Effect of phosphate availability on cyanophycin accumulation in *Synechocystis* sp. PCC 6803 and the production strain BW86

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A B S T R A C T

The cyanobacterial wild-type strain *Synechocystis* sp. PCC 6803 and its engineered strain BW86 were cultivated under defined conditions in photobioreactors to investigate the effect of phosphate availability on cyanophycin accumulation. Cyanophycin, multi-α-arginyl-poly-α-aspartate, can be deployed as amino acid source or can be chemically converted into polyaspartic acid, a biodegradable polymer. In previous studies it was demonstrated that a single point mutation in the PII signaling protein from the *Synechocystis* wild type is sufficient to unlock the arginine pathway causing an over accumulation of the biopolymer cyanophycin in BW86. One process strategy to evoke cyanophycin synthesis in *Synechocystis* is nutrient starvation. Therefore, different phosphate concentrations from 17.5 to 175 μM were tested. Progressive phosphate starvation resulted in an increased cyanophycin accumulation. The highest obtained cyanophycin amounts in g cyanophycin per g cell dry mass were 18% and 40% for *Synechocystis* sp. PCC 6803 wild type and BW86 respectively, demonstrating that phosphate starvation is an effective route for biotechnological cyanophycin production. By evaluating cyanophycin and phosphor quotas per cell dry mass, it was possible to determine the specific required amount of phosphor to accumulate cyanophycin and to initialize stationary growth phase. Phosphor quotas in the range of 4 to 1 mg phosphor per g cell dry mass were determined. Additionally, light kinetics was determined. Photon flux densities exceeding 46 μmol photons m−2 s−1 result in a maximum growth rate of 1.32 d−1.

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1. Introduction

Cyanobacteria are photosynthetic gram-negative prokaryotes. Besides their ability to obtain energy by oxygenic photosynthesis, they are known to synthesize reserve substances like cyanophycin, polyhydroxybutyrate (PHB) [1] and glycogen [2]. The accumulation of cyanophycin or multi-α-arginyl-poly-α-aspartate, a nitrogen and carbon reserve polymer, is unique for cyanobacterial metabolism and a few other heterotrophic bacteria [3–7].

Extracted cyanophycin can be chemically converted to polyaspartic acid, a biodegradable substitute for polycrylic acid which can be used for many technical and medical applications [8,9]. Furthermore, cyanophycin could be used as a possible amino acid source for arginine and asparagine. Cyanophycin-derived dipeptides as natural alternatives to the widely applied amino acid mixtures are under discussion [10].

Cyanophycin consists of a polyaspartic acid backbone, its α-carboxyl groups are linked via amid bonds to the α-amino group of α-arginine. Aspartic acid and arginine are usually present in equimolar amounts in cyanophycin. The molecular masses of the polymer range from 25 to 100 kDa [11–14]. Cyanophycin is located in the cytoplasm in form of optically opaque granules. It is soluble under acidic, pH < 2, or alkaline, pH > 9, conditions and insoluble at physiological pH [15,16]. Cyanophycin is non-ribosomally synthesized by the cyanophycin synthetase (CphA) and can be degraded by the enzyme cyanophycinase within the cell (CphE) or extracellularly by the cyanophycin hydrolizing enzyme CphE, which is excreted by other bacteria like *Pseudomonas anguilliseptica* [17]. It has to be mentioned that other proteases like pepsin, leucine aminopeptidase, chymotrypsin or α- and β-carboxypeptidases are not able to degrade cyanophycin [18–21].

The cyanophycin content of *Synechocystis* sp. PCC 6803 (from now on *Synechocystis* 6803) and other cyanobacteria varies depending on the growth phase and environmental conditions. During exponential growth, a cyanophycin content of roughly 1% per cell dry mass (CDM) is reported while higher contents up to 18% per CDM are synthesized under unbalanced conditions, as there are nutrient starvation e.g. of phosphate or sulfate, adverse light intensities and low temperature.
[22,23]. Simon [5] showed that chloramphenicol, a broad-spectrum antibiotic which inhibits protein synthesis, stimulates cyanophycin synthesis in Anabaena cylindrica. Cyanophycin contents up to 46% (w/w) were achieved in cultivations of Acinetobacter calcoaceticus by using a phosphate-limited medium spiked with arginine and chloramphenicol [24]. Generally, cyanophycin accumulation is depending on the availability of arginine [25].

It is also possible to obtain increased cyanophycin concentrations by genetic engineering. The key enzyme of the arginine pathway, acetyl glutamate kinase (NAGK), is controlled by the global carbon-nitrogen-energy-status sensing PII signaling protein, which is a member of the widely distributed family of PII signal transduction proteins, present in bacteria, plants and archaea [26]. PII proteins are involved in the regulation of nitrogen assimilation. Therefore PII senses the cellular energy level by binding ATP or ADP, as well as the carbon/nitrogen availability by 2-oxoglutarate [27–29]. Depending on the bound effector molecules and phosphorylation status, PII changes its conformation leading to a structural rearrangement of the T-loops, which are the major protein interaction structures [30]. Under nitrogen excess conditions, PII binds to NAGK [31] and strongly enhances its activity. A PII variant with a single amino acid replacement, Ile86 to Asp86, is responsible for a constitutively in vivo activation of NAGK, which leads to the accumulation of cyanophycin in high amounts in this strain, named BW86. Under phosphate-limited conditions this engineered strain accumulated an extremely high cyanophycin content per CDM, which is 57% [32]. To our knowledge this is the highest cellular cyanophycin content in a bacterial cell reported so far.

Here the engineered strain Synechocystis BW86 is used to investigate the quantitative effect of phosphate availability on cyanophycin accumulation. Experiments were carried out in photobioreactors under defined cultivation conditions. The wild-type strain Synechocystis 6803 was cultivated using the same process parameters as benchmark. Furthermore, light kinetics were determined for the wild type.

2. Materials and methods

2.1. Strain, medium and culture conditions

Liquid cultures of the strains Synechocystis 6803 and BW86 [32] were cultivated for 2 weeks in shaking flasks and incubated at 25 °C, 100 rpm, pH 7.5 and a photon flux density (PFD) of 135 μmol m⁻² s⁻¹ (PAR photons, photosynthetic active radiation) supplied by warm-white LED illumination. The culture medium was BG-11 [33]. In shaking flask cultures, 10 mM HEPES buffer was added to the medium. Phosphate concentrations in the photobioreactor experiments varied from 16.65, 8.32, 4.16, 3.33 and 2.08 to 1.66 mg L⁻¹. Reduced phosphate concentrations were achieved by using lower amounts of K₂HPO₄ in the culture medium. To prevent potassium starvation, KCl was added in equimolar amounts. To attain equal starting conditions for the respective experiments, the inoculum volumes were slightly adjusted depend- ing on the optical densities of the precultures. All chemicals used were of analytical grade (p.a.) and purchased from Carl Roth, Sigma Aldrich, VWR Chemicals and Merck.

2.2. Photobioreactor system

Experiments to investigate cyanophycin accumulation were carried out in flat plate photobioreactors (Midplate reactor, Fig. S2 in the supplementary material) with a working volume of 1 L and a temperature of 28 °C. This reactor system was described earlier by Dillenschneider et al. [34]. Light kinetics were determined in flat plate photobioreactors (Miniplate reactor) with a working volume of 0.2 L. The basic design is analogous to the Midplate reactor with inner reactor dimensions for height, width, depth of 140, 100, 20 mm. Gas flow was adjusted to 0.33 and 0.25vvm for Midi- and Miniplate reactors, respectively. Prior to inoculation the reactor system was saturated with 2 to 5% CO₂ for 24 h. In Miniplate reactors temperature was controlled by a climate chamber (25 °C, MKK1200, Flohr Instruments) and pH was buffered with 10 mM HEPES, while 1 M NaOH was used for pH adjustment in Midplate reactors. The pH value was set to 7.5. For experiments in Miniplate reactors, the amount of CO₂ and O₂ in the exhaust gas was measured by the gas analyzer M610, Maihak AG. PFD (PAR photons) was adjusted with a planar light sensor (LI-250, Li-Cor) by measuring the PFD directly behind the first glass plate where photons enter the liquid suspension. The amount of transmitted photons was measured behind the second glass plate. Illumination was supplied using warm-white LEDs.

2.3. Cyanophycin extraction and quantification

Cyanophycin extraction was carried out as described in the protocol from Elbahloul et al. [24] with some modifications. A sample volume of 15 mL was centrifuged (Rotina 420R, Hettich Zentrifugen) for 15 min with 11,000 rpm and 4 °C and supernatant discarded. The remaining pellet was dissolved in 1 mL absolute acetone and incubated at room temperature for 30 min at 900 rpm (orbital shaker KS 501 digital, IKA). After a second centrifugation step (10 min, 11,000 rpm, 4 °C) the acetone supernatant was discarded again, the pellet was resuspended in 1.2 mL 0.1 M HCl and incubated for 60 min at 60 °C and 900 rpm. A third centrifugation step (10 min, 11,000 rpm, 4 °C) was performed to remove cell debris as pellet. The supernatant contains the solubilized cyanophycin. Addition of 720 μL 0.1 M Tris/HCl, pH 8.0, to the supernatant and following incubation at 4 °C for 40 min leads to precipitation of cyanophycin. A further centrifugation step (15 min, 11,000 rpm, 4 °C) was carried out to obtain a cyanophycin pellet. The described precipitation step was repeated once more with the obtained pellet to ensure a higher purity of the product. After disposal of the supernatant, the pellet was resuspended in 500 μL 0.1 M HCl. Cyanophycin quantification was carried out according to Messineo [35] by using the Sakaguchi reaction, a detection method for arginine. l-arginine was used to generate a calibration curve. Each cyanophycin sample was measured in duplicates.

2.4. Ion chromatography

Concentrations of nitrate (NO₃⁻), phosphate (PO₄³⁻) and sulfate (SO₄²⁻) were determined by ion chromatography (Metrohm 882 Compact IC plus) equipped with a Metrosep A Supp 5 column 150/4.0, filling material polyvinyl-alcohol with quaternary ammonium groups, as stationary phase and a conductivity detector (Metrohm). The limit of quantification was 1.0 mg L⁻¹. Mobile phase was an elution buffer consisting of 3.2 mM Na₂CO₃, 1.0 mM NaHCO₃ and 12.5% (v/v) acetonitrile in water. Cyanobacterial samples were centrifuged (10 min, 11,000 rpm, 4 °C) and the supernatant was used for subsequent analyses. An autosampler unit (Metrohm Professional Sample Processor 858) diluted and injected the samples automatically. The phosphor concentration was calculated through the determined phosphate concentration by the particular molar mass of phosphor and phosphate, 0.3097 g mol⁻¹ and 94.97 g mol⁻¹.

2.5. Determination of cell dry mass concentration and cell density

For CDM determination, duplicates of 20 mL cyanobacterial samples were taken, centrifuged (15 min, 6000 rpm, 4 °C, Rotina 420R, Heraeus), washed and incubated in a drying chamber (48 h, 80 °C). The resulting pellets were cooled down to room temperature and weighed on an analytical balance (Kern & Sohn GmbH AB) 320–4). A synchronous measurement of the optical density at 750 nm (spectrophotometer Lambda 35, Perkin Elmer) enabled the generation of individual calibration curves for each experiment. A reproducible correlation factor of 0.179 g CDM L⁻¹ per OD₇₅₀nm, for wild type (R² = 0.997) and 0.177 g CDM L⁻¹ per OD₇₅₀ nm for BW86 (R² = 0.999) was determined. Cell density was measured via flow cytometry (Guava EasyCyte 6–2 L,
Merck Millipore). Cyanobacterial cells were counted by gating RED/RED2 fluorescence; RED = red laser, filter bandpass 690/50; RED2 = red laser, filter bandpass 661/19. The cell density of each sample was measured in triplicates.

2.6. Calculation of specific growth rate

The specific growth rate \( \mu \) in d\(^{-1} \) was determined from the initial exponential phase according to eq. (1) where \( c_{X2} \) is the cell concentration on time point \( t_2 \) and \( c_{X1} \) the cell concentration on time point \( t_1 \). Using the optical density at 750 nm instead of \( c_X \) would also be applicable.

\[
\mu = \ln\left(\frac{c_{X2}}{c_{X1}}\right) \cdot \frac{1}{t_2 - t_1}
\]

(1)

2.7. Calculation of photo conversion efficiency

Photo conversion efficiency (PCE) was calculated as the quotient of \( E_X \), the captured biomass energy, divided by \( E_{\text{abs,light}} \), the absorbed light energy (Eq. (2)).

\[
PCE = \frac{E_X}{E_{\text{abs,light}}} \times 100
\]

(2)

According to Dillschneider et al. [34], by assuming an average photon energy \( E_{\text{photon}} = 210.48 \text{ kJ mol}^{-1} \) for the illumination device and an combustion enthalpy of cyanobacterial biomass \( H_X \) of 22 kJ g\(^{-1} \) [36], \( E_X \) and \( E_{\text{abs,light}} \) can be defined as follows (eq. (3)):

\[
PCE = \frac{P_X \cdot \Delta H_X}{E_{\text{photon}} \cdot \left(I_0 - I_{\text{trans}}\right) \cdot \Delta t} \times 100\% 
\]

(3)

with \( P_X \) as the volumetric CDM productivity in g L\(^{-1} \) d\(^{-1} \), \( A_p \) in m\(^{-2} \) the illuminated surface area of the liquid reactor volume \( V_R \) in L, \( I_0 \) and \( I_{\text{trans}} \) in mol m\(^{-2} \) d\(^{-1} \) represents the incident and transmitted PFD. Start and end time of the PCE calculation interval is defined as \( \Delta t \) [d]. It has to be mentioned that \( \Delta H_X \) can change with biomass composition [34]. The measurements for \( I_0 \) and \( I_{\text{trans}} \) were performed in triplicates using a planar light sensor (Li-250, Li-Cor).

2.8. Calculation of cyanophycin and phosphor quota

The calculations of cyanophycin and phosphor quota are given in Eq. (4). Either cyanophycin \( c_{CP} \) (w/v) or phosphor concentration \( c_P \) (w/v) is used for \( c_i \) with reference to the measured CDM concentration \( c_X \) (w/v).

\[
q_{CX} = \frac{c_i}{c_X}
\]

(4)

By the evaluation of quotas (w/w), it is possible to determine the specific content of the investigated substance within the produced biomass. Quotas can be seen as an expression for theoretical balance boundary of cells.

2.9. Statistical analysis

One representative photobioreactor experiment was performed for each initial phosphate concentration and light intensity. The measurement of cyanophycin, optical density and cell density were conducted in duplicates, while PFD was measured in triplicates. A correlation curve enabled the determination of CDM in triplicates from optical density measurements. The software Microsoft Excel 2013 was used to calculate average and standard deviation values as well as one-way analysis of variance (ANOVA) with a statistical significance of \( p = 0.05 \). For IC analysis, an absolute analytical error of 10% was applied.

3. Results

3.1. Growth rates in dependency of photon flux densities

A light kinetics curve for the strain Synechocystis 6803 was determined in Miniplate photobioreactors to identify the reasonable irradiance for maximum growth (Fig. 1). Therefore, PFD were adjusted to 15, 25, 50, 100, 160, 250 and 450 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). For each irradiance used, one batch experiment was performed and specific growth rate \( \mu \) in d\(^{-1} \) was determined according to Eq. (1). The particular growth curve for each light intensity applied can be found in Fig. S2 in the supplementary material. Since low biomass concentrations <0.125 g CDM L\(^{-1} \) were used to study the effect of PFD on growth rate, the formation of a light gradient can be neglected. In our experiments the maximum growth rate was 1.32 d\(^{-1} \), resulting in a generation time of 0.52 d\(^{-1} \) (=ln(2)/1.32 d\(^{-1} \)) or a cell division rate of 1.91 d\(^{-1} \) (=1.32 d\(^{-1} \)/ln(2)). We observed that growth rate increased strongly when PFDs in the range of 15 to 50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) were applied, while in the range of 100 to 450 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) showed only a slight increase. The point of intersection between the dashed lines in Fig. 1 represents \( I_k \), the transition from photo-limited to the photo-saturated region. \( I_k \) was determined to be 46 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). This means that in the light-limited region, lower than 46 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), growth rate is directly proportional to irradiance whereas in the light-saturated region, above 46 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) this relation is no longer valid and the growth rate reaches its maximum at around 150 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). However, for future experiments it would be reasonable to use a PFD slightly higher than \( I_k \) to achieve high growth rates with the lowest possible light intensity supplied.

3.2. Cultivation of Synechocystis BW86 under phosphate starvation

Batch experiments in Midiplate reactors were performed with different concentrations of phosphate in the culture medium in order to investigate the potential of Synechocystis BW86 to accumulate the biopolymer cyanophycin. Initial phosphate concentrations varied from 16.65, 8.32, 4.16, 3.33 and 2.08 to 1.66 mg L\(^{-1} \) (corresponding to 5.43, 2.71, 1.36, 1.09 and 0.68 to 0.54 mg L\(^{-1} \) phosphor). Furthermore, the wild-type strain Synechocystis 6803 was cultivated under same conditions as benchmark. Since the phosphate availability affects both cyanophycin accumulation and metabolism maintenance, we were able to evaluate how the concentration of the internal cellular
phosphate, i.e. the phosphor quota $q_{P,X}$ (in mg initial phosphor per g CDM), specifically influences the cyanophycin concentration, i.e. the cyanophycin quota $q_{CP,X}$ (in g cyanophycin per g CDM), as well as the transition to stationary growth phase. $I_0$ was manually adjusted to 70 $\mu$mol m$^{-2}$ s$^{-1}$ prior to each experiment in accordance to $I_0$ (Fig. 1) and in order to facilitate the comparability with Watzer et al. [32]. The initial biomass concentration (CDM) for all experiments was 0.03 g L$^{-1}$.

Fig. 2 shows exemplarily a batch cultivation with an initial phosphate concentration of 3.33 mg L$^{-1}$ (corresponding to 1.09 mg L$^{-1}$ phosphor). Concentration gradients of CDM $c_X$, phosphor $c_P$, cyanophycin $c_{CP}$ and the PFD of transmitted photons $I_{trans}$ are given in Fig. 2A. Fig. 2A can be defined as the balance boundary “cell suspension”, showing volumetric quantities. Fig. 2B shows the cell concentration $c_{cells}$, phosphor quota $q_{P,X}$ and cyanophycin quota $q_{CP,X}$ and therefore can be defined as the balance boundary “cells”, showing specific quantities. During the first 4 days cells were growing exponentially while $I_{trans}$ decreased to 19 $\mu$mol m$^{-2}$ s$^{-1}$, corresponding to a decrease of 67%. From day 4 to 8 growth extends linearly and merges into the stationary phase from day 8 to 9. Due to the increasing biomass concentration, $I_{trans}$ decreased below 6 $\mu$mol m$^{-2}$ s$^{-1}$ until cultivation day 13. The cultivation was finished at day 13 with a maximum biomass concentration of 1.1 g L$^{-1}$. Concerning the low amount of transmitted light at the end of this experiment, the cells are light limited at this point. As can be seen in Fig. 2 light limitation usually leads to linear growth. Hence, with phosphate as the only depleted nutrient, stationary growth phase was induced due to phosphate limitation. The predetermined amount of phosphor was consumed within the first 24 h of cultivation as confirmed by ion chromatography (Fig. 3). Incident and transmitted PFD were measured to calculate the photo conversion efficiency (PCE, Table 1). The calculation of PCE was performed according to chapter 2.7 and yielded a value of 5.5% for this exemplary batch cultivation.

Cyanophycin concentration increased 8.5fold from 40 to 340 mg L$^{-1}$, resulting in a cyanophycin quota $q_{CP,X}$ of 0.14 and 0.34 g cyanophycin per g CDM, respectively. After entering the stationary phase, $c_{CP}$ did not increase anymore. By considering the progression of $q_{CP,X}$ in Fig. 2B an increase in cyanophycin content was observed during the linear phase. During the stationary phase, $q_{CP,X}$ decreased slightly from 0.34 to 0.32 g cyanophycin per g CDM. This decline can be attributed to the increase in $c_X$ from 1.0 to 1.1 g L$^{-1}$ despite steady cell concentrations $c_{cells}$, which indicates that cyanobacterial cells increase their weight, probably by accumulating proteins or storage compounds like lipids, PHB or glycogen. The amount of initial phosphor per CDM $q_{P,X}$

**Fig. 2.** Batch cultivation of Synechocystis BW86 under phosphate starvation in dependence of cultivation time $t_c$. A: cell dry mass (CDM) concentration $c_X$, media phosphor concentration $c_P$, cyanophycin concentration $c_{CP}$ and PFD of transmitted photons $I_{trans}$, B: cell concentration $c_{cells}$, phosphor quota $q_{P,X}$ [mg initial phosphor per g CDM] and cyanophycin quota $q_{CP,X}$ [g cyanophycin per g CDM]. Cultivation parameters: $I_0$ 70 $\mu$mol m$^{-2}$ s$^{-1}$, 28 °C, pH 7.5–8.0, 2% CO$_2$. Except for $q_{CP,X}$ no error bars are given since no significant difference regarding the standard deviation (<1%) was observed.
showed a reverse progression compared to \( c_{\text{cells}} \). At the end of the exponential phase, \( q_{\text{P,X}} \) was already reduced to 4.1 mg phosphor/g and reduced further to 1.2 and 1.0 mg phosphor/g at the end of linear and stationary phase, respectively. The data presented in Fig. 2B show that the highest increase in cyanophycin content occurred in the range of 4.1 to 1.2 mg phosphor per g CDM and that the stationary phase was initiated at 1.0 mg phosphor per g CDM. This value corresponds to a phosphor content of 5.5 ng phosphor per cell. Thus, according to the determined values for \( q_{\text{P,X}} \) and under consideration of the growth curves, the entry to the stationary phase presumably occurs at phosphor quotas of 1.0 mg phosphor per g CDM or 5.5 ng phosphor per cell. The accumulation of cyanophycin occurs between 4.1 and 1.2 mg phosphor per g cell or 16.6 to 6.0 ng phosphor per cell. The nutrients nitrate and sulfate were sufficiently available since their final concentrations at the end of the cultivation were estimated as 0.86 g nitrate L\(^{-1}\) and 12.1 mg sulfate L\(^{-1}\) (Fig. 3).

It was furthermore possible to evaluate the specific cyanophycin formation rate \( r_{\text{CP}} \) in d\(^{-1}\), and the specific growth rate \( \mu \) in d\(^{-1}\). \( r_{\text{CP}} \) was calculated according to Eq. (5) where \( C_{\text{cell,2}} \) is the cell concentration on time point \( t_2 \) and \( C_{\text{cell,1}} \) the cell concentration on time point \( t_1 \).

\[
r_{\text{CP}} = \frac{C_{\text{cell,2}} - C_{\text{cell,1}}}{t_2 - t_1} \cdot \frac{1}{C_{\text{cell,2}}} \tag{5}\]

A linear stoichiometric correlation between \( r_{\text{CP}} \) and \( \mu \) was observed. The slope of the estimated specific rates was determined to be 0.33 (correlation coefficient \( R^2 = 0.999 \), Fig. 4).

### 3.3. Cyanophycin accumulation of Synechocystis 6803 and BW86 under phosphate starvation

The engineered strain Synechocystis BW86 and the wild-type strain were used to investigate the effect of phosphate availability regarding cyanophycin accumulation. Therefore, several cultivations with different initial phosphate concentrations in BC-11 medium were performed, using Midiplate photobioreactors. A representative batch process for Synechocystis BW86 is illustrated in chapter 3.2. The combined results of all performed batch cultivations regarding the cyanophycin quota \( q_{\text{CP,X}} \) in dependence of initial phosphor concentration \( c_{\text{P,initial}} \) (applied as K\(_2\)HPO\(_4\) in the medium) and in dependence of phosphor quota \( q_{\text{P,X}} \) are shown in Fig. 5. With the reduction of the initial phosphate concentration in the medium (\( c_{\text{P,initial}} = 2.71 \text{ mg L}^{-1} \)), an increased cyanophycin quota was observed in the stationary phase (\( q_{\text{CP,end,X}} \)). From 2.71 to 0.54 mg phosphor/L a linear increase in \( q_{\text{CP,end,X}} \) for both Synechocystis 6803 and BW86 was detected. A maximum of 0.40 and 0.18 g cyanophycin per g CDM was achieved for mutant and wild-type cultures, respectively (Fig. 5A).

The diagram in Fig. 5B, representing phosphor quotas to corresponding cyanophycin quotas for Synechocystis BW86 and 6803, reveals that a certain amount of phosphor should not be exceeded to induce cyanophycin accumulation. For phosphor quotas higher than 4 mg phosphor/g, no increase in \( q_{\text{CP,X}} \) was found. In contrast, as soon as \( q_{\text{P,X}} \) falls below 4 mg phosphor per g CDM, cyanophycin accumulation occurs. For \( q_{\text{P,X}} \) values < 1 mg phosphor per g CDM no further increase of \( q_{\text{CP,X}} \) was observed because the stationary phase was usually reached at this point, most likely due to the ongoing decrease in \( q_{\text{P,X}} \) and the related

### Table 1

<table>
<thead>
<tr>
<th>( c_{\text{P,initial}} ) [mg L(^{-1})]</th>
<th>( c_{\text{P,end}} ) [g L(^{-1})]</th>
<th>( c_{\text{P,initial}} ) [mg L(^{-1})]</th>
<th>( q_{\text{P,end,X}} ) [g g(^{-1})]</th>
<th>( q_{\text{P,end,X}} ) [g g(^{-1})]</th>
<th>( q_{\text{P,end,X}} ) [g g(^{-1})]</th>
<th>( PCE ) [%]</th>
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<tr>
<td>( 5.43 \pm 0.54 )</td>
<td>( 2.1 )</td>
<td>( 2.3 )</td>
<td>( 4.0 \times 10^8 )</td>
<td>( 4.3 \times 10^8 )</td>
<td>( 0.156 \pm 0.016 )</td>
<td>( 0.002 \pm 0.000 )</td>
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<tr>
<td>( 2.71 \pm 0.27 )</td>
<td>( 2.2 )</td>
<td>( 2.4 )</td>
<td>( n.a. )</td>
<td>( 4.5 \times 10^8 )</td>
<td>( 0.159 \pm 0.016 )</td>
<td>( 0.016 \pm 0.002 )</td>
</tr>
<tr>
<td>( 1.36 \pm 0.14 )</td>
<td>( 1.6 )</td>
<td>( 2.0 )</td>
<td>( n.a. )</td>
<td>( 3.4 \times 10^8 )</td>
<td>( 0.250 \pm 0.025 )</td>
<td>( 0.110 \pm 0.011 )</td>
</tr>
<tr>
<td>( 1.09 \pm 0.11 )</td>
<td>( 1.1 )</td>
<td>( 1.7 )</td>
<td>( 2.0 \times 10^9 )</td>
<td>( 2.9 \times 10^8 )</td>
<td>( 0.331 \pm 0.033 )</td>
<td>( 0.135 \pm 0.014 )</td>
</tr>
<tr>
<td>( 0.68 \pm 0.07 )</td>
<td>( 0.7 )</td>
<td>( 1.1 )</td>
<td>( 1.5 \times 10^9 )</td>
<td>( 1.9 \times 10^8 )</td>
<td>( 0.360 \pm 0.036 )</td>
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<td>( 0.54 \pm 0.05 )</td>
<td>( 0.7 )</td>
<td>( 0.9 )</td>
<td>( 1.5 \times 10^9 )</td>
<td>( 8.2 \times 10^8 )</td>
<td>( 0.402 \pm 0.040 )</td>
<td>( 0.180 \pm 0.018 )</td>
</tr>
</tbody>
</table>

n.a. = not available
inability of synthesizing nucleic acids. The breakdown of the cell metabolism is attributable solely to phosphate limitation, not to light deficiency, as confirmed by the PCE data presented in Table 1. By reducing $c_{\text{P,initial}}$ lower than 1 mg phosphor L$^{-1}$, PCE is reduced as well. Therefore, based on the data presented here, it is assumed that firstly, cell growth of Synechocystis enters the stationary phase at phosphor quotas below 1 mg phosphor per g CDM and secondly, cyanophycin accumulation is triggered at phosphor quotas between 4 and 1 mg phosphor per g CDM.

The volumetric cyanophycin productivity $P_{\text{CP}}$ in Fig. 5D follows an analogue pattern compared to the cyanophycin quotas of Fig. 5B. For phosphor quotas higher than 4 mg phosphor L$^{-1}$, no increase in $P_{\text{CP}}$ was found whereas lower values for $q_{\text{P,X}}$ result in higher productivities up to 38.7 mg cyanophycin per L per day for Synechocystis BW68. However, low initial phosphor concentrations can lead to a reduction of the volumetric cyanophycin productivity in batch mode as shown in Fig. 5C.

A summary of the batch experiments from Fig. 5 regarding final biomass concentrations $c_{\text{X,end}}$, cell densities $c_{\text{cells,end}}$, final cyanophycin quotas $q_{\text{CP,end,X}}$ as well as PCE to the corresponding initial phosphor concentrations $c_{\text{P}}$ is given in Table 1. $c_{\text{X,end}}$ was determined gravimetric as indicated in chapter 2.5. Phosphor concentrations were calculated based on applied $K_2HPO_4$ in the culture medium. The wild type Synechocystis 6803 generally showed higher values for $c_{\text{X,end}}$ and $c_{\text{cells,end}}$ indicating a higher conversion of incorporated phosphor to biomass. In return, $q_{\text{CP,end,X}}$ in the wild type was lower compared to the strain BW68, suggesting that the engineered strain preferably uses the available resources for cyanophycin synthesis with an negative effect on biomass production. By considering the calculated PCE results, it is obvious that the wild-type strain is more efficient in converting absorbed energy to biomass. Except for the two lowest phosphor concentrations, the maximum PCE was between 6.6 and 7.3% for the wild type and between 5.5 and 5.8% for the mutant, indicating that Synechocystis BW68 is less effective in the conversion of photons, probably due to the introduced mutation. The calculation of PCE is given in chapter 2.6.

4. Discussion

The growth rate of Synechocystis 6803 in dependence on PFD was investigated in Miniplate photobioreactors (Fig. 1). Maximum growth rate was determined as 1.32 d$^{-1}$. A former study investigated the growth rate of Synechocystis minima at different PFD in light/dark cycles and different temperatures. Cultivation conditions of 32 °C and 125 μmol m$^{-2}$ s$^{-1}$ resulted in a similar maximum growth rate of 1.32 d$^{-1}$ [37]. Though Synechocystis minima and Synechocystis 6803 belong to the same genus, the maximum growth rates are not directly comparable. Bland and Angenent [38] cultivated Synechocystis 6803 at different light regimes and room temperature. White light did not induce photoinhibition even at high PFD up to 1000 μmol m$^{-2}$ s$^{-1}$. Photo-saturation occurred at 200 μmol m$^{-2}$ s$^{-1}$ with growth rates between 1.2 d$^{-1}$ and 1.44 d$^{-1}$ [38]. Hence, the data presented here confirm previous studies since maximum growth rates and the photo-saturated area are in the same order of magnitude although different temperatures, i.e. 25 °C and 32 °C, were applied. Since temperature presumably affects the growth rate, it would be worthwhile to investigate this impact on light kinetics in future studies.

To investigate the ratio of the energy output that can be obtained from the produced biomass to the supplied light energy, PCE was calculated for batch cultivations with Midipile photobioreactors, using different initial phosphorus concentrations (Table 1). A theoretical maximum PCE value of 12.4% is described in literature. In real cultures the PCE is lower depending on the photobioreactor system or cultivation environment. Values between 1.5 and 5.0% are cited although higher PCEs can be reached by means of favorable light distribution [39]. Touloupakis et al. [36] reported PCE values between 7.7 and 11.9% by growing Synechocystis 6803 in continuous cultures. The comparatively high PCE levels were achieved in particular by applying high dilution rates, indicating that the reduction or avoidance of dark zone formation in the reactor can lead to an increased PCE. Regarding the PCE information from literature, we assume that the utilized photobioreactor system is adequate for the cultivation of Synechocystis. PCE values between 5.5 and 7.3% as achieved in this study for initial phosphorus concentrations between 16.65 and 3.33 mg L$^{-1}$ (corresponding to 5.43 and 1.09 mg L$^{-1}$ phosphor) are common and have no adverse effect on phototrophic cells. In order to achieve higher PCE values continuous grown cultures might be an option.

In chapter 3.2 an exemplary growth curve was shown for a batch cultivation of Synechocystis BW68 under phosphate starvation (Fig. 2). We observed that cell growth proceeds despite phosphor depletion. For that reason we assume that polyphosphate granules were enriched within the first 24 h and supplied the organism with phosphor until the stationary phase was reached. Moreover, it is likely that the cells degraded their phycobilisomes. Bleaching of the culture from a blue-green to a yellowish color was observed during process time (unpublished data). This effect was already described earlier for Synechococcus sp. PCC 7942 grown under nitrogen, sulfur and phosphor deprivation in 50 mL culture tubes, bubbled with 3% CO$_2$ in air [40]. Nutrient deprivation of cyanobacterial cells leads to degradation of phycobilisomes after sulfur or nitrogen deprivation. Collier and Grossman [40] also reported a partial degradation of light-harvesting phycobiliproteins in cyanobacteria after phosphor deprivation. Hence, it is reasonable to run a cyanophycin production process at phosphor quotas $q_{\text{P,X}}$ in which a high PCE value is reached despite the fact that phycobilisomes are degraded, i.e. between 1 and 3 mg phosphor per g CDM (see Table 1 or Fig. 5B). Of course, nitrogen and sulfur deprivations have to be avoided. A further degradation of phycobilisomes due to nutrient deprivation would result in lower PCE values.

Nutrient consumption of Synechocystis BW68 revealed a linear decrease of NO$_3^-$ and SO$_4^{2-}$ concentrations (Fig. 3) until stationary growth phase was reached. In terms of cultivating this strain under phosphate starvation, the use of lower amounts of these nutrients in the medium would be feasible regarding the fact that only 26% of the available NO$_3^-$ and 50% of the available SO$_4^{2-}$ in the BG-11 medium were consumed. The linear correlation between $c_{\text{P}}$ and $\mu$ in Fig. 4 point out that considered phosphate depletion has no adverse effect on the growth efficiency of cells as additionally confirmed by the comparatively high PCE (Table 1). Progressing phosphate starvation force the cell to
cease nucleic acid synthesis. Consequently, cell division stops (see Fig. 2). The subsequent increase in CDM indicates that the remaining nutrients are used to form proteins or storage compounds.

The main focus of this research was to understand how phosphate availability affects the production of cyanophycin. Previous studies already described a relation between phosphate concentration and cyanophycin accumulation [24,32,41]. However, there was a lack of quantitative information regarding the suitable phosphate concentration, phosphor quota as well as corresponding PCE so far. The engineering approach and quantitative data of this work can consequently contribute to improve cyanophycin production with Synechocystis.

Elbhaloul et al. [24] characterized phosphate limitation as the most effective nutrient limitation for promoting cyanophycin biosynthesis and accumulation in Acinetobacter calcoaceticus. For small phosphate amounts of 42 μM, which corresponds to a phosphor concentration of 1.30 mg L$^{-1}$, higher cyanophycin contents were reported. This value corresponds to our results for Synechocystis (Fig. 5A). Stevens and Paone [41] describe the accumulation of cyanophycin granules as a result of phosphate limitation in Agmenellum quadruplicatum (now renamed as Synechococcus sp. PCC 7002). So far, heterologous expression systems like heterotrophic bacteria, genetically engineered yeast or plants harboring a cyanobacterial cyanophycin synthetase gene (CphA) were used for cyanophycin production in bench scale [3]. The resulting heterologous cyanophycin differs from native cyanophycin in the length of the polymer, amino acid composition and solubility. The unicellular cyanobacterium Synechocystis 6803 can accumulate native cyanophycin in response to unbalanced growth conditions.

The work of Watzer et al. [32] and the results presented here confirm an enhanced cyanophycin accumulation in cyanobacteria due to deficiency of the element phosphor, in particular for Synechocystis 6803 and the engineered strain BW86. Therefore, phosphate starvation can be an effective strategy to produce cyanophycin in high yields using photobioreactors. The flat plate reactors we used are geometrical similar to larger flat plate production systems and allow a scale-up to higher volumes. For open pond or bag systems the geometric differences should be considered. In terms of productivity, the applied phosphate concentration and production strategy such as batch or fed-batch is important to achieve a sufficient volumetric cyanophycin productivity (Fig. 5C and 5D).

Based on the data of this work it is furthermore possible to develop a cultivation process, preferably a fed-batch or continuous process, where the phosphate availability is controlled to induce an ongoing cyanophycin production with high cyanophycin yields. Genetic stabilization of the engineered production strain BW86 might be an issue in

![Figure 5](image-url)

**Fig. 5.** Effect of phosphate availability on cyanophycin accumulation and productivity in Synechocystis 6803 and BW86. A: cyanophycin quota in the stationary phase $q_{CP,end,X}$ [g cyanophycin per g cell dry mass] in dependence of initial phosphor concentration $c_{P,initial}$ [mg L$^{-1}$]; B: cyanophycin quota $q_{P,X}$ in dependence of phosphor quota $q_{P,X}$ [mg phosphor per g cell dry mass]; C: volumetric cyanophycin productivity in the stationary phase $P_{CP,end}$ [mg cyanophycin per L per day]; D: volumetric cyanophycin productivity $P_{CP}$ [mg cyanophycin per L per day]. The dashed line shows the trend of the respective quota and productivity. Cultivation parameters: $I_0$ 70 μmol m$^{-2}$ s$^{-1}$, 28 °C, pH 7.5–8.0, 2% CO$_2$. For reasons of clarity no error bars are shown in 4B and 4D, the absolute analytical error in 4A and 4C is 10%.
biotechnological applications, especially for continuous processes. Although Guerrero et al. [42] reported that an engineered Synechocystis 6803 strain for ethylene production was stable for >6 months in liquid culture, it has to be considered that the BW86 strain harbors solely a point mutation in the PII gene.

5. Conclusion

A quantitative regulation of phosphate availability in terms of different initial phosphate concentrations affects the cyanophycin accumulation. The engineered strain Synechocystis BW86 showed higher cyanophycin yields compared to the wild type due to the increased NAGK activity. However, the total produced biomass and PCE values were lower compared to cultivations of the wild type, indicating a less effective conversion of absorbed photons. The essential amount to induce cyanophycin accumulation is between 1 and 4 mg phosphate per g CDM, while stationary growth phase was reached around 1 mg phosphate per g CDM. To our knowledge, this is the first study analyzing the specific necessary phosphate quota to trigger cyanophycin accumulation and to initialize stationary growth phase of Synechocystis. A maximum growth rate of 1.32 d⁻¹ was found by applying a photon flux density of 150 μmol m⁻² s⁻¹ where the transition from photo-limited to photo-saturated region already occurred at 46 μmol m⁻² s⁻¹. The quantitative statements of this work allow a scale-up to larger production systems.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.algal.2016.10.005.

References