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Exploring the potential of high-density cultivation of cyanobacteria for the production of cyanophycin

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ABSTRACT

Photoautotrophic cyanobacteria and microalgae offer significant potential for the renewable synthesis of high-value products. As yet, however, the productivity of phototrophic cultures is limited due to the low cell densities that are typically obtained in current pilot scale photobioreactors. Here, we explore the use of ultrahigh-density cultivation of cyanobacteria for the production of cyanophycin, a non-ribosomally synthesized biopolymer of high biotechnological interest. We demonstrate that ultrahigh-density cultivation using a two-tier vessel with membrane-mediated CO_2 supply yields a cyanophycin content per cellular dry weight similar to previously reported values, while the volumetric productivity per culture volume is significantly increased. Already after 96 h of cultivation, the engineered production strain BW86 reached up to 1 g cyanophycin per liter culture, approximately a 4-fold increase over the previously reported maximal yield obtained after 12 days of cultivation. Under phosphate-limiting growth conditions, the wild-type strain *Synechcocystis* sp. PCC 6803 accumulates up to 0.6 g cyanophycin per L culture. Our results demonstrate that ultrahigh-density cultivation is a suitable strategy towards the development of viable phototrophic production processes for cyanophycin and possibly other products of interest.

1. Introduction

Cyanophycin (*multi-L-arginyl-poly-L-aspartate*) is a non-ribosomally synthesized amino acid polymer and is found in many cyanobacteria and few heterotrophic bacteria. Cyanophycin (CP) is of significant interest for a variety of biotechnological and medical applications, in particular as a source of polyaspartic acid - a biodegradable material that is functionally equivalent to petroleum-based nonbiodegradable polyacrylates [7,9]. The production and efficient isolation of CP using microorganisms is an area of active research [4].

In cyanobacteria, CP functions as an intracellular nitrogen reserve and is stored in the form of optically opaque granules in the cytoplasm. CP synthesis and accumulation is strongly dependent on growth phase and environmental conditions [10]. Accumulation of CP up to 18% of cellular dry weight (CDW) was reported for the transition to the stationary growth phase and under nutrient limitation, in particular under phosphate limitation, whereas CP content is typically < 1% of CDW during exponential growth [8].

Recently, metabolic pathway engineering in the cyanobacterial strain *Synechocystis* sp. PCC 6803 has resulted in overproduction of arginine and, correspondingly, to an improved production of CP. In particular, a single amino acid replacement, Ile86 to Asn86, in the P_{II} protein, the central regulator of nitrogen metabolism and a sensor of the carbon-to-nitrogen ratio in the cell, results in the constitutive in vivo activation of a key enzyme of arginine synthesis, the acetylglutamate kinase, and increased arginine levels [10]. The resulting strain, denoted as BW86, was reported to exhibit a CP content of up to 57% of CDW and is the most potent cyanobacterial CP production strain described to date. The results of Watzer et al. [10] were later confirmed by a quantitative evaluation of the effects of phosphate availability on CP accumulation under defined growth conditions [8]. Trautmann et al. [8] reported a CP content of up to 40% of CDW for the production strain BW86. In addition, the wild-type strain *Synechocystis* sp. PCC 6803 (denoted as Syn6803 in the following) was cultivated as a benchmark under identical conditions. For Syn6803, the study reported a maximal CP content of 18% (g CP per g CDW) under phosphate limitation. The results confirmed the suitability of phosphate limitation, as an effective strategy to produce CP with high yields using cyanobacteria.

Given the high CP yield in Syn6803 and BW86 under appropriate growth conditions, Trautmann et al. [8] argued that the development of

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a cultivation process with phosphate availability as a process parameter is possible. However, the overall volumetric productivity of photoautotrophic cyanobacterial cultures is still significantly limited due to the low cell densities that are obtained in current pilot scale photobioreactors. Therefore, in this short communication, we seek to explore and report the potential of high-density (HD) cultivation of cyanobacteria for the production of CP using the strains BW86 and Syn6803 as a benchmark.

2. High-density cultivation of cyanobacteria

In the following, we utilize a recently developed novel cultivation strategy that allows for HD cultivation of phototrophic microorganisms [1]. Typical photoautotrophic cultures involve a density of ~1 g CDW per L of culture [2]. These optically dense cultures give rise to light attenuation, such that most light quanta are absorbed in a thin surface layer. Hence, the cells in the surface layer are subject to light inhibition and exhibit low photosynthetic efficiency, whereas the remaining culture is light limited. In addition, the supply of inorganic carbon (in the form of CO_2 and HCO_3^-) is typically limiting in dense cultures. Culture media supplied with saturating concentrations of HCO_3^- may result in increased pH and reduced viability of the cells due to chronic photoinhibition and alkaline stress [2].

The cultivation strategy introduced by Bähr et al. [1] aims to overcome some of these intrinsic constraints on phototrophic growth. The suggested growth setup consists of a two-tier vessel, using a membrane-mediated CO_2 supply via a porous hydrophobic membrane, in combination with high photon flux density, rapid turbulent mixing, and optimized layer thickness of the culture. The setup is shown in Fig. 1 and detailed in the Section 5.3 “High-density cultivation setup”. The cultivation setup was previously shown to enable rapid growth of the model cyanobacteria *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 up to ultrahigh cell densities with biomass yields up to 30 g CDW per L culture [1]. Subsequently, the novel cultivation strategy was also evaluated for the HD cultivation of the filamentous terrestrial cyanobacterium *Nostoc punctiforme* PCC 73102, demonstrating the suitability of HD cultivation for the accelerated generation of biomass with filamentous cyanobacteria of the genus *Nostoc* and the induction of new secondary metabolites [5].

3. Results and discussion

We investigated the potential of HD cultivation of cyanobacteria for the production of CP, using the results of Trautmann et al. [8] as a benchmark. We considered two media compositions, phosphate-replete (P4) and phosphate-limited (P03) medium, for both strains, BW86 and Syn6803, respectively. Our growth setup did consist of 13 HD culture vessels, with triplicates for each condition (except BW86 in P4 medium with four replicates). Details are provided in the Section 5 “Materials and Methods”. Culture vessels shared a common CO_2-compartment and a common light source, enabling parallel cultivation. Cells were grown in batch culture for 6 days using an identical start optical density OD_{750nm} = 0.3. At selected timepoints, cells from individual culture vessels were harvested and the OD, CDW, and CP content was determined. CP accumulation and growth as a function of time are summarized in Fig. 2.

With respect to CDW, the results are in excellent agreement with the results of Trautmann et al. [8]: the strain Syn6803 grown in P-replete conditions accumulated only negligible amounts of CP. Under P-limiting conditions, Syn6803 accumulated CP up to 10% of CDW, a slightly lower percentage than the value reported by Trautmann et al. [8]. In contrast, the production strain BW86 accumulated high amounts of CP under both, P-replete and P-limiting, conditions. The maximum value of CP accumulation, 0.45 ± 0.3 g CP per g CDW, was again similar to the value (~0.4 g CP per g CDM) reported by Trautmann et al. [8].

Significant differences arise, however, when considering CP accumulation per culture volume, as well as CP production per culture volume per time. The strain BW86 reaches a maximum of 1 g CP per L culture already after 96 h cultivation. In contrast, conventional cultivation resulted in a maximum value of 0.28 g CP per L culture, obtained after 12 days of cultivation [8]. We note that, similar to the results of Trautmann et al. [8], we do not observe a biphasic process, but CP is steadily accumulated proportional to the CDW. The difference between HD cultures and conventional cultivation is even more pronounced for the WT strain Syn6803 under P-limiting conditions. As expected, the WT strain reached significantly higher CDW per L culture, corresponding to a yield of approximately 0.6 g CP per L culture after 96 h of cultivation.

4. Conclusions

Based on the observed CP accumulation, our key conclusions are: HD cultivation yields similar percentages of CP per CDW as reported previously. Accumulation of CP is not detrimentally affected by high cell densities. The volumetric yield per culture volume, however, was significantly increased due to the increase in CDW. Noteworthy, while the wild-type strain Syn6803 exhibits a significantly lower yield of CP per CDW even under P-limiting conditions, this difference was partly compensated by the substantially higher growth rate and higher overall culture density.

We argue that volumetric productivity is a crucial but often neglected parameter for phototrophic production scenarios, in particular with respect to the costs of liquid handling and harvesting. Given that CP accumulation was proportional to CDW during the entire growth phase, the time of harvesting, as well as overall culture conditions, can be further optimized to increase volumetric productivity per time. Our results demonstrate that HD cultivation is a suitable strategy for the growth of CP production strains and represents a promising approach to overcome some of the limitations of current photoautotrophic cultivation. We consider our results to be a strong incentive to further explore HD cultivation towards a viable production process for green biotechnology.

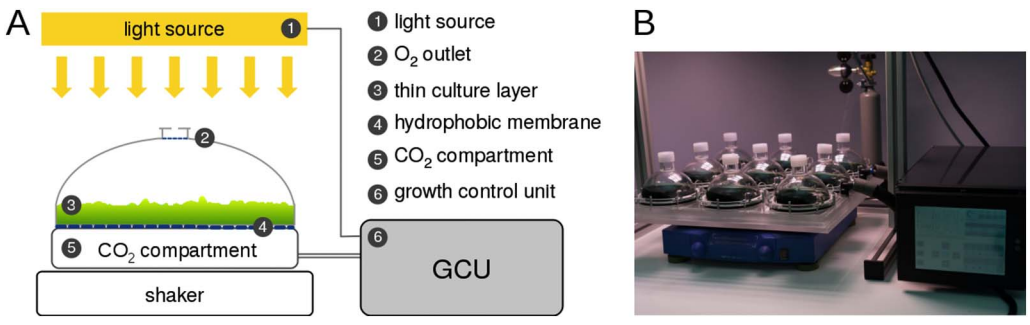

Fig. 1. High-density (HD) cultivation setup. Detachable 100 mL HD cultivators are connected via a CO_2-permeable hydrophobic membrane to a CO_2 compartment. The CO_2 partial pressure in the bottom compartment and the light intensity are controlled by a growth control unit (GCU). O_2 is released by a gas outlet membrane integrated in the cap. A thin horizontal culture layer (optical light path < 10 mm), in combination with high photon flux density and rapid turbulent mixing, allows for photoautotrophic growth of HD cultures. (B) Several HD cultivators can be attached to a common bottom CO_2 compartment in a 3 × 3 or 2 × 2 configuration, enabling parallel cultivation under identical conditions.