Trans-4-hydroxy-L-proline production by the cyanobacterium Synechocystis sp. PCC 6803

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ABSTRACT

Cyanobacteria play an important role in photobiotechnology. Yet, one of their key central metabolic pathways, the tricarboxylic acid (TCA) cycle, has a unique architecture compared to most heterotrophs and still remains largely unexploited. The conversion of 2-oxoglutarate to succinate via succinyl-CoA is absent but is by-passed by several other reactions. Overall, fluxes under phototrophic growth conditions through the TCA cycle are low, which has implications for the production of chemicals. In this study, we investigate the capacity of the TCA cycle of Synechocystis sp PCC 6803 for the production of trans-4-hydroxy-L-proline (Hyp), a valuable chiral building block for the pharmaceutical and cosmetic industries. For the first time, phototrophic Hyp production was achieved in a cyanobacterium expressing the gene for the L-proline-4-hydroxylase (P4H) from Dactylosporangium sp. strain RH1. Interestingly, while elevated intracellular Hyp concentrations could be detected in the recombinant Synechocystis strains under all tested conditions, detectable Hyp secretion into the medium was only observed when the pH of the medium exceeded 9.5 and mostly in the late phases of the cultivation. We compared the rates obtained for autotrophic Hyp production with published sugar-based production rates in E. coli. The land-use efficiency (space-time yield) of the phototrophic process is already in the same order of magnitude as the heterotrophic process considering sugar farming as well. But, the remarkable plasticity of the cyanobacterial TCA cycle promises the potential for a 23–55 fold increase in space-time yield when using Synechocystis. Altogether, these findings contribute to a better understanding of bioproduction from the TCA cycle in phototrophs and broaden the spectrum of chemicals produced in metabolically engineered cyanobacteria.

1. Introduction

When pursuing the development of a carbon-neutral global economy, fossil-based materials, fuels, and chemicals need to be replaced with compounds that are carbon neutral, meaning that their life cycle from cradle to grave has no net carbon emissions. One useful parameter to compare different production methods is space-time yield, which compares the amount of product per input of substrate over a period of time and on a given area (Janz and Wait, 1955). An essential step towards this is the use of photosynthesis as the basis of bioproduction. To date, several value-added compounds have already been produced in phototrophic cyanobacteria through genetic engineering (e.g. ethanol, isobutyraldehyde, isobutanol, 1-butanol, isoprene, ethylene, hexoses, cellulose, mannitol, lactic acid, fatty acids) (Ducat et al., 2011; Rosgaard et al., 2012; Savakis and Hellingwerf, 2015). However, there are currently two major limitations for the application of such systems. On the one hand, the space-time yields of phototrophic organisms are limited by the reactor technology available today. On the other hand, our understanding and ability to engineer photosynthetic bacteria, like cyanobacteria, is still limited, especially around central carbon metabolism. For instance, the tricarboxylic acid (TCA) cycle functions as one of the major biochemical hubs in most aerobic heterotrophs and runs at high rates due to its central role in energy metabolism. This pathway, therefore, has been a target for genetic engineering of heterotrophs for decades (Vuoristo et al., 2016). On the contrary, biotechnological processes that utilize the carbon flux through the cyanobacterial TCA cycle are currently scarce. One reason might be that, in contrast to heterotrophic bacteria, cyanobacteria cover their needs of chemical energy and...
reduction power mainly by photosynthesis. In addition, the cyanobacterial TCA cycle has for a long time been hypothesized to be incomplete (Pearce et al., 1969), as the enzyme 2-oxoglutarate dehydrogenase (2-OGDH) is absent (Hoare et al., 1967). Hence, it has been assumed that when photosynthesis supplies sufficient reducing equivalents and ATP, the TCA cycle is bifurcated and maintains only low anabolic fluxes compared to heterotrophs (Van et al., 2017). The first TCA-coupled production of a value-added compound in cyanobacteria was the production of ethylene via the introduction of the ethylene forming enzyme (Efe) (Ungerer et al., 2012). Subsequently, it could be shown that the introduction of Ef enhanced the flux through the TCA cycle from 13 to 37% of total fixed carbon, and interestingly, 10% of total fixed carbon are diverted to ethylene production (Xiong et al., 2015). This indicates an intriguing potential of the cyanobacterial TCA cycle for bioproduction.

Hydroxylated amino acids, such as trans-4-hydroxy-L-proline (Hyp), serve as valuable chiral building blocks for chemical synthesis, which are often not available at a reasonable expense by classical chemical means (Klein and Hüttel, 2011; Theodosiou et al., 2015). The non-canonical amino acid Hyp is a chiral building block and is important for the pharmaceutical and cosmetics industry. It is for instance processed into carboxenem antibiotics (Remuzon, 1996), antifungal echinocandins (Petrera et al., 2013), or neuroexcitatory kainoids (Selley et al., 2002), of which the latter are relevant in neuroscience research. Hyp is also a direct ingredient of several skincare products from the cosmetics industry (Wishart et al., 2018).

In nature, Hyp is formed by post-translational modifications of proteins, i.e. from the amino acid L-proline. A high Hyp content is found in mammalian collagen, where it accounts for approximately 13.5% of total amino acids (Neuman and Logan, 1950). Accordingly, the acidic hydrolysis of mammalian collagen is the most common method to produce Hyp on an industrial scale (Takeshi Shibasaki et al., 2000). Even though the process is economically feasible, acid hydrolysis of collagen requires a complex purification process and produces large volumes of downstream waste (Shibasaki et al., 2000; Takeshi Shibasaki et al., 2000). Enzymes offer an alternative for the conventional production of Hyp. The 2-oxoglutarate (2-OG) dependent L-proline-4-hydroxylase (P4H; EC 1.14.11.2) is Fe2+ dependent and uses L-proline, oxygen, and 2-OG as co-substrates for catalyzing the hydroxylation of proline to Hyp. When microbial cells are used as catalysts their TCA cycle continuously regenerates the substrate proline, as well as the co-substrate 2-OG. The by-product succinate enters back into the TCA cycle (Fig. 1). Recombinant P4H from Dactylosporangium has been shown to efficiently hydroxylate free L-proline regio- and stereospecifically in Escherichia coli (E. coli) by fermentation (glucose as substrate) and biotransformation (L-proline as substrate) (Shibasaki et al., 1999; Shibasaki et al., 2000; Theodosiou et al., 2017, 2015) as well as in Corynebacterium glutamicum by fermentation (Falcioni et al., 2015).

The biotechnological production of Hyp with heterotrophic bacteria or biotransformation requires organic carbon, which will usually be provided in the form of carbohydrate, i.e., phototrophic CO2 fixation into glucose. Producing sugar first and then running a heterotrophic process leads to substantial energy losses, considering photon efficiency and achievable yields per hectare. Hence, it is advantageous on a photons per mol product basis to produce a desired chemical directly from CO2 photo-autotrophically and skipping the intermediate fixation of CO2 into biomass (Ducat et al., 2011). Direct production based on photosynthetic CO2 fixation should therefore be preferred from an ecological point of view, especially in view of a bio-based economy (Van Dam et al., 2005).

Here, we report the first example of photosynthetic Hyp production directly from CO2, by genetic engineering of the cyanobacterium Synechocystis sp. PCC 6803 (hereafter Synechocystis). Upon expression of the p4h gene from Dactylosporangium Hyp formation with titers of 162 μM were observed. Interestingly, we found that the pH of the growth medium and the growth phase of the culture strongly impact Hyp production via a not yet characterized mechanism.

2. Material and methods

2.1. Cultivation

2.1.1. Media and chemicals

All chemicals used in this study were at least of analytical grade and obtained from AppliChem (Darmstadt, Germany), Sigma Aldrich (Steinheim, Germany), or Carl Roth (Karlsruhe, Germany) unless stated otherwise. Water used for medium preparation was Milli-Q® grade (18 MΩ cm).

Cultivations were performed on a range of chemically defined culture media based on BG11 medium (Rippka et al., 1979). Transformation and initial experiments were carried out in BG11 medium buffered with 10 mM 2-[(1,3-dihydroxy-2-hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid (TES; buffering capacity 6.8–8.2) adjusted to pH 8. For the cultivation at different initial pH, the concentration of the buffering substances was increased to 20 mM. Depending on the initial target pH of the cultivation the following buffer substances were added: TES adjusted to pH 7.5 (BG11–7.5); TES adjusted to pH 8 (BG11–8); 3-[(1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]propane-1-sulfonic acid (TAPS; buffering capacity 7.7–9.1); BG11–8.5) adjusted to pH 8.5; 2-(cyclohexylamino)ethanesulfonic acid (CHES; buffering capacity 8.6–10; BG11–9.5) adjusted to pH 9.5 using; 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS; buffering capacity 9.7–11.1; BG11-10) adjusted to pH 10. The pH was adjusted with 10 N NaOH.

Fig. 1. Phototrophic and heterotrophic production of Hyp.
Solid media were obtained by supplementation of BG11 liquid medium with 3 g L\(^{-1}\) sodium thiosulfate and solidified with 1.5% Bacto\(^{TM}\) agar.

2.1.2. Synechocystis strains and growth conditions

*Synechocystis sp.* PCC 6803 was obtained from the Pasteur Culture Collection of Cyanobacteria and served as the parental strain. Axenic cultures were grown at 30 °C, 150 rpm (25 mm orbital shaking), 75% relative humidity, and 50 μmol photons m\(^{-2}\) s\(^{-1}\) constant illumination. For incubation on solid medium, the relative humidity was increased to 80%. Growth media for mutant lines were supplemented with 25 μg mL\(^{-1}\) kanamycin (Km) or 5 μg mL\(^{-1}\) gentamycin (Gm).

2.2. Molecular biology

2.2.1. Strain engineering and transformation of Synechocystis

The construct for p4H expression in *Synechocystis* was generated in *silico* and then amplified by Eurofins Genomics GmbH (Ebersberg, Germany). The sequence of the p4H gene was used as described in a previous study (Falcioni et al., 2013). The gene was codon-optimized for *E. coli* and due to the high similarity of codon usage between *E. coli* and *Synechocystis* did not require further codon optimization (Heidorn et al., 2011). As the promoter, a sequence resembling the 336 bp upstream of *petE* start codon was fused to the 5′-end of the p4H coding sequence. A 3xFLAG tag-encoding sequence was added to the 3′-end thereby replacing the native stop codon. Moreover, the sequence resembling 100 bp downstream of the *petE* stop codon was added as a 3′UTR as well as the oop transcriptional terminator. Restriction sites for Xhol and Asel were added to the 5′- and 3′-end of the sequence, respectively. The whole sequence was synthesized (Eurofins Genomics) and cloned into the replicative plasmid pVZ322 (Zinchenko et al., 1999) using the added Xhol and Asel restriction sites.

For transformation, electro-competent *Synechocystis* cells were prepared by centrifugation of 50 mL culture with an OD\(_{750}\) of 1 (4 °C; 2000 g; 10 min). The cell pellet was washed three times with 10 mL of ice-cold HEPES buffer (1 mM, pH 7.5, sterile filtered). Finally, the cell pellet was re-suspended in 1 mL ice-cold HEPES buffer with 5% DMSO (sterile filtered) and stored in 100 μL aliquots at −80 °C. For transformation via electroporation, an aliquot of electro-competent cells was thawed on ice. 500 ng DNA was added carefully and gently mixed by stirring with a pipette tip. The cells were transferred to ice-cold electroporation cuvettes and transformed by conducting 2.5 kV, 25 μF, 200 Ω for approximately 5 ms (Eporator, Eppendorf). The cells were re-suspended in 1 mL of fresh BG11 medium and added to 50 mL BG11 medium. The culture was incubated for 24 h at standard conditions before the cells were harvested by centrifugation (30 °C; 2000 g; 10 min) and transferred to BG11 agar plates containing 5 μg Gm mL\(^{-1}\), and incubated at standard conditions with a reduced light intensity of 20 μmol photons m\(^{-2}\) s\(^{-1}\) constant light for the first 24 h. Single colonies were picked and transferred to a new BG11 plate containing 5 μg mL\(^{-1}\) Gm. The successful transformation was verified via PCR using primers (Table S1) and pVZ322_fw and pVZ322_rv for SynP4H.

2.3. Analytics

2.3.1. Sampling

1 mL samples were taken in 2 mL tubes and centrifuged for 5 min at 17,000 g and room temperature. The supernatant was transferred to a fresh tube and both supernatant and cell pellet were stored at −20 °C. The supernatant was used in the Hydroxyproline assay without further processing. Intracellular Hyp was extracted from cell pellets and the concentration was determined using the same assay.

2.3.2. Extraction of intracellular metabolites

Metabolite extraction was performed as described in (Eisenhut et al., 2008). Cell pellets stored at −20 °C were thawed on ice, resuspended in 300 μL of methanol, and incubated at 70 °C for 15 min under continuous shaking (2000 rpm). Afterward, the suspension was mixed with 200 μL of chloroform and further incubated at 37 °C and 1000 rpm for 5 min 200 μL of Milli-Q water were added to the mixture and the tubes were vortexed briefly. The samples were centrifuged at 17,000 g for 5 min. The resulting upper fraction was collected and stored at −20 °C. The extracts were not checked for metabolite leakage during cell harvest and the metabolism was not quenched before harvest. But all samples (including controls) were treated the same way and potential leakage or turnover of intracellular metabolites should affect all samples similarly, hence the provided data can be interpreted qualitatively.

2.3.3. Hydroxyproline assay

Hydroxyproline concentration was determined using a photometric assay developed to assess the quality of meat products, which was adapted to be performed in 96-well microtiter plates (Falcioni et al., 2013). In short, 100 μL of the sample was mixed with 50 μL oxidizing solution (14 g L\(^{-1}\) Chloramine T) and incubated shaking (amplitude 4 mm) at RT for 20 min. Subsequently, 50 μL of chromophore solution (100 g L\(^{-1}\) 4-dimethylamine-benzaldehyde) was added and the plate incubated at 60 °C for 20 min. After cooling down to RT for 20 min absorption was measured at 558 nm. Hyp concentration in the samples was determined against calibration standards ranging from 0 to 50 μM Hyp. Samples with higher concentrations were diluted accordingly. The concentration of Hyp has not been corrected for the evaporation losses of 0.2 mL day\(^{-1}\) during the long cultivation times (David et al., 2018).

2.3.4. Determination of cell dry weight (CDW)

CDW was measured as OD\(_{750}\) and calculated with a correlation factor determined experimentally. For each strain, triplicates of exactly 2 mL culture samples were taken daily for a duration of 14 d in pre-weight tubes. The samples were centrifuged at 17,000 g for 5 min. The cell pellets were washed twice with Milli-Q water and then dried completely at 60 °C. An additional sample was taken to determine the OD\(_{750}\) and to check the culture for contamination using light microscopy. Correlating the measured CDW with the corresponding OD\(_{750}\) values gave a correlation factor of 0.163 CDW OD\(_{750}\), which was used in all experiments to determine CDW from OD\(_{750}\) measurements.

2.3.5. Western blot analysis and SDS-PAGE

Cells were grown under copper-depleted conditions to an OD\(_{750}\) of 1.3 before p4H expression was induced by adding 1 μM CuSO\(_4\) to the cultures. Samples were taken before induction (0 h) and after 1, 2, 4, 8, and 24 h. Samples were centrifuged (4 °C; 3000 g; 15 min) and re-suspended in ice-cold TBS buffer with 1 mM PMSF added. Cells were disrupted using glass beads (2 × 1 mm, 140 mg 0.4–0.6 mm, 120 mg 0.09–0.15 mm) and a Precellys Evolution homogenizer (Bertin Technologies) at 10,000 rpm for 4 × 30 s with a break of 30 s. The temperature of the samples was kept at 0–4 °C with a Cryolys Advanced Temperature Controller containing liquid nitrogen. Protein content was determined using a Bradford Dye Reagent Solution (ready-to-use, Alfa Aesar, Karlsruhe, Germany). A total amount of 7.5 μg protein was separated on 12% SDS-PAGE (Laemmli, 1970) and transferred to a nitrocellulose membrane (pore size 0.45 μm, NitroBind). Recombinant P4H protein was detected using an ANTI-FLAG M2-HRP antibody (Sigma-Aldrich, Cat. No. A8592) following the manufacturer’s protocol.

2.3.6. Data analysis

All data visualization and statistical analysis were done using the Prism software (GraphPad). The level of quantification (LoQ) was determined to be ≤7 μM Hyp, using the Z-factor of Zhang and colleagues (Zhang et al., 1999). At a concentration of ≤7 μM Hyp the Z-factor is < 0, meaning that the signal variation and the control signal variation overlap, and the signal cannot be differentiated from random noise.
3. Results

3.1. Genetic engineering of a hyp producing Synechocystis strain

In order to produce Hyp photosynthetically, the p4h gene from *Dactylosporangium* was fused to the copper-inducible petE promoter (Englund et al., 2016), native to *Synechocystis* (Fig. 2A). The gene was amplified from the previously generated pET24a-p4h and already contained the p4h gene codon-optimized for *E. coli* (Theodosiou et al., 2015). Further optimization was not required due to the high similarity in codon usage between *E. coli* and *Synechocystis* (Heldorn et al., 2011). Moreover, the coding sequence of a 3xFLAG-tag was fused to the 3’ end of the p4h gene to enable detection of the gene product in *Synechocystis*. The recombinant promoter:gene construct was introduced into *Synechocystis* via the self-replicating vector pVZ322 (Zinchenko et al., 1999) (Fig. 2A). The new strain was designated *Synechocystis* pVZ322_PpetE:P4H (SynP4H). A control strain was designated *Synechocystis* pVZ322_PpetE (SynEV). Successful transformation of *Synechocystis* was verified via PCR (Fig. 2B).

Cu²⁺-inducible synthesis of the recombinant enzyme P4H was verified via Western blot using an antibody against the 3xFLAG-tag. As expected the protein abundance increased strongly over a time course of 24 h after the addition of 1 μM CuSO₄ (Fig. 2C). However, a significant amount of recombinant protein was also detectable prior to induction and even though cells were grown in medium without any added Cu²⁺, indicating a certain leakiness of the promoter. This leakiness can be explained by residual Cu²⁺ attached to the glassware or in the cells since it did not have any negative effect on final biomass concentration when compared to cells grown in standard BG11 (Fig. 2E). The half growth inhibitory concentration (IC₅₀) for Cu²⁺ has previously been reported to be 2 μM (Blasi et al., 2012). However, even at the highest concentration of 3 μM Cu²⁺, a negative effect on the final biomass concentration was not observed. Besides, no difference in growth was observed between SynP4H and SynEV (Fig. 2E).

3.2. Factors correlating with hyp production

It is generally known that cyanobacteria increase the pH when grown to high densities in shake flasks. Likely this is due to the large-scale uptake of HCO₃⁻ and subsequent release of OH⁻ inside the cell, which is then compensated by the uptake of protons (Badger and Price, 2003; Mangan et al., 2016; Prins et al., 1980). Furthermore, the pH is important for cellular processes like carbon uptake and the carbon concentrating mechanism (Mangan et al., 2016; Price et al., 2002). However, unlike light intensity or nutrient deprivation, the pH is usually not considered as an important parameter when evaluating factors to increase the growth and bioproduction of cyanobacteria (Hoschek et al., 2019; Koch et al., 2020; Ungerer et al., 2018). In an initial experiment using a 4 L flat-panel bio-reactor, it was noted that the daily production of Hyp was highest at a pH of ~10, while at a lower pH the production decreased strongly (Fig S5). For this reason, the effect of the pH on the production of Hyp was investigated by using media with different buffering substances and set to different initial pH (BG11-7.5, BG11-8, BG11-8.5, BG11-9.5, BG11-10). It has to be noted that using TES at an initial pH of 8 is almost out of the buffering range of the substance, which makes the medium...
essentially not buffered. In the modified BG11 media, none of the cultures maintained a stable pH (Fig. 3A–B). In BG11–7.5 medium, the pH increased to >10 within 14 days and decreased slightly to pH ~10. In BG11-8, the pH increased to ~11 within 7 days. From day 12 onwards, the pH decreased to ~9.5 until the end of the cultivation. In BG11–8, the pH was the most stable but also increased steadily to a pH > 9 until the end of the experiment. The pH of cultures buffered at pH 9.5 and 10 behaved similarly and increased to pH > 10 within 4 days, remained stable until day 14 before it decreased slightly to pH ~10. No difference in pH was observed between SynP4H and SynEV cultures.

Significant amounts of Hyp were first detected at day 7 in cultures grown in BG11-8 (Fig. 2C). Cultures incubated in BG11–7.5 accumulated significant amounts of Hyp from day 14 onwards. In both cases, the production correlates with an increase in the pH of the medium. Interestingly, cells cultivated in BG11–9 and BG11–10 showed no correlation of pH and Hyp production and produced most of the Hyp after day 14. Cultures grown in BG11–8.5, where the pH was most stable and does not increase above 9.5, did not produce significant amounts of Hyp throughout the experiment. SynEV control cultures did not produce Hyp, regardless of the medium used (Fig. 3D). The maximum Hyp titers were observed at the end of the experiment and were 78.61 ± 6.26 μM in BG11–7.5, 162.5 ± 11.38 μM in BG11–8, below the LoQ in BG11–8.5, 132.14 ± 12.71 μM in BG11–9.5, and 89.09 ± 8.43 μM in BG11–10 (Fig. 3C). Comparable to the previous experiments, all cultures showed similar growth behavior throughout the experiment, regardless of the initial pH, changes of the pH over time, or the buffering substance used (Fig. 3E and F).

As the pH was not stable in any culture, Hyp export rates and internal content of Hyp were determined in the same experiment for every day and plotted against the pH on that given day (Fig. 4). The maximum production rate observed was 30.77 μmol gCDW d⁻¹ at pH 10.18 (Fig. 4A). During the most stable period of production (BG11–8, days 5–13) the average production rate was 16.87 μmol gCDW d⁻¹ and the pH higher than 11 (Figs. 3C and 4A). Release of Hyp into the medium was detected only in the pH range of 9.5–11.3 (Fig. 4A). Hyp was never detected in amounts above the LoQ at a lower pH and hence no export rates could be calculated. However, also at a pH > 9.5, the production rates were not stable and sometimes close to zero or even below zero (Hyp import or degradation). During this experiment, Hyp was also extracted from cell pellets to analyze the intracellular Hyp pool. It has to be noted that the samples were neither quenched nor tested for metabolite leakage and should be seen as a qualitative measure for enzyme activity. Intracellular Hyp was detected in all samples except for days zero and two. Here the biomass amount was likely too low to extract significant amounts from the pellets. In contrast to the medium, Hyp could be detected independently of the external pH inside the cell (Fig. 4B).

We noticed that the late cultivation phases seemed more productive than earlier phases. To illustrate this, the data from Figs. 3 and 4 were pooled in early (days 1–10) and late (days 11–19) cultivation phases (Fig. S2). It was found that in the late cultivation phase the specific yield was indeed higher (Fig. S2A) and that the pH also increased generally throughout the cultivation (Fig. 4B). Interestingly, the internal concentration of Hyp was significantly lower (Fig. S2B). This indicates that more factors than the pH might influence Hyp production or that the pH is
simply correlating with the increase in specific yield but is not causing it. Nevertheless, Hyp was produced in the early cultivation phases as well (Fig. S2A). However, if Hyp was produced during the first days of an experiment it took place at a high pH (Fig. S2D).

4. Discussion

The outcome of this study is an important step towards using the cyanobacterial TCA cycle for the production of value-added chemicals. This study presents one of the few examples of a bioproduction directly from the TCA cycle and is the first report of the phototrophic bioproduction of Hyp directly from CO2 in a genetically modified organism. The maximum production rate observed for SynP4H was 30.77 μmol gCDW h−1. This study presents one of the few examples of a bioproduction directly from CO2 in a genetically modified organism. Operating such a bioreactor with SynP4H at cell densities reached in shake flasks experiment (2 gCDW L−1) SynP4H could potentially produce 4.06 kg Hyp ha−1 d−1 (average 2.10 kg Hyp ha−1 d−1) at the observed production rates (Table 1).

Apart from the biotransformation of proline, the current best method for the bioproduction of Hyp is a fed-batch fermentation with an E. coli strain expressing a P4H from Alteromonas mediterranea (Wang et al., 2018). Here, production rates of 1.27 gHyp L−1 h−1 and final titers of 45.83 gHyp L−1 in a sugar-based process have been reported. In this experiment, glucose has been used as a carbon source at a ratio of 3.36 gglc gHyp. However, when looking at a global transformation of a fossil-based economy to a bio-based economy, the volumetric production rates and yields are mainly important for operating costs, capital investment and down-stream processing. But overall, the total surface area needed to run a heterotrophic process also relies on the efficiency of a phototrophic process. The majority (75%) of the world’s sugar production (as sucrose) is derived from sugarcane (Shield, 2016). Assuming average yields for sugarcane of 70.63 t FW ha−1 a−1 and a sucrose content of 15% (FAO, 2020), the land-use efficiency of E. coli model derived from (Vickers et al., 2012) estimated using elementary flux mode analysis in an E. coli model derived from (Vickers et al., 2012), the land-use efficiency of E. coli under optimal conditions is 67.5 ha per ton Hyp. Additionally, the glucose needed for the heterotrophic process adds approximately 2355 $ tHyp−1 which is far less than what was observed for SynP4H in the shake flask experiment. In this theoretical scenario, space-time yields of 93.47–222.82 kg Hyp ha−1 d−1 would be possible in large scale photobioreactors (Table 1).

Reaching these hypothetical production rates would drastically reduce the land use per ton of Hyp produced from 246 to 476.6 ha (SynP4H) or 115.9 ha (E. coli) to 5–11 ha (Fig. 5). Even when assuming that E. coli could reach the theoretical maximum of around 2 gHyp gglc−1 estimated using elementary flux mode analysis in an E. coli model derived from (Vickers et al., 2012), the land-use efficiency of E. coli under optimal conditions is 67.5 ha per ton Hyp. Additionally, the glucose needed for the heterotrophic process adds approximately 2355 $ tHyp−1 to the production costs of the heterotrophic process. For the phototrophic Hyp production the major challenge will most likely relate to reactor scale-up and down-stream processing of diluted aqueous solutions of Hyp. In comparison, agriculture and heterotrophic bio-production is an established and well-understood process.

The question remains, how the calculated theoretical yields can be reached with SynP4H. The p4h gene in SynP4H was put under the Cu2+-.
inducible petE-promoter. The optimal concentration for induction was determined to be 1 μM Cu^{2+}. This is not in accordance with the results of Englund et al., who reported that the petE promoter is already fully expressed at the 0.3 μM Cu^{2+} normally present in BG11 (Englund et al., 2016). Our results indicate that already at the cell densities reached in shake flask experiments, the inducer concentrations get diluted to the point where expression levels of the p4H gene and in turn P4H amounts are already limited and additional Cu^{2+} is beneficial for the production. However, no further improvement of Hyp production could be observed at higher Cu^{2+} concentrations indicating that saturation of expression induction is reached at 1 μM. However, even under elevated concentrations of 1 μM Cu^{2+} the maximum titer of Hyp observed was 162 μM after 24 days of incubation. The maximum Hyp titers of SynP4H are 10-fold higher compared to our first approach (Fig. S3), where the p4H gene was under the control of the trc-promoter (Lan and Liao, 2011) and stably integrated into the sbp0168 neutral site on the chromosome (Kunert et al., 2000; Qi et al., 2013). This strain yielded about 16 μM Hyp.

As an increase in P4H amount seemed to be beneficial, additional gene copy numbers might further boost the production. This has been shown to work for ethylene production (Ungerer et al., 2012). Additionally, carbon flux can be streamlined towards the reaction of P4H by knockout or knockdown of one or several bypasses of the branched architecture of the TCA cycle. For instance, the major flux through 2-OG is for the synthesis of glutamate and further downstream tetrapyroles like for example chlorophyll via the GABA shunt (Vavilin and Vermaas, 2000; Qi et al., 2013). This strain yielded about 16 μM Hyp.

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extract 95% pure lactic acid from fermentation broth in a modular system at rates of 76–77 L m⁻² h⁻¹ (Pal and Dey, 2012). Adaption of these methods for the purification of Hyp is key for a continuous phototrophic production process. However, the cultivation of cells in a basic medium with a pH higher than 9.5 does not result in stable production of Hyp over the whole cultivation. This indicates that factors, other than the pH of the cultivation medium, are important for the production of Hyp. It was noted that the specific yield of Hyp was higher in the late phase of the cultivation (days 11–19) than in the early phase (days 1–10). A similar phenomenon was reported for the production of 1,2-propanediol in Synechocystis, which is linked to the turnover of intracellular glycogen (David et al., 2018). As the pH also increased over time, it is not clear if the pH is causing or correlating with the higher production of Hyp. At the same time, the internal concentration of Hyp decreased when comparing early and late cultivation phases. This might be an indication that the P4H enzyme from Dactylosporangium is not working as efficiently in Synechocystis as in E. coli (Shibasaki et al., 2000).

Although the production of Hyp has been increased 10-fold throughout this project, the final titers remain low. This is due to both production rates and the stability of production over time. However, the space-time yields are already in the same order of magnitude compared to those of processes using heterotrophic E. coli. They have the potential to be further increased by employing additional strain engineering and altered cultivation methods (e.g. higher cell densities). One of the greatest challenges remains the downstream processing of large quantities of culture with relatively low concentrations of product. If the activation of the TCA cycle published for the production of ethylene is generally also possible in cyanobacteria, SynP4H has the potential to outcompete E. coli with regards to the area needed per unit of product.

CRediT authorship contribution statement

Fabian Brandenburg: Investigation, Data curation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. Eleni Theodosiou: Investigation, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. Carolin Bertelmann: Investigation, Writing - review & editing. Marcel Grund: Investigation, Methodology, Writing - review & editing. Stephan Klahn: Conceptualization, Methodology, Supervision, Writing - review & editing. Andreas Schmid: Conceptualization, Funding acquisition, Writing - review & editing. Jens O. Krömer: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2020.e00155.

Table S1

| Primer | Sequence 5’→3’ |
|--------|---------------|
| PpetE_fw(XhoI) | ACTCGAGGAAGGATAGCAAGC |
| Toop_rev(AseI) | GATTAAATAAAGGCGCDGGGG |
| pVZ_fw | ACAAGGCGATGGTGTTC |
| pVZ rv | TGTCCTCGCGATGTACAC |

References

Badger, M.R., Price, G.D., 2003. CO2 concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution. J. Exp. Bot. 54, 609-622. https://doi.org/10.1093/jxb/erg076.

Blasi, B., Pecas, L., Vass, I., Kosi, P.B., 2012. Characterization of stress responses of heavy metal and metalloid inducible promoters in Synechocystis PCC6803. J. Microbiol. Biotechnol. 22, 166–169. https://doi.org/10.4141/jmb.11.06050.

David, C., Schmid, A., Adriam, L., Wilde, A., Bühler, K., 2018. Production of 1,2-propanediol in photoautotrophic Synechocystis is linked to glycogen turnover. Biotechnol. Bioeng. 115, 300–311. https://doi.org/10.1002/bit.26468.

Dienst, D., Wichmann, J., Mantovani, O., Rodrigues, J., Lindberg, P., 2019. High Density Cultivation for Efficient Sesquiterpenoid Biosynthesis in Synechocystis Sp. PCC 6803. bioRxiv.

Ducat, D.C., 2015. Metabolic engineering: kick-starting TCA cycling. Native Plants 1.

Ducat, D.C., Way, J.C., Silver, P.A., 2011. Engineering cyanobacteria to generate high-value products. Trends Biotechnol. 29, 95–103. https://doi.org/10.1016/j.tibte.2010.12.003.

Eisenhut, M., Huege, J., Schwarz, D., Bauwe, H., Kopka, J., Hagemann, M., 2008. Evaluation of promoters and ribosome binding sites for biotechnological applications in the unicellular cyanobacterium Synechocystis sp. PCC 6803. Sci. Rep. 6, 1–12. https://doi.org/10.1038/srep06646.

Falcioni, F., Blunk, L.M., Frick, O., Karau, A., Bühler, B., Schmid, A., 2013. Proline availability regulates proline-4-hydroxylation synthesis and substrate uptake in proline-hydroxylation recombinant Escherichia coli. Appl. Environ. Microbiol. 79, 3091–3100. https://doi.org/10.1128/AEM.03440-12.

Falcioni, F., Bühler, B., Schmid, A., 2015. Efficient hydroxyproline production from glucose in minimal media by Corynebacterium glutamicum. Biotechnol. Bioeng. 112, 322–330. https://doi.org/10.1002/bit.25442.

FAO, 2020. World Food and Agriculture - Statistical Yearbook 2020. FAO, Gafatar, T., Inshad, M., Anwar, Z., Apil, T., Zafir, Z., Tariq, A., Kamran, E., Ehna, N., Mehmoody, S., 2014. Recent trends in lactic acid biotechnology: a brief review on production to purification. J. Radiat. Res. Appl. Sci. 7, 222–229. https://doi.org/10.1016/j.jrras.2014.03.002.

Good, N.E., Winget, G.D., Winter, W., Connolly, T.N., Iwaza, S., Singh, R.M.M., 1966. Hydrogen ion buffers for biological Research. Biochemistry 5, 467–477. https://doi.org/10.1021/bi00860a011.

Heidorn, T., Cansdall, D., Huang, H., Lindberg, P., Lindblad, P., Oliveira, P., Stensjo, K., Stensjo, K., Lindblad, P., 2011. Synthetic biology in cyanobacteria: engineering and analyzing novel functions. Methods Enzymol. 497, 539–579. https://doi.org/10.1016/B978-0-12-385075-1.00024-X.

Hoare, D.S., Hoare, S.L., Moore, R.B., 1967. The photo-assimilation of organic compounds by autotrophic blue-green algeae. J. Gen. Microbiol. 109, 351–370. https://doi.org/10.1099/00221287-49-3-351.

Hoschek, A., Toepel, J., Hochkeppel, A., Karande, R., Bühler, B., Schmid, A., 2019. Light-dependent and aeration-independent gram-scale hydroxylation of cyclohexanol by CYP450 harboring Synechocystis sp. PCC 6803. Biotechnol. J. 14, 1–10. https://doi.org/10.1002/biot.201800724.

Janz, G.J., Wait, S.C., 1955. Space-time yield and reaction rate. J. Chem. Phys. 23, 1550–1551. https://doi.org/10.1063/1.1742539.

Kallas, T., Castenholz, R.W., 1982. Internal pH and ATP-ADP pools in the cyanobacterium Synechococcus sp. during exposure to growth-inhibiting low pH. J. Bacteriol. 149, 229–236.

Kaniya, T., Kizawa, A., Kawai-Yamada, M., Uchimiya, H., Kaneko, Y., Nishiyama, Y., Hihara, Y., 2013. Deletion of the transcriptional regulator cyAbrB2
Shibasaki, T., Mori, H., Chiba, S., Ozaki, A., 1999. Microbial proline 4-hydroxylase. Biopolym. Biophys. 40, 1135-1143. https://doi.org/10.1002/(SICI)1097-0282(199908)60:8<1135::AID-BBB4>3.0.CO;2-F.

Shibasaki, T., Mori, H., Ozaki, A., 2000. Membrane-bound proline 4-hydroxylase of Pseudomonas putida. Biochim. Biophys. Acta 1473, 162-169. https://doi.org/10.1016/S0006-291X(99)00183-6.

Shibasaki, T., Mori, H., Ozaki, A., 2000. Synthetic biology for microorganisms. J. Bioscience Bioeng. 90, 522-528. https://doi.org/10.1265/jbb.90.522.

Shibasaki, T., Mori, H., Ozaki, A., 2000. Engineering of proline 4-hydroxylase. Microb. Biotechnol. 3, 147-154. https://doi.org/10.1111/j.1757-9126.2010.00033.x.

Shibasaki, T., Mori, H., Ozaki, A., 2000. Engineered proline 4-hydroxylase for producing trans-4-hydroxy-L-proline. J. Biosci. Bioeng. 90, 522-528. https://doi.org/10.1265/jbb.90.522.

Shibasaki, T., Mori, H., Ozaki, A., 2000. Enzymatic production of trans-4-hydroxy-L-proline. Biotechnol. Bioeng. 70, 403-409. https://doi.org/10.1002/1097-0290(20000616)70:6<403::AID-BBI2>3.0.CO;2-7.

Shibasaki, T., Mori, H., Ozaki, A., 2000. Engineering proline 4-hydroxylase for production of (R)-4-hydroxy-L-proline. J. Bioscience Bioeng. 90, 522-528. https://doi.org/10.1265/jbb.90.522.

Shibasaki, T., Mori, H., Ozaki, A., 2000. Engineering proline 4-hydroxylase for production of (R)-4-hydroxy-L-proline. J. Bioscience Bioeng. 90, 522-528. https://doi.org/10.1265/jbb.90.522.

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Shibasaki, T., Mori, H., Ozaki, A., 2000. Engineering proline 4-hydroxylase for production of (R)-4-hydroxy-L-proline. J. Bioscience Bioeng. 90, 522-528. https://doi.org/10.1265/jbb.90.522.

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