11 General discussion

Angermayr SA
11.1 Tools for synthetic biology in cyanobacteria

Progress in synthetic biology of cyanobacteria relies on the successful modification of their metabolism. Generally, new biosynthetic routes are established through the insertion of new genetic elements, foremost gene cassettes intended for expression. The design and construction of a cyanobacterial cell factory employing a synthetic production pathway can represent a precursor to the (future) reconstruction of an artificial photosynthetic cell factory, which might also be envisioned as a complete synthetic cell ‘per design’. Although tools for (high) heterologous expression, such as strong promoters and expression from self-replicating plasmids in model cyanobacteria have been provided in the 1990’s (113, 304, 305) their employment for product formation has been less facilitated, until relatively recently. Significantly, the direct comparison, and objective characterization, is still lacking for most expression systems. However several recent reviews serve as excellent collective memory for the array of promoter systems that are in use for synthetic biology of cyanobacteria to date (147, 150, 306). It is evident, that the semi-artificial promoters originating from *E. coli*, *Ptac*, *PlacO1* allow strong expression in cyanobacteria, and can be regulated (by the addition of β-D-1-thiogalactopyranoside (IPTG), provided the *lac* repressor (LacI) is heterologously co-expressed and the operator sequence is present). A recent non-IPTG regulated artificial promoter system comprised of a modified version of *PR40*, likewise originating from *E. coli*, induced by anhydrotetracycline (aTc), provided the *tetR* repressor is co-expressed, has become available (307). Both systems have been subject to characterization with respect to the dynamic range and the stringency of control (141, 307). Most of the regulated promoters employed are native promoters which are responsive to metal ions. For example *PpetE* is induced by Cu$_2^+$, and *PisiAB* is repressed by iron. Additionally, a limited set of native non-metal responsive promoters is available for the expression of heterologous genes, such as the light responsive *PpsbA2* or the nitrogen-responsive *PnirA*. Not unmentioned should be, that a weak but constitutive expression has been achieved with *PrnpB* (94). Strong, native promoters are e.g. *PrbcL* from RuBisCO (94), and the promoters driving the genes which are coding for the abundant physobilisome-proteins (i.e. *PcpcBA*) (175). However, especially in the latter example, it is difficult to dissect the promoter strength from the translation efficiency, which calls for further characterization.

Interestingly, in some studies, native regulation systems are ‘hijacked’ for expression at a defined physiological state, e.g. through promoters from genes associated with CCM (selected based on transcription analysis) (308). It can easily be envisioned that such (and similar systems, responding to various environmental conditions), together with careful design of growth media, will provide powerful applications for heterologous pathway regulation in the future.

Further examples for the ‘fine tuning’ of gene-expression level and protein-level alterations in cyanobacteria are included in preliminary studies on the use of degradation tags
for proteins (147, 309), the comparison of different ribosomal binding sites (147), the modulation of the 5’-untranslated region (5’UTR) (310), amplified expression by a recombinant T7 polymerase and PT7 (146), most of which are ‘copy-pasted’ from *E. coli*.

Codon optimization of a foreign gene of interest for a new cyanobacterial host has been used widely, but systematic studies providing general guidelines do not exist, and the success has been evaluated on a case-to-case basis, and is likely dependent on the donor organism (99, 155) (and chapter 4). Generally codon optimization is applied to the whole open reading frame (ORF). Care must be taken at the 5’-end, which can influence the secondary structure formation and subsequently the access of this region to the ribosome. Like most prokaryotes (and also some eukaryotes), *Synechocystis* shows a 5’-end that is predicted to form a less stable secondary structure than the downstream mRNA (311). A less stable secondary structure at of the region close to the 5’-end has been correlated with increased expression employing combinations of GFP and mCherry reporter constructs in *E. coli*, emphasising that rare codons are enriched at those positions (312). In combination with the ribosomal binding site (147) the sequence (and resulting secondary structure formation) at the 5’UTR can also be utilized for regulatory purposes of translation initiation, e.g. by a riboswitch, which has been recently investigated using the firefly luciferase reporter in *Synechococcus elongatus* PCC 7942 (313).

Recently antisense RNAs (asRNAs) have been identified as a major level of regulation in *Synechocystis* (314). Some asRNA-systems have been characterized in more detail, such as the regulation system of IsiA (the iron stress-induced protein A) (315) and components of the flavodiiron-response to carbon limitation (316). Interestingly, complementing asRNA-based stabilization for the efficient translation of the D1 protein in *Synechocystis* (encoded by *psbA2*) was engineered by expressing the stabilizing asRNA ectopically through PrnpB (317).

The introduction of heterologous genes can be achieved by the incorporation of new DNA into the genome or on self-replicating, foreign plasmids. With minor strain-to-strain variations the majority of the ‘model cyanobacteria’ can be transformed with foreign DNA through natural transformation (utilizing endogenous DNA-uptake mechanisms), bacterial conjugation (utilizing a *E. coli* helper-strain) and through electroporation. Also, in ‘the early days’ of cyanobacterial transformation, an UV-based method independent of homologous recombination events has been proposed for *Synechocystis* (55). The ‘model cyanobacteria’ generally hold more than one copy of their genome per cell, which introduces the need for full genome segregation to obtain a homogenous genotype when targeting the main chromosome or one of the native (multiple-copy) plasmids (be it for the introduction of DNA or a deletion) (51, 114, 318, 319). Plasmids to be used essentially narrow down to the broad-host range plasmid RSF1010 and derivatives thereof (251, 305). Copy number of plasmid and genome, as well as effectiveness and expression form those distinct replicons, are still matter of debate and their numbers have not been addressed systematically enough to allow final
conclusions (94, 114, 318–320). However, chapter 4 shows evidence that the protein amounts, activity of the expressed enzyme, and the production rate originating from the same gene, but expressed in single-copy from genome and a plasmid, respectively, results in ~4 to 5-fold higher productivity for the plasmid borne expression, compared to the expression form the genome.

Detection of insertions is generally facilitated by the use of selective markers such as antibiotic resistance cassettes. Cyanobacterial mutant strains carrying up to four different antibiotic markers have been reported (321). Studies on the protein- or metabolic burden of the heterologous genes through modulated expression levels of those marker genes, or their (often energy consuming) mechanism for antibiotic resistance are, to the best of my knowledge, not available for cyanobacteria.

Especially for up-scaling and growth in mass-cultures in bioreactors or even outdoors, or for the construction of multiple gene knock-ins and knock-outs, markerless mutants are desired. Markerless systems, essentially adopted from *E. coli*, that are in place for cyanobacteria include the *sacB*-system, originating from *B. subtilis*, which enables the use of sucrose as negative selection marker (91, 322, 323) and the *mazF*-system, established in *E. coli*, employing a nickel-inducible promoter for the expression of a protein-synthesis inhibitor as counter-selection marker (324).

Apart from the construction guided by rational design parameters, the creation of a large mutant library might lead to the emergence of an altered genotype with the desired increased productivity. Examples of mutant libraries based on random mutagenesis in cyanobacteria are still rare. However, a 3-fold increase of poly-3-hydroxybutyrate (PHB) accumulation in *Synechocystis* has been achieved by a screen of a mutant isolated from a whole-genome deletion library. Briefly, the genome of *Synechocystis* has been subjected to random transposon insertion *in vitro* and was subsequently transformed to *Synechocystis*, relying on integration by homologous recombination for gene disruption with an accompanying marker (271). Other examples, employing the transposon mutagenesis approach, include a study of light-dependent motility in *Synechocystis* (325) and a study of circadian regulation mechanisms in *Synechococcus* sp. PCC 7942 (326). Further strategies include the *in vitro* fragmentation of genomic DNA, ligation of a resistance cassette to those fragments and subsequent transformation, again relying on integration by homologous recombination for gene disruption (115, 327). Recently UV- and mutagen-mediated (methyl methanesulfonate, MMS) mutagenesis, aiming at the introduction of point mutations, has been used for the generation of mutant strains of *Synechocystis* with a slight increase in temperature tolerance (328).

The construction of a mutant library relies on the capacity to read-out the desired phenotype, and, potentially, needs to be coupled to a high-throughput screening method, to characterize the large number of generated mutants. To date, only a few studies report, but do not evaluate, the use of 96-well titer plates (309, 310), which are widely used for larger
screens of enzyme activity and production strain with other organisms/systems. **Chapter 9** presents an alternative for the (future) screen of a large mutant library of cyanobacterial strains that excrete a product, which is the use of a single-cell screen based on micro-droplets, analysed and sorted in a microfluidic system (269). The construction of a deletion library through the methods briefly introduced above, in the background of a producing strain, aiming at finding a clone with higher productivity, can easily be envisioned. Alternatively, a mutant library consisting of the co-overexpression of a random set of genes (e.g. a fragmented genome library) in a strain where the control of the production is not embedded fully in the heterologous pathway, might lead to a clone with higher productivity, as exemplified by the rational design described in **Chapter 4**. Undoubtedly synthetic biology endeavors in cyanobacteria will benefit from (semi)high-throughput systems and screens.

Rational design will continue to play an important role in the development of strains with higher productivity and the understanding of the physiology of cyanobacteria. For example, targeted mutagenesis will benefit from *in silico* models that aim at the description of the metabolism of cyanobacteria (30). Such models allow the *in silico* modification and the test of different (genetic and environmental) scenarios employing a tool from systems biology, flux balance analysis (FBA), based on the genome sequence and its annotated functions (31).

Systems-level information has been integrated with strain optimization with respect to the resistance to toxic products. Transcriptome analysis in *Synechocystis*, e.g. upon ethanol and butanol addition, resulted in the identification of genes up-regulated as response to the stress (329, 330). Consequently, the overexpression of those target genes resulted in strains showing an improved tolerance phenotype (329).

As stated above, advances in the synthetic biology of cyanobacteria did benefit from the ‘copying/pasting’ of genetic elements originally described and defined in *E. coli*. It is not only because if this fact, that cyanobacteria have been termed ‘green *E. coli*’ (150). Significantly, whole biosynthetic pathways that have been realized and characterized in *E. coli* first, can then be ‘plugged’ into cyanobacteria with ease, now that the molecular tools have advanced. Interestingly, since relatively recently, commercially available ‘engineering kits’ for *Synechococcus* sp. PCC 7942 are available providing vectors for gene insertion, overexpression, including tags for protein identification and purification (331, 332).

This further illustrates the popularity (and need) of expanding research endeavors towards the understanding and utilization of cyanobacterial metabolism for synthetic biology projects towards a biotechnological application. Not unmentioned should be the entry of cyanobacteria into the ‘world’ of the International Genetically Engineered Machine (iGEM) competition on synthetic biology. Next to (genetic) parts for the ‘traditional’ laboratory and biotechnology model organisms, several parts, including expression vectors, promoters, different markers, etc. are available for cyanobacteria (compare also http://parts.igem.org/Main_Page).
For the deployment of cyanobacteria as a ‘chassis’ for a specific biotechnological application (i.e. use of a biosynthetic pathway leading to product formation) efficiency and minimized energy losses are important criteria for an economically viable approach and successful up-scaling.

Future developments might result in strains with a reduced number of genome copies and/or reduced (and purposefully designed) genomes, specific to a certain application. For organisms, designed for a specific purpose, the pathways needed for the adaption to a changing environment and systems that secure the fitness during the course of evolution, could potentially be omitted. The ‘streamlining’ of a metabolism might result in a higher efficiency of the production pathway. It can also be envisioned that an engineered strain lacking the stress-response pathways, and means of adaption, is not competitive in the ‘outside world’ which might be needed for genetically modified organisms (GMOs) to be ‘considered generally regarded as safe’ (GRAS).

11.2 Genetic (in)stability

The modification of an organism (or its genetic information), which has been shaped by evolution since the advent of life, might result in altered stability of the integrity of the genome, or the organism as a whole.

Conditions in the laboratory (or in a bioreactor) can be maintained relatively stable and it is matter of debate if a modified organism ‘needs’ certain properties for a certain biotechnological application, as opposed to its successful proliferation in a natural environment.

Nonetheless, it is evident from many of the projects on product formation with cyanobacteria that the redirection of metabolism can lead to an (unexpected) imbalance of cellular homeostasis. Examples include the loss of ethylene-forming capacity during subsequent culturing, facilitated by ‘mutational hotspots’ in the heterologous gene and the selection against the originally producing strain, which is disturbed in the nitrogen homeostasis (101). In chapter 2 a mutant solely expressing a soluble transhydrogenase is presented, that shows a slow-growing phenotype and, upon full segregation of the insertion into all the genome copies of a single cell, the emergence of suppressor mutants with disrupted transhydrogenase expression (133). In one of the mutants analyzed, duplication of a part of the heterologous gene resulted in a pre-mature stop codon. Further examples from the cyanobacterial ‘production literature’ include point mutations disrupting the enzymatic activity in the pathway towards isopropanol formation (221) and a frame shift mutation in an engineered mannitol biosynthesis pathway (222). It is unknown through which mechanisms those suppressor mutations in the genomic sequence emerge, i.e. whether this is based on the random mutation frequency provided by replication, a result of an ‘error-prone’ repair function, or any other mechanism that may lead to the introduction of spontaneous mutations. The error-prone repair might be facilitated by RecA, and other enzymes involved in
homologous recombination, which are involved genome rearrangements in the segregation of
genes into the multiple copies of the genome of cyanobacteria (compare Ref. (333) and
references therein). In *E. coli*, genetic stability, after the introduction of multiple copies of the
gene of interest, has been achieved e.g. by the deletion of RecA (334). Briefly, first the
recombination system, presumably facilitate by RecA, was used for gene multiplication for
the purpose of increased of PHB-production. To prevent the cells from subsequently lowering
the engineered gene dosage by means of homologous recombination, the final producer strain
was deleted in for *recA*. It is relevant to note that the deletion of the *Synechocysits recA*
homolog (149) led to a slow-growing, light-sensitive phenotype. This implies that other
targets for the engineering increased genetic stability in this organism must be found.

However, it is tempting to ask if a ‘simple’ redirection of carbon metabolism, without
dramatic alterations elsewhere, thus a cells with ‘wild type-like’ homeostasis, would lead to
an imbalanced and unstable phenotype. For further studies on genetic stability and
mechanisms to improvement thereof it might be relevant to distinguish between a strain that
is unstable because of a severe imbalance in the metabolic network, and a strain which is
‘balanced’ but slow growing, implying a ‘cell factory’, that nonetheless might be
outcompeted by a faster growing strain (assuming the heterologous constructs provide
optimal genetic stability, e.g. avoid internal repatitions, etc.).

11.3 Metabolic control analysis and rational pathway modification

In a multitude of studies on the heterologous product formation in cyanobacteria it has
been established that increased expression of the pathway resulted in the desired effect of
increased productivity (compare also chapter 7 and (216)). Examples of such studies include
the production of ethanol (140), 2,3-butanediol (310), *meso*-butanediol (129), isobutanol
(217), acetone (223), ethylene (219), and lactic acid (155) (and chapter 4). The strategies to
achieve overexpression in the listed examples include the use of promoters with different
strength, inducible promoters, codon optimization, gene duplication, and modification of the
5′-untranslated region (5′UTR) and the ribosomal binding site. Undoubtedly, the utilization
of (a) different enzyme(s) in a given pathway can lead to an altered productivity as shown
e.g. for 2,3-butanediol (148) and lactic acid (133) (and chapter 4). The systematic
comparison of engineered cyanobacteria is lagging behind that of the established laboratory
workhorses *E. coli* and yeast. Nonetheless, a few studies advance on the more detailed
characterization of the ‘bottlenecks’ of and in a heterologous pathway towards product
formation. An attempt to control expression and relate the expression level to the productivity
was performed for isobutanol production in *Synechocystis* (217). From those results it is
evident that the promoter used, *Ptac*, which is controlled by the heterologously co-expressed
lac repressor (LacI), is leaky, but transcription of the genes, comprising the pathway toward
isobutanol, can be enhanced upon the addition of β-D-1-thiogalactopyranoside (IPTG), a non-
metabolizable allosteric regulator for LacI. Transcript analysis by a semi-quantitative reverse

169
transcription (RT-PCR) analysis reveals that increasing IPTG concentrations correlate roughly with the increased transcription of at least one gene from the employed pathway. Strikingly, the maximum titer product titer is reached already without the addition of IPTG. Hence, it has been suggested that the lowest expression level of the two genes is sufficient for the productivity achieved with the employed enzymes. However, it has not been further investigated if the decrease of the measured titers is caused by the altered expression levels in any way. Isobutyraldehyde production in Synechococcus sp. PCC 7942 was achieved by the overexpression of a ketoisolvalerate decarboxylase (kivd) and three genes from the pathway leading to ketoisovalerate: alsS, ilvC and ilvD (compare also Figure 1 in chapter 7). The co-overexpression of RuBisCO resulted in an increase of productivity, implying that the introduced synthetic pathway towards isobutyraldehyde did hold overcapacity (95). The combinatorial overexpression of three genes from the heterologous biosynthetic pathway towards 2,3-butandediol - through modifications of the 5’UTR - aims at balancing the encoded activities to lower the effects of toxic intermediates and the potential loss of energy into an unnecessary protein burden. Highest titers were achieved with a relative low activity of the first enzyme from that pathway, acetolactate synthase (ALS), but high levels were necessary for the two later steps of the pathway, acetolactate decarboxylase (ALDe) and secondary alcohol dehydrogenase (sADH) (or acetoin reductase (AR), compare also Figure 1 in chapter 7). Significantly, the modulation of ALDe in a shortened pathway, by excluding the sADH specific for acetoin, holds the majority of the control over the productivity. The individual contribution of the separate enzymes to the production rate in the complete pathway, i.e. including the sADH, was not resolved. Interestingly, an in vitro assay of the enzyme activity suggests a huge overcapacity for the last step, the sADH. Similar results were obtained for meso-butane2,3-diol production in Synechocystis (129). The productivity of has been increased by increased levels of the two last steps (i.e. ALDe and AR). The activity of the preceding step, catalysed by ALS, could not be confirmed in vitro, but was assumed unaltered, because in both mutants it seems to be provided sufficiently by the native metabolism (129). This suggests that ALS does not hold significant control over the production rate of meso-butane2,3-diol in the employed synthetic pathway.

It has been suggested repeatedly in literature that the overexpression of the endogenous upstream parts of a synthetic pathway will benefit productivity. With the exception of the ectopic co-overexpression of the genes coding for the large and small subunit of the RuBisCO complex (95), the examples for a systematic underpinning of this strategy are rare. Both examples on butane2,3-diol formation (see above) essentially lead to the conclusion that the increase in heterologous ALS activity (next to the native ALS) do not support increased flux through the pathway. The control of the heterologous pathway has not (yet) been relocated (from the inserted pathway) to a reaction elsewhere in the endogenous metabolism of the cell.
General discussion

The systematic increase of the capacity to catalyze a specific reaction, and the concomitant analysis of the productivity, can be used for a metabolic control analysis (MCA). A positive linear correlation with a slope of 1 of the two entities, activity and productivity, leads to the conclusion that that particular reaction has a metabolic control coefficient of 1 (138). The extensive overexpression of the gene (ldh) coding for lactate dehydrogenase (LDH) of *L. lactis* resulted in mutant strains with a control coefficient lower than 1, suggesting that the control over the production pathway has been ‘relieved’ from the heterologous pathway towards one or more steps of the endogenous intermediary metabolism. Exactly in this strain, but not in the strains in which the lactate dehydrogenase still has a control coefficient of 1, the co-overexpression of pyruvate kinase (pk) which is the immediate upstream reaction in the native metabolic network, resulted in a higher productivity (see chapter 4).

The productivity of a pathway can be altered not only by the amount of participating enzymes, but also by the modulation of the activity of those enzymes. Interestingly, the *ldh* of *L. lactis* encodes an L-LDH that is allosterically activated by FBP (122), which leads to dimer formation. Apparently, a subsequent tetramer formation of LDHs is of lesser importance than this initial dimerization (335). 2,5-anhydromannitol (2,5-AHM) if phosphorylated twice, can act as non-metabolizable FBP analogue (336), and thus act as an allosteric activator for the *L. lactis* LDH, and can thereby increase lactic acid formation in this organism (337). Thus, provided that the expressed LDH is not saturated with intracellular FBP, a further activation of the enzyme could be accomplished by the addition of 2,5-AHM to increase lactic acid formation. Considering that the pathway of glycolysis yields pyruvate, it is important to note here that the *Synechocystis* genome contains two pyruvate kinase genes, *sll0587* and *sll1275*, but only the latter, encoding a PK-A (a AMP-activated PK, ATP-inhibited, not FBP-dependent), seems to be expressed (338). Figure 1 shows the effect of different concentrations of 2,5-AHM added to a lactic acid-producing *Synechocystis* strain. The addition of low amounts of 2,5-AHM (0.025 and 0.10 mM, respectively) leads to an increase in lactic acid production. The addition of 0.025 mM AHM roughly doubles the lactic acid titer after 1 week of culturing and the addition of 0.10 mM AHM roughly triples this titer, compared to the non-treated control culture.

Neither concentration of 2,5-AHM affects growth in this time span. Higher concentrations of 2,5-AHM result in a growth inhibition. Nevertheless, a ‘sweat spot’ for highest productivity exists, even if growth is negatively affected by the addition of 2,5-AHM. Hence, for the *L. lactis* LDH both, the further increase in protein level (155) (and chapter 4) and the increase in activity by allosteric regulation does lead to a higher lactic acid productivity. It is likely that independence from an allosteric regulator, might results in a simplified production system. By combining knowledge about the proteins’ structure, its catalytic properties and molecular modelling, the rational engineering towards the independence of an allosteric regulator should be achievable (339).
Figure 1: *Synechocystis* SAA023 (155) expressing the *ldh* of *L. lactis* from *Ptrec* supplemented with different concentrations of 2,5-anhydromannitol (2,5-AHM), which, if phosphorylated, resembles a non-metabolizable structural analogue of fructose-1,6-bisphosphate (FBP).

### 11.4 Carbon partitioning and homeostasis

As noted in the introduction, the fine-tuning of the rates of linear and cyclic e− transport serves the balanced energy requirement of the cellular synthetic machinery, which is needed for optimal growth and adaptation, especially to changing light intensities. Textbook knowledge holds that the generation of ATP and NADPH at the thylakoids results in a slight ‘surplus’ of NADPH over ATP, considering the ratio of 9:6 needed for the CBB cycle. This leads to the hypothesis that the utilization of NADPH elsewhere (e.g. by a introduced NADPH-dependent fermentation reaction, compare meso-butanediol production in Ref. (129)) might adjust the generation and utilization ratios of these two high-free-energy intermediates just right. In a cyanobacterial cell the fine-tuning of ATP- and NADPH-availability (including the ratio at which they are synthesized) and their ratios can be regulated by multiple alternative e− transport routes (30). All cyclic e− transport pathways around PSI results in ATP generation only, whereas the linear e− flow results in ATP and NADPH.

Many additional reactions in the cell also require ATP and/or NADPH, thus have an influence on the anticipated ‘balanced’ use of those free-energy carriers. Modelling approaches suggest that alternative e− flows are primarily needed to adjust to carbon limitation or to changes in light intensity or to changes in metabolism, and might be less likely tolerated if the ‘photosynthetic machinery of the cell’ would not show this adaptability and robustness (30). All living cells have to preserve a redox balance for physiological
activities, including growth, thus balance the pathways that produce and consume reducing power (12). It can therefore be considered a rational design principle to keep balancing redox co-factors when introducing production pathways (340).

It has been speculated that the most efficient heterologous biosynthetic routes will be those, that have been adjusted to the native stoichiometry of reducing equivalents and ATP equivalents in the cell, which are provided (and governed) by the photosystems in the thylakoid membrane (29). Consequently, an organism could be engineered that channels almost 100% of the fixed carbon into a product with the same requirements for reducing equivalents and ATP equivalents as the biomass, without a significant alteration of the homeostasis. Such an organism can be called a ‘true’ cell factory, but might still be considered unstable, because growth (rate) will essentially be reduced to zero, which would lead to a fast out-competition by a suppressor mutant emerging in a population of wild-type cells. An interesting speculation is based on those strains that carry pathways considered ‘carbon sinks’. A few strains, upon heterologous product formation (e.g. ethanol, butanediol, sucrose, compare chapter 7), seem to fix carbon at a higher rate than the wild type. In contrast to ‘cell factories’ that ‘simply’ channel the majority of their carbon into product, those strains might be more efficient with respect to the harvesting and utilizing of carbon than wild type. However, those strains still channel a significant fraction of their carbon into product rather than biomass, thus are actually also rather efficient ‘cell factories’ at the same time, resulting in slower growth than wild type. Unless product formation represents a beneficial situation for the producing cell, growth will remain a selection criterion against a ‘cell factory’. Hypothetically, instead of a ‘cell factory’ that is channeling almost 100% of the fixed carbon into the product, a production system strictly coupled to growth (be it minor) might result in a stable production system. Alternatively a regulated expression system that only allows product formation in a stationary growth phase might circumvent the disadvantage for a slow-growing (but producing) cell in the (competitive) growth phase in a culture. This emphasizes the need of a ‘holistic’ solution for a biotechnologically viable application, from product and host selection to the regulation genetic system, the growth conditions in a bioreactor and towards product recovery (7).

11.5 Concluding remarks on the optimization of a ‘cell factory’

As evident from the increasing list of products produced by and pathways employed in cyanobacteria, a plethora of mechanisms can be envisioned to be optimized. Undoubtedly, the near future will allow detailed characterization and optimization of specific pathways. To date only a few products and pathways have been investigated in multiple studies or by multiple research groups. Furthermore, generalized optimization strategies might have to give way to specific strategies, possibly coupled to downstream processing for a specific biotechnological application.
Plenty of mechanisms that deal with the efficient use of energy and energy substrates are present in every cell/organism. Those mechanisms have to help to support growth and also need to assure survival in a changing environment. Some react to the changing environmental conditions through the accumulation of storage compounds in times of abundance, others serve robustness and also adaption for the survival during the course of evolution. Several points have been raised in the General introduction to this thesis that balance the (photon) energy uptake through the light harvesting complexes, and many points-of-interest from metabolism have been suggested in various chapters throughout this thesis. Countless enzymes and metabolites can be involved in important ‘decision making’ on how to distribute energy and metabolite fluxes. When ‘re-programming’ cellular metabolism, changes are not only introduced in the respective local biochemical network, but they may also influence the cell as a whole. The capturing of (sun)light and efficient utilization of this energy holds a lot of control over the cells energy household and subsequent metabolism. Especially in larger cultures, where a cell might be exposed to a dynamically changing light regime due to turbulence in the (photobio)reactor, an optimization of the light harvesting complexes might hold potential for increased productivity. The feedback of metabolites on the photosynthesis-processes might be fine-tuned to fulfill the requirements of a production system, rather a homeostasis-driven system that is, without question, able to adapt and perform well when it comes to the survival of the fittest. For an efficient cyanobacterial ‘cell factory’, or a ‘microbial catalyst’ the optimization of metabolism might hold a lot of potential once regulatory circuits are fully understood and the coordination of carbon uptake, anabolism and catabolism becomes possible at will. Minimizing loss, optimizing carbon partitioning and maximizing production titers are, obviously, the desired goal. Combined with the understanding of the (genetic) regulation, understanding of the physiology and the metabolic network, the construction of virtually any desired microbial biosynthetic process can be envisioned. For the construction of an efficient ‘microbial catalyst’, both, (sequential) genome reduction and the de novo construction of any (biosynthetic) pathway in an ‘empty’ biological chassis, might be possible in the future (7).

11.6 Non-secreted products

The work in this thesis focused on products that diffuse from, or are excreted from, the cytosol to the extracellular growth medium. An optimized cyanobacterial cell factory can thus be defined as an entity that remains (almost) unchanged but processes the input (H₂O, CO₂ and light) into a product that is separated and detached from the catalyst (i.e. deposited in the extracellular compartment). A final detachment of the product from the cells is especially important for toxic products or products that lead to negative feedback regulation in metabolism.

Consequently, not under investigation in this thesis are products contained in the biomass, or products that are typically residing in the cell. Those products of (engineered)
cyanobacteria include pigments (91), fatty acids (322), alkanes (151), PHB (271), polysaccharides, etc. Especially medium chain-length alkanes gained considerable attention recently, because they are a major fraction of current petroleum-based jet fuels (341). Nonetheless, the export of some of those products might be facilitated (e.g. by a transporter, such as for sucrose (97)) indexing those products with the excreted products and thus make them products of a ‘cell factory’ as it is defined in this thesis.

As mentioned in the General introduction, cyanobacteria have not only gained interest for their utilization in (potentially large-scale) biotechnological applications for bulk chemical and biofuel generation (in order to contribute to a sustainable energy supply), but are also utilized for nutritional purposes and the production of several high value products (33, 36). Production of a large, and complex molecule, e.g. for the pharmaceutical industry, through enzyme catalysis (as opposed to chemical catalysis) can benefit from the mild conditions at which a biological process takes place, and from the selectivity of an enzyme. Significantly, the biosynthesis of compounds originating from reduction reactions is overshadowed by the need for (expensive) reducing equivalents (156). Through the light reactions cyanobacteria can provide a continuous supply of reducing equivalents for such reactions (342). Recently, the production of plant secondary metabolites, such as limonene from the isoprenoid pathway (343), and p-coumaric acid from the tryrosine biosynthesis (344), exemplified the relevance of cyanobacteria as a production system for higher-value (and more complex) molecules that are otherwise produced only at low yields by plant cell cultures and are unfeasible to produce through chemical synthesis.