the main transcriptional regulator of nitrogen assimilation [24]. Under nitrogen starvation α-ketoglutarate levels are increased leading to the upregulated expression of genes of the nitrogen assimilation machinery by NtcA. However, here we observed no difference in the intracellular α-ketoglutarate concentrations between wild type and deletion mutant, indicating that in this case bleaching is not due to a nitrogen limitation. Nevertheless, also a depletion of other nutrients like phosphor or sulfur, or limitations within the Calvin cycle can cause photobleaching in *Synechocystis* [40-42]. Possibly, *sll1625* is also involved in the regulation of bleaching effects in *Synechocystis* but these hypotheses needs to be further addressed in future studies.

4.4 Final conclusion

This work shows for the first time that it is possible to produce succinate from CO_2 under constant illumination utilizing the oxidative branch of the TCA cycle in *Synechocystis*. Deletion of either of the two ORFs encoding for subunit B, which are described to be functional equal, have a different effect on overall SDH activity. Whether this is due to functional differences of the gene products or due to regulation mechanisms, affecting overall carbon flux distribution or gene transcription of the corresponding enzymes remains speculative at this point.

Deletion of all three ORFs could not be accomplished, indicating a lethal mutation. The obtained single and double deletion mutants showed significantly altered succinate titers, while biomass formation appeared equal in wildtype and mutant strains when looking at OD measurements. However, deletion of *sll1625* prevented degradation of the photopigments and resulted in a non-bleaching phenotype under enhanced CO_2 concentrations. Furthermore, striking differences were observed in cell numbers and cell volumes when comparing the wildtype to the *Synechocystis_*Δ*sll1625.* In the mutant, cell division stalls already in the early exponential phase and cells only grow in volume, accompanied by an increase in glycogen concentration. It seems as if the TCA cycle and in particular SDH fulfills a much more complex function in central carbon metabolism of *Synechocystis* than assumed so far. A detailed characterization of its enzymes is necessary to create a basis for rational engineering of the TCA for productive catalysis, following the design principles of bioprocess engineering from analysis to synthesis to design.

Acknowledgement

Prof. Annegret Wilde, University of Freiburg, is gratefully acknowledged for supplying the strain *Synechocystis* sp. PCC 6803_moscow. Furthermore, we acknowledge the use of the facilities of the Centre for Biocatalysis (MiKat) at the Helmholtz Centre for Environmental Research, which is supported by European Regional Development Funds (EFRE, Europe funds Saxony) and the Helmholtz Association. This project was funded by the Bundesministerium für Bildung und Forschung (BMBF) and the Südzucker AG. Project ID: 031A217H

Declaration of competing interest

The authors declare no commercial or financial conflict of interest.

Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

Authors' agreement to authorship and submission

All the authors agreed to the authorship and submission of the manuscript to Algal Research.

Declaration of authors' contributions

MM and KB conceived the project. MM designed and performed the experiments. MM and KB wrote the manuscript. MM, KB, and AS discussed the results, revised the text, and approved the final manuscript.

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