Expression of Phenylalanine Ammonia Lyases in *Synechocystis* Sp. PCC 6803 and Subsequent Improvements of Sustainable Production of Phenylpropanoids

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Research Article

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Abstract

Background

Phenylpropanoids represent a diverse class of industrially important secondary metabolites, synthesized in plants from phenylalanine and tyrosine. Cyanobacteria have a great potential for sustainable production of phenylpropanoids directly from CO\(_2\), due to their photosynthetic lifestyle with a fast growth compared to plants, and the ease of generating genetically engineered strains. This study focuses on photosynthetic production of the first compounds in the phenylpropanoid pathway, trans-cinnamic acid and p-coumaric acid, in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis*). This was achieved firstly via heterologous overexpression of a selected set of phenylalanine ammonia lyase (PAL) enzymes from different organisms in *Synechocystis*. The resulting strains were evaluated for productivity to find the best performing candidate. Secondly, in order to further improve the titer of target compounds, we evaluated the use of stronger expression cassettes for increasing PAL protein levels, as well as knock-out of the laccase gene *slr1573*, as this was previously reported to prevent degradation of the target compounds in the cell. Finally, to investigate the effect of growth conditions on the production of trans-cinnamic acid and p-coumaric acid from *Synechocystis*, cultivation conditions promoting rapid, high density growth were tested.

Results

Results of comparative expression of PALs showed that the highest specific titer was achieved for the strain AtC, expressing a PAL from *Arabidopsis thaliana*, while a subsequent increase of protein level did not improve the productivity. In contrast to previous reports, the production of target compounds in strains where the *slr1573* laccase had been knocked out was found to be lower compared to strains with wild type background. Additionally, the Δ*slr1573* strains exhibited a strong phenotype of slower growth rate and lower pigment content. The application of a high-density cultivation system for the growth of production strains allowed reaching the highest total titers of trans-cinnamic acid and p-coumaric acid reported so far, at around 0.8 and 0.4 g/L, respectively, after 4 days.

Conclusions

The production of trans-cinnamic acid, unlike that of p-coumaric acid, is not limited by the protein level of heterologously expressed PAL in *Synechocystis*. High density cultivation led to higher titres of both products, while knocking out *slr1573* did not have a positive effect on production. This work contributes to capability of exploiting the primary metabolism of cyanobacteria for sustainable production of plant phenylpropanoids.

Background
Aromatic amino acids (AAA) and their derivatives phenylpropanoids, represent a large group of plant secondary metabolites that includes flavonoids, coumarins, stilbensenes, lignols, and catechin. These compounds are widely applicable as food additives, fragrances, cosmetics, nutraceuticals and for production of antibacterial, antitumor, antiviral drugs and other pharmaceuticals [1–4]. The huge market demand for plant bioactive secondary metabolites is mostly fulfilled through demanding and inefficient extraction from plant tissues, or through chemical synthesis based on fossil resources, which in many cases is difficult to achieve due to the structural complexity of the desired metabolites and often results in mixtures of isomers. Microbial synthesis of such compounds presents an attractive alternative. Engineered microorganisms can be designed to generate more of a desired product per cell than what can be achieved in plants, while at the same time, the biosynthetic pathways in the host microorganisms can be very specific to generate only one product, and the production will be scalable and more sustainable [5–7].

Engineered cyanobacteria exhibit a great potential for sustainable production of plant phenylpropanoids by direct conversion of CO₂. Just as other autotrophs, cyanobacteria possess a pathway for de novo biosynthesis of AAA phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp), from the common precursor chorismate through the shikimate pathway (Fig. 1). The pathway starts from the condensation of erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP), derived from CO₂ fixation in central carbon metabolism, to form the first compound in the shikimate pathway, 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase (DAHP). This first step is catalyzed by the enzyme DAHP synthase. This enzyme in microorganisms, supposedly including cyanobacteria, is subjected to tight regulation by feedback inhibition by the products of the pathway [8, 9]. Through six consecutive reactions, DAHP undergoes cyclisation to form chorismate, which in cyanobacteria is a common substrate for the three AAA, plastoquinone, and folic acid [10–12]. From chorismate, by one additional step, Phe can be non-oxidatively deaminated to form trans-cinnamic acid (tCA) by the action of an enzyme called phenylalanine ammonia lyase (PAL, EC 4.3.1.24). The PAL enzymatic reaction plays a key role in synthesis of phenylpropanoids, linking secondary metabolite synthesis with primary metabolism. In plants, tCA can be further converted to p-coumaric acid (pCou, or otherwise named hydroxycinnamic acid) by membrane bound cinnamate-4-hydroxylase (C4H) [13]. Alternatively to C4H, the enzyme tyrosine ammonia lyase (TAL, EC 4.3.1.25) which was discovered in bacteria, catalyzes a non-oxidative deamination to form pCou directly from Tyr. PAL and TAL are widely spread in nature, however in bacteria their role is connected to synthesis of photoactive yellow protein and antibiotics [14–16] rather than synthesis of flavonoids and coumarins as in plants [17]. PAL was also discovered in filamentous cyanobacteria, but not in unicellular strains, and yet its role in metabolism in these organisms has not been elucidated [18].

A distinct class of PALs is represented by bifunctional enzymes that can utilize both Phe and Tyr with close to similar efficiencies [19, 20]. The ability of an enzyme to use both substrates led to investigation of a possible region in the protein amino acid sequence that might be responsible for substrate preference. The discovery of such a region, called substrate selectivity switch, showed that a single
aminoacid substitution can dictate whether an enzyme will use Phe or Tyr as substrate [21]. PAL and TAL together with histidine ammonia lyase (HAL, EC:4.3.1.3) belong to the ammonia lyase family of proteins. All three of them share the common characteristic of a modified prosthetic group, 3,5-dihydro-5-methylidine-4H-imidazol-4-one (MIO), a unique prosthetic group which is formed autocatalytically from a highly conserved Ala-Ser-Gly tripeptide sequence (ASG motif) [22]. Crystal structure studies revealed that eukaryotic PALs differ from prokaryotic being ~20kDa larger by the presence of N-terminus extension and an additional insertion domain. This additional domain forms as arch-like structure over the active site, that proposedly is acting as shielding domain which restricts substrate entry to the active site, however its exact role is unknown [18, 23, 24].

Cyanobacterial AAA biosynthesis is less studied than that in plants, yeasts and some other bacterial hosts like E.coli, presenting a challenge for engineering. Nevertheless, the photosynthetic nature of cyanobacteria and their faster growth rate than that of plants make them good candidates for sustainable production of AAA and phenylpropanoids, and a few reports have described cyanobacteria engineered for this purpose. To date, cyanobacterial production of trans-cinnamic acid, caffeic acid [25], p-coumaric acid [26–28], and phenylethanol [29] has been demonstrated. The common strategies implemented to improve the productivity in cyanobacteria were enhancing the carbon flux into shikimate pathway by relieving the feedback inhibition of key steps in pathway, such as overexpression of feedback-resistant DAHP synthase and feedback-resistant chorismate mutase/prephenate dehydratase from E.coli. Xue and coworkers [26] discovered a putative laccase in Synechocystis sp. PCC 6803 encoded by slr1573, and deletion of this gene led to increased pCou titers, presumably due to involvement of the laccase in oxidation of phenolic compounds. Another strategy for productivity boost consisted in knocking-out of a competing pathway: deletion of arogenate dehydrogenase encoded by tyrA from the terminal branch of Tyr biosynthesis led to the enhancement of the two-step pCou biosynthesis pathway via Phe and tCA. However, it is not clear how the cells are able to survive a knockout of this supposed essential gene, unless other reactions leading to Tyr synthesis exist in cyanobacteria [30].

In this study we comparatively express and test several PALs originating from different organisms for the production of tCA in the model cyanobacterium Synechocystis sp. PCC 6803 (hereafter Synechocystis), in strains with and without the slr1573 laccase gene. We also test engineered strains under more optimal growth conditions; recently, a high-density cultivation system exhibited a great potential of reaching high cumulative titer of desired product in short period of time [31–33]. The reaction performed by PAL is of industrial interest, since it opens a gateway to generating a myriad of highly valuable secondary plant metabolites non-native to cyanobacteria. Furthermore, tCA as well as pCou can play the role of reporter molecules for exploring the function and regulation of the under-investigated native AAA biosynthesis pathways of cyanobacteria, since changes in cell metabolism affecting the relevant pathways would lead to changes in their titers.

**Results And Discussion**

**Comparative production of tCA**
In this study we aimed to use several PAL enzymes in order to test and select the one most favorable to tCA production in *Synechocystis*. For this, we studied the literature for the available kinetic data on purified PAL proteins, and from available data choose a set of genes with different origins and characteristics. Selected candidates for *pal* genes were from two plant species, *Petroselinum crispum* (*Pc*) [34] and *Arabidopsis thaliana* (*At*) [35], and two filamentous cyanobacteria *Nostoc punctiforme* (*Np*), *Anabaena variabilis* (*Av*) [18]. For these candidates, there were already in vitro studies on their properties. In addition to these already purified and described PAL enzymes, we also decided to include a fifth PAL candidate, based on the PAL evolutionary study of Hemmati [36]. In that study, a phylogenic tree generated for 369 aligned amino acid sequences of PAL showed that several PAL enzymes do not cluster together. As suggested by the author, those PAL enzymes are probably encoded by *pal* isozymes with unique functional genes. Among several options from such unclustered PAL candidates, our choice fell to the bacterial PAL from *Treponema socranskii subsp. paredis* ATCC 35535 (*Ts*). Partially the choice of this PAL candidate was based on the unusual amino acids residues at the positions 86 and 87. These residues correspond to the substrate specificity [21], and are phenylalanine and histidine in case of *Ts* PAL instead of the more canonical pair phenylalanine-leucine for PAL and histidine-leucine for TAL characteristic.

The five genes encoding the different PAL candidates were cloned in two types of expression vectors (Fig. 2): pEEK [37], and its derivative pEEKN, and successfully conjugated into *Synechocystis* (see Table 1 for a list of plasmid backbones used and strains created in this study). In this approach, each *pal* gene is expressed in two versions, with either an N- or a C-terminus Strep-tag in order to evaluate the protein expression and production of tCA in *Synechocystis*. An empty vector control strain was obtained by conjugating *Synechocystis* with the pEEK* plasmid, which only contains the Km antibiotic resistance.
# Table 1

List of plasmid backbones and *Synechocystis* strains used in this study

| Construct | Comment | Antibiotics resistance | Reference |
|-----------|---------|------------------------|-----------|
| pEEK*     | RSF 1010-based expression vector, harboring Km resistance cassette | Km | Englund, E., not published |
| pEEK | RSF 1010-based expression vector with cloning sites flanked by RBS*, promoter *Ptrc2O*, and C-terminal Strep-tag sequence | Km | [37] |
| pEEKN | Same as pEEK but with N-terminal Strep-tag sequence | Km | this study |
| pEEK3 | Based on pEEC3 [38], with promoter *Ptrc*, RiboJ and N-terminal Strep-tag sequence, but with Km resistance | Km | Englund, E., not published |
| pEEK3C | Same as pEEK3 but with C-terminal Strep-tag sequence | Km | this study |
| pEEERM3 | Empty backbone based on pJ344 vector backbone for integration into neutral site 1 (*slr1068*) of *Synechocystis* 6803, carrying P*nrsB* promotor and synthetic RBS, RBS* | Cm | [39] |
| p1573 | same as pEEERM3 but with homologous recombination regions to *slr1573* | Cm | this study |

| Strains | Comment | Antibiotics resistance | Reference |
|---------|---------|------------------------|-----------|
| EVC | Control strain of *Synechocystis* WT harboring pEEK* plasmid for Km resistance | Km | this study |
| PcC | *Petroselinum crispum* PAL expressed from *Ptrc* RBS* in pEEK | Km | this study |
| AtC | *Arabidopsis thaliana* PAL expressed from *Ptrc* RBS* in pEEK | Km | this study |
| NpC | *Nostoc punctiforme* PAL expressed from *Ptrc* RBS* in pEEK | Km | this study |
| AvC | *Anabaena variabilis* PAL expressed from *Ptrc* RBS* in pEEK | Km | this study |
| TsC | *Treponema socranskii* PAL expressed from *Ptrc* RBS* in pEEK | Km | this study |
| Construct | Comment | Antibiotics resistance | Reference |
|-----------|---------|-------------------------|-----------|
| TsC-H87L  | *Treponema socranskii* PAL with mutation H87L expressed from Prtc RBS* in pEEK | Km | this study |
| PcN       | *Petroselinum crispum* PAL expressed from Prtc RBS* in pEEKN | Km | this study |
| AtN       | *Arabidopsis thaliana* PAL expressed from Prtc RBS* in pEEKN | Km | this study |
| NpN       | *Nostoc punctiforme* PAL expressed from Prtc RBS* in pEEKN | Km | this study |
| AvN       | *Anabaena variabilis* PAL expressed from Prtc RBS* in pEEKN | Km | this study |
| TsN       | *Treponema socranskii* PAL expressed from Prtc RBS* in pEEKN | Km | this study |
| TsN-H87L  | *Treponema socranskii* PAL with mutation H87L expressed from Prtc RBS* in pEEKN | Km | this study |
| Pc3C      | *Petroselinum crispum* PAL expressed from Prtc RiboJ in pEEK3C | Km | this study |
| At3C      | *Arabidopsis thaliana* PAL expressed from Prtc RiboJ in pEEK3C | Km | this study |
| Np3C      | *Nostoc punctiforme* PAL expressed from Prtc RiboJ in pEEK3C | Km | this study |
| Av3C      | *Anabaena variabilis* PAL expressed from Prtc RiboJ in pEEK3C | Km | this study |
| Ts3C      | *Treponema socranskii* PAL expressed from Prtc RiboJ in pEEK3C | Km | this study |
| Ts3C-H87L | *Treponema socranskii* PAL with mutation H87L expressed from Prtc RiboJ in pEEK3C | Km | this study |
| Pc3N      | *Petroselinum crispum* PAL expressed from Prtc RiboJ in pEEK3 | Km | this study |
| At3N      | *Arabidopsis thaliana* PAL expressed from Prtc RiboJ in pEEK3 | Km | this study |
| Np3N      | *Nostoc punctiforme* PAL expressed from Prtc RiboJ in pEEK3 | Km | this study |
| Av3N      | *Anabaena variabilis* PAL expressed from Prtc RiboJ in pEEK3 | Km | this study |
| Ts3N      | *Treponema socranskii* PAL expressed from Prtc RiboJ in pEEK3 | Km | this study |
| Ts3N-H87L | *Treponema socranskii* PAL with mutation H87L expressed from Prtc RiboJ in pEEK3 | Km | this study |
### Table

| Construct   | Comment                                                                 | Antibiotics resistance | Reference |
|-------------|-------------------------------------------------------------------------|-------------------------|-----------|
| EVCΔslr1573 | Control strain of *Synechocystis* WT harboring pEEK* plasmid for Km resistance with knocked-out slr1573 | Km, Cm                  | this study |
| AtCΔslr1573 | *Arabidopsis thaliana* PAL expressed from Ptrc RBS* in pEEK with knocked-out slr1573 | Km, Cm                  | this study |
| AtNΔslr1573 | *Arabidopsis thaliana* PAL expressed from Ptrc RBS* in pEEKN with knocked-out slr1573 | Km, Cm                  | this study |
| TsCΔslr1573 | *Treponema socranskii* PAL expressed from Gttrc RBS* in pEEK with knocked-out slr1573 | Km, Cm                  | this study |
| TsNΔslr1573 | *Treponema socranskii* PAL expressed from Gttrc RBS* in pEEKN with knocked-out slr1573 | Km, Cm                  | this study |

Interestingly, when initially all the *pal* expression constructs were tested in *E.coli* for corresponding *tCA* of *pCou* presence in the culture medium, it was revealed that *Ts pal* encodes a Tyrosine ammonia lyase, producing *pCou* from tyrosine (data not shown). To explore a possibility that by switching the histidine to leucine at position 87 would change the substrate specificity from tyrosine to phenylalanine, we performed a site directed mutagenesis on the *Ts-tal* sequence. As a result, the mutation *Ts–H87L* successfully diverted the enzymes selectivity towards phenylalanine, as no *pCou* was accumulating in the medium (data not shown). The *Ts-H87L pal* was therefore included in the study, and was cloned and conjugated into *Synechocystis* the same way as the other *pal* CDS.

Cultures of the engineered *Synechocystis* strains were grown for 6 days under constant light, and samples for OD$_{750}$ and LC-MS analysis of *tCA* and *pCou* content in the culture supernatant were taken on days one, three and six (Fig. 3A and B). The highest specific production of *tCA* was observed by strain AtC, reaching the value of $40.2\pm5.6$ mg·L$^{-1}$·OD$^{-1}$ of *tCA* at day six (Table 2). Western-Immunoblot analysis of proteins in the engineered strains at the last day of the experiment (Fig. 3C) showed that expression levels of C-terminus tagged PAL proteins were higher than N-terminus tagged, which correlates with higher titers of the product in most of the cases. The cause of the observed differences in expression levels of the same PAL dependent on the tag position is uncertain. Unpredictability of expression levels is often caused by different 5 UTR’ sequences from combination of promoters and RBSs and together with different coding sequences can create the secondary structures restricting efficient ribosome binding [40, 41]. The N-terminus Strep-tag sequence may be expected to even out such context-dependent differences, however, our results showed weaker and more uneven protein expression levels for strains with N-terminus tagged PALs. Another possible explanation is a lower stability of PAL proteins when expressed with N-terminus tag. The differences in levels of production might also be due to differences in enzyme activity of expressed PALs due to the fused Strep-tag peptide, as the AtN and AtC strains demonstrated similar protein levels but different production of *tCA* per cell (Fig. 3A and C). The growth of engineered strains over the time of experiment (Fig. 4) showed a negative correlation with the *tCA* production, where growth was slower compared to the EvC for the engineered strains that produced more of the molecule of...
interest per cell. This result demonstrates that the expression of PAL protein, which consumes the central metabolite phenylalanine, negatively affects the growth.

Table 2

| CA production | mg·L⁻¹·OD_{750}⁻¹ | Strain | mg·L⁻¹·OD_{750}⁻¹ | mg·L⁻¹ | Strain | mg·L⁻¹·OD_{750}⁻¹ | mg·L⁻¹ |
|---------------|-----------------|--------|-----------------|--------|--------|-----------------|--------|
| EvC           | -               | PcC    | 15.8 ± 9.4      | 26.3 ± 13.4 | Pc3C | 20.7 ± 3.3 | 27.5 ± 4.7 |
| PnN           | 28.3 ± 2        | 37.3 ± 6.1 | AtC    | 40.2 ± 5.6 | 49.6 ± 8.7 | At3C | 32.5 ± 1.6 | 41.0 ± 4.9 |
| AtN           | 29.1 ± 4.3      | 35.8 ± 3 | At3N           | 32.6 ± 3.6 | 45.5 ± 3.2 |
| NpC           | 9.6 ± 1.4       | 14.1 ± 1.6 | NpN           | 2.6 ± 0.7 | 4.8 ± 1.4 | Np3C | 32.7 ± 5.4 | 38.0 ± 7.1 |
| NpN           | 2.6 ± 0.7       | 4.8 ± 1.4 | Np3N           | 22.8 ± 8.5 | 29.1 ± 10.6 |
| AvC           | 35.3 ± 4.6      | 46.3 ± 6.6 | Av3C           | 29.5 ± 5.1 | 29.3 ± 7.0 |
| AvN           | 16.8 ± 2.9      | 26.7 ± 5.5 | Av3N           | 30.7 ± 9.7 | 30.2 ± 13.7 |
| TsC/H87L      | 0.3 ± 0.3       | 3.8 ± 0.7 | Ts3C/H87L      | 4 ± 0.4   | 4.7 ± 0.7 |
| TsN/H87L      | 0.2 ± 0.3       | 2.5 ± 0.7 | Ts3N/H87L      | 2.7 ± 0.2 | 3.9 ± 0.7 |
| TsC           | trace amounts   | Ts3C   | trace amounts   | Ts3C/H87L | N/D |
| TsN           | trace amounts   | Ts3N   | trace amounts   | Ts3N/H87L | N/D |

pCou production

| Strain | mg·L⁻¹·OD_{750}⁻¹ | Strain | mg·L⁻¹·OD_{750}⁻¹ | mg·L⁻¹ |
|--------|-----------------|--------|-----------------|--------|
| TsC    | 16.2 ± 0.7      | Ts3C   | 23.5 ± 1.2      | 22.4 ± 3.2 |
| TsN    | 12.6 ± 1.5      | Ts3N   | 22.7 ± 2.4      | 28.3 ± 3.9 |
| TsC/H87L | N/D             | Ts3C/H87L | N/D           |        |
| TsN/H87L | N/D             | Ts3N/H87L | N/D           |        |

N/D – none detected.
The production of pCou by strains TsC and TsN showed similar values, although TsC protein levels were significantly higher than those of the TsN strain (Fig. 3B). Substitution of one amino acid residue, H87L, in TsTAL, had successfully switched the enzyme's substrate selectivity from tyrosine to phenylalanine. However, the activity of the mutated enzyme variant was decreased compared to a wild type variant, as the productivity per cell decreased nearly eight times, whereas the protein expression levels remained similar (Fig. 3).

**Enhancement of tCA production via increase of protein expression level**

As production levels in our first experiments seemed to correlate with protein expression levels, to further improve tCA production in *Synechocystis* we intended to increase protein expression levels of the PALs. For this, the *pal* genes were cloned into two vectors, pEEK3 and pEEK3C, and successfully conjugated into *Synechocystis*. In pEEK3, the expression is driven by the strong constitutive promotor *Ptrc core* followed by a sequence of self-cleaving ribozyme RiboJ [38, 42]. This way, the transcribed mRNA will undergo enzymatic processing of cleaving its own 5'UTR, which facilitates biding of the ribosome to RBS of the transcript. Application of RiboJ enables a strong predictable and reliable expression [38, 43].

Comparative growth experiment on these strains demonstrated a similar range of tCA production per cell among all strains, except for the Ts3-H87L strains (Fig. 5A). A normalized maximal titer of approx. 32 mg·L⁻¹·OD⁻¹ was reached by several strains, namely Pc3N, At3C, At3N, Np3C (Table 2). The pCou specific production titer was increased for Ts3C and Ts3N strains as compared to TsC and TsN strains (Fig. 5B and Table 2). In addition, Western immunoblot (Fig. 5C) also showed similar expression levels of different PALs with lesser variation dependent on the Strep-tag position. Compared to engineered strains based on overexpression in pEEK, the growth of all engineered strains based on overexpression in pEEK3 showed a larger growth impairment as compared to EvC (Fig. 6), however, the obtained productivity values were lower than the titer achieved for the AtC strain. These findings prompted us to perform a comparison of the protein expression level for each PAL in all engineered strains. Figure 7 shows the comparative expression of each PAL overexpression variant and we can observe that for all pEEK3 based strains, expression was higher than for corresponding pEEK based strains. The difference in the relative expression levels of the same pEEK3 strains in Fig. 5C and Fig. 7 might be due to variation between protein extracts from different biological replicates of same strain. Nonetheless, we can conclude that more PAL protein per cell did not lead to higher tCA productivity, and usage of RiboJ helped to decrease the differences between C- and N-terminus Strep-tag expression constructs. These results indicate that the PAL expression level does not seem to be the bottleneck of tCA production in *Synechocystis*. This is further supported by the fact that although all PALs used in this study possess different kinetic parameters as was shown on purified proteins [18, 34, 35], their overexpression with similar protein levels in *Synechocystis* resulted in nearly similar production titers of product tCA. The apparent limitation once a high enough protein level has been reached might thus be in the intracellular levels of the substrate phenylalanine and the metabolic regulation of its biosynthesis. The obtained production of 40.2±5.6 mg·L⁻¹·OD₇₅₀⁻¹ of tCA might represent an experimental maximum under the tested conditions,
corresponding to the “limit” of phenylalanine consumption before causing detrimental effect on cell fitness.

**Effect of laccase knockout on tCA production**

The discovery by Xue et al [26] of a laccase enzyme encoded by gene *slr1573* in *Synechocystis* was related to the role of this enzyme in the decomposition of *pCou* inside the cell. When the laccase gene was knocked out, the authors detected a 25-fold increase in *pCou* titer. To assess if the tCA can be decomposed in our engineered strains by the action of the laccase, we constructed an integration plasmid, p1573 (Fig. 2F), to knock out this gene in the most prominent producing strains in our study – AtC and AtN. TsC and TsN strains were also included, as control strains of *pCou* production. The EvC strain was also transformed with the p1573 plasmid to form strain EvCΔ*slr1573*, serving as a growth control strain (Table 1).

Comparative growth experiments with the original and laccase knockout strains were performed during four days under constant moderate light intensity of 45 μmol photons m\(^{-2}\) s\(^{-1}\). The productivity on the last days of experiment showed lower tCA and *pCou* titers for the Δ*slr1573* strains (Fig. 8). These results are different from what was observed before [26]. Moreover, on the LC-MS profile of *pCou* production by TsC and TsN strains we did not detect additional peaks that may correspond to the polymerization of 4-vinylphenol as observed by Xue et al [26]. This difference may however depend on different extraction procedures of *pCou*: extraction of media with ethyl acetate [26, 27] whereas in our study we subjected the supernatant from the culture directly to LC-MS analysis. With organic solvents extraction in those studies it was possible to extract polymeric compounds with low water solubility. Furthermore, we observed two unidentified peaks of high molecular weight (m/z 323) (Fig. S1) in the LC-MS profile of all the Δ*slr1573* strains including the control strain EvCΔ*slr1573* (Fig. 9). These peaks may correspond to accumulation of phenolic compounds or polymers due to the disruption of their native pathway in the cell.

We also observed a notable difference in phenotype of Δ*slr1573* strains compared to the strains with an intact *slr1573*. The color of the Δ*slr1573* strains was comparably more yellowish with less green (Fig. 10A). To address this phenotype difference, we performed a prolonged growth experiment for both control strains as well as pigments quantification. The growth of EvCΔ*slr1573* was considerably slower than that of EvC in the first half of the linear phase (Fig. 10B). Whole cell absorbance spectra were measured in the beginning of the growth experiment, before both cultures reached an OD\(_{750}\) of 1, as for older cultures a shading effect may contribute to the difference in pigment content. The recorded spectra showed distinct absorbance patterns for the two strains (Fig. 10C). Pigment content quantification was also carried out at the same time points as the whole spectra. The results (Fig. 10D-F) showed that EvCΔ*slr1573* strain has markedly less phycobiliproteins as well as less chlorophyll *a* per cell, while carotenoid content was higher, although only in the beginning of the experiment. All together, these data indicate that knock out of the *slr1573* laccase in *Synechocystis* has a clear impact on the cell metabolism, which has not been previously reported. Although laccases and laccases-like enzymes are
very abundant in nature and have many diverse functions, their role in bacteria, and especially in cyanobacteria has not been investigated extensively [44].

**Production of tCA under small-scale High Density Cultivation (HDC) conditions**

The specific production titers of tCA and pCou obtained in the above described experiments are lower compared to those that were reported previously, although we find it difficult to compare them due to different normalization and growth conditions used. The levels of tCA and pCou produced in cyanobacteria reported to date are: 114 mg·L$^{-1}$ (267±31 mg·gDW$^{-1}$) of tCA and 207 mg·L$^{-1}$ (470±70 mg·gDW$^{-1}$) of pCou obtained after growth in Multi-cultivator MC 1000OD, with air enriched with 3% (v/v) CO$_2$ [25]; 197 mg·L$^{-1}$ of pCou after 7 days of cultivation under 50 µmol photons m$^{-2}$ s$^{-1}$ [27]; 82.6 mg·L$^{-1}$ of pCou after 7 day cultivation under 50 µmol photons m$^{-2}$ s$^{-1}$ with 5 mM glucose supplementation in the media [26].

Therefore, we intended to test whether we can reach high volumetric titer of molecules of interest using improved growth conditions, such as small-scale high-density cultivation. We used a cultivation system from CellDEG (www.celldeg.com) (Fig. 11A), which has been used to overcome crucial high-density cultivation limitations such as CO$_2$ supply and uneven light distribution in dense cultures for successful production of cyanophycin and terpenoids reaching ultra-high cell densities in a short period of time [31–33].

For HDC experiments, we used the production strains based on the pEEK expression vectors. The Ts-H87L strains were not included since the productivity of tCA was the lowest for those. The cultures of engineered *Synechocystis* were grown for 4 days, and samples for LC-MS were taken on day two and four. Figure 11B-F displays the growth curves, where the culture density after four days of cultivation reached values in the range of OD$_{750}$ 25–32. Similarly to what was observed before (Fig. 4), the growth pattern of engineered strains that had the least productivity showed similar growth to the EvC (NpC and NpN) whereas the other reached lower OD compared to EvC. The productivity results (Fig. 12, Table 3) showed that the specific tCA and pCou titers at the end of experiment are lower than during the eflask cultivation for each corresponding strain. The relative expression of PALs (Fig. 12C) showed considerably higher protein levels in strains AvC and PcC than AtC, which presumably led to higher tCA titer per cell. The difference in productivity of PcC and PcN strains during HDC and standard cultivation experiments might be due to apparent distinction in relative PAL expression for these strains and variation between different biological replicates.

Although the specific production titer was compromised for HDC, as it was observed previously [32], the high biomass accumulation rate resulted in high total product yield of 797.8 ±153.3 mg·L$^{-1}$ and 411.6 ±94.9 mg·L$^{-1}$ of tCA and pCou respectively (Fig. 13, Table 3), which is the highest reported in cyanobacteria so far. In summary, this experiment shows a great potential in obtaining high volumetric concentrations of a product of interest via optimized growth conditions, allowing the cultures to reach very high biomass concentration within a short time period.
Table 3
\(\text{tCA and } p\text{Cou production by engineered }\text{Synechocystis}\) strains after 4 days of high-density cultivation

| Strain | \(\text{mg}\cdot\text{L}^{-1}\cdot\text{OD}_{750}^{-1}\) | \(\text{mg}\cdot\text{L}^{-1}\) |
|--------|----------------|----------------|
| EvC    | -              | -              |
| PcC    | 30.1 ± 3.3     | 746.6 ± 51.2   |
| PcN    | 13.1 ± 2.4     | 369.9 ± 83.8   |
| AtC    | 28.7 ± 8.2     | 771.3 ± 233.9  |
| AtN    | 17.4 ± 5.9     | 539.3 ± 134.1  |
| NpC    | 3.9 ± 0.5      | 123.4 ± 19.8   |
| NpN    | 1.3 ± 0.3      | 43 ± 9         |
| AvC    | 29.7 ± 6.1     | 797.8 ± 153.3  |
| AvN    | 7.9 ± 2.3      | 238.0 ± 62.5   |

Conclusions

In this study we aimed to express several \(\text{pal}\) genes in \text{Synechocystis} and subsequently evaluate the production of \(\text{tCA}\) by engineered strains to select the best performing candidate. Out of five selected \(\text{pal}\) genes for different organisms, \(\text{PAL}\) from \text{Treponema socranskii} turned out to be a TAL, but a single aminoacid substitution in substrate selectivity switch position was sufficient to change the substrate preference to Phe. Comparative growth and production experiments showed that the best performing stain was AtC with the specific production of \(40.2 ± 5.6 \text{mg}\cdot\text{L}^{-1}\cdot\text{OD}_{750}^{-1}\) in six days. Further improvement of productivity by the increase of expression levels of PAL proteins did not result in higher specific production titers, although several strains showed nearly similar productivity due to similar relative PALs expression. Knock-out of the \(\text{slr1573}\) laccase gene, which has been suggested to be active in the degradation of synthetized \(\text{tCA}\) and \(p\text{Cou}\), did not result in any improvement of productivity. However, we could observe strong phenotype of slower growth and lower pigments content for the strains lacking the \(\text{slr1573}\) laccase. The application of a high-density cultivation platform for growth of the engineered strains resulted in a remarkably high \(\text{tCA}\) volumetric titers of \(797.8 ± 153.3 \text{mg}\cdot\text{L}^{-1}\) by strain AvC, which is
in line with central role of Phe in cell metabolism. In summary, this work contributes to development of cyanobacteria as a cell factory for sustainable conversion of CO$_2$ into phenylpropanoids.

**Methods**

**Bacterial strains and growth conditions**

*Escherichia Coli* DH5 $\alpha$Z1 (Invitrogen) was used for subcloning and conjugation. *E.coli* cells were grown in LB medium at 37°C and supplemented with appropriate antibiotics to the final concentrations in the medium: 50μg·ml$^{-1}$ Kanamycin (Km) or/and 20μg·ml$^{-1}$ Chloramphenicol (Cm) (Sigma, Merk).

*Synechocystis* sp. PCC 6803, a unicellular glucose-tolerant strain was used in this study. Cultures were grown in BG11 medium [45] with respective antibiotics Km 25μg·ml$^{-1}$ and/or Cm 20μg·ml$^{-1}$ at 30°C under constant light.

**Construction of plasmids for gene expression**

The broad host range self-replicative vectors pEEK and pEEK3 [37] and Englund E., not published) which are based on the vector pPMQAK1 [46] were used in this study as shuttle vectors (Fig. 2). The pEEK vector carries a Strep-tag with glycine-serine linker at the C-terminus position and the anti-selection marker $ccdB$ between XbaI and BglII site. The expression is driven by strong constitutive promotor $Ptrc2O$ and the translation is initiated from the synthetic ribosomal binding site RBS$^*$[47]. In order to evaluate an optimal condition of PAL proteins activity, another vector pEEKN was constructed, which in contrast to pEEK, can be used to place a Strep-tag at the N-terminus, and the $ccdb$ sequence is flanked by BamHI and SpeI. Vector pEEK3 is different from pEEK and pEEKN by containing the strong constitutive promotor $Ptrc$ core and a RiboJ element to give strong reliable expression [42]. Based on pEEK3 another vector pEEK3C was constructed, where downstream of RiboJ sequence strong bacterial RBS [48] is placed, followed by XbaI and BglIII restriction sites flanking $ccdb$ sequence and a glycine-serine linker with Strep-tag placed at the C-terminus of the expressed protein.

$pal$ from *Nostoc punctiforme* ATCC 29133/PCC 73102 (UniProtKB - B2J528) (Np-pal) was amplified using genomic DNA as a template. The rest of the $pal$ genes used in this study, from *Arabidopsis thaliana* (UniProtKB - P45724), *Petroselinum crispum* (UniProtKB - P24481), *Anabaena variabilis* ATCC 29413/PCC 7937 (Trichormus variabilis) (UniProtKB - Q3M5Z3) and *Treponema socranskii subsp. paredis* ATCC 35535 (UniProtKB - S3JNX8), were codon optimized using Gene Designer (DNA2.0) software for heterogeneous expression in *Synechocystis* and synthesized by GenScript.

Genes were inserted into plasmids using SpeI and BamHI (pEEK), BamHI, and SpeI (pEEK3) restriction sites. For insertion into pEEK3C vector, genes were likewise digested with SpeI and BamHI, but ligated into XbaI and BglIII sites on the plasmid backbone, resulting in creation of the TCTAGT and GGATCT scars, respectively. For insertion into pEEKN, BglIII and SpeI restriction sites were used for $Pc$, $At$ and $Av$ pals, BamHI and SpeI for Np-pal, BglIII and PstI for *Ts tal*. 

Page 14/32
Site directed mutagenesis of *Ts tal* sequence was performed with *Ts tal* cloned into pEEK and pEEKN as template using 5’phosphorelated primers carrying overhang for substitution of two nucleotides at position Histidine 87 to create Leucine 87. The linear DNA fragment was then ligated and transformed in *E.coli*.

The laccase oxidase encoded by open reading frame slr1573 in *Synechocystis* [26] was knocked out by replacing the gene with a Cm resistance cassette. For this, 1kb upstream and downstream flanking regions of *slr1573* gene were amplified from *Synechocystis* genomic DNA and cloned into integrative vector pEERM3 [39] creating the plasmid p1573 (Fig. 2F).

**Conjugation and transformation of *Synechocystis***

For conjugation, overnight cultures of *E.coli* cargo cells and *E.coli* HB101 helper cells with pRL443-Amp<sup>R</sup> plasmid were centrifuged at 5000 rpm for 5 minutes and resuspended in fresh LB medium without antibiotics. A mixture of cargo cells (1 ml), helper cells (1 ml) and wild-type *Synechocystis* PCC 6803 (200 µL) was incubated under 100µmol photons m<sup>-2</sup>·s<sup>-1</sup> at 30°C for 2 hours. The mixture was then spread on a filter on a BG11 agar plate without antibiotics for another 24 hours incubation. For colony selection, the filters were changed onto new BG11 agar plate with 50µg·ml<sup>-1</sup> kanamycin. Colonies appeared after 1-2 weeks and were screened by PCR using gene specific primers and DreamTaq DNA polymerase (Thermo Fisher Scientific). Positive colonies were inoculated into fresh liquid BG11 medium with 25µg·ml<sup>-1</sup> Km.

For transformation, *Synechocystis* engineered strains possessing already the Km resistance, were transformed with p1573 as described previously [46]. Colonies that appeared after 10-14 days were analyzed using PCR and restreaked on plates repeatedly until full segregation.

**Determination of tCA and pCou by LC-MS**

Determination of tCA and pCou in the growth media was performed by LC-MS. For this 1 ml of supernatant was taken from *Synechocystis* cultures at certain days of experiment, samples were filtered through 0.2 µm pore PTFE filters (Fisherbrand) and subjected to HPLC analysis. Samples were stored at -20°C if not analyzed the same day.

For qualitative analysis of *E.coli* strains for the presence of corresponding compounds, 3 ml of liquid cultures were inoculated from overnight seed cultures, grown until OD<sub>600</sub> ~ 1 and induced with 1mM of IPTG (Sigma, Merk). After induction, cells were grown for another 3 hours and 1 ml of supernatant was collected. The samples were treated analogously as from *Synechocystis* cultures.

HPLC-MS analysis was performed using an Agilent 1290 Infinity II HPLC system equipped with a 1290 Infinity II High Speed pump and a 1260 II Infinity DAD HS UV-vis detector, using an InfinityLab POROSHELL SB-120 C18 column with dimensions of 50mm×2.1mm and 2.7µm particle size. The HPLC was coupled to an InfinityLab LC/MSD equipped with an ESI source as ionization. LC separation was performed using a water (A, 0.1% formic acid) and acetonitrile (B) eluent system using the method: 0-1
min 10% B; 1-10 min 10→90% B; 10-11 min 90% B; 11-11.1 min 90-10% B; 11.1-12 min 10% B; at the flow rate of 0.3 ml/min. The quantification of tCA and pCou in Synechocystis cultures was based on a linear calibration curve from standards measured in technical triplicates. Standards for tCA and pCou (Sigma, Merk) were prepared in BG11 medium in the range 1-100 μg·ml⁻¹ and filtered before analysis.

**Western Immunoblot (WB)**

Proteins from Synechocystis were extracted on the last day of the experiment as described by Ivleva and Golden 2007 [49]. Protein quantification was performed with DC protein assay (Bio-Rad), using albumin from bovine serum (Sigma) as standard. Soluble proteins were separated by SDS-PAGE, using Mini-PROTEAN TGX™ gels (Bio-Rad), and transferred to PVDF membrane (Bio-Rad.) Western immunoblot was performed according to standard techniques using Anti-Strep-tag II (Abcam) for the detection of Strep-tagged proteins.

**Pigments quantification**

To determine pigment content in strains EvC and EvCΔslr1573, the pre-cultures inoculated from cryostocks were grown in triplicates under 45 μmol photons m⁻² s⁻¹ for several days. Then, cultures were re-inoculated with fresh media to the starting OD₇₅₀ ~ 0.1. Triplicates of samples for chlorophyll a and carotenoids quantification were collected on the next day after starting the experiment (day 1) and the following two days (day 2 and day 3) until the cultures reached OD₇₅₀ ~ 1. Then, cells for centrifuged for 2 min at 15,000×g, supernatant removed and 1 mL of Methanol 100% (Alpha Aesar) was added. Samples were homogenized and incubated in dark at 4°C for at least 30 min. After incubation, samples we centrifuged for 10 min at 15,000×g at 4°C, and the supernatant was then used to measure absorbance at 470nm, 665 nm and 720 nm with Varian Cary 50 BIO spectrophotometer, using methanol as blank. Concentration of chlorophyll a and carotenoids were calculated according to Ritchie et al [50] and Wellburn et al [51] respectively.

For phycobiliproteins determination, culture samples (2 ml) in triplicates were collected at day 3 and centrifuged at 15,000×g for 2 min. The supernatant was discarded and acid-washed glass beads (425–600 μm diameter, Sigma-Aldrich) were added to the sample together with 200 µL of PBS solution. The cells were disrupted using the Precellys-24 Beadbeater (Bertin Instruments) using program 3×30s. Then 800µL of PBS was added to the sample, the mixture was mixed vigorously and incubated for at least 1 hour. After, the tubes were centrifuged for 5 min, the supernatant transferred to a new tube and centrifuged at maximum speed for 30 min at 4°C. The absorbance values at 652 nm and 615 nm were measured and concentration of allophycocyanin (APC) and phycocyanin (PC) was determined according to Bennet and Bogorad [52].

**High-density cultivation**

For the small-scale high-density cultivation an HDC 6.10 starter kit (CellDEG, Germany) was used. The kit consist of 10 ml cultivation vessels with porous hydrophobic membrane at the bottom and a buffer
reservoir which was filled with 200ml of a 3M KHCO\textsubscript{3}/3M K\textsubscript{2}CO\textsubscript{3} (9:1 ratio) solution to provide 90 mbar partial pressure of CO\textsubscript{2} (reference \( T=20^\circ C \), according to the manufacturer's recommendation). The nutrient-enriched media was prepared as described in Lippi et al [33] with slight modifications: as an iron source ammonium iron (III) citrate was used and sodium nitrate as a sole nitrate source.

The cultivation was carried out in a "Versatile Environmental Test Chamber" (Sanyo) w/o humidier at \( 30^\circ C \) under multidirectional illumination with fluorescent white light with increasing light intensities: 250 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) (0-24h), 490 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) (24-48h), 750 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) (48-96h). The CellDEG system was shaking constantly at 320 rpm (IKA KS 130 basic orbital shaker \( \varnothing = 4 \) mm).

**Abbreviations**

AAA: aromatic amino acids; APC: allophycocyanin; \textit{At}: \textit{Arabidopsis thaliana}; \textit{Av}: \textit{Anabaena variabilis}; \textit{C4H}, cinnamate-4-hydroxylase; CBB cycle, Calvin-Benson-Bassham cycle; Cm: chloramphenicol; DAHP: 3-deoxy-D-arabinohexaluronate 7-phosphate synthase; \textit{E.coli}: \textit{Escherichia coli}; E4P: erythrose-4-phosphate; EPSP, 5-enolpyruvylshikimate-3-phosphate synthase; HAL: histidine ammonia lyase; HDC: high-density cultivation; Km: kanamycin; MIO: 3,5-dihydro-5-methylidine-4H-imidazol-4-one; Np: \textit{Nostoc punctiforme}; PAL: phenylalanine ammonia lyase; \textit{Pc}: \textit{Petroselinum crispum}; PC: phycocyanin; pCou: \( p \)-coumaric acid; PEP: phosphoenolpyruvate; Phe: phenylalanine; PQ: plastoquinone; \textit{Synechocystis}: \textit{Synechocystis} sp. PCC 6803; TAL: tyrosine ammonia lyase; \( t\text{CA} \): \textit{trans}-cinnamic acid; Trp: tryptophan; \textit{Ts}: \textit{Treponema socranskii subsp. paredis} ATCC 35535; Tyr: tyrosine; WB: Western Immunoblot; WT: wild type.

**Declarations**

**Ethics approval and consent to participate**

Non applicable.

**Consent for publication**

Non applicable

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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Authors’ contributions

KK, and PL designed the study. KK performed experiments. PL supervised work in the project. KK and PL analyzed data and wrote the manuscript.

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**Figures**
Figure 1

Schematic overview of Shikimate pathway for biosynthesis of aromatic amino acids in Synechocystis. Abbreviations: C4H, Cinnamate-4-hydroxylase; CBB cycle, Calvin-Benson-Bassham cycle; DAHP, 3-Deoxy-D-arabinohexose phosphate 7-phosphate synthase; E4P, Erythrose-4-phosphate; EPSP, 5-enolpyruvylshikimate-3-phosphate synthase; L-Phe, L-Phenylalanine; L-Trp, L-Tryptophane; L-Tyr, L-Tyrosine; PAL, Phenylalanine ammonia lyase; pCou, p-Coumaric acid; PEP, Phosphoenolpyruvate; PQ, plastoquinone; TAL, Tyrosine ammonia lyase; tCA, trans-Cinnamic acid.
Figure 2

Schematic representation of genetic constructs used in this work. A: an illustration of pEEK* control construct, B – pEEK1, C - pEEKN, D – pEEK3, E – pEEK3C. F – illustration of integrative plasmid p1573, where US and DS are upstream and downstream regions for homologous recombination for knock-out of slr1573 gene in Synechocystis.
Figure 3

Productivity and WB analysis of Synechocystis strains overexpressing PAL or TAL from the pEEK vector. A tCA production in strains EvC, PcC, PcN, AtC, AtN, NpC, NpN, AvC, AvN, TsC-H87L and TsN-H87L. B pCou production in strains TsC and TsN. Samples of growth medium were taken on day one, three and six. The values are the means of three biological replicates and two technical replicates, error bars represent the standard deviation. C Western-immunoblot and SDS-PAGE of extracts from engineered Synechocystis strains (EvC, PcC, PcN, AtC, AtN, NpC, NpN, AvC, AvN, TsC-H87L, TsN-H87L, TsC and TsN) analyzed for presence of PAL proteins. Upper panel displays the Western-immunoblot using anti-Strep antibody; bottom panel shows the SDS-PAGE loaded with 5µg of protein crude extract of the different strains.
Figure 4

Growth of Synechocystis strains overexpressing PAL or TAL from the pEEK during 6 days experiment. A Strains EvC, PcC, PcN; B strains EvC, AtC, AtN; C strains EvC, NpC, NpN; D strains EvC, AvC, AvN; E strains EvC, TsC, TsN; F strains EvC, Ts3C-H87L and Ts3N-H87L. Results are the mean of three biological replicates, error bars represent standard deviation.
Figure 5

Productivity and WB analysis of Synechocystis strains overexpressing PAL or TAL from the pEEK3 vector. A Comparison of tCA production in engineered Synechocystis strains EvC, Pc3C, Pc3N, At3C, At3N, Np3C, Np3N, Av3C, Av3N, Ts3C-H87L and Ts3N-H87L. B pCou production in strains Ts3C and Ts3N. Samples of growth medium were taken on day one, three and six. The values are the means of three biological replicates and two technical replicates, error bars represent the standard deviation. C Western-immunoblot and SDS-PAGE of extracts from engineered Synechocystis strains (EvC, Pc3C, Pc3N, At3C, At3N, Np3C, Np3N, Av3C, Av3N, Ts3C-H87L and Ts3N-H87L) analyzed for presence of PAL proteins. Upper panel displays the Western-immunoblot using anti-Strep antibody; bottom panel shows the SDS-PAGE loaded with 3μg of protein crude extract of the different strains.
**Figure 6**

Growth of Synechocystis strains overexpressing PAL or TAL from the pEEK3 during 6 days experiment. A strains EvC, Pc3C, Pc3N; B strains EvC At3C, At3N; C strains EvC, Np3C, Np3N; D strains EvC, Av3C, Av3N; E strains EvC, Ts3C Ts3N; F strains EvC, Ts3C-H87L and Ts3N-H87L. Results are the mean of three biological replicates, error bars represent standard deviation.

**Figure 7**

PAL and TAL relative expression in cell extracts at day six of comparative growth experiments. Upper panel displays the Western-immunoblot using anti-Strep antibody; bottom panel shows the SDS-PAGE loaded with 5µg of protein crude extract of the different strains, except for strains TsC, TsN, Ts3C, Ts3N, TsC-H87L, TsN-H87L, Ts3C-H87L and Ts3N-H87L where 2µg of protein was loaded.
Comparisons of Δslr1573 and WT background strains. A Comparison of tCA production in engineered Synechocystis strains Atc, AtN, AtCΔslr1573 and AtNΔslr1573; B comparison of pCou production in strains TsC, TsN, TsCΔslr1573 and TsNΔslr1573. Samples of growth medium were taken on day four. The values are the means of three biological replicates and two technical replicates, error bars represent the standard deviation. C Western-immunoblot and SDS-PAGE of cell extracts at day four of comparative
growth experiments analyzed for presence of PAL proteins. Upper panel displays the Western-immunoblot using anti-Strep antibody; bottom panel shows the SDS-PAGE loaded with 5µg of protein crude extract of the different strains.

Figure 9

LC-MS profile of culture medium at 275 nm wavelength on day four from comparative experiment. A profile from EvC strain; B profile from EvCΔslr1573 strain.

Figure 10
Comparison of strains EvC to EvCΔslr1573. A Picture of both cultures at OD750 approx. 0.4, left – EvC, right – EvCΔslr1573; B growth curves during 24 days of cultivation; C whole cell absorbance spectra normalized at 750 nm; D: phycobiliprotein concentration normalized per OD750 taken at day 3; E: Chlorophyll a content normalized per OD750; F: total carotenoid content normalized per OD750.

Figure 11

Growth of Synechocystis engineered strains during high-density cultivation for 4 days. A schematic representation of the CellDeg HDC 6.10B system. The cultivation bath is filled with 3M carbonate buffer and constantly creates a CO2 rich atmosphere with diffuses through a porous hydrophobic membrane to the culture medium. Oxygen ventilation of growing cultures is provided by gas permeable membrane outlet on the lid of cultivation vessel. B growth of strains EvC, Pcc, PccN; C strains EvC AtC, AtN; D strains EvC, Npc, NpcN; E strains EvC, AvC, AvN; F strains EvC, TsC TsN. For each strain at least two independent cultivations experiments with biological triplicates was carried out. Error bars represent standard deviation.
Figure 12

tCA and pCou production and Western immunoblot analysis of engineered Synechocystis strains in HDC experiments. A tCA production in engineered Synechocystis strains EvC, PcC, PcN, AtC, AtN, NpC, NpN, AvC, AvN. B pCou production in strains TsC and TsN. Samples of growth medium were taken on day two and four. The values are the means of three biological replicates and two technical replicates, error bars represent the standard deviation. C Western-immunoblot and SDS-PAGE of extracts from engineered Synechocystis strains (EvC, PcC, PcN, AtC, AtN, NpC, NpN, AvC, AvN, TsC and TsN) analyzed for presence of PAL proteins taken on day 4 of HDC experiment. Upper panel displays the Western-immunoblot using anti-Strep antibody; bottom panel shows the SDS-PAGE loaded with 3µg of protein crude extract of the different strains.
Figure 13

Total tCA and pCou accumulated on the final day of standard and high-density cultivation experiments. The values are the means of three biological replicates and two technical replicates, error bars represent the standard deviation.

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