Chapter 5

Cyanophycin: A Nitrogen-Rich Reserve Polymer

Björn Watzer and Karl Forchhammer

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.77049

Abstract

Cyanophycin is a nitrogen/carbon reserve polymer present in most cyanobacteria as well as in a few heterotrophic bacteria. It is a non-ribosomally synthesized polyamide consisting of aspartate and arginine (multi-L-arginyl-poly-L-aspartic acid). The following chapter provides an overview of the characteristics and occurrence of cyanophycin in cyanobacteria. Information about the enzymes involved in cyanophycin metabolism and the regulation of cyanophycin accumulation is also summarized. Herein, we focus on the main regulator, the P_{II} signal transduction protein and its regulation of arginine biosynthesis. Since cyanophycin could be used in various medical or industrial applications, it is of high biotechnological interest. In the last few years, many studies were published aiming at the large-scale production of cyanophycin in different heterotrophic bacteria, yeasts and plants. Recently, a cyanobacterial production strain has been reported, which shows the highest so ever reported cyanophycin yield. The potential and possibilities of biotechnological cyanophycin production will be reviewed in this chapter.

Keywords: cyanophycin, cyanophycin synthetase, cyanophycinase, nitrogen reserve, polyamide, L-arginine, L-aspartate, P_{II} protein

1. Introduction

Cyanophycin, abbreviated CGP (cyanophycin granule peptide), is next to poly-γ-glutamic acid and poly-ε-lysine, the third polyamino acid known to occur in nature [1]. It serves as a nitrogen/carbon reserve polymer in many cyanobacterial strains as well as in a few heterotrophic bacteria. CGP consists of the two amino acids, aspartate and arginine, forming a poly-L-aspartic acid backbone with arginine side chains. The arginine residues are linked to the β-carboxyl group of every aspartyl moiety via isopeptide bond [2].

CGP was discovered in 1887 by the botanist Antonio Borzi during microscopic studies of filamentous cyanobacteria [3]. He observed opaque and light scattering inclusions by using light microscopy and created the name *cianoficina*. Early electron microscopic studies showed a strong structure variation of the CGP granules, depending on the fixatives and poststains used during electron microscopic examinations [4, 5]. This led to a controversy about the ultrastructure of these inclusions until the 1970s. Later, electron microscopic studies described CGP granules as membrane less, electron dense and highly structured cytoplasmic inclusions [6, 7].

With a C/N ratio of 2:1, CGP is extremely rich in nitrogen and consequently an excellent nitrogen storage compound. During the degradation of CGP and subsequent degradation of arginine, a function as energy source was also proposed [8].

2. CGP occurrence

Most cyanobacteria, including unicellular and filamentous, as well as diazotrophic and non-diazotrophic groups are able to accumulate CGP (**Figure 1**).

In non-diazotrophic cyanobacteria, the amount of CGP is usually less than 1% of the cell dry mass during exponential growth. CGP accumulates conspicuously under unbalanced growth conditions including stationary phase, light stress or nutrient limitation (sulfate, phosphate or potassium starvation) that do not involve nitrogen starvation [9, 10]. Under such unbalanced conditions, the amount of CGP may increase up to 18% of the cell dry mass [10]. During the recovery from nitrogen starvation by the addition of a usable nitrogen source, CGP is transiently accumulated [11, 12].

In the unicellular diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142, nitrogen fixation and photosynthesis can coexist in the same cell, but temporarily separated. The nitrogen-fixing enzyme, nitrogenase, is highly sensitive to oxygen. Nitrogen fixation occurs in dark periods and the fixed nitrogen is stored in CGP. In the light period, when photosynthesis is performed, the CGP is degraded to mobilize the fixed nitrogen [13]. Transient CPG accumulation during dark periods was also reported in the filamentous cyanobacterium *Trichodesmium* sp., which has a high abundance in tropical and subtropical seas and is an important contributor to global N and C cycling [14].

Furthermore, in heterocysts of diazotrophic cyanobacteria of the order *Nostocales,* polar nodules consisting of CGP are deposited at the contact site to adjacent vegetative cells [15] (**Figure 1**). The heterocystous CGP seems to be involved in transport of fixed nitrogen to the adjacent photosynthetically active vegetative cell. CGP catabolic enzymes are present at significantly higher levels in vegetative cells than in heterocysts. Moreover, CGP could serve as a sink for fixed nitrogen in the heterocyst to avoid feedback inhibition from soluble products of nitrogen fixation [16, 17]. In *Anabaena* sp. PCC 7120 and *Anabaena variabilis,* mutational studies have shown that strains lacking CGP synthetic genes are little affected in diazotrophic growth under standard laboratory conditions [15, 18]. However, a growth defect was observed under high light conditions [15]. Moreover, diazotrophic growth is significantly decreased in strains that are unable to degrade CGP [16, 18].

http://dx.doi.org/10.5772/intechopen.77049

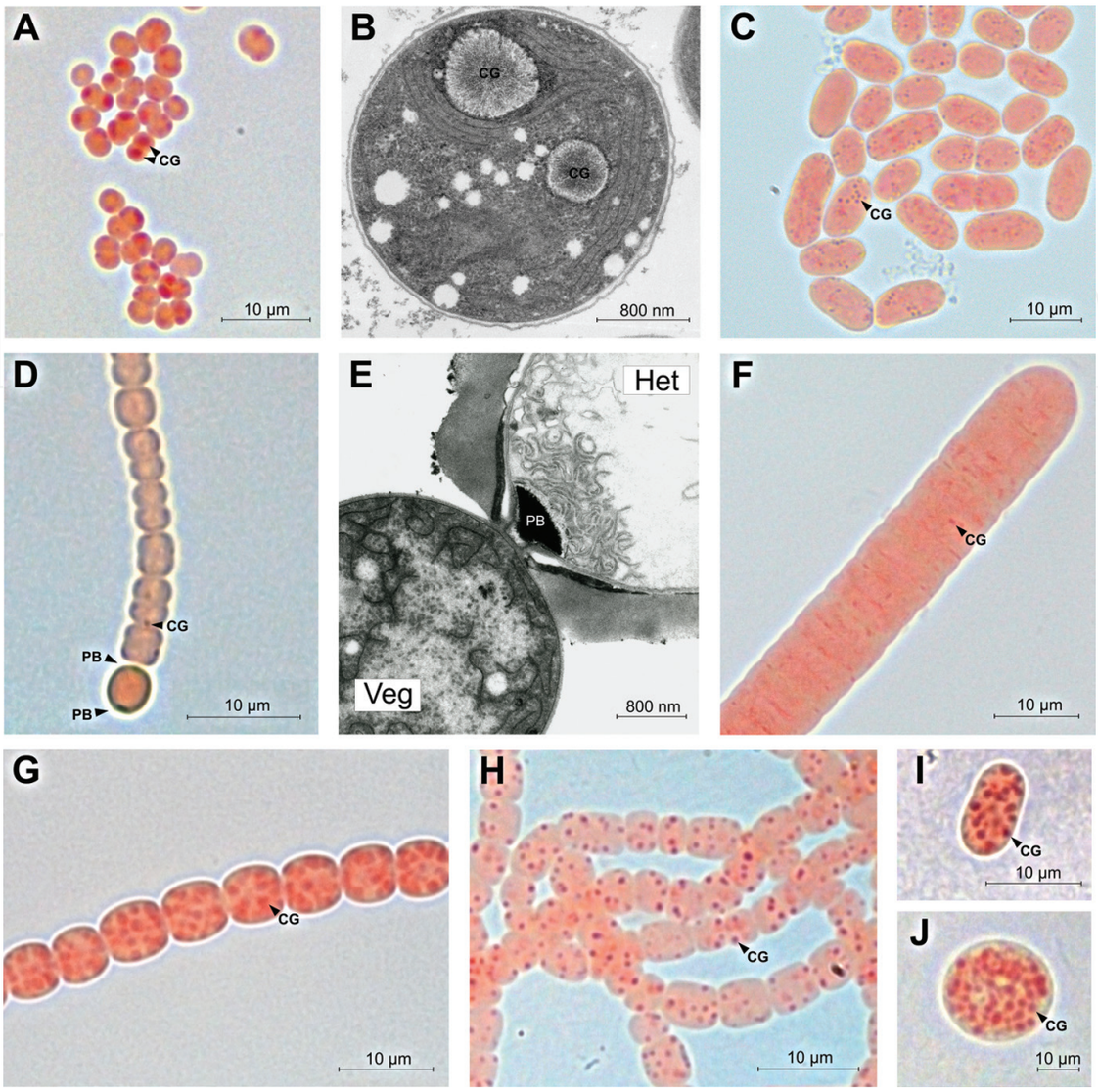

Figure 1. Light and electron microscopic pictures of CGP accumulating cyanobacteria. In light microscopic pictures, CGP was stained using the Sakaguchi reaction [10]. The intensity of the red color indicates the amount of arginine. Dark red to purple dots are CGP granules [CG]. (A) and (B) Phosphate starved *Synechocystis* sp. PCC 6803 in light and transmission electron microscopy, respectively. (C) *Cyanothece* sp. PCC 7424 cultivated in presence of nitrate and continuous light. (D) Filament of diazotrophic growing *Anabaena* sp. PCC 7120 with terminal heterocyst containing polar bodies [PB]. (E) Transmission electron micrographs of a heterocyst and adjacent vegetative cell from *Anabaena* sp. PCC 7120, showing a GCP consisting polar body [PB]. (F) *Oscillatoria* sp. cultivated with nitrate supplementation, showing small CGP granules. (G) Phosphate starved *Anabaena variabilis ATCC 29413* under nitrate supplemented growth. (H) *Nostoc punctiforme ATCC 29133* under phosphate starvation and nitrate supplementation. (I) and (J) Mature akinetes of *Anabaena variabilis ATCC 29413* and *Nostoc punctiforme ATCC 29133*, respectively.

Akinetes are resting spore-like cells of a subgroup of heterocyst-forming cyanobacteria for surviving long periods of unfavorable conditions. During akinete development, the cells transiently accumulate storage compounds, namely glycogen, lipid droplets and CGP [19, 20] (**Figure 1**). CGP granules also appear during germination of dormant akinetes [21]. *Anabaena variabilis* akinetes lacking CGP granules were also able to germinate. This behavior agrees with early observations that CGP is not the direct nitrogen source for protein biosynthesis and therefore not essential for akinete germination [21, 22].

CGP was formally thought to be unique in cyanobacteria. In 2002, Krehenbrink et al. and Ziegler et al. discovered through evaluation of obligate heterotrophic bacteria genomes that many heterotrophic bacteria possess CGP synthetase genes [23, 24]. Genes of CGP metabolism occur in a wide range of different phylogenetic taxa and not closely related to cyanobacteria [25].

3. CGP characteristics

In 1971, Robert Simon isolated CGP granules for the first time by using differential centrifugation. Along with this study, CGP has shown its special and unique solubility behavior [26]. CGP is insoluble at physiological ionic strength and at neutral pH, but soluble in solutions which are acidic, basic or highly ionic. In non-ionic detergents such as Triton X-100, CGP is insoluble; however, in ionic detergents like SDS, it is soluble [6]. Present-day CGP extraction methods are based on its solubility at low pH and insolubility at neutral pH [27].

The chemical structure of CGP was proposed in 1976 by Simon and Weathers [2]. According to this model, CGP has a polymer backbone consisting of α-linked aspartic acid residues. The α-amino group of arginine is linked via isopeptide bonds to the β-carboxylic group of every aspartyl moiety. Because every aspartate residue is linked to an arginine residue, CGP contains equimolar amounts of aspartate and arginine [2]. This structure has been confirmed via enzymatic degradation studies. CGP-degrading enzymes (see below) release β-Asp-Arg dipeptides [28]. CD spectroscopy data suggest that the acid-soluble and neutral insoluble forms of CGP have similar conformations. Both forms contain substantial fractions of β-pleated sheet structure [29].

Cyanobacterial CGP has a molecular weight and polydispersity ranging from 25 to 100 kDa [26]. In contrast, the native CGP producer *Acinetobacter* sp. ADP1 synthesizes CGP with a lower molecular weight ranging from 21 to 28 kDa [30]. Recombinant bacteria or genetically engineered yeast harboring heterologous expression of cyanobacterial CGP synthesis genes also show a lower molecular weight of 25–45 kDa [27, 31]. Transgenic plant-produced CGP also shows a reduced polydispersity between 20 and 35 kDa [32]. A possible explanation would be that cyanophycin synthesis in the native cyanobacterial background involves additional factors contributing the polymer length. These additional factors should also be absent in *Acinetobacter* sp. ADP1.

Native CGP is exclusively composed of aspartate and arginine. By contrast, in CGP isolated from recombinant *E. coli* expressing cyanophycin synthetase (see below) from *Synechocystis* sp. PCC 6803, besides aspartate and arginine, lysine has been found [33]. The amount of incorporated lysine in CGP influences its solubility behavior. Recombinant CGP with a high lysine amount (higher than 31 mol%) is soluble at neutral pH [34].

4. CGP metabolism

4.1. Cyanophycin synthetase

CGP is non-ribosomally synthesized from aspartate and arginine by cyanophycin synthetase (CphA1) (**Figure 2**). In 1976, CphA1 was enriched and characterized for the first time by

Simion [35]. The enzyme incorporates aspartate and arginine in an elongation reaction, which requires ATP, KCl, $MgCl_2$ and a sulfhydryl reagent (β-mercaptoethanol or DTT). For its activity, CphA1 needs a so far unknown CGP primer, as a starting point of the elongation reaction [35]. By using synthetically primers, Berg et al. could show that a single building block of CGP (β-Asp-Arg) does not serve as an efficient primer for CphA1 elongation reaction in vitro. The primers need to consist of at least three Asp-Arg building blocks $(\beta\text{-Asp-Arg})_3$ to detect CphA1 activity [36]. Other peptides, like cell wall or other cellular components, have been suggested to serve as an alternative priming substance for the CphA1 reaction [37]. This could be an explanation for the functionality of CGP synthesis in recombinant bacteria, without the ability to produce native CGP primers [38]. Interestingly, the CphA1 of *Thermosynechococcus elongatus* strain BP-1 shows primer-independent CGP synthesis [39].

Today, CphA1 enzymes from several bacteria, including cyanobacteria and heterotrophic bacteria, have been purified and characterized [33, 39–42]. The molecular mass of the characterized CphA1 enzymes ranges from 90 to 130 kDa. The active form of CphA1s from *Synechocystis* sp. PCC6308 and *Anabaena variabilis* PCC7937 is most likely homodimeric [33, 41], while the primer-independent CphA1 from *Thermosynechococcus elongatus* strain BP-1 forms a homotetramer [39]. The primary structure of cyanobacterial CphA1 can be divided into two regions [33]. The C-terminal region shows sequence similarities to peptide ligases that include murein ligases and folyl poly-γ-glutamate ligase. The N-terminal part of CphA1 shows sequence similarities with another superfamily of ATP-dependent ligases that include carboxylate-thiol

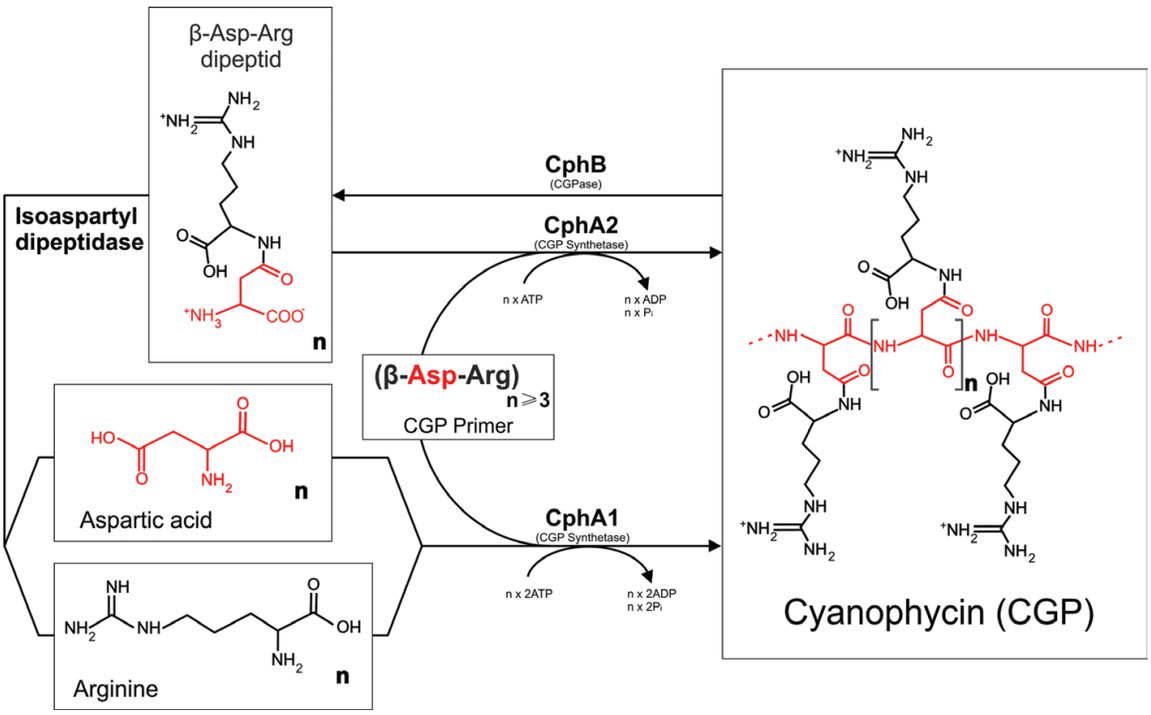

Figure 2. Schematic illustration of CGP metabolism in cyanobacteria. CGP is synthesized from aspartate and arginine by CGP synthetase (CphA1) in an ATP-depending elongation reaction using CGP primers, containing of at least three Asp-Arg building blocks. Intracellular CGP degradation is catalyzed by the CGPase (CphB). The β-Asp-Arg dipeptides resulting from cleavage of CGP are further hydrolyzed by isoaspartyl dipeptidase, releasing aspartate and arginine. In many nitrogen-fixing cyanobacteria, an additional CGP synthetase is present, termed CphA2. CphA2 can use β-aspartyl-arginine dipeptides to resynthesize CGP.

and carboxylate-amine ligase. Since the C- and N-terminal parts show similarity to different superfamilies of ATP-dependent ligases, two ATP-binding sites and two different active sites have been predicted [36]. In vitro experiments revealed that arginine is probably bound in the C-terminal and aspartate in the N-terminal active site [43].

The mechanism of CGP synthesis by CphA1 has been suggested by Berg et al. in 2000, by measuring the step-wise incorporation of amino acids to the C-terminus of the CGP primer. The putative CGP elongation cycle starts at the C-terminal end of the poly-aspartate backbone. First, the carboxylic acid group of the poly-aspartate backbone is activated by transfer of the γ-phosphoryl group of ATP. In the second step, one aspartate is bound at the C-terminus of the growing polymer by its amino group, forming a peptide bound. Subsequently, the intermediate (β-Asp-Arg)$_n$-Asp is transferred to the second active site of CphA1 and phosphorylated at the β-carboxyl group of the aspartate. Finally, the α-group of arginine is linked to the β-carboxyl group of aspartate, forming an isopeptide bound [36].

Various CphA1 enzymes have been characterized with respect to their substrate affinity and specificity. For CphA1 of *Synechocystis* sp. PCC 6308, apparent K_m values were determined to be 450 μM for aspartate, 49 μM for arginine, 200 μM for ATP and 35 μg/ml CGP as priming substance. The lower K_m of arginine compared to aspartate indicates a higher affinity of CphA1 towards arginine. During the in vitro reaction, CphA1 converts per mol incorporated amino acid 1.3 ± 0.1 mol ATP to ADP. The optimal reaction conditions of this enzyme were at pH 8.2 and 50°C [41].

CphA homologs are widely distributed in eubacteria. In silico analysis proposes 10 different groups of cyanophycin synthetases [25]. In cyanobacteria, cyanophycin synthetases of group I–III (CphA, CphA2 and CphA2′) can be found.

Recently, the function of a cyanophycin synthetase of group II (CphA2) has been characterized. Most non-diazotrophic cyanobacteria use a single type of cyanophycin synthetase (CphA1). However, in many nitrogen-fixing cyanobacteria, an additional version of CphA is present, termed CphA2. In 2016, Klemke et al. resolved the function of CphA2 [44]. Compared to CphA1, CphA2 has a reduced size and just one ATP-binding site. CphA2 uses the product of CGP hydrolysis, β-aspartyl-arginine dipeptide as substrate to resynthesize cyanophycin, consuming one molecule of ATP per elongation. A mutant lacking CphA2 shows only a minor decrease in the overall CGP content. However, a CphA2-deficient mutant displays similar defects under diazotrophic and high light conditions than a CphA1 mutant [15, 44]. This observation suggests that the apparent "futile cycle" of CGP hydrolysis and immediate repolymerization is probably of physiological significance in the context of nitrogen fixation [17].

4.2. Cyanophycinase

Since 1976, it is known that CGP is resistant against hydrolytic cleavage by several proteases or arginase [2, 45]. This resistance is probably due to the branched structure of CGP [38]. Therefore, the presence of a highly specified peptidase for CGP hydrolysis was suggested.

In 1999, Richter et al. reported a CGP hydrolyzing enzyme from the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, called CphB [28] (**Figure 2**). During this study, CphB was purified

and studied in detail. CphB is a 29.4 kDa C-terminal exopeptidase, catalyzing the hydrolyzation of CGP to β-Asp-Arg dipeptides [28]. Based on sequence analysis and inhibitor sensitivity to serine protease inhibitors, CphB appears to be a serine-type exopeptidase related to dipeptidase E (PepE) [28]. According to its sequence, CphB contains a serine residue within a lipase box motive (Gly-Xaa-Ser-Xaa-Gly). The serine residue together with a glutamic acid residue and a histidine residue forms the catalytic triad, which is typical for serine-type peptidases [28]. In 2009, the crystal structure has been solved at a resolution of 1.5 Å, showing that CphB forms a dimer. Site-directed mutagenesis confirms that CphB is a serine-type peptidase, consisting of a conserved pocket with the catalytic Ser at position 132 [46]. Structure modeling indicates that the cleavage specificity occurs due to an extended conformation in the active site pocket. The unique conformation of the active site pocket requires β-linked aspartyl peptides for binding and catalysis, preventing CphB from non-specific cleavage of other polypeptides next to CGP [46].

In addition to CphB, which catalyzes the intracellular cleavage of CGP, other versions of cyanophycinase exist, catalyzing the extracellular hydrolysis of CGP. In 2002, Obst et al. isolated several Gram-negative bacteria from different habitats, which were able to utilize CGP as a source of carbon and energy [47, 48]. One isolate was affiliated as *Pseudomonas anguilliseptica* strain BI. In the supernatant of a *Pseudomonas anguilliseptica* culture, a cyanophycinase was found and purified, called CphE [47]. CphE exhibits a high specificity for CGP; however, proteins were not or only marginally hydrolyzed. Degradation products of CphE are β-Asp-Arg dipeptides. Inhibitor sensitivity studies indicated that the catalytic mechanism of CphE is related to serine-type proteases. CphE from *Pseudomonas anguilliseptica* strain BI exhibits an amino acid sequence identity 27–28% to intracellular CphB enzymes of cyanobacteria [47]. Today, extracellular CGPases has been found in a high variety of bacteria including Gram-positive, Gram-negative, aerobic and anaerobic strains. This indicates that the extracellular cleavage and utilization of CGP as carbon, nitrogen and energy source is a common principle in nature [47–53].

In 2007, in silico analysis showed that CphB homologs are widely distributed in eubacteria, proposing eight different groups including intracellular and extracellular CGPases. CGPases from cyanobacteria belong to group I, II and partially group III ($CphB_{1–3}$). Groups IV–VIII, including CphE, are present in a large variety of non-photosynthetic bacteria [25].

4.3. Aspartyl-arginine dipeptidase

The last step in catabolism of CGP is the cleavage of β-Asp-Arg dipeptides to monomeric amino acids, arginine and aspartate (**Figure 2**). In 1999, Richter et al. found β-Asp-Arg dipeptides hydrolyzing activity in extracts of *Synechocystis* sp. PCC 6803 [28]. In *Synechocystis* sp. PCC 6803, the ORF sll0422 as well as ORF all3922 from *Anabaena* sp. PCC 7120 is annotated as "plant-type asparaginase," because of sequence similarities to the first cloned asparaginase from plants [54]. During characterization of plant-type asparaginase in general, including Sll0422 and All3922, Hejazi et al. were able to show that these enzymes are able to hydrolyze a wide range of isoaspartyl dipeptides [55]. Isoaspartyl peptides arise from two biological pathways: First, proteolytic degradation of modified proteins containing isoaspartyl residues and second, as primary degradation product of CGP cleavage from CGPases. Thus, the plant-type

asparaginases, Sll0422 and All3922, have not only a function in asparagine catabolism but also in the final step of CGP and protein degradation [55].

The mature isoaspartyl dipeptidases of *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 consist of two protein subunits that are generated by autocleavage of the primary translation product between Gly-172 and Thr-173 (numbering according to *Synechocystis* sp. PCC 6803) within the conserved consensus sequence GT(I/V)G [55]. The native molecular weight of approximately 70kD of this enzyme suggests that it has a subunit structure of $\alpha_2\beta_2$ (α derived from the N-terminal part and β from the C-terminal part of the precursor) [55].

In *Anabaena* sp. PCC 7120, all genes involved in CGP metabolism as well as the isoaspartyl dipeptidases All3922 are expressed in vegetative cells and heterocysts but in different expression levels. Both, CGP synthetases and CGPases are much higher expressed in heterocysts than in vegetative cells [56]. However, asparaginase All3922 is present in significantly lower levels in heterocysts than in vegetative cells [57]. A deletion of All3922 in *Anabaena* sp. PCC 7120 causes an increased accumulation of CGP and β-Asp-Arg dipeptides. Furthermore, a deletion mutant shows an impaired diazotrophic growth similar to the phenotype known from CphB deletion mutants in *Anabaena* sp. PCC 7120 [18, 57]. This observation implies that the first step of CGP catabolism, the cleavage catalyzed by CphB, takes place in the heterocyst. The released β-Asp-Arg dipeptides are transported to the adjacent vegetative cells. Isoaspartyl dipeptidase All3922, present in the vegetative cells, cleaves the β-Asp-Arg dipeptides and releases monomeric aspartate and arginine [57]. When CGP synthesis is not possible, due to a deletion of CphA, arginine and aspartate might be transferred directly from heterocysts. This explains the minor effects on diazotrophic growth in a CphA deletion mutant [15]. These results identified β-Asp-Arg dipeptides as nitrogen vehicle in diazotrophic heterocyst forming cyanobacteria, next to glutamine and arginine alone or with aspartate [57–59]. A benefit of β-Asp-Arg dipeptides as nitrogen transport substance is avoiding the release of free arginine and aspartate in the heterocyst. This indicates that CGP metabolism has evolved in multicellular heterocyst-forming cyanobacteria to increase the efficiency of nitrogen fixation [57].

5. CGP regulation

5.1. Genetic organization of CphA and CphB

Usually, genes involved in CGP metabolism are clustered. The organization of these clusters can be different, depending on the respective organism [25]. In *Synechocystis* sp. PCC 6803, *cphA* and *cphB* are adjacent; however, they are expressed independently [60]. A hypothetical protein named slr2003 is located downstream of *cphA* and is transcribed in a polycistronic unit with *cphA* [60]. However, the function of Slr2003 is unknown. In the gene of CphB (slr2001), a small antisense RNA was detected (transcriptional unit 1486) [60].

In *Anabaena* sp. PCC 7120, two clusters containing CphA and CphB were identified [18]. In the *cph1* cluster, *cphB1* and *cphA1* were expressed under ammonia and nitrate supplemented growth, but the expression of both genes was higher in the absence of combined nitrogen in

heterocysts and vegetative cells. In the *cph1* operon, *cphB1* and *cphA1* were cotranscribed. In addition, *cphA1* can be expressed from independent promoters, of which one is constitutive and the other regulated by the global nitrogen control transcriptional factor NtcA [18].

In cluster *cph2*, the *cphB2* and *cphA2* genes were found in opposite orientation and both genes were expressed monocistronically. The genes were expressed under conditions of ammonia, nitrate or N_2 supplementation, but the expression was higher in the absence of ammonia. Generally, the expression of the *cph2* is lower compared to *cph1* [18].

In addition to these two gene clusters, a third set of ORFs containing putative *cphA* and *cphB* genes was found in *Nostoc punctiforme* PCC 73102 and *Anabaena variabilis* ATCC 29413 [25].

5.2. Dependence of CGP metabolism on arginine biosynthesis

Generally, CGP accumulation is triggered by cell growth arresting stress conditions, such as entry into stationary phase, light or temperature stress, limitation of macronutrients (with the exception of nitrogen starvation) or inhibition of translation by adding antibiotics like chloramphenicol [9, 10, 61]. All of these CGP triggering conditions result in a reduced or arrested growth. In exponential growth phase the amino acids arginine and aspartate are mostly used for protein biosynthesis with the consequence of a low intracellular level of free amino acids. Under growth-limiting conditions, protein biosynthesis is slowed down, which yields an excess of monomeric amino acids in the cytoplasm, triggering the CGP biosynthesis [10].

CGP accumulation also requires an excess of nitrogen. For the filamentous cyanobacterium *Calothrix* sp. strain PCC 7601, it was shown that CGP accumulation occurs preferably in the presence of ammonia [62]. The addition of amino acids to the media further increased CGP formation [63]. During process optimization studies for heterotrophic CGP production in the strain *Acinetobacter calcoaceticus* ADP1, it was shown that addition of arginine to the medium as sole carbon source increased CGP accumulation drastically. When, in *A. calcoaceticus* strain ADP1, CGP synthesis is induced by phosphate starvation, it accounts to 3.5% (w/w) of the cell dry matter (CDM) with ammonia as nitrogen source. Additional supply of the medium with arginine increases the CGP amount to 41.4% (w/w) (CDM). Notably, a combined supply of arginine and aspartate has a much lower stimulating effect to CGP accumulation than arginine alone [30].

A potential link between regulation of arginine biosynthesis and GCP metabolism was suggested in many previous studies. In a transposon mutagenesis study in the filamentous cyanobacterium *Nostoc ellipsosporum*, an arginine biosynthesis gene, *argL*, was interrupted by a transposon. This mutation partially impairs arginine biosynthesis but does not strictly result in L-arginine auxotrophy. Without arginine supplementation, heterocysts failed to fix nitrogen, akinetes were unable to germinate and CGP granules did not appear. However, when both nitrate and arginine are present in the media, the impaired arginine biosynthesis is bypassed. Under this condition, the mutant could form CGP and was able to differentiate functional akinetes, which contained CGP granules [64].

In metabolic engineering studies of the CGP production strain *Acinetobacter calcoaceticus* ADP1, several genes related to the arginine biosyntheses or its regulation were modified to yield higher amounts of arginine. As a consequence, significant higher CGP production was observed [65].

Bacteria produce arginine from glutamate in eight steps. The first five steps involving N-acetylated intermediates lead to ornithine. The conversion of ornithine to arginine requires three additional steps [66]. The second enzyme of ornithine biosynthesis is the N-acetylglutamate kinase (NAGK), which catalyzes the phosphorylation of N-acetyl glutamate to N-acetylglutamylphosphate. NAGK catalyzes the controlling step in arginine biosynthesis [67]. NAGK activity is subjected to allosteric feedback inhibition by arginine and is, moreover, positively controlled by the P_{II} signal transduction protein (see below) [67, 68]. Maheswaran et al. showed that arginine production and the following CGP accumulation depend on the catalytic activation of NAGK by the signal transduction protein P_{II} [69]. In a P_{II}-deficient mutant of *Synechocystis* sp. PCC 6803, NAGK remained in a low activity state, which caused impaired CGP accumulation [69].

The nitrogen-regulated response regulator NrrA also has influence on arginine and CGP biosynthesis. An NrrA-deficient mutant in *Synechocystis* sp. PCC 6803 shows reduced intracellular arginine levels and, consequently, reduced CGP amount [70].

All these results and observations point towards arginine as main bottleneck of CGP biosynthesis, while aspartate plays a minor role. CGP accumulation occurs as a result of arginine enrichment in the cytoplasm. Reasons for increased arginine content in the cell are lowered protein biosynthesis as a result of various growth limiting conditions. Furthermore, an excess of nitrogen and energy sensed by P_{II} leads to NAGK activation and thereby increased arginine biosynthesis.

5.3. P_{II} regulation of arginine metabolism

The P_{II} signal transduction proteins are widely distributed in prokaryotes and chloroplasts, where they play a coordinating role in the regulation of nitrogen assimilatory processes [71–73]. For this purpose, P_{II} senses the energy status of the cell by binding ATP or ADP in a competitive way [74]. Binding of ATP and synergistic binding of 2-oxoglutarate (2-OG) allows P_{II} to sense the current carbon/nitrogen status of the cell [75]. 2-OG is the carbon skeleton for the GS/GOGAT reactions and thereby links the carbon and nitrogen metabolism in all domains of life [76, 77]. The pool size of 2-OG reacts quickly to changes in nitrogen availability, wherefore 2-OG is an indicator of the carbon/nitrogen balance [78, 79]. Depending on the nitrogen supply, P_{II} may be phosphorylated at the apex of the T-loop at position Ser49 [80, 81]. Binding of the effector molecules ATP, ADP and 2-OG as well as phosphorylation leads to conformational rearrangements of the large surface-exposed T-loop, P_{II}'s major protein-interaction structure [82]. These conformational states direct the interaction of P_{II} with its various interaction partners and thereby regulate the cellular C/N balance [83].

In cyanobacteria, P_{II} regulates the global nitrogen control transcriptional factor NtcA, through binding to the NtcA co-activator PipX [84]. In common with other bacteria, cyanobacterial P_{II} proteins can interact with the biotin carboxyl carrier protein (BCCP) of acetyl-CoA carboxylase (ACC) and thereby control the acetyl-CoA levels [85]. Furthermore, P_{II} controls arginine biosynthesis via regulation of NAGK [68, 69, 86].

P_{II} proteins form a cylindrical-shaped homotrimer with 12–13 kDa per subunits. The T-loop, a large and surface-exposed loop, protrudes from each subunit. The effector binding sites are positioned in the three inter-subunit clefts [87, 88]. If sufficient energy and nitrogen are available,

indicated by a high ATP and low 2-OG level, non-phosphorylated P_{II} forms an activating complex with NAGK.

The crystal structure of the P_{II}-NAGK complex from *Synechococcus elongatus* strain PCC 7942 revealed two P_{II} trimers sandwiching a NAGK homohexamer (trimer of dimers) [88]. Each P_{II} subunit contacts one NAGK subunit [88]. Two parts of P_{II} are involved in interaction with NAGK. The first structure, called B-loop, is located on the P_{II} body and interacts with the C-domain of NAGK subunit, involving residue Glu85. The interaction of the B-loop is the first step in complex formation. Second, the T-loop must adopt a bent conformation and insert into the interdomain cleft of NAGK [89]. This enhances the catalytic efficiency of NAGK, with the V_{max} increasing fourfold and the K_m for N-acetylglutamate decreasing by a factor of 10 [86]. Furthermore, feedback inhibition of NAGK by arginine is strongly decreased in the presence of P_{II} [86].

During P_{II} mutagenesis, a P_{II} variant was identified that binds constitutively NAGK in vitro. This P_{II} variant exhibits a single amino acid replacement, Ile86 to Asn86, hereafter referred as P_{II}(I86N) [89]. The crystal structure of P_{II}(I86N) has been solved, showing an almost identical backbone than wild-type P_{II}. However, the T-loop adopts a compact conformation, which is a structural mimic of P_{II} in the NAGK complex [89, 90]. Addition of 2-OG in the presence of ATP normally leads to a dissociation of the P_{II}-NAGK complex, however P_{II}(I86N) no longer responds to 2-OG [90].

The P_{II}(I86N) variant enables a novel approach of metabolic pathway engineering by using custom-tailored P_{II} signaling proteins. By replacing the wild-type P_{II} with a P_{II} carrying the mutation for I86N in *Synechocystis* sp. PCC 6803, it was possible to engineer the first cyanobacterial CGP overproducer strain. Strain BW86, containing the P_{II}(I86N) version, shows an increase of NAGK activity, which causes a more than 10-fold higher arginine content than the wild-type [10]. Under balanced growth conditions with nitrate as nitrogen source, strain BW86 accumulates up to 15.6 ± 5.4% CGP relative to the CDM, i.e., on average almost sixfold more than the wild type. Appropriate starvation conditions can further increase the CGP content of strain BW86 up to 47.4 ± 2.3% per CDM under phosphate starvation and 57.3 ± 11.1% per CDM under potassium starvation, without addition of arginine to the medium [10]. Furthermore, the CGP, which is produced by strain BW86, shows a high polydispersity ranging from 25 to 100 kDa, similar to the polydispersity of cyanobacterial wild-type CGP, which contrasts CGP from recombinant producer strains using heterologous expression systems with heterotrophic bacteria, yeasts or plants [10]. CGP isolated from those strains have a size ranging of 25–45 kDa [27, 31, 32].

6. Industrial applications

Industrial applications for CGP have previously mainly focused on chemical derivatives. CGP can be converted via hydrolytic β-cleavage to poly(α-L-aspartic acid) (PAA) and free arginine. PAA is biodegradable and has a high number of negatively charged carboxylic groups, making PAA to a possible substituent for polyacrylates [48, 50, 91]. PAA can be employed as antiscalant or dispersing ingredient in many fields of applications, including washing detergents or suntan lotions. Furthermore, PAA has potential application areas as an additive in paper, paint, building or oil industry [48, 50].

CGP can also serve as a source for dipeptides and amino acids in food, feed and pharmaceutical industry. The amino acids arginine (semi-essential), aspartate (non-essential) and lysine (essential) derived from CGP have a broad spectrum of nutritional or therapeutic applications. Large-scale production of these amino acids, as mixtures or dipeptides, is established in industry, with various commercial products already available on the market (reviewed by Sallam and Steinbuchel [92]).

Potential applications of non-modified CGP have been discussed but remain so far largely unexplored. This can partially be explained by the lack of research being conducted on the material properties of CGP. Recently in 2017, the first study regarding CGP material properties has been published. In this study, Khlystov et al. focused on the structural, thermal, mechanical and solution properties of CGP produced by recombinant *E. coli*, giving new insights in the nature of this polymer as bulk chemical [91]. They describe CGP as an amorphous, glassy polyzwitterion with high thermostability. The dry material is stiff and brittle. According to these properties, CGP could be used to synthesize zwitterionomeric copolymers or as reinforcing fillers [91].

7. Biotechnological production

Previous ventures to produce CGP in high amounts were mainly focused on heterotrophic bacteria, yeasts and plants as production host. These recombinant production hosts heterologously express CGP synthetase genes, mostly from cyanobacteria. In this way, heterotrophic bacteria, which are established in biotechnological industry including *E. coli*, *Corynebacterium glutamicum*, *Cupriavidus necator* (formally known as *Ralstonia eutropha*) and *Pseudomonas putida*, were used for heterologous production of CGP [93].

Strain *E. coli* DH1, containing *cphA* from *Synechocystis* sp. PCC6803, was used for large-scale production of CGP in a culture volume of up to 500 liter, allowing the isolation of CGP in a kilogram scale. During process optimization, the highest observed CGP content was 24% (w/w) per CDM. However, the synthesis of CGP was strongly dependent on the presence of complex components in the medium (terrific broth complex medium). In mineral salt medium, CGP accumulation only occurs in the presence of casamino acids [27]. An engineered version of CphA from *Nostoc ellipsosporum*, transformed in *E. coli*, shows a further increase in CGP production, up to 34.5% (w/w) of CDM. However, this production strain also requires expensive complex growth media to yield such a high amount of CGP [94].

Cupriavidus necator and *Pseudomonas putida* are known as model organisms for the industrial scale production of polyhydroxyalkanoates (PHA). Therefore, they have been considered as candidates for large scale CGP production [93, 95]. Metabolic engineering and process optimization studies of *Cupriavidus necator* and *Pseudomonas putida* harboring *cphA* from *Synechocystis* sp. PCC 6803 or *Anabaena* sp. PCC 7120 were performed. In these organisms, the accumulation of CGP is mainly depending on the origin of the *cphA* gene, the accumulation of other storage compounds like PHA as well as the addition of precursor components like arginine to the medium [96]. PHA-deficient mutants of *Cupriavidus necator* and *Pseudomonas putida* accumulate in general more CGP compared to the PHA containing strains [96]. During genetic modification of *cphA* expression in *Cupriavidus necator*, CGP accumulation turned out to be

strongly affected by the expression system. A stabilized multi-copy *cphA* expression system, using the KDPG-aldolase gene (*eda*)-dependent addiction system, allows cultivation without antibiotic selection. The multi-copy *cphA* expression results in a CGP yield between 26.9% and 40.0% (w/w) of CDM. The maximum amount of 40.0% (w/w) of CDM was observed in a 30- and 500-l pilot plant. In the absence of the amino acids arginine and aspartic acid in the medium, the CGP amount was still between 26.9% and 27.7% (w/w) of CDM [97].

The industrially established host *Saccharomyces cerevisiae* has also been used for CGP production, by expression of *cphA* from *Synechocystis* sp. PCC 6803. *S. cerevisiae* harboring *cphA* accumulated up to 6.9% (w/w) of CDM. Two CGP species were observed in this strain: water-soluble and the typical water-insoluble CGP. Furthermore, the isolated polymer from this transgenic yeast contained 2 mol% lysine, which can be increased up to 10 mol% when cultivation occurs with lysine in the medium [31]. During metabolic engineering studies, several arginine biosynthesis mutants have been analyzed concerning their CGP accumulation abilities. Surprisingly, strains with defects in arginine degradation accumulated only 4% CGP (w/w) of CDM; however, arginine auxotrophic strains were able to accumulate up to 15.3%. Depending on the cultivation conditions, between 30 and 90% of the extracted CGP was soluble at neutral pH. In addition to arginine, aspartate and lysine, further amino acids, such as citrulline and ornithine, have been detected in isolated CGP from different arginine biosynthesis mutants [98]. Furthermore, it was also possible to produce CGP and CGP derivates in *Pseudomonas putida* and the yeast *Pichia pastoris* [99, 100].

CGP and CGP derivates are important sources for β-dipeptides for several applications. A large-scale method was developed to convert CGP into its constituting β-dipeptides by using CphE from *Pseudomonas alcaligenes*. This allows the large-scale production of customized β-dipeptides, depending on the composition of the CGP derivates [92, 101].

Production of CGP has also been attempted in several transgenic plants. Here, ectopic expression of the primer-independent CphA from *Thermosynechococcus elongatus* BP-1 leads to an accumulation of CGP up to 6.8% (w/w) in tobacco leafs and to 7.5% (w/w) of CDM in potato tubers [102, 103]. CGP production and extraction in plants can be coupled with the production of other plant products like starch [103]. The peculiarities and challenges of plant-produced CGP have been reviewed by Nausch et al. [32].

Compared to bacteria that are used so far in biotechnological industry, cyanobacteria are unique as they use sunlight and CO_2 as energy and carbon source. Cyanobacteria have been identified as rich source of various biologically active compounds, biofertilizers, bioplastics, energy, food and feed [104]. Obviously, the importance of environmentally friendly production processes increases more and more. Hence, Cyanobacteria are expected to play a major role in future industry. *Synechocystis* sp. PCC 6803 strain BW86 is the first reported bulk chemical producing cyanobacterial strain in the literature. CGP production in *Synechocystis* BW86 does not require organic carbon or CGP precursor substances. Growth limiting conditions like phosphate and potassium starvation can further increase the CGP production up to 47.4 ± 2.3% and 57.3 ± 11.1% per CDM, respectively. The studies of Trautmann et al. showed that strain BW86 can be cultivated in flat plate photobioreactors (Midiplate reactor system [105]). During this optimization study, the optimal light intensity as well as the phosphate concentration was determined to maximize CGP synthesis. Under optimal production conditions, highest amount of CGP was around 40% of CDM with a total yield of 340 mg CGP per liter in 9 days [106].

The main bottleneck of CGP production in Cyanobacteria is the relatively slow growth rate, which is much lower than in biotechnologically established bacteria. Conventional cultivation methods of cyanobacteria reach a biomass of roughly 1 g dry mass per liter [107]. To overcome this limitation, a new cultivation method was developed, using a two-tier vessel with membrane-mediated CO_2 supply. By using this cultivation setup, it was possible to enable rapid growth of *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 up to 30 g CDM per liter [108]. *Synechocystis* sp. PCC 6803 strain BW86 was also used in this high-density cultivation setup. During this study, CGP amounts up to 1 g per liter were reached in 96 h. This is approximately four times higher compared to the maximum CGP yield observed during conventional cultivation after 12 days [106, 109].

In comparison, the recombinant *E. coli* strain DH1 harboring *cphA* from *Synechocystis* sp. PCC 6803 produces between 6.7 and 8.3 g CDM per liter culture in 16 h. CGP amounts during this fed-batch fermentations were between 21 and 24% of the CDM [27], resulting in a CGP production rate of 87.9 to 124.5 mg/l and hour. Although this exceeds the production rate in *Synechocystis* sp. PCC 6803 strain BW86 by a factor of 10, the recombinant *E. coli* requires terrific broth complex medium, while *Synechocystis* sp. PCC 6803 strain BW86 is cultivated in simple mineral medium and additionally sequesters hazardous greenhouse gas CO_2. Considering these super ordinate factors, production of biopolymers with cyanobacteria may in fact become an alternative to heterotrophic bacteria.

8. Conclusions

CGP is well researched and its occurrence in cyanobacteria is known for more than 100 years. However, many questions are still open. Most obviously, the cell biology of the CGP granules remains largely unknown. In the last decades, research on CGP mainly focused on biotechnological purposes, like strain or process optimization. Most work has been carried out with short-chain CGP from recombinant producer strains; however the biophysical properties of the long-chain native CGP remain largely unexplored. So far, heterotrophic bacteria were mainly used to produce industrial biocompounds including CGP. In this chapter, we discussed the possibility of a cyanobacterial CGP production strain. The main disadvantages of cyanobacteria, their slower growth and the low abundance of product can be compensated using genetic engineering together with appropriate production processes. Future industry has to cope with the manifold challenges to counteract environmental pollution and climate change. The use of cyanobacteria in CGP production and, more generally, in biotechnological applications for bioproduct synthesis provides an environmentally friendly alternative to conventional biotechnological approaches.

Acknowledgements

This work was supported by grants from the DFG (Fo195/9), the research training group GRK 1708 and the Baden-Württemberg foundation grant 7533-10-5-92B. We thank Iris Maldener for provision of the electron micrographs of *Anabaena* sp. PCC 7120 and *Synechocystis* sp. PCC 6803. We would also like to give thanks to Rebeca Pérez for provision of light micrographs of *Anabaena variabilis ATCC 29413* and *Nostoc punctiforme ATCC 29133.*

Conflict of interest

The authors declare that they have no competing interests.

Author details

Björn Watzer and Karl Forchhammer*

*Address all correspondence to: karl.forchhammer@uni-tuebingen.de

Interfaculty Institute of Microbiology and Infection Medicine Tübingen, Eberhard Karls Universität Tübingen, Tübingen, Germany

References

[1] Feng S, ZhiNan X. Microbial production of natural poly amino acid. Science in China Series B. 2007;**50**(3):291-303

[2] Simon RD, Weathers P. Determination of the structure of the novel polypeptide containing aspartic acid and arginine which is found in cyanobacteria. Biochimica et Biophysica Acta (BBA)—Protein Structure. 1976;**420**(1):165-176

[3] Borzi A. Le communicazioni intracellulari delle Nostochinee. Malpighia. 1887;**1**:28-74

[4] Wood P, Peat A, Whitton BA. Influence of phosphorus status on fine-structure of the cyanobacterium (blue-green-alga) calothrix-parietina. Cytobios. 1986;**47**(189):89-99

[5] Lang NJ. The fine structure of blue-green algae. Annual Review of Microbiology. 1968; **22**:15-46

[6] Lang NJ, Simon RD, Wolk CP. Correspondence of cyanophycin granules with structured granules in Anabaena-Cylindrica. Archiv fur Mikrobiologie. 1972;**83**(4):313

[7] Allen MM, Weathers PJ. Structure and composition of cyanophycin granules in the cyanobacterium Aphanocapsa 6308. Journal of Bacteriology. 1980;**141**(2):959-962

[8] Weathers PJ, Chee HL, Allen MM. Arginine catabolism in Aphanocapsa 6308. Archives of Microbiology. 1978;**118**(1):1-6

[9] Allen MM, Hutchison F, Weathers PJ. Cyanophycin granule polypeptide formation and degradation in the Cyanobacterium Aphanocapsa 6308. Journal of Bacteriology. 1980;**141**(2):687-693

[10] Watzer B, Engelbrecht A, Hauf W, Stahl M, Maldener I, Forchhammer K. Metabolic pathway engineering using the central signal processor PII. Microbial Cell Factories. 2015;**14**:192

[11] Allen MM, Hutchison F. Nitrogen limitation and recovery in the Cyanobacterium Aphanocapsa-6308. Archives of Microbiology. 1980;**128**(1):1-7

[12] Klotz A, Georg J, Budinska L, Watanabe S, Reimann V, Januszewski W, et al. Awakening of a dormant cyanobacterium from nitrogen chlorosis reveals a genetically determined program. Current Biology. 2016;**26**(21):2862-2872

[13] Sherman LA, Meunier P, Colon-Lopez MS. Diurnal rhythms in metabolism: A day in the life of a unicellular, diazotrophic cyanobacterium. Photosynthesis Research. 1998;**58**(1): 25-42

[14] Finzi-Hart JA, Pett-Ridge J, Weber PK, Popa R, Fallon SJ, Gunderson T, et al. Fixation and fate of C and N in the cyanobacterium Trichodesmium using nanometer-scale secondary ion mass spectrometry. Proceedings of the National Academy of Sciences of the United States of America. 2009;**106**(24):9931

[15] Ziegler K, Stephan DP, Pistorius EK, Ruppel HG, Lockau W. A mutant of the cyanobacterium Anabaena variabilis ATCC 29413 lacking cyanophycin synthetase: Growth properties and ultrastructural aspects. FEMS Microbiology Letters. 2001;**196**(1):13-18

[16] Burnat M, Herrero A, Flores E. Compartmentalized cyanophycin metabolism in the diazotrophic filaments of a heterocyst forming cyanobacterium. Proceedings of the National Academy of Sciences of the United States of America. 2014;**111**(10):3823-3828

[17] Forchhammer K, Watzer B. Closing a gap in cyanophycin metabolism. Microbiology. 2016;**162**:727-729

[18] Picossi S, Valladares A, Flores E, Herrero A. Nitrogen-regulated genes for the metabolism of cyanophycin, a bacterial nitrogen reserve polymer—Expression and mutational analysis of two cyanophycin synthetase and cyanophycinase gene clusters in the heterocyst-forming cyanobacterium Anabaena sp. PCC 7120. The Journal of Biological Chemistry. 2004;**279**(12):11582-11592

[19] Sukenik A, Maldener I, Delhaye T, Viner-Mozzini Y, Sela D, Bormans M. Carbon assimilation and accumulation of cyanophycin during the development of dormant cells (akinetes) in the cyanobacterium Aphanizomenon ovalisporum. Frontiers in Microbiology. 2015;**6**:1067

[20] Perez R, Forchhammer K, Salerno G, Maldener I. Clear differences in metabolic and morphological adaptations of akinetes of two Nostocales living in different habitats. Microbiology. 2016;**162**:214-223

[21] Perez R, Wormer L, Sass P, Maldener I. A highly asynchronous developmental program triggered during germination of dormant akinetes of the filamentous diazotrophic cyanobacteria. FEMS Microbiology Ecology. 2018;**94**(1)

[22] Sutherland JM, Reaston J, Stewart WDP, Herdman M. Akinetes of the Cyanobacterium Nostoc Pcc 7524—Macromolecular and biochemical-changes during Synchronous germination. Journal of General Microbiology. 1985;**131**(Nov):2855-2863

[23] Krehenbrink M, Oppermann-Sanio FB, Steinbuchel A. Evaluation of non-cyanobacterial genome sequences for occurrence of genes encoding proteins homologous to cyanophycin

synthetase and cloning of an active cyanophycin synthetase from Acinetobacter sp. strain DSM 587. Archives of Microbiology. 2002;**177**(5):371-380

[24] Ziegler K, Deutzmann R, Lockau W. Cyanophycin synthetase-like enzymes of non-cyanobacterial eubacteria: Characterization of the polymer produced by a recombinant synthetase of Desulfitobacterium hafniense. Zeitschrift für Naturforschung. Section C. 2002;**57**(5-6):522-529

[25] Fuser G, Steinbuchel A. Analysis of genome sequences for genes of cyanophycin metabolism: Identifying putative cyanophycin metabolizing prokaryotes. Macromolecular Bioscience. 2007;**7**(3):278-296

[26] Simon RD. Cyanophycin granules from the blue-green alga Anabaena cylindrica: A reserve material consisting of copolymers of aspartic acid and arginine. Proceedings of the National Academy of Sciences of the United States of America. 1971;**68**(2):265-267

[27] Frey KM, Oppermann-Sanio FB, Schmidt H, Steinbuchel A. Technical-scale production of cyanophycin with recombinant strains of Escherichia coli. Applied and Environmental Microbiology. 2002;**68**(7):3377-3384

[28] Richter R, Hejazi M, Kraft R, Ziegler K, Lockau W. Cyanophycinase, a peptidase degrading the cyanobacterial reserve material multi-L-arginyl-poly-L-aspartic acid (cyanophycin)—Molecular cloning of the gene of Synechocystis sp. PCC 6803, expression in *Escherichia coli*, and biochemical characterization of the purified enzyme. European Journal of Biochemistry. 1999;**263**(1):163-169

[29] Simon RD, Lawry NH, McLendon GL. Structural characterization of the cyanophycin granule polypeptide of Anabaena cylindrica by circular dichroism and Raman spectroscopy. Biochimica et Biophysica Acta. 1980;**626**(2):277-281

[30] Elbahloul Y, Krehenbrink M, Reichelt R, Steinbuchel A. Physiological conditions conducive to high cyanophycin content in biomass of Acinetobacter calcoaceticus strain ADP1. Applied and Environmental Microbiology. 2005;**71**(2):858-866

[31] Steinle A, Oppermann-Sanio FB, Reichelt R, Steinbuchel A. Synthesis and accumulation of cyanophycin in transgenic strains of *Saccharomyces cerevisiae*. Applied and Environmental Microbiology. 2008;**74**(11):3410-3418

[32] Nausch H, Huckauf J, Broer I. Peculiarities and impacts of expression of bacterial cyanophycin synthetases in plants. Applied Microbiology and Biotechnology. 2016;**100**(4): 1559-1565

[33] Ziegler K, Diener A, Herpin C, Richter R, Deutzmann R, Lockau W. Molecular characterization of cyanophycin synthetase, the enzyme catalyzing the biosynthesis of the cyanobacterial reserve material multi-L-arginyl-poly-L-aspartate (cyanophycin). European Journal of Biochemistry. 1998;**254**(1):154-159

[34] Wiefel L, Steinbuchel A. Solubility behavior of cyanophycin depending on lysine content. Applied and Environmental Microbiology. 2014;**80**(3):1091-1096

[35] Simon RD. The biosynthesis of multi-L-arginyl-poly(L-aspartic acid) in the filamentous cyanobacterium *Anabaena cylindrica*. Biochimica et Biophysica Acta. 1976;**422**(2):407-418

[36] Berg H, Ziegler K, Piotukh K, Baier K, Lockau W, Volkmer-Engert R. Biosynthesis of the cyanobacterial reserve polymer multi-L-arginyl-poly-L-aspartic acid (cyanophycin)—Mechanism of the cyanophycin synthetase reaction studied with synthetic primers. European Journal of Biochemistry. 2000;**267**(17):5561-5570

[37] Hai T, Oppermann-Sanio FB, Steinbuchel A. Molecular characterization of a thermostable cyanophycin synthetase from the thermophilic cyanobacterium Synechococcus sp strain MA19 and in vitro synthesis of cyanophycin and related polyamides. Applied and Environmental Microbiology. 2002;**68**(1):93-101

[38] Shively JM. Inclusions in Prokaryotes. Berlin ; New York: Springer; 2006. xii, 349 p

[39] Arai T, Kino K. A cyanophycin synthetase from Thermosynechococcus elongatus BP-1 catalyzes primer-independent cyanophycin synthesis. Applied Microbiology and Biotechnology. 2008;**81**(1):69-78

[40] Hai T, Oppermann-Sanio FB, Steinbuchel A. Purification and characterization of cyanophycin and cyanophycin synthetase from the thermophilic Synechococcus sp. MA19. FEMS Microbiology Letters. 1999;**181**(2):229-236

[41] Aboulmagd E, Sanio FBO, Steinbuchel A. Purification of Synechocystis sp strain PCC-6308 cyanophycin synthetase and its characterization with respect to substrate and primer specificity. Applied and Environmental Microbiology. 2001;**67**(5):2176-2182

[42] Krehenbrink M, Steinbuchel A. Partial purification and characterization of a non-cyanobacterial cyanophycin synthetase from Acinetobacter calcoaceticus strain ADP1 with regard to substrate specificity, substrate affinity and binding to cyanophycin. Microbiology. 2004;**150**(Pt 8):2599-2608

[43] Berg H. Untersuchungen zu Funktion und Struktur der Cyanophycin-Synthetase von Anabaena variabilis ATCC 29413. Germany: Dissertation Humboldt–Universität zu Berlin; 2003

[44] Klemke F, Nurnberg DJ, Ziegler K, Beyer G, Kahmann U, Lockau W, et al. CphA2 is a novel type of cyanophycin synthetase in N-2-fixing cyanobacteria. Microbiology. 2016;**162**:526-536

[45] Simon RD. Inclusion bodies in the cyanobacteria: Cyanophycin, polyphosphate, polyhedral bodies. In: Fay P, Van Baalen C, editors. The Cyanobacteria: Elsevier Science Publishers B.V.; 1987. p. 199-225

[46] Law AM, Lai SW, Tavares J, Kimber MS. The structural basis of beta-peptide-specific cleavage by the serine protease cyanophycinase. Journal of Molecular Biology. 2009;**392**(2):393-404

[47] Obst M, Oppermann-Sanio FB, Luftmann H, Steinbuchel A. Isolation of cyanophycin-degrading bacteria, cloning and characterization of an extracellular cyanophycinase gene (cphE) from Pseudomonas anguilliseptica strain BI. The cphE gene from P. anguilliseptica BI encodes a cyanophycinhydrolyzing enzyme. Journal of Biological Chemistry. 2002;**277**(28):25096-25105

[48] Rehm B. Microbial Production of Biopolymers and Polymer Precursors: Applications and Perspectives. Wymondham: Caister Academic; 2009. viii, 293 p, 1 p. of plates p.

[49] Obst M, Sallam A, Luftmann H, Steinbuchel A. Isolation and characterization of gram-positive cyanophycin-degrading bacteria—Kinetic studies on cyanophycin depolymerase activity in aerobic bacteria. Biomacromolecules. 2004;**5**(1):153-161

[50] Obst M, Steinbuchel A. Microbial degradation of poly(amino acid)s. Biomacromolecules. 2004;**5**(4):1166-1176

[51] Obst M, Krug A, Luftmann H, Steinbuchel A. Degradation of cyanophycin by Sedimentibacter hongkongensis strain KI and Citrobacter amalonaticus strain G isolated from an anaerobic bacterial consortium. Applied and Environmental Microbiology. 2005; **71**(7):3642-3652

[52] Sallam A, Steinbuchel A. Anaerobic and aerobic degradation of cyanophycin by the denitrifying bacterium Pseudomonas alcaligenes strain DIP1 and role of three other coisolates in a mixed bacterial consortium. Applied and Environmental Microbiology. 2008;**74**(11):3434-3443

[53] Sallam A, Steinbuchel A. Cyanophycin-degrading bacteria in digestive tracts of mammals, birds and fish and consequences for possible applications of cyanophycin and its dipeptides in nutrition and therapy. Journal of Applied Microbiology. 2009;**107**(2):474-484

[54] Lough TJ, Reddington BD, Grant MR, Hill DF, Reynolds PHS, Farnden KJF. The isolation and characterization of a Cdna clone encoding L-asparaginase from developing seeds of Lupin (Lupinus-Arboreus). Plant Molecular Biology. 1992;**19**(3):391-399

[55] Hejazi M, Piotukh K, Mattow J, Deutzmann R, Volkmer-Engert R, Lockau W. Isoaspartyl dipeptidase activity of plant-type asparaginases. The Biochemical Journal. 2002;**364**: 129-136

[56] Gupta M, Carr NG. Enzyme-activities related to cyanophycin metabolism in heterocysts and vegetative cells of anabaena Spp. Journal of General Microbiology. 1981;**125**(Jul):17-23

[57] Burnat M, Herrero A, Flores E. Compartmentalized cyanophycin metabolism in the diazotrophic filaments of a heterocyst-forming cyanobacterium. Proceedings of the National Academy of Sciences of the United States of America. 2014;**111**(10):3823-3828

[58] Wolk CP, Austin SM, Bortins J, Galonsky A. Autoradiographic localization of N-13 after fixation of N-13-labeled nitrogen gas by a heterocyst-forming blue-green-alga. The Journal of Cell Biology. 1974;**61**(2):440-453

[59] Thomas J, Meeks JC, Wolk CP, Shaffer PW, Austin SM, Chien WS. Formation of glutamine from [ammonia-N-13], [dinitrogen-N-13], and [glutamate-C-14] by heterocysts isolated from Anabaena-Cylindrica. Journal of Bacteriology. 1977;**129**(3):1545-1555

[60] Mitschke J, Georg J, Scholz I, Sharma CM, Dienst D, Bantscheff J, et al. An experimentally anchored map of transcriptional start sites in the model cyanobacterium Synechocystis sp PCC6803. Proceedings of the National Academy of Sciences of the United States of America. 2011;**108**(5):2124-2129

[61] Simon RD. The effect of chloramphenicol on the production of cyanophycin granule polypeptide in the blue green alga Anabaena cylindrica. Archiv für Mikrobiologie. 1973; **92**(2):115-122

[62] Liotenberg S, Campbell D, Rippka R, Houmard J, deMarsac NT. Effect of the nitrogen source on phycobiliprotein synthesis and cell reserves in a chromatically adapting filamentous cyanobacterium. Microbiology. 1996;**142**:611-622

[63] Sarma TA, Khattar JIS. Accumulation of cyanophycin and glycogen during sporulation in the blue-green-alga Anabaena torulosa. Biochemie und Physiologie der Pflanzen. 1986;**181**(3):155-164

[64] Leganes F, Fernandez-Pinas F, Wolk CP. A transposition-induced mutant of Nostoc ellipsosporum implicates an arginine-biosynthetic gene in the formation of cyanophycin granules and of functional heterocysts and akinetes. Microbiology. 1998;**144**:1799-1805

[65] Elbahloul Y, Steinbuchel A. Engineering the genotype of Acinetobacter sp strain ADP1 to enhance biosynthesis of cyanophycin. Applied and Environmental Microbiology. 2006;**72**(2):1410-1419

[66] Cunin R, Glansdorff N, Pierard A, Stalon V. Biosynthesis and metabolism of arginine in bacteria. Microbiological Reviews. 1986;**50**(3):314-352

[67] Caldovic L, Tuchman M. N-acetylglutamate and its changing role through evolution. The Biochemical Journal. 2003;**372**:279-290

[68] Heinrich A, Maheswaran M, Ruppert U, Forchhammer K. The Synechococcus elongatus P signal transduction protein controls arginine synthesis by complex formation with N-acetyl-L-glutamate kinase. Molecular Microbiology. 2004;**52**(5):1303-1314

[69] Maheswaran M, Ziegler K, Lockau W, Hagemann M, Forchhammer K. P-II-regulated arginine synthesis controls accumulation of cyanophycin in Synechocystis sp strain PCC 6803. Journal of Bacteriology. 2006;**188**(7):2730-2734

[70] Liu D, Yang C. The nitrogen-regulated response regulator NrrA controls cyanophycin synthesis and glycogen catabolism in the Cyanobacterium synechocystis sp PCC 6803. The Journal of Biological Chemistry. 2014;**289**(4):2055-2071

[71] Sant'Anna F, Trentini D, Weber SD, Cecagno R, da Silva SC, Schrank I. The PII superfamily revised: A novel group and evolutionary insights. Journal of Molecular Evolution. 2009;**68**(4):322-336

[72] Chellamuthu VR, Alva V, Forchhammer K. From cyanobacteria to plants: Conservation of PII functions during plastid evolution. Planta. 2013;**237**(2):451-462

[73] Forchhammer K. P(II) signal transducers: Novel functional and structural insights. Trends in Microbiology. 2008;**16**(2):65-72

[74] Zeth K, Fokina O, Forchhammer K. Structural basis and target-specific modulation of ADP sensing by the Synechococcus elongatus PII signaling protein. The Journal of Biological Chemistry. 2014;**289**(13):8960-8972

[75] Fokina O, Chellamuthu VR, Forchhammer K, Zeth K. Mechanism of 2-oxoglutarate signaling by the Synechococcus elongatus P-II signal transduction protein. Proceedings of the National Academy of Sciences of the United States of America. 2010;**107**(46):19760-19765

[76] Huergo LF, Dixon R. The emergence of 2-oxoglutarate as a master regulator metabolite. Microbiology and Molecular Biology Reviews. 2015;**79**(4):419-435

[77] Luddecke J, Francois L, Spat P, Watzer B, Chilczuk T, Poschet G, et al. PII protein-derived FRET sensors for quantification and live-cell imaging of 2-oxoglutarate. Scientific Reports. 2017;**7**(1):1437

[78] Yuan J, Doucette CD, Fowler WU, Feng XJ, Piazza M, Rabitz HA, et al. Metabolomics-driven quantitative analysis of ammonia assimilation in *E. coli*. Molecular Systems Biology. 2009;**5**:302

[79] Yan DL, Lenz P, Hwa T. Overcoming fluctuation and leakage problems in the quantification of intracellular 2-oxoglutarate levels in *Escherichia coli*. Applied and Environmental Microbiology. 2011;**77**(19):6763-6771

[80] Forchhammer K, Tandeau de Marsac N. Phosphorylation of the PII protein (glnB gene product) in the cyanobacterium Synechococcus sp. strain PCC 7942: Analysis of in vitro kinase activity. Journal of Bacteriology. 1995;**177**(20):5812-5817

[81] Forchhammer K, Hedler A. Phosphoprotein PII from cyanobacteria—Analysis of functional conservation with the PII signal-transduction protein from Escherichia coli. European Journal of Biochemistry. 1997;**244**(3):869-875

[82] Radchenko M, Merrick M. The role of effector molecules in signal transduction by PII proteins. Biochemical Society Transactions. 2011;**39**(1):189-194

[83] Forchhammer K, Luddecke J. Sensory properties of the PII signalling protein family. The FEBS Journal. 2016;**283**(3):425-437

[84] Espinosa J, Forchhammer K, Contreras A. Role of the Synechococcus PCC 7942 nitrogen regulator protein PipX in NtcA-controlled processes. Microbiology. 2007;**153**:711-718

[85] Hauf W, Schmid K, Gerhardt EC, Huergo LF, Forchhammer K. Interaction of the nitrogen regulatory protein GlnB (PII) with biotin carboxyl carrier protein (BCCP) controls acetyl-CoA levels in the Cyanobacterium synechocystis sp. PCC 6803. Frontiers in Microbiology. 2016;**7**:1700

[86] Maheswaran M, Urbanke C, Forchhammer K. Complex formation and catalytic activation by the P-II signaling protein of N-acetyl-L-glutamate kinase from Synechococcus elongatus strain PCC 7942. The Journal of Biological Chemistry. 2004;**279**(53):55202-55210

[87] Luddecke J, Forchhammer K. From PII signaling to metabolite sensing: A novel 2-oxoglutarate sensor that details PII-NAGK complex formation. PLoS One. 2013;**8**(12): e83181

[88] Llacer JL, Contreras A, Forchhammer K, Marco-Marin C, Gil-Ortiz F, Maldonado R, et al. The crystal structure of the complex of PII and acetylglutamate kinase reveals how

PII controls the storage of nitrogen as arginine. Proceedings of the National Academy of Sciences of the United States of America. 2007;**104**(45):17644-17649

[89] Fokina O, Chellamuthu VR, Zeth K, Forchhammer K. A novel signal transduction protein P(II) variant from Synechococcus elongatus PCC 7942 indicates a two-step process for NAGK-P(II) complex formation. Journal of Molecular Biology. 2010;**399**(3):410-421

[90] Zeth K, Fokina O, Forchhammer K. An engineered PII protein variant that senses a novel ligand: Atomic resolution structure of the complex with citrate. Acta Crystallographica Section D. 2012;**68**(8):901

[91] Khlystov NA, Chan WY, Kunjapur AM, Shi WC, Prather KU, Olsen BD. Material properties of the cyanobacterial reserve polymer multi-L-arginyl-poly-L-aspartate (cyanophycin). Polymer. 2017;**109**:238-245

[92] Sallam A, Steinbuchel A. Dipeptides in nutrition and therapy: Cyanophycin-derived dipeptides as natural alternatives and their biotechnological production. Applied Microbiology and Biotechnology. 2010;**87**(3):815-828

[93] Aboulmagd E, Voss I, Oppermann-Sanio FB, Steinbuchel A. Heterologous expression of cyanophycin synthetase and cyanophycin synthesis in the industrial relevant bacteria *Corynebacterium glutamicum* and *Ralstonia eutropha* and in *Pseudomonas putida*. Biomacromolecules. 2001;**2**(4):1338-1342

[94] Hai T, Frey KM, Steinbuchel A. Engineered cyanophycin synthetase (CphA) from Nostoc ellipsosporum confers enhanced CphA activity and cyanophycin accumulation to *Escherichia coli*. Applied and Environmental Microbiology. 2006;**72**(12):7652-7660

[95] Diniz SC, Voss I, Steinbuchel A. Optimization of cyanophycin production in recombinant strains of *Pseudomonas putida* and *Ralstonia eutropha* employing elementary mode analysis and statistical experimental design. Biotechnology and Bioengineering. 2006;**93**(4):698-717

[96] Voss I, Diniz SC, Aboulmagd E, Steinbuchel A. Identification of the Anabaena sp strain PCC7120 cyanophycin synthetase as suitable enzyme for production of cyanophycin in gram-negative bacteria like *Pseudomonas putida* and *Ralstonia eutropha*. Biomacromolecules. 2004;**5**(4):1588-1595

[97] Voss I, Steinbuchel A. Application of a KDPG-aldolase gene-dependent addiction system for enhanced production of cyanophycin in *Ralstonia eutropha* strain H16. Metabolic Engineering. 2006;**8**(1):66-78

[98] Steinle A, Bergander K, Steinbuchel A. Metabolic engineering of *Saccharomyces cerevisiae* for production of novel Cyanophycins with an extended range of constituent amino acids. Applied and Environmental Microbiology. 2009;**75**(11):3437-3446

[99] Wiefel L, Broker A, Steinbuchel A. Synthesis of a citrulline-rich cyanophycin by use of *Pseudomonas putida* ATCC 4359. Applied Microbiology and Biotechnology. 2011;**90**(5): 1755-1762

[100] Steinle A, Witthoff S, Krause JP, Steinbuchel A. Establishment of cyanophycin biosynthesis in *Pichia pastoris* and optimization by use of engineered cyanophycin synthetases. Applied and Environmental Microbiology. 2010;**76**(4):1062-1070

[101] Sallam A, Kast A, Przybilla S, Meiswinkel T, Steinbuchel A. Biotechnological process for production of beta-dipeptides from cyanophycin on a technical scale and its optimization. Applied and Environmental Microbiology. 2009;**75**(1):29-38

[102] Huhns M, Neumann K, Hausmann T, Ziegler K, Klemke F, Kahmann U, et al. Plastid targeting strategies for cyanophycin synthetase to achieve high-level polymer accumulation in *Nicotiana tabacum*. Plant Biotechnology Journal. 2008;**6**(4):321-336

[103] Huhns M, Neumann K, Hausmann T, Klemke F, Lockau W, Kahmann U, et al. Tuber-specific cphA expression to enhance cyanophycin production in potatoes. Plant Biotechnology Journal. 2009;**7**(9):883-898

[104] Abed RMM, Dobretsov S, Sudesh K. Applications of cyanobacteria in biotechnology. Journal of Applied Microbiology. 2009;**106**(1):1-12

[105] Dillschneider R, Steinweg C, Rosello-Sastre R, Posten C. Biofuels from microalgae: Photoconversion efficiency during lipid accumulation. Bioresource Technology. 2013; **142**:647-654

[106] Trautmann A, Watzer B, Wilde A, Forchhammer K, Posten C. Effect of phosphate availability on cyanophycin accumulation in Synechocystis sp PCC 6803 and the production strain BW86. Algal Research. 2016;**20**:189-196

[107] Beardall J, Raven JA. Limits to phototrophic growth in dense culture: CO_2 supply and light. In: Borowitzka MA, Moheimani NR, editors. Algae for Biofuels and Energy. Dordrecht: Springer Netherlands; 2013. pp. 91-97

[108] Bahr L, Wustenberg A, Ehwald R. Two-tier vessel for photoautotrophic high-density cultures. Journal of Applied Phycology. 2016;**28**(2):783-793

[109] Lippi L, Bähr L, Wüstenberg A, Wilde A, Steuer R. Exploring the potential of high-density cultivation of cyanobacteria for the production of cyanophycin. Algal Research. 2018;**31**:363-366

IntechOpen